

Variation of Polyphenols, Anthocyanins and Antioxidant Power in the Strawberry Grape (*Vitis labrusca*) after Simulated Gastro-Intestinal Transit and Evaluation of *in Vitro* Antimicrobial Activity

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ABSTRACT

The influence of a simulated digestive process on some biochemical and biological aspects of strawberry grape (*Vitis labrusca*) was investigated. The amount of total polyphenols and anthocyanins as well as the antioxidant power were evaluated. Results evidenced that the simulated gastrointestinal transit caused a decrease of the polyphenols content and total anthocyanins; these last, however, were more resistant than polyphenols, decreasing only of 50% respect to the initial value (31.50 µg/ml of extract). The extract exhibited an excellent antioxidant power (EC₅₀ 3.8 mg/ml), which decreased of about four times after the simulated gastrointestinal transit. The antimicrobial activity of the extract, evaluated against three *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus cereus* pathogen strains was enhanced by the simulated digestion, with an increase of the inhibition halo.

KEYWORDS

Strawberry Grape; Antioxidants; Polyphenols; Anthocyanins; Antimicrobial Activity

1. Introduction

Strawberry grape (*Vitis labrusca*) is the most ancient “American grape” introduced in Europe. Although it is poor resistant to fungi attacks, is very resistant to cold. For this reason, strawberry grape is diffused in most of the Italian territory [1,2]. Bunch is formed by several grapes linked to the stalk, which are characterized by an intense dark violet-black color, with an unmistakable strawberry aroma and taste. Strawberry grape contains several nutritionally relevant elements, such as vitamins, minerals, carbohydrates, fibers and phytochemicals. Polyphenols are the most abundant phytochemicals and confer to the product many beneficial effects. They include mainly proanthocyanidins, anthocyanins, flavanols, flavonols, and phenolic acids. Anthocyanins, being re-

sponsible of pigmentation, are present mainly in the skin, conversely to flavonoids, generally distributed into the pulp, in the seeds and into stalks, and represented basically by catechin, epicatechin, and polymers of procyanidin.

Polyphenols represent a complex family of about 5000 organic molecules, diffused in the vegetal kingdom. Their main feature, from a structural point of view, is represented by the presence of more phenolic groups, organized in more or less complex structures, generally with high molecular weight. The number and the characteristics of such structures affect also the physical, chemical and biological (metabolic, toxicological, antimicrobial, therapeutics, etc.) properties of the different classes of polyphenols.

Anthocyanins, water-soluble pigments, belonging to flavonoids, are present both in flowers and in fruits.

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Their color can vary from red to blue, depending on the environmental pH and on the linkage with heavy metals present in the vegetal tissues. Like all polyphenols, anthocyanins are essential for the physiology and biology of plants. They represent also a mechanism of defense of plants against biotic and abiotic stress. From a food and sensorial point of view, polyphenols and anthocyanins affect the acceptance and stability of food in terms of antioxidants and stabilizers, and influence their aroma. Some studies ascertained an inverse relationship between the consumption of grape and its derivatives and human mortality linked to pathologies such as cardiovascular diseases. These compounds have antioxidant, anticancer, anti-inflammatory, antimicrobial, anti-age properties [3]. Different studies demonstrated that a diet rich in grape juice, then rich in polyphenols, induced the platelet aggregation in rats and rabbit, as well as reduced the production of superoxide anion. A diet containing grape juice induces, after 15 days, an enhancement of the lipid profile, with an increase of HDL and a reduction of the oxidized LDL [4-6]. The antitumor effect is demonstrated, for example, *in vitro*, with an increase of the apoptosis events taking place in the cell lines of different kinds of cancer [6]. Anthocyanins, isoflavones and tannins from *Vitis labrusca* exhibit an antiseptic power and can be used as preventing agents of tooth decay [7]. The bioactive components of grape skin can be used for the development of functional foods or to enrich and fortify fruit juices without negatively affecting their sensorial characteristics [6]. Only in recent years something is known about the phytochemical composition of strawberry grape. An HPLC-DAD-ESI-MS study of the most important low molecular weight phenolic compounds (anthocyanins, pyranoanthocyanins, flavonols, and hydroxycinnamic acid derivatives) in hybrid grape cultivar Isabel (*Vitis labrusca*) red table wines was performed to look for differences between these and *V. vinifera* red wines [2]. A metabolic profiling of strawberry grape (cv. "Isabella") components by Nuclear Magnetic Resonance (NMR) was performed [1] and was also assessed the antioxidant and anti-proliferative properties of peel, pulp, seed, leaf, and stalk components of the plant. At least at our knowledge, nothing is known about the biochemical and biological changes of the extracts of strawberry grape giving rise after the digestion. In this study we evaluated the fate of polyphenols and anthocyanins after a simulated digestion performed on the extracts of the strawberry juice. The antioxidant activity and antimicrobial properties were also evaluated.

