REVIEW

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Anthocyanin- and proanthocyanidin-rich extracts of berries in food supplements – analysis with problems

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Received May 8, 2007, accepted June 4, 2007

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Pharmazie 62: 803–812 (2007) *doi: 10.1691/ph.2007.11.7621*

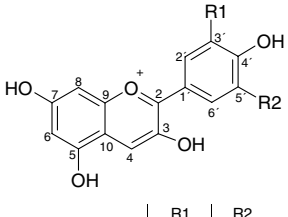
The fundamental nutritional benefit of fruit and vegetables in the prevention of degenerative diseases – especially in the light of the current "anti-aging wave" – has directed the attention of scientists and consumers to a variety of berry fruits and their constituents. Many of these fruits, e.g. blueberries, elderberries or cranberries, have a long tradition in European and North American folk medicine. Based on these experiences and due to the growing interest the number of food supplements on the market containing fruit powders, juice concentrates or extracts of these fruits has increased considerably. Advertising for these products mainly focusses on the phenolic compounds, especially the anthocyanins and proanthocyanidins and their preventive effects. Most of the preparations are combinations, e.g. of extracts of different fruits with vitamins and trace elements, etc. which are labelled in a way which does not allow a comparison of the products. Typically, information on the extraction solvent, the drug : extract ratio and the content of anthocyanins and proanthocyanidins is missing. Besides that, the analysis of these polyphenols causes additional problems. Whereas the quality control of herbal medicinal products is regulated in detail, no uniform requirements for food supplements are existing. A broad spectrum of methods is used for the assay of the constituents, leading to differing, incomparable results. In addition to that, the methods are quite interference-prone and consequently lead to over- or underestimation of the contents. This publication provides an overview of some selected berries (lingonberry, cranberry, black elderberry, black chokeberry, black currant, blueberry), their constituents and use. The analytical methods currently used for the identification and quantification of the polyphenols in these berries are described, including an evaluation of their advantages and disadvantages.

1. Chemistry and occurrence of anthocyanins and proanthocyanidins

Anthocyanins and proanthocyanidins are plant polyketides, which belong to the large group of phenylchroman derivatives.

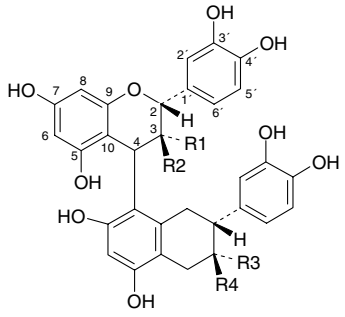
As flavylium salts, anthocyanidins in plants always occur as watersoluble glycosides (= anthocyanins, anthocyanosides). The genines, which up to date have been identified most frequently in higher plants, are shown in Figure 1. The distribution of these compounds in edible plant parts is about 50% cyanidin, approximately 12% each of pelargonidin, peonidin and delphinidin, as well as about 7% petunidin and malvidin. In most cases ubiquitous sugars, such as glucose, galactose, rhamnose and arabinose, are linked as mono-, di- or trisaccharides mainly in position 3 and/or position 5 of the genines (Kong et al. 2003). The sugar moieties, which improve solubility and stability of the substances, can additionally be acylated with hydroxycinnamic acid, hydroxbenzoic acid, acetic acid, etc.

The anthocyanins, broadly distributed in plants, are mainly found in flowers and fruits and there responsible for the intense colours. The colouration depends on the pH value: At



	R1	R2
Pelargonidin	H	H
Cyanidin	H	OH
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

Fig. 1: Structure of anthocyanidins



	R1	R2	R3	R4
Procyanidin B_1	OH	H	H	OH
Procyanidin B_2	OH	H	OH	H
Procyanidin B_3	H	OH	OH	H

Fig. 2: Structure of proanthocyanidins of the B-type (dimers)

pH < 3 the compounds have a red colour. By hydroxylation at C-2 the flavylium cation is transformed into the colourless carbinol pseudobase (pH between 4 and 5). In fruits, at physiological pH values there is equilibrium between colourless carbinol base and coloured flavylium cation. At pH values above 6 the quinoide form or an anhydro base is formed by cleavage of water, which leads to purple and further to blue colouration (pH value between 6 and 8) (Wrolstad et al. 2002). Temperature, light and other constituents in the extracts, such as sugars and ascorbic acid, also have an influence on the stability of the anthocyanins.

Proanthocyanidins (= condensed tannins, see Fig. 2) are the colourless precursors of anthocyanidins. They occur as dimers, oligomers or polymers of flavan-3-ols, which are linked via C−C bridges and formed by enzymatic condensation. With increasing degree of polymerisation astringent yellow to brown compounds are formed (Lea and Arnold 1978). Low-molecular compounds (MG < 7000 Dalton) are watersoluble. However, in plants insoluble higher polymers are predominant. In B- and C-type proanthocyanidins the monomers are linked by C−C bonds between C4→C8 or C4→C6. Representatives of the A-type possess an additional linkage of the single subunits via an ether bridge O7→C2 (Prior and Gu 2005).

Depending on the hydroxylation pattern of the monomers, the proanthocyanidins are divided into procyanidins, prodelphinidins, propelargonidins, etc., with procyanidins being the most common ones. Procyanidins are condensates exclusively consisting of catechin and epicatechin units (3′,4′-OH), whereas prodelphinidins are gallocatechin/epigallocatechin condensates (3′,4′,5′-OH) (Rohr et al. 2002).

