Non basta guardare,
occorre guardare con occhi che vogliono vedere,
che credono in quello che vedono
(Galileo Galilei)

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"DEVELOPMENT OF SELECTIVE MATERIALS AND METHODS FOR ANALYTICAL TECHNIQUES COMBINED WITH MASS SPECTROMETRY"

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❖ Euterpio MA, Pagano I, Piccinelli AL, Rastrelli L and Crescenzi C(2012), *Development and validation of method for determination of (E)-resveratrol and related phenolic compounds in beverages using molecularly imprinted solid phase extraction*, Journal of Agricultural and Food Chemistry , XXX, XXX-XXX DOI**:**10.1021/jf303251m

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Abstract

Mass spectrometry (MS) is a powerful detection technique that has become very important in several chemical disciplines for detection of both small molecules (environmental pollutants, small metabolites) and large biomolecules (proteins, peptides). The "heart" of the mass spectrometer is the analyzer, that uses electrical or magnetic fields, or combination of both, from the region where they are produced, to a detector, where they produce a signal which is amplified. This element separates the gas phase ions according to their m/z (mass to charge ratio) value.

MS is mostly used coupled to high performance liquid chromatography (HPLC) or ultra- high performance liquid chromatography (UHPLC) with an atmospheric pressure ionization (API) interface between the LC and MS. The most important API technique is electrospray ionization (ESI), with a wide range of application from small molecules to large molecules such as proteins or polymers.

Complex matrix, as environmental samples, biological fluids, food or tissue extracts, are not usually compatible with MS detection without extensive sample preparation. In facts, complex matrix could contains substances that can cause the detector signal to decrease or increase for a selected analyte compared to the signal from the same compound in a standard solution. ESI is the ionization technique in which this phenomenon is most common because in the ESI process ions compete for ionization/desorption. Matrix effect is observed in ESI when compounds co-elute with the analyte of interest: this affects sensitivity, linearity, accuracy, precision and the limit of detection (LOD).

Furthermore, the target compound(s) are often present at concentrations lower than their detection limits and require a preliminary concentration step. Therefore, the first step of an analysis is usually some kind of sample pretreatment to improve both the selectivity and sensitivity of the subsequent

detection. Many techniques are available for this purpose, the suitability of which depends primarily on the physical state of the sample, e.g. solid-phase extraction (SPE) for liquid and pressurized-liquid extraction for solid samples. Proper sample preparation is critical for MS analysis, because the quality and reproducibility of sample extraction and preparation significantly impact results from MS instruments.

In this Ph.D. research project improvements in the following extraction techniques were evaluated:

Solid-liquid extraction using pH controlled subcritical water to extract very low solubility

Solid phase extraction by molecularly imprinted polymers (MISPE) to selectively extract a class of natural compounds from beverages

Solid phase extraction (SPE) by porous graphitic carbon (PGC) coupled on line with LC/MS detection in order to extract anionic compounds from environmental water samples

For solid-liquid extraction was used pure water at elevated temperature as an extraction fluid for non-polar analytes. Pressurized hot water extraction (PHWE, also known as subcritical water extraction) is currently considered one of the most interesting recent developments in extraction technology. Unfortunately, the applicability of this technique could be limited by the very low water solubility of the target compounds, even at high temperature. In this study the scope of broadening the applicability of PHWE by adjusting the pH of the water used in extraction is dimostrate for the extraction of curcumin (which has very low water solubility) from untreated turmeric rhizomes.

In order to improve the selectivity of solid phase extraction a molecularly imprinted polymer was prepared using (E)-resveratrol as template and was evaluated for multicomponent multiclass analysis of polyphenolic compounds in complex matrices such as natural and alcoholic beverages. Chromatographic evaluation of the polymer exhibited high selectivity for (E)-

resveratrol and its structural analogues, quercetin, and other flavonoids. An analytical procedure based on molecularly imprinted solid phase extraction (MISPE) and high HPLC coupled to UV detector was developed and validated for determination of (E)-resveratrol and quercetin in wine and fruit juice samples. The specific binding capacity of the MIP was estimated as 80 μg g^{-1} polymer by the cartridge test. MISPE sample pretreatment allows an excellent sample cleanup, enormously decreasing the number of coextracted potentially interfering compounds. Under the described conditions, by extracting 2 mL samples a clean extract is obtained and (E)-resveratrol and quercetin could be easily identified at concentration levels of, respectively, 1.5 and 7.0 μg L^{-1}.

Finally, the peculiar characteristics of porous graphitic carbon was exploited to develop a method for the determination of perfluoroalkyl acids (PFAS). Because their ability to persist in the environment, bioaccumulate and interfere with the endocrine system these compounds are considered"emerging pollutants". It has been also demonstrated their animal mutagenicity, carcinogenicity and teratogenicity. The feasibility of using PGC for both online solid phase extraction and LC separation of these compounds was evaluated in combination with tamndem mass spectrometry detection in order to obtain structural information and/or achieve better selectivity and sensitivity for quantitative purposes. The optimized analytical procedure was applied to the analysis of real samples, such as drinking and ground waters.

INTRODUCTION

1.1 Mass spectrometry as detection technique for liquid chromatography

Introduced for the first time in 1912 by Sir J.J. Thomson (Thomson, 1913), Mass Spectrometry (MS) is an analytical technique widely used for identification, quantification and structural elucidation of different analytes. It is capable of ionizing, fragmenting, separating according to the mass to charge ratio (m/z), and finally detecting many classes of compounds in gas phase.

Since the studies that, in the first decade of the 20th century, have led to the electrons discovery, mass spectrometry has undergone numerous improvements in resolution, sensitivity, mass range and accuracy, becoming a fundamental tool with broad and heterogeneous application fields (Table 1).

Table 1:	Common applications and fields that use mass spectrometry.
Field of Study	**Applications**
Proteomics	•Determine protein structure, function, folding and interactions •Identify a protein from the mass of its peptide fragments •Detect specific post-translational modifications throughout complex biological mixtures •Quantitate (relative or absolute) proteins in a given sample •Monitor enzyme reactions, chemical modifications and protein digestion
Drug Discovery	•Determine structures of drugs and metabolites •Screen for metabolites in biological systems
Cliinical Testing	•Perform forensic analyses such as confirmation of drug abuse •Detect disease biomarkers (e.g., newborns screened for metabolic diseases)
Genomics	•Sequence oligonucleotides
Environment	•Test water quality or food contamination

| Geology | •Measure petroleum composition |
| | •Perform carbon dating |

After the construction of the first mass spectrometer (then called a parabola spectrograph), by Thomson, F. W. Aston developed the first mass spectrometer with velocity focusing, which improved MS resolving power (Aston, 1919). Around 1920, A. J. Dempster of the University of Chicago developed the electron ionization source and the first spectrometer with a sector-shaped magnet (180◦) with direction focusing (Dempster, 1918).

Afterwards a large number of mass spectrometers has been developed and, as the techniques continue to advance, the use of mass spectrometry continues to grow.

Always a mass spectrometer consists of the following four sections:

- sample inlet to introduce the compound that is analysed;

- ionization source to produce ions from the sample;

- one or several mass analysers to separate the various ions;

- a detector to 'count' the ions emerging.

Figure 1: Four section of Mass Spectrometer

In order to avoid any hindrance of the ions during the travelling in to the instrument, analyzer and detector and often the ionization source too, are maintained under high vacuum. The whole process performed by the mass spectrometer includes the ionization of the analytes into the ion source; depending on proton affinity and ionization method, both positively and negatively charged sample ions are created; the ions are then separated in the mass analyzer according to their mass-to charge ratio; eventually the ions can

fragmented and the fragments can pass through a second analyzer to undergo a second separation; the ions emerging from the last analyzer are finally measured by detector, that converts them into electrical signals transmitted to the computer.

Sample complexity and ionization method determine the kind of sample introduction into the ionization source, while the ionization method used should depend on the type of sample under investigation and the mass spectrometer available.

LC/MS is a hyphenated technique, combining the separation power of HPLC, with the detection power of mass spectrometry.

Liquid chromatography/mass spectrometry is now a robust androutinely used technique for bioanalysis. The technique can be coupledto a versatile chromatographic system and offers, selective and sensitivedetection. The development of atmospheric pressure ionization (API) techniques has enabled LC to be easily and reliably interfaced with MS. The API techniques, which include atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI), and the recently introduced atmospheric pressure photoionization (APPI) are generally referred to as soft ionization techniques, since they usually cause little or no fragmentation of the analyte ions. Several recent reviews are recommended for further reading regarding the applications of these techniques (Raffaelli & Saba, 2003; Hernàndez et al., 2005; Hewon, 2005).

ESI-MS is a commonly used technique in bioanalysis. It was originally developed in 1984 by John Fenn and M. Yamashita (Yamashita & Fenn, 1984a; Yamashita & Fenn, 1984b), based on the pioneering work of Malcolm Dole (Dole et al., 1968).

In electrospray ionization the analyte is dissolved in a volatile solvent then pushed through a very small, charged metal capillary. In the solution volatile acids, bases or buffers are often added and the analyte exists as an ion in

solution either in its anion or cation form. The liquid is pushes out of the capillary forming an aerosol of small droplets (about 10 μm). The aerosol is produced by a process involving the formation of a Taylor cone and a jet from the tip of this cone. An uncharged carrier gas such as nitrogen is sometimes used to help nebulizer the liquid and to help evaporate the neutral solvent in the droplets. As the solvent evaporates, the analyte molecules are forced closer together, repel each other, due to repulsive Coulombic forces between charged molecules, and split the droplets. The process repeats until the analyte is free of solvent and is a bare ion. The ions observed are created by the addition of a proton (a hydrogen ion) or of another cation such as sodium ion or potassium, or the removal of a proton. Multiply charged ions such are often observed especially for large macromolecules as proteins or larger peptides. The sensitivity in ESI-MS is affected by:

- analyte structure (how easy to protonate, polarity etc.);
- mobile phase pH, polarity, surface tension;
- tuning of ion source parameters, like voltages, nitrogen flow;
- mobile fase flow. In ESI w/o nebulizer gas at maximum 5-10μL/min, nebulizer gas about 200μL/min;
- ion suppression from sample matrix or mobile phase additives.

1.1.2 Frequent troubles in combining liquid chromatography and mass spectrometry

Many parameters affect the performance of the ESI process. To maximize sensitivity, the pH, solvent, solvent additives, and flow rates for the LC effluent must all be considered. Generally in ESI ion formation is most efficient at low flow rates, optimally around 5-10 μl/min (Voyksner, 1997). However, if the solvent evaporation is pneumatically assisted, often referred toas ion spray, the flow rates can be increased up to 1 ml/min, with an optimum flow rate of around 200 μl/min. Various LC/ESI devices and LC

instruments have been developed to meet the low flow rate criterion,and a practical consideration of the different devices and miniaturization is described by Abian (Abian et al., 1999). Suitable solvents for the LC mobile phase must permit formation of ions in solution. The additives used for the mobile phase should be volatile to avoid contamination or plugging of the sample orifice. Additives in the mobile phase are used to control the pH; a high pH should be used for negative-ion detection, and for analytes with acidic sites, while a low pH is normally used for positive ion-detection and analytes with basic sites. The additives may also act as ion-pairing agents for the LC separation, since ionic and polar compounds often have poor retention in the commonly used reversed phase chromatography. The ion-pairing agent should not be strong enough to neutralize the analyte and it should be added in a low concentration to avoid ion-suppression. Other issues to consider when combining LC with ESI are described more thoroughly in reference (Voyksner, 1997).

A number of aspects related to the retention of polar compounds by HPLC systems for subsequent ESI-MS analysis must also be considered. The composition of the mobile phase must be compatible with the ESI-MS detection system and allow chromatographic separation. Sometimes a compromise between the sensitivity and the retention might be necessary.

The separation efficiency of the column is important, even if the MS detector is selective and can separate compounds by their mass-to-chargeratios (m/z). Co-eluting peaks may give rise to severe suppression of the analyte response. A retention time long enough to separate the analyte from interferences, especially early eluting polar matrix interferences, is often advantageous.

1.1.3 Tandem mass spectrometry

Tandem mass spectrometry (MS-MS) is used to produce structural information about a compound by fragmenting specific sample ions inside the mass spectrometer and identifying the resulting fragment ions. This information can

then be pieced together to generate structural information regarding the intact molecule. Tandem mass spectrometry also enables specific compounds to be detected in complex mixtures on account of their specific and characteristic fragmentation patterns.

A tandem mass spectrometer is a mass spectrometer that has more than one analyser, in practice usually two. The two analysers are separated by a collision cell into which an inert gas (e.g. argon, xenon) is admitted to collide with the selected sample ions and bring about their fragmentation. The analysers can be of the same or of different types, the most common combinations being:

- quadrupole - quadrupole
- magnetic sector - quadrupole
- magnetic sector - magnetic sector
- quadrupole - time-of-flight.

Fragmentation experiments can also be performed on certain single analyser mass spectrometers such as ion trap and time-of-flight instruments, the latter type using a post-source decay experiment to effect the fragmentation of sample ions.

The basic modes of data acquisition for tandem mass spectrometry experiments are as follows:

a) **Product or daughter ion scanning**:

the first analyser is used to select user-specified sample ions arising from a particular component; usually the molecular-related (i.e. $(M+H)^+$ or $(M-H)^-$) ions. These chosen ions pass into the collision cell, are bombarded by the gas molecules which cause fragment ions to be formed, and these fragment ions are analysed i.e. separated according to their mass to charge ratios, by the second analyser. All the fragment ions arise directly from the precursor ions specified in the experiment, and thus produce a fingerprint pattern specific to the compound under investigation.

This type of experiment is particularly useful for providing structural information concerning small organic molecules and for generating peptide sequence information.

b) Precursor or parent ion scanning:

the first analyser allows the transmission of all sample ions, whilst the second analyser is set to monitor specific fragment ions, which are generated by bombardment of the sample ions with the collision gas in the collision cell. This type of experiment is particularly useful for monitoring groups of compounds contained within a mixture which fragment to produce common fragment ions, e.g. glycosylated peptides in a tryptic digest mixture, aliphatic hydrocarbons in an oil sample, or glucuronide conjugates in urine.

c) Constant neutral loss scanning:

this involves both analysers scanning, or collecting data, across the whole m/z range, but the two are off-set so that the second analyser allows only those ions which differ by a certain number of mass units (equivalent to a neutral fragment) from the ions transmitted through the first analyser. This type of experiment could be used to monitor all of the carboxylic acids in a mixture. Carboxylic acids tend to fragment by losing a (neutral) molecule of carbon dioxide, CO_2, which is equivalent to a loss of 44 Da or atomic mass units. All ions pass through the first analyser into the collision cell. The ions detected from the collision cell are those from which 44 Da have been lost.

d) Selected (SRM)/multiple reaction monitoring (MRM):

both of the analysers are static in this case as user-selected specific ions are transmitted through the first analyser and user-selected specific fragments arising from these ions are measured by the second analyser. The compound under scrutiny must be known and have been well-characterized previously before this type of experiment is undertaken. This methodology is used to confirm unambiguously the presence of a compound in a matrix e.g. drug

testing with blood or urine samples. It is not only a highly specific method but also has very high sensitivity.

1.1.2 *Matrix effects on the MS detection*

Matrix effects are problems associated with LC/ESI-MS, and have recently been reviewed (Taylor, 2005). Even if a selective scan method is used,such as SIM or SRM, which avoids the detection of most interfering compounds, such compounds may still affect the ionization efficiency.

ESI has proved to be generally more susceptible to matrix affects than APCI (Taylor, 2005; Dams et al., 2003). The matrix effect is detected as a suppression (most commonly) or enhancement of the analyte signal response arising from the co-elution of matrix components. The exact mechanism is still unclear, but probably involves competition between the analyte and the co-eluting components from the sample matrix for access to the droplet surface in the electrospray (Taylor, 2005). A mechanistic investigation of electrospray ionization was presented by King (King et al., 2000), who suggested that the ion suppression is probably not caused by reactions in the gas phase, but rather to arise from a high concentration of non volatile materials present in the ESI spray together with the analytes, inhibiting their transfer to the gas phase. Matrix effects on the ionization can be assessed by an addition of the analyte after extraction of the sample and to compare the resulting signals with those obtained from astandard solution. Different approaches for compensating for or reduce these problems have been presented, such as reducing the flow (Kloepfer et al., 2005) and using 2D-LC (Choi et al., 2001), where the primary problems associated with coeluting matrix components was attributed to column overload. The use of an internal standard may compensate for matrix effects, but this may also contribute to ion suppression. Liang et al. have presented a study showing that the use of co-eluting isotope-labelled internal standards suppressed the analyte signals (Liang et al., 2003). Another study

also reported problems with matrix effects when using a stable isotope analogue as internal standard (IS) (Jemal et al., 2003); here the ion suppression was greater for the IS than for the analyte, causing variations in the response ratios. The matrix effects can also be compensated for by using standards prepared in a blank matrix to create the calibration curve for quantification. However, such measures cannot avoid the loss of sensitivity due to ion suppression. The only solution for avoiding ion suppression effects is to improve the sample clean-up to remove co-eluting components.

1.2 Sample preparation techniques coupled with MS

Sample preparation is a crucial stepin the analysis of compounds in real samples, it maybe a source of imprecision and inaccuracy of the entire analysis.

In this project was evaluated these extraction techniques:

- Solid-liquid extraction to optimize a good solvent, water

- Solid phase extraction to optimize a good stationary phase, molecularly imprinted polymers (MIP)

- Solid phase extraction (SPE) on-line to optimize chromatographic condition using porous graphitic carbon (PGC) as stationary phase

1.2.1   Subcritical-water extraction and green chemistry

Subcritical-water extraction or superheated-water extraction (SWE), that is also called hot-water extraction (PHWE), is based on the same principles as pressurized-liquid extraction (PLE) in which pure water as the solvent and is an innovative green sample preparation technique.

This technique used at the expense of the classical methods of extraction, which involve the use of solvents that will, definitely, an increase of environmental pollution. The toxicological research has highlighted the health hazards arising from residues of processing that may be present in trace

amounts in foods and drugs. Thanks to advances in analytical chemistry has shown that it is extremely difficult to eliminate these residual traces, as for example in the case of organic solvents often used in the extractions. Some of these solvents are very toxic, even if taken in small amounts, and then, only when it is possible, are excluded from industrial practices. In other cases are imposed tolerance values extremely low hardly obtainable. Attempts are therefore, increasingly, to obtain natural extracts through methods of extractions based on technologies which avoid the use of solvents or hazardous reagents. Among the solvents that have proved toxic for the human body there are numerous distinctly apolar, used for the extraction of compounds not soluble in water or in ethanol. Examples are hexane, benzene, chloroform, dichloromethane and the carbon tetrachloride.

The technique was introduced in the mid-90s by Hawthorne's group (Hangeman et al., 1996; Yang et al., 1995; Hawthorne et al., 1994, 1998, 2000, 2001) and it exploits the large change to physico-chemical properties of water that occurs at high temperatures. When the temperature is increased, the polarity (measured by dielectric constant ε) decreases, and the solubility of organic compounds in the subcritical water increases.

This technique is an examples of a new ''green'' process.

Water is the most abundant molecule on the planet and is sometimes referred to as a benign ''universal solvent.''

Green Chemistry is a fundamental and important tool in accomplishing pollution prevention. Pollution prevention is an approach to addressing environmental issues that involve preventing waste from being formed so that it does not have to be dealt with later by treatment or disposal.

1.2.1.1 History

At the beginning of the new century, a shift in emphasis in chemistry is apparent with the desire to develop environmentally benign routes to a myriad of materials.

There is no doubt that over the past 20 years, the chemistry community, and in particular, the chemical industry, has made extensive efforts to reduce the risk associated with the manufacture and use of various chemicals. There have been innovative chemistries developed to treat chemical wastes and remediate hazardous waste sites.

1.2.1.2 Extraction of solid sample

Generally, the extraction of analytes, from aerial parts, from roots and rhizomes of plants, occurs using techniques involving the use of organic solvents. The solid-liquid techniques currently in use in the industry, it is to process large quantities of materials are essentially three different types: maceration, percolation's solid-liquid extraction or dynamic Naviglio extractor and the extraction by the Soxhlet.

The maceration is a simple and inexpensive technique, which does not require complex equipment and skilled personnel. The solid to be extracted is introduced into the container and is added a quantity of liquid which generally covers the same solid. The major limitation of this technique is given by inability to extract with water, because the majority of the vegetable substances goes decaying faster than the extraction process. The alternatives are the extraction with organic solvents, which, however, have environmental problems related to disposal, or reuse of these solvents.

The percolation, instead, is a technique of liquid-solid extraction, which is based on the phenomenon of diffusion and osmosis, but, which differs from the macerating as occurs dynamically.

The rapid solid-liquid extraction or dynamic Naviglio extractor base its efficiency of extraction of a pressure difference between the liquid extraction at the interior and the exterior of the solid matrix. The system is left under pressure for a time required for the liquid can effectively penetrate inside of the solid matrix. It is created a pressure difference between the interior and the exterior of the matrix itself, which forces the liquid to flow rapidly from the interior of the solid. The rapid displacement of the liquid contributes to the leakage of substances not chemically bound to the solid matrix. The limits of this technique are: the time, in fact, the whole process, it usually takes time of the order of hours, and that not all the analytes are water soluble.

The Soxhlet extraction apparatus consists of three basic components superimposed at the bottom of a flask with ground glass stopper, half the extractor itself and a condenser at the top. The solid material (consisting of the solute that you want to separate and impurities) is placed in an extraction chamber, generally in a filter thimble consisting of filter paper, permeable bythe solvent. The solvent is brought to boil, then passes into the extractor and in the condenser, condensate falling back as in the extraction chamber, where the charge of the solute, then flows through the filter paper carrying the solute, and is drawn off by a side duct where accumulate the solute at the bottom and at the top the solvent, and the solvent is re-circulated from here to the flask.

Alternatively, the solvent and the solute can be poured together into the flask, where the solute remains as a precipitate and is recovered at the end of operation. Subsequently, the solvent may be removed from the mixture solvent-solute with the aid of a rotavapor (or rotary evaporator). Since it is generally used in the ether, the operating temperature is relatively low, therefore, the ball is immersed in a thermostatic bath with a temperature regulated as a function of the boiling point of the solvent. Even in this case, as in previous extraction techniques, solvents are chosen assuming that they have an affinity towards the analyte to be extracted. In the case of the extraction of

curcumin from the rhizome, have been used various methods, such as, Soxhlet extraction, percolation, extraction with organic solvents. The solvents generally used, for example, in the Soxhlet are: ethanol or acetone and ethanol dichloride; despite the high extraction yields, there are several problems, such as, flammability, and high costs for the recovery of acetone, the production in extract fractions with a high boiling point, the part of the ethylene dichloride, and the need for washing with water to remove the solvent, extraction with ethanol. For all these problems, together with the toxicity of these solvents proved to be best and, more effectively, the extraction with subcritical water, which does not cause even the degradation of the molecule that, instead, it would have to supercritical values.

1.2.1.3 *Water as solvent*

Water is a good solvent with a high boiling point (100°C) for its mass, high dielectric constant and high polarity. It has a highly hydrogen-bonded structure that gives its unique property as solvent.

Figure 2: Water's molecules

Water-only separations have been evolving in the field of sample preparation by solvent-extraction for chemical analysis.

The purpose of this project was to use an extraction technique based on the use of a solvent harmless, versatile, economic and of low environmental impact which the water. It is well known that water is an excellent polar solvent, which, under certain conditions of temperature and pressure, acquires properties suitable to be able to dissolve, and in order to extract, analytes with

a different degree of polarity: as, for example, the polycyclic aromatics from the ground, essential oils, anthraquinones and nutraceuticals from different plant species (Di Corcia et al., 1999; Eikani & Golmohammad, 2007; Ozela, et al., 2003). In this work, we have shown, as it is easy to remove poorly water-soluble molecules such as, for example, Curcumin and curcuminoids, coming from the rhizome of the plant Curcuma longa, with a highly polar solvent, which is suitable with the principles of "Green chemistry". Curcumin is becoming more and more an object of study, which aim at new pharmaceutical products and food, so even for it, it requires the search for alternatives to the traditional ones practiced in the past. Generally, these substances, are extracted using highly toxic solvents, such as, for example: dimethyl sulfoxide, dichloromethane, methanol, with various solid-liquid extraction techniques. The technique most commonly used is that which provides for the use of the soxhlet, in which the boiler, there is a single solvent, or a mixture of several solvents. From various studies, however, we have seen that the yields obtained with this last extraction method are comparable with those of SWE, a result that could mark a radical change in methods of extraction, resulting in less environmental pollution.

1.2.1.3.1   *Physical and chemical characteristics of the water*

Water is essential in the nutrition of animals and plants, is also widely used in science and industry as a solvent, catalyst, as a standard practice in the representation of certain properties and physical quantities, as a means of Convention for the transport of materials and for the destruction of waste. In particular, industry water is used as the dispersing medium, as the coolant, such as cleaning agent, in the production and distribution of heat, and in the production of electricity. Being a clean liquid, harmless, cheap, versatile and environmentally friendly, it is preferred to other organic solvents. Water is a good solvent with a boiling temperature of 100 ° C and melting temperature of

0 ° C at a pressure of 760 mmHg, whose changes of state: liquid, vapor, solid, are described by the phase diagram, in which the abscissa shows the temperature and on the ordinate the pressure.
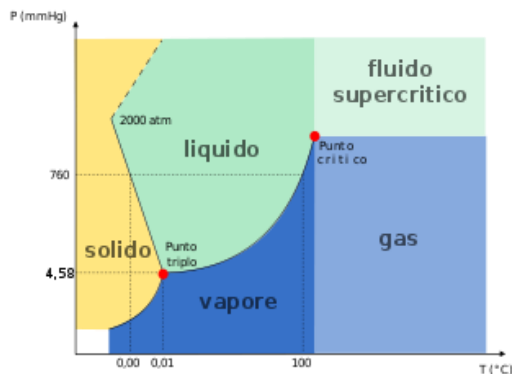
Figure 3: The phase diagram of water: the abscissa shows the temperature and on the ordinate the pressure.

The process of solidification of the water takes place with an increase of the specific volume. This implies that as the temperature decreases, the pressure corresponding to the passage solid, liquid increases significantly (negative slope); in particular for every hundredth of a degree celsius there is an increase of pressure of about 1 atm. This report has occurred up to a pressure of 2070 atm and at a temperature of -22 ° C. The point T, identified by the value of pressure equal to 4.58 mm Hg and the temperature of 0.01 ° C is the triple point where all three phases coexist solid, liquid, gaseous; furthermore, observing the graph, we see the presence of a specific point said critical point in which the characteristics of the vapor and liquid become the same; for example, for water, is at a temperature equal to 374 ° C at a pressure equal to 218 atm (22,064 MPa). Thanks to its high versatility, the water is used for the extraction of poorly water-soluble substances; changing the values of pressure and temperature, and remaining below, or above the critical values, we obtain

a fluid with different physico-chemical characteristics compared to those of departure, referred to as sub-supercritical fluid.

1.2.1.4 Sub- and supercritical water

Subcritical water is obtained at temperatures between100 and 374 °C (which is the water critical temperature) under high pressure (usually from 10 to 60 bar) which is high enough to maintain the liquid state (below the critical pressure of 22 MPa) (Ramos et al., 2002).

