ANALYSIS AND CHARACTERIZATION OF ANTHOCYANINS IN *MORUS NIGRA L.* FRUITS, HARVESTED FROM IASSY AREA, ROMANIA

ANALIZA ŞI CARACTERIZAREA CONŢINUTULUI ÎN ANTOCIANI AL FRUCTELOR SPECIEI *MORUS NIGRA L.* RECOLTATE DIN ZONA IAŞI, ROMÂNIA

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Abstract. *Used for centuries in folk medicine worldwide, black mulberries have an important content of anthocyanins, still unused to their real technological potential. Were analyzed the ethanolic extracts of Morus nigra L. fruit (mulberry) harvested from the experimental field of UASVM Iaşi, being determined spectrophotometrically total content of anthocyanins and phenolic compounds. The anthocyanin profile of the extracts was performed by HPLC-DAD technique and for an objective assessment of extracts color, were calculated chromatic parameters (L*a*b*), based on VIS spectrum. It was found a high anthocyanins content (159.26±0.17 mg/100 g fruit, expressed as equivalent of cy-3-gl), the main representative of anthocyanin profile was cyanidin-3-O-glucoside, with a rate of participation over 70% of total area. Knowing the type and quantities of pigment available in horticultural products, can be appreciate the technical and functional qualities (preventive and curative) of fruits, and also the structure and stability of the color obtained.*
Keywords: *Morus nigra* L., Iassy area, anthocyanins, HPLC-DAD, chromatic parameters.

Rezumat. *Folosite în medicina tradiţională mondială de secole, dudele negre au un conţinut important în antociani, încă neutilizat la adevăratul potenţial tehnologic. Au fost analizate extractele etanolice obţinute din fructele speciei Morus nigra L. provenite din câmpul experimental al USAMV Iaşi, fiind determinate spectrofotometric cantităţile totale de antociani şi compuşi fenolici. De asemenea, a fost realizat profilul antocianic al extractelor prin tehnica HPLC-DAD, iar pe baza spectrului VIS au fost calculaţi parametrii cromatici (L*a*b*) ai acestora, pentru o evaluare obiectivă a culorii extractelor. A fost identificat un conţinut ridicat în antociani (159.26±0.17 mg cianidină-3-O-glucozid/100 g fruct,), principalul reprezentant al profilului fiind cianidină-3-O-glucozid, cu un procent de participare de peste 70 % din aria totală corespunzătoare antocianilor. Cunoscând tipul şi cantităţile de pigment disponbile în produsele horticole, se pot aprecia calităţile tehnologice şi funcţionale (preventive şi curative) ale fructelor, dar şi structura şi stabilitatea culorii obţinute.*
Cuvinte cheie: *Morus nigra* L., arealul Iaşi, antociani, HPLC-DAD, parametri cromatici.

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INTRODUCTION

Interest in natural pigments increasedgradually in the last decade, as a consequence of global legislation in a permanent update and consumers reticence to synthetic food colorants. It became an immediate need to identify new sources of vegetal pigments, non-toxic and non-polluting such as anthocyanins, phenolic compounds that belong to flavonoid class, responsible for the red-blue-violet nuances of fruits, flowers, and certain vegetables. Anthocyanins are now considered the most reliable alternative in replacement of somered-blue synthetic food colorants (Liu et al., 2004).

Anthocyanins extracted from the fruits of *Morus nigra L.*currently have limited applications in food industry, although possess a very good stability (at 80 °C), being used as color additives in confectionery products or carbonated beverages(Mazza and Miniati, 1993), but beyond their coloringcapacity, they were reported as owning a large number of beneficial effects on the human body being considered anti-cancer, vaso-protective, anti-inflammatory and neuro-protective agents (Chen et al., 2006, Wu et al., 2011).

