Integration of pulsed electric fields technology in the biorefinery of agri-food wastes and microalgae

Daniele Carullo

UNIVERSITY OF SALERNO

DEPARTMENT OF INDUSTRIAL ENGINEERING

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INTEGRATION OF PULSED ELECTRIC FIELDS TECHNOLOGY IN THE BIOREFINERY OF AGRI-FOOD WASTES AND MICROALGAE

Supervisor   **Ph.D. student**
Prof. Eng. Giovanna Ferrari   *Daniele Carullo*

Scientific Referees
Eng. Gianpiero Pataro
Prof. Eugene Vorobiev

Ph.D. Course Coordinator
Prof. Ernesto Reverchon

Aknowledgements

HIGHLIGHTS

1. Pulsed Electric Fields technology represents a useful tool for a "green" valorisation of second generation biomasses (food wastes/by-products) with low energy expenditures.
(this thesis)

2. "Hurdle approaches" are essential to achieve selectivity and efficiency of cell disruption phases in microalgal biorefinery.
(this thesis)

3. Biomass coming out from PEF-assisted biorefinery processes may still be exploited for further applications.
(general conclusion)

4. The optimisation of downstream operations is strongly required to achieve feasibility of the whole biorefinery scheme.
(future perspectives)

List of contributions

Publications on international peer reviewed journals

- Pataro, G., **Carullo, D.**, Bobinaite, R., Ferrari G. (2017), Improving the extraction yield of juice and bioactive compounds from sweet cherries and their by – products by pulsed electric fields. Chemical Engineering Transactions, 57, 1717 – 1722, DOI : 10.3303/CET1757287.

- **Carullo, D.**, Abera, B. D., Casazza, A. A., Donsì, F., Perego, P., Ferrari, G., Pataro, G. (2018), Effect of Pulsed Electric Fields and High Pressure Homogenization on the acqueous extraction of intracellular compounds from the microalgae Chlorella vulgaris. Algal Research, 31, 60 – 69.

- Pataro, G., **Carullo, D.**, Siddique, M.A.B., Falcone, M., Donsì, F., Ferrari, G. (2018), Improved extractability of carotenoids from tomato peels as side benefits of PEF treatment of tomato fruit for more energy-efficient steam-assisted peeling. Journal of Food Engineering, 233, 65 – 73.

Publications on proceedings of international conferences

- Pataro, G., **Carullo, D.**, Donsì, G., Ferrari, G. "PEF-assisted green solvent extraction of high-added value compounds from agri-food by-products". Proceedings of the 2nd "Green Extraction of Natural Products" Conference (Turin, 31st May - 1st June, 2016).

- Pataro, G., **Carullo, D.**, Maresca, P., Ferrari, G. "Application of Pulsed Electric Fields technology for improving peeling of tomato fruits". Proceeding of the 18th World Congress on Food Science & Technology (Dublin, 21st - 25th August, 2016).

- **Carullo, D.**, Pataro, G., Apicella, E., Ferrari, G. "Extraction of valuable compounds from microalgae by pulsed electric field and high pressure CO_2". Proceedings of the 30th "EFFoST" Conference (Wien, 28th - 30th November, 2016).

- Pataro, G., **Carullo, D.**, Capitoli, M.M., Ferrari, G. "Application of Pulsed Electric Fields technology in tomato fruit processing".

Proceedings of the 30th "EFFoST" Conference (Wien, 28th - 30th November, 2016).

- Pataro, G., **Carullo**, **D.**, Abera, B.D., Donsì, F., Ferrari, G. "Pulsed Electric Fields and High Pressure Homogenization assisted extraction of valuable compounds from microalgae C. vulgaris". Proceedings of the FITEMI (Italian Forum on Microalgal Technologies, Palermo, 6th - 7th April, 2017).

- **Carullo, D.**, Pataro, G., Ferrari, G. "PEF assisted green solvent extraction of high – added value compounds from agri-food by-products". Proceedings of the "2nd World Congress on Electroporation and Pulsed Electric Fields in Biology, Medicine, Food & Environmental Technologies" (Norfolk, 24 – 28 September, 2017).

- **Carullo, D.**, Pataro, G., Abera, B.D., Scognamiglio, M., Donsì, F., Ferrari, G., "Application of Pulsed Electric Field and High Pressure Homogenisation in the Biorefinery of Microalgae". Proceedings of the "IFT-EFFoST 2018 International Nonthermal Processing Workshop and Short course" (Sorrento, 25 – 27 September, 2018).

- **Carullo, D.**, Pataro, G., Ferrari, G., "Influence of comminution degree on the PEF-assisted extraction of phenolic compounds from artichoke bracts". Proceedings of the "IFT-EFFoST 2018 International Nonthermal Processing Workshop and Short course" (Sorrento, 25 – 27 September, 2018).

- Pataro, G., **Carullo, D.**, Falcone, M., Ferrari, G., "Recovery of lycopene from industrial tomato processing wastes by pulsed electric fields". Proceedings of the "IFT-EFFoST 2018 International Nonthermal Processing Workshop and Short course" (Sorrento, 25 – 27 September, 2018).

- Frontuto, D., **Carullo, D.**, Brunton, N., Pataro, G., Ferrari, G., Lyng, J., "Optimization of PEF-assisted extraction of polyphenols from potato peels by using response surface methodology". Proceedings of the "IFT-EFFoST 2018 International Nonthermal Processing Workshop and Short course" (Sorrento, 25 – 27 September, 2018).

Summary

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Abstract

Recently, the idea of valorizing industrial agro-food wastes and microalgal biomass, through an efficient recovery of their major bioactive constituents to be used as ingredients in food, feed, pharmaceutical and cosmetic sectors, is gaining interest, due to the increasing demand of consumers for natural products.

However, the extraction of these compounds is problematic, due to the presence of a "physical barrier" (cell wall/membrane) when removing intracellular substances. Pre-treatment stages (thermal, mechanical or enzymatic) to permeabilise cellular tissues and facilitate the diffusion of the intracellular compounds in the external medium are required, together with the use of large amount of organic solvents and long contact times. This lead to high energy consumption, the formation of undesired compounds as well as to the eventual degradation of the target molecules to be recovered. Physical pretreatments operations have been proposed in the last years to overcome these problems and among them Pulsed electric fields (PEF) technology has shown promising results as to obtain a mild, sustainable and efficient permeabilisation of cell membranes with respect to conventional cell disintegration methods.

PEF consists in exposing biological cells to repetitive short voltage pulses (μs – ms in width) with an electric field strength in the range 0.5 - 50 kV/cm and an energy input up to 150 kJ/kg, mainly depending on cell size and morphology. This technique exploits its potential via the "electroporation" of membranes, which rapidly favors the leakage of valuable compounds from the cells.

Although the use of PEF has shown encouraging results in the last years in enhancing mass transfer rates from vegetative biological tissues, there was a strong need to perform a systematic study which may have considered not only the effect of the electroporation phase, but also the influence of upstream (granulometry, solid-liquid ratio) and downstream processes (extraction, purification) on the extraction yield and extract quality from treated biomass.

Therefore, the main aim of my PhD thesis was to propose a systematic approach for the development of a PEF-based biorefinery process for an efficient and sustainable valorization of agri-food wastes/by-products and microalgal biomass.

To this purpose, different food wastes (tomato skins, artichoke external bracts and stems) and microalgal strains (A. platensis, C. vulgaris) have been selected and subjected in the first instance to a study of the "electroporation" mechanism of cell membranes, carried out in lab-scale or pilot-scale specifically designed treatment chambers, by using several analytical

methods (in situ measurements of electrical impedance/conductivity, particle size distribution, optical and SEM microscopy), accompanied by the assessment of target intracellular components release (carotenoids, polyphenols, proteins, carbohydrates, lipids) after the application of PEF treatments of variable intensity.

Only in the case of microalgal biomass processing, in order to furtherly enhance the recovery yields of specific molecules from the cells, "hurdle approaches" have been proposed (PEF + mild heating, PEF + other disruptive technologies such as High Shear Homogenization or High Pressure Homogenization) and their effects compared with those achieved by the application of single treatments in terms of purity of extracts and energetic consumptions.

The results obtained highlighted the potentiality of PEF to unlock intracellular substances from the considered matrices in a "cell structure" dependent manner, with maximum effects detected at very low energy input $(1 - 5 \text{ kJ/kg})$ for tomato skins and artichoke wastes, while microalgal cells required the application of higher treatment severities (100 kJ/kg) to achieve proper disintegration degrees, due to a greater rigidity of the cell wall/membrane system.

Moreover, microscopy observations have shown that PEF represents a mild disintegration technique, since no formation of cell debris could be detected, which may potentially allow to avoid excessive costs for further refining operations (e.g. solid/liquid separation, purification of extracts).

The permeabilisation effect achieved after PEF application has promoted, consequently, the selective and efficient release of intracellular compounds from both food wastes and microalgal biomass during extraction phases, with significantly higher recovery yields in carotenoids from tomato skins, phenolic acids from artichoke wastes, carbohydrates, lipids and proteins from microalgal biomass, solubilized in extracting solvents (e.g. water, acetone, ethyl lactate, ethyl acetate) with low "environmental impact", which could grant the possibility to exploit the use of exhaust biomass, if adequately processed, for further applications.

The use of PEF in combined approaches for microalgal biomass processing showed interesting results in terms of both purity and yields of extracts, with significant reduction of operative costs needed for cell permeabilisation and recovery of high-added value intracellular compounds, with respect to the application of single PEF technology.

In conclusions, the optimization of PEF-assisted cell permeabilisation of food wastes/microalgae represents only the first step towards a sustainable biorefinery processing, while further research is needed for a better understanding of the role of PEF technology on downstream processing operations, which still need to be optimized to possibly move towards industrial applications.

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Chapter I
State of the art

I.1 Introduction

The huge rise in world population which has been occurring in the last decades has led to a dramatic increment in the demand for natural products to accomplish with human being nutritive requirements. The over-consumption of natural food resources available on our planet is intended to be accompanied by several negative effects like climate change, loss of fertile soils and increase in environmental pollution, thus gradually resulting in a rapid resources depletion. For these reasons, new policies of natural resources management are urgently needed so as to contribute to a more sustainable and economic social development.

In particular, the recovery and re-utilisation of industrial food wastes/by-products or the usage of renewable matter like microalgal biomass could be potentially helpful to this purpose, being both rich sources of valuable bioactive compounds, which may find large application in food, feed, pharmaceutical and cosmetic industries, as well as for the production of biofuels. The full exploitation of such biomasses may be achieved through the so-called "biorefinery" process, whose concept and purpose are described in the following section.

I.2 Concept of "biorefinery"

According to the International Energy Agency (IEA), the term "biorefinery" is referred to as "a sustainable and efficient processing of biomass into a spectrum of marketable products and energy" (IEA, 2009, Bioenergy Task 42 on Biorefineries). It consists of a cascade of sustainable processes that utilize biological sources to produce end products, pursuing the concept of zero-waste (Khan & Rashmi, 2010). In particular, it comprises upstream (biomass comminution/grinding), extraction/recovery and downstream processing (separation/purification) steps, aimed at fractionating biomass into multiple added-value products (Fitz Patrick *et al*., 2010; Khan &

Rashmi, 2010; Günerken *et al.*, 2015; Postma *et al.*, 2016b). Biorefineries could be chategorised in three different ways, depending on the starting feedstocks (Kamm and Kamm, 2007), as follows:

- First generation biorefinery, which uses whole crops such as cereals and maize;
- Second generation biorefinery, involving the use of industrial food wastes/ by-products which are generally discarded and disposed into the environment;
- Third generation biorefinery, which aims at selectively and efficiently recovering high-added value intracellular compounds from microalgal biomass, representing a valid alternative to both first and second generation biomasses.

I.3 Second and third generation biorefinery feedstocks

I.3.1 Agri-food wastes and food by-products

Along the whole food supply chain approximately one third of the edible part of food produced for human consumption, accounting for about 1.3 billion tn/year (Gustavsson *et al.*, 2011), gets lost (FAO, 2011). In particular, food wastage is related to specific stages of the food supply chain (Figure I.1), giving birth to the distinction into:

- "Food loss", which accounts for the decrease in edible food mass that is lost, discarded or degraded throughout the production, postharvest and processing stages;
- "Food waste", whose production occurs during the retail and final consumption stages due to the behavior of retailers and consumers. Moreover, these biomasses are distributed across a broad range of households and are very susceptible to deterioration.

Agri-food losses, generally recognized in the frame of scientific research as "agri-food wastes" (Figure I.2), representing also the most abundant part of generated biomass, are residues with high organic load resulting in liquid or solid form, which are usually produced during raw materials processing to end-products, thus being concentrated in few locations (Galanakis, 2012). They are considered as "one among the most generated bio-wastes around the globe" (Dahiya *et al.*, 2018), which may potentially be accompanied by dramatic economical losses as well as strong environmental impact (Gustavsson *et al.*, 2011) if remain inutilised (Al-Wandawi *et al.*, 1985).
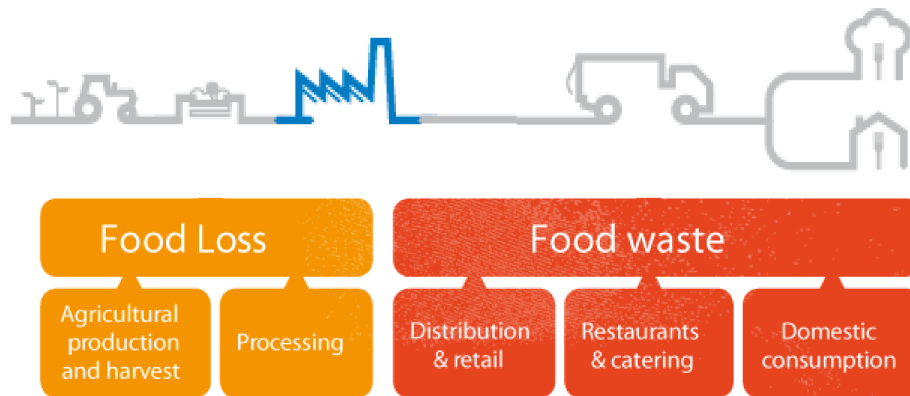
Figure I.1 *Schematics of food supply chain and related stages of food wastage production (FAO, 2011).*

Figure I.2 *Examples of industrial agri-food wastes: tomato peels (a), blueberry skins (b), grape pomace (c) and olive pomace (d).*

These biomasses currently find low-added value uses as animal feed and fertilizers (Knoblich *et al.*, 2005; Strati & Oreopoulou, 2014), or are directly sent to landfill (Rossini *et al.*, 2013), without achieving their full exploitation.

On the other hand, agri-food wastes offer the possibility to create new opportunities and markets since these substrates are of particular interest for food, pharmaceutical and cosmetic industries especially due to their

capability to retain large amount of "natural" high-added value reusable materials (Chandrasekaran, 2013). For this reason, nowadays these kind of "low cost" sources of bioactive compounds tend to be referred to as "food by-products" rather than food wastes, due to their possible re-integration in the food supply chain.

In particular, the great majority of by-products derives from fruit and vegetable processing, which account for about 50% by weight all around the world (Galanakis, 2015).

These sources have a great potential to be used for the recovery of value-added products, as illustrated in Table I.1.

Table I.1 *Wastes/by-products from fruit and vegetable processing and main retained bioactive compounds (adapted from Galanakis, 2015).*

Fruit & Vegetable	Food wastes/by-products	Target Compound	Reference
Mandarin	Peel	Flavonoids	Kim *et al.* (2004)
	Peel	Limonene	Lota *et al.* (2000)
	Leaf	Linalool	Lota *et al.* (2000)
Orange	Peel	Hesperidin	Di Mauro *et al.* (1999)
		Apocarotenoids	Chedea *et al.* (2010)
		Limonene	Farhat *et al.* (2011)
		Cellulose	Bicu & Mustata (2011)
Peach	Pomace	Pectin	Pagan *et al.* (1999)
Apple	Skin	Polyphenols	Schieber *et al.* (2001)
Grape	Skin	Anthocyanins	Pinelo *et al.* (2006)
Carrot	Peels & Pomace	β – Carotene & Polyphenols	Chantaro *et al.* (2008)

Tomato	Pomace	Lycopene	Lavecchia & Zuorro (2008)
	Peel	Carotenoids	Knoblich *et al.* (2005)
	Seed	Dietary Fibers	
Artichoke	External bract	Inulin	Ruiz-Aceituno *et al.* (2016)
	Stems	Dietary fibers	Boubaker *et al.* (2016)
	Leaves	Polyphenols	Zhu *et al.* (2004)
Potato	Peels	Polyphenols	Oreopoulou *et al.* (2007)

I.3.2 Microalgae

Microalgae are eukaryotic or prokaryotic microorganisms, with cell size ranging between 0.1 and 40 μm, which can grow rapidly and live in harsh conditions due to their unicellular or simple multi-cellular structure (Mata *et al.*, 2010).

In particular, they are able to synthesize organic materials from sunlight, CO_2 and water through the process of "photosynthesis", whose light-to-chemical energy conversion efficiency is 5-fold higher than that observed in common plants.

They are genetically a very diverse group of organisms with a wide range of physiological and biochemical characteristics. It has been estimated that about 200,000 - 800,000 microalgae species exist of which about 30,000 species are described (Starckx, 2012).

They contain numerous bioactive compounds (Cardozo *et al.*, 2006), such as proteins, lipids, carbohydrates, carotenoids and vitamins (Figure I.3), which could be recovered and used as additive in food, feed and cosmetic products as well as for energy production (Indira & Biswajit, 2012).
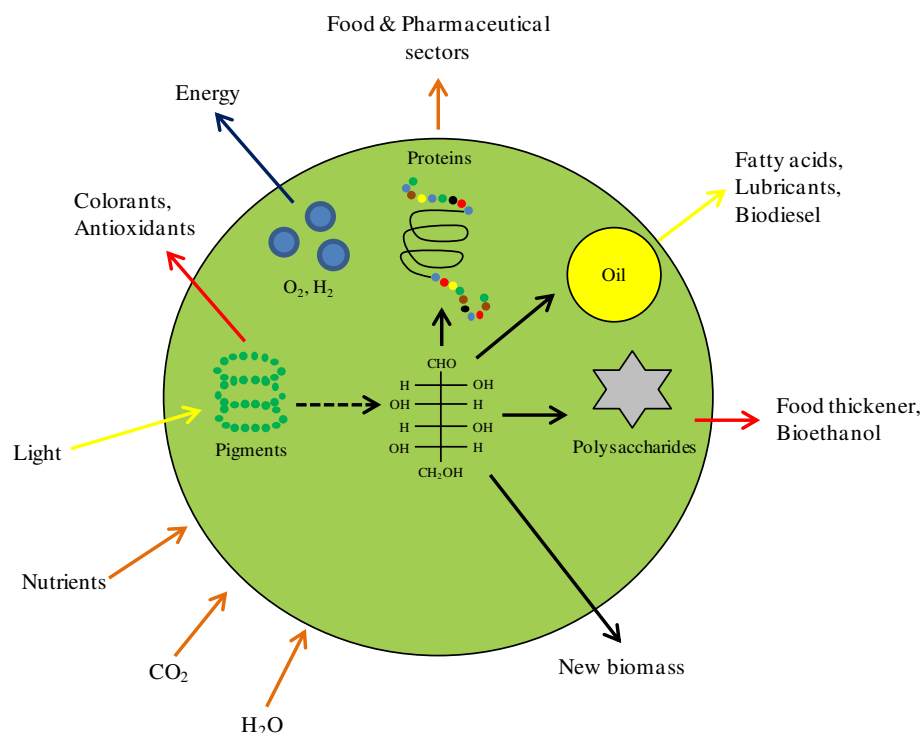
Figure I.3 *Microalgae as a source of different valuable compounds (adapted from Posten & Walter, 2013).*

Some of the most biotechnologically relevant strains are the green algae (Chlorophycea), namely *Chlorella vulgaris*, *Nannochloropsis oceanica*, *Haematococcus pluvialis*, *Dunaliella salina* and the Cyanobacteria *Arthrospira platensis* (Spirulina). They are already widely commercialized and used, mainly as nutritional supplements for humans and as animal feed additives (Gouveia *et al.*, 2008), with *Chlorella* and *Spirulina* being the first microalgae species to be commercialized as a healthy food in Japan, Taiwan and Mexico (Cuellar-Bermudez *et al.*, 2015).

Microalgal chemical composition is generally variable and this is due to a series of environmental and physiological factors, such as temperature, pH-value, mineral contents, CO_2 supply, population density and growth conditions.

As previously observed, microalgal biomass could be fully exploited for the recovery of intracellular compounds, such as:

- Proteins, being the most abundant constituents of microalgal cells. They are involved in key roles such as growth, repair and maintenance of the cell but also serve as cellular motors, chemical messengers, regulators of cellular activities and defense against foreign invaders (Safi *et al.*, 2014). Algal proteins comprise all the

20 amino acids and constitute up to 50% of dry weight in different cultures (Khan *et al.*, 2009). The purified proteins may find large application in the food, feed, health and bulk chemical market (Vanthoor-Koopmans *et al.,* 2013). For example, *Arthrospira platensis* (Spirulina) represents the main source of phycocyanin, a water-soluble pigmented protein belonging to phycobiliproteins family, which may be used as a natural ingredient for functional food formulation as well as cosmetic coloring agent (blue color extract) and biochemical tracer in immunoassays (Gouveia *et al.*, 2008; Perosa *et al.* 2015). Moreover, this protein exerts beneficial effects on human health due to its hepatoprotective, anti-inflammatory, and antioxidant properties (Martinez *et al.*, 2016);

- Carbohydrates, mainly consisting of mono- and di-saccharides but also energy storage polysaccharides (starch), which generally constitute the "building blocks" for cell biomass.
  In particular, some microalgal species (*Porphyridium cruentum*, *Chlamidomonas reinhardii*) are able to synthesize high molecular weight polysaccharides which act as gelling/thickening agents, thus being of great interest especially for food sector;

- Lipids, which are principally composed of fatty acids (FA), are recovered from microalgae by solvent extraction and transesterified to produce fatty acids methyl esters (biodiesel).
  Lipids yields from microalgal biomass are significantly higher than those deriving from conventional crops (e.g. corn, soybean, coconut) on the same basis of production area (Chisti, 2007);
  Polyunsaturated fatty acids (PUFAs) have high market value, especially ω-3 eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA), as well as ω-6 γ-linoleic acid, which may be involved in the formulation of infant products and as nutritional supplements (Gouveia *et al.*, 2008). Moreover, PUFAs are essential for the protection from chronic, degenerative and cardiovascular diseases but also from several kinds of cancer;

- Polyphenols, ranging from phenolic acids and other compounds with relatively simple chemical structures, to the more complex structures of phlorotannins (Ibanez *et al.*, 2011).
  In addition to their strong antioxidant activity, polyphenols from microalgae play also beneficial activities including chemopreventive (Kang *et al.*, 2003), UV-protective (Artan *et al.*, 2008), and antiproliferative effects (Kong *et al.*, 2009).
  Hence, there is increasing interest in using microalgae as natural antioxidants source for cosmetics (e.g. sun protecting) and functional food/nutraceuticals;

- Carotenoids, being thought to possess high antioxidant power and, thus, to allow prevention against cancer, cardiovascular and macular

diseases (Ibanez *et al.*, 2011), due to their ability to scavenge radical oxygen species.

Certain microalgal strains (e.g. *Dunaliella salina*, *Haematococcus pluvialis*) are able to accumulate large amount of β -carotene and astaxanthin (up to 5% DW, dry weight), respectively, when cultivated under unfavourable growth conditions. They serve as essential nutrients and have high demand in the market as natural food coloring agents, as additives to cosmetics and also as supplements in the formulation of functional foods;

- Microalgae represent a valuable source of nearly all important vitamins (e.g. A, B_1, B_2, B_6, B_{12}, C, E, folic acid and pantothenic acid) and have also a balanced mineral content (e.g. Na, K, Ca, Mg, Fe, Zn and other trace minerals), which improve the nutritional value of algal biomass (Gouveia *et al.*, 2008). For instance, the high levels of vitamin B_{12} and iron in some microalgae, like *Spirulina*, make this strain particularly suitable as a nutritional supplement for vegetarian individuals.

Table I.2 reports the average composition, in terms of the main intracellular constituents for the most industrially relevant microalgal species.

Table I.2 *Biomass composition of microalgae expressed in dry matter basis (adapted from Demirbas et al., 2010).*

Microalgal species	Proteins (%)	Carbohydrates (%)	Lipids (%)
Scenedesmus Obliquus	50 – 56	10 - 17	12 – 14
Scenedesmus Quadricauda	47	-	1.9
Scenedesmus Dimorphus	8 – 18	21 - 52	16 - 40
Chlamydomonas Rh.	48	17	21
Chlorella Vulgaris	51 - 58	12 - 17	14 - 22
Chlorella Pyrenoidosa	57	26	2
Spyrogira sp.	6 - 20	33 - 64	11 - 21
Dunaliella Salina	57	32	6
Porphyridium Cruentum	28 - 39	40 - 57	9 - 14

Nannochloropsis sp.	27 - 34	23 - 40	13 - 14
Arthrospira Platensis	46 - 63	8 - 14	4 - 9
Arhtrospira Maxima	60 - 71	13 - 16	6 - 7
Prymnesium parvum	28 - 45	23 - 33	22 - 38
Synechoccus sp.	63	15	11
Anabaena Cylindrica	43 - 56	25 - 30	4 – 7
Euglena Gracilis	39 - 61	14 - 18	14 - 20

I.4 Biorefinery of food wastes/by-products and microalgae

I.4.1 Biorefinery process

The valorization of second and third generation biomasses via the recovery of their main bioactive constituents may occur through the "biorefinery" process, which consists of a sequence of operations schematized in the block diagram of Figure I.4.

As reported in Galanakis (2012) and Gunerken *et al.* (2015), the main goals of the "biorefinery" processing of food wastes/by-products, as well as of microalgal biomass, are:

- To find a proper cell disintegration pre-treatment (upstream phase) which allows to maximize the yields of bioactive compounds recovery during the subsequent extraction phase;
- To selectively separate the high added-value ingredients from impurities and toxic compounds;
- To avoid deterioration and loss of functionality of the desired product during processing.

The "core" of the biorefinery scheme is represented by the extraction phase, which often requires the use of organic solvents with a certain affinity towards target compounds to be recovered, generally contained in the cytoplasmatic matrix or in the internal organelles of the biological cell (Gunerken *et al.*, 2015).
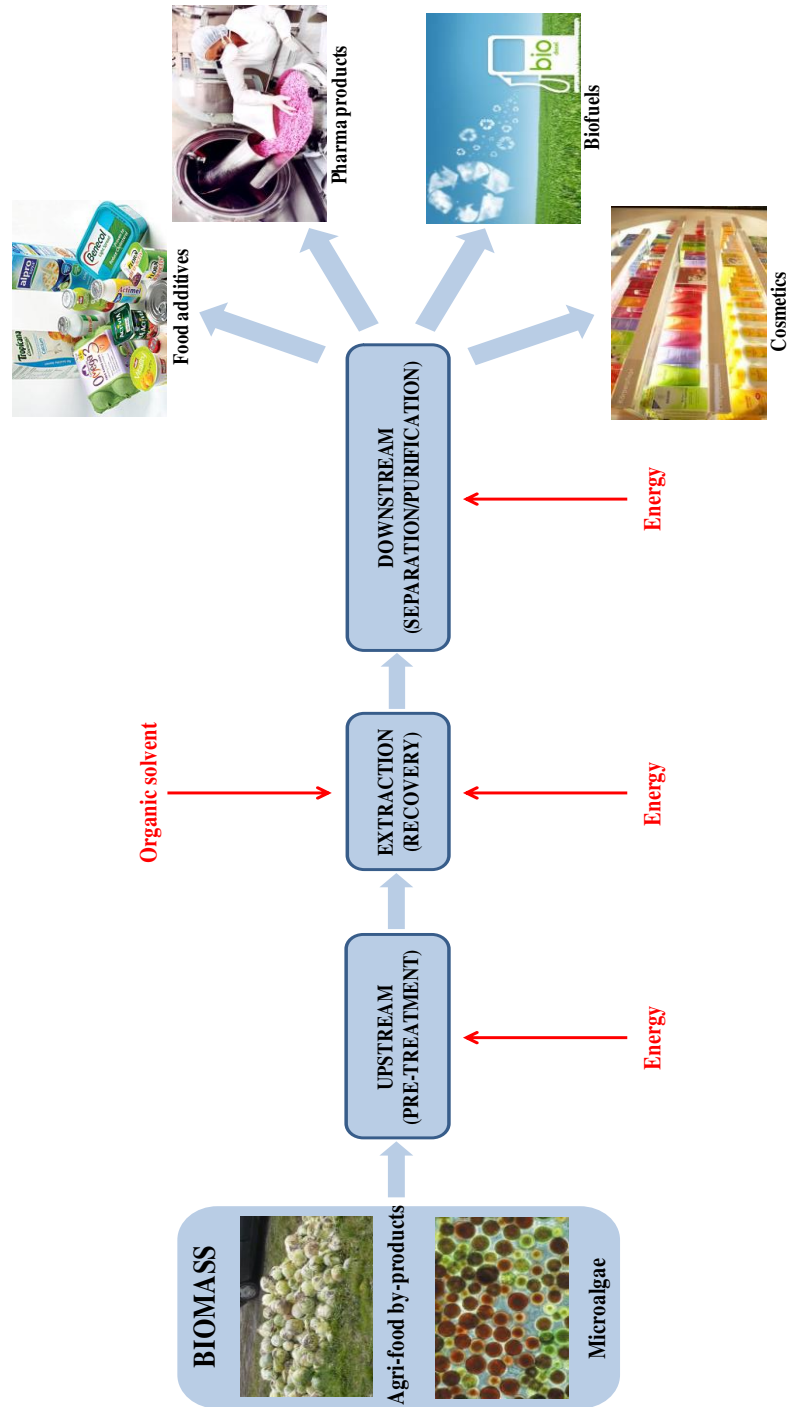
Figure I.4 *Schematisation of biorefinery processing of food wastes/by-products and microalgae.*
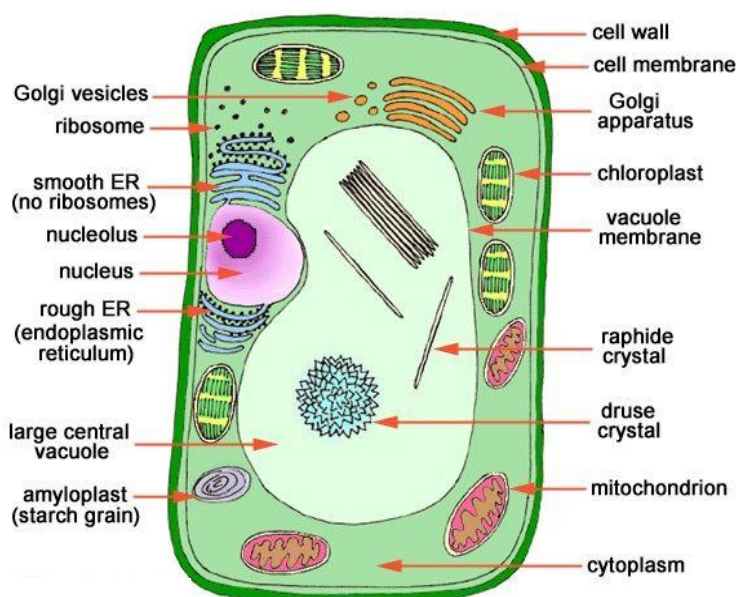
Figure I.5 *Cross section of a plant cell (Yadufashije, 2018).*

The presence of a "physical barrier", constituted by the cell wall/membrane system (Figure I.5), dramatically affects the efficiency of conventional extraction process, thus requiring the use of large volumes of polluting and harmful solvent as well as long maceration times (Luengo *et al.,* 2014b). Moreover, this process is generally characterized by very low recovery yields of target compounds and also poor selectivity, which negatively impact on the economics of the process, leading to high energetic consumption in downstream processing (Nobre *et al.*, 2009; Poojary *et al.*, 2016).

Therefore, a cell permeabilisation/disintegration pre-treatment, able to improve the mass transfer of target intracellular compounds with increases in the efficiency of the extraction step, both in term of recovery yields and consumption of solvent and energy, is of remarkable importance.

Conventional cell permeabilization techniques are based on the application of high pressure, grinding, cutting, drying and chemicals or enzyme cocktails to disintegrate cell wall/membrane facilitating the penetration of solvent into the intracellular space and the subsequent increase in mass transfer phenomena of solubilized molecules. However, they typically show a series of drawbacks such as degradation of sensitive compounds, especially when high temperatures are involved (drying), with subsequent reduction in extracts quality (Golberg *et al.*, 2016, Günerken *et al.*, 2015), low selectivity as well as high energy requirements, which dramatically disadvantage the feasibility of the biorefinery approach.

For this reason, there is an increasing interest of both research world and industry to scout different technologies that can substitute current permeabilization methods, exerting a positive impact in facilitating the extraction of valuable compounds from biological tissues in a sustainable way, avoiding to negatively affect the quality and the functionality of the recovered products (Vanthoor-Koopmans *et al.*, 2013).

I.4.2 Up-stream processing: size reduction and drying of biomass

The first step for the recovery of valuable compounds from food wastes/by-products consists of a macroscopic pretreatment, which is necessary to adequately prepare the raw materials for the following operations such as extraction, separation/purification, and product formation.

Among these pre-treatments, size reduction/comminution by grinding or cutting is generally conducted prior to solvent extraction step in order to increase the area/volume ratio and consequently to improve the liquid/solid contact, with positive effects on the recovery yield (Oreopoulou and Tzia, 2007).

Specifically, in the grinding process, raw materials are reduced in size by the action of mechanical components moving inside the grinding machine (Figure I.6). The resulting applied force in terms of compressive, impact, or shear force, as well as the processing time, affect the extent of cellular damages induced by grinding. For efficient comminution, the energy applied to the material should slightly exceed the minimum energy needed to achieve material rupture. Since the excess of energy is dissipated in heat, it is of utmost importance to keep this loss as low as practicable (Galanakis, 2015) to avoid undesired thermal effects which may potentially induce damages to compounds of interest.
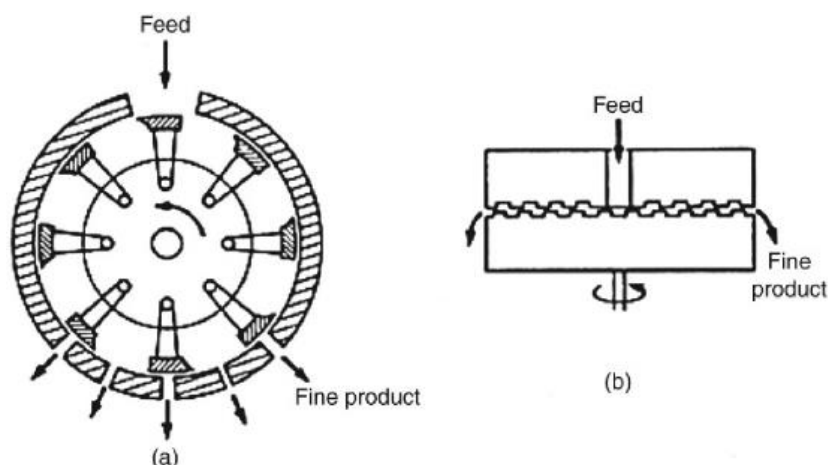
Figure I.6 *Simplified schematics of equipment used for size reduction of solid wastes: (a) hammer mill and (b) attrition mill (Galanakis, 2015).*

A considerable amount of heat may be generated in a mill, particularly if it operates at high speed.

Generally, processing costs of milling processes are very high (100 – 400 kJ/kg, Cao & Rosentrater, 2015), especially when wet products are treated (Galanakis, 2015).

Drying, instead, is an extremely energy intensive thermal treatment (3000 – 8000 kJ/kg, Singh *et al.*, 2012) due to the high water amount that must be removed from wet biomass, which allows lower storage volume, microbiologically stable derivatives and increased concentration level of by-products, thus facilitating the recovery during the extraction phase (Figure I.7).
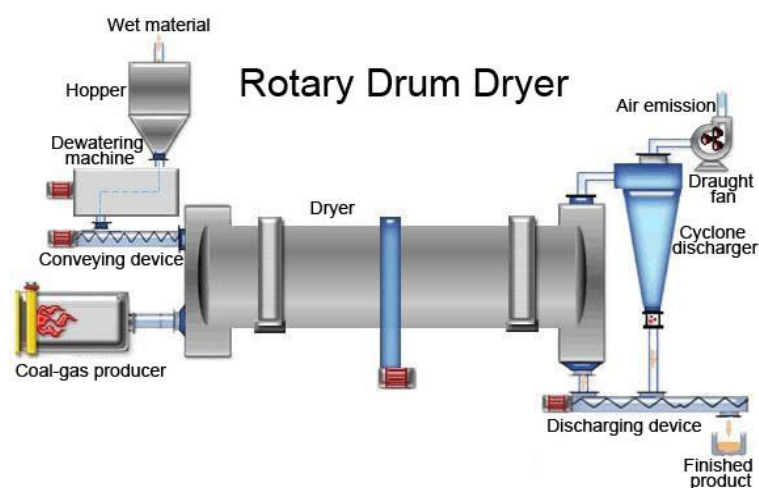
Figure I.7 *Schematisation of a rotary drum dryer (www.ft-dryer.com).*

I.4.3 Solvent extraction

Solvent extraction is the classical method to extract and separate compounds from their originary matrices, based on their relative solubility in the extracting medium. This process generally involves the following steps:

- Penetration of organic solvent through the cell wall/membrane system;
- Interaction of organic solvent with the target compounds;
- Formation of organic solvent-compound complex;
- Diffusion of organic solvent-compound complex across the cell wall/membrane system;
- Diffusion of organic solvent-compound complex across the static solvent film into the external bulk.

Since the selective nature of the solvent towards a given target compound to be solubilized is an important factor to take into consideration (Richardson *et al.*, 2002), the rate of compounds solubilisation in solid/liquid extraction (SLE) is controlled by diffusion and can be approximated based on Fick's law using the expression:

$$\frac{dc_E}{dt} = \frac{a\,D_E}{d}\,(c_S - c_E) \tag{1}$$

where c_E is the concentration of the solute in the extracting solution, a is the surface area of the solid sample, given as the ratio between the area and the volume of the particle or cell (m^2/m^3), D_E is the diffusion coefficient of the solute in the sample soaked with solvent, c_S is the current concentration of the solute in the sample and d is the diffusion layer thickness.

From Eq.1 it emerges that a larger difference between the concentration of the solute in the solid medium and in the extracting solution, achieved by increasing the amount of implied solvent, leads to a higher extraction efficiency but also to diluted extract, thus increasing the solvent consumption and the subsequent downstream processing costs.

The improvement of SLE performances may also be achieved either by reducing solid particle size (biomass pre-treatment), with consequent increases in the surface area available for mass transfer processes, or by raising the temperature of the extraction step, which leads to higher values of diffusion coefficient.

Generally, the stages of SLE may be carried out either as a batch or as a continuous process.

The extraction units are followed by distillation or similar unit operations in order to separate the solvent from the solute.

I.4.4 Down-stream processing: separation/purification of products

I.4.4.1 Chromatography

Chromatography is an important physical-chemical technique that enables the separation, identification and purification of the components of a mixture for quali-quantitative analysis (Coskun, 2016). This technique is generally conducted in packed column where the interaction between the molecules dissolved into a mobile phase, which is flowing through the system, and the packing material (stationary phase) occurs via adsorption/partition (liquid – solid interaction) phenomena. The residence time of a given compound inside the chromatographic system is a function of its affinity with the packed material. The purpose of applying chromatography is to achieve a satisfactory separation within a suitable time interval (Coskun, 2016).

Depending on the mobile phase nature, it is possible to discriminate between liquid chromatography (LC) and gas chromatography (GC). Separation of compounds via chromatography may occur by means of a series of principles (Galanakis, 2015), such as:

- Size (size exclusion chromatography, SEC);
- Charge (ion exchange chromatography, IEC);
- Hydrophobicity (reversed phase high performance liquid chromatography).

Because chromatographic systems are largely automated, basic runs can be performed with minimal training.

Table I.3 lists the principal advantages and limitations associated to the application of chromatographic technology.

Table I.3 *Advantages and limitations of chromatography for the separation/purification of target compounds recovered by means of a solid liquid extraction process.*

Advantages	Limitations
Capability of separating complex mixtures at low operating temperatures	Irreversible adsorption of materials creates problems
Possibility to separate delicate or heat labile compounds	Pre-filtration of feed material is usually required
Very pure products can be recovered	Periodic column re-packing / regeneration is required

I.4.4.2 Membrane Filtration

Membrane filtration processes are nowadays used as an alternative to conventional industrial separation methods such as distillation, centrifugation and extraction (Bowen & Genner, 1995), since they potentially offer several interesting perspectives and advantages in terms of absence of phase transition, low energy expenditures, easy scale-up, great separation efficiencies, low contamination risks and preservation of compounds bioactivity (Rabelo *et al*., 2016).

Basically, filtration is a pressure-driven process which allows the separation of the feed solution into a permeate stream, which contains all substances able to pass through membrane pores, and a retentate stream which, in turn,

is composed of all compounds retained by the membrane itself (Galanakis, 2015).

In particular, membrane technology operates under two types of filtration conditions, namely cross-flow or dead-end filtration (Igunnu & Chen, 2012), as illustrated in Figure I.8.
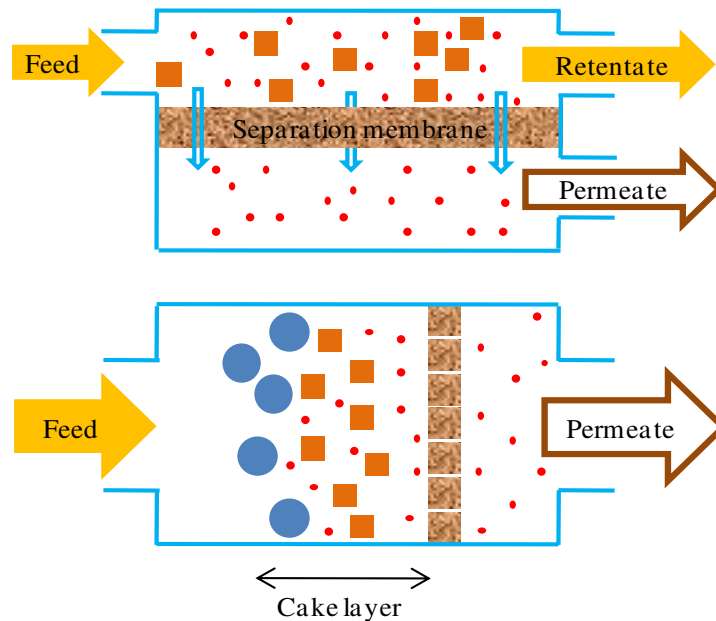
Figure I.8 *Configurations of membrane separation processes: cross-flow filtration (top) and dead end filtration (bottom).*

The separation mechanism is based on a sieving effect and compounds dissolved in the feeding stream are separated according to the membrane pore size (PS), leading to four established processes including microfiltration (MF, PS = 0.1 – 3 μm), ultrafiltration (UF, PS = 0.01 - 0.1 μm), nanofiltration (NF, PS = 0.1 - 1 nm) and reverse osmosys (RO, PS ≤ 0.1 nm). Moreover, as long as the membrane pore size decreases, separation processes become more difficult, thus requiring higher pressure gradients across the separation module (Figure I.9).
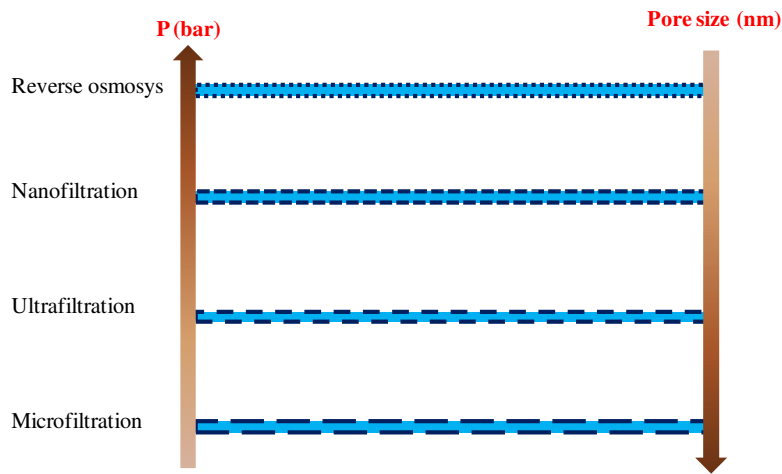
Figure I.9 *Classification of membrane filtration processes as a function of pore size and pressure gradient.*

Materials for fabrication of commercial membranes include synthetic polymers (polypropylene, perfluoropolymers, polyamides, polysulfones, etc.), cellulose derivatives, as well as ceramics, inorganics, and metals.

Despite the easiness of the membrane separation processes, a decline on the permeated flow throughout the operative period may occur, due to the accumulation or absorption of materials on the surfaces of the membrane (fouling effect) and/or within the porous structure.

The consequence of that is represented by the decrease of permeability and the need to increase the transmembrane pressure to maintain the desired flow, thus increasing energy consumption, operating costs and the frequency of cleaning (da Silva Biron *et al.*, 2018).

Moreover, although the cleaning of membrane is the main form of mitigation of the fouling phenomenon effects, a loss in the lifetime of the membrane will occur (Mohammad *et al.* 2015).

I.5 Conventional cell permeabilisation techniques for food wastes/by-products and microalgae

I.5.1 Bead milling

Bead mills are commonly applied in the chemical industry for the manufacture of paints/lacquers and size reduction of minerals (Kula and Schütte, 1987), but they have shown to be a useful technique especially for the disintegration of microbial cells like yeasts (Bunge *et al*., 1992), cyanobacteria (Balasundaram *et al*., 2012) and microalgae (Günerken *et al*., 2015; Postma *et al*., 2015).

During bead milling processing, a suspension of the biological material is put in contact with small steel, ceramic or glass beads under a rapid stirring condition. High shear forces are created and the direct impact of beads with cell suspension leads to cell rupture and release of intracellular compounds (Gunerken *et al*., 2015). The most common design for a bead mill disruption system is shown in Figure I.10.
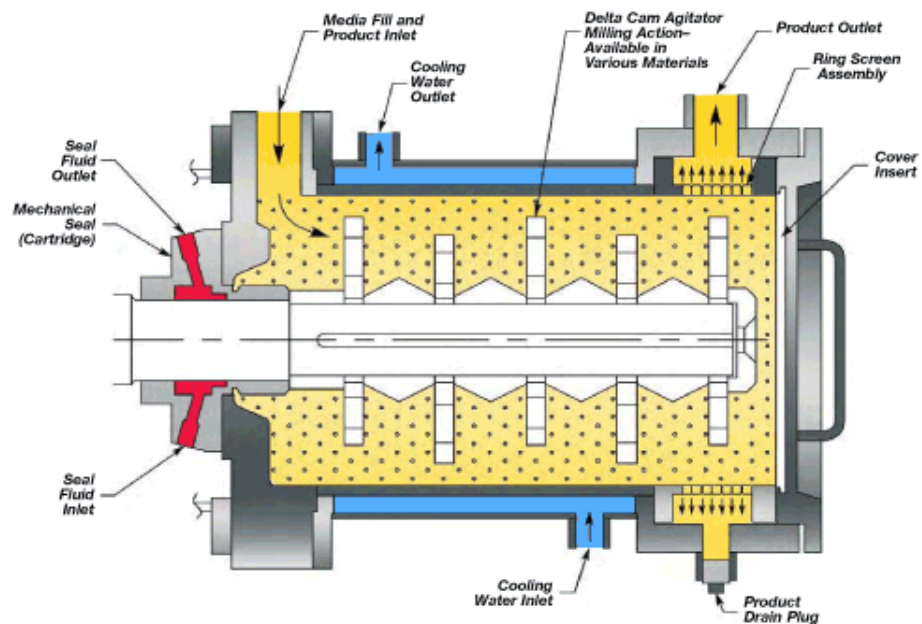
Figure I.10 *Configuration of a large scale bead mill (Gunerken et al., 2015).*

It consists of a horizontal or vertical grinding chamber with a shaft located at the very center, which may be equipped with different disks, rotors or agitators whose purpose is to transfer the kinetic energy to the beads.

A bead mill can be operated either under batch (recirculation) or continuous (single passage through milling chamber) flow conditions. At the end of the milling process, treated suspensions could flow out of the chamber while beads are retained by a sieve or axial slot.

The bead milling processing is typically affected by a series of factors, among which the biomass concentration and the agitator speed have been identified as the most influential variables on process duration, disintegration efficiency and energy consumption (Postma *et al*., 2015).

The efficacy of bead milling in disintegrating microalgae with the aim of improving the extraction yield of intracellular compounds has been proved in different research works, as reported in the following table (Table I.4).

Table I.4 *Impact of bead milling treatment of microalgal biomass on the cell disintegration and extractability of valuable compounds*

Microalgae	Treatment conditions	Notes	Energy requirement	Reference
Nannochloropsis oculata *Porphyridium cruentum*	Bead size: 0.2 – 2.15 mm, bead material: glass, Zirconia, shaft speed: 8 – 14 m/s, flow rate: 48 – 200 mL/min	Efficient microalgal cell disintegration . Nannochlorops. was less susceptible to bead milling treatment than Porphyridium	> 2800 kWh/kg$_{DW}$	Montalescot *et al*. (2015)
Chlorella Vulgaris *Neochloris Abundans* *Tetraselmis Suecica*	Bead size: 0.3 – 1 mm, shaft speed: 9 – 10 m/s	Optimal disintegration attained with smaller beads, leading to higher release of proteins and carbohydrates. Structure of Rubisco native protein was retained	0.47 kWh/kg$_{DW}$	Postma *et al*. (2016a)

Schyzochytrium S31	Bead size: 0.4 – 1 mm, biomass concentration: 1 – 7% DW, shaft speed: 1500 – 5500 RPM	Optimised conditions granted an efficient release of lipids (Y_{MAX} = 0.40)	73.3 – 146.66 kWh/kg$_{DW}$	Byreddi et al. (2016)
Nannochloropsis sp.	Bead size: 0.3 – 2 mm, shaft speed: 0.6 – 7.2 m/s, processing time: 10 – 90 min	Microalgal cells can be effectively disintegrated under optimum conditions	-	Pan et al. (2017)

Based on the results of the case studies briefly summarized in Table I.4, it emerges that increasing the treatment time, agitator tip speed and number of cycles may lead to positive effect on the microalgal cell disruption process (Byreddi et al., 2016; Pan et al., 2017). Moreover, the increase in dry cell weight and biomass flow rates positively affect the cost of the cell disintegration process by reducing the specific energy consumption.

For example, in the work of Postma et al. (2015) it has been demonstrated that the increase in biomass concentration in treated suspensions from 25 g/kg to 145 g/kg may grant a faster and almost complete disintegration process, with lower energy expenditures. Instead, Doucha & Livansky (2008) found that a decrease in the average retention time of *Chlorella vulgaris* biosuspensions allowed to significantly reduce processing costs associated to biomass disruption, independently of the utilized bead milling equipment.

Despite many positive characteristics, the inefficient energy transfer from the rotating shaft to the individual cells and energy conversion into heat (Doucha and Lívanský, 2008) requires an intensive cooling system to avoid loss of functionality of labile products (Gunerken et al., 2015).

Moreover, the production of large amount of cell debris, accompanied by a non-selective release of intracellular compounds, dramatically affects the downstream costs for separation/purification of different products streams.

I.5.2 High pressure homogenization (HPH)

High pressure homogenization (HPH) is a purely mechanical process, which consists in forcing a fluid product through a narrow gap (homogenizing nozzle or valve) at high pressure (150-200 MPa, or 350-400 MPa for ultra-high pressure homogenization, UHPH) (Stang *et al.*, 2001).

The mechanism of cell disruption by HPH is still not fully understood, even though several mutually interacting mechanisms have been proposed by different authors. Brookman (1975) suggested that the cell rupture is achieved due to the rapid pressure drop near the entrance of the valve. Doulah *et al.* (1975) rejected this hypothesis and believed that turbulence was the most important parameter for cell disruption. These authors hypothesised that most of the applied compression energy is converted into kinetic energy of the liquid while the remaining component is converted into friction energy. More recently, Save *et al.* (1994), instead, proposed that cavitation and shock waves/pressure impulses are responsible for cell disruption.
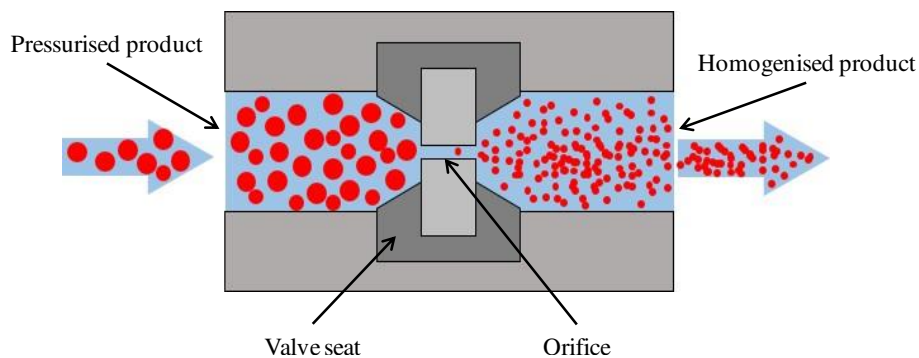
Figure I.11 *Basic principle of HPH cell disruption technology.*

Cell disruption by HPH is a particularly promising technique for microalgae, as it is effective in aqueous environments (eliminating the need for energy intensive drying) and can be scaled up to process large volumes (Samarasinghe *et al.*, 2012). In the case of food wastes/by-products, instead, a comminution step to reduce particle size prior to HPH processing, such as High Shear Homogenisation (HSH), is necessary. In this case, the HPH is effective in turning waste into functional homogenates, possibly exploitable in the formulation of blended juices and smoothies.

An overview of the main literature findings on the effect of HPH treatment on microalgal cells disruption and release of intracellular compounds is given in Table I.5 and discussed below.

