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ARTICLE

Polarographic Assay Based on Hydrogen Peroxide Scavenging in Determination of Antioxidant Activity of Strong Alcohol Beverages

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Total antioxidant (AO) activity of strong alcohol beverages such as wine and plum brandies, whiskeys, herbal and sweet fruit liqueurs have been assessed using a polarographic assay based on hydrogen peroxide scavenging (HPS). Rank of order of total AO activity, expressed as percentage of decrease of anodic oxidation current of hydrogen peroxide, was found analogous with total phenolic content estimated by Folin−Ciocalteau (FC) assay and radical scavenging capacity against the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). Application of the assay for surveying of a quarter century long maturation of plum brandy in oak barrel was demonstrated. In addition, influence of different storage conditions on preservation of AO activity of some herbal liqueurs was surveyed. Wide area of application of this simple, fast, low cost and reliable assay in analysis and quality monitoring of various strong alcohol beverages was confirmed.

KEYWORDS: Alcohol beverages; antioxidant activity; Bitter 54; hydrogen peroxide; phenolics; polarography

INTRODUCTION

Alcoholic beverages are an integral part of the human diet. Epidemiological studies have proved that moderate consumption of alcoholic beverages rich in phenolic compounds have a positive influence on coronary artery disease, improving lipid metabolism, increasing anticoagulant and antioxidant (AO) activity, and decreasing mortality from coronary disease, as well as on colorectal cancer (*1*, *2*). The "French paradox" demonstrates that for apparently the same level of risk factors, cardiovascular mortality rate is lower in France than in the European Northern countries. The low incidence of heart disease among the French population despite a high fat intake has been attributed to high consumption of red wine (*3*). This phenomenon is particularly evident in southwest France, a region where people do not drink more wine than elsewhere, but often drink Armagnac (*4*).

Among alcoholic beverages grape wine has been studied more in depth with respect to AO activity, phenolic compounds content and profile, and influence on consumer's health. Red grapes, red wines, and grape byproducts contain high amounts of phenolic compounds (500−4059 mg GAE/L) (*5*). The amount of total phenolics in berry and fruit wines and liqueurs ranges from 91 to 1820 mg GAE/L. The highest AO activity was found in red (Cabernet) wine and elderberry, blueberry and black currant wines; moderate in cherry, raspberry, cranberry and plum wines; and the lowest in apple, peach, icewine (from grapes), white (Chardonnay) and pear wines (*6*).

Antioxidant activity and various AOs present in different strong alcoholic beverages attracted increasing interest. Liqueurs made from berry and from fruit juices contain a wide range of flavonoids, especially anthocyanins. Phenolic compounds are present in lower amount than in red grape wines, due to a lower proportion of fruit juice in the final product (*5*, *7*). Different products labeled as bitters, herbal liqueurs or medicinal tonics, used as aperitifs or digestives, exhibit considerable AO capacity (*8*). Bitter 54, a bitter containing 46 herbs and 8 fruit extracts, is becoming more popular in Serbia as natural product. Small scale Bitter 55 with the same composition but enriched with a slice of fungus *Ganoderma lucidum*, known for its strong pharmacodynamic effect (*9*), has attracted attention too.

The distillates exhibited AO activity values that are intermediate between the corresponding red and white wines (*10*). The highest values of total phenolic content and AO activity were attributed to Armagnacs, Cognacs and bourbon whiskeys. Development of AO activity and the formation of the color and the flavor of the strong alcohol beverages have been related with aging in wood casks. Nonflavonoid ellagitannins, as well as ellagic acid, extracted from the wood during aging are important contributors to the overall AO activity of strong alcohol beverages (*11*). The flavor of the fresh beverages becomes changed

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during aging in wood through the extraction of hydrolyzed or ethanolyzed wood compounds and evaporation of ethanol, water and volatile compounds; slow oxidation reactions and other chemical and enzymatic changes can also contribute to the formation of new compounds (*12*). The AO activity of the phenolic compounds depends on their chemical structure, concentration and oxidation status. The latter two are mainly determined by the aging conditions, including the wooden barrel characteristics, such as wood botanical species, toasting or charring level, barrel size, and the cellar environment (*13*). Strong alcohol beverages with a low amount of phenolic compounds such as rum, vodka, gin and other similar distillates exhibited negligible AO activity (*10*).

Various assays have been employed in the analysis of AO activity of alcoholic beverages. Despite numerous spectrophotometric, fluorometric and electrochemical assays successfully used until now, there is still an interest to develop novel direct, less time-consuming and less costly assays. Assays with simple and rapid experimental procedures, with no need for sample pretreatment or exotic, nonphysiological radical species, are favored. Since electrochemical assays can satisfy these criteria, they are being used more often in determination of AO activity. Electrochemical determination of radical species commonly used in spectrophotometric assays has been established as well. Determination of AO activity of different beverages, including red wine based on the amperometric reduction of 1,1-diphenyl-2-picrylhydrazyl (DPPH) (*14*), as well as AO activity of red and white wines and brandies (*15*), including sherry brandies (Solera, Solera Reserva and Solera Gran Reserva), and several other commercial aged distillates (Cognacs, Armagnacs and Spanish, French and South African brandies) (*16*) based on the electrochemical oxidation of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) has been reported.

