ACTA UNIVERSITATIS AGRICULTURAE ET SILVICULTURAE MENDELIANAE BRUNENSIS

Volume LX 7 Number 8, 2012

EFFECT OF SAMPLE DILUTION ON ESTIMATED VALUES OF ANTIOXIDANT CAPACITY BY PHOTOCHEMILUMINISCENCE METHOD

P. Híc, J. Balík

Received: September 13, 2012

Abstract

HÍC, P., BALÍK J.: *Effect of sample dilution on estimated values of antioxidant capacity by photochemiluminiscence method.* Acta univ. agric. et silvic. Mendel. Brun., 2012, LX, No. 8, pp. 67–72

This paper describes principles of a proper dilution of samples used for the determination of antioxidant capacity by means of a photochemiluminiscence method using the instrument Photochem (Analytik Jena AG). The authors used the method ACL (Lipid-soluble Antioxidant Capacity), which is one of two methods enabling to measure in this instrument. It was demonstrated that values measured by the photochemiluminiscence method ACL were influenced by the degree of sample dilution. When studying effects of dilution of samples of wine and rutin, it was demonstrated that there is a non-linear correlation between the degree of dilution and measured values of antioxidant capacity. At low molar amount of substance (i.e. at 0.5 and 1.0 nmol), the measured value of rutin inhibition was higher than the same that molar amount of trolox. At higher molar amounts (i.e. 3; 4 and 5 nmol), the inhibition value of trolox was higher. This dependent change can be explained by means of a different effectiveness of antioxidants and a different stability of their products with radicals.

photochem, photochemiluminiscence, sample dilution, antioxidant capacity

There are two reactive forms of oxygen and nitrogen that occur normally in living organisms. These compounds can easily react with otherwise stable molecules and thus damage individual organisms (ŠTÍPEK *et al.*, 2000). In this way they markedly participate in processes of senescence (HARMAN, 1956). An increase in amounts of reactive forms of oxygen and nitrogen can lead to and result in the occurrence of many diseases, e.g. atherosclerosis, cancer, brain attacks, arthritis, heart attacks, liver damage etc. (PACKER *et al.*, 2001), (ZLOCH *et al.*, 2004). At the same time, however, they also can function as signal molecules for the expression of genes and/or as an important component of the hypersensitive reactions occurring within the process of programmed cell death (ĎURAČKOVÁ, 1998). This means that they are indispensable for the existence of living organisms. They can occur both endogenously and exogenously (ŠULC, 2007). The existence of free radicals forced the living organisms to create a defensive system, which inactivates these dangerous free radicals and protects them therefore against an uncontrolled oxidation (LONNROT *et al.*, 1996). The defence of living organisms is based on production of antioxidants. There are many, often very different, definitions of antioxidants. VELÍŠEK (2002), for example, defines antioxidants as compounds that in low concentrations protect foodstuffs against oxidation and increase their storability. As far as the human body is concerned, antioxidant can be defined as molecules that even in small concentrations can prevent (or reduce) negative effects of free radicals and thus prevent the damage of the organism. From the chemical point of view, antioxidants are compounds capable to inactivate free radicals without becoming one of them. Naturally, this antioxidant protection is not absolute and for that reason a certain form of damage can be always expected (AMES *et al.*, 1993).

There are several methods how to determine antioxidants and they all function on different

principles. Due to this fact the obtained results may be rather different and dependent above all on the analytical methods used (PAVLOVÁ *et al.*, 2004; STRATIL *et al.*, 2006). The method of sample processing shows also a great effect on measured values (HÍC, BALÍK, 2008). Different authors published different results (e.g. FERNANDÉZ-PACHÓN *et al.*, 2004; KJERSTI *et al.*, 2005). But not only these factors may influence the measured values. BRENZIE *et al.* (1999) mentioned that reduction capacity of ascorbic acid were directly proportional to its concentration. In case of peroxyl radical trapping tests of total antioxidant capacity the ascorbate stechiometry is dependent just on the sample concentration (WAYNER *et al.*, 1986). The assumption that concentration of antioxidants in the measured sample influences measured values represents another source of variability that influences obtained results and this fact reduces possibilities of a comparison of results published by different authors. In this study we tried to assess the dependence of antioxidant capacity values of rutin and red wine on sample concentrations. Measuring was performed using the Photochem instrument (Analytik Jena AG), which functioned on the principle of photochemiluminiscence. This method was elaborated by POPOV, LEWIN (1996) and it was already used in a number of studies (BALOGHA *et al.*, 2010).

