STUDIES ON THE IMPROVEMENT OF ANTHOCYANIN EXTRACTION FROM GRAPE SKINS

STUDII PRIVIND OPTIMIZAREA PROCESULUI DE EXTRACŢIE A ANTOCIANILOR DIN PIELIŢELE DE STRUGURI

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Abstract. *Anthocyanins, besides the fact that they are a source of natural coloring, represent a very complex subject of research due to their antioxidant, antibacterial and anticancer features. Thanks to the structural features specific to anthocyanins and by performing the extraction of raw material by solvents in static conditions we evaded the use of mechanical and magnetic stirring of samples to prevent oxidation of active substances. Extracts obtained after 24, 48, 72 and 96 hours from solvent addition, were separated from residues by decantation and/or filtration and further subjected to centrifugation for 5 – 10 minutes at a speed of 4000 – 8000 rpm and were stored in a cold place for additional tests. To assess the efficiency of the extraction process according to the vegetal origin we used grape skins of Fetească neagră, Băbească neagră, Arcaş, Negru de Drăgăşani, Cabernet Sauvignon, Merlot and Chambourcine varieties for each extract, the anthocyanin content (mg/L) being determined in each case.*

Key words: extract, anthocyanins, grape, skins.

Rezumat. *Antocianii, pe lângă faptul că reprezintă o sursă de coloranţi naturali, constituie un subiect amplu de cercetare datorită proprietăţilor antioxidante, antibacteriene şi antineoplazice. Datorită caracteristicilor structurale specifice ale antocianilor, prin efectuarea extracţiei materiei prime cu solvenţi în condiţii statice, s-a evitat utilizarea agitării mecanice şi magnetice a probelor pentru a nu favoriza procesele de oxidare ale substanţelor active. Extractele obţinute la 24, 48, 72 şi 96 ore de la adăugarea solventului, au fost separate de reziduuri prin decantare şi /sau filtrare şi supuse ulterior centrifugării timp de 5 – 10 min la viteza de 4000 – 8000 rotaţii pe minut şi păstrate la rece pentru analizele ulterioare. Pentru aprecierea eficienţei procesului extractiv funcţie de provenienţa vegetală au fost utilizate pieliţe de struguri aparţinând soiurilor Fetească neagră, Băbească neagră, Arcaş, Negru de Drăgăşani, Cabernet Sauvignon, Merlot şi Chambourcine, pentru fiecare extract fiind determinat conţinutul în antociani (mg/L).*

Cuvinte cheie: extract, antociani, struguri, pieliţe

INTRODUCTION

The anthocyanins from grapes are located in the cell vacuoles of skins' epidermis and hypodermis as well as in the cells near to the pulp. From chemical

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perspective anthocyanins (pigments) are heterocyclic, polyhydroxilic and/or metoxylic compounds characterized by a phenyl-benzopyril nucleus to which is attached one or two molecules of sugars (glucose, galactose, rhamnose). By acid hydrolysis anthocyanins release the sugarless colored component (aglycone), called anthocyanidine or anthocyanidol. This is the reason why grape pigments are recently called anthocyanidols (Colette Navarre, 2002). In the skin of grapes we identified 8 flavonoid monoglucosides and three diglycosides among the group of flavonoid compounds.

Anthocyanin extraction from vegetal materials depends on the chemical, structural features, the extraction method used, the sizes of vegetal material particles as well as on the presence of interfering substances. By correlating all data we have till now, we may draw the conclusion that the best procedure of anthocyanin extraction for all vegetal sources is not yet available. Usually, anthocyanin extraction from vegetal materials is carried out using methanol as dissolving agent also adding small amounts of hydrochloric acid. Considering the future biological applications of vegetal extracts, ethyl alcohol was selected as final extraction medium. Moreover, the dissolvent chosen was not acetous to prevent anthocyanin degradation (Escribano-Bailon M. et al, 2003; Mazza G. et al., 1983, Ribereau – Gayon P., 1965).

