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ANTIOXIDANT ACTIVITY ASSESSMENT IN FRUIT LIQUORS AND SPIRITS: METHODS COMPARISON

AVALIAÇÃO DA ATIVIDADE ANTIOXIDANTE EM LICORES DE FRUTOS E AGUARDENTES: COMPARAÇÃO DE MÉTODOS

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SUMMARY

During the last few years, Portugal has seen an increasing trend in the development of new fruit-based products, such as liquors and spirits. The antioxidant capacity of fruit liquors and spirits, sourced commercially or produced on a pilot-scale, was assessed using two simple spectrophotometric methods: the 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) assays. The major goal of this work was to find which of the two methods is the most suitable and accurate in determining antioxidant capacity for fruit liquors and spirits. In addition, the total phenolic compounds of both kinds of alcoholic beverages were determined by Folin-Ciocalteau method. Despite the widespread use of DPPH assay for distilled drinks, a weak Spearman correlation coefficient (r = 0.023) between ABTS and DPPH methods in spirits were found, as well as, between DPPH values and total phenolic compounds content (r = 0.239). This work highlights the limitations of DPPH assay when used for analysis of samples with very low antioxidant capacity, as distilled spirits.

RESUMO

Portugal, nos últimos anos, tem desenvolvido novos produtos à base de frutos, tais como aguardentes e licores. A capacidade antioxidante dos licores de frutos e aguardentes, obtidos comercialmente ou em produções à escala piloto, foi medida usando dois métodos espetrofotométricos: o 2,2´-azino-bis-(3-etilbenzotiazolina-6-ácido sulfónico) (ABTS) e o 1,1-difenil-2-picrilhidrazil (DPPH). O principal objetivo deste trabalho consistiu em determinar qual o método mais fiável na determinação da capacidade antioxidante total das aguardentes e licores. Adicionalmente, o conteúdo total em compostos fenólicos de ambos os tipos de bebidas alcoólicas foi determinado pelo método de Folin-Ciocalteau. Embora o DPPH seja o método mais utilizado neste tipo de análises, no presente trabalho, no entanto, verificou-se que o coeficiente de correlação de Spearman (r=0.023) entre o ABTS e DPPH é baixo, bem como entre os valores de DPPH e o teor de compostos fenólicos totais (r=0.239). Este trabalho destaca assim as limitações do método de DPPH quando usado em amostras com baixa capacidade antioxidante, como é o caso dos licores de frutos e das aguardentes.

Key words: fruit liquors and spirits, total antioxidant activity, ABTS, DPPH, total phenolic compounds.
Palavras-chave: licores de frutos e aguardentes, capacidade antioxidante total, ABTS, DPPH, compostos fenólicos totais.

INTRODUCTION

The production of distillates (spirits) from fermented fruits, as well as, fruit liquors has been widely practiced around the world, including Portugal, for several centuries.

There are several studies focused on the evaluation of total antioxidant compounds in fruits (Liu *et al.*, 2002; Céspedes *et al.*, 2010; Huang *et al.*, 2012). Antioxidant capacity is related to the ability to capture the free radicals, being an important defence mechanism of living systems, in opposition to oxidative stress (Valko *et al.*, 2006; Oliveira *et al.*, 2009). Abundant evidence suggests that oxidative stress is a major cause of aging and several chronic diseases, including cancer, diabetes, cardiovascular

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disease, Alzheimer's disease, and other neurodegenerative disorders (Halliwell, 1994; Giacco and Brownlee, 2011). Many antioxidant compounds, such as polyphenols, possess anti-inflammatory, antiatherosclerotic, antiproliferative, antimutagenic, anticarcinogenic, antibacterial, or antiviral activities to a greater or lesser extent (Liu *et al.*, 2002; Ratnam *et al.*, 2006).

