Foodomics for mulberry fruit (Morus spp.): Analytical fingerprint as antioxidants' and health properties' determination tool

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FOODOMICS FOR MULBERRY FRUIT (*MORUS SPP.*): ANALYTICAL FINGERPRINT AS ANTIOXIDANTS AND HEALTH PROPERTIES DETERMINATION TOOL

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ABSTRACT

Human nutrition science has greatly developed in the past decades, turning from the consideration of foods as simply energy sources to the recognition of their role in maintaining health and in reducing the disease risks: Foodomics is a new science that studies the Food and Nutrition domains through the application and integration of advanced “omics” technologies to improve consumer's well-being, health, and

knowledge. In recent years, wild food plants have become very attractive to the food industry, prompting their use as replacements for synthetic chemicals and nutraceuticals: in this sense, mulberry is a very important resource for its phytochemical composition, nutritional value, and antioxidant properties.

The aim of this study was to describe mulberry fruit quality traits and report on the level of potentially bioactive compounds (HPLC fingerprint) and their influence on total fruit phytocomplex and antioxidant activity in comparison to the most common fruits.

Mulberry was identified as a rich source of antioxidant compounds; the observed analytical fingerprint demonstrated that the species (and in particular the considered genotype) represents a rich source of phytochemicals, as organic acids, monoterpenes and polyphenolic compounds, especially flavonols and anthocyanins, which led to reasonably good overall fruit quality.

This study developed an important tool to assess mulberry quality, chemical composition, and bioactivity, using different chromatographic methods for comprehensive authentication and quality control of its fruits: this research showed that analytical fingerprinting could be an important tool for studies of Foodomics, helping to find new sources of natural health-promoting compounds.

Keywords: fruit species biodiversity, nutraceutical quality, bioactive compounds

1. INTRODUCTION

Mulberry (*Morus* spp., *Moraceae* family) has been domesticated over thousands of years and adapted to the wide area of tropical, subtropical, and temperate zones of the Northern hemisphere (Asia, Europe, North and South America, and Africa) (Chen, Kan, Tang, Cai, & Liu, 2012; Radojkovic, Zekovic, Vidovic, Kocar, & Maskovic, 2012) and it can grow in a wide range of climatic, topographic and soil conditions. (Özgen, Serçe, & Kaya, 2009). There are 24 species of *Morus*, with at least 100 known cultivars. The most commonly known species in the *Morus* genus are white mulberry (*Morus alba* L.), black mulberry (*Morus nigra* L.) and red mulberry (*Morus rubra* L.). *Morus alba* has white and purple fruits with a very sweet taste and low acidity. Its fruits are perishable and mostly used for fresh consumption. *M. rubra*, known as "red mulberry", is high in dry matter and has a sweet taste and low acidity. *M. nigra*, known as "black mulberry", has juicy fruits with extraordinary colour and a unique, slightly acidic flavouring (Özgen et al., 2009; Uzun & Bayir, 2012).

Mulberry is a deciduous tree growing to a 10 - 13 m height and is variable in form, including drooping and pyramidal shapes. The leaves are from 10 to 20 cm long (Kostic et al., 2013; Lin & Lay, 2013). Mulberries can be grown from seed, and this is often advised as seedling-grown trees are generally of better shape and health, but they are most often planted from large cuttings which root readily. The tree branches pruned during the fall season (after the leaves have fallen) are cut and used to make durable baskets supporting agriculture and animal husbandry (Lin & Lay, 2013). The plant yields dark purple-black edible fruits that are 2 - 3 cm long after they have matured. Mulberry present a very sweet fruit, with high levels of bioactive compounds, hence it has a very

important role in the food industry: the mulberry fruits, even from the same species, may contain different amounts of chemical composition as well as different antioxidant properties. The chemical composition and nutritional status of edible plant parts may be influenced by genetic, physiological and environmental factors, as genotype, soil chemistry and climatic conditions (Sadia et al., 2014). Moreover, these molecules can be affected by several agronomic conditions (agrotechniques, ripening stage at harvest) and technological factors (harvest method, post-harvest treatments, storage and processing conditions) (Donno, Beccaro, Mellano, Cerutti, et al., 2012; Donno, Beccaro, Mellano, Canterino, et al., 2013).

Mulberry trees have been traditionally cultivated for their leaves as food for silkworms. However currently and especially due to its nutritive value, mulberry fruits are consumed as both fresh and processed products, such as juices, fruit salads and dried fruits (Calin-Sanchez et al., 2013). Recently, the production and consumption of mulberry fruits are rapidly increasing because of their aromatic taste, nutritional value, bioactive compound content and biological activities (Liang et al., 2012).

Among the bioactive compounds, one of the most important constituent of mulberry fruit are represented by anthocyanins (Lee, Durst, & Wrolstad, 2005). Several studies have investigated the contents of phenolics as flavonoids and anthocyanins in mulberry extract. Along with these compounds, mulberry has been found to contain carotenoids (Arabshahi-Delouee & Urooj, 2007).

Thanks to these health-promoting compounds mulberry fruits are traditionally used as a worming agent and a laxative, odontalgic, anthelmintic, expectorant, hypoglycemic, and emetic agent; in traditional Chinese herbal medicine, mulberry fruit

has been used as a folk remedy to treat oral and dental diseases, diabetes, hypertension, arthritis and anemia (Liang et al., 2012).

In recent years, wild food plants have become very attractive to the food industry, prompting their use as replacements for synthetic chemicals and nutraceuticals (Donno, Beccaro, Mellano, Cerutti, & Bounous, 2013; Sadia et al., 2014), but neglected and underutilized natural food resources are suffering from less attention and research, and their nutritional, economic and socio-cultural potential are not fully exploited (Beccaro, Bonvegna, et al., 2014; Donno, Beccaro, Mellano, Cerutti, & Bounous, 2014a, 2014b):data on antioxidant properties of several plants, particularly those that are not used in nutrition and medicine, still lacks (Sadia et al., 2014). Therefore, investigation of such properties has been of interest mainly for finding new sources for natural antioxidants, functional foods and nutraceuticals: several researches investigated nutraceutical properties of *Morus* spp. fruits, studying their nutritional potentials, but a complete profile with quality traits, phytochemical composition and antioxidant activity evaluation still lacks. During the past several years, the quest for alternative crops with high nutritional value has increased interest in mulberry: previous studies have examined the total content of phenols, flavonids, anthocyanins and antioxidant activity of *Morus spp.* grown in different regions (Chen et al., 2012; Özgen et al., 2009; Uzun & Bayir, 2012), but TPC, antioxidant activity, and most of the potential health-promoting agents of mulberry still remain undescribed. Despite many researches on commonly available fruits, as blueberry, kiwifruit, orange and apple, on their TPC and antioxidant activity (Canterino, Donno, Mellano, Beccaro, & Bounous, 2012; Donno, Beccaro, Mellano,

Canterino, et al., 2013; Donno, Beccaro, Mellano, Torello-Marinoni, et al., 2012), little information is available for currently minor and underutilized fruits.

