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Faculty of Agricultural and Food Sciences and Environmental Management

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Risk assessment for forest honey's toxic element content

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SUMMARY

In our study, the micro element contents of 20 Hungarian forest honey samples had been determined by using ICP-MS. After the measurement, a risk analysis was carried out for the elements which have PTDI (Probable Tolerable Daily Intake) values announced by WHO/JECFA (Joint FAO/WHO Expert Committee on Food Additives) to find out whether these samples could have any adverse effect on human health or not. These elements were Al, As, Cd, Cu, Fe, Pb and Zn.

The risk analysis was calculated for consumers with a body weight of 30, 60 and 90 kg. As for the results, we can claim that these samples did not contain the above-mentioned elements in hazardous concentrations except for cadmium. Even by consuming 30 g of the sample which contained Cd in the highest concentration, children with a body weight of 30 kg could reach the level of consumption which indicates a possible risk. This means that during some specific periods, when the personal consumption of honey increases (winter months, illnesses, etc.), it could be possible to take in enough cadmium to trigger adverse effects.

Keywords: *honey, forest honey, nutrition, toxic element, risk assessment*

INTRODUCTION

Honey is a natural sweet substance produced by *Apis mellifera* bees from plant nectar and different plant saps by adding their own substances (Belay et al., 2013; MÉ 1-2-2001/110, 2002). Honey consumption is really high worldwide, which can be explained by its positive health effects. EU is the second biggest producer of the world, but it still needs to import this product. Hungary plays a significant role in the EU's honey production (I_1). Our country has a huge tradition of the production, therefore we are able to satisfy high quality requirements.

The beneficial effects of honey have been known for a long time, because it contains health-protecting compounds in high amounts, such as phenolic compounds, flavonoids, amino acids, organic acids, vitamins and minerals (Bilandzic et al., 2017). In spite of the possible health benefits and the high quality production, in some cases the product could get contaminated by substances, which could have adverse effects on our health – toxic elements, for instance. The accumulation of these elements could be originated to various factors, for example industrial pollution, environmental pollution, or wrong treatment and storage of the product (Czipa et al., 2015).

In our study, the micro element contents of 20 Hungarian forest honey samples had been examined, followed by a risk assessment for the toxic elements, which have PTDI (Probable Tolerable Daily Intake) values announced by WHO/JECFA (Joint FAO/WHO Expert Committee on Food Additives). The aim of the study was to determine, whether the collected samples could have any adverse effect on human health or not, based on their toxic element contents.

MATERIAL AND METHODS

The examination of 20 forest honey samples was carried out. These samples had been collected from different areas of Hungary, and been stored at room temperature in the dark.

For the sample preparation needed for the determination of micro element concentrations, the method of *Kovács et al. (1996)* has been applied. Determination of micro elements has been carried out by using ICP-MS (Inductively Coupled Plasma Mass Spectrometry) (Thermo Scientific XSeries 2, Bremen, Germany) equipment.

This procedure was followed by a risk assessment for those elements which have PTDI values announced by WHO/JECFA (Joint FAO/WHO Expert Committee on Food Additives). Calculations have been performed for consumers with a body weight of 30, 60 and 90 kg, by using the following equation:

$$\textbf{Risk} = \frac{\textbf{Tolerable Daily Intake}}{\textbf{Estimated Daily Intake}}$$

PTDI values were provided by WHO/JECFA. In case of the result is higher than 10, no risk can occur. If this value is between 1 and 10, there could be a slight risk. A value lower than 1 means that there is a risk.

RESULTS AND DISCUSSION

After the determination of the micro element contents, the elements found in *Table 1* have been chosen. Among these elements, the samples contained Fe in the highest concentrations in general (2625 µg kg^{-1}), followed by Zn (2210 µg kg^{-1}) and Al (1928 µg kg^{-1}). Cd was the element found in the lowest concentrations (8.17 µg kg^{-1}). Between the

concentrations of these elements, big differences can be observed in case of different samples. The highest Al concentration has been measured in sample no. 16 (4.89 mg kg^{-1}), while sample no. 9 contained this element only in a concentration of 0.309 mg kg^{-1}. In case of As, these values were 34.6 µg kg^{-1} (sample no. 11) and 1.43 µg kg^{-1} (sample no. 5), in case of Cd, they were 31.2 µg kg^{-1} (sample no. 16) and under the limit of detection (LoD=0.19 µg kg^{-1}). By observing the concentration of Cu, the highest was 895 µg kg^{-1} (sample no. 13), while the lowest was 640 µg kg^{-1} (sample no. 7), and by Fe-concentrations, 3823 µg kg^{-1} (sample no. 3) and 2020 µg kg^{-1} (sample no. 6). Sample no. 5 contained Pb in the highest concentration (89.9 µg kg^{-1}), whereas sample no. 10 had the lowest Pb-concentration (1.42 µg kg^{-1}). In case of Zn, the highest concentration was 3.72 mg kg^{-1} (sample no. 11), while the lowest was 1.02 mg kg^{-1} (sample no. 15). It can also be declared, that in case of sample no. 11, the concentrations of several toxic elements were higher than the concentrations measured in other samples.

Table 1

Toxic element contents of the analysed samples

Sample	Al 396.1 (mg kg^{-1})	As 75 (µg kg^{-1})	Cd 111 (µg kg^{-1})	Cu 65 (µg kg^{-1})	Fe 56 (µg kg^{-1})	Pb 206 (µg kg^{-1})	Zn 66 (mg kg^{-1})
1	1.77	19.1	2.97	659	2575	47.2	2.43
2	2.02	10.1	17.6	702	3027	28.8	1.13
3	2.51	3.08	2.54	766	3823	42.3	1.76
4	2.26	4.29	5.74	876	3538	37.6	2.33
5	0.475	1.43	6.46	665	2662	89.9	1.57
6	1.24	7.01	<LoD	649	2020	7.45	1.86
7	0.406	6.35	4.15	640	2997	61.4	2.12
8	0.584	8.99	7.35	671	2049	10.6	2.21
9	0.309	6.74	1.11	695	2668	4.26	1.58
10	0.863	5.81	2.71	674	2879	1.42	1.07
11	0.945	34.6	8.98	891	3212	12.1	3.72
12	3.52	10.9	16.5	879	2160	20.1	3.28
13	2.98	25.2	4.86	895	2215	8.05	2.66
14	3.45	18.8	13.5	892	2851	29.8	2.38
15	0.312	13.2	1.24	656	2065	6.21	1.02
16	4.89	8.69	31.2	852	2415	6.98	3.62
17	1.26	33.9	0.798	698	2098	16.4	1.09
18	3.64	10.9	17.4	889	2578	20.3	3.37
19	2.97	24.1	4.98	817	2198	8.19	2.59
20	2.15	4.19	5.17	887	2479	34.9	2.47

Those elements, which have a PTDI (Probable Tolerable Daily Intake) value announced by WHO/JECFA can be seen in *Table 2.*

Table 2

Tolerable Intake values announced by WHO/JECFA

Element	Year of determination	PTMI	PTWI	PTDI	Reference
Al	2011	8 mg $kgbw^{-1}$	2 mg $kgbw^{-1}$	0.267 mg $kgbw^{-1}$	TRS996-JECFA 74/7
As	2011		Withdrawn		TRS 959-JECFA 72
Cd	2013	25 µg $kgbw^{-1}$	6.25 µg $kgbw^{-1}$	0.833 µg $kgbw^{-1}$	TRS 983-JECFA 77
Cu	1982	15 mg $kgbw^{-1}$	3.5 mg $kgbw^{-1}$	0.5 mg $kgbw^{-1}$	TRS 683-JECFA 26/31
Fe	1983	24 mg $kgbw^{-1}$	5.6 mg $kgbw^{-1}$	0.8 mg $kgbw^{-1}$	TRS 696-JECFA 27/29
Pb	2011		Withdrawn		TRS 960-JECFA 73
Zn	1982	9-30 mg $kgbw^{-1}$	2.1-7 mg $kgbw^{-1}$	0.3-1 mg $kgbw^{-1}$	TRS 683-JECFA 26/32

bw – body weight

According to these values, the Tolerable Intake values have been calculated for consumers with a body weight of 30, 60 and 90 kg, indicated in *Table 3*. PTDI values of As and Pb had been withdrawn in 2011, nevertheless, we have calculated the risks for these elements too, based on their previous PTDI values (As: 2.14 µg $kgbw^{-1}$; Pb: 3.57 µg $kgbw^{-1}$).

Table 3

Tolerable Intake values of the elements for consumers with a body weight of 30, 60 and 90 kg

Element	30 kg bw			60 kg bw			90 kg bw		
	PTMI	PTWI	PTDI	PTMI	PTWI	PTDI	PTMI	PTWI	PTDI
Al (mg)	240	60	8.01	480	120	16	720	180	24
As (µg)			64.2			128			193
Cd (µg)	750	187.5	24.9	1500	375	50	2250	562,5	75
Cu (mg)	450	105	15	900	210	30	1350	315	45
Fe (mg)	720	168	24	1440	336	48	2160	504	72
Pb (µg)			107			214			321
Zn (mg)	270-900	63-210	9-30	540-1800	126-420	18-60	810-2700	189-630	27-90

The risk assessment was carried out by calculating with a daily honey consumption of 30 g. The element intake by consuming 30 grams of the analysed samples can be seen in *Table 4*.

Table 4

Element intake by consuming 30 grams of honey

Sample	Al (mg)	As (µg)	Cd (µg)	Cu (mg)	Fe (mg)	Pb (µg)	Zn (mg)
1	0.0532	0.574	0.0892	0.0198	0.0773	1.42	0.0729
2	0.0606	0.303	0.529	0.0211	0.0909	0.865	0.0339
3	0.0754	0.0925	0.0763	0.0230	0.115	1.27	0.0527
4	0.0679	0.129	0.172	0.0263	0.106	1.29	0.0700
5	0.0143	0.0429	0.194	0.0200	0.0799	2.70	0.0470
6	0.0372	0.211	-	0.0195	0.0607	0.224	0.0558
7	0.0122	0.191	0.125	0.0192	0.0900	1.84	0.0635
8	0.0175	0.270	0.221	0.0202	0.0615	0.318	0.0663
9	0.00928	0.202	0.0333	0.0209	0.0801	0.128	0.0475
10	0.0259	0.174	0.0814	0.0202	0.0865	0.0426	0.0320
11	0.0284	1.04	0.270	0.0268	0.0965	0.363	0.112
12	0.106	0.327	0.495	0.0264	0.0649	0.604	0.0985
13	0.0895	0.757	0.146	0.0269	0.0665	0.242	0.0799
14	0.104	0.565	0.405	0.0268	0.0856	0.895	0.0715
15	0.00967	0.396	0.0372	0.0197	0.0620	0.186	0.0307
16	0.147	0.261	0.937	0.0256	0.0725	0.210	0.109
17	0.0378	1.02	0.0240	0.0210	0.0630	0.492	0.0327
18	0.109	0.327	0.523	0.0267	0.0774	0.610	0.101
19	0.0892	0.724	0.150	0.0245	0.0660	0.246	0.0778
20	0.0646	0.126	0.155	0.0266	0.0744	1.05	0.0742

Risks could be calculated from the concentrations of these elements by consuming 30 grams of honey, and the tolerable daily intake values mentioned above. The results of the risk assessment can be observed in *Table 5.*

Table 5

Results of the risk assessment for 30 kg bw (body weight)

Sample	Risk							
	Al	**As**	**Cd**	**Cu**	**Fe**	**Pb**	**Zn (18)**	**Zn (60)**
1	151	112	279	758	310	75.5	123	411
2	132	212	47.1	712	264	124	265	884
3	106	694	326	652	209	84.2	171	569
4	118	498	144	570	226	94.8	129	429
5	562	1495	128	751	300	39.6	191	638
6	215	305	-	770	396	478	161	538
7	657	337	200	780	267	58.0	142	472
8	457	238	113	744	390	336	136	452
9	863	317	747	719	300	836	189	631
10	309	368	306	741	278	2509	281	938
11	282	61.8	92.3	561	249	294	80.6	269
12	75.8	196	50.3	568	370	177	91.4	305
13	89.5	84.8	171	558	361	443	113	376
14	77.3	114	61.4	560	280	120	126	420
15	855	162	669	761	387	574	293	977
16	54.5	246	26.6	586	331	510	82.8	276
17	212	63.1	1039	716	381	217	275	917
18	73.3	196	47.7	562	310	176	88.9	296
19	89.8	88.7	167	611	364	435	116	386
20	124	510	160	563	322	102	121	404

According to the results, it can be claimed that the intake of these toxic elements does not mean any risk even for children with a body weight of 30 kg by consuming 30 grams of these samples. Nevertheless, it is also important to point out that there were some samples which contained these elements in quite high concentrations. For instance, sample no. 16. showed a risk value of 26.6 in case of Cd, which is not much higher than the limit of 10.

In this case we still cannot claim that it could indicate any risk, but we also have to mention that we could have found samples even in Hungary or worldwide, that could have higher toxic element concentrations. What is more, there could be some periods when honey consumption increases, for example in the winter months, or in case of being sick, etc.

The risk assessment has been carried out for consumers with a body weight of 60 and 90 kg too. Results of the risk assessment for 60 kg bw can be seen in *Table 6.* These values were certainly higher than the results calculated for 30 kg bw, because the Tolerable Daily Intake values are much higher in these cases. Based on that, it can be declared that consuming 30 grams of these samples does not cause any risk on account of their toxic element contents.

The results of Di Bella et al. (2015) also confirm that the concentrations of Al and Pb are below the value which could indicate hazards. Based on the study of Czipa et al. (2015), Al, Pb, As and Cd concentrations in honey samples are very low, which means that they do not present any problems for human health.

Table 6

Results of the risk assessment for 60 kg bw

Sample	Risk							
	Al	As	Cd	Cu	Fe	Pb	Zn (18)	Zn (60)
1	301	224	561	1516	621	151	247	823
2	264	423	94,6	1423	528	248	530	1768
3	212	1388	656	1304	418	169	341	1138
4	236	997	290	1140	452	190	257	858
5	1122	2990	258	1502	600	79,3	383	1276
6	430	610	-	1539	791	957	323	1076
7	1312	673	401	1561	533	116	283	944
8	912	476	227	1489	780	673	271	905
9	1724	634	1500	1437	599	1674	379	1263
10	617	736	614	1482	555	5023	563	1876
11	564	124	185	1121	498	590	161	537
12	151	392	101	1137	740	355	183	609
13	179	170	343	1116	722	886	225	751
14	154	227	123	1120	561	239	252	839
15	1708	324	1343	1523	774	1149	586	1953
16	109	492	53,4	1173	662	1022	166	552
17	423	126	2086	1431	762	435	550	1833
18	146	392	95,7	1124	620	351	178	593
19	179	177	334	1223	727	871	231	771
20	248	1020	322	1126	645	204	243	809

CONCLUSIONS

In this study, the micro element concentrations of 20 Hungarian forest honey samples have been determined by using ICP-MS equipment. The measurement was followed by a risk assessment for those elements, which have PTDI values announced by WHO/JECFA, to examine whether the toxic element contents of these samples could mean any risk for the health of the consumers. The analysed elements were the following: Al, As, Cd, Cu, Fe, Pb, Zn.

In general, the samples contained Fe in the highest amount, followed by Zn and Al. Cd could be measured in the lowest concentrations. It can also be seen that these samples contained some of these elements in various concentrations.

The risk assessment has been carried out for consumers with a body weight of 30, 60 and 90 kg. According to the results, we can claim that the concentrations of these elements were not high enough to indicate any risk. In spite of this fact, we have to highlight that one of the samples contained Cd in a concentration which showed a risk value of 26.6 in case of a children with 30 kg bw. This is still higher than the limit of 10, but we have to mention that we could have found samples with higher toxic element concentrations. Furthermore, it is also possible that honey consumption could increase in some periods, such as winter months and illnesses, which could lead to adverse effects.

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Microbiological stability and subsequent hygienic quality of Slovak chocolates

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SUMMARY

Microbiological stability and hygienic quality is a considerable issue of the chocolate production process, even despite its low water activity. Outbreaks of various diseases originated in chocolate are just proof of the importance of this issue to be considered when examining the chocolate quality.

The aim of this study was, therefore, to analyse chocolate samples produced in the Slovak Republic for their microbiological stability and to identify its microbiota on the principle of mass spectroscopy. MALDI-TOF mass spectrometry has changed the process of microbial identification, as it provides a more precise and faster results than the conventional methods of microbial identification.

Total viable counts (colony forming units – CFU) were lower than 2.32 log CFU g^{-1} in all samples. Lactic acid bacteria were found only in one sample, with 0.69 log CFU g^{-1}. Yeasts were present in the samples only up to 2.51 log CFU g^{-1}, whereas microscopic filamentous fungi were detected only in one sample with almonds, which could have probably affected this result. Nevertheless, none of the examined samples contained coliform microorganisms, which is an important indicator of the hygienic quality.

Microscopic filamentous fungi were identified based on their morphological characteristics and were identified as Aspergillus *sp. Yeasts and bacteria were identified by mass spectrometry using MALDI-TOF MS Biotyper.* Candida parapsilosis *yeast was further identified in the samples.* Bacillus *genus occurred frequently and was identified as* B. pumilus, B. subtilis *and* B. mojavensis *species.* Bacillus *spores are able to survive technological steps such as fermentation and drying of the cocoa bean, moreover, roasting at temperatures above 100 °C.*

Keywords: *chocolate, microbiological stability, microbiological quality, hygiene, microorganisms, MALDI-TOF mass spectrometry.*

INTRODUCTION

Foods with less than 0.70 water activity (a_w) can be labelled as low-water activity foods (Blessington et al., 2013). The presence, survival, and heat resistance of bacterial pathogens in low-water activity foods provide a continuous challenge to the food producing companies (Podolak et al., 2010).

The survival of pathogens, such as *Salmonella* spp. or *Bacillus cereus* is well documented in low-water-activity foods like chocolate, nuts and other similar foodstuffs. It is perhaps best illustrated by numerous outbreaks of foodborne diseases caused by pathogens such as *Salmonella* (Scott et al., 2009). In the past two decades, however, the frequency of these outbreaks increased. This may be due to improved, more sensitive microbiological testing methods, better epidemiologic traceback investigations, and more proficient sampling plans (Gurtler et al., 2014).

Although *Salmonella* cannot grow in this kind of products, it can remain viable for a long time. This is mainly due to the low water activity (0.3-0.5) and high fat content (>20%) of chocolates. Moreover, these characteristics also provide high heat resistance to *Salmonella*. Furthermore, the high fat content protects the bacteria from gastric acids, resulting in an extremely low infectious dose requirement (Nascimiento et al., 2015).

Enterobacteriaceae and coliforms are widely used as universal hygiene indicators for various products. Identifying the possible entry points of *Enterobacteriaceae* contamination would enable the establishment of prevention or at least to minimalize the contamination risk of final products with *Salmonella*. Monitoring of *Enterobacteriaceae* family can also be used to predict deviations and defects in the hygiene control measures (ICMSF, 2011). The determination of *Enterobacteriaceae* is recommended for the chocolate industry, for in-process samples, processing environment as well as for end products (ICMSF, 2011).

To the best of our knowledge, there is only a limited number of studies investigating the microbiological stability and hygienic quality of chocolates and even fewer studies focus particularly on chocolates from Slovakia, if not none. For this purpose, the aim of the present study was to determine the microbiological stability and to evaluate the hygienic quality of chocolates produced in the Slovak Republic.

MATERIALS AND METHODS

Samples

The chocolate samples evaluated in this study were kindly supplied by two Slovakian chocolate-producing companies. We were working with samples of dark, milk and white chocolates. The characteristics and ingredients of the sample are stated in the *Table 1*.