During the production of alcoholic beverages from fruits or from fermented fruits, such as spirits or liquors, it will be expected that some antioxidant compounds, such as phenolic compounds, remain in these beverages (Mrvcic *et al.*, 2012). Furthermore, aging in wooden barrels is a major source of phenolic compounds in alcoholic beverages, such as rum, wine, whiskey, brandy and "cachaça" (Mosedale and Puech, 1998; Goldberg *et al.*, 1999; Arnous *et al.*, 2001; Da Silva *et al.*, 2009). These beverages have variable content of phenolic compounds that can be related to their antioxidant capacity (Vicente *et al.*, 2011). In fact, the presence of low molecular weight phenolic compounds, such as vanillin, syringaldehyde, syringic acid, vanillic acid, gallic acid, coumarin, scopoletin and furanic compounds has been reported (Goldberg *et al.*, 1999; Arnous *et al.*, 2001; Madrera *et al.*, 2003; De Aquino *et al.*, 2006; Da Silva *et al.*, 2009).

A wide range of spectrophotometric assays have been adopted to measure antioxidant capacity of food and the most popular alcoholic beverages, being the chemical methods using 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) assays the most commonly performed (Brand-Williams *et al.*, 1995; Kim *et al.*, 2002; Canas *et al.*, 2008; Floegel *et al.*, 2011).

Most of these chemical methods for determining antioxidant capacity employ the same principle: a synthetic coloured radical or redox-active compound is generated; and the ability of a biological sample to scavenge the radical or to reduce the redox-active compound is monitored using a UV-Vis, applying an appropriate standard to quantify antioxidant capacity. The approach is based on electron transfer and involves the reduction of a coloured oxidant, as $ABTS^{\bullet+}$ and $DPPH^{\bullet}$, which includes a change in colour that can be spectrophotometrically quantified (Brand-Williams *et al.*, 1995; Kim *et al.*, 2002). These easy and economic methods to evaluate radical scavenging activity of antioxidants, since the radical compounds are stable and need not be generated, are very convenient in their applications; nevertheless, they present several limitations, including the promiscuity of reactions of $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ as they use non physiological radicals (Floegel *et al.*, 2011). Also, $DPPH^{\bullet}$ is sensitive to some Lewis bases and solvent types, as well as, oxygen (Ancerewicz *et al.*, 1998).

Generally, DPPH assay has been used to assess antioxidant capacity of spirits such as Cognac (Da Porto *et al.*, 2000), Portuguese brandies (Canas *et al.*, 2008) and aged sugar cane spirits (Vicente *et al.*, 2011).

As far as we know from the scientific literature, a performance comparison of ABTS and DPPH has not been, previously, assessed using fruit liquors and spirits in similar, but adjusted, experimental conditions. Hence, the major goal of this research was to compare the efficiency of ABTS and DPPH methods to evaluate the antioxidant capacities of fruit liquors and spirits. In addition, the total phenolic compounds of both categories of alcoholic beverages studied were determined.

MATERIAL AND METHODS

Chemicals

2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$), 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), potassium persulfate and Folin-Ciocalteau reagent were obtained from Sigma–Aldrich (St. Louis, MO, USA). All the other chemicals used were of analytical grade.

Samples

The samples used in the assays are described in Table I. The ethanol content of spirits was determined by electronic densimetry (OIV, 2014) by using an electronic densimeter (Antoon Paar DMA 5000, 2002, Austria). The ethanol content of fruit liquors was determined by using an automatic distiller-extractor apparatus (Dujardin-Salleron, DE 2000 model, 2004, France) followed by aerometry of the obtained distillates.

Pilot-scale production of fruit spirits and liquors

Spirits were obtained, separately, in a pilot-scale production. The distillation was performed in a 16 dm^3 copper Charentais alembic. The first part with approximately 5% (above 70 % vol., with a strong, pungent and unpleasant flavour) of the distillates was collected as head fraction and was discarded. The heart fractions (spirits – Table I), obtained by single distillation, were collected when the ethanol concentration varied from 70 to 35 % v/v; finally, the tail fractions were obtained and discarded when the alcoholic content decreased below 35% v/v. The alcoholic fermentation of each fruit occurred under controlled conditions. The wine spirits were obtained from red table wine of Marselan grape variety with 14 % vol.

