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Chemometric approach to quality characterization of milk-based kombucha beverages

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Abstract

Milk-based kombucha beverages were obtained conducting kombucha lead fermentation of milk. In order to discriminate the analysed samples and to detect similarities or dissimilarities among them in the space of experimentally determined variables, hierarchical cluster analysis (HCA) and principal component analysis (PCA) were applied. Linear discriminant analysis (LDA) was conducted on the raw data set in order to find a rule for allocating a new sample of unknown origin to the correct group of samples. In the space of the variables analysed by HCA, the dominant discriminating factor for the studied samples of kombucha beverages is the milk fat (MF) content, followed by total unsaturated fatty acids content (TUFA), monounsaturated fatty acids content (MUFA) and polyunsaturated fatty acids content (PUFA). The samples with 0.8 and 1.6% milk fat belong to the same cluster in the space of the analysed variables due to similarities in their AA_{DPPH}. It was determined by LDA that there was the biggest difference in quality between the groups of products with winter savoury and stinging nettle, while the highest similarity is between groups of products with wild thyme and peppermint regarding their pH values and antioxidant activity expressed as AA_{DPPH}.

Key words: **kombucha; milk-based products; antioxidant activity; chemometric analysis**

Introduction

Kombucha is a symbiotic association of bacteria and yeasts. Traditionally, it is well known by its capability to ferment a simple substrate, black or green tea sweetened with sucrose, into non-alcoholic, slightly carbonated refreshing beverage (Kapp and Sumner, 2019). The main characteristic of the traditional kombucha beverage is the possibility of production in a home environment. Also, kombucha products are consumed worldwide (Jayabalan et al., 2016). Beside traditional substrate, kombucha successfully ferments sweetened herbal extracts, coca cola, beer, milk etc. (Jayabalan et al., 2014). Kombucha beverage is a product of natural origin with numerous potential health benefits (Jayabalan et al., 2014) that are related to the presence of vitamins, organic acids, polyphenols, and other components produced during fermentation (Jayabalan et al., 2015). Diverse chemical composition was the reason that kombucha was chosen to be used in the production of milk-based products (Malbaša et al., 2014; Sarkaya et al., 2020; Vitas et al., 2013a).

Milk-based kombucha beverages are obtained after milk inoculation with different kombucha starters and fermentation up to pH 4.50. It could be traditional kombucha inoculums or, for example, inoculums obtained by fermentation on wild thyme, stinging nettle, peppermint or winter savory water extracts sweetened with sucrose (Jayabalan et al., 2014; Malbaša et al., 2014, 2009; Vitas et al., 2018, 2013a). The main chemical composition and the antioxidant potential of kombucha milk-based beverages obtained using alternative starters was established (Brezo et al., 2011; Kravić et al., 2011; Lončar et al., 2013; Malbaša et al., 2014, 2011b; Vitas et al., 2018, 2016, 2013a, 2013b, 2011). Latest research suggests that kombucha milk products contain ACE-inhibitory peptides (Elkhtab et al., 2017), and the ACE inhibitory activity of this type of products was determined by Hrnjez et al. (2014). Also, Xia et al. (2019) concluded that kombucha enhanced in a significant amount the health-promoting effects of soymilk.

The novelty of this paper is related to the application of modern statistical and mathematical methods to quality evaluation of milk-based kombucha products. Statistical tools used pH value, milk fat and fatty acids content, as well as determined ***in vitro*** antioxidant capacity.

Various kombucha products possess antioxidant activity and it is one of the main characteristics which affect the physiological benefits of kombucha beverage. Antioxidant capacity of different kombucha beverages is the consequence of number of ingredients and metabolites such as tea polyphenols, vitamin C, B group vitamins, unsaturated fatty acids and some enzymes (Jayabalan et al., 2008; Vitas et al., 2013a).

Toward additionally clarification of the data which describe kombucha beverages obtained by cultivating milk with kombucha inoculum obtained by fermentation on four different extracts chemometric analysis was carried out.

A method for dividing a group of objects into classes so that similar objects are in the same class (cluster) is known as hierarchical cluster analysis (HCA) (Miller and Miller, 2010). This type of analysis searches for objects which are close together in the variable space. The cluster analysis result is displayed as a tree diagram called a dendrogram, where the horizontal axis represents the distance or dissimilarity between the clusters.

Principal component analysis (PCA) is the mostly used chemometric tool applied to reduce the amount of data and/or obtain orthogonal variables, especially when collinearity occurs (González-Díaz et al., 2007a, 2007b). It is suitable for big data sets giving the information regarding data that behave in a similar way. The result of PCA analysis is presented by scores and loadings plots. The new coordinates of the projected objects are scores and loadings reflect the direction with respect to the original variables. The results of PCA analysis could be used as input data for other multivariate techniques instead of original variables (Ciosek and Wróblewski, 2006).

Linear discriminant analysis (LDA), as a supervised pattern recognition method (Miller and Miller, 2010), uses a basically different mathematical approach for sample discrimination than HCA and PCA. Its purpose in this study was to find linear relationships that will be able to define the linear discriminant function (LDF), which linearly combines original variables (Miller and Miller, 2010). The boundary is estimated such that the variance between the groups is maximized and the variance within the individual groups is minimized (Otto, 2017).

Chemometric analysis covered HCA and PCA used to detect similarities or dissimilarities among analysed samples, and LDA was applied toward finding a rule for assigning a new sample of unknown origin to the correct group.

The chemometric approach for characterization and selection is used for textural characteristics of milk-based kombucha products (Malbaša et al., 2015) while it was not used for estimation of quality based on chemical characteristics and antioxidant potential.

The objective of this article is chemometric considerations of milk-based kombucha products with herbal extracts of winter savoury, peppermint, stinging nettle and wild thyme obtained by kombucha fermentation on milk with 2.8, 1.6 and 0.8 % milk fat at 37, 40 and 43 °C. Mathematical calculations with obtained experimental results determined the discriminating factor for the classification of the samples based on their quality.

