BUDAPESTI CORVINUS EGYETEM

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CHARACTERIZATION OF THE ANTIOXIDANT CAPACITY AND DETERMINATION OF SOME ANTIOXIDANT COMPOUNDS IN BERRY FRUITS

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1. INTRODUCTION

The healthy and well-balanced diet can play a significant role in the prevention of diseases associated with free radical-reactions. Comparing fruits and vegetables with different antioxidant assays, berries proved outstanding due to their high contents of vitamins and especially polyphenolic compounds.

Demand for the accurate determination of antioxidant capacity is gaining importance in most areas within the food industry; therefore several analytical methods and measuring systems have been developed. Most researchers use not only one, but more different methods for determining the antioxidant capacity of a given fruit or vegetable sample as each method is specific for certain antioxidants or for certain reactions.

According to above mentioned it is important and reasonable to get to know and to investigate the antioxidant characteristics of berry fruits grown in Hungary and get to know the quality and quantity of the compounds that play significant role in developing the antioxidant capacity.

2. OBJECTIVES

The outstanding antioxidant capacity of berry fruits is well-known on the strength of data of several international literatures. Contributing to this issue the aims of my research work were to assess and compare the antioxidant capacity of the berry species and cultivars grown in Hungary at the same conditions with special consideration to the compounds that are responsible for the certain differences.

1. My primary aim was to characterise comprehensively the current antioxidant capacity of the investigated fruits with the methods based on different principles (electron transfer, hydrogen transfer) or on the elimination of different free-radicals (DPPH, ABTS, superoxid-anion, hydroxyl radical). Moreover the connection and correlation of the results were also assessed.
2. Another important aim was to measure the ascorbic acid content and to determine the quantity and the quality of the polyphenolic compounds (including anthocyanins, phenolic acids, flavanols [epicatechin and catechin]) that can account for the different results given by the various antioxidant measuring methods.
3. One of my objects was the exploration of the relation between the results obtained by the applied antioxidant capacity methods and the quantity of the determined compounds.

4. In addition, the characterization of some standard antioxidant compounds and different berries in terms of their superoxid-anion scavenging ability was carried out by using a relatively new, commercially available instrument (Photochem, Analytic Jena) measuring based on photochemiluminescence. Within this issue the aims were to get to know the advantages and disadvantages of the applied method, and to test the usability of this tool as there is lack of literature data.

3. MATERIALS AND METHODS

The following berry fruit species and cultivars were investigated during the experiments: strawberry (*Fragaria x ananassa* DUCH.), 'Elsanta', 'Honeoye', 'Onebor', raspberry (*Rubus idaeus* L.), 'Malling Exploit', 'Fertődi Zamatos', 'Glen Ample', red currant (*Ribes rubrum* L.), 'Rondom', 'Detvan', 'Jonkheer van Tets' and black currant (*Ribes nigrum* L.), 'Fertődi 1', 'Fertődi 11', 'Titánia' and 'Otelo'. The samples derived from the same production site (Agárd, 2008) and their growing conditions were also the same. Lyophilised samples were used in all measurements.

The lipid-soluble (ACL) and watersoluble (ACW) antioxidant capacity of berry fruits and some standards (ascorbic acid, citric acid, gallic acid and trolox) were determined by a Photochem instrument (Analytic Jena, Germany). This photochemiluminescence method was described by Popov and Lewin (1994, 1996). The total scavenger capacity by chemiluminescence way of berry fruits was determined according to the methods of Blázovics et al. (1999) (λ=420 nm). The ferric reducing ability of samples were measured at λ=593 nm (Benzie and Strain, 1996). The total polyphenol content (TPC) was detected at λ=734 nm according to the describing of Singleton and Rossi (1965). The elimination of ABTS radical was tested at λ=734 nm according to a spectrophotometric (TEAC) method of Stratil et al (2006). The inhibition of DPPH radical was measured at λ=517 nm (Hatano et al., 1988).

