Comparative study of antioxidants in fresh and frozen blueberries and cranberries fruits

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Abstract

The beneficial effect of blueberries and cranberries consumption is largely due to the high content of biomolecules with antioxidant properties, the most important are vitamins (especially vitamin C and provitamins A - carotenoids), anthocyanins and phenolic compounds. The purpose of this work is to determine how the blueberry and cranberry preservation at -80ºC influence the antioxidant content of these fruits. The biochemical parameters analyzed were as follows: anthocyanin pigments (total anthocyanin extraction and dosing, anthocyanin profile by TLC and HPLC chromatography); carotenoid pigments (total carotenoid extraction and dosing, carotenoid profile by HPLC); determination of ascorbic acid and total phenolic compounds. Antioxidants profile is different in blueberry and cranberry, both of quality and quantitative point of view. Preserving berries by freezing them for a period of time between 1 and 3 months induce different changes in the content of specific antioxidants: the concentration of vitamin C and anthocyanin pigments decreases, simultaneously with an increase in concentration of polyphenols and carotenoids.

Key words*: blueberries, cranberries, antioxidants, freezing*

Introduction

Blueberry (*Vaccinium myrtillus*) and cranberry (*Vaccinium vitis*) are part of the *Ericaceae* family and are spread in mountain areas in Asia, Europe and North America. The berries contain water, sucrose, proteins, pectin substances, vitamins (C, A, PP, B1, B2), and mineral salts. Anthocyanins, flavones, phenolic acids, and proanthocyanins are the main secondary metabolites [Bunea et al., 2012].

Epidemiological and *in vitro* studies suggest that blueberries help maintain the health and act as a barrier to the effects of aging, in particular neurodegeneration and cognitive defects. There is evidence of their action on the prevention of cardiovascular disease and certain types of cancer. Supplementing feed with blueberry extracts can be used to prevent or treat Alzheimer's disease and possibly other neurodegenerative disorders [Garcia da Rocha Concenço et al., 2014].

Anthocyanins from blueberries and cranberries act as cardio protectors by maintaining vascular permeability, reducing inflammatory responses and platelet aggregation, providing superior vascular protection compared to other cardiovascular drugs [Zafra-Stone et al., 2007].

In vitro studies have suggested that phenols, the class of compounds present in these fruits, can affect the pathogenesis of cardiovascular disease by increasing LDL resistance to oxidation, preventing platelet aggregation and thrombosis, reducing blood pressure and/or inhibiting the inflammatory processes [McKay and Bulmberg, 2007].

Another very important effect of these fruits is the neuroprotective effect. According to a study in which a stroke was simulated in rats, it was observed that after treatment with a blueberry extract, oxidative stress-induced necrosis was reduced by 43%, and ischemia-induced necrosis was reduced by 49% [McKay and Bulmberg, 2007].

Cranberries have been used since the earliest times as cataplasms for wounds and septicemia, and cranberry juice has been widely used as a popular remedy for treating women's urinary tract infections (UTIs) and other gastrointestinal disorders in infections with *E. coli* and

other pathogens. In many clinical trials, a positive relationship has been established between the consumption of cranberries and the prevention of UTIs, an effect due to the bacteriostatic activity of hippuric acid, which is formed by the metabolic conversion of p-hydroxybenzoic acid into the liver. Hippuric acid excreted in the kidney system produces urinary acidification and prevents E. coli growth in the urinary tract [Vattem et al., 2005].

Blueberries and cranberries are also used to treat diabetes, due to the presence of anthocyanins that prevent free radical production, lipid peroxidation, increased insulin secretion, and improved insulin resistance. Both *in vivo* and *in vitro* studies demonstrated a decrease in oxidative stress markers and an increase in insulin production in patients with type 2 diabetes [Andrei et al., 2014].

Age-related macular degeneration (AMD) is another condition that can be treated by eating blueberries, the anthocyanins present in them can cross the blood-retina barrier and the blood-brain barrier, which can accumulate in the eye and cause some biological effects, also acting indirectly by increasing blood flow [Andrei et al., 2014].

Blueberries and cranberries are frequently consumed fresh or frozen. The beneficial effect of these fruits is largely due to the high content of biomolecules with antioxidant properties, the most important being vitamins (especially vitamin C and provitamins A - carotenoids), anthocyanins and phenolic compounds. The purpose of this study was to determine how fruit preservation by freezing at -80 ° C influences the content of antioxidants. The biochemical parameters analyzed were as follows: anthocyanin pigments (total anthocyanin extraction and dosing, anthocyanin profile by TLC and HPLC chromatography); carotenoid pigments (total carotenoid extraction and dosing, carotenoid profile by HPLC); determination of ascorbic acid and total phenolic compounds.

