beverages

Article

Chemical Composition, Physical Properties, and Aroma Profile of Ethanol Macerates of Mistletoe (*Viscum album*)

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Abstract: Maceration parameters such as alcohol and mistletoe content were studied to obtain high-quality macerates to produce *biska*, a traditional Istrian herbal spirit. An ethanol–water solution containing 25, 40, 55, and 70 v/v% ethanol and 20, 40, and 80 g/L mistletoe was macerated and pH, total acids, extract content, and color parameters were measured. Volatile compounds were determined by GC/MS. More than 150 different volatile compounds were detected. The composition of the ethanol–water solution and mistletoe content affects the values of total extract, total acids, and color. Samples with lower alcohol content have a higher number of different acids, aldehydes, alcohols, esters, and ketones, while macerates with higher alcohol content are rich in hydrocarbons and terpenes. Ethyl decanoate; ethyl hexynoate; ethyl octanoate; benzaldehyde; hexanal; octanal; and the terpene compounds limonene, cymene, menthone, humulene, eucalyptol, linalol, and borneol contribute significantly to the aroma of the macerates due to a low odor threshold. Ylangene and α- and β-bourbonene are tentatively determined new terpene molecules that are not found in the mistletoe literature, as well as thujone, which was also detected. For the preparation of aromatic macerates, 40–50 v/v% ethanol and about 40 g/L mistletoe should be used.

Keywords: mistletoe (*Viscum album*); maceration; herbal spirit; physicochemical parameters; volatile compounds

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1. Introduction

Herbal spirits are strong alcoholic beverages with a particular aromatic smell and taste, usually containing up to 40% ethanol by volume. They are generally made by macerating various medicinal, spice, and aromatic plants in alcohol; by distilling the alcohol in the presence of herbs; by adding herbal extracts to the distilled alcohol; or by combining some of these processes. They are made from one or more plant species, and the alcohol base is ethanol, wine distillate, or marc distillate. By maceration, a colored alcoholic beverage is obtained, which, in addition to all the flavors and colorants extracted from the plants, also contains other nutrients such as sugars, minerals, and phenolic substances known as antioxidants, which have a beneficial effect on the human body [1,2]. There are a number of strong alcoholic beverages made from various medicinal or aromatic herbs, including Orujo, Zubrowka, and Absinthe.

In different countries, herbal spirits differ mainly in the selection of herbs for their production. In the Mediterranean countries, which include Croatia, there is a favorable climate for the growth of various plant species and thus for wild and cultivated medicinal and aromatic herbs. Therefore, herbal liqueurs and flavored alcoholic distillates have been traditionally produced for centuries. Łuczaj et al. [3] conducted 295 interviews on 36 Dalmatian and Kvarner islands and summarized 114 herb species used by regional small-scale producers for the production of *travarica*, a marc distillate flavored with single or mixed herb species. The most commonly used species are *Foeniculum vulgare*, *Myrtus communis* L., *Salvia officinalis* L., *Ruta graveolens* L., *Juniperus oxycedrus* L., *Ceratonia siliqua*

L., *Juglans regia* L., *Citrus* spp., *Ficus carica* L., *Laurus nobilis* L., *Rosmarinus officinalis* L., *Artemisia absinthium* L., *Rosa centifolia* L., and *Mentha piperita* L.

Biska is a traditional Istrian herbal spirit made by macerating leaves and twigs of white mistletoe (*Viscum album*) in a water–ethanol base, usually *komovica* brandy (marc distillate). From the mistletoe–ethanol base are extracted various compounds that make up the characteristic aroma, color, and taste of *biska*, as well as biologically active compounds that carry the functional properties of *biska*. In ancient Greece and Rome, white mistletoe was considered a medicinal plant, and its extracts were used for medicinal and pharmaceutical purposes. Today, it is known that mistletoe is a rich source of biologically active compounds, the content of which depends mainly on the type of host tree on which it parasitizes, but also on the time of harvesting [4]. White mistletoe contains a number of phytochemicals such as lectins, viscotoxins, polysaccharides, alkaloids, terpenes, proteins, amines, peptides, polyphenols, phytosterols, and amino acids [5,6]. Most research has focused on aqueous extracts, which have been shown to have a wide range of biological effects [7]. Mistletoe also contains some volatiles, but these have been poorly studied. Wang et al. [8] investigated the volatile and nutritional properties of *Viscum articulatum*. Ćebović et al. [9] studied the nonpolar components of the extract of *Viscum album* obtained by supercritical CO_2 extraction and discovered some new terpene molecules.

Maceration, a technological process for the extraction of compounds from medicinal and aromatic plants on an alcoholic basis for the production of flavored herbal distillates, has not been fully investigated. It is known that, in addition to the quality of the plants used, the maceration parameters such as the ethanol content in the water–ethanol base, the plant–liquid ratio, the temperature, and the duration of the maceration affect the quality of the macerate [1,10–13]. However, herbal producers usually perform the process of plant maceration traditionally, without precise knowledge of the influence of maceration parameters on the amount and type of extracted volatile and biologically active compounds.

Since the parameters of mistletoe maceration in a water–ethanol base have not been researched thus far, the aim of this work is to describe the physicochemical parameters of the samples (pH, total acids, total extract content, color parameters, and aroma profile of the compounds) in order to obtain aromatic macerates with attractive color.

2. Materials and Methods

2.1. Preparation of Mistletoe (V. album) Macerates

Twelve samples of mistletoe macerates were prepared for the study, which differed by volume of ethanol in the ethanol–water solution (A: 25, B: 40, C: 55, and D: 70 v/v% of ethanol) and mistletoe concentrations (20, 40, and 80 g/L). The macerates obtained were analyzed for their physicochemical properties: pH, total extract, total acids, colors, and volatile compounds. Mistletoe (*V. album*) macerates were prepared by the maceration of dry, chopped plant material (mistletoe leaves and stems) in a hydroalcoholic base. Hydroalcoholic bases were prepared by mixing 96% ethyl alcohol of agricultural origin and water. Plant material was weighed and added to 500 mL of the hydroalcoholic base to obtain three different mistletoe concentrations (20 g/L, 40 g/L, and 80 g/L). Accordingly, the samples are named _20, _40, _80, with prior indication of the strength of the hydroalcoholic base (A, B, C, D). Maceration was conducted in bottles with plugs at room temperature and in darkness to avoid exposure to sunlight for 28 days. At the end of maceration, macerates were filtered through filter paper to remove plant material, which was additionally squeezed and stored at −20 °C. During the maceration, the samples were manually shaken daily. Plant material was obtained from a local phytotherapy producer (Croatia). Macerates were prepared in duplicate, and all analyses were performed in triplicate.