Table I.5 *Impact of high pressure homogenisation treatment of microalgal biomass on the cell disintegration and extractability of valuable compounds.*

Microalgae	Treatment conditions	Notes	Energy requirement	Reference
Nannochloropsis oculata *Chlorella sp.* *Tetraselmis suecica*	Pressure: 4 – 150 MPa, number of passes: 1 – 10	Almost all microalgal cells were disrupted after 5 passes. Nannochloropsis species resulted to be the most resistant strain	222.22 kWh/kg$_{DW}$	Spiden *et al.* (2013)
Nannochloropsis sp.	Pressure: 150 MPa, number of passes: 1 – 10	More than 90% of water soluble proteins were unlocked after HPH	25 kWh/kg$_{DW}$	Grimi *et al.* (2014)
Nannochloropsis sp.	Pressure: 30 - 150 MPa, biomass concentration: 0.25 – 25% DW	Cell disruption efficiency of HPH treatment was independent from cell concentration	< 1 kWh/kg$_{DW}$	Yap *et al.* (2015)
Chlorella Vulgaris	Pressure: 270 MPa, number of passes: 2	Efficient release of proteins but lower than that of bead milling	7.5 kWh/kg$_{DW}$	Safi *et al.* (2015)
Nannochloropsis oculata	Pressure: 75-230 MPa,	The release of water soluble compounds was strictly related		

	number of passes: 1 - 6	to processing conditions. HPH treatment was capable of reducing cell aggregation phenomenon	13 kWh/kg$_{DW}$	Shene *et al*. (2016)
Desmodesmus sp.	Pressure: 275 MPa, number of passes: 4	No effect of biomass concentration was detected in terms of pigment release from microalgae	3.4 kWh/kg$_{DW}$	Xie *et al*. (2016)

Generally, literature works report that cell disruption efficiency of microalgal cells dramatically increases with either working pressure and number of homogenization passes.

HPH is recognized, together with bead milling processing, one of the most effective methods for industrial scale microalgal cell disruption (Gunerken *et al*., 2015).

On the other hand this technology suffers from a series of drawbacks such as high energy consumption and huge generation of cell debris which remains solubilised in the extracts, thus requiring high separation/purification costs as well as non-selective release of intracellular compounds. Moreover, the usage of less concentrated microalgal suspensions dramatically leads to an increase of processing costs (Gunerken *et al*., 2015).

I.6 Innovative cell disintegration methods

As an alternative to the conventional disintegration technologies, the application of Ultrasounds (US), Microwaves (MW) and Pulsed Electric Fields (PEF) have increasingly been gaining attention in the last decades (Galanakis, 2012; Gunerken *et al.*, 2015) as faster, sustainable and efficient disruption techniques of biological cells able to foster the release of high-added value intracellular compounds during extraction processes.

I.6.1 Ultrasounds (US)

Ultrasound (US) is a special type of sound wave beyond human hearing in the range between 20 kHz and 100 MHz.
During an ultrasonic treatment (Figure I.12), the energy of high frequency acoustic waves initiates a "cavitation" process, which involves the production, growth and collapse of bubbles, and a propagating shock wave forms jet streams in the surrounding medium causing cell disruption by high shear forces (Gunerken *et al.*, 2015).
Bubble implosion and fragmentation during Ultrasounds-assisted extraction (UAE) processes locally produce extreme conditions with estimated temperatures of around 5000 °C and pressures up to 100 MPa (Gunerken *et al.*, 2015).
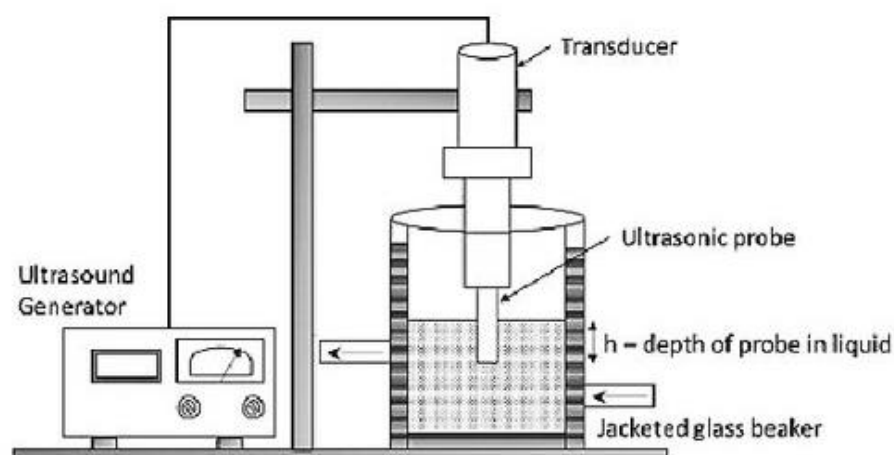
Figure I.12 *Schematic representation of an ultrasound-assisted extraction equipment (Castro-Lopez et al., 2016).*

Numerous authors have demonstrated the capability of US to improve extraction of bioactive compounds (e.g. dietary fibers, phenolics, sugars,

starches, pigments, essential oils, and organic acids) from microalgae and food wastes/by-products, as summarized in Tables I.6 – I.7.

Table I.6 *Impact of ultrasounds treatment of microalgal biomass on the cell disintegration and extractability of valuable compounds.*

Microalgae	Treatment condition	Notes	Energy requirement	Reference
Chlorella vulgaris	Frequency: 40 kHz, treatment intensity: 29.7 W/L	US-assisted Bligh and Dyer method resulted in the highest extraction of oil from *C. vulgaris*	0.4 kWh/kg$_{DW}$	Araujo *et al.* (2013)
Nannochloropsis sp.	Frequency: 24 kHz, treatment power: 100 – 400 W, duration: 0 – 30 min	US-assisted method granted higher yields than that of conventional water extraction, with minimum energetic costs	0.18 kWh/kg$_{DW}$	Parniakov *et al.* (2015a)
Chlorella Vulgaris *Nannochloropsis oculata* *Scenedesmus obliquus*	Treatment power: 720 W, duration: 3 h	Lipids, carbohydrates and proteins were unselectively extracted from raw microalgae. No cell disruption was observed	41.1 kWh/kg$_{DW}$	Ferreira *et al.* (2016)

	Frequency: 20 kHz, treatment intensity: 0 – 50 W/cm^2, duration: 0 – 10 min			
Heterochlorell a Luteoviridis	Frequency: 20 kHz, treatment intensity: 0 – 50 W/cm^2, duration: 0 – 10 min	The best extraction yield was obtained with 40–80% of ultrasound intensity	41.1 kWh/kg$_{DW}$	Jaeschke *et al.* (2017)
Mixture of microalgae *(Scenedesmus sp., Chlorococcum sp.)*	Frequency:30 kHz, treatment power: 50 W, duration: 5 – 60 min	US leads to microalgal cells disintegration with an improvement in the recovery of proteins, carbohydrates and lipids	1600 kWh/kg$_{DW}$	Keris–Sen *et al.* (2017)

Table I.7 *Impact of ultrasounds treatment of food wastes/by-products on the cell disintegration and extractability of valuable compounds.*

Food waste/ by-product	Treatment condition	Notes	Energy requirement	Reference
Dried tomato peels	Frequency: 20 kHz, treatment power: 50 - 125 W, duration: 0 – 10 min	UAE improved the extraction of carotenoids with reduced solvent (hexane) consumption	-	Luengo *et al.* (2014a)
Dried artichoke wastes	Frequency: 20 kHz, treatment power: 0 - 720 W, duration: 5 – 60 min	Improved extraction of phenolics, powers higher than 240W had no influence on process efficiency	-	Rabelo *et al.* (2016)

Olive waste	Frequency: 40 kHz, treatment power: 200 W, duration: 20 – 40 min	Improved extraction yield of phenolics	0.11 kWh/kg$_{DW}$	Wang *et al.* (2017)
Dried Pomegranate peels	Frequency: 20 kHz, treatment power: 130 W, duration: 10 – 60 min	Efficient extraction of carotenoids when optimal conditions were used	1.91 kWh/kg$_{DW}$	Goula *et al.* (2017)

UAE offers a process that reduces the dependence on solvents such as hexane, with improved economic and environmental benefits, mainly due to an increased yield of extracted components, increased extraction rate, reduced extraction time, and higher process throughput (Luengo *et al.*, 2014a).

However, since the ultrasounds processing is followed by drastic temperature and pressure increases, damages to more thermolabile intracellular compounds may be achieved (e.g. denaturation of proteins).

Moreover, cavitation also results in water thermolysis around the bubbles forming highly reactive free radicals that react with the substances in water, thus leading to their oxidation (Gunerken *et al.*, 2015).

I.6.2 Microwaves (MW)

The microwave-assisted extraction (MAE, Figure I.13) is considered a novel method for extracting soluble compounds into a fluid from a wide range of materials.

Microwaves are electro-magnetic waves in the frequency range between 300 MHz and 300 GHz, based on the combined effect of two perpendicular oscillating fields such as electric and magnetic ones (Angiolillo *et al.*, 2015).

When a substance is exposed to microwaves, the latter interact selectively with the dielectric or polar molecules (e.g., water), causing local increases in temperature due to frictional forces from inter- and intramolecular movements (Amarni and Kadi, 2010). As a result of this, subsequent increments in pressure on the cell wall of the biological source are induced, which then lead to cell disruption and consequent release of intracellular compounds towards the surrounding solvent (Liew *et al.*, 2016).

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It is a selective technique for the recovery of organic and organometallic compounds that are more intact, being also recognized as a green technology because it reduces the use of organic solvent (Azmir *et al*. 2013).

MAE may be affected by a large variety of factors, such as microwave power and frequency, treatment duration, moisture content and particle size of sample matrix, type and concentration of solvent, solid to liquid ratio, extraction temperature, extraction pressure and number of extraction cycles (Mandal *et al*., 2007).
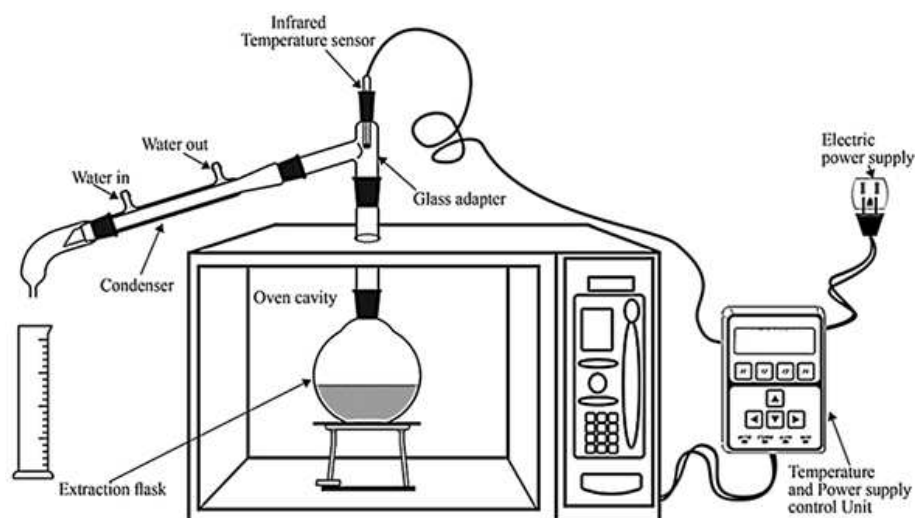
Figure I.13 *Schematic representation of a microwave-assisted extraction equipment used at laboratory scale (Castro-Lopez et al., 2016).*

In recent years, MAE has been successfully used to recover many bioactive compounds from microalgae and wastes/by-products of food industry, as summarized in Tables 1.8 – 1.9.

Table I.8 *Impact of microwaves treatment of microalgal biomass on the cell disintegration and extractability of valuable compounds.*

Microalgae	Treatment conditions	Notes	Energy requirement	Reference
Cylindrotheca closterium *Dunaliella tertioletica*	Irradiation power : 25–100 W, duration: 3–15 min	MAE granted efficient pigments extraction as well as homogeneous heating	84.18 kWh/kg$_{DW}$	Pasquet *et al*. (2011)

Chlorella sp.	Irradiation temperature: 80 – 120°C, duration: 5 – 10 min	The disruption level of cell walls increased as microwave treatment temperature increased	0.66 kWh/kg$_{DW}$	Cheng *et al.* (2013)
Nannochloropsis sp. *Tetraselmis sp*	Irradiation power: 500 W, temperature: 65°C	MW treatment allowed an enhancement in the recovery of lipids	-	Teo & Idris (2014)
Chlorella vulgaris .	Irradiation power: 25–100 W, duration: 3–15 min	MW treatment granted significant percentage increase over controls in terms of lipid recovery yield	2.39 kWh/kg$_{DW}$	Garoma & Janda (2016)

Table I.9 *Impact of microwaves treatment of food wastes/by-products on the cell disintegration and extractability of valuable compounds.*

Food waste/ by-product	Treatment conditions	Notes	Energy requirement	Reference
Dried mango peel	Irradiation power: 160–480 W, duration: 60–180 min	Significant recovery yields of pectin (28.86%) at optimal MW conditions	-	Prakash Maran *et al.* (2015)
Dried pomelo peels	Irradiation power: 350–650 W, duration: 4–	Significant recovery yields of pectin (38%) at optimal MW	6.86 kWh/kg$_{DW}$	Liew *et al.* (2016)

	12 min	conditions		
Dried dragon fruit peel	Irradiation power: 300–600 W, duration: 5–10 min, temperature: 70-100°C	MAE improved the rate of pectin recovery by giving a higher yield in shorter time over traditional solvent extraction	40 kWh/kg$_{DW}$	Tongkham *et al.* (2017)
Dried banana peels	Irradiation power:300–900 W, duration:100–300 s	Improvement of pectin yield at optimized conditions	12.63 kWh/kg$_{DW}$	Swamy *et al.* (2017)
Wet olive pomace	Irradiation power: 150–300 W	Improvement in oil yields at optimum conditions	57.36 kWh/kg$_{DW}$	Yanik (2017)

Even though MAE processes have the potential to increase the extraction yield and decrease the amount of solvent, they are also associated to numerous problems. In fact, this technique is limited to polar solvents and is not suitable for volatile target compounds (Zheng *et al.*, 2011).

In particular, especially in the case of food wastes/by-products assisted extraction step, the recovery of valuable compounds requires the application of a series of up-stream processes such as grinding, which, in turn, furtherly increase the overall processing costs, making this technology less favourable to the recovery of intracellular compounds from such biomasses.

Moreover, the formation of free radicals, as well as high temperature increases, may potentially lead to oxidation and degradation of thermolabile compounds during the extraction processes, with a subsequent loss of functionality (Gunerken *et al.*, 2015).

I.6.3 Pulsed Electric Fields (PEF)

I.6.3.1 Basic principles and main applications

Pulsed electric fields (PEF) is a non-thermal technology arising as alternative to traditional cell disintegration techniques, which can allow energy saving and gentle processing of biological matrices (Toepfl *et al.* 2007).

The PEF treatment consists of applying a series of short duration pulses (1µs - 1ms) of electric field (E = 0.1 – 50 kV/cm) to a biological matrix placed in contact with two electrodes of a batch or continuous flow treatment chamber, with a relatively low energy input (W_T = 1 – 150 kJ/kg).

Upon the application of an external electric field strength, biological cells are subjected to a "transmembrane potential" (V), which leads to a "reversible" or "irreversible" formation of pores on the cell membrane (Figure I.14) depending on the field strength intensity, thus allowing the leakage of intracellular compounds towards the external medium (Angersback *et al.*, 2002).
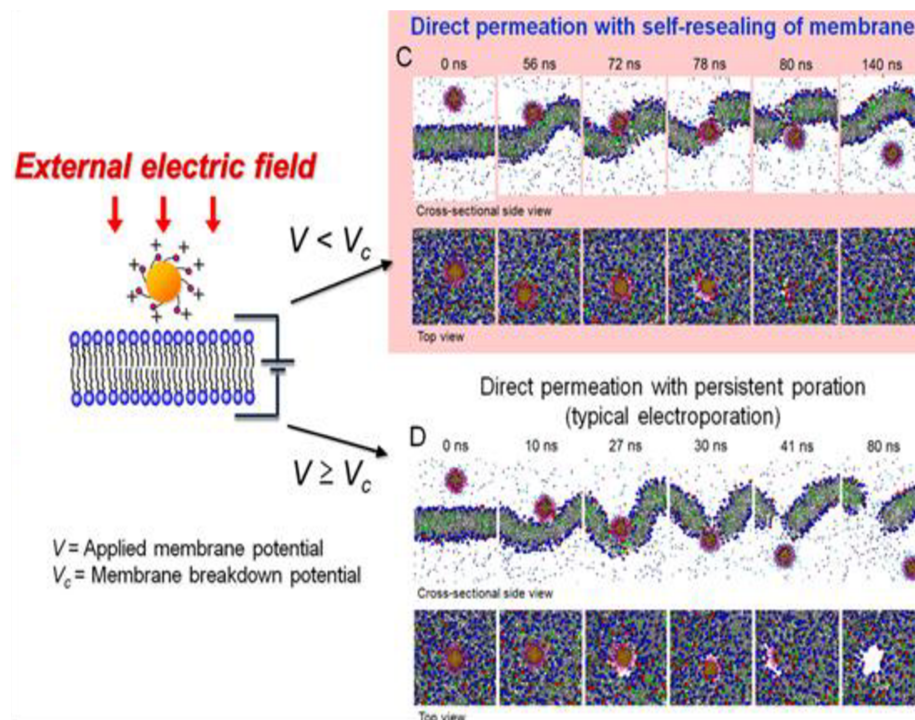
Figure I.14 *Basic principles of Pulsed Electric Fields (PEF) technology, with schematization of reversibile and irreversible electroporation phenomena (Shimizu et al., 2016).*

In general, the electroporation is considered as a dynamic process that can be divided into three main phases: the first one, which is not contributing to molecular transport, consists of a temporary destabilization of cell structure with subsequent pores formation, which lasts about 10 ns, during cellular polarization.

The second phase leads to pores expansions and their coalescence (for a time that goes from 100 µs to some ms).

The last phase, occurring after the pulses application, consists of pores closing and it can last from few seconds to many hours.

The molecular transport occurring through the permeabilized cell, associated to the electroporation phenomenon, may be observed starting from the pores formation and it ends only if the initial membrane structure is restored (Kanduser *et al.*, 2008).

Since the critical electric field intensity is strongly affected by the cell sizes, diminishing as the cellular radius increases (Neumann *et al.*, 1996), vegetal tissues cells (D = 100 µm) require a lower intensity electric field strenght (0.5 – 5 kV/cm) (Knorr, 1999) with respect to microalgal cells (D = 1 - 10 µm) in which the occurrence of electroplasmolysis requires higher values of field strength (10 – 50 kV/cm) (Barbosa-Canovas *et al.*, 1993).

In the last decades, the "electropermeabilisation" phenomenon associated to the application of PEF treatment has been attracting a great interest, especially in the food sector, as a mild and efficient alternative or to complement well established industrial processes (Figure I.15), including:

- "Microbial inactivation": PEF processing constitutes an alternative to traditional pasteurization treatments, having the advantage of non-thermal inactivation of vegetative microbial cells with no or minimal effect on sensorial, nutritional and health-promoting properties of processed products, thus completely accomplishing with consumers request for safe "fresh-like" products (Barba *et al.*, 2015a);

- "Structure modification": recent research has suggested that pulsed electric fields processing could significantly alter the microstructure and functional properties of biomacromolecules such as proteins and polysaccharides (Hong *et al.*, 2016a; Ma *et al.*, 2012), by reducing the amount of chemicals involved in the conventional modification methods such as acetilation and acid hydrolysis, as well as granting similar functionalities/outcomes in a cleaner and more efficient manner (Zhu, 2018);

- "Heat and mass transfer": PEF could be potentially used to enhance heat/mass exchange processes such as drying, with the possibility to facilitate the moisture transfer from the material resulting in an increase in the effective water diffusion coefficient, and freezing, thus avoiding the formation of large ice crystals inside the tissues which cause membrane damage and cell shrinkage (Barba *et al.*, 2015a).

In this frame, several authors have demonstrated that PEF pre-treatment of vegetables and fruits could positively impact on the reduction of operative times with respect to conventional processes, with no evident changes in macroscopic characteristics of the samples (Arevalo *et al.*, 2004; Ben Ammar *et al.*, 2010; Wiktor *et al.*, 2013, 2015).

The induction of permeabilization of plant cells membrane due to PEF technology may also be helpful as a pretreatment to increase the recovery yield of mass transfer based processes, such as pressing or expression, widely used in the production of fruit and vegetable juices (Schwartzberg, 1997), to accelerate the transfer of water and salts/sugars during osmotic dehydration processes (Amami *et al.*, 2006), as well as to enhance the extraction of valuable compounds (such as antioxidants, colorants or flavors) from the inner parts of the biological cells (Donsì *et al.*, 2010).
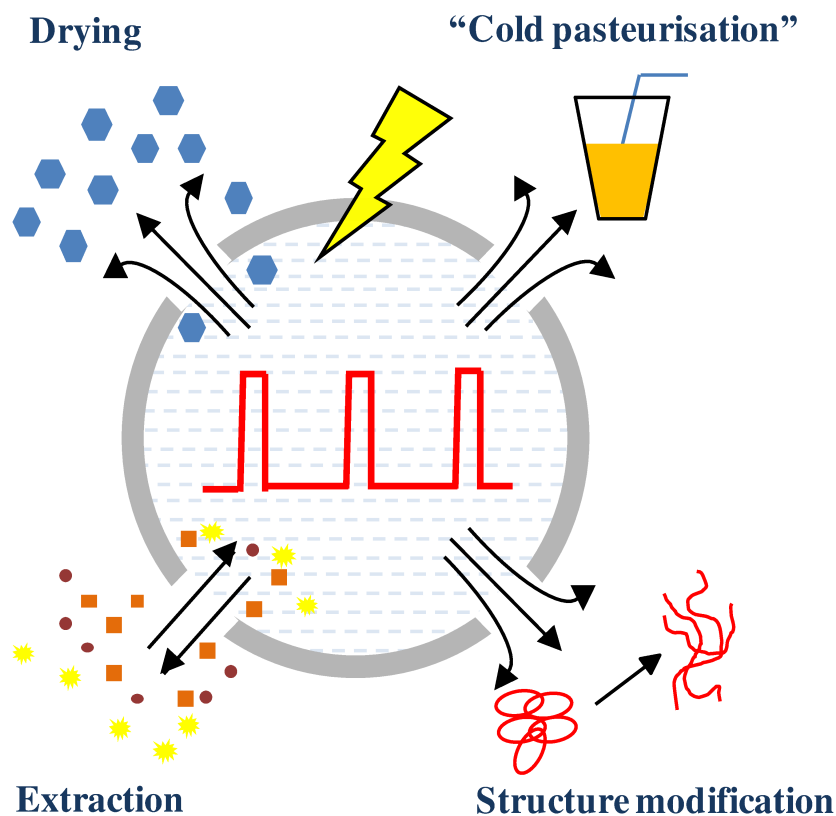
Figure I.15 *Potential applications of PEF technology.*

I.6.3.2 PEF system and principal processing parameters

A PEF system for the treatment of biological matrices (Figure I.16) essentially consists of three main parts:
- A high voltage pulse generator;
- A treatment chamber;
- A control system for process parameters administration.
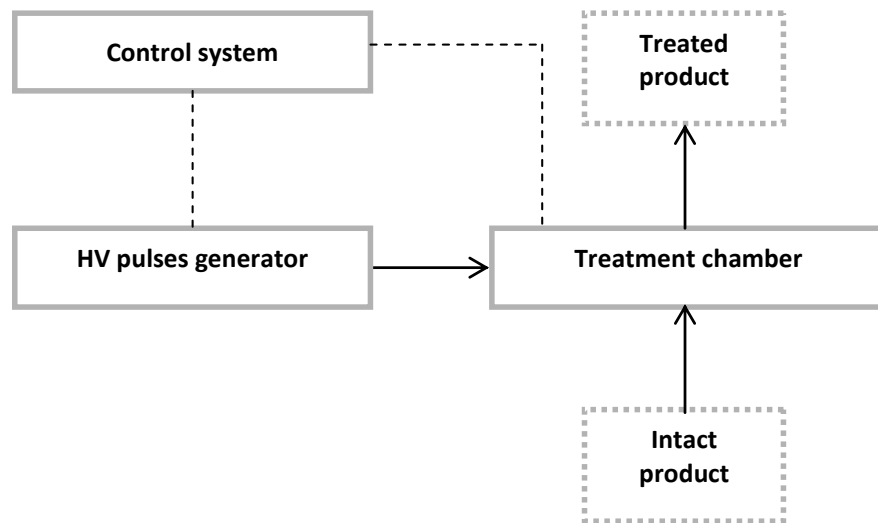
Figure I.16 *Simplified scheme of a PEF system.*

The high voltage pulse generator, which includes a power supply and a pulse forming network (PFN), has the aim to provide pulses of electric voltage according to the shape, duration, intensity and frequency required for the treatment. It has three essential functions:
- To convert the alternative current, provided on the civil or industrial power grid (220V - 380V), in continuous current to the voltage value required for the PEF treatment;
- To load the bank of capacitors and, therefore, accumulate energy;
- To deliver the energy stored in the capacitor bank through the treatment chamber via the PFN including a high voltage switch, with the aim of transferring high voltage electric pulses to the treated product.

One of the most important components of a PEF system is the treatment chamber, whose main function is to transfer, in a mostly uniform way, the high voltage electric pulses to a biological matrix placed in direct contact with two electrodes separated by a spacer insulator.

Therefore, treatment chambers should be designed in order to ensure treatment uniformity avoiding the occurrence of dielectrical breakdown of the treated matrices, which in turn leads to arc formation reducing the life

time of the electrodes while affecting the quality of the final product (Barbosa-Canovas *et al.*, 1993).

PEF treatment chambers can be operated in either batch (used in laboratory scale to carry out preliminary studies), or continuous flow (used for pilot and industrial applications) mode. Among the latter, the most common chamber configurations include:

- Parallel flat plates (Figure I.17a);
- Coaxial (Figure I.17b);
- Colinear (Figure I.17c).
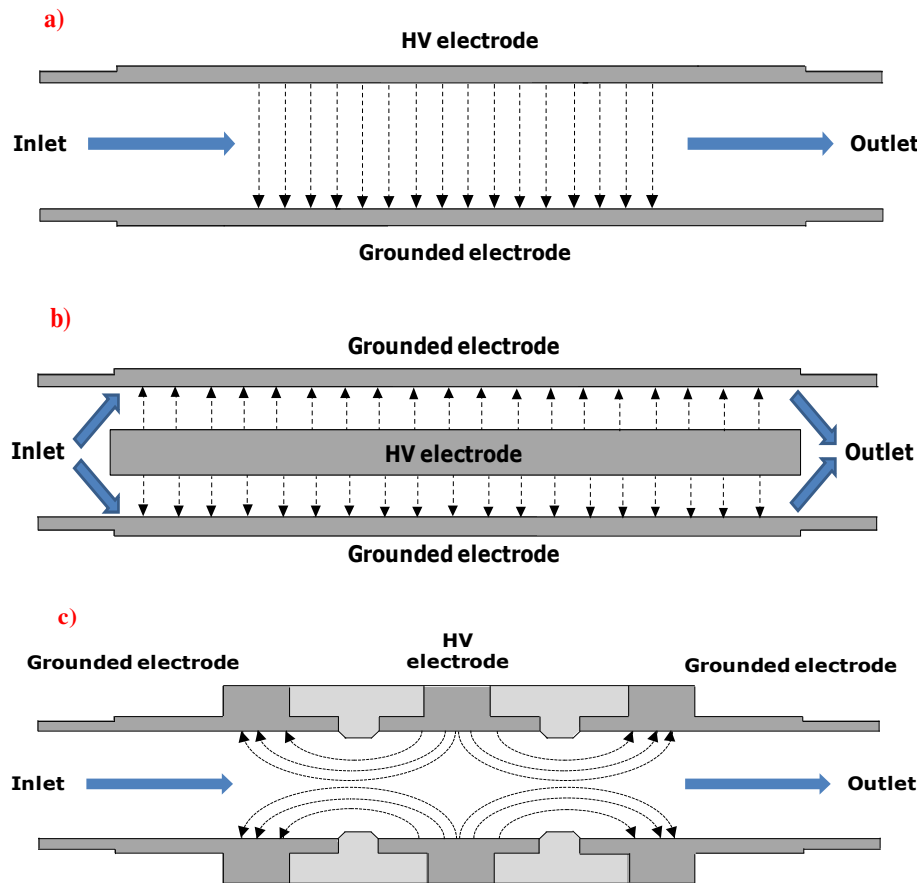
Figure I.17 *Common electrode configurations of continuous flow PEF treatment chambers: (a) parallel-plate, (b) coaxial and (c) colinear.*

For parallel plate (Figure I.17a) or coaxial (Figure I.17b) electrode configuration of batch or continuous treatment chambers, apart from some edge effects (Donsì *et al*., 2007), the electric field is homogeneous within the interelectrode space.

In contrast, other chamber configurations, such as co-linear electrode one (Figure I.17c), suffer from a non-uniform distribution of the electric field in the treatment zone, thus requiring the need for approaches based on numerical simulation procedures for obtaining a more accurate estimation of the actual field strength applied (Toepfl *et al*., 2007).

The process electrical parameters can be monitored with a control system allowing measurements, visualization and data acquisition, consisting of different parts: an oscilloscope, measurement probes for voltage and current through the treatment chamber and thermocouples for temperature monitoring into a batch cell or placed at inlet and outlet sections of a continuous treatment chamber.

Basically, the efficiency of the electroporation process is depending on a series of parameters such as:

- Electric field strength (E, kV/cm), typically estimated as the ratio between the applied voltage (kV) at the treatment chamber and the inter-electrodes space (cm);
- Specific energy input (W_T, kJ/kg), which provides an estimation of the energy consumption associated to the PEF treatment itself. This parameter could be calculated by means of the following formula (Eq. I.2):

$$W_T = \frac{n_P}{m} \int_0^{\tau_P} V(t)\, I(t)\, dt \qquad\qquad (Eq. I. 2)$$

  where m is the mass of the treated product, n_P is the number of applied pulses, V(t) and I(t) are the actual voltage and current signals at the treatment chamber, respectively;
- Pulse width (τ_P) (Figure I.18) and repetition rate (f, Hz);
- Pulse shape and polarity (monopolar, bipolar, Figure I.18).
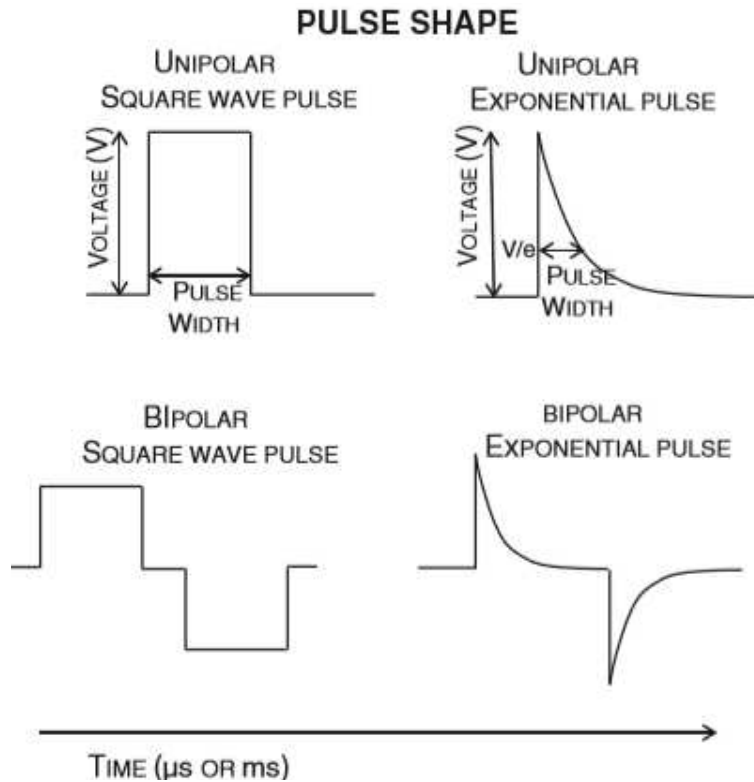
PULSE SHAPE

Figure I.18 *Pulse shapes commonly used in PEF treatments (Raso et al., 2016).*

A reliable and successful method to quantify the extent of cellular damages induced by the electroporation phenomena, defined as the ratio between the number of damaged cells and their total number, is typically accomplished by monitoring the variation of the electrical properties of the biological material upon the exposure to the electrical treatment via the measurements of either electrical conductivity (especially in the case of microbial cell suspension) or complex electrical impedance (as in the case of food tissue), as largely reported in the current literature (Angersbach *et al*., 2002; Donsì *et al*., 2010; Jaeger *et al*., 2012; Pataro *et al*., 2011a, Postma et al., 2016b).
In fact, once nanometric pores are formed at cell membrane level due to the application of a PEF treatment of given intensity, the leakage of small intracellular molecules (e.g. conductive ions) occurs, which then contribute to a decrease in the electrical impedance associated to the treated raw materials (Donsì *et al*., 2010).
The biological cells have electrically insulated structures (e.g. cell walls/membranes) which surround the whole cell as well as the internal organelles, where valuable compounds are stored. For this reason, the

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electrical behavior of an intact biological cell when subjected to an external electric field could be associated to a very complex ohmic-capacitive electrical circuit (Angersbach *et al.*, 1999), where cell membranes act as capacitors, while both the intracellular cytoplasmatic liquid and the liquid surrounding the cells are assumed to be electrical resistors.

When the electroporation phenomenon occurs (Figure I.19), the cell membrane loses its own capacitive behavior due to pores formation, thus allowing the flowing of electrical current, with a shift to only resistive behavior.
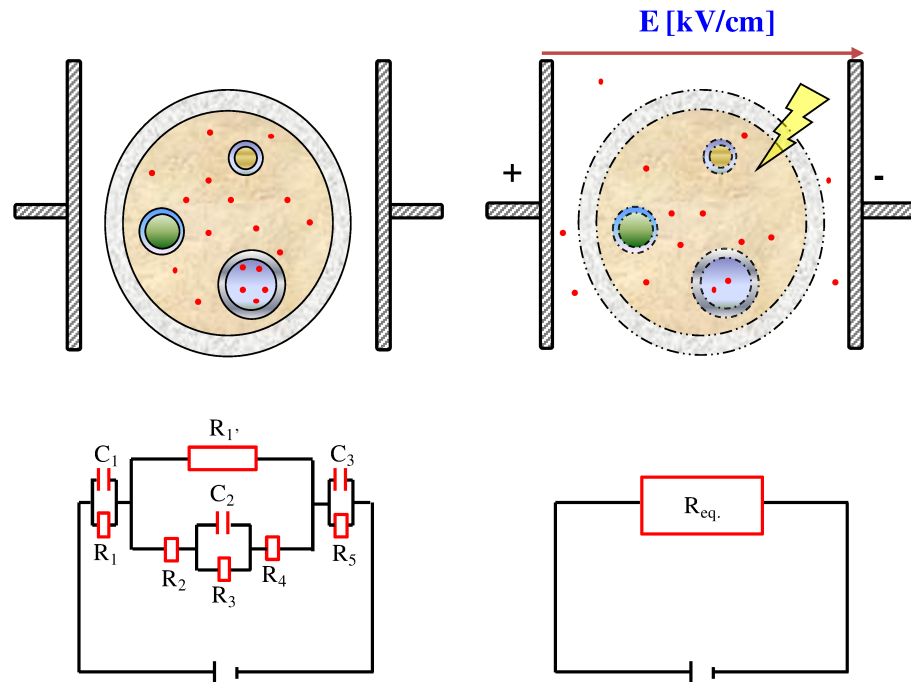
Figure I.19 *Electrical behavior of intact biological cell (left) and electroporated cell (right) after the application of an external field strength (E). Red dots represent intracellular ions (adapted from Pataro et al., 2009).*

Impedance measurement are generally performed in a system in which a sample, placed between two electrodes, is subjected to a sinusoidal or wave voltage signal of variable frequency (f) in the range of 3 – 50 MHz. Examples of results from impedance analysis are reported in Figure I. 20, showing the dependency of complex impedance modulus, Z, and phase angle, ϕ, associated to untreated and PEF treated artichoke bracts samples, upon the investigated frequency values (Battipaglia *et al.*, 2009).
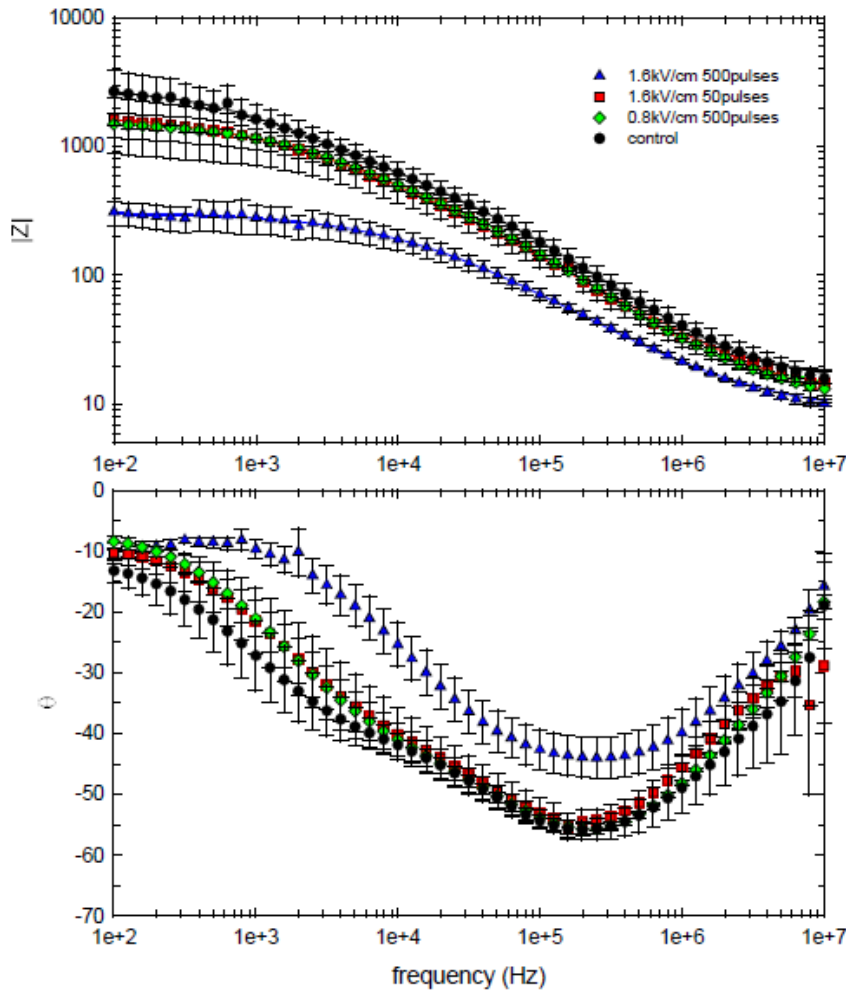
Figure I.20 *Changes with frequency of the absolute value of complex impedance (Z) and phase angle (φ) for artichoke bracts disks before and after PEF treatments of different intensity and duration (Battipaglia et al., 2009).*

The reported graphs clearly show the variation in the electrical behavior of samples when subjected to PEF treatment of increasing intensity.

In particular, when the permeabilisation of tissues is achieved, a reduction in the impedance modulus is observable in the low frequency range ($10^2 - 10^3$ Hz), which is correlable to an increase in the electrical conductivity of the samples.

The occurrence of the electroporation phenomenon may also be visualized in the graph of phase angles against the frequency, where the enhancement of cell membrane rupture leads to the passage from capacitive-ohmic behavior (negative values of φ) to pure resistive behavior ($φ \cong 0°$).

I.7 Principle applications of PEF in the frame of second and third generation biorefinery

The first study of PEF treatment effects on the enhancement of mass transfer processes from food tissues was carried out by Flaumenbaum (1968). The author found that the electroplasmolysis of apple mash allowed an increase in juice yield of 10–12 %, with the final products being lighter in color and less oxidized than after a heat or enzymatic pre-treatment (McLellan et al., 1991).

From these first interesting results it arose that the electroporation phenomenon induced by PEF technology could have potentially been exploited due to its ability to create conditions for the transport of small or large molecules through the cellular membrane of processed tissues, enhancing extraction/diffusion processes (Vorobiev & Lebovka, 2016).

PEF-assisted extraction by diffusion has been widely studied for different products and shown to be promising for the development of modern industrial technology for fresh food plants (Vorobiev & Lebovka, 2010).

In particular, it has been demonstrated that the use of PEF allowed to carry out "cold" and "green" extraction processes, due to the possibility to reduce diffusion temperature, and so the energy associated to the process, as well as to reduce or eventually eliminate dangerous and polluting solvents, encouraging the involvement of eco-friendly solvents like water (Barba *et al.*, 2015a). In the last decades, the interest of PEF technology as a physical pre-treatment prior to extraction processes has been shifted towards the valorization of second and third generation biomasses, with the aim of selectively recovering high-added value compounds stored in the inner part of cells. Recent literature studies suggest that PEF technique may grant an efficient and controlled permeabilisation of cellular tissues of food wastes/by-products and microalgal biomass (Tables I.10 – I.11) allowing to follow a "wet route", thus avoiding the need for a very energy intensive up-stream processing (drying, grinding).

Table I.10 *Impact of pulsed electric fields treatment of microalgal biomass on the cell disintegration and extractability of valuable compounds.*

Microalgae	Treatment conditions	Notes	Energy requirement	Reference
Chlorella Vulgaris	Electric field strength:15 kV/cm, specific energy input: 100 kJ/kg	525% higher recovery of total carotenoids as compared to conventional ball milling	0.93 kWh/kg$_{DW}$	Toepfl (2006)

Synechocystis PCC 6803	Specific energy input:17.9 – 71.7 kWh/m^3	Enhanced access to lipid molecules during solvent extraction	59.67–239 kWh/kg$_{DW}$	Sheng *et al.* (2011)
Auxenochlorell prototothecoides	Electric field strength: 15-35 kV/cm, specific energy input: 0–210 kJ/kg	Increasing the biomass concentration reduced the energy demand of PEF treatment	0.28 kWh/kg$_{DW}$	Goettel *et al.* (2013)
Auxenochlorell prototothecoides	Electric field strength: 35 kV/cm, specific energy input: 0–210 kJ/kg	Improved extraction of water-soluble cell components	0.42 kWh/kg$_{DW}$	Eing *et al.* (2013)
Ankistrodesmus falcatus	Electric field strength: 45 kV/cm, specific energy input: 42 kJ/kg	Over 2 - fold more lipid extraction with ethyl acetate-methanol after cell disruption by PEF	5.83 kWh/kg$_{DW}$	Zbinden *et al.* (2013)
Nannochloropsis sp.	Electric field strength: 20 kV/cm, number of pulses: 400	PEF improved extraction of proteins and pigments reducing solvents consumption	9.35 kWh/kg$_{DW}$	Parniakov *et al.* (2015b)

Arthrospira Platensis	Electric field strength: 46 kV/cm, number of pulses: 130	Cell disruption by PEF granted an extract with higher proteins purity than US treatment	-	Aouir *et al.* (2015)
Chlorella sp.	Electric field strength: 10-25 kV/cm, treatment duration: 60-900 ms	97% cell viability level at optimal conditions	0.75 kWh/kg$_{DW}$	Rego *et al.* (2015)
Arthrospira Platensis	Electric field strength: 10-25 kV/cm, specific energy input: 13.5-110.1 kJ/kg	Selective and efficient extraction of C-phycocyanin	18.75 kWh/kg$_{DW}$	Martinez *et al.* (2016)
Auxenochlorell protothecoides	Electric field strength: 40 kV/cm, specific energy input: 150 kJ/kg	Improved lipids extraction, with similar yields to conventional bead milling processes	0.42 kWh/kg$_{DW}$	Silve *et al.* (2017)
Chlorella Vulgaris *Neochloris Oleoabundans*	Electric field strength: 7.5-30 kV/cm, number of pulses: 1-40	Protein extraction by PEF was not comparable to those achieved by bead milling	0.05 – 150 kWh/kg$_{DW}$	't Lam *et al.* (2017a)

	Electric field strength: 27-35 kV/cm, specific energy input: 50-150 kJ/kg	PEF treatment increased the amount of carbohydrates and phenolic compounds released	0.34 – 1.02 kWh/kg$_{DW}$	Pataro *et al.* (2017c)
Chlorella Vulgaris				

Table I.11 *Impact of pulsed electric fields treatment of food wastes/by-products on the cell disintegration and extractability of valuable compounds.*

Food waste/ by-product	Treatment conditions	Notes	Energy requirement	Reference
Grape by-products	Electric field strength: 3 kV/cm, specific energy input: 10 kJ/kg	Selective extraction of anthocyanins, with higher yields than US treatment	0.01 kWh/kg$_{DW}$	Corrales *et al.* (2008)
Artichoke external bracts	Electric field strength: 0.8-1.6 kV/cm, number of pulses: 500	Enhanced polyphenols extraction, due to tissue disintegration	0.02 kWh/kg$_{DW}$	Battipaglia *et al.* (2009)
Orange peels	Electric field strength: 1-7 kV/cm, specific energy input: 10 kJ/kg	Improved extractability of phenolics during peel pressing	-	Luengo *et al.* (2013)
Flaxseed hulls	Electric field strength: 8-20 kV/cm, treatment duration: 0-20 ms	Efficient extraction of polyphenols at 20 kV/cm and at 10 ms treatment duration	2.16 kWh/kg$_{DW}$	Boussetta *et al.* (2014)

Fermented grape pomace	Electric field strength: 0-3 kV/cm, number of pulses: 200-2000	Selective extraction of phenolic compounds	0.01 kWh/kg$_{DW}$	Brianceau *et al.* (2014)
Tomato peels	Electric field strength: 3-7 kV/cm, number of pulses: 5-100	Improved extraction of carotenoids and reduced hexane percentage in solvent mixture	0.002 kWh/kg$_{DW}$	Luengo *et al.* (2014b)
Vine shoots	Electric field strength: 13.3 kV/cm, number of pulses: 0-1500	Significant enhancement in polyphenols extraction achieved	0.32 kWh/kg$_{DW}$	Rajha *et al.* (2014)
Papaya peels	Electric field strength: 13.3 kV/cm, number of pulses: 1-2000	Significant enhancement of the yields of carbohydrates, proteins and phenolics	0.96 kWh/kg$_{DW}$	Parniakov *et al.* (2014)
Mango peels	Electric field strength: 13.3 kV/cm, number of pulses: 1-2000	Improved recovery of valuable compounds avoiding solvents and reducing temperature	0.64 kWh/kg$_{DW}$	Parniakov *et al.* (2015c)

Papaya seeds	Electric field strength: 13.3 kV/cm, number of pulses: 1-2000	Noticeable enhancements of extraction yields and antioxidant capacities of extracts over conventional diffusion	0.77 kWh/kg$_{DW}$	Parniakov *et al.* (2015d)
Sesame seeds cakes	Electric field strength: 13.3 kV/cm, specific energy input: 42-191 kJ/kg	Enhanced recovery of intracellular compounds and lower solvent consumption	0.25 kWh/kg$_{DW}$	Sarkis *et al.* (2015)
Blueberry press-cake	Electric field strength: 1-5 kV/cm, specific energy input: 1-10 kJ/kg	Enhanced phenolic release from pressed blueberry cakes	-	Bobinaite *et al.* (2015)
Potato peels	Electric field strength: 0.25-1 kV/cm, number of pulses: 5-500	Optimal PEF conditions gave the highest alkaloids yields (2-fold greater than control)	-	Hossain *et al.* (2015)
Red prickly pear peels	Electric field strength: 8-20 kV/cm, number of pulses: 50-300	PEF granted higher yields and lower energetic consumptions than US treatments	2 kWh/kg$_{DW}$	Koubaa *et al.* (2016)

		PEF treatment of stacks of orange skins at		
Orange, pomelo and lemon peels	Electric field strength: 3-10 kV/cm	E = 10 kV/cm improved extraction of polyphenols over controls	-	El Kantar *et al.* (2017)

As emerged from the main literature findings, schematized in Tables I. 10 – 11, the application of a PEF treatment of microalgal biomass, as well as of food wastes/by-products, represents a valid alternative to conventional cell disruption methods due to its capability to enhance a selective release of intracellular compounds, thus leading to extracts with high purity, which positively affect from an economical point of view the downstream processing, requiring a lower amount of energy for the separation/purification phases.

Moreover, PEF technology may be easily scaled-up and combined with different biomass treatment methods (Gunerken *et al.*, 2015).

I.8 "Hurdle" approaches in second and third-generation biorefinery

From the analysis of Tables I.6 – I.11 it has derived that the application of alternative cell permeabilisation processes prior to solvent extraction phases has shown its potential in enhancing the recovery of intracellular compounds from food wastes/by-products and microalgal biomass, with significant results in terms of reduction in solvent consumptions and duration of extraction processes with respect to conventional methods.

However, there still exist a series of bottlenecks which need to be overcome for a successful implementation of a "biorefinery" scheme. In particular, the cell disintegration step should be performed without applying severe processing conditions to save energy and avoid any negative impact on quality and purity of the extracts, thus diminishing the product value (Postma *et al.*, 2016b). Moreover, when a non-selective release of intracellular compounds is achieved, a complication of downstream processing occurs, with subsequent increases in separation/purification economical expenditure.

Recently, the idea of using a sequence of conventional/innovative cell disruption technologies, as well as the application of a single permeabilisation step in combination with physical/chemical agents (pH, temperature, enzymes), has emerged with the aim of increasing extraction yields and purity, achieving the required degree of cellular damages in

milder and more energetically convenient processing conditions (Carciochi *et al.*, 2017).

In the last few years, a series of technological advancements have been obtained in the frame of "hurdle-approaches", whose main outcomes are reported in the following Tables (Tab. I.12 – I.13).

Table I.12 *Impact of hurdle approaches of microalgal biomass on the cell disintegration and extractability of valuable compounds.*

Microalgae	Treatment conditions	Notes	Energy requirement	Reference
Nannochloropsis sp.	PEF(20 kV/cm; 4 ms) + HVED (40 kV/cm; 4 ms) + US (200W, 4 min)+ HPH (150 MPa, 6 passes)	The greatest contribution to protein release was given by HPH treatment (91%), followed by PEF (5.2%)	> 30 kWh/kg$_{DW}$	Grimi *et al.* (2014)
Chlorella Vulgaris	HPH (270 MPa, 2 passes) + alkaline conditions (pH = 7 – 12)	98% of proteins can be released from cell using high pressure treatment and pH 12	11.53 kWh/kg$_{DW}$	Ursu *et al.* (2014)
Nannochloropsis sp.	PEF (20 kV/cm, 1 – 60 pulses) + alkaline conditions (pH = 8.5 – 12)	PEF allowed selective extraction of and water soluble proteins with respect to the simple diffusion	14.03 kWh/kg$_{DW}$	Parniakov *et al.* (2015e)

Neochloris oleoabundans	HPH (80 MPa; 1 pass)/US (600 W, 30 min) + enzyme (cellulase;3 h)	Highest disruption degree achieved by the combination of enzymatic hydrolysis and HPH	13.03 kWh/kg$_{DW}$	Wang *et al.* (2015)
Chlorella Vulgaris	PEF (20 kV/cm; 50-100 kJ/kg) + temperature (35-65°C)	PEF + temperature (35°C) granted the release of small ionic solutes and carbohydrates up to 75% and 39%, respectively	0.55 kWh/kg$_{DW}$	Postma *et al.* (2016b)
Clamydomonas reinhardtii	PEF (7.5 kV/cm; 5 pulses) + enzyme (protease; 6h)	Enzymatic weakening of cell wall resulted in higher protein yields after PEF	2 kWh/kg$_{DW}$	't Lam *et al.* (2017b)

Table I.13 *Impact of hurdle approaches of food wastes/by-products on the cell disintegration and extractability of valuable compounds.*

Food waste/ by-product	Treatment conditions	Notes	Energy requirement	Reference
Tomato peels	US (200W; 9 min) + enzyme (cellulase)	Synergistic effect on lycopene extraction yields	-	Konwarh *et al.* (2012)
Jujube waste	US (10-20 min) + MW (560W; 40 – 60s)	Combination of technologies at optimal conditions lead to the highest release of pectins (2%)	-	Bai *et al.* (2015)

Chapter II
Objectives of the work

From the literature study on Pulsed Electric Fields (PEF) technology, previously schematised in Tables 1.10 – 1.11, it has been shown that the exposure to an electric field can improve mass transfer based processes through "electroporation" of membranes of either microalgae and plant cells, thus allowing a sustainable and efficient recovery of high-added value intracellular compounds.

Despite the literature results would make PEF technology suitable to be used in the "biorefinery" (Figure I.3) of second and third generation biomasses, there are still a series of "bottlenecks" to be overcome.

In particular, current literature data focuses, in most of the cases, only on the optimization of the PEF pre-treatment conditions of agri-food wastes/by-products and microalgal suspensions, with the aim to induce the greatest cell disruption effect with the minimum energy consumption in order to facilitate the leakage of the intracellular compounds of interest during the subsequent extraction phase. Moreover, the observed results, which are generally obtained from lab-scale batch systems, are in some cases contradictory due to the differences occurring among the equipments and processing conditions used.

In addition, the use of PEF technology, applied on its own even at optimized conditions, may lead to recovery yields of target compounds which are considerably lower than those achieved by applying more disruptive conventional techniques, such as bead milling (BM) or high pressure homogenisation (HPH). To this purpose, further efforts in the frame of "hurdle approaches" are urgently needed in order to positively impact on both the energy consumptions and recovery yields, with the possibility to preserve the integrity of the extracted compounds.

Finally, it is worth noting that the optimization of cell permeabilisation phase represents only one of the steps of a "biorefinery" scheme, whose successful application in terms of sustainability, quality of the end products as well as on the economics of the whole process, is also related to the proper optimization of the upstream (e.g. grinding, preheating) and

downstream (purification of extracts, selective separation of target compounds) operations.

