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DOCTORAL DOMAIN: HORTICULTURE
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DOCTORAL THESIS

„STUDIES REGARDING SOME VEGETAL PIGMENTS WITH FUNCTIONAL VALUE FROM HORTICULTURAL PRODUCTS"

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ABSTRACT

Keywords: anthocyanins, phenolic compounds, horticultural products, functional foods, Iaşi area

Doctoral thesis theme is part of the research ensemble carried out worldwide on the development of obtaining food with health benefits (*functional foods*), resulting from the appearance in modern society of a new concept concerning the role of food in human health and reducing the risk of diseases to consumers.

In the last years, it has become a challenge for food industry to identify new, efficient, non-toxic and non-polluting sources of plant pigments, to replace synthetic dyes currently used widely because of their stability and low price.

This paper aims to study *anthocyanin pigments*, compounds with important functional value, from fruits of some horticultural species, harvested from the north-eastern area of Romania, Iasi city areal, in the years 2010 and 2011. Studies have been conducted on horticultural products with a high anthocyanin potential, less studied in our country, such as elderberries (*Sambucus nigra* L.), black mulberries (*Morus nigra* L.), black currants (*Ribes nigrum* L.) or species with limited consumption in fresh condition (*Prunus avium* L. and *Prunus cerasus* L.), but with a functional potential shown by previous research, mainly due to their chemical composition. Also, was collected the pomace derived from black grapes (*Vitis vinifera* L.) vinification, to determine the anthocyanin content and the recovery yield of these compounds with bioactive role, from the processed oenological material.

Were determined some physicochemical and structural characteristics of fruits at harvest, followed by determining the action of some factors with influence on phenolic content of fruits (enzymes from the oxidoreductases class). Analysis was continued by the obtaining and drawing of anthocyanin profile through liquid chromatography (HPLC-DAD), followed by the identification of the main representatives through mass spectrometry (ESI-MS) and their individual quantification (in equivalent cyanidin 3-O-glucoside). Knowing the type and quantities of pigments available in horticultural products, the main representatives and the relationship between them, can be evaluated the technological qualities, especially their functional properties, along with the structure and stability of the color obtained.

For this purpose, were established the following objectives:

1. ***Evaluation of physico-chemical properties of the analised fruits***, in order to know their chemical composition at harvest and the conditions in which the reported quantities of compounds and the anthocyanin profile of the samples were obtained.

2. ***Study of anthocyanins from selected horticultural products***, which begins with obtaining of ethanolic and methanolic extracts, being continued by the determination of the total amount of anthocyanin, separation, identification and quantification of individual anthocyanins of the profile and reanalyzing total anthocyanin content after 12 months of extracts storage.

3. ***Study of phenolic compounds from horticultural products considered***, including here the flavonoids class, which contains the anthocyanin pigments subclass, existing percentage relations or known values of some ratios between these classes and subclasses of phenolic compounds. It was monitored the quantitative determination of total flavonoid compounds content and total phenolic content of the fruits, and the evolution of these values throughout the period of storage.

4. ***Analysis of chromatic parameters***, which allows an objective estimation of samples color, its quality and the possibility to follow in dynamic the evolutionary phenomena, mainly related to structural and biochemical changes of anthocyanins, as main participants compounds in the formation of fruits red color. This analysis was completed by a computer simulation of extracts color, based on the chromatic parameters registered.

5. ***Study on some enzymes of oxidoreductases group*** (polyphenoloxidase, peroxidase, catalase), as direct or indirect factors of influence on anthocyanin content of the fruits.

6. ***Testing of the statistical hypothesis*** (*analysis of variance - ANOVA*) to highlight in what measure one or more factors (variety or genetic factor, year of harvest, solvent used for the extraction), influence on the resultative characteristic, represented by the total anthocyanin content of the samples.

Doctoral thesis contains 266 pages, which includes 91 tables, 107 figures and color photos, references containing 217 titles and 12 attached document, being structured in two parts. The first part of the thesis, *present state of research* regarding the approached issues, include *introduction* and three chapters, and the second part, that represents *personal researches* presented in four chapters including the final *conclusions and recommendations*.