Material and methods

Biological material:

The determinations were made on blueberries and cranberries, collected from the spontaneous flora (in the Băişoara Mountain region, Cluj county), during July - September. The determinations of the chemical parameters were performed immediately after harvesting. An aliquot of samples were subjected to freezing at -80ºC and is then analyzed at 1 month and 3 months after freezing. Thus, the analyzed samples were noted as follows: fresh blueberries = FB; fresh cranberries = FC; frozen blueberries 1 month = FrB1; frozen blueberries 3 months = FrB3; frozen cranberries 1 month = FrC1 and frozen cranberries 3 months = FrC3.

Extraction and determination of anthocyanins concentration:

The extraction of anthocyanins was carried out after homogenization with a mixture of acidified methanol (85:15 v/v, MeOH: HCl 0.03%). The total extract was evaporated to dryness at 40°C. The residues were taken up in 10 ml of methanol, centrifuged at 5000 rpm and filtered with a 0.45 μm Millipore filter [Bunea et al., 2011]. To determine the concentration of anthocyanins in the extracts was used the differential pH method proposed by Giusti and Wrolstad (2001).

Separation of anthocyanins by TLC and HPLC chromatography:

Extracts obtained from all types of fruit (fresh and frozen) were subjected to chromatographic separation, using two types of stationary phases, namely: paper chromatography and thin layer chromatography (TLC). Two different mobile phases were also tested. The methods were modified after Santos et al. (2013) for mobile phase 1 (ethyl acetate: acetic acid: formic acid: water - 100:11:11:26) and Halbwirth (2010) for mobile phase 2 (water: hydrochloric acid: acetic acid - 83:3: 5). The best results were achieved by TLC chromatography on silica gel and mobile phase 1.

In order to better characterize the profile of anthocyanin pigments in fresh and preserved fruits, on the total obtained extracts we performed the RP-HPLC chromatographic separation proposed by Bunea et al. (2011): Shimadzu chromatographic system equipped with LC-20 AT (Prominence) pumps, DGU-20 A3 (Prominence) degassing, photodetector SPDM20 A UV-VIS detector (DAD). For separation, column Luna Phenomenex C-18 column (5 μm, 25 cm x 4.6 mm) was used. The mobile phase consisted of two solvents: A - formic acid (4.5%) in bidistilled water and B - acetonitrile. The gradient separation system was as follows: 10% B, 0-9 min; 12% B, 9-17 min; 25% B 17-30 min; 90% B, 30-50 min; 10% B, 50-55 min. Separation was performed at a flow rate of 0.8 ml / min at 35°C. Chromatograms were monitored at 520 nm. Identification of the separated anthocyanins was based on retention time and UV-Vis spectra, by comparison with standard solutions and literature data.

Extraction and determination of total carotenoids:

The extraction of total carotenoids was performed using the method proposed by Breithaupt et al. (2000) and Bunea et al. (2012); with a mixture of methanol: ethyl acetate: petroleum ether (1: 1: 1). The partition of the extracts was carried out by the successive addition of distilled water, ethyl ether and saturated sodium chloride solution. The organic upper phase was separated, evaporated to dryness and the residue was dissolved in ethyl ether and saponified with a 30% methanolic KOH solution at room temperature for 12 hours. The saponified extract was then washed with large amounts of saturated sodium chloride solution and then water. The organic phase containing the extracted pigments was passed over anhydrous sodium sulfate and evaporated to dryness at 35°C. To determine the total carotenoid concentration, the formed residue was dissolved in 15 ml of petroleum ether and the absorption spectrum of the extracts was determined in the range 300-700 nm. The dosing was performed photometrically by reading the sample absorbance at 442 nm.

Separation of carotenoids by HPLC chromatography:

The separation of carotenoids was carried out using the method proposed by Bunea et al. (2012): Waters 990 chromatographic system with PDA detector, Kontron pumps and a reversed phase column C18 Zorbax ODS (250 mm × 4.6 mm, 3.5 μm). The mobile phase was a mixture of two solvents: acetonitrile: water (9: 1 with 0.25% triethylamine (solvent A) and ethyl acetate with 0.25% triethylamine (solvent B). The gradient program started at 15% B at 50% B from minute 0 to 16 minutes. The program was continued isocratic (16-30 minutes) with 50% solvent B.

