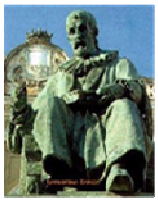
UNIVERSITY OF CALABRIA

FACULTY OF MATHEMATICAL, PHYSICAL AND NATURAL SCIENCES (S.M.F.N)

DEPARTMENT OF CHEMISTRY

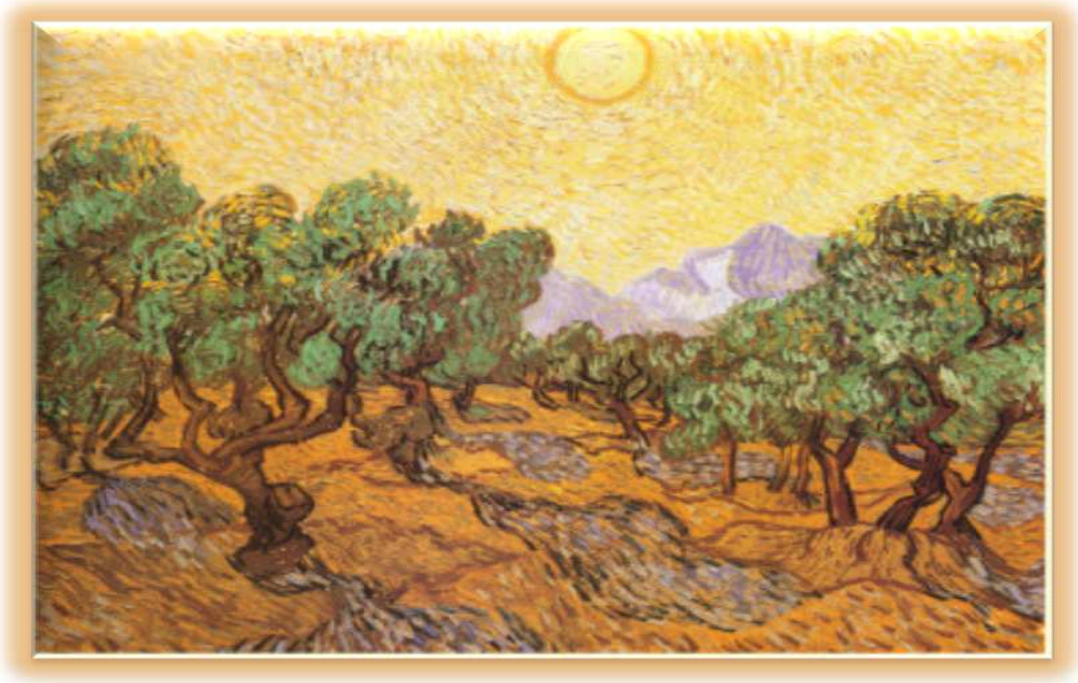
BERNARDINO TELESIO - DOCTORATE SCHOOL OF SCIENCE AND TECHNIQUE (BTSST)



OMPI - ORGANIC MATERIALS OF PHARMACOLOGICAL INTEREST

CHIM 06

XXIV CYCLE



AN INNOVATIVE APPROACH BY MASS SPECTROMETRY FOR THE RECOVERY OF HIGH-VALUE ANTIOXIDANT NUTRACEUTICALS FROM PLANTS, DEVELOPMENT OF NEW FUNCTIONAL FOODS, QUALITY AND SAFETY IN AGRI-FOOD.

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UNIVERSITÀ DELLA CALABRIA

SCUOLA DI DOTTORATO BERNARDINO TELESIO – SCUOLA DI SCIENZA E TECNICA

FACOLTÀ DI SCIENZE MATEMATICHE FISICHE E NATURALI (S.M.F.N.)

DIPARTIMENTO DI CHIMICA

OMPI – MATERIALI ORGANICI DI INTERESSE FARMACEUTICO

CHIM/06

XXIV CICLO

UN APPROCCIO INNOVATIVO DI SPETTROMETRIA DI MASSA PER IL RECUPERO DI NUTRACEUTICI AD ALTO VALORE ANTIOSSIDANTE DA PIANTE, SVILUPPO DI NUOVI ALIMENTI FUNZIONALI, QUALITÀ E SICUREZZA AGROALIMENTARE.

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Anno Accademico 2010/2011

Prefazione

Numerose ricerche hanno evidenziato gli effetti benefici per la salute dovuti alla "dieta Mediterranea" caratterizzata dal consumo di verdure, cereali, pesce e olio d'oliva. La gente che vive nel bacino del Mediterraneo è risultata più longeva e ha mostrato una minore incidenza di malattie cardiovascolari e cancro, grazie ad una alimentazione ricca in composti bioattivi antiossidanti come quelli biofenolici che non possono essere sintetizzati dagli esseri umani e di cui le piante sono particolarmente ricche.

Lo scopo del presente lavoro di dottorato è stato quello di recuperare composti nutraceutici da matrici alimentari (olio, olive e bergamotto) utilizzando delle procedure innovative e dei solventi non tossici e costosi. Il loro recupero oltre a favorire un abbassamento dei costi di smaltimento dei reflui agroalimentari, potrebbe trovare una diretta applicazione in campo cosmetico, farmaceutico e nello sviluppo di nuovi alimenti funzionali arricchiti in composti bioattivi.

Inoltre, le emergenze alimentari che si sono succedute negli ultimi anni, con grande rilievo mediatico, hanno sollecitato una maggiore attenzione ai temi della qualità e della sicurezza, sia nell'opinione pubblica che nel legislatore e negli stessi produttori, mostrando peraltro gli effetti disastrosi sul mercato della mancanza di fiducia nella sicurezza del cibo. Prima dell'adozione di specifiche leggi europee, alcuni Stati Membri – proprio con l'intento di recuperare la fiducia dei consumatori – avevano per lungo tempo legiferato autonomamente sulla qualità e sicurezza del cibo, ma applicando criteri di base diversi. Questa diversità di approcci, unitamente alla totale assenza di legislazione in materia in altri Stati Membri, iniziava però a minacciare seriamente il corretto funzionamento del Mercato Interno. Era necessaria, dunque, la definizione di un sistema di regole e di garanzie comune. È così che si giunse, fra le altre misure, alla creazione di un "sistema di allerta rapido" anche per i prodotti alimentari, sulla falsariga di quello già esistente sulla sicurezza generale dei prodotti (Direttiva (CEE) 29/06/1992 n.59). Un sistema che rendesse possibile un migliore coordinamento delle autorità preposte alla prevenzione e alla gestione delle emergenze e che, all'occorrenza, permettesse l'adozione di misure più efficaci sulla base di informazioni scientifiche precise. Il Parlamento europeo ed il Consiglio adottarono così il Regolamento (CE) 28/12/2002 n.178, in vigore dal Gennaio 2005, che traccia i principi generali e i requisiti di una nuova legislazione alimentare, istituendo l'Autorità Europea per la Sicurezza Alimentare (European Food Safety Agency = EFSA) e definendo procedure in materia di sicurezza degli alimenti. Una delle principali innovazioni del Regolamento consiste nell'imposizione all'industria alimentare della rintracciabilità degli alimenti durante tutte le fasi della filiera produttiva. Una misura atta a permettere, in caso di emergenza, ritiri dal mercato di lotti specifici di prodotti, evitando così interventi più drastici ed inutilmente distruttivi, e contribuendo nel contempo a trasmettere ai consumatori un'immagine di efficienza tale da arginare in qualche modo la loro progressiva perdita di fiducia. L'introduzione dell'obbligo di rintracciabilità, oltre a facilitare il compito delle autorità poste a garanzia della salute dei consumatori (Food Standards Agency), esercita anche un'importante funzione di deterrente a commettere irregolarità o imprudenze nei confronti di tutti gli operatori della filiera produttiva agroalimentare, consentendo di attribuire responsabilità precisamente definite a chiunque abbia preso parte al processo produttivo. A tale scopo, la spettrometria di massa risulta essere un metodo affidabile e sensibile per garantire la qualità e la sicurezza in campo alimentare.

In un'ottica di recupero di risorse e di sostenibilità dei processi produttivi il ritorno a protocolli

ecocompatibili può offrire una migliore qualità del prodotto abbassando i costi di smaltimento dei reflui attraverso il recupero di nutraceutici e nutricosmeceutici ad alto valore aggiunto. Nel campo dei prodotti alimentari, però, la qualità e sicurezza devono essere garantite: il consumatore che per motivi economici è costretto a fare la spesa nei discount o ad orientare i propri acquisti verso prodotti di seconda scelta, non deve trovarsi per questo ad esporre la propria salute a rischi maggiori rispetto a chi compra soltanto prodotti di qualità. Di fatto, nessuna delle norme comunitarie e nazionali in materia di sicurezza degli alimenti opera distinzioni sulla base della qualità o del prezzo: ogni consumatore, indipendentemente dal budget dedicato alla spesa alimentare, ha diritto allo stesso livello di tutela. La sicurezza e la salubrità degli alimenti, infatti, sono diritti di tutti, e sia le attività di prevenzione che di repressione volte a garantirle, devono interessare l'intera filiera di ogni prodotto immesso sul mercato.

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To myself

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Introduction

A number of researches have evidenced the beneficent effects on the health of the "Mediterranean diet" founded on the consumption of vegetables, cereals, fish and olive oil. Studies indicate people who eat a "Mediterranean diet" are less likely to have cardiovascular disease and cancer, and they live longer. It has been estimated that up to 70% of all cancer is attributed to diet.

Biophenolic compounds are natural phenolic biomolecules widely distributed in plants (principally fruits, and in minor amount, flowers, leaves and other vegetable organs) that offer protection against cancer and others disorders. They may occur in various forms: free or in a conjugated form, mainly with a sugar molecule or as esters. With the term biophenols we include not only compounds with phenolic molecular structures, but also related compounds as metabolites or degradation products. The plant phenols being regarded as those substances derived from the shikimate pathway and phenylpropanoid metabolism. Their metabolic pathways are particularly complex with multiple alternative metabolic fates that may vary markedly from tissue to tissue, from one growing condition to another, and in response to environmental stimuli. Factors contributing to the variability in phenolic distribution include the cultivar and genetics, maturity, climate, position on the tree, rootstock and agricultural practices. In the case of processed products, technological processes to which olive fruits are exposed may also impact significantly on the phenolic content.

However, over the past few years, following the growing interest in antioxidant bioactive compounds and their dietary sources has attracted attention as a result of its remarkable flavonoid content. The development of new methods of extraction of nutraceuticals compounds without use of toxic and expensive solvents is crucial for the introduction of new active principles as drugs. Therefore, in this thesys we want to validate modern appropriate extracting procedures, meeting pharmacopoeia requirements, to set-up safety protocols for the recovery of the bioactive compounds present in food (bergamot, olive oils and drupes) using water as extracting solvent. The recovery of compounds of high antioxidant value can be useful as raw materials for the direct application in cosmetics, for the production of food supplements and the development of new functional foods enriched in bioactive compounds.

Also, quality (e.g. olive oils) and safety control and the validation of origin are hot issues in the production of food and its distribution, and are of primary concern to food and agriculture organization. Quality and safety are the two main issues related to genuineness of processed and fresh aliments. In food safety, there are different problems related to chemicals in foodstuffs. Certain food has the potential of containing chemicals which, if eaten in sufficient quantities, are harmful to human health. Other food can be contaminated by illegal dyes. The White Paper on Food Safety outlines a comprehensive range of actions needed to complement and modernize existing EU food legislation. Moreover, the Food Standards Agency aims to protect the consumer from these chemicals, and for this reason must maintain the best knowledge base possible on the subject to provide the necessary tools to ensure that consumer exposure to these chemicals is kept as low as reasonably practicable.

The quantitative LC-MS/MS methodology is utilized in food safety, agricultural and forensic chemistry. The Modern mass spectrometry (MS) provides unique, reliable and affordable methodologies to approach with a high degree of scientific nature any problem which may be

posed in this field.

The production and consumption of food is central to any society, and has economic, social and, in many cases, environmental consequences. Although health protection must always take priority, these issues must also be taken into account in the development of food policy.

1. Liquid chromatographic separations and mass spectrometry

Mass spectrometry is an important tool virtually in all the application of atomic and molecular sciences. In some fields, the practice of mass spectrometry can be described as mature. In others, both the technology and the basic science associated with the application of mass spectrometry are rapidly evolving. The combination of chromatography and mass spectrometry is a subject that has attracted much interest over the last forty years or so because has had a tremendous impact on mass spectrometry with significant time and effort being expanded on improving the mating of the two techniques.[1] Similarly, developments in mass spectrometry have also had a dramatic effect in the separation sciences. One of the most important field in which this combination has had a strong improvement is certainly the agricultural sciences.[2] Tandem mass spectrometry has, in addition to single-stage mass spectrometry, become a particularly important analytical methodology in many application areas like a food safety or in the structure elucidation of unknown compounds from biological samples.[3-4]
Thus, mass spectrometry provides a perspective on the significant changes in strategies to solve specific problems in the life sciences.

1.1 Liquid chromatography

Liquid chromatography (LC), the generic name used to describe any chromatographic procedure in which the mobile phase is a liquid, is used for analysis of complex mixtures of unvolatile samples. Modern high resolution LC (HPLC), has now become firmly established at the forefront of chromatographic techniques. HPLC is used for a wide range of applications and offers significant advantages in the analysis of pharmaceutical formulations, biological fluids, environmental residues and trace element contaminants.[5] Volatility or thermal stability of the analytes is no longer a limit, as in the Gas Chromatographic (GC) applications, thus making LC the method of choice for polymers, polar, ionic and thermally unstable materials. Moreover, sample detection and quantitation can be achieved by means of continuous flow detectors; thus improving accuracy and precision of analysis.

1.1.1 HPLC general features

In HPLC, a liquid sample, or a solid sample dissolved in a suitable solvent, is carried through a chromatographic column by a liquid mobile phase. Separation is determined by solute/stationary-phase interactions.
Different types of columns are available for various types of separation techniques referred as, normal phase, reverse phase, size exclusion, Ion exchange and affinity chromatography. In each case, however, the basic instrumentation is essentially the same(fig 1.1.1).

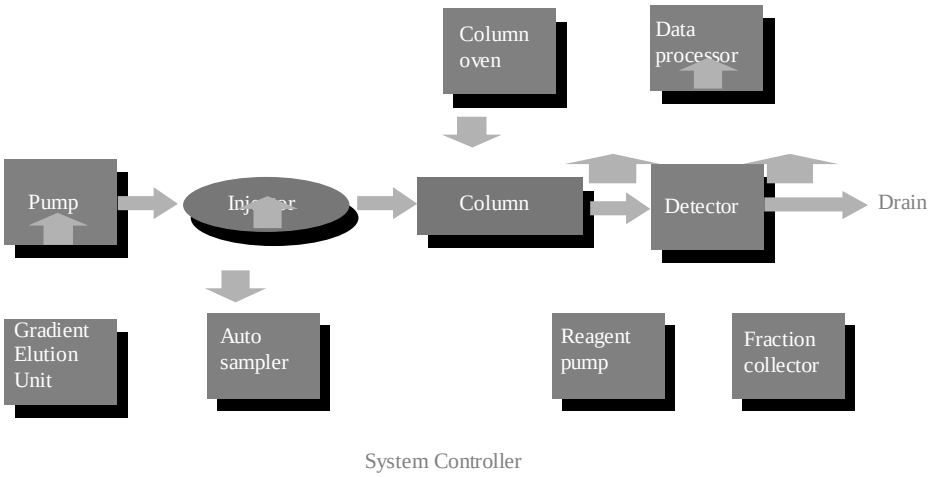

Figure 1.1.1 Schematic diagram of HPLC system.

In the normal phase mode, the retention is governed by the interaction of the polar parts of the stationary phase and solute. Retention occurs when the packing is more polar than the mobile phase with respect to the sample.
In the reverse phase approach, the packing material is relatively unpolar while the solvent is polar with respect to the sample. Retention is the result of interaction of the unpolar components of the solutes and the unpolar stationary phase. In the ion exchange column type, the mixture components are separated on the basis of attractive ionic forces between molecules carrying charged group of opposite charge on the stationary phase. Finally, affinity chromatography requires that an immobilized ligand, covalently coupled to the column's stationary phase, interacts specifically and reversibly with the solute of interest. The table 1.1.1, summarizes the different HPLC performances and reports the most commonly used packing materials.

LC mode	Packing materials	Mobile phase	Interaction
Normal phase	Silica gel	n-Hexane/IPE	Adsorbtion
Reversed phase	Silica C-18	MeOH/Water	Hydrofobic
Size exclusion	Porous polymer	THF	Gel permeation
Ion excange	Ion excange gel	Buffer sol.	Ion excange
Affinity	Packings with ligand	Buffer sol.	Affinity

Table 1.1.1. Common LC column characteristics.

Reversed phase separation[6-7] are about the most used HPLC method in food analysis and high-performance columns that provide minimum broadening of the separated bands are the heart of the modern LC system. Besides the nature of the packing material, an important role is played by the way how the columns are packed. They need also to be appropriately designed in order to minimize the dispersion and to allow the individual solutes to reach the detector, after they have been moved apart and separated. After injection into an HPLC column, any sample components that does not interact with the stationary phase would be eluted in the void volume (vo) which is characteristic for that column. This void volume represents both the interstitial volume between the particles of the bonded phase and the available volume within the particle pores themselves. The retention times,

tr(A) and tr(B), for the two sample components shown in Figure 1.1.2 are the times elapsed from injection to the times of maximum concentration in the eluted peaks. Similarly, the retention volumes are the amounts of solvent required for their elution. The basic principle of this separation techniques derive from various parameters that are summarized here:

- Theoretical plates
- HETP (height equivalent to a theoretical plate)
- Retention factor
- Selectivity
- Resolution

The number of theoretical plates (N) has traditionally been used as a measure of column efficiency.

$$N = 16(tr/W)^2 \qquad \text{(eq. 1)}$$

where tr is the retention time and W is the peak width at baseline (equation 1).

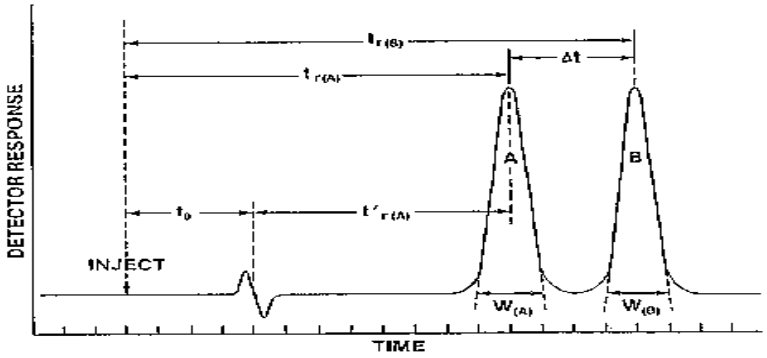

Figure 1.1.2. Important chromatographic parameters.

Generally, the measurement of peak width at half height has been found to be most useful, since it can be applied to peaks not completely resolved, that exhibit tailing , or that are otherwise asymmetrical in shape.

$$N = 5.54(tr/w½)^2 \qquad \text{(eq. 2)}$$

where w½ is peak width at half height (equation 2).

The value of N is a useful measure of the performance of a chromatographic column, and in general the more the theoretical plates, the better are the column performances. The number of theoretical plates can be calculated for any peak in a given separation, with each calculation resulting in a slightly different value. The value for N is to a first approximation, independent of retention time; however, it is proportional to column length. Therefore, height equivalent to a theoretical plate (HETP) is the better measure

of column efficiency since it allows for a comparison between columns of different lengths (equation 3):

$$H = HETP = L/N \qquad \text{(eq 3)}$$

where L is the length of the column, usually in mm.

The retention factor (k') is a measure of the degree of retention and can be calculated by

the following equation:

$$k' = (Vr - Vo)/Vo = (tr - to)/to \quad \text{(eq 4)}$$

where k' is the number of column volumes required to elute a particular solute Vo and to represent the void volume and void time, respectively.

A related concept is that of selectivity (α), which can be defined as the relative separation between

adjacent resolved peaks. This value is the ratio of the retention factors for the two peaks (equation 5):

$$\alpha = k'1/k'2 \qquad \text{(eq 5)}$$

Resolution is defined as the extent between separation of two chromatographic peaks.
It can be described as a measure of how well a given HPLC column separates the two components (equation 6).

$$Rs = 2(tr(B) - tr(A)) / (WA + WB) \qquad \text{(eq 6)}$$

Resolution can also be described in terms of an equation which includes three factors: the selectivity factor α, the capacity factor k', and the plate number, N (equation 6). Thus:

$$Rs = \alpha\ k'\ N \qquad \text{(eq 7)}$$

The characteristics of column are:

- *Column dimension* (length and internal diameter of packing bed)
- *Particle shape* (spherical or irregular)
- *Particle size* (average particle diameter, typically 3-20µm)
- *Surface Area* (sum of particle outer surface and interior pore surface, in m^2/gram)
- *Pore size* (average size of pores or cavities in particles, ranging from 60-10.000 Å
- *Bonding type* (monomeric: single-point attachment of bonded phase molecule; polymeric: multi-point attachment of bonded phase molecule.
- *Carbon load* (amount of bonded phase attached to base material, expressed as % C
- *Endcapping* (capping of exposed silanols with short hydrocarbon chains after the primary bonding step).

Column dimensions should be carefully chosen.[8,9] The *short* columns (30-50 mm length) offer short run times, fast equilibration, low backpressure and high sensitivity. *Long* columns (250-300 mm length) provide higher resolving power, but create more backpressure, lengthen analysis times and use more solvent. *Narrow* column (2.1 mm and smaller) beds inhibit sample diffusion and produce narrower, taller peaks and a lower limit of detection.
They may require instrument modification to minimize distortion of the chromatography. *Wider* columns (10-22 mm) offer the ability to load more sample.
The table 1.1.2 shows the relationship between column internal diameter and flow, mass load and volume injection parameters.

ID (mm)	Flow (ml/min)	Mass load (mg)	Injection vol (µl)
0,3-0,5	5-10 µl/min	0,00005-0,01	0,01-0,5
1	25-75 µl/min	0,005-0,05	0,2-5
2	0,15-0,25	0,002-0,3	1-20
3	0,3-0,6	0,1-1	2-40
4,6	0,5-1,5	0,2-5	5-100
10	2,5-10	1-40	20-50
21,2	15-40	5-200	100-2000
50	50-100	25-1000	600-10000
100	200-500	125-5000	2300-50000

Table 1.1.2. Setting of different parameters in HPLC.

1.1.2 Analytical and preparative chromatography

The difference between analytical and preparative HPLC concerns the aim of the separation. In analytical HPLC the aim is to separate all individual components of a mixture as completely as possible with subsequent identification of the peaks. In general, sample sizes are small. For 4 mm ID, typical sample sizes are 1 – 100 µg analyte per g adsorbent in normal phase columns and 10 – 1000 µg analyte per g adsorbent in RP columns. For columns with smaller inner diameters correspondingly smaller samples are applied. Thus analytical HPLC often requires maximum separation efficiency of a column. Due to the small inner diameter, expenses for solvents and packing are low, with the result that in analytical HPLC costs for separation time (solvent consumption) and packing material can be almost neglected for method development.
On the contrary, in preparative HPLC development of a separation often involves detailed economical-chemical optimization calculations.
Due to the column dimensions, costs for solvents and packing or prepacked columns become more and more important with increasing column diameters. The aim of HPLC now is isolation of the desired product with defined purity, in maximum amounts and with minimum time. The important parameter are called production rate and throughput. Definition of the production rate includes information about the required purity of the isolated product.
When speaking about the production rate of a preparative separation, the term loadability[10-12] of the column should be considered, too. According to general understanding, this is the maximum sample size (with defined sample mass and volume) under which a column still provides optimum selectivity.
The parameters which are important for the optimization of the mass loadability of a column can be described by the formula:

$$M = C_1 \pi r^2 l k d A_S [C_2 (d_P^2/l)]^2$$

M = maximum sample mass
C_1, C_2 = constants
r = column radius
l = column length
k = partition coefficient
d = packing density
A_S = adsorbent surface
d_P = particle diameter.
It is important to note how the mass loadability of the column decreases with increasing plate number (l/d_P^2 proportional to the plate number N).
If an increased loadability is required for a given separation efficiency, it is recommended to increase particle size and column length, the increase in column length being the square of the increase in particle diameter. Volume loadability can be related to the dead volume (V_0), the maximum overload volume (V_L), the relative retention (α), the plate number (N) and the capacity factors (k_A', k_B') by equation 8, hence it depends on the k' values of the components to be separated and on the separation efficiency of the column.

$$V_L = V_0 [(\alpha-1) k_A' - 2/N^{1/2} (2 + k_A' + k_B')] \quad \text{(eq. 8)}$$

The production rate is directly proportional to the column diameter, the linear flow velocity of the mobile phase, the concentration of the component to be isolated (unless under mass overload

conditions) and the term $[1/N–H_0 /l]1/2$, where H_0 is the plate height of the column under ideal conditions, l is the column length, and N is the plate number required for separation of the desired product with the purity required.[13]

1.1.2 Detectors

Although over the years a large number of LC detectors have been developed and described, the vast majority of all contemporary LC analyses are carried out mainly using four detectors.
The UV, in one of its different forms, the electrical conductivity, the fluorescence and the refractive index.
The widespread use of the UV spectrophotometer approach deserves a detailed description of the method. The coupling with mass analyzers will be treated in a different paragraph. UV absorption detectors respond to those substances that absorb light in the range 180 to 350 nm. Many (but not all) substances absorb light in this wavelength range, including those having one or more double bonds (p electrons) and unshared (unbounded) electrons, *e.g.* olefin and aromatic compounds and species containing C=O, C=S and –N=N– groups.
The sensor of a UV detector consists of a short cylindrical cell having a capacity between 1 and 10 ml through which passes the column eluant. UV light is arranged to pass through the cell and fall on a photo–electric cell (or array). The signal from the photocell is transmitted to a modifying amplifier and then to a recorder or data acquisition system.
The relationship between the intensity of UV light transmitted through a cell (I_T) and the concentration of solute contained by it (c) is given by Beer's Law (equation 9).

$$I_T = I_O e^{-klc}$$
$$\text{or } \ln (I_T) = \ln (I_O) – kcl \quad \text{(eq 9)}$$

where (I_O) is the intensity of the light entering the cell, (l) is the path length of the cell, and (k) is the molar extinction coefficient of the solute for the specific wavelength of the UV light.
UV detectors can be used with elution gradients, providing the solvents do not absorb significantly in the wavelength range used for the detection. The solvents usually employed in reversed phase chromatography are water, methanol, acetonitrile and tetrahydrofuran (THF), all of which are transparent to UV light over the total wavelength range normally used by UV detectors. In normal phase operation more care is necessary in eluant selection since many solvents that might be appropriate for a given chromatographic phase strongly absorb at the wavelengths used by the detector. The *n*-paraffin, methylene dichloride, aliphatic alcohols and THF are useful solvents that are transparent in the UV and can be used with a polar stationary phase such as silica gel.

1.2 Mass spectrometry

Mass spectrometry is essentially a technique for "weighing" molecules. Obviously, this is not done with a conventional balance. Instead, mass spectrometry is based upon the motion of a charged particle, called ion. Figure 1.2.1 is a block diagram that shows the basic parts of a mass spectrometer. The inlet transfers the sample into the vacuum of the mass spectrometer. In the source region, neutral sample molecules are ionized and then accelerated into the mass analyzer.
The mass analyzer is the heart of the mass spectrometer. This section separates ions, either in space or in time, according to their mass to charge ratio. After the ions are separated, they are detected and the signal is transferred to a data system for analysis. All mass spectrometers also have a vacuum system to maintain the low pressure, which is also called high vacuum, required for operation. A variety of ionization techniques are used for mass spectrometry.
Most ionization techniques excite the neutral analyte molecule which then ejects an electron to form a radical cation ($M^{+\bullet}$). Other ionization techniques involve ion molecule reactions that produce adduct ions (MH^+). High vacuum minimizes ion-molecule reactions, scattering, and neutralization of the ions.
The most important considerations are the physical state of the analyte and the ionization energy. Electron ionization and chemical ionization are only suitable for gas phase ionization. Electrospray ionisation (ESI)[14-15], and matrix assisted laser desorption (MALDI)[16] are used to ionize condensed phase samples.

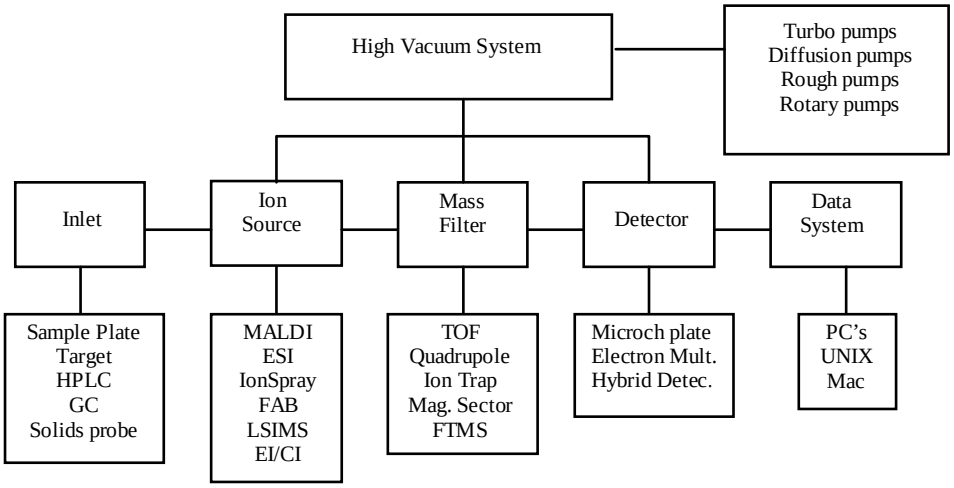

Figure 1.2.1. Mass spectrometry block diagram.

The ionization energy is significant because it controls the amount of fragmentation observed in the mass spectrum. Although this fragmentation complicates the mass spectrum, it provides structural information for the identification of unknown compounds. Some ionization techniques are very soft and only produce molecular ions, other techniques are very energetic and cause ions to undergo extensive fragmentation. Currently, API (ESI and APCI) and MALDI are the most commonly employed ionization methods and they opened doors to the widespread biological and biomedical application of mass spectrometry. These techniques are used to ionize thermally labile samples such as flavonoids, peptides, proteins and polymers directly from the condensed phase. Otherwise, the

selection of a mass analyzer is very important and depends upon the resolution, mass range, scan rate and detection limits required for an application.[17] Each analyzer has very different operating characteristics and the selection of an instrument involves important tradeoffs. Analyzers are typically described as either continuous or pulsed. Continuous analyzers include quadrupole filters and magnetic sectors while pulsed analyzers include time-of-flight, ion cyclotron resonance, and quadrupole ion trap mass spectrometers. There are also different hybrid analyzers; two popular of these are triple quadrupole and Qq-TOF. The former is utilized for quantitative analysis in tandem mass spectrometry, the latter is applied in high resolution analysis.

1.2.1 Electrospray Ionization (ESI) and atmospheric pressure chemical ionization (APCI)

The electrospray process involves the creation of a fine aerosol of highly charged micro droplets in a strong electric field. Electrospray as an ionization technique for mass spectrometry was developed by Dole and co-workers in the late 1960s[14] and considerably improved upon by Yamashita and Fenn who in 1984 coupled an electrospray source to a quadrupole mass analyser[15]. A continuous flow of solution containing the analyte from a highly charged (2–5 kV) capillary generates an electrospray. The solution elutes from the capillary into a chamber at atmospheric pressure, producing a fine spray of highly charged droplets due to the presence of the electric field (figure 1.2.2), a process called nebulisation. A combination of thermal and pneumatic means is used to desolvate the ions as they enter the ion source.