Assays based on hydrogen peroxide scavenging (HPS) have attracted attention. As a naturally present, stable reactive oxygen specie able to reproduce *in vivo* state conditions, hydrogen peroxide has an advantage over artificial radical species usually used in common spectrophotometric assays. Besides assays based on photometric or fluorometric determination of hydrogen peroxide (*17*), two electrochemical assays based on decrease of hydrogen peroxide monitored using either chronopotentiometry or polarography have been developed recently. Kinetics of elimination of peroxide after its injection into liquid samples has been measured by Karyakina (*18*) while decrease of anodic oxidation current of hydrogen peroxide upon addition of tested samples has been measured by Gorjanović (*19*, *20*). Both mentioned assays have been used in the analysis of red and white wines (*18*, *19*) while a polarographic assay has been used in the analysis of AO activity of beer samples and surveying of the brewing process (*20*).

Here, a comparative study of AO activity of different strong alcoholic beverages is reported. A recently developed AO assay based on polarographic surveying of hydrogen peroxide anodic oxidation current decrease has been applied for the first time to determine AO activity of strong alcoholic beverages such as plum and wine brandies, whiskeys as well as bitters and sweet fruit liqueurs. Commercial alcoholic beverages, either domestic or imported brands, as well as small scale products have been included in the study. Results have been correlated with total phenolic content estimated by Folin−Ciocalteau (FC) assay and DPPH scavenging, and correlation coefficients obtained have been discussed. In addition, changes of AO activity of some herbal liqueurs during storage under different conditions as well as a quarter century long aging of plum brandy in oak barrel have been surveyed.

MATERIALS AND METHODS

Chemicals and Strong Alcoholic Beverages. Hydrogen peroxide was from Merck (Darmstadt, Germany). Folin−Ciocalteau reagent was from Merck (Darmstadt, Germany). DPPH reagent was from Aldrich (Milwaukee, WI).

Commercial bitters, plum and wine brandies, and whiskeys were purchased from the local market while small scale beverages such as vermouth and liqueurs were obtained from Faculty of Agriculture, University of Belgrade, Serbia. The following beverages were included in the study:

No. 1. Commercial bitter herbal liqueur "Underberg" (44% vol of alcohol).

No. 2. Commercial herbal liqueur "Bitter 54" (35% vol of alcohol) natural product made from the following extracts of aromatic herbs and fruits: Linnean herbarium (*Paris quadrifolia L.*), Prostrate knotweed or knotgrass (*Polygonum aviculare L.*), Mountain germander (*Teucrium montanum L.*), Common sage (*Salvia officinalis L.*), Common Yarrow (*Achillea millefolium L.*), Peppermint (*Mentha piperita L.*), Wild Thyme or Creeping Thyme (*Thymus serpyllum L.*), Common Thyme (*Thymus vulgaris L.*), Camomile (*Matricaria chamomilla L.*), Wall germander (*Teucrium chamaedrys L.*), Grand Wormwood (*Artemisia absinthium L.*), Mellisa (*Melissa officinalis L.*), Hibiscus (Hawaiian hibiscus), Eugenia (*Eugenia caryophyllata L.*), Anise (*Pimpinella anisum L.*), Cinnamon (*Cinnamomum div.*), Vanilla (*Vanilla planifolia*), Dog Rose or rosehip (*Rosa canina L.*), Common Juniper (*Juniperus communis L.*), Carob tree (*Ceratonia siliqua L.*), Oregano or pot marjoram (*Origanum vulgare L.*), St John's wort (*Hypericum perforatum L.*), Ribwort Plantain (*Plantago lanceolata*), Uva (*Arctostaphylos uva ursi*), Mulberry (*Morus alba L.*), Rosemary (*Rosmarinus officinalis L.*), Lady's mantle (*Alchemilla vulgaris L.*), Basil (*Ocimum basilicum L.*), Elder or Elderberry (*Sambucus nigra L.*), Horsetails (*Equisetum arvense L.*), Shepherd's Purse (*Capsella bursa-pastoris L.*), Senna alexandrina (*Cassia officinalis*), Blackberries leaf (*Rubus fruticosus L.*), Birch (*Betula L.*), Hawthorn (*Crataegus oxyacantha L.*), European Mistletoe or Common Mistletoe (*Viscum album L.*), Fennel (*Foeniculum vulgare Mill.*), Centaury (*Erythraea centaurium Pers.*), Heartsease or Johnny Jump Up (*Viola tricolor L.*), Oak wood (*Quercus*), Pot Marigold or English Marigold (*Calendula officinalis L.*), Stinging nettle (*Utrica dioica L.*), Coltsfoot (*Tussilago farfara L.*), Pimpernel (*Anagallis arvensis L.*), Common Dandelion (*Taraxacum officinale Web.*), Cypress Spurge (*Euphorbia cyparissias L.*), Common fig (*Ficus carica L.*), grape (*Vitis vinifera*), plum (*Prunus domestica L.*), apple (*Pirus malus L.*), raspberry (*Rubus idaeus L.*), orange (*Citrus aurantium L.*), lemon (*Citrus limonum Risso*), grapefruit (*Citrus paradisi*). Bitter 54 contains 88.22 g/L total extract and 75 g/L sugar added. Additive E160d (caramel) was used to gain dark-brown color.