Table 1

Characteristics of studied chocolate samples

Code	Char.	Flavour	Cacao Mass (%)	Ingredients[a]
SK1	Dark	Sea buckthorn	60	Cocoa butter, cocoa powder, agave syrup, flavour
SK2	Dark	Almond	60	Cocoa butter, cocoa powder, agave syrup, flavour
SK3	Dark	Mulberry	65	Cocoa butter, cocoa powder, agave syrup, flavour
SK4	Dark	Currant	65	Cocoa butter, cocoa powder, agave syrup, flavour
SK5	Dark	Cherry	65	Cocoa butter, cocoa powder, agave syrup, flavour
SK6	Dark	-	80	Cocoa butter, cocoa powder, agave syrup
SK7	Dark	Origin Arauca	70	Cocoa mass, cocoa butter, cane sugar, V, SL
SK8	White	Origin Trapiche	40	Cane juice powder, cocoa butter, milk powder, SL, V
SK9	Dark	Origin Arauca[b]	70	Cocoa mass, cocoa butter, cane sugar, V, SL
SK10	Milk	Origin Melao	37	Cane juice powder, cocoa butter, MP, SL, CM, V

a CM – cocoa mass, MP – milk powder, SL – soy lecithin, V – vanilla
b sample before conching

Microbiological tests

Four different media were used for the microbiological quality assessment of samples according to Żyżelewicz et al. (2018) with minor modifications. For the total viable counts, tryptic glucose yeast agar (plate count agar) (Biolife, Italy) was used, consisting of 5.0 g tryptone, 2.5 g yeast extract, 1.0 g glucose and 15.0 g agar in 1 L of distilled water with final pH 7.0 ± 0.2 at 25°C. Violet Red Bile Lactose (VRBL) agar (Oxoid, England) was used for the detection and enumeration of coliforms in samples, consisting of 7.0 g peptone, 3.0 g yeast extract, 1.5 g bile salts No. 3, 5.0 g sodium chloride, 0.03 g neutral red, 0.002 g crystal violet, 10.0 g lactose and 12.0 g agar in 1 L of distilled water with final pH 7.4 ± 0.2 at 25°C. For lactic acid bacteria determination, MRS (de Man, Rogosa, Sharpe) agar was used, consisting of 10.0 g peptone, 8.0 g 'Lab-Lemco' powder, 4.0 g yeast extract, 20.0 g glucose, 1 mL sorbitan mono-oleate, 2.0 g dipotassium hydrogen phosphate, 5.0 g sodium acetate $3H_2O$, 2.0 g triammonium citrate, 0.2 g magnesium sulphate $7H_2O$, 0.05 g manganese sulphate $4H_2O$ and 10.0 g agar in 1 L of distilled water with final pH 6.2 ± 0.2 at 25°C. For the cultivation of yeasts and microscopic filamentous fungi, Malt Extract Agar (MEA) (Oxoid, England) was used. MEA consisted of 12.5 g maltose, 2.5 g dextrin, 1.0 g glycerol, 2.6 g peptocomplex and 17.0 g agar in 1 L of distilled water with final pH 4.6 ± 0.2 at 25°C.

45 mL of sterile saline (0.9% NaCl) was added to 5 g sample to prepare a 10^{-1} dilution. Samples were then left to shake in a shaker for 30 minutes. Prepared dilution thus was used for inoculating the culture media.

Table 2

Cultivation conditions of the different microbes

Type of cultivated microorganism	Temperature °C	Incubation Time
Total viable count	30	48-72 h
Coliform bacteria	37	24-48 h
Lactobacilli	37	48-72 h
Yeasts	25	5-7 days
Microscopic filamentous fungi	25	5-7 days

Different microorganisms were quantified with inoculating each sample into different media by pouring, in three replicates. Each colony was inoculated to the surface of the media for quality determination and subsequent identification of the cultivated microorganisms. Each surface-coated sample was prepared in three replicates as well. The cultivation conditions are summarized in *Table 2*.

Colonies were enumerated on the plates following incubation. For the calculation of CFU g^{-1} chocolate, the following formula was used (considering the dishes of two consecutive dilutions), $N = \Sigma C/([(n1 + 0,1n2).d])$, where ΣC is the sum of characteristic colonies on selected dishes, n1 is number of dishes of the 1st dilution used for the calculation, n2 is number of dishes from the 2nd dilution used for the calculation and d is dilution factor consistent with the 1st dilution used.

If the colonies were present only on the first dilution plates (10^{-1}), the CFU g^{-1} was determined by the ratio of the sum of the colonies and dishes (on which they were cultivated) and the subsequent multiplication by the inverted dilution value (i.e., 10).

Identification of the microorganisms

Identification of microorganisms was performed by mass spectrometry (MALDI-TOF MS Biotyper). This method was chosen because it is fast and highly reliable. MALDI-TOF mass spectrometry principle has changed the process of microbial identification, allowing a more precise and faster result than the conventional methods.

The most important step in the sample preparation for MALDI-TOF MS was the cultivation and the subsequent purification of microorganisms using a plate-streaking method and re-cultivation to obtain pure cultures. MALDI matrix was prepared by pipetting 250 µL of stock solution (50% acetonitrile, 47.5% distilled water and 2.5% trifluoroacetic acid) to „HCCA matrix portioned" (α-Cyano-4-hydroxycinnamic acid, ≥99.0% (HPLC), Sigma-Aldrich, Germany) vial. It was vortexed until all the crystals of the matrix were completely dissolved. 300 µl of distilled water was pipetted to a 1.5 mL Eppendorf tube. The biological material from the culture dish was then transferred into a water in the tube and mixed thoroughly. The amount of biological was ranged from individual colonies to 5-10 mg. Subsequently, 900 µL of ethanol was added and mixed thoroughly. The mixture was centrifuged (MPW-223e, MPW Medical Instruments, Poland) at maximum speed (2000 rpm) for two minutes and the supernatant was decanted. After repeated centrifugation, the remaining ethanol was removed carefully by pipetting. The pellet was let to dry for a few minutes at room temperature. Then the volume of 1 to 50 µL of 70% formic acid was added to the pellet (the amount of formic acid added was depended on the amount of biological material used, the less the material was, the less acid was used), and then thoroughly mixed by pipetting and vortexing. Next, the same amount of acetonitrile was added and mixed again thoroughly by pipetting. The samples were centrifuged again at maximum speed for two minutes. 1 µL of the supernatant was then pipetted to the plate and allowed to dry. Once dried, the supernatant was overlaid with 1 µL MALDI matrix solution. Upon complete drying of the sample covered by the matrix, samples were prepared for MALDI-TOF identification with competent software.

RESULTS AND DISCUSSION

Chocolate is often regarded as a microbiologically stable product because of its low water activity, but although the water activity in chocolates is not sufficiently low to prevent the growth of all microorganisms, for example yeasts (Copetti et al., 2014). Chocolate quality may be influenced by a variety of environmental, agronomic and technological factors. Among these factors, it is known that the microorganisms present in the fermentation play an essential role in the development of the sensorial characteristics of chocolate (Copetti et al., 2011). The intensity of microbial proliferation and subsequent presence of one or another group of microorganisms during processing steps are crucial for the development of particular characteristics in cocoa beans leading to a good quality final product (Schwan and Wheals, 2004).

The number of different groups of monitored microorganisms detected in the tested chocolate samples is summarized in *Table* 3. The total number of microorganisms in samples of Slovak chocolates ranged from 0.69 to 2.32 lg CFU g^{-1}. Lactic acid bacteria were not detected in the not tempered chocolate samples SK9 and SK7.

None of the examined samples contained any coliform, which are an indicator of hygienic quality (Hervert et al., 2016). Yeasts were present in the samples only up to a max 1.18 lg CFU g^{-1}, whereas microscopic filamentous fungi were detected only in SK2 sample. Its almond content may probably be the source of the moulds. The results of De Clercq et al. (2015) also concluded that nuts seemed to represent an important source of contamination of Belgian chocolates and factories, where they were produced. The final microbial population in the chocolates can be the result of several different factors. Essential factors influencing the qualitative and quantitative composition of the microbial population can be raw materials, a fermentation method of cocoa beans, the storage conditions, and finally the composition of the final product (Żyżelewicz et al., 2018). Cacao bean fermentation involves the successional growth of various yeasts species, lactic and acetic acid bacteria as well as *Bacillus* species and different filamentous fungi (De Vuyst et al., 2010).

Table 3

Colony forming units (CFU) of the different groups of microorganisms

Sample	Total Viable Counts (lg CFU g^{-1})	Lactic Acid Bacteria (lg CFU g^{-1})	Yeasts and Fungi (lg CFU g^{-1})	Coliforms (lg CFU g^{-1})
SK1	0.69	NP	0.69	NP
SK2	1.30	NP	1.18	NP
SK3	1.18	NP	1.00	NP
SK4	NP	NP	NP	NP
SK5	1.00	NP	NP	NP
SK6	1.18	NP	0.69	NP
SK7	NP	NP	NP	NP
SK8	NP	NP	NP	NP
SK9	2.32	0.69	NP	NP
SK10	0.69	NP	NP	NP

NP – not present

Microscopic filamentous fungi were identified based on morphological features. According to findings of Copetti et al. (2011), *Aspergillus* sp. was one of most often detected moulds, with the highest occurrence among cocoa bean samples. However, the fungal contamination should be reduced by the industrial processing in all fractions and fungi are normally not found in the final chocolate products.

Yeasts and bacteria were identified by mass spectrometry using the MALDI TOF MS-Biotyper instrument. *Candida parapsilosis* yeast was identified in the sample SK2, which was the most often isolated from the hands of healthy people (Bonassoli et al., 2005). *Bacillus* genus commonly occurred and *B. pumilus*, *B. subtilis* and *B. mojavensis* species were identified. Migueal et al. (2017) found *B. pumilus* and *B. subtilis* after both, spontaneous and inoculated fermentation processes of cocoa beans. On the other hand, *B. mojavensis* was found after the spontaneous fermentation process only. According to Schwan and Wheals (2004), spore-forming *Bacillus* bacteria are able to survive technological steps such as fermentation and drying of cocoa beans and even roasting at temperatures above 100°C. Nielsen et al. (2012) identified that *Bacillus pumilus* was able to survive in chocolate pralines. Erdem et al. (2014) reported that *Bacillus pumilus* had probiotic properties.

CONCLUSIONS

In this study, the microbiological stability and the hygienic quality of Slovak chocolates were evaluated. This work doesn't focus on only one type of chocolate, but analysis covered all types: dark, milk and white chocolates. Our results revealed that chocolates produced in the Slovak Republic by small companies had very good microbiological stability since only low numbers of colony forming units were detected in the samples. Moreover, there were no coliform bacteria found in any of the studied samples.

All identified microorganisms could be naturally found in the natural cocoa bean throughout the process of cocoa bean fermentation or later. These microorganisms can survive even temperatures used while roasting raw cocoa beans. Fungal contamination was present just in one case, in chocolate with added nuts. This addition could be the main culprit behind this result, which was also supported by other authors.

Therefore, it can be concluded that the studied chocolates had good microbial stability, which is a very important feature to ensure the health safety of consumers.

Since there is a very limited number of studies of the microbiological quality of the chocolates to this day, further research is recommended to gain more results and in this field of food microbiology and food processing.

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Application of MALDI-TOF MS for identification of food associated *Staphylococcus aureus*

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SUMMARY

Staphylococcus aureus *is recognized as one of the most common pathogens responsible for food poisoning and gives rise to various infections in humans and animals. Our aim was to look into the possibility of applying matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) techniques to the identification of coagulase-positive staphylococci with two sample preparation methods (direct sample preparation and formic acid suspension technique) to study the impact on the identification results. In the present study 20 food samples were collected from different food matrices and screened for food-borne staphylococci with a selective medium (Baird-Parker). The strains collected from food were identified by MALDI BioTyper, 19 out of 20 coagulase-positive* Staphylococcus *isolates were securely identified at a genus and within that 7 isolates were identified at a species level with a high probability. The two sample preparation methods (direct and formic acid suspension techniques) yielded the same identification evaluation results, thus both protocols seem to be adequate. Application of MALDI-TOF MS could be reduced the analysis time of identification of* Staphylococcus *spp. The developed method is able to identify* Staphylococcus aureus, *cheaper and more reliably in a routine diagnostic laboratory.*

Keywords: *Staphylococcus aureus, MALDI-TOF MS, foodborne pathogen*

INTRODUCTION

The matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) technique is based on coupling a laser ion source and a time-of-flight mass spectrometer. The energy of the laser emitted is absorbed by the matrix, which in turn ionises the target compounds of the sample, and thus they can enter the vacuum of the mass spectrometer and finally reach the detector. The obtained mass spectra provide information on the protein and macromolecule profiles of the sample (Pavlovic et al., 2013). This fingerprint pattern serves as a base for the routine identification of the microbes, compared to a validated database (Singhal et al., 2015) to uncover markers or marker sets which can reliably distinguish microorganisms from a wide variety of sources: for example isolates from clinical, livestock, food, feed or environmental sources (Sandrin et al., 2012).

Staphylococcus aureus belongs to the normal flora of mammals and birds' skin and mucous membranes. It is distributed in the environment through the action of its hosts and will survive for a long time. Several *S. aureus* biotypes have been isolated from different hosts (human, poultry, cattle and sheep/goat) have been described, which show a close adaptation of the microorganism to the host cell (Hennekinne et al., 2012). The most common cause of bovine subclinical mastitis is coagulase-positive staphylococci; however, other animals (swine, poultry, horse) may also be infected, so these microorganisms can often be included in foods (Rajic-Savic et al., 2015).

Staphylococcal food poisoning is one of the most common food-derived diseases. Staphylococcal enterotoxins that are produced by enterotoxigenic strains of coagulase-positive staphylococci, mainly *Staphylococcus aureus* and very occasionally by other staphylococci species such as *Staphylococcus intermedius*. Symptoms of staphylococcal food poisoning occur quickly (2-8 hours), including nausea, vomiting, abdominal cramps, with or without diarrhoea. The disease is usually self-restraint and is typically resolved within 24-48 hours after recovery (Kadariya et al., 2014). At times may be serious to provide hospital treatment, especially when infants or elderly weakened (Argudín et al., 2010).

Thus it is important to develop microbiological procedures and confirmation methods to decrease the time-to-result for the food and feed industry. One of the major steps in the analysis is the sample preparation which has to be simple and fast enough to provide timely results. Our aim was to look into the possibility of applying MALDI-TOF MS techniques to the identification of coagulase-positive staphylococci with two sample preparation methods (direct sample preparation and formic acid suspension technique) to study the impact on the identification results.

MATERIAL AND METHODS

2.1 Bacterial strains, samples, isolates and culture conditions

In the present study 20 food-borne *Staphylococcus* spp. isolates were collected from different food matrices. The isolates were isolated by culturing on selective and non-selective growth media according to the method described in the standard MSZ EN ISO 6888-1:2008. Staphylococci form typical colonies on the surface of Baird-Parker medium (Biokar, FR) and those strains showing positive coagulase reaction were selected. An additional coagulase test (Microgen Bio Product, UK) from colonies grown at 37 °C for 24±1 h on Columbia Blood agar (Neogen, UK) was also used for the coagulase test confirmation. The strains and their origins are indicated in *Table 1*.

Table 1

The origin of the different coagulase-positive staphylococci strains isolated from different foodstuffs

ID number	Type of food sample
HB-1	dried pasta
HB-2	milk
HB-3	beef
HB-4	pork chops
HB-5	pork sausage
HB-6	bacon
HB-7	duck meat
HB-8	beef
HB-9	pork shoulder
HB-10	cheese
HB-11	goose liver
HB-12	dried pasta
HB-13	duck liver
HB-14	pork sausage
HB-15	pork greaves
HB-16	dairy desserts
HB-17	beef
HB-18	dried pasta
HB-19	pork chops
HB-20	dried pasta

Sample preparation for MALDI-TOF MS analysis

For MALDI-TOF MS analysis the isolates were grown on Columbia Blood agar. Two different types of sample preparation for MALDI-TOF MS analysis were used. The direct sample preparation protocol was carried out according to Bruker Daltonic Inc. In this case samples were taken from colonies with sterile sampling loops, then applied directly onto the target plate and 1-1 µl 70 v/v% formic acid was added. After evaporation of the solvent, 1 µl α-HCCA (10 mg/ml) matrix solution was added to the sample and the spots were crystallized by air drying.

In the other sample preparation protocol formic acid suspending was used: a single colony was picked up with an inoculation loop and the culture was suspended in 40 µl of formic acid in an Eppendorf tube for 30 seconds. Following this 40 µl of acetonitrile was added to the suspension and mixed thoroughly. Finally 1 µl of the suspension was transferred onto the target plate. When dried, it was overlaid with 1 µl α-HCCA (10 mg/ml) matrix solution and left to dry again.

MALDI-TOF MS parameters and data evaluation

Mass spectra were obtained with Bruker Microflex LT MALDI-TOF mass spectrometer operating in positive linear mode, in the molecular mass range of 2.0-25 kDa. Identification of *Staphylococcus* spp. was carried out by MALDI BioTyper 3.1 software. More than 200 shots gave satisfactory spectra with good signal-to-noise ratio. Following calibration sample measurement with the usage of *Bruker Bacterial Test Standard*. With the aim of analysing mass spectra of *Staphylococcus aureus* serovars in detail, 640 shots were performed and mass data files were transferred to *flexAnalysis 3.4* software (Bruker Daltonics). Spectra were processed by baseline correction, Gaussian smoothing, and peak finding. The obtained mass spectra were internally calibrated using a specific biomarker ion. The spectra were analysed individually for characteristic peaks.

The 20 isolates were analysed in parallel, and results are reported as numeric score based on collective comparison of protein spectra obtained experimentally compared to MALDI Bruker's Biotyper-specific database. Scores below 1.699 reported as non-reliable genus ID, scores of 1.700–1.999 were classified as probable genus ID, scores of 2.000–2.299 were secure genus ID and scores of 2.300–3.000 designated as highly probable species ID.

RESULTS AND DISCUSSION

Identification of *Staphylococcus* spp.

With the use of the inbuilt MALDI-Biotyper database it was possible to identify accurately all 20 (100%) isolates by direct sample preparation. Seven coagulase-positive staphylococci isolates gave score values ≥2.300, twelve isolates had score value between 2.000 and 2.299, and one strain had a score in

the 1.700-1.999 range. The summary of the identification results of the direct sample preparation protocol including the best and second best match names and scores are shown in *Table 2*.

Table 2

The result of direct sample preparation

ID number	Organism (best match)	Score value	Organism (second best match)	Score value
HB-1	*Staphylococcus aureus*	2.135	*Staphylococcus aureus*	2.056
HB-2	*Staphylococcus aureus*	2.245	*Staphylococcus aureus*	2.214
HB-3	*Staphylococcus aureus*	2.098	*Staphylococcus aureus*	2.067
HB-4	*Staphylococcus aureus*	2.278	*Staphylococcus aureus*	2.136
HB-5	*Staphylococcus aureus*	2.012	*Staphylococcus aureus*	2.007
HB-6	*Staphylococcus aureus*	2.361	*Staphylococcus aureus*	2.287
HB-7	*Staphylococcus aureus*	2.596	*Staphylococcus aureus*	2.436
HB-8	*Staphylococcus aureus*	2.015	*Staphylococcus aureus*	2.002
HB-9	*Staphylococcus aureus*	2.472	*Staphylococcus aureus*	2.314
HB-10	*Staphylococcus aureus*	1.871	*Staphylococcus aureus*	1.856
HB-11	*Staphylococcus aureus*	2.156	*Staphylococcus aureus*	2.102
HB-12	*Staphylococcus aureus*	2.432	*Staphylococcus aureus*	2.367
HB-13	*Staphylococcus aureus*	2.324	*Staphylococcus aureus*	2.284
HB-14	*Staphylococcus aureus*	2.645	*Staphylococcus aureus*	2.559
HB-15	*Staphylococcus aureus*	2.233	*Staphylococcus aureus*	2.172
HB-16	*Staphylococcus aureus*	2.158	*Staphylococcus aureus*	2.141
HB-17	*Staphylococcus aureus*	2.255	*Staphylococcus aureus*	2.206
HB-18	*Staphylococcus aureus*	2.275	*Staphylococcus aureus*	2.223
HB-19	*Staphylococcus aureus*	2.226	*Staphylococcus aureus*	2.158
HB-20	*Staphylococcus aureus*	2.383	*Staphylococcus aureus*	2.189

The results for the formic acid suspension sample preparation did not differ significantly from the direct suspension method: seven coagulase-positive staphylococci isolates gave score values ≥2.300, twelve isolates had score value between 2.000 and 2.299, and one strain had a score in the 1.700-1.999 range (*Table 3*).