Black mulberries contain significant amounts of anthocyanins, with values within the range of 25.3 and 83.0 mg cy-3-gl/100 g fresh fruit (Yang and Tsai, 1994; Ozgen et al., 2009) orbetween 147.68 and 2725.46 mg cy-3-gl/L juice (Liu et al., 2004). Were identified in the black mulberries extract four main anthocyanins, cyanidin-3-O-rutinoside (cy-3-rut), cyanidin-3-O-glucoside (cy-3-gl), pelargonidin-3-O-rutinoside (pg-3-rut), pelargonidin-3-O-glucoside (pg-3-gl) (Du et al., 2008; Qin et al., 2010). It is obvious that, whatever the area of origin or method of analysis was, cy-3-gl remaines the main pigment in *Morus nigra L.*

MATERIAL AND METHOD

Fruits were harvested from the experimental field of the University of Agricultural Sciences and Veterinary Medicine (UASVM) Iassy,V. Adamachifarm, at maturity of consumption, identified as the moment when the fruit turn its color from green to dark purple,28-30 July, 2010 (Yang and Tsai, 1994; Özgen et al., 2009).

Were determined certain physico-chemical properties of the fruits at harvest: the average mass of a fruit (g), the moisture content (%), soluble dry substance (°Bx), titratable acidity, ascorbic acid (titration with 2,6-DI),pH, reducing sugars (Schoorl method),total dry substance (%), as factors that influence the anthocyanin content.

Mulberries were stored at -18±2°C until extraction (7 days), anthocyanin transformation beingconsidered as minimal for fruits kept in a frozen state (Mazza, Miniati, 1993).Were performed two phases of extraction at an interval of 12 hours, untill the depletion of vegetal material. Extraction system used was ethanol-HCl-water (96:1:3), the final ratiobetween plant material and solvent was 1:5 (m/v) (5 g fruits/25 mL solvent). Determination of total monomeric anthocyanins content (ACY) was performed by the pH differential method(J. AOAC Int., 2005):

$$A \text{ (absorbance)} = (A_{520\,nm} - A_{700\,nm})pH\ 0.68 - (A_{520\,nm} - A_{700\,nm})pH\ 3.56;$$

Results were expressed as mg equivalent cy-3-gl/100 g fruit:

$$ACY \text{ (mg cy-3-gl,mg/L)} = (A \times MW \times DF \times 10^3)/(\varepsilon \times l);$$

where: ACY - total anthocyanin content (cy-3-gl equivalents, mg/L), A - absorbance,MW - molecular weight (449.2 g/mol forcy-3- gl); DF - dilution factor;l- optical pathlength (1 cm); Ɛ- molar extinction coefficient (26 900L×mol^{-1}× cm^{-1}for cy-3-gl); 10^3- factor for conversion from g to mg.

To obtain the total phenolic compounds content (TPC) was used Folin-Ciocâlteu photocolorimetric method, by measuring absorbance at λmax (765 nm) (Singleton and Rossi, 1965), results were expressed in grams of gallic acid equivalents (g GAE) /100g fresh fruits. Measurements were made using a UV-VIS spectrophotometer, Analytik Jena Specord 200,the results represent the average of three determinations, having calculated standard deviation (±), and the coefficient of variability (CV) or uniformity of the data obtained.

Measuring colors in CIE-76 system consistsin determination of *L** (brightness coordinate or psychometric clarity), *a** (red-green coordinate) and *b** (yellow-blue coordinate), and parameters: C (chromaticity or color saturation), H (tone), Intensity (A_{420}+A_{520}+A_{620}) and color tint (A_{420}/A_{520}) (OIV, 2012). For an objective assessment, extract color was simulated using the software Digital Colour Atlas® 3.0 Demo version.

Using a Shimadzu LC 20 liquid chromatograph, with a Hypersil ODS C18 separation column (25 cm length) at 20 °C, was performed the separation of anthocyanins. Elution was achieved at a flow rate of 1.2 mL/min. As eluent A was used H_2O:HCOOH:CH_3CN (87:10:3) and as eluent B, H_2O:HCOOH:CH_3CN (40:10:50), increasing eluent B from 6% to 60%. Anthocyanin compounds were individualized with a diode array detector (DAD) Shimadzu at λ518 nm, and their identification was made according to Ozgen et al., 2009 and Qin et al., 2010 .

RESULTS AND DISCUSSIONS

Physico-chemical properties of *Morus nigra* fruits, showed lower values than those offered by the literature, regardingthe titratable acidity, at a pH 3.01and a content of reducing sugars in juice of 22.7 mg glucose/100 g fruits (tab. 1).

