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Omne Ignotum pro Magnifico: characterization of commercial Bilberry extracts to fight adulteration

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Abstract. **Thanks to their chemical composition, extracts from the bilberry are commonly used to manufacture food, health products, supplements and cosmetics.**

However, in this field, as in others, "the process is the product" and a careful characterization of the entire supply and production chain, from purity and quality of raw material to extraction procedures needs to be implemented by using validated, sensitive and specific techniques of analysis.

This position paper discusses the importance of the characterization of bilberry extracts, in order to fight adulteration.

Keywords:
Adulteration, Bilberry, Plant extracts, Production.

Introduction

Berries are rich in anthocyanin glycosides, which are responsible for the pigmentation of the fruits and for the natural anti-oxidant activity due to the positively charged oxygen atom making this molecule class a potent and distinct hydrogen donating antioxidant (Figure 1).

Belonging to the genus *Vaccinium* of the plant family *Ericaceae*, bilberry (*Vaccinium myrtillus L.*) is a low-growing shrub, up to 60 cm in height. The fruits are purplish-black, globe-shaped and 6-10 mm in diameter. Bilberry is widely distributed throughout Northern Europe and the mountain areas of Central and Southern Europe.

Among the different berry cultivars, bilberry contains the largest amount of anthocyanins (about 300-600 mg/100 g fruit)[1]. Five main anthocyanins are common in bilberry fruit extracts: cyanidin, delphinidin (these two compounds account for more than 60% of the total anthocyanin content), malvidin, peonidin, and petunidin. The glycoside forms of these anthocyanins are mainly 3-glucosides, 3-galactosides and 3-arabinosides (Figure 1). Of these, cyanidin-3-glucoside displayed the greatest level of antioxidant activity[2,3].

Thanks to their chemical composition, extracts from the rich dietary source of bilberry are commonly used to manufacture food, health products, supplements and cosmetics.

This review discusses the importance of the characterization of bilberry extracts, on the basis of existing evidence and personal interpretation of such evidence. The results of our analysis of literature are presented hereafter.

Benefits of Bilberry

The first clinical studies in the 60s suggested that bilberry extracts could be beneficial for the therapy of both vascular and vision problems, although those studies were affected by several methodological weaknesses[4]. More recent and accurate pharmacological evidence indicates that fruit extracts containing anthocyanins have a broad spectrum of anti-inflammatory, anti-angiogenic and anti-carcinogenic activities[5]. Other reports[2,6] show that bilberry extracts may decrease abnormal vascular permeability through an effect on vascular connective tissue (specifically by inhibiting elastase and collagenase activities), have vasorelaxant and antioxidant properties and inhibit platelet aggregation. Bilberry extracts also revealed anti-angiogenic effects, by inhibiting stress- and cytokine-induced VEGF (vascular endothelial growth factor) expression *in vitro* and *in vivo*[5]. Recently, it has been found that the bilberry anthocyanins inhibit the formation of amyloid fibrils and toxic intermediate molecular species, associated with various severe neurological disorders, including Alzheimer's, Parkinson's and Huntington's disease, thus, suggesting interesting therapeutic perspectives for bilberry extracts in the treatment of amyloid-related degenerative diseases[7].

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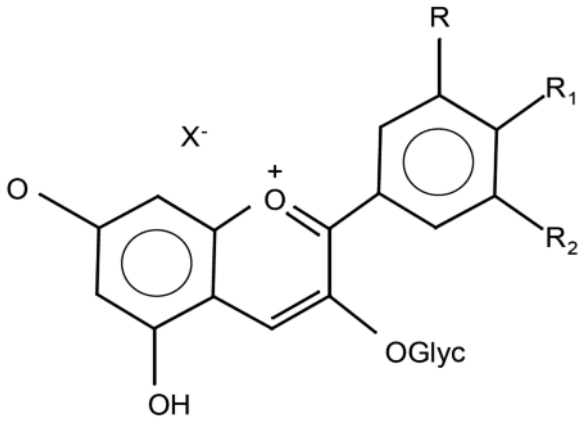

Figure 1. The basic structure of anthocyanins, also known as Flavylium cation. R = OH in Delphinidin, Cyanidin and Petunidin; OCH_3 in Peonidin and Malvidin. R_1 = OH in all anthocyanin. R_2 = OH in Delphinidin; H in Cyanidin and Peonidin; OCH_3 in Petunidin and Malvidin. OGlyc = 3-O-arabinoside, or 3-O-glucoside, or 3-O-galactoside.