Table 3

The result of formic acid suspending sample preparation

ID number	Organism (best match)	Score value	Organism (second best match)	Score value
HB-1	*Staphylococcus aureus*	2.241	*Staphylococcus aureus*	2.164
HB-2	*Staphylococcus aureus*	2.238	*Staphylococcus aureus*	2.216
HB-3	*Staphylococcus aureus*	2.103	*Staphylococcus aureus*	2.085
HB-4	*Staphylococcus aureus*	2.251	*Staphylococcus aureus*	2.227
HB-5	*Staphylococcus aureus*	2.162	*Staphylococcus aureus*	2.081
HB-6	*Staphylococcus aureus*	2.352	*Staphylococcus aureus*	2.311
HB-7	*Staphylococcus aureus*	2.473	*Staphylococcus aureus*	2.469
HB-8	*Staphylococcus aureus*	2.129	*Staphylococcus aureus*	2.097
HB-9	*Staphylococcus aureus*	2.487	*Staphylococcus aureus*	2.302
HB-10	*Staphylococcus aureus*	1.812	*Staphylococcus aureus*	1.802
HB-11	*Staphylococcus aureus*	2.182	*Staphylococcus aureus*	2.005
HB-12	*Staphylococcus aureus*	2.412	*Staphylococcus aureus*	2.128
HB-13	*Staphylococcus aureus*	2.353	*Staphylococcus aureus*	2.207
HB-14	*Staphylococcus aureus*	2.593	*Staphylococcus aureus*	2.249
HB-15	*Staphylococcus aureus*	2.193	*Staphylococcus aureus*	2.113
HB-16	*Staphylococcus aureus*	2.227	*Staphylococcus aureus*	2.028
HB-17	*Staphylococcus aureus*	2.144	*Staphylococcus aureus*	2.037
HB-18	*Staphylococcus aureus*	2.205	*Staphylococcus aureus*	2.136
HB-19	*Staphylococcus aureus*	2.269	*Staphylococcus aureus*	2.208
HB-20	*Staphylococcus aureus*	2.335	*Staphylococcus aureus*	2.155

The summary of identification results of the 20 *Staphylococcus* isolates are indicated in *Table 4*. These results of the direct sample preparation are similar to other published results with respect to identification of *Staphylococcus* isolates. In a study published by Zhu et al. (2015), 20 out of 21 *Staphylococcus* spp. isolates were identified with a > 2.000 score value, and 1 isolate has score values between 1.700-1.999 when using direct sample preparation. The method included direct sample preparation and 2 µl α-HCCA (10 mg/ml) matrix solution was added to the sample. In another study, Manukumar et al. (2017) have obtained similar results with direct sample preparation methods: 34 out of 36 *Staphylococcus* spp. isolates were identified with a >2.000 score value, and two isolates had score values between 1.700-1.999 when using the same direct sample preparation. In another study, Matsuda et al. (2012) 56 out of 64 *Staphylococcus* spp. isolates were identified with a > 2.300 score value.

Table 4

The summary of identification results of the 20 *Staphylococcus* isolates

ID number	Best score of formic acid suspension	Best score of direct sample preparation	Mean of score value	Standard deviation of score value
HB-1	2.241	2.135	2.188	0.075
HB-2	2.238	2.245	2.242	0.005
HB-3	2.103	2.098	2.101	0.004
HB-4	2.251	2.278	2.265	0.019
HB-5	2.162	2.012	2.087	0.106
HB-6	2.352	2.361	2.357	0.006
HB-7	2.473	2.596	2.535	0.087
HB-8	2.129	2.015	2.072	0.081
HB-9	2.487	2.472	2.161	0.011
HB-10	1.812	1.871	1.842	0.042
HB-11	2.182	2.156	2.169	0.018
HB-12	2.412	2.432	2.422	0.014
HB-13	2.353	2.324	2.339	0.021
HB-14	2.593	2.645	2.619	0.037
HB-15	2.193	2.233	2.213	0.028
HB-16	2.227	2.158	2.193	0.049
HB-17	2.144	2.255	2.200	0.078
HB-18	2.205	2.275	2.240	0.049
HB-19	2.269	2.226	2.248	0.030
HB-20	2.335	2.383	2.359	0.034

Furthermore *Table 4* summarizes the best score value of mean and standard deviation in case of different sample preparation. The best score value of mean and standard deviation were defined by Microsoft Excel 2007 software. The largest standard deviation was obtained in HB-5 sample (0.106), and the smallest in HB-3 sample (0.004). The largest mean of score values was determined in HB-14 sample (2.619), the lowest mean of score value was determined in HB-10 sample (1.871).

CONCLUSIONS

Based on the results of the study, MALDI-TOF MS technique can be used to assess the taxonomic position of coagulase-positive staphylococci from foodstuffs. In the present study, 19 out of 20 coagulase-positive *Staphylococcus* isolates were securely identified at a genus and within that 7 isolates were identified at a species level with a high probability. All isolates were evaluated as *Staphylococcus aureus* at the first and second best match level.

The two sample preparation methods (direct and formic acid suspension techniques) yielded the same identification evaluation results, thus both protocols seem to be adequate. As a summary, MALDI-TOF MS method is to be a simple, quick and exact tool for a more reliable and even faster identification and confirmation of the taxonomic position of coagulase-positive staphylococci as *Staphylococcus aureus* which was easily implemented into routine analysis because of its high throughput and relatively low-cost. In these terms it means that with the usage of MALDI-

TOF technique, streaking of typical or suspect colonies on nutrient agar and the classical biochemical confirmations could be replaced, thus the analysis time of *Staphylococcus* spp. could be reduced by at least 24 hours or even 48 hours. To improve results in the future, it is important to collect additional mass spectra of the same strains and use additional strains of coagulase-positive staphylococci and *Staphylococcus aureus* from food matrices.

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The effect of refrigeration storage condition on the most important sensory, chemical and microbiological characteristics of Pastirma

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SUMMARY

Pastirma samples manufactured from beef and covered with a layer of fenugreek, garlic, salt and spices were collected from different commercial markets in Latakia, Syria and transported to the laboratory. The samples were stored at commercial storage conditions (7±2 °C), which are usually the conditions for refrigeration storage in the markets, within prolonged storage time (0, 15, 30, 60, 120, and 180 days). Some of the most important sensory, chemical and microbiological characteristics were investigated. The results of the sensory analysis showed that storage negatively affects the sensory properties of the Pastirma and leads to significant loss especially after four months of storage. The most sensitive properties were: texture, appearance and smell. Moisture content decreased marginally from 43.476% to 40.989% after 180 days of cold storage. However, there was a significant (<0.05) difference in pH, acidity, fat, peroxide number, dissolved nitrogen (non-protein) and volatile nitrogen. The pH values, and fat decreased significantly from 6.14 to 5.51 and 7.475% to 5.835% respectively after 30 days of cold storage. However, after 30 days, acidity, peroxide number, dissolved nitrogen (non- protein) and volatile nitrogen increased significantly from 0.181 to 1.124%, 2.012 to 2.898 meq/kg, 0.169 to 0.268%, 0.005 to 0.035% respectively. The microbial results indicated that the Pastirma is free from pathogenic bacteria Salmonella and Escherichia coli. The results also showed a significant (p<0.05) increase in the number of aerobic bacteria, yeasts and moulds (10.182±0.85, 9.899±0.69) after 180days of storage respectively. However, they were within acceptable limits.

Keywords: *Pastirma, storage condition, sensory qualities, chemical and microbiological parameters.*

INTRODUCTION

Meat is one of the most important nutrients for humans as valuable sources of protein. Animal proteins contain about 20 amino acids (Al-aswad, 1980). Pastirma is considered one of the fermented semi-dry meat products, which is called in many countries as fermented sausages, made from different types of meat, including cattle, sheep, goats, buffalo, camels, poultry and fish (Mohammed et al., 2011). Pastirma is a type of uncooked, pressed and dried meat which is manufactured from beef and covered with a layer of fenugreek "*Trigonella foenum graecum*", garlic, salt and spices (Anonymous, 2005).

The Byzantines were the first who manufactured the Pastirma and followed by Turks until it spread in the Balkan and Mediterranean countries Nizamlioglu et al. (1998). There are various methods of eating Pastirma according to the regions and food habits. In Egypt and Palestine, it is fried with eggs or added to pizza. In Syria and Lebanon, it is consumed raw in the form of thin slices. (Anonymous, 2005).

Meat preservation involves the application of methods that delay or prevent changes that make meat unsuitable for consumption. Therefore, many techniques have been used to preserve meat such as salting, smoking, drying and fermenting (Kalalou et al., 2004). Unfortunately, different kinds of meat are good nutrition medium for microorganisms that allow these microorganisms to function and spoil the meat or excrete many compounds that cause food poisoning to the consumer and therefore they reduce some of meat's sensory, microbial and chemical properties (Philips et al., 2001). The aim of this research was to monitor changes in the most important chemical, microbial and sensory properties of the Pastirma in the market through chemical, microbial and sensory analysis within storage time (0, 15, 30, 60, 120, and 180 days).

MATERIALS AND METHODS

Pastirma samples

Three Pastirma samples were collected from different commercial markets in Latakia, Syria. They were transported to the laboratory in Tishreen University, Department of Food Sciences, Latakia. The samples were stored at commercial storage conditions (7±2 °C), within storage time (0, 15, 30, 60, 120, and 180 days). Some of the most important sensory, chemical and microbiological characteristics were investigated.

Sample preparation

The packaging cover of the Pastirma samples was cleaned from the outside, and then Pastirma samples were mixed and tested in three repetitions.

Assay procedures (AOAC, 2000)

Microbiological analysis

A sterile 10 g of each sample was taken and placed in a sterile dilution bottle containing 90 ml of peptone water (8,5g/L NaCl and 1g/L peptone in water). The necessary dilutions were performed in sterile conditions where the following tests were applied:

• Total count of aerobic bacteria using the nutrient agar media (NA) and incubation at 37 °C for 48 hours.
• Total count of yeast and moulds using potato agar media (PDA) and incubation at 25 °C for 3 days.
• Detection of *Escherichia coli* using Tryptone Bile X-Glucuronide agar (TBX), and incubation at 44.5 °C for 48 hours.
• *Salmonella* was detected using Salmonella Shigella Agar media, and incubation at 37 °C for 48-72 hours. Urease Test was carried out as confirmation for *Salmonella* presence.

Chemical Analysis (AOAC, 2000)

Moisture content: Determination the percentage of dry matter using drying method at 105 °C (Memmert, UNP400, Germany) until weight stability.
pH: Using pH-meter (PENCH meter, Mi150, Germany). By putting the device electrode in the filtrate of the Pastirma and moving quietly, then recording the resulting number after the stability with the temperature at which the number is recorded.
Acidity: By titration the Pastirma's filtrate with Sodium hydroxide (0.1 M) and some drops of phenolphthalein, until the appearance of pink color.
Fat content: Using Soxhlet method with (LG-6900 Extraction Apparatus, SOXHLET, Germany).
Peroxide number: By titration sodium thiosulfate, first, melting down the extracted fat from Soxhlet at 50 °C then, dissolving this fat with chloroform, and adding potassium iodide. The part of released iodine due to presence of peroxides is titrated by sodium thiosulfate with starch as an indicator.
The content of nitrogen total nitrogen, protein, soluble and volatile nitrogen were determined by Kjeldahl method (Gerhardt vepodest 45S-Gerhardt company, Germany).

Sensory Tests

Sensory tests were carried out by a committee of eight persons (graduated students and members of the teaching staff of the Food Science Department, Tishreen University). The grade range was adopted from 1 to 9, where grade 1 indicated the lowest level of the studied property and Grade 9 indicated the highest level of the studied property, as following Gök et al. (2008):

1-3 → not acceptable
4-5 → fairly acceptable
6-7 → good
8-9 → very good

Committee members were asked to evaluate the samples for the texture, appearance, smell, color and taste. The samples were presented under good light and in slices form, from day 0 to day 180 of storage.

Statistical analysis

Statistical analysis of the data was obtained by using three replicates with (GenStat12) program to get the mean and the standard deviation with significant difference at ($p<0.05$) using one sample t-test.

RESULTS AND DISCUSSION

Microbiological analysis

The results indicated that the Pastirma is free from pathogenic bacteria *Salmonella* and *Escherichia coli*. The decimal logarithm of the total count of aerobic bacteria per gram was 4.1 ± 0.2 $\log_{10}$ cfu g^{-1}, and the logarithm of the count of yeast and moulds per gram was 3.7 ± 0.1 $\log_{10}$ cfu g^{-1}. The total count of aerobic bacteria and the total count of yeast and moulds in the form of $\log_{10}$ cfu g^{-1} during storage at a temperature of 7 ± 2 °C within the proposed duration are shown in *Table1*.

Table 1

Microorganism count of Pastirma samples at different storage time

Storage time (days)	Aerobic bacteria count Mean+SD ($\log_{10}$ cfu g^{-1})	Yeast and moulds count Mean+SD ($\log_{10}$ cfu g^{-1})	*Salmonella* count ($\log_{10}$ cfu g^{-1})	*E.coli* count ($\log_{10}$ cfu g^{-1})
0	4.1±0.2	3.7±0.1	None	None
15	4.5±0.2	3.9±0.3	None	None
30	5.1±0.3	4.3±0.4	None	None
60	6.9±0.2	6.4±0.5	None	None
120	8.5±0.5	7.1±0.7	None	None
180	10.2±0.9	9.9±0.7	None	None
LSD*	0.6	0.3	-	-

* Least significant difference LSD at <0.05

Table 1 shows a significant increase in the number of aerobic bacteria within storage time, where the total number of aerobic bacteria after two months of storage was 6.9 $\log_{10}$ cfu g^{-1}. This is not consistent with the results of Gök et al. (2008), who found that with an increase in storage time of 4 °C, the total number of aerobic bacteria decreased from 7.8 to 7.1 due to the sloping of humidity.

The number of yeasts and moulds was increasing, and this is in the opposite to the results of Gök et al. (2008), who found that yeast and moulds counts were reduced from 5.8 to 4.8 as well as are not consistent with the results of Mohammed and Alzobaay (2012), who found that the number of yeast and moulds decreased due to the loss of moisture, which increases the concentration of some salts, thus inhibiting the growth of microorganisms.

Chemical analysis

Table 2 shows a slight decrease in moisture content, which decreased to 40.9% at the end of storage after 180 days, and this corresponds to Mohammad et al. (2011) findings. The percentage of fat decreased from 7.5% to 5.2% at the end of the storage period. This can be attributed to the activity of lipase and oxidative enzymes. There was an increase in the values of the peroxide number from 2.0 to 5.8 within storage time, this is due to increased activity of lipoxidase enzymes and it is consistent with Pleser et al. (2007) results. This increment value was not too much because of the activity of lactic acid bacteria which reduces the pH values and thus blocks the effectiveness of Lipase enzymes Al-Faydi (1996). The pH values have fallen below 6 and the acidity increased to 1.1% as a lactic acid after one month of storage, which is higher than the values that have reached by Al-Faydi (1996). After six months of storage the acidity was up to 2.3% as a lactic acid due to the activity of acidic bacteria, including lactic bacteria, and it is identical with the results of Al-Faydi (1996).

Table 2

Chemical changes of Pastirma samples within storage period

Storage time (days)	Moisture (%)	Fat (%)	Peroxide number (meq/kg)	Protein (%)	Acidity (%)	pH
0	43.5	7.5	2.0	26.5	0.2	6.1
15	42.9	5.9	2.5	24.9	0.6	6.0
30	42.5	5.8	2.9	24.4	1.1	5.5
60	41.9	5.7	3.3	21.4	1.7	5.3
120	41.4	5.5	4.2	20.9	1.9	5.0
180	40.9	5.2	5.8	17.3	2.3	4.9
LSD*	0.4	0.3	0.3	1.6	0.4	0.3

***** Least significant difference LSD at <0.05

Changes in total nitrogen, protein, soluble and volatile nitrogen

The importance of studying changes in nitrogen (total, protein, soluble, and volatile) is attributed to the fact that meat is a major source of protein. TVN (total volatile nitrogen) known as a group of compounds such as trimethylamine (TMA) which is produced by spoiled bacteria, dimethylamine (DMA) which is produced by autolysis enzymes within freezing storage, and ammonia (NH3) which is produced by removing the amine group from amino acids during meat spoiling Malle and Poumeyrol (1989). Soluble nitrogen (non-protein nitrogen NPN) is defined as amino acids, imidazolyl, dipeptide, triethanolamine, urea and betaine Sikorski and Pan (1994).

Table 3 shows changes in total nitrogen, protein, soluble and volatile nitrogen in the Pastirma while stored at a temperature of 7 ± 2 °C, according to the proposed time periods.

Table 3

Changes in nitrogen types (%) in Pastirma samples

Storage time (days)	Total nitrogen TN (%)	Protein nitrogen PN (%)	Soluble nitrogen (non-protein nitrogen NPN) (%)	Total volatile nitrogen TVN (%)
0	4.4	4.2	0.2	0.01
15	4.3	3.9	0.3	0.02
30	4.2	3.9	0.3	0.03
60	4.1	3.4	0.5	0.07
120	4.1	3.4	0.6	0.08
180	3.6	2.8	0.7	0.09
LSD*	0.2	0.2	0.04	0.003

***** Least significant difference LSD at <0.05

The ratio of TN and PN is decreasing while there was a significant increasing in the ratio of NPN and TVN in the stored Pastirma at a temperature of 7 ± 2 °C within storage time, especially after 30 days and that because of the activity of protease enzymes Veli Gök et al. (2008).

Sensory tests

Table 4 indicates the changes in the sensory properties of stored Pastirma as time progresses, with significant differences ($p<0.05$). This indicates that storage affects sensory properties, and that the greatest effect of this storage occurs after two months. It was found that the most sensitive sensory properties were the texture, appearance and smell, this is consistent with what was reached by Veli Gök et al. (2008), who found that most of the sensory properties (color, taste, appearance, texture and acceptability) for the Pastirma stored at 4 °C, fall to the lowest values at storage for 120 days.

Table 4

Changes in sensory properties of Pastirma samples

Storage time (days)	Color	Taste	Texture	Appearance	Smell
0	8.6	8.9	9.1	8.2	8.9
15	7.1	7.4	8.7	8.1	7.8
30	5.9	6.9	7.1	7.2	5.1
60	5.2	5.3	6.2	6.2	3.9
120	4.3	-	5.1	4.3	2.5
180	3.9	-	4.3	3.9	1.9
LSD*	0.6	0.5	0.7	0.7	0.9

***** Least significant difference LSD at <0.05

CONCLUSIONS

The results were obtained in the chemical, microbial and sensory tests on the Syrian Pastirma stored at a temperature of (7 ± 2 °C) within storage time (0, 15, 30, 60, 120, 180 days) are as following:

The microbiological, sensory and chemical properties of Pastirma become progressively worse with the increasing of storage time.

It is not recommended to keep the manufactured Pastirma at a temperature of 7 ± 2 °C for more than a month.