The total anthocyanin content (Giusti and Wrolstad, 1996) was determined spectrophotometricaly (λ=520 and λ=700 nm), the vitamin C content was measured on the basis on the developed method of Engel et al (2010). The characteristic phenolic acids (4-hydorxi-benzoic acid, t-cinnamic acid, p-cumaric acid, vanilic acid, gallic acid, caffeic acid, ferulic acid, siringic acid, sinapic acid and ellagic acid) and the quantity of catechin and epicatechin flavanols were determined by chromatographic technique, with standard addition (Harbaum et al, 2007). The mono- and diglycosides of berry fruits were investigated by nontarget HPLC-ESI-MS/MS three-step method applying MRM-Fullscan-EPI modes (Rak et al., 2010). The following standards were used: apigenin, daidsein, fisetin, genistein, hesperetin, kaempherol, quercetin, luteolin, myricetin,

naringenin. To the evaluation of data one-point variance analysis, correlation analysis and Tuckey's test were used. In connection with the flavonoid profile principle component analysis was used applying the program of Statistica 6.0 Stat Soft.

4. RESULTS

4.1. Testing the Photochem instrument

The following results and inferences can be concluded on the basis of the data measured by Photochem instrument.

The ascorbic acid was tested in ACW mode, in which the ascorbic acid was measured as a sample and the results were calculated on the reference ascorbic acid standard. To carrying out this test the work solution was diluted. The dilution factor was varied and the measured quantity of the sample was also changed. Applying this procedure the 1000 µg ascorbic acid was determined 920, 928, and 976 µg ascorbic acid at 100 times dilution, and 1017, 1042 and 1009 µg at 200-times dilution. In these cases the differences are below 10 per cent so it can be negligible. If the dilution factor is bigger (600 times) the differences are increased with 34-46, 5 % referred to the 1000 µg to the theoretical value. Namely the dilution has great effect to the obtained results. This shows that the samples should be measured in different volumes and with different dilution factors. The results show in spite of that the software is calculated on the differences between the lag phases the results do not agree with the lag phases derived from the different sample volumes. If the shape and the „run" of the lag phases are different the inflexion point of the curves are also different that can cause huge differences. In ACL mode there is similar problem. Ascorbic acid gives signal in ACL mode, therefore the software can calculate antioxidant capacity referred to trolox. This means that in ACL mode the results are not the capacity of the lipidsoluble antioxidants but also the capacity of some water soluble antioxidants; more punctually this capacity is methanol soluble antioxidant capacity. If the sample preparation is made according to the protocol, namely the sample is solved in methanol, then metanolsoluble-watersoluble compounds contribute to the results. On the other hand carotenoids can not solve in methanol. In ACL mode the shape of the curve is decisive, because it affects the obtained values. The dilution factor is the primer effect that can determine the results. The bigger the dilution factor the bigger the error. In the case of unknown sample it can cause serious problem and the suitable concentration rage can be found only with many measurements. In this mode also the sample volume has effect to the results like in ACW mode. In

the case of 10 µl sample the differences were above 30 %, whilst in the case of 20 or 30 µl the differences were below 16 %. An increase in the dilution factor apparently increased the antioxidant capacity.

4.1.1. Observations based on the measurements by Photochem instrument

1. The instrument was developed for measuring water and lipidsoluble antioxidant capacity

Water is offered for watersoluble- and methanol is offered for lipidsoluble antioxidant capacity measurements. There is a problem in lipidsoluble antioxidant measurements. The methanol that has to be used according to the protocol of the instrument, can soluble the watersoluble compounds. Therefore unknown quantity of watersoluble compounds contributes to the obtained results/values. Other solvent can not be used because it can cause change in the inner of the instrument that is not guaranteed by the manufacturer. I can advise the use of "metanolsoluble antioxidant capacity" naming.

2. The software can calculate automatically the antioxidant capacity based on the lag phase and the integrated area using the signal curve of the blank and the calibration curves.

The software provides data automatically if there is inflexion point of the investigated lag phase or there is curve among the blank and the calibration curves. Problems appeared if the curves are not „normal" for example in watersoluble antioxidant capacity measurement. It is quite common if the sample is complex or too concentrated. In lipidsoluble antioxidant capacity there is curves also in too diluted sample, and therefore the software can calculate area of the signal curve. If it is multiple with the dilution factor the antioxidant capacity referred to trolox will be false and overestimated.

