UNIVERSITÀ DEGLI STUDI DI MODENA E REGGIO EMILIA

International Doctoral Program in
AGRI-FOOD SCIENCES, TECHNOLOGIES AND BIOTECHNOLOGIES

CYCLE XXXIV

AGRI-FOOD PRODUCTS AND BY-PRODUCTS AS A SOURCE OF BIOACTIVE COMPOUNDS: EXTRACTION, CHARACTERIZATION AND BIOLOGICAL ACTIVITIES

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During the last few decades, a great attention has been paid to the use of botanical species, as well as their by-products, for the isolation of bioactive compounds. This interest is mainly linked to the growing demand for more sustainable and eco-compatible alternative processes leading to the production of preparations of plant origin. The by-products are mainly generated by agri-food and pharmaceutical industries. Their processing could be considered as an important opportunity to avoid the serious problems caused by their throwing into environment. Thus, it has been reported that these by-products are still rich in different classes of bioactive compounds with significant therapeutic properties.

The isolated bioactive compounds from plant products and byproducts could be valued according to their biological properties such as antioxidant, antimicrobial, antidiabetic and anticancer. In addition, they could have applications in the food industry as preservatives, colorants, anti-browning and thickeners. This study aims to the characterization of bioactive compounds from different plant species and the related by-products (*Lavandula angustifolia* Mill., *Lavandula x intermedia* Emeric ex Loisel, P*runus domestica* L. and *Solanum lycopersicum*), as well as the evaluation of biological activities and possible applications of the isolated compounds.

PRODOTTI E SOTTOPRODOTTI AGROALIMENTARI COME FONTE DI COMPOSTI BIOATTIVI: ESTRAZIONE, CARATTERIZZAZIONE E ATTIVITÀ BIOLOGICHE

Negli ultimi decenni è stata posta grande attenzione all'utilizzo di specie botaniche, nonché dei loro sottoprodotti, per l'isolamento di composti bioattivi. Questo interesse è principalmente legato alla crescente domanda di processi alternativi più sostenibili e ecocompatibili che portino alla produzione di preparati di origine vegetale. I sottoprodotti sono principalmente generati dalle industrie agroalimentari e farmaceutiche. La lavorazione di questi sottoprodotti potrebbe essere considerata un'importante opportunità per evitare i gravi problemi causati dal loro smaltimento nell'ambiente. Pertanto, è stato riportato che questi sottoprodotti sono ancora ricchi di diverse classi di composti bioattivi con proprietà terapeutiche significative.

I composti bioattivi isolati da prodotti e sottoprodotti vegetali potrebbero essere valutati in base alle loro proprietà biologiche come antiossidanti, antimicrobici, antidiabetici e antitumorali. Inoltre, potrebbero avere applicazioni nell'industria alimentare come conservanti, coloranti, anti-doratura e addensanti. Questo studio mira alla caratterizzazione dei composti bioattivi di diversi specie vegetali e i relativi sottoprodotti (*Lavandula angustifolia* Mill., *Lavandula x intermedia* Emeric ex Loisel, *Prunus domestica* L. e *Solanum lycopersicum*), nonché alla valutazione delle attività biologiche e delle possibili applicazioni dei composti isolati.

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PREFACE

"Where there is a will there is a way" **(Winston Churchill)**

"Imagination is more important than knowledge. For knowledge is limited, whereas imagination embraces the entire world, stimulating progress, giving birth to evolution"

(Albert Einstein)

For thousands of years, man has used plants available to him for food and healing. Most of these plants contain natural substances known for their nutritional properties and their therapeutic virtues. Their different uses are mainly linked to the presence of biomolecules which have a wide range of biological activities attributed to their great chemical diversity. Recently, several pharmaceutical companies are interested in plant-derived biomolecules, mainly due to the current widespread belief that "green medicine" is safe and more reliable with less expensive active ingredients than synthetics which are often associated with negative side effects. Nowadays, about 42% of the world's best-selling drugs are marketed either directly from natural products or derived from plant by-products. Furthermore, about 20% of the plants found in the world have been subjected to pharmacological or biological testing, and a significant number of new antibiotics introduced on the world market are obtained from natural or semi-synthetic resources. Some of these plants represent a rich source of antimicrobial agents. Political, economic, societal and environmental issues also encourage manufacturers to innovate technologies and to improve the energy and environmental efficiency of their processes, thanks to the development of so-called green chemistry to replace harmful chemical processes [1-6].

Biomolecules are natural metabolites synthesized by plants, of very diverse nature and chemical structure and they are distributed differently from one species to another. Thus, a biologically active (bioactive) substance characterizes any natural molecule possessing biological activity. This activity is closely related to the chemical composition of the biomolecule and it is expressed at the level of the living organism by a modification of its functioning and/or its structure. Indeed, the natural substances extracted from plant resources have aroused a lot of interest in the therapeutic field which has allowed in recent years, great advances due to their added value in the preparation of many products in particular in the agri-food, nutraceutical, pharmaceutical and cosmetics fields [7-9].

Plants are a valuable source of biomolecules divided mainly into two groups. The first one is macronutrients (carbohydrates, proteins and lipids) which have the capability of providing energy to organisms. The second group is micronutrients (vitamins, polyphenols, essential fatty acids, amino acids, minerals, trace elements and other compounds) which are essential to the living organism and also have biological activities of health interest [10-12].

The most common target compounds are polyphenols. They all have in common the presence of one or more benzene rings which have an aromatic ring containing one or more hydroxyl(s) and including different functional groups (ester derivatives, glycosides and others). The structure of phenolic compounds varies from simple molecules (simple phenols and phenolic acids) to highly polymerized molecules (condensed tannins, lignins). Phenolic compounds are conventionally subdivided into several classes based on their chemical structure and their biosynthetic pathway. They can range from simple molecules, like phenolic acids, flavonoids to highly polymerized compounds like tannins. Specifically, phenolic compounds could be classified into phenolic acids (hydroxybenzoic acids, hydroxycinnamic acids, coumarins), bioflavonoids (flavones, flavonols, flavanones, flavanonols, aurones, chalcones, dihydrochalcones, isoflavonoids, flavanols, flavanediols), anthocyanidins and anthocyanins, stilbenes and tannins (hydrolysable tannins and condensed tannins) [13,14]. Phenolic compounds are becoming more and more important due to the beneficial biological effects that they provide to the human organism. They are used in the food and cosmetics industry as antifungal and antibacterial agents in order to remedy the problem of the appearance of microbial strains that are multi-resistant to conventional drug treatments (example of stilbenes, especially trans resveratrol) [15,16]. Furthermore, they are considered as strong antioxidant agents since they can act in different ways in the process of regulation of oxidative stress. This is mainly manifested by their ability to complex with transition metals such as iron, thus preventing the Fenton reaction which results in the formation of the hydroxyl radical, and as inhibitors of the activity of certain enzymes responsible for the production of reactive oxygen species [14].

Flavonoids have been reported to be the most powerful antioxidant phenolic compounds [14]. Polyphenols have also a nutraceutical role in the form of functional foods which are able to reduce the risk of chronic diseases, in addition to fulfilling their nutritional functions. The consumption of fruits and vegetables rich in polyphenols can be a means of prevention against many diseases [17,18]. In the pharmaceutical field, we can cite the example of trans-resveratrol which show a cardio-protective effect. It acts on the inhibition of platelet aggregation and vasodilation [19]. It can also act as an anti-carcinogenic agent by inhibiting the initiation and growth of tumors (inhibits cyclo-oxygenase, ornithine decarboxylase and angiogenesis) [20]. Flavonoids possess anti-inflammatory properties by inhibiting the expression of inflammatory mediators such as histamine [21].

On the other hand, the valorization of fruits in the form of dried, concentrated orfrozen products leads to the appearance of a wide range of by-products and wastes composed in particular of fruit pulp, skins and stems. These by-products are easily degradable and their storage and use are subject to legal restrictions. To respond to environmental concerns regarding the recovery of by-products, the fruit processing industry is often confronted with several solutions which are mainly animal feed, spreading, composting, energy production, bio-adsorbents for wastewater treatment and the recovery of various constituents that can be incorporated into new agri-food, cosmetic or pharmaceutical products. The composition of by-products from the fruit processing industry is extremely variable. In general, the energy value of fruit residues is quite high due to the high content of digestible pectins and sugars [22-25].

Development of appropriate and economically productive processes for the isolation of bioactive compounds still remains the most important task. For that, various methodologies were performed in order to obtain the best possible yield in bioactive components from plant material. Thus, soxhlet extraction and maceration, which are the classic extraction methods, have been reported to be the most used methods because of to their availability, efficiency and the simplicity of their application.

From these conventional extraction methods, the progress of technology allowed the application of other modern techniques such as ultrasound-assisted extraction, microwave-assisted extraction, supercritical fluid extraction and accelerated solvent extraction [1,26].

Concerning the characterization and identification of isolated bioactive compounds, several techniques were carried, mainly spectrophotometric and chromatographic. Chromatographic techniques are currently widely applied for the qualitative and quantitative analysis of bioactive compounds since they are well-known for high sensitivity, selectivity and versatility. Gas chromatography is the most applied technique and it is used coupled with mass spectrometry (GC-MS) or flame ionization detector (GC–FID). The other used chromatographic techniques are thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC). This latter is usually coupled to different detectors such as mass spectrometry detector (MS), refractive index detector (RI), evaporative light scattering detector (ELSD), diode array detector (DAD) or ultra-visible detector (UV) [27-30].

The study we are conducting is part of the valorization of agri-food products and by-products though their application in different industrial fields. The objective is to demonstrate the richness in bioactive compounds of these materials thought the knowledge of the phytochemical and quantitative profile of these materials, as well as the identification of specific functional activities.

In **Chapter I**, the aim is to develop a product useful for intestinal function starting from a derivative of the process of transformation of plums for food use. Currently, this industrial by-product is used as a corrector for other foods, but the knowledge of its phytochemical and quantitative profile could lead to the identification of specific functional activities. The transformation and integration with targeted active or excipients, chosen appropriately for the aforementioned biological activity, could lead to the enhancement of this by-product and its application as a food supplement. Firstly, several techniques are applied for the qualitative and quantitative characterization of the industrial by-product (polyphenols, sugars, minerals

…). Then, given the presence of high quantities of reducing sugars, a fermentation process will be carried out in order to break down the concentration of glucose and fructose.

Chapter 2 will be dedicated to the characterization of the products and by-product issued from the steam distillation process of two lavender species: *Lavandula angustifolia* Mill. (LA) and *Lavandula x intermedia Emeric* ex Loisel (LI). Essential oils are analysed by gas chromatography coupled to mass spectrometry and flame ionization detector, whereas the analysis of phenolic compounds from residual material is performed by spectrophotometric assays and high-performance liquid chromatography coupled to mass spectrometry and diode array detectors. The isolated and characterized phenolic compounds will be tested for their possible antioxidant, acetylcholinesterase and tyrosinase activities.

The last part of this study (**Chapter 3**) focus on another topic related to agri-food wastes which is the secondary shelf life of food products. In this part, we are interested to the study of the effect of some related factors on the secondary shelf life of a dairy food product: tomato sauce. The studied parameters are total phenolic content, antioxidant activity and lycopene content.

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CHAPTER I

RECOVERY AND VALORISATION OF BIOACTIVE COMPOUNDS FROM PRUNE BY-PRODUCTS

"That's how you learn best, when you do something with so much fun that you can't see the time passing" **(Albert Einstein)**

"Anyone who has never made a mistake has never tried anything new"
(Albert Einstein)

I. INTRODUCTION

During the last decades, there has been an increasing demand for more sustainable alternative processes through the production of preparations of plant origin [1,2]. In this context, more attention has been directed towards the use of plant waste generated mainly by the food and pharmaceutical industries. The large quantities of these wastes, mostly unexploited, has attracted the attention of several researchers for the exploitation of sustainable production of bioactive compounds having relevant therapeutic applications [3-6].

According to the statics performed by the United Nation's Food and Agriculture Organization, the fruits production around the world in 2017 has been reported to be more one billion tones, thus inevitably leading to the generation of large amounts of by-products and wastes. For example, in Europe approximately 100 million tonnes of waste and by-products are generated each year, which consists mainly of outcome of drinks companies (26%) and fruits and vegetables industries (14.8%) [7].

Among the major problems facing the agri-food industries, the management of by-products issued from the fruits processing chains can be considered one of the most important, which is mainly due to their rich composition in biological compounds. These latter, when present with higher concentrations, could cause dangerous environmental problems such as phytotoxicity phenomena, thus leading to several issues like deterioration of drinking water quality, contamination of the aqueous media, plant growth interference, and inhibition of seed germination…[8,9] For that, and in order to resolve the serious environmental issues related to agri-food wastes, proper disposal and processing of these wastes can lead to the closure of bio-economical value chains though the application of a proper treatment using various extraction and analytical methods [10]. Fruit by-products could be considered as a low-cost raw material, allowing the recovery of several bioactive compounds available for transformation into value-added products for other industries [11,12].

Recently, several bioactive compounds were isolated from fruit wastes and showed significant uses in food, cosmetic, or pharma industries. The main targeted compounds were polyphenols, essential oils and polysaccharides [13,14]. In order to obtain these compounds from generated fruit by-products, various extraction techniques were carried out. Although traditional processes (solid-liquid extraction, maceration, soxhlet …) are still in use, novel sustainable methods are continuously developing though the improvement of the existing methods taking into consideration the principles of green chemistry. The most applied novel techniques are supercritical fluid extraction, microwave-assisted extraction and ultrasound-assisted extraction [15].

As examples for studied by-products, the most know are apple pomace, orange peels and grape pomace, skin and seeds. Phenolic compounds isolated from apple pomace by supercritical fluid extraction showed significant industrial applications including production of microbial oils and bioethanol and application as biofuel [16,17]. In the other hand, orange peels have been reported to be a rich source of essential oil, polyphenols and pectin. The presence of these bioactive compounds broadens the field of application of this by-product either in food (improvement of nutritional value, reducing of the caloric value, fructo-oligosaccharides production….) or pharmaceutical (agent for selective modulation of the gut microbiota) industries [18,19]. A wide variety of bioactive compounds were isolated from grape by-products (skin, pomace, seeds) using various extraction methods like classical extraction, ultrasound-assisted extraction, microwave-assisted extraction, supercritical fluid extraction and enzymatic extraction. These compounds are essentially polyphenols, anthocyanins, pectin, cellulose and pullulan which showed important applications in food, pharmaceutic, and cosmetic industries, as well as biofuel production and packaging materials [20-22]. In addition, by-products of exotic fruits (pericarp, peels or seeds) such as pineapple, papaya and mango, which are known for their large production, were demonstrated to contain significant amounts of phenolic compounds, carotenoids, dietary fiber and vitamins [7].

In this study, we are interested in the by-product of prune obtained from industrial company issued after the production of jams and dried fruits. The prune fruit belongs to the variety *Prunus domestica*, which is known also as European plum. This variety is probably originated from South-Eastern Europe and South Western Asia and is commercially cultivated mainly in China, India, USA, and several European countries [23]. Prune fruits may be consumed fresh, dried, or prepared into preserves, compotes, mousse, candied fruit, frozen fruit, jams and jelly products [24]. This wide range of products is essentially related to the benefits reported of this fruit such as nutritive, laxative, and digestive properties. In addition, prune has been reported to possess other beneficial effects since it could be considered as hypoglycemic, hypotensive, and hepatoprotective agent. All these benefits were demonstrated by the wide range of pharmacological activities proven by this fruit including antioxidant, anticancer, antibacterial, antihyperlipidemic, anti-osteoporosis and, anxiolytic. [23]

Thus, such a variety of pharmacological activities should be necessarily linked to a rich chemical composition. Several studies demonstrated that prunes are an important source of major nutrients like phenolic compounds, carbohydrates (fructose, sucrose, glucose, sorbitol), dietary fiber, amino acids, vitamins, and minerals [25-27]. The identification of phenolic compounds revealed the presence of polyphenols, flavonols and anthocyanins. The major ones are chlorogenic acid, neochlorogenic acid and caffeic acid. Previous researches reported that phenolic compounds may contribute to the laxative effect of prunes since it has been shown that chlorogenic acid promotes dissipation of the sodium electrochemical gradient, possibly causing glucose to pass from the jejunum to the bowel, and thus enhancing microbial fermentation, with a concomitant effect on laxation. Another important compound is sorbitol, which was reported to constitute about 15% of the sugar content in prunes. Sorbitol is poorly absorbed in the gastrointestinal tract and is hygroscopic, thus acting as an osmotic laxative [28].

II. EXPERIMENTAL

II.1. Plant material

Prune's by-products were obtained from the processing chain and the variety of prune used in the processing chain was *Prunus domestica* L. var d'Ente. The by-product consists of residual pomace after processing the fruits into jams and dried fruits.

II.2. Chemicals and standards

1,1-Diphenyl-2-picrylhydrazyl (DPPH), Quercetin, gallic acid, ascorbic acid, sodium sulphate (Na_2SO_4), and Folin–Ciocalteu reagent were purchased from Sigma-Aldrich (Milan, Italy). Aluminum chloride was provided by Carlo Erba Reagents (Milan, Italy).

Formic acid (FA), nitric acid (HNO_3, 69%), hydrogen peroxide (H_2O_2), methanol (MeOH) and ethanol (EtOH) were of LC–MS purity grade (Sigma-Aldrich). Ultrapure water was obtained using a Milli-Q Plus 185system from Millipore (Milford, Mass., U.S.A.)