Moreover, because mulberry fruit consumption is driven by both fresh market and processing industry requirements, it is crucial to fully characterize the fruit traits not only from a chemical point of view, but also to verify whether they fit current market demands for high-quality products (good qualitative and sensorial properties, high bioactive compound content) (Calin-Sanchez et al., 2013).

In the last years, food science greatly grew, developing new food products, designing processes to produce these foods, improving packaging materials, food shelf-life, and sensory characteristics (Capozzi & Bordoni, 2013; Donno, Beccaro, Mellano, Di Prima, et al., 2013). New analytical methods are mainly related to the holistic "omics" approach, implemented by ''high-throughput'' technologies (Tranchida et al., 2013). Thanks to the "omics" approach, researchers are now facing a new science, called Foodomics, which can connect food components, foods, diet, human health and diseases. It is presented as a global discipline in which food (including nutrition), advanced analytical techniques, and bioinformatics are combined (Capozzi & Bordoni, 2013).

Regarding analytical technologies used in Foodomics and in industrial quality control, the most common method for analytical controls is to spectrophotometrically quantify total bioactive compounds in fruits: spectrophotometric determination is a commonly adapted method because of its relatively milder conditions, rapidness and cost-effective nature; this method works very well where an estimation is needed rather than an accurate quantification of bioactive compounds: therefore, such methods are excellent tools for rapid screening of total nutraceutical contents in plant material as

fruits. Besides this, the spectroscopic method does not provide any specificity regarding a bioactive compound fingerprint in fresh fruits or food supplements(Canterino, Donno, Beccaro, & Bounous, 2009, 2010; Giusti & Wrolstad, 2001); for this reason, recently, the fingerprint approach was used for identification and direct analysis of plant material. Different kind of features can be referred to the overall fingerprint: genetic, quality, sensory or morphological features could be used to create a full fingerprint as showed in other studies (Beccaro et al., 2012; Canterino, Donno, & Mellano, 2010; Donno, Beccaro, Mellano, Torello-Marinoni, et al., 2012; Mellano et al., 2012).

In this study, bioactive compound composition was referred to a chemical fingerprint; the best practice of characterizing fruit extracts is by measuring the concentration of the main bioactive compounds, called "markers": with the development of analytical techniques, chromatographic fingerprints have been widely used for the authentication and quality control of fresh fruit and processed products (Donno, Beccaro, et al., 2014a, 2014b). By definition, a chromatographic fingerprint is a chromatographic pattern of the extract of the most common pharmacologically active compounds (Donno, Beccaro, Mellano, Cerutti, Marconi, et al., 2013). The chromatographic techniques could be used to obtain a relatively complete picture of the fruit extracts, which is usually called analytical fingerprint, in order to represent the so-called phytocomplex.

The aim of this research was to describe mulberry fruit quality traits and report on the level of potentially bioactive compounds and their influence on total fruit phytocomplex and antioxidant activity. This study focused on quality traits and health-promoting effects based on the nutraceutical fingerprint and antioxidant activity; the considered genotype is one of the most cultivated in small family-managed farms and

nurseries with commercial purposes. The research emphasizes that quality parameters are not enough for a full Foodomics evaluation of these fruits but it is also necessary to consider nutraceutical features, defining an effective chemical fingerprint, that could be also used as a quality control tool: as few information is currently available on the chemical fingerprint of mulberry fruits, the results of the present study may encourage a deeper evaluation of the effective nutraceutical value for the many hundreds of different fruit-bearing *Morus* spp. cultivars.

The growing worldwide interest in introducing the cultivation of *Morus* spp. to promote the differentiation of the cultivated agrobiodiversity could also be encouraged by the high rusticity of the species that could be managed with more environmentally friendly agrotechniques (if compared with the most commonly grown fruit species), and by the greater sustainability of its production (Beccaro, Cerutti, et al., 2014; Cerutti et al., 2013).

2. MATERIALS AND METHODS

2.1 Plant material

Samples of mulberry fruit (cv Kokuso) were picked up in a farm located in Lagnasco (Cuneo, Northern Italy) in June 2014; the fruits (0.5 kg for each plant) were manually picked from three plants for each replication. Imported from Korea, this berry is a seedless dark berry. It early ripens over a long period. It’s a very vigorous fast growing cultivar and begin to produce berries in the same year when it is planted with a high production.

The same analyses were performed on some common temperate fruit species grown in the same pedoclimatic conditions in order to understand if this species presents a real added nutritional value compared with others. All harvested fruits were collected randomly in the orchard from different plants and analyzed fresh or after being stored for few days at 4°C and 95% relative humidity (RH).

2.2 Solvents and chemicals

Sodium carbonate, Folin-Ciocalteu phenols reagent, sodium acetate, citric acid, potassium chloride, hydrochloric acid, iron(III) chloride hexahydrate, 2,4,6-tripyridyl-S-triazine (TPTZ), and 1,2-phenylenediamine dihydrochloride (OPDA) were purchased from Sigma Aldrich (St. Louis, MO, USA), while acetic acid was purchased from Fluka Biochemika, Buchs, Switzerland. Ethylenediaminetetraacetic acid (EDTA) disodium salt was purchased from AMRESCO (Solon, OH, USA), while sodium fluoride was purchased from Riedel-de Haen (Seelze, Germany).

Ethanol was purchased from Fluka Biochemika (Buchs, Switzerland). Analytic HPLC grade solvents, methanol, and formic acid were purchased from Sigma Aldrich and Fluka Biochemika, respectively; potassium dihydrogen phosphate, ammonium dihydrogen phosphate, and phosphoric acid were also purchased from Sigma Aldrich. Milli – Q ultrapure water was produced by using Sartorius Stedium Biotech mod. Arium (Sartorius, Goettingen, Germany).

Cetyltrimethylammonium bromide (cetrimide) was purchased from Extrasynthése (Genay, France), while 1,2-phenylenediamine dihydrochloride (OPDA) was purchased from Sigma Aldrich.

All polyphenolic and terpenic standards were purchased from Sigma Aldrich. Organic acids were purchased from Fluka Biochemika, while ascorbic acid and dehydroascorbic acid were purchased from Extrasynthése.

2.3 Qualitative analysis

2.3.1 Physical parameters

Average fruit weight (g) was evaluated by Mettler PM460 DeltaRange Electronic Balance (Mettler, Greifensee, Switzerland), while a digital caliper (Traceable Digital Caliper-6'', VWR International, Milano, Italy) was used for measuring fruit size (mm). For each analysis, three replications, each obtained from 15 fruits, were considered.

2.3.2 Chemical parameters

Total soluble solutes (TSS, °Brix) were recorded with a digital refractometer DBR35 (Tsingtao Unicom-Optics Instruments, Laixi, China); titratable acidity (TA, $meq \cdot L^{-1}$) and pH (pH-units) were determined by titrating 10 mL of pulp juice (rising to 100 ml final volume with Milli-Q water) with a solution of NaOH (0.2 $mol \cdot L^{-1}$), using an automatic titrator (Crison Titromatic 2S, Crison, Alella, Spain).