However, it was observed that we need extra experiments to determine the most appropriate conditions for Pastirma storage.

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Antioxidant activity and total phenolic content of wheat, barley, corn and rice

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SUMMARY

The aim of the present study was to determine the antioxidant activity (DPPH method and phosphomolybdenum method), total polyphenol, phenolic acid and flavonoid content in four cereals – wheat, barley, corn and rice.

Results of antioxidant activity showed that from analyzed cereals the sample of barley had the best activity tested by both methods (3.09±0.22 and 33.27 ±1.75 mg TEAC/g; TEAC – Trolox equivalent antioxidant activity). In this sample was also detected the highest content of total polyphenol (2.38 ±0.14 mg GAE/g; GAE – gallic acid equivalent) as well as total flavonoid content (0.11±0.01 mg QE/g; QE – quercetin equivalent) and phenolic acid content (1.38±0.03 mg CAE/g; CAE – caffeic acid equivalent). The lowest antioxidant activity and phenolic content was detected in sample of rice.

The obtained results confirmed that cereals are a good source of phytochemicals and their extracts should be developed as health supplement products.

Keywords: *cereals, polyphenols, DPPH, phenolic acids, flavonoids*

INTRODUCTION

Cereals belong to the most important food groups supplying significant amounts of energy, macronutrients, micronutrients and non-nutrients to the human diet. Epidemiological studies conclude that there exists a relationship between high intakes of whole grains and reduced incidence of chronic diseases such as cardiovascular disease, diabetes and cancer (Mikulajová et al., 2007). Cereals contain many phenolic compounds, having different chemical structures, of which phenolic acids are of great significance (Prajapati et al., 2013). Cereal grains are rich in phenolic acids phytosterines, saponins, and phytoestrogens, and flavonoids are present in small quantities. It has been suggested that these antioxidants may contribute to the health benefits of cereal-based foods in reducing the incidence of aging-related chronic diseases including heart diseases and some types of cancer (Djordjevic et al., 2011).

Wheat (*Triticum aestivum* L.) is an important agricultural commodity and a primary food ingredient worldwide. It contains important beneficial nutritional components. Wheat and wheat-based food ingredients rich in natural antioxidants can ideally serve as a basis for development of the functional foods designed to improve the health of millions of consumers. Growing evidence indicates that intake of whole wheat foods may associated with health benefits including the reduced risk of coronary heart diseases and certain type of cancers (Vaher et al., 2010).

Barley (*Hordeum vulgare* L.) is considered as a nutraceutical grain because it contains bioactive compounds like *β*-glucan, phenolic compounds, B-complex vitamins, tocotrienols, and tocopherols. Among the cereal grains barley has higher antioxidant activity as compared to the more widely consumed cereals wheat and rice. The risk imposed by the consumption of free radicals and oxidation products towards various forms of cancer and cardiovascular disease could be lowered by the intake of dietary phenolics (Sharma et al., 2012).

Corn (*Zea mays* L.) is one of the most important grains that provide food for most of world population. It is a source of macro and micronutrients and also is rich in phytochemicals such as phenolic acids and anthocyanins among others compound (Fabila-Garca et al. 2017).

Rice (*Oryza sativa* L.) is the staple food in several countries especially in Asian. Rice grains have a hard husk protecting the kernel inside. After the husk is removed, the remaining product is known as brown rice. After removal of the bran and embryo, the remaining endosperm is known as polished rice. Traditionally, polished rice is consumed. However, the rice bran fraction contains high levels of fibre and bioactive phytochemicals including tocopherols, tocotrienols, oryzanols, dietary fibres, vitamins, and phenolic compounds, which are beneficial to human health and well-being (Ghasemzadeh et al., 2018).

The objective of this study was to determine antioxidant activity as well as total polyphenol,

phenolic acid and flavonoid content of wheat, barley, corn and rice purchased from local market in Slovakia.

MATERIALS AND METHODS

Samples

Cereals – wheat, barley, corn and rice (polished rice) were purchased from local market in Slovakia. Sample of wheat, barley and corn was grown in Slovakia (west part), sample of rice was originated (grown) from China. Before the measurement samples were milled into whole meal flour (Perten 3100, Sweeden), and stored at room temperature (21°C) in closed containers.

Sample preparation

An amount of 0.5 g of sample was extracted with 40 mL of 80% ethanol for 2 hours. After centrifugation at 4000 g (Rotofix 32 A, Hettich, Germany) for 10 min, the supernatant was used for measurement (antioxidant activity, polyphenols, flavonoids, phenolic acids). Extraction was carried out in triplicate.

DPPH method – Radical scavenging activity

Radical scavenging activity of samples was measured using the 2,2-difenyl-1-picrylhydrazyl (DPPH) according to the procedures described by Yen and Chen (1995). The extracts (0.5 mL) were reacted with 2.5 mL of DPPH solution (0.025 g DPPH in 100 mL ethanol). Absorbance of the reaction mixture was determined using the spectrophotometer Jenway (6405 UV/Vis, England) at 515 nm. Radical scavenging activity of the samples was expressed as mg Trolox equivalent antioxidant capacity per g (mg TEAC/g).

Reducing power – Phosphomolybdenum method

Phosphomolybdenum method of extracts was determined by the method of Prieto et al. (1999) with slight modifications. The mixture of sample (1 mL), monopotassium phosphate (2.8 mL, 0.1 M), sulfuric acid (6 mL, 1 M), ammonium heptamolybdate (0.4 mL, 0.1 M) and distilled water (0.8 mL) was incubated at 90°C for 120 min, then rapidly cooled and detected by monitoring absorbance at 700 nm using the spectrophotometer Jenway (6405 UV/Vis, England). Reducing power of the samples was expressed as mg Trolox equivalent antioxidant capacity per g (mg TEAC/g).

Total polyphenol content

Total polyphenol content of cereal extracts was measured spectrophotometrically, using the modified Folin-Ciocalteu method as described by Singleton and Rossi (1965). An amount of 0.1 mL of each cereal extract was mixed with 0.1 mL of the Folin-Ciocalteau reagent and 1 mL of 20% sodium carbonate, and centrifuged at 11 000 g (Eppendorf MiniSpin) for 10 min. (centrifugation was necessary due to presence of turbidity). The supernatant was used for measured the absorbance at 700 nm using the spectrophotometer Jenway (6405 UV/Vis, England). Gallic acid was used as the standard and the results were expressed as mg gallic acid equivalent per g (mg GAE/g).

Total flavonoid content

Total flavonoid was determined using the modified method by Quettier-Deleu et al. (2000). An amount of 0.5 mL of cereal extract was mixed with 2 mL of 5% ethanolic solution of aluminum chloride and centrifuged at 11 000 g (Eppendorf MiniSpin) for 10 min. (centrifugation was necessary due to presence of turbidity). The supernatant was used for measured the absorbance at 405 nm using the spectrophotometer Jenway (6405 UV/Vis, England). Quercetin was used as the standard and the results were expressed as mg quercetin equivalent per g (mg QE/g).

Total phenolic acid content

Total phenolic acid content was determined using method of Farmakopea Polska (1999). A 0.5 mL of sample extract was mixed with 0.5 mL of 0.5 M hydrochloric acid, 0.5 mL Arnova reagent (10% $NaNO_2$ +10% Na_2MoO_4), 0.5 mL of 1 M sodium hydroxide (w/v) and 0.5 mL of water. Absorbance at 490 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Caffeic acid was used as a standard and the results were expressed mg caffeic acid equivalent per g (mg CAE/g).

Statistical analysis

All experiments were carried out in triplicate and the results reported are the results of those replicate determinations with standard deviations. Correlation coefficients were calculated by CORR analysis ($p \leq 0.05$) (SAS, 2009).

RESULTS AND DISCUSSION

Antioxidant activity

The scavenging effect of cereal extracts (*Table 1*) on DPPH radical expressed as mg TEAC/g decreased in this order: barley > wheat > corn > rice. These results indicated that all the extracts had a noticeable effect on scavenging free radical. The extract from barley had the strongest scavenging activity. From the literature it is known, that the barley is an excellent source of natural antioxidant either for food preservation (to inhibit lipid oxidation), or for disease prevention (Fardet et al., 2008). Liu and Yao (2007) determined scavenging activity of barley extracts and

found strong activity, which was dominant in 70% acetone and shown similar activity to BHT (butylated hydroxytoluene) at the amount of 200 µg. Strong activity was also observed in wheat. Yo et al. (2013) determined antioxidant activity by DPPH method in wheat refined and whole wheat flour. Refined flours in their study (range 4.59 to 5.00 µmol TE/g, mean 4.82 µmol TE/g) had slightly higher values than their whole wheat flour counterparts (range 4.28 to 4.77 µmol TE/g, mean 4.53 µmol TE/g), but the differences were not significant. These authors also published that the genetic and environmental factors, the processing conditions, as well as the extraction procedures can affect the antioxidant levels of cereal flour (Yo et al., 2013).

For measurement of the reductive ability (phosphomolybdenum method), the $Mo^{6+} \rightarrow Mo^{5+}$ transformation in the presence of cereal extracts was investigated. Increase in absorbance of the reaction mixture indicated the reducing power of the samples. Reducing power of cereal extracts expressed as mg TEAC/g exhibited the following order: barley > corn > wheat > rice. The reducing capacity of a compound may serve a significant indicator of its potential antioxidant activity (Liu and Yao, 2007). The reducing properties are generally associated with the presence of reductones (Pin-Der, 1998). It is reported that the antioxidant action of reductones is based on the breaking of the free radical chain by donating a hydrogen atom, or reacting with certain precursors of peroxide to prevent peroxide formation. It is presented that the phenolic compounds in cereals may act in a similar fashion as reductones by donating electrons and reacting with free radicals to convert them to more stable products and terminating the free radical chain reaction (Liu and Yao, 2007). Similar like DPPH assay, the extract from barley had the strongest reducing power in both years in all fractions. Liu and Yao (2007) determined reducing power of different solvent extracts of barley and found that 70% methanol extract exhibit the highest activity. Zhao et al. (2008) measured reducing power of malting barley extract and confirmed strong activity of barley. The high activity by this method was also determined in corn. Kijak and Grbeša (2015) reported that carotenoids are the dominant antioxidants in corn. In their study was determined antioxidant activity increased linearly with total carotenoid content. Lutein and *β*-carotene were the primary contributors to TEAC activity, while lutein, *β*-cryptoxanthin and *β*-carotene were primary contributors to TBARS activity (inhibition of linolenic acid peroxidation).

Table 1

Antioxidant activity of analyzed cereals

Sample	DPPH method [mg TEAC/g]	Phosphomolybdenum method [mg TEAC/g]
Wheat	2.61±0.14	17.79±1.42
Barley	3.09±0.22	33.27±1.85
Corn	2.54±0.18	18.42±1.41
Rice	1.31±0.04	4.15±0.53

TEAC – Trolox equivalent antioxidant capacity; mean ± standard deviation

Total polyphenol (TPC), flavonoid (TFC) and phenolic acid content (TPAC)

The total polyphenol content was determined by the Folin-Ciocalteau assay. The results are presented in *Table 2*. Total phenolic content of cereal extracts as mg GAE/ g decreased in this order: barley > wheat > corn > rice. The results showed that polyphenols were found in all cereal extracts. The sample of barley and wheat showed higher amounts of polyphenols than corn and rice.

The total flavonoid content of cereal extracts is presented in *Table 2*. Feng et al. (1998) published that flavonoids are dominant in bran layer of cereals and may be important for the miller because bran are introduced into flour during the milling process. Increasing amounts of bran will decrease the grade of the flour. In cereal extracts content of flavonoid expressed as mg QE/mg decreased in this order: barley > wheat > corn. In sample of rice amount of flavonoids was under limit of detection (minus value in spectrophotometer), whereas it was sample of polished rice, in which during technological process bran is removed. After removal of the bran and embryo, the remaining endosperm is known as polished rice. Traditionally, polished rice is consumed. From literature is known that flavonoids are concentrated mainly in pericarp.

The total phenolic acid content is presented in *Table 2*. In cereal extracts content of phenolic acid expressed as mg CAE/mg decreased in this order: barley > corn > wheat > rice. Our results are comparable with the results of Kandil et al. (2012) which determined total phenolic acid content in wheat (1.70 mg/g), barley (0.90 mg/g) and corn (1.25 mg/g). These authors also by HPLC method found that ferulic, coumaric, and protocatechuic acids were the major phenolic acids in wheat. Ferulic, coumaric, hydroxybenzoic, and gallic acids were predominant in

barley. In corn, ferulic, coumaric, gallic, and syringic acids were abundant.

TPC, TPAC and TFC have often been found to positively correlate with other antioxidant capacity assays such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) and reducing power (phosphomolybdenum method). In our study statistically significant correlation ($p \leq 0.05$) was observed between parameter DPPH method and TPC→ r=0.809; between phosphomolybdenum method and TFC→ r=0.941 and between phosphomolybdenum method and TPAC→ r=0.912.

Table 2

Total polyphenols, flavonoids and phenolic acids in analyzed cereal extracts

Sample	TPC [mg GAE/g]	TPAC [mg CAE/g]	TFC [mg QE/g]
Wheat	1.75±0.11	0.93±0.15	0.11±0.01
Barley	2.38±0.14	1.38±0.03	0.02±0.00
Corn	1.49±0.09	1.26±0.41	0.03±0.01
Rice	0.24±0.02	0.17±0.02	n.d.

TPC – total polyphenol content; TPAC – total phenolic acid content; TFC – total flavonoid content; GAE – gallic acid equivalent; CAE – caffeic acid equivalent; QE – quercetin equivalent; mean ±standard deviation

CONCLUSIONS

Cereal grains remain a staple component of diets worldwide. They provide significant levels of bioactive phytochemicals. In our study the best antioxidant activity was observed in sample of barley (3.09±0.22 and 33.27 ±1.75 mg TEAC/g). In this sample was also detected the highest content of total polyphenol (2.38 ±0.14 mg GAE) as well as total flavonoid content (0.11±0.01 mg QE/g). The strong biological activity in all observed parameters was detected in sample of corn. Correlations between antioxidant activities and polyphenol components were also confirmed in this study. Our results confirmed suggestion that cereal grains are rich for bioactive compounds with antioxidant activity and their consumption mainly in whole grain products can improve human health.

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Nutritional parameters of different cereal flour's bread

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SUMMARY

In this study 16 breads were examined. The breads were made from the following flours: 1-wheat flour 55, 2-wheat flour 80, 3-bio whole wheat flour, 4-graham wheat flour, 5-light rye flour, 6-whole grain rye flour, 7-fine rice flour, 8-whole grain fine brown rice flour, 9-white spelt wheat flour, 10-whole spelt wheat flour, 11-oat flour, 12-oatmeal flour, 13-corn flour, 14-sorghum flour, 15-barley flour, 16-millet flour.

The following nutritional parameters were measured: dry matter-, protein-, fat-, ash-, flavonoid- and total polyphenol (TP) content.

There was no significant difference in the dry matter content. In the protein content, the highest values (>8%) were measured in breads which were made from wheat and millet flours. The fat content of breads did not exceed 1.3% in any case. The ash content of the products was between 1.64-2.72%. There are several factors that influence this. The flavonoid and TP contents showed wide ranges. The highest value for both parameters was measured in the sorghum bread, and the lowest was found in the fine rice flour bread. However, it is important to note that the TPC and flavonoid values of samples 3, 4, 5, 6, 10, 11, 14 and 15 were high compared to the others.

Keywords: *bread, dry matter content, protein content, fat content, ash content, flavonoid content, total polyphenol content (TPC)*

INTRODUCTION

Since the beginning of the civilization, cereal products, especially bread are important sources of food for human species (Abdullah, 2008). This product is a source of carbohydrates, proteins, dietary fibres, vitamins, micronutrients and antioxidants (Rubel et al., 2015). Its quality depends on a number of physical and organoleptic characteristics which may be influenced by the following factors: type of the flour, quality of other ingredients, bread making process, fermentation, baking time and temperature. In recent years, the importance of bread has increased, because it is considered a possible functional food. Both industry and researchers are working to optimize bread-making technology. To improve its diversity, flavour and quality by adding ingredients that have functional properties (Dall'Asta et al., 2015).

MATERIALS AND METHODS

A 16 kinds of bread made from different flours were examined (*Table 1.*) The composition of bread: 300 g flour, 9 g yeast, 6 g salt, 1.5 g sugar and sufficient amount of water. After working of the ingredients into consistent dough, maturing, measuring, shaping, leavening and baking were executed (Szilágyi and Borbély, 1999).

Table 1

Flours used to make the breads, and Kjeldahl conversion factors

Marking	Flours used (100%)	Kjeldahl factor	Marking	Flours used (100%)	Kjeldahl factor
1	wheat flour 55	5.70	9	white spelt wheat flour	5.70
2	wheat flour 80	5.70	10	whole spelt wheat flour	5.70
3	bio whole wheat flour	5.70	11	oat flour	5.83
4	graham wheat flour	5.70	12	oatmeal flour	5.83
5	light rye flour	5.83	13	corn flour	6.25
6	whole grain rye flour	5.83	14	sorghum flour	6.25
7	fine rice flour	5.95	15	barley flour	5.83
8	whole grain fine brown rice flour	5.95	16	millet flour	6.31

After baking, breads have been cooled down (*Picture 1*) and measured. The determination of dry matter, protein, fat and ash content was performed according to standard MSZ 20501-1:2007. The measurements were carried out in triplicate.

Picture 1: **Top line: breads made from 1st, 2nd, 3rd flours**
Bottom line: breads made from 10th, 9th, 4th flours

Determination of dry matter content

Petri dishes were dried in an oven, then cooled down in a desiccator and their weight were measured. The analysed samples were homogenized before the analysis, then 5 grams of them were measured into the dishes. Then the dishes containing the samples had been put into the oven on 130±3 °C and dried to constant weight, then cooled in the desiccator. After cooling, the common weight of the dish and the sample was measured, then water content had been calculated by using the original and the dried weight of the dishes and the samples. The results were presented in percentage.

Determination of protein content

The samples were put into a nitrogen-free paper and digested with concentrated sulfuric acid in the presence of a catalyst. The breads nitrogen content was converted into ammonium salt under distillation. By titration, nitrogen content is obtained. The obtained value was multiplied by a specific factor (Kjeldahl conversion factor in *Table 1*), which gives the protein content.

Determination of fat content

Prepared samples were digested on 100 °C until 45 minutes with sulfuric acid and ethyl-alcohol. After cooling, fat content was extracted with petroleum ether. The mixture was left to stand for two hours, then the glass was filled up with water to separate the phases. The next day, 20 cm^3 of pure petroleum ether phase was separated and then evaporated. The fat content was calculated by a formula for the amount of residual fat.

Determination of ash content

Ash is determined by the cremation of samples on 550-600 °C. The total amount of residual material contains inorganic components, which causes white colour. The analysed samples were homogenized, then 5 grams of them were measured into ashing crucibles, then put into the incinerators on 200-220 °C, then on 500-550 °C. The ash content is related to the weighed mass, obtained in percentage by means of a formula.

Determination of antioxidant component

Total polyphenol content (TPC) was determined according to the Folin-Ciocalteu method (Singleton et al., 1999). Flavonoid content was measured by the method of Kim et al. (2003). Both methods include the samples soaked in a mixture of methanol:distilled water (80:20). After the filtration, different reagents were added.

In case of TPC, for the calibration gallic acid was used. The mixtures had to stand in the dark for two hours, then their absorbance was measured on 760 nm. The obtained results are reported in mgGAE/100 g.

In case of flavonoid content, for the calibration, catechin was used. The absorbance was measured on 510 nm. The results are expressed in mgCE/100 g.

RESULTS AND DISCUSSION

The details of the finished products can be found in the *Table 2*.