Table 1

The physico-chemical properties of black mulberries samples

Sample	M. fr.* (g)	M.* (%)	T. ac.* (g m. a.)	Asc.ac.* (mg %)	Rd. sg.* (gl. %)	pH (units)	SDS* (°Bx)	TDS* (%)
Morus nigra	1,72 ±0,15	81,62 ±1,12	0,56 ±0,02	14,84 ±0,54	22,72 ±0,32	3,01 ±0,03	12,92 ±0,11	18,38 ±0,22

Legend:*M. fr. -average mass of fruit; M (%) - moisture; T. ac. (g m.a.) - titratable acidity (g malic acid /100g); Asc. ac. (mg %) -ascorbic acid (mg/100 g); Rd. sg. (gl. %) - reducing sugars (mg glucose/100 g fruits); SDS (°Bx) - soluble dry substance (°Brix); TDS (%) - total dry substance (%);**CV<10;*p-value*>0,05.**

The content of ascorbic acid was considered low compared with other species rich in anthocyanins, only 14.84 mg/100 g fruits. The coefficient of variability (CV)was in all cases less than 10 (CV<10), indicating that the data are homogeneous and behave uniform according to the studied parameters.

Afterthe interpretation of the absorption spectra at λ 520 nm, specific of anthocyanins (fig. 1) and λ 765 nm for phenolic compounds with reducing properties, were determined total quantities of monomeric anthocyanins (ACY) and total phenolic compounds (TPC), summarized in table 2. Average values of these parameters were found to be higher than the data available in the literature for this specie, presented in the introductorychapter.

Percentage of total anthocyanins participation at total phenolic compounds, was 14.61% (tab. 2). This value is important in the context of evaluationon the biologically active capacity of *Morus nigraL.* fruits.

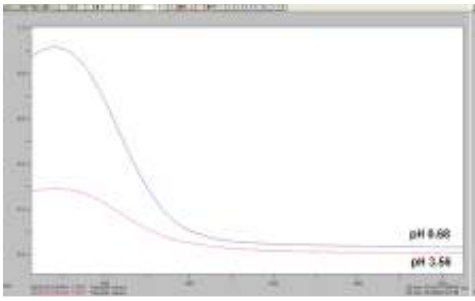

Fig. 1-Overlay of absorbance spectra at λ 520 nm, pH 0.68 and 3.56

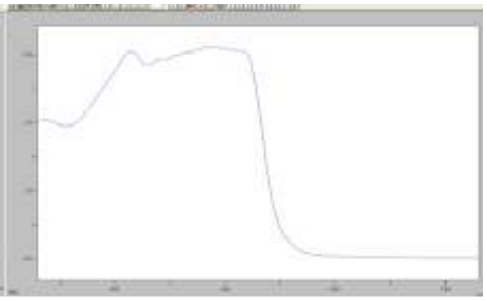

Fig. 2-VIS absorption spectrum of the black mulberriesextract

Table 2

ACYand TPCvalues of freshblack mulberries extract

Sample	ACY (mg cy-3-gl/100g)	TPC (g GAE/100g)	ACYofTPC (%)
Black mulberries	159.26±1.17[a]	1.0902±0.05[a]	14.61

CV<5;[a]*p-value*>0.05

To determine the stability of extracts and the anthocyanin losses by enzymatic and oxidative degradation, spectrophotometric measurements were repeated after 12 months storage of extracts at low temperature (4 °C) and in the dark. Mean values calculated are presented in table 3.

It is noted that the recorded values revealed a loss of anthocyanin pigments by degradation of 25.78 % and only 14.37 % for total phenolic compounds. These values are considered to be at a low level, indicating the high stability of mulberries anthocyanins. Degradation of anthocyanins can be attributed partially to acidic hydrolysis that may occur in the extract as a result of low pH (<1.0).

Table3

ACY and TPC values of black mulberries extract after 12 months storage

Sample	ACY (mg cy-3-gl/100g)	TPC (g GAE/100g)	ACYofTPC (%)
Black mulberries	118.20±0.84[a]	0.9335±0.08[a]	10.32

CV<5;[a]*p-value*>0.05

Anthocyanin profile of the extract includes four main anthocyanins, in which aglicons are represented by cyanidin and pelargonidin (fig. 3). Anthocyanin that participated in the highest proportion at black mulberries profile was cy-3-rut (70.30%), followed by cy-3-gl (29.65%), the othersidentified compounds influencein a very small proportion the anthocyanins area (tab. 4). By the analysis of UV-VIS spectra of peaks, obtained through chromatographic analysis, can be observedthe increase in

spectral line at λ 270-280 nm, specific to all phenolic compounds and at λ 515-530 nm, specific only to anthocyanin compounds (fig. 4, fig. 5).

