

Phenolic Compounds in Red Fruits Produced in Organic Farming at Maturation Stage

Susana M. A. Soutinho, Raquel P. F. Guiné, António M. Jordão, and Fernando J. Gonçalves

Abstract—The agricultural organic farming is different from conventional farming in a way that is aimed at providing a balanced and constructive action in agricultural systems. With the increase in intensive agriculture, undesirable changes were being observed in ecosystems with irreparable damage being caused to the natural equilibrium. This is the reason for the increasing interest in organic farming as an environment friendly agricultural production method. In the present work three red fruits produced in organic farming were analyzed, namely raspberry, gooseberry and blueberry. The samples were harvested in a local farm when at plain maturation. The results obtained allowed to conclude that the blueberry contained higher amounts of phenolic compounds, total tannins and total anthocyanins than raspberry and gooseberry. Furthermore, the HPLC analysis allowed to identify monomeric anthocyanins and phenolic acids in the three fruits studied.

Keywords—Blackberry, gooseberry, organic farming, phenolic compounds, raspberry.

I. INTRODUCTION

ORGANIC farming is distinguished from other production systems because it excludes almost all synthetic chemicals, uses cultural rotations, crop residues, animal manure, and all organic waste from the farm.

The red fruits are fairly complete foods and should be part of a balanced diet. Recent studies [1] show that the group of small fruits possesses characteristic and specific contents of phenolic compounds, consisting essentially of anthocyanins, flavonols, proanthocyanidins and phenolic acids, catechins and isoflavones, compounds known for their high antioxidant capacity [2].

The extraction is the main step in the recovery, isolation, characterization, and quantification of individual and total polyphenolics from various plant-based materials. Phenolic compounds are often most soluble in solvents less polar than water. The most common solvents are methanol, ethanol, acetone, ethyl acetate, and their aqueous solutions. In this work three red fruits, raspberry, gooseberry and blueberry, produced in organic farming, were characterized in relation to

their phenolic composition.

II. EXPERIMENTAL PROCEDURE

A. Sampling

The fruits studied in this work were raspberry, gooseberry and blueberry, all produced in organic farming and provided by a local producer. The samples were harvested for analysis at complete maturation stage.

B. Extraction Procedure

The extraction of phenolic compounds from red fruits was carried out successively, three times with methanol and then three times with acetone solution (40%, v/v), lasting one hour each.

C. Phenolic Content

The phenolic content of the different extracts was determined by Folin-Ciocalteu reagent [3]. Each sample (0.125mL) was added to 0.5mL of deionized water and 0.125mL of Folin-Ciocalteu reagent (Sigma). After 6min, 1.25mL of 7.5% solution of sodium carbonate and 1.0mL of deionized water were added. The mixture was left 90min at room temperature in the dark and the absorbance at 760nm was measured. A calibration curve was made with standard solutions of gallic acid. The results were expressed in equivalents of standard used. All analyses were done in triplicate.

D. Total Tannins

Total tannins (TT) were estimated according to Ribereau-Gayon and Stonestreet [4]. Briefly, the sample was diluted to 1/50 in water. 2.0mL of the previous solution was added to 1.0 mL of water and 3.0mL of 12M HCl. The content was divided into two tubes. One of them was heated for 30min in boiled water and cooled (tube A), while the last one stayed at room temperature (tube B). A 0.5mL amount of 95% ethyl alcohol was added. The absorbance was read at 550nm for each tube, AbstA and AbstB. Total tannins (g/L) were calculated using the equation:

$$TT = 19.33 \times (AbstA - AbstB).$$

E. Total Anthocyanins

Total anthocyanins (TA) were determined using the SO₂ bleaching method [5]. Each sample (1mL) was added to 1mL of ethanol acidified by 0.1% HCl and 20mL of 2% HCl solution. In one tube, 2mL of previous solution was added to 0.8mL of water (t1). In another tube (t2) were mixed 2mL of previous solution and 0.4mL of HNaSO₃ solution (15% w/v). After 20min at dark room temperature, the absorbance at 520nm was measured. The TA was calculated using the equation:

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$$TA = 875 \times (\text{abst1} - \text{abst2}),$$

and results expressed as mg/L of malvidin equivalents.

F. Fractionation of Phenolic Compounds

The fractionation of fruit phenolic compounds was based on the methodology proposed by Sun *et al.* [6]. The pH of phenolic extracts was adjusted at pH≈7.0. This solution was loaded into the column in a flow of less than 2mL/min. Phenolic acids fractions were eluted with 50mL of diluted pH 7.0 phosphate buffer (1/8, v/v). The column was then washed with distilled water and ethyl acetate. The fraction composed of anthocyanins and polymeric proanthocyanidins was removed from the column by elution with methanol acidified by 0.1% of HCl.

