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Nutraceuticals

The New Frontier

Edited by

Antonello Santini

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Nutraceuticals: The New Frontier

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Special Issue Editor

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About the Special Issue Editor

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Editorial

To Nutraceuticals and Back: Rethinking a Concept

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The concept of nutraceuticals as pharma-foods comes from far. This term is made from the two words "nutrient" and "pharmaceutical", was coined by Stephen DeFelice, and is defined as "a food or part of a food that provide medical or health benefits, including the prevention and/or treatment of a disease" [1].

This definition leads to a partial overlap with the definition of a food supplement. In fact, both claim beneficial effects for health; however, while nutraceuticals are made from food or part of a food, food supplements are single substances used alone or in mixtures with the scope of adding micronutrients when the body is in need of them.

The aspect outlined by DeFelice [1]—in particular the preventive aspect and the treatment of a disease—is absent in the definition and scope of food supplements, which can be an aid for the body but are not required to have a proven clinical efficacy on a health condition. Based on these considerations, it hence appears of utmost importance to develop a new definition for nutraceuticals foreseeing their use "beyond the diet, before the drugs" as tools which can be able to prevent or delay the onset of some asymptomatic long term pathological conditions (e.g. hypercholesterolemia, hypertriglyceridemia, etc.). The steps involved in a new nutraceutical formulation should start with the identification of the target pathologic condition, in a way similar to what happens for drugs.

Figure 1 shows the steps to take when assessing the possible use of a nutraceutical. It is of utmost importance the clinical target identification and the appropriate food matrix to use. The safety and the in vitro and in vivo tests are crucial. The differences between nutraceuticals and food supplements (e.g. mineral or protein food supplements) are also outlined, stressing the necessity of clinical evidences substantiating the health efficacy for nutraceuticals based on safety, efficacy, and known mechanism of action.

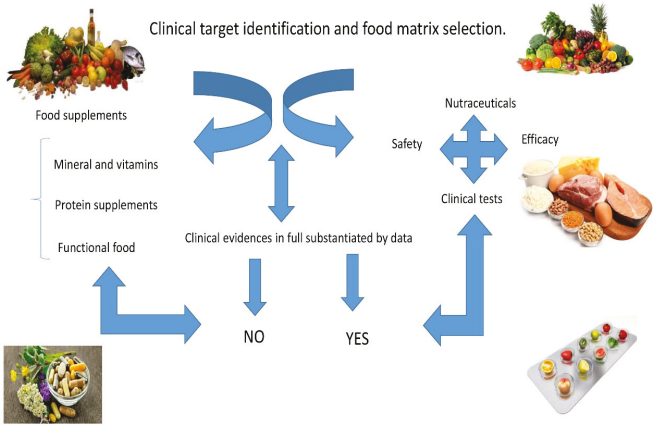

Figure 1. Food supplements and nutraceuticals.

Once a pathologic health condition target has been identified, formulation can be prepared from vegetal or animal matrices, and tested in vitro and in vivo, keeping in mind that safety and efficacy must be substantiated by clinical tests [2].

Nutraceuticals which are extracted from vegetable sources (phytocomplex) or which are the active metabolite complex (if of animal origin) should hence be understood as a set of pharmacologically active substances which have inherent therapeutic properties due to the natural active principles of recognized effectiveness which they contain. They should be administered in the appropriate pharmaceutical form (e.g., capsule, tablet, drink, etc.). Incidentally, these forms of administration coincide with those used for both drugs and food supplements.

The assessment of nutraceuticals' optimal conditions of use should be complementary with safety information as well as bioavailability and bioaccessibility information, so that they can propose themselves as a powerful toolbox to be used to prevent and cure some pathologic conditions in subjects who, for example, are not eligible for conventional pharmacological therapy.

For this reason, and due to their natural origin, a growing demand exists for nutraceuticals, which shade the frontier existing between pharmaceuticals and food, and this is also helping the producers to diversify their agriculture and promote research and innovation. Nonetheless, different country-specific regulations, safety, and health claim substantiation are the main challenges which the nutraceuticals are experiencing. The main challenge is the absence of a shared supra-national regulation for nutraceuticals, which would recognize their potential and possible role as therapeutic tools in some pathological conditions based on assessed safety, known mechanism of action, clinically proven efficacy in both reducing the risk of illness onset and enhancing overall well-being.

The labelling of marketed products is another source of confusion, and is often due to misinformation, which could induce false expectations regarding beneficial health effect and miss the target for a product to be effective as claimed. What may be considered a functional food under a given set of circumstances may be deemed a dietary supplement, medical food, food for special dietary use, a nutraceutical, or a drug under different circumstances, depending on its ingredients and the claims as reported on the label [3].

While the definition of food supplement is quite clear and understandable (see Table 1), the definition of nutraceutical still lies in between food, food supplement, and pharmaceutical, and the legitimate assessment of their potential in medicine is still contradictory and far from being shared and accepted worldwide [4].

Table 1. Some definitions.

Terms	Definitions	Reference
Food supplement	A product (other than tobacco) in the form of a capsule, powder, softgel, or gelcap intended to supplement the diet to enhance health that bears or contains one or more of the following dietary ingredients: a vitamin, mineral, amino acid, or other botanical or dietary substance.	United States Government Office, 1994 [5]
Nutraceuticals	Food or part of food that provides medical or health benefits, including the prevention and/or treatment of a disease.	De Felice, 1995 [1]
Nutraceuticals	Nutritional products that provide health and medical benefits, including the prevention and treatment of disease.	European Nutraceutical Association, 2016 [6]
Functional food	Any food or ingredient that has a positive impact on an individual's health, physical performance, or state of mind, in addition to its nutritive value.	Hardy, 2000 [7]

The definition of a food supplement often overlaps with the one accepted for nutraceuticals as present in the collective imagination, and the rationale behind their use is becoming a challenge of this millennium [8,9].

Food supplements should be, as per their micronutrients content, addressed to improve health if appropriately targeted to those in need. Nevertheless, many of the health claims which are currently

associated to food supplements, pro- and pre-biotics, as well as herbal products and functional foods are often not properly substantiated by in vivo data on safety, efficacy, and effect on health and/or on pathologic conditions. This is mainly due to the lack of in vivo and mechanism of action studies confirming the claimed health beneficial effect. Many literature data refer to in vitro studies, and focus on single food constituents (micronutrients). Any health beneficial effect for nutraceuticals is related to the fact that they derive from food or part of food, and consequently they can be considered safe or generally recognized as safe (GRAS). Safety is of utmost importance, since possible contaminants of inorganic [10] and organic origin [11] can contaminate these products and cause health issues.

It seems necessary to restructure the entire regulatory framework of dietary supplements and include nutraceuticals as a new category, by giving credit to their role in the prevention and cure of some pathological conditions. The pre-market approval system should be under any circumstance substantiated by in vivo clinical data to determine and assess their safety and efficacy. This approach could look similar to the one used for pharmaceuticals, which includes clinical trials to in vitro and safety tests. The likelihood of this happening in the foreseeable future is unfortunately quite low, but it seems reasonable to hypothesize that the competent national authorities could ask the manufacturers to provide data that substantiates safety, efficacy, and mechanism of action of any claims attributed to food supplements and nutraceuticals, avoiding any possible source of confusion.

Conflicts of Interest: The authors declare no conflict of interest.

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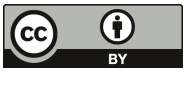

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Article

Chemical and Nutritional Characterization of Seed Oil from *Cucurbita maxima* L. (var. Berrettina) Pumpkin

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Abstract: Pumpkin (*Cucurbita* spp.) has received considerable attention in recent years because of the nutritional and health-protective value of seed oil. The nutritional composition of pumpkin native to central Italy, locally known as "Berrettina" (*Cucurbita maxima* L.), was evaluated. In particular, the lipid fraction of seed oil was characterized, and the triacylglycerol (TAG) was thoroughly studied by using a stereospecific procedure to obtain the intrapositional fatty acid composition of the three *sn*-positions of the glycerol backbone of TAG. Moreover, alkaline hydrolysis was carried out to study the main components of the unsaponifiable fraction, i.e., sterols and alcohols. It was observed that monounsaturated fatty acids and polyunsaturated fatty acids were the most abundant (41.7% and 37.2%, respectively) in Berrettina pumpkin seed oil, with high content of oleic and linoleic acid (41.4% and 37.0%, respectively). The main sterols of Berrettina pumpkin seed oil were $\Delta^{7,22,25}$-stigmastatrienol, $\Delta^{7,25}$-stigmastadienol, and spinasterol; with regard to the alcoholic fraction, triterpenic compounds were more abundant than aliphatic compounds (63.2% vs. 36.8%). The obtained data are useful to evaluate pumpkin seed oil from a nutritional point of view. The oil obtained from the seed could be used as a preservative and as a functional ingredient in different areas, e.g., cosmetics, foods, and nutraceuticals.

Keywords: pumpkin seed oil; fatty acids; stereospecific analysis; sterols; alcohols

1. Introduction

The pumpkin (*Cucurbita* spp.), one of the most popular vegetables consumed in the world, has been recently recognized as a functional food [1–3]. Pumpkin seeds, generally considered agro-industrial waste, are an extraordinarily rich source of bioactive compounds with interesting nutraceutical properties [4]. In recent years, several studies [5–7] have highlighted the health properties of pumpkin seed oil against many diseases, including hypertension, diabetes, and cancer. It also shows antibacterial, antioxidant, and anti-inflammatory properties [8,9]. Due to the presence of interesting natural bioactive compounds, such as carotenoids, tocopherols, and sterols, pumpkin-derived products have a wide spectrum of biological activity, proven by in vivo experiments [10].

Because of the positive health effects, research has been focused particularly on the content and composition of fatty acids (FA) and tocopherols in pumpkin seed oil, while, to a lesser extent, other lipid components, such as sterols, alcohols, and phenol acids, have been studied, as is done with other food matrices to identify specific markers characteristic of the plant varieties [11]. Among the

relevant aspects to be considered when dealing with this vegetable, the beneficial effects of using environmentally friendly natural herbicides [12] must be mentioned, since the content of bioactive compounds could be affected, and there could be possible contamination of this vegetable due to the presence of *Fusarium* spp. microfungi and their secondary metabolites [13], affecting the content of beneficial compounds of the vegetable itself.

Stevenson et al. [14] summarized FA composition and reported significant differences among various cultivars of pumpkin seed oil extracted from various pumpkin sources. Rezig et al. [15] studied the chemical composition and oil properties of seeds of a Tunisian variety of pumpkin, Béjaoui (*C. maxima*). They found that the major FA were oleic, linoleic, and palmitic acids and that the seed oil was rich in δ-tocopherol, while the sterol marker was β-sitosterol and the predominant phenolic acid was syringic acid. Siano et al. [16] highlighted that saturated FA (SFA) and monounsaturated FA (MUFA) of *C. maxima* produced in southern Italy showed similar values (25.20% and 25.54%, respectively), while the polyunsaturated FA (PUFA) content was 48.14%. Habib et al. [17] determined the proximate composition of powdered seed and the lipid composition of the oil of *C. maxima* collected in Bangladesh. They affirmed that the high degree of unsaturation makes the oil suitable for use as valuable drying agent, and lower free FA content indicates suitability of the oil for consumption as food.

Other researchers studied the chemical composition of pumpkin seed oils from *C. pepo* [5,18–21]. Due to the differences among the species and/or varieties of *Cucurbita* spp. grown in different areas of the world, the present study focused on characterizing a native Italian cultivar (*C. maxima*, var. Berrettina, locally known as "priest's hat"), paying attention to the lipid composition of the seed oil. Since there is little information about the lipid structure, the present research deepens understanding of the total FA content and intrapositional composition of Berrettina pumpkin by using stereospecific analysis, and pays attention to other minor lipid components such as sterols, alcohols, and carotenoids. Butinar et al. [22] proposed high-performance liquid chromatography (HPLC) analysis of triacylglycerol (TAG) as a useful technique to evaluate the genuineness of pumpkin seed oils produced in Slovenia, but to the best of our knowledge, there are no data in the literature dealing with stereospecific analysis of pumpkin seed TAG.

2. Materials and Methods

2.1. Materials and Chemicals

Methanol (MeOH), diethyl ether, petroleum ether, formic acid, hydrochloric acid, and acetone were purchased from J.T. Baker B.V. (Deventer, the Netherlands). Hexane, ethanol (EtOH), chloroform ($CHCl_3$), anhydrous sodium sulfate (Na_2SO_4), and potassium hydroxide (KOH) were bought from Carlo Erba Reagents (Milan, Italy). Deionized water (>18 MΩ cm resistivity) was obtained from a Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). Supelco™ 37 Component FAME Mix, containing the methyl esters of 37 fatty acids (Supelco, Bellefonte, PA, USA; catalog No. 47885-U), was used. Lipase from porcine pancreas (EC 3.1.1.3), *sn*-1,2-diacylglycerol kinase from *Escherichia coli* (DAGK; EC 2.7.1.107), cholesterol (≥99%), ergosterol (≥95%), stigmasterol (~95%), β-sitosterol (≥95%), 5-α-cholestane (≥97%), γ-linolenic acid (≥99%), 2′,7′-dichlorofluorescein, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), 1-octadecanol (99%), 1-docosanol (98%), 1-octacosanol (≥99%), lutein (≥97%), and β-carotene (≥97%) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Collection of Pumpkin Samples

Pumpkin belongs to the family *Cucurbitaceae*. The samples (*C. maxima* L., var. Berrettina) were taxonomically identified by Luigi Frassineti (Tuder Green Service, Todi, Italy). It is a leafy green vegetable with medium-large flattish fruits with green-gray, moderately hard knobby skin, edible yellow/orange flesh, and a central cavity with numerous plump, whitish-yellow seeds.

Three pumpkins cultivated in central Italy (Todi) and collected in autumn 2016 were selected for their uniformity of shape, weight, and color. The fresh pumpkin samples were weighed (about 2.5 kg each), peeled, and, after manual removal of seeds, cut into small pieces (1.5 cm × 1.5 cm × 1.5 cm) and analyzed. The seeds were cleaned to remove impurities and dried at 60 °C for 24 h in a hot-air fan oven. After that, the seeds were reweighed until the weight was constant. The samples were stored in a dry place in the dark at room temperature.

2.3. Determination of Pumpkin Chemical Composition

Crude fat, protein, moisture, and ash contents of pumpkin samples were determined according to the procedures described in the Association of Official Analytical Chemists (AOAC) method [23].

2.4. Seed Lipid Extraction

Dried pumpkin seed samples were ground using a kitchen grinder (Oster, model 869-50R, Lakewood, CA, USA). Extraction of lipid fraction of the pumpkin seeds was performed using petroleum ether as a solvent in a Soxhlet extractor, according to AOAC procedure [23]. The extract was dried over Na_2SO_4, and then the solvent was evaporated under reduced pressure using a rotary evaporator (Büchi Rotavapor B-480, Essen, Germany) at 40 °C. Finally, the residue was weighed and dissolved in hexane. The recovered oil was stored at 4 °C until use.

2.5. Isolation of TAG Fraction from Oil Samples

The TAG fraction was isolated by thin layer chromatography (TLC), according to the method described by Cossignani et al. [24], from total fat of pumpkin seed samples using silica gel plates (SIL G-25, 0.25 mm, 20 cm × 20 cm; Macherey-Nagel, Germany) and petroleum ether/diethyl ether/formic acid (70:30:1, *v/v/v*) as a developing solvent. The TAG fraction was scraped off, extracted with hexane/diethyl ether (1:1, *v/v*), subjected to transesterification, and analyzed by high-resolution gas chromatography (HRGC) as reported in Section 2.6 to obtain the constituent fatty acid methyl esters (FAME). The obtained data represent the total composition of FA esterified in all 3 *sn*-positions of TAG, named A_t.

2.5.1. Stereospecific Analysis of TAG

The stereospecific analysis procedure [25] carried out on TAG of pumpkin seed oil isolated as reported in the previous paragraph involved the following steps:

- The pancreatic lipase procedure (Section 2.5.2) to obtain the FA% intrapositional composition of *sn*-2 position of glycerol backbone of TAG, named A_2;
- Preparation of *sn*-1,3/*sn*-1,2(2,3)-diacylglycerol (DAG), followed by the DAGK enzymatic procedure (Section 2.5.3), to obtain the FA% intrapositional composition of *sn*-1 and *sn*-2 positions of glycerol backbone of TAG, named $A_{1,2}$.

2.5.2. Pancreatic Lipase Procedure

Hydrolysis of TAG was carried out according to the method provided by the Italian fat and derivate control standards (Norme Italiane per il Controllo dei Grassi e Derivati (NGD) method) [26]. Tris-HCl buffer (pH 8.08), bile salts, $CaCl_2$, and pancreatic lipase were added to an aliquot of TAG. The mixture was incubated under magnetic stirring in a water bath at 40 °C for 5 min, and then 6 M HCl and diethyl ether were added and the mixture was centrifuged. Diethyl ether was dried by anhydrous Na_2SO_4 and evaporated under nitrogen flow to small volume. The hydrolytic products were separated on TLC plates, and the developing solvent system was petroleum ether/diethyl ether/formic acid (70:30:1, *v/v/v*). The band corresponding to *sn*-2-monoacylglycerols (*sn*-2-MAG), visualized with 2′,7′-dichlorofluorescein spray, was scraped off, methylated, and analyzed by HRGC as

reported in Section 2.6 to obtain the constituent FAME. The obtained data represent the intrapositional composition of FA esterified in *sn*-2 position, named A_2.

2.5.3. *sn*-1,3/*sn*-1,2(2,3)-Diacylglycerol (DAG) Preparation

An aliquot of TAG was dissolved in anhydrous diethyl ether, and freshly prepared ethyl magnesium bromide in anhydrous diethyl ether was added. The mixture was shaken, and then pentane (0.1% acetic acid) and water were added. The solution was vortexed and centrifuged (ALC 4218 centrifuge, Thermo Scientific, Waltham, MA, USA). The water was removed and the organic phase was dried over anhydrous Na_2SO_4 and concentrated with solvent removal under nitrogen stream. The DAG mixture was applied to TLC plates previously treated with 5% boric acid solution (methanol/water, 80:20, *v/v*) and then activated for 1 h at 120 °C. The developing system was hexane/diethyl ether (60:40, *v/v*). The band containing the *sn*-1,2(2,3)-DAG fraction (Rf ≈ 0.30; Rf of the *sn*-1,3-DAG band ≈ 0.35), located by iodine vapor exposition, was scraped off and extracted with diethyl ether.

2.5.4. DAGK Enzymatic Procedure

The *sn*-1,2(2,3)-DAG ethereal solution was concentrated under nitrogen stream, then cardiolipin solution, buffered *sn*-1,2-DAGK, buffer, and Na_2ATP were added, mixing each time. After incubation at 40 °C for 90 min under constant stirring, chloroform/methanol (2:1, *v/v*) was added to the mixture to stop the reaction and extract the products of interest. The combined extracts were concentrated, treated with anhydrous Na_2SO_4, and applied to TLC plates. The developing system was chloroform/methanol/25% ammonia (65:25:5, *v/v/v*). The band of the *sn*-1,2-phosphatidic acids (*sn*-1,2-PA), visualized with 2′,7′-dichlorofluorescein spray (Rf ≈ 0.1), was scraped off, methylated, and analyzed by HRGC with flame ionization detection (FID) as reported in the following paragraph to obtain the constituent FAME. The obtained data represent the intrapositional composition of FA esterified in *sn*-1,2 positions, named $A_{1,2}$.

The FA composition at the *sn*-1- and *sn*-3-positions was obtained applying the following formulas:

$$A_1 = 2A_{1,2} - A_2 \quad (1)$$

$$A_3 = 3A_t - A_2 - A_1 \quad (2)$$

where A_1 = % intrapositional composition of FA esterified in *sn*-1 position; $A_{1,2}$ = % intrapositional composition of FA esterified in *sn*-1 and *sn*-2 positions; A_2 = % intrapositional composition of FA esterified in *sn*-2 position; A_t = % total composition of FA esterified in all 3 *sn*-positions of TAG; A_3 = % intrapositional composition of FA esterified in *sn*-3 position.

2.6. Preparation of FAME and HRGC-FID Analysis

The FAME of TAG, *sn*-2-MAG, and *sn*-1,2-PA fractions were prepared by transesterification. Hexane and 2 N methanolic KOH were added to the fraction and stirred for 3 min, then water was added. The organic phase (upper) containing the FAME was dried over anhydrous Na_2SO_4, then analyzed by HRGC. A DANI GC1000 DPC gas chromatograph (Norwalk, CT, USA) equipped with a split-splitless injector and a flame ionization detector (FID) was used. Separation was obtained using the CP-Select CB for FAME fused silica capillary column (50 m × 0.25 mm i.d., 0.25 μm f.t.; Varian, Superchrom, Milan, Italy). Chromatograms were acquired and processed using Clarity integration software (DataApex Ltd., Prague, Czech Republic). The injector and detector temperature was 250 °C. The oven temperature was held at 180 °C for 6 min, and raised to 250 °C at 3 °C/min; the final temperature was held for 10 min. Carrier gas (He) flow rate was 1 mL/min; the injection volume was 1 μL with a split ratio of 1:70. A standard solution containing 37 FAME was used to identify the individual FA. The percentage of each FA was calculated using the peak area of the samples. The data were normalized considering only the main reported FA (% mol mean values ≥0.1).

2.7. Alkaline Hydrolysis of Pumpkin Seed Oil

Alkaline hydrolysis of pumpkin seed oil was carried out according to the method reported by Cossignani et al. [27]. Prior to alkaline hydrolysis, 0.2% 5-α-cholestane in $CHCl_3$ and 1% 1-octacosanol (used as internal standards) were added to the oil samples, then the American Oil Chemists' Society (AOCS) method (Ch 6–91) was used [28]. The products obtained after alkaline reaction were applied to TLC silica gel plates previously treated with 0.2 N KOH in MeOH, then activated for 1 h at 100 °C. The developing system was hexane/diethyl ether (65:35, *v/v*). The band containing the sterols, visualized with 2′,7′-dichlorofluorescein spray, was scraped off and extracted with $CHCl_3$. Then the solvent was evaporated and removed under nitrogen stream.

2.8. Preparation of Trimethylsilyl Ether Derivatives and HRGC-FID Analysis

The silylation reaction was carried out as described by Lombardi et al. [29] with slight modifications. In brief, BSTFA and acetone were added to the sterol fraction and the reaction was carried out at 40 °C for 20 min to obtain trimethylsilyl ether (TMSE) derivatives. Sterols and alcohols, as TMSE derivatives, were analyzed using a DANI GC1000 DPC gas chromatograph equipped with a split-splitless injector and FID.

Separation of TMSE sterols and alcohols was obtained using the AT-1701 fused silica capillary column (25 m × 0.25 mm i.d., 0.2 μm f.t.; Alltech, Milan, Italy).

For analysis of TMSE sterols, the following chromatographic conditions were used: injector and detector temperature was 300 °C; oven temperature of 260 °C was held for 4 min, then increased to 300 °C at 1.5 °C/min, and the final temperature was held for 30 min; carrier gas (He) flow rate was 1.2 mL/min.

For analysis of TMSE alcohols, the following chromatographic conditions were used: injector and detector temperature was 290 °C; oven temperature was held at 180 °C for 3 min, raised to 260 °C at 6 °C/min for 15 min, then raised to 280 °C at 2 °C/min for 30 min.

Chromatograms were acquired and processed using Clarity integration software. The percentage of each sterol was calculated by using the peak area of the samples corrected with the correction factor equal to 1 as reported by Laakso [30]. TMSE sterols were also analyzed by HRGC coupled with mass spectrometry (MS) detector as described in Section 2.9.

2.9. HRGC-MS Analysis

A Shimadzu GCMS-QP2010 gas chromatograph equipped with a quadrupole mass spectrometer (Shimadzu, Milan, Italy) and split-splitless injector maintained at 300 °C was used. The following MS parameters were used: interface temperature 270 °C; MS ionization mode electron ionization; detector voltage 0.9 kV; acquisition mass range 50–500 u; scan speed 1000 u/s; acquisition mode full scan; scan interval 0.5 s; solvent delay 6 min. Data were collected by GC-MS Solution software (Shimadzu). The column and the chromatographic conditions were the same as those reported in Section 2.8.

TMSE sterols and alcohols were identified by comparing retention times and mass spectra to those of authentic TMSE-derivatized compounds. Confirmation of these structures was achieved by HRGC-MS using the National Institute of Standards and Technology (NIST; Gaithersburg, MD, USA) 2008 library to match mass spectral peaks of phytosterol standards to those found in pumpkin seed oil samples. Comparisons of parent molecular ion (M+) and fragmentation ions/patterns were employed to assist in elucidating the identities of the phytosterols. In addition to the presence of specific ion fragments, the relative intensity of the ion fragments was considered. Some compounds, for which commercial standards are not available, were tentatively identified by comparison of relative retention times, M+ values, and fragmentation patterns with data obtained from olive oil analysis or from MS spectra reported in the literature.

2.10. Carotenoid Analysis

The seed oil carotenoids were analyzed by HPLC with diode-array and mass spectrometry detection systems (DAD-MS). To perform this determination, a seed oil sample was diluted fourfold and injected into the HPLC system. Quali-quantitative determination of carotenoids was carried out using the HPLC-DAD-MS validated method described in Blasi et al. [31].

2.11. Statistical Analysis

FA, sterol, alcohol, and carotenoid composition data are reported as mean values and standard deviation (SD). HRGC analyses were carried out in duplicate. Data were processed and edited with Microsoft Excel 2016 (Microsoft, Redmond, MA, USA).

3. Results and Discussion

3.1. Nutritional Composition and Caloric Value

Data on nutritional composition and caloric value of pumpkin (*C. maxima*, var. Berrettina) are reported in Table 1. The samples had a high content of water (82.50%); in fact, a variety of vegetables have water composition in the range of 80–90%. The flesh is characterized by a low fat content. Simple sugars and ash showed similar values (0.82% and 0.84%, respectively), while the protein content was higher (1.28%). Data relative to moisture, ash, and protein are in good agreement with those reported by Kim et al. [32] for Korean pumpkin (*C. maxima*) flesh. Generally, the proximate composition is extremely variable [32–34], due to the differences among the species and/or varieties of *Cucurbita* spp. grown in different areas of the world. The low caloric value of pumpkin (*C. maxima*, var. Berrettina) is 25.35 kcal/100 g, according to data in the literature [35,36].

Table 1. Nutritional composition (g/100 g edible part) of pumpkin (*C. maxima*, var. Berrettina) (mean value ± standard deviation (SD), $n = 3$).

Component	Mean Value ± SD
Energy (kcal/100 g)	25.35
Moisture	82.50 ± 0.37
Dry matter	17.50 ± 0.14
Total ash	0.84 ± 0.04
Crude protein	1.28 ± 0.03
Crude oil	0.08 ± 0.01
Total sugars	4.90 ± 0.09
Starch	4.10 ± 0.05
Simple sugars	0.82 ± 0.03

3.2. Fatty Acid Composition of Seed Oils and Nutritional Quality Index

Total FA% compositions, corresponding to each FA component of oil and TAG fraction, are reported in Table 2. SFA with carbon chains shorter than 14 carbon atoms, called short- and medium-chain FA, was not found in pumpkin seed oil, as confirmed in other papers [14,20,37,38]. SFA was represented especially by palmitic (C16:0) and stearic (C18:0) acids, at 14.2% and 5.8%, respectively. It is reported that oils rich in myristic (C14:0) and palmitic acids affect the ratio of total to high-density lipoprotein (HDL) cholesterol only a little, and stearic acid slightly reduces this ratio [35]. PUFA and MUFA fractions were the most abundant (37.2% and 41.7%, respectively, for oil; 37.8% and 43.0%, respectively, for TAG); in fact, the main FA were oleic (C18:1*n*-9) and linoleic (C18:2*n*-6) acids. Berrettina pumpkin seed oil showed a higher content of oleic acid than linoleic acid (41.4% vs. 37.0% for oil); on the contrary, Procida et al. [20] reported a higher content of linoleic acid (44.30–51.58%) than oleic acid (34.16–42.59%) for three Italian samples of pumpkin (Crudigno, Pepo, and Winter). It has been reported by some authors [14] that oleic acid is the predominant FA (41–46%), followed

by linoleic acid (33.4–34.3%), in pumpkin seed oil from Italy and Libya. Siano et al. [16] found that the main FA of southern Italian pumpkin (*C. maxima*) seed oil were linoleic acid (47.45%), followed by oleic (25.54%) and palmitic (17.58%) acids. Habib et al. [17] found that pumpkin (*C. maxima*, known as "Misti Kumra") seed oil contained a high amount of oleic acid, 40.58%, while linoleic acid was 14.97%.

