UNIVERSITÀ DELLA CALABRIA

International doctorate programme "Bernardino Telesio School of Hard Sciences":

"Methodologies for the Development of Molecules of Pharmacological Interest"

(MDMP, *XXII cycle*)

Food Control Quality by Isotope Dilution Liquid Chromatography-Tandem Mass Spectrometry

Submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy, Department of Chemistry, University of Calabria, Italy

Supervisor

Prof. Bartolo Gabriele

Dr. Antonio Tagarelli

Coordinator

Prof. Bartolo Gabriele

Candidate

Hicham Benabdelkamel

Declaration

I, the undersigned, certify that this thesis submitted for the degree of PhD in chemistry is the result of my own research, except where otherwise acknowledged, and that this thesis (or any part of the same) has not been submitted for a higher degree to any other university or institution.

Signature Benabdelkamel Hicham Date 10/12/2009

DEDICATION

This thesis is dedicated to my beloved family

My father, Benabdelkamel Houssine

My mother, Benouzzi Naima

My sibling

Youness, Othmane

Soufian, Mehdi and Safaa

and

my beloved friend Koroma, Samuka C

ACKNOWLEDGEMENTS

In the name of Allah, most Gracious, most Merciful Only by His grace and mercy this thesis can be completed.

I would like to thank Professor Giovanni Sindona, for giving me the opportunity to prepare my thesis in his, mass spectroscopy laboratory at the University of Calabria . His expert knowledge has been a great support for my work. I was always encouraged to invest maximum effort due to his excellent ability to motivate resulting in important outcomes for further investigations. Furthermore, I would like to thank him for the many opportunities to participate in national and international congresses.

I wish to express my most sincere acknowledgment and deepest appreciation to my supervisor, Professor Bartolo Gabriele and Dr Antonio Tagarelli for supervising this work and their support during the whole thesis. I am grateful for their great ideas, them constructive and exciting discussions and for the chance to realise them into practice I would like to thank my colleagues for creating a helpful and friendly atmosphere.

I am thankful to professor Anna Napoli, Dr Leonardo DiDonna , Dr Fabio mazzotti (jimmi fountana),Dr Pierluigi Plastina, Monica Nardi, Rafaelle salerno for their help during my whole thesis.

I am also beholden to all staffs of Industry chemistry laboratory, University of Calabria, for their help and cooperation. Much appreciation also goes to professor G. Salerno, My gratitude goes to all my colleagues PhD, for their support and the good time we spent together during the work with the test persons. A great thank goes to our Technician Lab Mr.Santoro (zio franco..portare per me un cammello) for his help and making my time

enjoyable one. Furthermore, I would like to thank Professor El Mokhtar Essassi and professor Joseph Banoub (shakhra for him) for their help and encouragement

My deepest thanks go to my family and my closest friends for their love, encouragement and support during my PhD.

Summary and Aims of the thesis

The late part of the 20th century the advancement of knowledge regarding nutrition and disease prevention provided an opportunity for individuals to affect their own health. This expanding body of information helped people to understand how the environment and their own behaviour affected their body. People now had powerful tools to help maintaining and protecting their health. The understanding of how our diet affects our well being has dramatically changed the lifestyles and attitudes of people, who began to make menu and purchasing decisions based on how foods would affect their heath. A shift toward healthier lifestyles and healthier diets began. Food processors and marketers had to refocus their efforts from promoting foods for pleasure to promoting foods that fit in to a healthy diet. Primarily, the focus was on reducing fat and cholesterol in the diet and supplementing vitamins and minerals. Research began to demonstrate the presence of various phytochemicals in wine and juice make it from fruits and vegetables(such as stilbenes, falvoniods, polyphenols…) and specially in olive oil as (polyphenol in dialdehyed form: oleochanthal, hydroxyoleocanthal..) These compounds have come to be known as nutraceuticals. The list of nutraceuticals present in wine, fruit drink and olive oil that are believed to have positive biological properties has been expanding. Food processors and developers have become very interested in exploiting these nutraceuticals for the production of foods that are not only part of a healthy diet but also improve the consumer's health in another specific way. These foods have become known as "functional foods" means quality marker.

The aim of this thesis has been to develop a analytical methods to determine the concentration of a group of these nutraceuticals in food , such as, quantitative determination of resveratrol in wine, pterostilbene in blueberry juice and dialdehyde form in olive oil using a sensitive high-performance liquid chromatographic separation method coupling with tandem-mass and isotope dilution to order to optimal the conditions for the analysis method, such as extraction procedure, matrices, column, quality controls, wavelength, mobile phases, run time, optimal separation (gradient, retention times), temperature, capillary voltage, cone voltages, vacuum and labelled internal standards, resulting in the best sensitivity and selectivity,

The goodness and satisfactory of the method was performed according to, containing linear measuring range, quantification, lower limit of quantification (LLOQ), lower limit of detection (LLOD), quality controls, precision(RSD %), accuracy, recovery, stability and matrix effects.

In conclusion, the described high-performance liquid chromatographic separation method with tandem-mass spectrometry detection and isotope dilution showed a satisfactory overall analytical performance well suited for applications in food quality control.

CONTENTS

List of Terms and Abbreviations.

APCI	**Atmospheric Pressure Chemical Ionisation**
API	**Atmospheric Pressure Ionisation**
APPI	**Atmospheric Pressure Photoionisation**
CE	**Capillary Electrophoresis**
CHD	**Coronary Heart Disease**
CID	**Collision Induced Dissociation**
COX	**Cyclooxygenase**
D	**Deuterium**
DC	**Direct Current**
DDQ	**2,3-Dichloro-5,6-dicyano-1,4-benzoquinone**
DLI	**Direct Liquid Introduction**
DMSO	**Dimethyl Sulfoxide**
DNA	**Deoxyribonucleic Acid**
ESI+	**Electrospray Ionisation in positive ion mode**
FAB	**Fast-atom Bombardment**
FTICR	**Fourier Transform Ion Cyclotron Resonance**
HETP	**Height Equivalent to Theoretical Plate**
HILIC	**Hydrophilic Interaction Liquid Chromatography**
HLMs	**Human Liver Microsomes**
HPLC	**High Performance Liquid Chromatography**
k	**Capacity Factor**
K_m	**Michaelis Constant**
LC	**Liquid Chromatography**
LOD	**Limit of Detection**
LOQ	**Limit of Quantitation**
M/Z	**Mass-to-charge ratio**
MALDI	**Matrix Assisted Laser Desorption Ionisation**
MB	**Moving Belt**
MRM	**Multiple Reaction Monitoring**
MS	**Mass Spectrometry**
MS/MS	**Mass Spectrometry/ Mass Spectrometry**
NMR	**Nuclear Magnetic Resonance**
P	**Product**
P450	**Cytochrome P450**
QC	**Quality Control**

QToF	**Quadrupole Time-of-Flight**
R^2	**Correlation co-efficient**
RF	**Radio-frequency**
RSD%	**Reproducible**
S	**Substrate**
S/N	**Signal-to-noise ratio**
SPE	**Solid Phase Extraction**
SRM	**Selected Reaction Monitoring**
THF	**Tetrahydrofuran**
TLC	**Thin layer chromatography**
TMS	**Tandem Mass Spectrometry**
ToF	**Time-of-Flight**
t_R	**Retention time**
U	**A term describing a fixed DC potential applied to a quadrupole mass filter assembly.**
UV	**Ultra-violet**
V	**A term describing the amplitude of a radio-frequency.**
VOO	**virgin olive oil**

CHAPTER 1

Introduction

Chapter 1

Chapter 1

1.1 Introduction to Mass Spectrometry

1.1.1 The History of Mass Spectrometry

The developments that have lead to days modern mass spectrometers began in 1897, when J. J. Thomson (1856 – 1940) built an apparatus that allowed a stream of ions present in a positive ray discharge to be separated according to their mass-to-charge ratio (*m/z*). Subsequent work allowed Thomson to provide the first experimental evidence that stable elements consist of a number of isotopes (Griffiths, 1997). F. W. Aston (1877 – 1945) made considerable improvements to the work started by Thomson at the Cavendish Laboratory in Cambridge, resulting in the development of a mass spectrograph. However, the first instrumentation to be termed a *mass spectrometer* was developed by A. J. Dempster (1886 – 1950) in Chicago, where a focused beam of ions of selected *m/z* was allowed to passed through a slit and measured electrically (Chapman, 1997).

Until this point the use of mass spectrometry had been limited to the measurement of masses or relative abundance of isotope ratios. Amongst the first to use mass spectrometry for the analysis of complex mixture was A. O. Nier (1911 – 1994) and it was this work that lead to the commercial production of mass spectrometers in the United States, circa 1940. At about the same time, the Metropolitan Vickers Electrical Company (Trafford Park, Manchester) became involved in the production of mass spectrometers and seeded the mass spectrometry industry in Manchester, the location of approximately 20 mass spectrometry companies today (Chapman, 1997).

1.2 Mass Spectrometry.

The basis of mass spectrometry is the production of ions that are subsequently separated or filtered according to the their mass-to-charge (*m/z*) ratio. A mass spectrum is produced when the abundance of these mass analyzed ions is plotted against their *m/z* ratio. Regardless of their complexity, all mass spectrometers comprise of five principle components: an inlet, an ion source, a mass analyzer, a detector and a data system. The ion source generates ions from the sample supplied by the inlet and these are then passed to the analyzer. The trajectory of ions is controlled by magnetic or electrical fields,

depending on the type of analyzer, until they reach the detector where their arrival is recorded by a data system (Grayson, 2002).

1.2.1 Ionization.

The need to study organic molecules of wide ranging chemical and physical properties by mass spectrometry has resulted in many different methods of ion formation. Where the inlet system supplies the sample in a liquid i.e. an HPLC system, a problem arises due to the high gas load that will be generated by a liquid buffer or solvent. As the mass analysis and detection regions of the mass spectrometer operate under reduced pressure, the pumping system needs to cope with the large gas volumes generated. Several different source designs and approaches have been used to reduce this problem most commonly by reducing the amount of solvent/liquid entering the mass spectrometer via miniaturisation of the separation system or by splitting the flow.

Interface	Reference
Moving Belt (MB)	Alcock et al., 1982.
Direct Liquid Introduction (DLI)	Melera, 1980
Particle Beam (PB)	Willoughby and Browner, 1984 Winkler et al., 1988
Fast-atom Bombardment (FAB)	Caprioli et al., 1986
Matrix-assisted Laser Desorption Ionisation (MALDI)	Moyer and Cotter, 2002

Table 1.1 Some of the major techniques used to interface liquid chromatography with mass spectrometry.

Arguably, the most commonly used type of ion source used in the drug development and clinical settings operates at near atmospheric pressure; atmospheric pressure ionization (API). The generation of ions in this ways alleviates the gas-burden on the pumping system of the mass spectrometer by allowing sampling of ions from a region of space at a controlled rate. Two ionization techniques are commonly

Employed today, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APcI). A more recently developed technique known as atmospheric pressure photoionisation (APPI) may also become more common (Robb et al., 2000).

1.2.1.1 The Z-spray™ source.

The Z- spray source was developed by Micromass (Wythenshawe, Manchester, UK) in 1996 and is a cleaner, more efficient way of generating and separating analyte ions from solvent, mobile phase modifiers and sample matrices (Figure 1.1).

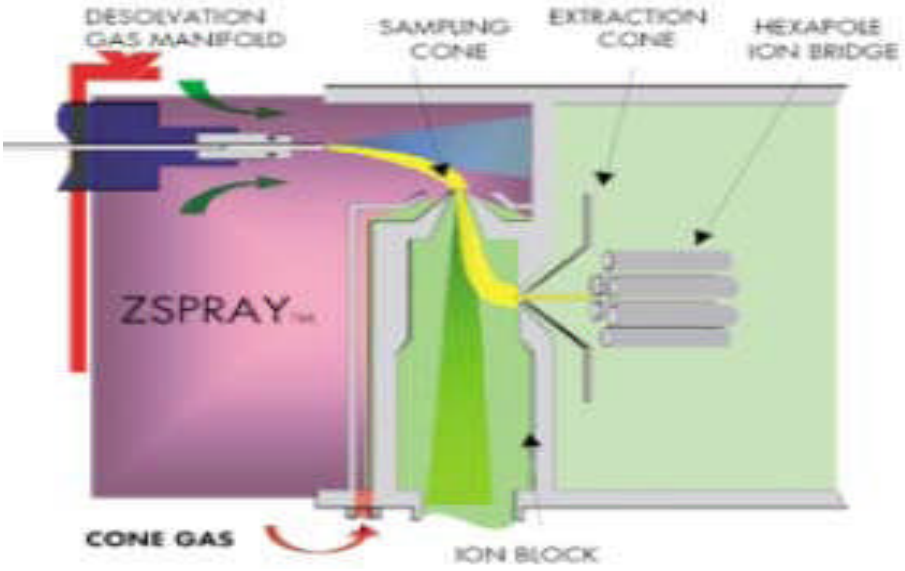


Figure 1.1 schematic diagram of the Z-spray™ ion source displaying the flattened Z-shaped path of ions entering the mass spectrometer courtesy of the Waters Corporation.

The droplets emerge from the inlet probe (ESI or APCI) in a cone-shaped spray. Low molecular weight components diffuse away from the central axis of the spray while higher molecular weight components continue along their origin trajectory, due to their higher momentum, until they reach the entrance to the mass spectrometer, the skimmer. In conventional electrospray sources, the ions produced travel along a linear trajectory from formation to entering the skimmer. Some of the neutral molecules contained in the spray strike the surface of the skimmer and are deposited there, where they accumulate and eventually block the entrance to the mass spectrometer (Herbert and Johnstone, 2003).

The Z-spray source draws the ion beam through a different trajectory that resembles a flattened Z-shape. A first skimmer orifice is positioned at right angles to the initial spray direction. As ions form in the background gas, they follow the gas stream toward the vacuum region of the mass spectrometer and pass through the skimmer orifice with some residual solvent vapor. A small voltage is applied to the skimmer cone that enhances the

extraction of ions from the initial spray. Further solvent removal occurs behind the skimmer and the ion beam is drawn through a further 90 degrees by a second skimmer, the extractor cone. Ions and background gas (typically nitrogen) are then drawn into the mass analyzer

1.1.2.2 Electrospray Ionization.

Electrospray ionization has gained unsurpassed acceptance as a liquid introduction technique for mass spectrometry. The first thorough descriptions of the technique were provided by Zeleny (Zeleny, 1914, 1915, 1917) in the early 20th century and the pioneering work using electrospray for the analysis of macro-ions using rudimentary MS was carried out by Dole et al. in the 1960's (Dole et al., 1968; Mack et al., 1970). This early work provided the inspiration for Fenn (Yamishita et al., 1984; Whitehouse et al., 1985) and Alexandrov (Alexandrov et al., 1984) who independently used the technique to generate gas phase ions for mass spectrometric detection successfully for the first time, demonstrating the potential of the technique. Bruins et al. introduced pneumatically assisted electrospray (or Ionspray) in 1987 in which a nebulising gas aids the formation of the charged aerosol (Bruins et al., 1987).

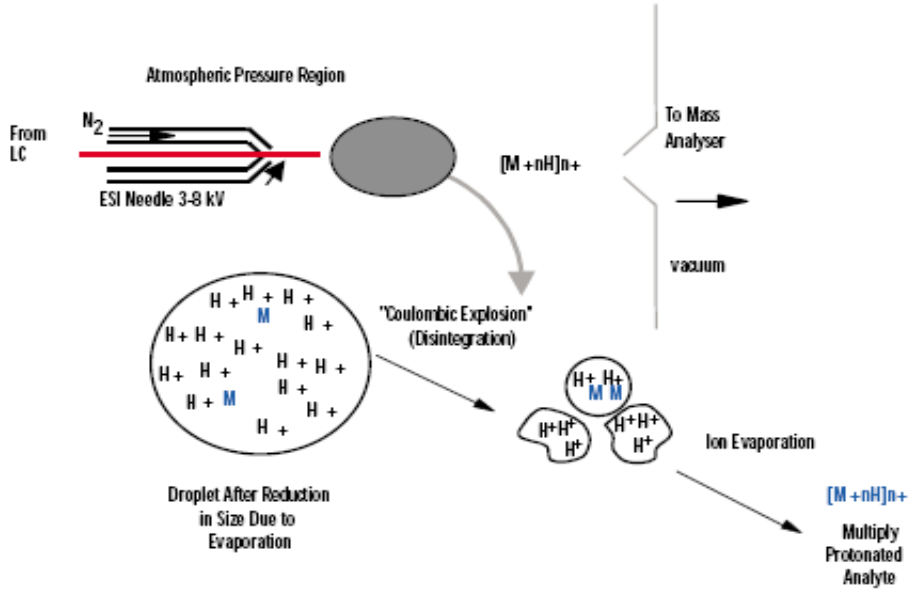


Figure 1.2 schematic diagram of the electrospray process; permission of the Waters Corporation.

The electrospray process when applied to ESI-MS can be divided into three stages: Nebulisation of a sample solution into electrically charged droplets, the formation of ions

from these droplets and transportation of ions from the source region (at atmospheric pressure) into the vacuum region of the mass spectrometer (Fenn et al., 1990; Kebarle and Tang, 1993; Bruins, 1998).

A sample solution is fed through a fine, stainless steel capillary to which a high electric potential is applied (Figure 1.2). In the case of positive ionization, the high electric field pulls positive charge toward the liquid front where electrostatic repulsion overcomes the surface tension of the liquid. At this point, small charged droplets leave the surface of the liquid and travel toward a counter electrode through the surrounding gas. The counter electrode is usually the entrance to the vacuum region of the mass spectrometer although electrospray may equally, though not commonly, be carried out with the sprayer tip at ground potential and the counter electrode at high potential.

Electrospray as described above may be considered as 'pure electrospray' and it is the electric field alone that results in droplet formation. Increases in surface tension, electrolyte concentration and flow rate will therefore impede the ability of the charge to form the desired aerosol for mass spectrometry. Several modifications to the simple electrospray system are aimed at increasing the tolerance toward these adverse effects. The addition of co-axial sheath flow of organic solvent serves to reduce the surface tension associated with samples present in highly aqueous media. This approach was originally used for the combination of capillary electrophoresis (Olivares et al., 1987) with mass spectrometry but later work has proven the value of liquid sheath flows during sample infusion and LC/MS. More commonly, the nebulisation process is adversely affected by the rate at which solvent emerges from the capillary tip. The use of a co-axial, high velocity gas is commonplace in electrospray mass spectrometry and the formation of droplets is carried out by the action of the gas stream on the sample bearing solvent. The capillary now serves only to charge the resulting droplets and such electrospray systems can manage higher flow rates of aqueous samples much more effectively than pure electrospray. Such assemblies are termed pneumatically assisted electrospray (Bruins, 1998).

When droplets emerge from the capillary tip, electrostatic repulsion has become greater than the cohesive properties that bind the liquid together. The droplet size (~1μm) is then reduced by evaporation of solvent molecules as they pass through a heated bath gas. The reduction in droplet size is accompanied with an increase in the charge density of the droplet and shear forces resulting from interaction with the relatively dense gas act upon it. The combination of these two effects deform the droplet giving rise to protrusions at which localized high

electric fields form. At points where the combination of the high electric field and deformation are sufficient, the surface tension of the droplet is overcome and the droplet becomes unstable. Smaller droplets (~0.1μm) are thus produced in turn and the process repeated until the droplets reach a size of ~ 10nm. Smaller droplets (~0.1μm) may also be produced as a jet of microdroplets. The upper limit of charge on a single droplet is termed the Rayleigh stability limit.

The precise mechanism by which ions are formed from the smallest droplets is not yet fully understood. However, two theories exist and the distinction between the details of the ion formation process has little or no effect on the use of ESI. In the smallest droplets, charge reduction at the Rayleigh stability limit does not take place by further fragmentation. Iribarne and Thomson propose that instead ions are emitted from the surface of the droplet into the surrounding gas, ion evaporation (Iribarne and Thomson, 1976 and 1979). Alternatively, Schmelzeisen-Redecker et al. (1989) propose that ions surrounded by one or more salvation shells separate from the parent droplet. Solvent molecules are then lost as this *nanodroplet* passes toward the entrance to the mass spectrometer. The mild ionization, high ion transmission, excellent sensitivity at low flow-rates, the ability to analyze large biomolecules with mass spectrometers with low upper mass ranges have led to widespread use of this technique in biological mass spectrometry (Chapman, 1996).

1.1.2.3 Atmospheric Pressure Chemical Ionization.

The first reported use of chemical ionization at atmospheric pressure coupled to mass spectrometry was carried out by Shahin (1966; 1967) in the 1960s. Horning et al. were the first to apply this as an ionization technique for LC/MS in 1973 (Horning et al., 1973). Further improvements to the technology were made by the groups of Kambara (1979; 1982), Henion (1982) and Covey (1986) during the late 1970s and early 1980s including the introduction of a heated pneumatic nebuliser.

APCI offers a soft ionization technique for the analysis of low to moderate polarity compounds having some volatility however, the high temperatures involved can restrict its use for some thermally labile compounds. The production of ions occurs via gas phase molecular reactions at atmospheric pressure. The sample inlet capillary is surrounded by a coaxial nebuliser capillary. Nebulisation takes place in a heated section in which a flow of

auxiliary gas minimizes interactions of the analytes with the tube wall as they are flash vaporized. As the resulting vapor emerges from the end of the probe, a corona discharge needle initiates a series of gas phase reactions, seeding the region with protonated reagent species, ultimately leading to sample ionization.

Ionization may take place by several routes depending on the mode of ionization; either *positive* or *negative*. In positive mode proton transfer, charge transfer or adduct formation may take place whereas in negative mode, proton abstraction, electron capture or anion attachment is commonly observed.

1.2.2 Mass Analyzer.

A variety of mass analyzers are commercially available such as: quadrupole; quadrupole ion trap; time-of-flight (ToF); Fourier transform ion cyclotron resonance (FTICR) and magnetic/electric sector instruments that offer application dependent benefits. The four parameters of greatest importance for a mass analyzer are: the upper mass limit governing the highest *m/z* ratio that can be measured; the transmission efficiency; the linear dynamic range for quantization and the resolution. The latter is the ability to yield distinct signals for ions with small differences in *m/z* ratio and is particularly applicable to time-of-flight (ToF) mass analyzers. The quadrupole mass analyzer has become the most widely used in the food control quality fields due to its overall robustness.

1.2.2.1 Quadrupole Mass Analyzers.

Quadrupole mass analyzers possess considerable advantages over other mass analyzers where resolving power is of minor importance. Their relatively small size for comparable resolving power, linear mass scale, fast scanning, simplicity of construction and ease of cleaning make them suitable for the routine, high-throughput environment. It is an entirely electrical device making it highly compatible to undertake complex automated tasks. The relatively low voltages under which these systems operate also renders them more amenable to atmospheric pressure ionization techniques as the risk of ‘arcing’ is reduced.

The quadrupole mass analyzer consists of four precisely machined, parallel rods or poles that are equidistant from one another. These have fixed DC voltages and alternating RF voltages applied to them. Depending on the field strengths applied, ions of a particular *m/z*

ratio can be selected and allowed to pass through the rod assembly whilst others are deflected away toward the poles or the vacuum system (Figure 1.3). The four poles are often cylindrical although theoretically these should be hyperbolic in cross-section; cylindrical poles offering adequate performance if correctly oriented. Two opposite poles have a potential + (U + V cos(ωt)) and the other two – (U + V cos (ωt)), where U is a fixed DC potential and V cos(ωt) represents a radio frequency (RF) field of amplitude (V) and frequency (ω/2π). Along the central (z) axis and the two planes shown in figure 1.3, the resultant electrical field is zero.

In the transverse direction in a quadrupole analyzer, an ion will oscillate between the poles in a complex fashion, depending on its mass, the voltages (U, V) and the angular frequency (ω) of the alternating RF potential. The theory behind the operation of the quadrupole is mathematically complex and will not be treated here. However, its operation is achieved by suitable choices of U, V and ω, it can be arranged that only ions of one mass will oscillate in a stable trajectory (resonant ions) about the central axis. In this case all other ions will oscillate with greater and greater amplitude until eventually they strike one of the poles and are lost; unstable trajectories. The operation of a quadrupole mass analyzer has resulted in it being regarded as more of a mass filter than an analyzer. Scanning of the quadrupole assembly is carried out by varying U and V such that U/V remains constant. This permits ions of increasing or decreasing *m/z* ratio to successively traverse the assembly.

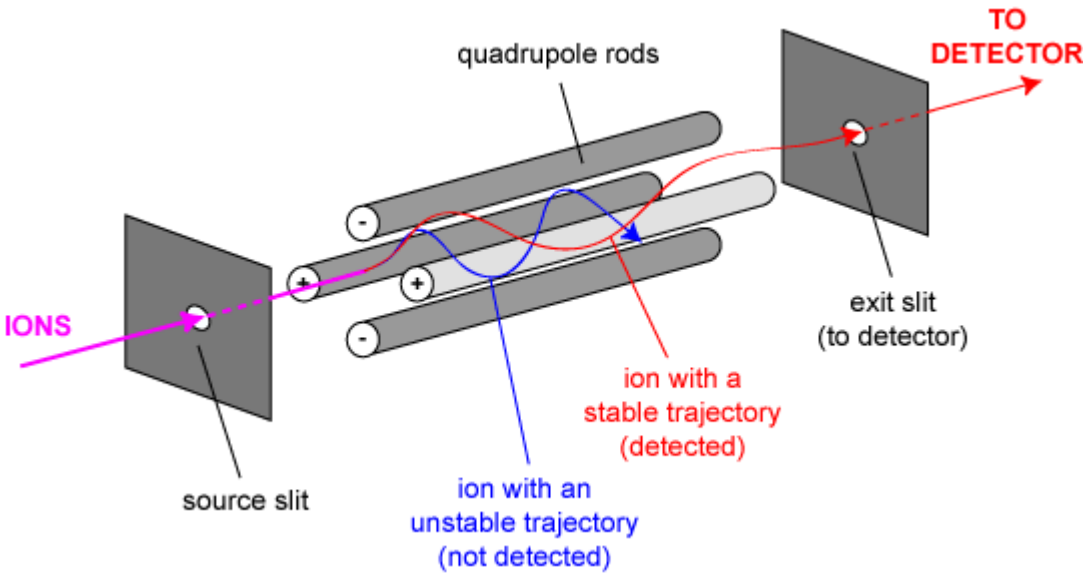


Figure 1.3 schematic diagram of a quadrupole mass analyzer showing the hypothetical trajectories of ions which are resonant and non-resonant; the resonant ion being passed through the assembly (Neissen and van der Greef, 1992).

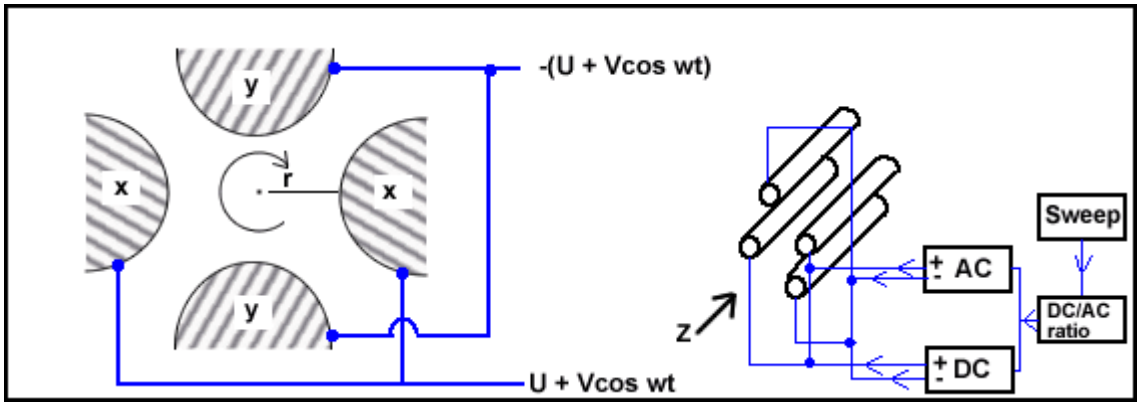


Figure 1.4 schematic diagram of a quadrupole assembly showing the planes through which the electric field strength is zero.

1.2.2.2. *Tandem Mass Spectroscopy (MS/MS) and High Resolution*

Desorption ionization techniques like FAB and MALDI and LC/MS ionization techniques like ESI and APCI facilitate the molecular weight determination of a wide range of polar and nonpolar, low- and high-molecular-weight compounds. However, the "soft" ionization character of these techniques means that most of the ion current is concentrated in molecular ions and few structurally significant fragment ions are formed. In order to enhance the amount of structural information in these mass spectra, collision-induced dissociation (CID) may be used to produce abundant fragment ions from molecular ion precursors formed and isolated during the first stage of mass spectrometry Then, a second mass spectrometry analysis may be used to characterize the resulting product ions. This process is called tandem mass spectrometry or MS/MS and is illustrated in Figure A.3A.4. Another advantage of the use of tandem mass spectrometry is the ability to isolate a particular ion such as the molecular ion of the analyte of interest during the first mass spectrometry stage. This precursor ion is essentially purified in the gas phase and is free of impurities such as solvent ions, matrix ions, or other analytes. Finally, the selected ion is fragmented using CID and analyzed using a second mass spectrometry stage. In this manner, the resulting tandem mass spectrum contains exclusively analyte ions without impurities that might interfere with the interpretation of the fragmentation patterns. In summary, CID may be used with LC/MS/MS or desorption ionization and MS/MS to obtain structural information such as amino acid sequences of peptides and sites of alkylation of nucleic acids, or to distinguish structural

isomers such as ®-carotene and lycopene. The most common types of MS/MS instruments available to researchers in food chemistry include triple quadrupole mass spectrometers and ion traps. Less common but commercially produced tandem mass spectrometers include magnetic sector instruments, Fourier transform ion cyclotron resonance (FTICR) mass spectrometers, and quadrupole time-offlight (QTOF) hybrid instruments (Table 1.2). Beginning in 2001, TOF-TOF tandem mass spectrometers became available from instrument manufacturers. These instruments have the potential to deliver high-resolution tandem mass spectra with high speed and should be compatible with the chip-based chromatography systems now under development. In addition to MS/MS with CID to obtain structural information, it is also useful to use high-resolution exact mass measurements to confirm the elemental compositions of ions. Essentially, exact mass measurements permit the unambiguous composition analysis of lowmolecular- weight compounds (mol. wt. <500) through precise and accurate m/z measurements. The types of mass spectrometers capable of exact mass measurements include magnetic sector mass spectrometers, QTOF hybrid mass spectrometers, reflectron TOF instruments, and FTICR mass spectrometers (Table 1.2). Some of these instruments permit the simultaneous use of tandem mass spectrometry and exact mass measurement of fragment ions. These include FTICR instruments, QTOF, and the TOF-TOF.

Table 1.2 *Types of Mass Spectrometers and Tandem Mass Spectrometers*

Instrument	Resolution	*m*/z Range	Tandem MS
Magnetic sector	100,000	12,000	Low resolution
Quadrupole	<4,000	4,000	None
Triple quadrupole	<4,000	4,000	Low resolution
TOF	15,000	>200,000	None
FTICR	>200,000	<10,000	High resolution
QTOF	12,000	4,000	High resolution
TOF-TOF	15,000	>10,000	High resolution

1.2.2.3 Triple Quadrupole Mass Spectrometers.

The triple quadrupole mass spectrometer, as its name suggests, is comprised of three quadrupoles; MS1, MS2 and MS3. The second quadrupole is not used as a mass separation device but as an RF-only collision cell where CID is performed. More recent developments in hardware have seen MS2 replaced by hexapole or ion-tunnel devices (Giles et al., 2004). Such devices offer much deeper potential energy wells to ions providing greater control and transmission efficiency.