2.4 Spectrophotometric analysis

2.4.1 Total polyphenolic compounds (TPC)

For the extraction of polyphenolic compounds, samples were placed in 50 mL test tubes, and 25 mL of extraction solution (a solution of methanol and water acidified with HCL 37%) were subsequently added to the weighed samples; after 60 minutes in the dark, the extracts were homogenized with an Ultra – Turrax (T25, IKA WERKE,

Staufen, Germany) for about 1 min and then centrifuged for 15 min at 50 Hz in an ALC Centrifuge PK 120 (ALC International, Cologno Monzese, Italy). The method used for the determination of total polyphenol content (TPC) was based on Folin-Ciocalteu phenol reagent and spectrophotometric determination at 765 nm (Slinkard & Singleton, 1977).

The standard calibration curve was plotted using gallic acid at concentrations of 0.02–0.1 $mg \cdot mL^{-1}$. The results were expressed as mg of gallic acid equivalents (GAE) per 100 g of fresh weight (FW).

2.4.2 Total anthocyanins

The total anthocyanin content (TAC) in the fruit extracts was directly determined using the pH-differential method (Giusti & Wrolstad, 2001; Lee et al., 2005). The extracts for TAC analysis were prepared using the previously described method used for quantification of total polyphenols.

Anthocyanins demonstrate maximum absorbance at 515 nm at pH 1.0 and also at 700 nm at pH 4.5. The colored oxonium form of anthocyanin predominates at pH 1.0, and the colorless hemiketal form at pH 4.5. The pH-differential method is based on the reaction producing oxonium forms. This method allows an accurate and rapid measurement of the total monomeric anthocyanins.

Absorbance was measured at 515 and 700 nm and the results, considered as the monomeric anthocyanin pigment, was expressed as milligrams of cyanidin-3-O-glucoside (C3G).

2.4.3 Antioxidant bioactivity

Antioxidant activity in the mulberry fruit pulp was evaluated by ferric reducing antioxidant power (FRAP) assay (Benzie & Strain, 1999). The extracts used for analysis were those used previously for quantification of total polyphenols.

The method was based on the reduction of the ferric (Fe^{3+}) TPTZ (2,4,6-tripyridyl-S-triazine) complex to its ferrous form (Fe^{2+}). Absorbance at 595 nm with a UV/Vis spectrophotometer (1600-PC, VWR International) was recorded.

The standard curve was obtained using $FeSO_4 \cdot 7H_2O$ (concentration range: 100–1000 $\mu mol \cdot L^{-1}$), and results were expressed as millimoles of Fe^{2+} equivalents per kilogram (solid food) of FW.

2.5 Chromatographic analysis

2.5.1 Sample preparation protocols

2.5.1.1 Polyphenolic compounds

Methanolic extracts used for the previous analysis were filtered with circular pre-injection filters (0.45 µm, polytetrafluoroethylene membrane, PTFE) and then stored for a few days at normal atmosphere (N.A.), 4 °C and 95% RH.

2.5.1.2 Monoterpenes and organic acids

For the extraction of organic acids and monoterpenes, three replications, each obtained from 30 fruits, were considered. Five grams of fruit pulp were put into a test tube and 25 mL of 95% ethanol solution, acidified with formic acid, were then added. After 10 min in the dark, the extracts were homogenized with an Ultra – Turrax (T25,

IKA WERKE, Staufen, Germany) for about 1 min and then centrifuged for 10 min at 66 Hz in an ALC Centrifuge PK 120 (ALC International, Cologno Monzese, Italy) (Donno, Beccaro, et al., 2014b).

Samples were then stored in at N.A., at 4°C and 95% R.H until analysis.

2.5.1.3 Vitamin C

Ten grams of fruit pulp were put into a test tube with 10 mL of extraction solution (0.1 $mol \cdot L^{-1}$ citric acid, 2 $mmol \cdot L^{-1}$ ethylenediaminetetraacetic acid (EDTA) disodium salt, and 4 $mmol \cdot L^{-1}$ sodium fluoride in methanol – water 5:95 v/v) were then added.

The extracts were homogenized with an Ultra – Turrax (IKA WERKE T25) for about 1 min and then centrifuged for 10 min at 66 Hz at room temperature in an ALC Centrifuge PK 120. The supernatants were recovered and transferred to a second test tube through filter cloth and then acidified with 4 $mol \cdot L^{-1}$ HCl to decrease the pH solution to a value of 2.2–2.4 (Canterino et al., 2012; Sanchez, Gil-Izquierdo, & Gil, 2003).

Acidified samples were centrifuged for 5 min at 200 Hz at 4°C with an ALC Multi Speed refrigerated centrifuge PK 121R (ALC International), and the supernatants were then filtered through a 0.45 µm filter (Titan 2 HPLC filter 17 mm PTFE Membrane); polyphenolic compounds were absorbed on a C_{18} cartridge for solid phase extraction (Sep-Pak® C-18, Waters, Milford, MA, USA). Then, 250 µL of OPDA solution (18.8 $mmol \cdot l^{-1}$) was added to 750 µL of extracted samples for DHAA derivatization into the fluorophore 3-(1,2-dihydroxyethyl)furo(3,4-b)quinoxalina-1-one

(DFQ). After 37 min in the dark, the samples were analyzed with the HPLC – DAD system (Gonzalez-Molina, Moreno, & Garcia-Viguera, 2008).

2.5.2 Standard preparation

Stock solutions of monoterpenes, ascorbic and dehydroascorbic acids, cinnamic acids, and flavonols with a concentration of 1.0 $mg \cdot mL^{-1}$ were prepared in methanol: four calibration standards were prepared by dilution with methanol; stock solutions of benzoic acids and catechins with a concentration of 1.0 $mg \cdot mL^{-1}$ were prepared in 95% methanol and 5% water. In this case, four calibration standards were prepared by dilution with 50% methanol–water.

Stock solutions of organic acids with a concentration of 1.0 $mg \cdot mL^{-1}$ were prepared in ultrapure water; from these solutions, four calibration standards were prepared by dilution with water.

Examples of the main botanical standards (chemical structure, chromatographic profile, UV-vis spectrum) are reported in Fig. 1.

2.5.3 Apparatus and chromatographic conditions

An Agilent 1200 High Performance Liquid Chromatograph, equipped with a G1311A quaternary pump, a manual injection valve, and a 20 μL sample loop, coupled to an Agilent GI315D UV-Vis diode array detector (Agilent Technologies, Santa Clara, CA, USA), was used for the analysis.

Five different chromatographic methods were used to analyse the samples, two for polyphenols and one for terpenic compounds, organic acids, and vitamins,

respectively. In this study, effective HPLC–DAD methods were used for fingerprint analysis and nutraceutical identification of mulberry fruit. The chromatographic conditions were setted to obtain an analytical fingerprint containing complete information of chemical composition with a good resolution and a reasonable analysis time. Different linear gradients in different slopes were used for optimizing the analyte separation; indeed, some compounds were similar in structure with each other in the same chemical class. Adding formic and phosphoric acid was necessary for enhancing the resolution and eliminating peak tailing because most of the compounds were also weakly acidic, according to other studies (Donno, Beccaro, Mellano, Cerutti, Marconi, et al., 2013). The wavelength selection was an important step for developing a reliable fingerprint; only selected wavelengths were suitable to achieve more specific peaks as well as a smooth baseline after a full-scan on the chromatogram from 190 to 400 nm, according to other similar research (Canterino et al., 2009; Canterino et al., 2012; Donno, Galizia, & Cerutti, 2010).

