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BARLEY GRAIN ENRICHEMENT WITH ESSENTIAL ELEMENTS BY AGRONOMIC BIOFORTIFICATION

Vesna D. Dragičević[1], Bogdan R. Nikolić[2], Milica M. Radosavljević[1], Nenad A. Đurić[3], Dejan B. Dodig[1], Milovan M. Stoiljković[4] and Natalija B. Kravić[1]*

[1] Maize Research Institute "Zemun Polje", Slobodana Bajića 1, 11185 Zemun Polje, Serbia
[2] Institute for Plant Protection and Environment, Teodora Drajzera 9, 11000 Belgrade, Serbia
[3] John Naisbitt University, Faculty for Biofarming, Maršala Tita 39, 24000 Bačka Topola, Serbia
[4] Vinča Institute of Nuclear Sciences, PO Box 522, 11001 Belgrade, Serbia

Barley grain is rich in mineral nutrients, but their bioavailability to humans depends on antinutrients that restrain bioavailability and promoters that promote bioavailability. The aim of this study was to examine composition of barley grain, including phytate and phenolics as antinutrients, carotenoids and glutathione as promoters and mineral elements, such as Ca, Mg, Fe, Si, Zn and Mn influenced by various non-standard foliar fertilizers (Zircon, Chitosan, Siliplant, Propikonazole), including some hormonal growth-stimulators (Epin Extra, Benzyladenine), as potential biofortification measure. Chitosan increased glutathione concentration in grain. Unfavorable meteorological conditions were partly mitigated by application of Benzyladenine and Siliplant, reflected through increased potential bioavailability of P, Mg, Ca and Fe.

KEY WORDS: barley grain composition, antioxidants, mineral elements, biofortification

INTRODUCTION

Wholegrain products are necessary part of healthy diets as sources of dietary fiber and mineral nutrients. Among cereals, barley grain is the main source of P, Ca, K, Mg, Na, Cu and Zn (1), as well as of Si, which showed the positive effect on bones (2). Barley health benefits are provided by a β-glucan fiber fraction, which is associated with lowering of blood cholesterol levels, glycemic index and weight loss (3).

High concentration of mineral elements in cereal grains does not mean that they are available for humans. Antinutrients, as essential part of grain, like phytate, phenolics, etc., limit the absorption of mineral elements. Grains also contain promoters, such as β-carotene, S-containing amino acids, etc., which enhance mineral nutrients bioavailability or decrease the activity of inhibitors (4, 5). Enrichment of grains with mineral elements and other important nutrients – biofortification is a very complex process. One of the strategies is the application of foliar fertilizers, which have also a positive effect on plant metabolism and grain yield (6).

* Corresponding author: Vesna D. Dragičević, Maize Research Institute "Zemun Polje", Slobodana Bajića 1, 11185 Belgrade-Zemun, Serbia, e-mail: vdragicevic@mrizp.rs

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There are many different substances which foliarly applied influence accumulation of mineral nutrients in grains. For instance, Si can improve crop production by increasing water uptake, maintaining nutrient balance, promoting photosynthetic rate, increasing the activities of antioxidants (7, 8). Nevertheless, hormones could also increase stress tolerance and uptake of mineral nutrients, like brassinosteroids (9). Propiconazole, as a brassinosteroid inhibitor (10) showed positive impact on barley and wheat grain yield and composition, decreasing phenolics and increasing carotenoids (11). Cytokinine, 6-benzyladenine expressed positive effect on photosynthesis and antioxidants in salt stressed plants (12). Chitosan has ability to mitigate stress and to chelate minerals and other nutrients, and it is widely used in phytoremediation for heavy metal removal (13).

The aim of this study was to test various foliar fertilizers, including some hormonal growth-stimulators, as potential biofortification measure on chemical composition of barley grain, including phytate and phenolics as antinutrients, β-carotene and glutathione as promoters and mineral elements, such as Ca, Mg, Fe, Zn and Mn.

EXPERIMENTAL

Field trial

The grain of hull-less barley (*Hordeum vulgare* L. *var. nudum*; cv. "Apolon") was produced in 2013 and 2014 at Zemun Polje (44°52'N, 20°20'E; 86 ± 3 m altitude). The experiment was set up in four replications. Foliar fertilizers were applied in recommended concentrations. Treatments included 0.335 ml L^{-1} of Epin Extra (24-epibrassinolide phytohormone with 0.025 g a.i. L^{-1}); 0.3 ml L^{-1} of Zircon (*Echinacea purpurea* extract, with 0.1 g L^{-1} of phenolic acids: 3,4-dihydroxycinnamic (caffeic) acid, chlorogenic and cichoric acid); 1 ml L^{-1} of Chitosan (0.5% of polysaccharide chitosan, 3-4% of organic C, 2-5% of organic N, 5% of amino acids and 10% of humic acids); 2 ml of 6-benzyladenine (technical grade 90%, syn. 6-BA or BAP); 3 ml of Siliplant (16.9±2.0 g L^{-1} of K, 0.13±0.05 g L^{-1} of Mg, 72.0±4.0 g L^{-1} of Si, 0.45±0.1 g L^{-1} of Fe, 0.32±0.09 g L^{-1} of Mn, 0.12±0.04 g L^{-1} of B, 0.08±0.03 g L^{-1} of Zn, 0.07±0.03 g L^{-1} of Cu and 0.02±0.01 g L^{-1} of Co); 0.2 ml of Propikonazole (PZR, technical grade 95%); control – without application of foliar fertilizers. First spraying for all treatments, except for BAP and PZR was performed 45 days after emergence, while the second one was 60 days after emergence (when BAP and PZR were applied).

Chemical analysis of barley grain

After harvesting the chemical composition of grain was determined. Phytic (P_{phy}) and inorganic (P_i) phosphorus were determined by the method of Dragičević et al. (14), and total glutathione (GSH) by the method of Sari Gorla et al. (15), after extraction with 5% trichloroacetic acid. The extract was centrifuged at 12,000 rpm for 15 min (Dynamica – Model Velocity 18R Versatile Centrifuge, Rotor TA15-24-2) at 4°C. P_{phy} was determined on a Biochrom Libra S 22 spectrophotometer, based on the pink color of the Wade reagent, which is formed upon the reaction of ferric ion and sulfosalicilic acid, and has an

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absorbance maximum at λ=500 nm. P_i was determined after adding of ammonium heptamolybdate + ammonium metavanadate solution to the extract and measuring the absorbance at λ=400 nm. GSH was determined by adding 0.2 M potassium phosphate buffer (pH = 8.0) and 10 mM DTNB (5.5'-dithio(2-nitrobenzoic acid)) to the extract and measuring the absorbance at 415 nm. Water soluble phenolics were determined by the method of Simić et al. (16), after extraction with double distilled water and centrifugation at 12000 rpm for 15 min, by adding 0.05 M $FeCl_3$ in 0.1 M HCl and 0.008 M $K_3Fe(CN)_6$ to sample solution; the absorbance was measured at λ=722 nm. Phenolic content was expressed in μg of ferulic acid (FAE) equivalent. Yellow pigment (β-carotene) concentration was determined according to the AACC procedure (17), after extraction with 1-butanol and centrifugation at 10,000 rpm for 5 min; the absorbance was measured at λ=436 nm.

After wet digestion with HNO_3 + $HClO_4$, the Ca, Mg, Fe, Mn, Zn and Si contents were determined by Inductively Coupled Plasma - Optical Emission Spectrometry (Spectro Analytical Instruments, Germany).

Statistical analysis

Chemical composition of the barley grain is present as mean ± standard deviation (SD). The ratios between P_{phy} and P_i, phytate, β-carotene, Ca, Mg, Fe, Zn and Mn could be considered as parameters of potential bioavailability of examined mineral elements and the differences between treatments means were tested by ANOVA, with Fisher's least significant difference (LSD) test at the 0.05 probability level.

RESULTS AND DISCUSSION

Meteorological conditions

Meteorological conditions had opposite trend in two experimental years. The difference in temperature between the seasons was negligible (Table 1), with the amount of precipitation being almost 2.5 times higher in 2014 than in 2013, that is near a 20-year average. The highest value of precipitation was obtained in May for both 2013 and 2014 (102.1 and 286.7 mm, respectively).

Table 1. Average monthly air temperatures and precipitation sums from March to July for 2013, 2014 and period 1991-2010, at Zemun Polje

Month		March	April	May	June	July	Average/Σ
T average (°C)	2013	7.3	14.8	19.5	21.3	24.1	17.4
	2014	11.3	14.1	17.0	21.5	23.3	17.5
	1991-2010	7.9	12.4	17.8	21.0	22.8	16.4
Σ precipitation (mm)	2013	95.5	21.7	102.1	49.8	2.7	271.8
	2014	48.6	85.4	286.7	59.5	250.0	730.2
	1991-2010	44.9	48.5	52.5	83.7	63.8	293.4

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Antioxidants in barley grain. Negative conditions present during 2014 affected concentrations of main antioxidants: yellow pigments, GSH and phenolics (Figure 1). Several times higher concentrations of yellow pigment and almost twice higher concentration of GSH and phenolics in 2013, compared to 2014 were obtained. The highest values of yellow pigment and GSH were in Chitosan treatment in 2014 (0.48 μg g^{-1} and 514.8 nmol g^{-1}, respectively), and in 2013 insignificantly higher in control (4.39 μg g^{-1} and 930.5 nmol g^{-1}, respectively). Phenolics had the highest value in Chitosan and in PZR treatment, in 2013 (2275.1 μg g^{-1} and 2325.0 μg g^{-1}, respectively) and in Siliplant and PZR treatments in 2014 (1758.9 μg g^{-1} and 1799.9 μg g^{-1}, respectively).

Concentrations of P_{phy} and P_i also showed differences among experimental seasons, with slightly higher P_{phy} and lower P_i values obtained in 2013, compared to 2014 (Figure 2). Slight variations among treatments indicated that higher P_{phy} values were obtained in control of both years (4.56 and 4.22 mg g^{-1} in 2013 and 2014), while the lowest values were in Chitosan treatment (4.17 and 3.93 mg g^{-1}, respectively). Epin Extra increased P_i values in 2013 and 2014 (0.86 and 1.05 mg g^{-1}, respectively). Brassinosteroids expressed the increased activity of enzymatic and nonenzymatic antioxidants, subsiding the harmful effect of salinity and heavy metal stress in bean plants (18).

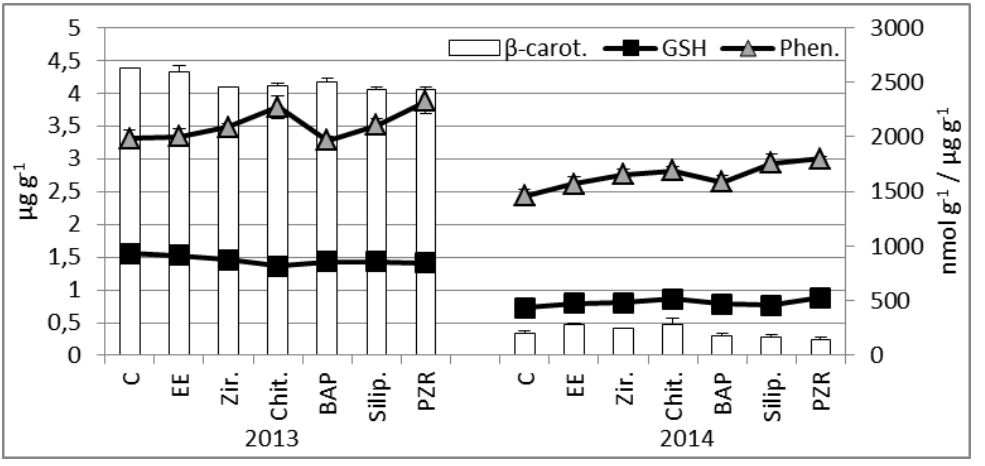

Figure 1. The effect of different foliar fertilizers on concentration of yellow pigment (β carot.), glutathione (GSH) and phenolics in barley grain (C – control, EE – Epin Extra, Zir. – Zircon, Chit. – Chitosan, BAP – Benzyladenine, Silip. – Siliplant, PZR – Propikonazole); Mean ± SD (standard deviation)

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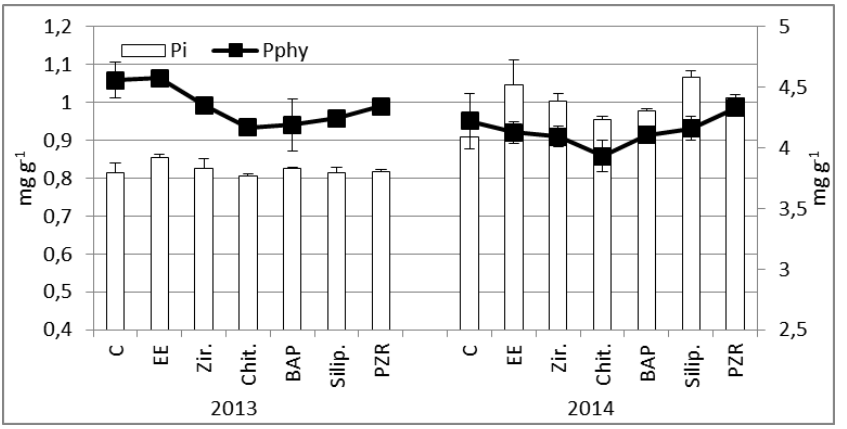

Figure 2. The effect of different foliar fertilizers on concentration of phytic (P_{phy}) and inorganic (P_i) phosphorus in barley grain (C – control, EE – Epin Extra, Zir. – Zircon, Chit. – Chitosan, BAP –Benzyladenine, Silip. – Siliplant, PZR – Propikonazole); Mean ± SD (standard deviation)

Mineral elements in barley grain. Meteorological conditions have also influenced accumulation of mineral elements in grain. This was especially true for Si, whose concentration was significantly (five times) lower in 2014 (Figure 3), and was opposite to the results of Ma et al. (2), who did not find significant impact of the year on Si variation in barley. Higher variations were observed among treatments in Ca concentration in 2014 compared to 2013.

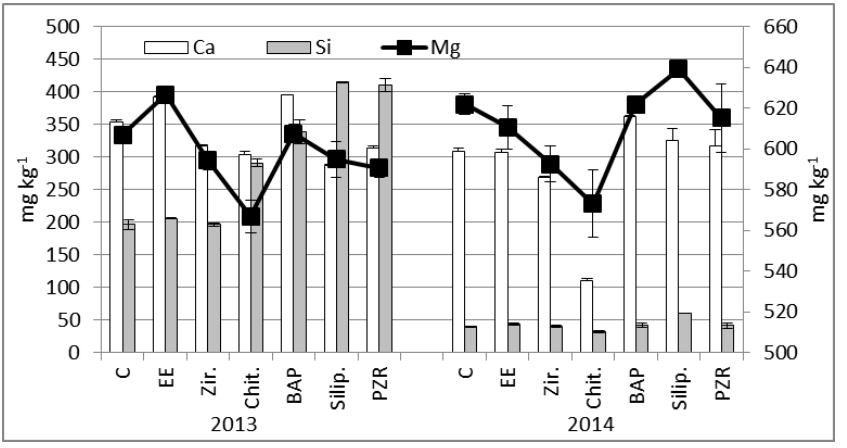

Figure 3. The effect of different foliar fertilizers on Ca, Mg and Si concentration in barley grain (C – control, EE – Epin Extra, Zir. – Zircon, Chit. – Chitosan, BAP – Benzyladenine, Silip. – Siliplant, PZR – Propikonazole); Mean ± SD (standard deviation)

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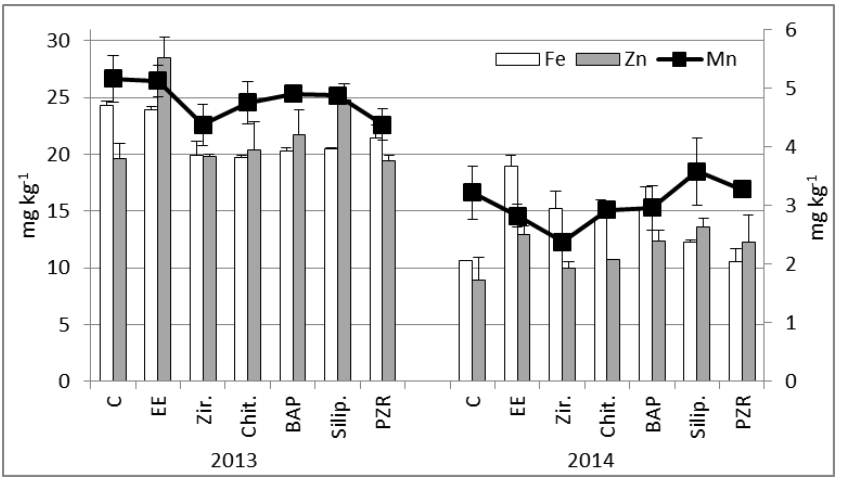

Figure 4. The effect of different foliar fertilizers on Fe, Zn and Mn concentration in barley grain (C – control, EE – Epin Extra, Zir. – Zircon, Chit. – Chitosan, BAP – Benzyladenine, Silip. – Siliplant, PZR – Propikonazole); Mean ± SD (standard deviation)

Epin Extra highly influenced Mg concentration, similarly to the results of Lachman et al. (19), as well as the concentrations of microelements, Fe, Zn and Mn (Figure 4), similarly to the results of Dragičević et al. (20), but under favorable meteorological conditions. Besides, higher Ca concentrations were observable in BAP treatment for 2013 and 2014 (395.0 and 362.8 mg kg^{-1}, respectively), according to the results of Gurmani et al. (21), who obtained higher Ca concentrations in wheat plants treated with BAP under saline and non-saline conditions. Furthermore, Siliplat was the most important fertilizer for Si increase in barley grain (414.7 and 60.4 mg kg^{-1}, for 2013 and 2014), as well as for Zn and Mn increase during unfavorable, 2014 (13.59 and 3.58 mg kg^{-1}, respectively), confirming that Si plays an important role in osmotic adjustment and regulation of the hormonal plant status under stress conditions (8).

Table 2. The effect of different foliar fertilizers on the relations between phytic and inorganic P, phytate, yellow pigment, Mg, Ca, Fe, Zn and Mn in barley (cv. Apolon) grain

Treatment	P_{phy}/P_i	Phy/yell.pigm.	Phy/Mg	Phy/Ca	Phy/Fe	Phy/Zn	Phy/Mn
Control	5.10 $^{**n.s.}$	5356.60 $^{n.s.}$	2.15 ^{b}	2.86 ^{ab}	107.34 ^{b}	40.22 $^{n.s.}$	74.1 ^{b}
Epin extra	4.58 $^{n.s.}$	5242.48 $^{n.s.}$	2.11 ^{ab}	2.68 ^{ab}	100.90 ^{b}	27.52 $^{n.s.}$	60.0 ^{a}
Zircon	4.62 $^{n.s.}$	5411.22 $^{n.s.}$	2.14 ^{a}	3.11 ^{b}	103.15 ^{b}	37.21 $^{n.s.}$	71.0 ^{ab}
Chitosan	4.60 $^{n.s.}$	5088.97 $^{n.s.}$	2.14 ^{a}	4.21 ^{c}	72.38 ^{a}	34.10 $^{n.s.}$	70.1 ^{ab}
Benzyladenine	4.60 $^{n.s.}$	5349.25 $^{n.s.}$	2.03 ^{a}	2.36 ^{a}	62.91 ^{a}	31.81 $^{n.s.}$	69.1 ^{ab}
Siliplant	4.47 $^{n.s.}$	5610.72 $^{n.s.}$	2.05 ^{ab}	2.96 ^{b}	51.13 ^{a}	28.70 $^{n.s.}$	76.1 ^{b}
Propikonazole	4.74 $^{n.s.}$	5828.46 $^{n.s.}$	2.16 ^{b}	2.96 ^{b}	55.46 ^{a}	35.80 $^{n.s.}$	80.3 ^{b}
LSD 0.05*	0.8	2397.6	0.11	0.58	26.27	15.66	10.43

* Least significant difference, P = 0.05 (n = 4); The values followed by same letters are not significantly different at the 0.05 level; $^{**n.s.}$The values are under the level of significance of 0.05.

The highest Fe concentration in barley grain was observed in Epin Extra treatment (23.9 and 18.9 mg kg^{-1}, for 2013 and 2014). Insignificantly higher Fe and Mn concentrations in control plants were obtained in 2013, due to the drought present during the grain filling period, similarly to the results of Hussein et al. (22) who also found lower concentrations of both elements in grain of barley sprayed with amino acid fertilizers in combination with water deficit. It could be supposed that foliar treatments, irrespective of the formulation applied, slightly reduced accumulation of some promoters, such yellow pigment and GSH (Figure 1), as well as some mineral elements (Fe and Mn; Figure 4) in dry conditions. However, positive impact of applied fertilizers on accumulation of examined elements was mainly related to hormone preparations, such as EE and BAP, as well as silicone fertilizer Siliplant, illustrating their complex role in plant metabolism and stress tolerance (8, 9, 12), which can also reflect on increased nutrients acquiring in barley grain.

Potential bioavailability of mineral elements. Considering the relations between Phy, P_i, yellow pigments, Mg, Ca, Fe, Zn and Mn, the highest values were mainly obtained in PZR treatment, with significant differences observed between the values of Phy/yellow pigment, Phy/Mg, Phy/Ca, Phy/Fe and Phy/Mn (Table 2). The lowest values of Phy/Zn and Phy/Mn were in hormone, Epin Extra treatment (25% and 23% lower in relation to PZR, respectively), while the significantly lowest values of Phy/Mg and Phy/Ca were in BAP treatment (6% and 44% lower in relation to PZR, respectively). Siliplant was characterized by the significantly lowest values of Phy/Fe in relation to control (12% and double, respectively) and Chitosan with significantly lowest Phy/yellow pigment (13% in relation to PZR). While Chitosan mainly decreased P_{phy} concentration, the increased concentrations of examined mineral elements influenced by Siliplant and hormone preparations Epin Extra and BAP were mainly reflected on decrease of the ratio between phytate and mineral elements, contributing to their better bioavailability to humans (4).

CONCLUSION

The obtained results indicate that the year of cultivation influenced the chemical composition of barley grain, mainly increasing concentrations of promoters, antinutrients and mineral elements during dry season. The highest impact of unfavorable conditions with high precipitation level was for Si, decreasing its concentration several times. Among applied treatments, Chitosan was the most effective for increasing of promoters' level, and reducing of Phy/yellow pigment ratio, thus increasing potential bioavailability of the examined mineral elements. Moreover, unfavorable meteorological conditions were partially mitigated by application of Siliplant and hormone preparations: EE and BAP, thus increasing potential bioavailability of P, Mg, Ca and Fe.

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ОБОГАЋИВАЊЕ ЗРНА ЈЕЧМА ЕСЕНЦИЈАЛНИМ ЕЛЕМЕНТИМА ПУТЕМ АГРОНОМСКЕ БИОФОРТИФИКАЦИЈЕ

Весна Д. Драгичевић[1], Богдан Р. Николић[2], Милица М. Радосављевић[1], Ненад А. Ђурић[3], Дејан Б. Додиг[1], Милован М. Стоиљковић[4] и Наталија Б. Кравић[1]

[1] Институт за кукуруз "Земун Поље", 11185 Земун Поље, Србија
[2] Институт за заштиту биља и животну средину, 11000 Београд, Србија
[3] Џон Незбит Универзитет, Факултет за биофарминг, 24000 Бачка Топола, Србија
[4] Институт за нуклеарне науке Винча, 11001 Београд, Србија

Зрно јечма је богато минералима, али њихова приступачност за људски организам зависи од антинутритива који инхибирају њихову апсорпцију и промотера који повећавају њихову приступачност. Циљ истраживања је да се испита састав зрна јечма, укључујући фитат и феноле као антинутритиве, каротеноиде и глутатион као промотере, као и минералне елементе Ca, Mg, Fe, Si, Zn и Mn, под утицајем нестандардих фолијарних ђубрива (Циркон, Хитосан, Силиплант, Пропиконазол), као и хормона (Епин Екстра, Бензиладенин), као потенцијалне мере за биофортификацију јечма. Хитосан је повећао концентрацију глутатиона. Неповољни метеоролошки услови су делимично превазиђени применом Бензиладенина и Силипланта, који су утицали на повећање потенцијалне приступачности P, Mg, Ca i Fe.

Кључне речи: хемијски састав зрна јечма, антиоксиданти, минерални елементи, биофортификација

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IMPROVEMENT OF PHYSICOCHEMICAL AND RHEOLOGICAL PROPERTIES OF KOMBUCHA FERMENTED MILK PRODUCTS BY ADDITION OF TRANSGLUTAMINASE AND WHEY PROTEIN CONCENTRATE

Mirela D. Iličić, Spasenija D. Milanović, Katarina G. Kanurić, Vladimir R. Vukić and Dajana V. Vukić*

University of Novi Sad, Faculty of Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia

The objective of this work was to investigate the effect of addition of transglutaminase (TG-0.02%, w/w) and whey protein concentrate (WPC-0.03%, w/w), on quality of kombucha fermented milk product. Samples were prepared from pasteurized semi-skim milk (0.9%, w/w fat) and kombucha inoculum (10%, v/v). The pH values were measured during the fermentation of milk (lasted until reached 4.5). Syneresis, water holding capacity and the product texture (firmness and consistency,) were assessed after production. Rheological properties of kombucha fermented milk samples were measured during ten days of storage.

The sample containing TG had the lowest syneresis (21 ml), the highest water holding capacity (62%) and the highest textural characteristics (firmness - 23.99g, consistency - 626.54gs) after production. The addition of WPC to milk improved the rheological properties, while the addition of TG improved it even to a significantly greater extent after the production and during 10 days of the storage.

KEY WORDS: fermented milk products, kombucha, transglutaminase, whey protein concentrate, rheology

INTRODUCTION

The use of kombucha as non-conventional starter culture for fermented dairy product, have been a topic of research in the resent years (1-5)**.** Kombucha is a sweetened tea beverage which is fermented by a symbiosis of bacteria and yeast embedded within cellulosic pelliculs. It has been shown that the major bacterial genus was *Gluconobacter* present <85% in the most samples, *Acetobacter* detected <2%. A prominent *Lactobacillus* population was also identified up to 30%. The yeast population was found to be dominated by *Zygosaccharomides* at 95% in kombucha (6). Some authors found that different starter cultures (yoghurt starter, probiotic or kombucha) had a significant effect on milk fermentation, textural characteristics and rheological properties of the final product. Milk

* Corresponding author: Mirela D. Iličić, University of Novi Sad, Faculty of Technology, Bulevar Cara Lazara 1, Novi Sad, Serbia, panim@uns.ac.rs

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fermentation with kombucha as starter is slower compared to the production of probiotic fermented milk beverages (4, 5, 7).

Many studies were aimed to analyse the textural characteristics and rheological changes in fermented milk products with addition of transglutaminase (8-10). The enzyme transglutaminase (TG) is a transferase which catalyses both inter and intra-molecular isopeptide bonds between proteins by cross-linking of the amino-acid residues of glutamine and lysine (8). Activity of TG and degree of polymerisation of product in milk depends on different factors during milk fermentation: type and composition of milk, temperature and time of fermentation, pH value. Neve et al. (2001) investigated the effect of TG treatment on microbiological and rheological characteristics of set skim milk yoghurt (9). They concluded that yoghurt starter culture requires longer fermentation time and gel strength of the enzyme treated yoghurt was increasing during the first 5 weeks of storage. They assumed that the TG is still active during this period.

Besides, other ingredients such as whey protein concentrate (WPC) are commonly used for fermented milk production with the aim to change the ratio of casein to whey proteins, which affects the mesh size and structure of protein network (10).

The main objective of this study was to determine the influence of TG and WPC on milk acidification and quality of kombucha fermented milk products (syneresis, water holding capacity, texture and rheological properties).

EXPERIMENTAL

Materials

Homogenized and pasteurised cow milk from AD Imlek, Division Novi Sad Dairy, was used for the production of kombucha fermented milk beverages. The composition of milk was as follows: fat content – 0.9 g/100g, total solids – 10.23 g/100g, total proteins – 3.15 g/100g and lactose – 4.74 g/100g.

Kombucha was cultivated on a black tea (*Camellia sinensis* – oxidized, 1.5 g/L) with saccharose concentration of 70 g/L. The tea was cooled to room temperature, after which inoculum from a previous fermentation was added in concentration of 100 mL/L. The incubation was performed at 29^{o}C for 7 days (2). The obtained kombucha tea was used as non-conventional starter culture for inoculating the milk.

The TG of microbial origin (*Streptoverticillium mobaraense*) preparation Activa MP, with declared activity of 100 U/g, was purchased from Ajinomoto Co. Inc. (Hamburg, Germany), and it was added to milk to the concentration of 0.02% (w/w).

WPC (Lactomin 80S, LACTOPROT, Aplenandisch Milchindustrie und Handels-GMBH, Germany**)** contained: 78.5% (w/w) proteins, 4.5% (w/w) ash, 4.0% (w/w) fat and 5.0% (w/w) moisture, was applied in a concentration of 0.3% (w/w).

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Methods

Kombucha fermented milk beverage manufacturing. Three kombucha fermented milk samples were manufactured: first from milk with TG, second from milk with the WPC and third control sample. TG was added to milk and activated at 40°C for two hours. After mixing, the sample with activated TG was treated at 80°C for 1 min, cooled to 43°C, and inoculated with the kombucha inoculum - 10% (v/v) (sample 10K, TG). The sample with WPC (10K, WPC) was treated at 80°C for 15 min, followed by cooling (43°C), was also inoculated with the kombucha inoculum. The control sample (10K) did not contain any of the two ingredients. In all cases, the fermentation was stopped when the pH 4.5 was reached. Finally, the gels were cooled to 8°C, mixed and packed in plastic containers. Each trial was repeated three times.

The following chemical properties: total solids (TS) (11), total protein (TP) by Kjeldahl method (12), fat by Gerber method (13) were determined in all samples after production. The pH was measured on a pH-meter (EcoScan pH 6 Eutech Instruments, Netherlands). Syneresis was measured by whey separation and it was expressed in mL of whey separated during filtration of 50 mL sample for 3 hours, at room temperature (14). Water-holding capacity (WHC) of the samples was determined according to a procedure introduced by Guzman-Gonzalez *et al.* (15).

Textural properties (firmness and consistency) of the kombucha fermented milk samples were analysed by Texture Analyser TA.HD.plus (Stable Micro System, Godalming, England) through a single compression test, using a back extrusion cell (A/BE) disc (diameter 35 mm; distance 30 mm; speed 0.001 m/s) and an extension bar, using 5 kg load cell at 5ºC. Using the options Return to Start, a trigger force of 10 g was applied.

Rheological measurement. Rheological properties of fermented milk samples were measured at 5°C using a viscometer HAAKE RheoStress 600HP (Karlsruhe, Germany), fitted with a PP60Ti sensor (gap 1 mm). The samples of kombucha fermented milk products were taken with a spatula and placed on the rheometer plate and were allowed to achieve temperature of 5°C. For each sample, replicate measurements were done independently, and data processing was performed using a RheoWin Pro software package (Version 2.94, Thermo Haake, Karlsruhe, Germany). The flow curves were obtained by registering shear stress at the shear rate which was increased from 0 to 200 s^{-1} in 180 s, held constant at 200 s^{-1} until the total system destruction, and decreased from 200 to 0 s^{-1} in 180 s. The other rheological parameters considered were the area under the upward (A_{up}) flow curve, the area difference under upward and downward (A_{down}) flow curves (ΔA or hysteresis loop area) (5). The magnitude of gels thixotropy was estimated as the coefficient of thixotropic breakdown, Kd, which is defined as the ratio of the hysteresis area to the area beneath the ascending shear curve $Kd=\Delta A/A_{up}$ (16). The rheological characteristics of samples were measured after production and during ten days of storage.

Statistical analysis. The data were expressed as means and standard deviation using the software program STATISTICA version 6 (2001) (StatSoft Inc, Tulsa, OK, USA) (17).

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RESULTS AND DISCUSSION

The effect of TG and WPC on physicochemical and textural characteristics of samples

The pH values of the samples (4.5) were uniform in all fermented milks after production. The fermentation time of control sample (10K) lasted 9.3 hours (Table 1). In the sample with TG the fermentation lasted 40 min longer and 10 min shorter compared to the fermentation in the control sample and sample with the WPC, respectively. These results are in accordance with the literature data (6, 18, 19) showing increased fermentation times for fermented milk samples with addition of TG. Chemical analyses showed that total solids of kombucha fermented milk products varied from 10.92% (sample 10K) to 11.17 4g/100g (sample 10K, WPC) (Table 1). The protein content in the samples ranged from 2.98% (10K) to 3.25% 10K, WPC).

The syneresis of the sample containing TG was 21 ml. In the other samples, the syneresis was higher by 4 ml (10K, WPC) and by 8 ml (control sample). A larger quantity of separated whey was the result of formation of the bonds in the protein matrix to give a non-homogenous gel. The sample with addition of TG had the highest value of WHC (62.5%) and the highest values of textural characteristics (firmness -23.99g, consistency 626.53 gs). It was the result of a stronger structure due to the addition of TG, which was also shown by the lower syneresis. These results are in accordance with the literature data (10, 20), where the authors found that WHC and textural characteristics of the control probiotic yoghurt produced from milk of 0.1% fat content significantly were lower compared to the same characteristics of the sample containing TG.

Table 1. Physicochemical and textural characteristics of kombucha fermented milk products manufactured using TG and WPC

Sample	Time (hour)	TS (%)	TP (%)	Syneresis (ml)	WHC (%)	Firmness (g)	Consistency (gs)
10K	9.3	10.92	2.98	29	35.5	13.60	383.88
10K, TG	10.0	10.97	3.20	21	62.5	23.99	626.53
10K, WPC	10.2	11.17	3.25	25	58.0	14.89	416.03

Rheological characteristics

The rheological characteristics of samples during 10 days of storage are presented in Fig. 1. The appearance of hysteresis area in the plot of shear rate meant that all samples exhibited typical shear thinning flow behaviour. The Kombucha fermented milk product containing TG had significantly higher yield stress (65.3Pa) than samples without TG addition – control sample (1.14 Pa).

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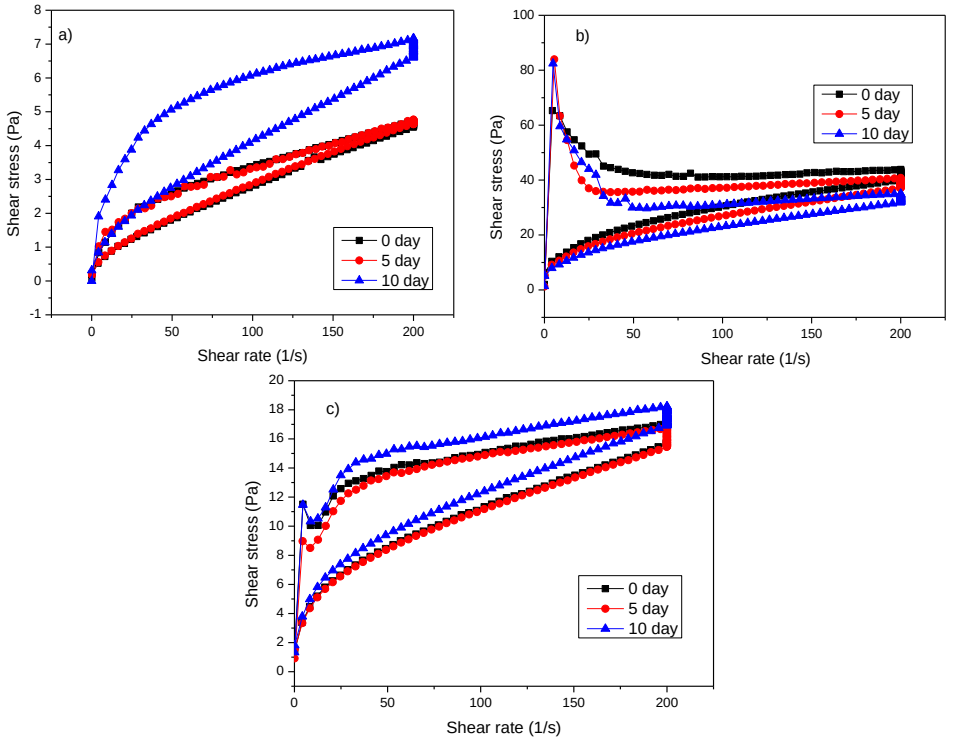

Figure 1. Flow curves of kombucha fermented milk products: a) Control sample (10K); b) Sample containing TG (10K, TG); c) Sample with addition of WPC

The hysteresis loop area (ΔA) is the indicator of the yoghurt structural breakdown and rebuilding (a degree of thixotropy) during shearing (5, 22). Fermented milk product containing TG produced with kombucha inoculum showed the highest hysteresis loop area (3000 Pa/s). The hysteresis loop area of kombucha fermented milk with WPC and control sample were lower compared with HLA of the sample containing TG (Fig. 2). The differences in hysteresis area between the samples were results of higher total proteins in sample with WPC (10K, WPC), as well as of the formation of strong intermolecular bonds in the protein matrix with addition of TG to the kombucha fermented milk product (10K, TG). The obtained results for the hysteresis loop area of fermented milk samples with TG with kombucha inoculum were higher compared with the literature data (4), where the values of hysteresis area of stirred probiotic yoghurt produced from milk of 0.9% fat with 0.02% TG addition varied from 1400 to 1500 Pa/s during ten days of storage. The magnitude of gels thixotropy was estimated also as the coefficient of thixotropic breakdown (Kd) or the relative hysteresis area (16). The coefficient of thixotropic breakdown is an index of the energy needed to destroy the structure of the system. The highest value of the coefficient of thixotropic breakdown was noticed for the samples

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produced with addition of TG (0.4). So, the sample with TG needed the highest energy to breakdown structure. The coefficient of thixotropic for the sample with addition of WPC was similar during ten days of storage. The experimental data indicated that the sample with TG and WPC during storage showed a structure which was more stable compared to the control sample.

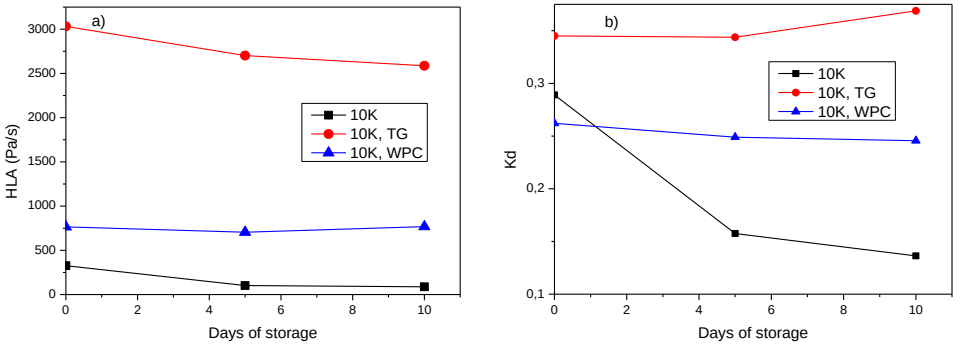

Figure 2. Rheological properties of kombucha fermented milk products during 10 days of storage**:** hysteresis loop area and b) coefficient of thixotropy

CONCLUSION

The fermentation time for kombucha fermented milk sample with addition of transglutaminase and whey protein concentrate lasted longer compared to the control sample. The addition of WPC to milk at a level of 0.3% contributed to the formation of kombucha fermented milk product with improved physical characteristics and better textural properties, while the minimal concentration of TG (0.02%, w/w) produced even more favourable effects. The addition of TG to the kombucha fermented milk sample improved significantly the rheological characteristics. During ten days of storage, the structure of the samples containing TG and WPC was more stable compared to the control fermented milk product.

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ПОБОЉШАЊЕ ФИЗИЧКОХЕМИЈСКИХ И РЕОЛОШКИХ КАРАКТЕРИСТИКА КОМБУХА ФЕРМЕНТИСАНОГ МЛЕЧНОГ ПРОИЗВОДА СА ДОДАТКОМ ТРАНСГЛУТАМИНАЗЕ И КОНЦЕНТРАТА ПРОТЕИНА СУРУТКЕ

Мирела Д. Иличић, Спасенија Д. Милановић, Катарина Г. Канурић, Владимир Р. Вукић и Дајана В. Вукић

Универзитет у Новом Саду, Технолошки факултет, Булевар Цара Лазара 1, 21000 Нови Сад, Србија

У раду је испитан утицај додатка ензима трансглутаминазе и концентрата протеина сурутке на физичкохемијске и текстуралне карактеристике, и реолошка својства комбуха ферментисаног млечног производа добијеног из млека са 0,9% млечне масти. Ензим трансглутаминаза коришћен је у концентрацији од 0,02% (w/w), док је концентрат протеина сурутке додат у млеко у концентрацији од 0,3%(w/w). Контролни узорак је произведен уз додатак 10% комбуха инокулума (без додатка наведених ингредијената). Ферментација млека је прекинута при pH вредности 4,5. Синерезис, способност везивања воде и текстурална својства варијанти комбуха ферментисаних млечних производа анализиране су након производње, а реолошке карактеристике су испитане током 10 дана складиштења.

Резултати анализе показују да. узорак који садржи трансглутаминазу има најмању вредност синерезиса (21mL). Додатак концентрата протеина сурутке и трансглутаминазе има значајан утицај на физичкохемијске и текстуралне карактеристике испитаних узорака након производње. Реолошки параметри квалитета показују, да је гел комбуха ферментисаног млечног производа са додатком трансглутаминазе значајно стабилнији током 10 дана складиштења у односу на контролни узорак и узорак произведен применом концентрата протеина сурутке.

Кључне речи: ферментисани млечни производи, комбуха, трансглутаминаза, концентрат протеина сурутке, реологија

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DETERMINATION OF PHENOLIC ACIDS IN SEEDS OF BLACK CUMIN, FLAX, POMEGRANATE AND PUMPKIN AND THEIR BY-PRODUCTS

Vera M. Krimer Malešević[1], Mihalj M. Poša[2], Žužana G. Stolić[3], Ljiljana M. Popović[3] and Ivana R. Peričin-Starčević[4]*

[1] Directorate of National Reference Laboratory, Batajnički drum 7/10, 11080 Zemun, Serbia
[2] University of Novi Sad, Faculty of Medicine, Hajduk Veljkova 3, 21000 Novi Sad, Serbia
[3] University of Novi Sad, Faculty of Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia
[4] Institute for Child and Youth Health Care of Vojvodina, Hajduk Veljkova 10, 21000 Novi Sad, Serbia

Ten phenolic acids, contained in the seeds of black cumin (Nigella sativa L.), flax (Linum usitatissimum L.), pomegranate (Punica granatum L.) and pumpkin (Cucurbita pepo L.) and their oil industry by-products, separated into the free, esterified, and insoluble-bound forms, were quantitatively analysed by reverse phase high performance liquid chromatography with photodiode array detector. The chromatographic data were interpreted using Principal Component Analysis (PCA). The PCA model with three principal components (PC1-PC2-PC3) fitted well with 12 examined plant samples, allowing their division into groups according to their origin. The total phenolic variables could be represented by two PCs and for the pattern recognition of the analysed samples, 13 phenolic variables are sufficient, including: free, esterified and insoluble-bound forms of gallic and syringic acids, free vanillic, insoluble bound p-coumaric, esterified p-hydroxybenzaldehide, and free and insoluble-bound forms of p-hydroxybenzoic and trans-synapic acids. This might have potential application in simplified screening of phenolic compounds in seeds and their oil industry by-products or in food component analysis or authenticity detection in such plant materials.

KEY WORDS: oilseeds, by-products, phenolic acids, chemometrics, *PCA*

INTRODUCTION

Cold pressed oils from seeds of black cumin (*Nigella sativa* L.), flax (*Linum usitatissimum* L.), pomegranate (*Punica granatum* L.) and pumpkin (*Cucurbita pepo* L.) are known for their therapeutic properties. Black cumin oil is usually used externally for treating psoriasis eczema or internally in treating asthma, allergies, diabetes, and digestion problems (1). A mixture of cumin oil with beeswax can be used for burns, skin infections, moisturizers, joint pain reliever, or as anti-wrinkle agent. Flax oil is known for high omega-3 fatty acids content, exerting an anti-inflammatory response (2). Punicic acid

* Corresponding author: Vera Krimer-Malešević, Directorate of National Reference Laboratory, Batajnički drum 7/10, 11080 Zemun, Serbia, e-mail: krimerv@orion.rs

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which is present in high concentration in pomegranate oil is rarely found in other plant oils. It has anti-inflammatory and anti-tumoural activity (in hormone dependent tumours such as breast tumour). Pomegranate oil is also used in luxury cosmetics (3). Pumpkin seed oil showed beneficial effects in the therapy of small disorders of the prostate gland and the urinary bladder (4).

In attempt to define new renewable resources, there has been a growing interest in studying phenolic acids in various crops, including oilseeds and their oil industry by-products (5-7). Phenolic acids have been reported to possess antioxidant, antimicrobial, antiproliferative and preservative properties (8, 9). Due to these bioactivities, they might have an important role as health-promoting substances, as well as in food deterioration prevention, applicable in food and pharmaceuticals. Hence, the focus on their characterization and utilization, especially from industrial wastes is of great interest. Skins and hulls of seeds represent valuable sources of these components, containing much higher concentration of phenolics than inner seed layers (10). Oilseeds meals/cakes are rich in proteins and phytochemicals, such as phenolic compounds, whose valorisation is of global economic and environmental interest (11-14). Additionally, after de-oiling process the meals/cakes contain more phenolic acids and in more easily extractable form than the corresponding seed (12).

Due to diversity of chemical structure and forms of phenolic acids in oilseeds or derived materials, the identification and quantification, performed usually by reverse phase high pressure liquid chromatography (RP-HPLC) with UV or PDA detectors, result in complex data sets. The interpreting and comprehensive comparison of different samples can be achieved by some of chemometric approaches, such as Principal Component Analysis (PCA). PCA is a dimensional reduction technique, which allows clustering different samples based on their shared features. It is commonly used to identify how one sample is different from another, which variables (i.e. features) contribute most to this difference, and whether variables are correlated or independent from each other. PCA has been applied for classification of vegetables, fruits, grains or nuts based on their genotype, varieties, bioactivity, volatile or antioxidant compounds (15-18), as well as for characterization, determination of geographic origin and quality control of food and beverages (19, 20).

Therefore, the focus of this study is a profiling of phenolic acids in 12 plant materials, including black cumin seed and meal, flaxseed and meal, pomegranate seed, meal and hull, hull-less pumpkin seed, skin and meal, and kernels and hull from hulled pumpkin seed. Free, esterified, and insoluble-bound forms of ten phenolic acids were quantitatively analysed by RP-HPLC with PDA detector and the chromatographic data were analysed by PCA.

EXPERIMENTAL

Plant samples

Black cumin (*Nigella sativa* L.) seed and meal; flax (*Linum usitatissimum* L.) seed and meal; and pomegranate (*Punica granatum* L.) seed, meal and hull were obtained

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from a 1ocal oil producer Suncokret (Hajdukovo, Serbia). Hull-less pumpkin seed (*Cucurbita pepo L. cv*. Olinka), oil cake and skin samples were obtained from Pan Union (Novi Sad, Serbia). Hulled seeds were purchased in a local store and prior to analysis dehulled by hand. All samples were stored at 4ºC, for the same length of time, until their analysis. All samples were ground in a coffee grinder for 3 min immediately before preparation, except for skin, which was ground in a mortar.

Phenolic compounds extraction and HPLC determination

Chemicals used and complete procedure for isolation of phenolic acids from the plant seeds, meals, hulls and skins are described in detail in previous papers (11, 12). Briefly, one gram of ground defatted meal was extracted three times with 70% methanol in ultrasound bath at room temperature. After centrifugation, the supernatants were analysed for free phenolic acids and soluble phenolic acid esters, and the residue was kept for the determination of insoluble-bound phenolic acids. The phenolic acids were separated from phenolic acid esters by diethyl ether - ethyl acetate (DE/EA) extraction. The phenolic acid esters and solid residue (insoluble-bound phenolic acids) were separately hydrolyzed with 4 N NaOH containing 1% w/v ascorbic acid, antioxidant, and 10 mM EDTA (added to prevent degradation of phenolic acids during alkaline hydrolysis), under nitrogen, in the dark and at room temperature. During the preparation, hexane clean-up was performed in order to get extracts free from residual oils, and hexane extracts were discarded. After hexane extraction the remaining water phases were extracted with DE/EA. The dried DE/EA extracts were redisolved in the initial HPLC mobile phase.

The prepared samples were analysed on a Thermo Finnigan Surveyor HPLC equipped with Surveyor quaternary pump, built-in vacuum degasser, Surveyor autosampling injector, column thermoregulator and Surveyor PDA detector. Separations were achieved using a C18 Hypurity Aquastar column (5.0 μm particle size, 250x4.6 mm i.d.) with a C18 Hypurity Aquastar guard column (5.0 μm particle size 10x4.6 mm i.d.); both Thermo Electron Corporation. HPLC analysis; identification and quantification of phenolic acids were carried out according to the previously described method (12). In short, gradient elution was carried out using the mobile phase A, water-acetic acid (98:2, v/v) and mobile phase B, methanol-acetonitrile (1:1, v/v). Detection of polyphenols by PDA was performed at 280 nm + spectral analysis (scanning from 220-360 nm). Identification of phenolic compounds was carried out by comparing retention times and spectral data with those of the standards. Quantification was done by external standard method, in triplicate.

Data treatment – pattern recognition

Plant samples were arranged as objects, while the contents of phenolic acids (mean value of triplicates) were taken as variables. The total 30 variables included free (1), esterified (2) and insoluble-bounded (3): *p*-hydroxy-benzaldehyde (HA1, HA2, HA3), caffeic (C1, C2, C3), *p*-coumaric (pC1, pC2, pC3), ferulic (F1, F2,F3), *trans*-synapic (S1, S2, S3), protocatehuic (P1, P2, P3), *p*-hydroxybenzoic (HB1, HB2, HB3), vanillic (V1,

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V2, V3), syringic (Sy1, Sy2, Sy3), and gallic (G1, G2, G3) acids. For pattern recognition and classification of the plant materials, PCA was used. Due to the scale differences among the variables, the correlation matrix was used to obtain the principal components (PCs). The use of correlation matrix automatically means that the data are standardized (autoscaling). The correlation matrix was formed from the original input matrix, wherein from each the variable the correspondent column average is subtracted and then, divided by the column standard deviations (autoscaling). The PCs are determined by NIPALS algorithm, while the significant PCs were assessed by *Screen* test (22, 24). All the chemometric analyses were performed using software *Statistica 10, Statsoft.*

RESULTS AND DISCUSSION

Principal Component Analysis

PCA was applied for the characterization of the phenolic acids with the aim to classify the plant materials by their similarity. According to the *Screen test*, four PCs were found to be significant for modelling the variables (Table 1).

Table 1. Significant eigenvalues of the correlation matrix

Value number	Eigenvalue	% Total variance	Cumulative eigenvalue	Cumulative variance %
1	8.815201	29.38400	8.81520	29.3840
2	6.231254	20.77085	15.04646	50.1549
3	4.247401	14.15800	19.29386	64.3129
4	2.762886	9.20962	22.05674	73.5225

As PCs with small eigenvalues explain the error of measurement (21), for the description of variation in the input matrix the first three PCs could be selected as sufficient (22), explaining >64 % total variance of the original data.

In the plane of PC loadings vectors the length of vectors determines the significance of the individual variables in the PC model, while the cosine of the angle between the variables vectors gives the correlation between the variables. Figure 1(A) shows significantly correlated variables in the same direction, revealing two major groups. The first group comprises all forms of ferulic acid (F3, F2, F1), pC2, C1 and C2. To the second group belong all forms of gallic acid (G3, G2, G1), Sy2, HA2, and V1. In the analysis of the phenolic variables the total free, esterified, insoluble-bonded, bonded and total content of phenolic acids (FT, ET, IT, BT, TT), which are linear combinations of the individual variables, were treated as supplementary variables and additionally projected in the plane of PCs. Since all total variables are highly correlated to the second group, they are mainly determined by the phenolic acids from this group. The variables from the first and second group carry two different characteristics of the analysed objects. It is worth to note that the first group contains only hydroxycinnamic acids, while the second compri-

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ses hydroxybenzoic acids, and this similarity between the variables could be understood based on their chemical structure. In the further studies, there was no need to evaluate all of the phenolic variables to achieve the same characterisation of the analysed plant materials; it was enough to determine one variable per group. In the PC1-PC3 loading plane (Figure 1(B)) the correlation among the phenolic variables are very similar to the PC1-PC2 plane. However, there is an increase of the angles between the vectors of the phenolic variables corresponding to the previously formed groups due to lower eigenvalue of the PC3; therefore there is a higher probability that PC3 explains the error of measurement compared to PC1 and PC2.

a) b)

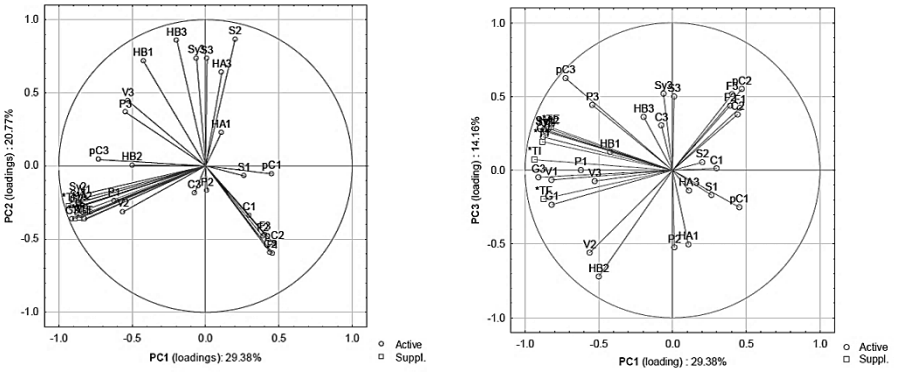

Figure 1. Principal components loadings of phenolic acids, a) PC1-PC2 and b) PC1-PC3 loading plane

Legend: *p*-Hydroxybenzaldehyde=HA; Caffeic=C; *trans p*-Coumaric=pC; Ferulic=F; *trans* Sinapic=S; Protocatechuic=P; *p*-Hydroxybenzoic=HB; Vanillic=V; Syringic=Sy; Gallic=G, Free total=TF; Esterified Total=TE; Insoluble-bound Total=TI; Bound Total=TB; Total Total=TT (Free=1; Esterified=2; Insoluble=3)

In the score plane of the PC1-PC2 (Figure 2) the objects are divided into four main groups – the clusters correspondent to the plant origin (the groups are formed comparing the total residual variance of groups and appended objects). The first group is consisted of the black cumin samples, the second group of pumpkin samples, etc. The pomegranate hull (object 5) does not belong to any of the groups, and it is an *outlier*, conformed also by *Hotelling* T^2 test for the model with three PCs. The classification of the analysed samples in the score plane of PC1-PC3 was confirmed in the same way as in the plane PC1-PC2 (not shown). According to the RP-HPLC analysis and score plots (Figure 2), the black cumin samples had higher contents of S2, S3, HB3, HB1 compared to other groups, while Sy3 was the highest in these samples, more than 93 mg/kg. Comparing the meal and seed, higher contents of G3, G2, G1, Sy1, HA2, V1 and pC3 was observed, especially of G2, Sy1 and V1, which were about three times higher in the meal. Only the content of Sy2 was almost equal. The second group consists of the pumpkin samples, and its general characteristic was the absence of gallic acid.

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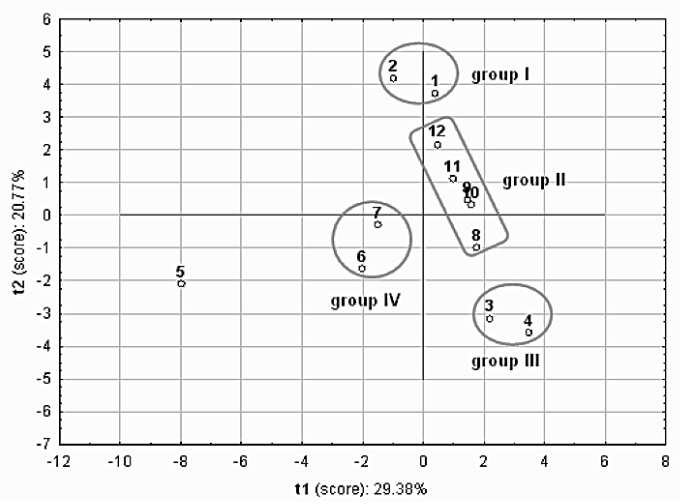

Figure 2. Principal components score (model: PC1+PC2)
Legend: 1: Black cumin seed; 2: Black cumin meal; 3: Flaxseed; 4: Flaxseed meal; 5: Pomegranate Hull; 6: Pomegranate meal; 7: Pomegranate seed; 8: Hull-less pumpkin seed; 9: Hull-less pumpkin seed meal; 10: Kernels from hulled pumpkin seed; 11: Hull-less pumpkin seed skin; 12: Hulls from hulled pumpkin seeds

According to Figure 2, the most similar are the pumpkin seed meal and kernels (objects 9 and 10). The skin and hull of pumpkin seed (objects 11 and 12) had the highest content of total phenolic acids. These samples had the highest content of free HB1, similar content of HA2 and V1; while pC3 was detected in the skin and Sy2 in the hull. The flaxseed samples comprise the third group. In this group, syringic acid was not detected, while the G3, V1, pC3 were lower in meal than in seed, which is verified by the position of objects 3 and 4. Also, no *trans*-sinapic acid was detected. The fourth group included the pomegranate meal and seed. In these samples *p*-coumaric and *trans*-sinapic acids were not detected, but very high content of G1, G2 and G3 was observed, which was higher in the meal. The pomegranate hull, defined as *outlier*, had an extremely high content of gallic acid (G1, G2 and G3). Pomegranate hull was so rich in hydrolyzable tannins, so that this by-product can be proposed as a potential and cheap commercial source of ellagic acid (23). Gallic acid, as one of the hydrolysis products of hydrolyzable tannins, which can possibly explain its unusually high content and statistical deviation of pomegranate hull from other samples.

Since the examined objects form also the same clusters in the PC1-PC2-PC3 space (Figure 3) as in the score planes, the first two PCs were sufficient for pattern recognition of these products. In addition, the significance of the variables – power of discrimination, could be evaluated from the biplot graph. Figure 4 shows that both PC1 and PC2 are important for the cluster formation (since the object scores are scattered on each side of the PC axis) and that the variables with high loadings and more parallel loadings axis to the axis of PC are important.

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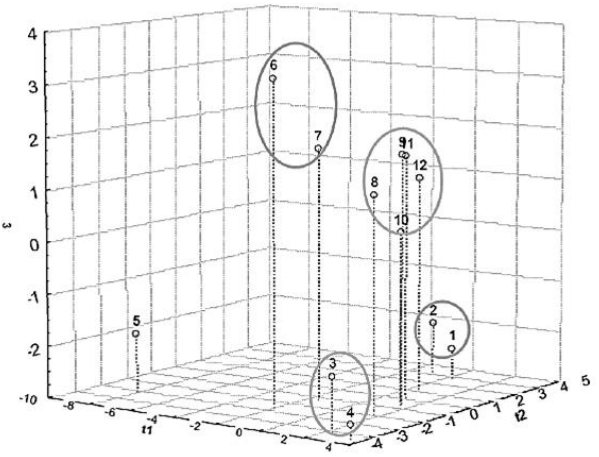

Figure 3. Principal components score (model: PC1+PC2+PC3)
Legend: 1: Black cumin seed; 2: Black cumin meal; 3: Flaxseed; 4: Flaxseed meal; 5: Pomegranate Hull; 6: Pomegranate meal; 7: Pomegranate seed; 8: Hull-less pumpkin seed; 9: Hull-less pumpkin seed meal; 10: Kernels from hulled pumpkin seed; 11: Hull-less pumpkin seed skin; 12: Hulls from hulled pumpkin seeds

This means that for the clustering of the analysed plant materials the phenolic variables G3, G2, G1, Sy2, Sy1, V1, pC3 and HA2 per PC1 axis and variables Sy3, HB3, HB1, S3 and S2 per PC2 axis are significant.

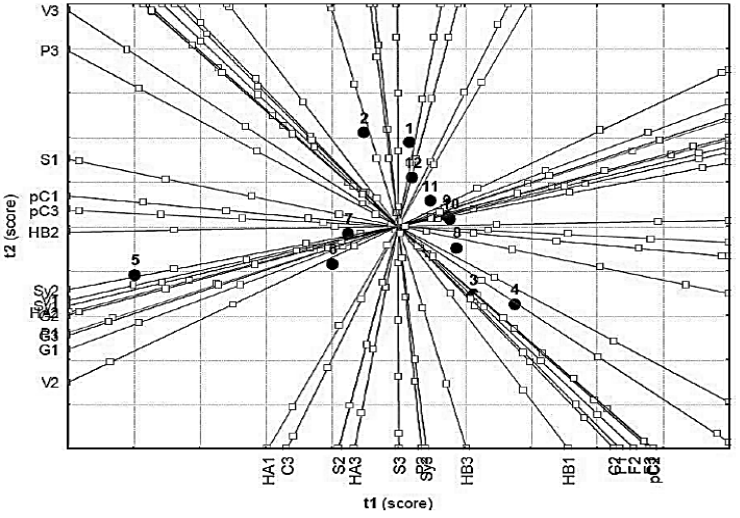

Figure 4. Biplot for the simultaneous characterization of the scores and loadings

CONCLUSION

Ten phenolic acids, contained in the seeds of 12 plant materials, including black cumin, flax, pomegranate and pumpkin and their oil industry by-products, separated in the free, esterified, and insoluble-bound forms, were quantified by RP-HPLC with PDA detector. Chromatographic data were explained by a PCA model, which allowed division of samples into groups according to their origin and decreased the number of phenolic acids required for their characterisation; instead of 30, only 13 phenolic acids were necessary. This might have potential application in simplified screening of phenolic acids or quality/authenticity assessment of these oilseeds and their by-products. The results also evaluated the examined oil crops and their by-products as valuable sources of phenolic acids.

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ФЕНОЛНЕ КИСЕЛИНЕ У СЕМЕНИМА И НУСПРОИЗВОДИМА ЦРНОГ КИМА, ЛАНА, НАРА И УЉАНЕ ТИКВЕ

Вера Кример Малешевић[1], Михаљ Поша[2], Жужана Вашташ[3], Љиљана Поповић[3], и Ивана Перичин-Старчевић[4]

[1] Директорат Националне референтне лабораторије, Батајнички друм 7/10, 11080 Земун, Србија
[2] Универзитет у Новом Саду, Медицински факултет, Хајдук Вељкова 3, 21000 Нови Сад, Србија
[3] Универзитет у Новом Саду, Технолошки факултет, Булевар Цара Лазара 1, 21000 Нови Сад, Србија
[4] Институт за здравствену заштиту деце и омладине Војводине, Хајдук Вељкова 10, 21000 Нови Сад, Србија

Десет фенолних киселина које су раздвојене на слободне, естерификоване и нерастворне-везане су квантификоване реверзно-фазном течном хроматографијом високих перформанси са детектором са низом диоде (photodiode array detector) у семенима црног кима (*Nigella sativa* L.), лана (*Linum usitatissimum* L.), нара (*Punica granatum* L.) и уљане тикве (*Cucurbita pepo* L.) као и нуспроизводима након цеђења уља. Хроматографски подаци су тумачени коришћењем принципалне анализе компоненти (Principal Component Analysis - *PCA*). *PCA* модел са три компоненте (*PC*1-*PC*2- *PC*3) се добро уклопио са 12 испитаних биљних узорака, омогућавајући њихову поделу на групе према пореклу. Укупне фенолне варијабле се могу представити са две *PC*, а за уочавање обрасца код анализираних узорака довољно је 13 фенолних варијабли, укључујући ту: слободне, естерификоване и нерастворне-везане облике галне и сирингинске киселине, слободну ванилинску киселину, нерастворну-везану *p*-кумаринску киселину, естерификован *p*-хидроксибензалдехид и слободне и нерастворне-везане *p*-хидроксибензоеву и *trans*-синапинску киселину. То би могло да има потенцијалну примену у поједностављеном скринингу фенолних једињења у семенима и нуспроизводима након цеђења уља, у анализи компонената хране као и утврђивању аутентичности у наведеном биљном материјалу.

Кључне речи: семена уљарица, нуспроизводи, фенолне киселине, хемометрија, *PCA*

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Original scientific paper

KEEPING QUALITY OF BEEF SAUSAGE USING ETHANOLIC EXTRACT OF GAMMA-IRRADIATED POMEGRANATE PEEL POWDER

Ibrahim Ali Soliman Mohammed[1], Fawzy Ramadan Hassanien[2], Kamal Mahfouz El Sahy[2], Sayed Ahmed Mohammed Afify[1] and Mohammed Hasan Mohammed Abdeldaiem[1]*

[1] Nuclear Research Center, Atomic Energy Authority, Cairo, Egypt
[2] Food Science Department, Faculty of Agriculture, Zagazig University, Zagazig 44519, Egypt

The present study aims to evaluate the antioxidant and antibacterial activity of ethanol extracts from gamma-irradiated pomegranate (Punica granatum) peel powder (PE) at the dose levels of 0, 3, 6, and 9 kGy. The antioxidant activity of the extracts was estimated using the radical scavenging activity against 2,2'-diphenyl-1-picrylhydrazyl (DPPH•), β-carotene/linoleic acid bleaching system, and ferric reducing antioxidant power (FRAP). Antibacterial activity of the extracts was assessed against Bacillus cereus, Staphylococcus aureus, Escherichia coli, Klebsilla penumoneae, Pseudomonas aeruginosa, and Salmonella typhumurium. The results showed that PE treated with 6 kGy had a higher content of total phenolic compounds (TPC), total flavonoid compounds (TFC), and antioxidant activity. Hence, the PE of 6 kGy-irradiated peels were selected to be added to beef sausage. Different concentrations of 6 KGy-irradiated PE were applied to improve beef sausage hygienic quality and extend the shelf life during cold storage (4°C). The results indicated that when 12 mL of the extract were added to one kg of beef sausage, the shelf-life of the sausage was extended from 15 days to 50 days (at 4°C), compared with the control, without changes of the microbiological, chemical, and sensory attributes.

KEY WORDS: pomegranate peels, polyphenol, flavonoid, antioxidant activity, DPPH•, β-carotene, γ- irradiation

INTRODUCTION

Pomegranate (*Punic granatum* L., family Punicaceae) is one of the oldest edible fruits. It has been cultivated in many tropical, and subtropical regions and its cultivation has increased considerably in recent years. Egypt is one of the main producers and exporters of pomegranate in the world. According to statistics of the Egyptian Ministry of Agriculture, the total pomegranate production of Egypt in 2011 was 64,574 tons (1). Pomegranate fruit is consumed fresh or as processed into juice, jams, syrup, and sauce. The edible part of the fruit constitutes about 52% of fruit weight. The composition of

* Corresponding author: Fawzy Ramadan Hassanien, Food Science Department, Faculty of Agriculture, Zagazig University, 44519 Zagazig, Egypt, e-mail: hassanienmohamed@gmail.com

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edible part consists of 78% of juice, and 22% of seeds (2). The fruit and its products showed effectiveness in retarding lipid oxidation *in vitro*, and *in vivo*. The homogenates prepared from whole fruit exhibited about 20-fold higher antioxidant potential than the level exhibited from the aril juice (3, 4). The waste remains after juice processing is composed mainly of pulp, and bagasses. Uses of these by-products are scarce, and their disposal represents environmental problem. However, due to its high content of phytochemicals (mainly phenolic acids and flavonoids), the pomegranate bagasses could be valorized as a source of bioactive compounds.

In order to overcome the stability problems of oils, and fats, synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and *tert*-butyl hydroquinone (TBHQ) are used as inhibitors of lipid oxidation (5). However, reports revealed that synthetic antioxidants may be implicated in many health risks. Due to safety concerns, there is an increasing interest to replace synthetic antioxidants with natural ones (6). Natural antioxidants such as phenolics, lignans, and terpenoids are found naturally in fruits, leaves, seeds, and oils (7) and they are known to protect easily-oxidizable constituents in food.

Parts of pomegranate fruit contain high concentrations of bioactive compounds. Li et al. (8) and Naveena *et al.* (9) reported that pomegranate peel had the highest antioxidant activity among the fruit parts (peel, pulp and seed) of 28 fruits. Pomegranate peel and rind are sources of tannins, anthocyanins, and flavonoids. Pomegranate peels extracts were inhibited the growth of several foodborne pathogens (10). Studies reported the efficacy of pomegranate extracts against the growth of Gram positive, and Gram negative bacteria (11).

Irradiation can affect the concentration of phytochemicals, and the capability of some plants to produce them. Under favorable conditions, the concentration of phytochemicals might be increased during extraction. These conditions include exposure to radiation, storage at low temperature, wounding, and exposure to high temperature (12). Gamma (γ) irradiation at 10 kGy dose increased phenolic acids content in cinnamon, and clove (13). Although, studies reported that γ irradiation can maintain or enhance the antioxidant activities, there are few examples wherein the antioxidant activities of the plant material were decreased (14).

The present study was carried out to (1) investigate the possibility of using pomegranate peel to extract natural antioxidants and antibacterial agents for food industry applications, (2) study the possibility of extraction of phenolic compounds from pomegranate peel with ethanol (50%), (3) study the effect of γ irradiation on the characteristics of phenolic compounds as a natural antioxidants, and (4) evaluate the antioxidant activity of pomegranate peel extracts (PE) as affected by γ irradiation compared to synthetic antioxidants in meat products.

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EXPERIMENTAL

Materials

Mature pomegranate fruits, having no visible external cuts or spoilage, were purchased from the local market (Zagazig, Egypt). Fruits were manually peeled to separate peels and arils. Butylated hydroxyl toluene (BHT), 2,2-Diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$), β-carotene, gallic acid, quercetin, Folin-Ciocalteu reagent, iron (III) chloride, and aluminum chloride were purchased from Sigma (St. Louis, MO, USA).