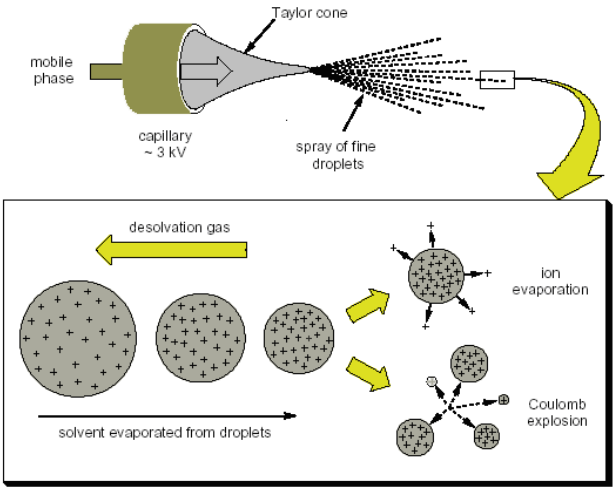

Figure 1.2.2. The desolvation process.

The solvent contained in the droplets is evaporated by a warm counter-flow of nitrogen gas until the charge density increases to a point at which the repulsion becomes of the same order as the surface tension. The droplet then may fragment in what is termed a 'Coulomb explosion', producing many daughter droplets that undergo the same process, ultimately resulting in bare analyte ions.
An alternative picture is one in which the ions 'evaporate' from the surface of the droplet. Whatever the exact mechanism, ESI is a very 'soft' means of ionization that causes little or no fragmentation of the sample.[20-21]The electrospray ion source is at very high pressure (atmospheric) with respect to the very low pressure that is required for ion separation by a mass analyzer, so the interface between the two involve a series of skimmer cones (acting as small orifices) between the various differentially pumped regions (figure 1.2.3). Early designs had the capillary exit pointing directly

into the mass analyzer but to limit contamination practically all modern designs have an orthogonal (or at least off-axis) spray direction.

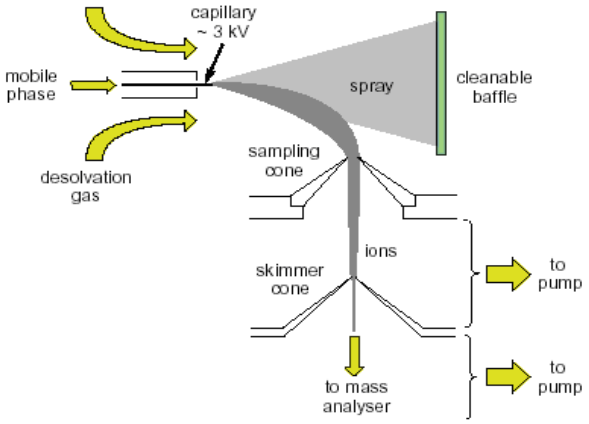

Figure 1.2.3. An electrospray source.

The ions are drawn into the spectrometer proper through the skimmer cones. A voltage can be applied (the cone voltage), which will accelerate the ions relative to the neutral gas molecules. This leads to energetic ion-neutral collisions and fragmentation due to what is termed collision induced dissociation (CID)[22]. The remaining bath gas is pumped away in stages (in order to attain the high vacuum necessary for separation of the ions) and the ions are focused through a lensing system into the mass analyzers.

The appearance of multiply charged species enables ESI to characterize compounds whose molecular weight would otherwise be far in excess of that accessible to most mass analysers[23-24]. Biological macromolecules tend to accumulate one unit of charge for every 1 – 2000 Da, so nearly all proteins, for example, produce signals in the region of 1 – 2000 m/z, regardless of their actual molecular weight. Ionization of a neutral analyte often occurs by protonation, or alternatively cationisation, with an adventitious cation present in the solvent used, such as Na^+, K^+ or NH_4^+. In some cases, adduct ions with several cations can occur, giving $[M + H]^+$, $[M + NH_4]^+$, $[M + Na]^+$ and $[M + K]^+$ ions. Atmospheric-pressure chemical ionization (APCI)[25-26] is another of the techniques in which the stream of liquid emerging from an HPLC column is dispersed into small droplets, in this case by the combination of heat and a nebulizing gas. In this case the liquid flow is passed through a pneumatic nebulizer where the droplets are both generated and desolvated. The successive neutral dried spray, obtained by a heated region, passed through a corona discharge were the analyte are ionized. The mechanism is a chemical ionization but an atmospheric pressure were is necessary, for initial gas ionization, utilized a corona discharge(fig. 1.2.4).

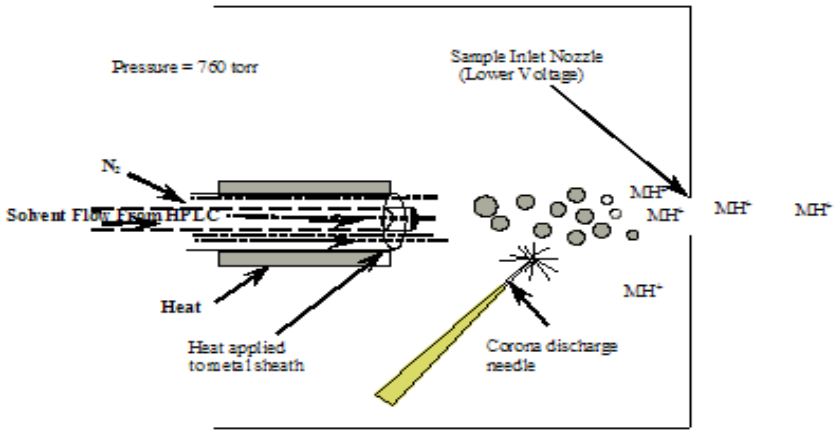

Figure 1.2.4. APCI source.

Then, the ions produced by the interaction of the electrons with the surrounding gas, undergo a number of reactions leading to the generation of reactive ions which interact with the analyte molecules present.
The reagent species in the positive-ion mode may be considered to be protonated solvent ions, and in the negative ion mode O_2^-, its hydrates and clusters. Is also possible the formation of cluster involving solvent molecules which can removed with use a "curtain gas". Finally, this technique can be applied both volatile and thermally stable and moderate polar compounds but the ionization regime is much more harsh that ESI and this precludes its use for the study of large biomolecules, with the mass limit for APCI being generally considered as below 2000 Da.

1.2.2 Common Mass analyzers: quadrupole and TOF

Quadrupole mass analyzers consist of four parallel rods arranged as in Figure 1.2.5. Applied between each pair of opposite and electrically connected rods are a DC voltage and a superimposed radio-frequency potential.
A positive ion entering the quadrupole will be drawn towards a negatively charged rod but if the field changes polarity before the ion reaches it, it will change direction. Under the influence of the combination of fields the ions undergo complex trajectories[27-29]. Within certain limits these trajectories are stable and so ions of a certain m/z are transmitted by the device, whereas ions with different m/z values will have an unstable trajectory and be lost by collision with the rods.
The operation of a quadrupole mass analyzer is usually treated in terms of a stability diagram that relates the applied DC potential (U), the applied rf potential (V) and the radio frequency (ω) to a stable vs unstable ion trajectory through the quadrupole rods.

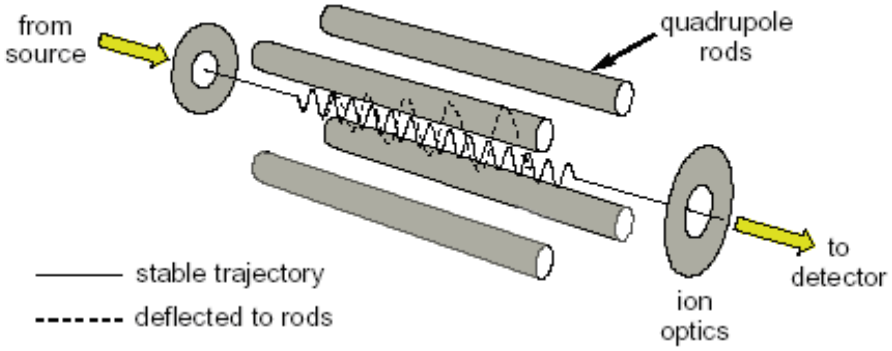

Figure 1.2.5. Diagram of a quadrupole mass analyzer.

A qualitative representation of a stability diagram for a given mass m is shown in figure 1.2.6; a and q are parameters that are proportional to U/m and V/m respectively: changing the slope of the scan line will change the resolution. Quadrupoles have other functions besides their use as a mass filter.

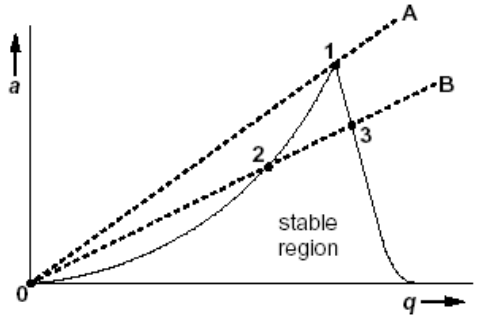

Figure 1.2.6. Stability diagram.

An rf-only quadrupole will act as an ion guide for ions within a broad mass range. In such applications, hexapoles or even octapoles are often employed.

The time-of-flight (TOF)[29-30] mass analyzer separates ions in time as they travel down a flight tube These instruments have high transmission efficiency, no upper *m/z* limit, very low detection limits, and fast scan rates. In the source of a TOF analyzer, a packet of ions is formed by a very fast (ns) ionization pulse. These ions are accelerated into the flight tube by an electric field (typically 2-25 kV) applied between the backing plate and the acceleration grid. Since all the ions are accelerated across the same distance by the same force, they have the same kinetic energy. Because velocity (*v*) is dependent upon the kinetic energy (E) and mass (*m*) lighter ions will travel faster.

The velocity of an ion (*v*) is determinate as a function of acceleration voltage and m/z value(equation 10):

$$v = \sqrt{\frac{2zeV}{m}} \qquad \text{(eq 10)}$$

After the ions accelerate, they enter a 1 to 2 meter flight tube(fig. 1.2.7). The ions drift through this field free region at the velocity reached during acceleration. At the end of the flight tube they strike a detector. The time delay (t) from the formation of the ions to the time they reach the detector dependents upon the length of the drift region (L), the mass to charge ratio of the ion, and the acceleration voltage in the source (equation 11):

$$t = \sqrt{\frac{mD}{2zeV}} \qquad \text{(eq 11)}$$

This second equation shows that low *m/z* ions will reach the detector first. The mass spectrum is obtained by measuring the detector signal as a function of time for each pulse of ions produced in the source region. Because all the ions are detected, TOF instruments have very high transmission efficiency which increases the S/N level.

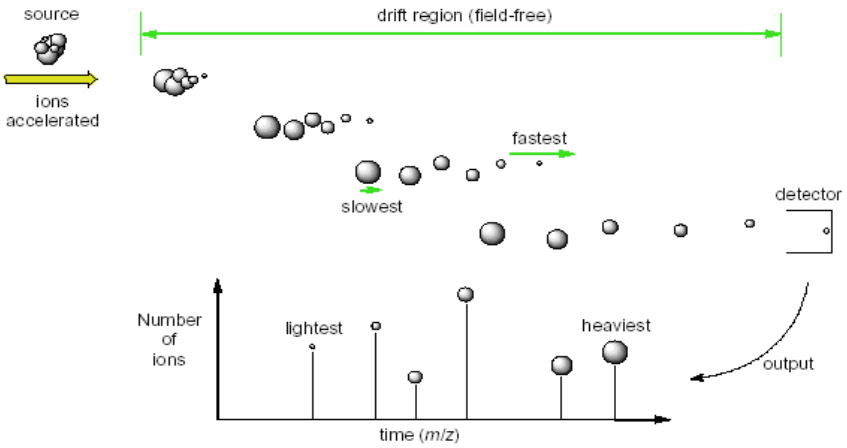

Figure 1.2.7. The essential of TOF optics.

The ions leaving the ion source of a time-of-flight mass spectrometer have neither exactly the same starting times nor exactly the same kinetic energies. Improvements in time of-flight mass spectrometer design have been introduced to compensate for these differences, and the most dramatic improvements in performance come with the use of a reflectron.[31]
This is an ion optic device in which ions in a time-of-flight mass spectrometer interact with an electronic ion mirror and their flight is reversed. Ions with greater kinetic energies penetrate deeper into the reflectron than ions with smaller kinetic energies. The reflectron will decrease the spread in the ion flight times, and therefore improve the resolution of the time-of-flight mass spectrometer.
Orthogonal TOF[32] analyzers are employed in conjunction with continuous ion sources, especially ESI[33], and in hybrid instruments. The continuous ion beam is subjected to a pulsed electric field gradient at right angles (orthogonal) to the direction of the ion beam. A section of the ion beam is thus pulsed away instantaneously and can be measured using a TOF analyzer (fig. 1.2.8).

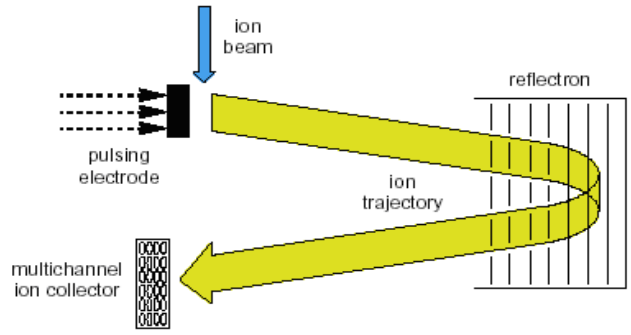

Figure 1.2.8. Orthogonal TOF.

Orthogonal TOF analyzers can accumulate tens of thousands of spectra per second and these are summed to provide spectra with high signal-to-noise ratios.

1.2.3 Hybrid mass analyzers: triple quadrupole and QqTOF

In a triple quadrupole, two mass analyzers are separated by an rf-only quadrupole used as a collision cell (fig. 1.2.9). MS/MS[34] experiments are easy to set up on triple quadrupoles, and are especially valuable when examining complex mixtures of ions. Each ion may be selected and individually fragmented to provide structural information exclusively on that ion, without any need for consideration of interference from other ions in the mixture.

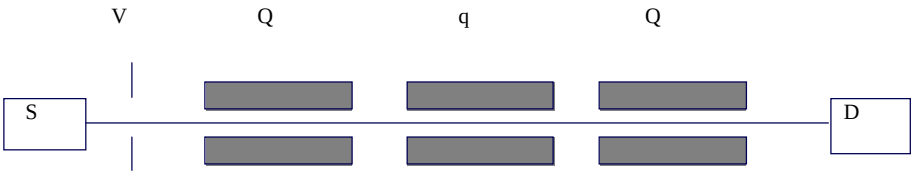

Figure 1.2.9. Scheme of triple quadrupole.

With this analyzer is possible to carry out different processes in tandem mass spectrometry.
The processes are:

- **Product ion scan;** the first quadrupole is set so that it passes only ions of a certain m/z value, the rf-only quadrupole contains an inert collision gas (typically argon) to assist in the creation of fragment ions and the third quadrupole scan the mass range of interst and generates a daughter ion spectrum.
- **Precursor ion scan;** MS1 scan through all ions, which are fragmented in the collision cell, and MS2 is set to detect only product ions of a certain mass.
- **Neutral loss scan;** this involves setting up MS1 and MS2 to scan in parallel but offset by a set mass difference, Δm.
- **Select reaction monitoring;** both MS1 and MS2 are settled on only one ion, father and daughter respectively.

The most popular Hybrid of TOF analyzer is certainly the quadrupole/oa-TOF[35]. In these instruments (fig. 1.2.10), the first mass analyzer (MS1) is a quadrupole, which acts simply as an rf-only ion guide in MS mode.

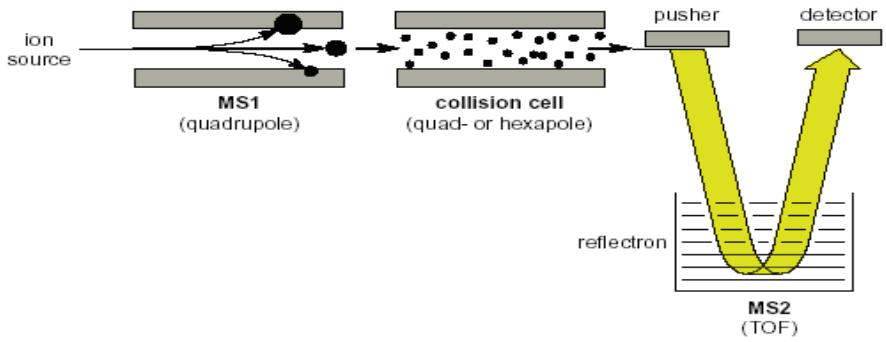

Figure 1.2.10. Quadrupole/oa TOF.

Next is a collision cell, followed by the oa-TOF (MS2). To collect MS/MS data, MS1 is used to select a single ion, which is then fragmented in the collision cell, and MS2 is used to collect the daughter ion spectrum. Fast, highly efficient, sensitive and capable of high resolution, the Q/TOF provides higher quality data than the popular triple quadrupole, though it is less suitable for quantitative work.

1.3 Liquid chromatography-Mass spectrometry

The development of new ionization methods as ESI and APCI, has been fundamental for coupling liquid chromatography to mass spectrometry.[1] They represents perfect LC-MS interfacing technologies. Even LC-nano ESI is feasible.[36] With these interfaces is possible to remove the incompatibility between HPLC, utilizing flow rates of ml/min of a liquid, and the mass spectrometer, which operates under conditions of high vacuum. It's due to the fact which ionization occurs directly from solution and consequently allows ionic and thermally labile compounds to be studied.
This combination allows more definitive identification and the quantitative determination of compounds that are not fully resolved chromatographically.
The major advantage of this coupling is the possibility to study a range of analyte, from low molecular-weight drugs and metabolites (<1000 Da) to high-molecular-weight biopolymers (>100 000 Da). Method development requires the analyst to identify the variables (factors) that are likely to affect the result of the analysis and to carry out experiments that allow those that have an effect on the final outcome to be identified. Having identified the factors of importance, experimental design finally allows the precise experimental conditions that give the 'best' result to be determined. This 'factors' could include for HPLC, the composition of the mobile phase, its pH and flow rate[37], the nature and concentration of any mobile-phase additive, buffer or ion-pair reagent, the make-up of the solution in which the sample is injected for the ionization technique[38], spray parameters for electrospray source, nebulizer temperature for APCI, nature and pressure of gas in the collision cell if MS–MS experiment is performed.[22]

1.3.1 LC-MS for low molecular weight compounds.

When 'low'-molecular-weight compounds are involved, both APCI and electrospray ionization are potentially of value and the first task is to decide which of these will give the more useful data. The choice of interface is relative to polarity of sample: APCI for low- to medium-polarity and electrospray for medium- to high-polarity.
In general terms, electrospray ionization is considered to be concentration sensitive at 'low' flow rates and mass-flow-sensitive at 'high' flow rates, while APCI is considered to be mass-flow-sensitive. Some compounds may be ionized very effectively under positive-ionization conditions, while others may require the formation of negative ions to allow analysis. Structural studies of low molecular weight compounds may require the extent of fragmentation to be maximized, but in this way is important to have an high resolution analyzer for calculate an exact mass so father as daughters ions. This factor is fundamental for reveal compounds which differ few mDa one another. Quantification may require the opposite, the efficient production of a small number of ions of different *m/z* ratios, in order to maximize sensitivity.

Quantification involves the comparison of the intensity of response from an analyte ('peak' height or area) in the sample under investigation with the intensity of response from known amounts of the analyte in standards measured under identical experimental conditions.

The attributes required of a method usually include good sensitivity, low limits of detection, and selectivity. Good selectivity allows the measured signal to be assigned, with certainty, to the analyte of interest rather than any interfering compounds which may be present. Low limits of detection allows to reveal very few quantities of compounds. There are three methods for quantification utilizing the standard solution: external standard, standard additions and internal standard. The former is the main method used when the detector is a mass spectrometer. Quantitative methodology employing mass spectrometry usually involves selected-ion monitoring or multiple reaction monitoring, sometimes using a labelled internal standard for accurate quantification.[39]

It is the role of the analyst to choose these ions/decompositions, in association with chromatographic performance, to provide sensitivity and selectivity such that when incorporated into a method the required analyses may be carried out with adequate precision and accuracy.

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2. LC/MS applications on food chemistry

The development of LC/MS techniques[1-2] has been fundamental for the analysis of mixtures of secondary metabolites from plants and fruits.[3-14] The powerful of this type of analysis is very important in order to improve the research in a life science.

Historically, consumption of particular fruits and vegetables was thought to prevent or cure aliments ranging from headaches to heart disease. In fact, early medicine revolved largely around the prescription of specific food concoctions for certain disorders. Until relatively recently, these attributes of vegetables and fruits were based more on beliefs than on scientific evidence, but during the recent years many studies have examined the relationship between vegetables fruit and health. It has been estimated that up to 70% of all cancer is attributed to diet.[15] Nowadays, the scientific evidence regarding a role for vegetable and fruit consumption in cancer prevention is generally consistent and is supportive of current dietary recommendations. A different type of micro-components called PPT (phenols, polyphenols and tannins) with various mechanisms, may help protect against cancer.[16-17] Continued attention to increasing vegetable and fruit intake is important.

For this reason the structural identification of new compounds derived from these matrixes was becoming an important field of research in which the development of hyphenated methods, like automated semi- or preparative LC-MS, given a very significant contribution.[18] For a more complete structure elucidation, the complementary information derived from NMR is indispensable.

Further developments may be expected with regards to miniaturization, that is, the coupling of micro- and/or nano-LC, to tandem-MS and NMR instruments: this should facilitate the analysis of minute samples, and help to create better operating conditions for NMR detection. However, the progress in this area has been slower than was expected several years ago.[19] In the near future, both, LC with tandem MS detection and preparative LC/MS, will continue to play a dominant role in this analysis.

On other hand, quantitative LC-MS/MS methodology is utilized in food safety, agricultural and forensic chemistry.[20-22] In food safety, there are different problems related to chemicals in foodstuffs. Quality and safety control and the validation of origin are hot issues in the production of food and its distribution, and are of primary concern to food and agriculture organization. Modern mass spectrometry (MS) provides unique, reliable and affordable methodologies to approach with a high degree of scientificity any problem which may be posed in this field. Quality and safety are the two main issues related to genuineness of processed and fresh aliments. The specificity and sensitivity of MS methodologies has become officially recognized by international quality-system control-bodies and the exploitation of multistage ion analysis has become mandatory to adhere to worldwide regulations regarding the recognition of fraud and bad practices in food manipulation. The production and consumption of food is central to any society, and has economic, social and, in many cases, environmental consequences. Although health protection must always take priority, these issues must also be taken into account in the development of food policy.

2.1 Flavonoids

Flavonoids constitute a large group of naturally occurring plant phenols that cannot be synthesized by humans. They are characterised by the carbon skeleton C6-C3-C6 derived from shikimic acid pathway. The basic structure of these compounds consists of two aromatic rings (commonly designated as A and B)linked by a three-carbon aliphatic chain which normally has been condensed to form a pyran (C) or, less commonly, a furan ring.

The main classes of flavonoids, including flavones, flavonols, isoflavones, flavonones (fig. 2.1.1) occur in all types of higher plant tissues.[23-24] Approximately 90 % of the flavonoids in plants occur as glycosides.[25] Usually the aglycones are more active in bulk phase,[26,27] as well as in phospholipid bilayers oxidation.[28] Many studies have suggested that flavonoids exhibit biological activities, including antiallergenic, antiviral, anti-inflammatory, and vasodilating actions. However, most interest has been devoted to the antioxidant activity of flavonoids, which is due to their ability to reduce free radical formation and to scavenge free radicals.[29-32] Other mechanisms of action of selected flavonoids include singlet oxygen quenching,[33-34] metal chelation,[35-36] as well as lipoxygenases inhibition.[37-40] The glycosides are less effective as antioxidants than are the aglycones.[41]

Flavonoids with free hydroxyl groups act as free-radical scavengers, and multiple hydroxyl groups, especially in the B-ring, enhance their antioxidant activity.[42] There is no evidence that flavonoid intake is protective against some types of cancer,[43] but they have a possible role again coronary heart disease as some epidemiological studies reported.[44]

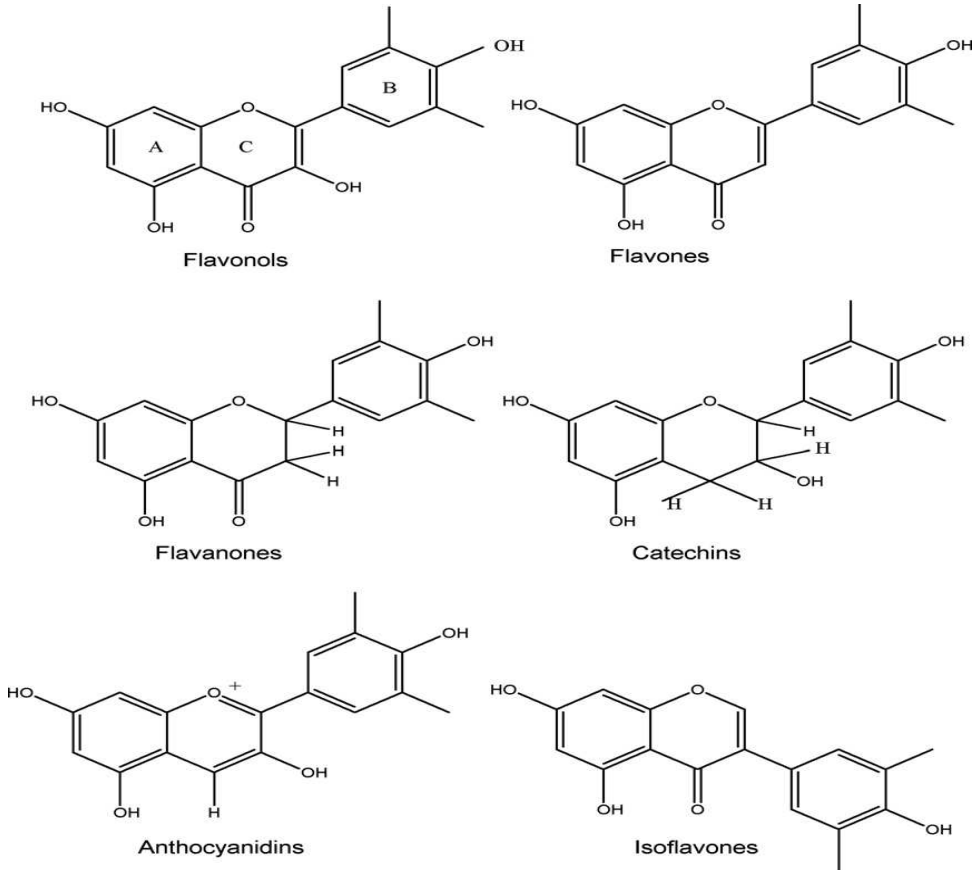


Fig. 1. Molecular structures of flavonoids. The basic structure consists of the fused A and C ring, with the phenyl ring B attached to through its 10 position to the 2-position of the C ring (numbered from the pyran oxygen).

The antioxidant efficacy of flavonoids in vivo is less documented, presumably because of the limited knowledge on their uptake in humans. For example only a weak but insignificant inverse correlation was observed for flavonoid consumption and coronary mortality.[45] They are generally poorly absorbed from food, and extensively degraded to various phenolic acids, some of which still possess a radical scavenging ability. Finally, accordingly, the present epidemiological data, far from conclusive, evidence a possible protective role of dietary flavonoids, thus making desirable a regular consumption of foods and beverages rich in flavonoids but is also important to change their role as health-promoting dietary antioxidants and place these observations in a broader context embracing other dietary phenols, and mechanisms other than simple radical scavenging and radical suppression.[46]

2.1.1 Flavonoids in food

All foods of plant origin potentially contain flavonoids[47,48] and over 4000 individual compounds have previously been identified.[49]

Catechins, flavonols, and proanthocyanidins are abundant in fruits. In contrast, flavanones and flavones are restricted to citrus varieties such as oranges and lemons; in some fruits (e.g., apples), flavonols are principally present in the skin and hence peeling significantly reduces levels unlike catechins which are found in the flesh of fruits.

Quercetin is the most common flavonol in fruits; although kaempferol and myricetin have also been identified in fruits such as peaches and pears, concentrations are generally too low to be readily quantified in the whole fruit. Often termed the citrus flavonoids, flavanones are only found in citrus fruits such as oranges, grapefruit, and lemons.

Allium, Brassica, and Lactuca varieties of vegetables are abundant sources of flavonols, primarily quercetin and kaempferol while catechins are often the most common flavonoids in beverages such as fruit juice, tea, and wine. Wine also contains a complex mix of catechins, flavonols, procyanidins, and flavanones. Procyanidins usually represent 50% of the flavonoids found in red wine, followed by catechins (37%). A similar profile is observed with beer where again procyanidins dominate accounting for 42% of total flavonoid content. Concentrations of flavonoids in foods can vary by many orders of magnitude due to the influence of numerous factors such as species, variety, climate, degree of ripeness, and post harvest storage.[48] The flavonoid content of plant foods may be affected by growing conditions. Flavonoid profiles are also influenced by irrigation, which, for example, modifies concentrations and types of anthocyanins and catechins in berries.[50]

Effects of varietal differences as flavonoid subclasses can vary widely between different cultivars of fruits and vegetables.[51] In general, industrially produced products such as tea, red wine, and fruit juice have significantly different flavonoid levels and profiles than the original fresh product.[52,53] Processing and preservation can expose fresh products to increased risk of oxidative damage and the activation of oxidative enzymes such as polyphenol oxidase.[54]Domestic preparation procedures may also affect flavonoid content.[55] Initial estimate of flavonoid intake of 1000 mg/day[48], has been increased including the aglycon form[51] and the different source related to different countries.[56]

2.1.2 Citrus flavonoids

Bergamot is the common name of the fruit Citrus bergamia Risso, which belongs to Rutaceae family, a subfamily of Esperidea. The uniqueness of bergamot trees is represented by a habitat that is virtually restricted to the coastal region of the Ionian Sea in the southern Calabrian region of Italy. This area presents favorable weather and pedoclimatic conditions for its cultivation. Three cultivars of bergamot ("Castagnaro", "Fantastico", and "Femminello") are commercially grown and then industrially processed, exclusively to extract their essential oils. The industrial processing uses an indiscriminate mix of the three cultivars. In the past, bergamot has been highly valued by the cosmetic and perfume industry since its essence is very rich in terpenes, esters, and alcohols possessing a very characteristic and intense fragrance. The development of synthetic essential oil production led to a drastic drop in commercial demand for bergamot. However, over the past few years, following the growing interest in antioxidant bioactive compounds and their dietary sources, such as Citrus juices, bergamot juice has attracted attention as a result of its remarkable flavonoid content.

Flavonoids identified in Citrus fruits cover over 60 types, divided in five classes: flavones, flavanones, flavonols, flavans and anthocyanins (the last only in blood oranges). In particular, this genus is characterized by the accumulation of large quantities of glycosylated flavanones, which are the first intermediaries in the flavonoid biosynthetic pathway. The most common sugar moieties include D-glucose and L-rhamnose. The glycosides are usually *O*-glycosides, with the sugar moiety bound generally to the aglycone hydroxyl group at C-7, or at the C-3 in some cases. In addition to these, *C*-glycosides have also been detected in various *Citrus* fruits or juices. **Table 1** presents the flavanone aglycones of recovered glycosides in *Citrus* juices. All these aglycones have a skeleton in which two hydroxyls are present at the C-5 and C-7 positions. In hesperetin (**1**) and isosakuranetin (**4**) the C-4' position is methoxylated. The flavanone taxifolin (**3**) contains a hydroxyl group in the C ring C-3 position, and can thus also be classified as a flavanol.

	Compound name	R_1	R_2	R_3
1	Hesperetin	H	OH	OMe
2	Naringenin	H	H	OH
3	Taxifolin	OH	OH	OH
4	Isosakuranetin	H	H	OMe
5	Eriodictyol	H	OH	OH

Table 1. Flavanone aglycones.

Flavone aglycones are summarized in **Table 2**. Acacetin (**6**) and diosmetin (**12**) present a methoxy moiety at C-4' position, whereas in chrysoeriol the methoxyl group is bound to C-3'. Kaempferol (**9**) and quercetin (**10**) both bear hydroxyl group at the 3 position and are often referred to as flavonols. **Table 3** shows the group of compounds classified as polymethoxyflavones (PMFs). These are usually found as components of the essential oils fraction of *Citrus* peels[57]. Hand-squeezed juices contain no detectable traces of this class of compounds[58]. Commercial juices, on the other hand, are rich in PMFs because the industrial processing of fruits leads to juices being contaminated with the peel constituents.