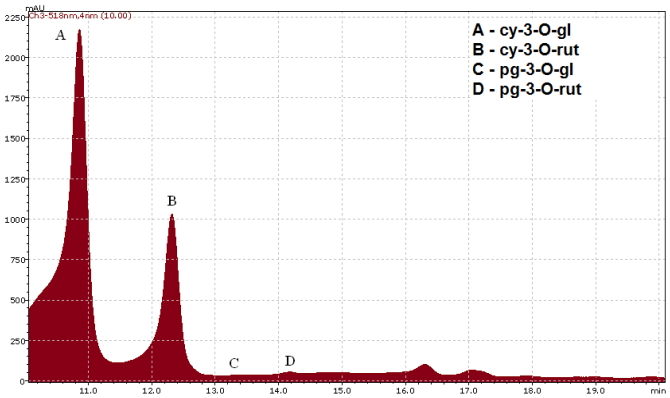

Fig. 3-HPLC chromatogram of black mulberries ethanolic extract

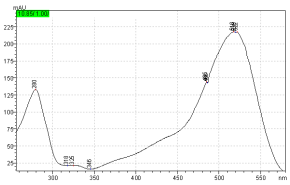

Fig. 4-UV-VIS spectra of cy-3-gl

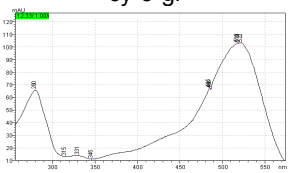

Fig. 5- UV-VIS spectra of cy-3-rut

Table4

Percentages of area corresponding to anthocyanins identified in black mulberries

Species	cy-3-gl	cy-3-rut	pg-3-gl	pg-3-rut	Total
Morus nigra L.	70.30	29.65	0.01	0.03	100.00

Color parameters of the black mulberries extract were calculated after obtaining the absorption spectrum in the visible domain (fig. 2). In table 5 were summarized the chromatic parametersvalues,average psychometric clarity (40.66) being pointed out also by the visual analysis of the extract.

Table5

Chromatic parameters of black mulberries extracts

Species	Color simulation	*L**	Color coordinates		C*	H°*	H*	I*
			*a**	*b**				
Morus nigra L.		40.66	73.86	65.71	98.68	41.66	5.24	0.69

Legend: *L – Brightness (clarity): 0 (opaque) - 100 (transparently); a – red (+) - green (-); b – yellow (+) - blue (-);C – Saturation; H° – Tone; H – Color tint; I – Intensity of color.

The parameter *a** has significant positive value (73.86), indicating the presence of red color given by the participation of anthocyanins, fact also sensorially observed by the analysis of extractcolor simulation (table 5). Sensation of bright red is more pronounced because of color hue, value which is closer to zero (5.24), the color being slightly influenced by yellow nuances. Considering that at H°=90° the corresponding color is yellow, we noticed an approachingto the angle of 0° of this parameter (41.66°), dominated by the blue shades. There is a general correlation between luminosity (*L**) and color intensity (*I**), thus in the intensely colored extracts, the psychometric clarity (*L**) is lower,due to a higher phenolic content, which is observed alsoin this situation.

CONCLUSIONS

1. Alcoholic extracts of *Morus nigra L.* fruits denoted an enormous therapeutic and commercial potential still insufficiently exploited.

2. The content of anthocyanins and total phenolic compounds had values higher than those existing in the literature for this species, the percentage of anthocyanins participation at the total phenolic compounds, was over 14 %. After 12 months of cold storage, ACY and TPC values remained high, suggesting the stability of these compounds, as an important technological feature.

3. Anthocyanin profile comprises four anthocyanins, represented by the cyanidin and pelargonidin, with rutinoside and glucoside forms, cyanidin-3-O-glucoside being the main anthocyanin of the profile.

4. Chromatic parameter *a**, presented significant positive values, indicating the presence of red color in the extract given bythe participation of anthocyanins in colour composition.

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