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Analysis of the amino acid profile of red and white graphs winery by-products from western Sicily

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ABSTRACT

Nowadays, research addressing the composition of nutrients and bioactive compounds in waste from the agri-food industry has increased due to environmental and economic interests. The wine industry produces enormous quantities of by-products every year along the production chain. The nitrogen content in wine grapes is important for the yeast's metabolism and growth, playing a key role in both grape and wine quality. Besides research in grapes and derived products, few studies are present in the literature on the characterization of the amino acid content of wine industry by-products. In this context, this preliminary research aims at the application of a methodological approach for the identification and quantification of amino acids profile from grape by-products (red and white skins, red and white defatted grape seeds) by HPLC-FLD analysis. The most abundant amino acid in red skins was leucine (3.163 g/100 g), while in white skins was histidine (1.886 g/100 g). Higher amounts of leucine, histidine and phenylalanine were found in red seeds, compared to white seeds. By-products of the wine industry, due to the presence of essential amino acids, together with other bioactive substances contained, can be used as functional ingredients for food fortification.

1. Introduction

The exponential growth of the world population and its negative environmental impact motivate the search for alternative protein sources for the human diet. Today, many of the environmental and economic interests are aimed at valorising the waste and by-products of the agri-food industry generated during the production chain [1]. With a view to sustainability and a zero-waste circular economy, in recent years new strategies have been developed for the recovery of waste from mechanical, chemical, or biological processes in the agro-industrial field. The circular economy plays a fundamental role in their valorization, promoting their sustainability and recovery for the production of new products and applications [2,3], in line with the 2030 Agenda which has the objective of conducting eco-sustainable economic activities aimed at a "zero-waste" society [4]. According to the "new Action Plan for the Circular Economy" of the European Commission, "for citizens, the circular economy could provide high-quality, functional and safe products".

In addition, agrifood chain by-products are rich in bioactive compounds; their reuse reduces problems of disposal in the perspective of an integrated valorization of biomass and virtuous and sustainable development paths [5]. Therefore, the circular economy can be defined as a model, in which the by-products are resources to be valorised and recycled, to obtain bioactive compounds such as amino acids and essential fatty acids, carbohydrate polymers, and substances with antioxidant action, and must be implemented expeditiously/ promptly across all fields to consider waste not as a problem but as a resource to be exploited, thus reducing the use of materials prime.

Animal proteins, most requested by consumers, have a worse environmental impact; this aims to find strategies to increase the intake of plant proteins and to seek alternative nutritional sources with the same protein quality. In recent years, alternative and sustainable plant-protein resources have been evaluated, including those coming from agro-industrial by-products and waste, which represent an excellent alternative for human nutrition and can also be used as food supplements [6].

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Protein and amino acid content in terms of essential and non-essential amino acids in agroindustrial by-products and their potential for human nutrition have recently been evaluated [7]. Today, consumers are showing a growing interest in plant proteins that replace animal proteins. Vegetable proteins can represent an economical alternative for fortifying foods by incorporating unconventional protein sources available in large quantities [8-11]. Non-traditional sources were therefore presented as having potential for human nutrition [12-16]. Plant proteins, even those derived from agroindustrial by-products, are good and potential sources of many essential amino acids and macronutrients. For these reasons, they represent valid alternatives to animal protein sources. A recent study showed that watermelon, pumpkin and paprika seeds could be used as a protein source for human consumption and for food fortification. Pumpkin meal contains up to 70 % protein, which is often discarded, with high protein digestibility [17]. Sunflower waste cake and its protein content were recently studied, and high protein digestibility was observed [13,18]. Rapeseed flour (a waste resulting from oil extraction) includes approximately 40 % protein, rich in lysine and sulfur amino acids with high nutritional value [19]. Another work showed that by-products of peanut oil extraction could reach 50 % protein content [20]. Peanuts, soybeans, cotton, hazelnuts and walnuts, grape, hemp, cotton, sunflower, sesame and canola seeds are the main oilseed crops that contain a high percentage of protein meals. The protein content varies depending on the processing of the oilseed flours, in fact, the hulled flours have a higher protein content and a lower fiber content [21]. By-products of cereals and legumes processing are also important for protein extraction and isolation. Rice bran is the most important protein source among cereals, as well as wheat bran, spent beer cereals and defatted wheat germ [22]. Commercial milling of legumes also produces approximately 25 % byproducts consisting of dust, peel, broken, shrivelled, and unprocessed seeds that have high nutritional value [23].