Previous studies pointed out the fact that the extraction process in a stable discontinuous system is the most effective for obtaining vegetal anthocyanic extracts as the temperature necessary is of only 30oC and does not trigger oxidation of active substances by lack of stirring the extraction system (Savin C. et al., 2007).

The objective of this study was to determine the optimum duration of the extraction process necessary for the depletion of vegetal material as far as anthocyanin content is concerned.

MATERIAL AND METHOD

Anthocyanic extraction from the skins of grape varieties such as Fetească neagră, Băbească neagră, Arcaş, Negru de Drăgăşani, Cabernet Sauvignon, Merlot and Chambourcine was performed by means of the discontinuous method, in stable context. The extraction processes were carried out in bottles with ground glass stopper and flat bottom, inside which the vegetal materials – grape skins were inserted also adding the dissolvent ethylic alcohol heated to 40°C. The extractions were performed at a temperature of 30°C observing the solid material / dissolvent ratio of 1/10. The anthocyanic extracts obtained after 24, 48, 72 and 96 hours since adding the dissolving agent were separated from deposits by decanting and/or filtration and were further subjected to centrifugation for 5-10 min at a speed of 4000–8000 revolutions per minute. For the tests the anthocyanic extracts obtained were stored in dark bottles at a temperature of 4°C, thus determining anthocyanin content (mg/L) by R. Gayon and Sonestreet method – 1965.

RESULTS AND DISCUSSIONS

Anthocyanic compounds have some features which influence the techniques of separation, analysis and conditioning of extracts. To assess the efficiency of the extraction process according to the vegetal origin we used the

skins of both local and international grape varieties (Fetească neagră, Băbească neagră, Arcaş, Negru de Drăgăşani, Cabernet Sauvignon, Merlot and Chambourcine).

The vegetal extracts obtained by discontinuous extraction, in stable context, were analyzed from the perspective of anthocyanin content. The data obtained are shown in the diagram from figure 1.

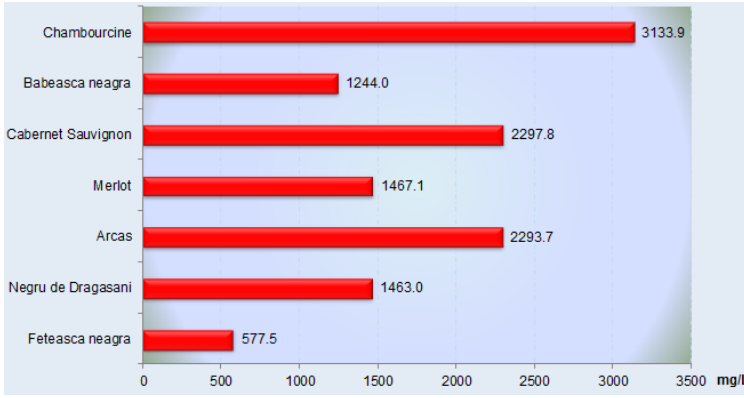

Fig. 1 - Anthocyanin content of vegetal extracts obtained

We noticed that among the vegetal materials tested the extracts obtained from the skins of Chambourcine variety have the highest anthocyanin content (3133.0 mg/L), being followed by Cabernet Sauvignon with 2297.8 mg/L and Arcaş with 2293.7 mg/L. The very similar anthocyanic content of Cabernet Sauvignon and Arcaş varieties, may be explained by the genetic origin of the latter, which is obtained by means of sexual hybridization between Cabernet Sauvignon and Băbească neagră varieties.

The lowest anthocyanin content was found for Fetească neagră variety with only 577.5 mg/L. The extracts obtained from the skins of Negru de Drăgăşani and Merlot varieties had almost similar values of anthocyanic content, namely 1463.0 and 1467.1 mg/L, being followed by Băbească neagră variety with 1244 mg/L.

