Chapter 6 Use of power ultrasound, supercritical fluids and membrane technology to obtain and/or preserve biological products for clinical use

Capítulo 6 Empleo de ultrasonidos de potencia, fluidos supercríticos y tecnología de membranas para la obtención y/o conservación de productos biológicos de uso clínico

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Abstract

The awareness of the population to acquire products based on natural components that provide health care benefits has become a necessity nowadays. From this situation arises the initiative to look for new and better alternatives to replace synthetic active ingredients with components obtained from biological extracts. Bioactive compounds are molecules that contain a variety of beneficial properties for people as they contribute to the prevention and treatment of diseases; therefore, obtaining and preserving these components requires processes that guarantee their functionality. The implementation of emerging technologies that do not require the use of heat or require it at low temperatures during the processes of extraction and/or microbial inactivation of biological products, is a solution to the global problem of replacing conventional methods that affect the quality of the products, in addition to negatively impacting the environment. During this work, a vast compilation of information from articles, books and theses on non-thermal technologies such as Power Ultrasound, Supercritical Fluids and Membrane Technologies was carried out. As a result, it was found that these technologies are suitable for the extraction, separation and microbial inactivation of biological products for clinical use, since they represent multiple advantages, such as time and energy savings during the processes, reduction of chemical waste, easy reproducibility at industrial level, higher yields of extracts, cost reduction and they are environmentally friendly. It could be said that the use of these emerging technologies is still new, but the results they have offered to date are really promising.

Bioactive compounds, High power ultrasounds, Supercritical fluids, Membrane technology

Resumen

La concientización de la población por adquirir productos a base de componentes naturales que aporten beneficios para el cuidado de su salud se ha vuelto una necesidad hoy en día. A partir de esta situación surge la iniciativa por buscar nuevas y mejores alternativas que sustituyan los componentes activos sintéticos que pueden ser perjudiciales para la salud, por componentes obtenidos de extractos biológicos. Los bioactivos son moléculas que contienen variedad de propiedades benéficas en las personas ya que coadyuvan a la prevención y tratamiento de enfermedades; por lo que la obtención y conservación de estos componentes requieren de procesos que garanticen su funcionalidad. La implementación de tecnologías emergentes que no requieran el uso de calor o bien, lo requieran a bajas temperaturas durante los procesos de extracción y/o inactivación microbiana de productos biológicos, es una solución a la problemática de carácter global de sustituir los métodos convencionales que afectan la calidad de los productos, además de impactar negativamente al medio ambiente. Durante este trabajo se realizó una vasta recopilación de información procedente de artículos, libros, tesis sobre tecnologías no térmicas como lo son los Ultrasonidos de Potencia, Fluidos Supercríticos y Tecnologías de Membrana, como resultado se encontró que estas tecnologías son adecuadas para la extracción, separación e inactivación microbiana de productos biológicos de uso clínico, ya que representan múltiples ventajas, como un ahorro de tiempo y energía durante los procesos, disminución de desechos químicos, fácil reproducibilidad a nivel industrial, mayores rendimientos de extractos, reducción de costos y son amigables con el medio ambiente. Se podría decir que el uso de estas tecnologías emergentes aún es novedoso, pero los resultados que han ofrecido hasta el día de hoy son realmente prometedores.

Compuestos bioactivos, Ultrasonidos de potencia, Fluidos supercríticos, Tecnología de membranas

6.1 Introduction

The distribution of safe and quality products has an impact on the processing and manufacturing of these products, the ingredients of which must contain a high biological value. The safety of biological products is limited by the application of efficient preparation methods, e.g. extraction, separation, purification, viability of microorganisms, etc. (Castillo et al., 2004).

Obtaining and preserving biological products is an ancient practice and the methods that have been applied for this purpose have had the capacity to offer functional and safe products, but these methods can compromise their biological integrity and, as collateral damage, bring negative consequences that impact the environment.

Conventional methods of microbial extraction and inactivation are mostly based on the use of high temperatures, addition of aggressive solvents and destructive physical processes, which, although they achieve their purpose, are not the best processing option. For this reason, researchers were interested in finding technologies and mechanisms that do not rely on the use of heat, solvents or any other agent that compromises the safety of the final product. Among the objectives, it is also sought that these non-thermal technologies are environmentally friendly, require less processing energy and reflect a low cost for manufacturers and buyers (Barbosa and Bermudez, 2010).

This paper provides a literature review of three non-thermal technologies: Power Ultrasonics, Supercritical Fluids and Membrane Technology. Each of them is based on different mechanisms of action, but their similarity is based on extraction, separation and microbial inactivation that does not affect the quality of the bioactives, in addition to providing cost, energy and processing time savings (Barbosa and Bermudez, 2010).

Non-thermal technologies represent a new processing system, which in the last 30 years have been studied on a global scale with rapid growth in different industries, such as food, pharmaceutical, agronomy and cosmetics. Another advantage is the possibility of combining two technologies to intensify the extraction, separation and/or inactivation processes, or to seek synergism between emerging and conventional technologies to optimize the overall quality of the products (Barbosa and Bermudez, 2010).

6.2 Biological products for clinical use

There are many definitions of biological products used for therapeutic purposes, but most of them refer to products originating from any living being, i.e., from any biological source: plants, animals, microorganisms or human derivatives (Royal Decree, 2007). According to the World Health Organization (WHO), biological products are considered to be drugs obtained from microorganisms, blood or other tissues, whose manufacturing methods may include the growth of strains of microorganisms on different types of substrate, use of eukaryotic cells, extraction of substances from biological tissues, including human, animal and plant tissues, products obtained by recombinant DNA or hybridomas, propagation of microorganisms in embryos or animals, among others (Pombo, 2008).

The nature of the components of the biological product is what differentiates it from traditional chemical drugs, which are made from a combination of chemical ingredients5. Table 6.1 shows the main differences of bioproducts compared to synthetic small molecule drugs (Strohl and Strohl, 2012).

Table 6.1 Characteristics of biologic and small molecule drugs.

Property	**Biological (non-monoclonal antibody)**	**Monoclonal antibody**	**Small molecules**
Composition	Hormone or enzymes	Protein-Antibodies	Synthetic organic compounds
Molecular weight	>700 Daltons	150 000 Daltons	<700 Daltons
Mode of activity	Substitution of a lost or decreased activity of peptides, proteins or enzymes.	Binds to extracellulartargets to antagonize,agonize, or decrease activity	Binds to extracellulartargets to antagonize, agonize, or decrease activity
Production	By cells of bacterial, yeast or mammalian origin	Using cells of mammalian origin	Chemical synthesis
Product defined by	Biological activity; biochemical by the manufacturingprocess	Biological activity; biochemical analysis; by the manufacturing process	Chemically
Specificity	High	Very high	Tends to be low
Route of administration	Intravenous or subcutaneous injection	Intravenous or subcutaneous injection	Usually oral

Source: "Therapeutic Antibody Engineering: Current and Future Advances Driving the Strongest Growth Area in the Pharmaceutical Industry" by Strohl W, Strohl L. 2012.

The purpose of the use of this type of compounds lies in prevention to improve the quality of life and/or in the treatment of multiple types of ailments or diseases. The use of this technology started at the end of the 20th century, around the 1980s, and since then, they have become the main treatments for many diseases, such as carcinomas, diabetes, multiple sclerosis, heart attacks, strokes and autoimmune diseases (IAPO, 2013).

6.2.1 Types of biological products for clinical use

Biological products are characterized by being synthesized by living organisms, they are very large and complex molecules, they are compounds with a very labile structure, they present complex manufacturing processes, it is difficult to achieve stabilization in order to store them and maintain their structure and biological efficacy (Cuñetti, 2012). Table 6.2 briefly defines the products that are considered to be of biological origin (Pombo, 2008).

Table 6.2 Definition of the different products of biological origin

Biologicalproduct	Definition	Reference
Vaccine	Antigenic preparation that induces immunity against infections [9]	Abbas A, Lichtmann A, Pillai S. Basic immunology: functions and disorders of the immune system. 4th ed. Barcelona, Spain: Elsevier; 2014.
Allergen	Product that elicits an immune response to anallergenic agent [3]	Royal Decree 1345/2007 of October 11, 2007, which regulates the procedure for theauthorization, registration and conditions of dispensing of industrially manufactured medicines for human use. (Official State Gazette, number 267, of 11-10-2007).
Antigen	Product that elicits an immune response to aforeign agent [10]	Rojas W, Anaya JM, Cano LE, Aristizábal B,Gómez LM, Lopera D. Rojas Immunology. 17th ed. Medellín, Colombia: Fondo Editorial; 2015.
Hormone	Tissue-produced chemical that has a specific effect on a target tissue [11]	Bishop M. Clinical chemistry: principles, procedures and correlations. 5th ed. Mexico: Mc Graw Hill; 2007.
Cytokine	Proteins mediating inflammatory and immunereactions [9]	Abbas A, Lichtmann A, Pillai S. Basic immunology: functions and disorders of the immune system. 4th ed. Barcelona, Spain: Elsevier; 2014.
Enzyme	Molecules that catalyze the conversion of compounds to one or more different compounds increase the rates of the reaction [12].	Rodwell V, Bender D, Botham K, Kennelly P, Weil P. Harper's illustrated biochemistry. 30th ed. Mexico: Mc Graw Hill; 2015.
Human blood and plasma derivatives	Blood components for therapeutic, diagnostic,preventive or research applications [13].	Norma Oficial Mexicana del 26 de octubre de 2012, Para la disposición de sangre humana y suscomponentes con fines terapéuticos (Poder ejecutivo, Secretaría de Salud, del 26-10-2012).
Immune sera	Intended polyclonal antibody sera. They are used for passive immunization of certain diseases [14].	Linares J. Immunohematology and transfusion: principles and procedures. Venezuela, Caracas: Cromotip; 1986.
Immunoglobulins	Proteins capable of behaving as antibodies [14].	Linares J. Immunohematology and transfusion: principles and procedures. Venezuela, Caracas: Cromotip; 1986.
Antibody	A glycoprotein produced by B lymphocytes that binds to antigens, often with a high degree of specificity and affinity [9].	Abbas A, Lichtmann A, Pillai S. Basic immunology: functions and disorders of the immune system. 4th ed. Barcelona, Spain:Elsevier; 2014.
In vitro diagnosticreagents	Reagent product, calibrator, made in control material, intended for the study of samples from the human body [15].	Diaz Roa K. Application of reactivevigilance and its advances in Colombia. [IV Workshop on Strengthening and Continuous Quality Improvement for the National Laboratory Network]. Bogotá D.C.: INVIMA; 2018.

