INFLUENCE OF EXTRACTION PARAMETERS ON ANTIOXIDANT COMPOUNDS RECOVERY FROM ELDERBERRY

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Abstract

The aim of this research was the optimization of parameters of elderberry fruit extraction to obtain the extracts with high antioxidant activity. Extraction was conducted at room temperature with ethanol as solvent used at different concentrations (40, 50 or 60%). The time of solvent contact with elderberry fruit was 4, 8, or 12 hours.

Antioxidant activity and total content of polyphenols were determined by colorimetric methods in the obtained extracts. The 8-h extraction with 60% ethanol was found to be the best process parameters, which allowed obtaining the highest antioxidant activity from that fruit. Meanwhile, the total polyphenols content increased as process duration and ethanol concentration rose.

Key words: *elderberry, extraction parameters, antioxidant activity, total polyphenol content*

1. INTRODUCTION

Therapeutical properties of plants have been exploited by human for centuries. The development in science allowed for conscious utilization of plant raw material and resulted in good knowledge about function of some bioactive compounds. It led to mass-utilization of plant material in food science, medicine and other branches of economy.

1.1 Bioactive compounds of plants and their pro-healthy properties

Active compounds are present in leaves, flowers, seeds and fruit. Their concentration depends on variety of plant, its maturity stage, season, climate, and soil type. Those substances are highly diversified in respect of both their chemical structure and concerning their functions. This group includes vitamins, alkaloids, ethereal oils, fiber, but the special attention should be paid to polyphenol compounds (Penny et al., 2002). Polyphenols are secondary metabolites of plants, and are divided into several classes according to their carbon skeleton: phenolic acids, flavonoids, lignans, and stilbens (Brusselmans et al., 2005).

Pro-healthy properties of polyphenols are connected mainly with their preventive action. Clinical and laboratory experiments demonstrated positive impact of polyphenols on human health. Those compounds caused the decreased risk of inflammatory diseases, cardiovascular system diseases, or tumor growth (Nijveldt et al., 2001).

It was observed, that the risk of lung cancer was reduced by 40-50% when food rich in antioxidants was consumed (Arabshahi et al., 2007). Polyphenols compounds are one of the most active antioxidants in human diet. They perform the preventive functions and help to fight free radicals and reactive oxygen species (ROS) (Sakihama et al., 2002). Another function of polyphenols is to prevent

peroxidation of LDL molecules, which is the cause of atherosclerosis. Antioxidants counteract also ROS-mediated damages of amino acids and proteins (Weinert and Timiras, 2003).

1.2 The recovery methods of active compounds from plant raw material

The active substances diversify during growth, maturing, and ageing of plants. Many of industries, such as pharmaceutical, medical, and food industry, are interested in the selective isolation of particular compounds or their efficient recovery from different plant sources. The main way of getting the bioactive substances is extraction, the process in which one or more compounds passes from solution (or solids) into solvent. Two main types of extraction, liquid-liquid and liquid-solid, are distinguished depending on the state of extractant and extracted substance (Naczk and Shahidi, 2004).

1.2.1 Extraction types

Among many variants of extraction, there are some fundamental.

Maceration (the classic method) is the extraction at room temperature, which consists in separation of compounds basing on its relative solubility in solvent. The duration of process and solvent type and concentration are selected individually as required (Palma and Taylor, 1999). They are conditioned by water amount in raw material and depend on properties of extracted substances.

Digestion is the maceration modification that is performed at higher temperature (45-60°C). It causes the acceleration of extraction and shortening of process duration. However, the quality of macerate obtained by digestion is usually worse than of those obtained by classic methods (Custodio et al., 2007).

Percolation is the improved and more efficient method of maceration. The liquid solvent in continuous movement is poured on the raw material. This technique has high efficiency of bioactive compound recovery in relatively short time (Lingyun et al., 2007).

The technological progress made possible to develop many methods that allow extract specified ingredients. One of them is solid phase extraction (SPE). It consists in adsorption of particular recovered constituents onto solid phase followed by their washing up with solvent (Dopico-Garcia et al., 2005). This technique allows for recovery of bioactive compounds; and the selectivity of process is regulated by different affinity to extractant and by process temperature.