Table 2. Total and intrapositional % fatty acid composition (% mol, mean value ± SD, $n = 3$) of oil and triacylglycerol (TAG) fraction of pumpkin (*C. maxima*, var. Berrettina) seed oil.

		Total Lipids	TAG	*sn*-1-	*sn*-2-	*sn*-3-
Yield (%)		29.0 ± 0.9				
Saturated fatty acids (SFA)						
Mystiric acid	C14:0	0.2 ± 0.0	0.1 ± 0.0	0.5 ± 0.0	–	–
Palmitic acid	C16:0	14.2 ± 0.4	12.2 ± 0.4	28.5 ± 0.0	1.0 ± 0.0	7.1 ± 0.0
Margaric acid	C17:0	0.2 ± 0.0	0.1 ± 0.0	0.5 ± 0.0	–	–
Stearic acid	C18:0	5.8 ± 0.2	6.2 ± 0.2	13.7 ± 0.0	0.4 ± 0.0	4.4 ± 0.0
Arachidic acid	C20:0	0.5 ± 0.0	0.4 ± 0.0	–	–	1.1 ± 0.0
Behenic acid	C22:0	0.1 ± 0.0	0.2 ± 0.0	1.5 ± 0.0	–	–
Lignoceric acid	C24:0	0.1± 0.0	–	–	–	–
Total SFA		21.1	19.2	44.7	1.5	11.6
Monounsaturated fatty acids (MUFA)						
Palmitoleic acid	C16:1*n*-7	0.2 ± 0.0	0.2 ± 0.0	–	0.1 ± 0.0	0.4 ± 0.0
Heptadecenoic acid	C17:1*n*-7	0.1 ± 0.0	0.1 ± 0.0	–	0.1 ± 0.0	0.3 ± 0.0
Oleic acid	C18:1*n*-9	41.4 ± 0.7	42.7 ± 0.7	46.5 ± 0.0	36.0 ± 0.0	44.6 ± 0.0
Eicosenoic acid	C20:1*n*-9	0.1 ± 0.0	–	–	–	–
Total MUFA		41.7	43.0	46.4	36.2	46.3
Polyunsaturated fatty acids (PUFA)						
Linoleic acid	C18:2*n*-6	37.0 ± 0.5	37.4 ± 0.5	7.7 ± 0.0	62.1 ± 0.0	42.0 ± 0.0
Linolenic acid	C18:3*n*-3	0.2 ± 0.0	0.4 ± 0.0	1.1 ± 0.0	0.3± 0.0	0.1 ± 0.0
Total PUFA		37.2	37.8	8.8	62.4	24.1
Index						
UFA/SFA		3.7	4.2	1.2	65.7	6.1
MUFA/SFA		2.0	2.2	1.0	24.1	4.0
PUFA/SFA		1.8	2.0	0.2	41.6	2.1
PUFA*n*-6/PUFA*n*-3		185	93.5	7.0	207	420
UI		116.3	118.9	65.2	161.3	129.6
AI		0.19	0.16	0.55	0.01	0.10
TI		0.50	0.44	1.38	0.03	0.32

–: <0.1%; UI: unsaturation index: Σ(mol % of each FA) × (number of double bonds of each FA); AI: atherogenic index (C12:0 + 4 × C14:0 + C16:0)/(MUFA + PUFA*n*-6 + PUFA*n*-3); TI: thrombogenic index (C14:0 + C16:0 + C18:0)/(0.5 × MUFA + 0.5 × PUFA*n*-6 + 3 × PUFA*n*-3 + (PUFA *n*-3/PUFA*n*-6)).

It was reported by Orsavova et al. [38] that MUFA may reduce low-density lipoprotein (LDL) cholesterol, while it may possibly increase HDL cholesterol, and that oleic acid (C18:1*n*-9) may promote insulin resistance contrary to PUFA, with protection against insulin resistance. The high content of linoleic acid is an important nutritional aspect, because it is an essential FA (EFA), together with linolenic acid (C18:3*n*-3), and a lack of either of the two leads to ill health and causes deficiency symptoms. In addition, several studies [39] have positively correlated EFA intake with reduction of numerous disorders (cardiovascular, neurological, visual, and cancerous).

Minor FA (contents lower than 0.5%) of Berrettina pumpkin seed oil were myristic, palmitoleic (C16:1*n*-7), margaric (C17:0), heptadecenoic (C17:1*n*-7), arachidic (C20:0), behenic (C22:0), and lignoceric (C24:0) acids. These data are in good agreement with similar studies [14,20,37,38].

The extreme variability of FA composition of pumpkin seeds, and consequently of the corresponding pumpkin seed oils, is affected not only by the variety of the cultivar, but also by the growth conditions and degree of ripeness [40].

In addition, the nutritional quality of pumpkin (*C. maxima*, var. Berrettina) cultivated in central Italy was evaluated, using different indices, based on the FA composition of the oils. It is known

that some FA can help to prevent or promote coronary thrombosis and atherosclerosis based on their effects on LDL concentration and serum cholesterol [41]. The equations proposed by Ulbricht and Southgate [41] for the atherogenic index (AI) and thrombogenic index (TI) showed that C12:0, C14:0, and C16:0 FA are atherogenic, while C14:0, C16:0, and C18:0 are thrombogenic. PUFA *n*-3, PUFA *n*-6, and MUFA are antiatherogenic and antithrombogenic. Atherogenic indices have been described as powerful indicators of the risk of cardiovascular disease; the higher the value, the higher the risk of developing the disease, and vice versa. The AI of pumpkin (*C. maxima*) seed oil was lower than that reported by Siano et al. [16] (0.19 for Berrettina vs. 0.34), while the TI was comparable (0.50 for Berrettina vs. 0.65).

3.3. Stereospecific Analysis Data

The indirect method of analyzing TAG was based on a chemical–enzymatic–instrumental (stereospecific analysis) procedure and allowed us to carry out qualitative and quantitative analysis of all molecular TAG species, including enantiomeric ones. In fact, it is known that in TAG molecules the positions esterified by FA are numbered relative to their stereospecific numbering (*sn*) as *sn*-1, *sn*-2, and *sn*-3. The procedure allowed us to evaluate the FA % composition of each of the three *sn*-positions of TAG (% intrapositional composition). These data could be used to obtain the distribution of FA among the three *sn*-positions of TAG [24,25,42,43].

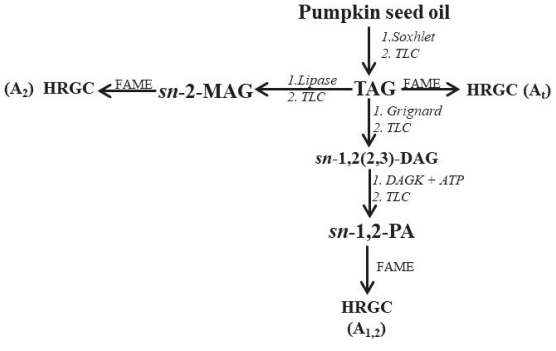

Figure 1. Scheme of the procedure used for stereospecific analysis of TAG from pumpkin (*C. maxima*, var. Berrettina) seed oil. HRGC: high-resolution gas chromatography; FAME: fatty acid methyl esters; TAG: triacylglycerol; *sn*-2-MAG: *sn*-2-monoacylglycerols; PA: phosphatidic acids; TLC: thin layer chromatography; DAG: diacylglycerol; DAGK: diacylglycerol kinase.

Initially, the lipid fraction was isolated by Soxhlet extraction, and then the TAG fraction was purified by TLC. As shown in Figure 1, several steps were carried out. Initially, the total FA% composition (A_t) was determined by HRGC. Figure 2a shows the characteristic HRGC profile of the FAME of the TAG fraction of pumpkin samples. Then, enzymatic hydrolysis of TAG with pancreatic lipase was used to obtain *sn*-2-MAG, and finally, after HRGC analysis of the FAME (Figure 2b), the acidic composition of *sn*-2-position (A_2) of the glycerol backbone of TAG was obtained. TAG was also subjected to chemical hydrolysis with Grignard reagent, then separation of enantiomeric *sn*-1,2(2,3)-DAG, realized by enzymatic synthesis of *sn*-1,2-PA, allowed us to obtain the acidic composition of the *sn*-1,2-positions ($A_{1,2}$) of the glycerol backbone of TAG, after HRGC analysis of the FAME (Figure 2c).

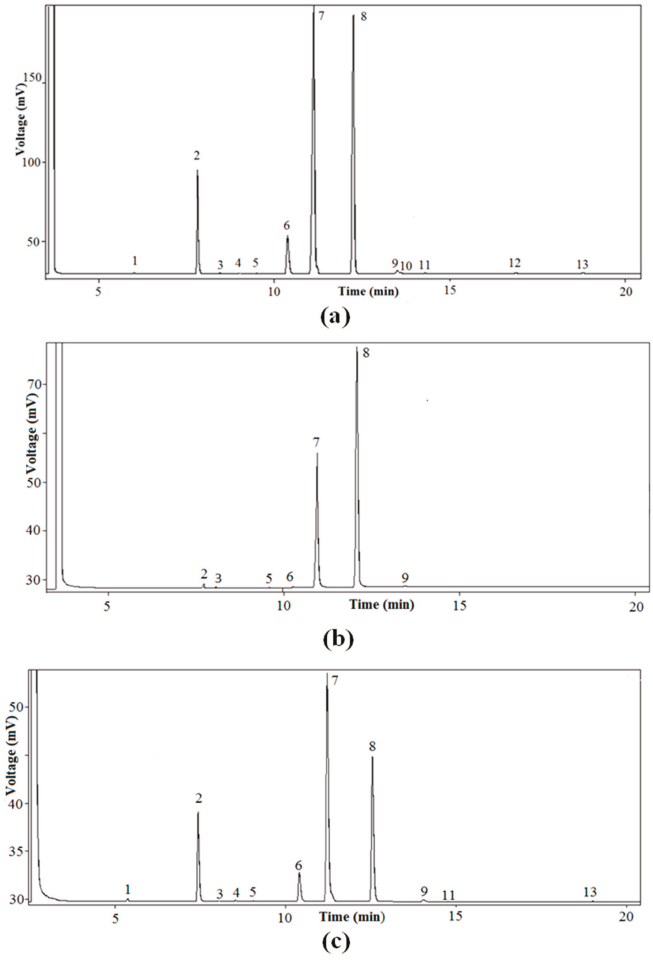

Figure 2. Characteristic high-resolution gas chromatography (HRGC) profiles of pumpkin (*C. maxima*, var. Berrettina) seed oil samples. (**a**) Fatty acid methyl esters (FAME) of TAG fraction; (**b**) FAME of monoacylglycerol (MAG) fraction; (**c**) FAME of PA fraction. 1. Myristic acid, 2. Palmitic acid, 3. Palmitoleic acid, 4. Margaric acid, 5. Heptadecenoic acid, 6. Stearic acid, 7. Oleic acid, 8. Linoleic acid, 9. Linolenic acid, 10. Arachidic acid, 11. Behenic acid, 12. Eicosenoic acid, 13. Lignoceric acid.

The stereospecific analysis represents a potent analytical-investigative procedure to give the fingerprint of TAG fraction for each botanical variety or animal species. The results of the stereospecific analysis procedure are shown in Table 2. It was observed that Berrettina pumpkin seed oil had a high percentage of UFA (98.5%) in *sn*-2 position, represented by MUFA (36.2%) and PUFA (62.4%). In *sn*-2 position, the main FA was linoleic acid (62.1%), followed by oleic acid (36.0%). SFA are preferentially esterified in *sn*-1 position (44.7%), represented essentially by palmitic and stearic acids (28.6% and 13.7%, respectively). Regarding the two primary positions, oleic acid was equally distributed between the *sn*-1 and *sn*-3 positions, while linoleic acid prefers the *sn*-3 position (42.2%).

3.4. Unsaponifiable Fraction

Another part of the research was analyzing the main components of unsaponifiable fractions, i.e., sterols and alcohols. Alkaline hydrolysis was carried out on pumpkin seed oils to obtain data relative to the qualitative composition of sterol and alcohol fractions. Phytosterols have been studied for their role in lowering cholesterol levels. In addition to this property, plant sterols have antiatherogenic, anti-inflammatory, anticancer, and antioxidation activities [44]. Together with the high content of linoleic acid, sterols can help in the treatment of lipid-associated disorders such as atherosclerosis.

In contrast to the other vegetable oils with Δ^5-sterols (β-sitosterol, campesterol, and stigmasterol) as the major components, Wenzl et al. [45] showed that pumpkin seed oil contains specific Δ^7-phytosterols, typical of only a few plant families (e.g., *Cucurbitaceae*), that provide a fingerprint for detection of adulteration. These Δ^7-sterols are supposed to give the pumpkin seed oil a beneficial effect in the treatment and prophylaxis of disorders of the prostate gland and the urinary bladder [46].

In this paper, sterol identification was carried out by HRGC-MS. Each peak was analyzed via detection of the parent molecular ion and the fragmentation pattern of the TMSE derivative. In addition to the presence of specific ion fragments, the relative intensity of the ion fragments was considered. Some TMSE sterols were identified by comparison with the NIST mass spectra library; typical fragmentation is reported in Table 3, together with sterol composition (% and mg/100 g). TMSE sterols give a molecular ion that is not abundant, while the first significant ion observed in the high mass range was usually equivalent to $[M\text{-}15]^+$, due to the loss of the methyl terminal group. Other main fragment ions useful for identifying the single sterol compounds are $[M\text{-}90]^+$, $[M\text{-}105]^+$, and $[M\text{-}129]^+$. They correspond to the loss of the trimethylsilanol, methyl group with trimethylsilanol, and fragmentation of the 1,2-cyclopenthanophenanthrene structure, respectively. The predominant sterols of Berrettina pumpkin seed oil are Δ^7-sterols, in particular $\Delta^{7,22,25}$-stigmastatrienol, $\Delta^{7,25}$-stigmastadienol, and spinasterol, which accounted for about 76.8% of the total sterols, followed by Δ^7-avenasterol and Δ^7-stigmastenol. The Δ^5-sterols were represented by campesterol, stigmasterol, and only a little cholesterol, as in other foodstuff [47]. Differences between the contents of Δ^5- and Δ^7-sterols could be attributed to the maturity stage of seeds or to the solvent used in the extraction procedure [15]. A total sterol content of 295 mg/100 g oil was measured in *C. maxima* seed oil; 15.7 mg/100 g was represented by Δ^5-sterols and 279.3 mg/100 g by Δ^7-sterols. The total content was in agreement with other studies [9,48], even if a wide range of variability is reported [9]. Hence, more detailed examinations of the composition of the sterol fraction of this oil could be of special interest. For example, analysis of a more extensive sampling is required for better characterization of pumpkin seed oils and for their authentication.

The alcoholic fraction (aliphatic and triterpenic classes) was also studied, after derivatization as TMSE and analysis by HRGC-MS; the typical fragmentation is reported in Table 4, together with the alcohol composition (% and mg/100 g). Some aliphatic alcohols (from C16 to C25 members of the 1-alkanol homologous series) with odd and even numbers of carbon atoms of the aliphatic chain were identified. The TMSE alcohols were identified by comparison with the NIST mass spectra library. TMSE alcohols give a molecular ion that is not abundant, while the first significant ion observed in the high mass range was equivalent to $[M\text{-}15]^+$, due to the loss of the methyl terminal group, and $[M\text{-}117]^+$, equivalent to the loss of $[(CH3)_3\text{-}Si\text{-}O]^+$, i.e., the OTMSi group. Moreover, HRGC-MS analysis of alcohols after BSTFA derivatization resulted in various peaks with MS fragments *m/z* 73, 75, 103, and 117 characteristics for OTMSi groups. The peak at *m/z* 129, corresponding to $[(CH3)_3\text{-}Si\text{-}O^+\text{=}CH\text{-}CH\text{=}CH_2]^+$, has been identified as the fragment originating from the breakdown of ring A along with the TMS moiety. Four main triterpenic alcohols were identified: butyrospermol, obtusifoliol, β-amyrine, and cycloartenol. The key fragmentation ions were molecular ion $[M]^+$, $[M\text{-}15]^+$, $[M\text{-}90]^+$, and $[M\text{-}105]^+$. Aliphatic alcohol content was 36.8%, of which hexadecanol and octadecanol were the most abundant (each about 4.4 mg/100 g oil), while triterpenic alcohol content was around 63.2%, of which obtusifoliol was the most abundant (11.9 mg/100 g oil).

Table 3. Fragmentation ions used for identification of trimethylsilyl ether (TMSE) sterols of pumpkin (*C. maxima*, var. Berrettina) seed oil and sterol composition (% and mg/100 g oil; mean value ± SD, *n* = 3).

Sterol	$[M]^+$	$[M-15]^+$	$[M-90]^+$	$[M-105]^+$	$[M-129]^+$	%	mg/100 g
Cholesterol	458	443	368	353	329	0.3 ± 0.1	0.9 ± 0.1
Campesterol	472	457	382	367	343	3.3 ± 1.0	10.3 ± 1.0
Stigmasterol	484	469	394	379	355	1.6 ± 0.4	4.5 ± 0.8
Spinasterol	484	469	394	379	–	20.5 ± 1.9	61.8 ± 4.2
$\Delta^{7,25}$-Stigmastadienol	484	469	394	379	–	26.4 ± 2.3	78.4 ± 5.5
$\Delta^{7,22,25}$-Stigmastatrienol	482	467	392	377	–	29.9 ± 2.5	91.2 ± 6.8
Δ^{7}-Stigmastenol	486	471	396	381	–	5.7 ± 1.0	15.1 ± 1.5
Δ^{7}-Avenasterol	484	469	394	379	–	12.3 ± 0.9	32.8 ± 2.8
Δ^{5}-Sterol (total)						5.2	15.7
Δ^{7}-Sterol (total)						94.8	279.3

$[M]^+$ indicates molecular ion. $[M-15]^+$, $[M-90]^+$, $[M-105]^+$, and $[M-129]^+$ correspond to loss of the methyl group, trimethylsilanol, methyl group with trimethylsilanol, and to fragmentation of the 1,2-cyclopenthanophenanthrene structure, respectively.

Table 4. Fragmentation ions used for identification of TMSE alcohols of pumpkin (*C. maxima*, var. Berrettina) seed oil and alcohol composition (% and mg/100 g oil; average value ± SD, *n* = 3).

Aliphatic Alcohols	$[M]^+$	$[M-15]^+$	$[M-90]^+$	$[M-117]^+$	%	mg/100 g
Hexadecanol	314	299	224	195	7.4 ± 2.0	4.3 ± 1.0
Heptadecanol	328	313	238	209	3.1 ± 0.9	1.9 ± 0.3
Octadecanol	342	327	252	223	7.5 ± 1.3	4.4 ± 0.9
Nonadecanol	356	341	266	237	3.4 ± 0.8	2.9 ± 1.5
Eicosanol	370	355	280	251	4.9 ± 1.0	2.0 ± 0.8
Docosanol	398	383	308	279	3.1 ± 0.9	2.2 ± 0.3
Tricosanol	412	397	322	293	1.8 ± 0.5	1.3 ± 0.3
Tetracosanol	426	411	336	307	3.6 ± 0.5	1.2 ± 0.2
Pentacosanol	440	425	350	321	2.3 ± 0.7	1.5 ± 0.9
Total					36.8	21.7
Triterpenic Alcohols	**$[M]^+$**	**$[M-15]^+$**	**$[M-90]^+$**	**$[M-105]^+$**		
Butyrospermol	498	483	408	393	13.7 ± 1.2	8.1 ± 2.1
Obtusifoliol	484	469	394	379	20.2 ± 1.5	11.9 ± 1.1
β-Amyrine	498	483	408	393	16.1 ± 1.1	9.5 ± 1.8
Cycloartenol	498	483	408	393	13.3 ± 1.0	7.8 ± 1.5
Total					63.2	37.3

$[M]^+$ indicates molecular ion. For aliphatic alcohols: $[M-15]^+$, $[M-90]^+$, and $[M-117]^+$ correspond to loss of the methyl group [$-CH_3$], trimethylsilanol [$-OSi(CH_3)_3$], and ethyl trimethylsilanol [$-CH_3CH_2-OSi(CH_3)_3$]. For triterpenic alcohols: $[M-15]^+$, $[M-90]^+$, and $[M-105]^+$ correspond to loss of the methyl group [$-CH_3$], trimethylsilanol [$-OSi(CH_3)_3$], and methyl group with trimethylsilanol [$-CH_3OSi(CH_3)_3$].

These minor compounds are also important constituents of edible oils and could be useful in distinguishing different pumpkin oil varieties.

3.5. Carotenoid Analysis

HPLC-DAD-MS analysis, performed directly on seed oils, detected two important carotenoids: lutein and β-carotene. Quantitative analysis showed a lutein concentration of 8 mg/L seed oil, while β-carotene concentration was 2.5 mg/L seed oil.

Carotenoids such as lycopene, lutein, β-carotene, and zeaxanthin have been studied by many researchers both analytically and biologically [49–51]. Carotenoids represent a significant added value for food: various health properties have been associated with these compounds, including antioxidant and anticancer activity, photoprotection, protection against cardiovascular diseases, and anti-inflammatory activity [52–54].

4. Conclusions

The results reported in this study confirm that pumpkin seed oils are interesting vegetable oils with important nutritional value, related to the presence of MUFA, PUFA, phytosterols, and carotenoids. A more extensive sampling for a better characterization of pumpkin seed oils and for authentication purposes is necessary. To the best of our knowledge, this is the first time stereospecific analysis data of pumpkin seed oil have been reported. Information obtained from this research could help to assess the potential of seed oil from this pumpkin cultivar to be commercially exploited for nutraceutical application, and incorporated into food formulations to benefit human health.

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Conflicts of Interest: The authors declare no conflict of interest.

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Italian *Opuntia ficus-indica* Cladodes as Rich Source of Bioactive Compounds with Health-Promoting Properties

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Abstract: Natural by-products, especially phenolic compounds, are in great demand by the nutra-pharmaceutical and biomedical industries. An analytical study was performed to investigate, for the first time, the presence of antioxidant constituents and the corresponding in vitro antioxidant activity in the extract of cladodes from Ficodindia di San Cono (*Opuntia ficus-indica*) protected designation of origin (PDO). The cladode extracts were analysed for target determination of selected constituents, i.e., β-polysaccharides and total phenolic content. Moreover, the antioxidant activity of hydro-alcoholic extracts was assessed by means of two different methods: α, α-diphenyl-β-picrylhydrazyl (DPPH) free radical scavenging method and ferric reducing antioxidant power (FRAP) assay. An untargeted UHPLC-ESI-QTOF-MS profiling approach was used to depict the phenolic profile of hydro-alcoholic cladode extracts. Interestingly, over 2 g/kg of polyphenols were detected in this matrix, and these compounds were mainly responsible for the antioxidant properties, as shown by the strong correlation between phenolic classes and antioxidant scores. Finally, this study provides basic information on the presence of bioactive compounds and in vitro antioxidant activities in cladode extracts from cactus that might recommend their novel applications at the industrial level in the field of nutraceutical products.

Keywords: food profiling; polyphenols; β-polysaccharides; antioxidant activity; *Opuntia ficus-indica*; cladodes

1. Introduction

The cactus (genus *Opuntia*, family Cactaceae) is a native plant of the American continent and is commonly found at every latitude although it is better adapted to arid areas. Prickly pear cacti can be found all over the world, with a wide climatic tolerance, being able to proliferate in rainfall regimes of 250 to 1200 mm per annum with very hot summers of over 40 °C, and cold winters with temperatures frequently falling below 0 °C for brief durations [1].

Nowadays, there are more than 250 species, distributed in Mediterranean Europe, India, the Middle East and in the American and African countries [2]. More than 90% of cultivated crops of the Italian production are grown in Sicily (southern Italy), representing an important food source [3]. In this regard, "Ficodindia di San Cono" (*Opuntia ficus-indica*) is a protected designation of origin (PDO) product from Sicily characterized by a large-sized fruit, with a weight varying from 105 to 270 g (with 5% tolerance). From a botanical point of view, the fruit presents a green or yellowish-orange colored skin for the "Surfarina" cultivar, whilst the "Sanguigna" and "Muscaredda" cultivars range from green to ruby-red and green to straw-white, respectively.

The main commercial exploitation of this plant remains the fruit, although the vegetative parts, i.e., cladodes, are generally used as animal feed [4,5], fodder or disposed in landfills, whilst in some countries they are also consumed as plants for human consumption [6]. In fact, not only the fruits but also young cladodes are used as a starting point to realize several consumer goods, such as candy, liqueurs, body lotions, creams, and shampoos [7]. Some authors [8] have calculated also the nutritional value of the Opuntia fruits, placing them between that of lettuce and spinach.

In recent years, the market has shown considerable interest in a variety of tissues of *Opuntia ficus-indica* since they can be used both in food and pharmaceutical areas [6]. In particular, attention is now focused on the difference between food/dietary supplements and nutraceuticals [9]. Nutraceuticals are closer to pharmaceuticals and can help in fighting some of the major health challenges of the century, such as metabolic syndrome, cardiovascular diseases and hypercholesterolemia [9]. In this regard, they are considered 'beyond the diet before the drugs', as pointed out in previous works [10].

Cactus cladodes contain high amounts of fiber, including pectin, mucilage, lignin, cellulose and hemicellulose, and generally these substances are able to bring wellbeing to the metabolism of lipids and sugars [11]. In particular, β-polysaccharides (i.e., glucose units linked (1→4)-β (as in cellulose) but interspersed with (1→3)-β-linkages), are characterized by an irregular linkage structure that prevents the formation of a crystalline structure leading to a water-soluble capacity [12]. These polysaccharides are generally classified as soluble dietary fiber, improving glucose control and modulate renal water and sodium handling in type 2 diabetes patients; therefore, the high dietary fiber content of cladodes has the capacity to absorb large amounts of water, forming viscous or gelatinous colloids, and determining the absorption of several kinds of organic molecules [13]. *Opuntia ficus-indica* cladodes can also be considered a rich source of bioactive and functional compounds, which make them an important candidate for the production of health-promoting and functional foods. In this regard, in recent years, the scientific world has paid particular attention to polyphenols as they have shown antioxidant properties in vitro, together with protective effects against cancer, and the ability to cure and prevent cardiovascular disorders, inflammatory and allergic diseases [14]. Cardiovascular disease is one of the major factors responsible for death in industrialized countries [15]; therefore, *Opuntia ficus-indica* cladodes could help in lowering cholesterol levels and in preventing hypercholesterolemia [16].

Several studies reported the potential of seeds and peels from *Opuntia* spp. as novel by-products with antioxidant activity and bioactive properties [17,18]. However, although traditionally used as a valuable health supporting nutrient, the vegetative parts of *Opuntia* spp. plants (i.e., the non-edible parts) have been scarcely studied, and nowadays there is a lack of information on their entire chemical and bioactive properties. Furthermore, antioxidant properties of *Opuntia* spp. genus have been described only for few species [19], and very little information is available about cultivars located in Italy, in particular about Ficodindia di San Cono PDO and its comprehensive screening of phenolic compounds.