Determination of ascorbic acid:

For vitamin C dosing the iodometric method was used [Moldovan et al., 2006], based on the oxidation of excess ascorbic acid with iodine.

Determination of total polyphenols concentration:

The amount of total polyphenol in the blueberry extracts was determined using modified Folin-Ciocalteu colorimetric method [Singleton et al., 1999]. The results were expressed as milligram of gallic acid (GAE) per 100 grams.

Results and discussion

The results obtained in determining the total anthocyanin concentration are detailed in Table 1 (mean and standard deviation). Concentration of anthocyanins is dependent on various factors, among which the most important is the species under consideration and its type (for example, whether it is cultured or spontaneous). The results obtained in this study are consistent with those presented by Bunea et al. (2011), according to which the concentration of the anthocyanins from wild blueberries harvested from Transylvania is between 250 and 300 mg /100g. In the case of fresh cranberries, the anthocyanin concentration is much lower compared to

blueberries, with an average value of 32.9 mg / 100g. These data are lower compared to those published by Celik et al. (2008).

Table 1: Concentration of total anthocyanins, total carotenoids, ascorbic acid and total polyphenol in fresh and frozen fruits (average and standard deviation; with different letters are significantly different at $P < 0.05$)

	Total anthocyanins mg/100g	**Total carotenoids µg/100 g**	**Ascorbic acid mg/100g**	**Total polyphenol mg GAE/100g**
FB	252.94±20.860	304.02±6.957	12.52±0.401	412.66±7.547
FrB1	211.78±8.533	353.12±19.786	8.73±0.224^{a}	520.46±12.817^{e}
FrB3	200.21±1.055	354.32±18.244	4.66±0.504^{b}	537.40±10.541^{f}
FC	30.17±2.110	189.77±8.892	15.67±0.851	342.45±20.066
FrC1	26.76±2.411	208.78±15.324	9.15±0.294^{c}	407.88±3.790^{g}
FrC3	26.21±2.648	209.34±7.273^{i}	7.33±0.270^{d}	416.98±10.395^{h}

According to them, the concentration of anthocyanins is dependent on the species but also the degree of maturation of the fruits. In their study, the concentration of anthocyanins in immature (light red) and mature (dark red) fruits was followed. These concentrations varied from 52 to 111 mg /100g. However, the data obtained by us are consistent with those presented by Duthie et al. (2006), according to which cranberries have an average anthocyanin content of 28.19 mg / 100g. As can be seen from the table, freezing processes cause a decrease in anthocyanin concentration in both blueberries and cranberries, the decrease being more pronounced in the first month of freezing.

In the case of anthocyanin pigments, it was of interest to carry out a comparative study of the profile of these pigments in the two types of fruit. A first step consisted of a TLC separation on SilicaGel (in Figure 1). The identification of these pigments was made by comparing the values of the specific retention factors, for the chromatographic system used, with literature data [Halbwirth, 2010; Santos et al., 2013]. From the figure we can see the different profile of anthocyanins in the two types of fruit. Two different pigments were identified in cranberry fruit: cyanidin 3-glycoside (2) and peonidine 3-glycoside (4) respectively. In the cranberry fruits, in addition to the two pigments mentioned above, there were also identified: delphinidin 3-glycoside (1); malvidin 3-glycoside (3) and petunidin 3-glycoside (5). We can therefore say that these fruits differ in both the type and the concentration of anthocyanins.

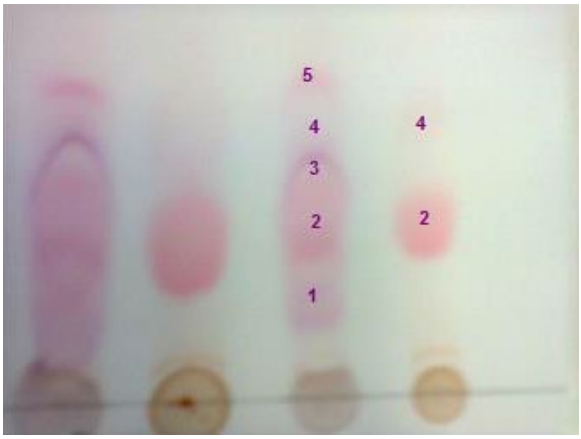

Figure 1: Separation of anthocyanins by TLC chromatography

A more accurate analysis of the qualitative profile of anthocyanins was performed by HPLC, the identification of separate peaks being performed by comparing retention times (Rt) with literature data for similar chromatographic systems [Bunea et al., 2011; Zheng and Wang, 2003; Prior et al., 2001]. Figure 2 shows chromatograms obtained in the separation of pigments from fresh fruit.