Methods

Preparation of pomegranate peel for extraction. Peels were rinsed with distilled water, and dried in oven at 45°C for 72 h, then ground to a fine powder in a coffee grinder for 2 min. To remove fatty materials, the ground peels were soaked in *n*-hexane (2:10, w/v), then the powder was dried at 45°C for 12 h. The obtained defatted dried peels powder was stored at -18°C in polyethylene bags (each bag contained 100 g).

Irradiation treatment. The irradiation treatment was carried out using a ^{60}Co Russian gamma chamber (dose rate 1.3 kGy/h) at the Nuclear Research Center (Atomic Energy Authority, Cairo, Egypt). The applied radiation doses were 3, 6, and 9 kGy for dried peels powder.

Preparation of PE extract (PE). Samples of non-irradiated and irradiated dry peels powder (20 g) treated with 3, 6, and 9 kGy were soaked individually into 200 mL ethanol (50%). All samples were shacked at room temperature for 7-8 h at a speed of 1000 vibration/min, then filtered through Whatman filter paper (No. 42). The residue was re-soaked again in 100 mL of solvent to ensure the complete extraction. The filtrate (extract) was subjected to rotary evaporator at 45°C to reduce the volume to 10 mL. The obtained extracts were stored at -18°C in brown bottles for further analyses.

Determination of TPC. TPC was determined by the Folin-Ciocalteu method according to Arabshahi-Delouee and Urooj (15). A volume of 200 μL of each extract was mixed with 1 mL of Folin-Ciocalteu's reagent (1 mL reagent with 9 mL distilled water). After 5 min, 1.5 mL of distilled water, and 1 mL of 75 g L^{-1} Na_2CO_3 solution were added. The mixture was incubated in a shaking incubator at ambient temperature for 60 min. The absorbance at 760 nm was measured. Gallic acid was used as a standard for the calibration curve (correlation coefficient R^2= 0.9944). TPC expressed as gallic acid equivalent (GAE) was calculated using the following linear equation based on the calibration curve:

$$y = 0.0045\ x + 0.0743$$

where y is the absorbance and x is the concentration (mg GAE g^{-1} extract).

Determination of TFC. TFC was determined according to Ordon et al. (16). An aliquot of 0.5 mL of $AlCl_3$ ethanol solution (20 g L^{-1}) was added to 0.5 mL of extracted solution. After 1 h, the absorbance of the mixture was measured at 420 nm. A yellow

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color indicated the presence of flavonoids. The extracted samples were evaluated at the final concentration of 0.1 mg mL^{-1}. TFC was expressed as quercetin equivalent (QE) (correlation coefficient R^2= 0.9853), and calculated using the following equation based on the calibration curve:

$$y= 0.0072\ x$$

where y is the concentration (mg QE g^{-1} extract), and x is the absorbance.

Antioxidant activity of extracts

The results of a single assay can provide a limited value of the antioxidant potential of a substance (17, 18). Thus, the antioxidant potential of the extracts was determined using three procedures.

DPPH Radical-scavenging activity. The electron donation ability of the extracts was measured by bleaching of DPPH˙ according to Hanato et al. (19). One hundred μL of each extract was added to 3 mL of 0.1 mM DPPH˙ dissolved in ethanol. After the incubation period of 0, 30, and 60 min at room temperature, the absorbance was determined at 517 nm against a control (20). The percentage of DPPH˙ antioxidant activity was calculated as follows:

$$\text{Inhibition (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

where $A_{control}$ is the absorbance of the control, and A_{sample} is the absorbance in the presence of the extract. BHT was used as a positive control, and the analysis was performed in triplicate.

β-Carotene/linoleic acid bleaching. The ability of the extracts and synthetic antioxidants to prevent the bleaching of β-carotene was assessed as described by Keyvan et al. (21). In brief, 0.2 mg of β-carotene in 1 mL of chloroform, 20 mg of linoleic acid, and 200 mg of Tween-20 were placed in a round-bottom flask. After removal of the chloroform, 50 mL of distilled water was added, and the resulting mixture was stirred vigorously. The aliquots (3 mL) of the emulsion were transferred to test tubes containing the extract or the synthetic antioxidant (BHT). Immediately after mixing, 0.5 mL of the extract, an aliquot from each tube was transferred to a cuvette, and the absorbance at 470 nm was recorded (Abs0). The remaining samples were placed in a water bath at 50°C for 2 h, then the absorbance at 470 nm was recorded (Abs 120). A control without extract was also analyzed. The antioxidant activity was calculated as follows:

$$\text{Antioxidant activity (\%)} = [1 - (Abs^{0}_{sample} - Abs^{120}_{sample})/(Abs^{0}_{control} - Abs^{120}_{control})] \times 100$$

where: Abs^{0}_{sample} is the absorbance of sample at zero time.
Abs^{120}_{sample} is the absorbance after 120 min.
$Abs^{0}_{control}$ is the absorbance of control at zero time.
$Abs^{120}_{control}$ is the absorbance of control after 120 min.

Ferric reducing antioxidant power (FRAP). The reducing power of the extracts was measured by the Oyaizu (22) method modified by Gülçin et al. (23). The reduction of

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Fe^{+3} to Fe^{+2} was determined by measuring absorbance of the Perl's Prussian blue complex. For this purpose, 0.1 mL of each extract was mixed with 1 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 1 mL (1%) of potassium ferricyanide [$K_3Fe(CN_6)$]. The mixture was incubated at 50°C. After 20 min of incubation, the reaction mixture was acidified with 1 mL of trichloroacetic acid (10%). Finally, 0.25 mL of $FeCl_3$ (0.1%) was added to this solution. Distilled water was used as the blank and control. The absorbance of the mixture was measured at 700 nm.

Antibacterial activity of the extracts

The antibacterial activity of the non-irradiated and irradiated PE was determined according to Baydar et al. (24). Nutrient broth cultures of *Bacillus cereus*, and *Staphylococcus aureus* as a Gram positive bacteria, and *Escherichia coli, Klebsilla penumoneae, Pseudomonas aeruginosa*, and *Salmonella typhumurium* as a Gram negative strains were incubated at 35ºC for 22 h. The test organisms used in this study was obtained from the Culture Collections of the Department of Microbiology, Cairo University (Egypt). Suspensions (250 µL) of the bacteria were added to the flasks containing 25 mL sterile nutrient agar at 43-45ºC, and poured into Petri dishes (10 cm diameter). The agar was allowed to solidify at 4ºC for 1 h. Wells (8 mm in diameter) were made in media using a sterilized stainless steel borer. Each well was filled with 70 µL of the extract. The plates were left at room temperature for 30 min to allow diffusion of the extract into the medium. The plates were incubated at 37°C for 18-24 h. The inhibition zones in mm (including the well diameter) around the wells were measured. Ethanol was used as a control sample (data not shown). The antibacterial activity was expressed as the diameter of the inhibition zones produced by the extracts against tested bacteria.

Application of 6 kGy-irradiated PE in beef sausage

Using of 6 kGy-irradiated PE in beef sausage as natural antioxidant and antimicrobial agents was studied. Fresh beef meat, mutton fat tissues, and lamb bowel (20 ± 2 mm diameter) were purchased from the local market. The hanks were washed with distilled water to remove the salt. The spices ingredients (fennel, black pepper, cardamom, cloves, cubeb, coriander, and laurel), salt, skim powder milk and garlic were purchased from the local market. Glucose, ascorbic acid, and sodium pyrophosphate were obtained from El-Gomhoria Company (Zagazig, Egypt). The sausage was formulated according to Modi et al. (25) protocol (Table 1).

The cuts of beef meat and mutton fat were minced in an electric mincer. Water (ice) was added in three stages to keep the temperature low. Salt, spices, and other additives were added in finality and the mixture was taken out of the bowl chopper. The obtained mixture was divided into three groups as follows:

1. First group was the control group.
2. Second group was divided into two parts: the first one corresponding to three concentration of 6 kGy-irradiated PE (3, 6, and 12 mL/kg), and the second group was

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divided into two parts corresponding to the γ irradiation doses (3, and 5 kGy) after packaging in the lamb hank.

3. The final mixture was packaged in the lamb hanks by packaging machine (sausage). The sausage samples were packaged into polyethylene bags, and sealed tightly by heat to prevent recontamination under good hygienic conditions, and cold stored (4°C) for periodical analyses.

Table 1. Formulation of beef sausage

Component	%	Spices mixture	%
Meat	70.6	Fennel	59.67
Mutton fat	14.0	Coriander	27.09
Water (as ice flakes)	7.00	Cubeb	3.19
Starch	4.65		
Sodium pyrophosphate	0.30	Black pepper	3.19
Salt (NaCl)	2.00		
Garlic	0.24	Cloves	3.19
Skim powder milk	0.40		
Glucose	0.10	Laurel	1.99
Ascorbic acid	0.04		
Spices mixture	0.66	Cardamom	1.59

Analysis of sausage

Thiobarbituric acid reactive substances (TBARS) content and total volatile basic nitrogen (TVBN) content. The evaluation of lipid stability was performed by measuring TBARS content every 5 days during cold storage following the method of Witte et al. (26). Ten g of sample were triturated with 25 mL of pre-cooled 20% trichloroacetic acid (TCA) in 2 M orthophosphoric acid solution for 2 min. The content was transferred into a beaker by rinsing with 25 mL of chilled distilled water. They were mixed and filtered through Whatman No. 1 filter paper. Three mL of TCA extract (filtrate) were mixed with 3 mL of TBA reagent (0.005 M) in test tubes, and heated in a boiling water bath for 30 min, then cooled. A blank sample was made by mixing 3 mL of 10% TCA, and 3 mL of 0.005 M TBA reagent. The absorbance was measured at 532 nm with a scanning range of 532 nm to 533 nm using a spectrophotometer (Unico, USA). The TBA value was calculated as mg malonaldehyde per kg of sample by multiplying the absorbance value by 5.2.

The TVBN was determined for muscle samples according to Mwansyemella (27).

Microbiological analysis

Preparation of samples. For each experimental patch, three bags were used in each treatment for microbiological examination. Each bag was sterilized using wetted cotton

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by hydrogen peroxide before opening. Under aseptic conditions, the bag was opened and 10 g of well mixed samples was transferred into 90 mL of sterilized saline solution (0.85% NaCl) in a conical flask. Two duplicate sets of Petri dishes were used and 1 mL aliquots from 10^{-1} to 10^{-6} dilutions pipetted in standard plate count agar (PCA, Biolife cod. No. 402145) and melted in the following steam. The agar was cooled to 44-46^{o}C, then poured into Petri dishes. Immediately, the aliquots were mixed with the agar medium by tilting and rotating the Petri dish. After solidification, the Petri dishes were returned and incubated at 37^{o}C for 48 h. The growing aerobic colonies were counted, and multiplied by the dilution factor (28).

Psychrophilic bacteria. Total psychrophilic bacteria were enumerated according to APHA (28), wherein the incubation was carried out at 7^{o}C for 5 days in refrigeration.

Coliform bacteria. Coliform bacteria were enumerated using Violet Red Bile Agar (VRBA, Biolife cod. No. 402185). Dilutions of 1 mL were poured into the plates, and incubated at 32^{o}C for 24 h (APHA, 1992).

Moulds and yeasts. An aliquot of 1 mL was promptly poured into the Petri dish of 10-15 mL containing Rose Bengal Chloromephenicol Agar (Biolife code No.401991 and Chloromephenicol antibacterial supplement cod. No. 421840003), and tempered to 44-46^{o}C. The aliquots, immediately mixed with the agar medium by tilting and rotating the Petri dishes, were incubated at 25^{o}C for 3-5 days. The number of cells is computed as cfu per g of samples (28).

Statistical analysis. All the analyses were performed using three bags per each separate replicate. The data were statistically analyzed using the general linear model procedure of the SAS software (29). The differences between the means were tested using Duncan's Multiple Range Test (30).

RESULTS AND DISCUSSION

Effect of irradiation on TPC and TFC of PE

Table 2 shows the TPC of non-irradiated and irradiated PE at 3, 6, and 9 kGy. The data revealed that 6 kGy dose produced an increase in the TPC of PE from 9323.17 mg GAE 100 g^{-1} DW (control) to 9323.17 mg GAE 100 g^{-1} DW. However, the irradiation of the PE samples at 3, and 9 kGy reduced their TPC to 9196.05, and 8988.17 mg GAE 100 g^{-1} DW, respectively. The data listed in Table 2 present the TFC of the non-irradiated and irradiated PE samples at 3, 6 and 9 kGy. The sample treated with 6 kGy had an increase in TFC, which was 3067.35 mg QE 100 g^{-1} DW, compared to TFC of the control, and with those treated with 3 kGy which were 2998.05 and 3005.52 mg QE 100 g^{-1} DW, respectively. However, the 9 kGy reduced the TFC to 1717.44 mg QE 100 g^{-1} DW.

This significant increase in TPC, and TFC could be due to the hydrolysis of tannins found in the pomegranate peels (having higher molecular weight), and the release of low molecular weight phenolic compounds (gallic acid, and tannic acid). The irradiation might break this complex to facilitate the release of bioactive ingredients, which could contribute to the increase in TPC (31). Bhatt et al. (32) observed a significant irradiation

dose-dependent increase in TPC by depolymerization of the cell wall polysaccharides, which is known to increase the activity of phenylalanine ammonialyase, the enzyme responsible for the synthesis of phenolic compounds.

Table 2. TPC (mg GAE 100 g^{-1} DW) and TFC (mg QE 100 g^{-1} DW) of the ethanol extract of γ-irradiated pomegranate peel samples

γ dose (kGy)	TPC	TFC
0	$9323.17 \pm 1.0401442^{B}$	$2998.05 \pm 1.706135985^{C}$
3	$9196.05 \pm 2.6137904^{C}$	$3005.52 \pm 0.790189851^{B}$
6	$9411.11 \pm 0.8544004^{A}$	$3067.35 \pm 1.2124356^{A}$
9	$8988.17 \pm 2.6401326^{D}$	$1717.44 \pm 1.3765537^{D}$

Means with the same letter in the same columns are not significantly different.

Antioxidant activity of PE

Tables 3, and 4 show the increase in the three parameters of antioxidant activity evaluated (DPPH, βCB and FRAP) at 6 kGy, which was the dose that induced a higher antioxidant activity compared to the control, 3, and 9 kGy. Kumari et al. (31) found an increase in gallic acid levels and TPC in the water extract of triphala (*Emblica officinalis),* due to irradiation that led to an increase in the antioxidant potential.

Table 3. DPPH radical scavenging activity (%) of PE of irradiated pomegranate peel samples

γ dose (kGy)	DPPH radical scavenging activity (%)		
	Time (min)		
	0	**30**	**60**
0	88.02	91.13	91.40
3	89.35	90.33	91.67
6	89.91	90.61	92.41
9	87.46	89.68	90.47
BHT (200 ppm)	25.09	48.56	61.97

Table 4. β-carotene/linoleic acid bleaching (βCB) system and ferric reducing power (FRAP) of PE of γ-irradiated pomegranate peel samples

γ-dose (kGy)	βCB	FRAP (O.D.)
	Peel	**Peel**
0	70.01	2.676
3	70.31	2.794
6	70.60	2.868
9	68.83	2.571
BHT (200 ppm)	60.85	1.019

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The irradiation caused either an increase or decrease in the antioxidant properties of fresh plants, which depended on the dose delivered, the raw material used, and exposure time. The enhanced antioxidant capacity (correlation with the TPC and antibacterial activity) of a plant after irradiation is mainly attributed either to the increase in enzyme activity (e.g., phenylalanine ammonialyase and peroxidase) or to the increased extractability of phenolics from the tissues (32). The increase or decrease in the antioxidants of irradiated samples might also be due to the solvents used in the extraction (33). However, depending on the technological criteria, the mechanism of the increase or decrease in the antioxidants can vary. The differences in the constituents of nutmeg oil and an increase in the levels of phenolic acids were detected after γ-irradiation, which was attributed to the degradation of tannins, and consequently to high extractability of phenolic acids (13). On the other hand, the decrease in the antioxidants caused in PE of irradiated peel at 3 and 9 kGy compared to 6 kGy could be attributed to the formation of radiation-induced degradation products or the formation of free radicals (34). Breitfellner et al. (35) reported that γ-irradiation (1-10 kGy) of strawberries resulted in degradation of phenolic acids. The decomposition of phenolic acids was attributed to the formation of free hydroxyl radicals ($OH^{\cdot}$) during treatment.

Antibacterial activity of PE

The efficiency of PE at the dose levels of 3, 6, and 9 kGy on the antibacterial activity is presented in Figure 1. All irradiated PE were effective against tested strains (*Bacillus cereus*, and *Staphylococcus aureus* as Gram positive, as well as *Escherichia coli, Klebsiella penumoneae, Pseudomonas aeruginosa,* and *Salmonella typhumurium* as Gram negative bacteria) with the differences between the strains variety and γ irradiation doses. This may be due to the strain sensitivity and effect of irradiation doses on the extract efficacy. A comparison of the antibacterial potential for the extracts shows that for all microorganisms the most effective was PE treated with 6 kGy, whereas 3 and 9 kGy had lower inhibitory activities. These results are in a good correlation with the TPC of PE. The 6 kGy-irradiated PE exhibited an increment in TPC, and TFC compared to 3 and 9 kGy treatment which caused reduction in TPC and TFC compared to the non-irradiated PE.

There has been no sufficient research in this field, to explain the effect of γ irradiation on antibacterial activity of pomegranate peel. Thus, Khattak et al. (36) showed that γ radiation did not have any effect on the antimicrobial activity of the *Nigella sativa* seed up to 10 kGy doses. Mishra et al. (37) found that the antimicrobial and sensory traits of tea samples were unaffected by radiation treatment up to 10 kGy.

Application of 6 kGy-irradiated PE in beef sausage

Physicochemical properties of sausage during cold storage. The analysis of TBARS measures the induction of secondary lipid oxidation products, mainly malonaldehyde, which might be responsible for the off-flavor of oxidized fat. Table 5 shows the effect of PE, and γ irradiation on TBARS (mg MDA/kg) of the sausages during cold storage. At

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the start, the TBARS of control sample was 0.456 mg MDA/kg, whereas it was increased in the treated samples, ranging between 0.474 and 0.516 mg MDA/kg for the sausage irradiated at 3 and 5 kGy, respectively. This increase caused by irradiation might be due to that fact that irradiation induced lipid oxidation through the hydroxyl radicals generated by ionizing irradiation in the meat products (38). The increase in TBARS values may be due to the indirect effect of γ radiation through the liberation of free radicals upon the radiolysis of water, which enhances lipid oxidation (39). On the other hand, the treatment with PE showed a decrease in the TBARS values compared to the control and irradiated samples at the starting time. In the beef sausage samples containing 3, 6, and 12 mL/kg of PE the TBARS values were lowered to 0.208, 0.188, and 0.179 mg MDA/kg, respectively. This reduction may be due to the presence of phenolic compounds in the PE, and their effect as antioxidants (Tables 2-4), which delayed lipid oxidation during the sausage formulation and storage.

Table 5. Effect of PE and γ irradiation on TBARS (mg malonaldehyde/kg on wet weight bases) of beef sausage during cold storage

Storage period (day)	control	PE concentration (mL/kg)			γ irradiation (kGy)	
		3	6	12	3	5
0	0.456 ± 0.010149 ^{Cd}	0.208 ± 0.004 ^{Dg}	0.188 ± 0.002 ^{Eh}	0.179 ± 0.002646 ^{Ej}	0.474 ± 0.011136 ^{Bd}	0.516 ± 0.013 ^{Ag}
5	0.595 ± 0.026458 ^{Ac}	0.228 ± 0.015588 ^{CEf}	0.207 ± 0.039038 ^{Ch}	0.196 ± 0.003606 ^{Cj}	0.500 ± 0.05 ^{Bcd}	0.601 ± 0.004583 ^{Af}
10	0.685 ± 0.055678 ^{Ab}	0.299 ± 0.009539 ^{Ce}	0.278 ± 0.01179 ^{CDg}	0.249 ± 0.026458 ^{Di}	0.551 ± 0.002646 ^{Bcd}	0.687 ± 0.006083 ^{Ae}
15	0.898 ± 0.021517 ^{Aa} R	0.365 ± 0.004359 ^{DEd}	0.333 ± 0.026458 ^{EDf}	0.297 ± 0.009165 ^{Eh}	0.619 ± 0.045826 ^{Cb}	0.734 ± 0.002646 ^{Bd}
20		0.437 ± 0.0043589 ^{Cc}	0.412 ± 0.0034641 ^{Ce}	0.386 ± 0.0051962 ^{Dg}	0.710 ± 0.052915 ^{Ba} R	0.805 ± 0.0026458 ^{Ac}
25		0.544 ± 0.005568 ^{Bb}	0.526 ± 0.002646 ^{Cd}	0.476 ± 0.004583 ^{Df}		0.889 ± 0.005292 ^{Ab}
30		0.645 ± 0.004359 ^{Ba} R	0.592 ± 0.005292 ^{Cc}	0.534 ± 0.002646 ^{De}		1.089 ± 0.003606 ^{Aa} R
35			0.670 ± 0.026458 ^{Ab}	0.644 ± 0.003464 ^{Ad}		
40			0.719 ± 0.005196 ^{Aa} R	0.710 ± 0.026458 ^{Ac}		
45				0.778 ± 0.004359 ^{b}		
50				0.881 ± 0.002646 ^{a} R		

Means with the same capital letter in the same rows are not significantly different,
Means with the same small letter in the same columns are not significantly different, R= rejected.

The results are in agreement with Ahn et al. (40) and Fernández-López et al. (41) who applied other natural antioxidants in cooked beef and pork. A relationship between TPC and antioxidant impact of PE has been mentioned by Negi and Jayaprakasha (42), and Rojas and Brewer (43) in beef, pork and chicken patties. In addition, Devatkal et al. (44) found a positive correlation between the TPC of pomegranate as well as kinnow by-products extracts, and the reduction of TBARS in cooked goat meat patties. Naveena et al. (45) found that pomegranate rind powder reduced the TBARS values in chicken patties. Even though the phenolic compounds reported to be mainly responsible for antioxidant traits in fruits, it is possible to assume a synergy between phenolic compounds.

In general, storage time had an impact on lipid oxidation in all investigated samples of beef sausage shown by an increase in TBARS values during cold storage. The data in Table 5 indicate that TBARS value of control sample was 0.898 mg MDA/kg at the rejected time (15 days). The increase in TBARS values in the PE-enriched samples was very slow, and remained the lowest (<0.9 mg MDA/kg sample) up to 50 days in the sample treated with 12 mL/kg PE. The high levels of phenolics contained in PE extract may cause its high antioxidant potential (8). Pomegranate peel phenolics may act in a similar way as a reducer by donating electrons, and reacting with free radicals to convert them to more stable products, and terminate free radical chain reactions (42). In addition, the TBARS values of the samples trod the same direction of increase as the effect of cold storage, and they were: 0.645, 0.719, 0.881, 0.710 and1.089 mg MDA/kg, corresponding to the rejected time of 30, 40, 50, 20, and 30 days of storage for sausage containing PE at concentrations of 3, 6, and 12 mL/kg, and irradiated at 3, and 5 kGy, respectively. There was a correlation between the phenolic content and antioxidant effect of PE, and its role in the resistance to lipid oxidation.

The results of the present study show that the addition of gamma-irradiated PE to beef sausage delayed lipid oxidation. This can be ascribed to the ability of the phenolic compounds to dump the process of the free radical formation, and the propagation of free radical reactions through the chelation of transition metal ions (46). The antioxidant potential of phenolics is associated with the hydroxyl group linked to the aromatic ring, which is capable of donating hydrogen atoms with electrons, and neutralizing free radicals, so this mechanism blocks degradation to more active oxidizing forms (47, 48).

TVBN. The contents of TVBN in the formulated beef sausage samples containing PE, irradiated and control sausage samples were determined at the starting time and during storage, as shown in Table 6. As can be seen from the table, the control samples of beef sausage had an initial content of 9.82 mg N/100g on wet weight basis for TVBN. The addition of PE at 3, 6, and 12 mL/kg, and irradiation treatment at 3, and 5 kGy had significant effects on the contents of TVBN in beef sausage samples as their contents amounted to 9.69, 9.60, 9.51, 9.44, and 9.65 mg N/100g on wet weight basis, respectively. Table 6 shows that TVBN values increased significantly in all samples during the storage, and it was the highest in the control. The amounts of TVBN reached 20.20, 19.61, 21.87, 21.63, 19.45, and 21.01 mg N/100g on wet weight basis after 15, 30, 40, 50, 20, and 30 days of storage, respectively.

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Table 6. Effect of PE and γ irradiation on TVBN (mg N/100g) on wet weight basis of beef sausage during cold storage

Storage period (day)	control	PE concentration (mL/kg)			γ irradiation (kGy)	
		3	6	12	3	5
0	09.82 ± 0.108167^{Ad}	09.69 ± 0.036056^{Bg}	09.60 ± 0.051962^{BCh}	09.51 ± 0.055678^{CDj}	09.44 ± 0.026458^{De}	09.65 ± 0.043589^{BCg}
5	16.22 ± 0.051962^{Ac}	11.34 ± 0.043589^{Df}	11.12 ± 0.075498^{Eg}	11.02 ± 0.026458^{Ei}	12.48 ± 0.091652^{Cd}	12.97 ± 0.026458^{Bf}
10	19.70 ± 0.052915^{Ab}	13.41 ± 0.08544^{De}	13.04 ± 0.034641^{Ef}	12.76 ± 0.045826^{Fh}	15.53 ± 0.026458^{Cc}	15.84 ± 0.036056^{Be}
15	20.20 ± 0.43589^{Aa} R	15.33 ± 0.060828^{Dd}	15.11 ± 0.026458^{De}	14.33 ± 0.036056^{Eg}	18.26 ± 0.052915^{Cb}	18.79 ± 0.034641^{Bd}
20		17.21 ± 0.045826^{Cc}	16.94 ± 0.10583^{Dd}	15.89 ± 0.06245 ^{Ef}	19.45 ± 0.079373^{Ba} R	19.93 ± 0.043589^{Ac}
25		18.11 ± 0.034641^{Bb}	17.80 ± 0.026458^{Cd}	16.54 ± 0.045826^{De}		20.41 ± 0.026458^{Ab}
30		19.61 ± 0.036056^{Ba} R	19.28 ± 0.045826^{Cc}	17.09 ± 0.043589^{Dd}		21.01 ± 0.173205^{Aa} R
35			20.07 ± 0.055678^{Ab}	18.87 ± 0.060828^{Bc}		
40			21.87 ± 0.052915^{Aa} R	19.28 ± 0.036056^{Bc}		
45				20.01 ± 0.985038^{b}		
50				21.63 ± 0.081854^{a} R		

The data for the TVBN contents in all analyzed samples reveal that the effects of irradiation on the formation of TVBN agree with those obtained by many investigators who found that γ irradiation, and pomegranate extracts had no remarkable effects on the contents of TVBN (49, 50). The TVBN is produced from degradation of proteins and non-protein nitrogenous compounds, mainly as a result of microbial activity (50). Moreover, the addition of PE, and application of γ irradiation lowered the rate of TVBN formation during cold storage of samples as compared with the control. This may be due to the inhibition of the microorganisms capable of producing those compounds through the application of PE and irradiation treatments.

Microbial quality of sausage during cold storage

Total bacterial count (cfu/g). The microbiological quality of meat products is dependent on a number of factors such as raw materials, hygienic conditions during the process, and storage period. The data in Table 7 show the effect of PE and γ irradiation on

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total bacterial counts of beef sausage during cold storage. It can be seen that the control samples of beef sausage had an initial total bacterial count of $7.8x10^5$ cfu/g. These results agree with those who found that sausage had an initial total bacterial count of more than 10^6 cfu/g (51). The formulation of sausage samples with PE at concentrations of 3, 6, and 12 mL/kg had almost similar initial counts for total bacterial count, and they were: $7.7x10^5$, $7.6x10^5$ and $7.5x10^5$ cfu/g, respectively, compared to the control ($7.8x10^5$ cfu/g). Meanwhile, the initial count of the irradiated sausage samples showed a gradual decrease depending on the irradiation dose ($4.9x10^4$ and $7.1x10^3$ cfu/g at 3 and 5 kGy, respectively), compared with the control sample.

Table 7. Effect of PE and γ irradiation on total bacterial count (cfu/g) of beef sausage during cold storage

Storage Period (day)	control	PE concentration (mL/kg)			γ irradiation (kGy)	
		3	6	12	3	5
0	$7.8x10^5$	$7.7x10^5$	$7.6x10^5$	$7.5x10^5$	$4.9x10^4$	$7.1x10^3$
5	$5.6x10^6$	$9.8x10^5$	$7.9x10^5$	$7.1x10^5$	$8.5x10^4$	$1.8x10^4$
10	$9.5x10^6$	$1.5x10^6$	$8.7x10^5$	$8.3x10^5$	$2.5x10^5$	$8.5x10^4$
15	$3.4x10^7$ R	$4.4x10^6$	$9.8x10^5$	$9.5x10^5$	$5.9x10^6$	$3.1x10^5$
20		$6.2x10^6$	$1.8x10^6$	$1.1x10^6$	$2.9x10^7$ R	$9.8x10^5$
25		$7.6x10^6$	$3.3x10^6$	$3.4x10^6$		$6.9x10^6$
30		$9.1x10^6$ R	$6.1x10^6$	$5.1x10^6$		$2.1x10^7$ R
35			$9.2x10^6$	$6.3x10^6$		
40			$4.1x10^7$ R	$7.9x10^6$		
45				$9.8x10^6$		
50				$3.3x10^7$ R		

R= rejected

The subsequent refrigerated storage increased the counts of the total bacterial in all sausage samples but at higher rates in the control, whereas, their initial count reached $3.4x10^7$ after 15 days of cold storage. At that time samples were rejected due to the increase in the total bacterial count to more than $1x10^7$ cfu/g and/or sensory evaluations. In addition, the formulation of sausages containing PE at concentrations of 3, 6, and 12 mL/kg, and their irradiation at 3, and 5 kGy prolonged the shelf-life from 15 days for the control sausage to a long storage period, even when the total bacterial count reached $9.1x10^6$, $4.1x10^7$, $3.3x10^7$, and $2.9x10^7$ $2.1x10^7$ cfu/g after 30, 40, 50, 20, and 30 days of cold storage, respectively. Similar results were also reported for fresh ground beef enriched with grape seed and pine bark extracts (52). Vaithiyanathan et al. (53) also reported a delay in the microbial growth in spent hen patties containing methyl gallate. Naveena et al. (45) reported an inhibition of microbial growth in buffalo meat streaks dipped in clove oil. PE was used to prolong the shelf life of chicken meat products by controlling bacterial growth and oxidative rancidity (54).

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Generally, depending on the antibacterial effect, the sausage samples containing PE at 12 mL/kg was the best treatment of samples formulated with PE, while the sausage samples irradiated at 5 kGy were better than those irradiated at 3 kGy. Moreover, the sausage samples formulated with PE at 12 mL/kg were better compared to the samples irradiated at 5 kGy. This may be due to the content of polyphenolic compounds in PE, and their effect as antimicrobial agent, as shown in Figure 1.

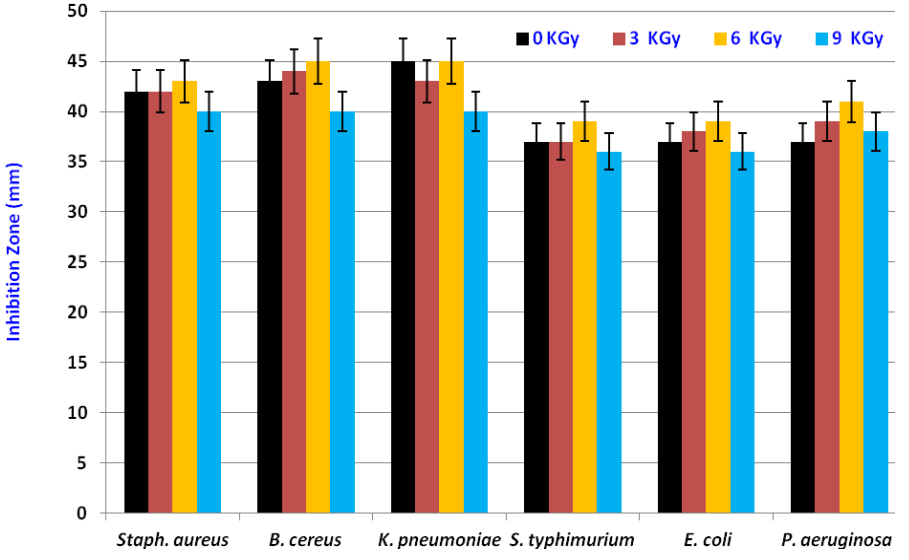

Figure 1. Antibacterial activity of γ-irradiated PE against bacterial strains

Therefore, the phenolics might have an effect on the microbial growth in the samples treated with PE, which may be due to the protein binding or enzyme inhibition (55). These results provide evidence on the presence of antimicrobial bioactive compounds in the PE. These bioactive compounds can degrade the cell wall, damage membrane proteins, disrupt the cytoplasmic membrane, and interfere with membrane-integrated enzymes, which may lead to the cell death (56). This antibacterial effect of all the extracts may be due to TPC which affect bacterial cell through the changes in the bacterial membrane physicochemical character, changes in the surface charge of cells to less negative values, compromise the integrity of the cytoplasmic membrane or alteration in the cytoplasmic membrane permeability and K^+ release (57). Phenolics have been reported to exhibit antibacterial activities with distinguished characteristics in their reactivity with proteins related polyamides (58). The inhibition of microorganisms by phenolics may be due to iron chelation or hydrogen bounding with vital proteins such as microbial enzymes (59). Phenolic compounds, notably proanthocyanidins (often called condensed tannins), are

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vulnerable to polymerization in air through oxidization reactions. Therefore, an important factor governing their toxicity is the extent of their polymerization. The oxidized condensation of phenols may result in the toxification of the microorganisms. On the other hand, polymerization can result in the detoxification of phenolics (60). This supports the fact that polyphenols may be responsible for the antimicrobial traits of the extracts.

Psychrophilic bacteria (cfu/g). Normally, the psychrophilic bacteria count in refrigerated foods was the main group among other bacteria group. The psychrophilic bacteria count is an important parameter of the microbiological quality of foods, especially refrigerated foods. The psychrophilic bacteria counts were determined in all samples containing PE at the concentrations of 3, 6, and 12 mL/kg, irradiated and control samples during storage, as shown in Table 8.

Table 8. Effect of PE and γ irradiation on total psychrophilic bacteria (cfu/g) of beef sausage during cold storage

Storage Period (day)	control	PE concentration (mL/kg)			γ irradiation (kGy)	
		3	6	12	3	5
0	$6.9x10^4$	$6.7x10^4$	$6.5x10^4$	$6.3x10^4$	$6.3x10^3$	$3.3x10^2$
5	$2.2x10^5$	$7.6X10^4$	$7.3X10^4$	$7.0X10^4$	$1.0x10^4$	$7.2x10^2$
10	$7.7x10^5$	$9.1x10^4$	$9.5x10^4$	$7.9x10^4$	$5.6x10^4$	$2.2x10^3$
15	$6.7x10^6$ R	$2.8x10^5$	$2.1x10^5$	$9.6x10^4$	$1.1x10^5$	$8.3x10^3$
20		$5.7x10^5$	$5.6x10^5$	$2.1x10^5$	$8.3x10^5$ R	$3.1x10^4$
25		$1.8x10^6$	$8.9x10^5$	$4.2x10^5$		$9.2x10^4$
30		$5.4x10^6$ R	$1.4x10^6$	$7.4x10^5$		$5.4x10^5$ R
35			$4.2x10^6$	$9.8x10^5$		
40			$8.6x10^6$ R	$2.2x10^6$		
45				$5.4x10^6$		
50				$8.3x10^6$ R		

R = rejected

The data reveal that the initial count of total psychrophilic bacteria was $6.9x10^4$ cfu/g in the control sample. The formulated beef sausage samples containing PE at the concentrations of 3, 6, and 12 mL/kg nearly had no almost changes in this initial count of the control samples at zero time, and were $6.7x10^4$, $6.5x10^4$ and $6.3x10^4$ cfu/g, respectively. Irradiation of the sausage samples at 3, and 5 kGy reduced the initial count of the control at zero time to $6.3x10^3$, $3.3x10^2$ cfu/g, respectively.

However, the storage of the samples at 4°C gradually increased the counts of the total psychrophilic bacteria for all sausage samples, with higher rates in the control, where it reached $6.7x10^6$ cfu/g. The count of psychrophilic bacteria increased to $5.4x10^6$, $8.6x10^6$, $8.3x10^6$, $8.3x10^5$, and $5.4\ x10^5$ cfu/g in the formulated beef sausage samples containing PE at the concentrations of 3, 6, and 12 mL/kg and those that received 3, and 5 kGy dose on day 30, 40, 50, 20, and 30 days of storage, respectively. At that time, the samples were rejected due to the increase in the total bacterial count to more than $1x10^7$ cfu/g and/or sensory evaluations. These results are close to those obtained by Badr (44); Badr and Ka-

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rema (51). The noticed variation in shelf-life between treatments based on the variation of the concentration of TPC added to the formulated sausage with PE, and its antimicrobial impact.

Total yeasts and molds (cfu/g). The molds, and yeasts counts are very important for indicated quality of foods. The data in Table 9 show the effect of addition of PE and irradiation on the yeasts and moulds counts during cold storage. The control had an initial count of $6.8x10^3$ for yeasts and moulds. The samples containing PE at the concentrations of 3, 6, and 12 mL/kg had no changes in the initial counts of the control samples at zero time, and these were: $6.5x10^3$, $6.3x10^3$, and $6.1x10^3$ cfu/g, respectively. The irradiation treatment of the sausage samples at 3, and 5 kGy reduced the initial count of control at zero time, to reach $5.2x10^2$, $8.9x10^1$ cfu/g, respectively.

Table 9. Effect of PE and γ irradiation on total yeasts and molds (cfu/g) of beef sausage during cold storage

Storage Period (day)	control	PE concentration (mL/kg)			γ irradiation (kGy)	
		3	6	12	3	5
0	$6.8x10^3$	$6.5x10^3$	$6.3x10^3$	$6.1x10^3$	$5.2x10^2$	$8.9x10^1$
5	$2.3x10^4$	$7.1x10^3$	$7.0x10^3$	$6.4x10^3$	$8.6x10^2$	$2.1x10^2$
10	$8.3x10^4$	$8.7x10^3$	$8.5x10^3$	$7.5x10^3$	$3.2x10^3$	$4.3x10^2$
15	$2.8x10^5$ R	$1.5x10^4$	$9.8x10^3$	$8.9x10^3$	$8.9x10^3$	$8.6x10^2$
20		$3.4x10^4$	$1.4x10^4$	$1.3x10^4$	$3.6x10^4$ R	$1.0x10^3$
25		$8.2x10^4$	$3.1x10^4$	$2.9x10^4$		$4.8x10^3$
30		$3.1x10^5$ R	$5.2x10^4$	$4.1x10^4$		$7.8x10^3$ R
35			$8.6x10^4$	$6.3x10^4$		
40			$2.5x10^5$ R	$8.8x10^5$		
45				$1.5x10^4$		
50				$3.9x10^5$ R		

R = rejected

During the storage, further gradual increases in the counts of total yeasts and molds were observed for both the control and treated samples. The count of total yeasts and molds at rejected time of 15, 30, 40, 50, 20 and 30 days were $2.8x10^5$, $3.1x10^5$, $2.5x10^5$, $3.9x10^5$, $3.6x10^4$, and $7.8x10^3$ cfu/g for the control and formulated beef sausage containing PE at the concentrations of 3, 6 and 12 mL/kg and irradiated at 3 and 5 kGy, respectively. The noticed variation in the shelf-life between the treatments are based on the variation in the concentration of TPC added to the formulated sausage as PE and their effect as antimicrobial agents.

Coliform group (cfu/g). The coliform group counts is a very important to indicator of the quality of foods. The coliform group counts were determined in beef sausage for all treatments during cold storage. The data in Table 10 indicate that the irradiation completely inhibited the coliform group counts in beef sausage samples treated with doses 3 and 5 KGy. However, the coliform group counts of the control sample were $3.6x10^2$ cfu/g

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at zero time. The samples of formulated beef sausage containing PE at the concentrations of 3, 6, and 12 mL/kg had almost no changes in the initial count zero time as they were: $3.6x10^2$, $3.4x10^2$ and $3.2x10^2$ cfu/g, respectively. The effects of irradiation of the sausage samples at 3, and 5 kGy were not detected during the storage period. The control sausage samples, and formulated sausage containing PE at the concentrations of 3, 6, and 12 mL/kg were rejected after 15, 30, 40, 50, 20, and 30 days of cold storage, at corresponding of the coliform group counts reaching $2.4x10^3$, $1.6x10^3$, $1.4x10^3$, and $2.1\ x10^3$ cfu/g, respectively, and were not detected for the samples irradiated with 3, and 5 kGy. The results of irradiation and cold storage were similar to those by Vural et al. (61) and Gumus et al. (62). The noticed variation in shelf-life between treatments are due to the variation of concentration of TPC added to formulated sausage as PE, and its antimicrobial effect.

Table 10. Effect of PE and γ irradiation on coliform group (cfu/g) of beef sausage during cold storage

Storage Period (day)	control	PE concentration (mL/kg)			γ irradiation (kGy)	
		3	6	12	3	5
0	$3.6x10^2$	$3.6x10^2$	$3.4x10^2$	$3.2x10^2$	ND	ND
5	$5.1x10^2$	$4.0x10^2$	$3.9x10^2$	$3.6x10^2$	ND	ND
10	$8.3x10^2$	$4.7x10^2$	$4.4x10^2$	$4.1x10^2$	ND	ND
15	$2.4x10^3$ R	$5.6x10^2$	$5.2x10^2$	$4.8x10^2$	ND	ND
20		$6.5x10^2$	$6.0x10^2$	$5.5x10^2$	ND R	ND
25		$8.9x10^2$	$7.2x10^2$	$6.3x10^2$		ND
30		$1.6x10^3$ R	$8.5x10^2$	$7.0x10^2$		ND R
35			$9.7x10^2$	$7.7x10^2$		
40			$1.4x10^3$ R	$8.3x10^2$		
45				$9.8x10^2$		
50				$2.1x10^3$ R		

R= rejected

CONCLUSION

The present study aimed to evaluate the antioxidant and antibacterial activity of ethanol extracts of irradiated pomegranate peel and the effect of PE on the shelf-life of beef patty. Generally, the obtained results indicate that irradiation of pomegranate peel at a dose level of 6 kGy enhanced the extraction efficiency, and extract efficacy. Ethanol extract of irradiated pomegranate peel at a dose level of 6 kGy may be considered as a good source of natural antioxidants with antibacterial activity which could be suitable as a potential ingredient for food products. Thus, these observations indicate that the selective extraction of the antioxidants from natural sources by appropriate solvent can be very important in obtaining fractions with high antioxidant activity. However, the random effect of irradiation treatment can be useful to enhance the antioxidant activity of pome-

granate peel shown at a 6 kGy dose. The addition of PE of irradiated pomegranate peel at 6 kGy dose to beef sausage led to the improvement in the microbiological properties of the sausage without remarkable changes in the physicochemical properties compared to the irradiation treatment.

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ОДРЖАВАЊЕ КВАЛИТЕТА ТЕЛЕЋЕ КОБАСИЦЕ ПРИМЕНОМ ЕТАНОЛНОГ ЕКСТРАКТА ПРАХА НАРА ОЗРАЧЕНОГ ГАМА-ЗРАЦИМА

Ибрахим Али Солиман Мохамед[1], Фавзи Рамадан Хасаниен[2], Камал Махфуз Ел Сахи[2], Сајед Ахмед Мохамед Афифи[1] и Мохамед Хасан Мохамед Абделдајем[1]*

[1] Нуклеарни истраживачки центар, Управа за атомску енергију, Каиро, Египат
[2] Департман науке о храни, Пољопривредни факултет, Универзитет у Загазигу, Загазиг, 44519 Египат

Циљ овог рада је био да се испита антиоксидативна и антибактеријска активност етанолног екстакта праха коре нара (*Punica granatum*) озраченог дозама гама-зрака од 0, 3, 6, и 9 kGy. Антиоксидативна активност екстракта је процењивана на основу његове активности као хватача радикала 2,2′-дифенил-1-пикрилхидразил (DPPH•), бељења система β-каротен/линолне киселине и способности редукције фери јона. Антибактеријска активност ектракта је тестирана на следеће родове бактерија: *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Klebsilla penumoneae, Pseudomonas aeruginosa*, and *Salmonella typhumurium*. Резултати су показали да прах коре нара третиран дозом од 6 kGy је имао већи садржај укупних фенолних једињења, укупних флавоноидних једињења и већу антиоксидативну активност. Због тога је прах коре нара озрачен дозом од 6 kGy одабран као додатак телећој кобасици. Испитан је утицај различитих концентрација овог праха са циљем побољшања хигијенског квалитета телеће кобасице и продужење рока трајања у току хладног складиштења (4°C). Резултати су показали да када се 12 mL екстракта дода у један килограм телеће кобасице њено време трајања се продужава са 15 на 50 дана (на 4°C), у поређењу са контролом, без промена њених микробиолошких, хемијских и сензорних карактеристика.

Кључне речи: кора нара, полифенол, флавоноид, антиоксидативна актиност, DPPH•, β-каротен, γ-зрачење

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MACRO- AND MICRO-ELEMENT ANALYSIS IN MILK SAMPLES BY INDUCTIVELY COUPLED PLASMA – OPTICAL EMISSION SPECTROMETRY

Sanja M. Petrović, Saša R. Savić and Živomir B. Petronijević*

University of Niš, Faculty of Technology, Bulevar Oslobodjenja 124, 16000 Leskovac, Serbia

The paper describes the determination of Ag, Al, B, Ba, Bi, Ca, Cd, Co, Cr, Cu, Fe, Ga, In, K, Li, Mg, Mn, Na, Ni, Pb, Sr, Tl and Zn, as well as total fat content of milk samples, originated from different sources. The analyzed milk samples were: human milk, fresh cow milk, pasteurized cow milk from a local market, and reconstituted powder milk. The milk samples were obtained from Jablanica District (Serbia) territory. Preparation of samples for macro- and micro-analyses was done by wet digestion. Concentrations of the elements after digestion were determined by inductively coupled plasma optical emission spectrometry (ICP-OES). Total fat content of milk samples was determinate by the Weibull and Stoldt method. The results showed that potassium and calcium concentrations were the highest in all samples: 1840.64 – 2993.26 mg/L and 456.05 – 1318.08 mg/L, respectively. Of all heavy metals from the examined milk samples (copper, zinc, manganese, nickel, cadmium, and lead), the most common were zinc and copper, with approximately similar content in the range of 5 – 12 mg/l, while cadmium nickel and manganese were not detected at all. Samples of fresh cow milk and human milk showed the highest fat content of 3.6 and 4.2 %, respectively. Results for total fat and macro- and micro-analyses showed that fresh cow milk has the highest contents of fat and calcium, making it the most nutritious.

KEY WORDS: milk, ICP-OES, minerals, fat

INTRODUCTION

Milk is a natural and almost the most complete source of food in the daily human diet. It contains highly valuable components as proteins, fats, sugars, vitamins and minerals in a form that the body can quickly use it in the easiest possible way. Cow, sheep, goat, buffalo and even mare's milk is used for human consumption. Of all the above mentioned types of milk, the most used is cow's milk. Mare's milk is the most consumed in Asia, particularly in the area of Kazakhstan and Turkmenistan, and more recently appeared in the United States. Nutritional value of milk originates from a large number of ingredients that are essential for the smooth running of the growth process, development and mainte-

∗ Corresponding author: Sanja M. Petrović, University of Niš, Faculty of Technology, Bulevar oslobodjenja 124, 16000 Leskovac, Serbia, e-mail: milenkovic_sanja@yahoo.com

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nance of the body. Milk is also an important factor in the nutrition of the elderly, sick and those who recover from some disease. It is the basis of many delicious dairy products and beverages such as sour milk, yogurt, acidophilus milk, kefir, cheese, butter, cream, sour cream and more. Milk is also an integral part of many nutritious foods adds of various types of milk bread, ice cream, chocolate, various types of test, candy, etc. In addition, reconstituted powder milk is frequently used as a milk substitute. It is especially used in industrial and commercial outputs. Its advantage is that it can be stored for a long time. Besides breasts milk, cow milk as well as powder milk are first and basic foods of very small children and children in general. Mature human milk is produced in the mother's breast, starting from the third or fourth week after the delivery. However, the milk composition changes during the day or during feeding (1-2). For example, in the morning it contains less fat. Use of reconstituted powder milk is also common in the children diet, and its use is possible after reconstituting in water in a proper volume. The value of this kind of milk is almost the same, as well as fresh. Reconstituted powder milk is obtained by evaporation of ordinary cow's milk or other. There are mainly two types: whole and skimmed milk powder. The skimmed milk powder is used for a diet food, particularly in gastric and intestinal diseases.

Mineral substances in milk, particularly macro-elements, play an important role in establishing the physical-chemical balance of the colloidal system, determining the properties of the milk. Particularly significant is calcium, which has a great influence on the size of the casein particles (2). Indirectly, it also influences the thermal stability of the milk. The amount of calcium in milk, as well as the state in which it is found, is of great importance in many processes as making cheeses. The micro-elements content of milk in most cases depends of animal feed and grazing. In most cases, animal feed contains sufficient amounts of these elements to meet the needs of the animal organism. In case of a deficit, some of these elements, as well as I, Co, Mn and others, have to be partially added to the diet, in order to avoid serious disturbances in the metabolism (decrease in productivity, decrease of reproducibility, loss of appetite (3)). In addition to the macro and micronutrients present in milk, fat is also important as the main energy source. Fats contain all the components that can be extracted by anhydrous ether, and that after one hour of drying in an oven at 100^{o}C do not evaporate. This extract, in addition to fat, contains also some other components, waxes, etc., and therefore this extract is called ether extract and crude fat. The determination of fat content in groceries is of great importance because it evaluates the energy value of foods and performs their classification. Milk contains the appropriate fatty acids required for the growth of the brain. The diet of animals or mothers may affect the level of fat in the milk. Human milk contains enzymes that are required for the dissolution of fats. In contrary to this, adapted milk does not contain digestive enzymes. The fat content of life groceries ranges from less than 0.1% (in vegetables) to nearly 100% (in natural fats and oils).

From all these reasons it is very important to evaluate content of macro- and micro-elements themselves, as well as fat content, because milk itself and milk products are widely consumed throughout the world. The content of Ag, Al, As, B, Ba, Bi, Ca, Cd, Co, Cr, Cu, Fe, Ga, In, K, Li, Mg, Mn, Na, Ni, Pb, Sr, Tl and Zn, as well as fat content in the milk samples are determinate in this work. The aim of the study was to determine the

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content of nutritive elements and trace elements in milk samples which are available on the markets from the territory of the Jablanica District (Serbia), by inductively coupled plasma optical emission spectrometry (ICP-OES).

EXPERIMENTAL

Reagents and standard solutions

In order to estimate the content of macro- and micro-elements the calibration standards were prepared. Multistandard IV - standard solution (Merck), which contained Al, Ag, B, Ba, Be, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Se, Ti, V and Zn at a concentration of 1000 ppm, was used for the preparation of calibration solutions. Distilled water, purified by Fisher Chemical (HPLC grade) was used for dilution of milk samples. The carrier gas was Argon 5.0 (99.999% purity). Standard solutions were prepared by diluting the multistandard IV, so that the concentrations of standards for the calibration chart were in the range of expected test elements concentrations. Table 1 shows the wavelength detection of each element in the samples, correlation coefficient (R^2), limit of detection (LOD), as well as the range of linearity.

Table 1. Calibration parameters: λ, nm; R^2; LD (μg/L) and the range of linearity (μg/L)

Element	Detection wavelength (nm)	Correlation coefficient (R^2)	Limit of detection (μg/L)	Linearity range (mg/L)
Ag	224.641 328.068	0.99993 0.99995	0.39	0.0145–12.00 0.000399–12.00
Al	167.078 394.401	0.99985 0.99998	7.6×10^{-2}	7.6×10^{-5}–2.40 0.00155–12.00
B	182.641 249.773	0.99999 0.99999	6.43	0.00737–12.00 0.00643–12.00
Ba	233.527	0.99995	0.183	0.000183–12.00
Bi	190.241 223.061	0.99991 0.99996	3.53	0.00353–12.00 0.00368–12.00
Ca	183.801 396.847	0.99995 0.99946	2.14	1.6–480.00 0.00214–2.41
Cd	214.438	0.99994	0.127	0.000127–12.00
Co	228.616	0.99980	0.327	0.000327–12.00
Cr	283.563	0.99998	0.435	0.000435–12.00
Cu	224.700 324.754	0.99999 0.99999	0.259	0.000907–12.00 0.000259–12.00

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Table 1. Continuation

Element	Detection wavelength (nm)	Correlation coefficient (R^2)	Limit of detection (μg/L)	Linearity range (mg/L)
Fe	259.941	0.99997	0.118	0.000118–12.00
Ga	417.206	0.99994	1.7	0.0017–12.00
In	325.609	1.00000	4.0	0.004–12.00
K	404.721 766.491	0.99998 0.99999	0.378	0.798–300.00 0.000378–1.20
Li	323.261 670.780	1.00000 0.99997	5.75×10^{-2}	0.0798–12.00 5.75×10^{-5}–1.20
Mg	279.553 285.213	0.99997 0.99994	0.115	0.000115–6.04 4.03–120.00
Mn	257.611	0.99992	3.57×10^{-2}	3.57×10^{-5}–12.00
Na	330.237 598.592	0.99994 0.99989	4.75	7.98–480.00 0.00475–12.00
Ni	231.604	0.99994	0.474	0.000474–12.00
Pb	220.353	0.99998	1.78	0.00178–12.00
Sr	407.771	0.99998	6.23×10^{-3}	6.23×10^{-6}–2.41
Tl	190.864	0.99998	1.72	0.00172–12.00
Zn	213.856	0.99997	8.2×10^{-2}	8.2×10^{-5}–12.00

Sampling and sample preparation

Four milk samples: human milk, fresh cow milk, pasteurized milk from a local market in Leskovac (Jablanica District), as well as reconstituted powder milk were taken for the analysis. Before the analysis, the milk powder was dissolved in distilled water (2 table spoons of powder in 100 ml of water). Preparation of the samples was carried out by wet digestion. To a 2-ml milk sample 2 ml of 98% nitric acid was added, and left for 24 h in digester for wet digestion. Before the ICP-OES analysis all samples were filtered (0.45 μm), and then diluted with distilled water purified by Fisher Chemical (HPLC grade).

Analysis of samples

The quantitative analysis of all samples was performed on ICP-OES (ARCOS FHE12, SPECTRO, Germany), according to the manufacturer's instructions. The instrument conditions and determined parameters are given in Table 2.

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Table 2. Operating conditions for ICP-OES (W)

Plasma Power (W)	1400
Gas flow (L/min) -*Coolant* -*Auxiliary*	 13.0 0.80
Nebulizer type	Cross flow
Nebulizer flow rate (L/min)	0.95
Pump speed	30
Stabilization time (s)	0
Number of probes for each measuring	3
Plasma observation	Axial

Determination of total fat content

The reference technique for measuring total fat content is the conventional acid hydrolysis followed by Soxhlet extraction (Weibull-Stoldt method) in petroleum ether (4-5). A volume of 20 ml of milk sample (human milk, fresh cow milk, pasteurized cow milk and prepared milk powder), together with 100 ml of distillate water and 60 ml of concentrated hydrochloric acid was heated in boiling water bath with occasional stirring for about 15 minutes. The glass vessel is then covered and left for about 30 min on the water bath at boiling temperature. Hot distillate water was then added to an approximately double volume. The solution was filtered through a wetted filter paper. After filtration, the filter paper was dried for 2-3 hours at 105ºC and then put directly into the desiccator. The dry residue is then extracted with petroleum ether for 1 h in the Soxhlet apparatus. After the fat extraction, the flask was dried in an oven at 105ºC for 1 hour to the constant weight.

Calculation:

$$\text{The total fat content (\%)} = (A\text{-}B) \times 100 / M, \quad [1]$$

where, A is the mass of the flask after drying, B is the mass of the empty flask, and M is the amount of the milk sample

RESULTS AND DISCUSSION

In order to estimate the nutritional value of different milk samples, content of 23 macro- and micro-elements are examined, and the results are shown in Table 3. The values for the particular elements in human and fresh cow milk samples were obtained as an average for three milk samples taken in the period of 24 h. The amounts of detected elements were compared between the samples as well as with the literature data, to see how the differences in milk sample origin influence the quality of the milk.

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Based on the results shown in Table 3, it can be noticed that potassium content in the milk samples was the highest of all investigated macroelements (K, Ca, Mg and Na). Potassium overage in milk samples compared to other macroelements is not unknown, since potassium is one of the most essential macroelements, which is very important for membrane transport, energy metabolism, and normal functioning of cells. Potassium content was also highest in the reconstituted powder milk sample, and its content was higher in cow milk samples compared to the human milk. Compared to blood, milk contains more potassium and calcium and less sodium and chloride, which is due to the sodium-potassium pump that regulates osmotic pressure between the cytoplasm of blood cells and milk (6).

Among the other macronutrients (Ca, Mg and Na), calcium was the most present, then the sodium, while magnesium was least present in all tested milk samples (Table 3). Some of the macro-elements occur bonded in protein in the milk, which increase the efficiency of the absorption (7). Calcium is the most present element in all kinds of milk. It provides bones rigidity while its ions play an important role in many metabolic processes. By absorption process or bone resorption calcium enters the extra-cellular fluid from the gastrointestinal tract, from which the kidney and skin embed it into the bone during their formation (8). Also, there is a steady flow of this element along the cell membrane. The lack of calcium in the body can lead to some diseases as osteoporosis. A recommended daily allowance of calcium is 200-1300 mg, depending of a human age (9). It has been found that the calcium content is the highest in goat milk compared to other animal kind of milk samples. It is also noted that calcium content is higher in cow milk than in human milk (6), which was found in our samples too (Table 3). Khatir et al. (10) measured calcium content of Sudanese mothers to be 388 mg/l, which is less than average calcium content of human milk of Serbian mother (Table 3).

Sodium is the part of the living bicarbonate buffer systems and it influences distribution of water through osmosis, and it is necessary for neuromuscular function because it takes part in the transport of glucose and other nutrients. Sodium content is higher at the end of lactation, and also depends of many different conditions such as food content. Our results showed that sodium content was higher in cow milk samples compared to the human milk, but the highest content was seen in reconstituted powder milk sample (Table 3).

Magnesium is necessary for the formation of the bone's minerals. As the content of the previous macroelements decreases continuously during the lactation, similar changes occur also in the magnesium content. Human milk sample showed magnesium content of 89.15 mg/l, which is also higher than that reported in the literature (7). Cow milk, as well as reconstituted milk powder sample showed higher magnesium content compared to the human milk. It is also seen that fresh cow milk is richer in all macronutrients (Ca, Mg and Na) compared to the pasteurized cow milk sample from a local market in Leskovac (Table 3).

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Table 3. Contents of the macro and micro-elements in milk samples (mg/L)

Element	Human milk Average value	Fresh cow milk Average value	Pasteurized cow milk	Reconstituted powder milk
Ag	0	0	0	0
Al	0.2505	0.86	1.722	1.601
B	0	0	0	0
Ba	0	0	0	0
Bi	0	0	0	0.01
Ca	456.05	1318.08	865.24	1099.46
Cd	0	0	0	0
Co	0	0	0	0
Cr	0	0	0	0
Cu	5.00	7.25	5.21	5.79
Fe	7.21	9.54	5.19	7.08
Ga	0	0	0	0
In	13.22	16.98	15.42	15.21
K	1840.64	2128.21	2448.03	2993.26
Li	0	0	0	0
Mg	89.15	278.87	148.45	206.41
Mn	0	0	0	0
Na	352.25	493.61	484.87	536.64
Ni	0	0	0	0
Pb	0.05	0.614	0.0428	0.022
Sr	0.644	1.708	0.701	1.611
Tl	0	0	0	0
Zn	5.22	12.13	8.17	5.49

Among the essential elements content (Cu, Fe, Ni and Zn), zinc showed the highest level in all milk samples. The concentrations of Zn and Cu must be monitored and compared with the values set by the EU and the WHO, because there is no legislation on their permitted maximum amount in Serbia. According to the literature data for cow milk, the average values of Cu and Zn are 0.43 mg/l and 4.96 mg/l, respectively (11). The amount of 12.13 mg/l is the highest zinc content, determined in the tested fresh cow milk sample. Zinc is very important because it is one of the trace elements, needed for normal growth. It has been proven that there was dramatic improvement of acrodermatitis enteropathica after zinc supplementation (12), which is of a great importance to human metabolism. Zinc can act as anti-arthritic, anti-infective, antiviral, astringent, immuno-stimulatory and healing agent, and in some cases the appearance of acne on the skin can be a symptom of a zinc deficiency in the body. Different zinc amount can be due to different nutrition of

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humans and animals, and also depends on the period of lactation. Of all essential elements mentioned above only presence of nickel was not detected.

Based on the detected content of essential elements in the tested milk samples it can be concluded that milk can be a good additional source of iron. In our samples, the highest iron content was observed in fresh cow milk sample, 9.54 mg/l. Iron, which is present in the body, is used for the activity of some enzymes that generate energy and its deficiency leads to microcytic and hypochromic anaemia (13). The higher iron content was observed also in human milk sample. Iron content in the samples taken directly after the childbirth was about 14 μmol/l, and it was established that the environment does not have an influence on the iron content, and no relationship was found between the mother age and number of children (7).

The presence of copper was also detected in all tested milk samples. The copper contents in human, pasteurized cow milk and reconstituted powder milk samples were slightly higher than 5 mg/l, while in the fresh cow milk it was the highest – 7.25 mg/l. Copper is an essential metal in the human body, animals and plants, and it appears in the form of element or mineral. It is essential for the haemoglobin production, and it is an essential ingredient in a large number of enzymes. It also helps the elasticity of fibres providing better skin structure support. The literature data for copper occurrence in cow and human milk are between 2-30 μg/100 g (6), which is more than 10 times less than the values in our samples. The literature data also showed that the lactation state had no significant effect on the copper concentration. On the other hand, it was also established that the copper content was significantly affected by the nutrition of the mothers (7).

Many other trace elements such as lithium, bromine, strontium, silver, lead, tin, vanadium, mercury, cadmium, rubidium and cesium also occur in milk, but they are not nutritionally important. Of all trace elements present in milk samples, aluminum was detected at the highest level. Nevertheless, its content was much higher than commonly detected in milk samples (46 μg/100 g) for cow milk and 0.06 μg/100 g for human milk (6). The total aluminum content in milk samples is affected by many different factors. First of all, aluminum is naturally present in water and also can be present in food. In the case of pasteurized cow milk sample, there is possibility that aluminum can be delivered through the packaging material made of aluminum (14). Finally, the presence of Ag, B, Mn, Co, Cr, Ga, Li, Cd and Tl was not detected.

The presence of lead was detected in milk samples, too. This can be a result of various factors such as water used for the preparation, production process and of the nutrition. FAO/WHO has defined the so-called acceptable daily heavy metal intake as 25 mg/kg of adult weight for lead (15). The detected content was much higher than the normal content, especially for the fresh cow milk sample that showed the highest lead value of 0.614 mg/l (Table 3). Therefore, keeping in mind that once absorbed in the bones, liver or kidneys, this metal has damaging effects (16), it is necessary to monitor and control its content. Lead that can be found in exhaust gases, and it can be absorbed when the animals graze near major transportation routes (17). Lead content of the mother's milk varies in the industrialized countries between 5-20 μg/l, and in strongly polluted areas it can be 20 times higher (18). However our human milk sample showed significant content of 0.05 mg/l. Of all heavy metals, European Union (2001) and International Dairy Fede-

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ration (IDF) allows lead concentration of 20 μg/kg. Whatsoever, by the old Regulations of the Socialist Federal Republic of Yugoslavia, the allowed lead content in milk was 0.1 mg/kg, which is five times higher than the content tolerated by the European Union legislation.

Strontium content was also detected in all milk samples, and the highest value was found in fresh cow milk sample, 17 mg/l. Stable forms of Sr might be beneficial and they were found to aid bone growth, increase bone density, and lessen vertebral, peripheral, and hip fractures (19). Sr content depends also of the period of lactation.

In summary, the content of heavy metals in human milk or three other milk samples depends on feed, their content of soil, environment contamination, as well as on the antagonistic bioelements heavy metals interaction, affecting their absorption and metabolism (20). In human milk samples their contents can be related to many factors as the effects of childbirth at young age, duration of the pregnancy, state of underfeeding of the mother, physical burden, various metabolic diseases, different nationality, lactation state, time of separation, milk amount, sampling techniques, environmental differences, social-cultural differences, smoking habits, nutrition, calcium and vitamin D supplementation, and other. The resent research has shown that, with the exception of youth motherhood and some metabolic diseases, no environmental or other factors have an effect on the calcium content in the human's milk (7, 20).

Total fat contents analysis in the milk samples are shown in Table 4. Fat content depends of many factors. Fat content in human milk depends on the mother's diet, amount of consumed meet per day, and period of lactation (6, 21). The fat content can be correlated to the macro- and micro-content too. According to Zamberlin (6), as the fat content in milk increases, the content of the major minerals in milk and dairy products decreases. The analyses of the capric (0.28), lauric (9.10), and miristic acid content (12.5%) of the milk fat in human milk showed that there is a relationship between the copper content and the amount of these three fatty acids (22). Copper-containing enzyme from milk, which is necessary for the synthesis of the middle-chain fatty acids, explains the relationship between the copper and the middle-chain fatty acids. On the other hand, no relationship could be found between the middle-chain fatty acids and the manganese content. A relationship was also found between cadmium and fat content. The decrease of the milk's cadmium content was in a close connection with the lower protein and fat content. Fat content in cow milk varies from 3.9 to 4.2% (20). The total fat content in human milk is noticeably less than in cow milk, which is also in agreement with data shown in Table 4. Our analysis of reconstituted powder milk showed low fat content, until pasteurized cow milk from a local market showed fat amount according to the manufacturer declaration.

Table 4. Total fat content in milk samples determinate by Weibull and Stoldt method

Sample type	Total fat content (%)
Human milk	3.60
Fresh cow milk	2.80
Pasteurized cow milk	4.20
Reconstituted powder milk	0.56

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CONCLUSION

Based on the results of the analysis of milk samples it can be concluded that the presence of most examined metals, i.e. macro- and micro-elements were within the permitted limits. In addition, from the detected amounts of certain elements such as potassium, calcium and magnesium, milk samples can be recommended for consumption because they can supply part of the daily requirement of these nutrients. However, on the other hand, the detected amounts of lead and iron in most samples were higher than allowed. Total fat analysis also showed a good quality of human, fresh cow milk and pasteurized cow milk from the examined territory, while the reconstituted powder milk did not satisfy the Regulations criteria. Since, total fat and macro- and micro-analyses showed that fresh cow milk has the highest yield/content of fat and calcium, it can be concluded that it is the most nutritious.

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АНАЛИЗА МАКРО- И МИКРО ЕЛЕМЕНАТА У УЗОРЦИМА МЛЕКА ПРИМЕНОМ ОПТИЧКЕ ЕМИСИОНЕ СПЕКТРОМЕТРИЈЕ СА ИНДУКТИВНО КУПЛОВАНОМ ПЛАЗМОМ

Сања М. Петровић, Саша Р. Савић и Живомир Б. Петронијевић*

Универзитет у Нишу, Технолошки факултет, Булевар Ослобођења 124, 16000 Лесковац, Србија

У овом раду је испитиван садржај минерала Ag, Al, B, Ba, Bi, Ca, Cd, Co, Cr, Cu, Fe, Ga, In, K, Li, Mg, Mn, Na, Ni, Pb, Sr, Tl и Zn као и садржај укупних масти у узорцима млека, који потичу са различитих извора. Анализирани узорци млека су: хумано млеко, свеже кравље млеко, пастеризовано кравље млеко са локалног тржишта у Лесковцу-Јабланички округ и реконституисано млеко у праху. Припрема узорака за макро- и микро анализу вршена је мокром дигестијом. Концентрације одабраних метала у растворима после дигестије одређиване су применом оптичке емисионе спектрометрије са индуктивно куплованом плазмом (ICP-OES). Укупни садржај масти у узорцима млека одређен је применом Weibull и Stoldt методе. У анализираним узорцима концентрације калијума и калцијума су биле највеће у опсегу од 1840,64 - 2993,26 мг/л и 456,05 - 1318,08 мг/л, редом. Од тешких метала који су испитивани (бакар, цинк, манган, никл, кадмијум и олово), у свим узорцима су највише били присутни цинк и бакар са приближно сличним износом у распону 5 - 12 мг/л, док присуство кадмијума, никла и мангана није детектовано у ниједном узорку. Узорци свежег крављег млека и хуманог млека су показали највиши садржај масти 3,6 и 4,2%, редом. Резултати тестова, садржаја масти и макро и микро елемената су показали да свеже кравље млеко има највиши принос масти и калцијума што га чини нутритивно вреднијим узорком млека у односу на остале анализиране узорке.

Кључне речи: млеко, ICP-OES, минерали, масти

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ENZYMATIC HYDROLYSIS OF EXTRUDED SOYBEAN MEAL AT HIGH SUBSTRATE CONCENTRATIONS

Anton Yu. Sharikov, Anna S. Sereda, Elena V. Kostyleva, Irina A. Velikoretskaya and Victor A. Polyakov*

All-Russia Research Institute of Food Biotechnology, Samokatnaya str. 4[b], 111033 Moscow, Russia

Extrusion as a pretreatment before enzymatic hydrolysis of soybean meal is an effective technique to eliminate antinutritional properties of the main thermostable soy proteins glycinin and β-conglycinin for production of feed ingredients with enhanced properties. In terms of economic efficiency, biotechnological processes are preferable to carry out at high substrate concentrations. The aim of the investigation was to evaluate the influence of high substrate concentrations in the range of 26 - 32% and enzyme dosages (0.4 - 3.1 PU/g) on efficiency of hydrolysis of extruded toasted soybean meal with bacterial protease. The results showed that maximum degree of hydrolysis was 42.1% at the enzyme dosage of 3.6 PU/g and at the substrate concentration of 29.0%. The increase in the substrate concentration had a strong effect on the deterioration of dynamic viscosity of the hydrolysates from 0.2 to 5.82 Pa·s. A combination of extrusion cooking at 120ºC and enzymatic treatment with „Protolad B" protease enabled hydrolysis of glycinin and β-conglycinin to peptides with molecular mass below 15 kDa.