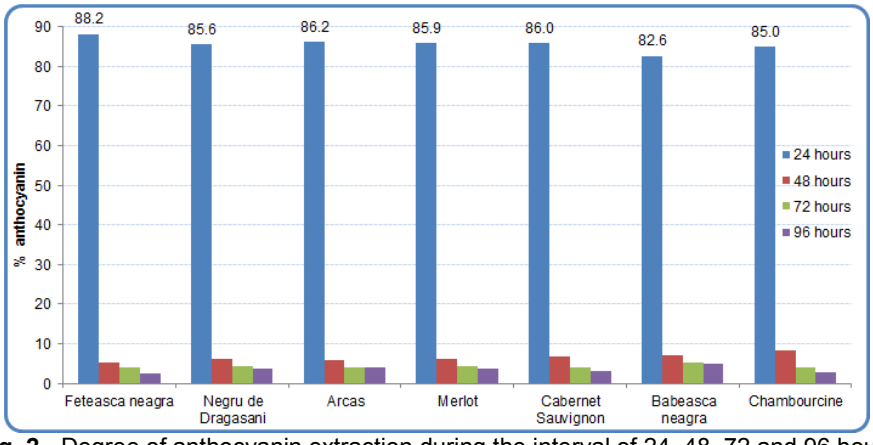

Fig. 2 - Degree of anthocyanin extraction during the interval of 24, 48, 72 and 96 hours

The extraction process carried out in discontinuous stable system was studied in dynamics, taking samples at various times (figure 2). According to the graphical representation of data the extracts obtained after 24 hours of contact between vegetal materials and dissolving agent had the highest anthocyanin content, over 82.6% of anthocyanins being extracted during this time period.

The low percentage of anthocyanins extracted in dissolvent after 48 hours (5.4 – 8.3%), 72 hours (3.9 – 5.2%) and respectively 96 hours (2.5 – 5.0%) does not justify the continuation of the extraction process. Moreover the high contact time between the vegetal material and the dissolvent may support the oxidation of active substances from extracts.

The extraction of anthocyanins from vegetal materials is not complete, this conclusion being also supported by data from literature. The probable explanation consists in the different solubility of anthocyanins but also the oxidative degradation during the extraction process.

CONCLUSIONS

1. We noticed that among the vegetal materials tested the extracts obtained from the skins of Chambourcine variety have the highest anthocyanin content (3133.0 mg/L), being followed by Cabernet Sauvignon with 2297.8 mg/L and Arcaş with 2293.7 mg/L.

2. The extracts obtained after 24 hours of contact between vegetal materials and dissolving agent had the highest anthocyanin content, over 82.6% of anthocyanins being extracted during this time period.

3. By means of stable discontinuous extraction method the vegetal material is not completely depleted as far as anthocyanin content is concerned.

Acknowledgments: *This work was cofinanced from the European Social Fund through Sectoral Operational Programme Human Resources Development 2007-2013, project number POSDRU/I.89/1.5/S62371 "Postdoctoral Schole in Agriculture and Veterinary Medicine area".*

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HPLC DETERMINATION OF OCHRATOXIN A IN BREAD AND CORN FLOUR

DETERMINAREA OCHRATOXINEI A DIN PÂINE ŞI MĂLAI PRIN METODA HPLC

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Abstract. *Ochratoxin A is a mycotoxin produced by different species of Aspergillus and Penicillinium fungi. Ochratoxin A has been found in peanuts, cereals, coffee, bread, flour, corn, peas, beans, beer, wine. The aim of this paper is to determine ochratoxin A in bread and corn flour. The samples purchased from markets and agro - food markets were processed and then analyzed by high performance liquid chromatography (HPLC) with fluorescence detection.*
Key words: ochratoxin A, bread, corn flour, HPLC

Rezumat. *Ochratoxina A este o micotoxină produsă de diferite specii de fungi din genurile Aspergillus şi Penicillinium. Ochratoxina A a fost identificată în alune, cereale, cafea, pâine, făină, mălai, mazăre, fasole, bere, vin. Scopul lucrării este de a determina ochratoxina A din probe de pâine şi mălai. Probele achiziţionate din reţeaua comercială şi pieţe agro-alimentare au fost prelucrate şi apoi supuse analizei prin cromatografie de lichide de înaltă performanţă (HPLC) cu detecţie în fluorescenţă.*
Cuvinte cheie: ochratoxina A, pâine, mălai, HPLC

INTRODUCTION

Ochratoxin A (fig. 1) is a toxic metabolite produced by species of *Aspergillus* genus in tropical and subtropical areas and species of *Penicillium* genus in temperate zones (Eskola M., 2002). For the first time, ochratoxin A was isolated from cultures of *Aspergillus ochraceus* by Van der Merwe et all. in 1965 while testing the toxin ability of fungal strains isolated from cereals and vegetables (Van der Merwe K.J. et all., 1965).