Materials and methods

Production of kombucha milk-based beverages

Samples were obtained according to the procedure described by Malbaša et al. (2014) and Vitas et al. (2013a, 2018). Briefly, milk with 0.8, 1.6 and 2.8 % milk fat was inoculated with kombucha starter obtained by kombucha cultivation on winter savoury, peppermint, stinging nettle and wild thyme water extract with dissolved sucrose. Fermentation of milk was conducted at 37, 40 and 43 °C until medium reached pH 4.50 in accordance to the references mentioned above or no longer than 1 7h. Samples were marked depending on the used initial substrate (winter savoury water extract - WS,

peppermint water extract - P, stinging nettle water extract - SN and wild thyme water extract - WT), applied process temperature and milk fat content of used milk.

Methods of analysis

pH values were determined by a pH-meter (PT-70, Boeco, Germany).

Milk fat content (MF, %) was measured by the Gerber method (IDF 105:1981) (Carić et al., 2000).

Monounsaturated fatty acids (MUFA, %) and polyunsaturated fatty acids (PUFA, %) content was determined by the gas chromatography-mass spectrometry (GC-MS) method (Vitas et al., 2018). The used method covers the extraction of fat, preparation of fatty acids methyl esters and the GC-MS analysis of these esters. Hewlett-Packard (HP) 5890 Series II gas chromatograph coupled an with HP 5971A mass spectrometer detector was applied. Capillary column Supelco fused silica SP-2560 (100 m x 0.25 mm; thickness of stationary liquid phase film 0.20 µm) was applied. Injection volume was 1 µL, injector temperature was set at 230 °C, and the split ratio was 1:40. The carrier gas was helium with 0.58 mL/min constant flow. The temperature program was set at: initial temperature of 100 °C, held for 5 min and increased for 6 °C/min until the final 240 °C was reached (20 min). MS possess an ionic source and works on the principle of electron ionization. The temperature of the quadrupole was 180 °C. Data acquisition was performed in scan mode (in range 50-400 m/z). The multi-Standard solution of 37 fatty acids methyl esters (37 component FAME Mix, 47885-U) from Supelco, Bellefonte, PA, USA was used, as well as the 'Wiley' commercial database of mass spectra. A modified method of 100 % was applied for MUFAs and PUFAs quantification. Total unsaturated fatty acids (TUFA, %) were presented as the sum of MUFA and PUFA. In this paper, values for MUFA, PUFA and TUFA were calculated as the percentage share in milk fat content of the obtained samples, since their presence in the kombucha milk-based beverages comes from milk fat of the milk used for fermentation.

Antioxidant activity to DPPH radical (AA_{DPPH}, %) was determined spectrophotometrically according to the (Vitas et al., 2018). Sample preparation covered absolute ethanol (Zorka Pharma Hemija d.o.o., Šabac, Serbia) addition (1:1, v/v) to the sample, after which it was kept for 20 min in the freezer and centrifuged (4000 g) for 30 min at 4 °C. The process was repeated. The obtained supernatant was used for the analysis. The volume of 1 mL of DPPH· radical standard (Sigma-Aldrich® CHEMIE GmbH, Steinheim, Germany) solution in methanol (120 µM) was added to 1.5 mL methanol and 0.5 mL of sample supernatant. The reaction tubes were kept in the dark for 45 min at 25 °C. Absorbance was measured at 515 nm. Antioxidant activity was expressed as inhibition percent (%).

Antioxidant activity to hydroxyl radical ($AA_{\cdot OH}$, %) was measured spectrophotometrically according to the (Deeseenthum and Pejovic, 2010). The 75 µL of the sample was mixed with 450 µL of sodium phosphate (Merck, Alkaloid, Skoplje, North Macedonia) buffer (0.2 mol/L, pH=7,00), 150 µL of 2-deoxyribose (Alfa Aesar GmbH & Co KG, Karlsruhe, Germany) (10 mmol/L), 150 µL of EDTA disodium salt dihydrate (Lach-Ner, Neratovice, Czech Republic) (10 mmol/L), 150 µL of $FeSO_4 \times 7H_2O$ (Zdravlje, Leskovac, Serbia) (10 mmol/L), 150 µL of H_2O_2 (Zorka Pharma, Šabac, Serbia) (10 mmol/L), and 525 µL of double-distilled water. Samples were incubated at 37 °C for 4 h after which 750 µL of trichloroacetic (J. T. Baker, Deventer, The Netherlands) (2.8 %) acid and 750 µL of thiobarbituric (Alfa Aesar GmbH & Co KG, Karlsruhe, Germany) (0.1 %) acid was added. Afterwards, the samples were kept in boiled water for 10 min. Absorbance was measured at 520 nm. 96 % ethanol (Reahem, Novi Sad, Serbia) was used as control. Antioxidant activity was expressed as inhibition percent (%).

Chemicals were of analytical and GC purity grade.

All results in this paper were given as mean values of three measurements ± standard deviation determined for each analysed sample.

Chemometric analysis

Chemometric tools HCA, PCA, and LDA were conducted by using Minitab 16.1.1. software. HCA was applied by using Statistica 12.0 and NCSS 2012 software. PCA was applied by using Statistica 12.0 software.

Results and discussion

Results of pH values were obtained in repeated fermentation processes. Results for milk fat content for samples with winter savoury obtained from milk with 1.6 % MF at 37, 40 and 43 °C were previously published in Vitas et al. (2011). Results for fatty acids content were previously published in several different publications (Brezo et al., 2011; Kravić et al., 2011; Lončar et al., 2013; Malbaša et al., 2014, 2011b; Vitas et al., 2018, 2016, 2013a, 2013b, 2011), where they were quantified by 100 % method, but they were not presented in the form that they are given in this article.

Antioxidant activity of the samples was not presented in this form in previous studies, but as difference between value obtained for product and value obtained for initial milk (Malbaša et al., 2014; Vitas et al., 2018, 2013a). Results for samples with winter savoury produced from milk with 1.6 % MF at 37, 40 and 43 °C were published in Vitas et al. (2016). Antioxidant capacity of samples with stinging nettle obtained using milk with 2.8 and 1.6 % MF at 37, 40 and 43 °C was published in Vitas et al. (2013b).

