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Cereal by-products as source of bioactive molecules for cosmetic applications: a sustainable approach based on eco-pharmacognostic research

Scientific/Disciplinary Sector (SDS) BIO/15

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Con la constatazione della finitezza ecologica, le società umane si vedono costrette a riaggiustare il loro processo di sviluppo, le loro tecniche di coltivazione e il loro sistema di riciclaggio. Fra tutti gli insegnamenti dati dall'ecologia, la presa di coscienza della finitezza e dell'inestendibilità dello spazio costituisce forse la rivoluzione più gravida di conseguenze, la più difficile da accettare.

Gilles Clément

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List of abbreviations

ALK-UAE:	Alkaline hydrolysis coupled with sonication
ALK:	Alkaline hydrolysis
BP:	Bound phenolics
BP+:	Bound phenolics obtained with alkaline hydrolysis coupled with sonication
CBM:	Cereal by-product matrices
CFU:	Colony-forming Unit
DPPH:	2,2-diphenyl-1-picrylhydrazyl
DW	Dry weight
Enz	Enzymatic hydrolysis
EVOO:	Extra virgin olive oil
FAE:	Feruloyl esterase
FAO:	Food and Agriculture Organization of the United Nations
FL:	Food losses
FP:	Free phenolics
FW:	Food waste
GAE:	Gallic acid equivalents
HPLC:	High Performance Liquid Chromatography
HPTLC:	High Performance Thin Layer Chromatography
MAE	Microwaves-assisted extraction
NP/PEG:	Natural Products-Polyethylene Glycol reagent
p-CA	para-Coumaric acid
R_f:	Retention factor
Rpm:	Revolutions per minute
rt:	Retention time
SC-CO_2:	Supercritical carbon hydroxide
SFE:	Supercritical fluids extraction
UAE	Ultrasound-assisted extraction
UAE-R	Ultrasound-assisted extraction residue
USP	Unsaponifiable matter

1

2 Introduction and research profile

Food waste recovery has become a hot topic in the field of natural product and pharmacognostic research in the past twenty years: however, it should not be surprising that the development of this research topic was born in conjunction with a series of ideologies and historical evidences.

From the early roots of Carl Linneus, Alexander von Humboldt, Alfred Russel Wallace, Charles Darwin, Ernst Haeckel and many more contributors, *ecology* has been recognized as a science and it started to spread all over the scientific world as the study of all those complex interactions between organisms and their environment.

From the first half of the twentieth century, ecology has reshaped the position of the human being that has become in biological equilibrium with the planet, now conceived as a finite space: the idea that the planetary biomass is limited makes life incapable of eternal renewal and subject to depletion (Clemént, 2013).

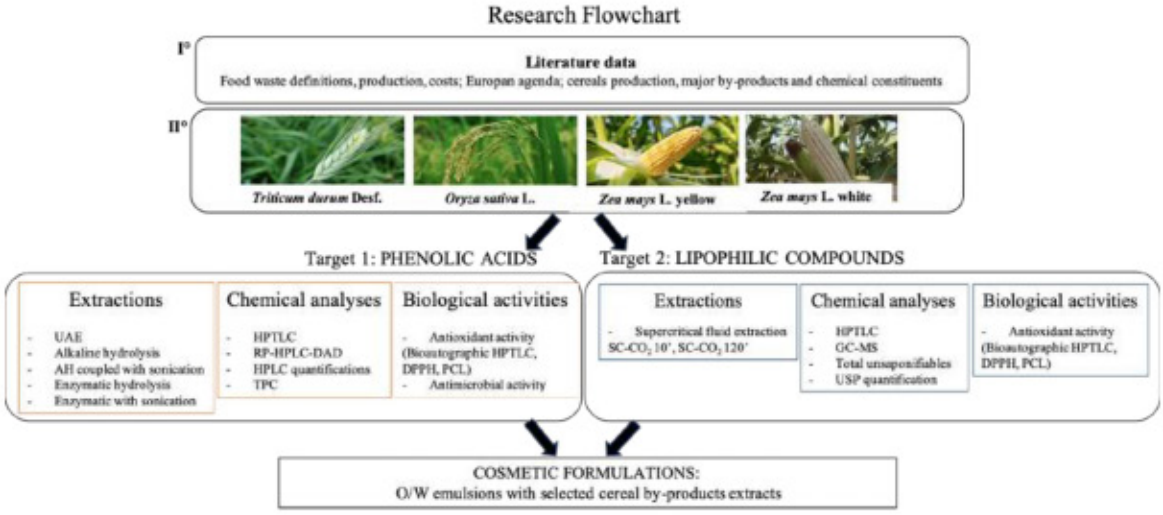
In 1981 Walter R. Stahel starts to develop the field of sustainability by supporting 'service-life extension of goods - reuse, repair, remanufacture, upgrade technologically' or what is known today as circular economy (Stahel, 1981). The purpose of circular economy, now supported by the European Commission action plans from 2015, is shifting from “taking, making, consuming, throwing away” linear scheme to a circular flow which maintain goods and resources in the economy as far as possible, reducing environmental impacts while maximizing resource efficiency (European Commission, 2015). The birth of Industrial Ecology, announced by Erkman in 1997, represent another useful tool for understanding the circulation of materials and energy flows and therefore the creation of alternative managements through reuse, repair, recycling and remanufacturing for the recovery of components and extend their lifecycle (Erkman, 1997; Saavedra, 2018).

The recovery of industrial by-products and the study of their potential applications began already before the First World War, accentuated in the first post-war period with the increase in the prices of basic necessities and developing further from the middle of the last century (Zitkowski, 1917; Woodruff and Miller, 1929; Symons, 1945); however, a significant interest increase in academic research, coincided, between the sixties and seventies, with a political awareness of the importance of minimizing waste (Mobius Loop is introduced as the symbol for Reduce, Reuse, Recycle in that period), thus numbers of

publications enriched literature with sustainable strategies of waste valorization (Pruthi et al., 1961; Rice, 1976; Braddock et al., 1978).

The recent updates given by the world's leading climate science during the UN Intergovernmental Panel on Climate Change (IPCC), held at the end of 2018, regarding the environmental risks of the planet said that urgent and unprecedented changes are needed to avoid catastrophic environmental breakdown. The management of the planet's resources and wastes, is, consequently, stimulating research to find useful and alternative strategies.

The exploitation of agri-food by-products, is the purpose of the present PhD project which, being part of a three years training program (D.R. 996/2016 Prot. 57324 of 24-6-16) financed by the Emilia Romagna Region (Italy), is aimed to valorize biomasses deriving from the cereal supply chain.



The present work is divided into two sections. The first section is focused on a general description of food waste definitions, production, extension and costs, with an in-depth analysis of the situation and the precautions taken by Europe and Italy as well as a detailed description of cereals production, major by-products and chemical constituents, updated to the most recent literature. In the second section the experimental part is reported, divided in Material and Methods, Results and Discussion and Conclusions chapters.

Wheat, rice and maize, known as the "big three" cereals, are the most produced crops in the world and thus they have been chosen as subject of the present research. The extractions of biomolecules from their main by-products (bran and germs) has been performed

following the guidelines of the green chemistry with the aim of minimizing environmental impact, costs and time of extraction while obtaining high added value extracts. In particular, the influence of green extraction technologies (such as ultrasounds) on the common extraction techniques of phenolics and vegetable oils has been investigated. Phenolic molecules and unsaponifiable fractions have been set as the two main extraction targets and extracts have been analyzed through qualitative (e.g. HP-TLC, HPLC-DAD, GC-MS, etc.) and quantitative assays (e.g. HPLC quantification, total phenolic content, total unsaponifiables, etc.). The potential application in the cosmetic industry of the obtained extract have been performed with various *in vitro* biological activity assays for the antioxidant (HP-TLC bioautographic assay, spectrophotometric DPPH assay) and antimicrobial activity evaluations.

Finally, in order to provide a practical example of a possible cosmetic application of the obtained extracts, four final products have been formulated in collaboration with Ambrosialab Srl (www.ambrosialab.it), evaluating the antioxidant activity of both final products and extracts with Photochemoluminescence (PCL) and testing the stability of final products.

3 Goals

The present PhD project is a three years training program (D.R. 996/2016 Prot. 57324 of 24-6-16) is aimed to valorize agri-food by-products, for the development of quality and safety control of processes and of innovative and sustainable cosmetic products.

In particular, the purpose of exploiting by-products focuses on the recovery of biomass deriving from the cereal supply chain.

For the enormous quantities of cereal production in Emilia Romagna Region and, consequently, for the disproportionate quantities of food waste that are still widely used only in the livestock sector, the need to find an alternative, and therefore a second life, for the management and recovery of these second-generation waste, became a priority, as underlined by the recent European Union directives for circular economy.

Being in line with the green proposals of natural products research to find alternative and sustainable solutions, this project aims to take stock of the current situation on the subject of food waste amounts and recovery, and to provide valuable alternative ideas to research applications in this area.

Therefore, the main goals of this project are:

- Summarize in detail the current literature data about food wastes amounts, management and recovery;
- Develop and apply innovative and green strategies for the extraction of bioactives from three of the major cereal biomasses in terms of production (durum wheat, rice and maize);
- Minimizing the environmental impact of conventionally used extractions by reducing, for example, the amount of solvents and extraction's time as prompt by the green chemistry;
- Identify the main extraction targets;
- Study the phytochemical profile of the extracts obtained in qualitative and quantitative terms using HPTLC, RP-HPLC-DAD, GC-MS, Folin-Ciocalteu's assay, etc.
- Evaluate the possible industrial application (cosmetic, phytotherapic, nutraceutical, etc.) of the obtained extracts by studying their biological activity: antioxidant and antimicrobial activities

- Formulate finished cosmetic products with the most interesting extracts in collaboration with Ambrosialab Srl (www.ambrosialab.it) as the company the applicative outcomes of the research project.

4 Food losses and wastes

4.1 Introduction and definitions

The issues of food losses and wastes are of high importance as they are directly liked to environmental, economic development, food quality and safety and on food security for developing countries. Hence, before going into the topic in details, few essential definitions are given below.

According to Fusions's "Estimates of European food wastes levels"[1], 'Food' is defined as "any substance or product, whether processed, partially processed or unprocessed, intended to be, or reasonably expected to be eaten by humans. Food includes drink, chewing gum and any substance, including water, intentionally incorporated into food during its manufacture, preparation or treatment".

In the same way, food wastes are: "fractions of food and inedible parts of food removed from the food supply chain to be recovered or disposed (including - composted, crops ploughed in/not harvested, anaerobic digestion, bioenergy production, co-generation, incineration, disposal to sewer, landfill or discarded to sea)" (Fusions, 2016).

A difference in meaning between 'food waste' and 'food losses' is given by the Food and Agricultural Organization of the United Nations (FAO): the former is considered as food loss which occurs at the end of food chain and is related to retailer's and consumer's behavior, the latter takes place at production, post-harvest and processing stages in the food supply chain (FAO, 2011).

4.2 Types of food waste and management

There are five stages in the food life cycle were waste is generated from both animal and vegetable sources, such as agricultural production, post-harvest handling and storage, processing, distribution and consumption: they were considered and described in the following table.

[1] FUSION' EU project is supported by the European Community's Seventh Framework Programme under Agreement no. 311972.

	Vegetable commodities and products	Animal commodities and products
Agricultural production	Losses for mechanical demage and/or spillage during harvest operations, crops separated post-harvest, etc	Animal death during breading (pork, bovine, poultry meat), fishing, dairy cow sickness for milk
Post-harvest handling and storage	Handling spillage and/or degradation, storage and transport during distribution	Death during transport, condemnation at slaughterhouse (pork, bovine, poultry meat); icing, parcking, storage and transportation (fish); spillage and degradation (milk)
Processing	Spillage and degradation during processing, separated crops not suitable to process or during washing, peeling, boiling or accidental spillage	Trimming splliage and industrial processing (pork, bovine, poultry meat); canning and smoking (fish); industrial milk tratment and processing
Distribution	Market system	Market system
Consumption	Household level	Household level

Table 1. Five system boundaries in food supply chain (FAO, 2011)

Until a few decades ago, food wastes were considered neither a cost nor a benefit: they were used as animal feed, brought to landfills or sent for composting. The growing environmental issues as well as the high disposal costs are two of the main reasons that brought countries to a more sustainable approach. According to the Directive 2008/98/EC, adopted by the New Waste Framework Directive, the prevention of waste generation has to be a priority, followed by processing for reuse and recycling, with disposal and landfill as the least favoured stages of waste management figure 1 (Ravindran, 2016).

Figure 1. Hierarchy for waste processing (adapted from Ki Lin et al., 2013)

Vision 2020, launched by ReFood in 2011, is a good example of credible initiative aimed to ban food waste from landfill by 2020 in UK (www.vision2020.info).

Besides their pollution and hazardous aspects, in many cases, food wastes might have a potential for recycling raw materials, converting to high added value products, or they could be raw materials for other industries.

Particularly, the recovery of food residues is receiving increased attention because of the growing awareness of the benefits deriving from potentially marketable components present in foods wastes which represent a possible and utilizable resource to obtain useful products (Laufenberg et al. 2003).

Two generations of food waste valorisation strategies are distinguished: the first-generation is aimed to use the complete material streams for animal feed, energy or compost production (e.g. bioenergy production); the second-generation valorisation rely on recovery and conversion of specific components in order to obtain various classes of products (e.g. chemicals, bioactives, biofuels, etc.).

Interesting sources of plant-derived food wastes can be found in each one of the previously described categories (table 1): from agricultural wastes until consumption (municipal waste), thus much of the efforts in waste processing have been focused on this topic.

During industrial processing a wide range of materials is generated and, apart from wastes that industry has to eliminate by disposal centres, incineration, or landfill, they can be distinguished in by-products and co-products.

According to Chemat et al. (2012), 'by-products' are residual products that appears during the extraction process: they are unintentional and unpredictable. They can be used directly or be ingredients to manufacture another finished product, they have economic value.

'Co-products' are materials, intentional and inevitable, produced along with the main product and with the same importance. Co-products must always meet specifications for their characteristics and may be used directly for a particular application (Chemat et al., 2012).

Figure 2 represents the Agri-Food system flowchart where it is shown how pre-harvest productions (A), such as plant, animal and fisheries productions, can be directed towards three different managements: the first (1) includes non-food chain (pet food, biomaterials, biofuels, etc.), the second (2) regards animal feedings and the third (3) concerns food supply chain (including post-harvest, manufacturing, wholesales, distributions and final consumptions, etc.). Food and inedible parts deriving from the food supply chain (B), can

have different destinations, such as: valorization and conversion, production of biomaterials, bioenergy, chemical and phytochemical compounds, up to incineration, landfill and composting.

Recent studies, focused on the exploitation of food waste, show many possibilities for food supply chain waste recovery such as the valorisation of wastes into high value chemicals: the topic of the present PhD project is focused on this type of management and particularly it is aimed to valorise by-products deriving from the cereals industries, as described in detail in afterwards.

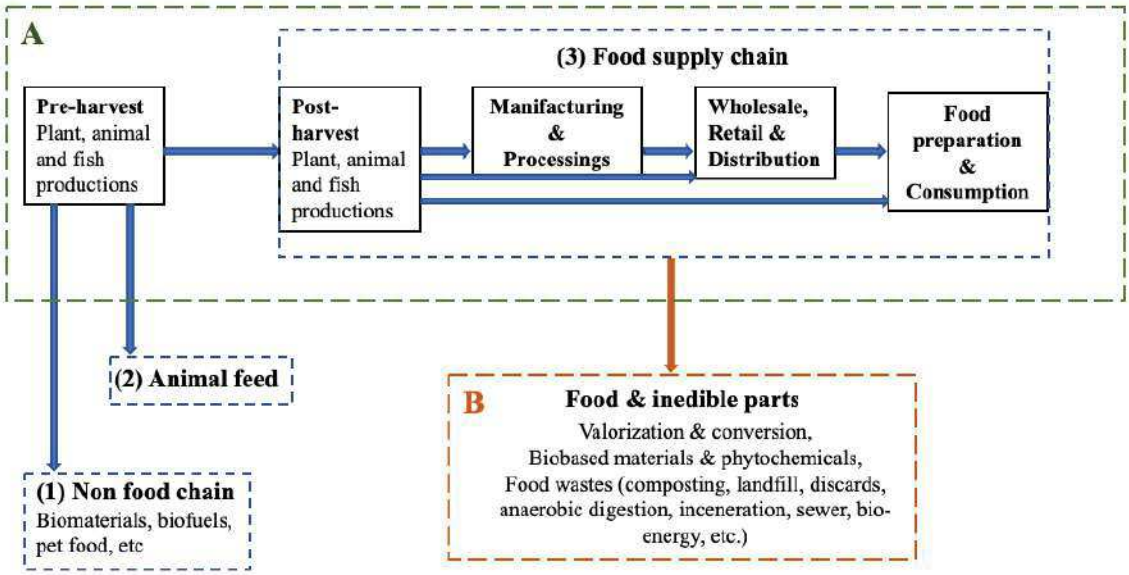


Figure 2. Resource flow in Agri-Food (adapted from: FUSIONS, 2016)

4.3 Extend, costs, and global concern

4.3.1 World

According to FAO, one-third of the edible parts of food produced for human consumption gets lost or wastes globally, this amount corresponds to 1.3 billion ton per year with a global cost of $750 billion annually (FAO, 2011). As previously described, food wastes and losses are produced throughout the whole food supply chain but dramatic differences between industrialized countries and developing countries are reported: low-income countries counted more than 40% of food losses during the post-harvest and processing levels, differently in medium and high-income countries more than 40% of food waste is produced at consumer level (222 million tons) meaning that food is thrown away even if is still suitable for human consumption. It must make us reflect that this amount is almost as high

as the total net food production in sub-Saharan Africa which counts 230 million tons (FAO, 2011).

FAO estimated that the societal costs of food wastage amount to about $2.6 trillion in 2014: 1 trillion of them are costs from economic losses, $700 billion are societal costs of environmental impact and $900 billion are due to individual well-being losses (FAO, 2014).

Beside the mere economic loss, food wastes are responsible for dramatic GHG emissions which, counting more than 8% of the total human GHG products, are almost as high as road transports ones. Climate changes and food wastes are closely related also in terms of water consumption, risks to biodiversity and soil erosions demonstrating the deep impact of this phenomenon to our society (IAI, 2017).

World food loss and waste % by manufacturing	
Cereals	4.1%
Roots and tubers	10.2%
Oil crops and pulses	5.9%
Fruits and vegetables	8.5%
Meat	4.8%
Fish	6.3%
Diary	1.4%

Table 2. Global food losses and food waste, FAO 2011

4.3.2 Europe and Italy

European amounts of food waste have been estimated for the year 2012 by Fusion's project "Estimates of European food waste levels" for the European Commission and published in 2016. Thanks to a combination of national waste statistics and literature data collected within EU member states permitted to quantify food waste including food and inedible parts associated with food. Resulting data confirmed the world estimations previously reported: in effect the sector contributing the most to food waste is household (47 million tons) followed by processing (17 million tons). These numbers tell us that 72% of EU food waste comes from the aforementioned two sectors, the remaining 28% is distributed among food service (11 million tons), production (9 million tons) and wholesale and retail (5 million tons). In 2012 EU production of food wastes corresponds to 173 kg per capita (88

million tons whose costs amount approximately to 143 billion euros) and it has been estimated that these numbers could increase up to 30% by 2020 if no action is taken.

The Italian Parliament, has recently approved a law against food waste (Law 166/2016 of 19 August 2016, n.166), that has been considered the last step of the National Food Waste Prevention Plan (Piano Nazionale di Prevenzione degli Sprechi Nazionali – PINPAS), launched in 2013, with the aim of promoting the recovery and donation of food surpluses for charitable purposes and minimising the negative impacts on the environment and on natural resources (reducing waste generation, encouraging reuse and recycle, extending products life). https://zerowasteeurope.eu/2016/10/the-italian-recipe-against-food-waste/
Italian food wastes have been counted around 5.1 million tons per year, and it has been expected that the new law will help to recover 1 million tons of food per year.
This new Italian approach to food waste is just one step aimed to fight this evergreen and growing issue: to give an idea of the severity of the problem, one quarter of the Italian forests serve just to absorb carbon dioxide emission produced as a result of food waste. Moreover, it has been calculated that if food waste was a country, Italy would be the third largest "emitter" of CO_2 worldwide (just behind the USA and China) (Food sustainability index, BCFN[2], 2013).

4.4 The EU agenda against food wastes: the "Circular Economy Action Plan"

The most relevant opportunity to rebalance the food supply chain and develop a sustainable, low carbon, resource efficient and competitive economy, at the European level, is represented by the so-called "circular economy".
Shifting from "taking, making, consuming, throwing away" traditional linear scheme to a circular model which closes the loop, is the main purpose of this action plan.
The Junker Commission of the European Parliament released a proposal for the circular economy in 2015, aimed to amend the already cited 2008 Waste Framework Directive: by maintaining products, materials and resources in the economy as far as possible, the circular

[2] Barilla centre for food & nutrition

economy will boost the EU's competitiveness in business, it will save energy and will reduce CO_2 emissions.

One key point stressed by the Commission to the European Parliament in 2015 refers to food waste as possible raw materials to be reuse and injected back to the economy: organic wastes, for example, could return back to soil as sustainable fertilisers. Biomass and bio-based materials are other possible candidates as they can provide alternatives to fossil-based products and energy: the bio-based sector is supported by the EU with investments and projects through research funding. The Horizon 2020 work programme 2016-2017 included the initiative: "Industry 2020 in the circular economy" with funding for over €650 million for projects that supported the circular economy package (European Commission, 2015).

In January 2017 a report on the implementation of the circular economy action plan has been presented by the Commission to the EU Parliament confirming its full commitment. Strategies on plastic recycling and reuse, chemicals and wastes facilitation of management as well as dialogue with stakeholders are essential actions for the 2017 in order to make the circular economy a reality (European Commission, 2017).

After intense debates among political groups, in March 2017 some relevant points have been prioritized: the need to give detailed definitions of "food waste", the need to reduce food waste up to 30% by 2025 and up to 50% by 2050 compared to the 2014 baseline, as well as the need to create an efficient monitoring system by 2017 (IAI, 2017).

Another report on the implementation of the Circular Economy Action Plan has been published in March 2019 and some relevant key point have been stressed: the importance of Circular Design and Production Processes to build circular models of production (Ecodesign Working Plan 2016-2019); empowering consumers who will be able to make informed choices based on reliable information; turning wastes into sources (revised waste legislative framework); new regulation for secondary raw material recover (Fertilising Products regulation); strategies for plastic lifecycle. All these points together with the already described actions, will accelerate the transition towards a greener, and climate-neutral economy (European Commission, 2019).

Finally, all these considerations and actions are considered in the so-called Agenda2030 (https://ec.europa.eu/europeaid/policies/european-development-policy/2030-agenda-

sustainable-development_en), where ‘sustainability’ and ‘circularity of processes’ are the key words for a sustainable development in many industrial and social fields.

5 Natural product research

5.1 A global interest: a focus on cosmetics

There has been a considerable rise in cosmetic products' demand in the past decades. Among various reasons there are a global economy growth, changing lifestyles, varying climatic conditions and also changing targets: in fact, the global cosmetic demand has been increasingly enhanced also by men lately (in 2015 it has been counted a 325% increase in men's cosmetic use since 1997). A shift of preference towards natural products, particularly in United States and Europe, promotes the growth of the cosmetics market. The increasing consumer awareness regarding natural ingredients' benefits is shifting the cosmetic market inclination towards greener products.

Figure 3, shows data collected by Allied Market Research (2016): among the main factors that have influenced the global cosmetics market in 2014, the most significant has been the change in lifestyles, followed by the second factor of greatest impact that has been the use of natural ingredients which will reach the first place within 2022.

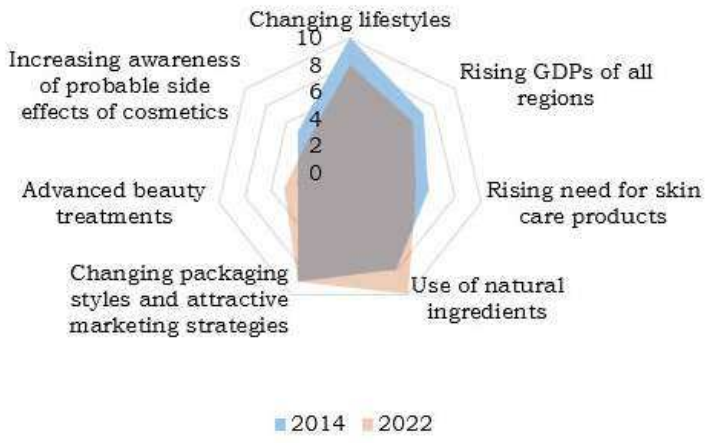
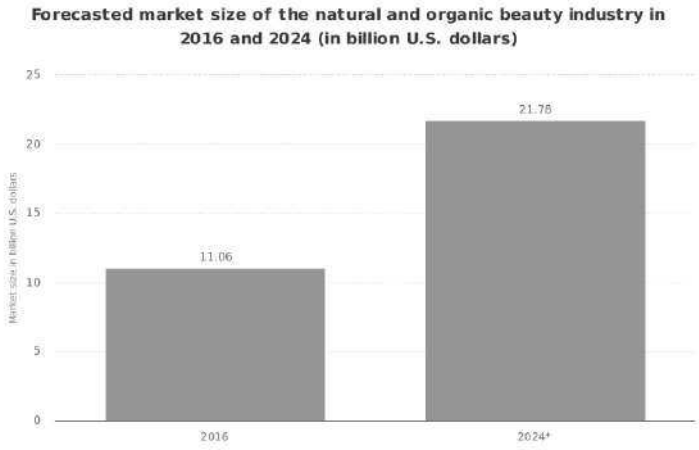


Figure 3. Top factors impacting Global cosmetics market in 2014 and 2022
www.alliedmarkedresearch.com

We are expected to register high growth rates between 2017 and 2026 in natural cosmetic use: with the largest numbers in North America, followed by Europe. The "Natural and Organic Personal Care Products Market: Global Industry Analysis 2013-2017 and Forecast 2018-2028" reported by Persistence Market Research (PMR), says that the global natural

and organic personal care products market is registering a healthy CAGR of 8.3% during the forecast period. A continual annual growth rate of 8-10%, which the industry has proven to be able to maintain over the last several years, predicts that the natural and organic beauty market will reach $22 billion in sales by 2024.

On the basis of application, the skin care segment is expected to register the highest growth: natural product research and innovation need to follow the numbers already reported with high quality works and high sustainable products (Allied Market Research, 2016; Persistence Market Research, 2017; Handler and Goldberg, 2018).



Graph 1. Forecasted market size of the natural and organic cosmetic industry in 2016 and 2024

www.statista.com

5.2 The Role of Natural Products Research

In the presence of a realistic threat against natural resources and biodiversity, the need to find green solution to tackle food waste and meet the growing demand for cosmetic products, natural products research has a crucial role in developing more and more sustainable strategies.

Pharmacognosy, defined as 'the study of biologically active natural products', is a broad-based science with many applications from agricultural to food industry; it is because of its multidisciplinary approach that it has become necessary to bring both philosophical and practical focuses on this science in order to address scientists' practices in green terms. According to Cordell (2017) 'the challenge for researchers is how to best develop, innovate, and apply new strategies using knowledge in a sustainable manner, by considering and integrating the relevant cognate technologies and ideas.

Use knowledge in a sustainable manner means also give intellectual property rights protection to indigenous who holds important traditional medicine lore: Nagoya Protocol[3] of 2010 is one of the major events of the past 50 years which has been thought to cover traditional knowledge associated with genetic resource.

It was out of these considerations, and many more, that the term "ecopharmacognosy" was proposed by Cordell in 2014 and defined as 'the study of sustainable, biologically active, natural resources', in order to underline the need to bring biodiversity and knowledge to posterity. Besides many other aspects of research, working as an 'ecopharmacognosist' means developing both new and established resources for nutraceuticals, cosmeticeuticals as well as foods. Moreover, the utilization of plant-based ingredients in industry, directly handed down by popular and ethnobotanical knowledge, could contribute to develop a green bioeconomy which have a global market with an annual growth of 11% and considering that 10-25% of today prescribed drugs contain at least one active compound isolated from plants (Devappa et al., 2015).

5.3 Biorefinery Concept: recovery of bioactive compounds

In addition to the aforementioned aspects of food waste management, other factors such as global warming, scarcity of resources and the constant lookout for natural biochemicals and products from both industries and market, are driving to a possible use of food wastes as source of biomaterials. Containing a variety of chemical components such as polysaccharides, proteins and lipids, food wastes have thousands of significant potential applications in the market as the research has demonstrated so far.

The novel concept of 'biorefinery' refers, analogously to the petroleum refinery, to the conversion of raw material into commercially valuable products: biofuel, bioplastics, nanoparticles and bioactives are some of the most relevant applications that have already demonstrated a valuable economic potential.

[3] The Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization to the Convention on Biological Diversity was adopted at the tenth meeting of the Conference of the Parties on 29 October 2010, in Nagoya, Japan.

Plant biomasses have been used for the production of biofuels for several decades, but recent developments permitted to use industrial organic wastes as possible source of bioethanol: Yang et al., (2014) prospected to use carbohydrates from noodle wastes to produce bioethanol; cooking oil wastes have been successfully converted into biodiesel by enzymatic transesterification (Seong et al., 2011; Lee et al., 2013). Food wastes and agricultural residues have been used as source of lignocellulose for the production of biodegradable plastics[4] (Ventaka and Venkateswar, 2013; Cesario et al., 2014).

Many other examples could be reported to emphasize the variety of applications of the biorefinery concept: between them, the use of food waste as renewable resource to produce valuable phytochemicals represents the main topic of this project.

Bioactive compounds are molecules with a certain effect upon a living organism and they naturally occur as secondary metabolites of plant; as they are already used as nutraceutical, cosmetic and phytotherapic constituents, their recovery from food waste rather than from cultivated plants represent a sustainable alternative to obtain them.

In addition, recovering the industrial food waste to obtain high added value molecules represents a significant economic potential for the industry. The conversion of citrus peel residues into high value products, in fact, would allow companies to increase their competitiveness in the market: citrus fruits, including oranges, lemons, limes, grapefruits and tangerines are sources of soluble sugars, cellulose and hemicellulose, pectin and D-limonene. D-Limonene, mainly used in essential oils as flavor and fragrance compound, it can be obtained after distillation of peel residues and used as a building block to generate compounds with similar structures (e.g. carveol, carvone, a-terpineol, perrillyl alcohol and perillic acid). Pectin, one of the most important food additives used as gelling agent and thickener, is a complex structural heteropolysaccharide found also in citrus fruits which contain 20–30% extractable pectin. Moreover, many flavonoids, including hesperidin, naringin and eriocitrin characterized the polyphenols profile of citrus peel and other solid residues of this species (Ki Lin et al., 2013).

Another good example of industrial agro-food waste exploitation is grape pomace: after orange production, *Vitis* sp. (Vitaceae), is the most produced crop in the world. Since grape

[4] Polyhydroxyalkanoates (PHAs) and Poly-3-hydroxybutyrate (PHB) are plastic-like materials which can replace petroleum-derived plastics.

pomace is approximately 20% of the weight of grapes processed, its valorization as a valuable by-product has been the subject of numerous studies recently (Tacchini et al., 2018). Flavonoids, anthocyanins, catechins, tannins and phenolic acids are some examples of phytochemicals with biological activity and potential health application: grape phenolics, for example, have been demonstrated to inhibit the oxidation of human low-density lipoproteins; grape pomace and seeds have been found to neutralize free radicals which are believed to contribute in the development of a number of health related problems like cardiovascular diseases and cancer (De Campos et al., 2008; Boussetta et al., 2011; Oliveira et at., 2013;).

In table 3 are listed some more examples of industrial agro-foods residues which have been subjected to exploitation in the last twenty years, many more are available in literature data including valorization studies on animal-derived wastes such as fish and meat products to obtain proteins, lipids, collagene and chitosans (Tahergorabi et al., 2011). Moreover, the production of functional foods enriched in biologically active compounds is becoming increasingly popular in many countries and the potential markets are enormous.

Source	Target phytochemical	References
Lemon by-product	Pectin	Schieber et al., 2011
	D-limonene	Schieber et al., 2011
Grape pomace and seeds	Dietary fibers	Schieber et al., 2011
	Polyphenols	Louanda et al., 2008
Orange peel	Flavonoids	Di Mauro et al., 1999
	Essential oils	Farhat et al., 2011
Potato peel	Phenols	Rodriguez de Sotillo, Hadley & Holm, 1994
Carror peel	Carotenoids	Chantaro et al, 2008
	Phenols	Chantaro et al, 2008
Tomato pomace	Lycopene	Naviglio et al., 2008
Olive pomace	Phenols	Obied et al., 2005
Rice bran	Insoluble dietary fibers	Nandi and Ghosh, 2015
Sesame husk	Insoluble dietary fibers	Nandi and Ghosh, 2015
Wheat bran	Fructans	Verspreet et al., 2015

Table 3. Examples of target phytochemicals from fruit and vegetable wastes

5.4 Emerging Technologies: Green Extractions

Returning to the concept of Cordell (Cordell, 2017) regarding the challenge for natural product researchers, research must necessarily consider sustainability in its development. Beside its sustainable aspect, the recovery of high value compounds must be economically interesting to be applied in industry. Conventional extraction processes are expensive, time-

consuming, not sustainable as they require amounts of energy and solvents and quite laborious (Cvjetko Bubalo et al., 2018), therefore in the past years various green alternatives which are safer, more efficient and in some cases economic, have been considered. Following the concept of green chemistry, which first appeared in 1991, six principles of green extractions have been given by Chemat et al., (2012) and summarized below.

I. Innovation by selection of varieties and use of renewable plant sources
II. Use of alternative solvents and principally water or agro-solvents
III. Reduce energy consumption by energy recovery and using innovative technologies
IV. Production of co-products instead of waste to include the bio and agro-refining industry
V. Reduce unit operations and favour safe, robust and controlled processes
VI. Aim for non-denatured and biodegradable extract without contaminants (heavy metals, mycotoxins, etc.)

EU environmental policy and legislation for the period 2010-2050 prioritized the use of eco-friendly solvents which needs to be safe for workers, process and environment and sustainable in their possibility to be reuse: green solvents must be in this sense chemically and physically stable, low volatile, easy to use and recycle (Cvjetko Bubalo et al., 2018).

5.4.1 Ultrasound-assisted extraction (UAE)

Ultrasound-assisted extraction has not only been used to extract bioactive compounds (e.g. polyphenolics, anthocyanins, aromatic compounds, polysaccharides and functional compounds such), but also essential oils, steroids, and lipids from plants.

Figure 4. Ultrasonic bath, Ultrasonik 104X, Ney Dental International, (photo: Ilaria Burlini)

Sound waves (from 20 kHz to 10 MHz) have been successfully utilized to extraction procedures using the cavitation effect. During the sonication process, longitudinal waves are created when a sonic wave meets a liquid medium, alternating compression and rarefaction waves. Sound waves create bubbles that grow and collapse: during the expansion cycle, these bubbles have a larger surface area causing the diffusion of gas and bubble to expand. During the compression cycle, in which the energy provided is not sufficient to retain the vapor phase in the bubble a rapid condensation occurs and large amounts of energy are released, creating shock waves. These shock waves create regions of very high temperature and pressure, inducing the penetration of solvent into cellular materials, by disrupting the plant cell walls and facilitate the release of extractable compounds.

During UAE extraction, several mechanisms of extraction have been identified and the results is the consequence of a combination of them: fragmentation, erosion, sonocapillarity effect, sonoporation, local shear stress and destruction of plant structures (Chemat et al., 2017).

Frequency and intensity of microwaves, product properties and ambient conditions (temperature and pressure) influence the UAE extraction ability. Ultrasound frequency can enhance the extraction yields with high reproducibility. Some other advantages of using ultrasounds are low temperatures, reduction of extraction time, amount of energy and CO_2 emissions and, in addition, the UAE apparatus is cheaper than that of other innovative technologies. Another advantage is that UAE has the capability for large commercial scale-up due to the availability of recently designed high units for large commercial operations (Vilkhu et al., 2008; Galanakis, 2012).

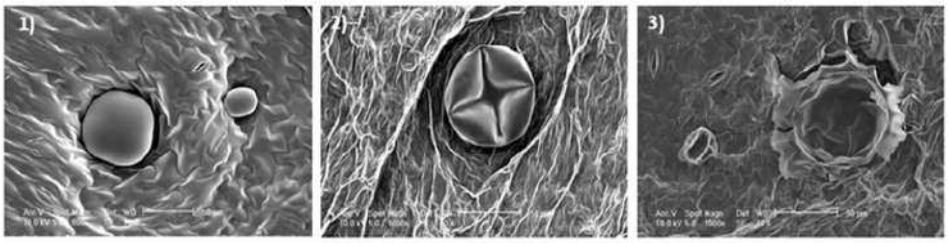


Figure 5. Effect of power ultrasound on boldo leaves: 1) control leaf surface, 2) leaf surface after conventional process, 3) leaf surface after UAE (Chemat et al., 2017).

5.4.2 Microwave-assisted extraction (MAE)

Microwave-assisted extraction work with electromagnetic radiations with a frequency from 0.3 to 300 GHz (generally 2.45 GHz). Microwaves possess electric and magnetic fields which are perpendicular to each other because of their electromagnetic nature. The electric field causes heating thanks to two mechanisms: dipolar rotation and ionic conduction.

The former is due to the alignment on the electric field of the molecules possessing a dipole moment in both the solvent and the solid sample: this oscillation produces molecules collisions and the liberation of thermal energy.

Unlike classical conductive heating methods, microwaves heat the whole sample simultaneously and this is one of the main advantages; another advantage of microwave heating is the disruption of weak hydrogen bounds promoted by the dipole rotation of the molecules; moreover, a higher viscosity of the medium lowers this mechanism by affecting molecular rotation and the migration of dissolved ions increases solvent penetration into the matrix facilitating the solvation of the sample.

The latter consists in ionic currents, which are also induced in the solution by the electric field: frictions which occur after the medium resists to these currents, causes heating liberation by a Joule effect. Size and charge of the ions present in the solution strongly influence this phenomenon. The effect of microwave energy is strongly dependent on the nature of both the solvent and the solid matrix and on the size and charge of the ions present in the solution. Solvents used can be polar and non-polar but the extracting selectivity and the ability of the medium to interact with microwaves can be modulated by using them in mixtures (Kauffman and Christen, 2002).

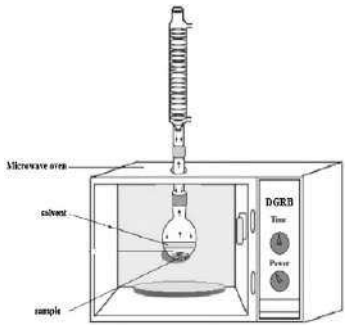


Figure 6. Schematic representation of a MAE (source: Thirugnanasambandham et al., 2015)

5.4.3 Pressurized solvent extraction (Naviglio® extractor)

Extractor Naviglio® has been presented as a technological innovation in the field of solid-liquid extractions and is the result of the application of a new principle called "Naviglio's Principle" (Naviglio, 2003).

The device, proposed by Daniele Naviglio in 2003, is a rapid and dynamic solid-liquid extractor that applies the "Naviglio's principle": 'in a suitable solvent, generating a negative pressure gradient and letting it to go to equilibrium between outside and inside of a solid matrix, that contains compounds that can be extracted in the solvent followed by a rapid equilibrium condition restoring, a forced extraction of the not chemically bound compounds contained in the solid matrix is produced' (Naviglio, 2003). Extractor Naviglio® can operate at room temperature or at sub-ambient temperature, and it works applying a pressure increase on the surface of the liquid phase containing the solid material (matrix) to be extracted; its device consists of one extracting chamber equipped with a cylinder and a piston where, at the bottom, one porous set let the liquid phase and liquid soluble substances pass through, while the solid particles are blocked. The solid raw material is put in the chamber that is filled with the solvent (organic, inorganic or a mixture). During the static phase, pressure gradient is applied allowing the system to reach equilibrium at a pressure of about 8-9 atm. When the piston is moved from its equilibrium position, the dynamic phase starts; this step is performed for five times and for a brief period of time with aim of remixing the solutions and to allow the diffusion of the extracted compounds. The movement of the piston and hence the static and dynamic steps alternate till the extraction process is efficiently completed. One extraction cycle is formed by one static and one dynamic step and by repeating more times these operations, complete exhausting of the solid matrix can be obtained.

The main advantages of Extractor Naviglio® are: the use of low or room temperature that reduces the thermal stress for any heat susceptible substances present in the matrix, it can be dimensioned according to the demand (from bench until industrial apparatus), large application possibilities (chemistry, medicine, agriculture, biology, etc.).

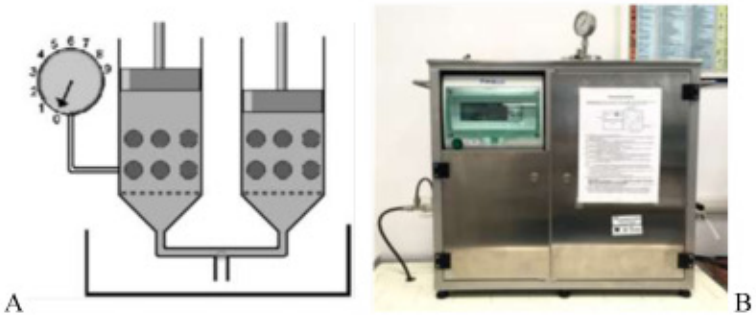
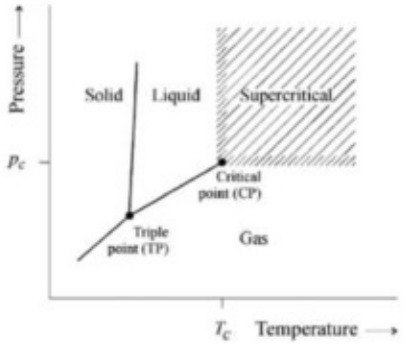


Figure 7. (A) Schematic representation of Naviglio® extractor; (B) Front view of extractor Naviglio®, laboratory model 500 cc (Atlas Filtri). (photo credit Ilaria Burlini)

5.4.4 Supercritical fluids extraction (SFE)

Supercritical fluid extraction is the process of separating one component from another (the matrix) using supercritical fluids as the extracting solvent. Extraction is usually from a solid matrix, but it can also be from liquids. More than 90% of SFE have been performed using carbon dioxide (CO_2) as a supercritical solvent, sometimes modified by co-solvents such as ethanol or methanol because it possesses low critical constants (extraction conditions for supercritical CO_2 are above the critical temperature of 31°C and critical pressure of 72 bar) (Cvjetko Bubalo et al., 2018).



Graph 2. Definition of supercritical state for a pure component (source: Sapkale et al., 2010)

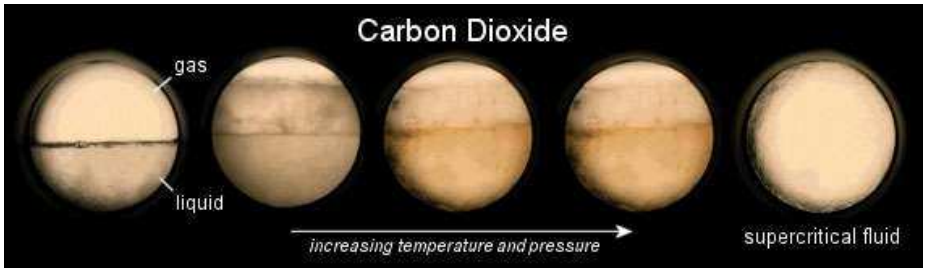


Figure 8. Supercritical carbon dioxide (source: www.avantech.it/)

The main advantages of the extraction process are: no solvent residue behind, the CO_2 is non-toxic, non-flammable, odorless, tasteless, inert, and inexpensive, various application possibilities in food, aromas, essential oils and nutraceutical industries (can be used as a sample preparation step for analytical purposes, or on a larger scale to either strip unwanted material from a product such as decaffeination or collect compounds as with essential oils) (Sapkale et al., 2010).

5.4.5 Subcritical water extraction (SWE)

Among the more recent developments of green extraction, water in its subcritical state has been identified as an effective solvent with numbers of advantages. At the temperature of 374°C and a pressure of above 220 bar, water is considered in its supercritical state, but subcritical water extraction is performed between 100°C and 374°C and high pressure to keep the water liquid. By altering water conditions, this solvent change its properties which become unique and adaptable for an effective and environmentally friendly extraction. In fact, at these conditions, water decrease its polarity and become suitable for both polar and non-polar compounds (this is due to a dramatic drop of the dielectric constant caused by the high temperature). As a low polar solvent, subcritical water is able to give high extraction yields and reductions of extraction time. At the same time viscosity and density of water decrease too and thus enhancing water penetration inside the sample matrix. Water is easily available, safe, low cost, non-toxic and non-inflammable and environmentally friendly: all these advantages of use brought subcritical water extraction to receive much attention among researchers from various research fields. Furthermore, the equipment easy to reproduce in a laboratory scale because of its uncomplicated design (Cvjetko Bubalo et al., 2018; Nastic et al., 2018).

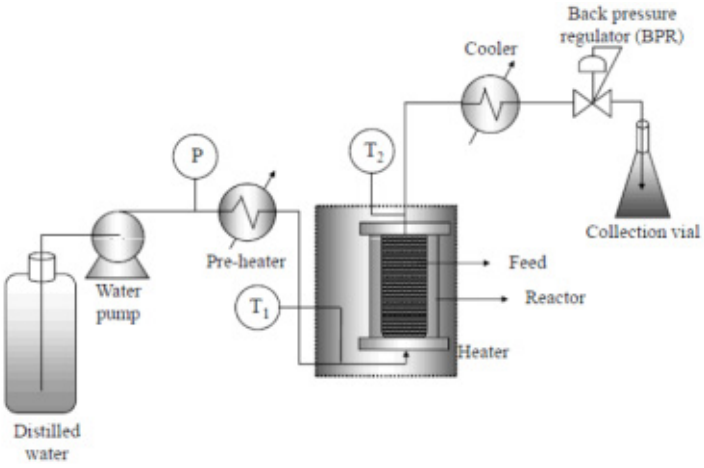


Figure 9. Schematic representation of a subcritical water extractor (source: Machmudah et al., 2015)

5.4.6 Natural deep eutectic solvents (NADESs)

First reported by Abbott in 2003 (Abbott et al., 2003), deep eutectic solvents (DESs) are now recognized as new sustainable solvents. Because of their similarity with ionic liquids, some of their properties are non-volatility, high viscosity, non-flammability. The preparation of DESs is simple: it is sufficient to mix organic salts such as quaternary ammonium or phospohonium salt, with metal salts or hydrogen bond donors (capable to create intramolecular hydrogen bonds between each other). NADESs (natural deep eutectic solvents) are DESs produced from primary metabolites common in living cells (choline, sugars, carboxylic alcohol, etc.) which are involved in the biosysthesis and storage of various non-polar compounds. They can be defined as green solvents because of their properties: low cost, simple to prepare, non-toxic, biodegradable, readily available and they can be tuned easily for specific applications. NADESs capacity to be good extractive solvents depends on their combination and physiochemical properties, water can also be added to modify polarity; it has been estimated that 10^8 are the possible combinations of NADESs their cost is comparable to the one of conventional solvents.

Disadvantages are also described: they are difficult to reuse of recover, the industrial scale up is possible only when the extract is used without purification steps and high energy could be require for stirring because of the high viscosity of NADESs (Zainal-Abidin et al., 2017; Cvjetko Bubalo et al., 2018).

5.4.7 Enzyme-assisted extraction

Enzyme-assisted extraction have gained much attention nowadays because of the need of green extraction technologies, even if the use of enzyme to extract bioactive compounds is already well established, its association with other technologies such as sonication and microwaves, is a promising tool. Enzymes, with their ability to disrupt cell walls, represent a good alternative to release compounds with higher yields comparing to conventional extraction methods. Particularly the use of enzymes in extraction procedures enable to reduce solvent amounts and also to extract compounds which are naturally found in a bound form with other plant structural components. Among the advantages of using enzyme-assisted extraction there are low extraction time, high yields and low energy and low solvent consumption.

Cellulases, pectinases and hemicelluloses are often required to hydrolyze cell wall components and thus increasing cell wall permeability; they can be derived from bacteria, fungi, animal organs or plant extracts. Appropriate operational conditions and enzymes combination are important parameters to obtain a successful result.

Enzymatic extractions are subjected to continuous research: good examples are the extraction of oils, polyphenols, phenolic acids, vanillin, polysaccharides and lycopene from tomatoes (Puri et al., 2012).

The release of bound polyphenols from plant cell walls represent a never-ending challenge among researches which needs to find green alternatives to the commonly used acid and alkaline hydrolyses conditions. Enzymes, such as feruloyl esterases, can be involved in the release of bound phenolics, in particular phenolic acid from cereal pericarps: ferulic acid exhibited numbers of possible applications such as in health, medicine, food and cosmetic fields (Sindhu and Emilia, 2004).

5.4.8 Hybrid techniques

Beside all the innovative or sustainable extraction strategies described above, the possibility of combining two or more of them, in order to optimize results, is receiving great attention nowadays. Various combinations of extraction procedures have been investigated so far on plant materials: in particular, the combination of ultrasounds with conventional or unconventional methods have demonstrated to be effective.

Ultrasound-assisted soxhlet extraction (Sono-Soxhlet), used by Djenni et al., (2012) combines the advantages of the soxhlet extraction (availability of fresh solvent) and of ultrasounds by enhancing mass transfer and reduction of extraction time. This system has been used for the extraction of lipids from seeds, sausage products, cheese and bakery products.

Another promising hybrid which is fast and efficient, is made by the combination of ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) and it was used for the first time by Cravotto et al. (2008). This combination allows to dramatically short extraction time with great potential industrial applications. The mechanical ultrasonic effect promotes the release of soluble compounds from the plant body by disrupting cell walls and microwaves heat the sample inducing the quick migration of molecules. The simultaneous irradiation increases the penetration of the solvent into the plant matrix and can increases the solubility of compounds. This technique has been used in the extraction of oils from vegetable sources (Cravotto & Binello, 2010).

Sonication have demonstrated also a positive effect when coupled with supercritical fluids (SC-CO_2) since UAE enhance the mass transfer of ginger to the solvent used for extraction of pungent compounds increasing the yields (Balachandran, 2006).

Between many other possible combinations of technologies that can be used to extract biomolecules following the green chemistry concepts, two other strategies need to be mentioned: ultrasound-assisted chemical hydrolysis and ultrasound-assisted enzymatic hydrolysis. These methods, recently used to extract bound phenolics and other bound compounds from various vegetable matrixes, allow to short extraction times and enhance the phenolic yields when compared to conventional hydrolysis conditions such as alkaline hydrolysis with NaOH 2N (Gonzales et al., 2014) and enzymatic treatments with feruloyl esterase (Wang et al., 2014). The advantages connected to these processes and a detailed description of methods will be described in the ‘Materials and Methods’ chapter as they have been used experimentally in the present PhD project.

Since the extraction technique’s choice of a desired metabolite has to be a result of a compromise between the efficiency and reproducibility of extraction, ease of procedure and considerations of cost, time, safety and degree of automation, the use of ultrasounds (alone or in combination with other techniques) has become nowadays, one of the most effective devices used to obtain green extracts (Chemat et al., 2017).

6 Cereals

6.1 Etymology and History

The etymology of 'Cereal' derives from the roman term 'Cerealia', the holidays celebrated by the Romans in honour of Ceres, the goodness of agriculture, granted with the best of the annual harvest. However, the importance of cereals in ancient civilization comes from much earlier: wheat was a sacred food for the Egyptians and the Greeks attributed to the mother goodness Demeter, the teaching of agriculture and wheat cultivation to mankind. Quinoa, called "mother of all grains" was considered sacred by the Incas likewise amaranth was considered sacred by the Aztecs; the Maya believed in gods who represented aspects of nature, society and professions: the maize god, Hun Hunahpu, was one of the most important and he is represented as a youthful, handsome man (Figure x). Moreover, there are mentions of spelled grains in the Old Testament.

Figure 10. Ancient finds that testify to the importance of cereals in Egyptian and Roman civilizations (source: wikipedia.org)

Figure 11. (left): Mexican figurine from the Metropolitan Museum of Arts (New York) from the 700s AD, showing the Maize God wearing jewelry and a headdress in a corn plant; (right): Maya's Maize God, stone bust in the British Museum, (London, England)

About 10,000 years ago, prehistoric communities started to make the transition from hunter-gathered to farmer, starting the so-called Neolithic Revolution: early Neolithic villages show evidence of the development of processing grain, particularly in the Levant

Region (equivalent to the historical region of Syria) where the ancestors of wheat, barley and peas were based. There are, in fact, evidences of cereal production in Syria approximately 9,000 years ago. The spread of agriculture supported the increase of population as well as the development of cities and political organization (in order to make decisions regarding labor, harvest, access rights to water and land). In the same period China began to farm rice and millet (The world's oldest known rice paddy fields, discovered in eastern China in 2007); sorghum and millets were also domesticated in sub-Saharan West Africa. Corn had to wait for natural genetic mutations to be selected for in its wild ancestor, teosinte[5] and the first directly dated corn cob dates only to around 5,500 years ago in North America ("The Development of Agriculture". National Geographic. Archived from original on 2016).

6.2 Botanical description

The term 'cereals' comprises every edible component of the caryopsis of a cultivated grass (herbaceous annuals from the Poaceae Family) but it doesn't represent a botanical precise classification; the term 'cereals' includes: wheat, oat, rice, corn, barley, sorghum, rye, millet and triticale. Pseudocereals includes edible grains which are included into other plant Families such as Polygonaceae (buckwheat), Amaranthaceae (quinoa) and Lamiaceae (chia).

6.2.1 The Poaceae Family

The Poaceae Family owes its name to John Hendley Barnhart in 1895 based on the tribe Poeae described in 1814 by Robert Brown and to the type genus Poa described by Linneus in 1753; the term derives also from the Ancient Greek "πόα" (fodder).

Also known as Graminaceae, the Poaceae Family, is a division of the order Poales and is a large and ubiquitous Family of monocotyledonous plants, commonly called grasses. It includes rhizomatous herbaceous plants or trees that can be annuals or perennials.

[5] Teosinte is an Aztec name meaning "mother of the mays"

With 780 genera and around 12,000 species of plants, Poaceae is the fifth largest plant Family (after Asteraceae, ~23,000, Orchideaceae, ~20,000, Fabaceae ~18,000 and Rubiaceae, ~18,000); they are the most abundant and important Family as they account for about 24 percent of the Earth's vegetation.

They grow on all continents, from the desert to freshwater and marine habitats, except high altitudes. Nearly three-quarters of Poaeceae genera are confined to one of seven centers of distribution: Africa, Australia, Eurasia north of the Himalayas, South and Southeast Asia, North America, temperate South America, and tropical America. About one-fifth have even broader distribution patterns throughout temperate or tropical regions of the world.

