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EVALUATION OF THE METHODS FOR DETERMINATION OF THE FREE RADICAL SCAVENGING ACTIVITY BY DPPH

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Abstract

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The survey of the methods for determination of free radical scavenging activity by DPPH has been done. The differences between methods conditions and their evaluation are presented. It was determined the effect of methods conditions by ruggedness testing of methods. It was specified that the accuracy of the method for determination of free radical scavenging activity is effected by the solvent used (ethanol or methanol) and the sample/reagent DPPH volume ratio. The coefficient of variation of the method with ethanol is twice lower that the respective one determined with using of methanol. The calibration curves with ascorbic acid (Vitamin C) and α-Tocopherol (Vitamin E) and solvent ethanol and methanol were plotted. They are characterized with very high regression coefficients. Based on the analysis and evaluation of the methods, the results of ruggedness testing of methods, coefficient of variations of determination with solvent ethanol and methanol and recommendations of some authors it was proposed modification of the method for determination of free radical scavenging activity of beer and beverages with DPPH. The modification of the method includes: 0.06 mM solution of DPPH in ethanol, reaction mixture 1.5 ml diluted sample and 1.5 ml DPPH solution, 30 minutes time of reaction in dark, measurement of absorbance at 517 nm, presentation of the results as equivalent of Vitamin C antioxidant activity. It was investigated the effect of malt and hops on the antioxidant activity of wort and beer. It was established that the main free radical scavenging activity of beer is attributed by the malt used. The hopping increases additionally the values of the parameter. During the different stages of the brewing process the free radical scavenging activity is changed. The differences between the free radical scavenging activity of laboratory and production beers indicated the very important role of raw materials and technology used. The free radical scavenging activity of beers determined by ethanol is higher (an average 8,2 - 38.9 % for the used beer samples) than the values obtained by solvent methanol.

Key words: method DPPH, modification of method, ethanol, methanol, malt, beer

Abreviations: DPPH - 2,2-Diphenyl-1-picrylhydrazyl, BHA – Butylated xydroxy anisole, BHT – Butylated hydroxy toluene, EVCAA – equivalent vitamin C antioxidant activity, EVEAA - equivalent vitamin E antioxidant activity, FRSA – free radical scavenging activity, EBC – European Brewery Convention

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Introduction

There is great number of methods for determination of antioxidant capacity of foods and beverages based on different principles: peroxyl radical scavenging (Oxygen Radical Absorbance capacity, ORAC); Total Radical-trapping Antioxidant Power (TRAP); metal reducing power (Ferric Reducing Antioxidant Power, FRAP); Cupric Reducing Antioxidant Power (CUPRAC); hydroxyl radical scavenging (deoxiribose assay); organic radical scavenging (2,2-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid, ABTS); 2,2-Diphenyl-1-picrylhydrazyl, DPPH); quantification of the products formed during the lipid peroxidation (Thiobarbituric Acid Reactive Substances, TRAPS); Low-density Lipoproteins (LDLs) oxidation, etc. (Pérez-Jiménez and Saura-Calixto, 2008). The most widely-used procedures for measurement of antioxidant capacity are FRAP, ABTS, TEAC (Trolox equivalent antioxidant capacity) , DPPH and ORAC (Pérez-Jiménez et al., 2008).