The anthocyanins identified in blueberries (whether fresh or frozen) were: (1) delphinidin-3-galactoside; (2) delphinidin-3-glucoside; (3) delphinidin-3-arabinoside; (4) petunidin-3-galactoside; (5) petunidin-3-glucoside; (6) petunidin-3-arabinoside; (7) peonidine-3-glucoside; (8) malvidin-3-galactoside and (9) malvidin-3-glucoside (Figure 2A). In the case of cranberry fruit, the number of pigments in the samples was lower compared to those in blueberries (Figure 2B), these being the following: (1) cyanidin-3-galactoside; (2) cyanidin-3-glucoside; (3) petunidin-3-glucoside; (4) peonidin-3-galactoside and (5) peonidin-3-glucoside.

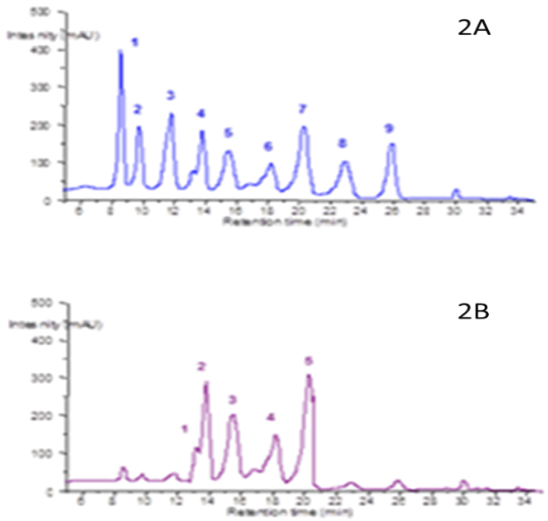

Figure 2: Separation of anthocyanins by HPLC chromatography

Carotenoid pigments are associated with a low risk of cardiovascular disease, muscle degeneration and cataracts, certain types of cancer, have immunostimulatory properties, and are involved in photo-protective mechanisms in the skin [Krinsky and Johnson, 2005; Andrei et al., 2014]. In the present study, it was of interest to determine the total concentration of these compounds in berries (Table 1) and the way in which freezing preservation influences these molecules. Concentration of carotenoids in blueberries was much higher compared to cranberries, both in the fruits analyzed immediately after harvesting and in those preserved by freezing. The results obtained in this study are consistent with those presented by Bunea et al. (2011), according to which the concentration of total carotenoid content of wild blueberries was in the range of 215–317 μg per 100 g of fruit. Fruit freezing induces an increase in carotenoid concentration, which can be explained by the fact that this freezing process causes a partial loss of water in the fruit, which facilitates the release and solubilization of these pigments.

The next step consisted in analyzing the carotenoid profile, in Figure 3 two of the chromatograms obtained were shown.

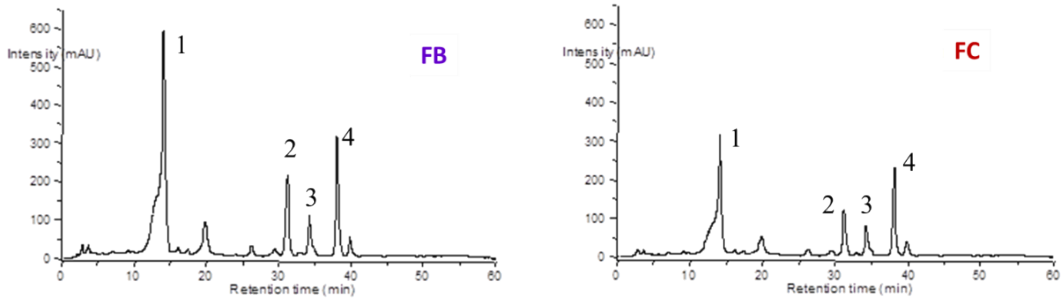

Figure 3: Separation of carotenoids by HPLC chromatography

Identification of separation peaks was performed by comparing retention times with literature data and based on absorption spectra [Bunea et al., 2012]. Thus, identified carotenoids are: lutein (pick 1); β-cryptoxanthin (pick 2); β-carotene (pick 3) and cis-β-carotene (pick 4). There is a very limited volume of data available on the composition of carotenoids in blueberries and cranberries. The data obtained for cranberry fruits are consistent with those presented by Bunea et al. (2012), according to which these fruits contain lutein, β-cryptoxanthin, and β-carotene. The profile obtained is different from that presented in the article published by Lashmanova et al. (2012). According to them, blueberry and cranberry fruits in the northern part of Europe contain neoxanthin; violaxanthin; anteraxanthin; lutein; zeaxanthin and β-carotene.