Therefore, in this work, β-polysaccharides, total phenolic content, antioxidant activity (as FRAP reducing power and DPPH radical scavenging), and the untargeted phenolic profile of extracts from fully-grown cladodes from Ficodindia di San Cono PDO were deeply investigated. The use of an untargeted ultra-high-pressure liquid chromatography coupled to quadrupole-time-of-flight mass spectrometer (UHPLC-ESI-QTOF-MS) system is expected to provide a much deeper investigation of the actual phenolic composition of these cladodes, as compared to classical targeted approaches. Moreover, the obtained fingerprint may be useful to better understand the nutraceutical traits of this species, considering both products and by-products obtainable from the processing of cladodes.

2. Materials and Methods

2.1. Cladodes Harvest and Preparation

Opuntia ficus-indica cladodes with an approximate length of 25–30 cm were manually harvested from the PDO consortium area, comprising the municipalities of San Cono, San Michele di Ganzaria, Piazza Armerina and Mazzarino (Catania and Enna, Sicily, Italy–altitude: from 200 to 400 m a.s.l.).

A sample per municipality (except the site of San Cono, where 2 samples were produced), was prepared by pooling 5 cladodes. In more detail, one apical, three central and one basal position fresh cactus cladodes were washed, and the thorns were removed manually. Then, they were individually cut into small pieces, peeled and homogenized by a mixer, obtaining five samples (replicates). The mean value for each target analyses has been obtained calculating the average among the five leaves. All samples were store at -18°C in freezer until further analysis.

2.2. Proximate Composition of Cladodes

The proximate composition analyses were carried out according to AOAC [20] for dry matter (DM; method 930.15), protein (method 976.05), ash (method 942.05) and lipid (method 954.02 without acid hydrolysis), while total carbohydrates were calculated by difference. Samples were also analysed for contents of different fiber fractions, i.e., neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL), according to the methods described by Van Soest et al. [21]. Finally, the results were expressed as grams of each compound per 100 g of cladodes.

2.3. Determination of β-polysaccharides Content

The β-polysaccharides content was determined colorimetrically, recording the absorbance at 540 nm, after reaction with Congo red dye, according to Eberendu et al. [22]. The spectrophotometric measurements were performed using a Perkin Elmer lambda 12 U*V*/*V*IS spectrophotometer (Ontario, Canada). The samples were extracted by 4 g of inner parenchyma in 10 mL of double distilled sterile water, homogenized and finally shaken using a horizontal shaker for 2 h. After that, 500 µL of 15 g/L KOH together with 2 mL of Congo red solutions (obtained by diluting a saturated aqueous solution 50 times) were added. The final reaction volume was left for 1 h prior the colorimetric reading at $\lambda = 540$ nm. In order to perform quantitative determinations, a pure β-glucan standard was used, and the respective results were expressed as β-glucan equivalents. Finally, measurements on five different cladode extracts from *Opuntia ficus-indica* di San Cono were performed.

2.4. Extraction and Profiling of Phenolic Compounds from Cladodes

Each replicate (five) was extracted from 4 g of representative sample of inner parenchyma in 20 mL of 0.1% formic acid in 80:20 (*v*/*v*) methanol/water (LCMS grade, VWR, Milan, Italy) using an Ultra-turrax (Ika T25, Staufen, Germany) at 25,000 rpm for 3 min. The extracts were then centrifuged at 313× *g* for 15 min at 4 °C. The resulting solutions were filtered using 0.22 µm cellulose syringe filters and collected in an amber vial for further use.

The total phenolic contents were investigated by using the Folin-Ciocalteu spectrophotometric assay, as previously reported [23], with small modifications. Aliquots of the sample (1 mL) were mixed with 2.5 mL of Folin-Ciocalteu reagent (Sigma, diluted five-fold) and 4 mL (75 g/L) sodium carbonate. Subsequently, the absorbance was recorded at 765 nm, after 40 min at 20 °C in dark conditions. Finally, a calibration curve was prepared in order to express final results; in particular, aliquots of gallic acid methanolic solutions were prepared to his purpose, and the results expressed as gallic acid equivalents (GAE).

After that, phenolic compounds were profiled through ultra-high-pressure liquid chromatography (a 1290 liquid chromatographic system equipped with binary pump, from Agilent Technologies, Santa Clara, CA, USA) coupled to a hybrid quadrupole-time-of-flight mass spectrometer (a G6550 mass spectrometer detector, from Agilent Technologies, Santa Clara, CA, USA), by using a JetStream dual electrospray as ionization source (UHPLC-ESI-QTOF-MS). UHPLC-ESI-QTOF-MS analytical conditions were set up on the basis of a previous experiment [24]. Briefly, the acquisition range of mass spectrometer was set from 50 to 1000 *m*/*z* with the instrument working in positive MS-only mode. In order to keep the mass axis calibrated, lock masses (121.0509 and 922.0098 *m*/*z*) were continuously infused during analyses using a separate electrospray. A reverse phase C18 column (Agilent Zorbax eclipse plus C18, 50 × 2.1 mm, 1.8 µm) with a water/methanol gradient elution was

used for chromatographic separation. In particular, the LC mobile phase A consisted of water (Milli-Q grade, Millipore, Bedford, MA, USA), while the mobile phase B was methanol (LCMS grade, VWR, Milan, Italy). Formic acid 0.1% (*v/v*) and ammonium formate (5 mM) (both from Sigma) were added to both mobile phases. The gradient was initiated with 5% B and increased to 90% B within 15 min, then held for 3 min. The LC mobile phase temperature was set to 35 °C, with a flow rate of 220 µL/min. Injection volume was 3 µL, and source conditions were the following: nitrogen was used as both sheath gas (10 L/min and 350 °C) and drying gas (8 L/min and 330 °C); nebulizer pressure was 60 psig, nozzle voltage was 300 V, and capillary voltage was 3.5 kV. Mass spectrometry raw data were analysed using the Agilent Profinder B.07 software, by using the 'find-by-formula' algorithm. In particular, compound identification was realized based on the Phenol-Explorer 3.6 database [25], and using the entire isotopic profile (monoisotopic accurate mass, isotope spacing and ratio) to increase the accuracy. An overall identification score above 85% and a mass accuracy below 5 ppm were adopted as minimum identifications criteria. The mass and retention time alignment with the compounds filtering (post-acquisition data processing) were conducted in Profinder B.07: only those compounds identified within 100% of the replications in at least one cladode sample were considered.

Furthermore, after a classification of polyphenols into phenolic classes and subclasses, cumulative intensities were calculated, and then quantitative values were determined by using methanolic standard solutions of pure phenolics (provided from Extrasyntese, Lyon, France) in order to provide more information. Particularly, the standards used were: matairesinol and sesamin (for dibenzylbutyrolactone and dihydroxydibenzylbutane, and furan and furofuran lignans, respectively), ferulic acid (for hydroxycinnamic acids and other phenolic acids), cyanidin (as representative of anthocyanins subclass), catechin (flavanols), luteolin (flavones and other remaining flavonoids), resveratrol (stilbenes), pentadecylresorcinol or cardol (for alkylresorcinols) and tyrosol (as representative of tyrosols and other remaining phenolics). Therefore, a calibration curve (not weighed and not forced to origin) was built and used for the quantization of phenolic compounds, considering five concentrations of standard over 5 orders of magnitude.

2.5. Assay of DPPH Radical Scavenging Activity

The radical scavenging ability of polyphenols against the stable radical DPPH was investigated by using the DPPH spectrophotometric assay, as previously described [23]. Briefly, 2 mL of each phenolic extract solution was placed in a cuvette together with 2 mL of a 1.0×10^{-4} mol/L daily-prepared ethanol solution of DPPH. The absorbance measurements were performed at 517 nm using a Perkin Elmer lambda 12 UV/VIS spectrophotometer (Ontario, Canada). The decrease in absorbance was determined continuously after the addition of the DPPH radical at five minute intervals. DPPH radical scavenging activity coefficients were calculated using gallic acid as reference compound, and results were finally expressed as gallic acid equivalents (GAE).

2.6. Assay of FRAP Reducing Antioxidant Power

The FRAP assay was carried out on the basis of the colorimetric method previously described by Benzie and Strain [26], with minor modification. In particular, the clinical auto-analyzer ILAB 600 (Instrumentation Laboratory, Lexington, MA, USA) was used to carry out the measurements. The FRAP working reagent was prepared daily by mixing a pH 3.6 acetate buffer 300 mM, a TPTZ (2,4,6-tripyridyl-*s*-triazine) 10 mM in 40 mM HCl solution and $FeCl_3$ 20 mM, in the ratio of 10:1:1. The phenolic extracts (100 µL) were mixed with 3000 µL of FRAP working reagent and the absorbance was measured at 600 nm, after 343 s of incubation at 37 °C. A calibration curve was built using gallic acid in ethanol before any session of analysis. Finally, the results were expressed as gallic acid equivalents (GAE).

2.7. Statistical Analysis

Data analysis were carried out with PASW Statistics 25.0 (SPSS Inc.: Chicago, IL, USA). Correlations among β-polysaccharides, total phenolic content, antioxidant activities and phenolic classes equivalents

of *Opuntia* cladode samples were obtained through Pearson's correlation coefficient ($p < 0.01$, two tailed). The elaboration of UHPLC-ESI-QTOF-MS data on phenolic profile of cladodes phenolic extracts were made using Agilent Mass Profiler Professional B.12.06 (Agilent Technologies, Santa Clara, CA, USA). Compounds' abundance was normalized at the 75th percentile and baselined to their median across all replicates.

3. Results and Discussion

3.1. Nutritional Composition, Total Phenolics and β-polysaccharides Contents

The nutritional composition of Ficodindia di San Cono cladodes is presented in Table 1.

Table 1. Chemical composition (on a fresh weight basis) of Ficodindia di San Cono cladodes. Data are presented as mean values ± standard deviation ($n = 5$). NDF = neutral detergent fiber; ADF = acid detergent fiber; ADL = acid detergent lignin. * = obtained by difference.

Parameters	San Cono cladodes
Moisture (g water/100 g cladodes)	92.33 ± 1.36
Protein (%)	0.58 ± 0.02
Ash (%)	0.50 ± 0.01
Lipid (%)	0.12 ± 0.02
Carbohydrates (%) *	3.05
NDF (%)	3.42 ± 0.63
ADF (%)	0.83 ± 0.14
ADL (%)	0.12 ± 0.02

These results showed that San Cono cladodes possessed low protein and lipid content, being 0.58 and 0.12 g/100 g, respectively. De Santiago et al. [27] reported a higher protein content (1.1 g/100 g) and a similar lipid content (<0.1 g/100 g) in fresh cactus cladodes (*Opuntia ficus-indica*) from Spain. San Cono cladodes were particularly abundant in fiber, being the NDF content of 3.42 g/100, and this finding fitted with results obtained by De Santiago et al. [27] and Guevara-Figueroa et al. [28]. Stintzing and Carle [7] reviewed the chemistry and technological use of Opuntia cladodes, showed that, on a fresh weight basis, the typical chemical composition of these matrices is characterized by 3–7 g carbohydrates, 1–2 g minerals, 0.5–1 g proteins, 0.2 g lipids and 1 g of fiber. However, the fact that cladode composition varies according to edaphic factors at the cultivation site, the season and the age of the plant should be taken into account; at this regard, young cladodes show higher carbohydrate, protein and water contents [7].

As regards polyphenols, these compounds are widely distributed in the plant kingdom and in recent years they have attracted much attention, due to their in vitro antioxidant capacity with potential beneficial implications in human health [29]. The total phenolic content (TPC), as assayed through the Folin-Ciocalteu approach, along with β-polysaccharides content and in vitro antioxidant activity (DPPH radical scavenging and FRAP reducing power) are reported in Table 2.

Overall, present findings showed that cladodes from Ficodindia di San Condo PDO possessed considerable nutritional value with health-promoting properties. It has been well studied that the phenolic content of plant materials is strongly correlated with their antioxidant activity [30]. Normally, lipophilic and hydrophilic compounds, such as carotenoids and polyphenols, contribute to the total in vitro antioxidant activity of fruits and vegetables [31,32]. In particular, phenolic compounds are the principal plant constituents with antioxidant properties, which exhibit an important function in neutralizing free radicals [33]. In the present study, the TPC in cladodes of Ficodindia di San Cono PDO was found around 2600 mg GAE/kg fresh weight (FW). This value was comparable to findings reported by De Santiago et al. [27] which obtained a GAE value of 1700 mg/kg FW for cactus cladodes (*Opuntia ficus-indica*) collected from Spain. Other works reported definitely lower TPC values; for example, Santos-Zea et al. [34] reported GAE values of 318 mg/kg DM for cladode

flours, considering the variety 'Jalpa' of *Opuntia ficus-indica*, whilst other varieties of *Opuntia* spp. showed on average GAE values around 700 mg/kg DM. Furthermore, Ramirez-Moreno et al. [35] determined the levels of polyphenols in cladodes of two species (*Opuntia ficus-indica*, cultivars 'Milpa' and 'Atlixco'), showing GAE values of 5710 and 3820 mg/kg DM, respectively. However, it is important to underline that these differences in TPC could arise above all from different climatic conditions [36]. In particular, looking at *Opuntia* spp. plants, all parts of the cactus are particularly rich in polyphenolic classes, such as various flavonoids and phenolic acids, as reviewed by El-Mostafa et al. [37]. However, the secondary metabolite accumulation in the plant depends on both biotic and abiotic factors. Since the Opuntia species used in this study were cultivated under the same environmental conditions (according to PDO product specifications), the amount and profile of polyphenols were characteristics of *Opuntia* spp. grown in this geographic area.

Table 2. Total β-polysaccharides, total phenolic content (TPC), and antioxidant activities (DPPH radical scavenging and FRAP reducing power) in cladodes extracts. The data are presented as mean values ± standard deviation (n = 5). Results for TPC, DPPH and FRAP are expressed as gallic acid equivalents (GAE), whilst results for β-polysaccharides are expressed as β-glucan equivalents. FW = fresh weight.

	β-Polysaccharides (mg β-Glucan Equivalents/kg FW)	*TPC (mg GAE/kg FW)*	*DPPH (mg GAE/kg FW)*	*FRAP (mg GAE/kg FW)*
Cladodes	2617.39 ± 225.58	2633.10 ± 214.78	1040.03 ± 112.42	1638.17 ± 41.30

Dietary fiber (DF) is considered a combination of chemically heterogeneous substances. Nowadays, the soluble/insoluble DF ratio is an important nutritional parameter, like TDF content, because of the different physiological and beneficial effects [38]. In particular, soluble dietary fiber (SDF) is characterized by compounds with high water holding activity, which can be considered health promoting substrates of intestinal and colonic microbiota. Some studies outlined that the majority component of cladode samples was DF [13]. In this study, the SDF content in cladodes was investigated colorimetrically, showing a value of 2617.39 mg β-glucan equivalents/kg FW. This value was even higher than the β-polysaccharides content of inner gel parenchyma from leaves of two different Aloe species, being on average 828 mg β-glucan equivalents/kg FW [39]. β-glucans, like some other β-polysaccharides, are considered the principal component of the soluble fiber in whole oats and barley, and their biological activities are strongly influenced by the molecular weight. Among cereals, the highest content of β-glucans (as g per 100 g dry weight) has been reported for barley 2–20 g and oats 3–8 g. The other cereals contain these compounds in much lower amounts, with the following values: sorghum 1.1–6.2 g, rye 1.3–2.7 g, maize 0.8–1.7 g, triticale 0.3–1.2 g, wheat 0.5–1.0 g, durum wheat 0.5–0.6 g, and rice 0.13 g [40]. Other studied sources of β-glucans include some types of seaweed and different species of mushrooms [40]. Cladodes from Ficodindia di San Cono PDO were then characterized by approximately 0.3 g/100 g DM of β-glucan equivalents, a value comparable to triticale, rice, and wheat. Therefore, the combination of high DF and associated phytochemicals (such as phenolic compounds) in a single matrix (such as cladodes) results in a food system with specific health related properties, suitable for different uses.

3.2. In vitro Antioxidant Activity of Cladodes

The in vitro antioxidant activity should not be determined based on a single antioxidant test [41]. Therefore, in the current work, the in vitro antioxidant activity of samples was evaluated by employing FRAP and DPPH assays, since the aforementioned methods are based on two different reaction mechanisms and kinetics [41]. Furthermore, the DPPH assay is currently considered a valid colorimetric method for the evaluation of antioxidant potential of plant extracts [42]. Furthermore, the literature presents a lack of uniformity in the standards used for calibration or regarding the best antioxidant assay performed; therefore, sometimes the comparison of spectrophotometric results with literature data becomes difficult.

Antioxidants are a group of compounds very different in chemistries and properties, therefore. the choice of assay could have a great effect upon the results obtained [43]. These antioxidant compounds may help to reduce the oxidative stress, preventing free radicals from damaging biomolecules such as proteins, DNA and lipids [44]. As shown in Table 2, San Cono Opuntia cladodes possessed GAE values of 1040 mg/kg FW considering DPPH radical scavenging, and 1638 mg/kg FW for FRAP reducing power. De Santiago et al. [27] obtained DPPH values in *Opuntia* spp. cladode extracts of 45.05 mg Trolox Equivalents/kg FW, while Astello-Garcia et al. [45] reported on average a DPPH value of 127,000 Trolox Equivalents/kg DW considering three different cultivars of the same species (*Opuntia ficus-indica*). However, these data regarding DPPH were not properly comparable due to the different expression of results. Considering FRAP reducing power of San Cono cladodes, the value obtained (962 μmol GAE/100 g) is higher than those reported by Rocchetti et al. [24], evaluating the antioxidant potential of common gluten-free flours containing polyphenols. Furthermore, cladode extracts also showed higher DPPH radical scavenging values than pumpkin and poppy seeds, being respectively 620 and 860 mg GAE/kg, as reported by Ghisoni et al. [46].

3.3. Evaluation of Phenolic Profile by UHPLC-ESI-QTOF-MS

An untargeted UHPLC-ESI/QTOF-MS approach was used to investigate the entire phenolic profile in cladodes extracts. Flavonoids were definitely the most frequent class of polyphenols detected, with 89 compounds annotated, followed by phenolic acids (54 compounds), tyrosols equivalents (27 compounds), and few other phenolics (lignans, alkylphenols and stilbenes derivatives). The phenolic profile of cladode extracts is shown in Figure 1 considering the cumulative intensity per phenolic class (as gained from UHPLC-ESI/QTOF-MS screening).

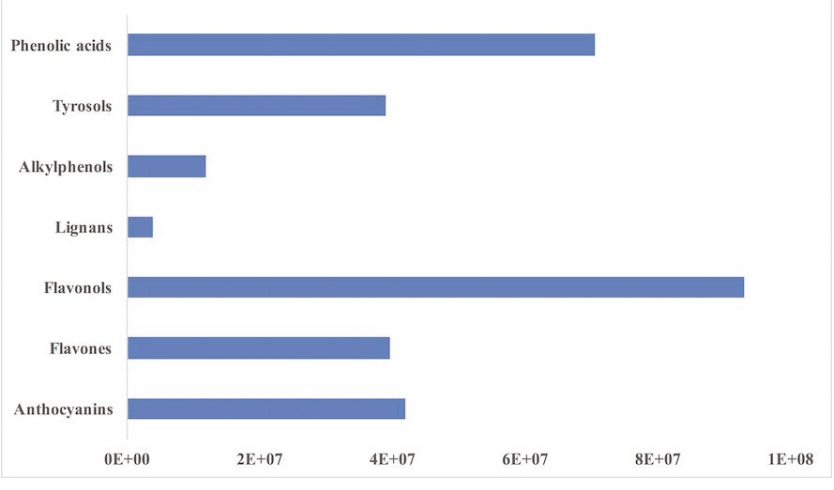

Figure 1. Abundance of different chemical classes of polyphenols in Ficodindia di San Cono PDO cladodes analysed (cumulative intensities as gained from UHPLC-ESI-QTOF-MS profiling).

Flavonols showed the highest intensity when compared to other phenolic subclasses, with approximately a difference of one order of magnitude. The entire list of phenolic compounds identified in cladodes extracts, together with annotations (raw formula, identification score, composite mass spectrum), is provided as supplementary material. Even though each compound possessed a different response factor at the electrospray ionization (ESI), the cumulative intensity of phenolic compounds in cladode extracts (when referred to semi-quantitative values) is generally in agreement with the Folin-Ciocalteu assay [24]. Nonetheless, results were elaborated and expressed as equivalents for

the main phenolic classes. In particular, as shown in Table 3, cladodes from San Cono PDO were very abundant in anthocyanins (1443.76 mg/kg cyanidin equivalents) and phenolic acids, expressed as ferulic acid equivalents, being 1453.84 mg/kg when referring to the sum of hydroxybenzoic, hydroxyphenylpropanoic and hydroxycinnamic acids. Glycosidic forms of kaempferol (a flavonol) were very abundant, being 241 mg/kg equivalents, together with alkylphenols equivalents which were strongly represented in this matrix being 65.04 mg/kg, followed by flavones equivalents (in particular glycosidic forms of apigenin). Lower values were obtained for both subclasses of lignans (furofurans and dibenzylbutyrolactones), being 6.52 and 3.47 mg/kg equivalents, respectively.

Table 3. Quantifications per classes of phenolics identified from untargeted UHPLC-ESI-QTOF-MS in Ficodindia di San Cono PDO cladodes. Results are expressed in mg/kg of phenolic equivalents. The data are presented as mean values ± standard deviation (n = 3). Glu = glucoside.

Phenolic Class	Phenolic Derivatives	mg/kg Equivalents
Flavonoids—Anthocyanins	Cyanidin-Glu	1058.55 ± 10.49
	Pelargonidin-Glu	187.97 ± 10.42
	Petunidin-Glu	186.55 ± 3.11
	Delphinidin-Glu	2.81 ± 0.31
	Malvidin-Glu	4.31 ± 0.08
Flavonoids—Flavones	Luteolin-Glu	5.14 ± 1.59
	Apigenin-Glu	40.69 ± 0.58
	Isoflavonoids	6.81 ± 0.52
Flavonoids—Flavonols	Myricetin-Glu	8.52 ± 0.55
	Quercetin-Glu	8.97 ± 0.69
	Kaempferol-Glu	241.68 ± 3.39
	Isorhamnetin-Glu	98.42 ± 8.52
Lignans	Furofurans	6.52 ± 2.24
	Dibenzylbutyrolactone	3.47 ± 0.02
Other phenolics	Alkylphenols	65.04 ± 10.43
	Hydroxybenzaldehydes	0.43 ± 0.06
	Hydroxycoumarins	7.81 ± 0.52
	Tyrosols	12.89 ± 1.02
Phenolic acids	Hydroxybenzoics	114.01 ± 3.34
	Hydroxyphenylpropanoics	91.58 ± 5.98
	Hydroxycinnamics	1248.24 ± 103.15

Looking at the great abundance in anthocyanins and phenolic acids, the health benefits of these two classes of compounds must be emphasized. In particular, anthocyanins are natural pigments in the plant kingdom, mainly responsible for the blue, purple, red, and orange colors of many fruits and vegetables. In the last year, the interest towards their absorption, metabolism, bioavailability and pharmacokinetics, has increased along with methods for their analytical determination. The interest to deepen the knowledge of the health benefits of anthocyanins has also strongly increased thanks to their anticancer, anti-inflammatory, antidiabetic, and neuroprotective activities together with prevention of cardiovascular diseases [47]. Concerning phenolic acids, they are usually divided into two classes: equivalents of benzoic acid and equivalents of cinnamic acid. The hydroxybenzoic acid content of plants is generally low when compared to hydroxycinnamic acids, with the exception of certain red fruits, black radish and onions [48]. The free form of hydroxycinnamic acids is rapidly absorbed by the small intestine after ingestion. Nevertheless, these compounds are naturally esterified in plant matrices, establishing chemical bonds with this latter, and esterification is able to reduce their absorption due to the lack of enzymes (esterases) able to hydrolyse phenolic acids in the intestinal mucosa, liver and plasma. The hydrolysis can be performed only by the microflora present into the colon; therefore, the polyphenols reaching the colon could promote an antioxidant environment after their hydrolysis

into aglycones by bacterial microflora [47]. Regarding San Cono PDO cladodes, phenolic extracts from this plant matrix could provide relevant quantities of these antioxidant and functional compounds.

Overall, present findings fitted with Astello-Garcia et al. [45], which identified phenolic compounds in cladode extracts considering *Opuntia* spp. cultivars with different domestication gradient. In particular, the phenolic profile showed major and minor compounds that were present only in wild or domesticated species. Among polyphenols, the most represented were conjugated forms of isorhamnetin, kaempferol, and quercetin, all belonging to the phenolic class of flavonoids. In another work, Guevara-Figueroa et al. [28] investigated the phenolic composition of commercial and wild leaves from *Opuntia* spp., showing that phenolic acids and flavonoids were the most represented compounds. In particular, these authors identified among phenolic acids ferulic, caffeic, gallic, coumaric, and 2-hydroxybenozoic (also known as salicylic acid) acids. Considering the flavonoids class, conjugated forms of quercetin (rutin and iso-quercitrin), kampferol-3-O-rutinoside (nicotiflorin) and two different forms of narcissin (isorhamnetin derivatives) were detected and quantified in different commercial and wild cladodes. The same findings were shown by El-Mostafa et al. [37], reviewing the possible use of cactus leaves as a source of bioactive compounds. Looking at semi-quantitative values, this work could be considered one of the first identifying anthocyanins as representative phenolic compounds in cladodes extracts from *Opuntia* spp. In particular, our findings showed that conjugated forms of cyanidin, pelargonidin and petunidin were very abundant in cladodes extracts with average values of 428.27, 161.39 and 114.08 mg/kg equivalents for cyanidin 3,5-*O*-diglucoside, pelargonidin 3-*O*-(6″-malonyl-glucoside) and petunidin 3-*O*-rutinoside, respectively (supplementary material). Finally, looking at Pearson's correlations, there were highly significant correlations between TPC and DPPH ($p < 0.01$), whilst flavones (luteolin equivalents) were well correlated to phenolic acids (ferulate equivalents) and flavanols (catechin equivalents) ($p < 0.05$). Interestingly, ferulate and luteolin equivalents were those phenolic compounds better explaining the DPPH radical scavenging activity ($p < 0.01$). However, all the correlation values recorded are shown in Table 4.

Table 4. Pearson's correlations coefficients between total phenolic content (TPC), DPPH radical scavenging, FRAP reducing power and phenolic subclasses equivalents. ** = $p < 0.01$; * = $p < 0.05$; n.s. = not significant.

	DPPH	FRAP	TPC	Cyanidin Eq.	Luteolin Eq.	Catechin Eq.	Ferulate Eq.	Matairesinol Eq.	Tyrosol Eq.	Cardol Eq.
DPPH	1	n.s.	1 **	0.84 *	0.95 **	0.92 *	0.96 **	n.s.	n.s.	0.59
FRAP	n.s.	1	n.s.	−0.74	n.s.	n.s.	n.s.	0.90	−0.84	−0.61
TPC	1 **	n.s.	1	−0.84	0.95	−0.92	0.96 **	n.s.	n.s.	0.59
Cyanidin Eq.	0.84 *	−0.74	−0.84	1	−0.64	0.56	−0.65	n.s.	n.s.	n.s.
Luteolin Eq.	0.95 **	n.s.	0.95	−0.64	1	−0.99 *	1 *	−0.45	0.56	0.80
Catechin Eq.	0.92 *	n.s.	−0.92	0.56	−0.99 *	1	−0.99	0.54	−0.64	−0.86
Ferulate Eq.	0.96 **	n.s.	0.96 **	−0.65	1 *	−0.99	1	−0.44	0.54	0.79
Matairesinol Eq.	n.s.	0.90	n.s.	n.s.	−0.45	0.54	−0.44	1	−0.99	−0.89
Tyrosol Eq.	n.s.	−0.84	n.s.	n.s.	0.56	−0.64	0.54	−0.99	1	0.94
Cardol Eq.	0.59	−0.61	0.59	n.s.	0.80	−0.86	0.79	−0.89	0.94	1

This work can be considered as the first assessing the entire phenolic profile and composition in cladodes of Ficondindia di San Cono PDO thanks to an untargeted UHPLC-ESI/QTOF mass spectrometry approach. The further study on the comparison of phenolic profile of different Opuntia species using a metabolomics-based approach (UHPLC-ESI/QTOF-MS) appears to be worthwhile, in order to select the best source of antioxidant compounds within this plant genus for industrial applications.