Human studies

In human clinical studies the use of bilberry fruit extracts has been successfully tested for the treatment of venous insufficiency, resulting from idiopathic varices or deep vein thrombosis and lower limb varicose syndrome. Several clinical trials indicate different degrees of improvement in the treatment or prevention of retinopathy. Additional clinical evidence from a number of different studies indicates that bilberry fruit extracts can be used to relieve the pain, itching, and burning associated with hemorrhoids and hemorrhoidectomy, to reduce occurrence and severity of post-operative hemorrhagic complications in otolaryngology surgery and for symptomatic relief of dysmenorrhea. No adverse effects were registered in these investigations and a systematic review of these clinical researches is available in the literature[3].

Based on the effectiveness shown by pharmacological and clinical studies, bilberry extracts are used to treat vascular insufficiency and associated symptoms such as edema, varicosities, paraesthesias, and cramping. Moreover, the antioxidant activity of anthocyanins has been shown to reverse age-related neuronal and behavioral alterations[8] and decreased visual acuity[9], as well as to reduce coronary heart disease[10].

Bilberry Market

Bilberry extracts have already been approved as active pharmaceutical ingredients (APIs) in many countries, e.g. in phytomedicines in the European Union (EU), or as dietary ingredients in food supplements in the United States. Bilberry extract as a dietary supplement ingredient is very popular in the United States, where it ranked 14th in the mainstream market in 2012[11], although its sales in this channel have dropped by about 10-11 percent per year since 2010 (Table I)[11-15], possibly because of the increased price of the biomass due to the poor harvest in the past years[16] and the substitution by extracts from cheaper berries.

Bilberry harvesting

Bilberries are difficult to grow and are seldom cultivated. The main source of intensively harvested bilberries is in Scandinavian and Eastern European countries. In particular, Norway, Sweden, Finland and Iceland have a cooperation agreement for developing research on plant biology and biodiversity and on market and quality issues as well[17].

In Russia and other Eastern countries wild-berry picking is less industrialized and more confined to individual activities among poor populations of rural areas. Interestingly, in some countries, like Austria, Finland, Norway, Sweden, Estonia, Latvia and Switzerland it is the "everyman's right" to collect bilberries irrespective of land ownership (private gardens and natural reserves excluded). This harvesting mode is also granted to commercial activities, through individual permissions from landowners or negotiations of exclusive rights with berry associations[17].

The structured network of harvesting stakeholders established in the various Scandinavian countries allowed to calculate the estimated harvest yields and the relative fluctuations, which may differ considerably among the various countries. The Finnish Forest Research Institute has estimated that in a ten year-period the annual potential yield of bilberry harvesting ranged from 92 to 312 million kilograms and has maintained relatively stable since the 80s, because wild berry picking is a solid social and cultural tradition in Finland. However, in other Scandinavian countries the situation of wild berry picking is different, with a negative trend, like in Sweden where the participation in berry collection and the harvesting yield decreased considerably in the last 20 years[18].

In Nordic European countries the cooperation of governments with 1,300 from small to large scale private companies allowed the development of a worldwide supremacy in the supply of bil-

Table I. Bilberry Product Sales Ranking, Sales Estimates and Percent of Change over Previous Years in Mass Market Channels in US 2007-2012*

Year	Sales rank	Retail sales	% ± Over previous year
2012	14	$ 1,407,746	– 11.04%
2011	15	$1,582,448	– 11.24%
2010	15	$1,784,932	– 9.95%
2009	15	$1,983,723	+ 7.41%
2008	17	$1,841,200	+ 1.92%
2007	15	$1,814,102	– 9.48%

*Data do not include sales in Wal-Mart stores. Information supplied for Food, Drug, and Mass Market retail stores, as compiled by Symphony IRI and published in ABC's annual Herb Market Report in *HerbalGram*[11-15].

berry as raw material. It is estimated that 750 Swedish, 350 Norwegian, and 200 Finnish companies are involved in harvesting wild berries. About half of the product harvested in Nordic European countries is exported to China and Japan, validated by genetic tests to exclude any contamination with other wild species. The issue of a high quality standard is one of the main research goals of the Nordic research project "Bilberry – Towards Functional Food Markets", coordinated by the University of Oulu (Finland) and including among partners the Arctic Flavours Association from Finland, the Swedish University of Agricultural Sciences, the Nordic Gene Bank, the University of Tromsø and Bioforsk from Norway and the Agricultural University of Iceland. The project aims at attaining a consistent wild berry quality within Nordic countries and at developing a uniform traceability system, to foster the future development of a Nordic wild berry brand as a guarantee of quality[19].