Therefore, the main aim of this Ph.D. thesis work was to propose a systematic approach for the development of a PEF-based biorefinery process for an efficient and sustainable valorization of agri-food wastes/by-products and microalgal biomass, ensuring the production of high-added value compounds, as well as to assess its potential feasibility by means of a techno-economical analysis of operative costs involved in the whole process. In order to achieve this goals, the Ph.D. project was articulated as schematised in the thesis outline reported in Table II. 1.

Table II.1 *Ph.D. thesis outline.*

INDUSTRIAL PROCESS	PRODUCED BIOMASS	TARGET COMPOUNDS	PROCESS DESIGN – "BIOREFINERY"						POTENTIAL APPLICATIONS	TECHNO-ECONOMICAL ASSESSMENTS
			Upstream		Extraction		Downstream			
			Lab-scale	Pilot-scale	Lab-scale	Pilot-scale	Lab-scale	Pilot-scale		
SECTION 1 *Food wastes biorefinery* — *Production of peeled tomatoes*	Tomato peels and seeds	Carotenoids (lycopene)			Solid liquid extraction (SLE) / PEF-assisted SLE		Solid liquid separation (SLS)		Food supplements	Operative costs of conventional and innovative processes
Production of canned artichoke heads	External bracts	Polyphenols (chlorogenic acid)	Cutting		SLE / PEF-assisted SLE		SLS		Food supplements	
	Stems		Cutting (grinding)				SLS +Nanofiltration			
SECTION 2 *Microalgae biorefinery* — *Microalgae cultivation*	*A. platensis*	Carbohydrates / Proteins	Biomass concentration		PEF-assisted SLE / HSH-assisted SLE / HSH +PEF assisted SLE		SLS		Food supplements	Schematization of PEF –based biorefinery plant
	C. vulgaris	Carbohydrates / Proteins / Lipids	Biomass concentration		PEF-assisted SLE / HPH-assisted SLE / PEF + HPH assisted SLE		SLS			

As reported in Table II.1, this thesis work has been structured in 2 different sections:

- **Section I - "Food wastes biorefinery" (Chapter IV - VI)**: in this section the integration of PEF technology in the biorefinery of tomato peels and artichoke (external bracts and stems) wastes with the aim of recovering high-added value compounds such as carotenoids and polyphenols, respectively, was studied.

  In particular, in **Chapter IV** the optimisation of the main PEF processing variables (electric field strength and specific energy input), as well as of the solvent extraction parameters (type of solvent, temperature, S/L ratio) on the recovery of valuable compounds from tomato peels was carried out and the main outcomes in terms of extract composition and its antioxidant power are presented and discussed.

  In **Chapter V**, instead, a systematic study on the effect of PEF treatments on the release of intracellular compounds from external bracts and stems of artichoke was performed.

  For the sake of comparison, the efficiency of PEF-assisted extraction tests were compared to those observed in conventional solvent diffusion processes.

  Results of Chapter V allowed to select artichoke stems, being the richest part of the artichoke wastes in terms of phenolic compounds, to be subjected to a "pilot-scale" biorefinery processing involving PEF and Nanofiltration (NF) technologies, aimed at increasing phenolics extraction yields and at concentrating liquid extracts, respectively, with the purpose of obtaining a solid product as reported and commented in **Chapter VI**.

- **Section II - "Microalgae biorefinery" (Chapters VII - VIII)**: in this section the focus was shifted from food wastes/by-products to microalgal biomass. The first major difference between plant tissue and microalgae cells lies in their average size, thus requiring the application of different electric parameters to achieve the proper permeabilisation effect.

  In **Chapter VII**, a preliminary study of the effect of PEF intensity $(E = 10 - 30 \text{ kV/cm}; W_T = 20 - 100 \text{ kJ/kg})$, and pulse polarity (mono/bipolar) on the extractability of proteins and carbohydrates from *A. platensis* microalgae, whose weak cellular structure makes it very susceptible to physical/mechanical stresses, was performed.

  The use of a more complex structured microalgae strain (*Chlorella vulgaris*), being more resistant to cellular breakages, was also considered in this thesis work. In particular, an intensive comparison of the effects induced by PEF method and the highly disruptive High Pressure Homogenisation (HPH) technology on the morphological aspect of microalgal cells, as well as on the release of the main

intracellular compounds (namely carbohydrates and proteins) during diffusion processes was investigated.

Optimal PEF processing conditions were carefully chosen to be used in a "cascade" permeabilisation step of microalgae (**Chapter VIII**), where a combination of PEF with other disruptive technologies (mild heating, High Shear Homogenisation, High Pressure Homogenisation) was applied for the whole exploitation of microalgae biomasses. Moreover, general consideration in terms of selectivity/efficiency of the designed combined processes are addressed.

General discussion and conclusions (Chapter IX): in this final chapter the major outcomes, bottlenecks and remaining knowledge gaps regarding the biorefinery processing of biomasses presented in this thesis work are discussed. Moreover, an evaluation of the specific energetic consumptions related to the proposed "biorefinery" processing of food wastes/by-products and microalgae was reported and discussed. Finally, future perspectives are presented, with the aim of opening new scenarios for subsequent research activities in the frame of downstream processing (purification/separation phases).
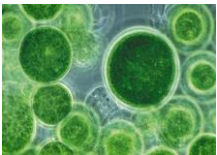
Chapter III
Materials & Methods

III.1 Raw materials

In this thesis work, three different biomasses have been selected and listed in the following table (Table III.1).

Table III.1 *Selected biomasses to be valorized by PEF-assisted extraction technology through the recovery of valuable compounds.*

Raw materials	Target Compound	Availability
Tomato skins	Carotenoids (lycopene)	Summer/Autumn (June – October)
Artichoke wastes	Phenolic compounds (Chlorogenic acid)	Spring (March – May)
Microalgae	Carbohydrates, Proteins, Lipids	All year long (cultivation in indoor systems)

The choice of these biomasses arises, first of all, from their amount and rich content of value-added derivatives. Secondly, they were selected because widely available in Campania Region, which is one of the major Italian producer of fresh and transformed tomatoes and artichokes and where one of the largest italian companies devoted to the cultivation and valorization of microalgae is located. Moreover, the selection of these biomasses was also performed taking into account their availability throughout the year. In particular, while microalgae biomass is cultivated all the year long, the availability of the two food by-products cover different period of the year, thus avoiding overlapping and dead periods during the experimental work.

III.2. Processing of wastes/by-products from food sector

III.2.1 Tomato peels and artichoke wastes

In a first set of experiments, tomatoes of "Datterino" variety were purchased from a local dealer and stored in the fridge at 4°C until required. Before of each experiment, tomatoes of the same colour and size (lenght = 5 cm; diameter = 3 cm) were manually selected and subsequently subjected to 1 min steam blanching at 70°C in a lab-scale steam oven (Minea, SO25P, France) in order to facilitate their manual peeling (Figure III.1a). Square shaped tomato peels (A = 1x1 cm^2) were produced and immediately processed.

In a second set of experiments, tomato peels were obtained from a local tomato processing factory (FPD S.r.l, Fisciano, Salerno, Italy). Peels of homogeneous size (Figure III.1b), approximable to rectangular slabs (L = 2.9 ± 0.3 cm; W = 1.4 ± 0.1 cm), and weight were selected and subjected to subsequent PEF-assisted extraction tests as described in § III.2.5.2.
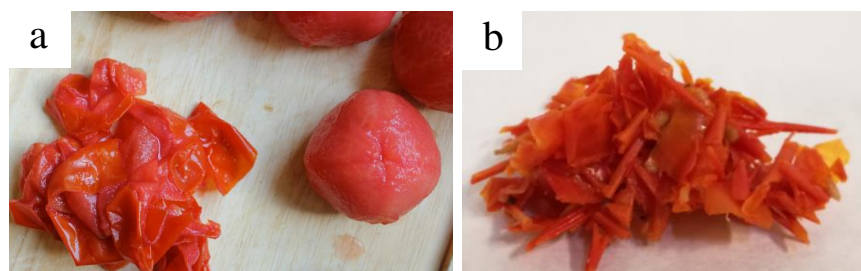
Figure III.1 *Picture of tomato skins deriving from the hand peeling of steam blanched tomato fruits (a) and real industrial tomato wastes (b) used in this thesis work.*

Fresh artichoke by-products, mainly composed of external bracts and floral stems (Figure III.2), belonging to "Cavaliere" variety, were supplied from a

local producer and stored under refrigerated conditions (T = 4°C) until their use.

Figure III.2 *Picture of artichoke external bracts and stems used in this thesis work.*

Prior to their processing, external bracts were selected by similar color and size and subsequently chopped in discs of constant diameter (D = 3 cm, Figure III.3) by means of a metallic cork borer.

Figure III.3 *Picture of artichoke external bract discs used for the extractability of phenolic compounds.*

Fresh artichoke stems, instead, were chopped in pieces of 10 cm lenght by means of a knife and subsequently put into a pilot dicer machine (Giulio Raiola, Angri, Italy, Figure III.4) to obtain 1 cm^3 cubic pieces, which were subjected to both PEF processing at lab-scale and pilot-scale, as described in detail in the following paragraphs.

Figure III.4 *Picture of the pilot dicer machine available at laboratories of ProdAl S.c.a.r.l (left) and artichoke stem cubes used in the experimental campaign (right).*

In order to reduce/slow down the occurrence of oxidation phenomena, both artichoke external bract discs and stem cubes were immediately immersed in a 1% citric acid solution until their processing. Initial moisture content of biomasses, evaluated by their drying at 105° for 24 h, was equal to 84% (w/w) for bracts and 88% (w/w) for stems.

III.2.2 Solvents and chemicals

For the characterisation and processing of tomato peels, HPLC grade methanol and acetonitrile as well as acetone, ethyl lactate and all-trans lycopene standard for HPLC analysis were purchased from Sigma-Aldrich (Steinheim, Germany). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was obtained from Acros Organics (Geel, Belgium).

As regarding artichoke wastes, HPLC grade methanol and phosphoric acid, as well as Folin-Ciocalteau's phenol reagents, ascorbic acid and chlorogenic acid standard for HPLC analyses were purchased from Sigma-Aldrich (Steinheim, Germany).

For both matrices, the evaluation of the antioxidant power of extracts required the usage of iron chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), 2,4,6-tripyridyl-s-triazine (TPTZ), provided by Sigma-Aldrich (Stenheim, Germany), as well as sodium acetate and acetic acid, which instead were purchased, respectively, from Panreac (Panreac Quimica, Barcelona, Spain) and Fisher (Fisher Scientific, Rodano, Italy).

III.2.3 Pulsed electric field (PEF) plant

Permeabilisation of tomato peels and artichoke wastes tissues has been performed by using a PEF system (Figure III.5), consisting of a high voltage pulsed power (20 kV-500 A) generator (Modulator PG, ScandiNova, Uppsala, Sweden) able to generate monopolar square wave pulses (3-25 μs, 1-450 Hz), connected to a batch or continuous flow treatment chamber.
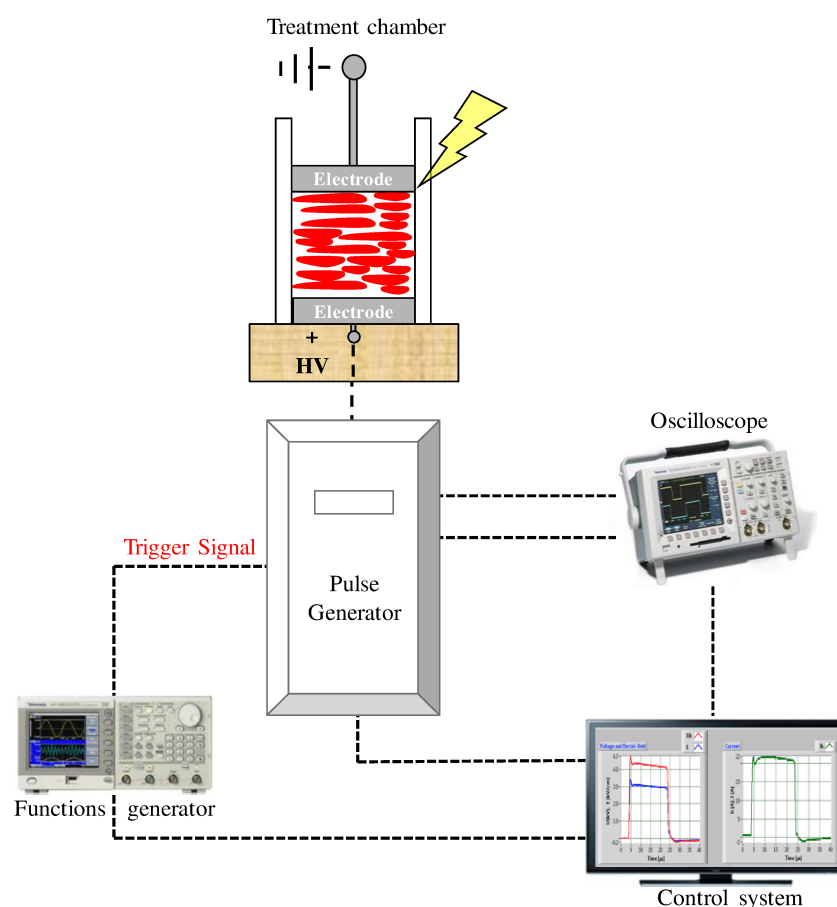
Figure III.5 *Simplified scheme of the batch PEF plant used for the processing of food waste biomasses.*

A wave shape/functions generator fulfills the generation of an external trigger signal that allows the remote control of the pulse generator. The actual voltage and current signals at the treatment chamber were measured using a high voltage probe (Tektronix, P6015A, Wilsonwille, OR, USA) and a Rogowsky coil (2-0.1 Stangenes, Inc., USA) connected to a 300 MHz digital oscilloscope (Tektronix, TDS 3034B, Wilsonwille, OR, USA).

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The peak of electric field (E, kV/cm) was calculated as the ratio between the peak voltage and the electrodes gap, while the specific energy input was evaluated as reported by Bobinaite *et al*. (2015).

III.2.4 Treatment chambers

Two different treatment chambers were used, specifically for lab-scale (Figure III.6) and pilot-scale (Figure III.7) PEF processing operations.
The batch chamber (Figure III.6), was essentially made of a polycarbonate vertical container, closed on both sides by two stainless steel cylindrical electrodes (D = 3 cm) with a contact area of 7.1 cm^2. The distance between the electrodes could be adjusted up to 5 cm, depending on the quantity of sample to be treated. This treatment chamber was used for carrying out both impedance analyses and PEF-assisted extraction tests of the investigated matrices (§ III.2.5).
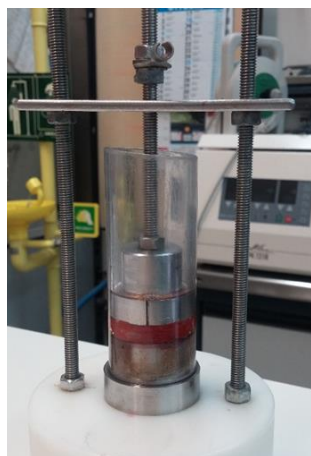
Figure III.6 *Batch treatment chamber with parallel plate cylindrical electrodes*.

The PEF treatment of larger quantities of artichoke wastes was, instead, achieved by using a specifically designed pilot-scale conveyor belt treatment chamber (Figure III.7). The belt consisted of a series of perforated baskets, used for the transportation of artichoke stem cubes. The flow rate can be changed from 22.5 to 375 kg/h by adjusting the belt speed between 0.21 and 2.78 cm/s. The PEF treatment zone, instead, consisted of four consecutive couples of parallel plate electrodes (A = 20 cm^2, Gap = 10 cm), which were alternatively disposed in vertical and horizontal way, in order to increase the efficiency and uniformity of the treatment. Moreover, to ensure electrical continuity between each couple of electrodes, the whole PEF chamber was filled with tap water.
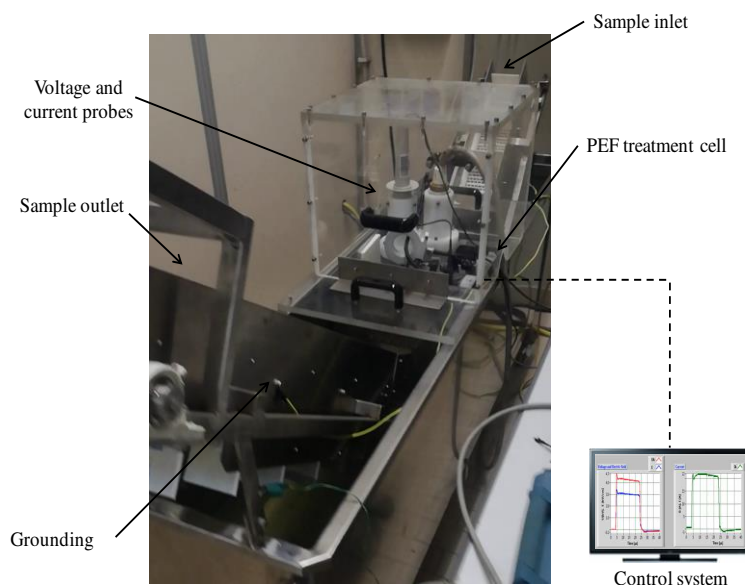
Figure III.7 *Picture of pilot-scale conveyor belt PEF treatment chamber.*

III.2.5 Experimental procedure

III.2.5.1 Evaluation of cell disintegration index (Z_P) via impedance analyses

Cell disintegration index (Z_P) was used to quantify the extent of cell membrane permeabilization of food wastes/by-products tissues due to the application of a PEF treatment of given intensity. Moreover, results were also used to define the optimal PEF treatment conditions enabling to achieve the highest cell membrane permeabilization with the minimum energy consumptions (W_T), to be subsequently applied for conducting PEF-assisted extraction experiments. The determination of Z_P is based on the treatment chamber geometry and on the frequency dependence of the absolute value (|Z|) of the electrical complex impedance of intact and permeabilized plant tissue (Donsì *et al*., 2010; Pataro *et al*., 2011a).

In particular, impedance analyses were conducted by loading 5 grams of either tomato peels or artichoke wastes in the PEF treatment chamber, which was then connected to an impedance analyzer (Solartron 1260, UK).

PEF treatments were carried out at different field strengths (E = 0.25 – 5 kV/cm) and energy inputs (W_T = 0.5 – 20 kJ/kg) at a constant pulse repetition frequency (10 Hz) and pulse width (20 μs). For each PEF treatment condition, a Z_P value was calculated at different frequencies (0.1 kHz and 1 MHz) as described by Pataro *et al*. (2011a), using the Equation 1:

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$$Z_p = \frac{\left|Z_{untr.(0,1\ kHz)}\right| - \left|Z_{tr.(0,1\ kHz)}\right|}{\left|Z_{untr.(0,1\ kHz)}\right| - \left|Z_{tr.(1\ MHz)}\right|} \qquad (III.1)$$

where the subscripts "untr" and "tr" refer, respectively, to untreated and PEF treated samples. The cell disintegration index was defined between 0 (intact tissue) and 1 (completely damaged tissue).

III.2.5.2 PEF-assisted extraction: lab-scale experiments

Figure III.8 shows a simplified scheme of the PEF-assisted extraction process of target compounds from either tomato or artichoke wastes.
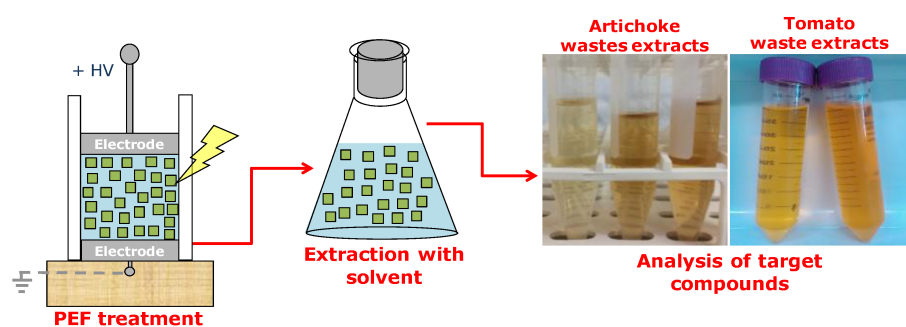
Figure III.8 *Simplification of the PEF-assisted extraction procedure carried out on both tomato peels and artichoke wastes.*

In particular, lab-scale PEF-assisted extraction experiments were performed by loading 5 g of food wastes into the lab-scale treatment cell, along with distilled water at constant S/L ratio of 1:1, in order to ensure electrical continuity with the electrodes of the chamber, and subsequently subjecting them to the optimal PEF treatments conditions, in terms of both field strengths (E) and total specific energy input (W_T) defined upon the impedance measurements (§ IV.3.1 and § V.3.1). Pulse repetition frequency and pulse width were kept constant at 10 Hz and 20 μs, respectively.

After the electrical treatment, the biomass was put into 200 mL flasks together with the extraction solvent (acetone or ethyl lactate for tomato peels, distilled water for artichoke wastes) at variable solid-to-liquid ratio (1:10 – 1:80 g/mL) and allowed to stand in an incubator (t = 0 – 6 h) under a gentle agitation (160 rpm) at variable diffusion temperature (T = 20 – 50 °C).

At the end of the extraction process, untreated and PEF treated biomasses were discarded and the extracts were centrifuged at 6500 rpm for 10 min (PK121R model, ALC International, Cologno Monzese, Milan, Italy) in

order to obtain clear supernatants, which were stored at − 20°C for further quali-quantitative analyses.

III.2.5.3 PEF-assisted extraction: Pilot-scale experiments

Pilot-scale PEF-assisted extraction process was carried out only on artichoke stems. During PEF treatment, artichoke stems were transported through the treatment zone at a flow rate of 150 kg/h and exposed to the same treatment intensity as for lab-scale tests. Approximately 500 g of stem cubes were loaded into each basket and a total amount of about 8 kg of raw material was processed. Afterwards, the untreated and treated artichokes stems were subjected to the aqueous extraction step into a 100 L agitated tank at constant solid-to-liquid ratio (1:10 g/mL) and temperature (25°C). Untreated samples were collected from the same system, but with the PEF system turned off.

III.2.5.3.1 Pilot-scale concentration of phenolic compounds from artichoke stem extracts via Nanofiltration (NF)

The water extracts from untreated and PEF treated artichoke stem cubes were clarified by means of a stack of sieves of decreasing pore size (500 – 25 μm) in order to remove any suspended solids, prior to be subjected to a NF process in a pilot-scale plant (Figure III.9) supplied by Sepra (Cesano Maderno, Italy).
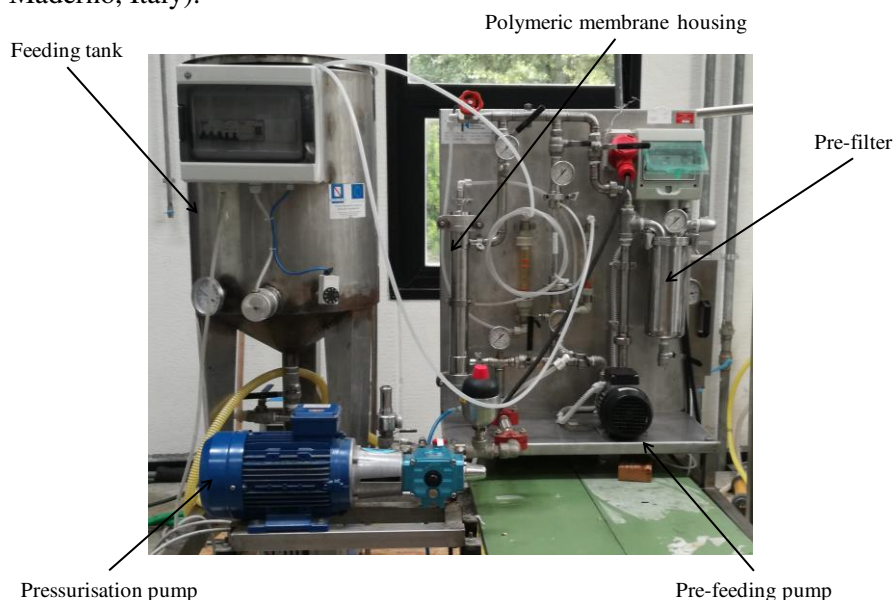
Figure III.9 *Picture of pilot-scale nanofiltration system used for the concentration of aqueous artichoke stem extracts.*

Briefly, the system consisted of a loading tank of 100 L capacity, equipped with a cooling coil to control the processing temperature, a stainless steel housing for both a pre-filter cartridge (20 μm of mean pore size) and a 18x1.2 in^2 spiral wound membrane module, a pre-feeding (P_{MAX} = 10 bar) and a pressurization pump (P_{MAX} = 45 bar) and a pressure control valve.

The concentration of phenolic compounds was carried out by using a NF polymeric membrane (GE Osmonics Desal DL 1812), supplied by Lenntech (Delfgauw, NE), whose main characteristics are reported in Table III.2.

Table III.2 *Main characteristics of DL nanofiltration membrane.*

Membrane type	Desal-DL
Producer	Lenntech
Polymeric material	Polyamide
Molecular cut-off (Da)	150 - 300
Membrane active area (m^2)	0.32
Maximum pressure (bar)	41.4
Maximum temperature (°C)	48.6
Average MgSO4 rejection percentage (%)	96
pH range	2 - 10

Nanofiltration tests were carried out by loading 80 L of water extracts from untreated and PEF treated stem cubes and subjecting them to phenolic concentration at constant temperature and pressure (T = 25°C; P = 20 bar), until reaching a volume reduction factor (VRF) of 4.

Aliquotes of concentrate and permeate streams were withdrawn each 60 min from the beginning of the nanofiltration process and subsequently stored in refrigerated conditions for further analyses.

The cleaning of NF plant was always carried out after each processing by circulating a detergent solution for up to 20 min. Afterwards, the system was shut down and the cleaning solution was kept for 20 min, before being rinsed with distilled water and flushed for 10 min.

The efficacy of NF process was assessed by the evaluation of both phenolic compounds (TPC) and antioxidant power (FRAP) retention rates, calculated as follows:

$$TPC\ retention\ rate = \left(1 - \frac{TPC_{per.}}{TPC_{ret.}}\right) * 100 \qquad (III.2)$$

$$FRAP\ retention\ rate = \left(1 - \frac{FRAP_{per.}}{FRAP_{ret.}}\right) * 100 \qquad (III.3)$$

where the subscript "*per.*" and "*ret.*" are referred, respectively, to the permeate and retentate streams.

III.2.5.3.2 Post-processing of retentate stream and product formation

At the end of the nanofiltration step, the retentate stream was recovered into a 20 L tank and furtherly concentrated by using a R-200/205 Rotavapor (BÜCHI Labortechnik AG, Flawil, Switzerland) until achieving a volume reduction up to 90%, prior being subjected to a freeze drying process into a 25 L VirTis Genesis freeze-drier (SP Scientific, USA, Figure III.10) at P = 50 mbar for 24h, by setting the plate temperature at 25°C.

Figure III.10 *Picture of freeze-drier used for the obtainment of solid extracts from artichoke waste retentate stream.*

The dried extract was then stored under refrigerated conditions to be furtherly characterised in terms of total phenolic compounds, according to the procedure illustrated in § III.2.6.2.

III.2.6 Analytical determinations of extracts from untreated and PEF treated tomato peels and artichoke wastes

III.2.6.1 Total carotenoids (TC) content of tomato peel extracts

The total carotenoids (TC) content of tomato peels extracts from untreated and permeabilised samples was determined according to the method described by Lichtenthaler and Wellburn (1983). The absorbance of undiluted extracts was measured at 470 nm (A_{470}), 645 nm (A_{645}), and 662 nm (A_{662}) in a V-650 UV-Vis spectrophotometer (Jasco Inc., Easton, USA). Absolute acetone was used as a blank. The total content of carotenoids, expressed in mg/100 g of fresh weight (FW) peels, was calculated from the following equations for 100% acetone:

$$C_a = 11.75\,A_{662} - 2.35\,A_{645} \qquad\qquad (III.4)$$

$$C_b = 18.61\,A_{645} - 3.96\,A_{662} \qquad\qquad (III.5)$$

$$C_{x+c} = (1000\,A_{470} - 2.27\,C_a - 81.4\,C_b)/227 \qquad\qquad (III.6)$$

where C_a is the content of chlorophyll a, C_b is the content of chlorophyll b, and C_{x+c} is the content of carotenoids.

III.2.6.2 Total phenolic compounds (TPC) of artichoke wastes extracts

Total phenolic content was measured by Folin–Ciocalteau method following the methodology proposed by Bobinaite *et al.* (2015), with some modifications. A sample of extract (1 mL) was mixed with Folin– Ciocalteau reagent (5 mL) and allowed to stand for 5 min at room temperature. Afterwards, sodium carbonate (7.5% w/v; 4 mL) was added to the mixture. After shaking, the mixture was incubated for 60 min. The absorbance of the reacting mixture was spectrophotometrically measured at 765 nm.
A standard curve was obtained by using solutions of gallic acid (GA) at different concentration (10 – 100 mg/L), which allowed to express the results in terms of milligrams of gallic acid equivalents per 100g of fresh weight artichoke wastes (mg_{GAE}/100g FW).

III.2.6.3 Ferric reducing antioxidant power (FRAP) of extracts from tomato peels and artichoke wastes

FRAP assay was carried out according to the method described by Benzie and Strain (1996) with some modification. Before the measurements, 0.3 M

sodium acetate buffer (pH 3.6) was prepared by dissolving 3.1 g of sodium acetate and 16 mL of acetic acid in 1000 mL of distilled water; 10 mM TPTZ solution was prepared by dissolving 0.031 g TPTZ in 10 mL of 40 mM HCl; 20 mM ferric solution was prepared by dissolving 0.054 g of FeCl3•6H2O in 10 mL of distilled water. The FRAP working solution was prepared by freshly mixing 0.3 M sodium acetate buffer, 10 mM TPTZ solution, and 20 mM ferric solution at a ratio of 10:1:1 (v/v/v).

For the analysis, 2.5 mL of freshly prepared FRAP working solution and 0.5 mL of undiluted extract from tomato peels/artichoke wastes were mixed and incubated for 10 min at ambient temperature. The change in absorbance due to the reduction of ferric-tripyridyltriazine (Fe III-TPTZ) complex by the antioxidants present in the samples was spectrophotometrically monitored at 593 nm. The absorptions of blank samples (applying the same analysis conditions) were tested each time before and after analysis.

Trolox and ascorbic acid were respectively used as the standards for the calibration curves of antioxidant capacity in acetone and water extracts and the final results were expressed as mmol of trolox equivalents (mmol TE) or mmol of ascorbic acid equivalent (mmol AAE) per 100 g of FW food waste.

III.2.6.4 HPLC analysis

HPLC analysis for single carotenoid and phenolic compounds determination were performed in a Waters 1525 Separation Module coupled to a photodiode array detector Waters 2996 (Waters Corporation, USA).

Before the injection, all the collected extracts were filtered with 0.45 μm filters. For lycopene determination, the mobile phase consisted of acetonitrile/methanol (10:90, v/v) and 9 mM TEA (triethilamine). The flow rate of the mobile phase through the column and the injection volume were 1 mL/min and 5 μL, respectively. The absorbance detection wavelength was set at 470 nm.

Instead, for chlorogenic acid determination, the mobile phase consisted of phosphoric acid (0.1 %, eluent A) and methanol (100%, eluent B). The gradient elution program was used as follows: 0-30 min from 5% B to 80% B, 30 – 33 min 80% B, 33 – 35 min from 80% B to 5% B. The flow rate of the mobile phase and the injection volume were respectively 0.8 ml/min and 5 μl. Chromatograms were acquired at the fixed wavelenght of 326 nm.

The identification of the lycopene in tomato peel extracts and chlorogenic acid in artichoke stems extracts was carried out by comparing their retention times and absorption spectra with those of the available commercial standards.

III.3 Microalgae processing

III.3.1 Arthrospira platensis: cultivation and harvesting

The strain *Arthrospira platensis* (PCC 8005, shown in Figure III.11) was supplied by a local factory (ATI Biotech, Castel Baronia, Italy) working in the field of research and production of microalgae and biomaterials.

Figure III.11 *Optical microscopy observation of A.platensis microalgae cells.*

This algae was cultivated in open pond systems, in which a maximum microalgal concentration of 4 g_{DW}/L_{SUSP} was achieved at the end of the exponential growth phase.

Afterwards, the microalgae suspension was pumped to a system of vibrating screens, which allowed to increase biomass concentration by dewatering up to 12% (120 g_{DW}/L_{SUSP}, Figure III.12). Then microalgae paste was packed in polyethylene bags and transported to the laboratories of ProdAl S.c.a.r.l. (University of Salerno, Fisciano, Italy).

Figure III.12 *Arthrospira platensis paste provided by ATI Biotech.*

Prior to their processing, microalgae paste was diluted with distilled water up to 2% DW, showing a mean electrical conductivity of 2.7 mS/cm at 25°C (Conductivity-meter HI 9033, Hanna Instrument, Milan, Italy).

The determination of dry weight amount of biosuspensions was conducted by their drying at 80°C until constant weigh was reached.

III.3.2 Chlorella vulgaris: cultivation and harvesting

C. vulgaris microalgae (CCAP 211, Figure III.13) was purchased from the Culture Collection of Algae and Protozoa (Argyll, UK) and cultivated at the Department of Civil, Chemical and Environmental Engineering of the University of Genova (Italy) in modified Bold's basal medium (Bischoff & Bold, 1963) at pH 7.0 ± 0.5, in a 5 L horizontal tubular photobioreactor illuminated by four 40 W fluorescent lamps from one side (Ortiz-Montoya *et al.*, 2014).

Figure III.13 *Optical microscopy observation of C.vulgaris microalgae cells.*

The composition (per liter of distilled water) of the modified medium was as follows: 1.5 g $NaNO_3$, 0.45 g $MgSO_4·7H_2O$, 0.15 g NaCl, 0.45 g $K_2HPO_4·3H_2O$, 1.05 g KH_2PO_4, 0.15 g $CaCl_2·2H_2O$, 0.003 g vitamin B_1, 7.5 10-6 g vitamin B_8, 7.5 10-6 g vitamin B_{12} and 6 mL of P-IV solution (Sigma Aldrich, Milan, Italy). The culture was aerated at a rate of 1000 cm^3/min with an air flow containing 2 % (v/v) carbon dioxide. Growth conditions were monitored by optical density (OD) measurements at 625 nm using a UV–Vis spectrophotometer (Lambda 25 model, Perkin Elmer, Milan, Italy).

Microalgae were harvested during the end of the exponential phase at a biomass concentration of about 3 g_{DW}/L of suspension and then concentrated by centrifugation (centrifuge model 42426, ALC, Milan, Italy) at 4000×g for 10 min at 20 °C up to a final concentration of 12 g_{DW}/L. The concentrated biomass was pre-packed in high-density polyethylene bottles (Nalgene) and transported in an EPS box under refrigerated conditions to the laboratories of ProdAl S.c.a.r.l. (University of Salerno, Italy) within 24 hours. The initial electrical conductivity of algae suspension was about 1.78±0.03 mS/cm at 25°C.

III.3.3 PEF system for permeabilisation of microalgal cells

PEF treatments of microalgal suspensions were conducted in a bench-scale continuous flow PEF unit (Figure III.14), described in detail in a previous work (Postma *et al.*, 2016b).
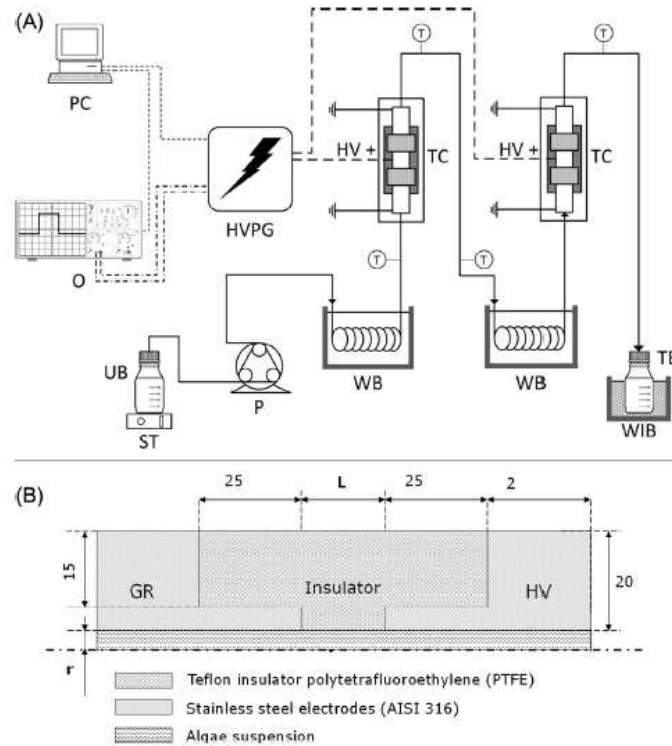
Figure III.14 *(A) Schematic overview of continuous flow PEF system. O: oscilloscope, UB: untreated biomass, ST: magnetic stirrer, HVPG: high voltage pulse generator, P: peristaltic pump, WB: water bath, HV+: high voltage, T: thermocouple, TC: treatment chamber, TB: treated biomass, WIB: water ice bath; and (B) dimensions and geometry of a single co-linear PEF treatment chamber in axis-symmetrical configuration. GR: ground electrode; HV: high voltage electrode; L: gap distance (4mm); r: inner radius (1,5mm), (adapted from Postma et al., 2016b).*

Briefly, the unit consisted of a peristaltic pump (Pump Drive PD5201, Heidolph Instruments GmbH, Germany) to set the flow rate of the algae suspension through the system. The inlet temperature of the algae suspension was controlled using a stainless steel coil (3.9 mm inner diameter, 1.2 mm thickness, 0.5 m length) immersed in a water heating bath (Thermo Haake DC 10, Henco srl, Italy). The PEF treatment zone consisted of two modules,

each made of two co-linear cylindrical treatment chambers hydraulically connected in series and made of stainless steel electrodes separated by Plexiglas insulator. The inner diameter of the treatment zone was 3.1 mm and the electrode gap was 4 mm.

The treatment chambers were connected to the output of a high voltage pulsed power (20 kV-100 A) generator (Diversified Technology Inc., Bedford, WA, USA) able to deliver both mono- and bipolar square wave pulses (1-10 μs, 1-1000 Hz), with a maximum average power of 25 kW. The peak electric field intensity (E, in kV/cm) and total specific energy input (W_T, in kJ/kg$_{SUSP.}$) were measured and calculated as reported in Postma *et al.* (2016b). Three T-thermocouples were used to measure the product temperature at the inlet and outlet of each module of the PEF chamber. Voltage and current signals at the treatment chambers were measured, respectively, by a high voltage probe (Tektronix, P6015A, Wilsonwille, OR, USA) and a rogowsky coil (2– 0.1 Stangenes, Inc., USA) and displayed on a 300 MHz digital oscilloscope (Tektronix, TDS 3034B, Wilsonwille, OR, USA). Independently on the considered microalgal strain, the experiments of biomass permeabilisation by PEF processing were executed at fixed suspension flow rate (2 L/h).

III.3.4 High Shear Homogenisation (HSH) pre-treatment of A. platensis microalgae

The high-speed homogenizer (Figure III.15) was used as a gentle pre-treatment prior to perform PEF treatments with the aim of disaggregating *A. platensis* cell clusters, which potentially lead to spontaneous separation of microalgal thricomes from the liquid medium.
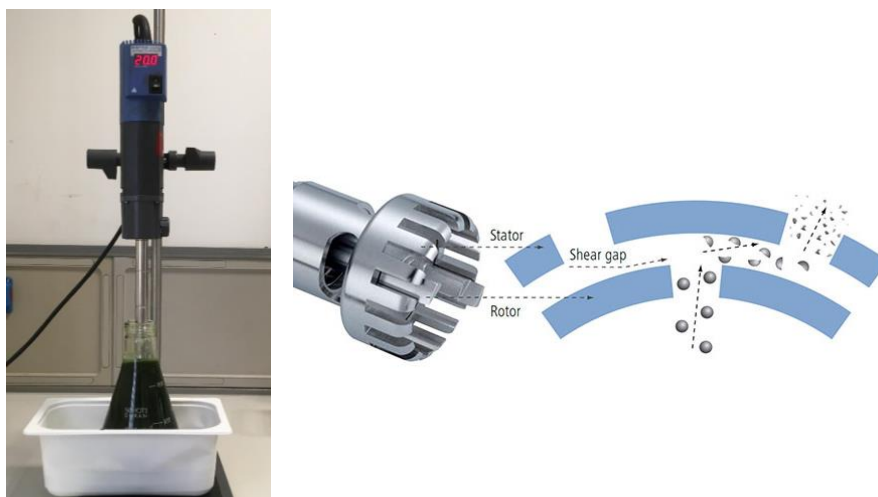
Figure III.15 *IKA T25 digital ULTRA TURRAX unit available at ProdAl laboratories (left) and schematisation of the rotor-stator principle (right).*

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The HSH system was essentially a stirring device at high speed (3000 - 25000 rpm, 800 W) which consisted of a stator-rotor assembly made of stainless steel. In order to avoid excessive temperature increases, the biosuspensions were kept in an ice-water bath during processing.

From preliminary results (data not shown) it was established that the application of a HSH treatment at 20000 rpm for 1 min of processing was capable of ensuring a high extent of *A. platensis* cell cluster disruption.

As a result, these conditions have been fixed for further application of "hurdle approaches", subsequently described in § III.3.6.3.

III.3.5 High Pressure Homogenisation (HPH) of microalgal biomass

The full disintegration of *A. platensis* and *C. vulgaris* cells has been performed in an in-house developed laboratory scale high-pressure homogenizer, shown in Figure III.16.
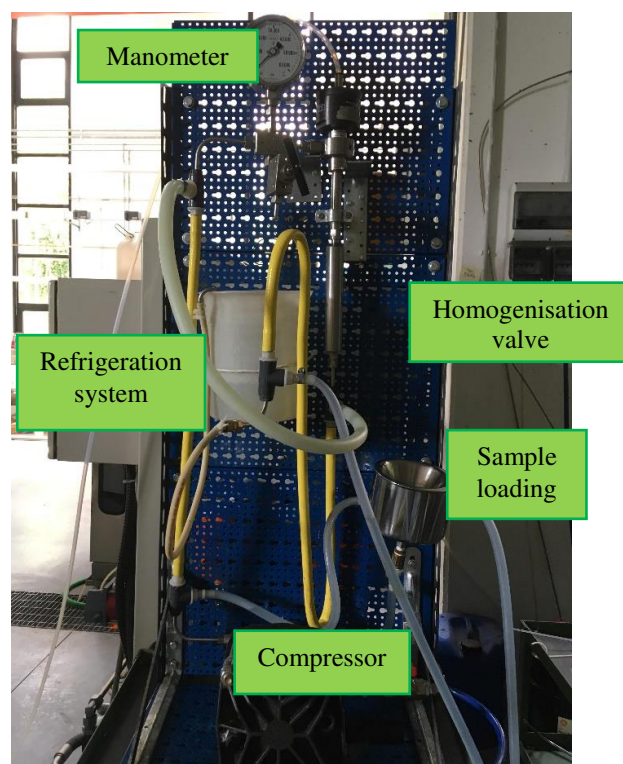
Figure III.16 *HPH plant available at ProdAl S.c.a.r.l. laboratories.*

The microalgal suspension was forced to pass through an orifice valve (model WS1973, Maximator JET GmbH, Schweinfurt, Germany) of

different size (80 – 150 μm) upon pressurization by means of an air driven Haskel pump (model DXHF-683, EGAR S.r.l., Milan, Italy).

The pressure drop across the orifice could be changed in the range between 100 and 200 MPa, while the volumetric flow rate of the suspension was 155 mL/min. In order to prevent excessive heating, after each pass, the suspensions were cooled at 25°C by flowing through a tube-in-tube exchanger, located downstream of the orifice valve.

In this thesis work, *A. platensis* microalgae were subjected to HPH processing at both variable pressure (P = 100 – 200 MPa) and number of passes (n_P = 1 – 3), while *C. vulgaris* cells were pressurised at 150 MPa for a wider range of cycles (n_P = 1 – 10). At the end of each treatment, samples were collected in plastic tubes and immediately centrifuged at 6500 rpm for 5 min (PK121R model, ALC International, Cologno Monzese, IT) due to the almost instantaneous release of intracellular compounds occurring upon the application of HPH treatment, with the supernatants being stored under refrigerated conditions for further characterisations.

Optimal HPH processing conditions for the highest release of intracellular compounds from *A. platensis* and *C. vulgaris* microalgae were identified and used during the combined PEF-HPH treatment of *C.vulgaris* (§ III.3.6.3.2) as well as for the energy analysis carried out in Chapter IX.

III.3.6 Extraction process assisted by PEF

III.3.6.1 Permeabilisation of microalgae cells by single PEF treatment

The effect of PEF treatment intensity and pulse polarity on the extent of induced cellular damages on microalgae was investigated.

To this purpose, a first screening of the main electric conditions (E = 10 – 30 kV/cm; W_T = 20 – 100 kJ/kg$_{SUSP}$) on the release of water soluble intracellular compounds was conducted, with the pulse width set at 5 μs, while the pulse repetition frequency was changed in order to deliver variable amounts of specific energy inputs.

Only in the case of *A. platensis* microalgae, in order to compare the permeabilisation effects induced by both monopolar and bipolar pulses, PEF treatments of constant intensity (E = 20 kV/cm; W_T = 100 kJ/kg$_{SUSP}$) were applied on microalgal biosuspensions at fixed pulse width (5 μs) and different delay time (1, 5, 10 and 20 μs) of square wave bipolar pulses.

The maximum temperature increase at the exit of each module due to Joule effect never exceeded 15 °C.

At the exit of the treatment chamber, treated and untreated (control) algae suspensions were collected in plastic tubes and placed in an ice water bath to be rapidly cooled up to a final temperature of 25 °C before undergoing the aqueous extraction process.

III.3.6.2 Water diffusion process

After processing, untreated and treated (PEF, HSH and HPH) samples were incubated at 25 °C under shaking at 160 rpm in order to grant the diffusion of water soluble compounds towards the external medium. Preliminary tests have shown that a 1h and 3h diffusion processes, respectively for *C. vulgaris* and *A. platensis* cells, were long enough to reach saturation in the kinetic release of target compounds (data not shown). At the end of this phase, samples were subjected to centrifugation at 6500 rpm at 4°C for 10 min in order to remove microalgal pellet from the supernatant, before analysis.

III.3.6.3 "Hurdle approaches" for microalgal biomass valorisation

The principal objective of this thesis work was not only to evaluate the efficacy of a single PEF treatment of given intensity on the release of valuable intracellular compounds from microalgal biomass, but also to assess the feasibility of a combination of treatments (PEF + T, HSH+PEF, PEF+HPH) to improve the extraction yields and selectivity of the compounds of interest, as well as to possibly reduce the energetic consumptions associated to the single applied technologies.

III.3.6.3.1 Combination of PEF with moderate temperature or HSH treatments in the biorefinery of *A. platensis* microalgae

As regarding *A. platensis* biosuspensions, two hurdle approaches were proposed, as follows:
- At first, PEF treatments with monopolar pulses at the optimal conditions defined during the experiments described in § III.3.6.1 was coupled with the application of a mild heating effect at the inlet of each PEF treatment chamber module set at 25, 35 and 45 °C;
- In a second set of experiments, the effect of combined HSH – PEF treatment applied in series on the recovery of proteins (e.g. C-phycocyanin) and carbohydrates from the intracellular medium was carried out. To this purpose, freshly prepared microalgal suspensions were subjected to a HSH pre-treatment, carried out at fixed rotor speed (20000 rpm) and for low processing times (t = 1 min). Subsequently, microalgae underwent a PEF treatment at fixed electric conditions (E = 20 kV/cm; W_T = 100 kJ/kg$_{SUSP.}$). The effects induced by the combined treatment were also compared to those achieved by single PEF and HSH treatments. A schematisation of the combined process has been reported in Figure III.17.
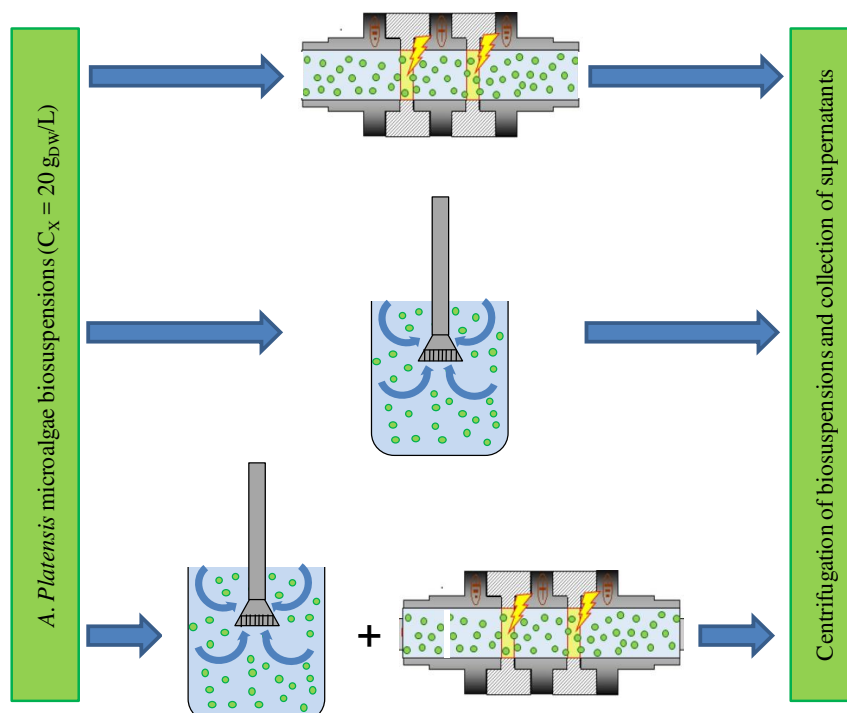
Figure III.17 *Schematisation of the combined HSH – PEF treatment proposed in this work for A. platensis microalgae suspensions.*

Untreated or treated (PEF, PEF + T, HSH, HSH+PEF) microalgal suspensions were collected in plastic tubes and subjected to water diffusion process as described in § III. 3.6.2, while the analyses on supernatants obtained after centrifugation followed the procedures reported in § III.3.7.2 – 5.

III.3.6.3.2 Combination of PEF with HPH treatments in the biorefinery of *C. vulgaris* microalgae

The whole exploitation of *C. vulgaris* microalgae for an efficient recovery of valuable intracellular compounds, namely proteins, carbohydrates and lipids, was achieved in a more complex "cascade approach", as schematised in Figure III. 18.
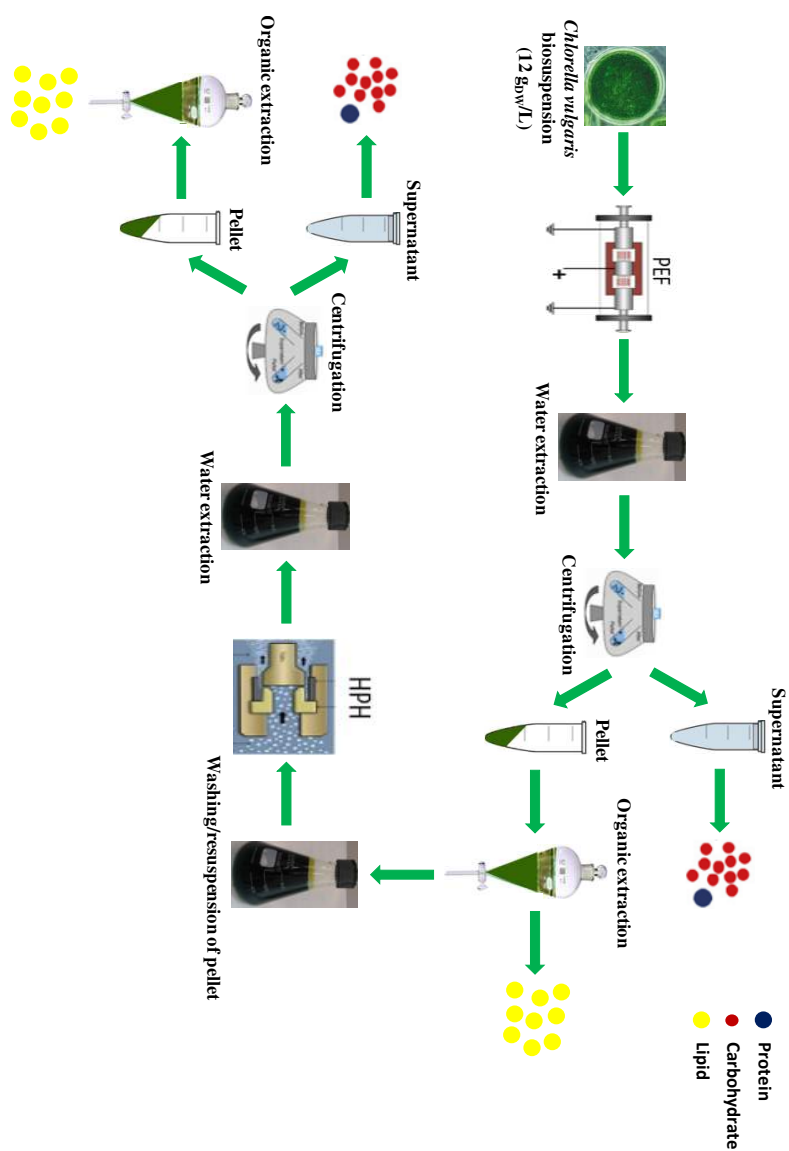
Figure III.18 *Schematic representation of the "cascade biorefinery approach" of C. vulgaris microalgae used in this study.*

More specifically, after the electrical treatment, carried out at 20 kV/cm and at 100 kJ/kg, microalgae suspensions were subjected to water diffusion (1h, 160 rpm) and subsequently centrifuged to recover on one side the aqueous phase, representing the first output of the whole proposed "biorefinery" scheme, and on the other side the pellet. The latter then was resuspended in water to be subjected to an organic extraction (t = 3 h, 160 rpm) as illustrated

in § III.3.8.6, aiming at obtaining a lipid rich phase as the second output of the process.

