

INFLUENCE OF THE STORAGE ON BIOACTIVE COMPOUNDS AND SENSORY ATTRIBUTES OF HERBAL LIQUEUR

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Stability of bioactive compounds present in a herbal liqueur, as well as its sensory attributes were surveyed during a year – long storage under various conditions. The antioxidant activity, the total content of phenolic and flavonoid compounds, their antioxidant effectiveness, as well as antibacterial and antifungal activity, were found dependent on storage conditions and duration. The storage in the opaque green and transparent white bottles caused statistically significant changes upon 60 and 15 days, respectively. Both decrease of antioxidant activity ($\approx 30\%$) and total content of phenolic compounds ($\approx 7\%$) upon one year storage in the original opaque bottles placed in cardboard box was 18 % lower than upon the storage in the white bottles exposed to daylight, while decrease of total flavonoid compounds ($\approx 16\%$) was 26 % lower. The most prominent changes were observed within the first six month of the storage. During the remaining storage time monitored parameters were found almost constant. Sensory analysis confirmed dynamics of changes that herbal liqueur experienced during the storage.

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1. Introduction

Herbal spirits are considered functional beverages. Extracts of aromatic and medicinal herbs used to produce herbal spirits have beneficial effects on human metabolism (Veljković and Stanković 2003). Health promoting effect of herbal spirits (when consumed moderately) has been attributed to bioactive compounds derived from plant material, mostly different polyphenolic antioxidants (AOs). Herbal liqueurs are defined as spirits with predominantly bitter taste, made from ethyl alcohol, sugar, water, fruit juices and herbal extracts with minimum 15 % volume of alcohol (Official journal of European Communities for spirit drinks 1989). They are consumed as an important part of traditional gastronomy in the southeast Europe.

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Various alcoholic beverages such as wine (Heinonen et al. 1998; Gorjanović et al. 2010a), beer (Gorjanović et al. 2010b), some brandies, distilled spirits and fruit liqueurs (Gorjanović et al. 2010c) have significant content of total phenolics (TPC), while rum, vodka, gin and other distillates are depleted of AOs (Schwarz et al. 2009; Gorjanović et al. 2010c). Substantial attention was focused on AOA of herbal liqueurs - cocktails of biologically active phytochemicals (Heinonen et al. 1998; Vacca et al. 2003; Alamprese et al. 2005; Gorjanović et al. 2010c; Li and Beta 2011, Komes et al. 2011). Strong alcoholic beverages commonly consumed in Serbia, including herbal and fruit liqueurs, were screened for AOA (Gorjanovic and other 2010c), using recently developed hydrogen peroxide scavenging (HPS) assay (Sužnjević et al. 2011). Radical scavenging activity against DPPH and HPS activity were in good correlation with TPC (Gorjanović et al. 2010c).

Antimicrobial activity (AMA) was proved to be linked to phenolic moiety. Alcoholic extracts of medicinal and aromatic plants displayed higher ABA and AFA than aqueous ones (Singh and Jain 2011), indicating that phytochemicals are more effective against microorganisms when combined with alcohol. Wines AMA (Radovanović et al. 2009) were investigated more thoroughly than effect of various other alcoholics containing phenolics. Addition of fruit and herb extracts further enforced wine AMA (Joshi and Siby 2002). Raspberry, cinnamon and peppermint-enriched wines, as well as raspberry enriched vodka, exhibited superior ABA compared to the plain beverages (Lin et al. 2005).

Effect of storage on phenolic content and AOA of conventional and ecological wines (Zafrilla et al. 2003; Mulero et al. 2009) and red myrtle liqueurs (Vacca et al. 2003) was surveyed. Until now, storage effect on AMA of alcoholic beverages was not reported. Stability of plant bioactive compounds was influenced by various storage parameters (Turker et al. 2004; Harbourne et al. 2011).

Determination of shelf life of beverages rich in phytochemicals and establishment of optimal storage conditions should be based on surveying of bioactive compounds changes. The aim of the present study was to evaluate influence of storage on TPC and TFC, AOA, ABA and AFA of commercial domestic herbal liqueur "Bitter 54", which is chosen as the represent of herbal liqueurs. Correlations between AOA, TPC and TFC were assessed. Antioxidant effectiveness of phenolics and flavonoids was calculated, in order to achieve more comprehensive understanding of changes occurred during the period of storage. Sensorial analysis was conducted, to enable better insight into the dynamics of investigated product changes.

2. Experimental

2.1 Chemicals

Methanol was obtained from Zorka-Pharma (Šabac, Serbia). Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazil (DPPH), gallic acid and rutin were obtained from Sigma (St. Louis, MO) while sodium carbonate, potassium acetate and aluminium chloride from Merck-Alkaloid (Skopje, FYR Macedonia).