Seven major groups (subfamilies) are distinguished depending on their structural features such as the anatomy of the leaves and their geographic distribution: Bambusoideae, Oryzoideae (e.g. *Oryza sativa*), Poideae (e.g. *Triticum durum*, *Triticum aestivum*, *Avena sativa*), Chloridoideae and Panicoideae, Arundinoideae (e.g. *Phragmites australis*), Centothecoideae and Stipoideae. The successful development of grasses resulted from their great tolerance of grazing herbivores and fire, their varied means of reproduction, and their versatility in photosynthesis.

Grasses are perennial or annual, usually terrestrial and free-standing; they are rarely vine or aquatic. The root system consists of fine, fibrous roots. Corms and bulbs are sometimes present and prop roots may develop from the lower nodes or joints of the stem (as in maize). Grass stems, called culms, are herbaceous or woody, and they range from about 2 cm to 40 m in height and 30 cm in diameter in bamboos. The stems of grasses range from fully erect to prostrate, they are solitary to densely clumped, as in the so-called bunch grasses. Many grasses produce horizontal stems, either below ground (rhizomes) or above ground (stolons). The internodes, or stem regions between the nodes, are usually round in cross section and either hollow or filled with a spongy pith. The structural strength of grasses comes from the leaf sheaths; the other major part of the grass leaf is the blade. Grass leaves are single at the nodes and arranged in two vertical ranks; structurally the point of leaf initiation alternates with each node; the leaf sheath grows to encircle the stem and overlap when the two points meet. Grass leaf blades (1-5 cm long) are usually long and narrow, with parallel margins, occasionally have a lance, egg, arrow or heart shapes. In grasses of arid areas, leaves roll up to form long and thin tubes in order to reduce water loss. The leaf veins are parallel; some special cells in the outer cell layer of the leaves contain silica

bodies, which shapes is used to distinguish large groups of grasses. Leaves have a ligule, a small flange or ring of hairs, at the junction of leaf sheath and blade (collar), and on the side facing the stem, used by the plant to prevent the entry of water into the leaf sheath. In some grasses (particularly Bambusoideae) the leaf is constricted and resembles a stalk or petiole at the base of the blade (pseudopetiole) that moves the leaf downward or upward at night.

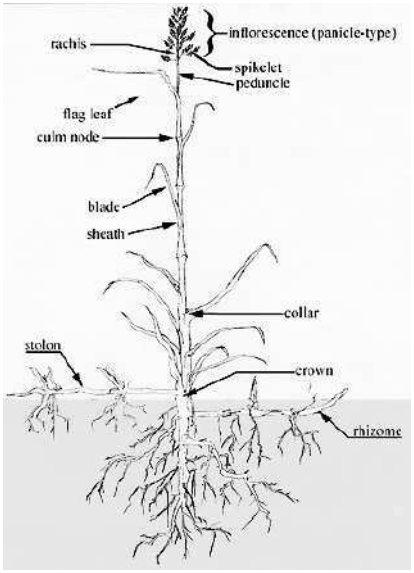


Figure 12. Grass schematic structure (source: forages.oregonstate.edu)

The primary inflorescence of grasses is the spikelet (highly useful in the identification of grass species and genera), a small structure consisting of a short axis, the rachilla, to which are attached chaffy, two-ranked, closely overlapping scales. There are three kinds of scales: the glumes, the lemma and the palea. Grass spikelets consist of usually 2 glumes and 1 to about 50 florets, depending on the species. The base of the spikelet may be hardened into a pointed and hairy callus, usually best developed in spikelets with an awn that twists when atmospheric humidity changes. Spikelets are the units of the secondary grass inflorencesce which differs in characteristics depending on the species: wheats have spikelets attached to a central axis without a stalk or pedicel; the bluegrasses of the genus *Poa*, in contrast, have a panicle inflorescence, with the spikelets borne on distinct pedicels. Grass flowers are minute and simple; in place of the petals there are translucent structures called lodicules. Flowers are adapted for wind-pollination since they don't attract animal pollinators, and they don't have nectar with pollen to be transported by animals. For this reason, there is an

abundance of pollen contained in a range of 3 to 120 anthers. Grass flowers may be bisexual or unisexual: flowers of wheat, barley, oat and rye are bisexual; the flowers of corn are unisexual, although inflorescences for pollen and others for fruit are on the same plant. The production of male or female gametes on separate individuals is rare in plants.

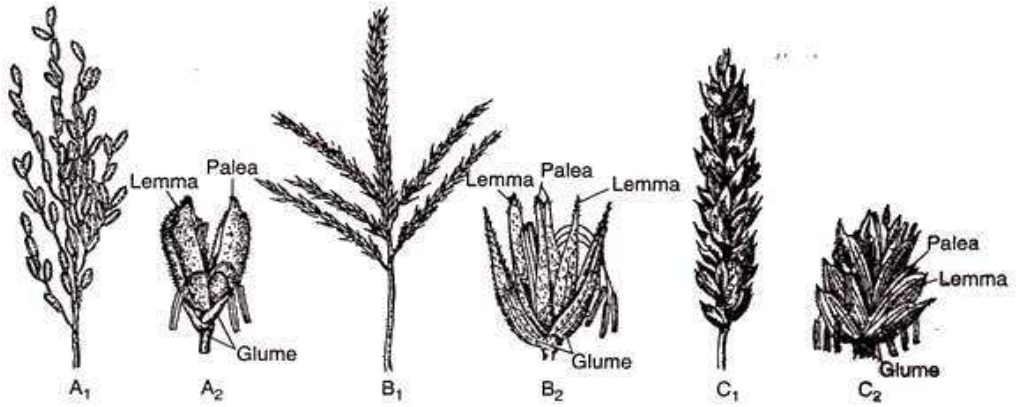


Figure 13. Inflorescence formed by spikelets (A1); inflorescence of rice (A2), spikelets of rice (B1); male inflorescence of maize (B2); two-flowered male spikelet of maize (C1); inflorescence of wheat (C2) (source: www.biologydiscussion.com)

Grass fruits, called caryopses (grain), are unusual among plants in that the fruit wall completely adheres to the single seed; caryopses are generally dry. The caryopsis, also known as kernel, is constituted by a large endosperm (aleurone external layer and endorsperm cells with starch granules), germ (embryo) and bran (testa and pericarp). The pericarp consists of a thin-walled, long, rectangular cells; the hypoderm vary in thickness. The cells of the outer part of the pericarp are elongated in the length-wise direction of the grain. The inner layer of the pericarp becomes torn during ripening and in the mature grains is represented by a layer of branching hyphalike cells known as “tube cells”. The testa (or seed coat) is a thin single or double layer with the inner part often pigmented and the hyaline layer is colorless. The bran of cereals comprises all outer structure of the kernel, including the aleurone layer; in wheat, the proportion of bran is more affected by the variety than by environment. Botanically, the aleurone layer is the outer layer of the endorsperm but the miller regards it as part of the bran. In wheat it is a single layer of thick cubical cells and almost completely surrounds the kernel over the starchy endosperm and germ and it is reported to be 60-70 μ thick; it represents the differentiated meristematic layer which by tangential division produced the radial rows of endosperm tissue during the first 10-14 days of development. Each aleurone cell contains one large nucleus and aleurone granules; in

wheat, maize, rye and oats the aleurone layer is one layer thick; in barley is two or three cell layers thick; in rice it is two or three cell layers thick in the species *indica* but five or six layers thick in the species *japonica* and the ventral side of the embryo is protected by a prolongation of the aleurone layer. The starchy endosperm consists of a thin-walled cells variable in shape, size and composition. The cells adjacent to the aleurone cells are small and cubical, the others are elongated in a radial direction, becoming large and polygonal in the center of the cheeks. The endosperm consists mainly of cells with starch and protein (the starch in granules and the protein filling the intergranular spaces). The endosperm of maize is variable; it has a crown region which is light in color and a horny region more intensely colored in yellow. The latter region contains more than double oil content than the former. The size and shape of starch granules vary from one cereal to another (simple or compound).

The embryo (germ) consists of scutellum, embryonic axis, plumule covered by coleptile, primary root covered by coleorhiza, secondary lateral roots and epiblast (Kent, 1966; Pomeranz, 1971; www.britannica.com).

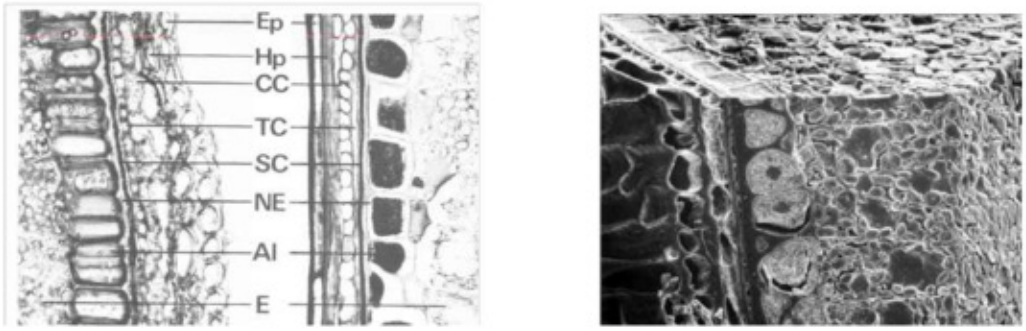


Figure 14. (left)Transection and longisection through pericarp and adjacent tissues (EP, epidermidis; Hp, hypodermis; CC, cross-cell; TC, tube cell; SC, seed coat; NE, nucellar epidermis; AL, aleurone; E, starchy endosperm. 200X) (source: Pomeranz, 1971); Fig. (right) Fig. (right) Scanning electron micrograph of the outer surface of a wheat grain, showing starchy endosperm, aleurone and overlying nucellar remnants, seed coat and inner pericarp (Joyner, 1985).

6.2.2 *Triticum durum* (Desf.)

Order: Poales

Family: Poaceae

Genus: *Triticum*

Species: *Triticum durum* (or *Triticum turgidum* subsp. *durum)*

Figure 15. Durum wheat (photo credit: Aldo De Bastiani)

Triticum durum, known as durum wheat, is an annual grass in the Poaceae (Graminaceae family), it is also called hard wheat because of the hardness of its caryopsis.
Because of its low gluten content, durum wheat is not used for bread and baked goods, but is wheat used in pasta, semolina, bulgur and couscous.

The word "wheat" derives from different locations, particularly from English, German and Welsh languages: the middle English term "whete" evolved from the Old English "hwæte". Similarly, the old German term "weizzi" for wheat, evolved from "hwīzandwīz", meaning white; the Welsh term gwenith, is very closely related to gwenn, all these terms mean "that which is white" due to wheat light color.

Wheat is a 6-12 dm tall, annual herbaceous plant made up of leaves surrounding a slender stalk that terminates in spikes, called ears, of grain at the top of wheat. Each spike, of grain is made up of spikelets, which encloses the wheat grain in between the lemma and the palea. The wheat grain has oval shape and responsible for wheat nutritional value. The grain may also vary in its length of brush hairs, either long or short. Cultivated wheat is most commonly grown with physical characteristics of fusiform spikes, with awns and easily threshed. Domestic wheats are also bread for strong seed heads which will not shatter during processing. More than 30,000 varieties of wheat exist today, divided into varieties such as common wheat (*Triticum aestivum*), Einkorn wheat (*Triticum monococcum*), Emmer (*Triticum turgidum* L. subsp. *dicoccon* (Schrank) Thell.) and Durum wheat (*Triticum durum*) and Spelt (*Triticum spelta*).

Hard wheat contains hard, small kernels while soft wheats contain larger, softer kernels. Durum wheats are completely different than both hard and soft wheats because its kernels are much larger and is has a unique shape than the other wheat varieties.

Durum wheat is a mid-tall annual grass with flat leaf blades and a terminal floral spike consisting of perfect flowers. The root system is composed of seminal roots which are produced by the young plant during germination and adventitious roots, which arise later from the basal nodes of the plant to become the permanent root system. The stem is cylindrical, erect, usually hollow, and subdivided into internodes, some durum wheats have solid stems. Culms arise from auxiliary buds at the basal nodes of the main stem; the number of culms formed depends on the variety, growing conditions, and planting density. As with other grasses, durum wheat leaves are composed of a basal portion which envelops the stem, and a terminal portion, which is linear with parallel veins and an acute apex. At the attachment of the leaf sheath is a thin, transparent membrane (ligule) with two small lateral appendices called auricles. The main stem and each culm produce a terminal inflorescence which is a spike with a rachis bearing spikelet separated by short internodes. Each spikelet consists of two glumes (bracts) enclosing two to five florets, all borne distichously on a rachilla. Each floret is enclosed by bract like structures called the lemma and the palea. Each floret is a perfect flower, containing three stamens with bilocular anthers and a pistil bearing two styles with feathery stigmas. Mature pollen is fusiform, normally containing three nuclei. Each floret has the potential to produce a one-seeded fruit called a caryopsis. The fruit, the kernel, is constituted by a large endosperm (aleurone external layer and endorsperm cells with starch granules), flatted germ (embryo) and bran (testa and pericarp) as shown in Figure 16 (Pignatti, 2017).

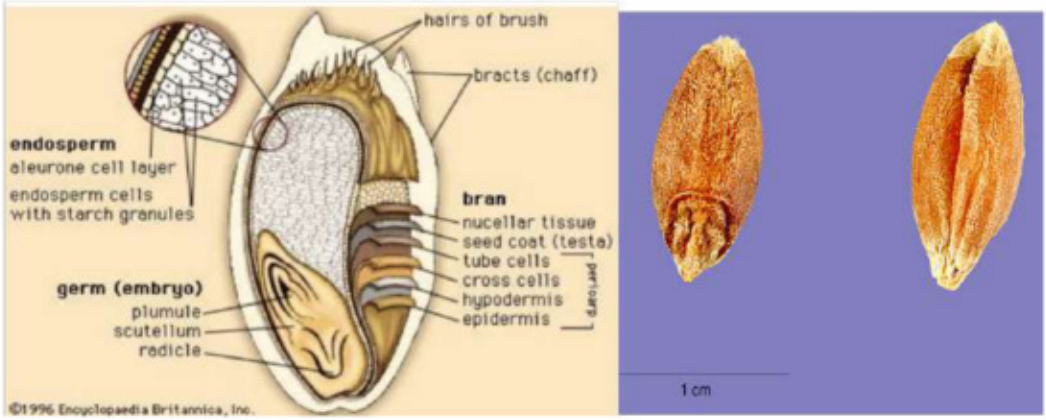


Figure 16. schematic representation and photograph of wheat kernel (source: Encyclopedia Britannica, 1996)

Geographical distribution

Durum wheat is grown in temperate climates worldwide (all types of wheat have been estimated to cover around 4% of the planet's agricultural land), particularly in the Mediterranean areas, United States, Canada and norther Mexico and some minor regions such as Russian Federation, China, Kazakhstan, India, Argentina and Australia. The Italian distribution of *Triticum durum* production is depicted in Figure 17.

Originated in the middle east regions of today Turkey, Syria, Iraq and Iran; with archaeological evidence of the cultivation of various species in the Fertile Crescent dating back to 9,600 B.C., wheat is one of the most ancient of domesticated crops.

Over 25,000 species have been developed into thousands of cultivars that differ in chromosome number: durum wheat is allotetraploid (two genomes: AABB) with 28 chromosomes, containing the full diploid complement of chromosomes from each of its progenitor species. Durum wheat was developed from wild emmer or kamut (*Triticum turgidum*) and has larger, harder grains and a higher protein content and lower gluten content that bread wheat (*Triticum aestivum* varieties).

Figure 17. Italian distribution of durum wheat cultivation (www.dryades.units.it/floritaly/)

6.2.3 *Oryza sativa* L.

Order: Poales

Family: Poaceae

Genus: *Oryza*

Species: *Oryza sativa*

Figure 18. Oryza sativa (Photo credit: right: Stefan Porembski; left:Shigeki Limura, Minden Pictures)

Oryza sativa L., most commonly known as Asian rice or simply rice, is an annual (although some varieties are perennial) herbaceous plant, 7-15 dm. It is the second most important cereal in term of production, second only to corn; its cultivation is mainly finalised to rice grain production and less commonly to rice flour used to produce gluten-free and children food products. Rice has also been important as a model system in plant biology since its genome has been the first to be fully mapped among all plant species (Encyclopedia of Life www.eol.org). The genus Oryza belongs to the tribe Oryzeae of the family Poaceae; there are 12 genera within the Oryzeae tribe and the genus Oryza contains approximately 22 species of which 20 are wild species and two, *O. sativa* and *O. glaberrima*, are cultivated. Rice is a typical grass, forming a fibrous root system bearing erect culms and developing long flat leaves. It has a semi-aquatic lifestyle, requiring water particularly during the reproductive growth phase. It forms multiple tillers, consisting of a culm and leaves, with or without a panicle; the panicle emerges on the uppermost node of a culm, from within a flag-leaf sheath and bears the flowers in spikelets. The culm consists of a number of nodes and internodes that increase in length and decrease in diameter up the length of the culm. Primary tillers emerge from nodes near the base of the main culm and secondary and

tertiary tillers emerge sequentially from these. Single leaves develop alternately on the culm, consisting of a sheath, which encloses the culm and a flat leaf blade. The leaf forms a collar between the sheath and blade and a ligule and two auricles develop on the inside of the collar and base of the leaf blade respectively. Cultivars can vary widely in the length, width, color and pubescence of the leaves. The fruit, the rice kernel, is constituted by endosperm (aleurone external layer and endorsperm cells with starch granules), germ (embryo), bran and husk, as shown in Figure 19. (Vaughan et al., 2003; Australian Government, Department of Health, 2005; Pignatti, 2017).

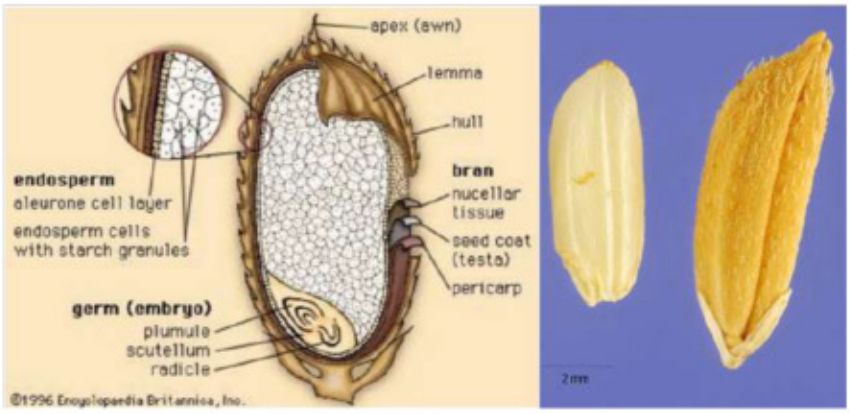


Figure 19. Schematic representation and photograph of rice kernel (source: Encyclopedia Britannica, 1996)

Geographical distribution

Figure 20. Italian distribution of rice cultivation (dryades.units.it/floritaly/)

It is originally from South-east Asia (India, Thailand and South China), it is now cultivated in wet tropical, semi-tropical, and warm temperate areas around the world for the production of rice grains. *O. sativa*, the most widely grown of two cultivated species, is grown worldwide, including in Asian, North and South American, European Union, Middle Eastern and African countries; *O. glaberrima* is grown solely in West African countries. The Italian distribution of *Oryza sativa* L. production is depicted in Figure 20.

The date of the first domestication estimated between 8,200 to 13,500 years ago.: while the older theory proposed in 2006 say that *O. sativa* var. *indica* was domesticated in easter India, Myanmar

and Thailand and that the var. *japonica* in southern China and Vietnam; in 2008 it has been determined a single domestication event for *O. sativa* happened in the region of the Yangtze River valley. Another theory from Korean archaeologists proposed the domestication of rice in Korea (dated to 13,000 BC). Cultivated in Babylon and in the Middle East by 2,000 years ago, it was finally spread to Europe during medieval times (Londo et al., 2006; Vaughan et al., 2008).

6.2.4 *Zea mais* L.

Order: Poales
Family: Poaceae
Genus: *Zea*
Species: *Zea mays*

Figure 21. Zea mais (Photo credit: right up: sciencenordic.com; right down: maveenseeds.com; left: thebalance.com)

Also known as corn, maize is an herbaceous annual plant 1,5-3m tall, originated from Central America. It has become the first cereal produced worldwide (before rice and wheat) but human consumption represents just a smallest final utilization: in fact, it is primary used for livestock feed, ethanol production and production of oil and sweeteners.
For human consumption corn is used fresh or it can be dried and ground to produce flour or meals. The oil, obtained from the grain, is used both for cooking and industrial uses; cornstarch can be processed enzymatically to make inexpensive corn syrup to replace sugar.

The word *maize* derives from the Spanish form of the indigenous Taíno word for the plant: *mahiz*; the world *corn* is considered less scientific since it can refer to any cereal crop and can vary by context and geographic region (Ensminger, A. H., 1994).

Each corn plant contains both male and female reproductive organs. Plants have staminate spikelets in long spike-like racemes forming large spreading terminal panicles and pistillate inflorescences in the leaf axils in which the spikelets occur in 8 to 16 rows on a thickened, almost woody axis. The whole structure (ear) is enclosed in numerous large foliaceous bracts and a mass of long styles (silks) protrude from the tip as a mass of silky threads to catch the pollen that is blowing in the wind; each ear of corn contains upwards of one thousand potential kernels. Fine hairs cover the end of the silks. The kernel has three parts: the pericarp with the bran, a thin layer of maternal tissues that encloses the entire seed and usually colorless but can be red, brown, orange, and cherry. The endosperm, or food storage organ, consists primarily of starch and of a thin outer layer known as the aleurone layer and the embryo (germ) (Hitchcock and Chase, 1997; Rueben & Casas, 2003; Pignatti, 2017).

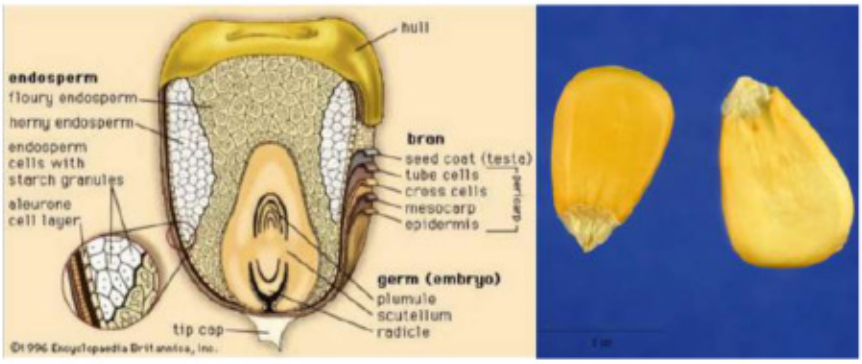


Figure 22. Schematic representation and photograph of maize kernel (source: Encyclopedia Britannica, 1996)

Geographical distribution

Maize is native to the western hemisphere, but its exact birthplace is still not certain. Archeological evidence of corn's early presence in the western hemisphere was identified from corn pollen grain considered to be 80,000 years old around Mexico. Even though historians believe that corn was domesticated in the Tehuacan Valley of Mexico, recent research now indicate the adjacent Mexican Balsas River Valley as the center of domestication. Corn began to spread in Europe after 1492 when Spanish discovered the grain in Cuba: at first, corn was only a garden curiosity, but within a few years, it spread

Figure 23. Italian distribution of maize cultivation (dryades.units.it/floritaly/)

throughout France, Italy, and all of southeastern Europe and northern Africa as valuable food crop. In 1575, corn was exported to western China, and after became important in the Philippines and the East Indies.

According to genetic studies, maize was introduced in South America from Mexico, in two waves: the first, more than 6000 years ago, spread through the Andes, the second, about 2000 years ago, through the lowlands of South America.

It is now the most completely domesticated of all field crops; its modern characteristics are far from similar to the ancient grains (teosinte - *Zea mays* ssp. *Parviglumis* or wild maize - *Z. mays* subsp. *mexicana*) and certainly it could not have existed as a wild plant in its present form (Benz, 2005; Iowa State University, 2014).

Maize is cultivated everywhere throughout the world, and the major producers are United States, China, Brazil, Argentina, Ukraine, India and Mexico (FAO, 2014). The Italian distribution of *Zea mais* L. production is depicted in Figure 23.

6.3 Cereal grains production

Cereals have been a primary source of nutrients since the domestication of the 'big three' (rice, corn and wheat) have started around 10,000 years ago and they have played an important role in shaping human civilization since that times. As mentioned above, the three most important crops in terms of production are, in order, corn (maize), wheat and rice as shown in table 4.

Cereal grain	Production in MMT (Million Metric Tonnes)
Corn	825
Wheat	650
Rice	680 (440*)
Barley	150
Sorghum	60

Table 4. Major world cereal grain production in 2010 (source: Awika, 2011)

**polished rice*

Corn, in 2010, was the major cereal grain produced in the world with 825 MMT, followed by wheat (650 MMT) and rice (440 MMT polished rice): these three crops contribute for more than a half of human dietary calories.

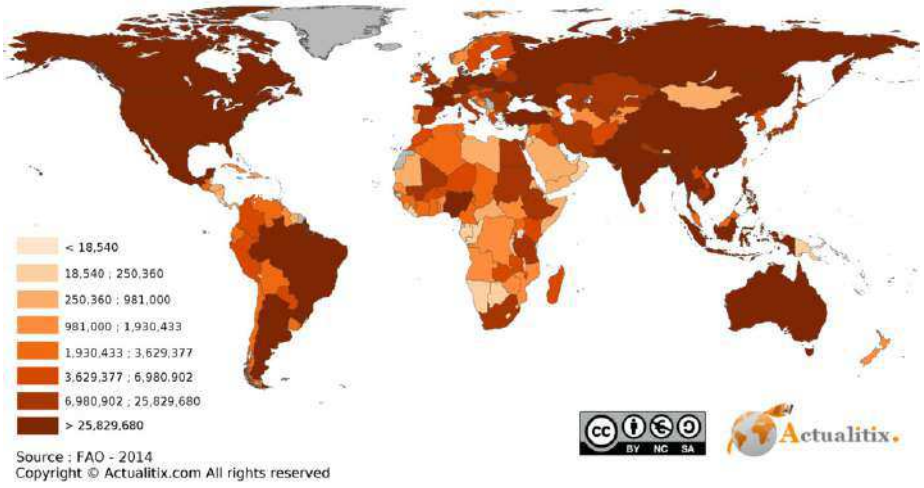


Figure 24. World cereal tons production in 2014 (source: it.actualitix.com from FAO)

World consumption and production is directly connected to geographical characteristics and water availability: for example, Burkina Faso and Niger, semi-arid African countries, introduce calories for more than 85% from cultivation and consumption of millet and sorghum (FAO, 2011).

Moreover, in developing countries more than 60% of intake calories come from cereal consumption (this percentage rises up to 80% in the poorest regions) and it decrease until 30% of intake calories in developed countries (Awika, 2011).

Cereal consumption seems to be contradictory in terms of human health: in developing countries where daily intake of carbohydrates is very high, malnutrition problems are high as well because of the low cereal content of proteins (lysine in particular) and because of the intake of refined grains which are poor in micronutrients. The second reason is connected to developed countries obesity: high contents of easily digestible carbohydrates have been cited as major contributors to this disease. The contradiction lies in the fact that certain grains consumption in developing countries demonstrated low incident of cancer and chronic diseases, particularly consuming high quantities of sorghum and millet. Numbers of publications are counted in literature about health benefits of cereals consumption (benefits against cardiovascular diseases, cancer, diabetes and other chronic

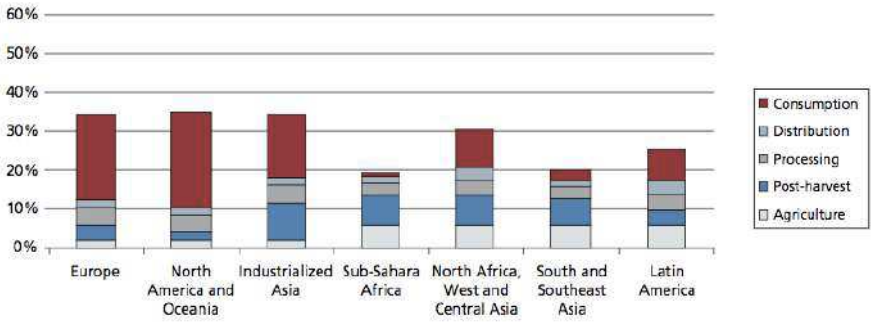
diseases) emphasizing the importance of whole grains intake which is, despite the recommendations, still very low (Anderson, 2003, Gani et al., 2012).

Whole grains have been defined as 'the intact, ground, cracked or flaked caryopsis, whose principle anatomical components (the starchy endosperm, germ and bran) are present in the same relative proportion as they exist in the intact caryopsis' (Gani et al., 2012). Whole grains are, in fact, important sources of carbohydrates, proteins, lipids, dietary fiber, vitamins (mainly B-complex and E), minerals and bioactive compounds. In particular, the outer layer of grains (brans and husks) have been shown to contain the highest levels of bioactive compounds such as phenolic compounds, phytosterols, tocols and carotenoids, responsible for the already mentioned beneficial effects; for these reasons the benefits of grains are mainly found in the seed coat.

Milling technologies, industrialized from 1800s, remove the brans and germs from the cereal grains efficiently, thus the refined flour obtained is mostly endosperm, poor of fibers, lipids, vitamins, minerals and phytochemicals which are removed during the milling processing too.

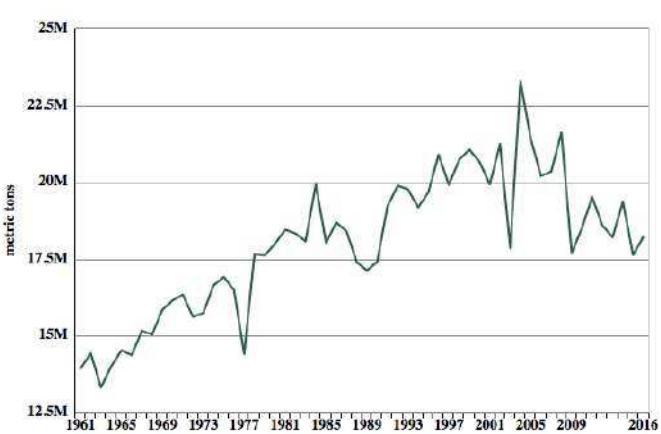
Beside the sensory appeal reasons which underlie the milling processes (bright color, smooth texture, etc.), the other advantage of this process is keeping the quality of flour by removing lipids (responsible for quick oxidation and rancidity). Even though it is very important to recommend whole grains consumption with its advantages, on the other hand it is unlikely that there will be a rapid change in the eating habits of the population, therefore, the recovery of milling by-products remain a possible solution.

In medium- and high-income countries the consumer phase is the stage of cereals supply chain with largest losses and wastes (40% and 50% of total cereal wastes) as show in the following graph (3); in low-income regions the main losses during cereals supply chain are represented by agricultural, production, post-harvested handling and storage. These data confirm the ones presented before about global food waste productions.



Graph 3. Cereals losses and wastes during food supply chain stages (source: FAO, 2011)

In the case of Italy, cereals production counts around 18 MMT, with a decrease in the past seven years of agriculture.



Date	Value	Change, %
2016	18,219,221	3.30 %
2015	17,637,215	-8.94 %
2014	19,368,014	6.33 %
2013	18,214,664	-2.04 %
2012	18,594,126	-4.75 %
2011	19,521,138	5.50 %
2010	18,502,904	4.50 %
2009	17,705,746	-18.08 %
2008	21,613,374	6.21 %
2007	20,350,618	0.71 %
2006	20,206,559	-5.68 %
2005	21,423,171	

Graph 4. Italian cereals production: from 1961 until 2016 (source: knoema.com/atlas/Italy/Cereal-production)

Nevertheless, Italy is still on the major cereal producer in Europe, counting in 2014 a production between 9 MMT and 24 MMT as shown in the below figure 25.

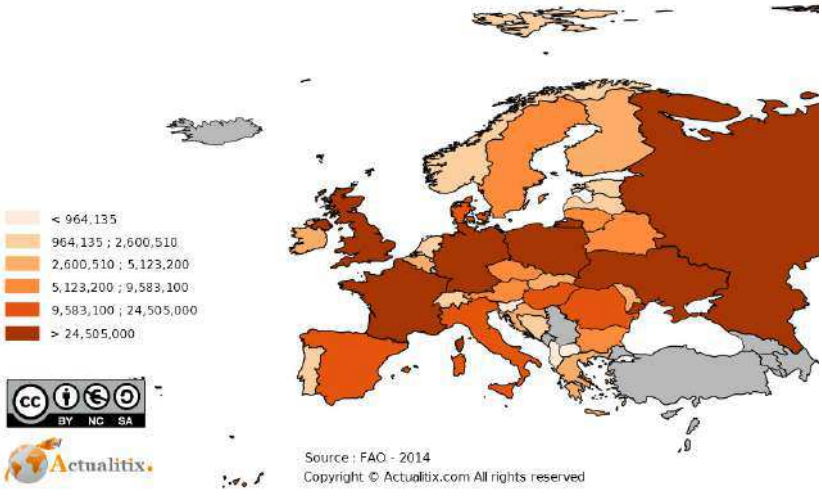


Figure 25. European cereals production in 2014 (source: FAO from www.actualitix.com)

ISTAT, the Italian National Institute of Statistics, reported Emilia Romagna as one of the first Italian regions in terms of total production quantities (fist in sorghum, third in rice, fourth in corn and durum wheat productions in 2016). Ferrara, in particular, is the Emilia Romagna's province with the highest total cereal production (2,707,794 MT of corn production in 2016).

	Total cereals production in 2016 (quintals)							
Region	Rice	Mayze	Sorghum	Common Wheat	Durum Wheat	Rye	Barley	Oats
Piemonte	8.292.300	14.415.437	115.435	5.209.225	166.435	9.482	1.008.322	15.637
Valle d'Aosta		1.400		150		160	60	30
Lombardia	6.597.770	18.034.970	215.187	3.791.730	1.422.591	27.477	1.170.539	10.785
Liguria		6.700		4.398			2.005	20
Trentino-Alto Adige		13.825	200	2.100		2.460	180	450
Veneto	210.850	17.914.190	172.150	5.824.805	1.236.894	3.424	1.033.605	8.452
Friuli-Venezia Giulia	1.050	6.238.384	32.885	596.604	21.091	2.237	297.950	920
Emilia Romagna	449.310	6.698.884	2.314.295	8.838.235	5.860.306	17.991	1.162.637	16.429
Toscana	20.630	1.275.298	107.086	706.518	2.895.829	4.564	567.329	312.556
Umbria		530.694	23.080	1.066.600	1.614.600	5.100	799.600	26.520
Marche		400.150	80.713	708.170	5.171.319		700.220	21.790
Lazio		1.371.000	12.300	579.500	1.474.800	4.090	386.500	43.900
Abruzzo		639.150	39.020	990.915	1.343.860	5.900	719.094	69.760
Molise		110.000	4.000	120.158	1.727.040		39.200	31.200
Campania		1.045.174	1.950	617.494	1.799.763	1.600	469.929	321.960
Puglia		65.200	4.500	496.500	13.289.000		591.000	629.100
Basilicata		39.429	6.516	189.743	3.619.920	10.451	417.885	339.530
Calabria	29.850	188.386	18.320	311.754	720.460	36.325	207.913	342.131
Sicilia	470	14.500		9.900	7.845.710	250	132.630	132.550
Sardegna	271.230	37.435	2.045	2.266	1.082.424		306.286	321.966
Italia	**15.873.460**	**69.040.206**	**3.149.682**	**30.066.757**	**51.292.050**	**133.446**	**10.014.984**	**2.645.804**

Table 5. Italian total cereal production in 2016 by regions (adapted from: www.istat.it)

	Total cereals production in 2016 (quintals)							
Province	Rice	Mayze	Sorghum	Coomon Wheat	Durum Wheat	Rye	Barley	Oats
Piacenza	6.130	1.040.000	26.000	1.165.500	359.600	336	171.500	2.010
Parma		410.400	46.500	695.200	483.900		80.030	2.115
Reggio nell'Emilia	400	560.500	45.600	592.100	47.600		58.600	800
Modena	15.270	746.450	496.400	1.263.750	150.500		109.120	2.296
Bologna	9.120	761.440	825.890	1.670.624	1.286.428	3.726	372.100	1.908
Ferrara	418.390	2.707.794	359.905	1.828.869	1.281.460	1.435	115.136	160
Ravenna		432.000	360.000	637.500	714.000	3.859	106.600	480
Forlì-Cesena		28.800	119.000	522.400	148.820	960	199.190	6.650
Rimini		10.500	35.000	193.800	139.200		78.000	1.890
Emilia Romagna	**449.310**	**6.698.884**	**2.314.295**	**8.596.743**	**4.611.508**	**10.372**	**1.290.276**	**18.309**

Table 6. Emilia Romagna and Ferrara cereal production in 2016 (adapted from: www.istat.it)

As already described above, high numbers of production translate into high volumes of losses and waste materials: these reasons, together with the need for competitive technological innovations in health sectors, brought Emilia Romagna region to invest in research (http://fesr.regione.emilia-romagna.it/opportunita/).

6.4 The milling process and by-products

Cereals processing is an important step of the production chain and milling represents the main procedure used in industry which produce large amounts of by-products. Milling is classified into dry milling, wet milling and brewing. The reason why this is a crucial part of production is because the nature of the obtained by-product is influenced, not only by the particular cereal concerned, but also by the exact conditions of processing.

Dry milling is the process by which cereal grains are ground into flour; before being grounded, a separation step of the grain into its constituents is performed: the bran and the germ layers are by-products and separated from the endosperm.

Pearling, an abrasive technique usually used for rice, oat and barley, is also considered part of a dry milling process and it permits to remove the seed coat (testa and pericarp), aleurone layers, and germ to obtain polished grain (e.g. polished rice). Wet-milling is used to produce starch and gluten, germ (for the oil-crushing industry) and bran. Lastly, malting in the process used to obtain beer and other alcoholic beverages by enzymatic fermentation of grain's starch.

During the dry milling process there are five working steps:

1. Storing: after the grain is collected, grain elevators are used to store the grain before the milling process. A truck full of grain is weighed, unloaded, and then weighed again to determine the amount of grain. An elevator manager will then determine the grade of the grain and consequently, the storage bin to place the load of grain in;
2. Cleaning: involves passing the grain through several machines that will remove any contamination in the batch of grain. Grain is also separated by size, shape and weight;
3. Conditioning: it takes place to produce a uniform moisture content, this will make the grain easier to mill without breaking up of the brans;
4. Gristing: different batches of cereals are blended together (gristed);
5. Milling process and sifting: separation of bran and germ from endosperm, and reduction of endosperm size to enhance uniformity.

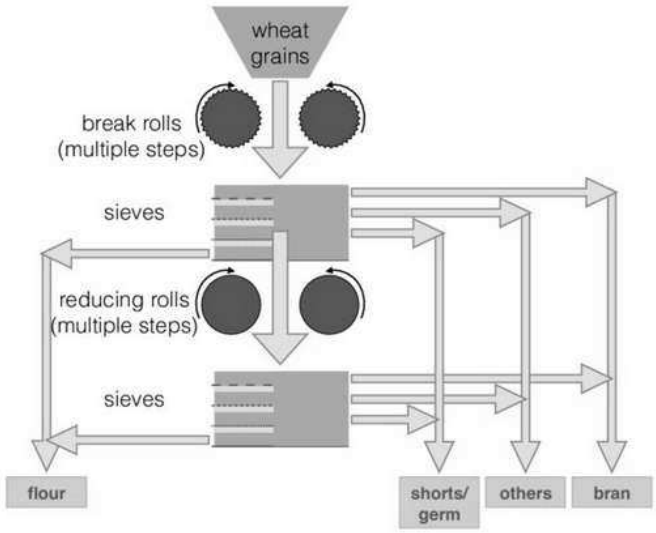


Figure 26. Sschematic representation of milling process (source: Heinze, 2017)

All of the different products resulting from the milling process are stored and can be blended to create different grades of flour: these blends can be treated with various enrichments before they are packaged and ready to ship; by-products are available to reuse and recycle processes (Galanakis, 2015).

Beside the general procedure steps of dry milling, each cereal has its own process adapted to best valorize product and by-products production. For this reason, a more detailed description of corn, rice and wheat milling and by-products, is reported.

6.4.1 Corn milling and by-products

Corn needs an extra step of process, degermination, which can be both wet or dry and is aimed to remove the germ if a low-fat finished product is needed. The outer coat of the maize grain is frequently removed during dry milling too. Dry milling of corn produces various by-products but a global terminology for dry-milled maize products is not yet standardized; pericarp, germ cake, standard meal and broken kernels are usually combined to produce the hominy feed (for animal feed of ethanol production). The maize germ is directed send to extract oil which very between 15% and 25%, depending on several variables such as the extraction procedure. The by-products obtained from wet milling are still rich in nutriments and they can be used by pharmaceutical industries as growth media for the antibiotic production (Papageorgiou and Skendi, 2018).

6.4.2 Rice milling and by-products

Rice treatment differs from the other cereal's procedures because of its physical and botanical peculiarities and because it is mainly consumed as intact grain.

Rice is first deawned in order to eliminate awns from the paddy grains (modern step); since the husk is not considered edible, a dehusking step is performed; the smooth brown rice obtained is still covered by its bran. The following steps are aimed to improve rice aesthetical appeal by pearling, polishing and grading: rice kernels are achieved with these steps their bright color and translucency. Rice flour is less commonly produced than other cereals, but it is becoming interesting to replace wheat flour in gluten-free products.

The main by-products of rice are rice husk (20%), rice bran (8%), germ (2%) and brewer's rice and they represent the 30% of paddy grains: rice bran consists mostly of pericarp, seed coat and aleurone layers (Papageorgiou and Skendi, 2018).

6.4.3 Wheat milling and by-products

Wheat is first cleaned and conditioned, after these steps, a breaking procedure is used to remove endosperm and germ from the pericarp obtaining also sizings, middlings and break flour fractions. The separation of wheat kernels is performed with sifters and purifiers, sievings and grinding follow the separation step obtaining wheat flour which can have various colors and ash content depending on wheat characteristics and milling procedure conditions.

Conventional wheat milling processes remove all bran layers together, whereas the debranning process (modern technology) takes of each individual bran layer in sequence and permits to obtain high value by-products. Wheat by-products are mainly bran, shorts (mixture of bran and germs), germ and middlings which are primary used as animal feed as source of energy and proteins (Papageorgiou and Skendi, 2018).

6.5 By-products and biomolecules of interest

Whole grain cereals contain significant amounts of phytochemicals (polyphenols such as phenolic acids and alkylresorcinols), vitamins (vitamin B and E group and folate), minerals (selenium, iron, zinc and magnesium) and fibers (β-glucans, lignans, soluble pantosans and arabinoxylans) which contribute to the health benefits of whole grains consumption.

In cereal grains most of phytochemicals, dietary fibers and micronutrients are located within brans and germ parts which corresponds to the main by-products of the milling industries and thus represent a valuable source of important biomolecules.

The bran and aleurone layers, which corresponds to the outer hard layers of cereal kernels, are constituted by dietary fibers, lipids, starch, protein, vitamins, minerals and phytochemicals such as phenolic acids. Dietary fibers include cellulose, hemicellulose, pectins, gums and other polysaccharides and oligosaccharides. The aleurone cells are surrounded by thick nonlignified walls (which correspond to 40% of total cell weight), constituted by nonstarch polysaccharides (mostly arabinoxylans and secondary β-glucans). Ferulic acid, the most represented phenolic compound in cereals bran, is esterified with arabinoxylans of the cell wall and in the form of diferulate is cross-linked between cell wall polysaccharides providing the structural properties of aleurone layer. Besides giving rigidity, phenolic acids are responsible for the protection against chronic disease radical scavenging activity associated with whole cereal consumption (Zaupa et al., 2014).

The germ of cereals, the reproductive part of the kernel, is usually produced as a by-product of the milling process and its primary use is finalized to the production of vegetable oils (from wheat and corn in particular), or it is directly used as ingredient. Since the germ is rich in polyunsaturated fats, it has the tendency to oxidize and to become rancid on storage and for this reason it is usually removed from the cereal flour production. Germs are source of important phytochemicals such as phenolic acids (ferulic acids, p-coumaric acids, etc.) flavonoids (kaempferol, quercetin, etc.), proanthocyanidins (cyaniding 3-O-glucoside) and lipids (phytosterols). This chemical composition makes cereal germ interesting not only for the production of vegetable oils but also for the recovery of biologically active phytochemicals.

6.5.1 Phenolic molecules

Phenolic acids belong to the polyphenols class and they are widely distributed in the plant kingdom: flavonoids, tannins, proanthocyanidins, stilbene and coumarins are other examples of compounds belonging to this category.

Even if there is no explicit ascription in literature regarding who coined the term polyphenols, it is known that Emil Fischer (1919), Karl Freudenberg (1920) and Theodore White (1957) carried out earliest historically significant laboratory studies on these

compounds. Between 1950 and 1994, in the United Kingdom, were carried out the first preliminary studies on the defensive roles of polyphenols in plants, with their nutritional benefits, chemical structures and properties (Adebooye et al., 2018).

The general definition of a phenolic compound is any compound with a benzene ring with one or more hydroxyl groups; phenolic acids are phenols with a single hydroxylated aromatic ring having a directly or indirectly attached carboxyl group.

They are synthesized in the plant as secondary metabolites as response to external stress like environmental conditions, insects, pathogens, physical lesions or UV radiations. In nature they have several functions such as giving appearance (colour), taste, odour, protection, structure and oxidative stability to the plant.

The chemical synthesis of phenolic acids starts with amino acids L-phenylananine and L-tyrosine, precursors which are converted initially into cinnamic acid and p-coumaric acid and after hydrolyzation and methylation reactions p-coumaric is converted into ferulic acid (Kumar et al., 2014).

As shown is figure 27, there are two classes of phenolic acids: hydrozybenzoic acids and hydroxycinnamic acids: the former class includes gallic, vanillic, syringic, p-hydroxybenzoic and protocatechuic acids, the latter includes *o*, *m*, *p*-coumaric, caffeic, chlorogenic, ferulic, isoferulic and sinapic acids (Xu, 2012).

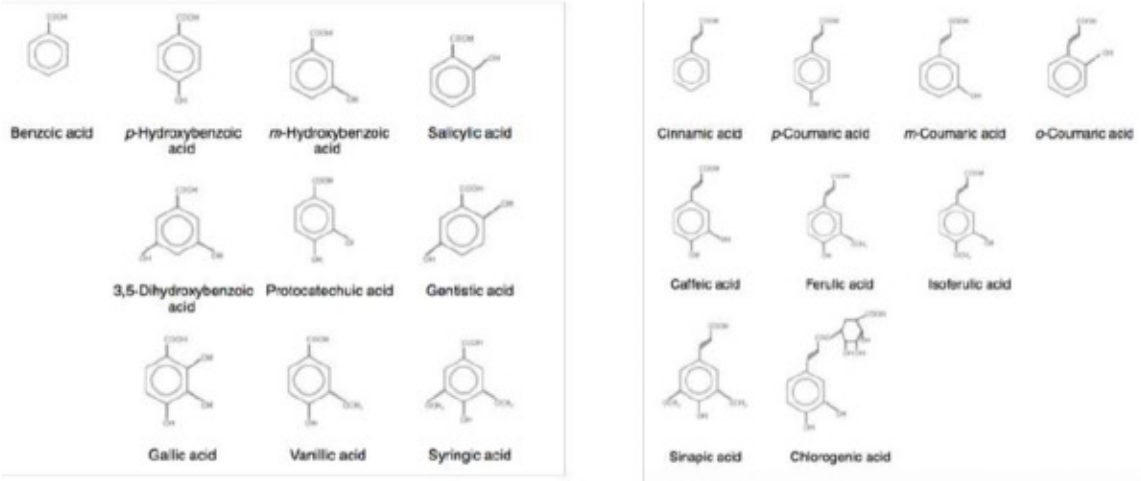


Fig 27. Chemical structure of hydroxybenzoic acids and hydroxycinnamic acids (Xu, 2012)

The potential health benefits of phenolic acids have been widely studied and literature data about this topic is very rich. The broad spectrum of applications in various fields such as biomedical, cosmetic, nutraceutical, epidemiological, have fascinated scientists since

decades. The hydroxyl group of the aromatic ring is responsible for phenolic acids' antioxidant activity (Xu, 2012); phenolic acids, (chlorogenic acid in particular) have been reported to give gastrointestinal benefits to the microflora (Azuma et al., 2000), caffeic acid have been proved to enhance immune system (Koshihara et al., 1984); ferulic acid have shown strong antioxidant and anti-inflammatory activity (Graf et al., 1992).

Phenolic acids are found as free, conjugated and bound (insoluble) in plants: they are most commonly found in their bound form in food and in cereal grains. Bound phenolics comprise, in average, 24% of the total phenolics in food, with picks of 88% in brown rice (Zhou, Robards, Helliwell & Blanchard, 2004). Acosta-Estrada (2014), reported an interesting table resuming several studies which reported percentages of bound phenolics in plants and cereals, the table 7, shows part of these results focusing on cereal grains phenolics.

Cereals	Insoluble bound phenolics in total phenolics (%)
Barley (Horderum vulgare L.)	70.08
Barley malt	51.59
Mayze (Zea mays)	85.00
Oat (Avena sativa)	75.00-88.04
Rice (Oryza sativa)	62.00
Brown rice	88.00
Red sorghum (Sorghum L.)	85.48
Wheat (Triticum spp.)	75.00
White wheat bran	83.18

Table 7. Insoluble bound phenolic in cereals (adapted from Acosta-Estrada et al., 2014)

Phenolic acids are found covalently bound to cell wall structural components such as hemicelluloses (arabinoxylans), celluloses, lignins, pectins and proteins. These strong bonds provide physical and chemical barriers, protection for the plant against pathogens as well as antibacterial, antifungal and antioxidant functions which also serve as protection against external attacks. Ferulic acid is attached to structural polysaccharides with ester linkage of its carboxylic group and ether linkages with lignin with its aromatic nucleus; moreover, ferulic acid oligomers form polysaccharide- polysaccharide cross-linkages limiting the biodegradability of the cell walls (Barberousse et al., 2008).
For these reasons phenolic acids in cereal grains are located mainly in the pericarp, including brans, husks and aleurone layers and their bonds are represented schematically in the following figures.

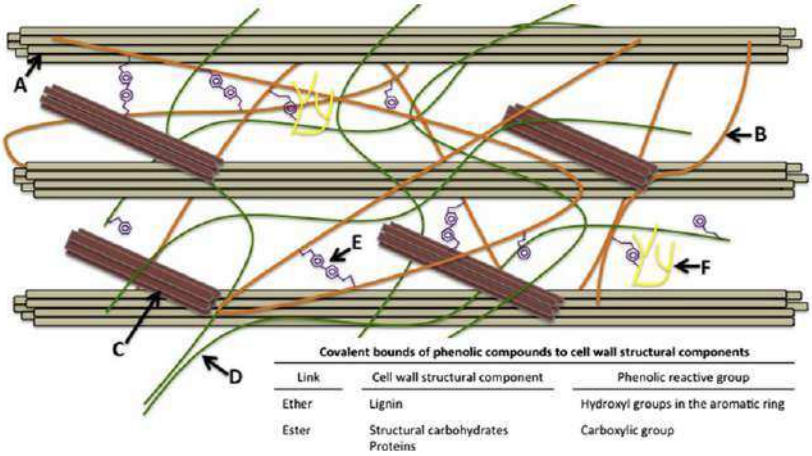


Figure 28. Schematic representation of bound phenolic bonds:(A) Cellulose (B) Hemicellulose (C) Structural protein (D) Pectin (E) Phenolic acids (F) Lignin (Acosta-Estrada et al., 2014)

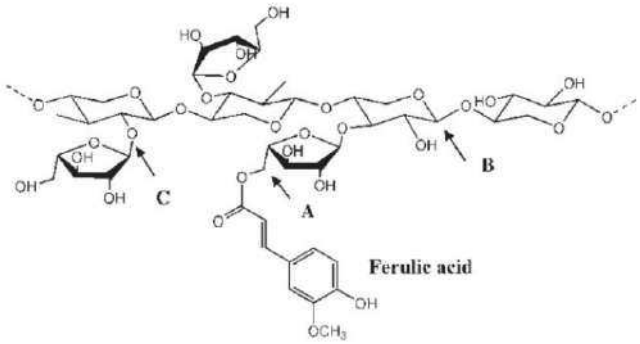


Figure 29. Structure of ferulic acid esterified to arabinoxylan: (A) ferulic linked to O-5 of arabinose chain of arabinoxylan. (B) b-1,4-linked xylan backbone. (C) a-1,2-linked L-arabinose (Buanafina, 2009)

Agro-industrial by-products are good sources of lignocellulosic materials which are rich in bound phenolics.

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is a derivative of cinnamic acid and it exists in both *cis* and *trans* isomeric forms. The double bond in the side chain is subjected to *cis-trans* isomerization stabilized phenoxy radical accounts for its antioxidant activity. With dihydroferulic acid, ferulic acid is the component of lignocelluloses responsible for the cell wall rigidity by making crosslink between lignin and polysaccharides. The covalent bonds already described make the release of ferulic acid available only after strong hydrolysis conditions.

Ferulic acid have demonstrated many possible applications in literature. Some of the main activities reported in literature have been summarized and listed by Mathew and Abraham

(2004) and by N. Kumar and V. Pruthi (2014): anti-diabetic, free radical scavenger, lipid lowering, antioxidant, UV absorber, anti-atherogenic, neuroprotective, anti-apoptotic, food preservative, anti-ageing, precursor of vanillin, anti-carcinogenic. Ferulic acid is, in fact, well known and it has been studied for decades: it is reported as antihepatotoxic and offers various benefits for the cardiovascular system (Kiso *et al*., 1983, Rukmini and Reghuram, 1991). It showed anti-inflammatory activity by suppressing the production of interleukin-8 (IL-8). Studies on its chemopreventive potential colon carcinogenesis showed that ferulic acid increases the activities of detoxifying enzymes such as glutathione S transferase and quinone reductase (Kawabata *et al*., 2000). Some of ferulic acid biological activities have a possible direct application in the cosmetic, phytotherapic and nutraceutical industries. In particular, the possible cosmetic application of this molecule can be considered in relation to the free radical scavenger, antioxidant, UV absorber activites. Ferulic acid structure is similar to the tyrosine one and this phenolic acid can inhibit the melanine formation by competition with tyrosine (Saija et al., 2000). Ferulic acid is a good candidate as topical protective agent against UV-induced skin damage because it was found to permeate through the stratum corneum thanks to its high lipophilicity (Shahrzad and Bitsch, 1996; Saija *et al*., 2000). Furthermore, it exhibits scavenging activity against nitric oxide secreted by human skin keratinocytes in response to UV A and B radiations. Moreover, it has been found that the incorporation of ferulic acid into a topical solution of 15% vitamin C and 1% vitamin E, not only improved chemical stability of the product, but also doubled the skin photoprotection to solar-simulated irradiation with a synergistic effect (Lin et al., 2005).