Fruit liquors (Table I) were obtained, both from pilot-scale production and commercial sources. The pilot-scale production was done with maceration and extraction between each fruit (previously washed and cut in small slices) and wine spirit (previously adjusted to 45 % vol.) without wood aging.

TABLE I

Codification of fruit liquors and spirits

Codificação dos licores de fruto e das aguardentes

Sample code	Description	Origin	Alcohol degree (% vol.)
Spirits			
S1	Wine spirit	Psp	40.0
S2	Wine spirit	Psp	55.0
S3	Apple	Psp	40.0
S4	Persimmon	Psp	49.8
S5	Fig	Psp	44.9
S6	Fig	Psp	44.3
S7	Cherry	Psp	53.4
S8	Passionfruit	Psp	45.0
S9	Pineapple with shell	Psp	37.6
S10	Pineapple shelled	Psp	42.4
S11	Mango with shell	Psp	42.0
S12	Mango shelled	Psp	39.5
S13	Banana	Psp	45.3
S14	Persimmon	Psp	40.0
S15	Persimmon	Psp	40.0
Fruit liquors			
L1	Green walnut	Psp	18.5
L2	Green walnut	Psp	18.5
L3	Green walnut	Psp	18.1
L4	Green walnut	Psp	18.1
L5	Fig	C	17.0
L6	Carob	C	17.0
L7	Carob	C	20.0
L8	Passionfruit	C	15.0
L9	Mulberry	C	20.0

C = commercial source; Psp = Pilot-scale production

Determination of antioxidant activity

ABTS assay

$ABTS^{\bullet+}$ radical-scavenging activity of the hydrophilic fractions was determined by a procedure reported by Miller and Rice-Evans (1997) with slight modifications. The $ABTS^{\bullet+}$ solution was prepared by mixing 7 $mmol.dm^{-3}$ of ABTS salt with 3 $mmol.dm^{-3}$ of potassium persulfate, and the final volume was adjusted to 25 cm^3 with distilled water. The solution was held at room temperature, in the dark, for 16 h before use. The $ABTS^{\bullet+}$ solution was diluted with water, in order to obtain an absorbance between 0.680 and 0.720 at 734 nm, using a Xion 500 spectrophotometer UV-Vis (Hach Lange, 2002, Germany). $ABTS^{\bullet+}$ solution was freshly prepared for each analysis. Antioxidant or standard solutions, 50 µL (corresponding to 2500 $mg.dm^{-3}$), were mixed with 1 mL of diluted $ABTS^{\bullet+}$ solution and incubated at 30 ºC (± 1 ºC) in a thermostatically-controlled water bath (Selecta, Precisterm 20L, 2008, Spain). The absorbance at 734 nm was read after 6 min under dim light conditions. Ethanol (95%) was used as a blank. A standard curve was performed for each assay, using Trolox (1 $mmol.dm^{-3}$; 0 150 $\mu mol.dm^{-3}$; $R^2 > 0.990$). All experiments were performed three times and in triplicate at controlled temperature of 30 ± 1 ºC. The free-radical scavenging activity was expressed as micromoles of Trolox per milliliter of sample ($\mu mol\ TE.cm^{-3}$).

DPPH assay

The antioxidant activity of the samples and standard (Trolox) was determined by the radical scavenging activity method using 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) as, previously described by Brand–Williams *et al.* (1995) with slight modifications. Briefly, 50 mm^3 aliquots of samples were added to 1.95 cm^3 of a $DPPH^{\bullet}$ methanolic solution (0.4% m/v). The blank sample consisted of 50 mm^3 of methanol added to 1.95 cm^3 of $DPPH^{\bullet}$. After a 30 min incubation period at room temperature (22.0 ± 1.0 ºC) in the dark, the absorbance was measured at 517 nm (Sharma and Bhat, 2009), using a Xion 500 spectrophotometer UV-Vis (Hach Lange, 2002, Germany) under dim light conditions. A standard curve was performed for each assay, using Trolox (1 $mmol.dm^{-3}$; 0 150 µmol; $R^2>0.990$). All experiments were performed three times and in triplicate and the free-radical scavenging activity was expressed as micromoles of Trolox per milliliter of sample ($\mu mol\ TE.cm^{-3}$).

