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Hyphenated approaches for the analysis of bioactive natural compounds in complex matrices

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आहारसम्भवं वस्तु रोगाश्चाहारसम्भवाः
हिताहितविशेषाच्च विशेषः सुखदुःखयोः

The body is formed from food

and even diseases also originate from food.

Conductive and nonconductive foods are responsible

for happiness and sorrow respectively.

Caraka Samhita Sutrasthana 28:45

The only way to keep your health is to eat what you don't want, drink what you don't like, and do what you'd rather not.

Mark Twain

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Abstract

Plants, animals and micro-organisms represent a reservoir of natural products, the so called "natural source-derived compounds". This is particularly true for the plant kingdom, as it offers a variety of species still used as remedies for several diseases in many parts of the world. Nevertheless, the bioactive potential of many plant species remains largely unexplored. Thus, biodiversity represents an unlimited source of chemical entities with potential beneficial effects on human health. These compounds are usually secondary metabolites often present in low quantity in plant material and their extraction, purification and quantitation still remain a great challenge for analytical scientists.

The research activity carried out during these three years of PhD Programme was focused on the development, validation and application of original methods aimed at the quali-quantitative analysis of compounds with potential bioactive interest in plant matrices, foods, drinks and related products, as well as the analytical screening of plant by-products from cosmetic manufacture. Bioactive substances, belonging to the classes of polyphenols, aminoacids, coumarins, triterpenes and phytohormones, have been investigated as authenticity markers, in order to identify high quality products and to valorise niche products. The study regarded herbs (i.e. *Argania spinosa*), fruits (i.e. *Citrus* × *myrtifolia*, *Punica granatum*) and berries (i.e. *Myrtus communis*) mainly used as folk medicines for their broad spectrum of supposed pharmacological and therapeutic effects. The analytical methods developed within this study are based on high performance liquid chromatography and ultra-high performance liquid chromatography coupled to spectrofluorometric detection, triple quadrupole and high-resolution triple quadrupole mass spectrometry (HPLC-F, LC-MS/MS and UHPLC-HRMS). Significant efforts have been put also into the development and optimisation of miniaturised sample pretreatment strategies, such as micro-solid phase extraction (μSPE) and micro-extraction by packed sorbent (MEPS), able to purify complex matrices of natural origin (whole fruits, fruit parts, leaves and their extracts) and derived commercial products (fruit juices, soft drinks and liqueurs).

Chapter I

Introduction

1. Bioactive compounds

The complex enzymatic system of organisms in nature has developed under an evolutionary driving force, resulting in a range of different biosynthetic pathways producing primary and, in particular, secondary metabolites with a large variety of basic structures and functional groups. The reason for this evolutionary trend is probably due to competition and coevolution between different organisms, not only for nutrients and living space, but also for communication, defence, synergism and predation.

In the search for bioactive compounds, since the beginning of the nineteenth century, emphasis has been put into isolation of pure components from the complex mixture of a bioactive extract. A typical example of this is the isolation of morphine by Sertürner [1], one of the first cases of bioassay-guided isolation of a pure bioactive natural compound.

For a long time thereafter, natural product research mainly focused on alkaloids, probably due to their often powerful biological activity and relative ease of isolation. Other secondary metabolites, e.g. flavonoids and monoterpenes, responsible for color and scent respectively, were considered to be waste products generally lacking important pharmacological activities and only occasionally explored for their supposed phylogenetic information. Today the scene has changed and many of these compound classes have received strong attention and have shown a diverse set of pharmacological activities.

1.1 Source and role

With the advancement of genomic research, new possibilities have opened up to study symbiotic bacteria, small and often highly specialized organisms involved in the biosynthesis of molecules, earlier believed to be produced by plants [2]. It is obvious that we are far from having enough knowledge about the biology of organisms in the field of natural product research.

In addition to the existence of still very little studied organisms, it seems that the vast majority of existing species on Earth are not yet known to science. Exploring this large yet understudied wealth, the question leaps to mind if it is still possible to discover not only new substance classes, but also new targets among organisms in nature. Traditionally, ethnopharmacological, ecological, or toxicological observations in nature have been the starting point for many research projects.

1.2 Nutraceuticals

For a long time natural products obtained mainly from plants have been used as a prominent source of agents for the prevention and treatment of diseases in humans and animals [3]. Hippocrates (460–370 BC) stated *"Let food be your medicine and medicine be your food"*. Nowadays, the relationship between food and drugs is getting closer. Thus, the term nutraceutical was firstly mentioned years ago to describe a union between nutrition and pharmaceutics, both key contributors to human wellness [4]. In the last 20 years many scientific publications have been devoted to the so-called "functional foods" and "nutraceuticals". Research into functional ingredients was showing promising prospects for the use of such ingredients in food products, thereby representing an important added value for manufacturers and benefits for consumer health [5]. Some studies are focused onto the beneficial properties of a particular natural matrix, other manuscripts paid attention to specific natural compounds like phytochemicals [6], proteins and peptides [7] or lipids [8], meanwhile other works showed the benefits of nutraceuticals against several diseases like atherosclerosis [9] and degenerative joint pathologies [10].

The capacity of some plant-derived foods to reduce the risk of chronic diseases has been associated, at least in part, to the occurrence of secondary metabolites (phytochemicals) that have been shown to exert a wide range of biological activities. In general, these metabolites have a low potency as bioactive compounds when compared to pharmaceutical drugs, but since they are ingested regularly and in significant amounts as part of the diet, they may have a noticeable long-term physiological effect [11]. There are numerous biological mechanisms by which nutraceuticals might be expected to exert favourable influences on pathophysiological processes. These products are safe and well tolerated, but interpretation of the collective results is hampered by study heterogeneity, inconsistent results and/or not well-designed investigations.

An additional problem, related to the production and consumption of nutraceuticals, is represented by the variability in composition and content of active constituents in plants (like in any other natural source), depending on

season, climate, temperature, humidity, soil and several other factors. For this reason collection, identification and maintenance of uniform quality, quantification and standardization are critical factors to be considered.

The development of advanced analytical methods is, therefore, necessary in nutraceutical research. It includes the identification of nutraceuticals, characterization of their chemical structure and bioactivity, quantification in the natural source, product development, quality control of their derived forms, etc. Due to the complexity of these natural matrices, the use of advanced analytical techniques is mandatory in order to properly carry out the mentioned studies. Some of these techniques are already applied for quality control, with the aim of confirming their composition from lot to lot and assuring the safety of the final product. Also, these strategies are typically used in a combined way for product development at the initial stages of their discovery, mainly for facing the challenge to analyse multiple components or multiple classes of components.

Moreover, the analytical method choice depends also on the target compounds and the matrix in which they can be found. For example, their physico-chemical properties (polarity, size, volatility) will have a strong influence onto the sample preparation procedure, separation mechanism and technique and the type of detector to be employed. Moreover, advanced analytical techniques are also needed to obtain a better understanding of the health promoting effects of the nutraceuticals and for knowing the body exposure and bioavailability after the intake of these compounds [12,13]. Important aspects during product development should include nutraceutical bioactivity and bioavailability studies thus, *in vitro*, *in vivo* and clinical trials should ideally be employed.

1.3 Signalling compounds

It is known that bacteria and fungi do not live isolated in nature, they exist in communities. While the production of compounds with antibiotic activity is consistent with the common perception that such diverse communities are highly competitive, microbial ones are also highly communicative. The two notions are not incompatible. The primary mechanism for controlling all cell functions is the regulation of transcription. It has been clearly demonstrated that at subinhibitory concentrations most antibiotics cause up- or down-expression of a large number of transcripts in different bacteria and fungi, many of which determine environmental interactions [14]. Since antibiotic use for therapeutic purposes is so strongly entrenched, the possibility that they have other roles, as for example as signalling molecules, has been ignored for long times. There must be an exponentially larger number of bioactive compounds produced naturally, probably by most types of living organisms; only a fraction of them have been identified to have biological activity in the laboratory.

Many small molecules have been isolated and/or used for other therapeutic purposes, as anticancer or antiparasitic agents, or for animal or plant growth promotion [15] but not much is known about the biology of small molecules; their biosynthesis is complex and the regulation of their production in the cell has been studied in detail in just a few cases. Furthermore, next to nothing is known of their evolutionary biology and ecology.

On the other hand, such communication systems based on the use of small molecules may represent new therapeutic targets towards bacteria and fungi pathogenic to humans. Quorum sensing (QS) is a system of stimuli and responses in relation to bacterial and fungal cell population density that regulates gene expression, including virulence determinants. Consequently, QS is an attractive target for the development of novel anti-infective agents and anti-QS is a promising strategy to combat bacterial infections, as unlikely to develop multidrug resistant pathogens, since does not impose any selection pressure. A number of anti-QS approaches have been documented and plant-based natural products have been extensively studied in this context. Plant

matter is one of the major sources of chemicals in use today in various industries, ranging from the pharmaceutical, cosmetic and food biotechnology to the textile industries. Just like animals and humans, plants are constantly exposed to bacterial infections; it is therefore logical to expect that plants have developed sophisticated chemical mechanisms to combat pathogens.

1.4 Classes and effects

Lipids

Lipids are a large group of natural compounds, which includes fats, waxes, sterols, fat-soluble vitamins (such as vitamins A, D, E and K), monoglycerides, diglycerides, phospholipids, carotenoids and others. Molecules such as fatty acids and their derivatives (including tri-, di- and monoglycerides and phospholipids), sterol-containing metabolites, such as cholesterol, are also grouped as lipids. The main biological functions of lipids include energy storage, structural components of cell membranes and important signalling molecules. Although humans and other mammals use various biosynthetic pathways to both break down and synthesize lipids, some essential lipids cannot be made this way and must be obtained from diet. Interestingly, many papers have discussed the health benefits that can be derived from some of these lipids.

Vitamins

Vitamins are a diverse group of organic compounds essential in trace amounts for the normal growth and maintenance of life. To ensure the adequate intake of vitamins the human diet can be completed with a high range of multivitamin tablets and food products enriched with vitamins, in other words, these compounds are usually administered as nutraceutical or functional ingredient. They are classified as either water-soluble or fat-soluble. In humans there are 13 vitamins: 4 fat-soluble (A, D, E and K) and 9 water-soluble (8 B vitamins and vitamin C). These compounds have diverse biochemical roles. Some have hormone-like functions as regulators of mineral metabolism (e.g. vitamin D), or regulators of cell and tissue growth and differentiation (e.g. some forms of vitamin A). Others work as antioxidants (e.g. vitamin E and sometimes vitamins B and C). The largest numbers of vitamins (e.g. B-complex vitamins) work as precursors of enzyme cofactors.

Proteins, peptides and aminoacids

According to the literature, there are several benefits for the human health that can be derived from protein, peptides and/or aminoacid consumption. They can have antibacterial, antioxidant, immunostimulating, antithrombotic and anti-inflammatory activities, thus could be used for prevention and treatment of hypertension, diabetes and hepatitis among other positive effects in the organism. All these health-promoting propertiess make these compounds of great relevance as nutraceuticals. Proteins, peptides and/or aminoacids are found in a great variety of matrices including animals, fungi, vegetables, cereals, etc. Their identification requires the use of advanced analytical methodologies, due to the complexity of both these substances and samples.

Carbohydrates, glycosides and related compounds

Carbohydrates perform numerous essential roles in living beings. Thus, monosaccharides are the major source of energy for metabolism, while polysaccharides serve for the storage of energy and can act as structural components. Moreover, other beneficial health effects have been linked to these compounds, including their prebiotic effect or antioxidant and antiinflammatory activity.

Phenolic compounds

Under the denomination "phenolic compounds" there are more than 4000 compounds, divided in 12 subclasses. Vegetables, fruits, fungi and some bacteria produce, as part of their secondary metabolism, a wide variety of phenolic compounds. Some of them are highly important for their physiological functions, others are used as a defence from stress situations or to attract or repeal other organisms. In the early 1960s, phenolic compounds were widely viewed as metabolic waste products that were stored in the plant vacuole. Whilst there was interest at that time in their function as flower colorants and in their distribution between plant taxa, the earliest investigations of their biosynthesis had just begun [16]. In foods this kind of compounds acts as pigments, antioxidants, flavor precursors, etc [16, 17] and, nowadays, as part of our diet they have been

associated with several health promoting activities such as: decreasing blood sugar levels, reducing body weight [18], anticarcinogenic [19,20], antiinflamatory, antiaging [21] and antithrombotic activity [22,23]. However, the main activity claimed for phenolic compounds has been as antioxidants. The main difference between bioactive phenolic compounds that can act as nutraceuticals and other phenolic compounds without noticeable bioactivity is their metabolic origin. The first ones are derived from two biosynthetic routes: shikimic acid and/or polyacetates routes [24]. Usually phenolic compounds bind sugars or other phenolic compounds.

A first classification of phenolic compounds could be done as follows [16,17]:

- Benzenediols: the simplest structures, based on the hidroxy phenol;
- Phenolic acids: derived from benzoic acid (C6-C1) or cinnamic acid (C6-C3), when associated as long polymers form tannins and lignans;
- Coumarins: with a basic structure of 2H-1-benzopiran-2-one;
- Flavonoids: with a basic structure of diarylpropane (C6-C3-C6), this group is the widest and includes subfamilies like catechins, flavones, flavonols, flavanones, isoflavonoids and anthocyanes [17].

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Chapter II

Aim of the PhD programme research

1. Products, matrices, samples

1.1 Raw foods

The study and application of natural bioactive substances and nutraceutical framework includes active ingredients, foods naturally rich in bioactive components, up to "designer" — engineered — food. This research is aimed at the analytical investigation of substances with potential beneficial activity in fruits and vegetables, still little studied from the nutraceutical point of view.

In order to provide scientific evidence on the role that active ingredients play in consumers' health (food constituent), it is necessary to address a complete quali-quantitative analysis of their bioactive compounds.

Raw food samples taken into account for this study included:

Citrus fruits and fruit parts

Citrus × *aurantium L.* (bitter orange), white and pink *C.* × *paradisi* Macfad. (grapefruit), *C.* × *aurantifolia* (Christm.) Swingle (lime), *C.* × *bergamia* Risso (bergamot), *C.* × *sinensis* Osbeck (sweet orange), *C. reticulata* Blanco (tangerine), *C.* × *limon* (L.) Osbeck (lemon), *Poncirus trifoliata* (L.) Raf. (trifoliate orange) and *Fortunella japonica* Swingle (kumquat). Some of these fruits had different geographic origins: *C.* × *aurantium* fruits were collected in Bologna, Bari and Cosenza (Italy); *C.* × *paradisi* fruits in Catania (Italy); *C.* × *aurantifolia* fruits in Bari, Cosenza and Palermo (Italy); *C.* × *bergamia* fruits in Reggio Calabria (Italy); *C.* × *sinensis*, *C. reticulata* and *C.* × *limon* fruits in Bari and Bologna (Italy); *P. trifoliata* fruits in Bologna, Ferrara and Catania (Italy); and *F. japonica* fruits from China. All fruits of the same kind were from the same source and at the same apparent ripeness stage in order to evaluate the natural variability of analyte content. Where possible, fruit parts were analysed by isolating the exocarp (flavedo) from the white mesocarp (albedo) and the endocarp (i.e., the "flesh" part, including the juice sacs and segment membranes) with the seeds.

Red fruits

Cherries *(Prunus avium)* were native of Chile and harvested in January; sour cherries (*Prunus cerasus)* from Gradara (PU, Italy) were harvested in July; blackberry (*Rubus ulmifolius)* samples from Trentino Alto Adige (Italy) were also collected in July; red currant (*Ribes alpinum)* and blueberries (*Vaccinium myrtillus*) were both from Trento (Italy); cranberries (*Vaccinium oxycoccus)* samples were from Russia and fox grape (*Vitis labrusca)* was from Vallarsa region (Trento, Italy); black carrots (*Daucus carota* ssp. *sativus* var. *atrorubens*) was bought in a local ethnical store; three commercial brands of dried goji berries (*Lycium barbarum* L.) were bought in specialised stores and an online retail shop.

Sardinia endemic berries

Four types of berries and leaves of plants belonging to endemic plant species of Sardinia: mastic (*Pistacia lentiscus)*, juniper (*Juniperus communis)*, strawberry tree (*Arbutus unedo)* and myrtle *(Myrtus communis*). Samples were collected in November 2014 by natural vegetation in the area of Urzulei (OG, Sardinia, Italy).

1.2 Processed foods and authenticity markers

Fruits and vegetables are well-recognized functional foods however, their beneficial ingredients can be extracted, purified and used as dietary supplements and consumed in concentrated form, or added to a different food product to exert an increased dietary value.

In order to provide a greater amount and variety of functional foods, beside the traditional natural products, food-manufacturing companies are working continuously on the development of novel products. This can either be in the form of modified raw ingredients, e.g. vegetables with an increased amount of phytochemicals, or in the form of adding desired bioactive ingredients to other food. Food fortification is a well-established production method and can be used

in application in numerous products, for example breakfast cereals with added vitamin, minerals or fruit juices fortified with ω-3-fatty acids.

Bioactive substances that can be used as authenticity markers of foods and beverages of natural origin have been investigated in this research, in order to enhance high quality products as well as niche products typical of peculiar geographic areas, under health-promoting and economical points of view.

Processed food samples taken into account for this study included:

Citrus × myrtifolia *and derived commercial soft-drinks*

Citrus × *myrtifolia* (chinotto) fresh fruits were from Savona (Liguria, Italy, 2013). Some of them were used as whole samples and other fruits were peeled, separating the endocarp with the seeds (where present) from the peel. Commercial "Chinotto" soft drinks from seven different manufacturers were bought on the Italian market: five of them reported on the label the presence of Chinotto extracts and infusions (brands 1–5), other two drinks reported the use of "flavours" (brands 6 and 7).

Red fruit-derived commercial liqueur and food supplements

Commercial "Maraschino" (a sour cherry-based distilled liqueur) was bought in a local store. Two dietary supplements were bought in a local pharmacy. One of them was based on quercetin and *Vaccinium myrtillus* extract; the other, produced by a French company, had as its main constituent *Prunus cerasus* extract in combination with B vitamins.

Pomegranate and derived commercial juices

Pomegranates (*Punica granatum*) fruits from the Bologna area were bought in local markets, as well as five commercial pomegranate-based juices: for three of them (brands 1, 2 and 3) the label stated the presence of 100% pomegranate juice with no further additives, while for two more commercial juices the label stated, besides pomegranate juice, also other juices and extracts (in particular, red grape and hibiscus in the first, black carrot and red grape in the second).

1.3 Food and cosmetic by-products

Large quantities of by-products from fruits & vegetables are discarded every year at processing plants resulting from the production, preparation and consumption of food and cosmetic products. These by-products represent a major disposal problem. Food processing residues are those end products of various food processing industries that cannot be recycled or used for other purposes. Industries simply dispose of these by-products by dumping, burning or land filling. This is neither good for the environment, nor is a sustainable behaviour. Serious environmental and public health problems can emerge by these residues, such as water pollution, unpleasant odors, explosions, fires, asphyxiation, vegetation damage and greenhouse gas emissions. Researchers have shown, however, that even residues can have therapeutic properties and thus health-promoting or disease-preventing effects. It has been fund that by-products can be a good source of various compounds with a wide range of pharmacological actions, including antitumor, antiviral, antibacterial, cardioprotective and antimutagenic ones. Thus, it is critical to evidence and emphasize the potential of food and cosmetic processing residues to be used as functional agents in cosmetics, medicines and functional food either as raw material for secondary processes, as operating supplies or as ingredients of new products

In this research work, an investigation and characterisation of bioactive phenolic fractions was carried out in both crude and aqueous extracts derived from argan (*Argania spinosa*) tree leaves as a by-product of argan oil production.

Argan leaves were collected on February 2014 (at the pre-flowering stage of development) in a wild area near to Agadir.

2. Bioactive profiling

Because of their remarkable antioxidant and bioactive potential, the samples and matrices explored in this research work have received increasing attention in the past two decades, especially in Europe and North America. A large number of studies on the physiological functions of chemical constituents of red fruits, citrus fruits and berries have been reported. However, information from systematic investigations on the antioxidant capacity and detailed composition of less known raw fruits and vegetables, and of derived commercial products is often poor.

This study was carried out in response to recent interest in the nutritional and health benefits of the samples taken into account. The objectives were to investigate and compare the total contents of flavonols, coumarins, catechins, phenolic acids, benzoic acids, amino acids and derivatives in whole fruits and fruit parts, in commercial derived products and in manufacture by-products (e.g. leaves).

The research will be helpful for analysing antioxidants from these berries, thus better understanding their health value for consumers, performing quality control, exploiting the bioactive potential of food and cosmetic by-products and also enhancing niche products from localised geographic areas.

3. Analytical approach

High performances liquid chromatography (HPLC) is the most powerful and frequently used technique for the separation of natural, low molecular weight compounds. HPLC offers significant advantages, with respect to other separation techniques, in terms of simplicity, speed, cost (although this would also depend on the type of detection method), sensitivity, specificity and precision.

3.1 Chromatographic separation

HPLC is versatile and adaptable for specific requirements through the use of adequate stationary phases, composition of mobile phases and the possibility to couple with a wide range of selective detectors. In addition, HPLC has the advantage of simultaneous separation and quantification of compounds without preliminary derivatization. However, because of the lack of standard compounds for many target analytes, especially for flavonoid glycosides and the enormous number of different bioactive compounds existing in nature, identification of these compounds is not a straightforward task. In the case of flavonoid glycosides, it has become an accepted practice to hydrolyse them into aglycones before HPLC analysis. A wide range of stationary and mobile phase combinations has been reported in the literature to obtain adequate resolution, since this is considered to be the main difficulty for the separation of flavonoids in a complex mixture. Normal phases (i.e., silica gel columns), currently seldom used, can be considered appropriate for the separation of nonpolar or weakly polar flavonoid aglycones, such as polymethoxylated flavones, flavanones, or isoflavones. Common chromatographic conditions include the use of reversed phase (RP) stationary phases, such as C8, C18 and PFP. and mobile phase mixtures composed of acidic aqueous buffers (acetic, phosphoric, sulfuric, or formic acids) and less polar organic solvents (methanol or acetonitrile).

3.2 Detection techniques

For the analysis of low molecular weight bioactive compounds in natural sources and complex matrices several different detection means can be used, mainly coupled to HPLC systems.

3.2.1 Mass spectrometry (MS, MS/MS, HRMS)

Mass spectrometry (MS) is the state-of-the-art detection technique in the analytical field in general and also in particlur for natural compound determination.

In most cases single-stage MS is used in combination with UV detection, to facilitate identity compound confirmation in a sample, with the help of analytical standards and reference data. For the identification of unknowns, tandem mass spectrometry (MS/MS or MSn) is used. Atmospheric pressure ionization interfaces (APCI) and electrospray ionization (ESI) are used almost exclusively today, both in positive and negative modes; ESI is more frequently used in flavonoid analysis. The composition of LC (gradient) eluent, its pH and the nature of the buffer components added, can have a distinct influence. In flavonoid analysis, the most common additives are acetic acid, formic acid, ammonium acetate and ammonium formate. In LC-MS, sets of flavonoids with the identical aglycon mass, e.g. the glucoside-malonate, the glucoside and the aglycone - also called satellite sets - are easily recognized. Flavonoids are reported to show very reproducible main fragmentation paths, even under different ionization methods and mass analysers. The emphasis lies on the expression “main fragmentation paths,” as the relative abundance of observed fragment ions change significantly between measurements with different instrumentations. Therefore, spectra interpretation should be based on the presence and not on the relative abundance of fragment ions, if using data from the literature, from other mass spectrometers or from standard reference compounds. Concerning LC-MS especially, triple-

quadrupole mass spectrometers are very often employed to screen a high number of samples. In fact, tandem MS allows, in addition to the full scan analysis, proof of identity via multiple reaction monitoring analysis or special investigative approaches like parent ion scan or neutral loss. On the other hand, high-resolution mass spectrometers, like Q-TOF mass spectrometers, are essential for the crucial quali-quantitative investigation in the field of "-omics".

3.2.2 Native fluorescence

Although the number of bioactive compounds that are naturally fluorescent is quite limited, fluorescence detection can provide satisfactory selectivity and sensitivity, allowing effective detection of flavonoids in complex mixtures.

The fluorescence of a particular compound is determined by the nature of the functional groups and their substitution pattern. Among flavonoids, only those that lack a hydroxyl group in C5 show strong native fluorescence. Other flavonoids, such as catechin or flavones with a hydroxyl group in position 3 or methoxylated flavones, have been described to possess native fluorescence. The nature of the functional groups and their substitution pattern determine whether a particular compound is fluorescent or not and sometimes Stokes' shifts can allow a high selectivity over residual fluorescence from the matrix, since it enables the use of distinctive emission wavelengths for detection.

3.2.3 UV-Vis Spectroscopy (UV, DAD)

The most feasible strategy for a preliminary screening of low molecular weight natural compounds is represented by UV-Visible (UV-Vis) spectrophotometry. Flavonoids, for example, have two characteristic absorption bands in the UV-Vis region, with absorption maxima in the 300-550 (Band I) and 240-285 (Band II) nm range, respectively. All flavonoids have an absorption maximum in the wavelength range corresponding to Band II, which is less specific and therefore

less useful than Band I for the their identification. Because of the lack of conjugation between A- and B-rings, flavanols, flavanones and isoflavones hardly show absorption in Band I, whereas anthocyanidins are characterized by the long wavelength position of this band (around 460-560 nm), flavones and flavonols have a peak in Band I in the region of 300-380 nm. Those problems caused by differences in the wavelengths for maximum UV absorption by individual flavonoids can be easly solved by the use of Diode Array Detection (DAD), since it allows simultaneous recording of chromatograms at different wavelengths, thus facilitating the selective detection of different flavonoid groups at their maximum wavelength to improve sensitivity. Although UV-Vis and DAD detections provide useful initial information for flavonoid analysis, the use of these conventional approaches based on UV-Vis spectra is often limited when samples contain very similar compounds. For complete structural identification, MS and MS/MS are quite necessary.

3.3 Sample pretreatment approaches

The analysis of bioactive compounds in natural sources, foods, beverages, and in general in complex matrices, requires adequate sample preparation to ensure sample purification and analyte quantitative extraction, thus avoiding degradation or modification of compound chemical structures. The initial steps for isolation and extraction usually are based on homogenization of solid samples, either fresh or after freezing, drying, or freeze-drying. It is usually advisable to avoid high temperatures that might lead to flavonoid thermal degradation, as well as working with fresh materials, since compounds can be modified during handling procedures, owing to their susceptibility to oxidation or to enzymatic hydrolysis.

Crushing of frozen materials, blending or milling are frequently used strategies for homogenization of plant materials, before solvent extraction. Maceration, stirring, ultrasound-assisted extraction, Soxhlet and reflux extraction are some of the most common processings based on the use of liquid solvents.

Beverages can be freeze-dried before extraction and analysis; sometimes they are directly analysed only after simple, rapid pre-purification steps (filtration or centrifugation). For both beverages and extracts obtained from solid foods, further purification steps are required, especially solid-phase extraction (SPE), microextracion by packed sorbent (MEPS) or micro-solid phase extraction (μSPE).

3.3.1 Preliminary extraction (L/L, S/L)

Liquid-liquid extraction (L/L) and solid-liquid extraction (S/L) can be considered preliminary steps for natural matix pretreatment. The extraction conditions applied to sample preparation can have an important influence on the type of flavonoids isolated, as well as on the extraction yield therefore, factors as the extraction solvent need to be carefully chosen. For example methanol and

methanol-water mixtures are the most commonly used solvents, often slightly acidified with hydrochloric or acetic acid. Alcohols cause cell membrane instability, thus facilitating the extraction of phenolic compounds; besides, they inactivate enzymes like polyphenol oxidase and contribute to increase the stability of the extracted compounds that can be further improved by the presence of antioxidants in the extraction media. Other factors to be considered when setting up extraction procedures are the sample particle size, number of extraction steps, sample-to-solvent ratio and pocess time and temperature. In general, high temperatures should be avoided to prevent labile compound degradation. In the case of beverages, such liquid samples are often treated by L/L or SPE after initial simple treatments like degassing (for gas-containing beverages), concentration under vacuum, filtration or centrifugation.

3.3.2 Sample clean-up

All the steps involved in analytical method development (sampling, extraction, analysis and data processing) greatly influence the whole analytical performance that can be achieved in terms of reliability, accuracy, precision and sensitivity, as well as the time and cost of the analysis. In several cases, over 80% of analysis time is spent on sampling and sample preparation steps, including homogenization, extraction, concentration and clean-up. This is necessary for several matrices, when the analytical technique cannot handle the sample complexity directly. Therefore, sample preparation has been recognized as the main bottleneck of the analytical process, particularly for the analysis of trace components.

In this sense, an ideal sample preparation procedure should include the following features:

- minimal sample loss and maximum recovery of the target analytes;
- elimination of coexisting components with a high yield;
- simple, fast and inexpensive procedures;
- compatibility with the analytical instruments;

- conformity with green chemistry demands.

Microextraction techniques, which use a minimal extractant amount (sorbent or liquid phase) offer these benefits and are becoming widely used in different fields, such as the biomedical, food, forensic and environmental ones.

The recent advances in terms of sample clean-up have converged on the miniaturization and integration of sample preparation with analytical instrumentation, in order to reduce laboratory workload and to increase analytical performance. From this perspective, microextraction techniques have emerged in the last few years, as powerful sample preparation approaches suitable for easy automation in conjunction with chromatographic systems applied in a diversity of bioanalytical areas.

3.3.3 Solid phase extraction (SPE)

Liquid samples or extracts obtained after solvent extraction can be submitted to SPE to eliminate interfering components or to enrich the extract. Solid phase extraction (SPE) is the most commonly used purification and preconcentration technique. Cartridges filled with C18 and C8 RP sorbent and also diol, phenyl and polymeric material allow rapid treatments with excellent recoveries. In all cases, cartridge conditioning, washing and elution steps have to be carefully optimised.

3.3.4 Microextraction by packed sorbent (MEPS)

Microextraction by packed sorbent (MEPS) is a miniaturization of conventional SPE, based on the use of a feasible device that can be connected online to HPLC without any further modifications. MEPS is composed approximately of 1-2 mg of sorbent, packed inside a syringe (100-250 µL) as a plug or between the barrel and the needle as a cartridge. Sample extraction takes place in this packed bed, which can be coated to provide selective and suitable sampling conditions. MEPS approach to sample preparation is suitable for reversed phases (extraction of hydrophobic analytes or polar organic analytes from aqueous matrices), normal phases (extraction of polar analytes from non-polar organic solvents) and mixed mode and ion exchange chemistries (extraction of charged analytes from aqueous or non-polar organic samples). There are several available MEPS sorbent materials, including reversed phase (C18, C8 and C2), normal phase (silica), restricted access material (RAM), HILIC (hydrophilic interaction liquid chromatography), carbon, polystyrene-divinylbenzene copolymer (PS-DVB), molecular imprinted polymers (MIPs), strong cation exchange (SCX) and mixed mode (C8/SCX). The packed sorbent can be used up to 100 times, even when using complex matrices, handling low sample volumes (10-250 µL). The analytes are eluted with small volumes of an organic solvent, such as methanol, or organic solvent mixtures, also granting a significant sample concentration if required. The combination of MEPS and chromatographic techniques, such as HPLC and LC-MS/MS is an excellent tool for the screening and determination of small molecules, biomarkers and bioactive compounds in biological and complex samples.

This approach to sample preparation is therefore very promising for many reasons:

- it is fast and easy to be used;
- it can be fully automated for online procedures;
- it reduces solvent and sample volumes, as well as waste produced;
- analysis costs are minimal when compared to conventional SPE approaches.

Overall, it is one of the most user- and environmental-friendly microextraction techniques available for sample extraction. MEPS is very simple and straightforward, but it nevertheless involves a wide range of optimization steps, to optimize the extraction of the target analytes by favouring a good interaction between analyte and sorbent. This involves pH adjustment (to reduce ionization of weak acids and bases for reversed-phase extraction), sample loading speed adjustment, number of extraction (draw-eject) cycles, washing and elution solvents.