1.2.2.4 *Collision-Induced Dissociation (CID).*

Upon entering the collision cell, mass analyzed ions undergo non-elastic collisions with a neutral gas typically argon (Herbert and Johnstone, 2003). The kinetic energy lost in these collisions is converted into internal energy (rotational, vibrational and electronic) within the ions of interest, the precursor ions. The amount of internal energy increases to such a point that it is great enough to break bonds within the molecule and this is termed fragmentation. The extent to which this occurs can be controlled by the nature of the gas within the cell, its pressure and the velocity with which the ion is passed through the cell. The latter is effected by varying the potential across the collision cell (figure 1.5). The function of the ion guide is to contain the parent and resulting fragment ions and pass them to the second stage of mass spectrometry. The collisions with neutral gas molecules gives rise to a scattering effect that should be minimized to maximize transmission efficiency, the multiple analyzer is an ideal device to efficiently focus these fragments and achieve high sensitivity. However, it is a scanning device which is inherently less efficient than some other mass analyzers, such as, time-of-flight and the storage device the quadrupole ion trap.

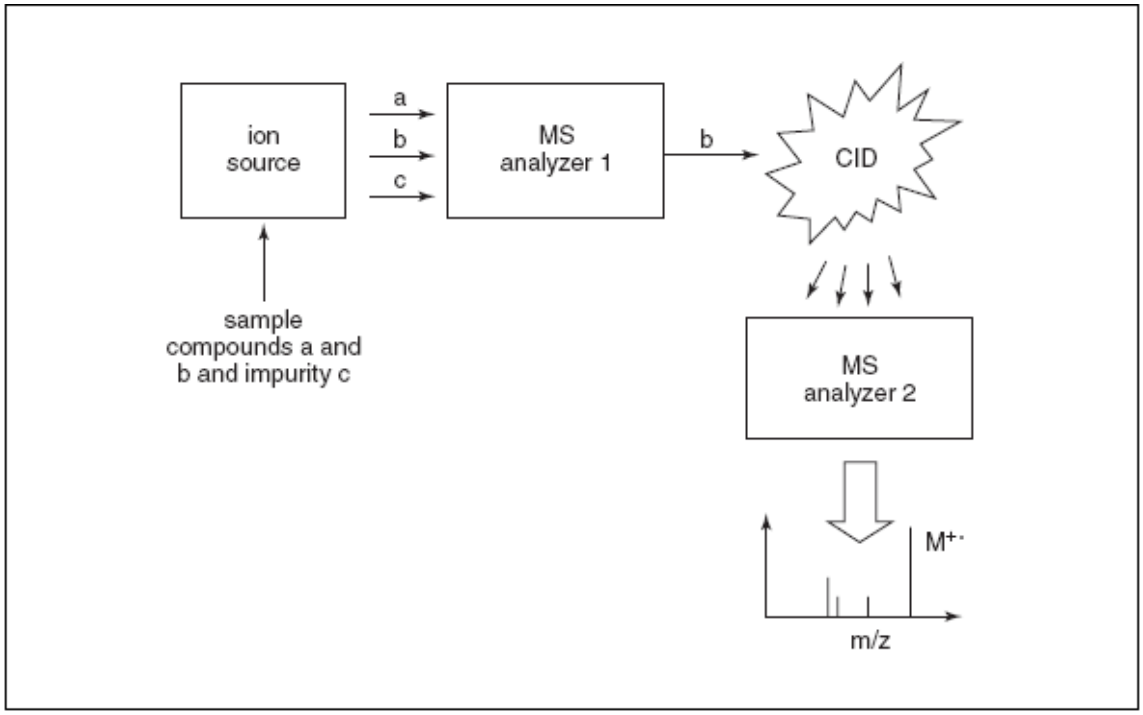


Figure 1.5. *Scheme illustrating the selectivity of MS/MS and the process by collision induced dissociation (CID) facilitates fragmentation of reselected ions*

1.2.2.5 Scanning Techniques in Tandem Mass Spectrometry

1.2.2.5.1 Product-Ion Scanning

This scanning mode is known as daughter-ion scanning. The first stage of mass spectrometry (MS1) is used to isolate an ion of interest and this ion is termed the precursor ion. This is commonly, though not always, the molecular ion of a species formed in the ion source. This ion is then subject to CID in the collision cell and the second stage of mass spectrometry is scanned to produce a spectrum of all the ions formed i.e. the product (fragment) ions. Such information can then be interrogated and the significance of the observed ions with respect to the structure of the precursor ion assessed. These experiments are also used to select a suitable product ion to utilize in a selected reaction monitoring (SRM) experiment.

1.2.2.5.2 Precursor Ion Scanning.

In this type of scan, the second stage of mass spectrometry (MS3) is set to pass only a particular product. The first stage of mass spectrometry (MS1) scans across the mass range

of interest sequentially passing ions to the collision cell to undergo CID. A signal is only passed to the detector when a precursor ion that forms the required product ion is passed to the collision cell. This type of scan is of particular benefit when a number of structurally related ions are studied and is especially widely used in neonatal screening applications (Rashed et al., 1995).

1.2.2.5.3 Constant Neutral Loss (CNL) Scanning.

The constant neutral loss scan is carried out be scanning both stages of mass spectrometry with a constant mass difference between them. In this way, a signal is only passed to the detector if a precursor ion enters the collision cell forming a product ion by loss of a neutral species that matches the mass offset of the two stages of MS. Again, this scanning mode is utilized to great extent in neonatal screening (Chace et al., 1993).

1.2.2.5.4 Selected Reaction Monitoring (SRM).

This scan mode introduces a high degree of selectivity into analyses performed by LC/MS/MS and is very sensitive. It may be regarded as product ion scanning in which only a single product ion is monitored. Each stage of MS is set to pass only ions of one *m/z*; MS1 the precursor ion and MS3 the product ion of interest. A response will only be recorded by the data system when a precursor ion of the selected *m/z* enters the collision cell forming a product ion of the selected *m/z*. This scan mode is widely used in the quantitative bioanalysis industry where trace levels of analytes are measured in complex matrices such as plasma, blood and urine.

1 1.2.3 Detection.

Tandem mass spectrometers utilize point ion detectors as opposed to array detectors such as micro-channel plates common in ToF-MS. The function of the detector is to convert the ion beam into an electrical signal to be recorded by the data system. The ion beam impinges upon a conversion dynode that produces a stream of electrons. The work in this thesis was carried out using Micro mass (Wythenshawe, Manchester, UK) tandem MS systems that employ photomultiplier detectors. First the ions are allowed to strike a conversion dynode, where ions resale secondary electrons from a metal surface. This stream of electrons is directed towards a phosphor screen and, upon contact with this, the electrons cause the emission of photons that are subsequently monitored in the sealed photomultiplier tube and

converted to an electrical signal. As this detector is sealed it essentially has an unlimited life and can withstand poor vacuum conditions.

1.3 Liquid Chromatography.

Despite the high selectivity of tandem mass spectrometry, some type of chromatography is usually necessary post sample preparation. Although the cumbersome and time-consuming baseline separation of analytes required for UV detection and other less specific techniques is not usually necessary when using tandem mass spectrometry, there are situations in which chromatography must be used to prevent interferences. This is especially true in the case of conjugated metabolites of drugs such as glucuronides (Vogheser et al., 2001a) and sulfates. Such conjugates can often degrade in the ion source to form the parent drug. If the parent drug and the metabolite are not separated chromatographically, falsely elevated signals corresponding to the parent drug will result.

Chromatography is also used to improve the yield of analyte ions from a sample by reducing the effects of ion suppression (Annesley, 2003; King et al., 2000; Dams et al., 2003). By allowing the compounds of interest to elute at a different time to the bulk of the sample matrix, competition for charge within the ion source is reduced. Co-elution of analytes once the adverse effects of ion suppression have been minimized is then preferable as several analytes may be monitored simultaneously in much shorter analysis times.

The Principles of Chromatography. Although the exact mechanisms of retention for the various types of chromatography differ, all are based upon the establishment of equilibrium between the stationary and mobile phase. Separations therefore occur due to differing distribution coefficients of individual analytes.

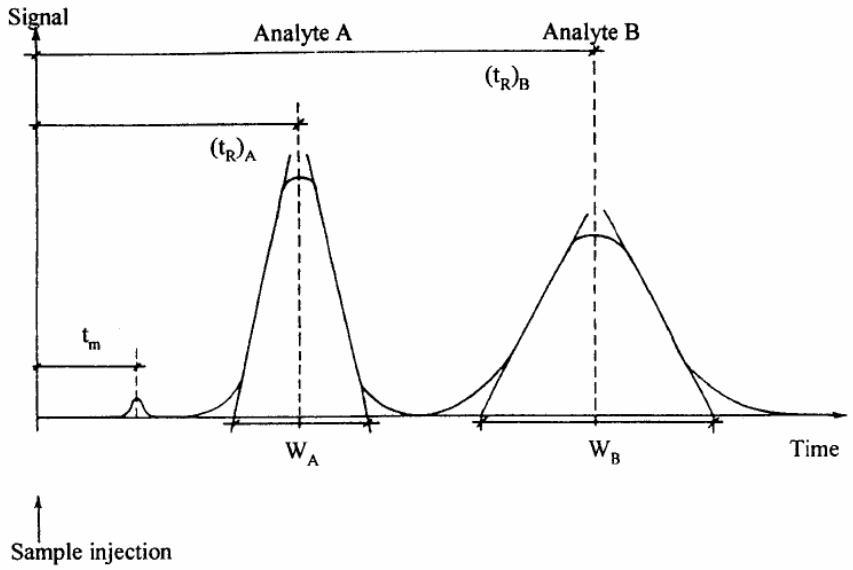
1.3.1 Retention, Resolution and Efficiency.

A number of fundamental equations have been derived to describe chromatographic parameters (Figure 1.6). Chromatographic retention is described in terms of t_R, retention time, and defines the time between injection of the analyte and the corresponding peak maximum. A preferred method for assessment of retention is the calculation of a capacity factor, k'. The capacity factor defines retention characteristics that are independent of the mobile phase velocity and column length. Resolution, R_S, is defined as the degree of separation between two analytes and may be calculated using t_R and the base width (W) of the analyte peaks . Baseline resolution i.e. complete separation of the analytes, is achieved

when $R_S \geq 1.5$. The selectivity of a column, α, measures the ability of a given column to separate two analytes and is calculated as ratio between the capacity factors for the analytes in question. Column efficiency is measured in terms of the number of theoretical plates (N) and may be calculated as shown in figure 1.6 using information from a chromatogram. Using this value, the height equivalent to a theoretical plate (HETP) may be calculated since it refers to the number of theoretical plates per unit length of the column (L). Column efficiency improves with the number of theoretical plates and decreasing plate height as each theoretical plate is deemed an equilibrium step.

1.3.2 Band Broadening.

Band broadening is a term that essentially refers to increasing chromatographic peak width thus increased HETP (or decreased efficiency). The largest contributions to this are found within the column itself however, careful reduction of dead volumes within ancillary tubing and connectors will reduce band



t_m = column dead time and corresponds to the time taken for an unretained compound to be detected.

$$\text{Capacity Factor, } k' = (t_R - t_m)/t_m$$

$$\text{Resolution, } R_S = 2\left[\frac{(t_R)_B - (t_R)_A}{W_A + W_B}\right]$$

$$\text{Selectivity, } \alpha = \frac{k'_B}{k'_A} = \left[\frac{(t_R)_B - t_m}{(t_R)_A - t_m}\right]$$

$$\text{Number of theoretical plates, N} = 16\left(\frac{t_R}{W}\right)^2$$

$$\text{Height equivalent to theoretical plate (HETP)} = \frac{L}{N}$$

Figure 1.6 Illustration of HPLC Parameters commonly used to determine capacity factors, selectivity, resolution and height equivalent to theoretical plate.

broadening. The van Deemter equation describes contributions to this effect in relation to the velocity of the mobile phase, μ, HETP = A + B/μ + (C_s + C_m)μ where A represents eddy diffusion, B represents molecular diffusion and C terms describe mass transfer effects in the mobile (C_m), and stationary (C_s) phases, respectively.

1.3.2 Reversed phase HPLC.

Reversed phase liquid chromatography accounts for ~90% of all HPLC separations performed in the drug development and clinical applications (Lee and Kerns, 1999). Hydrophobic analytes are retained more strongly than hydrophilic analytes and are eluted by changing the composition of the mobile phase, typically by increasing the percentage of organic solvent. The stationary phase typically consists of modified silica particles in which the a proportion of the surface silanol groups are reacted with alkyl- or aryl- chlorosilanes, arguably the most common of which is the octadecylsilane (ODS or C_{18}). A procedure termed *endcapping* is commonly carried out post-modification as unreacted silanol groups may remain (due to steric hinderance) that can result in severe peak tailing for basic analytes (Waters, 2003). Mobile phases typically consist of water and one or more organic solvents

such as methanol, acetonitrile, ethanol or tetrahydrofuran. Commonly, and especially in the LC/MS applications, the mobile phase may contain additives to improve the performance of the liquid chromatography and ionization process. Buffered mobile phases are used where ionization of functional groups (acidic or basic) may result in asymmetric chromatographic peak shape. Adjusting the pH of the mobile phase within two or three units of the analytes pK_a will reduce this effect in most instances. Weak organic acids such as formic or acetic are frequently used to lower pH whereas ammonium hydroxide is used to buffer solutions for basic analytes. Buffered solutions such as ammonium format or ammonium acetate also interact with unreacted silanol groups and minimize potential ion exchange interactions that may contribute to peak asymmetry; peak tailing for basic analytes.

1.3.3 Normal Phase HPLC.

Normal phase chromatography is the classical form of chromatography where polarities of the stationary and mobile phases are reversed. The functional groups of polar analytes interact with the polar groups on the surface of the stationary phase and a non-polar mobile phase. Unbonded alumina and silica were originally used however, these have been superseded by bonded stationary phases that equilibrate much faster and have a greater resistance to very small amounts of water that have a detrimental affect on this technique.
An extension of this technique is hydrophilic-interaction chromatography (HILIC) in which a polar stationary phase is used with an aqueous-organic mobile phase. Contrary to reversed phase chromatography, retention increases with the concentration of organic solvent in the mobile phase and analytes are eluted using the aqueous solvent (Grumbach et al., 2003).

1.4 Stable Isotope Dilution Assay-SIDA

In order to overcome matrix effects and related quantification problems external matrix calibration for each commodity tested was recommended so far, which is extremely time-consuming and proved to be very unpractical under routine conditions, where one is confronted with a variety of matrices every day. As an alternative approach the use of [stable] isotope labelled internal standards has been introduced recently(Sindona et al, 2009,2008,2006)). These substances are not present in real world samples but have identical properties to the analytes. Internal standards are substances which are highly similar to the analytical target substances, i.e. their molecular structure should be as close as possible to the target analyte, while the molecular weight has to be different (see

figure 1.7). Within the analytical process internal standards are added to both, the calibration solutions and analytical samples, and by comparing the peak area ratio of internal standard and analyte, the concentration of the analyte can be determined.

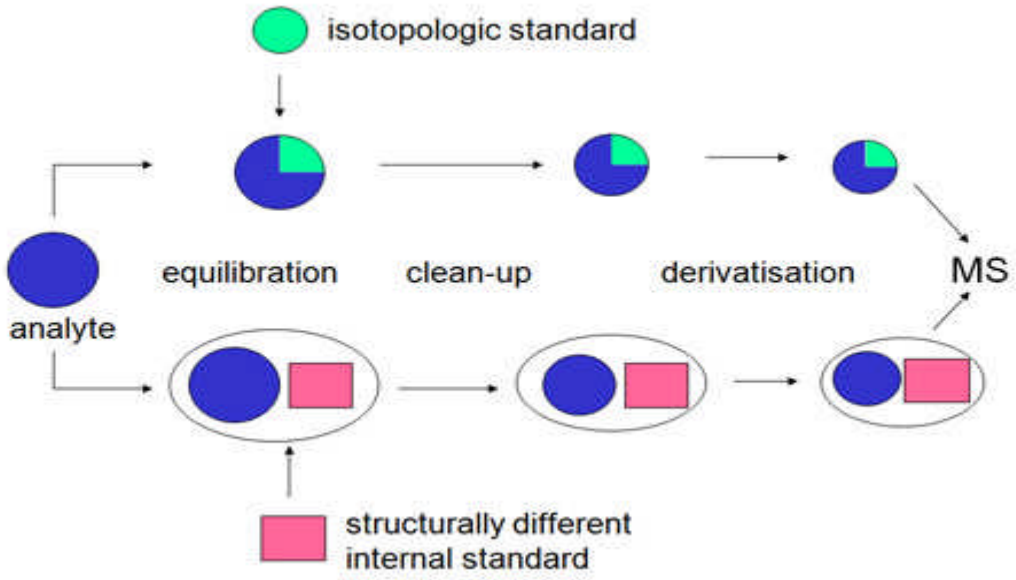


Figure 1.7

Ideal internal standards are isotope-marked molecules of a respective target analyte, which are usually prepared via organic synthesis by exchanging some of the hydrogen atoms by deuterium, or by exchanging carbon [12C] atoms by [13C]. Physico-chemical properties of such substances and especially their ionization potential is very similar to or nearly the same as of their naturally occurring target analytes, but because of their higher molecular weight (due to the incorporated isotopes) distinction between internal standard and target analyte is possible. Variations during sample preparation and clean-up as well as during ionization are compensated so that methods with especially high analytical accuracy and precision can be developed. Optimally these isotope labeled analogues must have a large enough mass difference to nullify the effect of natural abundance heavy isotopes in the analyte. This mass difference will depend generally on the molecular weight of the analyte itself, in case of molecules with a molecular weight range of 200 to 500, a minimum of three extra mass units might be required. Isotope labeled standards supplied by Biopure is fully labeled thus providing an optimum mass unit difference between labeled standard and target analyte. For example,

the [13C15]-DON standard, which is available as liquid calibrant (25mgl-1) was thoroughly characterized by Häubl et al. with regard to purity and isotope distribution and substitution, the latter being close to 99%. Fortification experiments with maize proved the excellent suitability of [13C15]-DON as internal standard indicating a correlation coefficient (R2) of 0.9977 and a recovery rate of 101% +/- 2.4%. The same analyses without considering the internal standard resulted in R2=0.9974 and a recovery rate of 76% +/- 1.9%, underlining the successful compensation for losses due to sample preparation and ion suppression effects by isotope labeled internal standards.

1.5 Conclusions

Direct coupling between a liquid phase separation technique such as liquid chromatography and mass spectrometry has been recognized as a powerful tool for analysis of highly complex mixtures. The main advantages include low detection limits, the ability to generate structural information, the requirement of minimal sample treatment and the possibility to cover a wide range of analytes differing in their polarities. Depending on the applied interface technique a wide range of organic compounds can be detected and flows up to 1.5ml/min can be handled. Despite their high sensitivity and selectivity LC/MS/MS instruments are limited to some extent due to matrix-induced differences in ionization efficiencies and signal intensities between calibrants and analytes; ion suppression/enhancement due to matrix compounds entering the mass spectrometer together with the analytes limit also ruggedness and accuracy and pose a potential source of systematic errors. Stable isotopes labeled internal standards have been proven to overcome these problems as well as to compensate also for fluctuations in sample preparation, extraction and clean-up. Numerous LC/MS/MS methods for the determination of nutraceuticals in food have been developed and published in recent years(sindona e al 2006,2007,2008,2009) however so far only a few were based on stable isotope labeled analytes,. For this reason we are interesting to development a methods being suitable for the quantitative determination of many types of analyte/matrix combinations poses a great challenge in food control quality in the future.

References

Alcock NJ, Eckers C, Games DE, Games MPL, Lant MS, McDowall MA, Rossiter M, Smith RW, Westwood SA, Wong H (1982) High-Performance Liquid Chromatography – Mass Spectrometry with Transport Interfaces *Journal of Chromatography A* **251**: 165 – 175.

Alexandrov ML, Gall LN, Krasnov MV, Nikolaev VI, Pavelenko VI, Shkurov VA (1984) (Title in Russian) *Zh. Anal. Khim.* **40**: 1570 – 1575.

Annesley T (2003) Ion suppression in Mass Spectrometry. *Clinical Chemistry* **49**: 1041 – 1044.

Bruins AP (1998) Mechanistic Aspects of Electrospray Ionisation *Journal of Chromatography A* **794**: 345 – 357.
Bruins AP, Covey TR, Henion JD (1987) An Ion Spray Interface for Combined Liquid Chromatography/Atmospheric Pressure Ionization Mass Spectrometry. *Analytical Chemistry* **59**: 2642 – 2646.

Caprioli RM, Fan T, Cotrell JS (1986) A Continuous Flow Sample Probe for Fast Atom Bombardment Mass Spectrometry. *Analytical Chemistry* **58**: 2949 – 2954. Chapter 1

Chace DH, Millington DS, Terada N, Kahler SG, Roe CR, Hofman LF (1993) Rapid Diagnosis of Phenylketonuria by Quantitative Analysis for Phenylalanine and Tyrosine in Neonatal Blood Spots by Tandem Mass Spectrometry. *Clinical Chemistry* **39**: 66 – 71.

Chapman JR, Errock GA, Race JA (1997) Science and Technology in Manchester: The Nurture of Mass Spectrometry. *Rapid Communications in Mass Spectrometry* **11**: 1575 – 1586.

Chapman JR (1996) Chapter 2: Mass Spectrometry: Ionization Methods and Instrument ation in *Protein and Peptide Analysis by Mass Spectrometry*, Humana Press, Totowa, NJ, USA.

Covey TR, Lee ED, Bruins AP, Henion JD (1986) Liquid Chromatography / Mass Spectrometry. *Analytical Chemistry* **58**: 1451A – 1459A

Dams R, Huestis MA, Lambert WE, Murphy CM (2003) Matrix effects in Bio-Analysis of Illicit Drugs with LC-MS/MS: Infleunce of Ionisation Type, Sample Preparation and Biofluid *Journal of the American Society for Mass Spectrometry* **14**: 1290 - 1294

Dole M, Mack LL, Hines RL, Mobley RC, Ferguson LC, and Alice MB (1968) Molecular Beams of Macroions. *Journal of Chemical Physics* **49**: 2240 - 2249
Fenn JB, Mann M, Meng CK, Wong SF (1990) Electrospray Ionization – Principles and Practice. *Mass Spectrometry Reviews* **9**: 37 – 70.

Frahnert C, Rao ML, Grasmader K (2003) Analysis of Eighteen Antidepressant, Four Atypical Antipsychotics and Active Metabolites in Serum by Liquid Chromatography: A Simple Tool for Therapeutic Drug Monitoring. *Journal of Chromatography B* **794**: 35 – 47.

Ghoshal AK, Soldin SJ (2003) Improved Method for Concurrent Quantification of Anti-Retrovirals by Liquid Chromatography – Mass Spectrometry. *Therapeutic Drug Monitoring* **25**: 541 – 543.

Giles K, Pringle SD, Worthington KR, Little D, Wildgoose JL, Bateman RH (2004) Applications of a Travelling Wave-Based Radio Frequency-Only Stacked Ring Ion Guide *Rapid Communicatiosn in Mass Spectrometry* **18**: 2401 – 2414.

Grayson MA (2002) Measuring Mass: From Positive Rays to Proteins, published by the *Chemical Heritage Association*, Philadelphia, USA.

Griffiths IW (1997) J. J.Thomson – The Centenary of His Discovery of the Electron and his Invention of Mass Spectrometry. *Rapid Communications in Mass Spectrometry* **11**: 2 – 16.

Grumbach ES, Diehl DM, Alden B, Iraneta P, Neue U, Mazzeo J (2003) Hydrophilic Interaction Chromatography (HILIC) for Small Molecules: Retaining Very Polar Analytes. *Poster presented at HPLC, Nice, France 15th -19th June.*

Henion JD, Thompson BA, Dawson PH (1982) Determination of Sulfa Drugs in Biological Fluids by Liquid Chromatography/Mass Spectrometry/Mass Spectrometry. *Analytical Chemistry* **54**: 451 – 455.

Herbert CG and Johnstone RAW (2003) Mass Spectrometry Basics, published by *CRC Press*, Boca Raton, Florida, USA.

Horning EC, Horning MG, Carroll DI., Dzidic I, Stillwell RN (1973) New Picogram Detection System Based on a Mass Spectrometer with an External Ionization Source at Atmospheric Pressure. *Analytical Chemistry* **45:** 936 – 943
Iribarne JV, Thompson BA (1976) On the Evaporation of Small Ions from Charged Droplets *Journal of Chemical Physics* **64**: 2287 – 2294.

Kambara H (1982) Sample Introduction System for Atmospheric Pressure Ionization Mass Spectrometry of Non-Volatile Compounds. *Analytical Chemistry* **54**: 143 – 146.

Kambara H, Mitsui Y, Kanomata I (1979) Identification of Clusters Produced in an Atmospheric Pressure Ionization Process by a Collisional Dissociation Method. *Analytical Chemistry* **51**: 1447 - 1452

Kebarle P, Tang L (1993) From Ions in Solution to Ions in the Gas Phase. *Analytical Chemistry* **65**: 972A – 986A.

King R, Bonfiglio R, Fernandez-Metzler C, Miller-Stein C, Olah T (2000) Mechanistic Investigation of Ionization Suppression in Electrospray Ionisation. *Journal of the American Society for Mass Spectrometry* **11**: 942 – 950.

Lee MS, Kerns EH (1999) LC/MS Applications in Drug Development. *Mass Spectrometry Reviews* **18**: 187 – 279.

Mack LL, Kralie P, Rehude A, Dole M (1970) Molecular Beams of Macroions II. *Journal of Chemical Physics* **52**: 4977 – 4986.

Moyer SC, Cotter RJ (2002) Atmospheric Pressure MALDI. *Analytical Chemistry* **74**: 469A – 476A.

Neissen WMA, van der Greef (1992) Liquid Chromatography – Mass Spectrometry *published by Marcel and Dekker, New York, USA.*

Olivares JA, Nguyen NT, Yonken CR, Smith RD (1987) On-line Mass Spectrometric Detection for Capillary Zone Electrophoresis *Analytical Chemistry* **59**: 1230 – 1232.

Rashed MS, Ozand PT, Bucknall MP, Little D (1995) Diagnosis of Inborn Errors of Metabolism from Blood Spots by Acylcarnitines and Amino Acids Profiling using Automated Electrospray Tandem Mass Spectrometry. *Peadiatric Research* **38**: 324 – 331.

Schmelzeisen-Redecker G, Butfering L, Rollgen FW (1989) Desolvation of Ions and Molecules in Thermospray Mass Spectrometry. *International Journal of Mass Spectrometry and Ion Processes* **90**: 139 – 150.

Shahin MM (1966) Mass Spectrometric Studies of Corona Discharges in Air at Atmospheic Pressure. *Journal of Chemical Physics* **45**: 2600 – 2605.

Shahin MM (1967) The Use of Corona Discharges for the Study of Ion-Molecule Reactions. *Journal of Chemical Physics* **47**: 4392 – 4398.

Thomson BA, Iribarne JV (1979) Field Induced Ion Evaporation from Liquid Surfaces at Atmospheric Pressure *Journal of Chemical Physics* **71**: 4451 – 4463.

Vogheser M, Zachoval R, Spohrer U, Jacob K (2001a) Potential Lack of Specificity Using Electrospray – Tandem Mass Spectrometry for the Analysis of Mycophenolic Acid in Serum. *Therapeutic Drug Monitoring* **23**: 722 – 724.

Waters Chromatography Columns and Supplies Catalogue (2003-2004), *Waters Corporation, Milford, MA, USA.*

Whitehouse CM, Dryer RN, Yamishita M, Fenn JB (1985) An Electrospray Interface for Liquid Chromatographs and Mass Spectrometers. *Analytical Chemistry* **57**: 675 – 679.

Willoughby RC, Browner RF (1984) Monodisperse Aerosol Generation Interface for Combining Liquid Chromatography with Mass Spectroscopy. *Analytical Chemistry* **56**: 2626 – 2631.

Winkler PC, Perkins DD, Williams WK, Browner RF (1988) Performance of an Improved Monodisperse Aerosol Generation Interface for Liquid Chromatography/Mass Spectrometry. *Analytical Chemistry* **60**: 489 – 493.

Yamishita M and Fenn JB (1984) Electrospray Ion Source: Another Variation of the Free-Jet Theme. *Journal of Physical Chemistry* **88**: 4451 - 4459.

Zeleny J (1914).The Electrical Discharge from Liquid Points, and a Hydrostatic Method of Measuring the Electric Intensity at Their Surfaces. *Physical Reviews* **3**: 69 – 72.

Zeleny J (1915) On the Conditions of Instability of Electrified Drops, with Applications to the Electrical Discharge from Liquid Points. *Proceedings of the Cambridge Philosophical Society*. **18**: 71 – 84.

Zeleny J (1917) Instability of Electrified Liquid Surfaces. *Physical Reviews* **10**: 1 – 6.

CHAPTER 2

Assay of Resveratrol by Tandem Mass Spectrometry and Isotope Dilution Method

2.1 Literature Review

2.1.2 Resveratrol. Resveratrol (3,5,4' -trihydroxystilbene) is a polyphenolic compound classified as a stilbene. It is synthesized from p-coumaroyl CoA and malonyl CoA by an enzyme called stilbene synthase. Its biosynthesis is stimulated by stress, including injury, infection or UV irradiation. It has been demonstrated to provide resistance in grapes to fungal diseases (Jeandet et al., 1995). It is synthesized almost exclusively in the skins of *V. vinifera* grapes but is also synthesized in the seeds of muscadine grapes (Jeandet et al., 1995; Ector et al., 1996). It exists in both trans and cis isomeric forms. The cis form is rarely found in Figure 1.1 Chemical structures. grapes in significant concentration, but it is found in substantial amounts in wines (Jeandet et al., 1995; Mattivi et al., 1995; Lamuela-Raventos et al., 1995). The reaction producing resveratrol in the plant is very similar to another reaction using the same products catalyzed by a similar enzyme, chalcone synthase (Goodwin et al., 2000). Chalcone synthase combines p-coumaroyl CoA and malonyl CoA to form chalcones including naringenin and eriodictyol which go on to form flavonoids which are responsible for the anthocyanins. Anthocyanins are a class of compounds that include the pigments in grapes (Croteau et al., 2000). Developmental studies have demonstrated that resveratrol concentration decreases with veraison, (pigment formation) in the grape (Jeandet et al., 1991; Strigler et al., 2005). It is suggested that the formation of chalcones to produce anthocyanins may come at the expense of resveratrol production by stilbene synthase. Resveratrol also exists in a glucoside form called piceid (5,4' dihydroxy-3- glucopyranosylstilbene). The two compounds are from a class of secondary metabolites called stilbenes. Studies have shown that piceid can exist in large amounts, sometimes exceeding resveratrol, in both wine and grapes (Mattivi et al., 1995; Romero-Perez et al, 1996a, 1996b; Romero-Perez et al., 2001). During fermentation there can be complex changes in the concentrations of the four monomers of resveratrol and piceid (Mattivi et al., 1995; Lamuela-Raventos et al., 1997; Romero-Perez et al., 1999). Many early studies only quantified trans resveratrol and sometimes the cis isomer. With the quantification of all four monomers, a more accurate representation of the beneficial properties of a wine or juice can be obtained.