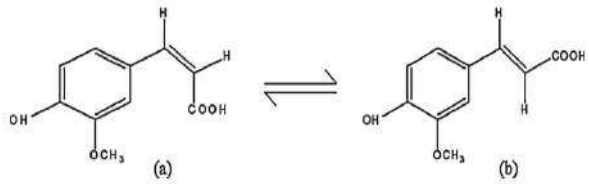


Figure 30. cis (a) and trans (b) ferulic acid structure (Kumar & Pruthi, 2014).

6.5.2 Unsaponifiable fraction

The unsaponifiable fraction is the whole quantity of substances present in oils or fats that after treated with saponification with potassium hydroxide are not volatile under the conditions of the tests. This fraction includes sterols, methyl sterols, high aliphatic alcohols,

tocopherols and tocotrienols, hydrocarbons and di- and tri-terpenes alcohols; lipophilic vitamins and some pigments are also part of this chemical class.

Sterols and methyl sterols

Sterols, also known as steroid alcohols, are a subgroup of the steroids and an important class of organic molecules. They occur naturally in plants, animals, and fungi, with the most familiar type of animal sterol being cholesterol.

Both sterols and methyl sterols are synthesized via the mevalonate pathway of isoprenoid metabolism and derive from cyclopenthanoperihydrophenanthrene: sterols have a hydroxyl in position 3 and a double bond between 5 and 6; methyl sterols are similar to sterols but with a methyl in position 4 (Capella et al., 1997).

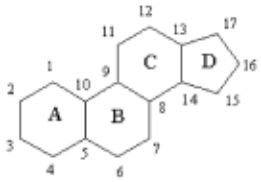


Figure 31. Chemical structure of cyclopenthanoperihydrophenanthrene

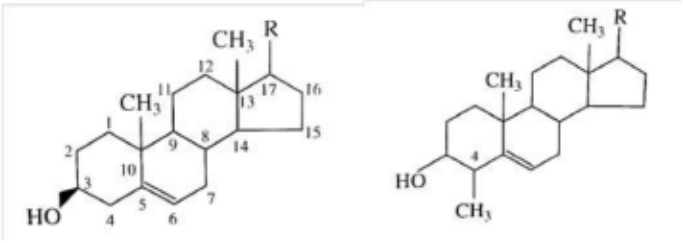


Figure 32. Sterols (left) and methyl sterols (right) chemical structures (Capella et al., 1997).

Sterols differ from each other by the structure of the R side chain; plants are characterized by some typical and most abundant sterols, such as β-sitosterol, campesterol and stigmasterol; some other phytosterols are Δ^7-stigmastenol, Δ^5-avenasterol and Δ^7-avenasterol.

Sterols have been quantified in plant oils and their range is valued between 0.1% and 0.5% of the unsaponifiable fraction.

Plant sterols are primary components of cellular membranes where they have a role in the regulation of fluidity and permeability; other sterols are precursors to steroid derivatives

and were only recently recognized as a new class of plant growth regulators called brassinosteroids (Schaller, 2003).

They are found in free form and conjugated form (as steryl esters, steryl glycosides and acyl steryl glycosides). Conjugated sterols are ubiquitously found in plants and their profile may change in response to developmental and environmental changes. Steryl esters play a central role in membrane sterol homeostasis and represent a storage of sterols in plant tissues. Moreover, there are evidences supporting a role of conjugated sterols in plant stress responses (Ferrer et al., 2017).

Methyl sterols are more recently studied and between the various identified compounds there are gramisterol, citrostradienol and isocitrostradienol.

Many studies on the biological activity of phytosterols agree on their ability to lower cholesterol levels with decreased risk of coronary heart diseases, moreover they exhibit anti-inflammatory activities, induction of apoptosis in cancer cells, disease prevention and treatment (Jiang and Wan, 2005).

Γ Orizanols

Orizanol is one of the main components of the unsaponifiable fraction of rice bran oil: it was first presumed to be a single component, but later is has been determined to be a mixture of ferulate esters of triterpene alcohols and sterols: cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, sitosteryl ferulate and campesteryl ferulate are the main components which characterize this fraction. It was first isolated from rice bran oil and this is where its name comes from: *Oryza sativa* L. (Sohail et al., 2016).

The unsaponifiable fraction (up to 5% mass of crude oil) contains approximately 0.9-2.9% of gamma oryzanols (Oliveira et al., 2012).

The interesting aspects of gamma-oryzanol in the health field, lies in the fact that this fraction have been reported to be effective against various disorders and diseases: it demonstrated antiatherogenic potential and hypocholesterolaemic-hypolipdipaemic effects (Kennedy and Burlingame, 2003; Tsuji et al., 2003); free-radical scavenging activity (Zhimin et al., 2001); anti-diabetic potential (Ohara et al., 2009; Son et al., 2011); anti-cancer (Nam et al., 2005; Revilla et al., 2013); antiinflammatory activity (Islam et al., 2008).

The antioxidant activity of gamma oryzanol have a potential in industry as stabilizer on fats and oils: brown rice, rice bran and oryzanol have already been used to stabilize food products with interesting application in develop functional foods (Bergman & Xu, 2003); rice bran oil extracts are also used in cosmetic formulation in the treatment of skin-related disorders and in sunscreen formulations as it absorbs UV radiations (Indira et al., 2004; Juliano et al., 2005).

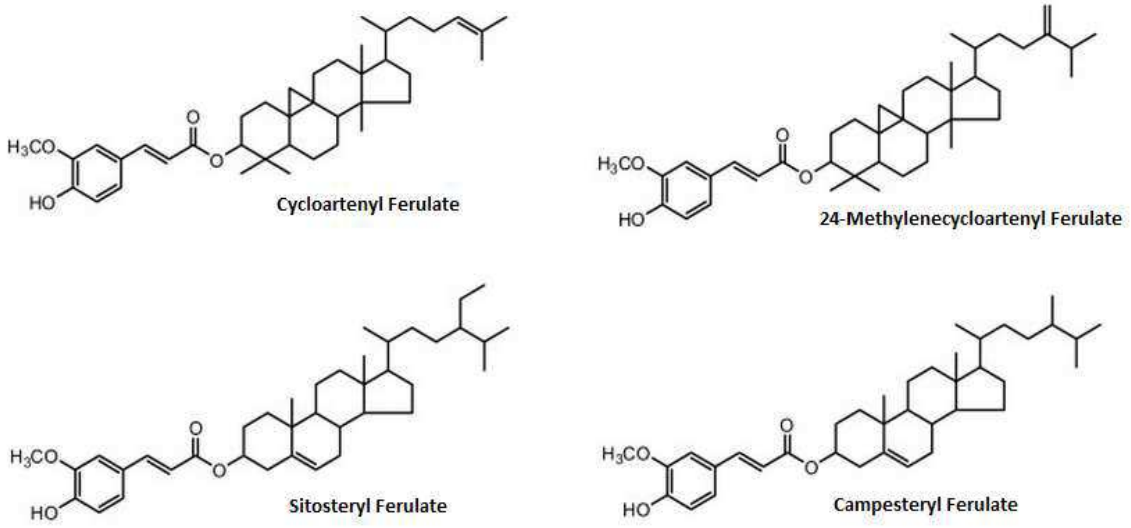


Figure 33. Chemical structure of the main components of γ-oryzanol (www.examine.com)

Tocopherols and tocotrienols

Tocopherol and tocotrienols comprise a group of closely related lipids (collectively known as tocols) that consist of a polar chromanol ring and a hydrophobic 16-carbon side chain attached to the ring via the C-2 atom. Tocopherols have saturated phytyl side chains while tocotrienols have isoprenyl side chains with three double bonds (Eitenmiller et al., 2004).

The main tocotrienols are: 5,7,8-trimethyltocotrienol (α-T3), 5,8- trimethyltocotrienol (β-T3), 7,8- trimethyltocotrienol (γ-T3), 8-metyltocotrienol (δ-T3).

The main tocopherols are: α-tocopherol (α-T) 5,7,8-trimetyltocol, β-tocopherol (β-T) 5,8-dimethyltocol, γ-tocopherol (γ-T) 7,8-dimethyltocol, δ-tocopherol (δ-T) 8-methyltocol, (5,7-T) 5,7-dimethyltocol, (7-T) 7-methyltocol. Only alfa, beta, gamma and delta-tocopherols exist in nature with alfa, beta and gamma-tocopherols as the more abundant.

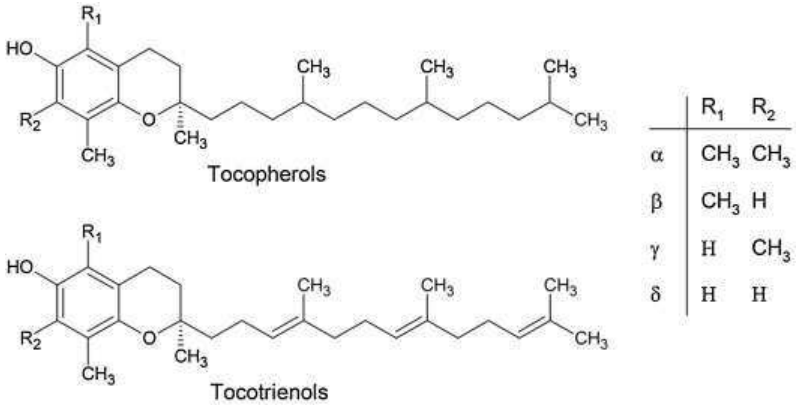


Figure 34. Tocols chemical structures (lipidlibrary.aocs.org)

The antioxidant activity of tocopherols and tocotrienols varies according to the molecule with the following order: α-T < α-T3 < γ-T < γ-T3 < β-T < β-T3 < δ-T < δ-T3 (Capella et al., 1997).

Tocols are antioxidants that scavenge lipid peroxyl radicals by donating hydrogen atoms, reduce oxidative stress and oppose development of degenerative diseases such as cardiovascular diseases and cancer; they also have been demonstrated to regulate cellular signaling, cell proliferation and gene expression (Sen et al., 2007).

Cereal grains are unique sources of tocols, in particular of Vitamin E which have an important role as natural preservative thanks to its radical scavenging activity: it is an alternative to, for example, the other synthetically derived butylated hydroxytoluene, butylated hydroxyanisole, tert-butylhydroquinone preservatives. The possibility of using vitamin E as a natural preservative emphasizes the importance of its extraction from natural sources and cereal grains which are rich in lipids particularly in the germ and represent a good starting matrix. Whole grain corn is the richest source of vitamin E among, rye, barley, sorghum, oat and millet (Fardet, Rock & Remesy, 2008). The extraction of vitamin E from cereal grains by-products (e.g. maize germ) have been performed in literature both with conventional and green extractions techniques: the levels of tocopherols extracted by SFE with supercritical CO_2 have been found to be similar to the results obtained with conventional extraction methods but with low content of phospholipids extracted by SC-CO_2 which is advantageous from the processing point of view (Rebollenda et al., 2012).

Wheat germ oil is a good source of tocopherols and tocotrienols which have been also successfully extracted with SC-CO_2 by Gelmez (2009) with 10 minutes of extraction (336

bar and 58°C) obtaining 0.33 mg tocopherol/g germ and suggesting a potential green extraction process which can be applied in cosmetic and food industries.

Experimental

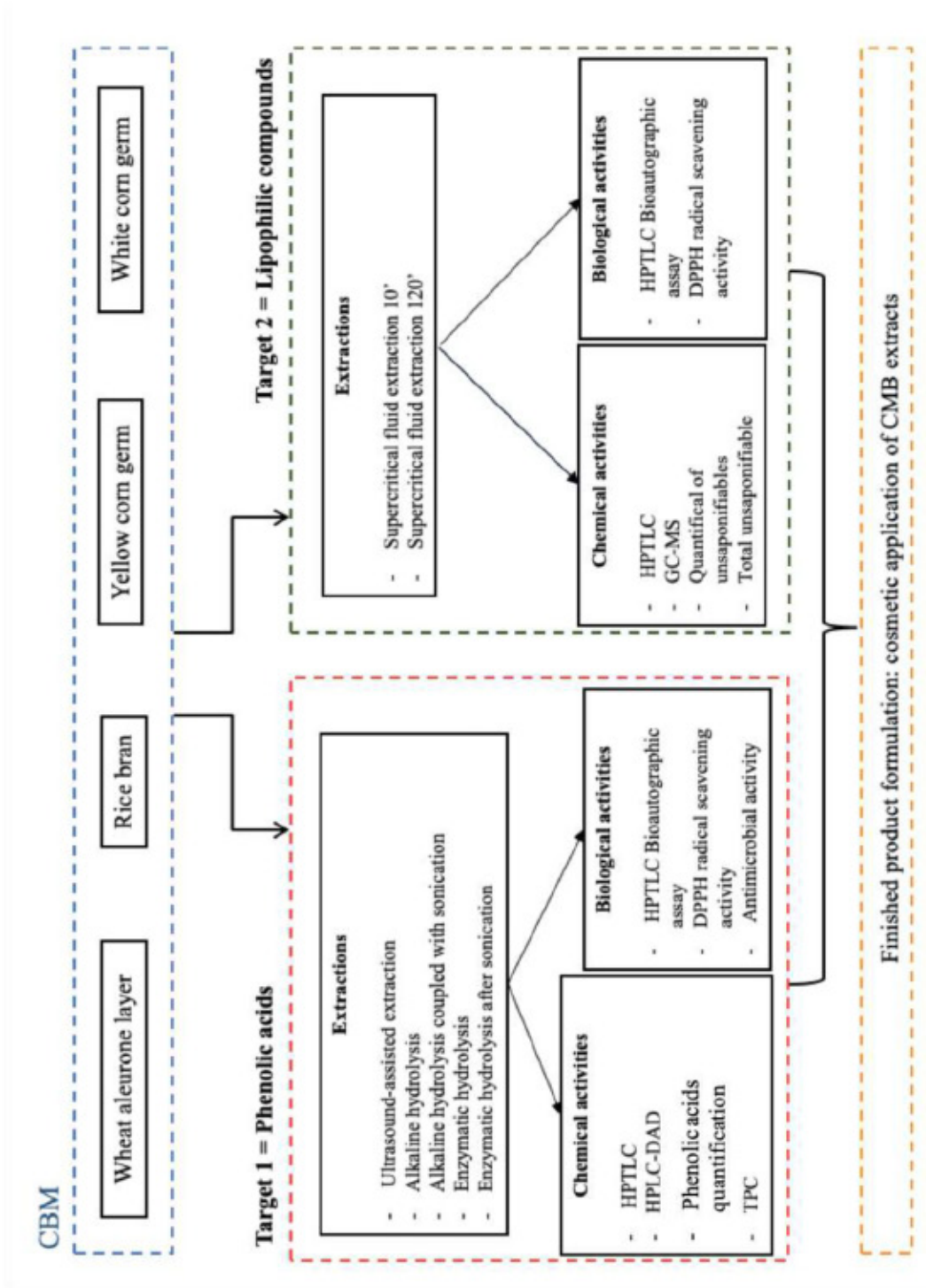


Figure 35. Schematic representation of the experimental work

7 Materials and Methods

7.1 Plant materials

The plant material studied for the present project consists of various cereal by-products obtained from the industrial milling processing, used to produce goods from cereals production (e.g. flours, pasta).

Triticum durum (Desf.) aleurone layer has been provided by Molino Grassi (Parma, Italy) and obtained after decortication of durum wheat caryopsis; *Oryza sativa* L. bran has been provided by Grandi Riso S.p.a (Ferrara, Italy) and obtained after the rice husking phase; *Zea mays* L. (both yellow and white varieties) germs have been provided by Corn Valley (Mantova, Italy) and obtained after the caryopsis decortication process.

All cereal by-products used for extraction have been procured thanks to the work of intermediation of Open Fields S.r.L. (Parma, Italy). After being received, all plant materials have been stocked at -20°C until used.

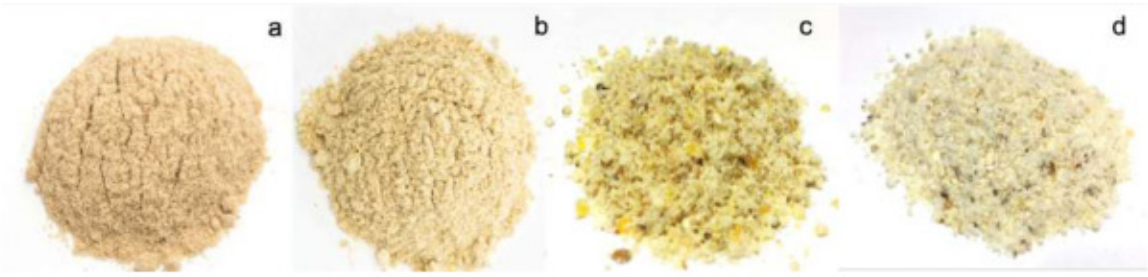


Figure 36. Milled plant material (a: wheat aleurone layer; b: rice bran; c: yellow corn germ; d: white corn germ) (photo credit a and b: Alessandro Grandini; photo credit c and d: Ilaria Burlini)

7.2 Chemicals

All the solvents and reagents employed for analyses were chromatographic grade. Ferulic acid standard, Folin-Ciocalteu reagent, Trolox, DPPH (1,1-diphenyl-2-picrylhydrazil), Methanol (≥99.9% for HPLC), Ethyl acetate, Ethanol absolute (≥99.8%) Formic acid (98% - 100% for HPLC), Acetic acid, Toluene, natural products-polyethylene glycol reagents (NP/PEG) and gallic acid (TraceCERT), were purchased from Sigma-Aldrich Italy (Milano, Italy). Feruloyl esterase (FAE) from *Clostridium thermocellum* and endo-1,4-β-Xylanase M1 from *Trichoderma viride* were obtained from Megazyme (Bray, Ireland). Extra virgin olive oil (EVOO) was purchased from COOP Italia (Bologna, Italy). Muller

Hinton Broth (MHB) and Muller Hinton Agar (MHA) were obtained from Oxoid S.A. (Madrid, Spain).

7.3 Microorganisms

Two clinical isolates of *Staphylococcus aureus* (MRSA 185087 and MSSA 185960) and one clinical isolate of *Staphylococcus epidermidis* (185240) have been obtained from the Hospital Universitario Reina Sofía (Córdoba, Spain). One reference strain of *Staphylococcus aureus* (ATCC 29213) Culti-Loops was obtained from Oxoid S.A. (Madrid, Spain). All antimicrobial agents were purchased from Oxoid S.A. (Madrid, Spain).

7.4 Extractions targets

All extraction techniques performed for the present PhD project have been chosen in order to maximize the extraction yields of the two extraction targets: phenolic acids and unsaponifiable compounds, the main biomolecules characterizing cereal by-products.
At the same time the extraction methods used were performed with the aim of minimizing as much as possible the environmental impact compared to other conventional methodologies.

- Ultrasound-assisted extraction (UAE), alkaline hydrolysis, alkaline hydrolysis coupled with sonication, enzymatic hydrolysis and enzymatic hydrolysis on sonicated materials, were performed to extract phenolic compounds which typically characterize cereal grains and germs;
- Supercritical fluid extractions (SC-CO_2) have been performed to extract the lipophilic compounds (unsaponifiables) which typically characterize cereal brans and germs.

Each extraction has been performed in triplicate. Prior to any extraction, all plant materials have been prepared to enhance accessibility of the substrate: this step requires grinding into fine powder the plant materials in order to disrupt cell walls and increase the solvent contact surface with the matrixes which have been milled through a 2 mm sieving ring of a Variable Speed Rotor Mill (Fritsch, Germany) and immediately stored at -20°C until further use.

7.5 Target 1: Phenolic acids

7.5.1 Extraction techniques

- Ultrasound-assisted extraction

Ultrasound-assisted extraction was performed in an ultrasonic cleaning bath (Ultrasonik 104X, Ney Dental International, MEDWOW, Cyprus) under a working frequency of 48 kHz.

In order to extract free phenolics from cereal by-products matrixes (CBM) 5 g of each sample were placed into a volumetric flask (100 mL), filled with a 65% ethanolic solution as extraction solvent and sonicated at room temperature (solvent/solid ratio of 20 mL/g of cereal matrix).

Literature reports two optimization methods for the extraction of phenolic molecules through UAE from wheat germ (Wang et al. 2008) and rice bran (Tabaraki et al. 2011), which were followed in the present project for the extraction of FP from cereal matrices. Since no literature data have been found about the most suitable ethanol content for corn UAE extraction, three extractions have been performed with three different percentages of hydroalcoholic solvent (100%, 50%, 0% of ethanolic solution). All the FP extractions are summarized in table 8. The obtained extracts have been filtered and lyophilized until further use.

n°	CBM	Parameters			
		Time (min)	Solvent	Solvent/solid ratio	note
1	Wheat aleurone	25	65% ethanolic solution	20/ 1	Residues of these extractions were used for BP extractions
2	Rice bran	45	65% ethanolic solution		
3	Yellow corn germ	25	100%, 50%, 0% ethanolic		
4	White corn germ				

Table 8. UAE extractions of FP performed on cereal by-products matrices (CBM)

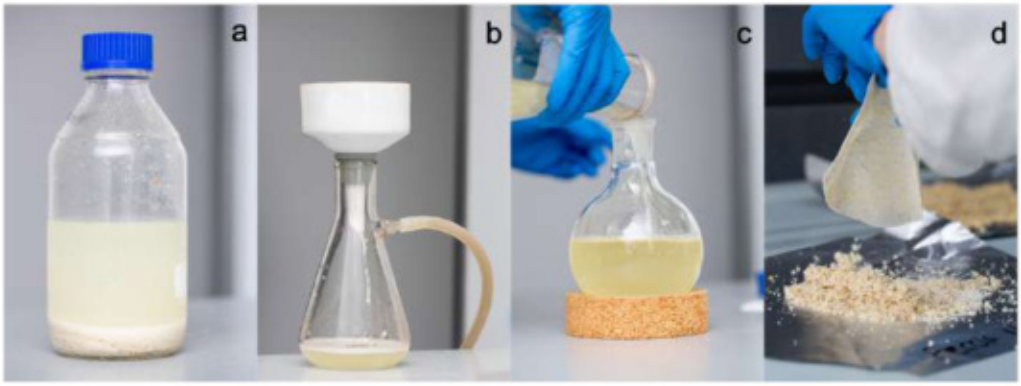


Figure 37. Ultrasound-assisted extraction steps (a:plat material ready to be extracted with hydroalcoholic solvent; b: filtration of the extract; c: filtrated extract; d: separation of the extraction residue) (photo credit, Alessandro Grandini)

- Alkaline hydrolysis

The bound phenolic (BP) compounds have been extracted as reported by Verma et al. (2009) with alkaline hydrolysis from all residues of ultrasound-assisted extractions (UAE-R), which have previously had the free phenolics (FP) removed. Alkaline hydrolysis was preferred to the acid one because it showed to be more efficient in literature (Kim et al. 2006) to break the phenolic bonds. Briefly, 2.5 g of each matrix has been hydrolyzed with 50 mL of 2M sodium hydroxide at room temperature for 1 h. After alkaline hydrolysis, the pH of the mixture was adjusted to 3 with 6N HCl. The BP samples were then extracted three times with ethyl acetate. The ethyl acetate extracts were evaporated to dryness with a rotary evaporator (Ica® RV10 digital) at room temperature and the residue was dissolved again in ethyl acetate, centrifuged 10 minutes at 4000 rpm to remove possible extraction residues. Samples were evaporated to dryness under a stream of nitrogen at room temperature and finally weighed to calculate the extraction yields.

- Alkaline hydrolysis coupled with sonication

The BP compounds have been extracted following a similar approach described for alkaline hydrolysis extraction with the addition of ultrasounds during the hydrolysis as described by Gonzales et al. (2014) for other plant matrices. 2.5g of each UAE-R has been hydrolyzed with 50 mL of 2 M sodium hydroxide in the ultrasonic bath (Ultrasonik 104X, Ney Dental International, MEDWOW, Cyprus) under a working frequency of 48 kHz. In the case of this extraction method the extraction time was halved at 30 min, instead of 60 min, in order to reduce the extraction time. After alkaline hydrolysis, the pH of the mixture was adjusted

to 3 with 6N HCl. The BP samples were then extracted three times with ethyl acetate. The ethyl acetate extracts were evaporated to dryness with a rotary evaporator (Ica® RV10 digital) at room temperature and the residue was dissolved again in ethyl acetate, centrifuged 10 minutes at 4000 rpm to remove possible extraction residues. Samples were evaporated to dryness under a stream of nitrogen at room temperature and finally weighed to calculate the extraction yields.

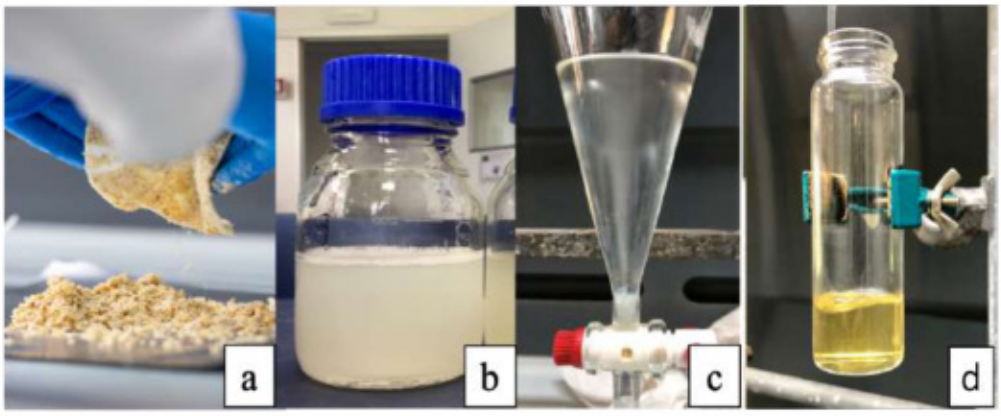


Figure 38. Main steps required for alkaline hydrolysis.
(a) UAE residue;(b) alkaline hydrolysis; (c) phenolics separation with ethyl acetate after being shaken; d*: hydrolyzed extract under nitrogen stream*
(photo a *and* b*: Alessandro Grandini; photo* c *and* d*: Ilaria Burlini)*

- Enzyme-assisted extraction

As reported in literature, enzyme-assisted extractions are gaining attention because of the need for eco-friendly technologies which have demonstrated to enable fast, low solvent, low energy and high recovery extractions (Puri et al., 2012).

For the present project, two different Feruloyl esterase (FAE) and one Xylanase enzymes were used for the hydrolysis of phenolic acids in cereal by-products in order to free ferulic acid. One FAE obtained from *Clostridium thermocellum* (FAEZCT) and one from rumen microorganism (FAERU) have been chosen for their ability to catalyze the hydrolysis of the feruloyl (4-hydroxy-3-methoxycinnamoyl) group from an esterified sugar. One Xylanase from *Trichoderma viride* has been used in association with both FAE enzymes because the endo- hydrolysis of (1,4)-β-D-xylosidic linkages in xylans performed by this enzyme is responsible for a significant enhancement of ferulic acid release when associated with FAE in literature reports (Faulds & Williamson, 1995; Wong, 2006).

Even if there are several literature methods about the enzymatic extraction of bound phenolics from cereal by-products, it was difficult to follow them scrupulously due to the peculiarities of the available enzymes (origin, synthesis, concentration, etc.). The method of Yu et al. (2002) was taken as a starting reference and it has been then adapted according to the needs of the present work.

Thus, to develop the most suitable method, various extractions have been performed starting on wheat bran aleurone and evaluating the extraction efficacy through HPTLC screenings. To do so, 0.1 g of wheat bran aleurone, have been hydrolyzed for 24 hours with two enzymatic blends, reported in table 9, in order to find the most suitable combination and the most performing FAE enzyme and against a blank.

n°	Sample	FAEZCT	FAERU	Xilanase	Time
1	Wheat bran aleurone	1% (0.49 U)	-	10 U	24 H
2		-	1% (12 U)	10 U	
3		Blank			
4					

Table 9. Enzymatic extractions performed in order to establish the most suitable enzymatic blend

Once FAEZCT has been selected as the most performing enzyme and the best enzyme combination has been chosen (1% FAEZCT and 10 U Xylanase), four more extraction times were tested in such a way as to verify if 24 hours were adequate time to extract the molecules and also to verify the possibility of speeding up the process without losing in extractive quality. Thus, 20 min, 60 min, 180 min and 48 hours have been tested as shown in table 10 and against a blank.

n°	Sample	FAEZCT	Xilanase	Time
1	Wheat bran aleurone	1% (0.49 U)	10 U	20 min
2				60 min
3				180 min
4				48 h
5		Blank		20 min
6				60 min
7				180 min

8					48 h

Table 10. Enzymatic extractions performed with 20 min, 60 min, 180 min and 48h

As shown in Results and Discussion chapter, we verified through HPTLC that reducing the extraction time up to 180 min allows us to obtain a comparable result to that obtained with 24 h, and this allowed us to proceed by making a more specific comparison between the extracts obtained after 180 min and 24 h.

Furthermore, for the same purposes, the possibility of performing enzymatic extractions starting from the UAE extraction residues (UAE-R) was evaluated to verify the contribution of the ultrasounds on the extractive effectiveness of the enzymatic mixture.

Finally, once the parameters of the extractions were decided, we also carried out the various extractions with the optimized parameters (24h, 180 min and 180 on UAE-R) starting from the remaining CBM (rice and maize matrices).

n°	**Sample**	**FAEZCT**	**Xilanase**	**Extraction time**	
1	CBM	1% (0.49 U)	10 U	180 min	24 h
2		Blank			
3	UAE-R	1% (0.49 U)	10 U	180 min	-
4		Blank			

Table 11. Enzymatic extractions performed for analyses

All hydrolyses have been performed with phosphate buffer, at pH 4.5 and temperature of 50°C and against a blank. To stop the reaction 100 µL of acetic acid were added to the solution. Phenolics were then extracted three times with ethyl acetate and separated through a separating funnel. The ethyl acetate extracts were evaporated to dryness with a rotary evaporator (Ica® RV10 digital) at room temperature and the residue was dissolved again in ethyl acetate, centrifuged 10 minutes at 4000 rpm to remove possible extraction residues. Samples were evaporated to dryness under a stream of nitrogen at room temperature and finally weighed to calculate the extraction yields.

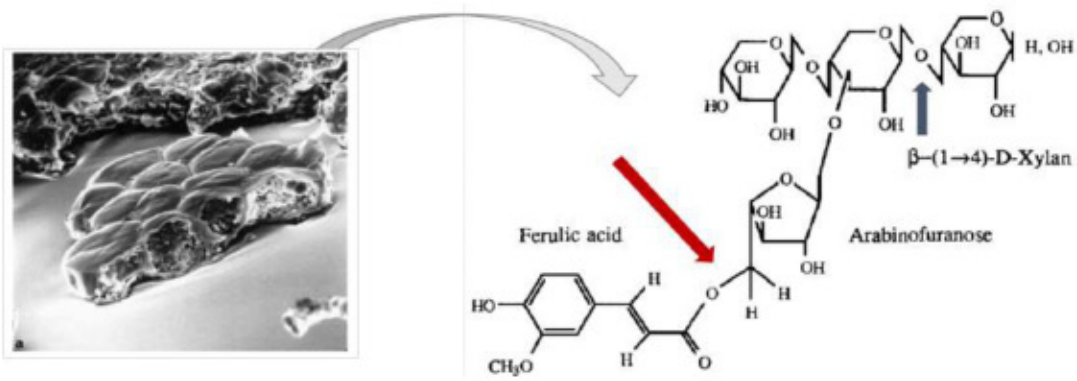


Figure 39. Scanning electron micrograph of aleurone layer and FA linkages (figure adapted from Rhodes, Sadek and Stone, 2002)

7.5.2 Separation of polyphenols from hydrolysed extracts

Since the extraction of bound phenolics, performed with the already described alkaline-hydrolysis methods, lead to obtain extracts with a considerable lipophilic fraction, a separation of polyphenols has been performed following the method described by Andrewes et al. (2003). Each hydrolysed extract has been dissolved in 2 mL of hexane and then extracted with ethanol/water (60:40) for 2 min. The hydroalcoholic phase was then washed again with 2 mL of hexane. The phenolic phase has been rotary evaporated at 35°C until the complete evaporation of ethanol and subsequently lyophilized to remove the water content. The dry residues have been used for analyses after confirming through HPTLC the occurred separation.

7.5.3 High performance thin layer chromatography (HPTLC)

High performance thin layer chromatography has been performed on all phenolic acid extracts in order to do a screening identification of the most performing extraction techniques and to check the extraction of phenolic molecules. Moreover, this rapid qualitative method has been used, as already described, for the choice of enzymatic extraction's parameters.

HPTLC silica gel 60 F_{254}-precoated high performance thin layer chromatographic plates (CAMAG, Muttenz, Swiss) with the Linomat V automatic sampler (CAMAG) and WinCATS Planar Chromatography Manager software (CAMAG) have been used for the analyses. Twin Trough Chambers (20x10 cm) have been used and pre-saturated for 20 min with the eluent mixtures. In order to better separate the phenolic molecules, different eluent mixtures have been used to perform the HPTLC for FP and BP.

Free phenolics extracts have been concentrated at 20 mg/mL in a 60% ethanolic solution and 8 µL of each extract have been put on the chromatographic plate using the automatic sampler. For the chromatographic separation of free phenolic acids, the following parameters have been chosen, following the guidelines of Wagner and Bladt (2009). A two steps elution has been performed: the first mobile phase was: ethyl acetate/formic acid/acetic acid/water (100:11:11:20); the second mobile phase was: toluene/ethyl acetate/acetic acid (100:90:10). After the elution, plates have been derivatized with NP/PEG solution and then photographed with TLC Visualizer (CAMAG) at 254 nm, 366 nm.

For the chromatographic separation of bound phenolic acids, the method described by Barberousse et al. (2008) has been chosen with some modifications to better separate the phenolic molecules. Bound phenolics have been concentrated at 7 mg/mL with ethyl acetate and 8 µL of each extract have been automatically put on the plate using the automatic sampler; the mobile phase used was: chloroform/ ethyl acetate/methanol (7:2.5:0.5) + 1% acetic acid. Plates have been captured at 254 nm and 366 nm before and after derivatization with NP/PEG in order to highlight the presence of phenolic compounds.

7.5.4 RP-HPLC-DAD analysis and quantification of phenolic acids

The analyses of the phenolic acid extracts and the quantification of phenolic acids were performed using a Waters modular HPLC system (MA, model 1525) coupled to a diode array detector (model 2998) linked to a 20-µL sampler loop, following the method described by Robbins & Bean (2004).

The separation of phenolic acids was achieved on a reversed phase C18Luna column (Phenomenex, 1504.6 mm; particle size 5mm). The mobile phase consisted of methanol (B) and 0.1% aqueous formic acid (A) as the binary solvent system. The solvent gradient in volumetric ratios was as follows: 5–30% B over 50 min, held at 30% B for an additional 15 min; at 65 min the gradient was increased to 100% B and held at 100% B for an additional 10 min to clean up the column. The column was thermostatically controlled at 30°C. Injection volume was set to 20 µl. Dedicated JASCO software (ChromNAV ver 2.02.01) was used to calculate peak area by integration.

For the quantification of phenolic acids, four different concentration of ferulic acid and p-coumaric standards were prepared in methanol within the range: 5÷500 µg/mL and each

solution were injected in triplicate. Following chromatogram recording, sample peaks identification was carried out by comparison of UV spectra and retention time with those of the pure standards. Each tested extract has been prepared in a methanolic solution (methanol/water, 80:20), filtered through a UNIFLO 25/0.2 PTFE pre-filter (Scheicher & Schuell) and a disposable syringe (Chemil s.r.l.) at the concentration of 0.5 mg/mL and analyzed under the same experimental conditions described for phenolic acids standards. The obtained calibration graphs allowed the determination of the ferulic acid and of p-coumaric acid concentrations, the most abundant phenolics characterizing cereal by-products.

Limit Of Detection (LOD) and Limit Of Quantitation (LOQ) were calculated following the approach based on the standard deviation of the response and the slope as presented in the "Note for guidance on validation of analytical procedures: text and methodology", European Medicine Agency ICH Topic Q2 (R1).

	FA	p-CA
r^2	0.9991	0.9993
LOD	0.342 µg/mL	0.221 µg/mL
LOQ	1.14 µg/mL	0.74 µg/mL

Table 12. RP-HPLC-DAD statistical parameters of phenolic acids quantification

7.5.5 Determination of total phenolic content (TPC)

The Folin-Ciocalteu spectrophotometric assay (Singleton, Orthofer, & Lamuela-Raventos, 1999) was used to determine the total phenolic content of all phenolic extracts with a ThermoSpectronic Helios-γ spectrophotometer and performed according to previously described methods (Vermerris and Nicholson, 2007; Tacchini et al., 2015). Samples were concentrated to 2 mg/mL and 0.1 mL of each solution was mixed 7.9 mL of distilled water and 0.5 ml of 0.2 N Folin–Ciocalteu reagent; after 2 min, 1.5 ml of a 20% Na_2CO_3 solution was added. After incubation at room temperature for 2 h, the absorbance of the reaction mixture was measured at 765 nm against a water blank. Gallic acid was used as standard to produce the calibration curve. The mean of three readings was used and the total phenolic content was expressed as mg of gallic acid equivalents (GAE)/ g of dried extract.

7.5.6 HPTLC DPPH bioautographic assay

HPTLC bioautographic assay has been employed to screen the radical scavenging activity of the extracts and to determine compounds responsible for this activity using the DPPH radical and following the method described by Rossi et al. (2011). Briefly, 8 µl of each extract were concentrated and eluted as described in the previous HPTLC sections were applied to a HPTLC plate as 7 mm wide bands with Linomat IV (Camag). After development, plates have been sprayed with a methanolic solution of 2,2-diphenyl-1-picryl-hydrazyl radical (DPPH, 2 mg/mL) to detect the antioxidant fractions and then photographed at visible light after 30 min.

7.5.7 DPPH assay: evaluation of the antioxidant activity

The DPPH assay has been developed by Blois (1958) and subsequently improved using a microplate reader (Cheng, Moore & Yu, 2006; Kedare & Singh, 2011). For the experiments, seven different concentrations of extracts have been tested with the microdilution method within ranges reported in table 13.

Extracts	**By-products**	**Tested concentration ranges (µg/mL)**
FP extracts	All CBM	48.88 - 3000
BP extracts	Wheat & rice brans	1.95 - 125
	Corn germs	7.81 - 500
BP+ extracts	Wheat & rice brans	0.49 - 31.25
	Corn germs	3.91 - 250
Enzymatic extracts	All CBM	7.81 - 500

Table 13. Concentration ranges used for the determination of the radical scavenging activity

After 40 minutes of incubation in the dark at room temperature and stirring, microplates were analyzed with a microplate reader (Biorad, 680 XL) and the absorbance was read in triplicate against a blank at 515 nm. The DPPH inhibition in percent was determined by the following formula: IDPPH% = [1-(A1/A2)] x 100. Where A1 was the DPPH absorbance with the extracts and A2 without extracts.

Eight different concentrations (0.16-20 µg/mL) of Trolox were prepared and used as positive control for FP, BP and BP+ extracts. Antioxidant activity of the extract was

expressed as IC_{50}, concentration providing 50% inhibition of the radical. All experiments were assessed in triplicate and values were reported as mean ±SD (Standard Deviation).

7.5.8 Antimicrobial activity of phenolic extracts

The antimicrobial susceptibility test and the antimicrobial microdilution assay for MIC calculation were performed in order to verify the possibility to reinsert some of the most performing cereal extracts in the dermo-functional and cosmetic market thanks to their high content of phenolic acids. Plants, in fact, have developed strategies to protect themselves from microbial attacks, including the production of antimicrobial agents. Ferulic acid, predominantly located in the outer layer of kernels, enhance the rigidity and strength of cell wall and could have a role also in the protection against microbial attacks. The antimicrobial properties of FA have been, in fact, recognized for twenty years (Lo and Chung, 1999). A recent study demonstrated that FA exhibits a potent antibacterial effect against Gram-positive bacteria but has no effect against Gram-negative bacteria (Takahashi et al., 2013), however Borges et al. (2013) reported that the Gram-positive *bacteria* were less susceptible to FA than Gram-negative. Several studies have also contributed to demonstrate the FA mechanism of action as an antimicrobial agent (Campos et al., 2009; Borges et al., 2013): it causes irreversible changes in membrane properties through hydrophobicity changes, decrease of negative surface charge, and occurrence of local rupture causing changes in charge, intra and extracellular permeability, and physicochemical properties and consequent leakage of intracellular constituents.

Staphilococcus aureus represent a large *bacteria* group with the shape of coccidia. Staphylococcus genus is divided into two groups: coagulase-positive (pathogenic) and coagulase-negative for the ability to produce coagulase enzyme. *S. aureus* and *S. intermedius* are coagulase-positive genres. All other staphylococci are coagulase-negative – CNS (Mierzejewski and Woźniak-Kosek, 2012).

S. aureus is the main member of the skin microbiota and in certain conditions can cause various skin disorders and infections by producing several extracellular enzymes which cause tissue damage and thus helping itself to spread into the deeper tissues. Moreover, it produces a series of exfoliative toxins such as enterotoxins A-E. Various disorders are associated with *S. aureus* virulence: impetigo, bacteremia, endocarditis, soft tissue infections, septicemia and food poisoning (Kumar et al., 2016). Moreover *S. aureus* is used

as an index of official evaluation concerning the efficiency of cosmetics preservation (Mierzejewski and Woźniak-Kosek, 2012).

S. epidermidis is a facultative anaerobe of skin microbiota which, even if is normally nonpathogenic, can turn into infectious with a role in acne lesions. *S. epidermidis* has several adhesion factors and has the ability to form a biofilm which favors the growth in anaerobic conditions of *Propionibacterium acne*. During the acne inflammation, lipases and delta-haemolysin are two virulence factors of *S. epidermidis* (Kumar et al., 2016).
These bacteria were chosen as targets of antimicrobial activity in this project due to the potential applications that a possible antimicrobial activity could have in the cosmetic field and more generally in the health industry.

Antimicrobial susceptibility test

The antimicrobial susceptibility of the *bacteria* was determined on Mueller–Hinton agar (Oxoid, Spain) using the disk diffusion method. Six different antimicrobial agents, widely used in human clinical, were studied: ampicillin (10 mg/disk), penicillin (10 mg/disk), chloramphenicol (30 mg/disk), kanamycin (30 mg/disk), ciprofloxacin (5 mg/disk) and doxycycline (30 mg/disk). *Staphylococcus aureus* reference strain ATCC 25923 was used as a quality control. Each antimicrobial agent has been tested against all bacteria strains and incubated overnight at 37°C. The measurement and interpretation of growth inhibition diameters was performed following the CLSI guidelines for human antimicrobial susceptibility tests for human pathogens (CLSI, 2015).

Antimicrobial microdilution assay

The Minimum Inhibitory Concentration (MIC) of alkaline hydrolyzed extracts have been determined using the microbroth dilution method. One reference strain (ATCC 29213), two clinical isolates strains (Methicillin-Resistant *Staphylococcus aureus*, MRSA 185087 and Methicillin-Sensitive *Staphylococcus aureus* MSSA 185960) of *Staphylococcus aureus* and one clinical isolate of *Staphylococcus epidermidis* (185240) have been used for the antimicrobial activity assay. The MICs were determined through microdilution method in accordance with the Clinical and Laboratory Standards Institute (CLSI, 2015).

All strains have been sub-cultured overnight in Muller-Hinton Agar (MHA) before testing. An inoculum has been prepared for each *bacterium* using a 0.85% saline solution and adjusting its OD_{595nm} to 0.08 ± 0.1 (1 x 10^8 CFU/mL). 100 µL of each extract has been put in a sterile u-shaped 96-well PS-microplate (Greiner bio-one, USA) and twelve different concentrations have been tested (2048-1 µg/mL); penicillin was used as positive control and eight different concentrations of it (4-0.06 µg/mL) were tested.

Bacteria suspensions without extracts and Muller-Hinton Broth (MHB) were used as controls. The microplates have been then incubated overnight at 37°C. MIC results have been determined as the lowest concentrations of extracts at which no *bacteria* growth has been detected. A re-count of *bacteria* concentration has been performed after each test by diluting 10 µL of each bacteria suspension (negative control) in 10 mL of sterile water: 100 µL of this final suspension have been put on MHA plate at 37°C for 24 hours in order to count the CFU. All tests have been performed in triplicate and compared with controls.

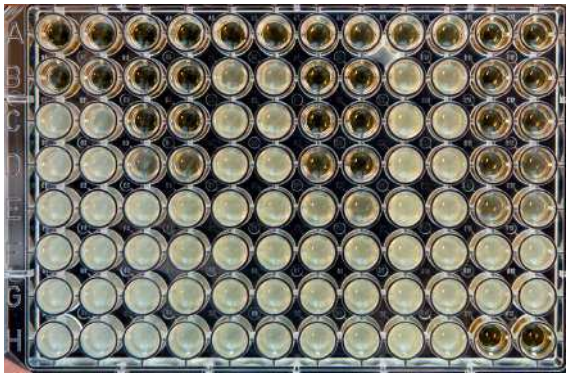


Figure 40. Microplate with antimicrobial activity assay

7.6 Target 2: Lipophilic compounds and USP fraction

7.6.1 Supercritical fluids extraction (SC-CO_2)

All samples were subjected to supercritical fluid extraction (SFE) using an Applied Separations (Allentown, PA, USA) model Speed SFE extractor. Extractions were performed on each type of CBM in order to extract the cereal fixed oils and the unsaponifiable fraction, in particular. For the extraction, 2g of each CBM have been extracted under the following operating conditions: carbon dioxide flow-rate of 2.5 mL/min, oven temperature was set at 40°C, restrictor temperature at 80°C, and pressure at 300 atm. Extraction parameters have been chosen from various literature reports as the

most suitable ones for the extraction of cereal by-products: from all literature reports, two of them have been chosen to be applied to CBM and compared as they differed only for the extraction times: Gelmez et al. (2009) propose 10 min of extraction and Kwon et al. (2010) proposed 120 min for the extraction of cereal fixed oils with SC-CO_2.

Figure 41. SFE apparatus Spe-edTM -Applied Separations (Photo credit: Ilaria Burlini)

7.6.2 HPTLC of fixed oils

For the HPTLC identification of fixed oils of SFE extracts, the method "HPTLC Identification of Fatty Oils (Fixed Oils)" from Camag laboratory was followed (Camag, 2014). HPTLC plates 10x10 cm RP-18 F_{254s}, (Merck) have been used for the analysis of SFE extracts with the Linomat V automatic sampler (CAMAG). WinCATS Planar Chromatography Manager software (CAMAG) and Twin Trough Chambers (10x10 cm) have been used. RP-18 F_{254s} plates have been pre-washed with dichloromethane and then oven dried at 120 °C for 10 min. The HPTLC pre-develop plate has been eluted with the following eluent mixture: dichloromethane/glacial acetic acid/acetone (20:40:50). 25 µl of all extracts were dissolved in 3 mL of dichloromethane, 2 µl of the diluted extracts have been automatically put on the plate and then eluted. For the fingerprint visualization of the SFE fixed oils the plate was dipped into phosphomolybdic acid reagent (derivatization reagent: 25 mg/mL of phosphomolybdic acid in 96% ethanol) for one second and oven heated for 3 minutes at 120°C, finally examined at visible light.

7.6.3 Determination of unsaponifiable (USP) composition with GC-MS

To determine the unsaponifiable composition and percentage of all 10 min SFE extracts, gas chromatography-mass spectrometry (GC-MS) was used. 10 mg of each extract have been cold saponified with 20 mL of 1 M methanolic KOH, the flask has been placed under constant agitation for 24 h at a temperature of 28°C. The solution has been then extracted twice with 2 mL of *n*-hexane and 0.2 mL of ethanol using a separator funnel. The *n*-hexane fractions have been then dried using a rotary evaporator after adding anhydrous sodium sulfate: the unsaponifiable fractions have been silanized at 80°C with 200 µL of N,O-Bis(trimethylsilyl)-trifluoroacetamide and trimethylchlorosilane (BSTFA /TMCS) and 200 µL of pyridine. After 1 h, the liquid has been evaporated under a nitrogen flow and then extracted with 0.3 mL of hexane. The products have been placed in ultrasounds for 2 min and centrifuged, the supernatant has been injected into the GC. Compounds have been identified by comparing their GC retention times and the MS fragmentation pattern with those of literature, with pure compounds (Sigma-Aldrich) and by matching the MS fragmentation patterns and retention indices with the mass spectra libraries NIST and literature.

The GC-analysis of the extracts have been performed using a Varian 3800 chromatograph interfaced with a Varian SATURN MS-4000 mass spectrometer, with electronic ionization in progress, provided with integrated control software with NIST library. For the GC analyses the following operating conditions have been used: column Varian VF-5 5% poly- and 95% phenyl-dimethyl-siloxane (0.25 mm; length, 30 m; film thickness, 0.25 µ m Agilent Technologies Inc., Santa Clara, California, USA). Injector temperature, 300°C; carrier helium, flow rate, 1.2 mL/min; and split ratio, 1:20. Oven temperature was increased from 230°C to 320°C at a rate of 5°C/min, followed by 7 min at 320°C. The MS conditions were: ionization voltage, 70 eV; emission current, 20 mAmp; scan rate, 1 scan/s; mass range, 40-650 Da; trap temperature, 150°C, transfer line temperature, 300°C.

7.6.4 HPTLC DPPH bioautographic assay

HPTLC bioautographic assay has been employed to determine antioxidant compounds on SFE extracts using the same method described for phenolic compounds. The SFE extracts have been prepared and eluted as described by the USP for the HPTLC analyses for fixed oils (Camag, 2014). After development, plates have been sprayed with a methanolic

solution of 2,2-diphenyl-1-picryl-hydrazyl radical (DPPH, 2 mg/mL) to detect the antioxidant fractions and then photographed at visible light after 30 min.

7.6.5 Radical scavenging activity of lipophilic extracts

The radical scavenging activity of vegetable oils was performed following the method described for phenolic extracts with some modifications for the application to lipophilic compounds as described by Radice et al. (2014).

SFE extracts have been diluted in *n*-hexane with the following concentration range: 0.08÷5 mg/mL and extra virgin olive oil (EVOO COOP) has been used as positive control for its well-known antioxidant activity. After 60 minutes of incubation in the dark at room temperature the microplates were analyzed with a microplate reader (Biorad, 680 XL) and the absorbance was read in triplicate against a blank at 515 nm. The DPPH inhibition in percent was determined by the following formula: IDPPH% = [1-(A1/A2)] x 100. Where A1 was the DPPH absorbance with the extracts and A2 without extracts.

Eight different concentrations (0.16÷20 mg/mL) of EVOO were prepared and used as positive control for SFE extracts. The antioxidant activity of the extract was expressed as IC_{50}, concentration providing 50% inhibition of the radical. All experiments were assessed in triplicate and values were reported as mean ±SD (Standard Deviation). A second EVOO control was used from literature reports for the high.

Since the quality of EVO oil depends on many variables including geographical origin and production, a second reference was taken from literature (Valavanidis et al., 2004) to compare the IC_{50} of our positive control EVOO and our results.

7.7 Cosmetic application

7.7.1 Products formulations

Starting from the interesting results we obtained with both phenolic and SFE extracts, we decided to select one extract from both targets to insert in finished dermo-cosmetic formulations in order to propose practical applications of the extracts obtained from cereal by-products in the health cosmetic market.

Based on the results obtained we decided to formulate the finished products using the following extracts:

1. Wheat bran aleurone BP+ extract (Formulation 1 – F1)
2. Yellow maize germ SFE extract (Formulation 2 – F2)

To continue the study, the investigation of the antioxidant activity of both finished products and extracts through PCL analyses, and the stability at ambient temperature and at 40°C (accelerate stability) of the finished dermo-cosmetic formulations were also evaluated.
The phenolic and SFE extracts were included in cosmetic formulations (O/W emulsion) with the INCI reported below. Two different percentages for each extract were tested to verify the best amount of active ingredients.
A common oil-in-water (O/W) base protocol was used for all formulations. Phase 1 (hydrophilic) was prepared by weighting each raw material and heating the phase up to 70°C. Phase 2 (lipophilic) was prepared and heated up to 40°C and subsequently added to phase 1 by pouring the lipophilic phase into the hydrophilic one with the use of a turboemulsifier mixer (Silverson®). Finally, the emulsion obtained was divided into aliquots to add phase 3 (extracts). The final pH was adjusted with NaOH 10% within the range 5-5.5 to which all preservative systems work best and to make the preparations suitable for facial application.

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Formulation 1 (F1): O/W with durum wheat aleurone phenolic extract
Final pH: from 3.91 to 5.04

	INCI Formulation 1	%
A.	PHASE 1	
1	Aqua	q.b. to 100
2	Glycerin	3
3	Benzyl alcohol, Ethylhexylglycerin	1
4	Xantham gum	0.2
B.	PHASE 2	
5	Glycerin stearate citrate	3.5
6	Cetearyl alcohol	2
7	Glyceryl stearate	2
8	Cocoglycerides	7
9	Coco-caprylate	7
C.	PHASE 3	

10	Aqua	3
11	Wheat bran phenolic extract	0.3-0.5
Total (all phases)		100

Formulation 2 (F2): O/W with yellow maize germ SFE extract

Final pH: from 3.85 to 5.4

	INCI Formulation 2	%
A.	PHASE 1	
1	Aqua	q.b. to 100
2	Glycerin	3
3	Benzyl alcohol, Ethylhexylglycerin	1
4	Xantham gum	0.2
B.	PHASE 2	
5	Glycerin stearate citrate	3.5
6	Cetearyl alcohol	2
7	Glyceryl stearate	2
8	Cocoglycerides	7
9	Coco-caprylate	7
C.	PHASE 3	
10	Yellow corn germ SFE extract	2.5-5
Total (all phases)		100

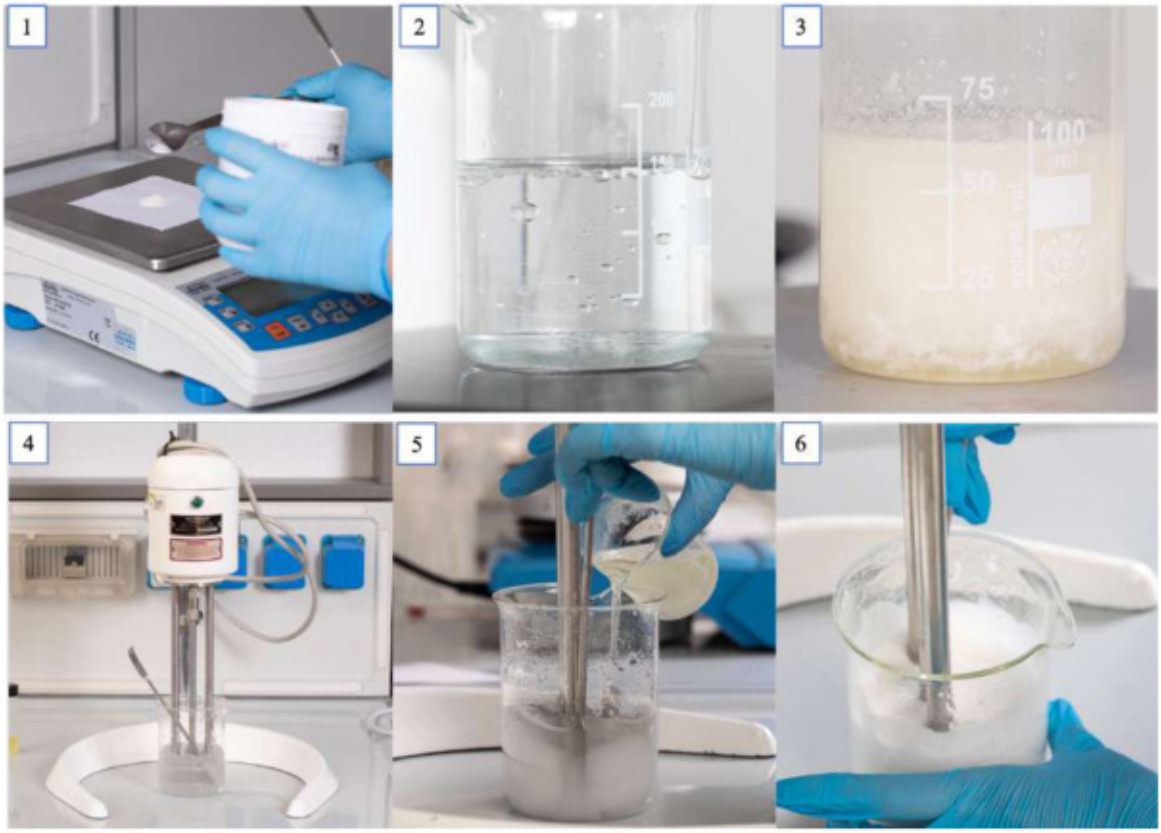


Figure 42. Step of O/W preparation: 1- weight of raw materials; 2- hydrophilic phase; 3- lipophilic phase; 4- turboemulsifier with hydrophilic phase; 5- O/W emulsion; 6-final product.