2.2 Herbal liqueur „Bitter 54“ preparation and conditions of storage

Commercial herbal liqueur "Bitter 54" contains following extracts of aromatic herbs and fruits: *Paris quadrifolia*, *Polygonum aviculare*, *Teucrium montanum*, *Salvia officinalis*, *Achillea millefolium*, *Mentha piperita*, *Thymus serpyllum*, *Thymus vulgaris*, *Matricaria chamomilla*, *Teucrium chamaedrys*, *Artemisia absinthium*, *Melissa officinalis*, *Hibiscus brackenridgei* Gray, *Eugenia caryophyllata*, *Pimpinella anisum*, *Cinnamomum zeylanicum*, *Vanilla planifolia* Jacks, *Rosa canina*, *Juniperus communis*, *Ceratonia siliqua*, *Origanum vulgare*, *Hypericum perforatum*, *Plantago lanceolata*, *Arctostaphylos uva ursi*, *Morus alba*, *Rosmarinus officinalis*, *Alchemilla vulgaris*, *Ocimum basilicum*, *Sambucus nigra*, *Equisetum arvense*, *Capsella bursa-pastoris*, *Sena alexandrina* Mill., *Rubus fruticosus*, *Betula* sp., *Crataegus oxyacantha*, *Viscum album*,

Foeniculum vulgare Mill., *Erythraea centaurium* Pers., *Viola tricolor*, *Quercus* sp., *Calendula officinalis*, *Urtica dioica*, *Tussilago farfara*, *Anagallis arvensis*, *Taraxacum officinale* Web., *Euphorbia cyparissias*, *Ficus carica*, *Vitis vinifera*, *Prunus domestica*, *Pirus malus*, *Rubus idaeus* *Citrus aurantium*, *Citrus limonum* Risso and *Citrus paradise* Macfad. „Bitter 54” contains 35 % volume of alcohol and 75 g/L of sugar. Total extract content is 88.2 g/L. Additive E160d (caramel) is used to gain dark-brown color. Freshly prepared commercial herbal liqueur “Bitter 54” has been obtained directly from producer (Top Spirit-Belgrade in cooperation with Faculty of Agriculture, University of Belgrade, Serbia).

In order to examine the effect of storage conditions on freshly prepared herbal liqueur “Bitter 54” (sample B54 first day of experiment) was compared with samples stored in: i) original packaging (opaque green bottles) in cardboard box (B54), ii) the original packaging with the presence of air (bottles filled to half volume, without cover, in a cardboard box (B54A), iii) the original packaging exposed to the effects of daylight (B54GL) and iv) bottles of white transparent glass exposed to daylight (B54WL). The experiment lasted one year. During the first four months measurements were done every fifteen days and after that once per a month. All samples were kept at ambient temperature.

2.3 Determination of total content of phenolic compounds (TPC)

The total content of phenolic compounds of “Bitter 54” was determined using Folin-Ciocalteu reagent (Singleton and Rossi 1965) with gallic acid as a standard. The reagent (1 mL) was mixed with liqueur diluted with methanol (1:4) (0.2 mL). Aqueous solution of sodium carbonate (0.8 mL, 7.5 %) was added. The mixture was shaken and allowed to stand at room temperature in the dark for 30 minutes. The absorbance was measured at 765 nm and TPC expressed as μg gallic acid equivalents (GAE) per mL.

2.4 Determination of total flavonoid compounds (TFC)

The total flavonoid compounds of “Bitter 54” was determined by the aluminum chloride colorimetric method (Chang et al. 2002), with rutin as standard. Liqueur samples diluted with methanol (1:4) (2 mL) were mixed with 10 % aluminum chloride (0.1 mL), 1 M potassium acetate (0.1 mL) and distilled water (2.8 mL). After incubation at room temperature for 30 minutes, absorbance was measured at 415 nm and TFC expressed as μg rutin equivalents (RE) per mL.

2.5 Antioxidant activity (AOA)

The stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was used to determine free radical-scavenging activity (Choi et al. 2002). Liqueur samples (2.5 mL) in concentrations from 20 to 100 μL liqueur/mL of the methanol solutions were mixed with 3×10^{-4} M DPPH solution in methanol (1.0 mL). After 30 minutes staying at room temperature in the dark the absorbance was measured at 517 nm. DPPH scavenging capacity (%) was calculated using the following equation:

$$\text{DPPH scavenging capacity (\%)} = 100 - \frac{(A_{\text{sample}} - A_{\text{blank}})}{A_{\text{control}}} \cdot 100$$

where A_{sample} , A_{blank} , A_{control} are absorbances of sample containing DPPH, methanol (1.0 mL) plus sample (2.5 mL), and DPPH solution (1.0 mL) plus methanol (2.5 mL), respectively. Efficient concentration (EC_{50}) was calculated as concentration necessary to provoke 50 % decrease of DPPH radical concentration.