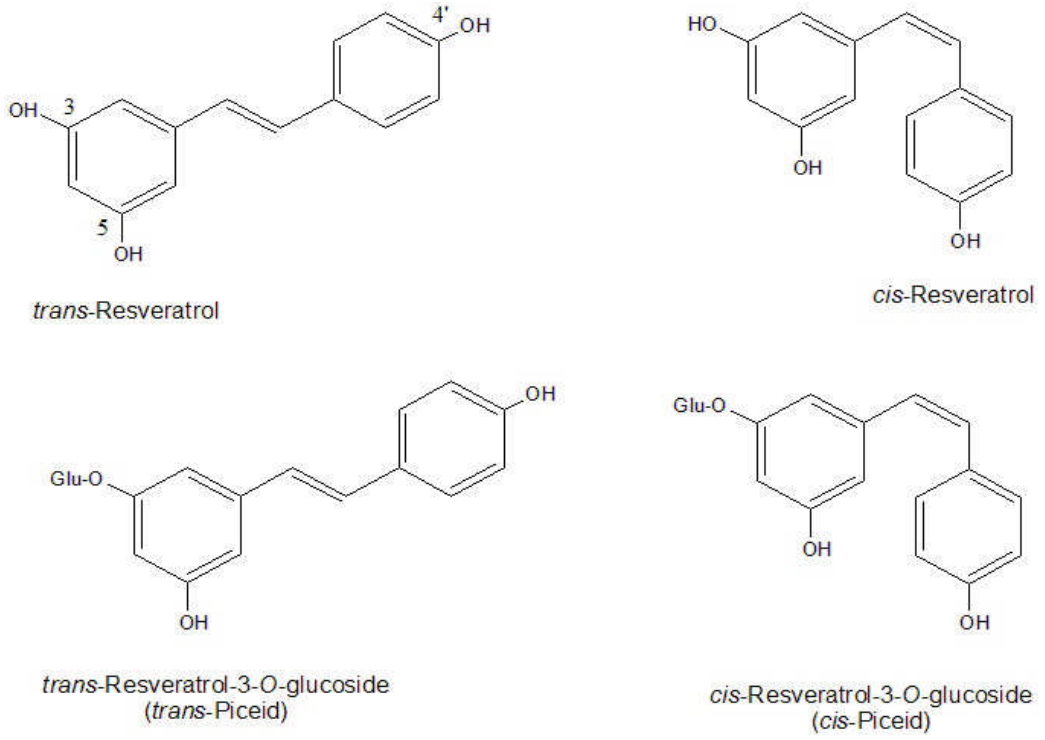


Figure 2.1 Chemical structures.

2.1.3 Health Benefits. During the 1980's, researchers looking into World Health Organization data from the United States and Europe noted an anomaly with regard to diet and mortality from heart disease (NRC, 1989). Data from the United States showed that generally with increased consumption of fatty foods there was a similar increase in the rate of coronary heart disease (CHD). Data from areas in France did not follow this pattern. In some areas where diets were traditionally high in fat, there were not similar elevations in CHD. This phenomenon has become known as “the French Paradox”. In 1992, Renaud and Lorgeril demonstrated that wine consumption was statistically the only factor correlated to the reduction in CHD. Subsequent data suggested that somehow wine consumption resulted in a larger reduction in CHD than did the consumption of beer and spirits. It was proposed that, although alcohol was a factor, there were other components in wine that were providing the protection. Prior to these investigations, a number of studies established that a phytoalexin called resveratrol (3,5,4' trihydroxystilbene) was present in grape skins (Langcake and Pryce, 1976; Pool et al., 1981; Jeandet et al., 1991). Initially, these studies focused on resveratrol’s presence as a marker for disease resistance. Some demonstrated that resveratrol metabolism can be stimulated by plant pathogen infection and by exposure to ultraviolet light (Langcake

and Price, 1976). Similarly, a group of scientists investigating a traditional Japanese folk remedy demonstrated that resveratrol was the primary active ingredient in a medicine composed of the dried powdered root of the Japanese knotweed (*Polygonum cuspidatum* Sieb. et Zucc.) (Arichi et al., 1982). For more than two decades, scientists have been reporting the various ways that resveratrol can positively effect our health (Arichi et al., 1982; Kimura et al., 1985; Kinsella et al., 1993; Jang et al., 1997; Lu and Serrero, 1999; De Santi, et al., 2000a, 2000b; Brakenhielm et al., 2001; El-Mowafy, 2002). By the mid to late nineties, work was underway around the world to both quantify resveratrol in wine and grapes and to verify its mode of action as a protective agent for human health. In 1985, Kimura et al. reported that resveratrol inhibited platelet aggregation in rats Jang et al., reported in 1997 that resveratrol acted as an antioxidant and as an anti-mutagen. Resveratrol reduced tumour formation in rats and reduced initiation and promotion of human cancer cells. In a 1999 publication, Cheong et al. reported that resveratrol had anti-allergenic properties. Resveratrol inhibited the release of hexosaminidase from mast cells. hexosaminidase is released along with histamine in response to allergic reactions. Resveratrol has also been reported to inhibit the growth of human breast cancer cells by acting as a estrogens receptor antagonist (Lu and Serrero, 1999). Tedesco et al. (2000) studied the effect of red wine extract and resveratrol singularly on red blood cells. They reported that the red wine extract acted as a strong antioxidant and that resveratrol by itself did not have as strong an effect. They suggested that the effects of red wine may be associated with the combined effect of the components of the wine and not with the individual compounds. Huang et al. (1999), proposed that resveratrol reduced tumor growth by inducing apoptosis (programmed cell death). Although there is a substantial amount of information about the effect of resveratrol *in vitro*, it is unclear how and how much of the compound is absorbed in the digestive tract. Kuhnle et al. (2000) studied the absorption of resveratrol in rat intestines. They reported that only small amounts were absorbed, but larger amounts of a resveratrol glucuronide was absorbed through the intestine. The authors suggested that resveratrol was converted to the glucuronide during absorption and postulated that the resulting molecule could be cleaved back into resveratrol in various organs of the body. The presence of flavanoids in products containing resveratrol may improve its bio-availability. Two studies in 2000 suggested that flavanoids inhibit the sulphation and

glucuronidation of resveratrol in the liver and therefore improve the bio-availability of the compound (De Santi et al., 2000a, 2000b). Kimura and Okuda reported in a 2001 study that resveratrol inhibited tumor growth in mice and inhibited angiogenesis in human umbilical cells which suggests a mechanism for the reduction in tumor growth. Another 2001 study demonstrated that resveratrol suppressed angiogenesis and tumor growth, but also reduced wound healing in bovine and mouse cells. Resveratrol has also been shown to inhibit human squalene monooxygenase, an enzyme that is part of the cholesterol biosynthetic pathway (Laden and Porter, 2001). A 2002 (El-Mowafy, 2002) study reports that resveratrol has vascular relaxation properties. The authors suggest that resveratrol could have significant effects on vascular disorders such as atherosclerosis, chronic hyperlipidemia and diabetes.

2.1.4 Early Studies. In 1976 Langcake and Pryce published a paper demonstrating that resveratrol was produced by "*Vitis vinifera* ... as a response to infection or injury". This work was focused exclusively on its effect on disease resistance. For the next 15 years, resveratrol was studied extensively in grapes (Langcake and Pryce, 1977; Creasy and Coffee, 1988; Derecks and Creasy, 1989; Jeandet et al., 1991). In 1992, Siemann and Creasy published a paper demonstrating that resveratrol (sum of trans and cis) was present in finished wine. They sampled 22 wines and found resveratrol (HPLC with UV detector) concentrations ranging from below detection to 0.7 mg/L. In general, red wines had higher resveratrol levels than white wines although this was not always the case. The authors proposed that since resveratrol is produced almost exclusively in the skins, wines with longer skin contact during vinification (red wines) should have higher levels of resveratrol. Their data also suggested that growing region had an effect on resveratrol concentration. Chardonnays from New York had higher resveratrol concentrations than Chardonnays from California. The authors proposed that since resveratrol production is stimulated by fungal attack, regions with greater fungal pressure would produce grapes with higher resveratrol concentration. In 1993, Jeandet et al. quantified resveratrol in Burgundy wines using gas chromatography and a mass spectrophotometer. Their results confirmed the findings of Siemann and Creasy. Resveratrol concentrations were higher in red wines than in white and they also found differences based on the growing conditions. Unlike Siemann and Creasy, they were able to quantify the trans and cis

isomers of resveratrol, separately. They were surprised to find, since it had not been found in grapes, that the cis isomer was the predominant form in the wines. The authors suggested that exposure to sunlight during processing or reactions occurring during vinification converted the trans isomer to the cis. This study found slightly greater amounts of resveratrol than did Siemann and Creasy. They found levels from not detected to 0.06 mg/L for Chardonnay to 0.4 to 2.0 mg/L for Pinot Noir. The previous two studies demonstrated conclusively that cis and trans resveratrol was present in finished wines. Although studies had concluded by then that resveratrol had biological activity (Frankel et al., 1993; Shan et al., 1990; Kimura et al., 1985; Kimura et al., 1983), there were still those who argued that the small amounts of resveratrol in wines were unlikely to have a meaningful effect on human health. A 1994 study by Waterhouse and Lamuela-Raventos demonstrated that grape berries contained not only resveratrol but also contained a 3-beta-glucoside of resveratrol (piceid). This compound could be converted to resveratrol during vinification and could also provide for a greater biological effect from wine if it survives processing and is present in the finished wine. Two studies (Lamuela-Raventos et al., 1995a, 1995b; Romero-Perez et al., 1996a, 1996b) subsequently demonstrated that piceid was present in wine. The 1995 article reports that resveratrol and piceid (stilbenes) were present in wines in proportions that agree with previous studies (Lamuela-Raventos and Waterhouse, 1993; McMurtey et al., 1997; Soleas et al., 1995). They report that Pinot Noir wines generally have the highest levels of stilbenes (9.39 mg/L) followed by Merlot (9.19 mg/L), Grenache (6.37 mg/L), Cabernet Sauvignon (3.23 mg/L) and Tempranillo (3.43mg/L). Romero-Perez et al., in a 1996 study of resveratrol and piceid isomers in Spanish white wines, reports that isomers of both compounds are present in all samples and the levels range from 0.46 to 1.24 mg/L total stilbenes. This is consistent with previous reports of lower levels in white wines. When considering trans and cis resveratrol and trans and cis piceid, the doses one would receive from a typical serving of wine is significantly greater than when only trans resveratrol was considered alone. Since glycosidase is known to be present in the digestive tract, it is possible that piceid could be converted to resveratrol and absorbed during digestion (Hackett, 1986). Several studies have demonstrated that piceid is itself biologically active in animal systems (Shan et al., 1990; Kimura et al., 1995).

2.1.5 Effect of UV Light on Resveratrol. A number of studies have demonstrated that UV light can induce the production of resveratrol in grapevine tissues (Langcake and Pryce, 1977; Jendet et al., 1997). In more recent years there have been several additional studies conducted in this area. In 1999, Douillet-Breuil et al. studied changes in the phytoalexin concentration of grape leaf tissue after exposure to UV light. The authors studied four *Vitis* species: three American species (*Vitis rupestris, Vitis cineria* and *Vitis labrusca)* and three cultivars of *Vitis vinifera.* All three American species showed a higher capacity for resveratrol synthesis than *V. vinifera*. Although, *V. rupestris* and *V. cineria* had higher resveratrol synthesis capacity than *V. labrusca.* All American species took longer to reach peak resveratrol concentration (30 to 45 15 hours) than *V. vinifera* (18 to 25 hours). The American species were considered to be more disease resistant than *V. vinifera*. The authors proposed that the results they obtained confirmed the role of resveratrol in defense of the plant against fungal attack. Adrian et al., in 2000, studied the concentration of various stilbenes in grape berries in response to UV light and level of infection of *Botrytis cinerea.* Three cultivars of *V. vinifera* ('Gamay', 'Pinot Noir' and 'Chardonnay') were studied. Five compounds were quantified trans piceid, cis piceid, trans resveratrol, ,-viniferin (resveratrol dimer) and pterostilbene (3,5methylated resveratrol). For 'Gamay' and 'Chardonnay', all compounds were detected in berries that were not UV elicited except for the highly infected berries. For the highly infected berries little or none of the compounds was quantified. This was probably due to the ability of the fungal organism to enzymatically degrade the compounds produced by the plant to defend itself. For the non-UV induced 'Pinot Noir', the only compounds detected were trans resveratrol and ,-viniferin and only in infected berries and those surrounding the infected berries. For UV elicited berries, neither trans nor cis piceid was detected in the 'Pinot Noir' cultivar and only very small amounts of pterostilbene was detected. Pterostilbene was only detected in very small amounts for all berries. Trans piceid, cis piceid, trans resveratrol and , viniferin were quantified in UV elicited berries of both 'Chardonnay' and 'Gamay' cultivars. Generally incubation of 48 hours after UV elicitation produced greater concentrations of the compounds than an incubation of only 24 hours. Like the non-induced berries, concentrations of all compounds were lower in the highly infected berries than for lesser infected or noninfected berries. Overall, UV elicitation increased the concentration of all compounds in the berries. The data reported

in this study demonstrate that both moderate fungal attack and UV 16 light can be strong elicitors of production of piceid, resveratrol and ,-viniferin and also that fungal organisms can eventually overwhelm the protective properties of these compounds A study published in 2000, by Cantos et al., studied the effect of cold storage and postharvest UV irradiation on 'Napoleon' table grapes. Both piceid and resveratrol were quantified using HPLC and a diode array detector. Cold storage (15 days) alone resulted in approximately 75% increase in piceid and a 300% increase in resveratrol. Cold storage in combination with UV irradiation increased piceid slightly more than cold storage alone, but resulted in a 900% increase for resveratrol. The authors suggested that one 200 g serving of 'Napoleon' table grapes after cold storage could provide the same dose (approximately 1 mg) of stilbenes (resveratrol+piceid) as a serving of red wine (200ml). The same grapes after UV irradiation could provide 2 to 3 times the dose of the cold storage grapes alone.

2.1.6 Factors Affecting Resveratrol. There has been a number of studies conducted on the effects of various factors on the concentration of resveratrol in wine. Several of these were centered on the type and time of fermentation. Jeandet et al., in 1995, studied the effect of maceration (exposure to skins during fermentation) on the resveratrol concentration of wines. Resveratrol concentration of wine increased with exposure to the skins. There was an approximate ten fold increase in resveratrol with skin exposure compared to the same wine made without exposure. Although the white wine prepared with maceration had much higher levels of resveratrol than without, these wines still had less than half the resveratrol of red wines with maceration. These data indicate for the cultivars used in this study [('Pinot Noir' (red) and 'Chardonnay'(white)] that there is still a significant effect of skin color on resveratrol concentration. These differences may be associated with these cultivars and not with the overall color of the grapes. This study also investigated the effect of botrytis infection on resveratrol concentration of wines. Previous studies demonstrated that fungal infection stimulates production of resveratrol in grapes (Langcake and Pryce, 1976), therefore, it would be expected that with highly infected fruit there would be higher resveratrol levels in the wine. The results of this study demonstrated that resveratrol levels decrease with high levels of botrytis infection. It is suggested by the authors that a fungal exo-cellular enzyme may aid in degrading

resveratrol after infection. Such an enzyme would lower resveratrol concentration in the grapes and would also be active in the wine must, lowering resveratrol in the finished wine. Although high resveratrol levels were found in wine with no infection, the highest levels of resveratrol were in wines made with grapes with approximately 10% infection. These grapes would have benefitted from a limited fungal attack that would stimulate resveratrol metabolism, but would lack the volume of fungal enzymes needed to degrade the compound. A 2000 study by Darias-Martin et al. also demonstrated a significant increase in resveratrol with fungal exposure to grape skins. Another 1995 study investigated the evolution of both isomers of resveratrol and piceid during fermentation (Mattivi et al.). In this study, levels of trans and cis-resveratrol and piceid were monitored during fermentation. In the initial must (crushed fruit), cis-piceid was the predominant monomer followed by trans piceid and trans-resveratrol. Cis-resveratrol was not present in the initial must. During the first four days of fermentation, cis-piceid declines while all other monomers increase. Trans resveratrol increases almost ten fold in the first four days while cis-resveratrol increases from not detected to 3.4 mg/L. Initially, most of the increase of the monomers is attributed to extraction from the skins. But after day four, both trans and cis-piceid decrease while trans and cis-resveratrol increase. The authors suggest that there is either an acid catalyzed or enzymatic hydrolysis of the glucosides to either of the two isomers of resveratrol. The final wine contained predominantly trans-resveratrol followed closely by cis-resveratrol and with small amounts of cis-piceid and trans-piceid. Although hydrolysis is proposed as the primary source of the trans and cis-resveratrol, isomerization is also probable since the isomers of resveratrol are less stable than the isomers of piceid. The authors suggest that the final concentration of trans-resveratrol is more related to extraction from the grape than from hydrolysis of the glucosides, whereas the concentration of cis-resveratrol in the final wine is likely exclusively produced by hydrolysis of the two glucosides. A 1997 study by Lamuela-Raventos et al. investigated the evolution of the four monomers of resveratrol during fermentation of ‘Merlot’ and ‘Cabernet Sauvignon’ grapes. The ‘Cabernet Sauvignon’ final wine had a similar distribution of the monomers as the Mattivi study, but there was an overall increase in all monomers during the fermentation. All four of the monomers increased during the entire fermentation for ‘Merlot’ as well, but the final ‘Merlot’ wine had a different distribution of the monomers. For ‘Merlot’,

trans-piceid was the predominant monomer followed by trans-resveratrol, cis-piceid and cis-resveratrol. For the 'Merlot' fermentation, there appeared to be less hydrolysis of the glucosides than in the 'Cabernet' fermentations. For 'Merlot' there were only slight decreases in the glucosides at the end of the fermentation, but for 'Cabernet' trans piceid decreased dramatically during the second half of the fermentation. This corresponded with an increase in both trans and cis-resveratrol. A 2000 study (Baveresco et al.) investigated the effect of cluster stems on resveratrol concentration of simulated wines. Resveratrol was extracted from cluster stems with methanol and in a hydro-alcoholic solution designed to mimic wine. The study only looked for trans and cis-resveratrol. Cis resveratrol was not detected. Three amounts of stems and four times of extraction were evaluated. The highest amount of stems (0.9 g/100ml) yielded the greatest amount of resveratrol for both the methanol (0.2 mg/L) and the hydro-alcoholic solution (1.4 mg/L). For the times of exposure (2,3,4 and 8 days), the greatest extraction was for the 4 day period for both the methanol extraction (0.2 mg/L) and for the hydro-alcoholic solution (1.2 mg/L) . There was a reduction in resveratrol for both extractions from 4 to 8 days. The authors suggested that oxidative degradation or transformation to an unknown compound may have been responsible for the decrease. The authors suggest that the addition of stem components to the must might be used as a method of increasing resveratrol concentration of 13 wine although they recognized that other undesirable compounds could be extracted from the stems during fermentation. Jeandet et al. (1991) studied the UV light induced production of resveratrol in grape berries of different developmental stages. The study suggested that the ability of the grape to produce resveratrol after UV elicitation declined with maturity. There was a gradual decline in resveratrol production from initiation to veraison with a rapid decline from veraison to maturity. These data suggest that the metabolic pathways responsible for resveratrol production diminish with maturity of the fruit. The study also investigated the possibility that the diminishing ability to produce resveratrol may be a result of the rise in UV absorbing anthocyanins that develop in the grape skin after veraison. Production of resveratrol was stimulated by UV light and by sucrose solution for both immature and mature grapes. Resveratrol production was reduced dramatically from immature to mature fruit for both elicitation factors. This suggests that the reduction with maturation in the ability to produce resveratrol is not a result of the production of

anthocyanins in the maturing fruit. In 2002, Magee et al. studied the effect of disease control spray program on resveratrol in muscadine berries. Resveratrol levels were determined for berry skins, juice/pulp and seeds separately from both fungicide treated and untreated vines. Resveratrol levels in the untreated vines were higher than the treated vines for all cultivars tested, although only three out of the five was the decrease statistically significant. For the two that were not significant, the overall levels of resveratrol were relatively low. There were no significant effect on the resveratrol values for the juice/pulp or for the seeds. The authors suggested that the fungicide treatment 14 resulted in less fungal pressure on the berries and therefore lower levels of elicitation of the metabolic pathway producing resveratrol. Gonzalez-Candela et al. in 2000 studied the effect of transgenic wine yeasts encoding a glycosyl-hydrolase enzyme on the concentration of the monomers of resveratrol and piceid. Wines made with the transgenic yeasts had trans-resveratrol levels four times that of the un transformed yeast and cis-resveratrol levels ten times that of the untransformed yeasts. Trans piceid was reduced by half for the transformed yeast, but cis-piceid was unaffected. The authors suggested that the enzyme encoded in the transformed yeast hydrolyzed the trans piceid into trans-resveratrol, but this would only explain a small part of the increase in trans resveratrol and none of the increase in cis-resveratrol. The authors speculate that either enzymes produced by the transformed yeast are providing more substrate for the hydrolysis enzyme from cell wall fragments or there are unknown conjugated forms of resveratrol present that have not been described.

2.2 Synthesis of d_4-trans-Resveratrol

A convenient, 6-steps synthesis of the so far unknown *trans*-resveratrol-d_4 **7** (*E*-3',4,5'-trihydroxy-2,3,5,6-tetradeuterostilbene), starting from commercially available phenol-d_6 **1**, with an overall yield of 25%, is described. The final labeled resveratrol was fully characterized by MS spectrometry and IR, ^{1}H NMR, and ^{13}C NMR spectroscopies. The isotopic distribution of the final product, determined by high resolution mass spectrometry, was as follows: d_4, 96%; d_3, 4%.

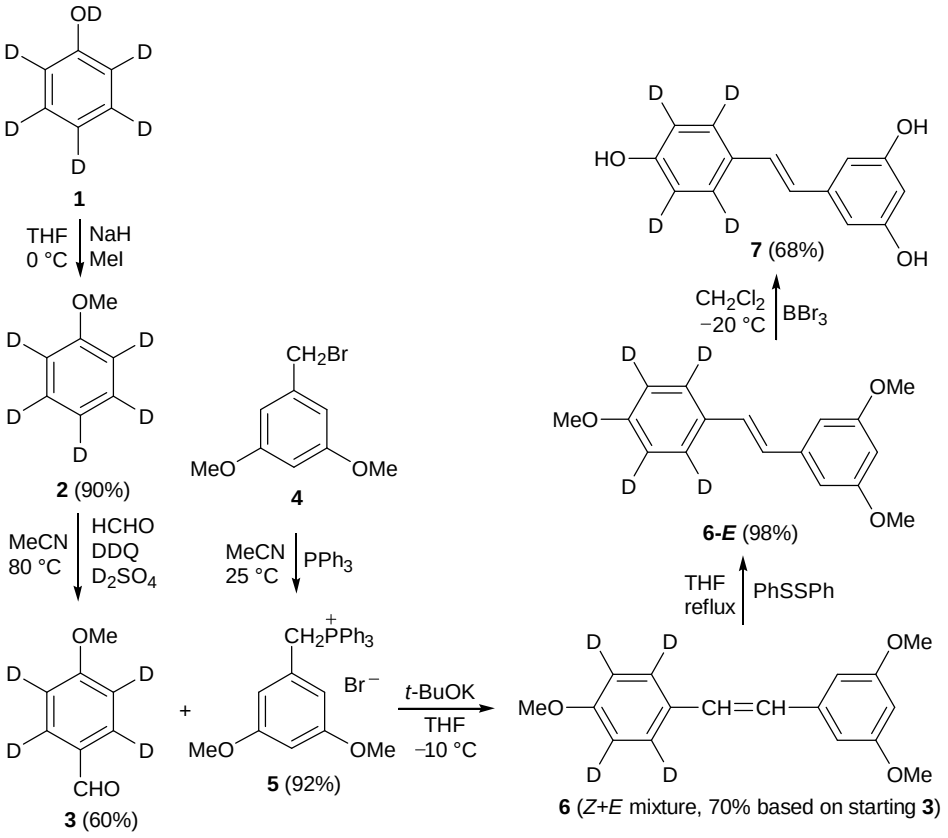
2.2.1 Introduction

Trans-Resveratrol (3,4',5-trihydroxystilbene) is a polyphenolic phytoalexin found in more than 70 plants and many foods, including grapes, peanuts, berries, and red wine(Burns J 2002, Siemann 1992). Resveratrol has recently attracted considerable attention in view of its significant biological activities, which include anti-oxidant and/or anti-inflammatory activity(Labisnkyy et al 2006). Recent data suggest that nutritional intake of resveratrol may contribute to the so-called “French paradox”, that is, the unexpectedly low incidence of coronary heart disease in the Mediterranean population, in spite of a relatively high intake of saturated fats. Resveratrol has been shown to inhibit LDL oxidation and prevent the oxidative stress in general, which results in an anti-aging effect (Labisnkyy 2006, Bureau 2008, Kirimlioglu 2008, Kumar 2007, Ou HC 2006, Jang 2003, Karla 2008, Fremont 1999). Moreover, resveratrol has been classified as a phytoestrogen, due to its ability to interact to estrogenic receptors, and has been shown to exert anti-proliferative and cancer-protective effects (Nicortra 2008, Trincheri 2007, Li Y 2006, Le Corre 2005,Bhat 2001,Nakagawa 2001, Bowrs 2000, Hisieh 1999, Mgbonyebi 1998, Gehm 1997, Jang 1997).

Considering the potential beneficial effects on human health of resveratrol, the development of new, reliable and sensitive techniques for its quantitative determination in foods appears to be of primary importance. One of the most promising methodologies for the highly sensitive quantitative determination of micro-components in food products is currently represented by stable isotope dilution assay (SIDA) (Rychlik 2008, Mosandal 1992). Thus, resveratrol could in principle be detected and quantified in food samples by means of SIDA using, for example, a deuterated derivative such as *E*-3',4,5'-trihydroxy-2,3,5,6-tetradeuterostilbene as internal standard, which, however, to our knowledge, has not been reported in the literature so far. Here we wish to present an easy and convenient synthesis for this derivative, starting from commercially available phenol-d_6, with an overall yield of 25% over 6 steps.

2.2.2 Results and discussion

E-3',4,5'-Trihydroxy-2,3,5,6-tetradeuterostilbene (*trans*-resveratrol-d_4) **7** has been prepared according to the convergent synthetic strategy shown in Scheme 2.1.



Scheme 2.1 Synthesis of E-3',4,5'-trihydroxy-2,3,5,6-tetradeuterostilbene

Commercially available phenol-d_6 **1** was converted into 2,3,4,5,6-pentadeuteroanisole **2** in 90% yield by deprotonation with NaH in THF at 0 °C followed by quenching with MeI. Formylation/oxidation of **2** with formaldehyde-D_2SO_4/DDQ in MeCN at 80 °C led to a mixture of 3,4,5,6-tetradeutero-2-methoxybenzaldehyde (20% yield) and 2,4,6,7-tetradeuteroanisaldehyde **3** (60% yield), which could be easily separated by column chromatography. On the other hand, commercially available 3,5-dimethoxybenzyl bromide **4** was easily converted into (3,5-dimethoxybenzyl)triphenylphosphonium bromide **5** in 92% yield by the reaction with an excess of PPh_3 in acetonitrile at room temperature. The key step of the synthesis then consisted in the olefination reaction between **3** and **5**, to give 3',4,5'-trimethoxy-2,3,5,6-tetradeuterostilbene **6** as a *E*/*Z*

mixture in 70% yield based on **3**. Double bond isomerization with a catalytic amount of diphenyl disulfide in refluxing THF to give the *trans* isomer **6-*E*** followed by deprotection (with BBr_3 in CH_2Cl_2 at –20°C) eventually led to the desired deuterated resveratrol **7** in 47% yield based on deuterated aldehyde **3**. The overall yield of **7** based on starting **1** was 25% over 6 steps.

No H/D exchange and/or redistribution was observed in the course of the synthesis, as confirmed by the spectroscopic characterization (MS, ^{1}H NMR, ^{13}C NMR, IR) of the key intermediates **2**, **3** and **6-*E***. The final deuterated resveratrol **7** was also isotopically stable and fully characterized by MS spectrometry and IR, ^{1}H NMR, and ^{13}C NMR spectroscopies. The isotopic distribution of **7** was determined by high resolution mass spectrometry, and gave the following result: d_4, 96%; d_3, 4%. The characteristics of the new labeled resveratrol **7** are therefore suitable in view of its utilization as internal standard for the quantitative determination of naturally occurring resveratrol in food samples.

2.2.3 Experimental

Phenol-d_6 **1** (99% atom D), sodium hydride (anhydrous, 95%), methyl iodide, paraformaldehyde, 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ), D_2SO_4 (96-98 wt. % in D_2O, 99.5 atom % D) 3,5-dimethoxybenzyl bromide **4**, triphenylphosphine, diphenyl disulfide, boron tribromide were commercially available (Aldrich) and were used as received.

Melting points were taken on a Reichert Thermovar apparatus are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DPX Avance 300 spectrometer at 25 °C in $CDCl_3$ or DMSO-d_6 solutions at 300 MHz and 75 MHz, respectively, with Me_4Si as internal standard. Chemical shifts (δ) and coupling constants (*J*) are given in ppm and in Hz, respectively. IR spectra were taken with a Perkin-Elmer Paragon 1000 PC FT-IR spectrometer. Mass spectra were obtained using a Shimadzu QP-2010 GC-MS apparatus at 70 eV ionization voltage. All reactions were analyzed by TLC on silica gel 60 F_{254} and by GLC using a Schimadzu GC-2010 gas chromatograph and capillary columns with polymethylsilicone + 5% phenylsilicone as the stationary phase. Column

chromatography was performed on silica gel 60 (Merck, 70-230 mesh). Evaporation refers to the removal of solvent under reduced pressure.

*2,3,4,5,6-Pentadeuteroanisole **2***

The method of (Kendall 2000) was employed. A solution of phenol-d_6 **1** (1.0 g, 9.99 mmol) in anhydrous THF (4 mL) was added dropwise over 1 h under nitrogen to a cooled suspension (0 °C) of sodium hydride (350.0 mg, 13.85 mmol) in anhydrous THF (3 mL). After additional stirring at 0 °C for 10 min, iodomethane (4.6 g, 32.4 mmol) was added rapidly. The reaction mixture was allowed to warm up to room temperature and then refluxed for 19 h. After cooling to room temperature, the reaction mixture was quenched with water (80 mL) and extracted with hexane (3 × 50 mL), and the collected organic layers were dried on Na_2SO_4, After removal of the solvent by distillation at atmospheric pressure, 2,3,4,5,6-pentadeuteroanisole **2** was obtained as a colorless liquid by short path distillation (bp 153-155 °C) (1.02 g, 90 % based on **1**). The spectroscopic properties of the product were in agreement with those previously reported (Vougioukalakis 2003).

*2,4,6,7-Tetradeuteroanisaldehyde **3***

The method of (Branytska 2004) was employed. A mixture of **2** (1.0 g, 8.84 mmol), paraformaldehyde (2.4 g, 79.92 mmol), D_2SO_4 (0.25 mg, ca. 2.5 mmol) and DDQ (3.61 g, 15.90 mmol) in MeCN (10 mL) was allowed to stir under nitrogen at 80 °C for 5 h. After cooling, AcOEt was added, the mixture was filtered and the solid washed with AcOEt. The solvent used for washing the solid was added to the filtrate. The collected AcOEt phases were evaporated, and the residue was purified by column chromatography on silica gel using 97:3 hexane- AcOEt as the eluent, to give pure 3,4,5,6-tetradeutero-2-methoxybenzaldehyde (colorless oil, 245.2 mg, 20% based on **2**) and 2,4,6,7-tetradeuteroanisaldehyde **3** (colorless oil, 746.1 mg, 60% based on **2**) in this order.