Chemometric analysis was carried out on the data (Table 1) which describe kombucha beverages obtained by cultivating milk with kombucha inoculum obtained by fermentation on winter savoury, peppermint, stinging nettle and wild thyme water extracts. Every sample was described by milk fat content, total unsaturated fatty acids content, monounsaturated fatty acids content, polyunsaturated fatty acids content, the antioxidant activity to DPPH and hydroxyl radical, pH value at

Table 1. The studied samples and their chemical (MF, TUFA, MUFA, PUFA, and pH) and antioxidant properties (AA_{DPPH} and $AA_{\cdot OH}$)

Sample	MF (%)	TUFA (%)	MUFA (%)	PUFA (%)	AA_{DPPH} (%)	$AA_{\cdot OH}$ (%)	pH_{start}	pH_{8h}	pH_{end}
WS-37-2.8	2.64±0.00	0.8931±0.0003	0.7416±0.0003	0.1515±0.0002	73.26±2.06	2.33±0.00	6.46±0.00	5.65±0.00	5.43±0.00
WS-40-2.8	2.64±0.00	0.8379±0.0003	0.7117±0.0003	0.1259±0.0001	60.80±1.21	3.34±0.20	6.43±0.00	5.87±0.00	4.50±0.00
WS-43-2.8	2.64±0.00	0.8514±0.0003	0.7384±0.0003	0.1133±0.0002	19.32±0.50	5.79±0.10	6.45±0.00	5.97±0.00	4.50±0.00
WS-37-1.6	1.54±0.00	0.5416±0.0002	0.4759±0.0001	0.0658±0.0001	81.04±2.74	3.71±0.04	6.47±0.00	5.92±0.00	5.09±0.00
WS-40-1.6	1.54±0.00	0.5025±0.0002	0.4441±0.0002	0.0585±0.0000	87.72±3.00	2.24±0.01	6.46±0.00	5.97±0.00	4.50±0.00
WS-43-1.6	1.54±0.00	0.5299±0.0002	0.4720±0.0002	0.0581±0.0001	63.22±2.55	2.59±0.01	6.43±0.00	5.21±0.00	4.50±0.00
WS-37-0.8	0.66±0.00	0.1851±0.0001	0.1651±0.0000	0.0200±0.0000	70.00±2.70	3.22±0.01	6.51±0.00	5.63±0.00	5.36±0.00
WS-40-0.8	0.66±0.00	0.1734±0.0001	0.1595±0.0000	0.0139±0.0000	57.10±1.67	1.25±0.00	6.40±0.00	5.53±0.00	4.68±0.00
WS-43-0.8	0.66±0.00	0.2178±0.0001	0.1819±0.0000	0.0360±0.0000	72.12±3.50	1.04±0.00	6.44±0.00	6.28±0.00	4.50±0.00
P-37-2.8	2.64±0.00	0.9021±0.0004	0.7487±0.0003	0.1531±0.0001	57.08±2.22	1.76±0.00	6.22±0.00	6.12±0.00	4.50±0.00
P-40-2.8	2.64±0.00	0.9082±0.0004	0.7764±0.0003	0.1315±0.0001	35.20±0.36	3.70±0.00	6.23±0.00	6.24±0.00	4.50±0.00
P-43-2.8	2.64±0.00	0.8694±0.0003	0.7524±0.0002	0.1170±0.0002	20.23±0.20	5.31±0.05	6.23±0.00	6.12±0.00	4.50±0.00
P-37-1.6	1.54±0.00	0.5384±0.0002	0.4657±0.0002	0.0725±0.0001	79.91±2.15	5.06±0.03	6.24±0.00	6.20±0.00	4.71±0.00
P-40-1.6	1.54±0.00	0.4736±0.0002	0.4215±0.0002	0.0521±0.0000	82.21±3.50	3.11±0.03	6.27±0.00	6.16±0.00	4.50±0.00
P-43-1.6	1.54±0.00	0.5276±0.0002	0.4609±0.0002	0.0667±0.0000	45.75±1.66	2.23±0.02	6.22±0.00	5.23±0.00	4.50±0.00
P-37-0.8	0.66±0.00	0.2091±0.0001	0.1827±0.0001	0.0264±0.0000	65.55±1.30	3.66±0.02	6.25±0.00	6.12±0.00	4.50±0.00
P-40-0.8	0.66±0.00	0.1749±0.0001	0.1547±0.0000	0.0202±0.0000	60.09±2.10	1.53±0.00	6.26±0.00	5.47±0.00	4.50±0.00
P-43-0.8	0.66±0.00	0.2026±0.0001	0.1797±0.0001	0.0229±0.0000	58.65±1.90	0.84±0.00	6.27±0.00	6.18±0.00	4.50±0.00
K-37-2.8	2.64±0.00	0.8419±0.0004	0.7186±0.0003	0.1233±0.0001	40.00±1.50	2.43±0.01	5.90±0.00	6.03±0.00	5.07±0.00
SN-40-2.8	2.64±0.00	0.9063±0.0005	0.7447±0.0003	0.1616±0.0001	15.73±0.17	4.28±0.02	5.87±0.00	6.01±0.00	4.50±0.00
SN-43-2.8	2.64±0.00	0.9554±0.0004	0.7806±0.0003	0.1748±0.0002	40.23±2.25	6.29±0.03	6.02±0.00	5.98±0.00	4.50±0.00
SN-37-1.6	1.54±0.00	0.5105±0.0002	0.4602±0.0002	0.0504±0.0001	64.12±1.57	5.50±0.07	5.95±0.00	6.00±0.00	4.60±0.00
SN-40-1.6	1.54±0.00	0.5094±0.0002	0.4480±0.0002	0.0614±0.0001	60.40±3.13	4.72±0.04	5.95±0.00	6.01±0.00	4.50±0.00
SN-43-1.6	1.54±0.00	0.5265±0.0002	0.4580±0.0002	0.0685±0.0001	65.06±3.03	3.01±0.09	5.93±0.00	5.37±0.00	4.50±0.00
SN-37-0.8	0.66±0.00	0.2116±0.0002	0.1760±0.0001	0.0356±0.0001	73.78±3.02	3.55±0.15	6.04±0.00	6.04±0.00	4.62±0.00
SN-40-0.8	0.66±0.00	0.1643±0.0000	0.1457±0.0000	0.0185±0.0000	57.94±2.33	1.60±0.55	6.04±0.00	5.43±0.00	4.50±0.00
SN-43-0.8	0.66±0.00	0.1599±0.0000	0.1460±0.0000	0.0139±0.0000	43.27±1.70	0.68±0.08	6.05±0.00	5.90±0.00	4.50±0.00
WT-37-2.8	2.64±0.00	0.9504±0.0003	0.7904±0.0003	0.1600±0.0002	65.17±2.55	0.78±0.05	6.27±0.00	6.31±0.00	4.50±0.00
WT-40-2.8	2.64±0.00	0.9082±0.0004	0.7519±0.0003	0.1566±0.0001	37.33±0.95	4.95±0.25	6.27±0.00	6.28±0.00	4.50±0.00
WT-43-2.8	2.64±0.00	0.8971±0.0004	0.7281±0.0003	0.1690±0.0002	41.36±1.09	6.41±0.20	6.29±0.00	6.25±0.00	4.50±0.00
WT-37-1.6	1.54±0.00	0.5335±0.0002	0.4709±0.0002	0.0625±0.0001	76.97±2.17	5.61±0.30	6.30±0.00	6.30±0.00	4.64±0.00
WT-40-1.6	1.54±0.00	0.5147±0.0003	0.4528±0.0002	0.0621±0.0001	77.44±2.01	2.76±0.10	6.30±0.00	6.24±0.00	4.50±0.00
WT-43-1.6	1.54±0.00	0.5432±0.0000	0.4759±0.0002	0.0673±0.0001	66.21±1.79	3.39±0.10	6.30±0.00	5.91±0.00	4.50±0.00
WT-37-0.8	0.66±0.00	0.1932±0.0000	0.1692±0.0001	0.0240±0.0000	73.33±2.55	3.75±0.20	6.33±0.00	6.17±0.00	4.57±0.00
WT-40-0.8	0.66±0.00	0.1725±0.0000	0.1533±0.0001	0.0192±0.0000	46.57±1.00	1.56±0.01	6.27±0.00	5.65±0.00	4.50±0.00
WT-43-0.8	0.66±0.00	0.1782±0.0000	0.1592±0.0001	0.0191±0.0000	55.13±1.07	1.08±0.00	6.24±0.00	6.13±0.00	4.50±0.00