The DPPH method is rapid, simple, accurate and inexpensive assay for measuring the ability of different compounds to act as free radical scavengers or hydrogen donors, and to evaluate the antioxidant activity of foods and beverages (Prakesh, 2001). The DPPH method is described as a simple, rapid and convenient method independant of sample polarity for screening of many samples for radical scavenging activity (Marxen et al., 2007). The method DPPH is widely used for measurement of free radical scavenging ability of antioxidants (Perez-Jimenez and Saura-Calixto, 2008; Perez-Jimenez et al., 2008). For determination of radical scavenging activity of different foods, beverages and substrates were elaborated a great variety of methods with utilisation of DPPH (1,1-Diphenyl-2-picrylhydrazyl). They are based on the original methods of Blois (1958) and Brand-Williams et al. (1995). The great diversity of methods and modifications is evident from its different names. It is known many methods using DPPH for determination of: ***the radical scavenging activity or free radical scavenging activity*** (Kumazawa and Nakayama, 2001; Okawa et al., 2001; Pavlov et al., 2002; Yang et al., 2004; Bankeblia, 2005; Kaukovirta-Norja et al., 2005; Kitao et al., 2005; De et al., 2007; Marxen et al., 2007; Aghar and Masood, 2008; Chen et al., 2009; Fukushima et al., 2009; Tabart et al., 2009; Gülcin et al., 2010; Ren et al., 2010; Uddin et al., 2010), ***the antioxidant activity*** (Schlesier et al., 2002; Molyneux, 2003; Potter at al., 2007; Butkhup and Samappito, 2008; Lachman et al., 2008; Belisario-Sanchez et al., 2009; Hua et al., 2009; Amit et al., 2010; Shikanga et al., 2010), the radical scavenging capacity or free radical scavenging capacity (Sanchez-Moreno et al., 1999; Dragović-Uzelac et al., 2007; Ivanov, 2007; Mihalev et al., 2007; Ting et al., 2008; Roy et al., 2010), ***the radical scavenging assay or free radical scavenging assay/method*** (Ismail and Hong, 2002; Liebenberg, 2004; Othman et al., 2005; Wang and Li, 2007; Morais et al., 2009), ***the antiradical activity*** (Brand-Williams et al., 2005; Sroka and Cisowski, 2005; Stoilova et al., 2005; Lahman et al., 2006), ***the antioxidant capacity*** (Freitas et al., 2006; Dvořáková et al., 2008; Perez-Jimenez et al., 2008), ***the DPPH scavenging amount*** (Jing et al., 2008a; Jing et al., 2008b), ***the total antioxidant activity*** (Tarozzi et al., 2004; Singh et al., 2008), ***the DPPH method/assay*** (Prakash, 2001; Kamkar et al., 2010), ***the DPPH scavenging assay*** (Gupta et al., 2007), ***the DPPH test/method*** (Kwon et al., 2003), ***the DPPH radical scavenging effect*** (Kim et al., 2002), ***the radical activity*** (Paulová et al., 2004), ***the free radical scavenging method*** (Qian and Nihorimbere, 2004), ***the decoloration of DPPH radical*** (Silva, 2004), ***the antioxidant content*** (Miller et al., 2000). The most used names of the method DPPH are the free radical scavenging activity and antioxidant activity. The most correct name, which described the mechanism of the reaction, is the first one. Not only the name but the conditions of the described in the literature methods are very different. The substantial differences are in sample preparation, extraction of antioxidants (solvent, temperature,

etc.), selection of end-points and expression of results. That means that the comparison between the values reported by different laboratories can be quite difficult (Pérez-Jiménez et al., 2008).

The goal of this investigation is critical analysis and evaluation of the used methods for determination of the free radical scavenging activity and suitable modification of the method DPPH for application in beer, wine, tee and others beverages.

Literature Review of the methods DPPH

Antioxidant compounds may be water-soluble, lipid-soluble, and insoluble or bound to cell walls (Prakash, 2001). The most utilised solvents for determination of the radical scavenging activity by DPPH are methanol and ethanol. **Methanol** as a solvent is used by Miller at al. (2000); Prakash (2001); Kim et al. (2002); Molyneux (2003); Tarozzi et al. (2004); Qian and Nihorimbere (2004); Benkeblia (2005); Kaukovirta-Norja et al. (2005); Sroka and Cisovski (2005); Lachman et al. (2006); Gupta et al. (2007); Ivanov (2007); Marxen et al. (2007); Mihalev et al. (2007); Butkhup and Samappito (2008); Dvořáková et al. (2008); Lachman et al. (2008); Pérez-Jiménez et al. (2008); Singh et al. (2008); Tabart et al. (2009); Kamkar et al. (2010); Shikanga et al. (2010), while **Ethanol** as a solvent is used by Pavlov et al. (2002); Kwon et al. (2003); Molyneux (2003); Liebenberg (2004); Paulová et al. (2004); Silva (2004); Yang et al. (2004); Kitao et al. (2005); Othman et al. (2005); Stoilova et al. (2005); Agshar and Masood (2008) and Gülcin et al. (2010). It is evident that 22 cited methods used methanol, while 12 prepared the DPPH solutions and samples with ethanol.