IR (film): ν = 1689, 1580, 1571, 1236 cm^{-1}

^{1}H NMR (300 MHz, $CDCl_3$): δ = 9.88 (s, 1 H, CHO); 3.88 (s, 3 H, OCH_3)

^{13}C NMR (75 MHz, $CDCl_3$): δ = 190.9 (CHO), 164.6 (C-4), 131.7 (t, J = 23.8 Hz, C-2 + C-6), 129.9 (C-1), 114.0 (t, J = 23.8 Hz, C-3 + C-5), 55.6 (OCH_3)

GC-MS (70 eV): m/z = 140 (M^+, 73), 139 (100), 111 (24), 96 (24), 81 (45), 80 (18), 69 (19), 68 (20), 66 (17).

(*3,5-Dimethoxybenzyl)triphenylphosphoniumbromide* ***4***

3,5-Dimethoxybenzyl bromide (2.0 g, 8.65 mmol) was added to a stirred solution of PPh_3 (3.0 g, 11,44 mmol) in MeCN (30 mL). The resulting mixture was allowed to stir at room temperature for 72 h. After removal of the solvent by evaporation, the residue was solubilized in CH_2Cl_2 (30 mL). Et_2O (10 mL) was slowly added without stirring, leading to the precipitation of pure **4** as colorless crystals, mp = 273-274 °C (3.92 g, 92% based on starting 3,5-dimethoxybenzyl bromide). The ^{1}H NMR data of the product were in good agreement with those previously reported (Gao 2006).

IR (KBr): ν = 1607, 1582, 1438, 1155 cm^{-1}

^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.98-7.88 (m, 3 H on phenyl rings), 7.82-7.71 (m, 12 H on phenyl rings), 6.44 (q, *J* = 2.2 Hz, 1 H, H-4), 6.22 (t, *J* = 2.2 Hz, 2 H, H-2 + H-6), 5.25 (d, *J* = 15.4 Hz, 2 H, CH_2P), 3.52 (s, 6 H, 2 OMe).

^{13}C NMR (75 MHz, DMSO-d_6): δ = 160.2 (d, *J* = 2.5 Hz, C-3 + C-5 on 3,5-dimethoxyphenyl ring), 135.0 (d, *J* = 3.8 Hz, C-4 on phenyl rings), 134.0 (d, *J* = 10.0, C-3 + C-5 on phenyl rings), 130.0 (d, *J* = 12.5 Hz, C-2 + C-6 on phenyl rings), 129.9 (d, *J* = 8.8 Hz, C-1 on 3,5-dimethoxyphenyl ring), 117.8 (d, *J* = 86.3 Hz, C-1 on phenyl rings), 108.9 (d, *J* = 6.3 Hz, C-2 + C-6 on 3,5-dimethoxyphenyl ring), 100.1 (d, *J* = 3.8, C-4 on 3,5-dimethoxyphenyl ring), 55.0 (2 OCH_3), 28.3 (d, *J* = 46.3 Hz, CH_2P).

(*E*)-*3',4,5'-Trimethoxy-2,3,5,6-tetradeuterostilbene* ***6-E***

The method of (Tsuda 1992) was employed. To a cooled (–10 °C), stirred mixture of (3,5-dimethoxybenzyl)triphenylphosphonium bromide (1.68 g, 3.41 mmol) and potassium *tert*-butoxide (381.5 mg, 3.40 mmol) in anhydrous THF (50 mL), was added under nitrogen 2,4,6,7-tetradeuteroanisaldehyde **3** (400 mg, 2.85 mmol) dropwise. After additional stirring at −10 °C under nitrogen for 1 h, the mixture was allowed to warm up to room temperature, and then it was poured into water (ca. 100 mL) and neutralized with 1 N HCl. The resulting mixture was extracted with diethyl ether (3 × 30 mL), and the collected organic phases were dried over Na_2SO_4. After filtration, the solvent was evaporated, and the residue purified by column chromatography on silica gel, using 8:2 hexane-AcOEt as eluent, to give 3',4,5'-trimethoxy-2,3,5,6-tetradeuterostilbene **6** as a *E*/*Z* mixture (544 mg, 70% based on starting aldehyde **3**). A mixture of the latter (400 mg, 1.46 mmol) and diphenyl disulfide (60 mg, 0.27 mmol) in anhydrous THF (50 mL)

was refluxed under nitrogen for 2 h. After cooling, the solvent was evaporated, and the residue was purified by column chromatography on silica gel, using 8:2 hexane-AcOEt as eluent, to give pure (*E*)-3',4,5'-trimethoxy-2,3,5,6-tetradeuterostilbene **6-*E*** as a colorless solid, mp 54-55 °C (390 mg, 98% based on the starting *E*-*Z* mixture).

IR (KBr): ν = 1591, 1456, 1204, 1151 cm^{-1}

^1H NMR (300 MHz, $CDCl_3$): δ = 7.04 (distorted d, *J* = 16.5, 1 H, *H*C=CH), 6.89 (distorted d, *J* = 16.5, 1 H, HC=C*H*), 6.68-6.64 (m, 2 H, H-2' + H-6'), 6.42-6.36 (m, 1 H, H-4'), 3.83 (s, 9 H, 3 OMe)

^{13}C NMR (75 MHz, $CDCl_3$): δ = 161.3 (C-3' + C-5' on 3,5-dimethoxyphenyl ring), 159.6 (C-4 on tetradeutero-4-methoxyphenyl ring), 140.0 (C-1' on 3,5-dimethoxyphenyl ring), 129.5 (C-1 on tetradeutero-4-methoxyphenyl ring), 128.9 (H*C*=CH), 127.5 (t, *J* = 23.4 Hz, C-2 + C-6 on tetradeutero-4-methoxyphenyl ring), 126.9 (HC=*C*H), 114.0 (t, *J* = 23.4 Hz, C-3 + C-5 on tetradeutero-4-methoxyphenyl ring), 104.8 (C-2' + C-6' on 3,5-dimethoxyphenyl ring), 100.0 (C-4' on 3,5-dimethoxyphenyl ring), 55.4 (2 OCH_3).

GC-MS (70 eV): *m/z* = 274 (M^+, 100), 273 (8), 259 (4), 243 (8), 228 (6), 216 (5), 200 (6), 185 (4), 173 (5), 157 (7), 156 (6), 155 (5), 145 (6), 137 (6), 119 (4).

(E)-3',4,5'-trihydroxy-2,3,5,6-tetradeuterostilbene (trans-resveratrol-d_4) ***7***

To a cooled (–20 °C), stirred solution of (*E*)-3',4,5'-trimethoxy-2,3,5,6-tetradeuterostilbene **6-*E*** (180.0 mg, 0.66 mmol) in anhydrous CH_2Cl_2 (20 mL) was added under nitrogen boron tribromide (1.30 g, 5.19 mmol) dropwise. The mixture was allowed to warm up to room temperature, then it was poured into ice-water, and extracted with AcOEt. The organic layer was washed with brine and dried over Na_2SO_4. After filtration, the residue was purified by column chromatography on silica gel using 6:4 hexane-AcOEt as eluent, to give pure (*E*)-3',4,5'-trihydroxy-2,3,5,6-tetradeuterostilbene (*trans*-resveratrol-d_4) **7** as a colorless solid, mp = 254-256 °C (105 mg, 68% based on **6-*E***).

IR (KBr): ν = 3294, 1590, 1407, 1345, 1155 cm^{-1}

^1H NMR (300 MHz, DMSO-d_6): δ = 9.53 (s, 1 H, OH at C-4), 9.18 (s, 2 H, OH at C-3' + OH at C-5'), 6.93 (distorted d, *J* = 16.5, 1 H, *H*C=CH), 6.81 (distorted d, *J* = 16.5, 1 H, HC=C*H*), 6.38 (s, 2 H, H-2' + H-6'), 6.12 (s, 1 H, H-4')

^{13}C NMR (75 MHz, DMSO-d_6): δ = 158.4 (C-3' + C-5' on 3,5-dihydroxyphenyl ring), 157.0 (C-4 on tetradeutero-4-hydroxyphenyl ring), 139.2 (C-1' on 3,5-dihydroxyphenyl ring), 127.8 (C-1 on tetradeutero-4-hydroxyphenyl ring), 127.7 (H*C*=CH), 127.3 (t, *J* = 22.2 Hz, C-2 + C-6 on tetradeutero-4-hydroxyphenyl ring), 125.5 (HC=*C*H), 115.0 (t, *J* = 19.0 Hz, C-3 + C-5 on tetradeutero-4-hydroxyphenyl ring), 104.2 (C-2' + C-6' on 3,5-dihydroxyphenyl ring), 101.7 (C-4' on 3,5-dihydroxyphenyl ring).

LC-MS (ES+): found: *m/z* = 233.18 [(M-d_4 + H)$^+$, 96%], 232.16 [(M-d_3 + H)$^+$, 4%]; calculated for (M-d_4+H)$^+$: 233.11, for (M-d_3+H)$^+$: 232.10.

2.2.4 Conclusion

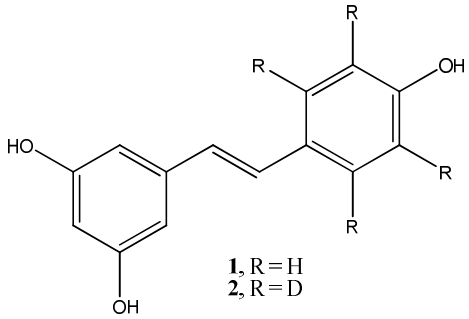
In conclusion, a convenient synthesis of a new labeled resveratrol derivative, *E*-3',4,5'-trihydroxy-2,3,5,6-tetradeuterostilbene **7**, with an overall yield of 25% over 6 steps and an isotopic purity of 96%, has been developed. The new labeled resveratrol is isotopically stable and therefore suitable for its utilization as internal standard for the quantitative determination of resveratrol in food samples be means of the stable isotope dilution assay technique.

.

2.3 Assay of Resveratrol by Tandem Mass Spectrometry and Isotope Dilution Method

2.3.1 Introduction

Resveratrol (*trans*-3,5,4'-trihydroxystilbene, **1**) is a secondary metabolite bearing powerful antioxidant properties produced by some plants for self-protection against environmental stresses(Langeake 1976).



1, R = H
2, R = D

It is found mostly in grapes, in particular in the skin, where it acts as shield against fungal diseases and sun damage. The amount of resveratrol in grape skins varies with the cultivar, its geographic origin, and exposure to fungal infection(Fremont 2000). Wines contain, therefore, appreciable amounts of resveratrol released from the grape skins(Msttivi 1995). The predominant form of resveratrol in grapes and grape juice is *trans*-resveratrol glucoside (*trans*-piceid), during fermentation, however, the action of specific glucosidases induces significant amounts of resveratrol aglycon in wines . The latter contains also detectable amounts of *cis*-resveratrol, which may be formed either during fermentation or released from viniferins (resveratrol polymers)(Goldberg 1995). The *cis* isomer can also be induced by photo- isomerization of the *trans*–form when the wine is exposed to light and oxygen (Cantos 2000). The potential health benefits of resveratrol, investigated in the early nineties when its presence in red wine was firstly reported (Siemann 1992), led to speculation referred as the “French Paradox” and related to the apparently low mortality from coronary heart disease (Criqui 1994, St Leger 1979) , despite the relatively high levels of dietary saturated fat and cigarette smoking. This fact led to the idea that the regular consumption of red wine might provide additional protection from cardiovascular disease. Inhibition of platelet aggregation, *in vitro(Kirk*

2000,Paceasciak 1995), anti-inflammatory and anti-estrogenic effects, and the prevention of cancer diseases(Jang 1997) are among those healing effects, recently, associated to the intake of functional foods containing resveratrol.

Various analytical methods have been developed for its quantitative determination in wine, most of which are based on liquid chromatography (LC) coupled to different detectors (Wang 2002,Careri 2003,2004, Pineiro 2006, Jerkovic 2008, Buiarelli 2006,Gerogiannaki 2006,Lo 2007,Gamoh 1999).

Resveratrol and its metabolites has been assayed successfully either in plasma and blood samples by means of ultraviolet (UV)(Hunang 2008,Boocock 2007,Chen 2007,He H 2006, Zhu 1999), diode array (DAD) (Wenzel 2005,Juan 2002) and mass spectrometric (MS) (Sindona 2001,Yang 2007) detection. None of them, however, was based on the use of the isotope dilution method(Duncan 2006,Mazzotti 2007,2009, De Nino 2005), which improves both precision and accuracy of the measurements by reducing drawbacks that may arise from calibration procedure, sample preparation, and matrix effects. Accordingly, we present now a fast determination of *trans* resveratrol in wine and plasma by LC-MS and LC-MS/MS using a labeled internal standard analogue. The labeled internal standard (**2**) was synthesized in our laboratory(Gabriele 2008); the measurements were carried out in SIM and MRM mode, by monitoring the ion current of the deprotonated molecular ion $[M-H]^-$, or by following the ion current generated by specific fragmentation pathways, respectively. The MRM approach is unsuitable when positive (+) ions are sampled because of the extensive isotopomerization of the original [$^2H_4$] isomer used as internal standard.

2.3.2 Experimental

Labeled [$^2H_4$]-*trans*-resveratrol has been synthesized starting from [$^2H_6$]-phenol.(Gabriele 2008); The isotopic distribution within the labeled analogue is reported in figure 1

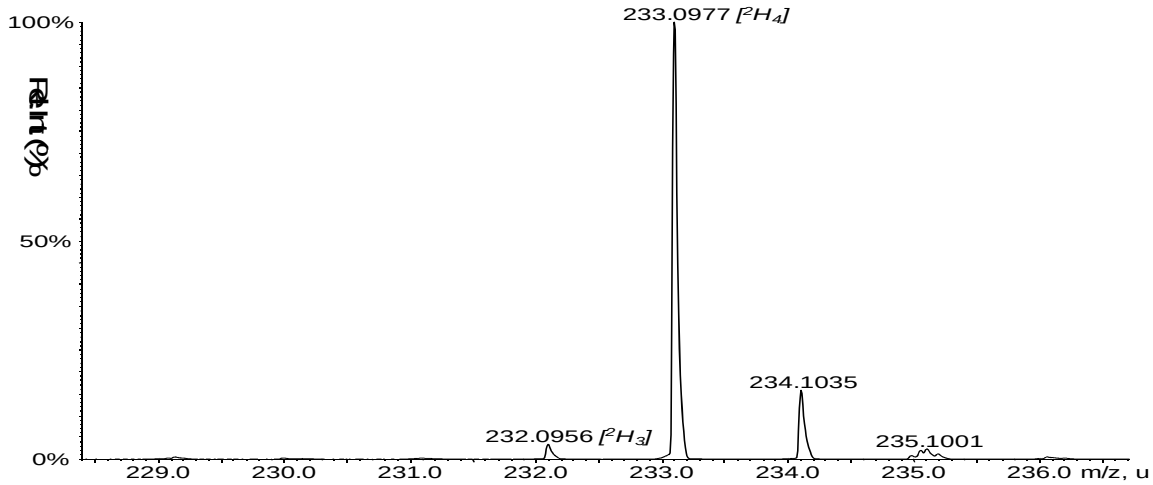


Figure 2.1 . *Isotopic distribution of [$^2H_4$]-resveratrol*

2.3.2.1 Sample Preparation.

2.3.3.1.a <u>Wine</u>:

1 mL of wine, diluted with water (1/10÷1/20), was mixed to 50 μL or 100 μL of 4 ppm solution of the internal standard dissolved in CH_3OH/H_2O 50/50; the resulting solution was directly injected into the instrument.

2.3.3.1.b <u>Plasma</u>:

To 1 mL of human plasma, spiked with compound **1** at concentration 0.015 and 0.150 ppm**,** were added 12.5 μL of 4 ppm solution of the internal standard, the mixture was treated with 1,5 mL of cold CH_3CN in order to precipitate the protein and then centrifuged at 8000 rpm for 5 min. The supernatant, transferred to an Eppendorf tubes, was evaporated to dryness under nitrogen gas and reconstituted with 0.5 mL of LC-MS mobile phase; after, it was filtered through a 0.22 μm HPLC filter and injected into HPLC (Remsberg 2007).

2.3.3.2 Mass Spectrometry.

The LC-MS and LC-MS/MS analyses were carried out with a triple-quadrupole mass spectrometer Quattro LC (Waters corporation, Milford, MA, USA), equipped with an electrospray (ESI) source interfaced with an HPLC 1100 (Agilent Technologies, Waldbronn, Germany). The chromatographic analyses were performed using a Discovery C_{18} column, 7.5 cm × 2.1 mm (Supelco, Saint Louis, MO). The flow rate was fixed at 0.25 mL/min and the following eluant and chromatographic conditions were used: solvent A (H_2O, 0.1% formic acid), solvent B (CH_3OH); from 30% B to 96% B in 6 min; 3 min at 96% B; from 96% B to 30% A in 3 min; 3 min re-equilibration time at 30% B. Mass spectrometer conditions were set up as following. Negative ionization, capillary voltage: 3.8 kV; cone voltage: 43 V; source temperature: 250°C; source block: 100°C. Nitrogen was used as drying and nebulizing gas at flow rates of 650 and 60 L/h, respectively. For MRM experiments the collision energies were set to 28 and 22 eV for the two transitions followed, while the collision gas (N_2) pressure was set at 1.9×10^{-3} mbar.

ESI MS and MS/MS spectra of resveratrol and of its labeled analogue were acquired on a hybrid Q-Star Pulsar-i (MSD Sciex Applied Biosystem, Toronto, Canada) mass spectrometer equipped with an ion spray ionization source at 8000 resolution. Analytes at 5 ppm concentration, dissolved in a solution of 0.1% acetic acid, CH_3OH/H_2O 50:50, were introduced by direct infusion (5 μL/min) at the optimum ion spray (IS) voltage of 4800 V. The source nitrogen (GS1) and the curtain gas (CUR) flows were set at pressures of 20 and 25 psi, respectively, whereas the first declustering potential (DP1), the focusing potential (FP), and the second declustering potential (DP2) were kept at 50, 220, and 10 V relative to ground, respectively. The MSMS spectra were acquired at a collision energy of 25 eV using N_2 as collision gas.

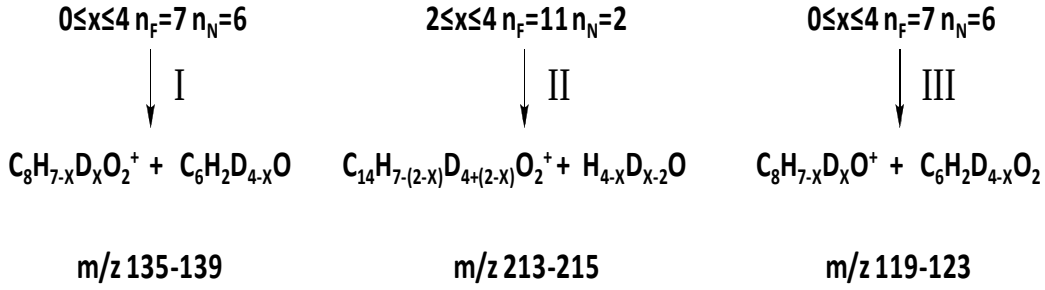
2.3.3.3 H/D isomerization in the dissociation of $[M+H]^+$ of $[^2H_4]$-resveratrol.

The isotopic distribution in the fragments formed by unimolecular gas-phase dissociation of the protonated molecular ion of the labeled reference **2** has been calculated according to eq. 1 (Kuck 2002)

$$P_x = \frac{\frac{n_F!}{(n_F - x)!\cdot x!} \cdot \frac{n_N!}{[a - (n_F - x)]!\cdot (b - x)!}}{\frac{n!}{a!\cdot b!}} \qquad \text{(eq. 1)}$$

where n_F is the number of sites occupied by those atoms remaining on the ionic fragment, n_N is the number of sites remained on the neutral fragment, n is the total number of interchanging sites and, for the case of two interchanging isotopes a and b, their sum (a + b) is equal to the sum of n_F and n_N , i.e. equal to n. The variable x corresponds to the number of atoms b in the ionic fragment, while P_X is the probabilities to find x atoms of b in the ionic fragment (Kuck 2002).

The reacting $[M+H]^+$ ion contains 13 interchangeable H/D atoms. The breakdown pattern can be described according to scheme 1. The experimental values are showed in Table 1 together with the theoretical values.



Scheme 2.1

	Experimental (%)	Statistical (%)
Pathway I		
$C_8H_7O_2^+ + C_6H_2D_4O$	4.7	2.7
$C_8H_6DO_2^+ + C_6H_3D_3O$	22.4	19.5
$C_8H_5D_2O_2^+ + C_6H_4D_2O$	41.0	44.0
$C_8H_4D_3O_2^+ + C_6H_5DO$	25.6	29.0
$C_8H_3D_4O_2^+ + C_6H_6O$	6.3	4.8
Pathway II		
$C_{14}H_7D_4O_2^+ + H_2O$	47.4	46.1
$C_{14}H_8D_3O_2^+ + HDO$	45.1	46.2
$C_{14}H_9D_2O_2^+ + D_2O$	7.5	7.7
Pathway III		
$C_8H_7O^+ + C_6H_2D_4O_2$	1.4	2.7
$C_8H_6DO^+ + C_6H_3D_3O_2$	11.8	19.5
$C_8H_5D_2O^+ + C_6H_4D_2O_2$	30.0	44.0
$C_8H_4D_3O^+ + C_6H_5DO_2$	42.4	29.0
$C_8H_3D_4O^+ + C_6H_6O_2$	14.4	4.8

Table 2.1. *Experimental and theoretical relative abundances of ions in isotopomeric clusters generated by fragmentation of* ***2***

2.3.4 Result and discussion

The ESI (+) MS/MS spectrum of resveratrol is characterized by a small number of fragments diagnostic of the structure (figure 2.3).

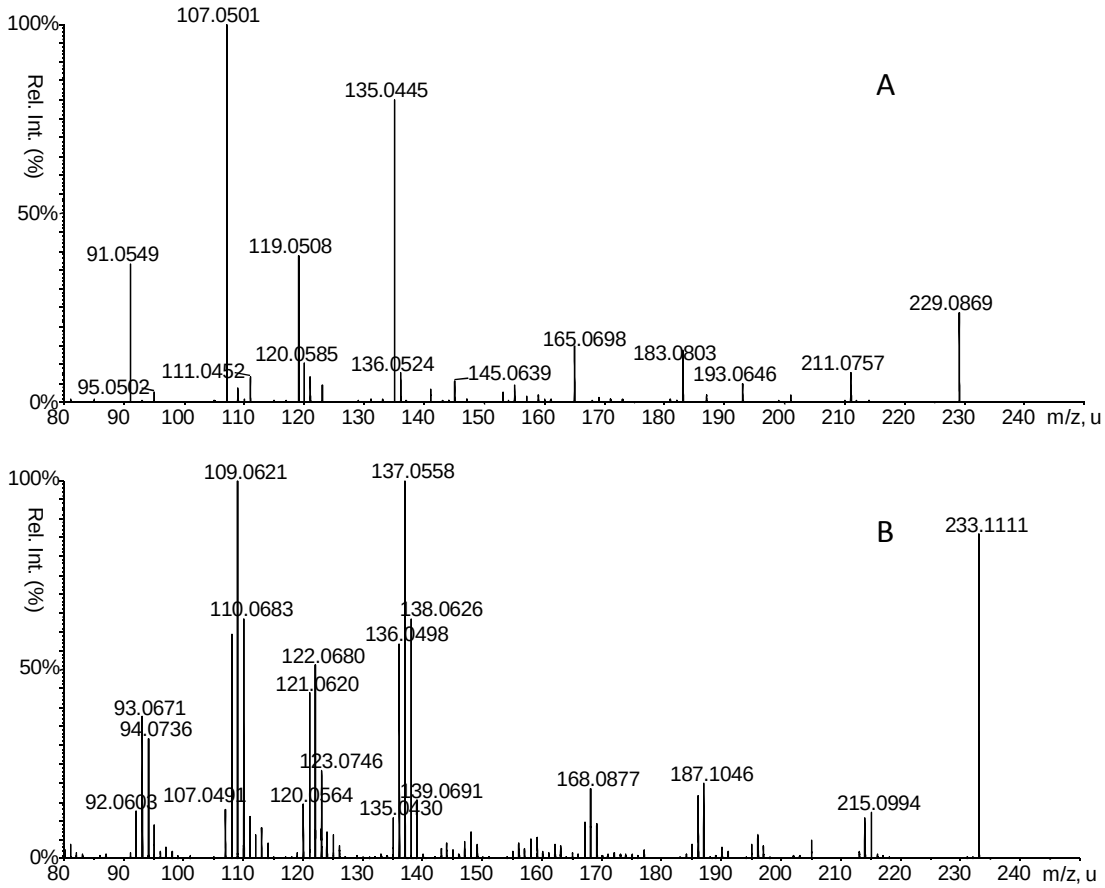
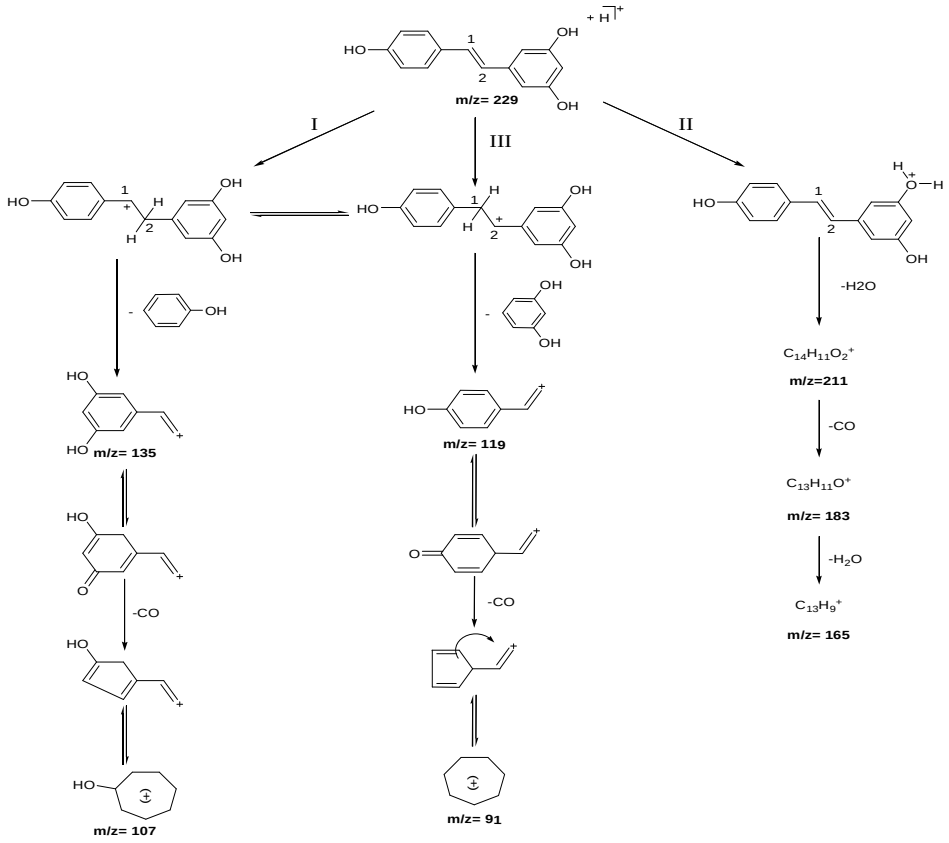


Figure 2.3. *HRESI (+) MSMS spectra of compound* ***1*** *(A) and* ***2*** *(B)*

Three different protonation sites could be suggested for the protonated molecular ion; the first one, located on the hydroxyl group of either phenol or resorcinol rings, could give rise to the product ion at *m/z* 211, which corresponds to formal loss of water from the precursor ion, followed by the formal loss of CO (*m/z* 183) and again water (*m/z* 165) (Scheme 2, pathway II).



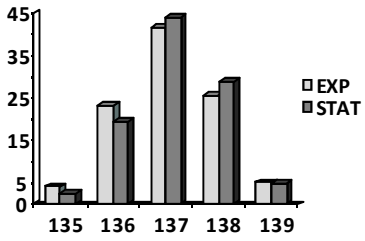
Scheme 2.2 Fragmentation pattern of $[M+H]^+$ ion of Resveratrol

The other protonation sites may be located at the stilbene double bond. In this case two possible fragmentation channels can be suggested depending on the regiochemistry of proton addition to the double bond. When carbon C_1 is protonated (scheme 2, pathway III), the fragmentation of the $[M+H]^+$ species leads to the formation of ions at *m/z* 119, through the loss of the resorcinol group, followed by CO elimination affording the species at *m/z* 91. In the other case the loss of phenol reasonably gives rise to the formation of the ion at *m/z* 135, which is followed by the formation of m/z at 107 due to the loss of CO (scheme 2, pathway I). The ESI (+) MS/MS spectrum of *[$^2H_4$]*-resveratrol shows extensive H/D isomerization during the gas-phase fragmentation, which occurs in the positive ion mode only (Figure 2B).

The gas-phase fragmentation gives rise to the isotopomeric clusters at *m/z* 212-215, *m/z* 135-139 and *m/z* 107-111 with different relative abundances (Scheme 3). The occurrence of H/D isomerization from ring-to-ring was supported by statistical calculations. Deuterium isomerization between aromatic rings linked by suitable bridges has been comprehensively described and the probability of the event is given by equation 1 (see experimental).

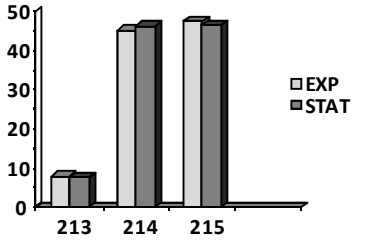
Pathway I

$C_8H_7O_2^+ + C_6H_2D_4O$
$C_8H_6DO_2 + C_6H_3D_3O$
$C_8H_5D_2O_2^+ + C_6H_4D_2O$
$C_8H_4D_3O_2^+ + C_6H_5DO$
$C_8H_3D_4O_2^+ + C_6H_6O$



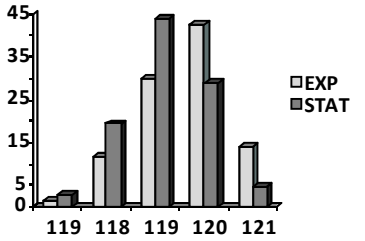
Pathway II

$C_{14}H_7D_4O_2^+ + H_2O$
$C_{14}H_8D_3O_2^+ + HDO$
$C_{14}H_9D_2O_2^+ + D_2O$



Pathway III

$C_8H_7O^+ + C_6H_2D_4O_2$
$C_8H_6DO^+ + C_6H_3D_3O_2$
$C_8H_5D_2O^+ + C_6H_4D_2O_2$
$C_8H_4D_3O^+ + C_6H_5DO_2$
$C_8H_3D_4O^+ + C_6H_6O_2$



Equation 1 was applied to the three reaction paths described in schemes 2 and 3. The relative abundances of the isotopomers related to the species at *m/z* 135-139 and *m/z* 212-215 formed from the protonated [$^2H_4$]-*trans*-resveratrol (scheme 3) are nearly identical to those expected by a complete H/D isomerization prior to fragmentation. The experimental results are relatively dissimilar from the statistical calculation in the case of isotopomers at *m/z* 107-111, only. This effect can be correlated with a kinetically driven fragmentation processes efficiently competing with the thermodynamic H/D full isomerization.

The possibility of quantifying resveratrol *via* positive tandem mass spectrometry has been therefore excluded as a consequence of the observed extensive H/D isomerization during the gas-phase fragmentation. Single-ion-monitoring (SIM) and multiple-reaction-methods (MRM) were therefore exploited in the assays of the analyte by the isotope dilution approach in the negative ionization mode, only.

