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Effect of *Vaccinium myrtillus* anthocyanin extract on lipid oxidation in cod liver oil

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SIMONA OANCEA[1], CRISTIAN GROSU[2]
[1] *Lucian Blaga University of Sibiu, Department of Agricultural Sciences, Food Industry and Environmental Protection, 7-9 I. Ratiu Street, 550012 Sibiu, Romania, Tel.: +40269-211338, Fax: +40269-212558, E-mail: simona.oancea@ulbsibiu.ro*
[2] *Lucian Blaga University of Sibiu, Department of Ecology and Environmental Protection, 5-7 I. Ratiu Street, 550012 Sibiu, Romania*
Corresponding author: SIMONA OANCEA, PhD, Assoc. Prof., Lucian Blaga University of Sibiu, Department of Agricultural Sciences, Food Industry and Environmental Protection, 7-9 I. Ratiu Street, 550012 Sibiu, Romania, Tel.: +40269-211338, Fax: +40269-212558, E-mail: simona.oancea@ulbsibiu.ro

Abstract

The present paper described the potential of anthocyanin extracts from bilberry (Vaccinium myrtillus) to stabilize polyunsaturated oil from cod liver (Gadus morhua) in reverse micelle system, by comparison with the effect of a tocopherols mixture on the same marine oil. Anthocyanins are plant bioactives known for their health benefits based on high antioxidant capacity. Also, the paper aimed to investigation of the storage stability at 15-17°C of cod oil samples with added anthocyanins. Our results showed that cod liver oil sample containing bilberry anthocyanin extract had lower levels of lipid oxidation at 30°C in comparison to the samples containing synthetic vitamin E and control samples. Also, our results showed that bilberry anthocyanin extracts may improve the storage stability of cod liver oil, as experimented here over a 42-day period at 15-17°C. These results may contribute to the future consideration of anthocyanins as new sources of natural antioxidants for use in marine oils formula and in food, cosmetic and pharmaceutical industry.

Key words: flavonoids, marine oils, lipid peroxidation, peroxide value

Introduction

Among worldwide dietary recommendations, an increased intake of omega-3 polyunsaturated fatty acids (PUFA) of marine origin is as important as consumption of fruits and vegetables because of their health benefits, in particular on decreasing the risk of chronic diseases.

Fish oils are known as good sources of bioactive long chain PUFAs, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Many fish oil formulas have been developed by the dietary supplement industry. One of the disadvantages of fish oils is that they are highly susceptible to oxidation that limits their shelf life. Consequently, such products need antioxidant protection. Oxidative degradation affects not only quality characteristics of lipids such as flavor (rancidity), color, texture, but also the nutritive value of the products. In addition, potentially toxic compounds may be generated (B. HALLIWELL & al. [1], E.N. FRANKEL [2], J.F. LIU & AL. [3], S. KUBOW [4], W. CHAIYASIT & al. [5]).

Generally, effective synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tertiary butylhydroquinone (TBHQ) are used to retard lipid oxidation, but the increased demand of the consumer for natural ingredients determined synthetic antioxidants to be replaced by the natural ones.

Unfortunately, in the last decades a reduced number of new antioxidant ingredients have been introduced on the market. Literature described some natural free radical scavengers such as tocopherols, carotenoids (including β-carotene, lycopene and lutein), polyphenols (including catechins and flavonoids), amino acids, peptides, proteins, urate, and ascorbate that act as singlet oxygen quenchers (E.A. DECKER [6]).

Natural antioxidants are present in a wide range of plants. Among them, anthocyanins are the most widely distributed water soluble pigments responsible for the bright colors red, purple or blue of flowers, skin, seeds, fruits and leaves (J.B. HARBONE & al. [7], F. DELGADO-VARGAS & al. [8]).

Structurally, anthocyanins are glycosides of salts of phenyl-2-benzopyrilium, composed of the aglycon called anthocyanidine and the carbohydrate residues (glucose, rhamnose, xylose, galactose, arabinose, rutinose) (J.B. HARBONE & al. [7]) (see general structure of anthocyanins in Fig. 1).