Source: "*Biological product" by Who 2008*

All these products are used in multiple circumstances; one of the practices employed daily around the world is the use of vaccines for disease prevention, also the use of blood and blood derivatives as treatment for multiple ailments, the supply of hormones, among many other cases that have the purpose of improving health.

The substances described above, which are used as the active fraction of the final product that is supplied to patients, are not originally found in pure form; they are a part of the total components of the raw material. This is why it is necessary to separate one or more components within a complex mixture (Pombo, 2008). In the following, different conventional extraction methods are described in contrast to the techniques under development.

6.2.2 Conventional methods for obtaining biological products

Extraction is the most important step to isolate different types of bioactive compounds (Yang et al., 2011). The desired compounds could be extracted by conventional methods, which are defined as classical techniques based on the extraction capacity of different solvents, application of heat and agitation (Viganó and Martínez, 2015).

The extraction technique is highly dependent on the type of solvents, energy input and agitation to improve chemical solubility and mass transfer efficiency (Yang et al., 2011). Some of the methods that have been used since ancient times are precipitation by addition of salts, by ionic potential (Harris, 1995), use of solvents (González et al., 2009), crushing and heat (Yang et al., 2007).

6.2.2.1 Precipitation by addition of salts or salting-out

Method applied to obtain products of protein origin. It is based on the decrease in solubility when the concentration of salts increases in excess, protein-protein interactions become stronger in comparison to protein-solvent interactions, so they precipitate (BQ Experimentals Blog, 2010). The salt mostly experimented for protein precipitation is ammonium sulfate, due to its high solubility and its stability at temperatures from 0 to 30°C (Rojas, 2009).

6.2.2.2 Isoelectric point precipitation

Method used for protein precipitation, in which the pH of the solution is adjusted to values close to or equal to the isoelectric point of the protein of interest (Rojas, 2009).

Proteins are made up of amino acids, which can be positively or negatively charged. If the isoelectric point is reached, these charges on the surface neutralize each other, preventing electrostatic repulsion with other molecules, and attraction between the dissolved proteins themselves, thus forming precipitates (Harris and Angal, 1995).

6.2.2.3 Addition of solvents

The use of solvents is mostly used to obtain lipid extracts (González et al., 2009). Extraction can be performed from a solid or liquid mixture to a liquid phase, where the solvents commonly used are organic solvents (UNP, 2020) such as hexane, ethanol, methanol (González et al., 2009).

The technique is based on the difference in solubility between the extraction solvent, the substance of interest and the other non-significant substances present in the initial compound. For a good choice of the solvent to be used, the desired product to be obtained, the volatility and toxicity of the solvent must be considered (UNP, 2020).

6.2.2.4 Crusher and heat

In addition to the addition of chemical products, mechanical procedures are necessary to assist in the extraction and obtaining of the product of interest, in order to cause mechanical damage to the raw material in which the compound to be isolated is present. Crushing as well as heat are intended to achieve the lysis of the starting material (Merino et al., 2019) in order to release the substances to be obtained through the different chemical methods mentioned above.

These are the mostly employed methods reported in the literature, but there is a diversity of techniques employed that aim to obtain bioproducts. These methods are widely used due to the advantages they offer, among them the easy handling, the acquisition costs of materials and reagents as well as the operating costs are economical (Yang et al., 2011). In contrast, conventional techniques also have disadvantages, mainly the treatment times are very long (Viganó and Martínez, 2015); large amounts of solvents are needed (Santos et al., 2013), which represents an environmental problem; another unfavorable point is the denaturation of the biological products themselves, due to the aggressiveness of the technologies used.

6.2.3 Conventional methods for the preservation of biological products

Throughout history, multiple techniques and procedures have been used to ensure the safety, quality and shelf life of products in multiple industries. In the present work we will be referring to products derived from biological sources and intended for clinical use, so it is understood the importance of complying with all the sanitation standards during all the processes and stages.

Temperature is one of the main elements that influence the viability and development of microorganisms; prolonged exposure to temperatures above the optimum produces structural changes, especially focused on proteins, enzymes, RNA, denaturation of membranes and/or nucleic acids (Hurst, 1977), in addition to functional alterations that end in a progressive decrease in the number of cells. (Perez et al., 2016).

Conventional preservation methods mainly include sterilization with the use of heat. Thermal processes are governed under two premises: the heat resistance of microorganisms for each specific product and the heating rate of the specific product (Awuah and Ramaswamy, 2006).

The use of thermal technologies for bacterial inactivation has proven to be effective in terms of product safety (Awuah and Ramaswamy, 2006), however, they are aggressive methods that lead to a decrease in the quality of the final product due to the denaturation of some biomolecules, affecting their initial functionality.

Currently, industries are opting for processing and preservation alternatives that are environmentally friendly, do not present health risks and provide high quality of the extracts used.

6.2.4 Non-thermal technologies for the production and preservation of biological products

The constant evolution of technology, the commitment to offer users better bioproducts and the awareness of reducing the environmental impact on our planet has led to the development and implementation of new and effective technologies for obtaining and/or preserving biological products. Thus, the so-called "emerging technologies" were born, which, although they do not completely replace conventional methods, complement each other to obtain more satisfactory results (Paniagua 2017).

The study of these new technologies has aroused particular interest thanks to all the benefits obtained in contrast to the use of conventional methods. And one of the main ones is that, by not requiring high temperatures during the processing of the raw material, many of the initial properties of the compounds remain unharmed; in turn, it significantly reduces costs, energy and production time, positively impacting the economic, social and environmental sphere (Bermúdez and Barbosa, 2010).

Within the group of these new technologies we can find High Intensity Pulsed Electric Fields, Light Pulses, Oscillating Magnetic Fields, Power Ultrasound (HPU), Supercritical Fluids (SF), Membrane Technology (MT), among others (Soleno, 2015). All of them are being tested and studied with the aim of being able to meet the constant demand for better products by consumers.

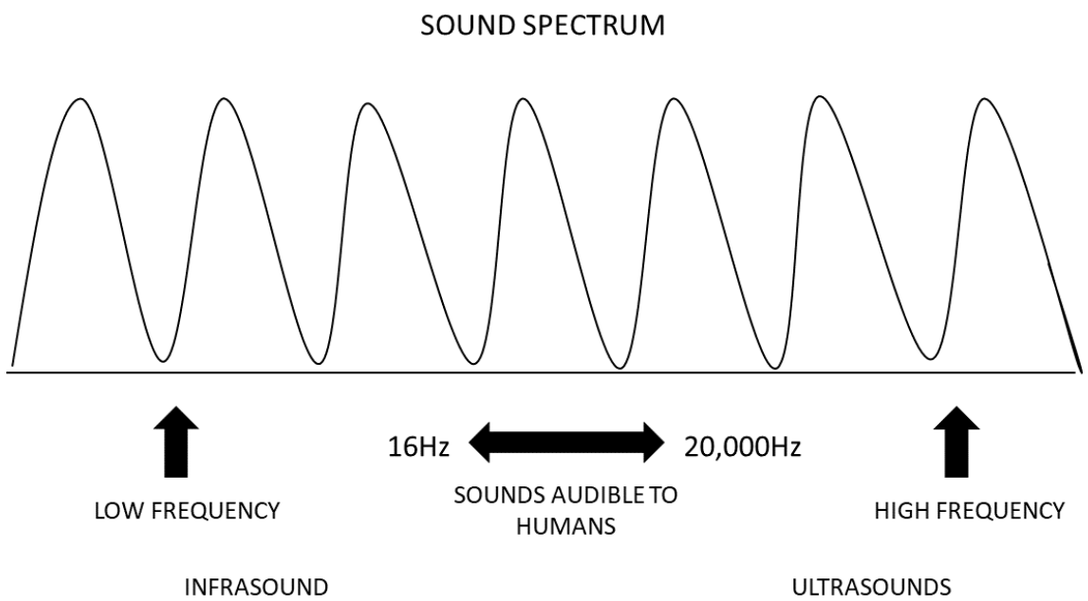
This paper reviews three technologies in particular: HPU, SF and MT.

6.3.1 Power Ultrasound

6.3.1.1 Ultrasound Generalities

We can begin by elucidating the term "ultrasound". It is made up of two words; -ultra: "beyond", "on the other side of"- (Real Academia Española, 2021), followed by -sound: "mechanical vibration transmitted by an elastic medium"- (Real Academia Española, 2021); being able to finally define the compound word as mechanical waves that propagate forcibly through an elastic medium longitudinally transmitting continuous vibrations between adjoining particles at a frequency above 20kHz up to 10MHz, thus exceeding the human audible spectrum. Figure 6.1 illustrates the boundaries between infrasound, audible range and ultrasound.