MATERIALS AND METHODS

Preparation of samples and measurement

For assays, red wine of the variety Blaufränkish, vintage 2008, cabinet dry, wine growing region Morava, Czech Republic was used. In this wine, the following parameters were estimated: titratable acids (5.8 g.dm^{-3}), content of alcohol (12.4 vol.%), content of residual sugar (3.8 g.dm^{-3}), and pH (3.45). Prior to measurements, wine was diluted in ratios 1:20; 1:30; 1:40; 1:50; 1:100 and 1:200. The volume of measured sample was always 20 µl. In case of the red wine, the measured total antioxidant capacity value (TAC) was expressed as trolox (mmol.dm^{-3}).

For measurements of the antioxidant capacity of rutin, a stock solution in 75% methanol was prepared with the concentration of 0.5 mmol.dm^{-3}. The following dilutions of this stock solution were used: 1:25; 1:50; 1:100; 1:150; 1:200 and 1:250 µmol. dm^{-3}. These rutin concentrations were measured in the Photochem instrument and the volume of measured samples was always 20 µl.

Estimation of the antioxidant capacity by photochemiluminescence method ACL (Lipid-soluble Antioxidant Capacity) in the Photochem instrument. In the ACL assay, the photochemical generation of free radicals was measured with a sensitive detector by using chemiluminescence and free radicals were produced from the luminol, which worked partly as a photosensitizer and partly as an oxygen radical detection reagent.

The lipophilic antioxidants were measured with the ACL kit. The working solution consisted of the following reagents: methanol (Reagent 1) 2.3 ml; buffer solution (Reagent 2) 200 µl and photosensitizer (Reagent 3) 25µl; these reagents were mixed with a vortex for 20–30 s. Altogether 500 µl of reagent 1 were poured into a vial containing reagent 4 and vortexes. Than the resulting solution was diluted 1:100 with methanol in order to prepare the trolox standard with the concentration of 100 µmol.dm^{-3}. For constructing a calibration curve were used 5; 10; 20; 30; 40 and 50 µl volumes. The TAC value was calculated as a linear dependence of the reciprocal value of antioxidant concentration on the value I^{-1}. The I value (inhibition) was calculated by the Photochem instrument as a ratio of different concentrations of the antioxidant trolox standard to the blank (POPOV, LEWIN, 1996).

Chemicals

Methanol p. a. (Riedel-de Haën)
ACL reagents (2, 3, 4) from (Analytik Jena AG)
Rutin – (Sigma Aldrich), assay : ≥94% (HPLC)

Equipment

PHOTOCHEM instrument (Analytik Jena AG)
Electromagnetic stirrer – IKA MS 3 digital IKA