In order to reduce the solvent consumption with simultaneous increase of extraction efficiency the method with microwaves application (MAE - microwave assisted extraction) was evolved. The energy of microwaves causes quick heating of extracted substances and results in time process shortening (Raghavan and Richards, 2007).

One of the modern methods of plant compounds recovery is supercritical fluid extraction (SFE). It enables obtaining constituents of low volatility and of high molecular weight as well as thermolabile compounds (Sovova et al., 2005). Carbon dioxide is the most frequently used gas because of its low price and relatively easy way to form supercritical fluid (0.52 MPa, -57°C) (Luque-Garcia and Luque de Castro, 2001). Another modern technique of valuable substances recovery is pressured liquid extraction (PLE). It is conducted with organic solvent, at raised temperature and pressure (80-200°C, 10-20 bar), that causes big acceleration of extraction process. Moreover, the high pressure maintains the solvent in liquid state, preventing evaporation into gas (Ong, 2004).

1.2.2 Solvents in different extraction methods

The high effectiveness of recovery of plant bioactive compounds can be achieved by learning about the properties of solvents and extracted constituents and by recognition the interactions between them (Brüggemann et al., 2004). During the extraction process, different solvents can be used, e.g. n-hexan,

chloroform, acetone, methanol, ethanol, acetonitrile, water, CO_2, and phosphoric acids. The chemical character of extracted molecules is very important. Depending on the solubility of extracted compound (water-, spirit- or lipid-soluble) the adequate solvent is matched: polar or non-polar (Yang et al., 2006).

Many studies and experiments show, that proper choice of recovery method guarantees the high process efficiency and good quality of the product. All the time the researches are conducted about optimization of extraction process parameters. They aim at reduction of necessary component consumptions and cost cutting. The standard methods combined with different modern innovations (ultrasounds, high pressure, high temperature, circulating maceration etc.) allow obtaining better results. In market economy, the existent competition forces continuous improvement of production methods and lowering their cost with simultaneous conservation of product quality (Lingyun et al., 2007).

The aim of the present study was the parameter optimization of process of bioactive compounds extraction from plant material and obtaining macerates of high antioxidant activity.

2. MATERIAL AND METHODS

The material for research was fresh fruits of elderberry (*Sambucus nigra*).

2.1. Chemicals

- Ethanol cz.d.a. 96%, Chempur (Poland)
- Trolox, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, Sigma-Aldrich
- ABTS – diammonium salt of the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic) acid, Sigma
- PBS - phosphate buffer, Sigma
- Potassium persulfate ($K_2S_20_8$) cz.d.a., POCh (Poland)
- Catechin, Sigma
- Folin-Ciocalteu reagent, Sigma

2.2. Maceration process

Fruits were washed, dried, and crushed in mortar. 5 g of pulp was poured with ethanol of different concentration (40, 50, 60% vol.) and extracted within 4, 8, or 12 hours. The extraction process was conducted on laboratory shaker (6 cpm), at room temperature, and under atmospheric pressure. The macerates were filtered (filter paper), fill up to initial volume and keep in dark until analysis.

2.3. Antioxidant activity assay

Antioxidant activity of the extracts was determined according to Tarko et al. (2009). ABTS radical was generated in the chemical reaction between the 7 mM diammonium salt of the 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid and the 2.45 mM potassium persulfate. In order to terminate the reaction and to stabilize the ABTS cation-radical the solution was kept overnight in darkness at ambient temperature. Prior to analysis the radical solution was diluted with phosphate buffer saline (pH 7.4) in such a way that allowed for obtaining the final absorbance of A = 0.70 ± 0.02 ($ABTS_{0.7}$) measured at 734 nm.

Samples of extracts (100 μL) or Trolox solution (conc. 1-10 mg/100 mL) were added to 1 mL of $ABTS_{0.7}$ and the absorbance was measured 6 min after mixing. Antioxidant capacity was calculated with the use of standard curve obtained by measuring the absorbance of synthetic vitamin E solutions (Trolox) and expressed in mg Trolox/100 g.