3. The instrument is suitable for rutin measurements of complex samples.

According to the above observations the instrument is not the most suitable tool for rutin measurements as there are problems even the measurements of standards. By the complex samples the matrix effect has to be also accounted for besides the problems of the not suitable dilution and the not suitable concentration range. In case of unknown samples it is time-consuming and also costly.

4. Working with the kits is simple and comfortable.

The system does not contain an autosampler. Measuring with this instrument is time-consuming and costly therefore it can not be recommended for routine analyses.

4.2. Results related to the berry fruits

4.2.1. The antioxidant capacity of berry fruits, correlations among the applied methods

13 cultivars of the four berry fruit species (**strawberry**: 'Elsanta', 'Honeoye', 'Onebor', **raspberry**: 'Malling Exploit', 'Fertődi Zamatos', 'Glen Ample', **red currant**: 'Rondom', 'Detvan', 'Jonkheer van Tets', **black currant**: 'Fertődi 1', 'Fertődi 11', 'Titánia', 'Otelo') were investigated by different antioxidant capacity methods.

According to the results obtained the berry fruits grown in Hungary have high antioxidant capacity measuring with different methods (different principles, different free radicals). Generally it can be stated that there are significant differences among the species and the cultivars. Black currant has higher antioxidant capacity (2-8 times) than the other investigated fruit species (red currant, raspberry, and strawberry) but the order among species is method dependent. Measuring with TEAC method the differences are only 30-40% amongthe other species and the black currants. In TRSC method there are no significant differences among the raspberries and red currants. The different order is due to that the species and cultivars contain compound in different quantity and quality that behave differently in the tests.

The correlations among the methods were significant ($p<0,05$) in several cases. There are strong correlations among the water-, and lipidsoluble antioxidant capacity and the results obtained by the elimination of hydroxyl, DPPH, ABTS radical and the ferric reducing ability. The explanation is probably that the polyphenols of berry fruits are decisive in developing the antioxidant capacity and their behaviour are similar against Fe^{3+}, Folin-Ciocalteu reagent, DPPH and hydroxyl radical. Among the TRSC and the other test there is negative correlation that is due to the different principle of the evaluation of data.

The correlations among the results obtained by the FRAP and TPC method ($r=0,761$) and among FRAP and TEAC ($r=0,730$) are significant. The tendencies of the results obtained by FRAP and TPC are the same in strawberry and raspberry cultivars but the results are outstanding in'Rondom' red currant and in'Titánia' black currant.

4.2.2. Determination the quantity of the compounds playing role in the development of the antioxidant capacity

In the case of vitamin C content the strawberry had also outstanding values besides the black currants. The red currant and raspberry cultivars gave lower values. The differences among the cultivars are 20-30%, among the species are double-threefold.

In black currants 3-5 times higher total anthocyanin could be detected then the other species. ‘Otelo’ possessed the most anthocyanin, while ‘Rondom’ red currant possessed the lowest value.

According with the literature data the ellagic acid and para-cumaric acid are dominant in strawberries but their quantities among cultivars are significantly different. These two acids are responsible for approximately the 75 % of the total investigated acids. The ratio of these two acids is the most spectacular: the ratio of ellagic acid: para-cumaric acid is 1:1 in ’Onebor’ 4:1 in Elsanta, and 2,5:1 in ’Honeoye’. Besides these acids the 4-hydorxi-benzoic acid, t-cinnamic acid and gallic acid were decisive. ’Elsanta’ is the richest in gallic acid and ellagic acid that consists of two gallic acid, which is outstanding antioxidant in tests. In strawberries the starting metabolites of the phenolic acid metabolite way are decisive.

On the basis of my measurements the raspberries have high ellagic acid content, besides the ferulic, gallic, and caffeic acid that were also observed other researchers. These acids are responsible for the 75 % of the investigated acids in raspberries. Within the investigated fruits raspberries contain the highest ferulic acid. Their 4-hydoxi-acid, cinnamic acid, and p-cumaric acid content are lower than in strawberries but their sinapic acid content is decisive. According to these measurements the more complex compounds (ferulic acid, sinapic acid) are dominant in raspberry on the other hand their ratio of the gallic acid/ ellagic acid shows that their non-condensed gallic acid ratio is higher.