The remaining pellet after organic extraction phase was washed three times in order to eliminate solvent traces in biomass and reconstituted to its initial volume with water before being fully permeabilised via HPH treatment (P = 150 MPa; n_P = 5). Extraction and phases separation after HPH were executed as for PEF treated samples, with two more outputs in terms of acqueous and organic phases, respectively. Results from the biorefinery scheme (PEF + HPH) were also compared to single PEF and HPH treatments, as reported in Chapter VIII.

III.3.7 Analytical determinations on A. platensis microalgal biomasses and their extracts

III.3.7.1 Optical Microscopy analysis

A first qualitative determination of the structural changes induced by the investigated disruption technologies on microalgal cells was performed by using an Eclipse TE2000-S microscope (Nikon Instrument, Amsterdam, Netherland, Figure III.19). Images of treated and untreated sample were captured in triplicate at three different magnitudes (10x, 20x and 40x).
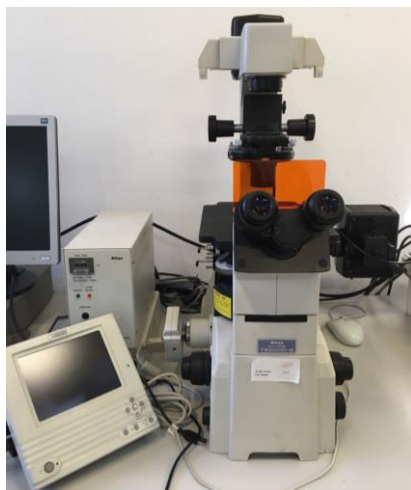
Figure III.19 *Optical microscope (Nikon Eclipse TE2000-S).*

III.3.7.2 Water soluble proteins analysis

The water soluble proteins concentration in the supernatants from microalgae was evaluated using the Lowry method (Lowry *et al*., 1951), with some modifications. The Folin-Ciocalteau reactive (Folin & Ciocalteau,

1927), purchased from Sigma Aldrich (Milan, Italy), was initially diluted in two volumes of ultra-pure water (1:2, v/v); then 0.5 mL of the diluted reactive were added to 1 mL of supernatant, previously mixed with 5 mL of the reactive "C" [50 volumes of reactive "A" [(2% (w/v) Na_2CO_3 + 0,1 N NaOH) + 1 volume of reactive "B" (1/2 volume of 0.5% (w/v) $CuSO_4$ •$5H_2O$ + 1/2 volume of 1% $KNaC_4H_4O_6$ ·$4H_2O$)] (Sigma Aldrich, Milan, Italy). Absorbance was measured at 750 nm against a blank (5 mL reactive "C" + 1 mL deionized water + 0.5 mL Folin-Ciocalteau reactants) 35 min after the start of the chemical reaction by using a V-650 Spectrophotometer (Jasco Inc. Easton, MD, USA).

Bovine serum albumin (BSA) (A7030, Sigma Aldrich, Milan, Italy) was used as standard and the results were expressed as mg equivalent of BSA per g of dry biomass.

III.3.7.3 C- phycocyanin and purity ratio of extracts

Quantification of C-phycocyanin content and purity ratio of extracts was performed according to the method of Bennet & Bogorad (1973), which is based on the measurements of the extracts absorbance (A) at two fixed wavelenghts (λ_1 = 615 nm; λ_2 = 652 nm). The C-phycocyanin concentration of extracts, expressed as mg/mL of supernatant, was evaluated as follows:

$$C - phy = \frac{(A_{615\ nm} - 0.474\ A_{652})}{5.34} \qquad (III.7)$$

Instead, the purity ratio of C-phycocyanin in the supernatants was spectrophotometrically evaluated, as reported in the following equation (Abelde et al., 1998):

$$Purity\ ratio = \frac{A_{615\ nm}}{A_{280\ nm}} \qquad (III.8)$$

where A_{615nm} and A_{280nm} indicate, respectively, the maximum C-phycocyanin absorption peak and the total concentration of proteins in the solution.

III.3.7.4 Carbohydrates Analysis

The total carbohydrates concentrations of the supernatants were analyzed according to the method of DuBois et al. (1957). 0.2 mL of 5% (w/w) phenol and 1 mL of concentrated sulfuric acid (Sigma Aldrich, St. Louis, USA) was added to 0.2 mL of diluted supernatant (Dilution Factor = 5). Samples were incubated at 35 °C for 30 min before reading the absorbance at 490 nm against a blank of 0.2 mL 5% (w/w) phenol, 1 mL concentrated sulfuric acid

and 0.2 mL of deionized water. D-Glucose (G8270, Sigma-Aldrich, Milan, Italy) was used as a standard and the results were expressed as equivalent mg of D-glucose per g of dry biomass.

III.3.7.5 SDS - PAGE analysis of extracts

A qualitative measurement of the protein profile of supernatants from single PEF, single HSH and HSH+PEF treated microalgal biosuspensions was achieved by means of SDS - PAGE analysis. Separation of proteins was performed in a TV200Y twin-plate mini-gel unit equipped with Apelex power supply unit (APELEX-Massy, France). The separating and stacking gels contained 15% and 6% of polyacrylamide, respectively. All supernatants and standards (25 μL) were diluted with the same amount of loading buffer (0.125 M Tris-HCl, 2% SDS, 10% glycerol, 0.02% bromophenol blue and 5% 2-mercaptoethanol, pH 6.8). The mixture was boiled for 2 minutes, centrifuged and loaded into the prepared gel, with the experiments run at constant current intensity (25 A). Gels were then stained with staining solution (0.2% Coomassie Brilliant Blue R 250, 10% acetic acid, 25% isopropanol) for overnight. Later de-staining solution (30% methanol, 10% acetic acid) was used until the background became clear.

III.3.8 Analytical determinations on C. vulgaris microalgal biomasses and their extracts

III.3.8.1 Electrical conductivity measurement

Changing of the electrical conductivity (σ) of untreated and treated (PEF, HPH) *C. vulgaris* algae suspensions was periodically monitored over time for up to 24 h by maintaining the samples in a water bath set at a constant temperature of 25 °C.
The collected data were elaborated also to evaluate the cell disintegration index (Z_P), which has been successfully used as a reliable macroscopic indicator of the degree of cell membrane permeabilization induced by PEF (Donsì *et al.*, 2010):

$$Z_P = \frac{\sigma_{PEF,t} - \sigma_0}{\sigma_{MAX} - \sigma_0} \qquad (III.9)$$

where $\sigma_{PEF,t}$ is the electrical conductivity of PEF treated biosuspensions measured at time t, σ_0 is the conductivity of untreated algae suspension at time 0, and σ_{MAX} is the conductivity of biosuspension with completely disrupted algae cells (HPH treatment: P = 150 MPa, n_P = 5).

III.3.8.2 Particle size distribution (PSD) analysis

PSD of untreated and treated (PEF or HPH) C. vulgaris suspensions were analyzed by laser diffraction at 25 °C, using a MasterSizer 2000 particle size analyzer (Malvern, United Kingdom). Using the Fraunhofer approximation, which does not require the knowledge of the optical properties of the sample, the size distribution of the algal suspension was determined, from which the mean particle size expressed as volume moment mean diameter ($D_{4,3}$) was evaluated for each processing condition. The parameters used in the determination of the PSD were the properties of water at 25 °C (refraction index = 1.33), which was used as dispersant medium.

III.3.8.3 Dry Matter (DM) content of supernatants analysis

Approximately 40 mL of the supernatants collected from the centrifugation of untreated and treated (PEF, HPH, PEF + HPH) algae suspension were placed in aluminum cups and dried in an oven (Heraeus, Germany) at 80 °C until constant mass was achieved. DM was gravimetrically determined by weighing the samples before and after drying on an analytical balance (Gibertini, Italy). The dry mass content was expressed as g of dry matter/kg of supernatant ($g_{DW}/kg_{SUP.}$).

III.3.8.4 Scanning Electron Microscopy (SEM) analysis

The morphological features and cellular details of C. vulgaris algae cells were analyzed by using a Scanning Electron Microscopy (SEM). Pellets derived from the centrifugation of untreated and treated (PEF, HPH, PEF+HPH) *C. vulgaris* suspensions were prepared as described by Kunrunmi *et al.* (2017) with some modifications. At first, samples were fixed by immersion in a 2 % (v/v) glutaraldehyde phosphate buffer solution. The buffer was then removed and the pellets were osmotically dehydrated with ethanol solutions of increasing concentration (25%, 50%, 75%, and 100% (v/v)). Afterwards, ethanol was removed from the pellet with supercritical CO_2 in a Quorum K850 critical point dryer (Quorum Technologies Ltd, London, UK) and the latter was then metallized by means of the Agar Auto Sputter Coater 103A (Agar Scientific Ltd, Stansted, UK), before being analysed in a high-resolution ZEISS HD15 Scanning Electron Microscope (Zeiss, Oberkochen, Germany).

III.3.8.5 Water soluble proteins and carbohydrates analysis

The analytical methods for the evaluation of water soluble proteins and carbohydrates of extracts from untreated and treated (PEF, HPH, PEF + HPH) suspensions were reported in § III 3.7.2 and § III 3.7.4.

III.3.8.6 Lipids analysis

Quantification of lipids was performed by following the method illustrated in the work of Zbinden *et al.* (2013), in which ethyl acetate was used as the main solvent for lipids extraction, in replacement of more toxic and high environmental impact solvents such as hexane and isopropanol (Capello *et al.*, 2007), which are commonly used for this purpose. The initial miscible solution of all solvents was obtained by mixing 10 mL of ethyl acetate and 5 mL of methanol, to which 4 mL of microalgal suspension were previously added. The extraction system was then put under gentle agitation at room temperature for 3 h. Separation of phases was carried out by adding 5 mL of ethyl acetate and 5 mL of water to the solution, right before being centrifuged (2,465 g for 10 min). After this stage, a two phase system was formed in which the bottom layer represented the water–methanol rich system and the top layer constituted the ethyl acetate, lipid-rich layer. The latter was then evaporated under a N_2 gas stream, by using a R-200/205 Rotavapor.

Lipid content was gravimetrically determined from the difference in weight of samples before and after drying and it was expressed as $g_{LIP.}/g_{DW}$ *C. vulgaris* biomass.

III.3.8.7 Spectra measurements of extracts

UV – Vis spectra of all acqueous and organic supernatants obtained respectively after water and lipid extraction were evaluated spectrophotometrically and their shape could be plotted as a function of the investigated range of wavelengths ($\lambda = 200 - 800$ nm). Both acqueous and organic supernatants were diluted (Dilution Factor = 10) right prior being analysed. Characteristics peaks of water soluble proteins, carotenoids and chlorophyll were determined at specific wavelengths from spectra measurements ($\lambda = 290, 435$ and 675 nm for water extracts; $\lambda = 260, 430$ and 662 nm for organic extracts).

III.4 Statistical analysis

All experiments and analysis of collected samples from both food wastes/by-products and microalgae were performed in triplicate from which the mean values and standard deviations (SD) of experimental data were calculated. Statistically significant differences ($p \leq 0.05$) among the averages were evaluated using one-way analysis of variance (ANOVA), and the Tukey's test, using the SPSS 20 (SPSS Inc., Chicago, USA) statistical package.

Section I

Innovative biorefinery of industrial food wastes/by-products

Chapter IV - *Recovery of carotenoids from tomato processing wastes by pulsed electric fields*

Chapter V - *Optimisation of lab-scale PEF-assisted extraction processes from artichoke wastes*

Chapter VI - *Pilot-scale biorefinery process of artichoke stems based on PEF and NF technologies*

Chapter IV
Recovery of carotenoids from tomato processing wastes by pulsed electric fields

Abstract - In this chapter, the efficiency of PEF-assisted extraction of carotenoid compounds from tomato peels achieved either in laboratory after steam blanching or after pressurized steam peeling at industrial level, was investigated. PEF treatments of variable intensity (E = 0.5 - 5 kV/cm; W_T = 0.5 - 20 kJ/kg) were carried out on tomato peels and the cell disintegration index (Z_P) was used to identify the optimal electric conditions for the subsequent PEF-assisted extraction experiments.

The latter were performed at different temperature (20-50 °C) by using two different solvents (acetone, ethyl lactate) in order to study also their efficacy in terms of carotenoids solubilisation. Extracts from untreated and PEF treated samples were quantified in terms of total carotenoids content and antioxidant power. Effect of PEF on the extractability of lycopene was also detected via HPLC analysis.

Results highlithed the capability of PEF to permeabilize cell membranes of tomato peels tissues. Compared to the untreated sample, the extracts from PEF pre-treated peels obtained after steam blanching of tomato fruits had a significantly higher total carotenoid content (+ 45%) and antioxidant activity (+53%).

The increase of extraction temperature did not significantly enhanced further improvements in the extraction yield of total carotenoids in the extracts, which, in turn, showed a slightly higher antioxidant power, potentially due to the co-extraction of other antioxidant intracellular compounds.

As regarding the valorization of industrial tomato wastes, PEF granted a significant increase in the recovery of lycopene (+38% on average over untreated samples), with acetone showing higher carotenoids solubilisation capabilities with respect to ethyl lactate.

Results obtained in this work have demonstrated that the PEF treatment of tomato by-products applied before the extraction process of valuable compounds could add new value to the tomato processing chain, improving economic performances and decreasing waste problems.

IV.1 Introduction

Tomato (*Solanum Lycopersicon*) is one of the most consumed vegetables all over the world, being a low caloric source of many bioactive compounds with high antioxidant activity such as carotenoids (lycopene, β-carotene, lutein), polyphenols and especially vitamins C and E, able to reduce the risk of cancer and cardiovascular diseases (Arora *et al.*, 2014; Kotíková *et al.*, 2011; Lenucci *et al.*, 2012; Shi & Le Maguer, 2000).

Italy is one of the world leading countries in the production of tomatoes, with an annual outcome of about 7 million tons in 2016, followed by Spain and Portugal in Europe (FAOSTAT, 2016). About 80% of produced tomatoes intended for human consumption is industrially processed into a great variety of final products such as purees, pastes, ketchup, sauces, salsas and soups (Shi & Le Maguer, 2000).

The core of the whole tomatoes transformation line is represented by the thermo-physical peeling phase, being critical for maximizing the efficiency of the processing equipment and ensuring uniform treatment of the product (Fellows, 2000). This stage is essentially conducted by subjecting tomato fruits to a rapid steam blanching and a subsequent vacuum cooling, necessary to facilitate the mechanical and complete removal of peels, which is carried out on pinch rollers or abrasive surfaces (Rock *et al.*, 2012).

Mass loss of tomatoes due to peels detachment from the fruits after thermo-physical peeling usually represents between 2% and 5% by weight of fresh product (Ruiz Celma *et al.*, 2009).

The generation of such waste is almost always associated with strong environmental and economical impacts due, respectively, to emission of greenhouse gases and dramatic disposal costs (Mohan *et al.*, 2017).

The tomato peels currently find low-added value uses as animal feed and fertilizers (Knoblich *et al.*, 2005; Strati & Oreopoulou, 2014), or are directly sent to landfill (Rossini *et al.*, 2013).

However, the relevant presence of phytochemicals in tomato by-products, such as carotenoids, polyphenols and pectin, strongly offers the possibility to re-integrate these biomasses at industrial level as a cheap source of high added-value compounds, thus contributing to create new commercial opportunities (Galanakis, 2015; Grassino *et al.*, 2016).

In particolar, the most abundant bioactive compound stored in the tissues of tomato peels is lycopene, belonging to the class of carotenoids, whose content is about five times greater than that observed in tomato pulp (Luengo *et al.*, 2014b; Poojary & Passamonti, 2014).

This molecule has been regarded as a potential agent in protecting against a series of illnesses (cardiovascular, cancers, cataract, macular degeneration), as well as in enhancing the immune system (Strati & Oreopoulou, 2011a; 2014). Moreover, the bright red coloration associated to lycopene allows its usage as a dyeing agent for several food products (Cadoni *et al.*, 2000; Ishida & Chapman, 2009), but also as a natural ingredient for the formulation of functional foods (Strati & Oreopoulou, 2014).

Solvent extraction is a well established method in the food industry for the recovery of valuable compounds from food wastes/by-products (Strati & Oreopoulou, 2011a; 2011b), due to its ease of operability and scalability. In general, recovery of fat soluble molecules (e.g. carotenoids) from tomato peels occurs by a simple diffusion phase in a non-polar organic solvent (hexane, petroleum ether, methylene chloride) which may result in high extraction efficiencies only when optimal processing conditions are applied (solvent-to-solid ratio, particle size, temperature, extraction duration). Despite this, most of the organic solvents involved in the extraction process are generally toxic and harmful, having adverse effects on human health due to their uncomplete removal (Ishida & Chapman, 2009), causing potential implication on extracts quality such as loss of functionality of the desired compounds when long maceration times are required (Luengo *et al.*, 2014b).

In order to overcome these problems, especially considering the safety issue related to the final products to be used as food supplements, the usage of less environmental impacting solvents like acetone or ethyl lactate, whose usage in food products is allowed by the U.S. Food and Drug Administration, being also completely biodegradable into CO_2 and water, has been suggested as an efficient alternative to the more hazardous organic solvents for the solubilisation of lycopene from tomato peels (Ishida & Chapman, 2009; Strati & Oreopoulou, 2011b).

To this purpose, Ishida & Chapman (2009) and Strati & Oreopoulou (2011b) found that the usage of ethyl lactate significantly increased the amount of extracted carotenoids from tomato peels with respect to ethyl acetate, a solvent which is primarily involved for food purposes, whose use is also restricted by a patent (Zelkha *et al.*, 1998). However, in these works the authors performed the solvent extraction step on dried/grinded tomato peels in order to achieve a fast and efficient recovery of lycopene from such biomasses, but with negative impacts on the economics of the process due to the high-energy intensive up-stream pre-processing of the biomass.

Pulsed electric fields (PEF) treatment is a cell disintegration technique that can be applied to wet biomass, thus avoiding highly energy consuming pretreatments like drying or grinding. To this purpose, as reported in Table I.11, PEF technology has been successfully applied as a mild and low energy cell disruption technique capable of leading to a series of remarkable improvements in food processing applications, including the increase in the extraction yields of many valuable compounds from foods and food

wastes/by-products, as well as a drastic reduction in the solvent consumption and extraction time (Barba *et al.*, 2015a; Bobinaite *et al.*, 2015; Kumari *et al.*, 2018; Luengo *et al.,* 2014b; Pataro *et al.*, 2017a; Putnik *et al.*, 2017).

However, to date, only the study of Luengo *et al.* (2014b) has been addressed to the valorisation of tomato wastes by PEF-assisted extraction of valuable compounds, where also a comparison among the extracting solvents used, in terms of their efficiencies in capturing interest compounds like carotenoids, has been carried out. Moreover, the authors used only tomato peels achieved after hand peeling of fresh tomatoes. In addition, no studies have been published on the extractability of carotenoids from tomato processed by-products (peels) after steam blanching (SB) of whole tomato fruits and, especially, no study has investigated the possibility of recovering carotenoids from industrial tomato wastes by applying PEF technology.

Therefore, in this work, the extractability of carotenoids compounds from both tomato peels achieved either after steam blanching and hand peeling of whole tomato or derived from industrial production of peeled tomatoes, was investigated.

As described in detail in Chapter III, tomato peels were first subjected to a systematic study of the influence of the main PEF parameters, namely electric field strength (E = 0.5 – 5 kV/cm) and specific energy input (W_T = 0.5 – 20 kJ/kg) on the peel tissue permeabilisation degree.

Then the effect of PEF pre-treatment, alone or in combination with different extraction temperatures (T = 20 – 50 °C), on the recovery of carotenoids from tomato peels, as well as on the antioxidant power of the extracts, was investigated.

Finally, the profile of carotenoids contained in extracts from untreated and PEF treated samples of industrial tomato peels was evaluated via HPLC analyses as a function of the extracting solvent (acetone, ethyl lactate).

IV.2 "Short" Materials and Methods

IV.2.1 Raw materials and sampling

Results reported in § IV.3.1 – 3 refer to the PEF processing of square shaped tomato peels (A = 1x1 cm^2), which were obtained from the steam assisted peeling of tomatoes of "Datterino" variety. Instead, as reported in § IV.3.4, PEF treatments were also performed on industrial tomato peels.

The preparation of raw materials and their sampling was accurately described in the first part of § III.2.1.

IV.2.2 PEF treatments of tomato peels

Both impedance analyses and PEF-assisted extraction tests of tomato peels were carried out in a batch cylindrical treatment chamber (§ III.2.4, Figure

III.6) connected to a pulse generator, as schematized in § III.2.3 (Figure III.5). In particular, impedance analyses (§ III.2.5.1) aimed at evaluating the optimal electric conditions (E; W_T) allowing to achieve the highest extent of cell membrane permeabilization with the minimum energy consumptions, to be subsequently applied for conducting PEF-assisted extraction experiments. To this purpose, the cell disintegration index (Z_P) of electrically treated samples was calculated according to Eq. III.1, as a function of both field strength (0.5 – 5 kV/cm) and specific energy input (0.5 – 20 kJ/kg).

Subsequently, PEF-assisted extraction experiments were performed according to the protocol illustrated in § III.2.5.2 (Figure III.8).

Briefly, after the application of electrical treatments of variable intensity (E = 0.5 – 5 kV/cm) and fixed energy input (5 kJ/kg) as clarified in § IV.3.1, tomato peels were subjected to a 6h extraction step in acetone or ethyl lactate, at variable diffusion temperature (20 – 50 °C).

IV.2.3 Analytical determinations

Clarified extracts from untreated and PEF treated tomato peels were quali-quantitatively analysed in terms of total carotenoid compounds (TC), antioxidant power (FRAP) and lycopene content (HPLC runs), as previously described in § III.2.6.1 and § III.2.6.3 – 4.

IV.3 Results and discussion

IV.3.1 Effect of PEF on the cell permeabilisation index of tomato peels achieved after hand peeling of steam blanched tomato fruits

The electroporation of biological cells, as well as the subsequent mass transfer processes, takes into account a series of parameters, like the interaction between the electric field applied and the material properties, which are also spatially dependent (Bobinaite *et al.*, 2015).

An easy method for the evaluation of the extent of cellular damages (Jemai & Vorobiev, 2002) is represented by the assessment of the cell disintegration index (Z_P), carried out via impedance measurements of untreated and PEF treated samples. This parameter has been successfully used as a reliable macroscopic indicator of the cell membrane disintegration of many fruits and vegetables (Angersbach *et al.*, 1999; De Vito *et al.*, 2008; Lebovka *et al.*, 2002). Figure IV.1 reports the permeabilisation index Z_P as a function of the specific energy input (W_T) delivered during the PEF treatment of tomato skins for different values of the applied electric field strength (E).
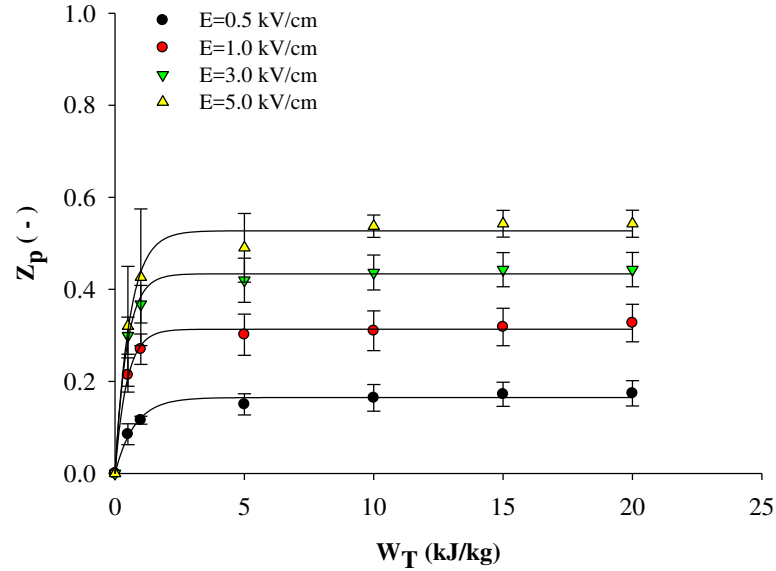
Figure IV.1 *Cell disintegration index (Z_P) of tomato peels as a function of electric field strength (E) and total specific energy input (W_T). Standard deviations were used as error bars (p≤0.05). Experimental data were fitted by exponential saturation models ($R^2 > 0.95$).*

For all the investigated values of E, the extent of cellular damages induced by the electrical treatment increased with the applied energy input up to reaching a constant value for very low energetic consumptions ($W_T = 5$ kJ/kg). The asymptotic behavior of Z_P curves as a function of the delivered number of pulses, or the specific energy input, is totally in agreement with the results shown in the work of Luengo *et al.* (2013).

These authors stated that, for each value of the electric field applied, the cell permeabilisation index increased as the specific energy input is increased until a threshold saturation value is reached.

In our case, further increases in the specific energy in the range between 5 kJ/kg and 20 kJ/kg did not lead to higher extent of tissue permeabilisation.

However, the electroporation phenomenon taking place in tissues of tomato skins is strongly affected also by the external electric field strength, since for all the investigated values of the specific energy input, the higher the electric field strength, the greater the Z_P value. In particular, for a fixed value of W_T, the cell permeabilisation index significantly increased (p < 0.05) as the treatment intensity was changed from 0.5 kV/cm ($Z_P = 0.15$) to 5 kV/cm ($Z_P = 0.49$). Results reported in the work of Luengo *et al.* (2014b) showed that the application of an electric field strength of increasing intensity from 3 kV to 7 kV/cm led to a gradual growth of the extent of cellular damages of tomato peel tissues, up to a maximum value of 0.3. The greater value of Z_P observed in our work may be ascribed to the different pre-processing of tomatoes, which in this case, were subjected to a steam blanching (T = 70°C)

step prior to their peeling, with the aim of simulating the thermophysical process typically used at industrial scale for tomato peeling.

It is likely that this pre-processing may have partially weaken the cellular structures of tomato skin tissues, making them more susceptible to the effect of the subsequent PEF treatment.

Similar behavior of the Z_P parameter have been previously observed in several literature works on different plant tissues (Brianceau *et al.* 2015; El Kantar *et al.*, 2017; Luengo *et al.*, 2014b; Segovia *et al.*, 2015), where the application of a PEF treatment of variable intensity favoured the formation of pores at cellular level which subsequently resulted in a better recovery of intracellular compounds during diffusion processes.

Further investigation of PEF pre-treatment on the extractability of valuable pigments (e.g. carotenoids) from tomato peels were carried out at 0.5, 1, 3 and 5 kV/cm with a constant energy input of 5 kJ/kg, which was set as the optimal condition to achieve the highest permeabilisation degree (Z_P).

IV.3.2 Effect of PEF on the extractability of carotenoids and on the antioxidant power of extracts from tomato peels obtained after hand peeling of steam blanched tomato fruits

Figure IV.2 shows the total carotenoids (TC) content of extracts obtained from untreated and PEF treated (E = 1 - 5 kV/cm; W_T = 5 kJ/kg) tomato peel samples.
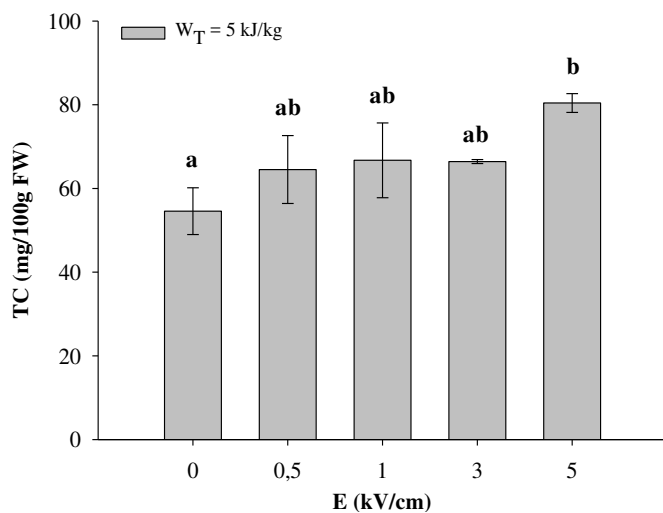
Figure IV.2 *Total carotenoids content (TC) of extracts obtained after a solvent (acetone) extraction at 20°C from untreated (0 kV/cm) and PEF-treated (W_T=5 kJ/kg) tomato peels, after 6 h of diffusion. Different letters above the bars indicate significant differences among the mean values (p≤0.05).*

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The concentration of carotenoids of extracts from control samples was equal to 54.6 mg/100 g FW tomato peels. In PEF treated samples, instead, the yield of carotenoids was higher than the control samples, due to the induced permeabilisation effect, accompanied by a better accessibility of the solvent through the chromoplasts of tomato peels cells, where carotenoids are stored (Pataro *et al*., 2015; Singh *et al*., 2015).

In particular, as shown in Figure IV.2, when applying an external field strength of 0.5 kV/cm, the mean carotenoids concentration in the extracts increased up to 64.5 mg/100 g FW, which in turn was not statistically different ($p > 0.05$) from that observed for samples subjected to the solvent maceration process alone. However, despite further increase in field strength up to 3 kV/cm induced a greater Z_P value (Figure IV.1), no significant increases in the extraction yield of carotenoids could be detected ($p > 0.05$).

A similar behavior has precedently been observed in the work of Brianceau *et al*. (2015), where the application of PEF treatment of variable field intensity to fermented grape pomace, respectively 1.2 kV/cm and 3 kV/cm, did not lead to gradual increases over the control samples in terms of polyphenols extraction yields, which therefore remained constant, despite the observed increase in permeabilization index ($Z_P = 0.36$ at 1.2 kV/cm and $Z_P = 1$ at 3 kV/cm).

Instead, the electroporation effect induced by the PEF treatment at the highest investigated intensity (E = 5 kV/cm; W_T = 5 kJ/kg) granted a significant ($p< 0.05$) enhancement in the recovery of carotenoids from tomato peels during the extraction phase (+ 44.5 % over controls), as also testified by the highest value of cell permeabilisation degree achieved (Figure IV.1).

It has already been proven that PEF-induced permeabilization of cell membranes is effective in improving pigments extractability from plant tissues, such as anthocyanins from grape pomace, blueberry press cake, purple-fleshed potato, red prickly pear peels and red cabbage (Barba *et al*., 2015; Bobinaite *et al*., 2015; Corrales *et al*., 2008; Gachovska *et al*., 2010; Koubaa *et al*. 2016; Pataro *et al*., 2017b; Puertolas *et al*., 2013), as well as betanin from red beets (Chalermchat *et al*., 2004; López *et al*., 2009).

Our results are in agreement to those observed in the work of Luengo *et al*. (2014b), who found that the application of a PEF treatment at 5 kV/cm for 90 μs led to a significant increase of about 50% in the carotenoids extraction yield (CEY) above the control samples.

Figure IV.3 reports the average values of the antioxidant power (FRAP) of acetone extracts from untreated and PEF treated samples of tomato peels.

It is worth noting that the observed data trend is very similar to that detected in Figure IV.2.

In fact, starting from an initial antioxidant power of the untreated samples equal to 2.54 mmol TE/ 100 g FW, the application of a PEF treatment allowed to achieve, respectively at 0.5 kV/cm, 1 kV/cm and 3 kV/cm,

increases in FRAP values of the extracts of about 30%, 33% and 32% with respect to the control samples. However, no statistical differences could be observed among the samples in this electric field intensity range ($p > 0.05$). Moreover, as previously observed for total carotenoids content, the highest investigated treatment intensity (E = 5 kV/cm; W_T = 5 kJ/kg) leads to the greatest and significant increase ($p < 0.05$) in the antioxidant power as compared to the controls (+ 53%).

The correlation existing between the total carotenoids contents in tomato peel extracts and their antioxidant powers could be described by means of a linear function (R^2 = 0.983, data not shown). This confirmed that carotenoids are the major antioxidant compounds contained in tomato peels.
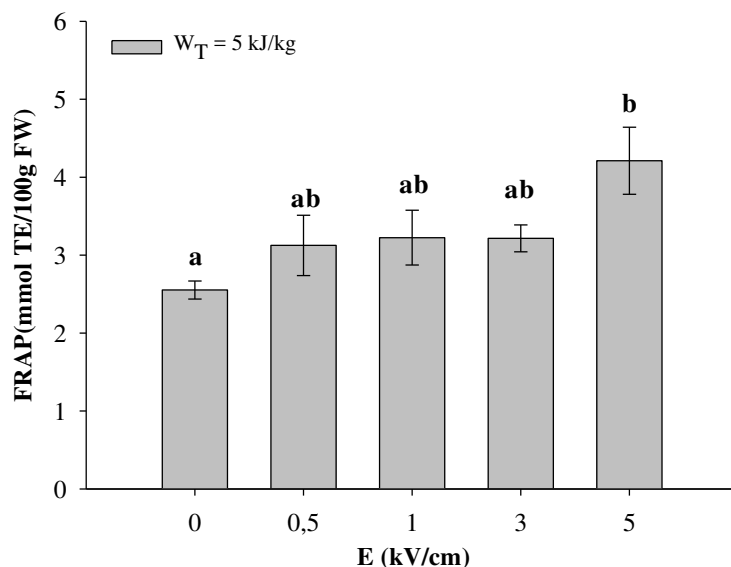
Figure IV.3 *Ferric Reducing Antioxidant Power (FRAP) of extracts obtained after a solvent (acetone) extraction at 20°C of untreated (0 kV/cm) and PEF-treated (W_T=5 kJ/kg) tomato peels, after 6 h of diffusion. Different letters above the bars indicate significant differences among the mean values ($p\leq0.05$).*

In the current literature, it has been proven that the application of a PEF treatment to crushed blueberries significantly increased the antioxidant power of blueberry press-cake extracts by 80% at 5 kV/cm and 10 kJ/kg (Bobinaite *et al.*, 2015).

Moreover, the effect of a PEF treatment on the extractability of phenolics with high antioxidant power from fresh orange peel was investigated by Luengo *et al.* (2013). The authors found that the application of high electric field strengths (E = 5 - 7 kV/cm) led to drastic increases in the antioxidant power of extracts obtained after the pressing of PEF treated orange peel,

respectively of 150 % and 190 %, which was totally correlable to the observed increases in the extraction yields of polyphenolic compounds.

From the overall analysis of Figures IV.2 - 3 it can be concluded that the most intense PEF condition (E = 5 kV/cm; W_T = 5 kJ/kg) allowed to reach maximum percentage increases in the extractability of carotenoids and antioxidant power as compared to the solid liquid extraction (SLE) process.

Therefore, this treatment condition was chosen as optimal and furtherly investigated both for tests of PEF-mild temperature (20°C, 35°C and 50°C) assisted pigments extraction of tomato peels obtained after steam blanching of tomato fruits, as well as for the study of the effect of the type of solvent (acetone, ethyl lactate) on the recovery of lycopene from industrial tomato peels.

IV.3.3. Effect of PEF and diffusion temperature on TC and FRAP of extracts from tomato peels achieved after hand peeling of steam blanched tomato fruits

In this work the effect of extraction temperature (20°C, 35°C and 50°C) on the total carotenoids yield obtained from both untreated and PEF treated (E = 5 kV/cm; W_T = 5 kJ/kg) tomato peel samples has also been investigated and the obtained results are shown in Figure IV.4.
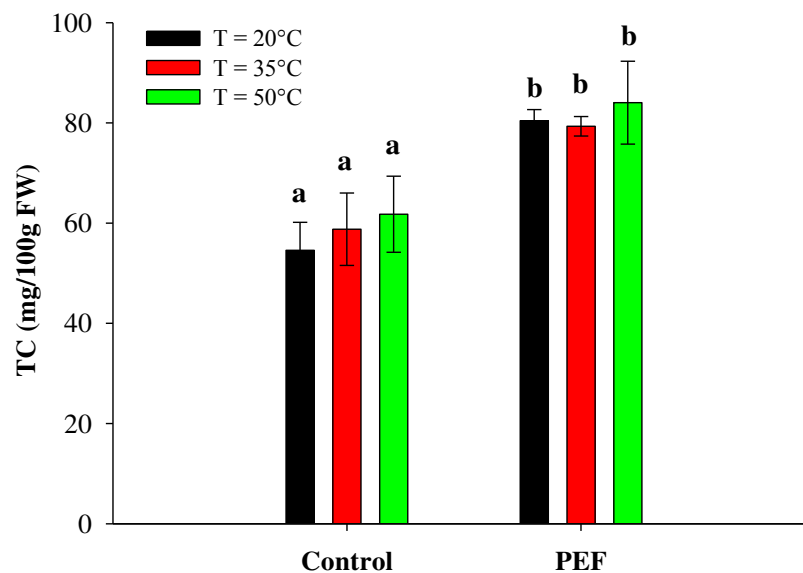
Figure IV.4 *Total carotenoids content (TC) of acetone extracts from untreated (0 kV/cm) and PEF-treated (E = 5 kV/cm; W_T=5 kJ/kg) tomato peels as a function of the diffusion temperature, after 6 h of diffusion. Different letters above the bars indicate significant differences among the mean values (p≤0.05).*

Total carotenoids content of control samples subjected to a 20°C solvent extraction was equal to 54.6 mg/100 g FW. The increase in the extraction temperature did not significantly affect the TC of extracts ($p > 0.05$) even if a slight enhancement in the extraction yields was observed at 35°C and 50°C, likely due to the increase of the diffusivity coefficient of solvent and intracellular compounds through the membranes of tissues of tomato peels. The application of the PEF treatment led to a marked and significant increase in the extraction of carotenoids from tomato peels with respect to the control samples ($p < 0.05$), with percentage increases of 35.1% (T = 35°C) and 36.1% (T = 50°C). However, no statistical differences could be detected among TC yields from PEF treated samples when the temperature was raised up to 50°C ($p > 0.05$). This behavior may be explained by considering the duration of the extraction phase (6 h) which was probably long enough to achieve the saturation of the solvent in terms of TC, thus masking the effect of diffusion temperature.
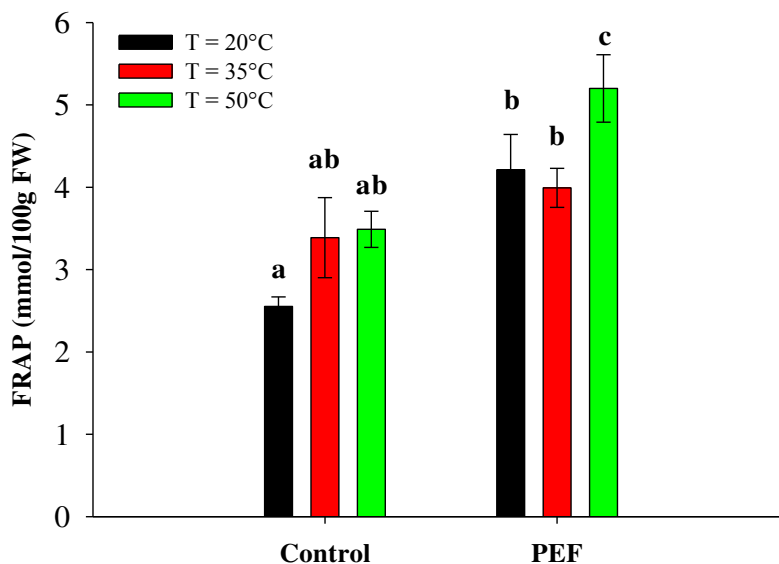
Figure IV.5 *Antioxidant power (FRAP) of acetone extracts from untreated (0 kV/cm) and PEF-treated (E = 5 kV/cm; W$_T$=5 kJ/kg) tomato peels as a function of the diffusion temperature, after 6 h of diffusion. Different letters above the bars indicate significant differences among the mean values (p≤0.05).*

The combined effect of PEF treatment and extraction temperature has been successfully investigated in previous studies (Brianceau *et al*. 2015; Puertolas *et al*., 2011; Segovia *et al*., 2015). For instance, Puertolas *et al*. (2011) studied the effect of both PEF treatment and maceration temperature on the anthocyanin composition of red wines obtained from the pressing of

Cabernet Sauvignon grapes. In this work, the authors showed that the increase in the maceration temperature from 4°C to 20°C, along with the application of a PEF treatment at 5 kV/cm and at 3.67 kJ/kg allowed to obtain a significant increase in the extraction of anthocyanins, leading to higher quality wines with respect to those obtained from the maceration of untreated grapes. Figure IV. 5 shows the FRAP of extracts from PEF treated (E = 5 kV/cm; W_T = 5 kJ/kg) and untreated tomato peels at variable extraction temperature (T = 20°C - 50°C). Results shows that, independently on the extraction temperature, the application of the PEF treatment led to a significant increase in the antioxidant power of the extracts in comparison with the untreated samples, with percentage increases of 18.2% and 45.7 %, respectively at 35°C and 50°C. The use of higher temperatures significantly improved the antioxidant power of the PEF extracts, probably due to both an enhancing in diffusion coefficient and the extraction of polyphenols, which are partially soluble in polar solvents such as acetone (Sharmin *et al.*, 2016). Therefore, in future works, the effect of extraction temperature should be deeply investigated, in order to improve the recovery yields and to better elucidate the effectiveness of PEF on the extractability of target intracellular compounds.

IV.3.4. Effect of PEF and type of solvent on the carotenoids composition of extracts obtained from industrial tomato peels

The composition of extracts, obtained after 6h of contact with acetone and ethyl lactate, from untreated and PEF treated (E = 5 kV/cm; W_T = 5 kJ/kg) samples of industrial tomato peels in terms of the main carotenoids compounds was carried out by HPLC analyses at fixed waveleghts (470 nm), with the chromatograms being reported in Figures IV.6 – IV.7, respectively.
Two main peaks could be observed, respectively related to the most abundant all-trans lycopene (peak 1) and cis-lycopene (peak 2) both in acetone and ethyl lactate extracts.
Regardless of the extracting solvent, PEF-assisted extraction process allowed a higher release of all-trans lycopene than the conventional SLE process (+40% in acetone, + 30% in ethyl lactate), as clearly shown by the increase of the area under the peak of lycopene (Figures IV.6 – IV.7), which is in agreement with the results previously shown in Figure IV.2 for hand-peeled tomatoes. However, no increases in terms of cis-lycopene could be detected after the application of PEF treatments, independently on the solvent used.
The micrographs of Figure IV.8 seem to confirm that the higher release of pigments from industrial tomato peel tissues subjected to PEF processing may be ascribed to the partial disintegration of the tomato peel cells, which leads to a faster diffusion process of solubilized intracellular compounds. However, as shown in Figure IV.8, the application of the electrical treatment did not affect the overall structure of the cells, which kept their original

shape. The lower concentration of lycopene in ethyl lactate extracts, testified by the smaller areas in the chromatograms of extracts from untreated and PEF treated tomato peels (Figure IV.7) with respect to those observed when acetone was used as a solvent (Figure IV.6), are representative of a lower capacity of such solvent in solubilising carotenoids, which is in contrast with the findings of Ishida & Chapman (2009) and Strati & Oreopoulou (2011b). Moreover, in agreement with previous findings (Luengo *et al*., 2014b; Pataro *et al*., 2017a), the application of the PEF treatment prior to the extraction phase did not alter the carotenoids spectrum, with no evidence of degradation/isomerisation phenomena of single compounds, probably associated with the mildness of the electric treatment.
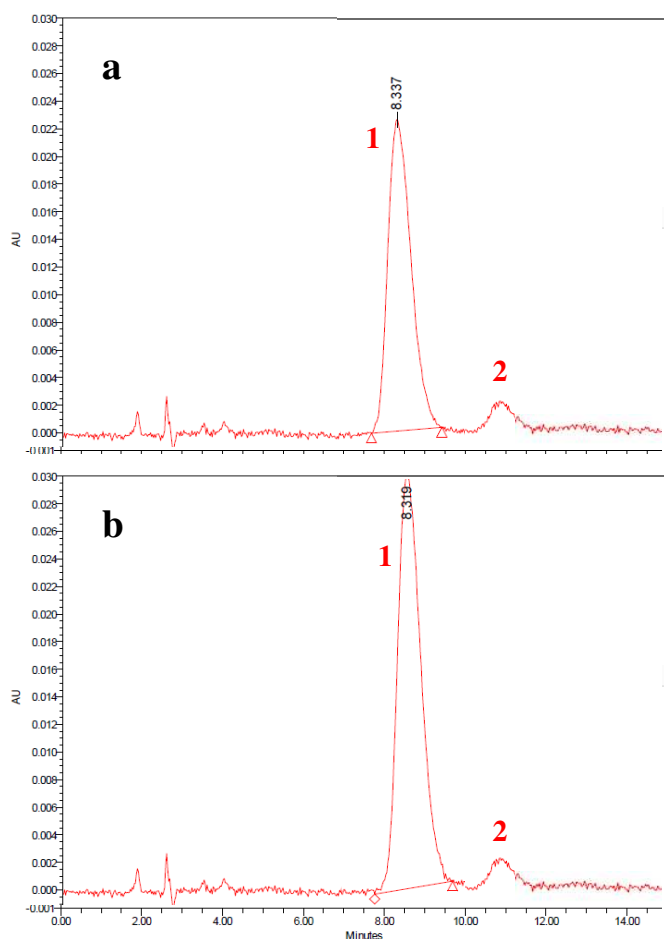
Figure IV.6 *HPLC-UV/Vis chromatograms at 470 nm of acetone extracts from untreated (a) and PEF-treated industrial tomato peels ($T_{extraction}$ = 20°C). Peak identification: (1) all-trans lycopene ($t_{elution}$: 8.3 min), cis-lycopene ($t_{elution}$: 10.7 min).*

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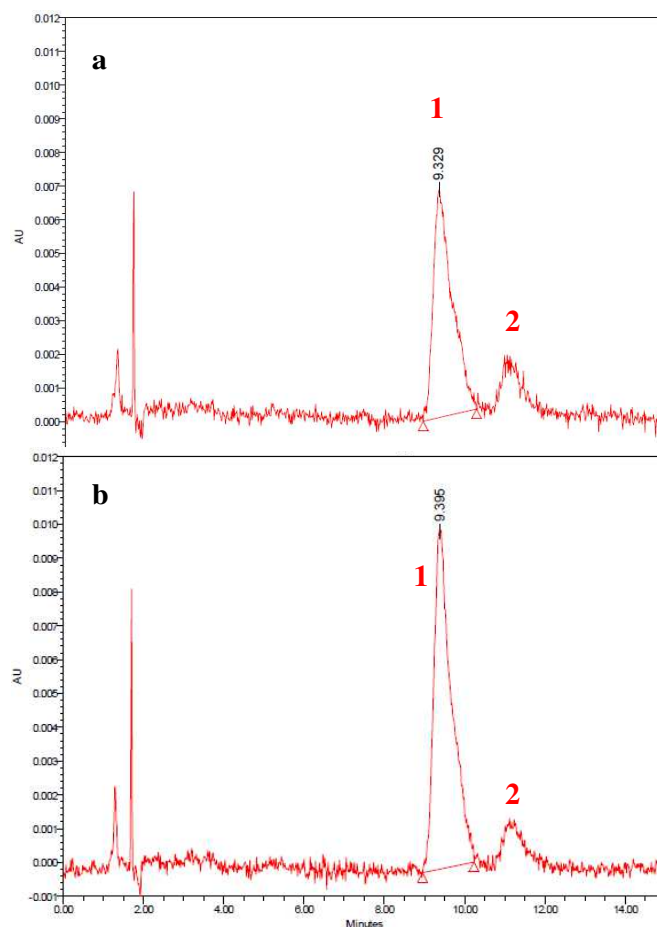
Figure IV.7 *HPLC-UV/Vis chromatograms at 470 nm of ethyl lactate extracts from untreated (a) and PEF-treated (b) industrial tomato peels ($T_{extraction}$ = 20°C). Peak identification: (1) all-trans lycopene ($t_{elution}$: 9.3 min), cis-lycopene ($t_{elution}$: 11.2 min).*
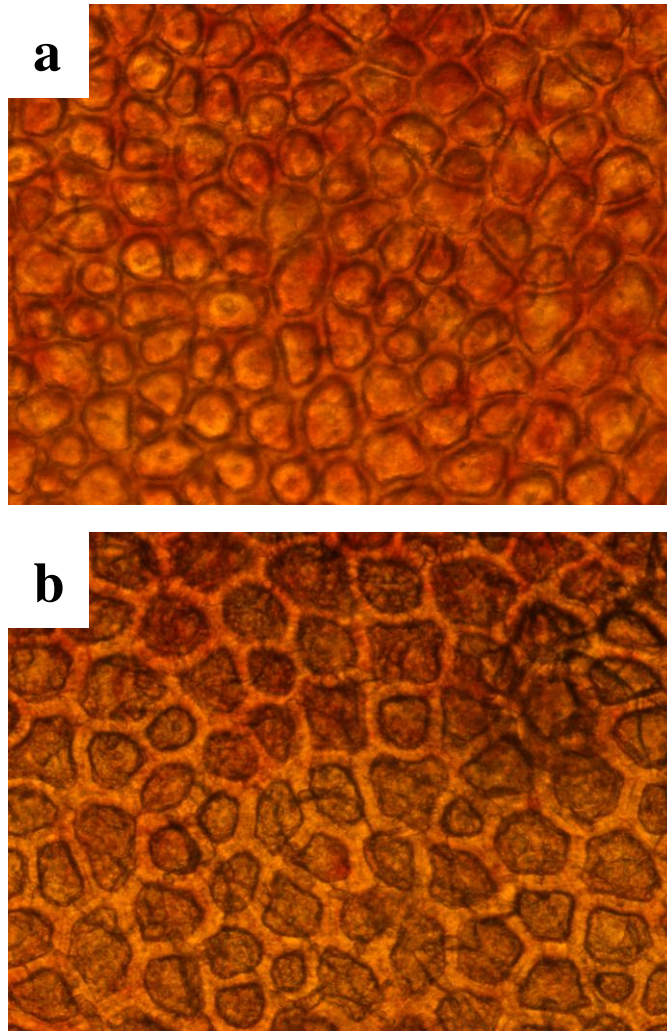
Figure IV.8 *Optical microscopy observations (40x) of untreated (a) and PEF (b) treated (E = 5 kV/cm; W_T = 5 kJ/kg) industrial tomato peels tissues.*

IV.4 Conclusions

In this work it has been demonstrated that PEF technology could potentially be exploited for the valorisation of tomato peels in terms of a better recovery of high-added value molecules by means of a conventional solvent extraction process (SLE).

Permeabilisation of tissues achieved by PEF has led to a more efficient recovery of carotenoids, especially lycopene, and enhanced the antioxidant power of extracts for very low energetic consumptions (W_T = 5 kJ/kg).

Moreover, for the first time, it has been demonstrated that the permeabilization effect of PEF is capable of improving the extraction yield of carotenoids from industrial tomato wastes. In this case, HPLC analysis also revelead that acetone was more efficient than ethyl lactate in penetrating tomato peel cells and in solubilising intracellular lipophilic compounds.

Thus, in view of the exploitation of tomato wastes derived from the industrial transformation of whole tomatoes, PEF technology could represent a valid alternative to the conventional pre-treatment of biomass like drying or grinding, which have negative effects both from environmental and economical points of view.

To this purpose, further investigations are required in order to evaluate the feasibility of PEF installation at industrial scale aiming at valorizing the tomato processing wastes, leading to a greater diversity of natural products to be used as food supplements or in cosmetics formulation, while lowering economical and environmental impact.

Chapter V
Optimisation of lab-scale PEF-assisted extraction processes from artichoke wastes

Abstract - Artichoke (*Cynara scolymus*) is an important and popular vegetable in Mediterranean countries which is rich of valuable phenolic compounds with high antioxidant properties.

Therefore, recovery of this kind of bioactives from wastes/by-products deriving from its industrial processing is of utmost importance for a possible exploitation in food, cosmetic and pharma applications.

In this work, the effect of PEF pre-treatments of different intensities (E = 0.25 – 5 kV/cm; W_T = 1 – 20 kJ/kg) on the disintegration degree of artichoke external bracts and stems tissues, as well as on total polyphenols content and antioxidant power of the extracts obtained after their acqueous extraction (S/L ratio: 1/10 w/w, T = 20-50°C, t = 6 h) was studied.

Results show that the permeabilization degree of artichoke tissue, properly quantified through the evaluation of the Z_P index, increased significantly when increasing the electric field strength up to a saturation value for a specific energy input of 5 kJ/kg.

The permeabilisation effect induced by the optimal PEF condition observed in this study (E = 3 kV/cm; W_T = 5 kJ/kg) led to significantly higher total polyphenols content (+ 350 % on average) and antioxidant power (+ 280% on average) of the aqueous extracts, as compared to the untreated samples.

From the overall results collected in this Chapter, artichoke stems, being more abundant in terms of phenolic compounds than external bracts, were selected and subjected to further studies of simulation of a pilot-scale biorefinery process, as described in Chapter VI.

V.1 Introduction

The re-utilisation of wastes/by-products deriving from food industries, due to their capability to retain large amounts of valuable compounds whose recovery and subsequent purification may allow to create new opportunities for several industrial sectors (Galanakis, 2015), represents an interesting approach to meet the bioeconomy challenges (Rocha *et al.*, 2018), due to their financially and environmentally sustainable valorisation.

An interesting case study is represented by the industrial processing of globe artichoke (*Cynara cardunculus L.*, var. *Scolymus*), whose production plays an important role in human nutrition and significantly contributes to the agricultural economics of the Mediterranean basin (Sihem *et al.*, 2015), where it is mostly cultivated (Ricceri *et al.*, 2016). Italy represents the leading country in terms of productivity (25.2% of total outcome), with a cultivated area of 43838 ha, followed by Egypt and Spain (Faostat, 2016). During harvesting and industrial processing phases, up to 60 – 80% by weight of the total raw material is discarded as a waste (Christaki *et al.*, 2012; Lattanzio *et al.*, 2009; Lavecchia *et al.*, 2018; Llorach *et al.*, 2002).

The majority of this biomass, whose currently generated amount exceeds one million tonnes per year (Rabelo *et al.*, 2016), is constituted by highly perishable artichoke external bracts and stems, representing a big burden for the processing industries, which need to ensure a proper management of this wastes either by affording huge disposal costs, with consequent increases in the induced environmental impact (Castro-Munoz *et al.*, 2016), or by using them as animal feed (Machado *et al.*, 2016) or fertilisers (Lopez-Molina *et al.*, 2005).