the start of the fermentation (pH_{start}), pH value after 8 hours of the fermentation (pH_{8h}) and the final pH value (pH_{end}).

In order to discriminate the analyzed samples and to detect similarities or dissimilarities among them in the space of aforementioned experimentally determined variables, HCA and PCA were applied. Prior to analysis, the variables were normalized by ***min-max*** normalization method so the values of the variables are scaled in the range 0.01-0.99. LDA was conducted on the raw data set in order to find a rule for allocating a new sample of unknown origin to the correct group of samples.

HCA was applied by using Statistica 12.0 and NCSS 2012 software. The HCA clustering was done on the basis of Ward's and Single linkage algorithms by calculating Euclidean distances.

The clustering was carried out in two steps. In the first step, all variables were taken into account so the grouping of the samples can be obtained based on chemical composition, antioxidant activity and pH values, whereas the second step included the clustering based only on antioxidant activity and pH values of the samples.

The results of the clustering of the samples on the basis of the whole set of variables are presented in Fig. 1a and Fig. 1b. In Fig. 1a, two well-separated clusters can be observed. According to the results, the samples were very well separated based on milk fat content. The cluster which contain the samples with the highest MF content is significantly separated from the other two. This could mean that these samples can be distinguished from the others not only because of the highest MF content but also on the basis of other determined properties. Considering the results obtained applying the Single linkage algorithm (Fig. 1b) the close placement of the samples which contain 0.8 and 1.6 % of MF could be observed as in the case of HCA where Ward's algorithm was applied. However, in the dendrogram presented in Fig. 1b it can be seen that WS-37-2.8 can be considered to be an outlier since it does not belong to any observable cluster. This sample actually had the highest pH_{end} value. According to the measured pH_{end} value (5.43) this sample could not be considered as fermented milk beverage. Kombucha milk-based beverages differ from the other types of kombucha products mainly because of the obtained product's pH value. Milk fermentation utilizing kombucha ends, similar to yoghurt fermentation, when pH value reaches 4.50, and is significantly shorter compared to the production of traditional kombucha products. These products obtained on black or green tea sub-

