UNIVERSITY OF CALABRIA

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Methodologies for the development of molecules of pharmacological interest

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Thesis

Integrated membrane operations for the recovery of bioactive compounds from juice and by-products of the *Citrus fruits* production

SSD CHIM/06 Organic chemistry

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Introduction

Foods characterised by protective and health-promoting potential, in addition to their nutritive value, are recognised as *functional foods*. The beneficial components in functional foods have been called by various terms such as phytochemicals, functional components and bioactive compounds. These components in fruit and vegetables have been receiving increased interest from consumers and researchers for their beneficial effects on human health. Epidemiological studies have consistently demonstrated that there is a clear significant positive association between the intake of fruit and vegetables and the reduced rate of heart disease mortality, common cancers and other degenerative diseases as well as ageing. The protection that fruit and vegetables provide against these diseases has been attributed to various bioactive compounds. In particular, most of the antioxidant capacity of fruit and vegetables can be attributed to polyphenolic compounds (such as flavonols, flavanols, anthocyanins and phenylpropanoids), other than vitamin C, vitamin E and β-carotene, which act as antioxidants or as agents of other mechanisms contributing to anticarcinogenic or cardioprotective action.

The world market of fruit and vegetables has remarkably increased in recent years and consumers have addressed their interest towards products characterised by:

- quality (maintenance or improvement of flavour, colour, texture);
- safety (microbiologically and chemically, materials and methodologies used during processing and preservation must not introduce hazardous compounds);
- formulation (new products starting from new and enabling raw material and processing);
- stability ("fresh food" with medium long term shelf-life).

Consequently, the food industry has focused on the development of processed items with increased shelf-life able to retain the peculiarity of fresh fruit as well as colour, aroma, nutritional value and structural characteristics as much as possible.

On the other hand, during the industrial transformation, a large part of the characteristics determining the quality of the fresh product undergoes a remarkable modification: the thermal damage and the chemical oxidation degrade the most sensitive components reducing the quality of fresh fruits.

In order to overcome these problems and to better preserve the properties of fresh fruits, several new “mild” technological processes have been proposed in the last years.

Within the agro-food industry, membrane technologies can work as well or better than the existing technology regarding product quality, energy consumption and environmental issues. The use of membrane separation, clarification, purification and concentration processes represents one of the most powerful tools for the agro-food industry to introduce innovative processes in order to pursue targets such as process intensification and reduction of production costs. In addition, membrane operations represent a valid alternative to thermal evaporation processes which cause the deterioration of heat sensitive compounds leading to a remarkable qualitative decline of the final product. On the other hand, current filtration of a wide variety of juices is performed by using fining agents such as gelatine, diatomaceous earth, bentonite and silica sol which cause problems of environmental impact due to their disposal.

Juice clarification, stabilisation, depectinization and concentration are typical steps where membrane processes such as microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and osmotic distillation (OD) have been successfully utilised and are today very efficient systems to preserve the nutritional and organoleptic properties of the fresh product owing to the possibility of operating at room temperature with low energy consumption and without chemical additives.

The aim of this work was to study the effect of different membrane processes in the separation, recovery and concentration of bioactive compounds in the juice of different varieties of Citrus fruit.

The possibility to obtain concentrated fractions enriched of antioxidant compounds from the by-products of the industrial citrus processing (such as press liquors coming from red orange peel) was also evaluated.

In the first chapter, a general introduction on membrane science and technologies, including their advantages over traditional separation processes, is reported. A description of the chemical composition of Citrus fruits and their bioactive compounds which are of interest for nutriaceutical applications, is reported in Chapter 2. An integrated membrane process for the production of bergamot juice with high nutritional and organoleptic properties is discussed in Chapter 3. The influence of the molecular weight cut-off of different ultrafiltration and nanofiltration membranes on the recovery

of polyphenols from bergamot juice is analysed in Chapter 4. Similarly, the possibility to recover flavonoids and anthocyanins from press liquors coming from pigmented oranges was investigated by using different membrane operations. Results concerning this study are discussed in Chapter 5. Finally, Chapter 6 discusses the results obtained in the clarification of mandarin juice by using hollow fibre membranes prepared in laboratory via the dry–wet spinning technique through the phase inversion process.

Some recovered fractions from Citrus juices and by-products of the citrus juice processing through the investigated membrane processes represent an ideal substrate for the formulation of new products with improved characteristics for food, pharmaceutical and nutriaceutical applications.

CHAPTER 1

MEMBRANE SEPARATION PROCESSES

1.1 Introduction

Membranes and membrane processes are not a recent invention. They are part of our daily life and play an essential role in nature but also in the modern industrial society.

The separation, concentration, and purification of molecular mixtures are major problems in the chemical industries. Efficient separation processes are also needed to obtain high-grade products in the food and pharmaceutical industries to supply communities and industry with high quality water and to remove or recover toxic or valuable components from industrial effluents. For this task a multitude of separation techniques such as distillation, precipitation, crystallization, extraction, adsorption, and ion-exchange are used today. More recently, these conventional separation methods have been supplemented with a family of processes utilizing semi-permeable membranes as separation barriers.

Membranes and membrane processes were first introduced as an analytical tool in chemical and biomedical laboratories; they developed very rapidly into industrial products and methods with significant technical and commercial impact.

The basic properties of membrane operations make them ideal for industrial production: they are generally athermal and do not involve phase changes or chemical additives; they are simple in concept and operation, modular and easy to scale-up; furthermore, they are characterized by a low energy consumption permitting a rational utilization of raw materials and recovery and reuse of by-products. Membrane technologies respond efficiently to the requirements of the so-called "*process intensification*", since they permit drastic improvements in manufacturing and processing, substantially decreasing the equipment-size/production-capacity ratio, the energy consumption, and/or the waste production and resulting in cheaper, sustainable technical solutions. The membranes used in various applications differ widely in their structure, in their function and in the

way they are operated. However, all membranes have several features in common that make them particularly attractive tools for the separation of molecular mixtures. This is mandatory for applications in artificial organs and in many drug delivery systems as well as in the food and drug industry or in downstream processing of bio-products where thermo-sensitive substances must often be handled [1,2].

1.2 Membranes: definition, preparation and properties

A *membrane* can be defined as a selective or non-selective barrier that separates and/or contacts two adjacent phases and promotes the exchange of matter, energy, and information between the phases in a specific or non specific manner. The separation of a mixture in a membrane process is the result of different transport rates of different components through the membrane. The function of a membrane in a separation process is determined by its transport properties for different components in a mixture. The transport rate of a component through a membrane is determined by its *permeability* in the membrane and by the *driving force*. Driving forces in membrane processes are gradients in the chemical and electrical potential, and in the hydrostatic pressure, resulting in a diffusion of individual molecules, a migration of ions, and a convection of mass, respectively.

The permeability of a certain component in a membrane is determined by its concentration and its mobility in the membrane structure. In a homogeneous polymer matrix, the concentration of a component in a membrane is determined by its solubility in the polymer. In a porous structure, the concentration of a component in the membrane is determined by its size and by the pore size of the structure. The mass transport through membranes can be described by various mathematical relations. Most of these are semi-empirical, postulating membrane models, such as Fick's law, Hagen-Poisseuille's law and Ohm's law. A more comprehensive description suitable for each membrane, which is independent of the membrane structure, is based on a phenomenological equation that connects the fluxes of the electrical charges, volume, that is, viscous flow, and individual components with the corresponding driving forces by a linear relation:

$$J_i = \sum_k L_{ik} * X_k \qquad (1.2.1)$$

Here J is a flux per unit area and X is a generalized driving force; the subscripts *i* and *k* refer to individual components, volume, and electrical charges; and L is a phenomenological coefficient relating the fluxes to the driving forces [3].

The molecular mixture which will be separated is referred to as *feed*, the mixture containing the components retained by the membranes is called *retentate* and the mixture composed of the components permeating the membrane is referred to as *permeate*.

Membranes can be classified according to different parameters as the materials (polymers, ceramics, glass, liquid), the structure (symmetric, asymmetric), and the configuration (flat-sheet, spiral wound, tubular, capillary, hollow fiber).

Based on their structure they can be classified in four groups:

1) porous membranes;
2) homogenous solid membranes;
3) solid membranes carrying electrical charges;
4) liquid or solid films containing selective carriers.

Figure 1.2-1 illustrates the morphology, materials and configuration of some technically relevant synthetic membranes [2].

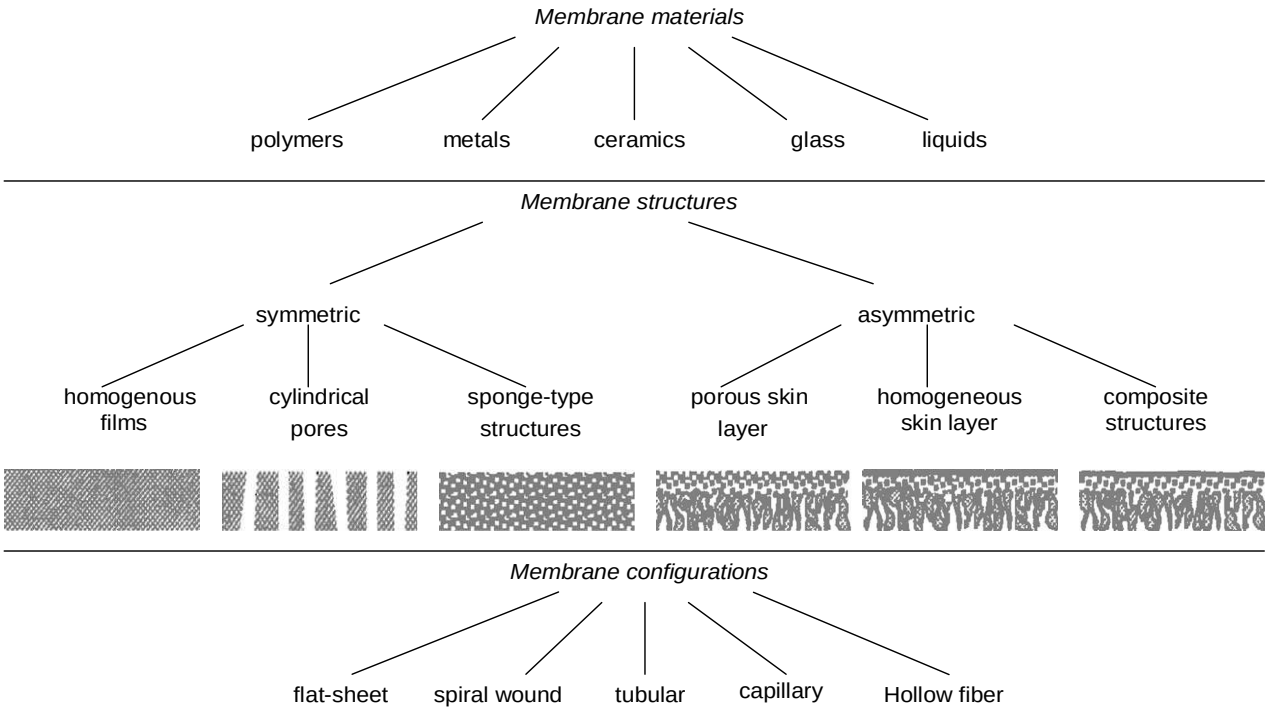

Figure 1.2-1 *Schematic representation of the various materials, structures and configurations of technically relevant synthetic membranes*

All kinds of different synthetic materials can be used for preparing membranes. Thus the material can be either inorganic, such as ceramic, glass or metal, or organic including all types of polymers. The basic principle involved is to modify the material in such a way by means of an appropriate technique so as to obtain a membrane structure with a morphology suitable for a specific separation. A number of different techniques is available to prepare synthetic membranes. Some of these techniques can be used to prepare organic (polymeric) as well as inorganic membranes. The most important techniques are sintering, stretching, track-etching, phase inversion and coating.

Sintering is a rather simple technique to obtain porous structures from organic as well as from inorganic materials. The method involves pressing a powder consisting of particles of a given size and sintering it at elevated temperature (Figure1.2-2). The required temperatures depend on the material used. The process yields a porous structure of relatively low porosity in the range of 10 to 40 % and a rather irregular porous structure with a very wide pore size distribution. The material selection for the preparation of sintered membranes is determined mainly by the required mechanical properties and the chemical and thermal stability of the material in the application of the final membrane. The particle size of the powder is the main parameter determining the pore size of the final membrane, typically ranging from 0.2 to 20 µm. The lower limit of the pore diameter is determined by the particle size of the powder [4].

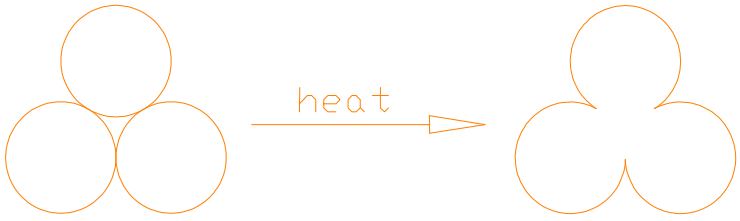

Figure 1.2-2 *Sintering process*

Another relatively simple procedure for preparing porous membranes is the *stretching* technique. In this method an extruded film made from a partially crystalline polymeric

material is stretched perpendicularly to the direction of the extrusion, so that the crystalline regions are located tangentially to the extrusion direction. When a mechanical stress is applied, a small rupture occurs and a porous structure is obtained with pore sizes of about 0.1-20 µm. Only crystalline polymeric materials can be used for this technique. The porosity of these membranes is much higher than one obtained by sintering, and values up to 90% can be obtained.

Porous membranes with very uniform, nearly perfectly round cylindrical pores are obtained by a process referred to as *track-etching* (Figure 1.2-3). The membrane is realised in a two-step process. A film or foil (polycarbonate) is subject to high energy particles applied perpendicularly to the film. The particles damage the polymeric matrix and create tracks. The film is then immersed in an acid (or alcaline) bath and the polymeric materials are etched away along the tracks to form uniform cylindrical pores with narrow pore size distribution. Pore sizes from 0.2 to10 µm are obtained, but the porosity is low (about 10%).

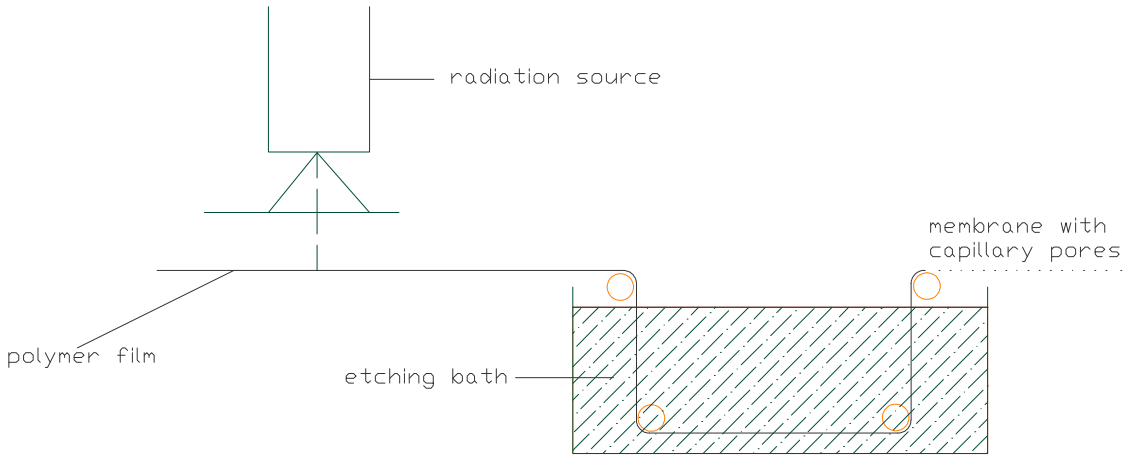

Figure 1.2-3 *Track-etching method*

The choice of the material depends mainly on the thickness of the film available and on the energy of the particles applied (usually about 1 MeV). The maximum penetration thickness of particles with this energy is about 20 µm. When the energy of the particles is increased, the film thickness can also be increased and even the inorganic materials

can be used. The porosity is mainly determined by the radiation time whereas the pore diameter is determined by the etching time.

The majority of polymeric membranes can be produced by a method known as *phase inversion.* This is a process in which a polymer is transformed, in a controlled method, from a liquid to a solid state. It is used particularly for the preparation of asymmetric membranes, characterised by a non uniform structure comprising an active top layer or skin supported by a porous support or sub-layer [5].

The concept of a phase inversion covers a range of different techniques such as solvent evaporation, precipitation by controlled evaporation, thermal precipitation, thermal precipitation from the vapour phases and immersion precipitation. The majority of phase inversion membranes is prepared by immersion precipitation.

In this technique a polymeric solution is cast on a suitable support and immersed in a coagulant bath containing a non solvent. Precipitation occurs because of the exchange of solvent and non-solvent. The membrane structure ultimately obtained results from a combination of mass transfer and phase separation.

Phase inversion membranes can be made from almost any polymer, which is soluble in an appropriate solvent and can be precipitated in a non solvent.

By varying the polymer, the polymer concentration, the precipitation medium, and the precipitation temperature, porous phase inversion membranes can be made with a very large variety of pore sizes and with varying chemical and mechanical properties.

1.3 Configuration of membrane modules

Membranes are usually packed in small units called membrane modules. They are quite different in design, mode of operation and productive costs.

The two important aspects of a membrane module are the material of the membrane and its configuration In particular, membrane modules are available in five basic designs: hollow fiber, spiral wound, tubular, plate and frame and capillary.

The choice of the module configuration, as well as the arrangement of modules in a system, is based on economic considerations, the type of separation, ease of cleaning, maintenance and operation.

Spiral wound modules consist of a sandwich of flat sheet membranes, spacers and porous permeate flow material wrapped around a central permeate collecting tube. Feed

solution passes axially along the sandwich in the channels formed by the spacers through the membrane to the feed solution, that is radially forwarded towards the central collecting tube.

In general two or more modules are fitted in series and suitably sealed into a pressure housing; the feed solution is introduced at one end and the retentate is collected at the other one (Figure 1.3-1).

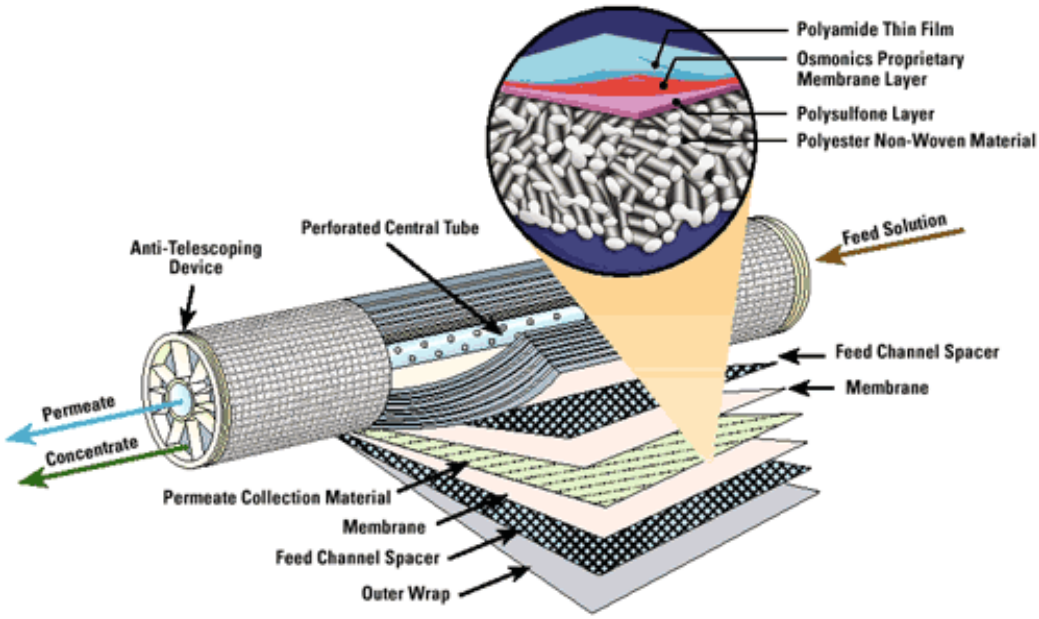

Figure 1.3-1 *Schematic representation of a spiral-wound membrane module*

Commercial spiral wound modules are about 1 meter long and have a diameter of 10 to 60 cm. They provide a relatively large membrane area per unit volume, they cannot be mechanically cleaned and require a pretreatment procedure of feed solution.

The *capillary membrane* modules consist of a large number of capillary membranes with an inner diameter of 0.2 to 3 mm arranged in a parallel bundle in a shell tube as shown in Figure 1.3-2. The feed solution is forwarded into the lumen of capillary membranes and the filtrate, which permeates the capillary wall, is collected in the shell tube. In the *hollow fiber membranes* the selective layer is on the outside of the fibers which are installed as a bundle of different fibers in a half loop with the free ends potted with an epoxy resin in a pressure tube. The filtrate passes through the fiber walls and flows up the bore to the open end of the fibers at the epoxy head. The main

disadvantage of the hollow fibers membrane module is the difficult control of polarization concentration and fouling. However this phenomenon can be controlled by feed pretreatment to remove particles, macromolecules or other materials that may be precipitated at the membrane surface.

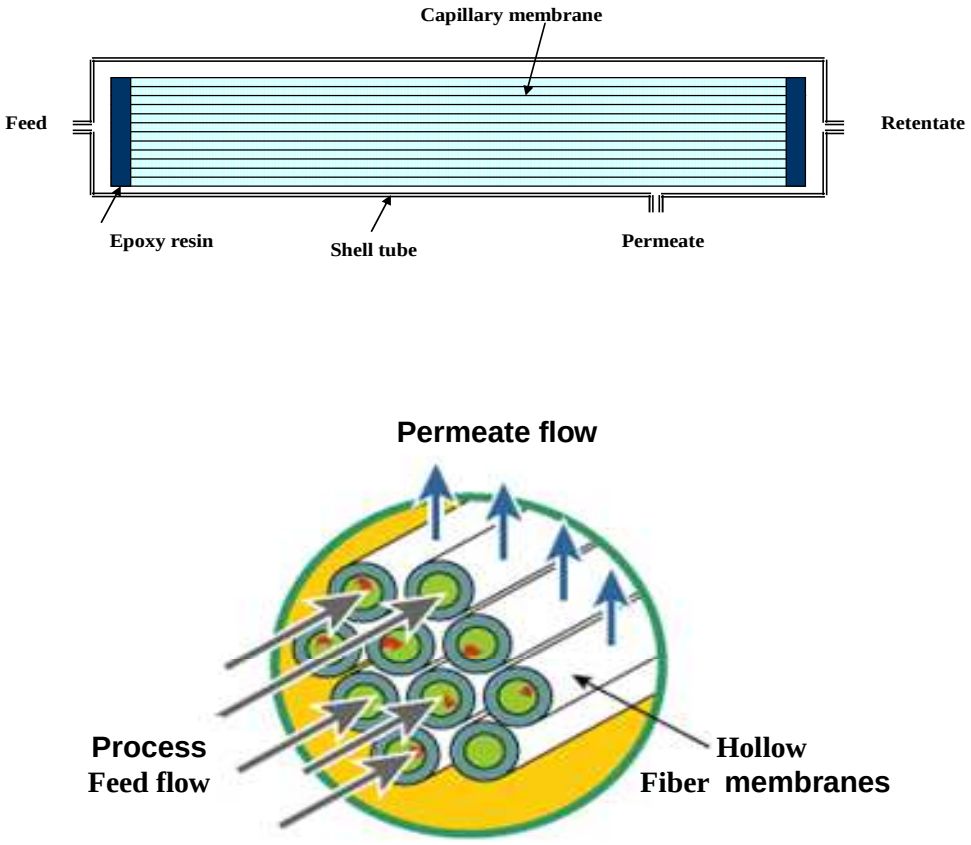

Figure 1.3-2 *Schematic representation of the capillary/hollow fiber module*

In the *tubular module* membranes are located according to the "shell and tube" geometry. The basic concept of a tubular module is a straight membrane tube surrounded by a porous support layer and a support tube. Feed flows internally along the tube and the permeate passes through the membrane into the porous support layer and through suitable pores in the support tube. Tube diameters are in the range of 1.2 to 2.4 cm and a number of tubes are placed in one pressure housing to increase module productivity. Main applications of tubular membranes are with feeds which cannot be

pretreated to remove potential foulants and when very hygienic conditions are required. The relatively large tube diameters permit the in situ mechanical cleaning method. Main applications are in ultrafiltration and microfiltration. In Figure 1.3-3 a schematic representation of a tubular membrane module is reported.

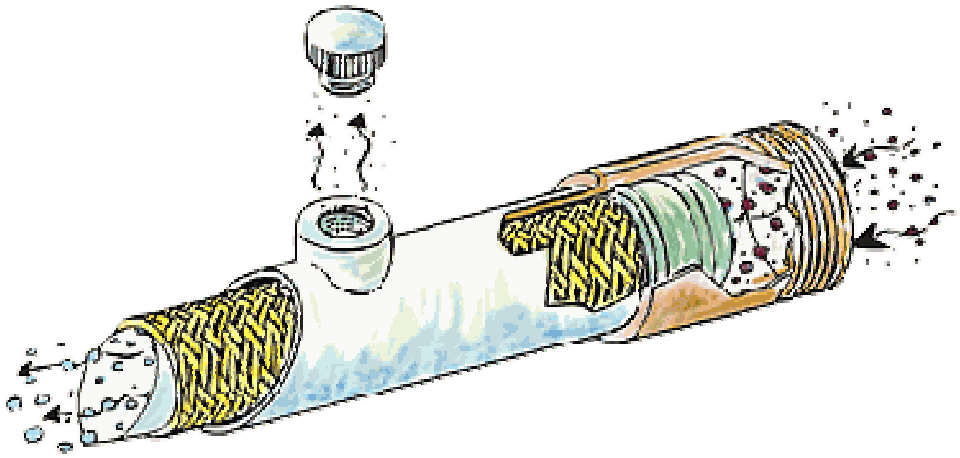

Figure 1.3-3 *Schematic representation of tubular membrane module*

Another module type is the *plate-and-frame.* Its design has its origin in the conventional filter press concept. The membranes, porous membrane support plates, and spacers forming the feed flow channel are clamped together and stacked between two endplates and placed in a housing. The feed solution is pressurized in the housing and forced across the surface of the membrane (Figure 1.3-4).

The permeate leaves the module through the permeate channel and it is collected in a central tube. Plate and frame units are mainly used in small-scale applications such as in the production of pharmaceuticals, bio-products or fine chemicals. The selection of the membrane module is mainly determined by economic considerations. This does not mean that the cheapest configuration is always the best choice because the type of application is also very important.

In Table 1.3-1 the characteristics of all the modules which must be considered in a system design, including packing density, investment cost, fouling tendency and ease of cleaning, are reported.

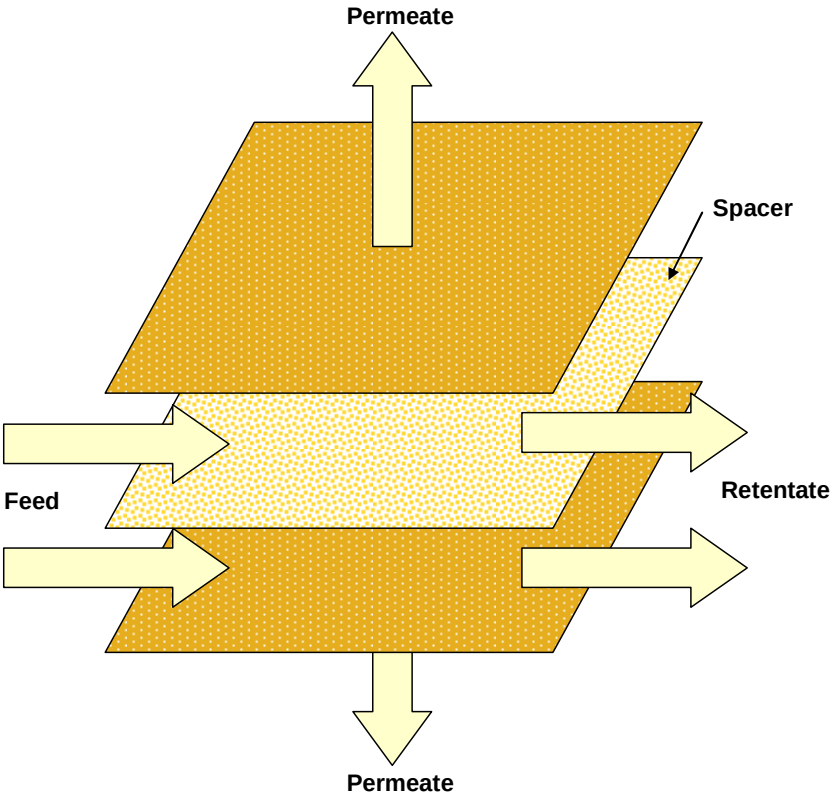

Figure 1.3-4 *Schematic representation of a plate and frame module*

Table 1.3-1 *Qualitative comparison of various membrane configurations*

Type of module	Membrane area per unit volume (m^2/m^3)	Membrane cost	Control of concentration polarization	Ease of cleaning	Packing density	Range of flux (l/m^2h)
Tubular	20-100	very high	very good	good	Low	20-100
Capillary	600-1200	low	very good	poor	high	20-50
Hollow fiber	2000-5000	very low	very poor	very poor	very high	-
Spiral wound	800-1200	low	good	medium	Medium	10-50
Plate and frame	400-800	medium	good	good	Low	20-100

The cost of the various modules varies appreciably; for instance the tubular module is the most expensive per installed membrane area. However it is suited to applications with a “high fouling tendency” because of its ease of operation and membrane cleaning. In contrast, the hollow fiber modules are very susceptible to fouling and are difficult to clean. Pretreatment of the feed stream is therefore an important factor in hollow fiber membranes.

Generally specific modules will generally tend to find their own field of application, although it is often possible to choose between two or more different types in specific applications [6].

1.4 Membrane processes

Membrane separation processes can differ greatly with regard to membranes, driving forces, areas of application, and industrial or economical relevance. Membrane processes can be classified according to the driving force applied to achieve the transport of specific components through the membrane into:

- hydrostatic pressure-driven processes such as reverse osmosis, nanofitration, ultrafiltration, microfiltration;
- concentration gradient or chemical potential driven processes such as dialysis, pervaporation and membrane contactors (membrane based solvent extraction, osmotic distillation);
- electrical potential driven processes such as electrodyalisis, electrofiltration;
- temperature-driven membrane processes such as membrane distillation.

Table 1.4-1 summarises the most important membrane separation processes.

The mechanism by which certain components are transported through a membrane can also be very different. In some membranes, for example, the transport is based on the viscous flow of a mixture through individual pores in the membrane, caused by hydrostatic pressure differences between the two phases separated by the membrane.

Components that permeate through the membrane are transported by convective flow through micropores under a gradient pressure as driving force and the separation occurs because of size exclusion.

If the transport is based on the solubility and diffusion of individual molecules in the non-porous membrane matrix, due to a concentration or chemical potential gradient, the

transport is referred to as *diffusion***.** The separation is possible because of different solubility and diffusivity of components into the membrane material [7].

Table 1.4-1 *Basic properties of membrane operations*

Membrane separation	Membrane type	Driving force	Mode of transport	Applications
Microfiltration	Symmetric macroporous	Hydrostatic pressure	Size exclusion convention	Clarification, sterile filtration
Ultrafiltration	Asymmetric microporous	Hydrostatic pressure	Size exclusion convention	Separations of macromolecular solutions
Nanofiltration	Asymmetric	Hydrostatic pressure	Size exclusion solution diffusion Donnan exclusion	Separation of small organic compounds and selected salts from solution
Reverse osmosis	Asymmetric, Skin-Type	Hydrostatic pressure	Solution diffusion mechanism	Separation of micro-solutes and salts from solutions
Dialysis	Symmetric microporous	Concentration gradient	Diffusion	Separation of micro-solutes and salts from macromolecular solutions
Gas separation	Asymmetric, composite, homogenous or porous polymer	Concentration gradient, hydrostatic pressure	Solution diffusion mechanism	Separation of gas mixtures
Pervaporation	Asymmetric, composite non porous	Concentration gradient, vapour pressure	Solution diffusion mechanism	Separation of mixtures of volatile liquids
Supported liquid membranes	Microporous	Concentration gradient	Diffusion	Separation, concentration
Membrane distillation	Microporous	Temperature gradient	Diffusion	Concentration

1.4-1 Microfiltration

The term microfiltration (MF) is used when particles with diameters of 0.1-10 μm are separated from a solvent or other low molecular components. The separation mechanism is based on a sieving effect and particles are separated according to their

dimensions. Membranes used for MF have pores of 0.1 to 10 μm in diameter. The hydrostatic pressure differences used are in the range of 0.05-0.2 MPa. The most used polymers for MF membranes are the hydrophobic polyviny1idenfluoride (PVDF), polypropylene (PP), polyethylene (PE), and the hydrophilic materials cellulose esters, polycarbonate (PC), polysulfone/polyethersulfone (PSf/PES), polyimide/polyetherimide and polyetheretherketone (PEEK). The ceramic membranes, which can be used both in micro- and ultrafiltration processes, have superior chemical, thermal, and mechanical stability compared to polymeric membranes, and the pore size can be more easily controlled.

MF membranes are prepared by sintering, track-etching, stretching, or phase inversion techniques. Module configurations include hollow fiber, tubular, plate and frame, spiral wound. The two standard modes of operation are dead-end and cross-flow configurations (Figure 1.4.1-1). In the dead-end method, the feed flow is perpendicular to the membrane surface. It is forced through the membrane, which causes the retained particles to accumulate and form a type of cake layer at the membrane surface. The thickness of the cake increases with the filtration time. The permeation rate decreases, therefore, with increased layer thickness.

In the cross-flow mode, the fluid to be filtered flows tangentially to the membrane surface and permeates through the membrane due to a pressure difference. The cross-flow reduces the formation of the filter cake to keep it at a low level.

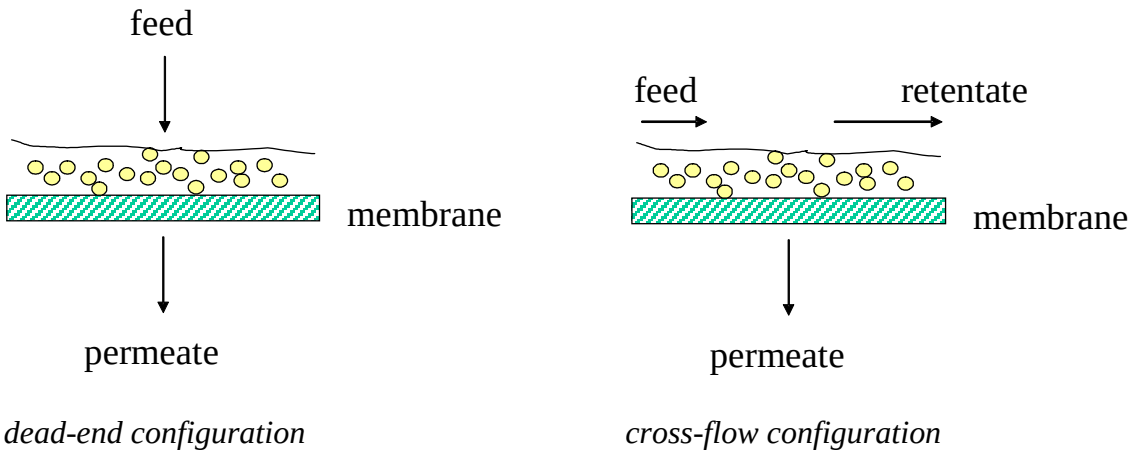

Figure 1.4.1-1 *Comparison between dead-end and cross-flow configurations*

MF is employed in both production and analytical applications. The technologically important applications are summarised as:

- removal of particles from liquid and gas streams coming from chemical, biological and food industry ;
- clarification and sterile filtration of heat sensitive solutions and beverages;
- production of pure water in the electronic industries;
- product purification, gas filtration, process solvent recovery in the chemical industries;
- waste water treatment.

In the food industry the use of MF for the retention of cellular components, microorganisms and other solids from alcoholic or non-alcoholic beverage, as well as clarification and concentration, is widely employed; different solutions can be processed including milk, beer, wine, whiskies, potable water, syrups, edible oils and vinegar.

The cold sterilisation of milk by MF is used to produce fresh milk with medium term conservation. In fact, this methodology allows to remove microorganisms while preserving the organoleptic properties of the milk components since the process is carried out at lower temperature compared to the one used in pasteurisation.