Apart from their potential uses to produce bioenergy (Fabbri *et al.*, 2014; Zuorro *et al.*, 2016), or to extract inulin, a heterogeneous blend of fructose polymers widely found in nature as plant storage carbohydrates, in the last decades the exploitation of artichoke wastes has been increasingly gaining interest especially for the recovery of antioxidant polyphenols (Ceccarelli *et al.*, 2010; Ruiz-Cano *et al.*, 2014). Among these compounds, flavones and phenolic acids (Schutz *et al.*, 2004), have shown a high capability of exerting a strong defense against pathogens (Balasundaram *et al.*, 2006), as well as of preventing from oxidative stresses and chronic diseases, such as diabetes, cancer and cardiovascular diseases (Barba *et al.*, 2017; Guida *et al.*, 2013; Pandino *et al.*, 2011). Additionally, the increasing consumer demand for natural products (Prasad *et al.*, 2009) to be used as food supplements testifies the relevant importance of phenolic compounds in many industrial fields of application (Bouras *et al.*, 2016).

Moreover, according to Zuorro *et al.* (2016), the phenolic content of artichoke bracts and stems is significantly higher than that found in grape pomace, carrot peels and spent coffee grounds.

These high-added value biomolecules, which are highly sensitive to heat, light exposure and chemical treatments (Khan *et al*., 2018), are generally found in the cytoplasmatic medium of cells of artichoke bracts and stems tissues as conjugated with mono- and polysaccharides. Therefore, for their recovery, a proper solvent able to penetrate the cellular structures and subsequently separate and dissolve the target compounds from the vegetal matrix is required (Carciochi *et al*., 2017).

Despite the ease of a solvent extraction process, granting high recovery yields only when optimal conditions are applied (Vorobiev & Lebovka, 2010), a series of detrimental effects on the final extracts could occur, due to the need for high contact times and huge amount of solvents (Barbosa-Pereira *et al*., 2018; Luengo *et al*., 2014b; Pataro *et al*., 2017a).

For instance, when a size reduction (slicing/grinding) or high temperature (drying) pre-treatment is carried out prior to the solvent extraction step, the structure of phenolic compounds could be irreversibly lost by means of oxidative phenomena catalysed by the polyphenoloxidase (PPO) enzyme, leading to browning of extracts and loss of phenolics scavenging activity (Aydemir, 2004; Icier *et al*., 2008).

Parallely, the release of undesirable compounds from the matrix clearly complicates the separation/purification step of extracts, thus burdening the economics of the "biorefinery" process.

In order to preserve the integrity of such antioxidant species, the need for a milder pre-treatment of wet biomass, able to improve the performances of the subsequent "green" solvent extraction step with a significant reduction in both duration and energy consumptions, is strongly required.

To this purpose the successfull application of PEF-assisted extraction as a sustainable and efficient process for the release of phenolic compounds from different food wastes such as grape pomace (Brianceau *et al*., 2015; Comuzzo *et al*., 2018; Saldana *et al*., 2017), citrus fruit residues (El Kantar *et al*., 2018; Peiro *et al*., 2017), vine shoots (Rajha *et al*., 2014), grape seeds (Boussetta *et al*., 2012) and red fruits skins (Bobinaite *et a*l., 2015; Pataro *et al*., 2017b) is well documented in current literature.

However, to date, the influence of a PEF-pretreatment on the extractability of polyphenols from artichoke wastes/by-products, namely involucral bracts, has been investigated only in the work of Battipaglia *et al*. (2009). In this preliminary work, the authors concluded that PEF treatment of artichoke external bracts at 1.6 kV/cm of electric field strength granted a significantly higher release of polyphenols (+166,67 %) with respect to the untreated ones during a diffusion process.

Moreover, the authors found that the usage of water as extracting solvent for the valorisation of PEF treated biomasses may have led to recovery yields of phenolic compounds similar to those detected when hydroalcoholic mixtures were used, thus avoiding the need for organic solvents and contributing to a more sustainable and "green" process.

Despite these encouraging results, only four different combinations of the main parameters, namely electric field strength and number of applied pulses were investigated (E = 0.8 – 1.6 kV/cm; n_{PULSES} = 50 – 500), thus requiring further studies for the optimisation of the PEF-assisted extraction protocol.

To the best of our knowledge, the work described in this chapter and in the next one (Chapter VI) represents the first attempt of full valorisation of artichoke wastes (external bracts and stems) through the application of PEF-assisted extraction process for the recovery of high-added value bioactive molecules.

Specifically, the aim of this preliminary study was to firstly investigate the impact of PEF pre-treatment of variable intensity (E, W_T) on the achieved cell permeabilisation degree of external bracts discs and stems cubes of artichoke tissues, as described in § III.2.5.1.

The results of the impedance analyses were used to define optimal PEF conditions to be used as pretreatment of the plant matrices with the aim of improving the mass transfer of phenolic compounds during the subsequent lab-scale extraction process in water (§ III.2.5.2).

V.2 "Short" Materials and methods

V.2.1 Raw materials and sampling

Prior to their processing, fresh artichoke external bracts and stems were properly selected and chopped in discs (3 cm in diameter) and cubes (1 cm^3 in volume) respectively, as explained in § III.2.1 (Figures III.3 – 4).

V.2.2 Permeabilisation of tissues

Both impedance analyses and PEF-assisted extraction tests of artichoke wastes were carried out in a batch cylindrical treatment chamber (§ III.2.4, Figure III.6) connected to a pulse generator, as schematized in § III.2.3 (Figure III.5). Impedance analyses were executed as described in § III.2.5.1.

Instead, PEF-assisted extraction tests (§ III.2.5.2) were carried out at fixed electric conditions (3 kV/cm; 5 kJ/kg) for both investigated matrices.

The subsequent extraction process was performed in distilled water at variable diffusion temperature (20 – 50 °C).

V.2.3 Analytical determinations

Clarified extracts from untreated and PEF treated artichoke external bracts and stems were quali-quantitatively analysed in terms of total phenolic compounds (TPC), antioxidant power (FRAP) and chlorogenic acid content (HPLC runs), as previously reported in § III.2.6.2 – 4.

V.3 Results and Discussion

V.3.1 Electrical characterisation of raw materials via impedance analysis

Impedance analysis of untreated and PEF treated samples has been carried out with the aim to assess the optimal treatment conditions, in terms of the minimum energy consumption (W_T, kJ/kg) able to maximize the cell membrane peremabiliztion (Z_p) of artichokes wastes tissue, which then have been applied for the subsequent PEF-assisted extraction experiments.

Figure V.1 shows the dependancy of Z_P on both the electric field strength (E) and the applied specific energy input (W_T), respectively for artichoke external bracts (Figure V.1a) and stems (Figure V.1b).

The results observed in Figure V.1 have evidenced similar behaviors to those precedently detected for tomato peels, as described in § IV.2.1.

In particular, for each field strength applied, the permeabilisation of the plant tissues increases with increasing the specific energy, achieving a constant value at about 5 kJ/kg, independently on the treated matrix.

Similar saturation tendencies of Z_P parameter have already been observed in previous studies (Luengo *et al*., 2013; 2014b). In the work of Battipaglia *et al*. (2009), it has been stated that the application of a PEF treatment of intermediate intensity (E = 1.6 kV/cm; n_P = 500 pulses) led to a significant decrease of the impedance modulus associated to the treated samples (strips of bracts) due to the occurrence of the tissues permeabilisation phenomenon, while lower values of the electric field strength (E = 0.8 kV/cm) and/or lower treatment durations (n_P = 50 pulses) were not able to induce significant permeabilisation effects.
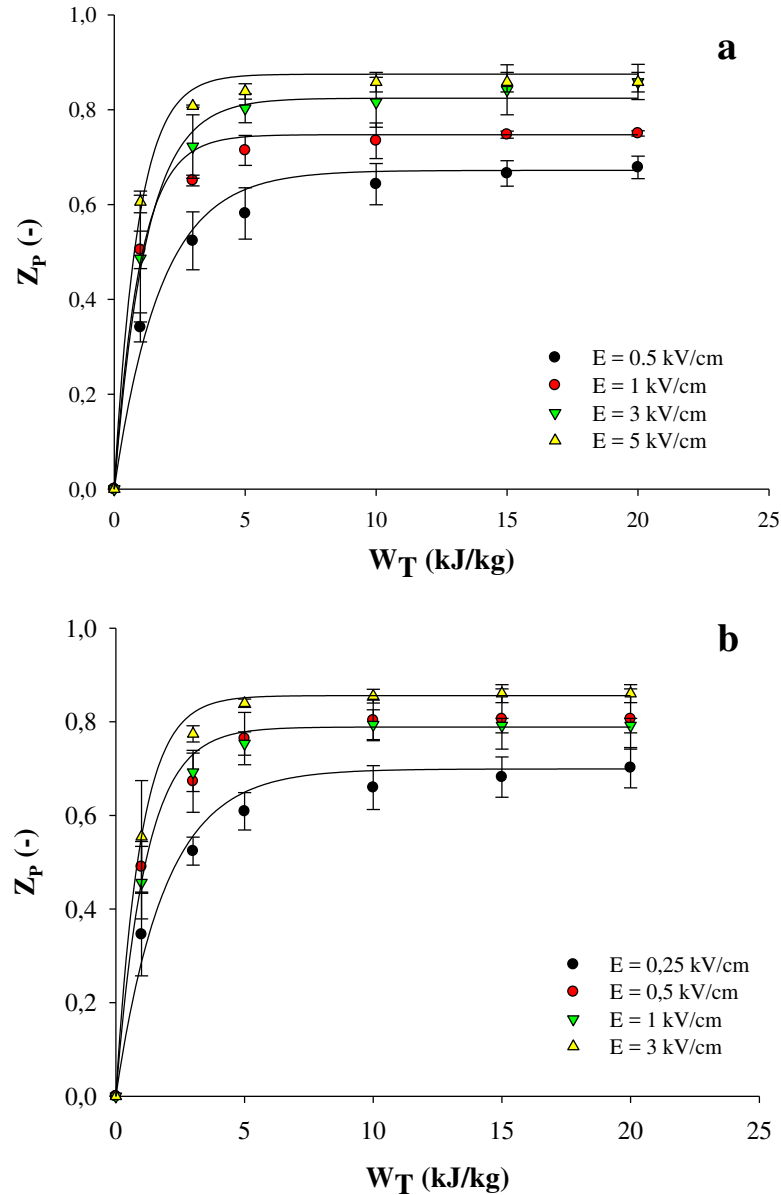
Figure V.1 *Cell disintegration index (Z_P) of artichoke external bracts (a) and stems (b) as a function of total specific energy input (W_T) and for different electric field strength (E). Standard deviations were used as error bars (p≤0.05). Experimental data were fitted by exponential saturation models ($R^2 > 0.97$).*

Results of Figures V.1 also show that the extent of tissue damages increases with the severity of the electric field strength, reaching maximum permeabilisation effects at E = 3 kV/cm for both treated matrices.

Based on the results of Figure V.1, a field strength of 3 kV/cm and a total energy input of 5 kJ/kg were selected as optimal PEF conditions capable of inducing the greatest permeabilization effect with the minimum treatment severity and furtherly applied to investigate the influence of PEF pre-treatment on the release of valuable intracellular compounds from both artichoke bracts and stems

V.3.2 Total Phenolic Compounds (TPC) and antioxidant power (FRAP) of extracts: effect of permeabilisation pre-treatment

In the following, the permeabilisation effect of cell membranes of artichoke bracts and stems due to the application of a PEF pre-treatment was evaluated by measuring the release of intracellular molecules (e.g. total phenolic compounds), as well as the antioxidant capacity of extracts obtained from the solvent diffusion process. Specifically, the effect of PEF-assisted extraction of phenolic compounds was evaluated using water as extraction solvent and only in the case of stems the effect of extraction temperature (T = 20 – 50°C) was also investigated as described in detail in Chapter III.

V.3.2.1 Artichoke external bracts discs

The evolution of TPC recovery during the aqueous diffusion process has been monitored for both untreated and PEF (3 kV/cm; 5 kJ/kg) treated samples, with the results reported in Figure V.2.

From Figure V.2 it is worth noting that the mass transfer resistance of intact cell membrane of untreated bract tissues leads to a very slow diffusion rate of phenolic compounds, granting negligible extraction yields (3.5 mg_{GAE}/100g FW) also after a long diffusion time (360 min).

The permeabilization effect induced by the application of a PEF pre-treatment , instead, markedly increased the extraction rate allowing to achieve maximum recoveries of phenolic compounds after about 6h of diffusion, which resulted to be significantly ($p < 0.05$) higher than those observed in the control samples (10-fold increase).
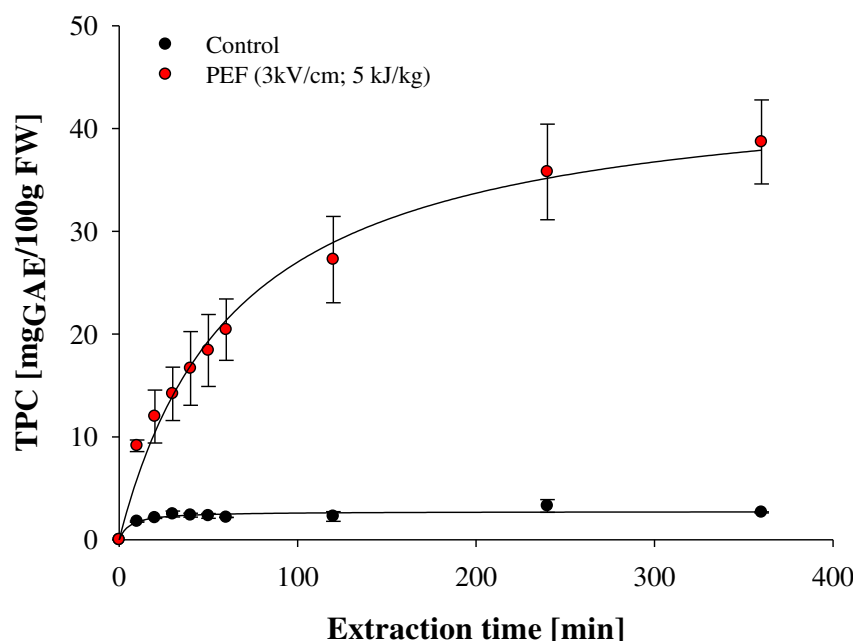
Figure V.2 *Kinetics of total polyphenols concentration (TPC, mg of gallic acid/100 g FW) in the extracts from untreated and PEF (E = 3 kV/cm; W_T = 5 kJ/kg) treated discs of artichoke bract samples at constant temperature (25 °C). Standard deviations were used as error bars (p≤0.05). Experimental data were fitted by exponential saturation models (R^2 > 0.95).*

Experimental data from kinetics of TPC extraction (Figure V.2) were well fitted (R^2 > 0.9) by the exponential model presented in Lebovka *et al.* (2004). Similar trends in terms of TPC extraction kinetics were observed in the work of Segovia *et al.* (2015), where the application of PEF treatments of different intensities (E = 2.5 – 5 kV/cm; W_T = 0.04 – 61.1 kJ/kg) led to a substantial increase in the extractability of phenolic compounds in acidified water (T = 25 °C) from borage leaves up to 1.3 – 6.6 folds as compared to untreated samples, with a significant decrease in the extraction process duration.

In the work of Bouras *et al.* (2016) it was demonstrated that the permeabilisation of norway spruce barks tissues induced by a high-intensity PEF treatment (E = 20 kV/cm; n_P = 25 – 400 pulses) could dramatically increase the rate of phenolic compounds recovery of about 8 times with respect to untreated samples, with no modification on the structure of the raw materials as well as on chemical functions of its constituents.

Pictures of extracts from untreated and PEF treated artichoke bract discs, which are shown in Figure V.3, seem to confirm the results of Figure V.2, being the greater darkness of the PEF extracts strictly related to the markedly higher release of phenolic compounds detected during the PEF-assisted extraction as compared to conventional SLE process (Bouras *et al.*, 2016).
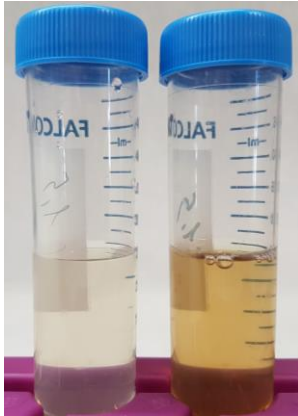
Figure V.3 *Aqueous extracts from untreated (left) and PEF treated (right) artichoke external bract discs.*

Enrichment of phenolic compounds in extracts is generally accompanied by the increase of their antioxidant power, thus favouring a stronger protection against oxidation phenomena (Luengo *et al*., 2013).
Figure V.4 shows the antioxidant power of extracts from both untreated and PEF treated artichoke bract discs.
Coherently with the results shown in Figure V.2, the application of a PEF treatment led to a significant ($p < 0.05$) increase of the antioxidant power of the extracts (+ 510% over controls).
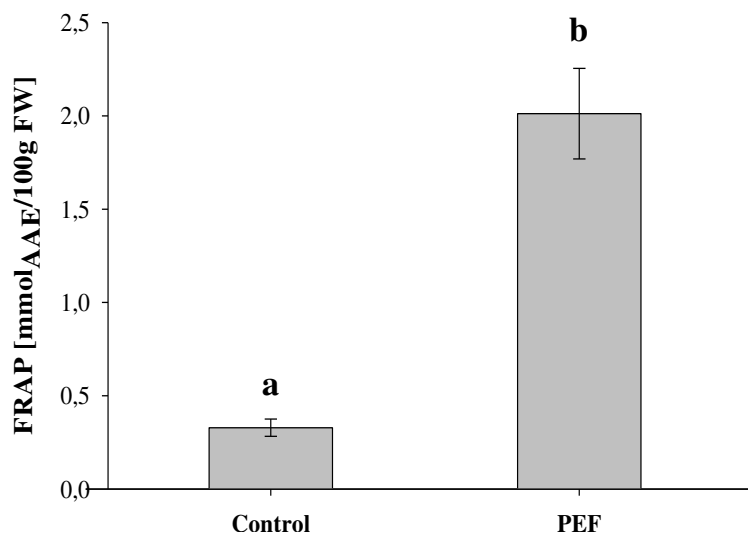
Figure V.4 *Antioxidant power (FRAP) of extracts from untreated and PEF treated (3 kV/cm; 5 kJ/kg) artichoke external bract discs after 6h of diffusion at constant temperature (25 °C). Standard deviations were used as error bars (p≤0.05).*

Similar enhancement of antioxidant power of extracts from several foods and food by-products by PEF has extensively reported in the literature (Bobinaite *et al.*, 2015; Grimi *et al.*, 2011; Luengo *et al.*, 2014b; Pataro *et al*, 2012).

V.3.2.2 Artichoke stem cubes

Figure V.5 shows the extraction kinetics of total phenolic compounds (TPC) from untreated and PEF (3 kV/cm – 5 kJ/kg) treated samples of artichoke stem cubes as a function of the diffusion temperature (20 – 50°C).

Independently on the investigated sample, a saturation value in the recovery yields of TPC was achieved after 2h of diffusion process, after which no appreciable additional leakage of phenolic compounds from the artichoke tissues could be detected.

Results show that a significantly faster extraction rate of polyphenols was detected when the artichoke tissues were electropermeabilised. Moreover, for both untreated and treated samples, the higher the extraction temperature, the lower the recovery yields. In particular, PEF treated samples showed a percentage increase of TPC over controls of 220%, 196% and 188%, respectively at 20, 35 and 50°C.

Moreover, it should be inferred that a browning phenomena was also observed for extracts obtained at the highest investigated extraction temperature (T = 50°C) and especially for PEF treated samples (data not shown).

Thus, it is likely that extraction temperatures higher than 20°C for long diffusion times might activate enzymatic and/or non enzymatic path-ways leading to degradation of phenolic compounds. In particular, the significant browning and phenolic compounds degradation induced at higher temperatures could be attributed to the decompartmentalization of oxidative enzymes and their phenolic substrates upon tissue permeabilization, leading to polymerized brown derivatives. Moreover, the combination of PEF pretreatment with the higher extraction temperature could have accentuated the decompartmentalising effect, making PPO no longer separated from phenolic compounds, which could have easily been oxidized.

Based on these results, for the following experiments and analyses carried out in this thesis work on the recovery of phenolic compounds from artichoke wastes, a diffusion temperature of 20°C was used.

In particular, HPLC analyses of extracts from untreated and PEF treated biomasses showed that the increase in diffusion temperature led to a significant lowering in the yields of chlorogenic acid, being the most abundant detected phenolic compound in the extract, until leading to its complete depletion at the highest investigated temperature (data not shown).

These results are in contrast with different findings in literature where the increase in diffusion temperature during solid/liquid extraction processes positively impacted on the solubility and diffusivity of phenolic compounds towards the extracting medium (Brianceau *et al.*, 2015; Luengo *et al.*, 2013; Segovia *et al.*, 2015).

In this specific case, in order to better intrepretate experimental data, further measurements of enzymatic activity in artichoke extracts from untreated and PEF treated samples as a function of the diffusion temperature are strongly required.

Figure V.6 shows the HPLC chromatogram profiles of untreated and PEF treated extracts at 20°C, from which it is evident that the application of a PEF pre-treatment significantly enhanced the release of phenolic compounds, and especially of chlorogenic acid, whose content in the extracts was 2.7-fold higher than that detected in the extracts collected after conventional SLE process.
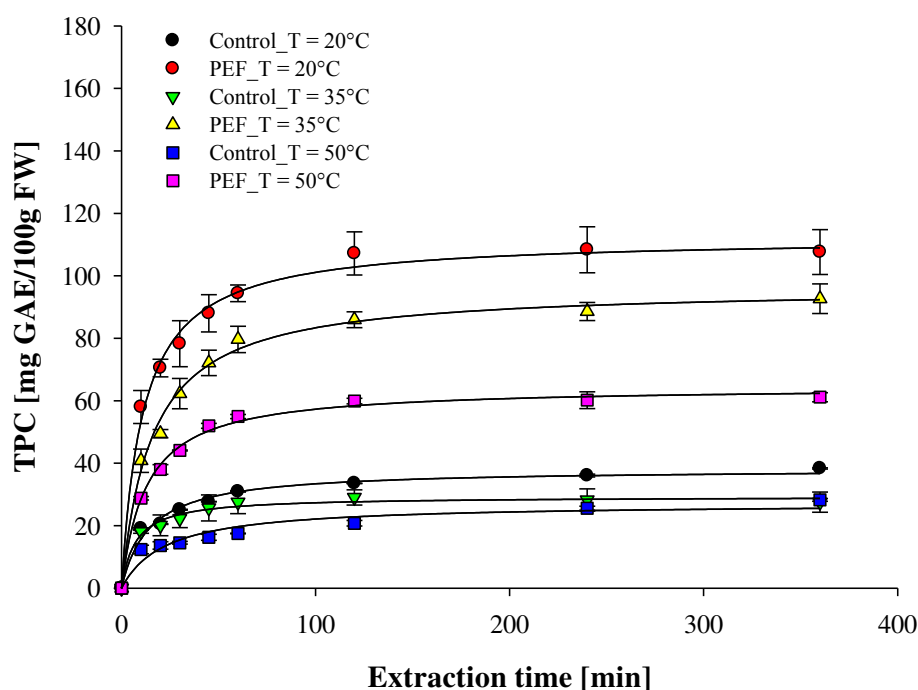
Figure V.5 *Extraction kinetics of total phenolic compounds (TPC) from untreated and PEF (3 kV/cm; 5 kJ/kg) treated artichoke stem cubes, as a function of diffusion temperature (T = 20 - 50 °C). Standard deviations were used as error bars (p≤0.05). Experimental data were fitted by exponential saturation models ($R^2 > 0.98$).*
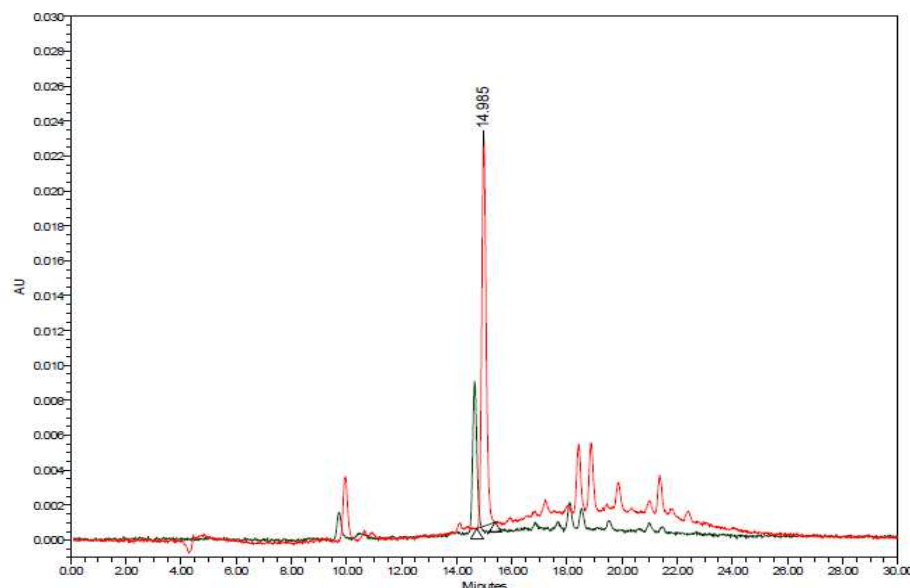
Figure V.6 *HPLC-UV/Vis chromatograms (λ = 326 nm) of extracts from untreated (green curve) and PEF treated (3 kV/cm, 5 kJ/kg, red curve) stem cubes. Peak identification: chlorogenic acid ($t_{elution}$ = 14.95 min).*

Moreover, as previously described by different authors, results of Figure V.6 clearly show that the mildness of PEF pre-treatment of vegetable tissues did not lead to any alteration (degradation/isomerisation) of the extracted compounds (Luengo *et al*., 2014b; Pataro *et al*., 2018).

The decrease in polyphenols level in extracts from control and PEF treated samples with increasing the extraction temperature is in agreement with the values of the FRAP antioxidant capacity depicted in Figure V.7, showing a linear correlation with phenolics yield after 2 h of extraction time (R^2 = 0.968, Figure V.8).
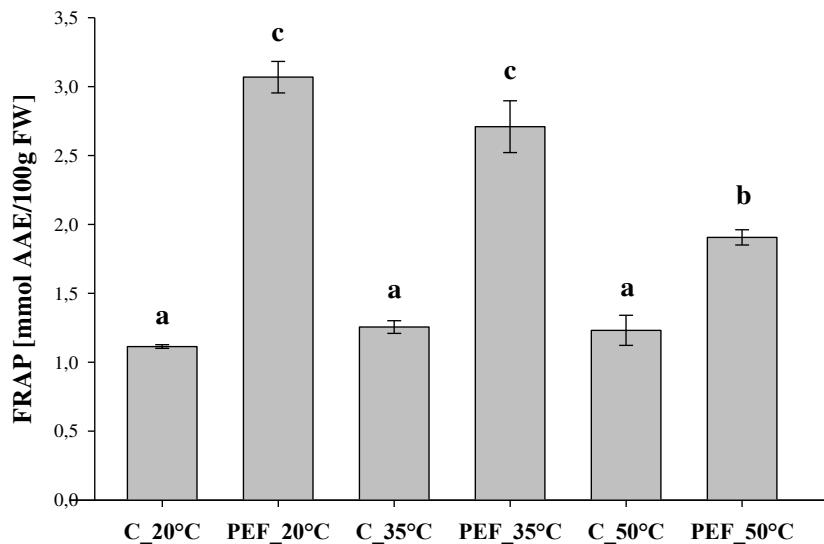
Figure V.7 *Antioxidant power (FRAP) of extracts from untreated and PEF treated (3 kV/cm; 5 kJ/kg) artichoke stem cubes, collected after 2h of diffusion at variable temperature (20 – 50 °C). Standard deviations were used as error bars (p≤0.05).*
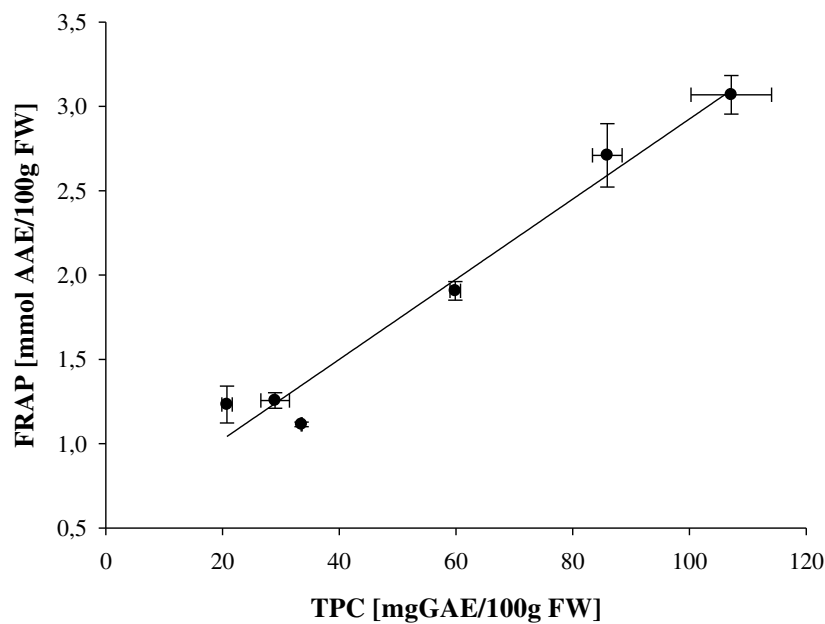
Figure V.8 *Linear relationship between the total phenolics content (TPC) and the antioxidant capacity of the extracts obtained from control and PEF-treated samples of artichoke stems (2h of diffusion). Standard deviations were used as error bars (p≤0.05).*

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The decrease in the antioxidant power resulted to be more pronounced in correspondence of extracts from PEF treated samples, with a significant reduction ($p < 0.05$) detected only at the highest investigated temperature level. As regarding control samples, no statistical differences could be noticed in terms of FRAP values as the temperature was changed ($p > 0.05$).

Due to the results obtained from this preliminary screening of operative conditions of the PEF assisted extraction process, a PEF pretreatment of 3 kV/cm and 5 kJ/kg as well as a diffusion temperature of 20°C and an extraction time of 2h were identified as optimal parameters for achieving the maximum release of valuable compounds from artichoke stems with the minimum energy consumption.

These optimal conditions have been subsequently used for carrying out further investigations of PEF-assisted extraction at pilot scale described in detail in Chapter VI.

V.4 Conclusions

Results obtained in this study have shown the possibility to exploit PEF-assisted extraction processes for valorising artichoke wastes through the recovery of intracellular phenolic compounds with a relatively low energy consumption, thus reducing both environmental and economic impact deriving from their disposal.

However, the extraction phase involving water represents a useful tool to reduce the risks associated with the large amount of organic solvents, which are generally implied to increase yields of processes.

More in details, it has been highlighted the capability of PEF technology to induce the permeabilisation of discs of artichoke bracts and cubes of stems in an intensity-dependent manner, improving the rate of diffusion of intracellular matters through cell membranes during extraction processes.

In particular, independently of the considered artichoke residue, the exposure to a PEF treatment at 3 kV/cm and 5 kJ/kg led to the maximum detected permeabilisation degree of tissues of bract discs, with a subsequent significant ($p < 0.05$) increase of both phenolics concentration (7-fold on average) and antioxidant power (3.5-fold on average) of the aqueous extracts, as compared to the control samples.

Similar results have been obtained in the case of artichoke stems, with 2.2-fold and 1.8-fold enhancements in phenolics recovery and antioxidant power of extracts over untreated biomass, respectively.

In this last case, the amount of phenolic compounds extracted suggested that artichoke stems should be preferred over external bracts in the frame of biomass valorization.

The results achieved in this Chapter have also allowed to gain insight in view of the set-up of a "biorefinery" scheme for the valorisation of artichoke stems, whose principal outcomes are reported in the following Chapter.

Chapter VI
Pilot-scale biorefinery process of artichoke stems based on PEF and NF technologies

Abstract - In this chapter, a pilot-scale "biorefinery" process of artichoke stems was designed, including a PEF (3 kV/cm; 5 kJ/kg) -assisted extraction (T = 20°C) step of phenolic compounds, followed by a nanofiltration step (20 bar) of extracts, and product formation by freeze drying.

Experimental results evidenced that, similarly to the results achieved at lab-scale (Chapter V), PEF-assisted extraction process at pilot-scale led to a marked increase in the extraction yield of phenolic compounds (+ 122%) and antioxidant power of the extracts (+158%) as compared to conventional SLE process.

HPLC analyses revelead that the main phenolic compound found in the extracts from untreated and PEF treated artichoke stems was chlorogenic acid.

Moreover, the nanofiltration step granted a high ability to retain phenolic compounds in the concentrate stream (> 95%), while no phenolic compounds were detected in the permeate, thus potentially exploiting it as a solvent for further extraction operations or as a washing liquid.

The subsequent freeze-drying of the retentate allowed to achieve a solid product containing a high amount of polyphenols (4% of DW extract), to be potentially used as a food supplement.

In conclusion, the biorefinery process at pilot scale of artichokes wastes based on an innovative extraction phase assisted by PEF, NF technology and freeze drying of phenolic extract has been successufully implemented and relatively high extraction yield and quality of the extracts have been achieved. More research is, however, necessary in order to further improve the process efficiency and demonstrate its feasibility at industrial scale, which in turn would contribute to reduce environmental problem, while increasing the value chain of the agri-food sector.

VI.1 Introduction

Artichoke (Cynara scolymus) is one among the main cultivated crops in Italy, whose production is strongly prevalent in the south of the country and islands, especially in Apulia, Sicily and Sardinia regions, accounting approximately for 90% of the national production (www.Istat.it).

Along the whole artichoke supply chain, only between 15% and 20% of the raw material is destined to human consumption (Lattanzio *et al.*, 2009), while the remaining mass is considered, together with blanching waters coming out from artichoke industrial processing, a low commercial value agri-food waste (Conidi *et al.*, 2015).

However, several clinical and epidemiological studies have demonstrated that the beneficial properties of artichoke wastes are to be ascribed to their high content of bioactive molecules, belonging to the class of polyphenols (Sanchez-Rabaneda *et al.*, 2003).

Phenolic compounds, which are produced during plant secondary metabolism, are generally recognised as powerful antioxidants due to their ability to scavenge reactive oxygen species (ROS), such as hydroxyl or oxide radicals (Alfano *et al.*, 2018; Cong Cong *et al.*, 2017; Rabelo *et al.*, 2016), thus protecting against abiotic and biotic stressors, as well as from the incurrance of chronic diseases and malfunctions (Pandino *et al.*, 2010; 2012).

Artichoke stems represent a rich source of caffeolquinic acids (chlorogenic acid, caffeic acid, cynarin), with great potential in the pharmaceutical sectors as hepatoprotective, anticarcinogenic and antibacterial agents, or as inhibitors of cholesterol biosynthesis (Conidi *et al.*, 2014; 2015; Ruiz-Cano *et al.*, 2014).

From the results shown in Chapter V, it has been proven that the use of a mild permeabilisation pre-treatment of artichoke stems by PEF technology ($E = 3$ kV/cm, $W_T = 5$ kJ/kg) could have beneficial effects in improving the recovery yields of phenolic compounds with high antioxidant activity, as well as to reduce the maceration time avoiding the use of organic solvents during the diffusion step.

However, as described in detail in Chapter I, apart from the extraction step, a typical biorefinery scheme should include also upstream operations, which are mainly aimed at preparing the raw plant matrices to be efficiently processed during the subsequent extraction phase, as well as downstream processing, which plays a crucial role for the separation/purification of the extracts before using product formation processes aimed at stabilizing the extracted compounds (Uyttebroek *et al.*, 2018). In particular, while grinding is widely used to increase the surface/volume ratio before the extraction phase, the choice of the best downstream processing steps should be performed also considering the need to preserve the nutritional value,

composition and overall quality of the final product, with the minimum energy consumptions (Nath *et al.*, 2018).

Over the last decades, the use of pressure driven membrane operations such as microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) has offered several interesting perspectives and advantages in terms of absence of phase transition, low energy expenditures, easy scale-up, great separation efficiencies, low contamination risks and preservation of compounds bioactivity (§ I.4.4.2).

As emerged from the literature study, several authors have demonstrated the benefits of membrane processes in facilitating the concentration of bioactive compounds from agri-food wastes/by-products, such as polyphenols from blueberry pomace (Avram *et al.*, 2017) and white vinasses (Diaz-Reinoso *et al.*, 2017), polysaccharides from wine lees (Giacobbo *et al.*, 2016) and pectins from olive mill wastewater (Galanakis *et al.*, 2010).

The recovery of valuable compounds from artichoke processing wastes, such as industrial blanching waters, by means of an integrated membrane process (microfiltration and nanofiltration) was extensively studied by Conidi *et al.* (2014, 2015). The authors found that the use of different membranes (Desal DL and NP030) could have led to different selectivities towards phenolic compounds and sugars, with the production of two fractions of remarkable interest for food and pharmaceutical industries and a clear permeate which could be reused as process water or for the cleaning of membranes, with a subsequent reduction in the overall operative costs of the biorefinery process.

To the best of our knowledge, only few works have been addressed to the combination of PEF and membrane technologies for the biorefinery of agri-food wastes (Liu *et al.*, 2011; Rajha *et al.*, 2015).

Moreover, all the aforementioned studies have been performed at lab-scale and using agri-food wastes different from artichoke residues.

Therefore, the aim of this work was to implement a pilot-scale biorefinery process of artichoke stems, whose schematisation is reported in Figure VI.1. In this simplified schematics, raw artichoke stems were first cut into cubic pieces before being subjected to PEF-assisted extraction process of phenolic acids with water at room temperature. Then, NF technology was used in downstream processing for the concentration of liquid extracts.

The last stage of the process is represented by the phenolic-rich product formation via freeze-drying of the retentate stream coming out as the main output from the NF phase. A detailed description of apparatus and processing conditions is described in Chapter III.
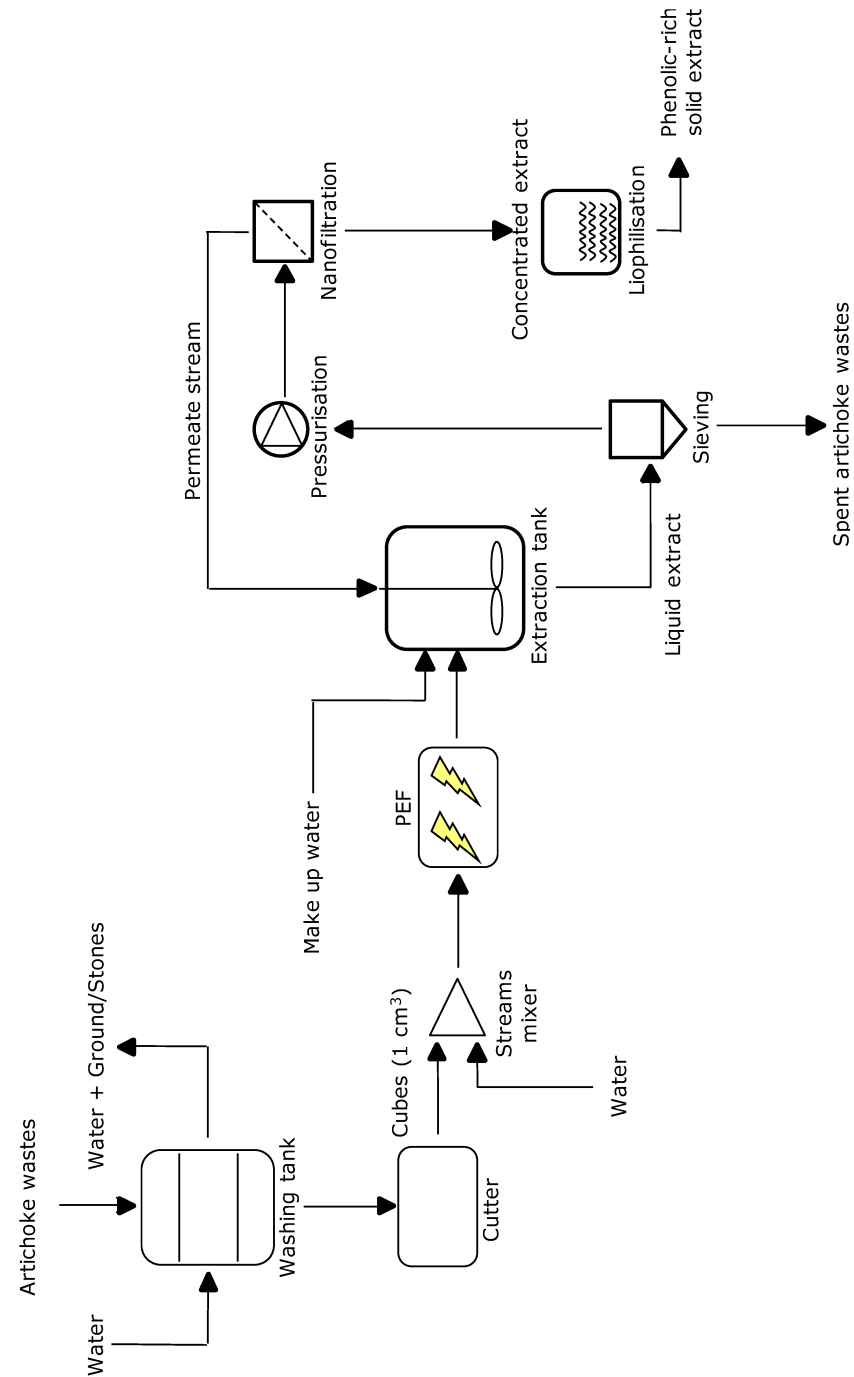
Figure VI.I. *Schematisation of the biorefinery process carried out on artichoke stems.*

VI.2 "Short" Materials and Methods

VI.2.1 Preparation of raw materials

Prior to their processing, fresh artichoke stems were chopped in cubes (1 cm^3 in volume) by means of a pilot dicer machine (§ III.2.1, Figure III.4) and subsequently stored in a 1% citric acid solution to slow down oxidation phenomena.

VI.2.2 Simulation of a PEF-based biorefinery process

According to the scheme reported in Figure VI.1, artichoke stem cubes were firstly electrically processed via a continuous PEF treatment cell (§ III.2.4, Figure III.7) at constant treatment intensity (3 kV/cm; 5 kJ/kg).
The subsequent water extraction procedure was conducted as reported in § III.2.5.3.
Afterwards, extracts from untreated and PEF treated samples were subjected to a nanofiltration process (NF) in a pilot scale plant (Figure III.9), by applying the processing conditions reported in § III.2.5.3.1.
Finally, the concentrated stream coming out from the NF plant was subjected to a freeze drying step in the equipment reported in Figure III.10, in order to obtain a stable phenolic-rich solid extract.

VI.2.3 Analytical determinations

Clarified extracts from untreated and PEF treated artichoke stems, as well as the solid extract obtained at the end of the freeze-drying process, were analysed in terms of total phenolic compounds (TPC), antioxidant power (FRAP) and chlorogenic acid content (HPLC runs), as previously reported in § III.2.6.2 – 4.

VI.3 Results and Discussion

VI.3.1. Influence of scale-up of PEF processing on the yield of polyphenols and antioxidant power of the extracts

When performing the scale-up of the PEF treatment chambers from small batch laboratory devices to larger sized continuous operating ones, it is of utmost importance to specifically design a series of process parameters such as electrodes material and geometry, electric field distribution, conductivity of the treated medium and average residence time in the treatment chamber, with the aim to reduce treatment inhomogeneities, thus reproducing performances of lab-scale facilities (Sack & Mueller, 2016; Toepfl, 2011).

Moreover, validation of pilot-scale facilities represent a remarkable step to be achieved for the subsequent industrialization of PEF processing technology (Jin *et al.*, 2015).

To this purpose, results of Figure VI.2 compare the phenolics yield (Figure VI.2 a) and antioxidant capacity (Figure VI.2 b) of extracts from tissues of artichoke stems obtained either at lab (§V.2.2.2) and pilot scale, achieved after 2 h of water diffusion.

Results clearly show that the scale-up of PEF chamber did not significantly affect the TPC extraction yield (Figure V.2), which showed a 3-fold for lab scale and 2.5-fold at pilot scale significant ($p < 0.05$) increase over untreated samples.

The slight but not significant reduction in TPC value observed for pilot scale PEF treated samples as compared to that detected at lab-scale, could be ascribed to a partial loss of intracellular compounds into the conductive medium (tap water) present in the continuous conveyor belt chamber, due to a longer duration of the pilot-scale PEF processing (120 s) with respect to that related to lab-scale experiments (5 s).
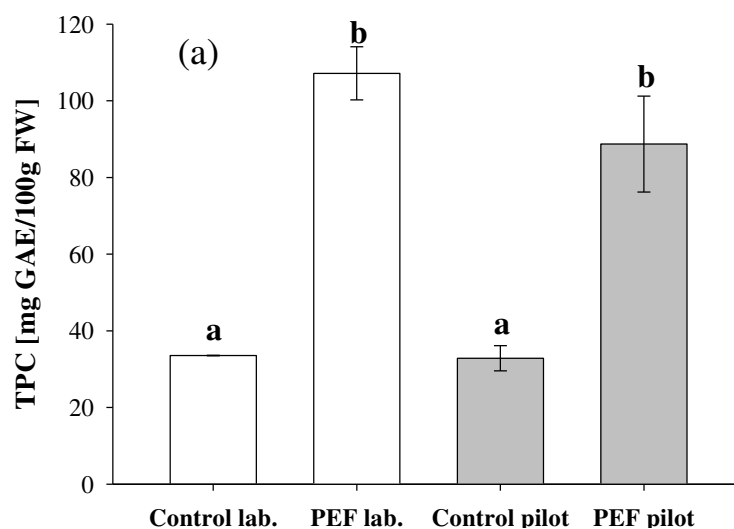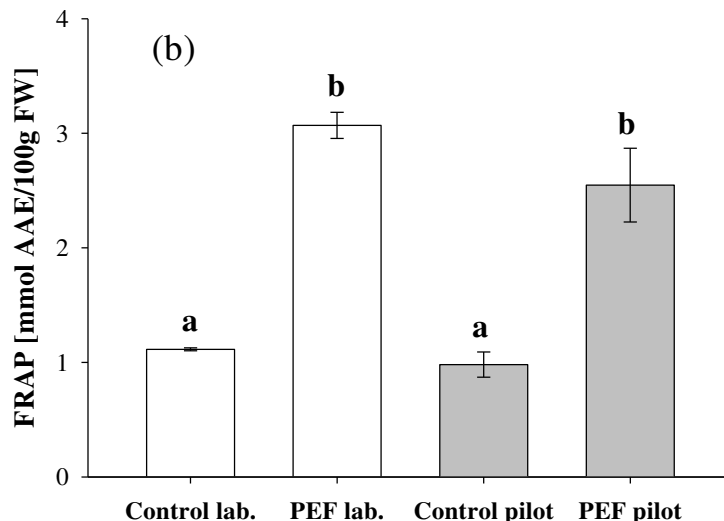
Figure VI.2 *Total phenolic compounds (a) and ferric reducing antioxidant power (b) of extracts from stem cubes obtained both from lab-scale and pilot-scale experiments. Standard deviations were used as error bars (p≤0.05).*

Coherently with the improved extractability of high-added value molecules due to electropermeabilisation of cell membrane of artichoke stem tissues, the antioxidant power of the extracts increased as well, with maximum detected values which were 177% for lab scale and 159% for pilot scale experiments higher as compared to the untreated samples.

It has been already proven in the current literature that the use of pilot scale PEF treatment chambers could lead to beneficial effects in different food industry applications such as the amelioration of vinification processes in terms of better extractabilities of valuable compounds (Sigler *et al.*, 2010), a reduction in acrylamide production during potato frying (Kern, 2012) and the possibility to inactivate microorganisms from pomegranate juice without altering their content in antioxidant compounds (Guo *et al.*, 2014).

Interesting outcomes have been reported by Loginova *et al.* (2011) in the frame of PEF-assisted extraction of sugar from beet cossettes. In their work, the authors demonstrated that the permeabilisation of tissues of sugar beets at relatively low electric conditions (E = 100 – 600 V/cm) could grant relevant advantages during the diffusion step, carried out in a 14 stages countercurrent pilot-scale extractor, both in terms of reduced processing temperatures, which dropped from 70°C to 30°C when an external field of 600 V/cm was applied, and of sucrose yield and purity of the obtained diffusion juice. Instead, in a more recent study performed by Puertolaz & De Maranon (2015) it has been shown that a PEF pre-treatment of olive paste at 2 kV/cm and at 11.25 kJ/kg could significantly (p < 0.05) enhance both the

extraction yield of oil over untreated samples (+ 13.3 %) and the content of antioxidant species in the product (+ 12% on average). Moreover, the oil extracted from permeabilised olive fruits showed similar chemical and sensorial characteristics of conventionally obtained olive oil, as required by EU legislation standards.

VI.3.2. Nanofiltration process: retention rates of TPC and FRAP

A "biorefinery" process envisages the possibility of efficiently and selectively separate compounds recovered during a solid/liquid extraction step, ensuring sustainability and low environmental impact, accompanied by low investment and operative costs.

It has previously been reported that the use of pressure driven separation technologies (MF, NF, RO) may potentially reduce energy consumptions, as well as to lead to processes with zero liquid discharge, due to the possibility of recycling the permeate stream as process water or for cleaning operations (Nath *et al.*, 2018).

Figure VI.3 reports the evolution of total phenolic compounds concentration (a) and antioxidant power (b) of extracts from pilot-scale PEF treated artichoke stem cubes as a function of the duration of the nanofiltration process, carried out at fixed processing conditions (P = 20 bar).

The usage of DL membranes, mainly characterized by the presence of a low volume fraction of small pores (Boussu *et al.*, 2008), is particularly indicated for the separation of low MW phenolic compounds with a considerable minimization in the fouling phenomena which, in turn, is reflected by the possibility of achieving negligible permeate flux drops along the filtration process, as previously observed by Conidi *et al.* (2014).

Results of Figure VI.3a highlight the presence of a substantial increase in the concentration of phenolic compounds during the NF process. In fact, a marked increase in TPC from 89.33 mg_{GAE}/100 g FW in the feed up to 268.63 mg_{GAE}/100 g FW in the final retentate stream occurred, with a TPC concentration factor of 3 and a volume reduction factor (VRF) of 4.

The non-linearity between the two parameters could be potentially explained by considering the loss of phenolic compounds deriving from oxidation phenomena or pores clogging due to adsorption of macromolecules (e.g. proteins) on the active surface of the membrane. This behavior has been previously reported by several authors working in the frame of nanofiltration for the concentration of polyphenols contained in extracts from apple pomace (Uyttebroek *et al.*, 2018), artichoke wastewaters (Conidi *et al.*, 2015) and blueberry pomace (Avram *et al.*, 2017).

Along with the results of Figure VI.3a, the increase in TPC with the filtration time is coherently accompanied by significantly ($p < 0.05$) higher values of chlorogenic acid contents in the retentate stream, which completely dropped to null values in the permeate one (Table VI.1). Similarly, the antioxidant

activity of extracts (Figure VI.3b) underwent a 2-fold increase (5.1 mmol AAE/100 g FW) with respect to that detected in the initial feed stream.

The trends observed in Figure VI.3 and Table VI.1 were also confirmed by the HPLC chromatograms reported in Figure VI.4, where a consistent increase in the peak associated to chlorogenic acid in the concentrate stream could be detected, with a negligible concentration of phenolic compounds in the permeate stream.
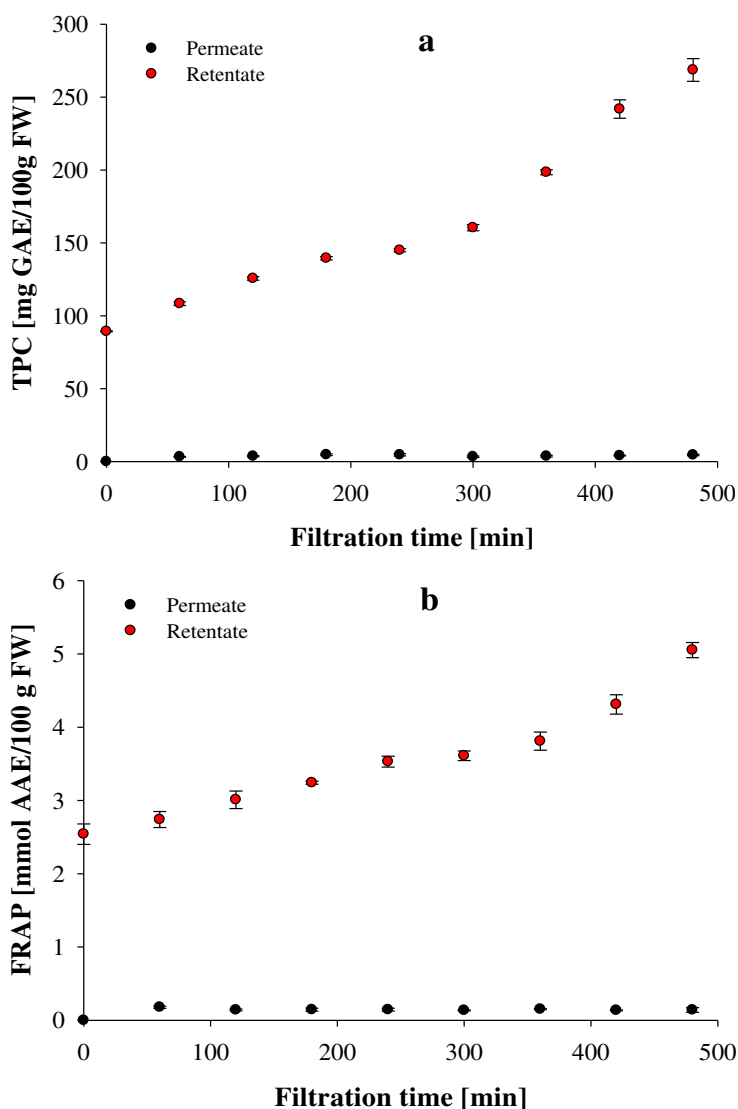
Figure VI.3 *Time course of TPC (a) and FRAP (b) during nanofiltration process of retentate and permeate streams. Standard deviations were used as error bars (p≤0.05).*

Table VI.1. *Concentration of chlorogenic acid, expressed in mg/100g of fresh weight artichoke stems, of both permeate and retentate streams, as a function of the filtration time.*

Sample	Chl. ac. [mg/100g FW]
PEF (t = 0h)	12.69 ± 0.54
PEF conc. 1h	13.63 ± 0.74
PEF conc. 2h	13.84 ± 0.46
PEF conc. 3h	15.11 ± 0.11
PEF conc. 4h	16.13 ± 0.67
PEF conc. 5h	17.25 ± 0.82
PEF conc. 6h	18.42 ± 1.01
PEF conc. 7h	22.85 ± 0.76
PEF conc. 8h	26.06 ± 1.26
PEF perm. 1h	0.105 ± 0.05
PEF perm. 2h	n.d.
PEF perm. 3h	n.d.
PEF perm. 4h	n.d.
PEF perm. 5h	n.d.
PEF perm. 6h	n.d.
PEF perm. 7h	n.d.
PEF perm. 8h	n.d.