In red currants the 4-hydroxi-benzoic acid is the dominant acid as it gives more than 1/3 part of the investigated phenolic acids. In the investigated cultivars the ratio of the more complex (hydroxylated) gallic acid is lower than in strawberry or raspberry cultivars. The ellagic acid is decisive only in ’Rondom’ cultivars. In red currant the starting metabolites of the phenolic acid metabolite way are decisive as the t-cinnamic acid, p-cumaric acid and caffeic acid could be detected in higher quantity.

In black currants 4-hydroxi-benzoic acid, p-cumaric acid, gallic acid and the caffeic acid are dominant, but the t-cinnamic acid, the sinapic acid, and the ellagic acid are also decisive. The differences among the cultivars are not so spectacular than in the case of strawberry and raspberry cultivars. On the other hand the ratio of the gallic acid:ellagic acid shows significant differences among the investigated cultivars.

The investigated total phenolic acid content was different significantly among the species and cultivars as in red currants its value is below 500 µg in black currant this value is higher than 4000 µg. The values of raspberries and strawberries are varied between 1000-3000 µg referred to 1 g dry matter content.

I had the possibility to determine the quantity of catechin and epicatechin flavanols. The quantity of catechin was below the detection limit in the investigated cultivars whilst the epicatechin content was below 16 µg in all samples.

The following correlation could be found between the antioxidant capacity values and the measured compounds:The correlation among the vitamin C content and the results obtained by the antioxidant test is lower than the correlation among and the total anthocyanin content, total phenolic acid content and the antioxidant capacities. Even though the ascorbic acid is a strong reducing substance there is no strong correlation among its content and the antioxidant capacities based on electron transfer. The following explanation can be accounted for: the other compounds in the sample can „mask" its expected effect. Therefore in berry fruits the vitamin C content plays not the primary role developing the antioxidant capacity but the polyphenolic content is the decisive in it. Polyphenols group consists of numberless compounds that show higher antioxidant capacity together than the vitamin C.

The anthocyanin content shows no significant correlation with the results of TPC method. Its reason can be that the role and the quantity of other polyphenolic compounds can be more decisive. The colourless compounds show stronger correlation that confirmed the previous observation. The quantity of the investigated phenolic acids shows no strong correlation with the results of FRAP and TPC method but the value of the correlation coefficient was high in case of DPPH, ACW and ACL methods.

It is due to that other compounds like phenolic acids, flavonoids etc. that could not be investigated/measured play also role developing the antioxidant capacity. The interferences (sugar etc.) can also be taken into account. 4-hydroxi-benzoic acid, t-cinnamic acid, gallic acid, caffeic acid and sinapic acid show strong correlation with all of the antioxidant capacities obtained by using different methods. Therefore their quantities are important parameter in developing the antioxidant capacity in the investigating methods.

My results confirm that the polyphenols are primarily responsible for the developing the antioxidant capacity in the investigated berry fruits. There were stronger correlations among the results obtained by the antioxidant capacity test and the total anthocyanin and total phenolic acid, and gallic acid, 4-hydroxi-benzoic acid, caffeic acid content than the vitamin C content of the fruits. The primary reason is that these compounds have high antioxidant capacity alone in the applied tests that can key to the number and the position of the hydroxyl groups.

4.2.3. Qualification of the flavonoids playing role in the development of antioxidant capacity

According to my measurements the quantity and also the quality are different in the investigated fruits. These differences arise on the one hand the differences of the concentration of the compounds on the other hand also from the genetic background.

In strawberry kaempherol-glycosides could also be detected besides the quercetin and apigenin-glycosides. In ’Onebor’ the number of confirmed compounds is the highest: 7, whilst in ’Honeoye’ it is only 4. Apigenin could be detected and confirmed in all of the strawberry cultivars.

In the investigated raspberries the quercetin derivates are decisive but in ’Fertődi Zamatos’ naringenin+hexose could also be confirmed. In red currants only quercetin-glycosides could be detected.