4. Conclusions

The demand for natural antioxidants, to be used for applications such as nutraceuticals, biopharmaceuticals, as well as food additives, is increasing due to consumers' preference. In this work, a new source of antioxidant compounds from Ficodindia di San Cono PDO cladodes extracts has been deeply investigated describing its entire phenolic profile. Very high antioxidant activity values have been observed in the hydro-alcoholic extracts of Ficodindia di San Cono cladodes. A high

correlation between total phenolic content and DPPH radical scavenging was observed ($p < 0.01$). Moreover, an elevated quantity of β-polysaccharides, included in the group of water-soluble fiber, was accounted. The UHPLC-ESI/QTOF-MS phenolic profiling allowed for the identification of the main phenolic classes and subclasses in cladode extracts, showing that they are good source of equivalent for anthocyanins and phenolic acids, followed by other phenolics. Overall, considering the results obtained, it would seem possible to use cactus cladodes as a source of natural and antioxidant compounds, possibly incorporating them into foods, cosmetics or pharmaceutical products. From a health-promoting perspective, these cladode extracts could be considered as new and very promising sources of natural antioxidants. Our findings provide a basis for developing a valuable food additive, based on *Opuntia ficus-indica* cladodes from San Cono (Sicily, Italy), thanks to their water-soluble fiber, phenolic composition and the related antioxidant activity.

Supplementary Materials: The following are available online at http://www.mdpi.com/2304-8158/7/2/24/s1. Table S1: Whole dataset of phenolic compounds identified in cladode extracts, together with relative abundances and annotations (raw formula, identification score, composite spectrum).

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A Comparative Study of Phenols in Apulian Italian Wines

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Abstract: Nutraceutics is a growing research field in which researchers study and attempt to improve the biological properties of metabolites in food. Wine is one of the most consumed products in the world and contains a plethora of molecules biologically relevant to human health. In this article, several polyphenols with potential antioxidant activity were measured in wines from Apulia, in Southeast Italy. Hydroxytyrosol, gallic and syringic acids, luteolin, quercetin, and *trans*-resveratrol were identified and quantified by HPLC. The amount of the analyzed metabolites in wines were largely dependent on their color, with red ones being the richest compared to white and rose wines. Gallic acid was the most abundant polyphenol, followed by syringic acid and luteolin. Nevertheless, significant amounts of hydroxytyrosol, quercetin, and *trans*-resveratrol were also found. The average concentration of polyphenols found in these wines could have potential health-promoting effects, especially if consumed in moderate quantities on a regular basis.

Keywords: HPLC; wine; polyphenols; antioxidants; hydroxytyrosol; *trans*-resveratrol

1. Introduction

Wine is not only one of the most ancient and consumed beverages, but it is also an important product from the nutritional point of view [1]. Among the variety of compounds which determine the overall quality of the final product, polyphenols contribute to several organoleptic properties, such as color, astringency, and flavor [2]. Furthermore, the healthy effect of polyphenols is well-known due to their antioxidant activity and contribute to a variety of biological processes, such as (a) inhibition of the oxidation of low-density lipoprotein (LDL) with a significant effect on atherosclerosis; (b) DNA protection from oxidative stress, with important consequences on the development of some age-related cancers; (c) protection of the vascular system by strengthening capillaries which carry oxygen and other essential nutrients to cells; (d) anti-thrombotic and anti-inflammatory effects; and (e) anti-microbial activity against viruses, bacteria, and hepatotoxins [3–6]. Polyphenols are produced by the shikimic pathway [7]. However, this path can be found in fungi and bacteria, but it is absent in animals; that is why humans have to introduce phenolic products and aromatic amino acids they need through diet. Phenolic compounds represent, after carbohydrates and acids, the most abundant species in grapes. Nevertheless, the amount and type of polyphenols in wine vary considerably depending on the grape type, environmental factors, production techniques, and conditions [8,9]. The total amount of polyphenols in red wine is usually about 1.5 g/L (80%–90% of which are flavonoids) [6]. Rose wine

contains about 400–800 mg/L of polyphenols (40%–60% of which are flavonoids); white wine has only 100–400 mg/L. Depending on the molecular structure and the number of carbon atoms, polyphenols can be roughly grouped into flavonoids, non-flavonoids, and hydroxystilbenes [1]. Flavonoids are found in the peel, seed, and petiole, while non-flavonoid compounds, such as hydroxybenzoic and cinnamic acid derivatives, can be found especially in the pulp. Gallic and syringic acids belong to the hydroxybenzoic acid class and have been shown to induce significant effects on neurodegenerative pathologies, such as Parkinson's and Alzheimer's diseases [10–12]. Quercetin, part of a subclass of flavonoids called flavonols, has received considerable attention because of its overwhelming presence in foods and its antioxidant activity and is believed to protect against several degenerative diseases by preventing lipid peroxidation [13]. Luteolin, another flavonoid derivative of the flavone subclass, was shown to possess a variety of pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial, and anticancer activities [14]. Hydroxytyrosol is one of the most powerful antioxidants in nature, and extensive research has been carried out trying to exploit its therapeutic effects on the cardiovascular system, neurodegenerative diseases, cancer treatment, osteoporosis, and diabetic neuropathy, to name a few [15–20]. The stilbene *trans*-resveratrol has been one of the most extensively studied non-flavonoids due to its well-known health benefits, such as cardioprotective and chemopreventive effects (by inhibiting LDL oxidation) and anti-inflammatory, antibacterial, antifungal, antiviral, neuroprotective, antiproliferative, and anti-angiogenic activities [21–25].

In this article, we measured the above-mentioned polyphenols in several red, white, and rose wines from Apulia and the results were associated with grape type. To the best of our knowledge, few characterization data have been reported in the literature regarding Apulian wines. In pioneering work from the groups of Fanizzi and Sacco, the authors determined several analytes by chemical, chromatographic, and ^{1}H NMR techniques in red wines and then managed to identify the origin of the unknown samples by multivariate statistical analysis [26–28]. Later, Crupi and colleagues identified and quantified several carotenoid and flavonoid compounds in red and white seedless grape varieties [29–31]; Siciliano and co-workers analyzed the volatile components of Negroamaro and Primitivo Apulian red wines [32,33]. Very recently, Barnaba et al. determined the profile of 61 phenols in two of the most common red grapes from Apulia, Primitivo di Manduria and Negroamaro [34]. Nevertheless, to date, quantification and comparison of some of these and other polyphenols in a much wider variety of local red, white, and rose wines has not been reported in the literature.

2. Materials and Methods

2.1. Chemicals

HPLC-grade reference standard of *trans*-resveratrol was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Analytical grade gallic acid, hydroxytyrosol, luteolin, quercetin, and syringic acid were purchased from Sigma-Aldrich (Milan, Italy). Acetic acid and HPLC-grade ethanol and methanol were purchased from J.T. Baker (Deventer, The Netherlands). HPLC-grade water was purchased from Carlo Erba Reagenti (Milan, Italy).

2.2. Samples

Analysis was conducted on a total of 72 commercially available wine bottles (27 red, 23 rose, and 22 white wines) from Apulia, a region in Southeast Italy (Figure 1). The most characteristic *Vitis vinifera* red grape (Negramaro, Primitivo, and Susumaniello), rose grape (Negramaro, Black Malvasia, and Primitivo), white grape (Bianco d'Alessano, Chardonnay, Falanghina, Fiano, Malvasia, Moscato, and Verdeca), and some combination of these varieties (blend samples) were studied. Details of number of samples, production year, and alcoholic grade for each grape type are given in Table 1. All samples had a denomination of origin (DOC) certification mark, which guarantees the quality and the geographical origin of the wines produced in the Apulia region in Italy.

Figure 1. Map of Italy with a zoom on a southern Apulia region (Salento, in red). The black dots represent the sites of production of the wines.

2.3. HPLC Analysis

Separation and identification of phenolic compounds were carried out using an HPLC 1220 Infinity (Agilent Technologies, Palo Alto, CA, USA) interfaced with a diode array detector (model G1315B DAD system; Agilent). A calibration curve was prepared using a standard solution at increasing concentrations of the analytes in ethanol. Fitting of the data was performed through a linear equation with zero intercept ($R^2 > 0.98$ for all samples). The concentration in mg/kg of the biomolecules in the analyzed samples was calculated through interpolation of the corresponding peak areas. Samples were collected directly from the bottle by a syringe injection through the cork cap. After filtration through a 0.45 µm pore size regenerated cellulose filters (VWR International, Milano, Italy), wine samples were directly injected into an Eclipse Plus C18 (particle size 5 µm; 4.6 × 250 mm, Agilent) stationary phase column. For the detection of *trans*-resveratrol, quercetin, and luteolin, a gradient system of water/methanol/acetic acid (75:20:5, *v:v:v*) (Solvent A) and water/methanol/acetic acid (50:45:5, *v:v:v*) (Solvent B) was used. The gradient parameters were: 0% B at 0 min, 100% B at 30 min, 0% B at 50 min. The solvent flow was maintained at 1.0 mL/min, the column temperature was set 25 °C, and the UV-Vis detection wavelength was set at 309 nm. Obtained retention times were: *trans*-resveratrol at Min 24.8, quercetin at Min 34.8, and luteolin at Min 42.3. For the detection of gallic acid, hydroxytyrosol, and syringic acid a gradient system of water/methanol/acetic acid (75:20:5, *v:v:v*) (Solvent A) and water/methanol/acetic acid (50:45:5, *v:v:v*) (Solvent B) was used. The gradient parameters were as follows: 0% B at 0 min, 100% B at 40 min, and 0% B at 50 min. The flow was maintained at 1.0 mL/min, the column temperature was set 25 °C, and the UV-Vis detection wavelength was set at 280 nm. Obtained retention times were as follows: gallic acid at Min 3.3, hydroxytyrosol at Min 4.3, and syringic acid at Min 10.1 (The original raw data are available from the Multilab—Chamber of Commerce of Lecce—upon request at multilab@le.camcom.it.).

Table 1. Characteristics and content of phenols in wines organized by type of grape.

Wine Color	Grape Type	*n* of Samples	Year [1]	Alcoholic Grade [2]	Gallic Acid	Syringic Acid	Hydroxytyrosol	Luteolin	Quercetin	*trans*-Resveratrol
					Content in mg/kg [2]					
Red	Blend	4	2010–2013	13.5 ± 0.4	23.7 ± 4.0	7.6 ± 4.2	2.9 ± 1.0	5.7 ± 3.2	5.8 ± 1.3	2.9 ± 0.3
	Negroamaro	16	2007–2013	13.6 ± 0.7	26.8 ± 7.4	7.2 ± 3.4	3.4 ± 1.8	6.3 ± 3.4	5.5 ± 2.7	2.8 ± 0.7
	Primitivo	5	2011–2012	13.6 ± 0.2	24.9 ± 7.0	7.8 ± 4.5	2.5 ± 1.1	6.0 ± 3.2	4.8 ± 3.4	2.8 ± 0.9
	Susumaniello	2	2012	13.3 ± 0.4	28.0 ± 2.2	5.2 ± 3.3	2.7 ± 2.1	7.2 ± 2.4	5.0 ± 1.4	4.3 ± 1.5
White	Bianco d'Alessano	1	2013	12.0	2.7	2.0	0.9	1.1	ND [3]	0.2
	Blend	4	2012–2013	12.6 ± 1.5	4.5 ± 1.1	2.2 ± 0.6	1.3 ± 0.9	0.8 ± 0.5	0.0 ± 0.0	0.2 ± 0.1
	Chardonnay	9	2012–2013	13.1 ± 0.8	5.5 ± 1.4	2.2 ± 1.7	2.3 ± 1.3	1.3 ± 0.7	1.2 ± 1.8	0.2 ± 0.1
	Falanghina	1	2013	12.5	5.3	2.3	0.8	0.7	ND	0.3
	Fiano	3	2013	13.0 ± 1.0	8.1 ± 4.1	1.8 ± 0.3	1.1 ± 0.3	0.6 ± 0.3	0.3 ± 0.6	0.1 ± 0.1
	Malvasia	1	2013	13.0	4.5	3.6	0.6	1.1	1.0	0.3
	Moscato	1	2012	14.5	12.5	2.0	0.2	4.5	1.0	0.3
	Negroamaro	1	2013	12.5	3.0	2.8	0.6	0.6	ND	0.1
	Verdecca	1	2013	12.5	5.5	2.7	0.4	0.5	ND	0.5
Rose	Negroamaro	18	2011–2013	12.9 ± 0.5	5.9 ± 2.1	2.7 ± 1.4	1.2 ± 0.8	2.3 ± 2.2	0.6 ± 0.8	0.6 ± 0.2
	Negroamaro and Malvasia Nera	4	2013	12.9 ± 0.6	7.4 ± 3.7	2.5 ± 0.7	1.1 ± 0.4	2.4 ± 1.6	1.6 ± 1.8	0.8 ± 0.4
	Primitivo	1	2013	13.5	5.5	3.6	1.1	1.8	ND	0.6

[1] Production year and alcoholic grade as reported on the bottle. [2] Values are expressed as mean ± standard deviation relatively to the different wine bottles analyzed. In some cases only one bottle per grape type was available and no standard deviation was reported. [3] Not detected, below the limit of detection.

2.4. Statistical Analysis

The amounts of polyphenols reported represent the mean values for a specific wine color and grape type. The reported standard deviation represents the difference among different samples from the same category. If just one sample for a specific grape type was available, no standard deviation has been reported. Standard deviation relatively to replicates of the same sample was always <5%. Obtained polyphenolic values were rounded to one decimal place. Multivariate statistical analysis was performed using STATISTICA 12.5 software (State-Ease Inc., Minneapolis, MN, USA). Principal component analysis (PCA) was performed using the detected polyphenols as variables (n = 6) and the wine samples as cases (n = 72), yielding a matrix of 432 data points. The analysis was based on correlation and the variances were computed as $SS/(N-1)$, where SS is the Sum of the Squares and N is the number of items in the list. In the 2D score plot, cases with sum of cosine$^2 \geq 0$ were reported. General discriminant analysis (GDA) was performed using the type of grape as a dependent variable, the wine color as a categorical predictor variable, and the analyzed polyphenols as continuous predictors.

3. Results and Discussion

The content of several polyphenols, namely gallic acid, hydroxytyrosol, luteolin, quercetin, *trans*-resveratrol, and syringic acid, was quantified in different types of commercially available Apulian wines, in Southeast Italy (Figure 1), and the results are shown in Figure 2. The data represent the mean values of the detected polyphenols grouped for wine color. In general, standard deviations are quite high for each data series because of the variability of the samples. In fact, each sample represents a commercially available bottle of a different brand, and even though some of them have the same grape composition, the high variability of all the production parameters led to a quite broad range of data. Nevertheless, all wines were prepared from grapes grown locally and from pure autochthonous varieties, although they were mixed in blend wines, as detailed later in the text. Average production year was about 2012 for red wines and about 2013 for white and rose wines. As expected, alcoholic grade was slightly higher for red wines (average 13.6% $\pm$ 0.6%), while white and rose ones presented almost the same value (about 13%).

All the 72 commercially available samples (27 red, 22 white, and 23 rose wine bottles) showed significant amounts of the investigated polyphenols. As expected, red wines contained the highest concentrations, while white and rose wines had less, with rose wines generally presenting slightly higher values compared to white ones.

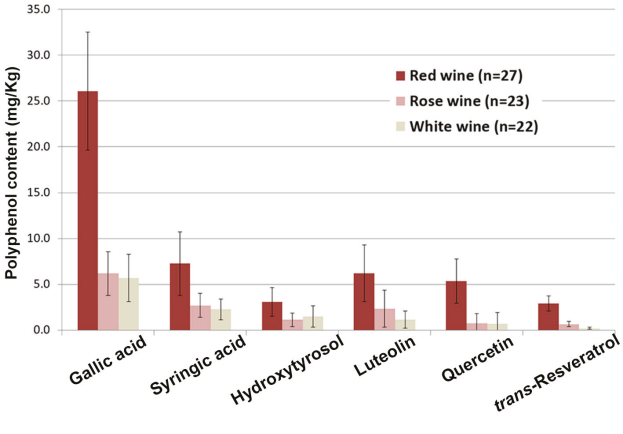

Figure 2. Histogram of the analyzed phenols grouped for wine color.

The results clearly indicate that gallic acid is the most abundant polyphenol in all wines, although there is quite a large difference when comparing their color. In fact, red wines averaged 26 mg/kg, about four times the quantity found in white and rose ones (around 6 mg/kg in both varieties). Considerable amounts of syringic acid, luteolin, and quercetin were also found in the red varieties (about 7, 6, and 5 mg/kg, respectively). Lower quantities of the same phenols were detected in white and rose wines (about 2–3 mg/kg of syringic acid and about 1–2 mg/kg of luteolin for white and rose wines, respectively). Finally, red wines also contained good amounts of hydroxytyrosol and *trans*-resveratrol (about 3 mg/kg each). On the other hand, half that amount of hydroxytyrosol was found in white and rose wines, and even less of *trans*-resveratrol. The results of a more detailed analysis of the quantified polyphenols grouped by type of grape are shown in Table 1.

3.1. Red Wines

A total of 27 red wines were analyzed, most of which were Negroamaro (16 samples), followed by Primitivo, blend, and Susumaniello grapes (5, 4, and 2 samples, respectively). The most abundant polyphenol observed was found to be gallic acid (between about 24 and 28 mg/kg in all samples). Lower amounts of syringic acid, luteolin, and quercetin were detected in all wines, with averages of about 7, 6, and 5 mg/kg, respectively (see Table 1 for details). The highest content of hydroxytyrosol was found in Negroamaro grapes, with an average content of 3.4 ± 1.8 mg/kg. Soon after, blend wines were shown to have 2.9 ± 1.0 mg/kg of hydroxytyrosol, not surprising if we consider that these wines were obtained mainly from Negroamaro grapes (percentages from 50% to 80%), and lower amounts were obtained from Malvasia Nera, Primitivo, and Monte Pulciano grapes. Similar concentrations were observed in Susumaniello and Primitivo grapes (about 2–3 mg/kg).

Finally, Susumaniello wines had the highest concentration of *trans*-resveratrol (4.3 ± 1.5 mg/kg), while Negroamaro, Primitivo, and blend wines averaged about 3 mg/kg, respectable amounts considering the antioxidant strength of this molecule. The results found are in line with values reported in the literature for this grape variety, although Barnaba and colleagues very recently found somewhat higher concentrations of gallic acid and slightly lower ones of hydroxytyrosol in Negroamaro and Primitivo wines from Apulia [34]. Nevertheless, these variations might be related to different cultivation areas and different production years and parameters.

3.2. White Wines

White wines were the ones with most grape varieties, with 8 monocultivar samples and 4 blends, mainly composed of different ratios of Malvasia Bianca with Chardonnay, Verdeca, and Trebbiano grapes. Surprisingly, Chardonnay yielded quite high values of hydroxytyrosol (2.3 ± 1.3 mg/kg), almost comparable to those found in red wines. Much lower amounts were found in Fiano, Bianco d'Alessano, Falanghina, Malvasia, and Negroamaro (from about 1.1 to 0.6 mg/kg). Blend wines contained about 1.3 ± 0.9 mg/kg, quite high values although quite variable too, probably because of the different type of grapes and the different mixing percentages used. Gallic acid was the most abundant analyte found. In particular, Moscato grapes yielded 12.5 mg/kg of gallic acid and Fiano about 8 mg/kg, while the remaining varieties yielded between about 3 and 6 mg/kg. Most notably, Moscato grapes were also shown to have the highest alcoholic grade (14.5%), quite high compared to average values in the analyzed white wines (between 12% and 13%). Malvasia grapes yielded the highest content of syringic acid (about 4 mg/kg), although not much higher than the other wines (about 2–3 mg/kg). Even smaller amounts of luteolin were generally detected (about 1 mg/kg if we exclude the only Moscato sample with 4.5 mg/kg). Verdecca showed to have the highest value of *trans*-resveratrol (0.5 mg/kg) among white wines, while the other grapes yielded about 0.1–0.3 mg/kg. Quercetin was detected only in Chardonnay, Malvasia, and Moscato (about 1 mg/kg), while it was present only in traces of the other white grapes.

3.3. Rose Wines

Most rose wines were composed of Negroamaro grape, with 18 monocultivar samples and 4 blends in which it was mixed in different percentages with Malvasia Nera. One sample of Primitivo was also present among rose wines. Nevertheless, all rose wines yielded similar results. Gallic acid was again the most abundant polyphenol found (about 6–7 mg/kg). Much smaller amounts of syringic acid were obtained (about 3 mg/kg), and even less of luteolin and hydroxytyrosol (about 2 and 1 mg/kg, respectively). Rose wines also contained higher values of trans-resveratrol compared to white wines (about 0.6–0.8 mg/kg in all samples), although still about a fourth of the average concentration found in red ones.

3.4. Statistical Multivariate Analysis

Principal component analysis (PCA) was performed using polyphenols data as active variables and wine samples as active cases. Two PCs were extracted: the first one describing 67.1% and the second one 13.4% of the sample variability, respectively. The first two PCs account for 80.5% of the total sample variability. At first glance, it can be seen from the scores plot that red samples spread from about −1 to −4 of PC1, while covering the entire PC2 axis (Figure 3a).

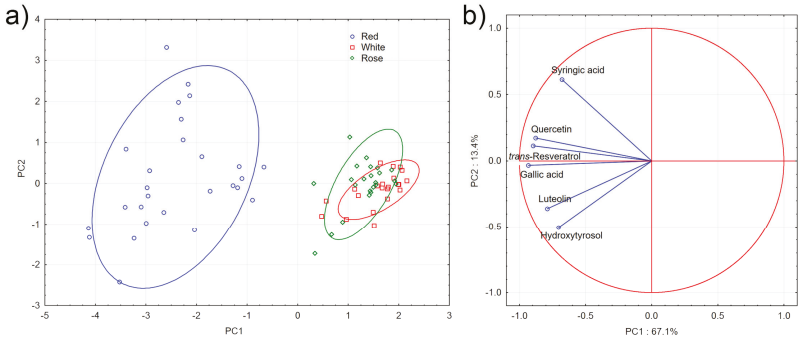

Figure 3. (**a**) Principal component analysis (PCA) scoreplot (PC1 vs. PC2) for red (blue circles), white (red squares), and rose (green diamonds) wines. Ellipses represent the density space of each group with a correlation coefficient of 0.95. (**b**) Loadings plot of PC1 vs. PC2.

By plotting the loadings of the variables, it can be seen that gallic acid, *trans*-resveratrol, and quercetin contribute the most to PC1, although not much difference was noted with respect to the contribution (on PC1) of the other variables. Syringic acid, hydroxytyrosol, and luteolin was shown to have a major influence on PC2 (Figure 3b). On the other hand, white and rose wines form two significantly merging groups with positive values along PC1 and average neutral ones along PC2, confirming that these wines in general contain much lower amounts of the detected polyphenols compared to red ones (Figure 3a). The two groups overlap significantly although minor differences can be noted through their density space, drawn by an ellipse with a correlation coefficient of 0.95. The three samples corresponding to the markers at the bottom of the rose wines' ellipse represent three Negroamaro characterized by a higher hydroxytyrosol concentration. The sample on top of the rose wines' ellipse corresponds to a Negroamaro sample with a higher concentration of syringic acid. White wines showed even lower variability than rose wines, meaning all samples were quite close to each other in the PCA score plot.

A further investigation was performed using a supervised general discriminant analysis (GDA) in order to determine the variables, allowing a better discrimination between rose and white wines. Complete separation between the two groups was obtained, as can be seen in the scatterplot of the correlation scores for Factors 1 and 2 in Figure 4a. *trans*-Resveratrol and, to a minor extent, luteolin

was shown to contribute the most to Factor 1, while gallic acid and hydroxytyrosol the most to Factor 2, as suggested by the respective coefficients. Nonetheless, appreciable separation of white and rose wines had already been noticed during the PCA in the scatter matrix plots for all the pairs of variables, in particular when plotting *trans*-resveratrol versus the other variables (data not shown). The discriminatory power of the model toward the two groups was also observed in the Mahalanobis distances scatter plot, as shown in Figure 4b.

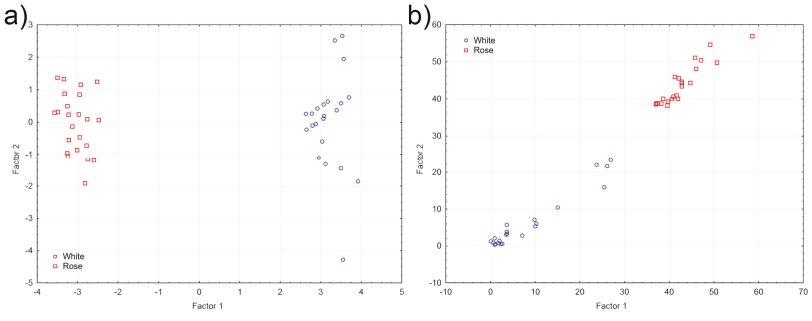

Figure 4. (**a**) GDA scoreplot; (**b**) Mahalanobis distances scatter plot for white (blue circles) and rose (red squares) wines.

4. Conclusions

Quantification of biomolecules with nutraceutical properties is of paramount importance, especially nowadays that food quality is being considered more and more. Wine is an aliment with important antioxidant activity due to the presence of a variety of biomolecules, some of which were here characterized by means of HPLC analysis in several commercially available red, white, and rose wines from Apulia, in Southeast Italy. Red grapes were shown to yield the healthiest wines, at least based on the analyzed polyphenols. Gallic acid was found to be the most abundant polyphenol. Nevertheless, red wines also contained high amounts of hydroxytyrosol, especially in those with Negroamaro grapes. High concentrations of hydroxytyrosol were also found in Chardonnay white wines, while the other white and rose grapes analyzed generally contained half those values or even less. Red grapes also produced significant amounts of syringic acid, luteolin, and quercetin. Much lower values of these polyphenols were found in white and rose wines. Red wines yielded good concentrations of *trans*-resveratrol, about 10 times those found in white wines, while rose wines showed an almost intermediate behavior. In general, the amounts of polyphenols found in this study are much lower compared to values used in research studies for curing, e.g., cardiovascular or cancer diseases; nevertheless, ingesting similar amounts of these biomolecules (especially *trans*-resveratrol) on a regular basis is at the origin of the French paradox assumption [35–37]. The results here reported were part of a study aiming at characterizing the nutraceutical properties of local Apulian wines, but they can be also exploited to modify the production parameters of these wines to improve their already beneficial properties. Furthermore, these data can be used to determine a metabolic profile of these grapes and can be exploited, in combination with other parameters, to guarantee their traceability and safety when analyzed by multivariate statistical analysis [26–28].

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Composition and Statistical Analysis of Biophenols in Apulian Italian EVOOs

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Abstract: Extra-virgin olive oil (EVOO) is among the basic constituents of the Mediterranean diet. Its nutraceutical properties are due mainly, but not only, to a plethora of molecules with antioxidant activity known as biophenols. In this article, several biophenols were measured in EVOOs from South Apulia, Italy. Hydroxytyrosol, tyrosol and their conjugated structures to elenolic acid in different forms were identified and quantified by high performance liquid chromatography (HPLC) together with lignans, luteolin and α-tocopherol. The concentration of the analyzed metabolites was quite high in all the *cultivars* studied, but it was still possible to discriminate them through multivariate statistical analysis (MVA). Furthermore, principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA) were also exploited for determining variances among samples depending on the interval time between harvesting and milling, on the age of the olive trees, and on the area where the olive trees were grown.