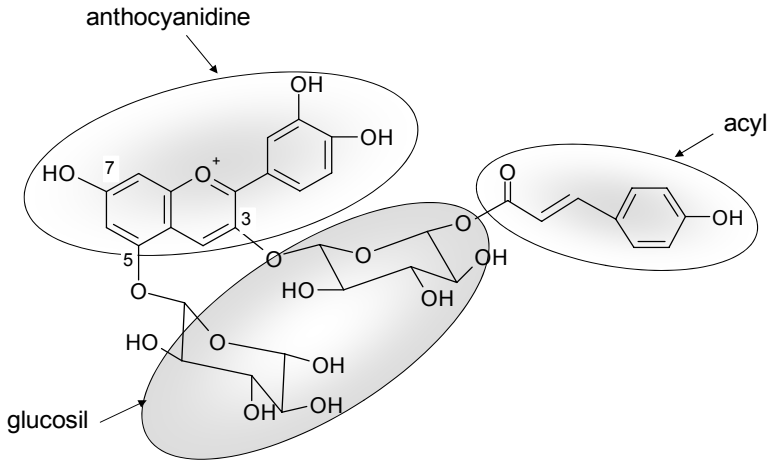

Figure 1. Chemical structure of acylated anthocyanin.

A large amount of literature dealing mainly with antioxidant mechanism and structure-activity relationship (SAR) of natural antioxidants has been published (N. YANISHLIEVA-MASLAROVA [9], I. RUŽIĆ, & al. [10], S. De MARINO & al. [11]).

Recent studies on anthocyanins are focused on their beneficial effects on human health, in particular in reducing the risk of heart diseases and cancer (N. MOTOHASHI & al. [12], M. BAGCHI & al. [13]). Anthocyanins are biologically active substances with strong antioxidant activities, which are able to capture dangerous free radicals such as superoxide, hydroxyl, hydrogen peroxide and singlet oxygen – chemical species that lead to lipid peroxidation of cell membranes. Antioxidant activity was closely related to the chemical structure of anthocyanins, mainly to the position and degree of hydroxylation of both rings of the basic structure (polyphenolic character) (T. TSUDA & al. [14], H. WANG & al. [15]). One limiting factor regarding incorporation of anthocyanins in foods is their low stability under specific conditions, the most important being the pH. Among studies regarding the application of anthocyanins in food industry, most of them were focused on their stability and color change in aqueous media (J. BAKKER & al. [16], L. CABRITA & al. [17]) and limited data regarding the behaviour of anthocyanins in apolar media is available. Therefore, in order to manufacture functional foods enriched with anthocyanin extracts it is desirable to stabilize these pigments in food systems.

The present paper describes a study of the effectiveness of a natural anthocyanin extract on the oxidative stability of cod oil, an essential step to prolong shelf life of fish oil formulas. The aim of this investigation was: (i) to determine the antioxidant effect of a crude anthocyanin extract from *Vacciunium myrtillus* L. added to fish oil from cod liver (*Gadus morhua*) in emulsion system; (ii) to compare the results to the same effect shown by cod oil treated with tocopherol (TOCOMIX) additive; (iii) to investigate the storage stability of the treated cod oil.

Materials and methods

Reagents and solutions

Chemical reagents of analytical grade without further purification were used for preparing the solutions. Ethanol (> 96% v/v), chloroform (min. 99%), clorhydric acid (37%) and glacial acetic acid were obtained from ADRACHIM (Bucharest, Romania), potassium iodide, potassium chloride, sodium acetate (trihydrate) and thiosulfate were obtained from CHIMOPAR (Bucharest, Romania), while starch of synthesis grade were purchased from SCHARLAU (Spain). Buffer solutions were prepared in distilled water.