As a measure of AOA, EC_{50} has the disadvantage that the higher AOA, the lower the value of EC_{50} . Thus, reciprocal value of EC_{50} (EC_{50}^{-1}) has been introduced. The phenol and flavonoid AO coefficients (PAC and FAC), i.e. their antioxidant effectiveness, were calculated as ratio of EC_{50}^{-1} (mL of solution/ μL liqueur), and TPC or TFC.

2.6 Antibacterial activity (ABA)

The modified microdilution method (Daouk et al. 1995) was applied to determine ABA of "Bitter 54" against Gram-negative bacteria *Listeria monocytogenes* (NCTC 7973), *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311) and *Enterobacter cloacae* (ATCC 13047), and Gram-positive bacteria *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240) and *Staphylococcus aureus* (ATCC 6538), obtained from the Institute for Biological Research "Siniša Stanković", Belgrade, Serbia. The bacterial suspensions were adjusted with sterile saline to concentration of 1.0×10^5 cfu/mL. The inocula were prepared daily and stored at 4 °C until use. Dilutions of the inocula were cultured on solid medium to verify the absence of contamination and to check the validity of the inoculum.

The minimum inhibitory and bactericidal concentration (MIC and MBC) was determined using 96-well microtiter plates. Samples were dissolved in broth LB medium with bacterial inoculum (1.0×10^4 cfu per well) to achieve concentration from 100 to 300 µL/mL. The microplates were incubated for 24 h at 37 °C. The lowest concentrations without visible growth (at the binocular microscope) were defined as MIC. Serial sub-cultivation of 2 µL of liqueur into microtitre plates containing 100 µL of broth per well were incubated for 72 h in order to determine MBC, defined as the lowest concentration with no visible growth (99.5 % killing of the original inoculums). The optical density of each well measured at 655 nm by Microplate manager 4.0 (Bio-Rad Laboratories) was compared with the blank (35 % ethanol) and the positive control (10 mg streptomycin/mL DMSO). Two replicates were done for each compound.

2.7 Antifungal activity (AFA)

"Bitter 54" AFA against *Aspergillus niger* (ATCC 6275), *A. ochraceus* (ATCC 12066), *A. versicolor* (ATCC 11730), *Penicillium funiculosum* (ATCC 36839), *P. ochrochloron* (ATCC 9112), *Cladosporium cladosporioides* (ATCC 13276), *C. fulvum* (TK 5318) and *Trichoderma viride* (IAM 5061) obtained from the Institute for Biological Research "Siniša Stanković" was determined using a modified microdilution technique (Daouk et al. 1995). The micromycetes were maintained on malt agar, the cultures were stored at 4 °C and sub-cultured once per a month. The fungal spores were washed from the surface of agar plates with sterile 0.85 % saline containing 0.1 % Tween 80 (v/v). The spore suspension was adjusted with the saline to a concentration of approximately 1.0×10^5 in a final volume of 100 µL per well. The inocula were stored at 4 °C for further use. Dilutions of the inocula were cultured on solid malt agar to verify the absence of contamination and to check the validity of the inoculum.

Serial dilutions using 96-well microtiter plates were prepared to determine MIC. Liqueur samples (100-300 µL/mL) were added in broth malt medium with inoculum. The microplates were incubated for 72 h at 28 °C, respectively. Serial subcultivation of 2 µL of liqueur into microtiter plates containing 100 µL of broth per well and further incubation 72 h at 28 °C was performed to determine minimal fungicidal concentrations (MFCs) (99.5 % killing of the original inoculums). Ethanol, 35 %, was used as a blank while positive control was commercial fungicide, 10 mg bifonazole per mL of diluted ethanol.

2.8 Sensory evaluation

The samples were subjected to sensory evaluation using the internal sensory panel of Faculty of Agriculture, University of Belgrade. The panel comprised of 15 highly trained members who had undergone extensive sensory training, and had previous experience in the assessment of herbal liquors and spirits. During one year storage five sessions were held. Liqueur (30 mL per evaluator) was served at 20 °C, in transparent glasses, covered with watch glass. Five attributes were evaluated: color intensity (max. 1 point), clarity (max. 1 point), bitter taste (max. 2 points), odour intensity (max. 6 points) and herbal-fruity taste (max. 10 points). The sensory properties were presented on a twenty -point scale (under 16 (very poor), 16 – 17 (moderate), 17 – 18 (good), over 18 (excellent)). The average point number for each of the attributes was calculated. In order

to present results by the radar charts the average point numbers are converted to the same maximum value (10).

2.9 Statistical analysis

All experiments were carried out in triplicates, except AMA which was carried out in duplicate. The results were presented as mean \pm standard deviation. Comparison of means was analyzed by Student's *t* test and differences were considered significant when $p < 0.05$.