In all of the used methods, bioactive compound separation was achieved on a KINETEX – C18 column (4.6 × 150 mm, 5 μm, Phenomenex, Torrance, CA, USA).

Different mobile phases were used: methanol and a solution of 40 mM potassium dihydrogen phosphate in water (pH 2.8, adjusted with phosphoric acid) with a flow rate of 1.0 $mL \cdot min^{-1}$ (method A, 60 minute gradient analysis of cinnamic acids and flavonols), a solution of methanol/water/formic acid (5:95:0.1 v/v/v) and a mix of methanol/formic acid (100:0.1 v/v) with a flow rate of 1.0 $mL \cdot min^{-1}$ (method B, 35 minute gradient analysis of benzoic acids and catechins), water and methanol with a flow rate of 1.0 $mL \cdot min^{-1}$ (method C, 75 minute gradient analysis of monoterpenes), 0.5% $(NH_4)H_2PO_4$

aqueous solution (pH 2.8, adjusted with phosphoric acid) with a flow rate of 0.5 $mL \cdot min^{-1}$ (method D, 20 minute isocratic analysis of organic acids), and methanol – water (5:95, v/v) containing 5 mM cetrimide and 50 mM potassium dihydrogen phosphate with a flow rate of 0.9 $mL \cdot min^{-1}$ (method E, 15 minute isocratic analysis of ascorbic and dehydroascorbic acids) (Donno, Beccaro, Mellano, Bonvegna, & Bounous, 2014).

UV spectra were recorded at 330 nm (A); 250, 280, and 320 nm (B); 220 and 235 nm (C); 214 nm (D); 261; and 348 nm (E) .

2.5.4 Identification and quantification of bioactive compounds

All single compounds were identified in samples by comparison and combination of their retention times and UV spectra with those of authentic standards in the same chromatographic conditions. The external standard method was used for quantitative determinations. Calibration curves in the 125–1000 $mg \cdot L^{-1}$ range with good linearity for a four point plot were used to determine the bioactive compound concentration in the fruit samples; the linearity for each compound was established by plotting the peak area (y) versus the concentration (x) of each biomarker. The limit of detection (LOD) and the limit of quantification (LOQ) of the five chromatographic methods were defined as the lowest amount of analyte that gives a reproducible peak with a signal-to-noise ratio (S/N) of 3 and 10, respectively. The main analytical method validation data are summarized in Table 1.

All samples were analysed in triplicate, and standard deviations are given in order to assess the repeatability of the used methods. Accuracy was checked by spiking

samples with a solution containing each bioactive compound in a concentration of 10 $mg \cdot mL^{-1}$.

According to "multi-marker approach", (Mok & Chau, 2006), total bioactive compound content (TBCC) was determined as the sum of the most important classes of bioactive compounds present in the samples. Bioactive markers were selected comparing mulberry health-promoting properties and the most important antioxidant and anti-inflammatory compounds in literature with an important role in the positive effects on human organism. Five polyphenolic classes were considered: benzoic acids (ellagic and gallic acids), catechins (catechin and epicatechin), cinnamic acids (caffeic, chlorogenic, coumaric, and ferulic acids), flavonols (hyperoside, isoquercitrin, quercetin, quercitrin, and rutin), and tannins (castalagin, vescalagin); one terpenic class was considered: monoterpenes (limonene, phellandrene, sabinene, γ-terpinene, and terpinolene). Organic acids (citric, malic, oxalic, quinic, succinic, and tartaric acids) and vitamin C (ascorbic and dehydroascorbic acids) were also considered to obtain a complete analytical fingerprint. All results were expressed as mg per 100 g of fresh weight (FW).

2.6 Statistical Analysis

Results were subjected to analysis of variance (ANOVA) test for mean comparison (SPSS 22.0 Software) and HSD Tukey multiple range test ($P < 0.05$). Principal component analysis (PCA) was performed on the chemical and nutraceutical data.

3. RESULTS

3.1 Chemical – nutraceutical analysis and antioxidant bioactivity

All quality data are reported in Table 2. Results showed that the fruit is quite cylindrical (27.46 mm in length and 12.07 mm in width), with a mean weight value of 3.09 g, and a black-purple color. Quality analysis reported a mean TSS value of 18.67°Brix, while TA ranged from 27.88 meq·L^{-1} to 30.61 meq·L^{-1} with a pH mean value of 5.77 pH-units.

G1, G2 and G3 represent different samples of the same genotype. The content of total polyphenolic compounds in the extracts is reported in Table 3a. TPC values ranged from 232.53 $mg_{GAE}/100g_{FW}$ (sample G1) to 243.31 $mg_{GAE}/100g_{FW}$ (sample G3). Moreover, the lowest FRAP value was observed in G1 (21.52 mmol $Fe^{2+}\cdot kg^{-1}$) and the highest in G3 (22.58 mmol $Fe^{2+}\cdot kg^{-1}$); sample G3 also showed the highest TAC value (91.05 $mg_{C3G}/100g_{FW}$), followed by G2 and G1 samples (Table 3a).

These analyses were also performed on some common temperate fruit species in order to compare mulberry qualitative, chemical and nutraceutical properties to other common species (Table 3b). Mulberry showed the highest TSS value (18.67°Brix), followed by blackcurrant (14.00°Brix) and apple (13.43°Brix), while raspberry (413.57 meq·L^{-1}) had the highest TA value, followed by orange (383.10 meq·L^{-1}) and strawberry (184.65 meq·L^{-1}). Mulberry extracts were the less acidic (TA value was 28.92 meq·$L^{-1}$and pH value was 5.77 pH-units).

The content of total polyphenolic compounds was statistically different among the different species. Apple contained small quantities of polyphenolic compounds (83.40 $mg_{GAE}/100g_{FW}$), while a significantly higher polyphenolic content was observed in blackcurrant (434.43 $mg_{GAE}/100g_{FW}$) and strawberry (323.39 $mg_{GAE}/100g_{FW}$). Mulberry (236.94 $mg_{GAE}/100g_{FW}$) was in a medium position among the considered fruit species.

The results showed large statistical variations among the different species in the values of the total antioxidant capacity, expressed as FRAP assay. Berries, in particular blackcurrant (76.86 mmol $Fe^{2+}\cdot kg^{-1}$), blackberry (64.96 mmol $Fe^{2+}\cdot kg^{-1}$) and blueberry (49.36 mmol $Fe^{2+}\cdot kg^{-1}$), showed the highest antioxidant capacity, while mulberry presented a higher FRAP value (22.12 mmol $Fe^{2+}\cdot kg^{-1}$) than raspberry, orange and apple.

The content of total anthocyanins was statistically different among the different species: mulberry presented a higher TAC value (80.02 $mg_{C3G}/100g_{FW}$) than strawberry, raspberry, orange and apple.