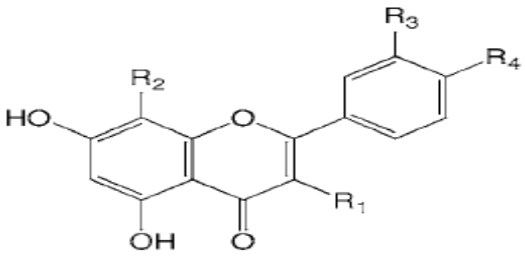


Table 2. Flavone aglycones.

	Compound name	R_1	R_2	R_3	R_4
6	Acacetin	H	H	H	OMe
7	Isoscutellarein	H	OH	H	OH
8	Luteolin	H	H	OH	OH
9	Kaempferol	OH	H	H	OH
10	Quercetin	OH	H	OH	OH
11	Apigenin	H	H	H	OH
12	Diosmetin	H	H	OH	OMe
13	Chrysoeriol	H	H	OMe	OH

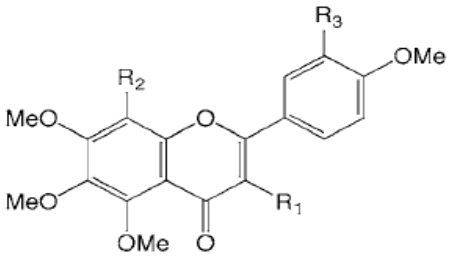


Table 3. Polymethoxyflavones.

	Compound name	R_1	R_2	R_3
14	Quercetogetin	OMe	H	OMe
15	3,3',4',5,6,7,8-Heptamethoxyflavone	OMe	OMe	OMe
16	Natsudaidain	OH	OMe	OMe
17	Nobiletin	H	OMe	OMe
18	Sinensetin	H	H	OMe
19	Tangeretin	H	OMe	H
20	Tetramethylscutellarein	H	H	H

The flavanone *O*-glycosides found so far in juices are listed in **Table 4.** These derivatives have a glycosyl substitution exclusively at the C-7 position (on ring A). Furthermore, only two disaccharides have so far been identified in this group of compounds, and both are L-rhamnosyl-D-glucosyl derivatives: rutinose, which presents a α-1,6 interglycosidic linkage, and neohesperidose, in which the two sugars are linked via a α-1,2 interglycosidic bond (**Figure 2**).

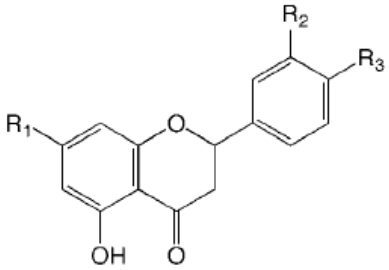
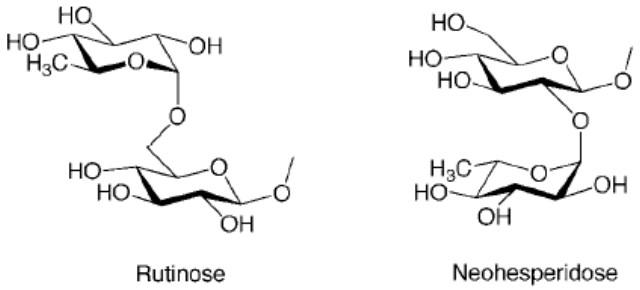


Table 4. Flavanone-*O*-glycosides.

	Compound name	R_1	R_2	R_3
21	Isosakuranetin 7-*O*-rutinoside (Didymin, Neoponcirin)	*O*-Ru[a]	H	OMe
22	Eriodictyol 7-*O*-rutinoside (Eriocitrin)	*O*-Ru[a]	OH	OH
23	Hesperetin 7-*O*-rutinoside (Hesperidin)	*O*-Ru[a]	OH	OMe
24	Naringenin 7-*O*-neohesperidoside (Naringin)	*O*-Nh[b]	H	OH
25	Naringenin 7-*O*-rutinoside (Narirutin)	*O*-Ru[a]	H	OH
26	Hesperetin 7-*O*-neohesperidoside (Neohesperidin)	*O*-Nh[b]	OH	OMe
27	Eriodictyol 7-*O*-neohesperidoside (Neoeriocitrin)	*O*-Nh[b]	OH	OH
28	Isosakuranetin 7-*O*-neohesperidoside (Poncirin)	*O*-Nh[b]	H	OMe

[a] *O*-Rutinose; [b] *O*-Neohesperidose.

Figure 2. Rutinose and neohesperidose.



Rutinose Neohesperidose

Flavone *O*-glycosides found in *Citrus* juices are generally 7-*O*-rutinosides or 7-*O*-neohesperidosides (**Table 5**), although a 3-*O*-rutinoside has also been reported, namely rutin (**39**). *Citrus* juices also contain a large number of di-*C*-glycosides, along with smaller amounts of mono-*C*-glycosides. For these compounds, substitution is generally on either the C-6 or the C-8, or on both positions[59-60].

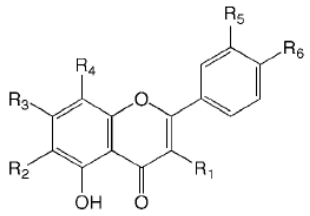


Table 5. Flavone-*C*-glucosides and flavone-*O*-glycosides.

	Compound name	R_1	R_2	R_3	R_4	R_5	R_6
29	Luteolin 6,8-di-*C*-glucoside (Lucenin-2)	H	Glu	OH	Glu	OH	OH
30	Apigenin 6,8-di-*C*-glucoside (Vicenin-2)	H	Glu	OH	Glu	H	OH
31	Chrysoeriol 6,8-di-*C*-glucoside (Stellarin-2)	H	Glu	OH	Glu	OMe	OH
32	Diosmetin 6,8-di-*C*-glucoside (Lucenin-2 4´-methyl ether)	H	Glu	OH	Glu	OH	OMe
33	Apigenin 7-*O*-neohesperidoside-4´-glucoside (Rhoifolin 4´-glucoside)	H	H	*O*-Nh[b]	H	OH	*O*-Glu
34	Chrysoeriol 7-*O*-neohesperidoside-4´-glucoside	H	H	*O*-Nh[b]	H	OMe	OH
35	Apigenin 6-*C*-glucoside (Isovitexin)	H	Glu	OH	H	H	OH
36	Luteolin 7-*O*-rutinoside	H	H	*O*-Ru[a]	H	OH	OH
37	Chrysoeriol 8-*C*-glucoside (Scoparin)	H	H	OH	Glu	OMe	OH
38	Diosmetin 8-*C*-glucoside (Orientin 4´-methyl ether)	H	H	OH	Glu	OH	OMe
39	Quercetin 3-*O*-rutinoside (Rutin)	*O*-Ru[a]	H	OH	H	OH	OH
40	Apigenin 7-*O*-neohesperidoside (Rhoifolin)	H	H	*O*-Nh[b]	H	OH	OH
41	Apigenin 7-*O*-rutinoside (Isorhoifolin)	H	H	*O*-Ru[a]	H	OH	OH
42	Chrysoeriol 7-*O*-neohesperidoside	H	H	*O*-Nh[b]	H	OMe	OH
43	Diosmetin 7-*O*-rutinoside (Diosmin)	H	H	*O*-Ru[a]	H	OH	OMe
44	Diosmetin 7-*O*-neohesperidoside (Neodiosmin)	H	H	*O*-Nh[b]	H	OH	OMe

[a] *O*-Rutinose; [b] *O*-Neohesperidose.

Recently, our research group has identified, in the whole fruit juice, the presence of two new statin-like flavonoids[61], Brutieridin and Melitidin (1, 2, chart), which have been isolated by liquid chromatography (LC) assisted by UV and Mass Spectrometric (MS) detection and whose structure was determined by extensive use of LC-MS/MS and high field Nuclear Magnetic Resonance (NMR) methods[8] **Figure 3**.

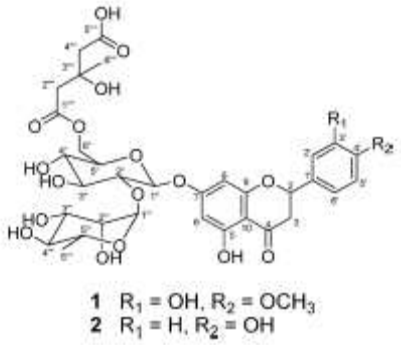


Figure 3: Brutieridin and Melitidin (1, 2, chart)

Brutieridin and melitidin, isolated and identified as HMG conjugates of neohesperidin and naringin, are present in bergamot fruit in concentrations ranges of approximately 300-500 and 150-300 ppm, respectively, as a function of the ripening stage; these compounds may be found either in the juice or in the albedo and flavedo of bergamot. To the best of our knowledge, the identification of similar HMG conjugates in plants has been described only for Roman chamomile (*Chamaemelum nobile*) [62-65].

Flavanone glycosyl compositions of peels and seeds are quite unlike those of juices. The Citrus peel and seeds are very rich in phenolic compounds, such as phenolic acids and flavonoids. The peels are richer in flavonoids than the seeds[66]. The seed and peel compositions are not always the same in Citrus fruits. The seeds of bergamot are the most important source of the glycosylated flavanones, naringin and neohesperidin[66-67]. The 7-O-glycosyl flavanones are the most abundant flavonoids in all Citrus fruits[68-69]. The neohesperidoside flavanones, naringin, neohesperidin and neoeriocitrin, are mainly present in bergamot, grapefruit and bitter orange juices, while rutinoside flavanones, hesperidin, narirutin and didymin, are present in bergamot, orange, mandarin and lemon juices[70]. Different tissues of the fruit also produce compounds such as diosmin and poncirin[71] . Flavanone chemical structures are specific for every species, which renders them markers of adulteration in commercial juices [72-74]

2.1.3 Liquid chromatography/mass spectrometry for separation and structural determination.

A number and variety of methods for the detection and quantification of flavonoid compounds in fruit have already been developed [75-77] . Several analytical procedures allow the simultaneous determination of the various kinds of flavonoid glycosides as flavanone-*O*-glycosides, flavone-*O*-glycosides, flavone-*C*-glucosides and polymethoxyflavones.

Qualitative and quantitative applications of high performance liquid chromatography (HPLC) for flavonoids analysis are nowadays, very common. These compounds can be separated, quantified, and identified in one operation by coupling HPLC to ultraviolet (UV), mass, or nuclear magnetic resonance (NMR) detectors. This excellent chromophore is, of course, UV active and provides the reason why flavonoids are so easy to detect. For the analytical HPLC of a given subclass of flavonoids (flavones, flavonols, isoflavones, anthocyanins, etc.), the stationary phase, solvent, and gradient have to be optimized. A very high proportion of separations are run on octadecylsilyl bonded (ODS, RP-18, or C18) phases. As solvents for application, acetonitrile–water or methanol–water mixtures, with or without small amounts of acid, are very common. These are compatible

with gradients and MS/UV detection. API are soft ionization methods and do not typically produce many fragments. This is useful in quantitative analysis or molecular mass determination but is of little use in structure elucidation.[78-80] For flavonoid aglycones and glycosides with a limited number of sugar units LC-MS provide only intense $[M+H]^+$ ions for the aglycones and weak $[M+H]^+$ ions of glycosides (mono- or disaccharide), together with intense fragment ions due to the loss of the saccharide units, lead to the aglycone moiety $[A+H]^+$. General type of fragmentations can be showed in fig 4 were the nomenclature proposed by Domon and Costello[81] is applied. Further structural characterization can be performed by LC-MS/MS and MS/MS analysis in high resolution.[82-85]

It's possible to establish the distribution of substituents between the A- and B-rings and also the determination of the nature and site of attachment of the sugars in O- and C- glycosides.[86-88] Online accurate mass measurements of all MS/MS fragments were obtained on the Q TOF instrument, allowing molecular formulae of compounds to be assessed directly. In order to obtain NMR structural elucidation must be have an adequate quantity of unknown compounds. For this reason is necessary to make a preparative steps for their isolation.

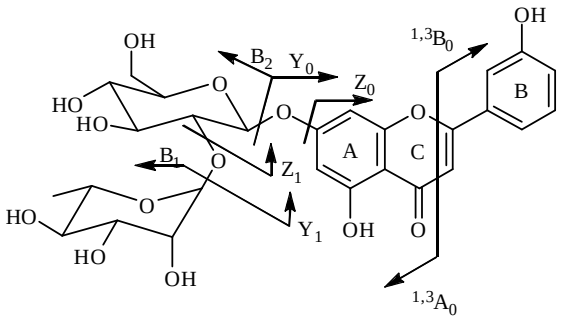


Figure 4. Fragmentations of flavonoid glycoside.[66]

After sample extraction ideal strategy consist in a previous medium-pressure liquid chromatography (MPLC)[89-90] and successive purification by semi-preparative HPLC. The former covers a wide range of column diameters, different granulometry packing materials, different pressures, and have a high loading capacity (1:25 sample-to-packing-material ratio[91]), the latter use columns of internal diameter 8 to 21 mm, often packed with 10 µm (or smaller) particles and both isocratic and gradient conditions are employed.[92-94]

Despite the intense research activity on the composition of flavonoid fraction, some questions still remain unanswered; many flavonoids present in low concentrations remain unidentified or only tentatively identified, due to the lack of standards, to the difficulty of isolating sufficient amounts of them from the matrix, and to the difficulty of synthesizing them due to their structural complexity. In recent years, interest in microcolumn LC has increased considerably. This is mainly due to the ability to work with small sample size, small volumetric flow-rates, and to the enhanced detection performance obtained with the use of concentration-sensitive detectors as a result of the reduced chromatographic dilution **[95-96]**. In the analysis of food samples, only few papers report the use of micro and capillary HPLC columns, although their usefulness in the characterization of minor components in food samples has been demonstrated **[97-98]**. The coupling of powerful and sensitive analytical techniques such as micro-LC with ESI/MS can permit more extensive investigations of

the flavonoid fraction in citrus juices and generally in many other natural complex matrices than conventional HPLC study. In this paper, microbore C18 columns have been used for the HPLC-ESI/MS analysis of flavonoids in five citrus juices: lemon, mandarin, sweet orange, grapefruit and bergamot (**Figure 5, Table 6**)[99].

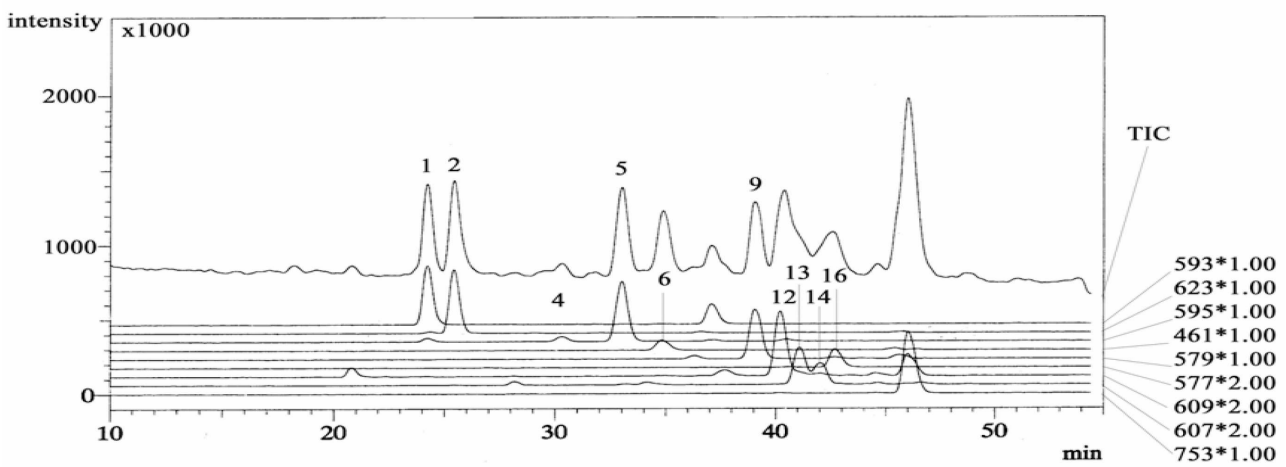


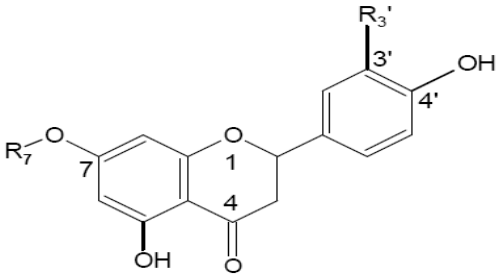
Figure 5. Micro HPLC-ESI/MS chromatogram of the flavonoids of a bergamot juice. TIC chromatogram and ion chromatograms extracted at m/z corresponding to the molecular weight of the detected flavonoids. For peak identification, see Table 6.

	Compound	RRt	MS	L	G	M	O	B
1.	**Apigenin 6,8-di-*C*-glucoside** 5,7,4′-trihydroxyflavone 6,8-di-*C*-β-D-glu	1	593	x	x	x	x	x
2.	**Diosmetin 6,8-di-*C*-glucoside** 5,7,3′-trihydroxy-4′-methoxy flavone 6,8-di-*C*-β-D-glu	1.06	623	x		x		x
3.	**Apigenin 7-(malonylapiosyl)-glucoside** 5,7,4′-trihydroxyflavone 7-*O*-(malonylapiosyl) glu	1.18	649	x	x		x	
4.	**Eriocitrin**[a] 5,7,3′,4′-tetrahydroxyflavanone 7-β-rutinoside	1.25	595/287	x	x	x[b]	x	x
5.	**Neoeriocitrin**[a] 5,7,3′,4′-tetrahydroxyflavanone 7-β- neohesperido-side	1.32	595		x[b]			x
6.	**Narirutin** a(Naringenin 7-β-rutinoside) 5,7,4′-trihydroxyflavanone 7-β-rutinoside	1.45	579/271		x[b]	x[b]	x	x
7.	**Rutin**[a] 3,5,7,3′,4′-pentahydroxyflavone 3-β-rutinoside	1.40	609/301	x				
8.	**Diosmetin-6-*C*-β-D-glucoside** 5,7,3′-trihydroxy-4′-methoxy flavone -6-*C*-β-D-glu	1.42	461	x				
9.	**Naringin**[a] 5,7,4′-trihydroxyflavanone-7-β-neohesperidoside	1.53	579		x			x
10.	**Hesperidin**[a] 5,7,3′-trihydroxy-4′-methoxyflavanone 7-β-rutinoside	1.56	609/301	x	x	x	x	
11.	**Naringenin 4′-*O*-xylosil-glucoside** 5,7,4′-trihydroxyflavanone 4′-*O*-xylosylglu	1.61	565				x	
12.	**Neohesperidin**[a] 5,7,3′-trihydroxy-4′-methoxyflavanone 7-β-neohesper-idoside	1.67	609	x	x			x
13.	**Diosmin**[a] 5,7,3′-trihydroxy-4′methoxyflavone 7-β-rutinoside	1.68	607/299			x		x
14.	**Neodiosmin**[a] 5,7,3′-trihydroxy-4′methoxyflavone 7-β-neohesperido-side	1.75	607				x	x
15.	**Apigenin 7-Rutinoside** 5,7,4′-trihydroxyflavone 7-β-rutinoside	1.68	577/269			x[b]		
16.	**Rhoifolin** (apigenin-7-neohesperidoside) 5,7,4′-trihydroxyflavone-7-β-neohesperidoside	1.75	577					x[b]
17.	**Iso-/Limocitrol 3-β D-glucoside** 3,5,7,3′-tetrahydroxy-6,8,3′-trimethoxyflavone 3-β-D-glu/3,5,7,3′-tetrahydroxy-6,8,4′-trimethoxyflavone 3-β-D-glu	1.66	537/375	x				
18.	**Limocitrin 3-β D-glucoside** 3,5,7,4′-tetrahydroxy-8,3′-dimethoxyflavone 3-β-D-glu	1.69	507	x				
19.	**Didimin**[a] 5,7-dihydroxy-4′-methoxyflavanone 7-*O*-rutinoside	2.04	593/285		x	x	x	
20.	**Poncirin**[a] 5,7-dihydroxy-4′-methoxyflavanone 7-*O*-neohesperi-doside	2.14	593		x			

Glu: glucoside.
[a] Identified with standard component.
[b] Identified only with the microHPLC column.
x identified for the first time.
L: lemon; G: grapefruit; M: mandarin; O: orange; B: bergamot.

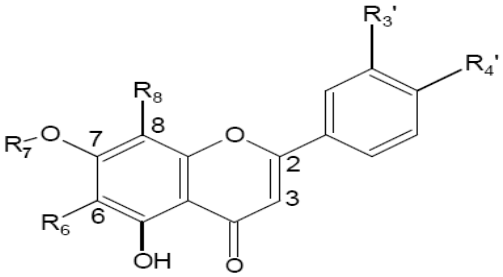
Table 6. Flavonoids in Citrus juices detected using a microbore column in HPLC-ESI/MS analysis.

Bergamot juice is a complex food matrix and the identity of its components can be correctly assessed by LC coupled with DAD and a tandem mass spectrometer. The identity of several flavonoids and furanocoumarins was assessed by co-chromatography, UV spectra and molecular weight comparison. The unknown compounds were dissociated by induced collision (CID-MS) and their identity established through the characteristic ions product. By this approach a complete profile of about twenty compounds (furano-coumarins, flavonoids C- and O-glycosides) present in BJ was obtained (**Figure 6-7**). Furthermore, three acylated flavanones, present in amounts of 20.1±1.1, 89.3±2.2 and 190.1±3.1 mg/L, respectively, and which seem to correspond to di-oxalate derivatives of neoeriocitrin, naringin and neohesperidin, were identified for the first time in BJ (**Table 7**)[100].



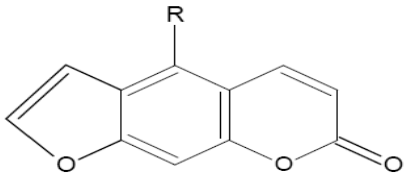
Peak	Flavanones	R_7	$R_{3'}$
1	eriocitrin	Rut	OH
2	neoeriocitrin	nH	OH
3	naringin	nH	H
4	neoeriocitrin-di-oxalate	nH-di-ox	OH
5	neohesperidin	nH	OCH_3
6	naringin-di-oxalate	nH-di-ox	H
7	neohesperidin-di-oxalate	nH-di-ox	OCH_3

Rut=rutinose, nH=neohesperidose, nH-di-ox = nH-di-oxalate



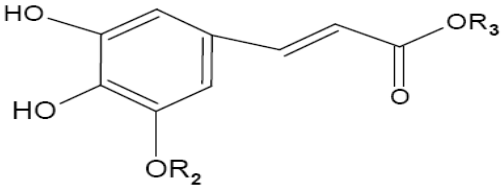
Peak	Flavones	R_6	R_7	R_8	$R_{3'}$	$R_{4'}$
A	lucenin-2	glc	H	glc	OH	OH
B	vicenin-2	glc	H	glc	H	OH
C	stellarin-2	glc	H	glc	OCH_3	OH
D	D-6,8-di-C-glc	glc	H	glc	OH	OCH_3
E	isovitexin	glc	H	H	H	OH
F	A-8-C-glc	H	H	glc	H	OH
G	scoparin	H	H	glc	OCH_3	OH
H	orientin	H	H	glc	OH	OCH_3
I	rhoifolin	H	-nH	H	H	OH
L	C-7-O-nH	H	-nH	H	OCH_3	OH
M	neodiosmin	H	-nH	H	OH	OCH_3

C: chrysoeriol, D: diosmetin, A: apigenin, glc: glucose



Furanocoumarins

Peak	Name	R
a	Bergapten	OCH_3
b	Bergamottin	$CH_2CHC(CH_3)CH_2C(CH_3)_2$



Caffeic acid derivatives

Peak	Name	R_1	R_2	R_3
X1	di-acetyl Caffeic acid	H	$COCH_3$	$COCH_3$
X2	Ferulic acid hexose	H	CH_3	hexose
X3	Sinapic acid hexose	OCH_3	CH_3	hexose

Figure 6. Structures of flavonoids, cinnamic acid derivatives and furanocoumarins founds in BJ.

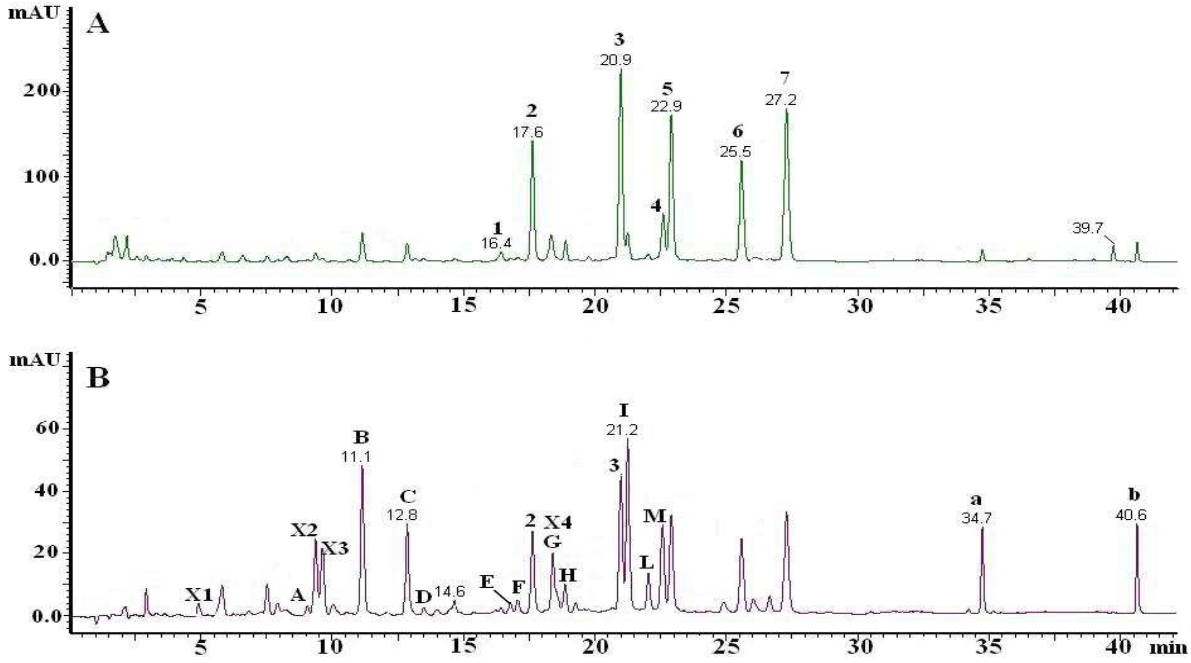


Figure 7. Typical chromatograms of bergamot juice at 282 nm (A) and 330 nm (B).

Peak	mg/L	Peak	mg/L
1	9.6±1.1	**F**	2.8±0.3
2	73.3±1.6	**G**	9.1±0.7
3	167.5±1.8	**H**	6.0±0.3
4	20.1±1.1	**I**	46.4±1.9
5	123.9±1.7	**L**	7.8±0.4
6	89.3±2.2	**M**	23.1±1.4
7	190.0±3.1	**a**	9.0±0.4
A	1.3±0.1	**b**	18.2±0.5
B	38.6±2.1	**X1**	1.6±0.2
C	25.8±1.3	**X2**	12.6±0.9
D	2.0±0.1	**X3**	12.1±0.8
E	2.1±0.1		

Table 7. Content of flavanone-O-glycosides (**1-7**), flavone-C-glucosides (**A-H**), flavone-O- neohesperidosides (**I-M**), cinnamic acid derivatives (**X1-3**) and furanocoumarins (**a-b**) in BJ.

2.2 Phenolic compounds in *Olea europaea L.*

Biophenolic compounds are natural phenolic biomolecules widely distributed in plants (principally fruits, and in minor amount, flowers, leaves and other vegetable organs) present in the Mediterranean diet. They may occur in various forms: free or in a conjugated form, mainly with a sugar molecule or as esters. With the term biophenols we include not only compounds with phenolic molecular structures, but also related compounds as metabolites or degradation products. Like many fruits and vegetables, olive drupes contain biophenols distributed in the olive mesocarp, the pulp, and also in the seed. The olive fruit is characterised by the epicarp (skin), with a soft, pulpy flesh (mesocarp), and the endocarp (stone), which contains the seed or kernel. In ripe olives, the seed makes up some 2–3% of the total mass, the stone 13–23% and the flesh or mesocarp some 84–90% but occasionally as low as 65%. The composition of the flesh, stone and seed components is given in **Table 8** but clearly the components of the flesh are quantitatively the more important. The flesh components pass either as is or transformed, to the oil, which is mainly composed of triacylglycerols with small quantities of free fatty acids, glycerols, phosphatides, pigments, carbohydrates, proteins, flavour compounds, phenols, sterols and unidentified resinous substances. Oil components from the seed, though a minor component, still become part of the olive oil but do not have the same composition as that from the flesh. Factors contributing to the variability in phenolic distribution include the cultivar and genetics, maturity, climate, position on the tree, rootstock and agricultural practices. In the case of processed products, technological processes to which olive fruits are exposed may also impact significantly on the phenolic content. There are approximately 2500 known varieties of olives, 250 of which are classified as commercial cultivars by the International Olive Oil Council (IOOC). These commercial cultivars are used for the production of either olive oil or table olives. The particular use of a given cultivar is determined by its oil content and size, with larger fruits (> 4 g) being favoured for table olive consumption [101-103].

Constituent	Flesh	Stone	Seed
Water	50–60	9.3	30.0
Oil	15–30	0.7	27.3
N matter	2–5	3.4	10.2
Sugars	3–75	41.0	26.6
Cellulose	3– 6	38.0	1.9
Ash	1–2	4.1	1.5
Phenolics	2–2.5	0.1	0.5–1.0
Intermediate		3.4	2.4

Table 8: Olive fruit composition

The virgin olive oil (VOO) hydrophilic phenols constitute a group of secondary plant metabolites showing peculiar organoleptic and healthy proprieties. They are not generally present in other oils and fats. VOO contains different classes of phenolic compounds (**Table 9, Figures 8 and 9**). Phenolic acids, represented by caffeic, vanillic, syringic, *p*-coumaric, *o*-coumaric, protocatechuic, sinapic, *p*-hydroxybenzoic and gallic acid, were the first group of phenols discovered in VOO.

Phenolic acids and derivatives	**Hydroxy-isocromans**
Vanillic acid	
Syringic acid	**Phenolic alcohols**
p-Coumaric acid	(3,4-Dihdroxyphenyl) ethanol (3,4 DHPEA)
o-Coumaric acid	(p-Hydroxyphenil) ethanol (p-HPEA)
Gallic acid	(3,4-Dihdroxyphenyl) ethanol-glucoside
Caffeic acid	
protocatechuic acid	
p-Hidroxybenzoic acid	
Ferulic acid	**Flavones**
Cinnamic acid	Apigenin
4-(acetoxyethil)-1,2-Dihydroxybenzene	Luteolin
Benzoic acid	
Lignans	
(+)-Acetoxypinoresinol	
(+)-Pinoresinol	
Secoiridoids	
Dialdehydic form of decarboxymethyl elenolic acid linked to 3,4-DHPEA (3,4 DHPEA-EDA)	
Dialdehydic form of decarboxymethyl elenolic acid linked to p-HPEA (*p*-HPEA-EDA)	
Oleuropein aglycon (3,4 DHPEA-EA)	
Ligstroside aglycon	
Oleuropein	
p-HPEA-derivative	
Dialdehydic form of oleuropein aglycon	
Dialdehydic form of ligstroside aglycon	

Table 9: phenolic composition of virgin olive oil (VOO)

Phenolic alcohols include (3,4-dihydroxyphenyl)ethanol (3,4-DHPEA) and (*p*-hydroxyphenyl)ethanol (*p*-HPEA); their concentration is generally low in fresh oils but increases during oil storage since the hydrolysis
of VOO secoiridoids such as 3,4-DHPEA-EDA, *p*- HPEA-EDA and 3,4-DHPEA-EA containing 3,4-DHPEA and *p*-HPEA in their molecular structures (**Figure 9**)[104]. Flavonoids like luteolin and apigenin were also reported as phenolic components of VOO by Rovellini *et al.* (1997)[105]. The lignans include (+)-1-acetoxypinoresinol and (+)-1- pinoresinol (**Figure 9**). These compounds are present in the olive pulp and in the woody portion of the seed; they are released in VOO during the mechanical extraction process without biochemical modification during the extraction.