No. 3. Small scale product "Bitter 55" (Ganoderma bitter) (Faculty of Agriculture, University of Belgrade, Serbia) (35% vol of alcohol) natural product made from Bitter 54 and medicinal mushroom *Ganoderma lucidum* (1 % w/v). In the bottle of Bitter 54 a slice of mushroom was added and extracted 30 days at ambient temperature.

No. 4. Commercial medicinal tonic "Pervivo" (40% vol of alcohol).

No. 5. Commercial sweet Raspberry liqueur (25% vol of alcohol), 250 g/L of sugar and 30% vol of raspberry juice. Raspberry juice was made using standard technology for production of clear fruit juices: crushing, depectinization, pressing and microfiltration.

No. 6. Small scale product Vermouth red (local name "Bermet") (Faculty of Agriculture, University of Belgrade, Serbia) (16.5% vol of alcohol) made from red wine (60% vol) and extract of 54 herbs and fruits. It contains 70 g/L of sugar. Red wine was prepared from two wine varieties: Cabernet Sauvignon and local variety Prokupac. The same herbal and fruit extract as in Bitter 54 was used (No. 2).

No. 7. Commercial sweet Quince liqueur (25% vol of alcohol) 250 g/L of sugar and 30% vol of quince juice. Quince juice

was made using standard technology for production of clear fruit juices: milling, depectinization, pressing, microfiltration.

No. 8. Commercial wine brandy "Vinjak" VSOP (Very Special Old Pale) (Rubin, Serbia) (40% vol of alcohol), 5 years old (used oak barrels of 500 L).

No. 9. Commercial wine brandy "Vecchia Romagna" (38% vol of alcohol) wine brandy aged in oak barrels.

No. 10. Commercial wine brandy "Vinjak" VS (Very Special) (Rubin, Serbia) (40% vol of alcohol), one year old (used oak barrels of 500 L).

No. 11. Commercial wine brandy "Metaxa", wine brandy (Metaxa, Greece) (38% vol of alcohol), 5 years old in oak barrel.

No. 12. Commercial wine brandy "Stock '84" VSOP (38% vol of alcohol) aged in oak barrel.

No. 13. Small scale product plum brandy "Mucenica 5" (slivovitz) (Faculty of Agriculture, University of Belgrade, Serbia) (45% vol of alcohol), 5 years old (new oak barrel of 500 L).

No.14. Commercial plum brandy "Stomaklija" (slivovitz) (Prokupac, Serbia) (40% vol of alcohol) with Grand Wormwood Extract added.

No. 15. Commercial plum brandy "Lincura" (slivovitz) (Miloduh, Serbia) (40% vol of alcohol) with Great Yellow Gentian (bitter root) extract added.

No. 16. Small scale product plum brandy "Mucenica" (slivovitz) (Faculty of Agriculture, University of Belgrade, Serbia) (45% vol of alcohol), stored in inox (stainless steel) tank.

No. 17. Whiskey, Grants, Black (38% vol of alcohol).

No. 18. Whiskey, Label 5, Classic Black (38% vol of alcohol).

Polarographic Instrumentation and Procedure. Hydrogen peroxide scavenging activity was estimated by direct current (DC) polarography, using a dropping mercury electrode (DME) as working electrode, with a programmed dropping time of 1 s. Saturated calomel electrode (SCE) served as a reference and a Pt-foil as auxiliary electrode. The current−potential ($i-E$) curves were recorded using the polarographic analyzer PAR (Princeton Applied Research Instrument), model 174A, equipped with X-Y recorder (Houston Omnigraphic 2000). The volume of the experimental solution in the electrolytic cell was 20 mL. Clark-Lubs (CL) buffer (pH 9.8) was prepared by mixing 25 mL of 0.4 M H_3BO_3, 25 mL of 0.4 M KCl and 40.8 mL of 0.2 M NaOH. Starting H_2O_2 concentration of 5.7 mM was obtained by addition of 10 μL of 35% H_2O_2 into 20 mL of CL buffer. Before each $i-E$ curve recording, the stream of the pure nitrogen was passed through the cell solution. During curve recording, the inert atmosphere was kept by passing the nitrogen above cell solution. The initial potentials were 0.10 or 0.15 V and potential scan rate was 10 mVs^{-1}. The DME current oscillations were filtered with low pass filter positioned at 3 s. All experiments were done at room temperature.

Determination of Hydrogen Peroxide Scavenging Activity of Strong Alcoholic Beverages. Anodic oxidation of H_2O_2 at the DME in alkaline solutions was investigated. At concentration of hydrogen peroxide higher than 1.5×10^{-4} M, instead of an ordinary shape DC wave a current peak was developed. The obtained polarographic curve of H_2O_2 can be explained by formation of the mixed mercury complex [(Hg(O_2H)(OH)]. The phenomenon of polarographic current attributed to the formation of the mixed mercury complex and its decrease that is proportional to the total AO activity of analyzed samples enabled HPS assay development. The anodic current of H_2O_2, i.e. initial i_l value (i_{l0}), obtained by recording 5.7 mM H_2O_2 solution, decreased upon addition of samples. Samples were gradually added (in 5 equal aliquots of 100, 500, or 1000 μL) into an electrolytic cell with buffered H_2O_2 solution. The decrease of hydrogen peroxide anodic oxidation current obtained upon addition of 500 μL of beverage samples (HPS500 (%)) was used as a criterion of AO activity (unless otherwise specified).