2.4. Estimation of total content of polyphenols

An amount of 5 mL of extract was diluted in 45 mL of redistilled water. Then, 0.25 mL of Folin-Ciocalteu reagent (water dissolved 1:1 v/v) and 0.5 mL of 7% Na_2CO_3 were added to the 5 mL of diluted extract. The mixture was left for 30 minutes in darkness. Then the absorbance was measured on a spectrophotometer (λ = 760 nm). The obtained results of total polyphenol content were expressed as mg catechin/100 g based on the standard plot.

2.5. Statistical analysis

There were minimum three replications of the whole analysis. The results were shown as an arithmetic mean (± standard deviation). A Kolmogorov-Smirnov test was applied to examine the normality of distribution. A single-factor Analysis of Variance test (ANOVA) with a *post hoc* Tukey test was applied to assess the significance of differences between means.

3. RESULT

In the study the assessment of efficiency of extraction with different concentration of ethanol and different time of process was performed. The efficiency was expressed by antioxidant activity level and total contents of polyphenol in macerates.

The Fig. 1 presents antioxidant properties of elderberry macerates received with different process parameters. The highest recovery of bioactive compounds was obtained in samples where 60% ethanol was used for extraction during 8 hours (5868 mg Trolox/100 g d.w.). Lower concentration of ethanol (40 and 50%) caused the reduction of this value by about 15%. It was shown also, that the optimal extraction time was 8 hours, independently the solvent concentration. The elongation of the process duration up to 12 hours contributes the decrease of antioxidant activity of received macerates.
A, B, a, b – the same letters mean no statistically significant differences ($P < 0.05$); small letters differences between samples macerated with the same concentration of solvent in different time; capital letters between samples macerated with different solvent concentration in the same time

Based on the results from total polyphenols content determined by Folin-Ciocalteu method (Fig. 2) it was shown that the concentration of alcohol had influenced the level of those compounds in macerates only in a slight degree. Elongation of extraction process (from 4 to 12 hours) increased the phenolic compounds recovery from elderberry. The highest polyphenols concentration was noted in samples extracted with 60% alcohol within 12 hours (1023 mg catechin/100 g d.w.).