The quality issue

The quality of bilberries is affected by weather and other conditions that may occur during the whole growth season among different years, while genetic differences contribute to significant variations among berries harvested in different regions during the same year. Moreover, harvest timing is an important factor in bilberry ripeness, which is another crucial factor for the quality of the products, because the concentration of flavonols and procyanidins decreases as the fruit ripens, while the concentrations of antioxidant anthocyanins increase. Altogether, these concurring factors make the quality of the harvested product very variable. However, high standards of bilberry quality are required by the food and pharmaceutical industry, so that wild berry suppliers need to be at the right locations at the right time during the berry-picking season. Also extraneous leafs and bud material may influence quality and must be cleaned from the bilberry harvest. According to the different areas in Europe, harvest of bilberry occurs between mid July to the end of September[17].

Also logistics plays a key role in the bilberry market, because the fruits ripen in wide geographic areas, often hardly approachable from the main roads, and the storage of the fresh product may be difficult. Thus, transport and freezing of bilberries needs to be run as quickly and efficiently as possible and logistical issues demand their share of attention[19].

Therefore, due to the high complexity of the bilberry market, there is an increased risk of distributing deteriorated supplements. For example, in Japan anthocyanin-containing supplements are classified as food with no appropriate regulation required. This may cause the risk of distribution of defective products to consumers and, therefore, a new index for detection of deteriorated products has been recently proposed. The degradation Index (DI) was based on ultra-high performance liquid chromatography (UHPLC), a variant of HPLC that uses smaller particle size columns, which provides significantly better separation than the traditional HPLC and enables the analysis time to be reduced. This technique is superior to spectrophotometry for identifying and quantifying individual anthocyanins and was defined as a useful indicator and quality control index for detecting deteriorated supplements[20].

Finally, the market of bilberry offers different quality grades. However, reproducible results for safety and efficacy of supplements and drugs based on bilberry extracts are dependent upon a consistent and reproducible quality. To obtain reproducible efficacy and safety for a specified health benefit the clinical studies must be conducted with bilberry extracts characterized on the basis of standardized anthocyanin content and reproducible phytochemical fingerprints[21,22].

Bilberry Adulteration

Bilberry extracts and the related commercial products are based on a relatively high-cost process, because the world's supply of the fruit is wild-harvested and cannot be successfully cultivat-

ed on a commercial scale, like instead in the case of blueberry. This latter fruit has a larger harvest yield because blueberry fruit clusters may have 30 or more fruits, compared to bilberry fruit clusters that usually produce no more than 1-2 fruits.

The European Medicines Agency (EMA) issued in 2006 the "Guidelines on Quality of Herbal Medicinal Products" establishing that the compliance with certain standards of quality is a necessary part of the marketing authorization dossier for a medicine based on herbal extracts[23]. On the contrary, in 1994 the United States Federal legislation issued the Dietary Supplement Health and Education Act that defines and regulates dietary supplements classified as foods instead of as drugs, therefore, without requiring proof of effectiveness or safety. Following the passage of this act, the herb product market increased exponentially, resulting in many new companies entering the herb market supply chain at both the wholesale and retail level. Well before this market boom, many standardized herb extracts available in the market were developed and started to be produced by well-established European firms, like Myrtocyan® and Mirtoselect® of Indena SpA (Milan, Italy), which also established the pharmacological and clinical research on such extracts[17]. Thus, the adulteration of bilberry supplies and extracts was relatively low until the booming of the global market for bilberry fueled the establishment of a highly competitive market, especially for the remarkably lower-priced extracts from Asian countries, particularly China. This marked change of the world market led as a consequence a more frequent and massive economic adulteration of bilberry extracts.

There are several examples of different means for bilberry adulteration. Extracts of other *Vaccinium (V.)* species, indicated as different varieties of bilberry, have been added to perform a deliberate adulteration of bilberry products. A study by the company Indena SpA identified adulterated species as *V. angustifolium* (low-bush blueberry), *V. corymbosum* (high-bush blueberry), and their hybrids and cultivars, *V. oxycoccos* (European cranberry) and *V. macrocarpon* (cranberry). Moreover, anthocyanosides from unrelated plants such as *Sambucus nigra* (elderberry, Adoxaceae), *Ribes nigrum* (black currant, Grossulariaceae), *Rubus idaeus* (raspberry, Rosaceae), and *Prunus avium* (wild cherry, Rosaceae) have also been found by the study as adulterants in bilberry extracts. In addition, it was identified also the adulteration with black soybean hull on the products sold in the retail market. Two qualities of black soybean hull extracts are available from Chinese market with 35% and 50% of anthocyanosides content, respectively[24].