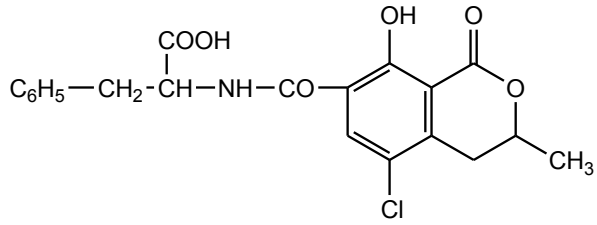

Fig.1 – Chemical structure of ochratoxin A

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Penicillium verrucosum is frequently isolated from cereal samples while *Aspergillus ochraceus* contaminates green coffee beans, spices, cocoa beans, soybeans and peanuts (Kuiper-Goodman T. et al., 1987).

Experimental studies demonstrated toxic effects of ochratoxin A: immunotoxic (Harvey R. B. et al., 1992), nephrotoxic (Vrabcheva T. et al., 2004), teratogenic (Wangikar P.B. et al., 2005). Due to the carcinogen action, shown on experimental animals, ochratoxin A has been included by the International Agency for Research on Cancer (1993) among the possible carcinogenic substances - 2B (IARC, 1993).

The aim of this study is to determine the content of ochratoxin A in two categories of food, bread and corn flour, given the high consumption of these foods by the population of Romania and reporting data obtained with the regulations set by the EU regarding the maximum limits permitted. Thus, the European legislation sets the maximum limits for ochratoxin A: 5 μg / kg for cereals and 3 μg / kg for cereal product (Commission Regulation, 2006).

MATERIAL AND METHOD

Reagents:

- Ochratoxina A from *Aspergillus ochraceus* (Sigma);
- Methanol Chromasolv ® min 99,9% (Sigma-Aldrich) ;
- Acetonitrile R Chromasolv ® min 99,8% (Riedel-de Haën);
- Glacial acid acetic 100% (Sigma-Aldrich);
- Purified water (resistivity 18,2 MΩ);
- Analytical balance Adam;
- Magnetic stirrer type AG-3;
- Filter Millipore 45 μm;
- Liquid chromatograph type HP 1090 Series II, equipped with a fluorescence detector type HP 1046 A;
- Phenomenex column, type Luna C18(2) 100Å (150 x 4,6 mm, 5μm).

Working procedure:

Analyzed samples (bread, corn flour) are subject to liquid-liquid extraction in acid medium to separate the ochratoxin A. Thus, 20 g of sample is shaken for 30 minutes with a magnetic stirrer in 100 mL mixture of chloroform and 10 mL phosphoric acid 0.1 M solution; filtered through the quantitative filter paper and the organic phase is evaporated to dryness. The residue is restarted with a volume of 0,5 mL methanol and subjected to analysis by high performance liquid chromatography (Langseth W. et al., 1989; Muscarella M. et al., 2004).

HPLC analysis was performed on high performance liquid chromatograph type HP 1090 Series II equipped with fluorescence detector type HP 1046 A. The analysis was performed at a Phenomenex column, Luna C18(2) 100Å (150 x 4,6 mm, 5μm) with a mobile phase formed by a mixture of acetonitrile : water : acetic acid (99 : 99 : 2), a flow of 0.7 mL/min; in the column compartment temperature was set at 25° C. For detection, the wavelenght of excitation was 228 nm and for emision was 423 nm.