Proanthocyanidins are, after lignin, the polyphenols with the widest distribution in plant kingdom. Hence, many vegetable foods such as cocoa, tea, nuts, fruit, legumes, spices, red wine, etc. contain proanthocyanidins. Of these, fruit is the most important source. In these matrices A-type proanthocyanidins are far less frequent than those of the B-type (Gu et al. 2003).

2. Anthocyanin and proanthocyanidin-rich preparations, their effects and use

In recent years interest has focussed on the antioxidant and radical scavenging effects of anthocyanins and proanthocyanidins, because since the 1980's, oxidative and anti-oxidative processes have been discussed intensively in relation to aging and degenerative diseases.

The preventive effects of these secondary plant metabolites in terms of cardiovascular diseases, arteriosclerosis and cancer are deduced from epidemiologic data as well as *in vitro-* and *in vivo* studies (Santos-Buelga and Scalbert 2000; Beattie et al. 2005; Rasmussen et al. 2005) and result in respective nutritional recommendations.

Despite numerous recommendations for regular consumption of a sufficient amount of fruit and vegetables, the average consumption is still relatively low in many European countries and in North America (Prior and Gu 2005; Wu et al. 2006). Thus, food supplements rich in polyphenols are increasingly promoted in recent years as "cell protectors", "radical scavengers", etc., and include a variety of preparations with extracts of different berries.

Preparations of the berries on which this paper focusses (Table 1) are used in therapy and prevention of different complaints, predominantly based on traditional use, but in part also supported by results of pharmacological investigations. The majority of the products is marketed as food supplements.

Anthocyanin-containing extracts of *blueberries* are the only preparations which – based on clinical evidence of vascular protection – are used as herbal medicinal products in peripheral vascular diseases and venous insufficiency of the legs as well as in ophthalmic disorders due

Table 1: Selected berry fruits and their phenolic constituents

English name	German name	Latin name	Phenolic constituents
Cowberry, Foxberry, Lingonberry, Red Whortleberry, Rock-Cranberry	Preiselbeere	*Vaccinium vitis-idaea* L. Ericaceae	Anthocyanins, flavonoids, phenol carboxylic acids, proanthocyanidins (Zheng 2003; Kähkönen 2001, 2003; Bomser 1996)
Cranberry, American Cranberry	Großfruchtige Moosbeere	*Vaccinium macrocarpon* Ait. Ericaceae	Anthocyanins, flavonoids, phenol carboxylic acids, proanthocyanidins (Zheng 2003; Foo 2000)
Elder, Elderberry, Common Elder	Schwarze Holunderbeere	*Sambucus nigra* L. Caprifoliaceae	Anthocyanins, phenol carboxylic acids, oligomeric catechins, flavonols (Malien-Aubert 2001)
Black Chokeberry, Wild Chokeberry	Kahle Apfelbeere	*Aronia melanocarpa* (Michx.) Elliot Rosaceae	Anthocyanins, flavonoids, proanthocyanidins, leucoanthocyanins, phenol carboxylic acids (Zheng 2003; Strigl 1995)
Black Currant	Schwarze Johannisbeere	*Ribes nigrum* L. Saxifragaceae	Anthocyanins, flavonol glycosides, hydroxycinnamic acids, proanthocyanidins (Kähkönen 2001; Määttä 2001, 2003)
Bilberry, Blueberry, Low Bush Blueberry, Whortleberry	Heidelbeere	*Vaccinium myrtillus* L. Ericaceae	Anthocyanins, flavonoids, phenol carboxylic acids, proanthocyanidins (Zheng 2003; Kähkönen 2001; Bomser 1996)