Another example of adulteration with different species occurred in the Chinese market, where extracts of two circumboreal species, *V. uliginosum* and *V. vitis-idaea*, present in northern areas of Europe, North America and Asia, and wild-harvested in Northeast China and Shaanxi Province of China, are offered to world markets as "homemade Chinese bilberry" and "Chinese domestic bilberry" extracts at prices as low as US$ 10/kg. In 2008, Chinese bilberry extract production (excluding "European bilberry", *V. myrtillus*) accounted to approximately 60 tons, 95% of which was exported, mostly to the United States[17].

In the dietary supplement industry the adulteration of bilberry extracts has been performed also by adding a chemical dye called amaranth. This color is not from the South American amaranth plant, the flowers of which produce a deep red dye. Instead it is a chemical coal tar-derived compound named amaranth, commonly known also as Red Dye No. 2, a synthetic dark red sulfonic acid based naphthylazo dye that was banned from the food supplies by FDA as a suspected carcinogen. Bilberry extracts adulterated with amaranth/Red Dye Nr. 2 are available on the market at a price of one-third off in the supplement market[17,22].

Methods for characterization of bilberry extracts and identification of adulterated products

Different analytical methods used for standardization of the bilberry extracts and their preparations are available from pharmacopeias and from the literature. Regarding the spectrophotometric assay for quantifying total anthocyanins in bilberry, herb and natural products industry had been alerted that this test, based on the calculations of total anthocyanin content as cyanidin-3-glucoside, was useful only if with other methods authenticity and purity of source material was assessed, because it does not detect intentional adulteration with added colorants[3].

An acid hydrolysis HPLC (high-performance liquid chromatography) method was developed for the characterization and standardization of raw material origin. This HPLC method has been deemed useful to quantify the five major individ-

ual anthocyanidins in bilberry (pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin), by complete separation of five anthocyanidin aglycones (compounds with no glucose structure attached)[25].

Interestingly, when identity tests were conducted on different bilberry sources, less expensive materials passed a general test for the anthocyanin content conducted by a single-wavelength spectrophotometric assay. However, when the sample was analyzed also by HPLC, it was found that the extract contained an anthocyanin-mimicking adulterant that was subsequently identified as amaranth by mass spectroscopy and nuclear magnetic resonance. This study revealed that, if deliberate adulteration occurs in an extract, a single-wavelength spectrophotometric assay is inadequate and the use of alternative, more sophisticated methods of analysis, such as HPLC with photodiode array detection, are required[22]. Other methods of assay have also been tested[26].

Based on the assumptions that free anthocyanidins are markers of poor product quality, a new liquid chromatography method with ultraviolet detection was developed by Indena SpA, an Italian company producing and researching bilberry extracts, for the identification and quantification of both anthocyanins and anthocyanidins in bilberry extracts and products. The technique is reproducible and highly specific and is suitable to unequivocally characterize the extract composition and the source of the raw materials used, thus ensuring a high degree of product consistency and quality[27]. This method was validated by Indena that confirmed that anthocyanosides are the major active ingredients in bilberry and discovered that the combination of delphinidin, cyanidin, malvidin, peonidin and petunidin contained in bilberry, when properly analyzed by liquid chromatography, reveals a unique pattern that allows to distinguish bilberry from all other sources of anthocyanosides, both of dietary and non-dietary origin[24]. The European Pharmacopoeia (EP) issued a monograph indicating an authentication method by thin-layer chromatography (TLC). For the identification and anthocyanidin quantitation EP adopted, with minor modifications (removal of molecular weight correction for the content calculation, use of primary or secondary references) the HPLC method established by Indena SpA as the official analytical method for bilberry. The analysis relies on EP reference standards of bilberry dry extract CRS (Community Resources for Science) and cyanidin chloride CRS and must be run within the framework of a suitable quality system, like Good Manifacturing Practices (GMP)[28].

Conclusions

Adulteration of bilberry extracts either with banned synthetic, potentially cancerous dye materials or with contamination of different species not only endangers the reputation of the natural products industry worldwide, but also affects the consumer confidence in bilberry as a dietary supplement ingredient in general. Hence, in this field, as in others, "the process is the product" and a careful characterization of the entire supply and production chain, from purity and quality of raw material to extraction procedures needs to be implemented by using validated, sensitive and specific techniques of analysis.

Interestingly, the most effective analytical methods developed to appropriately characterize bilberry extracts and to solve the problems of bilberry adulteration originated by the joint effort of university scientists and industrial analytical laboratories: Nature's Sunshine Products, MediHerb and Indena, respectively. These analytical procedures will contribute to eradicate bilberry adulteration if all companies operating in this market will offer retail consumer products complying with this analytical characterization and to current Good Manufacturing Practices.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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