II.3. Chemical composition of the by-product

II.3.1. Water, moisture and pectin content

- Water content: 2g of prune by-product were kept under agitation for 30 minutes at room temperature with 100 ml of distilled water. Then, the solution undergoes a centrifugation for 10 min at 5000 rpm. The supernatant was then filtrated and the filtrate was freeze-dried.
- Moisture content: 20g of prune by-product were mixed with 50 ml of distilled water and left under agitation for 30 minutes at room temperature. After centrifugation for 15 min at 5000 rpm, the residue was dried in oven at 50 °C up to a constant weight. The moisture content was estimated by calculating the percentage weight loss.
- Pectin content: 2 g of the by-product were kept under agitation with 50 ml of distilled water for 30 minutes at room temperature. After centrifugation (5000 rpm for 10 minutes) and filtration, the filtrate undergoes a precipitation with ethanol (20:80 v/v) followed by a centrifugation for 10 min at 5000 rpm. The pellet was then evaporated to

remove the residual amounts of ethanol and dissolved in a minimum amount of Milli-Q water for freeze-drying.

II.3.2. Metal content

The content of metals in prune by-product was estimated by Inductively coupled plasma mass spectrometry (ICP-MS) using a XSeries^{II} ICP-MS (Thermo Fisher Scientific) [29,30]. Before starting the experiments, all used glassware were rigorously rinsed with HNO_3 solution (1M) and ultrapure water in order to avoid metal contamination risk.

300 mg of each sample were mixed with 3 ml of nitric acid solution (HNO_3, 69%) and 500 µl of hydrogen peroxide (H_2O_2) in microwave tubes and then placed in microwave for 20 min. After cooling to room temperature, each solution was transferred into a 10 ml volumetric flask and filled up to 10 ml with Milli-Q water (by rinsing each tube). Blank solutions were also prepared with the same procedure without adding samples.

Thereafter, from each solution (samples and blanks), we prepared two dilutions of 1:10 (1 g of each solution and up to 10 g by Milli-Q H_2O) and 1:100 (100 µl of each solution and up to 10 ml by HNO_3 4%).

The amount of each metal (in mg by 100g of prune by-product) was determined according to the following equation:

$$\text{Metal amount } (mg/100g \text{ FW}) = \frac{\left(\frac{C_{metal}\ x\ F_d\ x\ V_t}{W}\right)}{1000}\ x\ 100$$

C_{metal} (µg/L) = $C_{sample} - C_{blank}$

F_d = Dilution factor (=100 for dilution 1:100 and =10 for dilution 1:10)

V_t = Total volume (L) (= 10 ml = 0.01 L in this assay)

W = initial weight (g)

II.3.3. Extraction of phenolic compounds

The extraction is a very important step before the quantitative and qualitative analysis, which is influenced by the chosen extraction method according to the phytochemicals to be studied. Generally, pH, temperature, time and material to solvent ratio are the main factors that influence the extraction procedure. Additional sample purification steps may be required to remove compounds such as waxes, fats, terpenes and chlorophylls.

In our study, for the analysis of phenolic compounds, solid-liquid extraction was carried out since it is one of the most known and commonly used industrial procedures due to their ease of application and their effectiveness. The most commonly used extraction solvents are alcohols (methanol, ethanol), acetone, ethyl ether and ethyl acetate. However, for very polar compounds such as phenolic acids that cannot be completely extracted with pure organic solvents, alcohol-water or acetone-water mixtures are recommended. Less polar solvents (dichloromethane, chloroform, hexane, benzene) are used to eliminate apolar compounds (waxes, oils, sterols, chlorophyll...) [31,32].

Alcoholic extract was obtained from prune by-product following to the method described by **Donovan *et al.*** [33]

Briefly, 25 g of prune by-product were added to 75 ml of a solution of water and ethanol (H_2O/EtOH, 20:80 v/v) saturated with NaCl. The mixture was left under agitation for 30 minutes at room temperature then centrifugated (4500 rpm/10 min) and filtered. The extraction was repeated twice then the filtrates were combined and concentrated by a rotary evaporator. Finally, the volume was adjusted up to 50 ml by Milli-Q water.

The extract was stored at 4 °C for further analysis.

II.3.4. Total phenolic content (TPC)

TPC was estimated using Folin-Ciocalteu assay. The test with the Folin–Ciocâlteu (FC) reagent is one of the most widespread methods for the quantification of phenolic compounds. It involves the oxidation in alkaline solution of phenols by the yellow-colored FC reagent and the colorimetric determination of the intensity of the blue molybdotungstophosphate formed. These blue pigments have an absorption maximum

depending on the pH of the solution (usually controlled using sodium carbonate) and the qualitative and/or quantitative composition of phenolic compounds [34].

For that, 400 µl of diluted ethanolic extract (40 µl extract+360 µl H_2O), gallic acid (standard) or double-distilled water (for blank) were placed in a volumetric flask of 10 ml. Then, 2 ml of Folin-Ciocalteu's reagent (diluted 1:10 with ultrapure water) and 1.6 ml of Na_2CO_3 (7.5% w/v) were added to the mixture and the volume was made up to the mark by H_2O.

After incubation for 2 hours at room temperature, the absorbance was determined at 765 nm with an UV-Visible spectrophotometer against the blank.

Standard solutions of gallic acid were prepared in Milli-Q water at concentrations ranging from 10 to 1000 µg/ml.

TPC was expressed as mg Gallic Acid Equivalents (GAE) per g of prune by-product.

II.3.5. Total flavonoid content (TFC)

TFC was determined by aluminum chlorate method [35]. For this assay, 400 µl of the extract or quercetin (standard) or Methanol (for blank) were mixed with 4 mL of Methanol and 200 µl of $AlCl_3$ solution (5%, m/v). Then, the solutions were completed up to 10 ml with Methanol and allowed to stand for 30 minutes at room temperature. The absorbance was read at 425 nm.

Quercetin solutions at concentrations ranging from 10 to 1000 µg/ml were taken as a reference. TFC was expressed as mg Quercetin Equivalents (QE) per g of prune by-product.

II.3.6. HPLC-ESI-MS and MS^2 analysis

In order to obtain a reliable identification of phenolic compounds present in the ethanolic extract of prune by-product, an analysis with LC-ESI-MS (Liquid Chromatography ElectroSpray Ionization Mass Spectrometry) and MS^2 experiments was performed both in the positive and in the negative ion mode [36].

The LC-ESI-MS and MS^2 analyses were carried out using an Agilent Technologies modular 1200 system coupled to an Agilent 6310A ion trap mass analyzer with an ESI ion source (Agilent, Waldbronn, Germany). HPLC analyses were performed on a Zorbax SB-C18 (15

cm × 4.6 mm i.d., 5 µm, Supelco, Bellefonte, PA, USA) column. The mobile phase consists of (A) water-formic acid (9:1 v/v) and (B) methanol-water-formic acid (5:4:1 v/v/v). The separation was carried out following the gradient elution: 0-15 min 10-20% (B), 15-35 min 20-30% (B), 35-45 min 30-50% (B). A period of 5 min was necessary for column equilibration, during which no MS data was recorded. The flow-rate was set at 1 mL/min and the injection volume was 10 µL. The ESI source operated in negative ionization mode and the experimental parameters were set as follows: the capillary voltage was 3.5 kV, the nebulizer (N_2) pressure was 32 psi, the drying gas temperature and flow were 350 °C and 10 L/min, respectively, and the skimmer voltage was 40 V.

Agilent 6300 Series Ion Trap LC/MS system software (version 6.2) was used for instrument control, data acquisition and qualitative analysis. The mass spectrometer was operated in full-scan mode in the m/z range 200–1200. MS^2 spectra were automatically performed by using the SmartFrag function with helium as the collision gas in the m/z range 50–1500.

II.3.7. Estimation of chlorogenic acid content by HPLC/DAD

Prunes have been reported to contain high amounts of chlorogenic acid which is well-known for its important role on the intestinal function. This role was mainly manifested by the inhibition of the growth of selected gut pathogens to a human gut cell, as well as the improvement of the probiotic *L. rhamnosus* proliferation.

Before injection, samples were filtered through a 0.45 µm filter and poured into the HPLC vials. Then, samples were analyzed by a 1100 Series HPLC system Agilent Technologies (Waldbronn, Germany) equipped with a quaternary pump, a degasser, a thermostatic autosampler and diode-array detector.

The chromatography was performed on a Zorbax SB-C18 (15 cm×4.6 mm i.d., 5 µm), at a flow rate of 1 mL/min and the injection volume was 5 µL. The mobile phase solvents consisted of (A) water-formic acid (9:1 v/v) and (B) methanol-water-formic acid (5:4:1 v/v/v). Separations were achieved at 30°C according to the following gradient: 10 to 20% (B)

in 15 min, 20 to 30% (B) in 20 min and 30 to 50% (B) in 10 min. A period of 5 min was necessary for column equilibration.

Detection and quantification of chlorogenic acid were carried out at 320 nm [37].

II.3.8. Determination of sugars by HPLC/ELSD

High Performance Liquid Chromatography (HPLC) was reported to be the most efficient method for the estimation of sugar content in plant extracts. For that, several detectors were coupled with HPLC like Evaporating Light Scattering Detection (ELSD), Refractive Index Detection (RID) and Charged Aerosol Detection (CAD). HPLC coupled with Mass Spectrometry (MS) and High-Performance Anion-exchange Chromatography (HPAEC) were also used for sugar analysis [38]. In this study, sugar analysis was performed using high performance liquid chromatography coupled ELSD detector.

Water extract was obtained by maintaining 2 g of prune by-product in agitation with 100 mL of distilled water for 2 hours at room temperature. Then, the solution was centrifuged for 10 min at 5000 rpm and the supernatant was filtrated. The extract was filtered through a 0.45 µm filter before injection.

Analysis was carried out using an Agilent 1290 Infinity Series LC system (Agilent Technologies, Germany) coupled to an Agilent evaporative light scattering detector (ELSD-380) (Agilent Technologies, UK). Separation was performed on Agilent Hi-Plex Ca (7.7 x 300 mm) column kept at room temperature. The mobile phase was composed only by Milli-Q water and the analysis time was set to 30 min.

The acquisition and quantification of data were performed by OpenLab software.

II.4. Evaluation of antioxidant activity

The total antioxidant capacity (TAC) of the ethanolic extract of prune by-product against DPPH radicals was measured as described by **Cosmulescu *et al.*** (2012) [34].

For that, 50 µL of each sample (ethanolic extract, standard or ethanol for blank) was mixed with 3 mL of ethanolic DPPH solution (0.004% m/v). Then, the solutions were incubated in dark for 30 min at room temperature. The absorbance was measured at 517 nm.

Ascorbic acid standards of various concentrations (0–20 µg/mL) were used as reference. Antioxidant capacity was expressed as milligrams of ascorbic acid equivalent per 100 g (mg AAE/100 g FW).

$$Inhibition\ (\%) = \frac{A_0 - A_s}{A_0}\ x\ 100$$

where A_0: absorbance of control (without sample)

A_s: absorbance of sample { A_s = A_s (with DPPH) - A_s (without DPPH) }

II.5. Reduction of sugar content by fermentation

The objective of this part is to investigate the fermentation of prune by-product using a yeast species (*Saccharomyces cerevisiae*) in order to reduce the sugar content (glucose and fructose) while maintaining or increasing the sorbitol content. Thus, it has been reported that higher sugar content may cause undesirable caramelization during thermal treatment in food processing [39]. For that, an amount of 50 g of the prune by-product were left under agitation with 500 ml of distilled water. Then, 1 g of a commercial Saccharomyces cerevisiae strain (baker's yeast) were added in order to start fermentation process which lasts 24 hours at room temperature. Samples were taken after a certain fermentation time (1h, 2h, 3h, 4h, 5h, 6h, 7h, 20h and 24h). The different solutions undergo a centrifugation for 10 min at 5000 rpm and then filtered. Final samples were filtered through a 0.45 µm filter before injection on HPLC/ELSD [39,40].

III. RESULTS AND DISCUSSIONS

III.1. Chemical composition

The chemical composition of prune by-product is reported in Table 1.

Indeed, it has been reported that the chemical composition in prunes differs according to the species, the place and the picking period.

Table 1. Chemical composition of prune by-product

	Method	**Fresh matter (FM)**	**Dry matter (DM)**
Water (g/100g)	Freeze-drying	73.97 ±2.75	-
Moisture (g/100g)	Oven drying	9.67 ±1.37	-
Pectin (%)	Precipitation EtOH	14.80 ±0.53	56.85 ±2.02
TPC (mg GAE/g)	Folin–Ciocalteu assay	3.32 ±0.66	12.77 ±2.55
TFC (mg QE/g)	Aluminum chlorate method	0.06 ±0.03	0.23 ±0.14
Chlorogenic acid (mg/g)	HPLC/DAD	0.53 ±0.12	2.04 ±0.48
Fructose (mg/g)	HPLC/ELSD	125.03 ±38.24	480.32 ±146.91
Sorbitol (mg/g)		44.04 ±9.50	169.17 ±36.50
Metals (mg/100g)	ICPMS		
K		583.79 ±82.16	2242.77 ±315.62
Ca		45.33 ±18.48	174.15 ±70.99
Mg		27.15 ±9.79	104.29 ±37.62
Na		25.58 ±18.50	98.26 ±71.09
Zn		24.31 ±8.84	93.41 ±33.96
B		2.2 ±0.73	8.49 ±2.81
Fe		0.97 ±0.46	3.73 ±1.78
Cu		0.84 ±0.53	3.22 ±2.03
Ni		0.65 ±0.50	2.49 ±1.93
W		0.48 ±0.33	1.83 ±1.27
S		0.34 ±0.19	1.31 ±0.74
Mn		0.19 ±0.12	0.71 ±0.45
Li		1.50 ±1.28	5.74 ±4.90

Pb	0.14 ±0.08	0.52 ±0.30
Cr	0.02 ±0.01	0.08 ±0.04
Co	Nd	Nd
Cd	Nd	Nd
As	Nd	Nd

III.1.1. Water, moisture, pectin and metal content

Firstly, prune by-product contains 73.97 ±2.75 g/100g FM of water, whereas the moisture content was 9.67 ±1.37 g/100g FM. By comparing these findings with those obtained for other fruit wastes, we can conclude that these values are in the same order of magnitude. The moisture content was found to be 8.59 ±0.43 g/100g for grape pomace [41], from 8.52 g/100g to 10.23±0.06 g/100g for the by-products of different citrus varieties [42,43], 9.00 g/100g for apple pomace [44], 10.55 g/100g for orange pomace [44], 4.30 to 5.43 g/100g for apricot (*Prunus armeniaca* L.) seeds [45] and 7.51 g/100g mango peels [46].

Concerning the composition of minerals, we can assert the presence of the most abundant metals in fruits. Thus, we notice that potassium is present up to the highest content (583.79 ±82.16 mg/100g FM) followed by calcium (45.33 ±18.48 mg/100g FM), magnesium (27.15 ±9.79 mg/100g FM), sodium (25.58 ±18.50 mg/100g FM) and zinc (24.31 ±8.84 mg/100g FM), while the other metals are present as traces.

III.1.2. Total phenolic and flavonoid content

Folin-Ciocalteu and aluminum chlorate assays (Figure 1) were performed to estimate the total phenolic content (TPC) and total flavonoid content (TFC) using gallic acid and quercetin as standards, respectively. The calibration curves of gallic acid and quercetin are illustrated in Figure 2 and Figure 3.

TPC and TFC of the ethanolic extract were found to be 3.32 ±0.66 mg GAE/g and 0.06 ±0.03 mg QE/g, respectively.

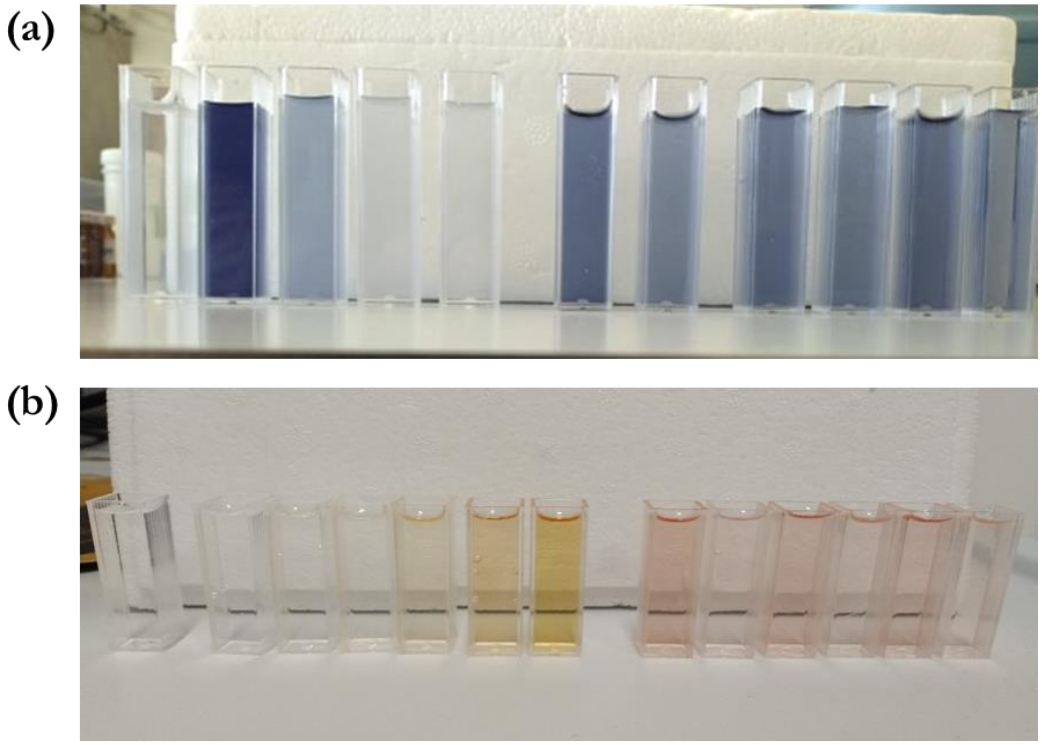

Figure 1. Determination of TPC and TFC by Folin-Ciocalteu (a) and aluminum chlorate (b) assays