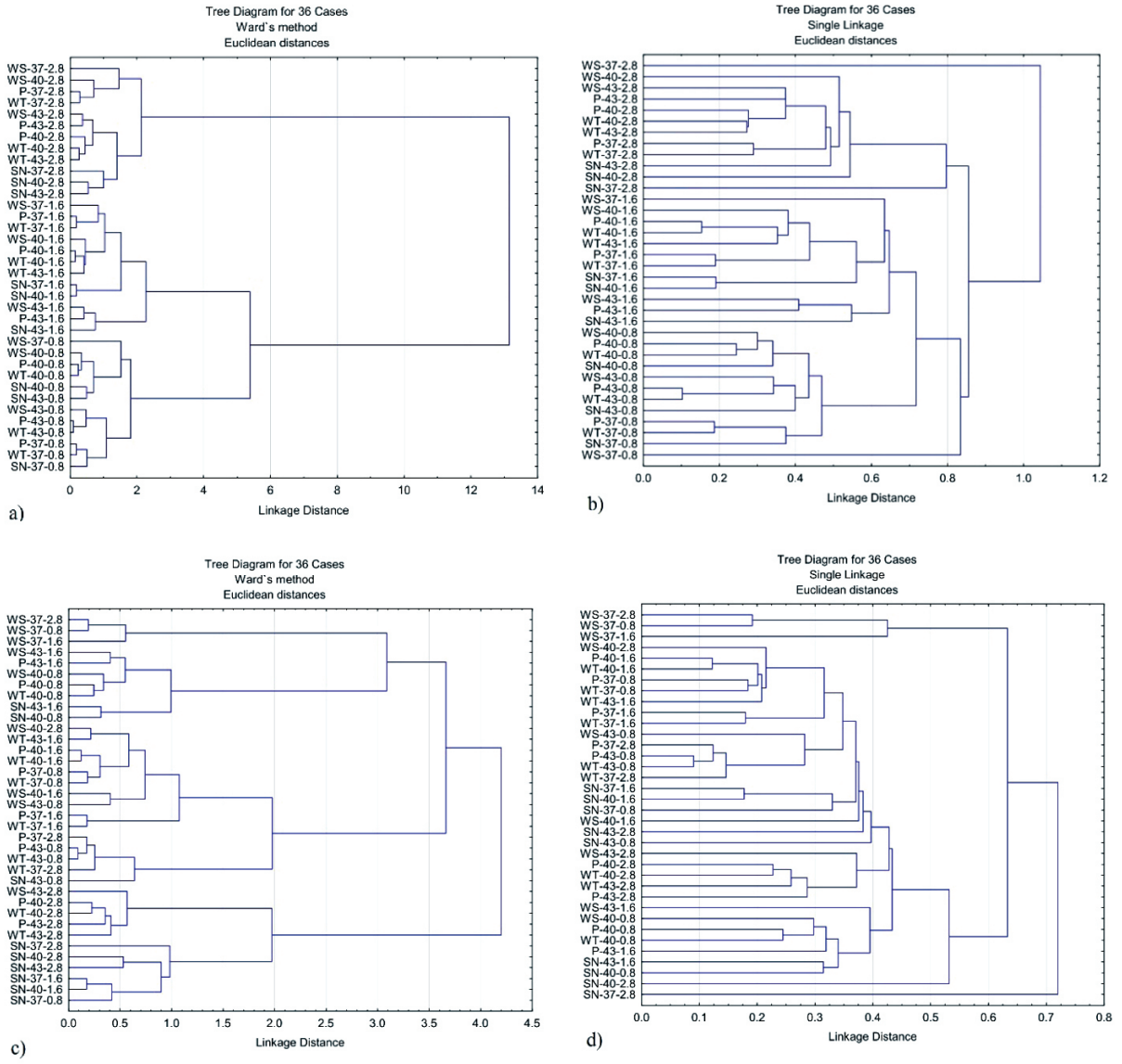

Figure 1. Clustering of the samples based on a) Ward's algorithm and all determined variables; b) Single linkage algorithm and all determined variables; c) Ward's algorithm and antioxidant activity and pH values; d) Single linkage algorithm and antioxidant activity and pH values

strate sweetened with sucrose have lower pH values, usually in the range of 3 to 3.5 (Malbaša et al., 2011a). The main criteria for the end of traditional fermentation, which lasts for 6 to 7 days are, the beverage's sensory characteristics. However, the approach to kombucha milk fermentation is entirely different. In comparison to traditional kombucha fermentations, the fermentation time required for reaching the pH of 4.50 is considerably shorter when milk is used. Besides that, the antioxidant activity of the obtained kombucha milk-based beverages is significantly different, too. The dendrograms presented in Fig. 1c and Fig. 1d are obtained when antioxidant activity and pH values of the samples were considered. In this case, the results indicated the significant separation of the samples cultivated on winter savory at 37 °C from the other samples regardless the algorithm used for HCA modelling. These samples are described by the highest pH_{start} and pH_{end} values, while their antioxidant activity expressed as AA_{DPPH} are among the highest. These samples also do not belong to the group of fermented milk products.

In order to analyse the grouping of both variables and samples on only one platform, the double dendrogram was defined on the basis of Ward's algorithm and Euclidean distances (Fig. 2). The grouping of the samples is almost the same as on dendrogram presented in Fig. 1a. Here it can be seen that the samples are very well separated based on MF, followed by TUFA, MUFA and PUFA. Particularly, the group of the samples which contains the highest MF is very well separated from the others (it is placed in the separate