Anthocyanin extract preparation

Samples of the wild blueberry fruits were obtained from Breaza - Brasov, Romania (900 m altitude). Fruits of *Vacciunium myrtillus* L. were homogenized and anthocyanins were extracted overnight in 70% (v/v) ethanol in water, at 4^{o}C. The extract was filtered and centrifuged at 4^{o}C at 8000 rpm for 10 minutes. The Nűve NF 800R refrigerated centrifuge was used.

Total pigments

The total content of anthocyanins in bilberry extract was spectrophotometrically determined by the pH differential method (M.M. GIUSTI & al.[18]). Absorbance was measured at 510 nm at two different pH levels (1.0 and 4.5) against blank sample. Measurements were done in two replicates. The T80 UV/VIS spectrophotometer (PG Instruments Ltd) was used. Total anthocyanins were expressed as cyanidin-3-O-glucoside (Cyn-3-O-G).

Physical-chemical characterization of extract

Moisture content of the sample was determined at 105^{o}C using the ML-50 moisture analyzer (A&D Company, Limited). Refractive index (n) and soluble dry matter of the *Vacciunium myrtillus* L. juice obtained by manually pressing, was determined by refractometric method using an Abbe AR2008 refractometer (Krüss) at a standardised temperature (21^{o}C). Values are expressed as refractometric total soluble dry substance (^{o}Brix).

Reverse micelles preparation

Reverse micelles (RM) were prepared by dissolving liquid soy lecithin in cod liver oil to obtain 0.5% concentration. Cod liver oil was obtained from Lysi (Reyjavik, Iceland). Tocopherols were not removed from cod liver oil samples. Soy lecithin (E322) is the most widely used ionic surfactant ingredient in the food industry. Then 50 □L of bilberry extract containing anthocyanins, were added, under vigourous stirring conditions, as water-soluble antioxidants. All emulsions were stored in duplicate in Petri dishes in a dark oven kept at 30^{o}C over a 14-day period. Two aliquots of each were removed periodically for PV analysis. Similar preparation was done by using tocopherols as fat-soluble antioxidants (Tocomix, liquid mixed tocopherols) added to cod oil samples, in concentration of 0.25%.

Lipid hydroperoxides determination

Peroxide values (PV) were determined by iodometric standard procedure and expressed as meq kg^{-1} (FARMACOPEEA ROMANA [19]). Data from the PV measurements were plotted against time.

Results and discussion

There are many studies that highlight the antioxidant activity of anthocyanins *in vivo* and *in vitro*. The researches have focused on the various investigative methodologies, such as oxygen radical absorbance capacity assay (ORAC), total reactive antioxidant potential (TRAP), ferric-reducing antioxidant power assay (FRAP), Trolox equivalent antioxidant capacity (TEAC), Folin-Ciocalteu (FC) or total phenolics assay, thiobarbituric acid reactive substances assay (TBARS), DPPH radical scavenging activity, β carotene-linoleic acid assay, generation of the radical anion superoxide with xanthine oxidase-hypoxanthine system, and generation of the hydroxyl radical by means of the system hydrogen peroxide-peroxide (W. LI & al. [20]). These assays are based either on the *1)* hydrogen atom transfer (HAT); or *2)* single-electron transfer (SET) reactions (D. HUANG & al.[21]).

In our work we investigated the antioxidant effect using a simple assay that estimate the peroxidation in an oil system. The assay has relevant practical applications because through this process it is possible both to determine the antioxidant potential of anthocyanins and to exploit their application in the food/dietary supplements industry to stabilize polyunsaturated oils.

As fish oils are important sources of PUFAs but are highly subject to oxidative deterioration, we investigated the stabilization potential of a *Vaccinium myrtillus* anthocyanin extract added to the cod liver oil samples. We selected bilberry extracts as natural antioxidants because they are rich in bioactive phytochemicals, which may preserve marine bioactives during storage.

In Table 1, nutritional information of cod liver oil is presented as described by the manufacturer. Tocopherols were not removed from the samples.

Table 1. The composition of fish oil from cod liver (*Gadus morhua*).