Keywords: HPLC; EVOO; biophenols; antioxidants; hydroxytyrosol; oleuropein; multivariate statistical analysis; OPLS-DA

1. Introduction

The nutritional and health-promoting properties of extra-virgin olive oils (EVOOs) are well-known and they have been extensively studied over recent decades. In particular, it has been demonstrated that phenolic derivatives in olive oils, also known as biophenols, have strong antioxidant activity which, apart from being responsible of the product shelf life, it also confers on EVOOs anti-inflammatory, chemopreventive, and anti-cancer effects, among others, yielding a product with very important nutraceutical properties [1].

Antioxidants in EVOOs can be mainly grouped in two categories depending on the solubility of the phenolic compounds in either organic or aqueous solvents [2]. Lipophilic phenols in EVOOs are mainly constituted by tocopherols, and in particular, their α-homologue, the most biologically active form of vitamin E, which represents over 90% of their total composition [3]. On the other hand, lignans and secoiridoids represent the mayor constituents of hydrophilic phenols in EVOOs, with the latter being only found in plants belonging to the family of *Oleaceae*. Among secoiridoids, *o*-diphenols, such as 3,4-DHPEA and its derivatives 3,4-DHPEA-EDA and 3,4-DHPEA-EA, were reported to have a higher antioxidant activity compared to *p*-HPEA and α-tocopherol (see Figure 1 for names and structures of these compounds) [4]. Nevertheless, all these molecules, together with other minor

compounds, such as carotenoids, and thanks to a well-balanced unsaturated-to-saturated fatty acid ratio, contribute to the high quality of EVOOs and its fundamental role in the Mediterranean diet.

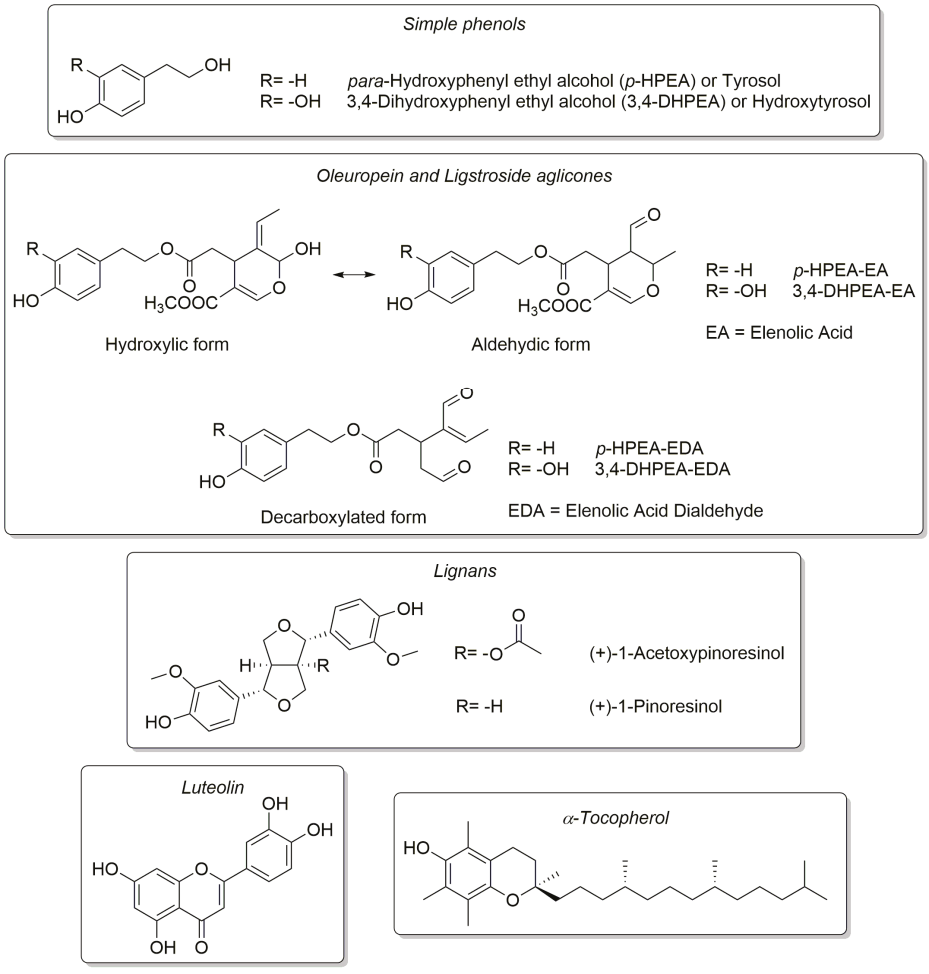

Figure 1. Chemical structures of the antioxidant molecules studied.

EVOOs from Apulia have been already studied in quite significant detail over recent years, also because they represent almost 40% of the total Italian production [5]. However, most of the studies examine *cultivar* from the central area of Apulia and only few investigate the southern part, that is, Salento. Back in 1999, Caponio et al., studied the phenolic composition of Ogliarola and Coratina EVOOs and correlated its variation to different technological variables [6]. They later investigated the influence of decanter working parameters on the quality of the oils [7]. Longobardi and colleagues classified Apulian olive oils based on their chemical composition, i.e., free acidity, peroxide value, spectrophotometric indexes, chlorophyll content, sterol, fatty acid, and triacylglycerol, and on the NMR profile combined with multivariate statistical analysis [8,9]. Gambacorta et al., also inspected the phenolic composition and the antioxidant activity of Collina di Brindisi oils and found out that the maturity degree of olives had a major influence on the phenol composition, followed by the extraction

system and the place of growth [10]. Over recent years Fanizzi and colleagues explored, in detail, the use of nuclear magnetic resonance (NMR) and used multivariate statistical analysis (MVA) to investigate various aspects of EVOOs from Apulia. They managed to correlate NMR spectra to genetic profile of the *cultivars*, to the characteristics of the soil where the trees were grown, to the age of the olive trees, as well as the production parameters employed and the weather influence over the years [11–14]. The NMR-based metabolomics was exploited to generate a comparable data set for fingerprinting olive oil *cultivars* from Apulia, thus allowing identification and traceability of commercial EVOOs [5,15–18]. They also applied the same technique to EVOOs from Salento, in particular to Ogliarola, Cellina, and blend samples [19]. Nevertheless, a detailed study regarding the phenolic composition in EVOOs from Salento has not been reported so far despite the commercial importance of these products.

We already reported the phenolic profile of several grape types from Salento and we now extend this study, by examining similar antioxidant molecules in local olive oils [20]. In this article, the total amount of biophenols, as well as the concentration of hydroxytyrosol and tyrosol and their derivatives conjugated to elenolic acid, lignans, luteolin, and α-tocopherol were quantified in several monovarietal *cultivars* and blend EVOOs. Furthermore, their concentrations were correlated to the type of *cultivar* and other agronomical parameters, such as the age of the trees and the areas they were located, as well as more technical variables, such as the interval time between harvesting and milling.

2. Materials and Methods

2.1. Chemicals

High performance liquid chromatography (HPLC)-grade *orto*-phosphoric acid (85% *v*/*v*) and reference standard of tyrosol, hydroxytyrosol, syringic acid, luteolin, and α-tocopherol were purchased from Sigma-Aldrich (Milan, Italy). HPLC-grade methanol, acetonitrile, isopropanol, and hexane were purchased from J.T. Baker (Deventer, The Netherlands). HPLC-grade water was purchased from Carlo Erba Reagenti (Milan, Italy).

2.2. Samples

Analysis was conducted on a total of 68 EVOO samples collected and produced between October and December 2013 by enterprises from Salento, a geographic region at the southern end of the region of Apulia, in South Italy (see Figure 2 for details). All samples were produced from olive trees grown locally and representing typical *cultivars* from Apulia, such as Ogliarola salentina (n = 25), Cellina di Nardò (n = 20), Leccino (n = 4), Frantoio (n = 2), Picholine (n = 2), and Cima di Melfi (n = 1). Furthermore, 14 blend samples, 9 of which composed of mixtures of Ogliarola and Cellina in different ratios (from 30 to 70%), and the other 5 same as the former with also a minor percentage (from 10 to 20%) of either Leccino, Frantoio, or Coratina, were also analyzed. The Protected Designation of Origin (PDO) *cultivars* were certified by the Chamber of Commerce of Lecce (IT), the competent Public Authority Control, while the non-PDO *cultivars* were assessed and certified based on the growers' declaration. Other technical info and production details were provided by the farmers and are reported in Table 1. When the sum of the number of samples in each category is lower to the total number of samples it means that not all the producers provided the corresponding information. All producers employed the same technical details additionally provided, such as the use of continuous crushers, malaxation at low temperatures (about 25 °C), and two-phases decanters, and as such they were not treated as variables. Samples were stored directly in dark glass bottles soon after production, and were kept as such in a cool, dark place until analyzed. All analyses were completed within 2014.

Figure 2. Map of Italy with a zoom on a southern Apulia region (Salento, in red). The colored shapes represent the sites of production of the extra-virgin olive oils (EVOOs) categorized according to their proximity either to the Adriatic (green circles) or Ionian Sea (red triangles), or to neither of them (blue squares).

Table 1. Technical info and production details about EVOO samples organized by type of *cultivar*. [1]

Cultivar	*n* of Samples	PDO "Terra d'Otranto" Certification [2]		Cultivation Area [3]			Interval Time between Harvesting and Milling (h) [4]		Age of Olive Trees (year)	
		Yes	No	Ionian	Center	Adriatic	≤12	>12	≤100	>100
Ogliarola salentina	25	19	2	10	13	2	25	-	2	15
Cellina di Nardò	20	17	1	5	8	6	13	7	7	11
Leccino	4	-	-	2	2	-	3	1	1	-
Picholine	2	-	-	2	-	-	2	-	-	-
Frantoio	2	-	-	2	-	-	2	-	-	-
Cima di Melfi	1	-	-	1	-	-	1	-	-	-
Blend	14	8	6	5	5	2	9	4	2	7
Total	68	44	9	27	28	10	55	12	12	33

[1] Extra-virgin olive oils (EVOOs) data were provided by the farmers. When the sum of the number of samples for each category is lower than the total it means that not all the producers provided the corresponding information. [2] Protected Designation of Origin (PDO) "Terra d'Otranto" certification is valid only for EVOOs from Ogliarola salentina and Cellina di Nardò *cultivars*, present alone or in combination, in varying percentages and not less than 60%. The remaining 40% can consist of other minor *cultivars* present in the olive grove of the production area [21]. [3] Location of the olive trees according to their proximity either to the Ionian or Adriatic Sea, or to neither of them. [4] Samples in the ">12" category were milled between 12 and 24 h from harvesting.

2.3. HPLC Analysis

Separation and identification of phenolic compounds were carried out using an HPLC 1220 Infinity (Agilent Technologies, Palo Alto, CA, USA) equipped with an Eclipse Plus C18 (particle size 5 µm; 4.6 × 250 mm, Agilent) stationary phase column interfaced with a diode array detector (model G1315B DAD system; Agilent).

2.3.1. Analysis of the Phenolic Compounds

Extraction and quantification of the phenolic compounds was carried out following an official methodology from the International Olive Council, see the original document for details and a representative spectrum [22]. Tyrosol and syringic acid in a methanol/water 80/20 (*v*/*v*) solution at a

concentration of 0.030 and 0.015 mg/mL, respectively, were used as external standards. Syringic acid in a methanol/water 80/20 (*v*/*v*) solution at a concentration of 0.015 mg/mL was used as internal standard. The concentration of the biomolecules in the analyzed samples was expressed as mg/kg of tyrosol.

Before analysis, 2.0 g of olive oil were put into a 10 mL vial and 1 mL of the internal standard solution was added. The vial was shaken on an orbital shaker (New Brunswick Innova 2050, Eppendorf, Germany) for 30 s and then 5 mL of a methanol/water 80/20 (*v*/*v*) solution were added. The sample was shaken for 1 min and then sonicated for 15 min at room temperature. The sample was centrifuged (Eppendorf 5804R Centrifuge, Eppendorf, Germany) at 5000 rpm for 25 min and an aliquot was extracted from the upper layer and filtered over polyvinylidene fluoride (PVDF) membrane filters with 0.45 μm pore size (VWR International, Milano, Italy). The solution obtained was ready for HPLC analysis.

A ternary gradient system with solvents (A) 0.2% H_3PO_4/H_2O (*v*/*v*), (B) methanol, and (C) acetonitrile was used. The gradient parameters were: 96% (A), 2% (B), 2% (C) at 0 min; 50% (A), 25% (B), 25% (C) at 40 min; 40% (A), 30% (B), 30% (C) at 45 min; 0% (A), 50% (B), 50% (C) at 60 min; 0% (A), 50% (B), 50% (C) at 70 min; 96% (A), 2% (B), 2% (C) at 72 min; 96% (A), 2% (B), 2% (C) at 82 min. The solvent flow was maintained at 1.0 mL/min, the column temperature was set 25 °C, and the ultraviolet-visible (UV-Vis) detection wavelength was set at 280 nm. The ratio of the response factors between syringic acid and tyrosol, $RRF_{syr/tyr}$, was calculated before sample analyses to be sure that it was in the range 5.1 ± 0.4. The concentration of the phenol *X* was calculated according to the equation:

$$X\ (\mathrm{mg/kg}) = ((\mathrm{A}_x) \times 1000 \times RRF_{syr/tyr} \times \mathrm{P}_{syr})/(\mathrm{A}_{syr} \times \mathrm{P}_{oil}),$$

where A_x is the area of the corresponding phenol *X*, P_{syr} is the weight in mg of the syringic acid utilized as internal standard, A_{syr} is the area of the peak of syringic acid, and P_{oil} is the exact weighted amount of oil utilized for the extraction. Obtained retention time for the identified phenols were: hydroxytyrosol, min 10.6; tyrosol, min 15.1; syringic acid, min 20.6; 3,4-DHPEA-EDA, min 31.7; 3,4-DHPEA-EA, min 34.4; *p*-HPEA-EDA, min 36.6; lignans, min 37.8; *p*-HPEA-EA, min 39.8; luteolin, min 40.9; 3,4-DHPEA-EA aldehydic and hydroxylic isomer, min 54.8; *p*-HPEA-EA aldehydic and hydroxylic isomer, min 57.7. The total amount of biophenols was calculated by integrating all the peaks in the spectrum in the range 9–59 min, i.e., those of the identified phenols plus other unidentified. The original raw data are available from the Multilab–Chamber of Commerce of Lecce—upon request at multilab@le.camcom.it.

2.3.2. Analysis of α-Tocopherol

Before analysis, 0.1 g of olive oil was put into a 10 mL vial and 5 mL of methanol were added. The vial was shaken on an orbital shaker for 2 min and then centrifuged at 3000 rpm for 5 min. An aliquot was extracted from the upper layer and filtered over PVDF filters with 0.45 μm pore size (VWR International, Milano, Italy). The solution obtained was ready for HPLC analysis.

A calibration curve was prepared using standard solutions at increasing concentration of α-tocopherol in methanol and recording the corresponding peak area. Fitting of the data was performed through a linear equation with zero intercept ($R^2 > 0.99$). The concentration in mg/kg of α-tocopherol in the analyzed sample was then calculated through interpolation of the corresponding peak area.

A binary gradient system with solvents (A) 0.5% isopropanol/hexane (*v*/*v*), (B) 10% isopropanol/hexane was used. The gradient parameters were: 100% (A), 0% (B) at 0 min; 100% (A), 0% (B) at 4 min; 60% (A), 40% (B) at 14 min; 40% (A), 60% (B) at 18 min; 100% (A), 0% (B) at 21 min; 100% (A), 0% (B) at 25 min. The solvent flow was maintained at 1.0 mL/min, the column temperature was set 25 °C, and the UV-Vis detection wavelength was set at 288 nm. Obtained retention time was 11.2 min.

2.4. Statistical Analysis

The amounts of phenols reported represent the mean values for a specific type of *cultivar*. The reported standard deviation represents the difference among different samples from the same

category. When just one sample for a specific type of *cultivar* was available, no standard deviation has been reported. Standard deviation relatively to replicates of the same sample was always <5%. Obtained values were rounded to one decimal place. Statistical analysis was performed using SIMCA 14.1 software (MKS Umetrics, Malmö, Sweden). Principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA) were performed using the detected biophenols, their total amount, and α-tocopherol as variables (n = 12) while the qualitative information, such as *cultivar*, interval time between harvesting and milling, age of the trees, location of the trees, and PDO certification, as classes according to the data reported in Table 1. Strong outliers were removed from the computation to obtain a better fit. R^2X(cum) and R^2Y(cum) are the cumulative Sum of Squares (SS) of the variation of the X or Y variables, respectively, explained by the extracted components of the model. Q^2(cum) is the cumulative variation of the X and Y variables predicted by the extracted components of the model. R^2X(cum) and R^2Y(cum) are parameters utilized for describing the goodness of the fit and their values are always between 0 and 1, the higher the better. Q^2(cum) is a parameter used to describe the predictive ability of the model and its value is usually between 0 and 1, the higher the better.

3. Results and Discussion

The content of several biophenols and their total amount was quantified in several EVOOs from Salento, in Southeast Italy (Figure 2). All EVOOs were prepared from olive trees grown locally from monovarietal species of Ogliarola, Cellina, Leccino, Picholine, Frantoio, and Cima di Melfi. Furthermore, Ogliarola and Cellina were mixed in blend samples, in percentages from 30 to 70%, plus a few samples wherein small amounts of other *cultivar* were added (see Section 2.2 for details). Oils were produced in late 2013, between October and December, and additional agronomical and technical information are reported in Table 1.

All samples showed significant amounts of the investigated biophenols, as well as very high quantities of α-tocopherol, as shown in Figure 3. *orto*-Diphenols conjugated to the elenolic acid in various forms, i.e., the dialdehydic 3,4-DHPEA-EA, the decarboxylated dialdehydic 3,4-DHPEA-EDA, and 3,4-DHPEA-EA in the mixed aldehydic and hydroxylic form, were found to be the most abundant compounds, followed by their mono-hydroxy homologous (the *p*-HPEA derivatives). Nevertheless, significant amounts of their simple phenols hydroxytyrosol and tyrosol (3,4-DHPEA and *p*-HPEA, respectively) as well as lignans and luteolin were also found.

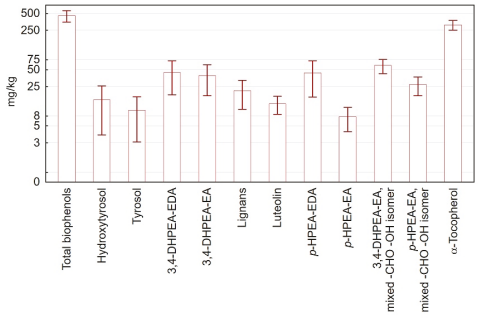

Figure 3. Histograms of the analyzed biophenols averaged for all samples. Bars represent standard deviations. A logarithmic scale was used on the Y axis.

3.1. Analysis by Cultivar

The average total amount of biophenols was about 452 mg/kg, with Frantoio being the *cultivar* with lowest quantity, as detailed in Table 2. On the other hand, Frantoio EVOOs had the highest concentration of hydroxytysosol and tyrosol (about 23 and 20 mg/kg, respectively), and lignans

(about 43 mg/kg). Nevertheless, all the other biophenols were present in modest quantities compared to the other *cultivars*. Ogliarola showed quite high concentrations of total biophenols and *p*-HPEA-EDA, all other values being on average. Cellina also had quantities of phenols analogous to average values, with the only exception being the amount of α-tocopherol, the highest among all *cultivars*. As expected, blend samples showed a trend similar to that of Ogliarola and Cellina, being they constituted completely by mixtures in different percentages of those two *cultivars*, or at least for 80% by them also when mixed with other varieties, such as Leccino, Frantoio, or Coratina. The only significant difference was the concentration of α-tocopherol, much lower than that in the pure parent varieties. Small amounts of *p*-HPEA-EDA and *p*-HPEA-EA were detected in Leccino EVOOs, while Picholine had a low concentration of hydroxytyrosol but very high quantities of *p*-HPEA-EA in the aldehydic and hydroxylic form. Finally, Cima di Melfi EVOOs showed quite high concentrations of biophenols compared to average, especially of 3,4-DHPEA-EDA, 3,4-DHPEA-EA, and lignans, while lower quantities of α-tocopherol were detected. However, it must be considered that only two samples each of Frantoio and Picholine and one of Cima di Melfi were analyzed, hence those average values might be quite variable on a broader pattern.

In order to check the presence of dominant constituents among the *cultivars*, principal component analysis (PCA) was carried out on the Cellina and Ogliarola samples, for a total of 45 observations, using the 10 detected phenols, α-tocopherol, and the total amount of biophenols as variables. Blend samples were initially excluded from the analysis because of their intrinsic variability being mixtures of Cellina and Ogliarola in various ratios and, in some cases, also with other *cultivars*, as already stated. On the other hand, Leccino, Picholine, Frantoio, and Cima di Melfi EVOOs were omitted due to low number of available samples for each category (4, 2, 2, and 1, respectively). The first two components of the model could explain 50.3% of the total population, however the resulting score plot did not show any dominant variable but the samples were quite spread with a high degree of overlap among them, as shown in Figure 4.

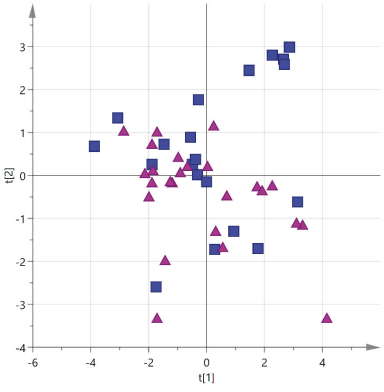

Figure 4. PCA score plot (PC1 vs. PC2) for EVOO samples from Salento categorized by *cultivar* (blue squares = Cellina; purple triangles = Ogliarola).

Much better results were obtained when a supervised multivariate method was used. Partial least-squares discriminant analysis (OPLS-DA) improved significantly separation of the *cultivars*, as shown in Figure 5, with cumulative R^2X and R^2Y of 0.60 and 0.83, respectively, and a respectable Q^2 of 0.66. The two groups were perfectly separated along the mayor component, while the variability among samples is probably responsible for their dispersion along the orthogonal component. According to the loadings, α-tocopherol and 3,4-DHPEA-EA contributed the most to the discrimination of Cellina EVOOs, while *p*-HPEA-EDA and *p*-HPEA-EA to that of Ogliarola samples.

Table 2. Content of phenols in EVOO samples organized by type of *cultivar*.

Cultivar	Total Biophenols	Hydroxytyrosol	Tyrosol	3,4-DHPEA-EDA	3,4-DHPEA-EA	Lignans	Luteolin	*p*-HPEA-EDA	*p*-HPEA-EA	3,4-DHPEA-EA, Mixed Isomer	*p*-HPEA-EA, Mixed Isomer	α-Tocopherol
	Content in mg/kg [1]											
Ogliarola salentin	468.7 ± 111.4	14.4 ± 8.9	8.2 ± 4.3	39.1 ± 26.7	30.4 ± 18.1	17.8 ± 6.4	12.7 ± 3.8	61.0 ± 32.2	7.9 ± 3.1	61.8 ± 17.4	26.8 ± 8.2	322.5 ± 39.4
Cellina di Nardò	449.1 ± 101.6	14.0 ± 11.6	8.6 ± 6.0	55.1 ± 26.6	44.9 ± 15.8	23.2 ± 11.0	14.8 ± 5.2	34.5 ± 17.4	6.1 ± 2.6	61.9 ± 21.8	25.4 ± 7.4	351.2 ± 52.6
Leccino	379.3 ± 35.8	13.8 ± 20.2	6.8 ± 5.6	44.0 ± 14.7	34.5 ± 11.2	16.5 ± 11.2	11.3 ± 1.3	14.8 ± 2.1	4.3 ± 2.2	63.8 ± 3.8	30.0 ± 7.7	297.8 ± 41.0
Picholine	463.0 ± 62.2	7.0 ± 2.8	6.9 ± 1.4	40.2 ± 22.6	47.5 ± 3.5	9.0 ± 9.9	11.1 ± 0.2	38.0 ± 17.0	4.5 ± 0.7	58.5 ± 2.1	52.3 ± 5.7	265.1 ± 13.1
Frantoio	308.0 ± 83.4	23.0 ± 26.9	20.2 ± 22.6	12.5 ± 2.1	16.0 ± 1.4	43.4 ± 7.1	7.3 ± 2.8	20.1 ± 5.7	5.0 ± 1.4	32.5 ± 7.8	24.5 ± 2.1	189.6 ± 25.1
Cima di Melfi	478.9	4.8	6.1	65.7	62.0	59.3	11.9	37.4	6.7	43.8	19.2	233.9
Blend	462.4 ± 96.8	16.2 ± 10.9	12.2 ± 8.5	40.3 ± 26.6	44.4 ± 28.6	20.9 ± 11.1	10.1 ± 3.4	38.1 ± 21.3	9.9 ± 3.9	54.9 ± 5.1	27.6 ± 12.6	280.4 ± 74.8
Avg total	451.6 ± 102.4	14.4 ± 11.2	9.3 ± 6.8	43.9 ± 26.4	38.2 ± 20.6	21.0 ± 11.3	12.5 ± 4.4	43.5 ± 27.6	7.4 ± 3.4	59.3 ± 16.9	27.3 ± 9.7	313.9 ± 61.9

[1] Values are expressed as mean ± standard deviation relatively to the different samples of EVOO analyzed in each category. Only one sample was available for the *cultivar* Cima di Melfi and no standard deviation has been reported.

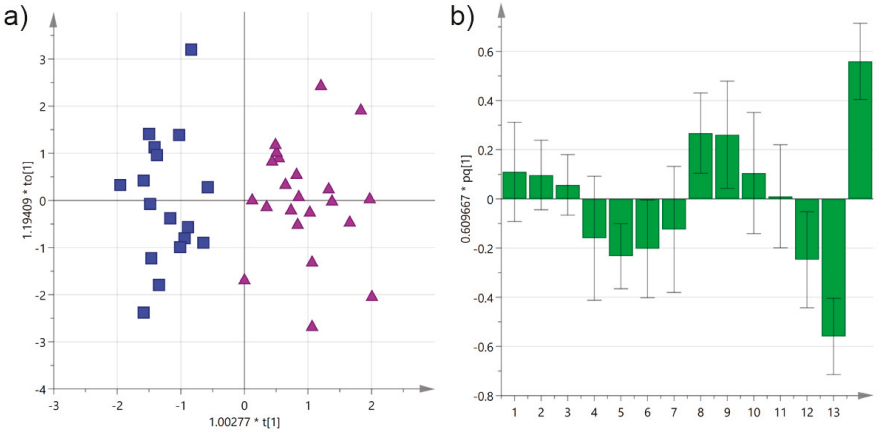

Figure 5. (**a**) Orthogonal partial least-squares discriminant analysis (OPLS-DA) score plot for EVOO samples from Salento categorized by *cultivar* (blue squares = Cellina; purple triangles = Ogliarola); (**b**) OPLS-DA loadings column plot for the first predictive component of the model (variables: 1 = total biophenols; 2 = hydroxytyrosol; 3 = tyrosol; 4 = 3,4-DHPEA-EDA; 5 = 3,4-DHPEA-EA; 6 = lignans; 7 = luteolin; 8 = *p*-HPEA-EDA; 9 = *p*-HPEA-EA; 10 = 3,4-DHPEA-EA in the mixed aldehydic and hydroxylic form; 11 = *p*-HPEA-EA in the mixed aldehydic and hydroxylic form; 12 = α-tocopherol; 13 = Cellina; 14 = Ogliarola).

In order to check the predictive ability of the model, blend EVOOs were used as prediction dataset. As expected, samples did not aggregate in any region of the plot but they spread along PC1 between the two extremes marked by the pure *cultivars*, probably according to the different percentages of Cellina and Ogliarola in their composition, as shown in Figure 6.