The concentration of the DPPH working solution in discussed methods ranges in a wide limits: from **0.05 mM** to **1.5 M** (Kim et al., 2002). Relatively often are used the concentration of **0.10 mM** (Miller et al., 2000; Kwon et al., 2003; Gupta et al., 2007; Asghar and Masood, 2008; Singh et al., 2008; Kamkar et al., 2010; Gülcin et al., 2010; Shikanga et al., 2010) **0.06 mM** (Qian and Nihorimbere, 2004; Lachman et al., 2006; Ivanov, 2007; Mihalev et al., 2007) **0.05 mM** (Ismail and Hong, 2002; Silva, 2004; Kitao et al., 2005; Othman et al., 2007) and **0.09 mM** (Liebenberg, 2004; Tarozi et al., 2004; Sroka and Cisowski, 2005). The DPPH concentration differences lead to the very substantial distinctions in the ratio between volumes of sample and reagent. In literature could be found ratios from **3:1** (Gupta et al., 2007; Gülcin et al., 2010) to **1:600** (Lachman et al., 2006). Almost every method used own volume ratio sample/reagent. The ratio **1:1** were used by five methods (Ismail and Hong, 2002; Molyneux, 2003; Kitao et al., 2005; Othman et al., 2007; Singh et al., 2008) the ration **1:7,5** were used by two methods (Ivanov, 2007; Mihalev et al., 2007), and the ratio 3:1 were used by another two methods (Gupta et al., 2007; Gülcin et al., 2010).

The duration of the reaction of radical scavenging activity between DPPH solutions and sample varied from **1 minute** (Sroka and Cisowski, 2005) to **240 minutes** (Miller et al., 2000; Prakash, 2001). Different authors used **5 min** (Lahman et al., 2006; Tabart et al., 2009), **10 min** (Lahman et al., 2008), **15 min** (Pavlov et al. 2002), **20 min** (Ismail and Hong, 2002; Kitao et al., 2005; Othman et al., 2005; Ivanov, 2007; Mihalev et al., 2007; Singh et al., 2008), **30 min** (Kim et al., 2002; Kwon et al., 2003; Molyneux, 2003; Tarozzi et al., 2004; Yang et al., 2004; Stoilova et al., 2005; Gupta et al., 2007; Wang and Li, 2007; Gülcin et al., 2010; Kamkar et al., 2010), **60 min** (Liebenberg, 2004; De et al., 2007), **90 min** (Asghar and Masood, 2008) and **120 min** (Dvořáková et al., 2008). The most frequently used duration of the reaction is 30 minutes (10 references) and 20 minutes (6 references).

The determination of radical scavenging activity by DPPH is effectuated under different wave lengths. The absorbances of the assays were measured between **492 nm** (Shikanga et al., 2010) and **540 nm** (Liebenberg, 2004). The wavelength **515 nm** were used by Brand-Williams et al. (1995);

Miller et al. (2000); Molyneaux (2003); He and Nihorimbere (2004); Benkeblia (2005); Kaukovirta-Norja et al. (2005); Sroka and Cisowski (2005); Lahman et al. (2006); Ivanov (2007); Mihalev et al. (2007); Dvořáková et al. (2008); Lahman et al. (2008); Pérez-Jiménez et al. (2008), **516 nm** by Molyneaux (2003); Kaukovirta-Norja et al. (2005), **517 nm** by Prakash (2001); Ismail and Hong (2002); Pavlov et al. (2002); Kwon et al. (2003); Molyneaux (2003); Silva (2004); Yang et al. (2004); Kitao et al. (2005); Kaukovirta-Norja et al. (2005); Othman et al. (2005); Paulová et al. (2005); Gupta et al. (2007); Wang and Li (2007); Asghar and Masood (2008); Singh et al. (2008); Tabart et al. (2009); Kamkar et al. (2010); Gülcin et al. (2010), **518 nm** by Molyneaux (2003); Stoilova et al. (2005), **520 nm** by Kim et al. (2002); Molyneaux (2003) and **525 nm** by Tarozzi et al. (2004). It is evident that the most utilised wave lengths for measurement of absorbance are 517 nm (18 references) and 515 nm (13 references).