Phenolic compounds evaluation

Total phenolic compounds were estimated by the Folin-Ciocalteau method, a colorimetric assay based on procedures described by Singleton and Rossi

(1965). Briefly, 50 mm^3 of sample was mixed with 0.95 cm^3 of 7.5 % sodium carbonate freshly prepared solution. After 30 seconds, 1 cm^3 of Folin Ciocalteu's phenol reagent was added. The reaction was kept in the dark, with the reaction tubes place on a thermostatically-controlled water bath at 40 ± 1.0 ºC for 30 min. The absorbance was read using glass cuvettes, at 765 nm, using a Xion 500 spectrophotometer UV-Vis (Hach Lange, 2002, Germany) under dim light conditions. Gallic acid (1 $mmol.dm^{-3}$) was used as a standard to construct a standard curve (0 20 µmol; $R^2 \geq 0.990$) and 20 mm^3 ethanol 95% m/v was added to the blank (30 mm^3 deionised water, mixed with 0.95 cm^3 of 7.5 % sodium carbonate and 1 cm^3 of Folin Ciocalteu's phenol reagent added after 30 s). All experiments were performed three times and in triplicate. Results were expressed as gallic acid equivalents per amount sample ($GAE.cm^{-3}$).

Statistics

Results are presented as mean ± SEM (standard error of the mean) of three independent experiments performed in triplicate. Spearman-Rho coefficients were calculated using the software IBM SPSS 20.0 (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

The ethanol contents of the fifteen spirits showed values ranging from 37.6 to 55.0 % v/v and all of them were above the legal limit level (≥ 37.5 % v/v). The ethanol contents of the nine fruit liquors showed values ranging from 15.0 to 20 % v/v and all of them were above the legal limit level (> 15.0 % v/v) (Regulation EC n. 110/2008).

Most techniques used for determining antioxidant activity, such as, ABTS, DPPH, FRAP (ferric reducing ability of plasma) and ORAC (oxygen radical absorbance capacity) assays, showed high correlation with total phenolic content in different fruits (and plant extracts) (Dudonné *et al.*, 2009; Floegel *et al.*, 2011). Phenolic compounds, are the most abundant secondary metabolites in fruits, responsible for their antioxidant activity (Macheix *et al.*, 1990). Also, high correlation between total phenolic content and antioxidant activity as determined by FRAP or electron spin resonance spectroscopy were reported in fruit juices (Gardner *et al.*, 2000). In the present work, the samples used are made from fruit and were not wood aged, for this reason, their antioxidant activity and total phenolic content derive only from the fruits.

Armagnac, Cognac and other aged brandies are rich in phenolic compounds due to their maturation in wooden barrels (Viriot *et al.*, 1993; Canas, 2003). DPPH method is commonly used to evaluate the antioxidant capacity of distilled drinks aged in wood (Da Porto *et al.*, 2000; Aoshima *et al.*, 2004). Nevertheless, it was verified that the use of this method in the present work for the evaluation of spirits antioxidant capacity was not successful, since samples with low amount of antioxidants and phenolic compounds assessed by Folin-Ciocalteau method (Singleton and Rossi, 1965) presented non detectable antioxidants using DPPH method (Table II).