Significant differences in vitamin C content were also recorded in the different species. Blackcurrant showed the highest vitamin C content (162.73 mg/100 g_{FW}), followed by orange (71.12 mg/100 g_{FW}), blackberry (45.07 mg/100 g_{FW}) and strawberry (57.95 mg/100 g_{FW}). The lowest vitamin C values were recorded in apple (3.91 mg/100 g_{FW}) and mulberry (2.97 mg/100 g_{FW}).

Principal component analysis was performed on all samples and it reduced the initial variables (TSS, TA, pH, TPC, antioxidant activity, TAC, and vitamin C content) into three principal components (86.76% of total variance) and divided samples in three groups (mulberry-A, berries-B, and no-berry fruit-C), confirming the statistically significant differences of the ANOVA test on quality and nutraceutical data (Fig. 2). The PCA graph showed a correlation between the nutraceutical variables (TPC, antioxidant activity, TAC and vitamin C content) and PC1 (46.18% of total variance), while TSS and TA presented a correlation with PC2 (28.94% of total variance). The pH was in an intermediate position between PC1 and PC2, in anti-correlation to total anthocyanins.

3.2 Total bioactive compound content (TBCC) and single compound profile

All data (with mean values) are reported in Table 4 (TBCC and single compounds).

The content of total bioactive compounds in the evaluated samples was calculated as the sum of the most important biologically active molecules detected in the extracts. The analysed samples showed a lower TBCC value of 3160.95 mg/100 g_{FW} (sample G2) and a higher value of 3316.54 mg/100 g_{FW} (sample G1).

Morus spp. samples showed the following bioactive compound composition: four cinnamic acids (caffeic acid, chlorogenic acid, coumaric acid, ferulic acid), three flavonol (hyperoside, quercetin, rutin), two benzoic acid (ellagic and gallic acids), two catechins (catechin, epicatechin), three monoterpenes (limonene, sabinene, terpinolene), five organic acids (citric acid, malic acid, oxalic acid, quinic acid, tartaric acid), and one vitamin (vitamin C expressed as the sum of ascorbic acid and dehydroascorbic acid); isoquercitrin, quercitrin, rutin, castalagin, vescalagin, phellandrene, γ-terpinene, and succinic acid were not detected. Single bioactive compound content ranged from 2.80 mg/100 g_{FW} (vitamin C, G1 sample) to 1078.74 mg/100 g_{FW} (citric acid, G1 sample).

Correlation among antioxidant activity and TPC, TAC, and single bioactive classes are reported in Table 5; monoterpenes showed a positive weak correlation (0.2252) with antioxidant activity, while organic acids presented a negative strong correlation (-0.8784). TPC (obtained by spectrophotometric measurements), TAC, polyphenols (obtained by HPLC analysis) and vitamins showed strong positive correlations with antioxidant capacity (0.8637, 0.7484, 0.8790 and 0.8233, respectively).

3.3 Fingerprinting

The chemical fingerprint of mulberry fruit was reported: in total, 20 bioactive compounds were identified by HPLC/DAD. By single bioactive compound profile, health-promoting agents were grouped into different classes to evaluate the single contribution of each class to total fruit phytocomplex composition.

The chemical fingerprint showed the prevalence of organic acids, monoterpenes and polyphenols (as the sum of anthocyanins, cinnamic acids, flavonols, benzoic acids, catechins, and tannins) in chemical composition of all the analyzed samples (mean values were considered); the most important class was organic acids (50.76%), followed by monoterpenes (40.25%), polyphenols (8.90%), and vitamins (0.09%) (Table 6).

Therefore, organic acids and monoterpenic compounds were two major groups of bioactive compounds in the evaluated *Morus spp.* fruit; in the polyphenol group, the most important classes were flavonols (3.61%) and anthocyanins (2.41%), followed by cinnamic acids, catechins and benzoic acids (all percentages refer to the total content of bioactive compounds). Tannins were not detected.

4. DISCUSSION

Many studies have shown the physiological functions of natural ingredients linked usually to the antioxidant activity of phenolic compounds and other phytochemicals (Kostic et al., 2013). In this study, the phytonutrient content and antioxidant capacity of a selected mulberry cultivar were characterized, comparing its nutraceutical traits with other common fruit species, and determined the strength of the relationships among commonly measured variables. *Morus spp.* fruits may contain a significant amount of

phytochemicals or even unique compounds that are health-promoting (Liang et al., 2012; Lin & Lay, 2013); this study showed that the analyzed parameters of the fruits of this species are comparable to those of other common fruit species that present an high nutraceutical value as *Vaccinium corymbosum*, *Ribes nigrum*, *Rubus idaeus*, and *Citrus sinensis*. Moreover, in addition to high antioxidant capacity, mulberry showed high anthocyanin and phenolic contents, which may increase its consumption.

In order to simplify the multivariate model based on the analysis of seven parameters (in particular, TPC, TAC, vitamin C and antioxidant activity) and classify the species according to their quality and nutraceutical characteristics, a PCA was carried out. As in other studies (Arabshahi-Delouee & Urooj, 2007; Radojkovic et al., 2012), results showed that mulberry is very different to other fruits (different PCA group). PCA also confirmed the scientific validity of the pH-differential method for TAC quantification; monomeric anthocyanins, indeed, undergo a reversible structural transformation as a function of pH (colored oxonium form at pH 1.0 and colorless hemiketal form at pH 4.5): PCA graph confirmed the relationship between monomeric anthocyanins and pH showing the anti-correlation between the content of total anthocyanins and the pH-values. Moreover, ANOVA test and PCA confirmed the TPC and antioxidant activity results of other authors (Arfan, Khan, Rybarczyk, & Amarowicz, 2012; Calin-Sanchez et al., 2013), significantly contributing to improve the knowledge of this species.

In this case, antioxidant activity and bioactive compound contribution to total fruit phytocomplex were also used to highlight mulberry nutraceutical properties; antioxidant activity was considered an important method to evaluate the nutraceutical properties of

fruit, as shown in other previous studies on other fruit species (Amaral, Mira, Nogueira, da Silva, & Florencio, 2009; Donno, Beccaro, et al., 2014a). In particular, in this study, the correlation between TPC/TAC and antioxidant activity was useful to show that the detected single compounds were strongly related to some nutraceutical properties (antioxidant capacity). Pearson correlation coefficient confirmed the individual biological activities of the different bioactive classes: polyphenols and vitamins showed the highest antioxidant activity values (R=0.86 and R=0.82, respectively), while the monoterpenes the lowest ones (R=0.23): terpenic compounds, indeed, are mainly characterized by anti-inflammatory activity; instead, organic acids presented no antioxidant activity (R=-0.88).