Figure 6.1 Sound spectrum



Source: (Morales and Martínez, 2015)

The range of the sound spectrum is determined by the frequency at which the mechanical waves that compose it oscillate, which are propagated longitudinally through a solid, liquid or gaseous medium. The wave frequency refers to the cycles completed per unit of time. This measurement is expressed in Hertz, where one Hertz corresponds to one complete cycle per second.

According to the frequency at which these acoustic waves vibrate, the sound spectrum is divided into three main groups: infrasound, sound and ultrasound.

Infrasound. They originate when the frequency of wave vibration is below 16 Hz below the threshold of human sensitivity. Although the infrasonic spectrum is imperceptible to the human ear, it does have an impact on the matter around us, producing contractions and dilations, including the particles of our body (Carcel et al., 2012). Thanks to the lower attenuation of infrasound these waves can travel a greater distance than sound or ultrasound, and this helps in the detection of large phenomena to prevent some natural disaster, such as earthquakes, earthquakes, volcanic explosions, turbulence, tornadoes Sound. Also called spectrum or human audible range. An individual is capable of perceiving an extensive range of sounds conditioned in a frequency range between 16 Hz to 20 KHz and, an amplitude range that goes from 0 to 140 dB. Although this hearing range varies depending on several factors, such as age. The perception of sound ranges from the threshold of audibility to the threshold of pain. The former, refers to the minimum signal pressure that is capable of stimulating an audible sensation to the human ear in the absence of noise.

Ultrasound. These are frequencies that exceed the human audible range, that is, higher than 20 KHz. However, ultrasound is perceived by some animals such as dogs. Depending on the frequency range and intensity, ultrasound can have applications such as in medicine, where one of the most common is ultrasound to observe different organs; or also used in multiple industrial areas.

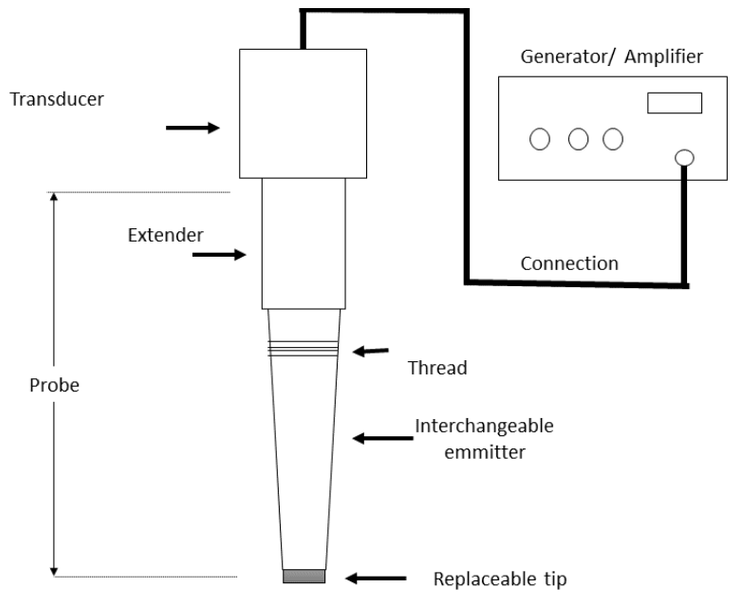
According to industrial applications, there are two classifications of ultrasounds, based on their frequency and intensity (McClements, 1997). The so-called signal ultrasounds are identified by being of low intensity (<1W/cm3), but high frequency (ranging between 100kHz and 20 MHz); because of these characteristics the power of this type of ultrasound is weak so it does not cause changes in the medium in which they expand, which is why they are used for non-invasive processes that require the destruction or transformation of components within the medium. On the contrary, there are power ultrasounds, which as its name refers, fluctuate at low frequencies (20 - 100kHz) and high intensities (higher than 1 W/cm^3) which gives them the property of making physical changes of some component suspended in the medium (Paniagua, 2017).

Due to the subject in question of this project, the present work focuses on power ultrasounds, since their previously mentioned qualities are necessary to meet the objective in the conservation and obtaining of bioproducts.

6.3.1.2 Power Ultrasonic Systems

Ultrasonic waves come from a source of acoustic energy, but initially this type of energy is not available, so its transformation is required. Ultrasound generation equipment is composed of three main elements, a generator, a transducer and an emitter (Robles and Ochoa, 2012). Broadly speaking, the generator is responsible for transforming the electrical signal from the network to the desired frequency; the transducer converts the high-frequency electrical signal into mechanical vibrations; and the emitter radiates the acoustic energy through the transducer to the medium to be treated (Cárcel, 2003). Figure 6.2 shows a schematic of the basic components of a probe-type ultrasound generator.

Figure 6.2 Schematic diagram of the ultrasonic generator equipment, probe type



Source: (Rayo, 2014)

Depending on the application needs required, the most appropriate ultrasonic system to be used will depend on the application requirements. Ultrasonic baths are the preferred systems to work with, due to their easy accessibility and low acquisition and processing costs (Povey and Mason, 1998). On the other hand, there are also probe systems, which must be manufactured with resistant materials to withstand the wear caused by cavitation generated in the medium. The probe systems are mostly used for research purposes, due to their convenience, and that is why we will be discussing experiments using this type of system.

The use of low frequency but high intensity ultrasound causes a transformation or damage of the matter in the medium that the ultrasonic waves pass through (Cárcel, 2003). These changes can be of a physical, chemical and biochemical nature that can be used in a number of applications in different industrial fields (Robles and Ochoa, 2012). One of the most studied and understood phenomena is cavitation.

6.3.1.3 Cavitation

In liquid media where mechanical sound waves propagate, microbubbles tend to be generated, and this is due to the fluctuation of the cycles giving rise to sudden pressure changes (Soria and Villamiel, 2010). During the low-pressure phase, gas spaces are created, which, in turn, grow as the pressure increases. This event results in continuous rarefaction and compression of the bubbles. During rarefaction the bubble increases in size, expands; while in compression it contracts and decreases in area, the amount of gas that the bubble gains in the expansion cycle is greater than that which it loses during the compression cycle. This means that in each pulse it will increase in size (Cárcel, 2003). Finally, the bubbles collapse and end up imploding, causing temperature rises of up to >4000°C and pressure of 1000 bar (Paniagua, 2017), although this environment has very short times of duration (fractions of a second). The intensity of cavitation and its effects will depend on the characteristics of the medium, such as its viscosity, process variables, ultrasonic intensity, frequency and pressure (Cárcel et al., 2012; Chandrapala et al., 2012). Cavitation is a mechanism that results in microbial inactivation due to high pressures and temperatures, inducing cell lysis.

6.3.2 Use of power ultrasound for obtaining and preserving biological products for clinical use

6.3.2.1 Obtaining biological products

In order to obtain the bioactives retained inside the cells, it is necessary to extract them by disrupting the cell wall. Generally, there are two mechanisms to achieve cell lysis: chemical and mechanical. The former usually consist of chemical treatment, osmotic shock and enzymatic treatment. While the latter, encompass other processes such as, high pressures, grinding, agitation, and ultrasonication (Lee et al., 2017). However, the advantage of ultrasound compared to traditional methods is the low energy consumption required, which, in turn, is reflected in decreased costs (Chemat et al., 2017) and decreased pollution to the environment.

Multiple studies have been conducted to test the efficiency of HPU as an extraction system for active compounds. Kuan et al. (2020), obtained the maximum values (recovery 95.08 ± 3.02%, extraction efficiency 99.74 ± 0.05% and partition coefficient 185.09 ± 4.78) of anthoxanthin, which is a potent antioxidant compared to other carotenoids, extracted from the microalgae *Haematococcus p*. It is worth mentioning, that for processing a HPU equipment was used (Bandelin Sonoplus UV2200, Germany), under conditions of 200 W/cm^2 and 20 kHz. After the sonication process, the cell morphology of the algae was observed, showing damage and rupture of the cell wall due to cavitation; at the same time, the rupture of the membranes favors the release of the anthoxanthin biomolecules. Therefore, it follows that ultrasonication is an effective technique for cell wall rupture of algae such as *Haematococcus p*. (Kuan et al., 2020).

One of the most promising sources for obtaining biomolecules that are intended for the manufacture of products with medicinal properties are plants. Medicinal plant extracts offer variety of biocompounds (Sun et al., 2019), such as alkaloids (Wang et al., 2018), anthraquinones (Jibril, et al., 2019), flavonoids (Zhou et al., 2019), glycosides (Dong et al., 2015), oils (Senrayan and Venkatachalam, 2019), pectins (Grassino et al., 2016), phenolic compounds (Um et al., 2018) and polysaccharides (Mandal et al., 2009). Lysis of the cell wall and membrane is necessary to release and obtain all these compounds (Sun et al., 2019). Traditionally, the mechanisms used have disadvantages, including long processing times, low bioactive purity and low efficiency (Mandal et al., 2009; Lu et al., 2012; Mura et al., 2015), which has led producers to search for new and better alternatives. As a solution to this problem, HPU was implemented as a technology that offers high efficiency, low energy consumption, higher quality and yield of extracts, as well as easy process automation (Wang et al., 2018).

In this context Sang et al. (2016), achieved the total extraction of anthocyanins (65.04 mg/100g) and polyphenols (947.39mg/100g), extracts with antioxidant, anti-inflammatory, anticancer and antimutagenic properties (Zhu et al.,2017; Huang et al., 2010); coming from Nitraria t. -raw material of active compounds and pigments- using HPU bath (TCX-600 S. Jiningtianyu Ultrasonic Instrument Co. Ltd., Shandong, China) at 30kHz and 300W/cm^2 conditions, also required a temperature of 70°C for 32 minutes by adding 51.15% ethanol (Sang et al., 2017).