Clarification of apple juice by MF has been practised for several years as a means of producing clean and sterile beverages [8].

In the pharmaceutical industry an important application of MF is virus and bacteria removal prior to final formulation of many products and in the clarification of fermentation broths to remove the suspended cell mass and other particles. In addition, MF membranes are used as passive substrates to which cells can attack and grow. Physiologically and anatomically, a microporous membrane is an ideal artificial surface for growing mammalian cells. A variety of polymeric membranes is currently used for cell culture depending upon the application [9].

1.4.2 Ultrafiltration

Ultrafiltration (UF) is a membrane process which is similar to MF in operation, but which uses asymmetric membranes with pores in the skin layer having a diameter of 2-10 nm. UF is a process of separating extremely small particles and dissolved molecules

from fluids. The primary basis for separation is molecular size although secondary factors, such as molecule shape and charge, can play an important role.

The molecular weight cut-off of ultrafiltration membranes is between 10^3 and 10^6 Dalton. Hydrostatic pressures of 0.1-0.5 MPa are used. The principle of operation is analogous to microfiltration and is based on “fine sieving”. The main hydrodynamic resistance of the membrane is offered by the top layer, while the supporting porous sublayer offers minimal hydraulic resistance.

UF membranes are prepared by phase inversion. Materials used are PSf, PVDF, PAN, PEEK and cellulosics such as cellulose acetate. Polymer blends, e.g. with polyvinylpyrrolidone (PVP) are commonly used to increase the hydrophilicity of the membranes. Also the UF process can be operated according to the dead-end and cross flow configurations.

UF is widely used for the recovery and concentration of enzymes and proteins produced by fermentations. The attraction of UF in protein concentration lies in the energy efficiency of the concentration of dilute fermentation broths and the gentle nature of separation which minimises protein denaturation, as the loss of protein activity. The UF of milk retains proteins, fats and insoluble and bound salts while it allows the permeation of lactose and soluble salts. The potentiality of UF is in the formulation of milk based beverages with a high content of calcium and a relatively low content of fat and cholesterol.

The addition of fining agents and then the decanting or filtering through pre-coat filter have been used in the traditional clarification of fruit juices. The application of UF simplifies the process by reducing time and labour and by increasing yield and juice quality [10].

UF is also used to remove particulate, microorganisms and colloidal material from drinking water and thus replace conventional clarification and disinfection and in the waster water treatment. UF is mainly used at industrial level as a method of fractionation, that is the separation of streams into two fractions on the basis of particulate size or molecular weight cut-off. The ability to separate soluble macromolecules from other soluble species and solvents is the major reason of the use of the UF in many industries such as pulp and paper industries [8].

1.4.3 Nanofiltration

Nanofiltration (NF) is a pressure-driven process situated between the separation capabilities of UF and reverse osmosis. In this process asymmetric mesoporous membranes are used to separate molecular mixtures and ions. Hydrostatic pressures of 0.3-3 MPa are used. The separations result from the contribution of different mechanisms such as size exclusion, diffusion and Donnan dialysis. Polymeric NF membranes contain ionisable groups, e.g. carboxylic or sulfonic acid groups, which result in a surface charge (positive or negative) in the presence of a feed solution. Therefore, the separation properties of NF membranes are determined in general by two distinct properties: the pore size of the membranes, which corresponds to a molecular weight cut-off value of about 200 Da, and the surface charge which can be positive or negative and affects the permeability of charged components such as ions. Due to electric interactions between ions and the membrane surface charge, for example, NF membranes are capable of separating monovalent from multivalent ions or neutral molecules [11].

NF is a unit operation that permits many applications such as solvent recovery from filtered oil, exchange of solvent in the chemical industry, water softening, desalination of dyestuffs, acid and caustic recovery, color removal, concentration and purification of ethanolic extract from different matrixes (e.g. xanthophylls, propolis) which are important in both pharmaceutical and food industries. NF is also a valid method for the fractionation and concentration of bioactive compounds from fruit juices. Some other specific applications are removal of cholesterol with applications in nutraceutics and nutritional supplements and for the wine and juice concentration [12].

NF is a relatively new process for the mineralisation of whey (and of milk). It is a competitive process comparable with electrodialysis or ion exchange for desalination. A degree of desalination up to 40% can be obtained by NF, which makes possible to utilise whey.

A NF process is used for recovering tannins and water from exhausted baths and their reuse as tanning agents [13]. Finally, NF membranes are used to recover polar or non-polar eluent phases coming from HPLC chromatographic processes. The dilute product is concentrated up to 10 %, while the contaminated stream is purified and recycled to the HPLC.

1.4.4 Reverse osmosis

Reverse osmosis (RO) is a pressure-driven process where particles, macromolecules, and low molecular mass compounds (salts and sugars) are separated from a solvent, usually water.

The mechanism of separation of species is based on processes relating to their size and shape, their ionic charge and their interactions with the membrane itself.

RO membranes are dense membranes; as a result, permeation is lower than the UF process and rejection is not a result of sieving, but of a solution-diffusion mechanism. The operating principle is that the surface layer of the membrane is a relaxed region of amorphous polymer in which solvent and solute dissolve and diffuse. To overcome the molecular friction between the permeate and the polymeric membrane during diffusion, large operating pressures are required in the range of 30 to 100 bar. The particle size range for applications of RO is approximately between 0.0001 and 0.001 micron and with solutes of molar mass greater than 300 Dalton a complete separation is achieved. RO membranes can be made of cellulose triacetate, aromatic polyamide or interfacial polymerization of polyamide and poly(ether urea). RO is employed in a wide range of applications in the processing of aqueous solutions:

- desalination of sea water;
- production of pure water for a variety of industries;
- concentration of solutions of food products, pharmaceutical solutions and chemical streams;
- waste water treatment.

A successful application of RO is found in the concentration of grape must prior to vinification. RO allows dewatering of must at room temperature avoiding losses of volatile compounds or damage of organoleptic properties. The must concentration has the scope of achieving proper sugars concentration for optimal fermentation and therefore high quality wine production [2-5]. The must concentrated by RO is rich in tannin and in organoleptic components, does not need addition of sugar and maintains the delicate balance of aroma compounds unchanged. In addition, the fruit juice concentration by RO has been of interest for the fruit processing industry for about 30 years. The advantages of RO over traditional evaporation are in low thermal damage to product, reduction in energy consumption and lower capital investments as the process

is carried out at low temperatures and it does not involve phase change for water removal [14].

In Figure 1.4.4 -1 the separation spectrum of pressure-driven membrane processes is reported.

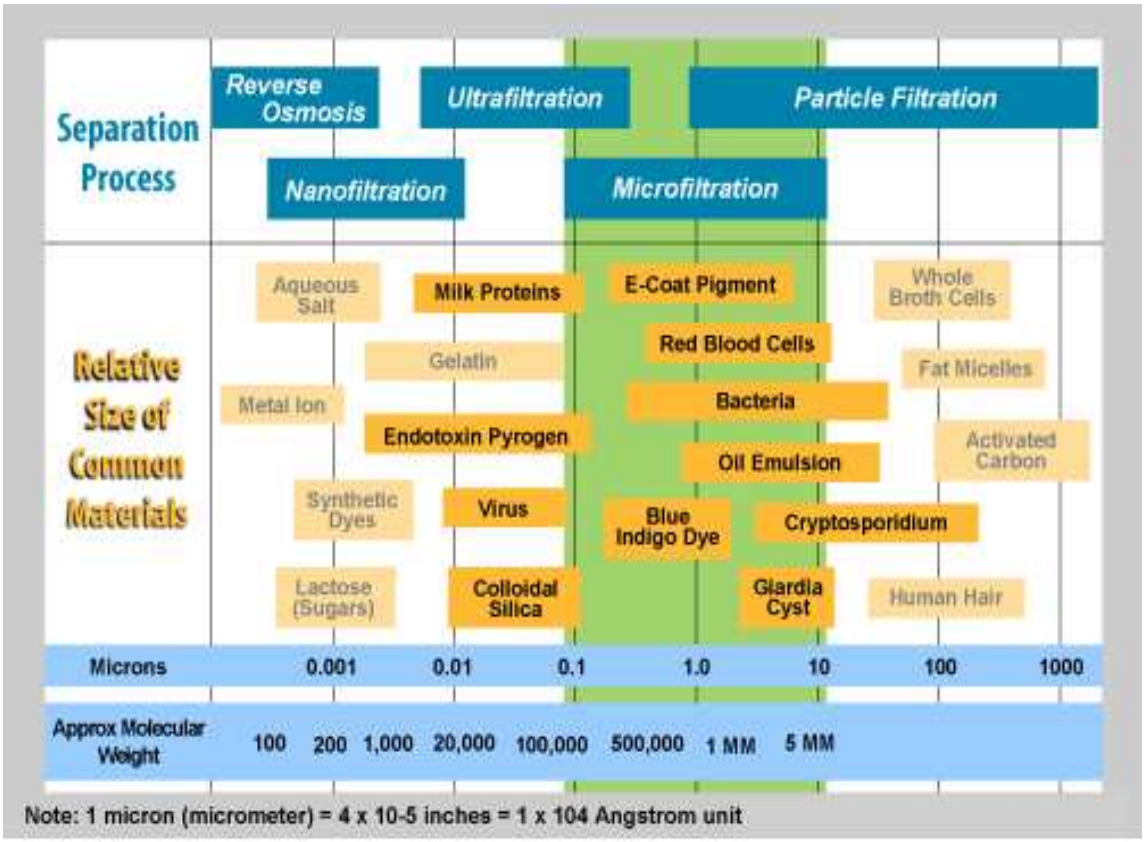

Figure 1.4.3.4 -1 *The filtration spectrum*

1.4.5 Membrane contactors

Membrane contactors (MCs) are systems where the membrane function is to facilitate diffusive mass transfer between two contacting phases (liquid–liquid, gas–liquid, gas–gas, etc.) without dispersion of one phase within another. This is accomplished by passing the fluids on opposite sides of a microporous membrane. By controlling the pressure difference between the fluids, one of them is immobilized in the pores of the membrane so that the fluid–fluid interface is located at the mouth of each pore. In a MC, generally, microporous hydrophobic membranes are used to promote mass transfer between phases. The membranes are not selective and represent an inert support by

which the contact between phases occurs. The mass transport takes place by a diffusive process through the membrane pores. With respect to conventional systems, MCs have some important advantages such as nondispersion of the phases in contact, independently variable flow rates without flooding limitations, lack of phase-density difference limitations, lack of phase separation requirements, higher surface area/volume ratios, and direct scale-up because of a modular design.

MCs offer a potential solution in a wide range of liquid/liquid and gas/liquid applications such as: liquid–liquid extraction, gas adsorption and stripping, dense gas extraction, fermentation and enzymatic transformation, pharmaceutical applications, protein extraction, wastewater treatment, chiral separations, semiconductor manufacturing, carbonation of beverages, metal ion extraction [15].

A number of commercial applications of MCs have been already successfully realized in beer production. Several industrial plants are using this technology for CO_2 removal followed by non dispersive nitrogenation in order to obtain a dense foam head. Another important field of application of MCs is the production of ultrapure water for electronic industry.

Membrane distillation and *osmotic distillation* can be considered examples of membrane contactors for carrying out the concentration of aqueous solution containing non-volatile solutes.

1.4.5.1 Membrane distillation

Membrane distillation (MD) is a relatively new membrane process in which two aqueous solutions, at different temperatures, are separated by a microporous hydrophobic membrane. In these conditions a net pure water flux from the warm side to the cold side occurs. The process takes place at an atmospheric pressure and at a temperature that may be much lower than the boiling point of the solutions. The driving force is the vapour pressure difference between the two solution–membrane interfaces due to the existing temperature gradient. The phenomenon can be described as a three phase sequence: (1) formation of a vapour gap at the warm solution–membrane interface; (2) transport of the vapour phase through the microporous system; (3) its condensation at the cold side membrane-solution interface.

The most suitable materials for MD membranes include PVDF, polytetrafluoethylene (PTFE) and polypropylene (PP). The size of micropores can range between 0.2 and 1.0 μm. The porosity of the membrane will range from 60% to 80% of the volume and the overall thickness from 80–250 μm, depending on the absence or presence of support. In general, the thinner the membrane and the greater the porosity of the membrane, the greater the flux rate. The membrane configurations used include flat sheet, spiral wound and hollow fiber; the latter has received the greatest attention. Because MD can be carried out at the atmospheric pressure and at a temperature which can be much lower than the boiling point of the solution, it can be used to concentrate solutes sensitive to high temperature (e.g.fruit juices), also at high osmotic pressure. Therefore MD has received a great attention as a technique for concentrating fruit juices [16,17].

1.4.5.2 Osmotic distillation

Osmotic distillation is a recent membrane process, also known as osmotic evaporation, membrane evaporation, isothermal membrane distillation or gas membrane extraction which has been successfully applied to the concentration of liquid foods such as milk, fruit and vegetable juice, instant coffee and tea and various non-food aqueous solutions. This technique can be used to extract selectively the water from aqueous solutions under atmospheric pressure and at room temperature, thus avoiding thermal degradation of the solutions. It is therefore particularly adapted to the concentration of heat-sensitive products like fruit juices.

The process involves the use of a microporous hydrophobic membrane to separate two circulating aqueous solutions at different solute concentrations: a dilute solution and a hypertonic salt solution. If the operating pressure is kept below the capillary penetration pressure of liquid into the pores, the membrane cannot be wetted by the solutions. The difference in solute concentrations, and consequently in water activity of both solutions, generates, at the vapour–liquid interface, a vapour pressure difference causing a vapour transfer from the dilute solution towards the stripping solution. The water transport through the membrane can be summarized in three steps: (1) evaporation of water at the dilute vapour–liquid interface; (2) diffusional or convective vapour transport through the membrane pore; (3) condensation of water vapor at the membrane/brine interface (Figure 1.4.5.2-1).

The typical OD process involves the use of a concentrated brine at the downstream side of the membrane as a stripping solution. A number of salts such as $MgSO_4$, $CaCl_2$, K_2HPO_4 are suitable. As compared with RO and MD process, the OD process has the potential advantage which might overcome the drawbacks of RO and MD for concentrating fruit juice, because RO suffers from high osmotic pressure limitation, while in MD some loss of volatile components and heat degradation may still occur due to the heat requirement for the feed stream in order to maintain the water vapour pressure gradient. OD, on the other hand, does not suffer from any of the problems mentioned above when operated at room temperature [18].

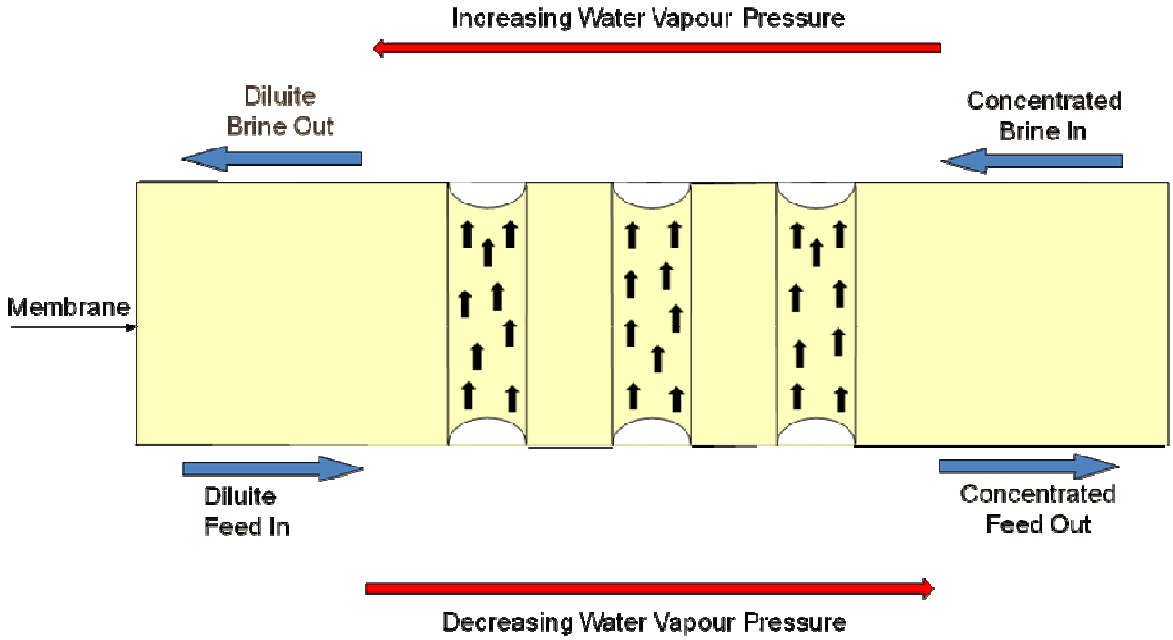

Figure 1.4.5.2-1 *Mechanism of osmotic distillation through a microporous hydrophobic membrane*

1.6 Concentration polarisation and fouling phenomena in membrane processes

During a separation process the membrane performance can significantly change with time, and often a typical flux-time behaviour may be observed: the flux through the membrane decreases over time. This behaviour is mainly due to concentration polarization and fouling, typical of pressure driven membrane processes. These two phenomena are aspects of the same problem, which is the build-up of retained components in the boundary layer of the membrane-solution interface. Both phenomena

induce additional resistances on the feed side to the transport across the membrane. When in a mass separation procedure a molecular mixture is brought to a membrane surface, some components will permeate the membrane under a given driving force, while others are retained. This leads to an accumulation and a formation of cake or gel layers by the feed solution constituents retained by the membrane which adds an additional hydrodynamic resistance to the membrane flux. This phenomenon is referred to as concentration polarization. It describes the concentration profile of the solutes in the liquid phase adjacent to the membrane resulting from the balance between different transport phenomena (convective flow and back diffusion) (Figure 1.6-1).

Concentration polarization is a reversible phenomenon, while fouling is irreversible and can be caused by several mechanisms: adsorption and constriction (deposit of material on the membrane surface), pore blocking (deposit inside the pores) and/or formation of a gel layer. Depending on the size of the particles and the membrane pore size, different cases of fouling can occur, giving different flux declines.

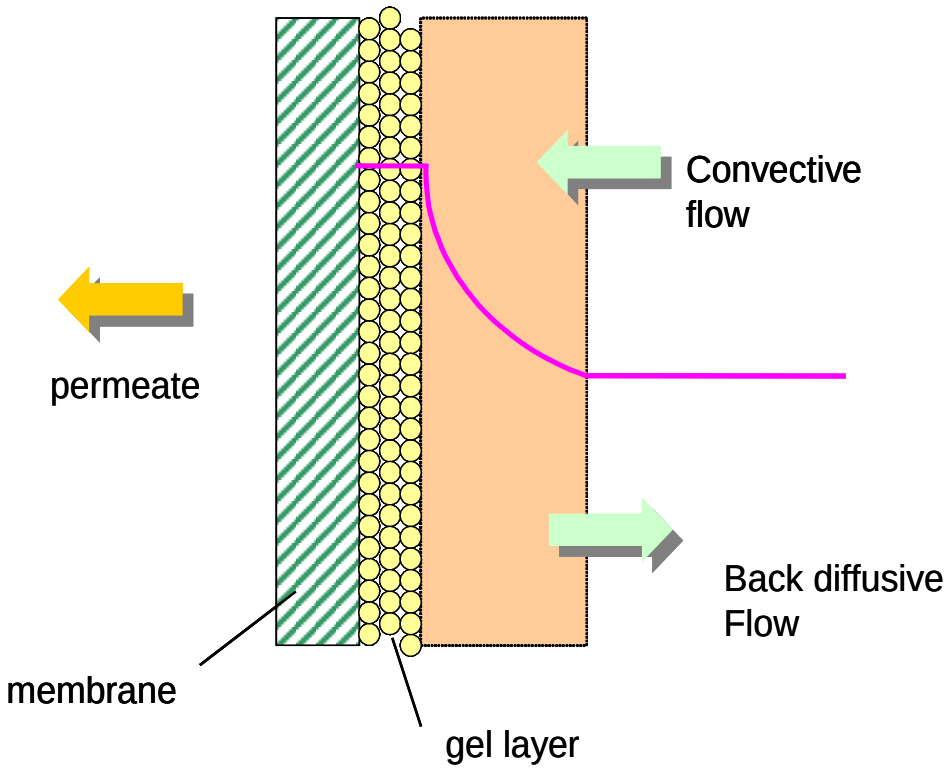

Figure 1.6 -1 *Schematic representation of the concentration polarization phenomenon*

The means of preventing or at least controlling membrane fouling effects are as heterogeneous as the different materials and mechanisms causing the fouling. The main procedures to avoid or controlling fouling involve:

- pretreatment of feed solution;
- membrane surface modifications;
- hydrodinamic optimization of the membrane module;
- membrane cleaning with the proper chemical agents.

A pre-treatment of the feed solution may include chemical precipitation, prefiltration, pH adjustment, chlorination or carbon adsorption. Membrane surface modifications include the introduction of hydrophilic moieties or charged groups in the membrane surface by chemical means or plasma deposition. High feed flow velocities and the proper module design are efficient tools in controlling membrane fouling.

Typical cleaning agents are acids and bases such as HNO_3, and NaOH, complexing agents, enzymes and detergents [19-20].

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CHAPTER 2

CITRUS FRUITS

2.1 Introduction

Citrus fruits are probably the best known and most widespread fruits all over the world, particularly appreciated for their fresh flavour and considered of high beneficial value for their high content in vitamin C and natural antioxidants, such as flavonoids and phenylpropanoids. Epidemiological studies on dietary Citrus flavonoids have been associated with a reduced risk of coronary heart disease; they have been also suggested as cancer-preventing agents. Therefore, the increased interest towards these compounds is due to their pharmacological activity as radical scavengers [1-3].

In addition, citrus by-products also represent a rich source of naturally occurring flavonoids: the peel, which represents almost one half of the fruit mass, contains high concentrations of flavonoids.

In contrast with other types of fruit, citrus fruits can be consumed mostly fresh or pressed to obtain a juice. The majority of citrus fruits are preferably eaten fresh-oranges, mandarins, grapefruits, clementines and tangerines. Oranges and grapefruits produce a very palatable juice and hence make for nutritious and popular breakfast. Lemons and limes can be processed into lemonades and pickles, and their juices can be also added to various food preparations to enhance flavour.

Furthermore, citrus fruits can be processed to obtain other food products such as dehydrated citrus products like jams, jellies or marmalades, which are very much appreciated. Citrus essential oil is another by-product of citrus derived from the citrus fruit peel. It is used in the food industry to give the flavour to drinks and foods; in the

pharmaceutical industry for the preparation of drugs and in the cosmetic industry for the preparation of soaps and perfumes and for home cleaning products. In particular, lemon oil is extensively used in furniture polish and bergamot is employed for making perfumes and massage oils [4-5].

In this chapter, after a description of *Citrus* origin, the chemical composition of citrus fruits and the most important functions of their nutrients in the human health are reported.

2.2 Origin, variety and production

The genus citrus, which includes few of the most important fruits worldwide, belongs to the family of Rutaceae, which comprises 140 genera and 1300 species in the world. Citrus cultivation originates in China and South-east Asia where it has been cultivated for more than 4000 years. Citrus is the second most important fruit in the world after apple, and accounts for the production of about 100 million tons with an area of cultivation spread over a massive 7.2 million hectares. It is a long-lived perennial crop and is grown in more than 100 countries across the world.

Favourable conditions for citrus cultivation are tropical and subtropical climates falling approximately within 40° latitude in each side of the equator, where temperatures are predominantly warm. Brazil, USA, Japan, Mexico, Pakistan and countries of the Mediterranean region are the major citrus producers (Figure 2.2-1).

In Italy the centre of Citrus growing is the South of the peninsula. Due to their favourable climatic conditions Sicily and Calabria are considered the “heart” of the Italian citrus fruit production.

In citrus species the plant is generally in the form of large shrubs or small trees reaching a height of 4 to 15 m. The genus citrus is closely related with other important genera of the family Rutaceae: *Fortunella, Poncirus, Microcitrus* and *Eremocitrus*. Major economically important species of citrus are: *C. sinensis* (Orange), *C. paradisy* (Grapefruit), *C. limon* (Lemon), *C. reticulate* (clementine), *C. aurantium* (sour orange), *C. medica* (Citron) and major citrus hybrids include *Citrange* (trifoliate orange X sweet orange), *Citrumelo* (trifoliate orange X grapefruit), C. *Bergamia Risso* (bergamot), *Tangor* (sweet orange X tangerine) and *Tangelo* (tangerine X grapefruit). Orange alone

accounts for 75 % of the total citrus fruit production worldwide followed by mandarin, grapefruit and lemon [5-6].

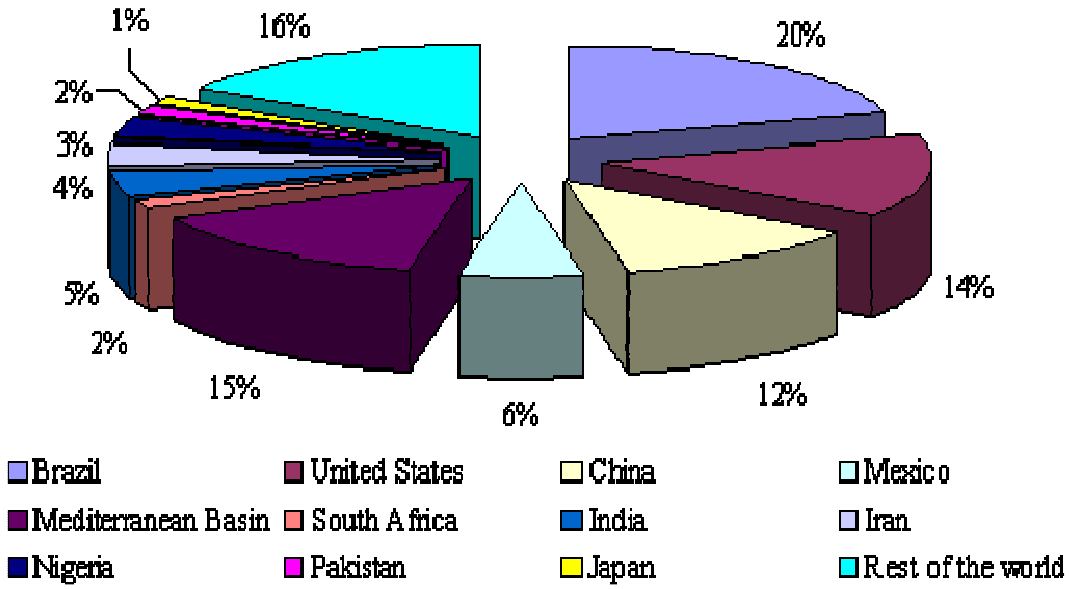


Figure 2.2-1 *Geographical distribution of fresh citrus production*

In Italy the most important and typical citrus varieties are:

- Orange: this variety which originates in China and Japan, was introduced in Italy by the Arabs in the 14th century. The red oranges of Sicily in the varieties *Tarocco*, *Moro* and *Sanguinello* are the best known in the world. The main characteristics of these varieties are their intense internal colouring (brought about by the anthocyanin pigments in the endocarp), their attractive external colour and lovely sweet taste. The areas of cultivation are located in numerous town districts in the provinces of Catania, Ragusa, Syracuse and Enna.
- Bergamot: is a natural hybrid fruit derived from bitter orange and lemon; it is produced almost exclusively in the Reggio Calabria area, where the cultivated area is about 1500 ha with an annual production of 25,000 tonnes. The origins of Bergamot remain obscure; probably bergamot was imported into Europe from the Canary Islands, where it had been introduced by Christopher Columbus. Whatever its origins, this strip of coastland in Calabria is the only place in the world where bergamot meets its optimal conditions for fructification.

- Clementines: are introduced in California in 1914. Clementines of Calabria region, cultivated in the provinces of Reggio Calabria, Catanzaro, Cosenza, Vibo Valentia and Crotone, are particularly well-known for their detectable flavor and remarkable freshness. The region cultivates the fruit of the common, Fedele, Hernandina, Marisol, Nules, Spinosa, SRA 63 and Tardiva varieties. The Calabrian clementina ripens earlier than the other varieties at the beginning of October.
- Citron (*Citrus medica*): this plant has an ancient origin; the most accredited provenance is from India but it probably arrived in Italy through the Hebrews who introduced the cultivation of the citron on the Calabrian coasts. The cultivars are divided into two groups: sweet and acidic citron. Among the acidic citron the most important variety is the Calabrian one, known as the Diamante citron. This plant finds the best conditions of cultivation in warm countries; in Calabria, the cultivation of citron extends along the coast of high Thyrrenium, from Diamante to Tortora, called Coast of Citron.

2.3 Structure

All varieties of citrus fruits are very similar in structure except for size and shape. They can be round, oblate, ellipsoid, spheroid, pyriform and ovoid.

The skin is constituted by an epicuticular wax layer. The quantity of waxes depends on the variety, the climatic conditions and growth rate. Immediately under the epidermis the flavedo is located characterized by a yellow, green or orange colour. It contains the oliferous vesicles that are constituted by a thin and fragile walls; inside the essential oil is characterised by a positive pressure, that permits the recovery by abrasion of flavedo layer. Under the flavedo there is the albedo constituted by tubular-like cells forming a network; it differs in thickness according to the variety and the cultivar.

The albedo is very rich in flavonoids that contribute to the bitter taste of the juice. Next layer is the endocarp, the edible part of the fruit with the segments containing juice vesicles: the juice can be considered as the liquor released by the cytoplasm and the vacuoles located inside the vesicles. The internal part is the core formed by a sponge tissue similar to the albedo (Figure 2.3-1) [7].

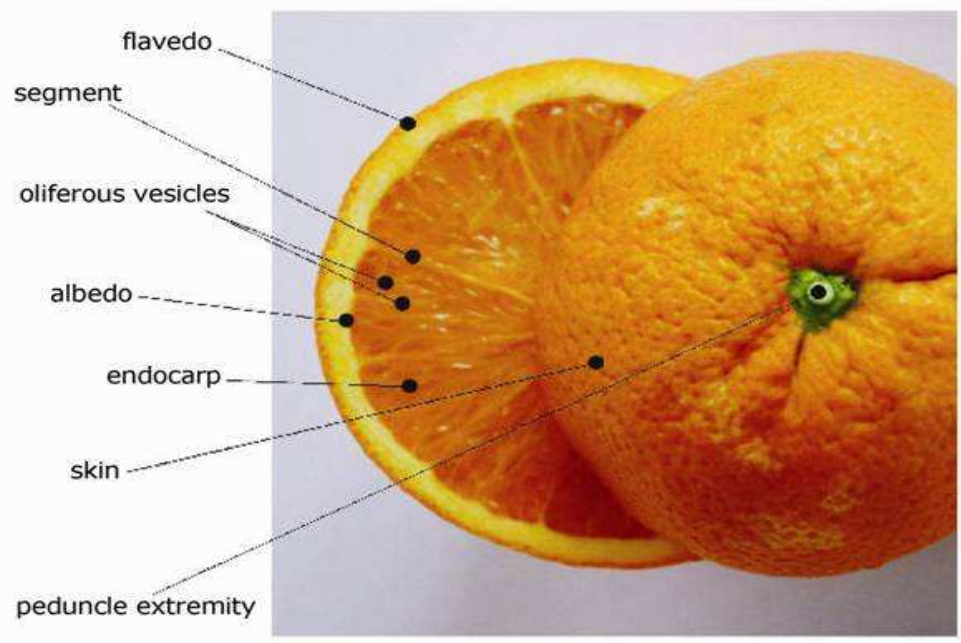


Figure 2.3-1 *Structure of Citrus fruits*

2.4 Chemical composition

Citrus is characterised by a high content of vitamin C (from 50 to 70 mg/100 g of edible fresh) and it also contains an impressive list of essential nutrients including: folic acid, vitamin B6, thiamine, riboflavin, organic acids, amino acids, minerals salts as potassium, calcium, cupper, phosphorus and iron. Furthermore, it is low in fat and contains no cholesterol; it appears, instead, as a good source of other phytochemicals and nutriaceuticals including polyphenols and carotenoids having antioxidant activity [8]. Studies performed on the nutrient density (a calculation frequently used by dietitians and nutritionists to reflect the nutritional value of a food) by Rampersaud et al. [9] of different commonly consumed fruits demonstrated that citrus juices, particularly orange and pink grapefruit, have the highest calculated nutrient density scores compared to apple, grape, pineapple and prune [10].

In Table 2.4-1 the chemical composition of citrus referred to 100 grams of edible portion is reported.

Table 2.4-1 *Chemical composition of Citrus fruit*

Chemical composition of Citrus	**Units**	**Value per 100 grams of edible portion**
Edible part	%	87
Water	g	86
Proteins	g	0.94
Lipids	g	0.12
Minerals		
Potassium	mg	0.181
Magnesium	mg	10
Iron	mg	0.10
Copper	mg	0.045
Vitamins		
Vitamin C	mg	70
Thiamin	mg	0.087
Riboflavin	mg	0.040
Niacin	mg	0.282
Folic acid	mg	30
Vitamin E	mg	0.240
Vitamin B6	mg	0.060
Tocopherol, alpha	mg	0.240
Amino Acids		
Tryptophan	mg	9
Threonine	mg	15
Isoleucine	mg	25
Leucine	mg	23
Methionine	mg	20
Phenylalanine	mg	31
Energy	**Kcal** **Kj**	**44** **184**

Listed below are some of the important nutrients described in citrus and their role in the safeguard of human health.

2.4.1 Dietary fibres

An excellent source of dietary fibres, Citrus fruits provide both soluble and insoluble forms. The predominant type of soluble fibre in citrus is pectin, making up 65 to 70 percent of the total fibre. The remaining fibres are the insoluble form constituted by cellulose, hemicellulose and trace amounts of gums. Citrus also contains lignin, a fibre-like component. The consumption of citrus fruits can contribute significant quantities of pectin in a diet. Dietary incorporation of pectin appears to affect several metabolic and digestive processes: pectin has been associated to numerous physiological effects

including the decreasing of glucose absorption and the improving of insulin response, the lowering of plasma LDL cholesterol concentrations and the binding to minerals to decrease their bioavailability. Pectin can also interfere with the reabsorption of bile acids which may help in lowering plasma cholesterol levels. In addition, observations indicate that citrus pectin possesses an anticancer potential and an immune modulatory effect.

A reasonable goal for dietary/fibre intake is 25 to 30 g/day, but in many developed countries the actual average intake is closer to 15 g. With one medium orange containing approximately 3.0 g of fibre, citrus fruit can make a valuable contribution to meeting the daily fibre goal [11-12].

2.4.2 Ascorbic Acid

Among the different substances contained in citrus fruits a primary role in the safeguard of the human health is carried out by Vitamin C.

Vitamin C (ascorbic acid) is an essential water-soluble vitamin, important for its antioxidant function. It is a six-carbon lactone (Figure 2.4.2-1) synthesized from glucose in most mammalian species, mainly in liver, but not in humans. The importance of ascorbate to humans is illustrated by the lethal nature of prolonged vitamin C privation, which causes scurvy. Ascorbic acid is a good reducing agent. Following the donation of an electron the semidehydroascorbate radical is obtained; loss of a second electron converts this radical to deydroascorbate, an unstable molecule. Metabolic pathways exist that can recycle ascorbyl radical and dehydroascorbate back to ascorbate, using NADH or GSH as sources of reducing power.

Vitamin C acts against oxidation of lipids, proteins and DNA, subsequently protecting their structure and biological function. The oxidation of these biomolecules generates measurable reaction products, such as 8-oxodeoxyguanosine from DNA, F2-isoprostanes from lipids, and carbonyl derivatives from proteins. Moreover, this can give a useful method to assess the antioxidant effect of vitamin C or other antioxidants.

In addition, vitamin C acts as an electron donor for different enzymes; it participates in collagen hydroxylation, is necessary for synthesis of carnitine, and participates in the biosynthesis of norepinephrine from dopamine and modulation of tyrosine metabolism.

It reduces the risk of heart disease by preventing the oxidation of low-density lipoprotein (LDL) cholesterol.