2.2. pH Assay, Total Acidity, and Total Extract

The measurements of pH value of all samples were done with an Oakton pH 5 plus Meter pH meter (Oakton Instruments). The system was calibrated by placing a pH probe in a buffer of pH 4. The total acidity was determined by titrating 25 mL of the macerate with

0.1 M NaOH until color change was reached, using phenolphthalein as an indicator. Results were expressed as milligrams of acetic acid per liter of macerate [14]. Total extract was determined by the gravimetric method and expressed as grams per liter of macerate [15].

2.3. HS-SPME and GC/MS Conditions and Analysis

Aliquots (7 mL) of spirit was transferred to glass vials (20 mL) and closed with a silicone septa. Sampling was performed using DVB/CAR/PDMS fiber (Divinylbenzene/Carboxen/Polydimethylsiloxane), 1 cm long, at 25 °C and an absorption time of 20 min using the SPME system (Solid Phase Extraction System). Before absorption of volatiles on the SPME fiber, 1 µL of internal standard (1,2-dichlorobenzene, 0.01% solution in methanol) was added to each sample. The fiber was inserted into the GC/MS (gas chromatography—mass spectrometry) injector, where desorption after 2 min took place (splitless mode). The apparatus used in the experiment was GCMS-QP2010 (Shimadzu Corportion, Japan). The separation of aroma compounds was performed with the use of Stabilwax (30 m × 0.25 mm × 0.25 µm, polyethelene glycol) Restek. The carrier gas was helium and the column flow was 0.65 mL/min. The column temperature program was as follows: an initial temperature of 35 °C (for 1 min) at a rate 5 °C/min to 220 °C (holding time of 2 min) and 220–240 °C at a rate of 10 °C/min. The total time of the analysis was 40 min. The interface and injector temperatures were 260 °C and 250 °C, respectively. Ion source temperature was 250 °C. Mass spectra were acquired in the electron impact mode (70 eV) using a full scan with a mass acquisition range of 30–450 m/z. The internal standard 1,2-dichlorobenzene was used to semi-quantify volatile compounds. Identification of aroma compounds was made on the basis of mass spectra libraries (NIST 2008, Wiley 175) and from literature data. All analyses were performed in triplicate.

2.4. Calculation of Odor Activity Value (OAV)

The calculation equation of OAV was as follows: OAV = Ci/OTi, where Ci is the concentration of each compound and OTi is its corresponding odor threshold value in a 10% *v*/*v* water–ethanol mixture. The threshold values were acquired from the relevant literature. Compounds with OAV $\geq$ 1 had a major contribution to the characteristic aroma of samples [16].

2.5. Chromatic Characteristics

The chromatic characteristics were determined on a Specord 50 Plus (Analytik Jena) and a 10 mm glass cell by measuring the transmittance of the sample every 10 nm from 380 to 770 nm, with a D65 illuminant. Based on the transmittance values, some parameters were calculated: luminosity (L*), saturation (C*), chromaticity coordinates (a* and b*), and hue (h*) [17].

2.6. Statistical Analysis

Statistical analysis was carried out using the MS Excel tool XLStat (Addinsoft, Paris, France) program. Data shown are mean values (n = 3). All data were subjected to analysis of variance (ANOVA). Principal component analysis (PCA) was used to visualize the volatile substances and differences in chromatic characteristics between samples.

3. Results

3.1. pH Assay, Total Extract, and Total Acidity

To obtain spirit drinks of the highest quality, it is necessary to control their physicochemical characteristics. Several analytical determinations, namely pH, total acidity, and total extract were done to characterize prepared macerates. The results show that the pH of mistletoe macerates varies between 5.5 and 6.25 (Figure 1), with the lowest pH measured in sample A_20 and the highest in sample D_40. It can be observed that the composition of the water–alcohol base significantly affects the differences in the pH of the macerates as a function of plant concentration. Indeed, the pH values of the aqueous alcohol solutions themselves differ significantly, with a higher proportion of ethanol leading to a neutral medium and a higher proportion of water leading to an acidic medium. Interestingly, the addition of plants to water–alcohol bases with lower ethanol concentrations (25% and 40% *v*/*v*) increases the pH, while the addition of plants to water–alcohol bases with higher ethanol concentrations (55% and 70% *v*/*v*) decreases the pH. This indicates that the maceration medium significantly affects the type and amount of compounds to be extracted.

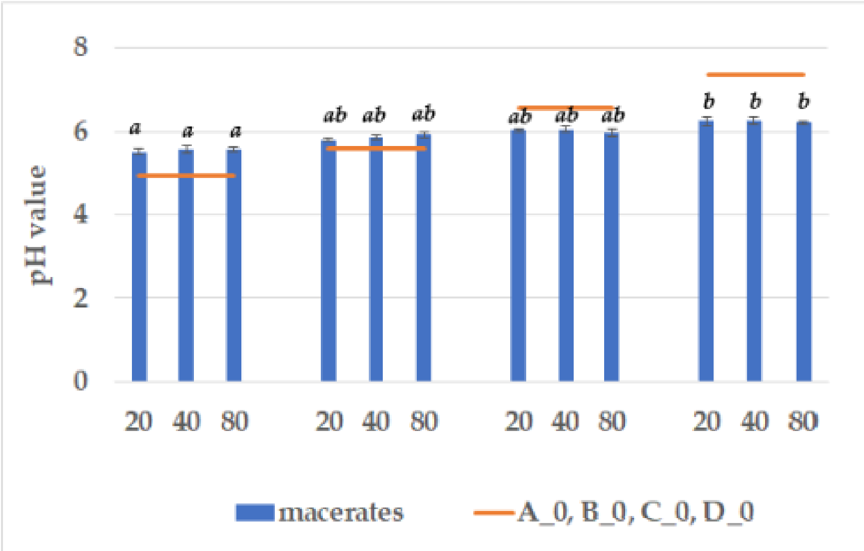

Figure 1. pH values of ethanol–water solution and mistletoe macerates prepared by maceration of 20 g/L, 40 g/L, and 80 g/L mistletoe in ethanol–water solution (A: 25%, B: 40%, C: 55%, and D: 70% *v*/*v* ethanol) at room temperature and in darkness, after 28 days of maceration. Different letters indicate significant differences between samples ($p < 0.05$).