Vitis vinifera currently occupies the first place among the most cultivated fruit species and about 80 % of the total harvest is dedicated to the production of wine. World grape production in 2014 stood at 75 million tons, and global wine production turned up 270 million hectoliters (mhl); of these 170 mhl are produced in Europe while Italian production stands at 44.2 mhl [1,24]. Based on the information collected on 91 countries, which account for 94 % of the global production in 2022, worldwide production in 2023 is estimated between 241.7 mhl and 246.6 mhl, while in Italy 43.9 mhl [25].

In light of this, the wine chain produces enormous quantities of waste including pomace, grape seeds, stems, leaves, lees, and cellar waste [26] Grape pomace is the solid residue derived from the processes of pressing and fermentation which constitutes 20–25 % w/w (weight/weight) of the total weight of the grape utilized for the production of wine. On average, for each hectoliter of wine, 20 kg of pomace and 3.85 kg of stems are produced [27-29].

Starting from these data, it is easy to understand how the wine industry generates huge quantities of waste every year with environmental and economic impacts. For this reason, it is important to adopt recovery and enhancement strategies for wine waste [28,29]. Grape by-products and waste from wine-making processes represent a rich source of compounds of nutritional and nutraceutical interest, of high-value bioactive compounds such as fibers, proteins, lipids, minerals and phenols, of metabolites such as anthocyanins, catechins, stilbenes, resveratrol and condensed tannins, characterized by antioxidant and antimicrobial properties [30,31]. All these bioactive compounds could be reused for the food industry and the enrichment of products included in the Mediterranean diet. It is estimated that about 70 % of the phenolic compounds are still present in the pomace [32]. For all of these reasons, the wine by-products represent an important waste-to-value, so a product with added value [33]. Among the bioactive constituents, high-value polyphenolic compounds such as gallic acid or ellagic acid, catechin, caffeic acid, and resveratrol play an important role in the reuse of grape pomace [34,35]. Other polyphenolic substances, as well as natural fibers, tannins, proteins and carbohydrates are in grape seeds [36]. This component of grapes has greater application importance because, from seeds, an oil with great nutritional value is extracted and widely used in the industrial, cosmetic and food fields. Grape seed oil is particularly rich in polyunsaturated fatty acids such as linoleic acids [37, 38]. In the study by Boso et al., the flavanol content of seed waste resulting from the production of white and red wines from grapes grown in northern Spain was examined. Procyanidin B1 and procyanidin B2 dimers were mostly highlighted, especially in the skins and seeds of white grapes [39].

Another reason that encourages to use of the by-products of the wine industry, concerns the presence of antioxidant dietary fiber in the pomace. Cronin et al. valuated foods rich in fiber; these are extremely important in the human diet as their intake gives benefits to the body, such as maintenance of the health of the gastrointestinal tract, weight control and improvement of the intestinal microbiota [40]. Another factor of importance is the content of nitrogen which plays a key role in determining the quality of grape and wine and has an influence on aromas during the maturing process [41]. Few studies are present in the literature on the characterization of the amino acid content of grape by-products [42,43].

The consumption of proteins of vegetable origin is growing increasingly [44] and food fortification represents a key way to combat malnutrition [45]. Finding adequate amounts of protein to feed humans and animals, without intensifying the overall environmental impact has become an important goal.

In this context, this research aims at the application of a methodological approach for the identification and quantification of amino acid profiles from grape by-products, by High Performance Liquid Chromatography - Fluorescence Detector (HPLC-FLD).

2. Materials and methods

2.1. Chemicals and reagents

For the pre-column derivatization process, the following reagents were used: hydrochloric acid 37 %, acetonitrile, acetone, heptylamine, *n*-hexane, 9-fluorenylmethoxycarbonyl chloride, (FMOC—Cl), boric acid, sodium chloride and sodium tetraborate acquired from Merck (Darmstadt, Germany). For the identification of individual amino acids a mix of 17 Amino acid standards containing L-alanine, L-arginine, L-aspartic acid, L-cystine, L-glutamic acid, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine and L-valine (0.5 μmol/L except L-cystine at 0.25 μmol/L, Supelco Bellefonte, PA, USA), L-tryptophan, L-asparagine, and L-glutamine pure standards (acquired from Merck) were used. Formic acid, acetonitrile and HPLC-grade water (Milli-Q Gradient A10 system, Millipore, Bedford, MA, USA) were used as mobile phases for HPLC separation.