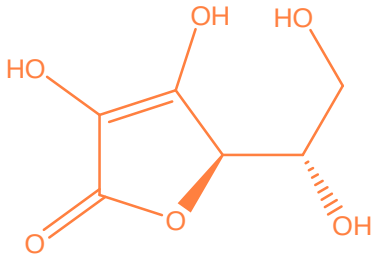


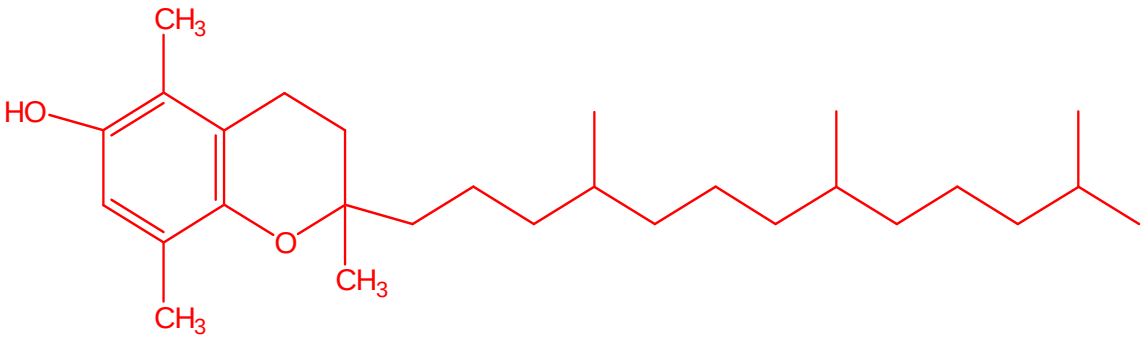
Figure 2.4.2-1 *Ascorbic acid*

Ascorbic acid plays a key role in the formation of collagen, a primary component of much of the connective tissue in the body. Adequate collagen synthesis is essential for strong ligaments, tendons, skin, blood vessels and for wound healing and tissue repair. The weakening of these tissues is a symptom of vitamin C deficiency. Vitamin C is an important aid in the absorption of inorganic iron: indeed it may aid in the reduction of dietary inorganic iron from the insoluble Fe (III) to the soluble Fe (II) form, which is more easily absorbed by the small intestine. Ascorbate has also been shown to aid in the treatment of anaemia and stress, and protect against intra-gastric nitrosamine formation.
Vitamin C is also a crucial factor in the eye's ability to deal with oxidative stress, and can delay the progression of advanced age-related macular degeneration (AMD) and vision-loss [13].
Only 10 mg of vitamin C per day are required to prevent vitamin C deficiency. However, for good health and sufficient body storage of vitamin C, 30 to 100 mg/day is generally recommended. Citrus fruits are a particularly good source of vitamin C, with one medium orange or grapefruit providing approximately 70 mg and 56 mg, respectively.

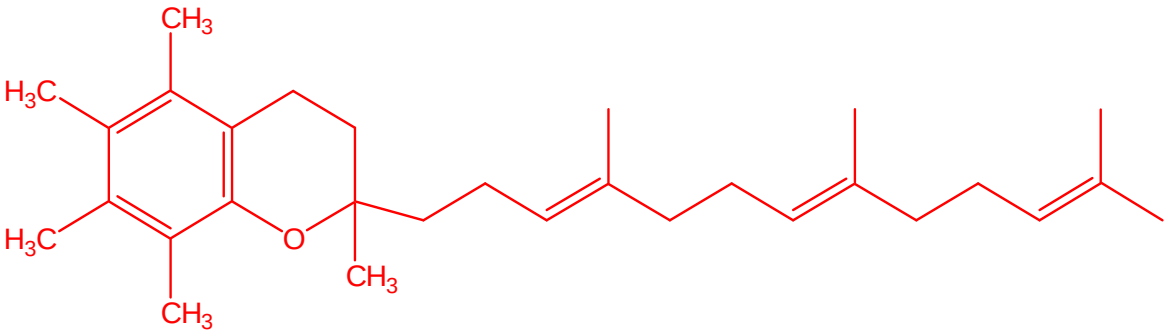
2.4.3 Vitamin E

Vitamin E is a lipophilic compound exhibiting a variety of biological activities.
It includes two groups, tocopherols (alfa, beta, gamma, delta) and tocotrienols (alfa, beta, gamma, delta) (Figure 2.4.3-1), not synthesized in humans and animals but

ingested with the diet; they are particularly present in vegetable oils and other plant-based food groups.



α–tocopherol



α–tocotrienol

Figure 2.4.3-1 *Structure of selected tocopherols and tocotrienols*

Vitamin E is considered as the most potent antioxidant of the lipid soluble type, inhibiting the propagation of lipid peroxidation, and thus preventing membranes or lipoproteins from oxidative damage.

The antioxidant activity of vitamin E is due to its ability to donate its hydrogen ions to lipid free radicals thereby neutralizing the radical and forming the tocopheroxy radical.

This constitutes an important biological function of vitamin E, since the deterioration of cellular membranes is associated to cellular dysfunction and because oxidative modification of lipoproteins plays a role in the formation of the atherosclerotic plaque. The elevation of plasma and tissue F2-isoprostanes (a reliable index of in vivo oxidative

lipid damage) in animals with vitamin E deficiency demonstrates the importance of vitamin E also in vivo.

Epidemiological studies suggest that vitamin E may reduce the risk of coronary heart disease, some cancers, cataracts and diabetes and slow the progression of neurological diseases. The health effects of vitamin E may be related to numerous mechanisms, including protections of cells from oxidative damage, protection of LDL from oxidation, enhancement of the immune system, reduction of cholesterol synthesis by inhibition of the enzyme HMG-CoA reductase.

Vitamin E plays an important role as a blocker of nitrosamine formation. This mechanism is implicated in the initiation stages of carcinogenesis. Effects on the immune system have also been hypothesized as a possible mechanism of action for vitamin E in the promotional stages of carcinogenesis and antitumor proliferation capacities possibly by modulating gene expression [14-16].

2.4.4 Folic acid

Folic acid (Figure 2.4.4-1) is a water soluble B-vitamin, known as B9 vitamin, which plays an important role in human nutrition. Citrus fruit and green vegetables such as broccoli, spinach and bell peppers are good sources of folate.

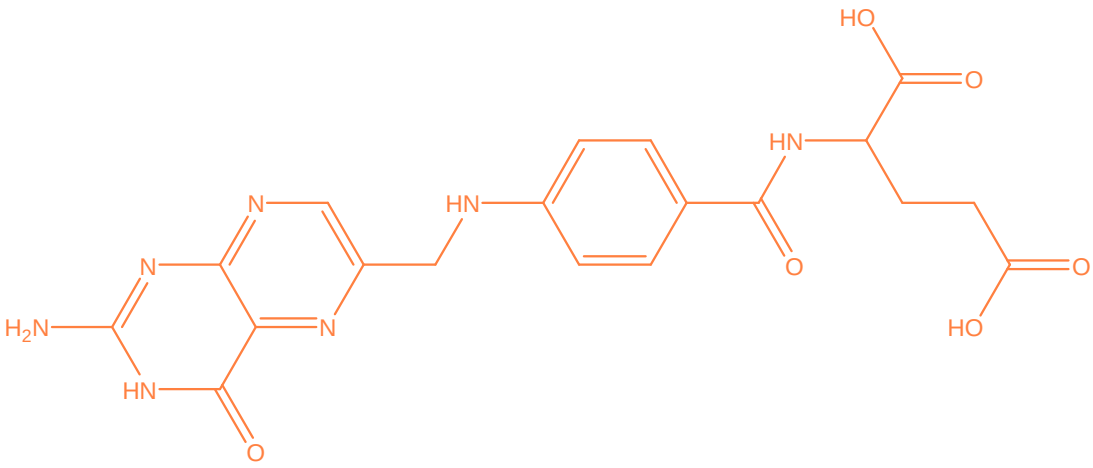


Figure 2.4.4-1 *Folic acid*

Folate compounds are important for DNA synthesis permitting to preserve the genetic information and for the production of amino-acids.

Folic acid plays an important role to maintain the levels of homocysteine in blood lower. Folate-rich diets have been associated to a decreased risk of cardiovascular disease.

Folate compounds prevent megaloblastic anemia, and recent studies implicate their deficiency in the etiology chronic diseases and coronary heart disease.

There is a strong scientific evidence supporting a link between folic acid intake and the prevention of neural tube defects in infants. The US Center for Disease Control and Prevention recommends a consume of about 0.4 mg/day in all women of childbearing age, and specially those who are planning a pregnancy.

Nowadays, folic acid-fortified beverages are used as a method to increase the intake of this vitamin in the pregnant population and their use is recommended [17-19].

2.4.5 Carotenoids

Carotenoids are a family of pigmented compounds that are synthesized by plants and microorganisms but not by animals. In plants, they contribute to the photosynthetic machinery and protect them against photo-damage. Fruit and vegetables constitute the major source of carotenoids in human diet. They are present as micro-components in fruits and vegetables and are responsible for their yellow, orange and red colors. In citrus fruits, carotenoids are mainly associated with pulp and its particles extracted in the juice. More than 600 carotenoids have so far been identified in nature. However, only about 40 are present in a typical human diet. Of these 40 about 20 carotenoids have been identified in human blood and tissues. Close to 90% of the carotenoids in the diet and the human body is represented by α–carotein, β–carotein, lycopene, lutein and cryptoxanthin. They contain a system of conjugated double bonds which allow them to interact efficiently with reactive oxygen species (Figure 2.4.5-1). β-Carotene, α-carotene, lutein as well as several other dietary carotenoids are efficient quenchers of singlet molecular oxygen and scavengers of peroxyl radicals.

In human studies, numerous associations between a low carotenoids intake or status and an increased risk for cancer, age-related macular degeneration, cataract and cardiovascular diseases have been observed [20].

The antioxidant properties of carotenoids have been suggested as the main mechanism by which they afford their beneficial effects. Recent studies are also showing that

carotenoids may mediate their effects via other mechanisms such as gap junction communication, cell growth regulation and modulating gene expression. However, carotenoids such as α and β carotein and α-cryptoxanthin also have the advantage to be converted to Vitamin A with its related role in the development and disease prevention.

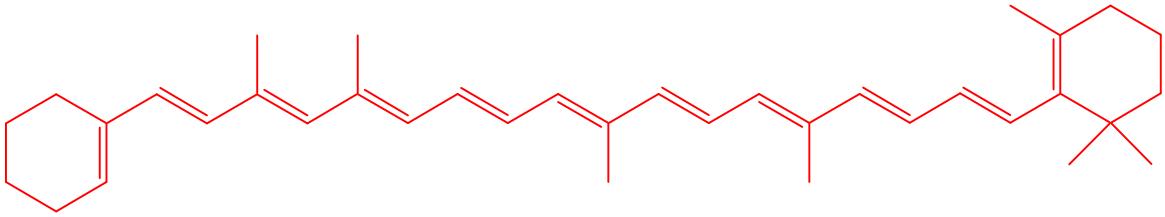


Figure 2.4.5-1 *β–carotene*

The role of carotenoids in the prevention of chronic diseases and their biological actions are summarized in Figure 2.4.5-1 [21-23].

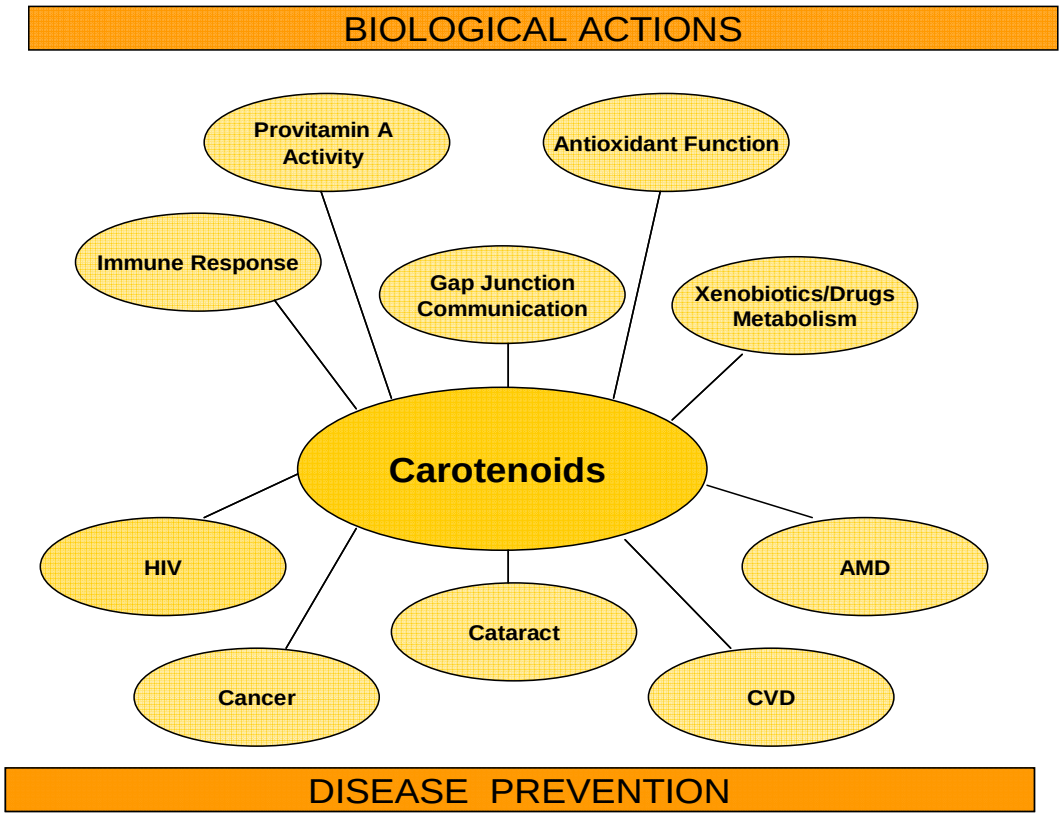


Figure 2.4.5-1 *Role of carotenoids in the prevention of chronic disease*

2.4.6 Flavonoids

Flavonoids are a group of polyphenolic compounds diverse in chemical structures and characteristics. They occur naturally in fruit, vegetables and nuts. Citrus fruits are the main source of dietary flavonoids.

They are characterized by a common benzo-γ-pyrone structure, which has been reported to act as an antioxidant in various biological systems. Multiple combinations of hydroxyl groups, sugars, oxygens and methyl groups attached to this structure generate the various classes of flavonoids: flavanols, flavanones, flavones, flavan-3-ols (catechins), anthocyanins, and isoflavones (Figure 2.4.6-1) [24].

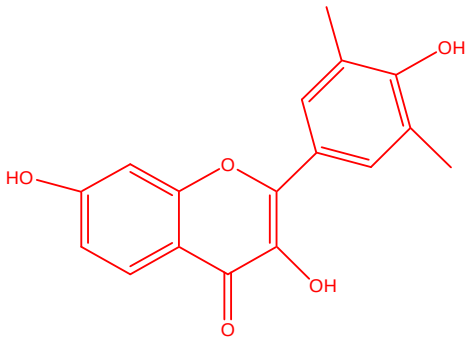
Citrus flavanones are present in the glycoside or aglycone foms. Among the aglycone forms naringenin and hesperidin are the most important flavanones.

Among the glycoside forms, two types are identified: neohesperidosides (naringin, neohesperidin and neoeriocitrin) and rutinosides (hesperidin, narirutin and didymin). The flavonoids composition is not always the same in Citrus fruits. For example, in the sweet orange juices (*Citrus sinensis*), the most abundant component is hesperidin, followed by narirutin and dydimin. The blood variety of sweet orange is characterised by the presence of anthocyanins.

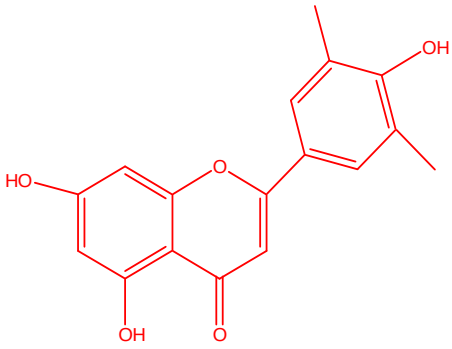
Anthocyanins constitute the colouring compounds of flowers and fruits. They are in the epicarp, but they also colour the mesocarp of oranges. The anthocyanin content is strongly dependent on the level of maturation.

Mandarin juices are quite similar to sweet orange, since they are characterised by the same distinctive flavanones. Lemon (*Citrus limon*) is characterised by the presence of significant amounts of hesperidin, eriocitrin and diosmin. Bergamot (*Citrus bergamia*) is extremely rich in flavonoids. Neoeritrocin, naringin, neoheperidin and hesperidin are the most abundant flavanones in juices and seeds but, recently new flavonoids have been identified as rhoifolin, diosmetin, luteolin, apigenin and chrysoeril glucosides [25].

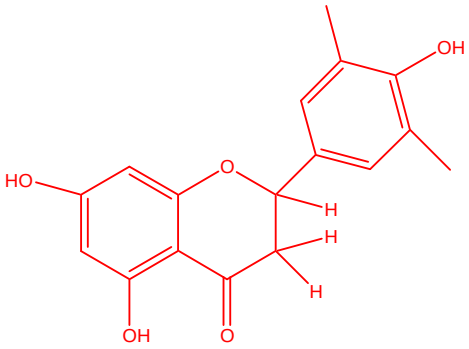
Grapefruit (*Citrus paradisi*) can generally be found in three different varieties, red, pink and white, their color depending on the presence (or absence) of lycopene. Its main component is the flavanone naringin and naringenin, which has always been recognized to be a distinctive component of grapefruit juices. Narirutin is also present in good amounts. Generally, white grapefruit juice is slightly richer in flavonoids than the pink and red varieties [26].



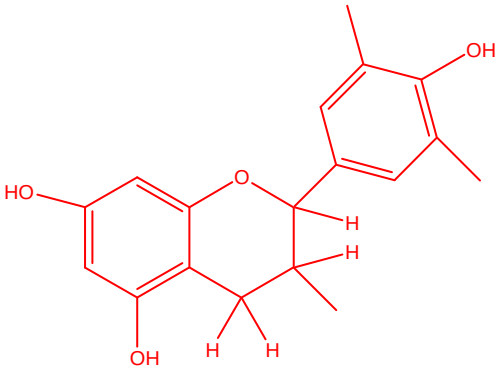
Flavonols



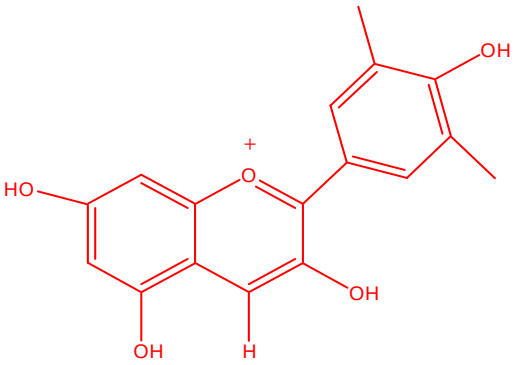
Flavones



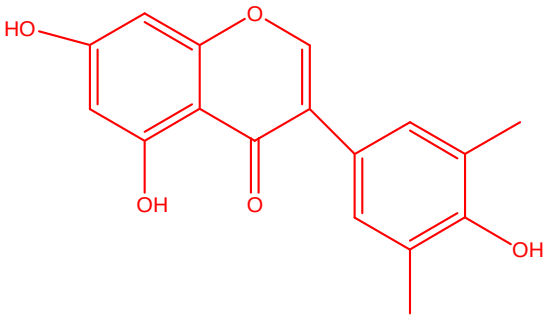
Flavanones



Flavanols



Anthocyanidins



Isoflavones

Figure 2.4.6-1 *Molecular structure of flavonoids*

Flavonoids play an important role in human health. They possess a variety of biological properties, including antiallergic, antiinflammatory, antiviral, antiproliferative, and anticarcinogenic activities. Flavonoids have received considerable attention because of their bene-ficial effects as antioxidants in the prevention of human diseases such as cancer and cardiovascular diseases, and some pathological disorders of gastric and duodenal ulcers, vascular fragility, and viral and bacterial infections [27].

Flavonoids protect against cancer through inhibition of oxidative damage and can exercise their antioxidant activity in several ways:

- antiradical activities: $OH^{\cdot}$ (hydroxyl), O_2, $^1O_2$, O_2^{-}(superoxide);
- anti-lipoperoxidation activities ($R^{\cdot}$; $ROO^{\cdot}$; $RO^{\cdot}$);
- activities of metal chelation.

Flavonoids have been shown to be able to act as antioxidants by scavenging free radicals, an activity related to their phenol rings containing hydroxyl groups. Flavonoids also have the ability to act as reducing agents capable of donating hydrogens to free radicals and causing their removal. Flavonoids can also act as singlet oxygen quenchers and as chelators of transition metal such as copper and iron, which are well-known pro-oxidants in foods. The ability of monomeric phenolics to act as antioxidants is dependent on extended conjugation, number and rearrangement of phenolics substituent and molecular weight.

The anti-inflammatory activity of flavonoids is due to the inhibition of cycloxygenase-2 (COX2) and inducible nitric oxide synthase. In particular, studies using mouse macrophage cells, have shown that *Citrus* hesperidin has an inhibitory effect on lipopolysaccharide (LPS)-induced over expression of cyclooxygenase-2, inducible nitric oxide synthase (iNOS), over-production of prostaglandin E2 and nitric oxide (NO) [28].

Indeed, citrus flavonoids are able to inhibit the kinases and phosphodiesterases essential for cellular signal transduction and activation. They also affect the activation of a number of cells involved in the immune response, including T and B lymphocytes.

Recent studies have focused on the Citrus flavonoids in the prevention of atherosclerosis by inhibiting the formation of atheroma in many steps of its pathogenesis. Particular flavonoids inhibit platelet aggregation and adhesion thus reducing thrombotic tendencies, by mediating increases in platelet cyclic AMP (cAMP) levels by either stimulation of adenylate cyclase or inhibiting of cAMP

phosphodiasterase (PDE) activity and by inhibiting the enzymes cyclo-oxygenase and lypoxigenase involved in arachidonic acid metabolism in platelets. In addition flavonoids have a considerable antithrombotic activity, because they maintain the right prostacyclin and NO endothelium levels.

Flavonoids appear to increase vasodilation by inducing vascular smooth muscle relaxation which may be mediated by the inhibition of protein kinase C or by a decreased cellular uptake of calcium.

Flavonoids intake has been inversely and significantly associated to death from coronary heart disease and showed an inverse relation with the incidence of myocardium infarction. In vitro, flavonoids inhibit the oxidation of low-density-lipoprotein (LDL) by macrophages, mainly by inhibiting the generation of hydroperoxide.

Recently, flavonoids have attracted attention as potentially important dietary cancer chemoprotective agents. In addition, the possible antitumor action of certain flavonoids has also generated interest. Flavonoids may act in all stages of the carcinogenesis process: damage to the DNA (initiation), tumor development (promotion) and invasion (proliferation).

An important mechanism by which flavonoids may exert their effects is through their interaction with phase I metabolizing enzymes (e.g., cytochrome P450), which metabolically activate a large number of pro-carcinogens to reactive intermediates that can interact with cellular nucleophiles and ultimately trigger carcinogenesis. Flavonoids are demonstrated to inhibit the activities of certain P450 isozymes such as CYP1A1 and CYP1A2.23,90,91. Thus, they are likely to have a protective role against the induction of cellular damage by the activation of carcinogens.

Moreover, some flavonoids have been reported as potent aromatase inhibitors. Substantial evidence supports the concept that estrogens are involved in mammary carcinomas. Estradiol, the most potent endogenous estrogen, is biosynthesized from androgens by the cytochrome P450 enzyme complex called aromatase. Inhibition of aromatase is an important approach for reducing growth stimulatory effects of estrogens in hormone-dependent breast cancer. Therefore, flavonoids could be considered potential agents against breast cancer through the inhibition of aromatase activity.

The molecular mechanism of antiproliferation may involve the inhibition of the prooxidant process that causes tumor promotion. It is generally believed that the formation of growth promoting oxidants (reactive oxygen species, ROS) is a major ‘‘catalyst’’ of the tumor promotion and progression stages, which follows the initiation stage (carcinogen metabolic activation to mutagens). The prooxidant enzymes induced or activated various tumor promoters. In addition, inhibition of polyamine biosynthesis could be a contributing mechanism to the antiproliferative activities of flavonoids. Ornithine decarboxylase is a rate-limiting enzyme in polyamine biosynthesis, which has been correlated with the rate of DNA synthesis and cell proliferation in several tissues. Several experiments show that flavonoids can inhibit ornithine decarboxylase induced by tumor promoters, and thus cause a subsequent decrease in polyamine and inhibition of DNA/protein synthesis. Furthermore, flavonoids are also effective at inhibiting signal transduction enzymes, for example, protein tyrosine kinase (PTK), protein kinase C (PKC), and phosphoinositide 3-kinases (PIP3), which are involved in the regulation of cell proliferation.

The abilities of particular flavonoids to block solid tumor growth may be due to their inhibition of the neoangiogenic process. Angiogenesis is a strictly controlled process in the healthy adult human body, which is regulated by a variety of endogenous angiogenic and angiostatic factors. However, pathological angiogenesis can occur in cancer. Angiogenesis inhibitors such as flavonoids are able to interfere with various steps of angiogenesis, like basement destruction of blood vessels, proliferation and migration of endothelial cells, or the lumen formation. Therefore, these compounds may have potential for the treatment of solid tumors.

2.4.7 Organic Acids

Organic acids play a very important role in fruits growth and, also, in citrus products sales. Total acidity, together with total sugar content is an important criterion to evaluate the ripening degree of oranges and grapefruits while for lemon juice it represents the primary factor for price definition.

Organic acids are a useful index of authenticity in fruits product. The organic acids composition of fruits is also of interest because of its important influence on the sensory

properties of the fruit juices. The main organic acids of citrus fruits are citric and malic acids. In addition, traces of benzoic, oxalic and succinic acids have been reported.

Organic acids are contained basically in the juice and their concentration in other parts of the fruit is very low.

Citric acid (Figure 2.4.7-1) is a commercially valuable product widely used in the food, pharmaceutical and beverage industries because it presents antibacterial and acidulant effects, reinforces the antioxidant action of other substances, and improves the flavors of juices, soft drinks, and syrups. As a food additive it is denoted by E number E330. Citrate salts of various metals are used to deliver those minerals in a biologically available form in many dietary supplements. In the processing of frozen foods, citric acid is used as a pH regulator; it is also able to optimize the stability of frozen food products by enhancing the activity of antioxidants and inactivating enzymes.

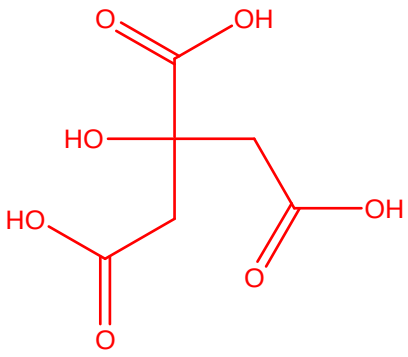


Figure 2.4.7-1 *Citric acid*

The buffering properties of citrates are used to control pH in household cleaners and pharmaceuticals. The ability of citric acid to chelate metals makes it useful in soaps and laundry detergents. By chelating the metals in hard water, it lets these cleaners produce foam and work better without need for water softening. In a similar manner, citric acid is used to regenerate the ion exchange materials used in water softeners by stripping off the accumulated metal ions as citrate complexes. The saturation point for citric acid and water is 59%. In biochemistry, citric acid is important as an intermediate in the citric acid cycle and therefore occurs in the metabolism of almost all living cells. It is

produced commercially by submerged fermentation of sucrose or molasse based medium [29].

Malic Acid (Figure 2.4.7-2) is one of the main fruit acids, and is produced naturally in a range of plants, most notably in apples. It is a non toxic and a natural product that, when ingested into the body as a supplement to a healthy diet has been shown to offer improved levels of energy, particularly during exercise.

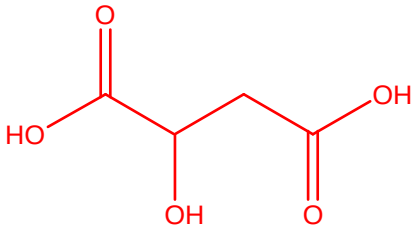


Figure 2.4.7-2 *Malic acid*

Recent medical testing has also shown links between the use of malic acid as a dietary supplement and a reduction in pain caused by the chronic condition fibromyalgia, which is a long term degenerative disorder that causes pain throughout the body, particularly in the joints.

Malic acid is prepared hydrolyzing maleic anhydride to maleic acid and, at elevated temperatures and pressures, forming an equilibrium mixture of maleic acid, fumaric acid and malic acid. The latter is isolated from the other two acids. Malic acid is used in a variety of products: it is the preferred acidulant in low-calories drink; in sugar free-drinks malic acid masks the off-tast produced by sugar substitutes [30].

2.4.8 Mineral salts

Citrus fruits are an important source of mineral salts such as potassium, copper, iron and magnesium. Minerals are naturally elements needed by the body and its vital activities. Each mineral is indispensable for important life functions; they are needed for the formation of hormones, enzymes and other body substances.

Copper is a mineral involved in different biological activities. It is found in a variety of enzymes: copper enzymes are widely distributed within the body; they perform several diverse functions including transport of oxygen and electrons, catalysis in oxidation

reduction reactions and the protection of the cell against damaging oxygen radicals. Copper plays an important role in the collagene formation and it plays a part in connective tissue maturation, a function of copper which is closely linked with the activity of lysyl oxidase, a copper dependent enzyme found almost exclusively in connective tissue and crucial for the optimal formation of a child’s brain and nervous system. The mineral is responsible for production and maintenance of myelin, the material that surrounds and protects nerve and brain cells. Copper also plays a role in making neurotransmitters. It also helps to sustain the elasticity of blood vessels, which allows maintenance of proper blood pressure.

The anti-inflammatory activity of copper complexes of various ligands such as amino-acid and salicylic acid has been demonstrated: these copper complexes are known to promote tissue repair.

Since copper is needed for healthy muscle tone and function, it also plays a vital role in the heart.

Potassium is an essential mineral that works to maintain the body's water and acid balance. As an important electrolyte, it plays a role in transmitting nerve impulses to muscles, in muscle contraction and in the maintenance of normal blood pressure. Potassium also plays an important role to mental function as well as to physical processes. It helps to promote efficient cognitive functioning by playing a significant role in getting oxygen to the brain. It has various roles in body functions and it is essential for the proper function of all cells, tissues, and organs. Among metabolic functions, potassium plays a role in the synthesis of proteins and in the biochemical transformations required for carbohydrate metabolism.

A high-potassium diet may also prevent or at least slow the progression of renal disease. An increased potassium intake lowers urinary calcium excretion and plays an important role in the management of hypercalciuria and kidney stones and is likely to decrease the risk of osteoporosis. Low serum potassium is strongly related to glucose intolerance, and increasing potassium intake may prevent the development of diabetes. Reduced serum potassium increases the risk of lethal ventricular arrhythmias in patients with ischemic heart disease. Epidemiological studies and outcome trials show that increasing potassium intake reduces cardiovascular disease mortality. This is mainly attributable to

the blood pressure-lowering effect and may also be partially because of the direct effects of potassium on the cardiovascular system [31].

Magnesium plays a critical role in human health and nutrition and is essential in numerous biochemical pathways. Divalent magnesium is the fourth most abundant metal ion found in cellular metabolism. About 90% of the intracellular magnesium ion is bound to the ribosome. Its biological functions include structural stabilisation of protein, nucleic acids and cell membranes. Magnesium ion is also required to promote specific structural or catalytic activities of proteins, enzymes or ribozymes.

Magnesium may play an important role in regulating blood pressure. Diets that provide plenty of potassium and magnesium are consistently associated with lower blood pressure. Magnesium deficiency can cause metabolic changes that may contribute to heart attacks and strokes, as well as an increased risk of abnormal heart rhythms. Population surveys have associated higher blood levels with lower risks of coronary heart diseases. Dietary survey have suggested that a higher magnesium intake is associated with a lower risk of stroke. Magnesium is also important in carbohydrates metabolism, and it may influence the release and activity of insulin.

The recommended daily allowance for an adult is in the range 380-420 mg of magnesium for day [32].

Citrus is a good source of *iron*. It is vital for almost all living organisms by participating in a wide variety of different processes. The metabolic functions of iron are the best-known of the micronutrients, given their key role in the structure and function of hemoglobin. The human adult contains about 3.5 g of iron, mostly (60%) in the form of hemoglobin in red blood cells[33]. The principal function of iron is thus coincident with the role of hemoglobin transporting oxygen from the lungs to metabolically active tissues. Iron is also present in myoglobin, an intracellular store of oxygen, in the cytochrome enzymes of the mitochondrial electron transport chain, in cytochrome P-450, which is involved in the metabolism of drugs and other foreign materials; in catalase and peroxidase, which prevent free radical-mediated cell damage; and in a number of other enzymes involved in energy metabolism, such as reduced nicotinamide adenine dinucleotide phosphate dehydrogenase [34].

The RDA for iron is 28-30 mg for day. An orange provides about 2mg of iron.

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CHAPTER 3

CLARIFICATION AND CONCENTRATION OF BERGAMOT JUICE BY UF/OD INTEGRATED PROCESSES

3.1 Introduction

Bergamot is mainly used for the production of the essential oil from peel which is widely employed in the cosmetic, pharmaceutical and food industries. The juice due to its better taste, has not found so far a real use in the food industry and it is considered a waste of the essential oil production. Therefore, it is interesting to investigate all the possible uses of the juice, in order to take advantage of the larger amount of this discarded product, considering the potentialities of its health promoting substances, especially in terms of ascorbic acid and flavonoids [1,2].

In order to preserve these components, the basic properties of "cold process" membrane technologies can be exploited. These technologies are ideal in the production of fruit juices with high quality, natural fresh taste and additive free since the separation process is athermal and does not involve phase change or chemical additives. In particular, membrane processes such as MF and UF represent a valid alternative to the use of traditional fining agents (gelatine, bentonite, silica sol). In these processes the juice is separated into a fibrous concentrated pulp and a clarified fraction free of spoilage microorganisms.

Membrane concentration processes such as RO, MD and OD are valid alternatives to the use of thermal evaporation which causes loss of thermo-sensitive compounds with a consequent remarkable qualitative decline of the final product [3-6].

The aim of this study was to evaluate, on laboratory scale, the potential of UF and OD processes for clarifying and concentrating depectinised bergamot juice. The performance of the membrane-based process was evaluated on the basis of the quality

of the products through the analytical measurements of the vitamin C, flavonoids and the total antioxidant activity (TAA).

3.2 Materials and methods

3.2.1 Juice extraction

Bergamot fruits were collected from plants growing in a cultivation area located in Reggio Calabria (Calabria, Italy). Fruits were halved and then squeezed by a domestic juicer (Aristarco S.r.l., Treviso, Italy). After pulping, sodium sulphite (Sigma–Aldrich, Milan, Italy) was added in order to inhibit the enzyme polyphenol oxidase that determines a browning of the pulp. A pectinase from *Aspergillus aculeatus* (Pectinex Ultra SP-L, Novo Nordisk A/S, Novo Allè, 2880 Bagsuaerd, Denmark) was also added in quantity of 10 g/kg. The enzyme is able to hydrolyse both high and low etherified pectins and also partially hydrolyze cellulose and hemicellulose. The puree was incubated for 4 h at room temperature in plastic tanks with a capacity of 5 l and then filtered with a nylon cloth. The extracting procedure gave an average juice yield of 41% (w/w). The juice was stored at -17°C and was defrosted to room temperature before use.

3.2.2 UF experimental plant and procedures

Bergamot fruit juice was clarified by using a laboratory bench plant equipped with a polysulphone hollow fibre membrane module prepared in laboratory. The bench plant consisted of a feed tank with a capacity of 5 liters, in which the juice is placed before the clarification step.

The plant was equipped with adequate devices for the regulation and control of operating parameters. The juice from the feed tank was circulated through the lumen side of the hollow fibre by using a gear pump. A thermometer placed in the feed tank was used for to control the juice temperature during the process. Two manometers located at the inlet (Pin) and outlet (Pout) of the membrane module were used to measure the inlet and outlet pressure and consequently the transmembrane pressure (TMP). The feed flow rate and the TMP value were regulated by a pressure control valve, on the retentate side, and by regulating the rpm of the gear pump. A tube heat exchanger fed with tap water was used in order to maintain constant the temperature of

the juice. A digital balance placed under the permeate tank permitted to measure the permeate flow rate and, consequently the permeate flux.