In another assay by He et al. (2016), phenolic compounds and anthocyanins were extracted from Vaccinium a. employing HPU equipment (Hong Xiang Long Biotechnology Co., Ltd; Beijing, China) at 400W/cm^2, temperature of 61.03°C, processing time of 23.67 minutes and addition of 70% ethanol and 0.01% hydrochloric acid. As a result, total anthocyanins of 4.11±0.01mg/g and total phenolic of 16.01±0.03mg/g of extracts were obtained, demonstrating better compound recovery results unlike conventional solvent extraction (He et al., 2016).

Recovery of flavonoids is also of interest due to their biological importance and their various antitumor, hepatoprotective, antibacterial, anti-inflammatory properties, and antioxidant activity (Yang et al., 2013). Wei et al. (2013), recovered apigenin, baicalin, and luteolin, major flavonoids familiar for their effect against various human cancer cell lines. The system used consisted of HPU (Branson B-33510E-DTH, USA) at 40kHz and 185W/cm^2, heat reflux assisted, 50°C temperature, 30 min processing, plus addition of 60% ethanol. This synergy of technologies was compared with the use of heat reflux alone at 60°C, 85% ethanol and 60 minutes processing, showing that ultrasound-assisted extraction obtained better extraction yields. Associated with savings in time, energy, addition of organic solvents and obtaining analytes with higher purity (Wei et al., 2013).

6.3.2.2 Preservation of biological products - microbial inactivation

The main mechanism of microbial inactivation of HPU is due to cavitation. Continuous cycles of microbubble compression and decompression cause the abrupt collapse of the microbubbles, alternating the aqueous medium where the microorganisms are located due to the high temperatures and pressures reached during a fraction of time (Carcel et al., 2012).

Microorganisms are able to aggregate into communities that grow in a matrix of proteinaceous material (Costerton et al., 1995), in turn, these bacteria are released from the matrix to continue colonizing new surfaces (Lasa et al., 2005). Webber et al. (2015), used HPU at 40kHz frequency, power of 81W/cm^2 for 10 minutes for biofilm removal of three Salmonella species. They also used vortex method for 2 minutes to perform the comparison between both methods; the results showed no significant difference in log cycle reduction between Salmonella species (Webber et al., 2015).

Within the food industry, multiple studies have been conducted on the inactivation of microorganisms using HPU technology. Perez (2020), analyzed the effectiveness of using HPU as a treatment for inactivation of goat and maternal milk microbiota, for HPU treatment a continuous flow system was used; a digital ultrasonic device (Hielscher model UP400St) at conditions of 400W/cm^2 and 24kHz at 40°C. In the treatment of goat milk, a temperature of 53.2°C was achieved at the same frequency and intensity conditions. Regarding aerobic mesophilic microorganisms, a reduction of 0.50 logarithmic cycles was observed; while for enterobacteria, a reduction of 0.66 logarithmic cycles was achieved. On the other hand, for treatment with breast milk at the same conditions, but at a final temperature of 52.9°C, an inactivation of aerobic mesophiles of 0.69 logarithmic cycles was recorded (Pérez, 2020). As a second trial, the effect of HPU coupled with SC-CO_2 technology on goat milk alone was studied. The details of the processing are described in the section Combined technologies.

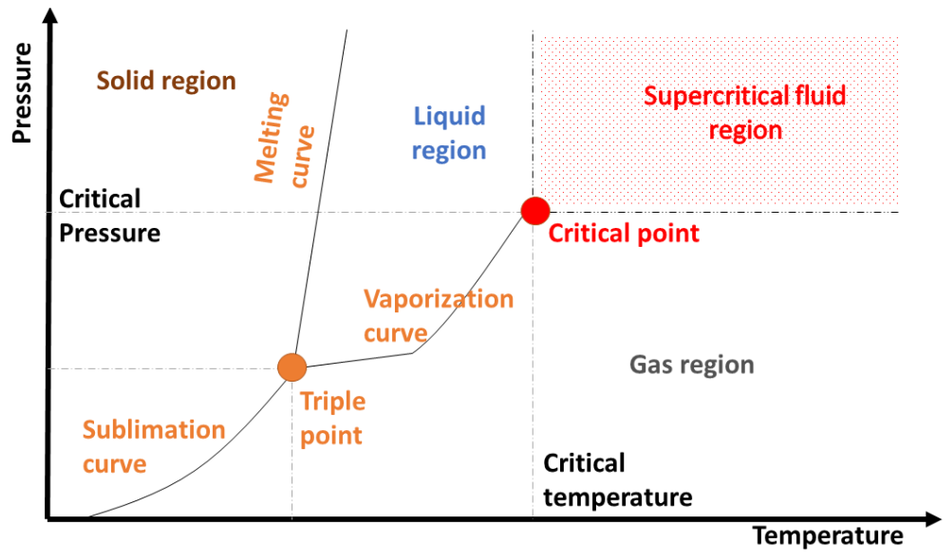
Likewise, other authors such as Gomez et al. (2020;2021) and Liao et al. (2018), have employed HPU-assisted microbial inactivation mechanism in combination with other technologies such as SF, Non-Thermal Plasma (NTP), etc. These, among other studies, have shown that HPU as the sole inactivation method does not achieve total inactivation of microorganisms (Li et al., 2016), however, the conjunction of this with other non-thermal technologies can obtain more effective results (Li et al., 2017). In the Combined Technologies section, the synergy of the use of HPU assisted by other methodologies can be analyzed.

6.3.3 Supercritical Fluids

As its name indicates, Supercritical Fluids (SF) are characterized by having pressure and temperature conditions beyond the critical point (McHug et al., 1994), being indistinguishable between a liquid and a gas, since some of its physicochemical properties oscillate between these two states of matter (Montañés 2009). Figure 6.3 shows the diagram of the different phases of a pure compound, visualizing the commonly known states of matter: solid, liquid and gas; it also shows a point where this triad converges, the so-called triple point (Montañés, 2009).

At the triple point all phases coexist. Scaling on the vaporization curve, there is a point where pressure and temperature are critical, thus modifying some of the physicochemical properties of the compounds, among the most important or of greatest interest are density, diffusivity and viscosity. The interest lies in the fact that they confer to this type of fluids the ability to be a better extraction solvent compared to if it were a simple liquid or gas (Paniagua, 2017).

Figure 6.3 Representative diagram of the different phases of a pure compound



Source: (Mendiola, 2008)

The use of Supercritical Carbon Dioxide (SC-CO_2) has generated great interest thanks to the pros resulting from its use as a solvent. SC-CO_2 is the ideal gas: it is inert, non-toxic and non-flammable; it is inexpensive and affordable, easily disposed of and reusable (Ortuño, 2014), it is also easy to control and is generally regarded as safe (Generally recognized as safe GRAS is a designation by the FDA that some chemical or substance added to food is safe).

Another advantage of SC-CO_2 is that both critical temperature (Tc) and critical pressure (Pc) are achieved without high energy demands. Pc is reached at 73.86 bar and Tc at only 31°C (Velasco et al., 2007). This property influences the preference of CO_2 over other solvents. In addition, many of the commonly used substances are more likely to leave product remnants, interfering with the purity of the desired extract.

Treatment with SC-CO_2 is revolutionizing the market in multiple industries, such as food, chemical, cleaning, pharmaceutical, agri-food, among others; this derived from the need to search for new and better methods of obtaining and/or preserving the active product of interest by manufacturers to consumers, who are increasingly aware and, therefore, demanding regarding the quality of the final product they purchase (Ozuna, 2014).

6.3.4 Use of supercritical CO2 in obtaining and preserving biological products for clinical use.

The characteristics of SC-CO_2 give it the potential to act as a solvent and extract active compounds or as an inactivator of the cell wall of microorganisms (Ortuño, 2014), contributing to the safety and preservation of the final product.

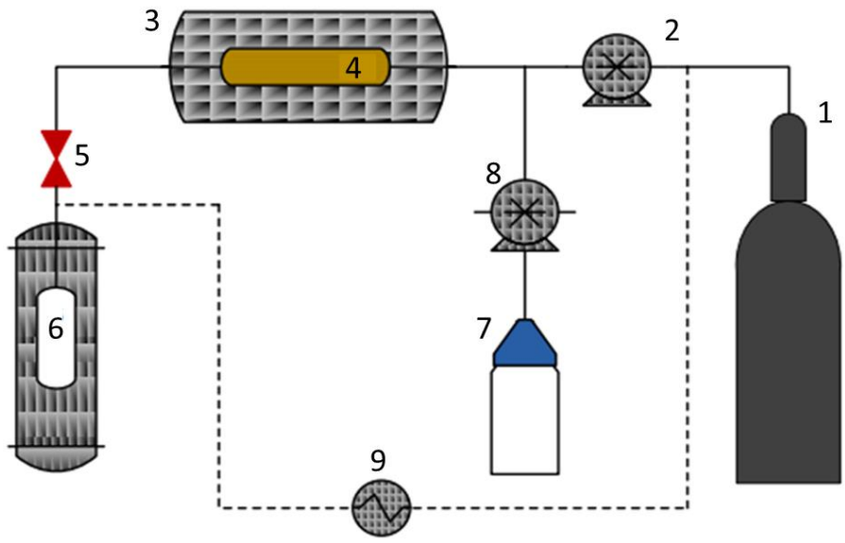
6.3.4.1 Obtaining bioproducts

The physicochemical properties of SC-CO_2 make it an effective extraction solvent. Its density, compared to that of a gas is 100 to 1000 times higher, but approaches liquid values (Paniagua et al., 2017), in addition to the fact that it can easily vary this parameter by increasing or decreasing pressure and temperature (Raventós et al., 2002).