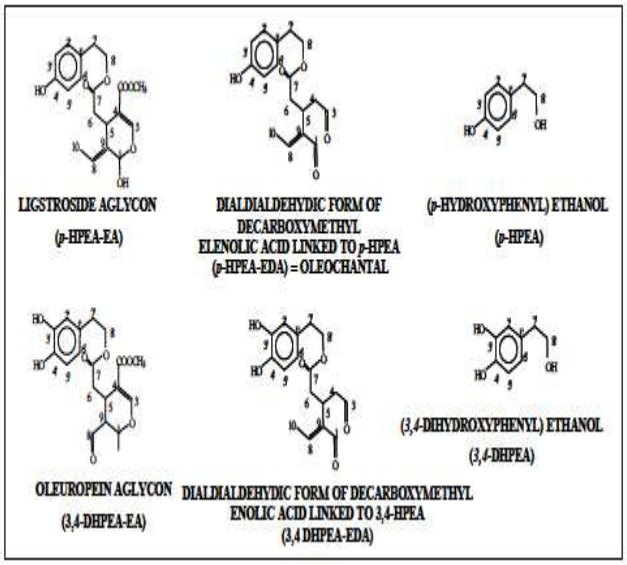


Figure 8. Chemical structures of secoiridoids derivates and phenyl alcohols of VOO

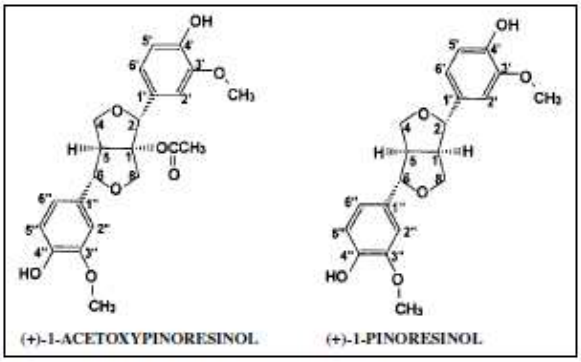


Figure 9. Chemical structures of lignans found in olives and VOO

Secoiridoids are main compounds of VOO. They are represented by the dialdehydic form of decarboxymethyl elenolic acid linked to 3,4-DHPEA or *p*-HPEA (3,4-DHPEA-EDA or *p*-HPEA-EDA), an isomer of oleuropein aglycone (3,4-DHPEA-EA) and the ligstroside aglycone (*p*-HPEA-EA).

These substances are aglycone derivatives of secoiridoid glucosides contained in the olive fruit, originating during oil mechanical extraction process, by hydrolysis of oleuropein, demethyloleuropein and ligstroside; these reactions are catalysed by endogenous b-glucosidases. Olives, in fact, contain high quantity of phenolic compounds the concentration of which can range between 1–3 % of the fresh pulp weight. Oleuropein, demethyloleuropein, ligstroside and nüzhenide are the most abundant secoiridoid glucosides in the olive

(**Figure 10**), that also contains, as main phenolic compounds, the verbascoside, a derivative of the hydroxicinnamic acid, having the 3,4-DHPEA in its molecular structure (**Figure 10**)[104].

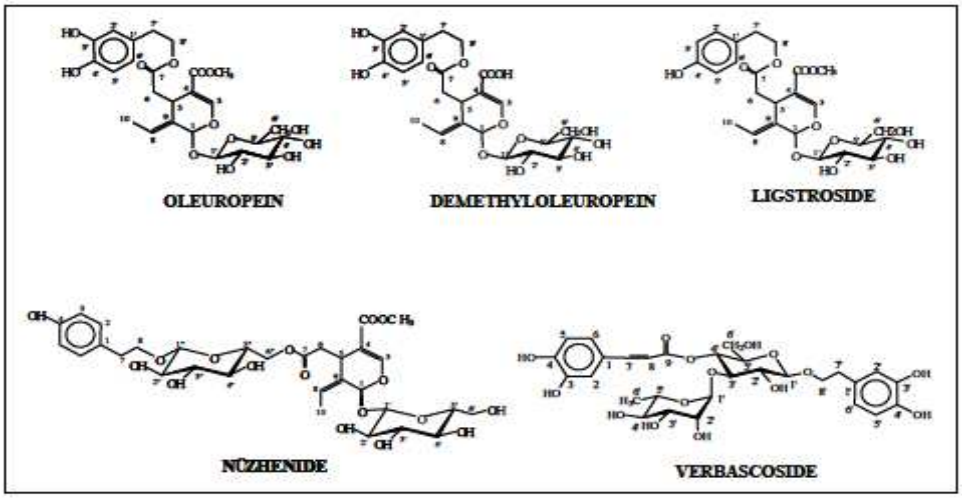


Figure 10. Chemical structures of secoiridoid derivates and phenyl alcohols of olives

2.2.1 Analytical methods for the separation, identification and quantification of phenolic compounds in Olea europaea L.

A number of extraction procedures and analytical methods for identifying and qualifying these compounds in olea europaea L. have so far been reported. The phenolic fraction of olive oil has traditionally been isolated by liquid–liquid extraction (LLE) [106-108]. This technique, however, is usually labour-intensive and sometimes more expensive; also, it often requires using large amounts of organic solvents. Some authors have used solid phase extraction (SPE) with C18, C8 [109-110] or -diol [111-112] to isolate phenolic compounds. Others have compared LLE and SPE for this purpose [113-114] , and they found LLE with hexane and methanol–water as mixture solvents and SPE with a normal phase to provide the best recoveries for these polar compounds. Due to the need to carry out an individual identification of each phenolic compound present in the extracts, the traditional methods were replaced with separative techniques [*e.g.* gas chromatography (GC) [115], high-performance liquid chromatography (HPLC) [116-117], and capillary electrophoresis (CE) [118]coupled to different detectors [119]. However, most of the discussions have been focused on the optimization of high-performance liquid chromatography (HPLC) methods, mainly with reversed phase C18 columns and different mobile phases and gradients, followed by ultraviolet (UV) [120], electrochemical [121], fluorescence [122] or mass spectrometric (MS) detection [118, 123]. Some authors have addressed the separation and quantitation of specific phenols by GC with various detection techniques including MS [124]and NMR [125]; however, the GC technique is less widely used for this purpose since the analytes are unstable at high temperatures and require a derivatization reaction for their determination. Recently, an improvement in chromatographic performance has been achieved by the introduction of rapid-resolution LC (RRLC) and ultra-performance LC (UPLC) [126]. These approaches use narrow-bore columns packed with very small particles (1.8µm) and high flow rate with delivery systems operating at high back-pressures. The major advantages of RRLC over conventional HPLC are improved resolution, shorter retention times, higher sensitivity, and better performance. Coupling RRLC with MS further offers a potent analytical alternative, which has been applied in recent publications characterizing food products [127-131]. The HPLC–MS couple has been used for the characterization of phenolic compounds in olive oil samples. The MS technique was used with electrospray ionization (ESI) [111,132, 133] and atmospheric pressure chemical ionization (APCI) [134]; and the MS analyser was a quadrupole (Q) or triple quadrupole (TQ) [123, 133], ion trap (IT) [132], or time-of-flight (TOF) type [135, 136]. Capillary electrophoresis (CE) has become one of the major choices for the separation of charged analytes and a solid alternative to LC, especially if we are speaking of polar or charged compounds using its different modalities (CZE, CEC, etc.) [137-140]. Mass spectrometers have gained increasing acceptance as supplements or replacements for conventional detectors in CE. CE–MS coupling combines the high efficiency and resolution power of CE with the high selectivity and sensitivity inherent in MS, thus providing a powerful, highly attractive analytical tool. While CE–MS is mostly performed with electrospray ionization, the soft-ionization technique can be used to obtain ions even from thermally labile, non-volatile, polar compounds [141-143]; however, CE–ESI-MS appears to have only been used very few times [135, 144, 145] to determine phenolic compounds in oils, using IT and TOFMS as analysers. Time ago, Lafont et al. [144]carried out a sensitive method for qualitative and quantitative analysis of several phenolic compounds (including ferulic and vanillic acids) in olive mill wastewater by CE–ESI-MS. More currently, Carrasco-Pancorbo et al. developed several methods for just the qualitative and semi-

quantitative determination of phenolic fraction (phenyl alcohols, phenyl acids, lignans, flavonoid and secoiridoids) of extra-VOO using CE–ESI-MS and CE–TOF-MS respectively [135,145]. Nevado et al. [146] developed a sensitive, reliable, off-line SPE CE–ESI-MS method for the analytical separation and determination for first time of phenolic acids such as hydroxyphenylacetic acid (HFA), gentisic (GEN), ferulic (FER) and vanillic (VAN) acids, including three isomer (*o*-, *m*-, and *p*-) of coumaric acid (COU) in extra and VOO samples. However, a considerable number of phenolic compounds have still not been completely characterized and many problems remain to be resolved. One of the reasons lying behind these difficulties is the absence of suitable pure standards, in particular secoiridoid molecules and lignans. Moreover, phenolic fraction of oil is quite heterogeneous and complex and the matrix in which phenols are found (i.e. olive oil) is also rather complicated; these two facts cannot facilitate their analysis.

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3. Experimental section

The growing interest in antioxidant bioactive compounds and their dietary sources has attracted attention as a result of its remarkable flavonoid content. The development of new methods of extraction of nutraceuticals compounds without use of toxic and expensive solvents is crucial for the introduction of new active principles as drugs. Therefore, in this thesys we want to validate modern appropriate extracting procedures, meeting pharmacopoeia requirements, to set-up safety protocols for the recovery of the bioactive compounds present in food (bergamot, olive oils and drupes) using water as extracting solvent. The recovery of compounds of high antioxidant value can be useful as raw materials for the direct application in cosmetics, for the production of food supplements and the development of new functional foods enriched in bioactive compounds.
Also, quality (e.g. olive oils) and safety control and the validation of origin are hot issues in the production of food and its distribution, and are of primary concern to food and agriculture organization. Quality and safety are the two main issues related to genuineness of processed and fresh aliments. In food safety, there are different problems related to chemicals in foodstuffs. Certain food has the potential of containing chemicals which, if eaten in sufficient quantities, are harmful to human health. Other food can be contaminated by illegal dyes. The White Paper on Food Safety outlines a comprehensive range of actions needed to complement and modernize existing EU food legislation. Moreover, the Food Standards Agency aims to protect the consumer from these chemicals, and for this reason must maintain the best knowledge base possible on the subject to provide the necessary tools to ensure that consumer exposure to these chemicals is kept as low as reasonably practicable.
The quantitative LC-MS/MS methodology is utilized in food safety, agricultural and forensic chemistry. The Modern mass spectrometry (MS) provides unique, reliable and affordable methodologies to approach with a high degree of scientific nature any problem which may be posed in this field.

3.1 Recovery of nutraceuticals of high antioxidant value from Citrus Bergamia Risso and development of new functional foods

The Citrus Bergamia Risso fruit is very rich in nutraceutical substances, with anticancer, antiatherogenic, antimicrobical and anti-infiammatory proprieties. The beneficial effects of the dietary Citrus fruits can in particular be attributed, not only to the vitamin C, folate, dietary fibre and carotenoids, but also to the antioxidant and radical scavenging activity of their flavonoids, responsible for reduced risk for certain chronic diseases, the prevention of some cardiovascular disorders, and certain types of cancerous processes. Flavonoids also exhibit antiviral, antimicrobial, and anti-inflammatory activities, effects on capillary fragility, an ability to inhibit human platelet aggregation, as well as antiulcer and antiallergenic properties.
Bergamot has yet to find an application in the food industry, despite its extremely high flavonoid content[1].
Recently, Pernice (2009) has reported the possibility to re-evaluate bergamot juice through its chemical characterization and its use to enrich and fortify fruit juices. To investigate this, apples

and apricots were used for the laboratory-scale production of fruit juice, following both the traditional industrial recipe and those with the addition of bergamot juice at 10% or 20%, together with or in order to replace the synthetic additives normally used in the industrial process (ascorbic acid and citric acid). The ascorbic acid content and the antioxidant activity were measured during the different steps of juice production and after storage at 37 °C for 15 days to evaluate juice shelf-life. Apricot and apple juices fortified with bergamot juice showed a significant increase in their antioxidant properties and a decreased reduction in ascorbic acid content after the typical production steps. All of the results obtained support the hypothesis that the addition of bergamot juice to juices preserves their ascorbic acid content from thermal degradation and contributes to enhance the antioxidant activity, ensuring a product much richer in antioxidants and ascorbic acid. A preliminary consumer test encouraged the production of bergamot fortified fruit juices [2].

Recently, our research group has identified, in the whole fruit juice, the presence of two new statin-like flavonoids[3], Brutieridin and Melitidin. The inhibitory effect on HMG-CoA reductase of these new peculiar flavonoids, bearing the 3-hydroxy-3-methyl-glutaric acid (HMG) moiety, was checked, in vitro, against commercial statin drugs with excellent results[4]. It can be anticipated, therefore, that the new natural molecules can play an important role in the control of the blood cholesterol level. Moreover, computational studies based on density functional theory have demonstrated that both molecules binds efficiently in the catalytic site of HMG-CoA reductase [5].

Miceli (2007)[6] has investigate the hypolipidemic effects of C. bergamia juice and its protective effect on liver of hyperlipidemic rats. Chronic administration of C. bergamia (1 mL/rat/day) provoked a significant reduction in serum cholesterol, triglycerides, and low-density lipoprotein (LDL) levels and an increase in high-density lipoprotein (HDL) levels; moreover, histopathological observations showed, in rats submitted to C. bergamia treatment, a protection of hepatic parenchyma. In addition, fecal neutral sterols and fecal bile acid excretion was found to be increased after C. bergamia treatment. These results suggest that the hypocholesterolemic effect of C. bergamia may be mediated by the increase in fecal neutral sterols and total bile acids excretion. In addition to the hypolipidemic effect, the juice shows radical scavenging activity in the diphenylpicrylhydrazyl (DPPH) test; probably the two effects are related. These observations suggest that the positive intake of C. bergamia may reduce the risk of some cardiovascular diseases through its radical scavenging function and hypocholesterolemic action.

The aim of my research has been the recovery of anticholaesterolaemic active principles from Citrus Bergamia Risso and the development of new functional foods. Different extraction procedures have been therefore exploited to assay the presence of Brutieridin and Melitidin in Bergamot using appropriate extracting procedures

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3.1.1 Recycling of industrial essential oil waste: Brutieridin and Melitidin, two anticholesterolaemic active principles from bergamot albedo

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A b s t r a c t

Bergamot albedo, the white tissues between the skin and the pulp, is a polluting waste in the production of the renowned fragrance and is easily available by simple industrial processes. Brutieridin and melitidin are found in bergamot albedo and possess statin-like activity. Their anticholesterolaemic effect has been proved in vitro. Different procedures were exploited to assay the presence of brutieridin and melitidin and to evaluate their availability in water extracts. The best results in terms of simplicity and accessibility of the method were obtained with hot water at 65 °C. Interestingly, tea beverages enriched with the two active principles have been obtained by simple addition of dried albedo into commercial tea bags. This is the first report on the availability of methodologies to extract active principles from citrus albedo wastes.

Keywords: Bergamot waste, Albedo, Brutieridin, Melitidin

Introduction

Bergamot (Citrus bergamia Risso) is the common name of the fruit of the genus Citrus belonging to the family Rutaceae, subfamily Esperidea, which is a very delicate plant that has its natural habitat in a narrow zone in the province of Reggio Calabria (Italy). The essential oil from the fruit flavedo is widely used in the cosmetics industry. However, bergamot juice and albedo, the white tissue between the skin and the pulp, have no important industrial applications, in evident disparity with other citrus fruits. The albedo layer, rich in pectin and several flavonoids, has never been investigated in detail, to see if it could provide value-added compounds, such as nutraceuticals. What remains after the extraction of the volatile fraction is considered, in fact, industrial waste with its relative economic and environmental disadvantages. Recently, our research group has identified, in the whole fruit juice, the presence of two new statin-like flavonoids brutieridin (1) and melitidin (2). The inhibitory effect on HMG-CoA reductase of these new flavonoids, bearing the 3-hydroxy-3-methyl-glutaric acid (HMG) moiety, was compared, in vitro, against commercial statin drugs with excellent results (Di Donna, Dolce, & Sindona, 2008). Preliminary results from animal testing against the commercial drug pravastatin have shown that the new natural molecules 1 and 2 (Fig. 1) can play an important role as natural active principles lowering blood cholesterol level (Sindona et al., unpublished results).

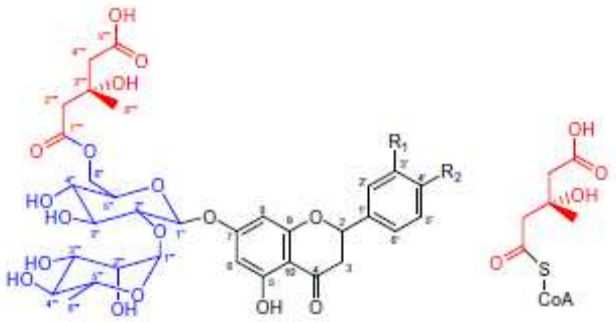

Fig. 1. Structures of brutieridin (1), melitidin (2) and HMG-CoA.

Moreover, computational studies based on density functional theory have demonstrated that both molecules bind efficiently to the catalytic site of HMG-CoA reductase (Leopoldini, Malaj, Toscano, Sindona, & Russo, in press). Calabrian folk medicine has considered, since the introduction of the plant in the middle of the 18th century, bergamot juice as a natural answer to the control of cholesterol level in blood. These important properties have always been associated with the effect of flavonoids, such as naringin, neoeriocitrin and neohesperidin, present in the order of several hundreds of ppm and to other minor species such as rhoifolin, neodiosmin, and chryosoeriol present at lower concentrations (Dugo et al., 2005;Gattuso, Barreca, Caristi, Gargiulli, & Luezzi, 2007; Gattuso et al.,2006). The association of anticholesterolaemic activity to flavonoid content lacks any scientific support. It cannot explain why other citrus fruits, containing the same species, do not exhibit the healing effect traditionally associated with bergamot. Recycling of waste from the citrus industry is limited to the transformation of the entire raw material into fertiliser (Van Heerden, Cronjé, Swart, & Kotzé, 2002) or animal fodder (Tripodo, Lanuzza, Micali, Coppolino, & Nucita, 2004). Considering that the cost of the albedo industrial waste is lower than the cost of the transportation to manufacturing plants, it is worthwhile to exploit its possible use as a source of anticholesterolaemic food supplements. Different procedures, therefore, are now presented aiming at optimizing the yields of those active principles present in properly treated albedo tissues. Safety procedures matching food supplements directives issued by European and International bodies were implemented (ICH, 2009).

Materials and methods

Chemicals

HPLC-grade methanol and 99% formic acid were purchased from Carlo Erba (Milan, Italy). Aqueous solutions were prepared using ultrapure water, with a resistivity of 18.2 MO cm, obtained from a Milli-Q plus system (Millipore, Bedford, MA). Brutieridin and melitidin used as calibration standards were purified in our laboratory.

Plant material

Frozen bergamot albedo was furnished by Goiasucchi s.r.l. (Gioia Tauro, Italy). Commercial Yellow Label Tea (Lipton) was used for the preparation of prototype albedo-containing tea bags.

Instrumentations and conditions

Microwave-assisted extraction was carried out using an Anton Paar Multiwave 3000 with programmable power control (maximum power 1400 W) and rotor XF100 (operating pressure up to 120 bar maximum; operating temperature 260 °C maximum; construction material PTFE-TFM for the vessel). In every vessel were put 3 g of homogenised whole albedo and 20 ml of water (4 vessels were used for each analysis) and a potential of 500W was applied for different duration times (1–5 min). This matrix/solvent ratio was maintained constant for warm/hot water extraction method. All water extracts were centrifuged at 4500 rpm for 5 min and filtered before instrumental analysis. LC–ESI-MS/UV analysis was performed using aWaters Fraction-Lynx system (Milford, MA) equipped with a ZMD mass spectrometer and a 486 UV detector. The column used for all our analyses was a Luna C18 (2) (250 x 4.6 mm; Phenomenex, Torrance, CA). The UV detector was set at 280 nm. The run time was 105 min, the flow rate was 1 ml/min, and the gradient was built using 0.1% HCOOH in H_2O (solvent A) and CH_3OH (solvent B) as mobile phases. The elution gradient was composed of the following steps: isocratic elution 80% A for 10 min; linear gradient from 80% A to 74% A in 2 min; linear gradient from 74% A to 31% A in 65 min; linear gradient from 31% A to 80% A in 18 min; equilibration of the column for 10 min. The calibration curve was constructed from the following solutions of brutieridin in methanol/water (1:1): 25, 12.5, 6.25 and 3.125 ppm.

Results and discussion

The peculiarity of bergamot lies in the presence of two new statin-like molecules 1 and 2 that are esters of 3-hydroxy-3-methylglutaric (HMG) acid which, when linked to CoA (3, Fig. 1), represents the key intermediate in cholesterol biosynthesis. The action of NADPH in the presence of HMG-CoA reductase is the accepted mechanism, which brings about the reduction of the thioester function to the corresponding alcohol and provides the biochemical tools to investigate the inhibitory effects of commercial statin drugs (Istvan & Deisenhofer, 2000, 2001; Istvan, Planitkar, Buchanan, & Deisenhofer, 2000; Tabernero, Bochar, Rodwell, & Stauffacher, 1999). Compounds 1 and 2 show an acceptable degree of inhibition against the same reduction system (Sindona et al., unpublished results). In this case in fact the ester function between positions 60 of the glucose and 1”’of the HMG moieties should be stable to the action of the NADPH reducing agent.

The content of active principles in the bergamot albedo

Mass spectrometric and UV analysis of the water extract (see Section 2.3) of bergamot albedo (Fig. 2A) displays a profile similar to that obtained with the juice, where the two active principles are present.

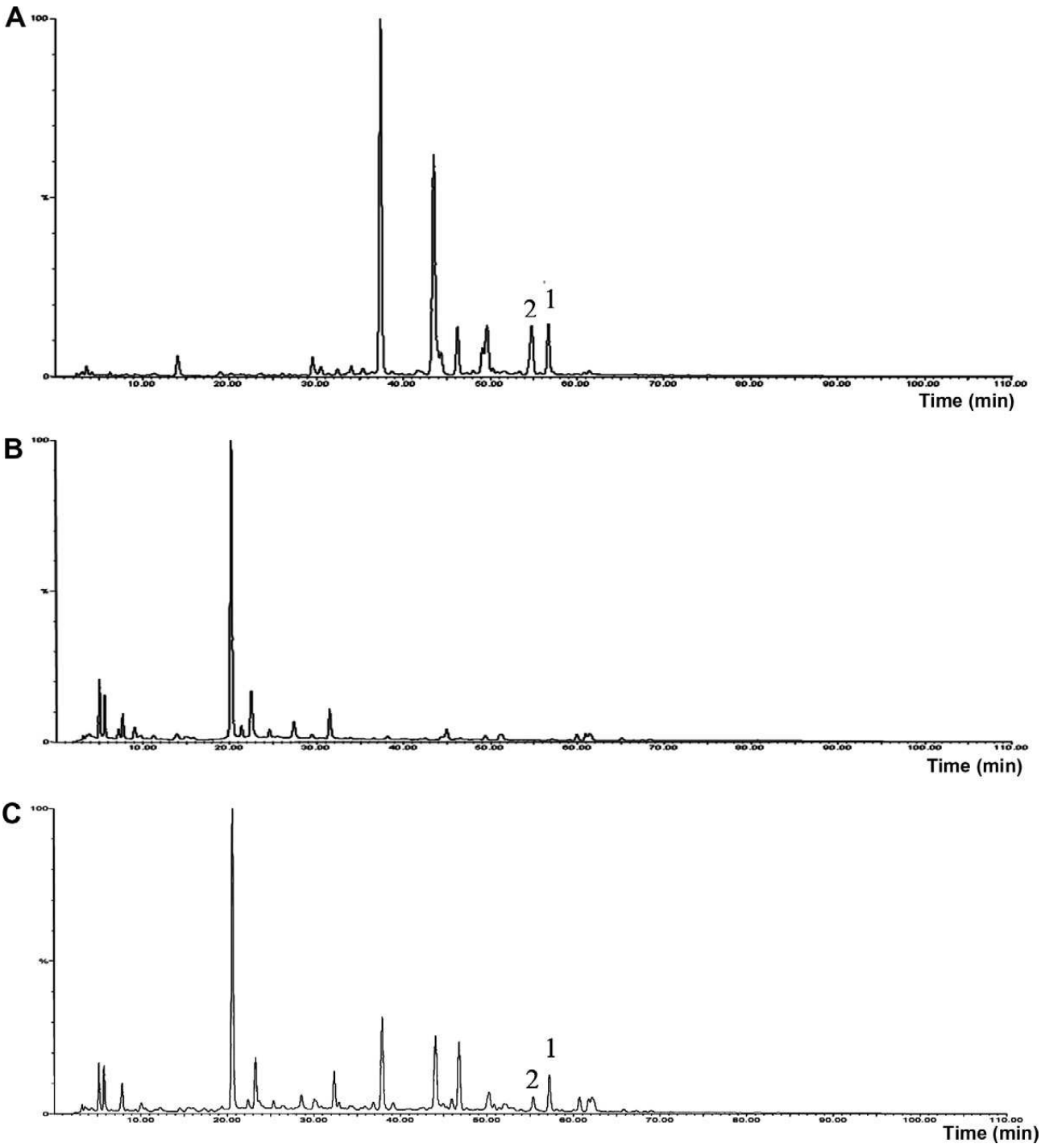

Fig. 2. UV profiles obtained from LC/UV/MS of albedo (A) water extract, (B) commercial tea and (C) dried albedo in commercial tea bag.

The optimization of the extraction procedure was exploited under different conditions: (i) water in a microwave (MW) oven operated at 500W or (ii) by simple extraction with warm-to-hot water. Water extracts taken at different time/temperature of the MW procedure were analyzed by LC/UV–MS (Di Donna et al., 2009) and the content of 1 and 2 was evaluated by UV (280 nm), using the same purified analytes (Di Donna et al., 2009) as external standard (r = 0.9999; Fig. 3).

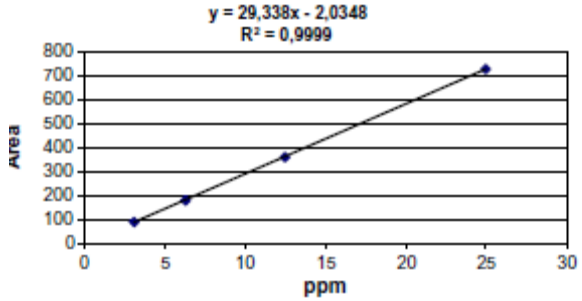

Fig. 3. Calibration curve for brutieridin standard.

The results are shown in Fig. 4A and the temperatures of each MW experiment are reported in Table 1.

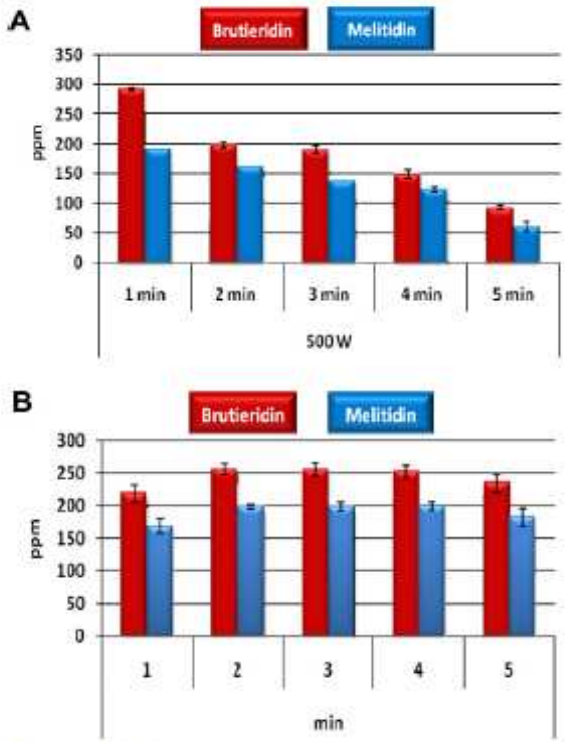

Fig. 4. Brutieridin (**1**) and melitidin (**2**) content (ppm) after five independent experiments (1–5) by (A) microwave extraction and (B) warm-to-hot water extraction. The mean values and the standard deviations were calculated from three measurements.

Table 1
Temperatures reached in microwave extraction and successively used in warm/hot water extraction method.

Time (min)	1	2	3	4	5
Temperature (°C)	48	58	68	75	82

In the MW extraction the temperature of water ranged from 48 °C to 82 °C with 1–5 min exposure (Table 1); however, the absolute amount of extracted species decreased almost linearly with increasing extraction time and solvent temperature. Therefore the best conditions are those corresponding to 1 min exposure of albedo in water in a 500-W microwave oven. The losses caused by prolonging the extraction time could be ascribed either to radiation or temperature effect on the active principles 1 and 2. It was, therefore, important to verify the behaviour of albedo under water extraction at temperatures equal to those experienced in the MW procedures. The results shown in Fig. 4B suggest that there is no thermal effect on the conventional extraction of the two statin-like molecules. What was observed in MW conditions may result from the exposure to microwaves, which probably degrade the compounds of interest, acting on the sugar or on the glutaric moieties. Actually, the amount of statin-like molecules in water extracts seems almost independent from the temperature, which can be conveniently set at a value of 65 °C in order to optimise the yields.

The content of active principles in whole albedo vs. dried albedo

Homogenised albedo (93.0 g) was oven-dried in two successive steps: at 30 °C for 16 h and then at 40 °C for 12 h. Dried tissue (23.2 g) was obtained, corresponding to a reduction to one-quarter of the original weight. The same quantity of whole and dried albedo was left, under gentle swirling, for four minutes in 100 ml of boiling water (100 °C); both extracts, submitted to conventional LC/UV/MS analysis showed that the relative yields of 1 and 2 were unaffected by the presence of water in the tissue. When dried matter is used, the absolute recovery of the natural statins is four times higher than that from whole albedo. Finally, a recovery test was performed, submitting albedo to the domestic procedure for preparing infusions from commercial tea bags. Dried albedo (0.503 g or 2.012 g of whole albedo) was added to commercial tea bags containing ca. 1.5 g black tea. The prototype tea bags were left, under weak agitation, for four minutes in a tea cup containing 200 ml of boiling water (100 °C). The same procedure was followed for bags containing whole albedo. In both cases 20 µl of the so prepared infusions, previously filtered using a 0.45-µm Teflon syringe filter, were injected into an LC/UV/MS. The results reported in Table 2 confirm the relative amount of extracted active principles does not depend on the presence of water and that the two statin-like molecules brutieridin and melitidin can be extracted easily by household procedures.

Table 2
Absolute content of brutieridin and melitidin in tea infusions.[a]

Sample	Brutieridin (ppm)	Melitidin (ppm)
Dried albedo in black tea	10.12 ± 0.16	7.01 ± 0.08
Whole albedo in black tea	10.56 ± 0.81	6.62 ± 0.34

[a] The mean values and the standard deviations were calculated over three measurements.

Conclusions

The results here presented throw light onto the possible use of an industrial waste as a natural resource in the control of blood cholesterol level. The active principles 1 and 2 can be easily obtained by simple extraction procedures.