A scheme of the UF bench plant is reported in the Figure 3.2.2-1

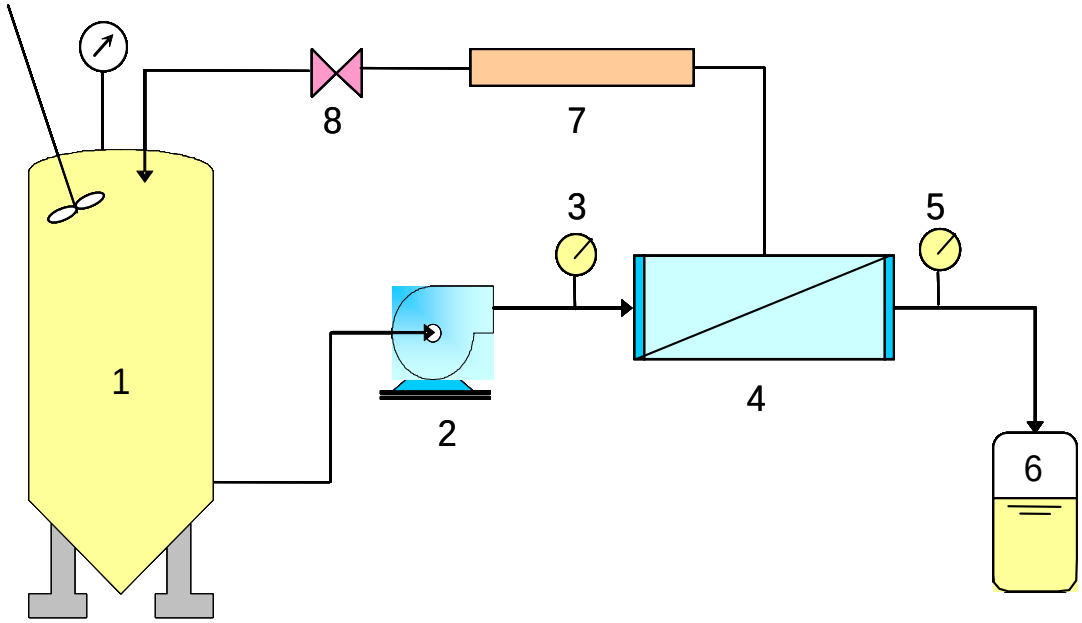

Figure 3.2.2-1 - *Scheme of ultrafiltration bench plant.*
(1-feed tank; 2-feed pump; 3,5- manometers; 4- membrane module; 6-permeate tank; 7-heat exchanger; 8-pressure valve; 9-thermometer)

Experiments were carried out according to the batch concentration procedure (Figure 3.2.2-2) in which the permeate is collected separately and the retentate is recycled to the feed tank. The UF system was operated at a TMP of 0.8 bar, an axial feed flow rate of 114 l/h and a temperature of 23.5°C up to reach a volume reduction factor (VRF, defined as the ratio between the initial feed volume and the volume of the resulting retentate) of 2.2.

The clarification process produced two fractions: a clarified juice (permeate) and a fibrous concentrated pulp (retentate).

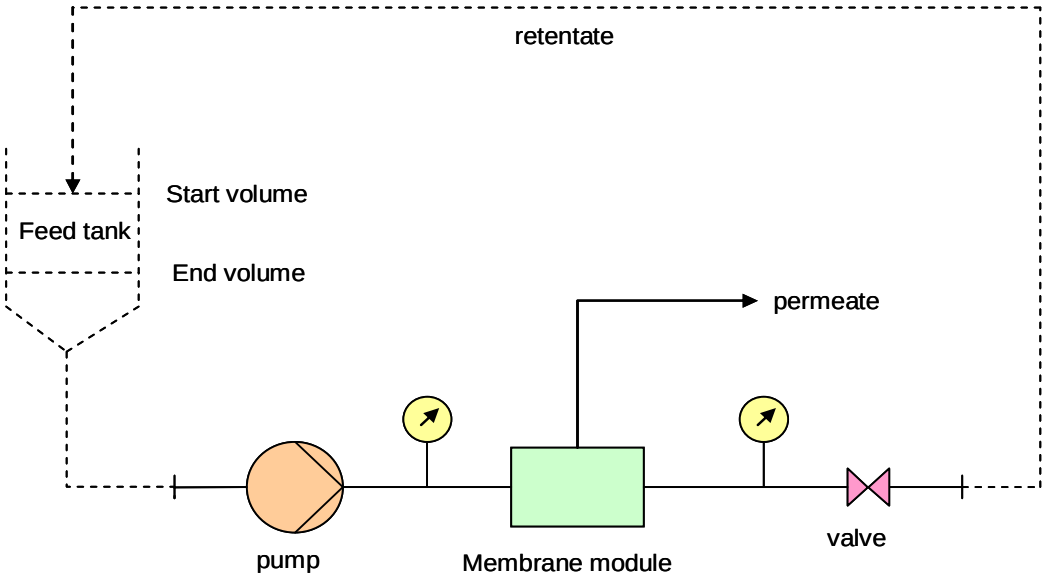

Figure 3.2.2-2 *Scheme of the batch concentration operation*

3.2.3 Characterisation of UF membranes with bidistilled water

HF membranes were characterised with bidistilled water in order to measure the hydraulic permeability.

The water permeability (L_p) was determined by feeding bidistilled water to the membranes module and measuring the water flux at different TMP values.

The water flux (J), was determined by measuring the volume of permeate (V permeate) collected in a certain time *t* through the membrane surface area, maintaining constant the feed flow rate and the feed temperature of the juice.

$$J = \frac{Vpermeate}{tA} \qquad (3.2.3\text{-}1)$$

The slope of the straight line obtained by plotting the water flux against the applied TMP, gives the measure of the water permeability [7].

The hydraulic permeability of the membrane module in the same fixed conditions was measured after each experimental run and after each cleaning treatment in order to evaluate the effect of the juice treatment on the membrane fouling.

Figure 3.2.3-1 shows the characterisation of the membrane with bidistilled water at 25 °C, at a feed flow rate of 80 l/h in the range of TMP values of 0-0.9 bar.

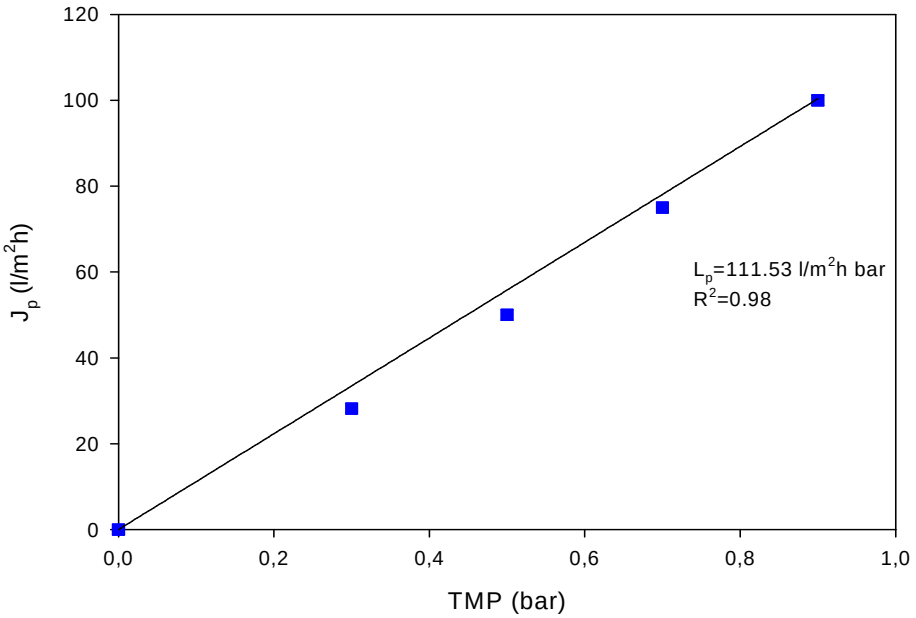

Figure 3.2.3-1 *Characterisation of UF membrane with bidistilled water*

3.2.4 Osmotic distillation unit and procedures

The permeate coming from the UF treatment was submitted to osmotic distillation (OD) experiments by using a laboratory plant supplied by Hoechst-Celanese Corporation (Wiesbaden, Germany). The plant, showed in Figure 3.2.4-1, is equipped with:

- two magnetic drive gear pumps for the circulation of both clarified juice and stripping solution in the shell side and in the lumen side (tube side) of the OD membrane module, respectively;
- four pressure gauges in order to register inlet and outlet pressures for both tube side and shell side streams;
- a digital balance (Gibertini Elettronica, Milan, Italy), placed under the juice tank, for the measure of the weight of extracted water; it was used to calculate the evaporation flux (J_w);
- two flow-meters for the measure of both brine solution and juice flow rate.

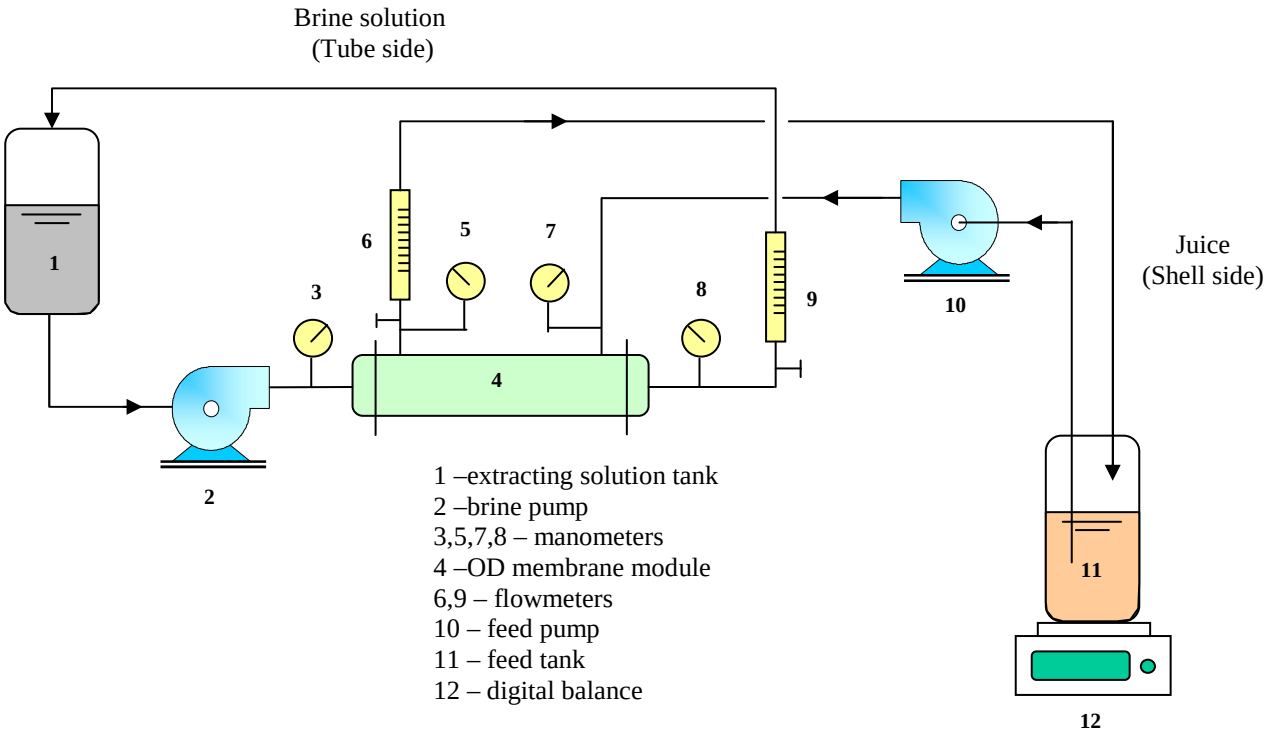

Figure 3.2.4 -1 *Scheme of the osmotic distillation bench plant*

The plant was equipped with a Liqui-Cel® Extra-Flow 2.5x8", membrane contactor supplied by Hoechst-Celanese Corporation (Wiesbaden, Germany). The OD membrane module is constituted by hydrophobic hollow fibre membranes with an external diameter of 300 μm and an internal diameter of 220 μm. Other characteristics of the OD membrane module are reported in Table 3.2.4-1.

The juice with an initial concentration of 10° Brix, was pumped through the shell side of the membrane module, while in the tube side flowed a 60 w/w% calcium chloride dehydrate (Fluka Chemie GmbH, Buchs, Switzerland) solution, in a counter current mode. It was chosen because it is not toxic and it is ready available at low cost.

Both solutions were re-circulated back to their reservoirs, after passing through the contactor, at a temperature of 28 °C±2°C.

Table 3.2.4-1 *Data sheet of Liqui-Cel® Extra-Flow 2.5x8" membrane contactor*

Fibres characteristics	Celgard® microporous
Fibre type	polypropylene hollow fibre
Cartridge Operating Limits	
Maximum Transmembrane Differential Pressure	4.2 kg/cm^2
Maximum Operating Temperature Range	40 °C
Cartridge Characteristics	
Cartridge Dimensions (DxL)	8x28 cm (2.5x8 in)
Effective Surface Area	1.4 m2
Effective Area/Volume	29.3 cm^2
Fiber Potting Material	Polyethylene

The initial weight of the stripping solution (generally 8 Kg) was two times higher compared to that of the juice, in order to prevent a significant dilution with consequent decreasing of the driving force during the process. OD system was generally operated with a slightly higher pressure on the shell side of the module than the lumen side bar in order to avoid the leakage of the brine strip into the product.

The flow rate of the extracted water, at various points during the concentration process, was calculated by measuring the weight loss of the juice over the time by a digital balance. Flow rates normalised by the membrane surface area (1.4m^2) gave the evaporation flux (J_w) values [3].

Experimental trials were performed in selective operating conditions up to reach the desired level of total soluble solids in the juice.

After each trial, the pilot plant was cleaned first by rinsing the tube side and the shell side with distilled water. Then a KOH solution at 2 w/w% was circulated for 1 h at 40 °C. After a short rinsing with distilled water, a citric acid solution at 2 w/w % was circulated for 1 h at 40 °C. Finally the circuit was rinsed with distilled water (Table 3.2.4-2).

Table 3.2.4-2 *Cleaning procedure for the OD membrane module*

Shell side	Tube side	Duration	Temperature
Distilled water	Distilled water	10-15 min	Room temperature
KOH solution at 2% (w/w)	Distilled water	60 min	40°C
Distilled water	Distilled water	10-15 min	Room temperature
Citric acid solution at 2% (w/w)	Distilled water	60 min	40°C
Distilled water	Distilled water	10-15 min	Room temperature

3.2.5 Analytical measurements

3.2.5.1 Total soluble solids

TSS measurements were carried out by using hand refractometers (Atago Co., Tokyo, Japan) with scale range of 0-32, 28-62 and 58-90 °Brix.

3.2.5.2 Total suspended solids

The suspended solids content was determined in relation to the total juice (w/w%) by centrifuging, at 2000 rpm for 20 min, 45 ml of a pre-weight sample; the weight of settled solids was determined after removing the surnatant.

3.2.5.3 Total antioxidant activity (TAA)

The total antioxidant activity was determined by an improved version of the 2,2'bis-azino-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) free radical decolouration assay in which the radical is generated by reaction with potassium persulphate before the addition of the antioxidant [8-10]. The decolouration of the blue/green $ABTS^{\cdot+}$ chromophore (radical cation) is measured as the percentage of inhibition of absorbance at 734 nm and it is referred to the reactivity of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), an analogous of vitamin E.

The concentration of antioxidants giving the same absorbance percentage inhibition of the radical cation at 734 nm as 1 mM Trolox was calculated in terms of Trolox equivalent antioxidant activity (TEAC).

The percentage of inhibition (%I) was calculated as:

$$\%I = \left(\frac{A_{ABTS} - A_{SAMPLE}}{A_{ABTS}} \right) x100 \qquad (3.2.5.3\text{-}1)$$

where :

A_{ABTS} is the is the mean value between initial and final absorbance of the $ABTS^{+}$working solution;

A_{SAMPLE} is the absorption value after 5 min of contact between the antioxidant and the ABTS solution.

A 2mM ABTS solution was prepared by dissolving the ABTS in water. The $ABTS^{\cdot}$ radical cation ($ABTS^{\cdot+}$) was produced by reacting 50 ml of ABTS, (diammonium salt, minimum 98%, Sigma Aldrich, Milan) with 500 µl of 70 mM potassium persulfate (minimum 99,0%, Sigma Ultra, Milan) solution and allowing the mixture to stand in the dark at room temperature for 6 h before use. The radical was stable in this form for more than two days. The work solution was prepared by diluting 1 ml of the $ABTS^{\cdot+}$ solution to 25 ml with phosphate buffer saline (PBS) (5mM NaH_2PO_4, 5 mM Na_2HPO_4, 9 g/l NaCl) to a final UV absorbance of 0.70±0.02 at 734 nm.

Different samples coming from the integrated UF-OD process were diluted with PBS buffer according to the following procedure:

- 40 µl of sample + 960 µl of PBS buffer ;
- 80 µl of sample + 920 µl of PBS buffer;
- 120 µl of sample + 880 µl of PBS buffer.

After this procedure samples were analysed according to the following method: addition of 1 ml of diluted ($ABTS^{\cdot+}$) solution to 10 µl of diluted sample; the absorbance reading was registered exactly 1 min after the initial mixing and up to 6 min. The absorbance value at 6 min was used to calculate the results reported as total antioxidant activity and expressed as mM trolox equivalent. Each determination was performed in triplicate. Results were expressed as means ± SD of three samples. In Figure 3.2.5-1 a scheme of the described method is reported.

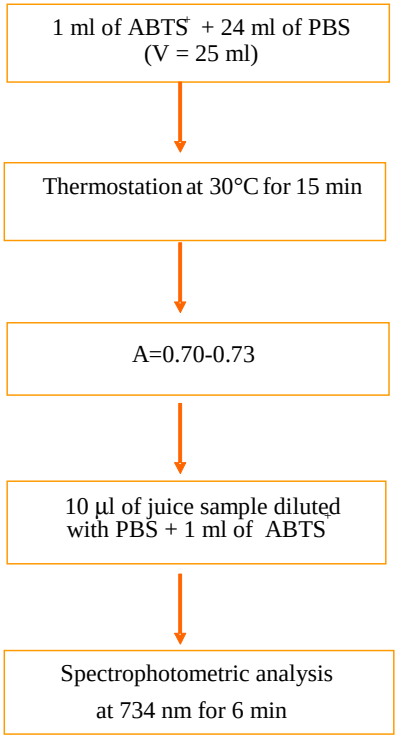

Figure 3.2.5.3 -1 *Scheme for the determination of TAA in samples of bergamot juice*

3.2.5.4 Determination of flavonoids and ascorbic acid

The concentration of flavonoids and ascorbic acid was determined by high-performance liquid chromatography (HPLC) by using a HPLC System (Agilent 1100 Series, USA) equipped with an UV detector, a quaternary pump and an HP-Chemstation data acquisition. Chromatographic separation is obtained by using a RP C 18(2) column 250*4.6mm, 5 µµ (Phenomenex, Torrance, CA, USA).

The identification and quantification of flavonoids and ascorbic acid in bergamot juice was based on the external standard method by comparing the retention times and their UV-Vis spectra with those of representative standards at different concentrations

For the evaluation of he flavonoids content, working solutions of hesperidin, naringin and neohesperidin were prepared by dissolving commercial flavonoids standards (Extrasynthese, Genay, France) in ethanol (Sigma Aldrich, Milan). Samples of fresh and concentrated bergamot juice were dissolved in a small quantity of DMSO (2ml) and 10 ml of ethanol were added later. The obtained solutions were filtered with 0.45 µm cellulose acetate filters and directly injected. For the chromatographic elution a solvent

gradient of KH_2PO_4 0.25 M (pH 3) (solvent A) and acetonitrile (solvent B) was used as indicated in Table 3.2.5.4-1. The following conditions were used: flux=1.2 ml/min; T=30°C; λ=284 nm.

Calibration curves with three different concentration of each flavonoids were used for the quantitative analysis. Each standard was injected three times.

Table 3.2.5.4- 1 *HPLC solvent gradient elution program*

Time (min)	Solvent A (%)	Solvent B (%)
0	100	0
5	75	25
30	58	42
34	58	42
34.1	0	100
39	0	100
39.1	100	0
49.0	100	0

For the evaluation of the ascorbic acid content, samples of fresh and clarified bergamot juice were directly injected (after filtration with 0.45 μm cellulose acetate HPLC filters). Concentrated samples were previously rediluted to the same concentration of the fresh juice (10°Brix). Samples were eluted in isocratic mode by using a mobile phase of H_3PO_4 0.05 M in the following conditions: flux=0.7 ml/min; T=25 °C; pressure=80 bar, λ= 205 nm.

3.3 Results and discussion

3.3.1 Clarification of the bergamot juice by UF

UF experiments carried out according to the batch concentration mode showed that the permeate flux decreased gradually with the operating times by increasing the VRF due to a concentration polarization and gel formation. The initial permeate flux of 55 l/m^2h decreased of about 75% when a final VRF value of 2.2 was reached (Figure 3.3.1-1).

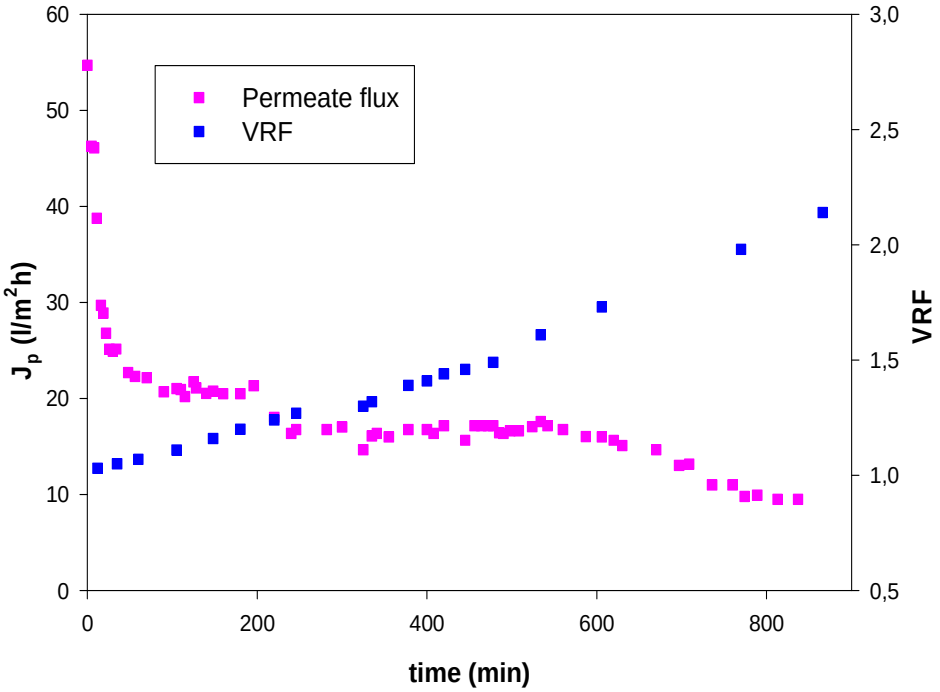

Figure 3.3.1 -1 *Clarification of bergamot juice. Time course of permeate flux and VRF. (T=23.5 °C; Qf=114 l/h; TMP=0.8 bar)*

The UF membrane retains microrganisms and large molecules as lipids, proteins and colloids, while small solutes such as vitamins, salts, sugars are allowed to flow through the membrane with water. Thus the possibility of microbilogical contamination in the permeate stream is minimised, avoiding thermal treatments and, consequently, loss of volatile aroma compounds. Moreover the UF step allowed to obtain a clarified juice more suitable for the following membrane based concentration step: indeed UF completely removed the suspended solids and the resulting clarified juice had lower viscosity and negligible turbidity [11,12].

The membrane module was rinsed with distilled water for 30 min after the treatment of the juice; then it was submitted to a cleaning procedures using NaOH (Carlo Erba, Milan) solution at a concentration of 0.5 w/w %. The cleaning solutions was circulated for 60 min at a temperature of 40°C. A final rinse of the system with distilled water for at least 20 min was carried out.

Figure 3.3.1-2 shows the characterisation of the membrane before and after cleaning procedures: a god restore of the initial hydraulic permeability was obtained after the alkaline cleaning.

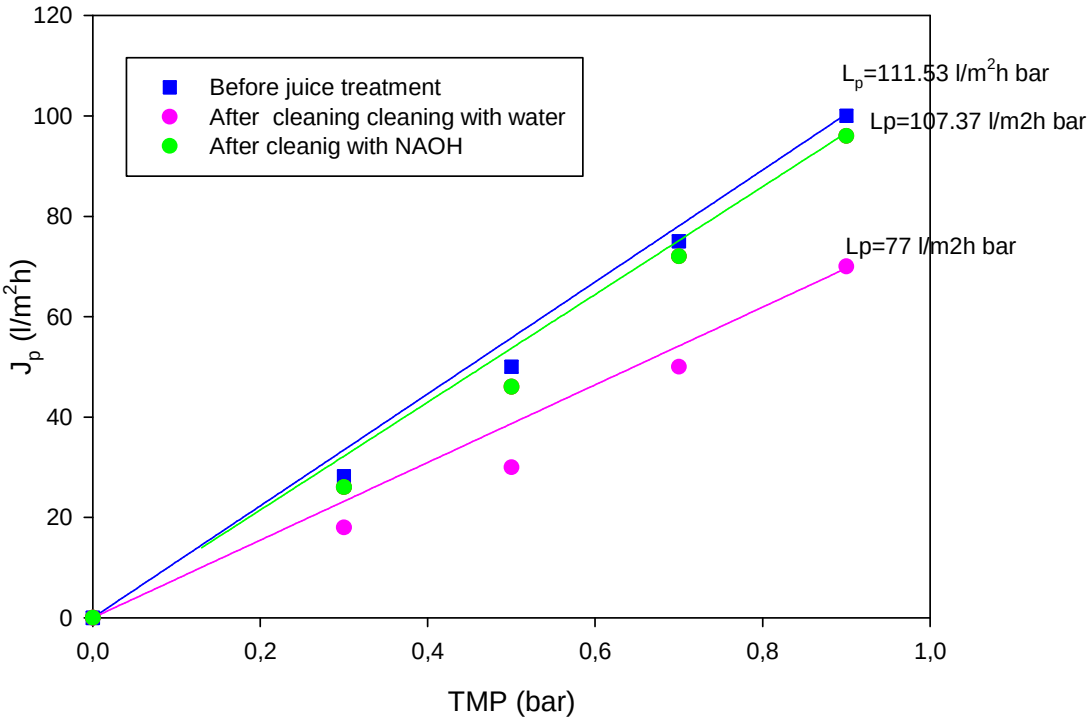

Figure 3.3.1-2 *Effect on membrane cleaning on the water permeability of the UF membrane (T=25°C)*

3.3.2 Concentration of clarified bergamot juice by osmotic distillation

Figure 3.3.2-1 shows the time course of the evaporation flux and the total soluble solids (TSS) for a generic run in which the clarified bergamot juice, with an initial concentration of 10.5°Brix, was concentrated up to 54°Brix. The juice and the brine were pumped through the shell and the tube side of the membrane module, respectively, at a flow rate of 33 l/h; both solutions were re-circulated back to their reservoirs, after passing through the contactor, at a temperature of 26°C. TMP was fixed at 0.48 bar. At first the brine concentration was 60% giving rise to an evaporation flux of about 1.4 Kg/m^{2}h. In the range 0-60 min a decrease of the evaporation flux was observed owing to the dilution of the stripping solution. In particular, a reduction of the stripping solution of 33%, and consequently to the driving force of the process, determined a 47% reduction of evaporation flux. In the range 60-190 min a further decline of the evaporation flux (39%) was observed. In this range the flux decay can be mainly attributed to the increase of the TSS concentration and consequently to the increase of the juice viscosity. The evaporation flux reached a value of 0.4 kg/m^{2}h when the juice TSS concentration was 54°Brix. These observations confirm data reported in the

literature for the concentration of sucrose solutions and passion fruit juice by osmotic distillation: at low TSS of the feed juice the flux decay is more attributable to the dilution of the stripping solution, at higher TSS concentrations it depends mainly on juice viscosity (viscous polarisation) and consequently, on juice concentration and temperature [14,15].

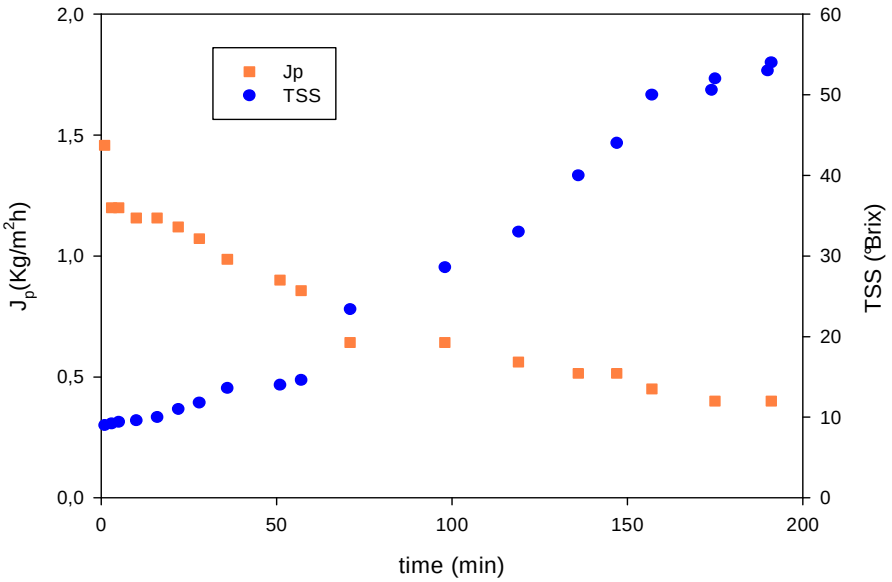

Figure 3.3.2-1 *Osmotic distillation of clarified bergamot juice. Time course of evaporation flux and TSS concentration*

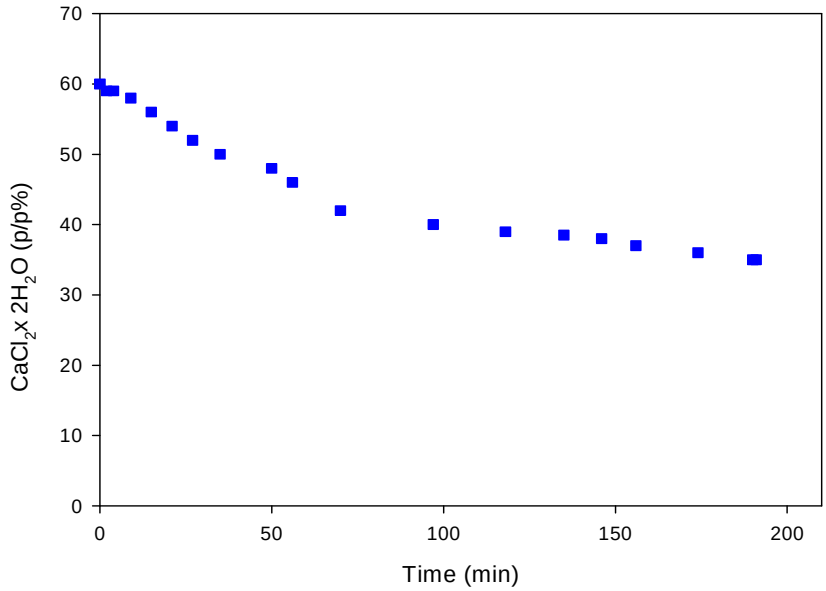

Figure 3.3.2-2 *Osmotic distillation of clarified bergamot juice. Time course of brine concentration*

3.3.3 Analytical evaluations

In order to evaluate the effect of the integrated UF-OD process on the juice quality and its TAA, analytical determinations were performed on samples collected during both membrane processes.

In Table 3.3.3-1 the evaluation of TSS, suspended solids and ascorbic acid in samples coming from the integrated UF- OD process is shown.

The rejection of the UF membrane towards these compounds was measured according to the following equation:

$$R = \left(1 - \frac{Cp}{Cf}\right) x100 \qquad (3.3.3\text{-}1)$$

where C_p is the solute concentration in the permeate and C_f is the solute concentration in the feed.

According to the equation 3.3.3-1 the rejection of the UF membrane towards the ascorbic acid and TSS was 12.3% and 4%, respectively. Suspended solids wew completely removed from the juice while ascorbic acid and TSS were recovered in the clarified fraction. In Table 3.3.3-2 a mass balance of the UF process for the ascorbic acid is reported. Considering a recovery factor, in terms of clarified juice, of 53.4%, the quantity of ascorbic acid in this fraction was 46.7%.

The retentate samples of the OD process, at different values of TSS concentration, showed the same content of ascorbic acid of the clarified juice: the OD process has no influence on the acid ascorbic content independently by the concentration degree achieved.

Table 3.3.3-3 shows the analytical evaluation of naringin, hesperidin and neohesperidin in samples of clarified and concentrated bergamot juice. The observed rejection of the UF membrane towards flavonoids was in the range 0.5-2.7%, a lower value if compared to that of the ascorbic acid.

Table 3.3.3-1 *Analytical evaluations in samples of bergamot juice coming from the UF-OD treatment*

Membrane	Sample	TSS (°Brix)	Suspended solids (%)	Ascorbic acid* (mg/L)
UF	Feed	10.5	11	252
	Permeate	10	-	221
	Retentate	12	94	224
OD	Feed	10	-	215
	Retentate1	20	-	212
	Retentate2	34	-	213
	Retentate3	54	-	212

***Value referred to the same TSS content of the fresh juice

Table 3.3.3-2 *Mass balance of the UF membrane for the ascorbic acid*

	Feed	Permeate		Retentate		Balance
Volume (liters)	4.02	2.14	53.2%	1.88	46.8%	100%
Ascorbic acid (mg)	1013.1	472.5	46.7%	459.2	45.3%	92%

The mass balance of the UF process for flavonoids is reported in Table3.3.3-4. It can be noted that the recovery of flavonoids in the permeate fraction is in the range of 51-53% when the recovery factor of the permeate is 53.2%. Therefore the mass balance of the UF process is in agreement with the measured rejection and with the observed recovery factor. During the OD process the flavonoids concentration in the retentate remained constant independently by the achieved value of TSS.

Table 3.3.3-3 *Analytical evaluations in samples of bergamot juice coming from the UF/OD process**

Membrane	Sample	TSS (°Brix)	Naringin (mg/L)	NeoHesperidin (mg/L)	Hesperidin (mg/L)
UF	Feed	10.5	48	75	5.6
	Permeate	10	46.7	74.6	5.52
	Retentate	12	48.86	76	5.6
OD	Feed	10	42	74	4.85
	Retentate1	20	41.5	74.3	4.70
	Retentate2	34	42.4	72.5	4.88
	Retentate3	54	42.4	73	4.88

*Values referred to the same °Brix of fresh juice

Table 3.3.3-4 *Mass balance of the UF membrane for flavonoids*

	Feed	Permeate		Retentate		Balance
Volume (liters)	4.02	2.138	53.2%	1.8820.	46.8%	100%
Hesperidin (mg)	22.5	11.8	52.4 %	10.53	46.8%	99.2
NeoHesperidin(mg)	301.2	160	53.1 %	143	47.47	100
Naringin (mg)	193	99.8	51.7 %	91.95	47.6	98.6

In Figure 3.3.3-1 the chromatographic profile of the flavonoids of the depectinised bergamot juice and of the OD retentate at 54° Brix is reported. The flavonoids profile of the depictinised juice shows typical components of the bergamot juice (neoeritrocin, naringin and neoheperidin). They are very well preserved in the concentrated juice.