Determination of Radical Scavenging Capacity of Strong Alcoholic Beverages against the Stable Free Radical DPPH. The antioxidant activity of samples of alcohol beverages was assessed using the stable radical DPPH. Series of samples with different dilutions were prepared in methanol as solvent. A mixture of 200 μL of samples and 1800 μL of DPPH solution (1×10^{-4} M in methanol) was placed in the dark at room temperature, and the absorbance (A_s) at 517 nm was measured after 30 min. Methanol (1800 μL) plus the sample solution (200 μL) was used as the blank (A_b), while the DPPH solution (1800 μL) plus methanol (200 μL) was used as the control (A_c). All determinations were performed in triplicate, and four different dilutions were used for every sample. The DPPH antiradical-scavenging activity, DPPH (%), was determined using the following equation:

$$\text{DPPH}\ (\%) = 100\left[1 - \left(\frac{A_s - A_b}{A_c}\right)\right] \quad (1)$$

where A_s is the absorbance in the presence of the samples in the DPPH solution, A_c is the absorbance of the control solution (containing only DPPH) and A_b is the absorbance of the sample solution without DPPH. EC_{50} values were obtained from the graph DPPH (%) = $f(c)$, where c is reciprocal value of dilution necessary to decrease absorption of DPPH radical for 50%. EC_{50} value is expressed in mL of sample per mL of total solution (dilution). This value, multiplied by 100, gives an also often used EC_{50} value as a percentage.

Determination of Total Phenolic Content. The concentration of total phenolic content in alcoholic beverages was determined by the Folin−Ciocalteau procedure. A volume of 200 μL of diluted sample was mixed with 1000 μL of FC reagent previously diluted with distilled water in a 1:10 ratio. Appropriate sample dilutions were experimentally found to satisfy linear dependence of absorbance from concentration to give absorbance on 740 nm in the range 0.2−0.7. After standing 6 min in the dark, 800 μL of 7.5% sodium carbonate solution was added, shaken and put in the dark for 2 h to react. Absorbance at 740 nm was then measured. Distilled water was used as a blank. All samples were done in triplicate. Four dilutions of each sample were used, and results were averaged. Each absorbance was adjusted for the value of blank probe. The same procedure was done with gallic acid standard (concentrations of 10, 25, 50, 100 mg/L), and a calibration curve was calculated. The total phenolic content is expressed in gallic acid equivalents (GAE) − concentration of gallic acid (mg/L) that corresponds to the dilution of sample with the same absorbance multiplied with the appropriate dilution factor.

Investigation of Bitter 54 and Bitter 55 Stability. Freshly prepared Bitter 54 and 55 were charged into dark and transparent bottles (0.7 L). After one year of storage at room temperature either in the dark or in the light, AO activity was determined using HPS assay.

Surveying of Plum Brandy Aging. Plum brandy maturated in used oak barrels for 1, 4, 14, and 24 years was analyzed using FC, HPS and DPPH assays. As a control, plum brandy stored in an inox (stainless steel) tank was used (designated as 0 years). Aliquots of 1000 μL were added into an electrolytic cell.

Statistic Analysis. Results were expressed as the mean value ± SD. Concerning the HPS assay, SD was obtained by linear regression analysis from dose-dependence curves by using OriginPro 6.1. In the FC and DPPH assays, SD was obtained from triplicates by using PASS 2008 statistical analysis software package.

RESULTS AND DISCUSSION

This study was conducted to determine total AO activity of strong alcohol beverages using a recently developed DC polarographic assay based on hydrogen peroxide scavenging (HPS). For the object of this study strong alcohol beverages such as whiskeys, wine and plum brandies aged in oak barrels, bitters and sweet liqueurs, medicinal tonic and aperitif wine vermouth were used. Beverages such as bitters (bitter herbal liqueurs), sweet fruit liqueurs, medicinal tonic and vermouth were added in five equal aliquots of 100 μL into initial peroxide solution. The optimal volume of distilled alcohol beverages such as plum and wine brandies or whiskeys was five times higher; these alcohol beverages were added in five equal aliquots of 500 μL. Polarograms of H_2O_2 before and after addition of samples were recorded. Anodic polarographic curves of hydrogen peroxide before and after gradual addition of herbal liqueur Bitter 54 are shown in **Figure 1**. Height of initial peroxide limiting current (i_{l0}) was compared with residual peroxide limiting current (i_{lr}) obtained upon gradual addition of tested samples. Percentage of i_l decrease (HPS (%)) was calculated upon each addition of tested samples