The dry matter content of the breads was between 59.6 and 76.4%. The lowest value was in the 12th, while the highest value was measured in the 16th bread. In case of wheat flours breads (1., 2., 3. and 4.) the dry matter contents decreased as the ash content increased in the flour. The same trend can be seen in the same plant flours (5.-6. and 9.-10.). In case of rice flours bread (7. and 8.), it can be seen that their dry matter content was almost the same. The dry matter contents of 11th, 13th and 14th, furthermore the 12th and 15th samples were similar. In case of oat flours products, the oat flour breads have higher dry matter content and ash content than the oatmeal flour bread.

Table 2

Nutritional parameters of the analysed breads

	Dry matter content (%)	Protein content (%)	Fat content (%)	Ash content (%)
1	68.2±0.3	8.37±0.21	not detected	1.89±0.01
2	67.7±0.0	9.03±0.16	0.255±0,012	1.89±0.02
3	64.1±0.5	8.71±0.12	0.336±0.021	2.32±0.11
4	63.7±0.3	8.72±0.13	0.363±0.022	2.46±0.09
5	65.6±0.1	6.10±0.10	0.178±0.011	2.22±0.05
6	63.6±0.2	7.20±0.23	0.444±0.020	2.43±0.06
7	67.7±0.2	4.47±0.12	0.080±0.001	1.64±0.01
8	67.9±0.3	5.58±0.10	0.164±0.013	2.44±0.05
9	67.3±0.3	8.07±0.20	0.213±0.011	1.80±0.04
10	65.4±0.2	8.27±0.16	0.079±0.001	2.35±0.10
11	66.5±0.3	7.75±0.16	1.16±0.05	2.20±0.05
12	59.6±0.0	7.63±0.15	0.641±0.011	2.02±0.08
13	65.7±0.3	3.83±0.15	0.051±0.000	1.72±0.04
14	67.3±0.2	7.12±0.29	0.877±0.015	2.42±0.07
15	61.1±0.1	5.89±0.22	0.122±0.001	2.03±0.01
16	76.4±0.1	9.50±0.12	1.22±0.04	2.72±0.06

mean ± standard deviation

Between the values of the protein content, bigger differences can be observed. Protein content of wheat flour was above 8% in every case (breads no. 1, 2, 3, 4). The protein content showed opposite trend than the dry matter content. As the ash content of the base material increases, the protein content also increases, except for the 2nd sample (9.03±0.16), because this value is higher than the 1st, 3rd and 4th. Using rye flours, the values were 6.10±0.10 (5.) and 7.20±0.23% (6.). In case of rice flours, the protein content was 4.47±0.12 (7.) and 5.58±0.10% (8). 9th (8.07±0.20%) and 10th (8.27±0.16%) samples values are above 8%. In case of rye, rice and spelt flours breads the protein content increases as the ash content increased in the flours. The protein content of products produced of oat flour (11.) 7.75±0.16% and oatmeal flour (12.) 7.63±0.15% was similar, but still the oat flour bread which has higher ash content, had higher protein content too. The lowest value was measured in corn flour bread 3.83±0.15% (13.), and the highest value was measured in millet flour bread 9.50±0.12% (16.). The protein content of sorghum and barley flours breads were 7.12±0.29% and 5.89±0.22%.

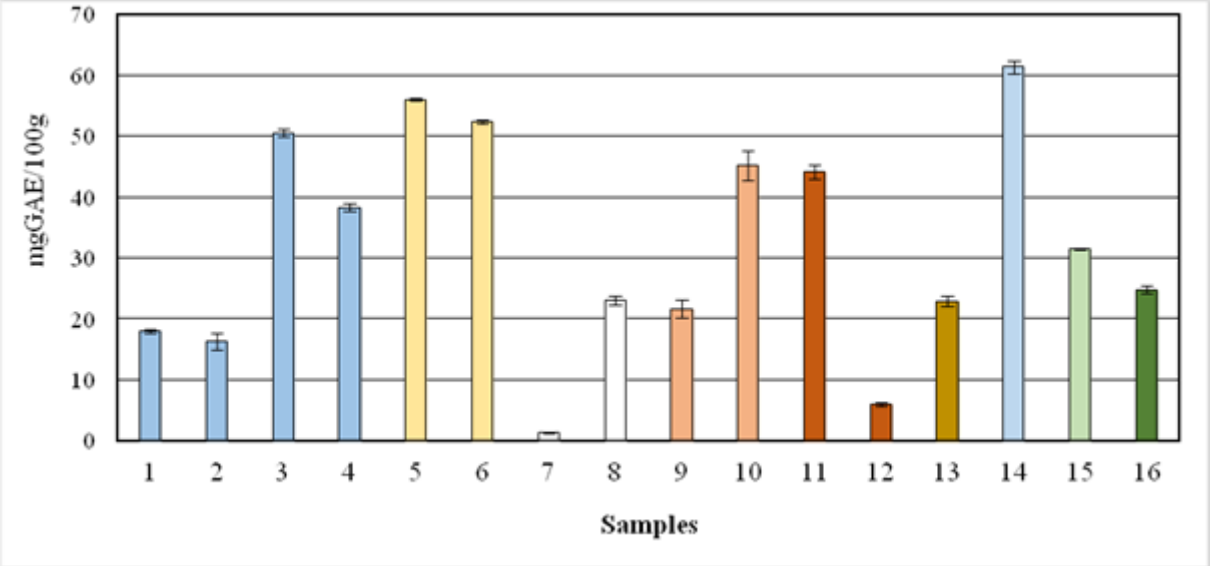
The fat content was low in every case. The highest value was only 1.22±0.04%, which was measured in the 16th product. This was followed by the 11th, 14th, 12th and 6th products. The fat content of the 1st bread could not be detected. The ash content also plays a role in the fat content. In case of wheat, rye, rice and oat flours, the fat content increases as the ash content increased in the flours. In case of spelt flours, the white spelt wheat flour bread's fat content was higher than the whole spelt wheat flour breads. The fat content of other samples was various. As shown in the table, the other results were under 0.363%.

The highest ash content was measured in product no. 16. The ash content the of the 3rd, 4th, 5th, 6th, 8th,10th, 11th, 12th, 14th and 15th breads were above 2.00%. The ash content of the 1st, 2nd, 7th, 9th, 13th samples were between 1.50 and 2.00%. The lowest value was found in the 7th product. The values in the second table show that the ash content effects the nutritional value. For example, sample 16 always showed the highest dry matter, protein and fat content besides the highest ash content.

The TPC (*Figure 1*) were different in the products. The lowest TPC (1.32±0.05 mgGAE/100 g) was found in the fine rice flour sample (7.). The highest value (61.3±1.1 mgGAE/100 g) was determined in the sorghum flours product (14.). Over 40.0 mgGAE/100 g TPC were measured in products made from the following flours: bio whole wheat flour (3.), light rye flour (5.), whole grain rye flour (6.), whole spelt wheat flour (10.), oat flour (11.). 30-40 mgGAE/100 g was measured only in graham wheat bread and barley bread. 20-30 mgGAE/100 g values were shown by whole grain fine brown rice, white spelt wheat, corn and millet products. The values between 10 and 20

mgGAE/100 g were measured in the 1st and 2nd products, while less than 10 mgGAE/100 g TPC were in the 12th and 7th breads. Whole grain flours were found to have higher total polyphenol content, but for samples 5 and 6, which were rye flour samples, the values were similar. In case of oat flours products, the oat flour breads had higher total polyphenol content than the oatmeal flour bread.

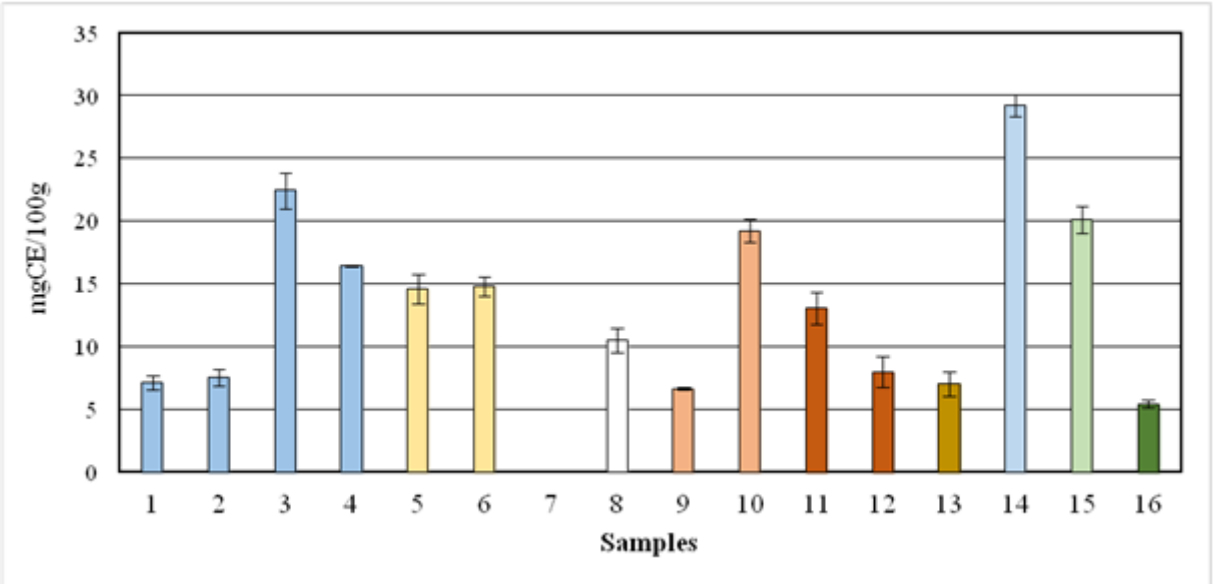
Figure 1: **The breads total polyphenol content**



The lowest flavonoid content (*Figure 2*) was measured in the 7th sample (<LOD), while the highest value was shown by the 14th bread (29.2±0.9 mgCE/100 g). 20-25 mgCE/100 g was measured only in the 3rd and the 15th bread. The value between 15 and 20 mgCE/100 g also had two products made from graham wheat flour and whole spelt wheat flour. In case of 5th, 6th, 8th and 11th breads, the flavonoid content was between 10-15 mgCE/100 g. In the 1st, 2nd, 9th, 12th, 13th and 16th samples, flavonoid content was determined under 10 mgCE/100 g. Whole grain flours were found to have higher flavonoid content, but for samples 5 and 6 the values were similar to the TPC. The 11th and 12th samples showed similar trend as in case of TPC.

Figure 2: **The breads flavonoid content**

(LOD=0.05 mgCE/100 g)



CONCLUSIONS

In this study, the nutritional parameters (dry matter-, protein-, fat-, ash-, flavonoid-and TP content) of 16 breads were examined.

As for the results, ash content also effects the dry matter content, the protein content and the fat content. There was no significant difference in the dry matter contents. For the first 10 samples of the same plant species, higher ash content can be related to lower dry matter content. It is also important to note that sample 16 has the highest dry matter content besides the highest ash content.

In the protein content, the highest values (>8%) were measured in breads which were made from wheat and millet flours. These values depend on the protein content of the flour, but are also related to the ash content. As the ash content increases in each sample, the protein content increases as well.

In any case, the fat content of bread did not exceed 1.3%. The result is influenced by the raw material and flour, as well as the value of the measured ash content. The exceptions are patterns 9 and 10, which would have a higher ash content with lower fat content.

The ash content of the products was between 1.64-2.72%. There are several factors that influence this. Among others, the type of plant, growing conditions and soil conditions.

The flavonoid and TP contents showed wide ranges. The highest value for both parameters was measured in the sorghum bread, and the lowest was found in the fine rice flour bread. However, it is important to note that the TPC and flavonoid values of samples 3, 4, 5, 6, 10, 11, 14 and 15 were high compared to the others.

This test was important for the selection of some types of flour that we would use for further research based on the appearance, behaviour and nutritional parameters of the finished breads.

In the future, besides wheat flour, rye flour would also be used for baking various products. We have decided to use these flours because the parameters we have measured are appropriate and these flours are used by the industry every day.

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Extraction of flavonoids from medicinal plants with use of various solvents systems

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SUMMARY

Extracts of three medicinal plants: yarrow (Achillea millefolium), agrimony (Agrimonia eupatoria) and three-lobe beggartricks (Bidens tripartita) made with use of micelle mediated extraction method were subjected for chromatographic evaluation for the content of flavonoid compounds. The results indicate, that aqueous surfactants solutions could be effective solvents for flavonoid aglycone extraction, whereas luteolin glucoside or present in trace amounts in such extracts. The highest content of luteolin was recorded in Agrimonia and Bidens micellar extracts made with Triton X-100 and Nonidet P-40. In contrast, in water-acetone extracts significant amount of luteolin glycoside was found (up to 50 µg/ml of Herba Bidentis extract), with much less content of flavonoid aglycones.

Keywords: *flavonoids, yarrow, agrimony, three-lobe begarrtricks, micelle mediated extraction*

INTRODUCTION

Plant secondary metabolites are a large group of compound biosynthesized by plants as dyes, attractants or signal compounds. Most of these chemicals have wide spectrum of bioactivities and find application in cosmetology, pharmaceutical or food industry. One important class of plant metabolites are flavonoids, based on the general 15-carbon structure of three rings: two phenyl and one hetrocyclic ring (C_6-C_3-C_6). Modifications of such a basic skeleton and the presence of various substituents in different positions led to the creation of an extremely diverse group of compounds that can be classified into several groups on the basis of structural structure, e.g. flavones, flavonols, flavanones, flavanonols, isoflavonoids or chalcones (Czaplińska et al., 2011). Flavonoids have an unusually broad spectrum of biological properties. Due to the presence of several hydroxyl groups, the carbonyl group and the double bond in the structure they are good antioxidants. In addition to scavenging free radicals and other reactive oxygen species (ROS), they also inhibit the activity of enzymes responsible for their formation (oxidases) and chelate transition metals, catalyzing the reactions of ROS formation. Moreover, anti-inflammatory, anti-atherosclerotic, anti-cancer, sealing and strengthening blood vessels, diuretics, platelet aggregation and antiallergic properties have also been demonstrated (Dai and Mumper, 2010; Czaplińska et al., 2011). Thanks to the mentioned properties flavonoids are important ingredients of functional or pro-health food.

Plant secondary metabolites are obtained from raw materials most often by solid-liquid extraction. In terms of efficiency or purity of the extract the selection of proper extraction solvent is crucial. In the case of isolation of flavonoids, the most common are polar solvents: simple aliphatic alcohols (methanol, ethanol, less butanol) as well as acetone or ethyl acetate and their mixtures with water in various proportions (Dai and Mumper, 2010). For example dandelion (*Taraxacum officinale*) flavonoids were extracted using ultrasound with pure methanol (Sun et al., 2014), in turn, 70% ethanol was used in the case of various methods of extraction of flavonoids from leaves and flowers of *Cassia angustifolia* (Laghari et al., 2011). In the literature, many examples of extraction system optimization can be found, in which acetone was recognized as the best solvent for the extraction of polyphenols. Parsley leaf extract made with 70% acetone showed the highest total phenolic content (Kuźma et al., 2014). Similar results were obtained in the extraction of polyphenols from dates (*Phoenix dactylifera*) (Kchaou et al., 2012).

One of modification of aqueous extraction is application of surfactants. It is a wide group of substances characterized by specific structure and properties. In the construction of their molecules, two parts can be distinguished: hydrophilic (polar), called "head" and hydrophobic (non-polar), called "tail". The consequence of this chemical structure is a series of characteristic properties of this type of compounds. Due to their two-part, asymmetric structure, surfactants show in aqueous solutions so-called surface activity. This phenomenon consists in lowering the surface tension of aqueous solutions and interfacial tension in the case of systems consisting of immiscible liquids. Another special feature of surfactants is the ability to associate in structures called micelles. In the polar environment, the "heads" of surfactant molecules are directed outside the micelle and "tails" inwards (Tadros, 2014). Surfactants have found application in extraction processes, as the so-called surfactant assisted extraction or micelle mediated extraction (MME). In this case, aqueous solutions of surfactants with concentrations above their CMC (critical micelle

concentration) value are used. Then micelles are forming in which various kinds of compounds can be solubilized. Due to the occurrence of the solubilization phenomenon in systems containing surface active compounds, a significant increase in the solubility of substances that are weakly soluble in pure water is observed. As the main advantages of extraction supported by surfactants, it should be mentioned its universal character (the possibility of applying to various types of analytes), relatively low process costs and, above all, reducing the consumption of organic solvents which are often toxic, volatile and flammable (Tomaszkiewicz-Potępa et al., 2010; Melnyk et al., 2015). Such technique of extraction can be use also for flavonoids, for example quercetin and rutin were successfully extracted from honeysuckle (*Lonicera japonica*) with use of 5% Triton X-100 (Jiang et al. 2011). Another surfactant, Genapol X-080 was used for isoflavones extraction form *Puerariae radix* (He et al., 2005). The daidzein content was the highest in the extract prepared with the surfactant solution, slightly lower in the methanol extracts (pure and 50% mixture with water), ethanol and the resulting mixture of dichloromethane and methanol. The acetic ether, hexane and cyclohexane were completely ineffective (He et al., 2005). Several flavonoids were determined among other compounds in apple extracts of several different varieties for which surfactants have been used (CTAB, Brij 35, SDS, Triton X-100 and PEG 2000) (Hosseinzadeh et al., 2013). Surfactants can also be used in the case of pressurized liquid extraction (PLE). For the isolation of flavonoids (rutin, quercitrin and quercetin) from crepe ginger flowers (*Costus speciosus*) by this method, Chang et al. (2011) used aqueous solutions of SDS and Triton X-100 in various concentrations. Flavonoids were determined by micellar electrokinetic chromatography (MEKC). The efficiency of the tested method was comparable to the control method (extraction in a Soxhlet apparatus, methanol 80%), however PLE is a technique that saves time, energy and reagents (Chang et al., 2011).

The aim of our study was to quantitate some flavonoids in extracts of several medicinal plants prepared with use of micelle mediated extraction and compare the results with aqueous acetone extract as a control.

MATERIALS AND METHODS

Plan material and extraction

As a plant material for extraction commercially available, standardized herbal raw materials were used: *Herba Agrimoniae* (*Agrimonia eupatoria*), *Herba Bidentis* (*Bidens tripartita*) (both from "Dary natury") and *Herba millefolii* (*Achillea millefolium*) ("Kawon"). 1 g of each raw material crushed in mortar was placed in screwed centrifuge tube (50 ml) and poured with 20 ml of a suitable solvent. Extraction was carried out in an ultrasonic bath (35 kHz) for 30 minutes and then the samples were centrifuged for 10 minutes at 6.500 × g. The supernatant was filtered through a filter paper (medium, POCH) and stored for further testing at 4°C. As solvents for extraction 2% aqueous solutions of Triton X-100 (molecular biology grade, Sigma Aldrich), Nonidet P-40 substitute (BioXtra, Sigma Aldrich) and Tween 20 (Ph. Eur., Roth) were used. The control sample was prepared with use of acetone (p. a., Chempur) and water mixture (1:1, *v/v*).

UHPLC-MS analyses

Obtained extracts were analyzed by ultra-high performance liquid chromatography coupled with mass spectrometry. The chromatographic separation was carried out on a Kinetex XB-C18 column (50 × 2.1 mm, 1.7 μm) with a precolumn. As mobile phases, acetonitrile and 0.05% formic acid in distilled water were used. A gradient elution program was used: 0 - 20 min ACN 10% -100%, 20 - 25 min ACN 100%. Mobile phase flow rate: 0.4 ml / min, sample injection volume: 2 μl. Prior to introduction into the chromatography system, all samples of the extracts were filtered through sterile membrane filters with a pore size of 0.22 μm. The extracts were analyzed for quantitative content of three flavonoid compounds: luteolin, luteolin-7-glycoside (cynaroside) and chrysoeriol. The quantification was carried out using the MRM method (multiple reaction monitoring), the appropriate fragmentation pairs were each time selected for a particular analyte using a direct injection of the standard solution, omitting the chromatographic column. Polyphenol standards were dissolved in methanol. All determinations were carried out in triplicate and the mean values were calculated based on prepared calibration curves of quantified analytes.