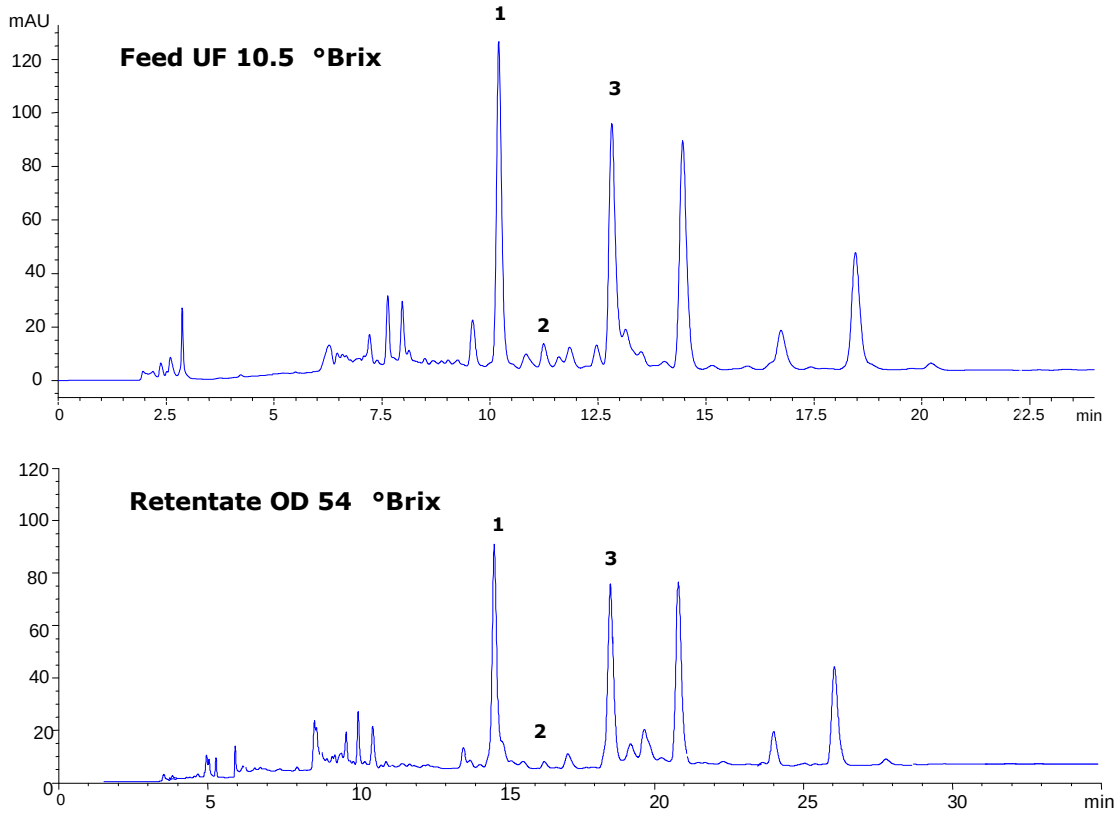

Figure 3.3-5 *HPLC chromatogram of flavonoids in feed, permeate and retentate. Peaks: 1, naringin; 2, hesperidin; 3, neohesperidin*

In Figure 3.3.3-2 the variation of the TAA during the integrated membrane process UF/OD is showed. Considering the UF process only a slight decrease of TAA in the permeate fraction was observed (7%). In the UF retentate the TAA was the same of the depectinised juice. The concentration treatment by OD did not induce other significant changes in the TAA value, independently by the TSS concentration achieved: the highly concentrated sample at 54°Brix, showed a high value of TAA (14 mM trolox), only 10% lower than the fresh juice.

The obtained result of the TAA are in agreement with the analytical results of ascorbic acid and flavonoids. These compounds contribute to the antioxidant activity of the juice and were very well preserved during the UF/OD integrated process.

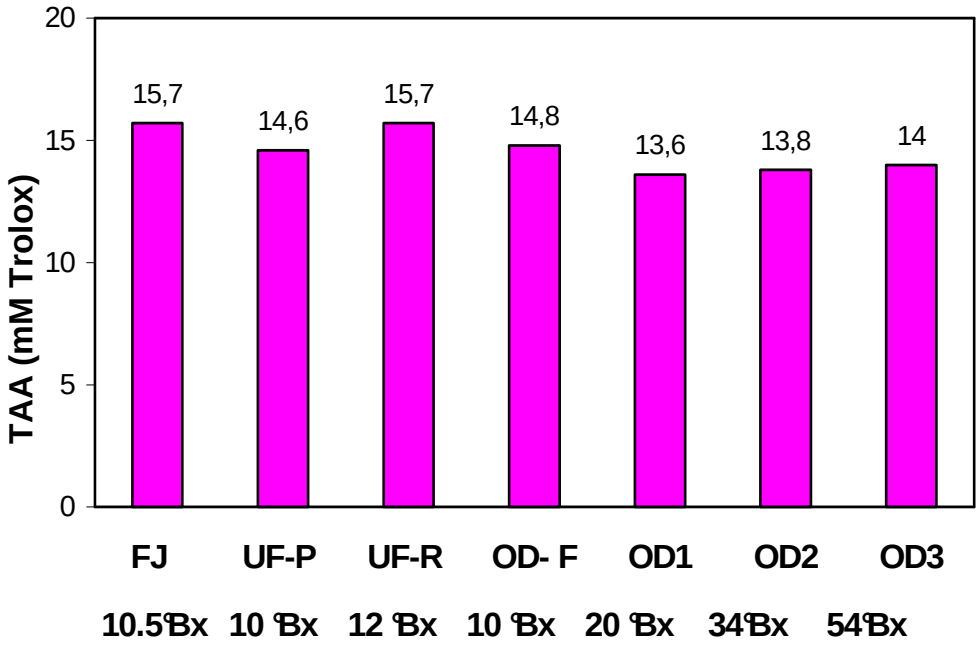

Figure 3.3.3-2 *Variation of TAA during the integrated UF-OD process*

On the basis of the results obtained on laboratory scale an integrated membrane process scheme (Figure 3.3.3-3) for producing a concentrated bergamot juice with high nutritional value was proposed. In this process UF and OD steps represent a valid alternative to the traditional clarification and concentration process based on the use of fining materials (gelatine, bentonite, silica sol) and thermal evaporation, respectively. The residual fibrous phase coming form the UF process (retentate) could be submitted to a stabilising treatment (pasteurisation, ohmic heating, high pressures) and successively added together with the water, to the final OD concentrate for the preparation of fibres enriched beverage [15]. Besides, the final retentate of the OD process is a good source of antioxidants and it can be used in foods and nutritional supplement formulations.

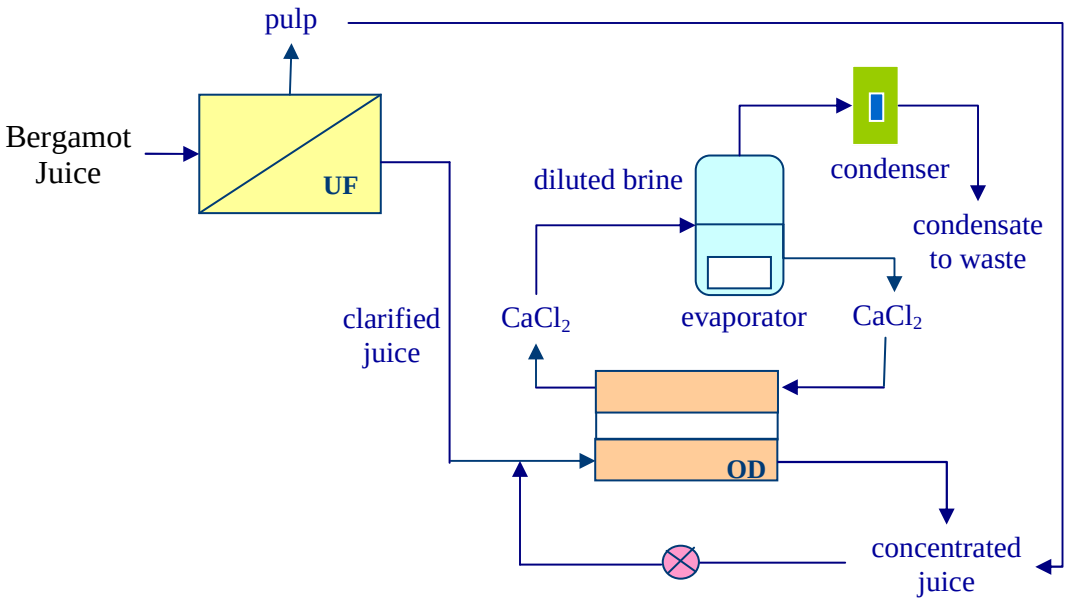

Figure 3.3.3-3 *Integrated membrane process for the production of concentrated bergamot juice*

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CHAPTER 4

RECOVERY OF POLYPHENOLS IN BERGAMOT JUICE BY INTEGRATED MEMBRANE PROCESS

4.1 Introduction

Current studies on bergamot juice revealed important pharmacological effects for the presence of different compounds able to reduce the cholesterol and the serum lipid levels. These properties can be attributed to the presence of a high content of polyphenols, and particularly flavonoids including flavanones, flavones and polymethoxyflavones [1-3].

The extraction of polyphenols from vegetable materials by using organic solvents is a classical operation applied to many industrial processes, particularly the pharmaceutical industry. This method is safe and efficient; however, it involves high capital cost and the high temperature required to increase the extraction rate may denature the polyphenols. Moreover, the extract may contain solvents which are considered unsafe for human consumption. Finally, the extraction with hydrophilic solvents is limited by the co-extraction of sugars from the fruit.

Therefore, technologies able to separate sugar from polyphenols are needed in order to concentrate the polyphenolic fraction for nutraceutical uses [4].

Within the agro-food industry membrane technologies can work as well as or better than the existing technology regarding product quality, energy consumption and environmental issue. They offer a competitive alternative to thermal processes which cause irreversible change of the aroma profile and colour degradation. On the other hand, current filtration of a wide variety of juices is performed by using fining agents such as gelatine, diatomaceous earth, bentonite and silica sol which cause problems of environmental impact due to their disposal.

In this work an integrated membrane process was also investigated for the separation and concentration of polyphenols in the bergamot juice, in order to develop a natural product enriched in polyphenols suitable for nutriaceutical applications. In particular, the bergamot juice, depectinised after an enzymatic treatment, was submitted to a preliminary ultrafiltration treatment devoted to the removal of suspended solids.

The clarified juice was then submitted to different ultrafiltration (UF) and nanofiltration (NF) processes in order to evaluate the effect of the nominal molecular weight cut-off (NMWCO) on the rejection of the membranes towards sugars and polyphenols (Figure 4.1-1). The separation process was monitored by comparing the concentration of total polyphenols, flavonoids, total soluble solids (TSS), organic acids and total antioxidants activity (TAA) in the permeate and retentate fractions with the composition of the fresh juice.

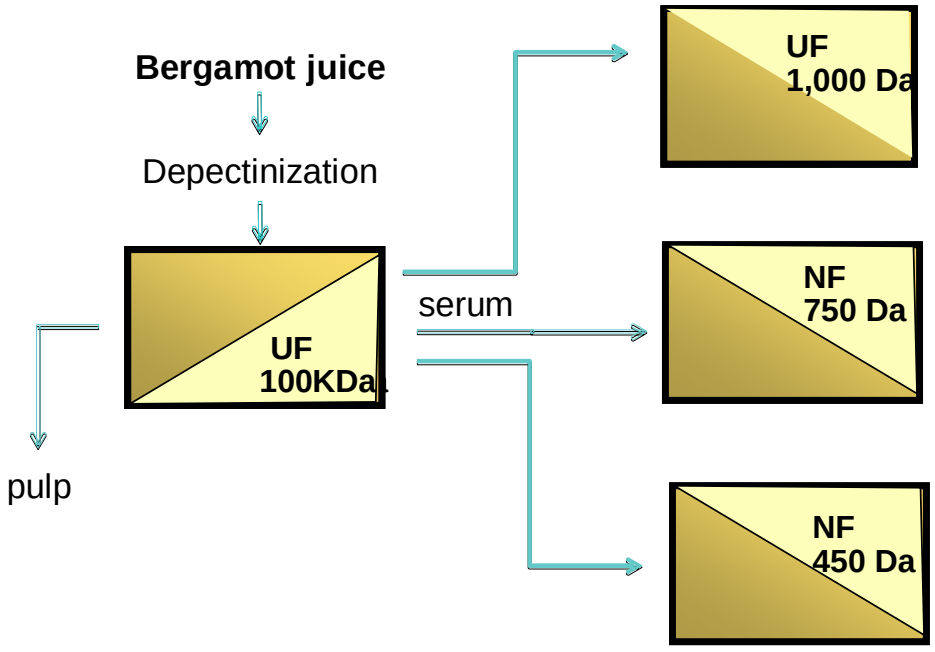

Figure 4.1-1 *General process scheme for the recovery of polyphenols from bergamot juice*

4.2 Material and methods

4.2.1 Bergamot juice

The bergamot juice was supplied by Gioia Succhi Srl (Rosarno, Reggio Calabria, Italy). Juice depectinization (treatment with pectinase from *Aspergillus aculeatus*, 10 g/kg of pulp, 4 h at room temperature) was described in the previous par. 3.2.1. After the enzymatic treatment the juice was filtered with nylon cloth and stored at -17 °C. It was defrosted to room temperature before use.

4.2.2 UF and NF equipment

UF experiments were performed by using the same equipment desribed in the previoud par.3.2.2. NF experiments were performed by using a laboratory bench plant equipped with 3 litres feed tank, a high pressure pump, a thermometer for the control of temperature, two manometers for the measure of the inlet and outlet pressures, a pressure control valve and a cooling coil fed with tap water used to maintain the feed temperature constant.

4.2.3 UF and NF membranes

Experimental trials were performed by using different UF and NF membranes. These membranes were selected on the basis of their particular molecular weight cut-off (MWCO). The depectinised juice was clarified by using a polysulphone hollow fibre membrane module supplied by China Blue Star membrane Technology Co., Ltd (Beijing China). The clarified juice was then submitted to an UF treatment by using a composite fluoro polymer flat sheet membrane with a NMWCO of 1,000 Da supplied by Alfa Laval (Lund, Sweden). The NF treatment of the clarified juice was performed by using two monotubular ceramic membranes with NMWCO of 750 and 40 DA, respectively, supplied by Inopor (Veilsdorf, Germany). In Table 4.2.3-1 the main properties of the selected membranes are reported.

Table 4.2.3-1 *Characteristics of the UF and NF membranes*

Type	DCQ	Etna01PP	Inopor	Inopor
Configuration	Hollow fibre	Flat-sheet	Tubular	Tubular
Membrane material	Polysulphone	Polymer	TiO_2	TiO_2
Operating pressure	1-1.5	1-10		
Operating temperature	0-40	0-60	350	350
Operating pH	2-9	1-11	0-14	0-14
Membrane surface area	0.16 m^2	38.46 cm^2	48 cm^2	48 cm^2

Membranes were characterised with distilled water, in fixed conditions of temperature (25°C) and at different values of TMP, in order to measure their water permeability. The water permeability was measured, in the same fixed conditions, before and after the experimental trials as well as after each cleaning procedure. In Figures 4.2-3-1, 4.2.3-2, 4.2.3-3 and 4.2.3-4 the hydraulic permeabilities of the selected membranes are reported. The water permeability indicated as L_p decreased by decreasing the NMWCO.

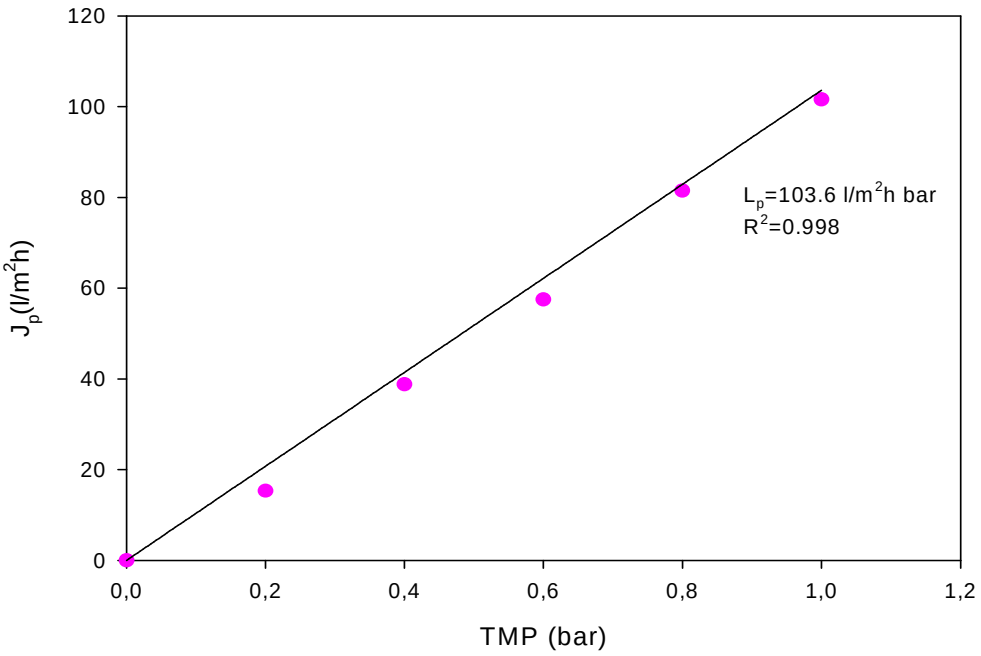

Figure 4.2.3-1 *Characterisation of 100 KDa UF membrane with distilled water .(T=25°C)*

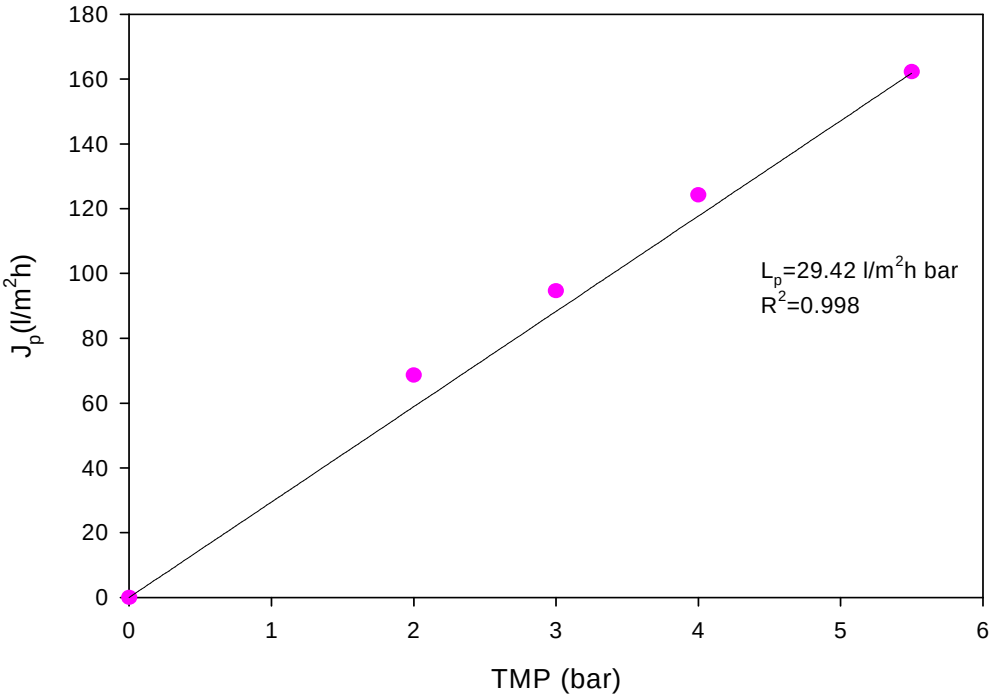

Figure 4.2.3-2 *Characterisation of 1000 Da UF membrane with distilled water. (T=25 °C)*

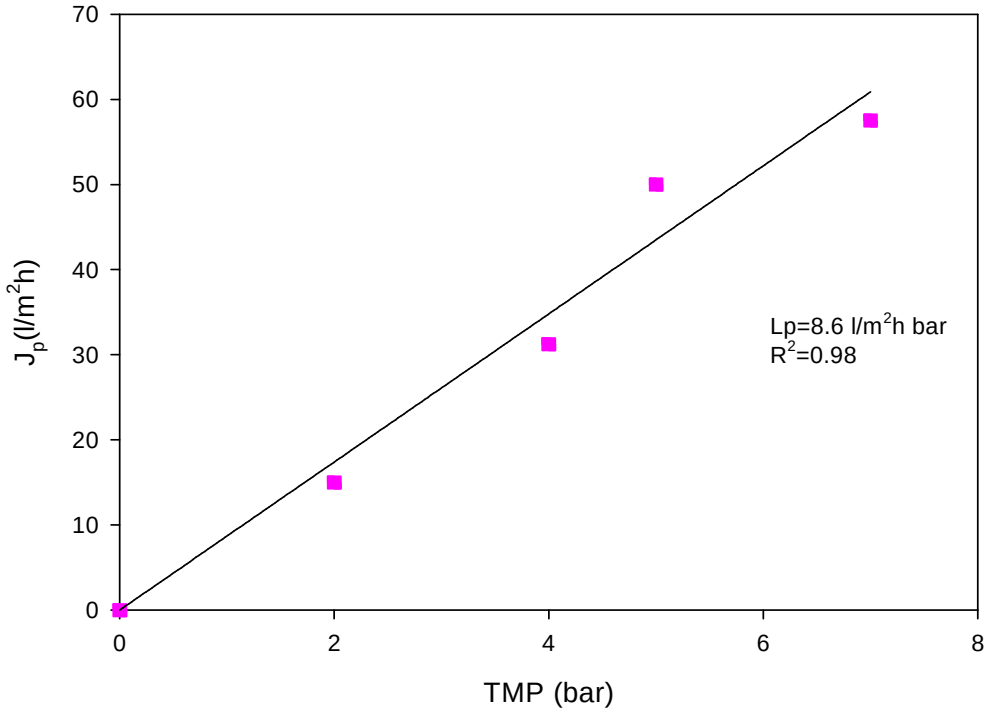

Figure 4.2.3-3 *Characterisation of 750 Da NF membrane with distilled water. (T=25 °C)*

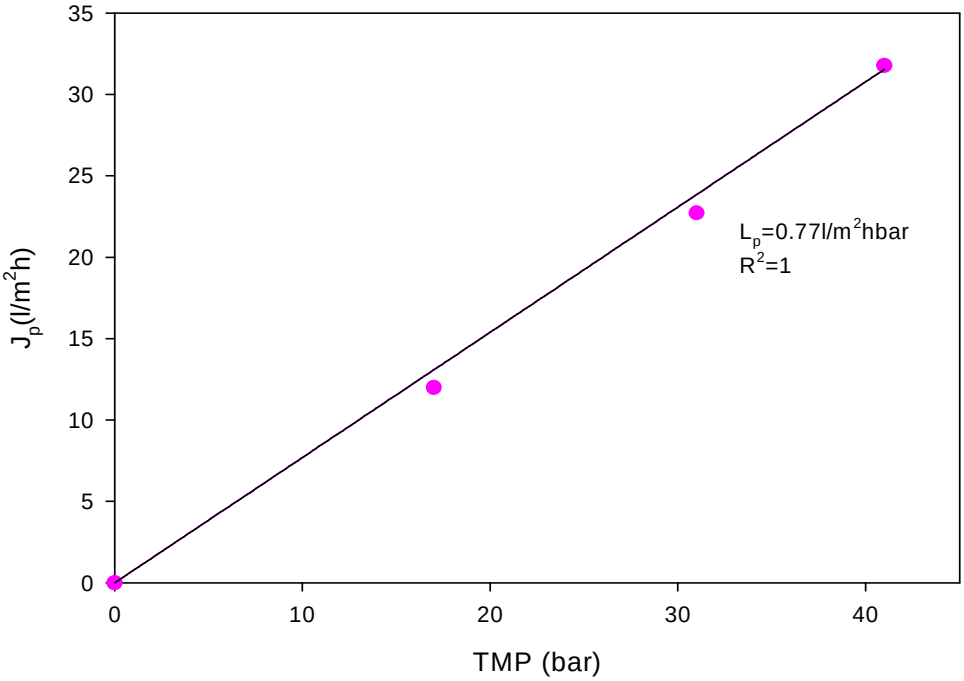

Figure 4.2.3- 4 *Characterisation of 450 Da NF membrane with distilled water. (T=25°C)*

4.2.4 Analytical determinations

Feed, permeate and retentate samples were analysed in relation to suspended solids, total soluble solids, pH, total antioxidant activity, organic acids and total polyphenols.

Suspended solids, total soluble solids and total antioxidant activity were evaluated according to the procedure reported in the previous chapter.

pH was measured by an Orion Expandable ion analyser EA 920 pH meter (Allometrics, Inc., LA, USA).

4.2.4.1 Total phenolics content

Total phenolics content was determined by using the Folin-Ciocalteau reagent (Sigma Aldrich, Milano, Italy) according to the method reported by Slinkard e Singleton [5]. The procedure is based on the observation that the phenolic substances are oxidised by the Folin-Ciocalteau reagent which contains a mixture of phoshotungstic acid and phosphomolybdic acid. The reagent becomes partly reduced resulting in the production of the complex molybden-tungsten blue, which is measured spectrophotometrically at 756 nm.

1.0 ml Folin-Coicalteau's phenol reagent diluted 1:10 with bidistilled water was added to 0.2 ml of bergamot juice. 0.8 ml of a 7.5 % sodium carbonate (Sigma Aldrich, Milano, Italy) was added to develop the color and the mixture was mixed for 1 min. After 30 min the absorbance was readed at 765 nm, against bidistilled water. The concentration of total phenols was calculated from the standard curve obtained by gallic acid solutions at different concentration (0, 5, 10, 20, 40, 60, 80, 100 mg/l). Results were expressed as gallic acid equivalents.

4.2.4.2 Flavonoids and organic acids

Flavonoids (hesperidin, naringin, neohesperidin and narirutin) were determined by using a HPLC system (Agilent 1100 Series, USA) equipped with a pump, an UV-Vis detector and a data acquisition system. Chromatographic separation was performed by using a Luna C 18(2) column (250x4.6mm, 5μm Phenomenex, Torrance, CA, USA); the following conditions were used: flux=1ml/min, T=25°C, pressure=100 bar, λ =284 nm. The mobile phase was a mixture of 80:20 water/KH_2PO_4 0.25 M (v/v) (solvent A) and a mixture of 46:4:50 water/KH_2PO_4 0.25 M/Acetonitrile (solvent B). A six-step linear gradient analysis for a total run time of 40 min was used.

The quantitative determination of ascorbic, malic and citric acids was carried out by using the same HPLC system equipped with an Alltima C18 HP 5U column (250x4.6 mm, 5 μm) (Alltech Associates, Inc., IL); samples were eluted in isocratic mode by using a 0.025 M KH_2PO_4 solution at pH 2.5. Operating conditions were as follows: flow rate 0.7 ml/min, temperature 25°C, pressure 80 bar. A sample volume of 10 μl was used. Analyses were monitored at 205 nm.

Prior to HPLC analysis all samples were filtered by using 0.45 μm cellulose acetate filters. All flavonoids and organic acids were identified by matching the retention time and their spectral characteristics against those of standards. Quantisation was made according to the linear calibration curves of standard compounds.

4.3 Results and discussion

4.3.1 Ultrafiltration of depectinised with 100 KDa membrane

UF experiments were performed at a TMP of 0.7 bar, an axial feed flow rate of 114 l/h and a temperature of 24°C according with the batch concentration mode. The UF

system was operated to clarify the juice up to a recovery factor of 87% corresponding to a volume reduction factor of 7.8.

The results showed that the permeate flux decreased gradually with the operating time by increasing the volume reduction factor due concentration polarization and gel formation. The initial permeate flux of 9 kg/m^{2}h decreased to about 3 kg/m^{2}h by increasing the operating time (Figure 4.3.1-1). The J_p/VRF curve can be divided into three periods. An initial period in which a rapid decrease of permeate flux occured; a second period up to VRF 3, corresponding to a smaller decrease of the permeate flux; a third period characterised by a small decrease of the permeate flux up to a steady state value [6,7].

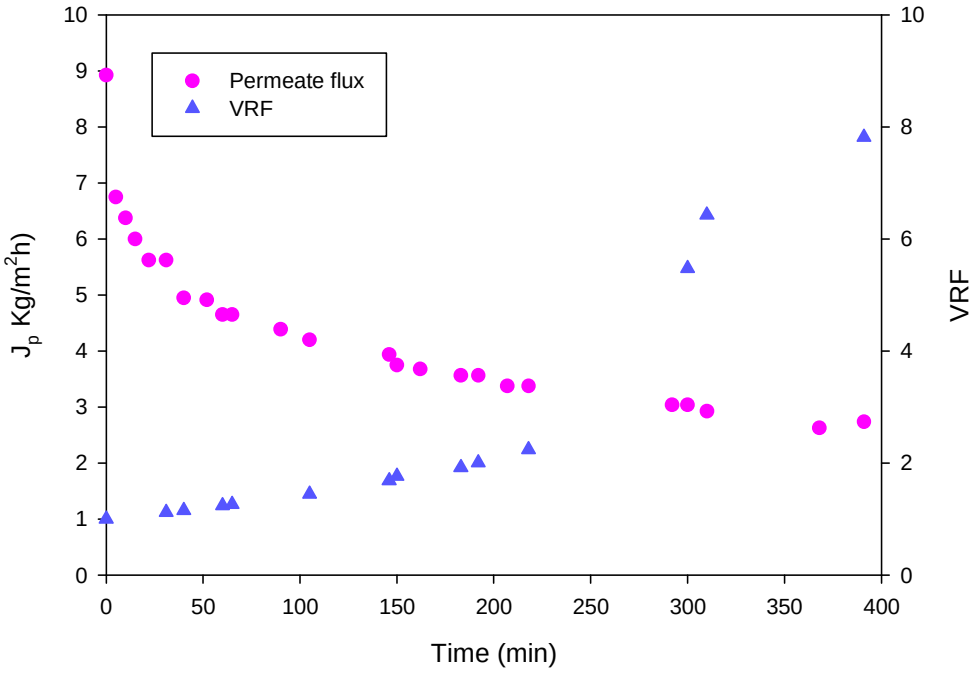

Figure 4.3.1-1 *Ultrafiltration of depectinised bergamot juice. Time course of permeate flux and VRF. (TMP= 0.7 bar; Qf = 114 ml/min; T= 24°C)*

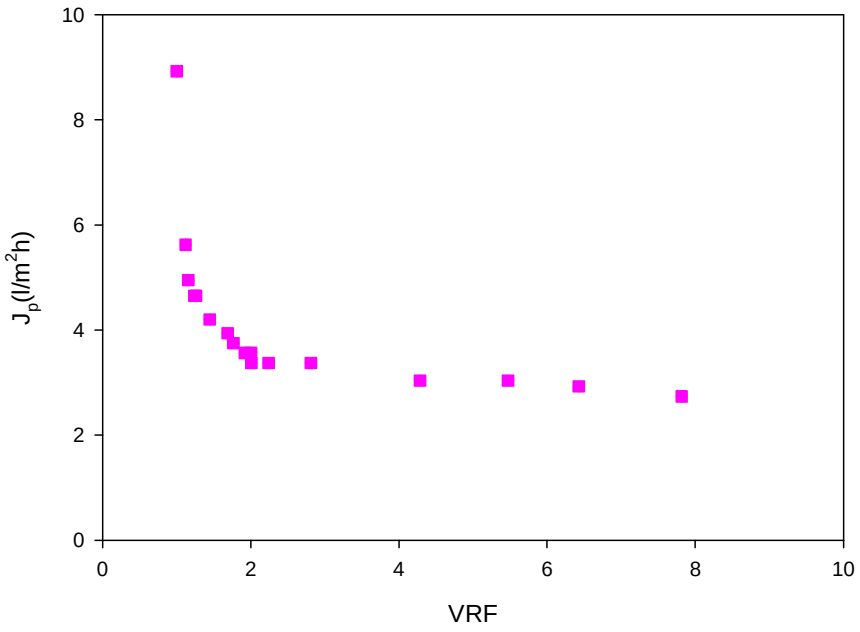

Figure 4.3.1-2 *Ultrafiltration of depectinised bergamot juice. Variation of permeate flux as a function of VRF. (TMP = 0.7 bar; Qf = 114 l/h; T= 24°C)*

In order to evaluate the degree of membrane fouling the water permeability of the UF membrane was measured after the treatment of the juice and compared with the initial value measured before the clarification step.

The membrane was then submitted to different cleaning procedures in order to restore the initial water permeability. Cleaning solutions were re-circulated in the UF plant for 60 minutes, according to the total recycle configuration, at a temperature of 40 °C at high flow rates and at low TMPs in order to avoid pore blocking phenomena. A good restore of the hydraulic permeability was observed after the following cleaning procedure:

- distilled water for 30 minutes after the treatment with the juice;
- NaOH solution at 0.1 % (w/w);
- enzymatic solution at 1% (w/w).

At the end of each cleaning procedure the membrane module was rinsed with distilled water for 20 min and the water permeability of the membrane in fixed conditions (T=24 °C; Q_f= 70 l/h) was measured.

Figure 4.3.1-3 shows the pure water flux of the membrane before and after cleaning treatments.

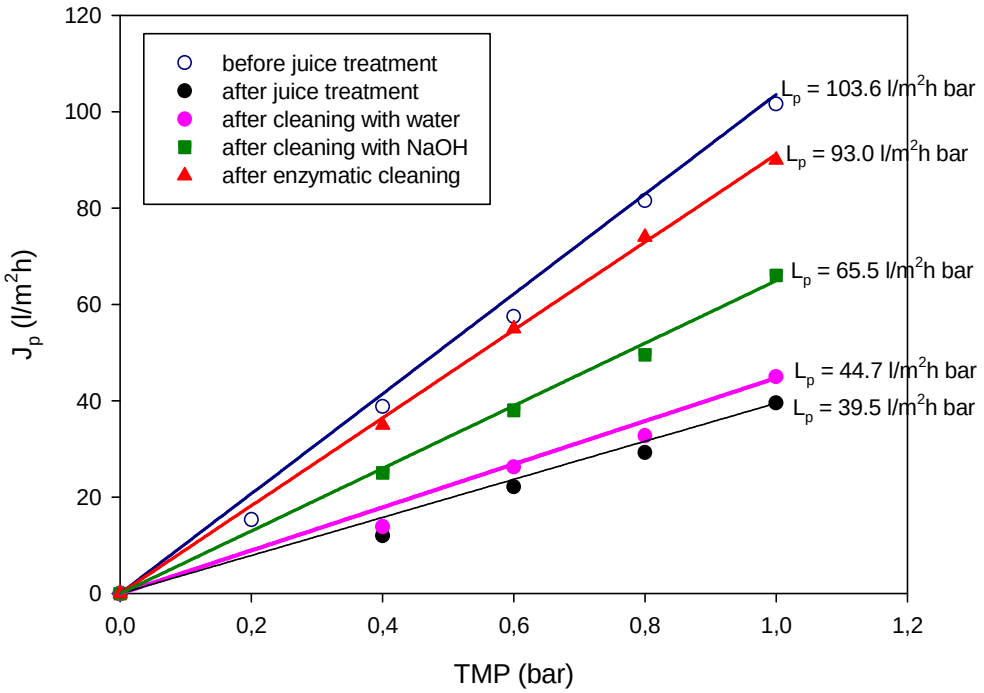

Figure 4.3.1-3 Pure water flux of the membrane before and after cleaning treatments. (operating conditions: T=24 °C; Q_f=70 l/h)

4.3.2 Ultrafiltration of clarified juice with 1000 Da membrane

Experimental trials with the 1,000 Da UF membrane were performed according two types of operating configuration: the total recycle and the batch concentration mode. In the former both permeate and retentate streams were recycled back to the feed tank to ensure a steady-state in the volume and composition of the feed.

Experimental trials were devoted to the investigation of the juice pH and TMP on the rejection of the UF membrane towards sugars and polyphenols. Figure 4.3.2-1 shows the effect of the TMP on the steady-state permeate flux in the range 3.75-12.5 bar in fixed conditions of feed flow rate (76 l/h) and of temperature (24°C).

As the pressure is increased the permeate flux shows a deviation from a linear flux-pressure behaviour and it becomes independent of pressure. In these conditions a limiting flux is reached at TMP value of about 10 bar and any further increase in pressure determines no significant increase of the permeate flux. The existence of a

limiting flux can be related to the concentration polarization phenomenon due to the build-up of rejected compounds on the membrane surface [6-9].