The method was validated by establishing the linearity on concetration range 6.25 - 50 ng / mL (fig. 2) (regression line equation is Peak area = 0.6339 x concentration + 6.7353), the system precision (RSD = 0.9645 %, n = 10, RSD = relative standard deviation, n = number of determinations), method precision (RSD = 2.4975%, n = 9, where RSD = relative standard deviation, n = number of determinations), accuracy (mean

recovery 100.1%), limit of detection (LD = 1.6 ng/mL) and limit of quantification (LQ = 4.6ng /mL).

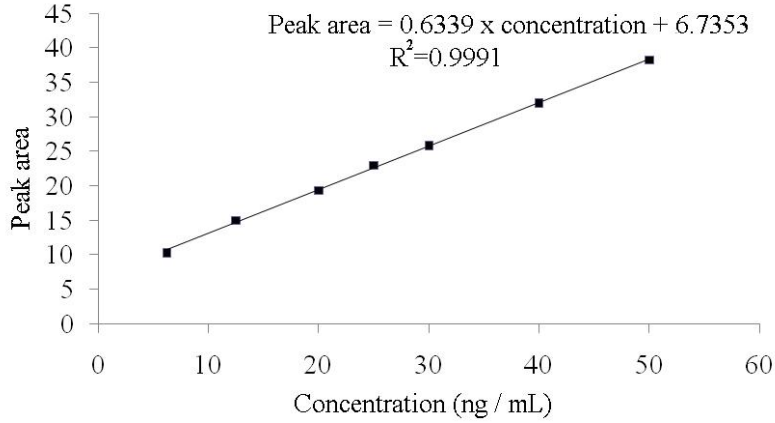

Fig. 2 – The calibration curve for ochratoxin A

The validated HPLC method was applied with good results in the determination of ochratoxin A in bread and corn flour samples.

Bread samples were purchased from commercial network of the Iasi area. We have analyzed 20 samples of bread of various kinds, from different manufacturers: 12 samples of white bread, 3 samples of graham bread, a sample of black bran bread, a rye black bread sample, 2 samples of rye bread and a sample wholemeal bread.

Samples of corn flour were purchased from open markets in the town of Paşcani (Iasi county), from private producers. 20 samples were analyzed.

RESULTS AND DISCUSSIONS

As a result of the analysis by high performance liquid chromatography chromatogram, the chromatograms that were obtained were processed and then the area of the peaks corresponding to ochratoxin A was established. The identification of ochratoxin A peak was done according to the retention time.

Figures 3 show a chromatogram obtained from the analysis of bread samples.

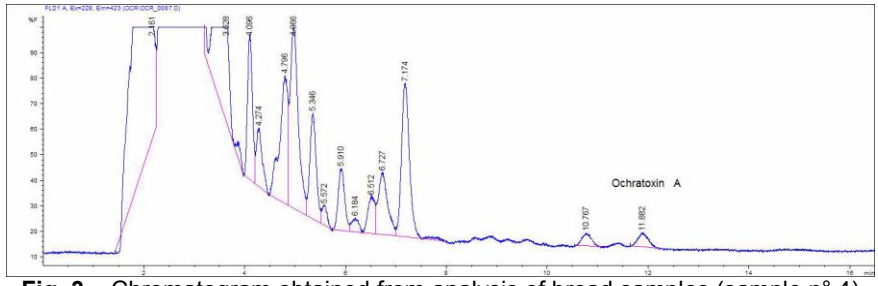

Fig. 3 – Chromatogram obtained from analysis of bread samples (sample n° 4)

Figure 4 show a chromatogram obtained from the analysis of corn samples.

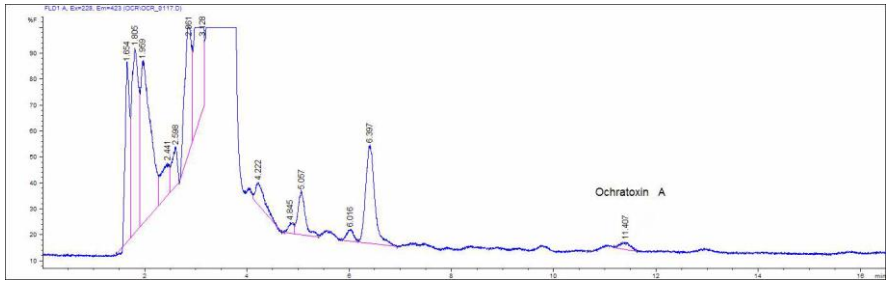

Fig. 4 - Chromatograms obtained from analysis of corn flour samples (sample n° 14)

Using the regression line equation (Area peak = 0.6339 x concentration + 6.7353) ochratoxin A content in bread and maize flour samples was calculated. The values obtained are shown in the tables below (table 1 and table 2).