If the temperature and pressure exceed 374°C and 221 bars, water becomes supercritical. Supercritical water has the dielectric constant 1 and becomes a good solvent for lipophilic compounds (Shaw et al., 1991)

Many extractions were carried out using supercritical water as a solvent (Yoda et al. 2003). Under these conditions, the properties of the fluid are in part similar to those of a liquid (such as density) and in part similar to those of a gas (e.g. viscosity). In addition, there is no surface tension in a supercritical fluid. The link with the temperature, however, is more complex, in fact, at constant density, the solubility increases with temperature. Then approaching the critical point, the density, and therefore also the solubility, may decrease drastically with a slight increase of the temperature to rise again (Yoshi et al., 2001). All supercritical fluids are completely miscible with each other, it can be deduced that it is possible to obtain a monophasic mixture of fluids if it is over the critical point of the desired mixture. The critical point of a binary mixture can be estimated using the arithmetic mean of the critical temperatures and pressures of the two components. In many work was used supercritical water as the mobile phase extractant for the rapidity of operation and for the low viscosity and diffusivity of supercritical fluids. The advantages of extraction with supercritical fluids (compared with liquid extraction) reside mainly in the speed of operation, given the low viscosity and high diffusivity (Brunner et al, 1994). The extractions can be made selective by controlling the

density of the medium and the extracted material is easily recovered by lowering the pressure, allowing the supercritical fluid to return to the gas phase and evaporate, leaving few traces of solvent. This is a technique widely used to decaffeinate the beans roasted coffee, for the extraction of hops for the production of beer and for the production of essential oils and pharmaceutical products derived from vegetable products (Rodrigues et al., 2001). The water at room temperature and ambient pressure has a dielectric constant of 80 and is therefore able to solubilize the compounds ionic or strongly polar. In supercritical conditions, instead, the dielectric constant can be lowered to a value much as 10 orders of magnitude: at 250 °C and pressure between 50 and 350 atm, the dielectric constant is equal to 27-29, a value similar to that of ethanol that pressure and at room temperature is equal to 24, and similar to that of the methanol that is instead equal to 33. The use of supercritical water is limited by a number of factors: it is corrosive, may cause damage to the cell extraction and it is not recommended for analytical purposes.

The temperatures required to reach the critical point (374 °C to 218 atm) are probably too high for the extraction of some thermolabile substances. In fact, the supercritical water is used frequently, with oxidizing agents, to cause degradation of organic substances dangerous (Kronholm et al., 2002).

1.2.1.5 Parameters affecting the SWE process

Raising the temperature increases the diffusion rates, the solubility of the analytes and the mass transfer, and decreases the viscosity and surface tension of the solvents.

High pressure helps to force the solvent into the matrix pores and to keep the solvent in the liquid state at the operating temperature. Of course, the thermal stability of the analytes of interest and, occasionally, the matrix can pose limitations on the experimental conditions.

Among the different properties of interest for an extraction process, the most useful is the change in the dielectric constant of the water with increasing temperature that leads water to behave like an organic solvent when heated at, for example, 498 K under at a constant pressure of 50 bar (constant decreases from 80 at 298 K to 27, which falls between those of methanol (33) and ethanol (24) at 298 K). (Miller & Hawthorne, 1998). When the temperature is raised as well as its permittivity also its viscosity and surface tension decrease rapidly. In fact, its increasing in diffusivity and density make it more useful for extraction.

Increases of temperature and pressure destroy the strong interactions existing between analyte and matrix as hydrogen bonds, Van der Waals forces and dipole-dipole attractions also increasing in this case the ability of the water as solvent.

The energy needed to heat the water from 25° C to 250° C at atmospheric pressure requires 2.869 kJ/kg, while to heat the water in the same temperature range at a pressure of 5 MPa requires only 976 kJ/kg . Therefore, the consumption of energy for extraction with subcritical water is lower than that required for the distillation in a current of steam.

The water is non-corrosive and, therefore, don't damage the cell extraction as could happen in the supercritical water extraction; in fact, only at temperatures above 350 °C were noted slight alterations to the cells in stainless steel after 40-50 extractions.

The extraction is fast because the diffusion of molecules, the solubility of analytes increases with temperature, but not all in the same way: for example, the extraction of essential oils from rosemary and coriander was faster of extraction of hydrocarbons. Therefore, the extraction with subcritical water is both selective and rapid,and for these qualities has been used to fractionate particulate material, for replacing organic solvents in many applications as for example for the extraction of PAH from the ground.
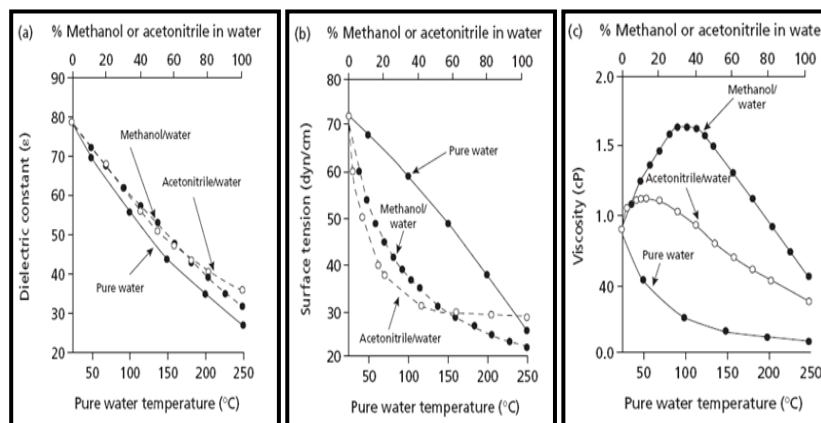
Figure 4: Control of solvent polarity (dielectric constant) (A), solvent surface tension (B) and solvent viscosity (C) by changing temperature (at 50 bar) with pure water compared to mixing water with methanol or acetonitrile at 25°C and ambient pressure (Yang et al., 1998)

1.2.1.6 SWE applications

Recent reports have demonstrated the ability of "subcritical" water to quantitatively remove a variety of polar andnonpolar organics from many matrices (Basile et al., 1998;Hartonen et al., 1997; Hawthorne et al., 1994; Yang et al.,1995, 1997, 1998).

The high pressures used may force water into areas of the matrices that would not be accessible to solvents under atmospheric pressure (Richter et al., 1996). The extraction efficiency is also affected by the extraction time, flow rate and the use of additives or modifiers (Kronholm et al., 2007).

Water is also used in chromatography (Yang, 2007; Pawlowsky & Poole, 1999) because showing properties between SFE and conventional liquid reversed phases.

Recently, there has been an increasing interest in the use of environmentally, clean technologies able to provide high quality and high activity extracts while precluding any toxicity associated to the solvents. In this sense, SWE is considered an alternative extraction as well as supercritical fluid extraction

(SFE), microwave extraction, dynamic sonication-assisted solvent extraction, and pressurized liquid extraction (Ramos et al., 2002; Kronholmet al., 2007; Herrero et al., 2006).

The conventional methods used currently for extraction of compounds from herbal plants are distillation and solventextraction. Low extraction efficiency and toxic solvent residues in the extracts occur when using these technologies. (He et al., 2012; Wang et al., 2012)

SWE exhibits a number of advantages over conventional extraction methods: the reduction of extractiontime, simplicity, less-expensive operation, higher qualityof the extracts and environmental-friendly (Herrero et al.,2006).

Table 2:   SWE advantages and disadvantages (Nerin et all, 2009)

Advantages	Disadvantages
Total absence of dangerous, toxic or expensive organic solvents	Handling of subcritical water is not easy
Non-flammable, non-toxic	Use of internal standard is
Readily available and environmentally friendly	highly recommended
	Initial cost of the extraction
Variable selectivity (polarity) simply by changingtemperature	system
	Intrinsic dilution of extracts
Easy to optimize (pressure has a little effect)	
Low extraction times	
Simple experimental set-up	
Potential for on-line coupling with different techniques, as HPLC	

1.2.2 *Solid phase extraction*

Solid phase extraction (SPE) is a very common type of clean-up technique for bioanalytical purposes, due to its simplicity and versatility.

SPE is well-established and widely used in many different areas of chemistry. Development of SPE methods and their use in analytical chemistry greatly increased during the late 1980s and the 1990s, although their history dates back to at least the early 1970s (Fritz, 1999).

SPE involves passing a liquid sample through a solid sorbent bed, usually consisting of modified silica particles. The aim is to retain the analytes in the sorbent bed, wash away interferences and finally elute the analytes as a clean extract in a small volume. The collected extract can then be analyzed by a suitable method, for instance, LC/MS. A wide range of different formats and sorbents for SPE applications is available, and the technique can be used either off-line or on-line.

SPE with tailored MIP sorbents (MISPE) is currently a rapidly growing field. This type of technique was evaluated in this thesis.

SPE was initially developed as a technique to complement or replace liquid-liquid extraction (LLE). The principle is very similar to that of LLE, except that the solutes are not partitioned between two immiscible liquid phases but between a solid phase (sorbent) and a liquid phase (sample matrix).

One advantage of SPE over LLE is that it requires lower amounts of organic solvents for the extraction (Huck & Bonn, 2000), which is important for environmental and health reasons. SPE also often offers higher selectivity for the target compound(s) due to the wide range of sorbent types available. Other advantages of SPE are its higher concentration factors and the fact that the extraction steps can readily be automated.

Consequently, the solid phase extraction has become the method of choice for the preparation of the sample (Hennion, 1999). Today, there are many types of sorbents for the SPE but for their lack of selectivity, they fail to efficiently separate the analytes from complex environmental matrices. The molecularly imprinted polymers (MIPs) in recent years have attracted the attention of many scientists, for the many benefits that characterize them.

The analyte can be retained by the sorbent surface by a range ofdifferent types of interaction, such as hydrogen bonds, dipole-dipole ordipole-induced dipole mechanisms, dispersive, charge transfer or ionicinteractions. Ionic interactions involve higher energies (50-200 kcal/mole) than both hydrogen bonds (5-10 kcal/mole) and the dipole typesof interactions (1-10 kcal/mole) (Handley & Mcdowall, 1999). The three main modes of SPE fororganic compounds utilizing one or several of these interactions are: reversed-phase, normal-phase, and ion-exchange systems. In reversed phaseSPE the aim is to extract non polar analytes from a polar sample using a sorbent of non polar character. For normal-phase mode an opposite approach is used; the sorbent consists of polar particles while the sample matrix is non polar and polar analytes are extracted. Ionic interaction occurs between an analyte carrying a charge (positive or negative) and a sorbent carrying the opposite charge. The SPE process usually consists of four distinct steps:

(1) conditioning/equilibration. Conditioning of the sorbent is important to promote good surfacecontact between the phases. This is usually done by wetting the sorbent with an organic solvent of appropriate polarity. This stepalso includes the equilibration of the sorbent with a solution as similar to the sample as possible, in terms of polarity, ionic strength and pH.

(2) adsorption/sample application: the sample is percolated through the sorbent bed, simply by gravity, or by the application of a gentle vacuum or pressure. Itis important to control the flow rate in this step to promote efficient mass transfer of the analytes to the sorbent. A suitable flow rate depends on the particle size distribution, the column dimensions and the packing characteristics of the sorbent.

(3) washing step, to wash off retained interferences from the sample matrix without eluting the analytes.

(4) elution step, that involves disrupting the retention of the analyte on the sorbent. A solvent offering a combination of the possible interactions is often

most effective. It is advantageous if the elution solvent volume is small enough for the analyte to be pre-concentrated. Furthermore, the solvent should ideally be compatible with the final analysis.
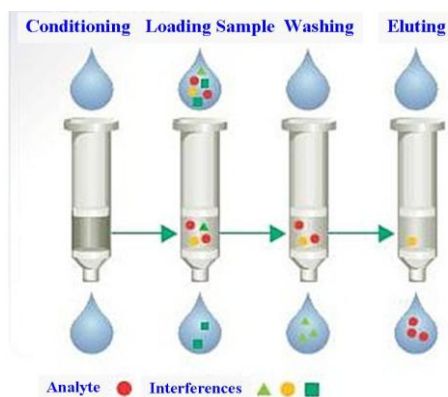
Figure 5: SPE process: (1) conditioning of the stationary phase, (2) loading of the sample, (3) washing phase, (4) elution step

An alternative approach for the SPE process is to retain the matrix components, while the analytes of interest pass through the sorbent bed.

1.2.2.1 Online and offline methods for sample preparation

The most common format for SPE materials is the cartridge, or SPE column, which consists of a reservoir, most often of polyethylene, with the sorbent bed fitted between two frits, as shown in Fig.5. Such columns are available with a wide range of different sorbents, sorbent sizes and reservoir volumes. Typical amounts of sorbents are between 25-500 mg with reservoirs volumes of 1-10 ml. This format is used for off-line applications, while for on-line use the sorbent is packed in so-called pre-columns. Typical amounts of sorbents in a pre-column are 20-100 mg. The on-line SPE process is similar to the off-line protocol, including conditioning, washing and application of the sample to the pre-column. The main difference is in the elution step, since the retained analytes are eluted directly onto the analytical column. The elution solution, then, acts as the mobile phase and has to be compatible with the following

separation and detection method. The most common on-line coupling of SPE when analyzing biological samples is with liquid chromatography (Hennion, 1999). This is explained by the good compatibility of the LC aqueous mobile phases with the SPE of biological samples, which are mainly aqueous. On-line coupling with gas chromatography is not as straight forward as SPE/LC for biological samples. Since GC is not normally compatible with water a drying step is usually required before elution witha solvent suitable for GC injection. On-line coupling of SPE to GC for the analysis of biological samples has recently been reviewed (Hyotylainen & Riekkola, 2005)

Another SPE format is in disks (Fritz, 1999), in which the sorbent is supported on a membrane. Such disks have a wide, thin bed, and contain smaller particles than those used for the columns. Advantages of disks, compared to the cartridges, are that higher flow rates can be applied due to the lower backpressure generated, and that the risk of channelling in the sorbent bed is reduced, since the sorbent particles are held closely together between the membrane filters. SPE sorbents are also available in 96-well plates, which were developed inthe early 1990s for high throughput and small-volume SPE in bioanalysis. These SPE blocks are now available for several types of sorbents and different sample volumes. Most plates are used in particle bed format, but they are also available as disk-based devices (Venn et al., 2005).

The development of these diverse formats for SPE has facilitatedautomated sample processing (Rossi & Zhang,2000).

The equipment that you can use to make a SPE off-line is the *Vacuum manifold* (Fig. 6).

Figure 6: Vacuum manifold

The procedures of solid phase extraction are used to extract traces of organic compounds from complex matrices but also to remove interfering components to obtain an extract cleaner containing the analytes of interest. Nowadays there is a considerable interest in the development of new methods selective and sensitive to extract components from environmental matrix's complex (Hennion, 1999).

Table 3:   SPE on- and off-line

SPE on-line	SPE off-line
Analysis of the total amount of analyte extracted	Analysis of a reduced rate of analyte extracted
Low volume of sample gives sufficient sensibility	Large samples volume needed (wather)
Matrix effect	Low matrix effect in mass spectrometry
Reusable cartridge	Disposable cartridges
Little flexibility, for most systems it is not possible to use different cartridges	Possibility of using a variety of cartrydge connected
Automation and minimal handling of the sample (better precision and accuracy)	Sample handling, possible contaminations (less precision and accuracy)
Direct and fast elution of the sample after preconcentration. Minimal degradation	Risk of contaminations (Longer analysis time)
Minimal consumption of organic solvent (HPLC mobile phase used for	Consumption of organic solvent for elution

elution)	
No analyte loss in the evaporation step	Possible anayte loss int he evaporation step
One analysis for each extract	Possibility of different analysis for each extract
Reduced analysis time (extraction and analysis in sequence)	Long analysis time
Limited portability of the system	Portable system
Expencive system	Cheap system

The most common types of SPE sorbents are bonded-phase silica particles. These particles consist of siloxanes chemically modified by molecules with appropriate functionalities for the intended application, e.g. hydrophobic carbon chains for reversed phase SPE: C18 (octadecyl modified silica) is by far the most widely used phase. Adisadvantage with silica-based sorbents is that they are not very stable outside the pH range 2-8. Another problem with silica sorbents is that the residues of free acidic silanol groups can give undesirable secondary interactions, for example hydrogen bonds with alcohols and amines.

Although, there is a wide range of different SPE sorbents commercially available they often lack in selectivity. This is a problem especially when the analytes occur in trace amounts and co-elute with interferences from the sample matrix. For biological samples this may necessitate an additional sample clean-up prior to final analysis. For this reason more selective sorbents, such as immunoaffinity sorbents and MIPs are often desirable.

Other common types of SPE phases are antibodies, molecularly imprinted polymers and various forms of carbon, such as graphitized carbon blacks (GCBs) and porous graphitic carbon (PGC).

1.2.3 Immunoaffinity extraction

The affinity chromatography is based on highly selective interactions of antigens with their antibodies, which are immobilized on a suitable support

called immunosorbent. This technique is based on the ability of antibodies to recognise and bind specifically the target analytes. Antibodies are produced by the mammalian immune system in response to the presence of foreign substances (antigens) (Venn, 2000).

Since the antigen-antibody interactions are established at short distance, the steric effects are involved in the coupling reaction. Only the antigen that will produce the immune response, or structurally closely related molecules, may bind to the antibody. In immunoaffinity extraction (IAE) antibodies are covalently boundonto an appropriate sorbent and packed into a solid-phase extractioncolumn or. The immunoextraction protocol consists of the same foursteps of SPE. The mechanisms of adsorption and desorptionare different from those involved in ordinary SPE. After the extraction a separation step, chromatographicor electrophoretic, is often performed. This may solve the problems associated with the cross-reactivity of the antibodies.
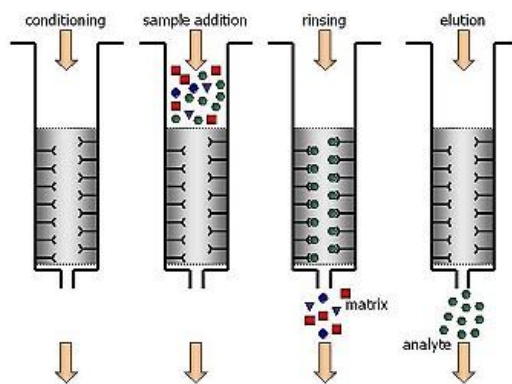
Figure 7: Various stages of affinity chromatography

Immunoaffinity extraction provides unique and powerful techniques, due to the high selectivity of the antibody-antigen interaction, which enable selective extraction and concentration of individual compounds or classes of compounds. Wide ranges of applications of solid-phase immunoaffinity

extraction have been developed, both off-line (Gam, 20003; Ferguson, 2001) and on-line (Deinl, 1999).

The drawbacks of immunosorbents include their cost and time-consuming development. The design and production of antibodies may take several months or even years and it can be difficult to obtain specific antibodies for low molecular weight molecules. Another drawback is the sensitivity of the antibodies to pH changes, elevated temperatures and organic solvents. These drawbacks have led to the development of synthetic antibody mimics, so-called molecularly imprinted polymers.

The selectivity is conferred to the polymer during the polymerization, when there is the formation of a binding pocket rigid, in which only the analyte or closely related compounds can be inserted (Muldoon & Stanker 1997).

Reported MIPs advantages on commonly used antibodies based immunosorbents are:

• Selective sites embedded in solid matrices stable at harsh condition: organic solvents, severe pH, relatively high temperature and pressure.

• Robust SPE materials preserving selective properties (possible regeneration and re-use).

• Higher load capacity due to several orders of magnitude higher number of selective sites.

1.2.4 Molecularly imprinted polymers (MIPs)

MIPs are a class of highly cross-linked polymer-based molecular recognition elements engineered to bind one target compound or a class of structurally related target compounds with high selectivity.

MIPs are prepared by first mixing a template molecule with one or more functional monomers.

The monomers form spontaneous complexes around the template. Upon complex formation, cross-linking monomers are then added with a suitable porogen to drive polymerization.
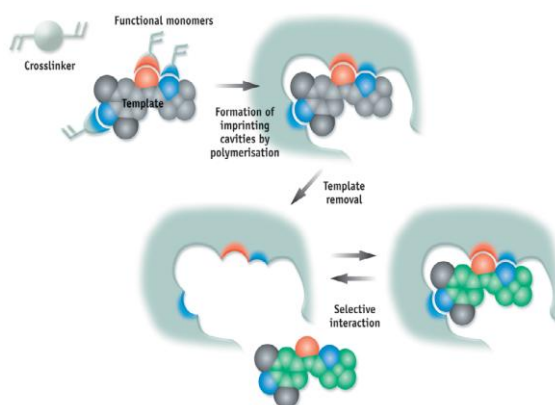
Figure 8: MIP preparation

The binding sites are formed around the template during the polymerization process and, at the moment when the template is removed, the remaining cavities retain a steric (size and shape), chemical (spatial arrangement of functional groups), and electronics 'memory' against the template. The cavity will be able to recognize and bind reversibly again the template and molecules similar (Shea & Sassaky, 1989; Rimmer, 1998)
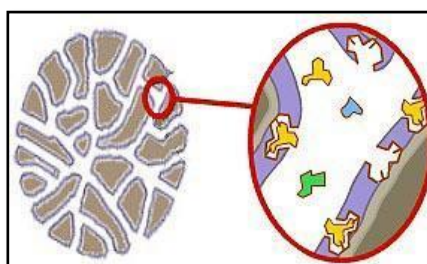
Figure 9: MIP cavities

The receptor and the ligand should be as complementary as possibleto have an efficient molecular recognition. It may establish various types of

intermolecular forces between the functional groups of the binding site and the ligand:

- hydrogen bond: electrostatic interactions directional, in which a hydrogen atom serves as a bridge between a group acceptor of hydrogen bond donating group and a hydrogen bond

- ionic interactions: are created between functional groups with a distribution of electrostatic charge

- Van der Waals forces, dipole-dipole or induced dipole-induced dipoleforces, the sum of contributions of electrostatic energies of dispersion and repulsion, type weak

- aromatic interactions and hydrophobic interactions: there is a transition metal or an aromatic group.

These types of forces are of considerable importance in the process of molecular recognition (March, 1997; Hunter, 1994).

1.2.4.1 MIP'S History

In 1977 Wulff was able to achieve a polymer that bound selectively a template, a sugar (Wulff et al., 1977). In the covalent imprinting it need to be conducted, before polymerization, a covalent bond between the functional monomer and the template, which is maintained during the polymerization. After curing the bond is broken and the template is removed. Between the template and the polymer molecule may have as a result of the formation of the same covalent bond. The technique has advantages and disadvantages.

The advantages:

- the monomer-binding template is stable and stoichiometrically defined

- the imprinting process and the molecular structure of the binding sites appear to be simple

- can be applied to various polymerization conditions thanks to the stability of the covalent bond.

The disadvantages:

- synthesis is difficult and costly
- the number of covalent bonds that are obtained is small
- the polymer may collapse, causing a rupture of covalent bonds and a decrease of the mold
- the bond and the release of the template are slow, because of the strength of the covalent bond.

In 1981 Mosbach succeeded in demonstrating that the covalent bond between the functional monomer and template was not strictly necessary, enough non-covalent bonds (Arshady & Mosbach, 1981; Vlatakis et al., 1993). To tie the functional monomer and the template is exploit non-covalent intermolecular interactions (hydrogen bonding, electrostatic interactions, etc.), So as to induce the formation of a complex by simply adding the template to the polymerization mixture. At the end of the polymerization the template, with a suitable solvent, is removed. Even the mold noncovalent has advantages and disadvantages.

The advantages:

- the synthesis of the monomer-conjugated templating agent is not necessary
- removal of the template is easy, given the strength of the bond, with continuous extraction
- the binding and release of the template are carried out quickly.

The disadvantages:

- the adduct monomer-template is not stoichiometrically defined
- the process of imprinting is unclear to maximize the formation of non-covalent adducts is important to study the polymerization conditions
- very often the functional monomers form non-specific binding sites that decrease the selectivity of the bond.
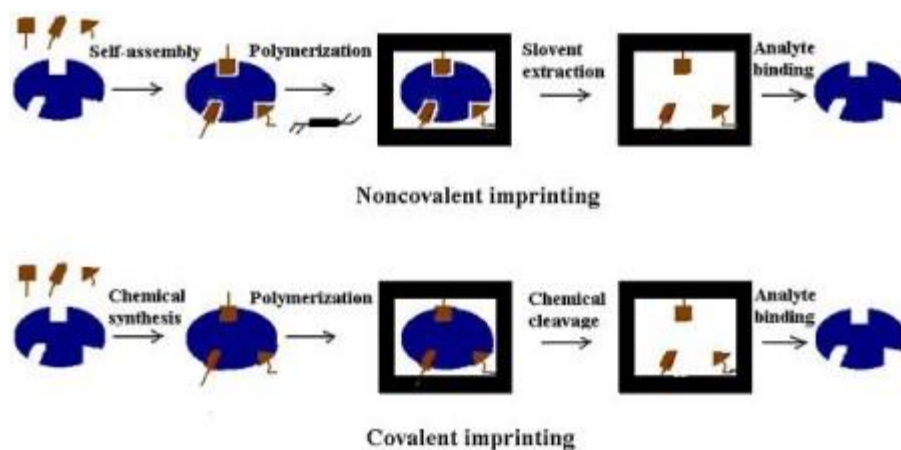
Figure 10: Covalent and non covalent synthesis of MIP

1.2.4.2 NIP and MIP

Complete removal of this template compound is necessary if the method is to be used for samples with the analyte in trace amounts. However, it has been shown that complete removal is difficult even if an extensive washing protocol is applied (Rashid et al., 1997; Andersson et al., 1997; Andersson et al., 2000) For this reason an approach to resolve this problem has been developed by Andersson (Andersson et al., 1997) based on the use of a structural analogue to the analyte as the template compound.

In some polymers prepared with the method non covalently is observed not a complete removal of the molecule and a template release its residual over time, defined bleeding. For this reason, some authors suggest to synthesize the MIP using as tempalte a structural analogue of the same template in place to prevent the phenomenon of bleeding interfere with the analysis. In any polymerization is a non imprinted polymer (NIP) synthesized in the same way as the MIP but in the absence of the template to be able to evaluate the effect of imprinting of the polymer, ie to determine the selectivity of the MIP compared to NIP molecule towards the template (Caro et al., 2006).