KEY WORDS: soybean meal, β-conglycinin, glycinin, extrusion cooking, protease

INTRODUCTION

Soybean meal (SBM) is one of the most economically effective sources of valuable protein in animal feed. However, the main soybean proteins – glycinin and β-conglycinin – exhibit antigenic properties and have a negative effect on the immune system (1, 2). Due to the compact structure and presence of disulphide bonds, they are thermostable, and are only partially denatured in the conventional process of SBM toasting. Complete denaturation of thermostable proteins can be provided by extrusion cooking which comprises a combination of moisture, pressure, temperature, and mechanical shear processing factors. Denaturation of β-conglycinin and glycinin during extrusion does not eliminate their allergenic properties caused by the characteristic amino acid sequences, but improves their accessibility for proteases action (3, 4). Marsman et al. (4) showed that only a combination of extrusion cooking with subsequent enzymatic hydrolysis using proteases allowed eliminating all glycinin and β-conglycinin subunits in toasted SBM (TSBM). It is

* Corresponding author: Anton Yu. Sharikov, All-Russia Research Institute of Food Biotechnology, Samokatnaya str. 4[b], 111033 Moscow, Russia, e-mail: anton.sharikov@gmail.com

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important to note that most of the investigations on SBM enzymatic hydrolysis were performed at substrate concentrations of lower than 10% (4-6). This may be explained by the challenging rheological properties of SBM/water suspensions at high substrate concentrations due to the characteristic property of glycinin and β-conglycinin to form gels through molecules aggregation caused by interactions of sulfhydryl and hydrophobic groups (7). Gelation impairs technological properties of the reaction mixture, impedes mass transfer, and reduces the effectiveness of the enzymatic hydrolysis.

At the same time, substrate concentration is one of the key economic factors of biotechnological processes (8). Hydrolysis at high solids reduces heating requirements, water losses, and energy consumption associated with water removal processes such as evaporation, concentration, drying. Volumetric productivity of the plant may be increased through more effective utilization of equipment.

The aim of this research was to evaluate the influence of high substrate concentrations (SC) and enzyme dosages (ED) on efficiency of extruded TSBM (ESBM) hydrolysis and completeness of glycinin and β-conglycinin elimination.

EXPERIMENTAL

Material

Commercial TSBM with crude protein content of 50% (dry matter basis) and 5.5% moisture content was used for the experiments. Enzyme preparation of *Bacillus licheniformis* protease – Protolad B (Mikrobioprom Ltd., Ukraine) with proteolytic activity 350 PU/g was used for protein hydrolysis. Proteolytic activity of Protolad B was assayed according to method developed by Sigma-Aldrich (9) using casein as a substrate.

Extrusion cooking

A co-rotating twin-screw extruder Werner & Pfleiderer Continua 37 (Stuttgart, Germany) was used for treatment of TSBM. The screws diameter was 37 mm with ratio of the screw length to its diameter (L/D) of 27:1. The screw configuration was as follows: 160 mm forwarding twin lead screw (FTLS) with 40 mm pitch, 2 × 10 mm kneading segments (KS), 120 mm FTLS with 40 mm pitch, 3 × 10 mm KS, 40 mm FTLS with 40 mm pitch, 15 mm reverse screw (RS) with 15 mm pitch, 160 mm FTLS with 40 mm pitch, 2 × 10 mm KS, 210 mm FTLS with 30 mm pitch, 2 × 10 mm KS, 60 mm FTLS with 20 mm pitch, 15 mm RS with 15 mm pitch, 120 mm FTLS with 20 mm pitch. The die had two openings each 2.5 mm in diameter. The barrel temperatures were 25ºC in the feed zone, 50ºC in the central zone and 120 – 160ºC at the die. The screw speed and feed rate were kept constant at 300 rpm and 14 kg/h, respectively. Moisture content of feed material in the process of extrusion cooking was adjusted to 20.0%. After drying, the extruded samples were maintained at room temperature and ground in a laboratory hammer mill with 1 mm sieve openings.

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Hydrolysis

Ground TSBM and ESBM samples were hydrolyzed by protease at 50°C, pH 6.1±0.1 (natural pH of SBM/water suspension without adjustment) for 5 hours. The substrates were mixed with water phase containing Protolad B in proper concentration, the reaction mixtures were stirred vigorously and then incubated without agitation until the end of the process. The hydrolyzed mixtures were heated to 85°C and incubated at this temperature for 10 min for protease inactivation. The resulting hydrolysates were dried at 55°C to constant weight for subsequent analyses.

Substrates and hydrolysates characterization

Crude protein content in TSBM and ESBM was measured according to Kjeldahl method using Turbotherm TT-625 digestion unit (Gerhardt, Germany) and DL 15 titrator (Mettler Toledo, Switzerland). Soluble protein concentration in hydrolysates was measured according to Lowry et al (10).

Protein solubility (PS) was determined as percentage of soluble protein content in relation to the total protein content of the sample. The degree of protein hydrolysis (DH) was determined as the percentage of content of protein soluble in 10% (w/v) trichloroacetic acid (TCA) in relation to the total protein content of the sample (11, 12).

Completeness of proteinaceous antinutritional factors (ANF) hydrolysis was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using Mini-Protean Tetra System (BioRad, USA). Samples were prepared as follows: 20 mg of dry hydrolysates were taken to Eppendorf tubes and extracted in 0.125 mL of SDS buffer for 1 h at constant stirring, then diluted 5-fold in the same SDS buffer with mercaptoethanol and incubated for 15 min at 100°C.

Determination of the rheological properties

Dynamic viscosity (DV) was measured by a sine-wave viscometer SV-10 (AND, Japan) with frequency of 30 Hz in 45-mL sample container (13). Rheological properties were also determined using a CT-3 Texture Analyzer (Brookfield Engineering, USA) equipped with a 4.5 kg load cell. The diameters of the back extrusion container and disk were 40 and 34 mm, respectively. Tests were carried out to a depth of 20 mm at 1 mm/s penetration speed. The maximum force was taken as a measurement of media firmness (14).

Experiment design and statistical analysis

The first step of the experiment was to determine the optimal extrusion conditions, i.e. optimal temperature of the die. In this step SC of 25% and ED of 2 PU/g were used for hydrolysis of TSBM after extrusion. At the next stage of the experiment, TSBM was extruded at the chosen temperature and then hydrolyzed at various SCs and EDs. SC and ED were used as independent variables (factors) for central composite rotatable design

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(CCRD). The ranges of factor values in this investigation were determined on the basis of preliminary research (not shown). Each variable in coded and real values is shown in Table 1. Protein solubility, degree of hydrolysis, dynamic viscosity, and firmness were investigated as response parameters of the design.

Student's t-test was carried out to determine the significance of the regression coefficients. The Fisher's variance ratio test was used for evaluation of the adequacy of the fitted regression models.

The regression coefficients estimation and statistical analysis were performed in accordance with Lazic (15) using Scilab 5.5 (Scilab Enterprises, France).

Table 1. Coded and real values of variables used in the CCRD

Coded value	Real value	
	Substrate concentration, %	Enzyme dosage, PU/ g of substrate
-α	26.0	0.4
-1	26.9	0.9
0	29.0	2.0
1	31.1	3.1
α	32.0	3.6

RESULTS AND DISCUSSION

Effect of extrusion temperature on enzymatic elimination of glycinin and β-conglycinin

Marsman et al. (4) reported that extrusion and the following hydrolysis with Neutrase enzyme preparation provided elimination of thermostable allergy-causing glycinin and β-conglycinin in TSBM. The results confirming the benefits of extrusion pretreatment for soybean proteins hydrolysis were also obtained by other authors (16-18). However, there is a lack of information about the influence of extrusion temperature level on completeness of the subsequent enzymatic hydrolysis of all glycinin and β-conglycinin subunits. Glycinin consists of basic polypeptide B (~20 kDa) and acidic polypeptide (~38 kDa) linked by a disulfide bond. β-Conglycinin is a trimeric glycoprotein composed of three types of subunits: α' (72 kDa), α (68 kDa), and β (52 kDa) (1). The completeness of glycinin and β-conglycinin elimination is usually estimated by the absence of the corresponding bands on SDS-PAGE electrophoregram. To define the optimal extrusion temperature TSBM was extruded at different die temperatures ranging from 120 to 160^{o}C, while the moisture content, screw speed, and feeding rate were kept constant. Samples extruded at different temperatures and unextruded TSBM were subjected to hydrolysis by 2 PU/g Protolad B, for 5 hours, at a substrate concentration of 25%.

The results of SDS-PAGE (Fig. 1) show that the changes in tertiary structure of TSBM proteins during extrusion promoted their hydrolysis by protease. This was confir-

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med by the absence of the pronounced bands corresponding to proteins with a molecular weight above 15 kDa in ESBM hydrolysates in contrast to TSBM hydrolysates, where the bands corresponding to antinutritional proteins subunits remained visible. However, a weak band corresponding to a protein with molecular weight of about 20 kDa was observed on SDS-PAGE of hydrolysates of ESBM obtained at 140–160^{o}C. It probably belongs to the residual amount of unhydrolysed subunit B of glycinin (Fig. 1, lanes 5-7). A decrease of extrusion temperature to 120–130^{o}C led to a more complete degradation of soy proteins to peptides with molecular weight under 15 kDa (Fig. 1, lanes 3, 4). For further experiment, the most energy-saving temperature mode (120^{o}C) providing complete soy proteins hydrolysis into short peptides, was chosen.

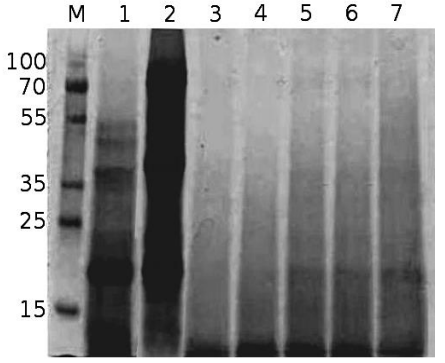

Figure 1. SDS-PAGE of processed TSBM. Lane M, molecular weight markers 100, 70, 55, 35, 25 and 15 kDa; lane 1, hydrolyzed TSBM; lane 2, TSBM; lanes 3,4,5,6,7, hydrolyzed ESBM, previously extruded at 120, 130, 140, 150, 160^{o}C, respectively

Influence of the substrate concentration and protease dosage on the ESBM protein hydrolysis and rheological properties of the slurries

Enzymatic hydrolysis at high solids is a promising way to enhance effectiveness of technological processes. It allows using bioreactors with increased productivity by reducing specific energy, water and materials costs. For this reason it was important to investigate the enzymatic hydrolysis of proteins in ESBM at high substrate concentrations, where gelation of the main soy proteins occurs, that impairs rheological properties and impedes mass transfer. On the basis of earlier studies (data not shown) we have found that the most dramatic change in the rheological properties of ESBM/water suspensions was observed at SCs close to 30%. Therefore, at the next stage of the experiment, the effect of SC in the range of 26–32% and proteolytic EDs on ESBM hydrolysis rate and rheological properties of the slurries was studied using response surface methodology. The final experimental data are presented in Table 2.

For the two independent variables, a second order polynomial regression model has been proposed as:

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$$y= \beta_0 +\sum\beta_i X_i+\sum\beta_{ii} X_{ii}^2+\sum\beta_{ij} X_i X_j \quad [1]$$

where 'y' is the response; 'β_0' is a constant; 'β_i', 'β_{ii}', and 'β_{ij}' are linear, quadratic and interaction regression coefficients, respectively; 'X_i' and 'X_j' are values of independent parameters.

Table 2. The experimental design and trial results

Treatment	Solids concentration (%)	Enzyme dosage (PU/ g)	PS (%)	DH (%)	DV (Pa·s)	Firmness (N)
1	26.9	0.9	37.33	32.56	0.40	3.29
2	31.1	0.9	34.98	33.34	3.32	7.43
3	26.9	3.1	42.98	40.25	0.20	1.69
4	31.1	3.1	40.16	37.44	2.46	6.47
5	26.0	2.0	40.36	37.86	0.20	1.88
6	32.0	2.0	38.72	35.98	5.82	10.52
7	29.0	0.4	29.10	27.04	2.42	6.61
8	29.0	3.6	42.68	42.10	0.69	3.82
9	29.0	2.0	39.12	37.63	1.69	4.53
10	29.0	2.0	40.78	36.36	1.82	5.53
11	29.0	2.0	40.32	38.06	1.21	5.12
12	29.0	2.0	40.80	37.00	1.80	5.55
13	29.0	2.0	42.00	38.05	2.04	5.00

PS – protein solubility; DH – degree of protein hydrolysis; DV – dynamic viscosity

On the base of each data set, regression coefficient for second order polynomial models and their statistical significance at 95% confidence were calculated (Table 3). The check of the lack of fit of the obtained regression models proved that all models were adequate with 95% confidence level.

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Table 3. Regression coefficients and model adequacy evaluation

Terms	PS (%)	DH (%)	DV (Pa·s)	Firmness (N)
Constant	39.9534	10.81	65.8231	26.6162
SC	-0.4458	0.50	-5.3475	-2.5458
ED	9.8648	19.52	2.4933	-2.0697
SC^2	-[a]	-[a]	0.1081	0.0632
ED^2	-1.6129	-1.12	-0.2087	-0.1750
SC×ED	-[a]	-0.39	-0.0709	0.0700
Adequacy of the regression model[b]	+	+	+	+
R^2	0.90	0.92	0.91	0.94

[a] insignificant at $p<0.05$
[b] at significance level $p<0.05$
PS – protein solubility; DH – degree of protein hydrolysis; DV – dynamic viscosity

Graphical interpretations of the estimated equations are shown in Figure 2. The character of contour lines for PS and DH equations shows that protease dosage was a more significant variable for these response parameters, especially in the local domain of low enzyme dosages. The increase of ED improved PS and HD. A maximum PS value in the investigated range of factor values was 42.98% at 26.9% solids and 3.1 PU/g protease dosage. The maximum DH was 42.1% at the starlike point with maximum enzyme dosage of 3.6 PU/g and 29.0% solids. The minimal ED (0.4 PU/g) provided 29.1% and 27.04% values for PS and DH, respectively.

Graphical analysis of contour plots for rheological properties of the hydrolysates (Figure 2) shows that DV and firmness correlate increasingly with the substrate concentration. The maximum DV and firmness values were observed at a starlike point of factorial space at 32.0% solids and 2.0 PU/g protease dosage and accounted to 5.82 Pa·s and 10.52 N, respectively. The increase in the substrate concentration from 26% to 32% resulted in a sharp increase of DV from 0.2 to 5.82 Pa·s. This indicated that the selected factor range was a domain of significant deterioration of rheological properties of the hydrolysates. This effect represents a challenging factor for developing a technological process in terms of selection of mixers and pumps for the process line. The increase of enzyme dosage had a slight effect on decrease of DV.

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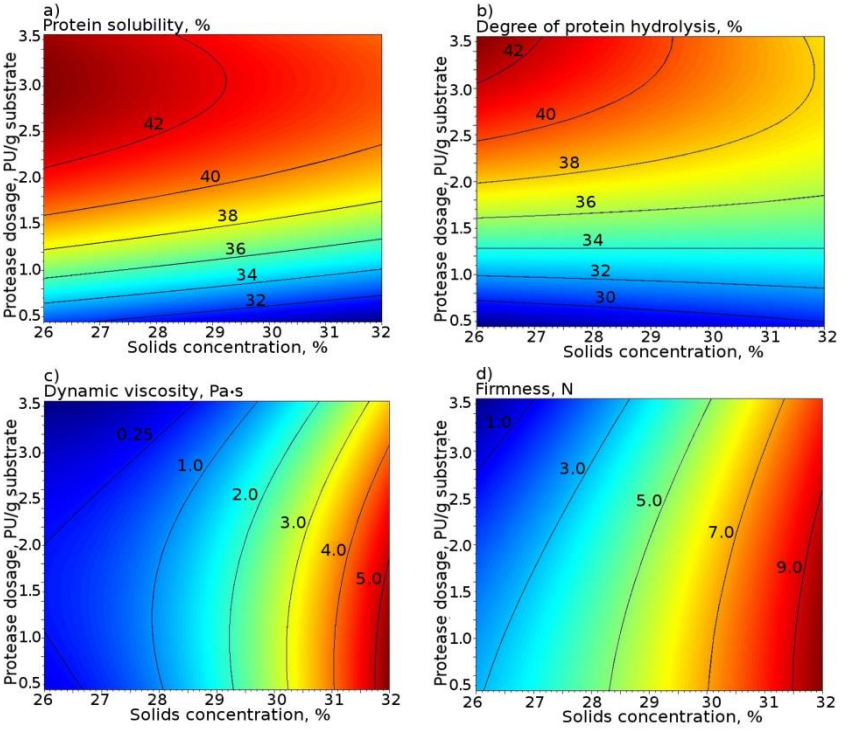

Figure 2. Effect of solids concentration (%) and enzyme dosage (PU/g) on protein solubility (a), degree of hydrolysis (b), dynamic viscosity (c), and firmness (d)

The efficiency of proteinaceous ANF elimination was estimated using SDS-PAGE analysis of the hydrolysates related to all points of factor space by the disappearance of protein bands with molecular mass above 15 kDa. Results of SDS-PAGE are shown in Figure 3.

Despite the high viscosity and adverse rheological properties of the reaction mass, almost all modes of hydrolysis provided complete hydrolysis of β-conglycinin and glycinin in ESBM with formation of the peptides with molecular weights under 15 kDa. Lane 7, corresponding to the minimum ED (0.4 PU/g), was characterized by the more intense staining, which may be explained by the presence of residual peptides with a molecular weight above 15 kDa, but lacked pronounced bands corresponding to subunits of the main anti-nutritional soybean proteins. In general, it can be assumed that Protolad B provides a sufficient hydrolysis of anti-nutritional soy proteins at high ESBM concentrations (26-32%).

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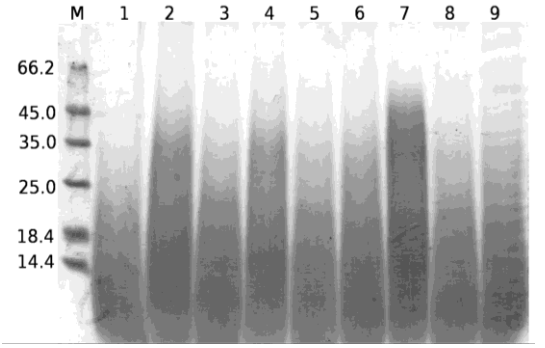

Figure 3. SDS-PAGE of hydrolysed ESBM. Lane M, molecular weight markers 66.2, 45.0, 35.0, 25.0, 18.4, 14.4 kDa; lanes 1-8 corresponding to treatments 1-8 in Table 2, respectively; lane 9 corresponding to treatment 10

CONCLUSION

The present study demonstrated a potential for combining of extrusion cooking technology and enzymatic hydrolysis at high substrate concentrations for processing of toasted soybean meal and elimination of its antinutritional proteins. The results obtained indicated that the extrusion cooking at 120–130ºC was an appropriate pretreatment for proteolytic hydrolysis of the extruded substrate. TSBM extruded at 120ºC underwent easily enzymatic treatment with protease at 50ºC and pH 6.0 within 5 hours at solids up to 32%. The observation of CCRD results and corresponding contour plots pointed out to the effect of substrate concentration on sharp deterioration of rheological properties of the hydrolysates. It was shown that enzyme dosage was more significant factor for the degree of hydrolysis and protein solubility compared to solids concentration. The results of SDS-PAGE of the hydrolysates indicated complete hydrolysis of glycinin and β-conglycinin fractions. The developed technology allows the obtaining of high quality protein feedstuff based on toasted soybean meal for animals with intolerance to glycinin and β-conglycinin.

Acknowledgement

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ЕНЗИМСКА ХИДРОЛИЗА ЕКСТРУДИРАНЕ СОЈИНЕ САЧМЕ ПРИ ВИСОКИМ КОНЦЕНТРАЦИЈАМА СУПСТРАТА

Антон Ју. Шариков, Ана С. Середа, Јелена В. Костиљова, Ирина А. Великорецкаја и Виктор А. Пољаков

Сверуски научно-истраживачки институт прехрамбене биотехнологије, ул. Самокатнаја 4б, 111033 Москва, Русија

Термостабилне, тешко хидролизирајуће сојине беланчевине глицинин и β-конглицинин имају имуногени утицај и негативно утичу на организам животиња. Комбинација екструзионе предобраде и ензимске хидролизе сојине сачме представља ефикасан начин одстрањивања антинутритивних својстава глицинина и β-конглицинина, са циљем добијања хранива са побољшаним својствима. Један од кључних фактора који одређују економску ефикасност биотехнолошких процеса је концентрација супстрата. Циљ овог рада био је проучавање утицаја дозирања ензимског препарата и концентрације супстрата, која се кретала у границама 26-32%, на ефикасност хидролизе беланчевина екструдата тостиране сојине сачме као и на реолошке карактеристике хидролизата. Истраживањем је утврђено да је максимални степен хидролизе износио 42,1% при највећој концентрији протеазе (3,6 PU/g) и при коцентрацији супстрата од 29%. Повећање концентрације супстрата доводило је до повећања вискозности суспензије од 0,2 до 5,82 Pa.s. Комбинација претходног екструдирања на 120ºC са даљом обрадом бактеријском протеазом „Протолад Б“ омогућује хидролизу глицинина и β-конглицинина до пептида са молекулском масом мањом од 15 kDa.

Кључне речи: сојина сачма, β-конглицинин, глицинин, екструдирање, протеаза

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EFFECT OF STARTER CULTURE ADDITION ON FATTY ACID PROFILE, OXIDATIVE AND SENSORY STABILITY OF TRADITIONAL FERMENTED SAUSAGE (*PETROVSKÁ KLOBÁSA*)

Branislav V. Šojić[1], Natalija R. Džinić[1], Vladimir M. Tomović[1], Predrag M. Ikonić[2], Marija R. Jokanović[1], Snežana Ž. Kravić[1], Tatjana A. Tasić[2] and Snežana B. Škaljac[1]*

[1] University of Novi Sad, Faculty of Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia
[2] University of Novi Sad, Institute of Food Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia

This work is concerned with the oxidative stability and sensory characteristics of traditional fermented sausage Petrovská klobása, produced with the addition of commercial starter culture (SC). Fatty acids profile, thiobarbituric acid-reactive substance (TBARS) values and sensory properties of odor and taste have been determined at the end of drying and after 2 and 5 months of storage. The sum of saturated, unsaturated and polyunsaturated fatty acids was not significantly different (P>0.05) in SC and control sausage at the end of storage. After 5 months of storage TBARS value of SC sausage amounted to 0.57 mg MDA/kg, and it was significantly lower (P<0.05) compared to control (0.84 mg MDA/kg). Also, sensory properties of odor and taste of SC sausage (3.66) were better in comparison to control (3.55). This study demonstrated that the addition of starter culture can hinder lipid oxidation and contribute to the preservation of desirable sensory characteristics of fermented sausages during a long storage period.

KEY WORDS: traditional sausage, starter cultures, lipid oxidation

INTRODUCTION

Petrovská klobása is a traditional fermented sausage produced in small-scale facilities in the municipality of Bački Petrovac (Republic of Serbia). This sausage has been produced during winter period for a long time by a traditional technique without the use of nitrate/nitrite, glucono-delta-lactone (GDL) and bacterial starters. Because of its specific and recognizable texture, color and aroma, *Petrovská klobása* has been protected as designation of origin at the national level, by the Serbian law (1-3).

Fermented sausages can undergo different chemical, sensory and microbiological deteriorations. The cause of this might be variability of the quality of used ingredients (meat, spices) and different processing and storage conditions. The most common chemical deterioration is lipid oxidation (2,4). One way to slow down the oxidation of lipids is the use of starter cultures. Bacterial starter cultures (Lactic acid bacteria - LAB and

* Corresponding author: Branislav V. Šojić, University of Novi Sad, Faculty of Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia, e-mail: bsojic@gmail.com

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Coagulase Negative Staphylococci - CNS) are widely used to accelerate the process of fermentation and ripening, as well as to improve the quality and safety of the final product (5-7). The protective effect of LAB starter cultures is in relation to pathogenic, as well as to spoilage bacteria through the antimicrobial properties of their metabolites (8-10). CNS participates in the development of red color and oxidative stability of final product. Hence, catalase and superoxide dismutase activities of CNS promote degradation of hydrogen peroxide and prevent lipid oxidation (6,11).

Small-scale production of *Petrovská klobása* is currently being transferred to industrial, and the main task is preservation of products quality in different stages of production, storage and distribution. Thus, the aim of this research was to investigate the effects of commercial starter culture addition on oxidative and sensory stability of fermented sausage (*Petrovská klobása*) during storage period. The results of this research can be of use to manufacturers of *Petrovská klobása*, as well as manufactures of similar traditional products of the type of fermented sausage.

EXPERIMENTAL

Sausage preparation

The batter for sausages was made according to a recipe described by Šojić et al. (2). Half of the obtained batter was inoculated with 0.015% of commercial starter culture (Quick-starter, Lay, Germany) which contained equal percentages of *Lactobacillus sakei*, *Pediococcus pentosaceus*, *Staphylococcus carnosus* and *Staphylococcus xylosus* (SC group). The other half of batter was assigned as control. Both groups of sausages were subjected to ripening process in an industrial room (average temperature, t=11.1 ± 4.58°C; average relative air humidity, rH = 76.1 ± 8.48%) for 2 months. After the ripening process, sausages were stored in the industrial room (t=10°C and rH=75%) for five months.

Methods

Fatty acid profile determination. Extraction of lipids was performed by the method of Folch et al. (12). The fatty acid composition was determined by gas chromatography, as described by Šojić et al. (2). Fatty acids methyl esters were quantified as percentage of total methyl esters.

TBARS determination. TBARS (2-thiobarbituric acid reactive substances) test was performed using the method of Bostoglou et al. (13). TBARS values were expressed as milligrams of malondialdehyde per kilogram of sample.

Sensory evaluation of odor and taste. Sensory evaluation was performed by 7 trained panelist, according to quantitative descriptive analysis (QDA), using a scale from 0 (visible mechanical or microbiological contamination, atypical product) to 5 (extraordinary, typical and optimal quality), with a sensitivity threshold of 0.25 points (4).

Statistical analysis. Statistical analysis was carried out using STATISTICA 12.0 (StatSoft, Inc., Tulsa, OK, USA). All data were presented as mean value with their stan-

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dard deviations (mean ± SD). Variance analysis (ANOVA) was performed, with a confidence interval of 95% ($P<0.05$). Means were compared by Duncan's multiple range test.

RESULTS AND DISCUSSION

Table 1 shows the fatty acid profile of the control and SC sausages after drying and after 2 and 5 months of storage. The addition of SC did not affected on fatty acid profile. Namely, at the end of drying and after 2 and 5 months of storage ΣSFA, ΣUFA and ΣPUFA were not significantly different for both tested sausages ($P>0.05$). In the sum of fatty acids, oleic acid (C18:1) content was the highest. During storage, the oleic acid content significantly decreased ($P<0.05$) for both groups of sausages. Decreasing content of oleic acid in fermented sausages was also reported by Ansorena and Astiasarán (14). Polyunsaturated fatty acids are very reactive and oxidize easily (15). During the storage period, both in SC and control sausages a downward trend of linoleic and linolenic acid (C18:3) contents was observed. This is likely due to oxidative changes. Similar results were also reported by Ansorena and Astiasarán (14) and Krkić et al. (16).

Table 1. Fatty acid profile of traditional sausage *Petrovská klobása*

Fatty acid	End of drying		2 months of storage		5 months of storage	
	Control	SC	Control	SC	Control	SC
C14:0	0.80 ± 0.04^{c}	0.86 ± 0.03^{c}	1.00 ± 0.03^{ab}	0.97 ± 0.04^{b}	1.04 ± 0.01^{a}	1.03 ± 0.04^{a}
C16:0	18.50 ± 0.41^{b}	19.00 ± 0.45^{b}	20.07 ± 0.53^{a}	20.02 ± 0.39^{a}	20.64 ± 0.10^{a}	20.80±0.21a
C17:0	0.11 ± 0.01^{c}	0.16 ± 0.01^{b}	0.16 ± 0.04^{b}	0.39 ± 0.11^{a}	0.16 ± 0.01^{b}	0.14 ± 0.02^{bc}
C18:0	11.30 ± 0.01^{a}	10.60 ± 0.03^{c}	10.57 ± 0.10^{c}	10.57 ± 0.07^{c}	10.75 ± 0.03^{b}	10.38 ± 0.08^{d}
C16:1	2.48 ± 0.04^{c}	2.19 ± 0.06^{d}	2.59 ± 0.03^{b}	3.05 ± 0.29^{a}	2.40 ± 0.05^{c}	2.42 ± 0.04^{c}
C17:1	0.14 ± 0.01^{b}	0.23 ± 0.03^{a}	0.19 ± 0.05^{ab}	0.15 ± 0.00^{b}	0.22 ± 0.02^{a}	0.25 ± 0.07^{a}
C18:1	44.42 ± 0.19^{a}	44.65 ± 0.18^{a}	43.96 ± 0.13^{b}	44.00 ± 0.04^{b}	43.74 ± 0.12^{c}	43.71 ± 0.24^{c}
C20:1	2.16 ± 0.04^{a}	1.99 ± 0.00^{b}	1.99 ± 0.02^{b}	1.71 ± 0.10^{c}	1.77 ± 0.03^{c}	2.02 ± 0.21^{b}
C18:2	18.55 ± 0.27^{a}	18.76 ± 0.12^{a}	17.77 ± 0.08^{c}	18.08 ± 0.06^{b}	17.86 ± 0.15^{c}	17.82 ± 0.20^{c}
C18:3	1.48 ± 0.01^{b}	1.55 ± 0.02^{b}	1.70 ± 0.30^{a}	1.04 ± 0.07^{d}	1.39 ± 0.07^{c}	1.42 ± 0.12^{c}
∑ SFA	30.71 ± 0.35^{c}	30.61 ± 0.52^{c}	31.79 ± 0.50^{b}	31.94 ± 0.23^{b}	32.59 ± 0.17^{a}	32.34 ± 0.09^{ab}
∑ UFA	69.23±0.31a	69.35±0.51a	68.18 ± 0.05^{b}	68.02 ± 0.22^{b}	67.37 ± 0.06^{c}	67.63 ± 0.09^{c}
∑ PUFA	20.03 ± 0.28^{a}	20.30 ± 0.10^{a}	19.47 ± 0.38^{b}	19.12 ± 0.11^{b}	19.25 ± 0.08^{b}	19.24 ± 0.08^{b}

[abc] The values of the same row significantly differ with 95% probability ($P<0.05$); SC – sausages produced with the addition of commercial starter culture

The thiobarbituric acid test (TBARS) is widely used to evaluate secondary lipid oxidation products in meat and meat products. TBARS values in sausages during storage are shown in Figure 1. At the end of drying, the TBARS values ranged from 0.21 mg MDA/kg (control) to 0.26 mg MDA/kg (SC). These values are in good correlation with the literature data registered in similar meat products (14, 15, 17).

During storage, there was an increase in TBARS values for both examined groups of sausages, probably as a result of lipid oxidation (18). After 5 months of storage, TBARS value in the SC samples amounted to 0.57 mg, and was significantly ($P<0.05$) lower,

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compared to the value measured for control (0.84 mg MDA/kg). The reducing lipid oxidation in fermented sausages could be attributed to the antioxidative activity of CNS (11).

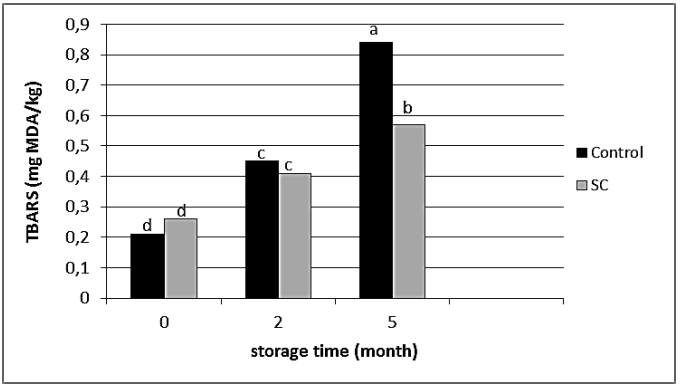

Figure 1. TBARS values in traditional sausage *Petrovská klobása*
Different letters abcd mark significantly different means with 95 % probability (P<0.05);
SC – sausages produced with the addition of commercial starter culture

Sensory evaluation of odor and taste of sausages during storage is shown in Figure 2. During two months of storage, no significant difference was observed between two examined groups of sausages (P>0.05).

After five months of storage, the score for odor and taste for SC sausage was 3.66, and it was higher (P<0.05) than the score for control (3.55).This result is in negative correlation with the TBARS values in SC and control sausage after 5 months of storage. Similar results were reported by Krkić et al. (16).

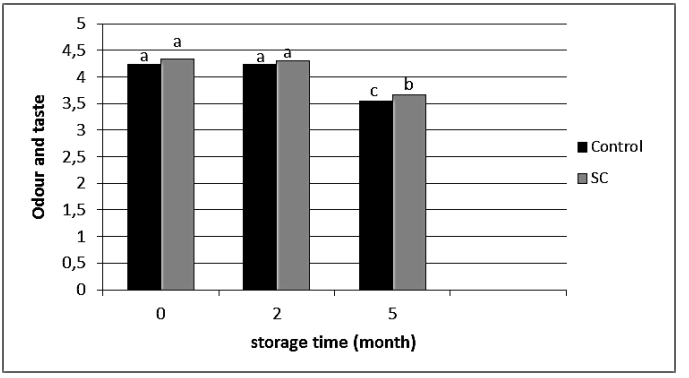

Figure 2. Sensory properties of odor and taste of traditional sausage *Petrovská klobása*
Different letters abc mark significantly different means with 95 % probability (P<0.05);
SC – sausages produced with the addition of commercial starter culture

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CONCLUSION

The addition of the commercial starter culture led to a lower lipid oxidation and improvement of the sensory properties of traditional fermented sausage *Petrovská klobása* during a long storage period (five months).

Acknowledgement

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УТИЦАЈ ДОДАТКА СТАРТЕР КУЛТУРЕ НА МАСНОКИСЕЛИНСКИ САСТАВ, ОКСИДАТИВНУ И СЕНЗОРСКУ СТАБИЛНОСТ ТРАДИЦИОНАЛНЕ ФЕРМЕНТИСАНЕ КОБАСИЦЕ (*PETROVSKÁ KLOBÁSA*)

Бранислав В. Шојић[1], Наталија Р. Џинић[1], Владимир М. Томовић[1], Предраг М. Иконић[2], Марија Р. Јокановић[1], Снежана Ж. Кравић[1], Татјана А. Тасић[2] и Снежана Б. Шкаљац[1]

[1]Универзитет у Новом Саду, Технолошли Факултет, Булевар Цара Лазара 1, 21000 Нови Сад, Србија
[2]Универзитет у Новом Саду, Институт за прехрамбене технологије, Булевар Цара Лазара 1, 21000 Нови Сад, Србија

У овом раду испитана је оксидативна стабилност и сензорска својства традиционалне ферментисане кобасице (Petrovská klobása), произведене са додатком комерцијалне стартер културе (SC). Маснокиселински састав, TBARS вредност и сензорска својства мириса и укуса испитана су на крају процеса сушења и након 2 и 5 ме-

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сеци чувања. Сума засићених, незасићених и полинезасићених масних киселина није била статистички значајно (P>0,05) различита у кобасици SC групе у поређењу са кобасицом контролне групе на крају периода чувања. Након 5 месеци чувања TBARS вредност кобасице SC групе износила је 0,57мг MDA/кг и била је статистички значајно (P>0,05) мања у поређењу са контролом (0,84 мг MDA/кг). Такође, сензорска својства мириса и укуса кобасице SC групе (3,66) била су бољa у поређењу са контролом. Ова студија указује да додатак стартер културе доводи до смањења оксидације липида и очувања сензорских својстава мириса и укуса традиционалне ферментисане кобасице (*Petrovská klobása*) током дужег времена складиштења.

Кључне речи: традиционална кобасица, стартер култура, оксидација липида

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CAROB FLOUR AND SUGAR BEET FIBER AS FUNCTIONAL ADDITIVES IN BREAD

Dragana M. Šoronja-Simović[1], Sonja Smole-Možina[2], Peter Raspor[2], Nikola R. Maravić[1], Jana J. Zahorec[1], Lucia Luskar[2] and Zita I. Šereš[1]*

[1] University of Novi Sad, Faculty of Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia
[2] University of Ljubljana, Biotechnical Faculty, Jamnikarjeva 101, Ljubljana, Slovenia

The effect of functional additives (carob flour and sugar beet fiber) on empirical rheological dough performance and bread quality was examined. Also the microbiological quality of bread was investigated during 16 days of storage. The study included 5 samples: control (CON), with preservative calcium propionate (CONP), with carob flour (CON-CAR), with sugar beet fiber (CON-SBF) and with a combination of carob flour and sugar beet fibers (CON-SBF-CAR). Samples with functional additives had a higher water holding capacity (2-10%) and extended dough development time due to the presence of dietary fiber. Dough resistance of these samples was significantly increased, especially in CON-CAR, in which the time of final fermentation is remarkably prolonged (20% in comparison to CON). The addition of the functional ingredients (due to hydration properties of dietary fiber) improved texture and sensory characteristics of bread. In sample CON-SBF crumb firmness was significantly reduced (by 70%) while elasticity was increased by 25% compared to CON. Positive effect of addition of sugar beet fiber was proved by improving the elasticity of the crumb and finer crumb structure (sample CON-SBF) in comparison with the addition of carob flour (sample CON-CAR). In bread sample with carob flour there was no microbiological contamination for 16 days of examination, which confirms the fact that carob flour can be used as a natural preservative.

KEY WORDS: carob flour, sugar beet fiber, bread quality, sensory properties, microbiological quality

INTRODUCTION

Functional foods are defined as those that have a positive physiological effect on human organism and in addition to the basic building function contribute to reducing the risk of diseases. For a long time, nutritionists have emphasized the importance of dietary fiber in achieving, maintaining and improving the health of people considering the deficiencies in the diet in highly developed countries (1). Bread is a component of daily nutrition in which by modification of raw material composition can be successfully adjusted

* Corresponding author: Jana J. Zahorec, University of Novi Sad, Faculty of Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia, e-mail: jana.zahorec@tf.uns.ac.rs

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to the deficit of nutrients and biologically valuable components. Presence of dietary fiber in bread enhances its nutritional and reduces its energy value. However, the addition of fiber causes a change in the rheological properties of dough and bread quality parameters (2). Earlier studies have shown that the addition of fiber generally leads to a decrease in volume of loaves, the result is a product with denser, less airy structure, firmer and darker crumb appearance. The influence of fiber on the quality of bread depends on the type (3, 4, 5), size, and amount of fiber (2).

The extracted sugar beet pulp produced during the technological processing of sugar beet is an excellent source of soluble and insoluble fiber (6). They are characterized by high water holding capacity, which is especially desirable in baking industry (7).

Carob (*Ceratonia siliqua* L.) belongs to the family of legumes and is widespread in the Mediterranean area (8). Carob seed pod has a high content of polyphenols with antioxidant properties (9). In addition, carob flour is rich in insoluble dietary fibers which take part in cholesterol lowering in animals (10) and in humans (11). Carob fiber is also an important raw material in the preparation of functional bakery products considering its chemical composition (4).

The aim of this study was to investigate the effect of addition of fiber from sugar beet and carob flour to the rheological properties of dough and bread quality, as well as their impact on the microbiological quality of the bread.

EXPERIMENTAL

Materials

Commercial wheat flour having moisture 13.3%, protein 11.1%, starch 79.9%, sugar 3.1%, fat 1.1%, total dietary fiber 3.1% and ash 0.46% was supplied by a local flour mill AD Danubius (Novi Sad, Serbia).

Commercial preservative calcium propionate - E282 (Propi San, PURATOS, Belgium) and commercial carob flour (MALINA IMPEX, Valjevo) were used. Sugar beet fibers were prepared according to Šoronja Simović et.al, 2010 (12).

Methods

The moisture, ash, fat, starch, reducing and total sugar content of wheat flour were determined according to methods described in AOAC, 2000 (13). Wheat flour was analyzed for gluten content according to standard AACC procedures (14). Total dietary fiber content was determined according to the AOAC method.

The empirical rheological properties of dough were determined by Brabender farinograph (Brabender SEW, Duisberg, Germany) according to AACC method (15). Extensibility of the dough and its resistance were determined according to the AACC method by Brabender extensograph (Brabender EXEK/7, Duisberg, Germany) (16).

Bread was baked according to the AACC method (17). The dough samples were prepared according to the following dough formula: wheat flour (100-80%), salt (1%), sugar

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(5%), yeast (2%), milk powder (2.5%) and bakery fat (5%). Five bread samples were used in examination and they were defined by the content of ingredients (Table 1):

Table 1. The content of additives in bread samples

Sample	Carob (%)	Sugar beet fiber (%)	Preservative (%)
CON	0	0	0
CONP	0	0	0.4
CON-CAR	20	0	0
CON-SBF	0	3	0.2
CON-SBF-CAR	10	3	0

CON – control, CONP – with preservative, CON-CAR – with carob flour, CON-SBF – with sugar beet fiber, CON-SBF-CAR – with sugar beet fiber and carob flour

All the ingredients were added to the mixer (MS-6). And then an optimal amount of water obtained from farinograph absorption, i.e. to 500 BU (49.5 – 60.0%) was added into mixing bowl and mixed for 4 minutes at a speed of 450 of rpm.

The dough was placed into a fermentation chamber at 30^{o}C for 60 minutes. The dough was then divided into three pieces of 350 g, and rounded to rest for 10 min. Afterwards samples were placed into the baking pans for proofing (50 min at 30^{o}C and 85% RH). Baking was carried out at 250^{o}C. The baking process was done when the bread lost 10% of its weight. The loaves were cooled to room temperature. The quality of bread was characterized by bread volume and crumb quality 24h after baking.

Bread volume was measured by the rapeseed displacement method (18). Crumb quality was evaluated by five experienced panelists.

Texture profile analysis (TPA) was performed using a TA-XT2i texturometer (Stable Microsystems, Surrey, UK) equipped with a 2.5 cm probe. Crumb slices of 2 cm were 50% compressed. Four replicates from two different sets of baking were analyzed and averaged. The recorded parameters were firmness and springiness.

The selected samples were tested on the 4, 8, 12 and 16th day after the baking. Dichloran Rose-Bengal Chloramphenicol Agar (DRBC) was used to detect molds and yeasts. The level of contamination was set by folowing formula:

$$LC=\frac{D^2}{R^2} \qquad [1]$$

where, LC represents level of contamination, D is diameter of colonies grown on petridish and R refers to diameter of petridish, as colony forming units were unable to count. The level of contamination was set by % of covered petri dish plate. The borders of low, medium and high contamination were set artificially. The corresponding intervals were used in order to present the obtained results in a concise and comparable form for easier understanding of differences between the samples.

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RESULTS AND DISCUSSION

Based on the results of rheological examination it can be seen that the addition of preservatives (sample CONP) did not significantly affect the tested rheological parameters. By adding 20% carob flour (sample CON-CAR) produced changes in all tested parameters. The water absorption was slightly increased, while the dough development was extended four times compared to the control sample (Figure 1). Farinograph data clearly show that the addition of sugar beet fiber (sample CON-SBF) affected hydration of the particles during the mixing, which confirms the long dough development (10 min). The above-mentioned parameter in CON-SBF sample is 5 times higher than that in control sample, and compared with CON-CAR up 20%. Increased water absorption by 8% (compared to the samples CON and CONP), or about 5% compared to the CON-CAR confirms that dietary fiber has a high water holding capacity (19).

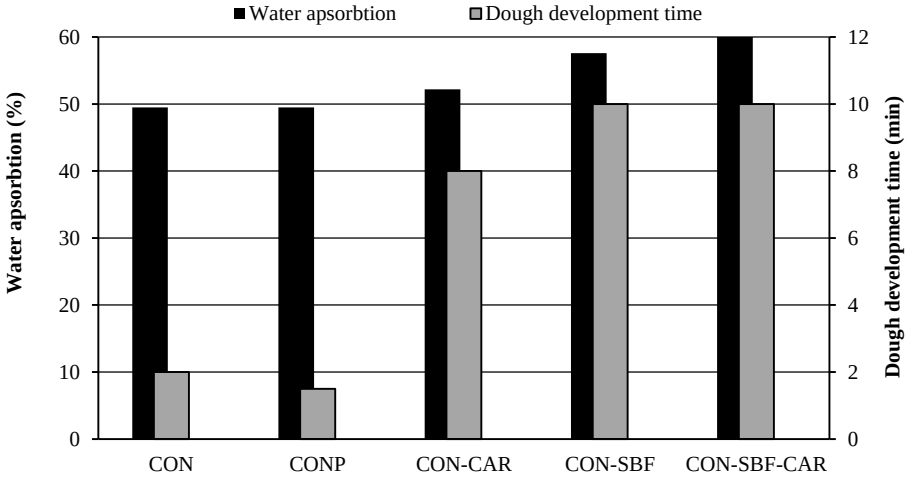

Figure 1. Effect of carob flour and sugar beet fiber on water absorption and dough development time

The changes of extensograph parameters in the sample CON-CAR were significant because there was an increase in energy for about 35% and resistance to extension by 70% (Table 2). The decrease of dough extensibility up to 40% caused the increase in the Ratio number of samples 3.5 (CON) and 3.3 (CONP) to as much as 10.2. These changes in the rheology parameters can be explained by the interactions between carob fiber and proteins with wheat gluten (20). Minor changes were observed in sample CON-SBF. The energy increased by 25%, also the resistance was higher by about 30% compared to the control sample, while it was by 15% lower compared with CON-CAR. The extensibility of the dough was, as well as of the sample CON-CAR, decreased up to 30%. The highest resistance and lowest extensibility was determined in CON-SBF-CAR, so that Ratio number for this sample increased by as much as 70%.

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Table 2. Results of extensograph examination

Sample	Energy (cm^2)	Resistance (Ej)	Extensibility (mm)	O/R*
CON	142.9	530	152	3.5
CONP	143.3	520	157	3.3
CON-CAR	196.4	920	90	10.2
CON-SBF	171.9	770	102	7.5
CON-SBF-CAR	183.6	1080	84	12.9

* O/R – ration number of resistance and extensibility
CON – control, CONP – with preservative, CON-CAR – with carob flour, CON-SBF – with sugar beet fiber, CON-SBF-CAR – with sugar beet fiber and carob flour

The maturograph parameters (Table 3) indicate that the addition of carob flour had a pronounced effect on the ability of dough to retain the gases during fermentation. Sample CON-CAR has lower resistance of dough up to 30% and elasticity by 25%, indicating reduced ability to retain created gas. The assumption mentioned is confirmed by the longer final fermentation time by as much as 20% compared to the CON. On the other hand, it can be noticed that the addition of fiber from sugar beet caused a slight increase in dough resistance, as well as a decrease in elasticity. The impact of carob flour, as well as sugar beet fiber, was observed in the sample CON-SBF-CAR, which is confirmed by the lower value of the dough resistance by about 7.5%, and a decline in elasticity by 30%.

Table 3. Results of maturograph examination

Sample	Final fermentation time (min)	Stability of fermentation (min)	Dough resistance (Mj)	Dough elasticity (Mj)
CON	68	6	665	270
CONP	70	2	625	250
CON-CAR	88	8	460	200
CON-SBF	69	2	720	220
CON-SBF-CAR	77	8	615	190

CON – control, CONP – with preservative, CON-CAR – with carob flour, CON-SBF – with sugar beet fiber, CON-SBF-CAR – with sugar beet fiber and carob flour

On the basis of the TPA test, it is evident that the addition of preservatives (sample CONP) did not affect the values of textural parameters of bread. The supplement of carob flour in an amount of 20% (sample CON-CAR) caused an increase in crumb firmness by 10%, while the crumb springiness decreased by 15% compared to the control (CON), which is in accordance to the findings of Salinas et al. (20). On the other hand, the supplementation of sugar beet fiber had an opposite effect on the firmness and springiness of the crumb compared with carob flour. A significant decrease in firmness of the crumb (70% compared to the control bread) was observed by adding sugar beet fiber (sample

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CON-SBF), whereas the springiness increased by 25%. The addition of sugar beet fiber in combination with carob flour (sample CON-SBF-CAR) showed a decreasing trend in the firmness, and also in the springiness of the crumb by 30% and 5%, respectively, compared to the control bread. Based on these results it can be concluded that sugar beet fiber had a more pronounced influence on the textural parameters, and it can be assumed that this is the result of their hydrating properties.

The results of sensory properties examination of bread samples confirmed that the addition of carob flour and sugar beet fiber had an effect on specific loaf volume (Figure 2), as well as on the elasticity of the crumb and the fineness of pore structure after 24 and 48 h (Figure 3).

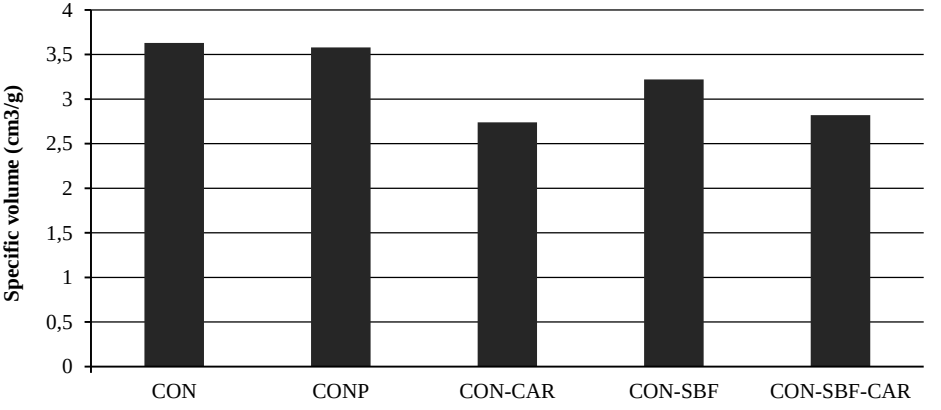

Figure 2. Effect of carob flour and sugar beet fiber on bread loaf volume

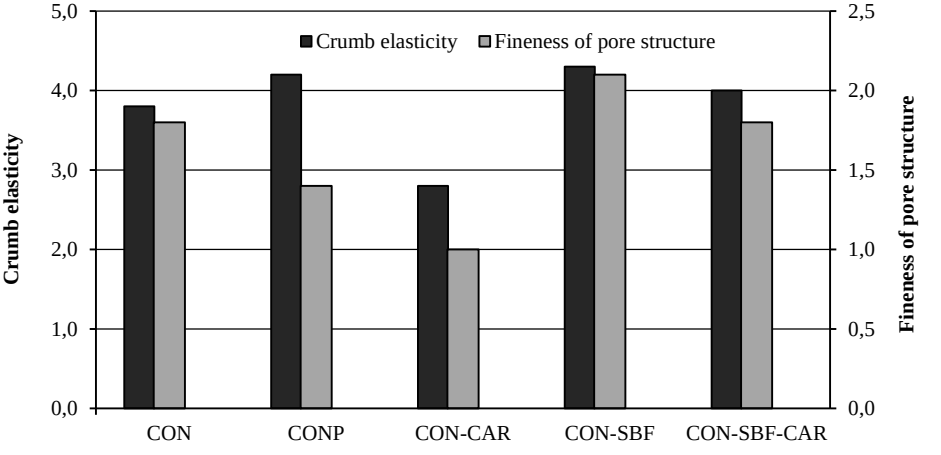

Figure 3. Effect of carob flour and sugar beet fiber on sensory properties of bread

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The addition of the preservative (sample CONP) had a positive effect on the pore structure and elasticity of the crumb. However, the specific loaf volume was slightly reduced compared to the control sample. The addition of carob flour in an amount of 20% (sample CON-CAR) had the biggest impact on reducing the specific loaf volume of the bread (by 25%), while the combined supplementation of 3% sugar beet fiber and 10% carob flour (sample CON-SBF-CAR) caused a decrease in the specific loaf volume by 20% compared to CON. The result of adding sugar beet fiber (sample CON-SBF) improved elasticity of the crumb, and the bread had a finer crumb structure compared with CON-CAR.

The effect of additional ingredients on the microbiological quality of bread during the 16 days of storage was also investigated, where the microbiological analysis was performed every fourth day, and the criteria of contamination was defined: low 0 - 0.1, medium 0.1 - 0.3 and high > 0.3.

By observing the development of molds and yeasts it was noticed that in all bread samples there was low contamination present, whereby the sample CONP retained the value of contamination to 0.1 for all 16 days, and the contamination of CON-CAR was negligible, which was probably a result of the antioxidant effect of carob flour (9). It was similar to the sample CON-SBF-CAR, except for the last day of sampling where the contamination was registered at the level of contamination of the control sample, which was due to the combined effects of both additional ingredients. Only the sample CON-SBF showed higher level of contamination compared to the CON (up to 0.9) as can be observed in Figure 4.

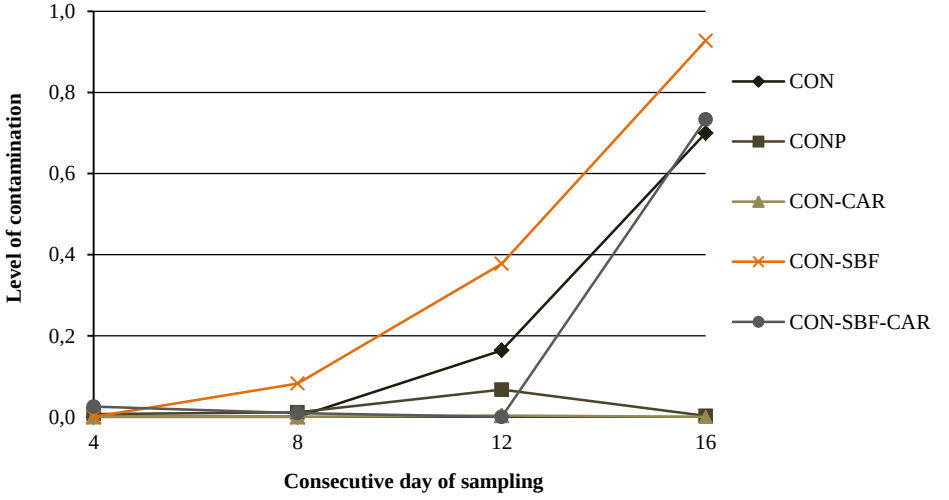

Figure 4. Level of contamination of bread by molds and yeasts

The bread samples after the sixteenth day were kept for additional 6 days, and then visually ascertained the changes (Figure 5). It is noticeable that molds did not develop

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only on the samples CONP and CON-CAR, indicating that carob flour has an antifungal effect, similar to a commercial preservative.

Figure 5. From left to the right: CON, CONP, CON-CAR, CON-SBF and CON-SBF-CAR

CONCLUSION

The addition of 20% carob flour slightly increased the water absorption and prolonged the dough development time by 4 times. Carob flour significantly increased the dough extension resistance by as much as 70%, which was resulted in a decreased ability of gas retention. The maturograph resistance and elasticity of the dough decreased by 30% and 25% respectively, while the final fermentation time was extended by 20%. The sample with 3% sugar beet fiber (CON-SBF) had 8% higher water absorption, due to the good hydrating properties, and 5 times longer dough development time in comparison to the sample without functional additives. The combined addition of 3% sugar beet fiber and 10% carob flour (CON-SBF-CAR) had an increased resistance and decreased extensibility of dough compared to the control sample.

The sample CON-SBF had the best bread quality, with the bread volume of 1013.33 ml, improved elasticity and fineness of crumb structure compared to the control sample, even to the sample CON-CAR. The volume of the sample CON-SBF-CAR was lower by 20%, while the minimum volume was obtained with carob flour (25% lower than CON). The textural characteristics of bread with carob flour were unfavorable, because of the crumb firmness increase by 10% and crumb elasticity decrease by 15%.

From the aspect of the microbiological quality of bread, the best results were achieved by adding 20% carob flour (CON-CAR). The highest level of contamination by molds and yeasts was observed for the sample CON-SBF, while the microbiological quality of the sample CON-SBF-CAR was preserved for 12 days.

The results confirm that the use of sugar beet fiber and carob flour in bread making is justified, because their effects on the rheological characteristics of the dough did not result in a significant deterioration of physical and sensory quality parameters of bread. Positive effects, primarily of carob flour on the preservation of microbiological quality of bread, confirm that its application as a natural preservative in bread making is justified and desirable.

Acknowledgement

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БРАШНО РОГАЧА И ВЛАКНА ШЕЋЕРНЕ РЕПЕ КАО ФУНКЦИОНАЛНИ ДОДАЦИ У ХЛЕБУ

Драгана М. Шороња-Симовић[1], Соња Смоле-Можина[2], Петер Распор[2], Никола Р. Маравић[1], Јана Ј. Захорец[1], Луција Лускар[2] и Зита И. Шереш[1]

[1] Универзитет у Новом Саду, Технолошки факултет, Булевар Цара Лазара 1, 21000 Нови Сад, Србија
[2] Универзитет у Љубљани, Биотехнички факултет, Јамникарјева 101, Љубљана, Словенија

У овом раду испитан је утицај функционалних додатака (брашно рогача и влакна шећерне репе) на емпиријске реолошке особине теста и квалитет хлеба. Квалитет хлеба дефинисан је одређивањем текстурних, сензорских и микробиолошких параметара. Испитивањима је обухваћено 5 узорака: контролни (CON), са 0,4% конзерванса (CONP), са 20% брашна рогача (CON-CAR), са 3% влакана шећерне репе (CON-SBF) и са комбинацијом 10% брашна рогача и 3% влакана шећерне репе (CON-SBF-CAR). Узорци са функционалним додацима имају повишену способност везивања воде до 10% и 4-5 пута продужен развој теста у односу на CON због присуства прехрамбених влакана са израженим хидратационим својствима. Код поменутих узорака значајно је повећан и отпор теста, нарочито код узорка CON-CAR. Код поменутог узорка продужено је и време завршне ферментације за 20% у односу на CON што је последица смањене способности задржавања гасова.

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Додатак функционалниих сировина утиче на побољшање текстурних и сензорских особина хлеба. Код узорка CON-SBF значајно је смањена тврдоћа средине (за чак 70%), док се еластичност повећала за 25% у односу на контролни узорак. Позитивни утицај додатка влакана шећерне репе (CON-SBF) манифестовао се побољшањем еластичности средине и постизањем финије структуре пора. Додатак брашна рогача (CON-CAR) узроковао је смањење запремине хлеба за чак 25% у доносу на контролни узорак и за 15% у односу на CON-SBF. Са друге стране код хлеба са додатком брашна рогача током 16 дана испитивања није дошло до микробиолошке контаминације па је микробиолошки квалитет на нивоу квалитета узорка са хемијским конзервансом (CONP). Поменути резултати су врло значајни јер указују да се брашно рогача може користити као природни конзерванс.

Кључне речи: брашно рогача, влакна шећерне репе, квалитет хлеба, сензорске особине, микробиолошки квалитет

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OPTIMIZATION OF THE COMPOSITION OF THE POWDERED CEREAL SPROUTS MIXTURES

Vesna T. Tumbas Šaponjac, Vanja R. Šeregelj, Gordana S. Ćetković, Jasna M. Čanadanović-Brunet and Sonja M. Đilas*

University of Novi Sad, Faculty of Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia

Sprouts of cereals have received significant attention as functional food due to their nutritional and functional value. Consumption of sprouts has become increasingly popular among people interested in improving and maintaining their health status by changing dietary habits. Cereal grains contain several classes of phytochemicals, i.e. phenolics, chlorophylls, and carotenoids. However, their nutritional and chemical profile is altered and improved during germination. The purpose of this study was to find the best ratio of the powdered wheat (WS), oat (OS) and barley (BS) sprouts for designing the cereal sprout mixture (CSM) with the highest total phenolic content (TPh) and antioxidant capacity (AC), using Simplex-Centroid experimental design and response surface methodology (RSM). Single- and multi-response optimizations showed that OS did not contribute to TPh or AC values of CSM and, therefore, was not included in any of the compositions of the optimized CSM. Single-response optimizations showed that the highest TPh was found for CSM containing 82% BS and 18% WS, while the best AC was found for pure BS. The predicted ratio of cereal sprout powders in CSM obtained by multi-response optimization was: 96% BS and 4% WS. This mixture possessed the highest predicted TPh and AC (372.32 mg GAE/100 g, 549.99 μmol TE/100 g, respectively), which was confirmed to be in accordance with the experimental values. Based on the results obtained in this study, a designed CSM is proposed as a convenient ingredient of functional food products, dietary supplements and nutraceuticals.

KEY WORDS: cereals, sprouts, mixture design, polyphenols, antioxidant capacity

INTRODUCTION

In the recent years, consumption of cereal sprouts has become an innovation in nutrition. They have received attention as a functional food due to many benefits they express on human health (1). These benefits have been mostly attributed to the content of dietary fiber, essential fatty acids, vitamins and antioxidant phytochemicals, including several phenolic compounds available in the cereals (2, 3). Antioxidants present in whole grain

* Corresponding author: Vesna T. Tumbas Šaponjac, University of Novi Sad, Faculty of Technology, Bulevar Cara Lazara 1, Novi Sad, Serbia, vesnat@uns.ac.rs

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cereals act in defense to remove the reactive oxygen species (ROS), thereby preventing and curing oxidative stress-related diseases (4).

As a simple technological method that is used to germinate seeds, sprouting has been reported to improve the nutritive value of seeds (5). Nutritional value of sprouted grain improves due to the conversion of complex compounds into simpler and essential form and by minimizing the effect of antinutritional factors during germination (6). The practice of sprouting of cereal grains has also become popular, and it is used in many different foods, for example, breakfast items, salads, soups, casseroles, pasta and baked products (7).

Among the plant foods, cereals are grown over 73.5% of the world harvested area, while oil seeds (10.8%), pulses and nuts (6.3%), roots and tubers (5.2%), sugar crops (2%), and fruits and vegetables (2.2%) occupy the remaining 26.5% of the planted area (8). Cereal grains contribute over 60% of the world food production and, along with pulses and oil seeds, form a major bulk of dietary proteins, calories, vitamins, and minerals to the world population in general, and to the developing world in particular (9). According to Eurostat statistics, among the cereals cultivated in Europe, wheat is the largest crop, followed by barley, with oats in the third place (10).

Response surface methodology (RSM) is a collection of statistical and mathematical techniques, useful for developing, improving and optimizing processes (11). Mixture designs are seen as the technology of quality to reach the excellence of a product. In the mixture design, two or more components are mixed in many different proportions and the characteristics of the resulting products, the responses, are recorded. They depended only on the proportion of components present in the mixtures (12, 13).

The aim of this study was to find the best ratio of powdered wheat, oat, and barley sprouts for designing the mixture with the highest total phenolic content and antioxidant capacity (AC), using Simplex-Centroid experimental design and RSM.

EXPERIMENTAL

Chemicals and instruments

Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazine (DPPH) and Trolox were purchased from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals and solvents were of the highest commercial grade and obtained from Lach-Ner (Brno, Czech Republic). Absorbances in spectrophotometrical assays were measured on a Multiskan GO microplate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Plant material and sprouting conditions

Three varieties of cereals, wheat cv. ‘Spelta’ (*Triticum aestivum var. spelta*), oat cv. ‘Jadar’ (*Avena sativa var. Jadar*) and barley cv. ‘Golozrni’ (*Hordeum vulgare var. nudum*) were kindly donated by the Institute of Field and Vegetable Crops (NS seme), Novi Sad, Serbia. For sprouting the cereal seeds, a modified method by Vale *et al.* (14)

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was used. Cereal seeds were sanitized with 5% H_2O_2 solution for 5 minutes, soaked in water for 6 h in the dark, drained and washed with distilled water. Sodden seeds (40 g) were distributed on the filter paper in pottery trays and left for 15 h in darkness. Germination was conducted consecutively in light mode (10.000 lux) at 21±1°C during 18 h, and dark mode at 17±1°C for 6 h, for six days. In total, germination lasted for seven days. During the first three days, the trays were covered with pierced parafilm and sprayed with water several times a day. On the fourth day, the parafilm was removed and ambient air humidifier was used till the end of germination. Excess liquid was decanted once a day. Harvested sprouts were freeze-dried (Alpha 2-4 LSC Martin Christ, Osterode, Germany) and ground. Powdered wheat (WS), oat (OS) and barley (BS) sprouts were packed in separate vacuumed plastic bags and stored at –20ºC pending further analysis.

Mixture design

In order to find the optimal cereal sprout mixture (CSM), response surface methodology (RSM) was used to optimize the content of each cereal sprout in the mixture. The experimental design adopted was a Simplex-Centroid design for three variables at four levels. The three independent variables were the share of OS, BS and WS in CSM. The tested values of the independent variables were 0, 0.33, 0.50 and 1. The complete design consisted of 8 experimental points (formulations) presented in Table 1.

Table 1. Experimental design for CSM

Formulation	Content (share) in CSM		
	WS	OS	BS
1	0	0.5	0.5
2	1	0	0
3	0.33	0.33	0.33
4	0.5	0.5	0
5	0	0	1
6	1	0	0
7	0.5	0	0.5
8	0	1	0

Extraction procedure

For spectrophotometrical analysis of total phenolic content as well as of AC of samples, a portion of 50 mg of CSM powder was extracted with 500 μl of methanol (70%, v/v) in an ultrasonic bath for 20 min, followed by agitation using a laboratory shaker at 200 rpm (Unimax 1010, Heidolph Instruments GmbH, Kelheim, Germany) under light protection for 2 h, at room temperature, and then filtered (Whatman paper No. 1).

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Total phenolic content (TPh)

Total phenolics were determined spectrophotometrically according to the Folin-Ciocalteu method (15). The content of total phenolics was expressed as mg of gallic acid equivalents (GAE) per 100g of CSM dry weight.

Antioxidant capacity by DPPH assay

The AC of the CSM extracts was assessed by evaluation of the free radical scavenging effect on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, according to the method proposed by Brand-Williams et al. (16). The ability to scavenge DPPH radicals, i.e. AC, was calculated following the formula:

$$AC\,(\%) = [(A_C - A_S) / A_C] \times 100$$

where A_C is the absorbance of the control, and A_S is the absorbance of the sample. The results were also expressed as μmol Trolox equivalents (TE) per 100 g CSM dry weight.

Statistical analysis

Data were reported as mean ± standard deviation (SD) of three independent experiments. Data were analyzed by one-way analysis of variance (ANOVA). The level of significance was 95% ($p \leq 0.05$). Data were analyzed by using OriginPro 8 SR2 software (OriginLab Corporation, MA, USA). Optimizations were carried out using Design-Expert version 10 (Stat-Ease, Inc, MN, USA).

RESULTS AND DISCUSSION

In this study, RSM was used in order to find the optimal ratio between wheat, oat and barley sprouts in the mixture with the highest functional characteristics, i.e. polyphenol content and antioxidant capacity. Testing the CSM formulations of OS, BS and WS powders, selected according to the Simplex-Centroid mixture design model (Table 1), yielded the values of dependent variables (TPh and AC) presented in Table 2.

Table 2. Total phenolic contents and antioxidant capacities of CSM formulations

Formulation	TPh (mg GAE/100g)	AC (μmol TE/100g)
1	316.62±1.33	419.92±10.47
2	269.80±2.07	318.66±1.91
3	337.15±15.77	355.94±3.26
4	314.88±7.48	274.24±2.16
5	367.71±11.28	568.73±9.05
6	261.32±1.09	340.32±3.26
7	372.32±3.82	406.20±3.43
8	279.60±4.57	224.81±6.53

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Phenolic compounds are considered as a major group of the phytochemicals that contribute to the antioxidant capacity of cereals (17). Eight formulations of CSM containing OS, BS and WS and their combinations had a TPh of between 261.32 and 372.32 mg GAE/100g (Table 1), where the mixtures containing BS and WS (formulation 7) had the highest TPh. Comparing the results for the formulations which consisted of only one variety of cereal sprout, it is evident that WS (formulations 2 and 6) had the lowest amount of phenolics, but not significantly different ($p>0.05$) than OS (formulation 8), while the pure BS samples (formulation 5) showed superior total phenolic values (367.71 32 mgGAE/100g). Hernanz et al. (18) have reported that barley sprouts contain a wide range of phenolic antioxidants, and amino phenolic compounds present in free and bound forms. Ilona et al. (19) found that TPh in barley seeds (var. Klass) is 196 mg GAE/100 g. In the study of Bleidere et al. (20), TPh values in barley seeds ranged from 143.6 to 262.1 mg GAE/100g. Significantly higher values obtained in this study are probably the result of sprouting, besides the differences among varieties, agronomic and postharvest techniques, sampling and extraction procedures, etc. However, Kruma et al. (21) observed higher values of TPh in barley seeds, similar to the ones obtained for barley sprouts in our study, ranging from 351 to 460 mg GAE/100g. It has been determined that, unlike other cereals, barley contains significant amounts of flavonoids, including flavan-3-ols (monomers, dimers and trimmers) and flavonoid-derived tannins (22). According to the reports of Chu et al. (23) oat seeds possessed lower TPh values than sprouts of oats tested in this study, ranging from 57 to 94 mg GAE/100g. It should be considered that the oat samples in the study of Chu et al. (23) were not sprouted, extracts were obtained with different solvents and under different conditions, and abovementioned factors such as climate, varieties and growing regions may also explain the differences. Total phenolic content of sprouted wheat, in the study of Alvarez-Jubete et al. (24), was 110 mg GAE/100g, which is significantly higher than TPh of wheat seeds in the same study (53.1 mg GAE/100g), pointing out to the effect of sprouting on phenolics in cereals. Also, the value for the WS in our study was significantly higher ($p<0.05$). Comparing the TPh values of CSM obtained by mixing 2 or 3 sprout varieties, the lowest TPh values were obtained when BS was excluded (formulation 4), while mixing WS and BS was favorable.