3.3.5 Micro-solid phase extraction (μSPE) and Stop-and-go extraction (StAGE) tips

In recent years, other forms of solid-phase extraction-based clan-up strategies have been developed and used in addition to the above-mentioned MEPS pretreatment. Sometimes, loosely packed SPE microtips (disposable solid-phase extraction, DPX) are used with repeated aspiration-eject cycles of a pipette. The loose structure, however, leads to a relatively low capacity and large elution volume, requires time for diffusion and might result in poor analyte recovery. In addition, it is difficult to remove contaminant particles or precipitates because the sample is loaded and eluted from the same side. Recently, Stop-and-go extraction (StAGE) tips have been developed: they are conceptually similar to SPE cartridges in which teflon embedded chromatographic beads are immobilized inside the tapered ends of pipette tips. StAGE tips allow for high loading capacity, high loading speed, small elution volumes and reproducible production. They are also very economical to produce as thousands of StAGE tips can be produced from a single, readily available teflon membrane containing the separation material of choice. A key property of the disks used for StAGE tips is that the beads are embedded in a teflon matrix and are therefore fixed in place. This prevents the formation of primary flow channels, which is the largest limitation of microcolumns based on loose beads. Furthermore, the teflon matrix allows for the creation of multi-functional columns without the risk that beads of

different functionality will mix at the interface. Disks containing beads with different functionalities, such as C18, C8, strong cation exchange (SCX), strong anion exchange (SAX), activated carbon and poly-(styrene-divinylbenzene) copolymer, can be stacked upon each other as needed.

3.4 Method validation

Validation is defined as the confirmation by examination and provision of objective evidence that the particular requirements for a specified intended use are fulfilled. In order to prove that the method conforms to its objectives, validation must respond to some important questions: Which analytes can be determined? In which matrices? In which concentration range? At which level of accuracy? The goal of method validation is thus to provide documents that will contain a description of the method, the numerical results of a set of experiments and the complete statistical calculations of data to establish the performance characteristics and methodology relevance. At the end of the validation protocol, it is possible to know whether the data are acceptable or not and the major criteria for any laboratory validation are summarized in Table II.3.1.

Table II.3.1. Usually accepted validation criteria

Criterion	Remarks
Scope of the method	Which analytes, which matrices, which equipment?
Response function	Calibration curve/linearity; sensitivity
Limit of detection	The lowest concentration of analyte that can be reliably detected
Limit of quantitation	The lowest concentration of analyte that can be quantitatively determined with an acceptable level of precision
Specificity/selectivity	Interferences/matrix effects
Trueness/accuracy	Reference materials, recovery test, proficiency scheme, alternative method
Precision	Repeatability/intermediate precision/reproducibility
Dosing range	Which concentration range?
Ruggedness/robustness	Capacity of the method to remain unaffected by small variations in operating parameters

There are three further important rules to know regarding method validation:

- The whole method must be validated and not only the final determination step. Also sample-preparation steps, for example, have to be validated.
- Validation must cover the whole range of considered concentrations, since sometimes a method may work very well at high concentration but be inadequate at low concentrations.
- The method must be validated over the whole range of studied matrices.

Chapter III

Development of analytical approaches

1. Quantitative evaluation of auraptene and umbelliferone, chemopreventive coumarins in *Citrus* fruits, by HPLC-UV-FL-MS/MS

1.1 Abstract

An analytical strategy, based on the development of two HPLC methods with spectrophotometric (UV), spectrofluorometric (F) and mass spectrometric (MS/MS) detection, has been developed to investigate the presence of and to quantitate two important chemopreventive coumarins, auraptene and umbelliferone, in foodstuffs.

The analytes were determined in fruits, and fruit parts, of plants belonging to the *Citrus*, *Poncirus* and *Fortunella* genera, to test their nutraceutical potential. The method validation has been carried out according to international guidelines, with good results in terms of precision (RSD < 6.9%) and absolute recovery (>91%).

Application to the quantitative analysis of auraptene and umbelliferone in several kinds of citrus fruits was successful, providing reliable and consistent data. Exploiting three different kinds of detection, the analytical methodology proposed herein has been demonstrated to be sound but versatile, as well as reliable.

Performances and results were compared and always found to be in good agreement among themselves. Thus, this approach is suitable for the identification and simultaneous quantitation of auraptene and umbelliferone in citrus fruits, with the aim of evaluating their nutraceutical potential.

1.2 Introduction

The fruits of many plants belonging to the *Citrus* genus and related genera such as *Poncirus* and *Fortunella*, are rich in health-promoting substances, such as vitamins, folates and fibers. However, other compounds found at lower (and even trace) levels can significantly contribute to the nutraceutical potential of citrus fruits.

Among these compounds, coumarins represent an important class of active substances possessing a range of different biological properties, including anticancer, antioxidant, anti-inflammatory, anticoagulant, antibacterial and analgesic effects [24] In recent years, some of these coumarins have been investigated as possible therapeutic or nutraceutical compounds.

Auraptene (7-((E)-3,7-dimethylocta-2,6-dienyloxy)-2H-chromen- 2-one, Figure III.1.1-a) is the main coumarin, which has been demonstrated to act as an anti-inflammatory, antibacterial and immunomodulatory agent [25] and to have positive effects on cholesterol metabolism [26]. It has been shown to protect rodents against chemically induced carcinogenesis [27,28] and to possess antiproliferative and proapoptotic activities in vitro on cancer cell lines from human hepatocellular carcinoma, colorectal adenocarcinoma and breast adenocarcinoma [27-30]. However, the first trials to test auraptene chemopreventive effects, with substance administration to animals and even human beings, are just starting to appear in the scientific literature [31].

Umbelliferone (7-hydroxychromen-2-one, Figure III.1.1-b) is another interesting coumarin, chemically related to auraptene, well-known for its strong UV absorbance and also seems to possess some potential chemopreventive activity as well, for instance, against hepatocarcinoma in rats [32].

Moreover, both auraptene and umbelliferone have shown interesting antioxidant properties. For example, auraptene suppresses superoxide generation in leukocytes [33], blocks the activation of the NADPH oxidase system [34] and inhibits reactive oxygen species (ROS) formation [35]; umbelliferone decreases lipid peroxidation markers, increases endogenous antioxidant

concentrations in rats [36] and inhibits ROS generation caused by γ radiation exposure [37].

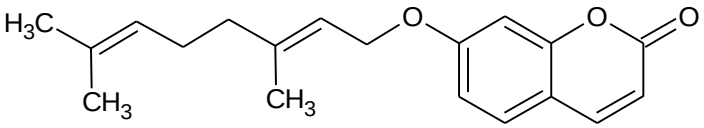
To correctly evaluate the real nutraceutical potential of auraptene and umbelliferone, an analytical approach is necessary, to reliably identify and quantitate both compounds in foodstuffs, such as fruits belonging to different species (or hybrids) of the *Citrus*, *Poncirus* and *Fortunella* genera, and also in their different parts (exocarp or flavedo, mesocarp or albedo, endocarp, seeds). This information could drive agronomic research toward the most promising species and cultivars to be hybridized, in the hope of obtaining fruits with pleasant taste and smell and also containing optimal levels of both coumarins.

In the past few years, much attention has been directed to the analysis of citrus and in particular of coumarins (not including auraptene or umbelliferone) in citrus fruits, with different purposes, for example, for the enantioselective analysis of chiral coumarins and psoralenes [38]; for the control of coumarin absence in bergamot essential oil [39]; for the determination of furocoumarins in citrus products [40]; and for the investigation of supercritical fluid extraction as an alternative to solvent extraction [41]. Several analytical methods based on HPLC can be found in the literature for the analysis of auraptene [42-44] or, alternatively, umbelliferone [45,46] in a variety of citrus plants. These methods are based on HPLC with diode array detection (HPLC-DAD) [42, 44-46] except one that exploits an HPLC-MS technique [43]. Other papers describe the simultaneous determination of both analytes [47-49]. However, of the latter group, two methods analysed auraptene and umbelliferone in animal matrices, that is, rat organs [47] and colorectal cell lines [48] and just one method [49], based on GC-MS, was applied to the original plant matrices, but in particular to a very limited selection of fruits (Egyptian cultivars of red grapefruit and sweet orange).

The aim of this study was the development and comparison of analytical methods, based on HPLC-UV-fluorescence (FL) and LC-MS/MS, for the identification and simultaneous determination of auraptene and umbelliferone in vegetal matrices such as citrus fruits and their parts, to test their nutraceutical potential. Because the analytes possess native fluorescence, the use of

spectrofluorometric detection could give many advantages: it is as feasible and almost as inexpensive as HPLC-UV or HPLC-DAD but grants much higher sensitivity and selectivity; however, none of the available methods is based on this technique. This is the first methodology that exploits and compares three detection means for auraptene and umbelliferone analysis with nutraceutical purposes and, in particular, the only one that features HPLC with spectrofluorometric detection. The availability of different analytical methods allows the choice of the one best suiting specific research needs.

a



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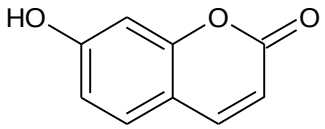

c

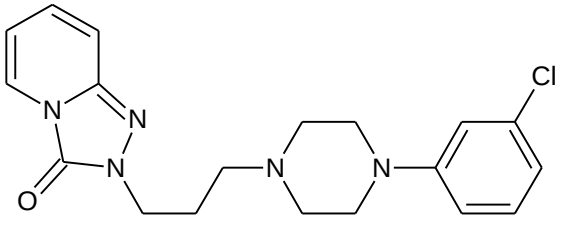

Figure III.1.1

Chemical structures of auraptene (a), umbelliferone (a) and trazodone (IS, c).

1.3 Materials and methods

1.3.1 Apparatus and chromatographic conditions

HPLC-UV-FL

The chromatographic system was composed of a Jasco (Tokyo, Japan) PU-980 isocratic pump, equipped with a Jasco UV-975 spectrophotometric detector and a serially connected Varian (Walnut Creek, CA, USA) 9075 fluorescence detector. The stationary phase was a 100 mm × 3.0 mm i.d., 3 µm, Pack Pro C18 column with a 4 mm × 3 mm i.d. guard column of the same material (YMC, Kyoto, Japan). The mobile phase was composed of a mixture of acetonitrile (60%, v/v) and a pH 2.7, 50 mM, aqueous phosphate buffer (40%, v/v). The flow rate was 0.5 mL/min and the injections were carried out through a 20 µL loop. UV absorbance was monitored at 330 nm and fluorescence intensity was monitored at 390 nm, with excitation at 330 nm.

LC-MS/MS

The chromatographic apparatus was a Waters (Milford, MA, USA) Alliance e2695 system coupled to a Waters Micromass Quattro Micro triple-quadrupole mass spectrometer. Separations were obtained on a 50 mm × 2.1 mm i.d., 1.8 µm, Zorbax Rapid Resolution SB-C18 column with a 4 mm × 2.1 mm i.d. guard column of the same material (Agilent, Waldbronn, Germany), using a mobile phase composed of 0.1% (v/v) formic acid in acetonitrile (A) and 0.1% (v/v) formic acid in water (B), flowing at 0.3 mL/min. The gradient program of the mobile phase composition started with a 5:95 (v/v) A/B ratio and then ramped up linearly to 95% (v/v) of A over 4 min; this ratio was maintained for 2 min and then ramped down linearly to 5% (v/v) of A over 1 min. The injection volume was 5 µL and injections were carried out through the autosampler integrated into the Alliance system.

Tandem mass spectrometry acquisition was carried out in multiple reaction monitoring (MRM) scan mode, using an electrospray ionization source operating in positive mode (ESI+). The working conditions were as follows: ion source

voltage, 3.4 kV; ion source temperature, 120 °C; desolvation temperature, 150 °C; desolvation gas flow, 150 L/h; cone gas flow, 50 L/h; extractor potential, 9 V; RF lens voltage, 0.7 V; collision entrance potential, 1 V; collision exit potential, 2 V; gas cell Pirani pressure, 4.4 × 10−3 mbar. Cone voltage was 24 V for auraptene and 56 V for umbelliferone. Collision energy was 12 V for auraptene and 54 V for umbelliferone. Nitrogen was used as the desolvation gas and was generated from pressurized air by an N_2 LC-MS (Claind, Lenno, Italy) nitrogen generator; collision gas was 99.995% argon (SIAD, Bergamo, Italy). The chosen analyte and IS transitions were m/z 299.4 → 163.1 for auraptene, m/z 163.1 → 77.3 for umbelliferone and m/z 372.9 → 176.1 for the IS. The dwell times were set at 300 ms for each transition.

1.3.2 Chemicals and Solutions

Auraptene was prepared from umbelliferone and geranyl bromide in the presence of K_2CO_3 as the base in acetone at 80 °C for 1 h following the reported methodology [50,51]. The compound was obtained in 95% yield and a purity >98%. Umbelliferone powder (≥98%) was purchased from Fluka (Milan, Italy). Trazodone (≥99.5% purity, 2-{3-[4-(3-chlorophenyl)piperazin-1-yl]propyl}[1,2,4]triazolo[4,3-a]pyridin-3(2H)-one,), used as the internal standard (IS), HPLC grade methanol and acetonitrile, phosphoric acid (85−87%, w/w), monobasic potassium phosphate (≥98%), formic acid (98%, w/w) and 0.1 M sodium hydroxide were manufactured by Sigma-Aldrich (Milan, Italy). Ultrapure water (18.2 MΩ cm) was obtained by means of a Milli-Q apparatus from Millipore (Milford, MA, USA). Stock solutions (1 mg/mL) of the analytes and the IS were prepared by dissolving 5 mg of each pure substance in 5 mL of methanol.

Standard solutions were obtained by diluting stock solutions with the mobile phase (for HPLC-UV-FL) or with methanol (for LC-MS/MS). When stored at −20 °C in the dark, stock solutions were stable for at least 1 month (as assessed by HPLC-UV); standard solutions were prepared fresh every day.

1.3.3 Citrus fruit pretreatment

Samples of fruits belonging to the following species and hybrids were analyzed: *Citrus* × *aurantium L.* (bitter orange), white and pink *C.* × *paradisi* Macfad. (grapefruit), *C.* × *aurantifolia* (Christm.) Swingle (lime), *C.* × *bergamia* Risso (bergamot), *C.* × *sinensis* Osbeck (sweet orange), *C. reticulata* Blanco (tangerine), *C.* × *limon* (L.) Osbeck (lemon), *Poncirus trifoliata* (L.) Raf. (trifoliate orange) and *Fortunella japonica* Swingle (kumquat). Some of these fruits had different geographic origins: *C.* × *aurantium* fruits were collected in Bologna, Bari and Cosenza (Italy); *C.* × *paradisi* fruits in Catania (Italy); *C.* × *aurantifolia* fruits in Bari, Cosenza and Palermo (Italy); *C.* × *bergamia* fruits in Reggio Calabria (Italy); *C.* × *sinensis*, *C. reticulata* and *C.* × *limon* fruits in Bari and Bologna

(Italy); *P. trifoliata* fruits in Bologna, Ferrara and Catania (Italy); *F. japonica* fruits from China.

At least three fruits of each kind were analysed, from the same source and at the same apparent ripeness stage, to evaluate also the natural variability of analyte content. All procedures were carried out using light-absorbing (high-actinic) containers and away from direct light sources. The fruits were individually weighed, then accurately peeled, removing (if possible) only the external, colored exocarp (flavedo); then, the white mesocarp (albedo) was removed, leaving the endocarp (i.e., the "flesh" part, including the juice sacs and segment membrane) with the seeds. The endocarp was cut in small pieces (collecting the juice and the pieces in the same glass bowl) and the seeds (if present) were removed and stored separately. However, it was not possible to separate all fruits in these different parts. The sample pretreatment was the same for all fruit parts: they were weighed, dried to constant weight in a ventilated oven, in the dark at 40 °C and finely ground to a powder. An amount of 100 mg of powder was extracted with 2 mL of methanol, vortexed for 10 min and centrifuged at 1400g for 3 min; the supernatant was separated. The extraction was repeated with the same volume of solvent and the supernatants were combined, dried (rotary evaporator), redissolved in 100 µL of mobile phase, suitably diluted with the mobile phase and injected into the chosen HPLC system. The two coumarin concentrations were obtained by interpolation on the respective calibration curves.

1.3.4 Method Validation

Calibration Curves

Analyte standard solutions at seven different concentrations, containing the IS at a constant concentration, were injected into the HPLC system. The procedure was carried out in triplicate for each concentration. The analyte concentrations were as follows: HPLC-UV, 10, 25, 50, 75, 100, 250 and 500 ng/mL for auraptene and 15, 30, 50, 75, 100, 250 and 500 ng/mL for umbelliferone; HPLC-FL, 1, 5, 10, 50, 100, 250 and 500 ng/mL for auraptene and 2, 10, 20, 50, 100, 250 and 500

ng/mL for umbelliferone; LC-MS/MS, 0.5, 2, 5, 10, 50, 100 and 250 ng/mL for both auraptene and umbelliferone. The analyte/IS peak area ratios (pure numbers) obtained were plotted against the corresponding concentrations of the analyte (expressed as ng/mL) and the calibration curves constructed by means of the least-squares method.

The values of the limit of quantitation (LOQ) and the limit of detection (LOD) (see the Supporting Information) were calculated according to 3rd AAPS/FDA Bioanalytical Workshop guidelines [52], as the analyte concentrations that give rise to peaks of heights of 10 and 3 times the baseline noise, respectively.

Absolute Recovery

Representative fruit part samples (*C.* × *aurantium* mesocarp, *C.* × *paradisi* endocarp and *P. trifoliata* exocarp) were subjected to the extraction procedure and analysed. Then, the same materials were subjected to the extraction and analysis procedure again. The results (analyte/IS peak area ratios) of the first extraction and analysis were compared with the corresponding results obtained from the sum of all extractions. Absolute recovery was considered to be complete when a further extraction, upon injection, produced analyte amounts lower than the LOD.

Precision

A fruit sample was analysed six times within the same day to obtain repeatability (intraday precision) and six times over different days to obtain intermediate precision (interday precision), both expressed as percentage relative standard deviation (RSD%) values.

Accuracy

Method accuracy was tested as follows: known amounts of standard solutions of the analytes and the IS were added to real fruit part samples, which had been already analysed. The added concentrations corresponded to the lower limit, an intermediate value and a high value of the respective calibration curves. The percentage recovery was obtained by comparing the added analyte concentration

to the difference between the total concentration obtained from the analysis and the original analyte concentration.

1.4 Results and discussion

1.4.1 HPLC-UV/F method development

The two analytes possess widely different lipophilicity characteristics, with auraptene (logP = 5.2) being much more lipophilic than umbelliferone (logP = 1.6) due to its geranyl side chain. For this reason, it was decided to use reversed-phase HPLC with a C18 column to simultaneously analyse both compounds within acceptable run times. Using this kind of sorbent and a mobile phase relatively rich in organic modifier (acetonitrile), both analytes and the IS (trazodone) are eluted within 9 min. The corresponding HPLC-UV chromatogram of a standard solution (containing 50 ng/mL of each analyte and 100 ng/mL of the IS) is shown in Figure III.1.2-a. As can be seen, the analytes and the IS are baseline resolved and symmetrical peaks were obtained.

The use of a column with small diameter (3.0 mm) and particle size (3 µm) grants good column efficiency and relatively short run times while also requiring reduced volumes (0.5 mL/min) of mobile phase and, thus, of organic solvents.

Preliminary spectrofluorometric assays ascertained that both analytes are natively fluorescent under the experimental conditions and this characteristic was exploited to obtain a HPLC method with tandem UV-FL detection. The spectrofluorimetric detection grants higher sensitivity and selectivity than UV while avoiding the need for complicated derivatization procedures. The chromatogram of a standard solution (containing 50 ng/mL of each analyte and 100 ng/mL of the IS) obtained by HPLC-FL analysis is reported in Figure III.1.2-b: the increase in sensitivity is apparent from the higher signal-to-noise ratio and the other chromatographic performance parameters remain substantially satisfactory, although significant dead volume, with some efficiency

loss, was introduced into the system when the two detectors were connected in series.

1.4.2 LC-MS/MS method development

With regard to the LC-MS/MS method, due to the extreme selectivity of the technique, a fast, linear composition gradient was set up to further shorten run times, which were thus reduced to 6 min. The chromatogram of a standard solution containing 10 ng/mL of each analyte and 20 ng/mL of the IS is reported in Figure III.1.3-a; again, peaks are symmetrical and complete resolution is achieved; although not strictly necessary in LC-MS/MS, analyte peak resolution is still desirable.

1.4.3 Development of the sample pretreatment procedure

Sample pretreatment was carried out on the dried matrix to reduce the water sample content, which is a source of variability; using the dried sample also greatly simplifies the application of water-miscible solvents. It was verified that all specimens were dry enough to be ground after being treated in a ventilated oven at 40 °C. Higher temperatures were tried to reduce the drying time, but significant analyte losses were observed. Light was also taken into account, because umbelliferone and other coumarins are known to be photolabile [53]; it was found that typical exposure of the analytes to normal laboratory light during sample handling causes a significant loss of umbelliferone. For this reason, all procedures were carried out away from direct light sources and using light-absorbing (high-actinic) containers. A solvent extraction was first tried as the sample pretreatment step, using different solvents and solvent mixtures, such as diethyl ether, ethyl acetate, methanol/ethyl acetate, methanol, methanol/water and water. No pH adjustment was tried, because the analytes do not possess easily ionisable functions. Most solvents proved to be unsatisfactory, due to either unequal extraction of the two analytes or insufficient purification of the extract (detected as interference in the HPLC-UV-FL method and as ionic

suppression in the LC-MS/MS method). However, methanol gave the most promising results: its relatively high hydrophilicity granted similar absolute recovery for both analytes. Extraction assays proved that two steps (with 2 mL of methanol per 100 mg of dried matter) were sufficient to completely solubilize both auraptene and umbelliferone: in fact, the third extraction step with methanol contained negligible analyte concentrations. Some fruit parts contained very high concentrations of the analytes, which would fall outside the calibration ranges if the corresponding extracts were injected as such; for this reason, the most concentrated samples were suitably diluted before injection.

1.4.4 Method validation

The methods were separately validated according to international regulatory guidelines (e.g., those of the U.S. Food and Drug Administration [54] as refined by the third AAPS/FDA Bioanalytical Workshop) [52,55]. Satisfactory linearity values (r^2 > 0.9990) were obtained on standard solutions for all analytes and the complete results for the two methods are reported in Table III.1.1. Absolute recovery and precision assays were carried out on fruit samples and the complete results for the two methods are reported in Table III.1.2. As one can see, mean absolute recovery values were good, always >91% for all analytes (>97% for the IS). Precision results, expressed as RSD, were always <5.6% (2.5% for the IS) for repeatability and <6.9% (4.5% for the IS) for intermediate precision.

Table III.1.1. Calibration data and LOQ and LOD values on standard solutions

Analyte	Method		Calibration range (ng/mL)	Linearity parameters ($y = ax + b$)			LOQ (ng/mL)	LOD (ng/mL)
				a	b	r^2		
Auraptene	HPLC	UV	10-500	0.0336	0.0335	0.9999	10	3
		FL	1-500	0.0292	-0.0547	0.9998	1	0.3
	LC-MS/MS		0.5-250	0.0553	0.0302	0.9992	0.5	0.2
Umbelliferone	HPLC	UV	15-500	0.0204	0.0254	0.9997	15	5
		FL	2-500	0.0162	-0.0108	0.9994	2	0.7
	LC-MS/MS		0.5-250	0.0500	-0.0221	0.9991	0.5	0.2

Table III.1.2. Absolute recovery and precision data for auraptene and umbelliferone in *Citrus* fruit parts.

Compound	Method		Fruit / part	Mean absolute recovery, % [(a)]	Repeatability, RSD% [(a)]	Intermediate precision, RSD% [(a)]
Auraptene	HPLC	UV	*C. x aurantium* / mesocarp	93.4	2.0	2.8
			C. x paradisi / endocarp	92.7	3.2	4.1
			P. trifoliata / exocarp	91.1	5.5	6.5
		FL	*C. x aurantium* / mesocarp	100.8	3.2	3.5
			C. x paradisi / endocarp	99.3	4.8	5.0
			P. trifoliata / exocarp	97.7	5.5	6.8
	LC-MS/MS		*C. x aurantium* / mesocarp	100.5	2.4	3.5
			C. x paradisi / endocarp	100.5	0.4	2.8
			P. trifoliata / exocarp	96.5	4.9	4.7
Umbelliferone	HPLC	UV	*C. x aurantium* / mesocarp	98.0	1.8	2.3
			C. x paradisi / endocarp	98.2	4.0	4.6
			P. trifoliata / exocarp	99.4	4.2	5.5
		FL	*C. x aurantium* / mesocarp	92.8	5.2	6.0
			C. x paradisi / endocarp	93.6	4.3	4.7
			P. trifoliata / exocarp	92.2	5.2	6.7
	LC-MS/MS		*C. x aurantium* / mesocarp	99.0	0.9	3.8
			C. x paradisi / endocarp	98.8	0.5	1.5
			P. trifoliata / exocarp	100.2	2.2	4.4
IS	HPLC	UV	100 ng/mL [(b)]	99.1	1.8	2.0
		FL	100 ng/mL [(b)]	99.3	1.3	3.9
	LC-MS/MS		20 ng/mL [(b)]	97.2	2.4	4.4

[(a)] $n = 6$.

[(b)] IS concentration added to the fruit part sample.

1.4.5 Analysis of real samples

After development and validation, the method was applied to the analysis of the parts of the following *Citrus*, *Fortunella* and *Poncirus* fruits: bitter orange, grapefruit, lime, bergamot, sweet orange, tangerine, lemon, trifoliate orange and kumquat. The results were very satisfactory: the two coumarins were identified and quantitated in all of them. As representative examples, the chromatograms obtained from the analysis of a *P. trifoliata* exocarp (by HPLC-UV), of a white *C.* × *paradise* endocarp (by HPLC-FL) and of a whole *F. japonica* fruit (LC-MS/MS) are shown in panels C and D of Figure III.1.2 and panel B of Figure III.1.3, respectively. As one can see, the matrices are remarkably clean and no evident interference is present. The results obtained with the different proposed methods were always in good agreement: the differences in the concentration estimates were always <7%. The complete data are reported in Table III.1.3. Although the data set was very limited, some simple statistical comparisons (independent two-sample Student t tests with $p < 0.01$) were made and the following results were obtained: (1) the exocarp, mesocarp and endocarp of *P. trifoliata* grown in warm climates contain auraptene levels that are significantly higher than those of all other studied fruit parts; (2) the differences in *P. trifoliata* auraptene content between warm and cold climates are significant; (3) *P. trifoliata* seeds contain significantly lower auraptene levels than the other fruit parts in all cases, without significant differences among climates; (4) *C.* × *aurantium* endocarp contains auraptene levels significantly higher than those of other fruits, excluding *P. trifoliata* (the auraptene content of other parts is not significantly different from that of other fruits); (5) the umbelliferone content of *C.* × *aurantium* exocarp is significantly higher than those of all other fruits and parts (including other *C.* × *aurantium* parts), but no significant difference exists between climates. In absolute terms, the highest concentration of auraptene (>5 mg/g) has been found in the trifoliate orange mesocarp and endocarp (i.e., the "flesh" part). On the other hand, the fruit part that contains the highest levels of umbelliferone (>1 mg/g) is bitter orange endocarp. These concentrations correspond to >10 mg of auraptene in a whole trifoliate orange fruit (mean weight

= 30 g) and >4 mg of umbelliferone in a whole bitter orange fruit (mean weight = 80 g). It seems that warmer climates (Catania vs Bologna, Ferrara) contribute to the high levels of auraptene in trifoliate orange. Interestingly, the analyzed white grapefruits seem to contain much higher coumarin levels than pink grapefruits (about 10 times, $p < 0.001$), although the highest auraptene levels are still much lower than those found in *P. trifoliata* fruits. Of course, these are just preliminary results and it is possible that the analyte concentrations will show different behaviors when a larger variety of samples and/or other kinds of citrus fruits are analyzed.

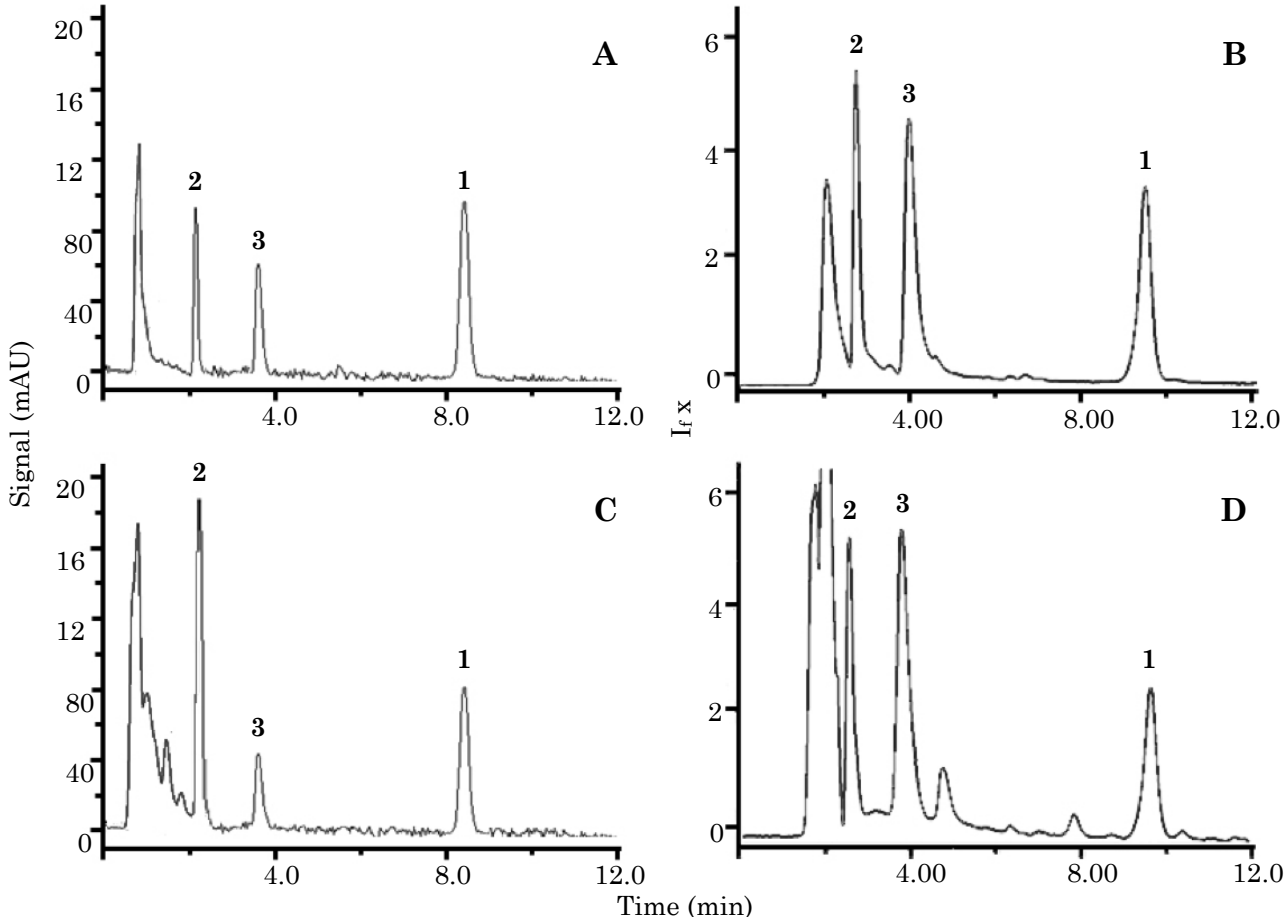

Figure III.1.2

Chromatograms obtained from (A) the HPLC-UV analysis of a standard solution (50 ng/mL of each analyte), (B) the HPLC-FL analysis of a standard solution (50 ng/mL of each analyte), (C) the HPLC-UV analysis of a *P. trifoliata* exocarp sample and (D) the HPLC-FL analysis of a white *C.* × *paradisi* endocarp sample. Peak identification: 1, auraptene; 2, umbelliferone; 3, internal standard.