The total extract is shown in Figure 2. As expected, the amount of extracted substances in the macerate (sugars, minerals, and other organic substances) increases with increasing plant content. Therefore, macerates with a higher plant content also have a greater amount of total extract. The results show that the highest amount of total extract is obtained for macerates with 25% volume and the highest plant concentration. At the highest plant concentration, the amount of total extract decreases as the ethanol concentration increases. The total extract at the lowest plant concentration does not depend on the alcohol content. At the lowest plant concentration, the concentration gradient is the largest, so with longer maceration time (28 days), mistletoe components are extracted regardless of the alcohol base. At a medium plant concentration, the sample with 40% ethanol had the highest concentration of extracted components.

Acid concentrations in the samples varied between 480 and 2880 mg/L (Figure 3). Sample B_20 had the lowest value and sample D_80 had the highest. As the amount of herbs increases, the total acidity of the macerate increases. The increase in the total acidity of the macerate with the addition of herbs shows the extraction of acids from the plants into the water–alcohol base. At low ethanol concentrations (25% *v*/*v*), extraction of polar compounds occurs due to the predominant polar nature of water. However, increasing the ethanol concentration in the macerates changes the polarity of the solvent and allows extraction of less polar compounds, which increase the concentration of total acids [18]. Mistletoe contains triterpene acids such as oleanolic, betulinic, and ursolic acids in its leaves and stem [19]. It is very rich in saturated and unsaturated fatty acids. It contains palmitic acid, arachidic, lignoceric, behenic, and cerotic acids from the saturated acids and oleic acid, linoleic acid, and linolenic acid from the unsaturated acids [7].

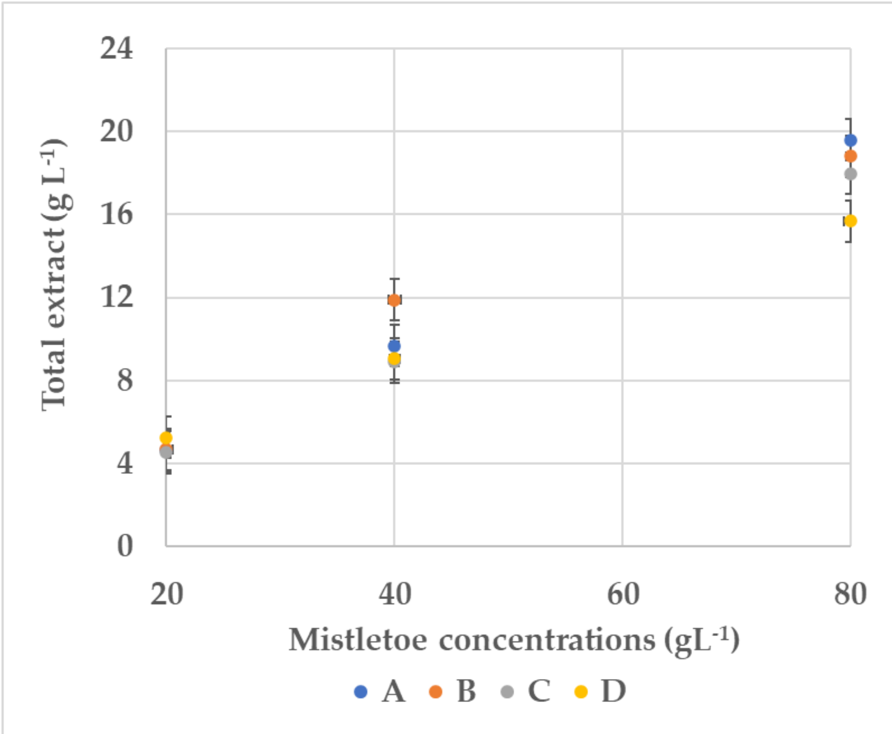

Figure 2. Total extract of mistletoe macerates prepared by maceration of 20 g/L, 40 g/L, and 80 g/L mistletoe in an ethanol–water solution (A: 25%, B: 40%, C: 55%, and D: 70% *v*/*v* ethanol) at room temperature and in darkness, after 28 days of maceration.