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay is a simple and precise method to determine the antioxidant activity of various plant extracts (25). The CSM containing pure BS, and combinations of BS with OS or WS (formulations 5, 1 and 7, respectively), expressed the best AC (568.73, 419.92 and 406.20 μmol TE/100g, respectively), which is in accordance with the superior TPh values in BS (Table 2). This can indicate that there is low incidence or lack of synergism between the phytochemicals present in tested sprouts. In contrast, the formulations containing pure OS, and combination of OS and WS (formulations 8 and 4, respectively), had the lowest AC values (224.81 and 274.24 μmol TE/100g, respectively), which is in line with their low TPh values.

Single and multi-response optimization was carried out in order to find the optimal CSM with the highest TPh and AC. These results are reported in Table 3.

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Table 3. Single and multi-response optimization of CSM

Optimization	Predicted share in CSM			Optimal responses	
	WS	OS	BS	TPh (mg GAE/100g)	AC (μmol TE/100g)
Single-response (TPh)	0.18		0.82	381.28	-
Single-response (AC)	0	0	1	-	559.66
Multi-response (TPh+AC)	0.04	0	0.96	372.32	549.99

Single-response optimization was performed to optimize the defined responses, total phenolic content and antioxidant capacity. The respective response surfaces are presented in Figure 1. Based on these results, the optimal mixture with maximum TPh (381.28 mg GAE/100g) can be obtained by mixing 82% BS, 0% OS and 18% WS (Figure 1a). On the other hand, it was found that pure BS shows maximum scavenging activity on DPPH radicals (559.66 μmol TE/100g) (Figure 1b).

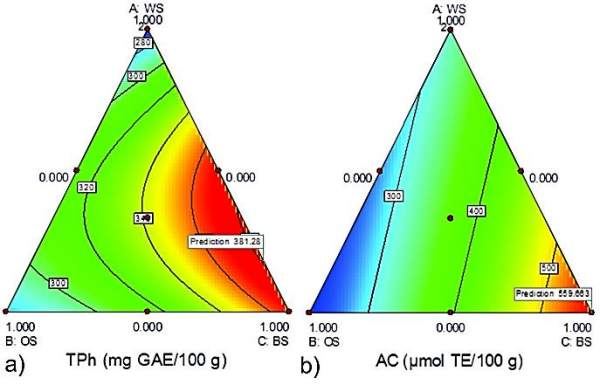

Figure 1. Mixture plots for TPh (a) and AC (b) values of CSM

For selecting the optimal mixture, a multi-response optimization was employed, where both responses, i.e. TPh and AC, are considered at the same time and the obtained result represents the optimal composition that provides maximum values of both responses, TPh and AC (Table 3). The designed optimal CSM included 96% BS and 4% WS, excluding OS as the component which did not contribute to the desirable functionality of CSM.

The CSM formulation predicted in multi-response optimization (Table 2) was prepared and experimentally characterized in terms of the TPh and AC, in order to confirm the

optimization model. The experimental values of this optimal mixture were 366.28 mg GAE/100 g for TPh and 557.23 μmol TE/100 g for AC, which was confirmed to be in accordance with the predicted values for these responses of the mixture, with no significant difference ($p>0.05$).

CONCLUSION

Response surface methodology and Simplex-Centroid mixture design were used to optimize the best composition of wheat, oat and barley sprouts, with the aim to achieve the best functional properties of the mixture, i.e. the highest total polyphenol contents and antioxidant capacity. Single-response, as well as multi-response optimization showed that oat sprouts did not contribute to any of the functional properties of mixtures and, therefore, was not included in the composition of the optimized mixtures. On the other hand, due to the barley sprouts' superiority in total polyphenol contents and antioxidant capacity, the combination of 96% barley sprouts and 4% wheat sprouts was proposed as optimized formulation with the most favorable functional parameters. The designed formulation with optimal composition was experimentally confirmed. The experimental data were in a good agreement with the predicted values, thus confirming the validity and adequacy of the predicted models and indicating that the proposed method is reliable for determining the optimum mixture formulation.

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ОПТИМИЗАЦИЈА САСТАВА СМЕШЕ ПРАШКАСТИХ КЛИЈАНАЦА ЖИТАРИЦА

Весна Т. Тумбас Шапоњац, Вања Р. Шерегељ, Гордана С. Ћетковић, Јасна М. Чанадановић-Брунет и Соња М. Ђилас

Универзитет у Новом Саду, Технолошки факултет, Булевар Цара Лазара 1, 21000 Нови Сад, Србија

Због своје нутритивне и функционалне вредности клијанци житарица привлаче пажњу као функционална храна. За употребу клијанаца у исхрани заинтересована је популација која је спремна да адаптира своје дијететске навике у циљу одржавања и побољшања здравственог статуса. Житарице садрже неколико класа фитохемикалија, као што су полифенолна једињења, хлорофили и каротеноиди. Међутим, њихов нутритивни и хемијски профил се мења и побољшава током клијања. Циљ овог истраживања био је да се пронађе најбољи однос прашкастих клијанаца пшенице (WS), овса (OS) и јечма (BS) у смеши клијанаца житарица (CSM) са највећим садржајем укупних полифенола (TPh) и антиоксидативним капацитетом (AC), коришћењем Simplex-Centroid експерименталног дизајна и методе одзивних површина (RSM). Оптимизације појединачних одзива и свих одзива истовремено показале су да OS не доприноси TPh и AC вредностима па стога ови клијанци нису укључени у оптимизоване смеше. Оптимизацијом појединачних одзива добијено је да максималан TPh поседује смеша 82% BS и 18% WS, док је најбољи AC показао чист BS. Смеша која је добијена истовременом оптимизацијом свих одзива састоји се од 96% BS и 4% WS. Предвиђене максималне вредности одзива за ову смешу износиле су: 372,32 mg GAE/100 g за TPh и 549,99 μmol TE/100 g за AC, што је и потврђено да је у сагласности са експерименталним вредностима. На основу резултата добијених у овом истраживању, оптимизована CSM има потенцијал примене у функционалној храни, дијететским суплементима и нутрацеутицима.

Кључне речи: житарице, клијанци, дизајн смеше, полифеноли, антиоксидативни капацитет

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Retraction: OSMOTIC DEHYDRATION OF FISH: PRINCIPAL COMPONENT ANALYSIS. APTEFF, 45 (2014) 45-53, DOI: 10.2298/APT1445045L

Editorial Board

Abstract:

On the proposition of the Editorial Board and with the consent of the authors, the paper entitled: OSMOTIC DEHYDRATION OF FISH: PRINCIPAL COMPONENT ANALYSIS, by the authors: Biljana Lj. Lončar (née Ćurćić), Lato L. Pezo, Ljubinko B. Lević, Vladimir S. Filipović, Milica R. Nićetin, Violeta M. Knežević and Tatjana A. Kuljanin, published in 2014 (Vol. 45, pp. 45-53, DOI: 10.2298/APT1445045L), is retracted because it is an autoplagiarism of the paper of the authors B. L. Ćurčić, L. L Pezo, V. S. Filipović, M.R. Nićetin and V. Knežević „OSMOTIC TREATMENT OF FISH IN TWO DIFFERENT SOLUTIONS – ARTIFICAL NEURAL NETWORK MODEL“, which was accepted for publication on May 9th 2014 (DOI: 10.1111/jfpp.12275) and published in 2015 in the Journal of Food Processing and Preservation (Vol. 39, pp. 671-680).

Уредништво

Сажетак:

На предлог Уредништва и уз сагласност аутора рад под називом: ОСМОТСКА ДЕХИДРАТАЦИЈА МЕСА РИБЕ – АНАЛИЗА ГЛАВНИХ КОМПОНЕНТИ, аутора: Биљана Љ. Лончар (рођ. Чурћић), Лато Л. Пезо, Љубинко Б. Левић, Владимир С. Филиповић, Милица Р. Нићетин, Виолета М. Кнежевић и Татјана А. Куљанин који је објављен у 2014. години (вол. 45, стр. 45-53, DOI: 10.2298/APT1445045L), повлачи се јер се ради о аутоплагијату рада аутора B. L. Ćurčić, L. L Pezo, V. S. Filipović, M.R. Nićetin and V. Knežević „OSMOTIC TREATMENT OF FISH IN TWO DIFFERENT SOLUTIONS – ARTIFICAL NEURAL NETWORK MODEL“ који је прихваћен за објављивање 9. маја 2014. године (DOI: 10.1111/jfpp.12275) и објављен 2015. године у часопису Journal of Food Processing and Preservation (вол. 39, стр. 671-680).

CHEMICAL TECHNOLOGY AND PROCESS ENGINEERING

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TAPPED DENSITY OPTIMISATION FOR FOUR AGRICULTURAL WASTES: PART I – TAGUCHI TECHNIQUE AND MEAN RESPONSE DETERMINATION

*Oluwaseyi Ayodele Ajibade[1], Johnson Olumuyiwa Agunsoye[1] and Sunday Ayoola Oke[2,3]**

[1] Department of Metallurgical and Materials Engineering, University of Lagos, Lagos, Nigeria
[2] Department of Mechanical Engineering, University of Lagos, Lagos, Nigeria
[3] Department of Mechanical Engineering, Covenant University, Ota, Nigeria

Environmental concerns are being intensified in composite manufacturing ventures, using agricultural fillers as strategic materials to improve environmentally-conscious performance. In this investigation, which is the first part of a series of papers, four agricultural wastes (particles of coconut, periwinkle, palm kernels and egg shells) were studied for their tapped density optimisation behaviour, being tested independently of matrices. The manual tapping method with measurements applied in successive taps of the cylinder containing the particles against solid surfaces and the experiments were carried out in the laboratory. Taguchi's method of the "lower-the-better" (LB) quality characteristic was used in this investigation to describe the performance of the experiments. The optimal parametric settings for the particles of coconut, periwinkle, palm kernels and egg shells were found to be $P_2Q_4R_4S_4$, $P_3Q_4R_1S_4$, $P_2Q_3R_1S_4$ and $P_1Q_4R_2S_4$, respectively. The optimal results would help in attaining good reinforcing fillers with better tapped density properties. The method of mean response investigation showed that either of geometric and quadratic methods of means could be used where the LB quality characteristic is required.

KEY WORDS: optimisation, agricultural wastes, tapped density, fillers

INTRODUCTION

The theory of tapped density (1, 2) and the associated experimental procedures are not new developments in science and engineering (3-6). However, they are new in their applied forms to agricultural wastes for composite developments. Agricultural wastes are becoming the central focus of many investigations on composite science and technology because of the immense benefits that they offer (7-9). The widely investigated area of natural-based biodegradable polymer (which includes poly (lactic acid), thermoplastic starch, polyhydroxy-alkanoate) as well as petroleum-based biodegradable polymer, see (10-11) are an example of the beneficial aspects of biodegradable composites. Agricultural wastes for composites are regarded as light-weight and very low density materials

* Corresponding author: Sunday Ayoola Oke, Department of Mechanical Engineering, Room 10, Mezzanine Complex, Faculty of Engineering, University of Lagos, Akoka-Yaba, Lagos, Nigeria, e-mail: sa_oke@yahoo.com

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(12) and hence fill the gap of providing an alternative to metals, which are relatively heavier, and have the disadvantage of high weight per unit area. Thus, agricultural wastes qualify for high-quality, customer-centred and customer desirable characteristics in reinforcing fillers used in composite fabrication. Second, agricultural wastes (13, 14) exhibit a relatively low range of processing temperatures as well as the curing temperatures when used as reinforcing materials in composites. Experience has shown that a commonly employed temperature is the room temperature and the composite outputs are known to perform satisfactorily.

Third, the manufacturing cost of composites that uses agricultural wastes as fillers is low (15). This is obvious as the reinforcing fillers are free of cost in many instances. Fourth, an outstanding characteristic of agricultural wastes is that they require low energy as inputs in the manufacturing process when used as reinforcing fillers for composites. Since we are in a contemporary era where energy-efficient composite manufacturing is the order-of-the-day, then, agricultural wastes are essential and inevitable in the manufacture of composites that are economically made. Fifth, they are bio-degradable and renewable and do not constitute environmental nuisance, unlike metals that cause environmental pollution and cannot decompose in soil. Sixth, they are freely available at sources and are abundant in quantities. Thus, noting the above tremendous benefits from the use of agricultural wastes, a significant effort should be invested in the study of such wastes for beneficial composite fabrication. In this respect, the current study has been motivated to study a range of four agricultural wastes, including the particles of coconut, periwinkle, palm kernels and egg shells in tapped density experiments, for preparation towards being used as reinforcing fillers for composites using any of epoxy, polyester, or vinyl ester resins as the matrix in composites. These agricultural wastes were chosen due to their abundance at collection sources, in the developing countries, particularly Nigeria.

Literature review

Since the motivation for the current work is geared towards carrying out tapped density optimisation measurements for some agricultural wastes, for use as fillers in composites, the literature search is directed towards what has been reported concerning the characterisation of these wastes (particulates of coconut shells, periwinkle shells, palm kernel shells and egg shells) and the composites that were produced using these agro-wastes as fillers.

Research relating to coconut shells. For some years now, reports from goal-directed research towards coconut waste-filled composite studies seem to have been given on the increase in literature. Barbosa et al. (16) investigated the impact strength, microstructural information and the water performance of tannin-phenolic polymers strengthened with fibers of coir. The introduction of coir fibers positively influenced on the impact strength as well as the storage moduli. Pradhan et al. (17) contributed a novel article that developed composite from polyethylene powder using coconut shell powder as the filler. Of interest was the determination of the compressive strength, impact test as well as fractograhic studies. While the compressive strengths of the specimens were below expectation, satisfactory results were obtained from the impact test and fractographic investi-

gations. Bledzki et al. (18) investigated the properties of hybrid coconut shell composite in terms of surface behaviour, chemical and physical characteristics. It was concluded that the coconut shell composite was stable in thermal perspectives when the temperature reached 195^{o}C. Salleh et al. (19) developed a composite having filler origin from activated carbon coconut shell. Improvement in the tensile strength was noticed as the content of activated carbon cocnut shell particles for sample weights of 4 and 8 wt% in composite. Sarki et al. (20) explored the fabrication of coconut shell-based composite with keen interest in understanding its mechanical as well as morphological characteristics. The conclusion was that with the employment of epoxy as well as particulate composite as fillers, the two mentioned properties were enhanced. Romli et al. (21) developed samples for coir fibre reinforced epoxy composite with interest in evaluating its tensile properties. It was the paper's conclusion that coir fibre volume fraction, curing time and coir fibre volume significantly influence the tensile dimensions of the composite. Bello et al. (22) developed coconut shell nanoparticle-based composite using a top-down approach by focusing on the milling period. It was concluded that the growth in the surface area of the filler led to decreased milling period.

Investigations of periwinkle shells. We now briefly review previous papers published with the use of periwinkle wastes. Very sparse documentation abounds on the papers on periwinkle as they relate to composite fabrication. Amaren et al. (23) utilised periwinkle in the preparation of brake pad. A developmental study on periwinkle was provided by Obot et al. (24). In the work, it was concluded that periwinkle shells potentially have attractive interfacial bonding of the waste and polymer resin. The morphology and properties of periwinkle shell brake pad was investigated by Yawas et al. (25). It was concluded that 125 μm sized periwinkle shell particulates rivalled in performance when compared with the commercially-available brake pad.

Studies concerning palm kernel shells. The third set of papers reviewed was focused on palm kernel wastes. The outstanding study by Dagwa et al. (26) is an important advance in the area palm kernel powder experiments linked to tapped density. The Nigerian-oriented details of the work make it unique but different from the current study in a number of perspectives, primarily as follows: (1) In our study, the samples collected were of two different sizes of 0.300 and 0.425 mm, however, no clear details about the size(s) of the composite filler used is given by Dagwa et al. (26); (2) There is no information given on how the tapping could be optimised in Dagwa et al. (26) but the optimisation adventure was rigorously pursued in the current work with the use of Taguchi's method; (3) In Dagwa et al. (26), since optimisation was not discussed, different methods of mean determination to derive the S/N response from the factor level combinations was absent but introduced in a novel manner in the current study. The reviewed study due to Dagwa and co-workers concluded that palm kernel shell is potentially useful as filler for composites. Still reflecting on the novel work by Dagwa et al. (26); the study was published about four years ago and since then, a growing number of studies have advocated for tapped density experimentation of agro-wastes, including Ajibade et al. (2), which is a recent call.

Other studies on palm kernel include Okoroigwe et al. (27) and Agunsoye et al. (28). As the first study focused on the reinforcement potentials of palm kernel shell, the second

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one utilised palm kernel shell in particulate form and studied the composite's mechanical properties. It is noteworthy that neither the first nor the second investigation addressed the tapped density concerns of the palm kernel shell in their analysis. Yet, it could greatly enhance understanding of the compactibility behaviour of the reinforcement and composites. Additional contributions to the advancement of palm kernel filler based composites include Nabinejab et al. (29), Tripathy et al. (30) and Daud et al. (31). The first research by Nabinejab and co-workers fabricated a polymer-oriented composite which employed particles of palm shell as fillers. It was concluded that as the powder size of palm kernel lessened, the tensile as well as flexural strength of the composite heightened. The second article by Tripathy and colleagues employed palm kernel in an experimental examination of its interactive behaviour with copper in composite development. The conclusion from the work was that the thermal stability of the specimens heightened when the volume fraction of copper composite increased up to 0.2. The third article by David and co-workers seemed to have focused on the influence of aminopropylttrimethoxysilane (AMEO) composite while palm kernel shell particles were loaded to it. It was concluded that an enhancement was noticeable in the rubber-filler association and was enhanced with the addition of AMEO. By considering these additional studies in detail, it was noticed that despite the employment of palm kernel shell in their experiments, no issues such as tapped density measurements and optimisation were examined. This presents a wide gap for investigation in composite fabrication, design and development.

Research relating to egg shells. The employment of egg shells in productive research is old and several studies are credited to egg shell usage in extractive compound purposes (32-35). Unfortunately, fewer studies have shown interest in the employment of egg shells as fillers in composites. Although some studies have recognised the power of egg shells in being dependable fillers of enhanced performance when combined with matrices and tested for mechanical and impact properties, a common gap in literature is the complete absence of studies that extensively showcase details of egg shell's tapped density characteristics in terms of measurements and optimisation. This gap should be urgently bridged. However, we review the specific literature that employed eggshell as fillers in the following details.

A few studies have been contributed on composites with the filler being eggshell. Zieleniewska et al. (36) developed a composite based on egg shell waste as filler. It was concluded that results from experiment yielded positive inclination towards extending the work to the cosmetics industry. Wang et al. (37) developed a completely novel composite with the employment of Ag^0 nanoparticles integrated with Artemia egg shell. It was concluded that a composite with enhanced performance was achieved. Chaithanyasai et al. (38) developed a composite using egg shell as the filler and aluminium 6061 grade alloy particulate as the matrix in a composite. The conclusion was that enhanced performance in hardness and an attractive union of the egg shell as well as Al 6061 alloy particulate were observed. Hassan and Aigbodion (39) studied the influence of egg shell particles on Al-Cu-Mg/egg shell's microstructures as well as properties. Using carbonised and uncarbonised samples of egg shell particles, it was concluded that samples of carbonised egg shell as fillers in the matrix yielded improved physical as well as mechanical properties in comparison with uncarbonised eggshell particles as fillers in the matrix.

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The following significant observations emerged from a crucial assessment of the literature, conducted on agricultural wastes involving coconut shell particulates, (CSPs), periwinkle shell particulates (PSPs), palm kernel shell particulates (PKSPs) and egg shell particulates (ESPs) based fillers and composites: (i) A large quantum of information exists, with studies applying the aforementioned agro-based fillers on composites. Diverse applications and the benefits of each fillers in the composites have also been reported; (ii) optimising the parameters of coconut, periwinkle, palm kernel and egg shell particle-based fillers is a great task with respect to tapped density experiments; (iii) There is scanty research on optimisation but the few available reports have focused on single filler among CSPs, PSPs, PKSPs and ESPs (iv) Report on a comparative analysis, on tapped density experiments, using particle fillers of CSPs, PSPs, PKSPs and ESPs with Taguchi method as the optimisation technique has not been reported in literature; (v) Reports relating to optimisation of tapped density process variables, applying the harmonic mean concept, and prioritization of S/N ratios according to vital few, trivial many, for the CSPs, PSPs, PKSPs and ESPs cannot be found in literature. As a consequence of the literature observations from the review, and the research gaps identified, the principal pursuit in this work is to optimise the tapped density parametric quantities of a set of four agro-wastes, namely CSPs, PSPs, PKSPs and ESPs and report on its comparative performance in optimisation.

From the above gaps identified, it appears to the current authors to pursue the optimisation of tapped density using a range of agricultural wastes. Thus, the purpose of this paper is to optimise the tapped density values of four agricultural wastes, namely particulates of coconut, periwinkle, palm kernels and egg shells. The starting point was to carry out laboratory experiments in which the powdered forms of the above-mentioned agricultural wastes are pored into a cylinder and tapped against solid surface, in a range of taps, such that the possible changes in the filling heights of the powdered will be notices. The results of these measurements are then used as inputs into the Taguchi method and the optimal parametric settings for each of the four agricultural wastes computed. It should be noticed that the Taguchi method utilised is a modified form of the traditional method, in which in the new method, the factor levels were determined using the harmonic mean method instead of the average method normally used. This method provides a good alternative to the current method.

EXPERIMENTAL

In this paper, four agricultural wastes are experimented upon, including particulates of coconut, periwinkle, palm kernel and egg shells. The details about collection and procedures shall be given in the second part of this paper. The tapped density experiment is carried out by pouring the particulate fillers into a 250 cm^3 measuring cylinder to a measured volume of 80 cm^3. The initial mass and volume of the material is used in obtaining the apparent density (D_a). The first four taps are applied by tapping the measuring cylinder against a fixed non-movable object. The new mass and volume of the filler particles are then measured to derive the tapped density (D_t) at four taps. Successive taps are applied in increase of four to the base of the measuring cylinder.

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For every application of taps, the mass and volume of the material are measured to derive the tapped density. An experimental run is completed with the application of 12 successive taps with a maximum of 48 applied taps. A complete run is characterized by a reduction in the volume of the material and a slight decrease in its initial mass. Different number of experimental runs was completed for the each of the filler particle sizes respectively, due to their available quantities. The average mass and volume obtained from the experiments were bifurcated using the harmonic method of mean to obtain the values of the factor levels. Taguchi method uses three quality characteristic to describe the performance of the experiment such as the "lower-the-better", identified as the (LB), "higher-the-better", described as the (HB) and "nominal-the-best", tagged the (NB). Results from the tapped density experiments shows that the tapped density of the materials grew under the application of taps. However, improved composite variety demands are for low stiffness and low density composites which also requires low tapped density. Therefore, the "lower-the-better" quality characteristic will be used in this investigation. From the foregoing details, it is essential to schematically represent the flow of this research and this is presented in Figure 1.

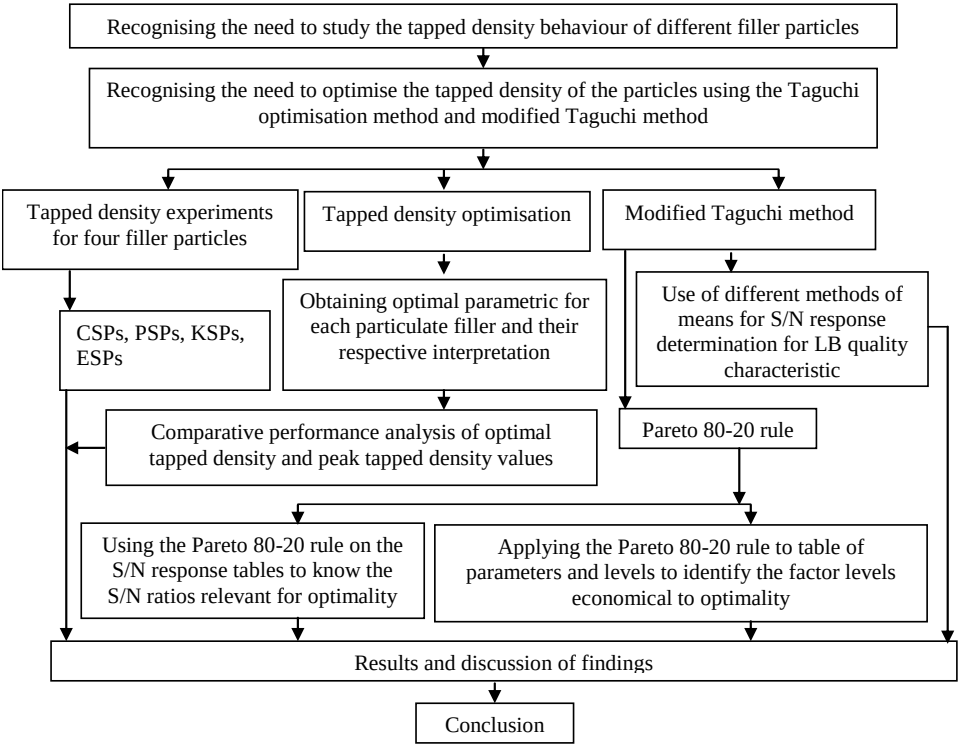

Figure 1. Research scheme for the whole work (Parts I and II)

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Taguchi method's orthogonal array makes use of a statistical system known as signal-to-noise (S/N) ratio to evaluate the behaviour of the system. Anthony and Anthony (40) defined S/N ratios as the output responses of the experiment, which indicates the degree of variation when uncontrolled factors are present within the system. For the purpose of obtaining particulates with low tapped density parameters in this work, the (LB) characteristic would be used. The (LB) quality characteristic is adapted from Zareh et al. (41), given as:

$$S/N = - 10 \text{ Log } (MSD) \quad [1]$$

where MSD refers to Mean Squared Deviation

$$MSD = \frac{1}{n} \sum_{i=1}^{n} y_i^2 \quad [2]$$

$$S/N = -10 \text{ Log } \frac{1}{n} \sum_{i=1}^{n} y_i^2 \quad [3]$$

where y_i is the obtained value of the (LB) quality characteristic and n is the total number of tests in a trial condition.

RESULTS AND DISCUSSION

Taguchi method uses a robust orthogonal array which depends on the initial factors and levels obtained from the experiment, which is described by Table 1(a) as follows.

Table 1(a). Factors and levels for agro-waste particulate tapped density

	Coconut shell particulates (CSPs)				**Egg shell particulates (ESPs)**			
Parameters/factors	Level 1	Level 2	Level 3	Level 4	Level 1	Level 2	Level 3	Level 4
P: 0.300 mass (g)	255.293	255.283	255.291	255.786	295.464	296.763	295.760	295.760
Q: 0.300 vol (cm^3)	75.681	69.707	67.553	66.332	76.641	72.831	71.298	70.298
R: 0.425 mass (g)	259.688	259.686	259.687	259.686	266.84	266.836	266.837	266.838
S: 0.425 vol (cm^3)	76.366	71.896	70.165	69.265	75.123	69.662	68.332	67.441
	Palm kernel shell particulates (PKSPs)				**Periwinkle shell particulates (PSPs)**			
P: 0.425 mass (g)	258.99	258.293	258.285	258.284	307.162	307.158	307.152	307.155
Q: 0.425 vol (cm^3)	76.49	72.663	71.431	70.081	75.753	71.898	71.099	70.183
R: 0.600 mass (g)	254.548	256.549	256.553	256.54	313.856	313.857	313.853	313.856
S: 0.600 vol (cm^3)	76.47	72.615	71.595	70.428	77.713	74.83	74.165	73.165

For a 4-factor, 4-level optimisation problem, an L_{16} (4^4) orthogonal array provides a framework for sixteen experimental trials to derive the optimal setting of the parameters. Minitab 16, commercially available software was used to generate the orthogonal array described by Table 1(b). This table is used for all the four wastes in the analysis concerning orthogonal arrays. The L_{16} orthogonal array comprising the different factor level combinations and obtained S/N ratios is described by Table 1(c).

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Table 1(b). L_{16} (4^4) Orthogonal array of factors and levels

S/No.	P	Q	R	S	S/No.	P	Q	R	S	S/No.	P	Q	R	S	S/No.	P	Q	R	S
1	1	1	1	1	5	2	1	2	3	9	3	1	3	4	13	4	1	4	2
2	1	2	2	2	6	2	2	1	4	10	3	2	4	3	14	4	2	3	1
3	1	3	3	3	7	2	3	4	1	11	3	3	1	2	15	4	3	2	4
4	1	4	4	4	8	2	4	3	2	12	3	4	2	1	16	4	4	1	3

Table 1(c). Orthogonal array containing tapped density experimental results and S/N ratios for filler particulates

	Coconut shell particulates					Periwinkle shell particulates				
S/No.	P	Q	R	S	S/N ratio	P	Q	R	S	S/N ratio
1	255.293	75.680	259.688	76.366	-45.568	307.162	75.753	313.856	77.713	-47.089
2	255.293	69.707	259.686	71.896	-45.522	307.162	71.898	313.857	74.83	-47.068
3	255.293	67.553	259.687	70.165	-45.505	307.162	71.099	313.853	74.165	-47.063
4	255.293	66.332	259.686	69.265	-45.496	307.162	70.183	313.856	73.165	-47.057
5	255.283	75.680	259.686	70.165	-45.541	307.158	75.753	313.857	74.165	-47.078
6	255.283	69.707	259.688	69.265	-45.510	307.158	71.898	313.856	69.265	-47.050
7	255.283	67.553	259.686	76.366	-45.533	307.158	71.099	313.856	76.366	-47.070
8	255.283	66.332	259.687	71.896	-45.508	307.158	70.183	313.853	71.896	-47.053
9	255.291	75.680	259.687	69.265	-45.537	307.152	75.753	313.853	69.265	-47.062
10	255.291	69.707	259.686	70.165	-45.514	307.152	71.898	313.856	70.165	-47.053
11	255.291	67.553	259.688	71.896	-45.513	307.152	71.099	313.856	71.896	-47.055
12	255.291	66.332	259.686	76.366	-45.528	307.152	70.183	313.857	76.366	-47.067
13	255.286	75.680	259.686	71.896	-45.548	307.155	75.753	313.856	71.896	-47.071
14	255.286	69.707	259.687	76.366	-45.542	307.155	71.898	313.853	76.366	-47.073
15	255.286	67.553	259.686	69.265	-45.501	307.155	71.099	313.857	69.265	-47.048
16	255.286	66.332	259.688	70.165	-45.500	307.155	70.183	313.856	70.165	-47.048

Table 1(c) (cont'd). Orthogonal array containing tapped density experimental results and S/N ratios for filler particles

	Palm kernel shell particulates					Egg shell particulates				
S/No	P	Q	R	S	S/N ratio	P	Q	R	S	S/N ratio
1	258.299	76.49	254.548	76.47	-45.539	295.464	76.641	266.84	76.47	-46.290
2	258.299	72.663	256.549	72.615	-45.535	295.464	72.831	266.836	72.615	-46.260
3	258.299	71.431	256.553	71.595	-45.526	295.464	71.298	266.837	71.595	-46.251
4	258.299	70.081	256.540	70.428	-45.515	295.464	70.298	266.838	70.428	-46.243
5	258.293	76.49	256.549	71.595	-45.548	296.763	76.641	256.549	71.595	-46.151
6	258.293	72.663	254.548	70.428	-45.495	296.763	72.831	254.548	70.428	-46.105
7	258.293	71.431	254.540	76.47	-45.516	296.763	71.298	254.54	76.47	-46.122
8	258.293	70.081	256.553	72.615	-45.524	296.763	70.298	256.553	72.615	-46.131
9	258.285	76.49	256.553	70.428	-45.543	295.76	76.641	256.553	70.428	-46.131
10	258.285	72.663	256.54	71.595	-45.531	295.76	72.831	256.54	71.595	-46.120
11	258.285	71.431	254.548	72.615	-45.499	295.76	71.298	254.548	72.615	-46.091
12	258.285	70.081	256.549	76.47	-45.541	295.76	70.298	256.549	76.470	-46.130
13	258.284	76.49	256.54	72.615	-45.552	295.76	76.641	256.54	72.615	-46.139
14	258.284	72.663	256.553	76.47	-45.553	295.76	72.831	256.553	76.470	-46.140
15	258.284	71.431	256.549	70.428	-45.520	295.76	71.298	256.549	70.428	-46.110
16	258.284	70.081	254.548	71.595	-45.488	295.76	70.298	254.548	71.595	-46.084

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Zareh et al. (2013) observed that irrespective of the quality characteristic used, a higher S/N ratio indicates superior quality characteristic. Therefore, the factor level combination with the highest S/N ratio is chosen as the optimal level in this investigation. Trials 1, 2, 3 and 4 are the experimental trials for factor P in the first column of the orthogonal array when it is set at level 1. The S/N response for P_1 is the mean S/N ratio obtained at the aforementioned experimental trials.

$$P_1 = (-45.568 + -45.522 + -45.505 + -45.496)/4 = -45.523;\ P_2 = -45.523;$$
$$P_3 = -45.523 \text{ and } P_4 = -45.523$$

In the same way, the S/N response for all other factor level combinations was obtained in Table 1(d). From the S/N response table described by Table 1(d), the optimal parametric setting for the tapped density of CSPs is given as $P_2Q_4R_1S_4$.

Table 1(d). S/N response table for CSPs tapped density using the harmonic mean

S/No	P	Q	R	S	S/No	P	Q	R	S
1	-45.523	-45.548	-45.523*	-45.543	3	-45.523	-45.513	-45.523	-45.518
2	-45.523*	-45.522	-45.523	-45.519	4	-45.523	-45.508*	-45.523	-45.511*

This translates to a mass and volume of 255.283 g and 66.332 cm^3 for the 0.300 mm CSPs as well as 259.688 g and 69.265 cm^3 for the 0.425 mm CSPs. Now, by following the same procedure as for coconut shell particulate, results on the same parameters as for coconut shell are obtained for the other four waste products, including particulates of periwinkle, palm kernel and egg shells. These are subsequently presented hereunder. The factors and levels of all the other waste products are described in Table 1(a) while the orthogonal array are described by Table 1(b) which gives an outline for sixteen experimental trials to be performed was generated using Minitab 16, a statistical software package. The experimental results and obtained S/N ratios by the orthogonal array. So, the S/N response for P_1 (periwinkle shell particulate) is the mean S/N ratio obtained at the aforementioned experimental trials.

$$P_1 = -47.069,\ P_2 = -47.063,\ P_3 = -47.056 \text{ and } P_4 = -47.060$$

In like manner, the S/N response for all other factor level combinations was obtained to give Table 1(e). From the S/N response table in Table 1(e), the optimal parameter setting for the tapped density of PSPs is given as $P_3Q_4R_1S_4$.

This can be translated as a mass and volume of 307.152 g and 70.183 cm^3 for the 0.425 mm PSPs as well as 313.856 g and 73.163 cm^3 for the 0.600 mm PSPs. For palm kernel shell particulates, the S/N response for P_1 is the mean S/N ratio obtained at the aforementioned experimental trials.

$$P_1 = -45.529,\ P_2 = -45.521,\ P_3 = -45.528 \text{ and } P_4 = -45.528$$

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Table 1(e). S/N response table for PSPs, PKSPs and ESPs tapped density

	S/N response table PSPs tapped density				S/N response table PKSPs tapped density			
S/No	P	Q	R	S	P	Q	R	S
1	-47.069	-47.075	-47.061*	-47.075	-45.529	-45.546	-45.505*	-45.537
2	-47.063	-47.061	-47.065	-47.062	-45.521*	-45.528	-45.536	-45.528
3	-47.060*	-47.060	-47.063	-47.060	-45.528	-45.515*	-45.536	-45.523
4	-47.060	-47.056*	-47.063	-47.055*	-45.528	-45.517	-45.528	-45.518*
	S/N response table ESPs tapped density							
1	-46.261	-46.178	-46.142*	-46.170				
2	-46.127	-46.156	-46.163	-46.155				
3	-46.118	-46.144*	-46.163	-46.151				
4	-46.118*	-46.147	-46.156	-46.147*				

The same mathematical operation is carried out to obtain the S/N responses for all other factor level combination. This produces the S/N response table. From Table 1(e), the optimal parametric setting for the PKSPs tapped density is given as $P_2Q_3R_1S_4$. This reads as 258.293 g and 71.431 cm^3 for the 0.425 mm PKSPs as well as 254.548 g and 70.428 cm^3 for the 0.600 mm PKSPs. For egg shell particulates, the S/N response for P_1 is the mean S/N ratios for trials 1, 2, 3 and 4.

$$P_1 = -46.261,\ P_2 = -46.127,\ P_3 = -46.118 \text{ and } P_4 = -46.118$$

The same operation was performed to obtain the S/N response for all factor level combination as shown in Table 1(e). The optimal parametric setting for the tapped density of ESPs is given as $P_4Q_3R_1S_4$. This can be translated as 295.760 g and 71.298 cm^3 for the mass and volume of 0.300 mm ESPs as well as 266.84 g and 67.441 cm^3 for the mass and volume of the 0.425 mm ESPs.

$$P_1 = -45.522,\ P_2 = -45.518,\ P_3 = -45.518 \text{ and } P_4 = -45.518$$

Using the same operation, the S/N response for all factor level combination was obtained as described by Table 1(e).

Method of mean response determination

In order to obtain lower optimal tapped density values of the filler particles, different methods of mean determination was used to derive the S/N response from the factor level combinations. This provides room for different comparative optimal results for each of the particulates which can be used for different composite varieties. In an earlier work by Ajibade et al. (42), which focused on optimising the moisture loss and drying properties of orange peels using Taguchi method's (HB) quality characteristic. It was observed that the harmonic mean responses could be used to obtain lower optimal results, while the quadratic mean responses could be used to derive higher optimal results when needed. This work seeks to further the investigation into the use of different methods of mean response when the (LB) quality characteristic is required.

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For the CSPs, the arithmetic and harmonic method of mean response produced optimal parametric settings of $P_2Q_4R_1S_4$ and $P_2Q_4R_4S_4$, respectively. However, these different optimal settings produced the same optimal tapped densities of 3.85 and 3.75 g/cm^3, respectively for the 0.300 and 0.425 mm particles. Using the geometric and quadratic methods of mean response, an optimal setting of $P_3Q_1R_3S_1$ was obtained. This gives optimal tapped density values of 3.37 and 3.4 g/cm^3 for the 0.300 and 0.425 mm particles. The optimal setting for the PSPs using the arithmetic and harmonic method of means was found to be $P_3Q_4R_1S_4$. This gives optimal tapped density values of 4.38 and 4.29 g/cm^3 for the 0.425 and 0.600 mm particles. The quadratic and geometric method of mean responses both produced the same optimal setting of $P_1Q_1R_2S_1$. This gives an optimal tapped density of 4.06 and 4.04 g/cm^3 for the 0.425 and 0.600 mm particles.

Using the arithmetic and harmonic method of mean response, the optimal parametric setting of $P_2Q_3R_1S_4$ was obtained for the PKSPs, which gives optimal tapped tapped density values of 3.62 and 3.61 g/cm3 respectively. The geometric and quadratic method of means response both produced an optimal setting of $P_1Q_1R_3S_1$, respectively. This translates to an optimal tapped density of 3.38 and 3.35 g/cm$_3$ for the 0.425 and 0.600 mm particles. The same trend of results was observed in the optimisation of the ESPs tapped density. An optimal setting of $P_1Q_4R_2S_4$ was obtained using the arithmetic and harmonic method of means, which produced optimal tapped density of 4.20 and 3.96 g/cm^3 for the 0.300 and 0.425 mm particles. On the other hand, the geometric and quadratic method of mean responses was used to obtain the same optimal setting of $P_2Q_1R_1S_1$. This gives lower optimal tapped densities of 3.87 and 3.55 g/cm3 for the 0.300 and 0.425 mm ESPs.

The investigation into the method of mean response showed how different ways of obtaining S/N response can be used in obtaining optimal results. In this investigation, the arithmetic and harmonic method of means produced higher optimal tapped density values than those obtained by the geometric and quadratic method of means. Therefore, for the purpose of obtaining lower optimal values of the tapped density values for the four filler particles in preparing improved composite variety demands where low density properties are required, the geometric and quadratic method of means can be adopted. The analysis is presented in Tables 2 (a) to (d).

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Table 2(a). Method of means S/N response determination for CSPs tapped density

Arithmetic method of means for S/N ratios response determination								
	Tapped density parametric levels				S/N response			
Parameters/factors	Level 1	Level 2	Level 3	Level 4	Level 1	Level 2	Level 3	Level 4
P: 0.300 mass (g)	255.293	255.283	255.291	255.286	-45.523	-45.523*	-45.523	-45.523
Q: 0.300 vol (cm^3)	75.680	69.707	67.553	66.332	-45.548	-45.522	-45.513	-45.508*
R: 0.425 mass (g)	259.688	259.686	259.687	259.686	-45.523*	-45.523	-45.523	-45.523
S: 0.425 vol (cm^3)	76.366	71.896	70.165	69.265	-45.543	-45.519	-45.518	-45.511*
Geometric method of means for S/N ratios response determination								
	Tapped density parametric levels				S/N response			
Parameters/factors	Level 1	Level 2	Level 3	Level 4	Level 1	Level 2	Level 3	Level 4
P: 0.300 mass (g)	255.293	255.283	255.291	255.286	45.523	45.523	45.523*	45.523
Q: 0.300 vol (cm^3)	75.68	69.707	67.553	66.332	45.548*	45.522	45.513	45.508
R: 0.425 mass (g)	259.688	259.686	259.687	259.686	45.523	45.523	45.523*	45.523
S: 0.425 vol (cm^3)	76.366	71.896	70.165	69.265	45.543*	45.519	45.518	45.511
Harmonic method of means for S/N ratios response determination								
	Tapped density parametric levels				S/N response			
Parameters/factors	Level 1	Level 2	Level 3	Level 4	Level 1	Level 2	Level 3	Level 4
P: 0.300 mass (g)	255.293	255.283	255.291	255.286	-45.523	-45.523*	-45.523	-45.523
Q: 0.300 vol (cm^3)	75.68	69.707	67.553	66.332	-45.548	-45.522	-45.513	-45.508*
R: 0.425 mass (g)	259.688	259.686	259.687	259.686	-45.523	-45.523	-45.523	-45.523*
S: 0.425 vol (cm^3)	76.366	71.896	70.165	69.265	-45.543	-45.519	-45.518	-45.511*
Quadratic method of means for S/N ratios response determination								
	Tapped density parametric levels				S/N response			
Parameters/factors	Level 1	Level 2	Level 3	Level 4	Level 1	Level 2	Level 3	Level 4
P: 0.300 mass (g)	255.293	255.283	255.291	255.286	45.523	45.523	45.523*	45.523
Q: 0.300 vol (cm^3)	75.68	69.707	67.553	66.332	45.548*	45.522	45.513	45.508
R: 0.425 mass (g)	259.688	259.686	259.687	259.686	45.523	45.523	45.523*	45.523
S: 0.425 vol (cm^3)	76.366	71.896	70.165	69.265	45.543*	45.519	45.518	45.511

* Optimal parametric level

Table 2(b). Method of means S/N response determination for PSPs tapped density

Arithmetic method of means for S/N ratios response determination								
	Tapped density parametric levels				S/N response			
Parameters/factors	Level 1	Level 2	Level 3	Level 4	Level 1	Level 2	Level 3	Level 4
P: 0.425 mass (g)	307.162	307.158	307.152	307.155	-47.070	-47.063	-47.060*	-47.060
Q: 0.425 vol (cm^3)	75.753	71.898	71.099	70.183	-47.075	-47.061	-47.059	-47.056*
R: 0.600 mass (g)	313.856	313.857	313.853	313.856	-47.061*	-47.065	-47.063	-47.063
S: 0.600 vol (cm^3)	77.713	74.83	74.165	73.165	-47.075	-47.062	-47.060	-47.055*
Geometric method of means for S/N ratios response determination								
	Tapped density parametric levels				S/N response			
Parameters/factors	Level 1	Level 2	Level 3	Level 4	Level 1	Level 2	Level 3	Level 4
P: 0.425 mass (g)	307.162	307.158	307.152	307.155	47.069*	47.063	47.060	47.060
Q: 0.425 vol (cm^3)	75.753	71.898	71.099	70.183	47.075*	47.061	47.059	47.056
R: 0.600 mass (g)	313.856	313.857	313.853	313.856	47.061	47.065*	47.063	47.063
S: 0.600 vol (cm^3)	77.713	74.83	74.165	73.165	47.075*	47.062	47.060	47.055

* Optimal parametric level

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Table 2(b) (cont'd). Method of means S/N response determination for PSPs tapped density

Harmonic method of means for S/N ratios response determination								
	Tapped density parametric levels				S/N response			
Parameters/factors	Level 1	Level 2	Level 3	Level 4	Level 1	Level 2	Level 3	Level 4
P: 0.425 mass (g)	307.162	307.158	307.152	307.155	-47.069	-47.063	-47.060*	-47.069
Q: 0.425 vol (cm^3)	75.753	71.898	71.099	70.183	-47.075	-47.061	-47.059	-47.056*
R: 0.600 mass (g)	313.856	313.857	313.853	313.856	-47.061*	-47.065	-47.063	-47.063
S: 0.600 vol (cm^3)	77.713	74.83	74.165	73.165	-47.075	-47.062	-47.060	-47.055*
Quadratic method of means for S/N ratios response determination								
	Tapped density parametric levels				S/N response			
Parameters/factors	Level 1	Level 2	Level 3	Level 4	Level 1	Level 2	Level 3	Level 4
P: 0.425 mass (g)	307.162	307.158	307.152	307.155	47.069*	47.063	47.060	47.060
Q: 0.425 vol (cm^3)	75.753	71.898	71.099	70.183	47.075*	47.061	47.059	47.056
R: 0.600 mass (g)	313.856	313.857	313.853	313.856	47.061	47.065*	47.063	47.063
S: 0.600 vol (cm^3)	77.713	74.83	74.165	73.165	47.075*	47.062	47.060	47.055

* Optimal parametric level

Table 2(c). Method of means S/N response determination for PKSPs tapped density

Arithmetic method of means for S/N ratios response determination								
	Tapped density parametric levels				S/N response			
Parameters/factors	Level 1	Level 2	Level 3	Level 4	Level 1	Level 2	Level 3	Level 4
P: 0.425 mass (g)	258.299	258.293	258.285	258.284	-45.529	-45.521	-45.528	-45.528
Q: 0.425 vol (cm^3)	76.49	72.663	71.431	70.081	-45.546	-45.528	-45.515	-45.517
R: 0.600 mass (g)	254.548	256.549	256.553	256.54	-45.505	-45.536	-45.536	-45.528
S: 0.600 vol (cm^3)	76.47	72.615	71.595	70.428	-45.537	-45.528	-45.523	-45.518
Geometric method of means for S/N ratios response determination								
	Tapped density parametric levels				S/N response			
Parameters/factors	Level 1	Level 2	Level 3	Level 4	Level 1	Level 2	Level 3	Level 4
P: 0.425 mass (g)	258.299	258.293	258.285	258.284	45.529	45.521	45.528	45.528
Q: 0.425 vol (cm^3)	76.49	72.663	71.431	70.081	45.546	45.528	45.515	45.517
R: 0.600 mass (g)	254.548	256.549	256.553	256.54	45.505	45.536	45.536	45.528
S: 0.600 vol (cm^3)	76.47	72.615	71.595	70.428	45.537	45.528	45.523	45.518
Harmonic method of means for S/N ratios response determination								
	Tapped density parametric levels				S/N response			
Parameters/factors	Level 1	Level 2	Level 3	Level 4	Level 1	Level 2	Level 3	Level 4
P: 0.425 mass (g)	258.299	258.293	258.285	258.284	-45.529	-45.521	-45.528	-45.528
Q: 0.425 vol (cm^3)	76.49	72.663	71.431	70.081	-45.546	-45.528	-45.515	-45.517
R: 0.600 mass (g)	254.548	256.549	256.553	256.54	-45.505	-45.536	-45.536	-45.528
S: 0.600 vol (cm^3)	76.47	72.615	71.595	70.428	-45.537	-45.528	-45.523	-45.518
Quadratic method of means for S/N ratios response determination								
	Tapped density parametric levels				S/N response			
Parameters/factors	Level 1	Level 2	Level 3	Level 4	Level 1	Level 2	Level 3	Level 4
P: 0.425 mass (g)	258.299	258.293	258.285	258.284	45.529	45.521	45.528	45.528
Q: 0.425 vol (cm^3)	76.49	72.663	71.431	70.081	45.546	45.528	45.515	45.517
R: 0.600 mass (g)	254.548	256.549	256.553	256.54	45.505	45.536	45.536	45.528
S: 0.600 vol (cm^3)	76.47	72.615	71.595	70.428	45.537	45.528	45.523	45.518

* Optimal parametric level

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Table 2(d). Method of means S/N response determination for ESPs tapped density

Arithmetic method of means for S/N ratios response determination								
	Tapped density parametric levels				S/N response			
Parameters/factors	Level 1	Level 2	Level 3	Level 4	Level 1	Level 2	Level 3	Level 4
P: 0.300 mass (g)	295.464	296.763	295.76	295.76	-46.261*	-46.281	-46.265	-46.265
Q: 0.300 vol (cm^3)	76.641	72.831	71.298	70.298	-46.283	-46.268	-46.263	-46.259*
R: 0.425 mass (g)	266.84	266.836	266.837	266.838	-46.268	-46.268*	-46.268	-46.268
S: 0.425 vol (cm^3)	75.123	69.662	68.332	67.441	-46.282	-46.267	-46.264	-46.259*
Geometric method of means for S/N ratios response determination								
	Tapped density parametric levels				S/N response			
Parameters/factors	Level 1	Level 2	Level 3	Level 4	Level 1	Level 2	Level 3	Level 4
P: 0.300 mass (g)	295.464	296.763	295.76	295.76	46.261	46.281*	46.265	46.265
Q: 0.300 vol (cm^3)	76.641	72.831	71.298	70.298	46.283*	46.268	46.263	46.259
R: 0.425 mass (g)	266.84	266.836	266.837	266.838	46.268*	46.268	46.268	46.268
S: 0.425 vol (cm^3)	75.123	69.662	68.332	67.441	46.282*	46.267	46.264	46.259
Harmonic method of means for S/N ratios response determination								
	Tapped density parametric levels				S/N response			
Parameters/factors	Level 1	Level 2	Level 3	Level 4	Level 1	Level 2	Level 3	Level 4
P: 0.300 mass (g)	295.464	296.763	295.76	295.76	-46.261*	-46.281	-46.26542	-46.265
Q: 0.300 vol (cm^3)	76.641	72.831	71.298	70.298	-46.283	-46.268	-46.26251	-46.259*
R: 0.425 mass (g)	266.84	266.836	266.837	266.838	-46.268	-46.268*	-46.26809	-46.268
S: 0.425 vol (cm^3)	75.123	69.662	68.332	67.441	-46.282	-46.267	-46.26357	-46.259*
Quadratic method of means for S/N ratios response determination								
	Tapped density parametric levels				S/N response			
Parameters/factors	Level 1	Level 2	Level 3	Level 4	Level 1	Level 2	Level 3	Level 4
P: 0.300 mass (g)	295.464	296.763	295.76	295.76	46.261	46.281*	46.265	46.265
Q: 0.300 vol (cm^3)	76.641	72.831	71.298	70.298	46.28277*	46.26819	46.26251	46.25888
R: 0.425 mass (g)	266.84	266.836	266.837	266.838	46.2681*	46.26806	46.26809	46.26808
S: 0.425 vol (cm^3)	75.123	69.662	68.332	67.441	46.28208*	46.26737	46.26357	46.25933

* Optimal parametric level

CONCLUSION

In this research, the tapped density behaviour of four different particulate fillers has been pursued through experimental studies. The second aspect of the work is the use of Taguchi method to optimise the process parameters of the tapped density experiment in order to find the optimal setting for the tapped density in each of the particles. From the aforementioned sections and discussion of results, findings have been made from the optimisation of tapped density parameters of these selected agro-wastes. These findings are presented based on the different steps carried out in the course of the work.

- Periwinkle was found to have the highest density of the particulate fillers. The 0.425 mm periwinkle particles have an apparent density of 3.839 g/cm^3, while the 0.600 mm has an apparent density of 3.923 g/cm^3. The peak densities for the 0.425 and 0.600 mm were found to be 4.38 and 4.30 g/cm^3 respectively, while their average densities were obtained as 4.24 and 4.29 g/cm^3, respectively.
- The 0.300 and 0.425 mm egg shells were found to have apparent densities of 3.69 and 3.34 g/cm3 respectively. The egg shells also have an average tapped density of 4.053 and 3.79 g/cm^3, respectively, for the 0.300 and 0.425 mm particles, while their highest tapped density was obtained as 4.14 and 3.95 g/cm3 respectively.

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- Coconut has an apparent tapped density of 3.19 and 3.25 g/cm^3 respectively, for the 0.300 and 0.425 mm particles. Their average tapped density values was obtained as 3.64 and 3.6 g/cm3 respectively for the 0.300 and 0.425 mm particles, while the highest tapped density values were obtained as 3.87 and 3.77 g/cm3 respectively, for the 0.300 and 0.425 mm particles.
- Palm kernel has an apparent density of 3.23 and 3.19 g/cm^3 for the 0.425 and 0.600 mm particles. Their highest tapped densities were obtained as 3.714 and 3.75 g/cm^3, respectively, while their average values were recorded as 3.55 and 3.59 g/cm^3, respectively, for the 0.425 and 0.600 mm particles.

The optimisation of the tapped density parameters of the four filler particles was done using the Taguchi method. The obtained parametric setting and their respective interpretation is presented as follows:

- Coconut shell particles has an optimal parametric setting of $P_2Q_4R_4S_4$, which reads as 255.283 g and 66.332 cm^3 for the mass and volume of the 0.300 mm particles as well as 259.686 g and 69.265 cm^3 for the 0.425 mm particles. This corresponds to a tapped density of 3.84 and 3.75 g/cm^3 for the 0.300 and 0.425 mm particles.
- The optimal parametric setting for the periwinkle shell particles was obtained as $P_3Q_3R_1S_4$, which can be interpreted as 307.152 g and 70.183 cm^3 for the 0.425 mm particles as well as 313.85 g and 73.165 cm^3 for the 0.600 mm particles. This gives optimal tapped density values of 4.37 and 4.03 g/cm^3 for the 0.425 and 0.600 mm particles, respectively.
- $P_2Q_3R_1S_4$ was obtained as the optimal parametric setting for the palm kernel particles. This translates to 258.293 g and 71.431 cm^3 for the 0.425 mm particles as well as 254.548 g and 70.428 cm^3 for the 0.600 mm particles. This produced optimal tapped densities of 3.62 and 3.61 g/cm^3 for the 0.425 and 0.600 mm particles, respectively.
- The optimal parametric setting for the egg shell particles is given as $P_1Q_4R_2S_4$. This can be read as 295.464 g and 70.298 cm^3 for the 0.300 mm as well as 266.836 g and 67.441 cm^3 for the 0.425 mm particles. This gives new tapped density values of 4.20 and 3.96 g/cm^3 respectively, for the 0.300 and 0.425 mm particles.
- The arithmetic and harmonic method of means response produced higher optimal tapped density values than those obtained by the geometric and quadratic methods. Therefore, in obtaining optimal results with (LB) quality characteristic, the geometric and quadratic method of means could be better preferred.

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ОПТИМИЗАЦИЈА СТРЕСЕНЕ ГУСТИНЕ ЧЕТИРИ ОТПАДНА МАТЕРИЈАЛА ПОЉОПРИВРЕДЕ: ДЕО I – ОДРЕЂИВАЊЕ МЕТОДАМА ТАГУЧИЈА И СРЕДИНЕ ОДЗИВА

Олувасеји Ајоделе Аџибаде[1], Џонсон Олумујива Агунсоје[1] и Сандеј Ајула Оке[2,3]

[1] Департман инжењерства металургије и материјала, Универзитет у Лагосу, Лагос, Нигерија
[2] Департман машинства, Универзитет у Лагосу, Лагос, Нигерија
[3] Департман машинства, Универзитет у Ковенанту, Ота, Нигерија

У оквиру бриге о околини интензивирано је интересовање за прављење композита уз коришћење филера од отпадака пољопривреде као стратешких материјала за побољшање перформанси у смислу односа према околини. У овом истраживању, које представља први део серије радова, испитивана су четири отпадна материјала пољопривреде: честице кокоса, зимзелени, палмине коштице и коре јајета, и да се

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изведе оптимизација понашања њихове стресене густине независно од матрикса. У лабораторијским експериментима је примењена метода ручног стресања, "лупкањем" мерног цилиндра који садржи честице о чврсте површину, а мерење је вршено после сваког низа сукцесивних стресања. За описивање перформанси експеримента примењена је Тагучијева метода и критеријум "мање-је-боље" (МБ) као карактеристика квалитета. Нађено је да су оптималне параметарске комбинације за честице кокоса, зимзелени, палминих коштица и љуски јајета $P_2Q_4R_4S_4$, $P_3Q_4R_1S_4$, $P_2Q_3R_1S_4$ и $P_1Q_4R_2S_4$, редом. Оптимални резултати могли би да буду од помоћи у добијању добрих армирајући пунилаца са бољим карактеристикама стресене густине. Метода средине одзива је показала да се могу применити било метода геометријских или квадратних средина када се захтева МБ карактеристика квалитета.

Кључне речи: оптимизација, пољопривредни отпад, стресена густина, пуниоци

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TAPPED DENSITY OPTIMISATION FOR FOUR AGRICULTURAL WASTES: PART II - PERFORMANCE ANALYSIS AND TAGUCHI-PARETO

*Oluwaseyi Ayodele Ajibade[1], Johnson Olumuyiwa Agunsoye[1] and Sunday Ayoola Oke[2,3]**

[1] Department of Metallurgical and Materials Engineering, University of Lagos, Lagos, Nigeria
[2] Department of Mechanical Engineering, University of Lagos, Lagos, Nigeria
[3] Department of Mechanical Engineering, Covenant University, Ota, Nigeria

In this attempt, which is a second part of discussions on tapped density optimisation for four agricultural wastes (particles of coconut, periwinkle, palm kernel and egg shells), performance analysis for comparative basis is made. This paper pioneers a study direction in which optimisation of process variables are pursued using Taguchi method integrated with the Pareto 80-20 rule. Negative percentage improvements resulted when the optimal tapped density was compared with the average tapped density. However, the performance analysis between optimal tapped density and the peak tapped density values yielded positive percentage improvements for the four filler particles. The performance analysis results validate the effectiveness of using the Taguchi method in improving the tapped density properties of the filler particles. The application of the Pareto 80-20 rule to the table of parameters and levels produced revised tables of parameters and levels which helped to identify the factor-levels position of each parameter that is economical to optimality. The Pareto 80-20 rule also produced revised S/N response tables which were used to know the relevant S/N ratios that are relevant to optimality.

KEY WORDS: optimisation, performance analysis, significance tests, pareto rule

INTRODUCTION

In the composite industry, concerns are often shown for the changing density values of reinforcing filling powders during transportation. Usually, discrepancies are often noticed in the values of the densities. As a solution to this, a scientific approach adopted to solve this dispute is the use of experimental tapped density. Still, this tapped density needs to optimised and the use of Taguchi becomes relevant since the investment of research efforts is just growing in this direction (1, 2). Taguchi method (TM) is an optimisation method based on a special design technique known as design of experiments (3, 4). Its uniqueness lies in the incorporation of statistical methods into various engineering process and its ability to use different factors at the same time, unlike conventional methods which uses one factor at the same time. This makes Taguchi method to be used

* Corresponding author: Sunday Ayoola Oke, Department of Mechanical Engineering, Room 10, Mezzanine Complex, Faculty of Engineering, University of Lagos, Akoka-Yaba, Lagos, Nigeria, e-mail: sa_oke@yahoo.com

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profitably in various industrial and engineering processes to maximise yield or profit under tough economic conditions. Taguchi method design of experiment uses a robust orthogonal array which allows for all possible combination of factors and levels. Parameters involved in the experiment are known as factors, while levels describe the possible conditions of the factors during the experiment.

A literature review was conducted on the four agricultural wastes to find out to what extent the wastes have been applied in practice (5, 6), as fillers to composites. The search of literature retuned information about some papers on orange peels and particulates as well as on coconut shell and particulates. A scanty number of studies were also sighted based on egg shell particles but composites or independent examinations of fillers of palm kernel shell and periwinkle shell particles are non-existent. The reason for the scanty and sometimes absence of studies of waste agricultural products as fillers is due to their filler characteristics and potentials not being fully appreciated. Another fact is the non- availability of scientific reporting on their properties, such as tapped density optimisation, which compels engineers and scientists to choose fillers that are well-studied and show reported rewards. It is not a surprise to note that majority of studies on orange peels and particulates are outside the composites fabrication domain.

EXPERIMENTAL

This section is dedicated to the materials used in the paper as well as the methods of analysis. The detailed information on the materials used for the experiments has been given in the first paper. Also, the details of the methods have been given in the schematic diagram concerning the research in the first paper also. The information from the schematic diagram (Figure 1 of the first paper) that concerns this work is principally the issue of Taguchi-Pareto. The claim of relevance of Taguchi-Pareto observation is made in this paper and in this section, justification concerning the choice of Taguchi scheme as well as Pareto analysis the necessity to integrate them is promoted. First, we start with the advantage of the Taguchi scheme. The Taguchi scheme principally has the advantage of tremendous cost savings compared with the basic mathematical optimisation steps in literature. This advantage stems from the fact that the required quantities of experimental runs to achieve acceptable results with the employment of Taguchi scheme is often very less in comparison with what is necessary using the basic mathematical optimisation steps.

Pareto, as a concept, has gained tremendous attention of quantitative researchers, particularly in industrial engineering as a realistic observation in most practical events that the majority of industrial activities, and in this case, issues concerning tapped density optimisation parametric values, are not distributed in an even manner, but according to an 80:20 percentage observation. The success of the Pareto analysis in industrial applications and theory has therefore motivated its use in our current work. However, it will be integrated with the Taguchi scheme to derive the most benefit from its synergy. The major advantage of Pareto lies in the understanding that it has the capability of prioritising certain problem causal agents in such a way that the most severe problems are

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distinguished and arranged in order of strength to the least severe problem. Synergy in the perspective of tapped density optimisation with respect to filler development in the composite industry is the integrated influence of Taguchi scheme and Pareto analysis that produces a situation in which the outcome of the integration is more beneficial to the system than the addition of the individual tools of Taguchi scheme as well as Pareto analysis employed alone to the tapped density optimisation process. The synergic effect of these two tools is positive as it will improve the optimisation results.

RESULTS AND DISCUSSION

Analysis of variance (ANOVA)

The use of the Taguchi method gives information for choosing the optimal parameter condition. It also makes it possible to analyse the relevance of each parameter for subsequent studies. The analysis of variance (ANOVA) makes it possible to measure the effect of the tapped density parameters on the quality characteristics of interest (7). The results of the ANOVA for the CSPs, PSPs, PKSPs and ESPs tapped density are presented in Table 1(a)-(d). Please note that F_{crit} means the critical F while $F_{0.05}$ means the F value at 95% level of significance. Also p-value is called the significant p.

Table 1(a). ANOVA table for CSPs tapped density using arithmetic, harmonic, geometric and quadratic S/N responses

	Source of Variance	SS	*df*	MS	F	p-value	F_{crit}
Arithmetic S/N response	Factors	1.69 E-12	3	5.63E-13	6.45E-09	1	3.862
	Levels	0.000746	3	0.000249	2.851281	0.097316	3.862
	Error	0.000784	9	8.72 E-05			
	Total		15				
Harmonic S/N response	Factors	2.27E-11	3	7.56E-12	8.67E-08	1	3.862
	Levels	0.000745	3	0.000248	2.846614	0.097638	3.862
	Error	0.000785	9	8.73E-05			
	Total	0.00153	15				
Geometric S/N response	Factors	1.5E-10	3	5E-11	5.73E-07	1	3.862
	Levels	0.000746	3	0.000249	2.849947	0.097408	3.862
	Error	0.000785	9	8.72E-05			
	Total	0.00153	15				
Quadratic S/N response	Factors	1.69E-10	3	5.63E-11	6.45E-07	1	3.862
	Levels	0.000746	3	0.000249	2.850482	0.097371	3.862
	Error	0.000785	9	8.72E-05			
	Total	0.00153	15				

* F_{crit} means Critical F

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Table 1(b). ANOVA table for PSPs tapped density using arithmetic, harmonic, geometric and quadratic S/N responses

	Source of Variance	SS	*df*	MS	F	p-value	F_{crit}
Arithmetic S/N response	Factors	3.746E-12	3	1.25E-12	5.97E-08	1	3.862
	Levels	0.0003042	3	0.000101	4.845557	0.028325	3.862
	Error	0.0001883	9	2.09E-05			
	Total	0.0004926	15				
Harmonic S/N response	Factors	1.87E-11	3	6.25E-12	2.99E-07	1	3.862
	Levels	0.000304	3	0.000101	4.85313	0.028209	3.862
	Error	0.000188	9	2.09E-05			
	Total	0.000493	15				
Geometric S/N response	Factors	1.87E-11	3	6.25E-12	2.99E-07	1	3.862
	Levels	0.000304	3	0.000101	4.850653	0.028247	3.862
	Error	0.000188	9	2.09E-05			
	Total	0.000493	15				
Quadratic S/N response	Factors	1.87E-11	3	6.25E-12	2.99E-07	1	3.862
	Levels	0.000304	3	0.000101	4.850653	0.028247	3.862
	Error	0.000188	9	2.09E-05			
	Total	0.000493	15				

* F_{crit} means Critical F

Table 1(c). ANOVA table for PKSPs tapped density using arithmetic, harmonic, geometric and quadratic S/N responses

	Source of Variance	SS	*df*	MS	F	p-value	F_{crit}
Arithmetic S/N Response	Factors	3.167E-11	3	1.056E-11	6.81E-08	1	3.862
	Levels	9.296E-05	3	3.099E-05	0.199988	0.893796	3.862
	Error		9	0.0001549			
	Total	0.0014874	15				
Harmonic S/N response	Factors	1.69E-10	3	5.63E-11	3.63E-07	1	3.862
	Levels	9.29E-05	3	3.1E-05	0.199926	0.893838	3.862
	Error		9	0.000155			
	Total	0.001487	15				
Geometric S/N response	Factors	2.19E-10	3	7.29E-11	4.71E-07	1	3.862
	Levels	9.3E-05	3	3.1E-05	0.20017	0.893672	3.862
	Error		9	0.000155			
	Total	0.001487	15				
Quadratic S/N response	Factors	5E-11	3	1.67E-11	1.08E-07	1	3.862
	Levels	9.28E-05	3	3.09E-05	0.199694	0.893996	3.862
	Error	0.001394	9	0.000155			
	Total	0.001487	15				

* F_{crit} means Critical F

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Table 1(d). ANOVA table for ESPs tapped density using arithmetic, harmonic, geometric and quadratic S/N responses

	Source of Variance	SS	*df*	MS	F	p-value	F_{crit}
Arithmetic S/N response	Factors	1.785E-08	3	5.951E-09	5.11E-06	1	3.862
	Levels	0.0055136	3	0.0018379	1.577182	0.261804	3.862
	Error	0.0104875	9	0.0011653			
	Total	0.0160012	15				
Harmonic S/N response	Factors	1.19E-10	3	5.951E-09	6.48E-07	1	3.862
	Levels	0.000298	3	0.0018379	1.627956	0.25076	3.862
	Error	0.00055	9	6.11E-05			
	Total	0.000849	15				
Geometric S/N response	Factors	1.88E-11	3	6.25E-12	1.02E-07	1	3.862
	Levels	0.000299	3	9.96E-05	1.629763	0.250377	3.862
	Error	0.00055	9	6.11E-05			
	Total	0.000849	15				
Quadratic S/N response	Factors	7.55E-11	3	2.5E-12	4.09E-07	1	3.862
	Levels	0.000298	3	9.95E-05	1.628067	0.250737	3.862
	Error	0.00055	9	6.11E-05			
	Total	0.000848	15				

* F_{crit} means Critical F

Table 2. Decision table for CSPs, PSPs, PKSPs and ESPs ANOVA

	Method of mean S/N response			
CSPs ANOVA	Description	Arithmetic	Harmonic	Geometric	Quadratic
	F_{crit}	3.86	3.86	3.86	3.862
	$F_{0.05}$	3.862	3.862	3.862	3.86
	Decision made	Rejection of null-hypothesis	Rejection of null-hypothesis	Rejection of null-hypothesis	Rejection of null-hypothesis
	Method of mean S/N response				
PSPs ANOVA	Description	Arithmetic	Harmonic	Geometric	Quadratic
	F_{crit}	3.86	3.86	3.86	3.862
	$F_{0.05}$	3.862	3.862	3.862	3.86
	Decision made	Rejection of null-hypothesis	Rejection of null-hypothesis	Rejection of null-hypothesis	Rejection of null-hypothesis

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Table 2. Continuation

PKSPs ANOVA	**Method of mean S/N response**				
	Description	Arithmetic	Harmonic	Geometric	Quadratic
	F_{crit}	3.86	3.86	3.86	3.862
	$F_{0.05}$	3.862	3.862	3.862	3.86
	Decision made	Rejection of null-hypothesis	Rejection of null-hypothesis	Rejection of null-hypothesis	Rejection of null-hypothesis
ESPs ANOVA	**Method of mean S/N response**				
	Description	Arithmetic	Harmonic	Geometric	Quadratic
	F_{crit}	3.86	3.86	3.86	3.862
	$F_{0.05}$	3.862	3.862	3.862	3.86
	Decision made	Rejection of null-hypothesis	Rejection of null-hypothesis	Rejection of null-hypothesis	Rejection of null-hypothesis

* F_{crit} means Critical F

The decision table for the ANOVA of the particulates tapped density using different methods of S/N mean response is described by Table 2. Since F_{crit} equals 3.862 which exceeds $F_{0.05}$ = 3.86, which is the value for 3 and 9 degrees of freedom for the numerator and denominator, respectively (8, 9). As a result of this difference, we can conclude that there is a difference in the mean values of the various factors for the different levels of the tapped density experimental values.

Performance analysis

The performance analysis of the Taguchi optimisation was measured in terms of percentage improvement of the optimal tapped density over the peak and average tapped density values of the particulates. This was done to find the effectiveness of the Taguchi method (10, 11) in providing lower tapped density values over the average and peak tapped density values obtained from the experiment. This is presented in Table 3(a) as follows.