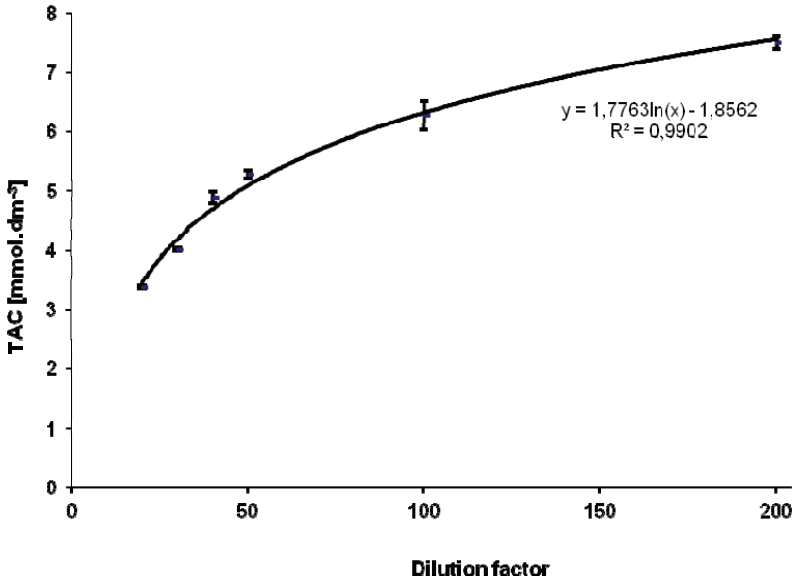
Statistical analysis

Analysed data represented mean values determined in five separate solutions. Analysis of variance (ANOVA) was carried out using the programme package Unistat for Excel 5.1. Significant differences were calculated according to the Tukey's test ($P \leq 0.05$).

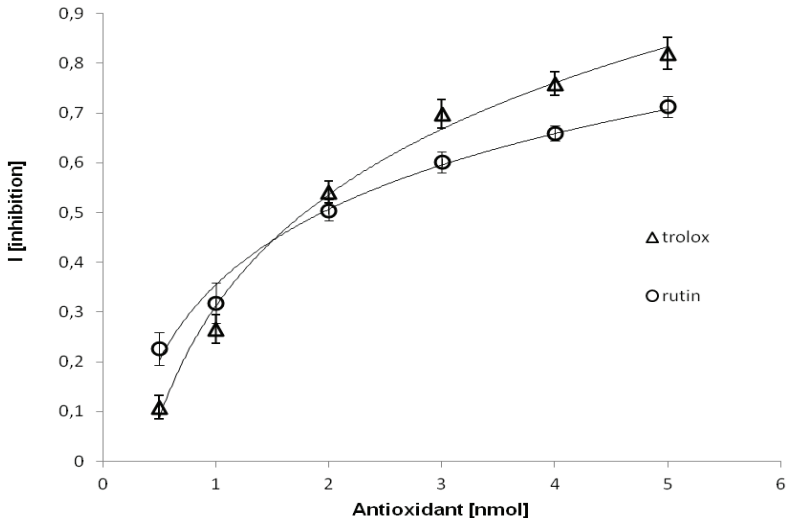
RESULTS AND DISCUSSION

It resulted from the analysis of data obtained in experiments with red wine that the measured values TAC increased in dependence on the increasing degree of dilution of wine samples (Fig. 1). These differences were statistically significant ($P \leq 0.05$) in all variants of dilution under study.

As shown in Fig. 1, at the dilution of red wine 1:20, the resulting antioxidant capacity was 3.38 ± 0.03 mmol of trolox.dm^{-3}. At the dilution 1:50, the measured value was 5.28 ± 0.02 mmol of trolox.dm^{-3} and at the dilution 1:200, the result was as much as 7.49 ± 0.11 mmol of trolox.dm^{-3}. The effect of dilution was not of random character; quite on the contrary, its course was relatively exact and could be expressed as a logarithmic curve ($R^2 = 0.9902$). When these measured values were evaluated as calibration points (similarly as in case of the trolox standard), it was possible to obtain an equation that was very similar to that for the trolox standard. The comparison of wine parameters with trolox calibration curves was not possible due to the fact that trolox units were expressed in nmol.dm^{-3} while those of wine were expressed in millilitres



1: *Effect of wine dilution on values TAC measured by means of the ACL method (n = 5; P ≤ 0.05)*



2: *A comparison of inhibition value of trolox and rutin depending in dependence on molar amount of substance (n = 5; P ≤ 0.05)*

(dilution factor). In the course of monitoring effects of rutin dilution on measured values of inhibition, the calibration series of trolox were compared with calibration data concerning rutin solutions (Fig. 2). Experimental results indicated a different course of calibration points even if the same molar concentrations were used. At low molar amount of substance (i.e. at 0.5 and 1.0 nmol), the measured value of rutin inhibition was higher than the same that molar amount of trolox. At higher molar amounts (i.e. 3; 4 and 5 nmol), the inhibition value of trolox was higher. A statistic analysis of the interaction existing between the effect of dilution and antioxidants under study indicated that one and the same dilution could influence on inhibition value under study in a different manner. POPOV, LEWIN (1996), who directly evaluated this method and its suitability for the estimation of antioxidant capacity, mentioned that the most exact result could be obtained in case that the value of inhibition value ranged from 0.5 to 0.7. This is a very strictly specified value that must be often obtained by means of a different solution of compared samples.