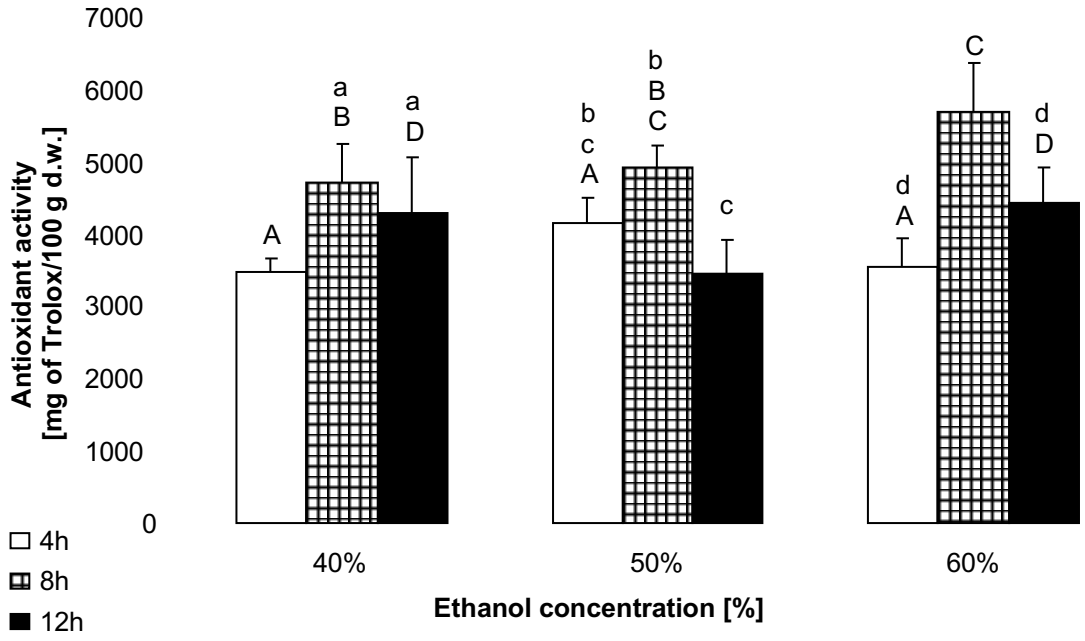
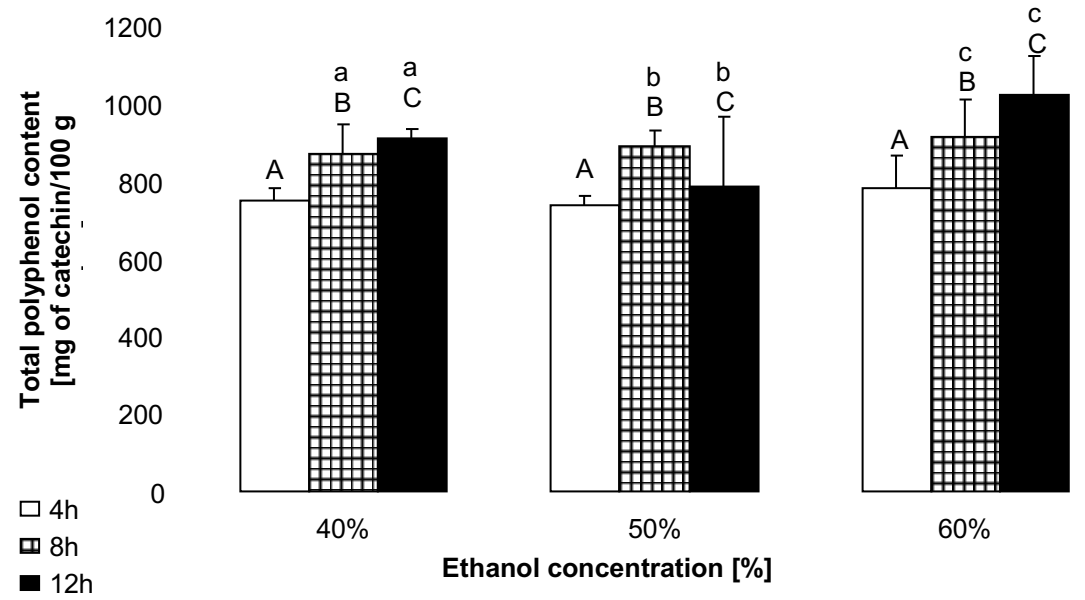


Fig. 1. The influence of extraction parameters on antioxidant activity of macerates produced from elderberry



A, a – the same letters mean no statistically significant differences ($P < 0.05$); small letters concern samples macerated with the same solvent concentration in different time, and capital letters concern samples macerated with different solvent concentration in the same time

Fig. 2. The influence of extraction parameters on total polyphenols content in macerates produced from elderberry

4. DISCUSSION

The recovery of polyphenols from plant material is not a simple process because of some reasons. In the first place, the form of appearance plays a crucial role; polyphenols are present in raw material either as conglomerates with proteins and sugars or as polymer-derivatives of differentiated solubility (Suhaj, 2006). Their chemical structure and interactions with other food components are not recognized to the end, although this information would be very important for proper solvent selection and optimization of process conditions. Secondly, polyphenols are susceptible for many external factors, such as oxygen or high temperature; they can also degrade in alkaline environment. Those factors cause the extraction process should be conducted with particular care (Tura and Robards, 2002).

Although the new techniques of recovery developed, classic methods still prevail in many laboratories. It is caused by their simplicity and low economical cost. Moreover, the efficiency can be adjusted by proper solvents selection and optimization of extraction parameters, especially duration and temperature (Brüggemanna et al., 2004). Many extractants are used in food industry. In this group, ethanol enjoys big popularity. Its concentration during extraction ranges from 40% to 90%, which is enough to lixiviate the aroma and taste compounds as well as antiradical components (Spingo et al., 2007). Ethanol is popular because is suitable for extraction of majority of chemical substances, is cheap and the consumption of ethanol residues is safe for human being (Suhaj, 2006).