The average tapped density values was obtained by finding the mean of the tapped density values across all the runs of the tapped density experiments, while the peak tapped density values is the highest tapped density obtained at the application of 48 taps. The optimal tapped density value was derived by dividing the mass by volume obtained from the optimal Taguchi setting. From Table 3(a), the performance analysis of the Taguchi optimal results was carried out by finding the percentage improvement in two directions. First is the percentage improvement of the optimal tapped density over the average tapped density of each particulate fillers. Second, is the percentage improvement of the optimal tapped density over the highest tapped density measurement for each of the particulate filler.

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Table 3(a). Performance analysis of optimal density with tapped density values of fillers

S/N	Filler particle size	Performance analysis			Performance analysis		
		Average tapped density (g/cm^3)	Optimal tapped density (g/cm^3)	Percentage improvement (%)	Peak tapped density (g/cm^3)	Optimal tapped density (g/cm^3)	Percentage improvement (%)
1	0.300 mm Coconut	3.64	3.85	-5.77	3.87	3.85	0.52
2	0.425 mm Coconut	3.6	3.75	-4.16	3.77	3.75	0.53
3	0.425 mm Periwinkle	4.24	4.38	-3.3	4.38	4.39	0.23
4	0.600 mm Periwinkle	4.18	4.29	-2.63	4.30	4.29	0.23
5	0.425 mm Palm kernel	3.55	3.62	-1.97	3.71	3.62	2.43
6	0.600 mm Palm kernel	3.52	3.61	-2.55	3.66	3.61	1.37
7	0.300 mm Egg shell	4.05	4.20	-3.70	4.23	4.20	0.71
8	0.425 mm Egg shell	3.79	3.96	-4.22	3.98	3.96	0.51

The particulate filler with the highest average tapped density is the 0.425 mm periwinkle with an average density of 4.24 g/cm^3, while the optimal tapped density from the Taguchi optimisation gave 4.38 g/cm^3. A negative percentage improvement of -3.3 % was obtained from the performance analysis. This indicates that the average tapped density value is not a reflection of the all the tapped density values obtained at the application of different number of taps. Hence, it cannot be optimised and it did not give a positive percentage improvement. On the other hand, the highest tapped density of the 0.425 mm was obtained as 4.39 g/cm^3 at the application of 48 taps. The performance analysis produced a positive improvement of 0.23 % for the optimal tapped density over the peak tapped density values of the 0.425 mm periwinkle. This means that the Taguchi method was effective in obtaining a lower tapped density value than the highest tapped density value of the particle. For all the particulate sizes, the performance analysis of the optimal values over the average tapped density values produced negative results. The performance analysis of the optimal tapped density values over the peak values of the filler particles gave positive percentage improvements. The effectiveness of the Taguchi method in obtaining lower tapped density results was significantly pronounced in the case of 0.425 mm palm kernel shell particles which had a percentage improvement of 2.43 %, followed by 0.300 mm egg shell particles which had an improvement of 1.89 % .

Taguchi-Pareto (80-20) rule analysis (factor-level dependent)

The Pareto 80-20 rule was used in finding the factor level values which makes the most significant contribution to the overall tapped density of the particulate fillers. This was done by arranging the factor levels in descending order from the highest to the lowest. For each of the filler particles, the overall sum of the factor level values was obtained. The percentage contribution of each factor level to the overall sum was also obtained. This was followed by finding the cumulative percentage contribution of each factor level to the overall sum. At the 80 % threshold, the factor levels are separated using the 80-20 rule. The factor levels from the 80 % threshold and above are rearranged to obtain a revised table of parameters and levels. The Taguchi method is applied to the

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revised tables to give a new optimal parametric setting. This new optimal setting is the Taguchi-Pareto optimal setting.

For the CSPs, the revised table is described by Table 3(b). The revised table shows that all the factor levels for 0.300 and 0.425 mm mass (P and R) are retained, while only one factor level was retained for 0.300 and 0.425 mm volume (Q_1 and S_1). The factor levels present in the revised table indicates that it is economical to pursue their optimality. On the other hand, the absence of factor levels from the revised table indicates that their contribution is not significant as revealed by the use of the Pareto 80-20 rule. Therefore, adequate attention may not be given to them. The geometric and quadratic methods of mean S/N response determination have been identified earlier in this investigation to obtain better optimal values than the conventional mean when the LB quality characteristics are required. Using these preferred methods, the Taguchi-Pareto optimal setting for CSPs tapped density is given as $P_4Q_1R_2S_1$. This gives optimal tapped densities of 3.37 and 3.40 g/cm^3 respectively, for the 0.300 and 0.425 mm CSPs particles. Although the Taguchi-Pareto optimal setting differs from the Taguchi optimal setting, both of them produced the same optimal densities for both particle sizes of the CSPs. The Pareto 80-20 rule helped to identify the factor levels of each parameter which are economical to optimise.

By applying the same principles of Pareto 80-20 rule to the PSPs tapped density parameters, a revised table of parameters and levels is obtained which is described by Table 3(c). The factor levels for 0.425 and 0.600 mm mass (P and R) respectively, were retained in the revised table, while only one from 0.600 volume (S_1) was available. However, no factor level was retained for the 0.425 mm volume (Q). This is an indication that it is not economical to go for its optimality, because the input of the parameter is not significant as shown by the Pareto 80-20 rule. Therefore, Taguchi-Pareto optimal setting is obtained as $P_1R_2S_1$. As a result, only the optimal tapped density for the 0.600 mm particles was obtained as 4.04 g/cm^3. The Taguchi-Pareto and Taguchi optimal settings were found to differ; they however produced the same optimal tapped density for the 0.600 mm particles. The application of the Pareto 80-20 rule showed that the contribution of Q parameter factor levels were less significant to the tapped density and not advisable for optimisation.

Using the same Pareto 80-20 rule for the PKSPs tapped density parameters, a revised table of parameters and levels was obtained which is given by Table 3(d). In the revised table, all the factor levels for 0.425 and 0.600 mm mass (P and R) are available while only one factor level for the 0.425 volume (Q_1) was retained. The revised table did not contain any factor for 0.600 volume (S). The implication of this is that it is not cost effective to go for its optimality because its contribution is not significant going by the use of the Pareto 80-20 rule. As a result of this, the Pareto-Taguchi optimal parametric setting is obtained as $P_1Q_1R_3$. This gives an optimal tapped density of 3.38 g/cm^3 for the 0.425 mm particles which agrees with that obtained by the Taguchi optimal setting while the optimal tapped density could not be determined due to application of the Pareto 80-20 rule. Applying the Pareto 80-20 rule to the ESPs, a revised table of parameters and levels was obtained which is given by Table 12d. From the revised table, all the factor levels for 0.300 and 0.425 mm particles were retained, while only one factor level was available for

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the for the 0.300 mm volume (Q_1). However, the 0.425 mm volume (S) did not have any factor level in the revised table. The absence of the S parameter from the revised table shows that its contribution to the overall tapped density is not significant going by the Pareto 80-20 rule. Therefore, it is not economical to go for its optimality. The resulting Pareto-Taguchi optimal setting is given as $P_2Q_4R_1$. The optimal tapped density of the 0.300 mm particles was obtained as 3.87 g/cm^3 which agree with the value obtained by the Taguchi method.

In Table 3, all the factor levels for the 0.425 and 0.600 mm mass (P and R) are retained, while only one factor level for the 0.425 mm volume (Q_1) was retained in the new table. None of the 0.600 mm volume (S) factor levels are however present in the revised table of parameters. Going by the Pareto 80-20 rule, the contribution of the S parameter is low and is not advisable to go for their optimality. Therefore, the Taguchi-Pareto optimal setting is given as $P_1Q_4R_1$. The Pareto-Taguchi optimal tapped density for the 0.425 mm particles is obtained as 3.30 g/cm^3 which is the same with the value obtained by the Taguchi method. Table 3(c) is the optimal parametric settings and tapped densities obtained by Taguchi and Taguchi-Pareto methods.

Table 3(b). Revised table of parameters and levels for particulate fillers

Levels	Parameters			
	CSPs tapped density			
	P: 0.300 mm mass(g)	Q: 0.300 mm vol (g/cm^3)	R: 0.425 mm mass(g)	S: 0.425 mm vol. (g/cm^3)
1	255.293	75.681	259.688	76.366
2	255.283		259.686	
3	255.291		259.687	
4	255.786		259.686	
	PSPs tapped density			
	P: 0.425 mm mass(g)	Q: 0.425 mm vol (g/cm^3)	R: 0.600 mm mass(g)	S: 0.600 mm vol. (g/cm^3)
1	307.162		313.856	77.713
2	307.158		313.857	
3	307.152		313.853	
4	307.155		313.856	
	PKSPs tapped density			
	P: 0.425 mm mass(g)	Q: 0.425 mm Vol (g/cm^3)	R: 0.600 mm mass(g)	S: 0.600 mm vol. (g/cm^3)
1	258.299	76.49	254.548	
2	258.293		256.549	
3	258.285		256.553	
4	258.284		256.54	
	ESPs tapped density			
	P: 0.300 mm mass(g)	Q: 0.300 mm vol (g/cm^3)	R: 0.425 mm mass(g)	S: 0.425 mm vol. (g/cm^3)
1	295.464	76.641	266.84	
2	295.763		266.836	
3	295.76		266.837	
4	295.76		266.838	

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Table 3(c). Optimal parametric settings and tapped densities obtained by Taguchi and Taguchi-Pareto methods

S/No.	Particulate filler	Optimal setting		Optimal tapped density (g/cm^3)	
		Taguchi	Taguchi-Pareto	Taguchi	Taguchi-Pareto
1	CSPs	$P_3Q_1R_3S_1$	$P_4Q_1R_2S_1$	(0.300 mm:3.37) (0.425 mm: 3.4)	(0.300 mm:3.37) (0.425 mm: 3.40)
2	PSPs	$P_1Q_1R_2S_1$	$P_1R_2S_1$	(0.425 mm: 4.06) (0.600 mm: 4.04)	(0.600 mm: 4.04)
3	PKSPs	$P_1Q_1R_3S_1$	$P_1Q_1R_3$	(0.425 mm: 3.38) (0.600 mm: 3.35)	(0.425 mm: 3.38)
4	ESPs	$P_2Q_1R_1S_1$	$P_2Q_1R_1$	(0.300 mm:3.87) (0.425 mm: 3.55)	(0.425 mm: 3.87)

Taguchi-Pareto 80-20 rule analysis (s/n ratio-based method)

The Pareto 80-20 rule was applied to the S/N response tables of the particulate fillers tapped density. This was done in order to know which of the S/N ratios are significant to the optimisation of each of the filler particles. All the S/N ratios in each response table for the respective particles were arranged in descending order from the largest to the smallest value. The overall sum of the S/N ratios was obtained and the percentage contribution of each S/N ratio to the total sum was calculated. The S/N ratios are separated using the 80-20 rule at 80 % threshold of the cumulative percentages.

For the CSPs, the revised S/N response table is given by Table 4(b). The Taguchi-Pareto optimal setting is consistent with the optimal setting obtained when the Pareto 80-20 rule was applied to the factor-level based method. However, the revised S/N response table shows the absence of S/N ratios from factor levels S_2, S_3 and S_4. These S/N ratios were cut off by the direct application of the Pareto 80-20 rule to the S/N response table. Applying the Pareto 80-20 rule to the PSPs S/N response table produced a revised table described by Table 4(c). The optimal setting was obtained as $P_1Q_2R_2S_1$ which differs from that obtained when the Pareto rule was applied to the table of factors and levels. The major difference is the inclusion of parameter Q which was left out of the revised table of parameters and levels in the new optimal setting. The S/N ratios of parameter Q were found to be significant to the optimisation of the PSPs tapped density using the Pareto 80-20 rule, although they were not considered when the Q parameter was left out of the revised table of parameters and levels. The S/N ratios absent from the revised S/N response table are from factor level positions S_2, S_3 and S_4. This is as a result of being demarcated by the Pareto 80-20 rule at the 80 % threshold.

Using the Pareto 80-20 rule on the PKSPs S/N ratios gave a revised S/N response table described in Table 4(d). From the table, the Taguchi-Pareto optimal setting is given as $P_1Q_1R_3S_1$. The major difference in the Pareto-Taguchi optimal setting is the presence of the S parameter which was left out in the revised table of parameters and levels. The revised S/N response table shows the S/N ratios from the factor levels that are significant

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for optimality. The S/N ratios from factor levels Q_2, Q_3 and Q_4 are considered not significant for optimality using the Pareto 80-20 rule and are not included in the revised S/N response table. The revised S/N response table for ESPs is described by Table 4(e). This was obtained after the application of the Pareto 80-20 rule to the S/N ratios of the ESPs response table. The new optimal setting is given as $P_2Q_1R_1S_1$ which varies with when the Pareto rule was applied to the table of parameters and levels. The main difference is the inclusion of the S parameter which was removed from the revised table of parameters and levels and from the optimal setting. The revised S/N response shows the presence of S/N ratios relevant for optimality, while the absence of S/N ratios from factor level positions Q_2, Q_3 and Q_4 indicates they are not relevant for optimality by the Pareto 80-20 rule.

A new optimal setting of $P_1Q_1R_1S_2$ was obtained which differs significantly when the Pareto 80-20 rule was applied to the parameters and levels. This is a result of the inclusion of the S parameter which was excluded from the revised table of parameters and levels. The S/N ratios present in the revised S/N response table are economical for optimality. The S/N ratios from factor level Q_2, Q_3 and Q_4 are absent from the revised S/N response table which indicates that they have been removed by the application of Pareto 80-20 rule. The application of the Pareto 80-20 rule to the S/N response table helps to identify the relevant S/N ratios that are economical for optimality for a given process. The S/N ratios not relevant for optimality were cut off by the Pareto 80-20 rule and are not included in the revised S/N response table. Hence, their significance is minimal and cannot be considered for optimality (Table 4).

Table 4. Revised S/N response tables for the particulate fillers

Levels	**Parameters**			
	Table 4b. Revised S/N response table for CSPs tapped density			
	P: 0.300 mm mass(g)	Q: 0.300 mm vol (g/cm^3)	R: 0.425 mm mass(g)	S: 0.425 mm vol. (g/cm^3)
1	45.296	45.436	45.298	45.438
2	45.298	45.254	45.300	
3	45.298	45.254	45.300	
4	45.306	45.254	45.300	
	Table 4c. Revised S/N response table for PSPs tapped density			
	P: 0.425 mm mass(g)	Q: 0.425 mm vol (g/cm^3)	R: 0.600 mm mass(g)	S: 0.600 mm vol. (g/cm^3)
1	46.865	46.865	46.865	46.966
2	46.865	46.865	46.865	
3	46.865	46.865	46.865	
4	46.865	46.8651244	46.8651309	

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Table 4. Continuation

Levels	**Table 4d.** Revised S/N response table for PKSPs tapped density			
	P: 0.425 mm mass(g)	Q: 0.425 mm vol (g/cm^3)	R: 0.600 mm mass(g)	S: 0.600 mm vol. (g/cm^3)
1	45.253	45.384	45.219	45.244
2	45.241		45.252	45.244
3	45.241		45.253	45.244
4	45.241		45.252	45.244
	Table 4e. Revised S/N response table for ESPs tapped density			
	P: 0.425 mm mass(g)	Q: 0.425 mm vol (g/cm^3)	R: 0.600 mm mass(g)	S: 0.600 mm vol. (g/cm^3)
1	46.021	46.151	46.028	46.028
2	46.042		46.028	46.028
3	46.026		46.028	46.028
4	46.026		46.028	46.028

CONCLUSION

In the current research, which is the second part of an article, the following conclusions are made. The analysis of variance (ANOVA) technique was used to calculate the individual contributions of each parameter to the tapped density of each particle. The performance analysis produced negative percentage improvements when the optimal tapped density was compared with the average tapped density except for orange peels which yielded 0%. However, the performance analysis between optimal tapped density and the peak tapped density values yielded positive percentage improvements for the four filler particles. The F_{crit} was found to exceed the $F_{0.05}$ value which implies that we can conclude that there is a difference in the mean values of the various factors for the different levels of the tapped density experimental values. The performance analysis results validate the effectiveness of using the Taguchi method in improving the tapped density properties of the filler particles.

The application of the Pareto 80-20 rule was carried in two main directions. First, it was applied to the table of parameters and levels to find the most significant factor levels that are economical for optimality of the particulate tapped density. Second, it was used on the S/N response tables to find the S/N ratios that are relevant to optimality of the particulate tapped density.

- The application of the Pareto 80-20 rule to tables of parameters and levels produced revised tables of parameters and levels. New optimal settings were obtained for the PSPs, PKSPs and ESPs tapped density, while that of CSPs remain unchanged. The new optimal settings obtained for the particulate fillers is due to the removal of one of the parameters in the revised table of parameters and levels by the Pareto 80-20 rule.

- The application of the Pareto 80-20 rule to the S/N response tables produced revised S/N response tables for CSPs, PSPs, PKSPs and ESPs tapped density. The revised S/N response tables for PSPs, PKSPs and ESPs contains the parameter that was removed by the application of the Pareto 80-20 rule to the tables of parameters and levels. This is majorly responsible for the new optimal settings for PSPs, PKSPs and ESPs tapped density. The revised S/N response tables retains the S/N ratios that are significant for optimality, while the S/N ratios that are absent indicates they are not relevant to optimality by the Pareto 80-20 rule.

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ОПТИМИЗАЦИЈА СТРЕСЕНЕ ГУСТИНЕ ЧЕТИРИ ОТПАДНА МАТЕРИЈАЛА ПОЉОПРИВРЕДЕ: ДЕО II – АНАЛИЗА ПЕРФОРМАНСИ МЕТОДАМА ТАГУЧИЈА И ПАРЕТА

Олувасеји Ајоделе Аџибаде [1], Џонсон Олумујива Агунсоје [2] и Сандеј Ајула Оке [2,3]

[1] Департман инжењерства металургије и материјала, Универзитет и Лагосу, Лагос, Нигерија
[2] Департман машинства, Универзитет у Лагосу, Лагос, Нигерија
[3] Департман машинства, Универзитет у Ковенанту, Ота, Нигерија

Овај рад, који је други део студије о оптимизацији стресене густине четири отпадна материјала пољопривреде: честице кокоса, зимзелени, палмине коштице и коре јајета, бави се компаративном анализом перформанси. Новина у раду је то да је оптимизација варијабли процеса извршена применом Тагучијеве методе у спрези са Паретовим правилом 80-20. Негативна побољшања су резултирала када је оптимална стресена густина упоређена са просечном стресеном густином. Међутим, анализа перформанси у којој су вредности оптималне стресене густине и пика стресене густине дала позитиван проценат побољшања за честице сва четири пуниоца. Резултати анализе перформанси валидирају успешност Тагучијеве методе у побољшању карактеристика стресене густине честица пунилаца. Примена Паретовог 80-20 правила на табелу параметара и нивое дала је ревидиране табеле параметара и нивоа на основу којих је било могуће идентификовати фактор-ниво позиције за сваки параметер који је економски оптималан. Паретово правило 80-20 је такође дало као резултат ревидиране табеле за S/N одговоре који су били коришћени за одређивање S/N односа који су релевантни за оптималност.

Кључне речи: оптимизација, анализа перформанси, тест значајности, Паретово правило

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DIRECT CHRONOPOTENTIOMETRIC ANALYSIS OF RIBOFLAVIN USING A GLASSY CARBON VESSEL AS THE WORKING ELECTRODE

Tanja Ž. Brezo[1], Zorica S. Stojanović[1], Zvonimir J. Suturović[1], Snežana Ž. Kravić[1], Jovana J. Kos[2], Spasenija D. Milanović[1] and Ana D. Đurović[1]*

[1] University of Novi Sad, Faculty of Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia
[2] University of Novi Sad, Institute of Food Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia

A new method for the determination of riboflavin (vitamin B_2) was developed based on chronopotentiometry with a glassy carbon process vessel macroelectrode. The method optimisation included investigation of the most important experimental parameters: type and concentration of the supporting electrolyte, initial potential, reduction current, and the working electrode surface area. The reduction signal of riboflavin appeared at about -0.12 V vs. Ag/AgCl (3.5 mol/dm^3 KCl) electrode in 0.025 mol/dm^3 HCl as the supporting electrolyte. A linear response was obtained in the the range of 0.05-4 mg/dm^3. The limit of detection and limit of quantitation were 0.018 mg/dm^3 and 0.054 mg/dm^3, respectively. Due to the use of specific working electrode, a significant enhancement of the method relative sensitivity of about 10 times was achieved. The accuracy of the defined method was confirmed by HPLC analyses. The developed method was successfully applied for the quantitation of riboflavin in various pharmaceutical multivitamin preparations.

KEY WORDS: vitamin B_2, glassy carbon vessel macroelectrode, chronopotentiometry

INTRODUCTION

Riboflavin (7,8-dimethyl-10-ribitylisoalloxazine) exists in three forms: free riboflavin and two cofactor forms, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Also known as vitamin B_2, it is a water-soluble vitamin crucial for metabolism and energy production from carbohydrates, fats and acids (1-3). In addition, several studies demonstrated that riboflavin derivatives may have antioxidant and anticancer activities and can be used in treating different diseases (4). Humans are not able to synthesize and store vitamin B_2 in their bodies. Therefore, it is necessary to provide sufficient amounts of this vitamin through a balanced diet (5). Recommended daily intakes for adults are 1.3 mg and 1.7 mg for women and men, respectively (3). Riboflavin deficiency is usually caused by inadequate dietary intake, disease, drugs, or alcohol abuse. Deficiency leads to skin and mucosal disorders (1).

* Corresponding author: Tanja Brezo, University of Novi Sad, Faculty of Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia, e-mail: tanja.brezo@tf.uns.ac.rs

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The principal objective of this study was to develop an alternative chronopotentiometric method for vitamin B_2 determination using a glassy carbon (GC) vessel as the working electrode. The GC vessel macroelectrode has been previously used for the determination of tocopherols (6) and mercury (7, 8), but its use for determination of riboflavin has not yet been demonstrated. Due to the large surface area of this macroelectrode, which depends on the volume of the analysed solution, enhancement in method sensitivity could be achieved. In order to optimise the method, the influence of the most important analytical parameters was investigated. The proposed method was applied for direct determination of vitamin B_2 in commercially available multivitamin pharmaceutical preparations.

EXPERIMENTAL

Chemicals and reagents

Riboflavin (VB_2), thiamine (VB_1) and pyridoxine (VB_6) were purchased from Sigma-Aldrich (Germany). VB_2 stock solution (0.5 g/dm^3) was prepared daily by dissolving appropriate amounts of solid standard in supporting electrolyte, while working standard solutions were prepared by appropriate dilutions of the stock solution with supporting electrolyte. All other chemicals used were of analytical grade purity. In all experiments, doubly distilled water was used.

Apparatus

All analyses were carried out using the analyser for potentiometric and chronopotentiometric stripping analysis of our own construction (6). The qualitative characteristic (reduction potential) and quantitative characteristic (transition time) of the analyte were determined automatically by the analyser. The output records were provided by an EPSON-570+ printer. A glassy carbon vessel, cylinder (Sigradur G, HTW, Germany) of an inner (active) surface area of 23.7 cm^2 (D_{in} = 1.9 cm, V = 9.92 cm^3) was used as the working electrode. An Ag/AgCl (3.5 mol/dm^3 KCl) electrode was used as the reference electrode. A platinum wire of total surface area of 2.75 cm^2, wrapped around the reference electrode, served as an auxiliary electrode. In order to renew the macroelectrode surface, the GC vessel was polished with a cotton tampon dipped in the aqueous suspension of aluminium oxide (grain size 0.5 μm). After polishing, the vessel was rinsed with distilled and double-distilled water. Prior to each analysis the glassy carbon vessel was electrochemically activated by a constant current of 48.2 μA in 10×99 potential cycles from 0.017 V to -0.2 V, in the solution usually used for electrode testing (0.018 mol/dm^3 H_2SO_4). After this pre-treatment, the analytical signal of VB_2 was higher and better defined. All laboratory accessories used were cleaned by rinsing first with nitric acid (1:1), then with distilled and double-distilled water. All experiments were carried out at ambient temperature (20 ± 2^{o}C).

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Samples and sample preparation procedures

Five samples of commercially available multivitamin pharmaceutical preparations were used, including vitamin B complex tablets, multivitamin tablets with minerals and multivitamin granules. Samples were purchased from local drugstores (Novi Sad, Vojvodina).

The sample preparation procedure has been described earlier (9), and it consisted of powdering, dissolution, sonication and filtration. Further, samples were diluted to the final content of VB_2 between 1 mg/dm^3 and 1.7 mg/dm^3 and directly analysed by chronopotentiometry.

RESULTS AND DISCUSSION

Method optimisation

Optimisation of the proposed electrochemical method for chronopotentiometric VB_2 determination using a GC vessel macroelectrode was performed by the examination of the most important experimental parameters described in the following sections, considering the values and reproducibility of riboflavin analytical signal.

Supporting electrolyte. The procedure for optimisation of the supporting electrolyte was done in our previous work (9), where the glassy carbon disc electrode was used as the working electrode. The 0.025 mol/dm^3 HCl solution was chosen as the optimal supporting electrolyte and used in all further analyses. In the optimal supporting electrolyte, the VB_2 reduction wave appeared at about - 0.12 V (vs. Ag/AgCl, 3.5 mol/dm^3).

Optimisation of the initial potential. The influence of the initial potential on the VB_2 determination using a GC vessel was investigated in the potential range from 0.7 to -0.1 V in model solutions of 1 mg/dm^3 of riboflavin in the supporting electrolyte. The reduction current applied was 48.2 μA, while the final potential was -0.21 V. More negative final potentials caused an extension of the chronopotentiogram. The initial potential of 0.017 V was chosen as optimal (RSD = 1.99%, n = 5).

Optimisation of the reduction current. The influence of the reduction current was investigated in the range from 36.3 μA to 48.2 μA for the VB_2 content of 0.5 mg/dm^3 and from 40.8 μA to 48.2 μA for the VB_2 content of 2 mg/dm^3. The transition time (τ) exponentially decreased with the reduction current (I) increase for the lower content of the vitamin ($\tau=7.72\times e^{-0.037I}$, r=0.9961, n=5).

For the higher content of riboflavin, the dependence was linear ($\tau = -0.15 \times I + 9.51$, r = 0.9956, n = 5). Considering the rectilinear sequence of the dependence $I\cdot\tau^{1/2} = f(I)$, the reduction current interval from 37.8 to 48.2 μA was selected as appropriate. In respect to the required sensitivity, an adequate value of current was chosen from the proposed range: smaller reduction currents were chosen for lower contents of the analyte and *vice versa*. The applied current did not affect the reduction potential of vitamin B_2, which was in the interval from -0.12 V to -0.14 V in all experiments (RSD = 2.09%, n = 5).

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Influence of the working electrode surface area. The influence of the working electrode surface area on the riboflavin transition time was investigated by changing the volume of the analysed solution. The measurements were made in the range from 9.15 cm^2 to 19.63 cm^2 (3-8 cm^3). The content of VB_2 was 0.5 mg/dm^3, and the reduction current applied was 48.2 μA (maximum value of the reduction current of the analyser). Figure 1a) represents the chronopotentiogram for 0.5 mg/dm^3 riboflavin using a glassy carbon disc electrode, whereas Figure 1b) shows the chronopotentiogram for the same content of the vitamin using a glassy carbon vessel electrode as the working electrode, indicating a significant increase of the relative sensitivity of about 10 times. The horizontal lines in Figures 1a) and 1b) show the position of the inflection points corresponding to the reduction time, i.e. analytical signal of VB_2.

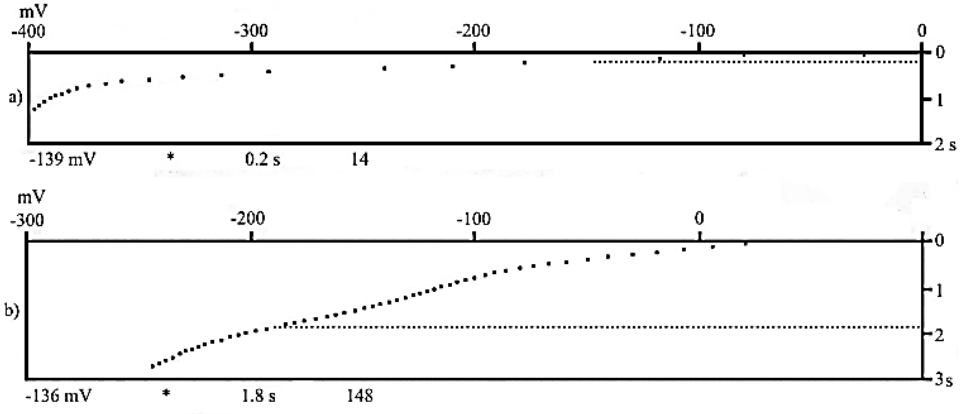

Figure 1. Chronopotentiogram of 0.5 mg/dm^3 riboflavin in 0.025 mol/dm^3 HCl:
a) using a glassy carbon disc electrode (I = 0.8 μA, $E_{Initial}$ = 0.023 V),
b) using a glassy carbon vessel electrode (I = 48.2 μA, $E_{Initial}$ = 0.017 V).
The first numerical value below the chronopotentiograms is the reduction potential, whereas the second and the third ones represent the transition time

For the macroelectrode surface area of 9.15 - 13.36 cm^2 (3-5 cm^3 of the analysed solution) the analytical signal increased with the increase of the working electrode surface area. With further increase of the working electrode area to approximately 15.47 cm^2 (V = 6 cm^3) the analytical signal was higher, but fragmented, possibly due to inappropriate ratio of the working electrode surface area and counter electrode surface area. Additionally, by increasing the working electrode surface area, the applied current density decreased, resulting in extension of the chronopotentiogram and decrease in the method reproducibility. Consequently, for 8 cm^3 of the analysed VB_2 solution (19.63 cm^2 of working electrode surface area) the chronopotentiogram was significantly extended and no analytical signal was observed. The macroelectrode surface area of approximately 13.36 cm^2 (V = 5 cm^3 of VB_2 solution) was chosen as optimal (RSD = 3.12%, n = 5).

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The influence of the macroelectrode surface area on the analytical signal of vitamin B_2 is presented in Table 1. It is important to emphasize that the working electrode surface area, as well as the value of the reduction current have to be adjusted according to the analysed content of VB_2.

Table 1. Influence of the macroelectrode surface area on the analytical signal of vitamin B_2

V(cm^3)	A (cm^2)	Transition time (s)	RSD (%)
3	9.15	0.44	4.99
5	13.36	0.89	3.12
6	15.47	1.03	18.48
8	19.63	No analytical signal observed.	

Method validation

The validation procedure of the optimised method was performed by evaluation of the following parameters: linearity, limit of detection (LOD), limit of quantitation (LOQ), precision, selectivity and accuracy.

Linearity. Dependence of the VB_2 analytical signal on the content was investigated in two ranges: 0.05 - 0.2 mg/dm^3 and 0.2 - 4 mg/dm^3, under the optimal conditions. The applied reduction current was 40 μA for the lower range and 45 μA for the higher range. Both experiments were conducted in five replicates. The reduction time (τ) - content (C_m) dependences were defined using the least-squares method and are presented in Table 2, indicating very good linearity.

Table 2. Linear concentration ranges for chronopotentiometric analysis of VB_2 using GC vessel electrode

Content range (mg/dm^3)	Dependence	S_a	S_b	r
0.05 - 0.20	$\hat{o} = 6.440 \times C_m + 0.418$	0.487	0.035	0.9981
0.20 - 4.00	$\hat{o} = 1.427 \times C_m + 0.034$	0.033	0.004	0.9984

S_a - standard deviation of the slope [s × dm^3/mg], $n = 5$;
S_b - standard deviation of the intercept [s], $n = 5$; r - correlation coefficient

LOD and LOQ. The LOD and LOQ values were calculated according to the ($3.3 \cdot S_b/a$) and ($10 \cdot S_b/a$) criteria, respectively (10), where S_b is the standard deviation of the intercept and a is the slope of the calibration curve defined for LOD concentration range (0.05 - 0.2 mg/dm^3). The calculated values of LOD and LOQ were 0.018 mg/dm^3 and 0.054 mg/dm^3 of VB_2, respectively, and they were in good agreement with the experimental ones. Compared to the LOD value for riboflavin determination using a GC disc electrode (9), the LOD obtained by GC vessel electrode used in this work was about four times lower. It is important to note that the LOD value using a GC vessel electrode could be further decreased by applying higher values of the reduction current, i.e. higher values of

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current density, which could minimise the extension of the chronopotentiogram. Therefore, measurement of the inflection points as well as the reduction time could be possible. Unfortunately, the analyser used in this study was not able to produce reduction currents higher than 50 μA. The limit of detection could be also decreased by automatic subtraction of the base line from the chronopotentiogram belonging to riboflavin.

Precision. The instrumental precision of the VB_2 chronopotentiometric determination using a GC vessel macroelectrode was tested by five times repeated analysis of the model solutions containing 0.2 mg/dm^3 and 2 mg/dm^3 of the vitamin. The RSD values of the chronopotentiometric signal were used for estimation of the instrumental precision.

The method precision (reproducibility and intermediate precision) was evaluated as well. Reproducibility was determined as the intra-day RSD by the analysis of five model solutions containing 0.2 mg/dm^3 and five model solutions containing 2 mg/dm^3 riboflavin. Intermediate precision was defined as the inter-day RSD value. The model solutions of the same concentrations of riboflavin were analysed every day in five consecutive days. The reduction currents applied in these experiments were 40.0 μA for the lower and 45.0 μA for the higher content of VB_2. As the RSD values of all experiments related to instrumental and method precision were less than 5%, it can be concluded that the precision of the proposed chronopotentiometric method was acceptable.

Interferences. Considering pharmaceutical and dietary multivitamin preparations, interference problems may come from other vitamins usually present in these products, as well as from filing materials such as different kinds of carbohydrates. The interference study was undertaken by analysing model solutions of VB_2 with and without addition of VB_1, VB_6, vitamin C (VC), nicotinic acid, sucrose and glucose, and comparing their VB_2 analytical signals. The influence of the interfering compounds was examined for two contents of VB_2, 0.5 mg/dm^3 and 1 mg/dm^3. The contents of the added interfering vitamins were 0.5, 1, 5 and 10 mg/dm^3, whereas the contents of sucrose and glucose were 5, 10, 15 and 20 g/dm^3. Similar to our previous work (9), the reduction time of VB_2 was not significantly affected by a 20-fold excess of the tested vitamins and 40000-fold excess of sucrose and glucose. In all experiments related to the interference study, the VB_2 analytical signal did not change more than 7%, indicating a very good selectivity of the proposed method.

HPLC analyses. HPLC parallel analyses of the same pharmaceutical and dietary multivitamin preparations were done in order to estimate the accuracy of the proposed method. The performed HPLC analysis was described in the previous study (9). The obtained results are shown in the following section.

Determination of vitamin B_2 in pharmaceuticals

The GC vessel macroelectrode was used for the determination of VB_2 in pharmaceutical and dietary supplement multivitamin preparations under optimum experimental conditions, using the standard addition method. The calibration curve method was used as well, but the results were not satisfactory, probably due to the intensive influence of the complex sample matrix. The results obtained using chronopotentiometry and HPLC are given in Table 3. The paired t-test (11) was used for evaluation of the obtained results.

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The contents of VB_2 in the analysed samples determined by the present method and HPLC method were in good agreement, i.e. for the 95% confidence level no statistically significant differences were observed ($|t| = 0.18 < t_{Critical} = 2.78$). The paired t-test also confirmed no statistically significant differences between the obtained results and the labeled specifications ($|t| = 0.35 < t_{Critical} = 2.78$).

Preliminary investigations showed that it is possible to determine VB_2 in various drinks after simple preparation step (decarbonation, filtration, addition of the supporting electrolyte, pH adjustment).

Table 3. Declared and found contents of VB_2 in pharmaceutical preparations calculated by the proposed method and by HPLC

Content of vitamin B_2(mg/tablet)[a]			
Sample	**CHA[b]-vessel electrode**	**HPLC[c]**	**Declared content**
1	3.28 ± 0.04	3.42 ± 0.04	3.40
2	1.48 ± 0.05	1.62 ± 0.05	1.60
3	5.20 ± 0.03	4.98 ± 0.08	5.00
4	3.50 ± 0.04	3.46 ± 0.15	3.40
Content of vitamin B_2 (mg/100 g)			
5	5.05 ± 0.03	5.09 ± 0.22	5.00

[a]mean value ± SD, n = 3; [b]chronopotentiometric analysis; [c]Reference method. Samples: 1 and 2 - B complex tablets, 3 and 4 - multivitamin with minerals tablets, 5 - multivitamnin granules

CONCLUSION

A rapid, convenient, accurate and precise chronopotentiometric method for determination of vitamin B_2 in pharmaceutical preparations was developed by using a glassy carbon vessel macroelectrode as a working electrode. The optimized experimental parameters were as follows: 0.017 V initial potential, -0.21 V final potential, reduction current interval from 37.8 μA to 48.2 μA in 5 cm^3 of 0.025 mol/dm^3 HCl supporting electrolyte solution. Under optimal experimental conditions, linear response of VB_2 was observed in the content range 0.05 - 4 mg/dm^3 with a relatively low detection limit of 0.018 mg/dm^3 provided by the large surface area of the used macroelectrode. LOD could be additionally decreased using an analyser able to impose higher values of reduction current, i.e. higher current densities, which could minimise chronopotentiogram extensions. The significant increase of the relative sensitivity of about 10 times (compared to the glassy carbon disc electrode) enables higher dilution of samples, which may decrease influences of the matrix. Therefore, smaller quantities of samples are needed for the analysis, contributing to the simplicity and fastness of the sample preparation procedure. The optimised method was successfully used to determine VB_2 in pharmaceutical and dietary multivitamin preparations. The obtained results were in very good statistical agreement with those obtained by a HPLC method. The proposed method could be further applied for VB_2 deter-

mination in various samples, including food products, after an appropriate sample preparation procedure.

Acknowledgement

The financial support of the Ministry of Education, Science and Technological Development of the Republic of Serbia (Project III 46009) is gratefully acknowledged.

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ДИРЕКТНО ХРОНОПОТЕНЦИОМЕТРИЈСКО ОДРЕЂИВАЊЕ РИБОФЛАВИНА УЗ ПРИМЕНУ ПРОЦЕСНЕ ПОСУДЕ ОД СТАКЛАСТОГ УГЉЕНИКА КАО РАДНЕ ЕЛЕКТРОДЕ

Тања Ж. Брезо[1], Зорица С. Стојановић[1], Звонимир Ј. Сутуровић[1], Снежана Ж. Кравић[1], Јована Ј. Кос[2], Спасенија Д. Милановић[1] и Ана Д. Ђуровић[1]

[1] Универзитет у Новом Саду, Технолошки факултет, Булевар Цара Лазара 1, 21000 Нови Сад, Србија
[2] Универзитет у Новом Саду, Институт за прехрамбене технологије, Булевар Цара Лазара 1, 21000 Нови Сад, Србија

Развијена је нова хронопотенциометријска метода за одређивање рибофлавина (витамина $Б_2$) уз примену процесне посуде од стакластог угљеника као радне електроде. Оптимизација методе је обухватила испитивање најважнијих експерименталних параметара: врсту и концентрацију помоћног електролита, потенцијал електролизе, струју растварања и површину радне електроде. Редукциони сигнал витамина се јављао на око 0,12V (Ag/AgCl, 3,5 mol/dm^3 KCl) у 0,025 mol/dm^3 HCl као помоћном електролиту. Линеарност аналитичког одзива је установљена у опсегу садржаја 0,05-0,4 mg/dm^3. Постигнута је граница детекције од 0,018 mg/dm^3 и граница квантитативног одређивања од 0,054 mg/dm^3. Захваљујући специфичности радне електроде, остварено је значајно повећање релативне осетљивости методе од око 10 пута. Тачност дефинисане методе потврђена је резултатима паралелне HPLC анализе. Развијена метода је успешно примењена за одређивање витамина $Б_2$ у различитим фармацеутским мултивитаминским препаратима.

Кључне речи: витамин $Б_2$, електрода у облику процесне посуде од стакластог угљеника, хронопотенциометрија

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ORGANIC-INORGANIC MEMBRANES FOR FILTRATION OF CORN DISTILLERY

Valeriy G. Myronchuk[1], Yuliya S. Dzyazko[2], Yurii G. Zmievskii[1], Anatoliy I. Ukrainets[1], Alexander V. Bildukevich[3], Ludmila V. Kornienko[1], Ludmila M. Rozhdestvenskaya[2] and Alexey V. Palchik[2]*

[1] National University of Food Technologies of the Ministry of Education and Science of Ukraine, Vladimirskaya str. 68, 01601, Kiev, Ukraine
[2] V.I. Vernadskii Institute of General & Inorganic Chemistry of the NAS of Ukraine, Palladin Pr. 32/34, 03142, Kiev, Ukraine
[3] Institute of Physico-Organic Chemistry, National Academy of Sciences of Belarus, Surganova Str., 13, Minsk 220072, Belarus

Organic-inorganic membranes were obtained by modification of polymer microfiltration membrane with inorganic ion-exchangers, which form secondary porosity inside macroporous substrate (zirconium hydrophosphate) or simultaneously in the macroporous substrate and active layer, depending of the particle size (from ≈50 nm up to several microns). Precipitation of the inorganic constituent is considered from the point of view of Ostwald-Freundlich equation. Such processes as pressing test in deionized water and filtration of corn distillery at 1-6 bar were investigated. Theoretical model allowing to establish fouling mechanism, was applied. It was found that the particles both in the substrate and active layer prevent fouling of the membrane with organics and provide rejection of colloidal particles.

KEY WORDS: corn distillery, organic-inorganic membranes, active layer, nanoparticles, baromembrane separation.

INTRODUCTION

Membrane technologies are widely used in the food industry (1-7): for concentration of valuable components, desalination, pH correction, decontamination of waste water and so on. Membranes play a key role in the processes and their proper choice is the main factor determining the efficiency of separation and the quality of the final product. Technological liquids and wastes of primary production in the food industry are complex multicomponent solutions containing both organic and inorganic compounds, as well as microorganisms. This variety of the composition causes fast fouling of the membranes. Thus, development of separators with antifouling properties is an important task (4, 5, 8,

* Corresponding author: Yurii G. Zmievskii, National University of Food Technologies of the Ministry of Education and Science of Ukraine, Vladimirskaya str. 68, 01601, Kiev, Ukraine, e-mail: yrazm@meta.ua

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9). A number of approaches are used in order to solve this problem: the membranes are modified by particles of inorganic ion-exchangers (4, 5) or coated by hydrophilic polymers (8, 9). Modification with inorganic particles looks more attractive, since they contain no hydrophobic regions, where adsorption of organics occurs.

Among liquid wastes of the food industry, grain distillery is rather complex object for membrane separation due to a wide range of particles: from ions to rather coarse species (10-18). It was found that a decrease in the pore size of active layer from 800 to 200 nm did not affect the permeate of grain distillery (15). Cake formation on outer surface of the membrane lowers its permittivity; however, the precipitate can be removed easily by hydrodynamic pulsation. Organic substrates can be deposited inside the membrane pores. In this case, the precipitate removal requires chemical reagents, which reduces life-time of the polymer separators.

Earlier we modified the track substrate containing no active layers: the size of pores was uniform (300 nm) throughout the thickness of the polymer separator. A disadvantage of the track membrane is rather low mechanical durability. More durable membranes consist of macroporous substrate coated with a thin active layer. During modification of the membrane with inorganic ion-exchanger, the particles can be deposited either in the substrate or in the active layer or simultaneously in the substrate and layer. As expected, different inorganic ion-exchangers form particles of various sizes, which evidently determine their location. At last, location of the modifier determines functional properties of the membranes.

The aim of the work was to establish the effect of the modifier on separation ability of the composite membranes and their stability against fouling by components of grain distillery.

EXPERIMENTAL

Mifil-0.2 microfiltration membranes (produced by the Institute of Physico-Organic Chemistry of the National Academy of Science of the Republic of Belarus) were used for the investigations. These materials consist of macroporous substrate (non-woven polyester) and thin active layer (aliphatic polyamides).

The sol of insoluble zirconium hydroxocomplexes containing 0.1 M Zr(IV) was prepared as described in (19). The membranes were degassed in deionized water under vacuum at 343 K and impregnated in the sol. Hydrated zirconium dioxide (HZD) or zirconium hydrophosphate (ZHP) were precipitated directly in the polymer with 0.1 solutions of NH_4OH or H_3PO_4 respectively. The membranes were washed with deionized water, dried at room temperature down to constant mass, treated in a Bandelin ultrasonic bath (Bandelin, Germany) at 30 kHz, and dried again. The separators were marked as Mifil-HZD and Mifil-ZHP. The pristine membrane was also used for a comparison. Different samples of the membranes were used for investigation of morphology, determination of cut-off, and filtration of corn distillery.

Morphology of the membranes and zirconium content in the modifier were investigated by scanning electron microscopy (SEM) using the equipment mentioned in (5, 19-21).

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The active layer was also removed from the outer surface of the membrane, crushed after cooling in liquid nitrogen and studied by transmission electron microscopy (TEM). The precipitate, which was removed from the outer surface of the membrane during ultrasonic treatment, was also investigated by TEM.

The experimental set-up for filtration (Fig. 1) involved a two-compartment flow-type cell (effective surface area of the membrane was $2.1 \cdot 10^{-3}$ m^2), liquid line (which provided circulation of liquids through the concentration compartment), thermostatic bath, and measuring instrumentation (manometer, rotameter). A buffer vessel filled with air was used to smooth-out of liquid pulsation.

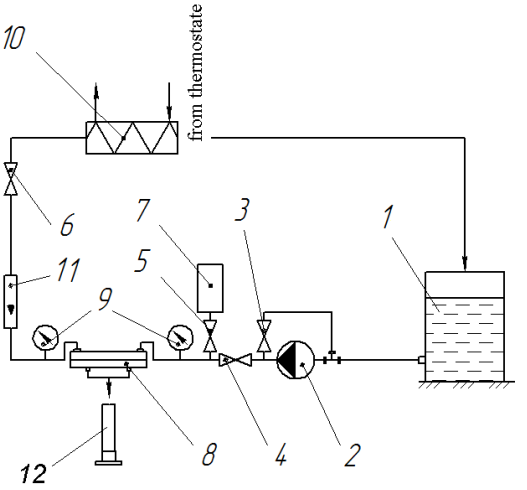

Figure 1. Experimental set-up: 1 - tank filled with a liquid, 2 - pump, 3-6 - valves, 7 - vessel for pulsation smooth-out, 8 - divided cell, 9 - manometers, 10 - heat-exchanger, 11 - rotameter, 12 - measuring cylinder

First of all, the membranes were pressed in deionized water at 298 K. The pressure was kept at the level of 0.6-8 bar; it was increased step by step from lower to higher pressure drop (ΔP) until a constant flow rate through the membrane at each ΔP magnitude (i.e. until steady state conditions). The volume of the permeate (V) was monitored. Each cycle of water filtration was repeated 4 times.

After the pressure test, the cut-off for the membranes was determined by filtration of the solution containing polyethylene glycol (PEG analytical standard, Aldrich Sigma) of 40 or 108 kDa. Filtration was performed at 1 bar, at room temperature. Both the initial solution and permeate were analyzed with modified Dragendorff reagent method (22).

Corn distillery (pH 4.1) was used for the investigations. The liquid contained (g dm^{-3}): total solids − 73, COD − 64. Preliminarily, the liquid was centrifuged on a LU-418 centrifuge (Laboratoriumi centrifuga gepkonyve, Hungary) and further filtered through the blue ribbon filter paper. In fact, the liquid for separation is related to semi-distillate.

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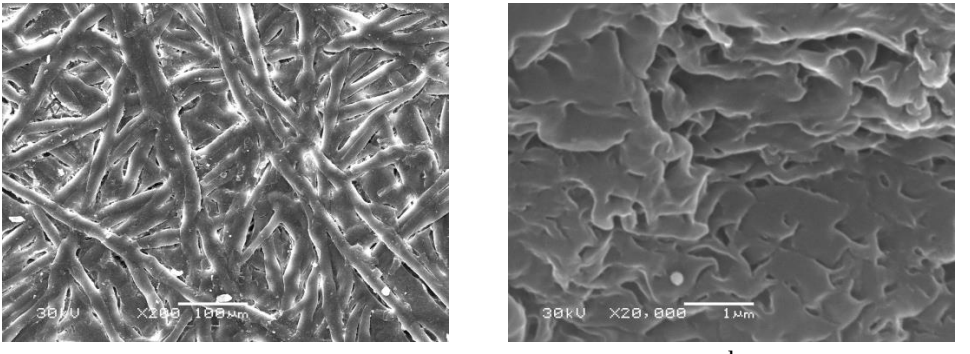
Before filtration of corn distillery, the samples were pressed in deionized water 4 times under different pressure as described above. Then, the corn distillery was inserted into the system instead of water. This liquid was circulated through the cell for 1 h at 333 K and 0.03-0.05 bar, this pressure provided no filtration. Then, the cycle of water filtration was repeated at different ΔP values. At last, corn distillery (1 dm^3) was filtered under different pressures. After the end of the process, water was forced though the membrane again. The content of organics in the grain distillery was determined using a URL-1 Model 1 refractometer (Analitpribor, Ukraine), calibrated using deionized water.

RESULTS AND DISCUSSION

Morphology of the membranes

The macroporous substrate was formed by interwoven fibers, a thickness of which was 20-30 μm (Fig. 2). The size of the pores between the fibers was up to ≈50 μm (Fig. 2a). The active layer was permeated with slit-shaped pores (100-300 nm), as seen in Fig. 2b.



a b

Figure 2. SEM images of fibrous substrate (a) and active layer (b) of the pristine microfiltration membrane

Modification with HZD caused precipitation both on the fibers (Fig. 3a) and in active layer (Figs. 3b, 3c). The size of the particle aggregates was 10 μm. These formations were much smaller than the pores between the fibers, so they cannot affect separation ability of the membrane. Simultaneously, a change of morphology of the active layer is visible, this layer contain aggregates of primary particles. The size of these primary particles was up to about 50 nm.

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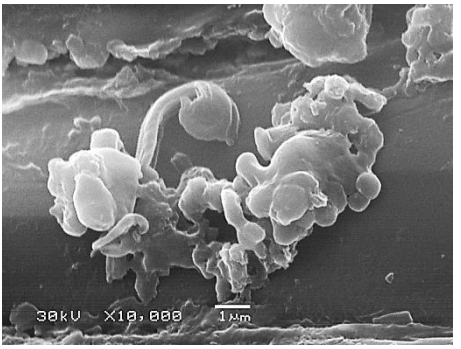

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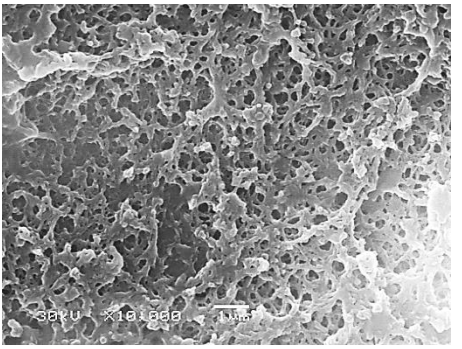

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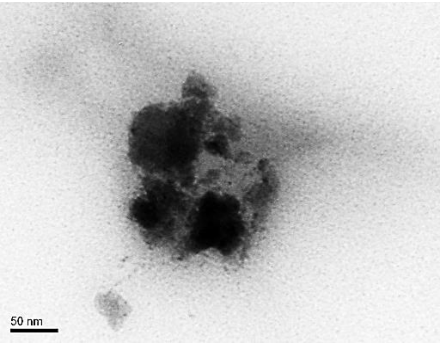
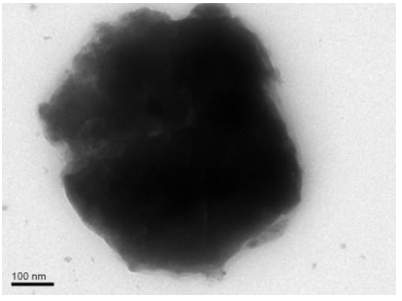


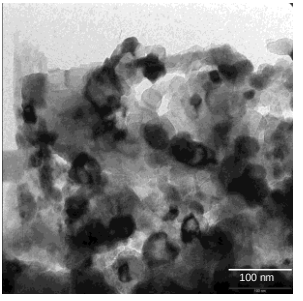
c

Figure 3. SEM (a, b) and TEM (c) images of the substrate (a) and active layer (b, c) of the Mifil-HZD membrane

The ZHP modifier was found to be precipitated mainly on the fibers due to a large size of the particles. Indeed, the TEM analysis of ZHP powder showed massive particles of micron size (Fig. 4a), at the same time, HZD was deposited in the form of aggregated nanoparticles (Fig. 4b).



a



b

Figure 4. TEM images of ZHP (a) and HZD (b) particles

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Different types of ZHP and HZD particles are evidently caused by the mechanism of their deposition from the sol. The sol contains nanoparticles of insoluble zirconium hydroxocomplexes (19), coordination arrangement of Zr involves mainly OH ligands (23). The particles are stabilized by counter-ions. The addition of an NH_4OH solution caused aggregation of the particles. On the other hand, the addition of H_3PO_4 to the sol caused evidently dissolution of the sol particles, followed by hydrophosphate deposition.

Deposition of inorganic particles

From the thermodynamical point of view, the smallest particles are dissolved during precipitation in accordance with the Ostwald-Freundlich equation (24):

$$\ln \frac{C}{C_\infty} = \frac{\beta \nu_m \sigma \cos\varphi}{RTr}. \qquad [1]$$

where C is the compound concentration, C_∞ is the concentration of saturated solution (the C and C_∞ values are extremely low), β is the shape factor, ν_m is the molar volume of the compound, σ is the surface tension of the solvent, φ is the wetting angle, R is the gas constant, T is the temperature, r is the particle radius. In other words, the particles with a radius r and less are dissolved and reprecipitated as larger particles. The compound of larger molar volume form larger particles.

Regarding amorphous ZHP and HZD, their exact chemical composition is indefinite. It is possible to make approximate estimations using data for crystalline materials. Molar mass and particle density of crystalline ZrO_2 are 123.2 g mol^{-1} and 5.68 g cm^{-3} respectively, thus ν_m = 21.7 cm^3 mol^{-1}. The ZHP deposited from the sol contains mainly hydrophosphate groups, similarly to crystalline α-ZHP (21). Since the particle density of this compound is 3.3 g cm^{-3} (25), its molar volume is 91 cm^3 mol^{-1}. Thus, larger particles are formed during ZHP deposition in comparison with HZD. In contrast to HZD, the ZHP particles, due to their size, can be located only in the macroporous substrate.

Pressure test

The flux of permeate (J) was determined as:

$$\frac{1}{A}\frac{dV}{d\tau}$$

where A is the membrane area, V is the cumulative volume of permeate, τ is the time. For example, the flux of deionized water through the Mifil-HZD membrane under steady state conditions is plotted in Fig. 5. The flux shows an increase with the increasing in pressure drop (ΔP), according to Darcy equation (26):

$$J = \frac{\Delta P}{\eta R_m} \qquad [2]$$

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where η is the dynamic viscosity of a liquid, R_m is the hydrodynamic resistance of the membrane. It is seen, that $J \neq 0$ at $\Delta P = 0$ due to deviation from the Darcy law at low pressure. The flux grows from the first to third cycle of pressure test, further no sufficient change of the J value is observed. Since no sufficient losses of Zr was found for the membrane after the pressure test, the increase of the flux from the first to third cycle can be caused by compaction of the particles inside the polymer active layer. Probably, this is possible to say about a decrease of thickness of the "incorporated inorganic active layer".

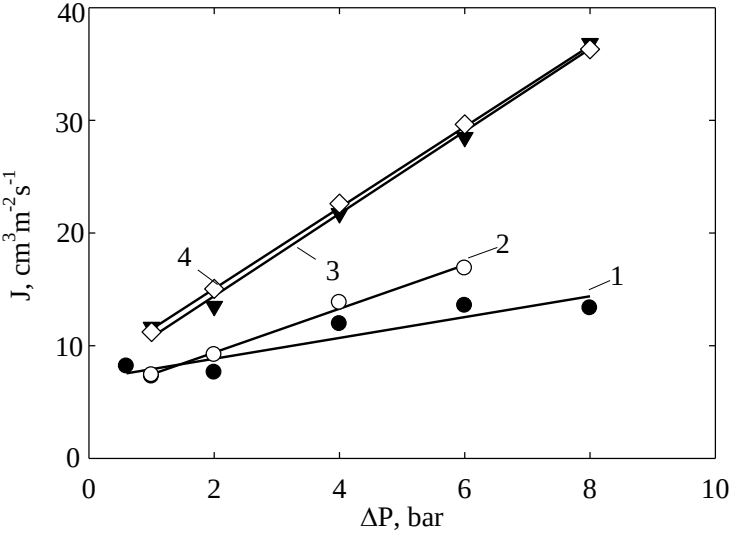

Figure 5. Flux of deionized water as a function of pressure drop through the Mifil−HZD membrane. The curve numbers correspond to the cycle of pressure test

The permeate flux increases with pressure also for other membranes. As seen from Table 1, the flux decreases in the order: Mifil-0.2 >Mifil-ZHP> Mifil-HZD. A lower rate of water flow through the Mifil-ZHP is due to ion-exchanger particles in the substrate, which provide higher hydrodynamic resistance. The lowest filtration rate for Mifil-HZD is due to the particles inside the active layer.

Table 1. Pressure test of the membranes

Membrane	**PEG rejection, %**		**Water flux, $cm^3m^{-2}s^{-1}$ (at 1 bar, 4th cycle of pressure test)**
	40 kDa	108 kDa	
Mifil-0.2	–	–	476
Mifil-ZHP	–	3	125
Mifil-HZD	10	94	12

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The cut-off value for the Mifil-HZD was 108 kDa, since rejection is higher than 90 %. No sufficient PEG rejection was found for other membranes.

Filtration of corn distillery

During filtration of corn distillery, the Mifil-0.2 and Mifil-ZHP membranes showed higher filtration rate than the Mifil−HZD separator (Fig. 6). However, the flux decreased after a certain induction period (Mifil-0.2 and Mifil−ZHP). In contrast to this, the Mifil−HZD membrane showed constant permeate rate over time, indicating stability of the membrane against fouling. No rejection of organics was found for the Mifil-ZHP membrane (this is the same for Mifil-0.2). The rejection reached 69 % for Mifil−HZD.

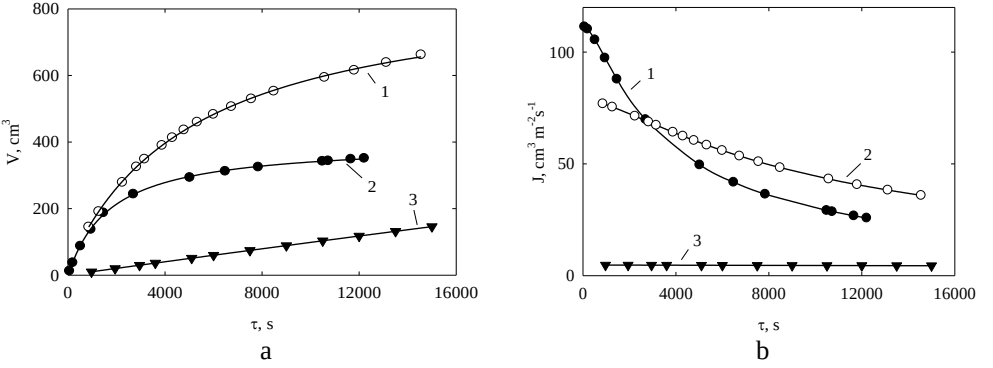

Figure 6. Filtration of corn distillery: cumulative volume (a) and flux of permeate (b) as function of time. Membranes: Mifil-0.2 (1), Mifil-ZHP (2), Mifil−HZD (3)

Adsorption of corn distillery without its filtration causes a decrease of water flux through this membrane from 12 down to 4.7 $cm^3m^{-2}s^{-1}$ (Fig. 7). Regarding the corn distillery, the permeate flux was even lower. Nevertheless, the flux of water after corn distillery filtration was practically the same as that before filtration of the biological liquid. The J - ΔP dependence is linear, however, their slopes to the abscissa axis is lower than those for water (see Fig. 5).

In order to explain the contradiction between the data of Fig. 6 (constant filtration rate) and Fig. 7 (the lowest flux of the permeate of corn distillery, the influence of this liquid on water filtration), a number of known theoretical models were applied to the process of corn distillery filtration as described further.

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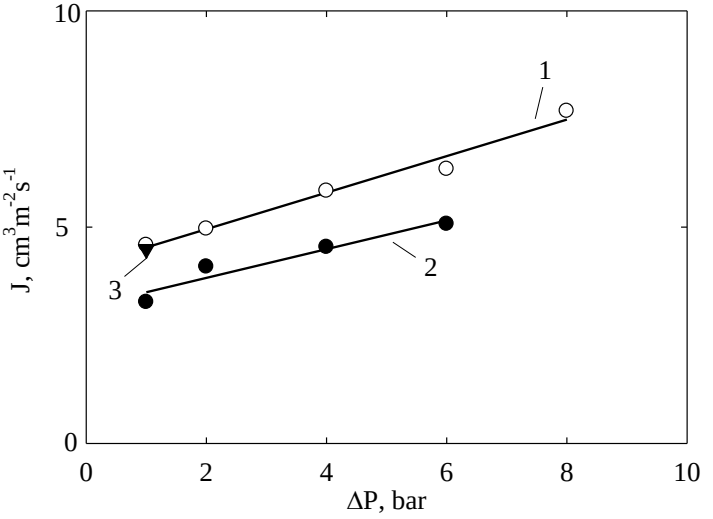

Figure 7. Permeate flux through the Mifil−HZD membrane as a function of pressure drop. The lines correspond to filtration of water after adsorption of corn distillery (1), filtration of corn distillery (2) followed by water filtration (3)

Fouling of the membranes during corn distillery filtration. Theoretical modeling

According to classical theoretical approaches, the fouling mechanism is pore blockage, if the permeate flux is proportional to time (27). The model of pore constriction (deposition on pore walls) considers the linearity of $\frac{\tau}{V} - \tau$. Finally, the linearity of the $\frac{\tau}{V} - V$ line corresponds to cake model (this approach is not suitable for water filtration).

As shown in Fig. 8, the application of pore blockage model shows linear regions of the $J - \tau$ dependence at the final stage of filtration (Mifil-0.2 and Mifil−ZHP). In the case of the Mifil−HZD membrane, the $J - \tau$ line is parallel to the abscissa axis, indicating no pore blockage. Similar feature is observed for the $\frac{\tau}{V} - \tau$ line, which corresponds to this membrane. Thus the model of pore constriction cannot be applied in this case. At the same time, the $\frac{\tau}{V} - \tau$ dependence is linear for other membranes. Finally, the linear regions are typical for the $\frac{\tau}{V} - V$ lines at the final stage of the process (all membranes).

Thus, in opposite to the pristine membrane and Mifil−ZHP, the membrane modified with HZD particles shows stability against accumulation of organics directly in the pores.

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Fouling is only due to cake formation on its outer surface. In fact, insertion of hydrophilic HZD into the active layer of polymer microfiltration membrane provides its stability against fouling with organics during filtration of corn distillery. The particles in the polymer matrix perform a function of additional active layer; they play the same role for composite inorganic membranes (19, 28).

The other membranes are poisoned with organic substrates, which are precipitated on the walls of pores, thus blocking them. The Mifil−ZHP membrane is more stable against fouling than Mifil-0.2, evidently due to ZHP particles, which protect the substrate. However, deposition of organics is possible inside the active layer of the membrane.

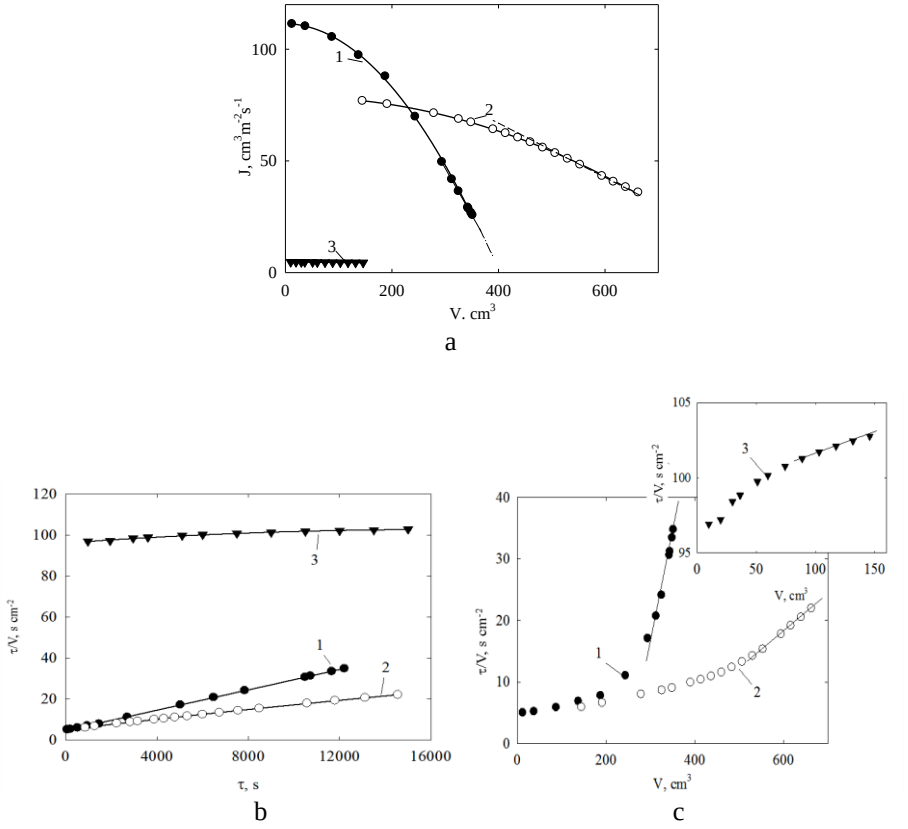

Figure 8. Filtration of corn distillery through: Mifil-0.2 (1), Mifil−ZHP (2) and Mifil−HZD (3). Application of the model: of pore blockage (a), pore constriction (b), and cake formation (c)

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CONCLUSION

The membrane containing HZD particles shows the best results: in this case no pore constriction and pore blockage occurs during filtration of corn distillery. Fouling takes place only by cake formation on outer surface of the membrane, which can be easily removed by hydrodynamical pulsations. This membrane demonstrates selectivity towards organics. These remarkable properties of the Mifil−HZD membrane can be due to the deposition of the ion-exchanger particles inside the polymer active layer. The particle location is possible due to their small size. The pores between primary particles, which form aggregates, provide rejection of the organics, such as PEG or components of corn distillery. In opposite to HZD, deposition of larger ZHP particles is possible from the point of view of thermodynamics. These large particles occupy mainly the fibrous substrate. As a result, the active layer is free from ZHP particles and cannot provide rejection of organic species.

The incorporated inorganic particles decrease the flux of the permeate. Thus, the next stage of the membrane development is a decrease of the thickness of secondary active layer, which is formed inside the pores. Improvement of selectivity requires a decrease of particle size, i.e. narrowing of the pores between them. The use of Ostwald-Freundlich equation is recommended for the control of particle size. The Mifil−HZD can be evidently applied to solution of various tasks in the food industry, for instance, to produce protein concentrate from milky whey, to clarify fruit and vegetable juices, etc.

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ОРГАНСКО-НЕОРГАНСКЕ МЕМБРАНЕ ЗА ФИЛТРАЦИЈУ У ДЕСТИЛАЦИЈИ КОМИНЕ КУКУРУЗА

Валерий Г. Мирончук[1], Јулија С. Ђазко[2], Јурий Г. Змијевскиј[1], Анатолиј И. Украињец[1], Александар В. Билдукевич[3], Људмила В. Корњенко[1], Људмила М. Рождественскаја[2] и Алексеј В. Палчик[2]

[1] Национални универзитет за технологију хране Министарства образовања и науке Украјине, Владимирска 68, 01601 Кијев, Украјина;
[2] Институт опште и неорганске хемије "В.И. Вернадски" Националне академије наука Украјине, Паладин пр. 32/34, 03142, Кијев, Украјина
[3] Институт физичко-органске хемије, Национална академија наука Белорусије, Сурганова ул., 13, Минск 220072, Белорусија

Органско-неорганске мембране су добијене модификовањем полимерних микрофилтрационих мембрана помоћу неорганских јонских измењивача који формирају секундарну порозност унутар макропорозног супстрата (цирконијум-хидрофосфата), или истовремено у макропорозном супстрату и активном слоју, у зависности од величине честица (од око 50 нанометара до неколико микрометара). Преципитација неорганског састојка се разматра са становишта Оствалд-Фројндлихове једначине. Испитивани су процеси као што је тест пресовања у дејонизованој води и филтрација кукурузног дестилата при 1-6 бара. Примењен је теоријски модел који омогућава установљење механизма онечишћења. Нађено је да честице, како у супстрату тако и у активном слоју, спречавају онечишћње мембране органским супстанцама и доводе до одбијања колоидних честица.