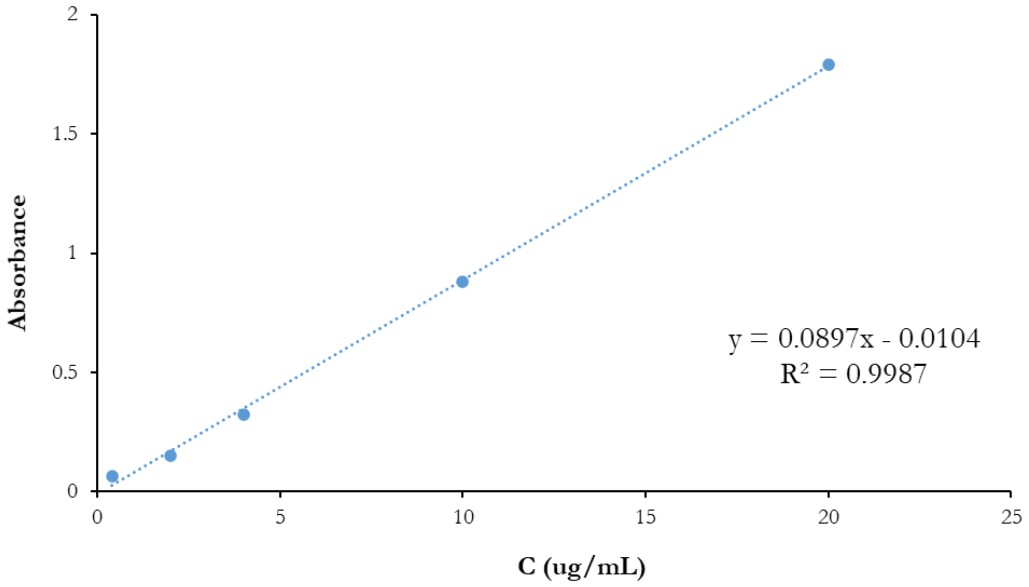

Figure 2. Calibration curve of gallic acid

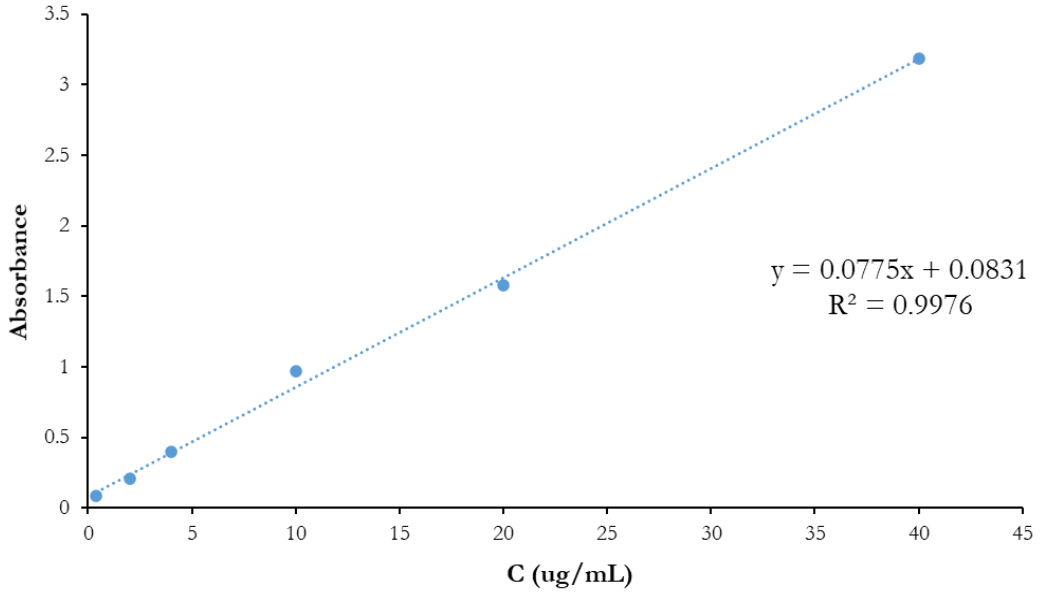

Figure 3. Calibration curve of quercetin

III.1.3. Composition of phenolic compounds

In order to obtain a reliable identification of phenolic compounds in the ethanolic extract, an analysis by LC-ESI-MS and MS^2 was performed both in the positive and in the negative ion mode. Figure 4 shows the obtained chromatograms, whereas the possible identifications are illustrated in Table 2.

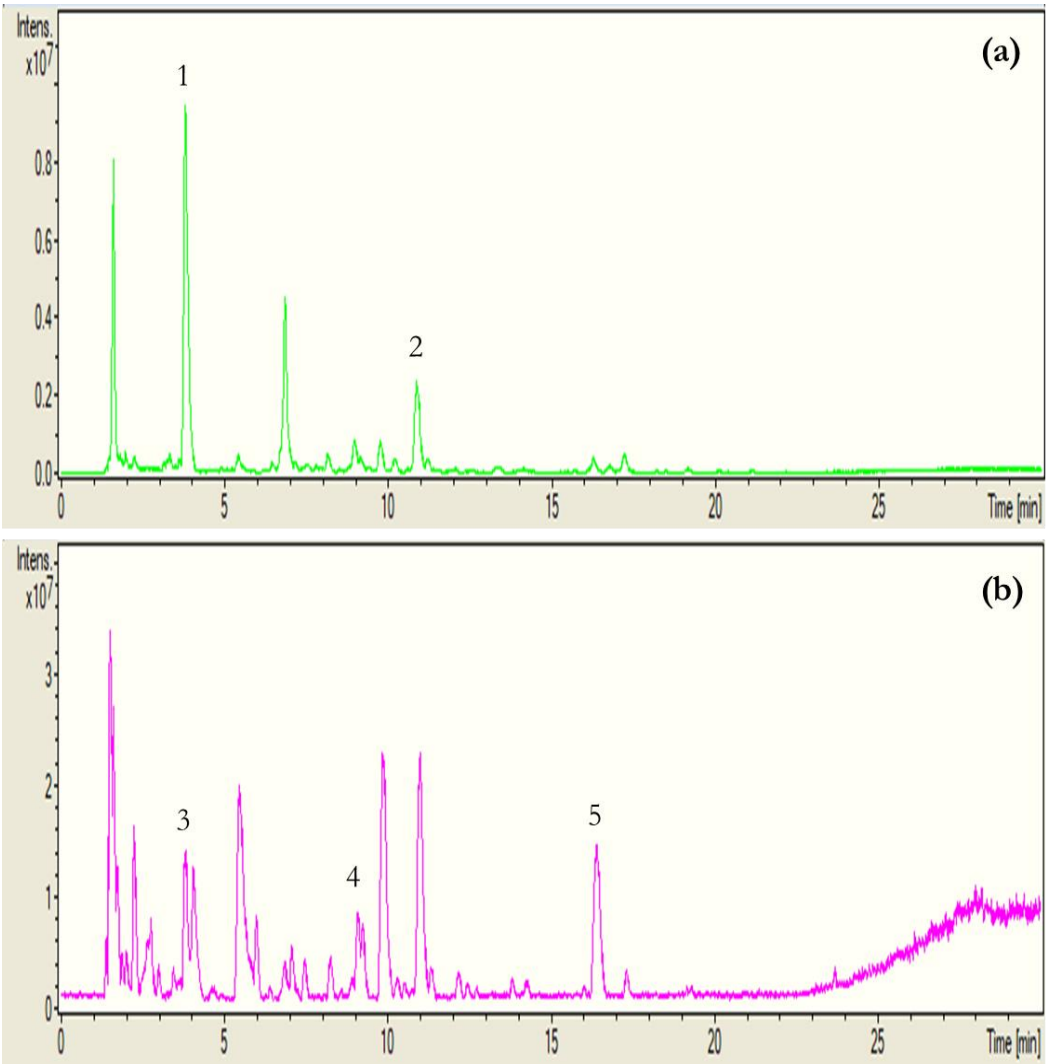

Figure 4. LC-ESI-MS and MS^2 analysis of the ethanolic extract from prune by-product **(a)** negative ion mode **(b)** positive ion mode

In negative ion mode, we observe a fragment at m/z 353.1 which is identified as caffeoylquinic acid isomers (chlorogenic acids) as described by **Fang *et al.*** [47]. Another fragment was observed at m/z 447.1 which confirms the presence of quercetin derivatives (quercetin-deoxyhexoside and quercetin-rhamnoside) as mentioned previously by **Jaiswal *et al.*** [48].

Otherwise, in positive ion mode, chlorogenic acid (5-O-caffeoylquinic acid) and neochlorogenic acid (3-O-caffeoylquinic acid) were identified with the same $[M+H]^+$ (m/z 355.2) and fragment (m/z 163.1) ions, which is in accordance and is consistent with previous research indicating that neochlorogenic acid is the predominant phenolic acid in plums [49] and that chlorogenic acid is also present in plums but in lower amounts than neochlorogenic acid [50].

Furthermore, cyanidin-3-O-glucoside and cyanidin-3-O-galactoside were also detected. Both compounds had the same $[M]^+$ (m/z 449.3). These anthocyanins were previously reported in some plum varieties such as *Prunus domestica* and *Prunus salicina* [49,50,51].

Concerning quercetins, only quercitin-3-O-rhamnoside has been detected at $[M+H]^+$ (m/z 449.3) [49].

Table 2. Possible identifications though LC-ESI-MS and MS^2 analysis

Negative ion mode			
Peak	**Rt (min)**	**$[M-H]^-$ (m/z)**	**Identification**
1	3.9	353.1	Caffeoylquinic acid isomers (chlorogenic acids)
2	10.9	447.1	Quercetin-deoxyhexoside Quercetin-rhamnoside
Positive ion mode			
Peak	**Rt (min)**	**$[M+H]^+$ (m/z)**	**Identification**
3	3.9	355.2	Chlorogenic acid (5'-caffeoylquinic acid) Neochlorogenic acid (3'-caffeoylquinic acid)
4	16.3	449.3	Cyanidin-3-O-glucoside Cyanidin-3-O-galactoside
5	16.3	449.3	Quercitin-3-O-rhamnoside

In addition, prunes have been reported to contain high amounts of chlorogenic acid which is well-known for its important role on the intestinal function. This role was mainly manifested by the inhibition of the growth of selected gut pathogens to a human gut cell, as well as the improvement of the probiotic L. rhamnosus proliferation [52]. Thus, the highest amounts of chlorogenic acid were found in prune fruits from France (39 mg/g FM) and USA (36 mg/g FM), whereas these amounts were lower in prunes from Chile and Argentina (13 mg/g FM) [52].

The analysis by HPLC/DAD revealed that prune by-product contains 0.53 ±0.12 mg/g FM of chlorogenic acid. This value is more important than that found by Michalska el al. for plum pomace obtained after juice extraction (0.33 mg/g FM) [53].

III.1.4. Composition of sugar

Furthermore, prune by-product was analyzed by HPLC-ELSD in order to estimate the exact content of the most evident sugar, and especially sorbitol (Table 1). The content of fructose and sorbitol were estimated using the calibration curves elaborated by HPLC analysis standards (Figure 5 and Figure 6).

The studied prune by-product contains 125.03 mg/g FM of fructose and 44.04 mg/g FM of sorbitol on wet weight basis (Figure 7).

In the light of the obtained results though the various analysis, and by comparing with those reported in the literature for prune fruits, prune juice and dried prunes, we can conclude that that the prune by-product is still rich in bioactive compounds [25,54,55].

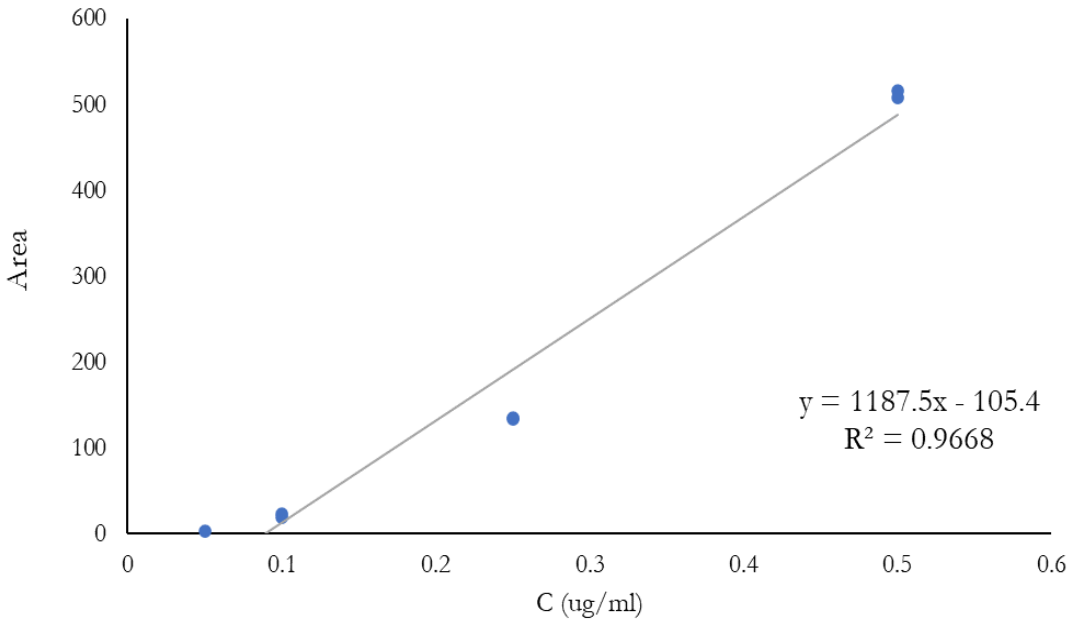

Figure 5. Calibration curve of fructose

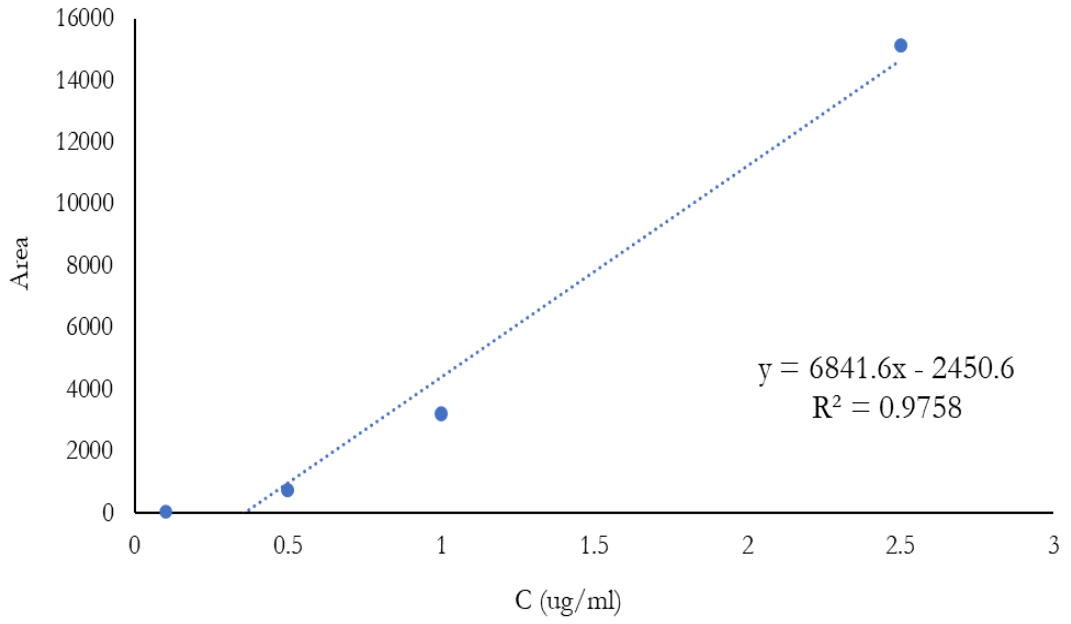

Figure 6. Calibration curve of sorbitol

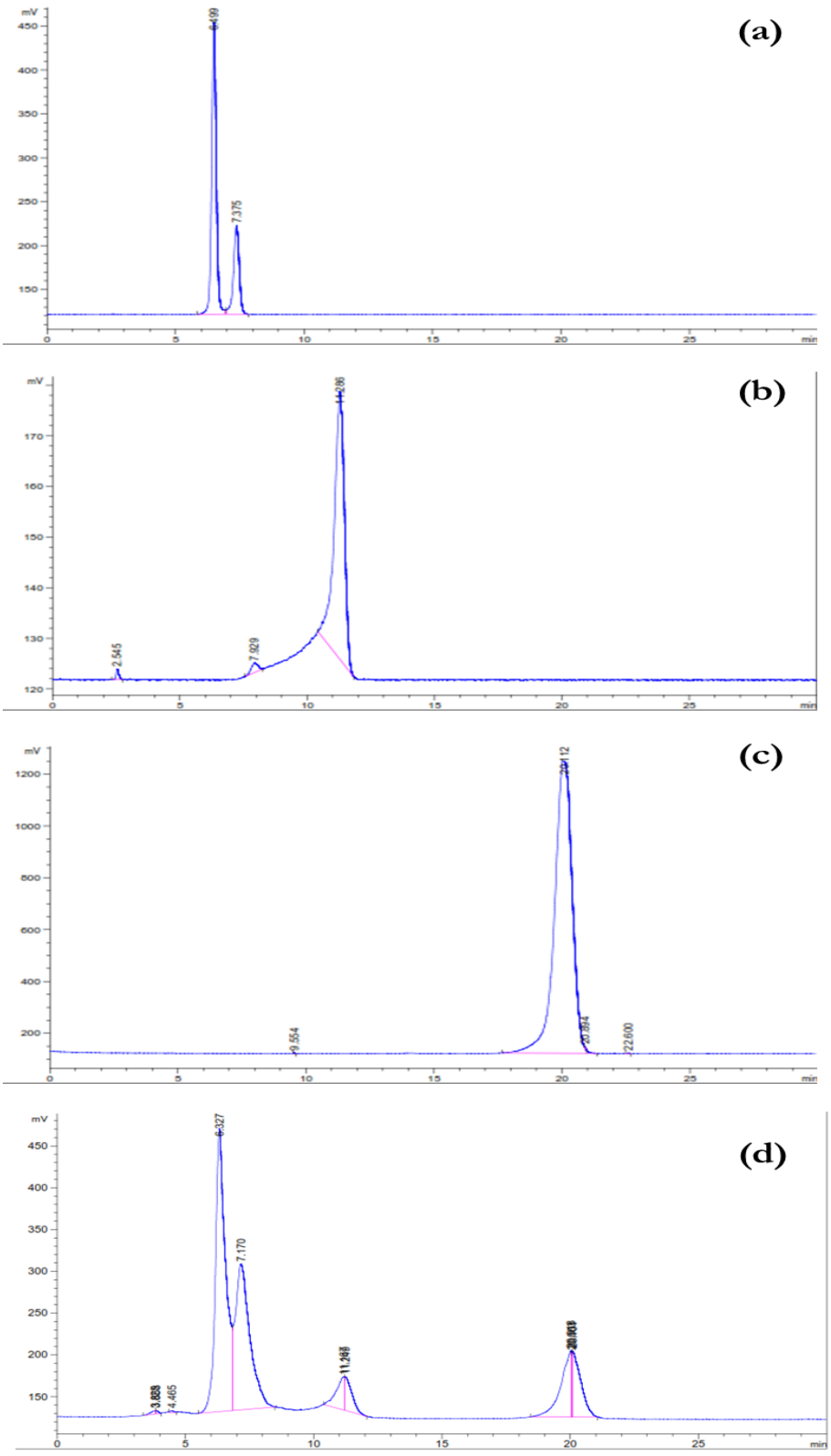

Figure 7. HPLC/ELSD chromatograms of (a) glucose (b) fructose (c) sorbitol and (d) prune by-product

III.2. Antioxidant activity

The effectiveness of plant's extracts against free radicals has been determined using several analytical procedures such as Radical Trapping Antioxidant potential (TRAP), β-carotene bleaching, Oxygen Radical Absorbance Capacity (ORAC), Ferric Reducing Antioxidant Power (FRAP), Superoxide anion radical scavenging assay, Hydroxyl radical scavenging assay and DPPH radical scavenging assay [56]. This latter has been used in this study to estimate the antioxidant capacity in prune byproduct.