Specific bioactive compounds can be used collectively as representative standards of a fruit extract in quantification (Donno, Cavanna, et al., 2013; Tsao & Yang, 2003), as done in this study. Chromatographic data can be used as TBCC for the quantification of health-promoting agents because HPLC methods give more information on individual compounds or groups of compounds than the TPC by the Folin-Ciocalteu method or the TAC by the pH-differential method (Giusti & Wrolstad, 2001; Tranchida et al., 2013). In this study, an innovative analytical approach has been applied to evaluate the mulberry fruit chemical composition and medicinal properties; a specific fingerprint, along with a multivariate data analysis, was used to show the single bioactive class contribution to the total fruit phytocomplex. Indeed, synergistic or additive biological effects of different bioactive compounds could contribute to disease prevention more than a single compound or a group of compounds (Bolwell, 1990). The main aim was to obtain a fingerprint of mulberry fresh fruits by reverse phase mode HPLC/DAD analyses. By different elution methods, the metabolites in the fruit extracts of the considered species

were simultaneously determined: the obtained fingerprint was useful for bioactivity evaluation and quality control; the UV–vis absorption spectra and the chromatographic retention times were used and combined for tentative identification of the selected biomarkers. The methods showed a good resolution for most peaks and could be routinely used to evaluate overall fruit quality; it could be also applied for other species and genera, as shown in other studies (Canterino et al., 2012; Donno, Beccaro, et al., 2014b; Donno, Beccaro, Mellano, Torello-Marinoni, et al., 2012).

Based on the obtained results, many studies pointed out that the identified polyphenolic compounds significantly contribute to the *Morus spp.* phytocomplex and antioxidant activity (Özgen et al., 2009; Radojkovic et al., 2012): the present study confirmed these results, adding organic acids, vitamins, and terpenic compounds also significantly contributed to the mulberry fruit phytocomplex, as antioxidant and anti-inflammatory health-promoting agents. No studies emphasized the complete identification of single bioactive compounds in *Morus spp.* fresh fruit by HPLC analysis.

This research is only a preliminary study on mulberry fruit chemical composition: genotype is an important variable to define the nutraceutical and quality traits (Beccaro et al., 2012) but, in this case, this research only focused on the antioxidant activity and chemical profile of a commercial cultivar. More detailed biological and pharmacological studies are still needed for additional information and better understanding of the heath benefits of anthocyanin – rich mulberries (Özgen et al., 2009). Finally, the diversity in total bioactive compound content and antioxidant activity between cultivars in other species (Canterino, Donno, & Mellano, 2010; Mellano et al., 2012) emphasizes the need

for additional screening to identify mulberry species and cultivars with high antioxidant capacity and health-promoting potential.

5. CONCLUSIONS

The high phenolic content and antioxidant activity of mulberry underline the nutritive and phytomedicinal potentials of the fruit: the results indicated that *Morus spp.* has the potential to be further developed into a nutritionally interesting raw material for food and beverage applications.

In this study, mulberry was identified as a rich source of antioxidant compounds; the observed analytical fingerprint demonstrated that the species represents a rich source of phytochemicals, as organic acids, monoterpenes and polyphenolic compounds, especially flavonols and anthocyanins, which led to reasonably good overall fruit quality; this research suggested that identified nutraceuticals might contribute to the total phytocomplex of these fruits. These results, demonstrating high quality and phytochemical traits of mulberries, may also provide a basis for planning breeding strategies as well as selecting cultivars with high phytonutrients profiles and antioxidant capacities as functional foods for consumers, but further studies are, however, required before the fruit extract can be utilized in the production of health foods and as an antioxidant carrier in pharmaceutical industries too.

Finally, few studies showed the complete profile with quality traits, phytochemical composition and antioxidant activity evaluation in mulberry fruits and in its extracts by HPLC analysis. Chromatography offers very powerful separation ability, such that the complex chemical components in fruit extracts can be separated into many

relatively simple sub-fractions. The recent approaches of applying hyphenated chromatography and spectrometry such as high-performance liquid chromatography–diode array detection (HPLC–DAD), gas chromatography–mass spectroscopy (GC–MS), capillary electrophoresis-diode array detection (CE-DAD), HPLC–MS and HPLC–NMR, could provide additional spectral information. This is very helpful for the qualitative analysis and for the on-line structural elucidation, but in this preliminary study HPLC-DAD coupled to multivariate statistical analysis (Principal Component Analysis) was a simply, rapid and effective approach to describe considered samples in relation to the research aim. This study developed an important tool to assess mulberry quality, chemical composition, and bioactivity, using different chromatographic methods for comprehensive authentication and quality control of its fruits: this research showed that analytical fingerprinting could be an important tool for studies of Foodomics, helping to find new sources of natural health-promoting compounds.

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Tables

Table 1. Calibration curve equations, R^2, LOD, and LOQ of the used chromatographic methods for each calibration standard (Donno, Beccaro, Mellano, Bonvegna, et al., 2014).

Class	Standard	Identification code	Method	Calibration curve equations (peak area = y; concentration = x)	R^2	LOD (mg/L)	LOQ (mg/L)
Cinnamic acids	caffeic acid	1	A	y = 10.155x + 13.008	0.985	1.232	4.107
	chlorogenic acid	2	A	y = 7.165x + 95.749	0.995	0.627	2.091
	coumaric acid	3	A	y = 10.904x + 187.144	0.999	1.037	3.456
	ferulic acid	4	A	y = 6.181x - 273.562	1.000	1.012	3.373
Flavonols	hyperoside	5	A	y = 14.315x - 262.753	1.000	0.549	1.829
	isoquercitrin	6	A	y = 11.437x + 100.974	0.998	0.475	1.585
	quercetin	7	A	y = 5.505x - 418.512	0.996	1.897	6.323
	quercitrin	8	A	y = 5.162x - 168.272	0.996	1.072	3.575
	rutin	9	A	y = 8.213x + 105.923	0.999	0.672	2.241
Benzoic acids	ellagic acid	10	B	y = 5.766x + 281.063	0.988	1.881	6.271
	gallic acid	11	B	y = 10.703x + 59.149	0.998	0.283	0.944
Catechins	catechin	12	B	y = 6.567x - 178.554	0.999	1.207	4.024
	epicatechin	13	B	y = 6.104x - 172.263	0.997	0.362	1.206
Tannins	castalagin	14	B	y = 3.261x - 65.994	0.995	1.755	5.850
	vescalagin	15	B	y = 19.124x - 42.783	0.996	1.749	5.829
Monoterpenes	limonene	16	C	y = 1.347x + 30.797	0.997	2.108	7.026
	phellandrene	17	C	y = 4.488x - 39.986	1.000	1.312	4.374
	sabinene	18	C	y = 29.237x - 296.283	1.000	0.026	0.087
	γ-terpinene	19	C	y = 2.461x + 205.211	0.993	2.758	9.194
	terpinolene	20	C	y = 0.056x - 1.809	0.995	7.479	24.930
Organic acids	citric acid	21	D	y = 1.695x + 16.075	1.000	1.065	3.549
	malic acid	22	D	y = 1.962x - 16.921	0.998	0.688	2.295
	oxalic acid	23	D	y = 20.034x + 287.523	0.999	0.098	0.328
	quinic acid	24	D	y = 1.193x - 3.232	1.000	2.054	6.845
	succinic acid	25	D	y = 0.845x + 47.492	0.997	1.492	4.972
	tartaric acid	26	D	y = 4.609x - 73.283	1.000	0.401	1.335
Vitamins	ascorbic acid	27	E	y = 40.541x - 798.702	0.998	0.236	0.786
	dehydroascorbic acid	28	E	y = 5.844x + 197.332	0.999	0.836	2.786

Table 2. Physical and chemical quality parameters in mulberry samples.