The radical scavenging activity could be calculated by using different standard solutions. The literature survey indicated that 5 standards were used for expression of the results. **Vitamin C** (Ascorbic acid) is used by Kwon et al. (2003); Molyneaux (2003); Liebenberg (2004); Paulová et al. (2004); Tarozzi et al. (2004); Othman et al. (2005); Lahman et al. (2006); Wang and Li (2007); Asghar and Masood (2008); Lahman et al. (2008); Shikanga et al. (2010). **Trolox** is selected by Miller et al. (2000); Prakash (2001); Liebenberg (2004); Paulová et al. (2004); Silva (2004); Dragović-Uzelac et al. (2007); Ivanov (2007); Mihalev et al. (2007); Tabart et al. (2009). **Vitamin E** (α-Tocopherol) is used by Ismail and Hong (2002); Molyneaux (2003); Silva (2004); Gupta et al. (2007); Marxen et al. (2007); Asghar and Masood (2008). Rarely are used **BHT** (Ismail and Hong, 2002; Asghar and Masood, 2008) and **BHA** (Singh et al., 2008). The most frequently used standards according the literature are ascorbic acid (11 references), Trolox (9 references) and α-tocopherol (6 references).

Last but not least different equations are used by the authors for calculation of the radical scavenging activity by DPPH or inhibition of DPPH which are presented below (in %):

$$A = \frac{Acontrol - Asample}{Acontrol} \times 100;$$

(Bankeblia, 2005; Kitao et al., 2005; Molyneaux, 2003; Pavlov et al, 2002; He and Nihorimbere, 2004; Singh et al., 2008; Wang and Li, 2007; Kamkar et al., 2010)

$$B = \left(1 - \frac{Asample}{Acontrol}\right) \times 100;$$

(Lahman et al., 2008; Lahman et al., 2006; Othman et al., 2005; Gülcin et al., 2010)

$$C = \left[1 - \frac{Asample - Ablank}{Acontrol}\right] \times 100);$$

(Stoilova et al., 2005; Yang et al., 2004)

$$D = 1 - \frac{Asample}{Acontrol} \times 100;$$

(Ismail and Hong, 2002)

$$E = \frac{Asample}{Acontrol} \times 100;$$ (Liebenberg, 2004)

$$F = Acontrol - Asample$$
(Sroka and Cisowski, 2005)

Some authors express the results as EC_{50} (efficient concentration value) – concentration of the substrate that causes 50 % loss of the DPPH activity (colour) (Kim et al., 2002; Kwon et al., 2003; Liebenberg, 2004; Asghar and Masood, 2008; Marxen et al., 2007; Prakash, 2001; Kaukovirta-Norja et al., 2005; Sanchez-Moreno et al., 1999; Pérez-Jiménez et al., 2008; Amit et al., 2010). Additional results as time taken to reach the steady state at EC_{50} (tEC_{50}) and antiradical efficiency (AE

= 1/ EC_{50} tEC_{50}) were proposed by Pérez-Jiménez et al. (2008).

Molyneux (2003) discussed profound the various methods using DPPH. He recommended for increasing the accuracy of the method for 1-cm pathlength spectrophotometric cuvettes with a maximum working volume 4 ml to use 2 ml DPPH solution and 2 ml sample (ratio 1:1), solvent methanol or ethanol (not water or acetone), concentration of the DPPH solution in the range 50 to 100 μM, reaction time 30 min and suitable standards or "positive controls" as ascorbic acid (Vitamin C) and α-tocopherol (Vitamin E). According him the EC_{50} has the drawback that the higher the antioxidant activity, the lower is the value of parameter.

Materials and Methods

The DPPH (1,1-Diphenyl-2-picrylhydrazyl) was obtain from Sigma-Aldrich Chemie GmbH, Germany; the L-(+)-Ascorbic acid and DL-alpha-Tocopherol from Dr. Ehrenstorfer GmbH, Germany; the methanol p.a. and ethanol p.a. from Merck Chemicals, Germany.

The congress wart was produced on the automatic laboratory device „Bender and Hobein". The malts used (from barley and wheat) were crop 2009. The worts were hopped by two doses hops pellets – from bitter variety Magnum and from aromatic variety Spalt Select in ratio 70:30 on the base of total 70 mg/l alpha-bitter acids. The boiling of worts were 90 minutes under reflux. For the main fermentation were used dry brewing yeasts – middle fermenting strain Saccaromyces carlsbergensis. The main fermentation was for three days at 15°C and four days at 10°C. The aging of beer was for two weeks at 4°C. Beer in bottles was purchased from the supermarket.

All measurements of free radical scavenging activity were performed in triplicate and standard deviation was calculated.