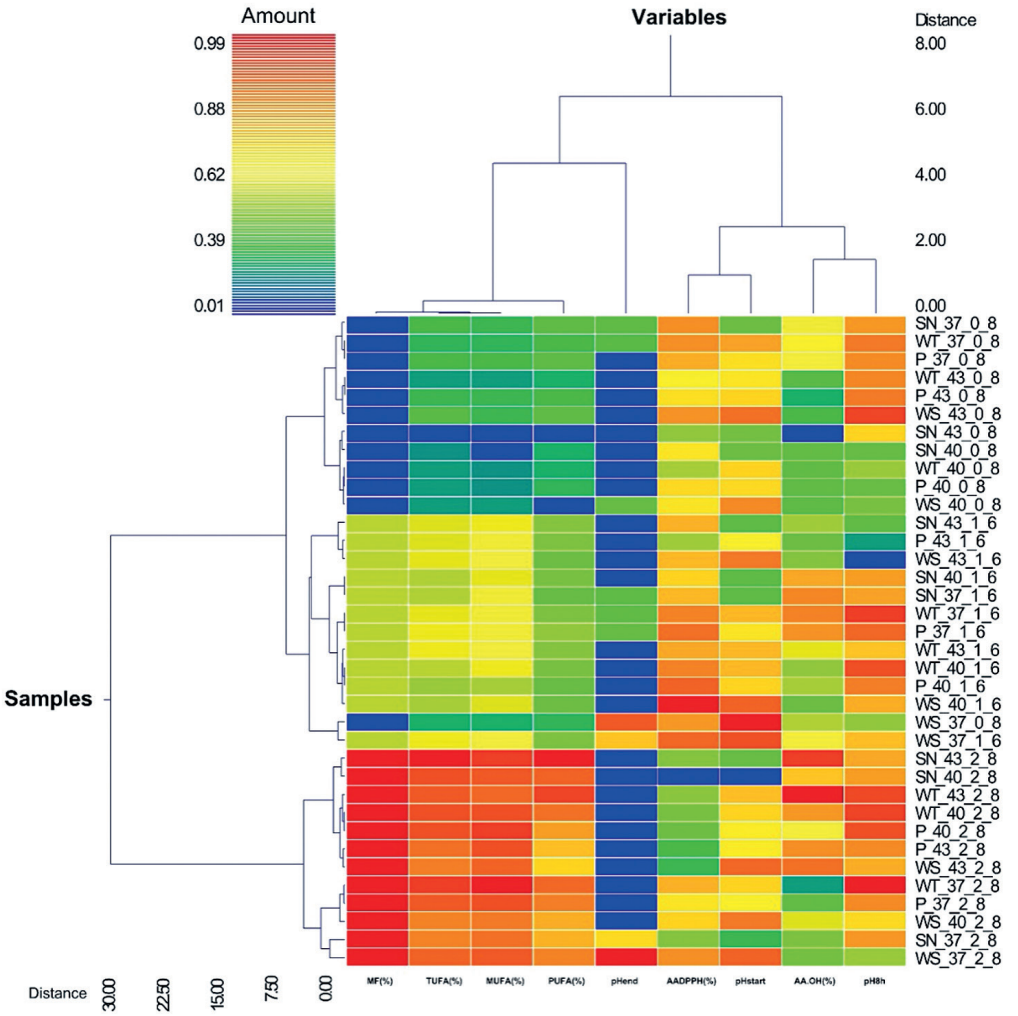

Figure 2. Double dendrogram of the samples based on Ward's algorithm and all determined variables

cluster). The majority of these samples had the lowest pH_{end} and lowest antioxidant activity expressed as AA_{DPPH}. These variables were grouped in the same cluster and could be considered as the main discriminating factor of the samples. The separation of the samples regarding the extracts used and fermentation temperature was not observed. The only overlap between the samples with 0.8 and 1.6 % of MF is the sample WS-37-0.8 which was classified together with the samples with 1.6 % of MF. The reason for this could be its antioxidant activity and pH values which were similar to the samples with 1.6 % of MF.

In order to gain an overview of distribution of the samples in the space of the analysed variables, PCA was applied by using Statistica 12.0 software. The model resulted in three PCs whose Eigenvalue was greater than 1 covering 79.1 % of total variance. The first PC contributes 50.93 % to the total variance, while the second one contributes 16.16 %. The third PC, which is also described with Eigenvalue higher than 1 contributes 12.01 % to the total variance (Table 2 and Fig. 3).

The score and loadings plots of the PCA are presented in Fig. 4. The results of PCA are in agreement with the results of the HCA, however they reveal more regarding similarities and dissimilarities among the samples. Going along the Factor 1 (PC1) axis, it can be seen that there is a quite good separation between the samples based on their MF content. Considering the distance between the samples, it can be noticed that the samples with the highest MF content are significantly separated from the negative end of the Factor 1 axis. This inequality between the distances among the samples is probably caused due to certain influence of $AA._{OH}$ and AA_{DPPH} and small influence of pH_{8h} variables on Factor 1 axis. Therefore, generally speaking, the samples which contain 0.8 % and 1.6 % MF are placed closer on the score plot mainly because of their similarities regarding $AA._{OH}$ and AA_{DPPH} values. The score and loadings plot indicate that the majority of the samples which contain 2.8 % MF have the lowest AA_{DPPH} and high $AA._{OH}$ potential. The presented values were different in comparison to traditional black or green tea kombucha beverages. The

Table 2. Eigenvalues and total variances of the PCA model

Factors (PCs)	Eigenvalue	% Total variance	Cumulative Eigenvalue	Cumulative %
1	4.58	50.93	4.58	50.93
2	1.45	16.16	6.04	67.09
3	1.08	12.01	7.12	79.10
4	0.76	8.49	7.88	87.59
5	0.60	6.64	8.48	94.23
6	0.46	5.14	8.94	99.37
7	0.05	0.58	9.00	99.95
8	0.004	0.05	9.00	100
9	0	0	9.00	100

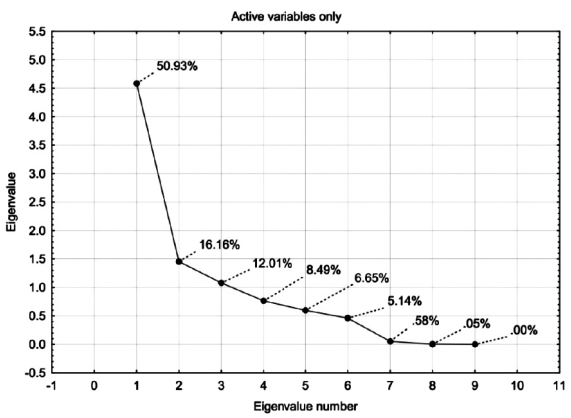

Figure 3. Eigenvalues of correlation matrix of the established PCs (Factors)

$AA._{OH}$ values of traditional kombucha beverages are much higher compared to milk-based ones. The highest $AA._{OH}$ value of the kombucha milk-based beverages was 6.41 % for the WT-43-2.8 (Table 1) while the $AA._{OH}$ values of traditional kombucha beverages were in a range of approximately 40 to 60 % (Malbaša et al., 2011a). The AA_{DPPH} of the kombucha milk-based beverages were in a wide range and varied from 15.73 to 87.72 % (Table 1). The average AA_{DPPH} of the traditional black or green tea kombucha beverages was around 40 % (Malbaša et al., 2011a). The forehead mentioned differences in the chemical composition of substrates and starters for the traditional and milk fermentation of kombucha affected $AA._{OH}$ greatly, while the differences of AA_{DPPH} were not much pronounced. The differences in antioxidant activities between kombucha milk-based beverages could be due to the different chemical compositions of the applied kombucha starters besides the different fermentation temperatures and milk fat percent in the substrate. Thymol and carvacrol are the main components of winter savoury and wild thyme. It is typical for the wild thyme that carvacrol content is higher than thymol content (de Oliveira et al., 2011; Zavatti et al., 2011; Fecka and Turek, 2008). The peppermint's main components are menthol, menthone, and limonene (Akdogan et al., 2004), while formic acid, histamine, serotonin, and acetylcholine are the typical components of stinging nettle (Cummings and Olsen, 2011; Hojnik et al, 2007).