This makes it a solvent of interest. On the other hand, the viscosity is 10 to 100 times lower than that of liquids, and conversely, the diffusivity 10 to 100 times higher compared also to liquids (Paniagua et al., 2017); viscosity and diffusivity being approximate to the values of gases. In addition, the surface tension of SFs is equal to zero, facilitates the extraction of substances contained in solid matrices. These properties (density, diffusivity and viscosity) cause an increase in the mass transfer rate of the solute in supercritical fluids (Brunner, 2005).

The use of SF extraction technology is relatively inexpensive when applied at an industrial level; Figure 6.4 shows the basic components of an SF extraction system (Montañes, 2009). According to Figure 6.4, the CO_2 coming from the bottle is pumped by the pump to the extraction cell where the raw material to be extracted is located, which is usually inside an oven that controls the temperature to be reached during extraction above the critical point. The components of the raw material dissolved or entrained by the CO_2 precipitate in the separator due to the decrease in the solvent power of the CO_2 as the pressure is reduced. If the addition of modifiers is necessary, they are usually mixed with the CO_2 stream by means of a pump before entering the extract. In addition, if CO_2 recirculation is required, it would be necessary to liquefy the CO_2 at the outlet of the separators by means of a cooling stage with a heat exchanger (Montañés, 2009).

Figure 6.4 Basic diagram of a supercritical fluid extractor



1.CO_2 container; 2. Pump; 3. Oven; 4. Extraction cell; 5; 7. Modifier container; 8. Pump; 9. Heat exchanger. (Montañés 2009)

It is worth mentioning that in most cases the feedstock requires grinding as a pretreatment to increase the extraction rate.

One of the major advantages of using this technology is that when the SC-CO_2 extraction and separation cycle is completed, it is reused, reducing waste generation, lowering production costs and environmental impact, considering the use of SC-CO_2 as a green solvent.

The recovery of active bioproducts is of great interest due to their development potential for the manufacture of functional compounds (Essien et al., 2020), which serve as replacements for active components of synthetic origin. These compounds are extracted from natural sources of animal or plant origin. The extraction of bioactives is a complex process, because other structures are protecting the desired functional moiety. In the case of plants, bioactives are surrounded by insoluble structures, such as vacuoles and lipoproteins, complicating extraction (Corrales et al., 2008).

The healing properties of plant extracts have been known for centuries: they work as treatment for infections, fever, repel insects, as food additives (Ragab and Raafat, 2016). Such is the case of Pulicaria j., which has been shown to possess antimicrobial and antifungal properties and has even been used as an anti-malaria and anti-insect treatment (Algabr et al., 2012; Fawzy et al., 2013; Ragab and Raafat, 2016).

Al-Maqtari et al. (2020), evaluated the total phenolic content, antioxidant activity by DPPH-, antioxidant capacity by ABTS-+ and antimicrobial activity of *Pulicaria j*. extract, using SC-CO_2 extraction and 10% ethanol (EtOH10%) as cosolvent; the addition of a cosolvent such as ethanol is necessary to increase the solubility of polar organic components, because the solubility of CO_2 alone is low. The equipment used for the assay was an SF extractor (Waters, Milford, MA), at temperature conditions of 40°C, 300 bar for 120 minutes. Extraction with SC-CO_2 - EtOH10% was compared with conventional extraction methods, such as stirring supported with two solvents (ethanol and water) at different concentrations (100, 70, 50, 50, 30 and 0% v/v). The results of this experiment showed that the best extraction yield was obtained with the shaking-EtOH50% method, followed to the shaking-EtOH70%, while the SC-CO_2 - EtOH10% treatment obtained the lowest extraction (Al-Maqtari et al., 2020). The authors conclude that the results could be due to the fact that SC-CO_2 extraction is not suitable for obtaining sugars, water-soluble compounds and some phenolic compounds (Roseiro et al., 2013).

In 2021, Al-Maqtari et al. performed another assay on extraction of four aromatic herbs (*Artemisia arborescens, Artemisia abyssinica, Pulicaria j*., and *Pulicaria p*.) to evaluate the antioxidant activity by DPPH-, antioxidant capacity by ABTS-+ and antimicrobial activity of the extract of these plants by SC-CO_2. The procedure was carried out in an SF extractor (Waters, Milford, MA); at 40°C temperature, 300 bar pressure and 120 min processing time, with addition of a cosolvent (ethanol 10%). The highest extraction yield (at 8.92±0.41) corresponds to *Artemisia abyssinica*., showing a significant difference compared to the other three plants (Al-Maqtari et al., 2020). This variation could be attributed to the difference in abundance of polar compounds between one species and another (Mustapa et al., 2015). However, as many other factors are also involved in the extraction yield rate of herbs, such as the difference in plant structures, size of leaves and stems, moisture, essential oils and volatile compounds contained. Despite the aforementioned data, when evaluating the antioxidant activity by DPPH-, antioxidant capacity by ABTS-+ and antimicrobial activity it was revealed that the extract coming from Pulicaria j. was significantly superior than that of the remaining three plants (Al-Maqtari et al., 2020).

On the other hand, one of the major applications in the use of SC-CO_2 has been the extraction and separation of lipids from multiple biological sources, such as plants and animals (Sohran et al., 2021). Lipids have diverse industrial and technological applications, such as polymers, coatings, pharmaceuticals, cosmetics, solvents, surfactants, lubricants, pesticides, biofuel, among others (Tao, 2007).

Olive oil, which is mostly known for its nutritional use, contains properties that also include it as an adjuvant in physiotherapy. Conventionally, prior to oil extraction, olive trees are crushed to reduce their size and to obtain the oil in a more efficient way; however, extraction by SC-CO_2 offers the possibility of obtaining the oil without previous crushing. Al-Otoom et al. (2014), studied the propensity of olive oil extraction with SC-CO_2 technology by testing different parameters regarding pressure, temperature and operation time. Subsequent to the tests they were able to evidence that, the maximum yield of 12.3% oil with respect to olive tree weight, was obtained at 2400 bar, 60°C and 150 minutes of treatment, thus demonstrating that the most effective parameter is extraction time, followed by temperature and, finally, operating pressure (Al-Otoom et al., 2014).

Although the main sources for obtaining lipids are of animal and plant origin, they can also be extracted from other microorganisms, such as bacteria or algae (Valenzuela, 2009). The extraction of lipids from microalgae is generally performed with the addition of organic solvents, however, this process cannot be replicated on an industrial scale because of the pollution it represents for the environment and the high toxicity for humans (Obeid et al., 2018); in addition to the fact that the organic solvent does not have good diffusivity on the penetration of the cell wall of microalgae, making diffusion into the interior of the cells difficult for a successful extraction of lipids. Obeid et al. (2018) studied the selective extraction of neutral lipids in freeze-dried *Nannochloropsis o*. and *Chlorella v*. microalgae.

The methodology was evaluated under different operating parameters (use of co-solvent, pressure and time), with a constant temperature of 50°C. The highest neutral lipid extraction (97%) observed for Chlorella v. was with the use of 10% ethanol as co-solvent, at 250 bar and 230 minutes of treatment; on the other hand, the maximum extraction recorded for *Nannochloropsis o.* was 83% under the same pressure conditions and 230 minutes of treatment. Microscopic observation showed that cell wall integrity was maintained during the extraction process (Obeid et al., 2018).

In addition to lipid extraction by microalgae, it is also possible to obtain other bioactive products, such as antioxidants (β-carotene) (Bhat and Madyastha, 2000), sulfated polysaccharides (antiviral) and sterols (antimicrobial) (Otles and Pire, 2001). Chatterje and Bhattacharjee, 2013 applied SC-CO_2 technology for the extraction of antioxidants from *Phormidium v*. The SC-CO2 extraction equipment was SPE-ED SFE 2 model of M/s Applied Separations, Pennsylvania, USA, at temperature of 50°C, 500 bar and 90 minutes processing time. As a result, it was observed that in 10g of *Phormidium v*. biomass, the best combination of phytochemical properties was extracted with a 93% reduction of anatoxin-a. It is worth mentioning, that the assay was also performed at 40°C, 350 bar and 90 min, and at these conditions the highest extract yield was seen, however, the desired properties were obtained at the above mentioned parameters (Chatterjee and Bhattacharjee, 2014).

6.3.4.2 Microbial inactivation

The inactivation of microorganisms by applying SC-CO_2 technology has been widely used within the food industry, where satisfactory results have been obtained. From the first time that SF was used as a non-thermal preservation technology by Fraser, (1951); Foster et al. (1962), to the present, a variety of research has been published on the inhibitory effect of SC-CO_2 on microorganisms such as viruses, bacteria, yeasts, fungi (Corwin and Shellhammer, 1992; Gasperi et a., 2009; Facroniet al., 2010).