2.2. Material for analysis and sample preparation

Red grapes var. “Sangiovese” and a mixture of white grape varieties “Trebiano” 25 %, “Catarratto” 20 %, “Insolia” 20 % and “Grillo” 20 % used to produce wine, were collected from a local winery in western Sicily (Italy). Grapes come from the provinces of Trapani, Agrigento and Palermo (100–250 m above sea level), cultivated in sunny conditions, mild temperatures and moderate ventilation. The harvest period goes from the end of August to the end of September 2020 at technological maturation. The harvested white grapes were subjected to “white fermentation” which involves the immediate separation of the must from the skins. Red grapes, however, were left in contact with the skins for a prolonged time, and subsequently separated. Grape pomace samples were manually separated into skins and seeds through a sieve. The grape seeds (from red and white grapes) were separately dried at a temperature of 24 °C for four days to reduce the humidity. Then they

were gradually put into an oil automatic cold press apparatus (Cgoldenwall CAN-684) for seed oil extraction. The defatted grape seeds (red and white respectively RSE and WSE) were ground until obtaining a fine powder. On the other hand, red skins (RSK) and white skins (WSK) were dried at 55 °C until completely dry (two days) and pulverized until fine powder was obtained.

2.3. Sample treatment, qualitative and quantitative determination of amino acids - derivatization process

Before the analysis of the amino acids, the hydrolysis of the proteins contained in samples was indispensable. The individual amino acids, in free form, were then chromatographically separated by HPLC-FLD method, identified and then quantified. The analysis of amino acids requires a pre-column derivatization process [46,47]. The derivatization method employed 9-fluorenylmethoxycarbonyl chloride (FMOC—Cl). The separation of the derivatized amino acids was performed on reversed-phase HPLC columns and the quantification was performed based on their fluorescent properties.

Approximately 1 g of sample (respectively Red defatted grape SEeds RSE, White defatted grape SEeds WSE, Red SKins RSK and White SKins WSK) was put to react in a clean hydrolysis tube with 1 mL of 6 M HCl at 110 - 120 °C for 24 h. After cooling, each sample was evaporated until to dryness at 60 °C. The residue was dissolved in 2 mL of high-purity water and filtered by 0.45 μm polytetrafluorethylene (PTFE) syringe filters. The aqueous mixture containing the amino acids in free form was then derivatized at room temperature with FMOC—Cl. Fifty μL of aqueous solution containing free amino acids were added to 200 μL of a borate buffer solution (pH 8) and 200 μL of a 3 mM FMOC—Cl solution in acetone. The resulting solution was heated to 70 °C for 10 min. It was necessary to eliminate the excess of FMOC—Cl by adding 50 μL of heptylamine solution (3 mL of heptylamine, 15 mL of acetonitrile (ACN) and 175 mL of 0.1 M HCl, pH 9) and then vortexed to 3 min. Eighty μL of the solution was withdrawn and 320 μl of ACN and 600 μl of hexane were added. Finally, 20 μL of the final solution containing the derivatized amino acids was injected into HPLC-FLD. Data were collected in triplicate and reported as mean ± *S*.D.

HPLC instrument from Agilent HPLC 1100 Series (Waldbronn, Germany), equipped with a binary pump (G1312A), a fluorescence detector (G1321A) and a PC with Agilent Chemstation software, were used to obtain the HPLC-FLD chromatographic amino acids fingerprints in grape by-products. Chromatographic separation was performed using Discovery HS C18 analytical column (150 mm x 4.6 mm i.d., 3.5 μm particle size) (Supelco, Bellefonte, PA, USA) fitted with guard column under gradient elution conditions employing 0.1 % formic acid aqueous solution (solvent A) and acetonitrile (solvent B) as the mobile phase components. The column operated at 40 °C. The program of gradient elution was as follows: 0–10 min 3 % B; 3–17 min linear increase to 10 % B; 17–47 min linear increase to 50 % B; 47–57 min linear increase to 100 % B, 57–60 min, hold 100 % B; 60–63 min return to the initial conditions and being equilibrated. The injection volume was 20 μL and the mobile phase flow rate was 1 mL min^{-1}. Amino acid chromatographic separation was recorded at an excitation wavelength of 254 nm and an emission wavelength of 330 nm.

For peak identification, the comparison between the retention time of the standards of the amino acids and those in samples was carried out and also fortification technique (spiking) was applied. For quantitation, an external standard method using calibration curves fitted by linear regression analysis was utilized. The results were expressed in grams of amino acids on 100 gs of sample. Standard mix solutions of amino acids were prepared in concentrations from 0.1 μmol/L to 1 μmol/L and from 0.25 μmol/L to 1.87 μmol/L for tryptophan, asparagine and glutamine, which were analyzed in triplicate.