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3.1.2 Purification of anticholesterolaemic nutraceuticals of Citrus juice flavonoids by flash chromatography

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Abstract

Recently we have isolated and characterized two new acyl flavonoids, namely brutieridin and melitidin, characterized by the presence of the 3-hydroxy-3-methyl glutaryl moiety (HMG):[1] these molecules resembles the active co-enzyme HMG-CoA, which is involved in the key step of the *in vivo* synthesis of cholesterol.
The latter molecule is a thioester that can be easily reduced by NADPH to the corresponding alcohol. On the contrary, the two active principles present in bergamot possess an ester moiety that hardly is reduced in mild condition. Furthermore, in vitro studies, and preliminary in vivo experiments demonstrates that the two isolated flavonoids posses a strong inhibition effect against the cholesterol synthesis.[2,3] In the present paper, the dry bergamot extract has been submitted to a purification step using the chromatographic system Supelco VersaFlash™, in order to obtain the enriched fraction of anticholesterolaemic nutraceuticals to use for the development of new functional foods.

Introduction

Bergamot is the common name of the fruit Citrus bergamia Risso, which belongs to the family Rutaceae, subfamily Esperidea. The uniqueness of bergamot trees is represented by a habitat that is virtually restricted to the coastal region of the Ionian Sea in the southern Calabrian region of Italy. This area presents favorable weather and pedoclimatic conditions for its cultivation. Three cultivars of bergamot (“Castagnaro”, “Fantastico”, and “Femminello”) are commercially grown and then industrially processed, exclusively to extract their essential oils. The industrial processing uses an indiscriminate mix of the three cultivars. In the past, bergamot has been highly valued by the cosmetic and perfume industry since its essence is very rich in terpenes, esters, and alcohols possessing a very characteristic and intense fragrance. The development of synthetic essential oil production led to a drastic drop in commercial demand for bergamot. However, over the past few years, following the growing interest in antioxidant bioactive compounds and their dietary sources, such as Citrus juices, bergamot juice has attracted attention as a result of its remarkable flavonoid content. Recently two new molecules have been isolated and identified as HMG conjugates (HMG= 3-hydroxy-3-methylglutaric acid) of neohesperidin and naringin, namely, brutieridin (m/z 755) and melitidin (m/z 725) from the Citrus bergamia risso juice by the research team cordinate by Prof Sindona (Figure 1). It is assumed that these new compounds can inhibit the activity dell'HMGR, as is the case for action of statins, since that portion of the structure of brutieridina and melitidina is also present in the structure of HMG-CoA and structure of statins (Figure 2).

Figure 1. LC/UV/ESI-MS of bergamot juice; C: UV at which correspond the m/z value (*[M+H]⁺;RT*): Apigenin 6,8-di-C-glucoside (*595; 15.42*), Diosmetin 6,8-di-C-glucoside (*625; 18.15*), Neoericitrin (*597; 23.49*), Narirutin (*581; 26.18*), Naringin (*581; 28.20*), Neohesperidin (*611; 30,95*), Rhoifolin 4'-glucoside (*741; 32.29*), 8) Rhoifolin (*579; 33.18*), Diosmir. (*609; 34.13*), Neodiosmin (*609; 34.88*), unknown 1 *(725; 38.13*), unknown 2 (*755, 40.69*), unknown 3 (*723, 43.66*), unknown 4 (*753, 44.62*). B: XIC of unknown 2; A: XIC of unknown 3.

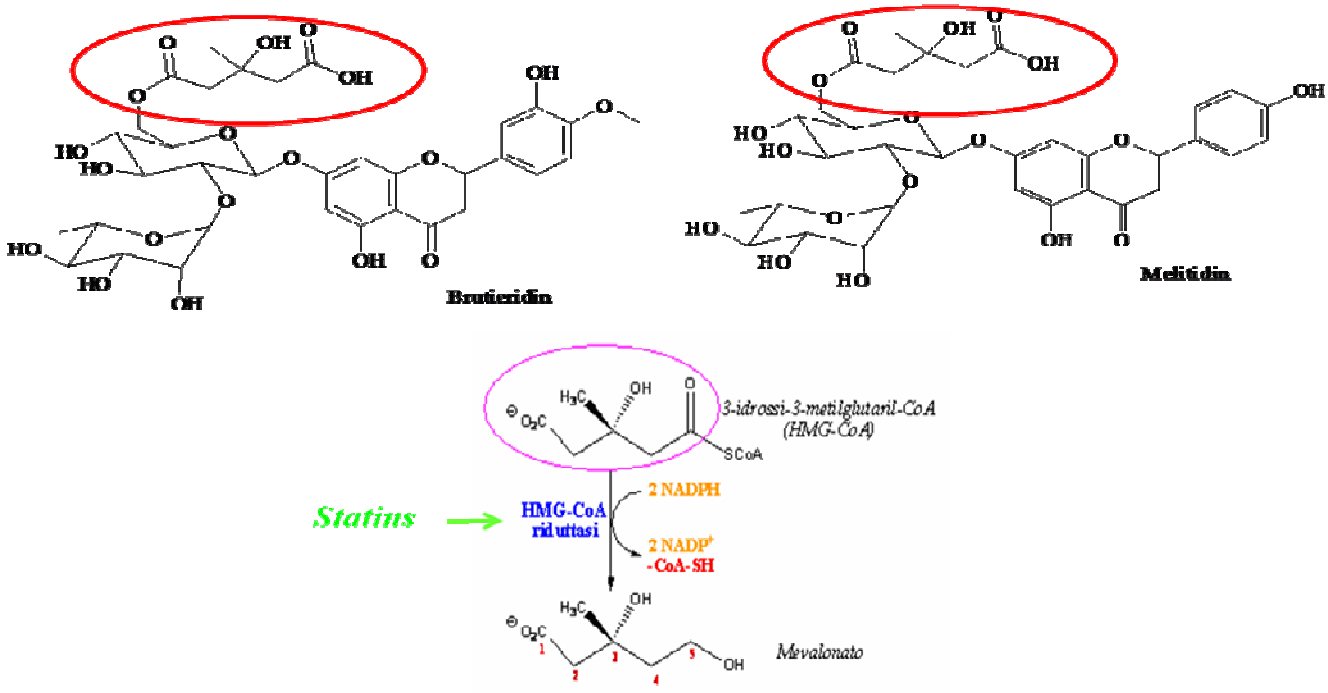

Figure 2. Probable anti-cholesterol activity

The aim of this work has been the Purification of citrus juice flavonoids using non-toxic solvents as water and ethanol (FUI XI). The adopted method includes different steps:1.SPE extraction of citrus juice using Water and EtOH; 2. Flash chromatography for purification using Water and EtOH; *3. LC-MS/UV* for analytical chromatography.
The recovery of bioactive compounds from bergamot juice would make in vivo studies on humans to verify the anti-cholesterol activity of the Brutieridin and Melitidin in order to create new drugs and the development of new functional foods enriched in nutraceuticals compounds.

Materials and methods

Chemicals

HPLC-grade methanol, Ethanol absolute and 99% formic acid were purchased from Carlo Erba (Milan, Italy). Aqueous solutions were prepared using ultrapure water, with a resistivity of 18.2 MO cm, obtained from a Milli-Q plus system (Millipore, Bedford, MA).

Plant material

The industrially processed bergamot juice was supplied by the Union Association, located at Condofuri Marina (RC, Italy). The juice was produced as a blend of the three cultivars of Citrus bergamia: Fantastico, Femminello and Castagnaro.

SPE extraction of citrus juice

The bergamot juice (100 mL) was passed through a C18 cartridge (Supelclean LC-18, 60 mL, 10 g; Supelco, St. Louis, MO), previously activated with EtOH and washed with water. The loaded material was washed with water (3x60 mL) and then eluted with EtOH (60 mL). The eluate was evaporated. The weight of dry extract was 2,4 g from 500 mL of bergamot juice. The extract was then divided into parts of 0.6 g and they were stored at -20°C until required for our study.

Flash Chromatography of dry extract

Flash chromatography is a type of preparative chromatography for rapid purification of compounds in a wide range of polarity.
The flavonoid extract was further separated using a Supelco VersaFlash High Throughput Flash Purification (HTFP), equipped with a C_{18} cartridge (40 x 75mm, VersaPak Cartridge, supelco), previously activated with a gradient of EtOH/Water (from 50:50, 25:75, 12.5: 87.5 – 0: 100).
The loaded material in the cartridge was eluted with a gradient of Water/EtOH. In order to obtain pure phenolic fractions, different tests were performed where the percentage composition of the solvents used was varied.

LC–ESI-MS/UV analysis

All fractions were centrifuged at 4500 rpm for 5 min and filtered before instrumental analysis. LC–ESI-MS/UV analysis was performed using a Waters Fraction-Lynx system (Milford, MA) equipped with a ZMD mass spectrometer and a 486 UV detector. The column used for all our analyses was a Luna C18 (2) (250 x 4.6 mm; Phenomenex, Torrance, CA). The UV detector was set at 280 nm. The run time was 105 min, the flow rate was 1 ml/min, and the gradient was built using 0.1% HCOOH in H_2O (solvent A) and CH_3OH (solvent B) as mobile phases. The elution gradient was composed of the following steps: isocratic elution 80% A for 10 min; linear gradient from 80% A to 74% A in 2 min; linear gradient from 74% A to 31% A in 65 min; linear gradient from 31% A to 80% A in 18 min; equilibration of the column for 10 min.

Results and discussion

For analytical chromatography a dry extract solution of 1000 ppm was injected into the LC-MS/UV. The profile shows the presence of many flavonoids already identified by LC-MS/UV analysis, together with some unknown components (Figure 3). The components identified are: 1, Neoriocitrin ; 2, Naringin; 3, Neohesperidin; 4, Neodiosmin; 5, Melitidin; 6, Brutieridin.

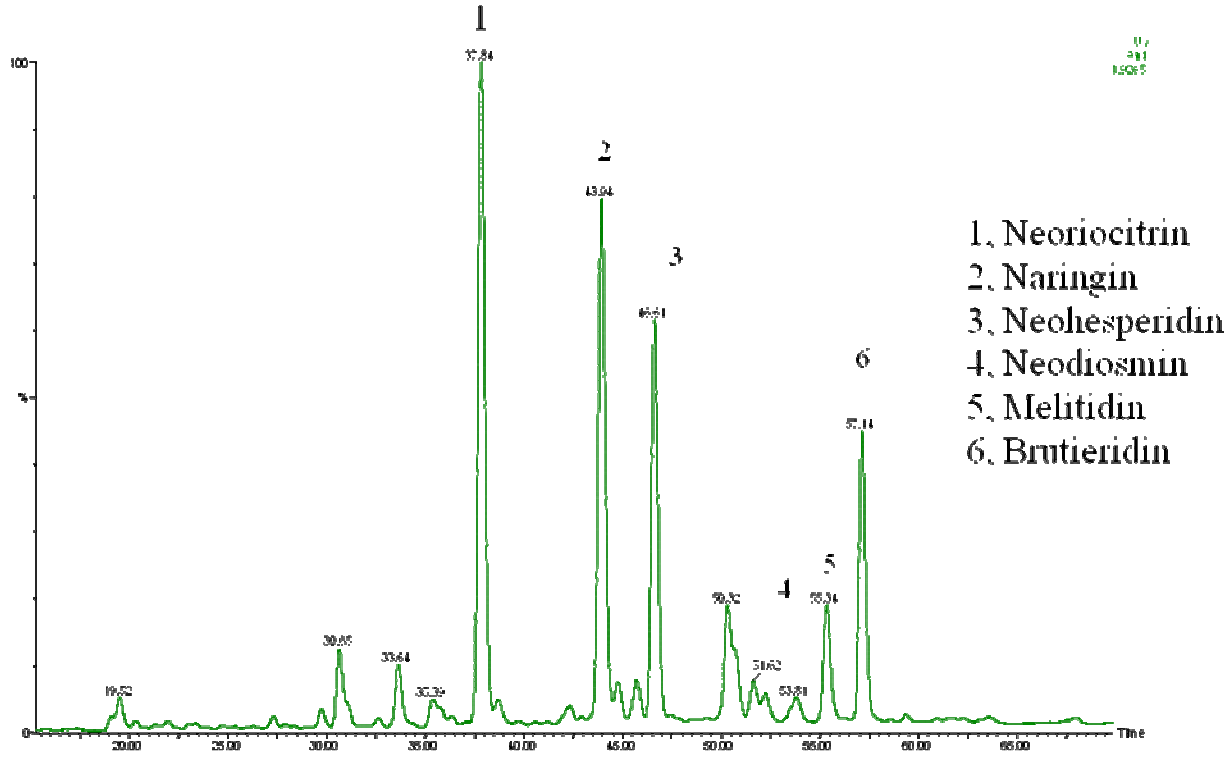

Figure 3. UV chromatogram of dry extract before the purification process by flash chromatography

After the Flash chromatography, different fractions were collected and stored at -20°C until the moment required by our study. In order to obtain pure phenolic extracts, different tests were performed where the percentage composition of the solvents used (Water/ EtOH) in VersaFlash system was varied. Optimal conditions were obtained at 25 mL / min. The duration process was 8 h.

VersaFlash Cartridge	C18 (40 x 75mm)
Amount of Loaded extract	0,6 g
Solvent System – Step Gradient, **25 mL/min**	5% EtOH for 6:30 10% EtOH for 1:30 30% EtOH for 5 min
Process duration	~8 h

Table 1. Used gradient in the versaflash system

Then, the fractions were analyzed by LC–ESI-MS/UV. Neoeriocitrin was the major compounds after 40 min of EtOH 5%; Naringin after 2:30 of EtOH 5 %; Neohesperidin after 5:30 min of EtOH 5%; Brutieridin after 2 min of ETOH 30% (Figure 4). Amount of recovered Brutieridin was 0,09g (Yield= 15%) with Purity of 80%.

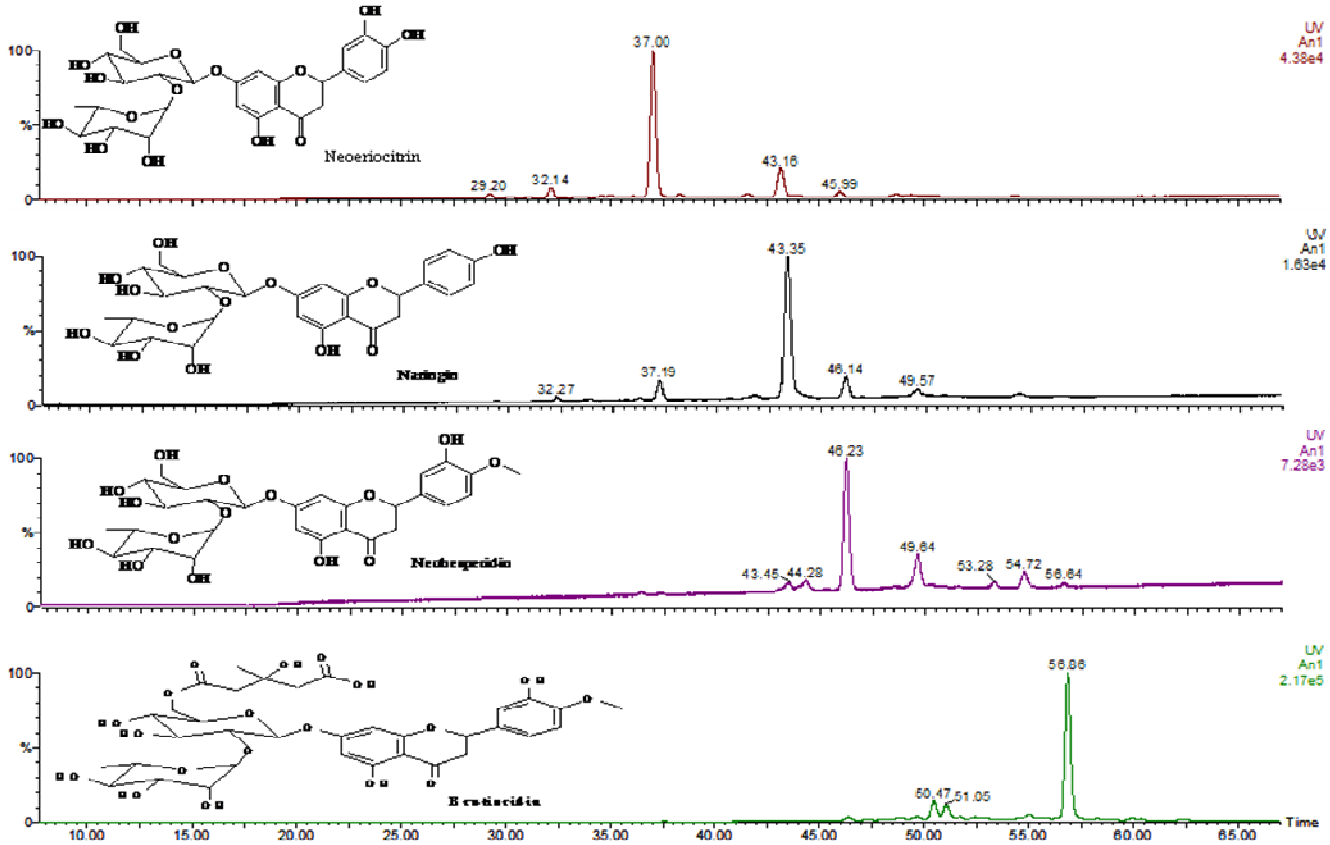

Figure 4. UV chromatograms of different bioactive compounds: Neoeriocitrin, Naringin, Neohesperidin, Brutieridin.

Conclusions

The growing interest in antioxidant bioactive compounds of bergamot juice has attracted attention as a result of its remarkable flavonoids content.
The growing interest in antioxidant bioactive compounds of bergamot juice has attracted attention as a result of its remarkable flavonoids content. The adopted method includes different steps:

1. SPE of the citrus juice;
2. Flash chromatography for purification of the raw extract;
3. LC–ESI-MS/UV of the collected fractions after the flash chromatography.

It has been possible to purify some different flavonoids in the Bergamot juice , some of those available in commerce as Certified standard (Neoeriocitrin, Naringin, Neohesperidin) and recover the Brutieridin, nutraceutical anticholesterolaemic compound, that is not in commerce as STD yet, with yield of 15%.

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3. Submitted for publication.

3.1.3 The selection of safe extraction methods of bioactive components from Bergamot fruit

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Abstract

The development of new methods of extraction of flavonoids and their conjugates without use of toxic and expensive solvents has become crucial for the introduction of some active principles as drugs. We have recently described the presence of statine-like drugs in different bergamot tissues [1], identified and isolated by classical methods. We now wish to validate modern appropriate procedures, meeting pharmacopoeia requirements [2], to set-up safety protocols for the recovery of the bioactive compounds. In this study we compare four different extraction methods (Soxhlet, heat reflux, microwave and temperature controlled extraction) in which the only solvent used is water. In order tocompare the efficiency of extraction methods the liquid extracts have been analized separately by LC-MS. This approach allowed the evaluation of the amount of bioactive flavonoids between flavedo and albedo layers.

Introduction

Bergamot (*Citrus bergamia* Risso) is a tree very sensitive to climatic conditions; it grows predominantly in a narrow area in province of Reggio Calabria (south Italy). Bergamot fruit has been widely employed in cosmetic, pharmaceutical and food industries as important source of essential oils contained in peel (flavedo). Bergamot juice, on the other hand, in contrast to other Citrus fruits is not used for food purposes because of its bitter taste and represent a waste product with its repercussions on environmental pollution. Recently, we have described the presence of two statin-like drugs in different bergamot tissues[1,2], identified and isolated by LC-MS, MS/MS and NMR techniques, providing new applications for bergamot juice.

The development of new methods of extraction of these two flavonoids and their conjugates without use of toxic and expensive solvents is crucial for the introduction of new active principles as drugs. Therefore, in this study we want to validate modern appropriate extracting procedures, meeting pharmacopoeia requirements[3], to set-up safety protocols for the recovery of the bioactive compounds present in albedo and flavedo compartments of bergamot fruit using water as extracting solvent.

Materials and methods

Flavedo and albedo layers of frozen bergamot have been separated and homogenized. The two separated portions have then been extracted using the following methods:

- Microwave-assisted extraction: 12 g of matrix and 80 ml of distilled water have been put in 4 vessels (3g of matrix and 20 ml of solvent for every vessel). This matrix/solvent ratio has been maintained constant for all the extracting methods used in this study. A potential of 500 W has been applicated for the following time durations (in min): 0.30, 1, 2, 3, 4, 5.
- Soxhlet extraction for 8 hours.
- Heat reflux extraction for 8 hours.
- Oil bath heating at constant temperature of 70°C. In this method we have monitored the extraction effectiveness during the following heating times (in minutes): 15, 30, 60, 180, 360 and 480.

20 µl of liquid extract, after centrifugation, have been injected into LC-MS/UV. The MS/MS of compounds, the TIC and the UV profiles of albedo and flavedo are reported in figures 1-2.

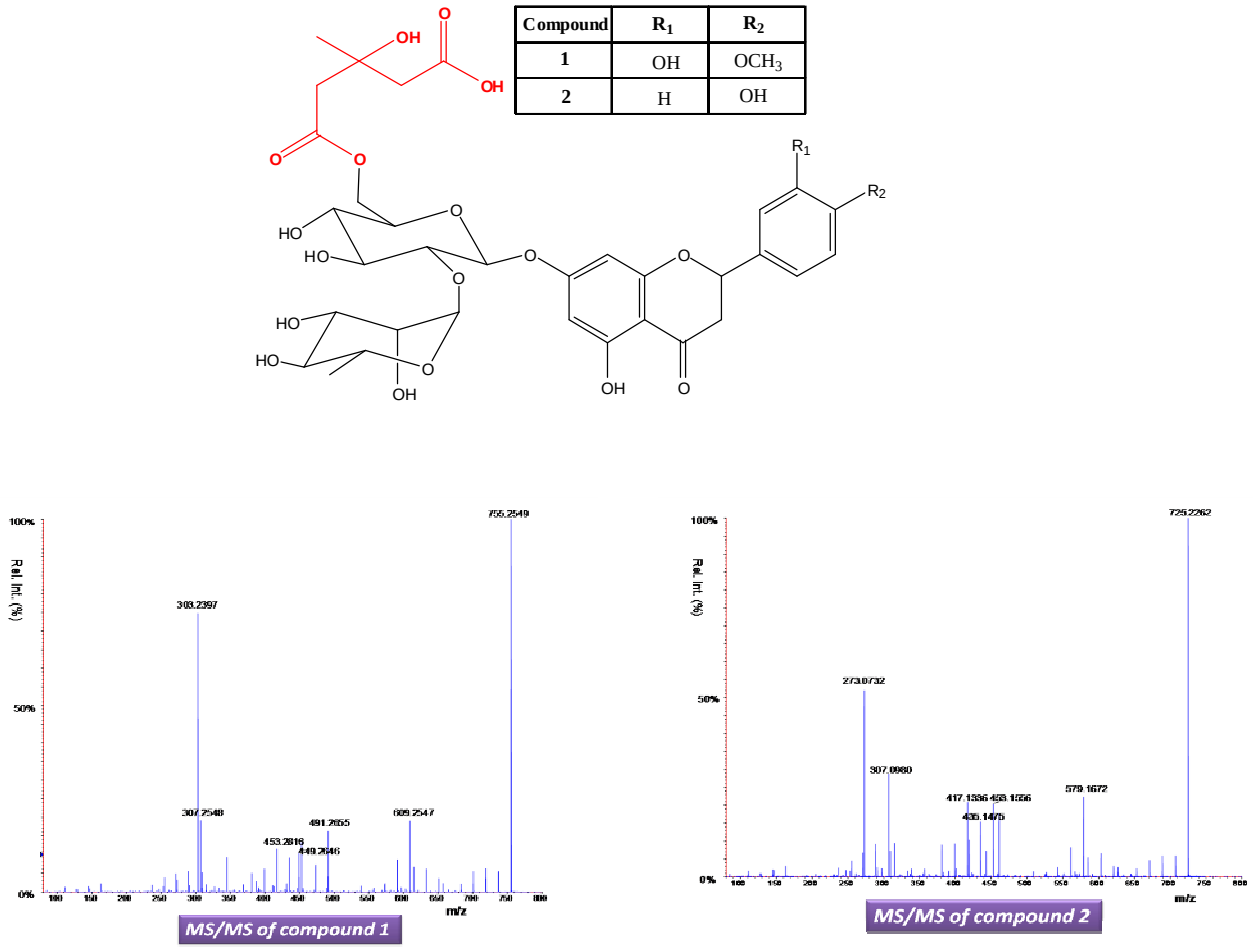

Figure 1. MS/MS of compounds 1 and 2.

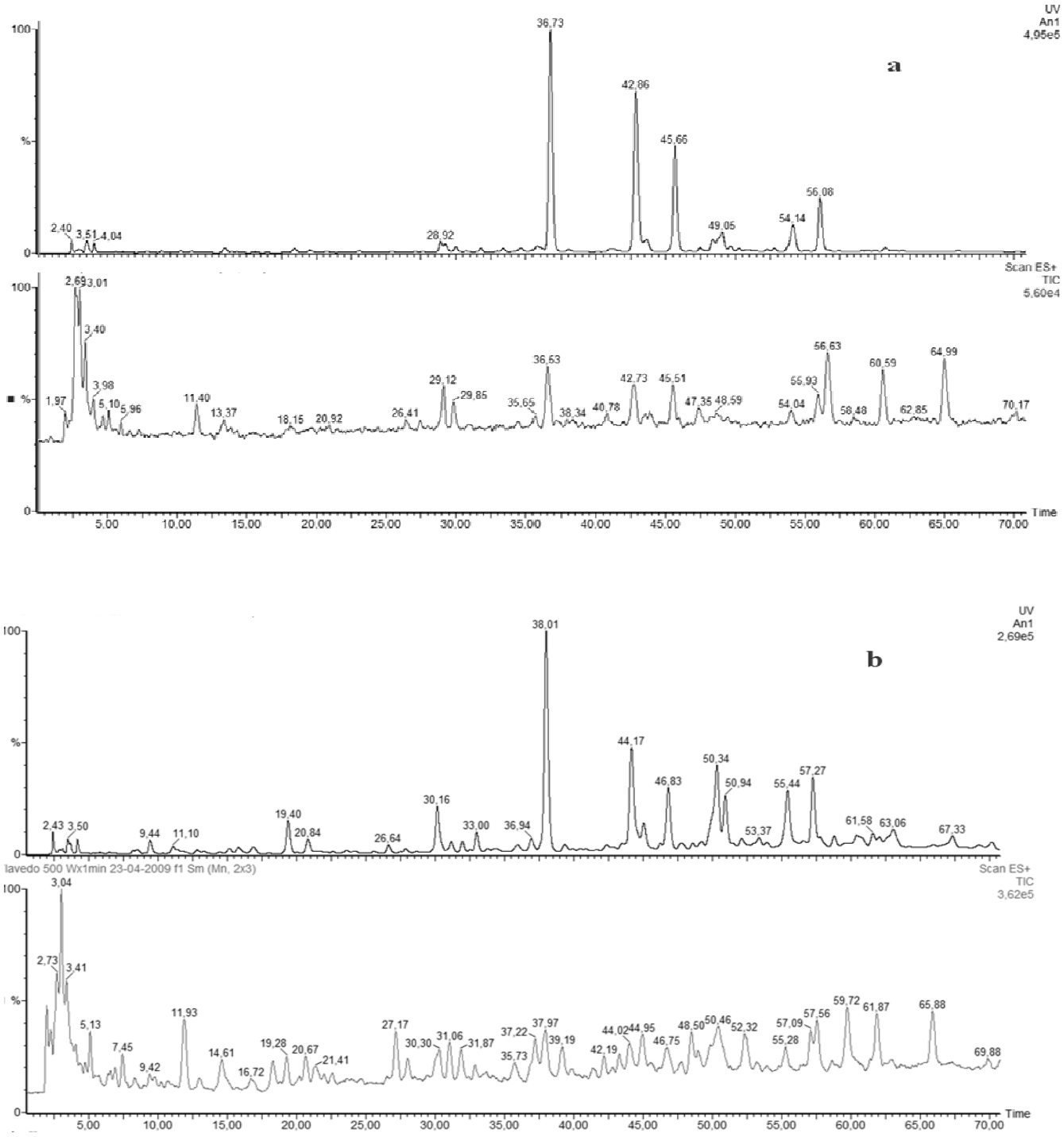

Figure 2. a) TIC and UV chromatogram of albedo and b) of flavedo. The areas of UV peaks at rt 55.56 (55.44 in b) and at rt 57.39 (57.27 in b), corresponding to compound 2 and 1 respectively, have been integrated and used to evaluate the effectiveness of extraction methods.

Results and discussion

The area of peaks corresponding to compound 1 and 2 have been integrated and reported in figures 3-6.

- Compounds 1 and 2 are best extracted by microwave extraction method, for both matrixes, at 500 W x 1 minute.
- The extraction effectiveness at 500 W, after the first minute, decrease continuosly with time.
- Compound 1 is more aboundant in albedo than compound 2 which is more aboundant in flavedo.
- Heat reflux, Soxhlet and oil bath heating methods after 8 h gave similar results. These methods are less effective than microwave extraction one. Oil bath heating at 70°C between 15 min and 6h is more effective than after 8 h.

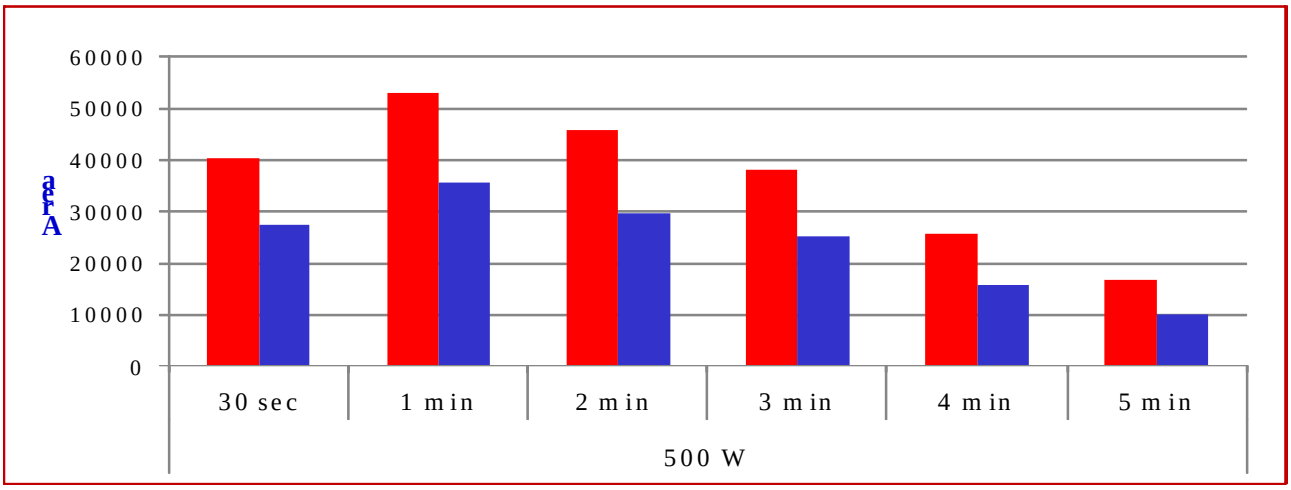

Figure 3. Extraction of compounds 1 (red) and 2 (blu) from albedo by microwave method.