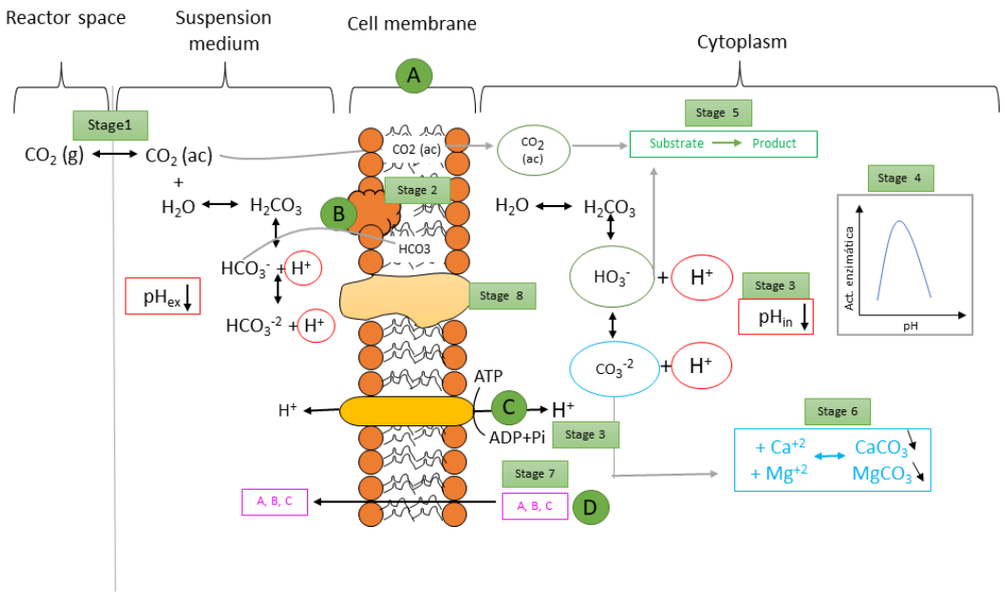
Dillow et al. (1999) subjected Gram-negative bacteria (*Salmonella s*., *Pseudomonas a*., *Escherichia c*., *Proteus v*. and *Legionella d*.) and Gram-positive bacteria (*Listeria i*. and *Staphylococcus a*.) to treatment with SC-CO_2 (205 bar at 34°C); resulting in higher resistance by Gram-positive bacteria compared to Gram-negative bacteria. This is due to the membrane composition of both classes of bacteria. Between the bilipid membrane of Gram-negative bacteria there is a thin layer of peptidoglycan (10%); in contrast, Gram-positive bacteria have a much thicker cell wall with respect to the percentage of peptidoglycan (90%), this property gives them greater resistance and lower permeability to external agents in advantage with Gram-negative bacteria that will be more susceptible due to their thin layer of peptidoglycan (Erkmen, 2012).

The rate of microbial inactivation depends on multiple factors, such as pressure, temperature, time and treatment medium, the nature of the microorganism, among others (Ortuño et al., 2014).

The model of the SC-CO_2 inactivation mechanism was proposed by Daniels et al. in 1985 and have been accepted by authors who have focused their research in this field of study (Dillow et al., 1999; Spilimbergo- Bertucco, 2003). Nowadays, these theories have been reinforced and better detailed thanks to technologies such as scanning electron microscopy and transmission electron microscopy, where it is possible to evaluate the condition of cells before and after treatment (Oulé et al., 2006). Thanks to previous research it is possible to compile the mechanisms of SC-CO_2 inactivation in a series of eight steps: (1, Figure 6.5) dissolution of pressurized CO_2 into the liquid phase where the cells are suspended and decrease in pH; (2, Figure 6.5) diffusion of CO_2 and modification of the cell membrane; (3, Figure 6.5) penetration of CO_2 into the cell interior and decrease in intracellular pH; (4, Figure 6.5) inhibition of cell metabolism due to inactivation of key enzymes; (5, Figure 6.5) direct inhibition of cell metabolism in response to the presence of CO_2 and formation of HCO^{3-}; (6, Figure 6.5) imbalance of intracellular electrolytes; (7, Figure 6.5) removal of intracellular components; (8, Figure 6.5) cell rupture (Damar-Balaban, 2006; Garcia-Gonzalez et al. , 2010).

Figure 6.5 schematizes the mechanism of microbial inactivation of SC-CO_2, emphasizing that this series of steps do not occur in a concatenated manner, but occur simultaneously.

Figure 6.5 Scheme of the stages of the inactivation mechanism by SC-CO_2: A, phospholipid bilayer; B, protein membrane; C, membrane; D, intracellular components



Source: (García González et al. 2007)

Qing et al, 2009 worked with a porcine acellular dermal matrix which was inoculated with a series of microorganisms, such as bacteria (*Enterobacter aerogenes*, *Staphylococcus cohnii*, *S. haemolyticus*, *Bacillus atrophaeus*), viruses [porcine encephalomyocarditis virus (EMC), porcine parvovirus (PPV), porcine pseudorabies virus (PRV) and murine leukemia retrovirus (LRV)], fungi (*Penicillium sp*, *Aspergillus sp*, *Verticillium sp*) and yeasts (*Debaryomyces hansenii*); all samples were treated with SC-CO_2 with the addition of a sterilizing agent such as paracetic acid (PAA). The different matrix samples inoculated with the different microorganisms were packed in a Tyvek® bag and treated in SC-CO_2 equipment (NovaSterilis, NY) with addition of PAA at temperature of 35-41°C, pressure of 94.1-100.3 bar, during variations from 1 to 30 minutes. At the end of sterilization, the biochemical and physical properties of the matrix were evaluated by enzymatic digestion and physical tests. The results showed that after 1 minute of treatment the vegetative forms of bacteria and yeast were reduced by 7 logarithmic cycles, while *Bacillus atrophaeus* was more resistant to the same treatment by reducing only 3 logarithmic cycles. For the sterilization of fungal forms, 5 minutes of treatment with SC-CO_2-PAA was used, obtaining a reduction of 6 logarithmic cycles, showing greater resistance than bacteria and yeast despite the longer sterilization time; however, fungi were more sensitive to the treatment compared to *Bacillus atrophaeus* due to a reduction of 2 logarithmic cycles. Post-sterilization, evaluations through enzymatic digestion and physical properties showed that SC-CO_2-PAA treatment does not cause significant changes in the structure and functionability of porcine acellular dermal acellular matrix (Qing et al., 2009).

6.3.5 Combined technologies

Techniques based on power ultrasound and supercritical carbon dioxide are useful for the extraction of substrates or for microbial inactivation; however, there are occasions in which they do not achieve a complete or optimal extraction or inactivation, without obtaining the desired results or prolonged process times are required to obtain satisfactory results.

Not obtaining the expected result on inactivation of aerobic mesophiles and enterobacteria in goat milk and human milk, Perez, (2020), attended HPU with SC-CO_2 equipment operating at 50W±5W/cm^2, at frequencies of 30±2kHz, at different variations of pressure and temperature, studying on the one hand the inactivation of aerobic mesophiles and on the other hand, the inactivation of enterobacteria. For the first class of microorganisms, in the treatment at low pressure and temperature (150 bar and 35°C), the aerobic mesophiles underwent a total inactivation of 4.72 logarithmic cycles in five minutes.

At high pressure and low temperature conditions (350 bar and 35°C) total inactivation of 4.39 logarithmic cycles was achieved in four minutes. Finally, in the last test, by increasing the temperature to 50°C and lowering the pressure to 150 bar again, total inactivation of 4.35 logarithmic cycles was observed to occur in only two minutes. This shows that at higher temperatures and lower pressures, in the case of mesophilic microorganisms, total inactivation is achieved in a shorter period of time compared to the previous conditions. In the case of Enterobacteriaceae, under conditions of low pressure and temperature (150 bar and 35°C) total inactivation of 3.3 logarithmic cycles was achieved in minute two; at conditions of 350 bar and 35°C total inactivation of 2.66 logarithmic cycles was witnessed at one minute of treatment, at 150 bar and 50°C a reduction of 3.29 logarithmic cycles was achieved in 2 minutes of treatment. Contrary to what happened with mesophilic microorganisms, enterobacteria achieved inactivation in less time using the parameters of low temperature at high pressures (Perez, 2020).

According to the results obtained through the previous work, it is possible to show that the HPU treatment does not show much effectiveness on its own; however, the HPU-SC-CO_2 combination shows a synergistic effect, achieving total inactivation of microorganisms.

Gomez-Gomez et al., (2020) tested the combined HPU-SC-CO_2 technology for pasteurization of lipid emulsions for clinical use on *B. diminuta*. and *E. coli* Lipid emulsions are a fatty acid preparation administered venously for those individuals who cannot obtain them orally through diet. The ultrasound system (Model WT210, Yogogawa, Japan) was programmed at power of 50 ±5 W/cm2 and frequency of 30 ±2 kHz. In conjunction, for the SC-CO_2 equipment (LDB, LEWA, Japan), a parameter variation of 100 and 350 bar and temperature of 35 and 50°C was used for 50 minutes of treatment. The experiment was also performed testing conventional thermal inactivation, which was carried out at the same 50°C and 50 minutes of treatment; this system only managed to obtain a reduction of 0.4 logarithmic cycles in E. coli and 0.9 logarithmic cycles in *B. diminuta*. The maximum reduction for E. coli using the combined HPU-SC-CO_2 technology at 350 bar, 50°C and 50 min processing conditions was about 7.5 logarithmic cycles.

In contrast, under the same conditions, *B. diminuta* was more sensitive to treatment, having a maximum reduction of about 8.4 log cycles. As a result of the multiple trials carried out, tested at different pressures and temperatures, it was evident that pressure had a significant effect on the inactivation of both genera of bacteria (Gómez-Gómez et al., 2020).

The synergistic bacterial inactivation power of HPU-SC-CO_2 could be essential not only for vegetative forms, but also for microorganisms with more resistant, complex and reinforced structures, such as spores (Dong et al., 2016). Bacteria form spores as a mechanism of resistance to extreme chemical and physical environments (Spilimbergo-Bertuco., 2003); therefore, processes are required that can achieve the necessary conditions for spore inactivation without compromising product safety. The use of HPU alone has proven to be ineffective for total spore inactivation (Mondal et al., 2015), however, the combination with SC-CO_2 improves the process of mass and heat transfer, together with the cavitation mechanism produced by HPU that would cause the degradation of the outer layers of the spore structure128, facilitating the penetration of SC-CO_2 inside the cells.