7.7.2 PCL (Photochemiluminescence) assay

The PCL assay, based on the methodology of Popov and Lewin (1994), was used to measure the antioxidant activity of cosmetic products and of the two selected extracts in collaboration with Ambrosialab Srl (www.ambrosialab.it). With this method, the antioxidant properties of the substances examined can be determined quickly and effectively, limiting the loss of activity related to degradation (Popov and Lewin, 1996).

The antioxidant activity was measured with a Photochem® apparatus (Analytik Jena, Leipzig, Germany) against superoxide anion radicals generated from luminol, a photo-sensitizer, when exposed to UV light (Double Bore® phosphorus lamp, output 351 nm, 3 mWatt/cm^2), using ACL (Antioxidant Capacity of Liposoluble substance) kit designed to measure the antioxidant activity lipophilic compounds (Popov and Lewin, 1994).

In ACL studies, the kinetic light emission curve was monitored for 180 s and expressed as micromoles of Trolox per gram of dry matter. The areas under the curves were calculated using the PCL soft control and analysis software. As greater concentrations of Trolox

working solutions were added to the assay medium, a marked reduction in the magnitude of the PCL signal and hence the area calculated from the integral was observed. This inhibition was used as a parameter for quantification and related to the decrease in the integral of PCL intensities caused by varying concentrations of Trolox. The observed inhibition of the signal was plotted against the concentration of Trolox added to the assay medium. The concentration of the added extract solution was such that the generated luminescence during the 180 s sampling interval fell within the limits of the standard curve. The extracts for ACL measurements were centrifuged (5 min at 16,000 g) prior to analysis. The antioxidant assay was carried out in triplicate for each sample, and 20 µL of the diluted extract (1:40, v/v) HPLC-grade methanol (ACL) was sufficient to correspond to the standard curve.

For the analyses the following samples were tested:

1. Wheat bran BP+ extract
2. O/W F1 (0.3%)
3. O/W F1 (0.5%)
4. Yellow maize germ SFE extract
5. O/W F2 (2.5%)
6. O/W F2 (5%)

7.7.3 Stability

The stability of the cosmetic products was evaluated at both ambient temperature (22-23°C) and at 40°C in oven for 1 month.

This is the first step of stability control that is normally done for cosmetic products which are usually also checked for 3 and 6 months but for reasons of time it has not been possible to continue.

Results and Discussion

7.8 Target 1: Phenolic acids

7.8.1 Yields and qualitative results

The extraction of phenolic molecules from cereal by-products has been one of our main targets because they are, with the unsaponifiable fraction, the main representative molecules of cereal by-products. The importance of extraction yields concerns the possibility of valorizing these by-products and of actually being able to reintroduce them to the market in an advantageous way. Yields of phenolic extracts (UAE, alkaline hydrolyzed and enzymatic hydrolyzed) are reported in the tables below.

7.8.1.1 Free phenolics

The highest yields were obtained with ultrasound-assisted extraction (UAE) for the extraction of free phenolics. These high percentages could be explained with the choice to use hydroalcoholic solutions as extracting solvent and with the sonication effects. Biomasses treated with sonication are, in fact, subjected to various forces, as previously described: the cavitation, fragmentation, erosion, sonocapillarity, sonoporation, local shear stress and destruction effects, which contribute to the extraction of various compounds without much specificity.

CBM	FP % yield
Wheat bran aleurone	17.29 ± 1.40
Rice bran	19.73 ± 1.45
Yellow corn germ	12.60 ± 0.80
White corn germ	12.40 ± 0.89

Table 14. Free phenolics extraction yields expressed in percentages on the dry weight of CMB

In table 14, free phenolics yields are reported for all CBM: as shown rice bran gave the highest result with a yield of 19.97 ± 1.45%, which is comparable and slightly greater than that obtained with the extractive optimization method in the literature of 18.2% (Tabaraki et al., 2011). The extraction of FP from both corn germs gave the lowest yield percentages: 12.6 ± 0.8% and 12.4 ± 0.89 % respectively. Yields obtained for maize germ can be

explained by two reasons. The first concerns the composition of the different matrices: the germ of the caryopses is, in fact, characterized by a greater percentage of fixed oils on the dry weight compared to the bran; the second reason may be due to the fact that there are two optimized extraction methods in literature for durum wheat and rice brans using ultrasounds-assisted extraction. The extraction optimization allowed us to extract free phenolics with optimal variables (temperature, percentage of solvent, etc.) for the target plant matrix. This optimization does not exist in literature for maize germ, whose FP extraction is therefore still optimizable and could be an interesting target for future studies. Because no optimized UAE extraction method was found in literature, we performed three extractions for each maize variety with three different percentages of hydroalcoholic solvent: 100% ethanolic, 50% ethanol/water and 100% water solutions. From the HPTLC analysis carried out on the extracts, we verified that the hydroalcoholic solvent is certainly the best choice among the three as can be seen from the HPTLC chromatogram in figure 43: both central runs in plate A (254 nm) and in plate B (366 nm), in fact, shows a richer phytocomplex than those obtained with 100% ethanol or 100% water.

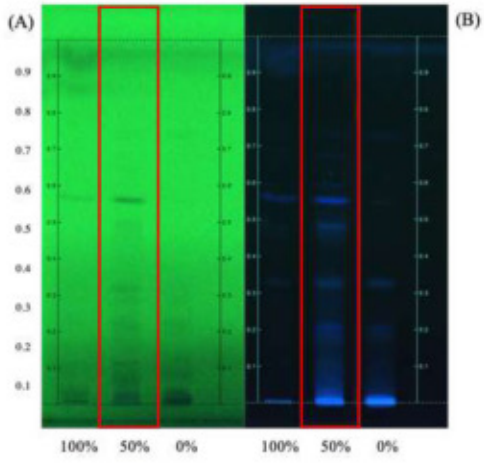


Figure 43. HPTLC plate. FP Extracts of yellow corn obtained with 100% ethanol, 50 % ethanol and 100% water solutions. Plate (A) was photographed at 254 nm and plate (B) at 366 nm after being derivatized with NP/PEG solution.

Free phenolics, as all other phenolic extracts, have been analysed with HPTLC in order to do a preliminary investigation on their phenolic content in qualitative terms. With HPTLC we have been able to exploit this analysis to verify the extractive effectiveness of each technique used. Being a fast analysis method, HPTLC has been used, in fact, to check the quality of the extracts and also to select the best extraction parameters.

HPTLC plate (figure 44) shows FP extracts that have been eluted in order to detect the presence of phenols inside the extracts. Firstly, the plate was photographed at 256 nm and secondly at 366 nm after being derivatized with NP/PEG solutions. Extracts exhibited a phenolic profile characterized by some flavonoids (highlighted by the presence of yellow and yellow-green spots) and phenolic acids or coumarins (highlighted by the presence of blue spots). The presence of saponarin (track 4), a flavone glucoside, has been hypothesized, both in wheat aleurone (W) and rice bran (R), by the evidence of a yellow-green spot in correspondence with R_f 0.16 which corresponds to the same color and R_f of the eluted reference standard. The presence of coumarins, such as esculin (track 13), has been hypothesized, in all extracts, by the evidence of a blue spot in correspondence with R_f 0.31 which corresponds to the same color and R_f of the eluted reference standard, present in wheat bran aleurone (W) track particularly. In the same way two flavonoids are likely present, rutin (track 5) and isoquercitrin (track 10) in white corn germ at R_f 0.25 and R_f 0.38 respectively. However, the presence of these molecules cannot be verified through this method and therefore needs further detailed analysis.

Finally, from the HPTLC plate it can be said that through UAE extraction it was not possible to extract ferulic acid (track 14) since in correspondence with the commercial standard eluted, at R_f 0.75, there is no presence of the molecule in the extracts.

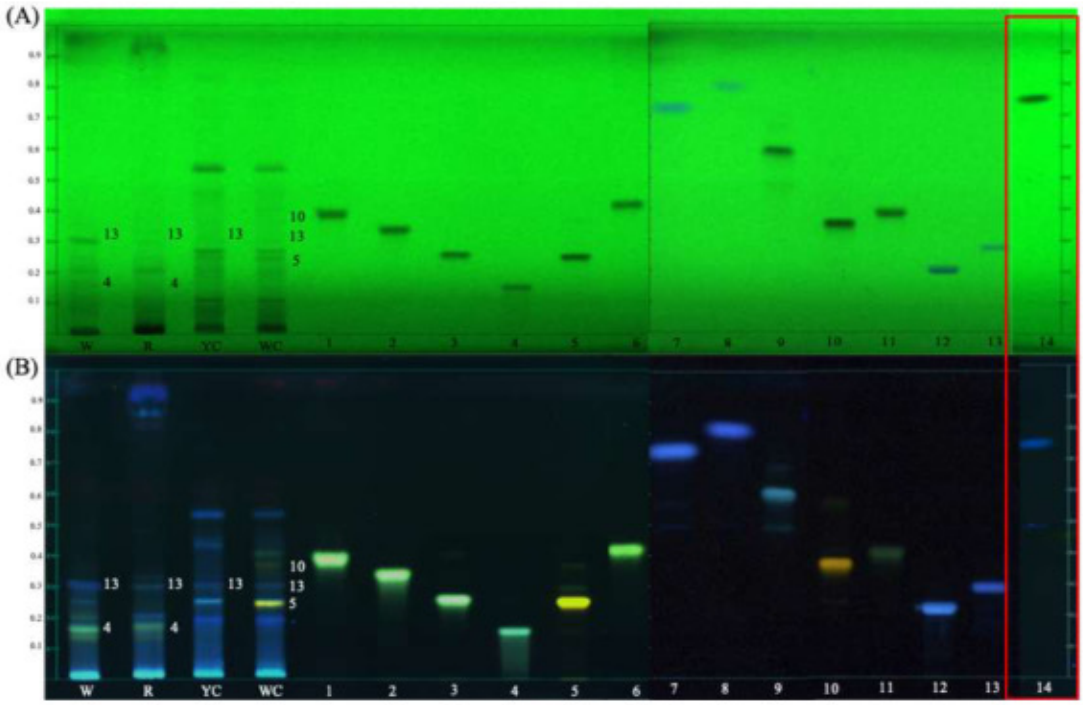


Figure 44. HPTLC plate. FP Extracts chromatograms. Detection at 254nm (A) and 366nm after derivatization with NP/PEG (B). Tracks: W- Wheat bran aleurone; R- Rice bran; YC- Yellow corn germ; WC- White corn germ; 1-vitexin; 2- isovitexin; 3- vitexina 2-O-rhamnoside; 4- saponarin; 5- rutin; 6; kaempferol-3-O-glucoside;7- scopoletin; 8-umbelliferon; 9- rosmarinic acid;10- isoquercitrin; 11-apigenin-7-O-glucoside; 12- fraxin; 13- esculin; 14- t-ferulic acid.

7.8.1.2 Bound phenolics

The extractions of bound phenolics have been performed using both chemical and enzymatic hydrolyses methods. Bound phenolics extractions were more selective than those of UAE and gave the lowest results in terms of yield percentage. These results might seem daunting if compared to those of free phenolics, but the yields obtained are valued, as it will be described later, by the high concentration of biomolecules that characterize the extracts.

CBM	Extracts	BP % yields	% After separation step
Wheat bran aleurone	BP	0.41 ± 0.04	nd
	BP+	0.48 ± 0.01	nd
Rice bran	BP	0.54 ± 0.09	nd
	BP+	0.54 ± 0.04	nd
Yellow corn germ	BP	3.75 ± 0.08	0.26 ± 0.01
	BP+	6.22 ± 0.22	0.51 ± 0.03
White corn germ	BP	2.29 ± 0.36	0.11 ± 0.01
	BP+	3.95 ± 0.39	0.6 ± 0.04

Table 15. BP and BP+ extraction yields expressed in percentages on the dry weight of UAE-R

The yields' results of alkaline hydrolyzed extracts (BP and BP+) are reported in table 15. Contrary to what reported for the FP results, both corn germ extracts obtained the highest extraction yields. The difference with rice and wheat aleurone brans' yields, are probably due to a larger amount of lipophilic component that is extracted together with BP during the corn hydrolysis. Thus, from maize germ alkaline hydrolysed extracts, a separation step was required to remove the high quantity of lipophilic fraction that has been extracted with the ethyl acetate. The separation was performed to better quantify the phenolic extracts in the hydrophilic fraction.

After the separation, the extracts have been analysed with HPTLC to verify the correct separation of the two phases and that no phenolic molecules were lost during the separation steps. Figure 45 represents the HPTLC elution of lipophilic and hydrophilic fraction of maize alkaline hydrolyzed extracts. Lipophilic tracks are marked with letter L and, as shown in the chromatogram, no phenolic acids, visible as blue spots at 366 nm, were detected and therefore lost.

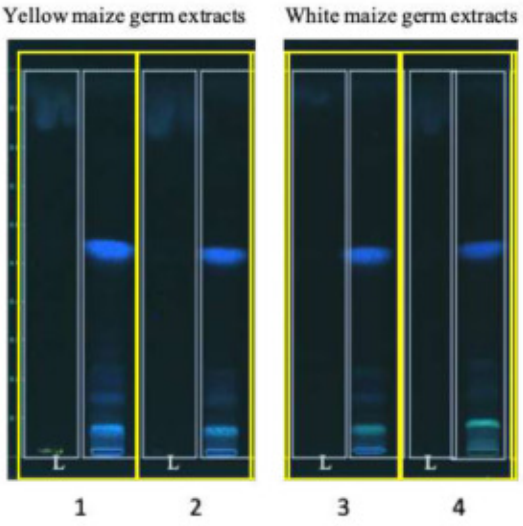


Figure 45. Polyphenol separation from maize alkaline hydrolyzed extracts: plate has been photographed at 366 nm and NP/PEG derivatized. Tracks: 1-4: hydrolyzed extracts. L- lipophilic fraction

By comparing the extraction techniques used (alkaline hydrolysis and alkaline hydrolysis coupled with sonication) we found an increase in percentage yield with the second extraction technique, particularly in the case of maize germs. The chemical hydrolysis of BP inside the ultrasonic bath allowed to facilitate the extraction of the molecules, by

degrading the plant cell walls, thanks to the various mechanisms of forces involved that allowed the sodium hydroxide to better break the ester linkages of phenolic acids.
All the following analyses were carried out considering only the hydrophilic part as maize germ alkaline hydrolyzed extract (BP and BP+).

HPTLC plate (figure 46) shows BP extracts, photographed at 254 nm and 366 nm after being derivatized with NP/PEG. The method employed for the HPTLC separation of bound phenolic acids, allowed us to better separate phenolic acids present in the extracts. Plate C reports the HPTLC runs of phenolic acid standards: *trans*-ferulic acid (R_f 0.55, track 1) and *p*-coumaric acid (R_f 0.5, track 2). In the extracts it is well visible at R_f 0.55 a light blue spot (plate B) whose characteristics correspond to those of the *t*-ferulic standard (FA), the presence of *p*-coumaric acid is less visible both because its color is dark blue and difficult to distinguish at 366 nm, and because the R_f of the standards are very similar and inside the phytocomplex they do not separate very well from each other. In fact, for its correct identification we used HPLC, as described later. Although this technique does not allow us to quantify the molecule precisely, FA is the molecule that appears to be present in greater quantities.

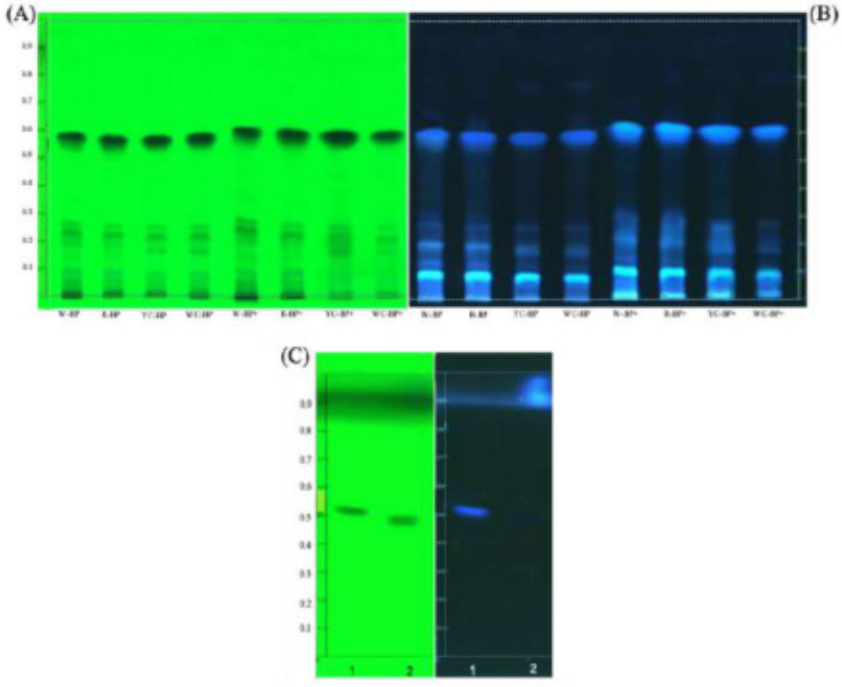


Figure 46. HPTLC plate (A) and (B): Chromatograms of BP extracts obtained with alkaline hydrolyses. Detection at 254nm (A) and 366nm after derivatization with NP/PEG (B). Tracks: W - Wheat bran aleurone; R- Rice bran; YC- Yellow corn germ; WC - White corn germ; BP – alkaline hydrolysis; BP+ -

alkaline hydrolysis coupled with sonication. HPTLC plate (C): Chromatograms of phenolic acids standards: track 1: t-*ferulic acid: track 2:* p-*coumaric acid*

Table 16 contains percentage yields obtained with the most performing extraction parameters used for the enzymatic treatment (24 h, 180 min, 180 min UAE-R). In the text, in order to facilitate the results reading, the three chosen enzymatic extraction will be named Enz 1 (180 min), Enz 2 (180 min on UAE-R) and Enz 3 (24 h).

The highest enzymatic yields were obtained for every matrix after 24 h of extraction (Enz 3), with yields of almost 2% in the case of yellow corn (1.92±0.08%) and durum wheat (1.83±0.15%). Comparing the two 180 min methods (Enz 1 and 2) the extractive yields, with the exception of durum wheat aleurone, saw an increase in percentage with the pre-sonicated material but still yields are much lower than that obtained at 24 h.

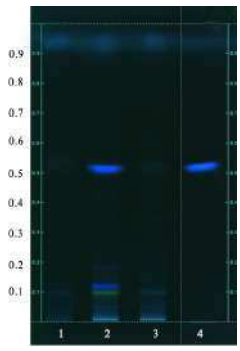
CBM	**Enzymatic extraction (Enz)**	**BP % yields**
Wheat bran aleurone	Enz 1	0.85 ± 0.05
	Enz 2	0.79 ± 0.11
	Enz 3	1.83 ± 0.15
Rice bran	Enz 1	0.95 ± 0.09
	Enz 2	1.09 ± 0.15
	Enz 3	1.49 ± 0.12
Yellow corn germ	Enz 1	0.98 ± 0.07
	Enz 2	1.41 ± 0.15
	Enz 3	1.92 ± 0.08
White corn germ	Enz 1	0.99 ± 0.09
	Enz 2	1.11 ± 0.16
	Enz 3	1.58 ± 0.13

Table 16. Enzymatic extraction yields expressed in percentages on the dry weight of cereal by-product

Among all the enzymatic-assisted extractions described in the Materials and Methods, the most interesting extracts were chosen on the basis of the preliminary HPTLC analyses.

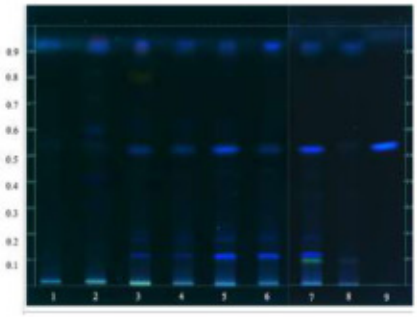
The choice of the most appropriate extractive parameters has been made, in the case of enzymatic extractions, starting from wheat bran aleurone on which several tests have been made and described below. Once the best extraction parameters have been decided, these have subsequently been applied also to other cereal matrices.

The following HPTLC plate (A) reports the enzymatic extracts of wheat bran aleurone obtained with the two enzymatic blends: blend 1, FAEZCT+Xylanase (track 2) and blend 2, FAERU+Xylanase (track 3) tested against a blank (track 1) for 24 h of extraction. As it can be seen from the HPTLC plate, track 2 demonstrated a richer phytocomplex compared to that of the blank and of the FAERU enzymatic blend, suggesting the best extractive efficacy of FAEZCT for our purpose. Furthermore, the extractive efficacy is also demonstrated by the presence of FA (R_f 0.55, track 4), extracted exclusively with feruloyl esterase from *Clostridium thermocellum* (FAEZCT). For these reasons the enzymatic blend 1 has been used for all the following enzymatic extractions.



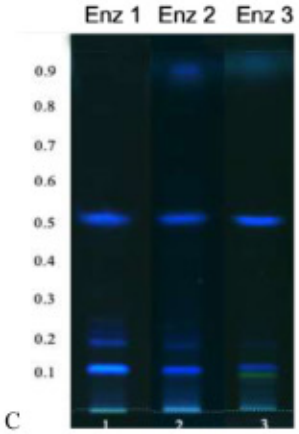
A

The HPTLC plate (B) reports the chromatographic plates of the enzymatic extracts obtained with enzymatic blend 1 after 20 min (track 1), 60 min (track 3), 180 min (track 5) and 48 h (track 7) and the respectively blanks (tracks 2,4,6,8). As can be seen, the tracks relative to the extracts obtained at 180 min (track 5) and at 48 h (track 7) showed the best phenolic profile in qualitative terms. In particular they are both characterized by the evident FA spot (track 9) at R_f 0.55, the main target of our phenolic extractions. Moreover, since the extract obtained after 48 h of extraction did not show a sufficient difference from that at 24 h or 180 min, it did not seem useful to continue the analyses on this extract also for the reasons of extractive optimization aimed at reducing the timing and consequently the impact of the proposed extractions.



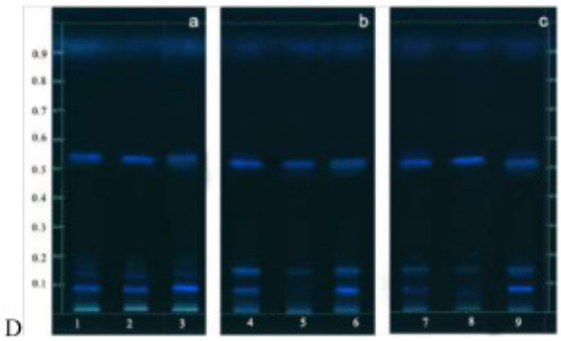
B

In the plate C it is shown the extract obtained from a pre-sonicated material (UAE-R, track 2) obtained with blend 1 after 180 min compared to the other most interesting extracts: 180 min (track 1) and 24 h (track 3).



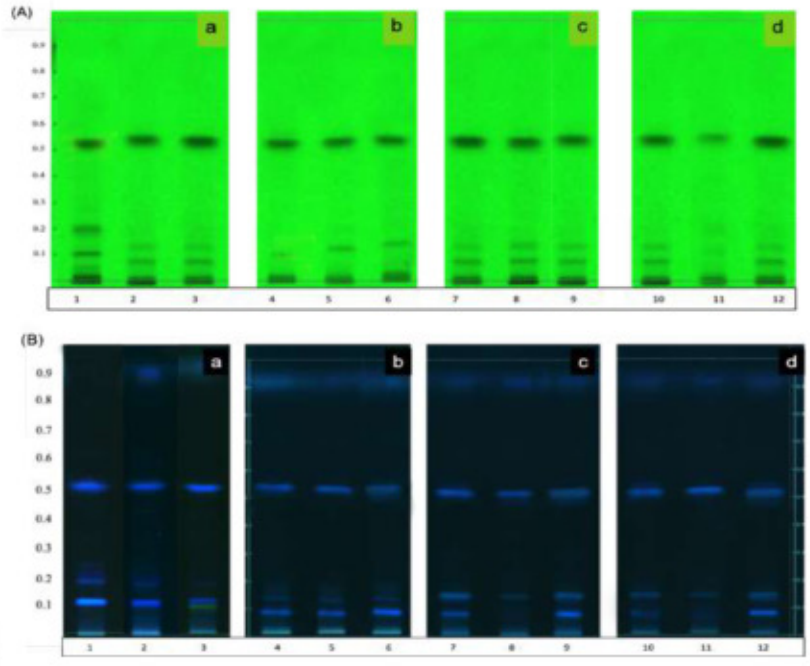
C

Plate D shows the three most significant enzymatic extractions for each other CBM - rice (a), yellow corn germ (b), white corn germ (c) - obtained with the three most interesting enzymatic extractions already described for wheat bran aleurone: Enz 1 (1), Enz 2 (2), Enz 3 (3). The influence of ultrasounds on the extraction efficiency of the enzymes-assisted extractions (Enz 1 and 2) should be read differently from the chemical hydrolyzed extracts. In fact, the two enzymatic extractions don't differ for the extraction technique used, but for the starting matrix: in the case of Enz 1, the starting matrix was the cereal by-product (e.g. wheat aleurone), in the case of Enz 2 the starting matrix was the residue of UAE. This means that the latter was previously subjected to ultrasounds for the extraction of the FP and thus it underwent two extraction steps (FP and enzymatic).



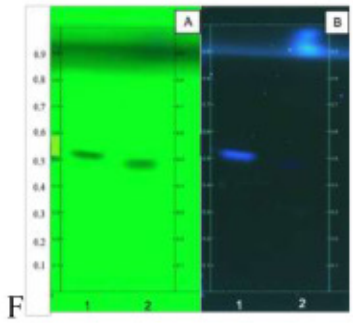
D

Finally, plate E summarizes the HPTLC of all CBM photographed both at 254 nm and at 366 nm. It can be seen that the spot at R_f 0.55 is more evident at 254 nm. This is due to the fact that FA has a R_f very similar to that of *p*-coumaric acid which is clearly visible at 254 nm as can be seen from the run of the single standard (Figure F, plate A) and less visible at 366 nm (Figure F, plate B) because of its very blue color dark, difficult to distinguish with this method but detected later in RP-HPLC-DAD.



E

Figure 47. HPTLC plate E. Chromatograms of enzymatic extracts Enz1 (1), Enz 2 UAE-R (2) and Enz 3 (3). Detection at 254nm (A) and 366nm after derivatization with NP/PEG (B). Plate a- wheat bran aleurone; b- rice bran; c-yellow corn germ; d- white corn germ.



F

Figure 48. HPTLC plate F. Chromatograms of ferulic acid (1) and p-coumaric acid (2) photographed at 254nm (A) and 366 nm (B)

From the HPTLC plates of hydrolyzed extracts, it is possible to compare the two hydrolysis techniques (chemical and enzymatic) that show similar phenolic profile from a qualitative point of view, but more evident spots characterize the chemical hydrolyzed extracts.

The RP-HPLC-DAD analyses have been performed for all hydrolyzed extracts in order to characterize and quantify the main peaks of the HPLC chromatograms.
Figure 49 reports the RP-HPLC-DAD chromatograms of BP and BP+ extracts with µV intensity of 2000000. Figure 50 reports the RP-HPLC-DAD chromatograms of enzymatic extracts with µV intensity of 200000. Both chromatograms' main peak, is marked with letter *a* and was identified as ferulic acid (FA). Peak marked with letter *b* was identified as p-coumaric acid. The peaks identifications were done by comparing the retention times and the absorption peaks with those of literature data.

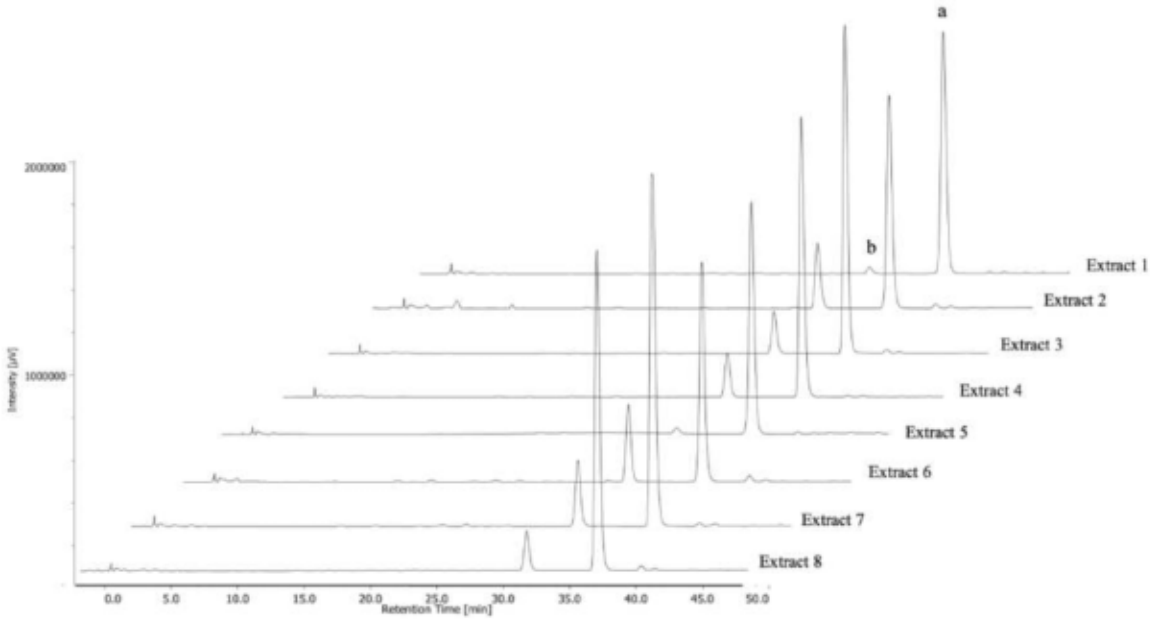


Figure 49. RP-HPLC-DAD chromatograms of BP and BP+ extracts. Peak a: FA; peak b: p-coumaric acid. Extracts: 1- Wheat BP; 2- Rice BP; 3-Yellow corn BP;4- White corn BP; 5- Wheat BP+; 6- Rice BP+; 7- Yellow corn BP+; 8- White corn BP+

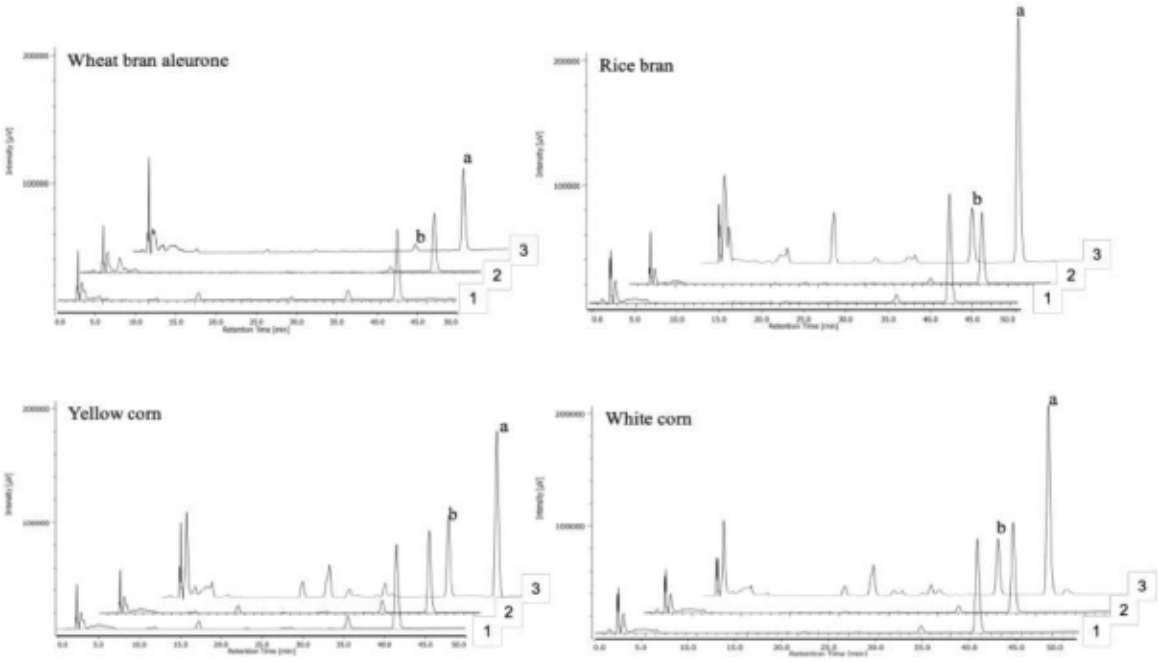


Figure 50. RP-HPLC-DAD chromatograms of enzymatic extracts. Peak a: FA; Peak b: p-*coumaric acid: Extracts: 1- Enz180; UAE; 2- Enz180 min; 3- Enz24h*

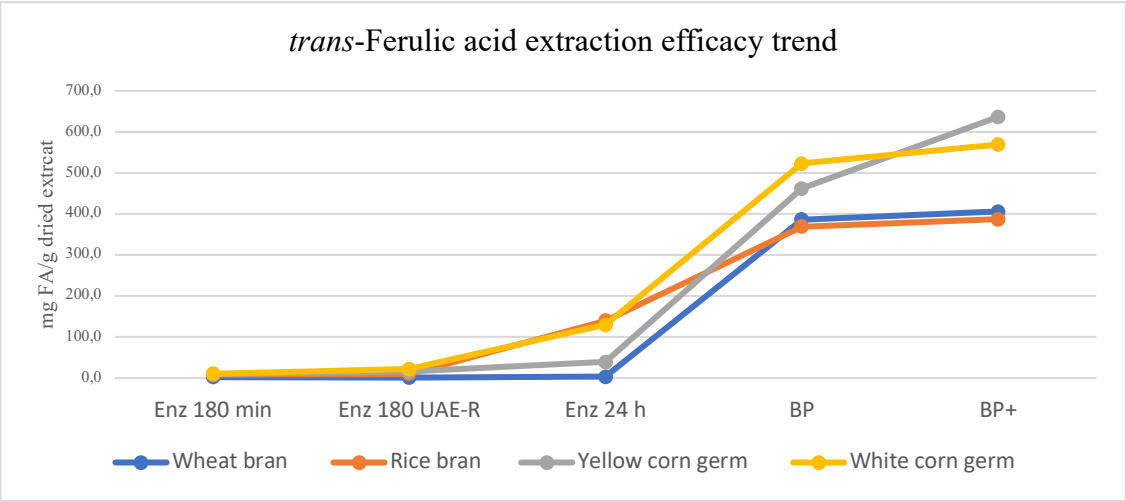
7.8.2 Quantification of phenolic acids

The quantification of the phenolic acids in hydrolyzed extracts (both chemical and enzymatic) with RP-HPLC-DAD is reported in table 17.

From the results it is clear how the results change drastically depending on the extractive technique used and, for this reason, it cannot be defined whether more FA is contained in durum wheat, rice or maize by-products. In general, it seems that more ferulic acid is contained in maize, but it is difficult to say with certainty whether more ferulic acid is contained in yellow or white maize germ. However, from white maize germ more FA was extracted with three methods out of four, particularly with BP+ (569.2 ± 1.7 mg FA/g dried extract). Yet the highest content of FA was obtained with BP+ extraction from yellow maize germ (636.5 ± 3.7 mg FA/g dried extract).

Moreover, the chemical hydrolysis was more effective in the extraction of FA compared to the enzymatic one for each extraction method. In particular, in the case of yellow and white maize germ, the BP extract has 461.89 ± 8.70 mg FA/g extract which increases to 636.53 ± 3.71 mg FA/g extract when associating ultrasounds to the chemical hydrolysis (BP+); in the case of white maize germ, the quantity of FA of BP extract, 522.99 ± 8.38 mg FA/g dried extract, increases to 569.23 ± 1.69 mg FA/g dried extract with the BP+ extraction. It can be noted that in all cases the plant material' sonication had a positive effect on the release of FA. This difference was detected mainly in the extracts obtained with chemical hydrolysis, while a minimal difference was encountered in the enzymatic extracts where the FA content, considerably lower than the BP and BP + extracts, has undergone a slight increase if extracted from pre-sonicated material. A higher content of FA was obtained with 24 h of enzymatic hydrolysis that, although it is more effective than the two other enzymatic treatment, still has a lower FA content than chemical hydrolysis. Even among the enzymatic extracts, both yellow and white maize germs had a larger quantity of FA compared to the other matrices.

A general trend in the extraction efficacy of the techniques used with respect to the ferulic acid content is shown in the graph 5 where it is easy to see how the alkaline hydrolysis associated with ultrasound is, for all the matrices, the most valid technique for obtaining a higher yield of the molecule. The trend of quantification reports also higher quantities of FA in the maize germs compared to wheat aleurone and rice bran.



Graph 5. Ferulic acid extraction efficacy with chemical and enzymatic hydrolysis

The quantification of *p*-coumaric acid is reported in the same table (17). A significant content of the molecule has been quantified only in the alkaline hydrolyzed extracts and in rice and maize germs 24h enzymatic extraction. Among all CBM, rice bran exhibited the highest content of *p*-CA (92.2 ± 0.1 mg *p*-CA /g BP+ extract), followed by yellow (81.9 ± 0.7 mg *p*-CA /g BP+ extract) and white (47.6 ± 0.9 mg *p*-CA /g BP+ extract) maize germs. In general, 180 min of enzymatic extraction, Enz-1 and Enz-2, were not enough to extract *p*-CA, whose quantity was often below the limit of quantification (LOQ).

Moreover, the sonication seemed to effectively contribute to the extraction of a greater quantity of the molecule also in the case of *p*-CA: all CBM, in fact, showed the highest content with the BP + extraction.

CBM	**Extracts**		**mg FA/g extract**	**mg *p*-CA/ g extract**
Wheat bran aleurone	Chemical hydrolysis	BP	386.2 ± 10.4	1.5 ± 0.1
		BP+	406.1 ± 0.7	1.1 ± 0,0
	Enzymatic hydrolysis	Enz 1	2.6 ± 0.2	*
		Enz 2	1.2 ± 0.1	2.3 ± 0.1
		Enz 3	3.5 ± 0.2	*
Rice bran	Chemical hydrolysis	BP	369.1 ± 4.9	82.9 ± 1.0
		BP+	387.4 ± 1.4	92.2 ± 0.1
	Enzymatic hydrolysis	Enz 1	8.2 ± 0.1	*
		Enz 2	10.6 ± 0.1	*
		Enz 3	140.0 ± 0.6	22.1 ± 0.0
Yellow corn germ	Chemical hydrolysis	BP	461.9 ± 8.7	48.5 ± 1.2
		BP+	636.5 ± 3,7	81.9 ± 0.7

	Enzymatic hydrolysis	Enz 1	9.9	±	0.2	1.6	± 0.0
		Enz 2	15.9	±	0.0	*	
		Enz 3	39.2	±	0.3	20.0	± 0.3
White corn germ	Chemical hydrolysis	BP	523.0	±	8.4	47.6	± 0.9
		BP+	569.2	±	1.7	47.2	± 0.1
	Enzymatic hydrolysis	Enz 1	10.3	±	0.1	0.7	± 0.0
		Enz 2	21.9	±	0.1	*	
		Enz 3	129.6	±	1.7	27.6	± 0.3

*Value < of Limit of Quantification (LOQ)

Table 17. RP-HPLC-DAD quantification of ferulic and p-coumaric acids in chemical and enzymatic hydrolyzed extracts

7.8.3 Determination of total phenolic content (TPC)

The total phenolic contents of the phenolic extracts, quantified with the Folin Ciocalteu' s spectrophotometric method, are reported in table 18. As expected, free phenolic extracts contained the lowest concentration of phenolic molecules; among them, wheat aleurone and rice brans exhibited the highest free TPC (80.90 ± 8.60 mg GAE/g dried extract and 79.02 ± 2.20 mg GAE/g dried extract respectively) since their extraction procedure followed optimized literature methods.

Each plant matrix extracted with alkaline hydrolysis exhibited a higher TPC compared with the other techniques. Corn germs, in particular the yellow variety, obtained the highest phenolic concentration in the extracts, followed by white corn germ, wheat aleurone and rice bran. Comparing the results of the two types of alkaline hydrolysis (BP and BP +), an increase in the phenolic quantity in the extracts occurred by associating the ultrasounds with the chemical reaction. The influence of mechanical sound waves, thus, affects the release of biomolecules despite the reduction in extraction time (30 min instead of 60 min). Yellow corn germ extracted with this method showed the best result with a TPC of 844.46 ± 64.60 mg GAE/g dried extract, which is a much greater quantity compared to the result obtained with the common alkaline hydrolysis technique 528.62 ± 18.40 mg GAE/g dried extract. Even white corn germ exhibited a high phenolic content which was 569.23 ± 1.69 mg GAE/g dried extract for BP+ and 522.99 ± 8.38 mg GAE/g dried extract. The TPC results of the enzymatic hydrolysis are decidedly lower than the chemical hydrolysis but among the three selected techniques, the 24h enzymatic treatment (Enz-3) exhibited the highest results, confirming to be the most effective of the three enzymatic times. The

highest Enz-3 TPC results is that of rice bran with a TPC of 349.9 ± 6.89 mg GAE/g dried extract.

CBM	Extraction technique	Extracts	TPC (mg GAE/g dried extract)
Wheat bran aleurone	UAE	FP	80.9 ± 8.6
	Chemical hydrolysis	BP	437.58 ± 9.9
		BP+	610.49 ± 57.6
	Enzymatic hydrolysis	Enz 1	149.31 ± 0.31
		Enz 2	158.79 ± 0.76
		Enz 3	167.92 ± 8.81
Rice bran	UAE	FP	79.02 ± 2.2
	Chemical hydrolysis	BP	506.8 ± 2.6
		BP+	552.8 ± 5.5
	Enzymatic hydrolysis	Enz 1	177.13 ± 2.29
		Enz 2	164.91 ± 3.81
		Enz 3	349.90 ± 6.89
Yellow corn germ	UAE	FP	49.54 ± 0.38
	Chemical hydrolysis	BP	528.62 ± 18.4
		BP+	844.46 ± 64.6
	Enzymatic hydrolysis	Enz 1	188.9 ± 0.76
		Enz 2	183.1 ± 4.5
		Enz 3	213.45 ± 6.98
White corn germ	UAE	FP	42.84 ± 2.23
	Chemical hydrolysis	BP	708.41 ± 41.7
		BP+	742.8 ± 15.44
	Enzymatic hydrolysis	Enz 1	172.3 ± 1.53
		Enz 2	187.92 ± 0.76
		Enz 3	332.84 ± 3.44

Table 18. TPC quantification of all phenolic extracts

7.8.4 Determination of radical scavenging activity

The antiradical scavenging activity of the extracts was tested both bioautographically and spectrophotometrically with DPPH radical: these methods allowed us to identity the main molecules responsible for the antioxidant activity and obtain the IC_{50} values of the extracts. The HPTLC plates below (figure 51) are related to the bioautographic screening test for the evaluation of antiradical activity.

The results of FP extracts are shown in plate A: as reported, there are no visible molecules with a particularly strong antioxidant activity. Some spots of discoloration in correspondence of R_f 0.85 (wheat), R_f 0.9 (rice), R_f 0.45 (corn) can be noted, probably corresponding to phenolic molecules as reported in the HPTLC results.

On the other hand, plates B and C reports results of hydrolyzed extracts: they both show some spots with apparently high antiradical activity. In particular, alkaline hydrolyzed extracts of wheat bran shows the highest difference in activity between the BP and BP+ with a high spot of discoloration at R_f 0.5-0.55, corresponding to those of FA and *p*-CA. Other phenolics with lower R_f (between the range of 0-0.3) have shown a certain degree of discoloration.

In plate C the enzymatic extracts did not show a great difference in activity between each other: all extracts are characterized by an active molecule (FA) at R_f 0.55 and only maize germs showed another spot of discoloration around R_f 0.9 corresponding to a molecule not yet identified. FA and *p*-CA exhibited the highest radical scavenging activity and thus they can be identified as the most active molecules in each hydrolyzed extract.

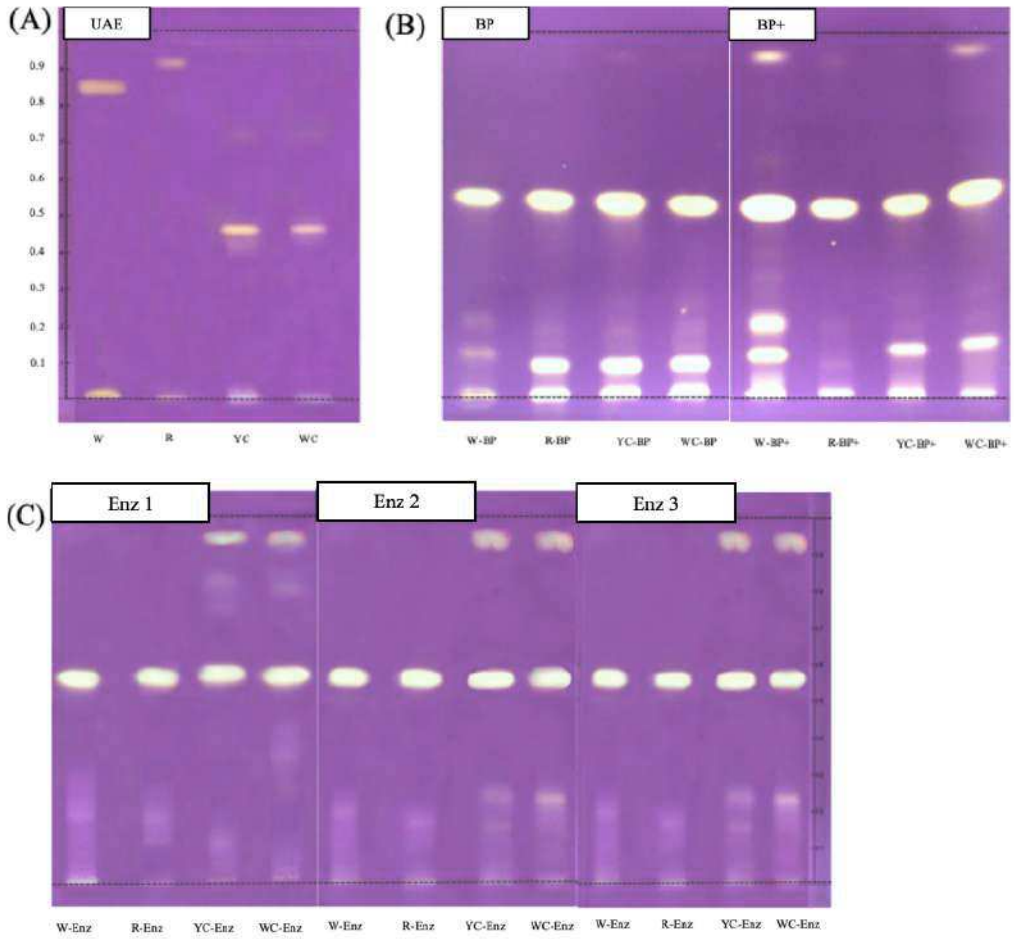


Figure 51. HPTLC bioautograms of FP extracts (A); alkaline hydrolyzed (B) and enzymatic (C). Detection at visible light after derivatization with DPPH. W- Wheat aleurone; R- Rice bran; YC- Yellow corn germ; WC- White corn germ; BP- alkaline hydrolysis; BP+ alkaline hydrolysis coupled with sonication; Enz – enzymatic hydrolysis.

The IC_{50} values of the radical scavenging activity of the extracts are reported in table 19, the FP extracts obtained the highest IC_{50} values, corresponding to a low antioxidant activity as initially predicted with the HPTLC bioautographic assay for their poor phenolic composition. Among FP extracts, rice bran gave the best results with an IC_{50} of 275.1 ± 13.79 µg/mL. In fact, rice bran FP extract has, with wheat aleurone, the richest free phenolic fraction (TPC of 79.02 ± 2.20 mg GAE/g dried extract) but this matrix is characterized by a greater lipophilic fraction compared to wheat bran (Jiang and Wang, 2005) which, during the UAE, is partially extracted with the hydroalcoholic solvent. Moreover, it is the food source with the highest phytosterol content, class of molecules that have demonstrated antiradical activity (Lerma-Garcia, 2009; Wang et al., 2002), and that could be partially responsible for the antioxidant activity of rice bran FP extract.

Among the hydrolyzed extracts, BP+ extracts gave the best antioxidant activity results confirming the effective contribution of ultrasound in the extraction process; the extracts obtained with enzymatic hydrolysis gave rather low activity values and this can be explained by the reduced amount of extracted ferulic acid. In fact, a higher concentration of FA, corresponds to a better antioxidant activity. The presence of CH=CH-COOH group in the hydroxycinnamic acids, such as FA, is considered as the main responsible for the higher antioxidant activity of this class of compounds compared to hydroxybenzoic acids which are characterized by the COOH (White & Xing, 1997).

Focusing the attention on the results obtained with the two methods of alkaline hydrolysis (BP and BP+), it can be noticed that the second type of extraction is more effective in extracting antioxidant molecules: in all cases, in fact, alkaline hydrolysis coupled with sonication, gave results up to ten times better than the common hydrolysis. In line with the results of HPTLC bioautographic assay, wheat bran aleurone showed the greatest difference between the two alkaline hydrolysis method: BP+ IC_{50} (3.61 ± 0.09 µg/mL) is ten times lower compared to the BP one (36.61 ± 0.65 µg/mL) and resulted the most active extract among all the cereal matrices. This result is comparable to that obtained with Trolox (3.45 ± 0.32 µg/mL) and is about twice the most active compared to the single ferulic acid standard (7.83 ± 0.26 µg/mL). The potential application of cereal antioxidant extracts is directly related to the content of ferulic acid. As previously described, FA has demonstrated various biological activities in correlation with its being radical scavenger: the antioxidant extracts can therefore be used as a source of FA with the potential to protect against UV A and B in formulations for topical use or as preservatives and stabilizers of natural origin.

CBM	**Extraction technique**	**Extracts**	**Antioxidant activity (IC_{50} µg/mL)**
Wheat bran aleurone	UAE	FP	1194.8 ± 44.93
	Chemical hydrolysis	BP	36.61 ± 0.65
		BP+	3.61 ± 0.09
	Enzymatic hydrolysis	Enz 1	363.2 ± 24.46
		Enz 2	373.1 ± 6.98
		Enz 3	388.8 ± 7.67
Rice bran	UAE	FP	275.1 ± 13.79
	Chemical hydrolysis	BP	55 ± 5.37
		BP+	38.01 ± 0.52

	Enzymatic hydrolysis	Enz 1	200.7	±	18.07
		Enz 2	332.6	±	8.78
		Enz 3	109.21	±	4.27
Yellow corn germ	UAE	FP	497.6	±	10.23
	Chemical hydrolysis	BP	29.19	±	2.85
		BP+	11.41	±	1.1
	Enzymatic hydrolysis	Enz 1	298.36	±	3.43
		Enz 2	199.9	±	17.27
		Enz 3	180.1	±	11.66
White corn germ	UAE	FP	680.2	±	25.52
	Chemical hydrolysis	BP	36.74	±	3.66
		BP+	14.33	±	0.48
	Enzymatic hydrolysis	Enz 1	148.4	±	2.18
		Enz 2	202.8	±	6.77
		Enz 3	102.74	±	0.86
Trolox			**3.45**	**±**	**0.32**
Ferulic acid			**7.83**	**±**	**0.26**

Table 19. Antioxidant activity of the extracts expressed as IC_{50} values (µg/mL)

7.8.5 Determination of antimicrobial activity

The antimicrobial activity tests, preceded by antimicrobial susceptibility tests, were used to hypothesize a further possible application of extracts, especially the most interesting ones, in the health sectors (e.g. phytocosmetic and phytotherapic).

The antimicrobial susceptibility test has been used to evaluate the susceptibility of the strains used against six antimicrobial agents commonly used to treat human infections with *S. aureus* and *S. epidermidis*. Figure 52 shows bacteria growth diameters for each bacterium, used to decide whether the bacterium was susceptible (S), intermediate (I) or resistant (R) to each antimicrobial agent.