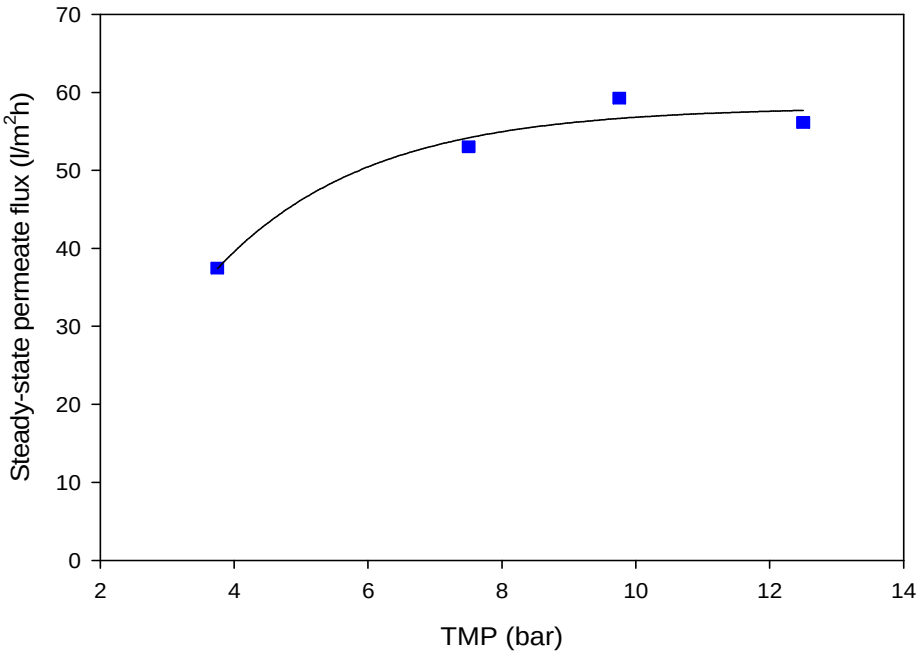

Figure 4.3.2-1 Effect of TMP on the permeate flux during UF of clarified juice (operating conditions: Q_f= 76 l/h; T=24°C)

Figure 4.3.2-2 shows the time course of the permeate flux at different pH values of the juice in selected operating conditions of temperature (25°C), feed flow-rate Q_f (1560ml/min) and TMP (7.5 bar). The initial value of the permeate fluxe was practically the same in the range of pH investigated and it decreased gradually with operating time. However, by increasing the pH of the juice from 2.8 to 8.5 a lower value of the permeate flux at steady–state was measured. This phenomenon could be attributed to an increase of fouling of the membrane, at high pH values, due to a change in the chemical composition of the juice; as reported by Chethanthe et al.[10], an increase in pH results in precipitation of polyphenols and the quantity of precipitate formed increases concurrently with increase in pH [11].

Figure 4.3.2-3 shows the time course of the permeate flux and of the VRF for a generic run carried out according to the batch concentration mode at an applied pressure TMP of 7.5 bar, at a temperature of 24 °C and at a feed flow rate of 97 l/h. The initial permeate flux was of about 80 kg/m^{2}h and only a 20% reduction of the initial flux was

obtained when the VRF reached the final value. At the end of the UF process a recovery factor of 88% was reached.

The preliminary treatment of the depectinised juice with the 100 KDa membrane limits the polarization concentration and fouling phenomena during the process producing higher permeate fluxes and steady state values [12-14].

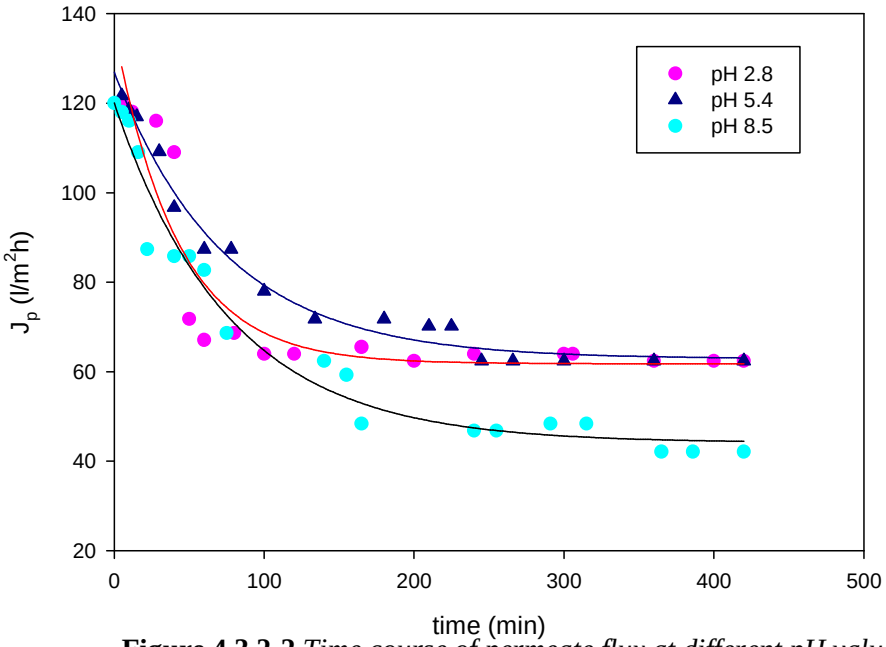

Figure 4.3.2-2 *Time course of permeate flux at different pH values (TMP = 7.5 bar; Qf = 1560 ml/min; T = 25°C)*

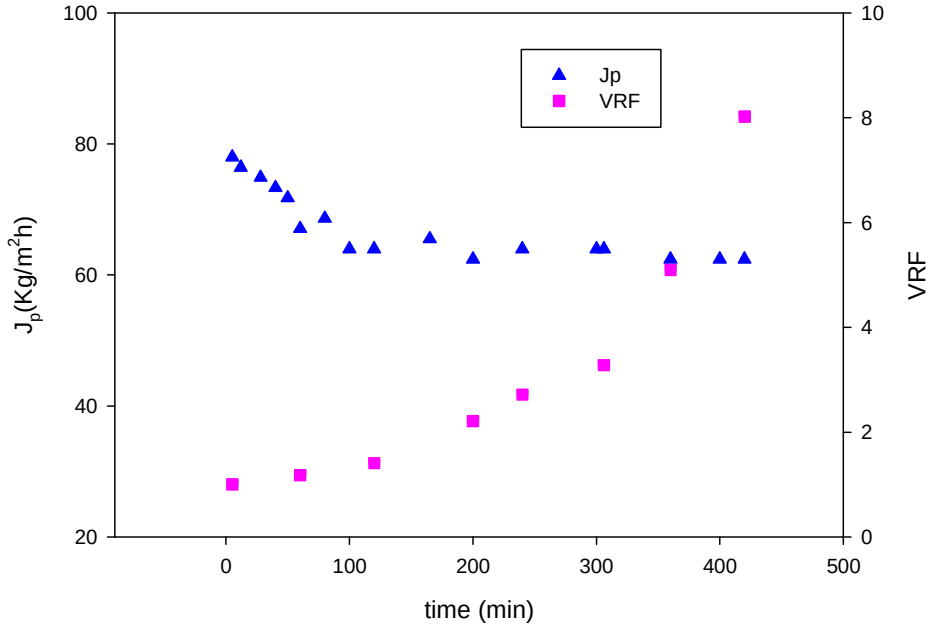

Figure 4.3.2-3 *Ultrafiltration of clarified bergamot juice. Variation of permeate flux as a function of VRF. (TMP=7.5 bar; Qf =97.6 l/h; T= 24°C)*

Figure 4.3.2-4 shows a hydraulic permeability values of the UF membrane after the treatment of the clarified juice and after a cleaning with a NaOH solution at 0.1%. The membrane water permeability dropped by 23 % after the juice treatment; a complete recovery (99%) of the hydraulic permeability was obtained after the cleaning with the alkaline cleaning solution.

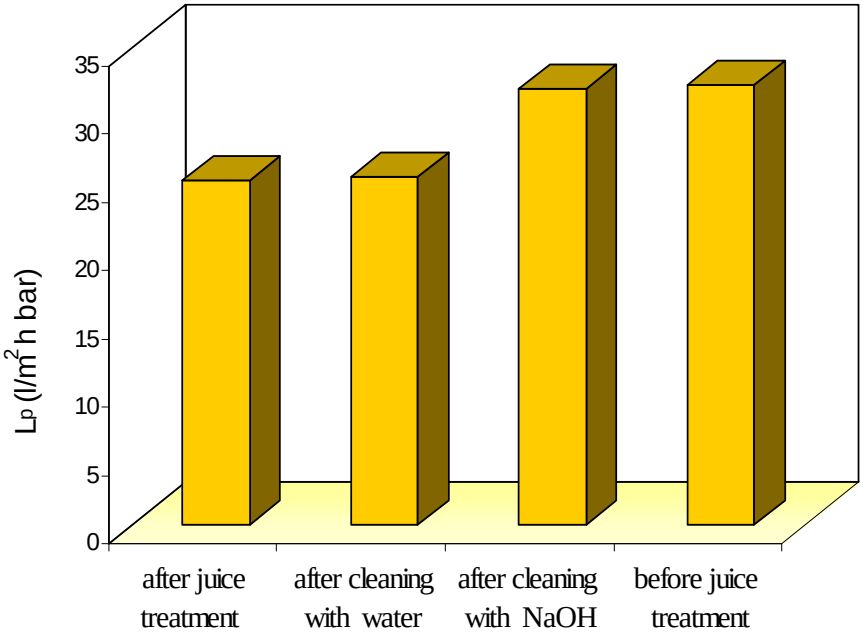

Figure 4.3.2-4 *Regeneration of water permeability in the UF membrane.* *(T=25 °C)*

4.3.3 Nanofiltration of clarified bergamot juice.

The clarified juice was processed by using two different NF membranes, with the same chemical and physical properties but different NMWCO. Experimental trials were carried out according to the batch concentration mode in selected operating conditions (TMP=7.5 bar; Qf =97.6 ml/min; T= 24°C for the NF 750 Da membrane; TMP=33 bar; Qf =100 ml/min; T= 24°C for the NF 450 Da membrane).

Figure 4.3.3-1 and 4.3.3-2 show the time course of the permeate flux observed for both the membranes. In the initial stage a higher decline of the permeate flux was observed for the 450 Da membrane; in particular, the initial permeate flux of about 40 l/m^{2}h descreased to 18 l/m^{2}h after 80 minutes and remain constant by increasing the operating time. For the 750 Da membrane a higher steady–state (about 25 l/m^{2}h)

was measured. The different phenomena of flux decline can be attributed to a different mechanism of polarization concentration and fouling.

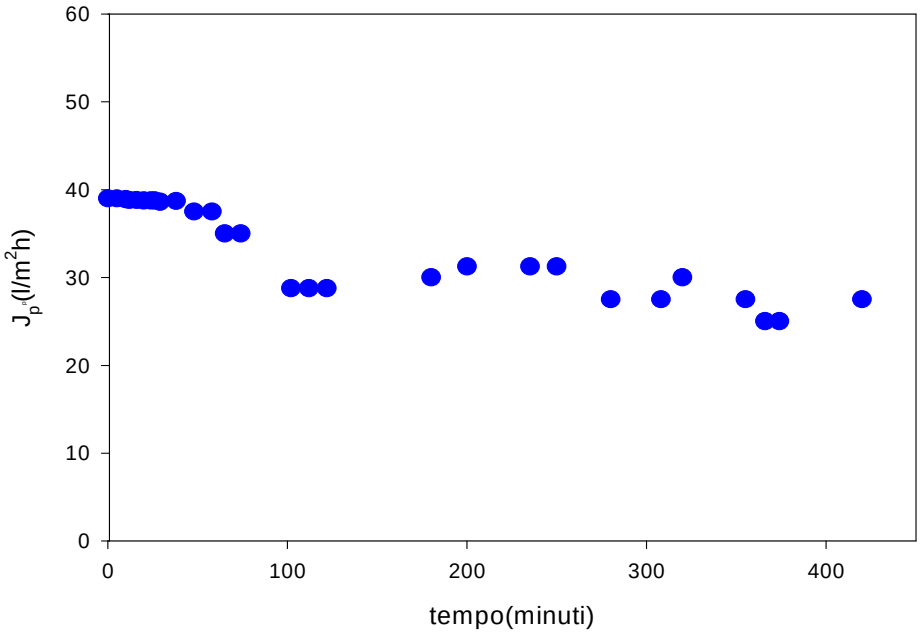

Figure 4.3.3-1 *Nanofiltration of clarified juice with 750 Da membrane. Time course of permeate flux. (TMP=7.5 bar; Qf =97.6 ml/min; T= 24°C)*

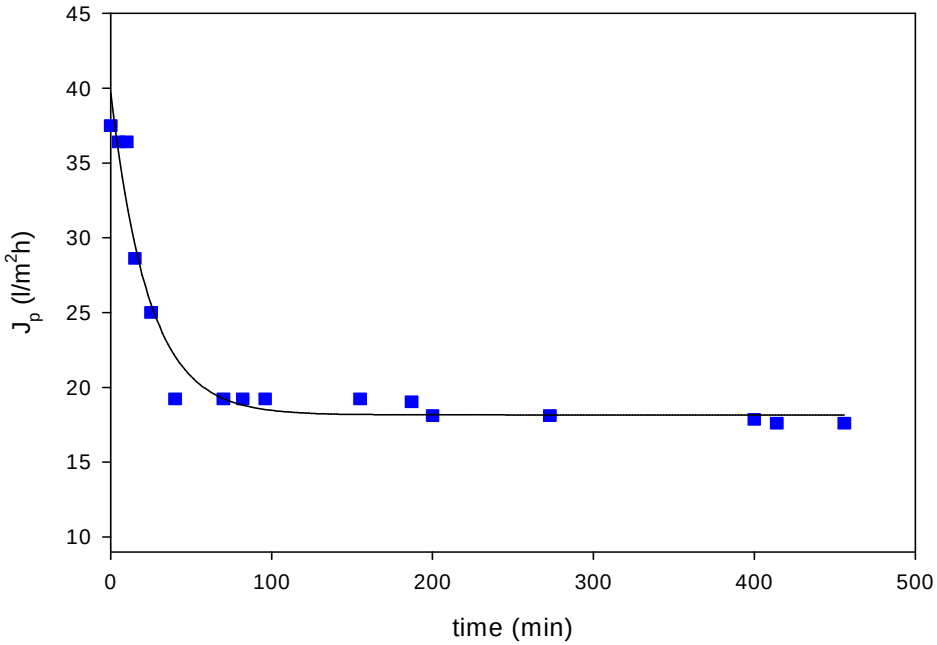

Figure 4.3.3-2 *Nanofiltration of clarified juice with 450 Da membrane. Time course of permeate flux. (TMP=33 bar; Qf =100 ml/min; T= 24°C)*

The water permeability of both membranes was completely restored after the cleaning with a NaOH solution at 1% (Figures 4.3.3-3 and 4.3.3-4).

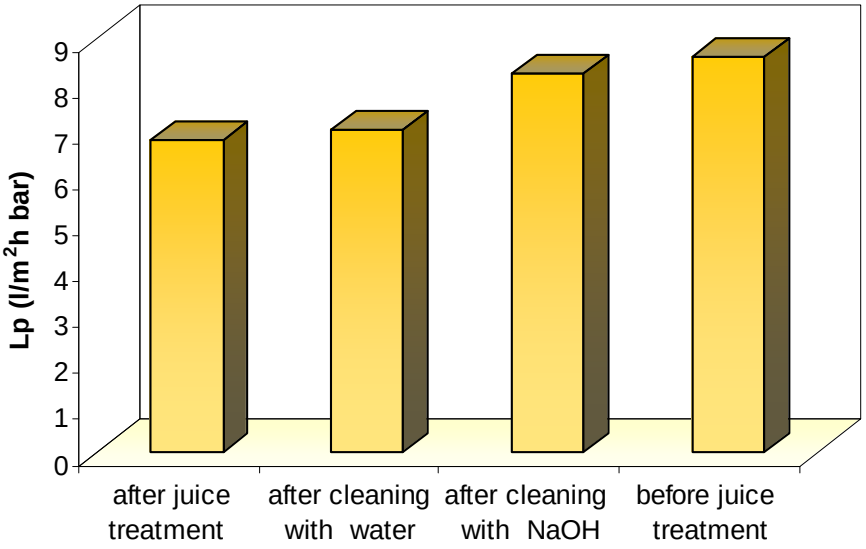

Figure 4.3.2-4 *Regeneration of water permeability in NF 750 Da membrane.* *(T= 25 °C)*

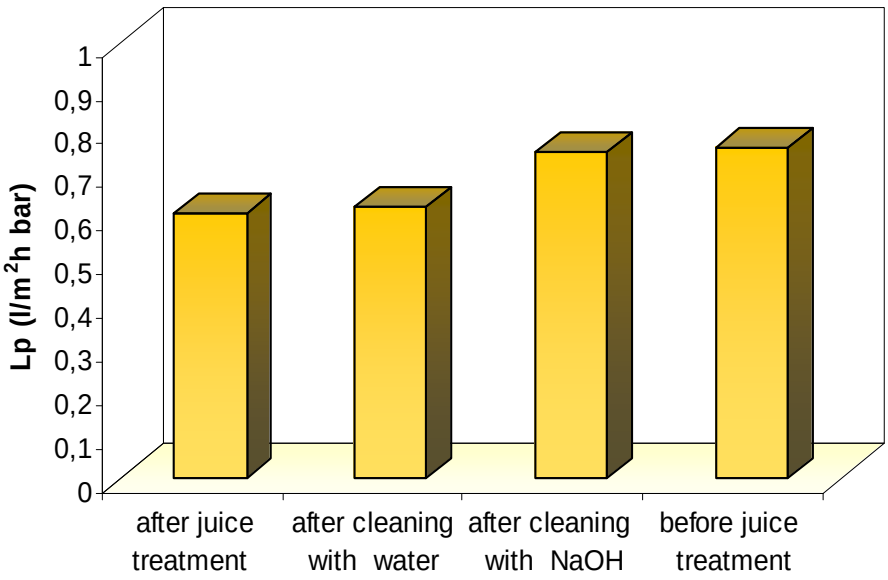

Figure 4.3.2-4 *Regeneration of water permeability in NF 450 Da membrane.* *(T= 25 °C)*

4.3.4 Analytical evaluations

Tables 4.3.4-1 and 4.3.4-2 show most of relevant physico-chemical determinations performed on samples coming from UF and NF treatments.

Suspended solids were completely removed by the 100 KDa UF membrane, while total soluble solids and pH remained practically unchanged in the clarified juice. Organic acids, flavonoids and polyphenols were recovered in the permeate of the UF process as showed also by the TAA value of the clarified juice which was only 9% lower than that of the depectinised juice.

Table 4.3.4 -1 *Physico-chemical characteristics of bergamot juice submitted to UF and NF treatments*

Membrane type	**Sample**	**TSS** (°Brix)	**pH**	**Suspended Solids** (%)	**Malic acid** (g/L)	**Ascorbic acid** (g/L)	**Citric acid** (g/L)	**TAA** (mM Trolox)
UF (100 KDa)	Feed	10	2.8	12	1.41	0.16	45	19.1
	Permeate	9.8	2.8	-	1.40	0.14	44.9	17.4
	Retentate	10.6	2.9	93	1.44	0.16	44.0	21.3
UF (1000 Da)	Feed	9.6	2.8	-	1.38	0.14	44.0	17.3
	Permeate	9.4	2.8	-	1.35	0.14	43.0	15.7
	Retentate	9.6	2.8	-	1.4	0.16	51.0	15.6
NF (750 Da)	Feed	8.9	2.97	-	1.9	0.3	57	17.6
	Permeate	6.2	2.95	-	1.8	0.26	53.74	8
	Retentate	9	2.95	-	2	0.25	55	16.7
NF (450 Da)	Feed	7.8	2.9	-	1.82	0.135	41.6	16
	Permeate	4	3.0	-	1.7	0.125	39.0	1.5
	Retentate	8	3.1	-	1.8	0.13	40.0	22

Table 4.3.4 -2 *Analysis of flavonoids and total polyphenols in samples of bergamot juice coming from UF and NF treatments*

Membrane type	**Sample**	**Narirutin** (mg/L)	**Naringin** (mg/L)	**Hesperidin** (mg/L)	**Neohesperidin** (mg/L)	**Total polyphenols** (mg/L gallic acid)
UF (100 KDa)	Feed	4.97	70.36	9.46	86.43	962
	Permeate	4.35	65.84	7.31	80.00	942
	Retentate	4.4	75.60	13.80	86.70	1056
UF (1000 Da)	Feed	4.6	65.0	10.2	80.0	868
	Permeate	4.33	64.5	10.0	78.0	860
	Retentate	4.0	72.4	10.0	78.0	947
NF (750 Da)	Feed	2.5	39.5	58.15	58.15	943
	Permeate	0.93	22.4	30.15	30.15	529
	Retentate	4.1	60.5	93.7	93.7	1477
NF (450 Da)	Feed	4.22	60.0	7.5	72.15	878.0
	Permeate	0.03	2.82	0.62	2.65	140.0
	Retentate	6.12	72.62	10.0	90.0	1102.0

Results obtained with the UF 1000 Da membrane showed that the physico-chemical properties of clarified juice were preserved during this process. Only a little reduction of TAA (9.2%) was observed in the permeate, in comparison with the initial feed.

The composition of flavonoids on permeate and retentate side was the same as indicated by the chromatographic profiles (Figure 4.3.4-1).

As reported in the previous section, the effect of the pH and of the TMP on the rejection of this membrane towards polyphenols was evaluated. As showed in Figure 4.3.4-2 an increasing of pH from 2.8 to 8.5 determines an increasing of the rejection from 0.9 % to 8.6%. This phenomenon could be explained assuming the formation of a cake layer on the membrane surface due to a precipitation of polyphenols when the pH of the juice is raised [15-16].

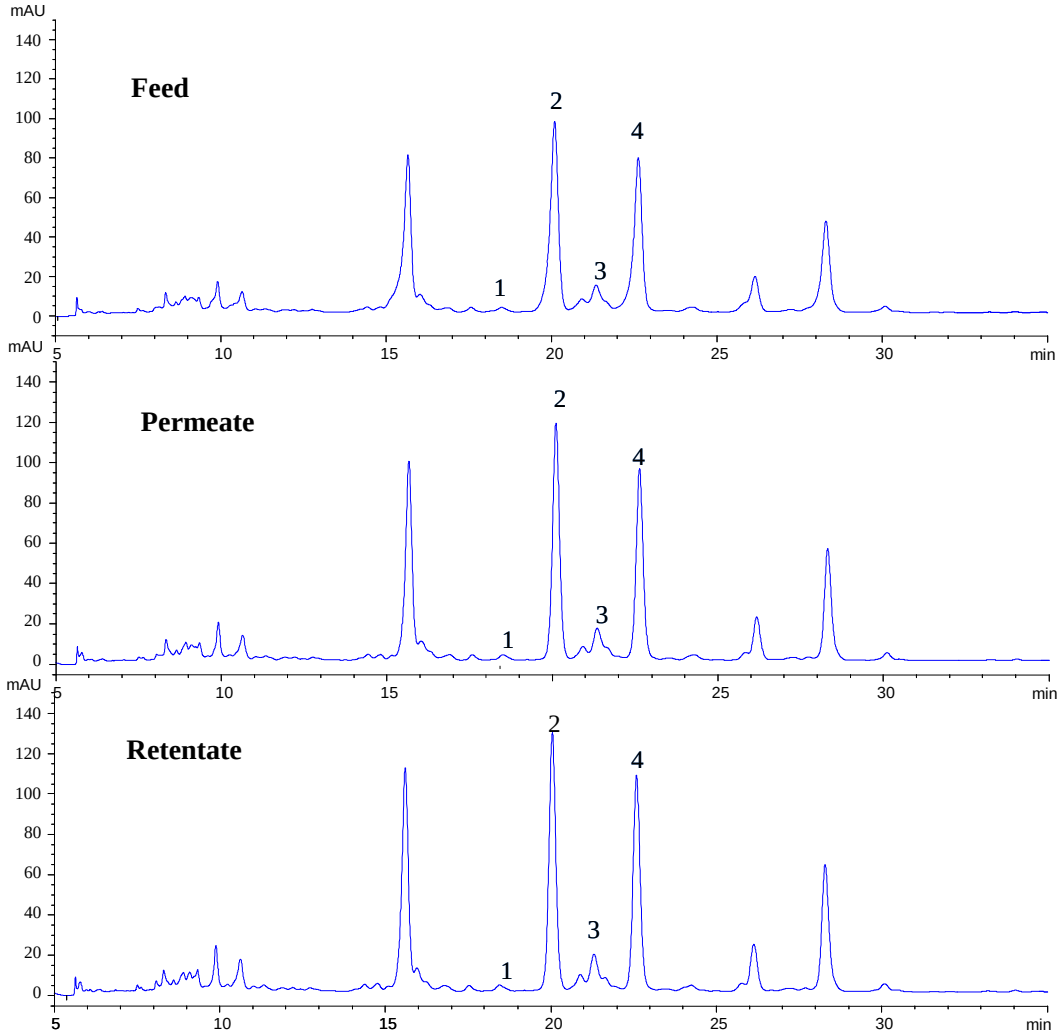

Figure 4.3.4-1 *HPLC chromatogram of flavonoids in feed, permeate and retentate coming from UF treatment of clarified bergamot juice with 1000 Dalton UF membrane. Peaks: 1, narirutin; 2, naringin; 3, hesperidin; 4, neohesperidin*

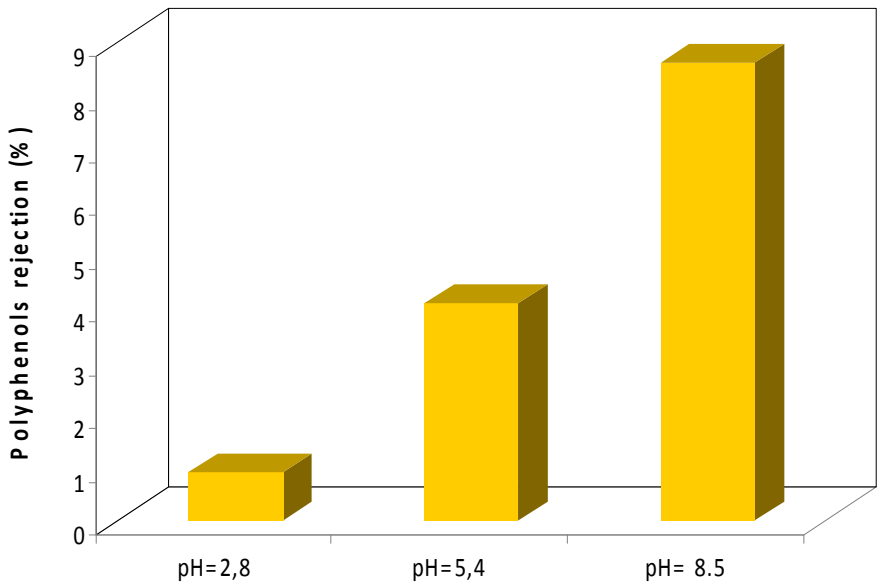

Figure 4.3.4-2 *Polyphenols rejection at different pH values. (1000 Da UF membrane)*

In Figure 4.3.4-3 the effect of TMP on the polyphenols rejection is showed. It can be noted that the rejection of the membrane towards polyphenols was increased in the range of investigated TMP values. Higher TMP values compressed the rejection solute into a thicker and denser fouling layer that is responsible for an additional resistance to the permeate flux, in addition to that of the UF membrane increasing the rejection of the membrane towards polyphenols.

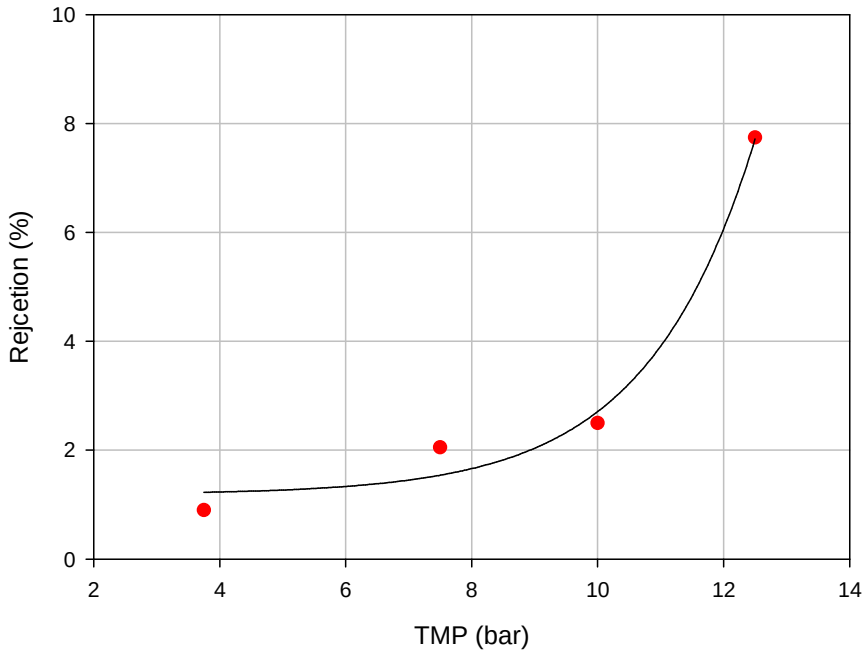

Figure 4.3.4-3 *Polyphenols rejection at different TMP values (1000 Da UF membrane)*

The NF 750 Da membrane showed a rejection towards flavonoids in the range of 43-62% and a rejection towards polyphenols of 44%. Therefore, phenolic fractions with molecular weight greater than 750 Da were retained on the retentate side of the membrane. Besides, flavonoids identified in the juice (naringin, narirutin, hesperidin and neohesperidin), with molecular weights from 550 to 610 Da, were also partially retained by the membrane. This behaviour can be attributed to a fouling phenomenon which determines a reduction of the membrane pore size. The observed rejection towards TSS was of about 30%. Consequently, despite the increased value of the rejection towards polyphenols and sugar, in comparison with the 1000 Da membrane, this membrane does not permit an efficient separation of both substances.

The observed rejection for the NF 750 Da membrane towards polyphenols is also confirmed by the lower value of the TAA in the permeate in comparison with the NF feed (the rejection of the NF membrane towards TAA was 54%). Finally, the content of organic acids in the NF permeate stream was practically similar to that of the clarified juice. As showed in Table 4.3.4-2, the rejection of the NF 450 Da membrane towards flavonoids was in the range 91-99%. Therefore flavonoids were retained on the retentate side of the membrane as also confirmed by the HPLC profiles (Figure 4.3.4 -3).

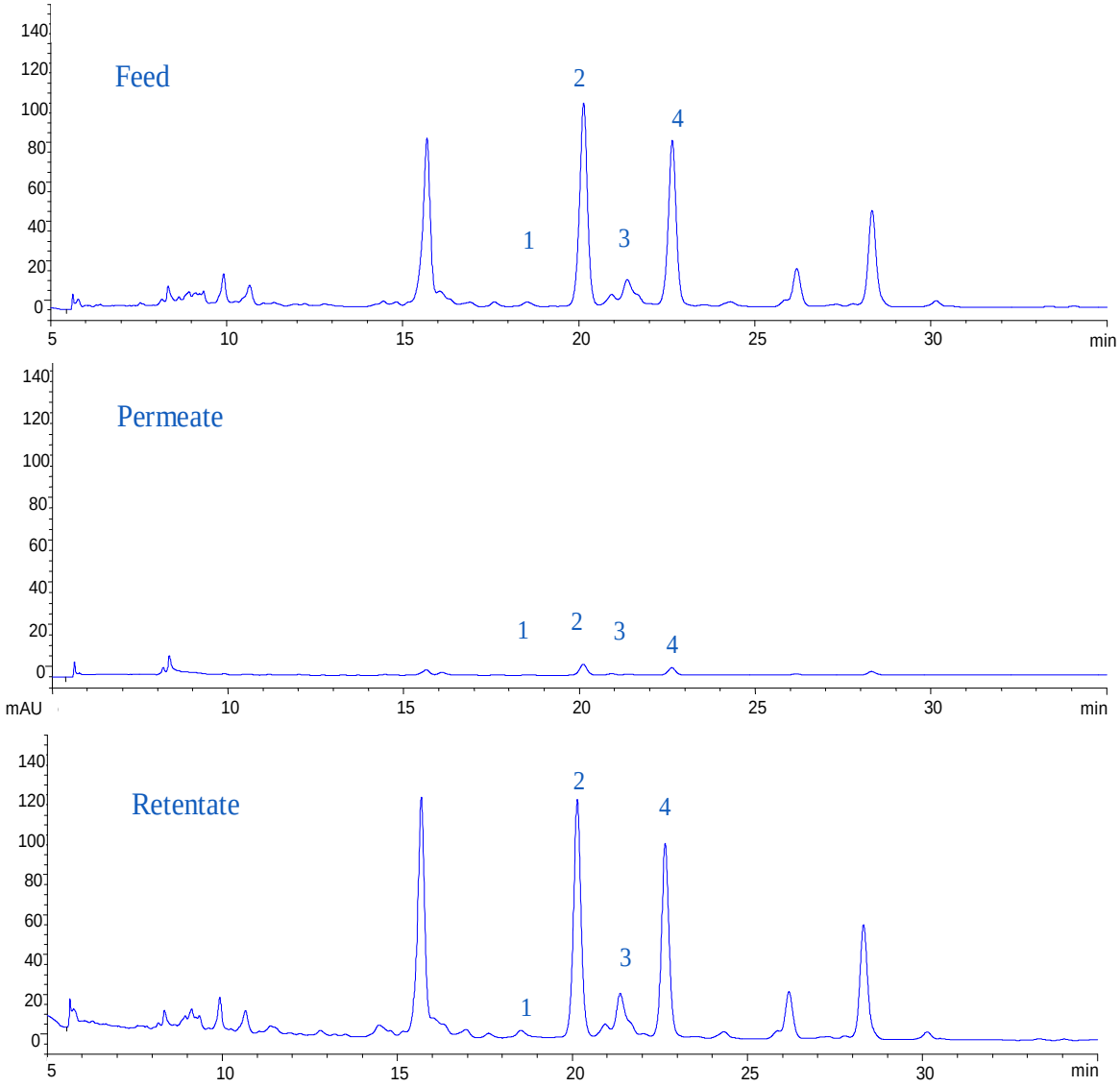

Figure 4.3.4-4 *HPLC chromatogram of flavonoids in feed, permeate and retentate coming from NF treatment of clarified bergamot juice with 450 Da NF membrane. Peaks:1,narirutin;2, naringin;3, hesperidin;4, neohesperidin*

The rejection towards TSS was of about 48% indicating for this membrane the best performance in terms of separation between sugars and flavonoids in the clarified juice. Figure 4.3.4-5 summarizes the effect of NMWCO on the rejection towards sugars and polyphenols for the selected UF and NF membranes. It can be noted that the difference in rejection between sugars and polyphenols was increased by decreasing the NMWCO.

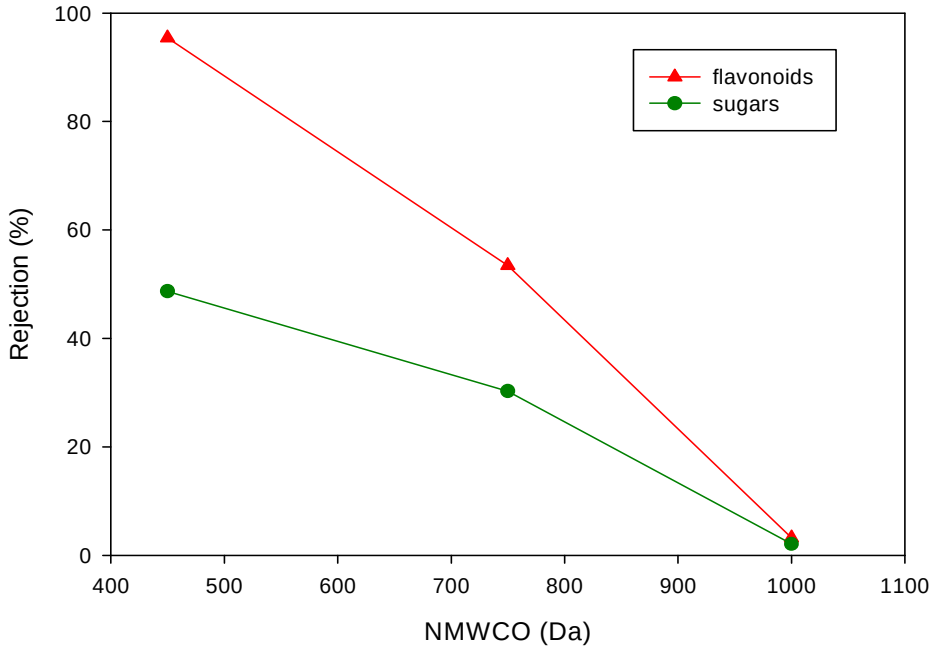

Figure 4.3.4-5 *Effect of NMWCO on sugars and flavonoids rejection*

The obtained results are in agreement with experimental data reported by Saleh et al. concerning the separation of health compounds in apple juice by using a 250 Da NF membrane [4].