Chapter I, entitled ***Present state of research regarding anthocyanin pigments***, presents a brief history of plant pigments, definition, classification and physico - chemical properties of anthocyanins, their presence in horticultural products and factors that influence their stability.

Anthocyanins (gr. *anthos* - flower and *kyanos* - blue) are natural pigments, vegetal active principles belonging to the flavonoid class, secondary products of plant metabolism, responsible for red-blue-purple shades of fruits, flowers and some vegetables, being the most important group of hydrosoluble pigments visible to the human eye (Davies, 2004).

In chapter II of the thesis, ***Present state of research regarding functional foods***, is presented a synthesis of knowledges at national and international level on the "*functional foods*" concept, being exposed the functional properties of anthocyanins from horticultural products. As the American Dietetics Association notified, the term "*functional*" implies that the food, through its components, has a specific value recognized, that leads to a health benefit, including the reduction of diseases risk to the consumer.

The final chapter of the first part of the thesis, entitled ***Present state of research regarding the methods of analysis used in the study of anthocyanins***, summarizes the issues related to the extraction and purification of anthocyanins, the use of spectrophotometric and chromatographic methods for their investigation and some specific elements of variational statistical analysis (ANOVA).

Chapter IV, ***Objectives of the research***, make the transition to the second part of the thesis, presenting a series of considerations on the purpose and objectives of the research, completed by the activities carried under each objective.

In chapter V, are presented the ***biological material*** and ***research methods*** used. In this chapter is exposed the organizational and institutional framework in which the experimental research were conducted and the area of biological material origin, being described the varieties selected for study and the methods used to achieve mentioned objectives.

Biological material came from the experimental field of Research and Development Station for Fruit growing (R.D.S.F.) Iasi, located as fruit growing area in the region VIII, of Moldavian Plateau, Iasi-Răducăneni basin and from the didactic - experimental field of "Vasile Adamachi" Farm, located in Copou area, Iasi, belonging to the Unversity of Agricultural Sciences and Veterinary Medicine (U.A.S.V.M.) "Ion Ionescu de la Brad", Iasi.

The most important part of instrumental measurements was carried out in the Laboratory of Chemistry from the Departament of Engineering for Innovation, University of Salento Lecce, Italy and in the Research Center for Oenology of the Romanian Academy, Iasi Branch.

Were selected for the experimental determinations six horticultural species:

• six varieties of sweet cherries (*Prunus avium* L.), to cover the variety of forms existing within species, three varieties of sweet cherries: Van, Stella, Maria, two hybrid elites of cherry:

Oana (sin. HC-840,860) and Radu (sin. HC-840836) and a variety of bitter cherries: Amar de Maxut, all grown in the experimental field of R.D.S.F. Iasi;

• four varieties of sour cherries (*Prunus cerasus* L.): Engleze timpurii, Meteor Korai, Crişana 2, Mocăneşti 16, with the same sourcing as sweet cherries;

• black mulberries (*Morus nigra* L.), from Copou Iasi, "V. Adamachi" farm;

• two indigenous varieties of blackcurrant (*Ribes nigrum* L.), recently introduced into assortment, Abanos and Ronix, grown in the experimental field of U.A.S.V.M. Iasi;

• two provenances of european elder (*Sambucus nigra* L.), fruits being harvested from Copou area, Iasi, located in the north of the city and Cetăţuia Forest, located in the south;

• three grape varieties (*Vitis vinifera* L.): a local variety, Fetească neagră and two cosmopolitan varieties, Merlot and Cabernet Sauvignon. It has also been recovered and examined the pomace resulted from the processes of winemaking, in fresh state and after drying.

To achieve the proposed objectives, research methods used included:

1. *Physical and structural determinations effectuate on samples at harvest*: average fruit weight, weight of 100 berries, the average weight of seed, number of berries.

2. *Chemical determinations effectuate on samples at harvest*: moisture, total dry substance, soluble dry substance, titratable acidity, ascorbic acid, pH and content in reducing sugars.

3. *Determination of some enzymes activity, belonging to the oxidoreductases group*: polyphenoloxidase (Ermakov method), peroxidase (Bergmeyer method) and catalase (gas meter method).