2. Materials and Methods

Organic strawberry (*Vitis labrusca*) was provided by an experimental plant located in Torre Annunziata (Naples,

Italy). Grapes were gently cleaned, weighted and soon used for the experiments.

2.1. Extraction of Polyphenols and Anthocyanins

Polyphenols were extracted by homogenization of 60 g of strawberry with 120 ml of ethanol 50% and keeping the resulting mixture for 24 h at room temperature into the dark. After filtration (0.80 µm Millipore, Milano, Italy), supernatant was recovered and concentrated to eliminate the alcoholic fraction, and stored at -30°C. Anthocyanins were extracted by mixing 60 g of strawberry with two volumes of methanol acidified with 0.1 M HCl. After incubation at room temperature into the dark, the supernatant was recovered and concentrated under air flow to eliminate the alcoholic fraction.

2.2. Simulation of Gastro-Intestinal Passage

The simulated digestive transit was performed following the method of [8] with some modifications. Briefly, artificial gastric juice was manufactured dissolving 3 g/l of pepsin in physiological solution at pH 2.0; pancreatic solution was prepared by dissolving 1 g/l pancreatin and 4.5 g/l bile salts in physiological solution; both solutions were sterile-filtered (0.22 µm, Millipore SpA, Milano, Italy). Strawberry extract was incubated in two volumes of gastric juice and kept at 37°C for 180 min; then, two volumes of pancreatic juice were added and the resulting solution was kept at 37°C for 60 min. Samples were centrifuged (15.700 × g, Biofuge Beckman Cassina de Pechi, Milano, Italy) and supernatants were recovered and frozen at -30°C until the analyses.

2.3. Biochemical Analysis

2.3.1. Colorimetric Analysis of Total Phenolics

The total phenolics present in strawberry grape were measured [9] using the Folin-Ciocalteu reagent and quercetin as a standard. The absorbance was evaluated at room temperature at $\lambda = 760$ nm using a Cary 50 Uv/Vis spectrophotometer (Varian-Agilent Italia, Cernusco sul Naviglio, Italy). Quantification was based on a standard curve generated with gallic acid. The results were expressed as the µmol of gallic acid equivalent (GAE)/g of sample.

2.3.2. Anthocyanin Content

The amount of anthocyanins present in strawberry grape was determined following the differential pH method [10,11]. Absorbance was measured with a UV-visible spectrophotometer (Cary 50 Uv/Vis, Varian-Agilent Italia, Cernusco sul Naviglio, Italy) simultaneously at $\lambda = 420$ and $\lambda = 700$ nm in buffers at pH 1.0 and 4.5, and the formula, $A = (A_{420} - A_{700})_{\text{pH 1.0}} - (A_{420} - A_{700})_{\text{pH 4.5}}$, was

used. A molar absorption of 26,900 l/mol cm was used for cyanidin-3-glucoside (molecular weight 449.2 g/mol). The results were expressed as milligrams of cyanidin-3-glucoside equivalents (C3GE)/g of sample. Three replicates were performed for each analysis.

2.3.3. Free Radical Scavenging Capacity

The free radical scavenging capability of the extract was determined using the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [12]. The analysis was performed in microplates by adding 7.5 μ L of extract to 303 μ L of a methanolic DPPH solution (153 mM). Next, the absorbance at $\lambda = 517$ nm was measured (Cary 50 MPR, Varian, USA). The absorbance of DPPH without antioxidant (control sample) was used as for baseline measurements. The scavenging activity was expressed as the 50% effective concentration (EC₅₀), which was defined as the sample concentration (mg) necessary to inhibit the DPPH radical activity by 50% during a 60-min incubation. These experiments were performed in triplicate, and the results are expressed as the mean values \pm standard deviation.