3. Results and discussion

3.1 Influence of storage conditions on bioactive compounds present in "Bitter 54"

Herbal liqueur "Bitter 54" is produced by combination of extraction and distillation of 46 herbs, with addition of 8 fruit juices (Vukosavljević et al. 2009). Aromatic and medicinal plants used in its production such as mint, basil, lemon balm, sage, oregano, plantain, St John's wort, common yarrow, grand wormwood, centaury and nettle are rich in bioactive compounds with strong AOA (Chrprová et al. 2010, Stanisavljević et al. 2008). As the starting point of the present study, TPC and TFC, as well as AOA of freshly prepared liqueur has been determined. It contains $412.7 \pm 4.7 \mu\text{g GAE/mL}$ and $321.1 \pm 5.7 \mu\text{g RE/mL}$. Range of TPC in various strong alcoholics, white and red wine, and various beers was 8.4 – 1031, 155 – 2314, 125 – 544 $\mu\text{g GAE/mL}$, respectively (Gorjanović et al. 2010c,a,b), while in fruit wines and liqueurs (Heinonen et al. 1998), nocino liqueurs (Alamprese et al. 2005) and Korean lotus liqueur (Lee et al. 2005) was 91–1820, 239–3884 and 1063 $\mu\text{g GAE/mL}$, respectively. Freshly prepared "Bitter 54" exhibits prominent AOA ($\text{EC}_{50} = 25.1 \pm 0.48 \mu\text{L liqueur /mL of solution}$; $\text{EC}_{50}^{-1} = 0.0399 \pm 0.0007 \text{ mL of solution}/\mu\text{L liqueur}$). Comparing to medicinal tonic "Pervivo", grape and plum brandies aged in wood barrels, and whiskies, AOA of "Bitter 54" is superior, but lower than herbal liqueur "Underberg", raspberry liqueur and red vermouth activity (Gorjanović et al. 2010c). "Bitter 54" AOA is about three times lower than trolox - water soluble analog of vitamin E ($\text{EC}_{50} = 8.28 \pm 0.17 \mu\text{g/mL}$) while Korean lotus liqueur had five times lower AOA than α -tocopherol (Lee et al. 2005). Myrtle liqueurs stored one year at different conditions had AOA between 12.28 and 10.07 mM of trolox (Vacca et al. 2003). Commercial and home-made nocino liqueurs AOA, determined using the DPPH assay, was between 0.5 and 0.01 μL^{-1} , while industrial myrtle liqueurs AOA was from 11.67 to 12.89 mM Trolox, and from 43.2 % to 76.5 % inhibition (Tuberoso et al. 2010).

Decrease of TPC and TFC is shown on Fig. 1. Upon a year of storage TPC reduction for B54 is 7 %, while upon the first 90 days is 5 %. The reduction is the most prominent (24 % lower comparing to the initial value) in B54WL. Student's *t*-test shows statistically significant difference ($p < 0.05$) between TPC present in samples kept under different conditions.

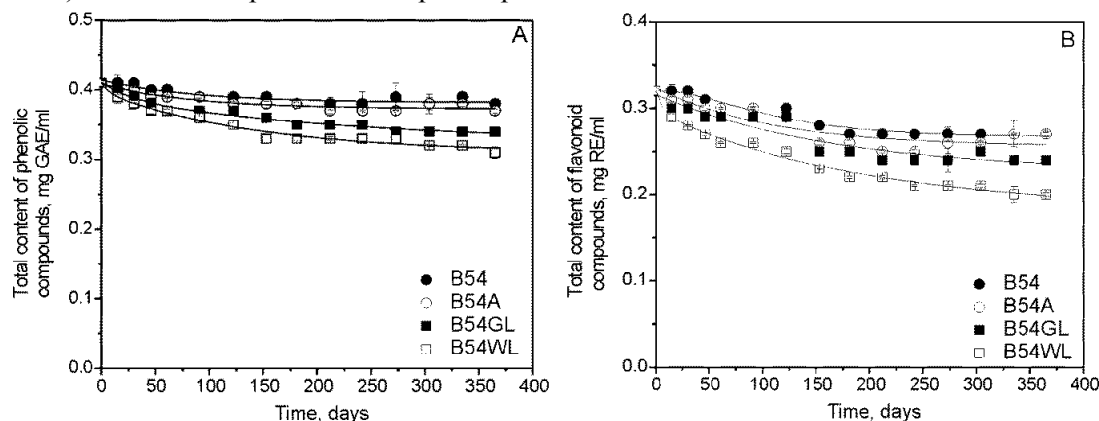


Fig. 1. The decrease of total phenolics (A) and flavonoids (B) present in freshly prepared herbal liqueur "Bitter 54" stored in the original opaque bottle (B54), exposed to daylight (B54GL), exposed to the presence of air (B54A), and in white transparent bottle exposed to daylight (B54WL), during one year period of storage.