Figure 4.3.4-6 shows the variation of the TAA in samples of feed, permeate and retentate coming from UF and NF treatments of clarified bergamot juice. It can be noted that the TAA is related to the flavonoids content. In particular, the lowest TAA value was measured in the permeate coming from the NF 450 process where flavonoids were practically absent. On the other hand the highest value was measured in the retentate of the same process due to the concentration of flavonoids.

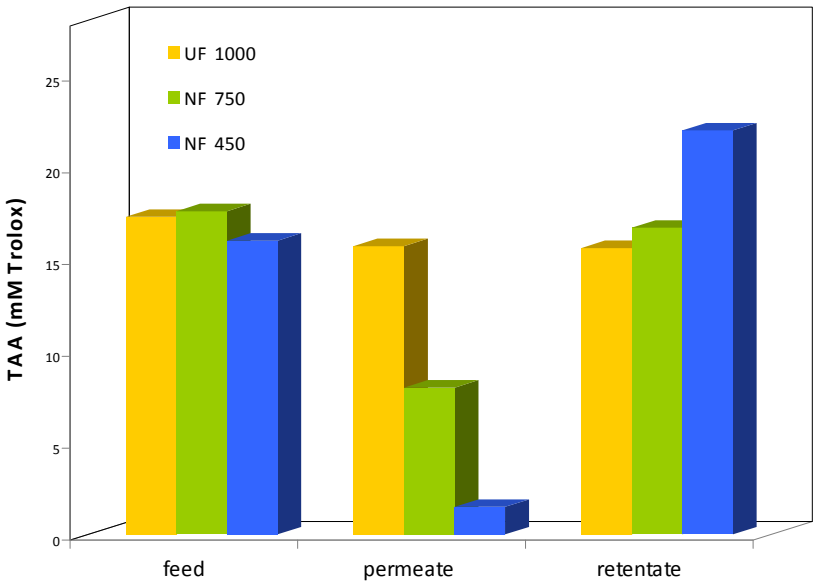

Figure 4.3.4-6 *Variation of the TAA in samples of clarified bergamot juice coming from the UF and NF processes.*

In conclusion the integrated UF-NF system can be considered a viable method to separate the phenolic fraction of bergamot juice in a suitable form which can be used as a functional ingredient.

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CHAPTER 5

CONCENTRATION OF FLAVONOIDS FROM RED ORANGE PRESS LIQUOR BY MEMBRANE PROCESSES

5.1 Introduction

The Italian citrus industry prevalently processes blood oranges producing both natural and concentrated juice, together with essential oil. Moreover, it discharges every year several hundred tons of peel, wet pulp and wastewater containing high quantities of bioactive compounds.

Disposal and purification of wastes require technological and economic efforts, so that the recovery and valorisation of products from residues has become a necessity, to balance costs and increase competitiveness by diversification of process. Among the bioactive compounds flavonoids (hesperidin, narirutin) and anthocyanins have found application in the food and pharmaceutical industries.

Recent interest in anthocyanins is due not only for their potential health benefits but also for their use as natural colorants. In particular, anthocyanins are extracted from red fruits and vegetables, to produce authorized food colorants (European Code, E 163) and nutraceuticals [1-3].

Extraction methods based on the use of solvents for separating bioactive compounds from fruit wastes are a classical operations applied in the pharmaceutical industry. It is obvious that medical interest in drugs obtained from plants has led to an increased need for ideal extraction methods, which could obtain the maximum of the bioactive constituents in a shortest processing time with a low cost [4].

This research was undertaken in order to evaluate the potentiality of membrane operations for the separation and concentration of flavonoids from press liquors obtained by pigmented oranges peels. In particular, the press liquor was submitted to a

preliminary ultrafiltration (UF) treatment, followed by a nanofiltration (NF) pre-concentration step and a further concentration of the NF retentate by osmotic distillation (OD). Permeate and retentate streams coming from membrane operations were analysed for their content in total flavonoids and anthocyanins.

5.2 Materials and methods

5.2.1 Press liquor coming from red orange peel processing

The press liquor coming from red orange peel processing was supplied by Citrech Snc (Messina, Italy). It was stored at -17°C and defrosted to room temperature before use.

5.2.2 UF experimental plant

The liquor was clarified by using a laboratory pilot (Verind S.pA, Milan, Italy) equipped with a polysulphone hollow fiber membrane module supplied by China Blue Star Membrane Technologies, Co., Ltd (Beijing, China). Characteristics of the membrane module are reported in Table 5.2.2-1. The equipment consists of a 25 l stainless steel feed tank, a feed pressure pump, two manometers (0-40 KPa) located at the inlet (P_{in}) and outlet (P_{out}) of the membrane module and a magnetic flow meter for the measure of the axial feed flow rate (Q_f). A tube and shell heat exchanger, placed after the feed pump, was used to maintain the temperature of the juice constant. A data acquisition system, permitting the continuous monitoring of the TMP and of the axial feed flow rate, was connected to the UF plant. A digital balance, connected to the system, was used to measure the permeate fluxes. A schematic of the UF plant is reported in the Figure 5.2.2-1.

Table 5.2.2-1 *Characteristics of the hollow fibres membrane module*

Type	***DCQ III-006C***
Configuration	Hollow fibre
Membrane material	Polysulphone
Dimension (mm)	90x522
Operating pressure (bar)	1-1.5
Operating temperature (°C)	0-40
Operating pH	2-13
Inner fiber diameter (mm)	2.1
Membrane surface area m^2	1.2
Nominal molecular weight cut-off (Da)	100.000

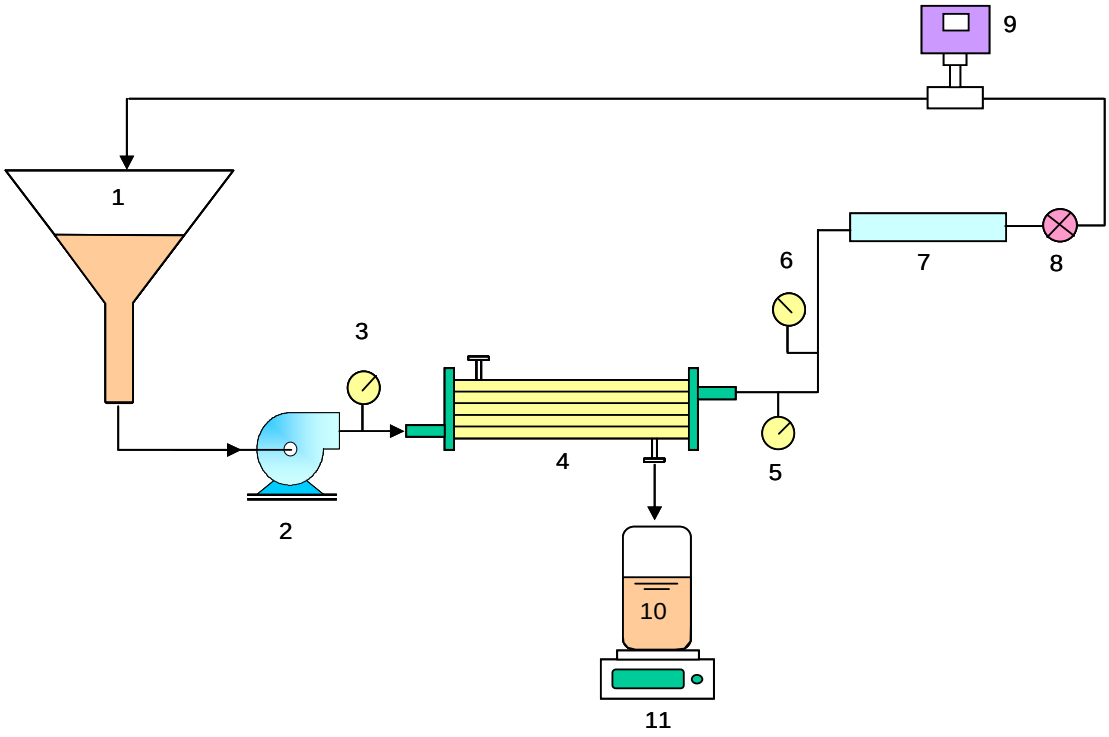

Figure 5.2.2-1 Scheme of the UF pilot laboratory plant.
(1-feed tank; 2-feed pump; 3,6-manometers; 4- membrane module; 5-thermomether; 7- heat exchanger; 8- pressure valve; 9- flowmeters; 10-permeate; 11- digital balance)

5.2.3 Nanofiltration procedure

The permeate coming from the UF treatment was submitted to a preliminary concentration by NF using a laboratory plant supplied by Matrix Desalination Inc. (Florida, USA). The equipment consists of a 12 l feed tank, a cooling coil working with tap water, a high pressure pump, a stainless steel housing, a permeate flowmeter and a pressure control system (Figure 5.2.3-1). The plant was equipped with a spiral wound membrane module (Nadir NF PES 10 2440 C) supplied by Microdin Nadir (Wiesbaden, Germany). The characteristics of the membrane module are reported in Table 5.2.3-1.

The NF experiments were performed according to the batch concentration mode

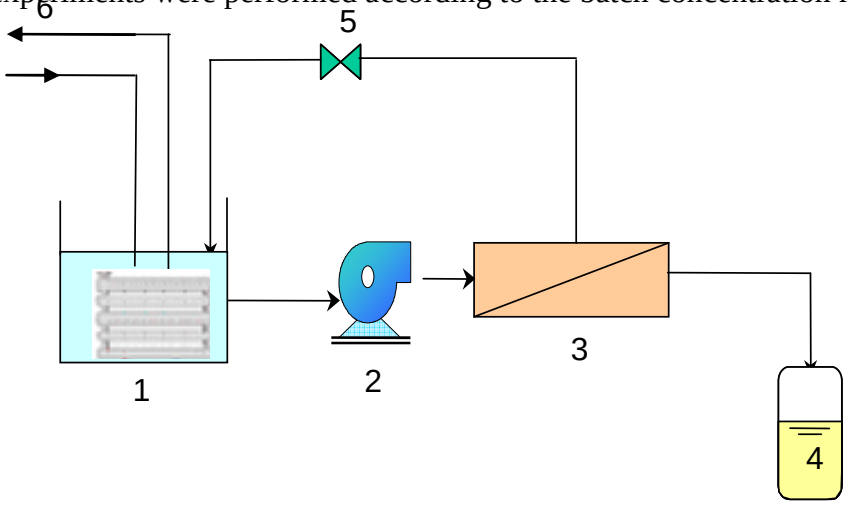

Figure 5.2.3-1 Scheme of the NF bench plant
(1-feed tank; 2-feed pump; 3-membrane module; 4-permeate; 5- pressure valve;6- cooling coil).

Table 5.2-3-1 *Characteristics of the Nadir NF PES 10 membrane*

Type	**Nadir NF-PES 10**
Configuration	Spiral wound
Membrane material	Poly-ether-ether-sulphone
Maximum operating pressure (bar)	40
Maximum operating temperature (°C)	50
Operating pH	2-9
Membrane surface area (m^2)	1.6
Na2SO4 rejection (%)	25-50
NaCl rejection (%)	5-15

5.2.4 Characterisation of the UF and NF membranes with water

The UF and NF membranes were characterised with water in order to evaluate their water permeabilities (L_p) according to the procedure previously described (par 3.2.3). The water flux was measured in fixed conditions of temperature (25°C) at different TMP values. This procedure was repeated after each UF and NF experiment and after the cleaning procedures. In Figures 5.2.3-1 and 5.2.3-2 the hydraulic permeabilities of both membranes are shown. The lower hydraulic permeability of the NF membrane (7.7 l/m^{2}h bar) can be attributed to its NMWCO (about 1000 Da) when compared with the NMWCO of the UF membrane (100 KDa).

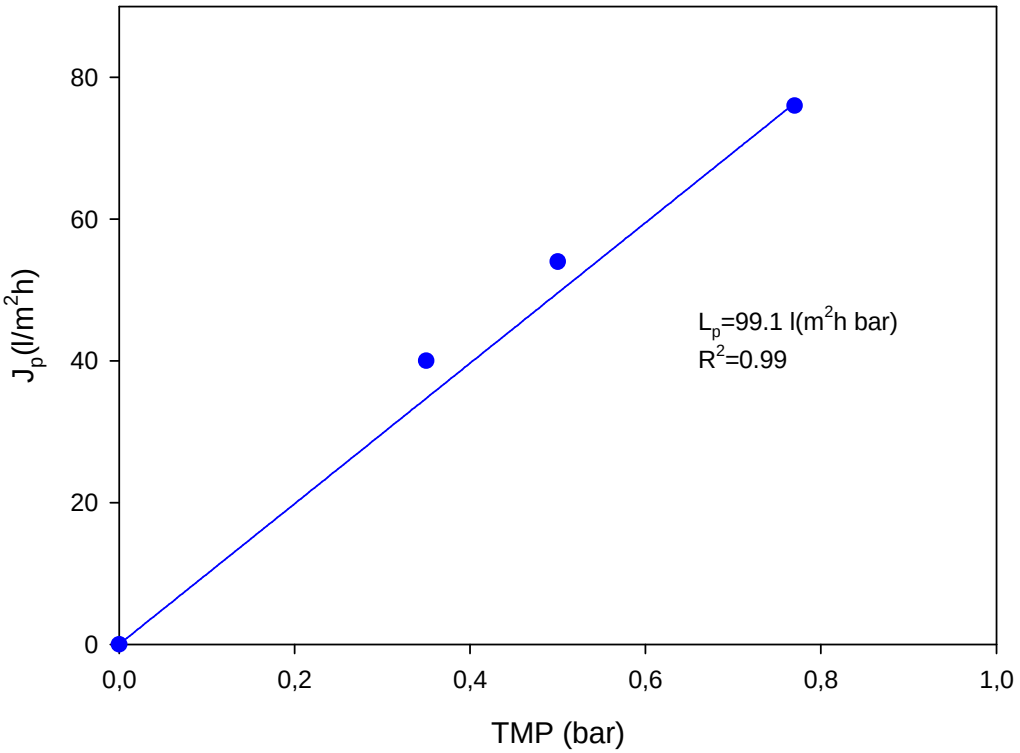

Figure 5.2.4 -1 *Characterisation of the UF membrane with water.* *(T=25°)*

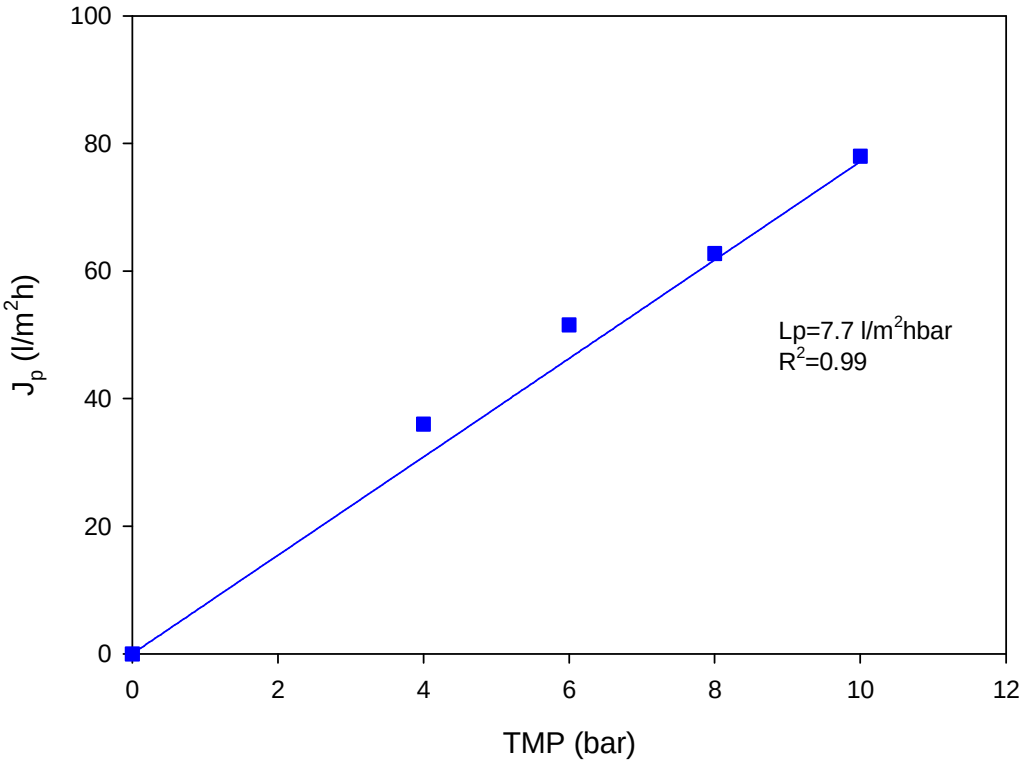

Figure 5.2.4 -2 *Characterisation of the NF membrane with water. (T=25°C)*

5.2.5 Osmotic distillation process

The retentate coming from the NF unit was submitted to an OD process using the laboratory bench plant previously described (par. 3.2.4). The liquor with an initial TSS concentration of 32 °Brix was pumped through the shell side of the membrane module, with an average flow rate of 30.0 l/h. The stripping solution (60 w/w% calcium chloride dehydrate) was circulated in the tube side whit an average flow rate of 30.3 l/h. The temperature of both, liquor and brine solutions was 28±2°C, whereas the average TMP was 0.28 bar.

The flow rate of the extracted water was measured with a digital balance and it was used to calculate the evaporation flux (J_w). After the experimental run, the plant was cleaned first by rinsing the tube side and the shell side with de-ionised water. Then, a NaOH solution at 2% was circulated for 1 h at 40°C. After a short rinsing with de-ionised

water, a citric acid solution at 2 w/w % was circulated for 1 h at 40 °C. Finally the circuit was rinsed with de-ionised water [5].

5.2.6 Analytical evaluations

Samples of feed, permeate and retentate coming from the integrated UF-NF-OD process were analysed for total flavonoids and anthocyanins content in order to evaluate the influence of the integrated membrane processes on the separation and concentration of flavonoids.

5.2.6.1 Total flavonoids content

The total flavonoids content of the samples was measured by using a modified colorometric method [6]. 3.5 ml of absolute ethanol was added to 0.5 ml of press liquor. After addition of 4 ml of 90 % diethylene glycol and mixing, the reaction was initiated by adding 0.1 ml of 4 M NaOH. The absorbance at 420 nm was measured, after 10 min of incubation at 40 °C, by using an UV-Vis Recording spectrophotometer (UV-160 A, Shimadzu Scientific Instruments, Inc, Japan). Hesperidin was used as standard and total flavonoids content was expressed as mg of hesperidin equivalents.

5.2.6.2 Total anthocyanins

The determination of the total amount of anthocyanins was carried out by spectrophotometric and HPLC analyses.

Spectrophotometric analyses were performed under the following conditions: at 5 ml of juice were added 40 ml of a EtOH/HCl mixture previously prepared mixing 79.3 ml of anhydrous ethyl alcohol with 20.3 ml of HCl (37%). The absorbance was measured at 535 nm. The calibration curve was obtained by measuring absorbance of standard solutions of pure cyanidin-3 glucoside [7,8].

HPLC analyses were carried out by using an HPLC-system (Agilent 1100 series, USA), equipped with a Luna C 18 column (250 ×4.6mm, 5µm, Phenomenex, Torrance) and an UV detector. The following conditions were used: V= 1ml/min; T= 25°C; λ=518 nm. The mobile phase was a mixture of H_2O/HCOOH (9:1) as solvent A and H_2O/HCOOH/CH_3CN (4/1/5) as solvent B. Anthocyanins separation was achieved by

using the following linear gradient: starting condition, 88 %A, 12 %B; 26 min, 70% A, 30% B; 35 min, 100% B; 43 min, 88% A, 12% B; 46 min 88% A, 12% B.

Anthocyanins were identified by matching the retention time and their spectral characteristics against those of standards (cyanin chloride, myrtillin chloride, cyanidin 3-glucoside chloride, peonidin-3-glucoside chloride). Quantification was made according to the linear calibration curves of standard compounds.

5.3 Results and discussion

5.3.1 Clarification of the press liquor by UF

The press liquor was clarified according to the batch concentration mode, at a temperature of 25°C, a feed flow rate of 500 l/h and a TMP of 0.54 bar. Figure 5.3.1-1 shows the time course of the permeate flux of a generic run in which starting from 39 litres of press liquor, 37.5 litres of clarified product were obtained (final VRF 26). The initial permeate flux of about 52 l/m^{2}h bar decreased gradually and reached a steady-state of 45 l/m^{2}h bar after 10 min of operation.The UF step was a fundamental pre-requisite in order to apply high flow-rate and maximize yield during the subsequent NF or OD treatment [9-12].

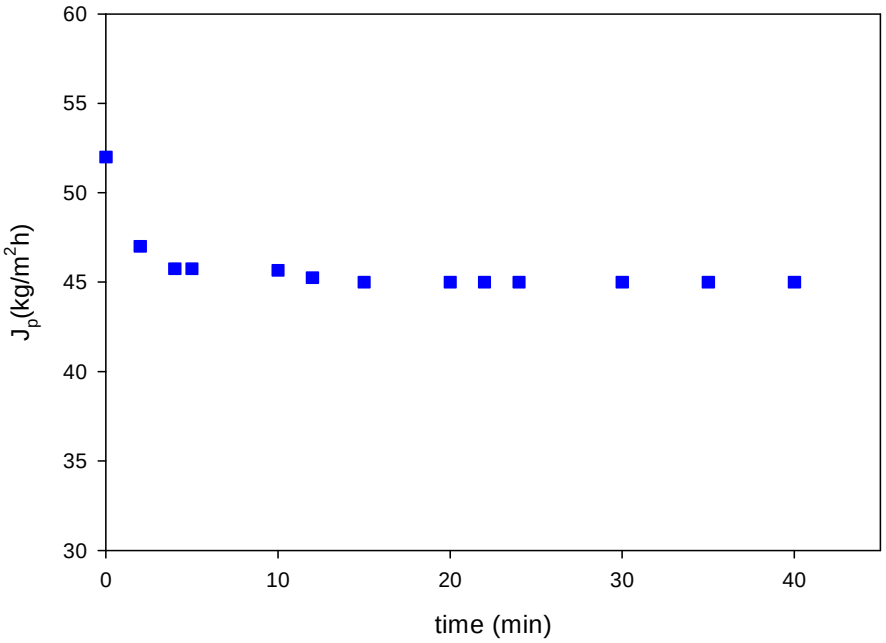

Figure 5.3.1-1 *Clarification of press liquor coming from red orange peel. Time course of permeate flux. (operating time: T=25°C; Qf=500 l/h, TMP=0.54 bar)*

The membrane module was rinsed with tap water for 30 min after the treatment of the press liquor; then it was submitted to a cleaning procedure with a NaOH solution at a concentration of 0.5 % w/w and at a temperature of 40°C for 60 min. A final rinse of the system with tap water for 20 min was carried out. Figure 5.3.1-2 shows the hydraulic permeability of the membrane module before and after the cleaning procedure: a good restore of the initial water flux was obtained after the alkaline cleaning.

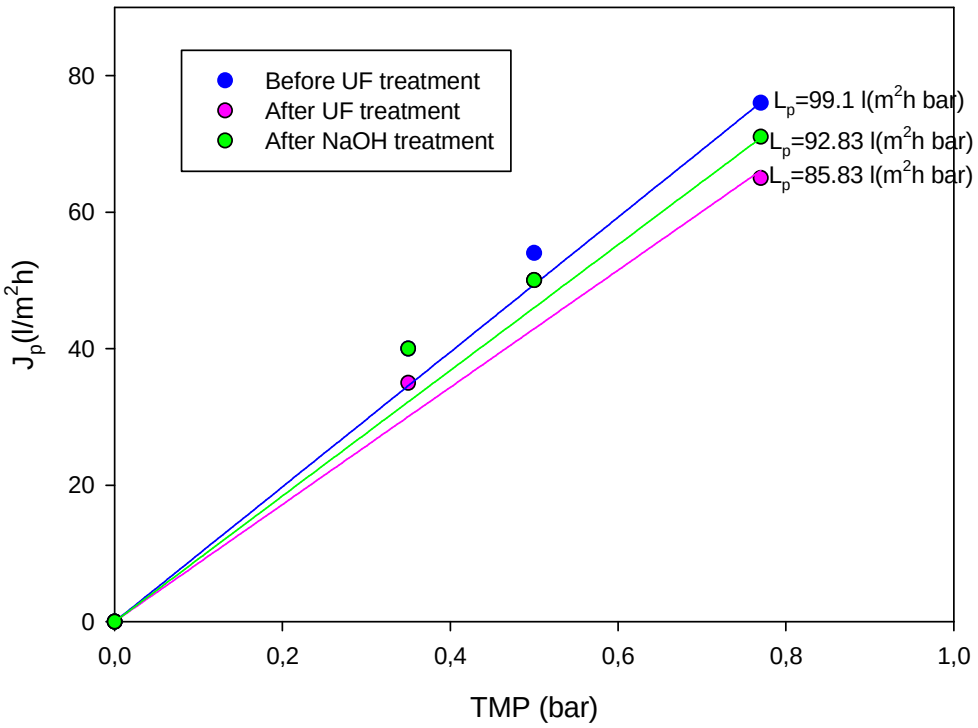

Figure 5.3.1-2 *Effect of membrane cleaning on the water permeability of the UF membrane (T=25°C)*

5.3.2 Concentration of clarified liquor by NF

Figure 5.3.2-1 shows the time course of the permeate flux and of the VRF for a generic NF run carried out according to the batch concentration mode at an applied TMP of 8 bar and a temperature of 20°C. Average permeate fluxes of about 0.6 l/m^{2}h were obtained. Starting from a clarified liquor with an initial TSS concentration of 5°Brix, a concentrated liquor with 32°Brix was produced.

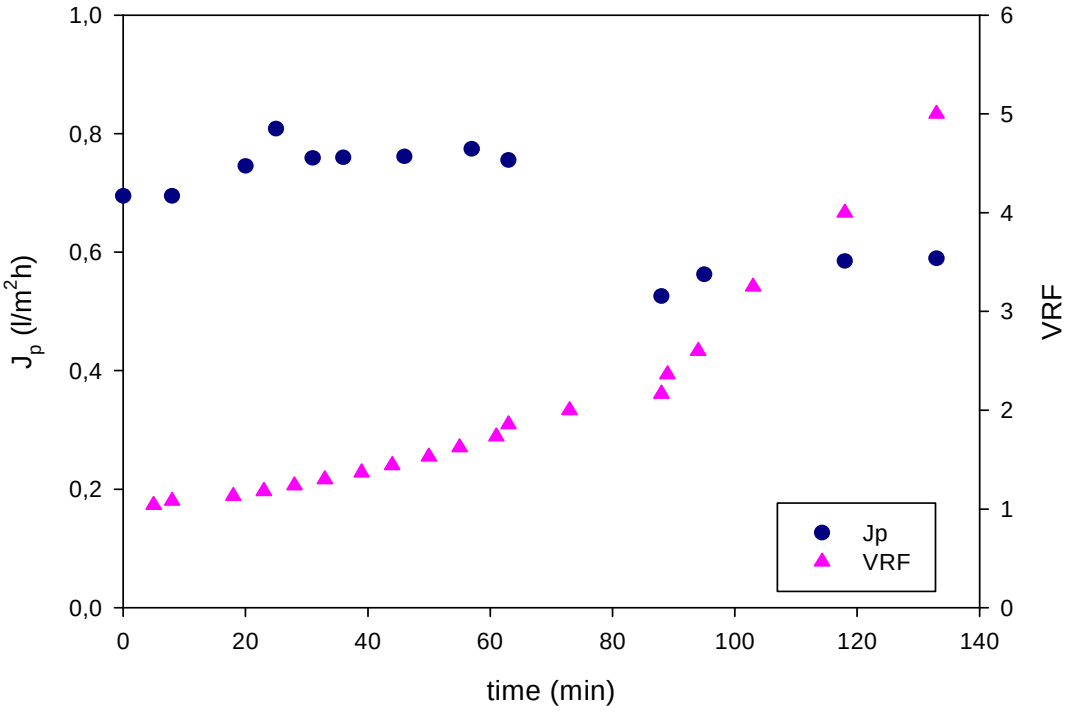

Figure 5.3.2-1 *Nanofiltration of clarified press liquor. Time course of permeate flux and VRF.(operating conditions: TMP = 8 bar; T = 20°C)*

5.3.3 *OD process*

The retentate coming from the NF process was then concentrated by OD. Figure 5.3.3-1 shows experimental results concerning a generic run in which the liquor with a TSS concentration of 32 °Brix, was concentrated up to 47 °Brix. At a brine concentration of 60 w/w % an initial evaporation flux of 1.3 Kg/m^{2}h was measured. The decrease of evaporation flux in the range 0-150 can be attributed to the dilution of the brine solution (Figure 5.3.3-2). In particular, a 33 % reduction of the stripping solution concentration and consequently the driving force of the process, determined a reduction of the evaporation flux of about 80%. In the range 150-250 min the evaporation flux remained unchanged despite the increasing in TSS concentration of the juice and its viscosity. On the contrary studies performed on the concentration of fruit juices by OD showed a decreasing of the evaporation flux when TSS concentration of the juice was higher than 30-35 °Brix. [13,14].

During the OD process samples of concentrated liquor at different level of concentration were collected for the analytical evaluations.

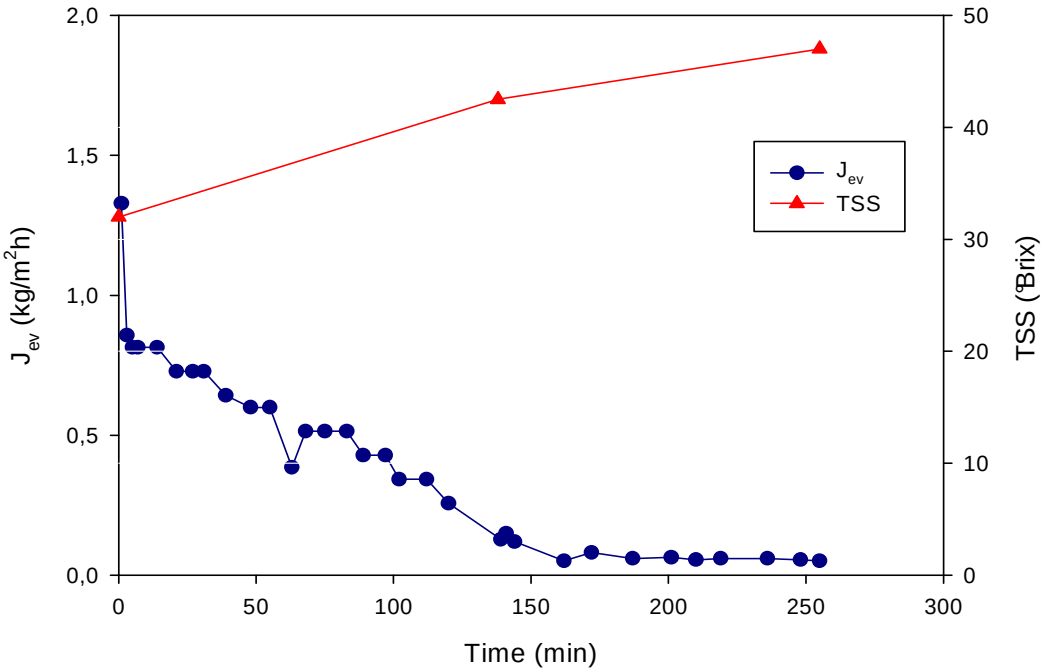

Figure 5.3.3-1 *OD of press liquors coming from a sequence UF-NF. Time course of evaporation flux and TSS concentration. (operating conditions: TMP = 0.28 bar; T = 28 ± 2°C ; Q_f=30 l/h)*

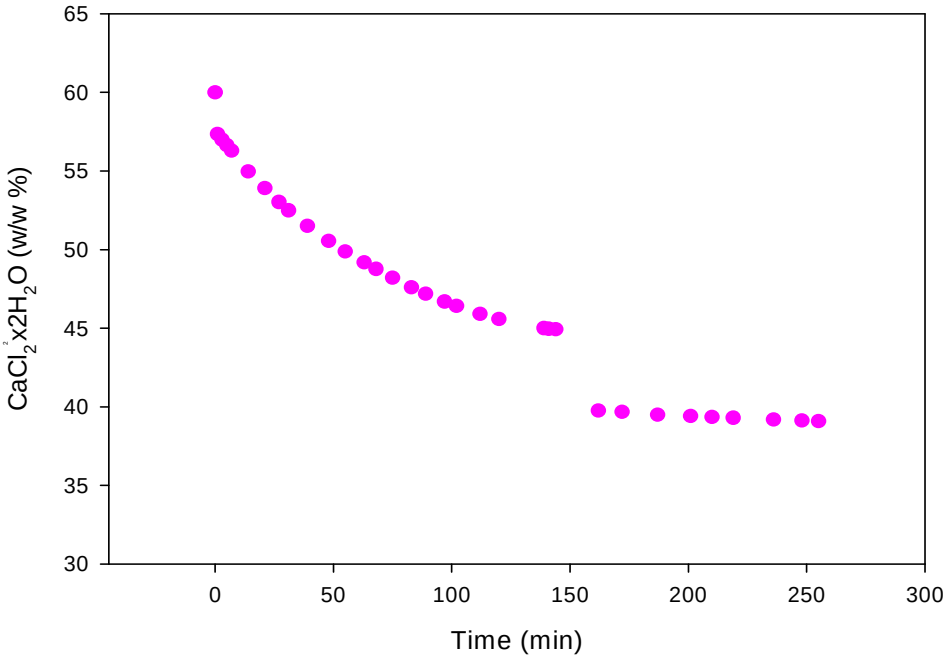

Figure 5.3.3 -2 *OD of press liquors coming from a sequence UF-NF. Time course of evaporation flux and TSS concentration. (operating conditions: TMP = 0.28 bar; T = 28 ± 2°C ;Q_f=30 l/h)*

5.3.4 Analytical results

Permeate and retentate samples coming from the integrates membrane process were submitted to analytical measurements in order to evaluate their content of flavonoids and anthocyanins and the effect of the UF-NF-OD sequence on the recovery of these compounds.

During the UF process the content of total anthocyanins and flavonoids remained practically unchanged in the clarified press liquor (Table 5.3.4-1) and a low rejection of the UF membrane towards these compounds was measured (R=1 %).

Table 5.3.4-1 *Analitycal evaluation of anthocyanins and flavonoids in samples o fpress liquor coming from the UF treatment*

Sample	TSS (°Brix)	Total Flavonoids (ppm)	Total Anthocyanins (ppm)
Feed UF	5.2	22770	1782
Permeate UF	5	22760	1780
Retentate UF	5.1	22785	1790

Results obtained with the NF membrane showed that the rejection towards anthocyanins was higher than that of flavonoids and the concentration of these compounds in the retentate fraction, increased by increasing the VRF. Nevertheless the ratio flavonoids/anthocyanins decrease by increasing the VRF (Figure 5.3.4-1).

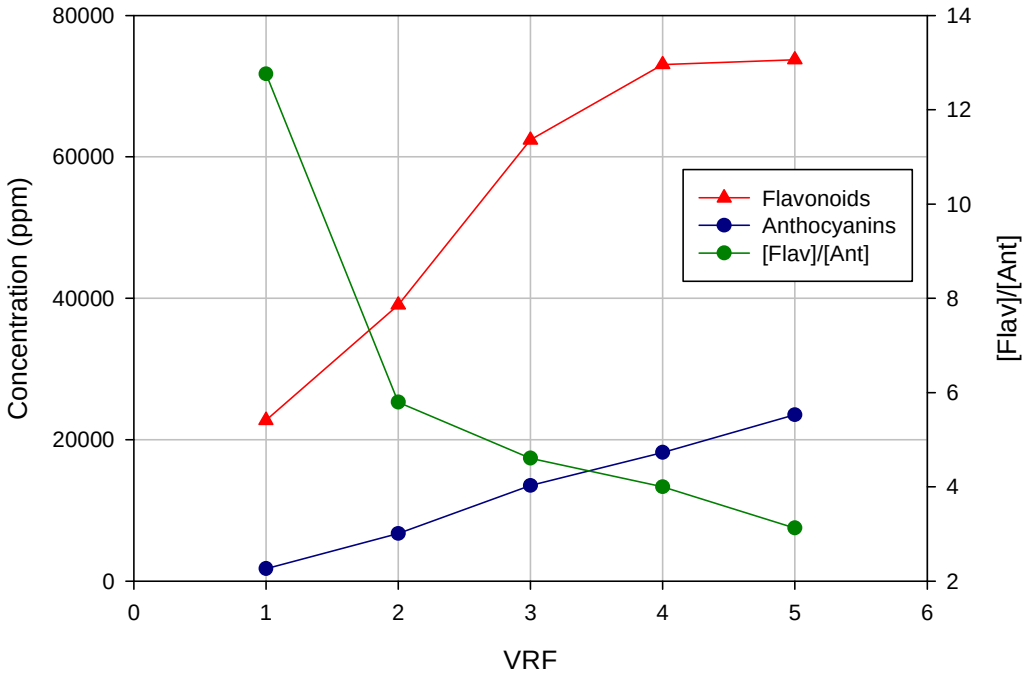

Figure 5.3.4-1 *Concentration of flavonoids and anthocyanins in NF retentate as a function of VRF*

In Table 5.3.4-2 the content of anthocyanins and total flavonoids coming from the integrated NF-OD process is reported.