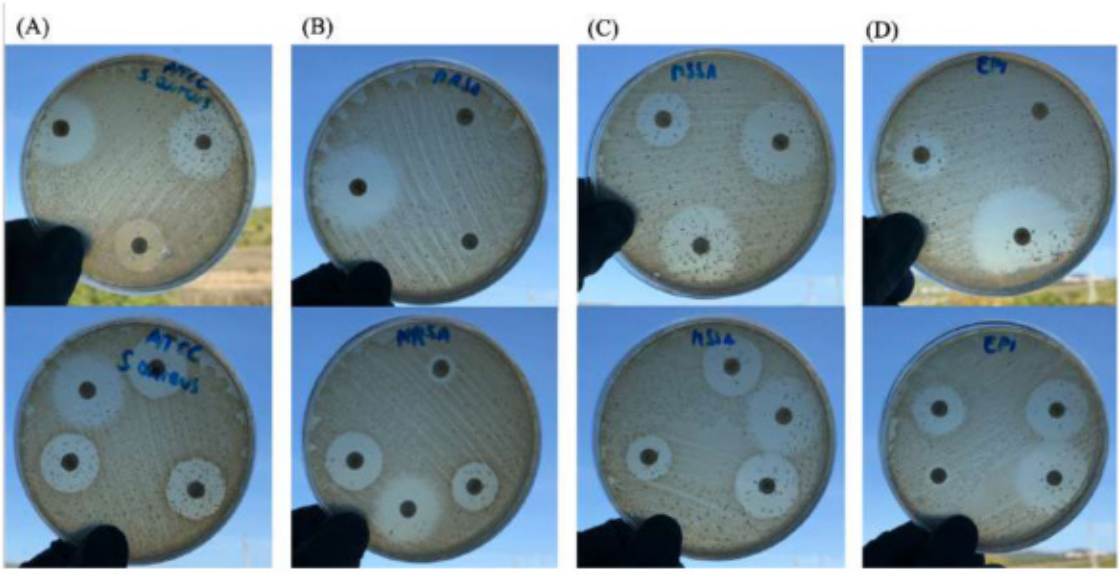


Figure 52. Bacteria *growth diameter of each bacterium evaluate with disk diffusion method. (A)*: S. aureus *ATCC; (B)*: S. aureus *MRSA; (C)*: S. aureus *MSSA; (D)*: S. epidermidis.

In table 20, results of the antimicrobial susceptibility tests are summarized for each antimicrobial agent: Ampicillin (AK30), Penicillin (P10), Chloramphenicol (C30), Kanamycin (K30), Ciprofloxacin (CIP 5), Doxycycline (DO30).

As reported, *S. aureus* MRSA and *S. epidermidis* demonstrated to be resistant to two or more antimicrobial agents. Every strain resulted resistant to Penicillin which is the antimicrobial agent used as positive control for the MICs calculations (CLSI, 2015).

Antimicrobial agent		***S. aureus* ATCC**	**S. aureus MRSA**	**S. aureus MSSA**	***S. epidermidis***
Ampicillin	AK 30	S	S	S	S
Penicillin	**P 10**	**R**	**R**	**R**	**R**
Chloramphenicol	C 30	S	S	S	S
Kanamycin	K 30	S	R	S	R
Ciprofloxacin	CIP 5	S	R	S	I
Doxycycline	DO 30	S	S	S	S

Table 20. Results of the antimicrobial susceptibility test. (S: susceptible; I: intermediate; R: resistant)

The Minimum Inhibitory Concentration (MIC) was evaluated by testing the phenolic extracts that demonstrated the most interesting and promising characteristics with the previous analyses: BP and BP+ extracts.

Table 21 shows results regarding the antimicrobial activity of the extracts tested on the three *S. aureus* strains and the *S. epidermidis* clinical isolated. The most interesting results were obtained with wheat bran aleurone and rice bran extracts which exhibited the lowest MICs with both BP and BP+: in particular rice and wheat extracts were active against the MRSA strain with a MIC of 16 µg/mL and 32 µg/mL respectively. In this case no difference was encountered in the use of sonicated extracts. The contribution of ultrasounds was instead effective in the case of the ATCC strain: BP+ extracts of wheat bran aleurone (128 µg/mL) and rice bran (128 µg/mL) showed greater activity than BP extracts (respectively 521 µg/mL and 256 µg/mL). The same result has been obtained comparing BP (128 µg/mL) and BP+ (256 µg/mL) wheat extract on MSSA clinical isolated strain.

Extracts tested on *S. epidermidis* showed a mild activity with high MIC values compared to the other results obtained (≥ 2048 µg/ml) and therefore not interesting from the application point of view.

Finally, according to CLSI guidelines (CLSI, 2015), all strains showed to be resistant to penicillin with MIC values above 0.25 µg/mL, as previously verified by the antimicrobial susceptibility test.

CBM	**Extract**	**MIC µg/mL**			
		S. aureus			***S. epidermidis***
		ATCC	**MRSA**	**MSSA**	
Wheat bran aleurone	BP	512	32	256	2048
	BP+	128	32	128	2048
Rice bran	BP	256	16	128	2048
	BP+	128	16	128	2048
Yellow corn germ	BP	128	512	128	≥ 2048
	BP+	128	512	128	2048
White corn germ	BP	128	512	128	≥ 2048
	BP+	128	512	128	≥ 2048
Penicillin		0.5	1	≥ 4	≥ 4

Table 21. Minimum Inhibitory Concentration (MIC) of alkaline hydrolyzed extracts

Our results on *S. aureus*, and particularly on the Methicillin-resistant (MRSA) strain, are interesting for their potential important role in cosmetic or in dermo-functional applications both as active ingredients and as natural preservatives. The interest lies on the resistant

nature of the strain to certain antibiotics and also on the growing importance of ingredients of natural origin in cosmetic formulations.

7.9 Target 2: lipophilic compounds and USP fraction

7.9.1 Yields and qualitative results

The same by-products matrices of durum wheat, rice bran and corn germs, have been considered for their considerable quantity of lipophilic compounds and in particular for their unsaponifiable fraction. The extraction method we have chosen for the extraction of these molecules (SC-CO_2) has proved to be particularly advantageous both for the extractive yields obtained and for the rapidity of its execution.

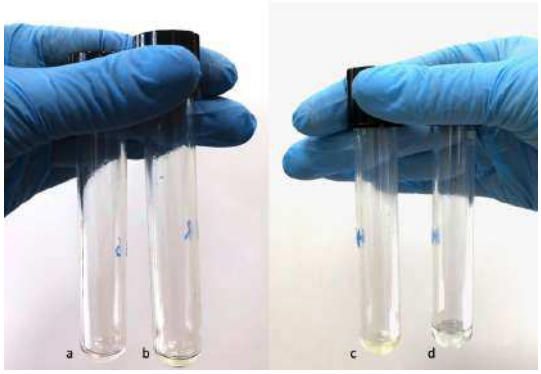


*Figure 53. SFE extracts (*a*: SFE wheat aleurone;* b*: SFE rice bran;* c*: yellow corn SFE;* d*: white corn SFE) (photo credit: Ilaria Burlini)*

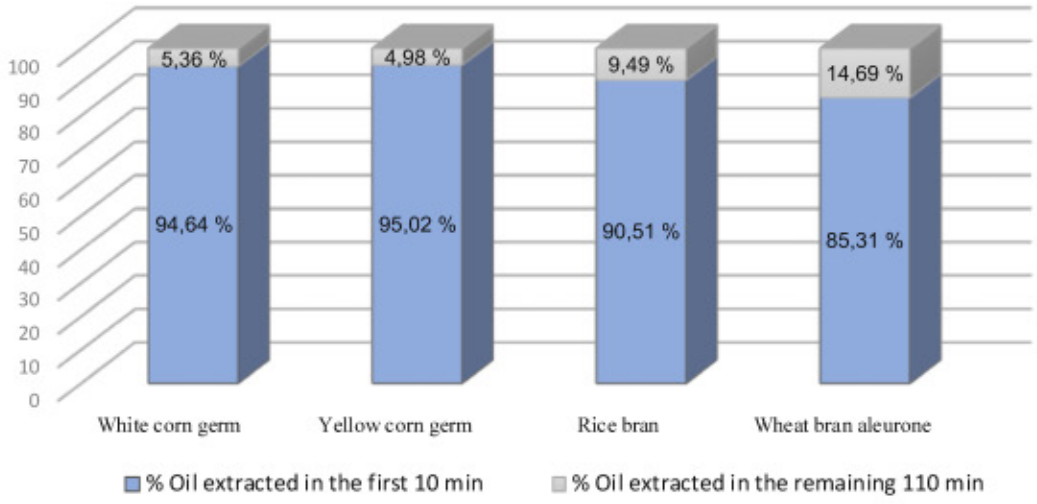
SFE extracts were obtained after 10 min and 120 min of extraction, following the two methods described in literature. In the table 22 the yields results are reported: yellow corn germ obtained the highest yields (19.98 ± 1.24 % and 20.10 ± 1.45 % with 10 and 120 min respectively). These results are in line with expectations and literature data: in fact, yellow corn germ is very rich in fixed oils which are used commercially for the production of vegetable oils; white corn white maize is instead used preferably for the production of gluten-free flour than for its lipophilic content, even if it has achieved high yield values.

CBM	Time (min)	SFE % yields
Wheat bran aleurone	10	4.55 ± 0.15
	120	5.33 ± 0.49
Rice bran	10	12.49 ± 0.77

Yellow corn germ	120	12.86 ± 0.89
	10	19.98 ± 1.24
	120	20.10 ± 1.45
White corn germ	10	13.91 ± 1.46
	120	15.70 ± 0.98

Table 22. SFE extraction yields expressed in percentages on the dry weight of CBM

Comparing the two method of extractions (10 min and 120 min), no significant increase in % yield has been found which could justify such different extraction times. As summarized in the graphic 6, in fact, between the 85% and the 95% of the total oil (extractable from CBM with this SFE method), is extracted in the first 10 min of extraction. Thus, SFE extracts obtained with only 10 min of extraction, have been chosen for the successive analyses. The extraction of cereal oils in such a short time also has several advantages from the point of view of the environmental impact of the extraction method that allows us to reduce the working times and the energy consumption.



Graph 6. Percentages of SFE yields extracted in 10 min and 120 min

Figure 54 shows HPTLC plates of SFE extracts obtained after 10 min (A) and 120 min (B) of extraction using SC-CO_2. This method has been performed in order to do a screening analysis and to discriminate the extraction technique and parameters used, together with the yields obtained. Since the two extraction methods described in literature differed greatly for the extraction time, after verifying that the extraction yields did not differ significantly, we went to qualitatively evaluate the phytocomplex. From the HPTLC chromatogram we

can say that there is no difference in the qualitative profile of SFE extracts fixed oils comparing the two extraction times. For this reason and for the yields obtained we decided to prefer the extract obtained with 10 min of extraction, thus enhancing the potential for energy and time savings that can have sustainable repercussions in the scale-up process.

According to CAMAG (2014), results of the HPTLC of fixed oils have to be compared to those of United States Pharmacopoeia (USP) references to check the quality of the phytocomplexes obtained; USP only reports the chromatograms related to various commercial fixed oils and among these is present the corn germ oil. As rice and wheat oil are not used commercially in the same way, they are not comparable with the USP standards and for this reason the HPTLC technique used has exclusively served as an internal qualitative screening to verify the extraction efficacy.

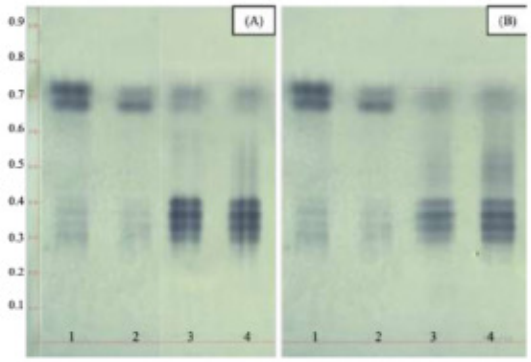


Figure 54. Detection of fixed oils in SFE extracts. (A) HPTLC chromatogram of 10' extracts at VIS light. (B) HPTLC chromatogram of 120' extracts at VIS light Tracks: 1-Wheat bran aleurone; 2-Rice bran; 3-Yellow corn germ; 4-White corn germ.

7.9.2 Determination of USP composition

In order to determine the most interesting lipophilic fraction among the available waste matrices, we have quantified the unsaponifiable (USP) fractions of the obtained extracts.

The determination of the total USP fraction has been evaluated on SFE extracts by calculating the % yields obtained after cold saponification and silanization. As reported in table 23, the highest total USP fraction has been obtained in rice bran extract (6.08 ± 0.04 %), followed by wheat aleurone (4.29 ± 0.42 %), yellow corn and white corn germs (respectively 2.41 ± 0.24 % and 1.85 ± 0.08 %).

Corn USP matter values are in line with those of literature which are around 2% (Majoni &Wang, 2010); rice bran oil USP value resulted higher than literature results, usually between 2.6 and 4.5% (Gopala Krishna et al., 2003) and wheat bran aleurone USP is

higher than that reported in literature which is 2.8% (Kumar & Krishna 2013). The high percentages of USP obtained through this extractive method could be interesting for a future application of the extracts: in fact, adding USP isolated from wheat germ and corn was found to retard oxidation in vegetable oils subjected to heating (Sims, Fioriti, & Kanuk, 1972).

Extract	Unsaponifiable value %
Wheat bran aleurone	4.29 ± 0.42
Rice bran	6.08 ± 0.04
Yellow corn germ	2.41 ± 0.24
White corn germ	1.85 ± 0.08

Table 23. Total unsaponifiable % fractions in SFE extracts

Each USP fraction has been then analyzed by GC-MS in order to characterize the main compounds as shown in following figure 55 and in particular phytosterols and triterpene alcohols.

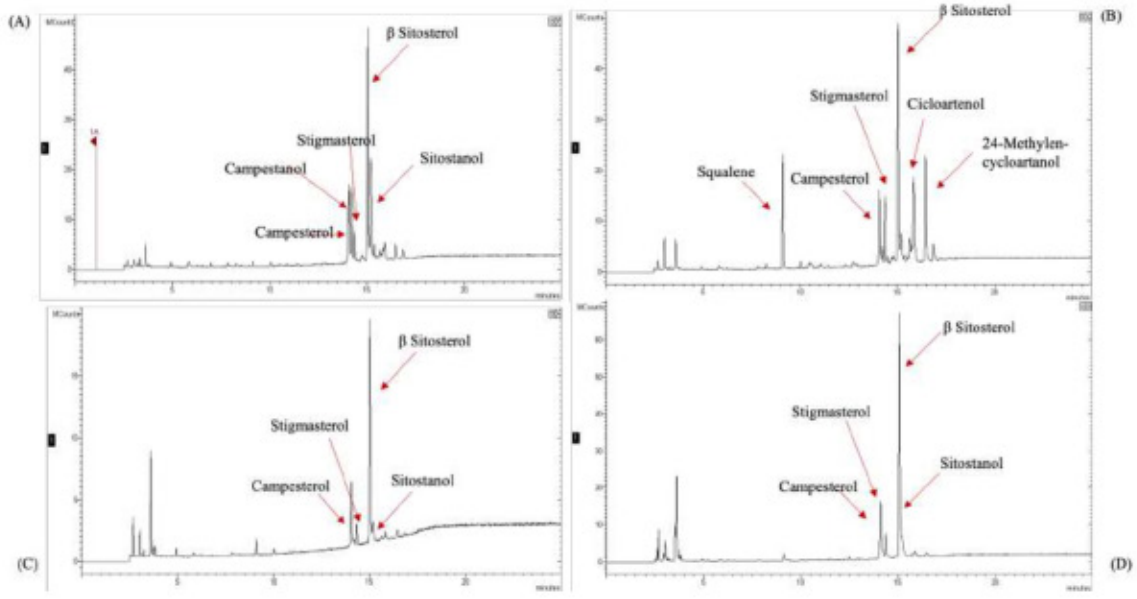


Figure 55. GC-MS SFE extracts: (A) wheat bran aleurone; (B) rice bran; (C) yellow corn germ; (D) white corn germ

The major ions mass spectra and retention times (RT) of the identified compounds are reported in table 24 for each by-product.

Sterols and triterpene alcohols	RT	Major ions in mass spectra
		Wheat bran aleurone
Campesterol	14.126	472 (50), 457 (16), 382 (88), **343 (100)**, 261 (14), 255 (19), 213 (12), 129 (85)
Campestanol	14.264	474 (55), 459 (74), 417 (22), 384 (28), 369 (49), 306 (31), **215 (100)**, 75 (98)
Stigmasterol	14.390	484 (61), 394 (60), 379 (22), 355 (29), 255 (63), 159 (33), 129 (87), **83 (100)**
Δ7-Campesterol	14.830	**472 (100)**, 457 (25), 386 (15), 367 (15), 255 (75), 229 (18), 213 (28), 75 (39)
β-Sitosterol	15.094	486 (54), 471 (17), 396 (97), 381 (43), **357 (100)**, 255 (19), 129 (76)
Sitostanol	15.232	488 (60), 473 (69), 431 (21), 398 (28), 383 (50), 357 (27), 305 (30), 257 (12), **215 (100)**, 147 (30), 75 (97)
Cicloartenol	15.884	498 (3), 483 (10), **408 (100)**, 393 (99), 365 (86), 339 (45), 69 (79)
24-Methylen-cycloartanol	16.500	512 (1), 497 (10), **422 (100)**, 407 (93), 379 (100), 353 (29), 300 (17)
Citrostadienol	16.890	498 (5), 483 (8), 400 (58), 393 (6), **357 (100)**
		Rice bran
Squalene	9.117	410, 341, 149, 136, 81, 69, 41
Campesterol	14.126	472 (50), 457 (16), 382 (88), **343 (100)**, 261 (14), 255 (19), 213 (12), 129 (85)
Campestanol	14.264	474 (55), 459 (74), 417 (22), 384 (28), 369 (49), 306 (31), **215 (100)**, 75 (98)
Stigmasterol	14.390	484 (61), 394 (60), 379 (22), 355 (29), 255 (63), 159 (33), 129 (87), **83 (100)**
Δ7-Campesterol	14.830	**472 (100)**, 457 (25), 386 (15), 367 (15), 255 (75), 229 (18), 213 (28), 75 (39)
β-Sitosterol	15.094	486 (54), 471 (17), 396 (97), 381 (43), **357 (100)**, 255 (19), 129 (76)
Sitostanol	15.232	488 (60), 473 (69), 431 (21), 398 (28), 383 (50), 357 (27), 305 (30), 257 (12), **215 (100)**, 147 (30), 75 (97)
Δ7- Avenasterolo	15,347	484 (3), 386 (48), 371 (7), **343 (100)**, 255 (10), 253 (14), 213 (13), 75 (25)
Cicloartenol	15.884	498 (3), 483 (10), **408 (100)**, 393 (99), 365 (86), 339 (45), 69 (79)
24-Methylen-cycloartanol	16.500	512 (1), 497 (10), **422 (100)**, 407 (93), 379 (100), 353 (29), 300 (17)
Citrostadienol	16.890	498 (5), 483 (8), 400 (58), 393 (6), **357 (100)**
		Yellow and white con germs
Campesterol	14.126	472 (50), 457 (16), 382 (88), **343 (100)**, 261 (14), 255 (19), 213 (12), 129 (85)
Campestanol	14.264	474 (55), 459 (74), 417 (22), 384 (28), 369 (49), 306 (31), **215 (100)**, 75 (98)
Stigmasterol	14.390	484 (61), 394 (60), 379 (22), 355 (29), 255 (63), 159 (33), 129 (87), **83 (100)**

β-Sitosterol	15.094	486 (54), 471 (17), 396 (97), 381 (43), **357 (100)**, 255 (19), 129 (76)
Sitostanol	15.232	488 (60), 473 (69), 431 (21), 398 (28), 383 (50), 357 (27), 305 (30), 257 (12), **215 (100)**, 147 (30), 75 (97)
Cicloartenol	15.884	498 (3), 483 (10), **408 (100)**, 393 (99), 365 (86), 339 (45), 69 (79)
24-Methylen-cycloartanol	16.500	512 (1), 497 (10), **422 (100)**, 407 (93), 379 (100), 353 (29), 300 (17)

Table 24. Major ions mass spectra and RT obtained with GC-MS

The following table (25) reports the results of the GC-MS quantification expressed in percentage of SFE silanized extracts.

Compound	**Wheat bran aleurone**	**Rice bran**	**Yellow corn germ**	**White corn germ**
Squalene	nd	11.59 ± 0.33	nd	nd
Campesterol	13.37 ± 0.36	10.75 ± 0.67	20.98 ± 0.26	19.3 ± 0.13
Campestanol	13.72 ± 0.37	3.02 ± 0.79	2.04 ± 0.58	2.48 ± 0.25
Stigmasterol	7.19 ± 0.19	8.36 ± 0.19	6.21 ± 0.06	6.99 ± 0.12
Δ7-Campesterol	0.51 ± 0.01	0.37 ± 0,04	nd	nd
β-Sitosterol	35.83 ± 0.96	30.46 ± 0.45	60.72 ± 0.49	59.5 ± 0.38
Sitostanol	22.28 ± 0.59	5.48 ± 0.54	7.79 ± 0.16	7.87 ± 0.13
Δ7 Avenasterol	nd	1.06 ± 0.01	nd	nd
Cicloartenol	1.41 ± 0.04	13.32 ± 0.03	1.19 ± 0.64	1.85 ± 0.12
24-Methylen-cycloartanol	3.19 ± 0.09	13.15 ± 0.14	1.08 ± 0.27	2.02 ± 0.12
Citrostadienol	0.65 ± 0.02	2.41 ± 0.13	nd	nd

Table 25. Major compounds in SFE extracts and their composition %

β-Sitosterol resulted the major compound of the unsaponifiable fraction of all SFE extracts: in particular it is between 30.46 ± 0.45 % and 35.86 ± 0.96 % of the total components in the wheat bran aleurone and rice bran extracts, while it is around 60% in both extracts of corn germs (60.72 ± 0.49% in the yellow variety and 59.5 ± 0.38 % in the white variety). This difference in percentage allows us to highlight a greater number of components that characterize the lipophilic phytocomplex of the first two extracts (wheat bran aleurone and rice bran). The lipophilic maize extracts, on the other hand, are characterized by a limited

number of molecules which are then distributed in greater quantities over 100% of the USP fraction.

Wheat bran aleurone is characterize by a high percentage of sitostanol 22.98 ± 0.59 %, followed by campestanol (13.72 ± 0.7 %), campesterol (13.37 ± 0.36 %), and stigmasterol (7.19 ± 0.19 %); rice bran is characterized by a number of molecules which are present in the extract in a range from 8-14%: cicloartenol (13.32 ± 0.03 %), 24-methylen-cycloartanol (13.15 ± 0.14 %), squalene (11.59 ± 0.33%), campesterol (10.75 ± 0.67 %), and stigmasterol (13.7 ± 0.37 %). Campesterol is the second most quantitatively present component in corn germs extracts 20.98 ± 0.26 % in the yellow variety and 19.3 ± 0.13 % in the white variety) followed by sitostanol and stigmasterol present in a range from 6 to 8%.

7.9.3 Determination of radical scavenging activity

Finally, even the lipophilic extracts were tested for their antiradical activity both through the bioautographic screening test and through the spectrophotometric one, both with the DPPH radical.

The HPTLC bioautogram reported below (figure 56) shows the DPPH bioautographic screening test results for the evaluation of antiradical activity of SFE extracts. As it can be weakly seen, there are few spots at R_f 0.65-0.66 (wheat and rice extracts) and at R_f 0.3-0.35 (yellow and white corn extracts) with a discoloration which apparently corresponds to a low antioxidant activity if compared to the previously presented HPTLC bioautographic results of BP extracts. The reason why there is such a difference in the visible activity lies in the range of activity reference that is commonly used to define whether an extract or a single compound is more or less active: for lipophilic extracts and compounds the refence standard used in literature is EVOO whose IC_{50} has a unit of measure of the order of mg/mL instead of µg/mL used for phenolics. For this reason, the results of this test do not give data comparable to the previous ones (phenolic extracts), but rather provide us with useful indications to determine the chemical components responsible for the activity subsequently quantified spectrophotometrically.

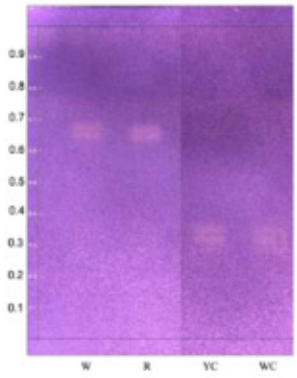


Figure 56. HPTLC bioautograms. Chromatograms of SFE extracts. Detection at visible light after derivatization with DPPH radical. Tracks: W- Wheat bran aleurone SFE extract; R- Rice bran SFE extract; YC- Yellow corn germ SFE extract; WC- White corn germ SFE extract.

In the table 26 are reported the IC_{50} results obtained for lipophilic extracts (SFE) using the spectrophotometric method described by Radice et al. (2014).

All the extracts showed a good antioxidant activity if compared with the controls: particularly yellow and white corn germ extracts obtained the lowest IC_{50} (1.29 ± 0.26 mg/mL and 1.33 ± 0.21 mg/mL) which are even better results compared to those of EVOO controls (5.89 ± 0.04 and 11.00 ± 0.06 mg/mL); wheat bran aleurone extract obtained a IC_{50} of 7.25 ± 0.66 mg/mL which is slightly higher to the tested control, while the rice bran extract obtained the highest IC_{50}, which corresponds to the lowest radical scavenging activity, but still comparable with the one of the literature control (11.00 ± 0.06 mg/mL).

CBM	**IC_{50} mg/mL**
Wheat bran aleurone	7.25 ± 0.66
Rice bran	11.17 ± 0.25
Yellow corn germ	1.29 ± 0.26
White corn germ	1.33 ± 0.21
EVOO (COOP)	5.89 ± 0.04
EVOO[6]	11.00 ± 0.06

Table 26. Radical scavenging activity (IC_{50}) of SFE extracts and controls

[6] Literature data (Valavanidis et al., 2004)

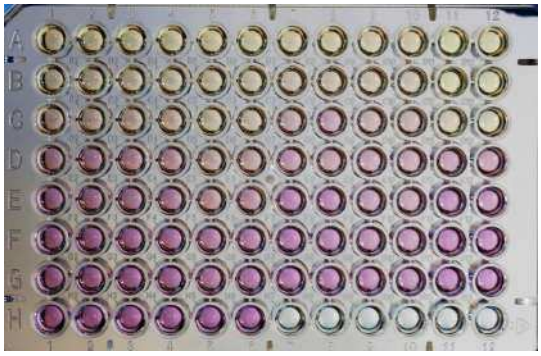


Figure 57. Microplate with DPPH radical scavenging activity

7.10 Cosmetic application results

The following results refer to the cosmetic formulations prepared with two selected extracts. The wheat extract BP + has revealed a good application potential as an antioxidant, while its preparation must be stabilized and improved. The SFE extract has not shown antioxidant activity with PCL but could be applied in cosmetic formulations with many other uses. INCI maize oil can be found as “Oil: Zea Mays Oil”, “Germs oil: Zea Mays Germ Oil” and “Unsaponifiables: Zea Mays Oil Unsaponifiables”, and it is used in cosmetic products as emollient since it demonstrated a good skin penetration and as emulsifier. Maize germ oil is, in fact, rich in fatty acids, especially linoleic acid and oleic acids, palmitic and stearic acids, which are renowned for their moisturizing qualities.

7.10.1 Formulations

In order to propose a practical application of the obtained extracts, in particular of those that have shown the most interesting results at qualitative-quantitative and bioactivity levels, four cosmetic preparations have been formulated (O/W emulsions); the antioxidant activity of both chosen extracts and final products by PCL and the stability at ambient temperature (22-23°C) and at 40°C have been evaluated.

Starting from a common base cream formulation protocol, in collaboration with Ambrosialab Srl (www.ambrosialab.it), two formulations were prepared with different percentages of extracts as described before.

The obtain cosmetic products are shown in figure 55. The inclusion of the lipophilic SFE extract with a straw yellow color, both 2.5% and 5%, did not alter the color of the O/W preparation. Instead the high quantity of ferulic acid contained in the phenolic extract BP

+, characterized by a deep yellow color, caused a slight alteration of the color of the final preparation, in particular in the 0.5% concentration. A slight color variation does not affect the pleasantness of the finished product; it is, in fact, an indicative characteristic of the presence of natural extracts and any color variations are not indication of a depletion of the product but enhance its naturalness. The fragrances of all finished product are characteristic and pleasant.

Figure 58. O/W formulations with wheat bran BP+ extract (A) and with SFE extract from yellow maize germ (B)

7.10.2 PCL results of antioxidant activity

Below, in table 27, the PCL values of antioxidant capacity of the samples are reported. The antioxidant activity values obtained are expressed expressed as µmoL equivalent of Trolox per gram of sample.

PCL TEST

Sample		**µmol Trolox/g**
1	Wheat bran BP+ extract	1728.2 ± 21.76
2	O/W F1 1 (0.3%)	4.65 ± 0.00025
3	O/W F1 1 (0.5%)	7.89 ± 0.3
4	Yellow maize germ SFE extract	no activity
5	O/W F2 (2.5%)	not tested
6	O/W F2 (5%)	not tested

Table 27. PCL results

The higher the value expressed in µmol TE/ gram the greater is the activity. From the results we can say that the extract wheat bran BP + is very powerful (1728.2 ± 21.76 µmol

Trolox/g) compared to other vegetable extracts (Vertuani et al. 2002) but inside the final product, it performs in proportion to the concentration. Having tested for reasons of extract availability and timings low extracts percentages, the formulation activity is therefore not very high. In order to obtain a very active product even compared to many cosmetic products on the market, it would be enough to increase the concentration of the extract to 1.5% thus obtaining a proportional activity around 20 µmol TE/ gram which is generally considered high.

Since yellow maize germ did not exhibit any antioxidant activity even at high concentrations, it was considered unnecessary to continue with the PCL analysis of lipophilic finished products that would have proved to be also inactive.

7.10.3 Stability results

The stability of the cosmetic formulations was tested at one month both at room temperature and at 40 ° C (accelerated stability).

Formulation 1 (0.3%) ambient temperature:

Color: slightly lightened

Parfum: pleasant and unchanged

Phase modification or separation: not occured

pH: 4.4

Formulation 1 (0.5%) ambient temperature:

Color: slightly lightened

Parfum: Parfum: pleasant and unchanged

Phase modification or separation: not occured

pH: 3.84

Formulation 2 (2.5%) ambient temperature:

Color: white

Parfum: unchanged

Phase modification or separation: not occured

pH: 5.0

Formulation 2 (5%) ambient temperature:

Color: white ivory

Parfum: unchanged

Phase modification or separation: not occured

pH: 5.14

Formulation 1 (0.3%) 40°C:

Color: pale straw yellow

Parfum: unchanged

Phase modification or separation: slight surfacing of the oily phase

pH: 5.5

Formulation 1 (0.5%) 40°C:

Color: slightly darkened, honey-colored

Parfum: unchanged

Phase modification or separation: slight surfacing of the oily phase

pH: 5.5

Formulation 2 (2.5%) 40°C:

Color: white ivory

Parfum: unchanged

Phase modification or separation: not occured

pH: 4.51

Formulation 2 (5%) 40°C:

Color: white ivory

Parfum: unchanged

Phase modification or separation: not occured

pH: 4.46

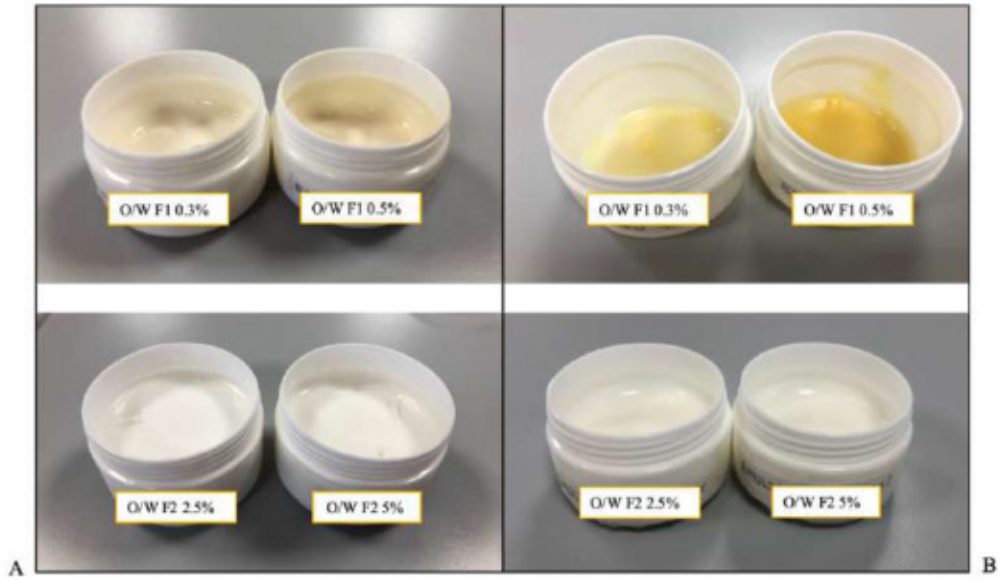


Figure 59. Stability results of F1 and F2 after 1 month at ambient temperature (A) and at 40°C (B) (Photo credit: Ilaria Burlini)

Emulsions containing the wheat phenolic extract (F 1) showed excellent stability at room temperature while a slight alteration occurred at 40° C, with a slight surfacing of the oily phase, probably due to the reduced quantity of product taken for the investigation. Even if the results will be published later, we are repeating accelerated stability analyses with a larger quantity of product.

Emulsions containing the lipophilic SFE extract (F2), on the other hand, have all shown excellent stability both at room temperature and at 40° C.

Furthermore, in all cases there has been a slight reduction in the pH which is in any case within the suggested limits for cosmetic formulations (pH: 3.5-6).

The pH of cosmetic preparations can range from a minimum of 2 for peeling products to over 12 for depilatory products: for this reason there are no restrictive guidelines for the pH of facial cosmetic formulations, even if the pH of the face is 5-5.5, the skin implements regulatory mechanisms that make products usable even with lower or higher pH.

8 Conclusions

The present PhD project, financed by Emilia Romagna region (D.R. 996/2016 Prot. 57324 of 24-6-16), was aimed to valorize cereal by-products studying the most effective extraction processes and finding possible applications in the health and cosmetic industries. The Circular Economy Action Plan represent one of the possible opportunities of the food supply chain to face the issues of food wastes and global demographic growth. The recovery of food residues is receiving increased attention because of the growing awareness of the benefits deriving from potentially marketable components of food wastes which might be recycled and exploit as high added-value products or raw materials for industrial applications. Among cereals, durum wheat, rice and maize represent the three main global productions. The high numbers of production translate into high numbers of wastes: cereals, in fact, are subjected to milling technologies which remove brans and germs from the caryopsis. The residual by-products are still rich in biomolecules such as phenolic acids and lipophilic compounds.

Two research targets have been set for the present project, considering the type of extraction product (bran and germ) and their main phytochemicals: phenolic acids (*trans*-ferulic acid in particular) and unsaponifiable compounds.

Free and bound phenolics have been extracted with ultrasound-assisted extraction (UAE), alkaline hydrolysis (BP), alkaline hydrolysis coupled with sonication (BP+) and enzymatic hydrolysis (Enz-1, Enz-2 Enz-3) testing the contribution of ultrasounds on the extraction processes. All extraction performed have been chosen in order to maximize the extraction yields and with the aim of minimizing the environmental impact compared to other conventional methodologies. Phenolic extracts have been analyzed for their qualitative composition with HPTLC and RP-HPLC-DAD and subsequently they have been quantitative analyzed in terms of total phenolic and phenolic acids contents. The radical scavenging activity of the extracts have been investigated both with HPTLC bioautographic assay and spectrophotometrically with DPPH radical. Finally, the antimicrobial activity of the most performing extracts was evaluated at the Department of Animal Health of the University of Cordoba (UCO) in Spain. The antimicrobial susceptibility test was performed on various antimicrobial agents, including Penicillin, and extracts have been tested against

three strains of *Staphylococcus aureus* and one clinical isolated of *Staphylococcus epidermidis* to calculate their minimum inhibitory concentration (MIC).

Unsaponifiable compounds, the second extraction target, were extracted using supercritical carbon dioxide (SFE), following and comparing two literature methods. Extracts have been analysed prior with HPTLC as screening for the extraction efficacy and afterwards with GC-MS to investigate the unsaponifiable content and percentage composition of the silanized extracts. The radical scavenging activity has been also assessed for the lipophilic extracts both bioautographically and spectrophotometrically with DPPH.

Target I

Among the various techniques used for the phenolic extraction, the chemical hydrolyses resulted more effective than the enzymatic one. Particularly, the most promising extracts were obtained by associating sonication with chemical hydrolysis. Despite the reduction of extraction time from 60 to 30 min, this technique allowed to obtain phenolic enriched extracts with enhanced quality compared to the conventionally used methods. The influence of mechanical sound waves, thus, affected the release of biomolecules, causing the acoustic cavitation effect on the plant matrix and permitting a greater penetration of the solvents into the sample.

Comparing the yields obtained, the highest results were those of free phenolic extracts thanks to the use an hydroalcoholic solvent together and to the sonication effects. Among them, rice bran showed the highest result (19.97 ± 1.45%) and both corn germs the lowest ones (12.6 ± 0.8% and 12.4 ± 0.89 % respectively). However high yields did not correspond to a rich phytocomplex as demonstrated by HPTLC analyses. In fact, through ultrasound-assisted extraction (UAE) it was not possible to extract ferulic acid (FA), the main phenolic molecule characterizing cereal matrices.

For what concern bound phenolic extracts, corn germ obtained the highest extraction yields, probably because of the high amounts of lipophilic compounds extracted during the hydrolysis. A phenolic separation step was required to remove the lipophilic fraction from maize bound phenolics extracts. Because of the high selectivity of bound phenolics extractions the yield results were lower than UAE: these yields are valued for the high concentration of biomolecules that characterize the extracts. By comparing the chemical

hydrolysis used we found an increase in percentage yield by coupling sonication to alkaline hydrolysis. The highest enzymatic yields were obtained after 24 h of extraction (Enz 3), with yields of almost 2% in the case of yellow corn (1.92 ± 0.08%) and durum wheat (1.83 ± 0.15%).

From the HPTLC analysis of bound phenolics FA was detected in all extracts and appeared to be the most concentrated phenolic molecule. The presence of *p*-coumaric acid was more visible at 254 nm than at 356 nm due to its dark blue color, but its identification has been subsequently confirmed with HPLC. HPTLC was also useful for the choice of enzymatic extraction parameters: a 1% Feruloyl esterase (FAZCT) and 10 U of Xylanase enzymes demonstrated to be the best enzymatic blend for the release of FA from cereal matrices obtained after 180 min and 24 h of extraction and from previously sonicated materials: extractive efficacy was demonstrated by the presence of FA.

The presence of FA was confirmed with RP-HPLC-DAD in all hydrolyzed extracts, but quantitative results changed drastically depending on the extractive technique. For this reason, it was not possible to defined whether more molecule was contained in durum wheat, rice or maize by-products but it appeared to characterize particularly maize by-products. In fact, three out of four extraction methods reported white maize germ as richest in FA, mainly with BP+ (569.2 ± 1.7 mg FA/g dried extract), but the highest content was obtained with BP+ extraction from yellow maize germ (636.5 ± 3.7 mg FA/g dried extract). In all cases the plant material' sonication had a positive effect on the release of the molecule. This difference was detected mainly in the extracts obtained with chemical hydrolysis, while a minimal difference encountered in the enzymatic extracts where the FA content, considerably lower than the BP and BP + extracts, has undergone a slight increase if extracted from pre-sonicated material. The association of ultrasounds to alkaline hydrolysis from yellow maize germ showed the highest FA content which was of 461.89 ± 8.70 mg FA/g extract BP extract and increased to 636.53 ± 3.71 mg FA/g with the BP+. Among enzymatic extracts, the highest content of FA was obtained with 24 h of enzymatic hydrolysis that, although it was more effective than the two other enzymatic treatment, still had a lower FA content than chemical hydrolysis.

A significant content of *p*-coumaric acid (*p*-Ca) was quantified only in the alkaline hydrolyzed extracts and in rice and maize germs 24h enzymatic extraction. The sonication seemed to effectively contribute to the extraction of a greater quantity of the molecule also

in this case. Among all matrices, rice bran exhibited the highest content of *p*-CA (92.2 ± 0.1 mg p-Ca/g BP+ extract), followed by yellow (81.9 ± 0.7 mg *p*-Ca /g BP+ extract) and white (47.6 ± 0.9 mg *p*-Ca /g BP+ extract) maize germs. In general, 180 min of enzymatic treatment (Enz-1 and Enz-2) were not enough to extract *p*-CA, whose quantity was often below the limit of quantification (LOQ).

In addition, the total phenolic content (TPC) of the extracts was quantified with the spectrophotometric assay Folin-Ciocalteu. As expected from the previous results, free phenolic extracts contained the lowest TPC; among them, wheat aleurone (80.90 ± 8.60 mg GAE/g dried extract) and rice brans (79.02 ± 2.20 mg GAE/g dried extract) exhibited the highest free TPC. Each plant matrix extracted with chemical hydrolysis exhibited a higher TPC compared with the other techniques. Comparing the two alkaline hydrolysis methods, an increase in the TPC was obtained by associating sonication. Yellow corn BP+ extract showed the highest result with a TPC of 844.46 ± 64.60 mg GAE/g dried extract, compared to 528.62 ± 18.40 mg GAE/g dried extract of BP one. Also white corn BP+ extract exhibited a higher phenolic content (569.23 ± 1.69 mg GAE/g dried extract) compared to the BP extract (522.99 ± 8.38 mg GAE/g dried extract). The TPC results of the enzymatic hydrolysis were decidedly lower than the chemical hydrolysis but among the three selected techniques, the 24h enzymatic treatment (Enz-3) exhibited the highest results, confirming to be the best of the three enzymatic times.

The radical scavenging activity of the extracts was tested both bioautographically and spectrophotometrically with DPPH radical. From the HPTLC bioautographic assay free phenolic extracts did not show molecules with a particularly strong antioxidant activity, this was later established with the spectrophotometric assay which showed a poor antioxidant activity of the extracts. Among FP extracts, rice bran gave the best results with an IC_{50} of 275.1 ± 13.79 µg/mL.

HPTLC bioautograms of hydrolyzed extracts showed some spots with apparently high antiradical activity. The IC_{50} of BP+ extracts gave the best antioxidant activity results confirming the effective contribution of ultrasounds in the extraction process; the extracts obtained with enzymatic hydrolysis gave rather low activity values and this can be explained by the reduced amount of extracted ferulic acid. Alkaline hydrolysis coupled with sonication showed resulted up to ten times higher than the common hydrolysis. In line with the results of HPTLC bioautographic assay, wheat bran aleurone showed the greatest

difference between the two alkaline hydrolysis method: BP+ IC_{50} (3.61 ± 0.09 µg/mL) is ten times lower compared to the BP one (36.61 ± 0.65 µg/mL) and resulted the most active extract among all the cereal matrices. This result was comparable to that obtained with Trolox (3.45 ± 0.32 µg/mL) and was about twice the most active compared to the single ferulic acid standard (7.83 ± 0.26 µg/mL).

From the antimicrobial susceptibility tests, *S. aureus* MRSA and *S. epidermidis* demonstrated to be resistant to two or more antimicrobial agents. Every strain resulted resistant to Penicillin, the positive control. The most interesting results obtained with the Minimum Inhibitory Concentration (MIC) calculation have been those of wheat bran and rice bran extracts which exhibited the lowest MICs with both BP and BP+. In particular, rice and wheat extracts were active against the MRSA strain with a MIC of 16 µg/mL and 32 µg/mL respectively. In this case no difference was encountered in the use of sonicated extracts. The contribution of ultrasounds has been instead effective in the case of the ATCC strain: BP+ extracts of wheat bran aleurone (128 µg/mL) and rice bran (128 µg/mL) showed greater activity than BP extracts (respectively 521 µg/mL and 256 µg/mL). The same result was obtained comparing BP (128 µg/mL) and BP+ (256 µg/mL) wheat extract on MSSA clinical isolated strain.

Extracts tested on *S. epidermidis* showed a mild activity with high MIC values compared to the other results obtained (≥ 2048 µg/ml) and therefore not interesting from the application point of view.

Target II

The second extraction target was the lipophilic fraction of cereal by-product matrices. Yellow maize germ obtained the highest yields in percentage (19.98 ± 1.24 % and 20.10 ± 1.45 % with 10 and 120 min respectively). Comparing the two method of extractions no significant yield increase was found which could justify such different extraction times: in fact, between the 85% and the 95% of the total oil was extracted within the first 10 min in all plant matrices. HPTLC was used to screen the obtained extracts from a qualitative point of view and to compare the extracts: the HPTLC chromatogram did not show a significant difference in the qualitative profile of SFE extracts fixed oils comparing the two extraction times. For this reason and for the yields obtained we selected the extracts obtained in 10 min for the following analyses; the choice was made also with the aim of enhancing the

potential for energy and time savings which could have sustainable repercussions in the scale-up process.

The highest unsaponifiable fraction was obtained in rice bran extract (6.08 ± 0.04 %), followed by wheat aleurone (4.29 ± 0.42 %), yellow corn (2.41 ± 0.24 %) and white corn germs (1.85 ± 0.08 %). Corn unsaponifiable matter values are in line with those of literature which are around 2% while rice bran oil and wheat bran's ones are higher than that reported in literature.

From the GC-MS qualitative determination, β-Sitosterol resulted the major compound of the unsaponifiable fraction of all extracts: its presence was between the 30 and 36 % of the total components in the wheat and rice bran extracts and around 60% in both corn extracts. Wheat bran was also characterized by a high percentage of sitostanol, followed by campestanol, campesterol and stigmasterol; rice bran was characterized by a number of molecules which are present in the extract in a range from 8-14%: cycloartenol, 24-methylen-cycloartanol, squalene, campesterol and stigmasterol. Campesterol was the second most quantitatively present component in corn germs extracts followed by sitostanol and stigmasterol present in a range from 6 to 8%.

The antioxidant activity was also evaluated for lipophilic extracts both with the bioautographic screening and with the spectrophotometric tests with DPPH radical.

The HPTLC bioautograms showed few spots of discoloration which apparently corresponds to a low antioxidant activity if compared to the HPTLC bioautographic results of BP extracts. All the extracts showed a good antioxidant activity if compared with extra virgin olive oils (EVOO) used as controls: particularly yellow and white corn germ extracts obtained the lowest IC_{50} (1.29 ± 0.26 mg/mL and 1.33 ± 0.21 mg/mL) which were even better results compared to those of EVOO controls (5.89 ± 0.04 and 11.00 ± 0.06 mg/mL); wheat bran extract obtained a IC_{50} of 7.25 ± 0.66 mg/mL which is slightly higher to the tested control, while the rice bran extract showed the highest IC_{50}, which corresponded to the lowest radical scavenging activity, but still comparable with the one of the literature control (11.00 ± 0.06 mg/mL).

Finally, a cosmetic application has been hypothesized thanks to the work of collaboration of Ambrosialab Srl, where an extract for each target has been formulated in a cosmetic product to propose a concrete application of the obtained extracts. The choice of the extracts to be included in the cosmetic formulations was guided by the previous results, taking into

account yields, biomolecules content, antimicrobial activity and, above all, antioxidant activity results. Thus, wheat bran BP+ extract and yellow maize germ SFE extracts were selected for several reasons. The former demonstrated a low oil content (no separation step required), high content of TPC (610.49 ± 57.6 mg GAE/g dried extract), high FA content (406.1 ± 0.7 mg FA/g dried extract) an interesting antimicrobial activity against *S. aureus* ATCC and MSSA (128 µg/mL) and MRSA (32 µg/mL) and finally the highest antioxidant activity (3.61 ± 0.09 µg/mL). The latter demonstrated the highest yield obtained in only 10 min of extraction (19.98 ± 1.24 %) and the highest antioxidant activity (1.29 ± 0.26 µg/mL).

All the cosmetic preparations obtained have shown good organoleptic characteristics. From the results of the PCL analysis a high value of antioxidant activity of the durum wheat BP+ extract was found (1728.2 ± 21.76 µmol Trolox/g), which is therefore confirmed to be an excellent active potential to be included in formulations with antioxidant purposes. However, to obtain a truly powerful product with an excellent market perspective, the concentration of the extract should be increased from 0.5% to 1.5%. Given the high amount of ferulic acid, the BP + extract could also be used in dermal-functional products as an alternative to pure ferulic acid generally obtained from synthesis or with common mining techniques less advantageous than that presented with the project.
The wheat extract BP + has revealed a good application potential as an antioxidant, while its preparation must be stabilized and improved. The SFE extract has not shown antioxidant activity with PCL but could be applied in cosmetic formulations as emollient or as emulsifier thanks to its high content of fatty acids, linoleic acid in particular.

In this project we applied a more effective, faster and consequently sustainable extraction method than those conventionally used in the literature for the extraction of cereals biomolecules. We have hypothesized a cosmetic application with extracts used as active ingredients, however another interesting possibility of use could be to insert them as excipients (stabilizers, emulsifiers or preservatives of natural derivation) given the demonstrated properties both in literature and in the project. Another interesting possibility of future application could be to insert cereal by-products extracts in food preparations (pasta, bread, etc.) with enhanced characteristics (functional foods).