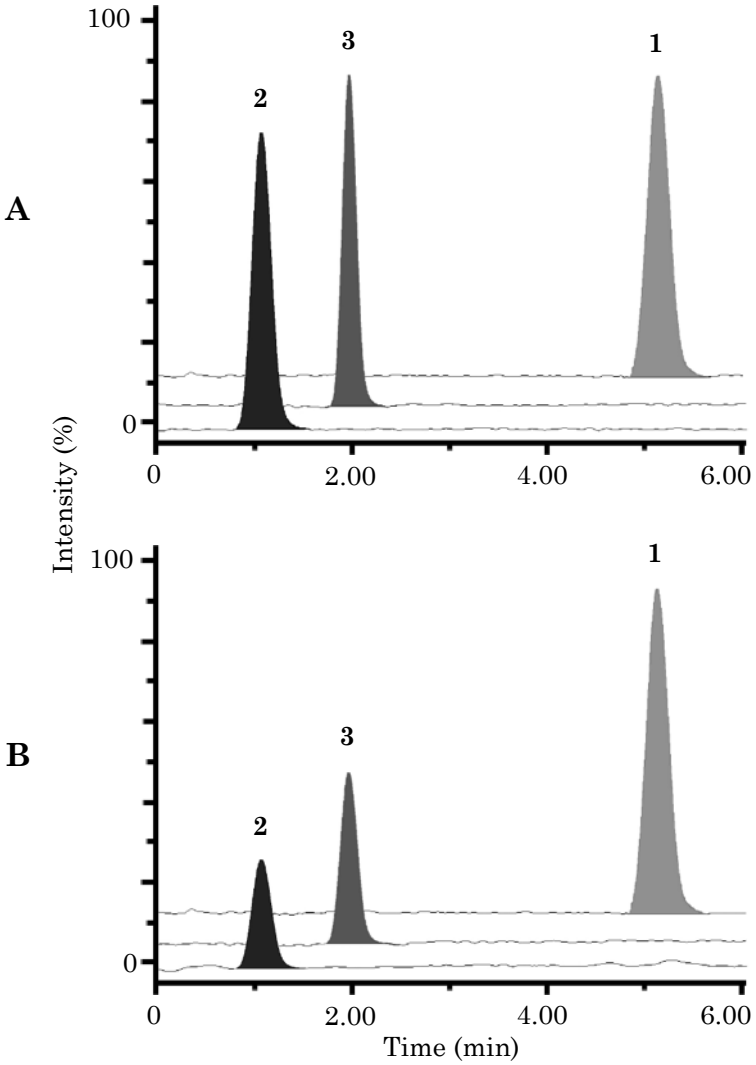

Figure III.1.3

Chromatograms obtained from (A) the LC-MS/MS analysis of a standard solution (10 ng/mL of each analyte) and (B) the LC-MS/MS analysis of a whole *F. japonica* fruit. Peak identification: 1, auraptene; 2, umbelliferone; 3, internal standard.

Table III.1.3. Analysis of different citrus fruits and fruit parts, for auraptene and umbelliferone.

Fruit	Fruit part	Analyte concentration (μg/g) ± SD [a]	
		Auraptene	Umbelliferone
Trifoliate orange (*P. trifoliata*) from Bologna	Exocarp	1211±20	113±4
	Mesocarp + endocarp	1072±21	19±0.8
	Seeds	663±19	12±0.5
Trifoliate orange (*P. trifoliata*) from Ferrara	Exocarp	1247±27	103±6
	Mesocarp + endocarp	1807±33	31±0.1
	Seeds	978±22	14±0.2
Trifoliate orange (*P. trifoliata*) from Catania	Exocarp	3432±59	44±2
	Mesocarp + endocarp	5786±102	29±0.8
	Seeds	980±15	15±0.8
Grapefruit (*C. x paradisi*), white, from Catania	Exocarp	18±1	11±0.6
	Mesocarp	262±11	122±8
	Endocarp	715±12	222±12
Grapefruit (*C. x paradisi*), pink, from Catania	Exocarp	1±0.05	0.8±0.05
	Mesocarp	25±1	17±1
	Endocarp	83±4	190±12
Kumquat (*F. margarita*), from China	Whole fruit	317±24	151±10
Lime (*C. x aurantifolia*) from Bari	Exocarp	243±10	35±1
	Mesocarp + endocarp	6±0.2	3±0.1
Lime (*C. x aurantifolia*) from Cosenza	Exocarp	331±29	44±0.8
	Mesocarp + endocarp	3±0.2	0.7±0.03
Lime (*C. x aurantifolia*) from Palermo	Exocarp	395±33	59±0.9
	Mesocarp + endocarp	8±0.4	5±0.1

(a) Each value is the mean of the results obtained by using the three detection means.

Table III.1.3. Continued

Fruit	Fruit part	Analyte concentration (µg/g) ± SD [(a) (b)]	
		Auraptene	Umbelliferone
Bergamot (*C. x bergamia*) from Reggio Calabria	Exocarp	9±0.5	0.9±0.5
	Mesocarp + endocarp	0.4±0.01	0.2±0.01
Bitter orange (*C. x aurantium*) from Bologna	Exocarp	338±18	31±2
	Mesocarp	244±10	72±3
	Endocarp	1205±66	1038±54
Bitter orange (*C. x aurantium*) from Bari	Exocarp	267±9	14±0.7
	Mesocarp	183±11	89±5
	Endocarp	1184±41	972±40
Bitter orange (*C. x aurantium*) from Cosenza	Exocarp	441±31	50±2
	Mesocarp	363±14	99±5
	Endocarp	1201±41	991±44
Sweet orange (*C. x sinensis*) from Bari	Exocarp	0.3±0.01	0.1±0.01
	Mesocarp	0.03±0.01	0.1±0.01
	Endocarp	0.4±0.01	0.1±0.01
Sweet orange (*C. x sinensis*) from Bologna	Exocarp	3.3±0.1	1.0±0.04
	Mesocarp	0.2±0.01	0.3±0.01
	Endocarp	0.6±0.02	0.2±0.01
Tangerine (*C. reticulata*) from Bari	Exocarp + mesocarp	0.1±0.01	0.1±0.02
	Endocarp	0.2±0.04	0.1±0.04
Tangerine (*C. reticulata*) from Bologna	Exocarp + mesocarp	0.2±0.07	0.2±0.04
	Endocarp	0.2±0.07	0.1±0.03
Lemon (*C. x limon*) from Bari	Exocarp	0.3±0.08	0.1±0.04
	Mesocarp	1±0.02	0.1±0.03
	Endocarp	1±0.02	0.5±0.01
Lemon (*C. x limon*) from Bologna	Exocarp	0.2±0.02	0.2±0.03
	Mesocarp	0.8±0.01	0.4±0.01
	Endocarp	0.5±0.04	0.1±0.01

(a) µg of analyte per g of fruit part.

(b) Each value is the mean of the results obtained by using the three detection means.

1.4.6 Accuracy

Method accuracy was evaluated by means of recovery assays, by adding three different concentrations of each analyte to already analyzed samples and calculating analyte recovery values. Mean recovery values were always between 95 and 105% for all matrices. Thus, method accuracy is satisfactory.

1.4.7 Method comparison

The comparison of performances and results obtained with the proposed methodology gives interesting insight into the options available for the analysis of auraptene and umbelliferone in citrus fruits. In particular, UV detection is feasible and inexpensive, but suitable only when coumarins are present at middle–high concentrations, being limited with regard to sensitivity and intrinsic selectivity. Thus, it could be best used for preliminary assays or when analyte levels are expected (or already known) to be relatively high. Coupling the HPLC method to spectrofluorimetric detection surely grants much better sensitivity and selectivity thanks to the native fluorescence of the analytes. Moreover, the HPLC-FL method retains satisfactory speed because there is no need for complicated and time-consuming derivatization procedures: thus, it could be a good choice for routine analysis and for basic research in this field. It should be noted that this is the first and only methodology that features HPLC-FL for simultaneous auraptene and umbelliferone quantitation with nutraceutical purposes. Finally, the HPLC-MS method granted the best results for most validation parameters and its high sensitivity and selectivity are great advantages in several situations. The fast chromatographic system combined with a fully automated setup achieves higher throughput and outstanding performance, at the cost of higher acquisition and maintenance expenses. The method could be used for advanced research purposes, granting the best results in many respects. Exploiting three different detection means, the developed methodology covers a wide range of needs and economic and scientific possibilities. The two methods, based on HPLC-UV-FL and HPLC-MS, have been

developed and validated for the identification and quantitation of auraptene and umbelliferone in several fruits (and parts) of the *Citrus*, *Fortunella* and *Poncirus* genera having different origins. The sample pretreatment procedure, based on a fast and feasible solvent extraction, grants very good absolute recovery (>91%) and matrix purification; validation assays also provided satisfactory results in terms of linearity and precision (RSD < 6.9%). It should be noted that, to the best of our knowledge, just one GC-MS method [49] is available for the simultaneous analysis of both auraptene and umbelliferone in fruits of two *Citrus* species (not in *Poncirus* or *Fortunella* fruits). With respect to this method, those proposed herein have been applied to many more kinds of fruits and also to their different parts; moreover, being based on three different detection principles, they can provide a wider choice to scientists and analysts. All other published methods either considered just one of the two analytes [42-46] or were not applied to plant materials [47,48]. Application of the proposed methods to real fruits gave interesting results: P. trifoliata fruits grown in warm climates are one of the best sources of auraptene and the highest umbelliferone concentrations are found in *C.* × *aurantium*. Because these fruits are seldom eaten as such, different approaches should be explored (e.g., preparation of processed foods and beverages, such as jams, candies and juices) to take full advantage of their nutraceutical potential. The study of new hybrids with *Citrus* species having more pleasant taste and relatively high coumarins content could also be a viable strategy. The proposed analytical methodology is a significant improvement and useful tool for further research on the nutraceutical properties of citrus fruits and foods prepared from them. The study of possible synergies between the biological activities of the two coumarins when present in the same foodstuff is one example of this kind of research; another example could be the evaluation of the long-term health effects of diets including fruits having a known content of auraptene and/or umbelliferone or the effectiveness of food supplements as sources of these compounds.

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QUALI-QUANTITATIVE EVALUATION OF CHEMOPREVENTIVE COUMARINS IN *CITRUS* FRUITS BY HPLC-UV/F/MS: COMPARISON OF ORIGINAL ANALYTICAL METHODS

Journal of Agriculture and Food Chemistry 61, 1694-1701 (2013) I.F.: 3.107

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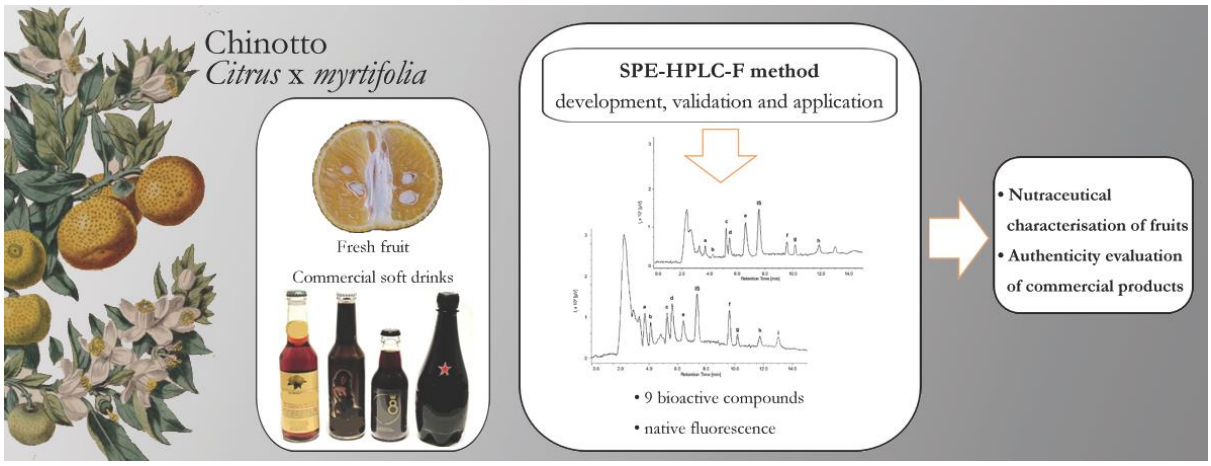
2. Bioactive molecules as authenticity markers of Italian Chinotto (*Citrus* × *myrtifolia*) fruits and beverages



2.1 Abstract

Chinotto (*Citrus* × *myrtifolia*) is an uncommon fruit belonging to the *Citrus* genus, mainly cultivated in small areas of the Italian territory, where the main use concerns the eponymous drink, marketed with the name "Chinotto". The lack of information about this fruit highlights the usefulness of nutraceutical compound characterization, as well as the need to identify genuineness markers in derived commercial products.

An analytical strategy based on SPE-HPLC-F was developed to identify and quantify different bioactive compounds in Chinotto (*Citrus* × *myrtifolia*) fruits and commercial beverages.

The method was fully validated and successfully applied to the analysis of nutraceutical compounds in chinotto fruits of Italian origin and in some chinotto-based beverages, granting reliable and consistent data. The obtained results provided preliminary key information about the bioactive profiling of *Citrus* × *myrtifolia* and proved the suitability of the selected compounds as authenticity markers of derived commercial soft drinks.

2.2 Introduction

Citrus × *myrtifolia* belongs to the *Citrus* genus of the Rutaceae family and is better known as chinotto. It is a tree that can reach a maximum height of three meters, with compact branches and small leaves that resemble those of the common myrtle, hence the Latin name [56]. The flowers are small, white and very fragrant, unripe fruits look like small green aromatic tangerines, while mature ones assume a very deep orange color with bitter and sour pulp. This fruit is native to China and has been imported in 1500 to Savona (Italy) where now is grown mainly on the Ligurian coast, but also in Calabria and Sicily. From the extract of *Citrus* × *myrtifolia* fruit a commercial carbonated soft drink, with a bitter taste and digestive properties, is produced and known with the eponymous name of "Chinotto". The drink has Italian origins and is considered a niche product, consumed mainly in this country, since the 50s. "Chinotto" soft drink is produced like a classic soda, but none of the producers describes in detail its preparation nor its recipe. To a small erextent, *Citrus* × *myrtifolia* fruits are used in the Italian confectionery industry to prepare jams and candies, while its essential oil is used in perfumery.

It is known that citrus fruits are rich in different phytochemicals, which all contribute to determine a high nutraceutical potential: several studies have shown that these compounds possess biological properties such as antioxidant, anti-inflammatory and analgesic effects, anti-viral and anti-bacterial activities and even anti-thrombotic, neuroprotective and anti-tumoral properties [57-71].

The most popular citrus fruits have long been the subject of scientific studies: some recent researches aim to investi-gate molecules belonging to the coumarin class in *Citrus*, *Poncirus* and *Fortunella* genera [72]. Further works concern quantitative determination of adrenergic amines in *Citrus aurantium* fruits [73].

Despite the increasing prevalence of the popular soft drink "Chinotto", just a few papers can be found in the scientific literature taking into consideration the qualitative and quantitative analysis of bioactive compounds in *Citrus* × *myrtifoli*a fruits [74-79] and namely tyramine derivatives [75]; flavonoids and furocumarins in fresh fruits, homogenate and juice [76-78] and betaines [79].

Furthermore, derived commercial products are not taken into account, as well as the test of their authenticity through identification of suitable markers.

The aim of this research is the development of an analytical method for the identification and the simultaneous determination of bioactive compounds in *Citrus* × *myrtifolia* fruit to test its nutraceutical potential and in derived commercial beverages, in order to verify their genuineness. This is the first methodology addressed to the determination of nutraceutical compounds in fresh Citrus × myrtifolia fruits and concurrently to the issue of authenticity investigation of derived "Chinotto" soft drinks. For this purpose, bioactive substances are studied as genuineness markers to verify the actual natural derivation of the extracts used to produce beverages. This approach could enhance product quality, or otherwise detect adulteration (addition of low quality or harmful substances), sophistication (food component alteration) or fraud (replacement of product components with respect to the ones stated in the label).

2.3 Materials and methods

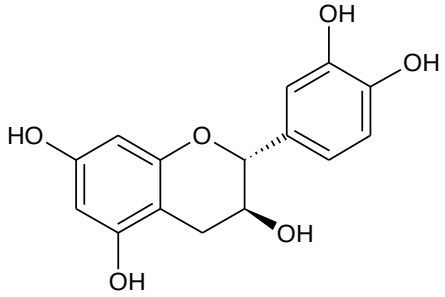
2.3.1 Chemicals

Pure powders of all the analytes (catechin, caffeic acid, epicatechin, ferulic acid, vanillin, umbelliferone, myricetin, quercetin and auraptene, Fig. III.2.1) and venlafaxine, used as the internal standard (IS), HPLC-grade methanol and acetonitrile, phosphoric acid (85–87%, w/w) and monobasic potassium phosphate (≥98%) were supplied by Sigma-Aldrich (Milan, Italy). Ultrapure water (18.2 MΩ cm) was obtained by means of a Millipore MilliQ apparatus (Milford, MA, USA). Stock solutions at the concentration of 1 mg/mL of the analytes and IS were prepared by dissolving suitable amounts of each pure substance in methanol and then diluted with the HPLC mobile phase to obtain the standard solutions. When stored at −20◦C in the dark, stock solutions were stable for at least one month, while standard solutions were prepared every day.

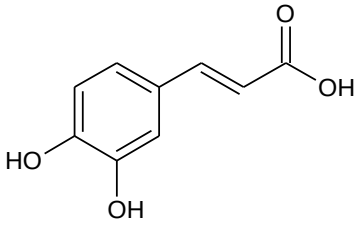
2.3.2 Analytical system and experimental conditions

Analysis was performed by means of a High Performance Liquid Chromatography system consisting of a Jasco (Tokyo, Japan) PU-2089 Plus chromatographic pump and a Jasco FP-2020 Plus fluorimetric detector (HPLC-F). The stationary phase was a Waters (Milford, Mass., USA) Sunfire C18 (100 mm × 4.6 mm i.d.; 3.5 μm) column. The mobile phase was composed of a mixture of 50 mM, pH 3.0 phosphate buffer (65%), acetonitrile (25%) and methanol (10%). All analyses were carried out using a linear flow gradient program, starting with a constant flow of 0.5 mL/min up to 8 min, then linearly ramping up to 0.9 mL/min over 9.5 min. This flow was then maintained until the end of the chromatographic run. Each injection had a volume of 20 μL. Fluorescence intensity was monitored at two different pairs of wavelengths: 330-390 nm for catechin, caffeic acid, epicatechin, ferulic acid, vanillin, umbelliferone and auraptene and 366-525 nm for myricetin and quercetin. Data processing was handled by means of a Jasco ChromNAV1.16 software.

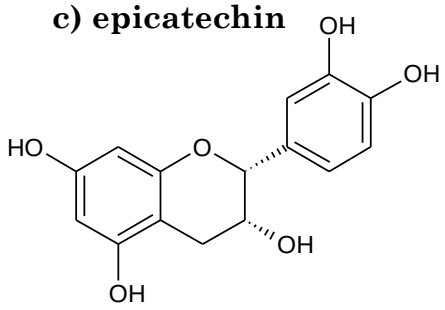
a) catechin



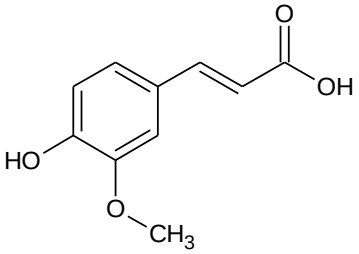
b) caffeic acid



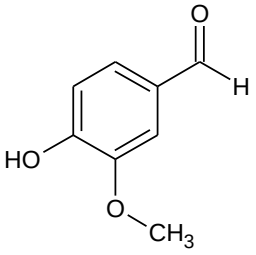
c) epicatechin



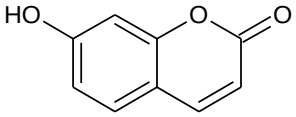
d) ferulic acid



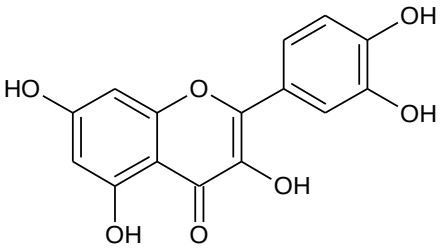
e) vanilllin



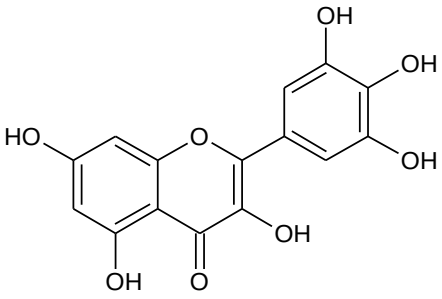
f) umbelliferone



g) myricetin



h) quercetin



i) auraptene

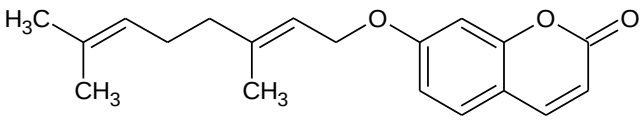


Figure III.2.1

Chemical structures of catechin (a), caffeic acid (b), epicatechin (c), ferulic acid (d), vanillin (e), umbelliferone (f), myricetin (g), quercetin (h) and auraptene (i).

2.3.3 Fruits and commercial beverages

The *Citrus* × *myrtifolia* fresh fruits were from Savona (Liguria, Italy, 2013). Some of them were used as whole samples and other fruits were peeled, separating the endocarp with the seeds (where present) from the peel. The sample pretreatment was the same for whole fruits and all fruit parts: they were weighed, dried to constant weight at 40°C and finely ground to a powder. An amount of 500 mg of powder was extracted with 10 mL of methanol, vortexed for 10 min and centrifuged at 1400 × g for 3 min; the supernatant was separated. The solid residue was subjected to two further extractions with the same volume of solvent and the supernatants were combined, dried under vacuum and re-dissolved in 10 mL of methanol.

Solid phase extraction (SPE) procedure was performed on a Macherey-Nagel (Düren, Germany) Chromabond® vacuum apparatus, using Biotage (Uppsala, Sweden) Isolute C2 cartridges (50 mg,1 mL), activated with 3 mL of methanol and then conditioned with the same volume of ultrapure water. To 500 µL of methanolic extract, 500 µL of ultrapure water and 10 µL of IS solution were added and the resulting mixture was loaded onto a cartridge. The cartridge was then washed with 2 mL of ultrapure water, 2 mL of a water/methanol mixture (80/20, v/v), with 100 µL of methanol and finally eluted with 1 mL of methanol. The eluate was dried under vacuum, redissolved with 500 µL of mobile phase and injected in tothe HPLC-F system. Commercial "Chinotto" soft drinks from seven different manufacturers were bought on the Italian market: five of them reported on the label the presence of Chinotto extracts and infusions (brands 1–5), other two drinks reported the use of "flavours" (brands 6 and 7). Beverages were filtered on 25 mm Ø, 0.45 µm pore size nylon syringe filters supplied by Sigma Aldrich (Milan, Italy), then suitably diluted with the mobile phase and injected into the HPLC-F system.

2.3.4 Validation

Linearity

Analyte standard solutions at seven different concentrations, containing the IS at a constant concentration, were injected in to the HPLC-F system. The procedure was carried out in triplicate for each concentration. The analyte/IS peak area ratios (pure numbers) obtained were plotted against the corresponding concentrations of the analyte (expressed as ng/mL) and the calibration curves constructed by means of the least-square method. The values of limit of quantitation (LOQ) and limit of detection (LOD) were calculated according to 3rd AAPS/FDA Bioanalytical Workshop [54,55] guidelines, as the analyte concentrations which give rise to peaks whose heights are 10 and 3 times the baseline noise, respectively.

Absolute recovery

Citrus × *myrtifolia* samples were subjected to the pretreatment procedures and analyzed. Then, the same materials were subjected to the pretreatment and analysis procedure again. Absolute recovery was considered complete when a further extraction, upon injection, produced analyte amounts lower than the LOD. The results (analyte/IS peak area ratios) of the first extraction were then compared with the corresponding results obtained from the sum of all extractions. Absolute recovery on beverage samples were evaluated by subjecting standard solutions of the analytes at known concentrations to the filtration procedure. The analyte peak areas were compared with those obtained by injecting standard solutions at the same theoretical concentrations and absolute recovery values were calculated.

Precision

Precision assays were carried out on standard solutions and real samples. Standard solutions of the analytes at three different concentrations, corresponding to a low, an intermediate and a high value of the linearity ranges and containing IS at a constant concentration were analyzed six times within the same day to obtain intraday precision and six times over six different days to obtain interday precision, both expressed as percentage relative standard deviation (RSD%). Similarly, fruits and commercial beverages were analyzed six times within the same day to test intraday precision and six times over six different days to obtain interday precision.

Accuracy

Method accuracy was tested by means of percentage recovery assays, adding known amounts of standard solutions of the analytes and the IS to fruits and commercial soft drinks, which had been already analyzed.

2.4 Results and discussion

2.4.1 Development of the analytical method

Preliminary tests were performed using a reversed phase C18 column as the stationary phase and a mobile phase composed of 50 mM, pH 2.7 phosphate buffer and acetonitrile at a 50/50 (v/v) ratio, using a constant flow rate of 0.5 mL/min, exciting at a wave-length of 330 and monitoring the emission at 390 nm.

Different wavelengths, namely 366 nm (excitation) and 525 nm (emission) were also tested. The optimized conditions were reached using a mobile phase consisting of 50 mM, pH 3.0 phosphate buffer, acetonitrile and methanol in the ratio 65/25/10 (v/v/v) and applying a linear flow gradient, starting with a constant flow of 0.5 mL/min up to 8 min, then linearly ramping up to 0.9 mL/min over 9.5 min. Fluorescence intensity was monitored at 390 nm exciting at 330 nm forcatechin, caffeic acid, epicatechin, ferulic acid, vanillin, umbelliferone and auraptene and at 525 nm exciting at 366 nm for myricetin and quercetin. Different compounds (Ziprasidone, Fluoxetine, Trazodone, Mirtazapine and Venlafaxine) with physicochemical properties comparable to those of the analytes were tested as potential internal standards (IS) and Venlafaxine has proved to be the best choice due to its chromatographic behavior and fluorescence response.

2.4.2 Sample pretreatment

Since the analyses were carried out on complex matrices, the development of a sample pretreatment procedure was necessary to avoid interferences. For this reason it was chosen a triple methanol extraction followed by solid phase extraction (SPE), which allows to purify the matrix, granting also good absolute recovery of the analytes. The method involves an initial washing of the fruit with ultrapure water and the subsequent complete drying to constant weight in a ventilated stove at 40°C. In particular, some fruits were dried as whole and others were peeled, separating the endocarp with the seeds from the peel and dried separately.

An amount of 500 mg of dried matrix was weighed and subjected to three subsequent extractions with 10 mL of methanol each. After filtration, the combined extracts were brought to dryness, redissolved in 10 mL of methanol and stored at −20°C before analysis.

An aliquot of 500 µL of the methanolic extract were loaded onto a C2 (50 mg, 1 mL) cartridge and subjected to the SPE procedure.

SPE development started by testing different sorbents such as:C1 (50 mg, 1 mL), C2 (50 mg, 1 mL), C8 (50 mg, 1 mL), C18 (100 mg,1 mL) and phenyl (PH, 50 mg, 1 mL). The best results were obtained using C2 cartridges, which provided high absolute recovery, implementing the following procedure: SPE cartridge was activated with 3 mL of methanol and then conditioned with the same volume of ultrapure water. To 500 µL of methanolic extract, 500 µL of ultrapure water and 10 µL of IS solution were added and the resulting mixture was loaded onto a cartridge. The cartridge was then washed with 2 mL of ultrapure water, 2 mL of a water/methanol mixture (80/20, v/v), with 100 µL of methanol and finally eluted with 1 mL of methanol. The eluate was dried under vacuum, redissolved with 500 µL of mobile phase and injected into the HPLC-F system.

2.4.3 Validation results

Method linearity was performed on standard solutions of the analytes at seven different concentrations, containing IS at a constant concentration (10 ng/mL). The resulting linearity ranges were 5–200 ng/mL for caffeic acid, 2.5–250 ng/mL for ferulic acid, 5–250 ng/mL for vanillin, 0.5–150 ng/mL for catechin, epicatechin and auraptene, 1–100 ng/mL for umbelliferone, 2.5–150 ng/mL for myricetin and 1.5–150 ng/mL for quercetin. Satisfactory linearity values (r^2 > 0.9971) were obtained on standard solutions for all analytes. LOD and LOQ values were found to be 2 and 5 ng/mL for caffeic acid and vanillin, 0.9 and 2.5 ng/mL for ferulic acid and myricetin, 0.2 and 0.5 ng/mL for catechin, epicatechin and auraptene, 0.4 and 1 ng/mL for umbelliferone and 0.5 and 1.5 ng/mL for quercetin, respectively. The complete linearity data are reported in Table III.2.1.

Absolute recovery and precision assays were carried out on fruit samples and commercial beverages and the complete results are reported in Table III.2.2. As one can see, mean absolute recovery values were good, always higher than 86% for all analytes (>93% for the IS).Precision results, expressed as RSD%, were always lower than6.3% for all analytes (<1.3% for the IS) for intraday precision and lower than 7.1% (<2.5% for the IS) for interday precision.

Table III.2.1. Linearity on standard solutions

Analyte	Linearity range (ng/mL)	Linearity parameters				LOQ (ng/mL)	LOD (ng/mL)
		$y = ax + b$		Linearity correlation coefficient (r^2)	Residual standard deviation		
		a	b				
Auraptene	0.5-150	0.5044	-1.2083	0.9998	0.91	0.5	0.2
Umbelliferone	1-100	0.5693	-0.5645	0.9994	1.71	1	0.4
Quercetin	1.5-150	1.1078	-0.5338	0.9984	2.70	1.5	0.5
Myricetin	2.5-150	1.1097	0.9714	0.9979	3.07	2.5	0.9
Caffeic acid	5-200	1.272	-0.6588	0.9988	2.14	5	2
Ferulic acid	2.5-250	1.106	-0.5248	0.9972	3.50	2.5	0.9
Catechin	0.5-150	0.5905	-1.3776	0.9995	2.18	0.5	0.2
Epicatechin	0.5-150	0.5864	0.6207	0.9997	1.19	0.5	0.2
Vanillin	5-250	0.4384	-1.7007	0.9975	3.36	5	2

Table III.2.2. Absolute recovery and precision on *Citrus* × *myrtifolia* whole fruits and "Chinotto" soft drinks

Compound	*Citrus* × *myrtifolia* whole fruit [a]			"Chinotto" soft drinks [b]		
	Mean extraction yield, % [c]	Intraday precision, RSD% [c]	Interday precision, RSD% [c]	Mean extraction yield, % [c]	Intraday precision, RSD% [c]	Interday precision, RSD% [c]
Auraptene	89	4.5	5.1	95	4.1	4.4
Umbelliferone	91	4.9	5.8	96	4.4	5.1
Quercetin	88	2.7	6.9	93	2.2	5.9
Myricetin	88	3.4	6.6	94	3.0	5.9
Caffeic acid	89	4.0	6.4	94	3.0	5.6
Ferulic acid	90	4.9	6.2	95	4.5	5.8
Catechin	87	3.3	6.1	91	2.9	5.7
Epicatechin	89	6.2	6.9	93	5.4	6.3
Vanillin	90	4.7	7.0	95	4.3	6.2
IS	94	1.2	2.4	95	1.1	2.2

(a) Each value is the mean of the results obtained from five fruits.

(b) Each value is the mean of the results obtained from five brands (1-5).

(c) n = 6.

2.4.4 Analysis of chinotto samples

The validated method has been applied to the analysis of *Citrus* × *myrtifolia* fruits from the area of Savona (Liguria, Italy, 2013) and of "Chinotto" commercial soft drinks of seven different Italian brands. In particular, five of them reported on the label the presence of Chinotto extracts and infusions (brands 1–5), the other two reported the use of "flavours" (brands 6 and 7).

Whole fruits were initially analyzed and subsequently fruit pulp and peel were analyzed separately, in order to determine exactly where the analytes were more concentrated. An example of a chromatogram obtained from the analysis of a whole chinotto fruit sample subjected to the SPE procedure and injected into the HPLC is shown in Fig. III.2.2-a. Quantitative results regarding the analyzed *Citrus* × *myrtifolia* samples are shown in Table III.2.3 and expressed as micrograms of compound contained in a gram of dried sample.

The collected results showed that *Citrus* × *myrtifolia* fruit contains all nine active compounds and that those present in greater amounts are caffeic acid, ferulic acid and vanillin, which are present both in the exocarp and in the endocarp in very similar concentrations. Moreover, significantly higher concentrations of quercetin were found in the peel of the fruit when compared to the pulp and the whole fruit; lower concentrations of myricetin were determined in the endocarp and in the exocarp. With regard to catechins, they were found at comparable levels in all fruit parts. Finally, concerning coumarins levels, auraptene has been found in slightly higher concentrations with respect to umbelliferone and mainly in the fruit peel. Comparing these data with those obtained in our previous work on coumarins, these levels are in good agreement with those found in citrus fruits such as sweet orange (*C.* × *sinensis*), tangerine (*C. reticulata*) and lemon (*C.* × *limon*) [72]. These are preliminary results and it is possible that the concentrations of the nutraceutical compounds show different behaviors when analysing a wider variety of samples, from different cultivars or from different climatic and geographic zones.