1.2.4.3 Variables in MIP's sinthesys

The synthesis of the MIPs, however, is rather complicated because of the many variables involved, including:

- the nature and levels of template
- functional monomer
- cross-linker
- porogen solvent
- initiator

   a) Template

The template is of fundamental importance and must be carefully considered because it may possess polymerizable groups, or it could interfere with the polymerization, inhibiting it, for example, and could also not resist high temperatures or be susceptible to ultraviolet light (UV). They are usually used small organic molecules as templates, since the irrigidity facilitates the synthesis of the polymer and recognition (Cormack & Elorza, 2004).

   b) Functional monomer

The choice of functional monomer depends on the characteristics of template. Template has to possess more functional groups capable of giving interaction with the functional monomers. Furthermore, in the crosslinking the strength of the interaction must be such as to confer an orientation stable (Robinson & Mosbach, 1989; Moradian & Mosbach, 1989). After the polymerization process of the functional groups should allow the removal of the greater number of template. For the covalent synthesis is used monomers such as esters or amides of acrylic and methacrylic acid; for the non-covalent synthesis is more indicated vinyl monomers, with various functional groups. An excess of monomer compared to the template is required to favor the formation of monomer-template complex and to maintain its integrity during the polymerization. A fraction of monomer is however incorporated randomly in the polymer and gives rise to non specific binding sites. A molar ratio 1:4

template-monomer complex ensures stability and guarantees the imprinting effect desired. You can also choose the monomers according to their chemical-physical characteristics (Fig. 11.):

• acidic monomers, such asmethacrylic acid (MAA) and acrylic acid(AA)

• Basic, such as 4-vinylpyridine (4-VP), the 2-vinylpyridine (2-VP) and the allylamine

• neutral, such as methyl methacrylate (MMA), acrylamide (AM) and styrene.
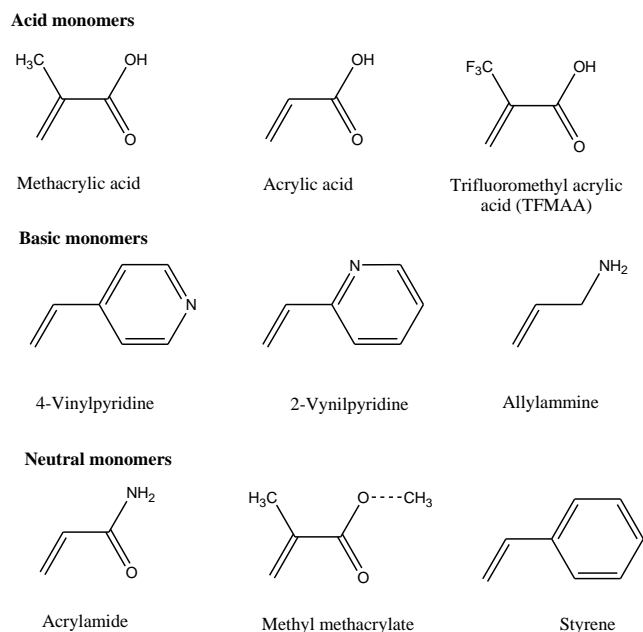
Figure 11: Examples of acid, basic and neutral monomers

It usually is used MAA with a basic template and 4-VP with an acid template. Both monomers are capable of establishing very strong hydrogen bonds (Beltran et al., 2010).

c) Crosslinking

The crosslinking agents weld binding sites in the structure, ensuring rigidity of the binding sites and giving selectivity and specificity to the polymer. The cross-linker control the morphology of the polymer matrix that may be gel-type, macroporous, micro-powder or gel. They make the polymer insoluble in

the solvents used. The reactivity of the crosslinking agent should also be as similar as possible to that of the functional monomer in order to avoid a predominant polymerization of the cross-linker or the monomer and to allow an efficient copolymerization. Very important is the molar ratio between the cross-linker and the monomer. If the ratios are too low, it will have binding sites too close to each other, so as not to be able to act independently. If the molar ratios are too high, you lose the efficiency of the bond. Furthermore it imparts mechanical stability to the polymer. The crosslinking agent is the component of the polymer present in greater amounts; usually employ amounts varying from 50 to 90% of the total moles of reaction (Richardson & Richardson, 1989). The most commonly used are cross-linker ethylene glycol dimethacrylate (EGDMA) and divinylbenzene (Fig. 12).

EGDMA   Divinylbenzene
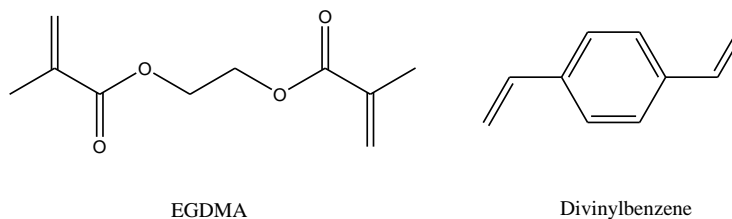
Figure 12: The most common cross-linker

d) Porogen

The solvent porogen should solubilize all the components of the polymerization mixture and give porosity to the polymer. It also dissipates heat of reaction which is generated during the polymerization, to avoid that the high temperature in some points might impede the formation of the complex template-monomer or the thermal degradation of the monomers. In the covalent synthesis can be used any type of solvent (Minton et al., 1992). In the case of non-covalent synthesis, the choice of the porogen is crucial. Usually, the high polarity of the solvent decreases the efficiency of molecular recognition of the MIP, and the polarity of the solvent negatively influence the formation of non-covalent bonds (Rose et al., 1985). The best solvents are

toluene and dichloromethane, and hydrophobic with low dielectric constants. Solvents with high dielectric constants, such as acetonitrile, are widely used but the polymers that are formed to show a low affinity to recognize the template. Protic solvents, such as water and methanol, are not recommended, as they are able to destroy the hydrogen bonds that develop between the monomer and the template. The solvent porogen determines the size, shape and spatial distribution of the pores (Rose et al., 1985). A low macroporosity allows a very efficient molecular recognition. The chloroform turns out to be the porogen better, because it dissolves all the monomers and the molecules mold and not hinders the existence of hydrogen bonding.

    e) Initiator

The initiatoris usually used at levels comparable to the monomer, such as 1% weight or 1% mole. There are various types of initiators, but the most widely used is α,α´-azoisobutyronitrile (AIBN) which can be easily decomposed by thermolysis or photolysis to give stable radicals and initiate polymerization (Cormack & Elorza, 2004).
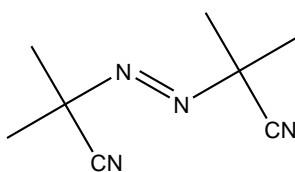
Figure 13: AIBN: an example of initiator

1.2.4.4 Other variables in MIP's sinthesys

MIP is a "black box": many questions remain unsolved on the synthesis and performance. Even today is practically impossible to propose a protocol reproducible and reliable for the preparation of a polymer with the properties required for a specific application. The important factors to be taken into consideration, in addition to the type and concentration of the monomer, the crosslinker and the solvent, are:

-        polymerization temperature

- pressure and swellingof the polymer
- time and polymerization conditions

The polymerization temperature plays a key role in the synthesis of the MIP and in its future performance because the affinity of the polymer and its specificity increases with decreasing temperature. In fact the polymer "memorizes" the temperature used in the polymerization, in a manner similar to what happens with the template and the polymerization solvent (Piletsky et al., 2002).

The polymerization pressure plays an equally important role, although indirect, on morphology and performance of the polymer, because it will affect the boiling temperature of the solvent porogen. It has a higher surface area, smaller pores and a higher density. For best performance it is also important to check the flexibility of the polymer and provide for binding conditions in which the template can adequately "sit" in the binding sites. So it is also important to assess the effect of swelling the polymer, which together with that of the polymerization pressure are responsible for the low reproducibility and the weak binding of the polymers synthesized with the template in different experimental conditions (Piletsky et al., 2004).

The polymerization time is important as increasing the time of the formation of a rigid polymer structure and the formation of cavities greater defined, but it is the initial period to be the most critical for the formation of the cavities of imprinting and then for determine the selectivity. Furthermore, important are the conditions of polymerization, UV and thermal. Various types of polymerizations correspond to different properties of the resulting polymer (Piletsky et al., 2005).

1.2.4.5 Types of polymerization

There are also various types of polymerization:

- synthesis in bulk, the most used (fast and easy) allows the polymerization by formation of a complex between functional monomer molecule and template, followed by polymerization and formation of a very hard block;

- synthesis for suspension, described for the first time by Mayes and Mosbach (Mayes et al., 1997), which does not require grinding, but forms aggregates of spherical particles, if the system is sufficiently diluted.

- Synthesis by precipitation (Cacho et al., 2004), which leads to the formation of precipitating particles because not soluble in the organic solvent.

- Polymerization in two steps swelling, developed by the teams of Hosoya and Haginaka (Haginaka et al., 2004a; Haginaka et al., 2004b), which requires more steps of initial swelling before the polymerization proceeds and the solvent is water, this produces monodisperse particles.

- Emulsion core-shell Polymerisation, which requires a complicated procedure to obtain spherical particles of controlled diameter with a morphology structured that allows the incorporation of additional properties to the heart of the particles without interfering with the structure of the polymer (Perez-Moral et al. 2004).

-

(A) Bulk Polymerisation

(B) Suspension Polymerisation

(C) Precipitation Polymerisation

(D) 2-Step SwellingPolymerisation

(E) Emulsion Core-Shell Polymerisation
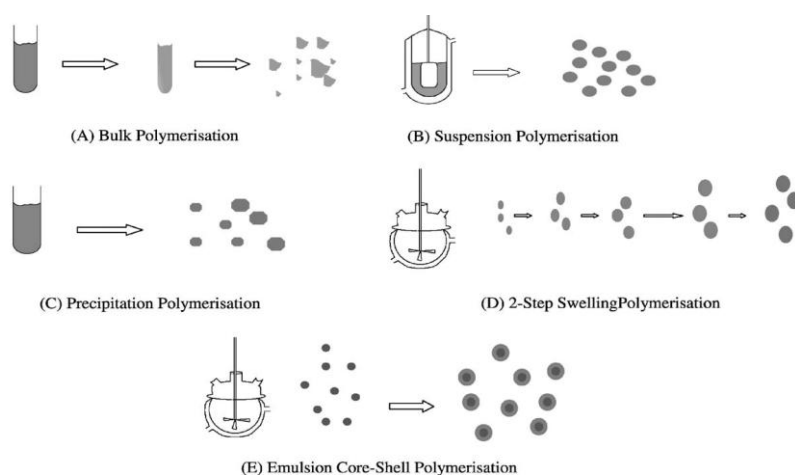
Figure 14: Methods of synthesis of MIPs (Perez-Moral et al., 2004)

1.2.4.6 MISPE

Before using the MIP as sorbent of Solid-Phase Extraction (MISPE) usually assess its molecular recognition properties for the selected target molecule with high performance liquid chromatography (HPLC). It thus examines its possible use as a stationary phase in chromatography, packing HPLC steel columns. The pressure that provides the column is an index of the quality of its packing, together with the symmetry of the chromatographic peaks that provides. The packed column is made to equilibrate with the passage of the mobile phase is not observed until a stable baseline of the signal of the detector connected with HPLC. It uses a small percentage of acetone to determine the dead volume of the column. It thus defines the capacity factor (K') and the imprinting factor (IF) of the MIPs (Mayes et al., 1994). These values are derived from the retention time of the template on a chromatographic column packed with MIP and the particles of a second column packed with particles NIP. If the MIP is imprinted, the analyte will be felt more strongly on the column compared to the MIP column NIP, because of selective interaction.

It is also possible to evaluate the cross-reactivity of the MIP verifying the retention of molecules similar to the template, structurally similar (Caro et al., 2003).

The benefits provided by MIP for SPE compared with conventional silica-based, and mixed-bed SPE columns, include:

- Cleaner extracts, reduced background interferences
- Improved sensitivity
- Consistently high target analyte recovery
- Reduced sample preparation time
- Fewer sample clean-up steps
- Elimination of ion-suppression in LC/MS applications
- MIPs can also be used for process-scale clean-ups.

In short, MIP delivers better results, greater productivity, and lower costs. MISPE is based on a conventional procedure SPE, and consists of the classical steps of conditioning, loading, washing and elution of the cartridges packed.
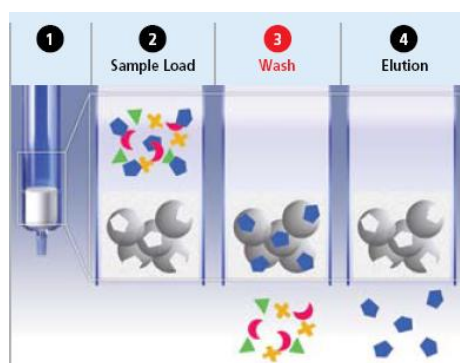
Figure 15: MISPE procedure

However, the principle of adsorption is based on a different mechanism, so a different method development strategy is required. Since the advantage of MISPE is its selectivity, it is important to optimize the selective retention of the target analyte(s) to the imprints and to suppress non-selective interactions to the polymer surface. Two different approaches can be used to obtain this selectivity; selective adsorption or non-selective adsorption followed by a selective desorption step. These two approaches are illustrated in Fig. 16.
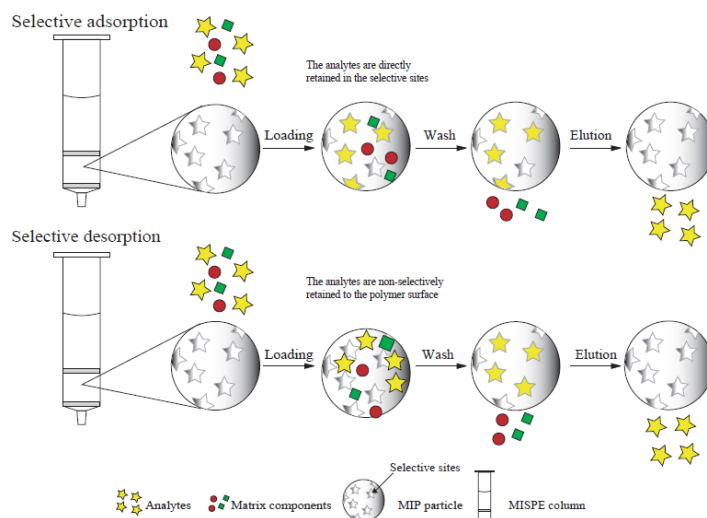
Figure 16: Illustration of the two different appoaches used for MISPE

- *Selective adsorption*

Most MIPs are prepared using an organic solvent as the diluent or porogen during the polymerisation reaction. As already mentioned, the interactions involved in the establishment of the pre-polymerisation complex are often of polar nature and hence stabilized in a non polar solvent. Thus, to achieve selective adsorption the sample needs tobe loaded on the MISPE column in an organic solvent of low to medium-polarity. It has been shown that the ideal solvent for selective rebinding to the imprints is often the same as the porogen (Muldoo, & Stanker, 1997; Stevenson, 1999). A selective adsorption approach to MISPE often requires extraction in an organic solvent prior to loading the sample (Muldoo, & Stanker, 1997; Turiel, 2004).

- *Selective desorption*

Samples that are suitable for MISPE are often of aqueous nature, such as biological fluids and environmental water samples.

However, when an aqueous sample is directly applied to the MIP sorbent, extensive non-selective adsorption to the polymer surface generally occurs. The polar interactions are weak in aqueous mediaand the hydrophobic forces

stronger. However, for relatively non polar compounds or compounds that can be retained by an ionexchange mechanism this non-selective adsorption is often strongand can be utilised for adsorption. The MIP sorbent then works as a conventional reversed phase or an ion-exchange SPE sorbent during the loading step. Furthermore, the non-selective adsorption can be converted to selective adsorption to the imprints by washing the cartridge with carefully chosen solutions, for instance the analytes may bind to the imprints in conditions that favour the interactions established during creation of the MIP. The porogen (Ferrer et al., 2000) or organic solvents with low polarity (Bereczki et al., 2001; Matsui et al., 2000) are often used in a "selective" wash step. This wash step should also remove the non-selective bound interferences from the sample matrix. If the non-selective adsorption also involves ionic interactions, a weak acidic or basic modifier could be added to the wash solvent in order to disrupt them (Blomgren, 2002).

Other approaches to improve the water compatibility and reduce non selective adsorption to the MIP have also been presented. For example, Dirion et al. (Dirion et al., 2003) increased the water compatibility by incorporating ahydrophilic co-monomer in an existing MIP protocol. Another approachis to use more polar solvents, such as mixtures of alcohols and water (Caro et al., 2004; Baggiani et al., 2001) as diluents for the MIP synthesis.

The hydrophobic parts of the detergent are adsorbed on the polymer surface as protecting layers, while the hydrophilic heads are in contact with the water. This layer hinders the interactions of the analyte with the polymer surface.

Using organic solvents of low polarity, such as dichloromethane, chloroform, toluene. However best results are obtained with a higher polarity solvent, such as acetonitrile or methanol. Some authors report that the best washing solvent is represented by the porogenic solvent used for the polymerization of the MIP, because it reproduces the environment established during synthesis (memory effect of the solvent) (Ferrer et al., 2000).

1.2.5   History of Graphitized Carbon as sorbent material

The use in gas chromatography of graphitized carbon black as solid deactivator and as solid stationary phase was introduced in the '60s by Kiselev (Kiselev, 1964), widely investigated by Halàsz and Horvàth (Halàsz et al. 1964) and later by Liberti (Liberti, 1965) The direct use of thermal carbon blacks, either graphitized or not, is very difficult in HPLC because their poor mechanical stability is much. In facts the particles are aggregates of polycrystaline microparticles 100-5000 A in size. These assemblages are destroyed by shear forces due to the Liquid flow during column operation or which appear during packing procedure or even during sieving (Colin et al., 1976).

The use of carbon-based sorbents for SPE began in the 1980s with the introduction of graphitized carbon blacks (GCBs) (Bacaloni, 1980). GCBs are obtained from heating carbon black in an inert atmosphere at 2700‑3000ºC. The heterogeneous surface of carbon black is covered with a layer of chemisorbed oxygen, which is not part of the surface structure of carbon black but is bound to large polycyclic molecules that are strongly adsorbed to the carbon surface. Heating carbon black results in the removal of most of the polycyclic molecules, and thus the chemisorbed oxygen from the surface, as well as forming a graphitic structure via the rearrangement of carbon atoms (Campanella et al., 1982).

Interest in using GCBs as SPE sorbents increased when their ability for isolating polar molecules, which have low affinity for most RP sorbents, was demonstrated. Compounds are retained on GCB as a result of the presence of two types of adsorption sites (Matisova et al., 1995). The most abundant sites are provided by the non-polar carbon atoms arranged in a graphite-like structure, which interact with compounds through van der Waals forces. However, GCB also has anion exchange properties due to the presence of positively charged chemical impurities on its surface. More specifically,

oxygen complexes with a chromene-like structure, originating from the heating of carbon black, rearrange to benzpyrylium salts in the presence of acidified water and are responsible for binding to anions via electrostatic forces (Campanella et al., 1982). As a consequence of the anion‑exchange properties' basic and neutral compounds can be separated from acidic compounds using GCB and suitable eluant mixtures. It is even possible to sub-fractionate acidic compounds according to their pKa values by including appropriate additives in the elution mixture (Di Corcia et al., 1993)

On the surface of GCB, there are also hydroquinone groups, which are in equilibrium with their oxidized forms, namely semiquinones and quinones (Campanella et al., 1982). Particular compounds can bind to the surface of GCB via quinone chemisorption, which makes their adsorption partially irreversible and results in low recoveries. This problem was overcome by passing an ascorbic acid solution through GCB and reducing the quinone groups to less reactive hydroquinones.

An important advantage of GCB, especially when dealing with large volumes of environmental samples, is its storage stability. It has been shown that problems associated with sample alteration during the period between sampling and analysis can be solved, and storage space saved, by storing analytes of interest on GCB (Sabik et al., 2000; Crescenzi et al., 19997). These sorbents are capable of trapping very polar analytes. However, the retention of some compounds can be excessive or irreversible, making their elution problematical.

1.2.5.1 *Porous Graphitic Carbon*

PGC is a macroporous material, unlike GBC, which retains compounds on the external sorbent surface, with high mechanical stability and is stable over the whole pH range. The surface of a PGC particle consists of a two-dimensional graphite structure, composed of layers of hexagonally arranged carbon atoms

with delocalized electrons, as illustrated in Fig.17. Its retention mechanism is different from that of silica and polymer-based sorbents. The analytes are retained by a combination of dispersive and stronger electrostatic interactions. The latter interactions are due to the high polarizability of the surface (Hennion, 2000).

The high capacity of graphitic materials trapping polar analytes was confirmed by the production of the first carbonaceous material can be used both as adsorbent in the SPE phase (also possible with the GCB) both as stationary phase HPLC: the porous grafitic carbon (PGC). Today the PGC is marketed by Thermo Fischer Scientific under the name Hypercarb. The characteristics of the material are:

• Particle Diameter: 3, 5 and 7 uM

• Average surface area: $120m^2/g$

• average pore diameter: 250 A

• Porosity of thumb: 75%

• Pressures tolerated: up to 400bar

• pH range tolerated: 0-14

Its surface is crystalline, uniform and stereoselective, then able to separate geometric isomers and structural. It is molecularly constituted by sheets of graphite, ie from C atoms arranged to form sp^2 hybridized to six cycles. The C atoms are held together by covalent bonds, the sheets, instead, by interactions of Van dar Waals. In principle there are no functional groups on the surface, since the C atoms have valence saturated. In fact, there are, however, undoubtedly C atoms at the edges of sheets of graphite having to have saturated valence have functional groups attached, type: hydroxyl, carboxyl and amino perhaps. However, the content of oxygen atoms at the surface is indeed very low, approximately 0.14%. The high-resolution electron microscope shows that the PGC more specifically consists of strips of graphite

braided, where each tape is composed of about 30 sheets (Fig.17) (Knox et al., 1986).
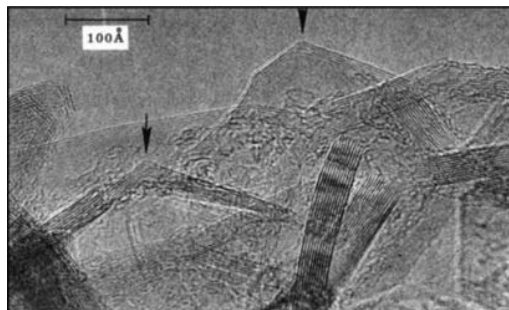
Figure 17: High-resolution electron microscopy of the surface of PGC

This structure has similarities with the GCB; in PGC, however, the tapes are woven, which makes account of its increased mechanical strength and thus of its possible use in HPLC. Has a two-dimensional graphite structure, which differs from graphite 3D, not being the successive layers regularly oriented (Fig.18) (Knox et al., 1986).
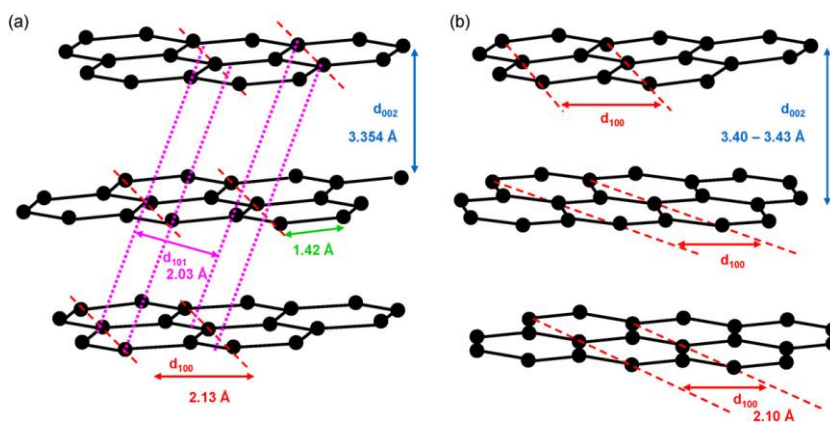
Figure 18: a) 3D Structure of graphite; b) 2D structure of the PGC

Therefore, the PGC has been called "two-dimensional graphite" or "graphite tubostratic" (West et al., 2010). Furthermore, while for the graphite sheets apart from each 3.354A, the PGC for the distance is between 3.40-3.43A.

Also PGC has imperfections: those functional groups bound to the C atoms to the edges of the sheets of graphite are, however, less than 1% of the surface, so that their effect on the chromatography is minimal.

The retention mechanisms of PGC mainly regulated by the solvofobicity of the analyte, the interactions of polarized groups or polarizable analyte with the graphitic surface and by steric effects.

The strength of the interactions that occur between the analyte and the surface is dependent on both the shape and the nature and type of functional groups of the analyte. Most polar analytes which interact closely with the surface of the PGC are strongly retained.
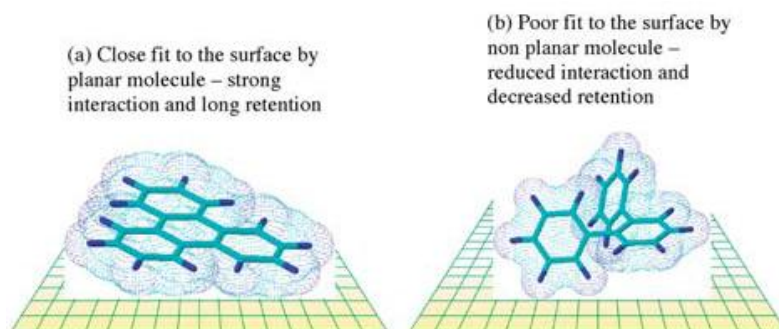
Figure 19: Strong and reduced interaction between PGC and analyte

1.2.5.2   *Knox and Ross Theory*

According to Knox and Ross, in a review published in 1997, the factors that determine retention are:

- Interactions between analyte-eluent, such as dispersive forces or hydrogen bonds, which favor the retention

- Repulsions hydrophobic eluent-analyte, typical eluents of polar and non-polar analytes and that favor the retention

- Interaction London dispersive type that you are generating between analyte and graphite surface and compete with those between surface and

eluent, very important for the selectivity despite the fact that they can both promote and disadvantage retention.

- Interaction induced charges between analyte and surface, offset by polar interactions analyte-eluent, whose strength increases in the case of a direct contact of the polar groups with the surface thanks to a steric effect.

Molecules which become more hydrophobic or more polar by addition of alkyl groups or groups capable of polarizing the surface, have an increased retention (West et al., 2000)

1.2.5.3 Reverse phase versus PGC

In a traditional reverse phase (RP) the retention of the analytes increases in direct proportion to their hydrophobicity, because of dispersive interactions that are generated between the stationary phase and analytes and with increasing polarity of the analytes retention decrease, because of the prevalence of analyte-solvent interactions. For PGC columns was observed that for very polar analytes the retention increases with the polarity of the analytes. We speak of "the polar retention effect on graphite" (PREG). From a simplified point of view when a polar molecule approaches the surface of the graphite induces a polarization of the same surface (Ross et al., 2000).
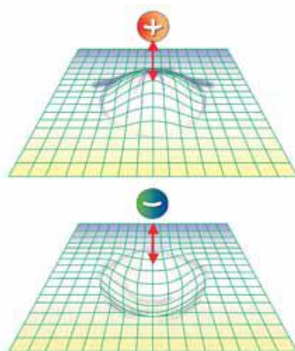
Figure 20: Schematic representation of the retention of polar molecules. a) Molecules positively charged and b) negatively charged molecules are close to the surface on the same inducing a dipole.

Interesting explanation of the mechanism of retention of this stationary phase is the theory of Hanai, according to which the center of the surface graphitic would electron-poor and the edges instead are electron-rich and thus positively charged (Hanai, 2003).

So according to the theory of Hanai polar compounds interact with the edges while non-polar with the center of that surface. These properties make the PGC a phase chromatographic unique in that it is appropriate to separate polar and non polar compounds.

1.2.5.4 Solvents for PGC

The elutropic solvent series associated with silica bonded phases does not always apply to PGC. The strength of organic solvents is solute-dependent and an important tool to adjust retention and selectivity. In general, methanol (MeOH) and acetonitrile (ACN) are similar in strength but weaker than 2-propanol (IPA) which in turn is weaker than either tetrahydrofuran (THF) or dichloromethane (DCM). The pressure difficulties associated with the use of IPA may be overcome by mixing it with ACN in a proportion of 1:1 or 3:1. This approach increases the relative elution strength of ACN whilst avoiding excessive backpressure. For the purpose of method development it can be assumed that the organic solvent elution strength increases as follows:

MeOH ≤ ACN < ACN:IPA(1:1) < ACN:IPA(1:3) <THF = DCM

Although MeOH and ACN have similar strength for most analyte types, in some cases they may provide different selectivity.

In non-aqueous analysis of hydrophobic solutes (ie., compounds with high hydrocarbon content) which are retained mainly by dispersive interactions, that chloroform and toluene are the strongest solvents, followed by DCM and THF (Gaudin et al., 2002).

Retention of polar compounds and ions on PGC is due to specific interactions between solutes and delocalized electrons on the graphite surface, which

behaves both as anelectron donor and electron acceptor. The type and concentration of electronic modifiers in the mobile phase can have a notice able effect on the retention, selectivity and also on the peak symmetry of solutes separated on PGC columns.

The most commonly used electronic modifier, trifluoroacetic acid (TFA), can act as a competitivemodifier which reduces polar retention. When separating ionizable acidic solutes, very often it is essential to use TFA in order to obtain elution in a reasonable run time. TFA competes with the acidic solutes for the graphite surface, preventing very strong retention of these. When analyzing ionizable basic solutes TFA behaves as an ion-pairing agent which improves peak shape at the same time as it increases retention; it is believed that in this case, TFA forms an ion pair with the analyte increasing retention moderately but also improving resolution and peak shape via electronic interactions with the graphite surface. Elfakir and Dreux compared the effect on solute retention of various acidic electronic modifier sand salts. They found that $HClO_4$> $NaClO_4$>TFA < KH_2PO_4, and that the resolution values were also dependent on the concentration of the electronic modifier (Elfakir & Dreux, 1996) .