Composition	**10 mL oil**
Saturated fatty acids (g)	1.6
Monounsaturated fatty acids (g)	4.6
Polyunsaturated fatty acids (g), from which	3.0
EPA (g)	0.7
DHA (g)	0.9
Vitamin A (□g)	460
Vitamin D (□g)	9.2
Vitamin E (mg)	9.2

Extraction of anthocyanins from wild bilberry *Vaccinium myrtillus* L. was conducted in ethanolic solution, as most of the biomedicinal use and bioactivities was associated with the ethanol extract. Quantitative analysis of pigments was performed by pH differential spectrophotometrical method. The obtained anthocyanin extract showed a high content of total monomeric anthocyanins (460.6 mg $100g^{-1}$ FW), as shown in Table 2.

Table 2. Moisture, refractive index, total soluble dry substance and total anthocyanin content of the studied bilberry extract.

Parameters	Sample
Moisture (g $100g^{-1}$)	83.3
Refractive index, n	1.3634
Total soluble dry substance (^{o}Brix)	13.7
Total anthocyanin content (mg $100g^{-1}$ fresh weight)	460.6

The antioxidant activity of the obtained anthocyanin extract as potential lipid peroxidation preventor was studied in the reverse micelles (RM), formed of cod liver oil and natural anthocyanin extract in the presence of soy lecithin. The inhibition of hydroperoxide formation was measured by peroxide values (PV). PV measurement is a well-established method (AOCS Official Method) for the determination of primary oxidation products in fats and related substances. In our study, as an index of lipid peroxidation, PV leveles were measured over a 14-day period at 30°C for the following samples: (1) untreated control; (2) samples treated with tocopherols; and (3) samples (RM) treated with bilberry anthocyanin extract.

Obtained results showed that cod liver oil sample containing bilberry anthocyanin extract had lower levels of lipid oxidation in comparison to the samples with added synthetic tocopherols (Tocomix), as shown in Figure 2. The inhibition of hydroperoxide formation in cod oil was increased from 20 to 50.7% with bilberry anthocyanin extract, and from 3 to 30.4% with the Tocomix, in the first four days. After 11 days, the inhibition of hydroperoxide formation seems to decrease drastically in cod oil with added Tocomix. Such negative effect of tocopherols on fish oils was shown by other workers (A.M. O'SULLIVAN & al.[22]). In RM samples with anthocyanin extract, a sinergistic effect of anthocyanins and vitamin E present in cod oil formula and which were not removed, might occur.

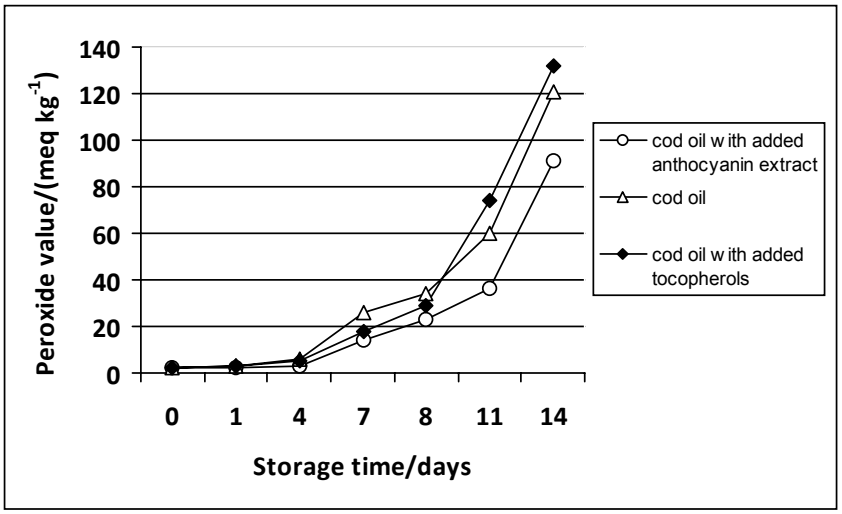

Figure 2. The inhibiting effect of added bilberry anthocyanin extract and added tocopherols (Tocomix) on the oxidation of cod liver oil (storage at 30^{o}C).