Table VI.2. *Retention rates of concentrate stream in terms of total phenolic compounds (TPC) and antioxidant power (FRAP) by NF with DL membrane.*

Filtration time [h]	TPC retention rate [%]	FRAP retention rate [%]
1	97.2	93.5
2	97.1	95.3
3	96.6	95.5
4	96.8	95.9
5	97.9	96.2
6	98.1	96
7	98.3	96.8
8	98.3	97.2

The great efficiency of the implied Desal-DL membrane in retaining phenolic compounds, as well as the antioxidant capacity of extracts in the concentrate stream, thus producing a permeate stream with a negligible amount of antioxidant species, is shown in Table VI.2.
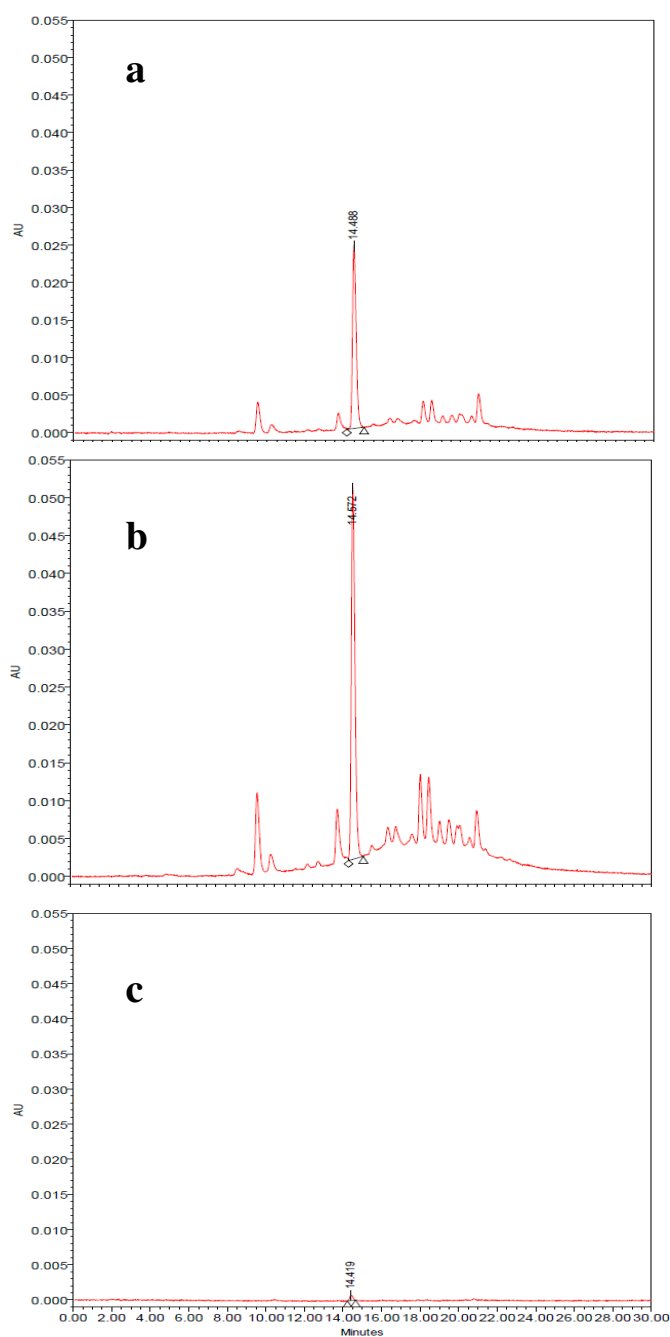
Figure VI.4 *HPLC-UV/Vis chromatograms (λ = 326 nm) of (a) extract from PEF treated (3 kV/cm; 5 kJ/kg) stem cubes, (b) retentate stream and (c) permeate stream after 8 h NF. Peak identification: chlorogenic acid ($t_{elution}$ = 14.88 min).*

Moreover, results of table VI.2 show that, regardless of the filtration time, 97.5 % of TPC and 95.8% of FRAP, on average, were detected in the retentate stream. These result are in agreement with those reported in the work of Cassano *et al.* (2016), which showed that the usage of a DL membrane was highly selective in splitting chlorogenic and caffeolquinic acids to the retentate stream, with rejection rates of 92% on average.

On the basis of the observed results, a possible role for the permeate stream in a potential artichoke waste biorefinery process may be hypothesized. This stream, being free of phenolic compounds, which were efficiently transferred to the retentate one, could be potentially recycled back as fresh extracting medium or as plants washing liquid, leading to a significant reduction in the operative costs by lowering the amount of water needed for the process (Cassano *et al.*, 2016, Seigworth *et al.*, 1995).

VI.3.3. Freeze-drying and product formation

The obtainment of a solid extract (Figure VI.5) from the retentate stream was achieved by a freeze-drying process, as described in detail in Chapter III (§ III.2.5.3.2).

Figure VI.5 *Picture of the phenolic compounds rich solid extract obtained after freeze-drying of the retentate stream coming out from the nanofiltration system.*

The characterization of the solid extract in terms of total phenolic compounds and carbohydrates is reported in Table VI.3.

Table VI.3 *Results of solid extract characterization from PEF treated sample.*

Composition [g of product]
Polyphenols (**4%**)
Carbohydrates (60%)
Ashes (30 %)

As it is clearly evident from the results of Table VI.3, the solid extract has a significant amount of phenolic compounds, which is comparable to those reported in the current commercialised products, generally obtained from a conventional SLE process performed on dried artichoke wastes, which, therefore, likely requires high energy consumptions.

Thus, in the frame of an industrial biorefinery of artichoke wastes, since our proposed PEF-based approach rely on very low energetic consumptions (5 kJ/kg), further efforts are required in conducting a full economical analysis so as to evaluate the real competitiveness of PEF technology to be fully integrated into existing processing lines.

VI.4 Conclusions

In this work, it has been demonstrated that PEF and NF technologies could be potentially useful in the frame of a second-generation biorefinery, granting the possibility to improve extraction process performances and to reduce the energy demand of downstream processing, respectively.

In particular, no significant efficiency losses could be detected when PEF processing (E = 3 kV/cm; W_T = 5 kJ/kg) of artichoke stems was carried out in a pilot-scale continuous treatment chamber, with respect to the results achieved when using small laboratory facilities.

Moreover, the usage of a DL membrane significantly enhanced the selective separation of small MW bioactives in the final retentate stream, showing an almost total retention capacity of phenolic compounds and antioxidant potential of these molecules.

Finally, HPLC results revealed that the chlorogenic acid was the most abundant recovered compound in the extract, which was completely absent in the permeate stream coming from NF processing, thus offering new opportunities for its exploitation in the biorefinery process.

Section II

Innovative biorefinery of microalgal biomass

Chapter VII - *Effect of pulsed electric fields and high pressure homogenization on the aqueous extraction of intracellular compounds from Arthrospira platensis and Chlorella vulgaris microalgae*

Chapter VIII – *Hurdle approach in the biorefinery of microalgae*

Chapter VII
Effect of pulsed electric fields and high pressure homogenization on the aqueous extraction of intracellular compounds from *Arthrospira platensis* and *Chlorella vulgaris* microalgae

Abstract - Pulsed Electric Fields (PEF) technology represents a promising and scalable cell disruption method of microalgae cells.

In this work, the permeabilization degree, morphological properties, and extractability of intracellular compounds from biosuspensions of *A. platensis* and *C. vulgaris* microalgae were investigated as a function of PEF treatment at different electric field strengths (10–30 kV/cm), pulse polarity (mono, bipolar pulses) and total specific energy inputs (20–100 kJ/kg).

For the sake of comparison, the effect of the more disruptive HPH treatment (150 MPa) at different number of passes (n_P=1-10) was also investigated.

Results collected in this chapter highlighted the efficacy of PEF in inducing the permeabilization of the microalgal cell membranes in an intensity-dependent manner, without the production of cell debris, with the possibility of facilitating the downstream seperation process.

Instead HPH treatment causes the total disruption of the algae cells into small fragments, independently on the examined strain.

Moreover, it has been demonstrated that the release of intracellular matters strictly depends on the microalgal cell structure, with greater recovery yields of carbohydrates (84% w/w of total carbohydrates) and water soluble

proteins (35% w/w of total proteins) detected in correspondance of the "weaker" structured *A. platensis* strain with respect to *C. vulgaris* microalgae, which in turn opposed more resistance to rupture, thus allowing only a substantial release of carbohydrates (36% w/w of total carbohydrates) and small ions, while the majority of proteins remained trapped into the microalgal cells or bounded to the cell wall.

From the overall analysis of the collected results, optimal PEF treatment conditions, granting the highest release of valuable intracellular compounds were chosen (E = 20 kV/cm; W_T = 100 kJ/kg for both microalgae) and subsequently applied in specifically designed multi-stage "microalgal biorefinery" processes, either in combination with HSH (t = 1 min; ω = 20000 RPM ; *A. platensis*) or HPH (P = 150 MPa, n_P = 5; *C. vulgaris*) technologies, as reported in Chapter VIII.

VII.1 Introduction

In the last decades there has been a growing interest in the exploitation of microalgal biomass for the recovery of high-added value intracellular compounds with interesting application in the food, feed, pharmaceutical, energy and cosmetic sectors as potential replacers of synthetically obtained products (Esquivel-Hernandez *et al.*, 2017).

Some of the most biotechnologically relevant strains are *Arthrospira platensis* and *Chlorella vulgaris*, not only for their remarkable content of bioactive molecules, but also for their worldwide abundance, being cultivated in both open and closed systems under controlled conditions.

A. platensis is a multicellular and filamentous blue-green alga with helical shape (trichomes of 50-500 μm in length and 3-4 μm in width), having a more fragile cell wall with respect to other microalgal species.

However, this cyanobacteria is one of the richest protein sources from microbial origin (60 – 70 % DW), with similar proteins levels of meat and soybeans (Lupatini *et al.*, 2016).

C-phycocyanin is the major water soluble pigmented protein accumulated inside *A. platensis* cells, identified as a potential natural dietary supplement having therapeutic properties including antioxidant, anti-inflammatory and anticancer activities, with possible positive effects towards diabetes and cardiovascular diseases, with both blood vessel-relaxing and blood lipid lowering effects (Chaiklahan *et al.*, 2012; Thangam *et al.*, 2013).

C. vulgaris is a freshwater eukaryotic microalga with a mean diameter ranging from 2.5 to 5 μm (Yamamoto *et al.*, 2004) belonging to the division of Chlorophyta. This algae is capable of accumulating large amounts of valuable components, especially proteins (51 – 58 %), but also polyunsaturated fatty acids (14 – 22 %), carbohydrates (12 – 17 %), vitamins and minerals (Demirbas, 2010; Song *et al.*, 2011). Moreover, it accumulates also chlorophyll (1-2%) that imparts the characteristic green color, masking

the presence of other less concentrated pigments, such as lutein and other carotenoids (Safi *et al*., 2014).

The extraction of all these intracellular compounds from the aforementioned microalgae strains is of utmost importance for achieving an economically feasible microalgae biorefinery (Gunerken *et al*., 2015).

In particolar, these valuable compounds are located in different parts of the cells, protected in some cases by a rigid cell wall, as in the case of *C. vulgaris*, and membranes surrounding the cytoplasm and the internal organelles (e.g., chloroplast), which greatly limit their mass transfer rates during conventional extraction processes.

The latter are often conducted from dry biomass with organic or aqueous solvents, depending on the polarity of the target compounds (Luengo *et al*., 2015; Pataro *et al*., 2017c). However, these methods suffer from several limitations, namely the long extraction times and the use of relatively large amounts of solvent, which may potentially lead to the co-extraction of undesirable components, with increased downstream processing costs (Gunerken *et al*., 2015; Poojary *et al*., 2016). In addition, the drying of microalgal biomass is reported to be one of the major energy-consuming steps within the overall process, being also responsible for significant losses of valuable compounds (Golberg *et al*., 2016; Gunerken *et al*., 2015).

For these reasons, the application of innovative cell disruption methods to wet biomass may considerably promote the implementation of the biorefinery concept on microalgae, enabling a faster and more efficient release of intracellular compounds at low temperature. This also contributes to limit the degradation of the extracts and promotes the reduction of energy costs, of solvent consumption, as well as of the extraction time (Gunerken *et al*., 2015; Poojary *et al*., 2016).

Among the cell disruption methods, Pulsed Electric Fields (PEF) treatments have emerged as promising tools for a mild permeabilisation of biological cells (Barba *et al*., 2015b; Grimi *et al*., 2014; Grosso *et al*., 2015; Joannes *et al*., 2015; Luengo *et al*., 2015; Pataro *et al*., 2017c; Poojary *et al*., 2016). Moreover, PEF technology can be easily scaled up to process large volumes of wet biomass in a wide range of solids concentration, thus avoiding the need for energy-intensive drying and possibly allowing to reduce the energy demand per unit biomass (Goettel *et al*., 2013; Golberg *et al*., 2016).

As previously observed in Table I.10 (Chapter I), several studies highlighted the effectiveness of PEF to enhance the selective recovery of intracellular compounds from wet microalgal biomass, including lipids (Lai *et al*., 2014; Zbinden *et al*., 2013), pigments (Grimi *et al*., 2014; Luengo *et al*., 2015; Parniakov *et al*., 2015b; Poojary *et al*., 2016), carbohydrates and water-soluble proteins of small molecular weight (Goettel *et al*., 2013; Pataro *et al*., 2017c; Postma *et al*., 2016b).

To date, very few works in the current literature have dealt with the effect of PEF technology on the extractability of water soluble compounds from *A*.

platensis microalgae (Aouir *et al.*, 2015; Martinez *et al.*, 2016) and, in particolar, no investigations have been carried out on the effect of pulse polarity on the permeabilisation of this microalgal strain.

Instead, as regarding *C. vulgaris* microalgae, it has been already proven by Postma *et al.* (2016b) that PEF was unable to release high quantities of large molecules such as protein (<5%) as compared to those achieved by conventional bead milling process, despite leading to a significant solubilisation of small ions and carbohydrates.

For this reason, the extraction of compounds of higher molecular weight, or more bounded to the intracellular structure (e.g., proteins) from hard structured microalgae (*C. vulgaris*), requires the application of more effective cell disruption techniques, such as High Pressure Homogenisation (Poojary *et al.*, 2016).

HPH is a purely mechanical process, during which a liquid dispersion of plant material or a cell biosuspension is forced by high pressure (50-300 MPa) through a micrometric disruption chamber, where the velocity increases rapidly and the pressure decreases to atmospheric conditions as the suspension exits the unit (Yap *et al.*, 2015). As a result, the biological cell suspension is subjected to extremely intense fluid-mechanical stresses (shear, elongation, turbulence and cavitation), which cause the physical disruption of the cell wall and membranes (Donsì *et al.*, 2009; 2013).

Due to its high cell disruption efficiency (Gunerken *et al.*, 2015), HPH is reported to markedly increase the extraction yield of several valuable compounds from microalgae (Safi *et al.*, 2015; 2017a; Shene *et al.*, 2016). However, the HPH treatment causes the non-selective release of intracellular matters, with the concurrent dispersion of cell debris, complicating the downstream separation processes (Grimi *et al.*, 2014). Moreover, because of the intense interfacial shear stresses and inherent heating occurring in the homogenization valve, which might induce the degradation of compounds such as proteins (Thomas & Geer, 2010), HPH treatments always requires an efficient heat dissipation at the homogenization valve.

Although several studies have already highlighted the potential of PEF and HPH pre-treatments in the microalgae biorefinery, to date, only the study of Safi *et al.* (2017a) has performed a comparison of their efficiencies in terms of cell disintegration degree and release of soluble proteins from microalgae *Nannochloropsis gaditana*. However, suspensions of this microalgae were prepared from a frozen paste and at different biomass concentration for PEF (15-60 g_{DW}/L) and HPH (100 g_{DW}/L) treatments.

Moreover, a deeper knowledge regarding the impact of PEF technology at micro and macro scale is required, which is thoroughly necessary in view of its use in a cascade biorefinery approach of microalgae, where the control of the degree of cell breakage could be exploited to enable the fine tuning of the recovery process of intracellular components (Gunerken *et al.*, 2015; Postma *et al.*, 2016b; Wijffels *et al.*, 2010).

Therefore, the aim of this study was to comparatively investigate the effects of the main process parameters of both PEF (E, W_T, pulse polarity) and HPH (pressure, number of passages) treatments on the cell disintegration degree and the release of intracellular compounds (ionic substances, proteins, and carbohydrates) from either fresh *A. platensis* or *C. vulgaris* acqueous suspension, in order to select, for each investigated technology, the best treatment conditions in the perspective of their implementation in a biorefinery scheme.

VII.2 "Short" Materials and methods

VII.2.1 Microalgae strains and permeabilisation techniques

In this work, *A. platensis (*§ III.3.1, Figure III.11) and *C. vulgaris* (§ III.3.2, Figure III.13) biosuspensions were separately subjected to permeabilisation by PEF in a continuous bench-scale system, as schematised in § III.3.3 (Figure III.14). As a sake of comparison, the full microalgal cell disruption was achieved by HPH treatments (§ III.3.5, Figure III.16).
The permeabilisation of biosuspensions by PEF has been executed according to the procedure and the operative conditions (E, W_T, pulse polarity) listed in § III.3.6.1.
Further water diffusion steps of untreated and PEF or HPH treated samples were performed as reported in § 3.6.2.

VII.2.2 Analytical determinations

For all the investigated samples (untreated, single PEF and single HPH treated), the analyses on both biosuspensions (optical and SEM microscopy) and aqueous extracts (dry matter of supernatants, water soluble proteins, C-phycocyanin and carbohydrates) were performed according to the procedures illustrated in § III.3.7 and § III.3.8.

VII.3. Results and Discussion

VII.3.1. Extraction of valuable compounds from A. platensis cells

VII.3.1.1 Impact of PEF and HPH treatments on the release of intracellular components

Figure VII.1 shows the effect of the main PEF treatment parameters (E, W_T) when monopolar pulses of 5 μs duration were applied, on the extraction yields of water soluble proteins and carbohydrates from microalgae *A. platensis*. For the sake of comparison, also the effect of HPH treatment (P = 150 MPa; n_P = 3) was reported.

A spontaneous release of both proteins and carbohydrates from intact microalgal thricomes occurred after 3h of extraction, leading to final yields of proteins and carbohydrates of 15.40 mg_{BSA}/g_{DW} and 23.40 $mg_{D.Glu.}/g_{DW}$, respectively. This indicates that the cytoplasmatic membrane, surrounding the intracellular medium, acts as a semipermeable barrier, thus limiting the release of intracellular molecules from *A. platensis* cells.

Instead, a marked increase in the diffusion phenomena from microalgae was detected after the application of a PEF pre-treatment, granting the electroporation of cell membranes, which facilitated the leakage of intracellular matters towards the external medium (Zimmermann, 1986).
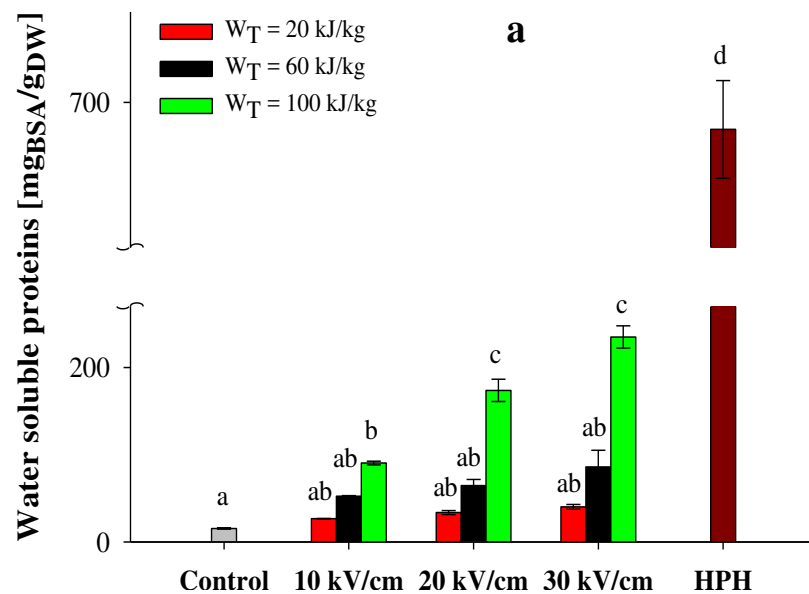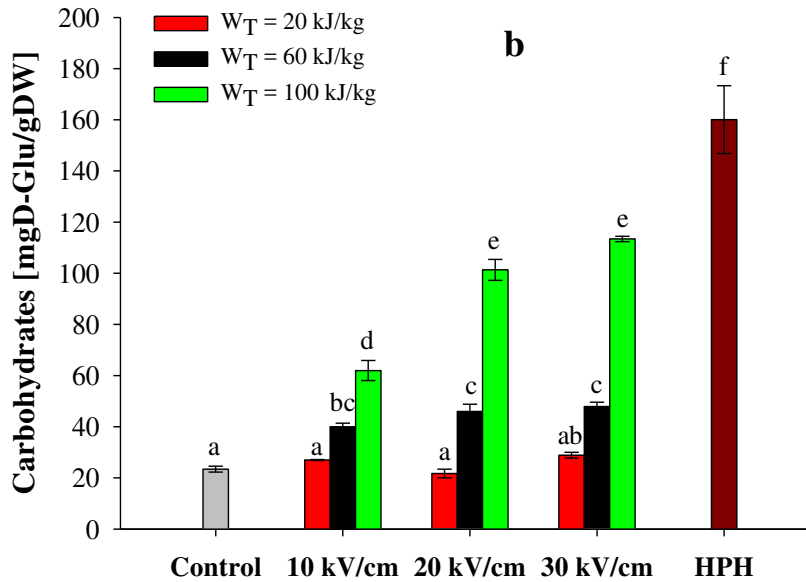
Figure VII.1 *Concentration of water soluble proteins (a) and carbohydrates (b) in the supernatant of untreated (control), PEF (E = 10-30 kV/cm, W_T = 20-100 kJ/kg) treated, and HPH (P = 150 MPa, n_P = 3) treated A. platensis suspensions, 3 h after water extraction. Biomass concentration: 2%. Different letters above the bars indicate significant differences among the mean values (p≤0.05).*

From both graphs of Figure VII.1 it emerges that, for $W_T \leq 60$ kJ/kg$_{SUSP.}$ the release of intracellular compounds upon PEF pre-treatment is mainly due to the effect of the specific energy input rather than that of the field strength.

These results are in agreement with the findings of Garcia *et al.* (2007) and Luengo *et al.* (2015), who concluded that the specific energy input plays an important role in the determination of the critical electric field strength causing an efficient cell disruption, required for a consistent leakage of valuable intracellular compounds.

Instead, when the highest energy input was applied (100 kJ/kg$_{SUSP.}$), the increase of E allowed to achieve significant higher releases of intracellular compounds, leading to 15.3-fold increase in proteins and 4.85-fold increase in carbohydrates, as compared to control samples, when the highest field strength (E = 30 kV/cm) was applied. However, as emerged from Figure VII.1, no statistical differences (p < 0.05) could be detected in terms of proteins or carbohydrates release from electropermeabilised microalgae for $E \geq 20$ kV/cm when the highest investigated energetic input was used.

The enhancement of intracellular compounds extraction from PEF treated *A. platensis* microalgae has been detected also by Aouir *et al.* (2015).

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These authors demostrated that the application of a PEF treatment of high intensity (46 kV/cm), carried out in a continuous system, allowed to speed up mass transfer rates through the electoporated membrane of the microalgae, leading to an average recovery yields of phycobiliproteins of 33.3% with respect to untreated samples.

Our results were also confirmed by visual observation of the clear supernatants obtained after centrifugation of PEF processed microalgae suspensions (Figure VII.2). In fact, while the supernatant obtained from centrifugation of fresh microalgal biosuspensions (control) appeared colorless, those obtained from PEF treatment were characterized by a blue color, due to the release of C-phycocyanin (water soluble protein), whose intensity increased with increasing the PEF treatment severity.
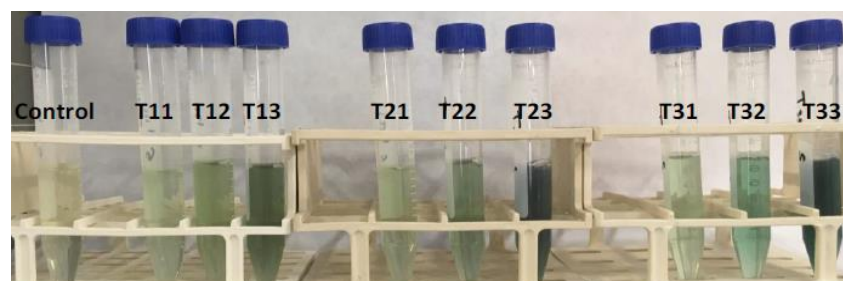
Figure VII.2 *Picture of supernatants obtained after centrifugation of untreated (Control) and PEF treated microalgal suspensions. Biomass concentration: 2%. T11: 10 kV/cm-20 kJ/kg, T12: 10 kV/cm – 60 kJ/kg, T13: 10 kV/cm -100 kJ/kg, T21: 20 kV/cm – 20 kJ/kg, T22: 20 kV/cm – 60 kJ/kg, T23: 20 kV/cm – 100 kJ/kg, T31: 30 kV/cm - 20kJ/kg, T32: 30 kV/cm – 60 kJ/kg, T33: 30 kV/cm – 100 kJ/kg.*

Complete cell disruption by HPH (P = 150 MPa, n_P = 3) revealed that proteins and carbohydrates amount of *A. platensis* cells were 68% DW and 16% DW, respectively (Figure VII.1).

Thus, as expected, it is likely that the cell disintegration degree induced by PEF was lower than that achieved upon the application of HPH treatment, due to a limited capability of favouring the mass transfer of larger molecules. Indeed, the amount of proteins released upon PEF treatment was 3-fold lower than that detected after HPH treatment.

On the other hand a considerable amount of smaller molecules like carbohydrates could be leaked out of the microalgal cells upon PEF application, leading to a percentage recovery of 82% as compared to the extraction yield of completely disintegrated biomass by HPH.

The different efficacy of PEF and HPH pre-treatment in inducing cell disintegration of *A. platensis* microalgal cells is also confirmed by the micrographs reported in Figure VII.3.

From the pictures it is possible to notice how the application of a PEF treatment led to a permeabilisation of membrane of algae cells with

subsequent release of intracellular compounds, without the formation of cell debris. In contrast, a complete disruption of the cells and the formation of small fragments was observed after HPH treatment.
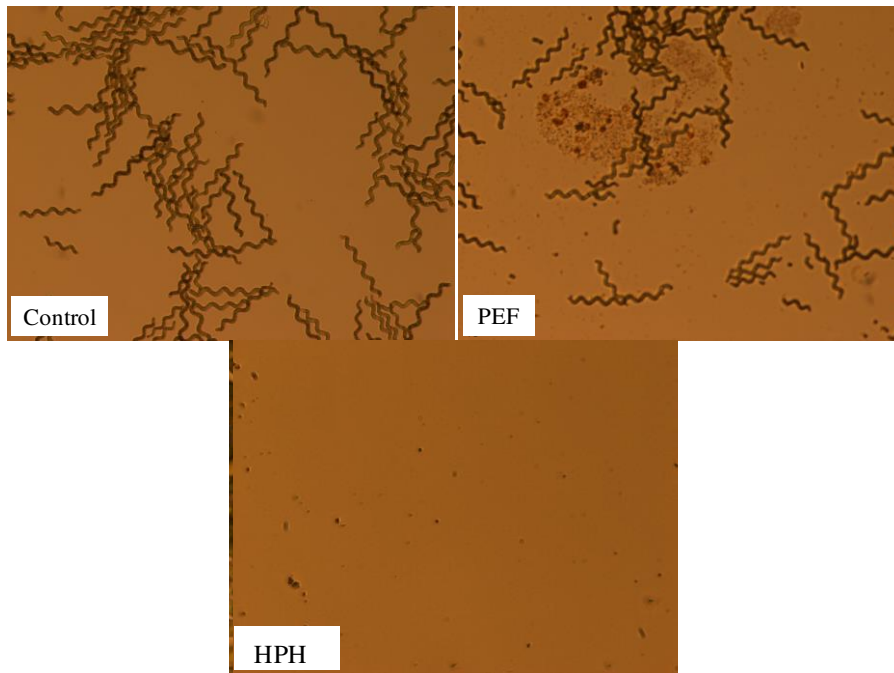
Figure VII.3 *Optical microscopy at 20x of Arthrospira platensis cells, before (control) and after either PEF (E = 20 kV/cm; W_T = 100 kJ/kg$_{SUSP.}$) or HPH (P = 150 MPa, n_P = 3) treatment.*

A similar behavior has been detected in the work of Martinez *et al.* (2016). The authors observed that while the application of a bead beating process was capable of fully disrupting *A. platensis* cells, PEF only lead to fragmentation of thricomes, with no visible effects on the whole structure of the microalgae.

This observation suggests that, despite a lower extraction efficiency with respect to conventional disintegration techniques (e.g. bead milling, high pressure homogenization), PEF technology may be helpful in granting a selective leakage of compounds from *A. platensis* microalgae towards the acqueous medium without the formation of large amounts of finely sized cell debris that negatively impact on the downstream separation process.

VII.3.1.2 Influence of pulse polarity and delaying time

In this study, the influence of pulse polarity and pulse delaying times on the permeabilisation efficiency of *A. platensis* microalgae has also been taken under consideration.

Despite the scarce literature evidence, previous studies on PEF technologies reported that the application of bipolar pulses appear to be more efficient than monopolar ones, involving lower energy consumption and reduced deposition of solids on the electrode surface, together with a decreased occurrence of electrolysis reactions (Chang, 1989; Qin *et al.*, 1994).

According to Brito *et al.* (2012), the use of bipolar pulses could likely increase the number of electroporated cells and improve PEF treatment performances due to a greater capability of the applied electric field to cover two different cell directions towards the electrodes. Moreover, reversing polarity may also be considered as an additional cause of cellular stress.

Figure VII.4 shows the content of water soluble proteins and carbohydrates in untreated and PEF treated extracts at constant intensity (20 kV/cm; 100 kJ/kg$_{SUSP.}$) with bipolar pulses and for variable delay times.
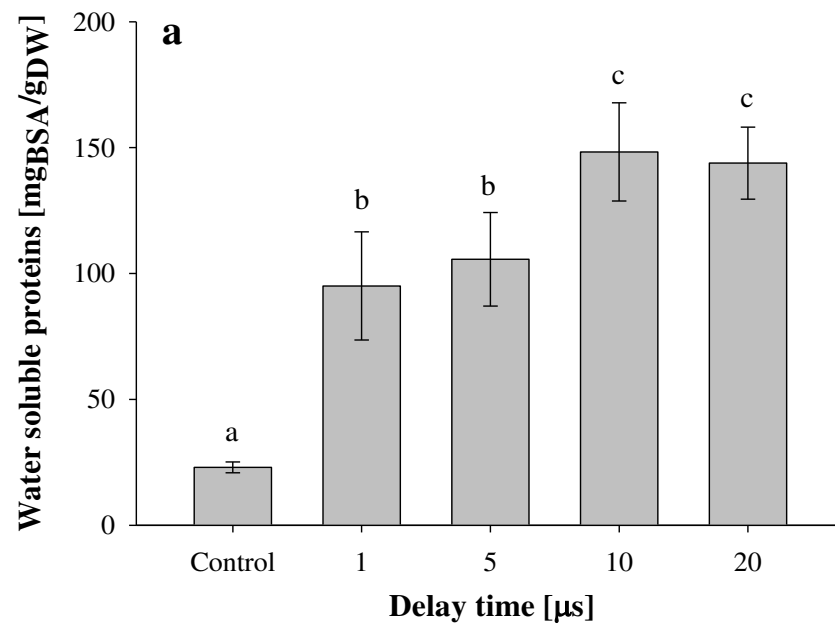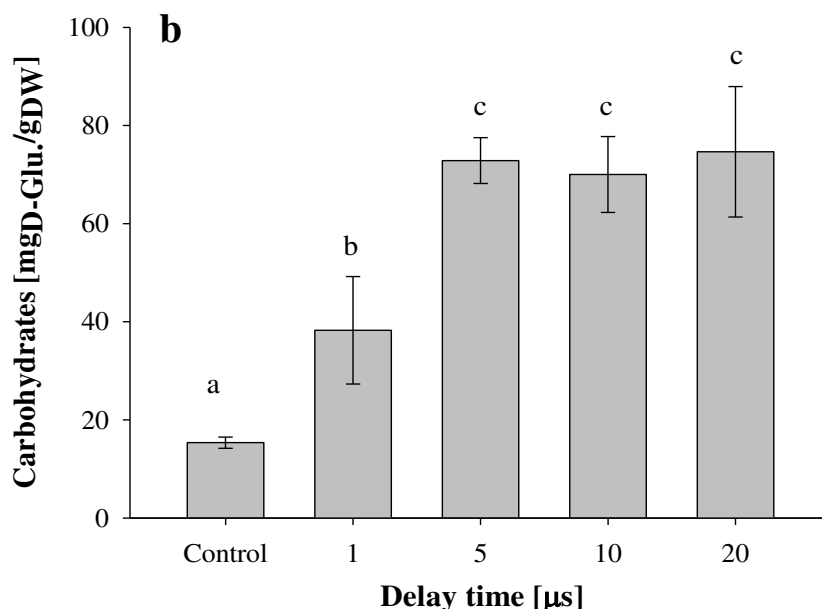
Figure VII.4 *Concentration of water soluble proteins (a) and carbohydrates (b) in the supernatant of untreated (control) and PEF (E = 20 kV/cm; W_T = 100 kJ/kg$_{SUSP}$) treated A. platensis suspensions with bipolar pulses, at variable delay time (1 – 20 μs). Different letters above the bars indicate significant differences among the mean values (p≤0.05).*

Results show an increase in the release of proteins and carbohydrates from PEF treated biosuspensions with respect to control samples, regardless of the applied delay time.

In particular, a delay time of 10 μs led to the maximum yield of water soluble proteins up to 148.27 mg$_{BSA}$/g$_{DW}$ (p < 0.05). No significant differences were revealed between 10 and 20 μs of delay time as well as between 1 and 5μs (p > 0.05). Instead, as regarding the carbohydrates concentration, the maximum yield was detected at 5μs of delay time, after which no additional effects could be detected.

However, when comparing the results of Figure VII. 4 with those obtained from the application of monopolar square wave pulses at the same PEF intensity (E= 20 kV/cm, W_T=100 kJ/kg$_{SUSP.}$ Figure VII.1), a significantly lower (p < 0.05) electroporation efficiency of bipolar pulses could be detected, with a 1.2-fold and a 1.79-fold reductions, respectively, in terms of water soluble proteins and carbohydrates extractability.

Different pulse delaying times yielded a differential amount of inactivation of *E. coli* O157:H7 inoculated into apple juice and skim milk as reported by

Evrendilek & Zhang (2005), which found that a 20 μs pulse delay was the most effective one among the investigated values (3 - 1430 μs).

Moreover, compared to untreated samples, PEF allowed to grant a 2.4 log reduction in the microbial load, on average, in the processed raw materials.

The authors explained these results concluding that most probably there was not enough time to charge capacitors after each discharging when the duration of pulse delay time was really short (3 - 5 μs), while as the pulse delay time was enlarged up to 1430 μs, it could be likely that such a long time interval between two pulses could adversely affect the performances of the treatment.

Our results seem to be consistent with current literature findings, where the usage of monopolar pulses has shown a greater capability of charging membrane of microorganisms, thus leading to higher cell disruption extents, with respect to bipolar ones (Beveridge *et al.*, 2002; Ibey *et al.*, 2014; Sweeney *et al.*, 2016).

From the overall analysis of the results presented in § VII.2.1, it is possible to state that the application of monopolar pulses allowed to obtain higher recovery yields of valuable compounds (e.g. proteins, carbohydrates) from *A. platensis* microalgae.

For this reason, further investigation on the proposed biorefinery scheme for the valorization of *A. platensis* biomass were conducted by using only monopolar pulses, as subsequently described in Chapter VIII (§ VIII.2.1.1-2).

VII.3.2 Extraction of valuable compounds from C. Vulgaris cells

VII.3.2.1 Impact of PEF and HPH treatments on the release of ionic intracellular components

The results of the electrical conductivity measurements of microalgae suspension have been successfully used as a valuable indicator to assess and quantify the amount of ionic intracellular components released from algae upon the application of the different cell disruption methods (Goettel *et al.*, 2013; Grimi *et al.*, 2014; Pataro *et al.*, 2017c; 't Lam *et al.*, 2017a).

Figure VII.5 shows the effect of PEF treatment intensity (E, W_T), as well as the number of HPH passes (n_P) on the conductivity profiles of *C. vulgaris* suspensions over time at 25 °C.
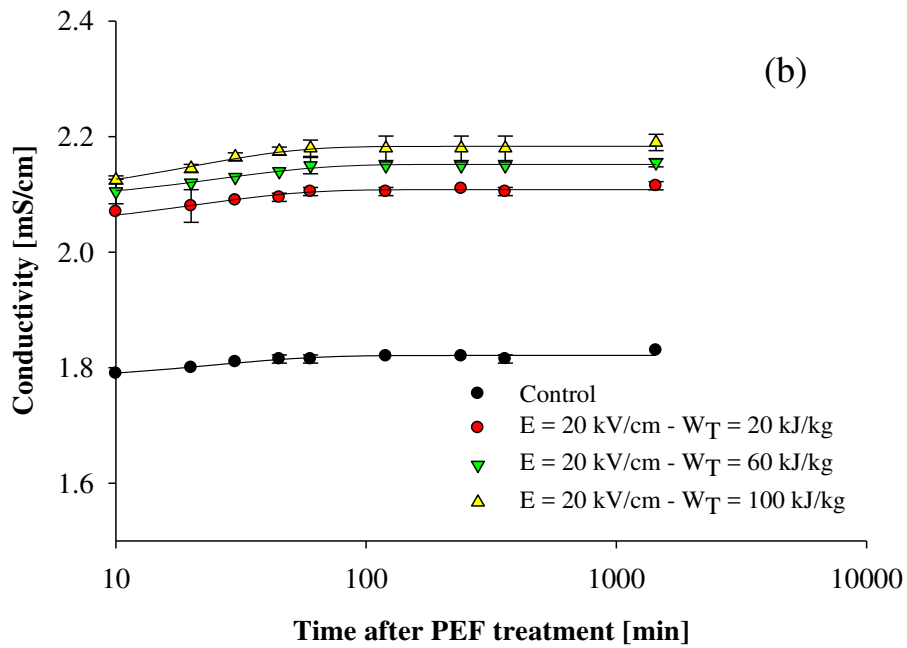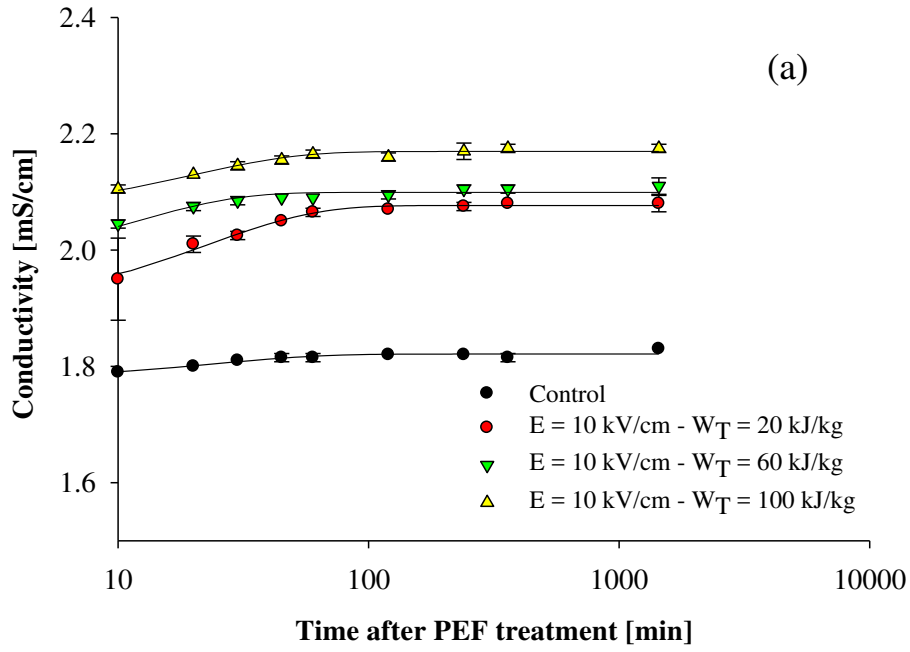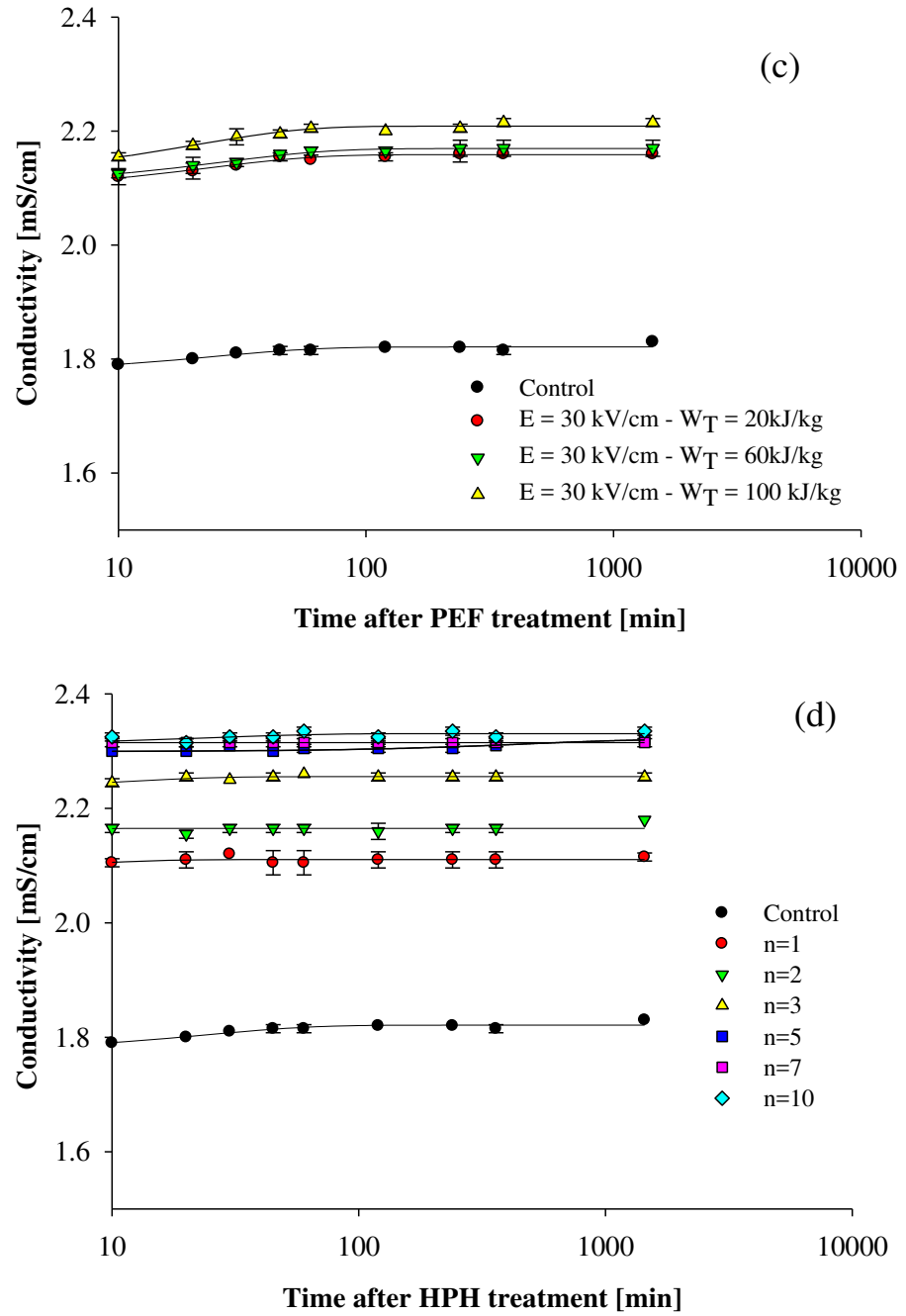
Figure VII.5 *Effect of incubation time after PEF and HPH treatment on electrical conductivity at 25 °C of (a-c) PEF (E=10-30 kV/cm; W_T=20-100 kJ/kg) and (d) HPH (150 MPa; np=1-10) treated C. vulgaris suspensions. Control means untreated suspension.*

For the sake of comparison, in the same graphs, also the time-conductivity profile of the untreated algae suspension is shown. Results demonstrate that the initial conductivity (1.78 mS/cm) of untreated suspension only slightly increased with the incubation time, likely due to a spontaneous release of a small fraction of intracellular ionic compounds, reaching a saturation value (1.82 mS/cm) already after 30 min of incubation.

The electroporation effect induced by the application of PEF treatment at different field strength (10-30 kV/cm) and energy input (20-100 kJ/kg) promoted a rapid release of the ionic intracellular compounds, which resulted in a substantial increase in the electrical conductivity, with respect to the untreated suspension (Figure VII.5a-c).

After PEF treatment, the saturation value, reached after 1 h of incubation, increased with the increase of the field strength and energy input, due to a faster diffusion of the ionic intracellular substances into the aqueous phase. A further increase of the incubation time did not cause any significant increase in the conductivity, which set to a final value in the range between 2.08 and 2.21 mS/cm, depending on the applied PEF treatment intensity.

A progressive increase of the content of ionic compounds in the extracellular medium when increasing the intensity of the PEF treatment was also observed by Goettel *et al.* (2013), which reported that 79% of the total released ions from *Auxenochlorella protothecoides* already occurred in the first hour after treatment. Similarly, Postma *et al.* (2016b) and Pataro *et al.* (2017c) reported that increasingly intense PEF treatments promoted the progressive permeabilization of the *C. vulgaris* cells and that an incubation time of 1 h was sufficient to allow small ions to diffuse out of the cells.

The data of Figures VII.5 a-c suggest the achievement of an irreversible electroporation after PEF treatment (Goettel *et al.*, 2013), by markedly improving the mass transfer rate of ionic compounds through the cell structure, which is partially damaged by the electrical treatment.

Coherently with this assumption, when compared to PEF treatments, the HPH treatments resulted in a significant increase in the conductivity of *C. vulgaris* suspension, whose extent was greater when increasing the number of HPH passes, as shown in Figure VII.5d. More specifically, the mechanical disruption of the algae cells appeared to be extremely fast, leading to an almost instantaneous diffusion of the intracellular compounds into the aqueous phase, as observed also by Safi *et al.* (2015).

Considering that HPH is a purely mechanical on-off disruption process, it is likely that after each pass a certain fraction of algae cells is completely broken, while the residual cells remain intact, in agreement with the observation of the significant extraction yield of ionic compounds after the multi-pass HPH treatment reported in Figure VII.5d.

Coherently, the results of Figure VII.5d also show that above 5 passes, the conductivity did not change significantly, and tended to an asymptotic value of 2.3 mS/cm, because the residual fraction of intact cells has become

extremely small. However, such asymptotic value was significantly higher than that measured after the application of the most intense PEF treatment, confirming that the release of ionic compounds by PEF is incomplete.

Thus, setting the conditions of $Z_P = 1$ in correspondence of 5 HPH passes, the cell disintegration efficiency of PEF varied in a range dependent on the treatment intensity applied: the lowest value of Z_P (0.47) was observed for a PEF treatment intensity of 10 kV/cm and 20 kJ/kg, whereas, increasing the electric field strength and energy input, a maximum Z_P value of 0.85 was recorded (data not shown).

VII.3.2.2 Effect of PEF and HPH treatment on C. vulgaris cell structure

In this work, particle size distribution (PSD) analyses and SEM observations were carried out in order to gain insight on the impact of PEF and HPH treatments on the size and structure of *C. vulgaris* cells.

Figure VII.6 depicts the mean particle size $D_{4,3}$ for untreated (control), PEF treated at variable field strength and energy inputs, and HPH ($n_P = 5$) treated microalgae suspensions.
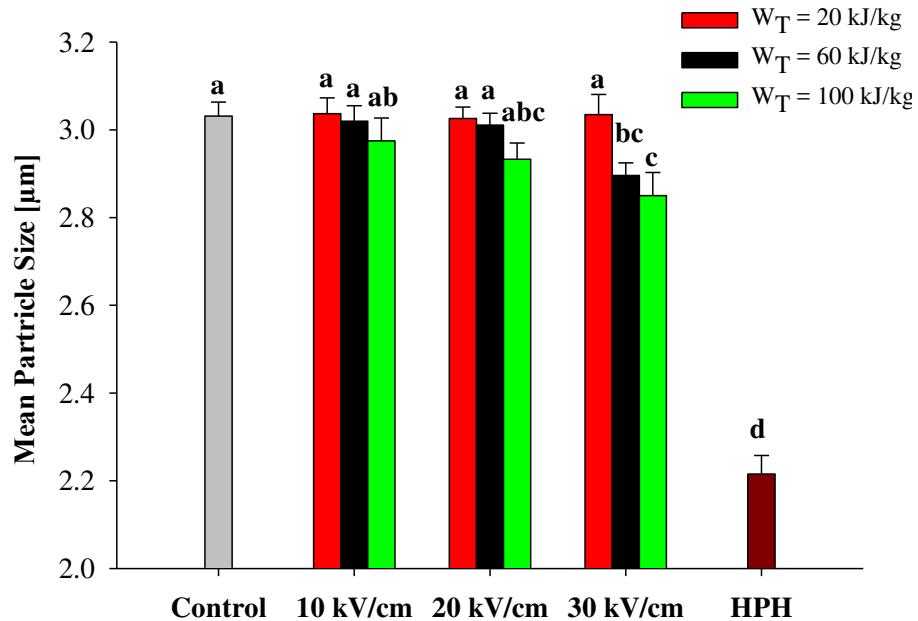
Figure VII.6 *Mean particle size of untreated (control), PEF treated (E=10-30 kV/cm; W_T=20-100 kJ/kg) and HPH treated (P = 150 MPa; $n_P = 5$) C. vulgaris suspension. Different letters above the bars indicate significant differences among the mean values of the samples (p≤0.05).*

The PSD curves of untreated algae suspension revealed the presence of a single peak between 1 and 10 μm (data not shown), which was characterized by a mean cell size of 3.03 μm (Figure VII. 6).

The size distribution curve of PEF-treated algae suspension was very similar to that of the untreated sample (data not shown), showing only a slight decrease of the mean cell size with increasing the treatment intensity (E and W_T). In fact, the value of the mean cell size significantly ($p \leq 0.05$) decreased by about 6% only upon the application of the most intense PEF treatment conditions (E=30 kV/cm, $W_T \geq 60$ kJ/kg) (Figure VII.6).

These results seem to confirm that PEF is a relatively mild cell disruption method, preserving the initial structure of the algae cells.

The application of 5 HPH passes, instead, leads to a significant change in the PSD curves of the microalgae suspension, highlighting a bimodal distribution, in which a second peak between 0.1 and 1 μm appeared (data not shown). As a result, a strong reduction in the mean cell size down to a value of 2.22 μm was observed, which is likely due to the complete cell disruption and the consequent formation of cell debris.

Partially in contrast with these results, Spiden *et al.* (2013) found that the application of a HPH treatment on *Chlorella* microalgae at different pressures (P = 30 – 107 MPa) only lead to a slight decrease in the mean cell size, which was in agreement with the only partial fragmentation achieved. Eventually, in our case, the application of a higher pressure (P = 150 MPa) was capable of inducing the complete disruption of the cells, which is in agreement with the previous findings of Safi *et al.* (2015). Similarly, Shene *et al.* (2016) and Samarasinghe *et al.* (2012), studying the effect of HPH processing (P = 70 – 310 MPa, n_P = 1 – 6) on *Nannochloropsis oceanica* microalgae, reported that the cells were fully disrupted in fragments, with a corresponding decrease in their mean particle size.

In order to better interpret the results of Figures VII.5 - 6, also SEM analyses were carried out on untreated, PEF-treated (E = 20 kV/cm; W_T = 20 – 100 kJ/kg), and HPH-treated (n_P=5) microalgae, as shown in Figure VII.7.

Untreated *C. vulgaris* cells exhibited a characteristic near-spherical shape and a diameter ranging from 1.5 and 4.5 μm, which relate to the findings reported in the current literature (Suali *et al.*, 2012).

The SEM images of Figure VII.7 highlight the different impact of PEF and HPH treatments on the microalgal cell structure.

Interestingly, the results clearly show, for the first time, the occurrence of a "shrinkage" phenomenon in PEF-treated algae cells, which gradually lose their initial near-spherical shape with increasing the applied energy input but were never disintegrated into cell debris. The observed shrinkage could be associated with the partial release of the intracellular compounds through the electroporated cell membranes (PEF 2), which led in some cases to cell collapse (PEF 3). Similar results were observed at different electric field strengths (data not shown).