A water-soluble antioxidant present in berries is vitamin C (Table 1). The data presented are similar to those published by Borges et al. (2010), according to which the berries are characterized by a rather low concentration of vitamin C, averaging 1107 nmol / g for cranberries and 115 nmol / g for blueberries. It can be noticed that, regardless of the fruits, freezing causes a sharp decrease in the concentration of this vitamin. During storage of food, the vitamin C content decreases, because ascorbic acid is oxidized to dehydroascorbic acid, which in turn degrades by hydrolysis and the opening of the lactonic ring with the formation of 2,3-dicetogulonic acid, which has no biological activity. Vitamin C can also be reduced by exposure to oxidases present in plant tissues [Andrei et al., 2014].

Numerous epidemiological studies suggest that a correct diet is significantly associated with reduced risk of cardiovascular disease. From the category of natural compounds, polyphenols have been shown to be associated with a decrease in the incidence of cardiovascular disease. Polyphenols are the most abundant class of antioxidants in the human diet, being present in various food products of vegetable origin: fruits, vegetables, cereals, olive oil, vegetables, chocolate and various beverages [Andrei et al., 2014]. Blueberries are a rich source of polyphenols, with a mean concentration of 412.6 mg/100 g (table 1). The data obtained from wild blueberry fruit are lower compared to those presented by Bunea et al. (2011). According to the studies presented by these authors, wild blueberry fruits were characterized by average concentrations between 672.59 and mg GAE /100g, while the fruits of culture ranged between 424.84 and 652.27 mg GAE /100g.

In this study, in the case of frozen fruits over a period of 1 to 3 months, an increase in the total polyphenol concentration was observed. The composition of phenolic compounds in fruits and vegetables is dependent on the product, the cultivar, the maturity stage and the post-harvest conditions. Because phenolic compounds are antioxidants, they are oxidized during storage and processing of food. The freezing preservative process inactivates the enzymes that cause the oxidation of phenols. [Rickman et al., 2006], which may explain the increase in the concentration observed in our study, in the frozen fruits for 1 to 3 months.

Conclusions

The antioxidant profile is different in fresh blueberry and cranberry fruits, both in qualitative and quantitative terms. Preserving the berries by freezing for a period of between 1 and 3 months induces different changes in the specific antioxidant content.

Freezing processes cause a decrease in the anthocyanin concentration in both blueberries and cranberries, the decrease being more pronounced in the first month of freezing.

The carotenoids identified in fresh and frozen fruits were: lutein; β-cryptoxanthin; β-carotene and cis-β-carotene. Fruit freezing induces an increase in carotenoid concentration, which can be explained by the fact that this freezing process causes a partial loss of water in the fruit, which facilitates the release and solubilization of these pigments.

Freezing causes a sharp decrease in vitamin C concentration, a variation that can be explained by two different mechanisms: ascorbic acid is oxidized to dehydroascorbic acid and vitamin C can also be reduced by exposure to oxidases present in plant tissues.

Blueberries are a rich source of polyphenols, while cranberry fruits are characterized by a lower concentration. In the case of frozen fruits over a period of 1 to 3 months, an increase in the total polyphenol concentration was observed.