Another factor influencing the efficiency of extraction is a temperature. When temperature is elevated the process yield grows, the cell membranes become more permeable, the diffusion factor increases and solvent viscosity reduces, and filtration and sedimentation proceed easier. However, the high temperature promotes the polyphenols degradation, so extraction process should be conducted at temperature lower than 25°C (Grajek, 2007).

Antioxidant activity of plants depends on many factors. The most important is the plant species, however within one species high differentiation could be also observed. It could be caused by climatic and soil condition. Spingo et al. (2007) demonstrated that increase of humidity and insolation raises the scavenging properties of plant. Big amounts of antioxidant compound are produced by plant infected with fungi or parasites (Slimestad et al., 2005).

The elderberries are abundant in anthocyanins (664-1816 mg/100 g depending on the variety) (Kaack and Austed, 1998). The main representatives of those substances are cyanidin-3-glucoside, cyanidin-3-sambubioside, cyaniding-3-sambubioside-5-glucoside, and cyanidin-3,5-diglucoside. Moreover, in fruits of elder flavones (e.g. rutin, isoquercetin, quercetin, astragaline), catechins and flavonones are present (Netzel et al., 2005). The organic acids (acetic, malic, valeric, tartaric, benzoic) and trace amounts of essential oils were identified too (Dawidowicz et al., 2006).

The effective recovery of polyphenols from plant material is conditioned by the application of appropriate extractive mixture. The solvent properties are closely connected with their polarity and polyphenols affinity to substances of different dielectric constant (Bazykina et al., 2002). Water, as solvent of very high polarity, allows recovering only little part of phenolic compounds (e.g. glycosides or some anthocyanins). Addition of less polar solvents causes increase in extraction efficiency because of higher share of polyphenols with affinity to those extractants (e.g. phenolic acids and their derivatives, anthocyanins, catechin, epicatechin etc.) (Bonilla et al., 1999). Many studies confirmed that mixtures of solvents with different polarity are better for extraction than those solvents use separately (Turkmen et al., 2006; Tabart et al., 2007). Pan et al. (2003) demonstrated that total polyphenols content had increased with the increase of ethanol concentration in solvent mixture. Extraction with water-ethanol mixture, when water share 10 to 30%, had caused effective growth of total polyphenol content (Spingo et al., 2007).

The results presented in this study indicate that increasing concentration of ethanol resulted in increased polyphenols level in macerates. However, the antioxidant activity of extracts fell if extraction had last more than 8 hours (Fig. 1, 2). Probably it was caused by reaction of aglycones with sugars that lead to formation of glycosides of lower antioxidant capacity. Important factor could be also the degradation of antioxidant vitamins, which occur in high concentration in the elderberry. Many sources gave out that vitamins C, E, and A, and β-carotene are present in elderberry (Szajdek and Borowska, 2004; Kotowski, 2006). They can neutralize free radicals and reactive oxygen species, but easy degrade under exposition to oxygen, light and temperature.

Vitamin A undergoes oxidation (even under the influence of atmospheric oxygen) to aldehydes retinal and 3,4-didehydroretinal, and further oxidation results in formation of retinoic acid and 3,4-didehydroretinoic acid. Products of oxidation of vitamin A and its provitamins show lower biological activity than the precursors (Moszczyński and Pyć, 1999), so can contribute to reduced antioxidant activity of macerates.

Vitamin C also can be degraded by oxidation. The rate of oxidation depends on temperature, amount of available oxygen and the presence of metals that catalyze the oxidation reactions (e.g. Cu^{2+} and Fe^{3+}). The role of oxidation lowers in higher temperature, and increases anaerobic destruction of ascorbic acid. Main products of the latter are furfural and CO_2 (at low pH). In the presence of fructose, the degradation of ascorbic acid is accelerated (Grajek, 2007).