TABLE II

Antioxidant capacity of spirits and their total phenolic content

Capacidade antioxidante e compostos fenólicos totais das aguardentes

	Antioxidant capacity (µmol TE.cm^{-3})		**Total Phenolic Content**
Sample code	**ABTS assay**	**DPPH assay**	**(µmol Eq GAE.cm^{-3})**
S1	959.4 ± 42.5	n.d.	556.4 ± 32.8
S2	989.8 ± 127.4	n.d.	489.0 ± 3.2
S3	97.6 ± 2.5	n.d.	193.7 ± 6.6
S4	50.2 ± 2.3	n.d.	72.3 ± 4.8
S5	359.4 ± 10.6	n.d.	365.7 ± 8.0
S6	88.6 ± 4.0	n.d.	164.5 ± 8.2
S7	80.1 ± 2.9	n.d.	199.6 ± 8.8
S8	199.3 ± 13.6	n.d.	250.1 ± 7.2
S9	139.5 ± 17.7	n.d.	250.2 ± 6.2
S10	290.0 ± 4.6	n.d.	419.1 ± 7.1
S11	154.4 ± 12.4	n.d.	441.4 ± 3.4
S12	221.3 ± 11.2	n.d.	195.2 ± 4.0
S13	185.9 ± 13.6	n.d.	216.0 ± 5.2
S14	70.1 ± 3.9	n.d.	57.1 ± 4.1
S15	71.4 ± 4.4	n.d.	39.6 ± 0.9

Data are mean value ± SEM; n.d. - not detected

Since pH was maintained in the range of 5.0 – 6.5 (Molyneux, 2004), its effects were discarded (Blois, 1958). Therefore, pH seemed not to influence the results achieved, conversely to some assays. This was not the first time we faced this problem with distilled spirits in our lab, so, we used different reagent sources for the different assessments and ethanol was used as the control without interference in the results obtained. Ascorbic acid was also used as standard; however due to its lability, the results were instead expressed in terms of Trolox equivalents (TE).

For fruit liquors containing a large content of phenolic compounds, the DPPH method detected antioxidant capacity with similar results to ABTS method (Table III). Nevertheless, the sample with lower content of phenolic compounds presented a non-detectable value for Trolox equivalents (L6, carob liquor with 17 % ethanol (v/v), obtained commercially). Likewise, in all the samples analyzed – sourced commercially or produced in a pilot-scale – the antioxidant activity evaluated with the DPPH method was not coherent with total phenolic content (Tables II and III). Therefore, a similar spectrophotometric method in which $ABTS^{\bullet+}$ is solubilized in distilled water was performed (Tables II and III).

TABLE III

Antioxidant capacity of fruit liquors and their total phenolic content

Capacidade antioxidante e compostos fenólicos totais dos licores de fruto

	Antioxidant capacity (µmol TE.cm^{-3})		Total Phenolic Content (µmol Eq GAE.cm^{-3})
Sample code	**ABTS assay**	**DPPH assay**	
L1	1285 ± 23.6	2371 ± 72.6	12800 ± 41.24
L2	1274 ± 23.6	2364 ± 72.8	12800 ± 55.94
L3	1275 ± 23.5	2395 ± 77.8	12800 ± 40.76
L4	1251 ± 27.6	2275 ± 101.5	12900 ± 35.66
L5	1272 ± 24.0	n.d.	12700 ± 16.41
L6	139.7 ± 10.6	n.d.	610 ± 13.33
L7	1307 ± 24.3	2543 ± 35.4	13200 ± 19.72
L8	1151 ± 28.2	659.7 ± 89.3	6900 ± 14.48
L9	1301 ± 23.7	2510 ± 56.5	13000 ± 23.78

Data are mean value ± SEM; n.d. - not detected.

Total phenolic content was correlated with antioxidant activity of fruit liquors and spirits and the Spearman-Rho coefficients were calculated (Tables IV and V).

TABLE IV

Spearman-Rho coefficients for the correlation between antioxidant capacities measured by ABTS, DPPH and total phenolic content of fifteen spirits

Coeficiente de correlação de Spearman-Rho entre a capacidade antioxidante obtida por ABTS, DPPH e compostos fenólicos totais em quinze aguardentes

	ABTS	DPPH	Total Phenolic Content
ABTS	1	0.023	0.448*
DPPH	0.023	1	0.239
Total Phenolic Content	0.448*	0.239	1

* Correlation is significant at α<0.05 (2-tailed).