Storage conditions considerably affect TFC (Fig. 1B). Upon the storage, TFC of B54A, B54GL and B54WL decreased 17, 24 and 38 %, respectively. Main reduction of TFC occurs during the first six months of storage. Decrease of TFC present in B54A, in the first four months is about 10 %, followed by 6 % during the remaining eight months. Student's *t*-test does not show statistically significant difference ($p < 0.05$) in TFC between B54 and B54A.

Changes of "Bitter 54" AOA during the storage under different conditions are shown in Figure 2. Upon one year storage in the original bottles in dark (B54) and white transparent bottles exposed to light (B54WL) EC_{50}^{-1} decreased 30 and 42 %, respectively. Dynamics of AOA decrease corroborates with TPC and TFC reduction. The decrease is found more prominent during the first months of storage, while during the remaining time stagnated. Total decrease of AOA after storage in the original bottles (B54GL) and in white transparent glass bottles exposed to daylight (B54WL) is 6 % and 18 % higher than decrease in the original bottle kept in cardboard box, respectively. Student's *t*-test does not show statistically significant differences ($p < 0.05$) between AOA of B54 and B54A.

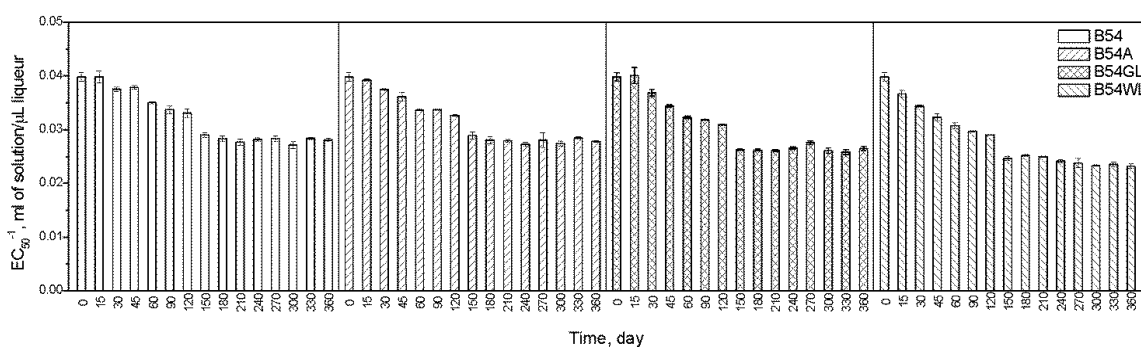


Fig. 2. The decrease of AO activity of freshly prepared herbal liqueur "Bitter 54" stored in the original opaque bottle (B54), exposed to daylight (B54GL), exposed to the presence of air (B54A), and in white transparent bottle exposed to daylight (B54WL), during one year period of storage.

The most prominent changes of AOA, TPC and TFC have been recorded during the storage in transparent white bottles exposed to daylight. Finding that flavonoids are the most sensitive corroborates with report on their prompt degradation under the influence of light and high temperatures (De la Rosa et al. 2009). Loss of anthocyanins has been found responsible for decrease of industrial red myrtle liqueur AOA during storage in the bottles with increasing headspace (Vacca et al. 2003). The most variable components during storage of conventional and ecological wines were anthocyanins as well (Zafrilla et al. 2003). While the content of anthocyanins decreased during storage, hydroxycinnamic derivatives, stilbenes and flavonols did not experience significant changes (Mulero et al. 2009). Under lower temperatures and away from light sources flavonoids can be preserved during longer period (Prasad 2010).

In order to follow AO effectiveness of phenolics and flavonoids during the storage, phenolics and flavonoids AO coefficients (PAC and FAC) are introduced. They are calculated as the ratio between EC_{50}^{-1} , and TPC or TFC. Decrease of PAC and FAC after 2, 4, 6 and 12 months storage has been given, in parallel with AOA, TPC and TFC (Table 1).

Table 1. Decrease of AO activity (EC_{50}^{-1}), total phenolics and flavonoids content (TPC and TFC), phenolic and flavonoid AO coefficients (PAC and FAC), upon 60, 120, 180 and 360 days of the storage.