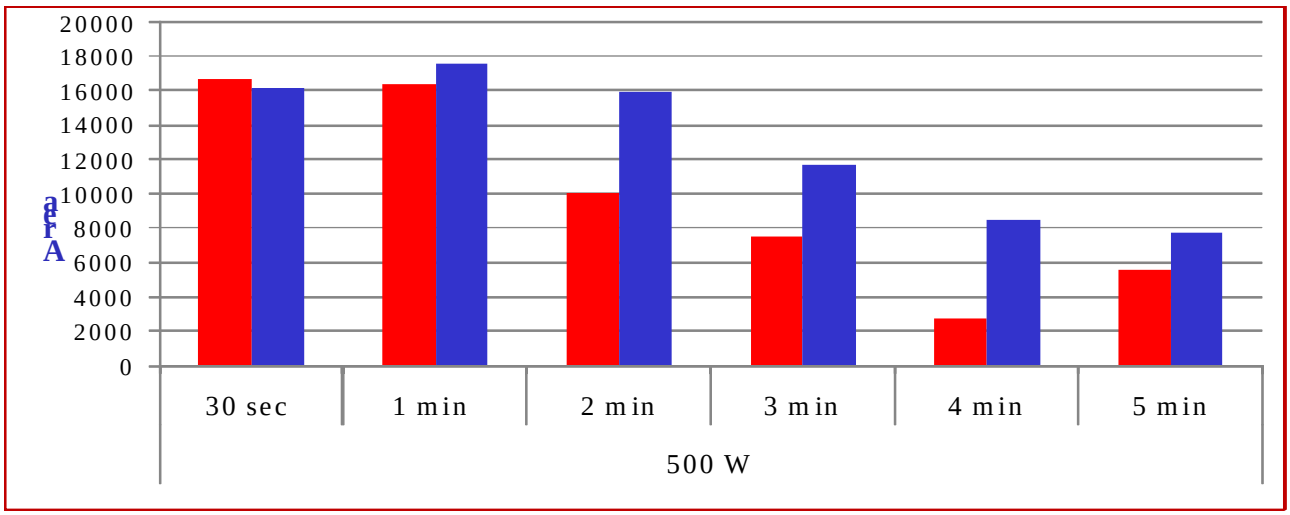

Figure 4. Extraction of compounds 1 (red) and 2 (blu) from flavedo by microwave method.

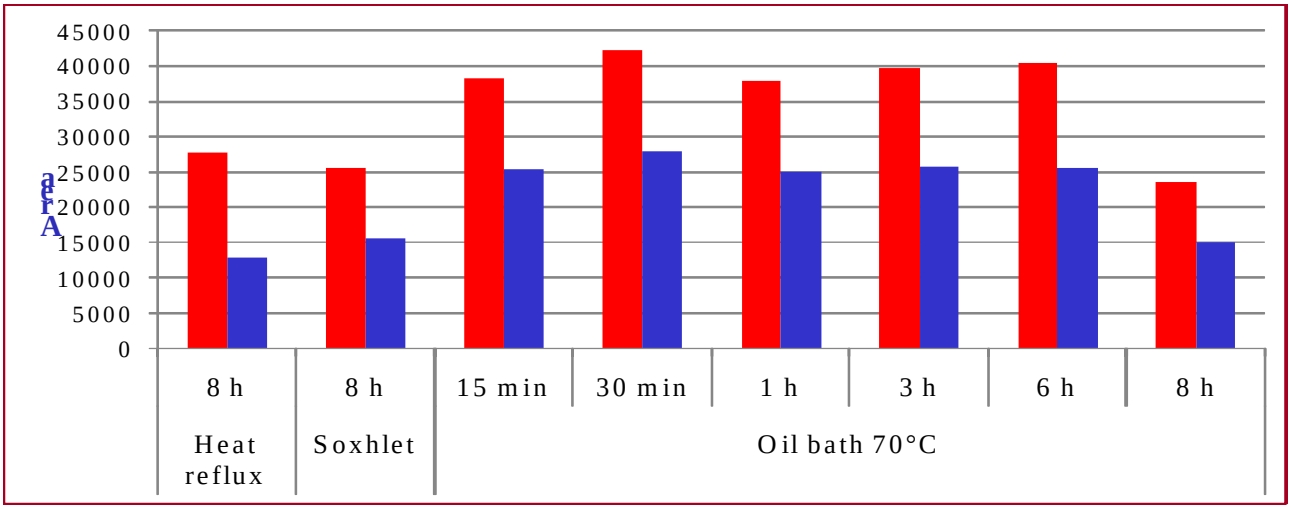

Figure 5. Extraction of compounds 1 (red) and 2 (blu) from albedo by heat reflux, Soxhlet and oil bath at 70°C.

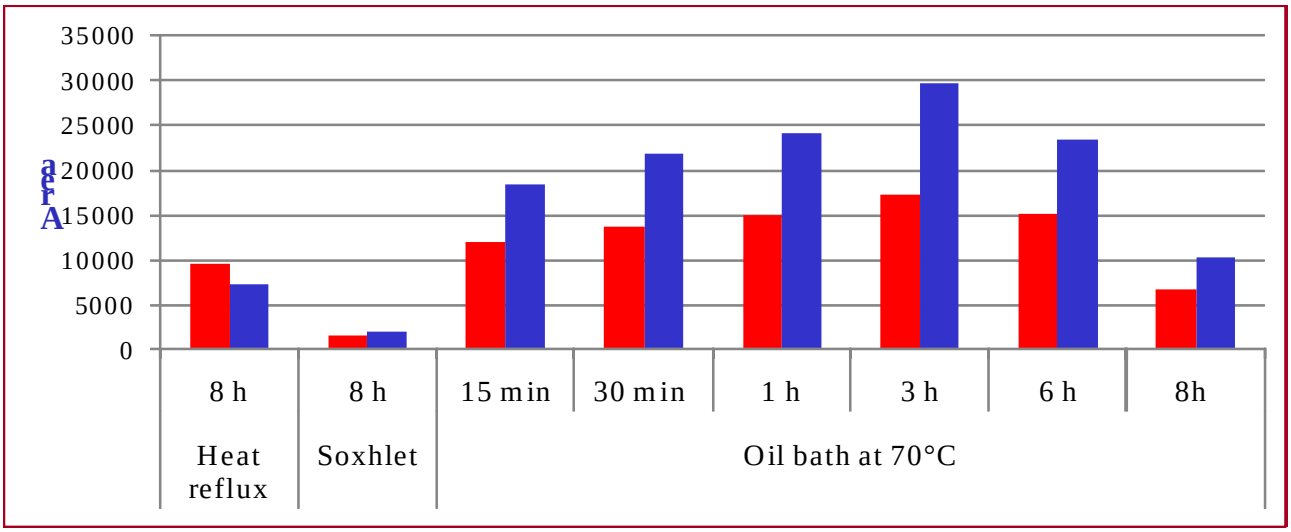

Figure 6. Extraction of compounds 1 (red) and 2 (blu) from flavedo by heat reflux, Soxhlet and oil bath at 70°C.

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3. EMEA/42371/2008 11 September **2008**.

3.2 Recovery of nutraceuticals of high antioxidant value from *Olea europaea L.* and its possible application in cosmetics or pharmaceuticals

Virgin olive oil is obtained from olive drupes (*Olea europaea*, L.), using exclusively mechanical procedures, without further treatments or chemical additions. This product is a genuine fruit juice, containing a high level of natural antioxidants, which, associated with an excellent fatty acid composition, confer it a high stability against oxidation and a recognized nutritional value.
Accumulating evidence indicates that adherence to Mediterranean diet (which consists of olive oil, fruits, vegetables and fish) is associated with lower prevalence of coronary heart disease, cancer and cognitive impairment, e.g., Alzheimer's disease (AD)[1-2]. Since reactive oxygen species (ROS) are implicated in these diseases, the benefits of the Mediterranean diet have been largely attributed to the antioxidant potential of polyphenols contained in the diet components, especially olive oil[3]. Indeed, olive oil phenols are efficient radical-scavengers in vitro[3] and can be well absorbed by the body (absorption >55–66 mol%)[4]. However, Vissers et al. (2004)[4] argued that the plasma concentration (<0.06 lM) of antioxidant phenols, resulting from dietary intake of olive oil, is too low to exert antioxidant effects. Moreover, increasing evidence suggests that the in vitro antioxidant potential can not necessarily be translated into in vivo therapeutic effects [5-7]. Therefore, it seems that, to elucidate the benefits of olive oil, we should go beyond antioxidants. Indeed, in recent years, some pharmacological effects other than antioxidant capacity have been reported for olive oil phenols. For instance, (-)-oleocanthal (Fig. 1), a component extracted from newly-pressed extra-virgin olive oil, possesses ibuprofen-like cyclooxygenases (COX-1 and -2) inhibitory ability[8]; hydroxytyrosol and hydroxy-isochromans (Fig. 1) are inhibitors of platelet aggregation [9,10] and oleuropein (Fig. 1) can form a non-covalent complex with amyloid-b (Ab) peptide or its oxidized form [11]. All of these effects help to explain the benefits of olive oil in preventing cardiovascular diseases, cancer and AD. Considering the fact that many polyphenols have been identified from olive drupes and olive oils, the aim of my study has been the Recovery of nutraceuticals of high antioxidant value from Olea Europaea L. for the possible application in cosmetics or pharmaceuticals.

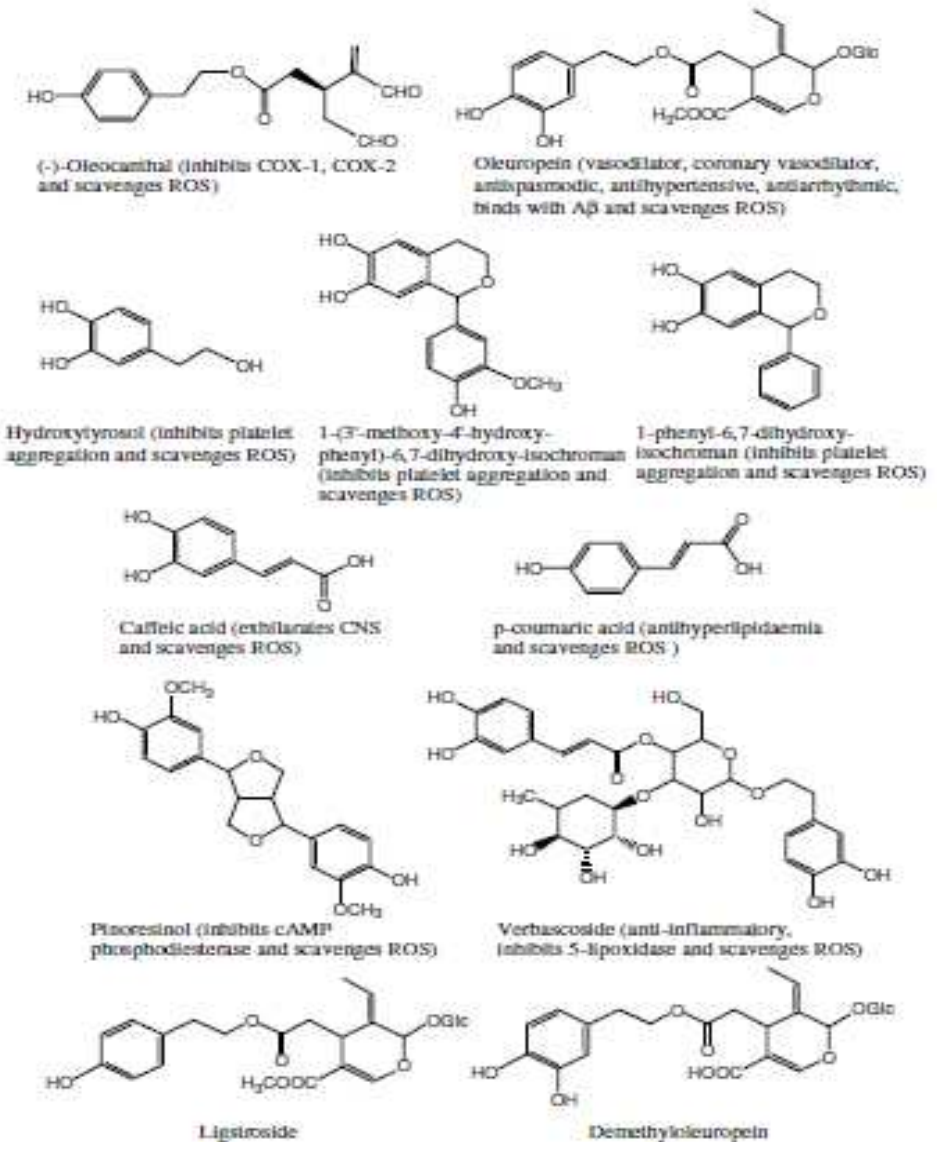

Figure 1. Structures and multiple pharmacological effects of olive phenols.

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3.2.1 Recovery of nutraceuticals of high antioxidant value from aqueous solution used during the debittering processes of drupes

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Proceeding "II Convegno Nazionale dell'Olivo e dell'Olio", Perugia 21-23 Settembre 2011, p. 125.
Italus Ortus, in press>>

Abstract

Most local industries and cooperatives that make table olives do not make the wastewater treatment. In fact, during the process of debittering of olives, the aqueous solutions utilised register a build-up of compounds characteristic of the bitterness of the fruits, most of them of phenolic nature. Sometimes, these waters are taken to the municipal sewage were the water purification is performed by paying a certain amount of money per cubic meter of liquid. For control purpose in case of inspections, the industries have their storage tanks to collect the wastewater even if most of it is distributed on the ground. In order to achieve a purification of these waters and a their easier disposal, the phenolic compounds could be extracted and used in food, pharmaceutical and cosmetic industries to produce antioxidant enriched products. The purpose of this research was, therefore, the daily monitoring of the concentration of some phenolic compounds accumulated during the debittering process of olives in aqueous solutions. In this way, in order to extract them, their highest expression in solution was extimated.

Keywords: table olives, phenolic coumpounds, tandem mass spectrometry.

Introduction

Olea europaea L. is an endemic plant of the Mediterranean basin and has been cultivated for the production of olive oil and for table olives, with significant efforts to improve the quality of products. Like many fruits and vegetables, the olive drupes contain biophenols distributed in the olive mesocarp, that is a soft and fleshy pulp, and in the endocarp (stone), which contains the seed. In ripe olives the seed makes up the 2–3% of the total mass, the stone the13–23% and the mesocarp the 84–90%. The concentration of the phenolic compounds is estimated to be 2-2.5% in the pulp, 0.5-1% in the seed and 0.1% in the core (*Gruenwald, 1998*; *Uccella, 2001; Ryan et al., 1998*). The factors that contributes to the variability in the phenolic distribution include the cultivar and genetics, maturity, climate, position on the tree, rootstock and agricultural practices. The growing

interest in the determination of phenolic compounds in vegetables and fruits is due to the natural antioxidant activity of these compounds *(Ryan et al., 2002)*. Phenolic compounds are secondary metabolites produced in plants as a mechanism of protection against microorganisms, pathogens and strong ultraviolet (UV) radiation. The antioxidant activity of phenolic compounds is due to their ability in quenching free radicals and metal chelation (*Saija et al. 1998)*. Some biophenols present in olive drupes seem to be involved in several activities such as prevention of coronary artery disease and atherosclerosis because of their ability to inhibit platelet aggregation *(Carluccio et al. 2003*), modulation of the arachidonic acid metabolism (*Kohyama et al. 1997*) and inhibition of low-density lipoprotein peroxidation (Visioli *et al.* 2002). Hydroxytyrosol and oleuropein act as potent radical scavengers (*Saija et al. 1998; Benavante-Garcia et al. 2000; Briante et al. 2001; Gordon et al. 2001; Saija and Uccella 2001; Paiva-Martins et al. 2003*). Some of the phenolic compounds in olive show antimicrobial activity by inhibiting the growth of a wide variety of bacteria, fungi (*Aziz et al. 1998*) and viruses (*Fredrickson 2000*).

In this context, one of the most crucial problems to solve is olive debittering. In fact, in olive leaf and fruit a bitter phenol glucoside, oleuropein *(Soler-Rivas et al., 2000)*, is accumulated as a defence mechanism against phytophatogens (*Amiot et al., 1989)*. In particular, when olive tissues are injured by phatogens or by mechanical damage, an enzyme specifically hydrolyses oleuropein producing highly reactive molecules *(Bianco et al, 1999)*.

The antioxidant and antimicrobial activities of oleuropein derivative molecules against herbivores and insect attacks has been demonstrated in plants *(Konno et al., 1999)* as well as against bacterial strains in vitro *(Bisignano et al., 2001; Kubo et al., 1995)*. The enzyme involved in this reaction is the β-glucosidase (E.C. 3.21.1.21) belongings to the glucohydrolase enzyme family 1 (GH 1); many components enzymes have been identified in plants where they play important roles in growth, development, detoxification, ripening and defence *(Esen, 1993)*. *Olea europaea* tissues also contain large amounts of β-glucosidase which specifically hydrolyses oleuropein *(Briante et al., 2002; Konno et al., 1999)*. Also during fruit ripening the β-glucosidases are involved in the progressive degradation of oleuropein, and in the release of glucose and the aglycones molecules, with the consequent physiological debittering of fruit tissues (*Morello et al., 2004 (A); Brenes Balbuena et al., 1992; Ryan et al., 1999)*. The detected changes in the β-glucosidase activity and in its products of enzymatic hydrolysis at different stages of fruit ripening *(Briante et al., 2002)* are strictly related to products quality; in fact, good-tasting table olives and olive oil are greatly influenced by the phenolic compounds *(Morello et al., 2004 (B); Angerosa et al., 1996; Cianfardini et al., 1994; Marsilio et al., 1996)*. Thus, debittering of green olives is a major challenge in the industrial processing of fruit. The most commonly employed methods involve dilute NaOH and/or low concentration of NaCl solutions. The NaOH treatments hydrolyse oleuropein into its derivatives and produces sugar. There is good evidence that the chemical treatments could be substituted by a microbiological procedure that would provide both debittering and fermentation steps by using oleuropeinolytic *Lactobacillus plantarum* strains (*Marsilio et al., 1996)*. In fact, the *L. plantarum* strains are able to hydrolyse oleuropein by means of a bacterial β-glucosidase reaction with the formation of aglycone and sugar. This treatment results in olives with better taste than alkali or salt treatments, due to the higher sugar and protein contents in the final products. In this context, investigations have been performed to test the efficiency of the enzymatic hydrolysis of oleuropein by the purified β-glucosidase from almond *(Capasso et al., 1996)* compared with the enzyme from the crude extract of olive fruit *(Bisignano et al., 2001; Konno et al., 1999)*. More recently an

immobilized recombinant β-glucosidase from the archaeon Sulfolobus solfataricus has been tried *(Briante et al., 2000). Bianco and Uccella (2000)* investigated the concentrations of different biophenolic compounds in olives in order to develop appropriate procedures for determination of these compounds in fresh and processed table olives and in the olive drupes for olive oil production. Despite the large number of studies on olive phenolic composition, not all these compounds have been identified, and, generally, the antioxidant activity is measured on methanolic extracts fractionated on a polarity basis (*McDonald et al. 2001*).
The aim of the present research was, therefore, the assay of some important phenolic compounds, such as oleuropein, hydroxytyrosol, tyrosol, verbascoside, luteolin and rutin, accumulated during the debittering process of olives in aqueous solutions. For this purpose, aqueous and acidic solutions containing whole, pitted and crushed olives were prepared. The assay of phenols was performed daily by means of an LC-MS/MS system. In this way, it was possible to have an estimates of their highest expression in the solutions to support their fruitful extraction.

Experimental

Chemicals

Certified standard of rutin, luteolin, verbascoside were purchased by Extrasynthese (Z.I Lyon Nord B.P 62 69726 Genay Cedex France); tyrosol, 3-Hydroxytyrosol and oleuropein were supplied from Sigma–Aldrich (Riedel-de Haën, Laborchemikalien, Seelze). Methanol, ethanol, formic acid were of LC/MS grade and purchased by VWR (VWR International s.r.l., via Stephenson 94, 20157 Milano); aqueous solution were prepared using ultrapure water, with a resistivity of 18.2 MΩ cm, obtained from a Milli-Q plus system (Millipore, Bedford, MA, USA).

Olives sampling and maceration process

Olive fruits of Coratina cultivar were collected by hand in November during the year crop 2010 in an olive grove located in Basilicata region and immediately stored at -25°C until analysis.
Three different types of olives were taken into account: whole, pitted and crushed fruits with the porpouse to evaluate the influence of the stone during the debittering processes of the olives.
Two trials were carried out: in the first trial a quantity of olives (12 g) were placed in a container fitted with a stopper where pure water (100 mL) was added; in the second trial a solution of water/ethanol (v/v, 80:20) was employed. The maceration process was carried out in the dark at a temperature of 4 °C except for the first day when the olives were subjected to two thermal shocks caused at first by passing from -25 °C to room temperature in the aqueous solutions and then for being frozen again to promote the membranes disintegration and the release of any bioactive compounds. Every day, for a total duration of 10 days, aliquotes of the water solutions were collected and their pH measured.

Preparation of standard solutions

Standard stock solutions were prepared dissolving the analytical standards in ethanol. Aliquots of these solutions were further diluted with water/0.1% formic acid to obtain calibration standards at concentrations range between 10-200 ng/mL for oleuropein and tyrosol; 10-100 ng/mL for 3-Hydroxytyrosol, rutin, luteolin; 10-500 ng/mL for verbascoside.

Instrumentation

Mass spectrometry

Sample analyses were performed using a MSD Sciex Applied Biosystem API 4000 Q-Trap mass spectrometer. The LC-MS was operated in the negative ion mode using multiple reaction monitoring (MRM) of the following transitions: *m/z 137 → m/z 137* (tyrosol); *m/z 153 → m/z 123* (Hydroxytyrosol); *m/z 285 → m/z 133* (luteolin); *m/z 539 → m/z 225* and *m/z 539 → m/z 275* (oleuropein); *m/z 609 → m/z 301* (rutin); *m/z 623 → m/z 161* and *m/z 623 → m/z 461* (verbascoside). The experimental conditions were as follow: ionspray voltage (IS) -4500 V; curtain gas 20 psi; temperature 400°C; ion source gas(1) 35 psi; ion source gas(2) 45 psi; collision gas thickness (CAD) medium. Entrance potential (EP), declustering potential (DP), collision energy (CE) and collision exit potential (CXP) were optimized for each transition monitored.

High performance liquid chromatography (HPLC)

HPLC was performed using an Agilent Technologies 1200 series liquid chromatography system equipped with G1379B degasser, G1312A pump, and G1329A autosampler. The analytes were separated on a Eclipse XDB-C8-A HPLC column [5 µm particle size, 150 mm length and 4.6 mm i.d. (Agilent Technologies, Santa Clara, California)] at a flow rate of 350 µL/min and an injection volume of 10 µL. The elution program was as follows: at the start 90% solvent A (0,1% aqueous formic acid) and 10% solvent B (methanol); the percentage of solvent B was linearly increased to 100% in 10 min, hold for 2 minute and ramped to original composition in 3 min. The total elution time was 25 minutes per injection.

Results and Discussions

Each day, before collecting aliquotes of the solutions under investigation (see *paragraph 3.2*) their pH was monitored. The aqueous solutions containing whole and crushed olives gave a pH value of 6 regardless if water/ethanol (v/v, 80:20) or pure water was employed. On the other hand, the pH value of aqueous solutions containing pitted olives was 5. Therefore, it seems that the presence of the stone is responsible of the changing of the pH of the solutions. Below, it follows the results obtained and the discussion for each compound analyzed summarised in Table 1 and Figure 1:

Oleuropein (olp). The solution containing pure water and whole olives, from day one, showed an accumulation of olp which reaches an average value of 423 mg/Kg. This content decreases during the 10 days of experimentation to reach the half of its initial value (228 mg/Kg). The solution containing water/ethanol (v/v, 80:20) since day one gave a lower content of olp (369

mg/Kg). This value decreases during the days until it reaches the mean value of 264 mg/Kg. The small amount of ethanol added to the solution seems to slow down the degradation process of the bioactive compound. The solution containing pure water and crushed olives from day one showed an olp content of 1118 mg/Kg. This value decreases according to a enzymatic kinetic to reach a value of 8 mg/Kg on the last day. Similar situation occurs in solutions containing pure water and pitted olives where the mean values of olp is 1187 and 60 mg/Kg in the first and last day respectively. The solution containing water/ethanol (v/v, 80:20) since day one gave a lower content of olp (665 mg/Kg for crushed olives and 754 mg/Kg for pitted olives). This value decreases during the days until it reaches the mean value of 139 mg/Kg and 162 mg/Kg for crushed and pitted olives respectively.

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

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

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

mg/Kg on the first and last day, respectively. The solution containing crushed and pitted olives in pure water showed an high content of verbascoside already on the first day of the experimentation (802 and 1319 mg/kg for crushed and pitted olives respectively). This values decrease over the time to almost disappeared (1 and 4 mg/Kg respectively).

Luteolin. Luteolin is responsible, along with other carotenoid compounds, of the colour of the drupes. Fermentation and debittering processes promote its release in solution that is pronounced if olives are damaged. Moreover, in the presence of solvents such as ethanol, this release is marked. The absence of light, also, preserve the colour of the final solutions. During the ten days of experimentation, depending on the olives utilized, different gradation of colour were obtained. Whole olives gave at the end of the experimentation a dark green colour, whereas crushed and pitted ones a green browny colour that was more evident in the ethanolic solutions. All solutions analysed showed an increase of the bioactive compound. In particular, in aqueous solution containing whole olives the luteolin content varies from 66 to 218 mg/Kg during the experimental period, whereas in the ethanol solution from 83 to 287 mg/Kg. The solutions containing crushed and pitted olives in pure water showed an higher content of luteolin during the first days of the experimentation; these values will not undergo through many changes over the period of the debittering process. In fact, the content of luteolin for crushed olives varies from 126 to 202 mg/Kg whereas for pitted olives from 110 to 190 mg/Kg. The ethanol solutions have a different pattern respect the previous ones examinated. In fact, from day one the solutions are very rich in luteolin that grows up during all the debittering process. At the end of the experimentation almost 700 mg/Kg were found in the solution containing crushed olives and almost 800 mg/Kg in the solution containing crushed ones.

Rutin. This glycoside flavone is stable in aqueous solution at neutral or alkaline pH; in an acid or by the action of enzymes specific hydrolase, the glycosidic bond is broken with the formation of anomeric hemiacetals. In the aqueous solutions analysed the concentration of rutin, from the first to the last day of experimentation does not change much and remains constant when whole olive are used (from 40 to 44 mg/Kg). A decrease of the compounds can be observed for crushed olives (from 110 to 31 mg/Kg) and for pitted olives (from 179-101 mg/Kg). The difference that can be noticed when ethanol is added, is that the content of rutin released is far greater but constant throughout the experimentation period: for whole olive the content of rutin is the same than in the aqueous solution (from 41 to 49 mg/Kg). The solutions containing pitted olives showed an increased content of compound than those containing crushed olives (from 144 to 132 mg/Kg and from 221 to 200 mg/Kg respectively).

Conclusions

From the results obtained (Fig. 1 and Table 1) it was possible to observe that these bioactive compounds were present in a very interesting amount already from the first three days of treatment.
Fermentation and debittering processes promote the release of bioactive compounds in solution that is pronounced if olives are damaged (crushed and pitted olives) and in water solution.
It seems that the presence of EtOH slows down the release in solution of the bioactive compounds and their complete degradation. This behavior is not true when luteolin is considered. The presence of stone (crushed olives) seems to improve more decay of analyzed compounds.

The content in OLP decreases over the time in every thesis analyzed.
The concentration in Htyr e Verbascoside increases over the time in whole olives macerated in pure water or in ethanolic solution, its decreases in pitted olives and crushed olives.
The content in Tyr and Luteolin increases during the maceration process in every thesis analyzed.
The concentration in Rutin in whole olives macerated in water solution varies from 28 ppm to 44 ppm, in ethanolic solution from 40 ppm to 53 ppm. In crushed olives macerated in pure water it decreases from 1101 ppm to 32 ppm, in ethanolic solution the range is between 97 ppm and 161 ppm. In pitted olives it decreases from 179 ppm to 101 ppm in water solution, in ethanolic solution the range is between 200 ppm and 288 ppm.
Each day, before collecting aliquotes of the solutions under investigation their pH was monitored. The aqueous solutions containing whole and crushed olives gave a pH value of 6 regardless if water/ethanol (v/v, 80:20) or pure water was employed. On the other hand, the pH value of aqueous solutions containing pitted olives was 5. Therefore, it seems that the presence of the stone is responsible of the changing of the pH of the solutions.
Safety procedures matching food supplements directives issued by European and International bodies were implemented (ICH, 2009). The solutions recovery from the extraction of olives obtained by both trials carried (100% water or solution of water/ethanol (v/v, 80:20)), can find direct application in cosmetics and useful as raw materials for the production of nutraceuticals.

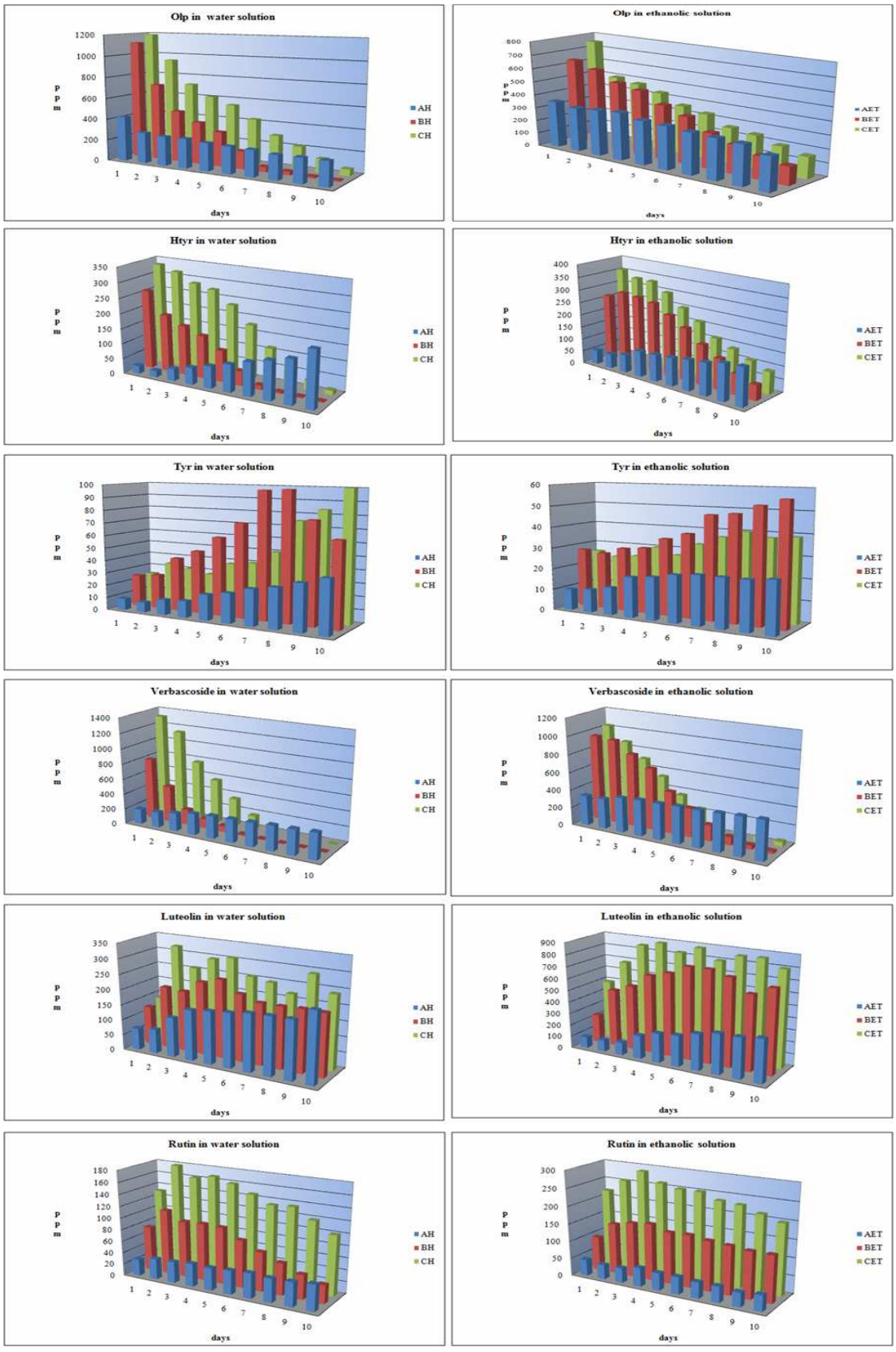

Figure 1. Trend of the debittering process in whole olives, crushed olives and pitted olives. Bar charts show the decay of bioactive antioxidant compounds in the three thesis with particular emphasis on the eluent phases utilised: water and water/ethanol (v/v 80/20).