The decomposition of labile compounds such as vitamins, can explain the fall in antioxidant activity of macerates produced in 12-h extraction. Long exposition to external factors (>8 h) caused loss of biological properties of ingredients included. Besides the vitamin degradation, antioxidant activity could be affected by sugars present in fruits. During extraction, the partial condensation occurs; the hydroxyl group of monosaccharide forms bond with non-sugar component (aglycone) forming glycosides. Hence, aglycones that possess free hydroxyl group are characterized by higher affinity to free radicals than its glycoside (Grajek, 2007).

5. CONCLUSION

The highest antioxidant activity was obtained for extraction performed with 60% ethanol within 8 hours. After 12 hours extraction the antioxidant activity of elderberry macerates was reduced, probably due to degradation of antioxidant vitamins present in fruit. The condensation of flavones and anthocyanins aglycones with monosaccharides also contributed in lowering of free radical scavenging potential. The total polyphenols content increased when extraction time was elongated and ethanol concentration was raised. The highest level of polyphenols was shown after 12 hours of extraction with 60% ethanol. Optimal choice of process parameters, such as duration or appropriate solvent, allows the effective recovery of bioactive compounds from plant raw material.

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REFERENCES

1. Arabshahi D.S., Vishalakshi D.D., Urooj A. Evaluation of antioxidant activity of some plant extracts and their heat, pH and storage stability. Food Chem 2007; 100: 1100–1105.
2. Bazykina N. I., Nikolaevskii A. N., Filippenko T. A., Kaloerova V. G. Optimization of conditions for the extraction of natural antioxidants from raw plant materials. Pharmac Chem J 2002; 36(2): 100-103.
3. Bonilla F., Mayen M., Merida J., Medina M. Extraction of phenolic compounds from red grape marc for use as food lipid antioxidants. Food Chem 1999; 66: 209-215.
4. Brüggemanna O., Visnjevski A., Burch R., Patel P. Selective extraction of antioxidants with molecularly imprinted polymers. Anal Chim Acta 2004; 504: 81-88.
5. Brusselmans K., Vrolix R., Verhoeven G., Swinnen J.V. Induction of cancer cell apoptosis by flavonoids is associated with their ability to inhibit fatty acid synthase activity. J Biol Chem 2005; 280(7): 5636–5645.
6. Custodio F.B., Tavares E., Gloria M.B.A. Extraction of bioactive amines from grated Parmesan cheese using acid, alkaline and organic solvents. J Food Comp Anal 2007; 20: 280-288.
7. Dawidowicz A.L, Wianowska B., Baraniak B. The antioxidant properties of alcoholic extracts from Sambucus nigra L. (antioxidant properties of extracts). LWT 2006; 39: 308–315.
8. Dopico-Garcia M.S., Lopez-Vilarino J.M., Gonzalez-Rodriguez M.V. Determination of antioxidants by solid-phase extraction method in aqueous food simulants. Talanta 2005; 66: 1103–1107.
9. Grajek W. Praca zbiorowa. Przeciwutleniacze w żywności, Aspekty zdrowotne, technologiczne, molekularne i analityczne. WNT Warszawa 2007: 512-524.
10. Kaack K., Austed T. Interaction of vitamin C and flavonoids in elderberry (*Sambucus nigra* L.) during juice processing. Plant Foods for Hum Nutr 1998; 52: 187–198.
11. Kotowski A. Rewolucja antocyjanowa. Esarom 2006; 2(47): 1-2.
12. Lingyun W., Wang J., Zheng X., Teng D., Yang Y., Cai Ch., Feng T., Zhang F. Studies on the extracting technical conditions of inulin from Jerusalem artichoke tubers. J Food Eng 2007; 79: 1087–1093.
13. Luque-Garcia J.L., Luque de Castro M.D. Extraction of fat-soluble vitamins. J Chromatography A 2001; 935: 3–11.
14. Moszczyński P., Pyć R. Witaminy lipofilne i kwas askorbinowy. Biochemia witamin. PWN Warszawa 1999: 76-77.
15. Naczk M., Shahidi F. Extraction and analysis of phenolics in food. J Chromatography A 2004; 1054: 95–111.
16. Netzel M., Strass G., Herbst M., Dietrich H., Bitsch R., Bitsch I., Frank T. The excretion and biological antioxidant activity of elderberry antioxidants in healthy humans. Food Res Internat 2005; 38: 905–910.
17. Nijveldt R.J., van Nood E., van Hoorn D.E.C., Boelens P.G., van Norren K., van Leeuwen P.A.M. Flavonoids: a review of probable mechanisms of action and potential applications. Am J Clin Nutr 2001; 74: 418–25.