2.3.4. Chromatographic Analysis

An ACQUITY Ultra Performance LCTM system (Waters, Milford, MA, USA) linked to a PDA 2996 photodiode array detector (Waters) was used for ultra high-performance liquid chromatography (UPLC) analysis of polyphenols extracts [13]. Empower software was used to control the instruments and for data acquisition and processing. The extracts and the standards (previously dissolved in methanol) were filtered (0.45 μ m, Waters, Milford, MA, USA) before analysis. The analyses were performed at 30°C using a reversed phase column (BEH C18, 1.7 μ m, 2.1 \times 100 mm, Waters). The mobile phase consisted of solvent A (7.5 mM acetic acid) and solvent B (acetonitrile) at a flow rate of 250 μ L·min⁻¹. Gradient elution was employed, starting with 5% B for 0.8 min, then 5% - 20% B over 5.2 min, isocratic 20% B for 0.5 min, 20% - 30% B for 1 min, isocratic 30% B for 0.2 min, 30% - 50% B over 2.3 min, 50% - 100% B over 1 min, isocratic 100% B for 1 min, and finally 100% - 5% B over 0.5 min. At the end of this sequence, the column was equilibrated under the initial conditions for 2.5 min. The pressure ranged from 6000 to 8000 psi during the chromatographic run. The effluent was introduced into an LC detector (scanning range: 210 - 400 nm, resolution: 1.2 nm). The injection volume was 5 μ L.

2.4. Antibacterial Activity

The inhibition halos test on agar plate was employed to investigate the antibacterial activity of the anthocyanins extracts before and after the simulated digestive process.

Samples were tested against the pathogenic Gram positive strain *Bacillus cereus* (DSM 4313) and Gram negative strains *Escherichia coli* DSM 8579 and *Pseudomonas aeruginosa* (ATCC 50071). All strains were purchased by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ Germany). Each strain was incubated at 37°C for 18 h into in Tryptone Yeast Extract (Oxoid, Milano, Italy). The microbial suspensions (1×10^8 Colony Forming Units-CFU-/mL) were uniformly spread onto the tryptone yeast extract agar plates ($\varnothing = 90$ mm dishes). Sterile Whatman n° 1 paper filter discs ($\varnothing = 5$ mm) were individually placed on the inoculated plates and impregnated with different amounts of the extracts, previously diluted 1:10 (v/v) in dimethylsulfoxide (DMSO) (final amount ranging from 0.1 to 0.3 μ g/paper disc). After 30 min under sterile conditions at room temperature, plates were incubated at 37°C for 24 - 48 h, depending on the strain. The diameter of the clear zone shown on plates was accurately measured and the antibacterial activity expressed in cm (not including disc diameter of 0.5 cm). DMSO (10 μ L/paper disc) was used as negative control. Gentamycin (8 μ g/paper disc) and tetracycline (7 μ g/paper disc), previously dissolved in physiological solution, served as positive controls.

3. Results and Discussion

3.1. Biochemical Analyses

Spectrophotometric analyses were carried out to get insight into the polyphenolic and anthocyanins concentration of strawberry grape extract grape. The results are shown in **Table 1**. *Vitis labrusca* exhibited 671.81 μ g GAE/g of product. Such value is lower if compared with other cultivars of *Vitis*, but, with all probability, the different behavior might be ascribable to the environment and to the different method of extraction, including the different temperature used [14]. The *in vitro* digestive process affected the polyphenol amount, with a decrease of nine-times respect to the initial value (**Table 1**), conversely, for example, to the tea extract which, if subjected to the double gastric + pancreatic juices action, exhibited a no so evident reduction in the polyphenols concentration [15], but in accordance with other works which reporting a loss of phenols under duodenal digestion conditions in different food matrices, such as orange juice, frozen strawberries, red cabbage, and chokeberries [16-20].

Anthocyanins exhibited a major resistance so that, after the complete simulated digestive process, the amount of such biomolecules decreased of 50% compared to the initial concentration (15.03 versus 31.50 mg/ml of extract, respectively), testifying that anthocyanins are “exalted”

Table 1. Total Polyphenols (TP), Total Anthocyanins (TA) and antioxidant activity of strawberry grape (*Vitis labrusca* L.).