Diethylamine (DEA) also behaves as an electronic modifier and is very useful when strong retentionon the graphite surface results in broad, asymmetrical peaks or very late eluting peaks,for basic or acidic analytes. In the separation of basic analytes DEA can produce sharp symmetrical peaks, even for compounds such as procainamides which are known for producing poor peak shapes. It is thought that DEA competes with the basic analytes for the surface of the graphite, and thus reduces retention. For acidic analytes, this modifiers's electronic interaction with the graphite surface will improve resolution and peak shape. 1-Methylpiperidine is another alternative electronic modifier for basic compounds, which improves peak symmetry.

PGC is the ideal stationary phase for the RP-LC/MS of polar analytes:

• PGC provides unique retention and separation of very polar compounds with MS compatible mobile phases such as 0.1% formic or acetic acid and low concentrations of volatile buffers such as ammonium acetate or ammonium formate;

• Polar compounds are strongly retained on PGC, therefore high concentrations of organic modifiers can be employed, which improves nebulization in atmospheric pressure ionization techniques, and thus improves sensitivity of the analysis;

• It allows shorter column lengths and smaller diameters to be used without compromising peak capacity, often with increased sensitivity;

• There is no modification of the porous graphitic carbon surface on PGC, thus it is stable with any mobile phase and there are no issues of phase bleed.

1.2.5.5 PGC application

Chromatographic applications of PGC have been recently reviewed (Pereira 2008; Forgács, 2002). Fewer application as on-line spe were reported

In 2000, Hennion reviewed PGC for SPE applications with emphasis on its capability to retain very polar and water soluble compounds (Hennion, 2000). Since then it has been employed for trapping various polar analytes like anatoxin-a (Dimitrakopoulos et al., 2010), herbicides (Carneiro, 2000) and degradation products of atrazine (Hennion, 2000) in water. Moreover, strong retention of polar analytes on PGC has been observed both with pure water and with high amounts of organic solvents in the mobile phase (Hennion, 2000). When evaluating PGC for separation of the AMPAs, Mercier et al. found that the compounds were totally retained with pure water as the mobile phase, but effectively eluted by the introduction of a carboxylate anion (Mercier et al., 1999).

The waters in environmental quality of complex matrices must be subjected to pretreatment to concentrate the analytes of interest and to eliminate all possible interferers. The use of the SPE online has made the development of the method faster, reducing the time of preparation of the sample. The processes of extraction and analysis occur in sequence. Conditioning, washing and elution are performed automatically, reducing operator intervention, the risk of any loss of the analyte, there is a greater sensitivity and can be analyzed the totality of the extract, you can reuse the cartridges, low-volume sample is sufficient to obtain an acceptable sensitivity; elution takes place fast and direct the sample after preconcentration; minimum consumption of organic solvents (the elution is carried out with the mobile phases used in HPLC). It must be stressed, however the lesser flexibility of the SPE on-line with respect to the SPE off-line, due to the inability to combine different types of cartridges and the so-called matrix effect, caused by co-elution of residues of the matrix components. So in the SPE on-line is coupled to the extraction step the analytical one.

AIM OF THE PROJECT

2.1　Challanges in sample pretreatment for LC/MS analysis

The aim of this Ph.D. thesis was the investigation of the major limitations of sample treatment techniques in order to improve methods selectivity. For liquid-solid extraction (section A) the feasibility of extending the applicability of subcritical water extraction to very poorly soluble compounds was explored. For solid phase extraction (section B) we assessed the effectiveness of a molecularly imprinted polymer in selectively extract classes of polyphenolic compound from beverages. Finally, the peculiar characteristics of porous graphitic carbon were evaluated for its use in online SPE-LC/MS determination of a class of emerging pollutants in environmental water (section C).

SECTION A

SWE AND CURCUMIN

2A.1   Poor water solubility and SWE

SWE is considered as an environmentally friendly extraction technique that could replace traditional methods. Then, the applicability of this technique is limited by the low solubility of some compounds and the instability of some compounds towards elevated temperatures.

Heated water could be as a useful solvent in the extraction of organic compounds because it is used in food and beverage preparation.

The scope of my work is to evaluate the SWE to extract curcumin, a molecule with very low water solubility, from turmeric rhizome also by adjusting pH water at extreme values.

Various study have explored the use of modifiers or additive in SWE extraction in order to improve the extraction yields.

In few cases, adjusted pH was used to improve recoveries of vary target compounds, as tetracycline and oxytetracycline antibiotics from animal feed.

Curcumin have a commercial importance for its useful pharmacological properties.

The developments in its extraction technology are monitored by various international authorities. Acetone, methanol, ethanol, and isopropanol are indicated by the FAO/WHO as solvents for curcumin extraction.

Then in theDirective 95/45/EC of the European Commision are indicated also hexane, ethyl acetate, dichloromethane, carbon dioxide and n-butanol.

This compound with very poor solubility are traditionally extracted with traditional techniques, as Soxhlet extraction, sonication and other methods that used organic solvents.

SWE has vary advantagesover traditionally techniques:

- Using water as a solvent the costs could dramatically reduce
- Wet samples can be extracted without any pretreatment
- High pressure may facilitate extraction from samples in which analytes are trapped in matrix

- Reduced risk of contamination with exogenous chemicals

- The superior activity to antioxidant extracted with SWE

2A.1 Curcumin

SWE is used in the extraction of curcumin (with very low water solubility) from untreated turmeric (Curcuma Longa L.) rhizome.

Curcumin, also known as diferuloylmethane, is a food dye, used to impart a yellow color to the preparations similar to that of saffron. According to Eureopean Food Safety Authority coloring numbering is identified by E 100.
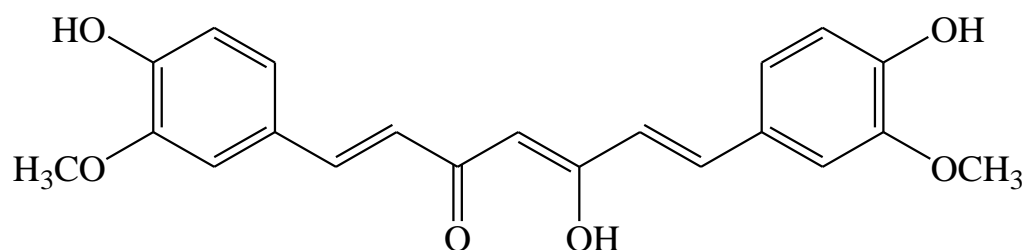
Figure 21: Curcumin structure

Curcumin is the main biologically active component of Curcuma Longa (Zingiberaceae), from which the curcumin is extracted and concentrated (Peret-Almeida et al., 2005). Desmethoxy curcumin and bismethoxy derivates are also present in varying proportions.

2A.2.1 Curcumin sources

The curcuminoids are also present in other species of turmeric, but in smaller percentages. Turmeric contains commercial curcuminoids: desmethoxyicurcumin, (typically 10-20%) and bisdesmethoxyicurcumin (usually less than 5%).
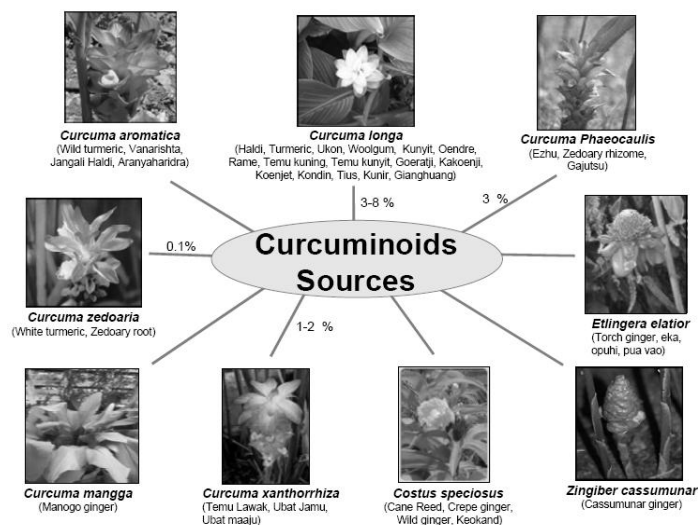
Figure 22: Curcumin sources

An yellow-orange powder, that is obtained from the root and the rhizome of the plant Curcuma longa, is used throughout the world as the main ingredient of curry and contains about 2% of curcumin (Sharma et al., 2005).

2A.2.2 History of curcumin use

The Turmeric is an ancient spice and a traditional remedy that has been used as a medicine in writings dating back more than 2000 years ago. Marco Polo, describing his travels in China, describes the Curcuma in the 13th century: "There is also a vegetable which has all the properties of true saffron, as well as the color, but that is not true saffron. 'Curcuma is held in high esteem, and is an ingredient in all their dishes".

Curcumin has been used for centuries by different cultures in Asia. For example, some Indian medicinal practices used curcumin to treat anorexia, cough, rheumatism, and other diseases (Zhang et al., 2009).

Hindu medicine still use curcumin to treat sprains and swelling. The traditional Chinese medicine uses this same compound for the treatment of diseases that are accompanied by abdominal pain.

Recently, moreover, Western medicine has recognized that curcumin may have anti-inflammatory, anti-oxidants, anti-bacterial, anti-venom, anti-HIV, as well as some activity against Alzheimer's, which may be due to keto-enol form (Balasubramanian, 2006). Some studies also show its effective in Cystic Fibrosis (Kunzelmann & Mall, 2004).

The Turmeric has been used in large quantities for hundreds of years without major adverse effects.

FDA (Food and Drug Administration) classifies Turmeric as GRAS substance (General Recognition And Safety), or "Generally Recognized Safe."

It is interesting to note that there are already products on the market with the most varied directions.

2A.2.3 Physico-chemical properties

Curcumin is the main biologically active component of the polyphenols of tumerico. Spectrophotometric investigation in UV showed that the maximum light absorptionoccurs at 425 nm. Its chemical structure is characterized by two-feruloil chromophores united by a methylene group identified in 1913. Curcumin is unstable at basic pH and degrades in 30 min in the compound trans-6-(40-hydroxy-30 methoxyphenyl)-2,4-dioxo-5-hexanal, ferulic acid, vanillin and feruloylmethane. The two aryl rings are symmetrically connected via ortho-methoxy phenolic groups in conjugation via a beta-diketone, which confers several interesting properties both in the solid state and in solution. The functionality of the beta-diketone is also responsible for the intramolecular transfer of the hydrogen atom that leads to form keto-enol tautomeric and different conformations (Priyadarsini, 2009).
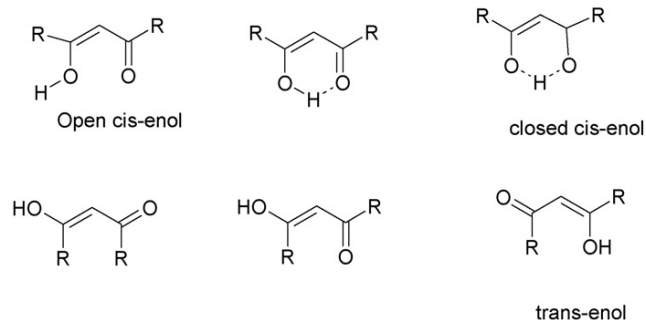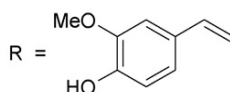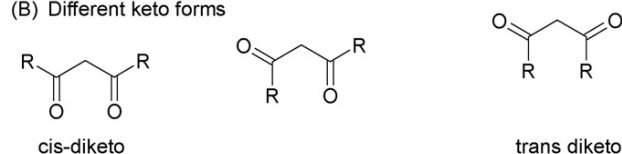
Figure 23: Differerent keto-enol conformation of curcumin

NMR studies of curcumin in $CDCl_3$ (Payton F. et al., 2007) have shown that, curcumin, in non-polar solvents and aprotic, exists in the enol form as a result of intramolecular transfer of the hydrogens.

Curcumin is stable at a low value of pH in aqueous alcoholic solutions, but, undergoes hydrolysis and chemical degradation, at basic pH (Tønnesen and Karlsen, 1985a), (Tønnesen and Karlsen, 1985b). Under physiological conditions of pH, in the presence of a phosphate buffer, the degradation was significant. Curcumin is also subject to photodegradation (Tønnesen and Karlsen, 2002), and therefore, exposure to sunlight of curcumin has been reported both in solution and in solid form with the formation of various degradation products, such as benzaldehyde and cinnamaldehyde.

Curcumin has three ionizable protons, two of the phenolic OH groups and a third of the enol form. So three acidity constants were estimated for curcumin both experimentally and theoretically.
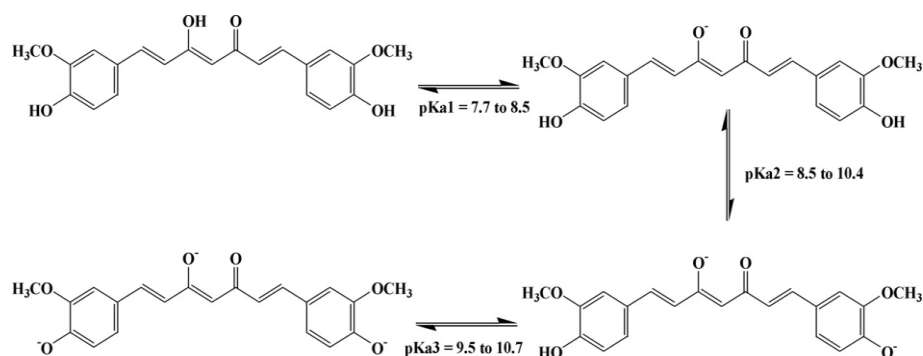
Figure 24: Three acidity constants of curcumin

2A.3   Aim

This work was divided into several stages, some of which have already been carried out previously:

1)   Characterization of the rhizome sample (Extraction of Curcumin by Soxhlet);

2)   Evaluation of the efficiency of extraction with pure water subcritical;

3)   Evaluation of the efficiency of extraction with subcritical water at controlled pH;

4)   Comparison of yields and optimization of extraction conditions;

SECTION B

MIP AND RESVERATROL

2B. 1 Molecularly imprinted polymers incorporating pharmacophore

Molecularly imprinted polymers (MIPs) are functional polymers generated by molecular imprinting, an efficient method for producing functional materials equipped with selective identification characteristics. The technique consists of self-assembly of a functional monomer and a template molecule in solution followed by co-polymerization of the functional monomer with an excess of an appropriate cross-linking monomer. After removal of the template, the resulting polymer exhibits high affinity for the molecule used as template and structural analogues. In the last decade MIPs used for solid phase extraction (MISPE) has been successfully applied to solve several challenging issue in food, biological and environmental analysis (Beltran,, 2010) and became commercially available.

In recent studies the MIP of resveratrol has been designed with molecular modelling to determine the optimal amount of template to associate with the functional monomer for the formation of the polymer and evaluated for the isolation and concentration of resveratrol, caffeic acid, (-)-catechin and (E)-piceid (Schwarz, 2011). Also flavonol (Suárez-Rodríguez, 2000; Gómez-Pineda, 2011) and quercetin imprinted polymer (Xie, 2001; Molinelli, 2002; Song, 2009; Yu, 2012; Song, 2009) are reported in literature.

The porpoise of this study was the evaluation of a resveratrol imprinted polymer for extraction of natural antioxidants from plant, food matrices and foodstuffs. The efficiency was evaluated comparing the behavior of few model compounds belonging to different chemical classes in some cases having structurally of functionally analogies to resveratrol. The potentiality of using such polymer for multiclass (multiresidue) analysis of selected polyphenolic structures from complex matrices was evaluated analyzing resveratrol and quercetin in wine and fruit juice.

2B.2   Resveratrol

All plants produce an amazing diversity of secondary metabolites. One of the most important groups of these metabolites are phenolic compounds that are characterized by at least one aromatic ring (C6) bearing one or more hydroxyl groups.

Phenolics compounds in nutraceuticals, functional foods and herbal products when consumed are known to play an important role in the human body.

The relevant amount of antioxidant compounds in foods and beverages such as wine and fruit juice are often referred to play a role in so called "French paradox" (the lower-than-expected coronary mortality in France) as well as other positive effects on health.

The (E)-Resveratrol (3,5,4 '- trihydroxy-trans-stilbene) is one of the major active compound of the stilbene phytoalexins (Schwarz, 2001). At first isolated from the roots of white hellebore and later from Polygonum cuspidatum, a member of the family Polygonaceae. Up to now resveratrol was found to be present in grapes, wine, peanuts and peanut products.

The molecule has three acid functions corresponding to the three phenolic OH groups and a conjugated system that allows the charge delocalization. Were carried out studies on the stability of the ions obtained deprotonando functions phenolic acid and it was observed that the function appears to be the more acidic positioned on the ring para substituted (Caruso et al., 2004) .
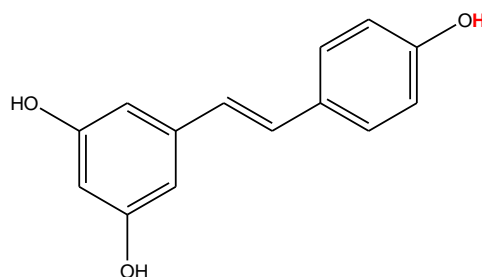
Figure 25: Resveratrol's structure

Resveratrol has been associated with several beneficial health effects such as antioxidative, anti-inflammatory and estrogenic effects as well as anticancer and chemopreventive activities (Jang, 1997; Neves, 2012). Epidemiological studies showed an inverse correlation between the consumption of red wine and the incidence of cardiovascular disease increasing the interest in monitoring the presence of resveratrol in wine. The effects of resveratrol observed in vivo and related possible evolutionary explanations, as well as development of human therapeutics based on either resveratrol itself or new, more potent compounds that mimic its effects has been recently reviewed (Baur, 2006). Despite to its particularly low bioavailability and rapid clearance from the circulation (Prasain, 2007), there is growing evidence that resveratrol can prevent or delay the onset of cancer, heart disease, ischaemic and chemically induced injuries, diabetes, pathological inflammation and viral infection (Baur, 2006). On the other hand, because the toxic effects observed administering resveratrol at or above 1 g per kg of body weight higher doses to improve efficacy might not be possible (Crowell, 2004). Moreover, due to the high current cost of resveratrol for a human weighing 75 kg a daily dose of 100 mg per kg (body weight) would cost US$6,800 year-1.

Therefore, there is a great interest over developing analogues with improved bioavailability, or finding new, more potent compounds that mimic its effects.

2B.2.1 Resveratrol's history

This molecule has been known for centuries in Asian medicine as Ko-jo-kon, and used as an anti-inflammatory drug (powdered root of Polygonum cuspidatum). The synthesis of resveratrol in plants is induced by exposure to microbial infections, ozone and ultraviolet radiation; the molecule is in fact a phytoalexin, a natural antibiotic that is produced by plants to cope with the attacks. Therefore, the production occurs in the skin of grapes (50-100 mg / g)

and in the epidermis of the leaves (50-400 mg / g), and, is in the woody parts of the plant, such as stems and seeds.

2B.2.2 Resveratrol in wine

The concentration of resveratrol in wine is influenced by several parameters:

- variety of climate and vine species are more sensitive to harmful agents and show a greater ability to synthesize the molecule, particularly in cold and humid climate, where there was an increase in the probability and frequency of fungal

- duration of contact with the skins during fermentation in red wines the concentration is higher than in white wines

- vinification process: barrel aging and certain types of filtration losses involve the content of resveratrol.

Wine and resveratrol in grapes are free as a species and as the glycosylated form, the 3β-glucoside of resveratrol, also called piceid (Fig. 26).
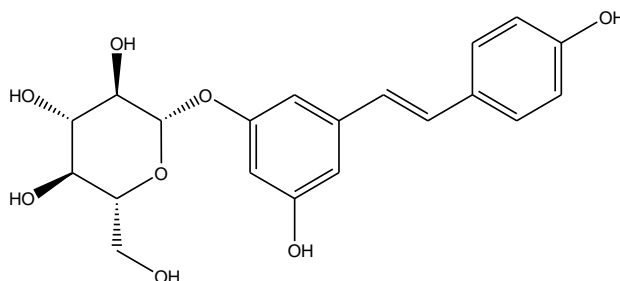
Figure 26: Piceid

SECTION C

PGC AND PFAS

2C.1   PFAs: emerging contaminant

Environmental issues relating to the protection of health is often dealt with priority to the aspects that have the greatest media coverage such as, for example, air pollution caused by emissions of industrial or domestic and motor vehicle traffic.

An "emerging contaminant" is a chemical or material that is characterized by a perceived, potential or real threat to human health or the environment or by a lack of published health standards. A contaminant may also be "emerging" because a new source or a new pathway to humans has been discovered or a new detection method or treatment technology has been developed.

Perfluorinated (fully fluorinated) organic compounds such as perfluorooctane sulfonate (PFOS) represent a class of compounds showing high thermal, chemical and biologicalinertness. They can be widely found in the environment primarily resulting fromanthropogenic sources (Lindstrom et al., 2011).

Polyfluorinated compounds (PFCs) represent a ubiquitous class of compounds whose effects are not yet completely understood. The CF bond makes these compounds resistant to hydrolysis, to photolysis, to heat (Giesy and Kannan 2001, Prevedouros 2006).

The perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) are chemical compounds with fluorinated surfactant properties that belong to the family of perfluoroalkylated substances (PFAS).
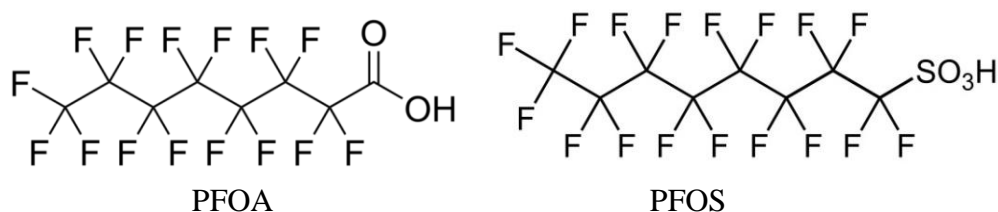
Figure 27: The structure of PFOA and PFOS

PFOS and PFOA are extremely persistent in the environment and can be transported long distances in air. As a result, they are widely distributed across the higher trophic levels and are found in soil, air and groundwater. The toxicity and bioaccumulation potential of PFOS and PFOA indicate a cause of concern for the environment and human health.

The compounds PFOS related are used in a wide variety of products and applications (OECD 2002, Calafat 2006, Kissa 2001), among which:

- waterproofing treatments of skin and soft tissues (16-30% total use);
- treatments dye and binders for non-woven fabrics;
- coating treatments of paper and cardboard (15-28% total use);
- detergents (as surfactant), wax floor polish (8% of total use);
- paints and varnishes (11 - 12% of total use);
- pesticides and insecticides;
- fire-fighting foams (16.3% in the UK, about 1% in Denmark);
- photographic film (about 1% in Denmark);
- semiconductor (about 2% of the total use);
- hydraulic oils for the aviation industry (antiknock, repellent);
- metallic surface treatments (to decrease the surface tension);
- agents gushedpiping

Starting from PFOA and PFOS are produced fluoropolymers and fluorotelomer chain more or less long. The polytetrafluoroethylene (PTFE), better known under the trade name Teflon ®, with non-stick properties and chemically inert (Davis 2007), is the ancestor of all fluoropolymers and has been developed for the first time in 1938 from DuPont, a company founded in 1802 that produces lubricants, paints, adhesives, additives, building materials, herbicides and packaging materials.

A further application is represented by Gore-Tex ®, durable, waterproof, used in the creation of technical clothing and sports-medical health care products and cosmetic surgery. The Gore-Tex ® is also used in the aerospace industry

for coating suits of astronauts, in industrial filtration for water purification and as an insulating material in electronic components (Martin 2004, Prevedouros 2006).

Emissions of PFOS and PFOA in the environment (mainly the compartments air and water) occur during the processes of production and processing of related products (Lehmler, 2005)

The presence of PFAS in the environment, is well documented in every region of the earth without excluding the remoter areas, and in any medium (air, water, etc..) (Steenland, 2010). They have been shown to be present, and the outcome of numerous monitoring, it is could also be argued that PFOS can be biomagnificato at the highest levels of the food chain.

2C.2 Toxicology and regulation

To date, the effects that cause PFAS on human health and the environment are unclear, which is why in recent years there has been several studies on animals and the environment in order to define more clearly the harmful effects of these molecules. Among the most important effects that have emerged from studies on fluorinated shows: the ability to interfere with the endocrine system animal, to cause hepatocellular adenoma, follicular cell adenoma of the thyroid, liver cancer in rats and teratogenicity in developing rodents (Austin et al., 2003; Seacat et al., 2003; Thibodeaux et al., 2004). For several years, many research groups have contributed, through their studies, to deepen the scientific knowledge necessary to be able to identify the effects of these substances (Kempter & Jempson, 2003; Biegel et al., 2001; Cook et al., 1992; Lau et al., 2005; Alexander et al., 2003).

With the Directive (76/769/EEC) of the Commission of the European Community in 1976 it was established that they were introduced restrictions on the marketing and use in the Member States of the Community of

dangerous substances and preparations, in the following years, Annex first, you will make even more restrictions as regards the use of PFOS.

In 1978, 3M stated that PFOA was found in the blood of its workers (Danish EPA 2005 - Project No. 1013) and DuPont expressed concerns about possible toxic effects of this compound (DuPont 1979).

In the early 80s were obtained further data on toxicological interest, but only in 2000 3M, under pressure of the Environmental Protection Agency (EPA), today announced the phasing out of PFOS and PFOA from the production to the environment.

In September 2002, the EPA began reviewing available data to understand the real danger associated with the use of PFOA. In the same year, the Organisation for Economic Co-operation and Development (OECD) stated that this molecule had a high persistence in the environment, tends to accumulate in the tissues of living organisms and is very toxic to mammals (OECD, 2002). The Scientific Committee of the European Environmental and Health Risks (SCHER) has classified PFOS as very toxic and highly persistent, bioaccumulative.

In April 2004, DuPont has launched a study comparing subjects exposed their company and non-occupationally exposed subjects in order to assess their health status and their liver function, the liver being a major target of toxic of fluorinated (DuPont, 2004). In June 2004, the EPA conducted a study to determine how PFOA in the environment is absorbed by the human body, the study took into account the mechanism of degradation of telomeres, the objective was to understand whether the PFOA environmental derives from the failure of the chain of carbon atoms of telomeres or by impurities in the production of the same (EWG 2004). In 2006, the European Parliament approved amendments to the Directive 76/769/EC, which include restrictions on the marketing and use of PFOS; these changes have been made on the basis of the information obtained through the evaluation of the OECD risk to human

health and the environment arising from the use of these molecules in various sectors.

The changes, in particular, provide a use of PFOS as components of preparations in a concentration equal to and not greater than 0.0005% by mass (Dir 76/769/EEC).

In October 2006, these molecules have been compared to persistent organic pollutants (POPs) for their potential to be transported long distances in the environment from harmful effects (Danish EPA 2005-Project No. 1013, Johansson et al., 2008) . PFOS in particular has the potential for long-range environmental transport, and thus satisfies all the criteria to be considered as such under the Stockholm Convention.

There is no estimate of global production of PFOS and related compounds, for this reason it is difficult to say exactly whether the use of these compounds is actually decreasing.