Analyte	Sample	Mean (SD) mg/Kg	RSD %	Sample	Mean (SD) mg/Kg	RSD %	Sample	Mean (SD) mg/Kg	RSD %	Sample	Mean (SD) mg/Kg	RSD %	Sample	Mean (SD) mg/Kg	RSD %	Sample	Mean (SD) mg/Kg	RSD %
Tyrosol	**AH1**	9,30 (0,02)	0,20	**BH1**	24,93 (0,04)	0,17	**CH1**	24,23 (0,22)	0,89	**AET1**	10,63 (0,05)	0,45	**BET1**	28,48 (0,25)	0,90	**CET1**	26,40 (0,02)	0,07
Tyrosol	**AH2**	8,44 (0,00)	0,04	**BH2**	26,60 (0,34)	1,27	**CH2**	34,01 (0,04)	0,12	**AET2**	9,72 (0,01)	0,07	**BET2**	27,27 (0,20)	0,70	**CET2**	24,11 (0,11)	0,44
Tyrosol	**AH3**	11,58 (0,05)	0,43	**BH3**	41,75 (0,62)	1,48	**CH3**	30,67 (0,05)	0,16	**AET3**	13,53 (0,00)	0,04	**BET3**	29,91 (0,28)	0,90	**CET3**	25,20 (0,25)	1,00
Tyrosol	**AH4**	13,24 (0,08)	0,63	**BH4**	48,90 (0,03)	0,06	**CH4**	2839 (0,01)	0,05	**AET4**	19,17 (0,18)	0,95	**BET4**	30,88 (0,01)	0,00	**CET4**	29,74 (0,04)	0,12
Tyrosol	**AH5**	19,83 (0,04)	0,22	**BH5**	61,09 (0,39)	0,64	**CH5**	37,82 (0,20)	0,52	**AET5**	20,43 (0,14)	0,70	**BET5**	35,95 (0,37)	1,00	**CET5**	26,92 (0,20)	0,74
Tyrosol	**AH6**	22,97 (0,17)	0,72	**BH6**	72,60 (0,83)	1,14	**CH6**	40,14 (0,66)	1,64	**AET6**	22,53 (0,19)	0,84	**BET6**	39,14 (0,43)	1,10	**CET6**	33,25 (0,22)	0,66
Tyrosol	**AH7**	28,10 (0,03)	0,11	**BH7**	97,80 (0,00)	0,00	**CH7**	50,22 (0,24)	0,48	**AET7**	22,68 (0,07)	0,32	**BET7**	48,05 (0,09)	0,20	**CET7**	36,68 (0,10)	0,28
Tyrosol	**AH8**	31,17 (0,08)	0,26	**BH8**	99,56 (2,49)	2,50	**CH8**	75,15 (0,19)	0,26	**AET8**	22,91 (0,13)	0,56	**BET8**	49,48 (0,14)	0,30	**CET8**	40,17 (0,54)	1,34
Tyrosol	**AH9**	36,04 (0,32)	0,90	**BH9**	78,17 (1,24)	1,59	**CH9**	83,73 (2,49)	2,97	**AET9**	23,63 (0,05)	0,21	**BET9**	53,17 (0,02)	0,00	**CET9**	38,15 (0,32)	0,84
Tyrosol	**AH10**	41,49 (0,01)	0,03	**BH10**	65,28 (2,07)	3,17	**CH10**	100,14 (0,05)	0,05	**AET10**	23,49 (0,25)	1,07	**BET10**	56,28 (0,22)	0,40	**CET10**	39,40 (0,21)	0,53
Hydroxytyrosol	**AH1**	26,32 (0,11)	0,43	**BH1**	260,76 (3,96)	1,50	**CH1**	339,31 (15,71)	4,63	**AET1**	55,21 (1,33)	2,40	**BET1**	264,26 (11,89)	4,87	**CET1**	356,72 (10,90)	3,10
Hydroxytyrosol	**AH2**	20,79 (0,00)	0,00	**BH2**	188,62 (2,97)	1,60	**CH2**	317,50 (0,99)	0,31	**AET2**	56,32 (1,26)	2,24	**BET2**	288,78 (9,91)	3,43	**CET2**	332,91 (20,80)	6,20
Hydroxytyrosol	**AH3**	39,77 (0,30)	0,75	**BH3**	163,40 (4,95)	3,00	**CH3**	286,68 (2,97)	1,04	**AET3**	70,10 (1,19)	1,70	**BET3**	283,87 (12,88)	4,54	**CET3**	332,20 (1,98)	0,60
Hydroxytyrosol	**AH4**	55,69 (1,61)	2,90	**BH4**	140,99 (4,95)	3,50	**CH4**	275,47 (2,97)	1,08	**AET4**	102,25 (0,89)	0,87	**BET4**	274,07 (0,99)	0,36	**CET4**	298,58 (3,96)	1,30
Hydroxytyrosol	**AH5**	72,76 (0,40)	0,54	**BH5**	104,51 (5,45)	5,20	**CH5**	239,14 (8,78)	3,67	**AET5**	104,96 (4,32)	4,12	**BET5**	241,15 (3,96)	1,64	**CET5**	252,36 (0,00)	0,00
Hydroxytyrosol	**AH6**	91,19 (3,45)	3,79	**BH6**	47,62 (1,00)	2,10	**CH6**	178,81 (14,86)	8,31	**AET6**	110,66 (3,27)	2,95	**BET6**	206,13 (1,98)	0,96	**CET6**	212,43 (0,99)	0,50
Hydroxytyrosol	**AH7**	111,32 (4,12)	3,70	**BH7**	13,45 (0,00)	0,00	**CH7**	113,53 (3,44)	3,03	**AET7**	120,60 (1,10)	0,90	**BET7**	157,80 (0,99)	0,63	**CET7**	162,70 (1,98)	1,20
Hydroxytyrosol	**AH8**	127,89 (5,84)	4,57	**BH8**	4,94 (0,04)	0,80	**CH8**	72,20 (0,79)	1,10	**AET8**	128,45 (3,27)	2,54	**BET8**	122,00 (5,65)	4,63	**CET8**	138,39 (1,68)	1,20
Hydroxytyrosol	**AH9**	145,19 (4,95)	3,41	**BH9**	3,26 (0,00)	0,00	**CH9**	33,05 (0,13)	0,38	**AET9**	144,49 (3,96)	2,74	**BET9**	82,07 (0,34)	0,42	**CET9**	111,22 (4,06)	3,70
Hydroxytyrosol	**AH10**	186,10 (8,50)	4,57	**BH10**	2,15 (0,00)	0,10	**CH10**	13,84 (0,10)	0,74	**AET10**	148,69 (1,98)	1,33	**BET10**	60,30 (0,40)	0,66	**CET10**	87,33 (3,05)	3,50
Oleuropein	**AH 1**	423,19 (11,22)	2,65	**BH1**	1118 (25)	2,20	**CH1**	1237 (10)	0,83	**AET 1**	351,62 (9,36)	2,66	**BET1**	638,75 (16,27)	2,55	**CET1**	754,18 (38,72)	5,13
Oleuropein	**AH 2**	288,51 (13,73)	4,76	**BH2**	721,82 (0,00)	0,00	**CH2**	943,34 (38,72)	4,10	**AET 2**	332,06 (4,22)	1,27	**BET2**	592,37 (7,08)	1,20	**CET2**	553,05 (21,78)	3,94
Oleuropein	**AH 3**	273,32 (10,36)	3,86	**BH3**	481,34 (23,86)	4,96	**CH3**	716,85 (14,08)	1,96	**AET 3**	346,00 (1,41)	0,41	**BET3**	517,73 (21,12)	4,08	**CET3**	478,66 (6,69)	1,40
Oleuropein	**AH 4**	276,31 (7,74)	2,80	**BH4**	392,79 (14,08)	3,58	**CH4**	609,82 (17,60)	2,89	**AET 4**	359,77 (12,44)	3,46	**BET4**	487,87 (0,00)	0,00	**CET4**	437,34 (13,73)	3,14
Oleuropein	**AH 5**	259,63 (11,62)	4,47	**BH5**	322,41 (11,54)	3,58	**CH5**	565,11 (17,47)	3,09	**AET 5**	331,07 (8,45)	2,55	**BET5**	409,47 (5,98)	1,46	**CET5**	367,65 (6,69)	1,82
Oleuropein	**AH 6**	252,17 (4,58)	1,81	**BH6**	167,01 (8,15)	4,88	**CH6**	427,64 (15,49)	3,62	**AET 6**	326,59 (4,93)	1,51	**BET6**	356,45 (11,97)	3,36	**CET6**	336,79 (2,46)	0,73
Oleuropein	**AH 7**	245,87 (11,36)	4,62	**BH7**	47,39 (1,92)	4,05	**CH7**	305,72 (11,20)	3,66	**AET 7**	313,89 (8,80)	2,80	**BET7**	267,60 (0,35)	0,13	**CET7**	266,11 (9,50)	3,57
Oleuropein	**AH 8**	227,38 (7,25)	3,19	**BH8**	32,83 (0,38)	1,15	**CH8**	216,33 (4,58)	2,12	**AET 8**	308,17 (3,52)	1,14	**BET8**	217,68 (10,71)	4,92	**CET8**	245,95 (2,11)	0,86
Oleuropein	**AH 9**	228,03 (7,04)	3,09	**BH9**	14,94 (0,38)	2,52	**CH9**	126,73 (1,06)	0,83	**AET 9**	301,70 (13,38)	4,43	**BET9**	171,12 (7,97)	4,66	**CET9**	200,15 (0,75)	0,35
Oleuropein	**AH 10**	227,07 (10,15)	4,47	**BH10**	11,51 (0,51)	4,46	**CH10**	60,27 (0,04)	0,06	**AET 10**	256,70 (12,40)	4,83	**BET10**	139,17 (2,46)	1,77	**CET10**	162,32 (11,26)	6,94

Analyte	Sample	Mean (SD) mg/Kg	RSD %	Sample	Mean (SD) mg/Kg	RSD %	Sample	Mean (SD) mg/Kg	RSD %	Sample	Mean (SD) mg/Kg	RSD %	Sample	Mean (SD) mg/Kg	RSD %	Sample	Mean (SD) mg/Kg	RSD %
Rutin	**AH1**	28,18 (0,27)	0,76	**BH1**	75,78 (0,94)	1,25	**CH1**	132,43 (7,55)	5,70	**AET1**	46,18 (0,43)	0,92	**BET1**	97,30 (1,42)	1,46	**CET1**	225,90 (7,39)	3,27
Rutin	**AH2**	35,20 (0,20)	0,72	**BH2**	109,75 (4,40)	4,01	**CH2**	179,11 (3,14)	1,76	**AET2**	41,12 (0,80)	1,95	**BET2**	144,48 (0,07)	0,05	**CET2**	266,47 (11,64)	4,37
Rutin	**AH3**	36,23 (0,05)	0,13	**BH3**	94,58 (0,08)	0,08	**CH3**	162,21 (5,66)	3,49	**AET3**	42,14 (0,68)	1,60	**BET3**	155,04 (0,40)	0,26	**CET3**	288,04 (12,58)	4,37
Rutin	**AH4**	39,07 (0,30)	0,77	**BH4**	97,36 (1,18)	1,21	**CH4**	167,55 (4,40)	2,63	**AET4**	53,29 (0,09)	0,18	**BET4**	160,88 (1,26)	0,78	**CET4**	261,80 (3,14)	1,20
Rutin	**AH5**	37,89 (0,05)	0,12	**BH5**	96,11 (0,20)	0,21	**CH5**	159,99 (8,80)	5,50	**AET5**	48,63 (0,49)	1,01	**BET5**	145,76 (5,03)	3,45	**CET5**	252,27 (16,35)	6,50
Rutin	**AH6**	39,61 (0,09)	0,24	**BH6**	80,05 (0,66)	0,83	**CH6**	146,65 (1,26)	0,86	**AET6**	48,93 (0,94)	1,92	**BET6**	147,54 (8,80)	5,97	**CET6**	251,58 (15,09)	6,00
Rutin	**AH7**	42,30 (0,41)	0,97	**BH7**	66,28 (1,75)	2,63	**CH7**	133,76 (5,04)	3,77	**AET7**	45,24 (0,32)	0,70	**BET7**	141,32 (5,03)	3,56	**CET7**	234,68 (13,83)	5,89
Rutin	**AH8**	39,99 (0,82)	2,04	**BH8**	53,63 (0,02)	0,03	**CH8**	135,54 (10,69)	7,89	**AET8**	44,84 (0,41)	0,91	**BET8**	136,87 (4,41)	3,22	**CET8**	230,68 (6,92)	3,00
Rutin	**AH9**	41,37 (0,09)	0,23	**BH9**	40,69 (0,80)	1,97	**CH9**	119,09 (1,26)	1,06	**AET9**	40,12 (0,25)	0,63	**BET9**	131,98 (0,94)	0,71	**CET9**	215,12 (2,52)	1,17
Rutin	**AH10**	44,27 (0,05)	0,10	**BH10**	30,97 (0,19)	0,60	**CH10**	101,30 (55,03)	4,97	**AET10**	44,62 (0,63)	1,42	**BET10**	131,54 (4,08)	3,10	**CET10**	199,56 (9,43)	4,73
Luteolin	**AH1**	71,23 (0,87)	1,22	**BH1**	126,17 (4,78)	3,79	**CH1**	140,18 (5,84)	4,17	**AET1**	92,67 (3,63)	3,92	**BET1**	240,22 (9,67)	4,03	**CET1**	544,45 (9,26)	1,70
Luteolin	**AH2**	78,89 (1,24)	1,57	**BH2**	201,80 (8,54)	4,23	**CH2**	321,67 (8,54)	2,66	**AET2**	97,17 (2,05)	2,11	**BET2**	535,27 (15,41)	2,88	**CET2**	586,33 (4,72)	0,80
Luteolin	**AH3**	128,88 (1,11)	0,86	**BH3**	198,42 (3,08)	1,55	**CH3**	274,08 (4,76)	1,74	**AET3**	107,07 (5,13)	4,79	**BET3**	503,75 (1,53)	0,30	**CET3**	875,66 (33,02)	3,77
Luteolin	**AH4**	165,06 (2,42)	1,46	**BH4**	236,60 (2,09)	0,88	**CH4**	296,29 (0,35)	0,12	**AET4**	197,70 (81,37)	0,69	**BET4**	657,33 (0,00)	0,00	**CET4**	886,91 (3,4)	0,39
Luteolin	**AH5**	171,61 (0,24)	0,14	**BH5**	254,52 (2,93)	1,15	**CH5**	307,41 (2,64)	0,86	**AET5**	246,99 (15,04)	6,09	**BET5**	698,41 (23,91)	3,43	**CET5**	863,91 (22,27)	2,58
Luteolin	**AH6**	177,88 (0,00)	0,00	**BH6**	217,75 (14,70)	6,75	**CH6**	256,90 (6,33)	2,46	**AET6**	260,04 (6,15)	2,37	**BET6**	770,91 (17,09)	2,22	**CET6**	884,49 (27,34)	3,09
Luteolin	**AH7**	190,06 (13,54)	7,12	**BH7**	200,10 (0,31)	0,15	**CH7**	247,24 (0,65)	0,26	**AET7**	304,51 (12,99)	4,26	**BET7**	773,33 (13,67)	1,77	**CET7**	802,32 (41,01)	5,11
Luteolin	**AH8**	187,55 (6,15)	3,28	**BH8**	199,63 (10,94)	5,48	**CH8**	220,93 (0,33)	0,15	**AET8**	338,34 (1,37)	0,40	**BET8**	709,66 (26,16)	3,69	**CET8**	860,32 (27,34)	3,18
Luteolin	**AH9**	191,17 (2,39)	1,25	**BH9**	202,77 (14,01)	6,91	**CH9**	301,22 (11,49)	3,81	**AET9**	340,03 (0,34)	0,10	**BET9**	625,91 (10,25)	1,64	**CET9**	865,16 (6,84)	0,79
Luteolin	**AH10**	229,35 (0,34)	0,15	**BH10**	202,29 (5,13)	2,53	**CH10**	242,01 (9,06)	3,74	**AET10**	361,08 (13,70)	3,80	**BET10**	698,41 (17,09)	2,45	**CET10**	802,32 (34,18)	4,26
Verbascoside	**AH1**	195,97 (4,81)	1,29	**BH1**	801,61 (20,42)	2,55	**CH1**	1319 (55)	4,19	**AET1**	337,82 (37,24)	11,02	**BET1**	960,45 (9,61)	1,00	**CET1**	1082 (18)	1,68
Verbascoside	**AH2**	203,38 (2,62)	2,45	**BH2**	523,73 (9,77)	1,87	**CH2**	1140 (2)	0,21	**AET2**	338,67 (7,22)	2,13	**BET2**	940,06 (0,00)	0,00	**CET2**	873,81 (38,44)	4,40
Verbascoside	**AH3**	235,89 (15,62)	6,62	**BH3**	201,92 (10,81)	5,35	**CH3**	772,73 (27,63)	3,58	**AET3**	388,79 (1,20)	0,31	**BET3**	812,65 (9,61)	1,18	**CET3**	660,72 (25,06)	3,79
Verbascoside	**AH4**	279,21 (0,00)	0,00	**BH4**	123,18 (3,79)	3,08	**CH4**	568,87 (10,81)	1,90	**AET4**	404,20 (5,07)	1,25	**BET4**	688,64 (4,81)	0,70	**CET4**	539,99 (22,82)	4,23
Verbascoside	**AH5**	296,11 (0,13)	0,04	**BH5**	75,27 (1,91)	2,54	**CH5**	374,06 (12,42)	3,32	**AET5**	404,93 (7,21)	1,78	**BET5**	466,94 (8,41)	1,80	**CET5**	361,61 (18,02)	4,98
Verbascoside	**AH6**	305,55 (22,82)	7,47	**BH6**	14,32 (0,02)	0,13	**CH6**	178,30 (0,25)	0,14	**AET6**	412,57 (15,62)	3,79	**BET6**	321,69 (9,61)	2,99	**CET6**	236,84 (7,34)	3,10
Verbascoside	**AH7**	314,04 (32,43)	10,33	**BH7**	4,01 (0,01)	0,18	**CH7**	60,68 (0,04)	0,06	**AET7**	414,27 (15,62)	3,77	**BET7**	175,58 (0,00)	0,00	**CET7**	149,34 (0,09)	0,06
Verbascoside	**AH8**	328,48 (12,01)	3,66	**BH8**	1,99 (0,00)	0,00	**CH8**	25,48 (0,13)	0,52	**AET8**	422,77 (18,02)	4,26	**BET8**	90,05 (0,11)	0,13	**CET8**	98,89 (0,03)	0,03
Verbascoside	**AH9**	332,73 (1,20)	0,36	**BH9**	1,15 (0,00)	0,00	**CH9**	8,01 (0,03)	0,43	**AET9**	433,79 (82,40)	0,55	**BET9**	43,68 (0,05)	0,12	**CET9**	62,36 (0,20)	0,32
Verbascoside	**AH10**	345,01 (0,16)	0,05	**BH10**	0,99 (0,00)	0,00	**CH10**	4,11 (0,00)	0,07	**AET10**	440,60 (4,78)	1,10	**BET10**	26,34 (0,12)	0,46	**CET10**	48,77 (0,94)	1,93

Table 1. The listed values are the average values obtained from three repetitions with the relative standard deviation (RSD). In parentheses is indicated the standard deviation (SD). The values are expressed in mg/Kg. The letters A, B and C are for whole olives, crushed olives and pitted olives respectively. H and ET are for water solution and 20% ethanolic solution respectively. The number 1 to 10 indicate the day of the experimentation.

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3.2.2 Composition of plant virgin oils obtained from *Oleaceae* fruits

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Summary

To increase the content of the secoiridoids compounds in the oil, some mixtures of olive fruits were made with those of other species of the Oleaceae family. The procedure applied for the last sample preparation and analytical investigation is patent pending. The same cultivar present in the Oleaceae oil has been used as control. Although the EU Regulation 61/2011 does not include the marketing of these oils labeled "virgin olive oils", recently a considerable interest is being manifested towards the use of oils used in cosmetics, while well-established markets exist for the marketing of food supplements rich secoiriion of tidoids activities in the Healthy for humans. The purpose of this research was to evaluate the composition of plant virgin oils obtained from *Oleaceae* fruits.

Abstract

Virgin oils are primary metabolites of many higher plants that are economically important as sources of food and industrial oils. Chemically, plant oils are rich of triglycerides, made of different fatty acids. The virgin olive oil contain about 96-98% of the saponificable fraction. Oleic acid is the principal component (70-80%) of this matrix, succeeding linoleic (12%) and palmitic (6.5%) acids. Unsaponificable fraction contains: terpenes, chlorophylls tocopherols, sterols and phenolic compounds. Secoiridoids, like aglycone derivatives of oleuropein, demethyloleuropein and ligstroside, are present in olive fruit as most abundant virgin olive oil phenolic antioxidants. Several important biological activities (antioxidant, anti-inflammatory, chemopreventive and anti-cancer) and the characteristic pungent and bitter tasty properties have been attributed to virgin olive oil phenols.
The olive tree is a member of the *Oleaceae* family, which contains the genera *Fraxinus*, *Forsythia*, *Forestiera*, *Ligustrum* and *Syringa*, as well as the genus *Olea*. Commercial olives are products of the *Olea europaea* L. species. Olive (*Olea europaea* L.) is a perennial evergreen tree that has grown naturally and been cultivated widely in the Mediterranean for centuries.

The aim of this study was to compare the phenolics and fatty acids composition in two different olive oils. Particularly, one sample is obtained from 'Coratina' cultivar and the other is obtained from virgin *Oleaceae* oil ('Coratina' + other *Oleaceae* fruit). The procedure applied for the last sample preparation and analytical investigation is patent pending.
Quali-quantitative analysis (performed by HPLC-MS/MS and GC-MS/MS) could be a useful tool to better correlate the typicity of the virgin *Oleaceae* oil with its phenolic compounds and fatty acids profile. Further studies are in progress to isolate unknown compounds and to further investigate the quality index of this food product.

Experimental

The current study has been led in the biennium 2009/2011 in two different oils: Oleaceae oil ('Coratina' cv + other *Oleaceae* fruit) and Olea europaea oil ('Coratina' cv).
Free acidity, peroxide index, UV spectrophotometric indices, fatty acids composition were analysed following the procedures of the EU Regulation 61/2011. The total phenols were analyzed by COI method (2009), while the individual phenols according to Montedoro and Owen methods (Montedoro et al., 1993; Owen et al., 2000). The oxidative stability (Rancimat method) has been performed following the methodology developed in the laboratories of the CRA-OLI. The sensory analysis (Panel test) accords to the Regulation CEE 2568/91. Analyses were conducted in triplicate.

Results and discussion

The results obtained show that the main parameters of the oil commodity are within the limits set by EEC Regulation EU Regulation 61/2011. There were no significant differences between oil and oil Coratina and oil Oleaceae (tables 1a nd 1b). Both show a sensory profile graded rating of 6.5 from the panel group and the two ascribed to the category of extra virgin oils (Figure 1).

	2009-2010		2010-2011	
	Olea europaea	***Oleaceae***	***Olea europaea***	***Oleaceae***
	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD
Free acidity (% as oleic acid; Limit: ≤ 0.8)	0,2±0,0	0,2±0,0	0,4±0,0	0,4±0,0
Peroxide index (mEq O2/kg oil; Limit: ≤ 20)	2,3±0,1	2,5±0,3	3,2±0,2	3,5±0,4
K232 (limit: ≤ 2,500)	1,943±0,074	2,033±0,072	1,720±0,069	1,737±0,015
K270 (limit: ≤ 0,200)	0,193±0,006	0,177±0,015	0,157±0,049	0,133±0,015
ΔK (limit: ≤ 0,001)	0,000±0,000	0,000±0,000	0,000±0,000	0,000±0,000

Table 1A: Free acidity, peroxide index and UV spectrophotometric indices.

	2009-2010		2010-2011	
	Olea europaea	***Oleaceae***	***Olea europaea***	***Oleaceae***
	Mean (%) ±SD	Mean (%) ±SD	Mean (%) ±SD	Mean (%) ±SD
C14:0	0,02±0,01	0,02±0,01	0,01±0,01	0,01±0,01
C16:0	11,31±0,17	11,14±0,38	7,59±0,12	9,08±1,26
C16:1	0,50±0,02	0,48±0,06	0,36±0,01	0,35±0,01
C17:0	0,05±0,01	0,04±0,01	0,03±0,01	0,04±0,01
C17:1	0,08±0,01	0,06±0,01	0,05±0,01	0,06±0,01
C18:0	2,05±0,05	2,16±0,02	2,09±0,02	2,16±0,01
C18:1	78,01±0,04	78,12±0,38	81,85±0,13	80,63±1,08
C18:2	6,35±0,11	6,43±0,10	6,12±0,03	5,79±0,14
C18:3	0,34±0,03	0,33±0,02	0,42±0,01	0,41±0,01
C20:0	0,82±0,12	0,80±0,06	0,90±0,06	0,86±0,07
C20:1	0,20±0,05	0,20±0,02	0,28±0,06	0,32±0,07
C22:0	0,10±0,02	0,10±0,01	0,14±0,03	0,14±0,03
C24:0	0,05±0,02	0,04±0,01	0,07±0,00	0,07±0,01
C18:1/C18:2	12,28±0,27	12,15±0,12	13,37±0,08	13,92±0,14

Table 1B: Fatty acids composition

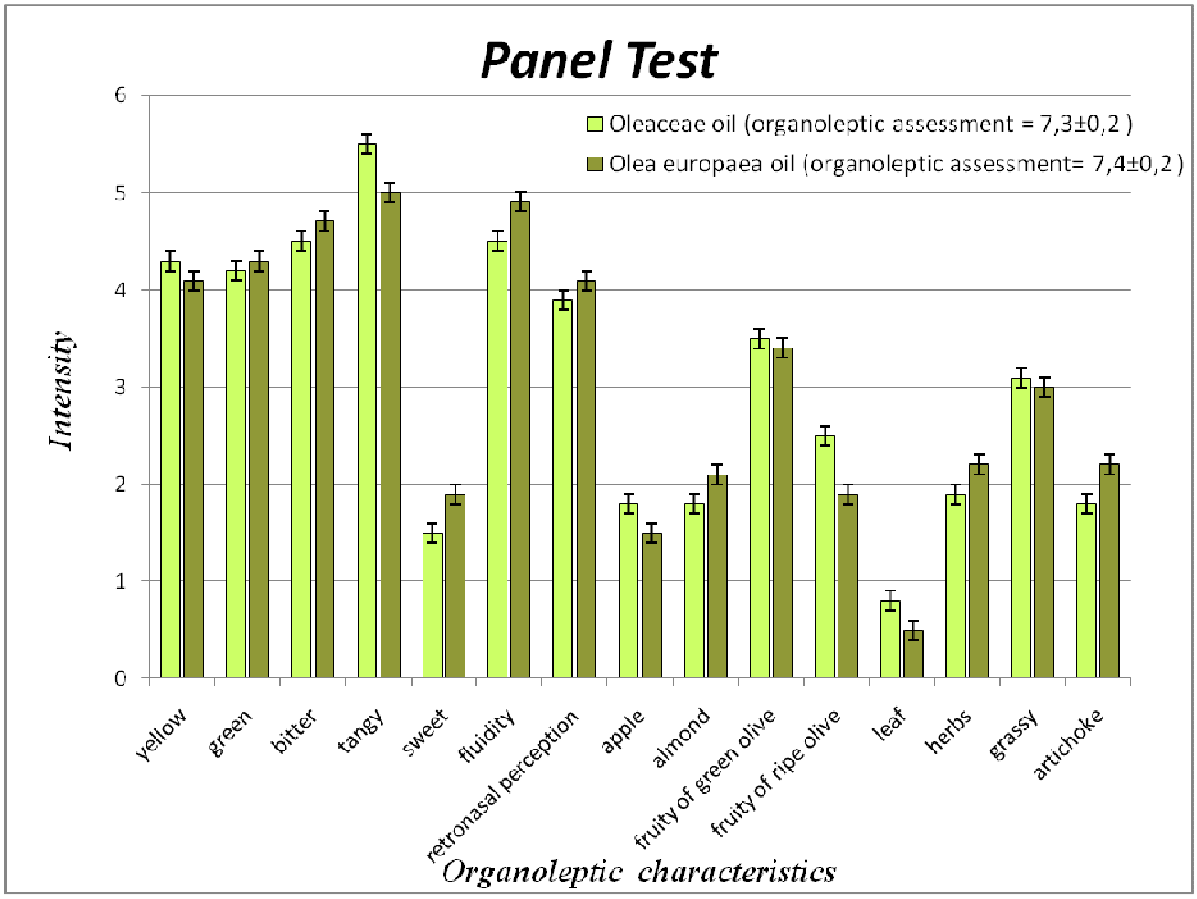

Figure 1: Panel Test

The main differences are related to their content of phenolic compounds (Table 2) and particularly the content of oleocanthal (dialdehyde of ligstroside, p-HPEA-EDA). This molecule in the Oleaceae oils appears to be double compared to those obtained from olives only. In addition, other phenolic compounds appear to be more present in the oil of Oleaceae, with the exception of tyrosol and pinoresinol. Among the phenolic compounds pinoresinol and oleocanthal are the most abundant. The different distribution of these two phenolic compounds in two different oils is interesting. In fact, in both years of oil production, the oils obtained from fruits of Oleaceae show an oleocanthal content nearly twice the corresponding oil obtained from olives only (430.80 ± 32.28 and 475.87 ± 57.69 against 220 , 38 ± 21.57 and 201.72 ± 5.31 mg / kg, respectively), while the content is about half pinoresinol (228.92 ± 20.81 and 254.26 ± 44.72 400.66 ± against 39.22 and 366.74 ± 9.66 mg / kg, respectively). The other phenolic compounds are mainly present in the oils obtained from fruits of the Oleaceae than olive oils, with the exception of tyrosol. The content of the simple phenol varies from 2.03 ± 0.05 to 2.21 ± 0.22 in the olive oil and a 1.07 ± 0.16 to 1.18 ± 0.20 mg / kg in Oleaceae oils.

The oils obtained from fruits of Oleaceae have a significantly higher total phenolic content. This oil, in fact, shows a content of phenolic compounds amounts to 883.27 ± 23,4 and 977.03 ± 111.62 mg / kg, oil production in the year 2009-'10 and 2010-'11 respectively. The

corresponding olive oil instead, shows a much lower content equal to 641.78 ± 62.8 and 587.44 ± 15.47 mg / kg olive oil in the same years.

	2009-2010		2010-2011	
	Olea europaea Mean ±SD	***Oleaceae*** Mean ±SD	***Olea europaea*** Mean ±SD	***Oleaceae*** Mean ±SD
RANCIMAT (Limit: ≥ 10h)	11,0±0,2	12,1±0,3	11,0±0,1	12,4±0,1
BIOPHENOLS (mg/kg of tyrosol)	641,78±62,8	883,27±23,4	587,44±15,47	977,03±111,62
3,4-DHPEA	0,65±0,06	0,73±0,20	0,59±0,02	0,81±0,26
p-HPEA	2,21±0,22	1,07±0,16	2,03±0,05	1,18±0,20
3,4-DHPEA-EDA	0,76±0,07	1,41±0,23	0,70±0,02	1,57±0,34
p-HPEA-EDA	220,38±21,57	430,80±32,28	201,72±5,31	475,87±57,69
EA	10,99±1,08	65,36±18,83	10,06±0,27	71,71±20,03
3,4-DHPEA-EA	2,00±0,20	14,97±0,97	1,84±0,05	16,58±2,35
p-HPEA-EA	2,26±0,22	95,03±1,33	2,07±0,05	105,05±10,67
ACETOXY-PINORESINOL	1,86±0,018	44,97±8,06	1,70±0,04	50,00±12,05
PINORESINOL	400,66±39,22	228,92±20,81	366,74±9,66	254,26±44,72

Table 2: Phenolic compounds and oxidative stability

In addition, the Rancimat method (Table 2), which allows to know the shelf life of an oil by determining the oxidative stability at elevated temperature (usually 110 ° C), showed significant differences between the oils produced from fruits and from those of Oleaceae Olea europaea. In both years,. induction time of about an hour to rancidity is more in oils of the fruits of the Oleaceae olive oil than that normal. However, the oils under study showed high values in the Rancimat, all over 10 hours to up to 12.4 hours.