to photosensitivity or changes in the microcirculation of the retina (ESCOP 2003; Canter and Ernst 2004).
There are, however, food supplements in the market containing blueberry extracts which are recommended as radical scavengers, claiming to improve visual capacity, e.g. in the case of nighttime driving, computer work, etc.
In contrast to blueberry products, extracts of lingonberries, cranberries, elderberries, chokeberries and black currants are exclusively marketed as food supplements. In such products extracts of different fruits are often combined with each other and/or with vitamins.
Cranberry orginates from North America and has widely been used by Indian tribes for nutrition purposes in winter due its high content of vitamin C. The berries were also traditionally used as an external and internal remedy.
Cranberry juice or extracts are recommended for prevention of recurrent infections of the urogenital tract (Cunningham et al. 2002). Several clinical studies related to the effect on infectious diseases of the urinary tract have been performed (e.g. Avorn et al. 1994; Kontiokari et al. 2001; Stothers 2002).
Particularly in more recent studies a decrease in the number of recurrent infections was observed in the verum groups and the use of antibiotics could be reduced in comparison with placebo. It was demonstrated by *in vitro* studies on *Escherichia coli* that oligomeric cranberry proanthocyanidins prevent the adhesion of the mannose-resistent P-fimbria type (assumed to be co-responsible for such diseases) to epithelial cells (Zafriri et al. 1989; Ahuja et al. 1998; Foo et al. 2000a, 2000b; Zirk et al. 2004; DiMartino et al. 2006; Liu et al. 2006). Positive effects have also been observed in investigations on the influence of cranberry proanthocyanidins on the growth and adhesion of *Escherichia coli* and *Staphylococcus aureus* to catheter material (Hui et al. 2004). The effects were ascribed to A-type proanthocyanidins (Foo et al. 2000a, 2000b; Howell et al. 2005).
In patients with a positive respiratory test for *Helicobacter pylori*, a decreased infection rate was observed after consumption of cranberry juice in comparison with placebo (Zhang et al. 2005). Recently a significant increase in HDL-cholesterol in obese men was observed after daily intake of cranberry juice over a period of four weeks (Ruel et al. 2006)
The claims for food supplements containing extracts of *lingonberries*, which like cranberries contain A-type proanthocyanidins (Cheng et al. 2005), are similar to those for cranberry juice or extracts, i.e. the prevention of urinary tract infections. Some of the products contain combinations with cranberry extracts.
Food supplements with anthocyanin-containing extracts of black chokeberries, black currants and elderberries are marketed as sources of polyphenols and several of the products are enriched with vitamins. For these products, consistent evidence of radical scavenging and antioxidant effects has been obtained *in vitro*. Thus, the majority of the labels contain claims such as "free radical protection", "cell protection" or similar claims.
Black chokeberry originally comes from the Eastern part of North America. Since the beginning of the 20th century it has been cultivated for fruit production mainly in Eastern Europe. Black chokeberry is among the berries with the highest anthocyanin and proanthocyanidin concentrations (Wu et al. 2004; Bermudez-Soto and Tomas-Barberan 2004; Prior and Gu 2005). Extracts are recommended due to the high polyphenol-, vitamin K- and vitamin C content. The majority of the phenolic substances consists of polymeric procyanidins and anthocyanins (Oszmianski and Wojdylo 2005). Gastro- and hepatoprotective effects as well as hypolipidemic and hypoglycemic activities of anthocyanin-rich black chokeberry extracts or juices have been demonstrated in animal studies (Niedworok et al. 1997; Matsumoto et al. 2004; Valcheva-Kuzmanova et al. 2004; Valcheva-Kuzmanova et al. 2005, 2007a, 2007b).
Black currants are another source rich in vitamin C and anthocyanins (Bermudez-Soto and Tomas-Barberan 2004; Benvenuti et al. 2004). Thus, the traditional use of these berries in common cold and, especially in France, for venous complaints, such as heavy legs or hemorrhoids, seems plausible. In recent years, in vitro studies have shown antiviral effects of extracts or isolated anthocyanidins against *Herpes simplex* Type 1 and *Influenza* A and B (Knox et al. 2001, 2003; Suzutani et al. 2003).
Extracts of *elderberries* are often combined with other extracts in food supplements. For a monopreparation, antiviral effects against different strains of the *Influenza* virus have been demonstrated. Two randomised, placebo-controlled, double blind studies have pointed to a reduced duration of the symptoms of *Influenza* A and B infections (Zakay-Rones et al. 1995, 2004). The authors of two other studies deduced an immuno-stimulating capability of an extract from the increase in the production of the inflammatory cytokines IL-1ß, TNFα, IL-6 and IL-8 as well as the anti-inflammatory cytokine IL-10 (Barak et al. 2001, 2002). Besides black chokeberries, elderberries are among those foods with the highest anthocyanin contents (Wu et al. 2006).
Food supplements are widely marketed under differing labelling which does not allow further conclusions concerning the composition of the extracts. Several examples in Table 2 illustrate the "confusion of tongues" concerning berry preparations which contain powdered fruits, extracts, juices etc. In most cases simply the amount of berry preparation per dose is stated without any drug to extract ratio (DER). Only for very few food supplements and for herbal medicinal products the concentrations of flavonoids or anthocyanins are given on the label.

3. Analysis of anthocyanins and proanthocyanidins in berries

The reactivity of these substances as well as the complex composition of the extracts require on one hand a very careful and diligent sample preparation and on the other hand the adaptation and validation of the methods used, depending on the respective plant material.

3.1. Pre-treatment of the plant material

Due to the instability of the polyphenols sparing conditions must be applied after harvesting the berries and during extraction.
For the analyses of anthocyanins, usually either fresh or frozen berries are used or the material is freeze-dried (Brenneisen and Steinegger 1981; Andersen et al. 1991; Inami et al. 1996; Froytlog et al. 1998; Prior et al. 1998; Häkkinen et al. 1999a, 1999b, 2000; Kähkönen et al. 2001; Määttä et al. 2001; Nyman and Kumpulainen 2001; Prior et al. 2001; Gu et al. 2002; Kandil et al. 2002; Moyer et al. 2002; Slimestad and Solheim 2002; Sun et al. 2002; Yan et al. 2002; Kähkönen et al. 2003; Määttä et al. 2003; Zheng and Wang 2003; Nakajima et al. 2004; Oszmianski and Wojdylo 2005; Wu and Prior 2005; Kapasakalidis et al. 2006; Koponen et al. 2007).
Accordingly, in analyses of proanthocyanidins the samples have to be treated with utmost care because oxidation, polymerisation processes and complex-formation with