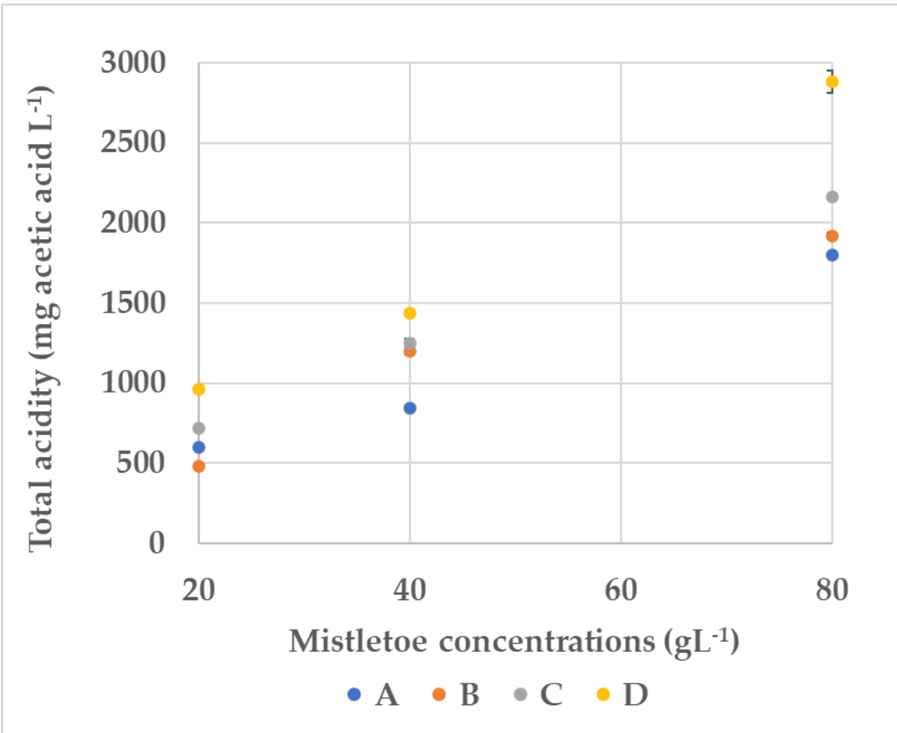

Figure 3. Total acidity of mistletoe macerates prepared by maceration of 20 g/L, 40 g/L, and 80 g/L mistletoe in ethanol–water solution (A: 25%, B: 40%, C: 55%, and D: 70% *v*/*v* ethanol) at room temperature and in darkness, after 28 days of maceration.

3.2. Macerate Volatile Compounds

Aromatic compounds are important sensory indicators and quality parameters for spirit drinks. All mistletoe macerates had a certain amount of volatile compounds. More than 150 different compounds were detected in all 12 macerates, belonging to six different chemical families: acids, alcohols, including terpene alcohols, aldehydes, esters, hydrocarbons, and terpenes. Analysis of the number of components in individual macerates showed that samples with lower alcohol content (A, B) have a higher number of different acids, aldehydes, alcohols, esters, and ketones, while macerates with higher alcohol content (C, D) are rich in hydrocarbons and terpenes. The same result is obtained if the analysis is performed according to the relative percentages of individual groups of volatile substances in the macerates (Figure 4).

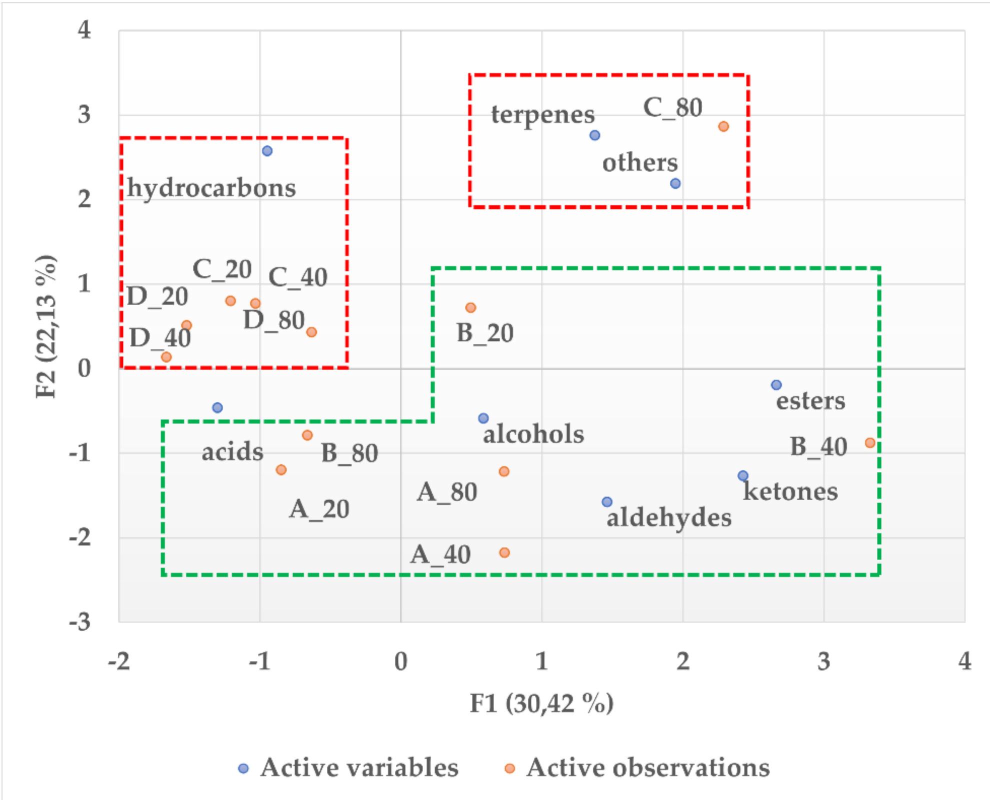

Figure 4. Principal component analysis (PCA) score plot for mistletoe macerates obtained after 28 days of mistletoe maceration (20 g/L, 40 g/L, and 80 g/L; ethanol–water solution A: 25%, B: 40%, C: 55%, and D: 70% v/v ethanol) at room temperature and in darkness, based on relative concentrations of individual groups of volatile substances in the macerates. Samples with lower ethanol content and characteristic volatiles are circled in green, samples with higher ethanol content and characteristic volatiles are circled in red.

Moreover, the relative concentration of volatile components partially follows the proportion of macerated plants. It was expected that samples with 80 g/L of plants would have the highest concentration of volatiles. However, sample B_40 had the highest concentration of volatiles, especially esters and ketones. It is possible that the saturation of the solution with other extractives (pigments, phenols, flavonoids) prevents the extraction of volatiles in highly concentrated plant samples. In addition, sample B_40 was a 40% water–alcohol-based sample in which both water- and alcohol-soluble volatile compounds were extracted, which could be the reason for the highest concentration of volatile compounds in this sample. Table 1 lists the compounds detected in sample B_40. In this sample, 42 compounds were detected. Results are expressed in relative percentages, as only a qualitative analysis has been carried out.

Table 1. Relative percentages of volatile compounds in ethanol–water mistletoe macerate. Mistletoe (40 g/L) was macerated in an alcoholic strength of 40% over 28 days (*n* = 3).