Table 1

The content of ochratoxin A in bread samples

N° sample	Sample name	Ochratoxin A (µg/Kg)
1.	Sliced graham bread	1,13
2.	Sliced graham bread	2,67
3.	Sliced graham bread	absent
4.	Sliced black bran bread	1,46
5.	Sliced black rye bread	absent
6.	Sliced rye bread	absent
7.	Sliced wholemeal bread	1,45
8.	Sliced white bread	absent
9.	Sliced white bread	trace
10.	Sliced white bread	trace
11.	Sliced white bread	1,20
12.	White bread	trace
13.	Sliced white bread	1,74
14.	Rye bread for sandwich	trace
15.	White bread for sandwich	absent
16.	White bread	trace
17.	Sliced white bread	1,15
18.	White bread	trace
19.	White bread	1,99
20.	Sliced white bread	1,46

Table2

The content of ochratoxin A in corn flour samples

N° sample	Ochratoxin A (µg/Kg)	N° sample	Ochratoxin A (µg/Kg)
1.	trace	11.	trace
2.	absent	12.	1,21
3.	trace	13.	9,08
4.	1,73	14.	2,41
5.	absent	15.	trace
6.	trace	16.	1,07
7.	1,69	17.	absent
8.	1,39	18.	1,13
9.	1,56	19.	trace
10.	1,45	20.	1,38

The analysis of the data obtained from the determination of ochratoxin A in the 20 bread samples has determined that no sample contains a higher level of ochratoxin A than the maximum permitted by applicable law (3 µg/kg); ochratoxin A is present, but within limits, in 45% of the samples, traced in 30% of the samples and absent in 25% of them (fig.5).

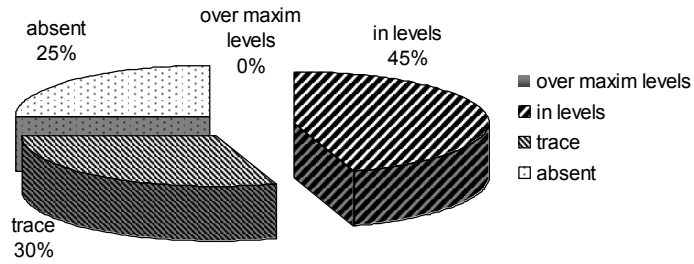

Fig. 5 -The content of ochratoxin A in bread samples

In corn flour samples ochratoxin A is present in a single sample in excess of the maximum level allowed by applicable law (5 µg/kg); in 50% of samples, ochratoxin A is within the maximum allowed limits, in 30% as trace and in 15% of the samples it is absent (fig. 6).

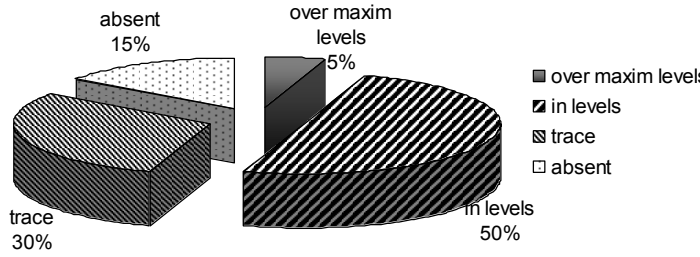

Fig. 6 - The content of ochratoxin A in corn flour samples

CONCLUSIONS

1. A validated HPLC method was applied for determination of ochratoxin A in bread and corn flour samples.

2. This paper contributes to the evaluation of the ochratoxin A present in agricultural products commercialized in open markets (maize flour) and food stores (bread) in the county of Iasi.

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