Results and Discussion

Determination of the methods conditions (ruggedness testing of methods)

Evaluation of the methods and modifications for determination of the radical scavenging activity by DPPH shows that the main factors influenced the reproducibility are the solvent, duration of the reaction, sample to reagent ratio and the wave length for absorbance measurement of the decolouration of the reaction mixture. The DPPH solution was prepared by dissolving 0.0024 g DPPH in 100 ml methanol or ethanol (0.06 mM). On the base of the most frequent use of the mentioned conditions, presented by the cited authors were chosen two possibilities. It was used the testing of the analytical procedure for ruggedness (ruggedness testing of methods) according to Analytica EBC (1998). The ruggedness testing determined the method's conditions, which render substantial effect on the accuracy. The chosen factors are presented in Table 1.

The effect of the identified variables on the method results is assessed using experimental designs referred as two level factorial and frac-

Table 1

Factors, effected the method of determination of radical scavenging activity

Factor	Name	-	+
A	Solvent	Methanol	Ethanol
B	Duration of reaction	20 min	30 min
C	Sample/Reagent Ratio	0.2 ml/1.5 ml	1.5 ml/1.5 ml
D	Wave length	515 nm	517 nm

tional factorial designs. Eight experimental trails are carried out in duplicate in a randomised manner at specific combinations of factor levels. The two level factorial design is described in terms of analysis of variance (ANOVA) and this is used to interpret the results of the trial and to examine the effects of variables. The results of the experimental design are presented in Table 2.

The following calculation estimated the effect of each factor. There were calculated the Total (T), the differences (D), the sum of the squares ($\sum D^2$), experimental error variance (Se^2), main effects error variance (Sm^2), standard error of the main effects (Sm), main effects E_A, E_B, E_C and E_D, confidence limits and confidence intervals.

$\sum D^2 = 0.002796$; $Se^2 = 0.00017475$;
$Sm^2 = 0.0000436875$;
$Sm = \pm 0.01522$; $E_A = 0.02855$;
$E_B = 0.007$; $E_C = 0.206$; $E_D = 0.005$

The effects of the main factors, the confidence limits and the confidence intervals are presented in Table 3.

The results in Table 3 indicated that the factors solvent and sample/reagent DPPH ratio influenced significantly the accuracy of the method. The other factors duration of the reaction and wave length include 0 for the confidence interval with 95 % confidence, which means that they did not effected statistically the accuracy of the method.

Determination of the effect of the DPPH and samples solvent

The antioxidant activity is usually measured in food extracts obtained with different aqueous-

Table 2

Results of the trials for determination of the effect of the factors

Number	Factor A	Factor B	Factor C	Factor D	Trial 1	Trial 2	T	D
1	Methanol	20 min	0.2 ml/1.5 ml	515 nm	0.313	0.318	0.631	- 0.005
2	Ethanol	20 min	0.2 ml/1.5 ml	517 nm	0.656	0.696	1.352	- 0.040
3	Methanol	30 min	0.2 ml/1.5 ml	517 nm	0.305	0.306	0.611	- 0.001
4	Ethanol	30 min	0.2 ml/1.5 ml	515 nm	0.653	0.678	1.331	- 0.025
5	Methanol	20 min	1.5 ml/1.5 ml	517 nm	0.186	0.186	0.372	0
6	Ethanol	20 min	1.5 ml/1.5 ml	515 nm	0.381	0.393	0.774	- 0.012
7	Methanol	30 min	1.5 ml/1.5 ml	515 nm	0.173	0.172	0.345	0.001
8	Ethanol	30 min	1.5 ml/1.5 ml	517 nm	0.403	0.383	0.786	0.020

Table 3

Effects of the factors (conditions) of the method for determination of the radical scavenging activity

Factor	Main effect	Confidence Limit	Confidence Interval	Effect
A Solvent	0.2855	± 0.01522	from 0.27026 to 0.30074	Yes
B Duration of the reaction	0.007	± 0.01522	from – 0.02224 to 0,00824	No
C Sample/Reagent ratio	– 0.206	± 0.01522	from – 0.19076 to – 0.22124	Yes
D Wave length	0.005	± 0.01522	from – 0.01024 to 0.02024	No

Table 4
Radical scavenging activity and variation coefficient for the solvent ethanol and methanol

Value	EVCAA, mmol/l ethanol	EVCAA, mmol/l, methanol
Maximum, mmol/l	1013.78	900.16
Minimum, mmol/l	902.16	724.10
Average, mmol/l	955.3133	818.5107
Standard deviation, mmol/l	28.85177	48.98722
Coeficient of variation, %	± 3.02	± 5.98

organic solvents (methanol, ethanol, acetone, chloroform etc.). There is not ideal solvent that would be entirely satisfactory for extraction of the total antioxidants, present in foods, especially those associated with complex carbohydrates and proteins (Pérez-Jiménez et al., 2008).