Table 2: Examples of "berry" preparations

Product	Labelled composition	Posology	Claim of the manufacturer
FS	320 mg Lingonberry powder containing anthocyanins 50 mg Vitamin C	2 × 1–2 capsules per day	Urinary tract infections, night vision, protection from free radicals
FS	400 mg Lingonberry extract 30 mg Vitamin C	1–4 × 1 capsule per day	Adjuvant treatment of infections of the urinary tract, acidification of the urine, thus protecting the bladder from bacteria, natural source of vitamin C
FS	Concentrate of lingonberry- and cranberry juice (at least 120 mg), Acerola powder (at least 120 mg) = 30 mg Vitamin C, Anthocyanins (at least 20 mg), Citric acid 75 mg	2–3 lozenges per day	Daily food supplement (prevention of urinary tract infections)
FS	Cranberry dry extract (Vaccinium macrocarpon) with 30% organic acids Vitamin C, Cranberry leaf powder	1–2 × 1 tablet per day	Herbal remedy for the bladder: strengthening of the bladder, helps to keep a beneficial balance of advantageous and disadvantageous bacteria in the bladder, remedy for use in bladder disorders, maintenance of good bladder function
FS	Cranberry concentrate, Acerola extract, Flavonoids, Vitamin C		Supports normal bladder function
FS	340 mg Black currant powder (standardised to 1.5% flavonoids)	2 capsules per day	Cell-protective, anti-inflammatory; rheumatism, gout, diuretic, radical scavenger (protection from negative influences of sunlight)
MP	100 mg Blueberry anthocyanins, 5 mg Betacarotene	3–4 × 1 coated tablet per day	Ophthalmicum, vasoprotective agent; traditionally used for the prevention of night blindness
MP	Standardised complex of anthocyanins from Vaccinium myrtillus, anthocyaninosidea ex vaccinio myrtillo 58 mg	2 × 1 capsule per day	Fragility and altered permeability of blood capillaries, microangiopathies, phlebopathies, post phlebitic symptoms, venous insufficiency, hemorrhoids, adjuvant in the therapy of arteriopathies, pre- and post-operative treatment in case of ORL operations and hemorrhoidectomy, diabetic retinopathy, hemeralopia
FS	400 mg Blueberry extract, 50 mg Vitamin C	1 × 1–2 tablets per day	Improvement of visual power in driving (night vision), cell-protection, radical scavenger
FS	400 mg Blueberry extract	1 × 1–2 tablets per day	Food supplement, improvement of visual power at night, e.g. car-drivers, elderly, those who read a lot and often watch TV
FS	Combination of Blueberry extract, Selenium and Vitamins A, C, E, B1, B2, B6		Food supplement, stabilisation of capillaries, antioxidant; visual capacity, computer work, nighttime driving, bright sunlight, frequent TV consumption
FS	100 mg Grape seed extract, 25 mg Blueberry extract, 150 mg Vitamin C, 200 µg Folic acid, 15 mg Vitamin E, 0.5 mg RE Vitamin A	2 tablets per day	Food supplement with bioflavonoids and vitamins for the eyes, inhibits production of free radicals and supports antioxidant protection of the eyes
FS	200 mg Blueberry extract (corresponding to 800 mg blueberries), 30 mg Vitamin C (50% RDA), 12 mg Zinc (80% RDA), 10 mg Vitamin E (100 % RDA), 6 mg Beta-Carotene, 6 mg Lutein, 3 mg Copper, 1 mg Lycopene, 750 µg Zeaxanthin, 60 µg Selenium	2 × 1 capsule per day	Food supplement with valuable blueberry extract, minerals and vitamins. Blueberry is well-known for "good vision", carotenoids are an important component of the retina and protect from free radicals
FS	80 mg Blueberry extract (Vaccinium myrtillus) containing 20 mg anthocyaninoside	3 × 1 capsule per day between meals or according to the recommendation of your nutritionist or doctor	Possible uses: diabetes mellitus/adjuvant therapy cataract, glaucoma, light-dark-adaptation, varicose veins, macular degeneration, oxidative stress-syndrome, varices, venous insufficiency

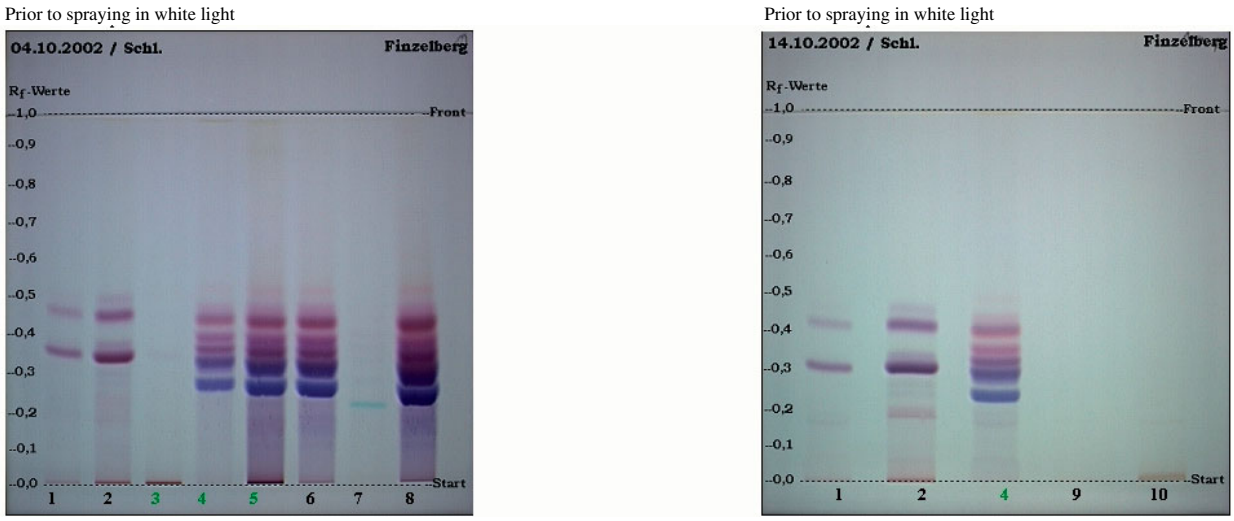
MP = medicinal product FS = food supplement

other biomolecules are rapidly initiated after destruction of the cells. It has been demonstrated that the results after freeze-drying corresponded to the results obtained with fresh materials. In contrary, air-drying under elevated temperatures leads to polymerisation and should not be applied. Long-term storage of the material has to be avoided as well (Rohr et al. 2000).