PFOS is widely distributed in the environment, even at long distances from emission sources primitive (Giesy and Kannan 2001). This has led to a growing attention to these compounds for which the European Union (EU) has taken steps to limit their production and use, published in December 2006 a European Directive relating to restrictions on the marketing of PFOS, not excluding the possibility of such a decision for PFOA (Directive 2006/122/EC). However, some exceptions have been established with regard to the use of PFOS as part of preparations but only for specific uses (for example: fire-fighting foams, hydraulic fluids for aviation, photolithography, the chromium plating industry).

In 2006, the United States Environmental Protection Agency (U.S. EPA) has required manufacturers of PFOA to 95% reduction in industrial emissions of the substance by 2010, with subsequent elimination with effect from 2015. It was also recommended replacement with alternative, although for some applications this proves impractical.
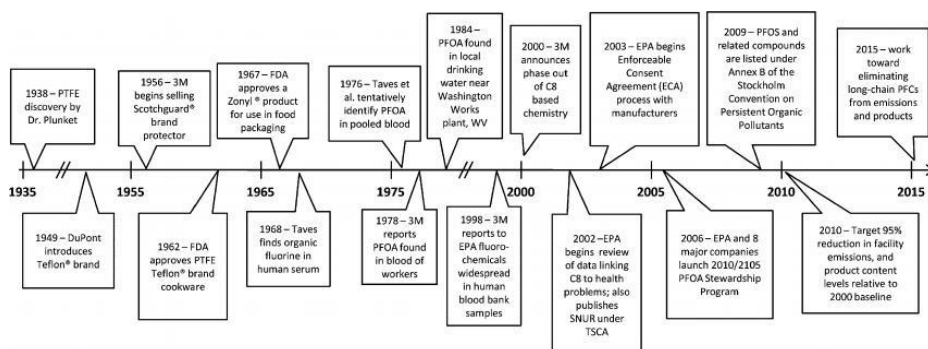
Figure 28: Timeline of important events in the history of polyfluorinated compounds (Lindstrom et al., 2011)

The alternatives to PFOS and PFOS-related compounds are essentially two: the fluorinated compounds of new generation (the Novec, compounds sulfonatesperfluorobutanici, the Polyfox, polyethers with short side chains (C2 or C3) perfluorinated) surfactants and non-fluorinated compounds (the polyether silicone, sulphosuccinates or sodium salts of 2-ethylhexyl sulfo succinate, the alcohol or aliphatic esters of ethoxylated alcohol and the aromatic propyl, in particular isopropilnaftaleni and isopropilbifenili).

As an alternative to PFAS, can be used as surfactants, compounds in which the main chains of carbon atoms are added perfluorinated alkyl chains: these "fluorinated polymers" are used as a coating for the surfaces (the axis adheres to the surface and the fluorinated chains align perpendicularly) (Moody et al., 2000).

2C.3   Degradation

The PFOS and compounds related to it are extremely difficult to destroy, because the link fluorocarbon is the strongest of organic chemistry, are extremely stable and have a high tendency to bioaccumulate.

The compounds PFOS related may be degraded to PFOS but this molecule is not further degradable as it is chemically and biologically inert. The

perfluorinated carbon chains are extremely resistant to heat, UV rays and the attack of acids, bases, oxidizing and reducing agents. The fluorinated organic polymers are very stable to hydrolysis, therefore are characterized by long half-lives ranging from five to five hundred years (3M 1999). However, the heating of fluorine polymers, such as PTFE, at a temperature greater than 350 ° C leads to degradation into smaller molecules among which is included the PFOA (0.01%) (Ellis et al., 2001).

The terminal functional group of the molecule (sulfonic or alcohol), however, is more readily degradable in the environment is that in organisms; in this way the more complex compounds can be degraded to PFOS and PFOA.

In a Canadian study has employed a computer program, that simulates the microbial degradation, it has been estimated that of 175 compounds PFOS-related, 109 would have been degraded to PFOS and PFOA 61 to (Key et al., 1998; Dimitrov et al., 2004).

2C.4   Human exposure

Among the different possible routes of absorption by the human body are: a) the inhaled; b) the ingestion of dust present in the environment, c) the consumption of drinking water and food.

The most relevant environmental exposure is that which is realized in the indoor, as these compounds are used in the textile sector as impregnating agents for fabrics, for garments, for carpets and curtains. In fact, analysis carried out in Japan on dust samples collected indoor, showed high levels of fluoropolymers and, in particular, levels of PFOA much higher than those of PFOS. It has been estimated that the daily intake of PFOS inhaled is between 10 and 100 pg absolute, which results in the plasma of exposed subjects, levels of 1.2-12 ng /L (Sasaki et al., 2003).

Another source of exposure is represented by food, in fact many perfluorinated compounds are present in food packaging, such as waterproofing agents of

paper and cardboard. A Canadian study carried out before 2000 on food or fast food has found that 55% of the composite food packaging containing fluorinated, with the level of concentration is highest (23.500 ng / g) in the pizza. (Titlemeier et al., 2003)

The fish and fish products seem to be an important source of exposure to these contaminants.

From studies carried out by the European Food Safety Authority (EFSA) has set a TDI index (estimate the amount of substance that can be ingested over a lifetime without appreciable risk to health) of 150 ng per kilogram of body weight per day; for the PFOA the TDI was set at 1500 ng per kilogram of body weight. The ESFA pointed out the existence of other routes desposizione related to food (such as drinking water for both compounds, packaging materials for food and non-stick cookware) may lead, in the case of PFOA, the excess values of TDI (EFSA, 2008).

Some researchers have turned their attention to some episodes of contamination of drinking water related to PFOA. The first, relating to 2004, he covered the area of Little Hocking (Ohio, USA) (LHWA 2005). The PFOA concentrations in the wells ranged from 1.9 to 10.1 g/L(2004), between 3.9 and 18.6 mg/L (January 2005), from 1.9 to 6.6 g/L (March 2005). The subsequent evaluation of the levels of PFOA in the serum of subjects exposed, as regular consumers of water coming from the exchange in question, has provided a median equal to 0.37 g/L. (Harada et al., 2003).

In the case of river water on the contamination of PFAS is reported that some Italian publications have reported high levels of PFAS in the waters of the Po (Loos, 2008) High levels of PFOA were recorded in Rivarone, in the province of Alessandria, where the values found are 10 to 200 times higher than those of the major European rivers, with a range of values ranging between 0.001 and 1.27 mg/L. For PFOS, however, the levels are much lower: 1-25 ng/L and the highest concentrations were found in the towns of San Zeno at Po and

Pizzighettone, respectively, in the province of Piacenza and Lodi. In some areas of the drainage basin of the Po, and more specifically in the river Tanaro and Bormida, were found even more alarming levels, (1.2 g/L), probably correlate with the presence in the province of Alessandria of a site industrial uses such compounds.

Data on concentrations of PFOS and PFOA in the serum of non-occupationally exposed groups in different countries of the world are highly variable: the highest levels of PFOS concern USA (Kentucky, New York, Michigan), the Netherlands and Germany, the highest in the PFOA are found in Korea and to follow in Kentucky and New York (Kannan et al., 2004).

The most influential factors on concentration levels are the area of residence and sex, while factors such as smoking and age are not factors.

As regards, instead, occupational exposure, perfluorinated compounds are found in the blood of workers for decades. The concentration in serum of workers engaged in the production of PFOS ranged between 2 and 12 mg/L. This concentration was 100-1000 times higher than that currently found in the general population (less than 50 g/L).

2C.5 Toxicity

The potential toxicity of PFOS and PFOA, the two PFAS most used at industrial level, has been studied in monkeys (Burris et al., 2002) and rats (Austin et al., 2003). The sub-chronic exposure to PFOS and PFOA leads to significant weight loss accompanied by hepatotoxicity and a reduction in serum cholesterol and thyroid hormones (U.S. Environmental Protection Agency, 2003). PFOS and PFOA, can cause dysfunction in liver (cause hypertrophy), kidney and bile; problems in the reproduction (Thibodeaux et al., 2003); interfere with intercellular communication; fact, due to the structure similar to that of the phospholipids (Fig. 29), can be inserted within cell membranes, increasing their permeability to toxic compounds which may

result in abnormal cell growth and therefore the development of tumors, they are carcinogenic, teratogenic, mutagenic, neurotoxic.

There are in fact some similarities between POPs substances and PFOS-related especially with regard to the stability and hydrophobicity.

The POPs, however, differ in that they are non-polar and lipophilic while the PFOS-related compounds have a hydrophilic head and the other end of the molecule hydrophobic and oleophobic (Fig. 29) (Key et al., 1997). The PFAS not accumulate in adipose tissue and are often dissociated as anions that interact with the polar sites of the membranes and sediment environments.
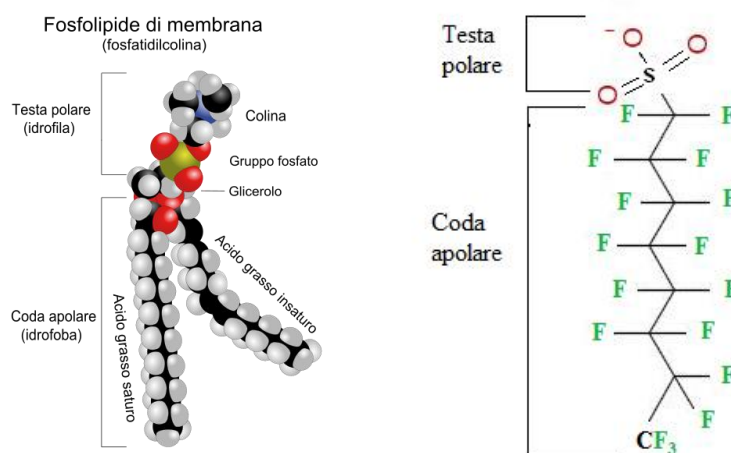
Figure 29: Structure of a phospholipid and PFOS.

The left figure represents the structure of a phospholipid membrane: it can be noticed the presence of a polar head (hydrophilic) consisting of a phosphate group and a tail apolar (hydrophobic) consisting of fatty acid molecules. In the figure on the right shows the structure of PFOS that in a similar manner to the phospholipid has a polar head group constituted by the sulphonic acid and an apolar tail consists of the hydrocarbon chain.

Despite the animal model has provided evidence of interference by the PFAS on the pathway of sex hormones and thyroid, are not available confirmations about similar effects in humans.

However, a study of individuals exposed in the workplace to perfluorinated (concentrations up to 3.9 mg/m^3) showed serum levels of organic fluorides 100 times higher (1-71 mg/L) than the general population and an increase in cancer mortality. (Alexander et al., 2003).

A similar study was carried out by conducted by Olsen and collaborators in the U.S. and in this case would show no correlation between cancer mortality and levels of perfluorinated in serum of workers (Olsen et al., 2004).

Overall, the few data in the literature are controversial and not fully defined.

Other studies have demonstrated the ability of PFAS to interfere with the hormones involved in reproduction compromising, therefore, the reproductive capacity of women and man. In women, the levels of PFAS detected, ranging between 6.4 and 106.4 ng/L, may cause difficulty in conceiving, irregularities in the menstrual cycle and a greater likelihood of infertility. Instead, reduce fertility in humans (Olsen et al., 1998).

RESULT AND DISCUSSION

SECTION A
SWE AND CURCUMIN

3A.1 Soxhlet extraction for rhizome characterization

The first test wasto observe the extraction yields with Soxhlet. Two tests were conducted, each of which lasted three hours, for three days, respectively, with isopropanol/ethanol (1:1) (Braga et al, 2007), and with acetone (Mandal et al., 2007).

The experiment was performed in triplicate. No significant differences were observed extracting between the two different procedures in terms of extraction yiels. In the following extraction experiments the content of curcumin determined by Soxhlet extraction was considered as 100% of extractable fraction.

Table 4: Yield of Soxhlet extraction

Solvents	Yield % (w/w)
Acetone	**1,350 ± 0,001**
isopropyl alcohol/ethanol(1:1)	**1,360 ± 0,001**

3A.2   Curcumin solubility

Various experiments have shown the solubility of this molecule in organic solvents, such as, acetone, ethanol, methanol, dimethyl sulfoxide and ethyl ether, with concentration values reported in Table 5.

Table 5: Curcumin solubility

Solvent	Solubility (mg/mL)
Acetone	0,066 ± 0,005
Ethanol	0,006 ± 0,002
Methanol	0,008 ± 0,000
Dimethyl sulfoxide	0,427 ± 0,019
Diethyl ether	0,003 ± 0,001

3A.2.1 Solubility in water

Curcumin was practically insoluble in water at room temperature; A solubility value of 11 mg/L in pH 5 buffered water is reported in a previous study (Tønnesen et al., 2002) investigating the effect of the complex formation with different cyclodextrins on solubility and stability of curcumin. In the present study, the feasibility of using plain water to extract curcumin was investigated by dissolving curcumin in water at different temperatures. The results, summarized in Table 6, clearly demonstrated that the solubility of curcumin increases rapidly, from 6.24 mg/L at 60 °C to almost 20 mg/L at 90 °C. Although this increase is significant (three times), these data never the less indicate that curcumin is not particularly soluble in water at temperatures below its atmospheric pressure boiling point.

This could be due to the decrease of the dielectric constant of water, from a value of 80 to a value dependent on the temperature, making it a solvent capable of being able to solubilize non-polar or slightly polar substances, such as curcumin. It has, therefore, the destruction of the strong interactions existing between analyte and matrix, such as, hydrogen bonds, Van der Waals forces and dipole-dipole attractions, which increases, even in this case, the solvent capacity of water.

Table 6:   Curcumin solubility as a temperature function;

Temperature (°C)	Solubility (mg/mL)
70	$0,006 \pm 0,005$
80	$0,011 \pm 0,067$
90	$0,020 \pm 0,08$

3A.3 SWE

The possibility of extracting curcumin rhizome by using water at temperatures above 100 °C was evaluated by carrying out the elution curves at different

temperatures. Fig.30 shows the elution curve obtained with water at 150 °C: it is evident a pronounced "tail".
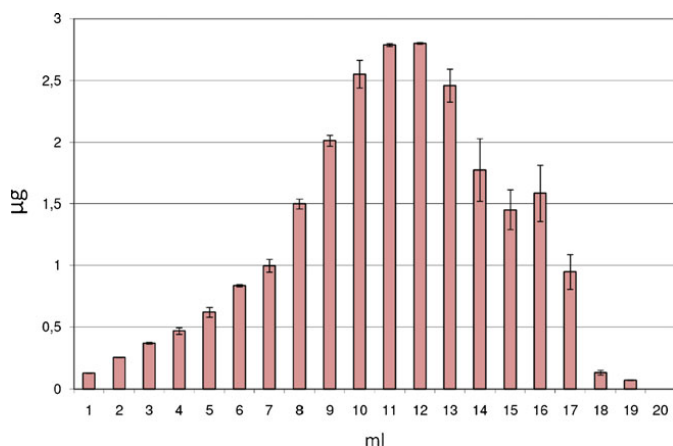
Figure 30: The quantity of curcuminin the fractions collected during dynamic pressurized hot water extraction with pure water at 150 °C and 50 bar on 10 mg turmeric samples (duplicate experiments)

The yields of the dynamic extraction procedure using dynamic PHW were then evaluated by duplicate extraction experiments performed at temperatures between 90 °C and 250 °C. In order to study the elution profile of curcumin under the extraction conditions, a series of 1 mL fractions were collected. An example of the elution curve profile obtained using pure water at the best conditions identified is shown in Fig.30. According to previous experiments performed under static conditions, the extraction yield increases with temperature, as can be seen in Table 6. Despite many attempts at optimizing the extraction conditions (including increasing the water flow rate at higher temperatures in order to reduce the residence time and subsequent curcumin degradation), the extraction yields did not exceed 18% (150 °C, flow rate of 0.5 mL/min, pressure of 50 bar). Below the boiling point of water (at 90 °C), a small amount of curcumin is extracted over a large amount of extractant fluid (28 mL). Increasing the temperature increase curcumin solubility, resulting in

a faster elution of a limited extractable fraction (6 mL). Further increase of the extraction temperature increases the extent of the extractable fraction. On the other hand, concurrently, at very high temperatures (i.e., above 200 °C), both curcumin and turmeric matrix were rapidly degraded and the extracted rhizome particles took on a deep dark brown color resulting in poor extraction yields. These results clearly demonstrate the limitations of using pure hot water for the extraction of thermolabile compounds and/or compounds with very low water solubility.

Table 7: PHWE yields from 10 mg turmeric samples using 50 mLpure water compared with that obtained by Soxhlet extraction

Temperature (°C)	Volume (mL)	Yields[a](%)
90	28	3,70 ± 0,13
120	6	9,60±0,59
150	18.5	17,00 ± 0,74
175	25	12,60 ± 0,59
250	8.5	1,5 ± 0,63

Flow rate 0.5 mL/min, volume of water (1 mL fraction) containing curcumin above LOQ
[a] Duplicate experiments

3A.4 The effect of pH on the extraction process

The addition of co-solvents or additives such as salts or buffers has also been found to improve extraction yields in various other studies.

In a previous study, heated phosphatebuffered water (0.5 mol/L, pH 7.5) proved to be much more efficient than pure water in the extraction of pesticides and their metabolites from a naturally aged soil sample. These increased extraction yields were attributed to the ability of phosphate ions to dissolve calcium and magnesium humate salts, allowing the hot water to dissolve the nowdesequestered chemicals (Di Corcia et al., 1999) . In previous studies (Crescenzi et al., 2000), (Turner et al., 2006), the phosphate buffer was

used as an additive in SWE. Different effects and benefits have been emphasized.

In the present study, we investigated the use of phosphate-buffered water in the extraction of curcumin, under the conditions described above but using acidic pH to avoid curcumin degradation. Data obtained in a preliminary set of duplicate extraction experiments performed at 200 °C (Fig. 33) indicate that the extraction yield increased steadily as the pH was decreased, down to a lower boundary of pH. Remarkably, huge improvements in the extraction yields were observed when the pH was adjusted to a very low value. Curcumin, in a pH range between 3 and 7, acts as a powerful donor atoms of hydrogen, given the presence between the two metoxyphenolic rings of a carbon atom highly activated. Then, under these very acidic conditions, curcumin is probably protonated. In Fig.32, shows the behavior of curcumin to vary the pH. At pH <1, the aqueous solution, show a red color, which indicates the presence of the protonated form (H_4A^+); instead, in a pH range between 1-7, most of the molecules, are in the neutral form (H3A), and the solution appears yellow; at pH> 7.5, the color turns red as curcumin degrades, whose pK_a were found to be, respectively, equal to 7.8, 8.5 and 9.0.

As described by Dyrssen (Dyrssen et al., 1972) and Tønnesen (Tønnesen & Karlsen, 1985), protonated curcumin, that is red, exhibits greatly increased water solubility (Fig. 31).
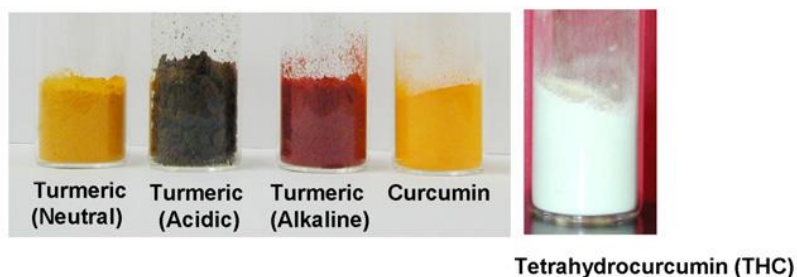
Figure 31: Colors of turmeric extract at different pH values

The red color of the extract obtained when using PHWE at low pH is consistent with this hypothesis. The improved extraction yields obtained in these experiments prompted us to consider the underlying mechanism in more detail and to investigate the influence of variables such as the pH and buffer concentration in order to identify optimum conditions. To this aim and to carefully analyze the effects of varying specific parameters, an experimental design was defined on the basis of this preliminary dataset, using a surface response methodology.
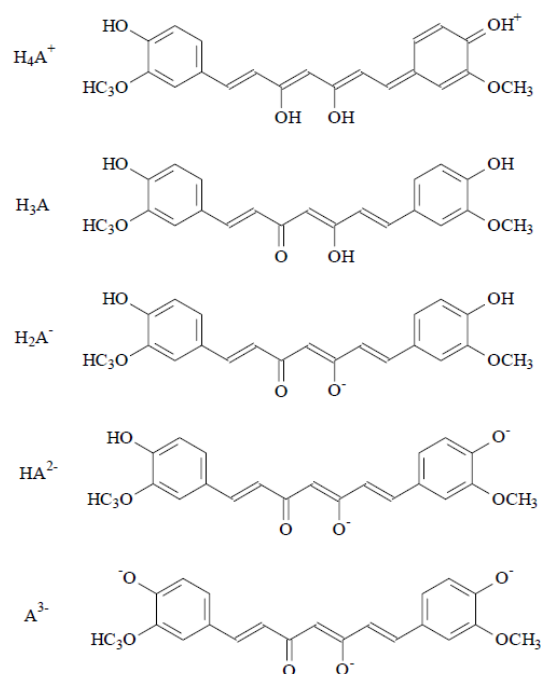
Figure 32: Protonated and deprotonated structures of curcumin at different pH values

To evaluate the effect of the variation of pH on the yield of the SWE of curcumin have been designed experiments from 180 and 220 ° C, by suitably varying the values of pH from 1 to 7.

At 200 ° C it was found that, the greater the percentage yield, this was achieved at pH 1.5, as indicated in Fig. 30. Yields were obtained at lower pH

100

values. A pH between 6.5 and 7.0, however, the yields decrease dramatically due to a more rapid degradation of the molecule. (Wang et al., 1997).

Curcumin extracted with the method "SWE" to 200°C and at different pH values;
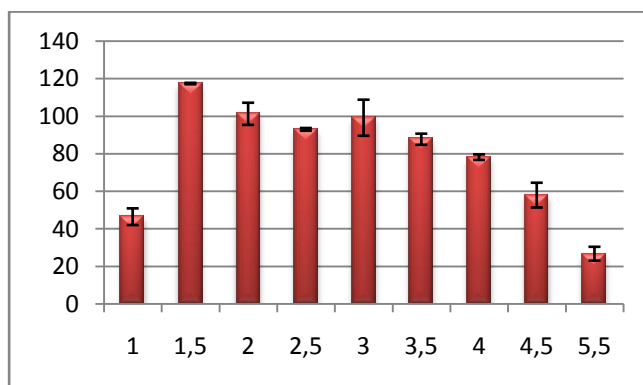
Figure 33:   SWE to 200°C and at different pH values

Curcumin extracted with the method "SWE" to 220 ° C at different pH;
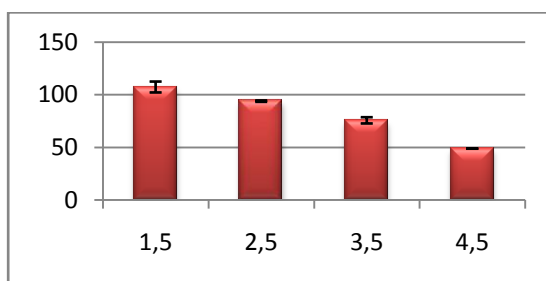
Figure 34: SWE to 220°C and at different pH values

The higher yield of extraction at pH values lower, has suggested other preliminary tests at 180 and 250 ° C. Contrary to what was observed in the extraction with pure water at these temperatures, have been recorded yields comparable to those obtained at a temperature of 200 °C (Fig. 30-31).

Curcumin extracted with the method "SWE" to 180 ° C at different pH;
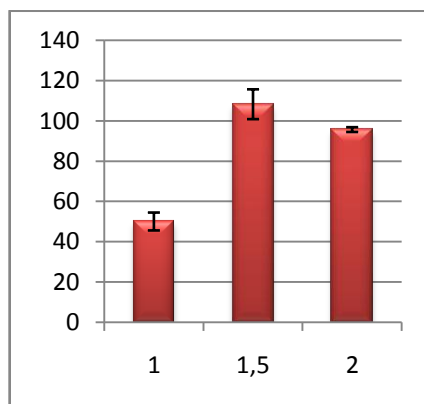
Figure 35: SWE to 180°C and at different pH values

Curcumin extracted with the method "SWE" to 250 ° C at different pH;
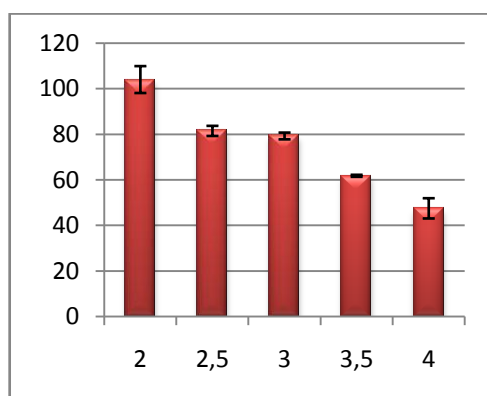
Figure 36: SWE to 250°C and at different pH values

In additional tests at 250° C, the extracted quantity of curcumin, is decreased proportionally with increasing pH; in fact, already at a value equal to 3.5 is observed a yield of the order of 0.834% (w/v). In these conditions it is however observed charring of the particles contained in the cell of the rhizome extraction.

2A.5   Parameter selection and surface response methodology

Increases in extraction efficiency are usually attributed to improvements in solubility, mass transfer effects, or disruption of surface equilibria. While the pressure and flow rate are often cited as influential factors in determining the yield of a given extraction, they are expected to be of little consequence in the case discussed herein and were therefore not considered further. Specifically, the pressure was discarded because several previous studies have established that it has little effect on the efficiency of PHWE extractions, which are much more sensitive to the temperature; the density of the water remains almost constant under the relevant conditions, and so varying the pressure has little effect (Meyer, 1993). In a previous study (Kubátová et al., 2002) on vegetable matrices, the extraction curves at different solvent flow rates were used to determine whether the extractions were primarily limited by partitioning thermodynamics or by desorption kinetics. The results indicated that for both non-polar and more polar compounds extracted from vegetable matrices, unlike supercritical carbon dioxide extraction, the efficiency of PHWE extraction is only mildly sensitive to variation of the flow rate, as factors were selected temperature, pH of extraction phase at 20 °C, and buffer concentration. Because the preliminary experiments provided evidence for a fairly strong effect, a Box–Behnken experimental design was used to reduce the number of experiments to be performed while still obtaining information on nonlinear effects and cross terms. The central point was set at 200 °C, pH 1.5, and 60 g/L buffer concentration. The experimental matrix and the extraction yields obtained are reported in Table 8. A model for extraction yield (Y) can be obtained by multilinear regression using an equation on the form

$$Y=b_0+b_1pH+b_2T+b_3C+b_4pH_2+b_5T_2+b_6C_2+b_7pHT+b_8pHC+b_9TC$$

where T is the temperature, pH is the pH of extraction phase measured at 20°C, and C is the buffer concentration.