G. HPLC Procedure

The individual phenolic acids and monomeric anthocyanins were analyzed using a HPLC Dionex Ultimate 3000 Chromatographic System (Sunnyvale, California, USA) equipped with a quaternary pump Model LPG-3400 A, a ACC-3000 auto sampler, having a thermostatted column compartment (adjusted to 30°C) and a multiple Wavelength Detector MWD-300. The column (250 x 4.6mm, particle size 5µm) was a C18 Acclaim® 120 (Dionex, Sunnyvale, California, USA) protected by a guard column of the same material.

1. Phenolic acids

The phosphate buffer fraction obtained by C18 sep-pak was used for chromatographic analysis of the phenolic acids. The solvents were (A) water/formic acid (95:5 v/v), and solvent (B) methanol. Analysis conditions were as follow: a linear gradient analysis for a total run time of 80min was used as follows: starting from 5% solvent B during 2min, increase to 80% solvent B over 68min and then isocratic for 8min, decreasing to 5% solvent B over 2min, and finally isocratic for 5min. The sample volume injected was 40µL, the flow rate was 1.0mL/min, and the column temperature was maintained at 30°C during the run.

The quantification of the individual hydroxybenzoic and hydroxycinnamic acids was made by a calibration curve obtained with standard solutions of gallic acid. The results were expressed in equivalents of gallic acid. The chromatographic peaks of all phenolic acids were identified by comparing their retention times with the retention time of standard compounds.

2. Monomeric Anthocyanins

The monomeric anthocyanins present in the sample solutions were analyzed by HPLC. The solvents were (A) 40% formic acid, (B) pure acetonitrile and (C) bidistilled water. The initial conditions were 25% A, 10% B, and 65% C, followed by a linear gradient from 10 to 30% B, and 65 to 45% C for 40min, with a flow rate of 0.7mL/min. The injection volume was 20µL. The detection was made at 520nm and a Chromeleon (version 6.8) software program (Sunnyvale, California, USA) was used.

The quantification of the individual anthocyanins was made by a calibration curve obtained with standard solutions of malvidin-3-glucoside. The anthocyanin structures were isolated and identified according to their UV-Vis spectrum [7].

III. RESULTS

In this study, three red fruits at maturation stage were characterized in regards with their phenolic composition. With the aim of removing the phenolic compounds present in the fruits: raspberry (RB), gooseberry (GB) and blueberry (BB), extractions were carried out with acidic methanol and acetone solutions, allowing to obtain two different extracts, Me-E and Ac-E, respectively. The ripening stage of the samples tested was plain maturation stage.

The phenolic compounds obtained in each extract for the different fruits are showed in Fig. 1. The total of phenolic compounds for each fruit was obtained by summing the phenolic compounds on both extracts.

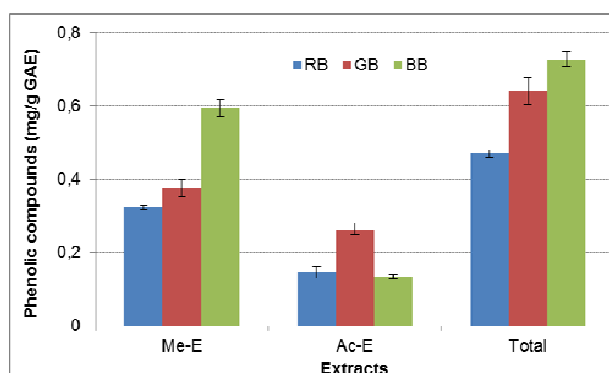


Fig. 1 Phenolic compounds present in each extract of the different fruits. RB: raspberry, GB: gooseberry, BB: blueberry

For all the studied fruits, the phenolic compounds were preferentially recovered in the methanolic extract when compared with the acetone extract, representing 59-82% of the total quantified. Regarding the methanolic extract, the higher amount of phenolic compounds was obtained for blueberry (0.59mg/g GAE), followed by gooseberry (0.38mg/g GAE) and raspberry (0.32mg/g GAE). Otherwise, in the acetone extract, the gooseberry contained a higher amount of phenolic compounds (0.26mg/g GAE). The total phenols present in each fruit showed a higher value for blueberry, reaching 0.73 mg/g. Gooseberry and raspberry exhibited 0.64 and 0.32mg/g GAE, respectively.

Fig. 2 shows the amount of total tannis and anthocyanins present in the different fruits.