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In contrast, a complete disruption of the cells and the formation of small fragments was observed after 5 passes of HPH treatment, which was consistent with the results of Figures VII.5 - 6.
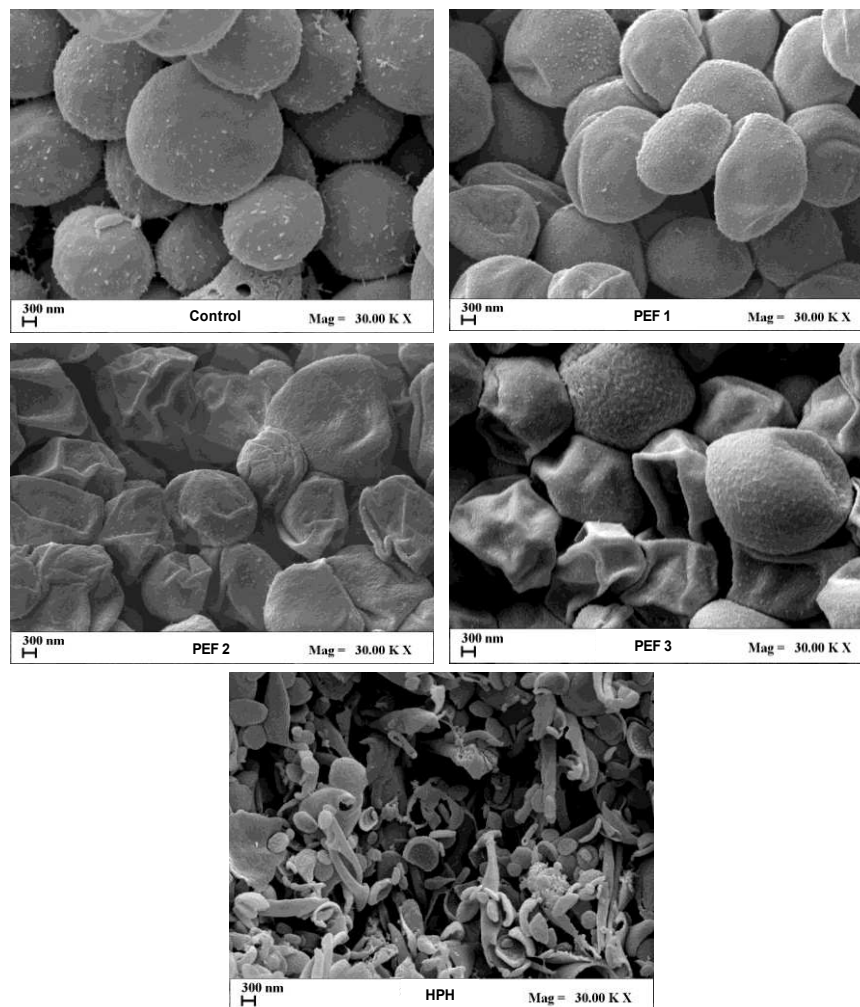
Figure VII.7 *Scanning electron microscopy (SEM) of C. vulgaris cells before (Control) and after PEF (20 kV/cm) at total specific energy input of 20 kJ/kg (PEF1), 60 kJ/kg (PEF2), 100 kJ/kg (PEF3), and after HPH (P = 150 MPa; n_P = 5) treatment of the microalgal suspension.*

Similarly, the formation of cell fragments was observed by other authors upon the application of HPH treatments to *Chlorella* (Choi & Lee, 2016; Safi *et al.*, 2015) and *Neochloris abundans* (Wang *et al.*, 2015) microalgae, highlighting the strong efficacy of HPH treatment as a method of complete cell disruption.

VII.3.2.3 Influence of PEF and HPH treatments on the release of intracellular compounds

The cell disruption efficiency of PEF and HPH treatments were also compared by monitoring the extractability of intracellular compounds by dry matter analyses and by measuring the amount of water-soluble compounds (proteins and carbohydrates) released into the supernatants obtained from untreated and treated (PEF, HPH) algae suspension.

The total amount of released intracellular compounds (Figure VII.8) was evaluated by measuring the dry matter content in the supernatant of untreated, PEF-treated at different field strength and energy inputs, and HPH-treated (n_P=5) microalgae suspensions.
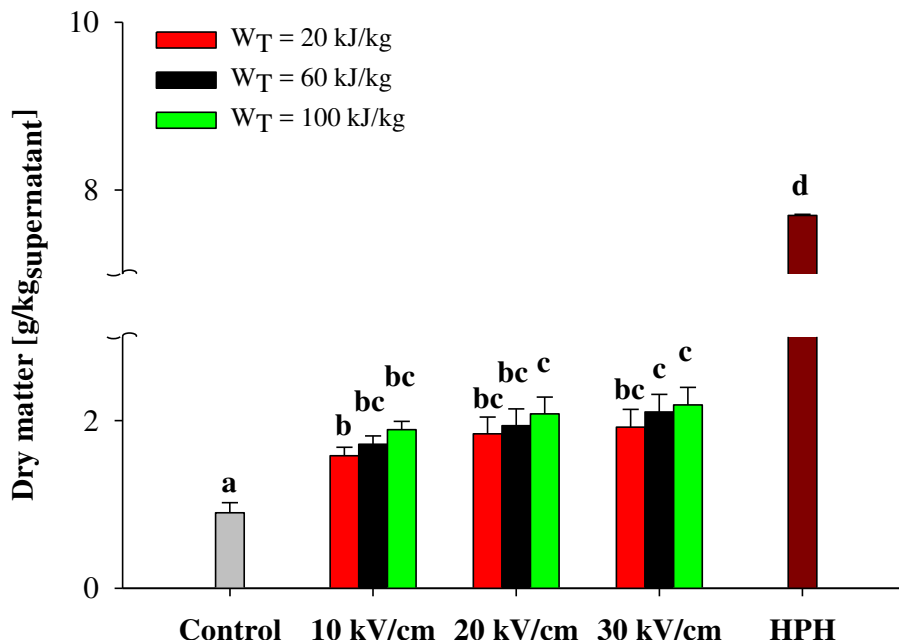
Figure VII.8 *Dry matter content in the supernatant of untreated (Control) and treated C. vulgaris suspension 1 h after PEF (E=10-30 kV/cm; W_T=20-100 kJ/kg) or after HPH (P = 150 MPa; n_P = 5) treatment. Different letters above the bars indicate significant differences among the mean values of the samples (p≤0.05).*

The results shown in Figure VII.8 are in agreement with the conductivity measurements of Figure VII.5. The application of PEF treatment markedly increased the dry matter content of supernatants, when compared with the untreated sample. Higher field strengths and energy inputs resulted in a greater extent of membrane permeabilization, leading to a significantly

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($p \leq 0.05$) higher release of intracellular compounds into the aqueous phase. The maximum value of dry matter content was detected at the most intense PEF treatment conditions (E = 30 kV/cm; W_T = 100 kJ/kg), which was 2.4 times higher than that detected in the supernatant of the untreated microalgae suspension. However, among PEF treated samples, statistically significant differences ($p < 0.05$) were observed only between samples treated at 10 kV/cm and 20 kJ/kg with those treated either at 20 kV/cm and 100 kJ/kg or at 30 kV/cm for an energy input greater than 20 kJ/kg.

Remarkably, the results of Figure VII.8 are in agreement with the previous findings of Goettel *et al*. (2013). The authors observed a continuous increase of cell components in the medium surrounding *Auxenochlorella prototothecoides* microalgae when the energy input was increased up to 200 kJ/kg at constant field strength (34 kV/cm). Moreover, in our case, the release of intracellular soluble compounds by PEF varied in the range 13 – 18 % of total cell dry weight, which is also in agreement with the results obtained by Goettel *et al*. (2013), who found that a PEF treatment at 30.5 kV/cm and 155 kJ/kg caused the spontaneous release of intracellular matter up to 15% of the initial biomass dry weight (109 g/kg$_{DW}$).

Pataro *et al*. (2017c) also observed a slightly higher leakage of intracellular matter from *C. vulgaris* cells with increasing the field strength (from 27 to 35 kV/cm) and energy input (from 50 to 150 kJ/kg).

The stronger cell disintegration effect, achieved after 5 passes of HPH treatment (Figures VII. 5 - 7), led to a highly efficient extraction of intracellular matter (Figure VII. 8), whose extent reached up to 64% of the total cell dry weight.

The results of Figure VII. 8 were also confirmed by visual observation of the supernatants. In fact, while the supernatants obtained from centrifugation of fresh and PEF treated microalgal suspensions appeared colorless, those obtained from HPH treated samples were characterized by a green color (data not shown). This was likely due to the presence of cell debris containing green pigments, which, being extremely reduced in size, did not precipitate in the pellet after centrifugation (Safi *et al*., 2015).

With this assumption, it can be stated that part of the supernatant dry matter content from the HPH treated cells could be due to the presence of submicrometric residues, which remained suspended in the aqueous phase, making the downstream separation processes more difficult.

Figure VII. 9 shows the concentration (on DW basis) of carbohydrates (a) and proteins (b) detected in the aqueous supernatant of untreated and PEF treated samples, 1 h after their collection, at different field strengths and energy inputs.
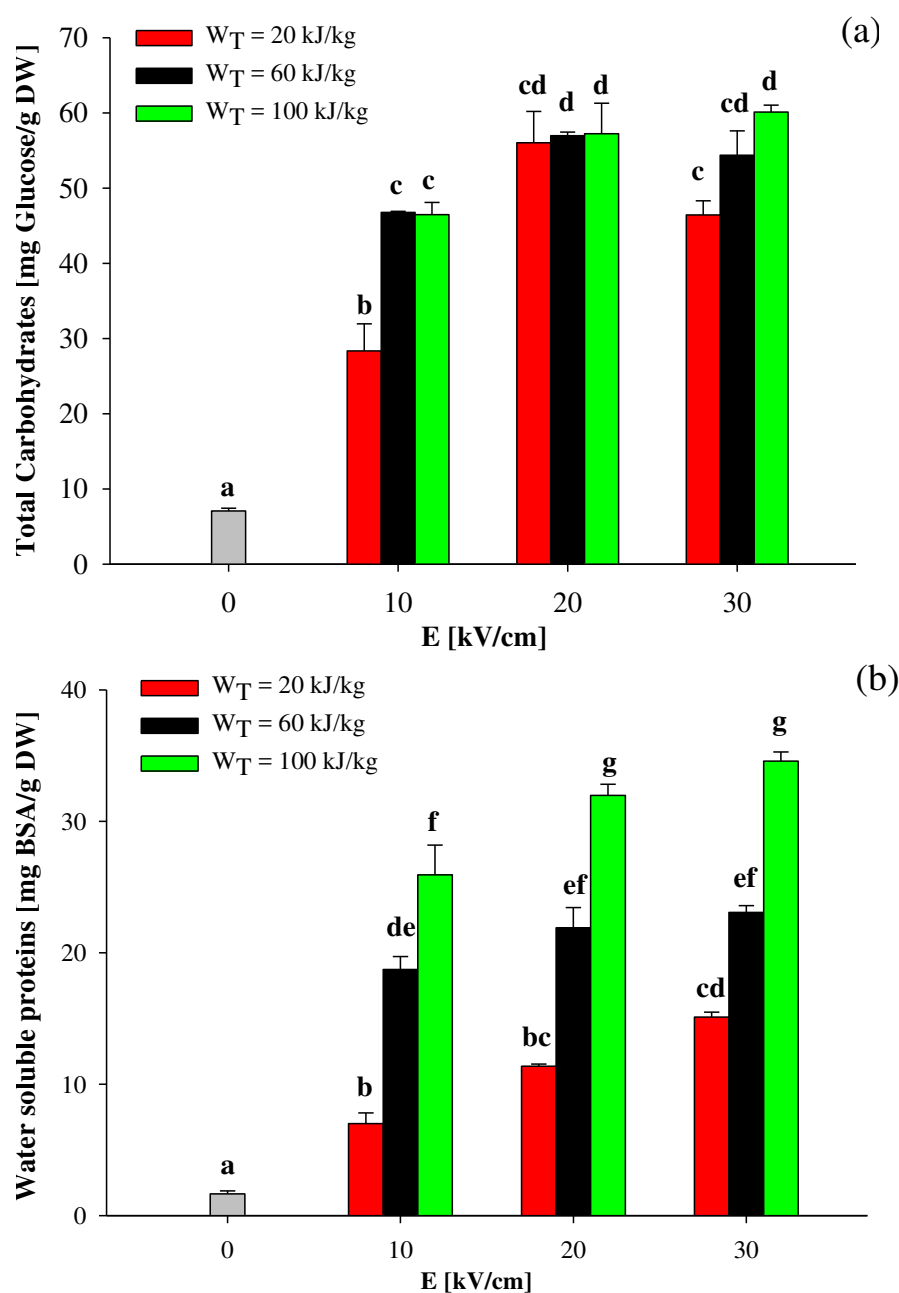
Figure VII.9 *Concentration of carbohydrates (a) and water soluble proteins (b) in the supernatant of untreated (0 kV/cm) and treated C. vulgaris suspension 1 h after PEF treatment as a function of the field strength and for different energy inputs. Different letters above the bars indicate significant differences among the mean values of the samples (p≤0.05).*

When no PEF treatment was applied, only very low amounts of carbohydrates (7.06 $mg_{D-Glu.}/g_{DW}$) and proteins (1.65 mg_{BSA}/g_{DW}) were released in the aqueous phase, which may be ascribed to either a concentration gradient across the intact cell membranes or to a spontaneous cell lysis.

The permeabilization effect of the cell membranes induced by the application of PEF treatment, instead, improved the mass transfer of intracellular compounds, leading to a significantly ($p \leq 0.05$) higher content of both carbohydrates and proteins, as compared to the untreated samples, being the extraction efficiency increased up to 20-fold for proteins and 8-fold for carbohydrates.

Among the PEF treated samples, the effect of the field strength applied (Figure VII. 9) appeared less important than that of the energy input within the investigated range, especially for the protein extraction, which is in agreement with the results of Figure VII.1 as well as with previous literature findings (Pataro *et al.*, 2017c; 't Lam *et al.*, 2017a).

In particular, a significant ($p \leq 0.05$) increase in the content of both intracellular compounds was detected only when the field strength was increased from 10 to 20 kV/cm and for a fixed energy input of 100 kJ/kg for proteins, and 20 kJ/kg for carbohydrates, respectively.

In contrast, while significant differences ($p \leq 0.05$) in the protein content were detected when PEF treatments were carried out at different energy inputs, regardless of the field strength applied, a slight effect of the energy input was observed for the extraction of carbohydrates, which was significant ($p \leq 0.05$) only when the energy input was increased from 20 to 60 kJ/kg at 10 kV/cm and between 20 and 100 kJ/kg at 30 kV/cm.

A slightly increasing trend when raising the energy input from 50 to 150 kJ/kg was previously observed by both Goettel *et al.* (2013) with the microalgae *A. protothecoides* at a fixed field strength applied of 34 kV/cm, and by Pataro *et al.* (2017c) with the microalgae *C. vulgaris* at a fixed field strength applied of 27 kV/cm. Postma *et al.* (2016b), instead, did not find any significant difference in the release of carbohydrates from *C. vulgaris* treated by PEF at 50 and 100 kJ/kg at 17.1 kV/cm.

From the results of Figure VII. 9 it can be concluded that a field strength of 20 kV/cm and an energy input of 100 kJ/kg could allow to achieve the maximum permeabilisation effect, due to an efficient release of carbohydrates and, to a lower extent, of water soluble proteins.

In particular, assuming a carbohydrates and proteins content of 16% and 61 % on DW for *C. vulgaris* microalgae, respectively (Postma *et al.*, 2016b), the amount of these compounds released after PEF treatment (20 kV/cm, 100 kJ/kg) was 35.8% (w/w) of total carbohydrates (approximately 5.7% DW biomass) and 5.2% (w/w) of total proteins (approximately 3.2% DW biomass). These values are in the same range of values reported by other authors (Barba *et al.*, 2015b; Goettel *et al.*, 2013; Grosso *et al.*, 2015;

Luengo *et al*., 2015; Safi *et al*., 2017a). In the study of Postma *et al*. (2016b), for example, it was observed that the application of a PEF treatment at room temperature resulted in an extraction yield of 22-24% for carbohydrates, and 3.2-3.6% for proteins, when the energy input was increased between 50 and 100 kJ/kg at a field strength applied of 17.1 kV/cm. Moreover, no further improvement of the diffusion kinetics of intracellular compounds was detected when PEF effect was combined with the thermal treatments at a higher temperature (Postma *et al*., 2016b) or elevated pH (Parniakov *et al*., 2015e).

These results suggest that PEF was successful in opening pores on membranes of *C. vulgaris* cells (Figures VII. 5 - 7), allowing the selective release of carbohydrates and small-sized cytoplasmic proteins, while simultaneously hindered the diffusion of the majority of proteins, which are likely larger and more bound to the cell structure. This hypothesis is supported by some literature evidence.

In fact, it has been reported that most of the proteins of *C. vulgaris* species have molecular weights ranging from 12 to 120 kDa (Safi *et al*., 2015) and that PEF was able to selectively enhance only the extraction of small protein materials, with molecular weight lower than 20 kDa, while larger molecules remained entrapped inside the cells, being unable to cross the permeabilized cell membrane (Postma *et al*., 2016b). In contrast, as suggested by the SEM images (Figure VII. 7), PEF merely electroporated the algae cells without altering the extremely resistant rigid cell wall of *C. vulgaris*, which represents a further barrier against the extraction of proteins (Coustets *et al*., 2013). Moreover, it is estimated that 20% of *C. vulgaris* proteins are bonded to the cell wall (Berliner, 1986), and therefore they likely remained entrapped in the pellet along with the water-insoluble fraction of proteins. This would contribute to furtherly explain the relatively low amount of proteins released after PEF (Figure VII. 9b).

Therefore, the disruption of the rigid cell wall of *Chlorella vulgaris* appears to be a crucial step to enhance the protein release (Safi *et al*., 2014), hence requiring a more effective cell disruption technique, such as high pressure homogenization (Poojary *et al*., 2016).

Figure VII. 10 reports the amount of carbohydrates and proteins released upon the application of HPH treatment (150 MPa) as a function of the number of passes. In agreement with the results of Figure VII. 5d, a significant fraction of *C. vulgaris* cells was already disrupted after 1 pass and water gained the access to the cytoplasmatic content, allowing the release of a certain amount of carbohydrates and proteins.
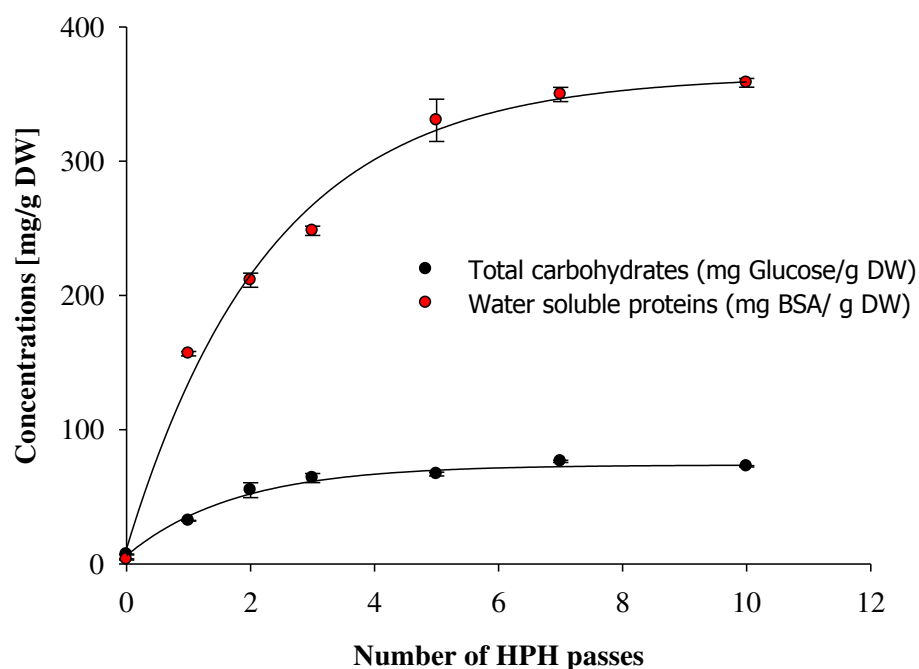
Figure VII.10 *Concentration of water soluble proteins and carbohydrates in the supernatant of untreated (n_P=0) and HPH (P = 150 MPa) treated C. vulgaris suspension as a function of the number of passes. Experimental data were fitted by exponential saturation models (R^2 > 0.95).*

The subsequent HPH passes led to the further release of carbohydrates and proteins, whose amount gradually increased up to reaching a saturation value after 5 cycles, which was, with respect to the control sample, 9-fold higher for carbohydrates and 200-fold higher for proteins.

An asymptotic behavior in the extraction yield of intracellular compounds, such as chlorophyll and carotenoids, as a result of the increased degree of cell disruption with increasing the number of passes has previously been shown by Xie *et al.* (2016). These authors reported that the release of these pigments from HPH-processed *Desmodesmus* microalgae could be enhanced by increasing the number of passes up to a saturation value above which no additional leakage of interest compounds could be achieved.

From the results of Figure VII. 10, using the same assumption for the composition of *C. vulgaris* cells used for PEF (Postma *et al.*, 2016b), the amount of carbohydrates and proteins released after 5 HPH passes was 41.9% (w/w) of total carbohydrates (approximately 6.7% DW biomass) and 54.1% (w/w) of total proteins (approximately 33.0% DW biomass).

Similarly, Safi *et al.* (2014; 2015) found that, among the different cell disruption techniques, including the chemical treatments, ultrasonication, and manual grinding, HPH was the most efficient one, and that after a HPH treatment (P=270 MPa, n_P=2) water gained rapid access to the cytoplasmic

proteins and infiltrated the chloroplast to recover 50-66% of the total protein content of *C. vulgaris* cells. However, even from these results it appears that, despite the higher cell disruption efficiency of the HPH treatment with respect to PEF, the complete release of all the proteins contained in the algae could not be reached, because of the rigidity of the cell wall (Scholtz *et al.*, 2014), as well as the insoluble nature of some proteins that remained in the pellet (Safi *et al.*, 2017b). In this frame, it has been demonstrated that the combination of higher HPH pressure than that used in our work with chemical cell lysis could furthermore improve the extractability of proteins from algae cells. In particular, Ursu *et al.* (2014) observed that 2 HPH passes at 270 MPa allowed the recovery of 98% of total proteins content of the microalgae *C. vulgaris* when the pH of the suspension was maintained at 12. The comparison between the results of Figures VII. 5 and 9 highlights the capacity of PEF to efficiently release low molecular weight molecules, such as carbohydrates, to an extent comparable to the one obtained from HPH treatment for a sufficiently high number of passes (85.4%).

This selectivity of PEF towards the carbohydrates could be advantageously exploited for specific applications ('t Lam *et al.*, 2017a).

In contrast, despite the huge increase in protein extraction caused by PEF processing with respect to untreated microalgae suspension, the obtained yields are still relatively low, being 10-fold lower than that detected in HPH treated samples.

VII.4 Conclusions

The present study provided additional insights into the impact of PEF and HPH technologies on the disintegration efficiency of microalgal cells and into the subsequent recovery of the main intracellular compounds, namely carbohydrates and proteins. General conclusions could be addressed to this study, respectively for each investigated microalgae, as follows:

- Case study 1 (*A. platensis*): Extraction yield of bioactive molecules from PEF treated microalgae resulted to be influenced by both the applied electric field (10-30 kV/cm) and energy input (20–100 kJ/kg), with monopolar pulses being more effective than bipolar ones.

   Moreover, independently on the applied treatment intensity, no cell debris was formed, thus allowing an easier solid/liquid separation in downstream processes.

   In conclusion, despite a lower proteins content of PEF treated extracts (34% DW on total proteins) with respect to the maximum value achieved upon HPH treatment, PEF was capable to induce the release of comparable amount of carbohydrates (84% DW of total carbohydrates) as for HPH.

-   Case study 2 (*C. vulgaris*): PEF resulted to be a relatively mild cell disruption method, which merely electroporates the algae cells, allowing to selectively enhance the extraction yield of small ionic substances and carbohydrates to an extent comparable to that achieved by HPH.

    The extraction efficiency of proteins, instead, was relatively low and did not exceed 5.2% of the total proteins amount.

    HPH, instead, was able to completely disrupt the microalgae cells, favoring an instantaneous and efficient release of all the intracellular material, including a large amount of proteins, whose release was 10.3 fold higher than that achieved by PEF.

    However, despite the higher extraction efficiency, the formation of large amounts of finely sized cell debris by HPH significantly complicates any downstream separation process.

From the overall results reported in this Chapter, optimal cell disruption conditions were identified for PEF (E = 20 kV/cm; W_T = 100 kJ/kg$_{SUSP}$), independently on the considered microalgal strain, and subsequently tested in a cascade biorefinery (Chapter VIII), in order to maximize in a selective and sustainable way the extraction yield of target compounds, by reducing the overall processing costs, which nowadays represent the main bottleneck to the full exploitation of microalgal biomass.

Chapter VIII
Hurdle approach in the biorefinery of microalgae

Abstract − In this chapter, a hurdle approach or cascade biorefinery of microalgae for the full exploitation of either *A. platensis* or *C. vulgaris* biosuspensions were specifically designed, based on the results achieved in Chapter VII. In particular, the application of a series of cell disintegration steps based on pulsed electric fields (PEF, E = 20 kV/cm; W_T = 100 kJ/kg$_{SUSP.}$) alternatively in combination with moderate heating (T = 25 – 45°C) or high shear homogenisation (t = 1 min, ω = 20000 rpm) for *A. platensis* and high pressure homogenisation (HPH, P = 150 MPa; n_P = 5) for *C. vulgaris* was carried out, in order to understand whether the achieved membrane permeabilization could potentially allow the selective and efficient release of intracellular compounds. As a sake of comparison, the effects of single PEF, mild heating, HSH and HPH treatments on the recobery yields of valuable molecules from both microalgal strains were compared to those observed in the cascade approach.

Based on the results obtained in this "biorefinery approach" study, the application of HSH prior to the PEF permeabilisation step of *A. platensis* microalgae showed a synergistic effect in the extractability of C-phycocyanin (C-PC, 94.24 mg/g$_{DW}$), whose recovery yield was significantly higher than that detected when coupling PEF with mild temperature (35°C).

Instead, the application of a series of PEF and HPH treatments granted a substantial release of small molecules like carbohydrates and lipids from *C. vulgaris* microalgae, with comparable or higher yields with respect to those obtained after a full permeabilisation stage achieved by HPH.

The results shown in this chapter suggest the possibility to apply PEF in combination with HSH or HPH technologies in hurdle approach, with the aim to valorise microalgal biomass by significantly reducing permeabilisation operative costs, as laterly shown in the energy analysis of Chapter IX.

VIII.1 Introduction

The algal biorefinery is a facility that integrates biomass conversion and separation processes to produce fuels, power, and value-added chemicals (Khan *et al.*, 2009), in which the objective is to obtain several products by using mild separation processes.

Microalgae contain high amounts of lipids, proteins and carbohydrates, of interest for different markets (Khan *et al.*, 2009; Vanthoor-Koopmans *et al.* 2013). In particular, lipids can be used as a source for biofuels, as building blocks in the chemical industry and as edible oils for the food and health market (Vanthoor-Koopmans *et al.* 2013).

Proteins and carbohydrates may find applications in the food, feed, health and bulk chemical market or for producing ethanol and chemicals, respectively (Radakovits *et al.*, 2010).

Given the differences in the properties of their intracellular compounds, microalgae may be suitable for being treated in a "biorefinery scheme", from which different classes of compounds may be recovered in a multi-stage processing operation (Wjiffels *et al.*, 2010).

According to the biorefinery approach, the first and most crucial step after microalgae harvesting is represented by cell disintegration pre-treatment, which aims to gently permeabilize the cell wall/membrane system in order to favour the extraction of valuable compounds from both cytoplasm and internal organelles (Gunerken *et al.*, 2015), without worsening the quality and purity of the extracts, and irreversibly reducing the product value (Postma *et al.*, 2016b).

For this reason, the need for a progressive permeabilisation strategy which may lead to a selective recovery of different compounds arises, with the aim to fully valorize microalgal biomass through an efficient and sustainable process of biorefinery, granting also a lowering of the energy requirements needed for the cell disintegration step.

For example, the work of Postma *et al.* (2016b) has highlighted that the electropermeabilisation effect induced by PEF can significantly increase the release of small molecules such as ions and carbohydrates from *Chlorella vulgaris* biosuspensions. However, the investigated electric conditions (E = 20 kV/cm; $W_T = 50 - 100$ kJ/kg$_{SUSP.}$) were not sufficient to furtherly enhance the extraction yields of more complex compounds (e.g. proteins).

Therefore, the authors suggested using PEF as the first step of microalgal permeabilisation, useful for recovering a large amount of carbohydrates, followed by a more disruptive and efficient technology such as bead milling (Postma *et al.*, 2014; 2016a) in a hypothetic cascade biorefinery approach. However, bead milling exhibits a series of disadvantages in terms of scalability (processing times) and product degradation.

In the work of Grimi *et al.* (2014), a series of permeabilisation steps based on both innovative (PEF, US, HVED) and conventional (HPH) technologies

has been performed with the aim to ameliorate the extraction rate of intracellular compounds from *Nannochloropsis spp.* microalgae.

The authors concluded that very high energy consumptions were needed, especially in correspondence of HPH treatments, for an efficient release of water soluble proteins (91%), with PEF giving a negligible contribution to cell permeabilisation. Moreover, the complete cell disruption due to HPH processing led to an undifferentiated release of intracellular compounds, thus requiring further economical efforts for an efficient downstream processing.

However, due to the possibility to exploit PEF technology in granting a selective and efficient release of small sized intracellular compounds (ions, carbohydrates, low molecular weight peptides, Chapter VII), it might be interesting to assess the potentiality of the electroporation phenomenon in a *ad hoc* designed "biorefinery scheme" where some other permeabilisation technologies could be applied, depending on the microalgal cell structure and the final products to be recovered, thus leading to the full valorization of the biomass (Gunerken *et al.*, 2015).

To the best of our knowledge, no works in literature are dealing with the design and reproduction of a PEF-based biorefinery process for achieving full valorization of microalgal biomass, despite different hypotheses have been formulated (Goettel *et al.*, 2013; Grimi *et al.*, 2014; Parniakov *et al.*, 2014b; Postma *et al.*, 2016b).

In this Chapter, two different approaches were adapted to the investigated microalgal strains in this thesis work, and the principal aims were:

1) To apply PEF technology in combination with either moderate temperature (25 – 45°C) or High Shear Homogenisation technique (t = 1 min; ω = 20000 rpm) in order to assess the efficacy of the combined treatment, in terms of selectivity and extraction yields of proteins (C-PC) from *A. platensis* as compared to the application of single PEF, mild temperature or HSH treatments (§ III 3.6.3.1);

2) To verify the efficacy of a cascade biorefinery scheme based on two consecutive extraction stages, namely PEF-assisted extraction followed by HPH-assisted extraction, on the selective recovery of proteins, carbohydrates and lipids from microalgae *C. vulgaris* suspension. Specifically, in the first extraction stage, PEF was used as a mild permeabilisation pre-treatment for the initial extraction of low molecular weight intracellular compounds (small proteins, carbohydrates and lipids), while in the second stage HPH treatment was conducted, in order to fully disrupt the microalgal cells, thus allowing the recovery of the intracellular compounds of interest that were still retained by the cells after the first extraction stage (§ III 3.6.3.2).

VIII.2 "Short" Materials and Methods

VIII.2.1 Hurdle approaches: combination of PEF with mild temperature, HSH or HPH technologies

In the previous chapter, optimal PEF conditions granting the highest release of water soluble compounds (mainly proteins and carbohydrates) from both investigated microalgal biosuspensions have been selected and applied in combination with physical (temperature) or mechanical (HSH, HPH) disruption stages, as clearly reported in § III.3.6.3 and schematized in Figures III.17 – 18.
The leakage of intracellular compounds from untreated or disrupted microalgae was achieved via water diffusion (§ III.3.6.2) while, only in the case of *C. vulgaris* cells, an organic diffusion step was performed with the aim of recovering lipids, by following the procedure reported in § III.3.8.6.

VIII.2.2 Analytical determinations

For all the investigated samples (untreated, single stage and multiple stage disrupted cells), the analyses on both biosuspensions (optical and SEM microscopy) and aqueous or organic extracts (dry matter of supernatants, water soluble proteins, C- phycocyanin, carbohydrates and lipids) were performed according to the procedures described in § III.3.7 and § III.3.8.

VIII.3 Results and Discussion

VIII.3.1 Use of PEF in a "Hurdle approach" for the biorefinery of A. platensis microalgae

In this section, the use of PEF in a hurdle approach with either moderate temperature or HSH pre-treatment on the biorefinery of *A. platensis* microalgae was investigated.
In particular, results expressed in terms of water soluble proteins and C-PC content, as well as of purity ratio of extracts are reported and compared with those achived upon the application of single PEF, mild heating and HSH treatment. Finally, a qualitative comparison of single and combined treatments based on the selective release of specific classes of compounds has been conducted by UV-Vis spectra analysis.

VIII.3.1.1 Effect of PEF-mild temperature treatments on the extractability of valuable compounds

Figure VIII.1 shows the C-PC yield and purity ratio of extracts obtained after either single mild heating at temperature from 25°C to 45°C and combined PEF (20 kV/cm; 100 kJ/kg$_{SUSP}$) + mild heating of *A. platensis* suspensions. Results show that only a small amount of C-PC was able to diffuse out of the microalgal cells of the untreated biosuspensions, in the investigated temperature range.
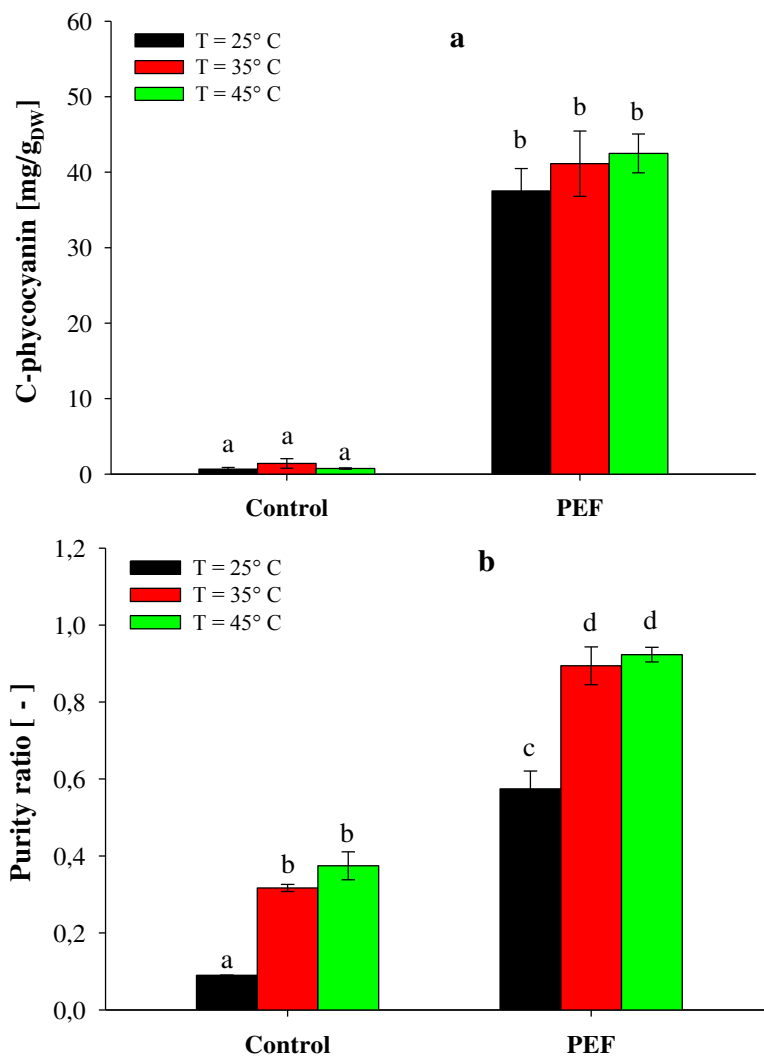
Figure VIII.1 *C-PC content (a) and purity ratio (b) in supernatants from untreated (Control) and PEF (20 kV/cm; 100 kJ/kg$_{SUSP.}$) treated A. platensis suspensions, at different inlet processing temperature (T = 25 – 45°C).*

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However, when PEF was applied in absence of a thermal pre-treatment, a significant (p < 0.05) improvement in the recovery of C-PC, yielding 37.52 mg/g$_{DW}$, could be obtained over intact microalgae (Figure VIII.1a).

Further increases of the processing temperature up to 45 °C, instead, showed a slight synergistic effect of the combined treatment, thus leading to a 10% increase of the C-PC content of supernatants (42mg/g$_{DW}$ on average) over single PEF treatment at 25°C.

A similar behavior has been observed in the work of Postma *et al.* (2016b). The authors reported that a slightly synergistic interaction between PEF and mild heating could be noted in the range of temperatures 25-45°C on the release of water soluble proteins from *Chlorella vulgaris* microalgae. However, the maximum recovery yield of water soluble proteins detected in their work were still dramatically lower than those achieved with the benchmark bead milling process, thus stating that the application of a PEF-temperature treatment did not sufficiently disintegrate *C. vulgaris* cells to grant an efficient release of intracellular compounds.

Martinez *et al.* (2016) studied the potential of PEF to selectively extract C-PC from fresh *A. platensis* biomass. The authors found that the increment of the temperature during PEF treatment allowed a significant reduction in both the electric field strength and the treatment time required to obtain a given yield of C-PC.

The maximum amount of extracted C-PC was obtained when *A. platensis* cells were previously treated at 40°C by the most intensive PEF treatment assayed (25 kV/cm, 150 μs), in complete accordance with the results observed in this paragraph.

Purity ratio (Figure VIII.1b) reflects the results obtained for C-PC (Figure VIII.1a). In fact, whatever the treatment temperature, a synergistic effect between PEF and mild heating has been reported. The highest value of purity ratio (0.89) has been detected from PEF treatment carried out at the intermediate investigated temperature.

The enhancement of pigments recovery from PEF treated microalgal cells has been widely reported in the literature (Luengo *et al.*, 2015; Parniakov *et al.*, 2015b; Poojary *et al.*, 2016).

For example, Luengo *et al.* (2015) investigated the effect of PEF of variable intensity (E = 10 – 25 kV/cm), coupled with a mild heating up to 40°C, on the release of valuable compounds from *C. vulgaris* microalgal cells via ethanolic extraction. The authors found that in addition to the electric field strength and specific energy input, treatment temperature was a critical parameter to influence the extent of the electroporation phenomena, thus furtherly allowing the extractability of lutein with respect to a single PEF treatment step. Instead, in our work, the main advantage is represented by the hydrophilic nature of C-PC, which potentially avoids the use of organic solvents and proteins denaturation phenomena, granting a more sustainable extraction process (Chemat *et al.*, 2012; Martinez *et al.*, 2016).

VIII.3.1.2 Effect of PEF-HSH treatments on the extractability of valuable compounds

Figure VIII.2 depicts the recovery yields of water soluble proteins (a), C-PC (b) and purity ratio (c) of extracts from intact, single PEF/HSH and HSH+PEF treated *A. platensis* microalgae suspensions.

The results show that the use of a cascade approach significantly promoted the diffusion processes of intracellular compounds towards the external medium, with an almost additive effect between the investigated technologies in correspondence of water soluble proteins (Figure VIII.2a) recovery yields, showing a maximum value of 460.1 mg_{BSA}/g_{DW}, which turned to be quite comparable to that achieved from HPH treatments (data not shown).
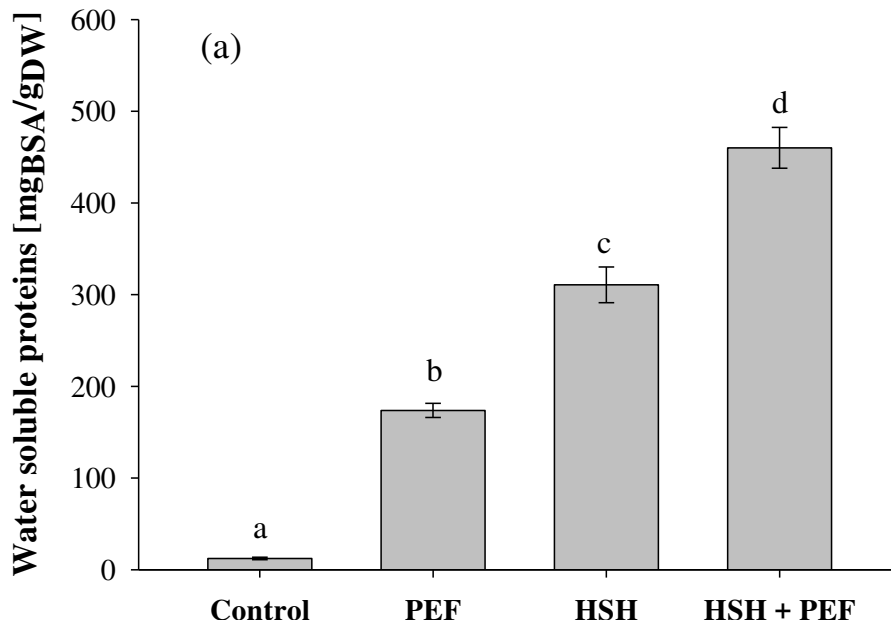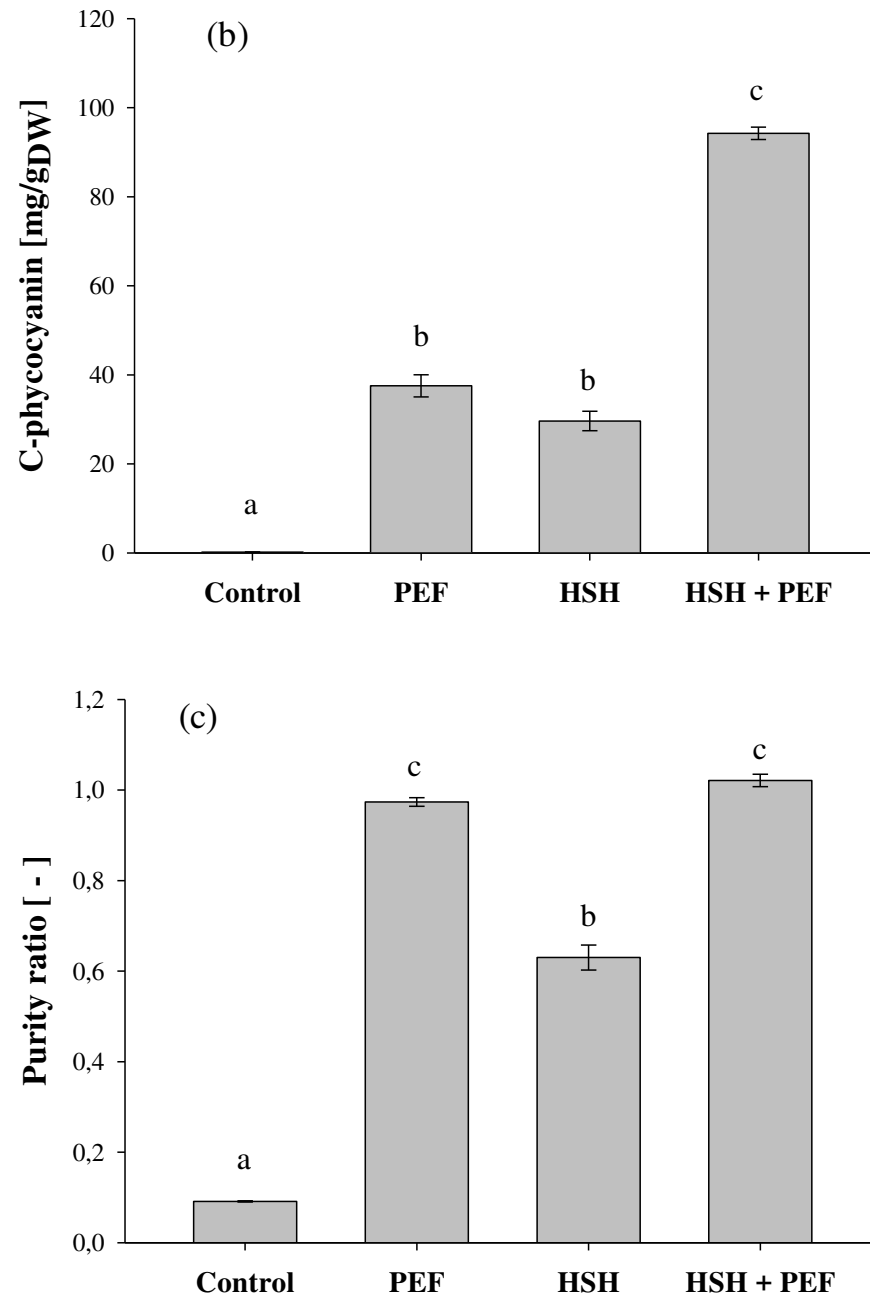
Figure VIII.2 *Water soluble proteins content (a), C-PC content (b) and purity ratio (c) of the supernatant obtained from untreated, single PEF treated, single HSH treated and combined (HSH + PEF) processed A. platensis microalgae. Standard deviations were used as error bars. Different letters above the bars indicate significant differences among the mean values (p≤0.05).*

More recently, the combination of PEF treatment with the use of either enzymes or high pH values to enhance the proteins solubilisation in the extracting medium (Parniakov *et al*., 2015e; 't Lam *et al*., 2017b) has been applied. In the first case, it has been demonstrated that a PEF pre-treatment of *Nannochloropsis sp*, carried out in basic conditions (pH=11) could improve the selective extraction of different intracellular components, while in the second case an enzymatic incubation with protease of *C. reinhardtii* before PEF (5 pulses of 0.1 ms at 7.5 kV/cm) resulted in a substantial increase in protein yields.

As regarding to C-PC recovery, results of Figure VIII.2b show that, while this compound is almost completely absent in the control samples, the combination of HSH and PEF technologies showed a synergistic effect on its extractability, which yielded up to 94.24 mg/g_{DW}, well above the sum of the amounts detected in the extracts from single HSH (29.63 mg/g_{DW}) and single PEF (37.52 mg/g_{DW}) treatments.

These findings could be explained taking into account the cell disintegration mechanisms occurring when single HSH and single PEF processes are applied. In fact, considering that HSH acts as a mild pre-treatment of *A. platensis* clusters breakage, the higher release of C-PC after the application of PEF in the cascade scheme could be likely ascribed to a more efficient processing due to the significant reduction in cell aggregates, whose presence may have affected the resistance of algae cells to PEF treatment.

To this purpose, it has been reported that the microrganism cells inside the clusters may be protected by the external layers of cells and therefore do not experience the same electric field strength as that acting on the external cells (Donsì *et al*., 2007). This would result in a lower extent of electroporation phenomena reached at the end of the PEF treatment.

However, as shown in Figure VIII.2c, the purity ratio of combined treatment was not significantly different from that granted by single PEF (p > 0.05). This means that in the combined treatment, PEF still exerts a positive impact on the selective extraction of C-PC, while the main effect of HSH pre-treatment is to quantitatively improve the recovery yields of intracellular compounds, as previously described.

In order to better characterize the supernatant obtained after cascade treatment of algae solutions, spectra measurements of single or combined processes were carried out, whose results are shown in Figure VIII.3.

The spectral data clearly evidence that the application of a single PEF treatment, having a gentle impact on the cellular structure of *A. platensis* cells, allows to selectively recover proteins and especially C-PC in the aqueous extracts, with the absence of peaks associated to light-absorbing yellow-red (420 nm) or green pigments (662 nm).

These results are in accordance with the findings of Grimi *et al*. (2014), who showed that PEF was unable to foster the diffusion of water insoluble pigments from *Nannochloropsis sp*. cells towards the external medium.

Instead, from the spectra of the extract achieved after HSH processing of microalgae, an undifferentiated release of intracellular compounds could occur, due to the presence of pigments which were kept suspended in the water phase, as also testified by the green coloration of the extract (data not shown).

Moreover, when the combined approach was used, the application of PEF increased both content of C-PC and water soluble proteins, due to the presence of higher peaks at 280 nm and at 615 nm. However, it is worth noting that the electropermeabilisation effect induced at the last processing stage did not enhance the extractability of other pigments.
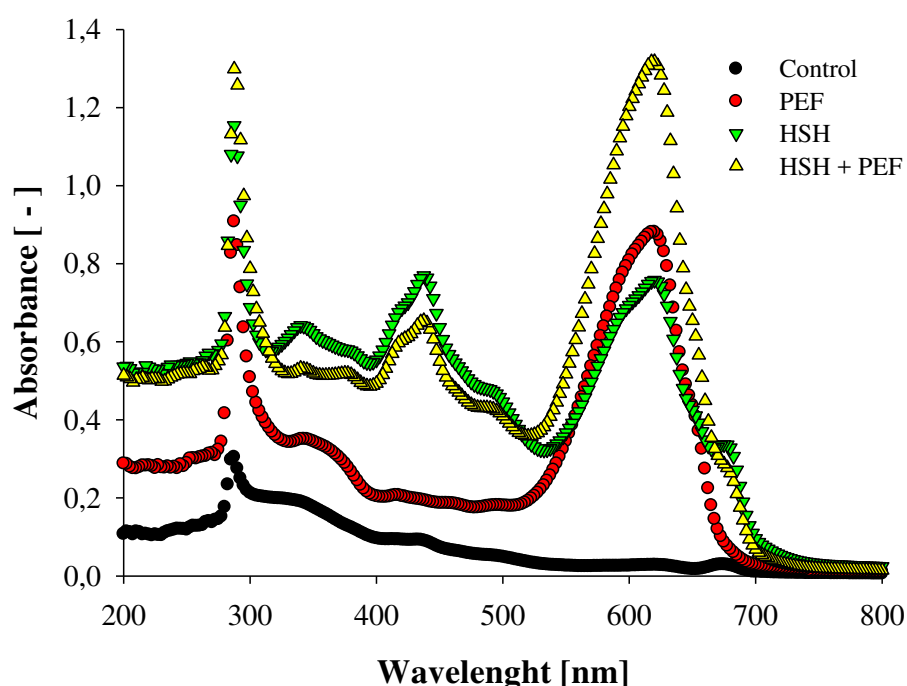
Figure VIII.3 *UV-Vis absorption spectra of supernatants from untreated, single PEF or HSH treated and combined (HSH + PEF) processed microalgal biosuspensions. Peaks identification: water soluble proteins (280nm), carotenoids (420 nm) and C-PC (615 nm).*

Proteins separation by SDS-PAGE can be used to estimate relative molecular mass, to determine the relative abundance of major proteins in a sample, as well as the distribution of proteins among fractions.

Figure VIII.4 shows the SDS-PAGE gel in which single PEF/HSH treatments (lanes 1 – 2) are compared with the HSH+PEF cascade treatment (lane 3) in terms of molecular weight (MW) of polypeptidic units recovered after processing.

In all the considered lanes, it can be observed that a wide range of protein subunits could be released (11 – 75 kDa), with the PEF treated sample showing two more distinct bands between 15 and 20 kDa, characteristics of α and β subunits of C-PC molecule (Kumar *et al.*, 2014).

Based on the Lowry method, used to quantify the amount of water soluble proteins in microalgal extracts, an equal amount of proteins was added per each lane. The more intense bands detected in lane 1 at 16 – 17 kDa are representative of a higher purity of C-PC from single PEF processing with respect to HSH processing (lane 2), which is in agreement with the results of Figures VIII.2 - 3.

As previously observed from the results of Figure VIII. 2b, the application of a sequential HSH-PEF treatment led to an increase in the intensity of the bands observed between 15 and 20 kDa, which is, once again, representative of an higher extractability of C-PC with respect to either single PEF and HSH treatments.
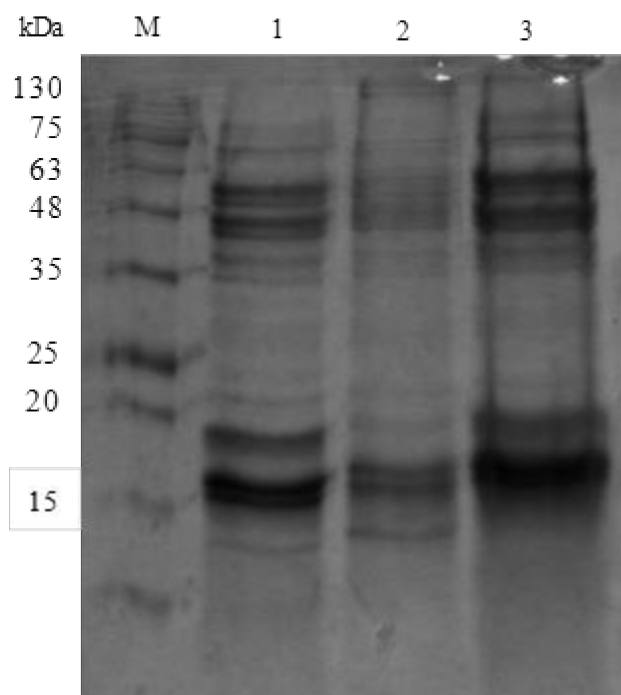
Figure VIII.4 *SDS-PAGE gel for single PEF (lane 1), single HSH (lane 2) and HSH+PEF (lane 3) treated suspensions of A. platensis microalgae. The letter M in the first lane stands for "marker".*

VIII.3.2 Use of PEF in a "Hurdle approach" for the biorefinery of C. vulgaris microalgae

The influence of the cascade treatments on the extractability of intracellular compounds (proteins, carbohydrates, and lipids) from microalgal suspensions of *Chlorella vulgaris* was also investigated in this work.