The distribution of the samples along the Factor 2 axis was mainly caused by pH_{end} and pH_{start} values, and to a lesser extent by AA_{DPPH} values, as it can be seen on the loadings plot. The samples with WS extract and fermentation temperature of 37 °C had the highest pH_{end} and pH_{start} values and among the highest antioxidant activities (AA_{DPPH}). On the other hand, the majority of the samples which contained stinging nettle extracts (SN) were placed at the end which implies low pH_{start} and pH_{end} values, regardless the MF content and fermentation temperature. However, these samples had low, moderate and high antioxidant activity (AA_{DPPH}) and could not be classified as the samples with only high nor only low AA_{DPPH} values (AA_{DPPH} express moderate influence on samples distribution on the score plot along the Factor 1 axis). The separation of other groups of the samples along the Factor 2 axis was not observed.

The samples were divided into four groups regarding the extract used in their fermentation: SN, WS, P and WT. The analysis was carried out considering the following variables: AA_{DPPH}, $AA._{OH}$, pH_{start}, pH_{8h} and pH_{end}. Since the normalization of the variables had no effect on the outcome of LDA (Miller and Miller, 2010), the analysis was done on the raw data. The validation of the established functions was conducted applying the cross-validation (CV) method.

The obtained results are presented in Table 3. One sample was put into specified group when Mahalanobis squared distance of observation to the group centre (mean) is minimum. The confusion matrix shows a success rate of 100 % (9 correct hits out of 9), 77.8 % (7 correct hits out of 9), 77.8 % (7 correct hits out of 9) and 100 % (9 correct hits out of 9) for the groups SN, WT, P and WS, respectively. The proportion of the correct hits is 88.9 %. When the leave-one-out (LOO) cross-validation is carried out 100 % (9 correct hits out of 9), 66.7 % (6 correct hits out of 9), 66.7 % (6 correct hits out of 9) and 100 % (9 correct hits out of 9) for the groups SN, WT,

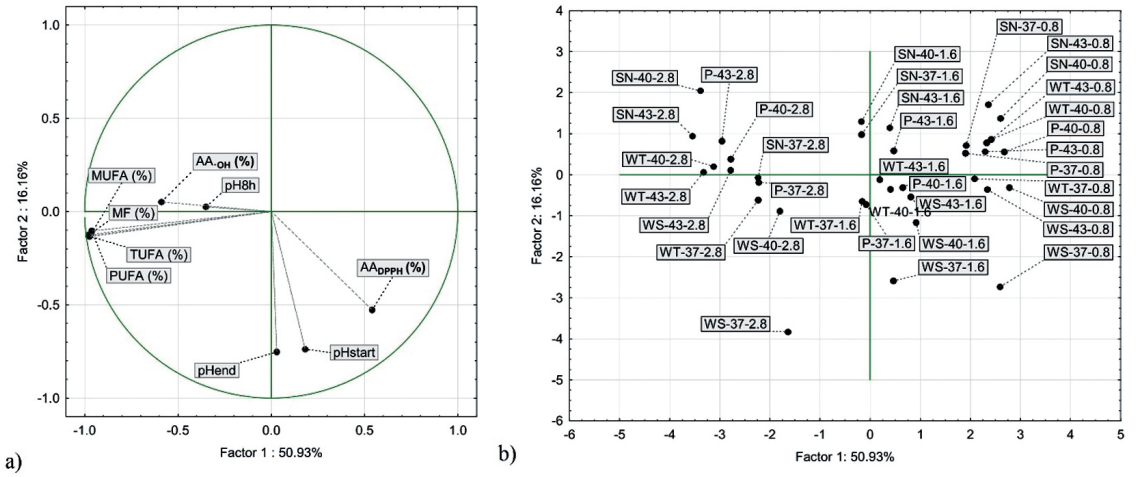

Figure 4. The loadings (a) and score plots (b) of the studied samples as a result of the PCA

P and WS, respectively. During the LOO-CV, the proportion of the correct hits was 83.3 %. This success in allocating an object correctly of 83.3 % could be considered satisfactory. The squared distances between the groups indicate that there was the biggest distance between groups WS and SN, while the highest similarity was between groups WT and P. This close placement of WT and P samples is reflected in the fact that false classifications occur only in these groups, as it is shown in Table 2.

The established linear discriminant functions used for linear discriminant scores calculations for the studied groups of samples are the following:

$$SN = -13144 - 4 \cdot AA_{DPPH} - 12 \cdot AA^{\cdot}_{OH} + 4304 \cdot pH_{start} + 86 \cdot pH_{8h} + 73 \cdot pH_{end} \quad (1)$$

$$WT = -14514 - 4 \cdot AA_{DPPH} - 13 \cdot AA^{\cdot}_{OH} + 4527 \cdot pH_{start} + 90 \cdot pH_{8h} + 70 \cdot pH_{end} \quad (2)$$

$$P = -14330 - 4 \cdot AA_{DPPH} - 13 \cdot AA^{\cdot}_{OH} + 4499 \cdot pH_{start} + 88 \cdot pH_{8h} + 70 \cdot pH_{end} \quad (3)$$

$$WS = -15241 - 4 \cdot AA_{DPPH} - 13 \cdot AA^{\cdot}_{OH} + 4643 \cdot pH_{start} + 86 \cdot pH_{8h} + 74 \cdot pH_{end} \quad (4)$$

The obtained results of LDA indicate that there was a significant separation between the studied kombucha products on the basis of the exact mathematical equations (1-4) which correlate antioxidant activity and pH values with a product type. Therefore, there was the function which reflects the difference between groups as much as possible. On the basis of the established equations 1-4 (linear discriminant function for groups), the highest probability (100 %) of the correct classification is evident in the group of kombucha beverages with stinging nettle (SN) and wild thyme tea extracts (WT).