2.3.4.1 Selected Ion Monitoring (SIM) approach

The SIM assay has been performed by monitoring the deprotonated molecular ion $[M-H]^-$ at *m/z* 227 for **1** and *m/z* 231 for the labeled internal standard (**2**). The calibration curve (y =1.3191x - 0.2974; R^2 = 0.9982), built using triplicate samples of six standard solution at different concentration of resveratrol and fixed amount of [$^2H_4$]-resveratrol, shows good linearity in the range from 0.050 to 1.000 ppm; the latter has been utilized for quantitative assay of either *cis* and *trans*-resveratrol in red wine. To highlight the presence of *cis*-resveratrol, a standard solution of the *trans* isomer was exposed for many hours to sunlight (Romero 1996) to induce the formation of the other geometrical isomer. The presence of the *cis* isomer was detected by LC-MS by the appearing of the SIM peak corresponding to *m/z* 227 at the retention time of 7.54 min (figure 2.3).
The separation of the isomers has been achieved in twelve minutes using a C_{18} column with a linear gradient of H_2O/CH_3OH (see experimental).

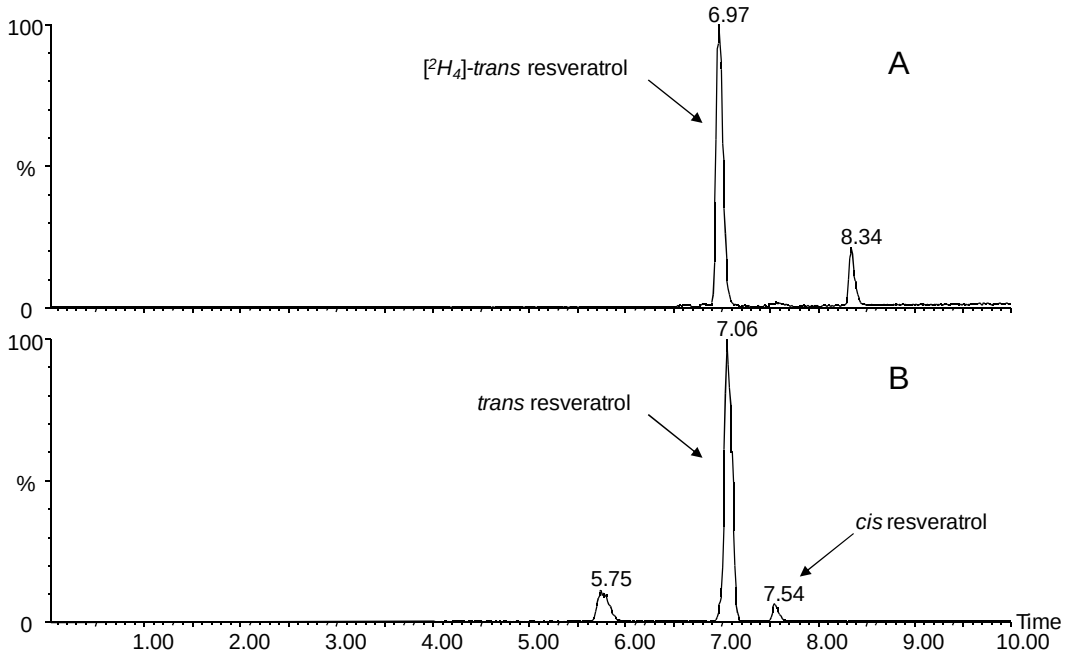


Figure 2.3. *SIM traces of "Riserva Lento" wine sample. (A) Ion chromatogram of m/z 231; (B) ion chromatogram of m/z 227I*

The specificity of the applied methodology is assured by the use of a labeled internal standard that elutes at the same retention time of the trans isomer. The RSD% value, in all case below 5%, demonstrates good repeatability of the measurements; in addition, the

accuracy of the method was determined from samples prepared by adding known quantities of analyte **1** to matrixes free of *trans*-resveratrol, i.e. controlled white wines (Table 2); the accuracy is higher than 96% in the two spiked wines which are representative of low and high samples containing *trans* resveratrol.

t-resveratrol (ppm)	Calculated concentration (ppm)	Accuracy % (average)	RSD% (average)
SIM approach in wine			
0.150	0.145 ± 0.003	96.7	2.07
0.700	0.722 ± 0.022	103.1	3.05
MRM approach in wine			
0.070	0.067 ± 0.005	99.0	7.46
0.700	0.684 ± 0.016	97.7	2.34
MRM approach in plasma			
0.015	0.016 ± 0.001	106.7	6.25
0.150	0.148 ± 0.013	98.7	8.78

Table 2.2. *Precision (RSD %) and accuracy for two distinct fortified samples for SIM and MRM assay in wine and MRM assay in plasma*

The methodology discussed above has been applied to different Mediterranean red wines available at local stores. The amount of *trans*-resveratrol ranged from 2 ppm to 20 ppm (table 3); to validate the data thus obtained, an alternative extended chromatographic run was performed in order to avoid possible co-elution of interfering species. In all cases, however, the analytical results did not change.

Wine	*trans*-resveratrol (ppm)	RSD %	*cis*-resveratrol (ppm)	RSD %
Dragone Lento	6.79 ± 0.49	7.25	1.43 ± 0.05	3.25
Federico II Lento	4.82 ± 0.41	8.46	1.64 ± 0.07	4.19
Riserva Lento	14.46 ± 0.32	2.19	2.46 ± 0.07	2.66
gaglioppo Statti	13.08 ± 0.90	6.89	2.86 ± 0.07	2.39
arvino Statti	10.78 ± 0.91	8.47	3.15 ± 0.19	6.14
I Gelsi Statti	20.39 ± 1.45	7.10	4.07 ± 0.23	5.72
Ceppereto	3.26 ± 0.16	4.87	1.09 ± 0.02	1.88
Cirò Classico	2.66 ± 0.03	0.98	----	----
Elios	8.48 ± 0.42	4.96	1.20 ± 0.03	2.59
Chianti	2.70 ± 0.20	7.50	----	----
Chateau Beni Chougrane (Algeria)	9.08 ± 0.54	5.98	1.53 ± 0.08	5.21
Sidi Brahim (Morocco)	2.02 ± 0.06	3.22	0.56 ± 0.02	3.68

Table2. 3. *Amount of resveratrol found in red wine by SIM approach*

The analytical parameters of LOQ, LOD, recovery and reproducibility are illustrated in Table 2.4.

SIM approach in wine		Reproducibility RSD %*	
LOQ (ppm)	0.020	0.150 ppm	0.700 ppm
LOD (ppm)	0.023		
		9.56	8.21
Recovery	>99%		
MRM approach in wine		Reproducibility RSD %*	
LOQ (ppm)	0.003	0.070 ppm	0.700 ppm
LOD (ppm)	0.005		
		5.71	3.21
Recovery	>99%		
MRM approach in plasma		Reproducibility RSD %*	
LOQ (ppm)	0.003	0.015 ppm	0.150 ppm
LOD (ppm)	0.004		
		6.21	7.23
Recovery[37]	>99%		

*The reproducibility of the measurements were performed by extracting three times each sample over a period of one week.

Table 2.4. *Accuracy, recovery, LOQ and LOD measurements; reproducibility of the methodologies applied*

The value of recovery is quantitative because no extraction procedure has been applied, while the reproducibility values, calculated by repeating the experiments in a period of one week, assess the validity of the proposed method. Very low values of LOQ and LOD corroborate the goodness of isotope dilution proposed approach.

2.3.4.1 Multiple reaction Monitoring (MRM) approach

The tandem mass spectrometric assay was applied to spiked sample of wine and plasma. The MRM methodology (figure 2.4) was carried out by monitoring the ion current of two different fragmentation pathways. The first reaction, used for the quantitation, corresponds to the breakdown of the deprotonated molecular ions of **1** and its [$^2H_4$] isomer **2** to give rise to the product ions at *m/z* 143 and *m/z* 147, respectively, through the formal consecutive losses of two molecules of ketene (Wang 2005).The other transitions leading to the ions *m/z* 185 and *m/z* 189 from unlabeled and labeled protonated molecular ions, respectively, were only used to confirm the detection of resveratrol.

Two different calibration curves were used for the assay of resveratrol in wine and plasma. For the analyses of the wine matrix, the curves were obtained by injecting the same standard solutions used for the SIM assay, i.e. resveratrol ranging from 0.050 to 1.000 ppm, and internal standard ([$^2H_4$]-resveratrol) kept constant at 0.200 ppm. The linearity was retained in the range of examined concentrations ($y = 1,8219x - 0,0088$, $R^2 = 0,9995$) as previously observed. The accuracy tests were carried out, by directly injecting two analyte-spiked samples of wine, respectively at 0.070 and 0.700 ppm. Table 2 shows that the value of accuracy for the two samples ranges from 98% to 99%.
The concentration of the reference solutions of resveratrol ranged from 0.005 to 0.200 ppm, while the internal standard was maintained at 0.050 ppm in the case of plasma matrix ($y = 1,9436x - 0,0084$, $R^2 = 0,9992$). The different amount of standard used for the curve is required by the lower amount of analyte that presumably can be found in plasma. The analyses of plasma samples spiked at 0.015 and 0.150 ppm showed accuracy values of 107% and 99%, respectively (table 2). Finally, LOQ and LOD values are in the low ppb range for both the examined matrixes, while a good repeatability is guaranteed by values always below 8% (table2.4).

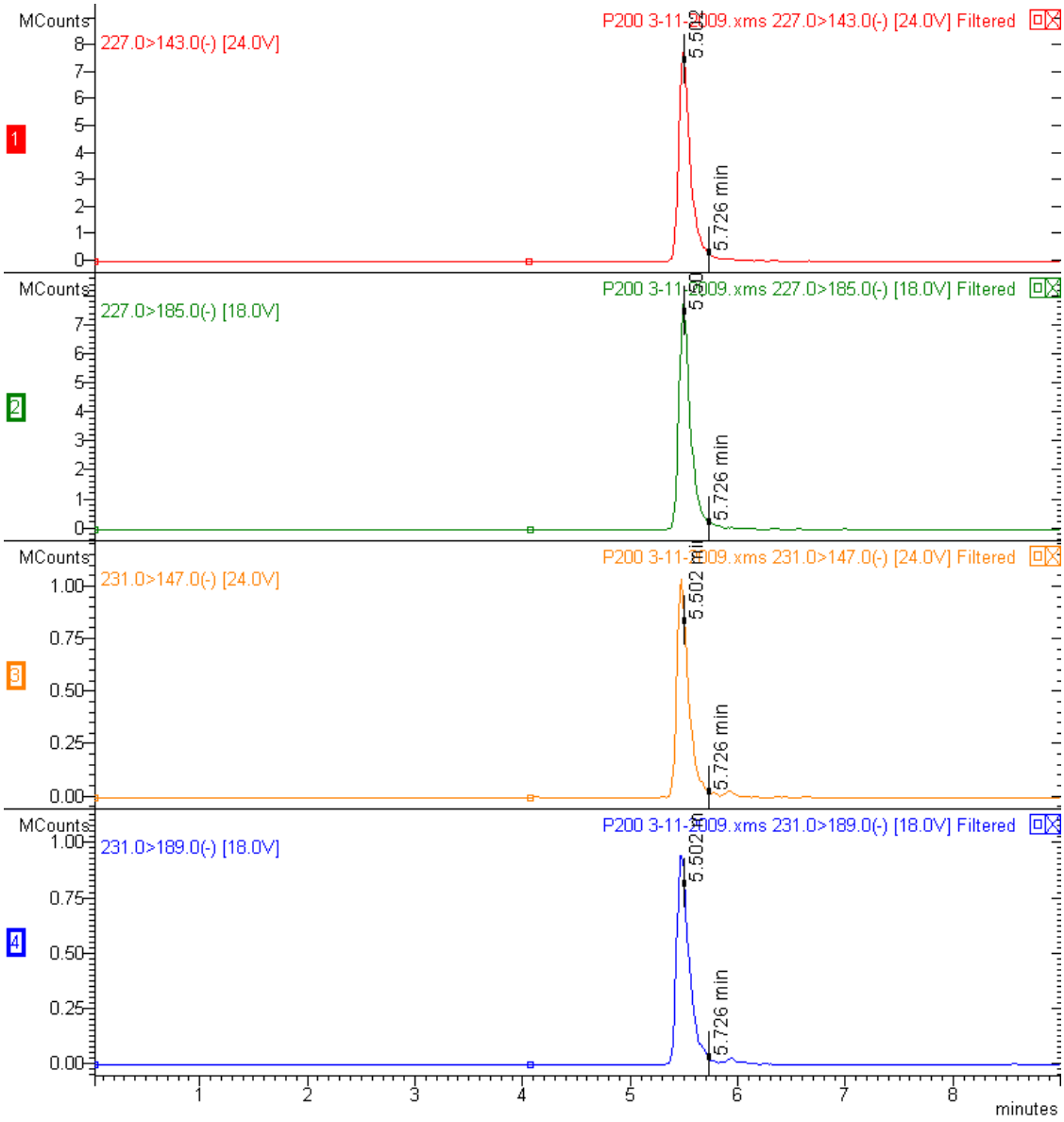


Figure 2.4 The representative mass chromatograms MRM in negative ion mode of compound 1 m/z 227 and 2 m/z 231 in plasma sample

2.3.5 Conclusions

The widespread use of the isotope dilution method in connection with mass spectrometric methodologies poses the problem of the retention of the isotope distribution in the reactive reference compound. This situation is particularly important in the case of molecules, such as resveratrol, where extensive conjugation allows a rapid

H/D equilibration. In the negative mode H/D ring walking is less probable, even if a reduced sensitivity is usually expected in quantitative determination. In the case of resveratrol, the low critical energy associated to the removal of the phenolic protons causes the formation of anions carrying enough ion current to favor the assay of the analyte with good sensitivity. Accordingly, it was possible to set up an easy and fast approach for the determination of resveratrol in wine and plasma through the combined use of SIM and MRM mass spectrometry and isotope dilution methods. As a corollary of the proposed procedure the assay of resveratrol was carried out for a number of commercial red wines produced in the Mediterranean basin.

References

Adrian, M., P. Jeandet, A. Douillet-Breuil, L. Tesson and, R. Bessis. 2000. Stilbene content of mature *Vitis vinifera* berries in response to UV-C elicitation. J. Agr. Food Chem. 48:6103-6105. 20

Ali MA, Kondo K, Tsuda Y(1992). Chem Pharm Bull; **40**: 1130-1136.

Arichi, H., Y. Kimura, H. Okuda, K. Baba, M. Kozawa and, S. Arichi. 1982. Effects of stilbene components of the roots of *Polygonum cuspidatum* Sieb. et Zucc. on lipid metabolism. Chem. Pharm. Bul. 30:1766-1770.

Bavaresco, L., C. Fregoni, M. Trevisan and, P. Forunati. 2000. Effect of cluster stems on resveratrol content in wine. Italian J. Food Sci. 12:103-108.

Bhat KPL, Pezzuto JM(2001). *Cancer Res*; **61**: 6137–6144.

Boocock, D. J.; Patel, K. R.; Faust, G. E. S.; Normolle, D. P.; Marczylo, T. H.; Crowell, J. A.; Brenner, D. E.; Booth, T. D.; Gescher, A.; Steward, W. P.(2007) *J. Chromatogr. B*, *848*, 182–187

Bowers JL, Tyulmenkov VV, Jernigan SC, Klinge CM(2000). *Endocrynology* ; **141**: 3657–3667

Brakenhielm, E., R. Cao and, Y. Cao. 2001. Suppression of angiogenesis, tumor growth and wound healing by resveratrol a natural compound in red wine and grapes. FASEB J. 15:1798-1800.

Branytska O, Neumann R(2004). *Synlett* ; 1575-1576.

Bureau G, Longpre F, Martinoli MG(2008). *J Neurosci Res*; **86**: 403–410.

Buiarelli, F.; Coccioli, F.; Jasionowska, R.; Merolle, M.; Terracciano(2006), A. *Chromatographia*, *64*, 475–481

Buiarelli, F.; Coccioli, F.; Jasionowska, R.; Merolle, M.; Terracciano, A(2007). *Rapid Commun. Mass Sp.*, *21*, 2955–2964.

Burns J, Yokota T, Ashihara H, Lean ME, Crozier A(2002). *J Agric Food Chem.***50**: 3337–3340.

Cantos, E., C. Garcia-Viguera, S. Pascual-Teresa and, F. Tomas-Barberan. 2000. Effect of postharvest ultraviolet irradiation on resveratrol and other phenolics of Cv. Napolean table grapes. J. Agr. Food Chem. 48:4606-4612.

Careri, M.; Corradini, C.; Elviri, L.; Nicoletti, I.; Zagnoni, I.(2003) *J. Agr. Food Chem.*, *51*, 5226–5231.

Careri, M.; Corradini, C.; Elviri, L.; Nicoletti, I.; Zagnoni, I(2004). *J. Agr. Food Chem., 52*, 6868–6874.

Chen, X. J.; He, H.; Wang,G. J.; Yang, B.; Ren, W. C.; Ma, L.; Yu, Q. L.(2007) *Biomed. Chromatogr., 21*, 257–265.

Cheong, H., S. Ryu and, K. Kim. 1999. Anti-allergic action of resveratrol and related hydroxystilbenes. Planta Medica. 65:266-268.

Creasy, L. and M. Coffee. 1988. Phytoalexin production potential of grape berries. J. Amer. Soc. Hort. Sci. 113:230-234.

Criqui, M. H.; Ringel, B. L.(1994) *Lancet 344*, 1719–1723

Croteau, R., T. Kutchan and, N. Lewis. 2000. Natural products (Secondary metabolites). In: Biochemistry and Molecular Biology of Plants. Amer. Soc. Plant Physiologists. Rockville, Md Darias-Mgbonyebi OP, Russo J, Russo IH(1998). *Int J Oncol* ; **12**: 865–869.

Martin, J., O. Rodriguez, E. Diaz and, R. Lamuela-Raventos. 2000. Effect of skin contact on the antioxidant phenolics in white wine. Food Chem. 71:483-487.

De Nino, A.; Di Donna, L.; Mazzotti, F.; Muzzalupo, E.; Perri, E.; Sindona, G.; Tagarelli,(2005) A. *Anal. Chem. 77*, 5961–5964.

De Santi, C., A. Pietrabissa, R. Spisni, F. Mosca and, G. Pacifici. 2000a. Sulphation of resveratrol, a natural compound present in wine, and its inhibition by natural flavonoids. Xenobiotica 30:857-866.

De Santi, C., A. Pietrabissa, R. Spisni, F. Mosca and, G. Pacifici. 2000b. Glucuronidation of resveratrol, a natural product present in grape and wine, in the human liver. Xenobiotica 30:1047-1054.

Douillet-Breuil, A., P. Jeandet, M. Adrian and, R. Bessis. 1999. Changes in the phytoalexin content of various *Vitis* Spp. in response to ultraviolet C elicitation. J. Agr. Food Chem. 47:4456-4461.

Duncan, M. W.; Jane Gale, P.; Yergey, A. L.(2006) *The Principles of Quantitative Mass Spectrometry;1st Ed;* Rockpool Productions LLC: Denver, p. 86.

Ector, B., J. Magee, C. Hegwood and, M. Coign. 1996. Resveratrol concentration in muscadine berries, juice, pomace, purees, seeds and wines. Amer. J. Enol. Viticult. 47:57-62. 21

El-Mowafy, A. 2002. Resveratrol activates membrane-bound guanylyl cyclase in coronary arterial smooth muscle: A novel signaling mechanism in support of coronary protection. Biochem. and Biophysical Res. Commun. 291:1218-1224.

Frankel, E., J. Kanner, J. German, E. Parks and, J. Kinsella. 1993. Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. Lancet 341:454-457.

Fremont L, Belguendouz L, Delpal S(1999). *Life Sci* ; **64**: 2511–2521.

Gabriele, B.; Benabdelkamel, H.; Plastina, P.; Fazio, A.; Sindona, G.; Di Donna, L(2008). *Synthesis Stuttgart* **.***18*, 2953–2956.

Gao M, Wang M, Miller KD, Sledge GW, Hutchins GD, Zheng QH(2006). *Bioorg Med Chem Lett* ; **16**: 5767-5772.

Gamoh, K.; Nakashima, K(1999). *Rapid Commun. Mass Sp.*, *13*, 1112–1115.

Gehm BD, McAndrews JM, Chien PY, Jameson JL(1997). *Proc Natl Aca. Sci USA*.**94**: 14138–14143.
Gerogiannaki-Christopoulou, M.; Athanasopoulos, P.; Kyriakidis, N.; Gerogiannaki, I. A.(2006); Spanos, M. *Food Control*, *17*, 700–706

Goldberg, D. M.; Karumanchiri, A.; Ng, E.; Yan, J.; Diamandis, E. P.; Soleas, G. J(1995) . *J. Agr. Food Chem. 43*, 1245–1250

Gonzalez-Candelas, L., J. Gil, R. Lamuela-Raventos and, D. Ramon. 2000. The use of transgenic yeasts expressing a gene encoding a glycosyl-hydrolase as a tool to increase resveratrol content in wine. Intl. J. of Food Microbiol. 59:179-183

Goodwin, P., T. Hsiang and, L. Erickson. 2000. A comparison of stilbene and chalcone synthases including a new stilbene synthase gene from *Vitis riparia* cv. Gloire de Montpellier. Plant Sci. 151:1-8.

Hackett, A. 1986. The metabolism of flavanoid compounds in mammals. Plant flavanoids in biology and medicine: Biochem., pharmacological and structure-activity relationships: Proceedings. Buffalo, NY. July. Eds. V. Cody, E. Middleton and J. Harbone. pp. 177- 194.

He, H.; Chen, X. J.; Wang, G. J.; Wang, J. P.; Davey, A. K.(2006) *J. Chromatogr. B*, *832*, 177–180.

Hsieh TC, Burfeind P, Laud K, Backer JM, Traganos F, Darzynkiewicz Z, Wu JM(1999) *Int J Oncol* ; **15**: 245–252.

Huang, C., W. Ma, A. Goranson and, Z. Dong. 1999. Resveratrol suppresses cell transformation and induces apoptosis through a p53-dependent pathway. Carcinognenesis. 20:237-242.

Huang, H. L.; Zhang, J. Q.; Chen, G. T.; Lu, Z. Q.; Wang, X. M.; Sha, N.; Shao, B.; Li, P.; Guo, D. A(2008). *Biomed. Chromatogr.*, *22*, 421–427

Jang JH, Surh YJ(2003). *Free Radical Bio Med* ; **34**: 1100–1110.

Jang, M., C, Lining, G. Udeani, K. Slowing, C. Thomas, C. Beecher, H. Fong, N. Farnsworth, A. Kinghorn, R. Mehta, R. Moon and, J. Pozzuto. 1997. Cancer chemopreventative activity of resveratrol, a natural product derived from grapes. Science 275:218-220

Jeandet, P., R. Bessis and, B. Gautheron. 1991. The production of resveratrol by grape berries in different developmental stages. Amer. J. Enol. Viticult. 42:41-46.

Jeandet, P. R. Bessis, B, Maume, P. Meunier, D. Peyron and, P. Trollat. 1995. Effect of enological practices on the resveratrol isomer content of wine. J. Agr. Food Chem. 43:316-319.

Jeandet, P., A. Breuil, M. Adrian, L. Weston, S. Debord, P. Meunier, B. Maume and, R. Bessis. 1997. HPLC analysis of grapevine phytoalexins coupling photodiode array detection and flourometry. Anal. Chem. 69:5172-5177. 22

Jerkovic, V.; Collin, S(2008). *J. Agr. Food Chem., 56*, 584–590

Juan, M. E.; Buenafuente, J.; Casals, I.; Planas, (2002)J. M. *Food Res. Int. 35*, 195–199.

Kalra N, Roy P, Prasad S, Shukla Y(2008). *Life Sci* ; **82**: 348–358.

Kandall JT. *J Label(2000) Compd Radiopharm* ; **43**: 917-924.

Katsagonis, A.; Atta-Politau, J.; Koupparis, M. A(2005). *J. Liq. Chromatogr. R. T., 28*, 1393–1405.

Kimura, Y., H. Okuda and, S. Arichi. 1985. Effects of stilbene on arachidonate metabolism in leukocytes. Biobhim. Biohys. Acta 834:275-278.

Kimura, Y., H. Okuda and, M. Kubo. 1995. Effects of stilbenes isolated from medicinal plants on arachidonate metabolism and degranulation in humna polymorhonuclear leukocytes. J. Ethnopharmacology 45:131-139.

Kimura, Y and H. Okuda. 2001. Resveratrol isolated from *Polygonum cuspidatum* roots prevents tumor growth and metastasis to lung and tumor-induced neovascularization in Lewis lung carcinoma-bearing mice. J. of Nutr. 131:1844-1849.

Kinsella, J., E. Frankel, B. German and, J. Kanner. 1993. Possible mechanisms for the protective role of antioxidants in wine and plant foods. Food Technol. April 1993.

Kuhnle, G., J. Spencer, G. Chowrimootoo, H. Schroeter, E. Debnam, S. Srai, C. Rice-Evans and, U.

Hahn. 2000. Resveratrol is absorbed in the small intestine as resveratrol glucuronide. Biochem. and Biophysical Res. Commun. 272:212-217.

Labisnkyy N, Csiszar, A, Veress G, Stef G, Pacher P, Oroszi G, Wu J, Ungvari Z C. *Curr(2006) Med Chem*; **13**: 989–996

Laden, B. and T. Porter. 2001. Resveratrol inhibits human squalene monooxyenase. Nutr. Res. 21:747-753.

Lamuela-Raventos, R. and A. Waterhouse. 1993. The occurance of resveratrol in selected California wines by a new HPLC method. J. Agr. Food Chem. 41:521-523.

Lamuela-Raventos, R., A. Romero-Perez, A. Waterhouse and, M. de la Torre-Boronat. 1995a. Direct HPLC analysis of cis and trans resveratrol and piceid isomers in Spanish red *Vitis vinifera* wines. J. Agr. Food Chem. 43:281-283.

Lamuela-Raventos, R., A. Romero-Perez, A. Waterhouse and, M. de la Torre-Boronat. 1995b. Resveratrol and piceid levels in wine production and in finished wines. p. 57-67. In: Wine: Nutritional and Theratpeutic Benefits. Amer. Chem. Soc.

Lamuela-Raventos, R., A. Romero-Perez, A. Waterhouse, M. Lloret and, M. de la Torre-Boronat. 1997. Resveratrol and Piceid Levels in Wine Production and in finished wine. p. 57-67. In: Wine: Nutritional and Theratpeutic Benefits. Amer. Chem. Soc.

Langcake, P. and R. Pryce. 1977. The production of resveratrol and the viniferins by grapevines in response to ultraviolet irradiation. Phytochemistry 16:1193-1196. 23

Langcake , P. and R. Pryce. 1976. The Production of resveratrol by Vitis vinifera and other members of the Vitaceae as a response to infection or injury. Physiol. Plant Pathol. 9:77-86.

Le Corre L, Chalabi N, Delort L, Bignon YJ, Bernard-Gallon DJ(2005). *Mol Nutr Food Res* ; **49**: 462–471

Li Y, Liu JY, Liu XP, Xing KF, Wang Y, Li FY, Yao LB. *Appl Biochem Biotechnol* 2006; **135**: 181–192.

Lu, R. and G. Serrero. 1999. Resveratrol, a natural product derived from grape, exhibits antiestrogenic activity and inhibits the growth of human breast cancer cells. J. Cellular Physiol. 179:297-304.

Magee, J. and B. Smith. 2002. Resveratrol content of muscadine berries is affected by disease control spray program. HortScience 37:358-361.

Mattivi, F. F. Reniero and, S. Korhammer. 1995. Isolation, characterization and evolution in red wine vinification of resveratrol monomers. J. Agr. Food. Chem. 43:1820-1823.

Martin, J., O. Rodriguez, E. Diaz and, R. Lamuela-Raventos. 2000. Effect of skin contact on the antioxidant phenolics in white wine. Food Chem. 71:483-487

Mazzotti, F.; Di Donna, L.; Maiuolo, L.; Napoli, A.; Salerno, R.; Sajjad, A.; Sindona, G.(2007) *J. Agric Food Chem. 56*, 63–67.

Mazzotti, F.; Di Donna, L.; Macchione, B.; Maiuolo, L.; Perri, E.; Sindona, G.(2009) *Rapid Commun. Mass Spectrom. 23*, 1515–1518.

Nakagawa H, Kiyozuka Y, Uemura Y, Senzaki H, Shibata N, Hioki K, Tsubura A(2001). *J Cancer Res Clin Oncol* ; 127: 258–264

Nicotra G, Peracchio C, Castino R, Isidoro C(2008). *Carcinogenesis* ; **29**: 381–389

Mosandl A.(1992) *J Chromatogr* ; **624**: 267–292

NRC. 1989. "Diet and health". Natl. Res. Council. National Academy Press, Washington, D. C.

Okuda, T. and K. Yokotsuka. 1996. trans-Resveratrol concentrations in berry skins and wines from grapes grown in Japan. Amer. J. Enol. Viticult. 47:93-99.

Ou HC, Chou FP, Sheen HM, Lin TM, Yang CH, Sheu WHH(2006). *Clin Chim Acta* ; **364**: 196–204.

Paceasciak, C. R.; Hahn, S.; Diamandis, E. P.; Soleas, G.; Goldberg, D. M(1995). *Clin. Chim. Acta*, *235*, 207–219.

Pineiro, Z.; Palma, M.; Barroso, C. G(2006). *J. Chromatogr. A*, *1110*, 61–65

Pinto, M. C.; Garcia-Barrado, J. A.; Macias, P.(1999) *J. Agr. Food Chem*, *47*, 4842–4846.

Pool, R., L. Creasy, L. and, A. Frackelton. 1981. Resveratrol and the viniferins, their application to screening for disease resistance in grape breeding programs. Vitis 20:136-145.

Kirimlioglu H, Ecevit A, Yilmaz S, Kirimlioglu V, Karabulut AB(2008). *Transpl* P; **40**: 285–289

Kirk, R. I.; Deitch, J. A.; Wu, J. M.; Lerea, K. M.(2000) *Blood Cell. Mol. Dis. 26*, 144–150.

Kuck, D.(2002) *Int. J. Mass Spectrom. 213*, 101–144.

Kumar A, Kaundal RK, Iyer S, Sharma SS(2007) *Life Sci*; **80**: 1236–1244

Raffaelli, A.; Perri, E.; Sindona, G.; Salvatori, P.(2001) Determination of Resveratrol in Red Wines by Flow Injection Analysis and Tandem Mass Spectrometry with Atmospheric Pressure Chemical Ionization. In *Advances in Mass Spectrometry*, VOL 15. E. Gelpi; John Wiley & Sons: Chirchester, pp 901–902

Remsberg, C. M.; Yáñez, J. A.; Roupe, K. A.; Davies N. M.(2007) *J. Pharmaceut. Biomed. , 43*, 250–254.

Renaud, S. and M. de Lorgeril. 1992. Wine, alcohol, platelets and the French paradox for coronary heart disease. The Lancet. 339:1523-1526.

Romero-Perez, A., R. Lamuela-Raventos, S. Buxaderas and, M. Torre-Boronat. 1996a. Resveratrol and piceid as varietal markers of white wine. J. Agr. Food Chem. 44:1975-1978.

Romero-Perez, A., R. Lamuela-Raventos, A. Waterhouse and, M. Torre-Boronat. 1996b. Levels of cis and trans resveratrol and their glucosides in white and rose *Vitis vinifera* wines from Spain. J. Agr. Food Chem. 44:2124-2128.