Gorinstein *et al.* (2010) reported a high correlation between polyphenols content in three exotic fruits and antioxidant capacities measured by ABTS and DPPH assays. Dudonné *et al.* (2009) reported a strong positive correlation between ABTS and DPPH assays with a Pearson correlation coefficient of r = 0.906 when used for 30 aqueous plant extracts.

TABLE V

Spearman-Rho coefficients for the correlation between antioxidant capacities measured by ABTS, DPPH and total phenolic content of nine fruit liquors

Coeficiente de correlação de Spearman-Rho entre a capacidade antioxidante obtida por ABTS, DPPH e compostos fenólicos totais em nove licores de fruto

	ABTS	DPPH	Total Phenolic Content
ABTS	1	0.883**	0.814**
DPPH	0.883**	1	0.712*
Total Phenolic Content	0.814**	0.712*	1

**Correlation is significant at α<0.01 (2-tailed); * Correlation is significant at α<0.05 (2-tailed).

In the present study, it was observed that using ABTS assay the antioxidant capacity of the spirit samples was consistent with total phenolic content (Tables II and IV). Similar results were observed for fruit liquor samples (Tables III and V).

In addition, in the present study a greater correlation between total phenolic content and ABTS assay than with DPPH assay was found. On the other hand, when the correlation between antioxidant capacities measured by ABTS, DPPH and total phenolic content in spirits was determined a weak correlation was found, between antioxidant capacity evaluated by ABTS and DPPH methods ($r = 0.023$) and DPPH antioxidant activity and total phenolic content ($r = 0.239$), evaluated by Folin-Ciocalteu method. Antioxidant activity determined using ABTS assay had a slightly better and significant correlation with total phenolic content ($r = 0.448$; $\alpha < 0.05$).

Towards fruit liquors (Table V), a Spearman-Rho correlation coefficient of $r = 0.883$ ($\alpha < 0.01$) for the relationship between ABTS and DPPH assays was found. Additionally, the correlation between ABTS and total phenolic content was significantly high ($r = 0.814$; $\alpha < 0.01$) and stronger than the correlation between ABTS and total phenolic content ($r = 0.712$; $\alpha < 0.05$).These results suggest that in the case of fruit liquors both methods, ABTS and DPPH, are suitable to determine the total antioxidant capacity.

Hence, in this study, the total antioxidant capacity of the fruit liquors and spirits revealed a good correlation with the total content of phenols and, thus, these phenolic compounds seem to be responsible for the antioxidant potential of the samples. Furthermore, our results suggest that, in agreement with Floegel *et al.* (2011), who studied fruits, vegetables and beverages (fruit juices, tea, beer and table wine), the ABTS assay provides a better antioxidant capacity estimate in spirits than the DPPH assay. Based on these results, the evaluation of antioxidant activity of several spirits, including spirits from *Arbutus unedo* L. fruits, using ABTS method, is an ongoing work that will be released in a further publication.

In the future, it would be desirable to expand the total antioxidant capacity and total phenolic compounds evaluation, using ABTS and Folin-Ciocalteu assays, to as many alcoholic fruit beverages as possible, with the objective of building a database and making this information widespread.

CONCLUSIONS

The antioxidant capacity of fruit liquors and spirits, sourced commercially or produced on a pilot-scale, was assessed using two simple spectrophotometric methods, ABTS and DPPH assays. Furthermore, the total phenolic compounds of both kinds of alcoholic beverages were determined by Folin-Ciocalteau method. A weak Spearman correlation coefficient ($r = 0.023$) between ABTS and DPPH methods in spirits were found, as well as, between DPPH values and total phenolic compounds content ($r = 0.239$). This work emphasises the limitations of DPPH assay when used for analysis of samples such as distilled spirits with very low total antioxidant capacity.

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