Sample	*Physical qualitative parameters*								
	weight (g)			*width (mm)*			*length (mm)*		
		mean	*SD*		*mean*	*SD*		*mean*	*SD*
G_1	3.07	3.09	0.02	11.77	12.07	0.26	27.86	27.46	0.43
G_2	3.10			12.18			27.52		
G_3	3.11			12.26			27.00		

Sample	*Chemical qualitative parameters*								
	total soluble solids (°Brix)			*titratable acidity (meq/L)*			*pH (upH)*		
		mean	*SD*		*mean*	*SD*		*mean*	*SD*
G_1	17.70	18.67	0.84	28.26	28.92	1.48	5.89	5.77	0.11
G_2	19.20			27.88			5.70		
G_3	19.10			30.61			5.71		

Table 3a. TPC and antioxidant activity data in analysed mulberry extracts.

Sample	TPC ($mg_{GAE}/100\ g_{FW}$)	Mean value ($mg_{GAE}/100_{FW}$)	SD	Antioxidant activity ($mmol\ Fe^{2+}/kg$)	Mean value ($mmol\ Fe^{2+}/kg$)	SD
G_1	232.53	236.94	5.65	21.52	22.12	0.55
G_2	234.98			22.26		
G_3	243.31			22.58		

Sample	TAC ($mg_{C3G}/100g_{FW}$)	Mean value ($mg_{C3G}/100g_{FW}$)	SD
G_1	74.29	80.02	9.56
G_2	74.71		
G_3	91.05		

Table 3b. Mulberry nutraceutical and quality traits compared to main common fruit. Mean values of each sample is given (N = 3). Different letters for each sample indicate the significant differences at $P < 0.05$.

Sample	TSS (°Brix)	Tukey test	SD	TA (meq/L)	Tukey test	SD	pH (upH)	Tukey test	SD
Apple	13.43	cd	0.53	49.01	a	3.28	3.81	d	0.04
Blackberry	12.81	cd	0.32	148.96	b	8.48	3.27	c	0.04
Blackcurrant	14.00	d	0.23	183.08	b	3.80	2.93	a	0.04
Blueberry	9.20	a	0.87	166.70	b	12.45	3.21	bc	0.12
Mulberry	18.67	e	0.84	28.92	a	1.48	5.77	e	0.11
Orange	12.53	c	0.21	383.10	c	20.54	3.36	c	0.05
Raspberry	10.70	b	0.36	413.57	c	51.62	3.03	ab	0.04
Strawberry	8.00	a	0.19	184.65	b	5.98	3.37	c	0.03

Sample	TPC ($mg_{GAE}/100_{FW}$)	Tukey test	SD	Antioxidant activity ($mmol\ Fe^{2+}/kg$)	Tukey test	SD	Anthocyanins (mgC3G/100gFW)	Tukey test	SD	Vitamin C ($mg/100_{FW}$)	Tukey test	SD
Apple	83.40	a	13.24	5.62	a	1.12	0.03	a	0.02	3.91	a	0.48
Blackberry	262.41	bc	9.57	64.96	e	4.18	99.93	b	5.08	45.07	c	5.82
Blackcurrant	434.43	d	99.66	76.86	f	8.55	225.22	c	29.06	162.73	f	7.17
Blueberry	299.60	c	44.12	49.36	d	5.05	230.63	c	15.74	12.60	a	2.79
Mulberry	236.94	bc	5.65	22.12	b	0.55	80.02	b	9.56	2.97	a	0.23
Orange	158.70	ab	1.91	12.43	ab	0.18	2.97	a	0.13	71.12	e	1.96
Raspberry	322.36	cd	7.15	13.02	ab	0.54	33.72	a	3.55	31.93	b	4.36
Strawberry	323.39	cd	57.80	35.43	c	4.69	35.16	a	8.53	57.95	d	2.60

Table 4. Single compound profile of analysed samples.

mg/100 g_{FW}	*Cinnamic acids*			
sample	***caffeic acid***	***chlorogenic acid***	***coumaric acid***	***ferulic acid***
G_1	4.04	16.29	2.99	15.55
G_2	8.03	23.27	2.81	16.64
G_3	5.45	24.99	4.24	14.90
Mean value	5.84	21.52	3.35	15.70
SD	2.02	4.61	0.78	0.88

mg/100 g_{FW}	*Flavonols*				
sample	***hyperoside***	***isoquercitrin***	***quercetin***	***quercitrin***	***rutin***
G_1	6.78	0.00	25.33	0.00	92.33
G_2	6.71	0.00	20.67	0.00	90.55
G_3	7.07	0.00	22.34	0.00	88.33
Mean value	6.85	0.00	22.78	0.00	90.40
SD	0.19	0.00	2.37	0.00	2.00

mg/100 g_{FW}	*Benzoic acids*		*Catechins*		*Tannins*	
sample	***ellagic acid***	***gallic acid***	***catechin***	***epicatechin***	***castalagin***	***vescalagin***
G_1	9.47	5.89	15.26	13.07	0.00	0.00
G_2	10.06	10.86	17.80	14.39	0.00	0.00
G_3	10.08	12.01	16.10	13.22	0.00	0.00
Mean value	9.87	9.58	16.39	13.56	0.00	0.00
SD	0.35	3.25	1.29	0.72	0.00	0.00

mg/100 g_{FW}	*Monoterpenes*				
sample	***limonene***	***phellandrene***	***sabinene***	***γ-terpinene***	***terpinolene***
G_1	849.10	0.00	4.89	0.00	493.29
G_2	820.35	0.00	4.81	0.00	396.01
G_3	918.75	0.00	4.95	0.00	521.25
Mean value	862.73	0.00	4.88	0.00	470.18
SD	50.59	0.00	0.07	0.00	65.74

mg/100 g_{FW}	*Organic acids*					
sample	***citric acid***	***malic acid***	***oxalic acid***	***quinic acid***	***succinic acid***	***tartaric acid***
G_1	1078.74	70.91	223.90	249.40	0.00	136.51
G_2	1037.45	84.91	234.63	182.86	0.00	175.27
G_3	1030.08	81.71	177.29	169.30	0.00	127.53
Mean value	1048.76	79.18	211.94	200.52	0.00	146.44
SD	26.22	7.34	30.48	42.87	0.00	25.37

mg/100 g_{FW}	**Vitamins**	*TBCC*
sample	***vitamin C***	
G_1	2.80	3316.54
G_2	2.87	3160.95
G_3	3.23	3252.83
Mean value	2.97	3243.44
SD	0.23	78.22

Table 5. Correlation among antioxidant activity and TPC\TAC\ all single bioactive compounds.