For determination of the solvent effect it was measured 15 times the radical scavenging activity by DPPH (prepared in ethanol or methanol) of the same beer sample, diluted by methanol or ethanol. The results were obtained by calibration graphs and the modification of the method DPPH described below. The results were expressed as EVCAA (equivalent vitamin C antioxidant activity) and as EVEAA (equivalent vitamin E antioxidant activity). Based on the results obtained were calculated the coefficient of variation for the solvents ethanol and methanol. The results for radical scavenging activity, expressed as mmol/l EVCAA are presented in Table 4.

The results from Table 4 could be summarized as follows:

- The coefficient of variation of the method for determination of the radical scavenging activity by DPPH and solvent ethanol is twice lower that the method with utilisation of the methanol as a solvent. That means that the reproducibility with ethanol is two times better than with methanol;

- The results obtained for the radical scavenging activity by DPPH and ethanol is higher than the results obtained by methanol (an average 16.7 % for the used beer sample). This probably is due to the better extraction by ethanol of the substances, which possessed antioxidant properties;

- The ethanol is natural component of the beer and wine, which means better solvent for these samples than methanol;

Calibration curves for determination of radical scavenging activity by DPPH

The most useful standard for preparation of calibration graphs is ascorbic acid (Vitamin C). Relatively often for the same purposes is used by different authors α-tocopherol (Vitamin E). The standard solutions were prepared by methanol and ethanol. The calibration graphs were obtained by

Table 5
Characteristics of the calibration curves

Equivalent	Solvent	R^2	y = ax + b
Vitamin C	Ethanol	0.9957	y = 159.06(Acontrol - Asample) + 6.0687
Vitamin C	Methanol	0.9958	y = 152.72(Acontrol - Asample) + 2.5337
Vitamin E	Ethanol	0.9995	y = 88.15(Acontrol - Asample) - 1.3005
Vitamin E	Methanol	0.9997	y = 80.776(Acontrol - Asample) - 0.7636

the modification of the method DPPH described below. They are presented on Table 5. The linear dependences were characterized by very high values of the regression coefficients. They are used for expression of the results as EVCAA and as EVEAA.

Modification of the method for determination of free radical scavenging activity in beer by DPPH

Based on the analyses and evaluation of the described methods, the results of the ruggedness test for determination of the conditions of the method, coefficients of variation and recommendations of some authors was elaborated modification of the method for determination of free radical scavenging activity by DPPH in beer and beverages.

Diluted sample: Dilute 13.3 ml degassed beer, attemperate to 20°C to 100 ml with water, attemperate to 20°C in volumetric flask and mix well. Pipette 2.5 ml diluted beer in volumetric flask 25 ml and adds 20 ml ethanol. Allow to stand 20 min to 20°C and fill up the flask to 25 ml with attemperated ethanol and mix well. Transfer the content to fluted filter. Filtered diluted sample store at 20°C before using.

Diluted blank: Dilute 2.5 ml water with ethanol in volumetric flask 25 ml. Attemperate 20 min to 20°C. If is necessary filter through fluted filter.

DPPH solution: Dilute 0.0024 g DPPH in 100 ml ethanol (0.06 mM). Attemperate 20 min to 20°C. Prepare fresh every day.

Determination

Sample: Add 1.5 ml diluted sample and 1.5 ml DPPH solution in test tube, stopper with a glass ball and mix well. Store 30 min in dark and determine the absorbance at 517 nm against diluted blank.

Control: Add 1.5 ml diluted blank and 1.5 ml DPPH solution in test tube and mix well. Store 30 min in dark and determine the absorbance at 517 nm against diluted blank.

Calculation of the results

The results are calculated by calibration curves, prepared with Ascorbic acid (Vitamin C) – (EVCAA, equivalent vitamin C antioxidant activity) and α-Tocopherol (Vitamin E) – (EVEAA, equivalent vitamin E antioxidant activity) rendered in account the dilution factor and expressed in mmol/l. For the beverages, produced on the plant basis as beer, wine, tea, fruit and vegetable juices it is advisable to express the results in equivalent Vitamin C. The results could be expressed so as % free radical scavenging activity (FRSA) with DPPH or as % inhibition of the free radical with DPPH accords the described formula A and C respectively.