Table 8: *Experimental design*

Exp. n°	Centre point	Temperature (°C)	pH pH units	Buffer Conc. g/l	Yield mg/100mg	% Soxhlet %
1	1	220	2	60	0,9568	70,9
2	0	200	1,5	60	1,591	117,9
3	1	220	1,5	75	1,078	79,9
4	1	200	1	45	0,5312	39,3
5	1	200	2	45	0,8988	66,6
6	0	200	1,5	60	1,579	117,0
7	1	200	1	75	0,6928	51,3
8	1	220	1,5	45	0,7408	54,9
9	1	180	1	60	0,676	50,1
10	1	180	1,5	45	1,0002	74,1
11	1	220	1	60	0,2894	21,4
12	1	180	2	60	1,2928	95,8
13	1	180	1,5	75	0,9104	67,4
14	1	200	2	75	1,227	90,9
15	0	200	1,5	60	1,4488	107,3

Was therefore possible to obtain response surfaces profile (Fig.37-38).
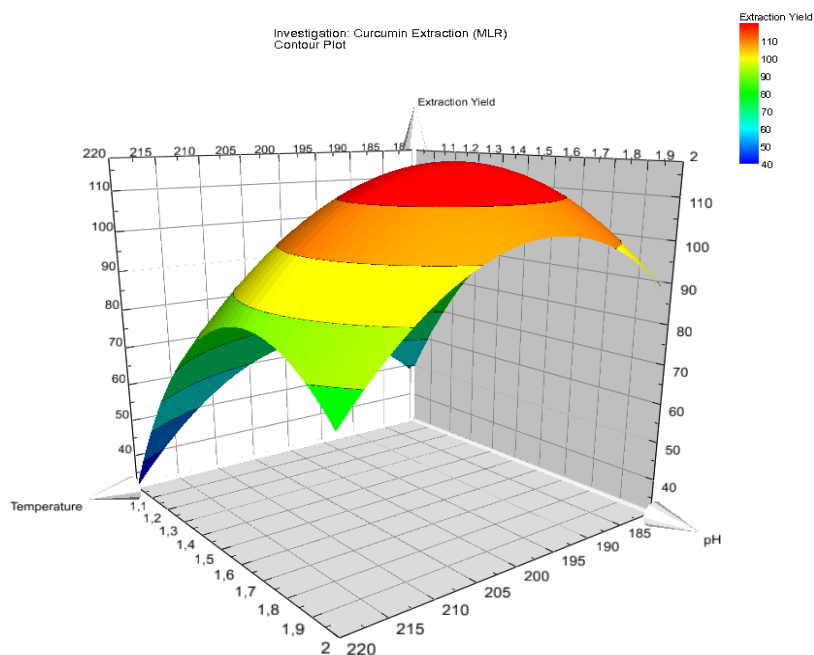
Figure 37: Response surface MLR contour plot showing curcumin extraction yields (as a percentage of the Soxhlet yield) versus extraction temperature (°C) and extraction phase pH at a buffer concentration of 60 g/L

3A.5.1 Response surfaces profile

Analyzing this predictive model is possible to identify a "sweet spot" in the conditions corresponding to: concentration phosphate buffer 62 g /L, pH 1.65 and extraction temperature 197° C. All parameters confirm that an excellent correspondence of the model obtained in terms of validity and reproducibility.

3A.5.2 Effects of selected parameters
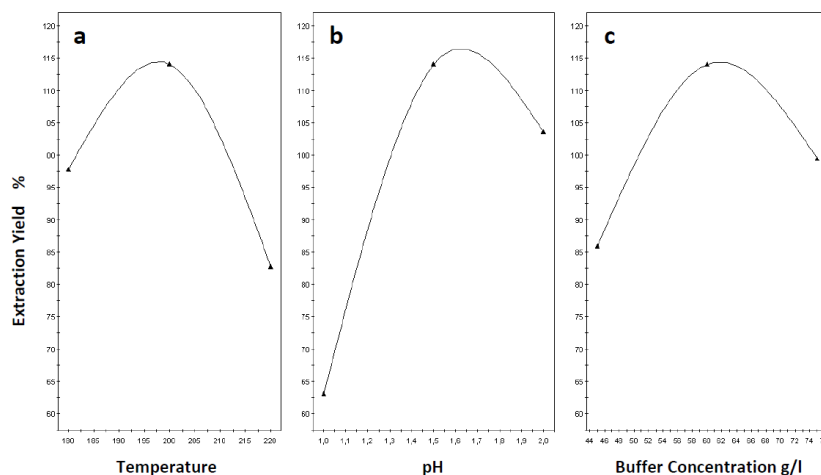
Figure 38: Response surface profiles for (a) temperature, (b) pH and (c) buffer concentration at the central point (200°C, pH 1.5 and 60 g/L).

The multilinear regression coefficients of the responsefunction obtained by statistical treatment of the data are shown in Table 9. It is apparent that at the 95% confidence level ($p<0.05$), all of the quadratic terms are significant. On the other hand, the interaction terms were not found to be significant. The response equations fit the experimental data with a high degree of significance. The high value of R^2 demonstrates that the model is capable of explaining most of the variation in the response. In the described conditions, optimum was estimated to occur when using 197 °C extraction temperature using 62 g/L buffer concentration at pH 1.6.

Table 9: Regression coefficients of the response function and significance at 95% confidence (R_2=0.957; R_2 adj.=0.879)

Term	Coefficient	Standard Error	P
Constant	76.9	3.8	5.6×10^{-6}
T	−5.09	2.3	0.081
pH	13.6	2.3	0.0020
C	4.60	2.3	0.10
$T \times T$	−16.0	3.4	0.0055
$pH \times pH$	−20.7	3.4	0.0018
$C \times C$	−14.3	3.4	0.0088
$T \times pH$	0.63	3.3	0.85
$T \times C$	5.33	3.3	0.16
$pH \times C$	2.08	3.3	0.55

3A.8.5.1 The effects of varying the temperature

Increase in the temperature was found to increase thesolubility of curcumin and thus to increase the extractionyields. However, at very high temperatures, the degradationof curcumin became a limiting factor; at temperatures above 250 °C, heavy degradation was observed at every pH examined.

3A.5.2.2 The effects of varying the pH

When considering the effects of the pH of the extraction medium, it is important to remember that, like many other properties, the ionization constant of water (K_w) and the dissociation constant of phosphoric acid are strongly temperature-dependent. They also exhibit a more modest pressure-dependence. Under the different conditions examined in this study, the negative logarithm (base 10) of the dissociation constant of water (pK_w) takes values ranging between 12.252 and 11.205 (Bandura & Lvov, 2006). For this reason to avoid confusion, all quoted values of the pH of a given extracting phase refer to those obtained under NIST standard conditions of temperature and pressure, i.e., 293.15 K (20 °C)and 101.325 kPa pressure (1.013247 bar).

At physiological conditions, the importance of the tautomerism of curcumin has been examined in a study investigating the molecular mechanism of the observed synergy between curcumin and water soluble antioxidants in cancer chemoprevention (Jovanovic et al., 2001). It has been observed that the curcumin radical preferentially exists as a phenoxyl-type species, which is more hydrophilic than the keto form. Being more hydrophilic, it is preferentially moved to the external side of the cell membrane; this effect is probably responsible of curcumin effectiveness as a scavenger of carcinogenic free radicals.The high solubility of curcumin under highly acidic (i.e., low pH) conditions is probably attributable to its protonation according to the equilibrium shown in Fig. 24.

3A.5.2.3 *The effects of varying the buffer concentration.*

The statistical data indicate that the concentration of thebuffer is a significant factor in the efficiency of extraction.This suggests that the buffer may play a role beyond simplystabilizing the pH; the nature of this role will beinvestigated in a future study. The optimal buffer concentration was found to be around 60 mg/L.

3A.6 Conclusions

The use of pure subcritical water extraction to extract curcumin, from Curcuma Longa rhizome revealed severe limitations. Maximum yields were 0,8% (w/w) of the fraction extractable by soxhlet .

Using phosphate buffer at controlled pH (under subcritical conditions) led to the extraction yields of 1,58% (w/w) higher than those obtained by Soxhlet order of 1,35% (w/w).

Because the relevance of results obtained using this approach further applications will be evaluated.

SECTION B
MIP AND RESVERATROL

3B.1 Chromatographic evaluation of MIP selectivity and rebinding capacity

Selective retention in the experiments of rebinding in conditions of dynamic flow of the template (E)-resveratrol on the polymer MIP compared with the polymer NIP clearly shows the presence of selective binding sites. Resveratrol is strongly retained on the MIP using other mobile phases, too.

Only the methanol rapidly eluted from the column.
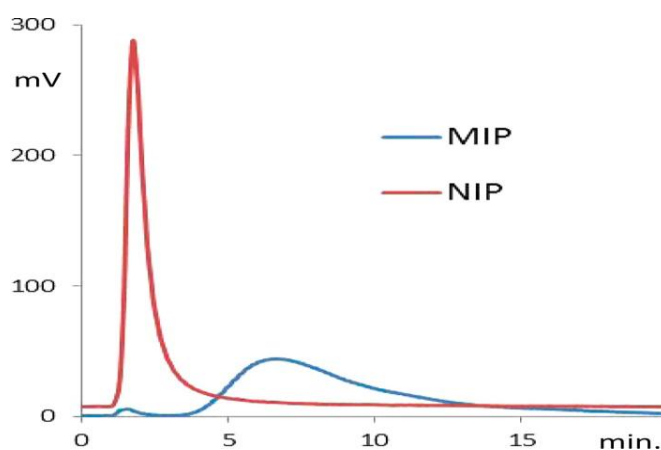
Figure 39: Retention time of resveratrol compared between the column packed with MIP (blue chromatogram) and column packed with NIP (chromatogram red). Methanol mobile phase, injection volume 50 ng

Selectivity of resveratrol MIP was investigated determining capacity factors and imprinting factors of selected model compounds (Fig.40) on both MIP and NIP packed in a short HPLC column.
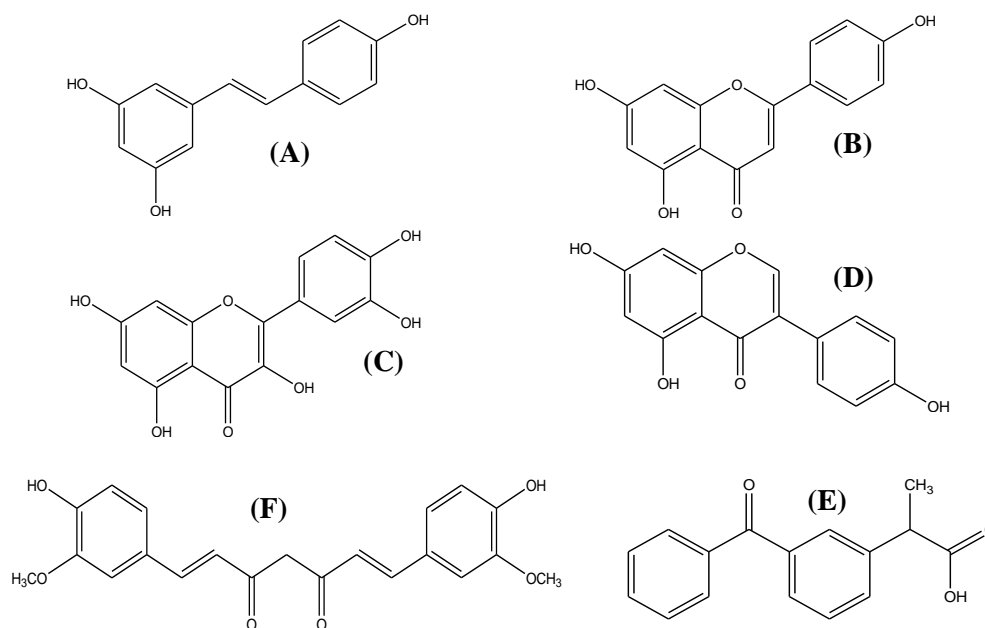
Figure 40: Selected model compound: Resveratrol (A), Quercetin (B), Apigenin (C), Genistein (D), Ketoprofen (E) and Curcumin (F)

Different solvents were used in order to verify MIP efficacy of such solvents as washing or elution phase for the considered analytes. Results are summarized in Table 10.

Table 10: Chemical properties and characteristics of selective retention on MIP for model compounds. Octanol-water partition coefficient, acidity in water and Imprinting Factor (calculated as MIP/NIP capacity factors ratio) in acetonitrile and methanol (elution solvent).

Compound	Log P*	pKa*	IF ACN	IF MeOH
Resveratrol (A)	3.08	9.07	n.c.	8.30
Quercetin (B)	1.48	7.17	n.c.	2.64

Apigenin (C)	3.02	7.12	n.c.	1.67
Genistein (D)	2.84	7.63	0.813	0.41
Ketoprofen (E)	3.01	4.25	0.735	0.32
Curcumin (F)	3.20	8.11	1.23	0.56

Chemical properties and characteristics of selective retention on MIP for model compounds. Octanol-water partition coefficient, acidity in water and Imprinting Factor (calculated as MIP/NIP capacity factors ratio) in acetonitrile and methanol (elution solvent).

Selection of model compounds was done bearing in mind a pharmacophore model containing four common chemical features in antioxidants agents: one aromatic ring and three hydrogen bond acceptors (Molinelli et al., 2002; Song et al., 2009). Template itself and two more compounds (quercetin and apigenin) had this specific features. Three more molecule were also evaluated for MIP retention effects as control compounds to emphasize the effects of non specific interaction such ion exchange or non specifically oriented hydrogen bond: genistein is an isoflavonoid then based on 3-phenylchromen-4-one structure instead of 3-phenylchromen-4-one; curcumin is a well known natural phenolic antioxidant, its phenols, are connected by two α,β-unsaturated carbonyl groups and the diketones form stable enols and enolates; ketoprofen is a compound with similar molecular weight and raw formula to resveratrol but pretty different molecular structure and physic-chemical properties, it is more acidic and, as well as resveratrol, it contain both H-bond donor acceptor sites.

It has been often shown that H-bond based MIPs exhibits higher selectivity in non protic solvents such as acetonitrile. Chromatographic rebinding

experiments results (Table 10) demonstrate that molecules with very similar pharmacophore triangles were not eluted in acetonitrile over 120 minutes making, from the practical point of view, the calculation of the retention factor impossible. Moreover, even in a protic solvent such as methanol, template molecule is hardly displaced from selective interaction sites (IF 8.30) and molecules with very similar H-bond donor stereochemistry are still significantly retained.

On the other hand it has been often observed that analytes can be retained by MIPs and NIPs through non-specific interactions assisted mainly by solvophobic effects. Differences in polymer characteristic (i.e. surface area) might be responsible for slightly stronger non specific retention of NIPs generating IF < 1 for molecules which are very different from template.

3B.2 Selectivity and rebinding capacity evaluation of MISPE cartridges

In order to investigate the feasibility of using resveratrol MIP to develop a multi-component multi-class method for polyphenolic compounds in food matrices and beverages, the two most abundant polyphenolic compounds present in many beverages were elected for evaluating the polymer selective retention effects.

In order to estimate the rebinding capacity of the polymer 50 mg cartridges were packed using both MIP and NIP particles in the size range 75-106 μm and used for breakthrough experiments.

Breakthrough volume was investigated extracting different size water and acetonitrile samples. This latter could be used as solvent for extracting polyphenols from solid matrices or washing filtrate from beverages.

As expected from the chromatographic retention experiments no relevant losses of these two analytes occurred for reasonable samples sizes for both solvents.

Table 11: Resveratrol breakthrough experiments from 2 μg spiked samples of different solvents. Extractions and elution performed using 50 mg MISPE cartridges.

Sample Volume	Water Recovery	(SD)	Acetonitrile Recovery	(SD)
mL	%		%	
5	101.6	(4.8)	101.0	(3.7)
10	-	-	82.7	(10)
25	100.3	(2.1)	78.0	(4.4)
50	99.6	(3.2)	72.8	(1.7)
100	100.0	(6.7)	-	-

The nonspecific hydrophobic interactions are considerably reduced in acetonitrile allowing the elution of substances not specifically retained by the MIP affinity binding sites. A protic solvent as methanol weaken the hydrogen bond reducing the retention effect allowing the elution of several analytes (results not shown). The presence of acetic acid was necessary, to achieve quantitative elution of most retained compounds (such as resveratrol and quercetin) from the MISPE column with a small amount of mobile phase.

The binding capacity of the MISPE cartridge was assessed by recovery measurements at high concentrations. No evidence of saturation effects on the MISPE cartridges were observed in recoveries up to 80 μg g-1. Some breakthrough was observed by loading higher than 100 μg g-1 samples.

3B.3 Validation of MISPE HPLC-UV method for the analysis of resveratrol and quercetin in beverages

The potential use of MISPE cartridges for determining polyphenolic compounds content in natural beverages was assessed by performing selected model compounds recovery experiments from different solvents and different concentration levels. As expected from preliminary experiments, no breakthrough was observed from water samples within reasonable size

volumes (see Table 11). In fact, non specific interactions play a major role in analytes retention mainly depending on water solubility, logP and pKa values. In acetonitrile, experiments performed by spiking samples at 2 µg (50% of calculated capacity), no losses occurred at 5 mL volume confirming preliminary experiments used to determine the washing step volumes in the MISPE procedure. At higher sample volume some breakthrough occur with moderate increase by increasing the sample volumes according to typical MIP binding isotherm. Table 12 reports separate recovery experiments for resveratrol and quercetin confirming acceptable accuracy and precision in the range of reasonable sample size and concentration.

Table 12: Recovery experiments using 50 mg MISPE cartridges at different concentration levels from 5 ml water and acetonitrile samples.

	Resveratrol				**Quercetin**			
	Water		**Acetonitrile**		**Water**		**Acetonitrile**	
Spike	**Recovery**	**SD**	**Recovery**	**SD**	**Recovery**	**SD**	**Recovery**	**SD**
µg	**%**		**%**		**%**		**%**	
0.5	98.2	(0.68)	98.6	(2.8)	97.5	(3.2)	91.4	(5.8)
1	101.6	(4.8)	99.9	(3.4)	98.7	(1.6)	98.8	(4.8)
2	101.6	(4.8)	100.9	(3.7)	86.4	(0.15)	85.9	(4.6)
4	100.7	(3.7)	-	-	-	-	-	-

Linearity was investigated in the range and 0.5 µg mL-1 - 5 µg mL-1. Pearson correlation coefficient R^2 =0.9998 was obtained for the curve of resveratrol (y= 188.5573 x) and R^2 = 0.9993 for the curve of quercetin (y= 50.39619 x). Limits of detection, defined as three time signal to noise, were estimated at 0.15 ng injected for resveratrol and 0.70 ng for quercetin. In these conditions, considering a sample size of 2 mL and concentrating the eluate up to 100 µL method detection limits (MDLs) were estimated as 1.5 µg L-1 e 7.0 µg L-1 respectively for resveratrol and for quercetin.

3B.4 Recovery and analysis of resveratrol and quercetin in real samples.

In order to carefully evaluate the efficacy of MISPE cartridges in the extraction from complex matrices, recovery of resveratrol and quercetin were performed from spiked samples of wine and "red fruits" juice. Excellent reproducibility was obtained in both cases as reported in Table 13.

Table 13: Evaluation of competitive effects from complex matrices. Resveratrol and quercetin recoveries from real samples spiked at different concentration levels.

	Resveratrol				Quercetin			
	Wine		Red Fruits Juice		Wine		Red Fruits Juice	
Spike (µg)	recovery %	SD	recovery %	SD	recovery %	S.D	recovery %	SD
0.5	95.2	(3.6)	92.3	(2.3)	91	(3.8)	97.4	(1.1)
1	-	-	99.5	(1.7)	88.2	(1.5)	98.8	(3.1)
1.5	97.4	(1.2)	99.3	(1.5)	94.7	(1.2)	94.7	(1 .3)
2	96.5	(1.5)	99.5	(2.5)	95.4	(0.8)	95.4	(0.62)
3	100.3	(0.095)	-	-	-	-	-	-

No losses were observed due the presence to the large amounts of possible competitors for binding sites demonstrating the robustness of the MISPE approach in the treatment of very complex matrices.
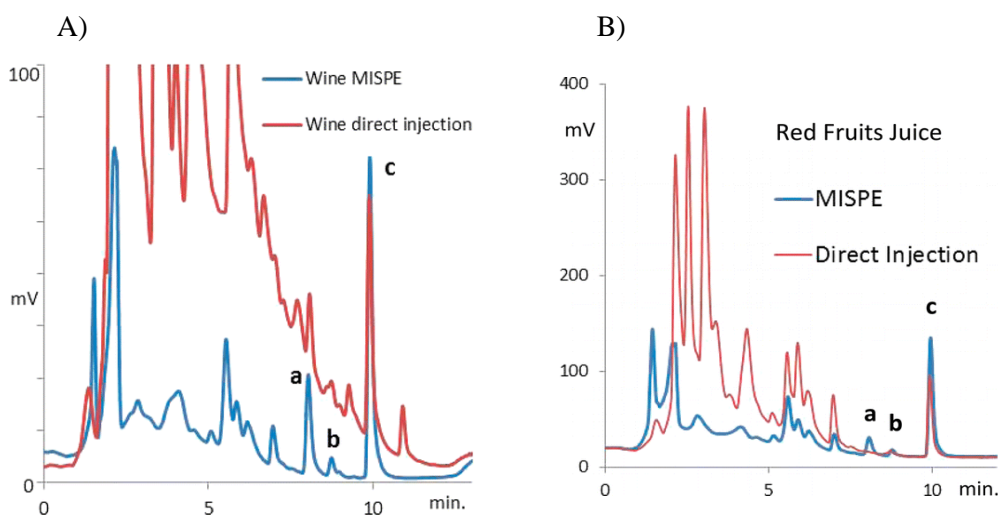
Figure 41: Red line: chromatograms of untreated red wine samples (A) and untreated red fruits juice (B). Blu line: MISPE extract: (a) resveratrol; (b) quercetin; (c) volumetric standard: genistein.
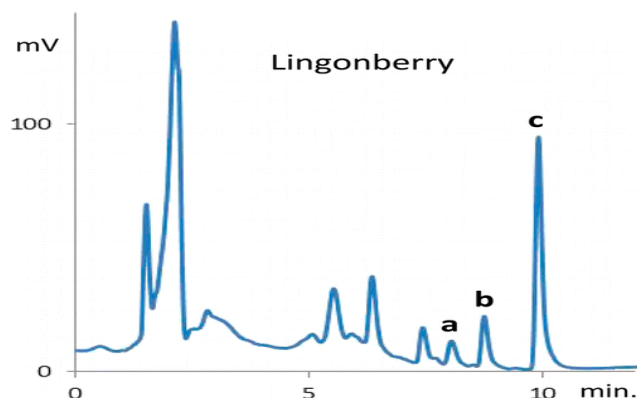
Figure 42: Chromatograms corresponding to analysis of lingonberry juice drink. (a) resveratrol; (b) quercetin; (c) volumetric standard: genistein.

The advantages of using MISPE is clearly shown in Fig. 40 and 41 where the chromatograms corresponding to, respectively, wine and fruit juice are overlapped to the analysis of the correspondent amount of untreated sample. Much cleaner baseline was obtained generating a lower LOD.

Finally the presented method was applied to few exemplary real sample, namely red wine, "red fruits" juice and Lingonberry (Vaccinium vitis-ideae, Ericaceae) juice selected because their high content of resveratrol and quercetin.

Table 14: Resveratrol and quercetin (more relevant natural polyphenols antioxidant) content in three beverage determined by MISPE HPLC-UV.

	Resveratrol		Quercetin	
	mg L^{-1}	SD	mg L^{-1}	SD
Wine	0.946	(0.007)	0.475	(0.010)
Red fruits juice	0.034	(0.001)	0.868	(0.016)
Lingonberry	0.161	(0.010)	1.871	(0.087)

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3B.5   Conclusions

In the present work the synthesis and the evaluation of an imprinted polymer based on resveratrol as template molecule is suggested as a valuable tool for SPE analysis of phenolic compounds from complex foodstuff matrices such as wine and fruit beverages. MISPE sample pretreatment allows an excellent sample clean up enormously decreasing the number of co-extracted potentially interfering compound. The presence of few peaks corresponding to strongly retained compounds in real samples warns about the possibility of using MISPE in the untargeted analysis of specific pharmacophores.

SECTION C

PGC AND PFAS

3C.1 Analytical strategy

The ability of PGC to strongly retain anion compound was exploited to concentrate analytes from water samples pumped by an auxiliary pump in a PGC precolumn. After sample extraction the precolumn was connected to analytical system by mean of a six port valve (Fig. 43). A suitable mobile phase should properly elute all the analytes from SPE trap and give rise to efficient chromatographic separation and a reproducible ESI response. Several different experiments were performed.

The system used for the online solid phase extraction and the subsequent analysis (LC-MS) is constituted by a binary HPLC pump, the auxiliary pump, a six-way valve, an auxiliary injector, the analytical column and the mass spectrometer equipped with an electrospray LC-MS interface (Fig. 43)
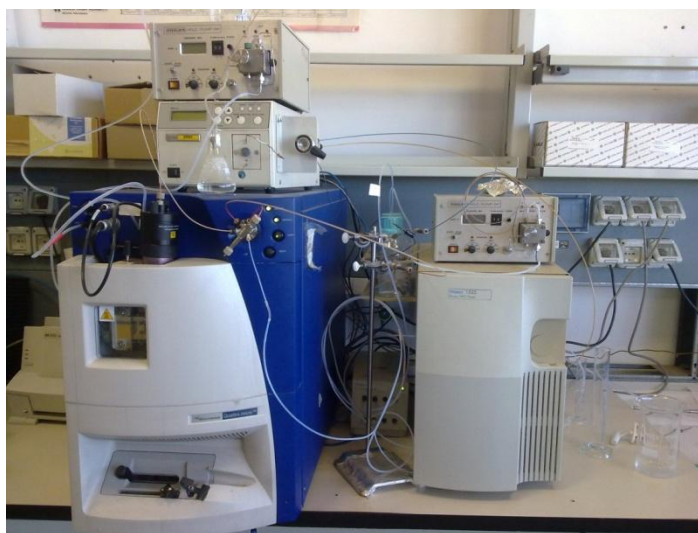
Figure 43: Analysis apparatus

3C.2 Determination of Breakthroug volume

In order to explore the breakthrough volume, preliminary experiments were performed simulating the extraction of a spiked sample, after injecting a known amount of the analytes in the precolumn. An exactly known amount of analytes mixture were injected in the auxiliary pump circuit prior the SPE trap

using an additional six-way valve. A sketch of the setup involved is reported in Fig. 44 and 45.

The rationale for this experimental setup was a reduction of possible variables including the risks of contaminations on glassware, tubing and other part of the pump due to relatively high analytes concentration of the preliminary experiments.

In order to remark analytes losses due to water sample elution, increasing size, 10, 50, 100 ml sample of Milli-Q water were pumped through the SPE trap after analytes deposition. The ratio of analytes peak areas and internal standard peak area were compared and results are reported in figure 46 and 47.
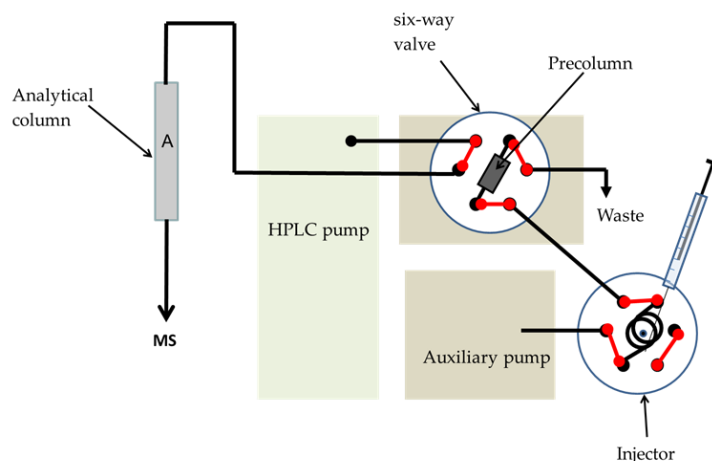
Figure 44: First step: extraction. Auxiliary pump flush water sample through the precolumn while the analytica pump equilibrate the analytical column.
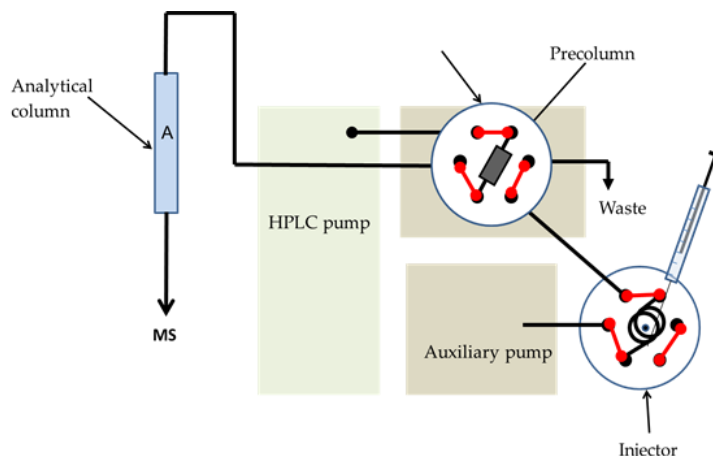
Figure 45: Second step: analysis step. by switching The six-way the precolumn is connected to the analytical system (LC-MS).