The antioxidant capacity of prune's ethanolic extracts, against DPPH radicals, is estimated as quantities in mg of AAE/100 g FM (Table 3).

From the obtained results, we notice that prune byproduct shows a significant antioxidant capacity which is in the order of 294.09 mg AAE/100 g FM. Thus, Cosmulescu *et al.* reported in a previous study that methanolic extracts from skin, pulp and fruits of Romanian plum cultivars had an antioxidant capacity of about 972.74 mg AAE/100 g FM, 38.17 mg AAE/100 g FM and 92.65 mg AAE/100 g FM, respectively [34].

Indeed, this significant antioxidant power could be mainly due to high content of polyphenolics and flavonoids remaining in our prune byproduct.

Table 3. Total antioxidant capacity of the ethanolic extract of prune byproduct

		TAC (mg AAE/100 g FM)
Prune by-product		294.09 ±65.85
Plum fruit [34]	Skin	163.62 ± 5.22 - 972.74 ± 13.36
	Pulp	10.38 ± 0.28 - 38.17 ± 0.82
	Fruit	22.17 ± 1.67 - 92.65 ± 3.56

III.3. Reduction of sugar content by enzymatic fermentation

Fermentation has been reported as of the suitable applications used for the biotransformation of fruit by-products into added-value products having low cost and high nutritional value, thus solving the problems related to their throw into environment. Generally, there is three types of fermentation processes that are mostly used: solid state, sub-merged and liquid fermentation. Selections of the fermentation methods are product specific. Solid state and sub-merged fermentation processes are applied to obtain bioactive compounds from by-products for further industrial interest [57].

In this part, we applied the fermentation process to prune by-product in order to reduce the sugar content (glucose and fructose), while retaining the sorbitol. The determination of sugars before and after fermentation was carried by HPLC-ELSD analysis. The concentrations of fructose and sorbitol during different times of fermentation are reported in Table 4. The results showed an increase of sorbitol content with the fermentation time, starting from 44.04 ±9.50 mg/g FW at the beginning of the process up to 77.45 ±12.04 mg/g FW after 24 hours of fermentation. Otherwise, the content of fructose, which was 125.03 ±38.24 mg/g FW initially, undergoes an increase after 1 hour of fermentation to 315.14 ±71.78 mg/g FW. Then, this value gradually decreases over time and became 12.97 ±2.19 mg/g FW after 24 hours of fermentation.

Thus, it has been reported in previous studies conducted on fruit products and by-products fermentation that there is a clear decrease in sugar content in the course of fermentation. In addition, it was observed a relatively fast degradation of glucose in the fermentation process, followed by degradation of fructose, whereas the degradation of sucrose has been reported to be much slower and could start even when glucose and fructose were nearly completely degraded [58,59]. This may explain the increase in fructose content after the first hour of fermentation, which is probably due to the degradation of glucose.

Concerning sorbitol, it has been reported that enzymatic fermentation of fruits boosts the production of sorbitol by hydrogenation of glucose [60]. Sorbitol could be also obtained by conversion from fructose by mean of fermentation via glucose-fructose oxidoreductase [61].

Table 4. Concentration of fructose and sorbitol during fermentation

Fermentation time (h)	Sorbitol (mg/g FW)	Fructose (mg/g FW)
0	44.04 ±9.50	125.03 ±38.24
1	52.68 ±8.31	315.14 ±71.78
2	53.74 ±7.33	254.55 ±22.72
3	55.83 ±3.55	228.09 ±46.53
4	57.69 ±13.47	189.24 ±36.99
5	62.85 ±12.16	119.06 ±17.14
6	63.08 ±10.80	72.24 ±7.30
7	65.87 ±9.29	46.37 ±7.29
20	79.70 ±7.23	20.12 ±5.04
24	77.45 ±12.04	12.97 ±2.19

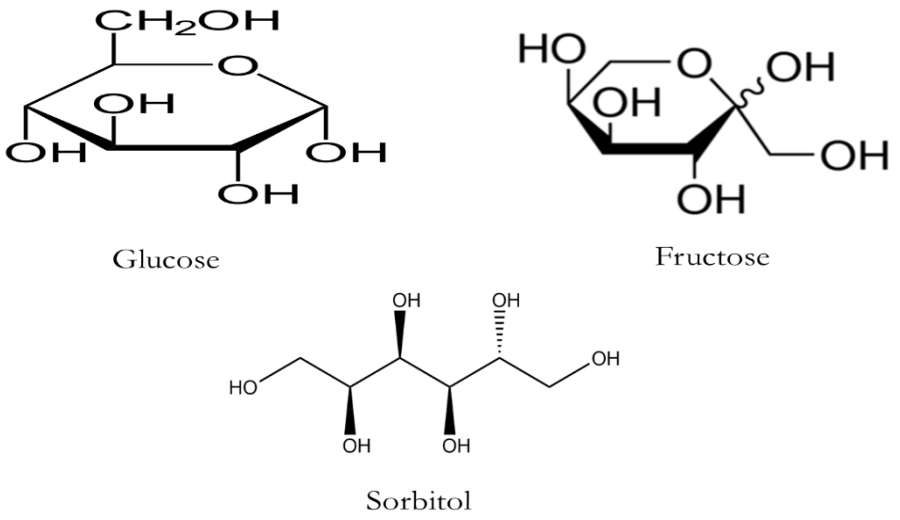

Figure 8. Chemical structure of glucose, fructose and sorbitol

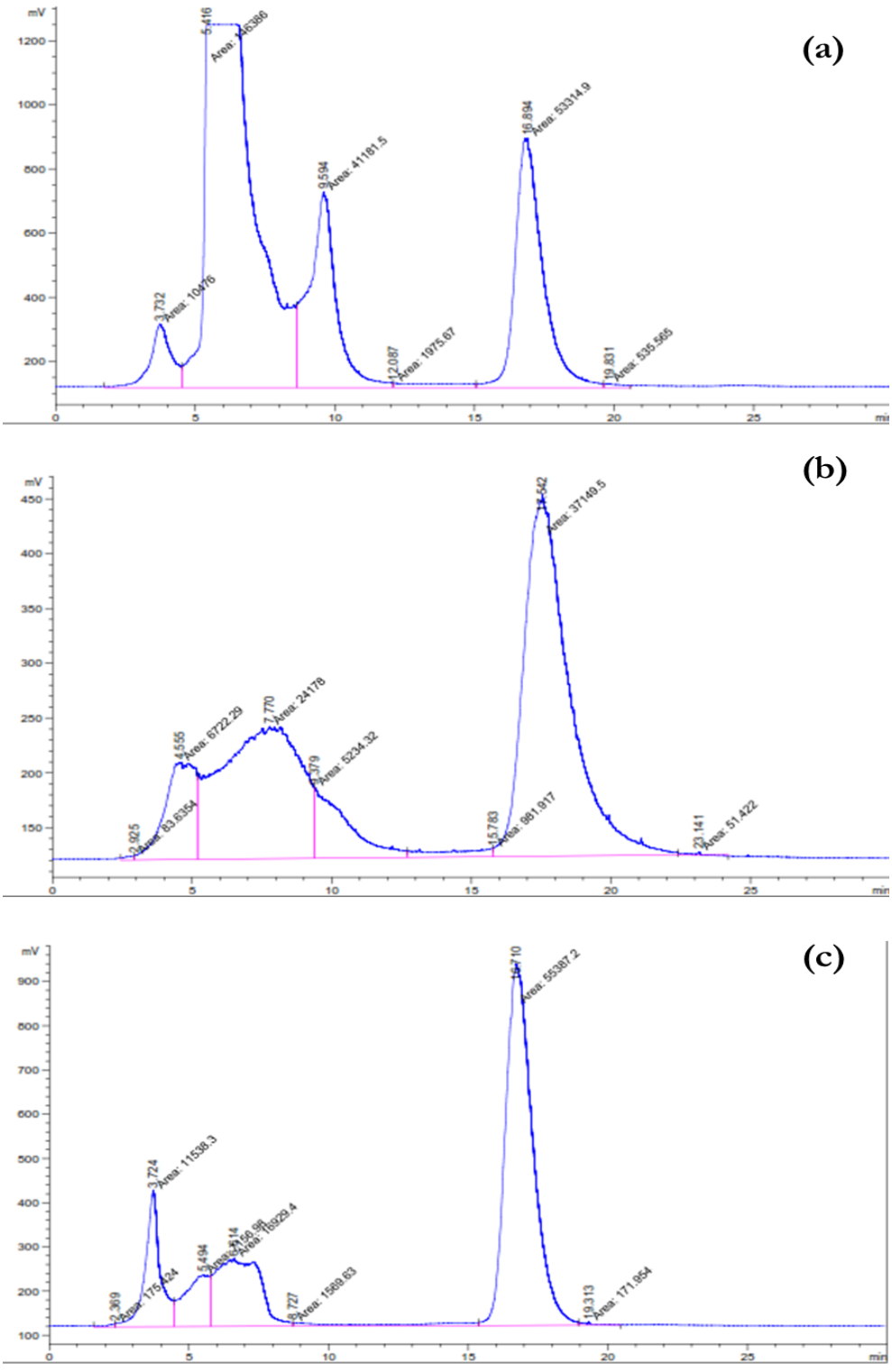

Figure 9. HPLC/ELSD chromatograms of prune by-product after (a) 1 hour (b) 7 hours and (c) 24 hours of enzymatic fermentation

IV. CONCLUSION

In this chapter, we were interested on the by-product of prune processing industry, which was generally considered as waste. Firstly, a study of the chemical composition of this by-product was performed using various analyses including spectrophotometric assays (Folin-Ciocalteu and aluminum chlorate) and chromatography techniques (LC-ESI-MS and MS^2, HPLC-DAD, HPLC-ELSD and ICP-MS). The results obtained by all these analyses revealed that the studied by-product still rich in bioactive compounds such as phenolic compounds (TPC= 3.32 and TFC=0.06 mg/g), sugars (sorbitol= 44.04 mg/g and fructose=125.03 mg/g) and minerals (especially potassium, calcium, magnesium, sodium and zinc). The detailed analysis of the phenolic composition asserted the presence of quercetin derivatives, cyanidin glycosides and particularly caffeoylquinic acids. Chlorogenic acid, which is reported to be the main phenolic compound in plums, was present in prune by-product by about 0.5 mg/g of wet weight, as estimated by HPLC-DAD analysis. Prune by-product showed also a significant antioxidant capacity (DPPH radical scavenging activity) compared to that obtained for plums.

Then, and in order to obtain derivative rich in sorbitol, chlorogenic acid and secondary metabolites, a fermentation was applied to the prune by-product. The main objective was to break down the concentration of reducing sugars since they are present in high quantities. For that, we resorted to an enzymatic fermentation using *Saccharomyces cerevisiae*. Preliminary results obtained by HPLC-DAD showed a significant decrease of glucose and fructose content and an increase of the sorbitol content after 24 hours of fermentation.

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CHAPITRE II

RESEARCH OF LAVENDER AND LAVANDIN ESSENTIAL OILS AND BY-PRODUCTS: CHARACTERIZATION AND BIOLOGICAL ACTIVITIES

"Learning is an experience. Everything else is just information" **(Albert Einstein)**

"Tell me and I forget. Teach me and I remember. Involve me and I learn"

(Benjamin Franklin)

I. INTRODUCTION

Aromatic medicinal plants (AMPs) have been used since antiquity as preservatives, colorants, flavoring agents and flavor enhancers. In addition, these plants have long been the basis of traditional medicine around the world for various purposes, including the treatment of infectious diseases. They have also been studied in the chemical, pharmaceutical and food industries because of their potential use to improve health. Thus, they have become "industrial products" with new concepts such as phytotherapy, aromatherapy, nutraceuticals and cosmeceuticals, thus broadening the field of their use. Over the last few decades, new innovative value-added applications have appeared including their use in functional foods, animal husbandry and plant protection in agriculture [1-4].

In addition, the attraction to medicinal and aromatic plants continues to grow due to increasing consumer demand and interest in these plants for culinary, medicinal and anthropogenic applications. As consumers are increasingly informed about food, health and nutrition issues, they are also becoming aware about the dangers associated to chemical preservatives used by food manufacturers. Recent reports revealed that some synthetic antioxidants widely used in industry food as potential inhibitors of lipid oxidation, may be implicated in many health risks, including liver damage, cancer and carcinogenesis [4-6].

These compounds have been reported to exhibit beneficial effects on the incidence of cancer and many chronic diseases, including cardiovascular disease, type II diabetes and neurodegenerative diseases such as Alzheimer's disease, due to their antioxidant properties having a protective role against the phenomenon of oxidative stress resulting from oxygenated radical species which are the main cause of these diseases [7-10].

Lavender, which has been reported to be a precious essential oil plant, belongs to the *Lamiaceae* family and is cultivated around the world. It is considered as a significant source of perfume and showed beneficial cosmetic and pharmaceutical effects of the high content and the best quality of its essential oil. This latter was found to be beneficial for use in nervous system stimulants, hypnotics, sedatives, tranquilizers and stress repellents.

Furthermore, Lavender oil has significant dermatological uses, especially in the treatment sunburn and skin rashes, as well as strong antibiotic (bacteria killing) and antiseptic (disinfectant) effects. These applications are mainly related to the beneficial effects of lavender essential oil including anti-inflammatory, sedative, analgesic, antidepressant, antiseptic and antispasmodic healing properties [11-14].

Lavender is a plant quite resistant to drought and temperature. Thus, there are 39 *Lavandula* species (*Lavandula sp.*). The majority of them are of Mediterranean origin, mainly France, Spain, Italy and Andorra). Yet, the most important three species are [15]:

- Lavender (*Lavandula angustifolia* Mill. syn., *L. officinalis* Chaix ex Vill syn., *L. vera* DC syn. *L. spica* (true lavender, fine lavender or English lavender).
- Lavandin (*Lavandula intermedia* Emeric ex Loisel syn. *L. hybrid,* a hybrid of *L. angustifolia* and *L. latifolia*).
- Spike lavender (*Lavandula spica or L. latifolia* Medicus).

The world production of lavender's essential oil is approximately 200 tons per year, with France, Bulgaria and UK as major producers. On the other hand, the world production of lavandin's essential oil is about 1200 tons per year and France was reported to have the highest production with a rate of 90% [14,15].

The rich composition of lavender in essential oils expand their field of application, either in food manufacturing industry as flavoring agent, preservative additives or in cosmetics and fragrance industry such as soaps, perfumes, skin lotions ... In particular, lavender essential oil find industrial application such as perfumery, pharmaceuticals and cosmetic because of the high levels in linalool and linalyl acetate, whereas lavandin essential oil is generally used in the formulation of hygiene products, household and industrial cleaner products and detergents due to high content of camphor [12,15].

Lavender and lavandin distilled straws, the by-products after oil isolation, have long been considered as waste. Recently, new researches reported that these by-products are also an important source of high added-value compounds, taking in consideration the large amounts of wastes generated by distillation process [13,14,16,17].

II. EXPERIMENTAL

II.1. Chemicals and reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH), quercetin, gallic acid, sodium sulphate (Na_2SO_4), ferrozine, Iron (III) and Iron (II) chlorides, ethylenediaminetetraacetic acid (EDTA), ascorbic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), trichloroacetic acid, potassium ferricyanide, Folin–Ciocalteu reagent, acetylcholinesterase (AChE) (electric eel, E.C. 3.1.1.7, type VI-S), acetylthiocholine iodide (ATCI), 5,5'- dithiobis (2-nitrobenzoic acid) (DTNB), galantamine hydrobromide, tyrosinase (mushroom, E.C. 1.14.18.1), L-tyrosine, and kojic acid were purchased from Sigma-Aldrich (Milan, Italy). Aluminum chloride was provided by Carlo Erba Reagents (Milan, Italy).