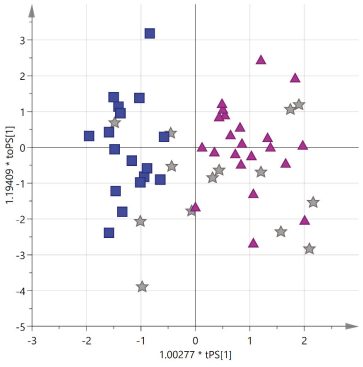

Figure 6. Predicted OPLS-DA score plot for blend EVOO samples from Salento with unknown percentages of Cellina/Ogliarola (blue squares = Cellina; purple triangles = Ogliarola, gray stars = blend predictions).

3.2. Analysis by Interval Time between Harvesting and Milling

It is well recognized that the amount of antioxidants in EVOOs is influenced, together with other factors, also by the interval time between harvesting and milling. In fact, it has been shown that the shorter this interval time the higher the concentration of biophenols and it is also the reason why, for example, PDO EVOOs are required to be processed by 48 h at the latest after being collected [21]. Samples were grouped in two classes, those which were milled by 12 h from harvesting and those for which that interval time was longer, and OPLS-DA was performed. Initially only Cellina EVOOs were considered to reduce the potential variability introduced by other *cultivars*, and the resulting score scatter plot is reported in Figure 7.

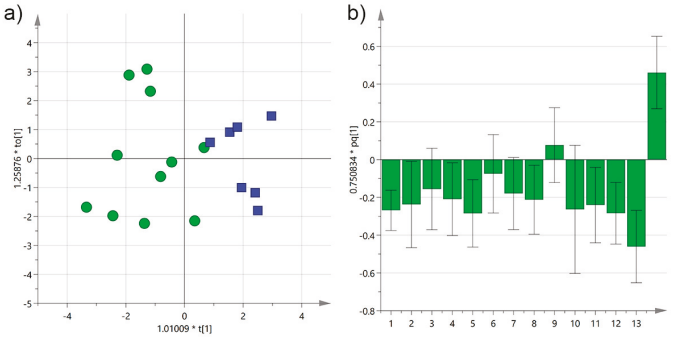

Figure 7. (**a**) OPLS-DA score plot for Cellina EVOO samples from Salento categorized by the interval time between harvesting and milling of the olives (green circles $\leq$ 12 h; blue squares > 12 h); (**b**) OPLS-DA loadings column plot for the first predictive component of the model (variables: 1 = total biophenols; 2 = hydroxytyrosol; 3 = tyrosol; 4 = 3,4-DHPEA-EDA; 5 = 3,4-DHPEA-EA; 6 = lignans; 7 = luteolin; 8 = *p*-HPEA-EDA; 9 = *p*-HPEA-EA; 10 = 3,4-DHPEA-EA in the mixed aldehydic and hydroxylic form; 11 = *p*-HPEA-EA in the mixed aldehydic and hydroxylic form; 12 = α-tocopherol; 13 $\leq$ 12 h; 14 > 12 h).

Discrimination was substantial and it could be observed from the loading plot that most variables contributed significantly to the separation of EVOOs with shorter interval time, especially the total concentration of biophenols, 3,4-DHPEA-EA, and α-tocopherol. On the other hand, only *p*-HPEA-EA appeared to give a slightly positive contribution to samples with an interval time longer than 12 h. However, this result is not surprising because it is known that degradation of antioxidant molecules starts soon after harvesting, and that is why a short interval time between harvesting and milling is a prerequisite for producing high quality EVOOs.

Performing the OPLS-DA on all EVOOs yielded a much worse model (R^2X(cum) = 0.38, R^2Y(cum) = 0.45, Q^2X(cum) = 0.28) compared to that obtained with only Cellina samples (R^2X(cum) = 0.56, R^2Y(cum) = 0.73, Q^2X(cum) = 0.54). This was expected given the randomness introduced by other variables, nevertheless a similar trend could be observed in the score scatter plot and in the loading column plot (Figure 8).

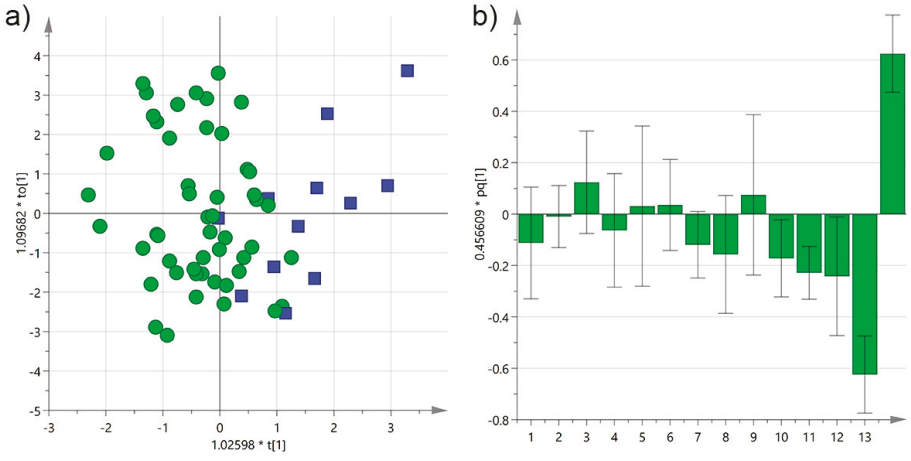

Figure 8. (**a**) OPLS-DA score plot for EVOO samples from Salento categorized by the interval time between harvesting and milling of the olives (green circles ≤ 12 h; blue squares ≥ 12 h); (**b**) OPLS-DA loadings column plot for the first predictive component of the model (variables: 1 = total biophenols; 2 = hydroxytyrosol; 3 = tyrosol; 4 = 3,4-DHPEA-EDA; 5 = 3,4-DHPEA-EA; 6 = lignans; 7 = luteolin; 8 = *p*-HPEA-EDA; 9 = *p*-HPEA-EA; 10 = 3,4-DHPEA-EA in the mixed aldehydic and hydroxylic form; 11 = *p*-HPEA-EA in the mixed aldehydic and hydroxylic form; 12 = α-tocopherol; 13 ≤ 12 h; 14 > 12 h).

3.3. Analysis by the Age of the Olive Trees

Salento is well-known for having a high number of secular olive trees, which have recently been regulated and surveyed because of their high historical value [23]. Furthermore, EVOOs from secular olive trees have been already studied and categorized by NMR due to their commercial importance [12]. We now tested if any statistical difference in the amount of biophenols could be detected between secular and younger olive trees. EVOOs were grouped depending on the age of their trees in >100 and <100-year-old classes and OPLS-DA was performed on Cellina samples. A quite good descriptive but not predictive model was obtained, as shown by the correlation coefficients (R^2X(cum) = 0.48, R^2Y(cum) = 0.70, Q^2(cum) = 0.02). According to the loadings, EVOOs from younger trees were significantly discriminated by the total amount of biophenols and *p*-HPEA-EDA, although many other variables also contributed to the separation, as shown in Figure 9. On the other hand, only tyrosol gave a slightly positive contribution to the discrimination of secular trees.

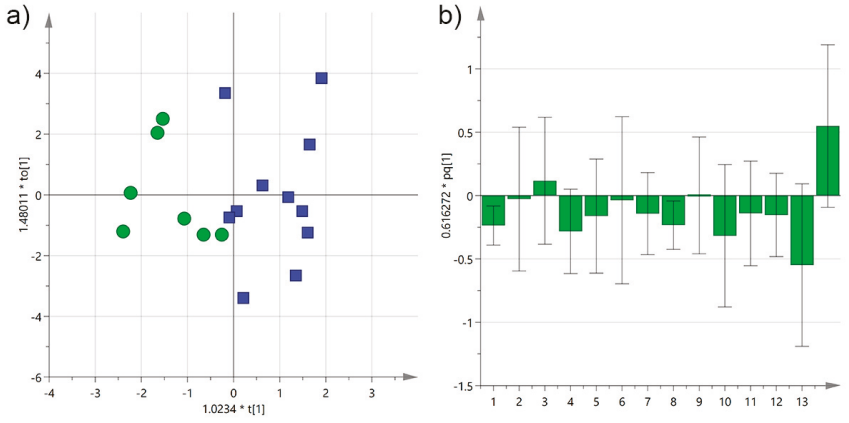

Figure 9. (**a**) OPLS-DA score plot for Cellina EVOO samples from Salento categorized by the age of the olive trees (green circles ≤ 100 years; blue squares > 100 years); (**b**) OPLS-DA loadings column plot for the first predictive component of the model (variables: 1 = total biophenols; 2 = hydroxytyrosol; 3 = tyrosol; 4 = 3,4-DHPEA-EDA; 5 = 3,4-DHPEA-EA; 6 = lignans; 7 = luteolin; 8 = *p*-HPEA-EDA; 9 = *p*-HPEA-EA; 10 = 3,4-DHPEA-EA in the mixed aldehydic and hydroxylic form; 11 = *p*-HPEA-EA in the mixed aldehydic and hydroxylic form; 12 = α-tocopherol; 13 ≤ 100 years; 14 > 100 years).

The OPLSA-DA was repeated on all EVOOs yielding, despite the different *cultivars* utilized, a model with very good both descriptive (R^2X(cum) = 0.74, R^2Y(cum) = 0.79) and predictive (Q^2(cum) = 0.65) ability, as shown in Figure 10. The reported loadings again confirmed that younger trees were richer of biophenols compared to secular ones, also emphasizing the role of 3,4-DHPEA derivatives and luteolin, while older ones were discriminated thanks to tyrosol and its derivatives conjugated to elenolic acid.

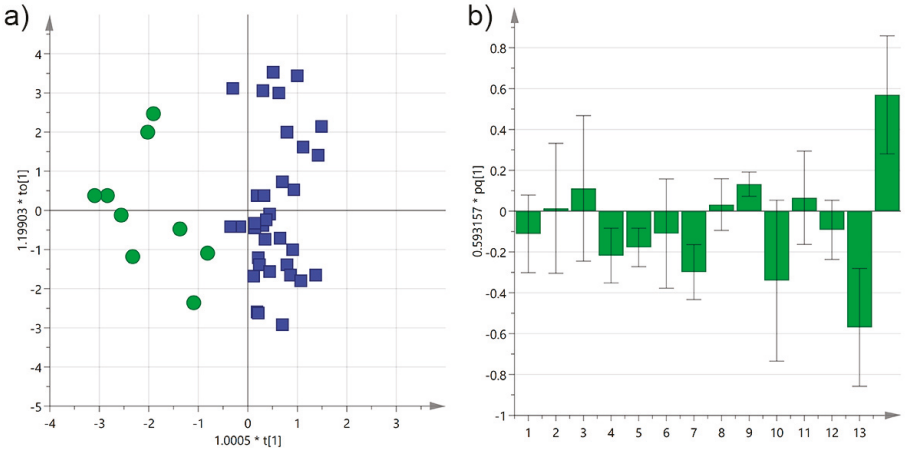

Figure 10. (**a**) OPLS-DA score plot for EVOO samples from Salento categorized by the age of the olive trees (green circles ≤ 100 years; blue squares > 100 years); (**b**) OPLS-DA loadings column plot for the first predictive component of the model (variables: 1 = total biophenols; 2 = hydroxytyrosol; 3 = tyrosol; 4 = 3,4-DHPEA-EDA; 5 = 3,4-DHPEA-EA; 6 = lignans; 7 = luteolin; 8 = *p*-HPEA-EDA; 9 = *p*-HPEA-EA; 10 = 3,4-DHPEA-EA in the mixed aldehydic and hydroxylic form; 11 = *p*-HPEA-EA in the mixed aldehydic and hydroxylic form; 12 = α-tocopherol; 13 ≤ 100 years; 14 > 100 years).

3.4. Analysis by the Cultivation Area

Climate and agronomical features are very important variables that influence noteworthy the final characteristics of olives and vegetables in general. Gambacorta et al., reported that olive maturity index and technology used had a greater influence with respect to the place of growth on the total amount of phenols [10]. Nevertheless, they found a variability of about 40% depending on the cultivation area. Similarly, we looked for any correlation between the area where the olive trees were grown and the concentration of the analyzed biophenols. OPLS-DA was performed on Cellina EVOOs grouped into 3 categories depending on the proximity of the trees either to the Adriatic or Ionian Sea, or to neither of them if in the central mainland. The EVOOs clustered quite nicely (R^2X(cum) = 0.85, R^2Y(cum) = 0.73) with samples from trees close to the Adriatic Sea being well separated on the positive axis because of most variables, especially luteolin, *p*-HPEA-EDA, lignans, and the total amount of biophenols, as shown in Figure 11. On the other hand, samples from the Ionian area and the central mainland reported both similar negative values along PC1, but were completely separated along PC2 mainly because of *p*-HPEA-EA the former and 3,4-DHPEA-EA in the mixed aldehydic and hydroxylic form the latter.

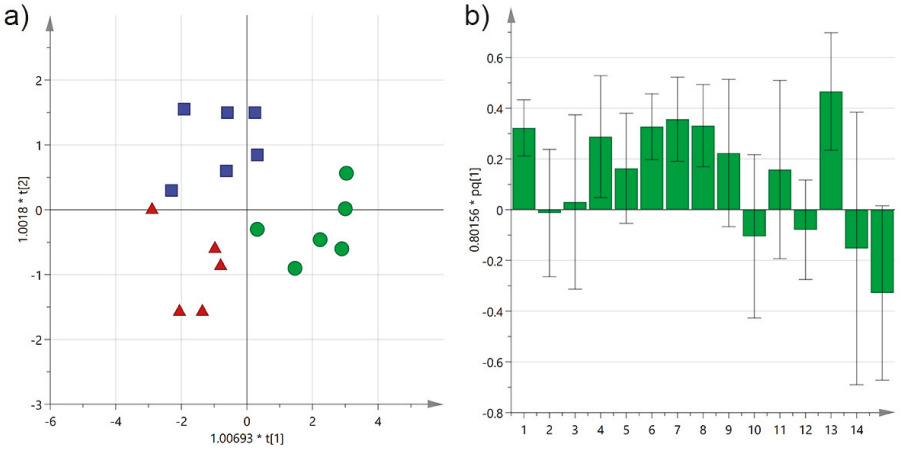

Figure 11. (**a**) OPLS-DA score plot for Cellina EVOO samples from Salento categorized by their proximity either to the Adriatic (green circles) or Ionian Sea (red triangles), or to neither of them (blue squares); (**b**) OPLS-DA loadings column plot for the first predictive component of the model (variables: 1 = total biophenols; 2 = hydroxytyrosol; 3 = tyrosol; 4 = 3,4-DHPEA-EDA; 5 = 3,4-DHPEA-EA; 6 = lignans; 7 = luteolin; 8 = *p*-HPEA-EDA; 9 = *p*-HPEA-EA; 10 = 3,4-DHPEA-EA in the mixed aldehydic and hydroxylic form; 11 = *p*-HPEA-EA in the mixed aldehydic and hydroxylic form; 12 = α-tocopherol; 13 = Adriatic area; 14 = central mainland; 15 = Ionian area).

OPLS-DA was also performed on Ogliarola EVOOs, but only samples from the Ionian Sea and the central mainland were considered, being only two those close the Adriatic area. A very good both descriptive (R^2X(cum) = 0.65, R^2Y(cum) = 0.89) and predictive (Q^2(cum) = 0.75) model was obtained, with samples close to the Ionian Sea being richer of *p*-HPEA-EDA and *p*-HPEA-EA, while those from the central mainland of 3,4-DHPEA-EA and α-tocopherol, quite in agreement with Cellina EVOOs from similar cultivation areas (Figure 12).

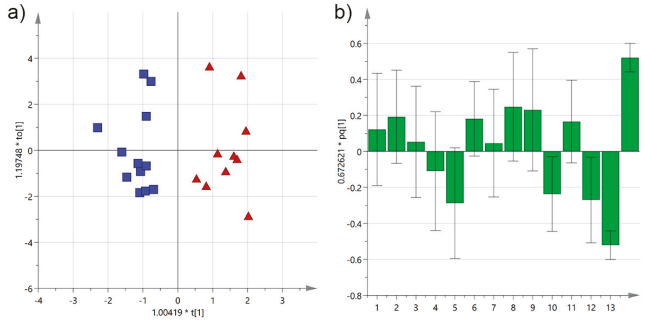

Figure 12. (**a**) OPLS-DA score plot for Ogliarola EVOO samples from Salento categorized by their proximity either to the Ionian Sea (red triangles), or to the central mainland (blue squares); (**b**) OPLS-DA loadings column plot for the first predictive component of the model (variables: 1 = total biophenols; 2 = hydroxytyrosol; 3 = tyrosol; 4 = 3,4-DHPEA-EDA; 5 = 3,4-DHPEA-EA; 6 = lignans; 7 = luteolin; 8 = *p*-HPEA-EDA; 9 = *p*-HPEA-EA; 10 = 3,4-DHPEA-EA in the mixed aldehydic and hydroxylic form; 11 = *p*-HPEA-EA in the mixed aldehydic and hydroxylic form; 12 = α-tocopherol; 13 = central mainland; 14 = Ionian area).

3.5. Analysis by PDO Certification

Protected Designation of Origin (PDO) "Terra d'Otranto" accreditation is a certificate given to EVOOs from the province of Lecce and part of the provinces of Brindisi and Taranto which satisfy determined agronomic and production characteristics which should guarantee healthful properties, among which a high content of antioxidants. Ogliarola and Cellina are the most popular *cultivars* in Salento and they are the mayor components of PDO EVOOs [21,24].

Most of the analyzed EVOOs in this study were PDO certified, however none of the pure *cultivars* studied contained a significant number of both PDO and non-PDO samples to perform a multivariate statistical analysis. Nevertheless, OPLS-DA analysis conducted on all EVOOs could descript (R^2X(cum) = 0.54, R^2Y(cum) = 0.77) and predict (Q^2X(cum) = 0.68) quite satisfactorily the two groups (Figure 13). The PDO samples were separated mainly because of a higher content of α-tocopherol and *p*-HPEA-EA in the mixed aldehydic and hydroxylic form, while smaller phenols, such as tyrosol and hydroxytyrosol, and *p*-HPEA-EA influenced the most the discrimination of samples without certification.

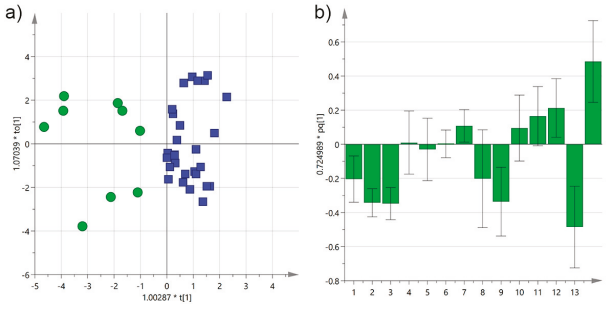

Figure 13. (**a**) OPLS-DA score plot for EVOO samples from Salento categorized by PDO certification (green circles = non-PDO; blue squares = PDO); (**b**) OPLS-DA loadings column plot for the first predictive component of the model (variables: 1 = total biophenols; 2 = hydroxytyrosol; 3 = tyrosol; 4 = 3,4-DHPEA-EDA; 5 = 3,4-DHPEA-EA; 6 = lignans; 7 = luteolin; 8 = *p*-HPEA-EDA; 9 = *p*-HPEA-EA; 10 = 3,4-DHPEA-EA in the mixed aldehydic and hydroxylic form; 11 = *p*-HPEA-EA in the mixed aldehydic and hydroxylic form; 12 = α-tocopherol; 13 = non-PDO; 14 = PDO).

4. Conclusions

Quantification of antioxidant molecules in EVOOs is of paramount importance because of their nutraceutical value, even though biophenols represent only minor constituents in olive oil. In this article, we quantified, by HPLC, the total amount of biophenols, several individual biophenols (i.e., tyrosol, hydroxytyrosol, and several more complex derivatives conjugated to elenolic acid in various forms), and α-tocopherol in a variety of pure *cultivars* and blend EVOOs from Salento, in South Apulia. Remarkable differences in the phenolic profile were evident among samples and a high variability was found within the *cultivars*, probably because of the influence of genotyping and other agro-climatic parameters. Nevertheless, all *cultivars* showed significant amounts of biophenols and supervised multivariate statistical analysis could detect differences between Cellina and Ogliarola EVOOs, and predict blend samples. Furthermore, the content of biophenols was correlated to production parameters, such the interval time between harvesting and milling, and to agronomical variables, such as the age of the olive trees and the area they were grown. OPLS-DA showed that shorter interval times lead to an increase in the total concentration of biophenols. Furthermore, it could distinguish between EVOOs from secular and from younger trees, and among samples from different cultivation areas, such as the proximity to the Adriatic or Ionian Sea, or to the central mainland. Eventually, these multivariate statistical analyses, besides yielding valuable information about the characteristics of the studied *cultivars*, could be exploited to improve the already beneficial properties of their EVOOs and to determine a metabolic profile that could be exploited, in combination with other parameters, to guarantee the originality and traceability of these products.

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Author Contributions: A.R. and M.M. conceived and designed the experiments; A.R., C.C., M.E.G., M.F.L., and P.F.M. performed the experiments; A.R., F.P.F., and M.M. analyzed the data; A.R., F.P.F., and M.M. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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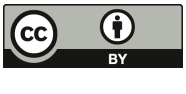

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Stimulatory Effects of Cinnamon Extract (*Cinnamomum cassia*) during the Initiation Stage of 3T3-L1 Adipocyte Differentiation

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Abstract: Cinnamon (*Cinnamomum cassia*) has an anti-diabetic effect by possibly increasing the lipid storage capacity of white adipocytes; however, this effect remains controversial. The aim of this study was to examine which stage of adipogenesis is critical for the stimulatory effect of cinnamon in adipogenesis using 3T3-L1 cells. Cells were treated with cinnamon extract during three different stages of adipogenesis. We found that genes related to adipogenesis and lipogenesis were enhanced when cinnamon extract was administered during the initiation stage of differentiation but not when administered during the preadipocyte and post stages of differentiation. At the same time, genes that were involved in the regulation of fatty acid oxidation were unexpectedly upregulated. Taken together, cinnamon may boost lipid storage in white adipocytes and increase the fatty acid oxidation capacity throughout the initiation stage of differentiation, which may be beneficial for the prevention of obesity-induced type II diabetes.

Keywords: cinnamon; white adipocyte differentiation; fatty acid oxidation; lipogenesis; adipogenesis

1. Introduction

White adipose tissue (WAT) is the primary site where extra calories are stored as fat for the body's energy reserve. WAT also secretes several hormones and cytokines that are critical for regulating nutrient metabolism [1]. However, excessive fat accumulation in WAT causes obesity and adipocyte dysfunction [2], resulting in a spillover of fatty acids into non-adipose organs, which further increases susceptibility for the development of type 2 diabetes mellitus, insulin resistance, and dyslipidemia [3]. Therefore, it is important for WAT to maintain its normal function and capacity to safely store fat, which protects other tissues against lipotoxicity [4]. This maintenance can be achieved by acquiring new adipocytes through adipocyte differentiation in WAT. Adipocyte differentiation is the process in which preadipocytes develop into mature white adipocytes that accumulate lipids as a single lipid droplet through early, intermediate, and terminal stages [5].

Various transcriptional factors and enzymes are involved in the adipogenesis process. Peroxisome proliferator-activated receptor gamma (PPARγ) is a primary transcriptional factor that regulates adipogenesis and also operates as a known therapeutic target for type 2 diabetes mellitus and dyslipidemia to increase the lipid storage capacity of WAT [6]. PPARγ agonists, such as thiazolidinediones (TZDs), increase the WAT storage capacity by activating PPARγ, subsequently improving insulin sensitivity [7]. However, various side effects of these drugs have demanded the development of alternative therapeutics from natural sources that activate PPARγ [6,8]. Cinnamon,

the bark of *Cinnamomum cassia*, has been used extensively as a traditional herb to manage numerous health conditions and as a spice in the food industry [9]. Previous studies have shown that cinnamon extract (CE) and cinnamaldehyde, a bioactive compound of cinnamon, improved insulin sensitivity and reduced plasma glucose levels, possibly via PPARγ [10–12].

However, some discrepancies remain regarding the anti-diabetic effect of cinnamon and whether cinnamon affects the fat storage capacity of adipocytes through PPARγ activation. Type II diabetic patients who took capsules containing cinnamon powder during a two-month period did not exhibit improvement in either plasma glucose or HbA1c levels [13]. In addition, Han and Huang et al. showed an inhibitory effect of cinnamon during adipogenesis [14,15]. Therefore, the aim of this study was to investigate the effects of CE in the three different stages of adipogenesis that regulate the fat storage capacity of adipocytes. These effects were defined by examining the expression profiles of genes related to adipogenesis, lipogenesis, and fatty acid oxidation in adipocytes that were fully differentiated and matured after CE was administered during the three different stages of adipogenesis.

2. Materials and Methods

2.1. Sample Preparation

CE (from *Cinnamomum cassia*) was purchased from New Age Botanicals (Melbourne, Australia). CE powder was diluted in culture media to final concentrations of 50, 100, and 200 μg/mL with 0.1% dimethyl sulfoxide (DMSO). The sample solution was then filtered using a syringe filter (0.22 μM). The nutritional value and phenolic content of CE powder are shown in the Supplementary Table S1.

2.2. Cell Culture

3T3-L1 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% bovine calf serum in a humidified cell culture incubator (37 °C and 5% CO_2). For experiments, cells were seeded at a density of 17,500 cells/cm^2 in 12-well plates with DMEM supplemented with 10% fetal bovine serum (FBS), indicated as day 0. On day 3, the culture medium was switched to differentiation medium (DMEM, 10% FBS, 1 μg/mL insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 0.25 mM dexamethasone), and the cultures were incubated for two additional days. On day 5, the differentiation medium was replaced with post differentiation medium (DMEM, 10% FBS, and 1 μg/mL insulin). The post differentiation medium was refreshed every other day until day 11. As shown in Figure 1, to determine the effects of CE on preadipocytes, the initiation of differentiationand its progress during the differentiation and maturation process, cells were treated with CE at concentrations of 50, 100, and 200 μg/mL on day 1 (preadipocyte stage), day 3 (initiation stage of differentiation) or days 5, 7, and 9 (post stages of differentiation) and were incubated for two additional days. The cells were then cultured and differentiated as described above until day 11. On day 11, cells were collected for total RNA extraction and Oil-Red O staining.

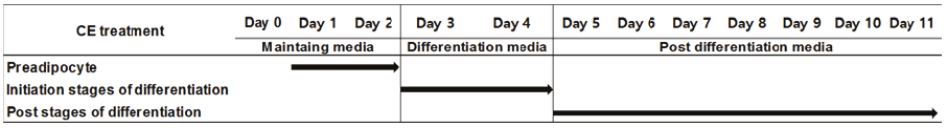

Figure 1. Experimental design to investigate the stimulatory effects of cinnamon extract (CE) treatments at different stages of 3T3-L1 adipocyte adipogenesis. 3T3-L1 adipocytes were treated with CE at the preadipocyte (days 1–2), initiation (days 3–4), and post stages (days 5–11) of differentiation during adipogenesis. When adipocytes were fully differentiated and matured on day 11, cells were harvested to extract total RNA for measuring gene expression using quantitative real-time PCR and were stained with Oil-Red O to visualize lipid accumulation as a marker of differentiation.

2.3. Cell Viability Assay

Cell viability was determined using a thiazolyl blue tetrazolium bromide (MTT) reduction assay according to the manufacturer's instructions (Cayman, Ann Arbor, MI, USA). Briefly, 3T3-L1 preadipocytes were seeded in a 96-well plate at a density of 17,500 cells/cm^2. The next day, the culture medium was replaced with fresh medium containing various concentrations of CE (0–1000 μg/mL). After 48 h of incubation, the media were replaced again with fresh culture medium containing 0.5 mg/mL MTT reagent, and cells were incubated for 3 h at 37 °C. Afterwards, the medium was discarded, and 100 μL of dimethyl sulfoxide (DMSO) was added to solubilize the purple formazan products. The absorbance was determined at 570 nm using a Synergy HT Microplate Reader (BioTek, Winooski, VT, USA), which indicates proportionally the number of viable cells. Cell viability was expressed as a percentage of the absorbance of cells treated with CE relative to the absorbance of cells that were not treated with CE. Experiments were performed in triplicate.