Кључне речи: алкохолни дестилат кукуруза, органско-неорганске мембране, активни слој, наночестице, баромембранска сепарација

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SEPARATION EFFICIENCY OF TWO WASTE POLYMER FIBERS FOR OILY WATER TREATMENT

Dunja S. Sokolović[1], Tatjana J. Vulić[2], Arpad I. Kiralj[2], Milica S. Hadnađev-Kostić[2] and Srđan S. Sokolović[3]*

[1] University of Novi Sad, Faculty of Technical Sciences, Trg Dositeja Obradovića 6, Novi Sad, Serbia
[2] University of Novi Sad, Faculty of Technology, Bulevar Cara Lazara 1, Novi Sad, Serbia
[3] NIS a.d. Novi Sad, Put Šajkaškog odreda 4, Novi Sad, Serbia

This work is concerned with the efficiency of two different waste polymeric materials as the filter media in a laboratory-scale bed coalescer in the horizontal fluid flow mode, operating in a steady-state regime. The applied materials are: waste polyethylene terephthalate from textile industry, BA1 and waste polypropylene from carpet industry, PP. Using these compressible fiber polymeric materials, high bed porosity (up to 98%) could be obtained. The investigation was carried out over a wide range of working conditions. Bed permeability was varied in the range from $0.18 \cdot 10^{-9}$ to $5.389 \cdot 10^{-9}$ m^2. Operating fluid velocity was varied from 19 to 80 m/h, until the critical velocity was reached. Different oily wastewaters were used in the experiments. Oily wastewater is defined as the oil-in-water emulsion model prepared using mineral oils of different physico-chemical characteristics: crude oil (A) from Vojvodina region, two vacuum distillation fractions (A1, A4), and blended petroleum product with a high paraffinic content (P1). Both applied polymeric materials, BA1 and PP, showed high separation efficiency for treatment of all investigated oily wastewater. However, the BA1 material showed higher efficiency in a wider range of bed permeability and physico-chemical characteristics of oil.

KEY WORDS: water reuse, oily wastewater, bed coalescence, waste polymer materials, sustainable development

INTRODUCTION

Various human activities produce oily wastewater. In addition to the industry (petrochemical, chemical, food, pharmaceutical, power plants, etc.) that certainly generates the highest amount of oily wastewater, they also arise from households, car wash, transportation, etc. Oily wastewater may contain lubricants, cutting liquids, heavy hydrocarbons such as tars, grease, crude oils, diesel oil, and light hydrocarbons such as kerosene, jet fuel and gasoline, as well as fats, vegetable oils or fatty acids (1-3).

* Corresponding author: Dunja S. Sokolović, University of Novi Sad, Faculty of Technical Sciences, Trg Dositeja Obradovića 6, 21000 Novi Sad, Serbia, e-mail: dunjaso@uns.ac.rs

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Water recycling is considered to be a sustainable approach and can be cost-effective if the adequate technology (method) for water treatment is chosen (4). Very often, the required standard can not be reached by the chosen method, if not taking into account diverse characteristics of oily wastewater, both chemical and physical, which are of crucial importance for the efficiency of the selected technology and unit design. For example, sedimentation is only effective when the dispersed oil droplets are larger than 100 μm. On the other hand, although the required standard could be reached by some of the methods, either the energy consumption is high or the methods are extremely expensive, and therefore not acceptable in terms of sustainable development (2, 5-8).

Treatment of oily wastewater by bed coalescence has been proven to be a highly effective method for separation of the oil phase from wastewater, particularly concerning extremely small droplets, even less than 5 μm. The benefits of coalescence filtration compared to other separation methods (such as sedimentation, centrifugation, adsorption), besides the low operating costs and high separation efficiency, are low energy consumption, low selectivity to chemical composition of the wastewater, as well as easy maintenance. Bed coalescer is mobile, small and it can be easily applied not just in industry, but also in households, car wash shops, petrol stations, ships, and everywhere where oily water is generated. Coalescence filtration is an environmental friendly method, not only because of the low energy demand, but based on the facts that no chemical agents are used, and that it can work continuously without washing and changing the filter media at all. Using waste polymeric fibers as filter media for bed coalescer make this separation method even more eco-friendly. By using waste polymeric materials savings are achieved in the consumption of crude oil as raw material for their production, simultaneously reducing the potential contamination of the environment, basically meeting the requirements of sustainable development.

This paper is concerned with a comparative investigation of the efficiency of coalescence filtration for treatment of different oily wastewaters, using two different waste polymeric materials, polyethylene terephthalate, BA1, polypropylene, PP, as the filter media. The aim was to elucidate the simultaneous effect of the polymeric fibres' nature, fiber bed permeability, and wide range of dispersed oil properties on the coalescence efficiency over different working velocities, in order to find out which polymeric material is most effective as filter media.

EXPERIMENTAL

The experiments were performed on a laboratory-scale bed coalescer in the horizontal fluid flow mode, operating in a steady-state regime. The experimental apparatus shown in Fig. 1 has been explained in detail in our previous publications (3, 9-14).

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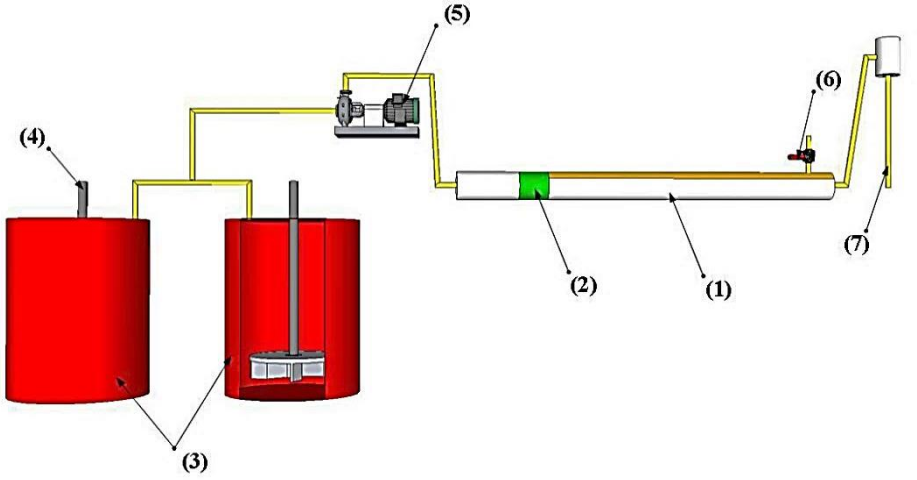

Figure 1. Schematic diagram of the experimental bed coalescer: (1) coalescer body; (2) filter media; (3) tanks; (4) stainless steel impellers; (5) pump; (6) valve for oil discharge

Four different model emulsions were prepared by using mineral oils of different physico-chemical characteristics: crude oil from Vojvodina region (A), two vacuum distillation fractions (A1, A4), and blended petroleum product with a high paraffinic content (P1). Properties of the investigated oils are presented in Table 1.

Table 1. Physico-chemical properties of dispersed oils

Property/Sample	A	A4	A1	P1
Density 20^{o}C, kg/m^{3}	915	918	905	879
Viscosity at 40^{o}C, mPa s	43	169	9	10
Neutralization number, mg KOH/l	1.42	1.71	1.13	0.13
Pour point, ^{o}C	-42	-3	-56	+3
Interfacial tension, mN/m	18.8	30.5	33.8	32.4
Surface tension, mN/m	26.56	27.72	28.91	30.16
Emulsivity % vol	100	70	56	54
Dielectric constant	0.16	0.19	0.13	0.06
Mean molecular mass, g/mol	410	520	150	300

Model emulsion was prepared prior to the experiment at the temperature of 20^{o}C, in two tanks of 80 liters volume (Fig. 1-3). One of four oils (A, A1, A4, P1) was dispersed in tap water by continuous stirring with stainless steel impeller (Fig. 1-4) at a rate of 650

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rpm for a period of 45 min. In order to maintain the average droplet diameter of 10 μm the stirring was maintained continuous throughout the whole duration of the experiment.

Coalescence of oil droplets was carried out by applying two different compressible fiber polymeric materials: polyethylene terephthalate, BA1, and polypropylene, PP, as filter media, whose properties are given in Table 2.

Table 2. Properties of the filter media

Material	Density kg/m^3	Melting point ^{0}C	Dielectric constant	Critical surface tension mN/m
Polyethylene terephthalate, BA1	1400	254.3	2.3	31
Polypropylene, PP	900	168.6	1.5	30.5

As previously mentioned, both applied polymer materials are waste materials. The BA1 fibers are thermo-materials used for stuffing jackets, while PP fibers are waste from the carpet production industry.

Using compressible polymeric fibers as filter media, a broad range of bed properties, especially porosity and permeability could easily be varied. Bed permeability was varied in the range from $0.18 \cdot 10^{-9}$ to $5.389 \cdot 10^{-9}$ m^2, as result of variation of the bed porosity ranging from 0.97 to 0.85. The bed length of 5 cm was kept constant in all experiments.

The operating fluid velocity was kept constant for 1 h and varied from 19 to 80 m/h, up to the critical velocity. Critical velocity is defined in as the velocity when the effluent oil concentration reaches 15 mg/l (9-14).

The experiments were carried out in the steady-state regime, achieved by pre-oiling of the fibers.

They were performed over constant inlet oil concentration of 500 mg/l at 20^{o}C. Composite samples, consisting of three samples taken at the outlet of the settling section after 45 min at 5-min intervals, were used for monitoring the effluent oil concentration.

The oil concentration was determined by FTIR spectrometry from a carbon tetrachloride extract.

The coalescence efficiency was calculated on the basis of the oil content in the influent (C_i) and effluent (C_e), using the expression [1]:

$$E = \frac{C_i - C_e}{C_i} \cdot 100\% \qquad [1]$$

RESULTS AND DISCUSSION

The obtained results are presented in contour diagrams (Figs. 2 and 3), which reveal the interdependence of bed permeability and nature of dispersed oil in wastewater on coalescence efficiency for both investigated materials, BA1 and PP, at different working velocities. The oil nature is defined by the viscosity.

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Figure 2 shows the interdependence of the investigated variables for both BA1 and PP material over the same fluid velocity of 30 m/h. By analysing these diagrams one can see that the separation efficiency is very high, over 95 %, for both investigated materials. However, at this working velocity, the BA1 exhibited higher coalescence efficiency over the low values of bed permeability ($0.180 \cdot 10^{-9}$ - $0.380 \cdot 10^{-9}$ m^2) for oil viscosity lower than 30 mPa s, compared to the PP material. At high bed permeability, independently of oil viscosity, both materials show very high coalescence efficiency, over 98 %.

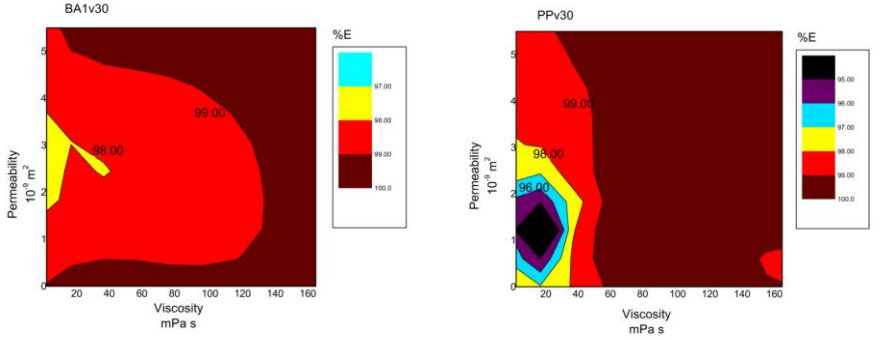

Figure 2. Coalescence efficiency at 30 m/h for: a) BA1 filter media, b) PP filter media

In order to determine the critical velocity for both investigated materials, further experiments with higher working velocities, up to 80 m/h, were performed. Critical velocity is defined as the velocity when the effluent oil concentration reaches 15 mg/l (9-14). The critical velocity is of crucial importance for the coalescer design because it defines the limiting reasonable value to be applied for defined conditions (12). Higher critical velocity contributes to smaller unit size of coalescer. The critical velocity for BA1 is 50 m/h, and for PP is 40 m/h.

Figure 3, illustrates the coalescence efficiency of BA1 and PP fiber materials at the critical velocities, for all investigated oils.

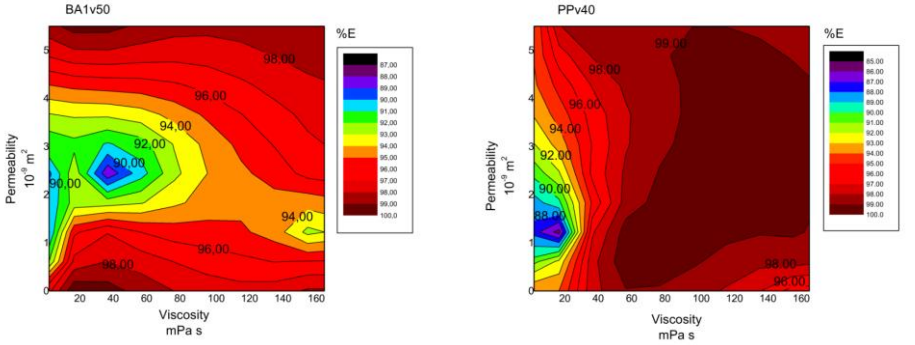

Figure 3. Coalescence efficiency at critical velocity for: a) BA1 filter media, b) PP filter media

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When BA1 fibers are applied as filter media in the bed coalescer, higher critical velocity could be obtained (50 m/h). This implies that the coalescer with the BA1 filter medium has a higher capacity compared to that of the PP filter medium of the same size. Nevertheless, the BA1 filter medium has lower coalescence efficiency, ranging from 87 to 96 %, over a wide range of permeability for all investigated oils, Fig. 3a). Only for very high or very low bed permeability, the efficiency is over 95 %. On the other hand, the PP filter media achieved coalescence efficiency over 95 % for almost all the values of permeability and all investigated oils, but at lower working velocity, 40 m/h, Fig. 3b). For the oils with the viscosity lower than 30 mPa s the PP filter media does not show high coalescence efficiency (85-95 %).

CONCLUSION

Both investigated filter bed materials, BA1 and PP, show very high coalescence efficiency (BA1 87-100%, PP 85-100%) for all investigated bed permeabilities, oil properties and working velocities. However, which material is more efficient in coalescence filtration it depends on the chemical composition of the dispersed oil in wastewater, the quantity of wastewater to be processed, as well as on the available space for coalescer installation. When chemical composition of wastewater does not vary over time, then BA1 is a better choice for the filter media, because smaller size unit with higher capacity could be installed due to higher critical velocity. In case when the chemical composition of wastewater varies over time and only if the dispersed oil has viscosity is higher than 30 mPa s, then PP fibers are a better choice as filter medum.

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ЕФИКАСНОСТ СЕПАРАЦИЈЕ ПРИМЕНОМ ДВА ОТПАДНА ВЛАКНАСТА ПОЛИМЕРА ЗА ТРЕТМАН ЗАУЉЕНИХ ВОДА

Дуња С. Соколовић[1], Татјана Ј. Вулић[2], Арпад И. Кираљ[2], Милица С. Хаднађев-Костић[2] и Срђан С. Соколовић[3]

[1] Универзитет у Новом Саду, Факултет техничких наука, Трг Доситеја Обрадовића 6, Нови Сад, Србија
[2] Универзитет у Новом Саду, Технолошки факултет, Булевар Цара Лазара 1, Нови Сад, Србија
[3] НИС а.д. Нови Сад, Пут шајкашког одреда 4, Нови Сад, Србија

Третман зауљене отпадне воде коалесцентном филтрацијом је изразито ефикасна метода, нарочито када су дисперговане капи уља у води ситне. Предности коалесцентне филтрације су: ниска потрошња енергије, ниски радни трошкови, висока ефикасност сепарације као и висока флексибилност у односу на хемијски састав отпадне воде. У овом раду испитивана је ефикасност два отпадна полимерна материјала као филтарског медијума у колесцеру. Коришћени материјали су: отпадни полиетилентерафталат из текстилне индустрије, PA1, и отпадни полипропилен који се користи у производњи тепиха, PP. Експерименти су реализовани на лаборато-

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ријском коалесцеру при хоризонталној оријентацији флуида у стационарном режиму. Истраживања су извођена варирањем широког опсега радних параметара. Применом компресибилних влакнастих полимерних материјала постиже се широки опсег пермеабилност слоја од $0.18 \cdot 10^{-9}$ do $5.389 \cdot 10^{-9}$ m^2 са максималном порозношћу слоја до 98 %. Радна брзина флуида варирана је у опсегу од 19 до 80 h/m, односно све до постизања критичне брзине флуида. Зауљена вода је модел емулзија уље у води припремљена коришћењем минералних уља различитих физичко-хемијских карактеристика: војвођанска сирова нафта (A), две вакуум дестилационе фракције исте нафте (A1, A4), и намешани нафтни производ са високим садржајем парафина без адитива (P1). Оба коришћена полимерна материјала, BA1 и PP, имају високу ефикасност сепарације за све испитиване отпадне воде. Међутим, материјал BA1 има већу ефикасност за шири опсег пермеабилности слоја за све испитиване отпадне воде.

Кључне речи: рециклирање воде, зауљене отпадне воде, коалесцер, отпадни полимери, одрживи развој

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INFLUENCE OF STEEPING TIME ON BIOLOGICAL ACTIVITY OF BLACK MULBERRY LEAVES TEA

Slađana R. Čestić[1], Marija M. Radojković[1], Aleksandra D. Cvetanović[1], Pavle Z. Mašković[2] and Saša D. Đurović[1]*

[1] University of Novi Sad, Faculty of Technology, Bulevar Cara Lazara 1, Novi Sad, Serbia
[2] University of Kragujevac, Faculty of Agronomy Čačak, Cara Dušana 34, Čačak, Serbia

Black mulberry leaves teas (BMLTs) were prepared using boiled water and different steeping time (5 10, 20, 35 and 45 minutes). In order to establish the connection between steeping time and tea quality, total phenolics content (TPC), total flavonoids content TFC contents were measured, as well as two antioxidant assays (DPPH and reducing power assays), alone with antimicrobial and cytotoxic tests. The obtained results showed that TPC, TFC, IC_{50} and EC_{50} values increased with the increase in steeping time, while antimicrobial and cytotoxic activity exhibited different tendency. Based on the obtained results, the 10-minute steeping time was the optimum for tea preparation and this tea was used for the determination of polyphenolic profile using HPLC-MS technique. The results showed that the main compounds in BMLT were chlorogenic and caffeic acids with the contents of 7226.00 and 537.52 μg/g, respectively.

KEY WORDS: black mulberry leaves, tea preparation, biological activity, steeping time

INTRODUCTION

Different medicinal plants have been used as a source of health benefit compounds for a long time. Awareness of the presence of such compounds in plants lead to increased popularity of teas all over the world (1). Herbal teas are very popular merchandise in the stores due to their fragrance, antioxidant properties and therapeutic applications, and can be found as pure or blended samples (2, 3). As a consequence of such popularity, hundreds of different herbal teas are sold in stores. Teas have been used for treatment of infections, ailments and diseases for centuries in many countries (4). These beverages may be prepared from almost every part of plant such as dry and fresh fruits, leaves, flowers, and sometimes even seeds and stems, and the process of preparation of teas is quite simple. They are prepared by pouring boiling water over the herbal material and allowing the steeping for a certain period of time, usually 5-15 minutes (5). The preparation technique relies on tradition and medical purpose. Teas are often prepared in different ways in different parts of the world. For example, teas may be infused several times, prepared

* Corresponding author: Slađana Čestić, University of Novi Sad, Faculty of Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia, e-mail: sladjanacestic@yahoo.com

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using water at different temperature or with addition of milk, lemon, honey or sugar (6). Studies have shown that medicinal plants exhibit antioxidant (7-9) and antimicrobial (10) activities. Due to such activity of the plant itself, preparation and usage of appropriate herbal tea can be very useful in the management of various diseases, such as Type 2 diabetes (11), various cardiovascular conditions (12), obesity (13), and Alzheimer's disease (14). Presence of polyphenolic compounds in the herbal beverages and teas is very significant due to biological activity of this class of compounds. They are known as potent antioxidants (3, 15) and are able to reduce the deleterious effects of reactive oxygen species (ROS) (16). Besides antioxidant activity, polyphenolics exhibit wide range of biological activities, such as antibacterial, anti-carcinogenic, anti-inflammatory, anti-viral, anti-allergic, estrogenic and immune-stimulating effects (17). Polyphenolics exert the antioxidant activity as a result of their redox properties. They are able to act as reducing agents, hydrogen atom donor and singlet oxygen quenchers, exhibiting also chelating potential toward metal ions (15).

Black mulberry (*Morus nigra*) belongs to the family *Moraceae*. It has been used for medicinal purposes for several centuries. It is cultivated commercially in China, India, Japan, and South Europe. A special characteristic of mulberries is that almost all the parts of the plant are pharmacologically used (18). Mulberry tea and its constituents exhibit a broad array of biological activities such as cytotoxic effects on cancer cell lines (19-21), reduce risk of atherosclerosis (22-24), anti-inflammatory effect (25, 26), hypolipidemic effect (27), neuroprotective effect (28), hypoglycemic effects (29,30) and antioxidant effect (31-34). To our best knowledge, black mulberry is not significantly presented in the Serbian market, with the exception of its fruit, which is used as food. Tea made from the leaves of this plant is still not popular in Serbia regardless of the presence and availability of the plant material itself. For such reasons, the study was conducted in terms of determining the leaves chemical composition and biological activity, in order to examine the possibility of using black mulberry leaves in the tea industry.

As polyphenolic compounds showed to be beneficial for human health, the process of tea preparation should be governed in such a way to ensure the highest possible content of these compounds in teas. Previous studies demonstrated that there was a correlation between tea preparation conditions and the content of extracted compounds from plant materials such as polyphenolic compounds (6, 35). In order to establish the connection between the time of steeping and content of polyphenols and flavonoids and biological activity of black mulberry beverages, total phenolic content (TPC) and total and total flavonoids content (TFC) were determined, as well as the antioxidant, antimicrobial and cytotoxic activities. HPLC analysis was also performed to evaluate polyphenolic profile of the obtained teas.

EXPERIMENTAL

Chemical reagents

Folin-Ciocalteu reagent, trichloroacetic acid, 1,1-diphenyl-2-picrylhydrazyl hydrate (DPPH), chlorogenic acid, gallic acid, dihydrobenzoic acid, sinapic acid, catechin, epica-

techin, vanillic acid, caffeic acid, quercetin, ferulic acid, resveratrol, ellagic acid, *p*-coumaric acid, caftaric acid, and rutin were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Aluminum chloride hexahydrate, sodium carbonate and sodium acetate trihydrate were purchased from Merck (Darmstadt, Germany). Potassium ferricyanide and ferric chloride were obtained from Zorka (Šabac, Serbia). Cirsimarin, resazurin, amaricin, nystatin, sabourand dextrose, Tween 80 and *cis*-diamminedichloroplatinum (*cis*-DDP) were purchased from Tedia Company (USA). Acetonitrile and water were products of Fisher Chemical (LC-MS and HPLC grade). Formic acid was purchased from Carlo Erba (Italy). All other chemicals and reagents were of analytical reagent grade.

Plant material

Dried plant material of the commercially available *Morus nigra* L. leaves (Adonis D.O.O., Sokobanja) was used. The samples of mulberry leaves were dried naturally (in the shade, on draft) during one month and ground in a blender before the extraction. Mean particle size was determined using sieve sets (Erweka, Germany). The mean particle size of the plant samples was 1.26 ± 0.07 mm.

Preparation of teas

Leaves of black mulberry (2 g) were topped with boiled water (200 mL). After certain steeping time (5, 10, 20, 35 and 45 minutes), the beverages were filtrated. The obtained black mulberry leaf teas (BMLT) were used for further analysis.

Determination of TPC and TFC

TPC in teas was determined using the Folin-Ciocalteu method (36). The reaction mixture was prepared by mixing 0.1 mL of the solution (concentration 50 mg/mL) of desired BMLT, 7.9 mL of distilled water, 0.5 mL of the Folin-Ciocalteu's reagent and 1.5 mL of 20% sodium carbonate. After 1 h, the absorbance at 750 nm (VIS spectrophotometer, Janwey 6300, Germany) was measured against the blank solution which was prepared in a similar manner, by replacing the extract with distilled water. The TPC, expressed as mg of chlorogenic acid equivalents per 200 mL of tea, was calculated using calibration curve of chlorogenic acid as standard.

The TFC in BMLTs were estimated according to previously described method (37). The reaction mixtures were prepared by mixing 5 mL of BMLTs, 1 mL of distilled water and 2.5 mL of $AlCl_3$ solution (26.6 mg $AlCl_3$ x $6H_2O$ and 80 mg CH_3COONa dissolved in 20 mL distilled water). A blank solution was prepared by replacing the BMLT with distilled water. The absorbance of probes and blank probe were measured immediately at 430 nm. The TFC, expressed as mg of rutin equivalents per 200 mL of tea, was calculated from a calibration curve using rutin as standard.

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Antioxidant activity

Antioxidant activity of the BMLTs was assessed using two methods: DPPH and reducing power assays. The free radical scavenging activity of BMLT was determined according to the method described by Espin et al. (38). A certain volume of diluted BMLTs was mixed with 95% methanol and 90 μM solution of 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical in order to obtain different final concentrations of teas. The blank probe was prepared by using proper extraction solvent instead of BMLT. After 60 min of incubation at room temperature, the absorbance was measured at 515 nm. The radical scavenging capacity (%RSC) was calculated using the Equation [1], where A_s is the absorbance of BMLT solution and A_b is the absorbance of the blank sample.

$$\%RSC=100-\left(\frac{A_s\times 100}{A_b}\right) \quad [1]$$

This activity was expressed as IC_{50}, which is the concentration of the solution tested required to obtain 50% of radical scavenging capacity.

Reducing power of the BMLTs was determined according to the assay based on the reduction of Fe^{3+} by polyphenol antioxidants (39). Different dilutions of BMLT (1 mL) were mixed with phosphate buffer (1 mL, 0.2 M, pH 6.6) and 1% potassium ferricyanide (1 mL) in glass tubes. Tubes were incubated at 50°C for 20 min. After incubation, 10% trichloroacetic acid solution (1 mL) was added to the reaction mixture. The tubes were then centrifuged at 3000 rpm for 10 min and the supernatant (2 mL) was further mixed with double distilled water (2 mL) and 0.1% $FeCl_3$ solution (0.4 mL). Absorbance was measured at 700 nm. The blank probe was prepared by using proper extraction solvent instead of BMLT. The reducing power was expressed as EC_{50} value (concentration in mg/mL), which causes reduction of 50% Fe^{3+} ions in the reaction mixture. The EC_{50} value was determined from the generated curve which represented the correlation between the BMLT concentration and measured absorbance. All experiments were performed in triplicate, and results were expressed as mean value.

Antimicrobial activity

Antimicrobial activity was determined according to the method described by Sarker at al. (40). The minimum inhibitory concentrations (MIC) of the BMLTs and cirsimarin against the test bacteria were determined by microdilution method in 96 multi-well microtiter plates. All tests were performed in Muller–Hinton broth (MHB), with the exception of the yeast, in which case Sabouraud dextrose broth was used. A volume of 100 μL stock solutions of oil (in methanol, 200 μL/mL) and cirsimarin (in 10% DMSO, 2 mg/mL) was pipetted into the first row of the plate. A volume of 50 μL of Mueller Hinton or Sabouraud dextrose broth (supplemented with Tween 80 at a 12 final concentration of 0.5% (v/v) for analysis of oil) was added to the other wells. The same volume (50 μL) from the first test well was pipetted into the second well of each microtiter line, and then 50 μL of scalar dilution was transferred from the second to the twelfth well. 10 μL of re-

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sazurin indicator solution (prepared by dissolution of a 270-mg tablet in 40 mL of sterile distilled water) and 30 μL of nutrient broth were added to each well. Finally, 10 μL of bacterial suspension (106 CFU/mL) and fungi suspension (3×104 CFU /mL) was added to each well. For each strain, the growth conditions and the sterility of the medium were checked. Standard antibiotic amracin was used to control the sensitivity of the tested bacteria, whereas nystatin was used as control against the tested fungi. The plates were wrapped loosely with cling film to ensure that bacteria, prepared in triplicate, did not become dehydrated, and then they were placed in an incubator at 37°C for 24 h for the bacteria and at 28°C for 48 h for the yeast. Subsequently, the colour change was assessed visually. Any colour change from purple to pink or colourless was recorded as positive. The lowest concentration at which colour change occurred was taken as the MIC value (mg/mL) for the tested BMLTs and standard drug.

Cytotoxic activity

The obtained BMLTs were evaluated for their cytotoxic activity through their influence on the growth of malignantly transformed cell lines using the MTT assay. Malignant cell lines used it this assay were cell line derived from human rhabdomyosarcoma (RD cell line), cell line derived from human cervix carcinoma (Hep2c cell line), and cell line derived from murine fibroblast (L2OB cell line). Cells were seeded (104 cell/mL; 100 μL/well) in 96-well cell culture plates (NUNC) in nutrient medium (MEM Eagle supplemented with 5% (for Hep2c) or 10% (for RD and L2OB), and grown at 37ºC in humidified atmosphere for 24 h. Then, the corresponding probe (stock solution: 5 mg of BMLT dissolved in 1 mL of absolute ethanol) and control (absolute ethanol), diluted with nutrient medium to desired concentrations, were added (100 μL/well) and cells were incubated at 37ºC in humidified atmosphere for 48 h. Pure nutrient medium (100 μL) represented positive control for each cell line. After the incubation period, the supernatants were discarded and 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) (dissolved in D-MEM (Dulbecco's modification of Eagle's medium) in concentration of 500 μg/mL) was added to each well (100 μL/well). After addition all wells were incubated at 37ºC in humidified atmosphere for 4h. The reactions were halted by the addition of 100 μL of sodium dodecyl sulphate (SDS) (10% in 10 mM HCl). After an overnight incubation at 37°C, absorbance was measured at 580 nm. The number of viable cells (NVC) per well was calculated from a standard curve, presenting the cell numbers against absorbance at 580 nm. The corresponding cells (grown in flasks), after cell count by hemocytometer, were used as standards. Standard suspensions were plated in serial dilution, centrifuged at 800 rpm for 10 min, and then treated with MTT/D-MEM and 10% SDS/10 mM HCl solutions in the same way as the experimental wells. The number of viable cells in each well was proportional to the intensity of the absorbed light, which was then read in an ELISA plate reader at 580 nm. Absorbance (A) at 580 nm was measured 24 h later. Cell survival (%) was calculated by dividing the absorbance of the sample with cells grown in the presence of various concentrations of the investigated extracts with control optical density (the A of control cells grown only in nutrient medium), and multiplying by 100. The blank absorbance was always subtracted from the absorbance of the corres-

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ponding BMLT with target cells. The IC_{50} concentration was defined as the concentration of an agent inhibiting cell survival by 50%, compared with a vehicle-treated control. The results of the measurements were expressed as the percentage of positive control growth taking the *cis*-diamminedichloroplatinum (*cis*-DDP), determined in positive control wells as 100% growth (41, 42). All experiments were done in triplicate.

HPLC-DAD-ESI/MS analysis

Quantification of individual phenolic compounds was performed using the Perkin Elmer PE200 HPLC system which was composed of a binary pump, a column thermostat and an autosampler. The mass spectrometer used was 3200 QTRAP MS/MS with ESI ionization (Applied Biosystems/MDS Sciex, Foster City, USA). The experimental conditions were: mobile phase A: 50% acetonitrile, 50% acetic acid (0.5%); mobile phase B: 2% acetic acid; gradient elution: 0 min 30% A, 70% B; 10 min 30% A, 70% B; 30 min 100% A, 0% B; 35 min 100% A, 0% B; 40 min 30% A, 70% B for reconditioning of the system; flow rate: 0.7 mL/min; injection volume: 20 μL; ionization: ESI negative; dwell time 50 ms; MRM transitions: gallic acid 169/125, dihydro-benzoic acid 153/109, sinapic acid 223/164, catechin and epicatechin 289/245,vanillic acid 167/123, caffeic acid 179/135, quercetin 301/151, chlorogenic acid 353/191, ferullic acid 193/134, resveratrol 227/185, ellagic acid 301/145, *p*-coumaric acid 163/119, caftaric acid 311/179. All solvents were HPLC grade and were filtered and degassed before their use.

RESULTS AND DISCUSSION

TPC and TFC

The TPC and TFC values were determined in BMLTs in order to establish the correlation between the steeping time and contents of these classes of compounds. The obtained results are presented in Figure 1 and Figure 2.

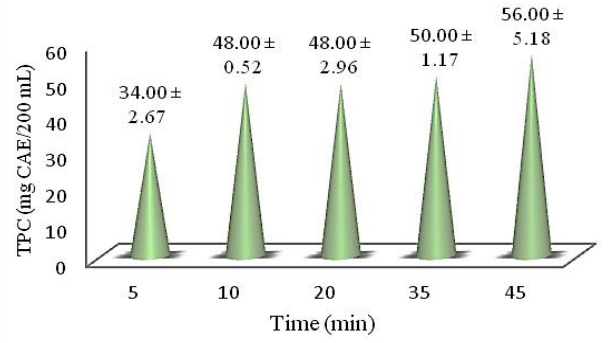

Figure 1. Dependence of TPC on steeping time

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The presented results for TPC confirmed previous findings regarding the correlation between content of extracted compounds and the steeping time of plant material (6, 35). The histogram presented in Figure 1 clearly shows that TPC increased with the steeping time. It can also be noticed that the biggest difference in TPC was observed between the BMLTs obtained after 5 and 10 minutes.

The obtained results for TFC are presented in Figure 2. By taking a closer look at the result, it can be noticed that TFC followed the trend of TPC. The biggest difference is again seen between the BMLTs obtained after 5 and 10 minutes, while the increase in TFC after 10 minutes was negligible. Taking the TPC and TFC results into account, it might be concluded that 10 minutes would be optimal steeping time for preparing the tea with high content of biologically active compounds.

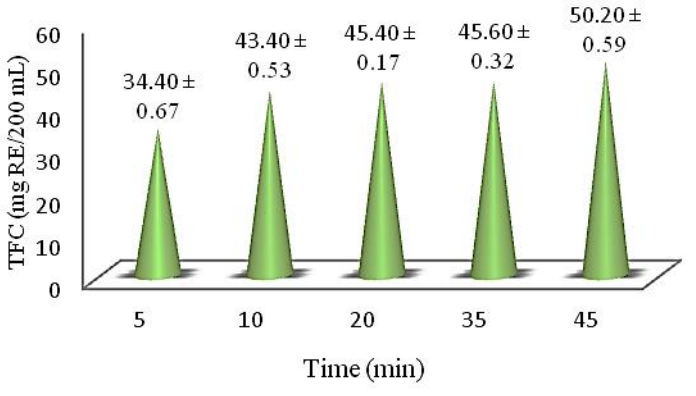

Figure 2. Dependence of TFC on steeping time

Antioxidant activity

The obtained results of antioxidant assays are presented in Table 1. The lower values of IC_{50} and EC_{50} indicate a higher activity. It can be noticed that the highest activity toward DPPH radical expressed BMLT obtained after 10 minutes, while BMLT obtained after 20 minutes showed slightly lower activity. The results of DPPH assay supported the previous conclusion that steeping time of 10 minutes should be sufficient for tea preparation. On the other hand, the highest reducing power exhibited the BMLT obtained after 20-minute steeping.

Table 1. Influence of steeping time on DPPH radical scavenging capacities and reducing power of BMLTs

Steeping time (min)	IC_{50} (mg/mL)	EC_{50} (mg/mL)
5	0.103 ± 0.005	2.668 ± 0.987
10	0.068 ± 0.015	2.406 ± 0.903
20	0.073 ± 0.002	2.205 ± 0.874

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Vidović et al. (1) determined antioxidant activity of medicinal plant extracts using DPPH assays, and obtained IC_{50} values in the range of 5.1-1943.7 mg/mL. By comparing these results with those presented in Table 1 it can be concluded that BMLT possessed a higher antioxidant activity. The presented results suggest that the EC_{50} values exhibited similar tendency as the IC_{50} values. Although the BMLT obtained after 20-minute steeping exhibited the strongest activity in this case, taking into account the results for TPC, TFC and IC_{50}, 10 minutes was taken as the optimum time.

The coefficients of correlation among the assays are presented in Table 2. It can be noticed that there is a very high correlation between TPC and TFC, TPC and IC_{50} value, as well as between TFC and IC_{50} values. The correlation between the IC_{50} and EC_{50} values is moderate, while the correlation among TFC and EC_{50} values, as well as among TPC and EC_{50} values, are rather weak.

Table 2. Pearson's correlation coefficients among TPC, TFC, IC_{50} and EC_{50}

r	**TPC**	**TFC**	**IC_{50}**	**EC_{50}**
EC_{50}	0.4532	0.2654	−0.6041	1
IC_{50}	−0.9842	−0.9287	1	
TFC	0.9797	1		
TPC	1			

The negative correlation between TPC, TFC and IC_{50} values indicates that the scavenging capacity of BMLTs toward DPPH radical increased with the increase in TPC and TFC. On the other hand, the negative correlation between IC_{50} and EC_{50} values indicates that these two tests are probably relying on different mechanisms, and that different compounds presented in obtained beverages exhibited different and specific activity under those assays. The difference of the presented correlation coefficients indicated the inefficiency of single assay to evaluate antioxidant activity of samples (43, 44), confirming the previous conclusion regarding the diversity and specificity in activity of different extracted compounds (45).

Antimicrobial and cytotoxic activities

The results of the measurement of the conducted antimicrobial activity, presented in Table 3 showed that BMLTs exhibited certain activity against all tested microbial stains. The highest activity regarding bacterial strains exhibited BMLT obtained after 20-minute steeping against *Escherichia coli* (19 μg/mL). It can be noticed that the activity against *Escherichia coli*, *Proteus vulgaris*, *Proteus mirabilis* and *Bacillus subtilis* increased with steeping time, while it was opposite in the case of the activities toward *Staphylococcus aureus* and *Klebsiella pneumoniae*. On the other hand, there is a difference in the activities against fungi. In the case of *Candida albicans*, the highest activity exhibited BMLT obtained after 10-minute steeping, while *Aspergillus niger* exhibited the highest sensitivity toward the BMLT obtained after 5 and 20 minutes of steeping.

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Table 3. Antimicrobial activities of BMLTs

Microbial strains	MIC (μg/mL)				
	Steeping time			Standard	
	5	10	20	Amracin	Nystatin
Staphylococcus aureus ATCC 25923	39.100	78.125	156.250	0.970	/
Klebsiella pneumoniae ATCC 13883	39.100	39.100	156.250	0.490	/
Escherichia coli ATCC 25922	156.250	78.125	19.530	0.970	/
Proteus vulgaris ATCC 13315	312.500	156.250	78.125	0.490	/
Proteus mirabilis ATCC 14153	156.250	156.250	78.125	0.490	/
Bacillus subtilis ATCC 6633	156.250	78.125	39.100	0.240	/
Candida albicans ATCC 10231	78.125	19.530	39.100	/	1.950
Aspergillus niger ATCC 16404	19.530	78.125	19.530	/	0.970

The cytotoxic activities of BMLTs are presented in Table 4. In the case of Hep2 and RD cell lines, the highest activity exhibited the BMLT obtained after 5-minute steeping, while in the case of L2OB cell line the most potent BMLT was the one obtained after 5-minute of steeping. According to the American National Cancer Institute, the criterion of cytotoxic activity for plant extracts was $IC_{50} < 30$ μg/mL (46). By taking a look at the presented results (Table 4), it can be noticed that the BMLT obtained after 5-minute steeping meets that requirement for all three cell lines, as well as the BMLT obtained after 10-minute steeping for Hep2 and RD cell lines.

Table 4. Cytotoxic activities of BMLTs

Steeping time	IC_{50} (μg/mL)		
	Hep2 cells	RD cells	L2OB cells
5	8.24 ± 0.85	21.25 ± 0.91	16.56 ± 1.05
10	22.25 ± 1.17	30.07 ± 0.63	101.25 ± 0.14
20	33.85 ± 0.64	51.36 ± 0.98	76.98 ± 0.25
(*cis*-DDP)	0.94 ± 0.55	1.40 ± 0.97	0.72 ± 0.64

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Polyphenolic profile of BMLTs

Taking into account that the 10-minute steeping time appeared be the optimal for BMLT preparation, the polyphenolic profile for that BMLT was obtained using HPLC-MS technique. The results of the performed analysis are presented in Table 5, and the corresponding chromatogram is shown in Figure 3.

Table 5. Polyphenolic profile of BMLT after 10-minute steeping

Compound	Content (μg/g)
Gallic acid	3.62
Sinapic acid	2.03
Vanillic acid	1.48
Caffeic acid	537.52
Chlorogenic acid	7226.00
Ferulic acid	4.87
p-Coumaric acid	11.87
Total	7787.39

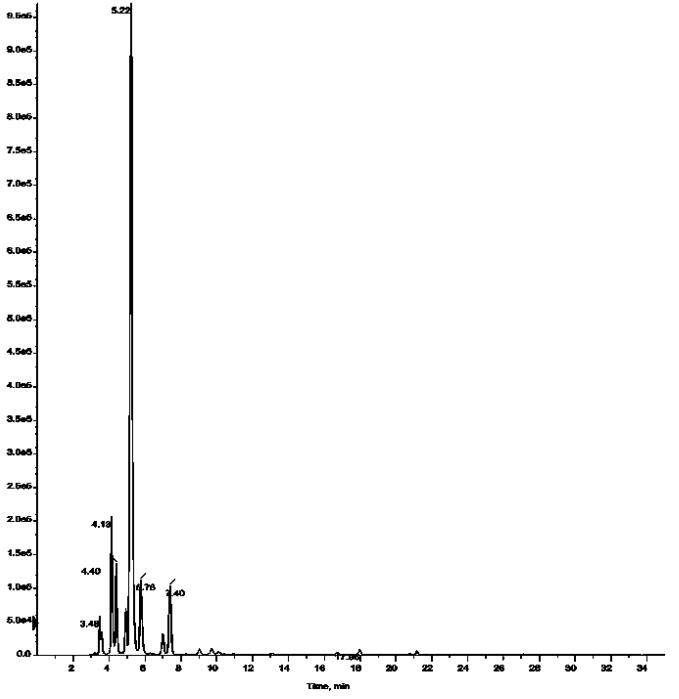

Figure 3. HPLC-DAD chromatogram of BMLT obtained after 10 minutes of steeping

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The most abundant compounds in BMLTs were chlorogenic and caffeic acids. This result is in correlation with previously obtained ones, where the presence of these two compounds was found in high amounts (33, 47). Memon et al. (33) found that chlorogenic acid was the main polyphenolic compound in black mulberry leaves extract, while Sánchez-Salcedo et al. (47) also detected high contents of this compound.

It is well known that polyphenolic compounds exhibit wide range of biological activities such as antioxidant, antibacterial, anti-carcinogenic, anti-inflammatory, anti-viral, anti-allergic, estrogenic and immune-stimulating effects (3, 15-17). Furthermore, several studies also showed that the presence of polyphenolic compounds is very important because these compounds delay aging and regulate fat metabolism in *Caenorhabditis elegans*, which is used as a model (48) and lower hepatic lipid accumulation (49).

CONCLUSION

The awareness of the presence of biologically active compounds and the significance of tea consuming is a rising phenomenon. A well-known contribution of tea consuming to the health should provide the mandatory usage of this beverage as a dietary supplement. This study showed that black mulberry leaves possess numerous health-beneficial compounds which may be used in the everyday diet as a supplement. The investigation of the correlation between the steeping time and the biological activity of BMLTs revealed that the contents of health-beneficial compounds in tea increase with the steeping time, as well as the antioxidant activity. On the other hand, the influence of steeping time exhibited different effect on the antimicrobial and cytotoxic activities of BMLTs. This implies that during the tea preparation degradation and/or other transformations of the biologically active compounds occur due to prolonged exposure to the high temperature. Due to possible degradations, it is important to determine optimal steeping time to prevent or reduce the influence of this process on the tea quality. Taking all results into account, 10-minute steeping time should be optimal for tea preparation.

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УТИЦАЈ ВРЕМЕНА ПРИПРЕМЕ НА БИОЛОШКУ АКТИВНОСТ ЧАЈА ЦРНОГ ДУДА

Слађана Р. Честић[1], Марија М. Радојковић[1], Александра Д. Цветановић[1], Павле З. Машковић[2] и Саша Д. Ђуровић[1]

[1] Универзитет у Новом Саду, Технолошки факултет, Булевар Цара Лазара 1, Нови Сад, Србија
[2] Универзитет у Крагујевцу, Агрономски факултет, Цара Душана 34, Чачак, Србија

Узорци чајева листа црног дуда припремљени су преливањем листа биљке кључалом водом и стајањем током одређеног временског периода (5, 10, 20, 35 и 45 минута). Овако припремљени узорци чајева употребљени су у даљим истраживањима како би се утврдио утицај времена екстракција на биолошку активност узорака. Сама биолошка активност је одређена применом DPPH теста, теста одређивања редукционе моћи и одређивањем антимикробне и цитотоксичне активности. Такође, спектрофотометријским методама одређени су и укупни садржаји полифенола (TPC) и флавоноида (TFC) у добијеним узорцима. На основу добијених резултата, утврђено је да је 10 минута оптимално време припреме чаја, при чему је тај узорак употребљен за одрешивање шполифенолног профила чаја дуда применом HPLC-MS технике. Резултати HPLC анализе показали су да су хлорогенска и кафена киселина најдоминантнија једињења у узорку чаја са садржајима од 7226,00 и 537,52 μg/g, редом.

Кључне речи: лист црног дуда, чај, биолошка активност, време припреме

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DETERMINATION OF OPTIMAL PARAMETERS OF BASIL SUPERCRITICAL FLUID EXTRACTION BY RESPONSE SURFACE METHODOLOGY

Snežana Dj. Filip, Senka S. Vidović, Branimir M. Pavlić and Zoran P. Zeković*

University of Novi Sad, Faculty of Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia

The supercritical fluid extraction of aroma compounds from basil (Ocimum basilicum L.) was studied. Response surface methodology was used to optimize the parameters of the process. Full factorial design was applied to evaluate the effects of two independent variables (pressure and temperature) on the extraction yield and linalool yield. From the response surface plots, pressure and temperature exhibited independent and interactive effect on the extraction yield. The optimal conditions to obtain the highest extraction yield (1.91%) of O. basilicum were the pressure of 29.7 MPa and temperature of 59.2°C, whereas the highest yield of linalool (1.998 g·kg^{-1}) was obtained at the pressure of 20 MPa and temperature of 40°C. The experimental values agreed with the predicted ones, indicating suitability of the response surface methodology for optimizing the extraction process.

KEY WORDS: basil, supercritical carbon dioxide extraction, linalool, response surface methodology

INTRODUCTION

In the recent years, there has been an increasing interest in essential oils and natural antioxidants extracted from different herbs and aromatic vegetable crops (1-3). Polyphenols, as biologically active compounds are used for preparation of dietary supplements, nutraceuticals, functional food ingredients or cosmeceuticals (4).

Basil (*Ocimum basilicum* L.) is one of the most popular plants grown extensively in many countries around the world (especially in Mediterranean countries). It is mostly used as culinary herb for its characteristic aroma. Besides its use in traditional medicine, it is a well-known source of food packaging and flavoring (5,6). Essential oil isolated from *O. basilicum* has antimicrobial, antiviral, anti-inflammatory and antioxidant activity (7-9). Also, basil extracts exhibit a wide spectrum of properties, including bactericidal, anti-inflammatory, antioxidant, chemopreventive, and anti-diabetic activities, and act as nervous system stimulatory (1, 10-12).

* Corresponding author: Snežana Dj. Filip, University of Novi Sad, Faculty of Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia, e-mail: filipsnezana@gmail.com

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Basil extracts are mostly obtained by different solvent extractions, using solvents with different polarity (such as methanol, ethanol and water, pure or mixed) and by supercritical fluid extraction (SFE) (13). Solvent extraction is limited by economic, environmental and safety problems. It is also non-selective, with large volumes of organic solvents used and long extraction times. The final products are with toxic organic residues and, most often, accompanied with degradation or loss of target compounds (14).

Among innovative process technologies, SFE has shown increasing importance. SFE can be a fast, clean and efficient method for the extraction of valuable natural compounds from plant materials (15). Carbon dioxide has been proved to be effective solvent for the application in the pharmaceutical, chemical and food industry. The solvating power of supercritical carbon dioxide can be summarized by a few rules (16): 1) it dissolves non-polar or slightly polar compounds; 2) the solvent power for low molecular weight compounds is high and decreases with increasing molecular weight; 3) free fatty acids and their glycerides exhibit low solubility; 4) pigments are even less soluble; 5) proteins and polysaccharides are insoluble and 6) supercritical CO_2 is capable of extracting compounds that are less volatile, having a higher molecular weight and/or a higher polarity as pressure increases.

In order to optimize the process, one-factor at-a-time approach is generally applied. Response surface methodology (RSM) is a collection of mathematical and statistical techniques used for developing, improving and optimizing process. That experimental methodology is based on the analysis of the effects of the independent variables, where influences of independent variables on responses are investigated one by one, while all the others are kept constant. For the possible industrial application, the optimization of the extraction process is essential. In the literature, the effects of supercritical extraction parameters on the basil extraction yields were poorly investigated.

In this study, the effect of pressure and temperature on the extraction yield and linalool yield were investigated. Response surface methodology was used to build a model between extraction yield and these independent factors, and to optimize the extraction conditions.

EXPERIMENTAL

Chemicals

Commercial carbon dioxide (Messer, Novi Sad, Serbia) purity >99.98% (w/w) was used for the laboratory SFE. The standard compound linalool, used as external standard, was purchased from Carl Roth GmbH (Germany). All other chemicals were of analytical reagent grade.

Plant material

Ocimum basilicum was cultivated at the Department for Organic Production and Biodiversity, Bački Petrovac, Serbia, in 2011. The collected plant material (leaves and

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flowering tops) were air dried and stored at room temperature. Moisture content of the dried plant was determined using standard procedure, i.e., after drying the plant sample at 105°C as 11.44% (w/w). The dried basil was ground and the particle size of ground material was determined using sieve sets (Erweka, Germany).

Supercritical carbon dioxide extraction

The extraction was carried out on a laboratory scale high pressure extraction plant (HPEP, NOVA-Swiss, Effertikon, Switzerland; Figure 1). The main plant parts and properties, by manufacturer specification were: gas cylinder with CO_2, the diaphragm type compressor with pressure range up to 100 MPa, extractor with heating jacket for heating medium with internal volume of 200 mL and maximum operating pressure of 70 MPa, separator with heating jacket for heating medium with internal volume of 200 mL and maximum operating pressure of 25 MPa, pressure control valve, temperature regulation system and regulation valves. Maximum carbon dioxide mass flow rate is 5.7 $kg \cdot h^{-1}$.

The ground sample of basil (50.0 g) mean particle size of 0.65 mm was placed in the extractor vessel. The extraction process was carried out and extraction yield was measured after 4 h of extraction. A flow rate of carbon dioxide, expressed under normal conditions, was 97.725 $dm^3 \cdot h^{-1}$. The investigated values of pressure were 10, 15, 20 and 30 MPa, and of temperature 40°C, 50°C and 60°C. The separator conditions were 1.5 MPa and 23°C. After each extraction, the obtained extract was placed in a glass bottle, sealed and stored at 4°C to prevent any possible degradation.

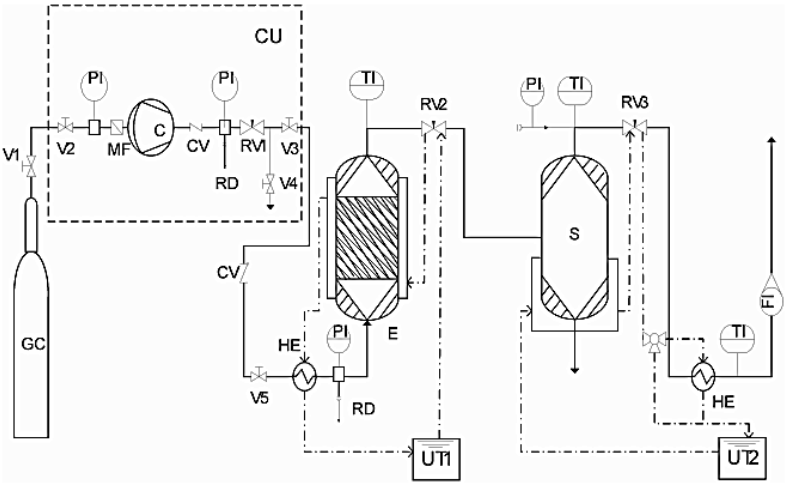

Figure 1. Schematic diagram of the apparatus used for supercritical fluid extraction
GC - gas cylinder, CU - compressor unit, C - compressor with diaphragm, E - extractor, S - separator, HE - heat exchanger, UT - ultra thermostat, RV - regulation valve, V - on-off valve, MF - microfilter, CV - cut-off valve, RD - rapture disc, PI - pressure indicator, TI - temperature indicator, FI - flow indicator.

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Chromatographic procedure

The GC/MS analysis was run on an Agilent GC 6890N system coupled to an Agilent MS 5795 mass spectrometer. An HP-5MS column (30 m length, 0.25 mm inner diameter and 0.25 μm film thickness) was used. The injected volume of sample solution in methanol was 5 μl with split ratio 30:1. The compounds were identified using the NIST 05 and Wiley 7n mass data base and by comparing their retention times to those in mass spectral libraries. Quantifications of the aromatic compound linalool was performed by FID detector and calibration curve for the compound. The percentage composition (relative amount) was calculated from the peak area. The GC/MS operating conditions were as follows: injector temperature 250°C, temperature program was: from 60°C to 150°C (4°C·min^{-1}), carrier gas He with flow rate 2 ml·min^{-1}. The GC/FID operating conditions were: injector temperature 250°C, temperature program from 60°C to 150°C (4°C·min^{-1}), detector temperature 300°C.

All experiments were performed in triplicate and results are given as mean values.

Experimental design and statistical analysis

In this study, the RSM and 3^2 factorial design were applied to determine the optimal extraction temperature and pressure for SFE. RSM is an optimization procedure based on physical experiments or computer experiments (simulations) and experimented observations (17, 18).

The experimental data were fitted to a second order response surface model (Eq. [1]) of the following form:

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i x_i + \sum_{i=1}^{4} \beta_{ii} x_i^2 + \sum \sum_{i<j=1}^{4} \beta_{ij} x_i x_j \qquad [1]$$

where Y represents the response variable, X_i and X_j are the independent variables affecting the response, and $\beta_0, \beta_i, \beta_{ii}, \beta_{ij}$ are the regression coefficients for the intercept, linear, quadratic and interaction terms, respectively.

If the response is defined by a linear function of independent parameters, then it is a first-order function that can be expressed as Eq. [2]:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 \qquad [2]$$

Generally, first-order equations cannot give the desired approximation to real experiments, so second-order models or models with more parameters are used.

In this study, two independent variables, pressure (X_1) and temperature (X_2) were evaluated to optimize the extraction yield (in percent) and linalool yield (in grams per kilogram). The carbon dioxide flow rate and extraction time were fixed values. The

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coded and uncoded independent variables used in the RSM design are shown in Table 1. Experiments were randomized, to maximize the effects of unexplained variability in the observed responses.

Table 1. The uncoded and coded independent variables used in RSM design

Independent variable	Symbol	Coded level		
		-1	0	+1
Pressure (bar)	X_1	100	200	300
Temperature (^oC)	X_2	40	50	60

The analysis was performed using commercial software Design-Expert v.7 Trial (Stat-Ease, Minneapolis, Minnesota, USA). The analysis of variance (ANOVA) was also used to evaluate the quality of the fitted model. The test of statistical difference was based on the total error criteria with confidence level of 95.0 %.

RESULTS AND DISCUSSION

Effect of SFE parameters on extraction yield

The effects of pressure (10-30 MPa) and temperature (40-60^{o}C) on the basil supercritical CO_2 extraction yield and linalool yield were investigated. The 3^2 factorial design was used to optimize important operating variable (pressure and temperature) in order to achieve the optimal yield of basil extract. The optimization of the experimental conditions represents the critical and most important step in the development of supercritical extraction method. The obtained experimental results of 9 runs are summarized in Table 2.

Table 2. Full factorial design (3^2) with independent variables and experimentally obtained results of the extraction yield (Y) and linalool content

Run order	Pressure (MPa)	Temperature (^{o}C)	Y* (%)	Linalool (g·kg^{-1})
1	10 (-1)	40 (-1)	0.719	1.194
2	20 (0)	40 (-1)	1.322	1.998**
3	30 (1)	40 (-1)	1.287	1.379**
4	10 (-1)	50 (0)	0.894	1.054
5	20 (0)	50 (0)	1.447	1.386**
6	30 (1)	50 (0)	1.715	1.739**
7	10 (-1)	60 (1)	0.382	0.382
8	20 (0)	60 (1)	1.666	1.609
9	30 (1)	60 (1)	1.879	1.313

Published data * (19) ** (20)

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The extraction yield after the supercritical extraction was in a range from 0.382 to 1.879 %. The highest extraction yield of 1.879 %, in the investigated conditions was achieved at a pressure of 30 MPa and temperature of 60ºC. Furthermore, it was observed that the extraction yield increased by increasing pressure from 10 to 30 MPa, at constant temperature. This can be explained by the increase in the solvent power of the supercritical CO_2, i.e. solvent density, with the increase in the pressure.

The analysis of variance (ANOVA) proved the suitability of the fitted model (Table 3). The coefficient of determination (R^2) of the model was 0.966, indicating that the model adequately represents the real relationship between parameters. This claim is supported by the relatively low value of the coefficient of variation, and also the good correlation between the predicted values by the mathematical model and experimental results obtained shown in Figure 2. Based on the p-value of the model ($p<0.05$), it can be concluded that the assumed model fits well to the experimental data.

Table 3. Analysis of variance (ANOVA) of the fitted second-order polynomial model

	SS	**Df**	**MS**	***F*-value**	***p*-value**
Model	1.9254	5	0.3850	17.3535	0.0201
Residual	0.0665	3	0.0221		
Total	1.9919	8			

SS - Sum of squares, Df - Degrees of freedom, MS - Mean square

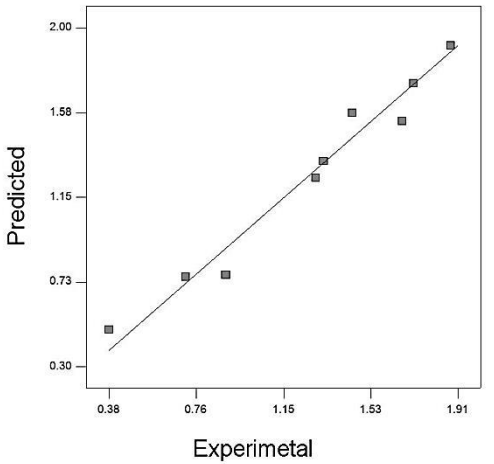

Figure 2. Distribution of the predicted and experimental values of extraction yield (Y)

The regression coefficients of the intercept, linear, quadratic and interaction terms of the model were calculated using the least squares technique. The degree of significance of each factor is presented in Table 4. It is evident that the linear parameter of extraction

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pressure has very significant ($p<0.05$) effect on the extraction yield, while the effect of the quadratic and interaction parameters on the extraction yield is significant ($p<0.10$).

Table 4. Regression coefficients of the fitted second-order polynomial model

Regression coefficient	**Y**
β_o	1.573*
Linear	
β_1	0.481*
β_2	0.099
Cross product	
β_{12}	0.232**
Quadratic	
β_{11}	-0.332**
β_{22}	-0.142
Coefficient of multiple determination R^2	0.966
Coefficient of variance [%]	11.9

* Significant at 5%; ** Significant at 10%

Figure 3 shows a three-dimensional plot of the response surface for the extraction yields of basil obtained by extraction with supercritical carbon dioxide. From the surface plot, it is evident that the extraction yield significantly increases with increased pressure and increased temperature, at the constant extraction time. The pressure had a positive linear effect on the extraction yield, however, the negative quadratic effect also became significant because the further increase in pressure resulted in a slight decline of the extraction yield. Also, it can be seen that the pressure influence was dominant comparing to the second independent variable. This can be explained by the fact that the increase in pressure can result in an increase in fluid density, which accelerates the mass transfer and improves extraction yield (15).

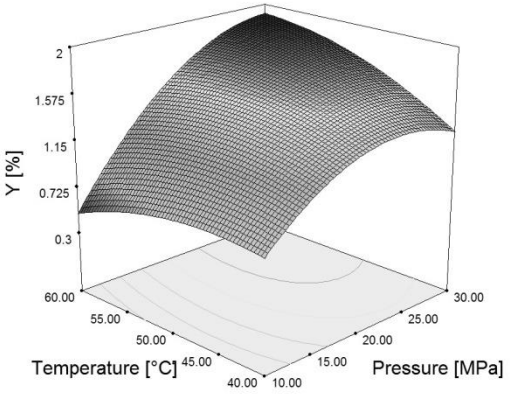

Figure 3. Response surface plot showing effects of the investigated parameters on the extraction yield (Y)

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The mathematical model representing the extraction yield of basil as a function of the independent variables within the region under investigation was expressed by the following equation:

$$Y = 1.573556 + 0.481\ p + 0.099833\ T + 0.23225\ pT - 0.33233\ p^2 - 0.14283\ T^2 \quad [3]$$

where p is the pressure and T is the temperature.

Using the above equation, the optimal conditions to obtain the highest extraction yield of basil were the pressure of 29.7 MPa and temperature of 59.2°C. Under these conditions, the calculated total extraction yield was 1.91%. As the experimentally obtained yield (1.879%) was not significantly different from the predicted value, at the 95% confidence level, it can be concluded that the optimization has been performed successfully.

These findings are in agreement with the results of other studies. In the supercritical extraction of basil reported by Mazutti et al. (21) the highest extraction yield of 1.95 % was obtained at a pressure of 25 MPa and temperature of 50°C during 3 h of extraction. Lachowicz et al. (22) reported that during the supercritical carbon dioxide extraction at 10.33 MPa and 40°C extraction yield was 0.51% and at 31 MPa and 40°C it was 0.97%. The extraction times in both processes were 2 h.

Effect of SFE parameters on linalool yield

In order to optimize the extraction parameters (temperature and pressure) to obtain the highest yield of linalool, the optimization could not be performed in the same manner, because the quadratic model (Eq. [1]) did not give the desired approximation to real experiments ($p>0.05$). It was found that the linear model (Eq. [2]) describes better the extraction system ($p<0.05$). The coefficient of determination (R^2) of the model was 0.85, indicating that the model had a certain deficiency. The predicted linear model used to express the yield of linalool (Y) obtained by supercritical carbon dioxide as a function of two independent variables is as follows:

$$Y = 1.79417 + 0.03003\ p - 0.021108\ T \quad [4]$$

The influence of the pressure and temperature on the linalool yield is presented in Figure 4. It can be observed that pressure had a positive effect on the yield of linalool, while temperature had a negative effect. The increase in pressure from 10 to 30 MPa at 40 and 60°C, led to the increase and then a slight decrease in the linalool yield. At the temperature of 50°C, the increase in pressure from 10 to 30 MPa (increasing in solvent density from 0.378 to 0.881 $kg \cdot m^{-3}$) resulted in the enhanced linalool yields. From the experimental results (Table 2), it can be seen that the highest yield of linalool (1.998 $g \cdot kg^{-1}$) was obtained at a pressure of 20 MPa and temperature of 40°C (fluid density 0.831 $kg \cdot m^{-3}$). So, the supercritical optimum operating conditions to achieve maximum yield of linalool were close to the fluid density of 0.8 $kg \cdot m^{-3}$.

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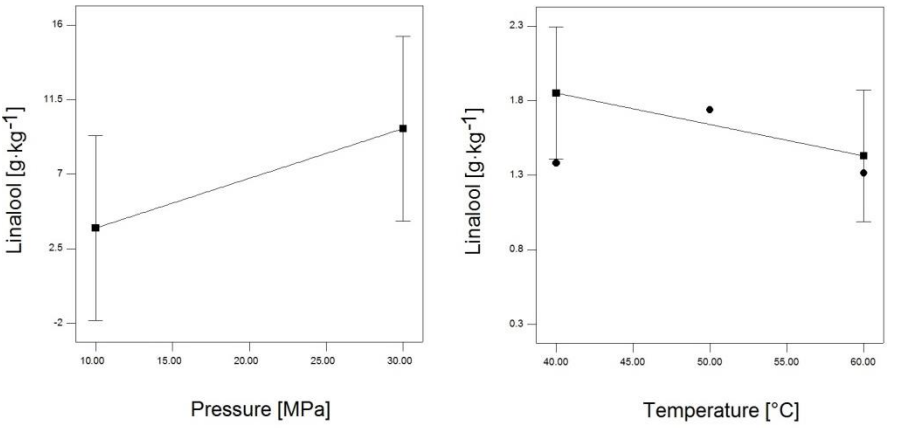

Figure 4. Linear influence of the independent variables on the linalool yield

CONCLUSION

Response surface methodology was successfully applied for optimization the parameters in SFE from *O. basilicum*. The results were very useful for the selection of pressure and temperature in order to obtain the highest extraction yield by supercritical carbon dioxide. The high correlation of the mathematical model indicated that a quadratic polynomial model could be employed to optimize supercritical carbon dioxide extraction of basil. From the response surface plots it can be concluded that pressure and temperature significantly influenced extraction yield, independently and interactively. The optimal conditions to obtain the highest yield were determined to be 29.7 MPa and 59.2^{o}C. Under the optimal conditions, the experimental values agreed with the predicted.

For optimization of the parameters leading to the highest yield of linalool, linear model was employed. The low correlation between experimental and predicted values indicated that further research must involve more independent variables in the non-linear model with the aim to better optimize this response.

This study can be useful in the development of industrial extraction process concerning the optimal sequential steps to enhance the efficacy of a large-scale extraction system.

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ОДРЕЂИВАЊЕ ОПТИМАЛНИХ ПАРАМЕТАРА ЗА СУПЕРКРИТИЧНУ ЕКСТРАКЦИЈУ БОСИЉКА МЕТОДОМ ОДЗИВНЕ ПОВРШИНЕ

Снежана Ђ. Филип, Сенка С. Видовић, Бранимир М. Павлић и Зоран П. Зековић

Универзитет у Новом Саду, Технолошки факултет, Булевар Цара Лазара 1, 21000 Нови Сад, Србија

У овом раду суперкритична екстракција испарљивих компонената босиљка (*Ocimum basilicum* L.) је испитивана. У циљу оптимизације параметара суперкритичне екстракције примењена је метода одзивне површине. Факторијални дизајн је употребљен за евалуацију ефеката две независно променљиве (притиска и температуре) на принос екстракције и принос линалоола. Утицај притиска на процес суперкритичне екстракције испитан је у опсегу притисака од 10 до 30 MPa, док је утицај температуре испитан у опсегу од 40 до 60°C. На основу одзивних површина, притисак и температура испољавају независни и међусобни утицај на принос екстракције. Оптимални услови при којима се добија највећи принос екстракције (1,91%) је притисак од 29,7 MPa и температура од 59,2°C, док је највећи принос линалоола (1.998 $g \cdot kg^{-1}$) добијен при притиску од 20 MPa и температури од 40°C. Експерименталне вредности које се одлично слажу са предвиђеним квадратним моделом, указују да је полиномска зависност посматраног одзива погодна за оптимизацију суперкритичне екстракције босиљка.

Кључне речи: босиљак, суперкритична екстракција, линалоол, метода одзивне површине

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ANTI-ATHEROGENIC PROPERTIES OF A FUNCTIONAL HERBAL MIXTURE

Stevan V. Miletić[1], Jaroslava V. Švarc Gajić[2], Ivan Lj. Milovanović[3], Maja Đ. Đurendić Brenesel[1], Anamarija I. Mandić[3], Vanja M. Tadić[4], Ivana A. Arsić[5] and Aleksandra Č. Mišan[3]*

[1] Clinical Centre of Vojvodina, Institute of Forensic Medicine, Hajduk Veljkova 7-9, 21000 Novi Sad, Serbia
[2] University of Novi Sad, Faculty of Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia
[3] University of Novi Sad, Institute of Food Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia
[4] Institute for Medicinal Plant Research "Dr Josif Pančić", Tadeuša Košćuška 1, 11 000 Belgrade, Serbia
[5] University of Niš, Faculty of Medicine, Department of Pharmacy, Bulevar Dr Zorana Đinđića 81, 18000 Niš, Serbia

In this work, anti-atherogenic and anti-hyperlipidemic effects of a herbal mixture, rich in polyphenols, and composed of 35% of buckthorn bark (Frangulae cortex), 20% of mint leaves (Menthae piperita folium), 20% of caraway fruit (Carvi fructus) and 25% of parsley fruit (Petroselinum fructus) were studied by monitoring biochemical parameters in experimental animals. Experimental animals (Wistar rats) were subjected to five different feeding regimes. Plasma levels of total cholesterol, triglycerides, cholesterol bound to high-density lipoproteins (HDL) and cholesterol linked to low-density lipoproteins (LDL) were monitored in different time periods. The feces of experimental animals was analysed for seven bile acids, as well as for total cholesterol. Simultaneously, the activities of alanine aminotranferase (ALT) and aspartate aminotransferase (AST) were measured. The introduction of herbal mixture into fatty food did not have significant influence on enzyme activities; however, the effect on induced hyperlipidemia was significant. Total atherogenic index was reduced by 43.3%, whereas total cholesterol and cholesterol bound to low density lipoproteins were reduced by 18.2% and 18.8%, respectively. Total bile acids concentrations dropped by 13.2%, whereas cholesterol was reduced by 33%.

KEY WORDS: herbal mixture, atherosclerosis, bile acids, anti-hyperlipidemic

INTRODUCTION

Atherosclerosis is a disease that causes more deaths and disabilities than any other disorder in the industrialized world, and more than all forms of cancer together (1). Compromised brain arteries due to atherosclerosis are the most common cause of stroke, while heart diseases are the result of coronary artery atherosclerosis. This health condition is caused by accumulation of cholesterol transported by lipoproteins in arteries,

* Corresponding author: Stevan Miletić, Clinical Centre of Vojvodina, Institute of Forensic Medicine, Hajduk Veljkova 7-9, 21000 Novi Sad, Serbia, e-mail: stevanmileticns@gmail.com

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leading to tissue damage, inflammation and the appearance of fibroproliferative scarring. Tissue damage of the inner arterial walls leads to the formation of thrombi that can cause heart attack or stroke (1).

Intensive medical research opened the possibilities for the prevention and treatment of atherosclerosis and currently the most commonly applied medical treatments include catheterization, stenting and bypass surgery (2). Nevertheless, it is necessary to stress the importance of the prevention and treatment of atherosclerosis through appropriate diet and lifestyle, as well as the role of drugs that affect balance of cholesterol transporting lipoproteins.