Romero-Perez, A., M. Ibern-Gomez, R. Lamuela-Raventos and, M. de la Torre-Boronat. 1999. Piceid, the major resveratrol derivative in grape juices. J. Agr. Food Chem. 47:1533- 1538. 24

Romero-Perez, A., R. Lamuela-Raventos, C. Andres-Lacueva and, M. Torre-Boronat. 2001. Method for the quantitative extraction of resveratrol and piceid isomers in grape berry skins. Effect of powdery mildew on the stilbene content. J. Agr. Food Chem. 49:210- 215.

Rychlik M, Asam S(2008). *Anal Bioanal Chem* ; **390**: 617–628.

Shan, C., S. Yang, H. He, S. Shao and, P. Zhang. 1990. Influences of 3,4,5-trihedroxystilbene- 3-beta-mono-D-glucoside on rabbits platelet aggregation and thromoxane B2 production in vitro. Acta Pharmacol. Sin. 11:527-530.

Siemann, EH, Creasy LL(1992). *Am J Enol Vitic* ; **43**: 49–52.

St Leger, A. S.; Cochrane, A. L.; Moore, F.(1979) *Lancet 314*, 1017–1020

Striegler, R., P. Carter, J. Morris, J. Calark, R. Threlfall and, L. Howard. 2005. Yield, quality and nutriceutical potential of selected muscadine cultivars grown in southwestern Arkansas. HortTechnology. 15:276-284.

Tedesco, I., M. Russo, P. Russo, G. Iacomino, G. Russo, A. Carraturo, C. Faruolo, L. Moio and, R. Palumbo. 2000. Antioxidant effect of red wine polyphenols on red blood cells. L. Nutr. Biochem. 11:114-119.

Trincheri NF, Nicotra G, Follo C, Castino R, Isidoro C(2007). *Carcinogenesis* ; **29**: 922–931.

Tinchieri NF, Follo C, Zhang W, Fei Z, Zhen HN, Zhang JN, Zhang X(2007). *J Neuro-oncol* , **8**: 231–240.

Vougioukalakis GC, Chronakis N, Orfanopoulos M(2003). *Org Lett* ; **5**: 4603-4606.

Wang, D.; Hang, T.; Wu, C.; Liu, W.(2005) *J. Chromatogr. B* **.***829*, 97–106.

Wang, Y.; Catana, F.; Yang, Y. N.; Roderick, R.; van Breemen,(2002) R. B. *J. Agr. Food Chem. 50*, 431–435.

Waterhouse, A. and R. Lamuela-Raventos. 1994. The occurance of piceid, a stilbene glucoside, in grape berries. Phytochemistry 37:571-573.

Wenzel, E.; Soldo, T.; Erbersdobler, H.; Somoza, V.(2005) *Mol. Nutr. Food Res. 49*, 482–494

Yang, R. T.; Zhou, S. Y.; Zhang, X.; Teng, Z. H.; Zhang, B. L.; Liu, X. Y.; Yang, X.;(2007) Zhang, L. J.; Mei, Q. B. *Chinese, J. Anal. Chem. 35*, 1309–1313.

Zhu, Z. M.; Klironomos, G.; Vacherau, A.; Neirinck, L.; Goodman, D. W(1999). *J. Chromatogr. B, 724*, 389–392

Urpi-Sarda, M.; Jauregui, O.; Lamuela-Raventos, R. M.; Jaeger, W.; Miksits, M.; Covas, M. I.; Andres-Lacueva, C(2005). *Anal. Chem. 77*, 3149–3155.

CHAPTER 3

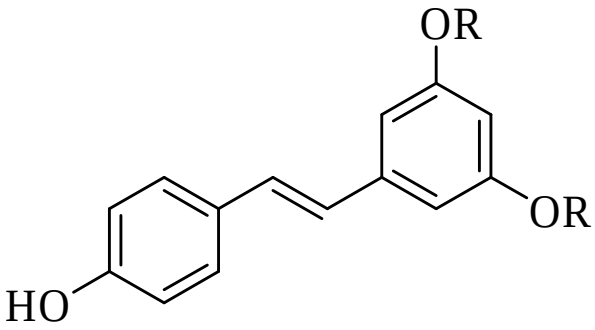
The assay of Pterostilbene in spiked matrices by liquid chromatography tandem mass spectrometry and isotope dilution method

Chapter 3

3.1 Introduction

Pterostilbene (trans-3,5-dimethoxy-4-hydroxystilbene), a dimethylether analogue of resveratrol is a naturally occurring phytoalexin identified in several plant species exhibiting important pharmacological properties (USDA Agricultural Research Magazine, 2006). It belongs to a group of phenolic compounds known as stilbenes, found in deerberry and rabbiteye blueberries (Rimando , Kalt, Magee, Dewey & Ballington, 2004), several varieties of grapes (Adrian, Jeandet, Douillet-Breuil, Tesson & Bessis, 2000; Douillet-Breuil, Jeandet, Adrian & Bessis, 1999); it has been also identified in the leaves of *Vitis vinifera* (Langcake, Cornford & Pryce, 1979) and in the Pterocarpus marsupium (Manickam, Ramanathan, Jahromi, Chansouria & Ray, 1997) and Guibourtia Tessmanii plant (Fuendjiep et al., 2002), used in traditional medicine. In particular the extract of Pterocarpus marsupium, used in Ayurvedic medicine, is known for its antiglycemic effect. It is a strong antioxidant, and is reported to scavenge 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radicals and to inhibit either the oxidation of citronellal, and lipid peroxidation in rat liver microsomes (Stivala et al., 2001). Pterostilbene is cytotoxic against a number of cancer cell lines, including human breast cancer and murine lymphoid neoplasma cells (Rimando, Cuendet, Desmarchelier, Mehta, Pezzuto & Duke, 2002; Rimando, Pezzuto, Farnsworth, Santisuk & Reutrakul, 1994); moreover it has been demonstrated that it may reduce cholesterol levels (Rimando, Nagmani, Feller & Yokoyama, 2005; Remsberg, Yáñez, Ohgami, Vega-Villa, Rimando & Davies, 2008). Animal studies have shown that this natural compound can lower blood glucose and may be a potent antidiabetic agent (Manickam, Ramanathan, Jahromi, Chansouria & Ray, 1997). The quantitation of pterostilbene in biological fluids is currently performed by HPLC coupled to fluorimetric detection (Remsberg, Yáñez, Roupe & Davies, 2007). In this work we present a rapid determination of pterostilbene in blueberries juice and human serum by LC MS under MRM condition and isotope dilution. The hexadeutero analogue of pterostilbene **2**, is used as labelled internal standard. Isotope dilution represents an extremely accurate method of quantitation of analytes in complex natural and biological matrices by mass spectrometry, and is best suited to improve precision and accuracy by reducing the

problems arising from calibration procedure, sample preparation, and matrix effects (De Nino et al., 2005; Mazzotti et al., 2008; Mazzotti, Di Donna, Macchione, Maiuolo, Perri & Sindona, 2009).



1. R=CH_3 Pterostilbene
2. R=CD_3 Pterostilbene-d_6

3.2 Experimental

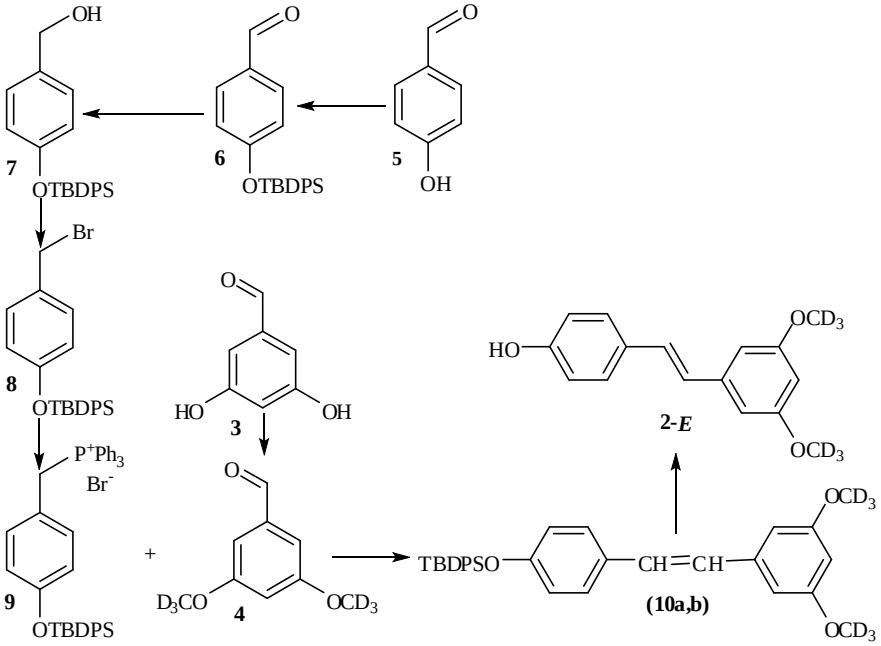
3.2.1 Synthesis of d_6-pterostilbene:

3,5 dimethoxybenzaldehayde (**4**): the commercially available 3,5-dihydroxybenzaldehyde (**3**) (1 g, 7.2 mmole) and anhydrous K_2CO_3 (20 g, 145 mmole) were stirred in dry acetone (50 mL); 7 mL (97 mmole) of CD_3I were then added. The mixture was heated under reflux for 45 min then cooled to room temperature, filtered and evaporated to dryness. The residue dissolved in CH_2Cl_2, was washed twice with water, dried over anhydrous Na_2SO_4, filtered and purified by flash chromatography (silica gel, hexane/ethyl acetate 4:1, v/v) affording **4** with 93% yield.

4-(tert butyldiphenylsilyloxy)-benzyltriphenylphosphonium bromide **9** (3 g, 11.9 mmole), synthesized as reported (Pettit et al., 2002) was treated at -78 °C, in anhydrous tetrahydroufan (35 mL), with n-butyllithuim (2.5 M, 3 mL). The dialdehyde **4** (1.5 g, 9.3 mmole) dissolved in 5 mL of tetrahydrofuran, was then added. The crude product purified by flash chromatography (silica gel, hexane/ethylacetate 8:2, v/v) yielded 90% of a mixture of E/Z isomers (10 a, b) .

Trans-pterostilbene-d_6 (**2**): To a 1.2 g (2.45 mmole) of (10a,b) dissolved in 20 ml of anhydrous tetrahydrofuran, 3.4 ml of tetrabutylammonium fluoride (1M) were added. The pale yellow solution, stirred for 45 min and poured into water was extracted with dichloromethane. The organic phase, dried on anhydrous sodium

sulphate, was evaporated to dryness to afford an E/Z *trans*-pterostilbene-d_6 isomeric mixture. The latter, after separation by flash chromatography (silica gel, hexane/ethyl acetate 9.5:0.5, v/v) yielded 70% , based on **4**, of pure trans-pterodtilbene-$d_{6,}$ (**2** scheme 1) (Pettit et al., 2002)



Scheme3.1 ***Synthesis of d_6-pterostilbene***

The isotopic distribution, checked by high resolution mass spectrometry, was d_5 = 2%, d_6 = 98%.

The structure of deuterated pterostilbene **2** was assigned by MS and ^{1}H NMR .

^{1}H NMR (300 MHz, $CDCl_3$): δ_H 7.39 (2H, m, *J* = 8.6 Hz, H-2',H-6'), δ_H 7.02 (1H, distorted d, *J* = 16.5 Hz, HC=CH), δ_H 6.87 (1H, distorted d, *J* = 16.5, HC=CH), δ_H 6.82 (2H, m, *J* = 8.6 Hz, H-2, H-6), δ_H 6.63 (2H, m, *J* = 8.6 Hz, H-3', H-5'), δ_H 6.37 (1H, m, *J* = 2.1 Hz, H-4), δ_H 5 (1H, br s, OH).

HRESI-MS: *m/z* 263.1553 $[M+H]^+$ calculated for $C_{16}H_{11}D_6O_3$ 263.1548.

***3.2.2 Sample Preparation*:**

***3.2.2.1 Juice**:* To 1 ml of juice diluted 1:2 with water 12.5 µl of 4 ppm solution of the internal standard was added; the resulting solution was passed trough 0.22 mm HPLC filter and used for subsequent analysis.

3.2.2.2 Plasma: To 1 ml of human plasma, spiked with compound **1**, 12.5 µl of 4 ppm solution of the internal standard were added; the mixture was treated with 1,5 ml of cold acetonitrile to precipitate the protein and centrifuged at 8000 rpm for 5min. The supernatant, transferred to other Eppendorf tubes, was evaporated to dryness under nitrogen gas, reconstituted with 0.5 ml of mobile phase, passed trough 0.22 mm HPLC filter and used in the next step. (Remsberg, Yáñez, Roupe & Davies, 2007)

3.2.3 Mass Spectrometry. The LC-MS analysis was carried out with a triple-quadrupole mass spectrometer LC 320 (Varian Inc., Palo Alto, CA), equipped with an ESI source interfaced with an HPLC Prostar 210 (Varian Inc.). The chromatographic analysis was performed with a C_{18} column (75 × 2.1 mm, Discovery, Supelco Bellefonte, PA). The flow rate was fixed at 0.25 mL min^{-1} using the following eluents and linear gradient: solvent A (H_2O, 0.025% Ammonium hydroxide), solvent B (MeOH); from 25% B to 98% B in 7 min; 2 min at 98% B isocratic; from 98% B to 25% B in 2 min. A 3 min of re-equilibration time was used after each analysis. The parameters of needle, shield and capillary were set at -4.5 KV, 600 V, and 70 V, respectively; whereas drying (N_2) and nebulizing gas (air), housing temperature and electron multiplier were set at 20 psi and 250 °C , 45 psi, at 60°C and at 1350 V, respectively. The dwell time was 0.200 s/scan, and the resolution was set using a mass peak width of 0.8 amu. The collision gas pressure (Ar) was fixed at 2 mTorr, and the collision energy was set to 30 eV for the transition *m/z* 255 → *m/z* 197 and *m/z* 261 → *m/z* 197, while for the transition *m/z* 255 → *m/z* 240 and *m/z* 261 → *m/z* 243 the collision energy was set to 18 eV.

High-resolution electrospray ionization (ESI) experiments were carried out in a hybrid Q-Star Pulsar-i (MSD Sciex Applied Biosystem, Toronto, Canada) mass

spectrometer equipped with an ion spray ionization source. Samples were introduced by direct infusion (3 μL/ min) of the sample containing the analyte (5 ppm), dissolved in a solution of 0.1% acetic acid, acetonitrile/water 50:50 at the optimum ion spray (IS) voltage of 4800 V. The source nitrogen (GS1) and the curtain gas (CUR) flows were set at pressures of 20 and 25 psi, respectively, whereas the first declustering potential (DP1), the focusing potential (FP), and the second declustering potential (DP2) were kept at 50, 220, and 10 V relative to ground, respectively.
Nuclear Magnetic Resonance. The NMR spectra were recorded from an AC 300 Spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) in $CDCl_3$; chemical shifts were measured in ppm and coupling constants (J) in Hz.

3.3 Analytical Parameters.

The limit of detection (LOD) and the limit of quantitation (LOQ) for each foodstuff were calculated by applying equations equations1 and 2, according to the directives of IUPAC and the American Chemical Society's Committee on Environmental Analytical Chemistry.

$S_{LOD} = S_{RB} + 3\sigma_{RB}$ (**1**)

$S_{LOQ} = S_{RB} + 10\sigma_{RB}$ (**2**)

S_{LOD} is the signal at the limit of detection, S_{LOQ} is the signal at the limit of quantitation, S_{RB} is the signal of the uncontaminated food matrixes (organic), and σ_{RB} is the standard deviation for uncontaminated matrixes calculated on seven measurements. The concentrations were obtained from the appropriate calibration curve

3.4 Results and discussion

Tandem mass analysis provides the specificity that allows unambiguous correlation between parent and product ions, moreover the good-to-excellent signal-to-noise (S/N) ratio confers the methodology the sensitivity to perform trace analysis in complex mixtures, through the well established multiple reaction monitoring (MRM)

technique. APCI-MS/MS has been recently applied to recognize the product of combinatorial synthesis of stilbenoids (Jerkovic, Nguyen & Nizet, 2007).

In the case under investigation ESI method was chosen to ionize the species eluted in the first chromatographic stage and both positive (+) and negative (-) modes were checked. As expected, the ESI (+) MS/MS spectrum of the $[M+H]^+$ unlabelled compound **1** provides a wealth of information displaying a complex fragmentation pattern, whose first diagnostic species was represented by the unimolecular elimination of methyl radical to give the species at *m/z* 242 (figure 3.1)

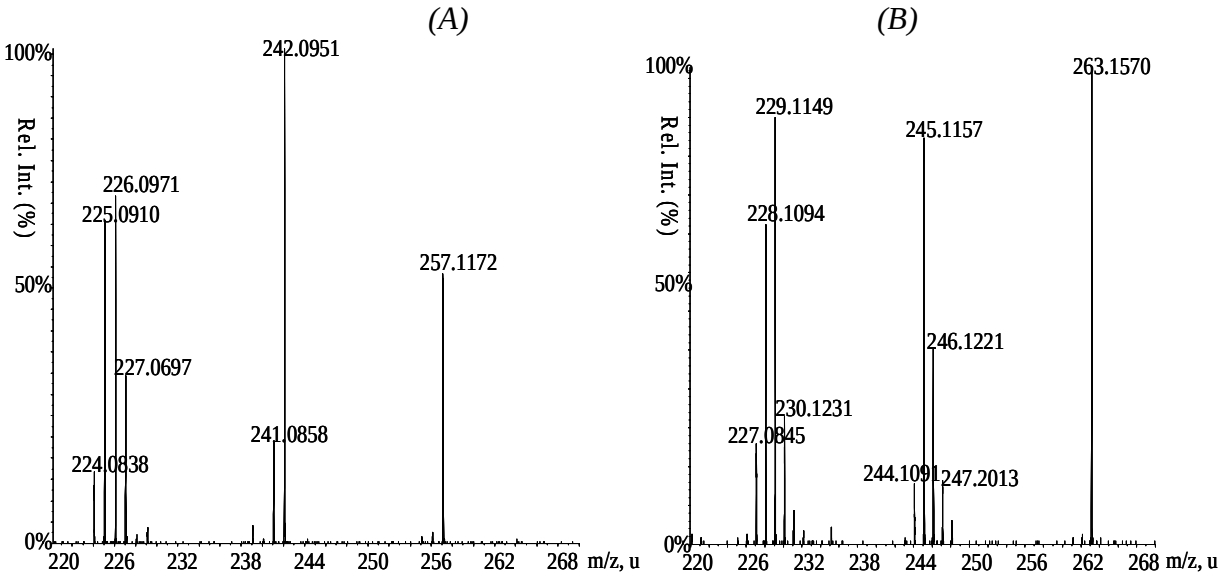
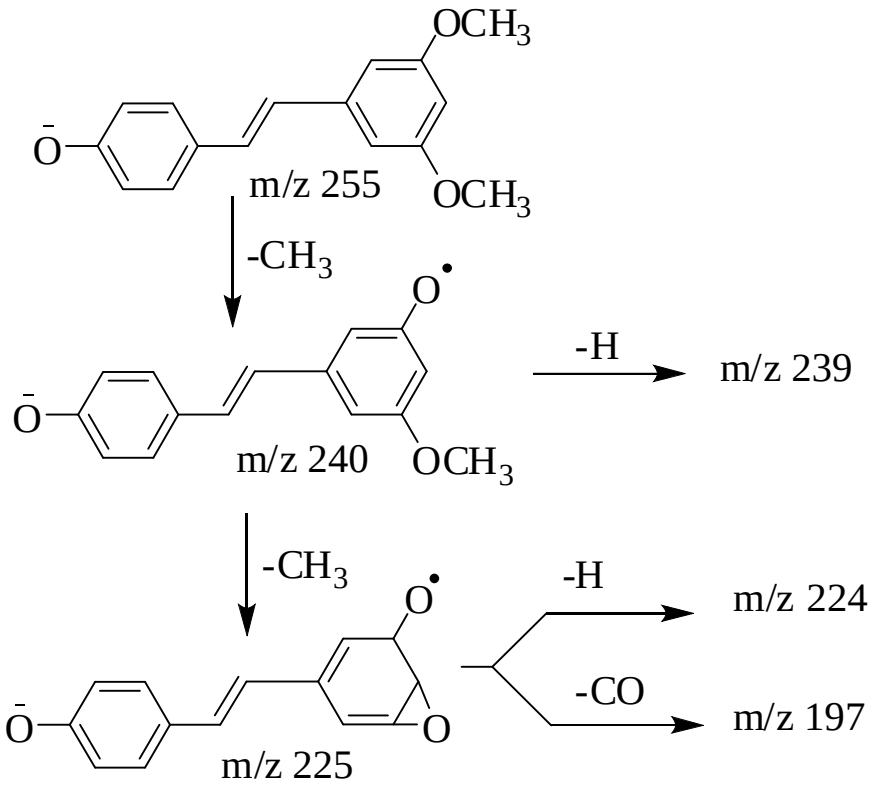


Figure3. 1. Partial ESI (+) MS spectra of compounds 1 (A) and 2 (B)

The number of peaks arising from competitive and consecutive hydrogen radical losses from the protonated molecular ions under investigations (figure 1 A), within reacting configurations which can be easily isomerise among them, represents the major drawback for a straightforward application of MRM quantitation method.

This observation is supported by the chemistry of the $[M+H]^+$ of the d_6 reference compound **2** (figure 3.1). It can be easily observed, in fact, that the elimination of d_3-methyl radical from the precursor specie at *m/z* 263, competes with an extensive H/D isomerization giving rise, besides the $[(M-CD_3)+H)]^{+\bullet}$ species at *m/z* 245, to the elimination of CHD_2 and CH_2D radicals. The fragmentation pattern is even more complex for the other consecutive and competitive reaction paths taken by the reactive species.

The ESI (-) MS/MS spectrum of the $[M-H]^-$ deprotonated ion of the unlabelled compound **1** shows only few diagnostic fragment ions at *m/z* 240, 239 225, 224 and 197. The gas phase chemistry of the labelled isomer 2 clearly shows that no hydrogen/deuterium isomerization competes with the observed fragmentation pathways (scheme 3.2 and figure 3.2).



Scheme3.2 Fragmentation pattern of $[M+H]^-$ ions of Pterostilbene

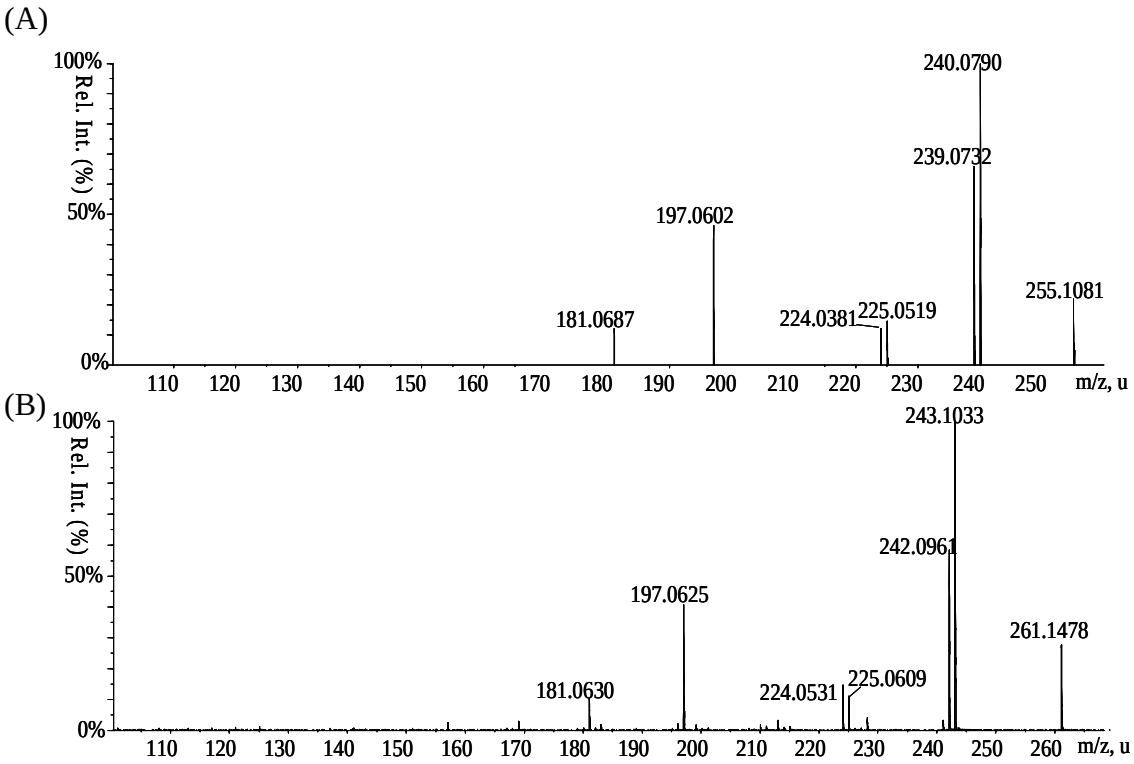


Figure3. 2 ESI (-) MS/MS spectra of compound 1 (A) and 2 (B)

The deprotonated species at *m/z* 255 undergoes competitive and consecutive radical and neutral losses not involving H/D isomerization. The elimination of CH_3 ($CD_3^{\cdot}$) radicals gives rise to a resonance stabilized distonic radical anion (Roth, 2004). The consecutive elimination of the second methyl group may likely lead to the distonic species suggested in scheme 2 which further fragments through carbon monoxide elimination to give the species at *m/z* 197, common to both labelled and unlabelled pterostilbene.

The assay of analyte **1** was, therefore, performed under MRM condition in the negative ion mode selecting the transitions with the highest specificity. Accordingly the following pathways: *m/z* 255 → *m/z* 240 and *m/z* 255 → *m/z* 197 for 1 and *m/z* 261 → *m/z* 243, *m/z* 261 → *m/z* 197 for the labelled internal standard were chosen, respectively. The assay of the analyte in the spiked matrices was performed by means of a calibration curve ($y = 1.3191x - 0.2974$; $R^2 = 0.9982$), built using triplicate samples of six standard solution at different standard concentration of pterostilbene ranged from 5 ppb to 200 ppb, while

those of the internal standard were fixed at 50 ppb. The new protocol has been applied to a spiked sample of blueberries juice and human plasma. The RSD% value was in all cases under 7%, thus showing a good repeatability of the measurements. The accuracy of the method was determined from samples prepared by adding known quantities of the analyte to blank matrices. In the two examined point (Table 1), which represent the borders of the calibration curve, the accuracy was higher than 95%.

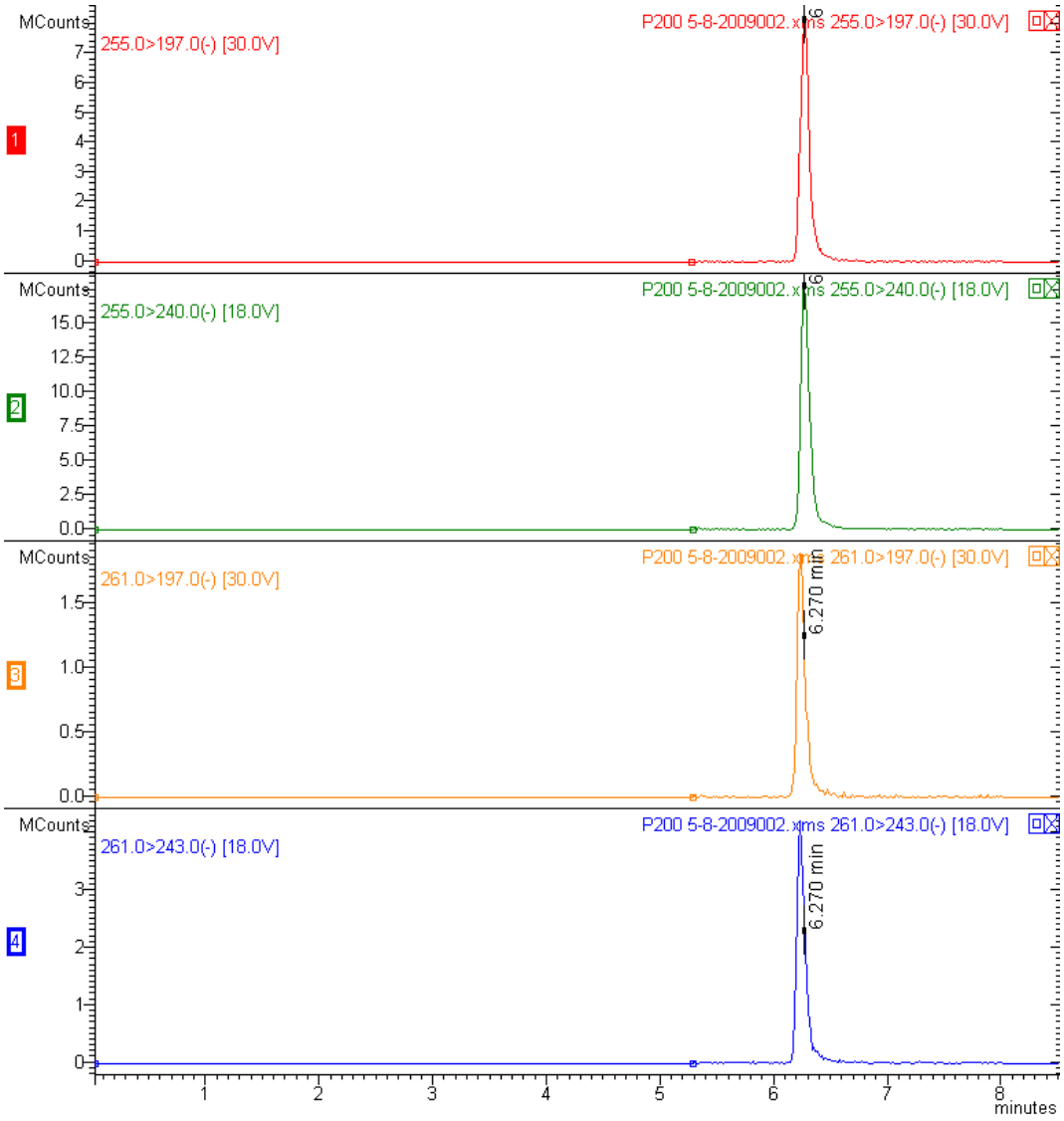


Figure3.3 The representative mass chromatograms MRM in negative ion mode of compound 1 m/z 255 and 2 m/z 261 in juice sample

***Table 3. 1**. Analytical parameters of precision and accuracy*

Ppb	Calculated concentration	RSD % (average)	Accuracy % (average)
		Juice	
15	16.0 ± 0.5	3.0	109.6
150	146.8 ± 4.7	4.7	97.9
		Plasma	
15	13.9 ± 0.9	6.4	92.6
150	152.3 ± 6.1	4.0	101.3

The standard deviation (RSD%) was calculated over three measurements.

The calculated analytical parameters confirm the appropriateness of this proposed approach. The values of LOD and LOQ, ranged from 1.5 ppb to 3.9 ppb for blueberry juice and 2.2 to 3.8 for human plasma, suggesting that the proposed technique is suitable for evaluating very low amounts of Pterostilbene in different matrices. The values of recovery are quantitative both for juice and for plasma (Remsberg, Yáñez, Roupe & Davies, 2007). In particular, in the case of juice, in fact, the samples are directly injected, after dilution, thus confirming the efficacy of the procedures; besides, the reproducibility is in all cases below 10% (table 3.2).