Acetonitrile (ACN), acetic acid (HAc), ethylacetate (EtOAc), n-hexane (Hex), and ethanol were of LC–MS purity grade (Sigma-Aldrich). Ultrapure water was obtained using a Milli-Q Plus 185system from Millipore (Milford, Mass., U.S.A.).

II.2. Plant material

Two plants were chosen to be the subject of this experimental work: *Lavandula angustifolia* Mill. and *Lavandula x intermedia* cv "Grosso". The flowers were harvested from two different farms located in the Italian Tuscan-Emilian Apennines (9X4J+7W map and 7XWH+3F map respectively). The aerial parts of the plants were hand-picked when the inflorescences were in full blooming during the summer 2021.

The freshly collected plant materials were dried on filter paper in the shade, at room temperature and in a dry place away from humidity.

II.3. Extraction of essential oils (EOs) and phenolic compounds

II.3.1. Isolation of EOs by steam distillation

The EOs were extracted from dried plant materials by steam distillation according to the method described by **European Pharmacopoeia X Ed**. [18]. Briefly, about 400 g of flowers were steam distilled for 1 hour by a stainless-steel distiller coupled with a Clevenger-type apparatus (Albrigi Luigi s.r.l., Stallavena, VR, Italy). The collected EO was separated from hydrosol and measured on an analytical scale. The percent yield of the EOs was calculated as weight of oil per weight of lavender flowers. The EOs were stored at 4 °C until analysis whereas the oil-exhausted biomasses were collected and dried at room temperature.

II.3.2. Hydrosol extraction

The volatile components were isolated from hydrosols as described by **Truzzi *et al.*** [19]. For that, 10 g of hydrosol were firstly extracted three times with 5 mL of ethyl acetate, then subsequently three times with 5 mL of hexane. The combined organic phases were washed with brine (solution of salt in water). The organic phase was then dried over Na_2SO_4 and concentrated at room temperature under vacuum. The residue was weighted, solubilized in hexane and analyzed by gas chromatography.

II.3.3. Isolation of phenolic compounds from residual material

Phenolic compounds were extracted by keeping 3.5 grams of oil-exhausted flowers under dynamic maceration for one night with 40 mL of a water:ethanol (50:50) solution. The solution was then filtered into a volumetric flask and the residue was extracted two times more with 35 mL of the same solvent for 1 hour. The filtrates were combined add the volume was adjusted at the final volume of 100 mL. The hydroalcoholic extract was stored at 4 °C until further analyses. Each extraction was performed in triplicate [20].

II.3.4. Isolation of phenolic compounds from waste water

The separation of phenolic compounds from waste water was performed by extraction of 500 mL with 100 mL of ethyl acetate. This procedure was repeated two times more. Then, the organic phases were combined and the solvent was removed by rotary evaporator. The final residue was weighted and completed up to 10 mL by EtOH (70%) [21].

II.4. Characterization of EOs and hydrosols

II.4.1. GC-MS analysis

Analyses were performed on a 7890A gas chromatograph coupled with a 5975C network mass spectrometer (GC-MS) (Agilent Technologies, Milan, Italy). Compounds were separated on an Agilent Technologies HP-5 MS cross-linked poly-5% diphenyl–95% dimethyl polysiloxane (30 m × 0.25 mm i.d., 0.25 μm film thickness) capillary column. The column temperature was initially set at 45 °C, then increased at a rate of 2 °C/min up to 100 °C, then raised to 250 °C at a rate of 5 °C/min and finally held for 5 min. The injection volume was 0.1 μL, with a split ratio 1:20. Helium was used as the carrier gas, at a flow rate of 0.7 mL/min. The injector, transfer line, and ion-source temperature was 250, 280, and 230 °C, respectively. MS detection was performed with electron ionization (EI) at 70 eV, operating in the full-scan acquisition mode in the m/z range 40-400. The EOs were diluted 1:20 (v/v) with n-hexane before GC-MS analysis.

II.4.2. GC-FID analysis

Chromatographic characterization of EOs was performed on a 7820 gas chromatograph (Agilent Technologies, Milan, Italy) with a flame ionization detector (FID). EOs and the mixture of aliphatic hydrocarbons (C_8–C_{40}) were diluted 1:20 (v/v) with Hex before GC-FID analysis. Helium was used as carrier gas at a flow rate of 1 mL/min with a pressure of 2.5 bar at the column head. The injector and detector temperatures were set at 250 and 300 °C, respectively. EO components were separated on an Agilent Technologies HP-5 crosslinked poly-5% diphenyl–95% dimethyl siloxane (30 m x 0.32 mm i.d., 0.25 mm film thickness) capillary column. The column temperature was initially set at 45 °C, then increased at a rate

of 2 °C/min up to 100 °C, then raised to 250 °C at a rate of 5 °C/min and finally maintained for 5 min. The injection volume was 1 µL, with a split ratio 1:20.

Compounds were identified by comparing the retention times of the chromato-graphic peaks with those of authentic reference standards run under the same conditions and by comparing the linear retention indices (*LRI*s) relative to C_8–C_{40} n-alkanes obtained on the HP-5 column under the above-mentioned conditions with the literature [22]. Peak enrichment by co-injection with authentic reference compounds was also carried out. Comparison of the MS-fragmentation pattern of the target analytes with those of pure components was performed, by using the National Institute of Standards and Technology (NIST version 2.0d, 2005) mass-spectral database.

The percentage relative amount of individual components was expressed as the percent peak area relative to the total peak area obtained by the GC/FID analysis. Semi-quantitative data were acquired from the mean of two analyses. The percentages of each compound are expressed as the mean ± standard deviation (SD) of the three replicates for each kind of treatment.

The data acquisition and processing were performed using the OpenLab CDS C.01.04 (Agilent Technologies, Santa Clara, CA) software.

II.5. Characterization of phenolic compounds

II.5.1. Total polyphenolic and flavonoid content

The total polyphenolic content (TPC) was determined by Folin–Ciocalteu method [13]. Briefly, 50 µL of the extract were mixed with 2.5 mL of 10% Folin-Ciocalteu reagent. Then, 2 mL of Na_2CO_3 saturated solution were added and the reaction mixture was incubated at 50 °C for 15 min. Finally, the absorbance of the solution was measured at 760 nm by using a UV/Vis spectrophotometer (UVmini-1240; Shimadzu Corp., Kyoto, Japan). The concentration of total polyphenolic compounds was calculated by using a standard curve prepared with gallic acid solution. The total polyphenolic content was expressed as milligrams of gallic acid equivalents (GAE) per gram of lavender flowers.

The total flavonoid content (TFC) was determined according to aluminum chloride method [13]. Briefly, 100 µL of sample were mixed with 1.9 mL of ethanol and 2 mL of 2% $AlCl_3$ solution. The reaction mixture was incubated for 30 min at room temperature in the dark and the absorbance was measured at 420 nm by a UV/Vis spectrophotometer (UVmini-1240). The concentration of total flavonoids was determined by using a standard curve prepared with quercetin solution. The total flavonoid content was expressed as milligrams of quercetin equivalents (QE) per gram of lavender flowers.

II.5.2. Identification of polyphenols by LC−ESI−MS and MS^2

The LC-ESI-MS and MS^2 analyses were carried out using an Agilent Technologies modular 1200 system coupled to an Agilent 6310A ion trap mass analyzer with an ESI ion source (Agilent, Waldbronn, Germany). HPLC analyses were performed on an Ascentis C_{18} column (250 mm × 4.6 mm I.D., 5 µm, Supelco, Bellefonte, PA, USA), with a mobile phase composed of (A) 0.3% acetic acid in water and (B) ACN. The gradient elution was set as follows: 0 min, 17% (B); 35 min, 23% (B); 52 min, 49% (B). The flow-rate was set at 1 mL/min and the injection volume was 20 µL. The ESI source operated in negative ionization mode and the experimental parameters were set as follows: the capillary voltage was 3.5 kV, the nebulizer (N_2) pressure was 32 psi, the drying gas temperature and flow were 350 °C and 10 L/min, respectively, and the skimmer voltage was 40 V.

Agilent 6300 Series Ion Trap LC/MS system software (version 6.2) was used for instrument control, data acquisition and qualitative analysis. The mass spectrometer was operated in full-scan mode in the m/z range 200–1200. MS^2 spectra were automatically performed by using the SmartFrag function with helium as the collision gas in the m/z range 50–1500.

II.5.3. Identification of polyphenols by HPLC-UV

A solution of the freeze-dried extract was prepared in 50% aqueous ethanol at a concentration of 1 mg/ml and then filtered using a 0.45 µm PTFE filter before HPLC injection.

Analysis was achieved using a HP 1100 Series HPLC system (Hewlett Packard, USA) coupled to an UV detector and an Ascentis C18 (250x4.6 mm, 5 um) column. The injection volume and the flow rate were 5 µL and 1 mL/min, respectively.

The chromatographic separation was carried out using mobile phase composed by a solution of 0.3% acetic acid in water (A) and acetonitrile (B) thought the following gradient: 0 min, 17% (B), 35 min, 23% (B) and 52 min, 49% (B) [23].

The quantification of phenolic compounds was performed at 280 nm and the concentrations were estimated against commercially available standards of chlorogenic acid, caffeic acid, 4-coumaric acid, ferulic acid, rosmarinic acid, luteolin and apigenin. Calibration curves were obtained with concentrations in the range of 10 and 300 µg/mL in methanol and each calibration point (n= 10) was replicated three times [23,24].

II.6. Biological activities

II.6.1. Evaluation of antioxidant activity of phenolic compounds

II.6.1.1. Determination of DPPH free radical-scavenging activity

The freshly-prepared extracts were diluted (1:10) with water:ethanol (50:50) solution and different aliquots of the obtained solution (ranged from 50 µL to 1.2 mL) were further diluted with ethanol to a final volume of 2.7 mL directly in cuvette. To each extract dilution, 300 µL of 0.04% DPPH ethanolic solution were added and the reaction mixtures were left to stand at room temperature for 15 min in the dark. The DPPH solution was freshly prepared daily, and stored in a flask covered with aluminum foil in the dark at 4 °C. A DPPH control sample (containing 2.7 mL of ethanol and 300 µL of DPPH solution) was prepared and measured daily. Finally, the absorbances were measured at 517 nm against blank extracts (without the addition of DPPH) by using a UV/Vis spectrophotometer (UVmini-1240, Shimadzu Corp., Kyoto, Japan) [13]. DPPH scavenging effect was calculated as follows:

$$(inhibition\ \%) = \frac{(A_{Control} - A_{Sample})}{A_{Control}} * 100$$

where $A_{Control}$ is the absorbance of the control reaction and A_{sample} is the absorbance of the sample.

Ethanolic solutions with different Trolox concentrations (ranged from 0.2 to 1.6 mM) were analyzed as positive control. The free radical scavenging capacity was expressed by IC50 values (mg of Trolox equivalents/g of biomass) extrapolated from dose-response curve.

II.6.1.2. Determination of Fe^{2+} chelating activity

To different aliquots (0.1 - 1.2 mL) of freshly-prepared extracts, 200 µL of 2 mM $FeCl_2$ solution and 200 µL of 5 mM ferrozine solution were added. The solutions were diluted with MilliQ water to 10 mL in a volumetric flask, and left to stand at room temperature for 10 min. The control sample was prepared in the same manner without the addition of the extract. Finally, the absorbances were measured at 562 nm against blank extracts (without the addition of $FeCl_2$ and ferrozine solutions) by using a UV/Vis spectrophotometer (UVmini-1240). The metal chelating effect was calculated as follows:

$$(inhibition\ \%) = \frac{(A_{Control} - A_{Sample})}{A_{Control}} * 100$$

where $A_{Control}$ is the absorbance of the control reaction and A_{sample} is the absorbance of the sample. EDTA was selected as positive control and different concentration (0.25 – 1 mg/mL) were analyzed. The metal chelating capacity was expressed by IC50 values (mg of EDTA equivalents/g of biomass) extrapolated from the dose-response curve.

II.6.1.3. Reducing power activity

The reducing power activity was performed according to **Papotti *et al.*** method with slight modifications [23]. The freshly-prepared extracts were diluted (1:10) with water:ethanol (50:50) solution, and different aliquots of the obtained solution (ranged from 100 to 500 µL) were further diluted with the same solvent up to 500 µL. Then, 2.5 mL of phosphate buffers solution (pH 6.6) and 2.5 mL of potassium ferricyanide 1% solution were added, and the solutions were incubated at 50 °C. After 20 min, 2.5 mL of Trichloroacetic acid 10% solution, 8 mL of water, and 1.6 mL of Iron (III) chloride 0.1% solution were added.

Finally, the 2 mL of the solutions were diluted with 2 mL of water, and the absorbances were measured at 700 nm. The slope of the dose-response curves indicated the reducing power of the extracts. Solutions of ascorbic acid with different concentrations (100 – 750 µg/mL) were prepared and analyzed as described above. The slope of the dose-response curves obtained for each ascorbic acid solution was plotted against the concentration, and the equation was used to determine the reducing power of the extracts (mg of ascorbic acid equivalents/g of biomass).

II.6.2. Evaluation of acetylcholinesterase inhibitory activity (IAChE)

The freeze-dried extracts of LA and LI were dissolved in PBS at the concentration of 10 mg/mL and different dilutions were prepared in the range of 0.5 – 10 mg/mL. For the inhibition of AChE, the extracts and the reagents were solubilized in PBS 100 mM at pH8.
The capacity of the extract in inhibiting the AChE was evaluated according to ***Costa et al.*** with minor modification [24]. The reaction solution was prepared by mixing 1 mL of DTNB 15mM, 200 µL of ATCI 3mM, 400 µL of PBS, and 200 µL of inhibitor solution (or PBS in the case of the enzymatic control) into a 1 mL cuvette. Then, 200 µL of AChE 0.115 U/mL were added, and the reaction was monitored for 5 min by recording the absorbance at 405 nm every 14 seconds using an UV/vis spectrophotometer (Jasco V-730, Easton, MD, USA). The absorbances were recorded against a blank solution composed by all the reactive without the enzyme. Galantamine was selected as reference inhibitor and it was tested in the range of 7 – 170 µg/mL under the same operative conditions.
The velocities (slopes, OD/min) of reaction were calculated for each inhibitor concentration tested, and the inhibition percentage was calculated as follows:

$$inhibition\ \% = \frac{(Slope_{CTRL} - Slope_{inhibitor})}{Slope_{CTRL}} * 100$$

Where $Slope_{CTRL}$ and $Slope_{inhibitor}$ are the velocities of the enzyme in absence or presence of the inhibitor respectively. The percentages of inhibition were plotted against the concentrations of the inhibitor and the curve was fitted to calculate the IC50 value.

II.6.3. Evaluation of Tyrosinase inhibition assay

For the inhibition of tyrosinase, PBS 20 mM at pH 6.8 was employed to prepare the solutions as described for the acetylcholinesterase inhibitory assay. The inhibition of tyrosinase was evaluated according to ***Fiocco et al.*** with minor changes [25]. The reaction solution was prepared by mixing 250 µL of tyrosine 1.66 mM, 700 µL of PBS (20 mM, pH 6.8), and 200 µL of inhibitor solution (or PBS in the case of the enzymatic control) in 1 mL cuvette. Then, 300 µL of tyrosinase 170 U/mL were added, and the reaction was monitored for 40 min by recording the absorbance at 475 nm every 14 seconds. Kojic acid was selected as reference inhibitor and it was tested in the range 7 – 70 µg/mL under the same operative conditions. the inhibition percentage was estimated following to the same procedure applied for the acetylcholinesterase inhibitory assay.

II.7. Statistical analysis

Student t-test was used to highlight significant differences between the two lavenders ($p<0.05$).

III. Results and discussions

The distillation process of lavender allows the obtention of one product which is EOs and three by-products: hydrosol, distillation waste water and solid residual of lavender (Figure 1).

III.1. Characterization of EOs and hydrosols

After the steam distillation of lavender (LA) and lavandin (LI), the obtained EOs were measured and the yield percentages were calculated. The content of EO was 1.87% and 2.74% (w/w) for LA and LI, respectively. Thus, lavandin has been reported to contain higher content of EOs compared to lavender due to the greater number of inflorescences on stems [26-28].

The fresh-distilled EOs were analyzed by GC-FID to quantify the relative percent abundance of the terpenes (Table 1). A total of 32 and 31 compounds were recognized in LA and LI EOs respectively.

Interestingly, the chemical composition of the two EOs did not differ as expected according to the European Pharmacopoeia [29], especially for the content of camphor, lavandulyl acetate, 1,8-cineole, borneol, and ocimene. The relative abundances of terpenes in LI EO resulted in agreement with those reported in the literature by several authors [30,31-33], while the composition of LA turned out to be extremely different [26,27].