The link between the development of coronary heart diseases, atherosclerosis and antioxidants is known for a long time. The formation and development of atherosclerotic lesions are affected both by fat in blood and antioxidants (3). Oxidation of low density lipoproteins (LDL) and consequent formation and growth of foam cells are responsible for the process of endothelial injury and arterial plaque deposition (4). Previous studies related to the influence of antioxidants focused mainly on examining the effects of dietary tocopherols (5,6) and carotenoids (7). Other studies also dealt with antioxidant effects of certain vitamins. The influence of vitamin E has been particularly emphasized (6). It was also proven that the levels of vitamin D were about 50% lower in the blood of people who suffered from atherosclerosis compared to healthy individuals (8).

Polyphenols are large group of naturally occurring compounds which have long been known to exhibit antioxidant properties (9). The effects of intake of foods rich in polyphenols on the development of atherosclerosis were studied in mice and hamsters with hereditary deficiency of apolipoprotein E (apoE - / -). Anti-atherogenic effects were observed for orally taken extracts of green and black tea (10, 11), pomegranate extract (12), grape extract (13) and red wine (14), thus previous studies mainly focused on catechin, anthocyanins and tannins. In this paper, anti-atherogenic effects of diet enriched with herbal mixture of parsley (*Petroselinum crispum*), peppermint (*Mentha piperita*), caraway (*Carum carvi*) and buckthorn (*Rhamnus frangula*) were studied by monitoring plasma levels of total cholesterol, cholesterol bound to low and high-density lipoproteins, atherogenic index and activities of aspartate aminotransferase and alanine aminotransferase in experimental animals.

EXPERIMENTAL

Chemicals and reagents

HPLC grade hexane, ethanol, methanol, formic and benzoic acid were obtained from Merck. Chemical sets for the determination of cholesterol, triglycerides and activities of alanine aminotransferase and aspartate aminotransferase were purchased from Sigma-Aldrich. Potassium hydroxide, magnesium chloride, sodium chloride and sodium carbonate were purchased from Centrohem (Stara Pazova). Bile acid and cholesterol standards were a kind gift of Prof. Mihalj Poša, from the Faculty of Medicine, Department of Pharmacy in Novi Sad.

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Instruments

The levels of total cholesterol (TC), triglycerides (TG), cholesterol bound to high-density lipoproteins (HDL-c), alanine transaminase activity (ALT) and aspartate transaminase activity (AST) were determined using automated enzymatic method (ELITECH Diagnostic, Sees, France). HPLC analysis was performed by using a liquid chromatograph, equipped with a diode array detector (DAD) and an evaporative light-scattering detector (ELSD) (Agilent G4218A LT-ELSD) on an Agilent, Eclipse XDB-C18, 1.8 μm, 4.6 x 50 mm column, at a flow-rate of 1.000 mL min^{-1}.

Materials and methods

Preparation of functional mixture. The herbal mixture was prepared by mixing 35% of buckthorn 35% of buckthorn bark (*Frangulae cortex*), 20% of mint leaves (*Menthae piperita folium*), 20% of caraway fruit (*Carvi fructus*), and 25% of parsley fruit (*Petroselinum fructus*). Control of individual plant sources included the determination of water content according to SRPS EN ISO 6540:2012, ash content according to SRPS EN ISO 2171:2012, and essential oil content according to SRPS E.38.018:1994 (Table 1).

Table 1. Characteristics of raw plant materials used for the preparation of functional mixture

Plant	Origin of plant	Part used	Moisture content (%)	Ash content (%)	Essential oil content (%)
Mint (*Mentha piperita*)	Serbia	Leave	9.30	5.31	2.90
Parsley (*Petroselinum crispum*)	Serbia	Fruit	12.03	10.87	2.26
Caraway (*Carum carvi*)	Lithuania	Fruit	5.73	4.95	2.35
Buckthorn (*Rhamnus frangula*)	Bosnia and Herzegovina	Bark	3.38	9.86	1.90

In the prepared herbal mixture, the moisture content was 7.91%, ash content 6.71% and granulation 0.75 mm. The microbiological quality, pesticide residues, mycotoxins content and radioactivity corresponded to the European Pharmacopoeia requirements (15).

Animal experiments. Standard feed for experimental animals (mixture of 20% protein powder) was acquired from the Veterinary Institute in Subotica. All experimental meals were prepared by extruding the feed mixture powder on the single screw extruder, at a temperature of 103°C, and the resulting granules had a diameter of 11.5 mm. Cholesterol (Sigma Grade, ≥99%; Sigma-Aldrich), and Na-cholate (98%, Sigma-Aldrich) were suspended in sunflower oil (Vital, Vrbas) and subsequently added to the granules by using vacuum coating technology. Animals had access to food and water ad libitum.

Experiment was conducted on 40 male Wistar rats 4 months old, weighing 295 to 388 g, in accordance with Ethics Commission standards. During the experiment, animals were kept in standard cages (two or three animals per cage) at a temperature of 24°C and twelve hours light/dark regime. During adaptation period, which lasted two weeks, all animals were fed with standard diet for laboratory rats. After this period the animals were

randomly divided into five groups. First group included eleven animals that had standard diet through the entire experiment period of 14 weeks. Second group consisted of six animals fed with standard diet supplemented with the herbal mixture in an amount of 5%. Third group consisted of eleven animals fed with high-fat content diet prepared by addition of sodium cholate, cholesterol and sunflower oil to a standard diet in amounts of 0.5%, 2% and 20% respectively. Fourth group of six rats was fed with the feed prepared in the same manner as for the third group, but with the addition of herbal mixture in an amount of 5%. Fifth group consisted of six animals which were on the same diet regime as the third group for the first six weeks, following eight weeks at the same diet regime as the fourth group.

Blood samples were collected at the beginning of the experiment, at 6 weeks, and at the end of the experiment (16). Faeces samples were collected at the end of the experiment (Table 2).

Table 2. Dynamics and number of samplings in animal groups

Sampling time	Group	Number of blood samples	Number of faucal samples
0 day	1st	3	-
After 6 weeks	1st	3	-
	3rd	5	-
After 14 weeks	1st	5	5
	2nd	6	2
	3rd	6	5
	4th	6	3
	5th	6	3

A number of animals were sacrificed at the beginning of the experiment after starvation during the night and a number after 6 and 14 weeks. The sacrifices were carried out with prior ether anesthesia. On this occasion, blood samples were collected in amounts from 5 to 6 ml of inferior vena cava in heparinized tubes, after which the blood plasma was separated by centrifugation for biochemical analysis (16).

Determination of cholesterol blood concentration. Levels of cholesterol bound to high-density lipoproteins were determined by precipitation with phosphotungstic acid and magnesium chloride (17). The method is based on the treatment of the serum with phosphotungstic acid in the presence of magnesium ions. Low-density lipoproteins, very-low-density lipoprotein (VLDL) and chylomicrons were precipitated from the serum. Remaining HDL cholesterol was further dissolved in the supernatant. The analysis of HDL cholesterol was carried out from the supernatant by enzymatic method.

Total cholesterol was determined by an enzymatic colorimetric method, hydrolyzing cholesterol esters to cholesterol with cholesterol esterase from *Pseudomonas sp.*, purchased from Sigma-Aldrich (21). Briefly, free cholesterol in the reaction with cholesterol oxidase forms 4-cholesten-3-one and hydrogen peroxide. Hydrogen peroxide was treated with peroxidase in the presence of phenol and 4-aminoantipyrine, forming the red com-

pound chinonimine. Serum cholesterol level was determined by measuring absorbance of the formed product at 500 nm. Total cholesterol was calculated as the ratio of sample absorbance (As) and standard absorbance (Ast) multiplied with 2.29 ((As/Ast) x 2.29). (17). Atherogenic index (AI) was calculated as (TC – HDL-c)/HDL-c. Cholesterol linked to low-density lipoproteins was calculated by using Friedewald's formula (16).

Determination of triglycerides. Due to the fact that triglycerides, like cholesterol, do not circulate freely in the blood, methods for their determination are based on previous alkaline or enzymatic hydrolysis to glycerol and free fatty acids, catalysed by lipase. The released glycerol was measured by enzymatic method (19).

Determination of aspartate aminotransferase activity. The assay is based on the ability of aspartate aminotransferase to catalyse reaction of aspartate and α-ketoglutarate to give oxaloacetate and glutamate. Oxaloacetate is converted to malate by malate dehydrogenase and NAD^+. The conversion of NADH to NAD^+ (measured at 450 nm) is proportional to the level of AST enzyme in the sample (20).

Determination of alanine aminotransferase activity. The method is based on the quantification of pyruvate produced by alanine aminotransferase. Pyruvate and nicotinamide adenine dinucleotide (NADH) are converted to lactate and NAD^+ respectively, with a lactate dehydrogenase (LDH). The decrease in absorbance of NADH at 340 nm is proportional to the activity of alanine transaminase (20).

Determination of cholesterol and bile acids in faeces. Faecal cholesterol was determined after sample saponification and the extraction of non-saponificated residue with hexane. Saponification was carried out by heating 500 mg of sample in 100 ml of 0.4 M solution of potassium hydroxide in methanol at 60^{o}C for one hour. Non-saponificated matter was extracted with 30 ml of n-hexane in order to separate the residual base. After evaporation, the dry residue was dissolved in ethanol and passed through a Teflon filter (0.45 μm pore size) prior to HPLC analysis. For the determination of bile acid, solid faecal samples (100 mg) were immersed in 1 ml of methanol, extracted in an ultrasonic bath for 5 minutes, and heated in an oven for half an hour at 80^{o}C. The supernatant was separated and the process was repeated two more times. The extracts were combined, evaporated to dryness and dissolved in 400 ml of methanol. To the prepared sample, 3.6 ml of 1% formic acid was added. Purification was carried out on Agilent SampliQ OPT Polymer columns with a loading of 60 mg.

Cholesterol and bile acids in faeces were determined by liquid chromatography using different detectors. The chromatographic analysis was performed on an Agilent 1200 Series chromatograph equipped with a Diode Array detector (DAD) and Evaporative Light Scattering Detector (ELSD). The separation was performed on a 4.6x50 mm Agilent Eclipse XDB-C18 column at a rate of 1000 ml/min, using methanol as the mobile phase. Cholesterol was determined with DAD at 212 nm with a reference wavelength set at 550 nm and the spectra were recorded in the range from 210 to 400 nm. The analysis time was 10 min with the post-time of 5 minutes.

For the bile acid determination a mixture of methanol and 1% (v/v) formic acid in water (75/25) was used as a mobile phase (21). The apparatus and the columns were the same as in the determination of cholesterol. Bile acids were determined using an ELS de-

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tector adjusted to the nitrogen carrier gas pressure of 3.5 bars and the temperature of 40°C.

Statistical analysis. The results were expressed as mean values ± standard deviation (SD). The statistical significance was determined using one-way analysis of variance (ANOVA). The differences between control and experimental diets were determined by the Tukey's test. Values of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Anti-atherogenic parameters in plasma

The first (control) and the second group of animals expressed an increase in the plasma triglycerides by 109% and 144%, respectively, throughout the experiment. The levels of triglycerides in the third, fourth and fifth group after 14 weeks were not significantly different from the values at the beginning of the experiment (Fig. 1).

After 6 weeks of the experiment, a significant difference was observed in TG, TC and LDL-c levels, and AI between the first and third group of animals (16).

The level of total plasma cholesterol in the first group increased by 34% after 14 weeks. In the second group, the total cholesterol increased by 25% at the end of the experiment. In the third group, which was fed with fatty food during entire experiment, the increase in cholesterol level was 101% after 6 weeks and 260% after 14 weeks, indicating induced hyperlipidemia. Fifth group of animals was on the same diet regime as the third group during the first six weeks. After that period, the diet of the fifth group was changed by adding the herbal mixture in an amount of 5%. During the last eight weeks of the experiment, the diets of the fourth and fifth groups of animals were the same.

The values for cholesterol linked to high density lipoproteins (HDL-c) show that in the first, second and third groups there were no significant changes after 14 weeks of the experiment. However, in the case of LDL-c the value for the third group of animals was significantly greater after 14 weeks (1753%) (Fig. 1) (16).

Addition of herbal mixture to the diet of the fifth group of animals, which was fed exclusively with fatty food during the first 6 weeks, reduced TC by 41%, LDL-c by 75%, and AI by 64%. Compared to the third group of animals, the increase of HDL-c was 33% ($p<0.05$). Also, it can be concluded that the addition of herbal mixture to the fatty food given to the fourth group of animals led to a decrease in TC by 25%, LDL-c by 64%, and in AI by 49%, and to an increase in the HDL-c by 33% in comparison to the third group ($p<0.05$) (Fig. 1).

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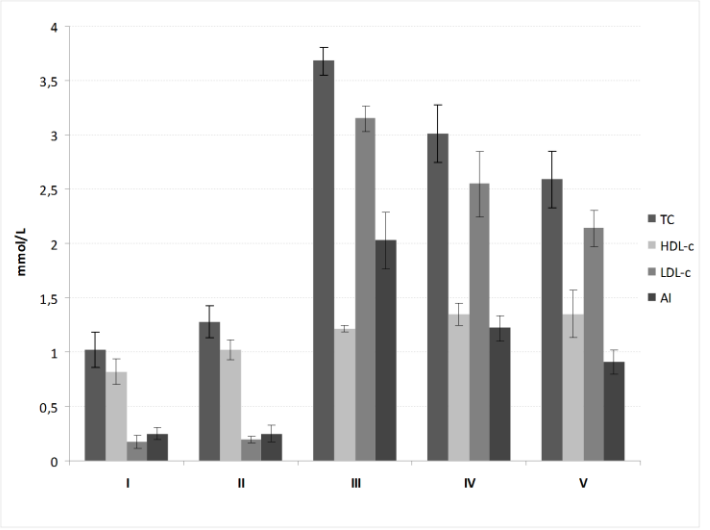

Figure 1. Changes of biochemical parameters evaluated in plasma in different animal groups after 14 weeks

For all tested animal groups there were no significant changes in the activity of aspartate aminotransferase, while alanine aminotransferase activity was significantly higher only for the third group (Fig. 2) (16).

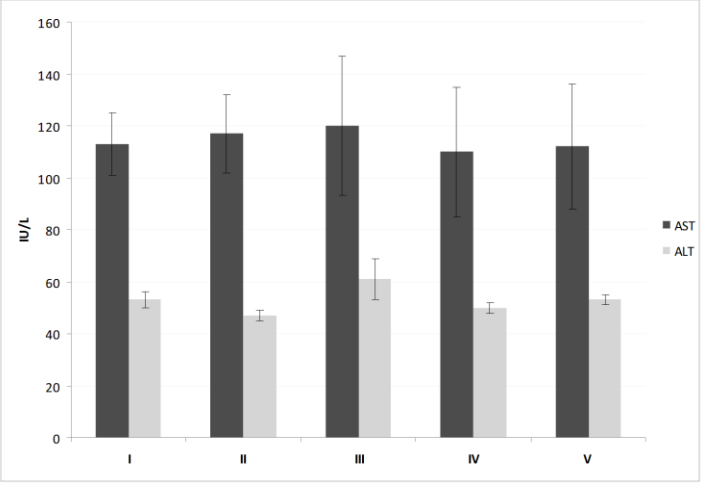

Figure 2. Alanine transaminase (ALT) and aspartate transaminase (AST) activity per groups after 14 weeks

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Faeces analysis

The data for the faeces content, gross energy value of diet and faeces, net caloric intake, cholesterol content of faeces, cholesterol excretion, protein and fat content of faeces, protein and fat excretion, apparent fat and protein absorption are presented in Table 3 (16).

Table 3. Faeces content, gross energy value of diet and faeces, net caloric intake, cholesterol content of faeces, cholesterol excretion, protein and fat content of faeces, protein and fat excretion, apparent fat and protein absorption in the control (I) and the experimental groups of rats: second (II), third (III), fourth (IV) and fifth (V), after 14 weeks of experiment

	I	II	III	IV	V
Faeces (g/day)	11.14 ± 1.45^{a}	17.52 ± 1.80^{b}	6.35 ± 0.99^{c}	7.77 ± 1.18^{d}	8.67 ± 1.63^{c}
Gross energy value of faeces (kJ/day)	158 ± 1.09^{a}	246 ± 8.67^{d}	116 ± 0.22^{b}	142 ± 1.47^{c}	161 ± 1.13^{a}
Gross energy value of diet (kJ/day)	409 ± 2.65^{c}	458 ± 0.564^{d}	397 ± 1.83^{a}	401 ± 0.30^{b}	401 ± 0.30^{b}
Net caloric intake (kJ/day)	251	212	281	259	240
Cholesterol content of faeces (mg/g)	0.817 ± 0.075^{a}	0.869 ± 0.004^{b}	19.14 ± 0.525^{c}	17.03 ± 0.590^{e}	13.07 ± 0.392^{d}
Cholesterol excretion (g/day)	9.10 ± 0.588^{a}	15.23 ± 0.044^{b}	119 ± 0.087^{e}	140 ± 0.555^{d}	120 ± 0.500^{d}
Protein content of faeces (%)	20.8 ± 0.26^{b}	20.6 ± 0.24^{b}	18.3 ± 0.41^{a}	16.5 ± 0.44^{c}	18.1 ± 0.5^{a}
Protein excretion (g/day)	2.32 ± 0.03^{d}	3.62 ± 0.04^{e}	1.16 ± 0.03^{a}	1.28 ± 0.02^{b}	1.60 ± 0.02^{c}
Apparent protein absorption (%)	104	39.9	140	134	89
Fat content of faeces (%)	2.81 ± 0.26^{a}	1.91 ± 0.19^{a}	11.2 ± 0.42^{c}	8.7 ± 0.31^{b}	11.4 ± 0.28^{b}
Fat excretion (g/day)	0.231 ± 0.028^{a}	0.336 ± 0.032^{b}	0.740 ± 0.027^{d}	0.679 ± 0.024^{c}	0.989 ± 0.024^{e}
Apparent fat absorption (%)	84	83	96	97	96

[a,b,c,d,e] Means in the same row not sharing a common superscript are significantly different ($p<0.05$) between groups

The faeces content is expressed in grams, as an averaged value per animal per group per day. It is obvious that the herbal mixture supplementation stimulated digestion of both the low- fat and high-fat diets, as significant differences in faces content are observed between the herbal mixture containing and non-containing groups ($p<0.05$). The gross energy value of diet refers to the averaged food energy intake per animal per day. Referring to the results, the herbal mixture supplementation increased food intake as significant differences appeared between the herbal mixture containing and non-containing low-fat and high-fat group counterparts ($p<0.05$). Contrary to this, the gross energy value of faeces was significantly increased by the herbal mixture presence in the diet. The net caloric intake presents the difference of the energy consumed and excreted. Although not statistically interpreted, the presented values clearly point out that the herbal mixture supplementation reduced the net caloric intake in both high and low fat fed groups. In our experiment, the cholesterol diet supplementation was used to induce hyperlipidemia in high fat fed groups (III, IV and V), and that fact explains a huge difference in the faecal cholesterol content between low and high fat fed groups. The results of cholesterol determination in faeces show that the contents were the highest in the third group that was fed with fatty food without addition of the herbal mixture. The values for the first and the second animal groups did not differ significantly, while those of the other groups was signi-

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ficantly higher ($p<0.05$). The values for the fifth group of the experimental animals were significantly lower than the values for the fourth group.

Based on the previously presented results on plasma cholesterol levels it is obvious that there was a strong correlation between blood and faeces cholesterol levels in all tested animal groups.

Regarding the cholesterol content, it could be concluded that the herbal mixture addition stimulated cholesterol absorption from high fat diet. However, if the mass of faeces is taken into account and cholesterol content of faeces is expressed as cholesterol excretion in g/day, it is clear that the herbal mixture addition stimulates cholesterol excretion regardless of the fat content in the chow. Regarding protein content of faeces, significant differences existed between the low-fat (I and II) and high-fat groups (III, IV and V). Referring to these results, as well as to those of protein excretion and apparent protein absorption, the herbal mixture supplementation seems to reduce protein absorption from the feed. A negative protein balance was noticed in the groups I, III and IV, meaning that the protein content of faeces was higher than the protein content of the control feed. According to Mahipala et al. (22), a large proportion of faecal protein is of bacterial and metabolic origin. The same authors concluded that faecal protein content does not accurately provide reliable quantitative prediction of the differences of the digestibility of dietary crude proteins. Regarding fat content of faeces, significant differences existed between the low- and high-fat fed groups. Fat excretion of the group II was increased in comparison with the control group I, indicating that herbal mixture stimulated fat excretion in low-fat fed animals. However, in high-fat fed groups, the increase existed in the group V, but a decrease in the group IV, in comparison with the group III. Unlike the apparent protein absorption, apparent fat absorption does not seem to be much influenced by the herbal mixture addition.

Digestion stimulating activity of the herbal mixture can be explained by the presence of *Rhamnus Frangula* L. bark in its composition. The alder buckthorn (*Rhamnus Frangula* L.) bark belongs to the stimulant laxatives. Emodin-9-anthrone is the most important metabolite, which is produced by the bacteria of the large intestine. The mode of action is based on two mechanisms. Firstly, colonic motility is increased leading to a reduced transit time. Secondly, the influence on the secretion processes by two concomitant mechanisms, namely inhibition of absorption of water and electrolytes (Na^+, Cl^-) into the colonic epithelial cells (antiabsorptive effect) and an increase in the leakiness of the tight junctions and stimulation of secretion of water and electrolytes into the lumen of the colon (secretagogue effect), results in enhanced concentrations of fluid and electrolytes in the lumen of the colon (23).

In order to examine if the herbal mixture influenced the enterohepatic circulation of bile acids, bile acid composition in the faeces of the control and the experimental groups of rats was performed and the results are presented in Table 4.

As expected, total bile acids showed no significant differences between the first and second group (Table 4). Looking at the values of individual bile acids it could be clearly seen that the contents were higher in experimental animals that were fed with food enriched with fats, except in the case of β-muricholic, hyocholic and hyodeoxycholic acids.

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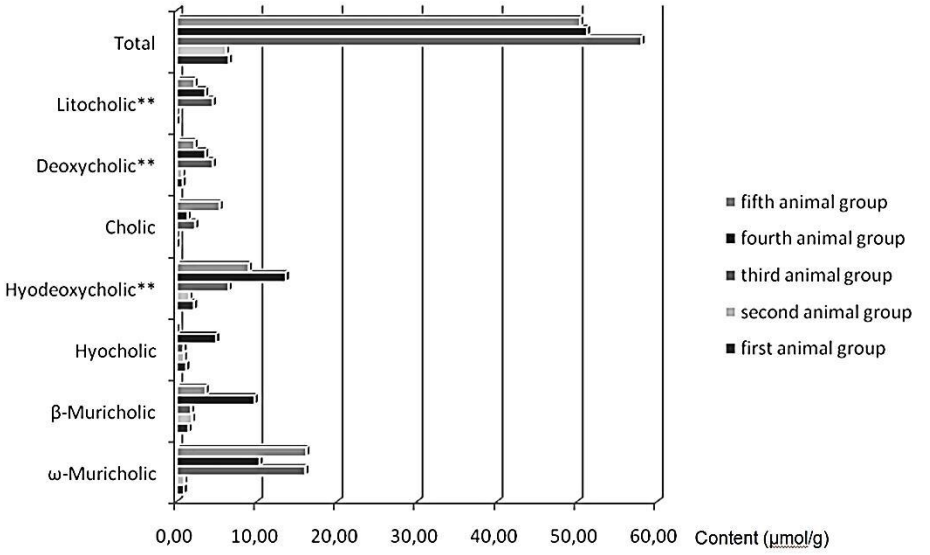
Table 4. The content of bile acids in faeces

Bile acid	first group	second group	third group	fourth group	fifth group
	Content (μmol/g)				
ω-Muricholic	0.90±0.19	0.98±0.17	16.08±0.09*	10.37±0.08*	16.23±0.90*
β-Muricholic	1.49±0.31	1.95±0.10	1.82±0.85	9.75±0.10*	3.63±0.18*
Hyocholic	1.21±0.22	0.94±0.20	0.86±0.05	4.95±0.04*	n.d.
Hyodeoxycholic**	2.18±0.24	1.60±0.34	6.49±0.19*	13.63±1.47*	9.03±0.99*
Cholic	n.d.	n.d.	2.32±0.02*	1.38±0.01*	5.38±0.38*
Deoxycholic**	0.75±0.00	0.72±0.11	25.99±0.83*	7.61±0.46*	13.91±1.39*
Lithocholic**	n.d.	n.d.	4.52±0.88*	3.58±0.34*	2.23±0.14*
Total	6.54±0.97	6.19±0.86	58.08±3.42*	51.27±1.92*	50.41±1.01*

Results are expressed as mean ± relative standard deviation with a confidence interval of 95%
* Values for the same parameter is statistically significantly different ($p < 0.05$)
** Secondary bile acids

As could also expected, the values for the third animal group differed significantly from the values for the first and second or the fourth and fifth groups. In contrast to cholesterol levels, the concentration of total bile acids in the faeces of the fourth and fifth groups did not differ significantly, indicating that the addition of the herbal mixture had the same effects independently on timing of the mixture introduction into the diet (Figure 3).



** secondary bile acids

Figure 3. Levels of individual bile acids in the faeces samples after 14 weeks

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In the faeces of experimental animals that were on a combined diet regime (fatty food with the addition of herbal mixture) the levels of β-muricholic, hyocholic (γ-muricholic) and hyodeoxycholic acids were higher than in the third group, which was fed only with fatty food. Comparing these values with total cholesterol and LDL-c levels in blood it could be concluded that in the case of the fourth group, the addition of herbal mixture to fatty foods led to lowering of blood cholesterol and increase of faecal bile acids; β-muricholic by 436% hyocholic by 475% and hyodeoxycholic by 110%. In the animals that were fed first six weeks with fatty food exclusively and last eight weeks with the fatty food enriched with herbal mixture (fifth group), there was an increase in fecal β-muricholic and hyodeoxycholic acid by 99% and 39%, respectively.

CONCLUSION

The analysis of blood and faeces biochemical parameters of experimental animals showed anti-atherogenic and antihyperlipidemic effects of the herbal mixture rich in natural phenolic compounds. The evaluated biochemical parameters showed that the introduction of herbal mixture into a high-fat content food had positive effects in induced hyperlipidemia, and the concentration of high density lipoproteins. Namely, high-fat content food enriched with 5% of herbal mixture lowered the total plasma cholesterol (41%) and low density lipoproteins (75%). Under the influence of tested herbal mixture, the atherogenic index dropped by 64%. Activities of aspartate transaminase and alanine transaminase showed that the diet did not have significant effects on liver functions. The introduction of the herbal mixture into the diet of experimental animals resulted in a decrease of cholesterol and bile acids concentrations in the faeces with the exception of β-miricholic, hyocholic (γ-muricholic) and hyodeoxycholic acids, whose concentrations increased with the introduction of the herbal mixture. In this respect, the diet had a more pronounced effect on cholesterol if administered after 6 weeks than when the herbal mixture was added to fatty foods from the start. On the other hand, in the case of biliary acids the timing of the diet introduction (at the start or after 6 weeks) had no effect.

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АНТИАТЕРОГЕНЕ ОСОБИНЕ ФУНКЦИОНАЛНЕ БИЉНЕ МЕШАВИНЕ

Стеван Милетић[1], Јарослава Шварц Гајић[2], Иван Миловановић[3], Маја Ђурендић Бренесел[1], Анамарија Мандић[3], Вања Тадић[4], Ивана Арсић[5] и Александра Мишан[3]

[1] Клинички центар Војводине, Институт за Судску Медицину, Хајдук Вељкова 7-9, 21000 Нови Сад, Србија
[2] Универзитет у Новом Саду, Технолошки факултет, Булевар Цара Лазара 1, 21000 Нови Сад, Србија
[3] Универзитет у Новом Саду, Институт за прехрамбене технологије, Булевар Цара Лазара 1, 21000 Нови Сад, Србија
[4] Институт за проучавање лековитог биља "Др Јосиф Панчић", Тадеуша Кошћушка 1, 11000 Београд, Србија
[5] Универзитет у Нишу, Медицински Факултет, Булевар др Зорана Ђинђића 81, 18000 Ниш, Србија

Кардиоваскуларна обољења су најбројнији узрок смрти и инвалидитета у индустријализованом свету. Најчешћи тип кардиоваскуларног обољења је атеросклероза која настаје због нагомилавања холестерола на одређеним местима унутар артерије. С обзиром на то да поред лекова на баланс холестерола велики утицај има и начин исхране, циљ овог истраживања је био да се утврди ефекат биљне мешавине на излучивање фекалних жучних киселина и апсорпцију холестерола. *In vivo* експеримент

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је спроведен на пет група мушких Вистар пацова који су били на различитим режимима хране током 14 недеља. За одређивање сирових протеина и масти су коришћене стандардне методе анализе, док је за сепарацију и одређивање фекалног холестерола и жучних киселина примењена течна хроматографија. Припрема фецеса код одређивања холестерола подразумевала је сапонификацију узорка и екстракцију несапонификоване материје хексаном а жучне киселине екстраховане су етанолом. Овако припремљени узорци су испитивани HPLC/DAD и HPLC/ELSD техником.

Кључне речи: биљна мешавина, атеросклероза, жучне киселине, антихиперлипидемијски

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CHARACTERISTICS OF W/O EMULSIONS CONTAINING POLYMERIC EMULSIFIER PEG 30-DIPOLYHYDROXYSTEARATE

Jelena R. Milinković, Lidija B. Petrović, Jadranka L. Fraj, Sandra Đ. Bučko and Jaroslav M. Katona*

University of Novi Sad, Faculty of Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia

Water-in-oil (W/O) emulsions are dispersed systems which are often used in the pharmaceutical, cosmetic and food industries as products, or as carriers of active substances. It is well known that they are very unstable, so that selection of the emulsifier and properties of the oil and water phase are main factors affecting their stability. The aim of this paper was to examine the possibility of application of a lipophilic, polymeric emulsifier, PEG 30-dipolyhydroxystearate (Cithrol™ DPHS), for stabilization of W/O emulsions. Behaviour of the emulsifier at W/O interfaces was determined by means of tensiometry. A series of emulsions were prepared with 20% (w/w) of water and different types of oil. Droplet size, droplet size distribution, viscosity, and sedimentation stability during 30 days of storage at room temperature of the emulsions prepared with paraffin oil, olive oil, grape seed oil, and medium-chain triglycerides, stabilized with 1% Cithrol™ DPHS, were determined. All investigated emulsions were stable for 30 days, except the one prepared with paraffin oil. The results of this study confirmed that PEG 30-dipolyhydroxylstearate is a good emulsifier and stabilizer of W/O emulsions which contain different types of oil.

KEY WORDS: water-in-oil emulsion, polymeric emulsifier, droplet size analysis, emulsion stability

INTRODUCTION

Water-in-oil (W/O) emulsions have different applications in the food, pharmaceutical and cosmetic industries (1, 2). A number of studies about emulsions focus on oil-in-water (O/W) systems, but there are few papers about liquid W/O emulsions. The reason for this is low stability of liquid W/O emulsions, which can easily sediment, flocculate or coalescence because of the high mobility of water droplets. A better understanding of interactions between water, oil and emulsifier at the interface and factors that affect emulsion stability would allow production of stable liquid W/O emulsions and, therefore, encourage the development of new products and applications (3, 4).

* Corresponding author: Jelena Milinković, University of Novi Sad, Faculty of Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia, e-mail: jelenamilinkovic@tf.uns.ac.rs

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The stability mechanism of W/O emulsions differs from the O/W emulsions, which can be stabilized by both steric and electrostatic repulsion. In the case of W/O emulsions, only steric forces are expected to stabilize the emulsion, due to low electrical conductivity of the continuous phase (5). For that reason, W/O emulsions generally present worse stability than O/W emulsions. Common destabilization mechanisms of emulsions are flocculation, coalescence, sedimentation and Ostwald ripening (2, 6). In general, emulsions are thermodynamically metastable systems, and therefore the main challenge for W/O emulsion production is to control the system stability, as well as to provide protection against destabilization (7 - 10). Selection of emulsifier is certainly the most important step in emulsions preparation (11, 12). Moreover, the bulk physicochemical characteristics are important for formation of W/O emulsions and their stability. For example, the density of continuous phase is decisive for stability against sedimentation, viscosity of the inner phase has influence on droplets breakup and their mobility, while the oil polarity can affect the interfacial tension and partitioning of the components at the interface (3). Therefore, in the last decade, better understanding of emulsion stabilization and destabilization mechanisms has become an increasingly interesting area for research (13, 14).

In addition, chemical structure of the oil, such as fatty acids chains length, degree of unsaturation and molecule configuration, influence on emulsion stability and its use. The grape seed oil is considered to be high quality dietary oil with a high concentration of unsaturated linoleic acid, vitamin E and phytosterols (15), which are associated with promotion of cardiovascular health by down-regulating low-density lipoprotein cholesterol production (16).

Olive oil, as main active components includes oleic acid, phenolic constituents, and squalene. The main phenolics include hydroxytyrosol, tyrosol and oleuropein, which have antioxidant activity (17).

Medium-chain triglycerides are liquid lipid (oil) of low viscosity at room temperature. Fatty acid composition is usually dominated by C8 fatty acids, followed by C6, C10 and C12. Due to its low toxicity to skin and mucous membrane, medium-chain triglycerides are commonly used in cosmetic and dermatological products, as emollient and drug solvent in peroral preparations (18).

Paraffin oil (mineral oil) is a purified mixture of liquid saturated hydrocarbons obtained from petroleum (19). As it can be concluded from the above, all of these oils and their emulsions have a great potential for application in pharmaceutical, cosmetic, and food industry.

The objective of the present study was a better understanding of the specific interactions between selected oils and surfactant and their effect on W/O emulsion stability. PEG 30-dipolyhydroxystearate (Cithrol^{TM} DPHS), a non-ionic, polymeric emulsifier suitable for double W/O/W emulsions (20) was selected as a surfactant and its interfacial properties were determined by means of tensiometry. Characteristics of the 20% (w/w) W/O emulsions such as droplet mean diameter, droplet size distribution, viscosity and sedimentation stability within 30 days of storage at room temperature were determined by means of dynamic light scattering measurement, optical microscopy, rheology and visual observation of the phase separation.

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EXPERIMENTAL

Materials

Different oils were used as continuous phase of the W/O emulsions: olive oil (Comcen, Zemun), medium-chain triglycerides or caprylic/capric triglyceride (Saboderm TCC, Comcen, Zemun), paraffin oil (Centrohem, Stara Pazova), grape seed oil (Olitalia, Forlì (FC) Italy). Oil-soluble emulsifier Cithrol^{TM} DPHS, which is PHS/PEO/PHS block copolymer i.e. PEG 30-dipolyhydroxystearate (Macrogol 30-dipolyhydroxystearate; Ph. Eur. 8), was donated by Croda, Belgium. Deionized water was used as aqueous phase.

Methods

Interfacial tension. Measurements of the interfacial tension between water and oil with various surfactant concentrations were carried out on a Sigma 703D tensiometer (KSV Instruments, Finland) using the Du Noüy ring method (21). Prior to the measurements, the ring was immersed in deionized water (below the surface), then the oil phase, slowly added on top and the interface, was left for 15 min to equilibrate. The interfacial tension of each system was measured at the point where the ring broke away from the interfacial layer between the two phases. In all measurements, the temperature was kept at 40^{o}C. The reported values of the interfacial tension were average of three measurements, at least. Concentrations of Cithrol^{TM} DPHS were varied from 0.00001% (w/w) up to 1% (w/w).

Preparation of W/O emulsions. The emulsions were prepared at the water – oil mass ratio 20:80, with different types of oil, while the aqueous phase was deionized water. Continuous phase of emulsions were prepared at room temperature by dissolving the emulsifier (1% w/w) in selected oil. Emulsions were prepared by dispersing a desired amount of water in the continuous phase at 40^{o}C by means of the homogenizer Ultra Turrax T-25 (IKA, Germany) at 20000 rpm during 10 min.

Rheological measurements. Viscosities of continuous phases and W/O emulsions were determined using an RS600 rheometer (Thermo Electron, GmbH, Germany). The cone-and-plate geometry was used (plate diameter, d = 60 mm, and cone angle $\theta = 1^{o}$, gap 0.052 mm). Measurements were carried out at the temperature of 25^{o}C. The shear rates were increased from zero to 200 s^{-1} and reversely. Viscosities of the emulsions were measured immediately after preparation.

Droplet size and polydispersity index analysis. Particle size analysis of freshly prepared emulsions and emulsions after 7 days of storage was performed by dynamic light scattering (DLS) measurements using a Malvern Zetasizer Nano ZS (Malvern Instruments, UK) at 25^{o}C. Prior to the measurements, all samples were diluted with oil that was used as the emulsion continuous phase, in order to yield a suitable scattering intensity. Refractive indexes of olive oil, medium-chain triglycerides, paraffin oil and grape seed oil were 1.475, 1.448, 1.462 and 1.475 respectively. DLS data were analysed using the general purpose mode, thus the hydrodynamic diameter (z-average) and the polydispersity index (PI) were obtained.

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After 30 days of storage, the droplet size of the emulsions was over the measuring range of DLS, so the determination was carried out by photomicrography, using of Leica QWin software. Photomicrographs were taken on an optical microscope, Biooptica BEL-3000, Germany at 40x magnification. Particle mean diameter, expressed as volume-surface mean value, d_{vs} (μm) and standard deviation σ (μm) were calculated from the experimental data:

$$d_{vs} = \sum n_i d_i^3 / \sum n_i d_i^2 \quad [1]$$

$$\sigma = (\sum n_i (d_i - d_{vs})^2 / \sum n_i)^{1/2} \quad [2]$$

where d_i is droplet diameter and n_i is number of droplets.

Emulsions stability test. For stability test, the emulsions were transferred into 10 ml graduated cylinders and stored at room temperatures for 30 days. The emulsions were observed for the changes in consistency, homogeneity and phase separation during storage. The oil phase separation from the emulsions was visually monitored at regular time intervals (immediately after preparation, after 7 days, and after 30 days). The total height of the emulsion, H_e, and the height of the oil layer, H_o, were measured with time laps. The extent of phase separation was characterized by the sedimentation index, *H*, given as:

$$H = 100\ H_o/H_e\ (\%) \quad [3]$$

A higher value of the sedimentation index indicates a worse emulsion stability.

RESULTS AND DISCUSSION

Interfacial tension measurements

Determination of the interfacial tension of the two-phase system can offer valuable information about stability of the emulsion prepared of the two phases. Furthermore, the rapid decrease in the interfacial tension, with the emulsifier addition, is important for formation of small droplets, which results in higher stability against the gravity force (2).

The behaviour of the emulsifier Cithrol™ DPHS at the W/O interface was determined by tensiometry. The surface tension at the water-grape seed oil, water-medium-chain triglycerides, water-paraffin oil and water-olive oil interface was 19.22, 18.36, 25.49 and 18.44 mN/m, respectively. The changes in the interfacial tension for different oils, with various Cithrol™ DPHS concentrations are presented in Fig. 1. As it can be observed, the interfacial tension for all investigated systems progressively decreased with an increase in the emulsifier concentration, reaching the lowest value at 0.1% Cithrol™ DPHS. The lowest value of the interface tension (0.16 mN/m) was obtained for the water – medium-chain triglycerides system. Above this concentration, the interfacial tension remained constant, i.e. the W/O interface was saturated with surfactant molecules, and micelles

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were formed in the bulk. As the values of interfacial tension were very low (from 0.16 to 0.95 mN/m), close to zero, it could be expected that Cithrol^{TM} DPHS is suitable for obtaining W/O emulsions of all oils used. For further examination of Cithrol^{TM} DPHS emulsifying properties, the W/O emulsions containing 20% of water, stabilized with 1% (w/w) of emulsifier, were prepared. Viscosities of the continuous phases and corresponding emulsions, droplet size, droplet size distribution, and sedimentation stability of the emulsions were studied in order to better understand the stabilization mechanism and characteristics of the water-in-oil systems.

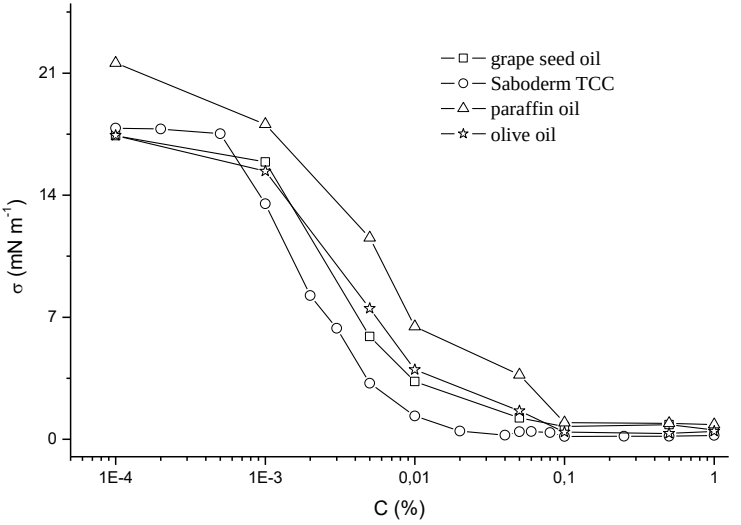

Figure 1. Interfacial tension as a function of Cithrol^{TM} DPHS concentration at 40^{o}C

Rheological investigations

Rheology of the continuous phases (containing emulsifier) and corresponding W/O emulsions, immediately after their preparation, was investigated at 25^{o}C. All emulsions and their continuous phases showed a Newtonian type of flow, and their viscosities are presented in Table 1. The low values of viscosity obtained for continuous phases, that contain Cithrol^{TM} DPHS in concentration of 1% (w/w) indicate that the addition of this polymeric emulsifier did not affect the viscosity of the oil. The obtained emulsions, as expected, were of low viscosities (from 40.4 to 115.2 mPas).

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Table 1. Viscosities of continuous phases and W/O emulsions at 25°C

Type of oil	Viscosity (mPas)		
	Oil	Continuous phase	Emulsion
Paraffin oil	33.93	36.82	40.8
Olive oil	67.42	69.37	115.2
Saboderm TCC	23.79	23.87	40.4
Grape seed oil	40.02	46.1	81.6

Emulsions droplet size and droplet size distribution

Besides the impact on the stability of emulsion, the size and size distribution of emulsion droplets are the parameters that greatly characterize the applied emulsifier. For this reasons, the determination of droplet size in W/O emulsions was performed immediately after the preparation and after 7 and 30 days of storage at room temperature. The droplets in all investigated emulsions, immediately after the preparation, were of nano dimensions, so that DLS was used for size determination. The results are presented in Table 2.

Table 2. Droplet size and PI with standard deviation for W/O emulsions with different type of oil, during storage at room temperature: z-average, d_{vs}-mean diameter, σ-standard deviation

Time (days)	0		7		30
Type of oil	Z-average±σ (nm)	PI±σ	Z-average±σ (nm)	PI±σ	d_{vs}±σ (μm)
Paraffin oil	980.6±44.94	0.26±0.21	1275±211.1	0.6±0.14	-
Olive oil	369.6±35.84	0.63±0.06	393.9±110.7	0.5±0.1	5.14±0.41
Medium-chain triglycerides	115.3±10.56	0.16±0.02	139±20.66	0.2±0.06	4.45±0.31
Grape seed oil	68.40±13.54	0.22±0.07	78.9±2.9	0.1±0.03	5.15±0.36

As it can be seen from Table 2, the smallest droplet diameter (z-average) was obtainned in the emulsions prepared with grape seed oil and medium-chain triglycerides (68.4 and 115.3 nm, respectively), while the droplet mean diameter of emulsion with paraffin oil was 980.6 nm. The PI was in the range of 0.16-0.63, which indicates the differences in size distribution. Namely, the particle populations with very narrow size distribution have PI values in the range of 0.02–0.05, while a PI higher than 0.5 indicates very wide droplet size distribution that can lead to layering of the emulsion (22). The emulsions prepared with medium-chain triglycerides, grape seed oil and paraffin oil showed a narrow, mono-

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modal droplet distribution (PI from 0.16 to 0.26), as it can be seen from Fig. 2. In the case of emulsion with olive oil, a bimodal size distribution was observed (PI = 0.63), with first peak at 201 nm and a second one around 4295 nm. Such finding indicates that the emulsion with olive oil stabilized by Cithrol^{TM} DPHS could be of lowest stability, which can lead to phase separation.

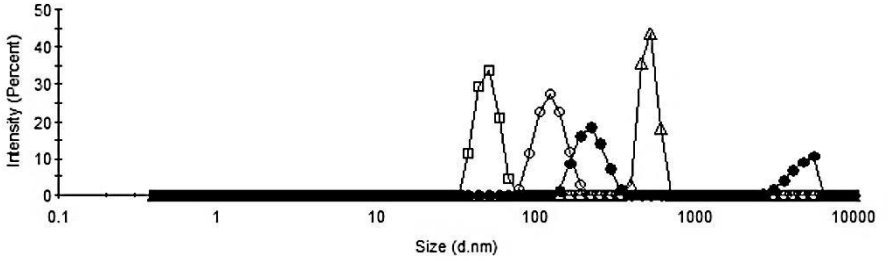

Figure 2. Droplets size distribution for W/O emulsions with medium-chain triglycerides (-○-), paraffin oil (-Δ-), grape seed oil (-□-) and olive oil (-●-), after preparation

The light microscope observation of emulsions after 7 days of storage at room temperature, showed the existence of small individual droplets. However, according to DLS analysis (Table 2), the mean droplets diameter increased in all investigated samples but still remaining in a nano range, except for the emulsion with paraffin oil. The emulsions with medium-chain triglycerides and grape seed oil showed a monomodal droplet size distribution with low PI (Fig. 3). Bimodal size distribution was observed for emulsions with olive oil (first peak at 190 nm and a second one near 5560 nm) and paraffin oil (first peak at 531 nm and at 5560 nm). Formation of larger droplets occurred as the result of coalescence due to a weak steric repulsion of the adsorbed layer at the interface.

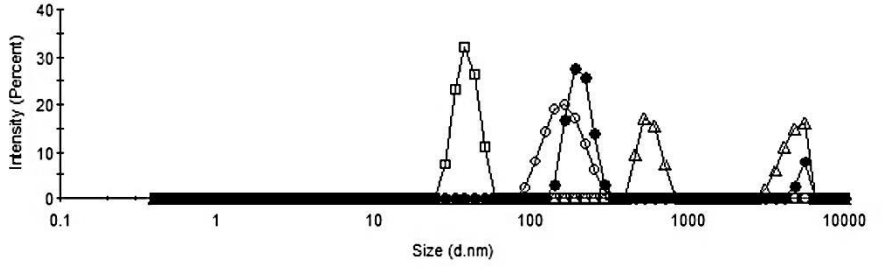

Figure 3. Droplets size distribution for W/O emulsions in medium-chain triglycerides (-○-), paraffin oil (-Δ-), grape seed oil (-□-) and olive oil (-●-), after 7 days

After 30 days of storage at room temperature, coalescence of droplets was enhanced in all emulsions, so that their size was determined by photomicrography. The mean droplets diameters (d_{vs}) and standard deviation are presented in Table 2. As it can be observed, the mean droplet diameter in all emulsions was around 5 μm. The existence of droplet aggregates was not observed, except for the emulsion with paraffin oil (Fig. 4). Deter-

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mination of droplet size in this emulsion was not possible due to the presence of closely packed aggregates of fine droplets around the large ones.

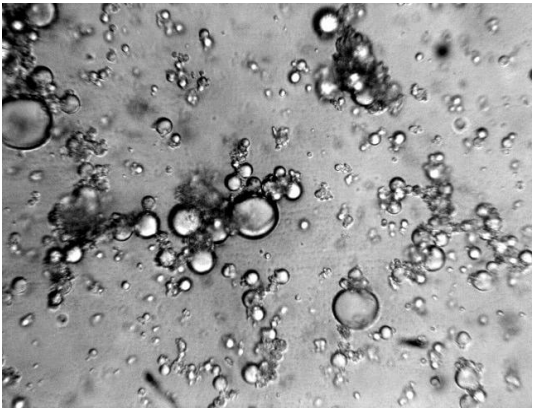

Figure 4. Photomicrograph of the W/O emulsion with paraffin oil after 30 days of storage at 25°C

Sedimentation stability of emulsions

The stability of the W/O emulsions was investigated during 30 days of storage at room temperature. The cylinders filled with emulsions, after 30 days of storage, are shown in Fig. 5.

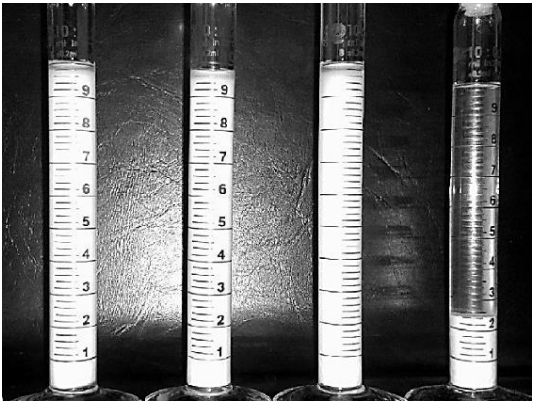

Figure 5. W/O emulsions prepared with different kinds of oil, from left to right: olive oil, grape seed oil, medium-chain triglycerides, paraffin oil, after 30 days of storage at 25°C

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It can be noticed that the emulsions obtained with olive oil, grape seed oil and medium-chain triglycerides were stable during 30 days, i.e. no separation of oil layer was observed. Considering that the coalescence of droplets with time in these emulsions was confirmed (Table 2.), the sedimentation stability could be due to the existence of single droplets and a small differences in viscosity of the oil and water phase.

In the paraffin oil emulsion phase separation appears with time, pointing to a decrease in its stability. The change in sedimentation index H with time is presented in Fig. 6.

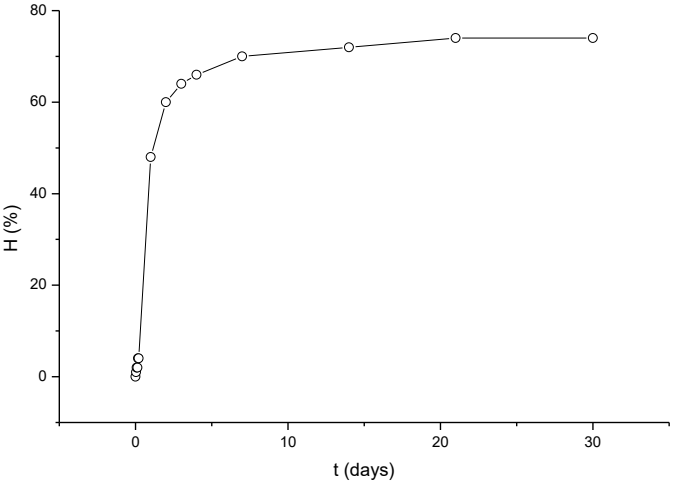

Figure 6. Sedimentation index (H) for the paraffin oil emulsion as a function of time

As it can be seen, the sedimentation index changes rapidly during the first 7 days of storage, reaching high value of 70%, and then remains almost unchanged. A bimodal distribution of droplets diameter, accompanied with formation of aggregates (Fig. 4) and very low viscosity of paraffin oil, could be a reason for such sedimentation instability, i.e. phase separation.

CONCLUSION

The investigation of the interfacial behaviour of PEG 30-dipolyhydroxystearate (Cithrol^{TM} DPHS) showed that its addition decreases the surface tension at the water-oil interface, for all examined types of oil, reaching the lowest value at a concentration of 0.1%. Freshly prepared W/O emulsions with 20% water and paraffin oil, olive oil, grape seed oil and medium-chain triglycerides, which were stabilized with 1% of Cithrol^{TM} DPHS, had submicron droplet size, low viscosity and showed Newtonian type of flow. The emulsions made with natural and medium-chain triglycerides oils were stable during 30 days of storage at room temperature. Namely, regardless of the increase in droplets

mean diameter of these emulsions with time, i.e. progressive coalescence, there was no phase separation. Formation of droplet aggregates was observed in the paraffin oil emulsion, thus phase separation occurred with time. The results of this study showed that Cithrol^{TM} DPHS, polymeric emulsifier could be used for obtaining stable W/O emulsions with natural and medium-chain triglycerides oils that could be suitable for pharmaceutical, cosmetic and food application.

Acknowledgement

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КАРАКТЕРИЗАЦИЈА В/У ЕМУЛЗИЈА КОЈЕ САДРЖЕ ПОЛИМЕРНИ ЕМУЛГАТОР PEG 30-ДИПОЛИХИДРОКСИ-СТЕАРАТ

Јелена Р. Милинковић, Лидија Б. Петровић, Јадранка Л. Фрај, Сандра Ђ. Бучко и Јарослав М. Катона

Универзитет у Новом Саду, Технолошки факултет, Булевар Цара Лазара 1, 21000 Нови Сад

Емулзије типа вода у уљу (В/У) су дисперзни системи, који се као готови производи, или носачи активних материја, често користе у козметичкој и прехрамбеној индустрији. Познато је да се ради о изузетно нестабилним системима, код којих често долази до сепарације фаза и других облика нестабилности, тако да избор емулгатора и особине уљне фазе представљају веома важне факторе који дефинишу њихову стабилност. Циљ овог рада био је испитивање могућности примене липофилног, полимерног емулгатора, Cithrol DPHS-а за стабилизацију емулзија В/У. Спроведена испитивања обухватила су дефинисање међуфазног понашања емулга-

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тора применом тензиометрије. Припремане су серије 20% (м/м) емулзија воде у различитим типовима уља. Испитиване су величина и расподела величина капи, вискозитет и седиментациона стабилност у току 30 дана емулзија припреманих у парафинском уљу, маслиновом уљу, уљу коштица грожђа и триглицеридима средње дужине ланца, стабилизованих са 1% Cithrol DPHS. Све испитиване емулзије, осим емулзије у парафинском уљу, су биле стабилне у току 30 дана. Резултати овог истраживања указују да Cithrol DPHS може бити веома добар емулгатор и стабилизатор В/О емулзија припреманих у различитим уљима.

Кључне речи: емулзија вода-у-уљу, полимерни емулгатор, анализа величине капи, стабилност емулзија

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OPTIMIZATION OF THE FLUX VALUES IN MULTICHANNEL CERAMIC MEMBRANE MICROFILTRATION OF BAKER`S YEAST SUSPENSION

Nemanja R. Milović, Aleksandar I. Jokić, Nataša Lj. Lukić, Jovana A. Grahovac, Jelena M. Dodić and Arpad I. Kiralj*

University of Novi Sad, Faculty of Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia

The objective of this work was to estimate the effects of the operating parameters on the baker's yeast microfiltration through multichannel ceramic membrane. The selected parameters were transmembrane pressure, suspension feed flow, and initial suspension concentration. In order to investigate the influence and interaction effects of these parameters on the microfiltration operation, two responses have been chosen: average permeate flux and flux decline. The Box-Behnken experimental design and response surface methodology was used for result processing and process optimization. According to the obtained results, the most important parameter influencing permeate flux during microfiltration is the initial suspension concentration. The maximum average flux value was achieved at an initial concentration of 0.1 g/L, pressure around 1.25 bars and a flow rate at 16 L/h.

KEY WORDS: microfiltration, baker's yeast, average flux, flux decline

INTRODUCTION

Microfiltration is a pressure-driven membrane process for the separation of fine particles, microorganisms, and emulsion droplets. The microfiltration membranes have a microporous structure which separates fine particles with a size in the range of 50–10^3 nm, or retain the particles from solvent or other components with a molecular weight of 100-1000 kDa (1, 2). Therefore, microfiltration can be placed between ultrafiltration and coarse filtration. During cross-flow microfiltration process the permeate flux decreases with time as the retained particles are accumulated on membrane surface and within porous structure, where they create additional resistance to permeate flow.

The biotech and pharmaceutical industries have a significant potential in the application of microfiltration (3). A typical industrial application of microfiltration is the separation of cells, separation and purification of enzymes, vaccines, and antibiotics for plasma and albumin treatment, and also in the removal of pyrogens, etc. (4). In addition, it is used in the separation process that needs to satisfy specific requirements, such as the absolute separation of seedlings**,** a continuous filtration without local contamination and

* Corresponding author: Nemanja R. Milović, University of Novi Sad, Faculty of Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia, e-mail: milovic@tf.uns.ac.rs

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separation without exposure to heat (5). Microporous membranes are the part of a new generation of bioreactors, where they serve as carriers of enzymes and microorganisms. They are used for aeration of fermentation broth without creating bubbles (bubble-free gassing), and for the filtration of small quantities of samples for measuring and control devices (4).

The interest in ceramic membranes has increased concurrently with new processes and new applications. Ceramic membranes have been widely used in the environmental, petrochemical and food industries because of their higher chemical, thermal and mechanical stability compared to organic membranes. Due to microfiltration, which is based on the mechanism of the sieve effect, the microstructure parameters of ceramic membranes have a great influence on the permeate flux and rejection. Ceramic membranes were initially used in the wastewater technology, but successful solutions and possible applications cover all industries where certain media are to be filtered (6).

Microfiltration of baker's yeast (*Saccharomyces cerevisiae*) suspension has been the topic of numerous studies. These studies were concerned with the individual impact of process parameters on the process of microfiltration, but with no quantification of these impacts, as well as their mutual interactions during microfiltration using multichannel ceramic membranes (6).

The aim of this work was to examine the influence of transmembrane pressure, initial concentration of the suspension and feed flow on the microfiltration of the baker's yeast suspension using multichannel ceramic membrane in a batch mode. To examine the mutual interaction of these process parameters, response surface methodology was used, which allows finding the appropriate polynomial model which can be used to optimize process in the observed range of process parameters.

EXPERIMENTAL

Experimental material

Baker's yeast was used to make the suspensions for the experiments. Prior to each experiment, the suspension was prepared by adding a given weight of dry yeast to a saline solution (0.9 g/l NaCl in distilled water). Yeast cells were almost spherical in shape with an average diameter of 5 μm. They were selected according to their well-defined granulometric properties.

The multichannel (19 channels) α-Al_2O_3 membrane (Pall, USA) with the pore diameter 450 nm was used in the experiments, and its characteristics are given in Figure 1. The experiments were carried out in a conventional cross-flow microfiltration unit (Figure 2).

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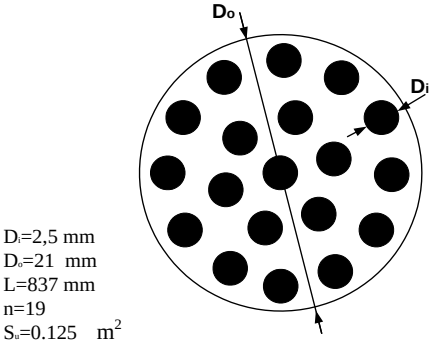

Figure 1. Characteristics of the membrane

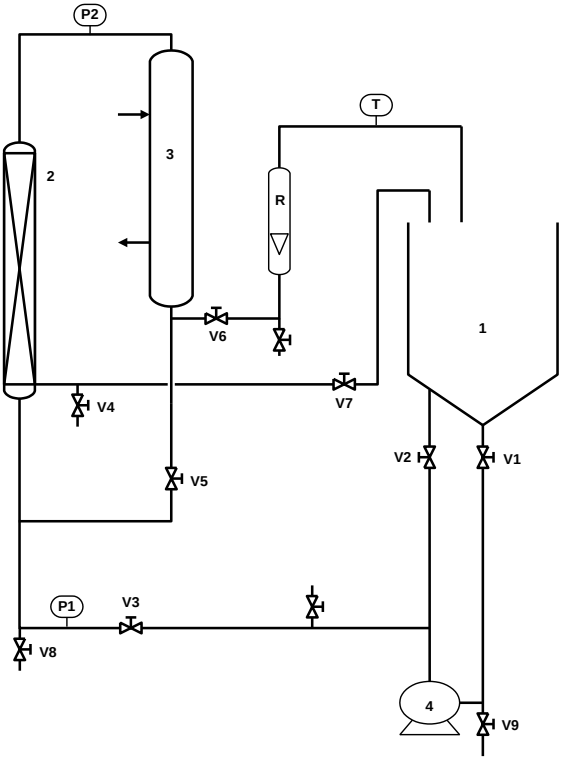

Figure 2. Schematic of the experimental setup for microfiltration; 1 – reservoir, 2 – membrane module, 3 – heat exchanger, 4 – centrifugal pump, P1 i P2 – manometers, T – digital thermometer, R – rotameter and V1…V9 – valves

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The feed was circulated by a centrifugal pump. During experiments, the retentate was recycled back to the suspension reservoir. The transmembrane pressure was adjusted by the regulation valve. After preparation, the suspension (22 l) was poured into the reservoir and stirred for 10 min. During mixing, the valves V1 and V2 were opened, and the valve V3 was closed, to prevent contact with the membrane. After opening the valve V3, the suspension was moving through the membrane in the membrane module. During the first minute, the values of the transmembrane pressure and feed flow were adjusted by the valves V2, V3 and V6.

At certain intervals, the permeate sample was collected at the valve V4. The permeate mass was measured by the technical digital scale connected to a personal computer. Microfiltration experiments were carried out at room temperature (25^{o}C), until a desired volume concentration factor was reached (VCF = 3). All measurements were carried out in triplicate, and the results were averaged. The membrane was cleaned before each experiment with 2% solution of NaOH and 1.5% solution of NaClO. The effectiveness in membrane cleaning was assessed by examining the water flux recovery. The cleaning procedure was repeated until the 95% of original water flux was restored.

Experimental design and response surface modeling

In order to investigate the main and interaction effects of the hydrodynamic factors upon the performance cross-flow microfiltration process, two responses have been derived from the experimental curves of permeate flux decline. The first response was the average permeate flux J_A that have been calculated by integration of the *J(t)* regression function from t_1 = 1 min up to t_n (corresponding VCF = 3), as follows (7):

$$J_A = \frac{1}{t_n} \int_{t_1}^{t_n} J(t)dt \qquad [1]$$

where *J(t)* is the regression functions of permeate flux given by regression analysis (data not shown).

The second response was chosen to quantify the intensity of fouling phenomena in dynamic conditions (11). In this respect, flux decline was considered as the most suitable response. The flux decline (FD) index can be defined as the percentage of the permeate flux decrease after the microfiltration operation (8):

$$FD = \frac{J_i - J_f}{J_i} \qquad [2]$$

where J_i and J_f are the permeate fluxes at time 1min and at the end of the process (VCF=3), respectively.

The Box-Behnken design with three factors at three levels and three replications in central point was used as the experimental design. It had 15 experiments, and the experimental results were fitted with the second order polynomial model (9, 10):

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$$Y = b_0 + \sum b_i X_i + \sum b_{ii} X_i^2 + \sum b_{ij} X_i X_j \quad [3]$$

where Y - response function, X_i - experiment parameter, b_0 - intercept, b_i - linear coefficient, b_{ii} - squared coefficient, and b_{ij} - interaction coefficient.

The factors and their levels are: transmembrane pressure (index 1), TMP, (0.5; 1 and 1.5 bar), suspension feed flow (index 2), Q, (4, 10 and 16 L/min), and the initial suspension concentration (index 3), C, (0.1; 5 and 10 g/L). The software package Statistica 12.6 was used for the results processing. Optimization of the parameters was carried out by desired function method, in the software package Design-Expert 7.1.5.

RESULTS AND DISCUSSION

The average flux during batch microfiltration can be used to optimize the hydrodynamic conditions performance of the process (11, 12). The fitting of the experimental results of the average permeate flux with the second order polynomial are shown in Table 1 and Table 2. The second order polynomial model approximates well the experimental results (R^2 = 0.965). The relatively high values of R^2 obtained for all responses indicate good fit of the experimental data to the equation. The most important linear factor influencing average permeate flux is the transmembrane pressure. The greatest impact among quadratic coefficients has the initial concentration. Judging from the interaction coefficients of highest importance is the interaction between the transmembrane pressure and initial concentration, while the remaining two interactions are much less significant.

Table 1. Results of fitting the experimental values of the average permeate flux and flux decline

Responses	**J_{av} (L/m^{2}h)**			**FD**		
Effects	Coefficient	t-value	p-value	Coefficient	t-value	p-value
Intercept						
b_0	-271.357	-1.431	0.963	0.439	1.725	0.423
Linear						
b_1	361.714	1.342	0.086	-0.361	-0.996	0.547
b_2	51.551	2.549	0.782	0.026	0.956	0.544
b_3	2.653	0.132	0.063	0.103	3.8	0.013
Quadratic						
b_{11}	2.454	0.254	0.235	-0.003	-0.193	0.6
b_{22}	-37.672	-3.221	0.897	0.012	0.751	0.585
b_{33}	-3.266	-3.351	0.042	-0.0004	-0.315	0.016
Interaction						
b_{12}	-39.994	-0.332	0.629	0.186	1.147	0.578
b_{13}	-1.339	-1.6	0.069	-0.002	-1.444	0.246
b_{23}	2.861	2.327	0.652	-0.006	-3.51	0.578

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The flux decline index is defined as the difference between the initial and the flux at the indicated value of the concentration factor (VCF = 3). The polynomial fitting results are shown in Table 1. The flux decline index results are also fitted with the second order polynomial (R^2 = 0.958). The most important linear factor influencing the flux decline is the initial suspension concentration. Among the quadratic coefficients, the greatest impact also has the initial concentration. The interaction between the transmembrane pressure and initial concentration is the most significant, while the other two interactions are less important.

Table 2. Analysis of variance (ANOVA) for the response surface model

Response	Residual			Model					
	DF	SS	MS	DF	SS	MS	*F-value*	*p-value*	R^2
J_{AV}	5	16758	3351.7	10	1021961	102196.1	30.491	0.000741	0.965
FD	5	0.03	0.0061	10	7.282	0.7282	120.126	0.000026	0.958

DF – Degrees of Freedom; SS – Sum of Squares; MS – Mean of Squares

The effects of the three selected operating parameters on the average permeate flow are presented in Figure 3. Only the interactions beetween the initial suspension concentration with the other two factors was selected, as presented by t-values (Table 1). Evidently, the most important factor influencing the average permeate flux is the initial suspension concentration and the figure shows that the highest average flux values were obtained at the lowest initial concentration (0.1 g/L). This can be explained by the membrane fouling. The feed concentration increase leads to the filtration cake formation and flux reduction, i.e. the higher the concentration, the lower the permeate flux. A completely different behaviour was observed at higher values of the initial suspension concentration. As it can be observed, the increase in the initial suspension concentration changes the way transmembrane pressure or feed flow affect the microfiltration process, in this case the average flux is reduced in contrast with low initial concentrations.

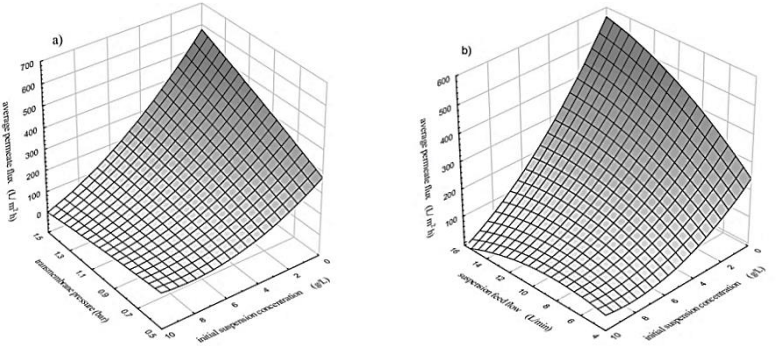

Figure 3. Dependence of the average permeate flux on: a) transmembrane pressure and initial suspension concentration, b) feed flow rate and initial suspension concentration

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Thus, the increase in the transmembrane presssure and flow rate, at the lowest initial concentration, increase significantly the average permeate flux. With increasing the initial concentration, the average flux decreases, eventually reaching a minimum value for the higher initial suspension concentration. Therefore, the maximum average permeate flow can be achieved at an initial concentration of 0.1 g/L, a pressure around 1.25 bars, and a flow rate of 16 L/h.

Figure 4 shows the response surface plots for flux decline index in dependence of the three operating parameters. As it can be noticed, the most important effect can be attributed to the initial suspension concentration. According to the response surface plots, the increment of the initial concentration leads to a more significant flux decline, especially for higher pressures and lower flow rates. The explanation for this is the same as in the previous section. The graphical response surface analysis (Figure 4) indicates that the increase in both the transmembrane pressure and flow rate did not significantly influence the flux decline, at least for the investigated region. Therefore, the lowest flux decline index can be achieved at lower values of the initial suspension concentration and transmembrane pressure, and at higher values of feed flow.

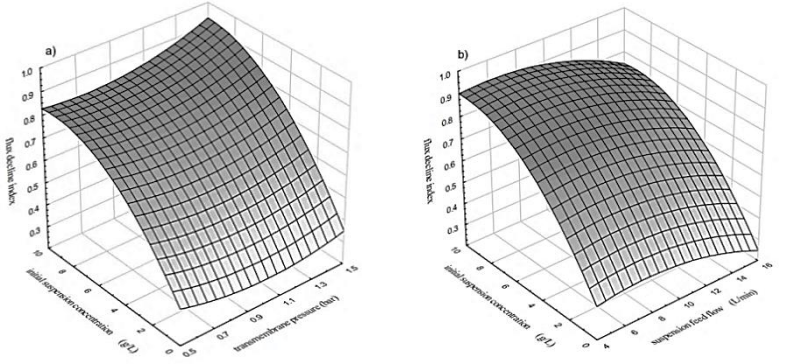

Figure 4. Dependence of the flux decline on: a) transmembrane pressure and initial suspension concentration, b) feed flow rate and initial suspension concentration

With the increase in the transmembrane pressure, the flux decline index increased for all the initial concentrations investigated. This behaviour can be attributed to the more intense cake formation as well as pore clogging due to the increase in driving force for the microfiltration (6). On the other hand, the increase in the feed flow reduces the values of flux decline index, as the higher values of feed flow can contribute to cleaning of the membrane surface. Higher values of feed flow are cause of more turbulent flow conditions in the membrane channels, which hinders the particles deposition on the membrane surface. In that way, cake formation is less pronounced, so the average flux is higher and, consequently, the flux decline index is lower. The time needed to reach the defined VCF value (VCF = 3) for the lowest flux decline value was 12 min, and for the highest flux decline value it was around 80 min.

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The ultimate goal of the response surface methodology is process optimization, so that proven models can be used for simulation and optimization. Several procedures can be applied for optimization of a process with two or more responses, but one of the most widespread is desirability function method (14).