Blueberries Juice		
LOQ (ppb)	3.9 ppb	Reproducibility (RSD%)
LOD (ppb)	1.5 ppb	9.2
Recovery	98%	
Plasma		
LOQ (ppb)	4.1 ppb	Reproducibility (RSD%)
LOD (ppb)	2.2 ppb	8.5
Recovery	98%	

Table 3.2. *Reproducibility (RSD %) and analytical parameters LOQ, LOD and recovery of the proposed method*

3.5 Conclusions

Pterostilbene (**1**) exhibits a wide spectrum of biological function. Assessment procedures for the determination of this natural drug in natural matrices are based on the conventional use of chromatographic techniques linked to fluorescence detection.[13] We now propose a different analytical protocol centred on the specificity of tandem mass spectrometry and on the reliability of the isotope dilution method. The procedure here presented allows the determination of the analyte with excellent specificity, due to the application of MRM methods, and excellent LOQ and LOD parameters, due to the use of the labelled internal standard **2**.

3.6 References

Adrian, M., Jeandet, P., Douillet-Breuil, A. C., Tesson, L., & Bessis, R. (2000) Stilbene content of mature Vitis vinifera berries in response to Uv-C elicitation. *Journal of Agricultural and Food Chemistry, 48*, 6103 6105.

De Nino, A., Di Donna, L., Mazzotti, F., Muzzalupo, E., Perri, E., Sindona, G., et al. (2005) Absolute method for the assay of oleuropein in olive oils by atmospheric pressure chemical ionization tandem mass spectrometry. *Analytical Chemistry, 77*, 5961 5964.

Douillet-Breuil, A. C., Jeandet, P., Adrian, M., & Bessis, R. (1999) Changes in the phytoalexin content of various Vitis spp. in response to ultraviolet C elicitation. *Journal of Agricultural and Food Chemistry, 47*, 4456 4461.

Fuendjiep, V., Wandji, J., Tillequin, F., Mulholland, D. A., Budzikiewicz, H.,

Fomum, Z. et al. (2002) Chalconoid and stilbenoid glycosides from Guibourtia tessmanii. *Phytochemistry, 60*, 803 806.
Jerkovic, V., Nguyen, F., Nizet, S., & Collin, S. (2007) Combinatorial synthesis, reversed-phase and normalphase high-performance liquid chromatography elution data and liquid chromatography/positive atmospheric pressure chemical ionization tandem mass spectra of methoxylated and glycosylated resveratrol analogues. *Rapid Communications in Mass Spectrometry, 21*, 2456 2466.

Langcake, P., Cornford, C. A., & Pryce, R. J. (1979) Identification of pterostilbene as a phytoalexin from *Vitis vinifera* leaves. *Phytochemistry, 18*,1025 1027.

Manickam, M., Ramanathan, M., Jahromi, M. A., Chansouria, J. P., & Ray, A. B. (1997) Antihyperglycemic activity of phenolics from Pterocarpus Marsupium. *Journal of Natural Products, 60*, 609 610.

Mazzotti, F., Di Donna, L., Maiuolo, L., Napoli, A., Salerno, R., Sajjad, A., et al. (2008) Assay of the set of all sudan azodye (I, II, III, IV, and para-red) contaminating agents by liquid chromatography-tandem mass spectrometry and isotope dilution methodology. *Journal of Agricultural and Food Chemistry, 56*, 63 67.

Mazzotti, F., Di Donna, L., Macchione, B., Maiuolo, L., Perri, E., & Sindona G. (2009) Screening of dimethoate in food by isotope dilution and electrospray ionization tandem mass spectrometry. *Rapid Communications in Mass Spectrometry, 23*, 1515 1518.

Pettit, G. R., Grealish, M. P., Jung, K., Hamel, E., Pettit, R. K., Chapuis, J. C., et al. (2002) Antineoplastic agents. 465. Structural modification of resveratrol: sodium resverastatin phosphate. *Journal of Medicinal Chemistry, 45*, 2534 2542.

Remsberg, M., Yáñez, J. A., Ohgami, Y., Vega-Villa, K. R., Rimando, A. M., & Davies N. M. (2008) Pharmacometrics of pterostilbene: preclinical pharmacokinetics and metabolism, anticancer, antiinflammatory, antioxidant and analgesic Activity. *Phytotherapy Research, 22*, 169 179.

Remsberg, C. M., Yáñez, J. A., Roupe, K. A., & Davies N. M. (2007) High-performance liquid chromatographic analysis of pterostilbene in biological fluids using fluorescence detection. *Journal of Pharmaceutical and Biomedical Analysis, 43*, 250 254.

Rimando, A. M., Cuendet, M., Desmarchelier, C., Mehta, R. G., Pezzuto, J. M., & Duke, S. O. (2002) Cancer chemopreventive and antioxidant activities of pterostilbene, a naturally occurring analogue of resveratrol. *Journal of Agricultural and Food Chemistry, 50*, 3453 3457.

Rimando, A. M., Kalt, W., Magee, J. B., Dewey, J., & Ballington, J. R. (2004) Resveratrol, pterostilbene, and piceatannol in vaccinium. *Journal of Agricultural and Food Chemistry, 52*, 4713 4719.

Rimando, A. M., Nagmani, R., Feller, D. R., & Yokoyama, W. (2005) Pterostilbene, a new agonist for the peroxisome proliferator-activated receptor alpha-isoform, lowers plasma lipoproteins and cholesterol in hypercholesterolemic hamsters. *Journal of Agricultural and Food Chemistry, 53*, 3403 3407.

Rimando, A. M., Pezzuto, J. M., Farnsworth, N. R., Santisuk, T., & Reutrakul, V. (1994) Revision of the NMR assignments of pterostilbene and of dihydrodehydrodiconiferyl alcohol: Cytotoxic constituents from Anogeissus acuminata. *Natural Product Letters*, *4*, 267 272.

Roth, H. D. (2004) Organic Radical Anions. In A. R. Moss, M. S. Platz, & M. Jones Jr, *Reactive Intermediate Chemistry* (pp. 205 272). New York: Wiley Interscience.

Stivala, L.A., Savio, M., Carafoli, F., Perucca, P., Bianchi, L., Maga, G., et al. (2002). Specific structural determinants are responsible for the antioxidant activity and the cell cycle effects of resveratrol. *Journal of Biological Chemistry, 276*, 22586 22594.

USDA Agricultural Research Magazine, 2006, *54*, 6.

CHAPTER 4

Screening of quality marker in olive oil by isotope dilution and mass spectroscopy

Chapter 4

4.1 Introduction:

The olive tree is a familiar feature of the Mediterranean landscape, however, may have been originated from Syria, Asia Minor, Ethiopia, Egypt, or India. Since ancient times, it has contributed, in practical and symbolic terms, to the economy, health and "haute cuisine" of the inhabitants of the Mediterranean. The culture of the olive tree has three aspects: the landscape itself, diet (consisting mainly of the use of oil), and the symbolic importance of the tree and its fruit. All these aspects have been the subject of intense discussion over recent decades (Polymerou-Kamilakis, 2006). Olive oil has been produced for over 6000 years, however, only in the last thirty years there has been a growing interest in use of olive oil in cooking due to its greater importance for Mediterranean food and an awareness of the healthy virtues of a Mediterranean diet, in particular of VOO (Grigg, 2001; Helsing, 1993). Among the different vegetable oils, VOO is unique since it is obtained from the olive fruit (Olea europaea L.) "(…) solely by mechanical or other physical methods of processing under conditions that help to avoid alteration of the oil without applying any treatments other than washing, decantation, centrifugation or filtration, by excluding oils obtained by using solvents or using adjuvant which have chemical or biochemical action, or by re-esterification process and any mixture with oils of other kinds (…)" (CR 1513/2001). Olive oil can be consumed in its natural unrefined state or as a refined product. The refined product is made either of VOO which considered being inedible for their chemical composition (lampante) and called refined olive oil (ROO) or olive pomace after solvent-extraction and refining which is called refined olive-pomace oil (ROPO). The ROOs and ROPOs are marked with edible VOOs after blending (CR 1513/2001). The chemical composition of VOO consists of major and minor components. The major components, that include glycerols, represent more than 98% of the total weight. Abundance of oleic acid, a monounsaturated fatty acid, is the feature that sets olive oil apart from other vegetable oils. In particular, oleic acid (18:1 n-9) ranges from 56 to 84% of total fatty acids (Rossell, 2001), while linoleic acid (18:2 n-6) is a major essential polyunsaturated fatty acid in human diet can vary between 3 and 21% Tiscornia *et al*., 1982; Visioli *et al*., 1998). Minor components, which represent about 2% of the total oil weight, include

more than 230 chemical compounds, such as aliphatic and triterpenic alcohols, sterols, hydrocarbons, volatile compounds, and antioxidants (Servili *et al*., 2002). Introduction 8 The main antioxidants of VOO are carotenes and phenolic compounds, including lipophilic and hydrophilic phenols (Boskou, 1996). The tocopherols which also represent lipophilic phenols can be found in other vegetables oils, while some hydrophilic phenols of VOO are not generally present in other oils and fats (Boskou, 1996; Shahidi, 1997).

4.2 Phenolic compounds in VOO

Polyphenols is a broad term used in the natural products literature to define substances that possess a benzene ring bearing one or more hydroxy groups, including functional derivatives (Harborne, 1989). Phenolic compounds present in VOO are also commonly named as biophenols (Uccella, 2001. According to Harborne *et al.* (1989) phenolic compounds are grouped into the following categories:

1. Phenols, phenolic acids, phenylacetic acids;
2. cinnamic acids, coumarins, isocoumarins and chromones;
3. lignans;
4. ten group of flavonoids;
5. lignins;
6. Tannins;
7. benzophenones, xanthones, and stilbenes;
8. quinones;
9. betacyanins.

Most phenolic compounds are found in nature in a conjugated form, mainly with sugar molecules. In case of VOOs, “polyphenols” are mostly referred to hydrolysis products of oleuropein and ligstroside, aglycones, and other related compounds. The phenolic fraction of VOO consists of a heterogeneous mixture of compounds; each one varies in chemical properties and has a particular influence on the quality of VOO (Psomadiou *et al*., 2003). The occurrence of hydrophilic phenols in VOO was observed more than 40 years ago by Cantarelli and Montedoro (1961; 1969). They established a set of research priorities related to polyphenols which remain practically unchanged up to date: – development of an analytical procedure to quantify phenolic compounds in oils; – estimation of phenolic compound levels in vegetables oils; – possible relationship

between these compounds and the characteristics of the olive fruit (variety, degree of ripeness); – effect of extraction technology and refining process on polyphenol levels; – importance of phenolic compounds as natural antioxidants; – possible role of polyphenols in justifying a considerable stability of olive oils with high peroxide values. Points mentioned above still have not been clear for many researchers and require much more work to be carried out. However, some recent interesting systematic studies have shown the development of the individual classes of hydrophilic phenols in VOO, accordingly, it is possible to claim that the composition of VOO is largely elucidated (Carrasco-Pancorbo *et al*., 2005). VOO contains different classes of phenolic compounds such as phenolic acids, phenolic alcohols, flavonoids, hydroxy-isocromans, secoiridoids, and lignans. Phenolic acids with basic chemical structure of C6-C1 (benzoic acids) and C6-C3 (cinnamic acids), such as caffeic, vanillic, syringic, *p*-coumaric, *o*-coumaric, protocatechuic, sinapic, and *p*-hydroxybenzoic acid, were the first group of phenols observed in VOO Montedoro, 1972; Vasquez-Roncero, 1978). Several authors confirmed the occurrence of phenolic acids as minor components in VOO (Cortesi *et al*., 1983; Solinas 1987; Montedoro *et al*., 1992; Tsimidou *et al*., 1996; Mannino *et al*., 1993; Carrasco-Pancorbo *et al*., 2004). Phenols present in VOO are secoiridoids, characterized by the presence of either elenolic acid or elenolic acid derivatives in their molecular structure (Garrido Fernandez Diez *et al*., 1997). These compounds, e.g., oleuropein, demethyloleuropein, and ligstroside, are derivatives of the secoiridoid glycosides of olive fruit. Breakdown products of two major phenolic constituents of the olive fruit, oleuropein and ligstroside derive form the majority of the phenolic fraction. The most abundant secoiridoids of VOO are the dialdehydic form of elenolic acid linked to hydroxytyrosol = (3,4-dihydroxyphenyl)-ethanol or tyrosol = (*p*-hydroxyphenyl)-ethanol (3,4-DHPEA-EDA or *p*-HPEA-EDA) and an isomer of the oleuropein aglycone (3,4-DHPEA-EA). These compounds were discovered by Montedoro *et al.* (1992) who also assigned their chemical structure (Montedoro *et al*., 1993) which was confirmed by other authors in late studies (Angerosa *et al*., 1996). Recent studies have also determined oleuropein and ligstroside present in VOO in glycosidic forms (Owen *et al*., 2000; Perri, 1999). Hydroxytyrosol and tyrosol are the main phenolic alcohols in VOO. It is also possible to find in VOO hydroxytyrosol acetate (Brenes *et al*., 1999), tyrosol acetate (Mateos *et al*., 2001), and a glucosidic form

of hydroxytyrosol (Bianco *et al*., 1998). Several authors have reported that flavonoids such as luteolin and apigenin were also phenolic components of VOO (Rovellini *et al*., 1997; Vazquez-Roncero *et al*., 1976). (+)- Taxifolin, a flavanonol, has recently been found in Spanish VOO (Carrasco-Pancorbo *et al*., 2004). The last group of phenols identified in VOO were lignans; Owen *et al.* (2000) and Brenes *et al.* (2000) have recently isolated and characterized (+)-1-acetoxypinoresinol, (+)- pinoresinol, and (+)-1-hydroxypinoresinol as the most frequent lignans in VOO. Some authors have indicated lignans as the main phenolic compounds in VOO. A new class of phenolic compounds, the hydroxy-isochromans, was found in different samples of Extra Virgin Olive Oil (EVOO).

4.3 The family of phenolic compounds : their antioxidant, health, and sensory properties

The antioxidant power of phenolic compounds in olive oil has also been a subject of considerable interest, due to their both chemoprotective effect on human health (Leenen *et al*., 2002; Vissers *et al*., 2001; Briante *et al*., 2001; Petroni *et al*., 1995; Caponio *et al*., 1999; Caponio *et al*., 2001) and being a major factor in high stability (shelf-life) of olive oils (Caponio *et al*., 1999; Caponio *et al*., 2001; Tsimidou, 1998; Baldioli *et al*., 1996; Velasco *et al*., 2002). The antioxidant activity of VOO components related to their ability to protect against important chronic and degenerative diseases such as coronary hearth diseases (CHD), ageing neuro-degenerative diseases, and tumours of different localizations (Soler *et al*., 1998; Franceschi *et al*., 1999; Hodge *et al*., 2004). Among these protective effects, it is possible to highlight the protection of low density lipoprotein (LDL) oxidation (Visioli *et al*., 1995); the reduced oxidative damage of human erythrocytes by 3, 4-DHPEA (Manna *et al*., 1999) and the low production of free radicals in the faecal matrix (Owen *et al*., 2000). Moreover, several studies affirmed that phenolic substances isolated and purified from olive oil were much more potent antioxidants than the classical *in-vivo* and *in-vitro* free radical scavengers such as vitamin E and dimethyl sulfoxide (Owen *et al*., 2000; Owen Introduction 11 *et al*., 2000; Owen *et al*., 2000; Gordon *et al*., 2001). Some other studies (Beauchamp *et al*., 2005) have reported not only preventive actions of phenolic compounds present in VOO

(named oleocanthal), also determined their anti-inflammatory actions. In particular, they disclosed that (-)-oleocanthal is a potent non-steroidal anti-inflammatory agent, similar to ibuprofen, and a powerful anti-oxidant such as α-tocopherol (Smith *et al*., 2005). Similar effects were observed by several researchers on throat irritation exerted by these two compounds (oleocanthal and ibuprofen). It was found that both enantiomers of oleocanthal act like ibuprofen by causing dose-dependent inhibition of the cyclooxygenase enzymes (COX-1 and COX-2) activities although had no effect on lipoxygenase *in vitro*. From the statement above is evident that long term consumption of oleocanthal may help to protect against some diseases by its ibuprofen-like COX-inhibiting activity, as well as the effect of ibuprofen on neoplasial risk reduction was reported earlier (Harris *et al*., 2005; Platz *et al*., 2005). However, some Italian scientists (Fogliano *et al*., 2006) claimed in Mol. Nutr. Food Res. that the attribution of the health effects of a diet to a single compound is always hazardous, and this is particularly can be critical for the oleocanthal present in the olive oil in low amount. As already anticipated, phenolic compounds contribute also to organoleptic properties of VOOs and commonly described as bitter and astringent (Tsimodou, 1998; Gutierrez- Rosales *et al*., 1992; Gutierrez-Rosales *et al*., 2003; Montedoro *et al*., 1992) and responsible for sensorial characteristics in general (Ryan *et al*., 1998). Less commonly, polyphenols are associated with pungency, which are peppery, burning, or hot sensations (Boskou, 1996; Tsimodou, 1998; Andrewes *et al*., 2003). However, relationship between individual hydrophilic phenols of VOO and its sensory characteristics are not totally defined. For instance, several authors associated off-flavour note of “fusty” with the presence of phenolic acids in VOO (Graciani-Costante *et al*., 1981), although other studies did not show any relation between bitter sensory note and phenolic acid content in VOO (Uccella *et al*., 2001). The relations between the secoiridoid derivatives and the bitterness of VOO have also been studied; first, interest was focused on two derivatives of oleuropein and demethyloleuropein, such as 3,4-DHPEA-EDA and *p*-DHPEA-EA (Kiritsakis, 1998; Garcia *et al*., 2001). In this case, García *et al.* (2001) studied the reduction of oil bitterness by heating olive fruits, and good correlation between oil bitterness and of hydroxytyrosol secoiridoid derivative’s content was found. Some recent studies have observed that a relationship between bitter and pungent sensory

properties and ligstroside derivative content (Tovar *et al*., 2001) or the amount of the aldehydic form of oleuropein aglycone (Mateos *et al*., 2004).

4.4 Importance of quantification of phenolic compounds in VOOs

The qualitative and quantitative composition of hydrophilic phenols in VOO is strongly affected by the agronomic and technological conditions of its production. Several agronomic parameters can modify the phenolic concentration of VOO. For these reasons, the identification and the quantification of the individual components of VOO have great interest. Many analytical procedures directed towards the determination of the complete phenolic profile have been proposed (spectrophotometer methods; biosensors; paper chromatography, TLC, GC with different detectors, and HPLC coupling with several detection systems; NMR and IR techniques for the characterization and identification of these compounds; capillary electrophoresis (CE); however, extraction techniques, chromatographic conditions, and quantification methods have contributed to find differences in reported levels of VOO phenolics. The direct comparison between the concentration of olive oil phenols reported in the literature is complicated, since reported concentrations often differ greatly (sometimes even in orders of magnitude). Several authors have explained this by the fact that there were numerous factors which affect phenolic compounds of VOO, such as various genetic characteristics of the olive cultivar (Tsimidou, 1998) or technological modifications during processing the olives (Ranalli *et al*., 1996). These reasons may partly, but not completely, explain these discrepancies. Pirisi and co-workers raised the question of whether discrepancies may be caused by the various analytical methods used and/or the expression of the results in various formats (Pirisi *et al*., 2000). In fact, individual phenolic compounds give different responses during UV detection after their separation in HPLC (Mateo *et al*., 2001). The use of different standard equivalent units in the case of the Folin-Ciocalteu colorimetric assay for total phenolic compounds, depending on the chosen calibration curve (e.g., caffeic acid, gallic acid, syringic acid, tyrosol, oleuropein equivalents) can also lead to confusion or mistakes. As Tsimidou proposed (1998), it would an interest to perform a possible collaborative study using the same analytical method to ensure that the differences in magnitude of phenol content depend mainly on the variety. Couple of

years later Pirisi *et al*. (2000) stated that before starting such studies, it would be necessary to investigate the influence of different milling conditions on the polyphenol content of oils in detail. Despite a general recognition of problems associated with the analysis and quantification of the phenolic compounds in olive oil, there have been some recent papers published to highlight the differences between various units used to express the levels of "olive phenolic compounds" (Hrncirik *et al*., 2004). In general, as it was mentioned before, an analytical procedure for the determination of individual phenolic compounds in VOO involves three basic steps: extraction from the oil sample, analytical separation, and quantification. These steps will be discussed in following sections.

4.5 Determination quantitative of antioxidant dialdehyde form (1, 2, 3, and 4) in virgin olive oil: an analytical approach method by chemical derivatization and Tandem mass spectrometry.

Much literature is available on the development of methods for the analysis, isolation, and identification of polyphenols in olives and oil olive (table4.1),but the complexity of phenolic extract is usually great, which is caused by the labile chemistry of the secoiridoid compounds that easily react with solvent, from nonconvalent adducts, and are easily converted in other compounds during extraction presses caused by the easy opening of the secroidoid ring after hydrolyzation of the glucose moiety (Dinino et al 2000) (scheme 1).

Table 4.1: Methods for the evaluation of the olive oil polyphenols content

Polyphenolic compound	Method employed	Phenol content	Reference
Total phenols	Enzymic assay	566·0–0·8 ppm (mg caffeic acid/kg oil)	Mosca *et al.* (2000)
Oleuropein and its isomers, ligstroside and oleuropein aglycones, deacetoxyligstroside and deacetoxyoleuropein aglycones, 10-hydroxy-oleuropein	APCI–MS		Caruso *et al.* (2000)
Hydroxytyrosol, tyrosol, vanillic, caffeic, syringic, *p*-coumaric, ferulic, cinnamic and elenolic acids	HPLC	Low concentration (total phenols 50–200 mg/kg); medium concentration (total phenols 200–500 mg/kg); high concentration (total phenols 500–1000 mg/kg)	Montedoro *et al.* (1992)

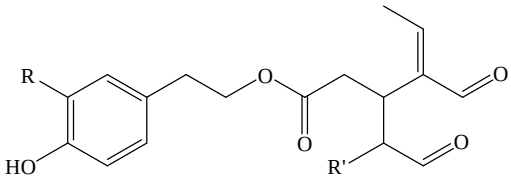
ppm, Parts per million; APCI, atmospheric pressure chemical ionisation.

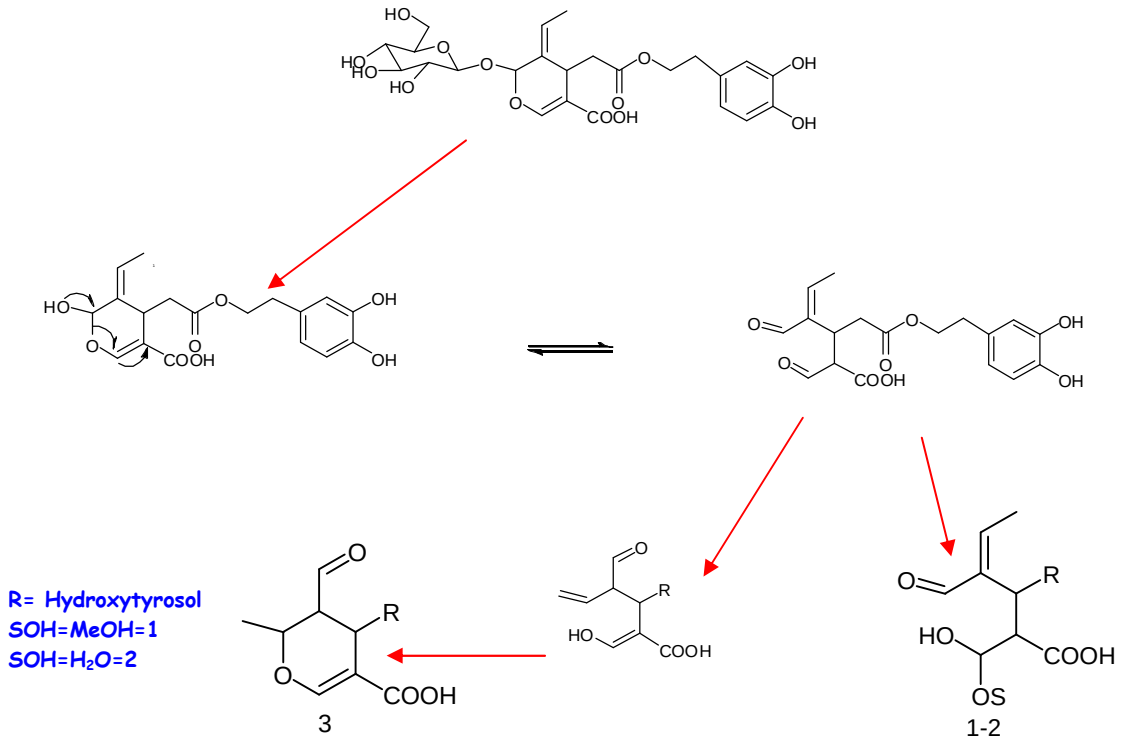
1; R = R’ = H; Oleocanthal

2, R = OH; R’ = H; Hidroxy-oleocanthal

3, R = H; R’ = $COOCH_3$; Ligstroside Aglycon

4; R = OH; R’ = $COOCH_3$; Oleuropein Aglycon





scheme 1.

For this reason we have chosen the method of chemical derivatization, using methoxyamine as agent of derivative, after derivatization, we could easily detect the derivatized, .using one of the most highly sensitive technique, that is stable isotope dilution assay, coupled with mass spectroscopy..

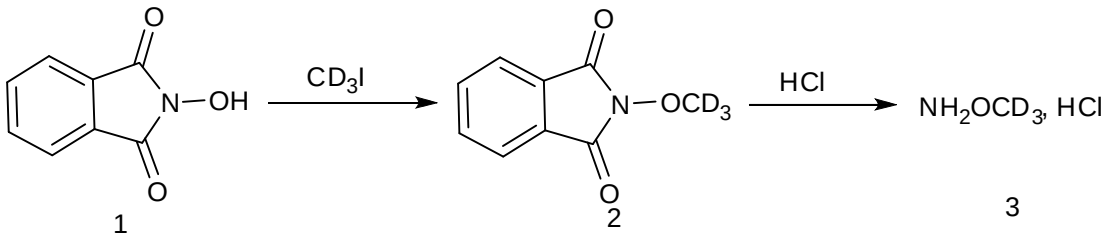
4.5.1 Experimental:

4.5.1.1 Syntheses of O-Methylhydroxylammonium chloride (CD3ONH2. HCl):

N-Methoxyphthalimide(2). Anhydrous potassium carbonate (2.21g, 16.0 mmol) was added slowly to a stirered solution of N-hydroxyphtalimide(1) (4.10g, 25.0 mmol) in dimethyl sulfoxide(37 cm^3).Methyl iodide (6.04g, 2.65 cm^3, 42.5 mmol) was added drop-wise to reddish brown solution at a rate such that the temperature did not exceed 30 °C .The

reaction was stirred at room temperature for 24h then poured into cold water and left to stand at 0-5 c for about 30 min.The residual with crystals was filtred and washed with water (3 x 20 cm^3).and dried aunder vacuum to a constant weight (3.34g, 18.9 mmol)

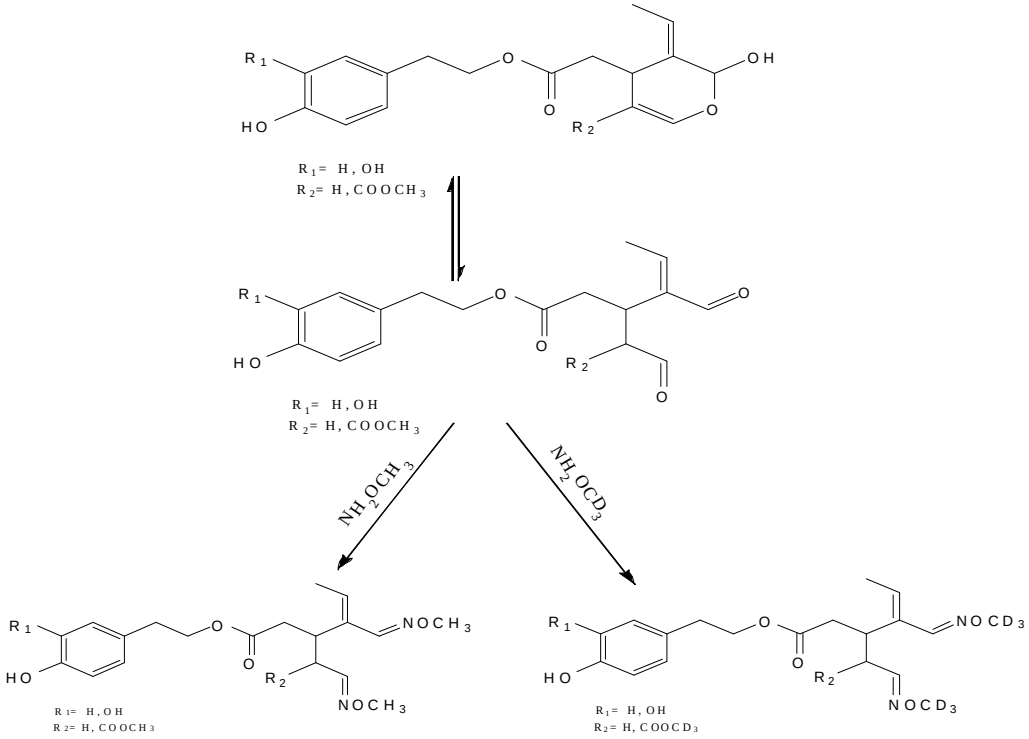
O-Methylhydroxylammonium chloride (CD3ONH2. HCl) (3) was synthesised according to (J. Ishwaras 2000)(scheme 4.1)). The isotopic distribution, checked by high resolution mass spectrometry, was d_2 = 1%, d_3 = 99%.



Sheme 4.1: Synthese of O-Methylhydroxylammonium chloride (CD_3NH_2. HCl)

4.5.1.2 Synthesis of standard derivative

The compound **2** and **4** are prepared by the method of (sindona e co-workers 2009), these compounds are utilized as precursor for the synthesis of labelled e non labelled standard after reaction with methoxyamine and methoxy-d_3-amine.(see scheme 4.2)



Scheme 4.2 : *The derivatization reaction of antioxidant dialdehyde 2, 4 with methoxyamine and methoxy-d_3-amine to using as internal standard.*

4.5.1.3 Sample preparation:

1g of the oil olive and excess amount of methoxyamine was stirred in 10 ml of methanol. The mixture was heated at 40 °C for 60 min, then known amount of internal standard was added, the product solution was portioned by (Hexane/ACN), the ACN phase was recovered and evaporated under vacuum, the residue was reconstituted with 1ml of methanol, and directly injected into the chromatography. The LC/MS analyses were carried out on a triple quadruple LC 320 (Varian inc) equipped with an ESI source

interfaced with an HPLC equipped with a C18 column. The linear gradient used was: 100% solvent A (H2O, 0.05% TFA) to 100% solvent B (CH3OH) in 12 min.

4.5.2 Results and discussion

4.5.2.1 Mass spectroscopy detection

The LC-MS analysis was carried out with a triple-quadrupole mass spectrometer LC 320 (Varian Inc., Palo Alto, CA), equipped with an ESI source interfaced with an HPLC Prostar 210 (Varian Inc.). The chromatographic analysis was performed with a C_{18} column (75 × 2.1 mm, Discovery, Supelco Bellefonte, PA). The flow rate was fixed at 0.25 mL min^{-1} using the following eluents and linear gradient used was: 100% solvent A (H2O, 0.05% TFA) to 100% solvent B (CH3OH) in 12 min.. The parameters of needle, shield and capillary were set at -4.8 KV, 600 V, and 70 V, respectively; whereas drying (N_2) and nebulizing gas (air), housing temperature and electron multiplier were set at 20 psi and 250 °C , 45 psi, at 60°C and at 1350 V, respectively. The dwell time was 0.200 s/scan, and the resolution was set using a mass peak width of 0.8 amu. The collision gas pressure (Ar) was fixed at 2 mTorr, and the collision energy was set to 9 eV for all of transition *m/z* 379 → *m/z* 137, *m/z* 363 → *m/z* 121, *m/z* 411 → *m/z* 137 and *m/z* 396 → *m/z* 121.