The cascade treatments were carried out in order to progressively increase the level of cell damage with sequential treatments of PEF and HPH, thus increasing the overall extraction yields and selectivity of the individual extraction steps. PSD and SEM analyses of microalgal biosuspensions were used to understand the impact of a single (PEF or HPH) or cascade treatment at cellular level. Subsequently, the amount of intracellular compounds released to aqueous and/or organic media was spectrophotometrically determined for the different classes of targeted compounds, together with the absorption spectra, and a systematic comparison of the different treatments, individually and in combination between them, was carried out.

VIII.3.2.1 Effect of single PEF, single HPH or combined treatment on morphological aspect of microalgae

The $D_{4,3}$ (mean particle size) and SEM images of untreated and processed microalgal cells (PEF, HPH or PEF+HPH) are reported in Figures VIII. 5 - 6, respectively.
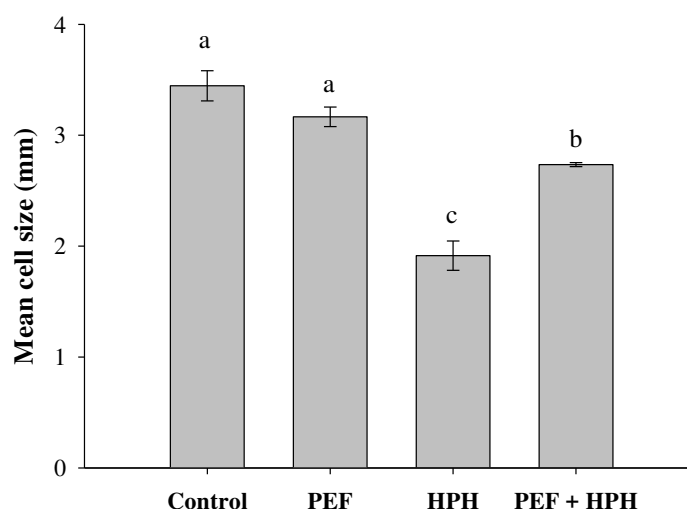
Figure VIII.5 *Mean particle size (MPS) of untreated (fresh sample), single PEF or HPH treated and cascade processed (PEF+HPH) microalgal biosuspensions. Standard deviations were used as error bars. Different letters above the bars indicate significant differences among the mean values (p≤0.05).*

The application of a single PEF treatment did not lead to a statistically significant (p > 0.05) change in the cell mean particle size ($D_{4,3}$ = 3.2 μm), which was only slightly reduced with respect to the value of untreated microalgae ($D_{4,3}$ = 3.45 μm). Coherently, also the particle size distribution curve did not show any significant change (data not shown), suggesting that PEF treatment only minimally affected the cell structure. This is evident also from the comparison of the SEM images of Figure VIII.6, which highlighted that most of the microalgal cells underwent a visible "shrinkage" phenomenon, probably due to the pore formation at cellular membrane level which then favoured the leakage of some intracellular compounds, as previously hypothesized in § VII 2.2.2. However, PEF treated cells substantially maintained their original shape and structure.

In contrast, results of Figure VIII. 5 show that when subjecting the microalgal suspension to a more drastic treatment, such as HPH, a significant decrease (p ≤ 0.05) in the mean particle size was obtained, with a drastic reduction of $D_{4,3}$ to 1.95 μm, due to the fragmentation of the cells and subsequent formation of cell debris (Safi *et al.*, 2014).

These observations are supported by the SEM image of Figure VIII.6, where it is clearly shown that the single HPH processed cells were completely disrupted, leaving only cell debris in the suspension. In the case of the combined treatment, the $D_{4,3}$ mean particle size lied in between those observed in correspondence of the individual treatments by PEF or HPH.
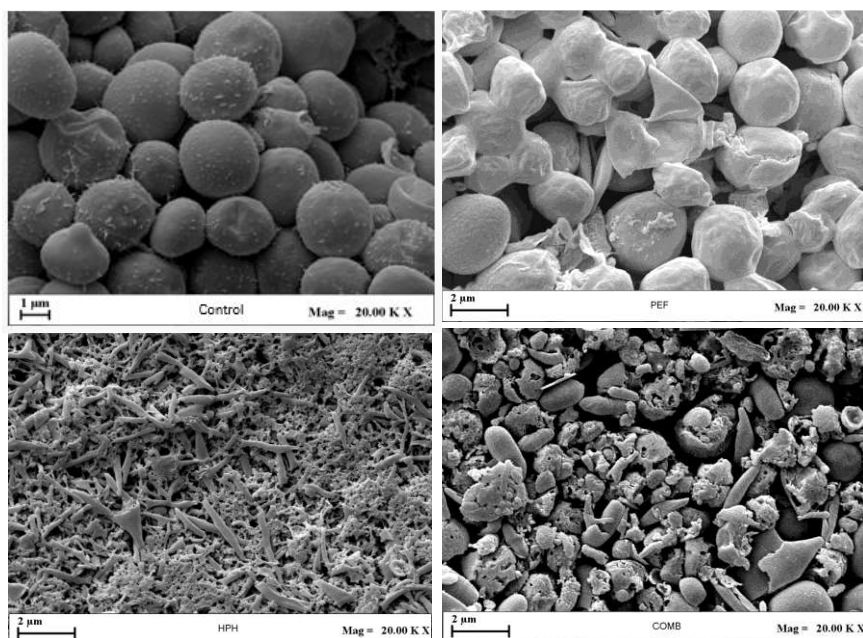
Figure VIII.6 *Scanning electron microscopy (SEM) of Chlorella vulgaris cells, before (control) and after the applications of PEF (20 kV/cm; 100 kJ/kg$_{SUSP.}$), HPH (P = 150 MPa; n_P = 5) and combined treatments (Comb.).*

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Despite the statistically significant decrease in $D_{4,3}$ with respect to control samples ($p \leq 0.05$), the particle size distribution curve of PEF+HPH treated biosuspensions showed only a slighter shift towards smaller sizes with respect to untreated cells (data not shown) than that shown by the individual HPH treatment, suggesting a lower cell disruption efficiency (Figure VIII.6). In particular, the larger mean size of the formed debris after the combined treatment than in the case of HPH treatment alone (Figure VIII.6) presumably might facilitate the separation phases in downstream processing.

Moreover, in the SEM picture of combined treated biomass it is worth noting the action of organic solvent, due to the lipid removal from cell walls of microalgae, which occurred prior to the final cell disintegration by HPH in the proposed biorefinery approach.

A decrease in cell disruption efficiency for the combined treatment with respect to HPH processing alone might be ascribed to the stress induced in the microalgal cells by the precedent electrical treatment (PEF) and to the contact with the organic solvent during the lipid extraction phase, which might have affected the cell structure, as well as its resistance to the fluid mechanical stresses delivered during the subsequent HPH treatment.

In particular, the release of intracellular compounds due to PEF treatment and lipid extraction, causing the cell shrinkage, might have increased the capability of the cell deformability, hence making the cell walls more resistant to the intense fluid dynamic fields generated in the HPH process.

In accordance with our results, Alvarez & Heinz (2007) found that the application of PEF pre-treatments for the inactivation of *Salmonella Senftenberg 775W* and *Listeria monocytogenes* increased the resistance to cell disruption when subsequently treated by US, thus reducing the efficacy of the combined approach with respect to single US treatments.

Another possible explanation of the reduced effect of HPH in the proposed cascade approach is the formation of cell clusters, which may have occurred during the resuspension in water of the pellet, immediately after the organic extraction. However, the formation of such cell aggregates was not confirmed by SEM analysis (Figure VIII.6), suggesting that the hypothesis of microalgae structural changes is more plausible.

However, more detailed studies are required to clarify this aspect.

VIII.3.2.2 Effect of single PEF, single HPH or combined treatment on the recovery of intracellular compounds

VIII.3.2.2.1 Extraction yields

The dry matter content (DM) of the aqueous supernatant from untreated, PEF alone, HPH alone and combined treated biomass is shown in Figure VIII.8. Instead, the appearance of the supernatant obtained in the different cases is shown in Figure VIII.7. Remarkably, the supernatant from the PEF-treated samples was as clear as in the case of the control, suggesting that no pigments were released (Figure VIII.7a-b). In contrast, the release of intracellular compounds significantly ($p \leq 0.05$) increased with the severity of the applied treatment, with a maximum value detected after HPH processing of microalgae (DM = 7.66 $g_{DM}/kg_{SUP.}$), which was significantly higher than that observed for PEF treated samples (DM = 2.79 $g_{DM}/kg_{SUP.}$). It is likely that the finest fraction of the cell debris might have remained in suspension in the supernatant after centrifugation, contributing to an overestimation of the real amount of dry matter content and to the opacity of the sample, as testified by the dark green coloration of the supernatant (Figure VIII.7c). Moreover, the complete cell disruption may have caused the release of different classes of intracellular compounds, including chlorophyll, thus making the HPH treatment an unsuitable method for a selective extraction of valuable molecules from microalgal biosuspensions.
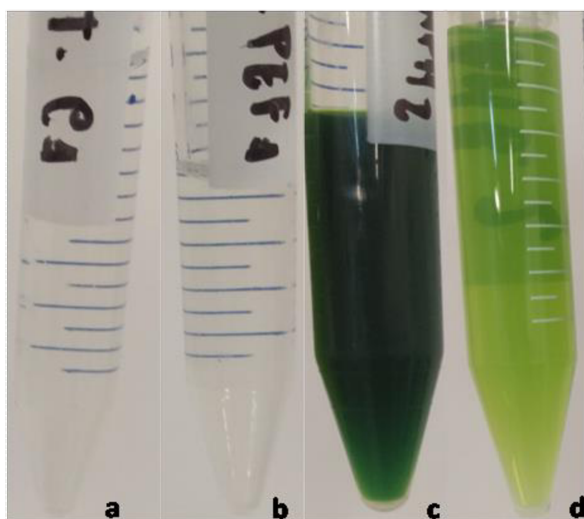
Figure VIII.7 *Images of the supernatant obtained after centrifugation of the untreated (a) , single PEF (b), single HPH (c), and Combined treated (d) aqueous microalgal suspensions.*
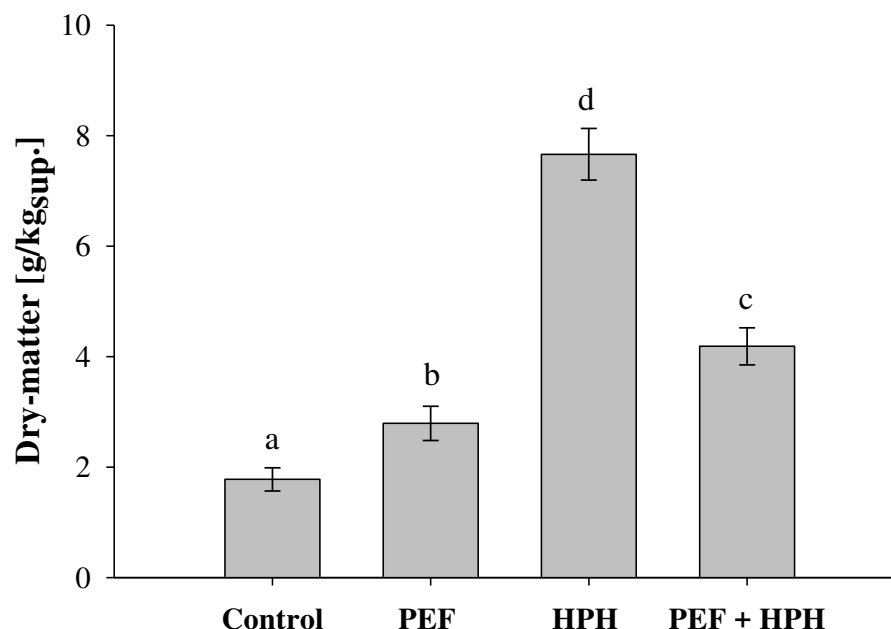
Figure VIII.8 *Dry matter content (DM) of supernatant from untreated (fresh sample), single PEF or HPH treated and cascade processed (PEF+HPH) microalgal biosuspensions. Standard deviations were used as error bars. Different letters above the bars indicate significant differences among the mean values (p≤0.05).*

The application of HPH in the cascade scheme (combined treatment) led to a small but significant (p ≤ 0.05) increase in the dry matter content of supernatant with respect to PEF treated samples, due to the further release of water-soluble compounds into the external medium. Correspondingly, the supernatant obtained from the combined treatment showed a light green coloration (Figure VIII. 7d).

In the case of the water-soluble proteins content of aqueous supernatant before and after individual PEF or HPH treatments, or their combination (Figure VIII.9a), it can be observed that PEF technology resulted in being scarcely efficient in extracting proteins from microalgal cells, especially when compared to a more intense treatment, such as bead milling or HPH, as shown here and observed also in previous works (Postma *et al.*, 2016a; 't Lam *et al.*, 2017a). It can be hypothesized that the pores formed on the cell wall/membrane of the microalgae during the application of PEF are not sufficiently large to permit the release of high molecular weight proteins, which may likely remain trapped inside the cell, or bounded to the cell wall which has not been influenced by the electrical treatment (Chapter VII). Our results seem to be consistent with those observed in the work of Grimi *et al.* (2014). In fact, when these authors performed a sequential treatment of

Nannochloropsis sp. biosuspensions (1% w/w) by means of a cascade of cell disintengration techniques (PEF, HVED, US, HPH), they found that HPH was the most efficient disruption method leading to the highest amount of extracted proteins ($Y_{HPH,WSP}$ = 91%), while PEF showed an efficiency of 5%, which in turn resulted to be higher when compared with the supplementary contributions of HVED and US.

In Figure VIII.9a, the amount of proteins extracted by the cascade approach (combined) was significantly ($p \leq 0.05$) smaller than that extracted by a single HPH treatment, despite showing a 6-fold increase in the extraction yield with respect to PEF treatment alone.

The reduction of the amount of extracted proteins with respect to single HPH treatment may have been caused by the contact with organic solvents during lipid extraction, that likely degraded and denatured them so that large parts of the soluble proteins might become insoluble, with a subsequent loss of their techno-functional properties, as previously hypothesised (Postma *et al.*, 2016c).

Smaller molecules like carbohydrates seem to easily move across the pores formed after the application of PEF treatment (Figure VIII.9b), which leads to a significant ($p \leq 0.05$) increase in the extraction yield with respect to the untreated samples. In this case, the amount of carbohydrates extracted during the cascade approach was slightly higher than that achieved after the individual HPH treatment ($p > 0.05$).
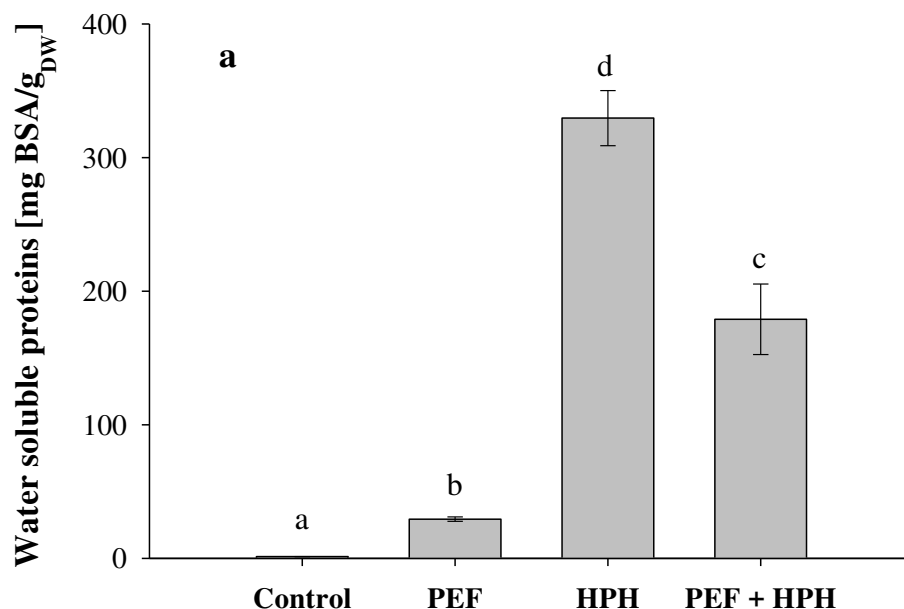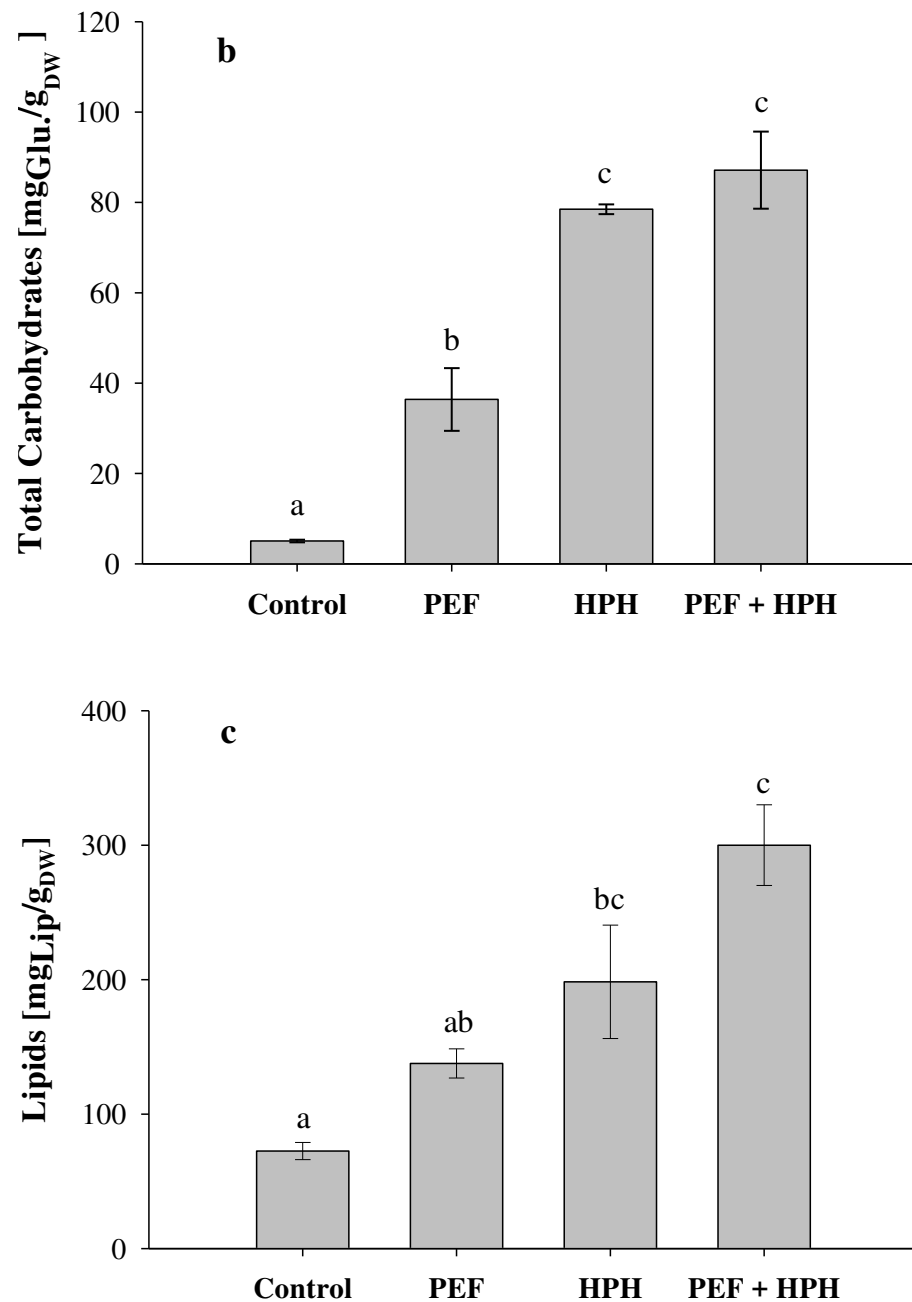
**Figure VIII.9** *Concentration of water soluble proteins (a), carbohydrates (b) and lipids (c) extracted from untreated (fresh sample), single PEF or HPH treated and cascade processed (PEF+HPH) microalgal biosuspensions. Standard deviations were used as error bars. Different letters above the bars indicate significant differences among the mean values (p≤0.05).*

Figure VIII.9c shows the amount of recovered lipids from the untreated samples, and those subjected to PEF alone, HPH alone and Combined treatments. PEF treatment caused a slight but not statistically significant ($p > 0.05$) increase in the extraction yield of lipids with respect to controls. Previous literature data also reports that the enhancement of lipids extraction by PEF is effective (Lai *et al*., 2014; Zbinden *et al*., 2013). For example, in the work of Lai *et al*. (2014) the application of a PEF treatment of constant intensity (30.6 KWh/m^3) on *Scenedesmus* biosuspensions yielded 3.1-fold more crude lipids and fatty acid methyl esters (FAME) after solvent extraction.

A further enhancement in the extraction of lipids was detected for the samples treated by HPH alone. Considering an average lipid content for microalgae *Chlorella vulgaris* in the range 20 – 25 % (g/g$_{DW}$), it can be observed that almost all of the lipids were extracted by the organic solvent after the complete cell disruption achieved by the HPH treatment.

The combined treatment led to an even higher yield of lipids ($Y_{COMB./LIP.} = 0.3$ g/g$_{DW}$), despite not statistically different ($p > 0.05$) from the HPH treatment alone. It is worth mentioning that, in the cascade approach, the lipid extraction was carried out twice, thus improving the lipid recovery. Moreover, it must be taken into account that in the repeated extraction procedure, the organic solvent may have become enriched also with some of the water-insoluble proteins, which naturally separated from the aqueous supernatant, together with the pellet, after the centrifugation of the biosuspension. Therefore, in future works, it is recommended that a Gas Chromatography – Mass Spectroscopy (GC – MS) analysis of the organic extracts is executed in order to better elucidate the effectiveness of alternative cell disruption methods such as PEF and HPH on the extraction yield of single lipidic compounds.

VIII.3.2.2.2 UV-Vis Absorption Spectra

Results from UV-Vis analyses are reported in Figure VIII.10, for aqueous (a) and organic (b) supernatants, respectively, belonging to all the investigated samples (Control, PEF, HPH, Combined).

Starting from a completely flat profile of the aqueous extracts from the fresh microalgal suspensions, the PEF treated samples showed the presence of a single peak, due to the extraction of a small amount of proteins, with complete absence of pigments extraction, thus evidencing its selectivity, despite a lower efficiency with respect to more destructive methods such as HPH.

HPH treatment alone, instead, leads to an instantaneous release of all the intracellular compounds, with absorption spectra showing the presence of proteins but also pigments (carotenoids and chlorophyll), which, therefore,

may not be considered a suitable method for a selective release of matter from microalgae, as also observed in the work of Grimi *et al*. (2014).
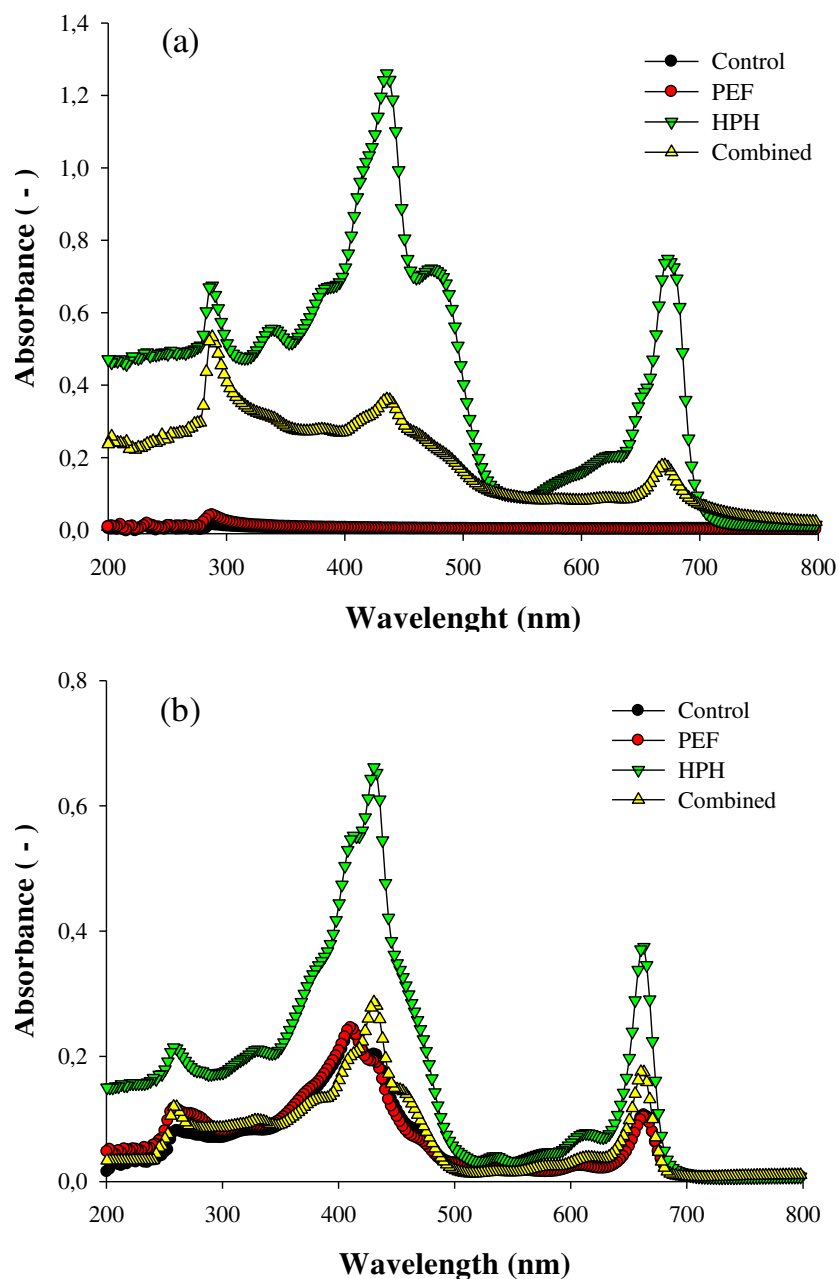
Figure VIII.10 *UV-Vis absorption spectra of aqueous (a) and organic (b) supernatants from untreated (fresh sample), single PEF or single HPH treated and cascade processed (Combined) microalgal biosuspensions.*

A lower amount of pigments could be detected from the spectra of the aqueous extracts after the combined treatments, due to their selective removal by the use of organic solvent during the intermediate lipid extraction phase. However, the spectra are in agreement with the supernatant images reported in Figure VIII.7.

As it is evident from the organic spectra of Figure VIII. 10b, the complete cell permeabilisation achieved upon HPH treatment also led to the highest extractability of the pigments, while similar spectra could be observed in the case of untreated and PEF treated samples. As in the case of Figure VIII.10a, the amount of pigments and proteins solubilized in the organic solvent resulted in being lower than that achieved for fully ruptured cells (HPH).

VIII.4 Conclusions

Results obtained in this study have shown the potentiality of PEF technology in the frame of microalgal biorefinery when used in either a hurdle approach (e.g., PEF + mild Temperature) or cascade operations (e.g., HSH-PEF, PEF-HPH) not only to efficiently extract valuable molecules from microalgae *A. platensis* and *C. vulgaris*, but also to allow a selective separation of distinct classes of compounds throughout the sequence of steps of the proposed biorefinery schemes.

In particular, as emerged from the results shown in paragraph VIII.2.1, the application of a combined treatment based on either PEF coupled with a mild heating or preceeded by HSH pre-treatment led to a synergistic effect on the extraction yield of C- PC from *A. platensis* microalgae, which in turn was comparable to those obtained upon full cell disintegration by HPH processing (data not shown).

On the other hand, as regarding the exploitation of *C. vulgaris* biomass, extraction yields of carbohydrates and lipids from the proposed cascade operations (PEF-HPH) seem to be comparable or even higher than those observed after the single HPH treatment.

However, further investigation needs to be carried out where special attention should be paid to the proteins extraction phase, which seems to be the most critical step during *C.vulgaris* biorefinery.

Interestingly, the formation of cell debris from combined approach seems to be reduced, thus potentially leading to an economical benefit during downstream separation phases.

Of course, apart from the extraction yields of valuable intracellular compounds, the feasibility of the integration in the microalgal biorefinery of a cascade of different cell disintegration techniques (PEF, HSH, HPH) should also take into account the energy requirement per kg of dry weight microalgal biomass.

To this purpose, a brief economical analysis related to the results obtained in this work will be presented and discussed in Chapter IX.

Chapter IX
General discussion and main conclusions

IX.1 Introduction

A projected global population increase from 7.5 to 9.7 billion people by 2050 will require an estimated 70% increase in food production, which will lead, as a consequence, to an increasing production of agri-food residues, which is a matter of concern due to their environmental, economic, ethical and social impact. At the same time, consumers have a growing interest in more "sustainable diets" based on alternative sources of proteins from agri-food wastes/ by-products, microalgae and seaweed. In parallel, there is also a growing demand for natural bioactive compounds (carotenoids, polyphenols, proteins, carbohydrates) to be used as functional ingredients in food, feed, nutraceuticals, cosmetics and pharmaceutical products.

The recovery of these valuable compounds, which are intracellularly locked, protected by a rigid structure (cell wall/membrane) of the aforementioned biomasses, should occur through a sustainable "green" biorefinery process, as described in Chapter I.

In this process, a crucial role is played by the cell disintegration technique used to improve the efficiency of the extraction step. In particular, the selected technique should allow a mild cell disruption of wet biomass (Galanakis, 2012; 2015; Gunerken *et al.*, 2015), in order to selectively improve the extraction efficiency, while reducing the processing times, temperature and amount of organic solvents implied in the diffusion process (Luengo *et al.*, 2014).

It is worth nothing that a full exploitation of biomass could be achieved only when the integrity of the extracted molecules is mantained, with no losses of functionality and decrease in product value (Postma *et al.*, 2016b).

In this frame, PEF technology has been selected in this thesis work as a mild and scalable cellular permeabilisation technique and successfully integrated,

alone or in combination with other factors (moderate temperature) or cell disruption methods (HSH, HPH), in the biorefinery process of food wastes/by-products, such as tomato peels and artichokes bracts and stems, and microalgae, namely *A. platensis* and *C. vulgaris*, for the selective recovery of high-added value compounds.

In this Chapter, an overview of the main results shown in Chapters IV – VIII is reported, along with an estimation of the energy requirements deriving from the integration of innovative extraction processes as compared with traditional SLE methods in the proposed biorefinery processes of food wastes and microalgae. Finally, the main conclusions and future perspectives are also reported.

IX.2 Biorefinery of food wastes tissues and microalgae by PEF

IX.2.1 Second generation PEF-assisted biorefinery

In Chapter IV, the influence of PEF treatment parameters on the extractability of carotenoids from industrial tomato peels was investigated. In particular, the use of "low impact" organic solvents (acetone, ethyl lactate), both listed in Class 3 by FDA ("regarded as less toxic and of lower risk to human health") was proposed for the solubilisation of non-polar intracellular compounds (e.g. lycopene), in replacement of the most used n-hexane which, despite showing the highest affinity with carotenoid compounds especially when used in combination with acetone and ethanol, is considered highly toxic and harmful for human health.
The permeabilization effect induced by PEF at level of cell membranes of tomato peel tissues was demonstrated to significantly increase the amount of recovered carotenoids with considerable antioxidant activities.
A higher sustainability of the second generation biorefinery process may be achieved by using a greener solvent extraction process as demonstrated in Chapter V, where the PEF pre-treatment of artichoke wastes (external bracts and floral stems) allowed an efficient extraction of high-added value phenolic compounds using water as solvent.
Moreover, the application of a pilot-scale biorefinery process of artichoke stems, involving the use of PEF and NF technologies, has shown interesting results in terms of process scalabity, leading to extraction yields of phenolics compounds which were comparable to those achieved at lab-scale, also allowing the production of a high added value solid extract with potential application as a food supplement (Chapter VI).
From the symultaneous analysis of the results shown in Chapters IV–VI, it emerges that the permeabilisation of food wastes/by-products tissues investigated in this thesis work could be achieved with relatively low electrical energy expenditure ($W_T = 5$ kJ/kg).

However, future works should also focus on the design of a biorefinery process able to exploit the whole biomass, thus pursuing the "zero wastes" concept.

For example, the presence of fibers and carbohydrates in the residual artichoke wastes make them suitable for additional recovery processing to produce food supplements or for animal feed or fertilizers, thus avoiding also the huge costs for their disposal and consequently lowering the environmental impact.

As regarding the case of tomato peels valorisation, a particular attention has to be paid to the downstream processing where the organic solvent needs to be efficiently removed from the extract in order to obtain a final product which can accomplish with the limit required by current legislation.

An interesting alternative to the organic solvents for the recovery of non-polar molecules from tomato peels could be represented by the usage of vegetable oils, capable of solubilising consistent amount of carotenoids from carrot purees (Roohinejad *et al.*, 2014), with the possibility to avoid additional downstream processing.

Despite the relevant results showed in Chapters IV – V, additional research activities should be focused on fulfilling the remaining gaps between the lab-scale and industrial-scale application, in view of the full exploitation of second generation biomasses.

IX.2.2 Third generation PEF-assisted biorefinery

Several authors in the literature have reported that PEF technology could represent a viable method for microalgal permeabilisation, allowing a relevant leakage of valuable intracellular compounds (Luengo *et al.*, 2015; Parniakov *et al.*, 2015e; Pataro *et al.*, 2017c; Poojary *et al.*, 2016).

However, the extraction yields of target molecules achieved by PEF are still far from those obtained when conventional disintegration techniques are used (bead milling, high pressure homogenisation).

As reported in Section 2, this statements is strictly related to the cellular structure and morphology of the investigated microalgal strain.

In fact, as described in Chapter VII, the application of a single PEF treatment of *A. platensis* and *C. vulgaris* microalgae lead to considerable differences in terms of intracellulars recovery during the acqueous diffusion step. In fact, the permeabilisation effects induced by the application of a PEF treatment of constant intensity (20 kV/cm; 100 kJ/kg$_{SUSP.}$) lead to higher extraction rates of water soluble proteins in the case of *A. platensis*, with a recovery yields of 33% of the average proteic content of this microalgae, while a negligible amount of water soluble proteins could be released from *C. vulgaris* (5.2 %) strain.

These results clearly reflect the different morphology of the two microalgal strains, which strongly affect their sensitivity to PEF treatment. In particular,

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it is likely that the presence of a fragile cell wall in *A. platensis* microalgae, mainly composed of murein and no cellulose (Lee, 2008; Lu *et al.*, 2006) might have not opposed appreciable resistance to the mass transfer of considerable amount of proteins through the electroporated cell membrane. Instead, the hard composition of *C. vulgaris* cell wall, formed by hemicellulose and cellulose (Payne & Rippingale, 2000) may have imparted the "barrier" behavior for the release of intracellular compounds, despite the pore formation on cytoplasmatic membrane upon the application of the PEF treatment. As a result, PEF application only increased the mass transfer of low MW compounds (e.g., ions, carbohydrates and small proteins), as previously stated by Postma *et al.* (2016b).

In Chapter VIII, due to the uncomplete disintegration effect achieved by single PEF processing it has been proposed a combined approach for the full valorisation of microalgal biomass.

More in details, the application of a mild HSH treatment, performed prior to PEF processing of *A. platensis* microalgae, has shown synergistic effects in terms of improved recovery of water soluble intracellular compounds.

The recovered stream, rich in proteins and carbohydrates, might be applied as a functional fraction, without further downstream processing.

Instead, in the case of *C. vulgaris* cells, PEF could be exploited only for the selective release of a water stream (consistent amount of carbohydrates accompanied by a small fraction of low molecular weight polypeptides) and a organic stream (rich in lipids) at the very beginning of the proposed biorefinery scheme. The unlocking of the majority of intracellular compounds could be achieved by further HPH processing, responsible for the complete microalgae disruption.

The remaining *C. vulgaris* pellet at the end of the whole proposed biorefinery process, mainly constitued by insoluble proteins and carbohydrates, could be potentially used as a nutritional fraction for food and feed sectors, thus allowing the complete usage of the originary biomass.

There still exists a series of processing parameters to be optimised, among which it is worth mentioning the biomass concentration of the suspensions, impacting on the overall efficiency and economics of the process.

Moreover, based on the selected microalgal strain, whose mean cell size and structural properties of the cell wall system affect the performances of the elctropermeabilisation step, a proper tuning of PEF electrical parameters as well as a specific design of the cascade operations could be required in view of the development of a potential biorefinery scheme.

IX.3 Operative costs of conventional and innovative extraction processes

The feasibility of a biorefinery process of biological biomass, including food wastes/by-products and microalgae, strictly depends on the integration of sustainable and efficient technologies able to provide high extraction yields of a wide range of marketable compounds, ensuring also high purities of the final extracts, with the minimum energy expenditure.

In this thesis work, a great attention has been paid on the potential integration of PEF technology in the biorefinery of food wastes/by-products and microalgae, with the main aim to improve the mass transfer and the overall efficiency of the subsequent SLE process.

The results described in Chapters IV-VIII have demonstrated the technical feasibility of the integration of PEF technology at both lab and pilot scale, granting beneficial effects not only on the performance of the extraction phase, but also on the following downstream separation processes.

In this last chapter, the evaluation of the operative costs derived from the application of PEF technology, in comparison with the classical SLE process for food wastes/by-products valorization, or with other disruptive technologies (mild heating, HSH or HPH) for microalgae processing has been performed, even though a more complete economical analysis, including also the impact of the additional investment costs deriving from the integration of the innovative extraction process, as well as the influence on the upstream and downstream phases, is necessary to demonstrate the full feasibility of the innovative approach.

More in details, the specific energy consumption associated with the application of either single or combined innovative cell disruption technologies before the conventional extraction process wil be reported and calculated as the energy input (in kWh) needed for the proper cell disintegration of 1 kg of dry (DW) biomass.

The principal outcomes in terms of extraction yields from both agri-food wastes and microalgal strains (Chapters IV – VII) investigated in this work, achieved upon the application of different permeabilisation technologies, are schematized in Tables IX.1 – 3.

Table IX.1 *Recovery yields of target compounds from tomato and artichoke wastes for either conventional or PEF-assisted SLE process.*

Food waste	Target compound	Process	Recovery yield ($mg_{PRODUCT}/kg_{waste}$)
Tomato peel	Carotenoids	SLE	**545.8**
		PEF + SLE	**804.2**
Artichoke stem	Polyphenols	SLE	**328.4**
		PEF + SLE	**887.1**

Table IX.2 *Recovery yields of target compounds (Water soluble proteins, WSP, C-PC) due to the application of either single or combined cell disruption technologies from A. platensis microalgae. When not specified, permeabilisation treatments were performed at 25°C.*

Disruption method	Recovery yields (mg/g_{DW})	
	WSP	C-PC
HPH	**685.14**	**65.04**
PEF	**173.74**	**37.52**
T (35°C)	**7.31**	**1.41**
PEF + T (35°C)	**285.74**	**41.12**
HSH	**310.62**	**29.63**
HSH + PEF	**460.10**	**94.23**

Table IX.3 *Recovery yields of target compounds (Water soluble proteins, WSP, carbohydrates and lipids) due to the application of either single or combined cell disruption technologies from C. vulgaris microalgae.*

Disruption method	Recovery yields (mg/g$_{DW}$)		
	WSP	Carbohydrates	Lipids
PEF	29.33	36.39	137.63
HPH	329.50	74.13	198.30
PEF + HPH	178.90	78.48	298.34

Instead, a summary of the results obtained from the energetical analysis is reported in Tables IX.4 – 7, where the operative costs derived from the integration of PEF treatment alone or in a hurdle approach before the conventional SLE process of the second (tomato peels/artichoke wastes) and third (microalgae) generation biomasses were reported, with reference to the optimal processing conditions found in Chapters IV – VII.

In particular, the energy requirement for each process unit was evaluated as follows:

Food wastes/by-products:
- Energy requirement for PEF treatment, E_{PEF}:

$$E_{PEF} = \frac{W_T}{DW_{Biomass} * 3600}$$

where:
- W_T is the specific energy consumption of PEF treatment (5 kJ/kg);
- $DW_{Biomass}$ is the dry matter content of tomato peels (0.358 kg$_{DW}$/kg$_{TOT}$) and artichoke stems (0.120 kg$_{DW}$/kg$_{TOT}$);
- 3600 is the conversion factor between kJ and kWh.

In this case, the performances of PEF-assisted extraction operations were compared with those deriving from a common SLE process.
To this purpose, the operative costs (€/kg) for the recovery of carotenoids and polyphenols from tomato peels and artichoke wastes, respectively, were calculated considering both the energetic requirements for biomass permeabilisation by PEF (5 kJ/kg) and the cost of the extracting solvent needed for the operation, by means of the following formula:

$$Operative\ cost\ \left(\frac{€}{kg_{PRODUCT}}\right) = \frac{E_{TECH.} * EL_{COST} + COST_{SOLVENT}}{Y_{PRODUCT}}$$

where:
- $E_{TECH.}$ is the specific energy demand (in kWh/kg$_{DW}$Biomass) of PEF technology;
- EL_{COST} is the average european industrial electricity cost (0.1204 €/kWh);
- $Y_{PRODUCT}$ is the recovery yield (mg/kg$_{DW}$) of interest compounds (e.g. polyphenols, carotenoids) from the conventional or PEF-assisted SLE (Table IX.1).
- $COST_{SOLVENT}$ is the cost of solvent used in the extraction of carotenoids from tomato peel (acetone, 515.78 €/m^3) and polyphenols from artichoke stems (water, 1.23 €/m^3).

Microalgae:
- Energy requirement for PEF treatment, E_{PEF}:

$$E_{PEF} = \frac{W_T}{C_X * 3600}$$

- Energy requirement for mild heating, E$_{HEAT}$:

$$E_{HEAT} = \frac{C_P * (T_{EXTR.} - T_{REF.})}{C_X * 3600}$$

- Energy requirement for HPH treatment, E_{HPH}:

$$E_{HPH} = \frac{P * n_P}{\mu_{PUMP} * C_X * \rho_{Biomass} * 3600}$$

- Energy requirement for HSH treatment, E_{HSH}:

$$E_{HSH} = \frac{W * t}{C_X * V * 3600}$$

where:
- W_T is the energy consumption of PEF treatment (100 kJ/kg);
- C_X is the biomass concentration (20 g$_{DW}$/L$_{SUSP.}$ for *A. platensis*, 12 g$_{DW}$/L $_{SUSP.}$ for *C. vulgaris*);
- C_P is the specific heat of microalgal suspensions (~ 4.186 kJ/kg);
- $T_{EXTR.}$ is the extraction temperature achieved after mild heating;

- $T_{REF.}$ is referred to room temperature (25°C);
- P is the pressure (150 MPa) and n_P is number of passes (3 for *A. platensis*, 5 for *C. vulgaris*) of HPH treatment;
- μ_{PUMP} is the efficiency of HPH pumping system (0.87);
- $\rho_{Biomass}$ is the density of microalgal suspensions ($\sim$ 1000 kg/m^3);
- W is the consumed power of HSH system (800 W);
- V is the volume of *A. platensis* biosuspensions treated by HSH (0.5 L)
- t is the HSH treatment time (60 s).

In this specific case, when a combination of technologies was applied (PEF+ mild T or HSH + PEF for *A. platensis*, PEF + HPH for *C. vulgaris*), the total energy was evaluated as the sum of each single energy contribution.

The operative cost (€/kg) of single target compounds extracted from microalgal biomass associated to a given cell disruption technology is evaluated according to the following formula:

$$Operative\ cost \left(\frac{€}{kg_{PRODUCT}}\right) = \frac{E_{TECH.} * EL_{COST}}{Y_{PRODUCT}}$$

where:
- $E_{TECH.}$ is the specific energy demand (in kWh/kg$_{DW}$Biomass) of single investigated technology (PEF, mild heating, HSH, HPH);
- EL_{COST} is the average european industrial electricity cost (0.1204 €/kWh);
- $Y_{PRODUCT}$ is the recovery yield (mg/kg$_{DW}$) of interest compounds (proteins, C-PC, carbohydrates and lipids) from the single or combined investigated technologies (Tables IX. 2 - 3).

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Table IX.4 *Operative costs for either conventional or PEF-assisted SLE process of target compounds from tomato and artichoke wastes.*

Food waste	Target compound	Process	Operative cost ($\text{€/kg}_{PRODUCT}$)
Tomato peel	Carotenoids	SLE	37798
		PEF + SLE	25654
Artichoke stem	Polyphenols	SLE	37.45
		PEF + SLE	13.86

From the results of Table IX.4 it emerges that the reduced energetic consumption involved during the permeabilisation step (5 kJ/kg), along with the consequent reduction in solvent consumption upon the application of PEF, significantly promotes the economical sustainability of a potential second generation biorefinery process, granting lower production costs for the recovery of a unitary amount of high-added value compounds with respect to SLE.

However, for a complete economical analysis the study and optimisation of up-stream and downstream processing is strictly required.

Table IX.5 *Specific energy consumptions and operative costs due to the application of either single or combined cell disruption technologies required for the recovery of target compounds (Water soluble proteins, WSP; C-Phycocyanin, C-PC) from A. platensis microalgae. When not specified, permeabilisation treatments were performed at 25°C.*

Disruption method	Specific energy consumption (kWh/kg_{DW})		Operative cost ($€/kg_{PRODUCT}$)	
	WSP	C-PC	WSP	C-PC
HPH	**10.48**	**110.52**	**1.26**	**13.31**
PEF	**7.99**	**37.01**	**0.96**	**4.46**
T (35°C)	**79.53**	**412.33**	**9.58**	**49.64**
PEF + T (35°C)	**6.89**	**47.91**	**0.83**	**5.77**
HSH	**4.29**	**44.99**	**0.52**	**5.42**
HSH + PEF	**5.91**	**28.88**	**0.71**	**3.48**

Results of Table IX.5 highlight the efficacy of each cell disruption technique in the frame of *A. platensis* biorefinery from both the energetical and economical points of view.

In spite of the highest proteins extraction yield, HPH showed the highest energy consumption (10.48 kWh/kg_{DW}) due to the high pressure and number of passes involved (P= 150 MPa, n_P=3).

On the other hand, although being characterised by relatively lower yields, the disruption of biomass by PEF technology required lower energy amounts while ensuring higher purity of extracts, as testified by the lower operative costs for obtaining 1 kg C-PC (3.42–fold lower than HPH).

The application of a combined PEF – mild heating exerted a positive impact only in terms of the reduction of operative costs for the extraction of 1 kg of water soluble proteins.

Instead, the disruption efficiency of single HSH treatment led to quite high yields of intracellular compounds (proteins, C-PC) with relatively low energy consumption, which led to comparable operative costs than PEF, even though granting extracts with a lower purity.

Interestingly, when HSH and PEF were combined, the resulted synergistic effect on the extractability of C-PC from *A. platensis* microalgae led to a significant lowering of the operative costs (0.71 $€/kg_{WSP}$, 3.48 $€/kg_{C-PC}$) with respect to the application of single disruption techniques.

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Table IX.6 *Specific energy consumptions due to the application of either single or combined cell disruption technologies required for the recovery of target compounds (WSP, carbohydrates and lipids) from C. vulgaris microalgae.*

Disruption method	Specific energy consumption (kWh/kg$_{DW}$)			
	Biomass	WSP	Carbohydrates	Lipids
PEF	2.31	78.92	63.61	16.78
HPH	19.95	60.54	269.19	100.60
PEF + HPH	22.26	124.48	283.76	74.2

Table IX.7 O*perative costs due to the application of either single or combined cell disruption technologies required for the recovery of target compounds (WSP, carbohydrates and lipids) from C. vulgaris microalgae.*

Disruption method	Operative cost (€/kg$_{PRODUCT}$)		
	WSP	Carbohydrates	Lipids
PEF	9.48	7.64	2.02
HPH	7.29	30.61	12.11
PEF + HPH	14.98	32.26	8.93

As precedently observed in Table IX.4, HPH turned to be an energy intensive cell disintegration technique, with a total consumed energy of 19.95 kWh/kg$_{DW}$, whereas PEF, despite the lower yields is characterized by a total consumed energy of 2.31 kWh/kg$_{DW}$.

These results are in contrast with the findings of Safi *et al.* (2017a), who demonstrated that PEF was energetically less efficient (10.42 kWh/kg$_{DW}$) than HPH (0.32 kWh/kg$_{DW}$) after only one passage at 100 MPa when applied for the recovery of proteins from suspensions of *Nannochloropsis gaditana* microalgae with a cell concentration of, respectively, 60 g/L and 100 g/L. Probably, this difference can be somehow explained in terms of the peculiarity of the tested microalgae, the different biomass concentrations as well as the differences in the PEF and HPH systems used.

For example, in agreement with previous findings (Pataro *et al.*, 2011b), it is likely that the energy efficiency of the continuous flow PEF system used in the present thesis work (Chapters VII-VIII) is higher than that of the batch chamber (electroporation cuvette with a maximum capacity of 400 μL) used in the work of Safi *et al.* (2017a). On the other hand, it has been reported that

processing biomass with higher solid concentrations than the diluted suspension used in our work, could positively affect the energy efficiency of both HPH and PEF treatment.

To this regard, for example, when Yap *et al.* (2015) processed suspensions of *Nannochloropsis sp.* by HPH at different concentrations, they found the same extent of cell rupture, but the energy demand of HPH was about 28 kWh/kg_{DW} at 0.25 % w/w solids and 0.28 kWh/kg_{DW} at 25 % w/w solids. Moreover, they also demonstrated that large scale HPH equipment is considerably more energy efficient than lab-scale apparatus.

Instead, Goettel *et al.* (2013) using a lab-scale PEF unit found that for an algae suspension containing 100 $g_{DW}/L_{SUSP.}$ algae the energy demand was 0.44 kWh/kg_{DW}, while for a suspension containing 167 g_{DW}/L_{SUSP} algae the energy demand of PEF was reduced up to 0.25 kWh/kg_{DW}.

The estimated operative costs for the recovery of different compounds by single or combined processing (Table IX. 7) clearly show that both carbohydrates and lipids could be efficiently recovered through PEF not only at comparable or lower costs than single HPH, but also with a higher degree of purity (Chapter VIII), thus positively affecting the economics of the further downstream processing.

The difficulty of recovering proteins by the application of single PEF technology had detrimental effects on the operative costs (14.98 $€/kg_{WSP}$), due to the lower extraction yields granted by the combined approach with respect to single HPH processing. However, PEF represents a viable option when considering the lower purity of HPH extracts and the need of more complex downstream purification processes.

It is worth remembering that a sustainable microalgae biorefinery should address for the cell disintegration, extraction and fractionation steps no more than 10% of the total available energy from the produced biomass (6.82 kWh/kg_{DW}) (Coons *et al.*, 2014).

It has been observed that the application of PEF technology, applied alone or in combination with HSH or HPH, despite showing advantages in terms of higher purity extracts, always required higher energetic consumption than the maximum allowable limit (0.682 kWh/kg_{DW}), which still results in the unfeasibility of both proposed biorefinery schemes.

For this reason, additional research is needed to optimize the processing condition and biomass concentration, possibly leading to lower energetic consumptions when PEF is applied in a combined approach, in view of its exploitation in microalgae biorefinery.

Moreover, as previously stated, also the impact of the integration of innovative extraction techniques on the performance and operative costs of the upstream and downstream phases should be included in the overall evaluation of the feasibility of the approach suggested in this work.

IX.4 Future perspectives

In this thesis work, the attention has been particularly focused on the implementation of innovative cell disintegration methods in the frame of a second and third generation biorefinery process.

More specifically, additional insights on the impact of Pulsed Electric Field (PEF) as a mild cell disruption technology of tomato peels/artichoke wastes and microalgal biomass for the selective extraction of high-added value compounds were provided, clarifying also the relationship between the applied process intensity (electric field strength, E and specific energy input, W_T) and the degree of cell permeabilisation, evaluating also the subsequent effect on the extent of intracellular compounds release during the further extraction processes.

Results shown in Chapters IV – VIII have highlighted the potentiality of PEF to unlock intracellular substances from the considered matrices in a "cell structure" dependent manner. In fact, while an efficient electroporation phenomenon of food wastes has been achieved at very low energetic consumptions (W_T = 5 kJ/kg), allowing a significant enhancement in the mass transfer processes involved during the conventional SLE, it has been shown that PEF alone did not allow the whole exploitation of microalgal biomass at sustainable processing conditions, especially in the case of strains whose complexity of the cell wall limits the release of target compounds.

To this purpose, the application of "hurdle approaches" (PEF+T, PEF + HSH/HPH) has been proposed in the frame of microalgal biorefinery to improve performances of the extraction process as well as to reduce the specific energy amount associated with the single cell disruption step.

In particular, only in the case of *A. platensis* processing it has been proved that the application of a mild HSH treatment prior to PEF permeabilisation step could grant a significant reduction in the operative costs for the recovery of C-phycocyanin, a water soluble pigmented protein, with yields comparable to those achieved from full biomass disruption processes (e.g. HPH).

Instead, the proposed biorefinery process of *C. vulgaris* showed interesting results in terms of purity of extracts, which may potentially avoid the need for excessive operative costs for further refinings.

The main drawback associated to this scheme is represented by the high energetic consumptions for cell disruption processing, mainly associated to the highly diluted biosuspensions considered in this work.

Future studies should, therefore, investigate the effect of biomass concentration of the microalgal suspensions on the energy requirements, as well as on the operative costs of the proposed innovative approach.

Furthermore, downstream processing operations (separation/purification steps), for example, should be also subjected to a more intensive and systematic study in order to identify, for each case study, the best unit

operation as well as the optimal processing conditions to achieve high separation efficiencies. Moreover, the choice of the best downstream processing steps should be performed also considering the need to preserve the nutritional value, composition and overall quality of the final product, ensuring low energy consumptions (Nath *et al.*, 2018).

The output of downstream processing is represented by a concentrated product, of which an example is reported in Chapter VI, which needs to be properly treated for further applications in several industrial sectors. Therefore, there is a strong need to implement also viable stabilisation processes, which may allow long-term preservation of the intrinsec properties of the product, with no degradation/loss of functionality of the main bioactive molecules.

Hopefully, the approach and the results achieved in the frame of this thesis work will represent a useful basis for opening up new scenarios in the frame of biorefinery processing, bearing in mind the "sustainability"concept to be fulfilled.

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