Among the black currant cultivars there are huge differences concerning the confirmed flavonoids as in some cases kaempherol, luteolin and naringenin glycosides could be confirmed. The most diverse profile was obtained in this berry fruit species. There were no differences among the ’Fertődi 1’ and ’Fertődi 11’ whilst in ’Titánia’ naringenin glycosides could also be detected, but the kaempherol and luteolin glycosides could not be confirmed. In ’Otelo’ kaempherol and naringenin derivates could not be detected.

The differences are spectacular in the case of ’Fertődi 1’ and ’Titánia’ concerning flavonoid profile. The quercetin aglycon eluated at 25,2 minutes in both cultivars. However in ’Fertődi 1’ there are more 3 peaks (17,2;17,4 and 19,4) whilst in ’Titánia’ there are 4 (17,2;17,4; 17,6 and 19,4) that means one more compounds (quercetin+hexose).

According to my result the differences are due to the quantity of the compounds and the genetic background as the growing conditions were the same. Quercetin and quercetin-glycosides are decisive in the investigated cultivars especially the quercetin+hexose and quercetin+hexose+deoxyhexose.

5. THESIS STATEMENTS

1. I have provided the following novel results regarding the the antioxidant capacity of 13 investigated berries and correlations among the applied methods:

Black currants possess the highest antioxidant capacity against hydroxyl radicals, superoxid anions, DPPH and ABTS free radicals. However, the extent of the differences among the cultivars within the same species are not always detectable by all of the applied methods.

There is significant correlation among the values/ results of lipid- and watersoluble antioxidant capacity assays based on the elimination of superoxid anions, the total scavenger capacity, the ferric reducing antioxidant power, the trolox equivalent antioxidant capacityand DPPH radical scavenging capacity.

2. Concerning the flavonoid profile of the berry fruits:

This is the first study of mapping the flavonoid-derivates of the 4 berry fruit species and their 13 cultivars (strawberry: 'Elsanta', 'Honeoye', 'Onebor', raspberry: 'Malling Exploit', 'Fertődi Zamatos', 'Glen Ample', red currant: 'Rondom', 'Detvan', 'Jonkheer van Tets', black currant: 'Fertődi 1', 'Fertődi 11', 'Titánia', 'Otelo').

I have concluded that there were greater differences among the species than among cultivars in terms of the quantity and quality of their flavonoids. In strawberries, apigenin-, quercetin- and kaempherol-; in raspberries, quercetin- and naringenin-derivates were identified. In red currant cultivars, only quercetin-; while in black currants, kaempherol-, quercetin-, luteolin-, myricetin- and naringenin-derivates could be identified.

This was the first time when apigenin-glycosides could be determined in all of the investigated strawberry cultivars ('Honeoye', 'Elsanta', 'Onebor') by HPLC-ESI-MS/MS.

3. Correlations among the antioxidant capacities of 13 investigated berries and the quantity of compounds contributing to the antioxidant capacity also provided valuable results:

There was no strong correlation among the results of the antioxidant capacity assays and vitamin C content. However, there are strong correlations among the quantities of anthocyanin, 4-hydroxi-benzoic acid, t-cinnamic acid, gallic acid, caffeic acid, sinapic acid and antioxidant capacity.

My measurements are confirmed that the outstanding antioxidant capacity of the black currant is due to gallic acid, myricetin and myricetin glycosides. The accumulation of these compounds is due to the genetic control of the flavonoid biosynthesis pathway.

4. Testing the Photochem instrument for measuring lipid- and water-soluble antioxidant capacities supplied the following observations:

I proved that due to the characteristics of methanol, that has to be used for the determination of lipidsoluble antioxidant capacity (according to the protocol of the supplier), the lipid soluble antioxidant capacity is influenced by watersoluble antioxidants, which presents an addition error.

There are contradictions in the data evaluation protocol elaborated by the manufacturer. In the ACL mode, results obtained by the evaluation of the signal curves converge to the signal curves of the blank, which results in an overestimated antioxidant capacity.

I have experimentally proved that the method is not robust enough as the dilution of the sample has a considerable effect on the results. The antioxidant capacity of the sample measured by the instrument does not change in the ratio of the dilution factor.

6. PUBLICATIONS

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