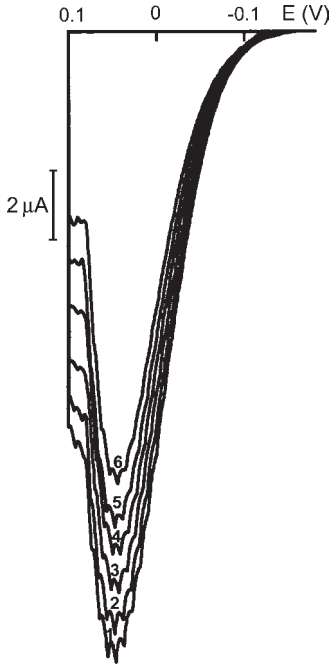

Figure 1. Anodic polarographic curves of buffered 5 mM hydrogen peroxide before (1) and after gradual addition of Bitter 54 in aliquots of 100 (2), 200 (3), 300 (4), 400 (5)and 500 μL (6).

according to eq 2:

$$\%\text{scavenged } [H_2O_2] = \left(1 - \frac{i_{lr}}{i_{l0}}\right)100 \qquad (2)$$

Prominent difference in hydrogen peroxide anodic oxidation current decrease between tested samples indicates assay potential applicability in determination of AO activity of alcohol beverages.

The following rank of order of AO activity was obtained for alcohol beverages added in aliquots of 100 μL: Underberg > Raspberry liqueur > Vermouth > Quince liqueur > Bitter 54 > Bitter 55 > Pervivo. The superior AO activity of herbal liqueur Underberg corroborates well with the data reported previously by Imark et al. (*8*). Using three different methods, linoleic acid oxidation, DPPH titration, and the TLC-fluorescent method, significant variations in AO activities between commercial samples of herbal strong alcohol beverages were revealed. Among 11 tested herbal beverages, Underberg exhibited the highest antiradical capacity (*8*). Herbal liqueurs Bitter 54 and 55 exhibit lower activity than Underberg. As expected no significant difference in AO activity between these two liqueurs was observed. Fungus *Ganoderma lucidum* known for its strong pharmacodynamic effect did not exhibit significant AO activity (*20*). Medicinal tonic Pervivo has shown the lowest AO activity among analyzed liqueurs.

Antioxidant activity of sweet fruit liqueurs has been found comparable with herbal liqueurs. Prominent AO activity of Raspberry liqueur can be explained by the presence of phenolic compounds with high AO activity. Antioxidants, the presence of which is considered as an important quality parameter for edible fruits, are present in fruit products such as juices, jams and liqueurs. Berries are particularly interesting as a source of AOs. Scavenging activity of berry crops on reactive oxygen species was found high (*17*). Raspberry (*Rubus idaeus*) contains a significant amount of ellagic acid and ellagitannins responsible for a good portion of the AO activity for all berries together with anthocyanins and proanthocyanidin-like tannins (*21*).

The AO activity of Quince liqueur was found lower than activity of Raspberry liqueur as expected. Phenolic content and AO activity of quince were investigated (*22*). The AO activity of phytochemicals identified in quince (*Cydonia vulgaris*) has been reported recently (*23*). Quince pulp, peel and jam extracts AO activity was correlated with the content of caffeoylquinic acids and total content of phenolics (*24*).

The AO activity of aperitif wine Vermouth, small scale product "Bermet" prepared from base wine by adding mixture of herbs and fruit extracts, was found high as expected. Antioxidants present in "Bermet" originate from both grape and extracts of herbs and fruits added, representing a complex mixture of different phenolic compounds.

Rank of order of AO activity obtained for strong alcoholic beverages added in aliquots of 500 μL (Vinjak VSOP > Metaxa > Vecchia > Stomaklija > Stock '84 > Mucenica 5 > Grants > Lincura > Vinjak VS > Label 5 > Mucenica) was related with aging in oak barrels, a key technological step for the formation of the color and the flavor, as well as the development of AO activity. Nonflavonoid ellagitannins, as well as ellagic acid, extracted from the wood during aging, are probably the most important contributors to the overall AO properties of alcoholic beverages such as cognacs, old fruit brandies and whiskeys (*11*). In this study, particularly illustrative are differences between AO activity of the same brand beverages such as two wine brandies "Vinjak" and two plum brandies "Mucenica". AO activity of five year old brandy "Vinjak" VSOP was found superior to "Vinjak" VS although both beverages have been produced from the same quality wine using the same technological procedure. Also, five year old plum brandy "Mucenica 5" has higher AO activity than "Mucenica" stored in an inox tank. The lowest AO activity among analyzed alcoholic beverages was attributed to "Mucenica". Addition of bitter root (Great Yellow Gebtian) and Grand Wormwood extract into the plum brandy showed some effect on AO activity as well.

The AO activity of two whiskeys examined was found low. Until now, the AO activity of whiskey was estimated using different assays such as EPR (*25*), DPPH (*26*), and reactive oxygen species scavenging activity (*27*). Significant AO activity dependent on time of maturation was reported (*25*−*27*).

AO activity of all tested beverages, expressed as percentage of i_l decrease obtained upon addition of 500 μL (HPS500 (%)), has been summarized in **Figure 2** in order to enable the reader to obtain comprehensive, overall insight into the comparison of AO activity of tested beverages.