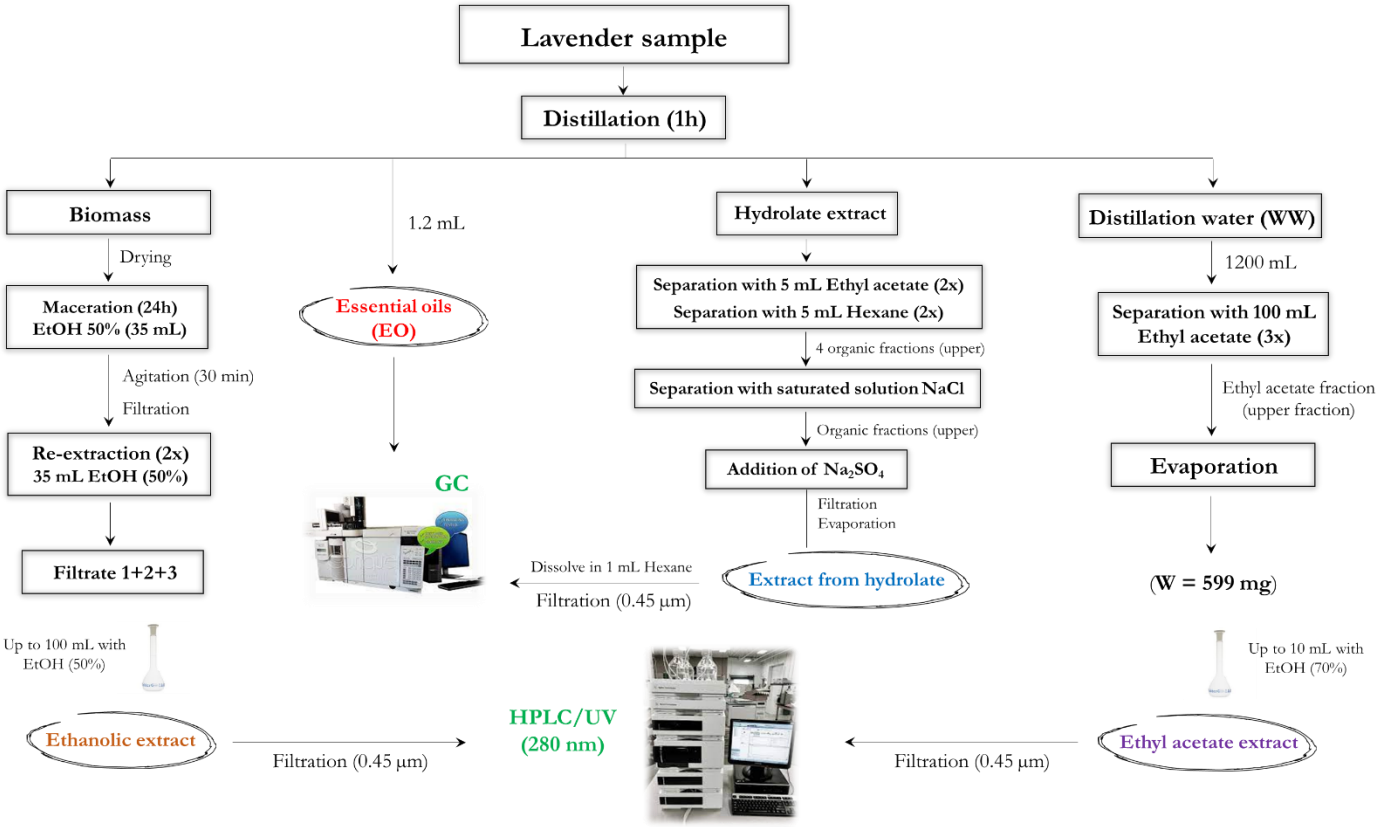

Figure 1. Isolation of EOs and by-products from lavender

In particular, high contents of camphor are rarely quantified in this valuable EO, as in the case of **Usano-Alemany et *al.*** work [34]. This diversity might be to several factors that affect the biosynthetic pathways, such as pedo-climatic conditions, light exposure, cropping technique, and physicochemical soil features [35-38].

The hydrosols collected after the steam distillation were extracted to isolate the volatile components dissolved in the water. Three replicates for each hydrosol were performed, and the component recovered by solvent extraction represented the 0.101 ± 0.005% and 0.078 ± 0.003 % (w/w) for LA and LI respectively. The GC results highlighted that the hydrosols from *Lavandula* species are different (Table 2). Both the hydrosols were rich in alcoholic monoterpenes, which represented more than 70% of the whole composition. This evidence is in agreement with the higher solubility of the alcohols in aqueous media compared to alkanes, esters, or ketones/aldehydes. Differently from EOs, linalyl acetate represented the 0.1% of the total composition, accordingly with its instability in water, where the hydrolysis to the alcohol occurs [19]. In addition, several alcohols were detected in the hydrosols but not in the EOs, such as menthol, neomenthol, p-cymen-8-ol, nerol, carvacrol and citronellol. Furthermore, the percentages of borneol, linalool oxides, α-terpineol, and terpinene-4-ol were higher in the hydrosols than in the EOs. These findings might be explained by the fact that their concentrations in the EOs were too low to be detected. On the contrary, due to the limited solubility of several terpenes, their relative abundances resulted higher in the hydrosols. Furthermore, part of them might derive from other monoterpenes after molecular rearrangement due to oxidation and cyclization processes.

Table 1. Semi-quantitative results of the percent chemical composition of LA and LI EOs

Compound	***LRI***	***LA***	***LI***
α-pinene	930	0.535	0.682
camphene	944	0.323	0.360
sabinene	970	0.177	0.242
β-pinene	973	0.555	0.818
oct-1-en-3-ol	977	0.141	-
myrcene	990	1.267	1.517
3-carene	1008	0.100	0.175
α-terpinene	1013	0.258	0.141
limonene	1027	0.648	0.541
1,8-cineole	1029	5.270	7.903
cis-ocimene	1037	1.388	1.649
trans-ocimene	1047	1.273	0.704
gamma terpinene	1056	0.102	0.168
cis linalool oxide	1071	0.152	0.117
trans linalool oxide	1086	0.344	0.328
linalool	1105	31.721	27.613
fenchol	1113	0.360	0.134
camphor	1144	5.684	6.779
borneol	1165	2.466	1.856
lavandulol	1168	0.401	0.270
terpinen-4-ol	1177	1.812	1.078
α-terpineol	1190	0.58	0.609
myrtenal	1193	0.278	0.194
carvone	1246	0.113	0.117
linalyl acetate	1265	33.857	35.631
lavandulyl acetate	1293	3.471	2.906
neryl acetate	1366	0.238	0.290
α-copaene	1385	0.486	0.591
β-caryophyllene	1423	1.562	1.532
α-humulene	1459	1.09	1.248
ar curcumene	1486	0.472	0.575
γ-cadinene	1519	0.201	0.305
Total		97.325	97.073

Table 2. Semi-quantitative results of the percent chemical composition of LA and LI hydrosols

Compound	***LRI***	***LA***	***LI***
α-pinene	944	0.26 ± 0.15	0.17 ± 0.01
camphene	948	0.26 ± 0.03	0.24 ± 0.02
β-pinene	977	0.2 ± 0.02	0.13 ± 0.00
1,8 cineole	1028	2.12 ± 2.06	1.80 ± 1.35
cis-ocimene	1037	0.57 ± 0.04	0.49 ± 0.11
cis linalool oxide	1070	4.46 ± 0.47	4.62 ± 1.14
trans linalool oxide	1087	4.25 ± 0.55	4.13 ± 1.00
linalool	1101	28.67 ± 0.63	32.12 ± 0.47
camphor	1143	13.38 ± 0.41	15.63 ± 0.99
borneol	1165	10.64 ± 1.31	10.42 ± 1.01
neomenthol	1169	1.96 ± 0.26	1.55 ± 0.22
lavandulol	1174	1.08 ± 0.06	0.92 ± 0.32
terpinen-4-ol	1177	5.58 ± 0.55	3.94 ± 0.14
menthol	1182	0.75 ± 0.09	1.00 ± 0.15
p-cymen-8-ol	1185	0.74 ± 0.13	1.1 ± 0.07
α-terpineol	1191	11.18 ± 0.28	9.78 ± 0.66
myrtenal	1195	0.21 ± 0.01	0.25 ± 0.02
verbenone	1208	0.44 ± 0.00	0.55 ± 0.04
nerol	1224	0.33 ± 0.03	0.31 ± 0.03
citronellol	1233	4.53 ± 0.04	2.48 ± 0.33
carvone	1243	0.18 ± 0.04	0.19 ± 0.01
neral	1255	0.15 ± 0.00	0.17 ± 0.02
piperitone	1259	1.94 ± 0.07	1.65 ± 0.27
linalyl acetate	1262	0.13 ± 0.02	0.10 ± 0.00
geranial	1276	0.34 ± 0.00	0.39 ± 0.11
lavandulyl acetate	1295	0.13 ± 0.00	0.10 ± 0.00
carvacrol	1303	0.66 ± 0.09	0.38 ± 0.01
Total		95.16 ± 1.28	94.49 ± 0.43

III.2. Phenolic composition in distilled straws

III.2.1. Total phenolic and flavonoid content

Previous studies reported that a mixture of ethanol and water (50:50, v/v) could be considered as the optimal solvent for the extraction of polyphenols from the oil-exhausted biomasses. Dry extracts were obtained from the liquid extracts by freeze-drying. The yields were 211.5 ±7.8 and 193.0 ±2.6 mg of dry solid per gram of biomass for LA and LI respectively.

Then, the estimation of TPC and TFC was carried out by means of Folin-Ciocalteu and aluminum chlorate assays, respectively. The two hydroalcoholic extracts did not show significant difference in both TPC and TFC contents (Table 3).

Table 3. Total phenolic content (TPC) and total flavonoid content (TFC) of LA and LI oil-exhausted biomasses

Residual material	***LA***	***LI***
TPC (mg GAE/g biomass)	19.22 ± 4.16	17.06 ± 3.31
TPC (mg GAE/mg dry extract)	9.09 ± 0.33	8.84 ± 0.12
TFC (mg QE/g biomass)	1.56 ± 0.21	1.41 ± 0.10
TFC (mg QE/mg dry extract)	0.74 ± 0.03	0.73 ± 0.01

Firstly, we can remark that the TFC represented about the 8% of the TPC in both the lavender extracts. In the literature, different results on *Lavandula* genus can be observed, and the diversity might be due to several factors.

As an example**, Spiridon *et al.*** reported higher values for the alcoholic extract from *Lavandula angustifolia* leaves and flowers. The TPC and TFC were found to be 50.6 ± 3.16 mg GAE/g and 27.6 ± 3.42 mg Rutin Equivalent/g, respectively [39].

On the other hand, **Duda *el al.* (2015)** showed similar TPC and TFC results by studying the whole biomass of *L. angustifolia* harvested in two different phenological periods (the beginning of flowering and the full bloom). They observed a TPC comprised between 12.44 and 18.16 mg GAE/g, and a TFC between 3.37 and 4.85 mg QE/g dry plant [40]. These differences might be due to the part of the biomass taken into account in the study. Indeed, by comparing the results from these studies, the leaves and the flowers of lavender seem to be richer in polyphenols than stems, which are evaluated in the extraction of the entire biomass. In addition, it has been reported that the TPC of lavender is strongly affected by the species, the harvest time, the growing conditions of the crops, as well as the age of the plant.

By comparing the results obtained in this study with other works concerning lavender wastes from distillation process, Turrini et al. observed higher levels of both TPC and TFC (40.15 ± 0.04 and 4.72 ± 3.56, respectively) in *Lavandula angustifolia* biomass after pulsed ultrasound-assisted extraction [41]. On the contrary, Slavov *et al.* noticed lower TPC , that ranged from 7.52 and 10.75 mg GAE/g in Bulgarian lavender (*Lavandula angustifolia*) waste [42]. Moreover, the obtained values are higher than those obtained in the research conducted by Méndez-Tovar *et al.* on the by-product of *Lavandula latifolia* essential oil distillation. The content of TPC in the studied samples vary between 1.89 ± 0.09 and 3.54 ± 0.22 mg GAE/g of dry flowers [43].

The dry extracts contained about the 9% and 0.7% of TPC and TFC respectively. These results suggested that the majority of the extracts were composed by other substances, such as fibers, lignin, organic acids, triterpenoids, and sugars [44,45].

III.2.2. LC−ESI−MS and MS^2 analysis

In order to have clearer idea on the composition of lavender extracts, an analysis by high performance liquid chromatography coupled to mass spectrometry was carried out. The detected compounds in the hydroalcoholic extract of the residual plant material were identified using data acquired by LC-ESI-MS of the parent ions and data-dependent MS/MS fragmentation.

A typical chromatogram of lavender extracts is displayed in Figure 2. The retention times, the molecular ions, fragmentation pattern, the tentatively identifications, molecular weights and formula are illustrated in Table 4. LC-ESI-MS and MS/MS analyses allowed the detection of the molecular ion for each compound and produced the fragmentation in negative mode. The tentatively identification of glycosides was supposed basing only on the fragmentation.

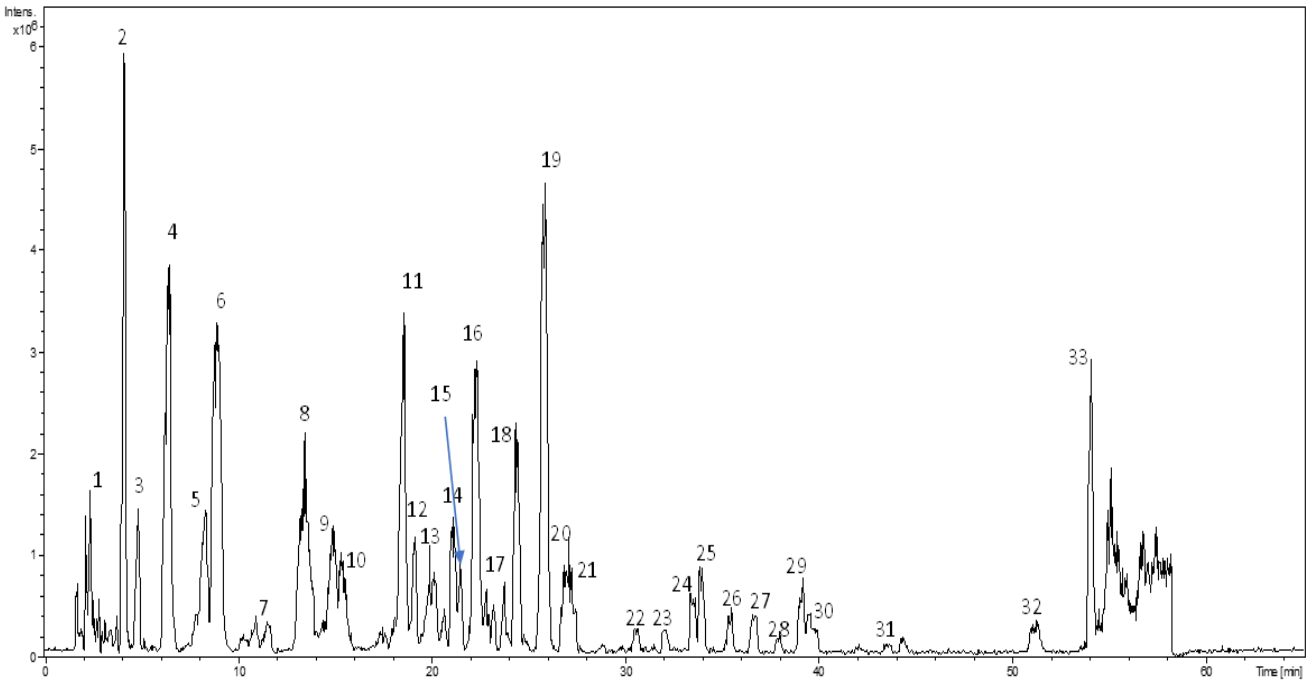

Figure 2. LC-MS base peak chromatogram of *Lavandula angustifolia* hydroalcoholic extract

Table 4. LC-ESI-MS and MS/MS data (negative ionization mode) of the tentatively identified compounds in *L. angustifolia* (LA) and *L. intermedia* extracts.

The symbols "+" and "−" indicate the presence or the absence of the compounds in the extracts

Peak number	Rt (min)	Tentative identification	$[M-H]^-$ (*m/z*)	Fragments (*m/z*)	Molecular weight (g/mol)	LA	LI
1	2.3	Caffeoyl aspartic acid	294.1	179	295.07	+	-
2	4.2	Danshensu	395 (2M–H) 197	178.9, 135	198.17	+	+
3	4.9	unknown	501	336.9, 295		+	+
4	6.5	*p*-coumaric acid hexose	651.3 (2M–H) 325	162.9, 119	326.10	+	+
5	8.2	unknown	387.2	369.2, 207.0		+	+
6	8.9	Ferulic acid hexose	711.3 (2M–H) 355.1	192.9, 148.9	356.32	+	+
7	11.5	Unknown	351.0	248.9, 231, 177, 113		+	-
8	13.3	*p*-coumaric acid hexose	651 (2M–H) 325.1	162.9, 119	326.10	+	-
9	14.7	Luteolin 7-O-diglucoronide	637.2	461.1, 284.9	638.11	+	-
10	15.5	Apigenin 7-O-diglucoronide	621	445.1, 268.9	622.12	+	-
11	18.4	Ferulic acid hexose	711 (2M–H) 355.1	192.9, 149	356.32	+	+
12	19.2	Unknown	521.2	358.9, 229.0, 285.0		+	-
13	20.7	Quercetin hexose	463	301, 178.9	464.095	+	-
14	21.0	Luteolin/kaempferol hexose	447.2	284.9	448.100	+	+
15	21.5	Unknown	441.2	395.3, 262.9		+	+
16	22.3	Luteolin glucuronide	461.1	284.9	462.4	+	+
17	23.7	Quercetin 3-O-rhamnoside	447.1	300.9, 151.0	448.101	+	-
18	24.3	Apigenin 7-O-glucoside	431.2	269	432.4	+	+
19	25.8	Rosmarinic acid	359.1	222.8, 196.9, 178.9, 160.9	360.31	+	+
20	26.7	Luteolin glucuronide	461	285	426.4	+	+
21	27.2	Apigenin 7-O-glucoronide	445.1	269, 174.9	446.4	+	+
22	30.5	rosmarinic acid methylester	373	178.9, 135	374.3	+	-
23	31.9	Kaempferol/Luteolin	285.1	254.8, 226.9	286.0477	+	-
24	33.4	Unknown	493.0	295.0, 269.1		+	+
25	33.9	Unknown	618.4	582.4, 462.3		+	-
26	35.3	Quercetin hexose	463.2	301.0		+	+
27	36.6	Unknown	507.3	345.2, 299.2		+	+
28	37.9	Unknown	329.2	221.0, 193.0, 170.9		+	+
29	39.1	Ellagic acid	301.2	283.4		+	-
30	39.6	Unknown	287.2	269.1		+	-
31	44.4	Unknown	307.2	289.0, 235.0, 185.0		+	-
32	51.0	Unknown	309.2	291.1, 208.9, 184.9		+	-
33	53.9	Unknown	487.5	469.4		+	-

From the obtained results, we notice that LA extract exhibited a higher abundance of phenolic acids and flavonoids. The main compounds found in the extracts were also reported in previous studies [41,44,45].