2.4. Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Total RNA was extracted from fully differentiated adipocytes (day 11) using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. RNA concentrations and purity were determined using a Take3 micro-volume plate equipped with a Synergy HT microplate reader (BioTek). RNA was transcribed to complementary DNA (cDNA) using XLAScript cDNA MasterMix (Exella GmbH, Feucht, Germany) according to the manufacturer's instructions. To determine the expression of target genes, first-strand cDNA was amplified using a Fast Start Essential DNA Green Light Master kit (Roche, Indianapolis, IN, USA) in a Light Cycler 90 (Roche). PCR conditions were as follows: 10 min at 95 °C, followed by 50 cycles of 10 s denaturation at 95 °C, annealing for 10 s at 60 °C, and extension for 10 s at 72 °C. The primers used are shown in Table 1. Ribosomal protein L 32 (RPL32) was used as a housekeeping gene. Cycle threshold (C_t) values were obtained. The expression level of each gene was calculated using the 2 delta C_t method by normalizing the C_t value of the targeted gene to that of the RPL32 gene. The data are presented as relative percentages of the expression level of each target gene when gene expression levels in adipocytes that were not treated with CE (control) were 100%.

Table 1. Primers designed for quantitative real-time PCR.

Gene	Forward Primer	Reverse Primer
ACC	TGCATTCTGACCTTCACGAC	ACATCCACTTCCACACACGA
C/EBPα	GGACAAGAACAGCAACGAGTA	GCAGTTGCCATGGCCTTGA
C/EBPβ	TGGACAAGCTGAGCGACGAG	TGTGCTGCGTCTCCAGGTTG
CIDEA	ATCACAACTGGCCTGGTTACG	TACTACCCGGTGTCCATTTCT
CPT1α	TTTGACTTTGAGAAATACCCTGATA	TGGATGAAATTCTCTCCCACAATAA
FAS	TGGGTTCTAGCCAGCAGAGT	ACCACCAGAGACCGTTATGC
PGC1α	TGCCCAGATCTTCCTGAACT	TCTGTGAGAACCGCTAGCAA
PPARγ	TTTGACTTTGAGAAATACCC	TGGATGAAATTCTCTCCAC
RPL32	CACCAGTCAGACCGATAT	TTCTCCGCACCCTGTTG
SREBP-1c	GAACAGACACTGGCCGAGAT	GAGGCCAGAGAAGCAGAAGAG

Abbreviations: ACC, acetyl-CoA carboxylase; C/EBPα, CCAAT-enhancer-binding protein α; C/EBPβ, CCAAT-enhancer-binding protein β; CIDEA, cell death-inducing DFFA-like effector; CPT1α, carnitine palmitoyltransferase 1α; FAS, fatty acid synthase; PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1α; PPARγ, peroxisome proliferator-activated receptor gamma; RPL32, ribosomal protein L 32; SREBP-1c, sterol regulatory element-binding protein 1.

2.5. Oil-Red O Staining

Intracellular lipid accumulation was determined using Oil-Red O staining (Thermo Fisher Scientific). Differentiated adipocytes (day 11) were rinsed with phosphate-buffered saline (PBS) twice and were fixed in 10% buffered formalin for 1 h at room temperature. After rinsing with 60%

isopropanol, cells were incubated with 60% isopropanol-based Oil-Red Solution by mixing 2 parts of deionized (DI) water and 3 parts of stock solution (350 mg of Oil-Red O in 100 mL of isopropanol) for 30 min at room temperature. Oil-Red O, an oil-soluble dye used to stain lipids, was removed and washed twice with DI water. Lipid droplets were visualized based on their red color using an Evos XL-microscope (Thermo Fisher Scientific).

2.6. Data Analysis

The data are presented as means ± standard error of mean (SEM). The comparisons were analyzed using one-way analysis of variance (ANOVA) with Tukey's post-hoc test in Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). All differences were considered significant at $p < 0.05$.

3. Results

3.1. Cytotoxicity of CE in Preadipocytes

CE did not induce cytotoxicity at or below 200 μg/mL (Figure 2). Thus, the concentration ranges of CE selected for treatment in 3T3-L1 cells were 50, 100, and 200 μg/mL.

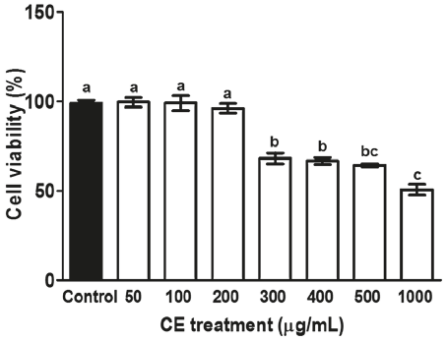

Figure 2. Cytotoxicity of CE in 3T3-L1 preadipocytes. Preadipocytes were treated with different concentrations of CE for 48 h. Cell viability was then measured using a thiazolyl blue tetrazolium bromide (MTT) reduction assay. The experiment was performed in triplicate. A different letter indicates a statistically significant difference ($p < 0.05$).

3.2. CE Increased Lipid Accumulation by Increasing the Expression of Adipogenic and Lipogenic Genes during the Initiation Stage of Differentiation

As shown in Figure 3A, CE treatment during the initiation or post stages of differentiation upregulated PPARγ gene expression during the adipogenesis process. PPARγ coordinately works with CCAAT/enhancer-binding protein alpha and beta (C/EBPα and β) to mediate a differentiation process that converts preadipocytes into mature adipocytes [16]. Figure 3B,C show that the C/EBPα and β genes were upregulated in adipocytes treated with CE in the initiation stage of differentiation during adipogenesis. Although C/EBPβ gene expression was increased slightly in adipocytes when CE was administered during the post stages of differentiation, there was no change in C/EBPα expression (Figure 3B,C). CE treatment during the preadipocyte stage did not alter the mRNA expression levels for any of the three genes related to adipogenesis, PPARγ, C/EBPα, or β, during adipogenesis (Figure 3A–C). Consistent with increased expression of genes that are involved in the regulation of adipogenesis, fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), and sterol regulatory element-binding protein 1c (SREBP-1c) genes, also involved in lipogenesis, were affected in adipocytes treated with CE during the different stages of adipogenesis (Figure 3D–F). The addition of CE in the preadipocyte stages barely affected the expression levels of FAS, ACC, or SREBP-1c genes when

the cells became fully mature, while CE treatment at 200 μg/mL exhibited an increase in SREBP-1c gene expression. Adipocytes treated with CE during the initiation stage of differentiation increased SREBP-1c, FAS, and ACC mRNA expression levels compared with control cells when they became mature adipocytes. CE treatment during the post differentiation stage did not affect FAS, ACC, or SREBP-1c gene expression. As shown in Figure 3G, the cell death-inducing DFFA-like effector (CIDEA) gene, which is known to play a role in the formation of lipid droplets, was also upregulated in mature adipocytes following treatment with 200 μg/mL CE during the initiation stage of differentiation. Figure 3H shows the increased lipid accumulation in mature adipocytes following treatment with CE during the initiation stage of differentiation.

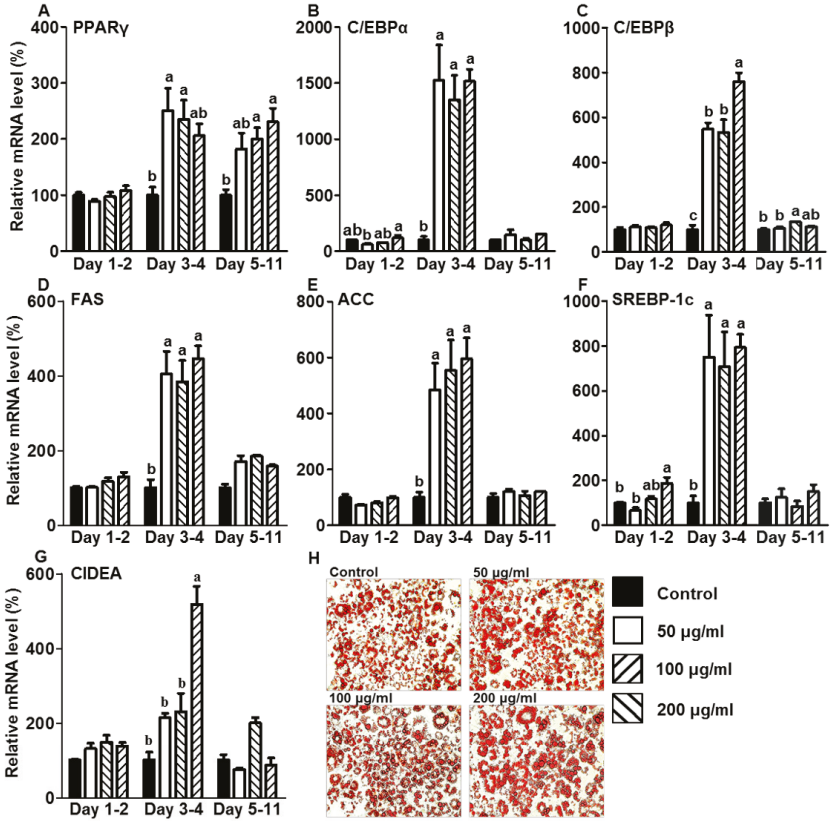

Figure 3. Effects of CE treatment at different stages of 3T3-L1 adipogenesis on adipogenic and lipogenic gene expression levels. Adipocytes were treated with CE at the preadipocyte (days 1–2), initiation (days 3–4), and post stages (days 5–11) of differentiation during adipogenesis. On day 11, the expression levels of genes related to adipogenesis (**A**) PPARγ, (**B**) C/EBPα, (**C**) C/EBPβ, and lipogenesis (**D**) FAS, (**E**) ACC and (**F**) SREBP-1c, and lipid droplet formation (**G**) CIDEA, were measured using a quantitative real-time PCR assay. (**H**) Representative images of Oil-Red O staining in fully matured adipocytes after adipocytes were treated with CE during the initiation stage of differentiation. The experiment was performed in triplicate. The statistical differences among the four CE concentrations (0, 50, 100, and 200 μg/mL) that were used to treat cells were separately analyzed for each experiment and each time window (e.g., day 1–2, day 3–4, and day 5–11) using one-way ANOVA with Tukey's post hoc test. A different letter indicates a statistically significant difference ($p < 0.05$).

3.3. CE Elevated the mRNA Expression Levels of Genes Related to Fatty Acid Oxidation in the Initiation Stage of Differentiation during Adipogenesis

Figure 4A,B show the effects of CE on the expression levels of genes related to the regulation of fatty acid oxidation. When preadipocytes were treated with 200 μg/mL CE, the expression of the PPARγ-coactivator 1 alpha (PGC1α) gene, which encodes a transcriptional factor to regulate energy metabolism including fatty acid oxidation, was increased in fully differentiated mature adipocytes after adipogenesis (Figure 4A). PGC1α gene induction was also observed in adipocytes treated with 200 μg/mL CE during the initiation stage of differentiation. However, PGC1α gene expression was not changed by CE treatment during the post differentiation stages (Figure 4A). Consistent with the stimulatory effect of CE treatment on the PGC1α gene at the preadipocyte and initiation stages of differentiation, treatment with 200 μg/mL CE during the initiation stage of differentiation increased mRNA expression levels of the carnitine palmitoyltransferase (CPT) 1α gene, which is involved in promoting fatty acid oxidation during adipogenesis (Figure 4B). However, there were no changes in CPT1α gene expression when preadipocytes were treated with concentrations of CE below 200 μg/mL. Treatment with CE at concentrations of 50, 100, and 200 μg/mL during the initiation stage of differentiation strongly elevated CPT1α mRNA levels when adipocytes were fully differentiated. Treatment with CE during the post differentiation stages did not affect CPT1α gene expression during adipogenesis (Figure 4B).

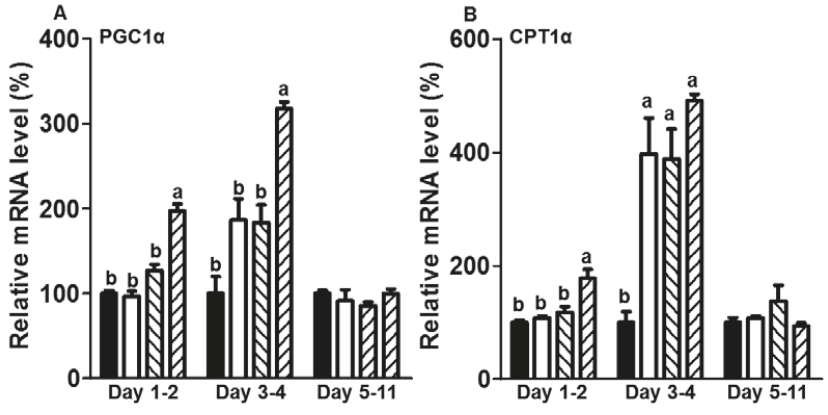

Figure 4. Effects of CE treatment at the different stages of 3T3-L1 adipogenesis on genes related to fatty acid oxidation. Adipocytes were treated with CE at the preadipocyte (days 1–2), initiation (days 3–4), and post stages (days 5–11) of differentiation during adipogenesis. On day 11, the expression levels of genes related to fatty acid oxidation (**A**) PGC1α and (**B**) CPT1α were measured using a quantitative real-time PCR assay. The experiment was performed in triplicate. The statistical differences among the four CE concentrations (0, 50, 100, and 200 μg/mL) that were used to treat cells were separately analyzed for each experiment and each time window (e.g., day 1–2, day 3–4, and day 5–11) using one-way ANOVA with Tukey's post-hoc test. A different letter indicates a statistically significant difference ($p < 0.05$).

4. Discussion

Increasing the lipid storage capacity of WAT with anti-diabetic medicines, such as TZDs, can reduce the development of type 2 diabetes by lowering circulating fatty acid and triglyceride levels [6,17]. Cinnamon is a functional food that may improve the lipid loading capacity of WAT by enhancing PPARγ activity [10,18]. However, the stimulatory effects of cinnamon on PPARγ remain controversial. It is also unclear which adipogenic stage is critical for the stimulatory effect of cinnamon

to increase the capacity of white adipocytes to store lipids. In the present study, we found that CE increased the lipid storage capacity during the initiation stage of differentiation by upregulating the expression levels of genes related to adipogenesis and lipogenesis. Additionally, CE showed potential for increasing fatty acid oxidation during adipogenesis, specifically as a result of CE treatment during the initiation stage of differentiation. Therefore, our present study demonstrated that the initiation of differentiation is a critical stage for cinnamon-stimulated effects in adipogenesis and lipogenesis to increase the lipid storage capacity in white adipocytes, as confirmed by increased lipid accumulation as a marker of promoted differentiation. We also observed that CE treatment during the initiation stage of differentiation could induce adipocytes to perform dual functions of lipid storage and utilization.

WAT contains adipocytes that are in various stages of development. For adipocytes to obtain the capacity to store lipids, a process called adipogenesis is required to differentiate preadipocytes into mature adipocytes. Although adipogenesis is regulated by coordinating with various transcriptional factors, e.g., PPARγ, C/EBPα, C/EBPβ, and adipocyte protein 2 (aP2), PPARγ is recognized as a primary regulator for adipogenesis [16]. C/EBPβ is induced at the early stage of differentiation and increases the activity of PPARγ and C/EBPα throughout the later stages of differentiation to promote differentiation, which further increases the utility of aP2 as a terminal marker of differentiation [16]. Consistent with the increased PPARγ, C/EBPα, and C/EBPβ gene expression levels noted in our present study, Sheng at al. observed similar stimulatory effects of CE during the induction of differentiation in 3T3-L1 cells by increasing PPARγ expression and transcriptional activity at a concentration of 600 μg/mL, which was cytotoxic in our present study [18]. In contrast, one study showed that 100 and 500 μg/mL CE inhibited adipogenesis and lipogenesis by decreasing PPARγ gene expression [14]. Another study also reported that cinnamaldehyde, one of the active compounds found in cinnamon, prevented PPARγ transcriptional activity [15]. It has been reported that cinnamon includes various dietary compounds, such as coumarin, 2-hydroxyl cinnamaldehyde, cinnamyl alcohol, cinnamic acid, cinnamaldehyde, 2-methoxy cinnamaldehyde, and eugenol [14]. The differences in the effects of CE between studies may depend on different concentrations of other major dietary compounds as a result of the different extraction methods used. As shown in our present study, the treatment stage was critical for acquiring the different effects of CE.

Along with adipogenesis, lipogenesis is also promoted to store lipids as triglycerides in adipocytes during differentiation. Consistent with the increased expression levels of the PPARγ, C/EBPα, and C/EBPβ genes, the FAS, ACC, and SREBP-1c genes, which are involved in the regulation of lipogenesis, were also increased when CE was administered during the initiation of differentiation. The increases in both adipogenesis and lipogenesis in response to CE treatment during the initiation stage of differentiation were further confirmed by increased lipid accumulation and larger-sized lipid droplets. Mice treated with a PPARγ agonist, rosiglitazone, exhibited upregulated CIDEA expression in WAT [19]. CIDEA plays a role in the enlargement of lipid droplets by transferring triglycerides between neighbored lipid droplets [20]. Transgenic mice expressing human CIDEA became obese by expanding the sizes of their adipose tissues, but had increased insulin sensitivity [21]. In contrast, CIDEA deficient-mice were lean and had small lipid droplets [22]. In the present study, the larger-sized lipid droplets produced in response to CE treatment during the initiation stage of differentiation may have resulted from increased expression of the CIDEA gene. It has also been reported that CIDEA expression was induced by PGC1α [23]. PGC1α is a positive regulator of catabolic metabolism, e.g., fatty acid oxidation, and is abundantly present in oxidative tissues, such as brown adipose tissue, muscle, and liver. Our present study showed that CE elevated PGC1α mRNA levels in adipocytes at the initiation stage of differentiation. Therefore, CE treatment during the initiation stage of differentiation may have increased CIDEA expression by activating PGC1α and PPARγ.

Although the fat storage capacity of WAT may positively improve diabetic conditions, expanding the size of WAT by accumulating excessive fat also presents some concern, e.g., obesity. However, the present study indicated that CE may improve mitochondrial fatty acid oxidation in the initiation of differentiation, likely in part due to the increased expression of CPT1α and PGC1α genes. C/EBPβ is

involved in not only adipogenesis but also energy metabolism [24]. When C/EBPβ was transiently overexpressed in 3T3-L1 cells, the cells displayed brown-like characteristics by increasing PGC1α expression, which is indicated as the browning of white adipocytes [24]. Therefore, increased expression of fatty acid oxidation genes, including CPT1α and PGC1α in response to treatment with CE at the initiation stage of differentiation, would be related to C/EBPβ upregulation.

5. Conclusions

In conclusion, the present study demonstrated that CE allows adipocytes to have enhanced lipid storage capacity and fatty acid oxidation in the initiation stage of differentiation during adipogenesis by simultaneously increasing the expression levels of genes related to adipogenesis, lipogenesis, and fatty acid oxidation. It is possible that the dual effects of CE on both the storage and utilization of fat would be beneficial for obesity related to type II diabetes by increasing insulin sensitivity through enhanced lipid storage capacity and by utilizing stored fat to avoid excess fat accumulation. Although further studies using animal models to understand the effect and efficiency of CE in vivo should be conducted, our findings would provide meaningful information for the development of new anti-diabetic drugs that act on specific target stages of adipogenesis.

Supplementary Materials: The following are available online at www.mdpi.com/2304-8158/5/4/83/s1, Table S1: *F*-values in the statistical analysis for gene expressions level by cinnamon extract (CE) treatments, Table S2: Nutritional components of 100 g cinnamon extract (CE) powder.

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Commentary

Red Yeast Rice

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Abstract: Red yeast rice (RYR), produced by the fermentation of the *Monascus purpureus* mold, has been used for a long time in Asian cuisine and traditional medicine. It consists of multiple bioactive substances, including monacolins, which potentially can be used as a nutraceutical. Monacolin K, which is chemically identical to lovastatin, has been recognized as responsible for the cholesterol-reducing effect of this compound. While the European Food Safety Authority maintains that the use of monacolin K from RYR preparations of at least 10 mg can produce a normal blood cholesterol level, the United States Food and Drug Administration considers monacolin K, due to its similarity with lovastatin, an unapproved drug, and therefore marketing of products that label the monacolin content is prohibited. This mini-review summarizes the benefit of RYR in hyperlipidemia, maintains RYR use as a food, and addresses the importance of regulation regarding RYR and the need for clinical data and clear label information for consumers with reference to a toxin-free, non-augmented, standardized amount of monacolins.

Keywords: red yeast rice; lovastatin; nutraceutical; safety; health

Red Yeast Rice as Nutraceutical

The term "nutraceutical" was coined in 1989 by Stephen DeFelice, founder and chairman of the Foundation for Innovation in Medicine, and it identifies a food or part of a food, which can be of vegetal or animal origin, that has a potential pharmaceutical activity [1]. The goal of assessing the possible role of a nutraceutical and its use in medicine is an important challenge for the future.

In general, any food, due to its content of active compounds, has the potential to go beyond its nutritional value as a source of macro- and micronutrients, and can also be used as a drug depending on the dose. Nonetheless, attention should be paid to potential risk factors related to the use of vegetal- or animal-origin foodstuffs as starting matrices to constitute a nutraceutical, e.g., the safety of the starting material, the presence of allergenic compounds, the absence of toxicity, the absence of exogenous and endogenous contaminants, the possible presence of toxic secondary metabolites and/or environmental pollutants, which could potentially cause a health threat [2,3].

Red yeast rice (RYR) is a nutraceutical made by fermenting white rice with the yeast *Monascus purpureus* and other related molds. RYR has been used as an herbal supplement and in the cuisine of East Asian countries including China, Japan, and Korea. It has been used for flavoring, coloring, and preservation of food and in traditional Chinese medicine for many years [4].

RYR consists of a multitude of compounds including polyketides, unsaturated fatty acids, phytosterols, pigments, and monacolins [5]. Monacolins inhibit HMG CoA (3-hydroxy-3-methyl-glutaryl-coenzyme A) reductase, the rate-limiting step in cholesterol synthesis. At least 13 monacolins have been isolated from RYR, of which monacolin K is chemically similar to lovastatin, a cholesterol-lowering drug [6].

Several clinical trials have been conducted to examine the efficacy and safety of RYR. A recent meta-analysis in 2015 examined 20 randomized trials consisting of 6663 patients and showed a reduction in low density lipoprotein (LDL) cholesterol when comparing RYR to placebo groups (−1.02 mmol/L (−1.20, −0.83)); there was no difference in LDL between RYR and statin therapy (0.03 mmol/L (−0.36, 0.41)), with an incidence of kidney injury and liver abnormalities of less than 5% in both the RYR and control groups [7]. RYR has been demonstrated to not only improve lipid metabolism, but it can also reduce blood pressure, and may possess anti-inflammatory, antidiabetic, anticancer, and osteogenic properties [5]. The largest randomized controlled trial examining RYR in secondary cardiovascular prevention consisted of 4870 Chinese patients and showed that RYR reduces nonfatal myocardial infarction, coronary disease mortality, coronary revasculization, and total mortality in patients with a history of myocardial infarction and moderate hypercholesterolemia [8].

Furthermore, a number of clinical trials have shown RYR to be effective in reducing cholesterol in those who are intolerant of statins because of statin-associated myalgias, gastrointestinal side effects, or elevated transaminase levels [4]. For those who are skeptical of drugs and are more interested in complementary and alternative medications, RYR has been used as a cholesterol-lowering option [9]. RYR in combination with other nutraceuticals including berberine, policosanol, astaxanthin, and coenzyme Q10 has been shown to be effective in reducing lipids and glucose. A recent meta-analysis of 14 randomized controlled trials showed that nutraceutical combinations containing RYR improve lipid and glucose levels [10]. Although statins have been shown to cause hyperglycemia, a 2014 meta-analysis of five trials consisting of 352 patients showed that RYR does not significantly increase glucose compared to placebo [8]. In addition, RYR in combination with antioxidants has been demonstrated to reduce high sensitivity C-reactive protein (hs-CRP) and endothelial dysfunction [11].

Adverse effects of RYR include gastrointestinal effects and may cause myopathy, hepatotoxicity, rhabdomyolysis, and anaphylaxis similar to the use of statins [9,12]. The mycotoxin citrinin, found in poorly produced RYR products, can pose a health risk as it may be mutagenic as found in animal models, genotoxic to human lymphocytes, and can cause kidney failure in animals, although acute toxicity is a rare event [13–16]. Furthermore, drug-herb interactions can potentially be harmful. Statins are metabolized by Cytochrome P450 (CYP) enzymes, and the administration of RYR with CYP enzyme inhibitors (e.g., ketoconazole, human immunodeficiency virus (HIV) protease inhibitors, erythromycin) can lead to worsening undesirable adverse effects, such as myopathy [5,17]. Nonetheless, the clinical studies that showed the effectiveness of RYR in dyslipidemia also demonstrated that it is a relatively safe product [7,18].

The European Food Safety Authority (EFSA) allows for health claims that RYR products can cause pharmacotherapeutic effects. The EFSA has established "a cause and effect relationship...between the consumption of monacolin K in red yeast rice preparations...and maintenance of normal blood LDL cholesterol concentrations", given that the daily dietary intake level is at least 10 mg of monacolin K from RYR [19]. This value is considered acceptable even though recently it has been called into question due to the variable contents of different RYR products, thus representing a health risk in the absence of appropriate information for the customer [17].

The U.S. Food and Drug Administration (FDA) maintains a different perspective. Because of its functional similarity to lovastatin, monacolin K is considered an unapproved drug by the U.S. FDA, and as such all RYR products that contain a specific amount of monacolin K are prohibited. In fact, RYR supplements on the market are not standardized and contain variable amounts of monacolins, including monacolin K, citrinin, and other contaminants. Manufacturers do not admit the monacolin content in their RYR products on the packaging for fear it would prompt regulatory action from the FDA [9,20].

In order to reduce the variability of the monacolin contents of different RYR preparations and to minimize toxic compounds, such as citrinin, quality control should be implemented and enforced by entities responsible for food and supplement oversight.

Due to its extensive use as a dietary supplement for many years before its medicinal purposes were discovered, RYR is a food and should be treated as such. Because of its wide potential health benefits, RYR should be produced as a standardized preparation for those who would potentially benefit from it. By not augmenting this product, it could be made available to consumers as a supplement and thus does not need to be rigorously regulated as a drug. Requiring a statement on the product label assuring a toxin-free, non-augmented, standardized amount of monacolins would be advantageous to consumers, allowing more predictable efficacy and better safety. In addition, a safety warning should also be included to caution those with myopathy, liver disease, or concomitant use of CYP inhibitors. Those who are taking this supplement should be advised to have regular follow-ups with a medical professional to monitor for potential side effects and interactions with drugs and other nutraceuticals.

Conflicts of Interest: The authors declare no conflict of interest.

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Review

Microalgae Nutraceuticals

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Abstract: Among the new entries in the food supplements sector, an important place must be assigned to nutraceuticals containing microalgae, nowadays accounting for a large and rapidly expanding market. The marketed products are mainly based on three production strains, i.e., Spirulina and Chlorella, followed at a distance by Klamath. It is a composite situation, since two of them are cyanobacteria and the second one is eukaryotic. The reality is that each presents similarities in shape and appearance concerning the marketed form and several utilizations, and peculiarities that need special attention and adequate studies. First, general information is reported about the current scientific knowledge on each microalga, in particular the nutritional value and properties in prevention and wellbeing. Second, original studies are presented concerning the quality control of marketed products. Quality control is a key argument in nutraceuticals validation. Microalgae are particular organisms that need specific approaches to confirm identity and validate properties. The proposed control of quality is based on microscopic analysis of the morphologic characteristics. The final parts of this paper are dedicated to the need for specificity in uses and claims and to considerations about the future of microalgae in food supplements.