Reliability of polarographic assay was validated though comparison with two standard spectrophotometric assays, FC assay for determination of total phenolic content and DPPH scavenging (**Table 1**). Folin−Ciocalteau assay was used in this study as the most commonly applied. The DPPH assay was chosen as one of the widespread method as well (*28*). Antiradical activity of various strong alcoholic beverages such as cognacs (*11*), nocino liqueur (*29*), lotus liqueur (*30*), herbal alcoholic beverages (*8*), whiskeys (*26*) and wine aged brandies (*13*) was evaluated using DPPH assay. In this report, reciprocal value of EC_{50} was used to make correlation with total phenolics and AO activity estimated using HPS assay. Reciprocal value reflects radical scavenging activity in a more comprehensive way since higher value corresponds to higher scavenging activity.

As seen in **Figure 3** high correlations have been obtained between total phenolic content (FC GAE) and both AO assays, HPS and DPPH scavenging. Results obtained corroborate well with already published data. Lugasi and Hovar demonstrated that AO properties of worldwide consumed beverages rich in phenolics, including commercial alcoholic beverages, correlated

with the total polyphenol content (*31*). The phenolic concentration of the herbal liqueurs correlated with the radical-scavenging activity (r^2 = 0.81) (*8*). A strong correlation between the AO activity and the total polyphenolic content of the brandies (r^2 = 0.99) was reported by Canas (*13*). The same authors revealed that AO activity has synergistic and antagonistic effects with other compounds present in the brandies, such as some volatile compounds. A strong correlation between antiradical activity measurements and high molecular weight polyphenols, ellagitannins, extracted from the wood and solubilized during aging, was revealed (*11*). The AO capacity of whiskey strongly correlated with the total phenol content, with ellagic and gallic acids (*25*) and lyoniresinol, the main polyphenolic compound in whiskey, increasing with maturation (*27*).

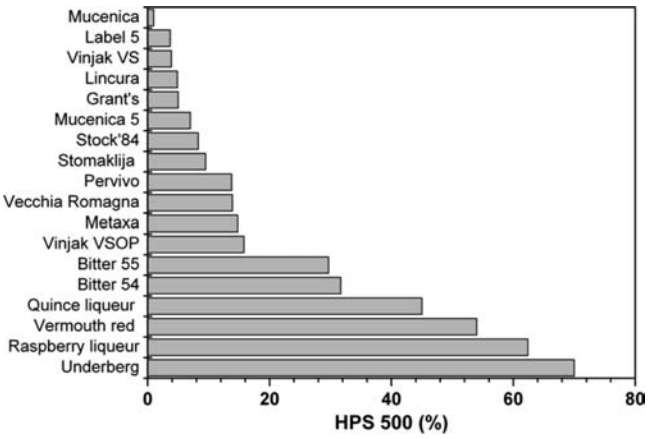

Figure 2. Comparative view of AO activity of analyzed beverages. Percentage of hydrogen peroxide decrease obtained upon addition of 500 μL of beverages (HPS500 (%)) was compared.

A high correlation coefficient between HPS and total phenolics for various red and white wines of different origin (0.997) as well as between DPPH and total phenolics (0.989) was obtained (*19*). The higher correlation between total phenolic content and HPS, than DPPH, obtained for both wines and strong alcoholic beverages confirms the previous suggestion (*19*) that the developed polarographic assay reflects the sum of activities of a wider range of phenolics. It has been demonstrated that DPPH radical is a weak oxidant not able to react rapidly and completely with polyphenolics (*32*).

Correlation between decrease of hydrogen peroxide anodic oxidation current upon addition of 500 μL of tested samples (HPS500 (%)) and DPPH scavenging activity was found high (r^2 = 0.921) (**Figure 4**). The correlation coefficient between HPS and DPPH reported for wines was higher (r^2 = 0.988) (*19*). Lower correlation coefficients obtained for alcoholic beverages may be explained by higher diversity of AO compounds originating either from various fruits and herbs or from wood barrels than those from grape only.

The second objective of this study was to demonstrate assay applicability to survey aging of plum brandy. The quality of plum and wine brandy, cognac and whiskey is improved remarkably by storage in wood barrels, commonly termed "maturing" or "aging". Polyphenols derived from barrel tannins and lignins have an important role in forming the matured flavor and taste, clustering ethanol and water and development of AO activity (*27*). According to Schwarz et al. (*16*), AO activity and polyphenolic content of commercial brandies increase with aging.

Decrease of hydrogen peroxide anodic oxidation current was monitored upon addition of five equal aliquots of 1 mL of plum

Table 1. Total Phenolic Content (FC GAE) and DPPH Scavenging in Comparison to AO Activity of Different Alcoholic Beverages Estimated Using HPS[a]