The method has also been applied to the analysis of commercial "Chinotto" beverages from seven different Italian manufacturers. Since five of these

companies claim in the product label the presence of Chinotto fruit extract or infusions (brands 1–5), the analysis has been extended to soft drinks in order to assess whether the bioactive substances detected in fruits were also present and their levels. Thus, these bioactive compounds were used as authenticity markers to evaluate beverage composition. A chromatogram obtained from the analysis of a "Chinotto" soft drink sample (Brand 2, which reported on the label the presence of Chinotto natural extract), is shown in Fig. III.2.2-b. Quantitative results regarding the beverage samples are reported in Table III.2.3 and expressed as micrograms of compound contained in one milliliter of sample.

The collected results showed that the analytes detected in fresh fruits were also present in beverages, thus confirming that the soft drinks actually contain chinotto fruit extract. In particular, even in beverages, the compounds found in greater amounts were ferulic acid and vanillin; on the other hand, caffeic acid was present at lower concentrations when compared to fruit. The levels of myricetin and quercetin found in beverages are comparable to those found in *Citrus* × *myrtifolia*, when analyzed as whole fruit, while catechins are present in slightly lower concentrations when compared with those found in different fruit parts. Finally, the levels of the two coumarins were found to be the lowest among the analytes taken into account, similarly to the fruit. The analysis was performed also on two samples from commercial "Chinotto-flavored" soft drinks where no use of natural extracts, but only of "flavours" was mentioned in the label (brands 6 and7). An example is shown in the chromatogram reported in Fig. III.2.2-c, where one can note that none of the analytes was detected; it is thus evident *that Citrus* × *myrtifolia* fruits have not been used in product manufacturing.

The obtained results show how the quali-quantitative determination of bioactive compounds can give key information concerning the nutraceutical potential of *Citrus* × *myrtifolia* fresh fruit and fruit parts. Furthermore, analyses prove that the same compounds can be used as authenticity markers of commercial products stating on label genuine fruit extracts and infusions, as opposed to for beverages declaring only the use of "flavours".

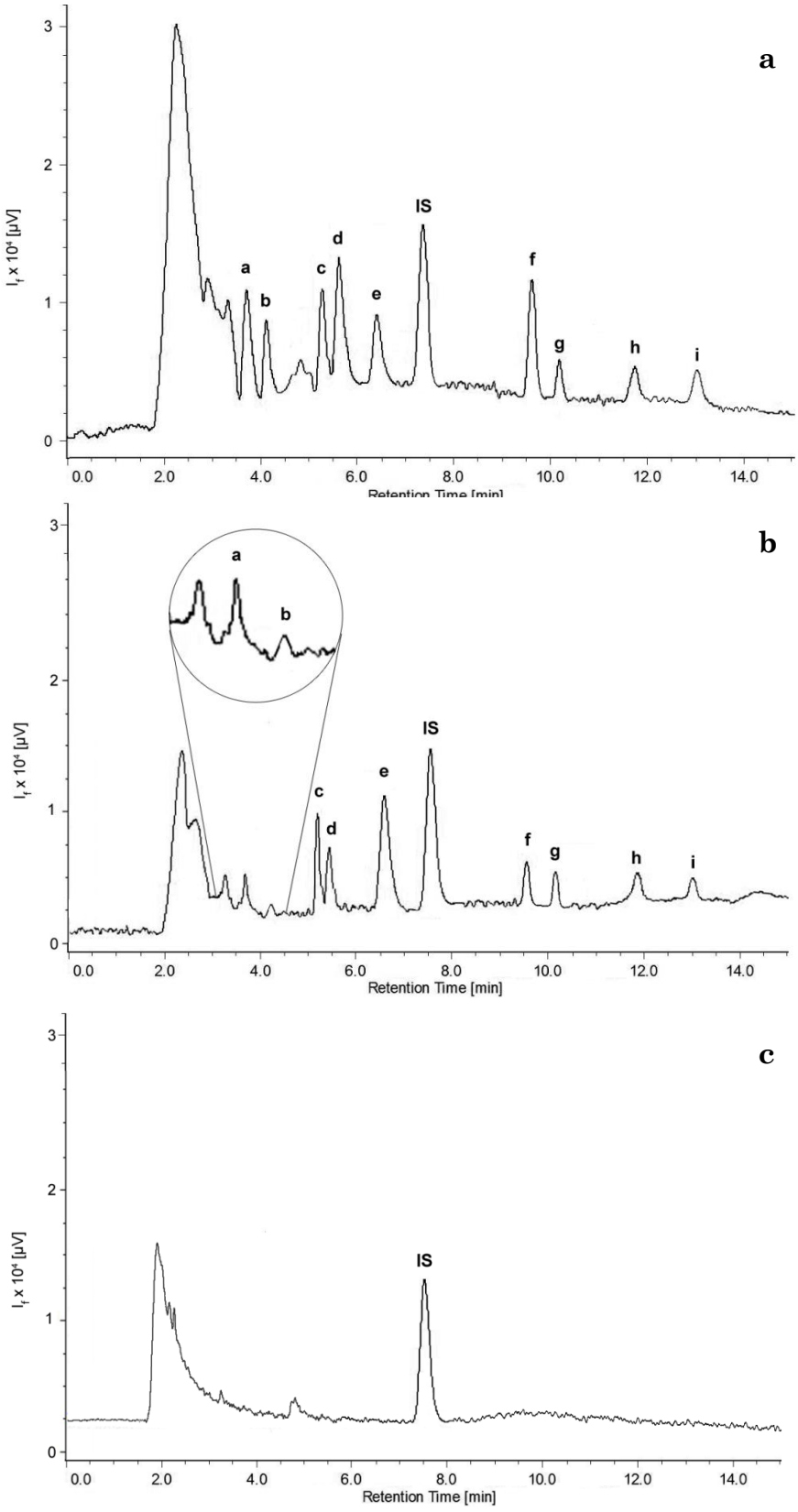


Figure III.2.2

Chromatogram of (a) a whole *Citrus* × *myrtifolia* fruit from Savona (Liguria,Italy, 2013); (b) a "Chinotto" commercial soft drink (brand 2) prepared from *Citrus* × *myrtifolia* fruit extract (as reported on the label); (c) a "Chinotto-flavoured" commercial soft drink (brand 6, no natural extract reported on the label).

Table III.2.3. Analysis of *Citrus* × *myrtifolia* fruit parts and "Chinotto" soft drinks.

Compound	*Citrus* × *myrtifolia* fruit: concentration (μg/g) ± SD [a,b]			"Chinotto" soft drinks: concentration (μg/mL) ± SD [b]				
	Whole fruit	Exocarp	Endocarp	Brand 1	Brand 2	Brand 3	Brand 4	Brand 5
Auraptene	0.60 ± 0.04	0.71 ± 0.03	0.21 ± 0.03	0.08 ± 0.01	0.09 ± 0.01	0.10 ± 0.02	0.12 ± 0.02	0.10 ± 0.01
Umbelliferone	0.21 ± 0.02	0.32 ± 0.02	0.11 ± 0.02	0.05 ± 0.01	0.05 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.06 ± 0.01
Quercetin	2.23 ± 0.04	5.14 ± 0.04	1.79 ± 0.03	2.01 ± 0.08	0.96 ± 0.06	1.99 ± 0.06	2.52 ± 0.06	2.34 ± 0.08
Myricetin	3.84 ± 0.05	1.91 ± 0.04	1.18 ± 0.05	3.51 ± 0.07	3.90 ± 0.06	4.21 ± 0.04	3.09 ± 0.05	4.03 ± 0.03
Caffeic acid	7.42 ± 0.06	3.34 ± 0.07	4.84 ± 0.10	1.53 ± 0.05	1.34 ± 0.05	1.18 ± 0.04	0.98 ± 0.04	1.95 ± 0.06
Ferulic acid	9.78 ± 0.09	5.88 ± 0.07	5.14 ± 0.09	7.34 ± 0.0	7.02 ± 0.08	5.24 ± 0.07	6.92 ± 0.05	5.78 ± 0.06
Catechin	0.72 ± 0.05	0.39 ± 0.03	0.31 ± 0.03	0.41 ± 0.03	0.39 ± 0.03	0.22 ± 0.03	0.19 ± 0.03	0.34 ± 0.03
Epicatechin	0.58 ± 0.05	0.31 ± 0.03	0.23 ± 0.03	0.39 ± 0.08	0.23 ± 0.03	0.32 ± 0.03	0.18 ± 0.03	0.13 ± 0.02
Vanillin	9.70 ± 0.08	6.24 ± 0.08	7.74 ± 0.07	5.34 ± 0.06	5.88 ± 0.05	6.63 ± 0.05	7.35 ± 0.06	4.34 ± 0.05

(a) Each value is the mean of the results obtained from the analysis of five fruits.

(b) n = 6

2.5 Conclusion

An original methodology based on SPE-HPLC-F has been developed and validated for the simultaneous analysis of nine bioactive compounds in *Citrus* × *myrtifolia* (Chinotto) fruits. The developed method was validated in terms of linearity, precision, absolute recovery and accuracy, with good results for all analytes, thus demonstrating reliability and suitability of this analytical strategy. After validation, the method was successfully applied to the analysis of samples of Chinotto fruits from the area of Savona (Liguria, Italy, 2013) and seven brands of "Chinotto" soft drinks from the Italian market. In particular, five of them reported on the label the presence of genuine Chinotto extracts and infusions, the other two reported the use of "flavorings". Examining the obtained results, it can be stated that auraptene, umbelliferone, quercetin, myricetin, catechin, epicatechin, caffeicacid, ferulic acid and vanillin can be considered good markers for the quality monitoring of the entire production chain, from fresh fruits to the commercial beverages and to investigate possible product adulteration, sophistication or fraud. Moreover these results provide preliminary key information about this still little-studied fruit, essential for its nutraceutical profiling, thus suggesting that *Citrus* × *myrtifolia* raw fruits and beverages are good sources of phytochemicals and may therefore provide health benefits. Nowadays these aspects are considered to be highly valuable for the commercial valorization of Chinotto as a fruit with high nutraceutical potential.

2.6 Acknowledgements

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The preliminary results obtained during this research were presented as a contribution to symposium:

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PROPRIETÀ NUTRACEUTICHE DEL CHINOTTO: ANALISI DI CUMARINE E ALTRI MARKER

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BIOACTIVE MOLECULES AS AUTHENTICITY MARKERS OF ITALIAN CHINOTTO (*CITRUS* × *MYRTIFOLIA*) FRUITS AND BEVERAGES

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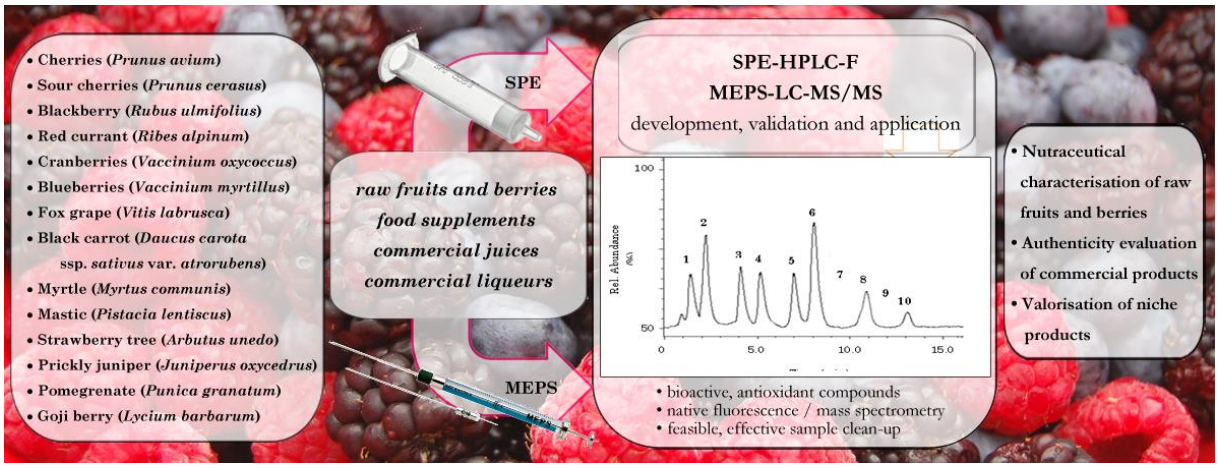
3. Antioxidant (poly)phenolic profile of niche and common raw foods and derived beverages by means of HPLC-F and LC-MS/MS coupled to SPE and MEPS sample pretreatment



3.1 Abstract

Several experimental, epidemiological and clinical studies highlighted how diet plays a major role in the prevention of chronic degenerative diseases. Therefore, great interest has been addressed in recent years towards the use and research of antioxidants obtained from natural sources (such as fruit, vegetables, berries and roots). For this reason, the interest of producers and consumers for products of this kind considerably escalated.

This research deals with the development of original analytical methods based on liquid chromatography coupled to spectrofluorimetric detection (HPLC-F) and tandem mass spectrometry (LC-MS/MS) for the simultaneous determination of several antioxidant and bioactive, phenol-derived compounds in various types of fruits, vegetables, berries and products derived from them, such as drinks and food supplements.

Due to the complexity of the matrices taken into account, effective sample pretreatment strategies based on solid phase extraction (SPE) and microextraction by packed sorbent (MEPS) have been developed and optimized *ad hoc* in order to obtain sound and reproducible analytical results.

After being validated according to international guidelines, methods have been applied to a consistent set of real samples of different nature and origin. Some of them are everyday consumption foods such as cherries, blueberries, red currant and pomegranate; others, such as cranberries, goji berries and black carrots, required a more careful search since they are better known in eastern countries; other still are simply more difficult to find on the market (sour cherry, fox grape).

Another important group of samples covered endemic plants and berries of the Mediterranean basin and particularly of Sardinia, such as myrtle, mastic, strawberry tree and prickly juniper. Where available, analysis was extended to derived products such as fruit juices, liqueurs and supplements, in order to deepen their knowledge from the nutraceutical perspective and investigate correlations between their bioactive profiles.

3.2 Introduction

Nowadays, only a part of the world population has access to medical care and healthcare system drugs. According to the World Health Organisation (WHO), more than 25% of the population in industrialized countries and more than 80% in developing countries take care of themselves with natural remedies. Most of them resort to plants and folk medicine extracts, which were the basis of medical sciences until a few decades ago, set aside with the subsequent advent of synthetic drugs.

Ethnopharmacological studies have provided, especially in the last decade, significant results for the search of active ingredients derived from fruits, berries, herbs, roots and this has increased the interest in manufacturers and consumers for products of this kind. For this reason, also in the pharmaceutical field, commercial interest has grown towards natural extracts. In this scenario, a wide range of bioactive compounds with antioxidant activities found in plant matrices seems to play an important role, confirmed by both clinical and epidemiological studies.

3.2.1 Red fruits and berries

The term "red fruit" brings together a broad class of foods, linked by the characteristic red color, of which the best known are: cherries (*Prunus avium*), sour cherries (*Prunus cerasus*), strawberries (*Fragaria vesca*), raspberries (*Rubus idaeus*), blackberry (*Rubus ulmifolius*), of the *Rosaceae* family; red currant (*Ribes alpinum*) of the *Grossulariaceae* family; cranberries (*Vaccinium oxycoccus*), blueberries (*Vaccinium myrtillus*) of the *Ericaceae* family. Moreover, fox grape (*Vitis labrusca*), otherwise known as Isabella grape, of the *Vitaceae* family, sports the characteristic deep red color.

It is known that such fruits are rich in a wide range of phytochemicals such as anthocyanins, the main compounds responsible for the color, flavonols (e.g. quercetin), flavanols (e.g. catechin and epicatechin), tannins or proanthocyanidins, hydroxycinnamic acids (e.g. caffeic acid and ferulic acid) and

hydroxybenzoic acids, but also vitamin A, C, E, folic acid and minerals. Several studies have shown that these compounds, particularly flavonoids, are endowed with bioactive properties such as antioxidant, antitumor [80], antiangiogenic [81], antibacterial (in particular against *E. coli* responsible for urinary tract infections [82] and against *Staphylococcus* and *Salmonella*, mainly responsible for intestinal infections [83]), anti-neurodegenerative and anti-inflammatory [84].

3.2.2 Pomegrenate

Pomegranate (*Punica granatum*) is considered to be originated in Iran and has been cultivated and naturalized over the whole Mediterranean region since ancient times. It has generated great interest in recent years and its consumption has been related to the decrease in the risk of certain diseases, including atherosclerosis and prostate cancer [85,86]. Pomegranate seeds are often consumed raw, being the edible part of the fruit; the juice is a popular drink in Middle Eastern cuisine and since the early '00s started to be widely distributed also among Western countries. With growing interest in the nutritional properties of this fruit, an increasing number of products derived from pomegranate are now being marketed. For example, several commercial juices are now available to consumers, although they contain different amounts of raw fruit. The composition in bioactive compounds is both complex and unique, thus being suitable as a fingerprint for quality control purposes. Moreover, the polyphenolic bioactive composition of pomegranate-derived products is strongly influenced by the cultivar, agronomical and climate conditions, harvest time and juice extraction methods.

3.2.3 Black carrot

Long before the orange carrot became established in the 16th century, the black carrot (*Daucus carota* ssp. *sativus* var. *atrorubens*) existed across Asia and eastern Mediterranean. The black carrot is still grown and consumed in countries such as Turkey, Egypt and India. It shows an attractive bluish-purple colour conferred by the high anthocyanins levels and its extracts can serve as a natural food dye, because of their high heat, light and pH stability. Today black carrot extracts are used in juices, confectionery and soft drinks and as a non-toxic alternative to synthetic dyes.

In recent years, several studies have shown that phenolic compounds and anthocyanins display a wide range of biological activities, including antioxidant, anti-inflammatory and anti-carcinogenic activities. They display also a variety of effects on blood vessels, platelets and lipoproteins, able to reduce the risk of coronary heart diseases. For these reasons, a complete evaluation of the content of these compounds is critical to understand the nutraceutical power of this ancient vegetable.

3.2.4 Goji berry

The Goji Berry is the fruit of *Lycium barbarum* and *Lycium chinense*, two very closely related species in the *Solanaceae* family, native of southeastern Europe and Asia.

Goji is one of the most interesting plants in Chinese herbal medicine. It is considered a national treasure in Tibet, where it is known as “the key to eternal youth”, thanks to its significant antioxidant properties. It is often cultivated for a variety of food and beverage applications within China, but increasingly today for export as dried berries, juice and pulp or powders. The wide-ranging properties of the Goji Berry can be explained by its composition: it is extremely rich in carotenoids, Vitamins C, B1, B2 and other vitamins, minerals, antioxidants and amino acids. Various studies have shown that Goji fruits possess numerous beneficial properties, including, antioxidant, immuno-potentiating,

antimutagenic, hypoglycaemic, hypolipemic, hypotensive, all of which contribute to the slowing down of the aging process.

In order to fully understand the composition of these potentially beneficial fruits, it is very useful to develop innovative analytical methods, able to provide reliable measurements of their content in bioactive substances.

3.2.5 Sardinian niche plants and berries

Endemic plant species of Sardinia are more than 200 and represent approximately 10% of the total flora, although this number is expected to gradually grow in response to the most recent phylogenetic and taxonomic studies [87]. Environmental isolation and the presence of geographical and biological barriers prevented the dispersion and diffusion of several species and has led to a genetic and morphological differentiation in response to the environmental conditions, providing uniqueness to Sardinian flora. As regards Sardinian popular tradition, the use of more than 800 plants has been documented, on humans and animals, either as medicinal products or in association with religious practices [88]. 397 of about 2800 species found in Sardinia are susceptible to pharmacological interest or are recognized as medications [89] and 20 are strictly officinal and recognized by the Official Italian Pharmacopoeia (F.U.I.) [90]. For this reason, the exploitation of these species has to be seen in a framework taking into account their potential use in many areas, ranging from new discoveries in the field of pharmacology; fine food productions; also new market opportunities and innovative products could find a common point in such plants.

For this purpose, four Sardinian endemic species have been chosen as a model of the study, e.g. myrtle (*Myrtus communis*) mastic (*Pistacia lentiscus*), strawberry tree (*Arbutus unedo*) and prickly juniper (*Juniperus oxycedrus*). The knowledge of the biological and nutraceutical traits of these plants represents an essential step to allow the development of their full potential. However, the available scientific information on their chemical and nutritional properties is

currently fragmented and therefore the need to deepen the studies and knowledge on the subject is increasingly high.

3.2.6 Aim of the research

In this study, liquid chromatographic methods coupled to spectrofluorimetric detection (HPLC-F), exploiting the native fluorescence of some analytes and to mass spectrometry (LC-MS/MS) has been developed and validated to simultaneously determine some significant representative antioxidant compounds belonging to the following classes: flavonols (quercetin, myricetin, apigenin, kaempferol, hesperetin, rutin, myricitrin, hesperidin), catechins (catechin, epicatechin), phenolic acids (caffeic, ferulic, coumaric and chlorogenic acid), benzoic acids and derivatives (vanillic, ellagic and gallic acids, vanillin), amino acids and derivatives (tryptophan, serotonin, melatonin).

Due to the complexity and heterogeneity of the matrices taken into account for this study, pretreatment procedures have been carefully developed, based on solid phase extraction (SPE) and novel and miniaturized variants like microextraction by packed sorbent (MEPS). The methods have been optimized specifically in order to ensure the best results in terms of analyte absolute recovery and purification of the sample from potential interfering compounds arising from matrices, with an eye to feasibility, effectiveness, use of small samples quantity and minimum organic solvent volumes. After being fully optimised and validated, methods have been applied to a significative set of real samples of different nature and origin. Such samples consisted in fruits, berries an plant parts (e.g. leaves) belonging to the species mentioned above with the aim to investigate their bioactive profile. Where available, analyses were extended to commercial and handmade products such as fruit juices, liqueurs and supplements in order to promote high-quality products and to valorise niche products typical of peculiar geographic areas.

3.3 Materials and methods

For the study presented here, two different analytical systems and pretreatment procedures have been developed, optimized and fully validated. In short, for the analysis of red fruits, Sardinian niche plants and berries, black carrot and their derived products, an analytical system consisting of high performance liquid chromatography coupled to spectrofluorometric detector (HPLC-F) has been used, taking advantage of the native fluorescence possessed by several of the compounds taken into account and a pre-treatment procedure based on solid phase extraction (SPE) has been optimized for matrix clean-up.

For the quali-quantitative analysis of bioactive compounds in pomegranate, goji berries and commercial products derived from them, a chromatographic system coupled to triple quadrupole mass spectrometry (LC-MS/MS) has been exploited and analyses were preceded by a pretreatment procedure based on microextraction by packed sorbent (MEPS).

3.3.1 Chemicals and Solutions

Pure powders of all the analytes (quercetin, myricetin, apigenin, kaempferol, rutin, quercitrin, myricitrin, catechin, epicatechin, caffeic, ferulic, coumaric, chlorogenic, vanillic, ellagic and gallic acids, tryptophan, serotonin, melatonin, resveratrol, zeaxanthin, ß-cryptoxanthin, ß-carotene and lutein) were supplied by Sigma-Aldrich (Milan, Italy), as well as HPLC-grade methanol and acetonitrile, phosphoric acid, formic acid, triethylamine and monobasic potassium phosphate. Ultrapure water was obtained by means of a Millipore MilliQ apparatus (Milford, MA, USA). Stock solutions at the concentration of 1 mg/mL of all the analytes were prepared in methanol and then diluted with mobile phase to obtain standard solutions. Stock solutions were stored at −20◦C in the dark while standard solutions were prepared every day.

3.3.2 HPLC-F and LC-MS/MS apparatus and conditions

HPLC-F

Analysis was performed on a Jasco (Tokyo, Japan) PU-2089 Plus chromatographic pump and a Jasco FP-2020 Plus fluorimetric detector. The stationary phase was a Phenomenex (Torrance, USA) Kinetex PFP (50 x 2.10 mm I.D., 2.6 µm) column, kept at room temperature. Mobile phase was composed of a mixture of 40 mM, pH 3.0 phosphate buffer containing 0.1% triethylamine (75%), acetonitrile (20%) and methanol (5%). All analyses were carried out using a linear flow gradient program, starting with a constant flow of 0.2 mL/min up to 8 min, then linearly ramping up to 0.4 mL/min over 9.5 min. This flow was then maintained until the end of the chromatographic run. Each injection had a volume of 20 µL. Three different pairs of wavelengths have been exploited for the simultaneous determination of analytes (λ1ex = 330 nm, λ1em = 390 nm; λ2ex = 366 nm, λ2em = 525 nm; λ3ex = 356 nm, λ3em = 450 nm), following a multi-wavelength automatic program (Table III.3.1).

Table III.3.1. HPLC-F multi-wavelength program

Wavelength	Analyte
λ_{ex} = 330 nm λ_{em} = 390 nm	Caffeic acid
	Vanillin
	Ferulic acid
	Melatonin
	t-Resveratrol
	c-Resveratrol
λ_{ex} = 366 nm λ_{em} = 525 nm	Myricetin
	Quercetin
λ_{ex} = 356 nm λ_{em} = 450 nm	Chlorogenic acid
	Hesperidin
	Rutin
	Apigenin

LC-MS/MS

Separations were obtained on a Waters XTerra MS C18 column (100 × 2.1 mm I.D., 3.5 µm), maintained at room temperature and equipped with a guard column. The mobile phase was a mixture of 0.1% aqueous formic acid (A) and acetonitrile (B), flowing at a constant rate of 0.3 mL/min. The gradient program of the mobile phase started with A:B (97:3, v:v), then ramped up to A:B (72:28, v:v) over 4.5 min; this ratio was maintained for 6 min, then ramped down to A:B (97:3, v:v) over 1.5 min and maintained for 2.5 min (Figure III.3.1) .

Multiple reaction monitoring (MRM) transitions acquired in negative ionisation mode (ESI-) were used. MS/MS parameters were optimised for the interested ions via direct infusion of each analyte. The optimised parameters were as follows: ion source voltage, 2.8 kV; ion source temperature, 110°C; desolvation temperature, 250°C; desolvation gas flow, 550 L/h; extractor potential, 2.4 V; collision exit potential, 1 V. Nitrogen was used as desolvation gas, while argon was used as collision gas. Precursor and product ions, with dwell time, cone voltage and collision energy, were optimised for each analyte.

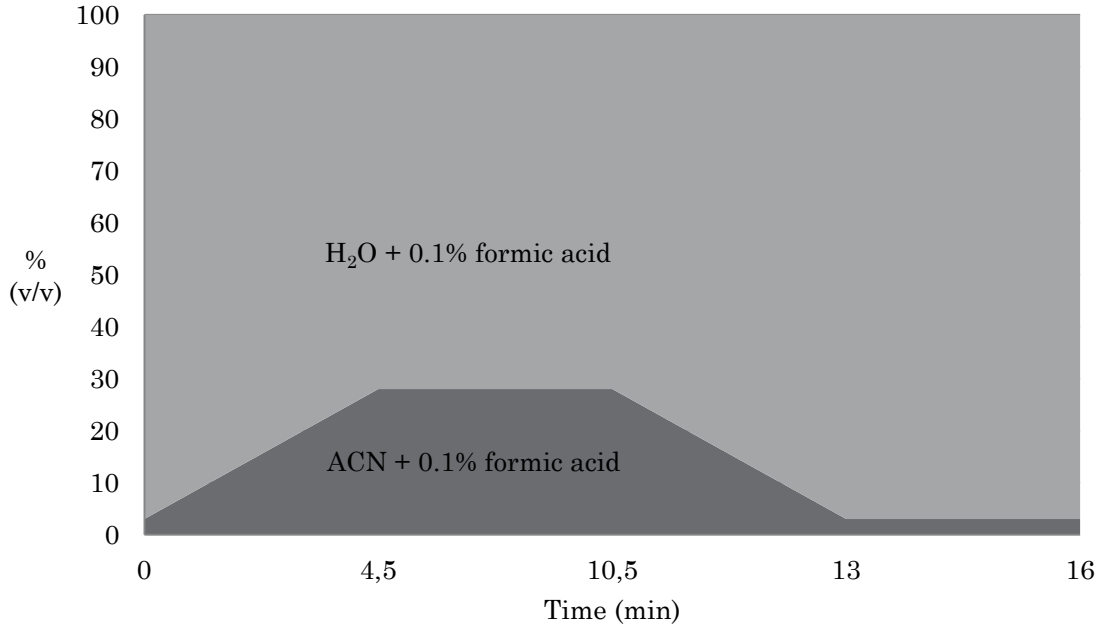


Figure III.3.1

LC-MS/MS mobile phase composition gradient program

3.3.3 Raw foods and derived product pretreatment

Preliminary procedures

A first fragmentation of solid matrices has been obtained with the aid of knife/scissors. Drying procedure was performed under mild conditions and in the dark, in order to avoid alteration (oxidation, photochemical and thermal degradation) of compounds to be extracted. The drying conditions were set to 40 °C, using a ventilated oven until constant weight. Samples were then crushed in a mortar in order to reduce dimensions to small particles

Extraction procedure was as follows: 500 mg of dried matrix were extracted with 5 mL of a methanol/water mixture (80/20, V/V), under magnetic stirring for 15 minutes. The supernatant was separated from the solid by filtration and dried under reduced pressure by means of a rotary evaporator, while the solid was subjected to two more extraction/drying steps. The dry residue of the combined extracts was redissolved in 5 mL of methanol. After filtration, extracts were stored at −20°C before sample clan-up procedures and analysis.

Beverages were filtered on 25 mm Ø, 0.45 µm pore size nylon syringe filters before being directly subjected to the solid phase extraction procedures.

As regards food supplements, the contents of a capsule was weighed, finely ground in a mortar, added with 2 mL of methanol, stirred in an ultrasonic bath for 15 minutes and subsequently centrifuged for 5 minutes at 4000 rpm. The supernatant solution was then filtered on 25 mm Ø, 0.45 µm pore size nylon syringe filters before being subjected to the solid phase extraction.

Solid phase extraction (SPE)

SPE procedure was performed using Agilent (Waldbronn, Germany), Bond Elut Phenyl (PH) cartridges (50 mg,1 mL), activated with 3 mL of methanol and then conditioned with the same volume of ultrapure water. To 500 µL of loading solution, 10 µL of IS and 500 µL of ultrapure water were added and the resulting mixture was loaded onto a cartridge. The cartridge was then washed with 1 mL of ultrapure water, 1 mL of a water/methanol mixture (90/10, v/v), with 50 µL of pure methanol and finally eluted with 1 mL of methanol. The eluate was dried under a gentle nitrogen steam, redissolved with 500 µL of mobile phase and injected into the HPLC-F system

Microextraction by packed sorbent (MEPS)

MEPS procedure was performed on a 100-µL HPLC syringe with a removable needle, fitted with a BIN (Barrel Insert and Needle) containing a PH (phenyl) sorbent. The sorbent was activated with 300 µL of methanol and then equilibrated with 300 µL of ultrapure water. The loading solution was a mixture of 100 µL of loading solution, 100 µL of ultrapure water and 5 µL of IS solution; the loading mixture was drawn into the syringe and discharged back 10 times. The sorbent was washed with 100 µL of water, 100 µL of a water/methanol mixture (90/10, V/V) and then eluted by drawing and discharging 500 µL of methanol. The eluate was dried under vacuum, re-dissolved with 100 µL of mobile phase and injected into the LC-MS/MS system.

3.4 Results and discussion

3.4.1 Chromatographic condition optimisation

HPLC-F

Preliminary tests were performed using reversed phase C8 and C18 columns and a mobile phase composed of 50 mM, pH 2.7 phosphate buffer and acetonitrile at a 80/20 (V/V) ratio, using a constant flow rate of 0.3 mL/min, exciting at a wavelength of 330 nm and monitoring the emission at 390 nm.

Then, a Kinetex PFP (pentafluorophenyl, 50 x 2.10 mm I.D., 2.6 µm) column was tested as stationary phase and showed a strong selectivity and resolving power for the considered compounds. It was therefore chosen as the column for all subsequent analysis. Then, the addition of a small percentage of methanol to the mobile phase showed an improvement in system resolution.

The optimized conditions were reached using a mobile phase consisting of 50 mM, pH 3.0 phosphate buffer, acetonitrile and methanol in the ratio 65/25/10 (v/v/v) and applying a linear flow gradient, starting with a constant flow of 0.5 mL/min up to 8 min, then linearly ramping up to 0.9 mL/min over 9.5 min.

Different wavelengths, namely 330, 356 and 366 nm for excitation and 390, 450 and 525 nm for emission were successfully tested, thus a multi-wavelenght automatic program was applied.