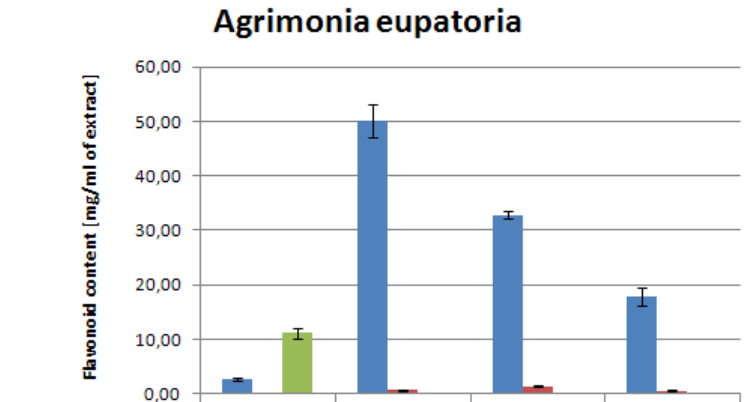
RESULTS AND DISCUSSION

Three flavonoids: luteolin, luteolin-7-glucoside and chrysoeriol were selected for quantitative analysis. According to literature, luteolin and its glycosides are common representatives of the flavonoid class present in the tissues of these plants (Wolniak et al., 2007; Vitalini et al., 2011; Lv and Zhang, 2013; Kyslychenko, 2014; Granica et al., 2015; Orhan et al., 2016). Chrysoeriol, as luteolin 3'-methoxy derivative, can also be biosynthesized by plants of the species, tested in this work.

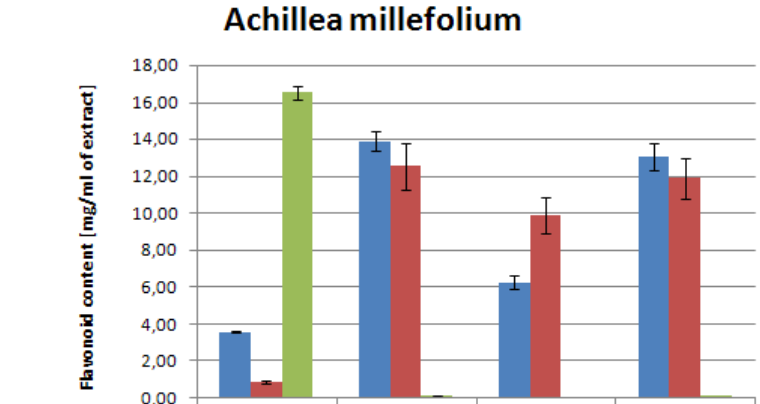
The results of the quantitative determination of selected flavonoids in extracts of the commercial pharmacognostic raw materials are presented in the diagram (*Figure 1*).

The results of the quantitative analysis clearly indicate a 50% mixture of acetone and distilled water, used in this case as a control sample, as an effective solvent for the extraction of luteolin glucoside. The concentrations of cynaroside in the obtained acetone extracts of all three tested raw materials were by far the highest and significantly exceeded the concentration of aglycone (luteolin) and chrysoeriol. In the case of micellar-assisted extraction, glycoside

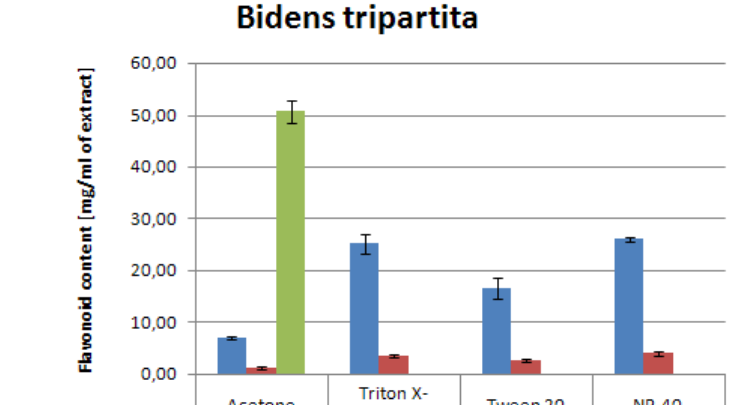
Figure 1: The d**etermined flavonoid contents in three medicinal plants extracts obtained by micellar-assisted extraction method in comparison with pure water-acetone extraction. Values are given as mean value (n=3) and the error bars are representing standard deviation values.**



	Acetone	Triton X-100	Tween 20	NP-40
Luteolin	2,57	50,10	32,83	17,90
Chrysoeriol	0,14	0,59	1,37	0,50
Luteolin-7-glucoside	11,13	0,06	0,01	0,05



	Acetone	Triton X-100	Tween 20	NP-40
Luteolin	3,58	13,90	6,26	13,07
Chrysoeriol	0,88	12,53	9,88	11,90
Luteolin-7-glucoside	16,50	0,09	0,03	0,08

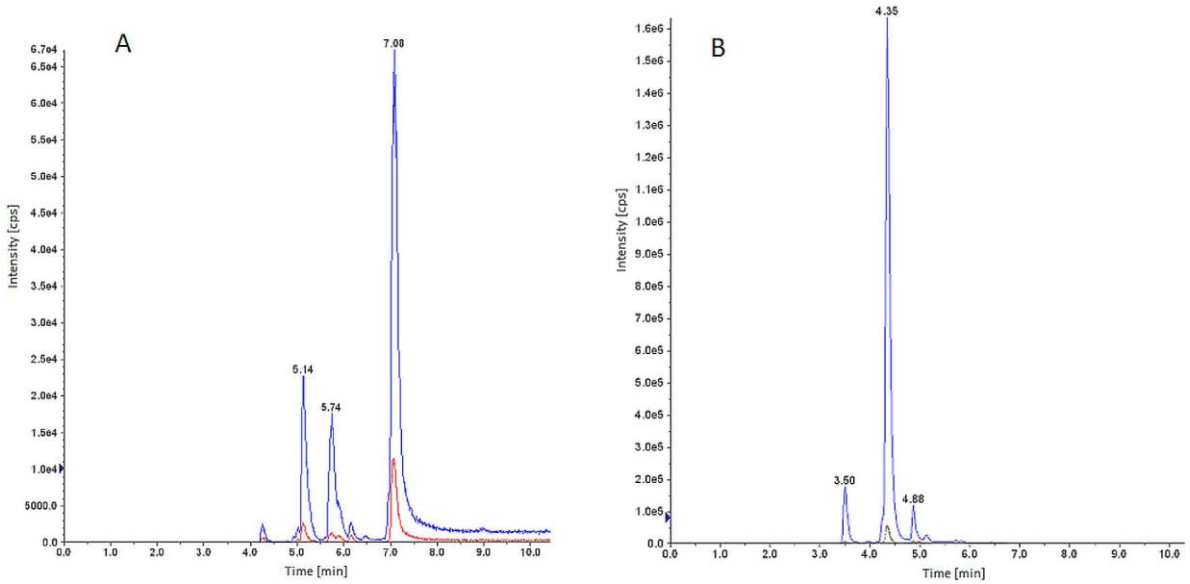


	Acetone	Triton X-100	Tween 20	NP-40
Luteolin	7,05	25,17	16,47	26,03
Chrysoeriol	1,25	3,60	2,68	4,07
Luteolin-7-glucoside	50,70	0,13	0,05	0,11

recovery is at a negligible level compared to free luteolin. The highest content of this glycoside was recorded in the three-lobe beggartricks (*Bidens tripartita*) extract. In all extracts from micellar extraction, luteolin predominates among the flavonoids, and in all the chrysoeriol was detected - in yarrow (*Achillea millefolium*), in concentrations comparable to luteolin. It confirms Ukrainian author's report on the considerable content of this flavonoid in tested plant (Kyslychenko, 2014). Scientific literature also informs about the presence of chrysoeriol in the flowers of other species of the genus *Achillea*: *A. asplentifolia*, *A. distans* and *A. collina* (Trendafilova et al., 2007). According to our knowledge, there are no reports of detection of this flavonoid in agrimony and three-lobe beggartricks.

In addition to free chrysoeriol, glycosylated forms are most likely present in all three analyzed aqueous-organic extracts, as indicated by the presence of additional peaks on MRM chromatograms occurring with shorter retention times than the aglycone (*Figure 2A*).

Figure 2: **MRM chromatograms of water-acetone *T. officinale* extract. A – chrysoeriol (main peak at 7.08 min) and minor glycosides peaks. B – Luteolin-7-glycoside (main peak at 4.35 min) and minor glycosides peaks.**



In extracts made with surfactant solutions the dominant form is free flavonoid, glycosides are present in trace amounts. Similarly, MRM chromatograms for luteolin and its 7-glucoside contain additional peaks that reveal the presence of other than the designated luteolin derivatives, probably different types of glycosides (Fig. 2B). It is worth noting that in extracts obtained as a result of the use of ultrasonically assisted micellar extraction, the content of luteolin and chrysoeriol is significantly higher than in water-acetone. This confirms observations that water-organic systems are more effective in relation to glycosides than to aglycones and is also proof of the high utility of aqueous solutions of surfactants at relatively low concentrations for the extraction of free flavonoids. Also the presence of other glycosides of luteolin and chrysoeriol in water-organic extracts proved the above conclusion.

CONCLUSIONS

Described research showed that aqueous surfactant solutions at low concentrations can be an effective solvent for flavonoid aglycones extraction. To obtain flavonoid glycosides a better solution is to use classical organic solvents (alcohols or acetone) in mixtures with distilled water. Moreover, it is possible to control the composition and properties of plant extracts by selection of suitable solvent system. When using surfactants as extraction solvents an important issue is removing the residues of such compound form extract, especially for food-related applications. The risk of adverse reactions should be taken into account – although surfactants are considered harmless, they can have a negative effect on organisms at cellular level.

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Analysis of flavonoids: Characterization, sample preparation for GC-MS

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SUMMARY

Recent research carried out in the field of natural antioxidants is increasing knowledge about naturally healthy compounds that are available in food. Their use in food products will increase quality and added value. Flavonoids from fruit, vegetables and beverages play an important role in human health, for example preventing cancer and cardiovascular diseases, and lowering the incidence of different diseases. This summary study features developments in the application of GC-MS for the determination of flavonoids, focusing on flavonols including quercetin, myricetin, and kaempferol from the perspective of sample preparation and GC-MS analysis.

Keywords: *GC-MS, quercetin, myricetin, kaempferol, flavonoids*

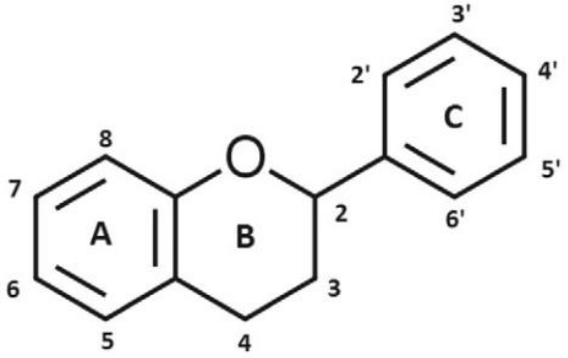
INTRODUCTION

Flavonoids are widely distributed phenolic compounds in the plant kingdom. They occur in all parts of plants as complex mixtures of different components. Flavonoids, as a class of diet-derived antioxidants, have received significant public attention for some decades due to their antioxidant and cell signalling modulation effects, which contribute to prevention against cancer, cardiovascular, and age-related diseases (Cao et al., 2008; Tsimogiannis et al., 2007). The search for new chemical entities and the quality control of flavonoid containing natural products require easy-to-use but reliable and robust analytical methodologies (Vukics and Guttmann, 2010). Several chromatography techniques have underpinned many chemical analysis methods, developed for superior flavonoid separation and identification. Among these, gas chromatography (GC) is one of the most powerful tools in separation science, providing precise measurement of a wide range of flavonoids (Nolvachai and Mariott, 2013). Combined with various detectors, such as MS (mass spectrometry), offers a sensitive and accurate tool for quantitative and qualitative flavonoids analysis. This summary study mainly on the flavonols, including quercetin, myricetin and kaempferol from the perspective of sample preparation and GC-MS analysis.

CHARACTERIZATION OF FLAVONOIDS

Flavonoids are naturally occurring polyphenolic secondary metabolites widely distributed in plants and foods of plant origin, such as vegetables, fruits. Flavonoids are a large group of phenolic plant constituents (Harborne and Williams, 2000). To date, more than 6000 flavonoids have been identified, although a much smaller number is important from a dietary point of view (Erlund, 2004; Spanakis et al., 2009). Flavonoids are divided to several subgroups, and it is important to keep in mind that the biological and chemical properties of flavonoids belonging to different subgroups can be quite different. Flavonoids, whose structures are based on a C_6-C_3-C_6 skeleton, are the most abundant group of phenolic compounds, and are subdivided into different classes differing in the oxidation state of the central heterocyclic ring. These comprise flavonols, flavones, isoflavones, flavanones, anthocyanins and flavanols (Kalili and Villiers, 2011). The basis of their nomenclature is the number and the position of the hidroxyl or methyl groups attached. The general structure of flavonoids are presented in *Figure 1.*

Figure 1: **General structure of flavonoids**



Flavonols

Flavonols are identified by the location of the alcohol group on the *C* ring. The principal dietary

flavonols are quercetin, myricetin and kaempferol. These components are present in many vegetables and fruits. Such as onions, tomatoes, grapes, blueberries, currants (Hertog et al., 1992).

One of the most important flavanols is quercetin. Quercetin 3, 3′, 4′, 5, 7-pentahydroxyflavone, is present in fruit and vegetables. In plants, it occurs mainly in leaves and the other parts of the plants as aglycones and glycosides, in which one or more sugar groups is bound to phenolic groups by glycosidic bond (Wach et al., 2007; Oroian and Escriche, 2015). Glucose is the most common sugar, with galactose and rhamnose also frequently found in composition with flavonoids. In general, quercetin glycosides contain a sugar group at the 3-position. A considerable amount of isoquercetin (quercetin-3-O-b-glucoside) has been found in apple and pear (Wach et al., 2007).

Myricetin is a member of the flavonoid class of polyphenolic compounds, with antioxidant properties. These compounds is structurally similar to luteolin and quercetin and is reported to have many of the same functions as these other members of the flavonol class of flavonoids (Ong and Khoo, 1997). It is a naturally occurring flavonol with hydroxyl substitutions at the 3, 5, 7, 3', 4' and 5' positions (*Figure 1*).

Kaempferol is a secondary metabolite found in many plants, plant-derived foods, and traditional medicines. This compound (3, 5, 7-trihydroxy-2-(4'-hydroxyphenyl)-4H-1-benzopyran-4-one) is a yellow compound that is commonly found in plant-derived foods and in plants used in traditional medicine (Calderon-Montano et al., 2011).

Flavones

Flavones are present in fruits and vegetables which we consume inadvertently in our daily diet and they have a positive impact on our health without any major side effects. In order to explore diverse roles of flavones, investigating various methods for their synthesis and structural modification of flavone ring have now become important goals of several research groups (Singh et al., 2014). Chemically, flavones lack a 3-hydroxygroup. The major part of the flavones is synthesis from two flavanones (naringenin and pinocembrin). These two flavones are synthesized from the condensation of one molecule of hydroxycinnamoyl-Coenzyme A and three molecules of malonyl-Coenzyme A. The flavones can be transformed into flavones by flavones synthetases I and II (Austin and Noel, 2002). As combinations of various modifications can occur, the number of different flavones is enormous (Oroian and Escriche, 2015).

Isoflavones

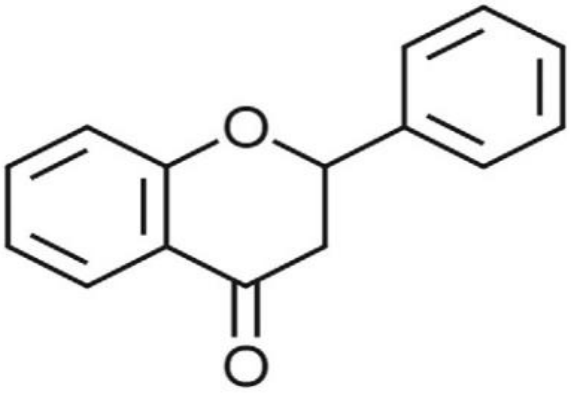
Many legumes are important sources of the isoflavone secondary metabolites, genistein, and daidzein, in addition to their being excellent sources of dietary protein. In one of these legumes, namely seedlings of soybeans, the isoflavones, including genistein and daidzein, are the predominant metabolites present (Kaufman et al., 1997; Balisterio et al., 2013).

Flavanones

A few decades ago, flavanones were considered as only minor flavonoids, like chalcones, dihydrochalcones, dihydroflavonols and aurones. However, in the last years, the total number of known flavanones has increased to the point that they are now considered a major flavonoid class like flavones, isoflavones, flavanols, flavonols and anthocyanidins (Veitch and Grayer, 2006).

Flavanones (*Figure 2.*) also can occur as *O*- or *C*-glycoside sand are especially abundant in citrus fruit and prunes. Naringenin (4′,5 ,7-trihydroxy flavanone) is a flavanone found predominantly in citrus fruit such as oranges and grapefruit (Ooghe et al., 1994; Khan et al., 2014; Oroian and Escriche 2015).

Figure 2: **Chemical structure of flavanone**

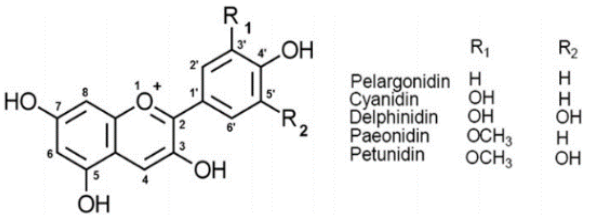


Anthocyanins

Anthocyanins are water-soluble vacuolar pigments that, depending on their pH, may appear red, purple, or blue. Food plants rich in anthocyanins include the blueberry, raspberry, black rice, and black soybean, among many others that are red, blue, purple, or black (Archetti et al., 2009; Zhang et al. 2015). They are sometimes present in other plant tissues such as roots, tubers, stems, bulbils and are also found in various gymnosperms and ferns (Cavalcanti et al., 2011; Andersen and Jordheim 2006).

Anthocyanins are part of the very large and widespread group of plant constituents known as flavonoids, which possess the same C_6–C_3–C_6 basic skeleton (Wilska-Jeszka, 2007). The aglycones are called anthocyanidins, which are usually penta-(3, 5, 7, 3′, 4′) or hexa-substituted (3, 5, 7, 3′, 4′, 5′). Among 31 monomeric anthocyanidins already properly identified more than 90% of all anthocyanins isolated in nature are based only on the following six anthocyanidins (*Figure 3*): pelargonidin, cyanidin, paeonidin, delphinidin and petunidin which differ in the number of hydroxyl and methoxyl groups in the B-ring of the flavylium cation (Cavalcanti et al., 2011).

Figure 3: **Anthocyanidins**



Flavanols (Flavon-3-ol)

Flavan-3-ols (or flavanols) are derivatives of flavans that use the 2-phenyl-3, 4-dihydro-2H-chromen-3-ol skeleton. Diets rich in flavan-3-ols have been associated with reduced risk of chronic diseases including cardiovascular diseases, certain cancers, diabetes, and obesity (Song et al., 2015). Therefore, the bioavailability, chemistry, and physiological responses of dietary flavan-3-ols have been the focus of recent studies. The flavanols are vulnerable to several dedragative reactions accelerated by heat, food ingredient, elevated pH conditions, and presence of dissolved oxygen or other reactive oxygen species (Hughes, 2008).