9 Bibliography

1. Abbott, A.P., Capper, G., Davies, D.L., Rasheed, R.K., Tambyrajah, V., (2003). Novel solvent properties of choline chloride/urea mixtures. Chemical Communication 1, 70–71
2. Acosta-Estrada B, Gutiérrez-Uribe J.A, Serna-Saldívar S.O. (2014). Bound phenolics in foods, a review. Food Chem. 152:46-55.
3. Adebooye, O.C., Alashi A. M., Aluko, R.E. (2018). A brief review on emerging trends in global polyphenol research. Journal of Food Biochemistry, e12519.
4. Anderson J.W., (2003). Whole grains protect against atherosclerotic cardiovascular disease. Procedeengs of the Nutrition Society 62, 135-142
5. Andrewes, P., Busch J.L., de Joode T., Groenewegen, A., Alexandre, H., (2003). Sensory Properties of Virgin Olive Oil Polyphenols: Identification of Deacetoxy-ligstroside Aglycon as a Key Contributor to Pungency. Journal of Agricultural and Food Chemistry 26, 51(5), 1415-20
6. Australian Government, Department of Health, Office of gene Technology Regulator. (2005). The Biology and Ecology of Rice (*Oryza sativa* L.) in Australia.
7. Awika, J.M, (2011). Advance in Cereal Science: Implication to Food Processing and Health Promotion. Chapter 1: Major Cereal Grains Production and Use around the World. American Chemical Society: Washington, DC.
8. Azuma, K., Ippoushi, K., Nakayam, M., Ito, H., Higashio, H., Terao, J. (2000). Adsorption of chlorogenic acid and caffeic acid in rats after oral administration. Journal of Agricultural & Food Chemistry 48, 5496–5500
9. Balachandran, S., Kentish, S.E., Mawson, R., Ashokkumar, M., (2006). Ultrasonic enhancement of the supercritical extraction from ginger. Ultrasonics Sonochemistry 13, 471–479
10. Barberousse, H., Roiseux, O., Robert, C., Paquot, M., Deroanne C. anD Blecker, C., (2008). Analytical methodologies for quantification of ferulic acid and its oligomers. Review. Journal of the Science of Food and 88, 1494–1511
11. Beerh, O.P., Raghuramaiah, B., Krishnamurthy, G. V. and Giridhar, N., (1976) Utilization of mango waste: recovery of juice from waste pulp and peel. Journal of Food Science and Technology (Mysore), 13(23), 138-141

12. Benz, B.F., (2005). Archeological evidence of teosinte domestication from Guilà Naquitz, Oaxaca. Proceedings of the National Academy of Sciences. National Academy of Sciences of the United States of America. 98 (4), 2104–2106
13. Bergman, C.J. and Xu, Z. (2003). Genotype and environment effects on tocopherols, tocotrienols and gamma-oryzanol contents of Southern US rice. Cereal Chemistry 80(4), 446- 449
14. Biology Document BIO2006-07: A companion document to Directive 94-08 (Dir94-08), Assessment Criteria for Determining Environmental Safety of Plant with Novel Traits
15. Blois, M.S. (1958). Antioxidant determinations by the use of a stable free radical. Nature, 29, 1199-1200
16. Borges A, Ferreira C, Saavedra MJ, Simoes M. (2013). Antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria. Microbial Drug Resistence, 19, 256–265
17. Boussetta N., Vorobiev, E., Deloison V., Pochez F., Falcimaigne-Cordin A., Lanoisellé J.-L. (2011). Valorisation of grape pomace by the extraction of phenolic antioxidants: Application of high voltage electrical discharges. Food Chemistry 128, 364–370
18. Braddock, R.J., Crandal, P.G. (1978). Properties and recovery of waste liquids from citrus pectin pomace manufacture. Journal of Food Science, 43 (6) 1678-1679
19. Buanafina, M.M de O. (2009). Feruloylation in Grasses: Current and Future Perspectives. Review article. Molecular Plant 1(5), 861–872
20. Camag (2014) HPTLC Identification of Fatty Oils (Fixed Oils). APPLICATION NOTES Camag laboratory, F-39
21. Campos, F.M., Couto, J.A., Figueiredo, A.R., Toth, I.V., Rangel, A.O.S.S., Hogg, T.A. (2009). Cell membrane damage induced by phenolic acids on wine lactic acid bacteria. International Journal of Food Microbiology; 135, 144–151
22. Capella, P., Fedeli, E., Bonaga, G., Lercker, G. (1997). Manuale degli Oli e dei Grassi. Ed. Tecniche nuove, Milano
23. Cesario, M.T., Raposo, M.S., De Almeida, MC., Van Keulen, F., Ferreira, BS., de Fonseca, MM. (2014). Enhanced bioproduction of poly-3-hydroxybutyrate from wheat straw lignocellulosic hydrolysates. New Biotechnology 25;31(1), 104-13

24. Chemat, F., Abert Vian, M., Cravotto, G. (2012). Green Extraction of Natural Products: Concept and Principles. International Journal of Molecular Sciences 13, 8615-8627
25. Chemat, F., Rombaut, N., Sicaire, A.G., Meullemiestre, A., Fabiano-Tixier, A.S., Abert-Vian, M. (2017). Ultrasound-assisted extraction of food and natural products. Mechanisms, techniques, combinations, protocols and applications. A review. Ultrasonics Sonochemistry 34, 540–560
26. Cheng Z, Moore J, Yu LL. (2006). High-Throughput Relative DPPH Radical Scavenging Capacity Assay. Journal of Agricultural and Food Chemistry, 54, 7429−7436
27. Clemént, G., (2013). Giardini, paesaggio e genio naturale. Quodlibet srl (Macerata), 19-21
28. Clinical and Laboratory Standards Institute (CLSI), (2015). Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement, M100-S25. Wayne, PA, 35 (3)
29. Cordell G.A., (2017). Cognate and cognitive ecopharmacognosy — in an anthropogenic era. Phytochemistry Letters 20, 540–549
30. Cravotto, G., Binello, A. (2010). Innovative techniques and equipments for flavours extraction. Application and effectiveness of ultrasound and microwaves. Household and Personal Care Today 1, 32–34
31. Cravotto, G., Boffa, L., Mantegna, S., Perego, P., Avogadro, M., Cintas, P., (2008). Improved extraction of vegetable oils under high-intensity ultrasound and/or microwaves. Ultrasonics Sonochemistry 15, 898–902
32. Cvjetko Bubalo, M., Vidovic, S., Radojcˇic´ Redovnikovic, I., Jokic, S. (2018). New perspective in extraction of plant biologically active compounds by green solvents. Food and Bioproducts Processing 109, 5–73
33. De Campos, L.M.A.S., Leimann, F.V., Curi Pedrosa, R., Ferreira, S.R.S. (2008). Free radical scavenging of grape pomace extracts from Cabernet sauvingnon (*Vitis vinifera*). Bioresource Technology 99, 8413–8420
34. Devappa, R.K., Rakshit, S.K., Dekker R.F.H. (2015). Forest biorefinery: Potential of poplar phytochemicals as value-added co-products. Biotechnology Advances 33, 681–716

35. Djenni, Z., Pingret, D., Mason, T.J., Chemat, F. (2012). Sono–Soxhlet: in situ ultrasound-assisted extraction of food products. Food Analytical Methods 6, 1229–1233
36. Eitenmiller, R.R. and Lee, J. (2004). Vitamin E, Food Chemistry, Composition, and Analysis, 540. Marcel Dekker Inc., New York, NY
37. Ensminger, A. H. (1994). Foods and Nutrition Encyclopedia, 2nd ed. CRC Press., 479
38. Erkman, S. (1997). Industrial Ecology: an historical view. Journal of Cleaner Production, 5 (1–2), 1-10
39. European Commission (2014). Preparatory Study on Food Waste across EU 27
40. European Commission (2015). Report from the Commission to the European Parliament, the Council, the European Economic and social Committee and the Committee of the Regions, "Closing the loop – An EU action plan for the Circular Economy". Brussels
41. European Commission (2017). Report from the Commission to the European Parliament, the Council, the European Economic and social Committee and the Committee of the Regions, "On implementation to the Circular Economy Action Plan", Brussels
42. European Commission (2019). Report from the Commission to the European Parliament, the Council, the European Economic and social Committee and the Committee of the Regions, "On implementation to the Circular Economy Action Plan", Brussels
43. Fang, N.B., Yu, S.G. and Badger, T.M. (2003). Characterization of triterpene alcohol and sterol ferulates in rice bran using LC–MS/MS. Journal of Agricultural & Food Chemistry 51, 3260-3267
44. FAO (2011). Global food losses and food waste - Extend, causes and prevention. Rome
45. FAO (2014). Food Wastage Foodprint - Full-cost Accounting 79
46. FAO, (2014). Maize production in 2014, Crops/Regions/Production Quantity from pick lists

47. Fardet, A., Rock, E., & Remesy, C. (2008). Is the *in vitro* antioxidant potential of whole-grain cereals and cereal products well reflected *in vivo*? Journal of Cereal Science 48, 258–276
48. Faulds, C. B., Williamson G. (1995). Release of ferulic acid from wheat bran by a ferulic acid esterase (FAE-III) from *Aspergillus niger*. Journal of Applied Microbiology and Biotechnology, 43, 1082-1087
49. Ferrer, A., Altabella, T., Arrò, M., Boronat, A. (2017). Emerging roles for conjugated sterols in plants. Progress in Lipid Research, 76, 27-37
50. Food sustainability index, Barilla Center for Food and Nutrition (2013)
51. Fusions (2016). Estimates of European food waste levels. Stockholm
52. Galanakis M.C. (2015), Food Waste Recovery: Processing Technologies and Industrial Techniques. Elsevier Inc.
53. Galanakis, C. (2012). Recovery of high added-value components from food wastes: Conventional, emerging technologies and commercialized applications. Trends in Food Science & Technology 26, 68-87
54. Gani A, Wani SM, Masoodi FA, Hameed G. (2012). Whole-Grain Cereal Bioactive Compounds and Their Health Benefits: A Review. Journal of Food Processings & Technology 3, 146
55. Gelmez, N., Kıncal, N.S., Yener, M.E. (2009). Optimization of supercritical carbon dioxide extraction of antioxidants from roasted wheat germ based on yield, total phenolic and tocopherol contents, and antioxidant activities of the extracts. Journal of Supercritical Fluids 48, 217–224
56. Gonzales, GB., Smagghe, G., Raes, K., Van Camp, J. (2014). Combined alkaline hydrolysis and ultrasound-assisted extraction for the release of nonextractable phenolics from cauliflower (*Brassica oleracea* var. *botrytis*) waste. Journal of Agricultural and Food Chemistry, 16, 62(15), 3371-6
57. Gopala Krishna, A.G., Prashanth, P.A., Pragasam, A., Raghavendra, K.V., Khatoon, S. (2003). Unsaponifiable matter and oxidative stability of commercially produced Indian rice bran oils. Journal of Food Lipids 10(4), 329-340
58. Graf, E. (1992). Antioxidant potential of ferulic acid: Review. Free Radical Biology and Medicine 13(4), 435-448

59. Handler, M.Z., Goldberg D.J. (2018). Cosmetic Concerns Among Men. Dermatologic Clinics, 36, 5-10
60. Heinze, K. (2017). Doctoral thesis: From Grain to Granule: The Biomechanics of Wheat Grain Fractionation with a Focus on the Role of Starch Granules
61. Hitchcock, A.S. and A. Chase. (1971). Manual of the grasses of the United States 2, 790-796. Dover Publications: N.Y.
62. Huerta, B., Maldonado, A., Ginel, P.J., Tarradas, C., Gomez-Gascon, L., Astorga, R.J., Luque, I. (2011). Risk factors associated with the antimicrobial resistance of staphylococci in canine pyoderma. Veterinary Microbiology, 150, 302–308
63. IAI (Istituto Affari Internazionali), (2017). D. Fattibene and M. Bianchi. Fighting against Food Losses and Waste: An EU Agenda, IAI working papers 17
64. Indira, T. N., Narayan, A. V., Barhate, R. S., Raghavarao, K. S., Khatoon, S., Channaiah, G., Council of Scientific and Industrial Research, assignee (2004). Production of oryzanol-enriched fraction useful in the treatment of melanin related disorders and for minimizing wrinkles involves saponification; dehydration and leaching of RBO soapstock. Patents Nos. US2004192948-A1, US6896911-B2 & JP2004300034-A
65. Islam, M. S., Murata, T., Fujisawa, M., Nagasaka, R., Ushio, H., Bari, A. M. (2008). Anti-inflammatory effects of phytosteryl ferulates in colitis induced by dextran sulphate sodium in mice. British Journal of Pharmacology, 154, 812–824
66. Jarudilokkul, S., Rungphetcharat, K., & Boonamnuayvitaya, V. (2004). Protein separation by colloidal gas aphrons using nonionic surfactant. Separation and Purification Technology, 35, 23-29
67. Jiang, Y. and Wang, T. (2005). Phytosterols in Cereal By-products. Journal of the Americal Oil Chemists'Society, 82 (6), 439-444
68. Joyner, S.J. (1985). Master thesis. La Trobe University, Melbourne
69. Juliano, C., Cossu, M., Alamanni, M. C., & Piu, L. (2005). Antioxidant activity of c-oryzanol: Mechanism of action and its effect on oxidative stability of pharmaceutical oils. International Journal of Pharmaceutics 299, 146–154
70. Kaufmann, B. and Christen, P. (2002). Recent Extraction Techniques for Natural Products: Microwave-assisted Extraction and Pressurised Solvent Extraction. Phytochemical analysis 13, 105–115

71. Kawabata, K., Yamamoto, T., Hara, A., Shimizu, M., Yamada, Y., Matsunaga, K., Tanaka, T., and Mori, H. (2000). Modifying effects of ferulic acid on azoxymethane induced colon carcinogenesis in F344 rats. Cancer Letters 157, 15–21
72. Kedare S.B., Singh R.P. (2011). Genesis and development of DPPH method of antioxidant assay. Journal of Food Science & Technology, 48, 412–422
73. Kennedy, G. and Burlingame, B. (2003). Analytical, nutritional and clinical methods analysis of food composition data on rice from a plant genetic resources perspective. Food Chemistry 80, 589-596
74. Kent, N.L. (1966). Technology of cereals with special reference to wheat. The Commonwheath and International Library of Science Technology Engineering and Liberal Studies. Pergamon Press Ltd.
75. Ki Lin C.S., Pfaltzgraff, L.A., Herrero-Davila, L., Mubofu, E.B., Abderrahim, S., Clark, J.K., Koutinas, A.A., Kopsahelis, N., Stamatelatou, N., Dickson, F., Thankappan, S., Mohamed, Z., Brocklesby, R. and Luque, R. (2013). Energy & Environmental Science 6, 426-464
76. Kiso, Y., Suzuki, Y., Watanabe, N., Oshima, Y., and Hikino, H. (1983). Validity of the oriental medicines. Part 53. Liver protective drugs, Part 8, Antihepatotoxic principles of Curcuma longa rhizomes. Planta Medica. 49(3): 185–187
77. Koshihara, Y., Neichi, T., Murota, S.I., Lao, A.N., Fujimoto, Y., Tatsuno, T. (1984). Caffeic acid is a selective inhibitor for leukotriene biosynthesis. Biochimica & Biophysica Acta 792, 92–97
78. Kumar, B., Pathak, R., B. Mary, D. Jha, K. Sardana and Gautam H.K. (2016). New insights into acne pathogenesis: Exploring the role of acne-associated microbial populations. Dermatologica Sinica 34, 67-73
79. Kumar, G. S. & Krishna, A.G.G. (2013). Studies on the nutraceutical composition of wheat derived oils wheat bran oil and wheat germ oil. Journal of Food Science and Technology, 52(2), 1145–1151.
80. Kumar, N. & Pruthi, V. (2014). Potential applications of ferulic acid from natural sources. Review. Biotechnology Reports, 4, 86-93
81. Kwon, K.T., Uddin, M.d.S., Jung, G-W., Sim, J-E., Chun, B-S. (2010). Supercritical Carbon Dioxide Extraction of Phenolics and Tocopherols Enriched Oil from Wheat Bran. International Journal of Nutrition and Food Engineering 4 (4), 188-193

82. Laufenberg, G., Kunz, B., Nystroem, M. (2003) Transformation of vegetable waste into value added products: (A) the upgrading concept; (B) practical implementations. Bioresource Technology 87, 167-198
83. Lee, M., Lee, D., Cho., J., Park C. (2013). Enzymatic Biodiesel Synthesis in Semi-Pilot Continuous Process in Near-Critical Carbon Dioxide. Applied Biochemistry and Biotechnology, 171(5)
84. Lerma-García, M.J., Herrero-Martínez, J.M., Simó-Alfonso, E.F., Mendonça R.B., Ramis-Ramos, G. (2009). Composition, industrial processing and applications of rice bran γ-oryzanol. Review. Food Chemistry 115(2), 15 389-404
85. Lin, F-H., Lin, J-Y., D. Gupta, R., Tournas, J.A Burch, J.A., Selim, M.A., Monteiro-Riviere, N.A., Grichnik, J.M., Zielinski, J. and PinnelL., S.R. (2005). Ferulic Acid Stabilizes a Solution of Vitamins C and E and Doubles its Photoprotection of Skin. The Journal of Investigative Dermatology, 125 (4), 826-832
86. Lo, H.H, Chung JG. (1999). The effects of plant phenolics, caffeic acid, chlorogenic acid and ferulic acid on arylamine N-acetyltransferase activities in human gastrointestinal micro-flora. Anticancer Research, 19, 133–139
87. Londo JP, Chiang YC, Hung KH, Chiang TY, Schaal BA. (2006). Phylogeography of Asian wild rice, *Oryza rufipogon*, reveals multiple independent domestications of cultivated rice, Oryza sativa. Proceedings of the Natural Academy of Science of U.S.A., 103 (25), 9578–83
88. Machmudah, S., Shiddiqi, QYA., Kharisma, AD., Widiyastuti, Wahyudiono, Kanda, H., Winardi, S. and Goto, M. (2014). Subcritical Water Extraction of Xanthone from Mangosteen (*Garcinia Mangostana* Linn) Pericarp. Journal of Advanced Chemical Engineering 5(1) 1000117
89. Majoni, S., Wang, T. (2010) Characterization of Oil Precipitate and Oil Extracted from Condensed Corn Distillers Solubles. Journal of the American Oil Chemists' Society 87, 205–213
90. Mathew, S. and Abraham, T.E. (2004). Ferulic Acid: An Antioxidant Found Naturally in Plant Cell Walls and Feruloyl Esterases Involved in its Release and Their Applications. Critical Reviews in Biotechnology 24(2–3), 59–83

91. Mierzejewski, J., Woźniak-Kosek, A. (2012). Microbes indicating the efficiency of cosmetics preservation. Part II. Staphylococcus aureus. Military Pharmacy and Medicine 3, 17-30
92. Nam, S.H., Choi, S.P., Kang, M.Y., Koh, H.J., Kozukue, N. and Friedman, B. (2005). Bran extracts from pigmented rice seeds inhibit tumor promotion in lymphoblastoid B cells by phorbol ester. Food & Chemical Toxicology 43, 741-745
93. Nastic, N., Švarc-Gajić, J., Delerue-Matos, C., Barroso., MS., Soares., C., Moreira, MM., Morais, S., Maškovic, P., Srček, VG., Slivac, I., Radoševic, K., Radojkovic, M. (2018). Subcritical water extraction as an environmentally-friendly technique to recover bioactive compounds from traditional Serbian medicinal plants. Industrial Crops & Products, 579–589
94. Naviglio, D. (2003). Naviglio's Principle and Presentation of an Innovative Solid–Liquid Extraction Technology: Extractor Naviglio. Analytical letters 36(8), 1647-1659
95. Ohara, K., Uchida, A., Nagasaka, R., Ushio, H. and Ohshima, T. (2009). The effects of hydroxycinnamic acid derivatives on adiponectin secretion. Phytomedicine 16, 130-7
96. Oliveira, D.A., Salvador, A.A., Smânia Jr, A., Smânia, E.S.A., Maraschin, M., Ferreira, S.R.S. (2013). Antimicrobial activity and composition profile of grape (*Vitis vinifera*) pomace extracts obtained by supercritical fluids. Journal of Biotechnology 164, 423–432
97. Oliveira, R., Oliveira, V., Kazue Aracava, K., da Costa Rodrigues C.E. (2012). Effects of the extraction conditions on the yield and composition of rice bran oil extracted with ethanol - A response surface approach. Food and Bioproducts Processing 90, 22-31
98. Origin, History and Uses of Corn. Iowa State University, Department of Agronomy. 2014
99. Papageorgiou, M. and Skendi, A. (2018). Sustainable Recovery and Reutilization of Cereal Processing By-Products. 1st Edition, Charles Galanakis, Woodhead Publishing, 1, 15

100. Peiqiang, Y., Maenz, DD, McKinnon, JJ., Racz, JJ and Christensen, DA. (2002). Release of Ferulic Acid from Oat Hulls by Aspergillus Ferulic Acid Esterase and Trichoderma Xylanase. Journal of Agricultural and Food Chemistry 50(6), 1625-30
101. Pignatti, S. (2017). Flora d'Italia. Edizione Agricole, 713
102. Pomeranz, Y. (1971). Wheat chemistry and technology, III. American association of cereal chemists, Incorporated (St. Paul, Minnesota)
103. Popov, I., Lewin, G. (1994). Photochemiluminescent detection of antiradical activity III: A simple assay of ascorbate in blood plasma. Journal of Biochemical and Biophysical Methods. 28, 277–282
104. Portal to the Flora of Italy. Available at http:/dryades.units.it/floritaly [Accessed: 04/10/2018]
105. Pruthi, J. S. and Parekh, C. M. and Girdhari, Lal. (1961) An integrated process for the recovery of essential oil and pectin from mandarin orange waste. Food Science 10, 372-378
106. Puri, M., Sharma, D., & Barrow, C. J. (2012). Enzyme-assisted extraction of bioactives from plants. Trends in Biotechnology 30(1), 37–44
107. Radice, M., Viafara, D., Neill, D., Asanza, M., Sacchetti, G., Guerrini, A., Maietti, S. 2014. Chemical Characterization and Antioxidant Activity of Amazonian (Ecuador) *Caryodendron orinocense.* Karst. and *Bactris gasipaes* Kunth Seed Oils. Journal of Oleo Science, 63, 12, 1243-1250
108. Ravindran, R., Jaiswal, A.K. (2016). Exploitation of Food Industry Wasta for High-Value Products. Review. Trends in Biotechnology 34 (1), 58-69
109. Rebollenda, S., Rubio, N., Beltràn, S., Sanz, M.T., Gonzales-Sanjosé, M.L. (2012). Supercritical fluid extraction of corn germ oil: Study of the influence of process parameters on the extraction yield and oil quality. Journal of Supercritical Fluids 72, 270-277
110. Revilla, E., Maria, C. S., Miramontes, E., Bautista, J., García-Martínez, A., Cremades, O., Cert, R. and Parrado, J. (2009). Nutraceutical composition, antioxidant activity and hypocholesterolemic effect of a water-soluble enzymatic extract from rice bran. Food Research International 42, 387–393
111. Rhodes, DI., Sadek, M., Stone, BA. (2002). Hydroxycinnamic Acids in Walls of Wheat Aleurone Cells. Journal of Cereal Science, 36(1), 67-81

112. Rice, A. C. (1976). Solid Waste Generation and By-Product Recovery Potential from Winery Residues. American Journal of Enology and Viticulture27, 21-26
113. Robbins, J.R., Bean, S.R. (2004). Development of a quantitative high-performance liquid chromatography-photodiode array detection measurement system for phenolic acids. Journal of Chromatography A 1038, 97–105
114. Rossi, D., Guerrini, A., Maietti, S., Bruni, R., Paganetto, G., Poli, F., Scalvenzi, L., Radice, M., Saro, K., Sacchetti, G. (2011). Chemical fingerprinting and bioactivity of Amazonian Ecuador *Croton lechleri* Müll. Arg. (Euphorbiaceae) stem bark essential oil: A new functional food ingredient? Food Chemistry 126, 837–848
115. Rueben, G.M. & Casa, I. (2003). Maize as a Food. In Encyclopedia of Food and Culture, Volume 2: Food Production to Nuts, 424-431
116. Rukmini, C., and Raghuram, T. C. (1991). Nutritional and biochemical aspects of the hypolipidemic action of rice bran oil: A review. Journal of American College Nutrition 10(6), 593–601
117. Saavedra, Y.M.B., Iritani, D. R., Pavan, A.L.R., & Ometto, A.R. (2018). Theoretical contribution of industrial ecology to circular economy. Journal of Cleaner Production, 170, 1514–1522
118. Saija, A., Tomaino, A., Trombetta, D., De Pasquale, A., Uccella, N., Barbuzzi T., Paolino D., Bonina, F. (2000). In vitro and in vivo evaluation of caffeic and ferulic acids as topical photoprotective agents. International Journal of Pharmaceutics, 199, 39-47
119. Sapkale, GN., Patil., SM., Surwasee US., Bhatbhage, PK. (2010). Supercritical fluid extraction – A Review. International Journal of Chemical Sciences, 8(2), 729-743
120. Schaller, H. (2003). The role of sterols in plant growth and development. Progress in Lipid Research, 42(3), 163-75
121. Sen, C.K., Khanna, S. and Roy, S. (2007). Tocotrienols in health and disease, The other half of the natural vitamin E family. Molecular Aspects of Medicine 28, 692-728
122. Seong, PJ., Jeon B.W., Lee, M., Cho, DH., Kim, DK., Jung KS., Kim, SW., Han, SO., Kim, YH., Park, C. (2011). Enzymatic coproduction of biodiesel and glycerol carbonate from soybean oil and dimethyl carbonate. Enzyme & Microbial Technology 6, 48(6-7), 505-9

123. Shahrzad, S., and Bitsch, I. (1996). Determination of some pharmacologically active phenolic acids in juices by high performance liquid chromatography. Journal of Chromatography A 741, 223–231
124. Sims, R. J., Fioriti, J. A., & Kanuk, M. J. (1972). Sterols additives as polymerisation inhibitors for frying oils. Journal of the American Oil Chemist's Society 4, 298
125. Sindhu M. and Emilia A. (2004). Ferulic acid: an antioxidant found naturally in plant cell walls and feruloyl esterases involved in its release and their applications. Critical Reviews in Biotechnology 24(2-3), 59-83
126. Singleton, V.L., Orthofer, R. and Lamuela-Raventos, R.M. (1999). Analysis of Total Phenols and Other Oxidation Substrates and Antioxidants by Means of Folin-Ciocalteu Reagent. Methods in Enzymology, 299, 152-178
127. Sohail, M., Rakha, A., Sadiq Butt, M., Muhammad, M., Iqbal J. & Rashid, S. (2016). Rice Bran Nutraceutics: A Comprehensive Review. Critical Reviews in Food Science and Nutrition, 3771-3780
128. Son, M.J., Rico, C.W. Nam, S.H. and Kang, M.Y. (2011). Effect of oryzanol and ferulic acid on the glucose metabolism of mice fed with a high-fat diet. Journal of Food Science 76, 7-10
129. Stahel, W.R., Reday-Mulvey, G. (1981). Jobs for tomorrow: the potential for substituting manpower for energy. Circular Economy and Sustainability – two faces of the same coin. Vantage Press, N.Y. 116
130. Symons, G.E. (1945). Industrial Waste Disposal. Sewage Works Journal, 17 (3) 558-572
131. Tabaraki, R., Neteghi, A. (2011). Optimization of ultrasonic-assisted extraction of natural antioxidants from rice bran using response surface methodology. Ultrasonics Sonochemistry 18, 1279-1286
132. Tacchini M, Spagnoletti A, Marieschi M, Caligiani A, Bruni R, Efferth T, Sacchetti G, Guerrini A. (2015). Phytochemical profile and bioactivity of traditional ayurvedic decoctions and hydro-alcoholic macerations of *Boerhaavia diffusa* L. and *Curculigo orchioides* Gaertn. Journal of Natural Products Research 29, 2071–2079
133. Tacchini, M., Burlini, I., Bernardi, T., De Risi, C., Massi, A., Guerrini, A., & Sacchetti, G. (2018). Chemical characterisation, antioxidant and antimicrobial

screening for the revaluation of wine supply chain by-products oriented to circular economy. Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology

134. Tahergorabi, R., Beamer, S. K., Matak, K. E., & Jaczynski, J. (2011). Effect of isoelectric solubilization/precipitation and titanium dioxide on whitening and texture of proteins recovered from dark chicken-meat processing by-products. LWT-Food Science and Technology, 44, 896-903
135. The Biology and Ecology of Rice (*Oryza sativa* L.) in Australia. (2005) Australian Government, Department of Health, Office of gene Technology Regulator
136. Thirugnanasambandham, T., Sivakumar, J., Prakash Maran, J. (2015). Microwave-assisted extraction of polysaccharides from mulberry leaves. International Journal of Biological Macromolecules 72, 1-5
137. Tsuji, E., Takahashi, M., Kinoshita, S., Tanaka, M. and Tsuji, K. (2003). Effects of different contents of gamma-oryzanol in rice bran oil on serum cholesterol levels. In XIIIth International Symposium on Atherosclerosis, Atherosclerosis Supplements 4, 278
138. Valavanidis, A., Nisiotou, C., Papageorgiou, Y., Kremli, I., Satravelas, N., Zinieris, N., Zygalaki, H. (2004). Comparison of the Radical Scavenging Potential of Polar and Lipidic Fractions of Olive Oil and Other Vegetable Oils under Normal Conditions and after Thermal Treatment. Journal of Agricultural and Food Chemistry, 52, 2358-2365
139. Vaughan, D.A., Morishima, H., Kadowaki, K. (2003). Diversity in the Oryzagenus. Current Opinion in Plant Biology, 6, 139-146
140. Vaughan, DA; Lu, B; Tomooka, N. (2008). The evolving story of rice evolution. Plant Science, 174 (4), 394–408
141. Ventaka, MS., Venkateswar, RM. (2013). Optimization of critical factors to enhance polyhydroxyalkanoates (PHA) synthesis by mixed culture using Taguchi design of experimental methodology. Bioresouce Technology 128, 409-16
142. Verma, B., Hucl, P., Chibbar R.N. (2009). Phenolic acid composition and antioxidant capacity of acid and alkali hydrolysed wheat bran fractions. Food Chemistry 116, 947–954

143. Vermerris, W., and Nicholson, R. (2007). Phenolic Compound Biochemistry. Springer Science & Business Media, 153-154
144. Vilkhu, K., Mawson, R., Simons L., Bates, D. (2008). Applications and opportunities for ultrasound assisted extraction in the food industry — A review. Innovative Food Science and Emerging Technologies 9, 161–169
145. Wagner, H. and Bladt, S. (2009). Plant drug analysis. A Thin Layer Chromatography. Atlas, Second Edition, Springer Verlag, Berlin
146. Wang, J., Sun, B., Cao, Y., Tian, Y., Li, X. (2008). Optimisation of ultrasound-assisted extraction of phenolic compounds from wheat bran. Food Chemistry, 106 (2), 804-810
147. Wang, J., Sun, B., Liu, Y., Zhang, H. (2014). Optimisation of ultrasound-assisted enzymatic extraction of arabinoxylan from wheat bran. Food Chemistry 150, 482–488
148. Wang, T., Hicks, K. B., & Moreau, R. (2002). Antioxidant activity of phytosterols, oryzanol, and other phytosterol conjugates. Journal of the American Oil Chemists' Society, 79(12), 1201–1206
149. White, P. J., & Xing, Y. (1997). Antioxidants from cereals and legumes. In F. Shahidi (Ed.), Natural antioxidants, chemistry, health effects, and application (25–63). Champaign IL: AOCC press
150. Wong, D. W. S. (2006). Feruloyl Esterase. A Key Enzyme in Biomass Degradation. Journal of Applied Biochemistry and Biotechnology 133, 87-112
151. Woodruff, S. and Miller, E.F. (1929). Studies upon Edible Cellulose. I. Recovery of Crude Fiber from Raw and Cooked Potato Cellulose. Journal of the American Dietetic Association 5, 23-7
152. Xu, P. Analysis of Antioxidant-Rich Phytochemicals, First Edition (2012)
153. Yang, X., Lee S.J., Yoo, H.Y., Choi, H.S., Park, C., Kim, S.W. (2014) Biorefinery of instant noodle waste to biofuels. Bioresource Technology, 159, 17–23
154. Yu, P., Maenz, D.D., McKinnon, J.J., Racz, V.J. and Christensen, D.A. (2002). Release of Ferulic Acid from Oat Hulls by Aspergillus Ferulic Acid Esterase and Trichoderma Xylanase. Journal of Agricultural and Food Chemistry 2002, 50, 1625−1630

155. Zainal-Abidin, M.H., Hayyan, M., Hayyan, A., Jayakumar, N.S. (2017). New horizons in the extraction of bioactive compounds using deep eutectic solvents: a review. Analytica Chimica Acta 979, 1–23
156. Zaupa, M., Scazzina, F., Dall'Asta, M., Calani, L., Del Rio, D., Bianchi, M.A., Melegari, C., De Albertis, P., Tribuzio, G., Pellegrini, N., Brighenti, F. (2014). In Vitro Bioaccessibility of Phenolics and Vitamins from Durum Wheat Aleurone Fractions. Journal of Agricultural and Food Chemistry, 62 (7), 1543–1549
157. Zhimin X., Godber, J. S. (2001). Antioxidant activities of major components of γ-oryzanol from rice bran using a linoleic acid model. Journal of the American Oil Chemists' Society 78, 645
158. Zhou, Z., Robards, K., Helliwell, S., & Blanchard, C. (2004). The distribution of phenolic acids in rice. Food Chemistry, 87(3), 401–406
159. Zitkowski, H.E. (1917). The Recovery of Potash from Beet-Sugar House Waste Liquors. Journal of Industrial and Engineering Chemistry, 9 (7), 692–694

10 Websites

http://fesr.regione.emilia-romagna.it/opportunita/

www.actualitix.com

www.alliedmarketresearch.com/cosmetics-market

www.avantech.it

www.biologydiscussion.com

www.britannica.com

www.examine.com

www.fao.org

www.faostat.fao.org

www.forages.oregonstate.edu

www.knoema.com

www.lipidlibrary.aocs.org

www.maveenseeds.com

www.persistencemarketresearch.com

www.sciencenordic.com

www.statista.com

www.thebalance.com

www.vision2020.info

www.zerowasteeurope.eu 2016

www.camag.com

11 Annex

11.1 Annex I: Publications

ECONOMIA CIRCOLARE: NON SOLO GEOMETRIE

La valorizzazione sostenibile degli scarti della filiera vitivinicola

La valorizzazione delle materie prime secondarie è oggi per l'industria un'occasione per incrementare la propria competitività convertendo i costi di smaltimento in nuove strategie di profitto, creando nuovi prodotti e favorendo l'occupazione di personale specializzato alla gestione dei nuovi processi produttivi. Le nuove politiche europee di governo sostenibile delle produzioni potrebbero così diventare strumento propulsivo di benessere sociale e contribuire al miglioramento della salubrità ambientale e alla conservazione e difesa della biodiversità.

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*Alessandro Grandini
**Tatiana Bernardi
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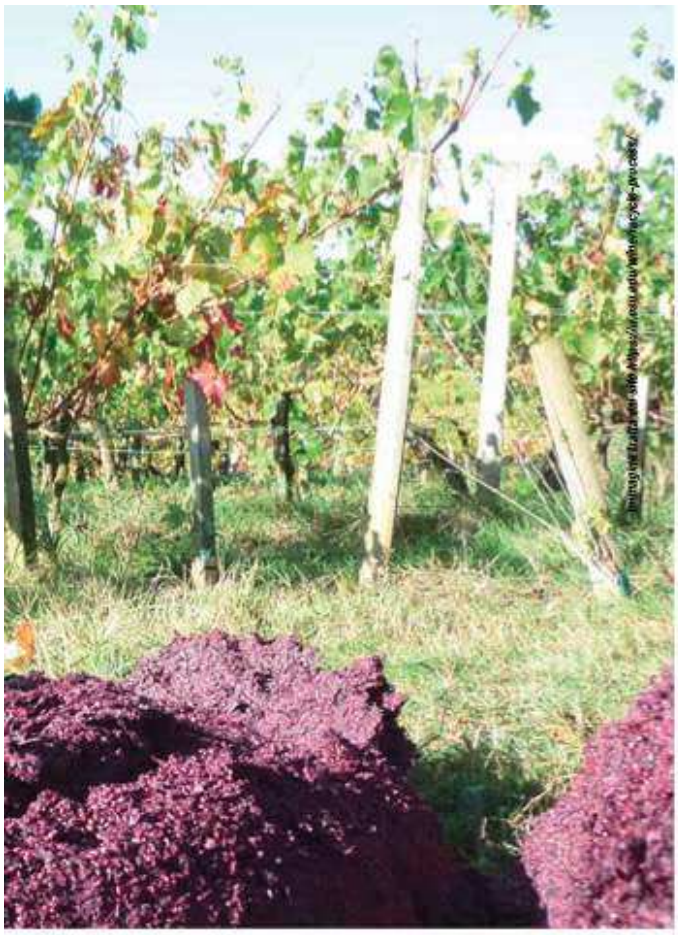
Il costante incremento dell'impatto antropico sull'ambiente in termini di inquinamento, sfruttamento delle risorse, impoverimento della biodiversità, aumento della popolazione globale, accompagnato da una conseguente crescita costante della domanda in termini di energia, materie prime e prodotti, ha stimolato istituzioni di governo e di ricerca a perseguire obiettivi focalizzati su nuovi approcci produttivi, alternativi allo sfruttamento diretto delle risorse prime, contribuendo a costruire e a consolidare un nuovo paradigma, ovvero quello dell'economia circolare concretizzato industrialmente nel concetto di bio-raffineria.



Mentre il concetto di economia circolare è definibile - in termini sintetici ma certamente esaustivi - come un sistema in grado di auto-alimentarsi e sostenersi economicamente, il termine di bio-raffineria concretizza lo stesso concetto a livello di processi produttivi industriali dove vengono massimizzate le rese di *output* (energia, prodotti con riferimento a ogni campo industriale) rispetto all'utilizzo delle materie prime, minimizzando o annullando la produzione di scarti. In sostanza, ciò che tradizionalmente veniva considerato *scarto della produzione*, con l'approccio della bio-raffineria diventa sottoprodotto, *materia prima secondaria* convertita in nuovi prodotti commerciabili utilizzando un panel di strategie produttive a basso impatto ambientale e sostenibili rispetto alla circolarità dell'economia *bio* che li alimenta (Lin *et al.*, 2013).

Le materie prime secondarie: nuove risorse al servizio della sostenibilità e dell'innovazione industriale

Questo nuovo paradigma di ricerca e di produzione ha generato e consolidato l'interazione dei saperi e delle competenze, da quella chimica a quella biologica, da quella ambientale a quella dell'ingegneria impiantistica, per rendere sempre più concreta la realtà della bio-raffineria, oggi sempre più consolidata in un modello industriale *bio-based*. Il passaggio dell'industria verso una maggiore sostenibilità per migliorare l'efficacia in termini di costi, l'efficienza dei processi e le credenziali ecologiche rende economicamente valido lo sviluppo di strategie sostenibili e innovative per il riutilizzo degli sprechi. Questa visione produttiva, di orizzonte globale e multidisciplinare, si accompagna necessariamente alla presa di coscienza delle istituzioni governative, chiamate a regolamentare la riduzione degli sprechi e dell'impatto sull'ambiente delle produzioni non virtuose – talvolta con modalità emergenziali (per es. il problema dell'inquinamento da agro-farmaci) – sostenendo un nuovo sviluppo sociale e industriale nella promozione della cultura del riuso e dell'annullamento dello scarto (*cradle-to-cradle policy*). Sulla base di queste premesse, l'Europa ha posto in essere, con progressiva scalarità virtuosa e rigidità nell'applicazione, diversi *drivers* normativi che vanno dalle *Landfill directives* alla più recente revisione della *Waste Policy Legislation* (http://ec.europa.eu/environment/waste/target_review.htm).

I rifiuti prodotti dalle aziende di trasformazione alimentare sono un buon esempio di un tipo di rifiuti – anche *pre-consumer* – generati su larga scala e a livello globale. Questo tipo di scarti sta diventando sempre più problematico in quanto in alcuni casi può rappresentare oltre il 50% del totale dei rifiuti prodotti nei Paesi, di cui almeno il 60-70% è costituito in media da sostanze organiche.

Da qui, la scala e il ritmo con cui le nostre filiere di trasformazione agroalimentare producono scarti

organici, per loro natura fermentabili e putrescibili, determinano inevitabilmente un problema normativo, di smaltimento, di logistica e in ultima analisi di costi per l'industria stessa.
La valorizzazione delle materie prime secondarie – non più scarti – è invece oggi per l'industria un'occasione per incrementare la propria competitività convertendo i propri costi di smaltimento in nuove strategie di profitto (nuovi prodotti), per favorire l'occupazione assumendo personale specializzato alla gestione profittevole dei nuovi processi produttivi, per adeguarsi alle nuove politiche europee di governo sostenibile delle produzioni diventando strumento propulsivo di benessere sociale e contribuendo al miglioramento della salubrità ambientale e alla conservazione e difesa della biodiversità.

Le strategie di valorizzazione e il progetto VALSOVIT (POR-FESR Emilia Romagna)

In generale, lo stato dell'arte della valorizzazione degli scarti agroalimentari consiste sostanzialmente in due tipi di approccio (Lin *et al.*, 2013):
- ***Approccio di prima generazione.*** Gli scarti vengono indirizzati al compostaggio, alla produzione di energia (digestione anaerobia) o trasferiti in discarica;
- ***Approccio di seconda generazione.*** Gli scarti vengono indirizzati verso processi con un più alto livello tecnologico e con elevata sostenibilità e un ridotto impatto ambientale (per es. tecnologie estrattive con basso o nullo impiego di solventi organici; strategie biotecnologiche e trasformative integrate; utilizzo di sistemi catalitici ad alta efficienza in processi a cascata) per ottenere (bio) sostanze ad alto valore aggiunto e con ricadute diversificate in più settori di mercato.

Sulla base di questi paradigmi, il laboratorio in rete Terra&Acqua Tech del Tecnopolo dell'Università di Ferrara, il Centro Interdipartimentale di Ricerca Industriale Energia e Ambiente (CIRI-EA) dell'Università di Bologna, il Centro Ricerche Produzioni Animali (C.R.P.A. Lab.) di Reggio Emilia, il Consorzio Laboratorio Energia Ambiente (L.E.A.P.) di Piacenza, Caviro Distillerie (Faenza, RA; http://www.caviro.com/it/), Eridania Sadam SpA (Parma; https://www.sadam.it), CBC (Europe) Srl (Biogard Division; Cesena; http://www.biogard.it/index.php/it/), AmbrosiaLab Srl (Ferrara, http://www.ambrosialab.it/it/) hanno insieme costituito una partnership che si è coagulata attorno a un progetto di ricerca industriale strategica, rivolto agli ambiti prioritari della Strategia di Specializzazione Intelligente (POR-FESR 2014-2020) della Regione Emilia Romagna, dal titolo ***Valorizzazione sostenibile degli scarti della filiera vitivinicola per l'industria chimica e salutistica*** (acronimo: VALSOVIT).
La natura del partenariato, caratterizzata da soggetti industriali di rilevante importanza nel panorama produttivo della regione Emilia Romagna e da istituti di ricerca specializzati nel trasferimento tecnologico, si inserisce in un contesto regionale di filiera vitivinicola che allo stato attuale considera principalmente processi di valorizzazione di prima generazione degli scarti (raspi freschi, bucce, vinaccia bianca, feccia, teste e code di distillazione dell'etanolo). Le finalità progettuali di Valsovit invece si rivolgono in particolare a una valorizzazione di seconda generazione per ricercare importanti ricadute per l'industria chimica e salutistica, nonché della difesa delle piante in agricoltura promuovendo, con un approccio operativo ispirato a un modello di simbiosi industriale, uno sviluppo tecnologico sostenibile della filiera. Per gli aspetti di progetto relativi alla valorizzazione rispetto a ricadute nel comparto chimico (produzione di poliidrossialcanoati, bio-anidride maleica, etc.) si veda Massi *et al.* (2018). In questo contesto, invece, verranno descritte le strategie di approccio, le attività e i risultati ottenuti relativamente all'ottimizzazione dei processi estrattivi applicati ai sottoprodotti della lavorazione delle uve per ottenere estratti, frazioni e biomolecole biologicamente attive per una valorizzazione industriale nutraceutica, cosmetica e per la difesa delle piante coltivate veicolata a parte del partenariato aziendale specificatamente legato a questi settori di mercato. Per quanto attiene nello specifico alle valutazioni di attività biologica, in questo articolo saranno riportati i dati preliminari che sono stati di indirizzo per i riscontri ottenuti da altri partner di progetto e riportati in Caliceti *et al.* (2018, in stampa).

MATERIALI E METODI

Il materiale vegetale: le materie prime secondarie

Gli scarti della lavorazione delle uve erano costituiti da vinacce (da uve bianche, VCB; e rosse, VCR) e da vinaccioli isolati, forniti dal partner di progetto Caviro Distillerie (Faenza, Ravenna) che provenivano dalla vendemmia del 2016 e 2017.
Le vinacce da uve rosse e bianche dealcolate contenevano rispettivamente il 51% e il 36% di umidità. All'uscita dei processi di lavorazione aziendale, i campioni di vinacce sono stati posti in stufa a 70 °C per 24 ore ed essiccati fino a peso costante. Una volta essiccati, i campioni di vinacce sono stati macinati in mulino con rotore a velocità variabile e griglia di macinazione con pori da 2 mm (Fritsch, Germania).
I campioni così polverati sono stati conservati a -20 °C fino al momento delle analisi. I campioni di vinaccioli invece (VLB, vinaccioli da uve bianche; VLR,

vinaccioli da uve rosse), caratterizzandosi per un contenuto d'umidità inferiore al 5%, sono stati posti immediatamente a -20 °C sin dal momento della consegna e mantenuti in quella condizione fino al momento delle estrazioni e analisi. Parte dei campioni di vinacce, sia bianche (VCB) sia rosse (VCR), non sono state invece sottoposte a essiccamento ma invece immediatamente sottoposte a distillazione in corrente di vapore per valutare la qualità e la quantità della componente volatile.

I reagenti chimici impiegati

Tutti i solventi e i reagenti impiegati per le analisi chimiche erano di qualità cromatografica, mentre tutti i reagenti impiegati per le valutazioni biologiche erano coerenti per qualità e purezza con quanto riportato in letteratura. Lo standard malvidina-3-O-glucoside è stato acquistato da Extrasynthese (Genay, Francia). Trolox, DPPH (1,1-difenil-2-picrylhydrazil), metanolo deuterato (CD_3OD), cloroformio deuterato ($CDCl_3$), metanolo, etilacetato, etanolo, acido formico, acido acetico, NP / PEG e acido gallico sono stati acquistati da Sigma-Aldrich Italia (Milano, Italia). Toluene è stato acquistato da Carlo Erba Reagents (Milano, Italia).

Le strategie estrattive

Sono state effettuate estrazioni su campioni essiccati e polverizzati di vinacce bianche (VCB) e rosse (VCR), utilizzando le seguenti tecniche di estrazione: estrazione di fluidi supercritici (SFE), estrazione assistita da ultrasuoni (UAE) e estrazione di fluido pressurizzato tramite estrattore Naviglio® (PFE-NAV).

Campioni non essiccati di vinacce bianche (VCB) e rosse (VCR) sono stati sottoposti a distillazione in corrente di vapore d'acqua (DIS). Ogni estrazione è stata effettuata in triplicato. Gli estratti ottenuti con UAE e PFE-NAV sono stati liofilizzati e conservati a -20 ° C fino al momento delle analisi. Gli estratti SFE sono stati conservati a -20 °C tal quali in quanto privi della frazione solvente, mentre gli estratti ottenuti per distillazione in corrente di vapore d'acqua sono stati immediatamente analizzati per gas cromatografia.

Estrazione assistita da ultrasuoni (UAE)

L'estrazione assistita da ultrasuoni è stata eseguita utilizzando un bagno a ultrasuoni (Ultrasonik 104X, Ney Dental International, MEDWOW, Cipro) impostato a una frequenza operativa di 48 kHz. Le condizioni estrattive non sono riportate per ragioni di riservatezza vincolate da un accordo di partenariato.

Estrazione con fluidi pressurizzati (PFE: Naviglio®)

L'estrattore Naviglio® (mod. 500 cc; Atlas Filtri, Italia) è stato utilizzato per estrarre materiale solido con un metodo di estrazione con solvente pressurizzato (Naviglio, 2003).

Le condizioni estrattive non sono riportate per ragioni di riservatezza vincolate da un accordo di partenariato.

Estrazione di fluidi supercritici (SFE)

I campioni sono stati sottoposti a estrazione con fluidi supercritici (CO_2; SFE) utilizzando un estrattore Speed SFE modello Applied Separations (Allentown, PA, USA).

Le condizioni estrattive non sono riportate per ragioni di riservatezza vincolate da un accordo di partenariato.

Estrazione per distillazione in corrente di vapore d'acqua

Campioni non essiccati di vinacce bianche (VCB) e rosse (VCR), e di vinaccioli da uve bianche (VLB) e rosse (VLR) sono stati sottoposti a distillazione in corrente di vapore.

Le distillazioni sono state effettuate seguendo le metodiche riportate in Scalvenzi *et al.* (2017) opportunamente modificate.

La caratterizzazione chimica

La caratterizzazione chimica degli estratti è stata focalizzata sulla rilevazione di classi di composti generalmente noti per proprietà funzionalmente utili nei contesti di ricaduta della ricerca, in particolare: polifenoli, fenoli semplici, flavonoidi e derivati, acidi organici, eventualmente attesi con rilevante abbondanza negli estratti idroalcolici (UAE, PFE-NAV); acidi grassi, steroli e sostanze lipofile come stereoisomeri della vitamina E, eventualmente attesi con rilevante abbondanza negli estratti con CO_2 supercritica (SFE); composti terpenici e derivati (mono-, sesqui-, di-terpeni) eventualmente attesi con rilevante abbondanza negli estratti ottenuti con la distillazione in corrente di vapore d'acqua (DIS).

Analisi (HP)TLC: cromatografia su strato sottile ad alta risoluzione

Le analisi sono state eseguite su una lastra di gel di silice HPTLC 60F254 (10 cm 20 cm; Camag, Swizerland). 8 µl di una soluzione etanolica al 50% degli estratti (20 mg/mL) sono stati depositati sulla lastra in bande di 6 mm di ampiezza utilizzando un microdepositore Linomat V (Camag, Swizerland) in flusso di azoto. Le bande depositate sono state eluite in due step e successivamente derivatizzate con soluzione NP-PEG (Wagner e Bladt, 2009).

Contenuto in polifenoli e proantocianidine totali

La determinazione del contenuto polifenolico totale negli estratti è stata eseguita utilizzando uno spettrofotometro Helios-Gamma, ThermoSpectronic seguendo le metodiche precedentemente descritte in Tacchini *et al.* (2015). I risultati sono stati espressi come mg di acido gallico/g di estratto secco per la quantificazione dei polifenoli totali e mg di cianidin cloruro/g di estratto secco per la quantificazione delle proantocianidine totali.

Immagine tratta dal sito http://nordestboulevard.it/2013/10/grappa-e-design/

Analisi HPLC: Cromatografia liquida ad alta prestazione

La caratterizzazione dei campioni finalizzata alla rilevazione dei principali flavonoidi è stata eseguita sui campioni di estrazione idroalcolica utilizzando un sistema HPLC modulare JASCO (Tokyo, Giappone, modello PU 2089) accoppiato a un detector a fotodiodi (MD 2010 Plus). Per l'analisi si è fatto riferimento a quanto riportato in Kammerer *et al.* (2004).

Analisi GC-MS: gas cromatografia accoppiata a spettrometria di massa

Le analisi gas cromatografiche sono state sviluppate sui campioni ottenuti dalla distillazione di matrici (VCA, VCB, VLB, VLR) fresche e non essiccate. Le analisi sono state sviluppate seguendo le indicazioni riportate in Tardugno *et al.* (2018).

L'attività biologica

Proprietà antiossidanti

Le proprietà antiossidanti sono state valutate per poter verificare in via preliminare gli estratti potenzialmente utili a una proiezione salutistica nutraceutica e/o cosmetica.

L'attività antiossidante degli estratti è stata valutata sia mediante analisi bioautografica (DPPH-(HP)TLC-bioautographic assay) sia mediante saggio spettrofotometrico (Spettrofotometro UV-Vis Helios) impiegando per entrambi i test il radicale DPPH (1,1-diphenyl-2-picrylhydrazil). I saggi sono stati sviluppati come riportato in Nostro *et al.* (2016). Tutti gli esperimenti sono stati effettuati in triplicato e i risultati ottenuti sono stati riportati come media ± deviazione standard.

L'attività antimicrobica

L'attività antimicrobica è stata valutata sia verso batteri sia verso funghi fitopatogeni per verificare in via preliminare quegli estratti potenzialmente utilizzabili per proprietà fitoiatriche.

Per valutare l'attività antibatte-

Pulitura dello strizzo idraulico

Le vinacce dopo la strizzatura

rica degli estratti vegetali è stato utilizzato un batterio, *Pseudomonas syringae* pv. *syringae* van Hall ATCC 19310. Gli esperimenti di attività antibatterica (MIC, Minima Concentrazione Inibente; MCB, Minima Concentrazione Battericida) sono stati allestiti seguendo le indicazioni riportate dal National Committee for Clinical Laboratory Standards (NCCLS, standard M7-A6).

Per la valutazione dell'attività antifungina sono stati invece utilizzati due funghi fitopatogeni, *Sclerotinia minor* e *Sclerotinia sclerotiorum*; l'allestimento delle colture dei funghi filamentosi e gli esperimenti di attività antifungina sono stati condotti seguendo le indicazioni riportate in Guerrini et al. (2009). La Minima Concentrazione Inibente la crescita fungina (MIC) e la Minima Concentrazione Fungicida (MCF) sono state determinate seguendole indicazioni riportate in Cavalerio *et al.* (2005). Infine, è stata calcolata la concentrazione efficace in grado di dare il 50% di effetto massimale (EC_{50}).

RISULTATI E DISCUSSIONE

Le strategie estrattive bioguidate da fingerprinting HP-TLC

Le matrici di scarto della lavorazione delle uve sono state fornite dall'azienda Caviro Distillerie (Faenza, RA; http://www.caviro.com/it/), partner aziendale di progetto, ed erano caratterizzate da vinacce rosse (VCR), vinacce bianche (VCB), vinaccioli da uve rosse (VLR) e bianche (VLB) (Figura 1).

Per le estrazioni, si è scelto di selezionare quelle metodiche che meglio potessero coagulare, come finalità di principio, criteri di efficienza (alta resa quali-quantitativa di biomolecole attive), sostenibilità (basso o nullo impiego di solventi organici) e opportunità di trasferimento tecnologico (*scale up* laboratorio vs. industria). In relazione a questi aspetti, peraltro caratterizzanti la finalità di valorizzazione sostenibile del progetto Valsovit, si è quindi optato per individuare nell'ampio e moderno contesto della *green chemistry* l'estrazione con ultrasuoni (UAE), l'estrazione con fluidi sotto pressione con strumentazione Naviglio®, l'estrazione con CO_2 supercritica (SFE) e la distillazione in corrente di vapore d'acqua (DIS) (Baiano, 2014). Ciascuna matrice di scarto è stata dunque sottoposta ad ogni metodica estrattiva individuata per verificare quale risultasse la più performante rispetto alle finalità di principio sopra descritte (Tabella 1).

Prima di procedere alle estrazioni UAE, PFE-NAV, SFE, le matrici da vinacce rosse (VCR) e da vinacce bianche (VCB) sono state sottoposte a essiccazione in stufa a 70 °C per 24 ore fino a ridurre il contenuto di acqua a valori inferiori al 5%, seguendo un protocollo di trattamento degli scarti normalmente adottato in azienda. I vinaccioli da uve rosse (VLR) e da uve bianche (VLB) presentavano già un tenore di umidità coerente con le esigenze di conservazione ed estrazione (<5%). Tutti i campioni sono stati successivamente polverizzati in mulino refrigerato per ottimizzare e uniformare le interazioni matrice-solvente in ciascuna delle metodiche estrattive adottate. Per le distillazioni in corrente di vapore d'acqua, invece, tutti i campioni sono stati utilizzati allo stato fresco minimizzando in questo modo la perdita dei composti più volatili. Le molecole target per l'ottimizzazione delle estrazioni erano rappresentate da polifenoli, fenoli semplici, flavonoidi e derivati, acidi organici, eventualmente attesi con rilevante abbondanza negli estratti idroalcolici (UAE, PFE-NAV); acidi grassi, steroli e sostanze lipofile come stereoisomeri della vitamina E, eventualmente attesi con rilevante abbondanza negli estratti con CO_2 supercritica (SFE); composti terpenici e derivati (mono-, sesqui-, di-terpeni) eventualmente attesi con rilevante abbondanza negli estratti ottenuti con la distillazione in corrente di vapore d'acqua (DIS). Per le metodiche UAE e PFE-NAV si è scelto di operare con solvente idroalcolico (etanolo-acqua) individuando sperimentalmente nel rapporto 50:50 la combinazione più efficace rispetto alle categorie chimiche estratte, valutate per numero e ampiezza delle bande su lastre per cromatografia su strato sottile ad alte prestazioni (HP-TLC), confermando così anche le indicazioni di letteratura (Da Porto *et al.*, 2013). Analogamente, si è operato con SFE individuando le condizioni di *set up* strumentale e tempistiche di estrazione statica e dinamica più adeguate. Per DIS, si è fatto invece riferimento a quanto riportato in Scalvenzi *et al.* (2017) opportunamente modificato (Figura 2).

Per ciascuna categoria di campioni, l'efficienza estrattiva in termini di resa quantitativa di estratto totale è risultata ottimale con UAE, più ridotta (fino anche al 60%) con PFE-NAV, e ulteriormente più bassa con SFE. Estremamente bassa è risultata la resa in olio essenziale (0.02-0,008%) ottenuto con la distillazione in

Figura 1. Gli scarti della lavorazione delle uve sottoposti ad estrazione. A, vinacce bianche essiccate (VCR); B, vinacce rosse essiccate (VCR); C, vinaccioli da uve bianche e rosse (VLB, VLR).

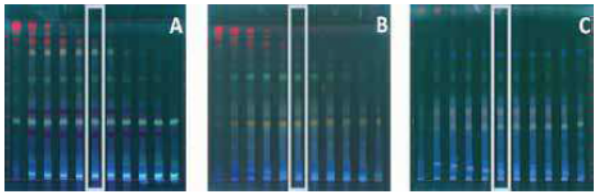


Figura 2. Cromatografia su strato sottile ad alta risoluzione (HP-TLC) degli estratti idroalcolici (a titolo d'esempio) per l'individuazione delle condizioni ottimali per ottenere un profilo fitochimico di compromesso qualitativamente e quantitativamente ottimale rispetto alle categorie chimiche di interesse. Bande blu-azzurre: polifenoli; bande giallo-verdi: flavonoidi; bande rosse: clorofille. A, campioni VCR; B, campioni VCB; C, campioni VLB. La regione circoscritta in ciascuna lastra corrisponde all'eluizione di un estratto idroalcolico al 50%.

corrente di vapore d'acqua (DIS, Tabella 1), rendendo sostanzialmente impraticabile l'ipotesi di scale-up industriale di processo per uno sfruttamento razionale dei distillati. Il dato quantitativo riflette quanto riportato in letteratura per quanto attiene alle rese totali di estrazioni idroalcoliche e SFE, mentre sostanzialmente inedito è il dato relativo agli estratti DIS.

Tabella 1. Rese di estrazione (riportate come valori percentuali medi), relativamente ad estrazione assistita con ultrasuoni (UAE), estrazione con fluidi sotto pressione con metodo Naviglio® (PFE-NAV), estrazione con fluidi supercritici (SFE) e distillazione in corrente di vapore (DIS; su matrici non essiccate). VCR, vinacce rosse essiccate; VCB, vinacce bianche essiccate; VLB, vinaccioli da uve bianche; VLR, vinaccioli da uve rosse. Dev. St., deviazione standard.

La caratterizzazione chimica

I campioni di vinacce di uve rosse (VCR) e bianche (VCB) e di vinaccioli da uve rosse (VLR) e da uve bianche (VLB) ottenuti con le estrazioni con solvente idroalcolico mediante ultrasuoni (UAE) e con fluidi pressurizzati con strumentazione Naviglio® ottimizzate per la migliore resa quantitativa in polifenoli, sono stati valutati con metodo spettrofotometrico per la quantificazione dei polifenoli totali (Figura 3).

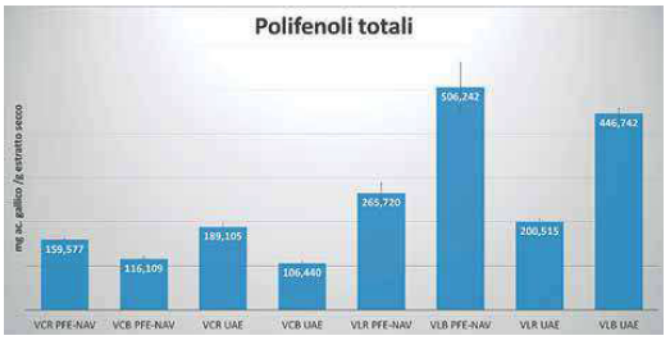


Figura 3. Nel grafico sono riportati i valori dei polifenoli totali (mg acido gallico/g estratto secco) degli estratti (VCR, vinacce rosse; VCB, vinacce bianche; VLR, vinaccioli da uve rosse; VLB, vinaccioli da uve bianche) ottenuti con le metodiche UAE (estrazione con ultrasuoni) e PFE-NAV (estrazione con fluidi sotto pressione con strumentazione Naviglio ®) ottimizzate per la migliore rilevazione qualitativa e quantitativa. I dati sono relativi a quanto ottenuto con solvente idroalcolico al 50%.