LC-MS/MS

LC conditions, including column and mobile phase composition, were optimised to achieve the simultaneous separation of the selected bioactive compounds. XTerra MS C18 column (100 x 2.1 mm I.D., 3.5 µm) was selected to obtain the best efficiency and resolution. Various mobile phases (0.1-0.2% formic acid-methanol, 0.1-0.2% formic acid-acetonitrile, 0.1-0.2% acetic acid-methanol and 0.1-0.2% acetic acid-acetonitrile). 0.1% formic acid-acetonitrile was then used as the mobile phase. In order to obtain short retention times, analyte resolution and separation from residual matrix components, a composition gradient elution was carefully developed, flowing at a constant rate of 0.3 mL. Injection volume

was 10 µL. Under these conditions, a good separation was achieved and a complete chromatographic run took less than 16 minutes.

3.4.2 Pretreatment procedure development

The first pretreatment step included a thorough washing of the sample with ultrapure water in order to remove potential contaminants from the external environment, followed by drying in a ventilated stove in the dark at 40 °C until constant weight, best compromise between a complete sample dehydration without analyte thermal degradation. Solid samples were previously chopped, so as to optimize dehydration. Subsequently, the dry matrix was further milled in a mortar to increase the contact surface of with the extraction solvent, weighted (500 mg) and subjected to extraction. In order to maximize analyte extraction, several solvents (methanol, acetonitrile, water) and mixtures were tested in different proportions. Among all the tested combinations, the best results in term of absolute recovery and matrix purification have been obtained with three extraction cycles with 5 mL of a methanol/water mixture (80:20, v/v), under magnetic stirring for 15 minutes. Extracts were then pooled, brought to dryness and reconstituted with 5 mL of methanol, prior to SPE/MEPS procedures.

For HPLC-F analysis, 500 µL of the obtained extract were mixed with 500 µL of ultrapure water and loaded onto phenyl-type sorbent cartridge (PH SPE, 50 mg, 1 mL) that allowed to satisfactorily purify the matrix obtaining a satisfactory extraction. Cartridges of different nature were tested such as: CN (50 mg, 1 mL), C2 (50 mg, 1 mL), C8 (50 mg, 1 mL), Oasis-HLB (50 mg, 1 mL) and phenyl (PH, 50 mg, 1 mL). By comparing such assays, it was established that the best results were obtained using PH cartridges, which ensured higher absolute recovery and a better purification of the extracts. A careful optimization of the washing and elution steps was carried out to obtain the best results in terms of sample clean-up, minimising analyte losses. The optimized procedure then included washings with 1 mL of ultrapure water, 1 mL of a water/methanol mixture (90/10, v/v) and with 50 µL of methanol; then, elution with 1 mL of methanol was performed. The

eluate was dried under a gentle nitrogen stream, reconstituted in 500 µL of mobile phase and injected into the HPLC-F system.

For LC-MS/MS, 100 µL of loading solution were mixed with 100 µL of ultrapure water before MEPS procedure. Performance of different kinds of sorbents such as C2, C8, C18, PH and mixed mode (C8+SCX) was evaluated in terms of extraction efficiency and reproducibility. Mixed mode, C2 and PH sorbents provided the best absolute recovery in comparison with C8 and C18; however PH sorbent was chosen for the MEPS procedure because also provided better results in terms of purification from interfering compounds. The main steps of the MEPS procedure (loading, washing and elution) were optimised: retention was satisfactory after 10 draw/discharge cycles of the loading mixture at a speed of 5 µL/s. Different pure solvents and mixtures were tested for the washing and elution steps: good purification was obtained by washing the sorbent with 100 µL of ultrapure water followed by 100 µL of a methanol/ultrapure water 90/10 (v/v) mixture at a speed of 10 µL/s; an elution step with 500 µL of pure methanol at a speed of 5 µL/s proved to be sufficient for the complete elution of the analytes. The eluate was dried under vacuum and redissolved with 100 µL of mobile phase prior injection into LC system.

3.4.3 Method validation

Standard solutions were prepared at seven different concentrations for each analyte in order to set up a calibration curve for each analyte using the least squares method. Limit of quantification (LOQ) and limit of detection (LOD) values for each analyte were calculated according to international, respectively, as 10 and 3 times the background noise. In order to obtain intraday and interday precision (RSD%) values, standard solutions of the analytes at 3 different concentrations, corresponding to a low, an intermediate and a high concentration of the linearity range of each analyte were prepared and analysed. Each solution was analysed six times on the same day for intraday precision and six times in six days for interday precision. SPE extraction efficacy was tested and optimised through pilot tests on standard solutions in order to exclude losses (or excessive retention) of the compounds during the loading phase. The absolute recovery from solid samples was evaluated by performing consecutive extractions with organic solvent followed by SPE clean-up and injection onto the chromatographic systems. The extraction was considered quantitative (100%) when a further extraction/SPE/analysis cycle, provided analyte concentrations below method LOQ.

3.4.4 Analysis of real samples

After being fully validated, HPLC-F and LC/MS/MS methods have been applied to the simultaneous analysis of significant representative bioactive compounds belonging to the classes of flavonols, catechins, phenolic acids, benzoic acids, amino acids and derivatives. The samples taken into account for method application are summarised in III.3.2.

Table III.3.2. Summary of analysed samples

Sample	Sample type	System
Cherries (*Prunus avium*)	raw	HPLC-F
Sour cherries (*Prunus cerasus*)	raw, food supplement, commercial liqueur	HPLC-F
Blackberry (*Rubus ulmifolius*)	raw	HPLC-F
Red currant (*Ribes alpinum*)	raw	HPLC-F
Cranberries (*Vaccinium oxycoccus*)	raw	HPLC-F
Blueberries (*Vaccinium myrtillus*)	raw, food supplement	HPLC-F
Fox grape (*Vitis labrusca*)	raw	HPLC-F
Black carrot (*Daucus carota* ssp. *sativus* var. *atrorubens*)	raw, commercial juice	HPLC-F
Myrtle (*Myrtus communis*)	raw berries, leaves, commercial liqueur	HPLC-F
Mastic (*Pistacia lentiscus*)	raw berries, leaves	HPLC-F
Strawberry tree (*Arbutus unedo*)	raw berries, leaves	HPLC-F
Prickly juniper (*Juniperus oxycedrus*)	raw berries, leaves	HPLC-F
Pomegrenate (*Punica granatum*)	raw, three commercial juices	LC-MS/MS
Goji berry (*Lycium barbarum*)	three commercial dried products	LC-MS/MS

Two examples of chromatograms obtained from the analysis of red fruits subjected to SPE procedure and injected into HPLC-F are shown in Figure III.3.2: in particular, (a) shows the chromatogram related to a sour cherries sample, while (b) shows a cranberries sample.

Quantitative results referring to the analysed red fruit samples are shown in Table III.3.3 and expressed as micrograms (µg) of compound contained in one gram (g) of dried sample. In particular, the table shows the levels found in a *Prunus avium* sample native of Chile and harvested in January; of a *Prunus Cerasus* sample from Gradara harvested in July; a *Rubus ulmifolius* sample from Trentino Alto Adige, also collected in July; the analyte concentrations present respectively in *Ribes alpinum* and *Vaccinium myrtillus*, both from Trento are reported; results from a *Vaccinium oxycoccus* sample from Russia and those relating to *Vitis labrusca.*

The collected results show that the red fruit most rich in serotonin (5.60 mg/g), tryptophan (29.0 µg/g), catechin (204 µg/g), epicatechin (78.0 µg/g) and ferulic acid (85.0 µg/g) was *Vaccinium oxycoccus*, while *Prunus cerasus* showed the highest melatonin levels with an amount of 0.24 µg/g. The specie with the highest levels and the greatest number of amino acid-derived antioxidant was found to be *Vaccinium oxycoccus*, while the poorest were *Prunus avium* and *Vaccinium myrtillus*. Moreover, as regards the application of the HPLC-F method to commercial derived products, it is noteworthy as in "Maraschino", sour cherries-based liqueur, the higher number of compounds was found, with significant concentrations of all analytes and in particular of umbelliferone and melatonin, as shown in Figure III.3.3-a. A further application of the method concerns two dietary supplements on the market. One of the them was based on quercetin and blueberry extract; the other, produced by a French company, had as its main constituent *Prunus cerasus* extract in combination with B vitamins. An example of a supplement sample subjected to the SPE procedure and injected into HPLC-F is shown in Figure III.3.3-b, in particular referred to blueberry-based food supplement. As can be seen in Table III.3.4, the richer supplement was found to be the one derived from *Prunus cerasus* extract.

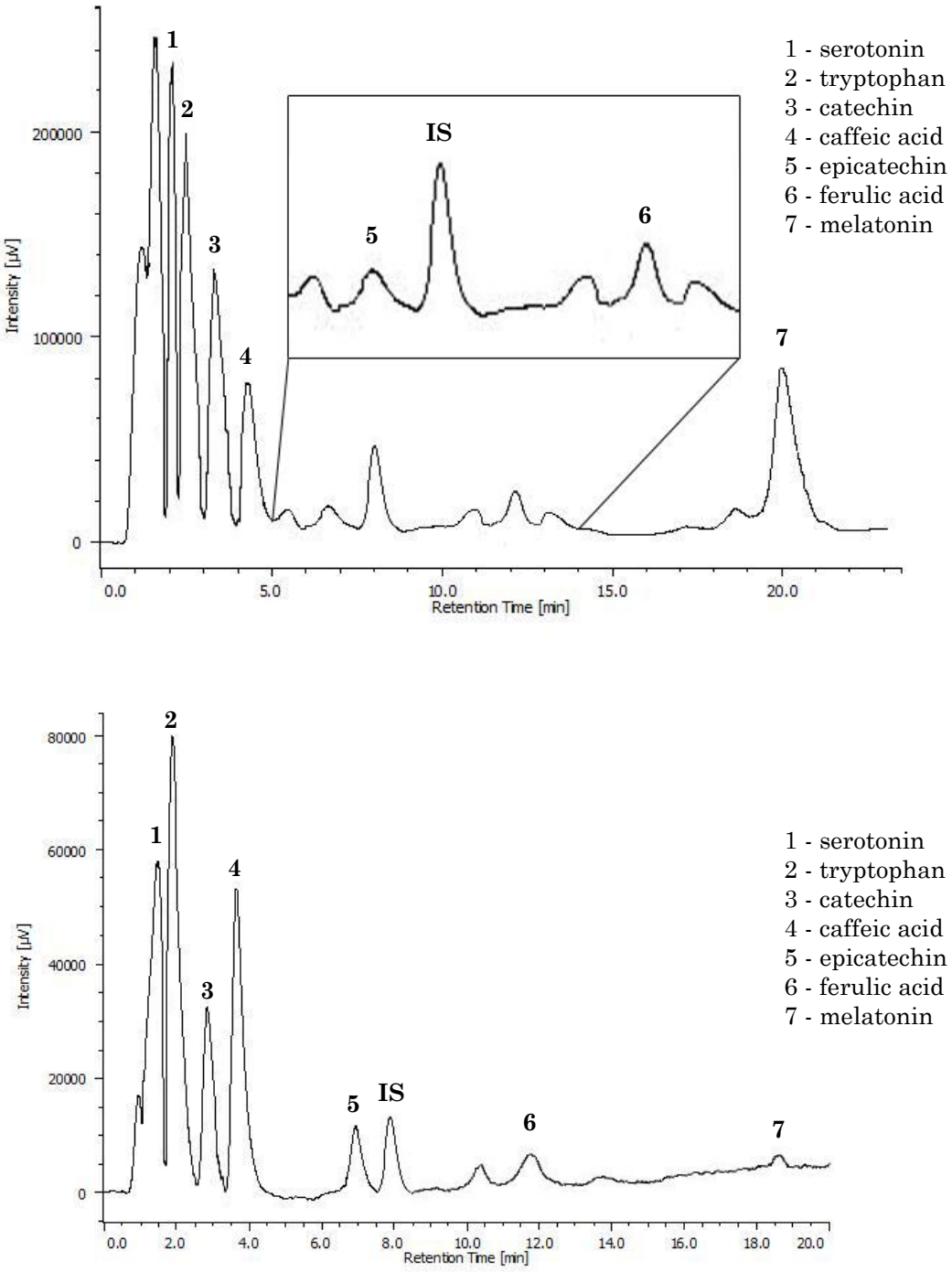


Figure III.3.2

HPLC-F chromatograms obtained from (a) a sour cherries (*Prunus Cerasus)* and (b) a cranberries (*Vaccinium oxycoccus*). Both samples were subjected to the optimised SPE procedure

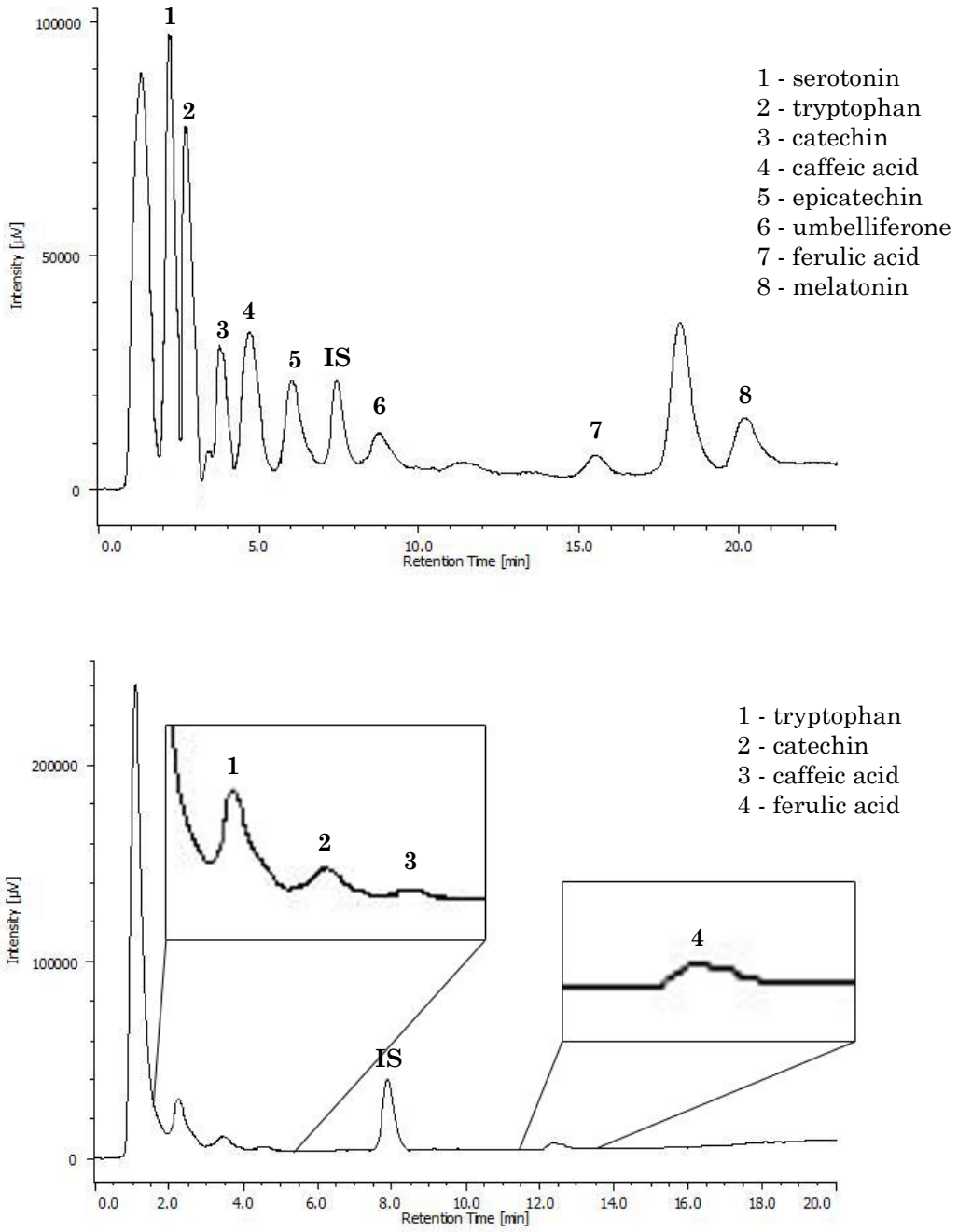


Figure III.3.3

HPLC-F chromatograms obtained from (a) a sour cherries (*Prunus Cerasus)* and (b) a cranberries (*Vaccinium oxycoccus*). Both samples were subjected to the optimised SPE procedure

Table III.3.3. Quantitative results obtained on red fruits samples and derived products

Analyte	Concentration (µg/g, dry weight)				
	Cherries	Blackberry	Red currant	Cranberries	Fox grape
Serotonin	0.12	0.02	0.51	5.60	0.47
Tryptophan	0.60	0.50	2.20	29.0	2.60
Catechin	0.55	1.70	/	204	0.75
Caffeic acid	/	1.40	7.90	2.22	29.0
Epicatechin	/	2.00	0.41	78.0	/
Umbelliferone	0.03	/	0.01	0.16	0.09
Ferulic acid	0.15	0.61	24.0	85.0	1.08
Melatonin	0.05	0.05	0.06	0.13	0.10

Table III.3.3. (Continued)

Analyte	Concentration (μg/g, DW)					
	Sour cherries			Blueberries		Black Carrot
	Raw	"Maraschino" liqueur	Food supplement	Raw	Food supplement	
Serotonin	0.13	0.15	/	/	/	
Tryptophan	0.01	0.65	/	5.30	0.19	5.81
Catechin	44.0	8.66	250	66.0	16.0	12.1
Caffeic acid	32.0	23.3	30.0	24.0	3.7	5.71
Epicatechin	6.80	0.09	1.34	/	/	10.8
Umbelliferone	/	20.0	54.0	/	/	/
Ferulic acid	1.84	13.3	9.79	0.34	2.99	36.0
Melatonin	0.24	0.63	0.41	0.07	/	0.23

Method application has also involved the analysis of four types of berries and leaves of plants belonging to endemic plant species of Sardinia: mastic, juniper, strawberry tree, myrtle (*Pistacia Lentiscus, Juniperus communis, Arbutus unedo* and *Myrtus communis*) and a commercial liqueur made from myrtle extract ("Mirto"). In Figure III.3.4, the chromatogram of a sample obtained from the analysis of juniper (*Juniperus communis*) is shown, while Figure III.3.5 shows chromatograms extrapolated from three different wavelength pairs used for the HPLC-F method and applied to a sample of myrtle-based commercial liqueur.

Quantitative results for each analysed plant and plant part are shown in Table III.3.4 and expressed as micrograms of compound contained in one gram of dried sample. It can be said from the data comparison that the analytes occurring with most frequency among the samples were chlorogenic acid, rutin, apigenin and hesperidin. As can be seen from the obtained data, the highest content of hesperidin was detected in strawberry tree leaves. Regarding myrtle we can note that apigenin was found in both samples: berries (1847 µg/g) and leaves (17.5 µg/g) while regarding rutin it is noticeable how in fresh myrtle berries it is contained at quite high concentration (3402 µg/g). The results collected show that, as for the leaves, also in myrtle-based liqueur the analyte found in higher concentration turned out to be hesperidin. The levels of hesperidin found in the liqueur are higher than those of the berries while levels of apigenin are lower in liqueur and medium in berries. Regarding strawberry tree, one can see that the same analytes found in berries are identified also in leaves. 142 µg/g for chlorogenic acid, 96.8 µg/g for rutin, 2553 µg/g for apigenin in berries, while in leaves the same analytes were as follows: chlorogenic acid 41.2 µg/g, rutin 178 µg/g and apigenin 3192 µg/g. The results for of juniper berries and leaves were as follows: rutin and hesperidin were predominant in the leaves with a concentration of rutin of 18.4 mg/g, hesperidin 1.0 mg/g and apigenin 7.0 mg/g. For mastic, berries appear to be rich in phenolic antioxidants and especially rutin (63.5 µg/g), hesperidin (270 µg/g) and apigenin (244 µg/g). Even leaves show comparable analyte levels: rutin 31.6 µg/g, hesperidin 102 µg/g and apigenin 115 µg/g.

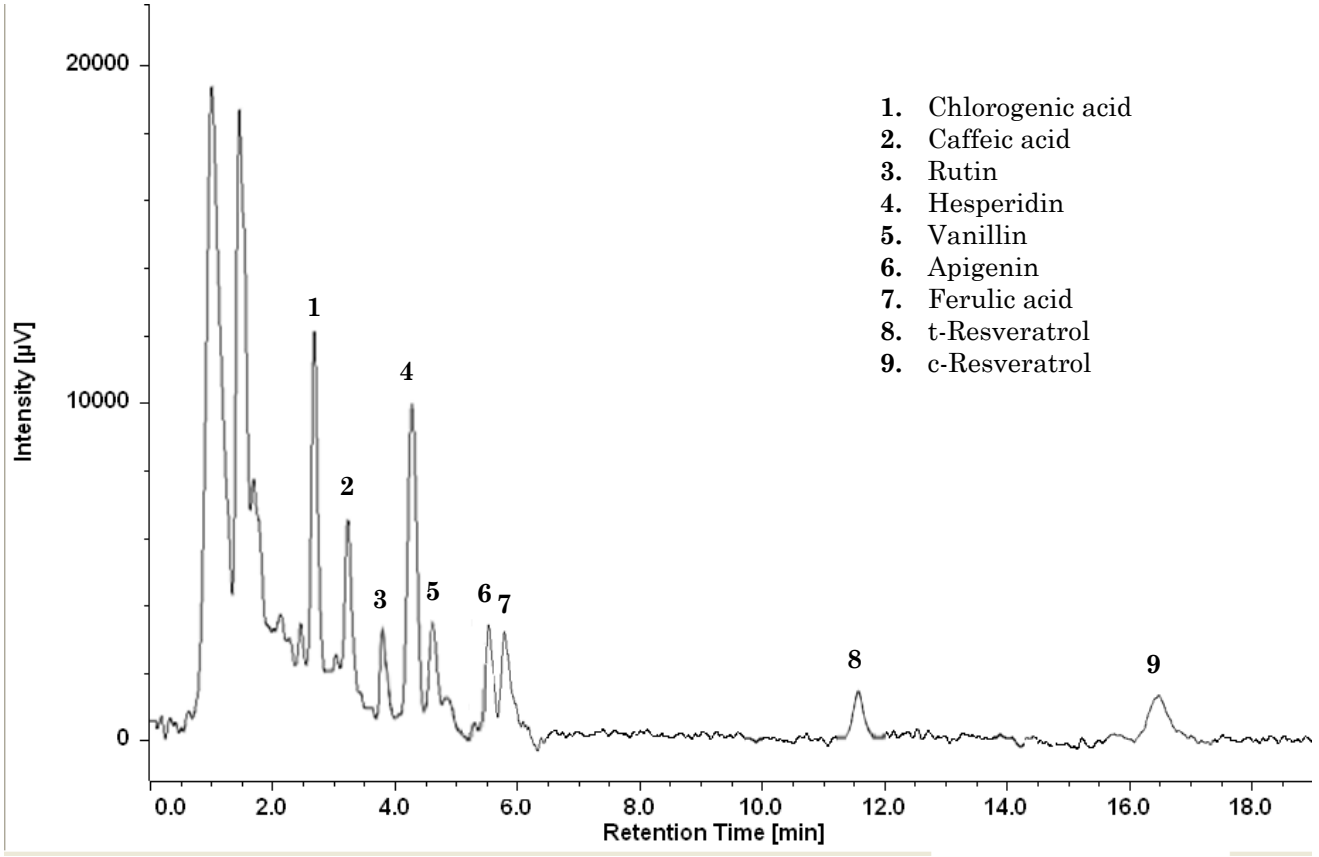


Figure III.3.4

Chromatogram of a sample obtained from the analysis of juniper (*Juniperus communis*) leaves subjected to SPE procedure and injected into the HPLC-F system.

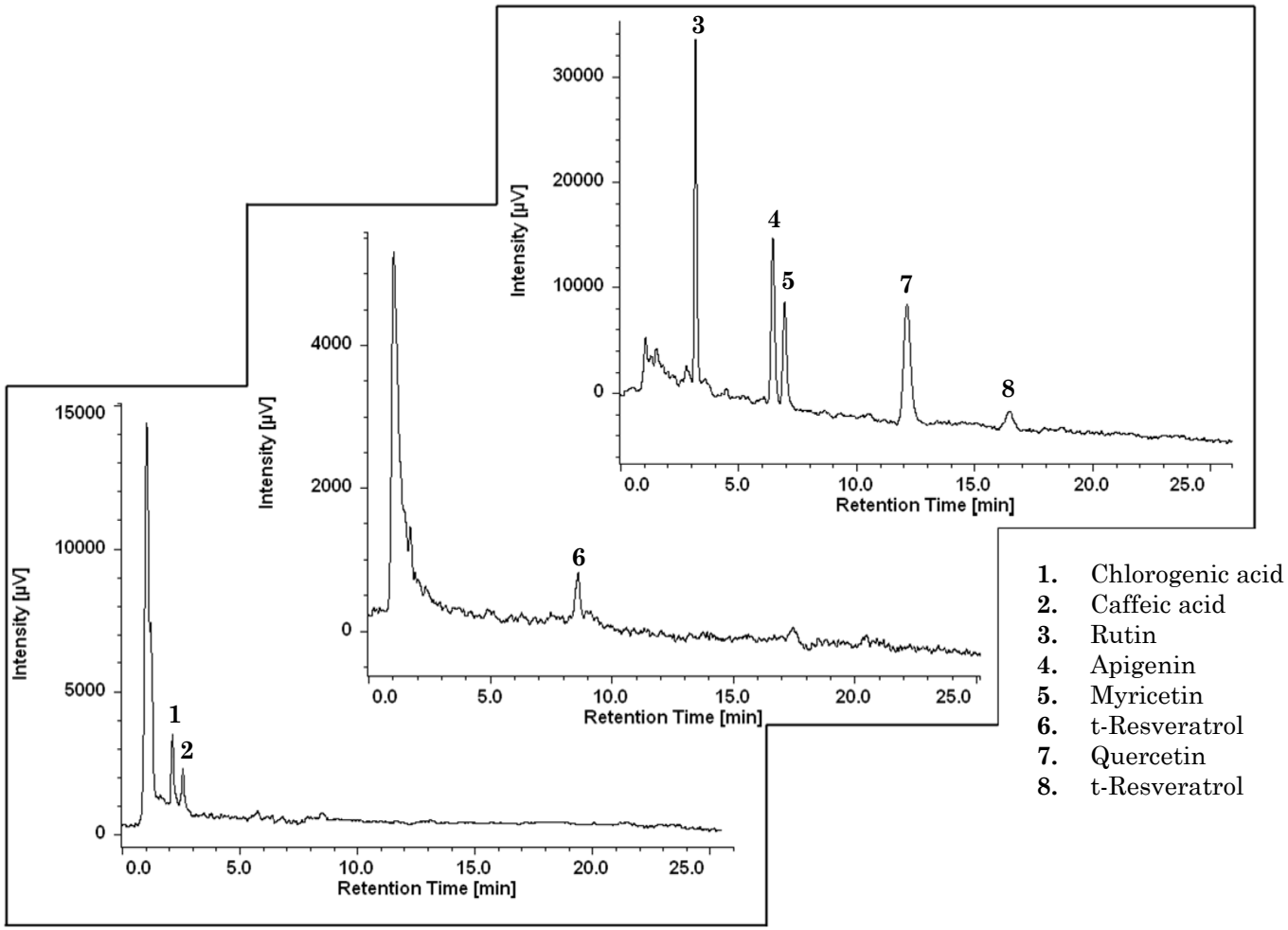


Figure III.3.5

Chromatogram at three different wavelength pairs used for the HPLC-F method and referred to a sample of myrtle-based commercial liqueur.

Table III.3.4. Quantitative results obtained on Sardinia plants (berries and leaves) and derived products

Analyte	Concentration (μg/g, DW)								
	Pistacia lentiscus		*Juniperus communis*		*Arbutus unedo*		*Myrtus communis*		
	Leaves	Berries	Leaves	Berries	Leaves	Berries	Leaves	Berries	Commercial liqueur
Chlorogenic acid	/	0.61	14.5	/	41.2	142	1.10	8.48	/
Caffeic acid	/	/	97.1	12.9	/	/	/	5.10	/
Rutin	31.6	63.5	18400	1204	178	96.8	/	3402	369
Hesperidin	102	270	1000	276	20261	3.78	407	/	443
Vanillin	/	/	642	/	/	2.09	/	/	39.6
Apigenin	115	244	7000	/	3192	27.5	17.5	1847	/
Ferulic acid	2.38	/	10.7	/	/	/	/	/	22.2
Melatonin	9.00	/	/	304	/	8.72	/	/	3.46
Myricetin	42.5	/	/	28.4	250	3.27	773	28.9	960
t-Resveratrol	/	/	1.39	11.1	/	0.11	/	1.18	6.28
Quercetin	27.7	5.06	/	3.32	/	18.6	/	61.2	1385
c-Resveratrol	/	/	2.41	7.21	/	0.09	/	0.62	4.63

Figure III.3.6 represents an LC-MS/MS chromatogram showing Total Ion Current (TIC) mode as the sum of MRM transitions of individual analytes and referred to a sample of pomegranate coming from the Bologna area. Table III.3.5 shows quantitative results: it is possible to observe how, among the analytes taken into account, there is a clear prevalence of catechins and phenolic acids, in particular ellagic and gallic acid. As regards tryptophan pathway compounds, namely tryptophan, serotonin and melatonin, they were found in significant concentrations (although in minor amounts than the other analytes), comparable to foods known for the presence of such molecules. Moreover, with the same LC-MS/MS system, different commercial beverages were analysed after applying the same MEPS pretreatment procedure adopted for the fresh fruits. As regards Brand 1, Brand 2 and Brand 3 commercial juices, the labels stated the presence of 100% pomegranate juice with no further additives: in fact, all analytes present in fresh fruit were identified and, albeit in different concentrations, they had the same ratio. The predominant compounds, in fact, were again catechins, ellagic acid and gallic acid. Brand 4 and Brand 5 samples are referred to commercial beverages for which the label stated, besides pomegranate juice, also other juices and extracts, (in particular, red grape and hibiscus in the former, black carrot and red grape in the latter). It is possible to observe some substantial differences: the presence of serotonin or melatonin was not detected in any of the two juices, while tryptophan was at very low levels in a case and even under the method LOQ in the other, while the compounds observed at highest concentrations were ferulic acid and quercetin. The histograms shown in Figure III.3.7 graphically summarize the results obtained: it is evident from Brand 1, Brand 2 and Brand 3 samples that the same relative analyte proportions are mantained when compared to fresh fruit, albeit in different concentrations; while in Brand 4 and Brand 5 such proportions are missing and this seems due to the contribution of others extracts and juices used in the manufacturing.

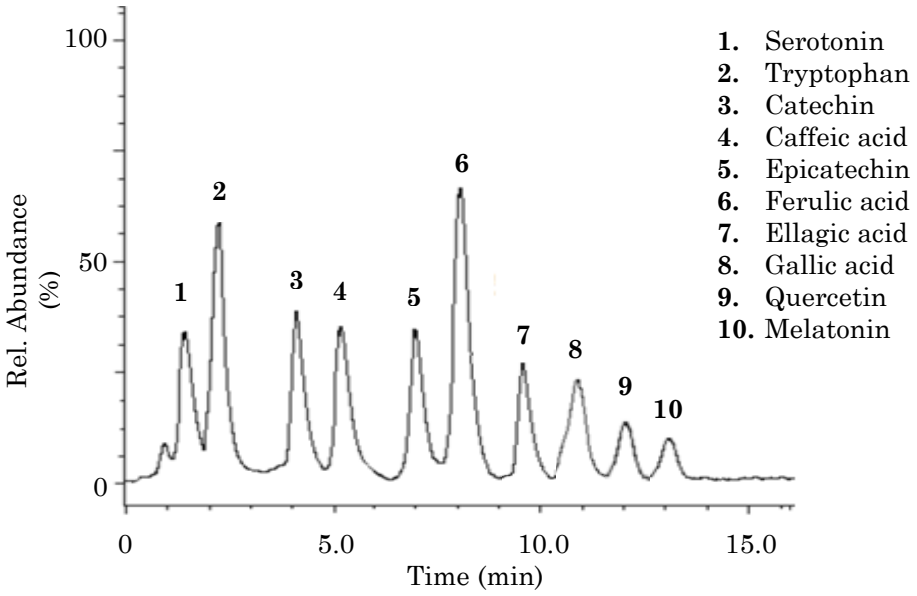


Figure III.3.6

LC-MS/MS chromatogram showing Total Ion Current (TIC) for a fresh pomegranate sample.