3.2. Extraction

For the quantification of the phenolic constituents the berries can be extracted with various solvents.

In the examination of anthocyanins and proanthocyanidins usually aqueous-organic solvents with small amounts of acid are used for the extraction. In recent publications



Position	Name of sample
1	Aronia juice – concentrate
2	Aronia juice – concentrate 3,4% anthocyanins
3	Aronia extract "enriched"
4	Blueberry – primary extract > 2% anthocyanins
5	Blueberry – primary extract > 20% anthocyanins
6	Blueberry – extract – capsules > 25% anthocyanins
7	Blueberry – extract – capsules; fermented
8	Blueberry – extract > 20% anthocyanins
9	Aronia – spissum extract
10	Aronia – dry extract

Fig. 3: TLC fingerprint chromatograms for anthocyanins in aronia and blueberry extracts and the respective preparations (stationary phase: silicagel F254; mobile phase: ethyl acetate-water-anhydrous formic acid 100+30+20)

most frequently acetone or methanol mixed with up to 50% of water were applied or acetonitrile. As acidic components acetic acid, formic acid, citric acid, trifluoroacetic acid (TFA) or hydrochloric acid proved suitable.

The extraction of the anthocyanins can be improved and accelerated by ultrasound or turbo extraction. In proanthocyanidin analysis the use of turbomixers increases the risk of oxidation processes. To avoid any thermic load extractions usually are performed at 0 °C, 4 °C or at room temperature. Ascorbic acid, sodium pyrosulfite or t-butylhydroquinone may be added as antioxidants; however, these agents interfere with the determinations of the total phenol content based on redox reactions (Fuleki and Francis 1968; Hong and Wrolstad 1990b; Froytlog et al. 1998; Prior et al. 1998; Häkkinen et al. 1999a, 1999b, 2000; Degenhardt et al. 2000; Kähkönen et al. 2001; Nyman and Kumpulainen 2001; Gu et al. 2002; Kandil et al. 2002; Slimestad and Solheim 2002; Sun et al. 2002; Kähkönen et al. 2003; Zheng and Wang 2003; Wu and Prior 2005; Kapasakalidis et al. 2006; Koponen et al. 2007).

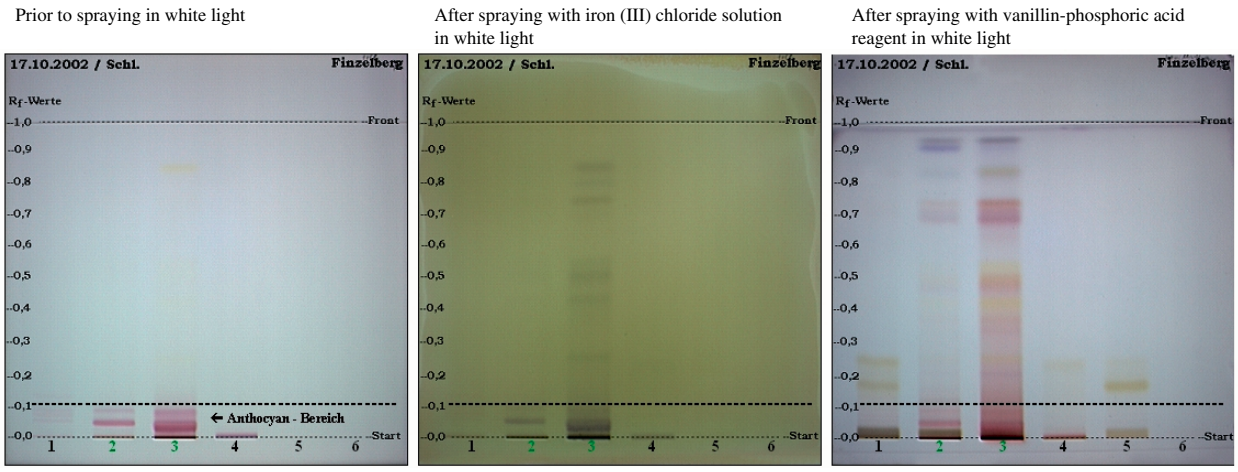
In recent extensive investigations on the optimisation of the extraction of black currants, 70% acetone was identified as the most efficient solvent for anthocyanins, hydroxycinnamic acids and ellagitannins, whereas 60% methanol turned out to be the better solvent for proanthocyanidines and flavonols (Kähkönen et al. 2001).

Although the use of acetone ensures good reproducibility in the quantification of anthocyanins (Kong et al. 2003), formation of artefacts of anthocyanins, i.e. pyranoanthocyanidins or furanoanthocyanidins, may occur to some extent (Wu et al. 2004).