One of possible explanations why the antioxidant capacity estimated by means of the photochemiluminiscence method in the Photochem instrument was dependent on the antioxidant concentration is based on the Guldberg-Waage law. This law expresses the magnitude of the reaction. Reactions of radicals and antioxidants gave relatively stable products. The difference between these products and radicals consists probably only in stability. Regarding the possibility that the magnitude of the reciprocal reaction of radical-

antioxidant products in case of trolox and rutin, it can be expected that reactions taking place in the course of dilution will be different in the inhibition values (Fig. 2).

BENZIE *et al.* (1999) mentioned that when using photochemiluminiscence in assays of ascorbic acid, the magnitude of the response was dependent on concentration and efficiency of the antioxidant. Differences in measured values of antioxidants under study could be caused also by a decreasing response of the instrument used for these measurements (BALOGHA *et al.,* 2010). WAYNER *et al.* (1986) studied also the dependence of antioxidant effects of ascorbic acid on its concentration. HENGST (2009) wrote about a non-linear behaviour of the photochemiluminiscence method in measurements of a calibration series of gallic acid. He also mentioned that the non-linear dependence of measured values on concentrations had not been adequately explained yet. The theoretical assumption that the measured values of the antioxidant capacity are influenced by the antioxidant concentration and the magnitude of the reciprocal reaction could be corroborated also by means of a mathematical model construction that would exactly describe the measured values. However, the construction of such a model is rather complicated due to an insufficient description of processes taking place in the Photochem instrument.

CONCLUSION

It was demonstrated that values measured by the photochemiluminiscence method were influenced by the degree of sample dilution. When studying effects of dilution of samples of wine and rutin, it was confirmed that there is a non-linear correlation between the degree of dilution and measured values of antioxidant capacity. The effect of dilution was not of random character; quite on the contrary, its course was relatively exact and could be expressed as a logarithmic curve. This behaviour could be probably explained by means of differences in the magnitude of the reverse decomposition of products of the reaction antioxidant-radical.

SUMMARY

When measuring the antioxidant capacity by means of photochemiluminiscence method in the Photochem instrument it is important to use constantly the same method of estimation and to describe the preparation of samples in detail. Of the same importance, however, is also to mention dilutions used for the assayed material because the results may be influenced by the degree of dilution. For that reason it is not possible to compare samples that were diluted in different ratios. When studying the effect of wine and the antioxidant rutin dilutions on measured values, it was demonstrated that the dependence of results on the degree of dilution was non-linear. In red wine, the antioxidant capacity increased due to the degree of dilution.

When comparing rutin with the calibration trolox solution, it was demonstrated that at low molar amount of substance (0.5 and 1.0 nmol) the measured values of rutin inhibition were higher than the antioxidant capacity of a trolox solution with the same molarity.

At higher molar amount of substance (3; 4 and 5 nmol), the inhibition value of trolox was higher. This behaviour could be probably explained by means of differences in the magnitude of the reverse decomposition of products of the reaction antioxidant-radical. In case that this assumption would be correct, it could be possible to compare the efficiency of antioxidants also on the base of reverse reactions of individual antioxidants. This means that important are not only antioxidant capacity of individual compounds but also the stability of final products and the capacity to prevent their decomposition to radicals and antioxidants. A detailed description of antioxidants and stability of products occurring after their reaction with radicals could further contribute to our knowledge in the field of antioxidant research.

Acknowledgment

This research was supported by Agency of the Ministry of Agriculture of the Czech Republic (Grant No. QI91B094).

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Address

Ing. Pavel Híc, doc. Ing. Josef Balík, Ph.D., Ústav posklizňové technologie zahradnických produktů, Mendelova univerzita v Brně, Valtická 337, 691 44 Lednice, Česká republika, e-mail: pavel.hic@mendelu.cz, balikj@zf.mendelu.cz