Linearity was determined by the least square regression (R^2). For all standard calibration curves, the coefficient of regression exceeded 0.934. Amino acid derivatives showed good linearity under the proposed conditions (Table S1).

2.4. Statistical analysis

Statistical analysis of amino acid content was carried out using an unpaired student's *t*-test. A *p*-value less < 0.05 is statistically significant.

3. Results and discussion

The amino acids profile of Red and White SKins (RSK and WSK, respectively) and Red and White defatted grape SEeds (RSE and WSE, respectively) was experimentally determined through the use of a chromatographic technique with a fluorescence detector (HPLC-FLD) and expressed as g of amino acid on 100 g of sample ± SD, as reported in Table 1 and Table 2.

Analyzes of amino acids profile carried out on red and white skins (Table 1) showed higher total amino acid values for RSK (11.782 ± 0.366 g/100 g) compared to WSK (8.253 ± 0.279 g/100 g).

The content of essential amino acids reflects the same trend as total amino acids. Higher values were highlighted in RSK compared to WSK (5.072 g/100 g and 1.925 g/100 g, respectively).

A substantial difference between RSK and WSK concerns the concentration of leucine, an essential amino acid, found to be more abundant in RSK (3.163 g/100 g) compared to white skins (1.015 ± 0.020 g/100 g). In WSK the amino acid at the highest concentration was histidine (1.886 g/100 g). Furthermore, RSK showed higher amounts of cystine (1.71 g/100 g), phenylalanine (0.894 g/100 g), and lysine (1.015 g/100 g) compared to WSK, which showed higher values of alanine (1.028 g/100 g), arginine (0.099 g/100 g), asparagine (0.754 g/100 g) and glutamine (1.153 g/100 g).

Non-quantifiable traces of serine, aspartic acid, glutamic acid, threonine, isoleucine, tyrosine, methionine, tryptophan, and proline were found in both types of samples.

The reported values were a bit different from those reported in the literature, such as Chikwanha et al. work [48], which analyzed pomace powder from three varieties of *Vitis vinifera*: two with red grapes ("Pinotage" and "Shiraz") and one with white grapes ("Sauvignon Blanc"). They recorded glutamine values of 1.77 - 1.98 g/100 g for the red grape skin powder and 3.88 g/100 g for the white grape skin powder, higher values found in this study. Also in their work, lower quantities of alanine (0.37 - 0.41 g/100 g) and lysine (0.34 - 0.38 g/100 g) in red grape skins flour, and lower quantities of asparagine (0.19 g/100 g), histidine (0.19 g/100 g) and leucine (0.60 g/100 g) in white grape skins powder were detected. From this comparison, data obtained from analysis of winery by-products seem very promising regarding total amino acid content.

Table 1
Amino acids profile of Red skins (RSK) and White skins (WSK). The data reported corresponds to the mean ± *S*.D. which was determined from triplicate experiments, ($n = 3$). * Level of significance of unpaired-samples t –test. *p*-value less $<$ 0.05 is statistically significant.

Amino acids	RSK	WSK	t student test
	g/100 *g* ± *S*.D.		*p* value*
ARGININE	0.004 ± 0.001	0.099 ± 0.003	0.0001
GLUTAMINE	1.311 ± 0.11	1.153 ± 0.082	0.1168
ASPARAGINE	0.889 ± 0.080	0.754 ± 0.076	0.1015
ALANINE	0.944 ± 0.019	1.028 ± 0.021	0.0068
LEUCINE	3.163 ± 0.053	1.015 ± 0.020	0.0001
PHENYLALANINE	0.894 ± 0.018	0.188 ± 0.005	0.0001
LYSINE	1.015 ± 0.020	0.722 ± 0.015	0.0001
HISTIDINE	1.848 ± 0.034	1.886 ± 0.030	0.2203
CYSTINE	1.714 ± 0.031	1.408 ± 0.027	0.002
TOT. AA	*11.782 ± 0.366*	*8.253 ± 0.279*	*0.0003*
TOT. ESSENTIAL AA	*5.072 ± 0.091*	*1.925 ± 0.04*	*0.0001*

Table 2
Amino acids profile of Red defatted grape SEeds (RSE) and White defatted grape SEeds (WSE). The data reported corresponded to the mean ± *S*.D. which was determined from triplicate experiments, ($n = 3$). * Level of significance of unpaired-samples t–test, *p*-value less < 0.05 is statistically significant.