Investigation on the effect of malt and hops on the antioxidant activity of wart and beer

It was investigated the effect of malt and hops on the antioxidant activity – free radical scaveng-

Table 6

Free radical scavenging activity of laboratory sweet wort, hopped wort, young beer and final beer produced from malted barley

Sample	EVCAA, mmol/l	EVEAA, mmol/l	FRSA, %	Inhibition, %
Sweet wort	542.93 ± 11.70	181.71 ± 6.18	9.13 ± 0.24	8.50 ± 0.24
Hopped wort	609.27 ± 15.31	216.81 ± 8.00	10.49 ± 0.32	9.86 ± 0.31
Young beer	573.55 ± 4.42	197.71 ± 2.34	9.75 ± 0.09	9.13 ± 0.08
Final beer	591.41 ± 4.42	207.36 ± 2.34	11.53 ± 0.10	10.81 ± 0.10

Table 7

Free radical scavenging activity of laboratory sweet wort, hopped wort, young beer and final produced from malted wheat

Sample	EVCAA, mmol/l	EVEAA, mmol/l	FRSA, %	Inhibition, %
Sweet wort	571.00 ± 7.66	196.56 ±4.05	9.70 ± 0.16	9.08 ± 0.16
Hopped wort	685.82 ± 7.66	257.29 ± 4.05	12.05 ± 0.16	11.42 ± 0.16
Young beer	599.07 ± 4.42	211.41 ± 2.94	10.28 ± 0.09	9.65 ± 0.09
Final beer	688.37 ± 4.42	258.64 ± 2.39	13.79 ± 0.10	13.07 ± 0.10

ing activity of wort and beer. In Table 6 and Table 7 are presented the results for free radical scavenging activity of worts, young beers and final beers prepared from malted barley and malted wheat. The utilized solvent was methanol. The results are expressed as EVCAA, EVEAA, FRSA according formula A and as inhibition according formula C, render an account of the dilution factors.

The results in Table 6 and Table 7 indicated that the sweet wort has relatively high free radical scavenging activity. The sweet wort produced from the malted wheat presented higher antioxidant activity. This is due very likely to the higher antioxidant content in malted wheat and weaker oxidation in the course of brewing process. It is evident that the antioxidant activity of wort and beer is mainly effected by the free radical scavenging activity of the malt. The hops doses increase the antioxidant activity of hopped wort produced from malted wheat additionally by 20.1 %, and of hopped wort produced from malted barley by 12.2 %, expressed as EVCAA. These raise in antioxidant activity is directly connected with the increasing of polyphenols and other antioxidants content due to the hopping. After the main fermentation the free radical scavenging activity of young beer lightly decreased respectively by 5.9 % for this produced from the malted barley and by 12.6 % for this produced from the malted wheat, expressed as EVCAA. These reductions probably are result of the certain oxidation of the antioxidants of young beer. In final beer, as result of the reducing activity of brewing yeast during the aging and limited oxygen access, the beer antioxidant activity is again higher. During all of the production stages the antioxidant content of wort and beer from malted wheat is higher than from the malted barley one.

Investigation of the free radical scavenging activity of different beers

Table 8 and Table 9 presented the free radical scavenging activity of the production final beer, brewed from malted barley and malted wheat. For the dilution of the beer sample and DPPH solution preparation were used methanol and ethanol. The

Table 8

Free radical scavenging activity of laboratory final beer produced from malted barley and malted wheat (using methanol as a solvent)

Sample	EVCAA, mmol/l	EVEAA, mmol/l	FRSA, %	Inhibition, %
Malted barley	586.31 ± 15.31	204.66 ± 8.10	11.41 ± 0.36	10.70 ± 0.36
Malted wheat	688.37 ± 15.94	258.64 ± 8.43	13.78 ± 0.37	13.07 ± 0.37

Table 9

Free radical scavenging activity of laboratory final beer produced from malted barley and malted wheat (using ethanol as a solvent)

Sample	EVCAA, mmol/l	EVEAA, mmol/l	FRSA, %	Inhibition, %
Malted barley	814.46 ± 7.97	217.61 ± 4.42	13.12 ± 0.19	12.17 ± 0.19
Malted wheat	883.55 ± 4.60	258.89 ± 2.55	14.77 ± 0.11	13.74 ± 0.12