In order to explain the differences between the samples when pH value was taken into account, the pH_{end} value was observed, as the most important. If this parameter's value was 4.50, it indicated that the analysed sample was a fermented milk product. Therefore, samples WS-37-2.8, WS-37-1.6, WS-37-0.8, WS-40-0.8, P-37-1.6, K-37-2.8, SN-37-1.6, SN-37-0.8, WT-37-1.6, and WT-37- 0.8 were not the products that could be consumed, and that was the main difference in comparison to other samples. Based on these results, it could be seen that the main influencing factor to the obtaining of the kombucha fermented milk product was the fermentation temperature of 37 °C, as the least suitable one. Fermentation was stopped after 17h of duration, the longest, for all samples that did not reach the pH value of 4.50. Samples P-37-2.8 and P-37-0.8 reached a pH value of 4.50 after 17 h. The production process of samples WS-43-1.6 and P-43-1.6 was the shortest and lasted for about 10 h.

Higher values of the antioxidant activity to DPPH and hydroxyl radical indicate the higher quality of the certain sample, and this fact was chosen in order to explain differences among samples regarding their antioxidant capacity. These values are not given in regulation, and based on the results given in Table 1, it can be established that the best antioxidant quality characteristics possess samples WS-40-1.6 (AA_{DPPH} was 87.72 %) and WT-43-2.8 ($AA^{\cdot}_{OH}$ was 6.41 %). These results suggest that higher process temperatures and milk fat content lead to a better quality of kombucha fermented products. Hrnjez et al. (2014) determined the radical scavenging ability of DPPH and ABTS radical of kombucha milk-based beverages with black tea during the 14 days long storage. The AA_{DPPH} (%) was the highest after the production and amounted to around 18%, while the ABTS (%) was around 41 %. Therefore, the kombucha fermented milk products with different herbs (peppermint, wild thyme, stinging nettle, and winter savory) possess stronger antioxidant capacity than the kombucha fermented milk products with black tea. The exception was sample SN-40-2.8, whose AA_{DPPH} was 15.73 %.

Conclusions

The general conclusion was that the chemometric analysis was successfully applied in the classification of the kombucha products, based on their quality characteristics. The purpose

Table 3. Linear discriminant analysis of the analysed samples

Group	SN	WT	P	WS
Count	9	9	9	9
Summary of classification (confusion matrix)				
	True group			
Put into Group	SN	WT	P	WS
SN	9	0	0	0
WT	0	7	2	0
P	0	2	7	0
WS	0	0	0	9
Total N	9	9	9	9
N correct	9	7	7	9
Proportion	100%	77.8%	77.8%	100 %
N Total = 36; N Correct = 32; Proportion Correct = 88.9 %				
Summary of classification with LOO cross-validation (confusion matrix)				
	True group			
Put into group	SN	WT	P	WS
SN	9	0	0	0
WT	0	6	3	0
P	0	3	6	0
WS	0	0	0	9
Total N	9	9	9	9
N correct	9	6	6	9
Proportion	100%	66.7%	66.7%	100%
N Total = 36; N Correct = 30; Proportion Correct = 83.3 %				
Squared distance between groups				
	SN	WT	P	WS
SN	0	69.965	53.016	159.250
WT	69.965	0	1.406	21.149
P	53.016	1.406	0	30.235
WS	159.250	21.149	30.235	0

of the applied chemometric tools was the comprehensive approach to the selection of products among different groups. This is important for the production process of beverages that have similar characteristics; there is a justification for the production of the group with the shorter duration of the fermentation.

The presented results of chemometric analysis indicate that in the space of the variables analysed by HCA, the dominant discriminating factor for the studied samples of kombucha beverages is MF% content, followed by total unsaturated fatty acids content (TUFA, %), monounsaturated fatty acids content (MUFA, %) and polyunsaturated fatty acids content (PUFA, %). It was determined that the samples with 0.8 % MF and 1.6 % MF belong to the same cluster in the space of the analysed variables due to similarities in their antioxidant activity (AA_{DPPH}). The samples fermented at 37 °C with winter savoury extracts are significantly separated from the others based on their highest pH_{start} and pH_{end} values, while their antioxidant activity expressed as AA_{DPPH} is among the highest, but these samples are not fermented milk products and cannot be consumed. The differences in quality of the studied samples are more pronounced regarding their chemical composition than antioxidant activity.

LDA successfully found functions that characterizes the analysed samples and successfully allocate new samples of unknown group to the correct group of samples. Also, by LDA was determined that there is the biggest difference in quality between the groups WS and SN, while the highest similarity is between groups WT and P regarding their pH values and antioxidant activity expressed as AA_{DPPH}.

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Kemometrijski pristup karakterizaciji kvalitete mliječnih kombuča napitaka

Sažetak

Mliječni kombuča napici su dobiveni fermentacijom mlijeka kombučom. Hijerarhijska klaster analiza (HCA) i analiza glavnih komponenti (PCA) primijenjene su u cilju utvrđivanja razlika između analiziranih uzoraka, kao i identifikacije sličnosti ili različitosti između njih, u prostoru eksperimentalno određenih vrijednosti varijabli. Linearna diskriminantna analiza (LDA) provedena je na neobrađenim podacima u cilju pronalaženja pravila za dodjeljivanje novog uzorka nepoznatog porijekla odgovarajućoj grupi uzoraka. Hijerarhijska klaster analiza pokazala je da je dominantan faktor razlikovanja analiziranih uzoraka kombuča napitaka sadržaj mliječne masti, a zatim slijede sadržaji ukupnih nezasićenih, jednostruko nezasićenih (MUFA) i višestruko nezasićenih (PUFA) masnih kiselina. Uzorci s 0,8 i 1,6 % mliječne masti pripadaju istom klasteru u prostoru analiziranih varijabli zbog sličnosti u njihovoj antioksidativnoj aktivnosti prema DPPH radikalu. Linearnom diskriminantnom analizom utvrđeno je da je najveća razlika u kvaliteti kada se u obzir uzmu vrijednosti pH i antioksidativna aktivnost prema DPPH radikalu između grupa proizvoda sa čajem primorskog vriska i koprivom, dok je najveća sličnost između grupa proizvoda s majčinom dušicom i paprenom metvicom.

Ključne riječi: kombuča; mliječni proizvodi; antioksidativna aktivnost; kemometrijska analiza

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