Sample	Time, day	EC_{50}^{-1} decrease, %	TPC decrease, %	PAC decrease, %	TFC decrease, %	FAC decrease, %
B54	60	5.01	2.01	3.09	2.58	2.42
	120	17.04	5.60	12.37	6.73	10.48
	180	29.07	7.27	23.71	15.88	15.32
	360	29.57	7.60	24.74	16.07	16.13
B54A	60	9.52	4.97	5.15	6.85	2.42
	120	18.05	8.09	11.34	8.53	10.48
	180	29.57	8.99	22.68	17.07	12.10
	360	30.33	9.63	24.80	18.87	15.32
B54GL	60	13.53	7.20	7.22	8.03	5.65
	120	22.31	10.10	14.43	10.50	12.90
	180	34.34	14.20	23.71	23.64	13.71
	360	35.58	17.76	26.59	24.29	14.10
B54WL	60	19.05	9.40	11.34	16.16	3.23
	120	27.32	15.02	14.43	21.30	7.26
	180	36.84	19.89	21.65	30.08	9.68
	360	41.85	23.89	30.71	38.24	10.65

During the first months of storage PAC and FAC decreased. Remaining phenolics and flavonoids seems well preserved during the following six months. All presented parameters are in good corroboration. High correlation between AOA, TPC ($R^2 = 0.901$) and TFC ($R^2 = 0.953$) for all analyzed samples has been shown on Figure 3.

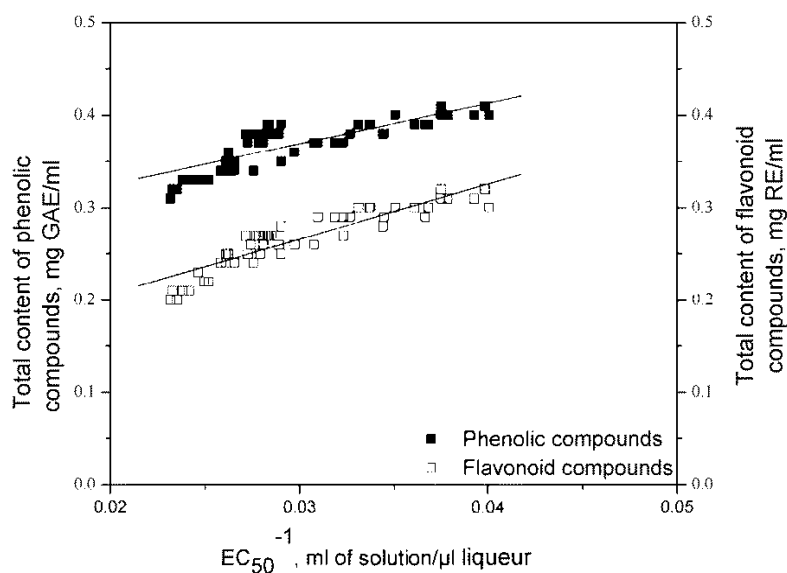


Fig. 3. The correlation between AO activity of herbal liqueur "Bitter 54" and the content of total phenolic and flavonoid compounds.

Good correlations between TPC and AOA were also demonstrated for cocoa (Redovniković et al. 2009), nocino (Alamprese et al. 2005) and some commercial herbal liqueurs (Imark et al. 2001), cognacs (Da Porto et al. 2000), different commercial brandies (Schwarz et al. 2009), red, white, and rose wines (Brenna and Pagliarini 2001; Gorjanović et al. 2010a), while Heinonen et al. (1998) showed low correlation for berry and fruit wines and liqueurs.

3.2 Influence of the storage conditions on antibacterial activity of “Bitter 54”

Table 2. Antibacterial activity of herbal liqueur “Bitter 54” determined by microdilution method (MIC and MBC in $\mu\text{L}/\text{mL}$)

Test microorganisms	B54		B54 ³⁶⁵		B54A ³⁶⁵		B54GL ³⁶⁵		B54WL ³⁶⁵		Streptomycin ^a	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC
<i>Bacillus Cereus</i>	200	250	250	250	250	250	250	250	150	250	50	50
<i>Micrococcus flavus</i>	150	150	150	250	150	250	150	250	150	250	50	100
<i>Staphylococcus aureus</i>	150	150	150	250	150	250	150	250	150	300	100	100
<i>Listeria monocytogenes</i>	250	250	250	300	250	300	250	300	250	300	100	200
<i>Escherichia Coli</i>	125	150	150	200	150	200	150	200	150	200	50	100
<i>Enterobacter cloacae</i>	150	200	200	250	200	250	200	250	200	250	50	100
<i>Pseudomonas aeruginosa</i>	125	150	150	150	150	150	150	150	150	200	100	200
<i>Salmonella typhimurium</i>	125	150	150	200	150	200	150	150	150	200	100	100

Freshly prepared herbal liqueur "Bitter 54" in the original opaque bottle placed in cardboard box on the first day of experiment (B54) and upon 365 days of storage (B54³⁶⁵), exposed to the presence of air (opened bottle) (B54A³⁶⁵), exposed to daylight (B54GL³⁶⁵), and in the white bottle exposed to daylight (B54WL³⁶⁵). ^a Streptomycin, 10 $\mu\text{g}/\text{mL}$

Extracts of medicinal and aromatic plants, known for high AMA (Perumal Samy and Gopalakrishnakone 2010; Krishnaiah et al. 2011), can be effectively combined in alcoholic beverages. High AMA of alcoholics enriched with plants extract was reported by Lin et al. (2005). All samples of “Bitter 54” showed high AMA against food contaminants, plant, animal and human pathogens (Table 2).