Frequently, the extracts were subjected to immediate analysis after centrifugation. However, for removal of concomitant substances such as sugars, amino acids, proteins etc. from anthocyanin- and/or proanthocyanidin-containing extracts different adsorbents were tested (Kraemer-Schafthalter et al. 1998). Using Amberlite XAD-7, the anthocyanin fraction was eluted with acidified ethanol (Nakajima et al. 2004), and in case of Sep-Pak C-18 cartridges elution was performed with methanol acidified with hydrochloric acid (Watson et al 2004). Sephadex® LH-20 is as well suitable for the purification of anthocyanins (Ichiyanagi et al. 2004) and proanthocyanidines (Gu et al. 2003; Määttä-Riihinen et al. 2005).

3.3. Analytical methods

As polyphenol mixtures in fruits are composed of different substance groups, e.g. flavonoids, anthocyanins, proanthocyanidins, phenolcarboxylic acids etc., the total phenol content is one criterion within quality control. In scientific investigations of berries usually both the total anthocyanin and/or total proanthocyanidin concentrations are quantified, almost exclusively by spectrophotometric methods. These determinations, however, possess a number of shortcomings (see below). The separation of the complex mix-



Position	Name of sample
1	Lingonberry – capsules
2	Lingonberry – spissum extract (primary extract)
3	Lingonberry – dry extract (secondary extract)
4	Lingonberry – lozenges
5	Lingonberry – capsules
6	Lingonberry – granulate

Fig. 4: TLC fingerprint chromatograms for proanthocyanidins in lingonberry extracts and the respective preparations (stationary phase: silicagel F254; mobile phase: ethyl acetate-water-anhydrous formic acid 100+40+10)

tures is preferably performed with chromatographic methods. Thin layer chromatography allows fast and simple qualitative comparisons of extracts and preparations on the basis of the anthocyanin or proanthocyanidin fingerprint (see Figs. 3 and 4). The "state of the art" for the separation and quantification of anthocyanins and oligomeric proanthocyanidins in berries is HPLC.
Below, the advantages and disadvantages of the different methods, including their significance, are summarized.

3.3.1. Total phenol content

Despite a number of disadvantages, the assay of the total phenol content in the above-mentioned berries is usually performed with the method according to Folin and Ciocalteau. Reaction of the phenols with sodium wolframate and sodium molybdate leads to the formation of blue polyphenol complexes, which are determined spectrophotometrically. The mechanism of reaction is not yet fully understood. Additionally, precipitations and interferences with non-phenolic substances may occur (Schofield et al. 2001), especially with oxidizable concomitant substances, e.g. ascorbic acid (Rohr et al. 2000). The content almost exclusively is declared as gallic acid equivalents (Costantino et al. 1992; Kähkönen et al. 2001; Prior et al. 2001; Schofield et al. 2001; Moyer et al., 2002; Sun et al. 2002; Katsube et al. 2003; Zeng and Wang 2003; Bermudez-Soto and Tomas-Barberan 2004; Wu et al. 2004; Kapasakalidis et al. 2006).

3.3.2. Total anthocyanin content

The most simple assay is based on the determination of the total anthocyanin content by measurement of the absorption at a wavelength between 490 and 550 nm, where all anthocyanins show a maximum. By this method, however, degradation products produced by browning reactions are co-determined and lead to false results for the anthocyanin content.
Therefore, for the analysis of anthocyanins in different foodstuffs, such as berries, the *pH difference method* should be preferred (Bronnum-Hansen and Hansen 1983; Hong and Wrolstad 1990a; Costantino et al. 1992; Strigl et al. 1995; Abuja et al. 1998; Prior et al. 1998; Prior et al. 2001; Moyer et al. 2002; Zheng and Wang 2003).
By this method the absorption of the extract solution is measured at pH 1 (anthocyanins as coloured oxonium salts) as well as at pH 4.5 (anthocyanins as colourless hemiketals). Thus a falsification of the total anthocyanin content by browning products, which are coloured at pH 4.5, is avoided. However, due to incomplete release of the anthocyanin monomers, the results of these measurements are sometimes too low (Prodanov et al. 2005).
As the berries contain mixtures of different anthocyanins, calculation of the concentration of anthocyanin monomers is usually based either on the molecular weight of the main component of the examined material or on the molecular weight of cyanidin-3-*O*-glucoside, the anthocyanin with the widest distribution in plant kingdom. For all quantifications the molecular weight (MW) and the molar extinction coefficient (ε) underlying the calculation should be given, because the differences in the molecular weights

Table 3: Total anthocyanin concentration (in %) of different extracts, calculated as delphinidin or malvidin

Extract	% calculated as delphinidin	% calculated as malvidin
Blueberry extract	26,0%	55%
Aronia juice concentrate	3,4%	10%
Elderberry concentrate	5,4%	17%

of the anthocyanins and the influence of the solvent on ε considerably distort the results (Wrolstadt et al. 2002). For example, the total anthocyanin content calculated as cyanidin-3-*O*-glucoside of berries containing mainly delphinidin glycosides lies significantly below the real value (Kähkönen et al. 2003). Investigations of blueberry, chokeberry and elderberry have shown that calculation of the anthocyanins as malvidin leads to 2 to 3 fold higher results than calculation as delphinidin (Table 3).
In a study of 20 food supplements containing extracts of cranberry, elderberry, chokeberry and blueberry, the total anthocyanin contents obtained with the pH value difference method were in good accordance with the results obtained with an HPLC method, when calculated as cyanidin-3-*O*-glucosides for both methods (Wrolstadt et al. 2002).