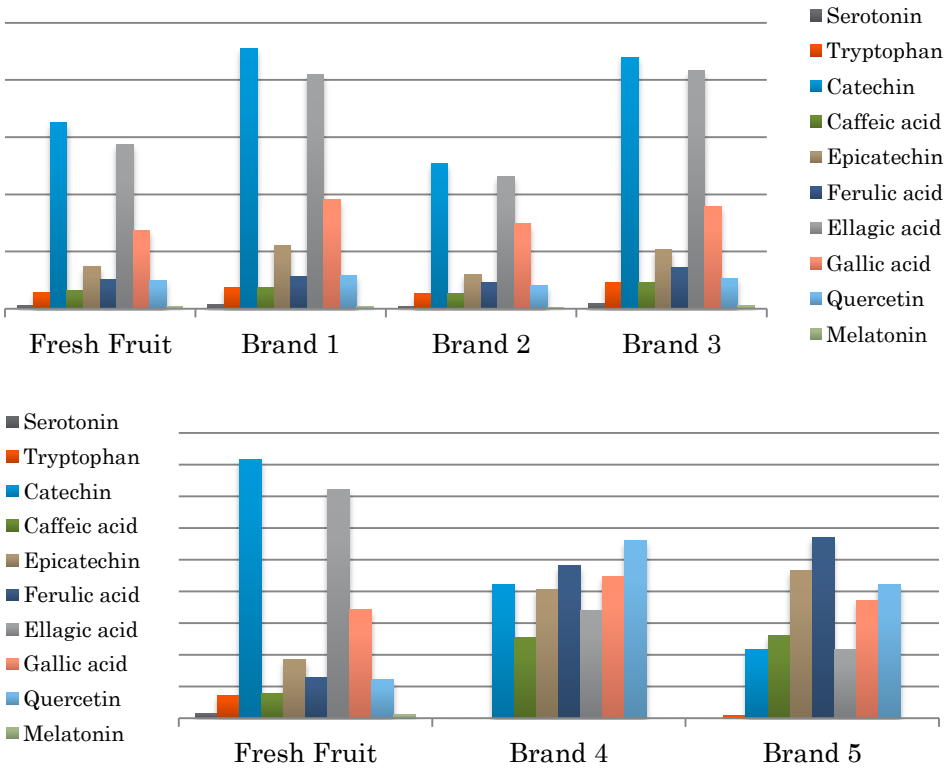


Figure III.3.7

Graphical representation of the quali-quantitative results obtained from LC-MS/MS analysis of pomegranate and derived commercial juices.

Table III.3.5. Quantitative results obtained from pomegranate and derived products

Analyte	Concentration (μg/g)					
	Pomegranate fresh fruit	Brand 1 Labeled:100% pure pomegranate juice	Brand 2 Labeled:100% pure pomegranate juice	Brand 3 Labeled:100% pure pomegranate juice	Brand 4 Labeled: pomegrenade juice, red grape juice, hibiscus extract	Brand 5 Labeled: pomegrenade juice, red grape juice, black carrot extract
Serotonin	0.30	0.42	0.25	0.45	N.D	N.D.
Tryptophan	1.4	1.9	1.3	2.3	N.D.	0.15
Catechin	16.3	22.8	12.7	22.0	8.4	4.3
Caffeic acid	1.6	1.9	1.4	2.3	5.1	5.2
Epicatechin	3.7	5.5	3.0	5.2	8.1	9.3
Ferulic acid	2.5	2.8	2.3	3.7	9.6	11.4
Ellagic acid	15.4	20.5	11.6	20.8	6.8	4.3
Gallic acid	6.8	9.6	7.4	8.9	8.9	7.4
Quercetin	2.5	2.9	2.0	3.7	10.2	8.4
Melatonin	0.21	0.23	0.15	0.32	N.D.	N.D.

Table III.3.6. Quantitative results obtained from three brands of commercial dried goji berry samples.

	Concentration (μg/g, dry weight)		
Analyte	Brand 1	Brand 2	Brand 3
Quercetin	941	1023	977
Myricetin	104	129	98
Apigenin	492	521	506
Kaempferol	76	83	91
Rutin	1128	1283	1099
Myricitrin	13	10	12
Quercitrin	11	12	10
Catechin	1341	1294	1129
Epicatechin	2180	2003	1984
Caffeic acid	899	931	926
Ferulic acid	931	978	927
Coumaric acid	985	943	994
Chlorogenic acid	10740	11039	11931
Vanillic acid	18	15	17
Ellagic acid	17	14	17
Gallic acid	130	129	116
Tryptophan	198	233	229
Serotonin	121	139	114
Melatonin	65 [a]	55 [a]	63 [a]
Zeaxanthin	750	832	793
ß-Cryptoxanthin	65	61	54
ß-Carotene	34	40	29
Lutein	45	57	33

[a] ng/mg

3.5 Conclusion

This research work has been aimed at the development and validation of two chromatographic methods, the first one equipped with fluorescence detection (HPLC-F) and the second coupled to mass spectrometry (LC-MS/MS), for the simultaneous analysis of significant representative bioactive compounds belonging to the classes of flavonols, catechins, phenolic acids, benzoic acids, amino acids and derivatives. Native fluorescence possessed by many substances among those considered was exploited in order to reach high sensitivity and selectivity, features also guaranteed by the employment of tandem mass spectrometer operating in MRM mode.

Because of the complexity of the matrices taken into account, effective sample pretreatment strategies based on SPE and MEPS have been developed and optimized in order to obtain sound and reproducible analytical results. Both developed methods have been validated in terms of linearity, sensitivity, precision and absolute recovery.

For method application, a set of real samples of different nature and origin was taken into account. Some of them are everyday consumption foods such as raw cherries, blueberries, red currant and pomegranate, others required a more careful search since they are better known in eastern countries as cranberries, goji berries and black carrots, or are more difficult to find on the market (sour cherry, fox grape). Another important set of samples covered endemic plants and berries of the Mediterranean basin and particularly of Sardinia, such as myrtle, mastic, strawberry tree and prickly juniper. Where available, analysis was extended to derived products such as fruit juices, liqueurs and supplements, in order to deepen their knowledge from the nutraceutical perspective and investigate correlations between their bioactive profiles.

Method application provided satisfactory results that allowed a detialed bioactive profiling of several fruits and berries, some of them commonly used, others typical of certain geographical areas only, less common or still poorly studied. The comparison of the profiles obtained from the analysis of

commercial products such as liqueurs, fruit juices and food supplements confirmed the quantitative analysis of bioactive compounds a promising tool for both authenticity control, for the characterization of products with high beneficial potential on human organism and for the exploitation of niche products.

3.6 Acknowledgements

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The results obtained during this research were presented as contributions to symposium:

Laura Mercolini, Roberto Mandrioli, Michele Protti, Maria Augusta Raggi

AROMI E POTENZIALE NUTRACEUTICO DI UVA E FRUTTI ROSSI: ANALISI DI AMMINOACIDI E DERIVATI

3° National Congress SINut (Società Italiana di Nutraceutica)
Milano, September 20-21, 2013. (abstract book p.56)

Michele Protti, Laura Mercolini, Viviana Spinelli, Nadia Ghedini, Maria Augusta Raggi

EXPLORING THE NUTRACEUTICAL POWER OF TIBETAN GOJI BERRIES: AN ANALYTICAL STUDY

13° Sigma-Aldrich Young Chemists Symposium (SAYCS)
Riccione, 2013, October 28-30 (Abstract Book NU5)

Michele Protti, Laura Mercolini, Elena Capelli, Anna Ferranti, Maria Augusta Raggi

THE BLACK CARROT: REDISCOVERING AN ANCIENT VEGETABLE FROM A NUTRACEUTICAL POINT OF VIEW

Flash Communication

13° Sigma-Aldrich Young Chemists Symposium (SAYCS)
Riccione, 2013, October 28-30 (Abstract Book NU6)

Michele Protti, Laura Mercolini, Maria Augusta Raggi

ANALYTICAL EVALUATION OF POMEGRANATE NUTRACEUTICAL POWER AND AUTHENTICITY ASSESSMENT OF DERIVED PRODUCTS

Oral Communication

VIII Meeting: Nuove Prospettive in Chimica Farmaceutica - NPCF8
Parma, 2014, June 9-11(Abstract Book O-22)

Michele Protti, Marco Maltesi, Roberto Mandrioli, Laura Mercolini

NUTRACEUTICAL INVESTIGATION ON MEDITERRANEAN BERRIES AND DERIVED NICHE PRODUCTS

Flash Communication

XIV Sigma-Aldrich Young Chemists Symposium - SAYCS 2014
Riccione, 2014, October 27-29 (Abstract Book FL-23)

3.7 References

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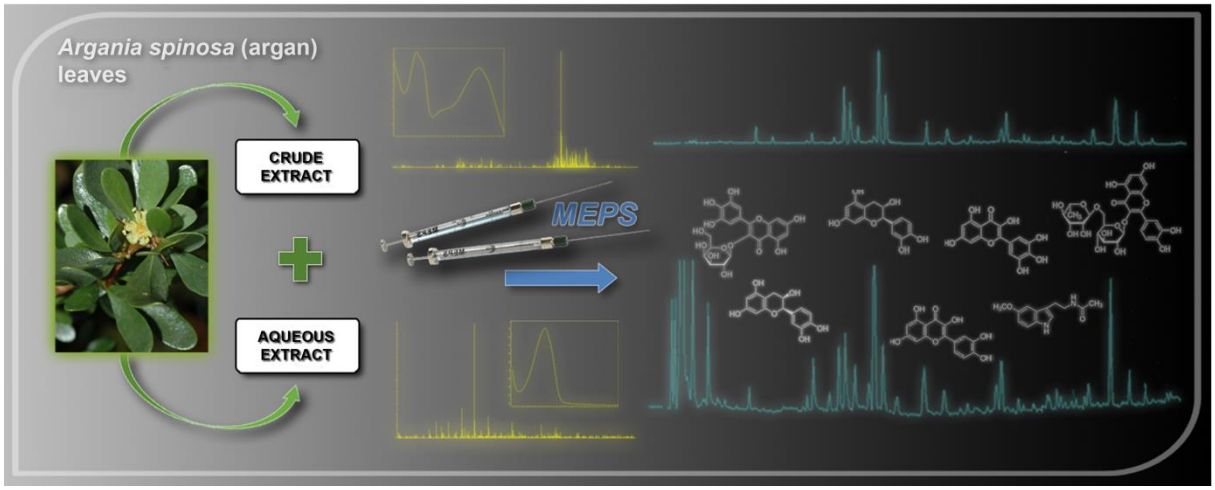
4. Analytical profiling of bioactive phenolic compounds in argan (*Argania spinosa*) leaves by combined microextraction by packed sorbent (MEPS) and LC-DAD-MS/MS



4.1 Abstract

The argan tree (*Argania spinosa*) is an endemic species from southwestern Morocco. Argan-based preparations have been widely used in the Moroccan traditional medicine for their biological properties, as well as for several cosmetic purposes. Whereas kernel, pulp of fruit and trunk have been extensively studied for their nutritional and pharmacological effects, relatively little is known about argan tree leaves.

The main purpose of the present study is to investigate and characterise the bioactive phenolic fractions in both crude and aqueous extracts derived from argan tree leaves.

A qualitative profile of the antioxidant phenolic compounds in argan leaves was obtained by means of structural hypotheses based on UV spectra and mass spectrometric fragmentation patterns. Moreover, selected phenolics were quantified in argan leaves by using a fully validated method based on liquid chromatography coupled to diode array detection and tandem mass spectrometry (LC-DAD-MS/MS). All the extracts were purified by a fast and reliable microextraction by packed sorbent (MEPS) procedure, before analysing them by LC-MS/MS.

Based on retention times, mass spectrometric fragmentation and UV spectra, 13 phenolic compounds were identified or tentatively elucidated from crude and aqueous extracts derived from *A. spinosa* leaves, while 7 compounds were quantified in both extracts.

The obtained results could represent a first step towards a complete characterisation of the argan plant, its bioactive profiling and the valorisation of its by-products as a source of potentially beneficial bioactive molecules.

4.2 Introduction

The argan tree (*Argania spinosa* (L.) Skeels; Sapotaceae) is an endemic species from southwestern Morocco with an essential role in its micro-economy. In the traditional Moroccan medicine argan oil-based preparations, obtained from *A. spinosa* fruit kernels, have been used by Berbers for centuries due to their hepatoprotective, hypocholesterolemic and anti-atherosclerotic effects, as well as to threat rheumatism and dermatological diseases [91]. Antibacterial and antifungal properties of the oil have also been demonstrated [92]. Indeed, the increasing popularity of argan oil, in particular for its cosmetic or phytoprotective application [92], has prompted the Moroccan government to triple annual production (2500-4000 tons) by 2020 [93]. Since the whole argan tree has many remarkable properties, it could be considered as a source of several phytochemically valuable compounds. In this respect, the identification and isolation of the potentially bioactive components of argan by-products, other than kernels, is a prerequisite.

This study has a double aim, which mainly involves the *A. spinosa* leaves:
1) to investigate the presence of phenolic compounds with specific antioxidant properties;
2) to assess the concentrations of these compounds in both crude and aqueous extracts derived from argan tree leaves.

According to what is reported in the scientific literature, a large diversity of compounds, including triterpenes, sterols, flavonoids, volatile compounds, phenols and saponins, have been isolated so far from various parts of the argan tree. For example, the chemical composition of argan oil has been deeply investigated and there are consistent data about fatty acids and derivatives, triglycerides, carotenoid pigments, tocopherols, sterols and stanols, triterpenic alcohols or polyphenols [95-99]. Marfil and colleagues pointed out that virgin argan oil contains higher levels of phenolic compounds than other edible oils such as olive oil [100]. Recently, one paper investigated the presence of pentacyclic triterpenic acids (ursolic acid was the main compound) in fruits and leaves from *A. spinosa* [91], by gas chromatography coupled with flame

ionization detector (GC-FID). During the last two decades, several studies have been addressed to the isolation and characterisation of saponins in *A. spinosa* kernel [101], fruit pulp [102] and shells [103]. To date, the only quali-quantitative evaluation of phenolic compounds in *A. spinosa* leaves concerns the structural identification of some compounds in mixed leaves, stems and thorns by NMR, the determination of total flavonoids by colorimetric assays and their disposition through histochemical tests [104,105]. Moreover, in these papers an analytical method based on HPLC coupled to photodiode array detection (HPLC-PDA) was exploited for the quantitative determination of four of the identified flavonol glycosides (namely quercitrin, myricitrin, hyperoside and myricetin-3-*O*-galactoside), but unfortunately it has not been validated. In another study, the chemical structure of four flavonoids isolated from the leaves of *A. spinosa* were elucidated by spectral data and their antimicrobial activity was studied [106]. Most of these studies are summarized in a review [107].

To the best of our knowledge, the one presented herein is the first study addressed to the elucidation of qualitative profile of antioxidant phenolic compounds in argan leaves, by means of a structural hypothesis based on UV spectra and MS/MS fragmentation patterns. Moreover, an original analytical method, based on liquid chromatography coupled to diode array detection and mass spectrometry (LC-DAD-MS/MS), was developed and fully validated in order to quantify for the first time selected phenolics in leaf extracts, after purification by a novel, fast and reliable microextraction by packed sorbent (MEPS) procedure.

4.3 Experimental

4.3.1 Chemicals

Powders (≥ 94%, w/w) of target analytes, quantified in argan leaf extracts ((-)-epicatechin, (+)-catechin, myricetin, quercetin, rutin, melatonin and myricetin-3-*O*-galactoside; Fig. III.4.1-a-g) and curcumin, used as the internal standard (IS, Fig. III.4.1-h), as well as HPLC grade acetonitrile, methanol, ethanol, chloroform (> 99.8%, w/w) and formic acid (98%, w/w), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water (18.2 MΩ cm) was obtained by means of a MilliQ apparatus by Millipore (Milford, MA, USA). Stock solutions (1 mg/mL) of the analytes and the IS were prepared by dissolving suitable amounts of pure powders in methanol. Standard solutions of the analytes and the IS were obtained by diluting stock solutions with methanol and were prepared weekly. All solutions were stored protected from light in amber glass vials from Waters Corporation (Milford, MA, USA).

4.3.2 Instruments

LC-DAD-MS/MS analysis was performed on a Waters Alliance e2695 chromatographic system with autosampler coupled to a Waters 2998 photo diode array detector and a Waters Micromass Quattro Micro triple-quadrupole mass spectrometer equipped with an electrospray ion source (ESI), working in both positive and negative ion mode. Data processing was performed using Waters MassLynx 4.1 software. A Crison (Barcelona, Spain) Basic 20 pHmeter, a Velp (Usmate, Italy) RX3 vortex mixer and an Elma (Singen, Germany) Sonic T310 Trans ultrasonic bath, were also used. Plant material was grinded in an IKA A11-1 analytical mill (Staufen, Germany). Plant extracts were dried under vacuum in a Büchi (Flawil, Switzerland) R-205 rotavapor equipped with a V-800 vacuum controller and a B-490 heating bath.

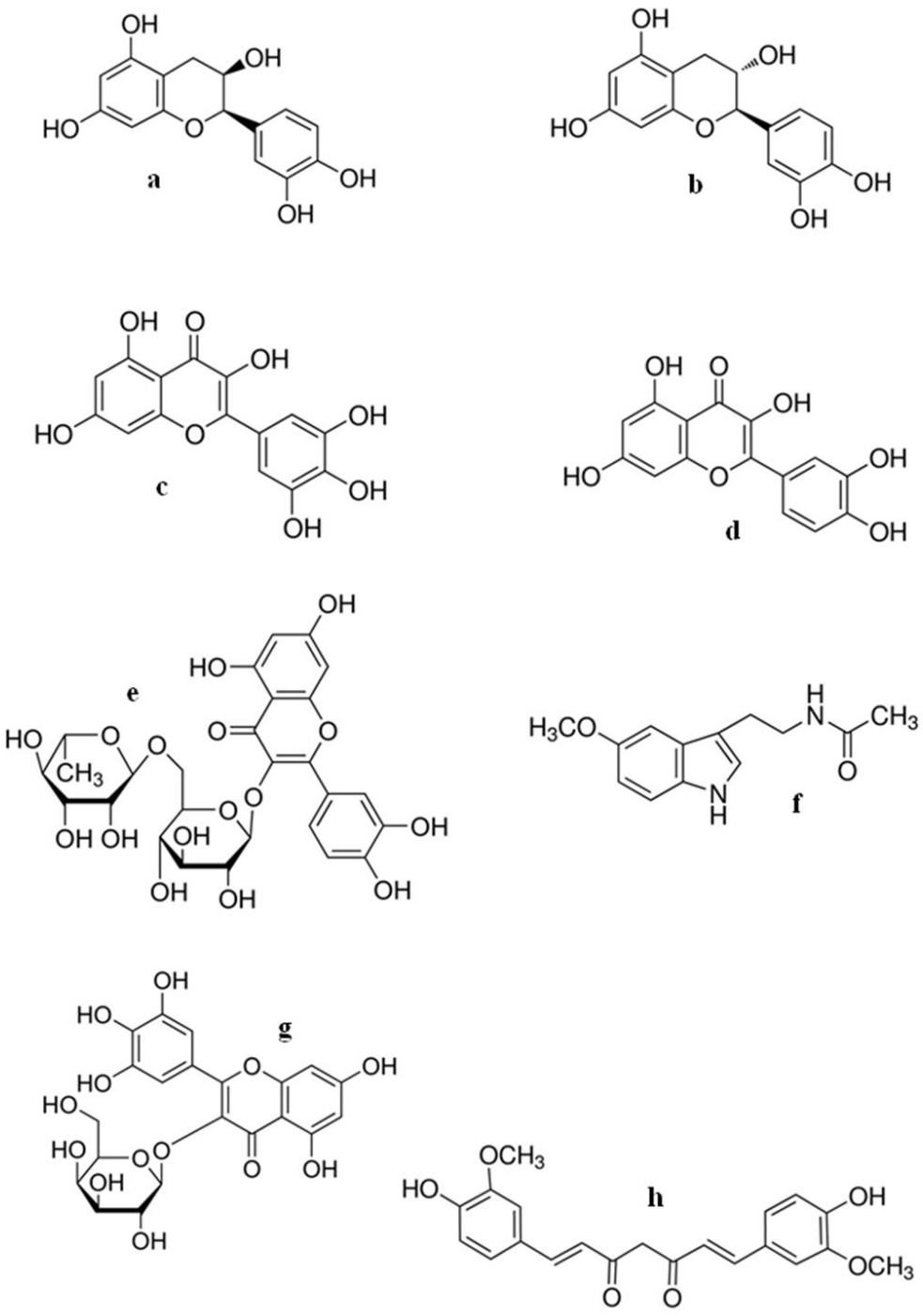


Figure III.4.1

Chemical structures of seven phenolics quantified by LC-DAD-MS/MS: (a) (-)-epicatechin, (b) (+)-catechin, (c) myricetin, (d) quercetin, (e) rutin, (f) melatonin, (g) myricetin-3-O-galactoside and (h) curcumin, used as the internal standard (IS).

4.3.3 Chromatographic conditions

Separations were obtained on a Waters XTerra MS C18 column (100 × 2.1 mm I.D., 3.5 µm), maintained at room temperature and equipped with a guard column. The mobile phase was a mixture of 0.2% aqueous formic acid (A) and acetonitrile (B), flowing at a constant rate of 0.3 mL/min. The gradient program of the mobile phase started with A:B (98:2, v:v), then ramped up to A:B (75:25, v:v) over 5 min; this ratio was maintained for 5 min, then ramped down to A:B (98:2, v:v) over 1 min and maintained for 2 min. The two components of the mobile phase were filtered through Sartorius (Göttingen, Germany) membrane filters (47 mm diameter, polyamide, 0.2 µm pore size) and degassed by an ultrasonic bath. The injection volume was 10 µL.

4.3.4 DAD and MS/MS parameters: identification and quantitation of phenolic compounds

For the identification of phenolic compounds, the DAD detector scanned from 220 to 400 nm; while MS parameters were as follows: the cone and capillary voltages were set at -30.0 V and -2.6 kV, respectively; the source temperature was 143 °C and the desolvation temperature was 350 °C. MS/MS spectra were obtained using argon as the collision gas and setting the collision energy between 10 and 45 V, within a mass range of m/z 50-1000 (scan duration of 0.5 s). Each sample was analysed in both positive (ESI+) and negative (ESI-) modes to provide abundant information for structural identification.

For LC-MS/MS quantitative analysis of some representative phenolic compounds, multiple reaction monitoring (MRM) transitions were used, acquiring in negative ionisation mode (ESI-). All the MS parameters were optimised for maximum abundances of the interested ions *via* direct infusion of each analyte (1 µg/mL methanolic solutions) at 20 µL/min. The optimised parameters were as follows: ion source voltage, 3 kV; ion source temperature, 120°C; desolvation temperature, 350°C; desolvation gas flow, 600 L/h;

extractor potential, 3 V; collision exit potential, 1 V. Nitrogen was used as the desolvation gas, while argon was used as the collision gas. The precursor ion and the product ion, with dwell time, cone voltage and collision energy, were optimised for each analyte.

4.3.5 Plant materials and sample preparation

Argan leaves were collected on February 2014 (at the pre-flowering stage of development) in a wild area near to Agadir by Dr. Fatiha Sattih and a voucher specimen (BOLO0508219) was deposited in the Herbarium of the University Museum System (SMA - Bologna University, Italy). Since the leaves were the object of investigation, the pre-flowering stage of development was chosen, in order to reasonably find a good secondary metabolites content in these organs.

Leaves were dried in a stove at 40°C, ground in an electric mill and then 25 g of powdered material were extracted with 250 mL of 70% ethanol for 24 hours under continuous stirring. The extract was filtered on a Büchner funnel and dried under reduced pressure, thus producing a crude leaf extract. The procedure was repeated three times and the obtained crude extracts were pooled (total extraction yield of 22.4%, w/w).

The obtained crude extract was solubilised in 50 mL of water and partitioned in a separatory funnel, using 50 mL of chloroform; the procedure was repeated three times, then the aqueous and organic phases were dried in a rotary evaporator. This procedure allows to obtain an aqueous extract enriched in hydrophilic compounds (yield of 57.5%, w/w) and an organic one containing lipophilic compounds.

Suitable amounts of both crude and aqueous extracts were dissolved in methanol, in order to obtain working solutions at the concentration of 1 mg/mL. The obtained solutions were then centrifuged, the supernatant was filtered on 25 mm Ø, 0.45 µm pore size nylon syringe filters supplied by Sigma-Aldrich, then appropriately diluted and subjected to the sample clean-up MEPS procedure, before the instrumental analysis.

4.3.6 Sample pretreatment: MEPS procedure

MEPS procedure on leaf extracts was performed on a SGE Analytical Science (Melbourne, VIC, Australia) apparatus, consisting of a 100 µL HPLC syringe with a removable needle, fitted with a BIN (Barrel Insert and Needle) containing an M1 (C8+SCX mixed mode) sorbent. The sorbent was activated with 200 µL of methanol and then equilibrated with 200 µL of ultrapure water. The loading solution was a mixture of 100 µL of diluted methanolic extracts, 100 µL of ultrapure water and 5 µL of IS solution; the loading mixture was drawn into the syringe and discharged back 10 times. The sorbent was washed with 100 µL of water, 100 µL of a water/methanol mixture (90:10, v:v) and then eluted by drawing and discharging 500 µL of methanol. The eluate was dried under vacuum, redissolved with 100 µL of methanol and injected into the LC-DAD-MS/MS system.

4.3.7 Quantitative analysis of phenolic compounds

Linearity

Analyte standard solutions of seven representative phenolic compounds (i.e. (-)-epicatechin, (+)-catechin, myricetin, quercetin, rutin, melatonin, myricetin-3-*O*-galactoside) at seven different concentrations, containing the IS (i.e. curcumin) at a constant concentration, were injected into the LC-DAD-MS/MS system. The procedure was carried out in triplicate for each concentration. The analyte/IS peak area ratios were plotted against the corresponding analyte concentration (ng/mL) and the calibration curves were constructed by means of the least-square method.

LOQ and LOD were calculated according to international regulatory guidelines as the analyte concentrations which give rise to peaks whose heights are 10 and 3 times the baseline noise.

Absolute recovery

Absolute recovery was evaluated by subjecting methanolic standard solutions of the phenolic compounds (at known concentrations) to the MEPS procedure previously described. The analyte peak areas were compared with those obtained by injecting standard solutions at the same theoretical concentrations.

Precision

Precision assays were carried out on phenolic standard solutions and *A. spinosa* leaf extracts. Standard solutions of the selected phenolic compounds at three different concentrations were analysed six times within the same day to obtain intraday precision and six times over six different days to obtain interday precision, both expressed as percentage relative standard deviation (RSD%).

Similarly, leaf extracts were analysed six times within the same day to test intraday precision and six times over six different days to obtain interday precision.

Accuracy

Method accuracy was tested by means of percentage recovery assays, adding known amounts of standard solutions of the analytes and the IS to leaf extracts, which had been already analysed.

4.4 Results and discussion

4.4.1 Chromatographic condition optimisation

LC conditions, including column and mobile phase composition, were optimised to achieve the simultaneous separation of the main representative phenolic compounds, i.e. epicatechin, catechin, myricetin, quercetin, rutin, melatonin and myricetin-3-*O*-galactoside. XTerra MS C18 column (100 x 2.1 mm I.D., 3.5 µm) was selected to obtain the best efficiency, most symmetrical chromatographic peaks and good baseline separation of the analytes. Various mobile phases (0.1% formic acid–methanol, 0.1% formic acid–acetonitrile, 0.1% phosphoric acid–acetonitrile, 0.1% acetic acid–acetonitrile and 0.2% formic acid–acetonitrile) were investigated. In general, because it gave sharp peak, better resolution and stable baselines, 0.2% formic acid-acetonitrile was used as the mobile phase. In order to obtain short retention times (while avoiding co-elution with the residual matrix components), a gradient elution starting at 2% organic modifier and going up to 25% over 5 min and flowing at a constant rate of 0.3 mL min was used. Injection volume was 10 µL. Under these conditions, a good separation was achieved and a complete chromatographic run took less than 18 minutes. The selected antioxidant phenolic compounds have strong UV absorption at λ close to 275, 325 and 370 nm and a few peaks of impurities were detected at these wavelengths. Therefore, 275, 325 and 370 nm were selected LC-DAD quantitative analysis.

Different compounds with physico-chemical properties comparable to those of the analytes were tested as potential internal standards (IS) and curcumin has proved to be the best choice.

4.4.2 MEPS procedure development

In order to optimise MEPS procedure, some factors affecting the recovery such as the kind of sorbent, the number of extraction cycles, the volumes and the nature of washing and elution solvents were carefully investigated. A careful sorbent selection was important to achieve acceptable clean-up and absolute recovery, therefore the performance of different kinds of sorbents such as C2, C8, C18 and M1 (mixed mode: C8 + strong cation exchange or SCX) was evaluated in terms of extraction efficiency and reproducibility. M1, C2 and C8 sorbents provided the best absolute recovery in comparison with C18 one; however M1 sorbent was chosen for the MEPS procedure because also provided better results in terms of purification from interfering compounds, when compared to both C2 and C8 sorbents. All the main steps of the MEPS procedure (loading, washing and elution) were optimised: the retention was satisfactory after 10 draw/discharge cycles of the loading mixture at a speed of 5 µL/s. Different pure solvents and mixtures were tested for the washing and elution steps: good purification was obtained by washing the sorbent with 100 µL of ultrapure water followed by 100 µL of a methanol/ultrapure water 90/10 (v/v) mixture at a speed of 10 µL/s; an elution step consisting in two cycles with 250 µL of pure methanol at a speed of 5 µL/s proved to be sufficient for the complete elution (> 87%) of all analytes.

After each extraction, a sorbent cleaning step was carried out with 200 µL of methanol and 200 µL of water. This steps prevented carryover effects while acting as conditioning steps for the next extraction. The eluate was dried under vacuum and redissolved with 100 µL of methanol and then injected into LC system. Using this innovative and fast MEPS pretreatment procedure, good absolute recovery of the analytes and IS were reached, while eliminating potentially interfering matrix components.

4.4.3 Identification of phenolics in A. spinosa *leaf extracts*

The most intensely studied natural phenols are the flavonoids, which include several thousand compounds, among them the flavonols (e.g. quercetin), flavones, flavan-3ol (e.g. catechins), anthocyanidins and isoflavonoids. Many plants store chemicals in the form of inactive glycosides (e.g. rutin, myricitrin, quercitrin, hyperoside) in which a sugar group (glycone) is bonded through its anomeric carbon to another group (aglycone) *via* a glycosidic bond. Herein, the DAD spectra and MS fragmentation pattern of the parent compounds in argan leaf extract would provide useful information for predicting the structures of some antioxidant phenolics.

The analytical method proposed herein was employed for the identification of the chromatographic peaks of the main phenolics in both crude and aqueous extracts from *A. spinosa* leaves. The negative electrospray ionisation (ESI-) mode affords higher sensitivity compared with the positive electrospray ionisation (ESI+) mode; therefore, the latter was used for the analysis. By comparing the retention time (t_R), UV absorption and MS/MS fragments with reference standards and some literature data, 13 chromatographic peaks were identified or tentatively identified, including 8 peaks for flavonol glycosides, 2 peaks for flavonols, 2 for flavanols and one peak for indoleamines. The DAD chromatogram recorded at 275 nm and the total ion chromatogram (ESI- TIC) of the *A. spinosa* aqueous extract are shown in Fig. Figure III.4.2-a and Figure III.4.2-b, respectively. Chromatographic peaks named as peaks 1, 3, 4, 9, 11, 12 and 13 were identified as quercetin-3-*O*-rutinoside (rutin), catechin, epicatechin, melatonin, myricetin-3-*O*-galactoside, myricetin and quercetin, by comparing t_R values, UV data and MS/MS fragments with the corresponding reference standard compounds under the same analytical conditions. On the other hand chromatographic peaks named as peaks 2, 5, 6, 7, 8 and 10 were tentatively identified as quercetin-3-*O*-galactoside (hyperoside), quercetin-*O*-pentoside, myricetin-3-*O*-rhamnoside (myricitrin), myricetin-3-*O*-glucoside, myricetin-*O*-pentoside and quercetin-3-*O*-rhamnoside (quercitrin), by

comparing t_R values, UV data and MS/MS fragments with literature data. The information for identification of the 13 phenolics are listed in Table III.4.1.