Gómez-Gómez. et al. 2021 evaluated the combination of these two technologies for the inactivation of spores from *Bacillus s.*, *Bacillus p*. and *Geobacillus s*. The system consisted of a SC-CO_2 extractor (LDB, LEWA, Japan) and an ultrasound system (WT210, Yokogawa Electric Corporation, Tokyo, Japan) at conditions of 35±5 W/cm^2 power, frequency of 30±2 kHz. For *Bacillus s*. the treatment was carried out at conditions of 85-95°C, 350 bar and 20 minutes of processing. On the other hand, for *Bacillus p*. and *Geobacillus s*. the temperature was set at 95°C, also changing the pressure to 550 bar for *Geobacillus s*. The results obtained were compared with the conventional thermal treatment, showing that inactivation with HPU-SC-CO_2 was higher by 2.5 and two for Bacillus s and Bacillus p., respectively. On the other hand, the same system was not effective for *Geobacillus s*., as there was no significant difference between the use of HPU-SC-CO_2 versus conventional heat treatment (Gómez-Gómez et al., 2021).

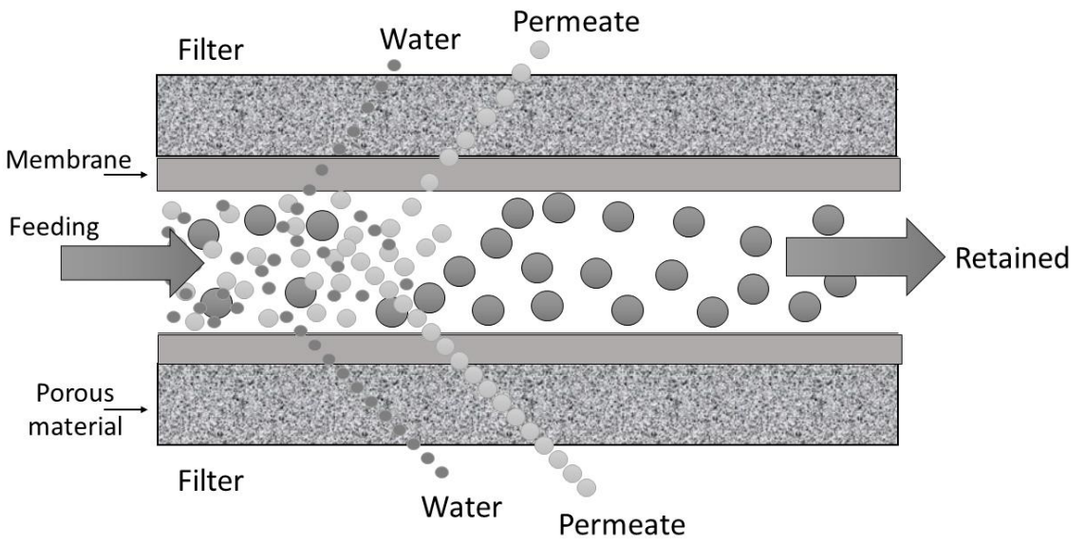
In addition to the combination with SC-CO_2, other technologies that intensify the power of HPU have been experimented. One example is the use of non-thermal plasma (NTP). Liao X. et al., 2018 employed this mechanism in conjunction NTP-HPU for the inactivation of *Staphylococcus a*. After several trials with different methodologies the highest reduction of 4.04 log cycles of *Staphylococcus a*. was obtained at 5 minutes of pretreatment with NTP followed by 10 minutes of HPU at 20 kHz and 200W/cm^2. In contrast, the use of HPU without pretreatment achieved only a 0.55 log cycle reduction in 20 minutes of treatment (Liao et al., 2018).

Reviewing the HPU and SF sections alone and, subsequently, comparing them with the combined technologies section, it can be analyzed that the use of the latter technologies represents better results than the former. The synergy of their mechanisms achieves good yields for both extraction and microbial inactivation.

6.3.6 Membrane Technology Processes

Membrane Technology (MT) is a method that consists of the separation or filtration of solutes present in a liquid; this separation is based on the difference in size and molecular weight between the particles suspended in the fluid (Sastre et al., 2009). The classification of MT processes is defined on the basis of the diameter of the pores of the membrane, which allow the concentration by retention of the larger components with respect to the pore itself, this product is called retained; on the other hand, substances smaller than the diameter of the pore, which are able to pass through it, are called permeate (Peña, 2006). Figure 6.6 shows an example of the membrane technology process.

Figure 6.6 Separation scheme with semi-permeable membranes



Source: (Solís et al., 2016)

The membrane process to be applied depends on the product to be obtained or, alternatively, the product to be removed. Table 6.3 summarizes the types of processes, pore diameter and examples of products to be separated (Pandolfi, 2008).

Table 6.3 Membrane processes

Type of process	Pore diameter	Examples
Microfiltration	10 - 0.1µm	Microbial cells, large colloids, small particles, etc.
Ultrafiltration	<0.1µm - 5nm	Proteins, emulsions, macromolecular colloids
Nanofiltration	Approx. 1nm	Organic compounds and dissolved salts
Reverse osmosis	<1nm	Small organic compounds, dissolved salts
Electrodialysis	<5nm	Dissolved salts
Dialysis	<5nm	Clinical treatment of renal failure

Source: "Membrane processes" by Pandolfi, 2008

6.3.6.1 Use of membrane technology to obtain biological products for clinical use

Separation processes are used in many industries to purify a compound of interest from the rest of the mixture, or otherwise to eliminate that component in order to increase the purity of the original solution. Whatever the case, the separation is based on the differences in the physical and chemical properties of these components (Mohsenin, 1980).

The obtaining of natural products has been of great relevance during the last few years, and this is due to the fact that consumers demand better quality products, which maintain their biological properties intact without changing their physical form. Researchers have opted to try new natural resources from which these bioactives can be obtained, sources that until a few years ago had not been exploited. Such is the case of algae, capable of producing various types of biomolecules such as carotenoids, lipids, polysaccharides, proteins, amino acids and carbohydrates (Khoo et al., 2020).

Denis et al. (2009) tested the feasibility of implementing ultrafiltration as the only separation step. For this, they used a tubular polyethersulfone membrane (PCI-MT600), surface area of 0.033 m^2 and molecular weight cut-off of 25-30 kDa. The permeate flux obtained was 35.1 Lh-1 m-2 at 7°C and 4 bar, allowing the retention of 100% of R-phycoerythrin pigment from the macroalgae *Grateloupia t*. Many times, obtaining and separating the desired product requires more than one process to achieve good purity, Safi C et al. (2014), obtained different biomolecules from *Tetraselmis s*. microalgae extract by two consecutive stages of ultrafiltration treatment. The test was performed with two membranes (TFF system Millipore, USA) of different molecular weight cut-off, during the first ultrafiltration it was possible to separate the starch present in the mixture with a cut-off of 100kDa, pressure of 2.07 bar for 30 minutes allowing a flux of 47.83kg h-1 m-2. In a second continuous stage, retention of proteins and sugars of interest was possible under the same pressure and time conditions, with a membrane cut-off of 10kDa, allowing a flux of 42.8±1.3kg h-1 m-2(Safi et al., 2014).

The recovery of value-added compounds involves different stages: macroscopic extraction, separation of macro- and micromolecules, extraction, purification and the final product (Galanakis, 2013). For the second and fourth stage processes, mainly microfiltration, ultrafiltration and nanofiltration techniques have been preferred, because their membrane characteristics allow for easy removal of suspended solids, concentration of molecules and clarification of smaller analytes178. Particularly, the MT of choice is UF, as it requires very low transmembrane pressure, inferring in better performance, lower energy expenditure and costs (Li et al., 2011). UF is regularly used for the separation of protein compounds. Modi et al., (2019) achieved separation of lysosome, trypsin, pepsin, human serum albumin, gamma globulin and fibrinogen with rejection values of 92.9±1.3%, 94.5±1.1%, 96.9±1.2%, 99.5±0.5%, 100% and 100%, respectively. Separation was performed with a polyethersulfone hollow fiber ultrafiltration membrane with a flux 110.0±3.8 L/m^2h, with a high flux recovery of 97.8%. UF was assisted by iron oxide nanoparticles-nanosheets decorated with carboxylated graphene oxide, this synergy proved to be efficient for the separation of biomolecules, especially proteins (Modi et al., 2019).

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The obtaining of natural products has been of great relevance during the last few years, and this is due to the fact that consumers demand better quality products, which maintain their biological properties intact without changing their physical form. Researchers have opted to try new natural resources from which these bioactives can be obtained, sources that until a few years ago had not been exploited. Such is the case of algae, capable of producing various types of biomolecules such as carotenoids, lipids, polysaccharides, proteins, amino acids and carbohydrates (Khoo et al., 2020).

Denis et al. (2009) tested the feasibility of implementing ultrafiltration as the only separation step. For this, they used a tubular polyethersulfone membrane (PCI-MT600), surface area of 0.033 m^2 and molecular weight cut-off of 25-30 kDa. The permeate flux obtained was 35.1 Lh-1 m-2 at 7°C and 4 bar, allowing the retention of 100% of R-phycoerythrin pigment from the macroalgae *Grateloupia t*. Many times, obtaining and separating the desired product requires more than one process to achieve good purity, Safi C et al. (2014), obtained different biomolecules from *Tetraselmis s*. microalgae extract by two consecutive stages of ultrafiltration treatment.