18. Ong E.S. Extraction methods and chemical standardization of botanicals and herbal preparations. J Chromatography B 2004; 812: 23–33.
19. Palma M., Taylor L.T. Extraction of polyphenolic compounds from grape seeds with near critical carbon dioxide. J Chromatography A 1999; 849: 117–124.
20. Pan X., Guoguang N., Huizhou L. Microwave-assisted extraction of tea polyphenols and tea caffeine from green tea leaves. Chem Eng Processing 2003; 42: 129-133.
21. Penny M. Etherton K., Hecker K.D., Bonanome A., Coval S.M., Binkoski A.E., Hilpert K.F., Griel A.E., Etherton T.D. Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. Am J Medic 2002; 113(9B): 71-88.
22. Raghavan S., Richards M.P. Comparison of solvent and microwave extracts of cranberry press cake on the inhibition of lipid oxidation in mechanically separated turkey. Food Chem 2007; 102: 818–826.
23. Sakihama Y., Cohen M.F., Grace S.C., Yamasaki H. Plant phenolic antioxidant and prooxidant activities: phenolics-induced oxidative damage mediated by metals in plants. Toxicology 2002; 177: 67–80.
24. Slimestad R., Torskangerpoll K., Nateland H.S., Johannessen T., Giske N.H. Flavonoids from black chokeberries, Aronia melanocarpa. J Food Composit Anal 2005; 18: 61–68.
25. Sovova H., Jez J., Bartlova M., Stastova J. Supercritical carbon dioxide extraction of black pepper. J Supercritical Fluids 2005; 8: 295-301.
26. Spingo G., Tramelli L., De Faveri M.D. Effects of extraction time, temperature and solvent on concentration and antioxidant activity of grape marc phenolics. J Food Engin 2007; 81: 200–208.
27. Suhaj M. Spice antioxidants isolation and their antiradical activity: a review. J Food Composit Analys 2006; 19: 531–537.
28. Szajdek A., Borowska J. Właściwości przeciwutleniające żywności pochodzenia roślinnego. Żywność - Nauka. Technologia. Jakość 2004; 4(41)S: 5-28.
29. Tabart J., Kevers C., Sipel A., Pincemail J., Defraigne J.-O., Dommes J. Optimisation of extraction of phenolics and antioxidants from black currant leaves and buds and of stability during storage. Food Chem 2007; 105: 1268–1275.
30. Tarko T., Duda-Chodak A., Tuszyński T. Simulation of Phenolic Compounds Transformations and Interactions in an In Vitro Model of the Human Alimentary Tract. Food Sci Technol Internat 2009; 15(3): 235-241.
31. Tura D., Robards K. Sample handling strategies for the determination of biophenols in food and plants. J Chromatography A 2002; 975: 71-93.
32. Turkmen N., Ferda S., Sedat V. Effects of extraction solvents on concentration and antioxidant activity of black and black mate tea polyphenols determined by ferrous tartrate and Folin–Ciocalteu methods. Food Chem 2006; 99: 835–841.
33. Weinert B.T., Timiras P.S. Theories of aging. J Appl Physiol 2003; 95(4): 1706-1716.
34. Yang D.Y., Kakuda Y., Subden R.E. Higher alcohols, diacetyl, acetoin and 2,3-butanediol biosynthesis in grapes undergoing carbonic maceration. Food Res Internat 2006; 39: 112-116.