In details, peak 1 presented spectral features with UV λ_{max} at 257 and 355 nm, ESI-MS pseudomolecular ion at *m/z* 609.52 $[M\text{-}H]^-$, fragment ions at *m/z* 301.52 and 255.52 and its t_R was at 3.51 min. Compared to the standard reference compound, peak 1 was identified as quercetin-3-*O*-rutinoside (rutin).

Peak 2 eluted at 5.50 min, showed UV λ_{max} at 258 and 361 nm, a $[M-H]^-$ ion at *m/z* 463.37 and provided a main fragment at *m/z* 301.38 due to the neutral loss of galactosyl group (162 Da) in MS^2. Spectral features similar to peak 2 has been observed previously [107,108] and has been tentatively ascribed to quercetin-3-*O*-galactoside (hyperoside).

Peak 5 (t_R = 7.19 min) presented UV λ_{max} at 356 nm, ESI-MS pseudomolecular ion at *m/z* 433.41 $[M\text{-}H]^-$ and a principal fragment ion at 301.39, generated from a loss of a pentoside moiety $[M–H–132]^-$ [107,109], thus peak 5 identity has been ascribed to a quercetin-*O*-pentoside.

For peak 10, UV spectra were obtained with a maximum at 350 nm and a shoulder at 255 nm, typical of quercetin derivatives; ESI-MS showed a pseudomolecular ion $[M\text{-}H]^-$ at *m/z* 447.36 and a daughter ion at *m/z* 301.38 in MS^2 corresponding to quercetin aglycone derived from the loss of neutral rhamnose fragment. Similar spectral characteristics have been previously observed [110,111], thus peak 10 (t_R 12.09 min) was tentatively identified as quercetin-3-*O*-rhamnoside (quercitrin).

Peak 11 showed spectral characteristics with UV λ_{max} at 257 and 358 nm, ESI-MS pseudomolecular ion at *m/z* 479.39 $[M\text{-}H]^-$, fragment ions at *m/z* 317.30 and 171.12 and its t_R was at 12.28 min. Compared to the pure reference standard, this compound was unambiguously identified as myricetin-3-*O*-galactoside.

For peaks 6 (t_R = 7.84 min), 7 (t_R = 8.19 min) and 8 (t_R = 9.53 min), UV spectra with a maximum at 356 nm and a shoulder at 260 nm, typical of myricetin derivatives, was observed; ESI-MS provided parent ions $[M\text{-}H]^-$ at *m/z* 463.36, 479.41 and 449.39 respectively, while all three peaks presented a daughter ion at *m/z* 317.30 corresponding to myricetin aglycone, due to the

loss of neutral rhamnose, glucose and pentose fragments, respectively. According to these features also observed in the literature [109,110], peaks 6, 7 and 8 were tentatively identified as myricetin-3-*O*-rhamnoside (myricitrin), myricetin-3-*O*-glucoside and myricetin-*O*-pentoside.

Based exclusively on spectral data, discrimination between compound 7 and compound 11 would not have been possible as they are glycosides deriving from the same aglycone (myricetin) linked to isomeric monosaccharides (glucose and galactose, respectively). Such discrimination was possible since, under the analytical conditions described above, the two compounds showed different chromatographic behaviours: due to an unambiguous identity assignation for myricetin-3-*O*-galactoside (peak 11) by comparison with the reference standard compound, peak 7 was tentatively identified as myricetin-3-*O*-glucoside.

Peaks 3 and 4 were eluted at 6.58 min and 6.83 min and showed UV absorption spectral characteristics with λ_{max} at 279 nm. The pseudomolecular ion *m/z* 289.27 $[M\text{-}H]^-$ and the main fragment ions *m/z* 245.27 and 139.12 were observed in MS and MS/MS spectra for those 2 peaks and they were unambiguously identified as catechin and epicatechin, compared with the authentic standards.

Peak 9 presented spectral characteristics with UV λ_{max} at 279 nm, ESI-MS pseudomolecular ion at *m/z* 231.32 $[M\text{-}H]^-$, fragment ions at *m/z* 128.32 and 159.17 and its t_R was at 10.25 min. Compared to the reference standard, this compound was identified as melatonin.

Peak 12 eluted at 15.26 min and showed UV absorption characteristics with λ maxima at 255 and 378 nm. The parent ion *m/z* 317.24 $[M\text{-}H]^-$, the main fragment ions 151.11 *m/z* and 137.11 were detected in MS and MS/MS spectra for peak 12, thus it was identified as myricetin by comparison with the reference standard compound under the same analytical conditions.

Finally, for peak 13 (eluted at 16.01 min), UV spectra with maximum at 250 and 370 nm were observed, ESI-MS provided a parent ion $[M\text{-}H]^-$ at *m/z* 301.24, while the main daughter ions were observed at *m/z* 255.52 and 179.49. Being such features fully comparable with those observed analysing quercetin

standard reference compound under the same conditions, peak 12 was identified as quercetin.

The UV and MS data for some representative phenolics quantified in argan leaf extracts are shown in Fig. III.4.3.

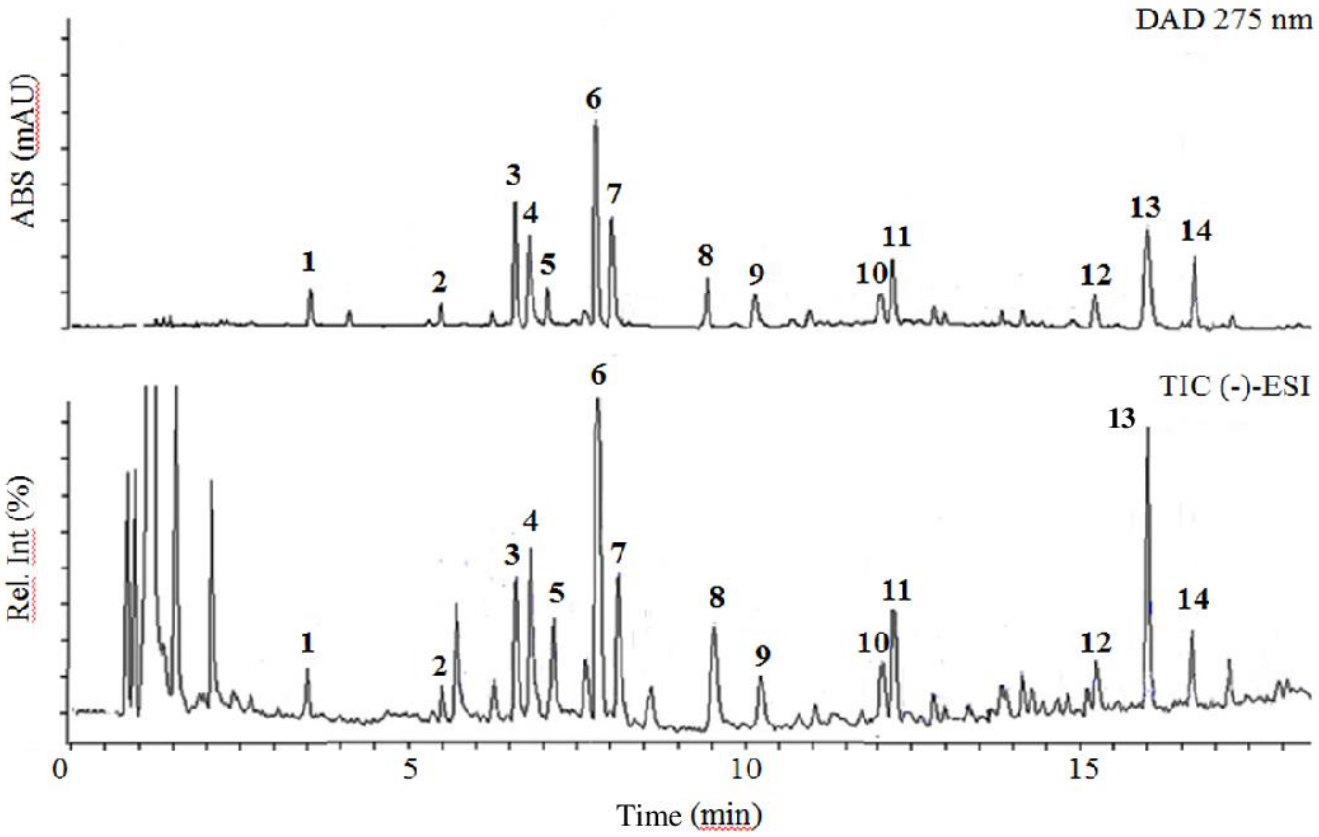


Figure III.4.2

Typical (a) DAD chromatogram at 275 nm and (b) total ion chromatogram (TIC) in negative mode of 13 phenolics from the A. spinosa aqueous extract; 1 = Quercetin-3-O-rutinoside (Rutin), 2 = Quercetin-3-O-galactoside (Hyperoside), 3 = Catechin, 4 = Epicatechin, 5 = Quercetin-O-pentoside, 6 = Myricetin-3-O-rhamnoside (Myricitrin), 7 = Myricetin-3-O-glucoside, 8 = Myricetin-O-pentoside, 9 = Melatonin, 10 = Quercetin-3-O-rhamnoside (Quercitrin), 11 = Myricetin-3-O-galactoside, 12 = Myricetin, 13 = Quercetin, 14 = IS.

Table III.4.1. Structural and spectral data of 13 phenolic compounds identified in *Argania spinosa* leaf extracts.

Peak #	t_R (min)	Identification	Formula	MW	DAD λ (nm)	[M-H] (*m*/*z*)	MS/MS fragments (*m*/*z*)	Ref.
1	3.51	quercetin-3-O-rutinoside (rutin)	$C_{27}H_{30}O_{16}$	610.52	257, 355	609.52	301.52, 255.52	STD
2	5.50	quercetin-3-O-galactoside (hyperoside)	$C_{21}H_{20}O_{12}$	464.38	258, 361	463.37	301.38	[108,109]
3	6.58	catechin	$C_{15}H_{14}O_6$	290.26	279	289.27	245.27, 139.12	STD
4	6.83	epicatechin	$C_{15}H_{14}O_6$	290.26	279	289.27	245.27, 139.12	STD
5	7.19	quercetin-O-pentoside	$C_{20}H_{18}O_{10}$	434.40	356	433.41	301.39	[108,110]
6	7.84	myricetin-3-O-rhamnoside (myricitrin)	$C_{21}H_{20}O_{12}$	464.37	260, 356	463.36	317.30	[110,111]
7	8.19	myricetin-3-O-glucoside	$C_{21}H_{20}O_{13}$	480.38	260, 356	479.41	317.30	[110,111]
8	9.53	myricetin-O-pentoside	$C_{20}H_{18}O_{12}$	450.35	260, 356	449.39	317.30	[110,111]
9	10.25	melatonin	$C_{13}H_{16}N_2O_2$	232.28	279	231.32	128.32, 159.17	STD
10	12.09	quercetin-3-O-rhamnoside (quercitrin)	$C_{21}H_{20}O_{11}$	448.38	255, 350	447.36	301.38	[110,111]
11	12.31	myricetin-3-O-galactoside	$C_{21}H_{20}O_{13}$	480.38	257, 358	479.39	317.30, 171.12	STD
12	15.26	myricetin	$C_{15}H_{10}O_8$	318.24	255, 378	317.24	151.11, 137.11	STD
13	16.01	quercetin	$C_{15}H_{10}O_7$	302.24	250, 370	301.24	255.52, 179.49	STD

STD = comparison with the reference standard compound.

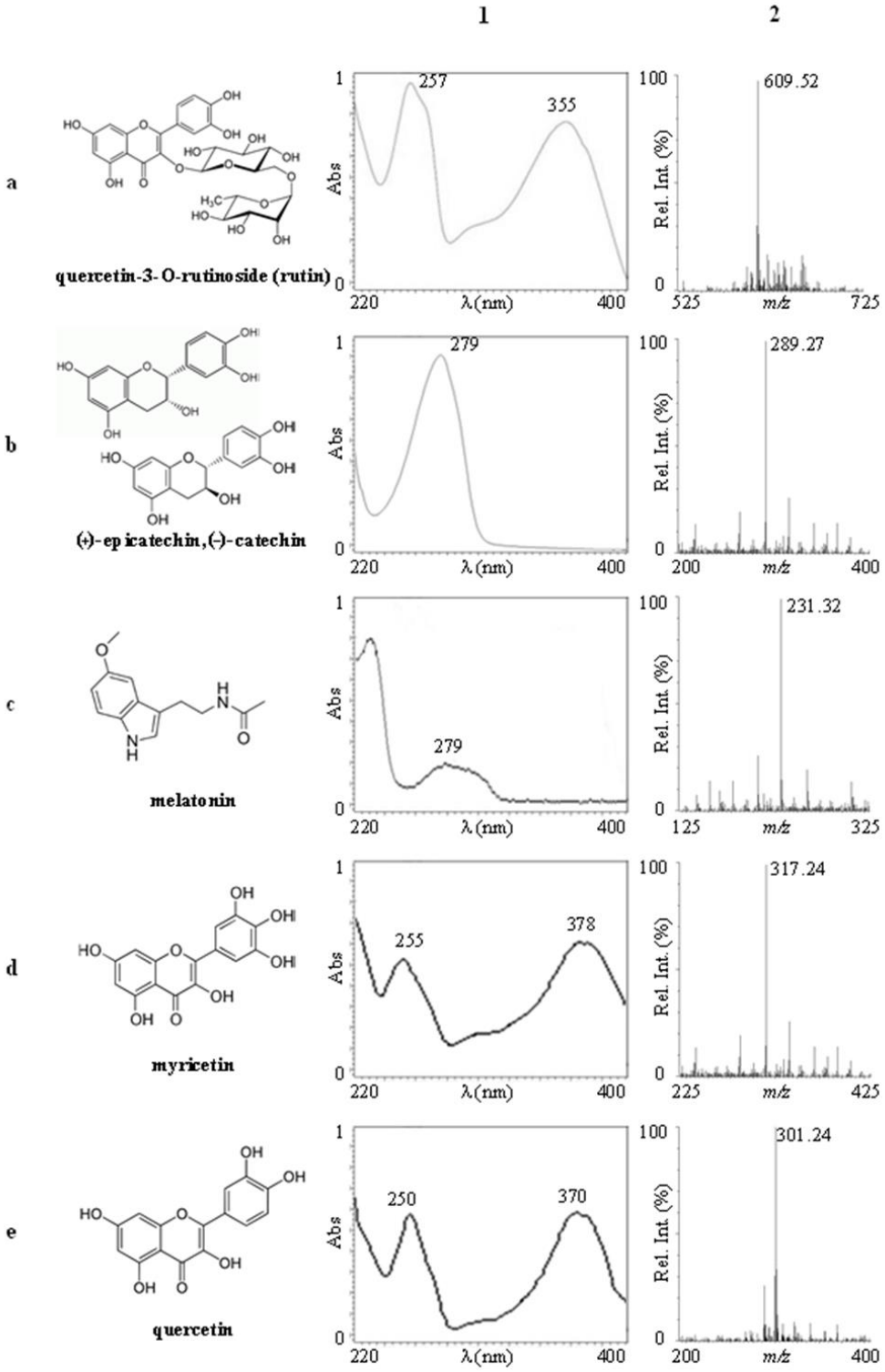


Figure III.4.3

1) UV and (2) MS spectra of some representative phenolic compounds quantified in *Argania spinosa* aqueous leaf extract: (a) catechin and epicatechin, (b) myricetin, (c) quercetin, (d) rutin, (e) melatonin.

4.4.4 Quantitation of phenolics in A. spinosa *leaf extracts*

Preliminary assays

Catechin, epicatechin, myricetin, quercetin, rutin, melatonin and myricetin-3-*O*-galactoside mass spectra were first acquired in full-scan mode (40-600 *m/z*) by infusion of reference methanolic solutions at 1 µg/mL. The chosen MRM transitions for LC-MS/MS quantitative analysis were: 289.27 → 245.27 for catechin and epicatechin (289.27 → 139.12 for qualitative purposes); 317.24 → 151.11 for myricetin (317.24 → 137.11 for qualitative purposes); 301.24 → 255.52 for quercetin (301.24 → 179.49 for qualitative purposes); 609.52 → 301.52 for rutin (609.52 → 255.52 for qualitative purposes); 231.32 → 128.32 for melatonin (231.32 → 159.17 for qualitative purposes); 479.39 → 317.30 for myricetin-3-*O*-galactoside (479.39 → 171.12 for qualitative purposes); 367.38 → 217.39 for the IS. The precursor ion and the product ion, together with optimised mass spectrometry parameters (dwell time, cone voltage and collision energy) for each compound are given in Table III.4.2.

Method validation

The whole methodology was validated in terms of linearity range, sensitivity, precision, absolute recovery and accuracy, demonstrating reliability and suitability of this analytical strategy.

Good linearity (r^2 > 0.9991) was obtained over the 0.3-100 ng/mL concentration range for all the analytes. LOQ and the LOD were were found as 0.3 ng/mL and 0.1 ng/mL, respectively, for all the analytes. Precision assays were carried out at 0.3, 50 and 100 ng/mL and IS at 1 ng/mL, obtaining good results. RSD values for intraday precision were always lower than 3.7%, interday precision was also satisfactory, with RSD values always lower than 3.9% for all the analytes.

Absolute recovery and precision assays were carried out subjecting to the MEPS procedure previously described, methanolic standard solutions of the analytes corresponding to a low, middle and high point of the respective linearity ranges. Mean absolute recovery was always higher than 87% for all analytes (>90% for the IS). The complete results of these assays are shown in Table III.4.3.

Method accuracy was assessed by means of recovery assays, by adding three different concentrations of each analyte to already analysed samples and calculating analyte recovery values. Mean recovery values were always higher than 95%; thus, method accuracy is satisfactory.

Table III.4.2. Multiple Reaction Monitoring (MRM) optimised parameters (triple quadrupole, ESI-) for the quantitation of 7 representative phenolic compounds in *Argania spinosa* leaf extracts.

Analyte	Parent ion (m/z)	Qualifier daughter ion (m/z)	Quantifier daughter ion (m/z)	Cone voltage (V)	Collision energy (V)
Catechin	289.27	139.12	245.27	43.00	29.00
Epicatechin	289.27	139.12	245.27	45.00	31.00
Myricetin	317.24	137.11	151.11	35.00	23.00
Quercetin	301.24	179.49	255.52	57.00	45.00
Rutin	609.52	255.52	301.52	57.00	39.00
Myricetin-3-*O*-galactoside	479.39	171.12	317.30	43.00	35.00
Melatonin	231.32	159.17	128.32	47.00	27.00
IS (curcumin)	367.38	/	217.39	39.00	27.00

Table III.4.3. Absolute recovery and precision on methanolic standard solutions of 7 selected phenolics.

Compound	Mean extraction yield, % [a]	Intraday precision, RSD% [a]	Interday precision, RSD% [a]
Epicatechin	93	4.1	5.4
Catechin	92	4.3	5.1
Myricetin	92	4.5	5.2
Quercetin	91	4.7	5.3
Rutin	93	3.9	4.8
Myricetin-3-*O*-galactoside	91	4.6	5.0
Melatonin	91	4.9	5.4
Curcumin (IS)	95	3.6	4.4

[a] n = 6

Table III.4.4 Contents of 7 representative phenolic antioxidants in *Argania spinosa* crude and aqueous leaf extracts.

Compound	Crude extract: concentration (µg/g) ± RSD% [a]	Aqueous extract: concentration (µg/g) ± RSD% [a]
Epicatechin	40.3 ± 6.3	55.9 ± 5.7
Catechin	7.7 ± 6.0	10.6 ± 5.5
Myricetin	6.1 ± 6.1	8.7 ± 5.8
Quercetin	4.2 ± 6.3	6.1 ± 5.9
Rutin	2.3 ± 5.8	3.2 ± 5.2
Myricetin-3-*O*-galactoside	16.2 ± 6.2	23.1 ± 5.9
Melatonin	0.2 ± 6.4	0.3 ± 6.0

[a] n = 6

Quantitative results

The validated method was applied to the analysis of *A. spinosa* leaf extracts (both crude and aqueous ones). Quantitative results regarding the analysed leaf extracts are shown in Table III.4.4 and expressed as micrograms of compound contained in a gram of dried sample. Each value is the mean of the results obtained from six analyses.

Briefly, we firstly identified 13 phenolics in two different argan leaf extracts and mainly the crude one and the phenolic-enriched aqueous one. Generally, a range of phenolic-enriched plant extracts is widely marketed for their antioxidant properties and the associated beneficial health properties. Interaction of phenolic constituents with free radical provides different perspectives on their antioxidant/pro-oxidant properties.

As shown in Table III.4.4, argan leaf is rich in phenolics and in particular the aqueous extract is richer than the crude one: 7 phenols of 13 compounds identified (i.e. epicatechin, catechin, myricetin, quercetin, rutin, myricetin 3-*O*-galactoside and melatonin) were quantified by LC-MS/MS analysis in both leaf extracts. Epicathechin and myricetin 3-*O*-galactoside were the most abundant of total phenols, up to 55.9% and 23.1%, respectively.

Within the framework of this research, melatonin has been found in argan leaves for the first time, while its presence is already reported in other vegetal matrices [112-114]. Considering that melatonin seems to possess antioxidant and free radical scavenging actions, which are independent from those mediated by receptors, it can be regarded as an important index to the quality control of argan products.

The obtained results show how the quali-quantitative analysis of certain compounds such as phenolics can give key information concerning the bioactive potential of argan plant parts other than kernel, pulp of fruit and trunk. In this perspective, the compounds identified and quantified in *A. spinosa* leaves could contribute to a further and complete understanding of the bioactive profiling of this plant and to the valorisation of its by-products as a source of potentially beneficial bioactive molecules.

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The preliminary results obtained during this research were presented as contributions to symposium:

Laura Mercolini, Michele Protti, Maria Addolorata Saracino, Manuela Mandrone, Ferruccio Poli

BIOACTIVE PROFILING OF ARGAN (*ARGANIA SPINOSA*) LEAF EXTRACTS BY MASS SPECTROMETRY

XIV Sigma-Aldrich Young Chemists Symposium - SAYCS 2014
Riccione, 2014, October 27-29 (Abstract Book POS-33)

Michele Protti, Maria Addolorata Saracino, Ferruccio Poli, Laura Mercolini

MEPS-HPLC-DAD-MS/MS FOR THE ANALYSIS OF BIOACTIVE TRITERPENIC AND PHENOLIC COMPOUNDS IN *ARGANIA SPINOSA* LEAVES

XIV Giornata di Chimica dell'Emilia Romagna
Verso l'Expo 2015: nutrizione e sicurezza alimentare
Parma, 2014, December 18, Centro Congressi Campus Universitario, Parco Area delle Scienze (Abstract book P45)

Complete results of this research were published as a full paper:

Laura Mercolini, Michele Protti, Maria Addolorata Saracino, Manuela Mandrone, Fabiana Antognoni, Ferruccio Poli

ANALYTICAL PROFILING OF BIOACTIVE PHENOLIC COMPOUNDS IN ARGAN (*ARGANIA SPINOSA*) LEAVES BY COMBINED MICROEXTRACTION BY PACKED SORBENT (MEPS) AND LC-DAD-MS/MS

Phytochemical Analysis 27, 41-49 (2015)
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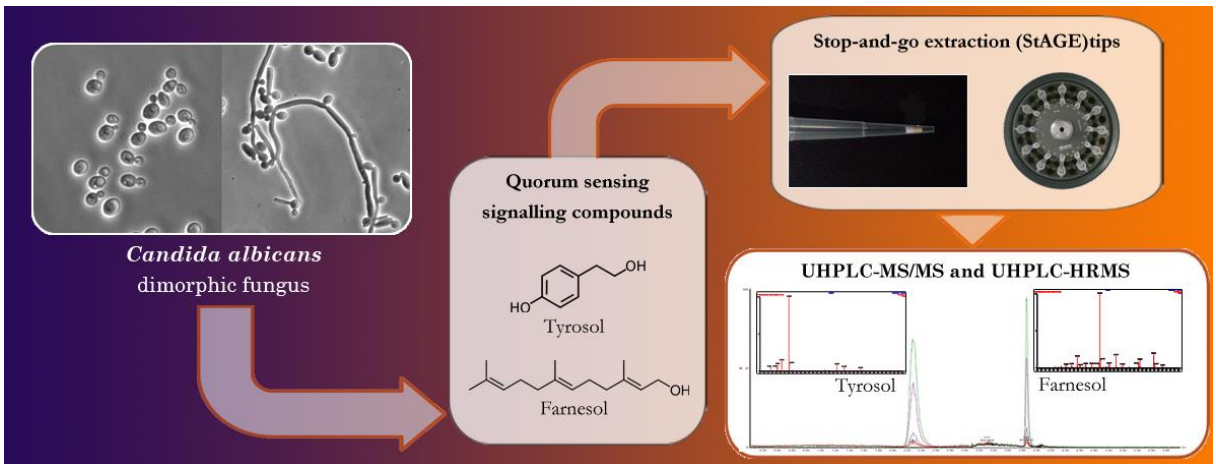
5. Development of StAGE tips sample pretreatment for UHPLC-MS/MS and UHPLC-HRMS analysis of *Candida albicans* quorum-sensing molecules



5.1 Abstract

Candida albicans is one of the fungal pathogens most commonly isolated from the human body. It is a frequent cause of nosocomial infections, bloodstream infections, urinary infections and mucosal infections of the oral cavity and the vagina. *C. albicans* can grow as hyphae, pseudohyphae, or budding yeast. Morphological conversion of a yeast form to pseudohyphal or hyphal one is often characterized by the change from the commensal status to an invasive form. Farnesol and tyrosol can participate in these transformation processes as quorum sensing molecules together with some other physical-chemical factors.

A new analytical method for the identification and quantification of the biologically active substances farnesol and tyrosol was developed using ultra high performance liquid chromatography (UHPLC) in connection with tandem mass spectrometry in multiple reaction monitoring (MRM) mode and high resolution mass spectrometry (HRMS).

The analytes were separated on an Acquity BEH C18 analytical column using an optimised composition gradient program in order to shorten total analysis times.

Moreover, a novel and high-throughput sample pretreatment approach based on stop-and-go extraction (StAGE) was developed and optimised.

The method was validated in terms of linearity (>0.9994), precision (0.5–3.8% RSD), accuracy (78.9–106.0%), LOD (limit of detection) and LOQ (limit of quantitation). The method can be considered an analytical tool for the detection and determination of quorum-sensing molecules in biological samples.

5.2 Introduction

The polymorphic fungus *Candida albicans* is one of the most important yeast in medicine. It is a member of the indigenous microbiota of mucosa and skin in humans and animals and is thought to be acquired during passage through the birth canal. *C. albicans* has been recently used as a model for studying the basic biology of fungi as well. For fungi exhibiting yeast-mycelium dimorphism the dependence of cell morphology on initial cell density has been termed an inoculum size effect. The inoculum size effect in the dimorphic yeast results from the production of quorum-sensing molecules (QSMs). The QSMs indentified in *C. albicans* are farnesol, farnesic acid and tyrosol [114-119].

QSMs are extracellular chemical signals and are produced continuously in response to an increasing density of microbial population to coordinate cell activity. Their production is usually not dependent on the type of carbon source or nitrogen source or on the chemical nature of the growth medium. In general, these signals can regulate some important virulence, morphological and physiological properties through the activation of suitable genes. Majority of studies concern the QSMs of bacteria [120,121], but there is a growing number of reports about QSMs in fungi, especially yeasts. The main two QSMs in *Candida* under study are farnesol and tyrosol; their structures are shown in Figure III.5.1. While farnesol blocks the yeast-to-mycelial dimophic transition of *C. albicans*, tyrosol supports the development of the filamentous form of this yeast [118,119,122].

Originally, analytical methods for the determination of farnesol and tyrosol were developed separately. Farnesol was first discovered to be a QSM of *C. albicans* [114], while tyrosol function in the QS was described later [115]. It would be however highly convenient to develop one analytical method being able of simultaneous determination of farnesol and tyrosol, because such method would give direct information about the concentrations of both QSM independently of sample origin and thus enable better identification of cell morphology and description of invasive stadium. This could be helpful in diagnostic and treatment approaches, e.g. in vaginal candidosis.

Farnesol is a volatile molecule of terpenoid structure, therefore GC-MS (gas chromatography with mass spectrometry detection) was often a method of choice for its determination [114,123,124]. Both chemical ionization and electron ionization were employed. The cited methods however were developed only for qualitative purposes to confirm a presence of farnesol molecule in various matrices including *C. albicans* strains [114] or tobacco smoke [124]. Further, HPLC-UV was used for identification purposes using retention times [114], however in complicated matrices this approach might lack in selectivity. Further, farnesol metabolites (farnesyl-glucuronide) and farnesol with other related compounds were determined using HPLC in connection with ESI-MS (electrospray ionization mass spectrometry) [125,126]. Ionization was typically performed in negative ion mode. $[M-H]^-$ served as a precursor ion for further MS/MS quantitation of farnesol or its glucuronide. According to our knowledge, only one method was developed for quantitative purposes and fully validated for the determination of farnesol in rat liver and testis [125]. ESI-MS/MS is highly advantageous from selectivity and sensitivity point of view, but the procedure reports a necessary derivatization step in order to reach sufficient sensitivity, which complicates the method and makes the application time-consuming. No fast, simple and sensitive method for the qualitative and quantitative analyses of farnesol was found in the literature.

Tyrosol was often determined with other phenolic compounds in virgin olive oils [127] and [128] or in human LDL-fraction after digestion of olive oil [129] and [130] or in olive mill wastewaters [131]. HPLC using UV (typically 280 nm) or MS detection was a method of choice [127-135]. MS detection is unequivocally more convenient, because it provides better sensitivity and selectivity especially in complicated matrices. Most of HPLC methods were developed and fully validated for quantitative purposes using ESI-MS/MS the provides very high selectivity in complicated matrices [129,131-133]. ESI was performed in negative ion mode using $[M-H]^-$ as a precursor ion for further SRM (selected reaction monitoring) quantitation. The typical sensitivity of such methods reached LOQ

values of about 1 ng/ml. Other methods for the determination of tyrosol employed GC-MS with EI [21] or MEKC (micellar electrokinetic chromatography) [135].

The aim of this work was to develop fast, reliable, sensitive and selective analytical method for the simultaneous determination of farnesol and tyrosol as quorum-sensing molecules of *C. albicans* using UHPLC-MS/MS method.

Moreover, a simple yet effective micro-solid phase extraction (μSPE) based on stop-and-go extraction (StAGE) workflow [136] was developed and optimised for the first time in order to be applied for the simultaneous extraction and purification of QSM from biological samples.

Such a method is necessary to evaluate useful correlations of QSM concentration with clinical status of patients with candidosis. High selectivity and sensitivity was assured by MS/MS while UHPLC increased method speed and efficiency.

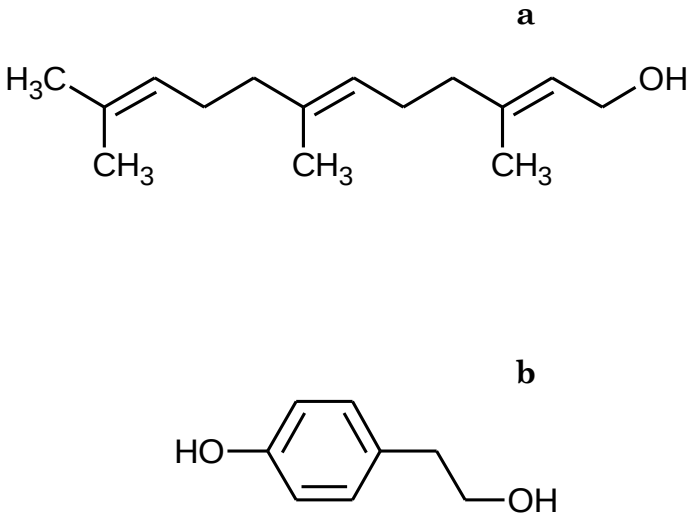


Figure III.5.1

Chemical structures of farnesol (a) and tyrosol (b).

5.3 Experimental

5.3.1 Chemicals and standard solutions

Working standards of farnesol (trans,trans-3,7,11-trimethyl-2,6,10-dodecatrien-1-ol) and tyrosol (2-(4-hydroxyphenyl) ethanol), ≥99.5%, were obtained from Sigma-Aldrich (Prague, Czech Republic).

Acetic acid and formic acid reagent grade and acetonitrile LC–MS grade, were purchased from Sigma-Aldrich. HPLC grade water was prepared by Milli-Q reverse osmosis Millipore (Bedford, MA, USA).