The test was performed with two membranes (TFF system Millipore, USA) of different molecular weight cut-off, during the first ultrafiltration it was possible to separate the starch present in the mixture with a cut-off of 100kDa, pressure of 2.07 bar for 30 minutes allowing a flux of 47.83kg h-1 m-2. In a second continuous stage, retention of proteins and sugars of interest was possible under the same pressure and time conditions, with a membrane cut-off of 10kDa, allowing a flux of 42.8±1.3kg h-1 m-2(Safi et al., 2014).

The recovery of value-added compounds involves different stages: macroscopic extraction, separation of macro- and micromolecules, extraction, purification and the final product (Galanakis, 2013). For the second and fourth stage processes, mainly microfiltration, ultrafiltration and nanofiltration techniques have been preferred, because their membrane characteristics allow for easy removal of suspended solids, concentration of molecules and clarification of smaller analytes178. Particularly, the MT of choice is UF, as it requires very low transmembrane pressure, inferring in better performance, lower energy expenditure and costs (Li et al., 2011). UF is regularly used for the separation of protein compounds. Modi et al., (2019) achieved separation of lysosome, trypsin, pepsin, human serum albumin, gamma globulin and fibrinogen with rejection values of 92.9±1.3%, 94.5±1.1%, 96.9±1.2%, 99.5±0.5%, 100% and 100%, respectively. Separation was performed with a polyethersulfone hollow fiber ultrafiltration membrane with a flux 110.0±3.8 L/m^2h, with a high flux recovery of 97.8%. UF was assisted by iron oxide nanoparticles-nanosheets decorated with carboxylated graphene oxide, this synergy proved to be efficient for the separation of biomolecules, especially proteins (Modi et al., 2019). Research dates that UF and NF processes have also been employed for the separation of compounds from plant extracts (Cassaro et al., 2014). UF has a molecular weight cutoff of 1-300kDa, while NF is in the range of 200-1000Da (Baker et al., 2004). In addition to the molecular weight separation process, there are other membrane properties that also influence filtration, such as solute interactions, charge interactions, bridging and hydrophobic interactions. Among the plant extracts of greatest interest are phenols, these compounds possess antioxidant, anticancer, antimutagenic and anti-inflammatory properties (Huang et al., 2010). Conidi et al., 2017 report the purification of phenolic compounds from pomegranate juice. For processing, four types of membrane with different molecular weight cutoffs and different fabrication materials were compared at the same conditions of 10 bar transmembrane pressure at 25±1°C. At the end of the different tests, it was observed that the Desal GK membrane type composed of a thin film and a molecular weight cut-off of 2kDa, was the one that obtained the best flux yields of 11.3kg h-1 m-2. The phenolic compound in the retained in the highest amount was anthocyanin of 84.4% and 90.7%, respectively Conidi et al., 2017). Another work reporting satisfactory separation of polyphenols is that of Liu et al., (2011); where they used high voltage electrical discharges to extract polyphenolic compounds from grape seeds and subsequently subjected the extract to UF purification with the use of three different membranes: 15PP membrane of floury polymer and pore size of 0. 15µm, UP150 polyethersulfone membrane and 150kDa molecular weight cut-off gave a rejection rate of 87% and UV050 polyvinylidene fluoride membrane and 50kDa molecular weight cut-off gave a rejection rate of 91% (Liu et al., 2011).

Membrane Technology (MT) is a separation process that has the advantage of low temperature and energy requirements, it is considered a cheap system since it does not require many solvents and chemicals, and those used can be instantly recycled, it also achieves a clean separation, keeping the components in their native state, and it does not require precipitation, centrifugation and dialysis stages that can dilute the samples (Marella et al., 2013).

6.4 Conclusion

According to the literature review analyzed throughout this work, it can be observed that non-thermal technologies, such as Power Ultrasound, Supercritical Fluids, in particular Supercritical Carbon Dioxide, and Membrane Technologies are tools that, compared to conventional extraction methods, have marked advantages, among which are lower energy consumption, shorter extraction time, less damage to the active components and high extraction yields.

The synergy of the technologies not only showed good results during the extraction of extracts; it also favored the logarithmic reductions of the different microorganisms subjected to sterilization, marking a gap between the use of a particular technology and the combination between them.

The study of these new technologies is relatively new and in spite of this it has had an accelerated growth in many parts of the world due to the satisfactory results that have been reported by multiple researchers from different industrial areas, however, more research is required to take these technologies to a large scale, since many experiments have only been carried out at laboratory level. Even so, the future of non-thermal technologies seems to be very promising and beneficial in every sense.

6.5 Glossary

ABTS-. 2,2 azino bis(3-ethylbenzo thiazolin-6 sulfonic acid), a chemical compound involved in redox reactions.

Nucleic acids. Structurally composed of a nitrogenous base, a pentose and a phosphate group, they constitute the genetic material of organisms, storing their genetic information.

Human serum albumin. Main protein present in plasma with multiple functions, where the main one is to transport or inactivate substances such as heavy metals, drugs, fatty acids, hormones and enzymes, besides being an excellent expander of plasma volume.

Alkaloid. Nitrogenous organic compound of vegetable origin with good pharmacological activity at low doses, since they have a high toxicity.

Allergen. A product that elicits an immunological response to an allergenic agent.

Antibody. A glycoprotein produced by B-lymphocytes that binds to antigens, often with a high degree of specificity and affinity.

Antigen. A product that elicits an immune response to a foreign agent.

Anthocyanin. A plant pigment belonging to the flavonoid group that produces the red to blue colors in fruits and vegetables. It is attributed with a reduction in coronary heart disease, anticancer, anti-inflammatory and antidiabetic effects, improvement of visual acuity and cognitive behavior.

Anthoxanthin. A colorless, astringent compound derived from the flavonoid group with antioxidant properties.

Anthraquinone. Main group of natural quinones. Aromatic polyhydroxylated compound, if it has OH in positions 1 and 2 it presents coloring properties, on the other hand, if the OH are in positions 1 and 8 the effect is laxative.

Apigenin. A type of flavonoid that reduces oxidative stress, has sedative, anxiolytic, antimutagenic, antitumor, antiallergic and anti-inflammatory effects, as well as regulating different signaling pathways.

Baicalin. A type of flavonoid with diverse pharmacological activities, such as antitumor, antimicrobial and antioxidant.

Carotenoid. Tetraterpenes that leak as natural liposoluble pigments synthesized by plants, algae and photosynthetic bacteria, with antioxidant properties.

Cytokine. Proteins that mediate inflammatory and immune reactions.

Derivatives of human blood and plasma. Blood components for therapeutic, diagnostic, preventive or research applications.

DPPH-. 2,2-diphenyl-1-picrylhydrazyl is a free radical that determines the antioxidant capacity of foods and synthetic compounds characterized by the yielding of a hydrogen provided by the antioxidant agent.

Lipid emulsions. fatty acid preparation administered venously for those individuals who cannot obtain them orally through the diet.

Enzyme. Molecules that catalyze the conversion of compounds to one or more different compounds by increasing the rates of the reaction.

Sterols. Derivatives of steroids with a cyclopentanohydrophenanthrene base nucleus, an OH group at carbon 3, and most, a side chain of 8 or more carbons at carbon 17. Compounds of interest for the production of steroid drugs.

Fibrinogen. Protein that functions as factor I of the coagulation cascade and the final substrate from which the clot is produced.

Flavonoids. The most abundant group of polyphenols, derived from phenylalanine and tyrosine. They decrease the incidence of cardiovascular diseases.

Gamma globulin. A positively charged globular protein present in blood serum. The main gamma globulins are immunoglobulins or antibodies.

Glycoside. Each of the organic substances, existing in many vegetables, which by hydrolysis produced by the action of dilute acids give, as products of decomposition, glucose and other bodies, many of which are energetic poisons and, in very small doses, are used as drugs.

Hormone. A chemical produced by tissues that has a specific effect on a target tissue.

Immunoglobulins. Proteins capable of behaving as an antibody.

Lysosome. Cellular organelles containing digestive enzymes, which aid in the elimination of viruses and bacteria. They are also responsible for recycling cellular waste and participate in apoptosis.

Luteolin. A type of flavonoid produced by various medicinal plants, with antioxidant, free radical scavenger, anti-inflammatory, neuroprotective, anticarcinogenic and antiallergic properties.

Pectin. Vegetable heteropolysaccharide contained mainly by D-galacturonic acid; purifying properties are attributed to it as it eliminates residues and toxins from the organism.

Pepsin. Enzyme produced by the stomach that breaks down food proteins during digestion.

Polyphenols. Compounds made up of one or more aromatic rings with at least one hydroxyl group linked, originated by the secondary metabolism of plants with diverse functions, such as nutrient assimilation, protein synthesis, enzymatic activity, formation of structural components and marked antioxidant activity; in addition to anti-inflammatory, antiallergic, antimicrobial, antineoplastic and anticarcinogenic properties.

Reagents for in vitro diagnostics. Reagent product, calibrator, made in control material, intended for the study of samples from the human body.

R-phycoerythrin. A class of phycobilin contained in microalgae are used as cell markers, cell analysis and immunoassays due to their fluorescence emission properties.

RNA. Ribonucleic acid, interferes with the transfer of information contained in DNA into cellular compartments.

Immune sera. Serum containing polyclonal antibodies. They are used for passive immunization of certain diseases.

Trypsin. A proteolytic enzyme produced by the pancreas and secreted into the duodenum, it cleaves peptide bonds of proteins to obtain small peptides and amino acids.

Vaccine. Antigenic preparation that induces immunity against infections.

β-carotene. A type of carotenoid with provitamin A activity.

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