Amino acids	RSE	WSE	t student test
	g/100 *g* ± *S*.D.		*p* value*
ALANINE	1.080 ± 0.021	1.080 ± 0.016	1.000
LEUCINE	0.887 ± 0.018	0.680 ± 0.014	0.0001
PHENYLALANINE	0.030 ± 0.001	0.003 ± 0.001	0.0001
HISTIDINE	2.031 ± 0.037	1.870 ± 0.034	0.0052
CYSTINE	1.356 ± 0.062	1.436 ± 0.030	0.1937
TOT. AA	*5.448 ± 0.139*	*5.070 ± 0.095*	*0.0177*
TOT. ESSENTIAL AA	*0.917 ± 0.019*	*0.683 ± 0.015*	*0.0001*

Table 2 shows the amino acid profile of Red defatted grape SEeds (RSE) and White defatted grape SEeds (WSE).

HPLC-FLD analyzes of the defatted seeds showed a lower concentration of amino acid profile compared to the skins. Total essential amino acid content values in RSE and WSE were 0.917 g/100 g and 0.683 g/100 g, respectively. Histidine was the amino acid present in the greatest quantity, 2.031 g/100 g and 1.87 g/100 g for RSE and WSE, respectively.

Higher amounts of leucine (0.887 g/100 g) and phenylalanine (0.03 g/100 g) were found in RSE compared to WSE. Cystine was higher in WSE (1.436 ± 0.030 g/100 g). In conclusion, data evaluation shows no quantitative difference in alanine concentration (1.080 g/100 g) in both RSE and WSE.

The total amino acid content found was 5.448 g/100 g and 5.070 g/100 g in RSE and WSE, respectively. These data were slightly lower than those obtained by Alvarez-Ossorio et al. [49] in the analysis of the amino acid profile carried out on grape seed powder from Spanish cultivars. Also, Chowdhary et al. [50] highlighted a higher protein content in grape seed powder, ranging from 13 to 20 %, with a high content of essential amino acids such as lysine.

However, in winery by-products an important amino acid profile including essential amino acids has been found, making them a potential high-quality plant-based protein source. The results highlighted quantities of leucine in red skin from winery by-products equal to 3163 g/100 g. This amino acid in combination with the energy availability signal from insulin stimulates muscle protein synthesis. In all other samples histidine and cystine were the most represented amino acids. Histidine plays particularly important roles in the active site of enzymes, and cystine serves as a site of redox reactions and a mechanical linkage that allows proteins to retain their three-dimensional structure.

The amino acid profile studied, with the presence of other bioactive substances such as essential fatty acids and polyphenols, make these wine production wastes a rich source of nutritional and nutraceutical interest compounds, characterized by antioxidant and antimicrobial properties [51].

Feed fortification in broiler feed has been studied, demonstrating that these by-products have the potential to improve certain blood parameters without affecting animal performance [52].

5. Conclusions

Every year, the production of wine and grape juices gives rise to tons of pomace and grape seeds as by-products. This preliminary research represents one of the first studies on amino acid characterization of grape by-products. A methodological approach by HPLC-FLD was assessed and applied. Total amino acids content in RSK and WSK were found 8.253 g/100 g and 11.782 g/100 g, respectively. HPLC-FLD analyzes of seeds showed an amino acid profile that is less rich than skins (5.448 g/100 g and 5.070 g/100 g in RSE and WSE, respectively). Essential amino acids such as lysine, leucine, and phenylalanine, together with other bioactive substances contained, make these by-products useful for technological functionalities. Wine industry waste can be used as food ingredients, thickeners, gelling agents, and fillers, as well as in the production of edible films.

In summary, the increase in demand for plant-based proteins is expected to be a consolidated trend in the coming years, as the international community searches for new sources of protein to meet the needs of a growing population. The healthy properties described for the by-products of wine processing, together with the high abundance of phytochemical substances with antioxidant and anti-inflammatory activity, make these by-products ideal candidates for human and animal food fortification.

CRediT authorship contribution statement

Carla Buzzanca: Writing – original draft, Supervision, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Manuela Mauro:** Supervision, Formal analysis. **Mirella Vazzana:** Supervision, Data curation. **Aldo Todaro:** Writing – original draft, Validation, Methodology, Data curation. **Vincenzo Arizza:** Writing – review & editing, Conceptualization. **Massimo Lucarini:** Writing – review & editing, Data curation. **Alessandra Durazzo:** Supervision, Conceptualization. **Vita Di Stefano:** Writing – original draft, Supervision, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.meafoo.2024.100174.

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