Group	Compounds Detected	Relative Percentages
Acid	Decanoic acid	0.317 ± 0.01
Alcohols	1-Butanol, 3-methyl-, acetate	0.359 ± 0.01
	Cyclohexanol, 1-methyl-4-(1-ethylethenyl)-	1.152 ± 0.02
	1,8-Cineole	1.587 ± 0.06
	1-Butanol, 3-methyl	0.282 ± 0.02
	1-Hexanol	0.317 ± 0.01
	3-Hexen-1-ol	0.021 ± 0.00
	1-Octen-3-ol	0.269 ± 0.00
	Cyclooctanol, acetate	0.372 ± 0.01
	2-Furanmethanol	0.531 ± 0.00
	Oxime-, methoxy-phenyl-	3.187 ± 0.10
	Benzenemethanol	0.435 ± 0.01
	1,2,3-Propanetriol	2.270 ± 0.09
Aldehydes	Acetaldehyde	0.552 ± 0.01
	Hexanal	1.607 ± 0.02
	Octanal	0.593 ± 0.02
	Benzaldehyde	2.208 ± 0.05
Esters	Formic acid, ethenyl ester	9.928 ± 0.22
	Butanoic acid, ethyl ester	1.621 ± 0.31
	Butanoic acid, 2-methyl-, ethyl ester	5.678 ± 0.05
	Pentanoic acid, ethyl ester	2.049 ± 0.03
	Hexanoic acid, ethyl ester	7.651 ± 0.21
	Heptanoic acid, ethyl ester	0.690 ± 0.03
	Octanoic acid, ethyl ester	4.070 ± 0.22
	Nonanoic acid, ethyl ester	3.036 ± 0.02
	Decanoic acid, ethyl ester	14.956 ± 0.30
	Methyl Salicylate	1.649 ± 0.02
	Dodecanoic acid, ethyl ester	1.725 ± 0.02
	Hexadecanoic acid, methyl ester	0.538 ± 0.00
	Hexadecanoic acid, ethyl ester	0.359 ± 0.02
Hydrocarbons	Undecane	1.980 ± 0.04
Ketones	2,2-Dimethyl-3-heptanone	0.338 ± 0.01
	Fenchone	1.283 ± 0.05
	alpha-Thujone	3.615 ± 0.09
	beta-Thujone	2.532 ± 0.06
	Cyclohexanone,5-methyl-2-(1-methylethyl)-, cis-	4.250 ± 0.13
	Theaspirane	4.629 ± 0.12
	2,6,10,10-Tetramethyl-1-oxa-spiro [4.5]dec-6-ene	1.877 ± 0.04
Others	l-Alanine ethylamide, (S)-	0.973 ± 0.02
	Methane, tetranitro-	1.690 ± 0.03
	Butane, 1,1-diethoxy-3-methyl-	5.775 ± 0.30
	Benzene, 1-methyl-3-(1-methylethyl)-	0.772 ± 0.02
Sum		100.00 ± 2.80

Volatile acids were generally the least abundant compounds in macerates, while esters were the most abundant. Volatile acids were detected only sporadically in the macerates studied. Only nine different acids were detected, including heptanoic acid, nonanoic acid, and decanoic acid, whose ethyl esters were also detected in the macerates. A total of 40 esters were detected, of which 21 different esters were detected in more than one macerate (Figure S1).

Esters qualitatively represent the most important class of flavors in spirits because they have a low sensory threshold value. Their contribution to flavor depends strongly on their concentration.

Ethyl esters of short- and medium-chain acids were the most abundant. Hexanoate imparts a fruity aroma to the spirit, so its presence is beneficial to the spirit. Ethyl octanoate is more pungent and less fragrant, whilst decanoate is less intense and imparts greasy tones [20].

Ethyl esters of long-chain fatty acids are only important if they are present in higher concentrations. They can then contribute to odors that impart a candle wax and stearic tone to spirits. Esters from this group are also poorly soluble in water. Therefore, samples with higher ethanol concentration are richer in esters of long-chain acids, while macerates with lower ethanol content are richer in esters of short- and medium-chain acids: butyric, pentanoic, hexanoic, octanoic, and decanoic acid esters (Figure 5). Due to the high concentration of ethyl formate in sample D_80, this sample was exceptionally not grouped.