Bibliography

1. ANDREI SANDA, BUNEA ANDREA, PINTEA ADELA, 2014, *Stresul oxidativ şi antioxidanţi naturali*, Editura AcademicPres, Cluj-Napoca, p:250, 256
2. BORGES G., DEGENEVE A., MULLEN W., CROZIER A., 2010, *Identification of Flavonoid and Phenolic Antioxidants in Black Currants, Blueberries, Raspberries, Red Currants, and Cranberries*, J. Agric. Food Chem., 58, 3901–3909
3. BREITHAUPT DE, SCHWACK W, SCHWACK W, 2000, *Determination of free and bound carotenoids in paprika (Capsicum annuum L.) by LC/MS*, Eur Food Res Technol, 21(1):52–55
4. BUNEA A., RUGINĂ O.D., PINTEA A. M., Z. SCONŢA, C. I. BUNEA, C.SOCACIU, 2011, *Comparative Polyphenolic Content and Antioxidant Activities of Some Wild and Cultivated Blueberries from Romania*, Not Bot Horti Agrobo, 39(2):70-76
5. BUNEA A., D.RUGINA, A. PINTEA, S. ANDREI, C. BUNEA, R.POP, C BELE, 2012, *Carotenoid and fatty acid profiles of bilberries and cultivated blueberries from Romania*, Chemical Papers 66 (10) 935–939
6. CELIK H., OZGEN M., SERCE S., C. KAYA, 2008, *Phytochemical accumulation and antioxidant capacity at four maturity stages of cranberry fruit*, Scientia Horticulturae 117 345–348
7. DUTHIE S.J., JENKINSON A., A. CROZIER, W. MULLEN, L. PIRIE, J.KYLE, L.YAP, P. CHRISTEN, DUTHIE G.G., 2006, *The effects of cranberry juice consumption on antioxidant status and biomarkers relating to heart disease and cancer in healthy human volunteers*, Eur J Nutr 45 : 113–122
8. GARCIA DA ROCHA CONCENÇO F.I., P.C. STRINGHETA, A.M. RAMOS, I. H.OLIVEIRA, 2014, *Blueberry: Functional Traits and Obtention of Bioactive Compounds*, American Journal of Plant Sciences, 5, 2633-2645
9. GIUSTI M.M., WROLSTAD R.E., 2001, *Current Protocols in Food Analytical Chemistry UNIT F1.2 Characterization and Measurement of Anthocyanins by UV-Visible Spectroscopy*, DOI: 10.1002/0471142913.faf0102s00
10. HALBWIRTH H. , 2010, *The Creation and Physiological Relevance of Divergent Hydroxylation Patterns in the Flavonoid Pathway*, Int. J. Mol. Sci., 11, 595-621; doi:10.3390/ijms11020595
11. KRINSKY N., JOHNSON E. J., 2005, *Carotenoid actions and their relation to health and disease*, Molec. Aspect Med. 26, 459-516
12. LASHMANOVA K.A. , KUZIVANOVA O.A., DYMOVA O.V. , 2012, *Northern berries as a source of carotenoids*, Acta Biochimica Polonica, Vol. 59, No 1, 133–134
13. MC KAY D. L., BLUMBERG J.B., 2007, *Cranberries (Vaccinium macrocarpon) and Cardiovascular Disease Risk Factors*, Nutrition Reviews, Vol. 65, No. 11, 490-502
14. MOLDOVAN, P., TOŞA, M.I., LEŢ, D., MAJDIK, C., PAIZS, C., IRIMIE, F. D. , 2006, *Aplicaţii pentru laboratorul de biochimie*, Editura Napoca Star, 110-115

15. PRIOR R.L., S.A. LAZARUS, G.CAO, H.MUCCITELLI, JO. F. HAMMERSTONE, 2001, *Identification of Procyanidins and Anthocyanins in Blueberries and Cranberries (Vaccinium Spp.) Using High-Performance Liquid Chromatography/Mass Spectrometry*, J. Agric. Food Chem. 49, 1270-1276
16. RICKMAN J.C., BARRETT D.M., BRUHN C.M., 2006, *Nutritional comparison of fresh, frozen and canned fruits and vegetables. Part 1. Vitamins C and B and phenolic compounds*, J Sci Food Agric 0022–5142
17. SANTOS D.T., CAVALCANTI R.N., M.A. ROSTAGNO, C.L. QUEIROGA,. N. EBERLIN, M. A. MEIRELES, 2013, *Extraction of Polyphenols and Anthocyanins from the Jambul (Syzygium cumini) Fruit Peels*, Food and Public Health, 3(1): 12-20
18. SINGLETON V.L., ORTHOFER R., LAMUELA-RAVENTÓS R.M., LESTER P., 1999, *Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent*. Meth Enzymol 299:152-178
19. VATTEM D.A., LINA Y.T., GHAEDIANB R., SHETTY K., 2005, *Cranberry synergies for dietary management of Helicobacter pylori infections*, Process Biochemistry, Volume 40, Issue 5, Pages 1583–1592
20. ZAFRA-STONE S., T.YASMIN, M. BAGCHI, A.CHATTERJEE, J.A. VINSON, D. BAGCH, 2007, *Berry anthocyanins as novel antioxidants in human health and disease prevention*, Mol. Nutr. Food Res.,51, 675–683
21. ZHENG W., WANG S. Y., 2003, *Oxygen Radical Absorbing Capacity of Phenolics in Blueberries, Cranberries, Chokeberries, and Lingonberries*, J. Agric. Food Chem. , 51 (2), 502-509