SAMPLE PREPARATION

Several methods have been developed to analyse various antioxidants. However, adequate sample preparation is required for these tests, for the identification and quantitative determination of the components. Such sample preparation steps include various extractions and derivatizations. This involves using extraction with solvents, supercritical fluid-, solid phase- and microwave-assisted extraction.

Sample extraction

The main goal in sample extraction is to purify or isolate the target analyte from an interfering matrix. An increased number of extraction steps can result in higher selectivity (and potentially improved sensitivity) in GC analysis (Stalikas, 2010).

The extraction yield of antioxidant compounds from plant material is influenced mainly by the conditions under which the process of liquid-solid extraction is carried out. Because each plant material has unique properties in terms of structure and composition, when they are combined with solvents the behaviour of the resulting material-solvent system is unpredictable (Gonzalez-Montelongo et al., 2010, Oroian and Escriche 2015).

Extraction with solvents or liquid-liquid extraction (LLE)

Solvent extraction is a separation operation which applies a solvent to extract/separate a desired component from solid food. It can be described as a technique, resting on a strong scientific foundation. Solvent extraction is used in numerous chemical industries to produce pure chemical compounds ranging from pharmaceuticals and biomedicals to heavy organics and metals, in analytical chemistry and in environmental waste purification.

An ideal solvent should have the following desirable features: it should have a high capacity for the solute being separated into it, it should be selective, dissolving the specific component to a large extent while having a minimum capacity for the other components, it should be chemically stable (no irreversible reactions with contacting components), it should be regenerable, and it should have low viscosity for easy pumping and transportation (Rydberg et al., 2004). Ethanol is one of the most used solvents for antioxidant extraction, because it is: cheap, reusable, nontoxic and the extracts can be used in the food industry (Chew et al., 2011). Aqueous solutions of ethanol offer many advantages as: hydrophilic and lipophilic active compounds are recovered together in different proportions and synergistic interactions occur inside the medium. Methanol is cheaper than ethanol but due to its toxicity it is not favored in the food industry. On the other hand, carotenoids such as tomato lycopene are more liposoluble and there by polar aprotic or non-polar solvents (i. e., acetone or ethyl acetate, respectively) are preferred (Strati and Oreopoulou, 2011). Fiamegos et al. (2004) reported dichloromethane to be superior to ethyl acetate due to its higher volatility, better immiscibility with aqueous phase and a decrease in interfering peaks.

Extraction generally is conducted at room temperature over a sufficient timeperiod. Although this extraction is convenient, relatively simple and versatile, some of its limitations include incomplete phase separation, lengthy procedures, high solvent usage, and health and environmental concerns. To reduce high solvent consumption in the solvent extraction, alternative techniques have been developed, e.g. ionic liquid extraction, liquid-phase microextraction, supported liquid membrane extraction, and single-drop extraction (Nolvachai and Mariott, 2013).

Supercritical fluids extraction (SFE)

Supercritical Fluid Extraction (SFE) is the process of separating one component (the extractant) from another (the matrix) using supercritical fluids as the extracting solvent. Supercritical fluids exhibit desirable transport properties that enhance their adaptability as solvents for extraction processes.

Supercritical fluids have been investigated since last century, with the strongest commercial interest initially focusing on the use of supercritical toluene in petroleum and shale oil refining during the 1970s. The biggest interest for the last decade has been the applications of supercritical carbon dioxide (Sapkale et al., 2010). The extraction efficiency of polar compounds with CO_2 can be improved by the addition of small quantities of polar organic solvents used as modifiers. CO_2 is non-toxic, nonflammable, and requires a minimum amount of solvent (5–10 ml). Extraction is fast (10–60 min), selective, requires no

additional clean up and can be carried out with small amounts of sample (Anouar et al., 2015). Disadvantages CO_2 cannot extract compounds with high molecular weight (e.g., anthocyanins) because of their lipophilic and nonpolar nature. To eliminate these drawbacks co-solvents are used to enhance the solubility of target compounds and to increase the extraction selectivity. Supercritical CO_2 has been applied for the extraction of antioxidant compounds from rosemary leaves (Caldera et al., 2012), seeds oils of sunflower (Casas et al., 2007), grapes and wine (Bousetta et al., 2012; Wijngaard et al., 2012).

Solid phase extraction (SPE)

In solid phase extraction, a liquid sample is passed through sorbent particles (the solid phase) to which the analytes have a greater affinity than the bulk liquid (Lombardi, 2015). Based on specific interaction between analytes (or impurities) and the stationary phase with a given mobile phase composition, SPE is often the crucial step to concentrate and purify the sample prior to GC analysis.

Solid phase extraction has a few advantages that have led to its rapid development and increasing usefulness as a sample preparation technique. These benefits include faster, less labour-intensive sample manipulation, reduced solvent use, and higher concentration factors (Lombardi, 2015; Buszewski and Szultka, 2012).

Details for flavonoid extraction can be found in many studies (Thurmann and Mills, 1998; Andersen and Jordheim 2006). For example, less polar flavonoids (e.g. isoflavones, flavanones, and flavonols) are normally extracted with chloroform, dichloromethane, diethyl ether, or ethyl-acetate, while polar flavonoid glycosides are extracted with alcohols or alcohol-water mixtures.

Apart from the popular C_{18} material, other types of stationary phases, e.g. poly(styrene/divinylbenzene) for the preparative isolation of flavones, flavanones, and isoflavones were reported. Many types of flavonoids, such as quercetin, catechin, genistein isorhamnetin, kaempferol, and rutin imprinted poly-(vinylpyridine-co-ethyleneglycol dimethacrylate) polymers, were reported for the extraction of these molecules in a wide range of samples, e.g. red wine, Chinese traditional herbs, Hippophae rhamnoides Linn, orange juice, and tea (Weiss et al., 2002; Molinelli et al., 2002; Xie et al., 2001; Theodoridis et al., 2006).

Microwave-assisted extraction (MAE)

The fundamentals of the microwave-assisted extraction (MAE) process are different from those of conventional methods (solid–liquid or simply extraction) because the extraction occurs as the result of changes in the cell structure caused by electromagnetic waves. Although in conventional extraction the heat is transferred from the heating medium to the interior of the sample, in MAE the heat is dissipated volumetrically inside the irradiated medium. (Veggi et al., 2013). Microwaves are non-ionising electromagnetic (EM) waves located between the radio frequency range at the lower frequency and infrared at the higher frequency in the electromagnetic spectrum, within the frequency band of 300 MHz to 300 GHz (Routray and Orsat, 2011; Leonelli et al., 2013). Microwave-assisted extraction (MAE) is a very popular technique in the last decade due to the reduction of extraction time and solvent used.

Advantages of MAE, that can be used with or without the addition of any solvent. Solvent -free microwave extraction (SFME) performed at atmospheric pressure. This is based on a combination of microwave heating and dry distillation which does not need any addition of solvent (Michel et al., 2011).

Subcritical water extraction (SWE) is a highly efficient method for the extraction of environmental samples, and the extraction of natural products from herbs, plants and food stuffs (Plaza et al., 2010). For example, pomegranate seeds, red grape or skin of grape, potato peel, onion skin and citrus peel (He et al., 2012; Ko et al., 2011; Singh and Saldana, 2011; Cheigh et al., 2012).

Ultrasound is a key-technology in achieving the objective of sustainable "green" chemistry and extraction. Ultrasound is well known to have a significant effect on the rate of various processes in the chemical and food industry. Using ultrasound, full extractions can now be completed in minutes with high reproducibility, reducing the consumption of solvent, simplifying manipulation and work-up, giving higher purity of the final product, eliminating post-treatment of waste water (Chemat et al., 2017). Ultrasonic extraction is applied in the case of flavonoids from grapes, orange peel, blueberry wine pomace and spruce wood bark (Khan et al., 2010; He et al., 2016; Ghitescu et al., 2015).

Derivatization for GC-MS

In order to improve volatility, peak shapes, and detection limits of flavonoids for GC analysis, low-volatility compounds such as flavonoids (the boiling points of which exceed 300°C) must be derivatized to their volatile derivatives. Almost all reactions that are used for derivatization are organic syntheses adapted to the micro-scale. This approach makes full use of an advantageous property of GC, namely the need to take only very small amounts of the sample for the analysis, but on the other hand, it makes heavy demands on the quality of the materials used and the precision of the operating procedures (Drozd, 1981).

The compounds in the essential oils are excellent for GC-MS analysis without derivatization. However, due to the polar properties of flavonoids, they should be derivatized to low volatility, thermally stable derivatives. The reagent may be a silylating or methylating agent, for example N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA), N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA), trimethylsilyl-chloride (TMCS), N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide and trimethyl-anilinium hydroxide (TMAH). Flavonoids

prior to GC analysis can be simply performed by vortex mixing a dry prepared sample with a suitable amount of derivatization reagent, followed by mixture incubation, e.g. at 60-70°C for 20-120 min (Chu et al., 2001), 80°C for 45 min (Proestos and Kromaitis, 2013), or 37°C for 30 min (Mari et al., 2013).

CONCLUSIONS

This study analysis of flavonoids, including quercetin, myricetin and kaempferol, using GC and related techniques. The techniques required for GC-MS analysis include extraction with solvents, supercritical fluid-, solid phase- and microwave-assisted extraction. The flavonoids are low-volatility (the boiling points of which exceed 300°C), therefore GC-MS analysis of these compounds are only possible after derivatization.

New methodologies of extraction, purification, identification and quantification of antioxidants using eco friendly techniques need to be developed to improve the extraction yields.

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Characterisation of *Staphylococcus aureus* strains isolated from raw milk from vending machines in Hajdú-Bihar County, Hungary

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SUMMARY

Using vending machines makes possible for dairy farms to deliver the produced raw milk directly to consumers. Raw milk from vending machines should be boiled before consumption, since raw milk may contain various microorganisms, including pathogens such as Staphylococcus aureus. *It is one of the most important microorganisms in dairy farms that can cause mastitis in cows. So it has got great economic importance in the dairy industry and its public health significance is considerable too.*

The aim of this study was to investigate the occurrence of S. aureus *in raw milk from 13 milk vending machines in Hajdú-Bihar County, Hungary, and after identifying the* S. aureus *strains isolated from the samples by the coagulase test, the characteristics (tellurite reducing ability, lecithinase activity, haemolysin production ability) and the antibiotic resistance of isolates were determined.*

Based on the results, S. aureus *occurred in raw milk from all vending machines, and the mean* S. aureus *colony forming unit (CFU) ranged between 0.8 and 2.9 log_{10} CFU/ml. There was no significant difference between the* S. aureus *CFU of the samples (P>0.05). In the samples of 3 out of 13 vending machines, the* S. aureus *count exceeded the refusal limit (M=2.7 log_{10} CFU/ml) set in the regulation of the Hungarian Ministry of Health 4/1998 (XI.11). A total of 26 S. aureus strains were chosen (1-3 from each vending machine) from raw milk samples for further investigations. All these strains produced coagulase enzyme, had tellurite reducing ability and lecithinase activity. Among the 26 strains, 9 strains were nonhaemolytic, 12 strains showed weak haemolysis, 3 strains showed α and β haemolysis, and 2 strains showed β haemolysis on blood agar. In antibiotic resistance testing, it was found that all strains were sensitive to cefoxitin, gentamicin, trimethoprim/sulphamethoxazole, 34.6% of the strains were resistant to penicillin, 23.1% were resistant to tetracycline, and 3.8%-3.8% were resistant to chloramphenicol, clindamycin and erythromycin.*

The results of our studies confirmed the requirement to boil raw milk from vending machines before consumption. Since S. aureus *was present in all samples, the consumption of raw milk from vending machines without heat treatment could be unsafe from a public health point of view. Our results also corroborate previous results, indicating the continuous increasing of the proportion of resistant strains in the world and in Hungary.*

Keywords: *microbiology, Staphylococcus aureus, raw milk, vending machine*

INTRODUCTION

Natural foods are currently the focus of attention. The so-called "consuming natural" trend becomes popular, and therefore the demand for raw milk consumption is also increasing as raw milk consumption is attributed to health benefits that can be lost by the heat treatment of milk (Claeys et al., 2013).

Using raw milk vending machines makes possible for customers to buy raw milk delivered from dairy farms directly. Raw milk from vending machines should be boiled before consumption, since raw milk may contain various microorganisms, including pathogens such as *Staphylococcus aureus* (Galičič et al., 2015).

The *S. aureus* is a ubiquitous microbe, and may occur in humans, animals and in the environment (e.g. soil, water, plants). It is a facultative pathogen, that mostly harmless in the body, but in certain circumstances it may become pathogenic. It can cause a variety of diseases, e.g. mastitis in milk producing animals, toxic shock syndrome or even (especially the enterotoxin-producing strains) food poisoning (Deák, 2006; Verraes et al., 2015).

S. aureus is one of the most important microorganisms in dairy farms that can cause mastitis in cows (Kovács et al., 2013). In the case of dairy cows, *S. aureus* causes 5-30% of clinical mastitis and 5-10% of subclinical mastitis (Peton and Loir, 2014). According to Visciano et al. (2014), it can be an effective method for controlling mastitis by improving milking hygiene and/or using antimicrobial agents. *Staphylococci*, however, show resistance to some antibiotics, which have two disadvantages. One is that healing rates may be reduced after such treatments and the other is that resistant bacteria can reach consumers through the food chain (Visciano et al., 2014). It is a problem that more and more antibiotic-resistant bacteria occur (Barcs, 2009). Foods (e.g. milk) that contain antibiotic-resistant microorganisms are ideal

for the delivery of antibiotic resistant strains to consumers (Angulo et al., 2004). Antibiotic resistance is an important public health problem worldwide (Carmeli et al., 1999; Cosgrove et al., 2006).

S. aureus in the milk may be originated from the udder itself, from water, from the hands of the milkers or from the devices used during milking (e.g. machines, wiping cloths, etc.), and the milking environment plays a major role in the spread of the bacterium (Markus, 2001).

The aim of this study was to investigate the occurrence of *S. aureus* in raw milk from 13 milk vending machines in Hajdú-Bihar County, Hungary. First, the isolated *S. aureus* strains were identified by the coagulase test, than they were characterized with testing tellurite reducing ability, lecithinase activity, and haemolysin production ability. Finally the antibiotic resistance of isolates were determined.

MATERIALS AND METHODS

During the research, raw milk samples were collected aseptically in sterile tubes from 13 vending machines (VM) in Hajdú-Bihar County (Hungary), in February 2016. The collected samples were delivered in a cooler bag equipped with ice batteries within two hours into the Microbiological Laboratory of the Institute of Food Science at the University of Debrecen, where the samples were processed as soon as possible.

Samples were prepared in accordance with the MSZ EN ISO 6887-1:2000 standard. The determination of *S. aureus* was performed according to MSZ EN ISO 6888-1:2008 standard, and Baird-Parker Agar (Biolab Ltd., Hungary) supplemented with sterile Egg Yolk Tellurite Emulsion (LAB-KA Ltd., Hungary) was used. Samples were incubated for 48 hours at 37 °C and colony forming unit (CFU) was determined. Coagulase test was performed to confirm suspect *S. aureus* colonies. The chosen strains (1-3 from each vending machine) were stored at -80 °C until further studies, which were performed in January and February 2018.

Haemolysis tests were performed on Columbia Blood Agar (Biolab Ltd., Hungary). The inoculated media were incubated at 37 °C for 24 hours.

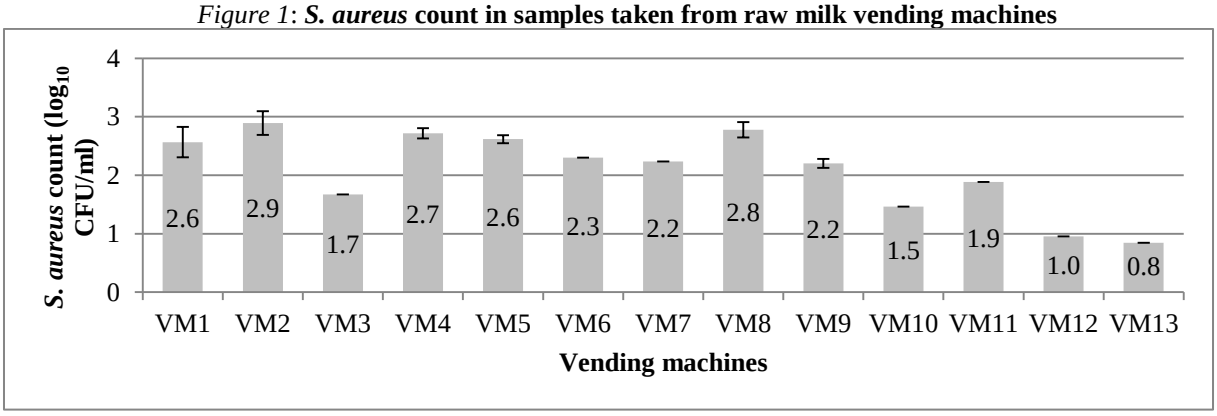
The antibiotic resistance test of the isolated strains was performed by agar diffusion method according to the Clinical and Laboratory Standards Institute guidelines (2017). The bacterial suspension was adjusted to 0.5 McFarland and was spread on Mueller-Hinton Agar (Biolab Ltd., Hungary) with sterile cotton swab, and then the antibiotic-soaked paper disks were placed on the surface of the inoculated media. Plates were incubated at 35 °C for 18 hours. The following antibiotic disks were used for the study: cefoxitin (30 µg/disk), chloramphenicol (30 µg/disk), clindamycin (2 µg/disk), erythromycin (15 µg/disk), gentamicin (10 µg/disk), penicillin G (10U), tetracycline (30 µg/disk), trimethoprim/sulphamethoxazole (1.25+23.75 µg/disk) (Biolab Ltd., Hungary).

Calculation of averages, standard deviations (SD), logarithmic transformation of the amount of microorganisms and variance analysis were performed using the SPSS v.22.0 (SPSS 2013) software.

RESULTS AND DISCUSSION

Based on the results, *S. aureus* occurred in raw milk samples from all 13 vending machines; the mean values ranged from 0.8 to 2.9 $\log_{10}$ CFU/ml (*Figure 1*). The difference between the *S. aureus* counts of raw milk samples taken from different vending machines was not significant (P>0.05). In 8 (VM1, VM2, VM4, VM5, VM6, VM7, VM8, VM9) out of 13 samples, the *S. aureus* count exceeded the limit (m=2.0 $\log_{10}$ CFU/ml) set in the regulation of the Hungarian Ministry of Health 4/1998 (XI.11). The *S. aureus* count exceeded the refusal limit (M=2.7 $\log_{10}$ CFU/ml) in 3 vending machines samples (VM2, VM4, VM8). The mean *S. aureus* CFU in the raw milk from these vending machines were 2.9 $\log_{10}$ CFU/ml; 2.7 $\log_{10}$ CFU/ml and 2.8 $\log_{10}$ CFU/ml, respectively.

Figure 1: **S. aureus count in samples taken from raw milk vending machines**



Altogether 26 *S. aureus* strains were chosen (1 to 3 from each vending machine) from raw milk samples for further investigations. 4 strains were grey and 22 of them were black on Baird-Parker Agar (BPA) supplemented with Egg Yolk Tellurite Emulsion, indicating that tellurite was reduced to tellurium by the bacteria (*Table 1*). All strains had lecithinase activity, which was derived from the observation of

zones around the colonies. Regarding the haemolysin production ability of strains, 9 of the 26 studied strains were nonhaemolytic, and 12 strains expressed weak haemolysis, while 3 strains showed both α and β haemolysis, and 2 strains showed β haemolysis on blood agar. *Table 1* also shows that *S. aureus* strains taken from the same vending machines had different characteristics, except the samples from VM2, VM8 and VM1.