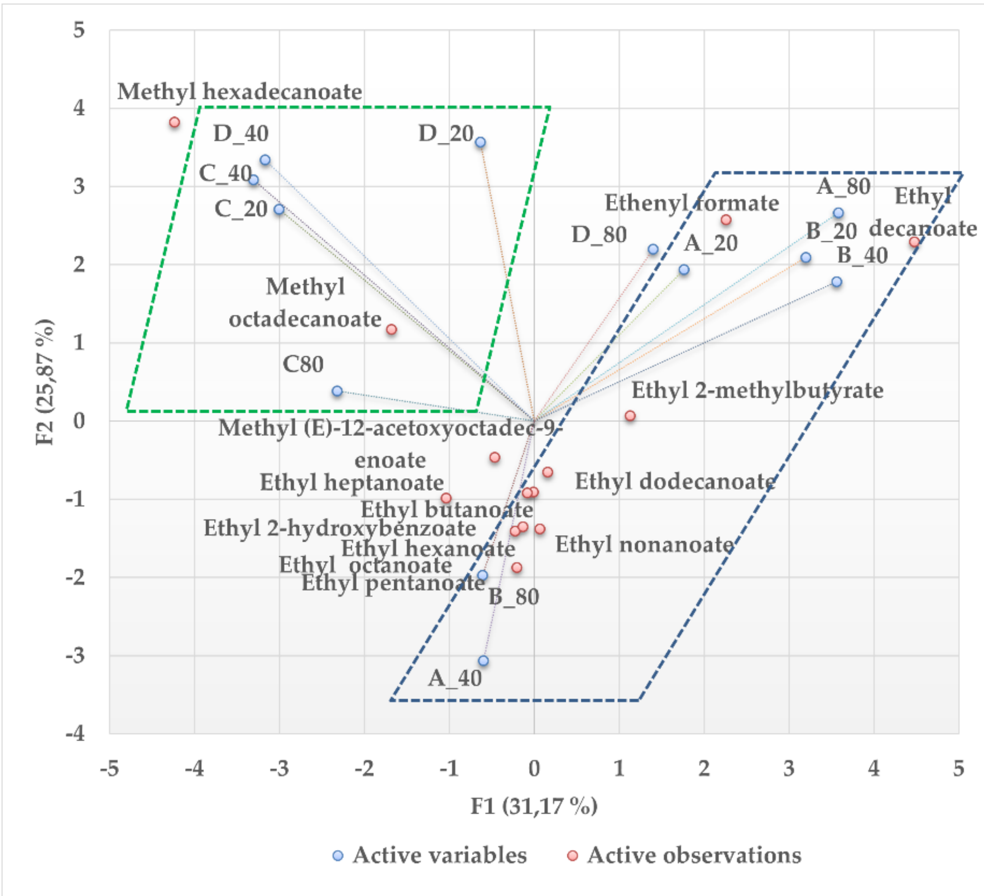

Figure 5. Principal component analysis (PCA) for mistletoe macerates obtained after 28 days of mistletoe maceration (20 g/L, 40 g/L, and 80 g/L; ethanol–water solution A: 25%, B: 40%, C: 55%, and D: 70% v/v ethanol) at room temperature and in darkness, based on the esters represented in each macerate. Samples with lower ethanol content and characteristic esters are circled in blue, samples with higher ethanol content and characteristic esters are circled in green.

Besides esters, alcohols were the dominant compounds. Thirty-nine alcohols were detected, fourteen of them in more than one macerate, noting that the alcohols were more abundant in the lower-percentage ethanolic samples. As mentioned earlier, sample C_80 was the richest in terpene compounds because terpenes are more soluble in multi-percent alcohol. Table 2 shows the terpenes and terpene alcohols characteristic of *biska* macerates. In contrast to the terpenes, which are more soluble in ethanol and more abundant in macerates with higher percentages, the terpene alcohols were present in the lower-percentage macerates, too. 1,8-cineole and linalool, although soluble in alcohol, also had good solubility in water (3500 mg/L for 1,8-cineole), possibly justifying this result. 1,8-cineole was present in almost all samples, as well as the alkaloid oxime, methoxy-phenyl. Oximes (R1R2C=NOH) are nitrogen-containing chemical constituents that are formed in plants, where oximes are positioned at important metabolic points. The majority of plant oximes are amino acid-derived metabolites.

Table 2. Relative concentrations, odor description, threshold, and OAV of key terpenes and terpen alcohols detected in the macerates.

Terpene	Odor Description	Concentration * (mg/L)	Threshold (mg/L)	OAV
β-pinene	turpentine	0.56	0.752	0.745
p-cymene	slightly woody, citrus, green pepper and oregano	0.56	10	0.056
l-limonene	**lemon-like**	1.23	0.2	**6.15**
m-cymene	**hints of citrus, earth, and wood**	2.06	0.12	**17.16**
trans-menthone	**minty-refreshing**	6.50	0.476	**13.65**
ylangene	pepper, spicy	1.41	-	-
β-bourbonene	herbal, woody, floral, balsamic	1.58	-	-
Camphor	warm, minty	0.70	1	0.70
α-humulene	**woody**	0.84	0.39	**2.15**
α-bourbonene	-	7.50	-	-
isomenthone	**peppermint**	1.14	0.63	**1.81**
α-bergamotene	pepper with woody and spicy undertones	0.13	-	-
trans-caryophyllene	sweet, woody, spice, clove	0.11	0.15	0.73
1,8-cineole/eucalyptol	**mint, eucalyptus, sweet, cooling, fresh, chemical pine**	0.201	0.064	**3.14**
linalool	**orange flowers, floral, rose, aniseed**	0.059	0.05	**1.18**
borneol	**pine, woody, camphor, balsamic**	0.134	0.08	**1.675**
menthol	menthol	0.29	1	0.29

* The relative concentrations are given in terms of equivalents to the internal standard. The relative concentration was calculated as the mean of relative concentrations of the compound present in all macerates. Key components whose OAV is greater than one is in bold. Threshold data from [21].

Of the ketone compounds, alpha-thujone, beta-thujone, and fenchone were the most common. Thujone is characteristic of plants such as wormwood, sage, thyme, and rosemary. Because of its toxicity, the maximum amount in beverages is prescribed. In the macerates in which it was detected, the sum of alpha-thujone and beta-thujone was a maximum of 2.1 mg/L, which is significantly lower than the prescribed limits for alcoholic beverages. Some others such as theaspirane and p-menthone were detected. Of the aldehydes, benzaldehyde was present in all A and B macerates at concentrations above the threshold, indicating that it contributes to the aroma of the macerates at lower alcoholic levels.

3.3. Macerate Color Parameters

All macerates are colored but differ in their chromatic characteristics (Figure 6). Samples prepared with a lower plant concentration (20 g/L) and a lower ethanol concentration showed the highest brightness. With increasing ethanol concentration, the brightness values decrease (Figure 6, highlighted line). The samples are also less bright with increasing plant concentration (40 g/L and 80 g/L).