Table 5.3.4-2-*Analitycal evaluations of anthocyanins and flavonoids in samples of clarified liquor from NF-OD treatments*

Sample	TSS (°Brix)	Cyanin chloride (ppm)	Cyanidin 3-glucoside chloride (ppm)	Myrtillin chloride (ppm)	Peonidin-3-glucoside chloride (ppm)	Total Flavonoids (Hesperidin) (ppm)
Feed NF	5	14.83	155.6	35.23	33.04	22750
Retentate NF	32	-	1304.34	300.51	213.20	73750
Retentate OD	47	639.19	1787.70	400.63	399.67	93250.6

As expected, the concentration factor of these compounds in the final OD retentate was in agreement with that of the TSS compounds due to the water removal.

Figure 5.3.4-2 shows the chromatographic profile of anthocyanins in the samples of NF feed, OD feed and OD retentate at different levels of TSS concentration. It can be noted that the cyanidin-3-glucoside chloride, corresponding to the peak 3, is the most important compound in the different fractions and as for other anthocyanic compounds, it was retained by the NF membrane and furtherly concentrated in the OD process.

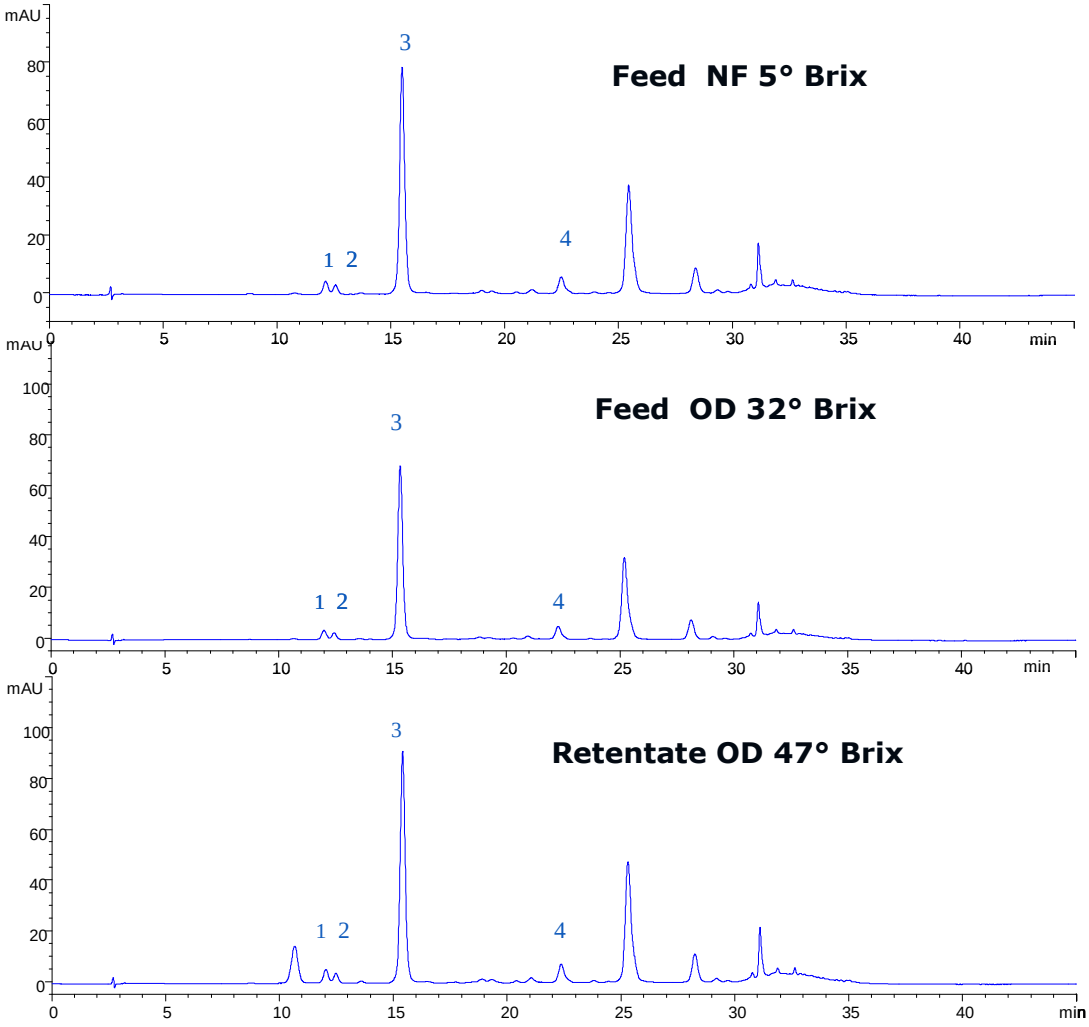

Figure 5.3.4-2 *HPLC chromatogram of anthocyanins.*
Peaks: 1 Cyanin chloride, 2: Myrtillin chloride,
3:Cyanidin-3-glucoside chloride, 4:Peonidin-3-glucoside chloride

The concentrated solution coming from the integrated NF-OD membrane process can be used as an industrial colorant (as alternative to the use of artificials colorants) or for the preparation of pharmaceuticals and nutriaceuticals.

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CHAPTER 6

ULTRAFILTRATION OF CLEMENTINE MANDARIN JUICE BY HOLLOW FIBRE MEMBRANES

6.1 Introduction

Clarified fruit juices can be used in different products such as cocktails of fruit juices, liqueurs, fizzy beverages, flavoured mineral water, etc.. Moreover, natural antioxidants occurring in food formulations may be used as components of composite food formulations for their stabilization or may be extracted and added to foods. Within the agro-food industry, membrane technologies can work as well or better than the existing technology regarding product quality, energy consumption and environmental issues. They offer a competitive and attractive alternative to thermal processes which cause irreversible change of the aroma profile, colour degradation and "cooked" notes recognized as off-flavours [1].

On the other hand, current filtration of a wide variety of juices is performed by using fining agents such as gelatine, diatomaceous earth, perlite, bentonite and silica sol which cause problems of environmental impact due to their disposal [2-3]. The application of membrane processes for fruit juices has been investigated by many authors [4-9] and commercial processes have been already been implemented for juices like apple and orange. However, there are almost no scientific references dealing with the clarification of mandarin juice by membranes.

This study was undertaken in order to evaluate the effect of the ultrafiltration of the mandarin juice by using modified poly(ether ether ketone) (PEEKWC) and polysulphone (PSU) hollow fibre membranes, prepared in laboratory, on the quality of the clarified juice in terms of suspended solids content, total soluble solids (TSS),

colour, clarity, pH, acidity, total phenols, organic acid and total antioxidant activity (TAA). The performance of membranes in terms of permeate flux was also evaluated.

6.2 Material and methods

6.2.1 Juice extraction

Clementine mandarins (Citrus clementina), of Calabria origin, were purchased from a local open market (Cosenza, Italy). Fruits were halved and squeezed with a domestic juicer. The juice was depectinised by using a pectinase (Sigma-Aldrich, Milan) from Aspergillus aculeatus (10 g/kg of pulp, 4 h at room temperature) and then filtered with nylon cloth (Figure 6.2.1-1). The extracting procedure gave an average juice yield of 48%w/w with a TSS content of about 11°Brix. The juice was stored at -17°C and was defrosted to room temperature before use.

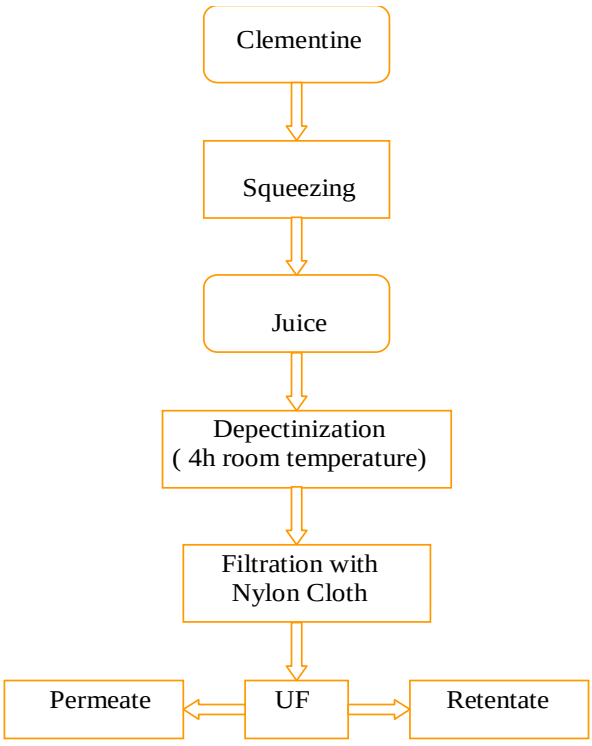

Figure 6.2.1-1 Process of the extraction of clementine mandarin juice

6.2.2 HF membranes and module preparation

PEEKWC and PSU polymer solutions were prepared by addition of the polymer to DMF or DMA under mechanical stirring at room temperature. The solution was then stored in a thermostated vessel, kept at 30 °C throughout the entire spinning run. HF membranes were prepared by extruding the polymer solution through the spinneret whose outer and inner diameters were 2.0 and 1.0 mm, respectively, according to the dry-wet spinning process[10]. PEEKWC and PSU membrane modules were prepared by embedding four HF membranes inside a 20-cm long glass tube (effective membrane length 18 cm) with epoxy resin. In Table 6.2.2-1 characteristics and membrane surface area of membrane modules are reported.

Table 6.2.2-1 Characteristics of PEEK-WC and PSU membrane modules

	PEEKWC membrane module	PSU membrane module
Number of HF membranes	4	4
Length of the HF membranes (cm)	18	18
Internal diameter of the HF membranes (mm)	1.64	1.43
Membrane surface area (cm^2)	37.0	32.0
Cross-flow area (mm^2)	8.45	6.42

6.2.3 HF membranes characterisation

6.2.3.1 Hydraulic permeability measurements

HF membranes were characterised with bi-distilled water in order to measure their hydraulic permeability. It was determined according to the method previously described (par. 3.2.3). Figures 6.2.3.1-1 and 6.2.3.1-2 show the characterization of the PEEWC and PSU membranes with bi-distilled water at 25°C. The water permeability was measured, in the same operating conditions, before and after experimental trials, as well as after each cleaning procedure.

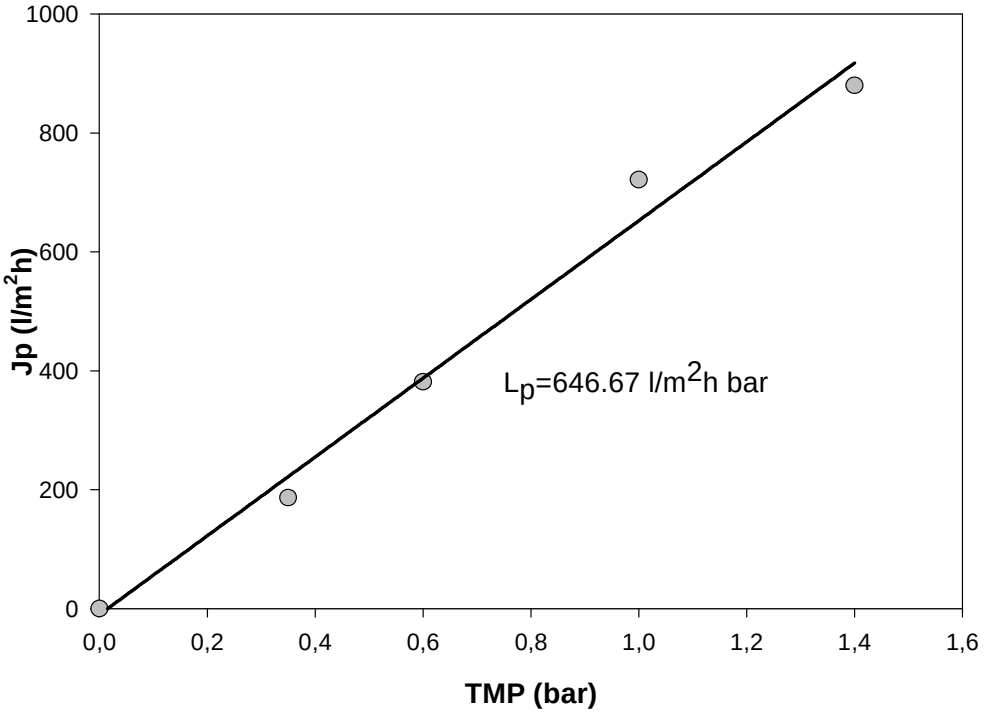

Figure 6.2.3.1 -1 Characterisation of PEEKWC membrane with bi-distilled water

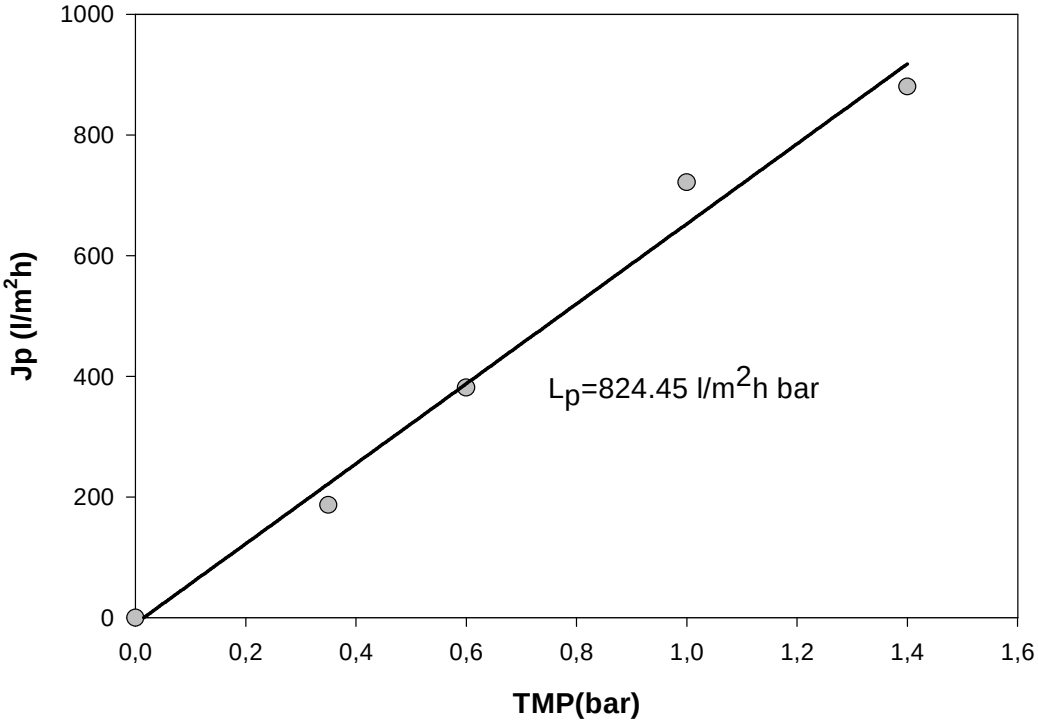

Figure 6.2.3.2 -2 Characterisation of PSU membrane with bi-distilled water

6.2.3.2 Dextran rejection

The dextran rejection was determined by feeding a 0.2 g/L aqueous dextran solution (at a TMP of 0.5 bar and a feed flow rate of 40 L/h) and measuring the dextran concentration in the permeate and feed stream after the test was running for 1 h. The dextran concentration was determined by a colorimetric method, according to the procedure of Dubois et al. [11]. The rejection was calculated as:

$$R(\%) = \frac{C_f - C_p}{C_f} x100 \qquad (6.2.3.2\text{ -}1)$$

where C_f and C_p are the dextran concentration in the feed and permeate solution, respectively. Figure 6.2.3.2-1 reports the dextran rejection of both membranes.

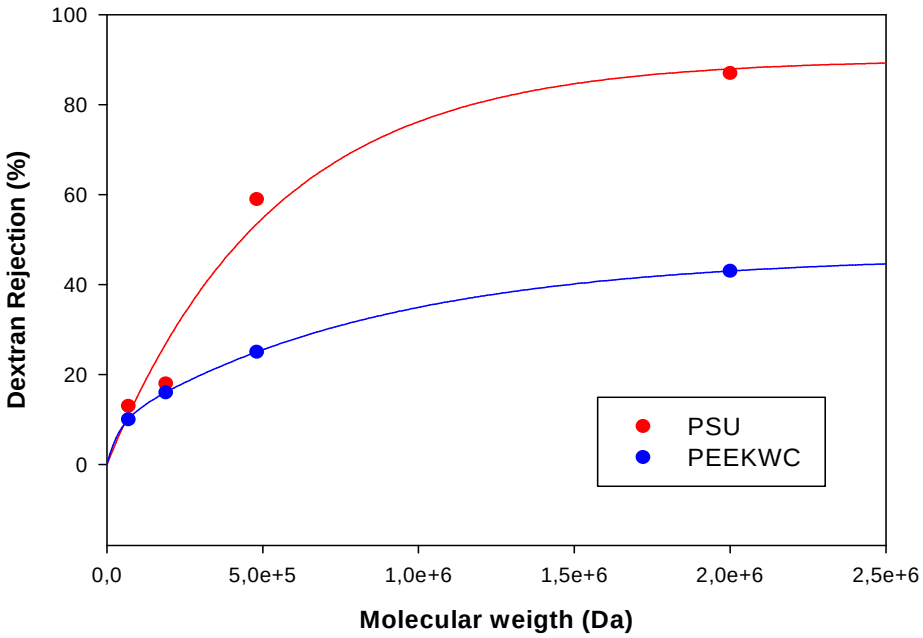

Figure 6.2.3-3 *Dextran rejection of PSU and PEEKWC membranes***.**

A lower rejection towards dextrans at different molecular weight was observed for PEEKWC membranes when compared with PSU membranes. In particular, the rejection towards dextran 2,000,000 Da for PEEKWC and PSU membranes was 50% and 87%, respectively.

6.2.4 UF experimental set-up

The clarification of mandarin juice was performed by using a laboratory bench plant (DSS LabUnit M10, Danish Separation System AS, Denmark) equipped with a HF membrane module. The equipment consists of a feed tank, a gear pump, two pressure gauges (0-2.5 bar) located at the inlet (P_{in}) and outlet (P_{out}) of the membrane module, a pressure control valve and a multitube heat exchanger fed with tap water.

Experiments were performed according to the batch concentration mode at a TMP of 0.3 bar and a temperature of 25±2 °C up to a final volume reduction factor (VRF) of 2. The feed flow rate through the HF membranes was 90 l/h which was equivalent to a mean velocity of 2.96 m/s for the PEEKWC membranes and 3.89 m/s for the PSU membranes.

6.2.5 Determination of physiochemical characteristics

Feed, permeate and retentate samples were analysed in relation to suspended solids content, TSS, pH, TAA, total flavonoids, polyphenols and organic acids content according to the procedures reported in the previous chapters.

Colour (as absorbance at 420nm) and clarity (as percentage of transmittance at 660 nm) were measured by using a Shimadzu UV-VIS Recording Spectrophotometer (UV-160A, Shimadzu Scientific Instrument, Inc., Japan). Acids were titrated to pH 8.2 with 0.1 N NaOH and expressed as percent citric acid.

6.3 Results and discussion

6.3.1 Juice clarification

Batch concentration experiments showed that the permeate flux, in the selected operating conditions, decreased gradually with operating times by increasing the VRF due to concentration polarization and fouling phenomena (Figure 6.3.1-1 and 6.2.3-2). In the initial stage the rapid flux decline, more evident for the PSU membranes, can be attributed to the adsorption and growth of a polarized layer formed by leftover pectin, protein and high molecular weight compounds present in the juice. However, internal fouling due to pore plugging at the early stage of the process can also occur. The slower decline towards a quasi-steady state can be attributed to a fouling phenomenon due to pore blocking and cake build-up [12, 13]. Permeate flux values observed with

PEEKWC membranes were lower than those observed with PSU membranes. Steady-state permeate fluxes were 38 L/m^{2}h and 42 L/m^{2}h, respectively.

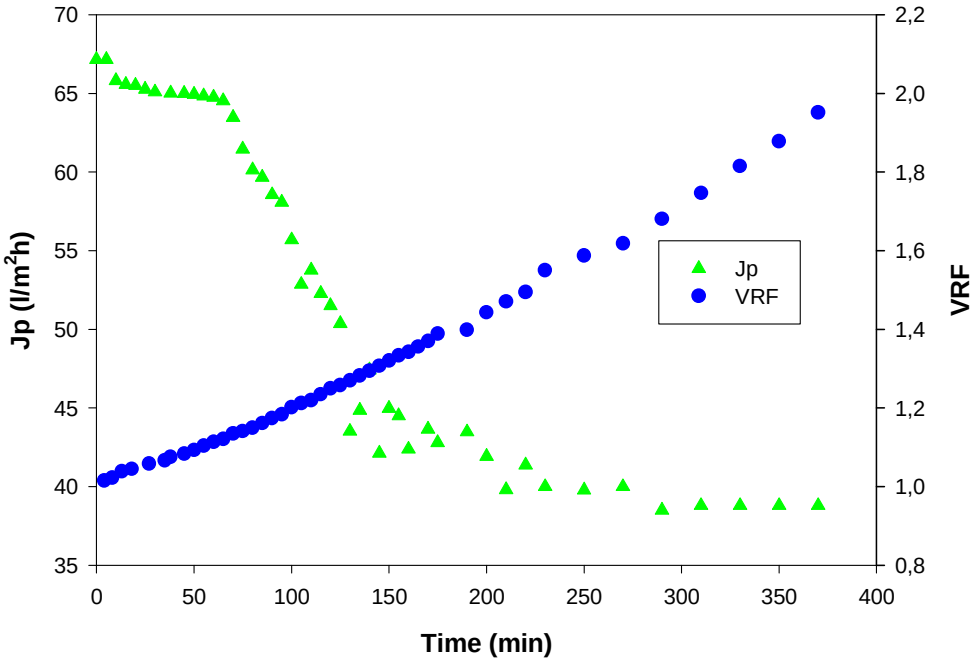

Figure 6.3.1-1 *Clarification of mandarin juice by PEEKWC hollow fibre membranes. Time course of permeate flux and VRF (TMP=0.3 bar; T=25±3°C)*

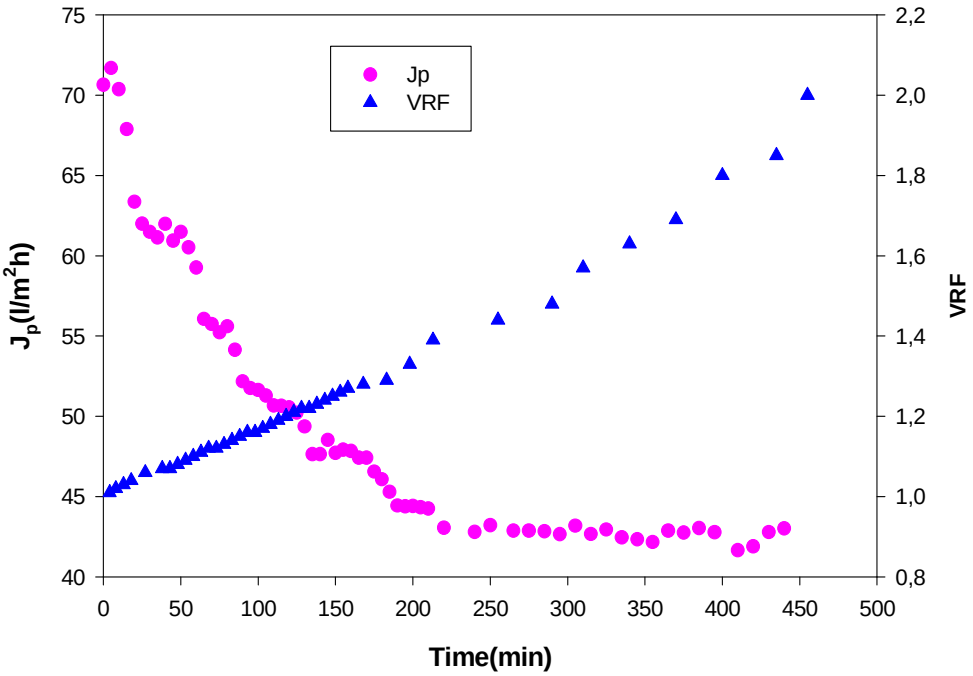

Figure 6.3.1-2 *Clarification of mandarin juice by PSU hollow fibre membranes Time course of permeate flux and VRF (TMP=0.3 bar; T=25±3°C)*

The hollow fibre membranes were rinsed with bi-distilled water for 30 min after the treatment of the juice; then they were submitted to a cleaning procedures using a 4000 ppm NAClO solution. The cleaning solution was circulated for 40 min at a temperature of 40 °C. Then the hollow fibres were submitted to a final rinsing with bi-distilled water. Figure 6.3.1-3 shows the characterization of PSU hollow fibre membranes before and after the cleaning procedures: the initial water permeability was completely restored after the chemical cleaning.

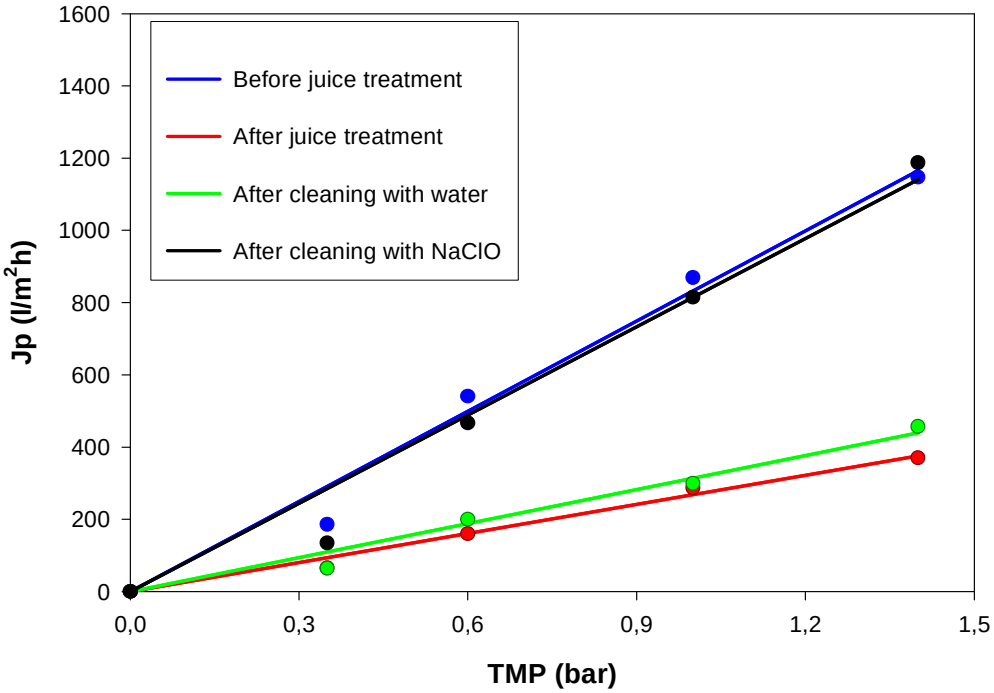

Figure 6.3.1-3 *Effect of membrane cleaning on the water permeability of the PSU hollow fibre membranes*

6.3.2 Analytical evaluations

Tables 6.3.2-1 and 6.3.2-2 show the influence of the clarification treatment with PSU and PEEKWC membranes, respectively, on the juice composition. The permeate of the depectinised mandarin juice treated with both membranes contains most soluble solids and acids (in terms of citric acid) of the initial feed. The pH remains unchanged in the clarified juice of both membranes.

Colour and clarity of the juice were improved after filtration because of the removal of suspended colloidal particles and higher molecular weight soluble solids of the juice.

The decrease in total soluble solids is due to the removal of suspended solids: it is known that the presence of suspended and soluble solids increases refractometric readings [14].

Table 6.3.2-1 *Analytical measurements on samples coming from UF of mandarin juice with PSU membranes*

Parameter	Feed	Permeate	Retentate
Colour (A_{420})	2.406	0.064	2.492
Clarity($\%T_{660}$)	1.67	98.62	0.78
TSS (°Brix)	11.2	10.6	11
Suspended solids (w/w,%)	4.9	0.0	-
pH	3.37	3.39	3.49
Acidity (% citric acid)	0.8	0.8	0.79
Total phenolics (as GAE) (mg/L)	3.05	2.55	3.62
Total Flavonoids (mg Hesperidin/L)	566	204.4	709.25
TAA (mM Trolox)	5	3.4	4.7
Malic Acid (g/L)	2.4	1.47	3.3
Ascorbic Acid (g/L)	0.3	0.246	0.285
Citric Acid (g/L)	7.75	6.4	8

Table 6.3.2-2 *Analytical measurements on samples coming from UF of mandarin juice with PEEKWC membranes*

Parameter	Feed	Permeate	Retentate
Colour (A_{420})	2.385	0.078	2.495
Clarity($\%T_{660}$)	1.69	98.85	0.73
TSS (°Brix)	11.0	10.6	10.6
Suspended solids (w/w,%)	5	0.0	-
pH	3.35	3.42	3.45
Acidity (% citric acid)	0.78	0.76	0.7
Total phenolics (as GAE) (mg/L)	2.67	2.45	2.39
Total Flavonoids (mg Hesperidin/L)	524.33	236	723.84
TAA (mM Trolox)	5.5	4.3	5.2
Malic Acid (g/L)	3	2.4	3.74
Ascorbic Acid (g/L)	0.365	0.419	0.304
Citric Acid (g/L)	7	6.65	7

Figure 6.3.2-1 shows rejections of both membranes towards analysed compounds. A higher rejection towards total flavonoids was observed for the PSU membranes (64%) in comparison with the PEEWC membranes (55%); this phenomenon can be attributed to the strong association of these compounds to the colloidal fraction of the juice which is completely rejected by the membranes. Since total flavonoids contributed to the TAA of the juice a consequent reduction of this activity in the clarified juice was observed. PEEKWC membranes showed also a lower rejection towards phenolics compound, vitamin C, malic and citric acids in comparison with the PSU membranes. This behaviour was in agreement with the lower rejection observed for PEEKWC membranes towards dextrans.

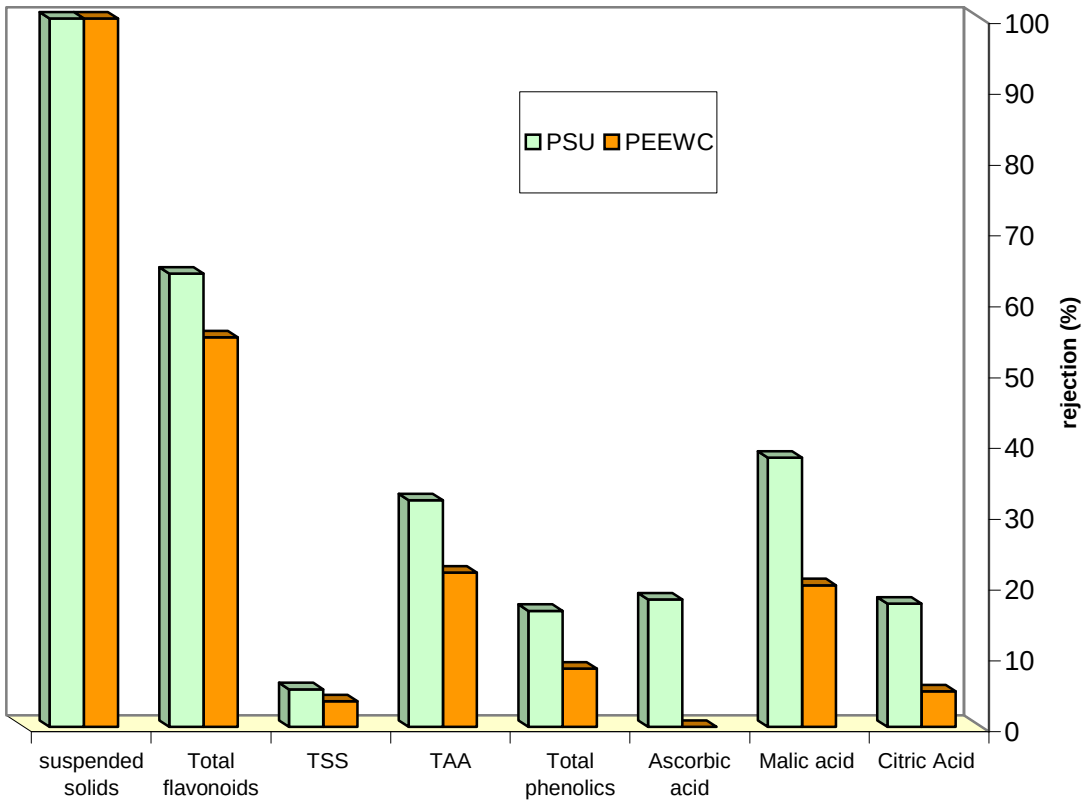

Figure 6.3.2-1 *Rejection of PSU and PEEKWC membranes towards analysed compounds*

In conclusion the treatment of mandarin juice with PEEKWC and PSU hollow fibre membranes prepared in laboratory, permits to obtain a clarified juice able to retain the food value of the original juice. However, in the clarified juice obtained with PEEKWC membranes was observed a higher recovery of antioxidant compounds, expecially in terms of vitamin C and total phenolic compounds, if compared with the PSU membranes.

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Conclusions

Membrane operations can be considered as a viable approach for the separation, recovery and concentration of bioactive compounds in Citrus fruits and their by-products.

The clarification and concentration of bergamot juice by using ultrafiltration and osmotic distillation processes represents an interesting alternative to the traditional use of fining agents and thermal evaporation to produce a juice with high nutritional content able to retain the peculiarity of the fresh juice. Experimental results confirm the possibility to recover most ascorbic acid, hesperidin, naringin, neohesperidin in the clarified bergamot juice, due to the low rejection of the UF membrane towards these compounds (3-12%). Suspended solids were completely removed during the ultrafiltration process. In the OD process a little reduction of antioxidant compounds (12%) was observed in comparison with the fresh juice.

The integrated membrane process UF/NF can be considered as a valid approach for the recovery of the phenolic fraction from the bergamot juice in a suitable form for the use as a functional ingredient. NF membranes characterised by different rejections towards phenolic and sugar compounds have been identified; these membranes separate the clarified juice in a permeate fraction containing 50% of the initial sugar content and in a retentate fraction, enriched in polyphenols, characterised by a high TAA value. The latter has a potential application in the pharmaceutical industry due to the recent statin-like active principles identified in the bergamot juice and their anticholesterolemic activity.

A polyphenolic concentrate can be obtained from the press liquor coming from pigmented orange peels by using an UF-NF-OD sequence. It can be potentially used as an industrial colorant (as an alternative to the use of artificial ones) or for the preparation of pharmaceutical and nutriaceutical products.

Finally, PEEK UF membranes prepared in laboratory by using the phase-inversion technique, permit to obtain a higher recovery of antioxidant compounds, especially in terms of total phenolic compounds, if compared with PSU membranes.

The obtained results confirm the efficiency of membrane operations in the treatment of Citrus fruits and their by-products aiming at the selective removal of bioactive

compounds and the formulation of products for nutriaceutical applications. These processes may be proposed as valuable alternatives to traditional operations to redesign transformation cycles of fruit and vegetables in order to improve the quality of the final products, to recover compounds with high added value (antioxidants and bioactive compounds) from by-products, to reduce energy consumption and environmental impact. As in other industrial sectors, the possibility to realise integrated membrane operations in which all the productive steps are based on molecular membrane separations can be considered as a valid approach for a sustainable industrial growth within the *process intensification* strategy.