3.3.3. Anthocyanin composition

The method of choice for the determination of the content of single anthocyanins in berries is HPLC (see Fig. 5). HPLC analysis and quantification of the anthocyanins in lingonberries, cranberries, chokeberries, black currants, elderberries and blueberries is performed almost exclusively by RP-HPLC on C_{18} phases, mainly endcapped materials, under gradient elution. Suitable mobile phases are aqueous acids and acetonitrile, methanol or tetrahydrofuran, the acid components being e.g. formic acid, acetic acid, phosphoric acid or trifluoroacetic acid (Bronnum-Hansen and Hansen 1983; Oszmianski and Sapis 1988; Hong and Wrolstad 1990a, 1990b; Goiffon et al. 1991; Krawczyk and Petri 1992; Strigl et al. 1995; Inami et al. 1996; Froytlog et al. 1998; Watanabe et al. 1998; Häkkinen et al. 1999a, 1999b, 2000; Degenhardt et al. 2000; Chandra et al. 2001; Kähkönen et al. 2001; Malien-Aubert et al. 2001; Määttä et al. 2001; Nyman and Kumpulainen 2001; Prior et al. 2001; Gu et al. 2002; Slimestad and Solheim 2002; Yan et al. 2002; Kähkönen et al. 2003; Määttä et al. 2003; Zheng and Wang 2003; Bermudez-Soto and Tomas-Barberan 2004; Seeram et al. 2004; Oszmianski and Wojdylo 2005; Wu and Prior 2005; Kapasakalidis et al. 2006; Seeram et al. 2006, Koponen et al. 2007).

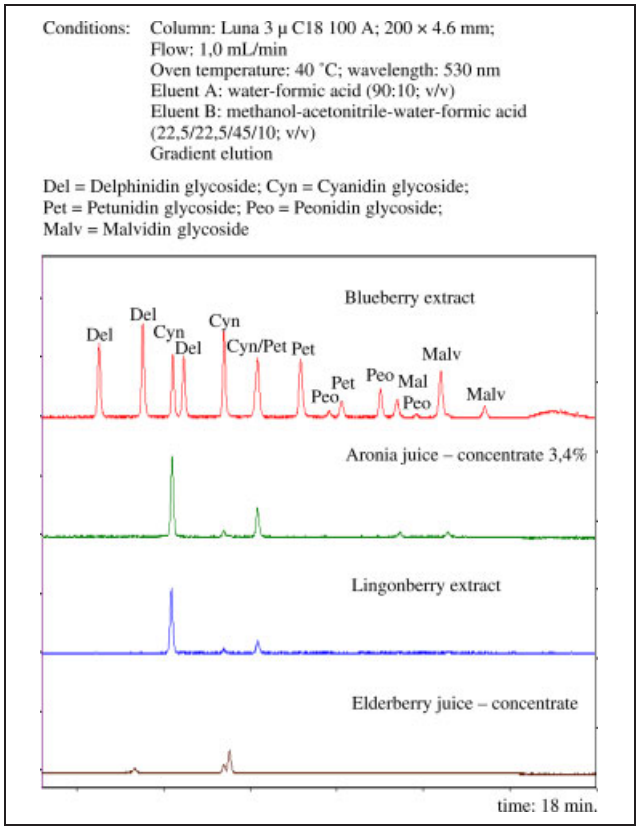


Fig. 5: Examples of HPLC fingerprint chromatograms of blueberry and lingonberry extracts as well as aronia and elderberry juices

In few studies the assay was performed by ion pair chromatography using ammonium dihydrogenphosphate/*o*-phosphoric acid buffer with acetonitrile as mobile phase (Häkkinen et al. 1999a, 1999b; Kähkönen et al., 2001).
For the detection of the anthocyanins, besides diode array detection, mass spectroscopy has been established as "state-of-the-art" in recent years (Nakajima et al. 2004; Wu and Prior 2005; Wu et al. 2006; Koponen et al. 2007).
Despite the ionic structure of the anthocyanins until now capillary electrophoresis has not been used frequently for the analysis of the above-named berries: For the separation of the anthocyanins from currants, blueberries and cranberries sodium phosphate buffers, partly under addition of cyclodextrin, were applied (Da Costa et al. 1998; Ichiyanagi et al. 2004; Watson et al. 2004). Micellar electrokinetic capillary chromatography (MECC) using sodium dodecylsulphate in phosphate borate buffer was applied for the analysis of the anthocyanins in elderberry (Watanabe et al. 1998).