The percentages of recovery are evaluated for extractions from 50.0 mL and 100.0 mL with Milli-Q water normalized to an extraction reference with 10.0 mL of Milli-Q water considered as 100% recovery.
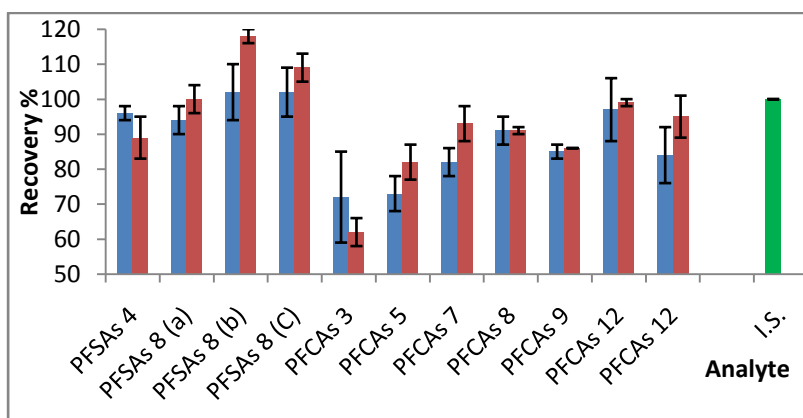
Figure 46: Results of Breakthrough experiments at 555 ng individual analytes level; extraction with 50.0 mL and 100.0 mL of Milli-Q water (triplicate).

The most striking result of this series of experiments is the high variability (standard deviation), nevertheless it is clear that this phenomenon is due to the excessive instability of the interface electrospry under this chromatographic conditions. The apparent tendency to breaktrough for the first eluted analyte already at 50.0 mL of

water does not seem to be confirmed by doubling the volume of the sample. The values reported in Fig. 46 show that the PGC is able to retain quantitatively the analytes considered at least up to a volume of 100.0 mL. This volume is sufficient for a system of solid phase extraction on-line, whereas with this type of system is injected to 100% of the extracted sample and not only a fraction.

Analogs recovery experiments were carried out with a quantity of analyte injected much lower (10 ng) using MRM mode using appropriately diluted standard mixture and passing 100.0 mL of Milli-Q water through the SPE cartridge.
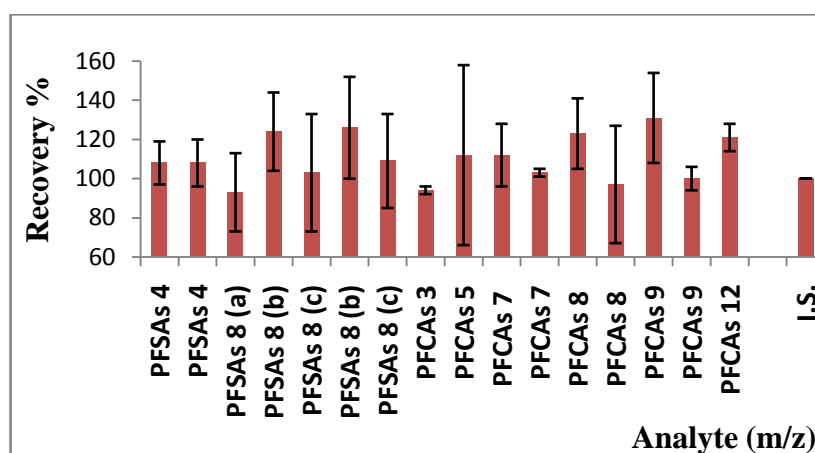
Figure 47: Results recovery experiments. Injection 10 ng of individual analytes; extraction with 100.0 mL of Milli-Q water in triplicate.

Results shown in Fig. 47 confirm previous observations. If breakthrough was occurring a much lower recovery is to be expected by doubling the sample volume. confirm previous observations. The most critical aspect of these experiments seems to be that of detection.

3C.3 Analysis of blank samples

The analysis of the whites was carried out by extraction of 100.0 mL of Milli-Q water with the SPE system on-line and subsequent analysis LC-MS. From these experiments was not detected the presence of the analytes above the limit of detection.

3C.4 Analysis of real samples

Some real samples were analyzed in order to verify the effectiveness of the method. In particular, we examined the following samples: ground water (Bellizzi well), bottled mineral water ("Spring"), spring water (Giffoni VP), municipal water (Giffoni VP). Under the experimental conditions described no evidence of the considered PFAs contamination was observed.

3C.5 Experiments recovery from real samples

For these experiments 1.0 L of a real ground water sample (Bellizzi) was artificially contaminated with a known amount of analyte.(50.0 mL of the Mix-10 ST 2.0 3.10 mg/mL). The contaminated sample thus obtained had a concentration of 100 ng/mL for each analyte.

In Table 15 shows the results of the experiment.

Table 15: Results of the recovery experiments (duplicate) of a water sample (Bellizzi) contaminated with a known amount (10 ng) of each individual analyte. Volume extracted 100.0 mL.

Analyte	Parent ion and its fragmentation (m/z)	Recovery % (± Variation coeff.)
PFSAs 4	298.9 > 80	0 ± 9
PFSAs 4	298.9 > 99	1 ± 70
PFSAs 8	498.9 > 80	51 ± 33
PFSAs 8	498.9 > 80	72 ± 32
PFSAs 8	498.9 > 80	61 ± 36
PFSAs 8	498.9 > 99	0 < 1
PFSAs 8	498.9 > 99	62 ± 27
PFSAs 8	498.9 > 99	60 ± 31

PFCAs 3	162,9 > 119	0 < 1
PFCAs 5	262.9 > 219	0 < 1
PFCAs 7	362.9 > 169	2 ± 97
PFCAs 7	362.9 > 319	4 ± 44
PFCAs 8	412.9 > 169	21 ± 20
PFCAs 8	412.9 > 369	28 ± 42
PFCAs 9	462.9 > 219	102 ± 14
PFCAs 9	462.9 > 419	132 ± 34
PFCAs 12	612.9 > 569	0 < 1
PFCAs 12	612.9 > 569	56 ± 64
I.S.	427 > 80	100 < 1

In order to exclude that teh very recovery percentage was due to the presence of electrolytes in the sample, the experiment was repeated contaminating a sample of Milli-Q water with the same amount of analyte in the sample of ground water (100 ng / L). The results of the experiment are reported in Table 16.

Table 16: Results of the experiments recovery (duplicate) of a sample of Milli-Q water contaminated with a known amount (10 ng) of each individual analyte.

Analyte	Parent ion and its fragmentation (m/z)	Recovery % (± Variation coeff.)
PFSAs 4	298.9 > 80	1 ± 36
PFSAs 4	298.9 > 99	1 ± 25
PFSAs 8	498.9 > 80	47 ± 9
PFSAs 8	498.9 > 80	75 ± 11
PFSAs 8	498.9 > 80	74 ± 17

PFSAs 8	498.9 > 99	88 ± 48
PFSAs 8	498.9 > 99	64 ± 4
PFSAs 8	498.9 > 99	68 ± 7
PFCAs 3	162,9 > 119	0 < 1
PFCAs 5	262.9 > 219	0 < 1
PFCAs 7	362.9 > 169	8 ± 39
PFCAs 7	362.9 > 319	5 ± 14
PFCAs 8	412.9 > 169	39 ± 46
PFCAs 8	412.9 > 369	20 ± 1
PFCAs 9	462.9 > 219	65 ± 35
PFCAs 9	462.9 > 419	44 ± 23
PFCAs 12	612.9 > 569	0 < 1
PFCAs 12	612.9 > 569	20 ± 59
I.S.	427 > 80	100 < 1

From the results shown in Table 16 it can be seen that the recovery percentages of the analytes in the case of water Milli-Q does not differ significantly from recoveries obtained for the ground water. Then, the low percentages of recovery in the sample of contaminated groundwater cannot be due by the presence of electrolytes within the sample.

3C.6   *Evaluation of chromatographic conditions*

The problems observed during recovery experiment induced us to further investigate PGC chromatography in order to optimize a more suitable mobile phase condition for the online desorbtion, separation and detection step.

Because of the strong electrostatic interactions between PFAS and the stationary phase, the elution of these analytes requires the presence of electrolytes.

In order to verify the best elution conditions different combination have been explored.

Test n ° 1

For the elution of the analytes have been used the following mobile phases:

A) 49.5% H_2O, 9.9% CH_3OH, 39.6% ACN and 1.0% CH_2Cl_2.

B) 73.0% CH_3OH, 25.0% ACN and 2.0% toluene.

The experiment was conducted with the same procedure described for the reference (extraction with 10mL total of Milli-Q water and analysis LC-MS) by injecting 5.0 mL of a mixture of PFAs. The separation of the analytes was carried out in gradient used the program chromatographic reported in Table 17.

Table 17: Chromatographic conditions for test 1. Flow 0.20 mL/min. The elution is carried out in linear gradient.

N° step	Time	% B
0	0,00	0,0
1	15,00	90,0
2	30,00	90,0
3	31,00	0,0
4	40,00	0,0

The detection was conducted in SIR mode using the parameters described above.

In these conditions it is observed that the PFAS are not eluted, while PFOS are separated and eluted in the form of very broad peak.

Test n°2

A further attempt was performed in the same way described in the first test using the program chromatographic reported in table 18 and the and following mobile phases:

A) 100,0% ACN

B) 25,0% ACN e 75,0% toluene.

Table 18: Chromatographic conditions for test 2. Flow 0.20 mL/min. The elution is carried out in linear gradient.

N° step	Time	% B
0	0,00	0,0
1	15,00	90,0
2	30,00	90,0
3	31,00	0,0
4	40,00	0,0

Under these conditions it was observed that when using not protic solvents , polar or ionic analytes are retained much more than in the presence of small percentages of protic solvents such as methanol or water, once again demonstrating the difference between the PGC and the conventional phase reverse. No analyte (PFCAs and PFOS) were eluted in these conditions.

Test n°3

We proceeded in the same way described in the first test using the elution steps program reported in table 19 and the and following mobile phases:

A)   100,0% ACN

B)   25,0% ACN, 30,0% CH_3OH e 45,0% toluene.

Table 19: Chromatographic conditions for test 3. Flow 0.20 mL/min. The elution is carried out in linear gradient.

N° step	Time	% B
0	0,00	0,0

1	20,00	90,0
2	30,00	90,0
3	31,00	0,0
4	40,00	0,0

In these conditions is observed that the presence of methanol (polar protic solvent) allows to elute both classes of analytes (PFCAs and PFOS). However, it was observed a loss of selectivity and all of the analytes are co-eluted. The reason for this loss of selectivity might be due to the effect of deactivation of the toluene on the surface of the PGC.

Test 4 microbore column

In order to reduce the analysis time and operate on a smaller scale a microbore column was packed and used for further investigations..

The column was connected to the input microbore, passing through the injector, the HPLC pump and the exit to the mass.

Using this systemthe following eluents phases were evaluated:

1)

A) 190 mM aqueous phase $HCOONH_4$

B) ACN/CH_3OH (80:20) 190 mM $HCOONH_4$ with 5% H_2O (in the absence of water are formed of crystals).

2)

A) Phase aqueous 100 mM $HCOONH_4$

B) ACN/CH_3OH (80:20) 100 mM $HCOONH_4$ with 5% H_2O (in the absence of water are formed of crystals).

3)

A) Aqueous phase 10 mM $HCOONH_4$

B) ACN/CH_3OH (80:20) 10 mM $HCOONH_4$ with 5% H_2O (in the absence of water are formed of crystals).

The preliminary data from these experiments confirm that several different combinations of solvent and additives can be used in order to correctly elute PFAs from PGC stationary phase.

3C.7 Conclusions

From the results obtained in this study is confirmed the potential of the use of the PGC for the determination of anionic surfactants, in particular of PFCAs, PFOS. The use of an on-line strategy has considerable advantages in terms of poor sample handling, automation possibilities, extraction and analysis of large volumes of the totality of the extract.

It has been demonstrated that the PGC is able to retain quantitatively the analytes considered up to a volume of at least 100.0 mL of water extracted. Poor reproducibly was observed in the ESI MS detection very likely due to the adopted chromatographic condition. Further investigations will be performed using the different conditions optimized sing a the microbore column.

MATERIALS AND

METHODS

SECTION A

SWE AND CURCUMIN

Based on the article:**Euterpio MA,** Cavaliere C, Capriotti AL, Crescenzi C (2011), *Extending the applicability of pressurized hot water extraction to compounds exhibiting limited water solubility by pH control: curcumin from the turmeric rhizome*, Anal Bioanal Chem. , **401** (9): 2679-2792

4A.1   Chemicals and standards

Water (HPLC grade) was obtained using a Milli-Q Plus apparatus (Millipore, Bedford, MA). Acetonitrile (RS forHPLC), methanol (RS for HPLC), sodium dihydrogenphosphate- 1-hydrate, hydrochloric acid, and sodium hydroxide were purchased from Carlo Erba (Milan, Italy). Sand (seasand, 40–200 mesh) was obtained from Fluka (AG, Buchs,Switzerland). Turmeric rhizome was purchased from Laboratori Biokyma S.r.l. (Anghiari, AR, Italy). Curcumin (97%purity) purchased from Farmalabor S.r.l. (Milan, Italy) was used as a standard. Phosphate buffer was prepared by dissolving sodium dihydrogen-phosphate-1-hydrate in water; its pH was adjusted by adding appropriate quantities of asolution of hydrochloric acid (0.1 mol/L) or sodium hydroxide (0.1 mol/L).

4A.2   Soxhlet

A homogeneous reference sample of C. longa rhizome was obtained by grinding 50 g of chopped turmeric using a coffee grinder and collecting particle fraction between 20 and 40 mesh. The concentrations of curcumin in the reference sample were determined by Soxhlet extraction, using two different solvents. The efficiency of amixture ethanol/isopropyl alcohol was compared with that achieved with pure acetone by using a classical 300 mL Soxhlet apparatus to extract 3 g of accurately weighed turmeric powder, with a solid to solvent ratio of 1:100. When acetone was used as the extracting solvent, extraction was conducted for 8 h/day for 3 days. Every day, the extract of the previous day was removed and 300 mL of fresh acetone was added. The experiment was performed intriplicate. The total extraction yield of curcumin, calculated as the mass of each single compound obtained divided by the mass of turmeric.

Soxhlet extractions were carried out using for three hours per day, in three days. 1 g of powdered rhizome of Curcuma Longa has been inserted in a

thimble, while 100 mL of solvent were poured into appropiate boiler of 250 mL. The experiments were carried out, respectively, with isopropanol/ethanol (1:1) (Braga et al, 2007), and with acetone (Mandal et al., 2007). Every day solvent, contained in the boiler, was replaced with fresh solvent, and in the end fractions were combined and analyzed by HPLC/UV. All experiments were performed in triplicate.

Figure 48: Soxhlet apparatus

4A.3 Study of curcumin's solubility

*S*olubility of curcumin was analyzed in water at various temperatures. These studies were conducted using a standard of curcumin to 95%. The first step was to construct a calibration curve with a HPLC chromatographic system able to work at high pressures coupled to a UV detector. The chromatograms were recorded at a wavelength of 425 nm (Péret-Almeida et al., 2005), which is selective for the curcuminoids (Fig.49). The calibration curve was obtained by injecting the standard sample to decreasing concentrations obtained from a parent substance of 1 g/mL. Preliminary water solubility tests were performed in a beaker, using water and solid curcumin under stirring at a controlled temperature and in darkness for 24 h. At the end of the extraction period, the extract was filtered at the extraction temperature and diluted with methanol (1:1) in order to ensure that any extracted curcumin remained insolution at room temperature. The extract was then analyzed by

HPLC-MS. These operations were carried out in triplicate for each temperature.
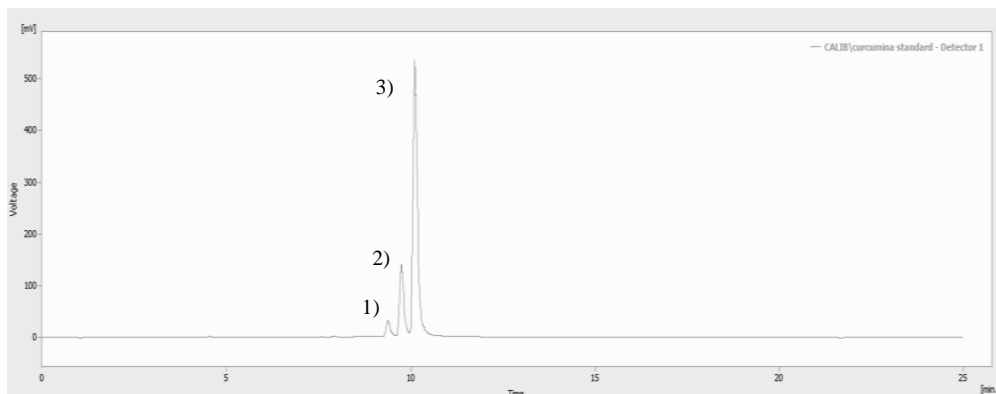
Figure 49: Chromatogram of (1) Bis-demethoxy-curcumin, (2)-demethoxy curcumin, and (3) Curcumin.

4A.4   SWE apparatus

SWE was conducted in the lab with a system consisting of a thermostated GC oven, inside which was assembled a long stainless steel coil, connected to the cell extraction with 0.5 um frit, disposed in input and output ; from a container of solvent; by a HPLC pump; by a pressure regulator and from the tube in which they were collected extracts (Yang et al., 1999). The pressure regulator, connected directly to the steel tube in output, has allowed us to maintain a pressure between 3.4 and 5.0 MPa. The column was packed with 100 mg of rhizome of Curcuma longa and triturated with 900 mg of silica, and was placed vertically in the oven for the water to flow from top to bottom, and this was done to allow for an optimal analyte elution (Fig. 50)
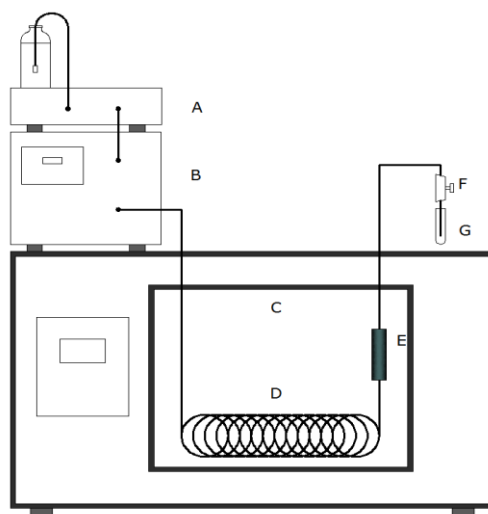
Figure 50: Schema extraction: (A) water degasser, (B) HPLC pump, (C) GC oven; (D) stainless steel coil, (E) cell extraction, (F) pressure regulator (G) tube for the collection of fractions.

4A.5   SWE Procedure

The rhizome of Curcuma Longa was ground and then sieved to obtain a powder of uniform size to be used for packing the cell extraction.

Unlike SFE, PHWE is (obviously) not affected by presence of water in the samples. Consequently, unless differently described, 100 mg of ground rhizome was directly mixed with 900 mg of sea sand and packed in the extraction cell. The empty space was filled with approximately 1 g of sea sand. The cell was then connected to the extraction system, with the plain seasand at the bottom, and the pump was started. The rate at which water flowed through the extraction cell was set at 0.5 mL/min, and the pressure was maintained at 5.0 MPa by regulatingthe BPR (Rodriguez-Meizoso et al., 2006). The cell was pre-washed for 10 min at room temperature, then the flow was stopped, and the cell was pre-heated and equilibrated for 5 min at 90 °C. After this equilibration time, the water pump was re-started and the temperature was

increased to the specified value. Fractions were collected in vials and analyzed by HPLC-UV.

The extractions were performed at different temperatures with this process: 90, 120, 150, 175° C. There has been an increase in the yield of extraction with the phase of the equilibrium at 90° C. After the extraction with water, the particles in the cell' s extraction were soaked in acetone and the solution appeared of a deep yellow color, and this made me think that it was still possible to extract a high percentage of curcumin. Finally, to try to obtain a higher yield of extraction, it was decided to operate at a temperature equal to 250° C, which, however, has resulted in a blackening of the particles present in the column. It is then noted, also, a decrease in the volumes of extraction; these fractions were combined and dried in a rotavapor, obtaining an orange powder subjected to maceration in acetone; the extract was analyzed by HPLC-UV showed the presence of a small amount of curcumin not degraded by the temperature rise. Each experiment was conducted in duplicate.
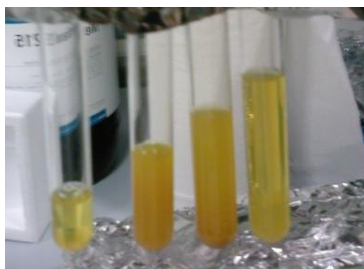
Figure 51: Different fraction of an extraction

A next test has been to change temperature and pH value.

For determination of curcumin concentration in the extracts a subsample of 100 μL was taken from each collectedfraction and mixed with 900 μL of acetonitrile in a 1.5-mLEppendorf vial. The mixture was then centrifuged at13,000 rpm using a Minispin Eppendorf centrifuge (Eppendorf Italia S.r.l., Milan, Italy) in order to separatestarch. The supernatant was collected and 5 μL wasinjected into the HPLC system for analysis.

4A.6   Chromatographic conditions

The analysis was conducted using an Accela autosampler and pump; UV detection was performed using a Spectra Physics UV2000 instrument. Both instruments were obtained from Thermo Scientific (Milan, Italy). The chromatographic system wasequipped with a Phenomenex Luna C18, 2.5 μm particles, 100×2.00 mm i.d. HPLC column purchased from Phenomenex (Bologna, Italy). As it has been reported by other authors (Marczylo et al., 2009), the addition of acetic acid afforded better separation and improved peak shapes, particularly in separating curcumin from the other curcuminoids. The mobile phases used with this column were water containing 2% acetic acid (solvent A) and acetonitrile (solvent B). Isocratic elution was realized at 30% solvent B, the flow rate was set at 300 μL/min. UV absorbance detection was performed at 425 nm.

4A.6.1 UPLC analysis

Acceptable separation of curcumin from major curcuminoidshas been described by several authors. However, inorder to reduce the consumption of organic solvents, theuse of an analytical column with a reduced internal diameter is recommended. Moreover, the availability of columns packed with particles having a diameter of 2 μm (or less) has generated greatly increased chromatographic performance, allowing the use of much shorter HPLC columns than was previously possible. In this case, by exploiting the advantages of narrow-bore columns packed with sub-2-μm particles, it was possible to use a 5-cmcolumn to achieve a resolution that previously required the use of a 25-cm column packed with 5 μm particles. The use of a shorter column significantly reduces both chromatographic run and equilibration times (resulting in so-called "fast chromatography"), allowing for greatly reduced solvent consumption. Using a Hypersil Gold C18, 1.9 μm particles, 50×2.00 mm i.d. HPLC column (purchased from Thermo Scientific), the

separation and quantification of curcumin and other curcuminoids was achieved using gradient elution. The initial composition of the mobile phase was 30% B; this was increased linearly to 60% over 10 min. The mobile phases used were water containing 2% acetic acid (solvent A) and acetonitrile (solvent B). The flow rate was set at 500 µL/min.

4A.6.2 LC-MS analysis

LC-MS confirmation analysis was performed under the chromatographic conditions described above, using an LCQ Advantage ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) equipped with an electrospray (ESI) source. Analyses were performed with the ESI interface in the positive ion mode. Data were acquiredin the full scan and MS/MS scanning modes. The maximum injection time was 50 ms, the number of microscans was three, and for the MS/MS scanning mode, the percentage of the collision energy was 20–45%. The ionization conditions were optimized, and the following values of the relevant parameters were maintained: capillary temperature, 200 °C; capillary voltage, 29 V; spray voltage, 4.50 kV; sheath gas flow rate, 30 (arbitrary units); auxiliary gas flow rate, 5(arbitrary units); scan range, m/z 200–700. Nitrogen was used as the sheath and auxiliary gas. Both the LC and MS systems were controlled using the Xcalibur 3.1 software package. The identity of the different analytes was confirmed by ESI-MS on the basis of the previously reported results (Jiang et al., 2006).

4A.7 Experimental design (DOE)

The fastest and most efficient way to explore the possibility of any process or to optimize the conditions is to apply an appropriate experimental design. The designs are so called because multifactorial analyzing multiple independent variables or factors. Through these drawings it is possible to analyze both the main effect of each independent variable, and the interaction between these.

The main effect indicates the effect that an independent variable has on the dependent variable apart from the effects of other independent variables. It has an interaction when the effect that an independent variable has on the dependent variable is not the same for all levels of the other independent variables. Factorial designs become more complicated if we have more than two independent variables, or whether each factor has multiple levels. For example, a 2x2x2 design means that we have three independent variables, each at two levels, then eight experimental conditions, a 2X3 design means that you have two independent variables, the first two levels, the second with three, and six experimental conditions . The application of a method of "Response Surface Design" will allow us to the realization of a model able to approximate locally the surface of the system response.

This knowledge will allow us to predict the conditions for the maximization of the response (in this case the yield of extraction) and/or identify the operating conditions more convenient.

The effects of three operating variables (temperature, pH and buffer concentration) were investigated using a 3-factor Box–Behnken response surface method design. Calculations, experimental matrices, graphs, and plots were generated using the MODDE 9.0 software package (Umetrics AB, Umeå, Sweden).

Table 20:   Property and level of factors considered in the experimental design

Data Coding		Low	High
Temperature	(°C)	180	220
pH	(pH units)	1	3
Buffer conc.	(mol/l)	45	75

4A.7.1 Response surface methodology (RSM)

Use a fractional factorial plan with three levels entails the risk of having to drastically increase the number of experiments needed to grow the factors. To this is often accompanied by an increase of the redundancy.

That of Box and Behnken is a type of independent quadratic design that does not contain an intrinsic or fractional factorial design. Actually consists of a selection of points. The selected points are midpoints of the edges of the experimental space. These designs require three levels for each factor.

Box and Behnken suggested how to select some points of the plane full factorial three levels sufficient to properly estimate the coefficients of the first and second order of the mathematical model. In this way the floor is much more efficient and cost of the corresponding complete plans 3^k, especially in the case of a high number of variables
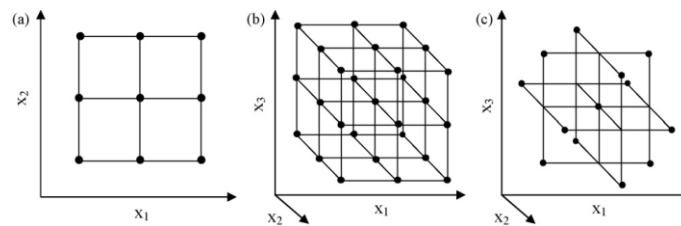
Figure 52: Comparison of factorial experimental designs based on the study of variables at three levels: a) for two variables, b) for three variables, c) plan for Box-Behnken three variables.

In the plane of Box-Behnken, the experimental points are taken at the edge of the experimental space of un'ipersfera equidistant the center point (as shown in Fig.52 for a case to three factors).