5.3.2 Chromatography

Acquity UPLC (ACQ) (Waters, Prague, Czech Republic) system for UHPLC (ultra high performance liquid chromatography) was used for the purposes of this study. It consisted of ACQ-binary solvent manager, ACQ-sample manager and ACQ-tunable UV detector. All UHPLC analyses were performed on BEH C18 analytical column (100 mm × 2.1 mm, 1.7 µm, Waters, Prague, Czech Republic) based on Bridged Ethyl Hybrid (BEH) particles. Mobile phase was composed of 0.075% (v/v) formic acid in acetonitrile (A) and 0.075% (v/v) formic acid in water (B), flowing at 0.20 mL/min. The gradient program of the mobile phase composition started with a 8:92 (v/v) A/B ratio and maintained for 2.0 min, then ramped up linearly to 92% (v/v) of A over 0.2 min; this ratio was maintained for 0.9 min and then ramped down linearly to 8% (v/v) of A over 0.2 min. This ratio was then maintained for 1.5 minutes for column reconditioning.

The analytical column was kept at 35 °C by column oven. Partial loop with needle overfill injection mode was set up to inject 3 µl using 5 µl injection loop. Acetonitrile was used as a strong wash and 20% acetonitrile in water was used as a weak wash solvent.

5.3.3 Mass spectrometry

A Quattro Micro (Micromass, Manchester, UK) triple quadrupole tandem mass spectrometer equipped with a multi-mode ionization source (ESCI) was used in this study. This ion source enables high-speed switching between electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) within one ion source. Ion source set-up was tuned as follows: capillary voltage: 3200 V, ion source temperature: 130 °C, extractor: 3.0 V, RF lens: 1.0 V. The desolvation gas was nitrogen at flow 500 l/h and at the temperature 450 °C. Cone voltage was set up individually for each analyte. Nitrogen was used also as a cone gas (120 l/h) to prevent the contamination of sample cone. Quantitation of all analytes was performed in ESI positive ion mode using SRM (selected reaction monitoring) experiment. Two specific transitions were optimized for each molecule in order to increase method selectivity. Argon was used as collision gas and collision energy was optimized for each analyte individually.

Concurrently, a high resolution mass spectrometer (HRMS) Synapt G2Si (Waters, Manchester, UK) based on IM-Q-TOF-MS was used for comparison in order to investigate its potential in terms of sensitivity and selectivity for the simultaneous determination of farnesol and tyrosol. The HRMS setup was the following: electrospray voltage 0.8 kV; cone voltage 40 V; ion source temperature 140 °C; cone gas flow 50 L/h; desolvation gas 800 L/h; EDC in traveling wave ion guide was set to 410 m/z. Synapt G2Si was operated in V optics mode (sensitivity mode) with resolution greater than 10 000 at full-width at half-maximum. MS was calibrated to less than 2 mDa mass error prior to each batch. All analyses were acquired using LockSpray to ensure accuracy and reproducibility Leu-enkephalin and sodium formate were used as a lock mass reference and calibrant, respectively; data acquisition rate was 0.15 s/scan, with a 0.024 s interscan delay; spectra were detected within the range 50 - 1200 m/z. The analytes were identified by exact mass of precursor ion and retention time. Tolerance was set to 30 mDa for the precursor ion and ±0.2 min for the retention time. The following ions were chosen: tyrosol at m/z 121.0592 with retention time 2.68 min and farnesol at m/z 205.1792 with retention time 3.87 min. All ions are

the $[M+H-H_2O]^+$ molecular entity. MassLynx 4.1 Data System was used for data MS control and data gathering. QuanLynx software was used for data processing and quantitation-regression analysis of standard curves and calculation of concentrations.

Chromatographic setup was the same for both mass spectrometric systems, as described in section 5.3.2.

5.3.4 Preparation of standard solutions

The stock solutions of standards were prepared by dissolving of the amount corresponding to 1 mg/mL concentration of appropriate working standard into 1.0 ml of pure acetonitrile. Stock solutions were further diluted with a mixture composed of 0.075% formic acid in water and acetonitrile (80/20, V/V) to achieve a concentration of 100 ng/mL for SST (system suitability tests) measurements and to get individual points of calibration curve in the range 12.5-1000 ng/mL for tyrosol and 20-1000 ng/mL for farnesol, using seven calibration points.

5.3.5 StAGE tips preparation

StAGE tips were prepared by punching 3M Empore material (Minneapolis, MN) with a gauge needle and stacking the disks into 200 µL pipette tips. For this research, self-produced StAGE tips composed by three layers of poly (styrene-divinylbenzene) material and three layers of C8 materials were used.

For this work, 1.5 mL plastic microtubes were suitably perforated and used as pipette tip adaptors to simultaneously process multiple samples by centrifugation, making the whole unit spinnable.

In order to apply the pretreatment procedure through StAGE tips, the obtained cartridges have been embedded within a 1.5 mL plastic microtubes through suitably perforated cap. All pretreatment steps (loading, washing and elution) were performed by loading the liquid from the top of the pipette tips. Then, the devices were housed inside a table centrifuge and the flow of solutions through the absorbent bed was obtained through centrifugal force. Solutions were directly collected inside the microtubes and discarded or collected after each centrifugation step.

Type and amount of the absorbent material layers, volume and nature of the used solutions, centrifugation time and speed were carefully optimised in order to obtain the best results in terms of sample purification and absolute recovery of the analytes considered for this work.

5.3.6 Sample pretreatment

Biological samples were obtained in 20 mM, pH 5.0 phosphate buffer veiculum and stored at -80 °C until analysis. 100 µl of sample was used for each analysis, prepared in triplicate and subjected to StAGE procedure as follows: packed tips were embedded into 1.5 mL microtubes and activated by loading 300 µL of methanol into the tips, spin at 4500 rpm for 1 min, then conditioned with 300 µL ultrapure water containing 0.075% formic acid, spin at 4500 rpm for 2 min; 100 µl of sample were loaded into the tip and spinned at 3500 rpm for 5 min;

samples were then washed in two steps with 100 µl of ultrapure water and 100 µl of a mixture containing 0.075% formic acid in water and methanol (98:2, v/v), spinning at 4500 rpm for 2 min; elution was performend with 200 µl of a mixture containing 0.075% formic acid in water and acetonitrile (5:95, v/v) spinned at 3500 rpm for 5 min; the eluate was then transferred in a 250 µL low volume insert vial, brought to dryness by means of a Mini-Vap evaporator/concentrator (Sigma–Aldrich, Prague, Czech Republic) and reconstituted with a mixture containing 0.075% formic acid in water and acetonitrile (80:20, v/v) before injection into UHPLC system. The centrifuge temperature was maintained at 4°C throughout the whole pretreatment procedure.

5.3.7 Method validation

Calibration curves of both analytes in the concentration range 12.5-1000 ng/mL for tyrosol and 20-1000 ng/mL for farnesol were measured using mixed standard solutions in a mixture composed of 0.075% formic acid in water and acetonitrile (80/20, V/V). Lower limit of quantitation and limit of detection were established based on signal-to-noise (S/N) ratio approach. Limit of detection was expressed as S/N = 3, lower limit of quantitation was expressed as S/N = 10. Method precision and method recovery were established using StAGE tips pretreatment procedure as an inherent part of the method. For precision, simulated biological samples (veiculum, 10 mM, pH 5.0 phosphate buffer) spiked at three different concentration levels in the linearity range, were subjected to the pretreatment procedure described previously and measured in three replicates to calculate RSD%. Method accuracy was determined by spiking already analysed samples withanalyte solutions at three different concentration levels in three replicates in order to establish the closeness of agreement between the true and measured value.

5.4. Results and discussion

5.4.1 Ultra high performance liquid chromatography and (high resolution) mass spectrometry

UHPLC was used as separation method for the analysis of farnesol and tyrosol under isocratic conditions and an Acquity BEH C18 was chosen for the separation, taking into account the physical–chemical properties of the analytes. Because of the large physical-chemical differences of the two analytes, a mobile phase composition gradient program was carefully optimized: a highly hydrophilic ratio was necessary as the starting point of the gradient in order to delay the peak of tyrosol from the solvent front. thus, the starting point chosen was H_2O/ACN 92/8 (V/V); this ratio allowed the elution of tyrosol at about 2.5 minutes, an acceptable time with the perpective of method application to biological samples, where the more hydrophilic matrix components have to elute without causing interference. Several composition gradients were tested in order to anticipate as much as possible farnesol peak and reduce the overall chromatographic run time. Only few additives are volatile enough to enable sensitive mass spectrometric response. Formic acid and acetic acid at different concentrations were tested in this study. In compromise of the response of mass spectrometer, analysis time and resolution, formic acid 0.075% (v/v) added to both components was finally chosen as mobile phase additive. The flow-rate was 0.2 ml/min in accordance with the requirements of electrospray ionization.

As regards solvent composition of the injected solutions, it was observed that a mixture composed of 0.075% formic acid in water and acetonitrile (80/20, V/V) was the best compromise for peak shape, resolution and signal-to-noise ratio. In fact, assays carried on using lower contents of organic solvent led to lower signal intensity for farnesol, while more hydrophilic solvents resulted in tyrosol peak shape worsening.

Since farnesol and tyrosol are small molecules of not substantial polarity, both ESI +/− ionization modes could be expected as convenient. As reported previously [137] and confirmed in this study, the protonated molecule $[M+H]^+$ was not

observed in full-scan spectra. The analytes directly fragmented in the ion source even under mild ionization conditions, thus both farnesol and tyrosol provided a precursor ion $[M+H-H2O]^+$ in positive ion mode, while in negative ion mode $[M-H]^-$ was observed. The best response and S/N ratio was obtained in ESI positive ion mode, thus precursor ion $[M+H-H^2O]^+$ was further used for quantitation and fine tuning of all parameters of mass spectrometer (see Figure III.5.2).

As regards triple quadrupole mass spectrometry (MS/MS), quantitation of both analytes was performed in ESI positive ion mode using MRM mode. Two specific transitions (quantitative and confirmatory) were optimized for both molecules in order to increase selectivity and identification value of the method. Product ions were chosen according to the fragmentation pathways in product ion scan mode. Cone voltage and collision energy were optimized for each analyte and for each of its two transitions individually in order to obtain the highest sensitivity (see Table III.5.1). Figure III.5.3 shows an LC-MS/MS chromatogram of standard mixtures containg tyrosol and farnesol at different concentrations and injected under the optimised conditions

As regards high-resolution mass spectrometry (HRMS), electrospray ionization combined with time of flight mass spectrometry (ESI-TOF-MS) is a powerful technique to analyse and identify many different compounds like peptides, proteins, phytonutrients and carbohydrates as present in complex mixtures. With the ion mobility (IM) cell, the range of molecules that can be analysed is extended to the analysis of isomeric compounds and the combination with an electron transfer dissociation (ETD) cell also can improve the identification of multiple charged molecules.

Table III.5.1. Optimized mass spectrometry parameters of tyrosol and farnesol

Compound	Tyrosol MW= 138.07		Farnesol MW= 222.20	
Precursor	121.2		205.3	
Precursor type	$[M+H-H2O]^+$		$[M+H-H2O]^+$	
SRM	SRM1	SRM2	SRM1	SRM2
Fragment	77.1	93.0	121.1	109.1
Dwell time	0.2	0.2	0.2	0.2
Cone voltage	25	25	20	20
Collision energy	20	10	10	10

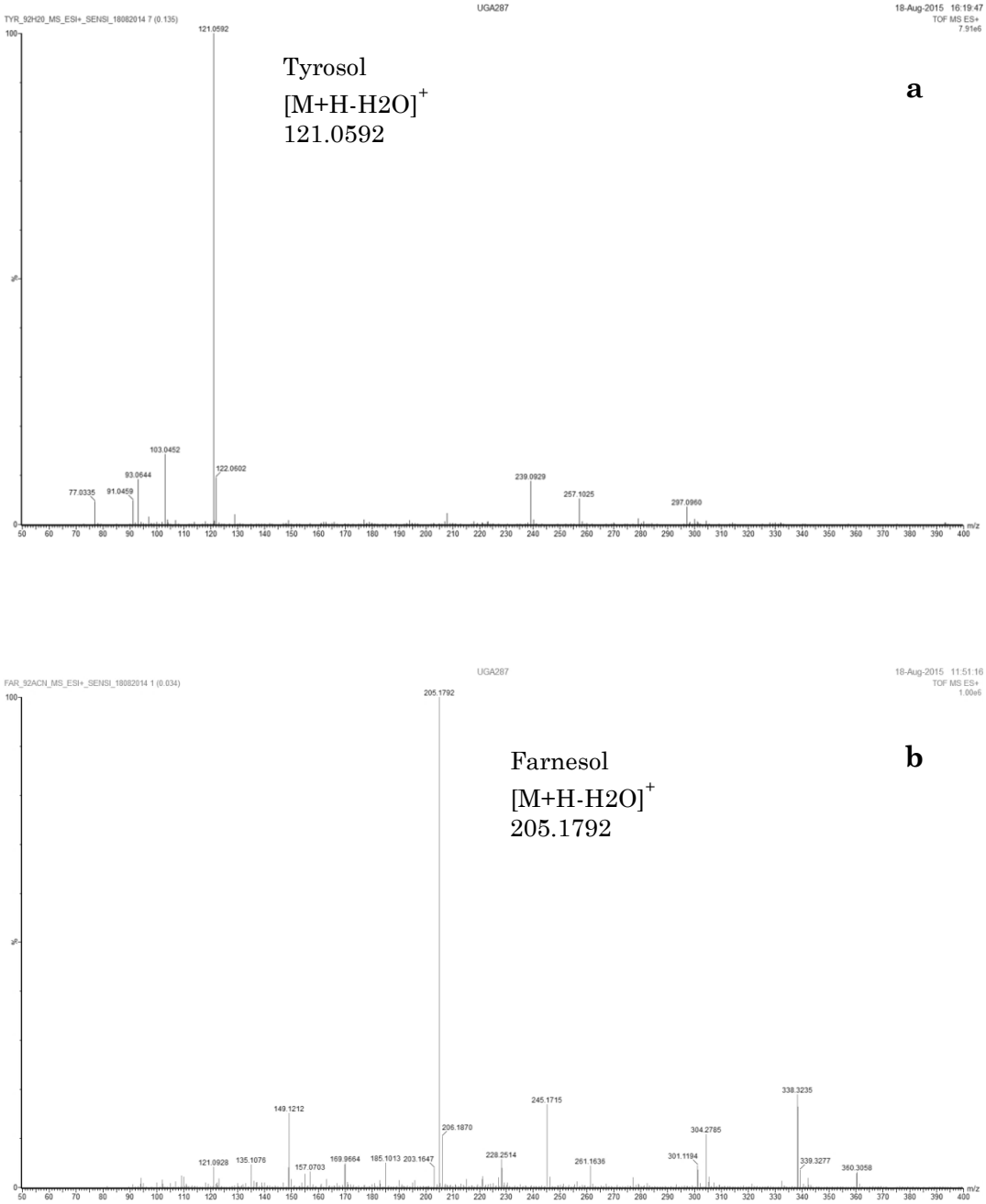


Figure III.5.2

Full MS spectra obtained by direct infusion of (a) tyrosol and (b) farnesol into the IM-Q-TOF-MS system

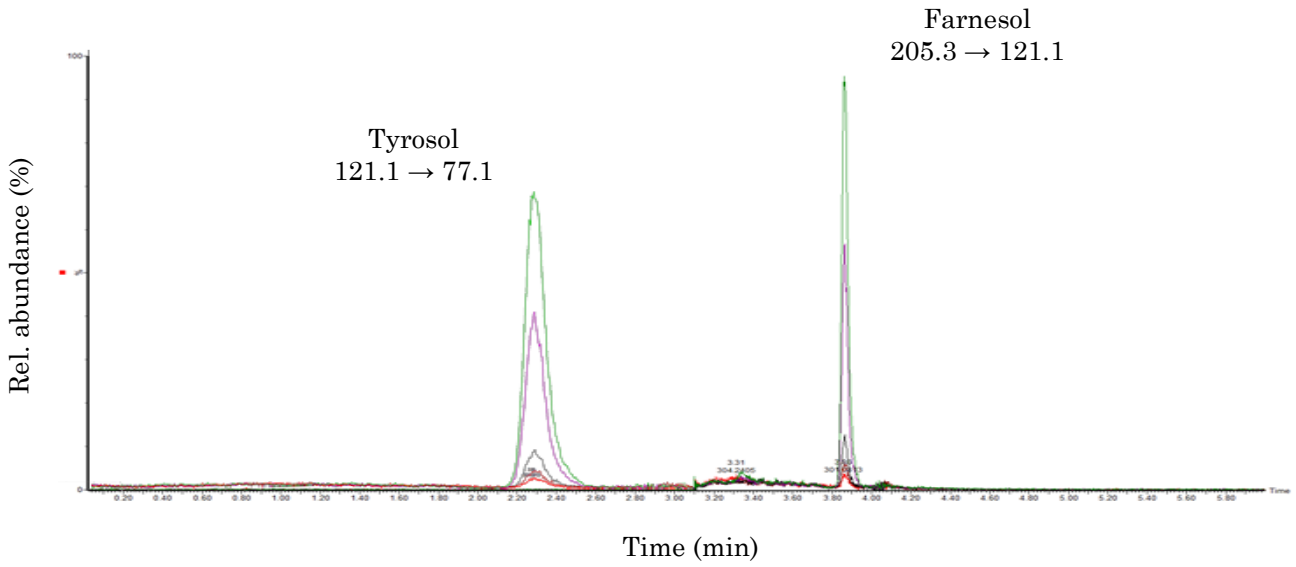


Figure III.5.3

LC-MS/MS chromatogram of standard mixtures containg tyrosol and farnesol at different concentrations

5.4.2 StAGE tips procedure optimisation

Stop-and-go extraction (StAGE) tips are conceptually similar to solid phase extraction cartridges in which teflon embedded chromatographic beads are immobilized inside the tapered ends of pipette tips.

To process samples using StAGE tips, originally a plastic syringe was commonly used to manually force solutions through the tips. This sounds relatively easy when dealing with only a few samples (<10). However, when tens of samples have to be processed, manual push with a syringe pump is impractical and impairs throughput. Thus, or this work, 1.5 mL plastic microtubes were suitably perforated and used as pipette tip adaptors to simultaneously process multiple samples by centrifugation, making the whole unit spinnable.

StAGE tips allow for high loading capacity, high loading speed, small elution volumes and reproducible production. They are also very economical to produce as thousands of StAGE tips can be produced from a single, readily available teflon membrane containing the separation material of choice. A key property of the disks used for StAGE tips is that the beads are embedded in a teflon matrix and are therefore fixed in place. This prevents the formation of primary flow channels, which is the largest limitation of microcolumns based on loose beads. Furthermore, the teflon matrix allows for the creation of multi-functional columns without the risk that beads of different functionality will mix at the interface. Disks containing beads with different functionalities, such as C18, C8, strong cation exchange (SCX), strong anion exchange (SAX), activated carbon and poly-(styrene-divinylbenzene) copolymer, can be stacked upon each other as needed.

For this study, type and amount of the absorbent material layers, volume and nature of the used solutions, centrifugation time and speed were carefully optimised in order to obtain the best results in terms of sample purification and absolute recovery of the analytes considered for this work.

Several types of sorbent materials, alone and in combination were tested, as well as the effect of the number of layers stacked within the pipette tips.

Furthermore, the performances obtained from handcrafted devices were compared with those of commercially available ones.

The best results in terms of absolute recovery and sample clean-up were obtained with handcrafted devices and it is noteworthy that the latter showed better stability during the pretreatment step under the action of centrifugal force, while for commercial devices it was often observable partial losses of the sorbent bed, leading to unsatisfactory assays.

As regards sorbent type, the best results were obtained with StAGE tips composed by three layers of C8 sorbent in combination with three layers of poly-(styrene-divinylbenzene) sorbent. This hydrophilic-lipophilic combination has proved to be the best compromise for the simultaneous isolation of compounds characterized by different lipophilicity as tyrosol and farnesol. In fact, other combinations tested were found to be unbalanced towards only one analyte.

All the parameters involved in the pretreatment steps have been carefully optimized, i.e. volume of loaded sample; washing cycle number, nature and volume; elution solvent characteristics. The best results in terms of sample clean-up, absolute recovery and total analysis time were obtained with a protocol involving 100 µL of sample loading, two washings consisting of 100 µl of ultrapure water and 100 µl of a mixture containing 0.075% formic acid in water and methanol (98:2, v/v) respectively and finally the elution with 200 µl of a mixture containing 0.075% formic acid in water and acetonitrile (5:95, v/v). All pretreatment parameter optimization assays and results are summarized in Figure III.5.4. Centrifuge speed and time for each step were optimized based on the volumes and nature of loaded solutions. The evaluated parameters were the StAGE tips and sorbent bed stability and total analysis time, without compromising analyte absolute recovery. It was observed that a speed between 3500 and 4500 rpm for a time comprised between 2 and 5 minutes was enough to complete all sample pretreatment steps. This was true for handcrafted StAGE tips, while commercially ones often showed to be unstable with partial loss of the sorbent bed. Centrifuge temperature was always maintained at 4 °C to ensure maximum sample stability. Finally, it is noteworthy that long drying times at reduced pressure led to a reduction in absolute recovery, probably due to the

volatile nature of the analytes. This phenomenon has been minimized through the use of an effective nitrogen flow by means of a mini-vap apparatus, thus limiting drying time as much as possible.

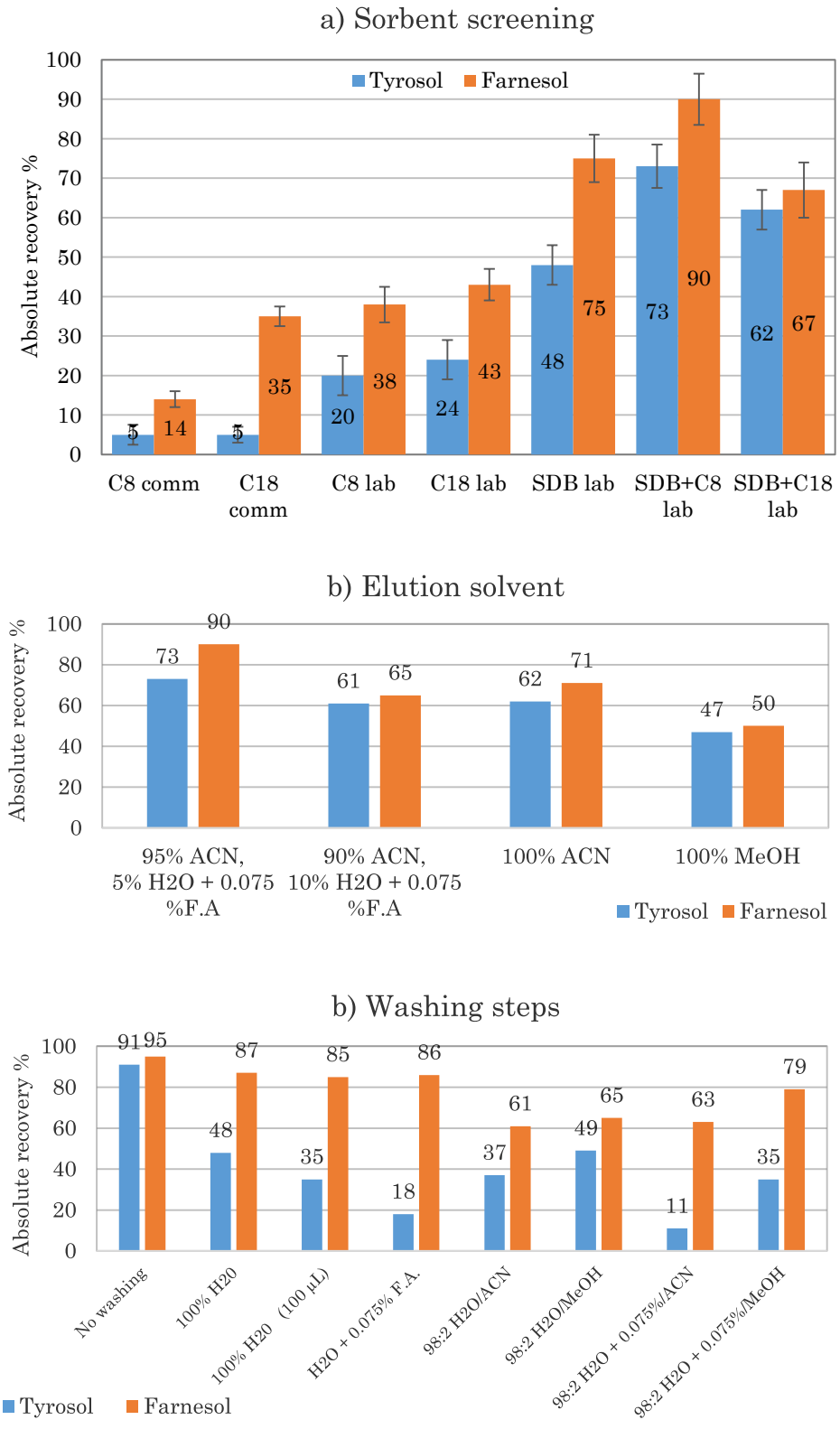


Figure III.5.4

StAGE pretreatment parameter optimization

5.4.3 System suitability test and validation

The SST was performed by 10 subsequent injections of standard mixture of farnesol and tyrosol at the concentration 50 ng/mL. The repeatability of retention times and peak areas was checked and it was expressed as RSD in %. Excellent repeatability of injection was obtained for both retention time (RSD < 1%) and for peak area (RSD < 4%) (see Table III.5.2).

Linearity-calibration range

Calibration curves of farnesol and tyrosol were measured in the concentration range 12.5–1000 ng/mL for tyrosol and 20–1000 ng/mL for farnesol, using seven calibration points. For both analytes the response was linear in tested concentration range ($r^2 > 0.9994$) as it can be seen in Table III.5.2.

Limits of detection and quantitation

LOD and LOQ were calculated based on S/N ratio. They were established first using standard solutions in mobile phase by the injection of the smallest amounts which provided S/N = 3 for LOD and S/N = 10 for LOQ. Subsequently this was confirmed by measurements in real matrix, which provided similar values. The results are displayed in Table III.5.2. The method had good sensitivity to be able to perform the determination of farnesol and tyrosol in biological samples reaching LOQ 12.5 ng/mL for tyrosol and 20 ng/mL for farnesol and LOD 4 ng/mL for tyrosol and 6 ng/mL for farnesol.

Recovery and precision

Recovery and precision were established by spiking surrogate matrix (buffered veiculum) at three concentration levels of the calibration curve using the sample preparation steps described. Through the optimized StAGE tips pretreatment procedure, it was possible to obtain absolute recovery always higher than 67% for tyrosol and 72% for farnesol. Method precision was determined as intra-day variability of three determinations at three different concentration levels expressed as RSD%, see Table III.5.2. Intra-day precision was generally within

4% RSD for both analytes farnesol and tyrosol. Method accuracy ranged from 94.1 to 106.0% for farnesol and 78.9–83.2% for tyrosol.

Table III.5.2. System suitability and validation results

System suitability test		Tyrosol	Farnesol
t_R (min)		2.39	3.86
Repeatability t_R (RSD%)		0.41	0.12
Repeatability A (RSD%)		2.9	2.2
Method validation		**Tyrosol**	**Farnesol**
Linearity (r^2)		0.9993	0.9998
Repeatability of slope (RSD%)		6.2	5.8
LOD (ng/mL)		4.0	6.0
LOQ (ng(mL)		12.5	20.0
Accuracy (%)	L1	84.3	89.3
	L2	86.2	91.0
	L3	88.6	92.1
Precision (RSD%)	L1	4.1	4.9
	L2	2.7	3.4
	L3	2.2	1.9

5.5 Conclusion

An UHPLC-MS/MS method was developed for the simultaneous determination of farnesol and tyrosol. The method is fast, sensitive and selective and it was proven to be applicable for the analysis of biological samples. MS/MS detection utilized two m/z transitions for each compound to ensure high selectivity and reliability of the method.

In order to minimize the required amount of biological samples and maximize sample preparation throughput, a feasible yet advanced miniaturized pretreatment procedure based on StAGE tips was developed, as a novel, simple yet effective strategy for complex matrix clean-up analysis. The method was validated according to the requirements of ICH with good results for linearity (>0.9994), precision (RSD < 4% for both analytes), absolute recovery (>67% for tyrosol and >72% for farnesol) and accuracy (79–106%). Analytes could be quantified with typical LOQ 12.5 ng/mL for tyrosol and 20 ng/mL for farnesol. The sensitivity was satisfactory for the intended purpose, as monitored QSM were present in biological samples at sufficient concentrations.

Method applicability was successfully tested in some real samples (vaginal washings) and provided results which can be helpful in the study of role of QSMs in potentially pathogenic yeasts.

5.5 Acknowledgements

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The preliminary results obtained during this research were presented as a contribution to symposium:

Michele Protti, Veronika Pilařová, Pavel Svoboda, Laura Mercolini, Lucie Nováková

DEVELOPMENT OF STAGE TIPS SAMPLE PRETREATMENT FOR UHPLC-MS/MS ANALYSIS OF CANDIDA ALBICANS QUORUM-SENSING MOLECULES

Oral Communication

XV Sigma-Aldrich Young Chemists Symposium - SAYCS 2015
Rimini, 2015, October 27-29 (Abstract Book p. 51, OR-34)

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Chapter IV

Conclusion

The research activity performed during this PhD Programme was devoted to the development, validation and application of original methods for the quali-quantitative analysis of compounds with potential biological activity in natural matrices, foods, drinks and related products, as well as the analytical evaluation of plant by-products from cosmetic manufacture. The study regarded herbs (i.e. *Argania spinosa*), fruits (i.e. *Citrus* × *myrtifolia*, *Punica granatum*) and berries (i.e. *Myrtus communis*) analyzed by high performance liquid chromatography and ultra-high performance liquid chromatography coupled to spectrofluorometric detection, triple quadrupole and high-resolution triple quadrupole mass spectrometry (HPLC-F, LC-MS/MS and UHPLC-HRMS). Significant efforts have been put also into the development and optimisation of miniaturised sample pretreatment strategies, such as micro-solid phase extraction (μSPE) and micro-extraction by packed sorbent (MEPS), for effectively purify complex matrices of natural origin (whole fruits, fruit parts, leaves and their extracts) and derived commercial products (fruit juices, soft drinks and liqueurs).

Citrus fruits, fruit parts and beverages were investigated by SPE-HPLC-F and LC-MS/MS in order to test their nutraceutical potential and to verify their genuineness. Bioactive substances have been identified as markers to verify the actual natural derivation of the extracts used to produce the related soft drinks. Such investigation could valorise niche products, enhance food quality, or otherwise detect adulteration, sophistication or fraud.

Several classes of active compounds such as aminoacids, phenolic acids, flavanols, flavonols and phytohormones were quantified in red fruits and berries of everyday consumption or more difficult to find on the market. The obtained results provided preliminary key information about raw fruits, essential for their nutraceutical profiling, thus suggesting that these fruits are good sources of phytochemicals, and may therefore provide health benefits. Moreover, bioactive

composition is both complex and unique, thus being suitable as a fingerprint for quality control purposes: the analytes taken into account can be considered good as markers for the quality monitoring of the entire production chain, from fresh fruits to the commercial beverages.

Moreover, an analytical investigation was carried out on the bioactive composition of argan tree leaves: in particular crude and hydrophilic extracts have been studied in order to obtain a qualitative profile of antioxidant phenolic compounds, by means of structural elucidation based on MS/MS fragmentation patterns. This evaluation could provide important information for a possible use as raw material for cosmetics and food supplements. Moreover, the characterization of phenolic antioxidants represents a first step towards a complete bioactive profiling of this high potential healthy plant, thus suggesting that argan leaf extracts can be considered of interest in the antibacterial chemotherapy of human infections and can also be exploited for a better profitability of the plant.

In the framework of a research period abroad at the Charles University (Hradec Králové, Czech Republic), a project was carried out in order to develop and optimise an advanced, fast and reliable analytical method, based on UHPLC-MS/MS and UHPLC-HRMS for the simultaneous determination of farnesol and tyrosol as quorum-sensing molecules of *C. albicans*. In order to minimize the required amount of biological samples and maximize sample preparation throughput, a feasible yet advanced miniaturized pretreatment procedure based on StAGE tips was developed, as a novel powerful µSPE approach to complex matrix analysis. As *C. albicans* morphological conversion is often characterized by the shift from commensal status to an invasive form and quorum sensing molecules (QSMs) are extracellular chemical signals participating in such transformation processes, the application of the developed method, could allow to study the role of quorum sensing molecules in potentially pathogenic yeasts.