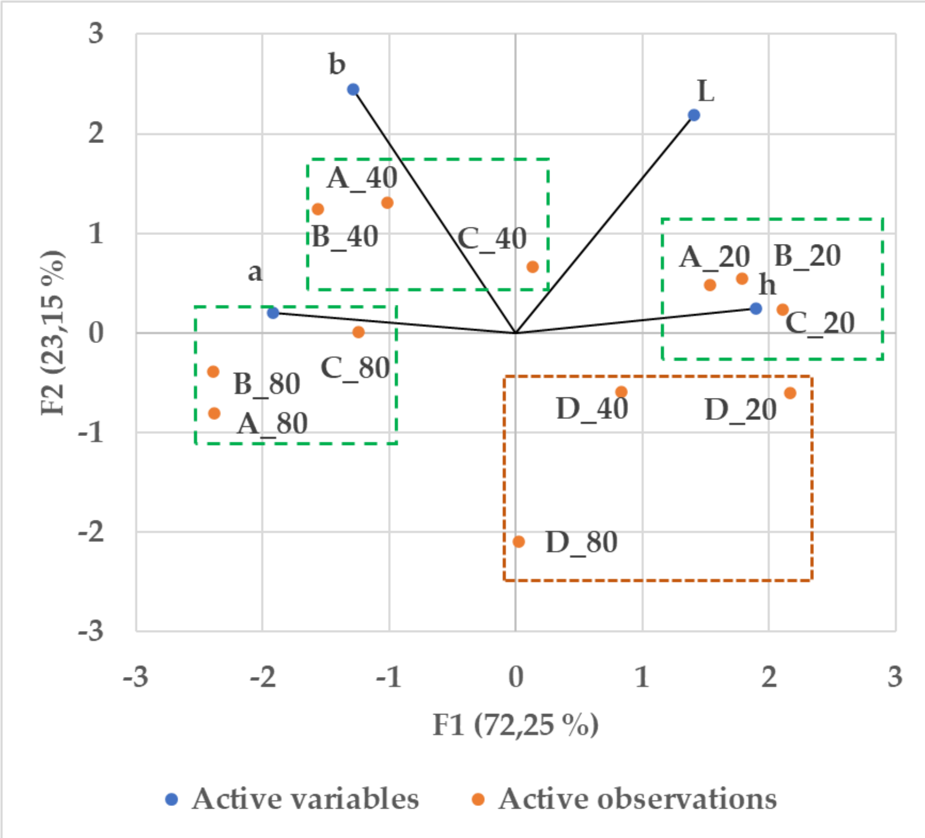

Figure 6. Principal component analysis (PCA) score plot for mistletoe macerates obtained after 28 days of mistletoe maceration (20 g/L, 40 g/L, and 80 g/L; ethanol–water solution A: 25%, B: 40%, C: 55%, and D: 70% *v*/*v* ethanol) at room temperature and in darkness, based on macerate chromate characteristics: L-luminosity, a, b-chromaticity coordinates, h-hue. Highlighted in red - samples with increasing ethanol concentration and decreasing brightness values.

Lower amounts of plants extract a smaller amount of substances that can affect color and brightness. As the amount of plants increases, various pigments and substances such as phenols, minerals, and acids are extracted from the plants into the water–alcohol base, decreasing the brightness values and making the macerates darker and cloudier [22]. Moreover, as the ethanol content in the aqueous alcohol base of the macerate increases, the proportion of ethanol-soluble substances increases.

In addition, all mistletoe macerates have positive a and b values; i.e., they are samples with red and yellow hues. Component a values are highest in samples with a high plant concentration (80 g/L). Component b has the highest values in samples with a plant concentration of 40 g/L. The values of component b are much higher than those of component a, which means that yellow hues predominate in the samples. Very similar results were shown by Hanousek Čiča et al. [1] for carob macerates, where yellow hues predominate over red hues in most samples.

The samples with the lowest plant concentration have the highest values of the hue angle (h) and are closest to 90°, which is why the color of the macerate has yellow hues with different brightness. As the plant concentration in the macerate increases, the parameter h decreases, causing the colors of the macerate to approach the red-purple spectrum, although they also contain yellow shades. All samples have h values below 90°, which means that they are in the red-violet and yellow color spectrum.

4. Discussion

Herbal spirits are special strong alcoholic drinks obtained by maceration of aromatic and medicinal herbs in spirits and are also, like liqueurs, traditional household products. Recently, they have been used not only for their smell and taste, but also because of the bioactive substances contained in them [7]. The herbal spirits are expected to have a decent smell and a slight yellow to green color. According to these requirements, it is necessary to adjust the production conditions, in terms of the amount of plants, ethanol strength, and maceration time. Because of that, the influence of maceration parameters on the physicochemical properties and volatile compounds of mistletoe macerates for the preparation of *biska* spirit is studied here.

The results show that both the ethanol content in the ethanol–water solution and the content of macerated plants influence the pH values of the macerate (Figure 1). Jovanović et al. [11] consider that the most suitable solvent for extraction is mixture of solvents of different polarity, as it facilitates the extraction of plant compounds of different polarity. Aqueous extracts have a higher proportion of polar compounds, while organic solvents have a higher proportion of less-polar compounds. The obtained results show that mistletoe contains different compounds (both basic and acidic) extracted during maceration in ethanol–water solution, so different functional and physicochemical properties of the obtained macerates can also be expected. Hanousek Čiča et al. [23] measured slightly lower pH values in commercial samples of *biska* made with grape marc spirit as a base, while *biska* made with ethyl alcohol of agricultural origin had pH values similar to the results in this paper. Indeed, distillates made from fruit or fruit pomace have a higher acidity, which ultimately affects the pH of the herbal spirits made from them.

The composition of the ethanol–water solution also affects the values of total extract and total acids (Figures 2 and 3). In accordance with Snoussi et al. [24], higher values of total extract were measured in macerates with lower ethanol concentration. Mistletoe contains significant amounts of glucose, fructose, and sucrose, with sucrose predominating. Of the polysaccharides, it contains pectin, highly methylated homogalacturonan, arabinogalactan, and α-1,4-methyl ester of galacturonic acid [7,25,26]. The decrease in total extract with increasing ethanol content can be explained by a decrease in the solubility of sugars in solution with increasing ethanol concentration [27]. Sugars with five and six hydroxyl groups form more hydrogen bonds with water than with ethanol. As ethanol content increases, the number of hydrogen bonds in the solvent decreases, reducing solubility.

Mistletoe is reported to contain oleanolic acid, to which cytotoxic properties are attributed [25]. Wojciak-Kosior et al. [28] determined the content of triterpenic acids, betulinic acid, and oleanolic acids in *V. album* from different hosts and in various seasons. High variance in the acids' content was noted. However, oleanolic acid was a dominant triterpenic acid. Also, unsaturated fatty acids, mainly oleic and linoleic acids, were identified in nine lipophylic extracts obtained from *V. album* samples growing on different host trees in Turkey [29]. Mistletoe is rich in these non-volatile acids, while the volatile acids were sporadically present in our macerates with lower alcohol content. Hanousek Čiča et al. [23] also detected only myristic acid and oleic acid in comercial *biska* spirits.