Further analyzing the obtained results, compound **1** was identified as caffeoyl aspartic acid due to the fragment at m/z 179 with the relative loss of 115 Da, which correspond to the aspartic acid moiety [46]. Compound **2** was recognized as danshensu (3,4-dihydroxyphenyl lactic acid), the derivative of caffeic acid, which showed the precursor ion of m/z 197 and the fragments m/z 178.9 and 135, caused by the loss of hydroxylic (18 Da) and carboxylic (44 Da) groups respectively [47]. Compounds **4**, **8**, and **6**, **11** were classified as hexose-derivative of p-coumaric acid and ferulic acid respectively, due to the loss of glucosyl moiety (162 Da) and the characteristic fragment at m/z 119 and m/z 149 (loss of carboxylic group, 44 Da) [48]. Finally, rosmarinic acid (compound **19**) was characterized by the unprotonated molecular ions $[M-H]^-$ ion at m/z 359.1, which fragmented to m/z 196.9, 178.9, and 160.9, due to the losses of danshensu and caffeic acid moieties, and further molecules of water respectively [49]. Compound **22** was tentatively identified as derivative of rosmarinic acid due to the presence of the fragments m/z 179 and 135 [50,51].

Regarding the flavonoids, the identified compounds were derivative of quercetin, apigenin, luteolin/kaempferol. Luteolin and kaempferol could not be distinguishable due to the same fragmentation patterns and scarce sensibility of ion trap mass spectrometer. The derivatives were identified due to the losses of glycosidic (162 Da), rhamnosidic (146 Da), and glucuronic (176 Da) moieties. The aglycones were classified basing on the characteristic product ions. Luteolin and kaempferol derivatives exhibited the fragment at m/z 284.9 [48], quercetin the fragments at m/z 301, 179, and 151 [52], and apigenin the fragments m/z 289 and 175 [53]. Finally, the compound **29** was tentatively identified as ellagic acid, due to the precursor ion at m/z 301 and the product ion at m/z 283 [54].

III.3. Biological activities

III.3.1. Antioxidant activity

The antioxidant properties of the two extracts were evaluated by calculating the direct neutralization of free radicals generated by DPPH, and the prooxidant activities related to the interaction with iron ions. The prooxidant activity is exerted by the chelation of Iron (II) and the reduction of Iron (III), both involved as catalyst in Fenton reaction. Indeed, the chelation or reduction of iron ions prevents the conversion of hydrogen peroxide to hydroxyl radicals [55]. The IC50 values of the DPPH inhibition were 0.17 ± 0.02 and 0.17 ± 0.01 mg of biomass for LA and LI respectively. The amount of the standard reference Trolox that gave the same inhibition was 16.40 ± 0.41 µg. Regarding the iron chelation, the IC50 values were 22.17 ± 0.42 mg of biomass, 15.77 ± 0.10 mg of biomass, and 33.00 ± 0.34 µg of LA, LI, and EDTA respectively. The reducing power of the extracts was calculated as described in method section, and it was esteemed equal to that obtained by the AA solutions at the concentration of 0.313 ± 0.014 and 0.261 ± 0.010 mg/mL for LA and LI respectively.

In order to evaluate the antioxidant strength of lavender biomasses, the mg equivalents of the references per gram of biomass were calculated (Table 5).

Table 5. Antioxidant activities of hydroalcoholic extracts of *L. angustifolia* and *L. x intermedia* expressed as mean ± standard deviation milligrams of positive control per gram of lavender biomass

	LA	***LI***
Antiradical activity (mg eqT/g of biomass)	94.17 ± 6.29	94.51 ± 2.85
Chelation activity (mg eqEDTA/g of biomass)	1.49 ± 0.03	2.10 ± 0.13
Fe^{3+} reduction capacity (mg eqAA/g of biomass)	89.36 ± 3.92	74.53 ± 2.74

T, Trolox; AA, ascorbic acid

The results highlighted the marked antioxidant activities of the extracts of lavender biomasses. These evidences were in contrast to Miliauskas et al. findings, where acetone extract of LA did not exhibit remarkable antioxidant activities [56]. Conversely, several other authors highlighted strong dose-dependent scavenging, chelating, and reducing activities of lavender and lavandin extracts [41,42,57,58]. Specifically, the two lavenders exhibited similar free scavenging activities, while the chelating and reducing activities demonstrated opposite trends. LA showed a significantly higher content of eqAA/g ($p<0.0001$), suggesting a greater capability in reducing ferric ions, as reported by Blažeković et *al.* [59]. The higher activity of LA compared to LI in oxidation-reduction reactions resulted in agreement with the greater total phenolic content (Table 3). In addition, besides the phenolic acids and flavonoids, several other compounds might contribute to the antioxidant power of the extracts, such as organic acids which have been reported in *Lavandula* genus [45]. On the contrary, LI exhibited a significantly higher activity ($p<0.01$) in chelating ferrous ions, correlated to the major content of eqEDTA/g. Similar evidences were also reported by Robu et al., where LI biomass displayed a greater chelating activity than LA biomass [60]. This result might be due to the major concentration of polyphenols with more than one chelating site or with greater stability constants of the complex. Indeed, the metal chelation potential of polyphenols is strongly related to the catechol moieties and the combination of hydroxyl and carbonyl groups, characteristic of the flavonoid structure [61]. Therefore, even though the TPC and TFC of LI were slightly smaller than LA, these results suggested the presence of higher concentration of stronger chelating polyphenols, such as rosmarinic acid, luteolin, and kaempferol.

III.3.2. In vitro AChE and tyrosinase inhibition assay

Nowadays, being the cholinesterase inhibition the most widely used approach for the treatment of Alzheimer's disease, several efforts have been made to discover new sources of inhibitors. Different plants have been tested to understand their effectiveness on AChE [62-64]. Indeed, flavonoids and phenolic acids have been reported to fit into the gorge of the active site of the enzyme [65].

Furthermore, these compounds demonstrated strong tyrosinase inhibiting properties, conferring them the features for several application in the food, cosmetic, and pharmaceutical industries [66-68]. Indeed, Tyrosinase is a widespread enzyme in food, fungi, bacteria, and animals. Tyrosinase is the enzyme responsible of food browning, and in humans it causes melanogenesis and skin pigmentation [68].

The freeze-dried extracts of LA and LI were tested to evaluate their anti-cholinesterase and anti-tyrosinase activities (Table 6). Both the extracts demonstrated to be effective in the inhibition of the enzymes. In particular, LA extracts showed significantly lower IC50 values than LI extracts, suggesting to exert a stronger inhibition ($p<0.01$ and 0.001 for AChE and Tyrosinase, respectively). The higher inhibition capacity of LA extract might be related to the highest content of both polyphenols and flavonoids.

Table 6. Acetylcholinesterase (AChE) and tyrosinase inhibition activities of *Lavandula angustifolia* (LA), *Lavandula x intermedia*, and reference inhibitors galantamine and kojic acid. The results are expressed as IC50 values

	AChE	**Tyrosinase**
LA	5.35 ± 0.47 mg/mL	5.26 ± 0.02 mg/mL
LI	6.67 ± 0.12 mg/mL	6.56 ± 0.16 mg/mL
Galantamine	18.83 ± 1.05 µg/mL	-
Kojic acid	-	18.13 ± 0.45 µg/mL

In the literature, the studies that aimed at the evaluation of the activity of *Lavandula* on AChE employed the whole fresh aerial parts of the plant to prepare the extracts. Thus, the extracts were composed by both volatile terpenes and polyphenols. No studies aimed at the evaluation of the inhibitory activity on waste biomass. Being terpenes well-recognized inhibitor of AChE [69-71], a direct comparison with the results of other authors might be difficult.

Vladimir-Knežević and co-authors evaluated the anti-cholinesterase capacity of ethanolic extracts of medicinal plants from *Lamiaceae* family. In their work, *Lavandula angustifolia* showed an inhibition of 50% at the concentration of 1 mg/mL, while galantamine exhibited an IC50 value of 0.122 µg/mL. In addition, the authors highlighted the essential role of certain polyphenols in the inhibition in combination with terpenes [72]. In another report, Costa et al. affirm that supercritical fluid extracts of *Lavandula viridis* exerted an IC50 value of 1.975 mg/mL, proving a central role of the monoterpenes of the EO. In their study, the author stated that the IC50 of the reference standard galantamine was 2.20 µg/mL under the same test condition of *L. viridis* extract [24].

Regarding the anti-tyrosinase activity of *Lavandula* extracts, few studies are present in the literature. Hsu and co-workers tested water extracts of different species of *Lavandula*, demonstrating that the strength of the inhibition was species-dependent. Furthermore, in contrast with our results, all the inhibitory capacities of the extracts were impaired by the freeze-drying process. The authors explained these evidences suggesting that the inhibitory effects of their extracts were related to the action of the enzyme polyphenol esterase, which degraded during the drying process [73].

IV. CONCLUSION

The solid by-products from steam distillation process of *L. angustifolia* and *L. intermedia* demonstrated to represent an interesting source of bioactive compounds, such as phenolic acids and flavonoids. The developed method of extraction of the oil-exhausted biomasses proved to be a promising strategy for the recovery of polyphenols by using food-grade solvents (ethanol and water).

The several properties of lavender extracts from oil-exhausted biomass confer them the suitability of their employment in different fields. Indeed, the antioxidant and the anti-tyrosinase activities might be exploited in the food and cosmetic industries to prevent the browning and the degeneration of active compounds, and ameliorate the conservation of the final products.

Furthermore, these extracts might be used by the pharmaceutical industry also due to the anti-enzymatic capabilities here demonstrated. In that, they might represent a valid therapeutic alternative for the prevention and treatment of Alzheimer's disease, hyperpigmentation, and other chronic diseases where radicals play a central role. In conclusion, lavender biomasses proved to be important solid by-products that deserve further studies to reuse them prior the biofuel production.

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CHAPTER III

STUDY OF THE "SECONDARY SHELF LIFE" OF TOMATO SAUCE

"If you want to succeed you should strike out on new paths, rather than travel the worn paths of accepted success" **(John D. Rockefeller)**

"In the Middle of Difficulty Lies Opportunity" **(Albert Einstein)**

I. INTRODUCTION

Nowadays, an important topic related to packed foods is generally discussed: shelf life and related issues. Shelf life can be defined as a determined time after the production and packaging during which the food product conserves a required level of quality under well-defined conditions of storage. Thus, after the opening these products by consumers or at industrial level, a significant acceleration of the degradation of product quality is observed due to the modification of the environmental conditions such as temperature change, variation in atmosphere composition, high level of oxygen or moisture, loss of sterility... This phenomenon is called "secondary shelf life" (SSL), which is defined as the period of time after the opening of a product. During this period, the food product keeps an acceptable level of quality asserting specific properties like taste, aroma, appearance, and any specific qualities that are related to the product [1].

The factors that affect the food shelf life could be classified as intrinsic such as the ingredients of the product (proteins, fats, carbohydrates, vitamins, phenolic compounds), water activity, pH, enzymes, microflora or extrinsic including processing procedures (homogenization, centrifugation, heat treatment, high pressure…), storage conditions (humidity, temperature, oxygen, light), packaging, logistics and retail issues [2].

During shelf life, various changes of food quality may occur and could be physical, chemical, biochemical, microbiological or light-induced. Table 1 illustrate the different types of these changes with some examples of foods products.

Table 1. Food quality changes during shelf life

Type of change	Effects	Examples
Physical	- Moisture gain or loss	Fresh fruits and vegetables, cheeses
	- Water vapor transfer, ice sublimation	Frozen products, e.g., ice cream
	- Loss of characteristic flavor, development of undesired odor or flavor	Juices, chocolate, tea leaves
	- Loss of carbon dioxide	Carbonated drinks
	- Crystallization and textural changes	Milk powders, toffee
	-Emulsion destabilizing and breakdown	Butter, margarine
	- Syneresis	Yoghurt products, jams, sauces
Chemical/biochemical	- Chilling injury	Fruits and vegetables
	- Hydrolysis of fatty acids	Butter, milk powder, cheeses, cereal grains
	- Oxidation of fats, proteins, pigments, vitamins	Cereals, oilseeds (soya), fish, meat, milk
	- Nonenzymic browning	Dehydrated fruits/vegetables, dry milk products
	- Enzymic browning	Precut fruits and vegetables
	- Off-flavors, oxidative discoloration, interactions between food and packaging	Foods in tinplate cans
Microbiological	- Growth of pathogenic microorganisms, microbial toxins	Fish, meat products, poultry, milk, cheeses
	- Microbial spoilage, spore-forming bacteria, Enterobacteriaceae, yeasts, molds	Fish, meat products, poultry, milk, cheeses
Light-induced	- Photo-oxidation of vitamins, light induced oxidation, color fading	Milk, snacks, bakery products

In this study, we are interested in well-known food products as tomato sauce.

Tomato is considered as one of the most important ingredients in human's food since it is cultivated in most parts of the world. This is mainly related to its rich composition in several nutrients including dietary fiber, lycopene, anthocyanin, monounsaturated fatty acids, antioxidants, carotene, minerals vitamins, in addition to low fat and zero cholesterol content. Tomato has been consumed in raw or under processed forms such as sauces, purees, ketchup..Nevertheless, tomato has been reported to have a relatively less shelf life because of several factors like the increased respiration which improves fruit ripening and leads to premature fruit decay [3-6].

The objective is to study the influence of some factors such as the brand, storage conditions and conservation time on the composition of phenolic compounds, the lycopene content and the antioxidant activity of tomato sauce.

II. EXPERIMENTAL

II.1. Chemicals and reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH), gallic acid, sodium carbonate (Na_2CO_3), ascorbic acid and Folin–Ciocalteu reagent were purchased from Sigma-Aldrich (Milan, Italy). Aluminum chloride was provided by Carlo Erba Reagents (Milan, Italy).

Acetone, n-hexane and ethanol were of LC–MS purity grade (Sigma-Aldrich).

Ultrapure water was obtained using a Milli-Q Plus 185system from Millipore (Milford, Mass., U.S.A.).

II.2. Analysis of tomato sauce

II.2.1. Samples

In this study, three different brands of tomato sauce were analyzed (Mutti, Cirio and Coop) by carrying out weekly sampling over the 28 days following the opening of the bottles. In addition, the samples were stored in five different fridges. Each week, a sample of the sauce was withdrawn and analyzed.

II.2.2. Preparation of the extracts

The extraction was performed using classical method. For that, 2.5 g of each tomato sauce sample were mixed with 20 mL of a 70% aqueous ethanol solution. The mixture was kept under agitation for 10 min then centrifuged for 5 min at 5000 rpm and filtrated. Finally, the filtrate was concentrated by a rotary evaporator and the volume was made up to 10 mL by Milli-Q H_2O [7].

II.2.3. Total phenolic content (TPC)

Total phenolic content (TPC) was estimated by Folin-Ciocalteu assay [7]. Briefly, 400 µl of ethanolic extract were placed in a volumetric flask of 10 mL and 2 mL of Folin-Ciocalteu's reagent (diluted 1:10 with ultrapure water) were added. After 3 min, 1.6 mL of Na_2CO_3 (20%, w/v) were added to the mixture and the volume was made up to the mark by ultrapure water. After incubation for 2 hours at room temperature, the absorbance was determined at 760 nm with an UV-Visible spectrophotometer (UVmini-1240, Shimadzu Corp., Kyoto, Japan) against a calibration curve made by gallic acid.

TPC was expressed as milligram of gallic acid equivalents per gram of fresh tomato sauce (mg GAE/g FW).

II.2.4. Lycopene content

Lycopene content was estimated spectrophotometrically according to the method proposed by **Suwanaruang (2016)**. 100 mg of tomato sauce were mixed with 8 mL of a solution of hexane:ethanol:acetone (2:1:1). The mixture was shaken and then incubated out of bright light. After at least 10 minutes, 1 mL of water was added and then solution was vortexed again. Then, the solution was left to stand for 10 minutes in order to allow phases to separate and all air bubbles to disappear. Finally, the absorbance of the upper layer was read at 503 nm (A_{503}) UV-Visible spectrophotometer using (UVmini-1240, Shimadzu Corp., Kyoto, Japan) against hexane as blank [8].

$$\text{Lycopene } (mg/kg\ FW) = \frac{A_{503}\ x\ M_{Lycop}\ x\ V_{sol}\ x\ 0.55}{W_s\ x\ E}$$

with M_{Lycop} = Molecular weight of lycopene (= 537 g/mole)

V_{sol} = Volume of mixed solvent Hexane:EtOH:Acetone (mL)

W_s = Weight or volume of tomato sample (g or mL)

E = Extinction coefficient for lycopene in hexane (= 172 mM^{-1})

$$\text{Lycopene } (mg/kg\ FW) = \frac{A_{503}\ x\ V_{sol}\ x\ 1.717}{W_s}$$

II.2.5. Antioxidant activity

The antioxidant activity of ethanolic extracts from tomato sauce was determined though measuring the scavenging activity of DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical [9]. For that, 50 µL of each extract was mixed with 3 mL of ethanolic DPPH solution (40 mg/L). Then, the solutions were incubated in dark for 30 min at room temperature. The absorbance was measured at 517 nm using UV-Visible spectrophotometer using (UVmini-1240, Shimadzu Corp., Kyoto, Japan).