Benché l'estrazione UAE sia risultata più performante rispetto alla variabile della resa totale di estratto (Tabella 1), relativamente al contenuto totale in polifenoli non si è dimostrata altrettanto efficace. Infatti, per tutte le matrici, tranne che per i campioni VCR dove peraltro lo scarto è risultato ridotto al 15%, la metodica PFE-NAV è risultata più performante nell'estrazione dei polifenoli totali. In particolare, i campioni che si sono rilevati più ricchi in polifenoli totali sono risultati sempre gli estratti da vinaccioli. In particolare, i vinaccioli da uve bianche (VLB) hanno dato in assoluto i risultati migliori con entrambi i metodi estrattivi ed evidenziando uno scarto di circa il 48% rispetto ai campioni VLR a parità di metodo estrattivo più performante (PFE-NAV). Il contenuto negli estratti VLB con PFE-NAV è risultato sensibilmente superiore a quello con UAE (12% circa). Maggiore lo scarto quantitativo (25% circa) tra gli estratti PFE-NAV e UAE per i campioni VLR. Decisamente più ridotti i risultati relativi alle vinacce, benché gli estratti da vinacce rosse (VCR) siano risultati con il maggior contenuto in polifenoli totali rispetto ai campioni VCB, con uno scarto medio del 39% rispetto al dato riferito al metodo estrattivo più performan-

te. Benché in letteratura siano numerosi gli esempi relativi al contenuto polifenolico di scarti della lavorazione delle uve compatibili con quelli da noi usati per quanto attiene alla loro tipizzazione, non è invece possibile effettuare un confronto costruttivo in quanto non ne viene mai indicata la provenienza rispetto ai diversi *step* di lavorazione in filiera. Infatti, dalla nostra esperienza, condivisa con la partnership di progetto, il contenuto in composti polifenolici può ragionevolmente e significativamente cambiare in relazione al momento di processo in cui le matrici vegetali vengono considerate scarti e quindi indirizzabili ad altri processi di lavorazione. Pertanto, per ragionevoli motivazioni di accordo di partenariato e di potenziale ricaduta applicativa, i risultati prodotti e qui riportati sono da ritenersi puramente indicativi e non sostanziali per quanto attiene al dato fitochimico, ma utili sul piano del riscontro della strategia operativa ed estrattiva più adeguata all'individuazione del momento più adatto al prelievo dei sottoprodotti in filiera come adeguate fonti di polifenoli.

Analoghe premesse e considerazioni sono alla base dei risultati spettrofotometrici ottenuti per le proantocianidine totali (Figura 4). In particolare, va sottolineato che a differenza del dato relativo ai polifenoli totali, per tutti i campioni la metodica estrattiva più performante rispetto al contenuto totale di proantocianidine è risultata quella con ultrasuoni UAE (Figura 4). In particolare, la differenza a parità di qualità di campione tra i metodi estrattivi (PFE-NAV, UAE) rispetto alla quantità totale di proantocianidine era estremamente diversificata ma sempre piuttosto rilevante, ovvero: del 42% per VCR, del 64% per VCB, dell'89% per VLR e del 93% per VLB. I campioni più ricchi in assoluto di proantocianidine sono risultati comunque i vinaccioli, sia da uve rosse (VLR) sia da uve bianche (VLB). Come per i polifenoli totali, però, anche per il contenuto totale in proantocianidine i campioni VLB sono risultati i più ricchi con uno scarto rispetto agli stessi campioni da uve rosse di circa il 29% a parità di processo estrattivo più performante (UAE).

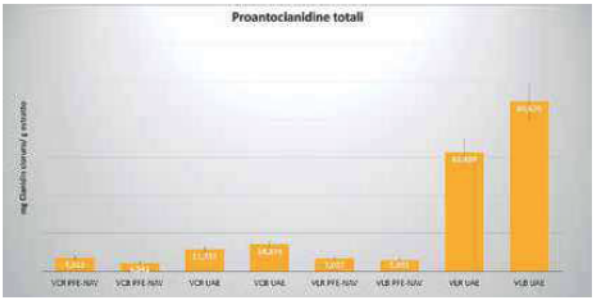


Figura 4. Nel grafico sono riportati i valori delle proantocianidine totali (mg di cianidin cloruro/g di estratto secco) relativi agli estratti (VCR, vinacce rosse; VCB, vinacce bianche; VLR, vinaccioli da uve rosse; VLB, vinaccioli da uve bianche) ottenuti con le metodiche UAE (estrazione con ultrasuoni) e PFE-NAV (estrazione con fluidi sotto pressione con strumentazione Naviglio ®) ottimizzate per la migliore rilevazione qualitativa e quantitativa. I dati sono relativi a quanto ottenuto con solvente idroalcolico al 50%.

Gli estratti idroalcolici UAE e PFE-NAV dei campioni VCR, VCB, VLR e VLB sono stati analizzati qualitativamente via HPLC anche per il contenuto di flavonoidi (Figura 5).

In generale, derivati glicosilati della quercetina e del kaempferolo con relativi agliconi, e rutina sono risultati sempre presenti in tutti i campioni, sebbene con sensibili variazioni quantitative di profilo cromatografico.

In figura viene riportato, a titolo di esempio, il profilo cromatografico relativo ai campioni di vinacce bianche (VCB) ottenuti con metodo estrattivo con ultrasuoni (UAE).

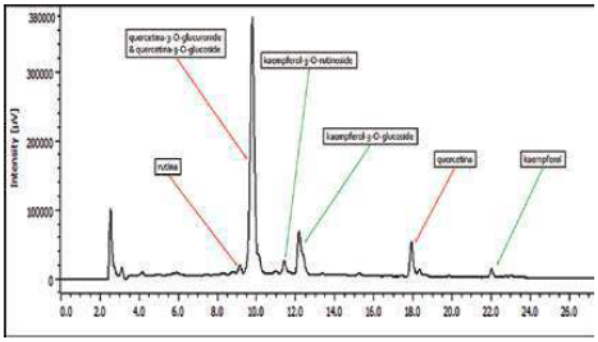


Figura 5. Profilo cromatografico di un estratto da vinacce bianche (a titolo di esempio) ottenuto con analisi HPLC in fase inversa e con detector a fotodiodi (RP-HPLC-DAD) da cui è possibile evincere i composti flavonoidici rilevati glicosilati e non glicosilati più abbondanti.

Sono state poi effettuate analisi gas cromatografiche degli estratti con CO_2 supercritica per valutare la qualità degli estratti lipofili con specifico riferimento al contenuto di acidi grassi e alla composizione della frazione insaponificabile (Figura 6). In particolare, particolarmente interessante sono risultati i campioni di vinaccioli, già peraltro noti per essere fonte di un olio dalle pregiate qualità nutrizionali e per cui emerge la prevalenza dell'acido linoleico confermando peraltro i dati di letteratura relativi a estratti ottenuti con la medesima tecnica (Passos *et al.*, 2010).
Per quanto riguarda gli estratti ottenuti per distillazione in corrente di vapore (DIS), l'analisi gas cromatografica ha rivelato una composizione di tutti i campioni prevalentemente caratterizzata da acidi grassi (Tabella 2) con tracce di composti sesquiterpenici (dati non presentati). I campioni hanno presentato tutti un profilo qualitativo sostanzialmente simile, mentre da un punto di vista di quantità relative sono emerse differenze interessanti. In particolare nei campioni di vinacce rosse (VCR) i composti più abbondanti espressi per area % sono risultati l'acido palmitico (53,7%), acido miristico (7,6%), acido dodecanoico (6,1%), acido oleico (5,2%). Nei campioni di vinacce bianche (VCB) erano presenti l'acido palmitico (41,3%), acido palmitoleico (6,7%), acido oleico (5,49%), acido miristico (4,8%). I campioni di distillato di vinaccioli invece sia da uve bianche e sia da uve rosse sono risultati con un profilo quali-quantitativo sostanzialmente analogo caratterizzato da acido palmitico (29,6%), acido linoleico (19,4%), etilestere dell'acido palmitico (11,1%), acido alfa linolenico (10,5%), acido oleico (6,9%). Non ci sono dati di letteratura che ci permettono di confrontare questi risultati relativamente a distillati di scarti, tuttavia è possibile fare alcune considerazioni rispetto al dato oggettivo. Innanzitutto, il dato non era atteso né per tipologia di composti né per la quantità. Il dato atteso era piuttosto quello di un fitocomplesso prevalentemente terpenico, mentre invece i composti rilevati erano presenti sostanzialmente con una abbondanza inferiore all'1%. Questo aspetto è probabilmente spiegabile con il fatto che le vinacce fresche, prima di essere fornite per le analisi, avevano subito condizioni per cui la maggior parte dei componenti volatili era scomparsa. Ha sorpreso invece la composizione in acidi grassi che per i nostri campioni è risultata sia qualitativamente sia quantitativamente più ricca rispetto ai campioni ottenuti con SFE. Dal momento che i campioni estratti con SFE erano stati essiccati mentre gli stessi campioni distillati erano stati estratti freschi (con la speranza fossero ricchi nella componente volatile terpenica) è possibile ipotizzare che il pre-trattamento in stufa a 70 °C abbia degradato la gran parte della componente di acidi grassi, rimasta invece nelle matrici fresche.

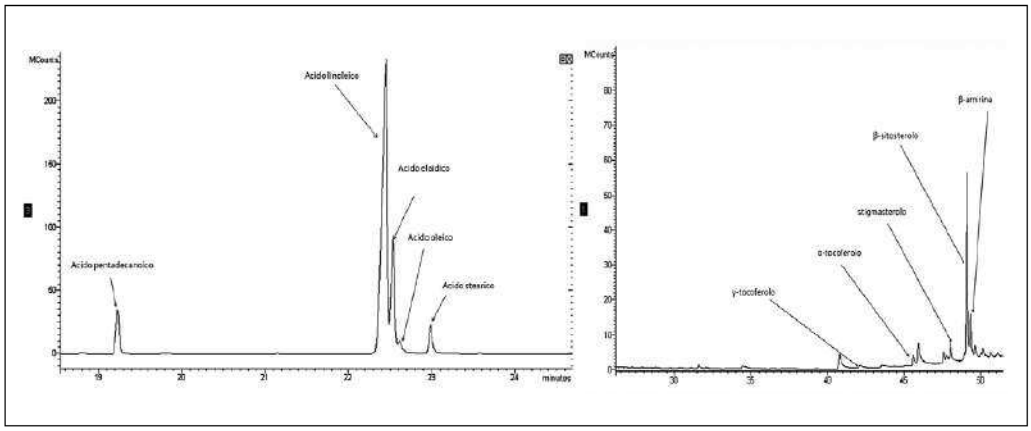


Figura 6. Estratti SFE. Profili gas cromatografici di un estratto SFE di vinaccioli da uve bianche (VLB). A, profilo gas cromatografico dove vengono evidenziati i principali acidi grassi. B, profilo gas cromatografico della frazione insaponificabile.

Composto	Area%
Octanoic acid, ethyl ester	0,14
Octanoic acid	0,89
Decanoic acid	1,24
Nonanoic acid, 9-oxo-, ethyl ester	1,48
Dodecanoic acid, ethyl ester	0,28
Dodecanoic acid	2,04
Octanedioic acid, bis	0,21
myristic acid, ethyl ester	0,41
Azelaic acid, bis	2,67
2-Pentadecanone, 6,10,14-trimethyl-	0,29
myristic acid	**4,83**
cis-10-Pentadecenoic acid	0,73
n-Pentadecanoic acid	**1,18**
palmitoleic ethyl ester	2,64
palmitic acid, ethyl ester	5,21
Palmitoleic acid	**6,73**
palmitic acid	**41,33**
Linoleic acid ethyl ester	0,37
oleic acid, ethyl ester	1,81
elaidic acid, ethyl ester	0,40
stearic acid ethyl ester	0,76
linoleic acid	0,95
oleic acid	**5,49**
elaidic acid	0,91
stearic acid	1,57
Tritetracontane	0,18
1-Decanol, 2-hexyl-	0,50
Eicosen-1-ol, cis-9-	0,26
Tritetracontane	0,35
(Z)-14-Tricosenyl formate	0,63

Tabella 2. Estratti ottenuti per distillazione in corrente di vapore d'acqua (DIS). A titolo di esempio viene riportata la composizione dell'estratto ottenuto dalla distillazione di vinacce bianche (VCB) dal momento che, benché estremamente bassa, hanno rivelato una resa complessivamente più elevata (0,05%).

I risultati preliminari sull'attività biologica e le possibili proiezioni applicative

Gli estratti di vinacce rosse e bianche (VCR, VCB) e da vinaccioli da uve rosse e bianche (VLR, VLB) sono stati valutati preliminarmente per le proprietà antiossidanti con metodo spettrofotometrico del DPPH, e antimicrobiche *in vitro*. La scelta rispetto a questa tipologia di saggi è stata guidata dal fatto che da un punto di vista preliminare l'attività antiossidante è senz'altro una proprietà di sicuro interesse per le possibili proiezioni nutraceutiche e cosmetiche del progetto Valsovit. Per le proprietà antiossidanti con test DPPH sono stati presi in considerazione gli estratti idroalcolici ottenuti con metodo PFE-NAV e UAE in quanto caratterizzati dalla componente polifenolica più abbondante e normalmente presa a riferimento per esprimere attività antiossidante (Figura 7). Non si è osservata una corrispondenza diretta tra il contenuto di polifenoli, proantocianidine e flavonoidi con l'attività antiossidante espressa nel saggio spettrofotometrico, se non che i campioni vinaccioli da uve bianche (VCB) hanno espresso i risultati di maggior rilievo. In questo caso, l'attività rilevante degli estratti VCB UAE era certamente attesa ma non risulta direttamente correlabile per esempio con il dato dei polifenoli delle proantocianidine totali rapportato al metodo estrattivo, in quanto i campioni maggiormente ricchi di polifenoli erano quelli ottenuti con il metodo PFE-NAV. Questo aspetto è evincibile anche per gli altri estratti, per esempio VCR UAE e VLR UAE. In ogni caso e come premesso, il dato è del tutto preliminare e necessita di ulteriori approfondimenti non solo dal punto di vista della bioattività, ma anche dal punto di vista fitochimico per poter meglio correlare i dati di caratterizzazione chimica e di attività biologica, ponendo in luce eventuali aspetti sinergici che al momento sembrano essere alla base delle apparenti discrepanze.

Per quanto riguarda invece l'attivi-

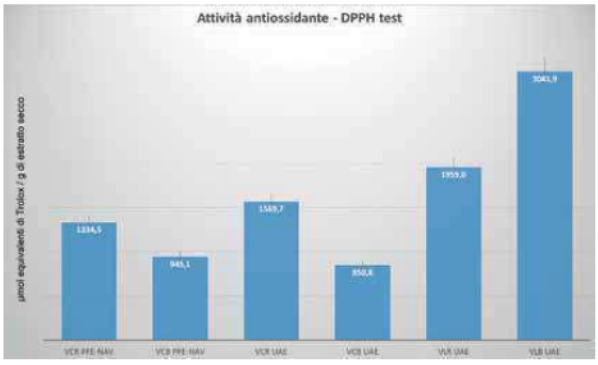


Figura 7. Attività antiossidante con metodo spettrofotometrico del DPPH. Nel grafico sono riportati gli estratti che hanno evidenziato le attività più rilevanti. I valori sono riportati come mmol equivalenti di Trolox / g di estratto secco.

Tabella 3. Attività antimicrobica di estratti idroalcolici di vinacce da uve rosse e bianche (VCR, VCB) e da vinaccioli da uve rosse e bianche (VLR, VLB). I risultati sono espressi come Minima concentrazione Inibente la crescita (MIC µg/ml) per l'attività antibatterica e come concentrazione efficace in grado di dare il 50% di effetto massimale (EC50) per quanto riguarda l'attività antifungina.
n.d.: non definite. In questi casi il ceppo ha evidenziato invece un forte incremento di crescita anziché un'inibizione, probabilmente dovuta alla componente zuccherina degli estratti. Controlli positivi: Delan 70 WG, per Pseudomonas syringae pv. syringae); Heliocuivre S, per Sclerotinia sp.)

tà antimicrobica, sono stati allestiti saggi preliminari *in vitro* con il preciso scopo di individuare estratti eventualmente promettenti per ulteriori approfondimenti rispetto all'attività fitoiatrica, ovvero per la difesa sostenibile delle coltivazioni come ulteriore ricaduta applicativa prevista dal progetto (Tabella 3).
Sono stati saggiati, anche in questo caso in via del tutto preliminare, gli estratti idroalcolici da matrici VCR, VCB, VLR, VLB. Come evincibile, non sono state registrate attività degne di nota. Anzi, con particolare riferimento all'attività antifungina, si è osservato un incremento di crescita dovuto con ogni probabilità alla componente zuccherina presente negli estratti.
In relazione a queste evidenze, le prospettive di progetto riguarderanno la valutazione degli stessi estratti privati della frazione zuccherina, nonché delle frazioni o eventuali molecole isolate putative di un'attività degna di una proiezione applicativa su scala industriale.

Conclusioni

La valorizzazione delle materie prime secondarie delle filiere agro-alimentari vede innanzitutto nella scelta e ottimizzazione del processo estrattivo un fattore chiave e determinante nel miglioramento sostenibile dei processi e nella loro conversione rispetto a un profilo industriale *bio-based* e di economia circolare. La scelta del metodo estrattivo dipende innanzitutto dal tipo di matrice vegetale (coriacea o meno), dalla sua condizione (umida o secca), dalla sua pezzatura (frantumata, triturata, polverata), e dal tipo di biomolecole target che si intendono ottenere per nuovi prodotti finiti. Nell'ambito del progetto Valsovit, trattandosi di un contesto di valorizzazione legato anche alla sostenibilità ambientale, è stato necessario selezionare, tra i processi individuati in relazione alle già elencate variabili, quelle strategie estrattive ispirate alla *green chemistry* che perseguono l'ottenimento di estratti minimizzando l'impiego di solventi organici, il cui smaltimento rappresenterebbe a livello industriale un costo sia economico sia ambientale ingente, per di più vincolato da strette regolamentazioni normative (Baiano, 2014).
Gli estratti ottenuti, caratterizzati da un punto di vista chimico in via preliminare, hanno dimostrato che gli scarti della filiera vitivinicola possono risultare un'ottima fonte di biomolecole utili a contesti applicativi, ma hanno altresì messo in evidenza l'importanza della conoscenza del grado di sfruttamento delle risorse primarie, ovvero del grado di lavorazione che hanno subito le uve prima di essere considerate come materie prime secondarie. Questo aspetto è sottovalutato dalla letteratura scientifica specializzata e riduce l'impatto applicativo delle evidenze riportate. In questo contesto, il merito del progetto Valsovit e dell'attività di ricerca applicata che lo caratterizza è senz'altro quello di porre in evidenza questo parametro per poter concretizzare realisticamente lo sfruttamento delle risorse in un'ottica di economia circolare.
In aggiunta a queste considerazioni, le valutazioni di bioattività preliminari correlate alle evidenze chimiche, impongono la necessità di approfondire la caratterizzazione chimica degli estratti, per poter meglio individuare eventuali molecole o frazioni chimiche degli estratti stessi responsabili delle bioattività, benché deboli, con proiezioni nutraceutiche, cosmetiche e/o fitoiatriche. Questo porterebbe, in un'ottica di processo bio-

La valorizzazione delle materie prime secondarie – non più scarti – è invece oggi per l'industria un'occasione per incrementare la propria competitività

guidato, a modificare le condizioni estrattive individuando quei parametri che determinano l'ottenimento di estratti arricchiti in principi attivi con le attività biologiche desiderate rendendo il processo di sfruttamento delle materie prime secondarie realisticamente e concretamente operativo anche per uno *scale up* industriale.

UNIVERSITÀ DEGLI STUDI DI FERRARA
°Dipartimento di Scienze della Vita e Biotecnologie
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Valorizzazione sostenibile degli scarti della filiera vitivinicola per l'industria chimica e salutistica (VALSOVIT): il progetto Valsovit è finanziato dalla Regione Emilia Romagna (POR-FESR) ed ha come finalità lo sfruttamento di seconda generazione dei sottoprodotti della filiera vitivinicola attraverso processi sostenibili per ottenere biomolecole ad alto valore aggiunto.

Bibliografia

Baiano, A. 2014. Recovery of Biomolecules from Food Wastes — A Review. *Molecules*, 19, 14821-14842.

Caliceti, C., Calabria, D., Porru, E., Franco, P., Fiori, J., Zangheri, M., Guardigli, M., Mirasoli, M. 2018. Valorizzazione sostenibile degli scarti della filiera vitivinicola come fonte di nutraceutici per l'industria salutistica - focus sulla disfunzione endoteliale (Sustainable recovery of wine by-products as a source of nutraceuticals for the healthcare industry - focus on endothelial dysfunction). *Pharmanutrition & Functional Foods*, (in stampa).

Cavaleiro, C; Pinto, E; Gonc¸alves, M J; and Salgueiro, L. Antifungal activity of Juniperus essential oils against dermatophyte, Aspergillus and Candida strains. *Journal of Applied Microbiology* (2006), Vol. 100 pp 1333-38

Da Porto, C., Porretto, E., Decorti D. 2013. Comparison of ultrasound-assisted extraction with conventional extraction methods of oil and polyphenols from grape (*Vitis vinifera* L.) seeds. *Ultrasonics Sonochemistry*, 20 (2013) 1076–1080.

Favretto, D., Flamini, R. 2000. Application of Electrospray Ionization Mass Spectrometry to the Study of Grape Anthocyanins. *American Journal of Enology and Viticulture*, 51, 55-64.

Guerrini, A., Sacchetti, G., Rossi, D., Paganetto, G., Muzzoli, M., Andreotti, E., Tognolini, M., Maldonado, M.E., Bruni, R. 2009. Bioactivities of *Piper aduncum* L. and *Piper obliquum* Ruiz & Pavon (Piperaceae) essential oils from Eastern Ecuador. *Environmental Toxicology and Pharmacology*, 27, 39–48.

Kammerer, D., Claus, A., Carle, R., Schieber, A. 2004. Polyphenol Screening of Pomace from Red and White Grape Varieties (Vitis vinifera L.) by HPLC-DAD-MS/MS. *Journal of Agricultural and Food Chemistry*, 52, 4360–4367.

Lin, C.S.K., Pfaltzgraff, L.A., Herrero-Davila, L., Mubofu, E.B., Abderrahim, S., Clark, J.H., Koutinas, A.A., Kopsahelis, N., Stamatelatou, K., Dickson, F., Thankappan, S., Mohamed, Z., Brocklesbye, R., Luque, R. 2013. Food waste as a valuable resource for the production of chemicals, materials and fuels. Current situation and global perspective. *Energy & Environmental Science*, 6, 426–464.

Maietti, S., Rossi, D., Guerrini, A., Useli, C., Romagnoli, C., Poli, F., Bruni R., Sacchetti, G. 2013. A multivariate analysis approach to the study of chemical and functional properties of chemodiverse plant derivatives: lavender essential oils. *Flavour and Fragrance Journal*, 28(3), 144-154

Massi, A., Sacchetti, G., Galletti, P., Bertin, L., Mazzoni, R., Viganò, F., Righi, S., Passarini, F., Soldano, M., Labartino, N. 2018. Dagli scarti delle uve una risorsa per l'industria chimica: il progetto Valsovit. La Chimica e l'Industria - Newsletter, 5(4), 16-24.

Naviglio, D. 2003. Naviglio's Principle and Presentation of an Innovative Solid–Liquid Extraction Technology: Extractor Naviglio®. *Analytical Letters*, 36(8), 1647–1659.

Nostro, A., Guerrini, A., Marino, A., Tacchini, M., Di Giulio, M., Grandini, A., ... Saraçoğlu, H. T. 2016. In vitro activity of plant extracts against biofilm-producing food-related bacteria. *International Journal of Food Microbiology*, 238, 33–39.

Passos, C.P., Silva, R.M., Da Silva, F.A., Coimbra, M.A., Silva, C.M. 2010. Supercritical fluid extraction of grape seed (*Vitis vinifera* L.) oil. Effect of the operating conditions upon oil composition and antioxidant capacity. *Chemical Engineering Journal*, 160, 634–640.

Rossi, D., Guerrini, A., Bruni, R., Brognara, E., Borgatti, M., Gambari, R., Sacchetti, G. 2012. Trans-resveratrol in nutraceuticals: issues in retail quality and effectiveness. *Molecules*, 17(10), 12393–12405.

Scalvenzi, L., Grandini, A., Spagnoletti, A., Tacchini,M., Neill, D., Ballesteros Lara, J.L., Sacchetti, G., Guerrini, A. 2017. *Myrcia fallax* (Rich.) DC. (Myrtaceae) essential oil from Amazonian Ecuador: a chemical characterization and bioactivity profile. *Molecules*, 22(7), art. no. 1163.

Tacchini, M., Spagnoletti, A., Marieschi, M., Caligiani, A., Bruni, R., Efferth, T., ... Guerrini, A. 2015. Phytochemical profile and bioactivity of traditional ayurvedic decoctions and hydro-alcoholic macerations of *Boerhaavia diffusa* L. and *Curculigo orchioides* Gaertn. *Natural Product Research*, 29(22), 2071–2079.

Tardugno, R., Spagnoletti, A., Grandini, A., Maresca, I., Sacchetti, G., Pellati, F., Benvenuti, S. 2018. Chemical profile and biological activities of *Cedrelopsis grevei* H. Baillon bark essential oil. *Plant Biosystems*, 152(1, 2), 120-129.

Wagner, H., Bladt S., 2009. Plant drug analysis. A Thin Layer Chromatography Atlas, second Edition, Springer Verlag, Berlin.

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Chemical characterisation, antioxidant and antimicrobial screening for the revaluation of wine supply chain by-products oriented to circular economy

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ABSTRACT

Aim of the project was the bioassay guided optimisation of extraction methods applied to wine chain by-product to obtain extracts, fractions and biologically active biomolecules with a possible use in the nutraceutical and cosmeceutical industry. Exhausted red and white grape marc were extracted using water:ethanol 50:50 with ultrasound assisted extraction and Naviglio® technology; and also with supercritical fluid extraction (SFE) and steam-distillation obtaining different phytocomplexes. Each extract was characterised by different molecular category: exhausted red grape marc (VCR) by anthocyanins, exhausted white grape marc (VCB) by flavonoids, and grapeseed (VIN) by proanthocyanins. SFE and steam-distillation, instead, highlighted the presence of fatty acids and their ester in every matrix, but terpenoids were not revealed at level major or equal than 0.1%, except for manoyl oxide in VCR (2.89%). VIN was the most abundant matrix in polyphenols (506.24 ± 55.91 mg gallic acid/g dried extract), and it showed the highest antioxidant activity (IC_{50} of 4.30 μg/mL). Regarding the antimicrobial activity, the hydroalcoholic extracts from VCR, VCB and VIN were tested but no noteworthy activities have been recorded.

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Waste-revaluation; exhausted grape marc (EGM); ultrasound; Naviglio®; steam-distillation; supercritical fluid extraction (SFE); antioxidant; antimicrobial

Introduction

The constant increase in the anthropic impact on the environment in terms of pollution and exploitation of resources has stimulated governments and research institutions to focus on new production approaches, consolidating the new paradigm of the circular economy, concretized industrially in the concept of bio-refinery. While the concept of circular economy can be defined as a self-sustaining system, the term bio-refinery concretizes the same concept at the level of industrial production processes, in which the output yields are maximized, minimizing or eliminating the production of waste. In this context, what traditionally was considered a production waste becomes a secondary raw material converted into new marketable products using a panel of production strategies with low environmental impact (Lin et al. 2013). This new paradigm of research and production has generated and consolidated the interaction of knowledge and skills to make the reality of bio-refinery more and more concrete. The shift of industry towards greater sustainability, to improve cost-effectiveness, process efficiency and ecological credentials, makes the development of sustainable and innovative waste-re-use strategies economically viable. Waste produced by food processing companies is an example of a type of waste generated on a large scale and globally. This type of waste is becoming increasingly problematic, in some cases it can represent more than 50% of the total waste produced in the countries, of which at least 60–70% is made up by organic substances. In line with this data, the case of the wine industry is exemplificative: in 2016, the International Organisation of Vine and Wine (OIV) indicate that 55% of the global grape production (75.8 million of tons) was wine grape. The volume of the wine industry waste is comprehensibly of the same order of magnitude, it is estimated that for each 6 L of wine, 1 kg of grape pomace is produced (Mendes et al. 2013). The valorization of this secondary raw material – no longer waste – could be an opportunity for the industry to increase its competitiveness by converting its disposal costs into new profit strategies (new products). In general, the state of the art of exploiting agri-food waste essentially consists of two types of approaches (Lin et al. 2013): (a) first generation approach: the waste is sent to composting, used for energy production (anaerobic digestion) or transferred to landfill; (b) second-generation approach: the waste is directed towards processes characterised by high technological level, high sustainability and reduced environmental impact to obtain high value (bio) chemicals. Emerging evidences in the last 20 years have suggested that vinification waste can be a relevant source of useful molecules in the contest of human well-being. Therefore, among the plethora of recycle action implemented by the wine industry, the extraction and the recovery of phenolic compounds also took place (Louli et al.

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2004, Tournour et al. 2015). As mentioned above, one of the main solid by-products produced during the wine-making process is grape pomace, and this, in accordance to the European Council Regulation 1493/1999 on the common organisation of the wine market, must be sent to alcohol distilleries, producing alcohol and tartrates, obtaining another by-product, named exhausted grape marc (EGM). In this contest, the aim of the research was the optimisation of the extraction methods applied to this by-product to obtain extracts and fractions, rich in biologically active biomolecules, with a possible use in the nutraceutical and cosmeceutical industry.

Materials and methods

Plant material

EGM from white (cultivar Trebbiano) and red (cultivar Lambrusco) Vitis vinifera L. was provided from Caviro Distillerie (Faenza, Ravenna – Italy) after being harvested in 2016. At the time of delivery, dealcoholized red and white EGM had, respectively, the 51% and the 36% moisture content. They were oven dried at 70 °C for 24 h until constant weight with a high-performance oven (mod. 2100), milled through a 2-mm sieving ring of a Variable Speed Rotor Mill (Fritsch, Germany) and immediately stored at −20 °C until further use. Part of the marc samples, either white (VCB) or red (VCR), was not dried but immediately steam distilled to evaluate the quality and quantity of the volatile component.

Chemicals

All the solvents and reagents employed for analyses were chromatographic grade. Standard malvidin-3-O-glucoside was purchased from Extrasynthese (Genay, France). Trolox, DPPH (1,1-diphenyl-2-picrylhydrazil), methanol deuterate, chloroform deuterate, methanol, ethyl acetate, ethanol, formic acid, acetic acid, toluene, natural products-polyethylene glycol reagents (NP/PEG) and gallic acid were purchased from Sigma-Aldrich Italy (Milano, Italy).

Ultrasound-assisted extraction of EGM and grapeseed

Ultrasound-assisted extraction was performed in an ultrasonic cleaning bath (Ultrasonik 104X, Ney Dental International, MEDWOW, Cyprus) under a working frequency of 48 kHz. Fifteen grams of each sample were placed into a volumetric flask (200 mL), filled with 195 mL of a 50% ethanolic solution as extraction solvent and sonicated for 80 min at room temperature (solvent/solid ratio of 13 mL/g of dried pomace). The extracts were filtered and lyophilized.

Naviglio® extractions of EGM and grapeseed

Naviglio® extractor (Atlas Filtri, Italy) was used to extract solid material with a pressurized solvent extraction method (Naviglio 2003). Briefly, 30 g of each samples were placed in a bag made of 60 Lm filtering membrane and transferred into the chamber of the Naviglio extractor, and 400 mL of a 50% ethanolic solution were added. Static phase was set for 5 min, while the dynamic phase was set for 3 min for a total extraction time of 80 min to complete 10 cycles. Each extraction was made in triplicate.

Supercritical fluids extraction of EGM and grapeseed

Samples were subjected to supercritical fluid extraction (SFE) using an Applied Separations (Allentown, PA) model Speed SFE extractor. Extractions were performed on each type of solid matrix (2 g) under the following operating conditions: carbon dioxide flow-rate of 2.5 L/min; oven temperature was set at 42 °C, restrictor temperature at 62 °C, and pressure at 150 atm.

Steam distillation of EGM and grapeseed

Fresh EGM (100 g) was used to obtain essential oils by 4 h steam distillation (DIS) with a Clevenger apparatus according to European Pharmacopoeia methods. The extract yield was determined on a volume to dry weight basis, obtaining the data reported in Table 1. The samples were dried over anhydrous sodium sulphate and stored in glass vials with Teflon-sealed caps at −18 ± 0.5 °C in the absence of light until analysis.

HPTLC analysis of the extracts

Analyses were performed on a high-performance thin layer chromatography (HPTLC) silica gel $60F_{254}$ (10 cm × 20 cm) glass plate (Camag, Swizerland); 50% ethanolic solution of the extracts (20 mg/mL) were applied using Linomat V (Camag, Swizerland). Spots were eluted in two steps with different eluents. First step: ethyl acetate/formic acid/acetic acid/water (100/11/11/20), second step: toluene/ethyl acetate/acetic acid (100/90/10), in two chromatographic chambers (Wagner and Bladt 2009). After development, the chromatogram was derivatized with NP/PEG solution (Wagner and Bladt 2009). The developed plate was dried at room temperature visualized with the Visualizer (Camag, Swizerland).

HPLC analysis of EGM and grape seeds extracts

The analyses of anthocyanins in VCR were performed using a Waters modular HPLC system (MA, model 1525) coupled to a diode array detector (model 2998) linked to a 20-LL sampler loop. The column used was a Luna C18 column (260 mm

Table 1. Procyanidins characterised by ESI-MS^2 in VIN extracts.

Compounds	$[M-H]^-$ (m/z)	MS/MS (m/z)
Epi/catechin	289	
Procyanidin dimer B	577	533, 439, 425, 269
Procyanidin dimer gallate	729	577, 451, 425, 289
Procyanidin trimer	865	739, 695, 577, 407
Procyanidin dimer digallate B	881	729, 577, 559, 407
Procyanidin trimer gallate	1017	865, 729, 695, 577
Procyanidin tetramer	1153	865, 983, 695, 577
Procyanidin trimer digallate	1169	1017, 881, 729, 577
Procyanidin tetramer digallate	1457	1306, 1153, 865, 729

×4.6mm, 5 mm; Phenomenex) at a flow rate of 1.0 mL/min. The mobile phase consisted of solvent solution B (methanol, water and formic acid, 50:40:10 v/v/v) and A (water and formic acid 90:10). The gradient system adopted was a suitably modified version of the one described by Favretto and Flamini (2000). Briefly (1) B raised progressively from 15 to 45% in 25 min; (2) B then raised to 70% at 45 min; (3) B achieved 90% at 55 min and 99% at 60 min. Individual stock solutions of standards of anthocyanins were prepared in methanol acidified with 0.5% HCl. Six calibration solutions were prepared within the range: 10•100 µg/mL. Each calibration solution was injected into HPLC in triplicate. The calibration graphs were provided by the regression analysis of peak area of the analytes versus the related concentrations (Table 2). The characterisation of flavonoids in VCB was performed using an Eclipse-PLUS-C18 (250 mm × 4.6mm, 5 mm; Phenomenex) column, at a flow rate of 1.0 mL/min. Conditions are reported in Tacchini et al. (2015). Following chromatogram recording, sample peaks identification was carried out by comparison of UV spectra and retention time with those of pure standards. The analyses of the phytocomplexes (10 mg/mL) were performed under the same experimental conditions. The obtained calibration graphs allowed the determination of the concentration of the three components. Three batches of extractions were tested.

GC-MS analysis

The samples obtained from steam-distillation were checked with NMR (data not shown) that evidenced a relevant amount of free fatty acids. For this reason, an aliquot of few milligrams of the extracts have been mixed with 200 µL of BSTFA (1% TMCS) (bis(trimethylsilyl)trifluoroacetamide + trimethylchlorosilane) (Sigma-Aldrich) for 45 min at 80 °C with the purpose of obtaining the trimethylsilylethers (TMS) of free fatty acids. Then 1 µL of solution was directly injected in gas chromatography (GC). GC analysis was performed by a Varian GC-3800 gas chromatograph equipped with a Varian FactorFour VF-5ms poly-5% phenyl-95%-dimethyl siloxane bonded phase column (i.d., 0.25 mm; length, 30 m; film thickness, 0.25 mm), a Varian MS-4000 mass spectrometer using electron impact (EI) and hooked to NIST library. TMS were identified by comparing their GC retention time and the MS fragmentation pattern with those of pure components (Sigma-Aldrich), the methylesters of fatty acids with 37 pure component FAME mix (Supelco, Sigma-Aldrich), and others molecules matching the retention indices (AI) with those in the literature (Adams 2007). Operating conditions are reported in Radice et al. 2014. Briefly: injector temperature, 300 °C; carrier (helium) flow rate, 1,2 mL/min and split ratio, 1:50. Oven temperature was initially increased from 130 °C to 200 °C at a rate of 1 °C/min, then from 200 °C to 250 °C at a rate of 5 °C/min and from 250 °C to 320 °C at a rate of 10 °C/min, finally for 3 min the temperature was maintained at 320 °C. The MS conditions were: ionization voltage, 70 eV; emission current, 10 mAmp; scan rate, 1 scan/s; mass range, 29•600 Da; trap temperature, 150 °C, transfer line temperature, 300 °C. One microliter of each sample was injected. Samples were analysed in GC-FID (GC-flame ionization detector) for quantitative determination through the normalization method, without using correction factors: the relative peak areas for individual constituents were averaged on three different chromatograms of three independent reactions. The relative percentages were determined using a ThermoQuest GC-Trace gas-chromatograph equipped with a FID detector maintained at 300 °C; all the others GC conditions were the same of GC-MS method.

Table 2. Statistical data of the evaluation of linearity regression, LOD and LOQ in the anthocyanins quantification by RP-HPLC-DAD.

Compound	R^2	Regression equation Linearity range 10-100 µg/mL	LOD µg/mL	LOQ µg/mL
Cyanidin-3-O-glucoside	0.9981	y = 63632x + 185474	0.28949	0.87726
Delphinidin-3-O-glucoside	0.9990	y = 29673x-16816	0.53933	1.63434
Malvidin-3-O-glucoside	0.9928	y = 65789x-126525	0.40976	1.24171

Mass spectroscopy

Lyophilized extracts were dissolved in a 50% ethanol•water solution and directly infused in a Mass Spectrometer Thermo Finningan (Ringoes) ESI Q-Duo linear trap set as follow: spray voltage 4,5 KV, sheath gas flow rate 20, capillary voltage 10 V, capillary temperature 160 °C and ion negative and positive modes. The samples were opportunely diluted and filtered before infusion. Opportune collision energies were used for the fragmentation and the simultaneous monitoring of the parent ions. The same conditions were applied for the mass analysis after chromatographic separation. Three batches of extractions were tested.

Total polyphenols quantification

The determination of the total polyphenolic contents in active extracts were performed using a ThermoSpectronic Helios-γ spectrophotometer and performed according to previously described methods (Rossi et al. 2012; Tacchini et al. 2015). The formers results are expressed as milligram of gallic acid per gram of crude. Each experiment was made in triplicate.

DPPH assay, evaluation of the antioxidant properties of ethanolic extracts

The DPPH assay was performed following the method by Cheng et al. (2006). After 30 min of incubation in the dark at room temperature, the microplates were analysed with a microplate reader (Biorad, 680 XL) and the absorbance was read in triplicate against a blank at 515 nm. The DPPH inhibition in percentage was determined by the following formula: IDPPH% = [1 • (A1/A2)] × 100; where A1 was the DPPH absorbance with the extracts and A2 without extracts. Eight different concentrations (range 20•0.16 µg/mL) of Trolox were prepared and used as positive control. Antioxidant activity of the extract was expressed as IC_{50}, concentration providing 50% inhibition of the radical, and calculated as described in Nostro et al. (2016). All experiments were assessed in triplicate and values were reported as mean-± standard deviation.

Antimicrobial activity

The antimicrobial activity has been evaluated against phytopathogenic fungi and bacteria to preliminarily verify phytoiatric properties. **Pseudomonas syringae** pv. **syringae** van Hall ATCC 19310 was used to evaluate the antibacterial activity. The experiments (minimum inhibitory concentration [MIC] and minimal bactericidal concentration [MCB]) were set up following the indications given by the National Committee for Clinical Laboratory Standards (NCCLS, standard M7-A6). For the evaluation of the antifungal activity, **Sclerotinia minor** and **Sclerotinia sclerotiorum** were used. The preparation of the filamentous fungi cultures and the experiments of antifungal activity were carried out following the indications reported in Guerrini et al. (2009). The MIC of fungal growth and minimum fungicide concentration were determined following the indications reported in Cavaleiro et al. (2006). Finally, the effective concentration able to give a 50% maximal effect (EC_{50}) was calculated. Each experiment was made in triplicate.

Results

Extraction yield and total polyphenols

The first target of the study was the isolation and quantitation of polyphenols, and the solvent system that showed the best compromise between water-soluble and alcohol-soluble molecules, obtaining the richest fingerprinting, resulted to be water:ethanol 50:50 (Figure 1). As reported in Table 3, ultrasound assisted extraction (UAE) showed the highest yields among the extraction processes: 25.13 ± 3.71% against 10.42 ± 2.38% of NAV for VCR and 27.59 ± 1.68% against 13.42 ± 6.25% for VCB.

On the other side, the extraction yields achieved by SFE and DIS were much lower than the previous, with the highest value obtained by VIN for SFE (8.36%) and VCR for DIS (0.05%). The UAE extraction was more performing than NAV regarding the total extraction yield and the total polyphenol content (Table 3), except for VIN, in which NAV method showed the same efficiency. The polyphenols richest samples were the extracts of grapeseed performed with both methods. Moreover, Student t test indicated significant differences in polyphenols content between the grape pomace extracts and grape seeds extract ($p = .0006$), and this was also valid for the antioxidant activity evaluation ($p = .0339$).

HPLC/MS analysis of UAE and NAV extracts

The mass spectrometry (MS) analysis, in positive-ion mode, and the reversed phase liquid chromatography (RP-HPLC-DAD) performed on VCR extractions highlighted the presence of anthocyanins, in particular: malvidin-3-O-glucoside (**m/z** 493), the most abundant; peonidin (**m/z** 301), malvidin (**m/z** 331), cyanidin-3-O-glucoside (**m/z** 449), peonidin-3-O-glucoside (**m/z** 463), delphinidin-3-O-glucoside (**m/z** 465), malvidin-3-O-(6-O-acetyl)glucoside (**m/z** 535) and malvidin-3-O-(6-O-p-coumaroyl)glucoside (**m/z** 639). Being the malvidin-3-O-glucoside the most abundant molecule (it had a relative abundance of 12,68% and 12,05% respectively in UAE and NAV), we expressed the quantification of the whole unknown anthocyanins fraction in grams of malvidin-3-O-glucoside/100 g of crude extract (Table 4).

As regards VCB extracts (both UAE and NAV), the most abundant molecular categories were represented by flavonols. The RP-HPLC-DAD analysis, confirmed by MS, highlighted the presence of seven main flavonoids: three glycosylate derivatives of quercetin (quercetin-3-O-rutinoside, quercetin-3-O-glucuronide and quercetin-3-O-galactoside), two glycosylate derivatives of kaempferol (kaempferol-3-O-rutinoside, kaempferol-3-O-glucoside) and the two free aglycons (Figure 2). The MS and the HPTLC analyses permitted to discriminate the co-elution of quercetin-3-O-glucuronide and quercetin-3-O-galactoside (data not shown).

The VIN extracts were mainly characterised by procyanidins (PAs). The PAs were identified by direct infusion of the extracts in electrospray ionization-MS, negative-ion mode without performing chromatographic separation. The analysis highlighted the presence of epi/catechin monomer and oligomers (Table 1). Each molecule was confirmed by MS/MS analyses.

GC-MS analysis of SFE and DIS extracts

To complete the overview of these exhausted matrix content, the lipophilic part of the biomass was investigated by SFE and DIS followed by GC-MS analyses. Results of the chromatographic analysis performed on SFEs extracts (data not shown) highlighted the presence of the characteristics fatty acids of grape seeds: linoleic, elaidic, oleic and stearic acid were the most abundant; and myristic, palmitoleic, 11-

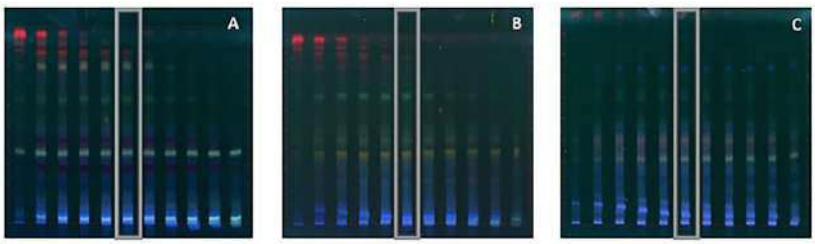

Figure 1. HPTLC analyses of the extractions of red grape (A), white grape (B) and grapeseed (C) performed with different percentages of ethanol•water (from left to right, form 100% ethanol to 100% water in step of 10%, for each matrix).

eicosanoic and arachidonic acids were detected in smaller quantities.

GC-MS analysis performed on the DIS extracts confirmed the data obtained by SFE, highlighting samples predominantly characterized by fatty acids (Table 5). All presented samples showed a substantially similar qualitative profile, while from a relative quantification point of view some differences emerged: in VCR, the most abundant compounds (expressed per area%) were palmitic acid (53.69%), myristic acid (7.60%), lauric acid (6.13%) and oleic acid (5.21%); while VCB exhibited the highest quantity of palmitic acid (43.04%), palmitoleic acid (7.01%), oleic acid (5.72%) and myristic acid (5.03%). The distillate of VIN exhibited a chemical profile mainly characterized by palmitic acid (29.60%), linoleic acid ethyl ester (19.4%), palmitic acid ethyl ester (11.1%), linoleic acid (10.48%) and oleic acid (6.86%).

Table 3. Yields % of the extraction processes, spectrophotometric quantitation of the total phenolic content and evaluation of antioxidant activity through DPPH test (expressed as half maximal inhibition concentration, IC_{50}).

Sample	Extraction methods	Extraction yield (%)	Total phenolic content (milligram gallic acid per gram of dried extract)	Antioxidant activity • DPPH • IC50 (μg/mL)
VCR	UAE	25.13 ± 3.71	189.11 ± 5.95	10.99 ± 1.74
	NAV	10.42 ± 2.38	159.58 ± 1.57	15.31 ± 4.95
VCB	UAE	27.59 ± 1.68	116.44 ± 3.49	22.44 ± 2.30
	NAV	13.41 ± 6.25	106.11 ± 5.46	20.82 ± 3.72
VIN	UAE	11.61 ± 4.39	446.72 ± 22.16	5.44 ± 0.40
	NAV	9.52 ± 0.76	506.24 ± 55.91	4.30 ± 0.31

Table 4. Anthocyanins quantification by RP-HPLC-DAD, expressed as milligram of standards per gram of dried extract.

	Red grape (mg/100g dried extract)	
	VCR NAV	VCR UAE
Cyanidin-3-O-glucoside	14.50 ± 0.64	15.88 ± 1.03
Delphinidin-3-O-glucoside	108.60 ± 4.80	102.10 ± 9.50
Malvidin-3-O-glucoside	259.40 ± 14.62	276.80 ± 12.10
Unknown anthocyanins	1770.07 ± 115.03	1788.08 ± 109.11
Total	2152.67	2182.86

The unknown anthocyanins are quantified as malvidin-3-O-glucoside.

Antioxidant and antimicrobial evaluation

Extracts of EGM (VCR, VCB) and grape seeds (VIN) have been preliminarily evaluated for the IN VITRO antioxidant (DPPH assay), and antimicrobial properties. Regarding the antioxidant properties (property of sure interest for future possible

Table 5. Chemical analyses of VCB, VCR and VIN steam distillation by GC-MS and GC-FID, expressed in area % of the total.

Compound[a]	VCR %	VCB %	VIN %	ID method[b]
Caprylic acid ethyl ester	•	0.15	•	GC-MS, AI
3-Octenoic acid	•	0.11	•	GC-MS
Caprylic acid	0.28	0.93	•	GC-MS, Co-GC
Nonanoic acid	•	0.16	•	GC-MS, Co-GC
Capric acid	0.21	1.29	0.14	GC-MS, Co-GC
9-Oxo-nonanoic acid ethyl ester	0.41	1.54	•	GC-MS
Lauric acid, ethyl ester	0.47	0.29	•	GC-MS
Lauric acid	6.13	2.12	2.23	GC-MS, Co-GC
Octanedioic acid	•	0.22	•	GC-MS
Myristic acid ethyl ester	0.74	0.43	0.30	GC-MS, AI
Azelaic acid	0.66	2.78	•	GC-MS
6,10,14-trimethyl-2-pentadecanone	0.18	0.30	0.13	GC-MS, AI
Myristic acid methyl ester	•	•	3.93	GC-MS, Co-GC
Myristic acid	7.60	5.03	•	GC-MS, Co-GC
Cis-10-pentadecenoic acid	0.13	0.76	0.14	GC-MS, Co-GC
n-Pentadecanoic acid	0.86	1.23	0.33	GC-MS, Co-GC
Palmitoleic acid ethyl ester	0.28	2.74	0.67	GC-MS
Manoyl oxide	2.89	1.85	•	GC-MS, AI
Palmitic acid ethyl ester	7.78	5.42	11.10	GC-MS, AI
Palmitoleic acid	0.58	7.01	1.44	GC-MS, Co-GC
Palmitic acid	53.69	43.04	29.60	GC-MS, Co-GC
Linoleic acid methyl ester	1.25	0.38	0.27	GC-MS, Co-GC
Oleic acid methyl ester	1.94	1.88	0.21	GC-MS, Co-GC
Linoleic acid ethyl ester	•	•	19.40	GC-MS
Linolenic acid ethyl ester	•	•	2.60	GC-MS
Oleic acid ethyl ester	•	•	7.24	GC-MS
Elaidic acid ethyl ester	•	0.42	0.35	GC-MS
Stearic acid ethyl ester	0.43	0.80	1.25	GC-MS, AI
Linoleic acid	2.42	0.99	10.48	GC-MS, Co-GC
Oleic acid	5.21	5.72	6.86	GC-MS, Co-GC
Elaidic acid	0.36	0.94	0.19	GC-MS, Co-GC
Stearic acid	0.90	1.64	0.33	GC-MS, Co-GC

[a]Free fatty acids were determined as trimethylsilylethers.
[b]GC-MS: gas-chromatography-mass spectrum; Co-GC: co-injection with authentic compound; AI: experimental retention indices compared with literature.

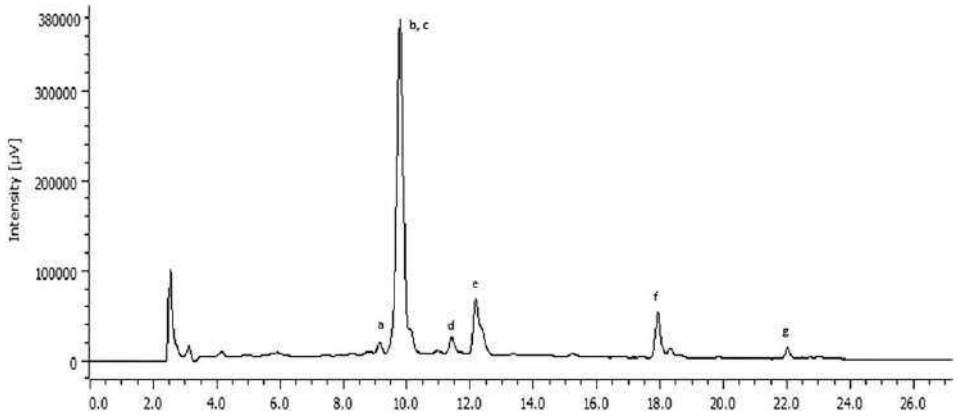

Figure 2. RP-HPLC-DAD chromatogram of the white grape extract, indicating the presence of quercetin-3-O-rutinoside (a), quercetin-3-O-gucoronide (b), quercetin-3-O-galactoside (c), kaempferol-3-O-rutinoside (d), kaempferol-3-O-glcoside (e), quercetin (f) and kaempferol (g).

Table 6. Antimicrobial activity of hydroalcoholic extracts of pomace from red and white grapes (VCR, VCB) and grape seeds (VIN).

	Antibacterial (MIC mg/mL)	Antifungal (EC_{50} mg/mL)	
	Pseudomonas syringae pv. syringae	Sclerotinia minor	Sclerotinia sclerotiorum
VCR UAE	>1000	n.d.	n.d.
VCR NAV	>1000	n.d.	n.d.
VCB UAE	>1000	n.d.	n.d.
VCB NAV	>1000	n.d.	n.d.
VIN UAE	>1000	>15	>15
VIN NAV	>1000	>15	>15
Positive Control	125	<0.5	<0.5

The results are expressed as MIC growth (μg/mL) for antibacterial activity and as an effective concentration able to give 50% maximal effect (EC_{50}) as regards the antifungal activity. n.d.: not defined; the strain showed a strong increase in growth instead of an inhibition, probably due to the sugary component of the extracts. Positive control: Delan 70 WG, per Pseudomonas syringae pv. syringae); Heliocuivre S, per Sclerotinia sp.

nutraceutical and cosmetic projections), the hydroalcoholic extracts obtained with the NAV and UAE method were considered because they are characterized by the most abundant polyphenolic component and normally taken as reference for expressing antioxidant activity (Table 3).

As far as the antimicrobial activity is concerned, the hydroalcoholic extracts from VCR, VCB and VIN matrices were tested (Table 6). As a fact, no noteworthy activities have been recorded. On the contrary, with reference to antifungal activity, an increase in growth was observed.

Discussion

The selection and optimization of the extraction processes are key factors for the valorization of secondary raw materials of the agro-food supply chains with respect to a bio-based industrial profile and circular economy. For these reasons, the choice of the extraction methods was driven by efficiency criteria (high yield), sustainability (low or no use of organic solvents) and technological transfer opportunities (scale up from laboratory towards industry), but they were also selected considering the type of vegetable matrix, its condition and size (powdered or not), and the type of target biomolecules that would characterise the new finished products. In relation to these aspects, it was decided to glean in the ample and modern context of the **green chemistry** the UAE, the pressurised fluid extraction with Naviglio® technology (NAV), the supercritical CO_2 extraction (SFE) and the steam distillation (DIS) (Baiano 2014).