Table 1	TP ($\mu\text{g GAE/gr} \pm \text{SD}$)	TA ($\mu\text{g/ml} \pm \text{SD}$)	Antioxidant power ($\text{EC}_{50} \pm \text{SD}$)
Before digestion	671.8 \pm 29.1	31.5 \pm 1.7	3.80 \pm 1.6
After digestion	65.5 \pm 0.3	15.03 \pm 0.3	12.92 \pm 1.0

The results of anthocyanins are expressed as milligrams of cyanidin-3-glucoside equivalents (C3GE)/g of sample and represent the mean of three independent experiments \pm Standard Deviation.

by the presence of an acidic environment like the gastric one and that, conversely to other *in Vitro* digestive processes [20], the presence of pancreatin did not negatively modified their amount by modifying their structure. Previous works reported a different influence of the simulated digestion on antioxidant activity and total polyphenols content [21]. In our case, although observing an evident loss of polyphenols, a not so remarkable decrease of anthocyanins was observed, that, in a certain manner, “protected” the antioxidant activity of the extract of strawberry grape, so that, after the simulated digestion, the amount of extract need to inhibit at 50% 1 ml of DPPH was of 12.92 mg, a value that, in our opinion, could be considered interesting, in terms of capability of the extract to act against the attack of free radical action.

UPLC polyphenols profiles of strawberry extract before and after the *in vitro* digestion are shown in Figures 1 and 2, respectively. We identified two phenolic acids, basically gallic and chlorogenic acid, which amount is shown in Table 2. The level of chlorogenic acid decreased from 147 $\mu\text{g/ml}$ to 45 $\mu\text{g/ml}$, with a loss of about three times. The level of gallic acid increased of four times, testifying that the molecule is generally one of the most stable present in the plant extracts also after the digestive process [15]. UPLC allowed us also to identify the presence of the flavone naringenin (2 $\mu\text{g/ml}$) which, however, disappeared completely after digestion, in contrast with the fate of such flavones after simulated gastrointestinal digestion of cocoa. Such contrasting behavior could be probably related to the low content of fat which, high in cocoa, is capable to exert a protective effect on different polyphenols [22].

3.2. Antimicrobial Activity

Plant polyphenols are recognized as well known antibacterial agents [23-26]. Grape wine inhibited mainly *Escherichia coli* growth, with a direct relationship between the microbial inhibition and amount of alcohol-free product [25]. The extracts of alcohol-free red and white wine exhibited antimicrobial activity to some pathogens such as *Staphylococcus aureus*, *Escherichia coli* and *Candida-*

Table 2. Quantitative determination of single polyphenols present in strawberry grape (*Vitis labrusca* L.) by UPLC analysis.

Table 2	Before digestion micrograms/ml \pm SD	After digestion micrograms/ml \pm SD
Gallic acid	13.28 \pm 1.23	51.19 \pm 5.12
Chlorogenic acid	147.73 \pm 15.45	45.17 \pm 3.34
Naringenin	2.07 \pm 0.84	ND

The results are expressed as micrograms of molecules/ml of extract from sample and represent the mean of three independent experiments \pm Standard Deviation.

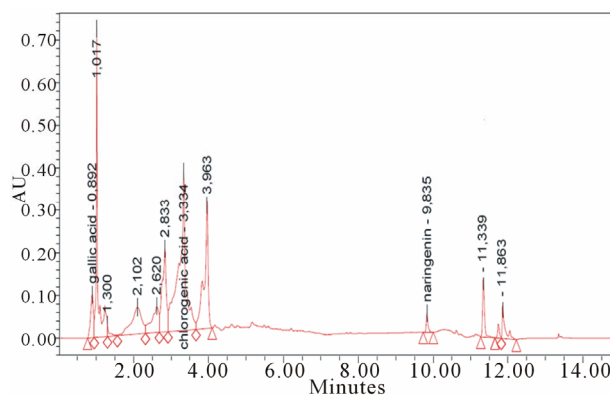


Figure 1. UPLC analysis of polyphenols extract of strawberry grape (*Vitis labrusca* L.) before simulated digestion.

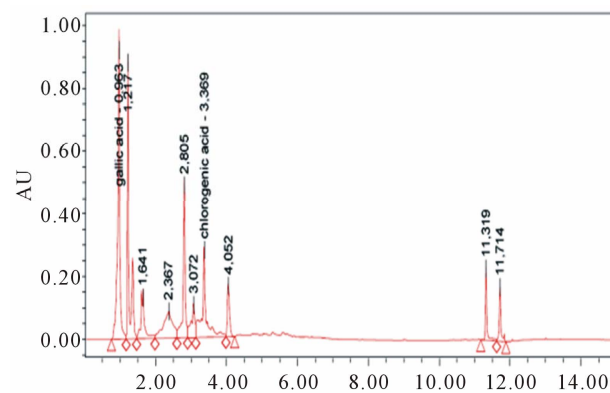


Figure 2. UPLC analysis of polyphenols extract of strawberry grape (*Vitis labrusca* L.) after simulated digestion.