4. *Analysis and characterization of anthocyanins*: determination of total monomeric anthocyanin by pH differential method, their chromatographic individualization and separation (λ = 520 nm), identification by mass spectrometry (electrospray ionization, positive ion mode) and quantification of individual anthocyanins identified based on standard solutions.

5. *Determination of total flavonoids* (Dowd method) and *phenolic compounds* (Folin-Ciocalteu photocolorimetric method).

6. *Determination of the chromatic parameters*, the method CIE *L*a*b* - 1976.*

Chapter VI, ***Results and discussion***, contains the largest part of the thesis and present experimental results recorded for each species, and finally the statistical analysis of the data and their interpretation.

Fruits of the six species analyzed, presented a series of physicochemical properties with specific values for each variety, which could not be correlated with a variation of anthocyanins and total phenolic content. Destructive action of ascorbic acid on anthocyanins was highlighted

in sour cherries, pigment decomposition being accelerated by the presence of ascorbic acid. In the same context, significant quantities of ascorbic acid in black currants (140-160 mg/ 100 g fruit) could not be correlated with low concentration of fruits anthocyanins.

The most significant amounts of anthocyanins in the study were recorded at species *Vitis vinifera* L., in fresh skins of Cabernet Sauvignon grapes, exceeding 1560 mg cy-3-gl/100 g, at the extraction with methanol. The following species as importance, in terms of anthocyanin content from methanolic extracts of fresh fruits, were: *Ribes nigrum* L., variety Ronix (452.82±6.15 mg cy-3-gl/100 g fruit), *Sambucus nigra* L., provenance Copou (428.60 ±6.02 mg cy-3-gl/100 g fruit), *Prunus avium* L., Amar de Maxut variety (193.07±0.54 mg cy-3-gl/100 g fruit), *Morus nigra* L. (159.26±1.17 mg cy-3-gl/100 g fruit) and *Prunus cerasus* L., Engleze timpurii variety (66.29±0.17 mg cy-3-gl/100 g fruit).

With the use of methanol as a solvent, the quantities of extracted anthocyanins were in all cases higher than those reported for ethanolic extracts. As percentage, additional quantities determined did not justify the use of this toxic compound in all cases. Thus, at black mulberries, additional quantities of anthocyanins extracted with methanol did not exceed 5%, at elderberries was between 6 and 8.5%, and in the case of grapes, ranging between 20 and 25%.

A positive correlation was detected between total flavonoid compounds content, total phenolic and the values of anthocyanin content, except extracts obtained from sour cherries, where varieties with an important content of total phenolic compounds, does not held also the highest amounts of anthocyanins, the ratio between those two classes of compounds being specific to each variety.

In the case of *Vitis vinifera* L. species, fresh pomace of Merlot variety retained the most important percentage of anthocyanins after grapes pressing, between 43 and 46% of existing concentration in the raw material. The variation between years of study may be due to the winemaking technologies, more specifically, to the inability of obtaining a constant yield of grapes pressing from year to year, in the experimental system of research.

Along with drying under natural conditions of elder and black currant fruits, as well as fresh pomace obtained from the grapes processing, occurred a concentration of phenolic compounds quantities, with the specification, that the drying efficiency of the fresh samples ranged around 20%, with an average of remanent moisture 7%. Dry pomace of Cabernet Sauvignon has concentrated the most important quantities of total phenolic compounds, 5.1074 ±0.03 g GAE/100 g dry product, anthocyanins owned 1568.87±3.13 mg cy-3-gl/100 g dry product.

Major negative correlations were found between anthocyanin content and the activity of peroxidase and polyphenoloxidase (except the species *Prunus cerasus* L.), a higher enzyme activity corresponded to a low concentration in anthocyanins, demonstrating the negative action of enzymes belonging to oxidoreductases group on this class of phenolic compounds. In the case of catalase for varieties with a high content of anthocyanins, its activity was also important, being inversely related with the activity of the other enzymes analyzed, due to its ability to decompose peroxides, compounds with a destructive action on the structure of anthocyanins.