The first two extraction methods (UAE and NAV) were performed using water and ethanol in different proportions, in accordance to the principle of the green chemistry. Figure 1 highlighted how a 50:50 mixture had the highest affinity for the extraction of polyphenols, obtaining extracts fingerprinting that showed the best compromise between water-soluble and alcohol-soluble molecules for all production waste of the wine production chain. Regarding the extraction yield, the UAE method was the most performing for all matrixes, except for VIN extracts, were the results of UAE was in line with the data obtained by the NAV extraction (9.74 ± 1.47% of UAE compared with 9.52 ± 0.76% of NAV). Going more in detail, VIN NAV extract obtained the lowest extraction yield, but, in comparison between extraction yield and quantity of total polyphenols where VCR and VCB showed a direct correspondence between extraction yield and polyphenol content, VIN NAV extract differs from this trend, showing the highest polyphenol content. The high abundance of PAs that characterise these extracts (Table 1) could explain these results. This molecular category is well known for its use in nutritional supplements, functional foods, in the cosmetic and pharmaceutical products (Nagasako-Akazome 2014; Xia et al. 2014; Panickar 2015; Martinez et al. 2017) and these extraction strategies could implement the revaluation of this by-product, making it a new profitable resource. VCR extracts, in turn, showed higher polyphenolic content than VCB samples, with an average difference of 39%, most probably because of the presence of anthocyanins. This is by far the most characterising molecular category of VCR extraction, and both extraction strategies considered (UAE and NAV) showed the same specificity towards these compounds (Table 4), in the range of the standard deviation. VCB extracts, instead, exhibited the presence of flavonols, most probably because white grape varieties do not undergo maceration during winemaking, leaving part of these molecules in the vinification wastes. Although the literature reports numerous examples of polyphenolic quantification of extracts obtained by grape wastes compatible with those considered in this article (Tournour et al. 2015; Ferri et al. 2016; Trikas et al. 2016), it is not possible to make a constructive comparison because of the grape cultivar chosen for the research, and because it is difficult to identify the point of the supply chain where they come from.

Considering the extractions of the lipophilic part of the vinification by-products achieved by SFE and DIS, they showed extractions yields much lower than the previous obtained by UAE and NAV (e.g. 0.05% was the highest yield among all the waste matrixes), making the hypothesis of industrial scale-up of these processes substantially impractical. With regard to the steam distillation of the considered by-products, there are no literature data that allowed us to compare these results; however, it is possible to make some considerations with respect to the predetermined target. The expected phytocomplex would be mainly characterised by terpenoids, whereas these compounds were present in small quantities just in VCR with a relative presence of 2.89%. This aspect could probably be explained by the fact that the fresh pomace, before being supplied for the analyses, had undergone conditions for which most of the volatile components had disappeared. Instead, the fatty acid composition was both qualitatively and quantitatively richer than the extracts obtained by SFE. Since the samples extracted with SFE had been dried, while the same distilled samples had been extracted fresh (hoping to extract the volatile terpene component), it is possible to hypothesize that the pre-treatment in stove at 70 °C degraded most of the fatty acid component, which remained in the fresh matrices instead.

In the preliminary evaluation of the antioxidant activity with DPPH assay, VIN NAV extract showed the highest antioxidant capacity and it showed a directly correlation with the polyphenols content, since the richest samples in

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Polyphenols From *Vitis vinifera* Lambrusco By-Products (Leaves From Pruning): Extraction Parameters Evaluation Through Design of Experiment

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Abstract

Vitis vinifera L. leaves from pruning are by-products of the wine industry and represent an important source of secondary raw material, thanks to their polyphenols content. Optimization of the extraction processes is a key factor for their valorization, and Design of Experiment (DOE) could be a tool to obtain the most performing extract in terms of polyphenols quality/quantity and bioactivity. *Vitis vinifera* Lambrusco leaves were subjected to ultrasound-assisted extractions guided by a 2^3 factorial design. Three independent parameters (% solvent, time of extraction, and solvent:solid ratio) were considered to evaluate the extraction process by analyzing the extraction yield, the total phenolic content (Folin-Ciocalteu assay), and the antioxidant capacity (DPPH assay). Moreover, the content of the main molecules was identified and quantified by reversed-phase high-performance liquid chromatography coupled with diode array detection and mass spectrometry. The DOE highlighted the best extraction conditions that showed slight changes considering the different evaluating parameters. The highest extraction yield was obtained by extraction with 100% water, 60 minutes of extraction time, and 30:1 solvent:solid ratio, but it was neither the richest in polyphenols nor antioxidant capacity. The latter 2 characteristics were associated with the extraction performed using 50% ethanol, 35 minutes of extraction time, and a 20:1 solvent:solid ratio. That extract also exhibited the highest quantity of flavonols.

Keywords

polyphenols, ultrasound-assisted extraction, design of experiment, chromatography, grape leaves, lambrusco, DPPH, by-products

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The concept of circular economy can be defined—in synthetic, but certainly exhaustive terms—as a self-sustaining system in which the output yields are maximized, minimizing the production of waste. What traditionally is considered a waste becomes a by-product, a secondary raw material converted into new marketable products using a panel of sustainable strategies with low environmental impact with respect to the circularity of the organic economy.[1] In this context, *Vitis vinifera* L. (Vitaceae) leaves from pruning are a promising example of a by-product rich in high-value biomolecules that show interesting biological activities.[2-6] Among the various examples found in the literature, the antioxidant capacity exhibited by polyphenols is well documented, and, *in vivo*, seems also to be closely related to vital biological functions such as antimutagenicity, anticarcinogenity, and anti-aging.[7,8] Flavonoids, in particular, are known to exhibit antioxidant properties and, therefore, they represent essential elements in a well-balanced human diet. Moreover, this molecular category showed promising effects in agriculture, controlling weed growth, insect pests, and spread of diseases.[9] To obtain a rich flavonoids extract, the present study considered a by-product of the wine industry represented by the leaves from the pruning of *V. vinifera* Lambrusco. The key factors of the project were the selection and optimization of the extraction processes. In this

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Table 1. Matrix of the Experimental Design 2^3 (Coded and Real Values) With Responses in Terms of Extract Yield, TPC, and Antioxidant Activity.

Extract	Solvents ratio (EtOH:H_2O)	Extraction time (minutes)	Solvent: solid ratio	Extract yield (%)	TPC (mg gallic acid equivalent/g of dried extract)	Antioxidant activity (IC_{50}, μg/mL)
1	-1 (0:100)	-1 (10)	-1 (10:1)	14.0 ± 0.6	106.3 ± 0. 9	25.4 ± 2.3
2	1 (100:0)	-1 (10)	-1 (10:1)	2.7 ± 0.2	103.3 ± 2.7	68.4 ± 1.7
3	-1 (0:100)	1 (60)	-1 (10:1)	14.0 ± 0.4	101.0 ± 2.6	21.3 ± 1.7
4	1 (100:0)	1 (60)	-1 (10:1)	3.9 ± 0.2	89.1 ± 1.0	32.8 ± 0.8
5	-1 (0:100)	-1 (10)	1 (30:1)	17.4 ± 0.9	98.1 ± 1.1	21.2 ± 0.6
6	1 (100:0)	-1 (10)	1 (30:1)	4.0 ± 0.3	112.6 ± 2.9	54.8 ± 3.4
7	-1 (0:100)	1 (60)	1 (30:1)	18.6 ± 0.4	110.7 ± 1.4	22.5 ± 2.4
8	1 (100:0)	1 (60)	1 (30:1)	5.7 ± 0.3	109.5 ± 1.7	43.1 ± 3.0
9	0 (50:50)	0 (35)	0 (20:1)	16.3 ± 0.7	180.7 ± 6.2	7.9 ± 0.1
10	0 (50:50)	0 (35)	0 (20:1)	16.3 ± 0.4	186.9 ± 2.1	7.7 ± 0.1
11	0 (50:50)	0 (35)	0 (20:1)	14.5 ± 1.9	164.0 ± 3.0	8.6 ± 1.0

TPC, total polyphenolic content. IC_{50}, half-maximal inhibitory concentration.

perspective, ultrasound-assisted extraction (UAE) using water, ethanol, and their mixture was the chosen method, guided by a Design of Experiments (DOE), to improve the quality of the product and efficiency of the process. DOE enabled the estimation of the influence of 3 independent variables on the result: solvent composition (%), solvent/solid ratio, and time of extraction (minutes), in order to evaluate the effects of the extraction process parameters to maximize yields, polyphenols content, and, in consequence, increment the biological activity in terms of antioxidant capacity. Moreover, these targets were achieved following the guidelines of green chemistry oriented to the minimization of the use of organic solvents, the disposal of which would represent enormous economic and environmental costs.[10]

The results reported in Table 1 highlight the significant yield difference between the extractions of the leaves obtained by modifying the parameters. Analyzing the data with the aid of the Pareto chart (Figure 1a), the ethanol-to-water ratio showed a significant negative effect, indicating that the absence of ethanol led to higher yields. However, the solvent-to-solid ratio exhibited a significant positive effect, showing that when a higher ratio is used the yield increases. Time, however, did not show a significant effect. The maximum yield (18.6%) was achieved by the extraction performed with 100% water, the highest solvent/solid ratio (30:1) and the longest extraction time (60 minutes). On the other hand, the phytocomplexes obtained using the parameters at the central point of the research (extracts 9, 10, and 11) showed a slightly lower extraction yield (16.3%) compared with the aqueous extracts, most likely due to the increase of ethanol in the solvent mixture and to the decrease in the solvent/solid ratio. However, the lower extraction yield of the latter extracts was balanced by the highest sensitivity toward polyphenols extraction (Table 1).

The Pareto chart describing the effect of the studied variables on the phenolic extraction (Figure 1b) indicated the solvent-solid ratio as the only variable capable of influencing the polyphenols concentration of the extracts. This variable had a significant positive effect ($P < 0.1$), showing that the increase of solvent to solid ratio led to higher phenolic contents. Nevertheless, the results reported in Table 1 displayed the highest phenolic content (186.9 mg GAE/g dried extract) in extract 10, performed with ethanol 50% (central points of the DOE), suggesting that polyphenols were more abundant using this solvent mixture. Therefore, the solvent % could have a

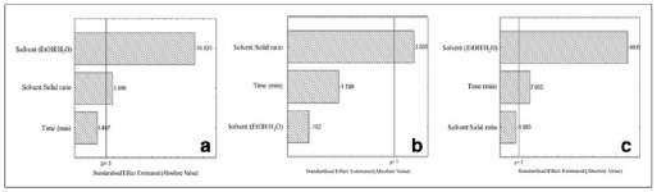

Figure 1. Pareto chart of the effects of the independent studied variables on (a) extract yield ($P < 0.1$); (b) quantification of TPC (mg gallic acid/g dried extract) ($P < 0.1$); (c) antioxidant activity expressed in μmol Trolox equivalent/1 g of dried extract ($P < 0.1$). Experimental data and conditions are shown in Table 1.

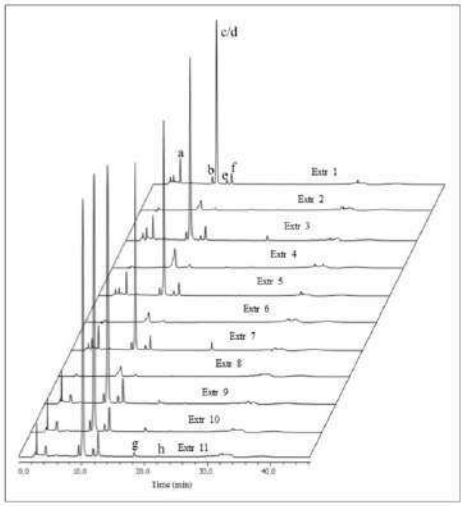

Figure 2. Reversed-phase high-performance liquid chromatography diode array detection chromatograms of the performed Lambrusco leaves extracts (Extr 1, 3, 5, and 7 in 100% water; Extr 2, 4, 6, and 8 in 100% ethanol; Extr 9, 10, and 11 in ethanol: water 50%). Peak a, caftaric acid; b, quercetin-3-*O*-rutinoside; c/d, coelution of quercetin-3-*O*-glucuronide and quercetin-3-*O*-glucopyranoside; e, kaempferol-3-*O*-rutinoside; f, kaempferol-3-*O*-glucoside; g, quercetin; h, kaempferol.

relevance in the polyphenols extraction. This hypothesis was supported by the results obtained by the total polyphenolic content (TPC) quantification of extracts 7 and 8 (highest solvent-solid ratio and highest time of extraction, but, respectively, 100% water and 100% ethanol) that exhibited lower content than the extractions performed with 50% ethanol. To the best of our knowledge, the literature does not report extractions performed with the same conditions in terms of type of solvents, method, and time of extraction. Moreover, the leaves of this grape variety were never considered, even if it is among the ten most produced in Italy.[11]

After the quantification performed using spectrophotometric assay (Folin-Ciocalteu method), the composition of *V. vinifera* Lambrusco grape leaves extracts was analyzed by liquid chromatography coupled with a diode array detector (DAD) and mass spectroscopy (MS) detector (Figure 2).

Extracts were found to be rich in flavonoids, in particular glycosylated flavonols, but one phenolic acid was also detected. All the molecules' identification was based on the comparison of their retention times, mass, and UV spectral data, with those of standard compounds and published data. In order of elution, *trans*-caffeoyltartaric acid (caftaric acid) was the first to be identified. It exhibited a single charged ion at m/z 311 (ms^2 m/z 179), and an UV absorption with λ_{max} of 331 nm. Peak 2 showed an ion, in negative mode, at m/z 609, an ms^2 of 301, and a UV spectrum that could lead to a flavonoid profile. Comparing its retention time with that of the pure molecules and due to the results of the enrichment process, it was identified as quercetin-3-*O*-rutinoside. The major peak (Figure 2, peak c/d) presented 2 peaks in the ESI-MS spectra: m/z 477 and 463, indicating a probable coelution of 2 molecules. The ms^2 analyses highlight the same fragmentation pattern with the presence of the fragment at m/z 301 (main ms^3 fragments at m/z 243, 179, and 151) identified as quercetin, therefore indicating them as quercetin glycosides. Column chromatographic separation was performed to isolate the compounds and make their identification possible. This approach does not, in most cases, separate complex mixtures of flavonoids present in crude plant extracts as well as other chromatographic techniques; nevertheless, where large quantities of the flavonoids are required, as in this case for the ^{1}H nuclear magnetic resonance (NMR) analyses, column chromatography is the method of choice. The chromatographic separation was able to isolate the 2 molecules in 1 fraction and the ^{1}H NMR analysis allowed the precise identification of the compounds as quercetin-3-*O*-β-glucuronide and quercetin-3-*O*-β-glucopyranoside. Peak 4 and peak 5 presented an m/z of 593 and 447, respectively. Their ms^2 analyses highlighted the same fragment at m/z 285. Comparing their retention times and UV spectra with those of 2 standards, they were identified as kaempferol-3-*O*-rutinoside and kaempferol-3-*O*-glucoside, respectively. To the best of our knowledge, kaemferol-3-*O*-rutinoside is here reported for the first time in extracts of *V. vinifera* leaves. At higher retention times, quercetin and kaempferol were identified, but not quantified because they were under the limit of quantitation (LOQ) in most of the extracts. Table 2 shows the data concerning the reversed-phase high-performance liquid chromatography diode array detection (RP-HPLC-DAD) quantification of the identified molecules. Caftaric acid and quercetin-3-*O*-β-rutinoside (rutin) were not quantifiable in the 100% ethanol extractions, and, therefore, the influence of the different parameters on their extraction could not be established by the statistical software. Nevertheless, while the yield of rutin exhibited its highest value in 50% ethanol and decreased in 100% water, those of caftaric acid reached their best in the 100% water extraction, reflecting the solubility behavior of the 2 molecules in the considered solvents.[12] The literature does not report results of analysis of by-products derived from this particular grape cultivar, but it presents data of chemical characterization of other grape leaf varieties. Moreover, each study described a different extraction method with different polyphenolic quantifications. Dresch et al[13] reported quercetin-3-*O*-glucuronide as the most abundant compound, but did not record the presence of kaempferol and its glycosylated derivatives. Pacifico et al,[14] instead, indicated the presence of other molecules compared with those

Table 2. Content of Caftaric Acid and Flavonols in *Vitis Vinifera* Lambrusco Leaves Expressed in Milligrams of Standard Per Gram of Dried Matrix.

	Extr.1	Extr.2	Extr.3	Extr.4	Extr.5	Extr.6	Extr.7	Extr.8	Extr.9	Extr.10	Extr.11
	Mg of standard / g of dried leaves ± SD										
caftaric acid	0.514 ± 0.001	-	0.637 ± 0.009	-	0.583 ± 0.005	-	0.830 ± 0.003	-	0.375 ± 0.018	0.428 ± 0.012	0.365 ± 0.012
quercetin-3-O-rutinoside	0.211 ± 0.019	-	0.246 ± 0.014	-	0.261 ± 0.013	-	0.333 ± 0.017	-	0.364 ± 0.007	0.435 ± 0.024	0.342 ± 0.005
quercetin-3-O-glucuronide / quercetin 3-O-galactoside	3.584 ± 0.395	0.098 ± 0.003	4.068 ± 0.280	0.284 ± 0.005	4.978 ± 0.220	0.158 ± 0.005	5.708 ± 0.235	0.206 ± 0.004	7.633 ± 0.052	8.070 ± 0.103	6.936 ± 0.021
kaempferol-3-O-rutinoside	0.189 ± 0.024	0.013 ± 0.000	0.262 ± 0.023	0.031 ± 0.000	0.276 ± 0.010	0.018 ± 0.000	0.368 ± 0.023	0.025 ± 0.000	0.438 ± 0.010	0.490 ± 0.012	0.404 ± 0.006
kaempferol-3-O-glucoside	0.295 ± 0.045	0.021 ± 0.001	0.380 ± 0.038	0.057 ± 0.000	0.470 ± 0.023	0.033 ± 0.000	0.543 ± 0.019	0.044 ± 0.001	0.779 ± 0.006	0.853 ± 0.026	0.713 ± 0.005

Experimental conditions are shown in Table 1.

identified in this research, most probably because of the use of a different solvent and a different grape variety. The Pareto chart, describing the effects of the independent studied variables on the antioxidant activity (Figure 1c), showed the significant contribution of the content of ethanol in the extraction solvent, and of the time of extraction in the evaluation of the bioactivity. The radical scavenging capacity is increased by the reduction of the ethanol proportion in the solvent mixture and by the increase of the extraction time. In effect, Lambrusco leaf extracts exhibited different bioactivity results (Table 1) when these 2 variables were modified, reaching the highest antioxidant activity with 50% ethanolic solution as solvent and 35 minutes of extraction time. In particular, extract 10, performed following the parameters described above, showed a half-maximal inhibitory concentration (IC_{50}) of 7.69 ± 0.06 µg/mL, in line with the value of the positive control that exhibited an IC_{50} of 3.83 ± 0.14 µg/mL. In contrast, extracts prepared with 100% ethanol exhibited the lowest antioxidant activity, particularly when subjected to 10 minutes of extraction (IC_{50} 68.40 ± 1.70 µg/mL), suggesting the need to use a percentage of water in the solvent mixture to obtain a richer extract in antioxidant compounds. On the other side, the sole use of water as extraction solvent gives better results when compared with the extraction performed with 100% ethanol, showing IC_{50} values for the radical scavenging activity ranging from 21.24 to 25.42 µg/mL, but still far from the values obtained with 50% ethanol. As reported in Table 2, aqueous extracts are characterized by the highest contents of caftaric acid, molecules with known antioxidant activity, which could be partially responsible for the bioactivity.

In contrast, the low activity exhibited by the 100% ethanol extracts could be due to the low quantity of this compound and of quercetin-3-O-rutinoside (both under the limit of detection [LOD]), molecules present in every other extract. To the best of our knowledge, no data on the radical scavenging activity of *V. vinifera* Lambrusco leaves' ultrasound-assisted extracts are present in the literature, but studies reporting the activity of leaf extracts of other grape varieties showed higher IC_{50} values (therefore lower activity) if compared with the present results. Férnandes et al[15] evaluated the antiradical capacity of *V. vinifera* leaves with DPPH assay and found an IC_{50} between 148 and 780 µg/mL; L. Pari and A. Suresh[16] reported a 50% inhibition of DPPH of 116.25 ± 2.74 mg/mL. The same paper showed a lower TPC compared with *V. vinifera* Lambrusco leaves, supporting the evidence that the extraction parameters of 50% ethanolic solution as solvent, 20:1 solvent/solid ratio, and 35 minutes of extraction time could be the most suitable for the extraction of antioxidant compounds from Lambrusco grape leaves.

Experimental

Plant Material

Vitis vinifera L. Lambrusco leaves were provided from CRPA Lab (Reggio Emilia, Italy) after being harvested in September 2016. Leaves were oven dried at 70°C for 24 hours until constant weight with a high-performance oven mod. 2100, milled through a 2 mm sieving ring of a Variable Speed Rotor Mill (Fritsch, Idar-Oberstein, Germany), and immediately stored at −20°C until further use.

Chemicals

All the solvents and reagents employed for analyses were chromatographic grade. Trolox, DPPH (1,1-diphenyl-2-picrylhydrazil), Folin-Ciocalteu reagent, methanol (≥99.9% for HPLC), acetonitrile (≥99.93% for HPLC), formic acid (98% - 100% for HPLC), ethyl acetate (≥99.8% for HPLC), acetic acid (≥99.9% for HPLC), deuterium oxide, deuterated methanol, gallic acid (TraceCERT), and silica gel were purchased from Sigma-Aldrich Italy (Milano, Italy). Ethanol absolute (≥99.8%

Table 3. Retention Time (RT), Calibration Range, Correlation Coefficient, Limit of Detection (LOD), and Limit of Quantitation (LOQ) of the Reversed-Phase High-Performance Liquid Chromatography Diode Array Detection Analysis.

Compound	RT	Calibration range (μg/mL)	Correlation coefficient (r^2)	LOD (μg/mL)	LOQ (μg/mL)
Caftaric acid	4.180	1-50	0.99934	0.403	1.221
Quercetin-3-*O*-rutinoside	9.153	1-50	0.99993	0.133	0.402
Quercetin-3-*O*-glucuronide	9.783	20-400	0.99841	4.372	13.247
Kaempferol-3-*O*-rutinoside	11.437	1-50	0.99979	0.228	0.692
Kaempferol-3-*O*-glucoside	12.217	1-50	0.99976	0.245	0.742

Normapur) was purchased from VWR International Srl (Milano, Italy).

Ultrasound-Assisted Extraction

UAE was performed with Ultrasonik 104X (Ney Dental International, Bloomfield, USA) under a working frequency of 48 kHz, at room temperature. Fifteen grams of dried and milled *V. vinifera* Lambrusco leaves were sonicated with various percentages of hydroethanolic solvents (100% water, 50% ethanol in water, 100% ethanol) for different times, following the DOE directions. A total of 11 extracts were obtained, and each extract was prepared in triplicate. They were then filtered through Whatman No. 40 paper under vacuum, and the solutions were lyophilized and stored at −20°C until analysis.

Design of Experiments

With the objective of determining experimental conditions to lead to a maximum extraction yield, a 2^3 factorial experimental design, including 3 repetitions at the central point, was carried out, totalling 11 runs.[17,18] The effects of ethanol-to-water ratio (0%-100%), solvent-to-solid ratio (10-30), and time (10, 60 minutes) were evaluated, keeping fixed the extraction temperature and the ultrasound working frequency. All the analyses were performed in triplicate. The software Statistica 10.0 (Statsoft Inc., Tulsa, Oklahoma, USA) was used to assist the design and the statistical analysis of experimental information, adopting a confidence level of 90% ($P < 0.1$). Data are present in Pareto charts that highlight the effects of the evaluated parameters.

Determination of TPC

The determination of the TPC in grape leaves extracts was determined using Folin-Ciocalteu reagent. Each was performed in triplicate using a ThermoSpectronic Helios-γ spectrophotometer (Waltham, Massachusetts, USA), according to a previously described method.[19] The results of TPC are expressed as milligram gallic acid equivalents per g of dry extract.

HPLC Analysis

Vitis vinifera leaves extracts were subjected to RP-HPLC analysis to identify and quantify their main phytomarkers. Their characterization was performed using a JASCO modular HPLC system (Tokyo, Japan, model PU 2089) coupled to an LCQ ion trap mass spectrometer (Thermo Finnigan, Ringoes, USA), and to a diode array apparatus (MD 2010 Plus). The HPLC was equipped with an injection valve with a 20 μL sampler loop. The column used was an Eclipse-PLUS-C18 (25 mm × 0.46 cm, 5 μm; Phenomenex, Bologna, Italy) at a flow rate of 1.0 mL/min. The mobile phase consisted of water and 0.5% of formic acid (A), and acetonitrile and methanol, in equal proportions, with 0.5% of formic acid (B); the gradient evolved from an initial condition of 25% B, to 60% B at 25 minutes, 100% B at 30 minutes; it remained isocratic for 5 minutes and returned to the initial conditions, for a total run duration of 45 minutes. The various peaks were identified by comparing their UV spectra and retention times with those of pure standards. Dedicated JASCO software (ChromNAV ver 2.02.01) was used to calculate peak area by integration.

Standard Solution and Calibration Procedure

Individual stock solutions of caftaric acid, quercetin-3-*O*-β-rutinoside, quercetin-3-*O*-β-glucuronide, kaempferol-3-*O*-β-rutinoside, and kaempferol-3-*O*-β-glucoside were prepared in methanol. Six different calibration levels were prepared within the following range: 1-50 μg/mL for caftaric acid, quercetin-3-*O*-β-rutinoside, kaempferol-3-*O*-β-rutinoside and kaempferol-3-*O*-β-glucoside, and 10-400 μg/mL for quercetin-3-*O*-β-glucuronide. Each calibration solution was injected into the HPLC in triplicate. The calibration graphs were provided by the regression analysis of peak area of the analytes versus the related concentrations. The analyses of the phytocomplexes (8 mg/mL) were performed under the same experimental conditions. Three batches of extractions were tested. LOD and LOQ were calculated following the approach based on the standard deviation of the response and the slope as presented in the "Note for guidance on validation of analytical procedures: text and methodology," European Medicine Agency ICH Topic Q2 (R1) (Table 3).[20]

Mass Spectrometry

The RP-HPLC-DAD was coupled to a Mass Spectrometer Thermo Finnigan (Ringoes, USA) LCQ ion trap, and the analyses were performed using the following parameters: spray voltage 4.5 kV, sheath gas flow rate 30, auxiliary gas flow rate 5, capillary voltage 10 V, capillary temperature 200°C, in ion negative mode. During the MS/MS experiments, an opportune energy was applied for the simultaneous monitoring of precursor and fragmented ions.

Column Chromatography

Extract 10 (highest TPC) was subjected to separation in a chromatographic column using silica gel (Sigma Aldrich, Milano Italy) to characterize better the main compounds that coeluted in the HPLC analyses. A mixture of ethyl acetate, formic acid, acetic acid, and water was used for the separation (300:11:11:20). The fractions were collected, analyzed by HPTLC, dried, and stored at -20°C until further analyses.

^{1}H NMR Spectroscopy

The ^{1}H NMR spectra were recorded on a Varian Gemini-400 spectrometer at 399.97 MHz at a temperature of 303 K. Extracts (8 mg/mL) were dissolved in D_2O:CD_3OD (1:2) in a 5 mm NMR tube. ^{1}H NMR spectra were run using a standard pulse sequence s2pul, with 45.0 degrees pulse, 3.00 seconds acquisition time, 8 repetitions, 6400 Hz spectral width, and 0.33 Hz Fid resolution.

Spectrophotometric DPPH Assay

The DPPH assay was performed following the method described by Nostro et al.[21] Antioxidant activity was expressed as concentration providing DPPH 50% inhibition (IC_{50}), and as µmol of Trolox equivalents/g of extract (data not shown) in order to complete the statistical analysis with the software Statistica 10.0 (Statsoft Inc., Tulsa, Oklahoma, USA) (Figure 1). All experiments were assessed in triplicate and values are reported as mean ± SD.

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Declaration of Conflicting Interests

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References

1. CSK L, Pfaltzgraff LA, Herrero-Davila L, et al. Food waste as a valuable resource for the production of chemicals, materials and fuels. Current situation and global perspective. *Energy Environ Sci.* 2013;6(2):426-464.
2. Katalinic V, Mozina SS, Generalic I, Skroza D, Ljubenkov I, Klancnik A. Phenolic profile, antioxidant capacity, and antimicrobial activity of leaf extracts from six *Vitis vinifera* L. varieties. *Int J Food Prop.* 2013;16(1):45-60.
3. Kosar M, Küpeli E, Malyer H, Uylaser V, Türkben C, Baser KHC. Effect of brining on biological activity of leaves of *Vitis vinifera* L. (Cv. Sultani Cekirdeksiz) from Turkey. *J Agric Food Chem.* 2007;55(11):4596-4603.
4. Deliorman Orhan D, Orhan N, Özçelik B, Ergun F. Biological activities of *Vitis vinifera* L. leaves. *Turk J Biol.* 2009;33:341-348.
5. Dani C, Oliboni LS, Agostini F, et al. Phenolic content of grapevine leaves (*Vitis labrusca* var. Bordo) and its neuroprotective effect against peroxide damage. *Toxicol In Vitro.* 2010;24(1):148-153.
6. Latouche G, Bellow S, Poutaraud A, Meyer S, Cerovic ZG. Influence of constitutive phenolic compounds on the response of grapevine (*Vitis vinifera* L.) leaves to infection by *Plasmopara viticola*. *Planta.* 2013;237(1):351-361.
7. Leborgne L, Mazière JC, Mazière C, Andrejak M. Oxidative stress, atherogenesis and cardiovascular risk factors. *Arch Mal Coeur Vaiss.* 2002;95(9):805-814.
8. Del Rio D, Rodriguez-Mateos A, Spencer JPE, Tognolini M, Borges G, Crozier A. Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxid Redox Signal.* 2013;18(14):1818-1892.
9. Palma-Tenago M, Soto-Hernández M, Aguirre- Hernández E. Ed. Flavonoids in Agriculture. In: *In Flavonoids, from Biosynthesis to Human Health.* 1st ed. London, UK: IntechOPEN; 2017:189-201.
10. Baiano A. Recovery of biomolecules from food wastes--a review. *Molecules.* 2014;19(9):14821-14842.
11. Miglietta PP, Morrone D, Lamastra L. Water footprint and economic water productivity of Italian wines with appellation of origin: managing sustainability through an integrated approach. *Sci Total Environ.* 2018;633:1280-1286.
12. O'Neil MJ. *The Merck Index.* Cambridge, UK: Royal Society of Chemistry; 2013.
13. Dresch RR, Dresch MK, Guerreiro AF, et al. Phenolic compounds from the leaves of *Vitis labrusca* and *Vitis vinifera* L. as a source of waste byproducts: development and validation of LC method and antichemotactic activity. *Food Anal Methods.* 2014;7(3):527-539.
14. Pacifico S, D'Abrosca B, Scognamiglio M, et al. Antioxidant polyphenolic constituents of *Vitis* × *Labruscana* cv. 'Isabella' leaves. *Open Nat Prod J.* 2013;5(1):5-11.
15. Férnandes F, Ramalhosa E, Pires P, et al. *Vitis vinifera* leaves towards bioactivity. *Ind Crops Prod.* 2013;43:434-440.
16. Pari L, Suresh A. Effect of grape (Vitis vinifera L.) leaf extract on alcohol induced oxidative stress in rats. *Food Chem Toxicol.* 2008;46(5):1627-1634.

17. Rodrigues MI, Iemma AF. *Experimental Design and Process Optimization*. Campinas, Brazil: Casa do Espírito Amigo Fraternidade Fé e Amor; 2012.
18. Montgomery DC. *Design and Analysis of Experiments*. New York, NY: John Wiley and Sons; 1991.
19. Tacchini M, Spagnoletti A, Marieschi M, et al. Phytochemical profile and bioactivity of traditional ayurvedic decoctions and hydro-alcoholic macerations of *Boerhaavia diffusa* L. and *Cuculiga orchioides* Gaertn. *Nat Prod Res*. 2015;29(22):2071-2079.
20. Agency EM. *ICH Topic Q2: Validation of Analytical Procedures: Text and Methodology. Step 5. Note for guidance on validation of analytical procedures: Text and methodology*; 1995.
21. Nostro A, Guerrini A, Marino A, et al. *In vitro* activity of plant extracts against biofilm-producing food-related bacteria. *Int J Food Microbiol*. 2016;238:33-39.

11.2 Annex II: Congress Abstracts

5th International Phytocosmetics and Phytotherapy Congress, Linking Health & Beauty.
University of Patras, 15-17 May 2017. Patras, Greece

Winner of Cáceres Award

Valorisation of grape pomace and seeds: a comparison between UAE and Naviglio extracts

I. Burlini, M. Tacchini, A. Grandini, I. Maresca, T. Bernardi, A. Massi, A. Guerrini, G. Sacchetti

Background: Food waste accounts for more than 50% of total waste production. Nine million tons of winery industrial wastes, known as grape pomace, are produced every year in the world, counting about 20% w/w of total grape use for vinification. Recycling biomass from agro-food chain waste could be a way of valorisation of internal resources and it represents an eco-friendly solution for the grape pomace valorisation. Methods: Hydro-alcoholic extractions of red and white grape (*Vitis vinifera* L.) pomace and seeds, were performed using ultrasound and Naviglio®. In addition, with the aim of removing sugars, a selective extraction was performed (Wagner, 2009). Qualitative analyses were performed with 1H-NMR and HP-TLC, including HP-TLC Bioautographic assay, used also to investigate the antioxidant activity. In order to evaluate grape pomace phenolic content, quantitative analysis on total polyphenol content was performed with Folin-Ciocalteu method. Results and Discussion: In a preliminary HP-TLC analysis, both ultrasound-assisted extractions (UAE) and Naviglio® extracts showed the best fingerprinting using 50% ethanolic solution as extraction solvent. Assuming that all the extracts did not exhibit a rich profile, UAE lyophilized extracts showed the highest yields (29,27%) and Naviglio® lyophilized extracts showed the richest phytocomplex in HP-TLC evaluation. HP-TLC Bioautographic assay demonstrated radical scavenger activity of the extracts, for both ABTS and DPPH assays, between Rf 0 and 0.5. Another active fraction, undetectable with the UV visualizer, was found at Rf 0.65. In general UAE showed higher total phenolic content (white seeds extracts exhibited the best yield, 446.74 ± 10.74 mg GAE/g of dried extract) than Naviglio®. Comparing the red grape pomace with the white one, the former showed higher polyphenol content than the latter (189.11 ± 5.95 mg GAE/g of dried extract; 116.11 ± 5.46 mg GAE/g of dried extract). Finally, extraction with ethyl acetate, aimed to obtain a sugar-free fraction. ^{1}H-NMR analyses were performed in order to better describe the fingerprinting of our extracts. Conclusions: Even if phenolic composition of grape pomace and its radical scavenging activity have been widely studied in literature, we performed the first comparison between a conventional extraction method (UAE) and Naviglio® on grape pomace and seeds. Further studies will focus on the complete chemical characterization and the bioactivities evaluation in terms of antimicrobial and anti-cholinesterase activities.

International Phytocosmetics and Phytotherapy Congress

15-17 May 2017
University of Patras

Certificate of Appreciation

Awarded to:

Ilaria Burlini

Winner of the Cáceres Award

For presenting

"Valorisation of grape pomace and seeds: a comparison between UAE and Naviglio extracts"

Jalal Ghaemghami, Ph.D.
IPPC-Greece, Chairman

Plant Extract with anticancer activity: *Hemidesmus indicus*– a case study

Massimo Tacchini, **Ilaria Burlini**, Guglielmo Paganetto, Giulia Greco, Carmela Fimognari, Gianni Sacchetti and Alessandra Guerrini

Background: The great interest that natural products draw is often based on their intrinsic complexity, that allows them to interact with numerous molecular targets. The decoction of the roots of *Hemidesmus indicus* (L.)R.Br.(Asclepiadaceae) is widely used in the Ayurvedic traditional medicine for the treatment of various diseases. The goal of this ongoing study is achieving simple fraction(s) or pure compound(s) responsible of the anticancer activity. Methods: Starting from the formulation of two traditional preparations (decoction and hydro-alcoholic extracts), different polarity extractions were performedwith the aim of increasing the bio-efficacy (cytotoxic activity towards cancer cells). The obtained extracts were chemically characterised by RP-HPLC-DAD, GC-MS and 1H-NMR following fully validated methods. In order to follow a bioassay-guided strategy, cytotoxicity tests were performed with several cancer cell lines, to evaluate the biological potential of H.indicus. Results and Discussion: Since 2013 our results highlighted the great multi-target potential of *H.indicus* decoction in term of cytodifferentiation and apoptosis induction. In 2015, we showed the anti-angiogenic activity of this traditional preparation and the cytotoxic potential of another traditional preparation (hydro-alcoholic extract, HE) against eight cancer cell lines. HE, the most performing extract, was studied in order to identify fraction(s) or pure compound(s) responsible of the bioactivity. The resazurin tests performed after incubation of HE with CCRF-CEM and CEM-ADR5000, showed IC50 value dropping down after the elimination of sugars (from IC50=84, 85±3, 34 μg/ml to 69,70±1, 22 μg/ml against CEM-ADR5000), and even more after soxhlet extraction (IC_{50}=5,76±0, 01 μg/ml). The main chemical components of the soxhlet extract are vanillin derivatives. During the extraction process their concentration is increased, but they did not exhibit cytotoxic activity against leukaemic cells, neither tested alone, nor considered together. Conclusions: H. indicus soxhlet extract proved to be an interesting phytocomplex showing remarkable antileukaemic activity, which fractionation will be the next step to pinpoint the molecule(s) responsible of this biological activity. Moreover, it would be interesting to verify if H. indicus extracts could exhibit activity also interacting with non-canonical cell death pathway, confirming its importance in prevention of cancer relapses and pharmacological resistance.

Hyssopus officinalis subsp. *aristatus* (Godr.) Nyman from Abruzzo region: morphological features, chemical and biological characterization of essential oil and hydroalcoholic extract

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Aerial parts of hyssop wildly grown in natural habitat of the Abruzzo region (Italy) were classified as *Hyssopus officinalis* subsp. *aristatus* (Godr.) Nyman (synonym *H. officinalis* subsp. *pilifer* (Pant.) Murb.) and then characterized on the basis of its essential oil composition and hydroalcoholic extract. *Hyssopus officinalis* L. (Lamiaceae), is a polymorphous species, well known for its pleasant aromatic scent, as an ornamental and bee-attracting plant, and in food industry as a condiment and spice or as a minty flavor (1). In Italy, the subspecies *aristatus* is found in Lombardia, Trentino-Alto Adige, Veneto, Friuli-Venezia Giulia, Umbria, Lazio, Abruzzo, Molise, Campania, Basilicata and Calabria. The literature (2,3) reports the peculiar features of the subsp. *aristatus* as floreal leaves ending with an arista of 1-3 mm, a calyx divided into teeth of 2–3 mm with lanceolate or ovate-lanceolate shape and a 7-9 mm blue-violet corolla. We confirmed these morphological aspects (Fig.1) and observed microscopic features.

The chemical characterization of essential oil, performed with GC-MS and NMR analyses, revealed an uncommon composition, never described in literature: limonen-10-yl acetate (67.9%) was the main compound, followed by 1,8-cineole (15.5%) and limonene (5.8%) (4,5,6). The fingerprinting (HPLC DAD, HPTLC) of hydroalcoholic extract showed chlorogenic and rosmarinic acid among the main secondary metabolites.

Regarding the antioxidant activity, performed by DPPH test, hydroalcoholic extract evidenced interesting IC_{50} value (38.9±2.12 µg/mL), if compared to Trolox (4,28±0.48 µg/mL), due to chlorogenic and rosmarinic acid (IC_{50} of 7.29±0.17 µg/mL, 4.31±0.34 µg/mL, respectively) as confirmed by DPPH bioautographic assay (Fig.2). The essential oil did not show radical scavenging capacity. The antimicrobial bioautographic and microdilution tests against *Staphylococcus aureus* are currently in progress.

Hydroalcoholic extract was assayed for biological activities employing two cell lines. The antiproliferative effects and the modulation of oxidative stress were investigated on C2C12 and HCT116 cell lines by MTT test and ROS production. In range concentration 50-300 µg/ml, the extract resulted well borne by both cell lines.

On HCT116 cell line, at concentrations higher than 150µg/mL, the extract is able to completely revert the induced ROS production.

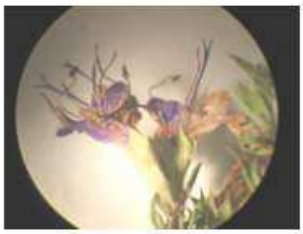

Fig. 1. Aerial parts of *H. officinalis* subsp. *aristatus*

Fig. 2. DPPH bioautographic assay of hydroalcoholic extract

Bibliography

1) S. Dragland, H. Senoo, K. Wake, K. Holte, R. Blomhoff (2003) Journal of Nutrition, 133, 1286–1290
2) S. Pignatti (2003) Flora d'Italia. Edizione Agricole,486
3) V.H. Heywood, R.A. De Filippis (1972) Flora Europea. Eds. Flora Europea, Cambridge, 170–171
4) A. Venditti, A. Bianco, C. Frezza, F. Conti, L. Maleci Bini, C. Giuliani, M. Bramucci, L. Quassinti, S. Damiano, G. Lupidi, D. Beghelli, S. Caterbi, D. Petrelli, L. A. Vitali, F. Papa, G. Caprioli, F. Maggi (2015) Industrial Crops and Products, 77, 353–363
5) A. M. Dzamić, M. D. Soković, M. Novaković, M. Jadranin, M. S. Ristić, V. Tesević, P. D. Marina (2013) Industrial Crops and Products, 51, 401–407
6) A.Hajdari, A. Giorgi, G. Beretta, F. Gelmini, S. Buratti, S. Benedetti, A. Merkouri, X. Mala, S. Kabashi, D. Pentimalli, B.Pulaj, B.Mustafa (2018) European Food Research and Technology

New extraction procedure for the release of bound phenolics from cereal by-products value chain coupling sonication and alkaline hydrolysis

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Background: Recovering agro-food by-products as sources of biomolecules for different large-scale applications (e.g. cosmetics, functional foods, etc.) is a current industrial trend inspired by the so-called bio-based economy. On these premises, by-products derived from cereal value chain represent a promising source of health-promoting phytochemicals, phenolic acids (ferulic acid in particular), predominantly esterified to cell walls of the caryopsis' bran tissues. Since approximately 60% of phenolics are bound in wheat bran, their effective extraction contributes to the cereal co-products valorization for health and food industries. Methods: Different extraction strategies were performed using ultrasound-assisted extraction, alkaline hydrolysis and their combination to find the best set-up conditions for improving phenolic compounds final yield through breaking covalent bonds of bound phenolics. HP-TLC bioautographic assay has been used as a semi-quantitative screening method to identify the most active chemical fractions and to bio-guide the extraction to obtain enriched extracts. Folin-Ciocalteu method, followed by HP-TLC and HPLC-DAD analyses, was used to determine the phenolic content. Spectrophotometric DPPH and ABTS assays were performed to check the antioxidant activity of the extracts.
Results and discussions: Extracts obtained by associating alkaline hydrolysis with sonication evidenced the highest polyphenol content (610.49±57.63 mg GAE/ g dried extract) and antioxidant activity both with DPPH (IC50: 3.61±0.09 µg/mL) and ABTS (IC50: 3.73±0.14 µg/mL) assays; hydrolysed extract without sonication showed a lower polyphenol content (437,58±9.93 mg GAE/ g dried extract) and a moderate antioxidant activity (IC50: 36.611±0.65 µg/mL with DPPH method); ultrasound-assisted extract exhibited the lowest polyphenol content (84.93±8,59 mg GAE/ g dried extract) and no antioxidant activity was detected. HP-TLC and HPLC-DAD analyses as well as HP-TLC bioautographic assay supported previous results, highlighting a prevalence of phenolic acids in both hydrolysed extracts. Conclusions: Alkaline hydrolysis with sonication allowed 50% reduction of extraction time, leading to a benefit in terms of total phenolics yield, and processing sustainability of cereal by-products processing through lowering time and energy consumption. To improve cosmetic applications of cereal by-products derivatives, further studies will be carried on through specific assays.

BIODIVERSITY WITHIN WILD *HYSSOPUS OFFICINALIS* SUBSP. *ARISTATUS* (GODR.) NYMAN FROM ABRUZZO REGION

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Aerial parts of *Hyssopus officinalis* subsp. *Aristatus* (Godr.) Nyman (synonym *H. officinalis* subsp. *pilifer* (Pant.) Murb.) harvested in different natural habitats (09/2016: Civitaretenga, L'Aquila; 09/2018: Navelli, L'Aquila; 10/2018: Majella, Chieti) of the Abruzzo region (Italy) were characterized based on essential oils and hydro-alcoholic extracts composition. In Italy, this subspecies is found in Lombardia, Trentino-Alto Adige, Veneto, Friuli-Venezia Giulia, Umbria, Lazio, Abruzzo, Molise, Campania, Basilicata and Calabria[1]. The chemical characterization of essential oils, performed with GC-MS and NMR analyses, revealed an uncommon composition for the specimen collected in Civitaretenga in 2016, never described in literature: (*l*) limonen-10-yl acetate (67.9%) was the main compound, followed by 1,8-cineole (15.5%) and (*l*) limonene (5.8%)[2,3,4]. On the other hand, the essential oil belonging to plants harvested in 2018 in the Navelli area (near Civitaretenga) showed a composition comparable to the data published by Hajdari *et al.* (2018) about plants of Western Balkans: *cis*-pinocamphone (43.2%), methyleugenol (15.8%), *trans*-pinocamphone (11.0%), 1,8-cineole (4.4%). The last specimen, collected in Majella, was characterized by essential oil rich in methyleugenol (41.5%), 1,8-cineole (39.7%) and (*l*) limonene (7.6%), as described by Piccaglia *et al.* (1999)[5]. The hydro-alcoholic extracts showed a similar fingerprinting for all specimens, characterized by the main presence of chlorogenic, rosmarinic and caftaric acid (RP-HPLC-DAD) and they also exhibited an interesting antioxidant capacity (DPPH). The plant collected in Civitaretenga in 2016 showed the greatest abundance of every compound. In particular, the quantity of chlorogenic acid was about twice as much as in the other specimens. Since a preliminary screening showed high cytotoxicity of the latter extract against A549 cell line, migration, invasion and wound healing assays were performed but with negative outcomes.

References

[1] S. Dragland, H. Senoo, K. Wake, K. Holte, R. Blomhoff (2003) Journal of Nutrition, 133, 1286–1290
[2] A. Venditti, A. Bianco, C. Frezza, F. Conti, L. Maleci Bini, C. Giuliani, M. Bramucci, L. Quassinti, S. Damiano, G. Lupidi, D. Beghelli, S. Caterbi, D. Petrelli, L. A. Vitali, F. Papa, G. Caprioli, F. Maggi (2015) Industrial Crops and Products, 77, 353–363
[3] A. M. Dzamić, M. D. Soković, M. Novaković, M. Jadranin, M. S. Ristić, V. Tesević, P. D. Marina (2013) Industrial Crops and Products, 51, 401–407
[4] A.Hajdari,A. Giorgi, G. Beretta, F. Gelmini, S. Buratti, S. Benedetti, A. Merkouri, X. Mala, S. Kabashi, D. Pentimalli, B.Pulaj, B.Mustafa (2018) European Food Research and Technology, 244, 1313-1327
[5] R. Piccaglia, L. Pace, F. Tammaro (1999) Journal of Essential Oil Reserch, 11(6), 693-699.

VI International Plant Science Conference (IPSC) con il 114° Congresso della Società Botanica Italiana,
Padova, 4-7 September 2019

Sustainable strategies for cereal by-products valorization

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Triticum durum (Desf.), *Oryza sativa* L. and *Zea mais* L. are the so-called "big three" cereal plants as they represent the largest productions worldwide. Their large amounts of by-products suggest the possibility of recovering them as they are important source of health-promoting phytochemicals. According to FAO, one-third of the edible parts of food produced for human consumption gets lost or wastes globally; italian food wastes have been counted around 5.1 MT per year and, beside the mere economic loss, these numbers are closely related to environmental risks (1, 2). The purpose of the present project is to recover wastes from the cereal industry in order to reinsert them within the dermo-functional cosmetic market as high-added value materials, embracing the concept of circular economy. Phenolic acids, mostly bound to cell-walls, are the most representative bioactives in cereals brans and germs; ferulic acid, the main phenolic in literature, demonstrated several applications in both health and food industries as antioxidant, UV absorber, photoprotective and preservative (3, 4). For these reasons, we have developed more effective and greener extraction strategies, compared to those commonly used. The plant materials used have been selected as the main by-products obtained from the milling industry: brans of wheat and rice and germs of yellow and white corn. To develop the project, we extracted ferulic acid both through chemical and enzymatic hydrolysis, verifying the contribution of ultrasound waves to extractive efficacy (alkaline hydrolysis, alkaline hydrolysis coupled with sonication, enzymatic hydrolysis and enzymatic hydrolysis on sonicated material). Therefore, we performed an extraction efficacy screening with HPTLC to evaluate yields and the phenolic molecules extracted. It has followed a RP-HPLC-DAD analysis and quantification of ferulic acid in all obtained extracts and a spectrophotometric quantification of total phenolic content which allowed us to identify the most performing extractions; analyses have confirmed ferulic acid as the main compound in the extracts. In particular, by associating ultrasound waves to yellow corn germ alkaline hydrolysis, we have obtained the best results regarding yields (6.22 ± 0.22%), content of ferulic acid (636.53 ± 3.71 µg FA/mg dried extract), and total polyphenols (844.46 ± 64.60 mg GAE/g dried extract). A screening test was then performed to evaluate the antioxidant activity and the main molecules involved through the DPPH bioautographic assay. The same activity has been investigated through the *in vitro* DPPH spectrophotometric assay evaluating the IC_{50} of the extracts: results obtained with alkaline

hydrolysis coupled with sonication, gave the lowest IC_{50} values, in particular with wheat bran (3.61 ± 0.09 µg/mL).

Finally the antimicrobial activity was tested on the most performing extracts, calculating the MICs on three clinical isolated bacterial strains (*S. aureus* MRSA 185087 and MSSA 185960, *S. epidermidis* 185240) and a reference strain of *S. aureus* (ATCC 25923): wheat and rice bran extracts gave the lowest MIC values against the *S. aureus* MRSA strain (32 µg/mL and 16 µg/mL respectively).

The results of this research project allow us to hypothesize possible cosmetic applications of the phenolic extracts obtained: the formulation of one or more dermo-functional products with antioxidant, anti-inflammatory and dermo-protective activities is, in fact, our next research goal.

1) FAO (2011). Global food losses and food waste - Extend, causes and prevention. Rome
2) IAI (2017) D. Fattibene and M. Bianchi. Fighting against Food Losses and Waste: An EU Agenda, IAI working papers 17
3) Kumar, N. & Pruthi, V. (2014). Potential applications of ferulic acid from natural sources. Review. Biotechnology Reports, 4, 86-93
4) Lin, F-H., Lin, J-Y., D. Gupta, R., Tournas, J.A Burch, J.A., Selim, M.A., Monteiro-Riviere, N.A., Grichnik, J.M., Zielinski, J. and Pinnel L., S.R., (2005). Ferulic Acid Stabilizes a Solution of Vitamins C and E and Doubles its Photoprotection of Skin. The Journal of Investigative Dermatology, 125 (4), 826-832

VI International Plant Science Conference (IPSC) con il 114° Congresso della Società Botanica Italiana,
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Ocimum campechianum Mill. leaves: an Amazonian promising drug

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Leaves of *Ocimum campechianum* Mill. (syn. *Ocimum micranthum* Willd.) were collected from a wild population in the Ecuadorian Amazonia, authenticated by Dr. David Neill and a voucher specimen was deposited at the Herbarium ECUAMZ of the Amazonian State University (UEA) in Ecuador. *O. campechianum* is a plant species of the Lamiaceae family, widespread across Central and South America, and it was chosen because of its broad spectrum of biological activities. It is traditionally used as ingredient for infusions and beverages or as flavouring agent for foods, against cough, bronchitis and general infections or as anti-inflammatory, antipyretic, to treat conjunctivitis and even as diuretic and emmenagogue (1, 2, 3). Literature reported also a good antioxidant capacity (4) and, recently, a larvicidal activity *in vitro* (5) for its essential oil. The plant crude drug was characterized based on essential oil, methanolic and hydro-alcoholic (ethanol 70%) extracts composition. The chemical characterization of essential oils, performed with GC-MS analyses, revealed a chemical profile characterized mainly by eugenol (44.1%), β-caryophyllene (10.4 %), β-elemene (6.9%) and 1,8-cineole (7.7%). The methanolic and hydro-alcoholic extracts, chemically characterized for the first time in this research, showed a similar fingerprinting, with the main presence of rosmarinic acid, followed by caftaric, chlorogenic acids as minor components (RP-HPLC-DAD, NMR). The ethanolic extract exhibited the greatest abundance of every compound. In particular, the quantities of caftaric and chlorogenic acid were about twice as much as in the other extract, while rosmarinic acid was nearly four times more abundant. A preliminary screening of the extracts against A549 cell line were performed but with negative outcomes. On the other hand, extracts did not show any cytotoxicity against HaCat cell line, giving indication of safety of use of the extracts. The antibacterial activity against *Pseudomonas syringae* pv. *syringae* was also tested, and essential oil resulted the most active preparation with MIC of 2.5mg/mL. All extracts exhibited a noteworthy antioxidant capacity (DPPH). The highest activity was showed, once again, by the essential oil with an IC_{50} of 7.7±0.1 μg/ml, close to the value of the positive control (Trolox). The hydroalcoholic extract exhibited a bioactivity close to the one of essential oil (11.1±0.01μg/ml), while the methanolic displayed a weaker activity when compared to the previous extracts (52.3±2.7 μg/ml). Rosmarinic acid and

eugenol, tested as main components of extracts and essential oil, respectively, showed values close to Trolox. The results confirmed very promising properties for the formulation of cosmetic and food supplements products.

1) L.C. Di Stasi, G.P. Oliveira, M.A. Carvalhaes, M. Queiroz-Junior, O.S. Tien, S.H: Kakinami, M.S. Reis. (2002) Fitoterapia, *73*, 69-91
2) R.E. Schultes, R. Raffauf (1990) *The Healing Forest*, Dioscorides Press: Portland, OR
3) R.F. Vieira, J.E. Simon. (2000) Econ. Bot., 54, 207-216.
4) G. Sacchetti, A. Medici, S. Maietti, M. Radice, M. Muzzoli, S. Manfredini, E Braccioli, R. Bruni. (2004) J. Agric. Food Chem., 52,3486-3491
5) L. Scalvenzi, M. Radice, L. Toma, F. Severini, D. Boccolini, A. Bella, A. Guerrini, M. Tacchini, G. Sacchetti, M. Chiurato, R. Romi, M. Di Luca (2019) Parasite 26, 23