albicans [25]. Due to the evident loss of polyphenols observed after the *in vitro* digestion of the strawberry grape extract, we monitored the antimicrobial activity exhibited by the anthocyanins, before and after the simulated digestive process. Such activity was evaluated using as tester strains the food-borne pathogen *B. cereus* and *E. coli* and against the Gram negative *Ps. aeruginosa*, which is increasingly recognized as an emerging opportunistic pathogen of clinical relevance. The results are shown in Table 3. The extract was capable to inhibit the

Table 3. Antimicrobial activity of anthocyanins extracted from strawberry grape (*Vitis labrusca* L.) against *B. cereus*, *E. coli* and *Ps. aeruginosa* measured before and after simulated digestion.

Table 3	<i>B. cereus</i>	<i>E. coli</i>	<i>Ps. aeruginosa</i>
Before digestion			
(0.162 µg)	0.21 ± 0.12	0.42 ± 0.12	0.20 ± 0.12
(0.323 µg)	0.53 ± 0.12	1.25 ± 0.12	0.7 ± 0.12
After digestion			
(0.162 µg)	0.72 ± 0.12	0.45 ± 0.12	0.68 ± 0.12
(0.323 µg)	1.6 ± 0.12	1.2 ± 0.12	1.28 ± 0.12
Gentamycin 8 µg	1.77 ± 0.12	1.57 ± 0.12	1.53 ± 0,06
Tetracycline 7 µg	1.03 ± 0.06	1.27 ± 0.12	0.97 ± 0.06
DMSO	-	-	-

The activity was measured in cm. the data are reported as cm of inhibition halo ± Standard Deviation.

growth, on plate, of all pathogen strains. Before the simulated digestion, the most sensitive strain was *E. coli*: just 0.16 µg of total anthocyanins were, in fact, capable to create a inhibition halo of 0.42 cm; this increased up to 1.25 cm with the double concentration. Such result was in accordance with [26], which observed a inhibition halo ranging from 1.6 to 2.2 cm. *B. cereus* and *Ps. aeruginosa*, although more resistant, were on the other hand inhibited by the two concentrations of anthocyanins used, with halos ranging from 0.2 cm until 0.7 cm. After the simulated digestion, the antimicrobial activity exhibited by the anthocyanins extracts was different. The action against *E. coli* was the same respect to that observed before treatment of extract with gastrointestinal juice: in fact, the dimensions of the inhibition halos arrived to be 0.45 cm and 1.2 cm, using 0.16 and 0.32 mg/ml of extract, respectively. Interestingly, the antimicrobial activity of the extract seemed to be enhanced after the digestive process against *B. cereus* and *Ps. aeruginosa*. In fact, the test performed using 0.32 mg of extract against *B. cereus* gave rise to inhibition halos of 1.6 cm, very closer to 1.77 cm, that is the dimension of the inhibition halo formed by using 8 µg of gentamycin; the activity was indeed superior to that exhibited by 7 µg of tetracycline. This unexpected result let us open new and interesting windows in the search of new natural biocides to be used for food or safety purposes. *Ps. aeruginosa* was more sensitive to the action of the digested extract too, giving inhibition halos ranging from 0.68 to 1.28 cm, this last value still superior respect to that obtained using tetracycline too.

4. Conclusions

Strawberry grape, like all purple foods, has all potentialities to be considered widely beneficial for human health. Our attention was called mainly on amount of its well known bioactive components polyphenols and anthocya-

nins. We thought to evaluate such aspects before and after a simulated digestive process, and try to contribute towards a better knowledge of such phytochemicals when ingested. The peculiar features of strawberry grape can be thus resumed:

- The product has a good amount of polyphenols, in particular anthocyanins;
- It has a good antioxidant power which is enough preserved also after the simulated digestion;
- Anthocyanins are capable to exert an inhibitory effect against Gram positive and Gram negative pathogens, such as *E. coli*, *Ps. aeruginosa* e *B. cereus*. Such effect is enhanced after digestion and should be further studied to know the molecular mechanisms of interactions between polyphenols and bacteria which give rise to an enhanced antimicrobial activity.

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