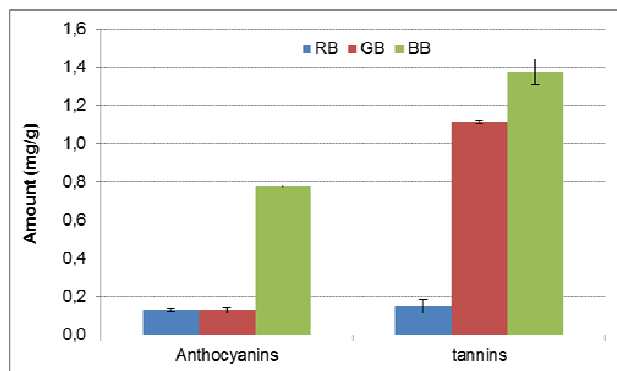


Fig. 2 Total anthocyanins (mg/g) and total tannins (mg/g) present in the different fruits. RB: raspberry, GB: gooseberry, BB: blueberry

The blueberry and gooseberry contained the highest amounts of tannins, 1.38 and 1.12mg/g, respectively. The blueberry fruit contained a significantly higher value of total anthocyanins (0.78mg/g MvGE) when compared with raspberry and gooseberry fruits. This value was similar to that described in literature for blueberries (0.81mg/g MvGE) [8]. The amount of anthocyanins quantified in gooseberry and raspberry was 0.13mg/g MvGE.

Results from the analysis performed to the three fruits relating to copigmented, monomeric and polymeric anthocyanins are presented in Fig. 3. The anthocyanins in raspberry were preferentially in the monomeric form (67%), while the copigmented and polymeric forms represented 18 and 15%. Otherwise, gooseberry contained 67% of anthocyanins in copigmented form. Regarding blueberry, the anthocyanins were mainly in the copigmented (48%) and monomeric (46%) forms.

Table I shows the monomeric anthocyanins identified by HPLC in the different fruits. From the results obtained it was possible to identify six anthocyanins in blueberry, and two in raspberry and gooseberry.

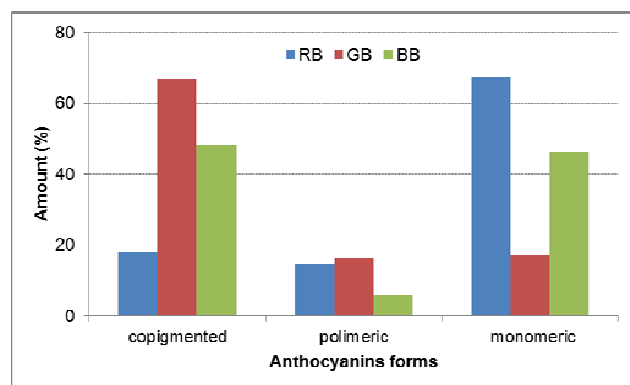


Fig. 3 Copigmented, polymeric and monomeric forms of anthocyanins present in the different fruits. RB: raspberry, GB: gooseberry, BB: blueberry

TABLE I
MONOMERIC ANTHOCYANINS COMPOSITION OF THE DIFFERENT FRUITS

Monomeric anthocyanins	raspberry	gooseberry	blueberry
Delphinidin 3-glucoside	106.3	---	---
Cyanidin 3-glucoside	3.9	4.8	17.8
Petunidin 3-glucoside	1.4	21.9	8.4
Peonidin 3-glucoside	---	---	45.2
Malvidin 3-glucoside	---	---	24.9
Petunidin 3-acetylglucoside	---	---	1.4
Malvidin 3-acetylglucoside	---	---	1.1

Results are expressed as $\mu\text{g/g}$ MvGE

The total amount of monomeric anthocyanins quantified in raspberry, gooseberry and blueberry was 111.6, 26.7 and 98.8 $\mu\text{g/g}$ MvGE, respectively. In raspberry, the delphinidin 3-glucoside was present in higher amount, which was in accordance with the findings of Obón *et al.* [9]. The gooseberry was composed of 21.9 $\mu\text{g/g}$ MvGE of petunidin 3-glucoside and 4.8 $\mu\text{g/g}$ MvGE of cyanidin 3-glucoside. The presence of these anthocyanins was previously described in gooseberry [10], [11]. The blueberry was mainly composed by peonidin 3-glucoside (45.2 $\mu\text{g/g}$ MvGE), malvidin 3-glucoside (24.9 $\mu\text{g/g}$ MvGE) and cyanidin 3-glucoside (17.8 $\mu\text{g/g}$ MvGE). The remaining quantified anthocyanins accounted for 10.9 $\mu\text{g/g}$ MvGE. The presence of these anthocyanins was previously described in literature [12].