		FC GAE (mg/L)	DPPH EC_{50} (mL/mL)[b]	DPPH $EC_{50}{}^{-1}$[c]	HPS500 (%)[d]
			Bitter Herbal Liqueurs		
1	Underberg	1205.7 ± 14.2	0.0505 ± 0.0013	19.80	70.0 ± 3.0
2	Bitter 54	477.9 ± 7.1	0.1754 ± 0.0049	5.59	31.7 ± 0.8
3	Bitter 55 (Ganoderma)	445.1 ± 13.4	0.1811 ± 0.0038	5.52	29.7 ± 0.5
4	Pervivo	247.8 ± 4.4	0.4466 ± 0.0074	2.24	13.8 ± 0.1
			Sweet Liqueurs and Vermouth		
5	Raspberry liqueur	1031.4 ± 2.8	0.0562 ± 0.0021	17.79	62.4 ± 2.6
6	Vermouth red	920.4 ± 14.9	0.0616 ± 0.0012	16.23	54.0 ± 2.1
7	Quince liqueur	511.9 ± 13.1	0.1883 ± 0.0030	5.31	45.0 ± 0.5
			Grape Brandies		
8	Brandy "Vinjak" VSOP	327.4 ± 1.8	0.1174 ± 0.0020	8.55	15.8 ± 0.8
9	Vecchia Romagna	145.9 ± 4.4	0.3077 ± 0.0084	3.21	13.9 ± 0.9
10	Brandy "Vinjak" VS	89.0 ± 2.5	0.4861 ± 0.0237	2.06	3.9 ± 0.2
11	Metaxa	67.6 ± 3.5	1.0000 ± 0.0000	1.00	14.8 ± 0.9
12	Stock '84	61.1 ± 3.6	1.0000 ± 0.0000	1.00	8.3 ± 0.4
			Plum Brandies		
13	"Mucenica 5"	153.7 ± 3.5	0.2727 ± 0.0053	3.67	7.0 ± 0.1
14	"Stomaklija"	86.2 ± 1.5	0.8219 ± 0.0270	1.22	9.5 ± 1.3
15	"Lincura"	43.4 ± 1.5	1.0000 ± 0.0000	1.00	4.9 ± 0.2
16	"Mucenica"	8.4 ± 0.9	1.0000 ± 0.0000	1.00	1.0 ± 0.0
			Whiskeys		
17	Grants	74.3 ± 1.1	1.0000 ± 0.0000	1.00	5.0 ± 0.2
18	Label 5	40.1 ± 3.5	1.0000 ± 0.0000	1.00	3.7 ± 0.0

[a] Data are presented as means ± SD. [b] % - mL of sample per mL of solution. [c] Reciprocal value of dilution corresponding to 50% decrease of DPPH radical absorption. [d] Decrease of anodic oxidation current of hydrogen peroxide obtained upon addition of 500 μL of beverages.

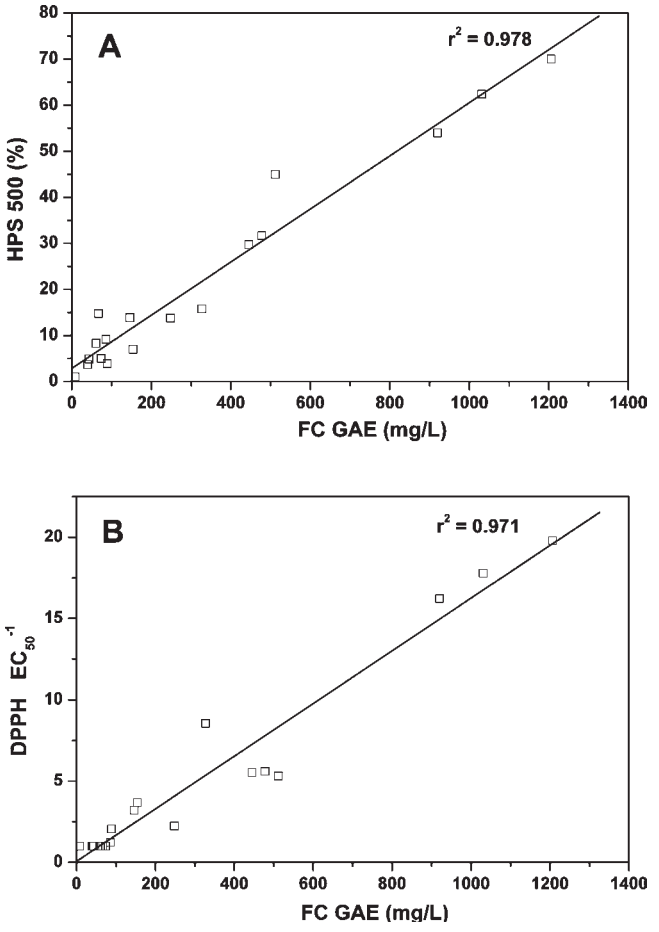

Figure 3. (**A**) Relationship between total phenolic content (FC GAE) and decrease of hydrogen peroxide anodic oxidation current upon addition of 500 μL of beverages (HPS500 (%)) (r^2 = 0.978) (**B**) Relationship between total phenolic content (FC GAE) and DPPH scavenging activity (r^2 = 0.971).