Keywords: microalgae; Spirulina; Chlorella; Klamath; food supplement; quality control

1. Introduction

International agencies like the FAO have announced the goal of food to feed everyone in the world [1,2]. However, to gain this success against any Malthusian prophecy, we must consider two main aspects, production and quality. Besides the eternal challenge concerning the production of a necessary quantity of food, recently quality is becoming fundamental for health maintaining and lifestyle improvements, as evidenced by the global obesity phenomenon [3–5]. Both aspects can be considered as decisive characteristics of the so-called nutritional environment. The nutritional environment is actually at the center of everybody's attention, from governments to ordinary people, in consideration of the related problems including health and social costs.

Recently, rapid changes have impacted the food scenario, involving the appearance of several new products. These products—nutraceuticals, botanicals, and others—deeply influenced the market for their hybrid nature, located somewhere between ordinary food and medical drugs [6,7]. Actually, they are considered part of the food supplements sector, although the situation is continuously subject to changes and new interpretations [8–10]. The result is a complex, dynamic situation, needing a careful study and information about each of the different aspects [11].

A clear example of the actual and future situation of food, including food supplements, is offered by microalgae [12–15], nowadays accounting for a large and rapidly expanding market [16–18]. As a matter of fact, microalgae chemical composition is a complex mixture of minerals, vitamins, and primary and secondary products, offering a large spectrum of possible applications and utilizations for humans, from nutritional properties to antioxidant and anti-aging, also considering the preventative effects. In other words, microalgae are a case study in nutraceuticals.

On this occasion, after a set of necessary information on the raw materials, three arguments concerning microalgae products will be considered in detail:

(a) Quality control, a problem involving the whole food supplements market.
(b) The specificity of use and claim, since the derived products in food supplements are still not sufficiently tailored in terms of possible utilization.
(c) The real need of microalgae for mankind, in particular as nutraceuticals.

2. The Evolution of Food Supplements

At the beginning of their appearance in the market, food supplements were considered as concentrated nutrients useful to support nutritional needs and/or supply alimentary deficiencies in the ordinary diet and, consequently, their composition clearly evidenced the presence of vitamins, minerals, proteins, and carbohydrates [8]. Later, their composition showed a massive introduction of "other substances," mainly consisting of extracts of medicinal plants. Composition, targets, and marketing clearly changed, as well as denominations, with possible names including nutraceuticals (the most frequently used, but still not officially recognized), dietary supplements, medical devices, herbal drug preparations, traditional medicine herbal products, botanical food supplements, botanical supplements, or simply botanicals, in the case of utilization of raw plant materials [10,11]. The main difference consists in the organic composition, since food supplements of the first generation mainly contain substances of primary metabolism, like carbohydrates, vitamins, and proteins, whereas other substances, typical of nutraceuticals, are secondary products, like flavonoids, terpenes, polyphenols, organic acids, pigments, etc., usually present in raw plant materials or extracts. This simplified scheme is contradicted by microalgae, which are a rich source of both types of substances, as evidenced by the reported chemical analyses. Similar to food supplements evolution, for a long time seaweed was mainly considered a source of animal feed and human alimentation, but the introduction in the market of nutraceuticals radically changed the scenario. To deal with this particular situation, the attractive term "superfood" was suggested [19]. The so-called superfoods are now present in supermarkets and herbal shops, although their composition and nature were not determined and clarified (Figure 1).

Figure 1. So-called superfoods can now be easily found in supermarkets.

The third type of food supplements, now emerging, is functional foods or pharmafoods, based on the addition of special ingredients with certain physiological properties to ordinary foods. This is probably the best future use of microalgae, bypassing the limits of attractiveness in the current utilizations and opening the way for a wide variety of different products, like green pasta or special desserts.

3. Aquatic Autotrophics

Life depends on water. Seaweed, together with animals and plants, is fundamental to the current food scenario [20,21]. Ordinary people may not aware, but a standard diet is full of seaweed products, directly or indirectly, and not even considering that planet life largely depends on the photosynthetic work of phytoplankton and that most living organisms are marine. The main biomass of seaweeds present in the planet are microscopic unicellular organisms named microalgae. Microalgae are not only naturally abundant in the sea and terrestrial water, but can be cultivated easily and in large quantities, giving rise to a low-cost raw material with many potential uses. Food supplements were able to enlarge the application horizon of microalgae, but this is probably only the beginning.

For a long time, seaweed use, including microalgae, was limited to livestock feed and fertilizer. Recently, new important uses for microalgae have been emerging, fuelled by increasing interest and curiosity from consumers. Again, technology has a fundamental role in transforming raw materials into a myriad of different products. From food to biodiesel, the microalgae empire is coming [22–27].

Algae are a polyphyletic group of autotrophic marine organisms (alga in Latin means marine plant), erroneously considered as plants due to the common photosynthetic trophic pathway. Generally speaking, they are known as seaweed; however, "seaweed" is a colloquial term and lacks a formal definition. In addition, some tuft-forming blue-green algae are sometimes considered to be algae, but they must be linked to prokaryotes (cyanobacteria) [28,29]. The classic classification of algae, now obsolete, is based on colour, being the most direct evident morphologic character. Colourations derive from different pigments associated with the photosynthetic process, according to the water depth where they live. Therefore, we have blue-green, yellow, green, brown, and red algae. Algae can be unicellular or multicellular, microscopic or giant, simple or complex, similar in form to plants or to bacteria. Inside these organisms, we can read the complete story of autotrophic living beings, from the beginning until 450 million years ago, when life left the water. Primordial microalgae were very similar to those present in our seas. Although primordial habitats were totally different from current ones, these cells have been able to survive practically unchanged until now and are still the main part of the biomass of the planet. Considering their massive distribution, they are still dominating the planet.

The potential use of microalgae is enormous: three-quarters of the planet's surface is occupied by water and most marine water is available for life. The space habitable by marine organisms is far greater to that available for terrestrial plants. Microalgae, which cover almost 75% of algae species, contribute approximately 40% of the oxygen in the atmosphere. Despite the apparent simplicity of their cells, at least 40,000 species of microalgae phytoplankton have been identified [29]. The key of this success is in the metabolism, i.e., in the completeness of substances present in the cytoplasm. This is the key to their importance in nutraceuticals. For this reason, in this paper the analytical part is a particular focus.

4. Cyanobacteria in Nutraceuticals

The market for microalgae nutraceuticals is dominated by two cyanobacteria, universally known as Spirulina and Klamath, and the chloroficean Chlorella. To understand cyanobacteria, we must start with the outset of life, 4.5 billion years ago, when these microscopic cells started the experiment of life [20]. From that beginning, organic life was separated by the trophic level. On one side were the autotrophic cyanobacteria, and on the other side were the heterotrophic consumers, the Eubacteria, which use the organic substances produced by the former. Besides them, Archaea prokaryotes were experimenting with other methods of producing energy and organic matter. We are here because that

model, despite the apparent simplicity, was (and still is) successful; its capacity is nowadays testified to by its presence. Despite the absence of a nucleus, bacteria possess all the biochemical tools to produce any kind of products, including precious essential amino acids and enzyme supply [30–32].

Cyanobacteria are also known as blue-green algae and are traditionally associated with seaweed, in consideration of the trophic level, the environmental condition, and several other similarities. The evolutionary line leading to modern plants started from that model based on the chlorophyll work in autotrophic algae. Following the evolutionary pathways, we focused on advanced organisms, considering them a major source of food and medicinal drugs. It is now time to reverse this attitude. Again, the first signal of novelty comes from nutraceuticals. Microalgae are heavily used as raw materials in food supplements. The claim is to obtain both equilibrium in the diet and a specific activity, in accordance with the characters of the functional food.

Production of microalgae must be carefully considered. Microalgae need some special conditions to produce large quantities of biomass. This can be obtained in natural, as well as artificial, habitats. Attention must be focused on algal bloom, which in some periods can interest particular species in certain seas, as the result of optimal biological, physical, and chemical conditions. The resulting toxic water contamination can be dangerous directly or in the food chain [33]. On the other side, certain species of microalgae can be successfully used to clean contaminated gas or water, in particular from industrial production [23].

5. Spiralated Cianobacteria

5.1. Spirulina

Spirulina (classified as *Arthrospira* sp.) is a cyanobacterium present in free-floating filaments in the form of an open left-hand helix characterized by cylindrical multicellular trichromes (Figures 2 and 3). It occurs naturally in tropical and subtropical alkaline hot lakes with high pH values and high salt concentrations, like carbonate and bicarbonate. Two species, *S. platensis* and *S. maxima*, are mainly present, the first occurring in Africa, Asia, and South America, whereas the second is confined to Central America. Cultivation of Spirulina on a large scale started 30 years ago in Mexico and China, and later in other parts of the world, owing to the easy conditions for cultivation. Most cultivated Spirulina is produced in open channel raceway ponds, with paddle-wheels used to agitate the water. The largest commercial producers of Spirulina are located in the USA, Thailand, India, Taiwan, China, Bangladesh, Pakistan, Burma (Myanmar), Greece, and Chile. Spirulina is primarily known across the world for its potential nutritional value. It is one of the rare edible bacteria, due to its low purine concentration, which allows it to pose a minimal risk of uric acid build-up in the body [22]. The food industry classifies *A. platensis* as a single-celled protein, meaning that it is an edible microbe with a high food value [32]. The nutritional value of Spirulina was already known to the Aztecs, who harvested the alga from Texcoco Lake, near Mexico City. Spanish soldiers led by Cortes described its use as a daily food and the sale as cakes [33,34]. It is rich in vitamins, minerals, β-carotene, essential fatty acids, and antioxidants, all of which have facilitated its commercial production as a human food supplement over the course of the past decade [35,36]. Its consumption has been shown to have cardiovascular positive effects, lowering blood pressure and reducing cholesterol [36]. In consideration of its anti-carcinogenic properties, it was used to treat radiation sickness in people that were affected by the 1986 Chernobyl nuclear accident [37–40].

Nowadays, it is used in food supplements, in the form of tablets or power, alone or in association with other algae or plant extracts, for human or animal uses. Actually, Spirulina is considered a good source of vitamins and essential amino acids. It also has very high protein content with a well-balanced composition, making it even more desirable as a food supplement [31,32]. It is also noteworthy for its oil content, in quantity (7%) and in quality (α-linolenic acid (ALA), linoleic acid (LA), stearidonic acid (SDA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (AA)).

The content in vitamins, like those of the group B, and hydrocarbons is considered relevant and complete [31].

Figure 2. In Spirulina, cells are aggregated into filaments that tend to form spirals. Note the blue-green colour typical of cyanobacteria.

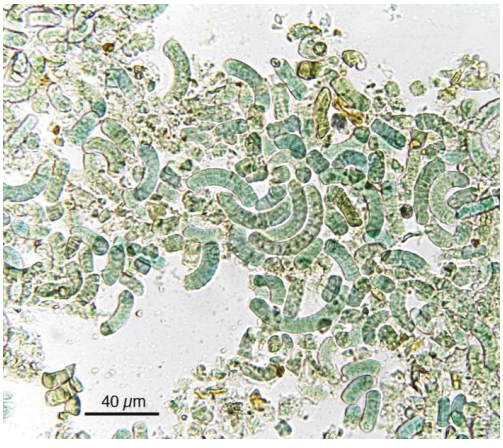

Figure 3. Most Spirulina cells, after their exsiccation and transformation into marketed products, are fragmented, but the original characteristics useful for identification are still evident.

6. Algae in Environmental Niches

6.1. Alga Klamath

Klamath is the name of a lake in Oregon (USA, perimeter 161 km, medium deep 2.4 m), where the cyanobacterium *Aphanizomenon flos-aquae* grows spontaneously and therefore is commonly known as alga Klamath (Figure 4). Several other *Aphanizomenon* spp. occur in other parts of the planet, but Lake Klamath allows for good production and easy harvesting. It can be considered a living fossil, survived into a particular ecological niche [41]. Therefore, alga Klamath's environmental condition is very different from that of Spirulina. Spirulina algae can be grown in controlled conditions that do not exclude completely contaminations, but Klamath microalgae are in a natural and very large place, open to the presence of other microalgae and microorganisms and subjected to seasonal cycles. In March, the *Fragilaria* spp. dominate the phytoplankton of the lake, followed by *A. flos-aquae* between May and July. In the same period, also *Anabaena flos-aquae* (less that 1% of the total algae) and later in July–October *Microcystis aeruginosa* and *Coelopshaerium* are present. Therefore, during the collection of Spirulina, several algal species can be collected, giving rise to warnings about the presence of neurotoxins produced by other algae, in particular about *Anabaena*, *Microcystis*, and *Oscillatoria* spp. that are normally present in the lake and can produce toxins. However, so far there is not complete and reliable information about the production of toxins, like microcystins, from *A. flos aque*; analyses

on Lake Klamath phytotoxins, as well as on marketed products, are a controversial matter, including the analytic method, although all reports only concern the possibility of contamination and toxic effects [42].

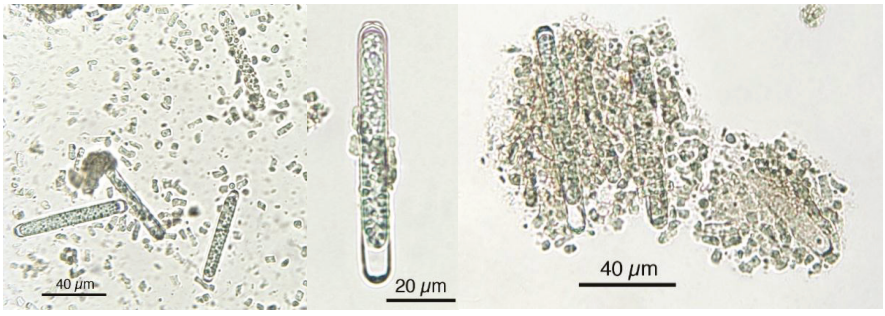

Figure 4. Microphotographs of Klamath power showing fragmented as well as intact cells with resistant cell wall and characteristic form.

Both Spirulina and alga Klamath are marketed with suggestive adjectives, such as *superfood* or *the food of the future*. Several activities are reported and in part confirmed by different types of experiments and clinical trials; however, as for several food supplements, the scientific validation is not considered complete. In the first place, despite the poorly inviting taste, the nutritional value and the anti-obesity effect are the object of considerable marketing appeals.

6.2. Chlorella

Chlorella is a microscopic unicellular seaweed pertaining to the green algae (Chlorophyta) (Figure 5). The evident green pigmentation is due to the presence of the two chlorophylls, *a* and *b*, the same ones in terrestrial plants. It can be easily cultivated in simple conditions, producing enormous quantities of biomass in little time. It needs only water, CO_2, light, and a small quantity of minerals. Considering its quantity of proteins, amino acids, minerals, vitamins, and pigments [43,44], it should be considered the ideal food [45].

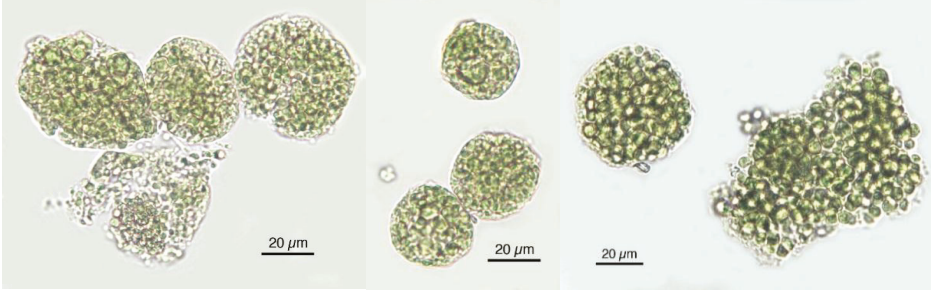

Figure 5. The microscopic unicellular Chlorella cells tend to agglomerate also in food supplements.

Its properties and capacities have been idealized and emphasized in various ways. Yury Viktorovich Romanenko, cosmonaut, twice hero of the Soviet Union, holds the record for the longest stay in space, with a total of 430 days, 20 h, 21 min, 30 s in several missions, before the space station came about (Figure 6). During his time in space, he was able to perform a series of important experiments on seaweed of the *Chlorella* genus, in consideration of its future utilization as a food in

long space journeys. However, simple chemistry is not the only consideration. The flavour of this seaweed, as well as of other ones, is not enjoyable in comparison with ordinary dishes. Also, in this case, there is a long list of assigned health activities and properties, including the detoxicant action and the stimulation of the immune system. On the side of wellbeing, evidence of improved digestion and normalization of sugar metabolism has been reported.

Figure 6. The soviet cosmonaut Yury Viktorovich Romanenko tested the utilization of Chlorella as an ideal food in long space journeys.

7. Quality Control in Microalgae

There is a concern about toxins produced by cyanobacteria [33]. Microcystins, produced by *Microcystis* sp., were in the news as they caused acute liver failure in more than 100 Brazilian haemodialysis patients. The problem arose due to the use of a contaminated water reservoir, whose filters and carbon adsorption tanks had not been changed for a long time. Another typical alarm comes from the consumption of saltwater mussels (*Mytilus edulis*), which feed on *Microcystis* and accumulate microcystins persisting for several days after the transfer of the mussels into clean water [46]. In Southern Italy, there is a tradition of consumption of fresh mussels, without any form of cooking, causing in some cases acute gastrointestinal problems and dysentery, albeit in some cases it is unclear that the problems were attributable to the mussels. When production is operated in clean water and controls are performed, no alarms or problems were registered. Another case comes from another species, *Pfiesteria* sp., which produces neurotoxins. In 1997 in Maryland, USA, the so-called "*Pfisteria* panic" produced a 210% reduction in sales of fish and shellfish due to a public perception of danger [47,48]. Harmful seaweed blooms are caused by the unusual proliferation of certain toxic microalgae, which are regular constituents of the plankton microflora found in the Austral ecosystem of Chile, when environmental conditions are favourable to their blooming [49–51]. They can be due to the presence of toxins, as occurs with the dinoflagellates responsible for paralytic shellfish poison and diarrhoeic shellfish poison. Therefore, some of the marine organisms that filter microalgae, such as bivalve shellfish, concentrate these toxins. Consumption of these organisms may seriously harm human health and may even be lethal.

In food supplements, only the reported microalgae species are utilized and they are considered devoid of any toxin production. They are used alone or together. The marketed raw material consists of lyophilized or dried seaweed as a fine green powder, so that is very difficult to ascertain at a glance the identity of the utilised species. A control is necessary, not least because the cost of each one is very different and therefore the combination is not always reported on the label, but the real danger comes from the incidental occurrence of toxic algae.

As evidenced in Figure 7, each microalga utilized in nutraceuticals possesses a distinct microscopic morphologic shape. The morphological analysis at the microscope is very useful and the results prove the presence of alien species. Figure 8 shows the presence of *Oscillatoria* sp. in a Spirulina commercial

sample, whereas in Figure 9 the co-occurrence of Spirulina and Chlorella is shown. However, to obtain a reliable result, the quality control needs a specialized treatment, a good instrument, and a specialist in algology able to identify the species, in particular in case of the presence of alien toxic microalgae that can be easily cultivated, or casually present, together with the required ones. Furthermore, during the process of preparation of tablets, the delicate original form of the microalgae can be partially destroyed. In particular, high-quality images should be obtained and presented, as those reported here. Also, a sufficient number of analyses is necessary to ascertain the amount of contamination. In case of contamination, it is necessary to identify the alien species, but the presence of a contaminant species, like in Figure 8, must be considered only as an indication of the possible presence of toxins in the marketed products.

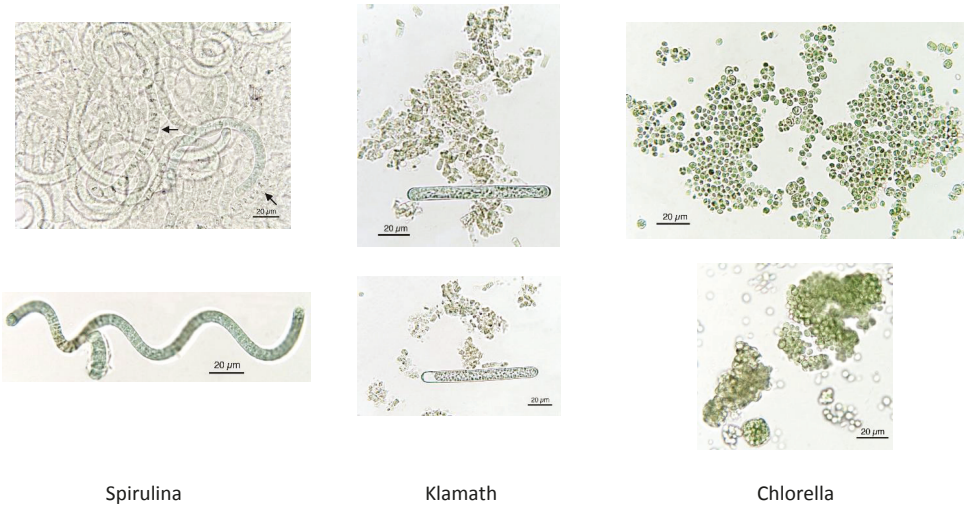

Figure 7. Microscopic characteristics of each microalga, allowing for identification.

Figure 8. Spirulina product showing the presence of *Oscillatoria* sp. (long linear cells).

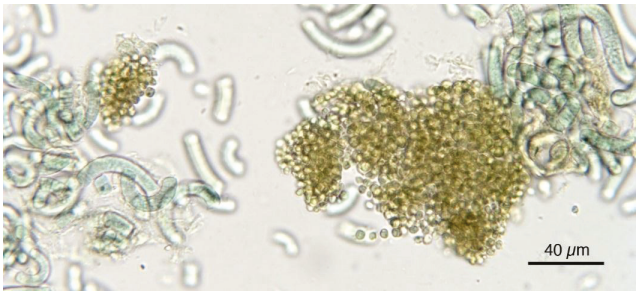

Figure 9. Spirulina product showing the presence of *Chlorella*-like and/or unicellular cyanobacterial cells.

8. The Importance of Microalgae in Food Supplements

Nowadays, microalgae are mainly utilized to feed livestock and pets. However, human consumption is increasing, in particular in food supplements. There are several cases of interest in the use of microalgae in food supplements. Vegans adopt a diet characterized by the practice of abstaining from the use of animal products [52]. Generally, the diet is associated with a philosophy that rejects the commodity status of animals and other ethical tendencies [53]. Therefore, vegans and omnivores can get into confrontations and debates [52,54]. So far, vegans seem to be victorious, with their numbers constantly increasing. Vegans account for ca. 5% of the total population in Israel, 2% in the United Kingdom and United States, and 1% in Germany and in Italy, which means anywhere from several thousand to seven million inhabitants.

The vegan diet is often considered nutrient-deficient, due to unbalanced protein sources and a low intake of some vitamins and minerals. Recently, some scientific data has been produced that gives substance to the debates. A recent study reports the results of a comparison between a group of vegetarians and a group of non-vegetarians for an average period of eight years [55]. The study stated: "A vegan diet should involve a balanced intake of whole grain products, legumes, seeds and sources of proteins, as well as vegetables, fruits, berries and unsaturated fats. In addition, vegans should consume calcium-fortified drinks and use vitamin B12, vitamin D and iodine supplements to complete their diet." The results mean that vegans face nutritional problems due to a shortage of vitamin B12, 25-hydroxyvitamin D, selenium, and long-chain omega-3 fatty acids.

Factors that influence vegetarian food intake should include knowledge of a balanced diet, vegetarian food variety, as well as the use of enriched food items and food supplements. Among the available food supplements, for their content microalgae seem one of the best candidates to supplement vegans' and vegetarians' diet.

The vegan diet debate, including the recent WHO alert concerning the need for limiting meat intake, evidences the tendency to obtain health implements by a hypocaloric intake. This aspect has been recently considered in a study reported by an interdisciplinary group [56–60]. The paper, published in *Cell Metabolism* in 2015, showed that in mice a diet alternating prolonged fasting (PF) and a nutrient-rich medium resulted in extended yeast lifespan (+20%), independently of established pro-longevity genes [60]. Besides extended middle-age longevity, bi-monthly FMD cycles of four days of a diet that mimics fasting (FMD), developed to minimize the burden of PF, lowered visceral fat, reduced cancer incidence and skin lesions, rejuvenated the immune system, and retarded bone mineral density loss. In old mice, FMD cycles promoted hippocampal neurogenesis, lowered IGF-1 levels and PKA activity, elevated NeuroD1, and improved cognitive performance. In a pilot clinical trial, three FMD cycles decreased risk factors/biomarkers for aging, diabetes, cardiovascular disease, and cancer without major adverse effects, providing support for the use of FMDs to promote health span. The results and conclusions of the research find an independent confirmation in Laron Syndrome.

The Laron population lives in remote villages of Ecuador. They are very small people, suffering from an incredibly rare genetic disorder that stops them from growing taller than 4 feet and produces elderly features even in children. The syndrome is a consequence of low IGF-1 levels, but the same factor also seems to protect them against cancer and diabetes, and maybe even heart disease and Alzheimer's.

Therefore, the mima hypocaloric diet shows that: (a) a high protein intake is linked to increased cancer, diabetes, and overall mortality; (b) high IGF-1 levels increase the relationship between mortality and high protein levels; (c) higher protein consumption may be protective for older adults; and (d) plant-derived proteins are associated with lower mortality than animal-derived proteins. Therefore, in this case also, microalgae can play a role in the diet.

9. Conclusions

As already reported, algae food supplements can be particularly useful to support some diets. However, some aspects need to be considered and research should play a central role. Microalgae are considered more or less at the same level of utilization in nutraceuticals, albeit with several differences. Dried spirulina used in food supplements contains about 60% (51%–71%) protein, with a composition rich in all essential amino acids, though with reduced amounts of methionine, cysteine, and lysine when compared to meat, eggs, and milk, although superior to typical plant protein, such as that from legumes [32,61–63]. Furthermore, an interesting debate concerns vitamin B12. Most edible cyanobacteria, like spirulina, do not naturally contain vitamin B12, but predominantly contain pseudovitamin B12, which is inactive in humans [64,65]. Therefore, the American Dietetic Association and Dieticians of Canada, in their position paper on vegetarian diets, state that spirulina cannot be counted on as a reliable source of active vitamin B12 [66]. However, companies that grow and market spirulina have claimed it to be a significant source of the vitamin on the basis of alternative, unpublished assays, although their claims are not accepted by independent scientific organizations. However, there is a general rule to be considered: plant drugs contain plenty of secondary metabolites acting as non-active biochemical precursors, usually named pro-drugs, from alliin in garlic to THC-A in cannabis, as reported in Table 1. There are several reported reasons for its situation: active products are precious and must be preserved or the metabolite must act on the right please, etc.

Table 1. Examples of plant pro-drugs.

Prodrug	Active Substances
Glucosinolates	Isothiocyanates
Alliin	Allicin, ajoenes
Cumaroylglucoside	Coumarin
Arbutin	Hydroquinone
Salicin	Saligenin, salicylic acid
Bi-desmosidic saponin	Mono-desmosidic saponins
Ranunculin	Protoanemonin
THC-A	THC
Proto-vitamin B12	Vitamin B12
Cyanogenic glucoside	HCN
Rhein, sennosides	Antraquinonic aglucone
Hennosides	Lawsone
Vanilloside	Vanillin
Gein	Eugenol
Methylazoxymethanol	Cycasin

The vitamin B12 debate is only an example of the general debate concerning the real necessity and effectiveness of nutraceuticals consumption. Probably, more research is needed on these as on many other food supplements, in order to obtain the right utilization. Food supplements, so far fuelled by a billion-dollar market, urgently need scientific validation.

Conflicts of Interest: The author declares no conflict of interest.

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