Table 1

Characteristics of *S. aureus* strains isolated from raw milk from vending machines

#	Strain code	Characterisation				Antibiotic resistance[a]
		Strain color on BPA (tellurite reduction)	Lecithinase activity test	Coagulase test	Haemolysis	
1	**VM1/1**	black	positive	positive	-	R (P)
2	**VM1/2**	black	positive	positive	β	S
3	**VM2/1**	black	positive	positive	weak	S
4	**VM2/2**	black	positive	positive	weak	S
5	**VM3/1**	black	positive	positive	weak	S
6	**VM3/2**	black	positive	positive	-	S
7	**VM4/1**	black	positive	positive	weak	R (P; TE)
8	**VM4/2**	black	positive	positive	weak	S
9	**VM5/1**	black	positive	positive	weak	S
10	**VM5/2**	black	positive	positive	weak	R (P; TE)
11	**VM6/1**	black	positive	positive	α+β	S
12	**VM6/2**	black	positive	positive	weak	R (P; TE)
13	**VM7/1**	black	positive	positive	weak	S
14	**VM7/2**	black	positive	positive	weak	S
15	**VM7/3**	black	positive	positive	weak	R (P)
16	**VM8/1**	black	positive	positive	-	R (P; TE)
17	**VM8/2**	black	positive	positive	-	R (P; TE)
18	**VM9/1**	grey	positive	positive	α+β	R (P)
19	**VM9/2**	grey	positive	positive	β	R (C; DA)
20	**VM10/1**	grey	positive	positive	α+β	S
21	**VM10/2**	grey	positive	positive	-	S
22	**VM11/1**	black	positive	positive	-	R (E)
23	**VM11/2**	black	positive	positive	-	R (P; TE)
24	**VM12/1**	black	positive	positive	-	S
25	**VM12/2**	black	positive	positive	-	S
26	**VM13/1**	black	positive	positive	weak	S

[a] R: resistant; S: sensitive; P: penicillin; TE: tetracycline; C: chloramphenicol; DA: clindamycin; E: erythromycin

In antibiotic resistance test, it was found, that all strains were sensitive to cefoxitin, gentamicin and trimethoprim/sulphamethoxazole. Similar results have been previously reported by several authors: strains tested by Abo-Shama (2014) were sensitive to cefoxitin, Visciano et al. (2014) reported gentamicin sensitive strains, while Peles et al. (2007a, b) trimethoprim/sulphamethoxazole sensitive ones. In this study, 9 strains (34.6%) were resistant to penicillin, which is similar to the results Peles et al. (2007a, b) detected (30.5%) in their study. In case of tetracycline, 6 of our strains (23.1%) were resistant, which is higher rate, than in the study of Visciano et al. (2014) (12.0%) and Peles et al. (2007a, b) (0.0%). Furthermore, based on our results, 1-1 strains (3.8-3.8%) were resistant to chloramphenicol, clindamycin and erythromycin (*Table 2*). Jamali et al. (2015) described higher resistance to chloramphenicol (4.9%) and clindamycin (13.6%), furthermore the strains tested by Abo-Shama (2014) showed higher resistance

to erythromycin (20.5%). 15 strains (57.7%) proved to be sensitive to all antibiotics, 4 strains (15.4%) were resistant to 1 antibiotic, and 7 strains (26.9%) were resistant to 2 antibiotics (*Table 1*).

Table 2

Antibiotic resistance of *S. aureus* strains (n=26) isolated from raw milk from vending machines

Antimicrobials (concentration)	Antimicrobial susceptibility					
	Resistant		Intermediate		Sensitive	
	n	%	n	%	n	%
Cefoxitin (30 µg/disk)	0	0.0	-	-	26	100.0
Chloramphenicol (30 µg/disk)	1	3.8	0	0	25	96.2
Clindamycin (2 µg/disk)	1	3.8	0	0	25	96.2
Erythromycin (15 µg/disk)	1	3.8	0	0	25	96.2
Gentamicin (10 µg/disk)	0	0.0	0	0	26	100.0
Penicillin G 10U	9	34.6	-	-	17	65.4
Tetracycline (30 µg/disk)	6	23.1	0	0	20	76.9
Trimethoprim/Sulphamethoxazole (1.25+23.75 µg/disk)	0	0.0	0	0	26	100.0

In the case of the penicillin resistance tests, that 54% of the strains were to found produce β-lactamase enzyme since the edge of inhibition zones around the disks were sharp („cliff"). Based on the results of cefoxitin resistance with agar diffusion method according to the CLSI, it can be said that in this study, all strains were sensitive to methicillin. Furthermore, based on the CLSI requirements, the cefoxitin resistance results can also provide an image of the *mecA*-mediated oxacillin resistance of the strains. Our studied *S. aureus* strains isolated from raw milk from vending machines were sensitive to *mecA*-mediated oxacillin.

CONCLUSIONS

The results of our studies confirm the requirement to boil raw milk from vending machines before consumption. Since *S. aureus* was present in all samples, the consumption of raw milk from vending machines without heat treatment could be unsafe from a public health point of view. The same conclusion was drawn by Medveďová et al. (2013) in their study. Our results also corroborate previous results, indicating the continuous increasing of the proportion of resistant strains in the world and in Hungary.

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Microbiological quality of raw milk and udder surface samples from Dorper, Merino and Cigaja sheep breeds

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SUMMARY

Sheep milk has been used by man since the beginning of sheep domestication due to its high nutritive value, and its health potential. However, the consumption of raw milk is an actual microbiological risk to consumers due to the possible presence of undesirable food spoilage or pathogenic bacteria. The aim of this study was to examine the microbiological status of raw milk and udder surface samples from Dorper, Merino and Cigaja sheep breeds. Total Plate Counts (TPC), Enterobacteriaceae count (EBC), Staphylococcus aureus count (SAC) and coagulase-negative staphylococci count (CNSC) in ewe raw milk and environmental samples (udder surface) were examined.

During the experiment, 24 milk samples and 24 environmental samples (udder surface) were collected and studied from a sheep farm in Hajdú-Bihar County from Dorper, Merino and Cigaja sheep at different times. The sampling, the preparation of samples and decimal dilutions were based on international standards and methods.

Our results revealed that, the mean of TPC were 3.25, 3.60 and 3.81 lg cfu/ml, and CNSC were 3.08, 3.62 and 3.08 lg cfu/ml in milk samples from Dorper, Merino and Cigaja, respectively. EBC and SAC were less than 10 cfu/ml. Statistically, there was no significant difference between milk samples taken from different ewe breeds. The mean TPC of udder surface samples were 3.46, 3.75, 4.04 lg cfu/cm^2; CNSC were 3.76, 3.95 and 3.91 lg cfu/cm^2 and EBC were 1.71, 1.05 and 2.14 lg cfu/cm^2 from Dorper, Merino and Cigaja breeds, respectively. There were significant difference between udder surface samples for TPC, and no significant difference for CNSC and EBC.

It was concluded that in case of all microbiological parameters examined in this study, there was no statistically significant difference between the ewe breeds. Hence, in the case of ewes, the breed had no significant effect on the microbiological status of raw milk. However, in most cases the higher microbial contamination of the udder surface resulted in higher milk contamination. Therefore, it can be recommended to implement hygiene practices in the sheep farm to minimize microbiological contamination of the raw milk.

Keywords: *Sheep breeds, raw milk, hygiene, microbiology*

INTRODUCTION

Dairy sheep farming is highly significant for the national economy in many countries in the Mediterranean and Middle East region (Park *et al.*, 2007). In the world, the highest amount of sheep milk is produced in China (12.2%), while in Europe is Greece is the most producer (8.7%) followed by Romania (7.2%), and Italy (6.1%) (Barłowska *et al.*, 2011). In Europe, ewe milk production was approximately 10.1 million tons in 2013 and the demand for sheep milk and its products is increasing (FAO, 2016). In Hungary, the production of sheep milk was 1.744 tons in 2015 (http://www.fao.org/faostat/en/#data).

Nowadays, the interest for sheep milk is increasing in many countries and its products have gained market size due to the products quality, high yield, higher concentration of proteins, fats, vitamins, and minerals than milks from cow and goat (Park *et al.*, 2007; Milani and Wendorff, 2011). However, its high nutritive value facilitates the growth of microbial spoilage (Quigley *et al.*, 2013). Contamination during and after milking, method of milking, health condition of ewes, type of feeds and feeding, season and the hygienical condition of farms can affect microbiological quality of sheep milk (Alexopoulos *et al.*, 2011).

Unlike cow's milk, which has rigorous hygiene and quality regulations, microbiological standards for the production and distribution of ewe milk are less strict, although there is increasing demands by consumers. Ewe's milk can be, similar to cow's milk, source of undesirable bacteria including *Enterobacteriaceae*, *Staphylococcus aureus* and coagulase-negative staphylococci (Muehlherr *et al.*, 2003). A better knowledge of the hygienic quality of raw ewe's milk will contribute to further research aimed at the improvement of its quality. Therefore, the objective of this study was to study the microbiological quality of raw milk and udder surface samples from Merino, Cigaja and Dorper sheep breeds in Hajdú-Bihar County sheep farm near to Debrecen (Hungary).

MATERIALS AND METHODS

Sampling

Twenty four milk samples and twenty four udder surface samples were collected and studied from a sheep farm in Hajdú-Bihar County from two Dorper, two Merino and four Cigaja ewes, three times between March and April 2018. At the beginning of sampling, udder surface samples were taken from a 20 cm^2 area of the udder. Before manual milking, ewe's teats were carefully cleaned with cotton drenched with 70% of ethanol alcohol. After the first three to four streams of milk were discarded, the milk samples were collected in sterile tubes and placed in a plastic bag with ice packs at less than 4 ºC and transported to the microbiological laboratory. The samples were tested immediately upon arrival in the microbiology laboratories of the Institute of Food Science, University of Debrecen.

Microbiological analysis

Total plate count (TPC) was performed according to ISO 4833-1:2013. It is a good indicator of contamination which was examined on plate count agar (PCA) medium (Biolab Ltd., Hungary) and was inoculated in duplicate with 1 ml of the diluted sample. All plates were incubated at 30 ºC for 72 hrs. *Enterobacteriaceae* count (EBC) was carried out according to ISO 21528-2:2004 on Violet Red Bile Glucose (VRBG) agar (Biolab Ltd., Hungary) medium and was inoculated in duplicate with 1ml of diluted sample, and colony forming unit (cfu) was calculated from the results. All plates were incubated at 37 ºC for 24 hrs. *Staphylococcus aureus* count (SAC) and coagulase-negative staphylococci count (CNSC) were performed according to MSZ EN ISO 6888-1:2008 standard. From the dilutions 0.1 ml was spread in duplicate on the surface of Baird Parker (BP) agar plates (Biolab Ltd., Hungary) supplemented with sterile egg yolk tellurite emulsion (LAB-KA Ltd., Hungary) and incubated at 37 ºC for 48 hrs. Latex agglutination test was done to identify the *Staphylococcus aureus*. The samples from udder surface were examined for the same microbiological parameters accordingly and the results were divided by 20.

Statistical analysis

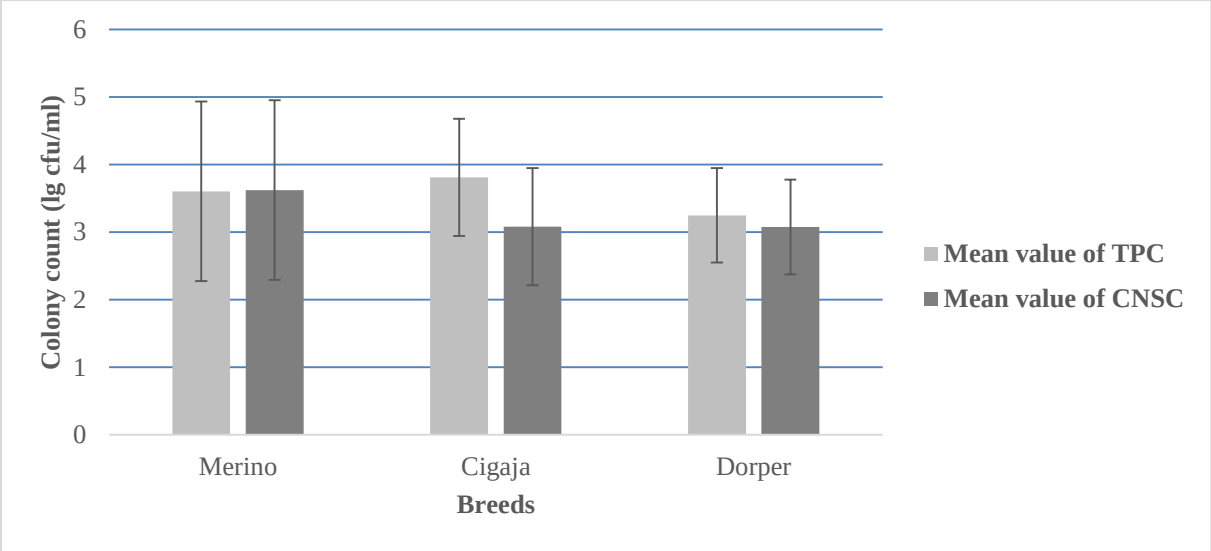
Microbial counts were log-transformed before analysis. A statistical analysis was carried out using programs GraphPad Prism 3.02 and MS excel.

RESULTS AND DISCUSSION

The total plate count ranged from 1.8 to 5.3 lg cfu/ml, 1.5 lg to 6.0 lg cfu/ml and 2.0 to 4.4 lg cfu/ml in ewe milk from Merino, Cigaja and Dorper, respectively. The TPC is considered as a general indicator of the overall product quality and it reveals the conditions of production, particularly hygienic practices during milking (Bouazza *et al.*, 2015). The mean value of TPC were 3.25, 3.60 and 3.81 lg cfu/ml, and CNSC were 3.08, 3.62 and 3.08 lg cfu/ml in milk samples from Dorper, Merino and Cigaja, respectively (*Figure 1*). Alexopoulos *et al.* (2011) reported the average TPC of 5.48 lg CFU/ml in Greece, which was higher than our finding. Talevski *et al.* (2009) from Macedonia also had higher values of TPC using the same milking system. There was no significant difference ($P>0.05$) between the three different ewe breeds for total plate count as shown in *Figure 1*. This is in lines with the finding of Alexopoulos *et al.* (2011), which conformed that there was no significant difference between ewe breeds.

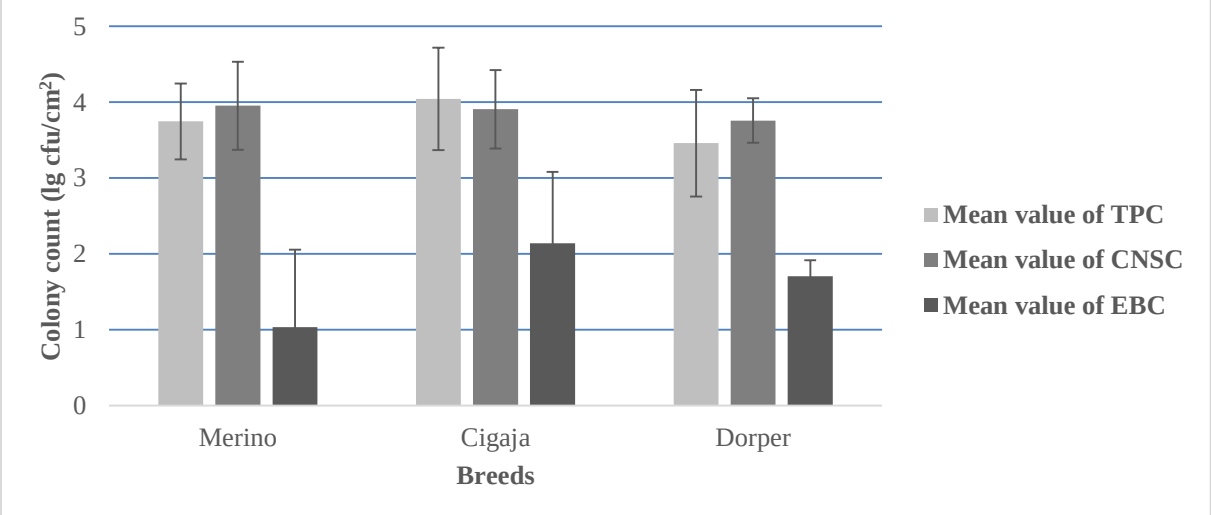
In our finding, both *Enterobacteriaceae* count (EBC) and *Staphylococcus aureus* count (SAC) were less than 10 cfu/ml in ewe raw milk. Latex agglutination test confirmed that there was no *S. aureus* in the samples. This was the evidence that the presence of these bacteria's in raw milk is influenced by environmental factors, such as dirty bed and poor quality feed, water, which are less likely to occur on this particular farm. However, Fatima *et al.* (2013) from Algeria reported 1.1 x 10^3 cfu/ml of EBC and 1.7 x 10^3 cfu/ml of SAC in ewe raw milk. The mean values of coagulase-negative staphylococci count (CNSC) were 3.08, 3.62 and 3.08 lg cfu/ml in raw milk from Dorper, Merino and Cigaja, respectively. The statistical analysis revealed that there was no significant differences between breeds for CNSC as indicated in *Figure 1*. Similarly, Alexopoulos *et al.* (2011) reported that there was no significant difference between ewe breeds for CNSC.

Figure 1: **Mean count of total plate count and coagulase-negative staphylococci count in raw milk from different ewe breeds**



The presence of microorganisms in ewe milk could be the result of contamination from various sources, mainly the farm environment and body of the animal to the milk. Specifically, udder surface of ewes can contribute microorganisms that are naturally associated with the skin of the animal as well as microorganisms that are derived from the environment in which the ewe is housed and milked (Quigley *et al.*, 2013). Hence, samples from udder surfaces of ewes were taken and examined for TPC, EBC, SAC and CNSC. The environmental sample from udder surface was due to an udder surface of ewes inevitably become soiled while they are lying in stalls or when allowed in muddy barnyards. The mean TPC of udder surface samples were 3.46, 3.75, 4.04 lg cfu/cm^2; CNSC were 3.76, 3.95 and 3.91 lg cfu/cm^2 and EBC were 1.71, 1.05 and 2.14 lg cfu/cm^2 from Dorper, Merino and Cigaja breeds, respectively. The value of TPC was ranged from 3.0 to 4.6, 2.9 to 5.1 and 2.5 to 4.8 lg/cm^2 in udder surface sample from Merino, Cigaja and Dorper, respectively. There was significant difference between udder surface samples taken from different breeds for TPC (*Figure 2*). The TPC was highest in udder surface samples from Cigaja breed, this was may be due to long hairs on udder surface which stick with dusts and feaces in case of Cigaja breeds. Even though there was no significant difference in case of CNSC and EBC (*Figure 2*), EBC was highest from Cigaja and CNCS was highest in udder samples from Merino. According to El Zubeir and Ahmed (2007) report in Sudan, the presence of these bacteria especially *Enterobacteriaceae* indicates the potential fecal contamination. At present, there are no legislative limits for the *Enterobacteriaceae* and coagulase-negative staphylococci, both groups of bacteria are indicators of hygienic conditions in primary ewe milk production (Bogdanovičova *et al.*, 2016).

Figure 2: **Mean count of total plate counts, *Enterobacteriaceae* count and coagulase-negative staphylococci count on udder surface samples from different ewe breeds**



CONCLUSIONS

This study revealed that in case of total plate count, *Enterobacteriaceae* count, *Staphylococcus aureus* count and coagulase-negative staphylococci count of raw milk there was no significant difference between the different ewe breeds. However, in most cases the higher microbial contamination of the udder surface resulted in higher milk contamination. Hence, cleanness or dirtiness of the udder surface affects the microbiological quality of milk in the farm. Therefore, it is necessary to minimize microbial contamination which can be achieved through healthy ewe and milkers, good hygienic practices and cleaning of teat and udder surface before milking.

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