Conclusions

In conclusion, as regards the first aspect, it is to be noted that preliminary work on varietal screening (choice of cultivars of olive trees and Oleaceae), optimization of olive varieties (weight ratio and weight olive drupe fruits Oleaceae) and conditions in oil to mini mill (time and temperature of crushing, malaxation and the centrifugation) were crucial for obtaining a high quality oil classified as "extra virgin". While, on the differences between the oils, it is to highlight that these are attributable only to the fruits of the Oleaceae which oil is extracted. In fact, the olive trees used for the extraction of both oils, have a common origin, the field collection of CRA-OIL in Rende. This allows us to assert that the differences we found (Rancimat and phenolic compounds) are actually related to the mixture of the fruits of the Oleaceae and not to the climatic conditions or farming techniques adopted for the cultivation of olive trees. Toxicological studies are currently underway.

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3.3 Quality and safety in agri-food

Agri-food chains and networks play an increasingly important role in providing access to markets for producers in developing countries. Globalization of trade and integration of supply chains lead to new demands regarding food quality and safety.

Quality and safety control and the validation of origin are hot issues in the production of food and its distribution, and are of primary concern to food and agriculture organization. Modern mass spectrometry (MS) provides unique, reliable and affordable methodologies to approach with a high degree of scientific nature any problem which may be posed in this field. The specificity and sensitivity of MS methodologies has become officially recognized by international quality-system control-bodies and the exploitation of multistage ion analysis has become mandatory to adhere to worldwide regulations regarding the recognition of fraud and bad practices in food manipulation.

On the other hand, quantitative LC-MS/MS methodology is utilized in food safety, agricultural and forensic chemistry. In food safety, there are different problems related to chemicals in foodstuffs. Certain food has the potential of containing chemicals which, if eaten in sufficient quantities, are harmful to human health. Other food can be contaminated by illegal dyes. The White Paper on Food Safety outlines a comprehensive range of actions needed to complement and modernize existing EU food legislation. Moreover, the Food Standards Agency aims to protect the consumer from these chemicals, and for this reason must maintain the best knowledge base possible on the subject to provide the necessary tools to ensure that consumer exposure to these chemicals is kept as low as reasonably practicable. The production and consumption of food is central to any society, and has economic, social and, in many cases, environmental consequences. Although health protection must always take priority, these issues must also be taken into account in the development of food policy[1].

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3.3.1 Identification of new Phenolic Compounds in Extra Virgin Olive Oils by Applications of Different Mass Spectrometry Scan Modes

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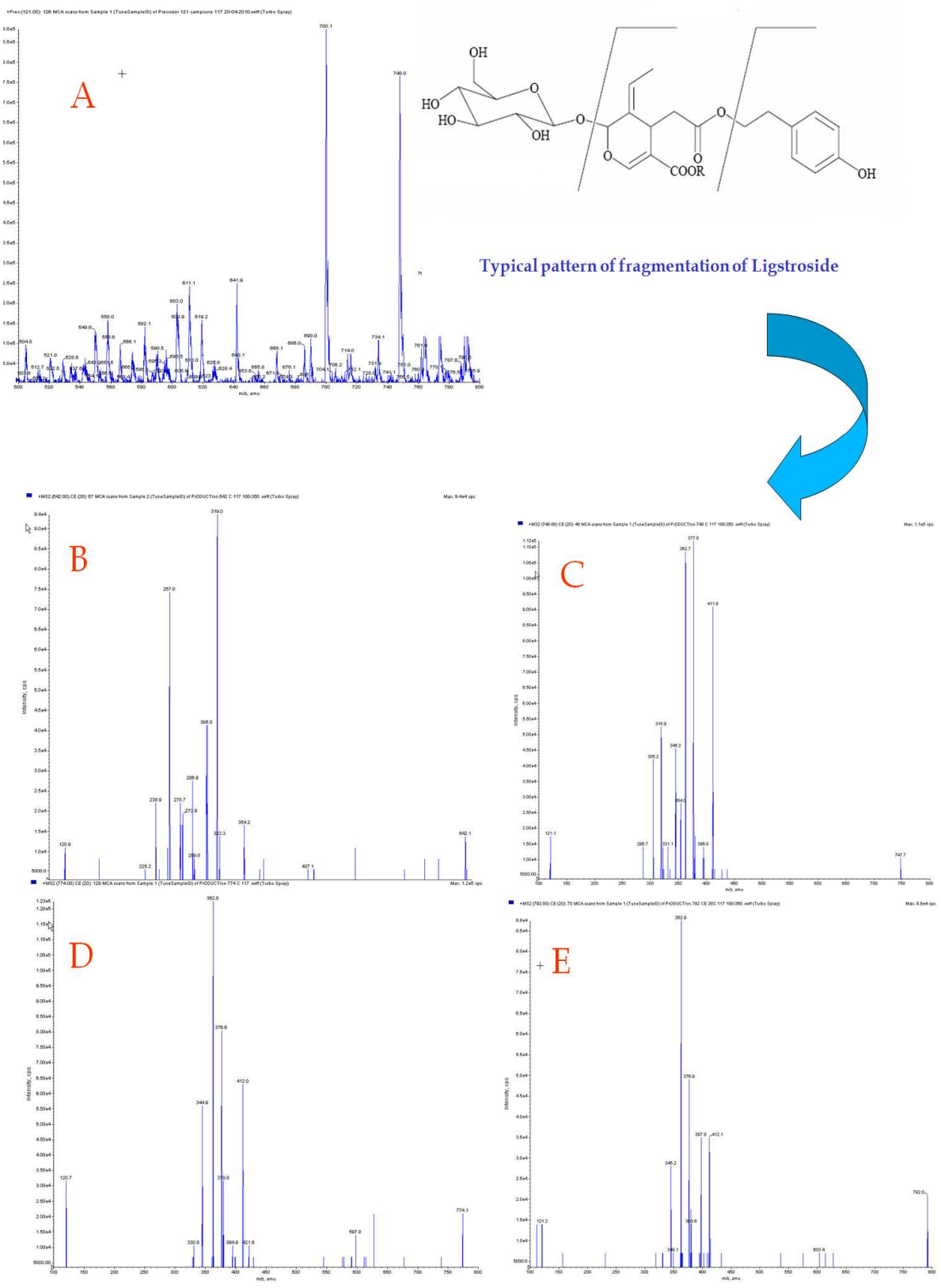
VIII Congresso Nazionale di Chimica degli Alimenti "Qualità e tipicità degli alimenti mediterranei: alimentazione e salute" 20-24 Settembre, 2010-Marsala (Trapani), 66-67

Abstract

Virgin oils are primary metabolites of many higher plants that are economically important as sources of food and industrial oils. Secoiridoids, like aglycone derivatives of oleuropein, demethyloleuropein and ligstroside, are present in olive fruit as most abundant virgin olive oil phenolic antioxidants. Several important biological activities (antioxidant, anti-inflammatory, chemopreventive and anti-cancer) and the characteristic pungent and bitter tasty properties have been attributed to virgin olive oil phenols [(1-2)]. Methanolic extracts of fresh extra virgin olive oil samples of Coratina cultivar were 0,0001% diluited in an acetonitrile and methanol 20 mM ammonium acetate mixture. ESI (+) Precursor ion of m/z 121 which correspond to tyrosol fragment gave information about the presence of ligstroside like molecules respectively at m/z 642, 748, 774 and 792 m/z. ESI(+) Product Ion confirm the possible pattern of fragmentation respectively in a glycosidic, aglycone and tyrosol fragments [(3-4)]. Future perspective of the work here presented will be isolation, purification and complete characterization of these new molecules identified.

Materials and methods

All experiments were carried out using an API 4000 QTRAP mass spectrometer equipped with an ESI source (Applied Biosystems, Foster City, CA), working in positive mode. Methanolic extracts of fresh extra virgin olive oil samples of Coratina cultivar were 0,0001% diluited in an acetonitrile and methanol (8/2) 20 mM ammonium acetate mixture. The ionization was carried with a ionospray voltage of 4800V , and a source temperature (TEM) of 250°C. The curtain gas (CUR) and source gases (GS1, GS2) were set to 20, 12 and 30psi, respectively, while the declustering potential (DP), and entrance potential (EP) were kept at 100, and 10V, respectively. The collision energy was switched for optimize the response of each analyte.


A
OH
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COOR
OH
Typical pattern of fragmentation of Ligstroside
B
C
D
E

Results and Discussion

ESI(+) MS2 mass spectra of ligsstroside is characterized by the fragment at m/z 345 corresonding to the elenoic acid aglycone and fragment at m/z 121 corresponding to the tyrosolic moiety. Mass spectra were acquired by direct injection of Coratina cultivar monovarietal extra virgin olive oil methanolic freshly extracts in precursor ion of m/z 121 (figure 1A) scan mode. ESI(+)/MS2 Mass spectra of the most abboundant ions at m/z 642, 748, 774, and 792 (figure 1B-E) confirm the presence of ligstroside like molecules. Particularly, from the pattern of fragmentation it seem that dimeric linkage of differently substitute glycosidic moieties are present. These preliminary results will be elucidated by isolation, purification and complete characterization of these new molecules by high resolution mass spectrometry and nuclear magnetic resonance.

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3.3.2 Approach to the phenolic profile of a superintensive Arbosana cultivar by mass spectrometry

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Afines, 11-13 maggio Jaèn (Spagna), Reference number: IND-49.

ITALUS ORTUS, IN PRESS>>

Introduction

Phenolic compounds have a high importance in olive oil because of their effects on shelf life and sensorial properties. Phenolic compounds are natural antioxidants and important factors to consider in evaluating the quality of an extra virgin olive oil since they are partly responsible for its auto-oxidation stability and organoleptic characteristics (Robards et al., 1999; Visioli et al., 1998; De Nino et al., 2005). The coupling of HPLC-MS with atmospheric pressure ionization techniques, such as electrospray ionization (ESI) (Sánchez-Rabaleda et al., 2003), atmospheric pressure chemical ionization (APCI) (Visioli et al., 1998; Perri et al., 1999; Caruso et al., 2000; De Nino et al., 2008) is a powerful tool for the identification of natural products in crude plant extracts because of their soft ionization. Therefore, this technique is useful to identify the phenolic compounds in olive oil. Also, the amounts of these chemical antioxidants are influenced by the cultivar, soil, climate, irrigation, degree of ripeness and processing methods (Morelló et al., 2004). The super-high-density system (1500–2500 trees per/ha) was developed within the past decade to use over the row mechanical harvesters to reduce the costs of hand harvesting and to bring orchards into production within only a few years after planting. The modern cultivation techniques used in the super-high-density (SHD) system are being rapidly disseminated in countries where olive-growing is a traditional crop, such as Spain, and in a series of new and interesting productive areas. This work aimed, therefore, to study the phenolic profile of Arbosana variety olive oils grown under a superintensive plantation located in Spain in Zaragoza province.

Material and methods

Fruit sampling

The trial was carried out in 2009 in a super-intensive orchard (*Olea europea* L. Cv. Arbosana) located in Aula Dei Experimental Station-CSIC at Zaragoza (Spain). Samples from Arbosana trees were handpicked from 60 trees (20 for each line) in perfect sanitary conditions.

Oil extraction process

Oil extraction was performed using an Abencor laboratory oil mill (MC2 Ingenierías y sistemas, Sevilla, Spain) according to the method described by Martínez et al., (1975). Olives from Arbequina variety were cleaned from leaves, crushed with a hammer crusher, and the paste mixed at a temperature of about 26º C for 30 min, and then centrifugated in a decanter (3500 rpm over 3 min).

Extraction of phenolic compounds

Phenolic compounds were extracted from virgin olive oil according to the method described by Gutfinger (1981). Oil (10 g), were dissolved in hexane (50 ml) and the solution was extracted successively with three 20-ml portions of 60% aqueous methanol. The combined extracts were brought to dryness in a rotary evaporator at 40º C and the residue was dissolved in 5 ml of methanol, filtered though a 0.45-µm filter and submitted to mass spectrometry analysis.

Mass spectrometry analysis

Sample analysis were performed using a triple quadrupole mass spectrometer (API 4000 Q-trap LC/MS/MS system, Applied Biosystems/MDS Sciex Toronto, Canada; software, Analyst version 1.2) interfaced with an HPLC system (Agilent Technologies 1200 series) equipped with a G1379B degasser, a G1312A pump, and a G1329A autosampler. The methanolic extracts of olive oil were acquired in full scan by means of a turbo ion spray source in negative mode. The range considered were between m/z 100 and 800 Da. The separation was performed using a gradient of methanol and water containing 0.1% formic acid.

Results and discussion

Figure 1 shows the LC-MS ion chromatogram obtained from the methanolic extract of Arbosana olive oil. The chromatogram shows the most abundant peak at elution time of 17.28 min, that is relative to the decarboxymethyl oleuropein aglycone (m/z 319).

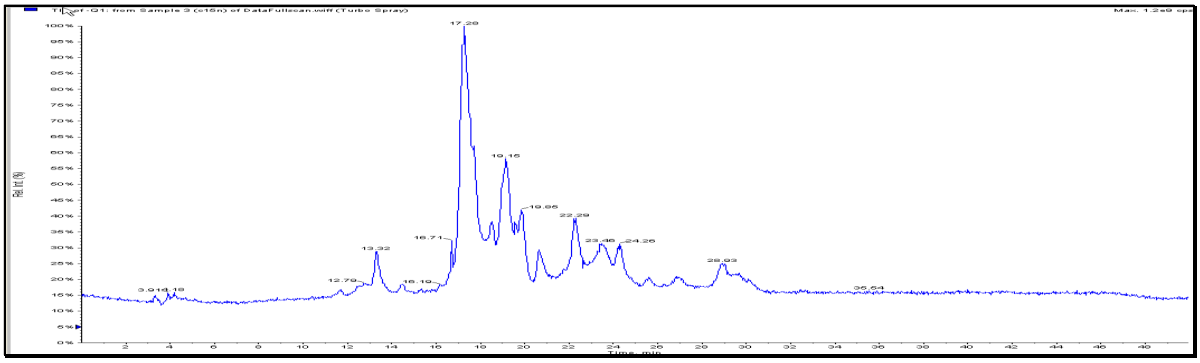

Figure 1. Total ion cromatogram obtained from 20 µl of a methanolic extract of olive oil. Total ion current in the range 100-800 units plotted vs. elution time.

Other peaks are eluted at 9.15 min and between 22.29 and 28.93 min corresponding to the decarboxymethyl ligstroside aglycone (m/z 303) and to the oleuropein aglycone species (m/z 377) respectively (Figure 2).

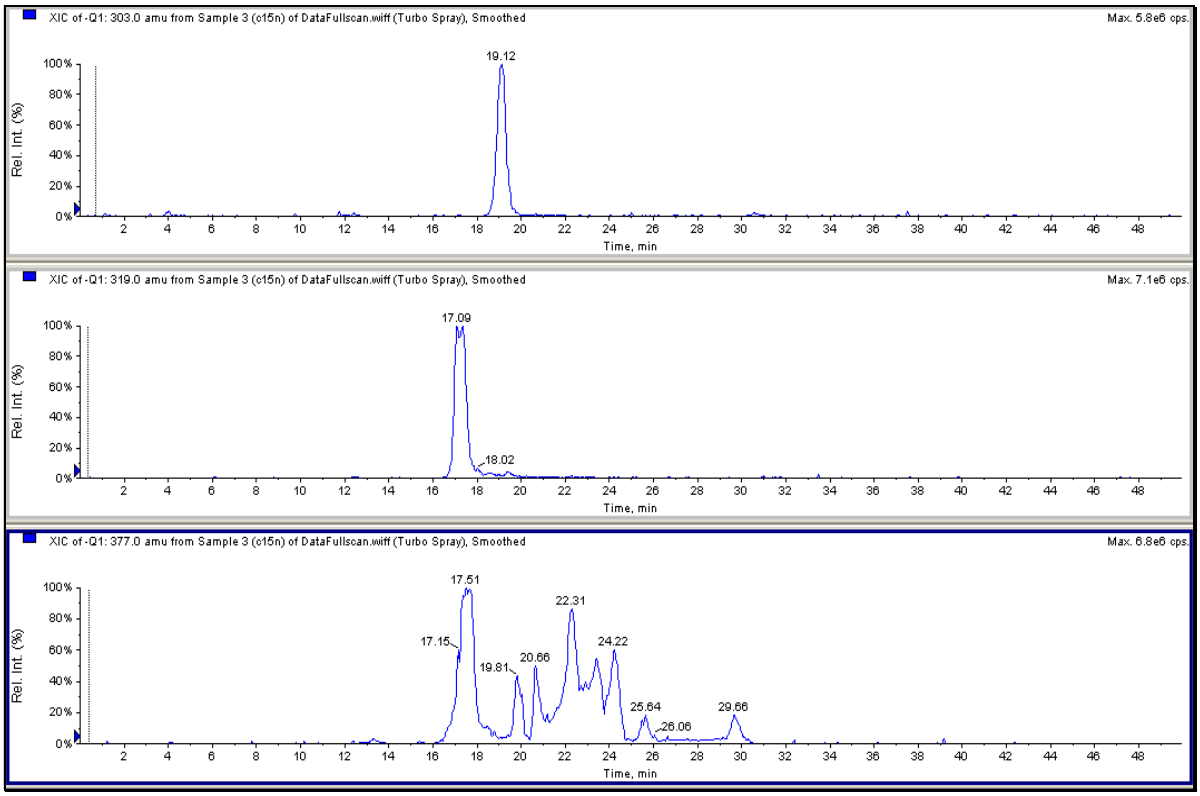

Figure 2. Extracted ion chromatograms of the species at m/z 303 (decarboxymethyl ligstroside aglycone), 319 (decarboxymethyl oleuropein aglycone) and 377 (oleuropein aglycone).

A peak at elution time of 11.81 min is also present and it is relative to the hydroxytyrosol of m/z 153 (Figure 3).

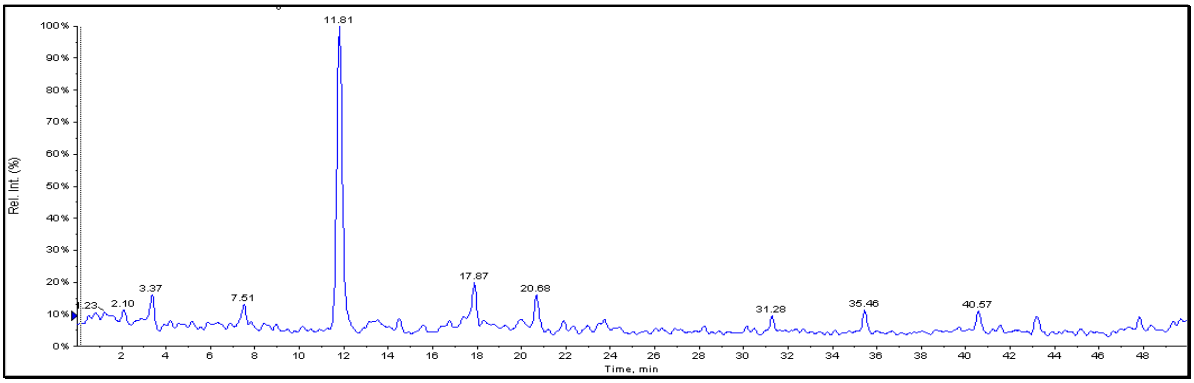

Figure 3. Extracted ion chromatograms of the species at m/z 153 (hydroxytyrosol) eluted at retention time of 11.81min.

Figure 4 shows the mass spectra relative to all the species previously described. The preliminary results obtained in this work have confirmed the presence of the species that best characterize the antioxidant properties of an extra virgin olive oil. This oil, obtained from Olea europea cv Arbosana under a superintensive plantation, showed a profile that is very rich in different derivates of oleuropein and ligstroside like decarboxymethyl ligstroside aglycone, known as oleocanthal, that was demonstrated to have strong anti-inflammatory properties (Beauchamp et al., 2005; Smith et al., 2005; Cicerale et al., 2009; Attya et al., 2010; Iacono et al., 2010) and a high influence in the pungency of olive oil (Lavelli and Bondesan 2005; Franconi et al., 2006; Impellizzeri and Lin 2006; Konstantinidou et al., 2010; Peyrot et al., 2011). Moreover, the presence of oleuropein aglycones, involved in the sensorial characteristics of the olive oil, could provide to obtain olive oils with a powerful taste and a plentiful sensorial profile.

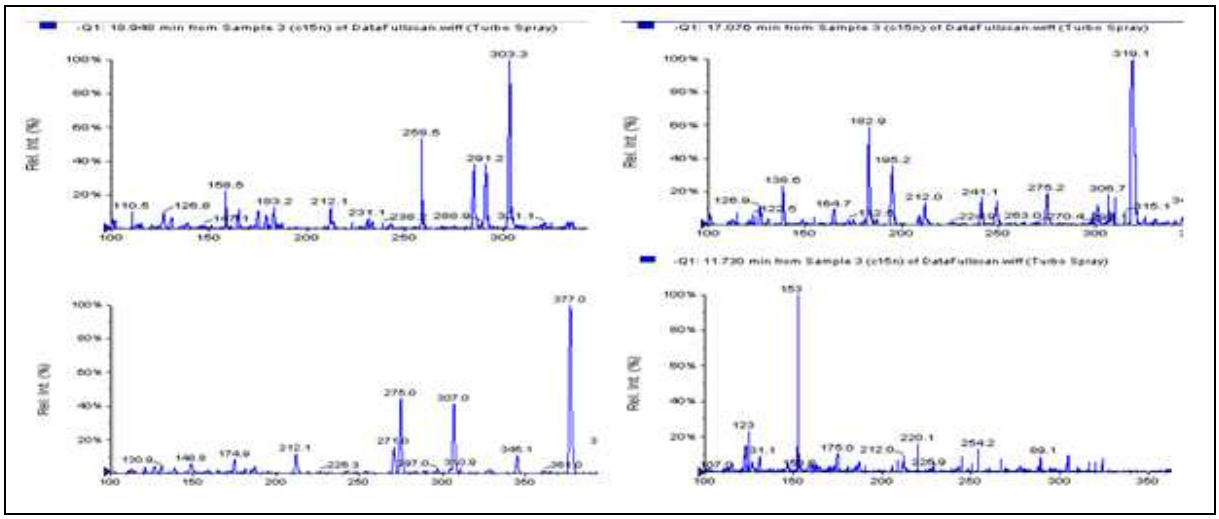

Figure 4. Spectrum relative to the ion chromatogram obtained from 20 □l of a methanolic extract of olive oil from Olea europea cv Arbosana at m/z 303, 319, 377 and 153.

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3.3.3 Determination of spinosad residues in olive drupes using high-performance liquid chromatography/ tandem mass spectrometry

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XIV Simposium Científico-Técnico de EXPOLIVA , 13-15 May 2009, Jaén (Spain). Forum on the olive oil industry, technology and quality, 14TH Scientific-Technical Symposium Expoliva 2009 Jaén, 13-15 May 2009 FORO DE LA INDUSTRIA OLEÍCOLA, TECNOLOGÍA Y CALIDAD: IND-47

Abstract

The determination of spinosyns A, D and K in acetonitrile extracts of olive drupes was carried out by on-line liquid chromatography/electrospray ionization tandem mass spectrometry. For the quantitative analysis of samples an external calibration curve was build in a concentration range of 25-400 ng/mL. Results from spike and recovery experiments at levels of 50 and 300 ng/ml gave mean recoveries ranging from 76–115% with satisfactory precision. The excellent selectivity and sensitivity allow quantification and identification of low levels of spinosad on olives (limits of quantitation (LOQs) 0.071-0.287 μg/g).

Key Words: *Spinosad; Spinosyn A, D, K; Pesticide residues; Mass spectrometry.*

Introduction

Modern conventional agricultural production depends heavily on the use of pesticides and their residues can persist to the harvest stage, making possible the contamination of the final product. In opposite, organic farming systems do not allow pesticides from chemical synthesis, though contamination may occur. Environmental safeguard, human health and rural development are the main goals of European Community Agricultural Policy. Moreover, foodstuffs quality and security understood as absence of chemical residues, represent for Italian Agricultural Policy and public opinion indisputable benefits to gain.[1] Both European Union and the Codex Alimentarius Commission of the Food and Agriculture Organization of the United Nations have established maximum pesticide residues limits (MRLs). Organic farming, regulated by the EEC Regulation No. 2092/91 and its updates recently reformulated, is an agricultural management system based on a variety of farming practices aimed at promoting sustainable agricultural development.[2] The key-point is to minimise pesticides inputs in agroecosystems increasing selectivity on target pests favouring pest control strategies aimed at environmental health safeguard. [3]

Organic farming regulation allows exclusively the use of plant defence products listed in annex II B previously authorized by the Member State, according to the Council Directive (Dir 91/414/EEC). Among them Spinosad has been applied to over 200 different crop in order to control many pest[14-15] and, in particular, many Tephritid fruit flies. [4-7] It has been recently utilised also in olive groves in order to perform *Bactrocera oleae* (Rossi, 1790) control [8, 9]. Spinosyns are the active ingredients in an insect control agent produced by fermentation of *S. spinosa*. The most active components of the spinosyn family of compounds are spinosyns A and D; other factors in this family have different levels of methylation and are significantly less active. [10] Analytical methods were needed to determine the magnitude of residues in the fruit and processed commodities treated with spinosad. Residue methods have been previously reported for spinosad in cottonseed and cottonseed processed commodities, [11] in soil, sediment, and water ,[12] in leafy vegetables, peppers, and tomatoes, [13] and in meat, milk, cream, and eggs.[14] Thus, the following work is presented for the identification and quantification of spinosyns A, D and K in olive drupes by using an on-line liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) method.

Materials and methods

Applications of Spintor Fly (GF-120) (1L/5L of water) were weekly carried out from the end of August to the end of October 2007. The solution was applied on the southern side of the tree canopy, on a spot area of 40 cm2. On each tree 25 mL of the solution were sprayed in order to apply 0.24 g/ha of Spinosad.

In order to evaluate the field persistence of Spinosad, olive drupe samples (1 Kg) were randomly collected immediately after the field application of the product on treated trees and after 2 days and 1 week. A control sample was collected on untreated trees. Drupes of treated samples were picked up only from the sprayed spot area. After sample collection, olive drupes were stored at -20 °C until required for analysis.

Olive samples (30 g), were quantitatively transferred into a flask and acetonitrile (50 mL) added. The solutions were blended for 30 minutes by means of a magnetic stirrer. Fortified recovery samples were prepared by adding appropriate different volumes of standard solution to result in samples fortified with all three analytes at concentrations of 50 and 300 ng/mL. A reagent blank (containing no sample or analytes) and an untreated control sample (containing no analytes) were also prepared.

Chemicals and apparatus

Certified reference spinosad (purity 90.4%) was obtained from Dow AgroSciences LLC (Indianapolis); acetonitrile and formic acid were of LC/MS grade and were supplied from Sigma-Aldrich (Riedel-de Haën, Laborchemikalien, Seelze); aqueous solution were prepared using ultrapure water, with a resistivity of 18.2 MX cm, obtained from a Milli-Q plus system (Millipore, Bedford, MA, USA).
The experimental work was carried out using a MSD Sciex Applied Biosystem API 4000 Q-Trap mass spectrometer interfaced with an Agilent Technologies 1200 series liquid chromatography system equipped with G1379B degasser, G1312A pump, and G1329A autosampler.
The positive ion spectra were obtained in "Multiple Reaction Monitoring" mode monitoring the following transitions: for spinosyn A: *m/z* 732.8 → *m/z* 142.3; for spinosyn D: *m/z 746.8* → *m/z* 142.4 and for spinosyn K: *m/z 718.8* → *m/z 142.3*.
The analytes were separated on a Polaris C18-A HPLC column [3 μm particle size, 50 mm length and 2 mm i.d. (Varian Inc., USA)] at a flow rate of 300 μL/min. The elution program started with 90% of solvent A (0,1% aqueous formic acid) and 10% solvent B (acetonitrile); the percentage of solvent B was linearly increased to 100% in 4.0 minutes, hold for 1 minute and ramped to original composition in 2 minutes. The total elution time was 10 minutes per injection.

Results and discussion

The ion spectra for spinosyn A, D and K gave the protonated molecule [M-H]+ as the most prominent ion. For the quantitative analysis of samples an external calibration curve was build in a concentration range of 25-400 ng/mL. Standard solutions were prepared by diluting the solution of certified reference spinosad. The correlation coefficient ($r2$) for the least-squares equations describing the detector response as a function of concentration was 0.997 for spinosyn A, 0.998 for spinosyn D and 0.999 for spinosyn K.
For recovery experiments two appropriate different volumes of standard solution where added to a sample of olive and extracted as previously described in the sample preparation section in order to obtain fortification standards at concentrations of 50 and 300 ng/mL. The extraction was performed three times per each fortification level. Sample data were processed by the external standard technique.
The limit of determination (LOD) and the limit of quantitation (LOQ) were defined as the amount equivalent to three and ten times the method noise, which included the instrument noise and background signal contributed by the matrix blank respectively. The calculated LODs were 0.001 μg/g for spinosyns A and D and 0.002 μg/g for spinosyn K.

These calculated values support a method LOD of 0.003 μg/g. Likewise, the calculated LOQs were 0.005 μg/g for spinosyns A, 0.003 μg/g for spinosyn D and 0.008 μg/g for spinosyn K. These calculated values support a method LOQ of 0.01 μg/g. Results from spike and recovery experiments at levels of 50 and 300 ng/mL were in the range 91–108% (Table 1).

	LOD (μg/g)	LOQ (μg/g)	Fortified (ng/mL)	Range	(Mean ± RSD)[a]	Recovery %	Fortified (ng/mL)	Range	(Mean ± RSD)[a]	Recovery %
Spinosyn A	0,001	0,005	50	44-58	51 ± 13	102	300	255-312	283 ± 10	94
Spinosyn D	0,001	0,003	50	38-49	44 ± 12	91	300	285-305	297 ± 11	101
Spinosyn K	0,002	0,008	50	44-52	50 ± 10	108	300	274-322	291 ± 9	108

[a] Each value is the mean of three determinations, RSD = relative standard deviation.

Table 1. LOD and LOQ values, recoveries and precision (RSD) of spinosyns A, D and K.

Results obtained analysing the olive drupe samples are listed in Table 2. There were no residues found in the control sample, where no treatment was performed. The olive drupes collected straight after performing the insecticidal treatment showed a concentration of spinosyns between 64 and 80 ng/g. Samples collected the fifth and the seventh day after performing the insecticidal treatment showed a concentration of spinosyns between 18 and 29 ng/g.

However, we must point out that only treated drupe were collected for analysis and presence of the residues were expected.

Spinosyn A	Olive sample	Control	Control	after the treatment	after the treatment	after 5 days	after 5 days	after 7 days	after 7 days
	standard addition (ng/mL)	–	120	–	100	–	100	–	100
	concentrazione (ng/g) found	–	126	78	165	29	130	20	121
Spinosyn D	Olive sample	Control	Control	after the treatment	after the treatment	after 5 days	after 5 days	after 7 days	after 7 days
	standard addition (ng/mL)	–	120	–	100	–	100	–	100
	concentrazione (ng/g) found	–	108	80	190	25	130	19	123
Spinosyn K	Olive sample	Control	Control	after the treatment	after the treatment	after 5 days	after 5 days	after 7 days	after 7 days
	standard addition (ng/mL)	–	120	–	100	–	100	–	100
	concentrazione (ng/g) found	–	113	64	154	25	115	18	120

Table 2. Spinosad residue concentrations in olive drupes under investigation.

Conclusions

In this paper we proposed a simple and rapid method for the simultaneous determination of spinosyns A, D and K in acetonitrile extracts of olive drupes by on-line liquid chromatography/electrospray ionization tandem mass spectrometry.
Although residue methodology exists for spinosad in food, feed, and environmental matrices using immunoassay,[15, 16] HPLC-UV, [11-14; 17, 18] and/or LC-MS, [19-24] the method here presented has been developed and validated for the determination and/or confirmation of spinosad and its major metabolites on olives.
The method had showed an excellent selectivity and sensitivity, achieving limits of quantitation very low allowing quantification and identification of low levels of pesticide in olive oils (LOQs 0.005-0.098 μg/g). No clean-up of extracts was performed and the run time was only 10 minutes.

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