High-resolution electrospray ionization (ESI) experiments were carried out in a hybrid Q-Star Pulsar-i (MSD Sciex Applied Biosystem, Toronto, Canada) mass spectrometer equipped with an ion spray ionization source. Samples were introduced by direct infusion (3 μL/ min) of the sample containing the analyte (5 ppm), dissolved in a solution of 0.1% acetic acid, acetonitrile/water 50:50 at the optimum ion spray (IS) voltage of 4800 V. The source nitrogen (GS1) and the curtain gas (CUR) flows were set at pressures of 20 and 25 psi, respectively, whereas the first declustering potential (DP1), the focusing potential (FP), and the second declustering potential (DP2) were kept at 50, 220, and 10 V relative to ground, respectively.(figure 4.2)

The Quattro triple quadropole mass spectrometr was operated under the positive-elctrospray-ionisation mod (ESP+) .The products of the derivatization reaction, dimethoxyl-iminato-oleocanthal, dimethoxyl-iminato-hidroxyoleocanthal, dimethoxyl-iminato- Ligstroside Aglycon and dimethoxyl-iminato- Oleuropein Aglycon, were

detected by ESI-MS/MS in MRM mod (Figure 4.1).The quantification of these compounds in olive oil samples was carried out by monitoring one of the major fragments, using dimethoxyl-iminato-hidroxyoleocanthal (*m/z* 379>137) together with that of dimethoxyl-D6-iminato-hydroxyloleocanthal (*m/z* 385>137) as internal standard using for the quantification of *hidroxy-olecanthal* 2.and dimethoxyl-iminato-*Oleocanthal* (363>121) together with that of dimethoxyl-D6-iminato-hidroxyoleocanthal (*m/z* 385>137) as internal standart using for quantification of *oleocanthal* 1

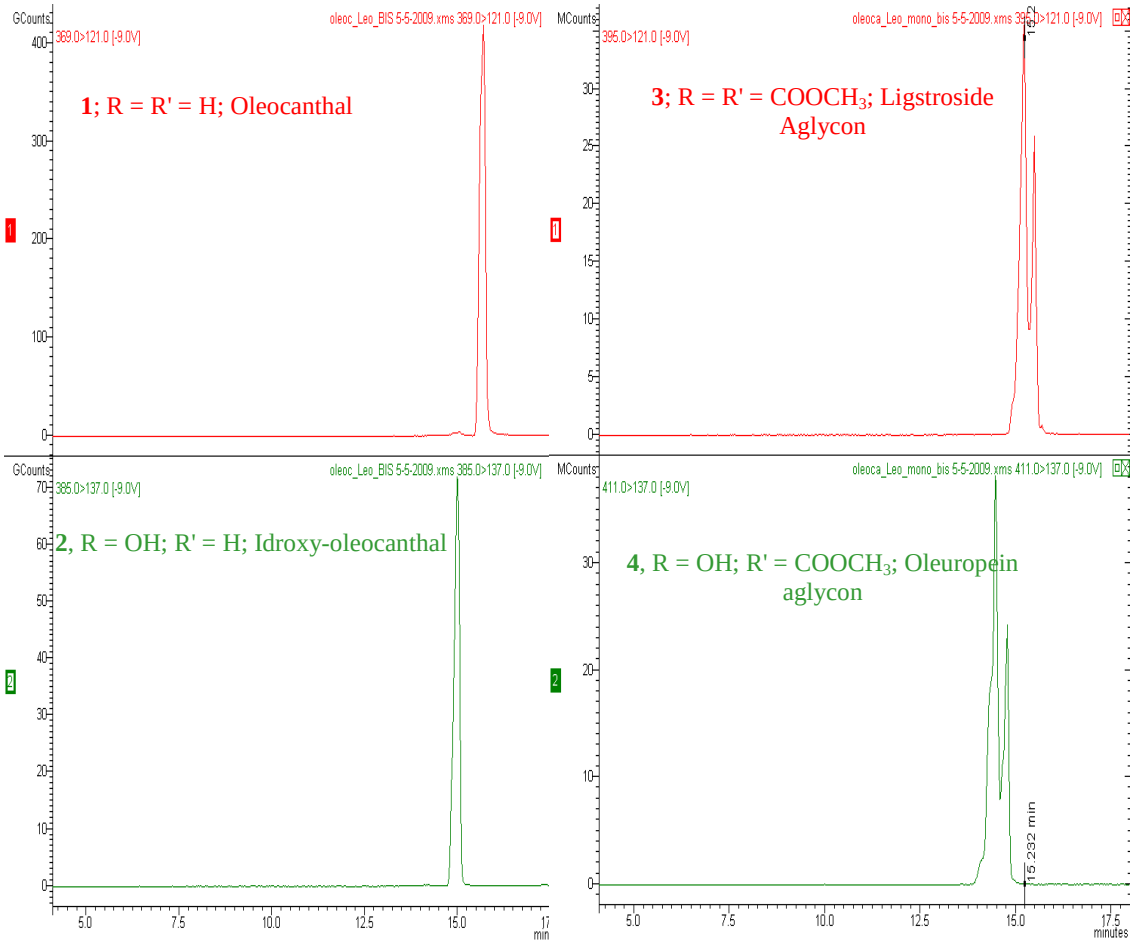


Figure4.1. The representative mass chromatograms(MRM mod) of derivativatization reaction of antioxidant dialdehyde1, 2,3,4 in olive oil sample.

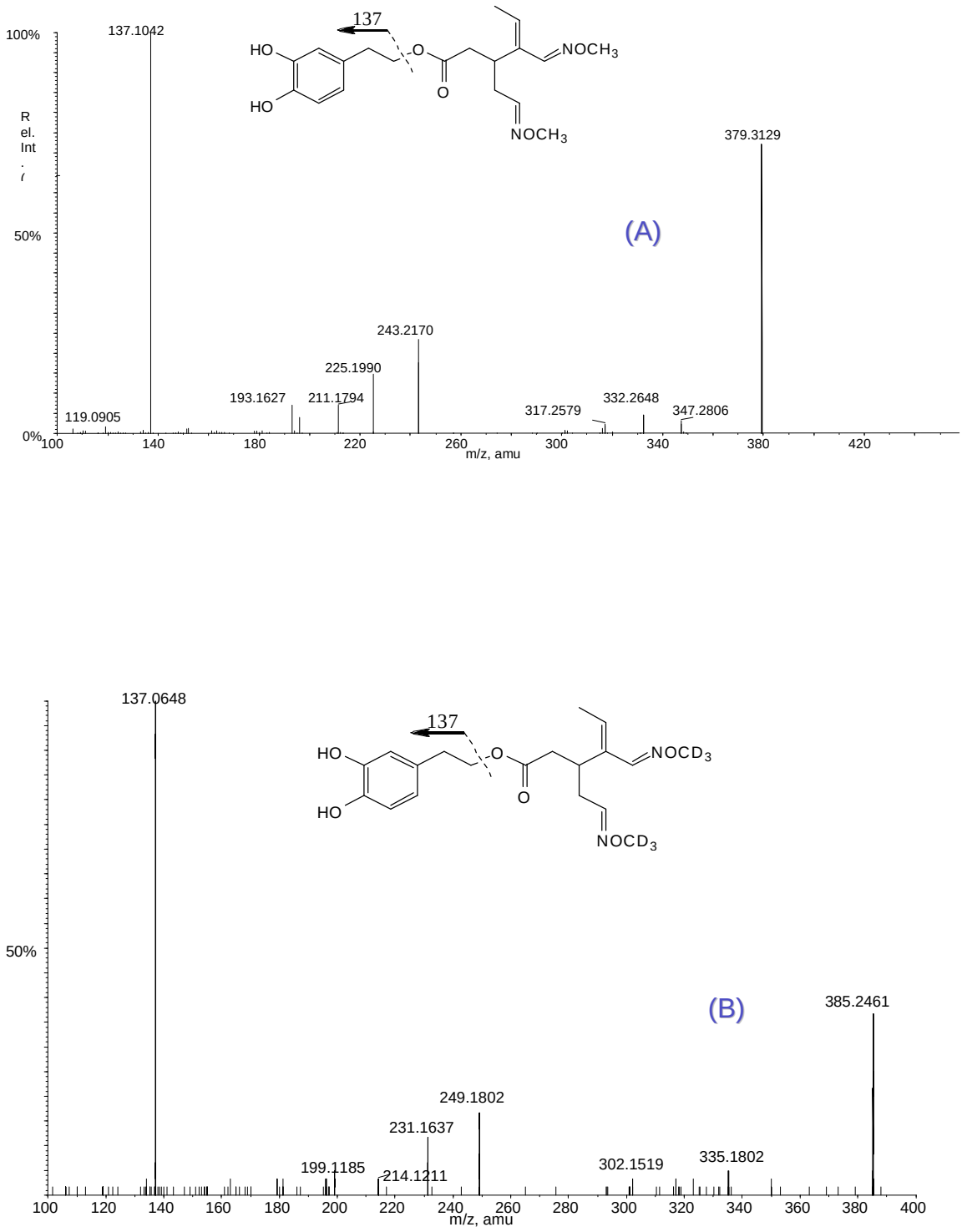


Figure 4.2 ESI (+) MS/MS spectra of the derivative of Hidroxy-oleocanthal 2 (A) and his labeled Internal standard (B) in positive ion mode

4.5.2.2 perspective

The above method developed will be used for quantitative determination of the unknown amount of antioxidant dialdehayde form (1,2,3,4) in olive oil in different cultivar area in Calabria region

Referances

Andrewes P., J.L.H.C. Busch, T. de Joode, A. Groenewegen, H. Alexandre. Sensory properties of virgin olive oil polyphenols: Identification of deacetoxy-ligstroside aglycon as a key contributor to pungency. *J. Agric. Food Chem.* **2003**, 51, 1415–1420.

Angerosa F. Influence of volatile compounds on virgin olive oil quality evaluated by analytical approaches and sensor panels. *Eur J Lipid Sci Technol*. **2002**, 104, 639-660.

Angerosa F., M. Servili, R. Selvaggini. Review, Volatile Compounds in Virgin Olive Oil: Occurrence and Their Relationship With the Quality, *J Chromatogr A*, **2004**, 1054, 17-31.

Angerosa F., N. d'Alessandro, F. Corana, G. Mellerio. Characterization of phenolic and secoiridoid aglycons present in virgin olive oil by gas chromatography-chemical ionization mass spectrometry. *J. Chromatogr. A* **1996**, 736, 195–203.

Angerosa F., R. Mostallino, C. Basti, R. Vito, A. Serraiocco, Virgin Olive Oil Differentiation in Relation to Extraction Methodologies, *J. Sci. Food Agric.*, **2000**, 80, 2190-2195.

Baldioli M., M. Servili, G. Perreti, G. F. Montedoro. Antioxidant activity of tocopherols and phenolic compounds of virgin olive oil. *J. Am. Oil Chem. Soc*. **1996**, 73, 1589–1593.

Beauchamp G.K., R. S. J. Keast, D. Morel, J. Lin, J. Pika, Q. Han, C.-H. Lee, A. B. Smith, P. A. S. Breslin. Ibuprofen-like activity in extra-virgin olive oil. *Nature* **2005** 437, 45–46.

Bianco A., F. Coccioli, M. Guiso, C. Marra. The occurrence in olive oil of a new class of phenolic compounds: hydroxy-isochromans. *Food Chem.* **2001**, 77, 405–411.

Bianco A.D., L. Muzzalupo, G. Romeo, M.L. Scarpati, A. Soriero, N. Uccella. Microcomponents of olive oil—III. Glucosides of 2(3,4-dihydroxy-phenyl)ethanol. *Food Chem.* **1998**, 63, 461–464.

Boskou D. *Olive oil: Chemistry and Technology*. AOCS Press, Champaign, IL, **1996**, 52– 83.

Boskou D. Storage and packaging. In *Olive Oil Chemistry and Technology*. Boskou, D., Ed. Champaign, IL, **2006**, 233-241.

Brenes M., A. García, P. García, J.J. Ríos, A. Garrido. Phenolic Compounds in Spanish Olive Oils. *J. Agric. Food Chem.* **1999**, 47, 3535–3540.

Brenes M., F.J. Hidalgo, A. García, J.J. Ríos, P. García, R. Zamora, A. Garrido. Pinoresinol and 1-acetoxypinoresinol, two new phenolic compounds identified in olive oil. *J. Am. Oil Chem. Soc*. **2000**, 77, 715–720.

Briante R., F. La Cara, M.P. Tonziello, F. Frebbraio, R. Nucci. Antioxidant activity of the main bioactive derivatives from oleuropein hydrolysis by hyperthermophilic betaglycosidase. *J. Agric. Food Chem.* **2001**, 49, 3198–3203.

Cantarelli C. Sui polifenoli presenti nella drupa e nell'olio di oliva. *Riv. Ital. Sost. Grasse* **1961**, 38, 69–72.

Caponio F., T. Gomes, A. Pasqualone. Phenolic compounds in virgin olive oils: influence of the degree of olive ripeness on organoleptic characteristics and shelf-life. *Eur. Food Res. Technol*. **2001**, 212, 329–333.

Caponio F., V. Alloggio, T. Gomes. Phenolic compounds of virgin olive oil: influence of paste preparation techniques. *Food Chem*. **1999**, 64, 203–209.

Carrasco-Pancorbo A., C. Cruces-Blanco, A. Segura Carretero, A. Fernandez Gutiérrez. Sensitive determination of phenolic acids in extra-virgin olive oil by capillary zone electrophoresis. *J. Agric. Food. Chem.* **2004**, 52, 6687–6693.

Carrasco-Pancorbo A., L. Cerretani, A. Bendini, A. Segura-Carretero, M. Del Carlo, T. Gallina Toschi, G. Lercker, D. Compagnone, A. Fernandez-Gutierrez. Evaluation of the antioxidant capacity of individual phenolic compounds in virgin olive oil. *J. Agric. Food Chem*.**, 2005**, 53, 8918-8925.

Cortesi N., E. Fedeli. I composti polari di oli di oliva vergine. Nota I. *Riv. Ital. Sostanze Grasse* **1983**, 60, 341–351.

Fogliano V., R. Sacchi. Oleocanthal in olive oil: between myth and reality. *Mol. Nutr. Food Res.* **2006**, 50, 5-6.

Franceschi S., A. Bavero, E. Conti, R. Salamini, R. Volpe, E. Negri, L. Barman, C. La Vecchia, Food groups, oils and butter, and cancer of the oral cavity and pharynx. *Br. J. Cancer* **1999**, 80, 614–620.

García J.M., K. Yousfi, R. Mateos, M. Olmo, A. Cert. Reduction of oil bitterness by heating of olive (Olea europaea) fruits. *J. Agric. Food. Chem.* **2001**, 49, 4231–4235.

Garrido Fernández Díez M.J., M. R. Adamos, *Table olives*. Chapman & Hall, London (UK), **1997**, 67–109.

Gordon M.H., F. Paiva-Martins, M. Almeida. Antioxidant activity of hydroxytyrosol acetate compared with that of other olive oil polyphenols. *J. Agric. Food. Chem*. **2001**, 49, 2480–2485.

Graciani-Costante E., A. Vázquez-Roncero. Estudio de los componentes polares del aceite de oliva por cromatografia liquida de alta eficacia. *Grasas Aceites* **1981**, 32, 365–371.

Grigg D. Olive oil, the Mediterranean and the world. *GeoJournal* **2001**, 53, 163–172.
Gutierrez F, T. Arnaud, A. Miguel, M.A. Albi. Influence of ecological cultivation on virgin olive oil quality. *J Am Oil Chem Soc* **1999,** 76, 617–621.

Gutierrez Gonzales-Quijano R., J. M. Olias Jimenez. Packing of Olive Oil in Commercial Type Containers, II, Packaging in G*lass, Tin, Polyethylene and Polyvinylchloride Containers. Peroxide Number Evolution*, Grasas Aceites**, 1970**, 21, 217-221.

Gutiérrez-Rosales F., J.J. Ríos, M.L. Gómez-Rey. Main polyphenols in the bitter taste of virgin olive oil. Structural confirmation by on-line high-performance liquid chromatography electrospray ionization mass spectrometry. *J. Agric. Food Chem*. **2003**, 51, 6021–6025.

Gutiérrez-Rosales F., S. Perdiguero, R. Gutiérrez, J.M. Olías. Evaluation of. the bitter taste in virgin olive oil. *J. Am. Oil Chem*. *Soc*. **1992**, 69, 394–395.

Harborne J.B., P.M. Dey. *Methods in Plant Biochemistry*. J.B. Harborne (Ed.). Academic Press, London (UK), **1989**. Harris R.E., J. Beebe-Donk, H. Doss, D. Burr Doss. Aspirin, ibuprofen, and other nonsteroidal anti-inflammatory drugs in cancer prevention: a critical review of non-selective COX-2 blockade. *Oncol. Rep.* **2005**, 13, 559–583.

Helsing E. Trends in fat consumption in Europe and their influence on the Mediterranean diet. *Eur. J. Clin. Nutr.* **1993**, 47, S4–S12.

Hodge E., D.R. English, M.R.E. McCredie, G. Severi, P.Boyle, J.L. Hopper, G.G. Giles. Foods, nutrients and prostate cancer. *Cancer Causes Control* **2004**, 15, 11–22.

Hornick S.B. Factors affecting the nutritional quality of crops. *Am J Altern Agric* **1992**, 7, 63–68

Hrncirik K., S. Fritsche, Comparability and reliability of different techniques for the determination of phenolic compounds in virgin olive *Eur. J. Lipid Sci. Technol*. **2004**, 106, 540–549.

International Olive Oil Council (IOOC). Sensory analysis of olive oil – Method – Organoleptic assessment of virgin olive oil. **1996**, COI/T.20/Doc. no. 15/Rev.1.

Ishwara J, William C, Howard M, Mark J, Elsegood, Lain D, Menmer and Peter CM. *J. Chem, PerkinTrans2. **2000**, 1435-1446*

Janer del Valle C., A. Vázquez-Roncero. Estudio de los componentes polares del aceite de oliva por cromatografia gaseosa. *Grasas Aceites* **1980**, 5, 309–316.
Kiritsakis A.K. Flavor components of olive oil. - A review. *J. Am. Oil Chem*. Soc. **1998**, 75, 673–681.

Kiritsakis A.K., R. J. Hernandez. Packaging and Bottling of Olive Oil, in *Olive Oil*,

Kiritsakis. B. A. P., Lenart E, Willet,W.C and Hernandez R.J.,Eds. Trumbull, Connecticut, *Food and Nutrition Press, Inc*.**, 1998**, 209-226.

Leenen R., A.J.C. Roodenburg, M.N. Vissers, J.A.E. Schuurbiers, K.P.A.M. van Putte, S.A. Viseman, F.H.M.M. van de Put. Supplementation of plasma with olive oil phenols and extracts: influence on LDL oxidation. *J. Agric. Food Chem.* **2002**, 50, 1290–1297.

Manna C., V. Galletti, P. Cucciolla, G.F. Montedoro, V. Zappia. Olive oil hydroxytyrosol protects human erythrocytes against oxidative damages. *J. Nutr. Biochem*. **1999**, 10, 159– 165.

Mannino S., M.S. Cosio, M. Bertuccioli. High performance liquid chromatography of phenolic compounds in virgin olive oil using amperometric detector. *Ital. J. Food. Sci.* **1993**, 4, 363–370.

Mateos R., A. Cert, M.C. Pérez-Camino, J.M. García, Evaluation of virgin olive oil bitterness by quantification of secoiridoid derivatives *J. Am. Oil Chem. Soc*. **2004**, 81, 71– 75.

Mateos R., J.L. Espartero, M. Trujillo, J.J. Ríos, M. León-Camacho, F. Alcudia, A. Cert. Determination of phenols, flavones, and lignans in virgin olive oils by Solid-Phase Extraction and High-Performance Liquid Chromatography with Diode Array Ultraviolet Detection. *J. Agric. Food Chem.* **2001**, 49, 2185–2192.

Mendez A.I., E. Falque. Influence of Container Type and Storage Time on Olive Marc Oil Quality. Electron. *J. Environ. Agric. Food Chem*. **2002** ISSN 1579-4377

Montedoro G. F., L. Garofolo, M. Bertuccioli. Influence of the Cultivars and Pedoclimatic Conditions on the Virgin Olive Oil Quality. Proceedings of the 6th International Flavor Conference, Rethymnon, Crete, Greece, 5-7 July 1989, Ed. G. Charalambous, Elsevier Science Publishers B.V., Amsterdam (The Netherlands), **1989**, 881-891.

Montedoro G. F., M. Servili, I Parametri di Qualita Dell'olio di Oliva ed i Fattori Agronomici e Tecnologici Che li Condizionano, *Riv. Ital. delle Sost. Grasse***, 1992**, 69, 563- 573.

Montedoro G.F. I costituenti fenolici presenti negli oli vergini di oliva. Nota 1: identificazione di alcuni acidi fenolici e loro potere antiossidante. *Sci. Tecnol. Aliment.* **1972**, 3, 177–186.

Montedoro G.F., C. Cantarelli. Indagine sulle sostanze fenoliche presenti nell'olio di oliva. *Riv. Ital. Sost. Grasse* **1969**, 46, 115–124.

Montedoro G.F., M. Baldioli, M. Servili. I composti fenolici dell'olio di oliva e la loro importanza sensoriale, nutrizionale e merceologica. *Giornale Ital. Nutriz. Clin. Prev*. **1992**, 1, 19–32.

Montedoro G.F., M. Servili, M. Baldioli, E. Miniati. Simple and hydrolyzable phenolic compounds in virgin olive oil. 1. Their extraction, separation, and quantitative and semiquantitative evaluation by HPLC. *J. Agric. Food. Chem.* **1992**, 40, 1571–1576.

Montedoro G.F., M. Servili, M. Baldioli, E. Miniati. Simple and hydrolyzable phenolic compounds in virgin olive oil. 2. Initial characterization of the hydrolyzable fraction. *J. Agric. Food. Chem.* **1992**, 40, 1577–1580.

Montedoro G.F., M. Servili, M. Baldioli, R. Selvaggini, E. Miniati, A. Macchioni. Simple and hydrolyzable compounds in virgin olive oil. 3. Spectroscopic characterizations of the secoiridoid derivatives. *J. Agric. Food. Chem.* **1993**, 41, 2228–2234.

Owen R.W., A. Giacosa, W.E. Hull, R. Haubner, B. Spiegelhalder, H. Bartsch. The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. *Eur. J. Cancer* **2000**, 36, 1235–1247.

Owen R.W., A. Giacosa, W.E. Hull, R. Haubner, G. Wurtele, B. Spiegelhaldar, H. Bartsch. Olive-oil consumption and health: the possible role of antioxidants. *Lancet* **2000**, 1, 107– 112.

Owen R.W., W. Mier, A. Giacosa, W.E. Hull, B. Spiegelhalder, H. Bartsch. Phenolic compounds and squalene in olive oils: the concentration and antioxidant potential of total phenols, simple phenols, secoiridoids, lignans and squalene. *Food Chem. Toxicol.* **2000**, 38, 647– 659.

Owen R.W., W. Mier, A. Giacosa, W.E. Hull, B. Spiegelhalder, H. Bartsch. Identification of lignans as major components in the phenolic fraction of olive oil. *Clin. Chem.* **2000**, 46, 976–988.

Perri E., A. Raffaelli, G. Sindona. Quantitation of oleuropein in virgin olive oil by ionspray mass spectrometry-selected reaction monitoring. *J. Agric. Food Chem.* **1999**, 47, 4156– 4160.

Petroni A., M. Blasevich, M. Salami, N. Napini, G.F. Montedoro, C. Galli. Inhibition of platelet-aggregation and eicosanoid production by phenolic components of olive oil. *Thromb. Res.* **1995**, 78, 151–160.

Pirisi F.M., P. Cabras, C. Falqui Cao, M. Migliorini, M. Suggelli. Phenolic compounds in virgin olive oil. 2. Reappraisal of the extraction, HPLC separation, and quantification procedures. *J. Agric. Food Chem*. **2000**, 48, 1191–1196.

Polymerou-Kamilakis A. The Culture of the Olive Tree (Mediterranean World) In *Olive Oil Chemistry and Technology*. Boskou, D., Ed. Champaign, IL, **2006**, 1-12.
Psomiadou E., M. Tsimidou, Stability of Virgin Olive Oil 2 Photo-oxidation Studies. *J Agric Food Chem*. **2002**, 50, 722-727.

Procopio, A.; Sindona, G.; Gaspari, M.; Costa, N.; Nardi. M. Metodo Chimico-Catalitico per la manipolazione dell'oleuropeina per la sintesi del suo aglicone. Italian Patent MI2007A000904;**2009**

Procopio, Sindona, G.; Gaspari, M.; Costa, N.; Nardi "Chemical-catalytic method for the peracylation of oleuropein and its products of hydrolysis" Italian Patent MI2007A000903, request International Patent. PCT/IT2008/000303 **2009**

Psomiadou E., X. Konstantinos, K.G. Blekas, M.Z. Tsimidou, D. Boskou. Proposed parameters for monitoring quality of virgin olive oil (Koroneiki cv). *Eur. J. Lipid Sci. Technol.* **2003**, 105, 403–408.

Ranalli A., F. Angerosa. Integral centrifuges for olive oil extraction. The qualitative characteristics of products. *J. Am. Oil Chem. Soc*. **1996**, 73, 417–422.

Ranalli, A., N. Costantini, G. De Mattia, M. L. Ferrante: Evaluation of two kinds of centrifuged virgin oils arising from continuous olive processing. *J Sci Food Agric***. 2000**, 80, 673-683.

Rovellini P., N. Cortesi, Determination of Phenolic Compounds in Different Cultivars During Olive Drupe Ripening by Liquid Chromatography-mass Spectrometry, *Olivae*, **2003**, 95, 32-38.

Rovellini P., N. Cortesi, E. Fedeli. Analysis of flavonoids from *Olea europaea* by hplc-uv and HPLC-electrospray-MS. *Riv. Ital. Sostanze Grasse* **1997**, 74, 273–279.

Ryan D., K. Robards. Phenolic compounds in olives. *Analyst* **1998**, 123, 31R–44R. Ryan D., M. Antolovich, P. Prenzler. Biotransformations of Phenolic Compounds in Olea europea L. Scientia Horticulturae, **2002**, 92, 147-176.

Servili M., G. Montedoro. Contribution of phenolic compounds to virgin olive oil quality. *Eur. J. Lipid Sci. Technol.* **2002**,104, 602–613.

Servili M., R. Selvaggini, S. Esposto, *et al*., Review, Health and Sensory of Virgin Olive Oil Hydrophilic Phenols: Agronomic and Technological Aspects of Production That Affect Their Occurrence in the Oil, *J Chromatogr A*, **2004**, 1054, 113-127.

Servili M., S. Esposto, E. Lodolini, R. Selvaggini, A. Taticchi, S. Urbani, G. Montedoro, M. Serravalle, R. Gucciservili. Irrigation Effects on Quality, Phenolic Composition, and Selected Volatiles of Virgin Olive Oils Cv. Leccino. . *J. Agric. Food Chem.* **2007**, 55, 6609-6618.

Shahidi F. *Natural antioxidants: Chemistry, health effects and applications*. AOCS Press, Champaign, IL (USA), **1997**, 97–149.

Smith A.B., Q. Han, P. A. S. Breslin, G. K. Beauchamp. Synthesis and assignment of absolute configuration of (-)-oleocanthal: a potent, naturally occurring non-steroidal anti-inflammatory and anti-oxidant agent derived from extra virgin olive oils. *Org. Lett.* **2005**, 22, 5075–5078.

Smith B. Organic foods versus supermarket food: element levels. *J Appl Nutr*. **1993**, 45, 35–39.

Soler M., L. Chatenaud, C. La Vecchia, S. Franceschi, S. Negri. Diet, alcohol, coffee and pancreatic cancer: final results from an Italian study. *Eur. J. Cancer Prev.* **1998**, 7, 455– 460.

Solinas M. Analisi HRGC delle sostanze fenoliche di oli vergini di oliva in relazione al grado di maturazione e alla varietà delle olive. *Riv. Ital. Sostanze Grasse* **1987**, 64, 255–262.

Solinas M., A. Cichelli. Sulla determinazione delle sostanze fenoliche dell'olio di oliva. *Riv. Ital. Sostanze Grasse* **1981**, 58, 159–164.

Tiscornia E., M. Forina, F. Evangelisti. Composizione chimica dell' olio di oliva e sue variazioni indotte dal processo di rettificazione. *Riv. Ital. Sostanze Grasse* **1982**, 59, 519– 556.

Tovar M.J., M.J. Motilva, M. Paz Romero, Changes in the phenolic composition of virgin olive oil from young trees (Olea europaea L. cv. Arbequina) grown under linear irrigation strategies. *J. Agric. Food. Chem.* **2001**, 49, 5502–5508.

Tovar M.J., M.P. Romero, J. Girona, M.J. Motilva. L-phenylalanine ammonia-lyase activity and concentration of phenolics in developing olive (Olea europaea L cv Arbequina) fruit grown under different irrigation regimes. *J. Sci. Food Agric.* **2002**, 82, 892–898.

Tsimidou M. Polyphenols and quality of virgin olive oil in retrospect. *Ital. J. Food Sci*. **1998**, 10, 99–116.

Tsimidou M., M. Lytridou, D. Boskou, A. Pappa-Louis, F. Kotsifaki, C. Petrakis. Sobre la determinación de ácidos fenólicos minoritarios de aceite de oliva virgen mediante RPHPLC. *Grasas Aceites* **1996**, 47, 151–157.

Uccella N. The olive biophenols: hedonic-sensory descriptors of evoo and wotos in the Mediterranean aliment culture. In *Food flavours and Chemistry: Advances of the New Millennium*. A. H. Spanier, F. Shahidi, T.H. Parliment, C. J. Mussinan, C. T. Ho, E. Tratras Contis Eds. The Royal Society of Chemistry Publishers, Cambridge, UK, **2001**, 253-265.

Vazquez Roncero A. Les polyphenols de l'huile d'olive et leur influence sur les caracteristiques de l'huile. *Rev. Fr. Corps Gras.* **1978**, 25, 21–26.

Vázquez-Roncero A., C. Janer, M.L. Janer. Componentes fenólicos de la aceituna. III. *Grasas Aceites* **1976**, 27, 185–191.

Velasco J., C. Dobarganes. Oxidative stability of virgin olive oil. *Eur. J. Lipid Sci. Technol.* **2002**, 104, 661–676.

Visioli F., C. Galli. Olive oil phenols and their potential effects on human health. *J. Agric. Food Chem.* **1998**, 46, 4292–4296.

Visioli F., G. Bellomo, G.F. Montedoro, C. Galli. Low-density-lipoprotein oxidation is inhibited in-vitro by olive oil constituents. *Atherosclerosis* **1995**, 117, 25–32.

Vissers M.N., P.L. Zock, R. Leenen, A.J.C. Roodenburg, K.P.A.M. van Putte, M.B. Katan. Effect of consumption of phenols from olives and extra virgin olive oil on LDL oxidizability in healthy humans. *Free Radical Res.* **2001**, 35, 619–629.

.