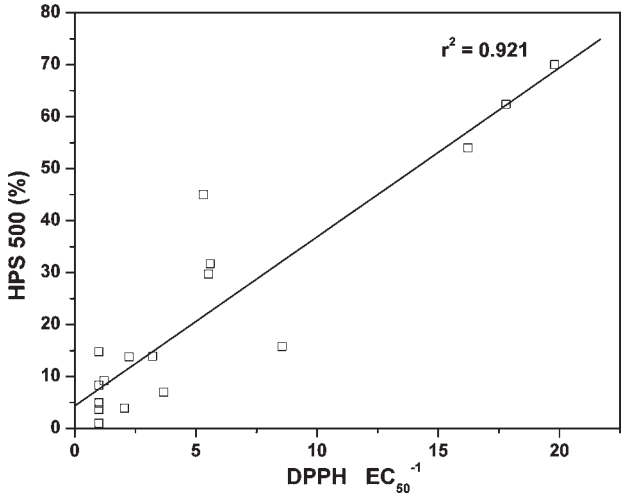

Figure 4. Relationship between decrease of hydrogen peroxide anodic oxidation current upon addition of 500 μL of tested samples (HPS500 (%)) and DPPH scavenging activity (r^2 = 0.921).

brandies aged for different periods of time (**Figure 5**). Higher volumes of aliquots have been chosen as optimal since plum brandy aged in already used oak barrels. Effect of plum brandies aged for various aging periods in oak barrels on peroxide anodic current decrease was compared with DPPH scavenging activity

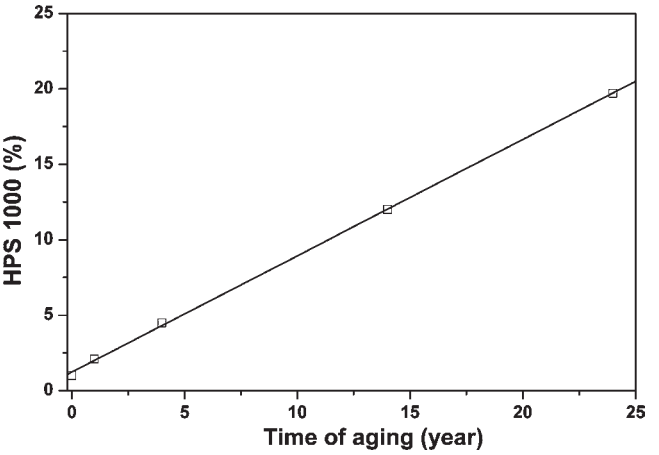

Figure 5. Aging of plum brandy in oak barrels followed by HPS assay (HPS1000 (%) vs time of aging).

Table 2. Aging of Plum Brandy in Oak Barrels for 24, 14, 4, and 1 Year Followed by FC, DPPH and HPS Assay in Comparison with Plum Brandy Stored in Inox Tank (Control)

aging (year)	FC GAE (mg/L)	DPPH EC_{50} (mL/mL)	DPPH EC_{50}^{-1}	HPS1000 (%)
24	138.1 ± 2.7	0.3793 ± 0.0053	2.64	19.7 ± 1.2
14	86.6 ± 3.6	0.6633 ± 0.0067	1.51	12.0 ± 1.1
4	34.9 ± 2.1	1.0000 ± 0.0000	1.00	5.6 ± 1.0
1	13.4 ± 1.4	1.0000 ± 0.0000	1.00	2.1 ± 0.3
0	8.0 ± 1.2	1.0000 ± 0.0000	1.00	1.0 ± 0.0

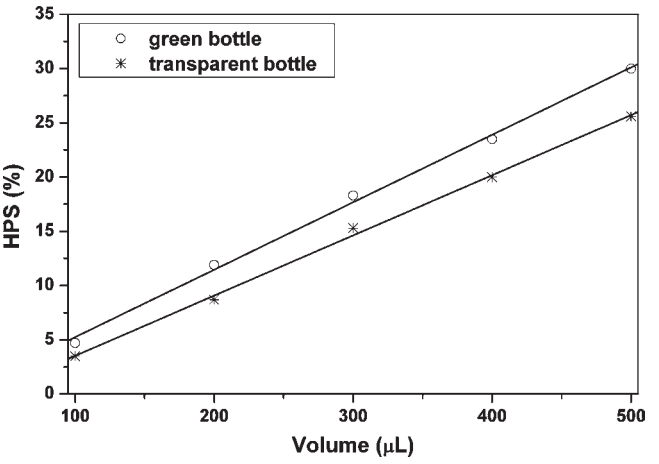

Figure 6. Effect of light on hydrogen peroxide anodic oxidation current (HPS (%)) of herbal liqueur Bitter 54 stored in green and transparent bottles.

and total phenolic content (**Table 2**). The increase of AO activity estimated by both assays is in accordance with total phenolics estimated by FC.

Increase of HPS1000 (%) as a function of time of aging in old oak barrels is shown in **Figure 5**. A linear relationship between total AO activity, expressed as HPS1000 (%), and time of aging was observed. Efficiency of the maturation process with respect to development of AO activity might be estimated based on slope of obtained linear curve of aging. The slope of the curve may depend on aging conditions related to both barrel wood and cellar environment.

The influence of bottle storage on the AO activity of herbal liqueurs was investigated in order to establish the effect of storage conditions. Applicability of the assay to follow changes of AO activity during storage in green or transparent bottles under

different conditions was demonstrated. After one year storage in the light, Bitter 54 (**Figure 6**) as well as Bitter 55 charged in a transparent bottle loses its activity. The AO activity of both Bitter 54 and Bitter 55 was preserved in a dark bottle either in the light or in the dark during a one year period. Results of our current research, related to surveying of changes of phenolic compounds and anthocyanins during twelve month storage of different liqueurs in different conditions, are under preparation.

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