3.3.4. Total proanthocyanidin content

Different colourimetric methods are used for the quantification of the total proanthocyanidin content in herbal material:
In the *proanthocyanidin assay* (acid butanol assay) acid treatment in butanol leads to autoxidative cleavage of the proanthocyanidins to anthocyanidins. The formation of the coloured monomers depends on numerous parameters, e.g. the substitution pattern and degree of polymerisation of the analytes, concomitant substances, water content in the reaction mixture, temperature, solvent, presence of oxidants etc. Reproducible results can only be expected for purified proanthocyanidin fractions with low concentrations of concomitant substances and under respective calibration (Rohr et al. 2000; Schofield et al. 2001).
Condensation of resorcin- or phloroglucin partial structures of flavonols with vanillin in acidic medium leads to the formation of coloured carbonium ions in the *vanillin assay.* This method of quantification is also very susceptible to external influences, especially differences in temperature and water in the solvent. The reaction time strongly depends on the structure of the analytes. The presence of anthocyanins or ascorbic acid in extract solutions from berries is another disturbing factor. Normally (+)-catechin is used for standardisation, in some cases leading to an overestimation of the proanthocyanidin content up to the two-fold (Rohr et al. 2000; Schofield et al. 2001; Cunningham et al. 2002).
The same reaction mechanism as in the vanillin assay is used in the *dimethylamino-cinnamicaldehyde assay* (DMACA assay), in which only the terminal units of the proanthocyanidins react with the reagent. Therefore, the molar extinction coefficients of oligomeric and polymeric procyanidins are very similar to those of the monomers. This fact readily leads to underestimation of polymers. Interferences with concomitant substances in the extracts are, however, less frequent than in the vanillin assay. Besides higher specificity further advantages of the DMACA assay are better sensitivity and much less sensitivity to

temperature and consequently an easier handling. Nevertheless, the determination of polymeric proanthocyanidins may be accompanied by undesirable precipitations (Prior and Gu 2005). This method also mainly uses (+)-catechin as a reference standard.
Numerous different protocols for the implementation of the method were published, each of them requiring adaptation to and validation for the respective sample material. For cranberries, pre-purification of the extracts or juices over Sephadex® LH 20 has proven to be very supportive; anthocyanins and flavonols are removed with water or 50% ethanol. The proanthocyanidin fraction is then obtained with 80% acetone and determined by spectrophotometry after DMACA treatment. With these steps interferences in the quantification of proanthocyanidins are minimised (Cunningham et al. 2002).

3.3.5. Proanthocyanidin composition

The main problems in the analysis of the proanthocyanidin composition in plant material are caused by the fact that plants contain complex mixtures of structurally different substances with differing degree of polymerisation. Usually HPLC is applied, normal phase HPLC provides separations depending on the polymerisation grade. Separations of the groups of oligomers up to the maximum of decamers have been described. Depending on the sample material however, significant peak broadening and overlapping may occur, leading to baseline-shifts, which complicate quantification (Gu et al. 2002). Despite these facts, separation of the oligomer groups as well as separation of the A-type and B-type proanthocyanidins within these groups could be achieved by the use of a special stationary phase and fluorescence as well as MS/MS detection (Gu et al. 2003).
Up to date only very few data are available for proanthocyanidin contents in the above mentioned berries (Gu et al. 2004; Määttä-Riihinen et al. 2004; Wu et al. 2004). The highest total contents were determined in chokeberries, the lowest in elderberries; the percentage of high molecular proanthocyanidins (polymerisation grade > 10) ranged between 55% and slightly over 80% of the total content. In elderberries high molecular proanthocyanidins were detected only marginally (Prior and Gu 1005).
The problems mentioned above related to the separation of proanthocyanidin groups, i.e. peak broadening, overlapping, etc. also complicate the determination of single proanthocyanidins by RP-HPLC. The single components may be separated up to trimers or at most tetramers. In recent years, characterisation of the single substances usually is performed by LC-MS-MS, whereas for quantification purposes diode array detection is preferred (Määttä-Riihinen et al. 2005). For the chromatographic behaviour of the low molecular proanthocyanidins the degree of polymerisation is less important than the stereochemistry, the substitution pattern in ring B and the polarity as well as the composition of the solvent.
Proanthocyanidins can also be examined by RP-HPLC on end-capped C-18 columns after thiolysis with benzylmercaptan (Gu et al. 2002; Oszmianski and Wojdylo 2005). During thiolysis only the terminal unit is released as free flavan-3-ol. The other sub-units of the proanthocyanidins are subsequently separated as the corresponding benzyl ethers by HPLC and quantified. Thiolysis, however, does not proceed fully quantitative (Santos-Buelga and Scalbert 2000; Schofield et al. 2001). The method serves for the analysis of proanthocyanidins in foods, for the compilation of a special proanthocyanidin database (Prior and Gu 2005).

4. Conclusions

Nowadays, numerous herbal food supplements containing preparations from different berries can be found in the market. Due to insufficient declaration and the described problems in analysis, consumers can hardly distiniguish between preparations and assess their quality. Standards for uniform declaration should be implemented in order to increase transparency concerning these food supplements and to facilitate the evaluation of the consumers. Thus, at least the determination of the total anthocyanin and/or total proanthocyanidin content of berry preparations with the two most robust methods is desirable.
Within quality control of monopreparations containing extracts of elderberries, chokeberries, black currants or blueberries, the total anthocyanin content should be determined with the *pH difference method* and calculated as the main anthocyanin of the respective plant material.
In case of combination products, as a compromise, cyanidin-3-*O*-glucoside, the anthocyanin with the widest distribution, should serve as the basis for calculation.
As type-A proanthocyanidins in lingonberries and cranberries evidentially contribute to the effects on the urinary tract, assay of the total proanthocyanidin content via *dimethylamino cinnamicaldehyde assay* (DMACA assay) should be required for the respective preparations.
For the qualitative and quantitative determination of the composition of the anthocyanin complex in mono- as well as in combination products, HPLC analysis on "end-capped" C_{18}-phases under gradient elution with aqueous acid and acetonitrile or methanol as mobile phase is indispensable.
Furthermore, fingerprint comparison with HPLC or TLC provides fast and valuable information on the quality of the berry products.

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