A calibration curve was conducted with the same procedure using ascorbic acid as standard. The antioxidant capacity was expressed as milligrams of ascorbic acid equivalent per 100 g of fresh tomato sauce (mg AAE/100 g FW).

$$Inhibition\ (\%) = \frac{A_0 - A_s}{A_0}\ x\ 100$$

With A_0: absorbance of control (without sample)

A_s: absorbance of sample { A_s = A_s (with DPPH) - A_s (without DPPH) }

III. RESULTS

The different samples were evaluated for total phenolic content (TPC), lycopene content and antioxidant activity applying the assay of DPPH radical scavenging capacity. The samples were compared according to the brands, as well as the freezers in which they were stored.

III.1. Comparison according to the brands

According to the obtained results (Figure 1), we notice first that, at the opening of the different samples, the three brands have very similar values for TPC, lycopene and DPPH radical scavenging activity. These values vary between 34.56 and 45.21 mg GAE/100g for polyphenols, between 13.99 and 20.52 mg/100g for lycopene and between 38.04 and 62.29 mg AAE/100g.

The present findings are in agreement with those reported previously in literature which are in the range of 26.34 to 141.98 mg GAE/100 g for TPC and 4.31 to 29.99 mg/100 g for lycopene [10,11].

Otherwise, the TPC value of the three brands decreases over time up to 21 days. After 28 days, the value of TPC of the brands Cirio and Coop increases over the 28 days, while that of Mutti brand decreases further.

Concerning lycopene content, the values decrease over the 28 days for the brands Coop and Mutti. For the brand Cirio, the lycopene content decrease after 7 days then increases for 14 and 21 days and decreases again over the 28 days.

Regarding the antioxidant activity, the results showed fairly close values of DPPH radical scavenging capacity for the three brands up to 14 days (57.50 to 62.29 mg AAE/100g). After 21 days, these values decrease significantly for the three brands and reached 38.04, 41.05 and 41.72 mg AAE/100g for Cirio, Coop and Mutti, respectively. Finally, the antioxidant activity values increased again after up to 28 days and was about 56 mg AAE/100g for all the brands.

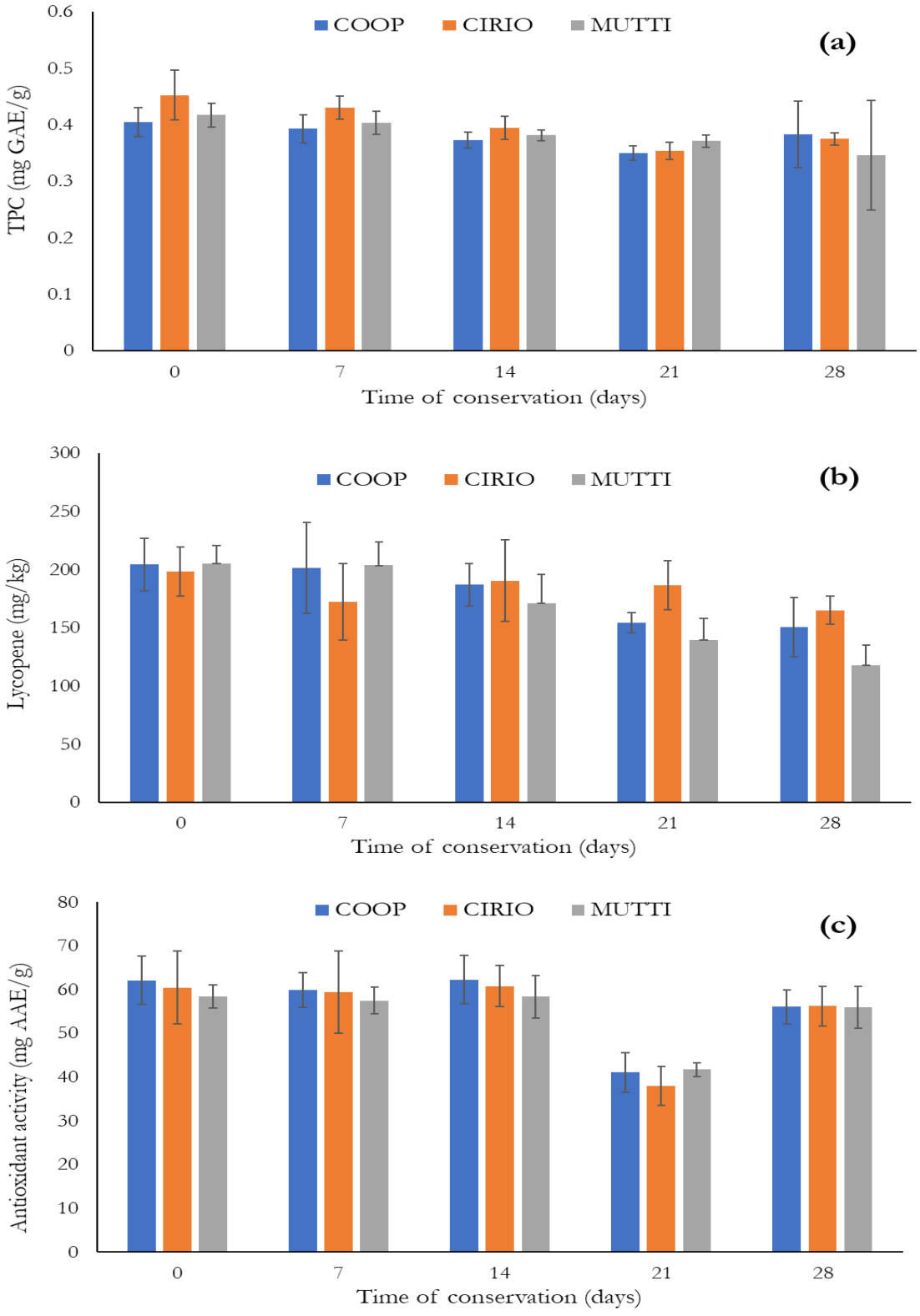

Figure 1. Effect of the conservation time on the properties of different brands of tomato sauce :(a) Total phenolic content (b) Lycopene content (c) Antioxidant activity

III.2. Comparison according to storage conditions (different refrigerators)

The studied samples showed close values of TPC in each time (Figure 2), with the exception of the sample in the refrigerator 3 at the first opening which has a higher value of TPC (46.62 mg GAE/100g) and the sample in the refrigerator 5 after 28 days which has a lower value of TPC (28.88 mg GAE/100g). TPC were about 41, 40, 38, 35 and 38 mg GAE/100g after 0, 7, 14, 21 and 28 days, respectively.

Unlike TPC, there are some differences in the values of lycopene and antioxidant activity. Samples from fridge 3 and 5 showed the highest lycopene values on days 0, 7 and 14, while sample in fridge 2 had the highest lycopene content after 21 and 28 days.

For the antioxidant activity, the highest DPPH radical scavenging capacity was observed for the sample of fridge 1 in 0 (65.06 mg AAE/100g), 7 (65.69 mg AAE/100g) and 14 days (62.31 mg AAE/100g). In this time, the other samples showed close values of antioxidant activity (56 to 60 mg AAE/100g). After 21 days, we notice a significant decrease in the activity which reached about 40 mg AAE/100g. Finally, an increase in the DPPH radical scavenging activity was observed over the 28 days.

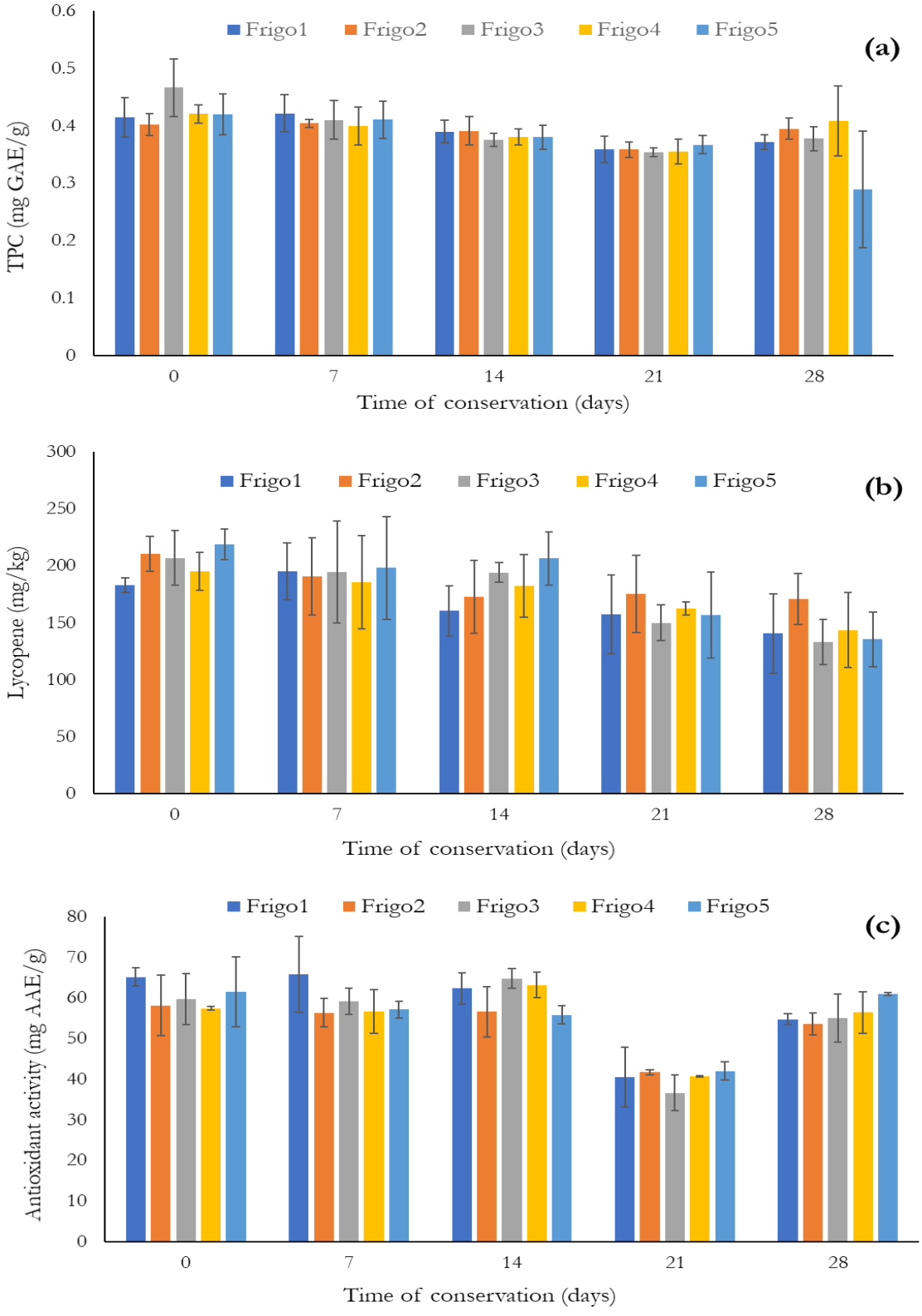

Figure 2. Effect of the conservation time and storage refrigerator on the properties of tomato sauce :(a) Total phenolic content (b) Lycopene content (c) Antioxidant activity

III.3. Discussion

In the light of the results obtained in this part, we can conclude that at the first opening of samples (0 days), the different brands of tomato sauce have close values in polyphenols, lycopene and DPPH radical scavenging activity. Moreover, even if the values decrease with time, the contents of polyphenols and antioxidant activity remain more or less close for the different brands, whereas the contents of lycopene decrease significantly and the difference becomes remarkable between the various samples.

A study conducted by **Garcia-Alonso et al.** focused on the effect of storage conditions on the stability of antioxidant compounds of tomato juices [12]. The studied parameters were time (0 to 12 months), temperature (8, 22 and 37 °C) and packaging materials (glass bottles and Tetra pack). Results revealed that the final losses were always about 20% in the total lycopene content after 12 months at different temperatures, either in glass bottles or Tetra pack. Concerning phenolic compounds, authors observed that the total phenolic content remained practically stable during storage with a slight enhancement after 8 months only for the sample in the Tetra pack stored at 37 °C, which is probably related to the formation of Maillard reaction products, which have the capacity to react with Folin-Ciocalteu's phenol reagent [12,13].

Otherwise, others researchers found final losses of about 70% and 65% in lycopene content for tomato juice stored in polypropylene bottle at 4 °C for 3 months and 3.7 months, respectively [14,15]. **Lin et *al.*** observed also approximately 65% lycopene losses in canned tomato juice stored for 3 months at 4, 25, and 37 °C [16].

IV. CONCLUSION

During this part, we tried to study the effect of some factors on the properties of tomato sauce, thus having a close relationship with its "Secondary Shelf Life". The factors involved were the time of conservation, the commercial brand and the storage place after opening (refrigerators), whereas the studied properties were total phenolic content, lycopene content and antioxidant activity. Regarding the effect of the brands, it was observed that the three studied brands had practically equal polyphenol contents and antioxidant capacities during the entire period of conservation. After 28 days, the different samples presented values comparable to those of the opening day. On the contrary, lycopene contents varied remarkably from one brand to another in particular from the seventh day of conservation. We noticed also that the lycopene content decreases considerably after 28 days. Concerning the storage refrigerators, we observed close values of TPC for all the studied samples in each time of conservation, whereas some differences were noticed for lycopene content and antioxidant activity.

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GENERAL CONCLUSIONS

"Life is not a problem to be solved, but a reality to be experienced" **(Soren Kierkegaard)**

"A real decision is measured by the fact that you've taken a new action. If there's no action, you haven't truly decided" **(Tony Robbins)**

Plants constitute an immense reservoir of chemical compounds which have the advantage of a great structural diversity and biological activities. Their therapeutic and dietary use by human dates back to antiquity. Nowadays, a large number of aromatic and medicinal plants find many applications in various fields, namely medicine, pharmacy, cosmetology and food industry. Among these molecules, essential oils, phenolic compounds and carbohydrates which play very important roles due to their pharmacological properties which are mainly attributable to their antioxidant capacities. Furthermore, agri-food products have been reported to exhibit large quantities of residues or by-products which are easily degradable and can contribute to increased levels of environmental pollution.

This study was carried out with the aim of valorizing some agri-food products and by-products with a view to better exploiting their potential in the food, nutraceutical and pharmaceutical fields, thus avoiding problems related to environmental issues.

The first studied by-product was prune waste issued from plum processing industry. Chemical analysis of this by-product revealed that it is still rich in bioactive compounds including polyphenols (TPC= 3.32 mg GAE/g), chlorogenic acid (0.5 mg/g), sorbitol (44.04 mg/g), fructose (125.03 mg/g) and minerals. The important content of polyphenols was the origin of the significant observed antioxidant capacity by scavenging DPPH radical. A fermentation study was also conducted in order to reduce the reducing sugar content (glucose and fructose), thus enabling this product to be a promising ingredient in food formulations useful for intestinal function.

As perspectives for this study, an optimization of the sugar analysis by HPLC-ELSD could be performed in order to obtain a quantitative data of the major sugars in prune by-product. In addition, we will try the application of some drying techniques (spray drying, lyophilization …) in order to generate a powder that can be readily packaged, stored, commercialized and handled, since it has been reported that the low water activity of the powdered product prevents the growth of microorganisms and the occurrence of biochemical reactions.

By-products obtained from lavender distillation process were also studied. The residual materials from steam distillation of two lavender species (*Lavandula angustifolia* and *Lavandula x intermedia*) were investigated for their phenolic composition and their possible biological activities. Chromatographic analyses showed a rich composition in phenolic compounds, with high abundance of phenolic acids and glycosylated flavonoids. In addition, ethanolic extracts from residual materials exihibited significant antioxidant activities (DPPH radical scavenging, chelation of Iron (II) and the reduction of Iron (III)), as well as an important anti-enzymatic activity (acetylcholinesterase and tyrosinase). These results proved that the oil-exhausted biomasses could be an important source of bioactive compounds, suitable for several application in the food, cosmetic, and pharmaceutical industries.

Further studies on this subject will focus on the quantitative analysis of phenolic compounds present in the residual lavender so as to know the most influencing compounds on each biological activity.

The last topic of this research was dedicated to study of the "Secondary Shelf Life" of tomato sauce, by the examination of the influence of different commercial brands, the conservation time and the storage condition on some properties of the tomato sauce including total phenolic content, lycopene content and antioxidant activity. The obtained results showed that the studied factors doesn't greatly affect the total phenolic content, whereas some differences were observed on lycopene content and DPPH radical scavenging capacity.

The study of the influence of these factors on other tomato sauce properties will be the main objective of this research.

APPENDIX I

SCIENTIFIC PUBLICATIONS

- **Chaouch, M. A.**, & Benvenuti, S. (2020). The role of fruit by-products as bioactive compounds for intestinal health. ***Foods***, 9(11), 1716. https://doi.org/10.3390/foods9111716

- Truzzi, E., **Chaouch**, **M. A.**, Rossi, G., Tagliazucchi, L., Bertelli, D., Benvenuti, S. (2022). Characterization and valorization of the agricultural waste obtained from Lavandula steam distillation for its reuse in the food and pharmaceutical fields. ***Molecules***, 27(5), 1613 https://doi.org/10.3390/molecules27051613

APPENDIX II

CONTRIBUTION TO CONGRESSESS

Oral communications

Poster communications

- **Mohamed Aymen Chaouch**, Virginia Brighenti, Federica Pellati, Davide Bertelli, Stefania Benvenuti. "*Chemical composition of bioactive compounds in prunes* (*Prunus domestica* L.) *waste product*. **XVI Congress of the Italian Society of Phytochemistry Jointly with the 2nd International Congress on Edible, Medicinal and Aromatic Plants "ICEMAP 2019**", Alghero (SS), 19-21 June 2019.

Participations

- Food Structure & Functionality Forum Online Mini Symposium, 20 October 2020.
- Oli essenziali in Fitopatologia, Microbiologia alimentare e Medicina veterinaria, S.I.R.O.E, 20 November 2020
- Aggiornamenti chimico-normativi su Cannabis sativa L.", SIF, 27 November 2020
- "Genes Webinar | Human Gut Microbiome", 22 November 2021