Table 10

Free radical scavenging activity determined with methanol of the ordinary pilsner beer from aging tanks of pub brewery

Sample	EVCAA, mmol/l	EVEAA, mmol/l	FRSA, %	Inhibition., %
Aging tank 1	1037.96 ± 7.66	443.54 ± 4.05	19.25 ± 0.16	18.62 ± 0,16
Aging tank.3	989.48 ± 4.42	417.90 ± 2.33	18.26 ± 0.09	17.63 ± 0.09

Table 11

Free radical scavening activity determined with ethanol of the laboratory final beer, produced from malted barley from different regions of country, crop 2008

Sample	EVCAA, mmol/l	EVEAA, mmol/l	FRSA, %	Inhibition., %
Region Lom	1446.98 ± 4.60	568.13 ± 2.55	28,72 ± 0,11	28,35 ± 0,12
Region V. Tarnovo	1481.18 ± 7,97	588.75 ± 4,42	29.64 ± 0,20	29.25 ± 0,20
Region St. Zagora	1436.34 ± 7.98	562.24 ± 4.42	28.46 ± 0.20	28.06,2 ± 0.20
Mix of the regions	1462.92 ±4.60	576.97 ± 2.55	29.12 ± 0.12	28.72 ± 0.11

results obtained confirmed again that the antioxidant activity determined with ethanol solution is higher than this obtained with solvent methanol (an average 28.4 % - 38.9 % for the used beer samples). Similar to the results of Table 6 and Table 7, the free radical scavenging activity is again higher for the beer, produced from malted wheat.

The free radical scavenging activity of ordinary pilsner beer, produced in pub brewery was determined. The solvent used was methanol. The results are presented in Table 10. The free radical scavenging activity of beer was relatively high, about 1000 mmol/l EVCAA.

Table 11 presented free radical scavenging activity of laboratory beers, produced from malted barleys from different regions of the country. They showed very high (above 1400 mmol/l equivalent vit. C) and very similar values of the free radical scavenging activity.

Table 12 and Table 13 presented the free radical scavenging activity of two beers from the supermarket. They showed very big differences in the free radical scavenging activity. The Almus beer had with 52% higher free radical scavenging activity than Zagorka beer, expressed as EVCAA. These results indicated the very important role of the raw materials and technology used for the beer production. The results obtained confirmed

Table 12

Free radical scavenging activity of beers from supermarket (using methanol as a solvent)

Sample	EVCAA, mmol/l	EVEAA, mmol/l	FRSA, %	Inhibition, %
Almus, glass bottle 0.5 l, shelf life 16.07.2009	1298.23 ± 7.66	581.21 ± 4.05	49.43 ± 0.29	43.47 ± 0.29
Zagorka, glass bottle 0.5 l shelf life 25.01.2010	851.69 ± 4.42	345.02 ± 2.34	15.84 ± 0.10	15.67 ± 0.10

Table 13

Free radical scavenging activity of beers from supermarket (using ethanol as a solvent)

Sample	EVCAA, mmol/l	EVEAA, mmol/l	FRSA, %	Inhibition, %
Almus, glass bottle 0.5 l, shelf life 16.07.2009	1404.45 ± 7.07	544.57 ± 4.42	46.24 ± 0.27	37.10 ± 0.27
Zagorka, glass bottle 0.5 l shelf life 25.01.2010	942.02 ± 7.96	288.29 ± 4.42	14.92 ± 0.19	14.73 ± 0.19

again that the antioxidant activity determined with ethanol solution is higher than this obtained with solvent methanol (an average 8.2 % - 10.6 % for the used beer samples).

Conclusions

The literature review of the methods for determination of free radical scavenging activity by DPPH shown that there are substantial differences in used solvents, concentration of DPPH working solutions, ratio between volumes of sample/reagent, duration of reaction, wave length of absorbance measurement, standard solutions and equations for calculation of the results. Determination of the effect of methods conditions by ruggedness testing of methods indicated that the accuracy of the method for determination of free radical scavenging activity is effected by the solvent used (ethanol or methanol) and the sample/reagent DPPH volume ratio. Based on the results obtained and review of the methods it was proposed modification of the method for determination of free radical scavenging activity of beer and beverages with DPPH. The values of free radical scavenging activity of beer determined by using of ethanol are higher and more precise than the respective ones determined by methanol. The antioxidant activity of the beer is attributed mainly by the raw materials, especially malt and the technology used.

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