Also, we have investigated the storage stability of cod liver oil in the presence of natural anthocyanin extracts, in terms of storage shelves conditions. The change of PV with storage time is presented in Figure 3. Our results showed that bilberry anthocyanin extract may improve the storage stability of cod oil, as experimented here over a 42-day period at 15-17°C.

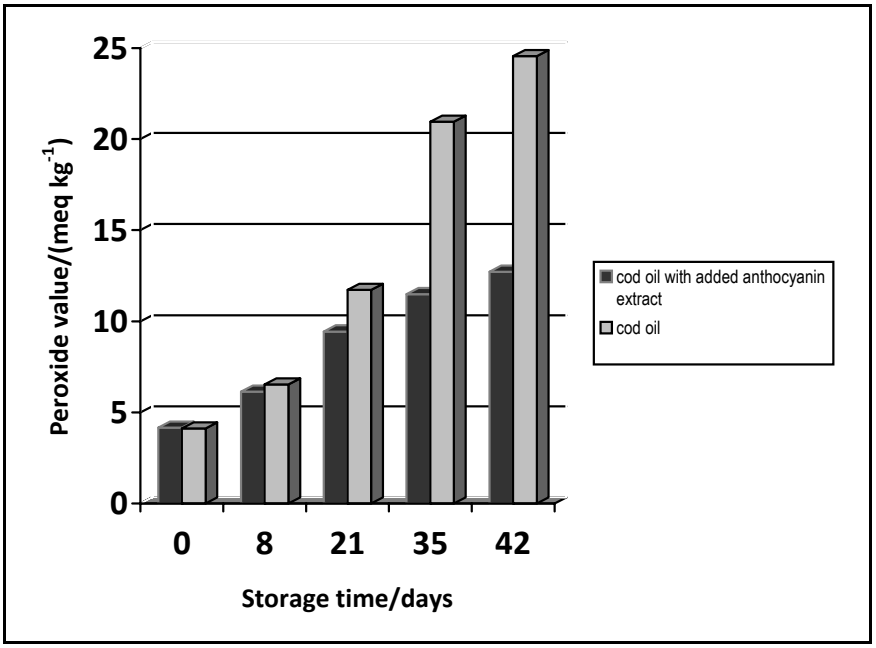

Figure 3. The stability of cod liver oil at 15-17°C by addition of bilberry anthocyanin extract.

Our results showed that anthocyanins have great potential to preserve polyunsaturated oils as fish oil formulas, based on their experimented antioxidant activity. Literature is scarce in such studies on anthocyanins, but successful attempts were done with other natural antioxidants such as tea catechins (A.M. O'SULLIVAN & al.[22]) or rosemary extract added to fish oils (H. K. SHIN & al. [23]), and mulberry leaves extract added to rice bran oil (L.G. ROY & al. [24]).

Conclusions

Due to the necessity of replacing synthetic antioxidants by natural ones in edible oils, this paper described the potential of anthocyanin extracts obtained from bilberry (*Vaccinium myrtillus*) to stabilize polyunsaturated oil from cod liver (*Gadus morhua*) in reverse micelle system.

The obtained results showed that anthocyanin extracts of *Vaccinium myrtillus* proved efficient antioxidant potential to stabilize cod liver oil formulas, compared to synthetic vitamin E added to the oil. The inhibition of hydroperoxide formation in cod oil was increased from 20 to 50.7% with bilberry anthocyanin extract, and from 3 to 30.4% with the Tocomix, in the first four days. After 11 days, the inhibition of hydroperoxide formation seems to decrease drastically with Tocomix tocopherols.

Also, in terms of storage shelves conditions, our results showed that bilberry anthocyanin extract may improve the storage stability of cod oil, as experimented here over a 42-day period at 15-17°C.

The results may contribute to future applications of anthocyanins to stabilize edible oils or dietary supplements with nutritionally significant amounts of PUFAs.

Acknowledgements

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