The volatile components are responsible for the organoleptic quality and safety of spirits, depending on their composition, concentration and sensory characteristics, which are crucial for their acceptance by the customer. Reports on mistletoe volatile extract composition have appeared in literature [9,30], but detailed reports have not been published. Most investigations on *V. album* are based on aqueous mistletoe extracts, which contain cytotoxic and immunomodulatory proteins such as lectins and viscotoxins [6]. Moreover, these are short extraction procedures of up to 24 h, while we performed maceration for 28 days. The end of maceration was determined by the maximum concentration of biologically active compounds (data not shown). In this paper, all detected volatile compounds were derived exclusively from mistletoe, as ethyl alcohol of agricultural origin was used as the alcohol base. Data on aromatic components in herbal liqueurs and herbs can be found in the literature, but in these accounts, the aroma of certain beverages is often complex due to volatile compounds derived from the base spirit, usually wine distillate or grape pomace distillate [10]. In our previous work investigating the physical properties and aroma compounds of fourteen commercial mistletoe-based spirits, the composition of the aroma compounds was very complex and did not provide an accurate insight into the aroma compounds originating from mistletoe. However, according to the results presented here, it is possible to define some aroma compounds characteristic of mistletoe that are transferred to the ethanol–water solutions during maceration. The results showed that macerates with lower alcohol content were richer in alcohols, esters, aldehydes, and ketones, while macerates with higher alcohol content were richer in terpenes and hydrocarbons (Figure 4). Medium plant concentration should be preferred since in macerates with high plant concentration there is less extraction of volatile components. Ethyl esters of short- and medium-chain acids are the main esters in strong alcoholic beverages and are responsible for their "fruity" and "floral" sensory properties. In agreement with the Hanousek Čiča et al. [23], these esters are most important in both commercial *biska* samples as well as in these macerates. However, the order of occurrence is somewhat different. In commercial samples, octanoate is the most abundant, followed by decanoate, hexanoate, dodecanoate, and hexadecanoate. In macerates, decanoate is most abundant, followed by others listed, and additionally formic acid ethenyl ester, which was not detected in commercial samples (Figure S1). The differences in the order of predominant esters can be explained by the differences in the base used for maceration and *biska* production as well as by the amount of macerate/plant used in the preparation of the commercial samples. In addition, ethyl decanoate has a shorter half-life than ethyl octanoate, and depending on the age of the spirit, it may be reasonable for ethyl octanoate to become the more common ethyl ester in macerates over time. Considering the low threshold of these esters (10–250 ppb) [31], it can be concluded that they are aromas that contribute significantly to the aroma of mistletoe macerate. Wang et al. [8] gave a comprehensive review of the volatiles of mistletoe compounds, but in an extract obtained with hot water, they did not detect our ethyl esters; nor did Vlad et al. [32], who analyzed the volatile components in the aqueous and alcoholic extract. It can be assumed that these ethyl esters are formed during maceration by esterification with mistletoe acids.

In accordance with our results, Wang et al. [8] detected the aldehydes, benzaldehyde, octanal, and a number of terpenes. The aldehydes benzaldehyde, hexanal, and octanal, as well as the esters and terpene compounds, have a low odor threshold [31] and also contribute significantly to the aroma of the macerates (Table 2). Hanousek Čiča et al. [23] detected limonene as a mistletoe-derived terpene in commercial *biska* samples, while numerous terpenes besides limonene were isolated in macerates at concentrations above the threshold, so we can say that these terpenes contribute significantly to the mistletoe macerate aroma. Ylangene, α-bourbonene, and β-bourbonene are some tentatively determined terpenes that we did not find in the literature on mestletoe, nor thujone, so we can consider them as a special contribution to this work. Other terpenes listed in Table 2 have been detected in stems and leaves of mistletoe [30]. Wang et al. [8] mentioned linalool and ionone as the most significant, while Ćebović et al. [9] identified trans-α-bergamotene, trans-β-farnesene, loliolide, and vomifoliol as terpene compounds that had not been previously detected as constituents in mistletoe. Hanousek Čiča et al. [23] also detected thujone in commercial *biska* samples, confirming that mistletoe contains thujone. The macerates with high ethanol content were rich in hydrocarbons, especially unsaturated aliphatic hydrocarbons with 10–17 carbon atoms. Some of them, such as 1-undecene, 1-dodecene, 3-dodecene, or saturated heptadecane, are volatile components with a pleasant odor and are found in the composition of some essential oils. As mentioned above, there are few data in the literature on volatile components in alcoholic mistletoe extracts. Moreover, the data analyzed are very variable, since the mistletoe itself is a parasitic plant whose composition depends strongly on the host tree, as well as on the harvesting and drying conditions [4,5], which certainly affects the composition of the volatiles in the extracts.

Measuring the chromatic characteristics of macerates, i.e., strong alcoholic beverages, is important because it has a psychological effect on the consumer. It is also an indicator of quality. Factors such as the drying process of the herbs or enzymatic and non-enzymatic browning affect the final color of the herbs and thus the color of the macerate [33]. Ethanol strength, maceration time, and quantity of fruits and plants affect the extraction of pigments (e.g., chlorophyll) from the plant into the ethanol–water solutions and are associated with color variations [1]. The degradation of these pigments leads to non-enzymatic browning and discoloration of the macerate. The darker color of macerates can also be explained by the presence of the enzyme polyphenol oxidase in the plant, which causes the formation of melanin and benzoquinone from phenol, resulting in brown coloration [34].

5. Conclusions

Croatia is traditionally a tourist country. Rural and island tourism have developed, and spirits are part of the gastronomic offer in this area. Traditional homemade alcoholic beverages are a part of the traditional food culture and have an important traditional and cultural value. Therefore, it is necessary to research the spirits in order to improve their quality, but at the same time preserving the traditional values: recipes, production methods, and general traditional knowledge of individual drinks such as *biska*, to which this work contributes. Based on the volatiles detected, *biska* is expected to have a fruity and floral odor due to the presence of short- and medium-chain ethyl esters. The sensory properties of citrus, earth, wood, and mint are due to the contributions of terpenes and terpene alcohols. We can suggest that ethyl alcohol of agricultural origin with 40–50% by volume is used for the preparation of aromatic macerates, because it contains volatile substances of mistletoe, which are soluble in both alcohol and water. In addition, the ratio of herbs to base should be about 40 g/L, since too high a concentration of herbs will result in macerates with lower volatile content due to extraction of other substances. These parameters should be considered and matched with the optimal parameters for extraction of biologically active compounds, in which mistletoe is also rich and which we will also report.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/beverages8030046/s1, Figure S1: Average value and standard deviation of concentrations of esters detected in ethanol/water mistletoe macerate.

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