Table II presents the phenolic acids identified in the different fruits. The raspberry (52.10 $\mu\text{g/g}$ GAE) and gooseberry (42.58 $\mu\text{g/g}$ GAE) contained higher amounts of phenolic acids when compared with blueberry (5.45 $\mu\text{g/g}$ GAE). In case of raspberry and gooseberry was possible to identify three hydroxybenzoic acids and four and five hydroxycinnamic acids. For blueberry was possible to identify four hydroxybenzoic acids and one hydroxycinnamic acid.

The total of hydroxybenzoic acids present in raspberry accounted for 14.48 $\mu\text{g/g}$ GAE, of which vanilic acid represented 75%. The hydroxycinnamic acids represented 37.62 $\mu\text{g/g}$ GAE, of which 79% were ferulic acid. Gooseberry contained 10.54 $\mu\text{g/g}$ GAE, of hydroxybenzoic and 32.04 $\mu\text{g/g}$ GAE of hydroxycinnamic acids.

TABLE II
PHENOLIC ACIDS (HYDROXYBENZOIC AND HYDROXYCINNAMIC) PRESENT IN THE DIFFERENT FRUITS

Phenolic acids	raspberry	gooseberry	blueberry
Hydroxybenzoic acids			
Protocatechuic	1.10	7.98	---
vanilic	10.94	1.27	0.58
syringic	2.44	1.29	0.91
Gallic	---	---	1.27
p-hydroxybenzoic	---	---	0.54
Hydroxycinnamic acids			
chlorogenic	3.07	9.58	2.15
p-Coumaric	6.23	2.10	---
Ferulic	27.26	7.97	---
Cafataric	1.06	3.58	---
p-hydroxycinnamic	---	8.81	---

Results are expressed as $\mu\text{g/g}$ GAE

IV. CONCLUSION

The results showed that at maturation the blueberry, among the fruits studied, was the one where the higher content of phenolics, anthocyanins and tannins was quantified.

HPLC analysis allowed to identify the presence of monomeric anthocyanins and phenolic acids (benzoic and cinnamic) in raspberry, gooseberry and the blueberry.

REFERENCES

- [1] Su M-S, Chien P-J (2007) Antioxidant activity, anthocyanins, and phenolics of rabbiteye blueberry (*Vaccinium ashei*) fluid products as affected by fermentation. *Food Chemistry*, 104, 182-187.
- [2] Koca I, Karadeniz B (2009) Antioxidant properties of blackberry and blueberry fruits grown in the Black Sea Region of Turkey. *Journal Scientia Horticulturae* 121, 447-450.
- [3] Singleton, V. L., & Rossi, A. (1965). Colorimetric of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, 16, 144-158.
- [4] Ribereau-Gayon, P., & Stonestreet, E. (1966). Dosage des tanins du vin rouge et détermination de leur structure. *Chimie Anal*, 48, 188-196.
- [5] Boulton, R. (2001). The copigmentation of anthocyanins and its role in the color of red wine: A critical review. *American Journal of Enology and Viticulture*, 52(2), 67-87.
- [6] Sun, B., Leandro, M. C., de Freitas, V., & Spranger, M. I. (2006). Fractionation of red wine polyphenols by solid-phase extraction and liquid chromatography. *Journal of Chromatography A*, 1128, 27-38.
- [7] Dallas, C., & Laureano, O. (1994). Effects of pH, sulfur-dioxide, alcohol content, temperature and storage time on color composition of a young portuguese red table wine. *Journal of the Science of Food and Agriculture*, 65(4), 477-485.
- [8] Pertuzatti PB, Jacques AC, Zambiasi RC (2007) Relação de Fitoquímicos na casca e polpa de mirtilo (*Vaccinium ashei* Reade). XVI Congresso de Iniciação Científica. Faculdade de Agronomia Eliseu Maciel.
- [9] Obón JM, Díaz-García MC, Castellar MR (2011) Red fruit juice quality and authenticity control by HPLC. *Journal of Food Composition and Analysis*. 1-12.
- [10] Goiffon JP, Mouly PP, Gaydou EM (1999) Anthocyanic pigment determination in red fruit juices, concentrated juices and syrups using liquid chromatography. *Analytica Chimica Acta* 382, 39-50.
- [11] Wu X, Prior R (2005) Systematic identification and characterization of anthocyanins by HPLC-ESI-MS/MS in common fruits in the United States: fruits and berries. *Journal of Agricultural and Food Chemistry*, 53, 2589-2599.
- [12] Del Rio D, Borges G, Crozier A (2010) Berry flavonoids and phenolics: bioavailability and evidence of protective effects. *British Journal of Nutrition* 104.