Anthocyanins isolated from plants are unstable and very disposed to degradation. Because the color stability of a product is one of the essential factors in his approval to sale, were recorded the anthocyanins degradations of unpurified ethanolic extracts after 12 months of storage in optimal conditions of pH (1) and temperature (4±1 °C) and in the dark. Within the study, extracts with the highest stability of anthocyanin were obtained from the fruit of *Prunus cerasus* L. varieties (degradation 13-23%), followed by the species *Morus nigra* L. (26%) and *Ribes nigrum* L. (29%). The high stability of anthocyanins could be correlated with higher initial concentration of the solutions, with some exceptions. Thus, the Cabernet Sauvignon grape skins, although they held the major anthocyanin concentrations of the study, have suffered the most significant degradation of these compounds after the storage period (76%), in the context of a high percentage of acylated anthocyanins (19% of total anthocyanins), however, the final quantities remained significant (773.51±5.86 mg cy-3-gl/100 g).

Anthocyanin profile of methanolic extracts determined by the LC/MS technique, was unique, characteristic for every species, area percentages and the individual quantities of anthocyanin identified were specific for each variety, but with general trends within the species.

Anthocyanin cyanidin 3-O-glucoside, the most widespread in nature and on the basis of which were quantified individual and total amounts of anthocyanins was identified in the profile of all species selected for the study, holding the position of main anthocyanin only in the fruits of *Morus nigra* L. and *Sambucus nigra* L.

Diglycosidic forms of anthocyanins have been identified in the profile of species *Prunus cerasus* L. (cyanidin 3-O-glucosyl-rutinoside) and *Sambucus nigra* L. (cyanidin 3-O-sambubiosyl-5-O-glucoside and cyanidin 3,5-O-diglucoside), all having in composition cyanidin as aglycone.

It was observed a selective degradation of anthocyanins along with drying of plant material, indicating a higher stability of some anthocyanin species, such as malvidin 3-O-

glucoside, delphinidin-3-O-rutinoside, pelargonidin 3-O-glucoside, as a decisive technological feature in selection of anthocyanin species used in functional food recipes.

Anthocyanin content values obtained by spectrophotometric method, were, at the majority of species studied (excluding *Morus nigra* L.), higher than the individual values cumulated, obtained by liquid chromatographic technique, with an overall percentage reaching a maximum of 15% at fresh samples and 30% at dried samples.

Along with increasing of the phenolic concentration in extracts, psychometric clarity values were negatively affected, fact that can be observed also through the sensorial analysis of computerized simulations of color. All samples showed positive values, medium and high, of the parameter *a**, corresponding to the complementary coordinate of red color, confirming the participation of anthocyanins as the main components of fruits color. Parameter *b** values were in generally positives, shades of yellow being predominant beside the blue ones. Once with the use of methanol, values of the chromatic parameters underwent important changes, taking place a additional extraction of phenolic compounds other than anthocyanins (yellow flavonoid pigments, tannins), ethanol being more selective for other phenolic compounds, extracting predominantly anthocyanins.

Statistical analysis of variance (ANOVA) applied on the experimental values of total anthocyanin content, at a 5% probability of transgression, suggested that at all species, values were influenced *insignificant* by the year of study (*p-value* > p = 0.05, F calc. < F crit.) and in relation to genetic factors were *significant* (grapes) and *very significant* (cherries) (*p-value* < p = 0.001, F calc. >> F crit.) variations of anthocyanin concentration. The solvent used for the extraction of anthocyanins influenced *significant* and *distinctly significant* (*p-value* < p = 0.01) total anthocyanins content of sweet cherries and sour cherries respectively.

Chapter VII of the thesis, presents the ***final conclusions and recommendations*** arising from the analysis of experimental data.

The important quantities of anthocyanins identified in the fruits of analyzed species, justifies the extraction of these phenolic compounds from the considered material and demonstrates that fruits from Iasi area, in fresh and dried state or as waste from their processing, are valuable and accessible source of pigment, with a huge functional potential, with the use in food and pharmaceutical industries, being successfully able to replace the synthetic compounds, extracts maintaining their biologically active properties even after long periods of storage.