Our finding that Gram positive bacteria are more sensitive than Gram negative is in accordance with previously published study focused on AMA of various red and white wines (Radovanović et al. 2009). Ethanol (35 %), known to cause mild dehydration of microorganisms, can give only slightly contribution to ABA of samples. As supposed by Lin et al. (2005) phytochemicals present in alcoholic beverages might damage cell membrane, making cell more sensitive to ethanol. “Bitter 54” ABA could be attributed to high TFA, ethanol concentration and low pH (4.53). Wine ABA was related to synergistic effect of phenolics, ethanol and low pH, while ethanol or low pH showed insignificant ABA (Carneiro et al. 2008; Boban et al. 2010).

“Bitter 54” is the most efficient against *E. coli*, *P. aeruginosa* and *S. typhimurium*. The most resistant bacterial species is *L. monocytogenes*. The highest ABA can be ascribed to freshly prepared “Bitter 54” (B54), while B54WL showed the lowest activity. Samples B54 and B54A showed the same ABA, only slightly lower than freshly prepared liqueur. Obviously, negligible loss of active compounds bearing AMA occurs in oxygen presence.

3.3 Influence of the storage conditions on antifungal activity of “Bitter 54”

Generally, influence of storage conditions on AFA has been found more prominent than on ABA. All samples of “Bitter 54” showed significant AFA (Table 3). “Bitter 54” showed the highest activity (MIC 100 μ L /mL; MFC 100-150 μ L/mL), similar to 10 mg/mL bifonazole.

Exposing to daylight caused significant loss of AFA. Both MIC and MFC values of B54WL and B54GL increase. Exposure to air does not have influence. Part of AFA can be attributed to 35 % ethanol (MIC 200 μ L/mL).

Table 3. Antifungal activity of herbal liqueur “Bitter 54” determined by microdilution method (MIC and MFC in μ L/mL)

Test microorganisms	B54		B54 ³⁶⁵		B54A ³⁶⁵		B54GL ³⁶⁵		B54WL ³⁶⁵		Bifonazol ^a	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
<i>Aspergillus niger</i>	100	150	150	150	150	150	150	200	150	250	100	100
<i>Aspergillus versicolor</i>	100	100	100	100	100	100	100	200	100	200	50	100
<i>Aspergillus ochraceus</i>	100	125	150	150	150	150	100	200	100	250	50	100
<i>Cladosporium cladosporioides</i>	100	100	100	150	100	150	100	150	150	200	50	100
<i>Cladosporium fulvum</i>	100	100	100	150	100	150	100	150	150	200	50	100
<i>Penicillium ochrochloron</i>	100	125	150	150	150	150	100	200	150	200	50	100
<i>Penicillium funiculosum</i>	100	125	100	150	100	150	100	200	150	200	50	100
<i>Trichoderma viride</i>	100	100	100	150	100	150	150	200	150	150	50	100

Freshly prepared herbal liqueur "Bitter 54" in the original opaque bottle placed in cardboard box on the first day of experiment (B54) and upon 365 days of storage (B54³⁶⁵), exposed to the presence of air (opened bottle) (B54A³⁶⁵), exposed to daylight (B54GL³⁶⁵), and in the white bottle exposed to daylight (B54WL³⁶⁵). ^aBifonazol, 10 mg/ mL

3.4 Influence of the storage conditions on sensory attributes of “Bitter 54”

The visual and olfactory properties of “Bitter 54” have been evaluated with the aim to obtain an objective insight in the sensory attributes. Changes that phenolics and flavonoids experience during the storage influence sensory properties such as color, clarity, bitter taste, odour and herbal-fruity taste. The means of scores for each attribute are presented in radar charts for each sense (Fig. 4).