In summary, the main features are:

1)   It requires a number of experiments N = 2k (k-1) + cp, where k is the number of factors and cp the number of center points;

2)   All levels of the factors must correspond to three levels (-1, 0, +1) at equal intervals.
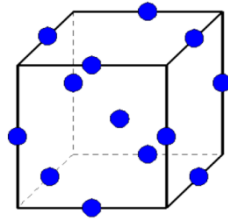
Figure 53: An example of Box-Behnken design for three factors (as in the case considered).

SECTION B

MIP AND RESVERATROL

4B.1 Materials

Chemicals and materials. The reagents used for the synthesis of the polymer molecularly imprinted were various

Acetonitrile HPLC-MS grade, Ethanol ACS-For Analysis Reag. Ph. Eur. Reag.USP, Methanol HPLC-MS, Acetone RS for HPLC, Acetic acid, Formic acid, Ethylene glycol dimethacrylate (EGDMA) and 4-vinylpyridine (4-VP) were obtained from Carlo Erba (Milan, Italy). HPLC grade purified water was obtained by using a Millipore Milli-Q water purification system (Millipore, Bedford, MA). (E)-resveratrol (98 %) purchased by Farmalabor (Canosa di Puglia (BT) Italy). Acetonitrile and Methanol employed for the HPLC-UV-MS analyses were of HPLC super gradient quality (Romil Ltd., Cambridge, UK). PBS phosphate buffer powder 0,1 M, free radical iniziator α,α´-azoisobutyronitrile (AIBN), Genistein (5,7-Dihydroxy-3-(4-hydroxyphenyl)chromen-4-one) synthetic 98% HPLC powder, Apigenin (5,7-Dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) 95% HPLC powder, Curcumin ((1E,6E)-1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) powder from Curcuma Longa (Turmeric), (S)-Ketoprofen ((S)-2-(3-Benzoylphenyl)propionic acid) 99% and Quercetin dihydrate 98% HPLC powder were obtained from Sigma-Aldrich. Syringe filter 0.45 μm PVDF (Alltech Italia Srl, I-20017 Passirana di Rho MI, Italy). Aglianico red wine, blended "red fruits" juices and lingonberry juice drink were collected at local markets.

4B.2 Standard solutions and samples preparation

A stock standard solution of 1 mg mL-1 of each compound was prepared in methanol and stored at 4°C in dark conditions. Working standard solutions were prepared by diluting the stock standard solutions with purified water or mobile phases.

Prior to extraction, real samples were filtered using PVDF 0.45 µm syringe filters. One mL of filtered samples was then diluted with 3 mL of HPLC water and 1 mL of pH 7 phosphate buffer (PBS) 0,1 mol L-1.

4B.3   Instrumentation

The high performance chromatographic system consists of an autosampler Accela Autosampler (Thermo Scientific), by a loop from 25 mL, from a compartment thermostatted for the column, by a pump Accela Pump (Thermo Scientific), an UV detector (Varian) Variable wavelength UV-VIS detector and a digital system of data collection and integration Clarity DataApex. For the analysis was used a HPLC column Luna 2.5 u C18 (2)-HST (100 x 2.0 mm) (Phenomenex). Upstream of the column was fitted with a pre-column Security Guard HPLC Guard Cartridge System (Phenomenex). The steel columns (20 x 2 mm) used for the evaluation of the selectivity of the materials (imprinting factor) containing the polymer and simple mold molecular were packaged using a pump Solvent Delivery System 9012 (Varian). For the heat curing is used is a thermoblock Grant QBT 1 Heating Block (Grant). For the experiments exploratory mining MISPE you used the apparatus SPE Vacuum Manifold, 12-port manifold (AHO-6023) (Phenomenex), 3 mL SPE cartridges made of polypropylene, polyethylene porous frits. For the operations of ultrasonication was used ultrasonicator LCD Series (Falc). To dry the polymer molecular mold was used the vacuum desiccator ČSN Simax. For the grinding of blocks of polymers are used is a ball mill. For the sieving has been used on vibrating screen machine Retsch AS 200 Control 'g', equipped with sieves Retsch Test Sieves.

4B.4   MIP preparation

In recent studies the MIP of (E)-resveratrol has been designed with the help of molecular modeling to determine the optimum amount of template to associate

with the functional monomer for the formation of the polymer (Schwarz et al., 2011). On the basis of these studies was prepared the MIP-resveratrol with the procedure of mold non-covalent (non-covalent imprinting) combining resveratrol with the functional monomer 4-vinylpyridine (4-VP) in a molar ratio of 1:3. The optimal conditions were predicted mediating the cited modeling studies and were allowed to select between various types of functional monomer as a candidate for the 4-VP and pred ire the molar ratio right in order to realize a complex of pre-polymerization stable (Fig. 54). The figure shows the hydrogen bonding that is to be established between resveratrol and three units of 4-VP.
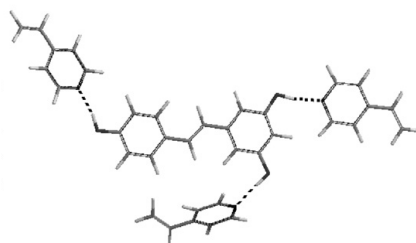
Figure 54: Molecular modeling of the complex between the (E)-resveratrol and three units of 4-VP shows the hydrogen bonding OH .... N (dashed lines)

The choice of the monomer agrees, furthermore, with the studies reported in the literature (Beltran et al., 2010) according to which it is preferred, usually, reacting a monomer with basic characteristics, such as the 4-VP, with a template with characteristics acid, such as resveratrol in my case. In the presence of the crosslinking agent was observed the formation of a rigid block copolymer shows that molecular recognition properties in respect of resveratrol, when compared with the copolymer not imprinted.

Resveratrol has been chosen as the best candidate for the preparation of a selective material that may in the future be used for the selective capture and separation of some polyphenols from any matrix. The (E)-resveratrol is a small molecule that has three rigid phenolic groups capable of establishing

hydrogen bonds and two aromatic rings capable of forming hydrophobic interactions and π-π stacking.

4B.4.1 Sythesis of (E)-resveratrol MIP

The MIP was prepared by bulk polymerization. The template (E)-resveratrol (1mmol, 228 mg) was dissolved in acetonitrile:ethanol (5 mL ,5:1 v/v) in a glass tube with screw cap. The functional monomer 4-vinylpyridine (0,322 mL, 3mmol) was added and the mixture was sparged with N2 gas and sonicated for 10 minutes. Then the cross-linker EGDMA (2.314 mL, 15 mmol) was added followed by the free radical iniziator AIBN (51 mg, 0,31 mmol) previously dissolved in acetonitrile:ethanol (1 mL,5:1 v/v). This pre-polymerisation mixture was sparged with N_2 gas for 5 min and then placed in a thermoblock at 50° C for 24 h and a further 24 h at 60° C. The same procedure without adding template was used to prepare NIP. The glass tubes were shaken and rotated periodically to ensure homogenous polymerization. The polymers were then removed from the glass tubes, crushed in a mortar and ground using a ball mill for 30 min speed 5.

Figure 55: Image of the block of NIP before undergoing the process of grinding in ball mill

The ground particles were subsequently sieved using sieve shaker (Retsch AS 200 control 'g') (amplitude 1.50 mm/'g', interval time 30 sec, time 10 min). In all experiments particles of 75-106 μm size were employed. The polymer was then sedimented to eliminate fines. The sedimentation was done in acetone by

repeated cycles of suspension, decanting the supernatant and fresh acetone adding to the precipitated particles. The (E)-resveratrol template was removed from the MIP by intense washings in methanol containing 10% acetic acid with gentle stirring. The washings were monitored by HPLC-UV at 306 nm and continued until the template could no longer be detected at 306 nm. Polymer particles were then washed with methanol to remove traces of acetic acid and dried in vacuum for 48h.

4B.5   Chromatographic evaluation of MIP rebinding capacity and Imprinting Factors (IF)

The molecular recognition properties of produced polymers were evaluated by using the polymers as HPLC stationary phases. MIP and NIP particles (75-106 μm) were packed into a stainless-steel column (20 mm x 2 mm i.d.) Direct-Connect™ Refillable Guard Column (Alltech Italia Srl, I-20017 Passirana di Rho MI, Italy) by slurry packing method using acetonitrile-water (8:2, v/v) at a costant pressure of 20 mPa. The amount of dry polymer packed was measured as 19.0 ± 0.8 mg.
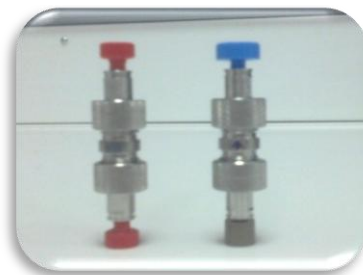
Figure 56: Image of MIP (red) and NIP (blu) column

4B.5.1 MIP and NIP column

In order to remove any trace of template bleeding the packed columns were washed with methanol at 100 μL/min until a stable baseline (306 nm) was obtained. After equilibration, 50 ng of selected model compounds standard solution were injected at the same flow rate. Each analysis was repeated three

times to evaluate repeatability. The experiments were repeated using different solvents and combination of solvents as mobile phase. Methanol was used to wash the column after each injection. Detection was performed at a suitable wavelength for each analyte. Column void volumes were measured by injecting 5 µL of acetone 1% v/v. All the measurements were performed at 30°C.

The capacity factors were calculated from the equation $k' = (t_r - t_0)/t_0$ where t_r and t_0 are the retention times of the analytes and acetone, respectively. The imprinting factor was then calculated as ratio of the MIP/NIP capacity factors.

4B.6   MISPE cartridges preparation

The polymer particles, 75-106 µm in diameter were suspended in methanol. MIP and NIP cartridges were prepared by packing 50 mg of the respective polymer particles into empty 3 mL SPE cartridge (Supelco, Bellefonte, PA, USA) and secured by polyethylene frits at the top and at the bottom. The extraction was performed using a 12 port vacuum manifold from Phenomenex. The eluent was forced to pass through the system by regulating the vacuum to obtain a flow rate of 1 mL/min and 15 KPa.

Before each experiment the cartridges were conditioned with 2 mL methanol and 2 mL acetonitrile. The SPE cartridges were then loaded with the sample. Triplicate cartridges were used for each extraction, to evaluate the repeatability of the extraction protocol. The cartridges were washed with 3mL acetonitrile. Elution was performed by passing 6 mL of a solution containing 10% acetic acid in methanol.

The eluate was then concentrated to 500 µL of water/ methanol and 5 µg of genistein was added as internal standard . Sample were analyzed by HPLC-ESI-MS when confirmation was necessary. Regeneration of the SPE columns was performed by washing the polymer with 2 mL methanol followed by 2

mL of a solution acetonitrile. All the experiments were carried out in triplicates.

4B.7 HPLC analysis

The quantification of phenolic compounds was performed using HPLC system consisting of Accela pump (Thermo Finnigan, San Jose, CA, USA) and Accela autosampler coupled to UV (Varian) Variable wavelength UV-VIS Detector . LC-ESI/MS confirmatory analysis were performed using a Agilent (Palo Alto, CA, USA) 1100 HPLC system coupled to an Applied Biosystems API2000 triple quadrupole instrument (Applied Biosystems, Foster City, CA, USA).

Separations were carried out with a Luna 2.5 u C18 (2)-HST reverse phase column (100 x 2.0 mm) equipped with Security Guard HPLC Guard Cartridge System (Phenomenex) using acetonitrile and water containing 0.1 % formic acid as mobile phase at flow rate 200 µL min -1. The injection volume was 25 µL. The initial percentage of B was 20%, and this was linearly increased to 90% over 15 min. Resveratrol and Quercetin was detected respectively at wavelengths 306 and 270 nm, and data acquisition was performer using Clarity DataApex software. The recoveries were determined by comparing the analyte/internal standard peak area ratios with those of calibration curve. Standards solutions for the calibration curve were prepared by dilution of the stock solution with concentration of 1 g L-1 in methanol in a solution of methanol:water 1:1.

The binding capacities of the MISPE cartridges were observed by recovery measurements at high concentrations.

4B.8 Experiments recovery from aqueous samples

Thought recovery tests is measured is the percentage recovery of resveratrol on the MIP, in order to evaluate efficiency and selectivity. It shall be arranged

recovery preliminary tests that take into account the effects of the volume of the sample, the concentration of the analyte and the effect of the washing solvent on the recovery of the analytes themselves. In this thesis, are some of the most relevant results.

It was extracted a sample of 5 mL of water containing 1 µg of resveratrol. The cartridges MIP and NIP have been regenerated after each experiment to submit them to other extractions.

After determining the calibration curve of resveratrol has been possible to calculate the recovery of the analyte. All calibration lines were determined by performing three replications for each point for all seven points. The recovery was calculated as the ratio between the response (peak area) of the analyte and the response (peak area) of the internal standard volumetric respect to the relationship between the response (peak area) of the analyte in the reference and the response (peak area) of the internal standard in the reference volume.

The statistical parameters related to the calibration curve are: LOD, which is the lower limit for which an analytical signal is statistically different from the values of the bottom and is equal to three times the signal/noise ratio (S/N); LOQ, that represents the minimum detectable concentration with an acceptable error and is equal to ten times the signal/noise. LOD and LOQ are: LOD: 0.15 ng; LOQ: 0.5 ng.

4B.9 Experiments recovery from real samples
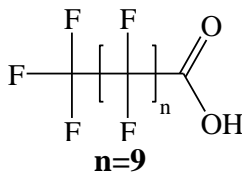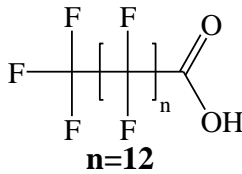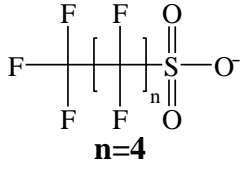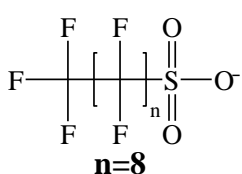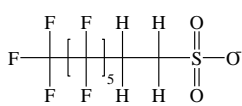
Prior to extraction, real samples were filtered using PVDF 0.45 µm syringe filters. One mL of filtered samples was then diluted with 3 mL of HPLC water and 1 mL of pH 7 phosphate buffer (PBS) 0,1 mol L^{-1}.

SECTION C
PGC AND PFAS

4C.1   Chemicals And Standards

Table 21:   List of PFAS standards

Name, acronym and formula	Parent Ion	Product ion	Structure
Perfluoropropionic acid **PFPeA/PFCAs 3** $C_3F_7CO_2^-$	**162.9**	**119.9**	(structure, n=3)
Perfluoropentanoic acid **PFPeA/PFCAs 5** $C_4F_9CO_2^-$	**262.9**	**218.8**	(structure, n=5)
Perfluorodecanoic acid **PFHxA/PFCAs 6** $C_5F_{11}CO_2^-$	**312.9**	**268.9** **118.9**	(structure, n=6)
Perfluoroheptanoic acid **PFHpA/PFCAs 7** $C_6F_{13}CO_2^-$	**362.9**	**318.8** **168.8**	(structure, n=7)
Perfluorooctanoic acid **PFOA/PFCAs 8** $C_7H_{15}CO_2^-$	**412.9**	**368.9** **168.9**	(structure, n=8)

Perfluorononanoic acid PFNA/PFCAs 9 $C_8H_{17}CO_2^-$	462.9	418.9 168.9	n=9
Perfluorododecanoic acid PFDoA/PFCAs 12 $C_{11}F_{23}CO_2^-$	612.9	568.9 168.9	n=12
Perfluorobutanesulfonic acid PFBS/PFSAs4 $C_4F_9SO_3^-$	298.9	79.9 98.9	n=4
Perfluorooctanesulfonic acid PFOS/PFSAs 8 $C_8H_{17}SO_3^-$	498.9	79.9 98.9	n=8
1H,1H,2H,2H- Perfluorooctanesulfonic acid (Internal standard) PFSAs 8 $C_8H_4F_{13}SO_3^-$	426.9	79.9 98.9	

The individual standard solutions of all analytes for fluorinated, were prepared by dissolving each compound in acetonitrile at a concentration of 1 mg/mL. Starting from the standard solutions were prepared for fluorinated two working solution not containing the IS

- Mix ST 0111 mg/mL

- Mix 10 ST 0.1 mg/mL

From these various mixtures were prepared:

- ST1: PFAS 0.050 mg/mL (ACN and water in equal volume)
- ST2: PFAS 2 mg/mL in ACN
- ST3: PFAS 4 ng/mL (90% water and 10% ACN: MeOH (80:20))
- ST4: PFAS 4 ng/mL (5% water and 95% ACN: MeOH (80:20))
- I.S.: 2.0 ng / mL in ACN

The standard solutions singular were stored at +4 ° C.

In the preparation of standards for the operations of extraction, analysis and HPLC were used:

• Formic acid RS-HPLC (Carlo Erba Reagents - Milan, IT);

• Toluene (Romil)

• Solution of ammonium hydroxide at 33% (Riedel-de Haen)

• Water Milli-Q (deionized water further purified);

• Methanol RS-HPLC (Romil well chemistry);

• Dichloromethane (Romil pure chemistry);

• Acetonitrile (ACN) RS-HPLC (Romil pure chemistry);

4C.2 Materials
• Waters 1525 Binary HPLC Pump

• Micromass 4 micro

• Knauer HPLC pump 64

4C.3 Optimizing detection conditions by ESI-MS
The detection of the analytes is carried out by mass spectrometry in tandem in negative mode. Via direct infusion of a mixture of standards of known concentration analytes, the source is optimized for the following parameters:

- the capillary voltage: 3.3 kV
- Extractor: 0 V

- RF Lens: 0.2 V

- Temperature of the source: 120 °C

- desolvation temperature: 350 °C

- cone gas flow: 80 L/h

- desolvation gas flow: 350 L/h

- pressure that collision gas (Argon): $3 \cdot 10^{-3}$ mbar.

4C.3.1 Selected Ion Recording (SIR) modality

The operation of a mass spectrometer in the selected ion mode Recording (SIR) allows the detection of specific analytes with greater sensitivity than the full scan mode. In SIR mode are focused, thanks to the first quadrupole, only certain ratios m/zWhen the instrument is properly configured and calibrated, the SIR mode the sensitivity can be increased by a factor of 10 to 100 compared to the Full Scan. The improvement of sensibilitàè due to the fact that is collected more ions of the analyte, for which, increases the intensitàdel signal and improves the signal/noise ratio.

The optimization of the parameters of the SIR mode is achieved by direct infusion (FIA-MS), using the mixture of standards of known concentration analytes and LAS. In Table 22 are reported, for each individual analyte, the optimized values related to the voltage of the cone and the dwell (e.g. the time of scanning of a single analyte).

Table 22: MS parameter in SIR modality optimized for each analyte

Analyte	m/z	tr (s)	Cone (V)	LDL (injectedng)	MDL (ng/L)
PFCAs 3	162,90	3,5	18,00	2,32	232
PFCAs 5	262,90	3,8	10,00	0,80	80
LAS C10	297,15	-	35,00	-	-
PFSAs 4	298,90	5,5	45,00	0,50	50

LAS C11	311,17	-	35,00	-	-
LAS C12	325,18	-	12,00	-	-
LAS C13	339,20	-	30,00	-	-
LAS C14	353,22	-	30,00	-	-
PFCAs 7	362.90	6,1	13,00	1,10	110
PFCAs 8	412,90	7,3	14,00	2,77	277
S.I.	427,00	6,7	35,00	1,20	120
PFCAs 9	462,90	8,11 8,4	14,00	1,73	173
PFSAs 8	498,90	9,5 10,1	40,00	0,12	12
PFCAs 12	613,00	11,2	18,00	0,78	78

4C.3.2 MS-MS modality

The operation of a mass spectrometer in mode Multiple Reaction Monitoring (MRM) allows to improve the selectivity (as it is possible to distinguish substances that have the same mass / charge ratio and the same retention time) and increased sensitivity (increased signal / noise ratio because it reduces the background noise) than the SIR mode.

In a similar way as done for the SIR mode, the parameters have been optimized for each individual analyte introduced by direct infusion (FIA-MS), relatively to the voltage of the cone, the energy of collision and to dwell for the MS-mode MS reported in Table 23.

Table 23: MS-MS parameter optimized for each analyte

Analyte	m/z parent ion	m/z fragment	Cone (V)	Collision energy (eV)	LOD (ng injected)	MDL (ng/L)

PFCAs 3	162,90	119,00	18,00	9,00	0,084	8,4
PFCAs 5	262,90	219,00	10,00	9,00	0,033	3,3
PFSAs 4	298.90	80,00	45,00	29,00	0,033	3,3
PFSAs 4	298.90	99,00	45,00	27,00	0,080	8,0
PFCAs 7	362.90	169,00	13,00	17,00	0,127	12,7
PFCAs 7	362.90	319,00	13,00	9,00	0,273	27,3
PFCAs 8	412,90	169,00	14,00	18,00	0,203	20,3
PFCAs 8	412,90	369,00	14,00	10,00	0,166	16,6
S.I.	427,00	80,00	35,00	35,00	0,136	13,6
PFCAs 9	462,90	219,00	14,00	17,00	0,375	37,5
PFCAs 9	462,90	419,00	14,00	10,00	0,110	11,0
PFSAs 8	498,90	80,00	40,00	50,00	0,020	2,0
PFSAs 8	498,90	99,00	40,00	40,00	0,044	4,4
PFCAs 12	613,00	569,00	18,00	12,00	0,375	37,5

4C.4   Simulated extractions

To achieve the extraction of the analytes is positioned the six-way valve in position "Load" and it operates in the following way:

• For the reference are injected 5.0 mL of a mixture of perfluorinated, is passed through the SPE cartridge, 1.0 mL of Milli-Q water by means of the auxiliary pump at a flow rate of 1.0 mL / min. At this point we inject 5.0 mL of internal standard and falsely as other 9.0 mL of Milli-Q water by means of the auxiliary pump at a flow rate of 1.0 mL / min. The total volume of extract is 10.0 mL.

• For the extraction of 50.0 mL of sample are injected 5.0 mL of a mixture of perfluorinated and pretend to be, through the SPE cartridge, 41.0 mL of Milli-Q water by means of the auxiliary pump at a flow rate of 1.0 mL/min. At this point we inject 5.0 mL of internal standard and falsely as other 9.0 mL of Milli-Q water by means of the auxiliary pump at a flow rate of 1.0 mL/min.

• For the extraction of 100 mL of sample are injected 5.0 mL of a mixture of perfluorinated, are made to pass through the SPE cartridge, 91.0 ml of Milli-Q water by means of the auxiliary pump at a flow rate of 1, 0 mL/min. At this point we inject 5.0 mL of internal standard and falsely as other 9.0 ml of Milli-Q water by means of the auxiliary pump at a flow rate of 1.0 mL/min.

After extraction you change the position of the valve in "inject" to connect the pre-column to the analytical system LC-MS.

The elution is carried out in gradient with a flow rate of 0.2 mL/min using as mobile phases:

A) 190 mM NH_4OH aqueous phase

B) ACN/MeOH (80:20) 190 mM NH_4OH

In Table 24 shows the chromatographic program used.

Table 24: Chromatographic program. Flow 0.20 mL / min. Linear gradient.

N° step	Time (min)	% B
0	0,00	10,0
1	15,00	95,0
2	30,00	95,0
3	31,00	10,0
4	40,00	10,0

At the end of the analytical step, the precolumn is disconnected from the analytical system by returning the six-way valve in position "Load".

4C.5   Analysis of real sample

After positioning the six-way valve in position "Load", it operates in the following manner: by means of the second auxiliary pump you send the sample in the pre-column and elute make 100.0 ml of sample at a flow rate of 1.0 mL / min. After extraction you change the position of the valve in "inject" to connect the pre-column to the analytical system LC-MS. The mobile phase, the program chromatographic and mass parameters are the same as previously used for the extraction simulated.

4C.6   Experiments recovery from real samples

For the analysis, after having conditioned the second auxiliary pump disconnected from the extraction system by passing 200.0 mL of sample, it positions the six-way valve in position "Load" and it operates in the following manner: by means of the second auxiliary pump sending the sample through the SPE cartridge and falsely as 91.0 mL of sample at a flow rate of 1.0 mL / min. At this point we inject 5.0 mL of internal standard (IS) and falsely as other 9.0 ml of the sample by the second auxiliary pump at a flow rate of 1.0 mL/min. The total volume extracted is of 100.0 mL. After extraction you change the position of the valve in "inject" to connect the pre-column to the analytical system LC-MS. The mobile phase, the program chromatographic and mass parameters are the same as previously used for the extraction simulated.

4C.7 Preparation column microbore

The following describes the procedure used to obtain the column microbore.

A tube made of peek of length 5.0 cm and diameter of 0.75 mm is connected at one end to a metal frit (output column), which presents a strainer metal on which rests the stationary phase and prevents it from escaping, and the other a frit peek (input column).

The stationary phase (PGC), suspended in acetone, is inserted into a steel tube (internal diameter 4.6 mm) that serves as a reservoir and which is connected to the input of the column.

With a HPLC pump, which allows to pump within the column a mobile phase of water / methane (50:50), is pushed the stationary phase within the column. The mobile phase is pumped until it reaches a pressure of 250 atm. Finally, disconnect the columninlet from the tank.

The column microbore is connected to the input, passing through the injector, the HPLC pump and the exit to the mass.

Figure 57: Microbore column

LIST OF ABBREVIATIONS

AA	Acrylic acid
ACN	Acetonitrile
AIBN	α,α´-azoisobutyronitrile
AM	Acrylamide
APCI	Atmospheric Pressure Chemical Ionisation
API	Atmospheric Pressure Ionisation
APPI	Atmospheric Pressure Photo-Ionisation
CI	Chemical Ionisation
DCM	Dichloromethane
DEA	Diethylamine
DMSO	Dimethyl Sulfoxide
DOE	Experimental Design
EGDMA	Ethylene Glycol Dimethacrylate
EI	Electronic Impact
EPA	Environmental Protection Agency
ESI	Electrospray Ionisation
FAB	Fast Atom Bombardament
GCB	Graphitized Carbon Black
HPLC	High Performance Liquid Chromatography
IF	Imprinting Factor
IPA	2-Propanol
IS	Internal Standard
LC	Liquid Chromatography
LLE	Liquid-liquid Extraction
LOD	Limit Of Detection
LOQ	Limit Of Quantification
m/z	Mass-To-Charge Ratio
MAA	Methacrylic Acid
MALDI	Matrix Assisted Laser Desorption Ionisation
MeOH	Methanol

MIP	Molecularly Imprinted Polymer
MISPE	Molecularly Imprinted Solid Phase Extraction
MMA	Methyl Methacrylate
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
NCI	Negative Chemical Ionisation
NIP	Non Imprinted Polymer
NOAEL	No-Observed Adverse- Effect
OECD	Organisation For Economic Co-Operation And Development
PBS	Phosphate Buffer Solution
PFAS	Perfluoroalkylated Substance
PFC	Polyfluorinated Compound
PFOA	Perfluorooctanoic Acid
PFOS	Perfluorooctanesulfonic Acid
PGC	Porous Graphitic Carbon
PHWE	Pressurized Hot Water Extraction
PHW	Pressurized Hot Water
PLE	Pressurized Liquid Extraction
PREG	Polar Retention Effect On Graphite
PS-DVB	Polystyrene-Divinylbenzene
PTFE	Polytetrafluoroethylene
Q	Quadrupole
RP	Reverse Phase
RSM	Response Surface Methodology
SFE	Supercritical Fluid Extraction
SIR	Selected Ion Recording
SPE	Solid Phase Extraction
SRM	Selected Reaction Monitoring
SWE	Subcritical-Water Extraction
TDI	Trophic Diatom Index

TFA	Trifluoroacetic Acid
THF	Tetrahydrofuran
TOF	Time-Of-Flight
UPLC	Ultra Performance Liquid Chromatography
UV	Ultraviolet
2-VP	2-Vinylpyridine
4-VP	4-Vinylpyridine

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