	Pearson correlation coefficient (R)					
	TPC	***TAC***	***Polyphenols***	***Monoterpenes***	***Organic acids***	***Vitamins***
Antioxidant activity	0.8637	0.7484	0.8790	0.2252	-0.8784	0.8233
correlation	positive strong	positive strong	positive strong	positive weak	negative strong	positive strong

Tab. 6. Contribution of antioxidant classes to the fruit phytocomplex in analysed extracts.

mg/100 g_{FW}	***Cinnamic acids***	***Flavonols***	***Benzoic acids***	***Catechins***	***Tannins***	***Anthocyanins***	***Monoterpenes***	***Organic acids***	***Vitamins***
G_1	38.88	124.44	15.35	28.33	0.00	74.29	1347.29	1759.46	2.80
G_2	50.75	117.93	20.92	32.19	0.00	74.71	1221.17	1715.12	2.87
G_3	49.59	117.74	22.09	29.32	0.00	91.05	1444.95	1585.91	3.23
Mean value	46.40	120.04	19.45	29.95	0.00	80.02	1337.80	1686.83	2.97
Phytocomplex	1.40%	3.61%	0.59%	0.90%	0.00%	2.41%	40.25%	50.76%	0.09%

Figures

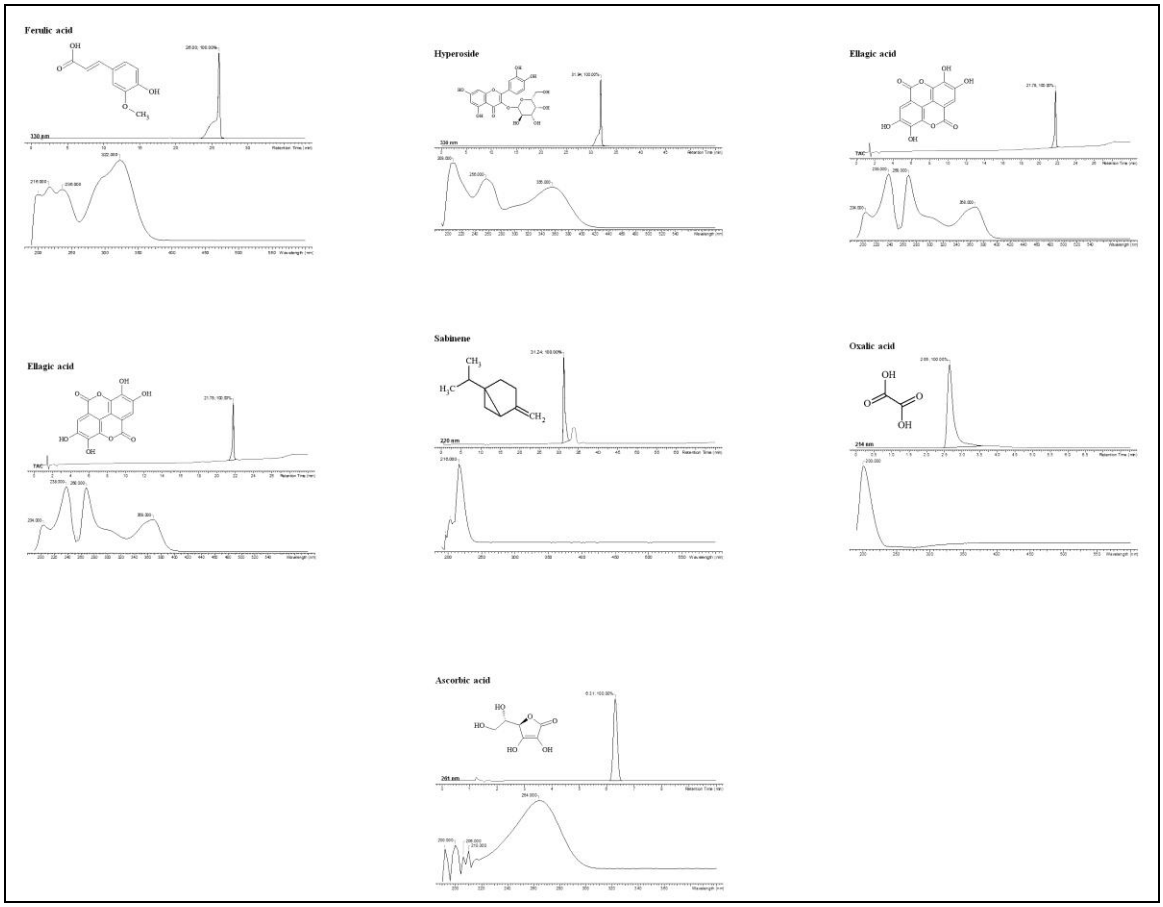

Fig. 1. Principal botanical standards (chemical structure, chromatographic profile, UV-vis spectrum).

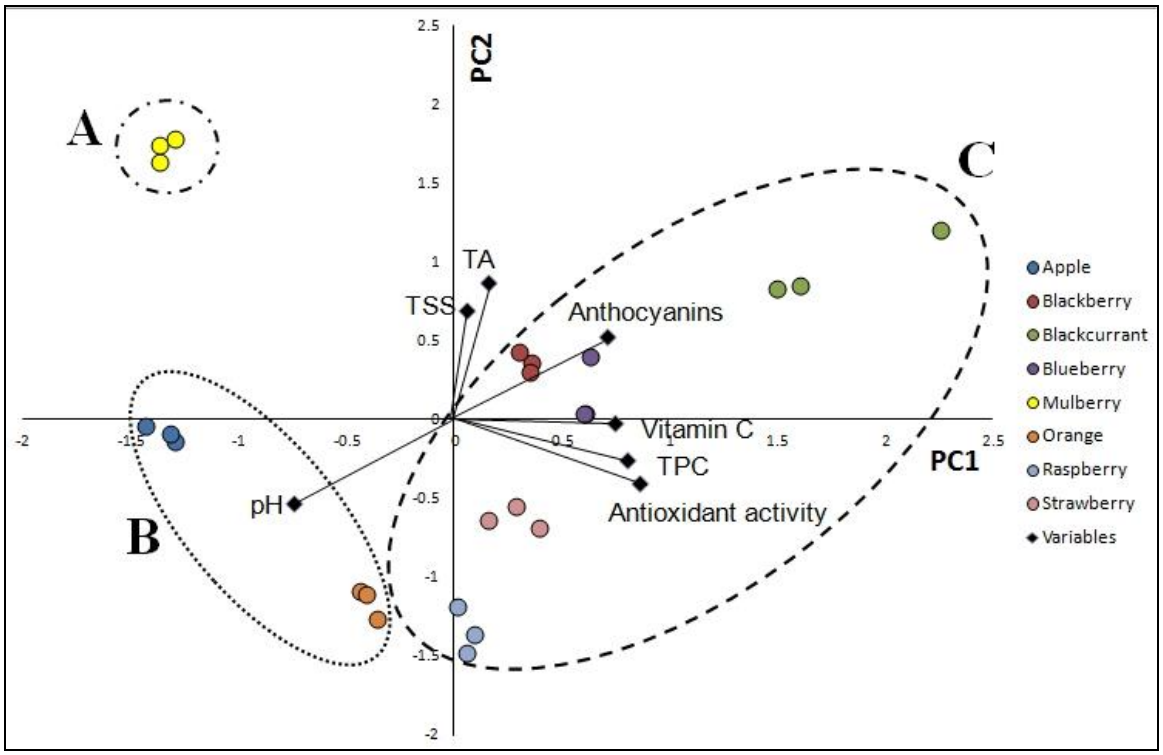

Fig. 2. PCA individual/variable graphs of fruit extract samples.