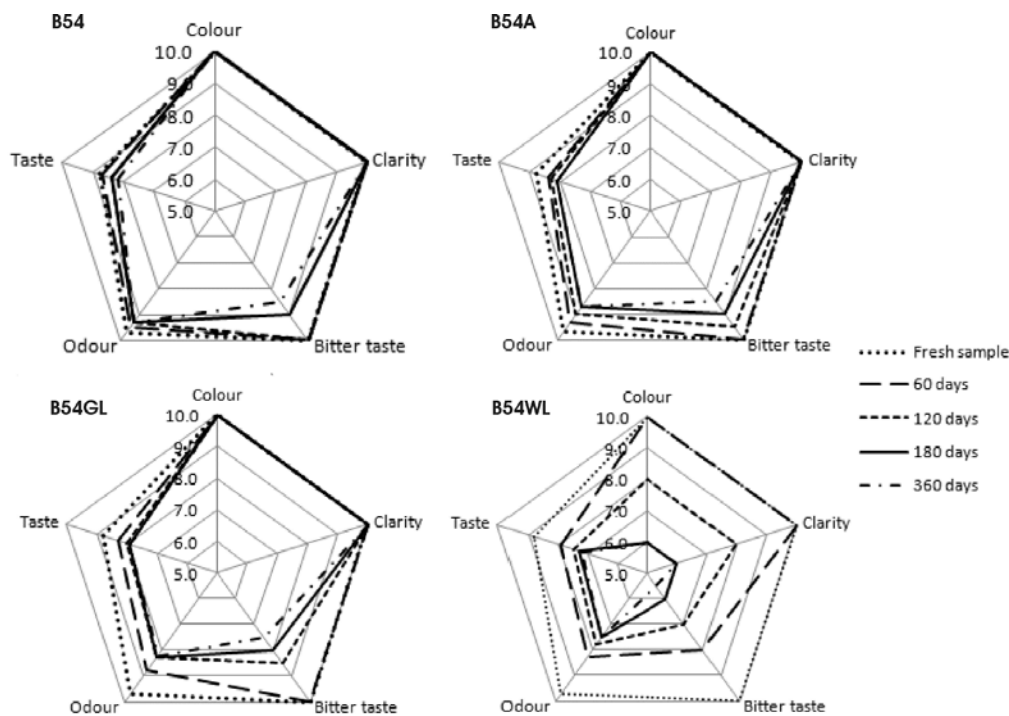


Fig. 4. Radar chart representing mean scores of the sensory analyses for the freshly prepared herbal liqueur “Bitter 54” stored in the original opaque bottle (B54), exposed to daylight (B54GL), exposed to the presence of air (B54A) and in white transparent bottle exposed to daylight (B54WL) during one year period of storage.

Results of sensorial analysis confirm previously noticed dynamics of investigated product change. Freshly prepared beverage has excellent quality and keeps it during the first months of the storage under optimal conditions (B54) (Fig. 5). Satisfied quality of B54 lasts till the end of the storage.

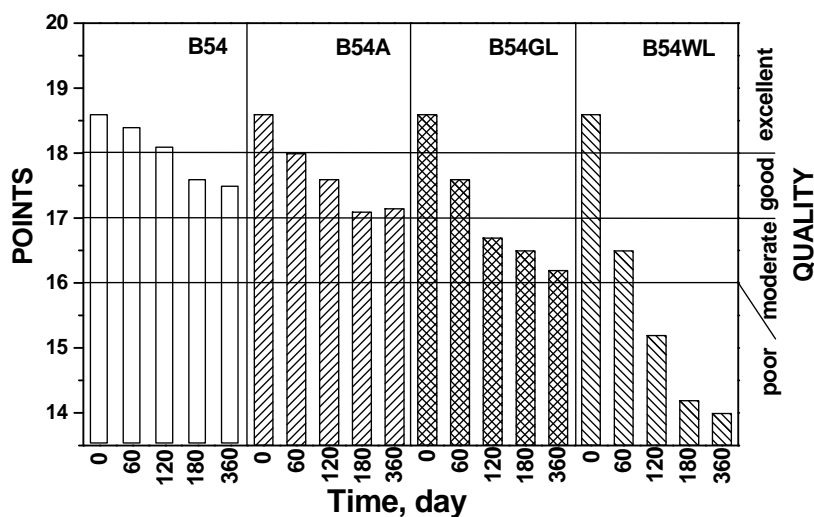


Fig. 5. Sensory quality of freshly prepared herbal liqueur “Bitter 54” stored in the original opaque bottle (B54), exposed to daylight (B54GL), exposed to the presence of air (B54A) and in white transparent bottle exposed to daylight (B54WL) during one year period of storage, expressed as sum of evaluated sensory attributes.

4. Conclusion

Content and activity of bioactive compounds present in investigated herbal liqueur and its sensory attributes have been found dependent on the storage conditions. Protection from light effectively reduces degradation of bioactive phenolic compounds possessing AOA and AMA. The preservation of biologically active phytochemicals has not been found dependent on air exposure. Decrease of AOA, TPC and TFC, and AO effectiveness has been found prominent during the first part of the storage, while during the remaining time all measured values have been found almost constant. According to monitored parameters related to beneficial effect on moderate consumers' health, as well as sensory characteristics, top-level quality of freshly prepared liqueur declines during the first part of the storage. However, the product keeps satisfying quality during the whole period of shelf life, if under adequate storage conditions.

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