In this paper, selected responses for the optimization of baker's yeast suspension multichannel ceramic membrane microfiltration, in terms of feed suspension concentration, were average flux and flux decline. The optimization objective was to select the initial suspension concentration, suspension feed flow and transmembrane pressure for which the responses will be maximal (average permeate flux) or minimal (flux decline index), or their individual desired functions have the highest possible value, ideally 1. Table 3 shows a survey of the optimal values of the varying parameters and corresponding optimized responses. As might be expected, the lowest total desired function value have been obtained for the highest initial suspension concentration (10 g/l), while the highest values have been obtained at the lowest initial suspension concentration (0.1 g/l). The optimal conditions for performing the baker's yeast suspension microfiltration indicate that the process of microfiltration by using multichannel ceramic membrane is highly dependent on the initial suspension concentration. It should be noted that the process must be conducted at the upper limit of the experimental values for suspension feed flow, which is about 16 L/h, in all experimental cases. On the other hand, the values of transmembrane pressure depend on the values of initial suspension concentration. For low initial concentration of feed suspension, the average flux has the highest values (around 630 L/m^2h), and at the same time, the flux decline index has the lowest value, around 0.25. This can be explained by the fact that even though during batch mode microfiltration the concentration of particulates increases, cake formation and consequent flux decrease are not high as in the case of higher initial suspension concentrations. Thus, for an approximately same transmembrane pressure and a feed flow around 1.25 bar and 16 L/h respectively, at an initial concentration of 0.1 g/L the obtained average flux value was 636 L/m^2h, and the flux decline 0.25, while at the initial suspension concentration of 5 g/L, the average flux falls to 240 L/m^2h, and the flux decline is 0.65.

Table 3. Optimization results

Initial suspension concentration (g/L)	Transmembrane pressure (bar)	Suspension feed flow (L/h)	J_{AV} (L/m^2h)	Flux decline index	Desirability function
0.1	1.25	15.58	635.98	0.25	1.00
5.05	1.31	15.89	240.55	0.65	0.39
10	0.56	15.75	85.00	0.84	0.12

CONCLUSION

On the basis of the experimental results it can be conducted that the most important effect on the baker's yeast microfiltration using multichannel ceramic membrane has the

initial suspension concentration. The maximum values of permeate flux were obtained at the lowest initial concentration. The higher the concentration, the lower the permeate flux. The transmembrane pressure and flow rate have no significant influence on the flux change at higher initial concentrations, while increase of these two parameters at low initial concentration caused an increase in the permeate flux. The optimal conditions were established by means of response surface methodology for two selected responses, i.e. average permeate flux (J_A) and flux decline index (FD). These conditions can be used to estimate microfiltration parameters in respect to the initial suspension concentration. For lower initial concentrations of baker's yeast suspensions, optimal microfiltration conditions include higher transmembrane pressures as well as suspension feed flow. On the other hand, with higher initial suspension concentrations, the process should be done at high feed flows and at a lower transmembrane pressure. The model obtained for the microfiltration by multichannel ceramic membrane can be used for determining process parameters depending on the initial suspension concentration.

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ОПТИМИЗАЦИЈА МИКРОФИЛТРАЦИЈЕ СУСПЕНЗИЈЕ ПЕКАРСКОГ КВАСЦА КРОЗ МУЛТИКАНАЛНУ КЕРАМИЧКУ МЕМБРАНУ

Немања Р. Миловић, Александар И. Јокић, Наташа Љ. Лукић, Јована А. Граховац, Јелена М. Додић и Арпад И. Кираљ

Универзитет у Новом Саду, Технолошки факултет, Булевар цара Лазара 1, 21000 Нови Сад, Србија

Циљ овог рада је оптимизација процеса микрофилтрације суспензије пекарског квасца кроз мултиканалну керамичку мембрану. Одабрани процесни параметри су трансмембрански притисак, почетна концентрација суспензије и проток суспензије. Посматрани одзиви за оптимизацију процеса су средњи флукс пермеата и пад флукса. За обраду добијених експерименталних резултата и оптимизацију коришћен је метод одзивних површина. Добијени резултати указују на то да је најзначајнији параметар за процес микрофилтрације почетна концентрација суспезије, и да је при ниским концентрацијама флукс пермета већи, док трансмембрански притисак и проток суспензије немају значајнији утицај при вишим концентрацијама, док при нижим концентрацијама њихово повећавање условљава повећање флукса пермеата. Оптимални услови за извођење микрофилтрације су: почетна концентрација суспензије од 0.1 g/L, протоку суспензије од 16 L/h и трансмембранском притиску од 1.25 бара.

Кључне речи: микрофилтрација, пекарски квасац, средњи флукс, пад флукса

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Original scientific paper

HOMOLOGY MODELLING AND DOCKING ANALYSIS OF L-LACTATE DEHYDROGENASE FROM *STREPTOCOCCUS THERMOPILUS*

Vladimir R. Vukić, Spasenija D. Milanović, Mirela D. Iličić, Katarina G. Kanurić and Dajana V. Vukić*

University of Novi Sad, Faculty of Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia

The aim of this research was to create a three-dimensional model of L-lactate dehydrogenase from the main yoghurt starter culture – Streptococcus thermopilus, to analyse its structural features and investigate substrate binding in the active site. NCBI BlastP was used against the Protein Data Bank database in order to identify the template for construction of homology models. Multiple sequence alignment was performed using the program MUSCULE within the UGENE 1.11.3 program. Homology models were constructed using the program Modeller v. 9.17. The obtained 3D model was verified by Ramachandran plots. Molecular docking simulations were performed using the program Surflex-Dock. The highest sequence similarity was observed with L-lactate dehydrogenase from Lactobacillus casei subsp. casei, with 69% identity. Therefore, its structure (PDB ID: 2ZQY:A) was selected as a modelling template for homology modelling. Active residues are by sequence similarity predicted: S. thermophilus – HIS181 and S. aureus – HIS179. Binding energy of pyruvate to L-lactate dehydrogenase of S. thermopilus was -7.874 kcal/mol. Pyruvate in L-lactate dehydrogenase of S. thermopilus makes H bonds with catalytic HIS181 (1.9 Å), as well as with THR235 (3.6 Å). Although our results indicate similar position of substrates between L-lactate dehydrogenase of S. thermopilus and S. aureus, differences in substrate distances and binding energy values could influence the reaction rate. Based on these results, the L-lactate dehydrogenase model proposed here could be used as a guide for further research, such as transition states of the reaction through molecular dynamics.

KEY WORDS: L-lactate dehydrogenase, *Streptococcus thermopilus*, homology modelling, molecular docking

INTRODUCTION

L-lactate dehydrogenase (EC: 1.1.1.27) belongs to the family of 2-hydroxy acid oxidoreductases and catalyses the conversion of pyruvic acid to lactate and back, as it converts NADH to NAD^+ and back. Anaerobe microorganisms use lactate dehydrogenase to oxidise NADH to NAD, which is an essential reaction in fermentation processes.

* Corresponding author: Vladimir Vukić, University of Novi Sad, Faculty of Technology, Bulevar Cara Lazara 1, Novi Sad, Serbia, vukicv@tf.uns.ac.rs

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Structural analysis of lactate dehydrogenase from microorganisms include several crystal structures: *Staphylococcus aureus* (unpublished work), *Bacillus stearothermophilus* (1), *Bifidobacterium longum,* (2), *Lactobacillus casei* (3), *Plasmodium falciparum* (4), etc. The diversity of amino acid sequences between each of these proteins is relatively low. Also, high level of structural similarity is preserved. Regarding milk fermentation, there are crystal structures of lactate dehydrogenases from some strains used as starter cultures - *Lactobacillus casei* subsp. *casei* (3) and *Bifidobacterium longum subsp. longum (2)*. However, crystal structure of the L-lactate dehydrogenase from yoghurt starter culture *Streptococcus thermopilus* is not known. Molecular modelling methods have been successfully used in research of enzyme structure, reaction mechanisms, and engineering (5). Modelling methods include prediction of protein stability, enzyme specificity and selectivity, mechanism, solubility, and interaction with interfaces. Molecular modelling methods were used to investigate the relationship between the structure of enzymes and their biochemical properties (6). Numerous approaches have been tested to stabilise proteins and improve their activity by using modelling strategies such as optimisation of electrostatic interactions (7) improvement of side chain packing (8), or balancing the ratio between hydrophilic and hydrophobic residues (9). The molecular modelling methods have been successfully applied to engineering a lipase into an aldolase (10) or into enzymes that catalyse Baeyer–Villiger oxidation with hydrogen peroxide (11). Therefore, the aim of this research was to create a three-dimensional model of L-lactate dehydrogenase from the main yoghurt starter culture - *Streptococcus thermopilus* (strain CNRZ 1066), to analyse its structural features and investigate substrate binding in the active sites.

MATERIALS AND METHODS

NCBI BlastP [12] was used against the Protein Data Bank (PDB) database [13] in order to identify template L-lactate dehydrogenase structures with the highest sequence similarity to the L-lactate dehydrogenase sequences from *Streptococcus thermophilus*. Because the present study focuses on the initial steps in substrate binding to L-lactate dehydrogenase, we selected template crystal structure without substrates in the active site. Three-dimensional coordinates of L-lactate dehydrogenase used as template structure was retrieved from PDB data base (PDB ID: 2ZQY:A). The molecular structure of lactate and NADH were extracted from crystal structure of L-lactate dehydrogenase from *Staphylococcus aureus* subsp. *aureus* (PDB ID: 3H3J:A). Energy minimization was performed using a Trypos force field. Protein sequences for alignment and construction of homology model were retrieved from the National Center for Biotechnology Information (NCBI) (*Lactobacillus casei* subsp. *casei* - GenBank: BAA02133.1; *Streptococcus thermophilus* - GenBank: AAV62824.1; *Staphylococcus aureus* subsp. *aureus* - GenBank: AAW38779.1). Multiple sequence alignment was performed using the program MUSCULE within the UGENE 1.11.3 program (14, 15). Highly conserved regions and potential active site residue were identified by sequence alignment.

Homology models were constructed using the program Modeller v. 9.17 (16). The best model was selected by considering the smallest value of the normalised discrete optimized molecule energy (DOPE) (17). The obtained 3D model was verified by Rama-

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chandran plots and using the Structural Analysis and Verification Server (18). The modelled protein structure was subjected to energy minimization using an AMBER7 force field and AMBER-derived atomic charges. The Powell method, distance dependent dielectric constant and convergence gradient method with a convergence criterion of 0.005 kcal/mol were applied. The modelled protein structure was prepared for molecular docking simulation using the following parameters: polar hydrogen atoms were added, protonation types and termini treatment was enabled. Docking simulations were performed using the program Surflex-Dock with flexible H atoms (19). The docking results were visualised using the program PyMol (20).

RESULTS AND DISCUSSION

BlastP analysis of L-lactate dehydrogenase from *S. thermophilus* revealed a high degree of sequence similarity with several X-ray crystal structures retrieved from the PDB. The most important PDB structures are presented in Table 1. The associated E-values confirm a correlation between query and reference sequences. The highest sequence similarity was observed with L-lactate dehydrogenase from *Lactobacillus casei* subsp. *casei*, with 69% identity. Therefore, its structure (PDB ID: 2ZQY:A) was selected as a modelling template for homology modelling. The structure of L-lactate dehydrogenase from *Staphylococcus aureus* subsp. *aureus* was experimentally determined with both substrates (NADH and pyruvate) in the active site (PDB ID: 3H3J:A). Therefore, it reveals the early steps of substrate recognition. It has high sequence similarity with the template sequence (58%), which makes it appropriate for evaluations of the docking results.

Table 1. BlastP results of query L-lactate dehydrogenase sequence against the PDB

Query sequence	PDB sequence	Query cover	E value	Identity
S. thermophilus	2ZQY:A (*Lactobacillus casei* subsp. *casei*)	95%	$3e^{-159}$	69%
	3H3J:A (*Staphylococcus aureus* subsp. *aureus*)	93%	$1e^{-119}$	58%

* E value - probability of the alignment occurring by chance

Active residue of L-lactate dehydrogenase of *L. casei* has been determined as HIS181 (21). The sequence alignment revealed highly conserved HIS residue which acts as an active residue (Fig. 1). The active residues are by sequence similarity predicted: *S. thermopilus* – HIS181 and *S. aureus* – HIS179. Furthermore, there are highly conservative regions around the active residue, which is crucial for highly accurate homology modelling of the enzyme.

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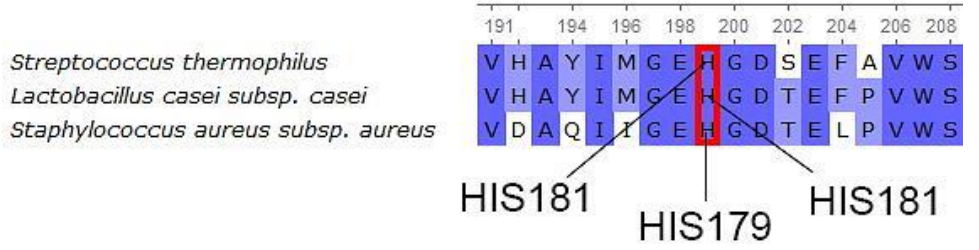

Figure 1. Sequence alignment of active site residues from the selected L-lactate dehydrogenases

Homology models of L-lactate dehydrogenase from *S. thermophilus* was constructed using Modeller program and the best model was chosen using the DOPE value (results not shown). Ramachandran plots were constructed for the modelled enzyme (Fig. 2). Based on the Ramachandran plot analysis, most residues of the modelled enzyme are in the favoured region, and only a few residues are in unfavourable conformations (Fig. 2a). A detailed analysis of residues in the active site revealed that all residues involved in substrate bindings are in the favoured region (Fig. 2b). These results revealed that the enzyme residues have favourable bond angles, which is extremely important for molecular docking simulations, and suggests that the model is suitable for analysing of substrates binding.

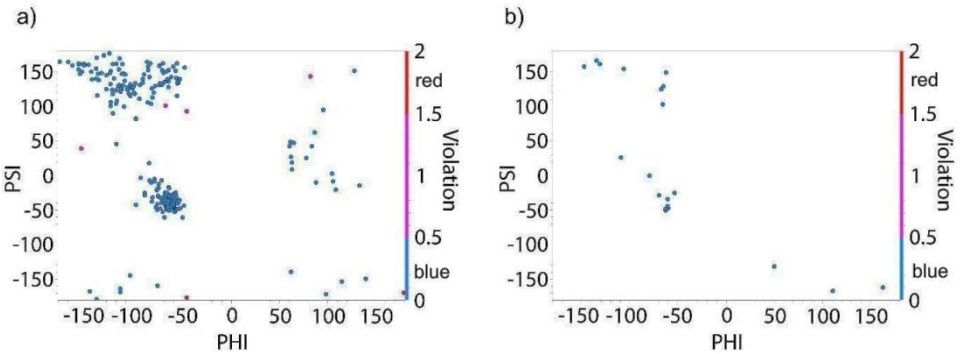

Figure 2. Ramachandran plot validation of the *S. thermophilus* L-lactate dehydrogenase homology model. Plot of phi/psi angles (-150→150) vs. violation (gradient, blue→red) a) homology model of *S. thermophilus* L-lactate dehydrogenase; b) Active site residues of *S. thermophilus* L-lactate dehydrogenase

The modelled enzyme was further validated by structural superposition with its corresponding template sequence (Fig. 3). The model showed a high degree of three-dimensional similarity with its template structure, with the Cα Root Mean Square Deviation (RMSD) of 0.78Å.

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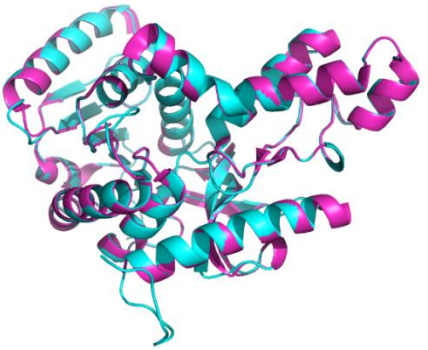

Figure 3. Superposition of L-lactate dehydrogenase homology model (cyan) with its corresponding template structure (magenta)

Using our molecular docking protocol, pyruvate was successfully re-docked with the crystal structure of *Staphylococcus aureus* subsp. *aureus* L-lactate dehydrogenase (RMSD of 0.249 Å), suggesting that the methods used in the present study are appropriate. Molecular docking simulation revealed position of substrates in the active site of L-lactate dehydrogenase (Fig. 4a). The binding energy of pyruvate to L-lactate dehydrogenase of *S. thermopilus* was -7.874 kcal/mol, while binding energy to L-lactate dehydrogenase of *S. aureus* was -7.325 kcal/mol. These results indicate stronger binding of pyruvate to the modelled enzyme than to *S. aureus*. The pyruvate in L-lactate dehydrogenase of *S. thermopilus* forms H bonds with catalytic HIS181 (1.9 Å), as well as with THR235 (3.6 Å). Threonine residue is also involved in pyruvate binding of some other L-lactate dehydrogenases with high sequence similarity with the enzyme of *S. thermopilus*, including L-lactate dehydrogenase from *S. aureus* subsp. *aureus* (Fig. 4b). A comparison of our molecular docking simulation results with the crystal structure of L-lactate dehydrogenase from *S. aureus* subsp. *aureus* revealed similar substrate positions. Nevertheless, the pyruvate in the enzyme from *S. thermopilus* is closer to the catalytic residue HIS181 than to the substrate binding residue THR235, while NADH is at a similar distance from the catalytic residue. Although our results indicate similar position of the substrates, the differences in substrate distances and binding energy values could influence the reaction rate. Although predicted docking energies are not reliable for modelling enzyme efficiency, they do correlate with protein-ligand complex stability and overall reaction rates. Based on these results, the L-lactate dehydrogenase model proposed here could be used as a guide for further research. Detailed reaction mechanism could be investigated through molecular dynamics simulations. Quantum mechanical calculations could reveal reactions highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) which could identify transition states of the reaction. These results could lead to rational design and desired modifications of the enzyme that could be used for production of lactic acid.

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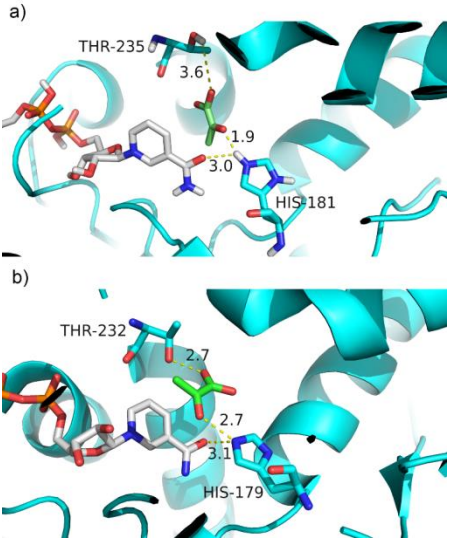

Figure 4. Substrate binding (pyruvate and NADH) to L-lactate dehydrogenase: a) Molecular docking simulation of *S. thermophilus* (homology model); and b) crystal structure of *Staphylococcus aureus* subsp. *aureus* (PDB ID: 3H3J:A)

CONCLUSION

The BlastP analysis of L-lactate dehydrogenase from *S. thermophilus* revealed the highest degree of sequence similarity with X-ray crystal structure of L-lactate dehydrogenase from *Lactobacillus casei* subsp. *casei* with 69% identity. Active residues are by sequence similarity predicted: *S. thermopilus* – HIS181 and *S. aureus* – HIS179. Homology models of L-lactate dehydrogenase from *S. thermophilus* was constructed and validated. The modelled structure showed a high degree of three-dimensional similarity with its template structure, with the Cα RMSD of 0.78Å. Molecular docking simulation revealed that pyruvate forms H bonds with catalytic HIS181 (1.9 Å), as well as with THR235 (3.6 Å). Although our results indicate similar position of substrates of L-lactate dehydrogenase from *S. thermopilus* and *S. aureus* subsp. *aureus*, the differences in substrate distances could influence the reaction rate. Based on these results, the L-lactate dehydrogenase model proposed here could be used for molecular dynamics simulations and quantum mechanical calculations that could lead to rational design of the enzyme and improvement of lactic acid production.

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ХОМОЛОГО МОДЕЛОВАЊЕ И ДОКИНГ АНАЛИЗА Л-ЛАКТАТ ДЕХИДРОГЕНАЗЕ *STREPTOCOCCUS THERMOPILUS*-А

Владимир Р. Вукић, Спасенија Д. Милановић, Мирела Д. Иличић, Катарина Г. Канурић и Дајана В. Вукић

Универзитет у Новом Саду, Технолошки факултет, Булевар Цара Лазара 1, 21000 Нови Сад, Србија

Циљ овог истраживања био је конструкција тродимензионалног модела L-лактат дехидрогеназе јогуртне културе *Streptococcus thermopilus*, као и анализа структуралних особина и везивање субстрата у активном месту. NCBI BlastP претрага PDB базе података је коришћена за проналажење шаблон структуре за конструкцију хомологих модела. Вишеструко поравнање секвенци је урађено помоћу програма MUSCULE у оквиру UGENE 1.11.3 програма. Хомологи модели су конструисани помоћу програма Modeller v. 9.17. Добијени модели су верификовани кроз Рамачандранове плотове. Симулације молекуларног докинга је урађена применом Surflex-Dock програма. Највећа сличност секвенци је пронађена са L-лактат дехидрогеназом из *Lactobacillus casei* подврста *цasei*, са сличношћу од 69%, услед чега је одабрана као шаблон за конструкцију хомологих модела (PDB ID: 2ZQY:A). Активне резидуе се предвиђене путем сличности секвенци: *S. thermopilus* – хистидин 181 и *S. aureus* – хистидин 179. Енергија везивања пирувата за активно место L-лактат дехидрогеназе *S. thermopilus*-а износи -7,874 kcal/mol. Пируват гради водоничне везе са каталитичком резидуом хистидина (1,9 Å), као и са треонином 235 (3,6 Å). Иако наши резултати указују на сличне позиције супстрата - лактат дехидрогеназа *S. Thermopilus*-а и *S. aureus*-а, разлике у удаљеностима супстрата од резидуа, ако и енергија везивања, указују на могуће разлике у брзини реакције. Резултати добијени у овом раду могу бити коришћени у даљим истраживањима, као што је идентификација прелазних стања реакције применом молекуларне динамике.

Кључне речи: L-лактат дехидрогеназа**,** *Streptococcus thermopilus***,** хомолого моделовање, молекуларни докинг

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OPTIMIZATION OF ULTRASOUND-ASSISTED EXTRACTION OF POLYPHENOLIC COMPOUNDS FROM CORIANDER SEEDS USING RESPONSE SURFACE METHODOLOGY

Zoran P. Zeković, Saša D. Đurović and Branimir M. Pavlić*

University of Novi Sad, Faculty of Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia

Coriandrum sativum L. (coriander) seeds (CS) were used for preparation of extracts with high content of biologically active compounds. In order to optimize ultrasound-assisted extraction process, three levels and three variables of Box-Behnken experimental design (BBD) in combination with response surface methodology (RSM) were applied, yielding maximized total phenolics (TP) and flavonoids (TF) content and antioxidant activity (IC_{50} and EC_{50} values). Independent variables were temperature (40-80^oC), extraction time (40-80 min) and ultrasonic power (96-216 W). Experimental results were fitted to a second-order polynomial model with multiple regression, while the analysis of variance (ANOVA) was employed to assess the model fitness and determine optimal conditions for TP (79.60^oC, 49.20 min, 96.69 W), TF (79.40^oC, 43.60 min, 216.00 W), IC_{50} (80.00^oC, 60.40 min, 216.00 W) and EC_{50} (78.40^oC, 68.60 min, 214.80 W). On the basis of the obtained mathematical models, three-dimensional surface plots were generated. The predicted values for TP, TF, IC_{50} and EC_{50} were: 382.68 mg GAE/100 g CS, 216 mg CE/100 g CS, 0.03764 mg/mL and 0.1425 mg/mL, respectively.

KEY WORDS: coriander seeds, ultrasound-assisted extraction, optimization, response surface methodology

INTRODUCTION

Nowadays, aromatic and medicinal plants are attracting an increasing amount of attention due to their potential application in various industry fields and for health benefits. One of these plants is coriander (*Coriandrum sativum* L.), which belongs to the *Apiaceae* botanical family, and is widely cultivated and distributed in Mediterranean countries (1). Seeds contain up to 1% of essential oil with the main component of monoterpenoid linalool (>50%) (2), while limonene, camphor and geraniol are also present in significant quantity (3). Coriander seeds also contain vegetal oil with a high concentration of monounsaturated fatty acids, particularly petroselinic acid (4). Coriander has also been recognized as a medical agent which has been used against worms, rheumatism and pain in the joints (2). Several studies have demonstrated hypoglycemic action and effect

* Corresponding author: Saša Đurović, University of Novi Sad, Faculty of technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia, e-mail: sasatfns@uns.ac.rs

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on carbohydrate metabolism (2, 5). It has been reported that volatile components in essential oil from seeds and leaves of coriander have antimicrobial activity (1, 2, 6, 7), as well as anticancerous and antimutagenic (5, 8), antioxidant (2, 8-10) and antidiabetic (8, 11) activities.

Recently, reports on the application of ultrasound as a method for extraction of biological active compounds form plant material have been published. It has been showed that ultrasound-assisted extraction (UAE) technique can be especially suitable due to low equipment requirements and its high economic efficiency (12), offering at the same time high reproducibility, simplified manipulation, and reduced solvent and energy consumption (13). Beside these advantages, the usage of ultrasound for extraction diminishes the danger of thermal degradation of desired compounds and reduces significantly the time needed for the process itself (14). Cavitation, which occurs in the solvent due to the creation, growth and implosion of gas bubbles (15) and mechanical effect of ultrasound which provides a greater penetration of solvent into cellular material (12, 14), are the main benefits of using this method. Like many other processes, this process also possesses certain disadvantages. They are manifested in the form of the effect causing the changes in chemical composition and degradation of desired compounds, as well as in formation of free radical species inside of gas bubbles (16).

Optimization of any technological process is a very important task, aiming to gain maximum of the process with minimal losses at the same time. In the case of UAE there is a need for optimization of the process temperature, time and ultrasonic power. The most frequent technique employed for optimization of these parameters is the response surface methodology (RSM), which represent a collection of statistical and mathematical methods suitable to perform this important task (17). This technique is based on the influence of several different variables on the response of interest, aiming at the optimization of the described response (18).

The aim of this study was to evaluate the influence of extraction time, temperature and ultrasonic power on the extraction process of coriander seeds. After evaluation, UAE was optimized applying the RSM in order to obtain the liquid extracts with the highest phenolics content and antioxidant activity.

EXPERIMENTAL

Plant material

Coriander seeds were acquired from the Institute of Field and Vegetable Crops, Novi Sad, Republic of Serbia (year 2013). Plant material was air-dried and stored at room temperature. Dried seeds were milled in the blender, and mean particle size (0.6493 mm) was determined by CISA Cedaceria Industrial sieve set (Spain).

Chemicals

1,1-Diphenyl-2-picryl-hydrazyl-hydrate (DPPH), Folin-Ciocalteu reagent, gallic acid and (±)-catechin were purchased from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). All other chemicals and reagents were of analytical reagent grade.

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Ultrasound-assisted extraction procedure

In all experimental runs, 10.0 g of powdered coriander seeds were mixed with 100 mL of 63% ethanol (ratio 1:10) in 300 mL glass flasks. Ultrasound-assisted extraction was performed in sonication water bath (EUP540A, Euinstruments, France) at a fixed frequency of 40 kHz. Ultrasonic power, temperature and extraction time were controlled from the panel of the instrument. Flasks were always positioned at the same distance from the transducer, and no agitation was applied. After extraction, the extracts were immediately filtered through filter paper under vacuum. The extracts were collected into glass flasks and stored at 4^{o}C until the analysis. After filtration, 10 mL of liquid extract were used for the extraction yield determination by removing the solvent and drying to the constant mass, to measure mass of the dry residue. The solvent was also removed from the rest of the extract on a rotary vacuum evaporator (Devarot, Elektromedicina, Ljubljana, Slovenia), and dried at 110^{o}C to constant mass. The total extraction yield was expressed as gram of dry extract per 100 grams of coriander seeds (g/100 g CS).

Total phenolics content

The total phenolics (TP) content in the obtained coriander extracts was determined by Folin-Ciocalteu procedure (19, 20), using gallic acid as a standard. Absorbance was measured at 750 nm. Content of phenolic compounds was expressed as milligrams of gallic acid equivalent (GAE) per 100 grams of *C. sativum* seeds (mg GAE/100 g CS). All experiments were performed in three replicates, and the results were expressed as mean values.

Total flavonoids content

The total flavonoids (TF) content was determined using aluminum chloride colorimetric assay (21). The results were expressed as milligrams of catechin equivalents (CE) per 100 g of coriander seeds (mg CE/100 g CS). All experiments were performed in three replicates and the results were expressed as a mean value.

DPPH assay

The free radical scavenging activity of coriander liquid extracts was determined as described by Espín et al. (22). A certain volume of diluted liquid extract was mixed with 95% methanol and 90 μM solution of 2,2-diphenyl-1-picryl-hydrazyl (DPPH) in order to obtain different final concentrations of extract. The blank probe was prepared by using proper extraction solvent instead of sample. After 60 min of incubation at room temperature, the absorbance was measured at 515 nm and the result was expressed as radical scavenging capacity (%RSC), which was calculated by the following equation:

$$\%RSC=100-\frac{(A_{sample}\times 100)}{A_{blank}} \quad [1]$$

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where: A_{sample} is the absorbance of sample solution and A_{blank} is the absorbance of the blank probe. Antioxidant activity was expressed as the inhibition concentration at RSC value 50% (IC_{50}), which represents the concentration of the test solution required to obtain 50% of radical scavenging capacity expressed as mg per mL (mg/mL). All experiments were performed in three replicates, and the results were expressed as a mean value.

Reducing power assay

Reducing power of the samples was determined according to the assay based on the reduction of Fe^{3+} by polyphenol antioxidants (23). Different dilutions of liquid extract (1 mL) were mixed with phosphate buffer (1 mL, 0.2 M, pH 6.6) and 1% potassium ferricyanide (1 mL) in glass tubes. The tubes were incubated on 50°C for 20 min. After incubation, 10% trichloroacetic acid solution (1 mL) was added to the reaction mixture. Then, the solution was centrifuged at 3000 rpm for 10 min, and the supernatant (2 mL) was further mixed with double distilled water (2 mL) and 0.1% $FeCl_3$ solution (0.4 mL). Absorbance was measured at 700 nm. The blank probe was prepared by using proper extraction solvent instead of sample. Reducing power was expressed as the EC_{50} value (concentration in mg/mL at the absorbance of 0.5), which caused the reduction of 50% Fe^{3+} ions in the reaction mixture. The EC_{50} value was determined from the generated curve which represented the relationship between the sample concentration and the absorbance. All experiments were performed in triplicate, and the results were expressed as a mean value.

Experimental design

The RSM was employed to evaluate the effects of extraction parameters and optimize conditions for various responses administering the Box-Behnken experimental design (BBD) with three numeric factors on three levels. The design consisted of seventeen randomized runs with five replicates at the central point. Independent variables used in the experimental design were the temperature (X_1, 40-80°C), extraction time (X_2, 40-80 min) and ultrasonic power (X_3, 96-216 W). The ranges of variables were determined according to the available literature data (15, 24). In order to normalize the parameters, each of the coded variables was forced to range from -1 to 1, so that they all affect the response more evenly, and so the units of the parameters are irrelevant (18). The variables were coded according to the following equation (25):

$$X=\frac{X_i-X_0}{\Delta X} \qquad [2]$$

where X is the coded value, X_i is the corresponding actual value, X_0 is the actual value in the center of the domain, and ΔX is the increment of x_i corresponding to the variation of 1 unit of X. The natural and coded values of independent variables used in BBD are presented in Table 1.

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Table 1. Natural and coded levels of independent variables used in the RSM design

Variable	Coded levels		
	-1	0	1
	Natural levels		
Temperature (^oC)	40	60	80
Extraction time (min)	40	60	80
Ultrasonic power (W)	96	156	216

The response variables were fitted to the following second-order polynomial model, which is generally able to describe the relationship between the responses and the independent variables (26):

$$Y=\beta_0+\sum_{i=1}^{3}\beta_i X_i+\sum_{i=1}^{3}\beta_{ii}X_i^2+\sum\sum_{i<j=1}^{3}\beta_{ij}X_iX_j \quad [3]$$

where Y represents the measured response; β_0 is a constant, b_j, b_{jj}, b_{ij} are the linear, quadratic and interactive coefficients of the model, respectively; X_i and X_j are the levels of the independent variables. Optimal extraction conditions were determined considering TP and TF contents, radical scavenging activity and reducing power as responses. The experimental design and multiple linear regression analysis were performed using Design-Expert v.7 Trial (Stat-Ease, Minneapolis, Minnesota, USA). The results were statistically tested by the analysis of variance (ANOVA) with the significance levels of 0.05. The adequacy of the model(s) was evaluated by the coefficient of multiple determination (R^2), coefficient of variance (CV) and *p*-values for the model and lack of fit testing.

RESULTS AND DISCUSSION

Model fitting

The influence of temperature (40-80^{o}C), extraction time (40-80 min), and ultrasonic power (96-216 W) on investigated responses (TP, TF, and antioxidant activity determined by DPPH and reducing power) was optimized using RSM. The results of the experimentally obtained responses using the Box-Behnken experimental design are presented in Table 2. The analysis of variance (ANOVA) was used for determining regression coefficients of the linear, quadratic and interaction terms for each response, and the results are presented in Table 3. The influence of each term was described as statistically significant ($p < 0.05$) and insignificant ($p > 0.05$). The coefficient of multiple determination (R^2) was used as first indicator of the model adequacy, together with ANOVA, and the calculated statistical parameters are presented in Table 4. The relatively high values of R^2 (> 0.90) for TP and TF, indicate that the second-order polynomial model represents a good approximation of the experimental results. On the other hand, R^2 for the antioxidant activity parameters (IC_{50} and EC_{50}) was slightly lower (0.829 and 0.834, respecti-

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vely). The ANOVA was used to provide detailed information about statistical signify-cance of the investigated models. The experimental results of all investigated responses showed good fitting with mathematical models, since the regression for the model was significant ($p < 0.05$), and the lack of fit testing was insignificant ($p > 0.05$) (Table 4). Therefore, the regression equations could be successfully used as predictors of these responses in the investigated experimental domain.

Table 2. Experimental conditions for the Box-Behnken design including natural and coded levels and experimentally obtained values of measured responses, including yield, TP, TF, IC_{50} and EC_{50} values

Independent variables			Measured responses				
X_1 Temperature (°C)	X_2 Time (min)	X_3 Ultrasonic power (W)	Y (g/100 g CS)	TP (mg GAE/ 100 g CS)	TF (mg CE/ 100 g CS)	IC_{50} (mg/mL)	EC_{50} (mg/mL)
0	0	0	8.80	287.76±15.89	150.40±0.00	0.04344±0.0056	0.1523±0.0064
0	-1	1	8.58	307.28±4.23	203.40±2.76	0.04516±0.0045	0.1582±0.0084
1	-1	0	11.06	350.82±28.03	203.90±23.33	0.05398±0.0030	0.1651±0.0084
0	0	0	8.37	282.67±1.56	160.40±4.31	0.04798±0.0030	0.1483±0.0076
0	1	1	9.14	296.61±10.09	162.904.03	0.05105±0.0098	0.1577±0.0095
-1	1	0	7.19	222.32±2.11	124.60±3.32	0.05392±0.0098	0.1634±0.0093
1	0	1	10.55	364.74±15.91	192.60±7.70	0.03569±0.0085	0.1448±0.0063
-1	-1	0	6.52	221.53±2.64	126.90±1.91	0.05094±0.0014	0.1545±0.0011
0	0	0	8.69	288.17±6.90	164.70±2.05	0.04990±0.0008	0.1566±0.0016
-1	0	1	8.21	260.05±11.14	145.70±0.99	0.04820±0.0021	0.1552±0.0050
0	0	0	8.85	265.60±24.32	155.40±7.71	0.04889±0.0019	0.1584±0.0089
0	-1	-1	9.68	326.05±2.64	190.50±2.76	0.05212±0.0021	0.1650±0.0089
1	0	-1	10.91	374.25±13.30	198.70±7.70	0.04862±0.0022	0.1474±0.0060
0	1	-1	9.29	310.64±12.20	165.01±1.77	0.05252±0.0022	0.1583±0.0075
-1	0	-1	7.34	240.85±4.20	138.90±1.27	0.04881±0.0000	0.1483±0.0024
1	1	0	11.18	372.10±3.72	199.50±5.80	0.04868±0.0001	0.1436±0.0020
0	0	0	8.59	287.46±1.04	149.30±1.77	0.04886±0.0001	0.1464±0.0060

Table 3. Estimated regression coefficients of the fitted second-order polynomial model for all investigated responses

Coefficient	Response			
	TP	TF	IC_{50}	EC_{50}
β_0	282.24	156.04	0.048	0.150
Linear				
β_1	64.64	32.32	$-1.863\cdot10^{-3}$	$-2.563\cdot10^{-3}$
β_2	-0.50	-9.09	$4.963\cdot10^{-4}$	$-2.475\cdot10^{-3}$
β_3	-2.89	1.44	$-2.746\cdot10^{-3}$	$-3.875\cdot10^{-4}$
Interaction				
β_{12}	5.12	-0.52	$-2.070\cdot10^{-3}$	$-7.600\cdot10^{-3}$
β_{13}	-7.18	-3.22	$-3.080\cdot10^{-3}$	$-2.375\cdot10^{-3}$
β_{23}	1.19	-3.75	$1.373\cdot10^{-3}$	$1.550\cdot10^{-3}$

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Table 3. Continuation

Coefficient	Response			
	TP	TF	IC_{50}	EC_{50}
Quadratic				
β_{11}	4.64	-1.90	$-4.083 \cdot 10^{-4}$	$-3.313 \cdot 10^{-3}$
β_{22}	4.81	9.58	$4.474 \cdot 10^{-3}$	$7.562 \cdot 10^{-3}$
β_{33}	23.09	14.83	$-2.076 \cdot 10^{-3}$	$-1.625 \cdot 10^{-4}$

Table 4. Analysis of the ANOVA of the fitted second-order polynomial models

Source	Sum of Squares	DF	Mean square	*F*-value	*p*-value
Total phenols content					
Model	36378.25	9	4042.03	25.94	0.0001
Residual	1090.77	7	155.82		
Lack of fit	725.40	3	241.80	2.65	0.1852
Pure error	365.37	4	91.34		
Total	37469.02	16			
$R^2 = 0.971$					
Total flavonoids content					
Model	10516.00	9	1168.44	9.30	0.0038
Residual	879.17	7	125.60		
Lack of fit	707.52	3	235.84	5.50	0.0666
Pure error	171.65	4	42.91		
Total	11395.17	16			
$R^2 = 0.923$					
IC_{50}					
Model	$2.516 \cdot 10^{-4}$	9	$2.795 \cdot 10^{-5}$	3.76	0.0474
Residual	$5.207 \cdot 10^{-5}$	7	$7.439 \cdot 10^{-6}$		
Lack of fit	$2.631 \cdot 10^{-5}$	3	$8.771 \cdot 10^{-6}$	1.36	0.3743
Pure error	$2.576 \cdot 10^{-5}$	4	$6.441 \cdot 10^{-6}$		
Total	$3.037 \cdot 10^{-4}$	16			
$R^2 = 0.829$					
EC_{50}					
Model	$6.426 \cdot 10^{-4}$	9	$7.140 \cdot 10^{-5}$	3.91	0.0429
Residual	$1.278 \cdot 10^{-4}$	7	$1.825 \cdot 10^{-5}$		
Lack of fit	$2.131 \cdot 10^{-5}$	3	$7.102 \cdot 10^{-6}$	0.27	0.8468
Pure error	$1.065 \cdot 10^{-4}$	4	$2.662 \cdot 10^{-5}$		
Total	$7.704 \cdot 10^{-4}$	16			
$R^2 = 0.834$					

Total extraction yield

The experimentally obtained values for the yield are presented in Table 2. They varied in the range of 6.52 to 11.18 g/100 g CS. The highest yield was obtained at 80°C, for

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80 min, and ultrasonic power of 156 W. On the other hand, the lowest yield was obtained under the following conditions: 40°C, 40 minutes, and ultrasonic power of 156 W. As the values were obtained under the same ultrasonic power but at the different values of temperature and time, this indicates the importance of those two independent variables on the yield.

TP content

The values obtained for the TP content are presented in Table 2, and they ranged from 221.53 to 374.25 mg GAE/100 g CS. The previously conducted UAE extraction of CS raffinate obtained after performed the SFE revealed the TP content in the range of 110.53-222.08 mg GAE/100 g of CS (in 70% ethanol as solvent) and 161.82-308.55 mg GAE/100 g CS (water as solvent) (27). These results were lower than those obtained in this study, but indicated the importance of the solvent selection. Comparing to the results obtained by Zeković et al. (28), where the maximal obtained value of TP content was 2629.70 mg GAE/100 g CS, these results present lower content, indicating that subcritical water extraction (SWE) is a more suitable method for extraction of phenolic compound than UAE. On the other hand, Gallo et al. (29) applied the UAE and microwave-assisted extraction (MAE) techniques and obtained 41.81 and 82.09 mg GAE/100 g CS, which presents a lower yield of these compounds comparing to those obtained in this study. The optimization of the MAE extraction process of CS gave the TP content in the range of 136.92-384.54 mg GAE/100g CS (30), which is similar to the results obtained in this study. This indicates the importance of extraction conditions and their significant influence ($p < 0.05$) on the yield of phenolic compounds.

The effects of UAE parameters on TP is presented in Figure 1a, while their significance was determined by RSM influence analysis expressed as regression coefficients from Eq. [3] (Table 3). According to ANOVA, only linear term of temperature and quadratic term of ultrasonic power exhibited significant influence ($p < 0.05$) on TP. The positive influence of the liner term of temperature was rather expected, since temperature affects mass transfer by increasing diffusion, causing degradation of the plant matrix and improving physical solvent properties in terms of penetration and solubility power (15). A negative influence of the linear term of ultrasonic and positive influence of its quadratic term indicate that TP decrease with the increase of ultrasonic power up to a certain value, then TP shows again a slight increase. The predicted second-order polynomial model for TP content is:

$$TP=282.24+64.64X_1-0.50X_2-2.89X_3+5.12X_1X_2-7.18X_1X_3+1.19X_2X_3+ \\ +4.64X_1^2+4.81X_2^2+23.09X_3^2 \quad [4]$$

TF content

The yields of TF are presented in Table 2. The previously performed UAE of CS raffinate obtained after SFE, showed the TF content in the range of 64.50-153.74 mg

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CE/100 g SC (70% ethanol as solvent) and 80.86-297.63 mg CE/100 g SC (water as solvent) (27). The results show that the TF content in the water UAE extract was higher than achieved in this study. Comparing the results for TF obtained in this study with the results obtained by Zeković et al. (28), where TF content in CS extracts varied from 231.15 to 628.00 mg CE/100 g CS, as it was the case with TP content, which indicates once again the superiority of the SWE method over the UAE method. The optimization of MAE process gave the TF content in the range of 94.50-211.83 mg CE/100 g SC (30), which is in accordance with the TF content obtained in this study.

According to the data presented in Table 3, the linear term of temperature and quadratic term of ultrasonic power exhibited a significant influence on the TF content ($p < 0.05$). The presented regression coefficients (Table 3) suggest that the influence of these three parameters on TF content should be similar as for the TP content (Figure 1.b). The predicted second-order polynomial model for the TF content is:

$$TP=156.04+32.32X_1-9.09X_2+1.44X_3-0.52X_1X_2-3.22X_1X_3-3.75X_2X_3-1.90X_1^2+9.58X_2^2+14.83X_3^2 \quad [5]$$

DPPH and reducing power assays

The results obtained assaying DPPH assay are presented in Table 2, and the measured values of IC_{50} ranged from 0.03569 to 0.05398 mg/ml. Comparing these results with those from the previous study (28), reporting the IC_{50} values of CS extracts in the range from 0.01706 to 0.06336 mg/mL, there is no big difference between the obtained values. Namely, the obtained the highest values of antioxidant activity at the high temperature were the same as in this study. Wangensteen et al. (2) reported the antioxidant activity of 0.51000 mg/mL of CS ethanolic extract obtained by solid-liquid extraction technique, which is lower compared to the result obtained in this research. The antioxidant activity of UAE extracts of CS (raffinate after the SFE process) against DPPH was in the range of 0.02478-0.04183 mg/mL (for 70% ethanolic extracts) and 0.07499-0.10770 mg/mL (for water extracts) (27). Ethanolic extracts exhibited higher activity against this radical than the extracts obtained in this study. The extracts obtained during the optimized MAE process of CS showed an activity in the range of 0.03020-0.06650 mg/mL (30), which is higher than in the case of UAE.

In the case of reducing power assay (Table 2), the obtained values ranged between 0.1436 and 0.1651 mg/mL. The highest value of EC_{50} indicates the lowest antioxidant activity while the lowest EC_{50} indicates the highest antioxidant activity, as well as it was the case of IC_{50} values in the DPPH assay. Comparing these results with those obtained during the optimization of MAE process (30), where obtained EC_{50} values were in the range of 0.1153-0.1824 mg/mL, the MAE extracts showed once again higher antioxidant activity.

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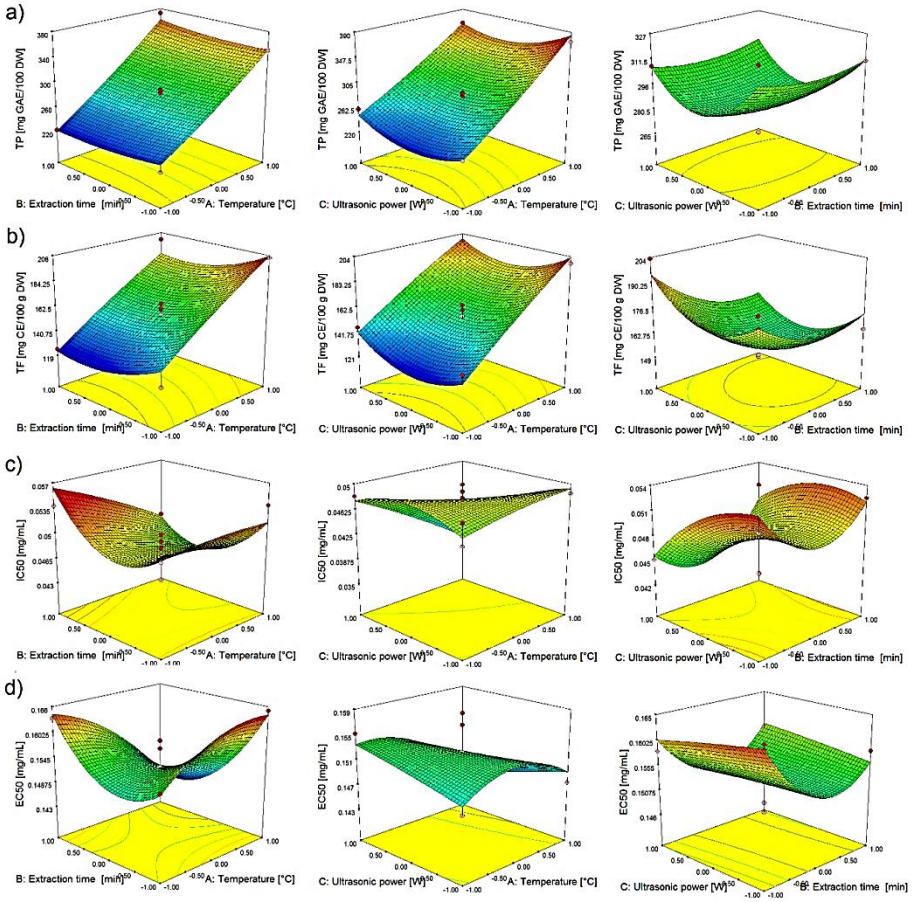

Figure 1. Response surface plots showing combined effects of UAE parameters on: a) TP, b) TF, c) IC_{50} and d) EC_{50}

The influence of the parameters on the DPPH assay is presented in Figure 1c, while the influence on the reducing power is shown in Figure 1d. The predicted second-order polynomial models for DPPH assay (Eq. [6]) and reducing power test (Eq. [7]) are:

$$IC_{50}=0.048-1.863E\text{-}0.03X_1+4.963E\text{-}0.04X_2-2.746E\text{-}0.03X_3-$$
$$-2.070E\text{-}0.03X_1X_2-3.080E\text{-}0.03X_1X_3+1.373E\text{-}0.03X_2X_3-$$
$$-4.083E\text{-}0.04X_1^2+4.474E\text{-}0.03X_2^2-2.076E\text{-}0.03X_3^2 \quad [6]$$

$$EC_{50}=0.15+2.563E\text{-}0.03X_1+2.475E\text{-}0.03X_2-3.875E\text{-}0.04X_3-$$
$$-7.600E\text{-}0.03X_1X_2-2.375E\text{-}0.03X_1X_3+1.550E\text{-}0.03X_2X_3-$$
$$-3.313E\text{-}0.03X_1^2+7.562E\text{-}0.03X_2^2-1.625E\text{-}0.04X_3^2 \quad [7]$$

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In the case of the DPPH assay, the linear term of ultrasonic power and quadratic term of extraction time exhibited a significant influence on the IC_{50} value ($p < 0.05$). With the increase in the ultrasonic power, the IC_{50} value was constant first, to decrease at higher values of ultrasonic power.

This phenomenon is connected with the influence of this parameter on the TP and TF contents. With the increase in ultrasonic power, the TP and TF content decreased in the beginning, while the IC_{50} value was constant. After certain value of ultrasonic power there were increases in the TP and TF contents, while the IC_{50} value sharply decreased, consequently causing an increase in the antioxidant activity. On the other hand, in the beginning of the extraction, the IC_{50} value decreased, and then started to increase. The decrease in the antioxidant activity after certain time could be explained by the heating effect or overexposure to ultrasound irradiation, when decomposition of the antioxidant agent in the extract might occur (31). Similar effects of ultrasonic power and extraction time on DPPH assay have been reported previously (32, 33). In the case of interaction of ultrasound power and time, the IC_{50} value decreased with both extraction time and ultrasonic power in the beginning of the extraction process, and the lowest IC_{50} value (highest antioxidant activity) were achieved at approximately half of the extraction process and ultrasonic power range (Figure 1c).

As for the reducing power, significant influence exhibited only the quadratic term of time and interaction between temperature and time ($p < 0.05$). The influence of the time was the same as in the case of DPPH assay. This means that both antioxidant tests rely on the reaction with the same compounds in the extracts and that their decomposition occurred due to prolonged exposure to high temperature or ultrasonic power.

Pearson's correlation coefficients among Y, TP, TF, IC_{50} and EC_{50} values are presented in Table 5. There was high correlation between Y and TP and between TP and TF ($r > 0.9$). The correlation among Y and TF was good ($r = 0.8710$).

Table 5. Pearson's correlation coefficients among Y, TP, TF, IC_{50} and EC_{50}

r	**Y**	**TP**	**TF**	**IC_{50}**	**EC_{50}**
EC_{50}	−0.1553	−0.2523	−0.0419	0.6024	1
IC_{50}	−0.1906	−0.2956	−0.2291	1	
TF	0.8710	0.9198	1		
TP	0.9671	1			
Y	1				

On the other hand, the correlation among the antioxidant assays (DPPH and RP) was moderate ($r = 0.6024$), while the correlation among Y TP, TF and antioxidant assays was very week ($r < 0.5$) and negative. A good correlation among Y, TP and TF was rather expected, and indicated the increases in TP and TF with the increase in Y.

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Optimization of UAE

As the aim of this research was the optimization id the extraction process to obtain maximal TP and TF contents and maximal antioxidant activity, each of the individual responses was optimized. This optimization was based on previously obtained experimental results and performed statistical analysis. The estimated optimal conditions and predicted values of individual responses are presented in Table 6.

Table 6. Predicted maximal values of individual responses and estimated values of optimal conditions

Optimal conditions	Investigated responses			
	TP (mg GAE/100 g CS)	TF (mg CE/100 g CS)	IC_{50} (mg/mL)	EC_{50} (mg/mL)
Predicted value	382.68	216.06	0.03764	0.1425
Temperature (^oC)	79.60	79.40	80.00	78.40
Time (min)	49.20	43.60	60.40	68.60
Ultrasonic power (W)	96.60	216.00	216.00	214.80

Observing the data from Table 6 it can be noticed that the conditions for both antioxidant activity tests were similar. On the other hand, there were differences in ultrasonic power for TP and TF contents. In the case of TP content, the maximum value required almost minimal ultrasonic power, while it was vice versa in the case of TF content, where maximal ultrasonic power was required. The desirability for all cases was 1.00 except for the IC_{50} value, which was 0.893.

CONCLUSION

Preparation of extracts with high antioxidant activity and maximum content of biological active compound (phenolic and flavonoid compounds) require careful selection of extraction parameters. In order to optimize process parameters, response surface methodology was successfully applied. It was shown that a second-order polynomial model was able to successfully describe the extraction process of polyphenolic compound and antioxidant activity. The obtained results showed that temperature and ultrasonic power had the strongest influence on total phenolics and total flavonoids content, while extraction time was crucial parameter in the case of DPPH and reducing power assays. Regarding the DPPH assay, ultrasonic power appeared to be an important factor for the optimization process. The temperature and time profiles were similar for all four measured responses, but in the case of ultrasonic power maximum value was predicted for all responses with exception of total phenolics content, where minimum of ultrasonic power was required.

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ОПТИМИЗАЦИЈА УЛТРАЗВУЧНЕ ЕКСТРАКЦИЈЕ ПОЛИФЕНОЛНИХ ЈЕДИЊЕЊА ИЗ СЕМЕНА КОРИЈАНДЕРА МЕТОДОМ ОДЗИВНЕ ПОВРШИНЕ

Зоран П. Зековић, Саша Д. Ђуровић и Бранимир М. Павлић

Универзитет у Новом Саду, Технолошки факултет, Булевар Цара Лазара 1, 21000 Нови Сад, Србија

Ултразвучном екстракцијом добијени су екстракти семена коријандера са високим садржајем биолошки активних једињења. Како би се оптимизовао процес екстракције, употребљена је метода одзивне површине (RSM) у циљу добијања екстраката са максималним садржајем укупних фенолних (TP) и флавоноидних (TF) једињења, као и максималном антиоксидативном активношћу (IC_{50} и EC_{50}). Независне промењиве у овом случају биле су температура (40-80^{o}C), време трајања екстракције (40-80 min) и снага ултразвука (96-216 W). Добијени експериментални резултати су фитовани полиномним моделом другог реда са вишеструком регресијом, а анализа варијанси (ANOVA) је примењена ради процене модела и добијања оптималних услова за максималан принос TP (79,60^{o}C, 49,20 min, 96,69 W), TF (79,40^{o}C, 43,60 min, 216,00 W), IC_{50} (80,00^{o}C, 60,40 min, 216,00 W) и EC_{50} (78,40^{o}C, 68,60 min, 214,80 W). Предвиђене максималне вредности при оптималним условима за TP, TF, IC_{50} и EC_{50} биле су: 382,68 mg GAE/100 g CS, 216 mg CE/100 g CS, 0,03764 mg/ml и 0,1425 mg/ml.

Кључне речи: семе коријандера, ултразвучна екстракција, оптимизација, метода одзивне површине

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Retraction: IDENTIFICATION OF AFLATOXIGENIC FUNGI POLYMERASE CHAIN REACTION- BASED ASSAY. APTEFF, 45 (2014) 259-269. DOI: 10.2298/APT1445259S

Vladislava M. Šošo[1], *Marija M. Škrinjar*[1], *Nevena T. Blagojev*[1] *and Slavica M. Vesković-Moračanin*[2]

[1] University of Novi Sad, Faculty of Technology, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia
[2] Institute of Meat Hygiene and Technology, Kaćanskog 13, 11000 Belgrade, Serbia

Abstract:

On the proposition of the authors, and with the consent of the Editorial Board, the paper entitled: IDENTIFICATION OF AFLATOXIGENIC FUNGI POLYMERASE CHAIN REACTION-BASED ASSAY, by Vladislava M. Šošo, Marija M. Škrinjar, Nevena T. Blagojev, Slavica M. Vesković Moračanin, which was published in 2014 (Vol. 45, pp. 259-269, DOI: 10.2298/APT1445259S), is retracted. The Editorial Board has found that the paper in question is to a lower extent a plagiarism (mild plagiarism), and put a proposition to the authors to make an appropriate correction that was to be published. However, the authors did not accept this proposition, requesting from the Editorial Board to retract the paper, to which the Editorial Board consented.

Владислава В. Шошо, Марија М. Шкрињар, Невена Т. Благојев и Славица М. Весковић Морачанин

Сажетак:

На предлог аутора и уз сагласност Уредништва рад под називом: ИДЕНТИФИКАЦИЈА АФЛАТОКСИГЕНИХ ПЛЕСНИ ПОМОЋУ МЕТОДА ЗАСНОВАНИХ НА ПРИНЦИПУ ЛАНЧАНЕ РЕАКЦИЈЕ, аутора: Владислава В. Шошо, Марија М. Шкрињар, Невена Т. Благојев и Славица М. Весковић Морачанин, који је објављен у 2014. години (волумен 45, стр. 259-269, DOI: 10.2298/APT1445259S) се повлачи. Уредништво је оценило да је оспорени рад плагијат мањих размера (благи плагијат) и предложило ауторима рада да се изврши одговарајућа исправка која ће бити објављена. Међутим, аутори нису прихватили наведени предлог, већ су предложили Уредништву да се рад повуче, са чим се Уредништво сагласило.

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Thesis: Linstead, J.B.: Linstead, J.B. Effects of adding natural antioxidants on colour stability of paprika. Ph.D. (or M.S.) Thesis, University of Glasgow, November 2006.

Patent: Lenssen, K. C.; Jantscheff, P.; Kiedrowski, G.; Massing, U. Cationic Lipids with Serine Backbone for Transfecting Biological Molecules. Eur. Pat. Appl. 1457483, 2004.

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Главни и одговорни уредник не сме имати било какав сукоб интереса у вези са рукописима који се разматрају. Ако такав сукоб интереса постоји, о избору рецензената и судбини рукописа одлучује Уредништво часописа. Главни и одговорни уредник и чланови Уредништва су дужни да благовремено пријаве постојање сукоба интереса.

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Главни и одговорни уредник и чланови Уредништва часописа не смеју да користе необјављен материјал из предатих рукописа за своја истраживања без изричите писане дозволе аутора, а информације и идеје изнесене у предатим рукописима морају се чувати као поверљиве и не смеју се користити за стицање личне користи.

Главни и одговорни уредник и чланови Уредништва часописа дужни су да предузму све разумне мере како би идентитет рецензената остао непознат ауторима пре, током и након поступка рецензије.

Обавезе аутора

Аутори гарантују да рукопис представља њихов оригиналан допринос, да није објављен раније и да се не разматра за објављивање на другом месту. Истовремено предавање истог рукописа у више часописа представља кршење етичких стандарда. Такав рукопис се моментално искључује из даљег разматрања.

Аутори такође гарантују да након објављивања у часопису ***Acta Periodica Technologica***, рукопис неће бити објављен у другој публикацији на било ком језику без сагласности издавача.

У случају да је послати рукопис резултат научноистраживачког пројекта или да је, у претходној верзији, био изложен на скупу у виду усменог саопштења (под истим или сличним насловом), детаљнији подаци о пројекту, конференцији и слично, наводе се у Захвалности на крају рукописа, а испред списка литературе. Рад који је већ објављен у неком часопису не може бити прештампан у часопису ***Acta Periodica Tehnologica***.

Аутори су дужни да се придржавају етичких стандарда који се односе на научноистраживачки рад. Аутори гарантују и да рукопис не садржи неосноване или незаконите тврдње и не крши права других. Издавач неће сносити никакву одговорност у случају испостављања било каквих захтева за накнаду штете.

Садржај рада

Рад треба да садржи довољно детаља и референци како би се рецензентима, а потом и читаоцима омогућило да провере тврдње које су у њему изнесене.

Аутори сносе сву одговорност за садржај предатих рукописа и дужни су да, ако је то потребно, пре њиховог објављивања прибаве сагласност свих лица или институција које су непосредно учествовале у истраживању које је у рукопису представљено.

Аутори који желе да у рад укључе илустрације, табеле или друге материјале који су већ негде објављени дужни су да за то прибаве сагласност носилаца ауторских права. Материјал за који такви докази нису достављени сматраће се оригиналним делом аутора.

Ауторство

Аутори су дужни да као ауторе наведу само она лица која су значајно допринела садржају рукописа, односно дужни су да сва лица која су значајно допринела садржају рукописа наведу као ауторе. Ако су у битним аспектима истраживачког пројекта и припреме рукописа учествовала и друга лица која нису аутори, њихов допринос треба поменути у захвалници.

Навођење извора

Аутори су дужни да исправно цитирају изворе који су битно утицали на садржај истраживања и рукописа. Информације које су добили у приватном разговору или кореспонденцији са трећим лицима, приликом рецензирања пријава пројеката или рукописа и слично, не смеју се користити без изричите писане дозволе извора.

Плагијаризам

Плагирање, односно преузимање туђих идеја, речи или других облика креативног израза и представљње као својих, представља грубо кршење научне и издавачке етике. Плагирање може да укључује и кршење ауторских права, што је законом кажњиво.

Плагијат обухвата следеће:

- дословно или готово дословно преузимање или смишљено парафразирање (у циљу прикривања плагијата) делова текстова других аутора без јасног указивања на извор или обележавање копираних фрагмената (на пример, коришћењем наводника);
- копирање слика или табела из туђих радова без правилног навођења извора и/или без дозволе аутора или носилаца ауторских права.

Упозоравамо ауторе да се сваки рукопис темељно проверава да ли је плагијат. У ту сврху, ради што веће објективности, користи се више софтверских решења.

Рукописи код којих постоје јасне индиције да се ради о плагијату биће аутоматски одбијени, а ауторима рада ће бити привремено забрањено објављивање у часопису.

Ако се установи да је рад који је објављен у часопису плагијат, исти ће бити повучен у складу са процедуром описаном под *Повлачење већ објављених радова,* а ауторима рада ће бити привремено забрањено објављивање у часопису.

Сукоб интереса

Аутори су дужни да у раду укажу на финансијске или било које друге сукобе интереса који би могли да утичу на изнесене резултате и интерпретације.

Грешке у објављеним радовима

У случају да аутори открију важну грешку у свом раду након његовог објављивања, дужни су да моментално о томе обавесте уредника или издавача и да са њима сарађују како би се рад повукао или исправио.

Предавањем рукописа редакцији часописа ***Acta Periodica Technologica*** аутори се обавезују на поштовање наведених обавеза.

Обавезе рецензената

Рецензенти су дужни да стручно, аргументовано, непристрасно и у задатим роковима доставе уреднику оцену научне вредности рукописа.

Рецензенти евалуирају радове у односу на усклађеност теме рада са профилом часописа, релевантност истраживане области и примењених метода, оригиналност и научну релевантност података изнесених у рукопису, стил научног излагања и опремљеност текста научним апаратом.

Рецензент који има основане сумње или сазнања о кршењу етичких стандарда од стране аутора дужан је да о томе обавести уредника. Рецензент треба да препозна важне објављене радове које аутори нису цитирали. Он треба да упозори уредника и на битне сличности и подударности између рукописа који се разматра и било којег другог објављеног рада или рукописа који је у поступку рецензије у неком другом часопису, ако о томе има лична сазнања. Ако има сазнања да се исти рукопис разматра у више часописа у исто време, рецензент је дужан да о томе обавести уредника.

Рецензент не сме да буде у сукобу интереса са ауторима или финансијером истраживања. Уколико постоји сукоб интереса, рецензент је дужан да о томе моментално обавести уредника.

Рецензент који себе сматра некомпетентним за тему или област којом се рукопис бави дужан је да о томе обавести уредника.

Рецензија мора бити објективна. Коментари који се тичу личности аутора сматрају се непримереним. Суд рецензената мора бити јасан и поткрепљен аргументима.

Рукописи који су послати рецензенту сматрају се поверљивим документима. Рецензенти не смеју да користе необјављен материјал из предатих рукописа за своја истраживања без изричите писане дозволе аутора, а информације и идеје изнесене у предатим рукописима морају се чувати као поверљиве и не смеју се користити за стицање личне користи.

Поступак рецензије

Примљени радови подлежу рецензији код најмање два независна рецензента. Рецензија је једнострано анонимна (идентитет аутора је познат рецензентима, а идентитет рецензената није познат ауторима). Рецензенти су обавезни да, уколико прихвате рецензирање рада, рецензију достављеног им рукописа изврше у року од 2-4 недеље. Коначну одлуку о публиковању рукописа доноси Уредништво часописа.

Циљ рецензије је да уреднику помогне у доношењу одлуке о томе да ли рад треба прихватити или одбити и да кроз процес комуникације са ауторима побољша квалитет рукописа.

Избор рецензената спада у дискрециона права главног и одговорног уредника часописа. Рецензенти морају да располажу релевантним знањима у вези са облашћу којом се рукопис бави и не смеју бити из исте институције као аутор, нити то смеју бити аутори који су у скорије време објављивали публикације заједно (као коаутори) са било којим од аутора поднесеног рада.

У главној фази рецензије, главни уредник шаље поднесени рад најмање двојици стручњака за научну област којом се рад бави. Рецензентски образац садржи низ питања на која треба одговорити, а која рецензентима указују који су то аспекти које треба обухватити како би се донела одлука о судбини једног рукописа. У завршном делу обрасца, рецензенти морају да наведу своја запажања и предлоге како да се поднети рукопис побољша.

Током читавог процеса, рецензенти делују независно једни од других. Рецензентима није познат идентитет других рецензената. Ако одлуке рецензената нису исте (прихватити / одбити), главни уредник може да тражи мишљење других рецензената.

Током поступка рецензије уредник може да захтева од аутора да доставе додатне информације (укључујући и примарне податке), ако су оне потребне за доношење суда о научном доприносу рукописа. Уредник и рецензенти морају да чувају такве информације као поверљиве и не смеју их користити за стицање личне користи.

Уредништво је дужно да обезбеди контролу квалитета рецензије. У случају да аутори имају озбиљне и основане замерке на рачун рецензије, уредништво ће проверити да ли је рецензија објективна и да ли задовољава академске стандарде. Ако се појави сумња у објективност или квалитет рецензије, главни и одговорни уредник ће тражити мишљење других рецензената.

Разрешавање спорних ситуација

Сваки појединац или институција могу у било ком тренутку да главном и одговорном уреднику и/или уредништву и пријаве сазнања о кршењу етичких стандарда и другим неправилностима и да о томе доставе неопходне информације/доказе.

Провера изнесених навода и доказа

Главни и одговорни уредник ће у договору са уредништвом часописа одлучити о покретању поступка који има за циљ проверу изнесених навода и доказа.

Током тог поступка сви изнесени докази сматраће се поверљивим материјалом и биће предочени само оним лицима која су директно укључена у поступак.

Лицима за која се сумња да су прекршила етичке стандарде биће дата могућност да одговоре на оптужбе изнесене против њих.

Ако се установи да је заиста дошло до неправилности, процениће се да ли их треба окарактерисати ако мањи прекршај или грубо кршење етичких стандарда.

Мањи прекршај

Ситуације окарактерисане као мањи прекршај решаваће се у директној комуникацији са лицима која су прекршај учинила, без укључивања трећих лица, нпр.:

- обавештавањем аутора/рецензената да је дошло до мањег прекршаја који је проистекао из неразумевања или погрешне примене академских стандарда;
- писмо упозорења аутору/рецензенту који је учнио мањи прекршај.

Грубо кршење етичких стандарда

Одлуке у вези са грубим кршењем етичких стандарда доноси главни и одговорни уредник у сарадњи са Уређивачким одбором часописа и, ако је то потребно, малом групом стручњака. Мере које ће предузети могу бити следеће (и могу се примењивати појединачно или истовремено):

- објављивање саопштења или уводника у ком се описује случај кршења етичких стандарда;
- слање службеног обавештења руководиоцима или послодавцима аутора/рецензента;
- повлачење објављеног рада у складу са процедуром описаном под *Повлачење већ објављених радова*;
- ауторима ће бити забрањено да током одређеног периода шаљу радове у часопис;
- упознавање релевантних стручних организација или надлежних органа са случајем како би могли да предузму одговарајуће мере.

Приликом разрешавања спорних ситуација Уредништво часописа се руководи смерницама и препорукама Одбора за етику у издаваштву (Committee on Publication Ethics – COPE): http://publicationethics.org/resources/.

Повлачење већ објављених радова

У случају кршења права издавача, носилаца ауторских права или аутора, повреде професионалних етичких кодекса, тј. у случају слања истог рукописа у више часописа у исто време, лажне тврдње о ауторству, плагијата, манипулације подацима у циљу преваре, као и у свим другим случајевима грубог кршења етиких стандарда, објављени рад се мора повући. У неким случајевима већ објављени рад се може повући и како би се исправиле накнадно уочене грешке.

Стандарди за разрешавање ситуација када мора доћи до повлачења рада дефинисани су од стране библиотека и научних тела, а иста пракса је усвојена и од стране часописа ***Acta Periodica Technologica***: у електронској верзији изворног чланка (оног који се повлачи) успоставља се веза (HTML линк) са обавештењем о повлачењу. Повучени чланак се чува у изворној форми, али са воденим жигом на PDF документу, на свакој страници, који указује да је чланак повучен (RETRACTED).

Отворени приступ

Часопис ***Acta Periodica Technologica*** је доступан у режиму отвореног приступа. Чланци објављени у часопису могу се бесплатно преузети са сајтова:

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Поступак предавања рукописа, рецензија и објављивање радова су бесплатни.

Самоархивирање

Часопис ***Acta Periodica Technologica*** омогућава ауторима да финалну, објављену верзију рукописа у PDF формату депонују у институционални репозиторијум и/или некомерцијалне базе података, као што су *PubMed Central*, *Europe PMC* или arXiv, или да га објаве на личним веб страницама (укључујући и профиле на друштвеним мрежама за научнике, као што су ResearchGate, Academia.edu итд.) и/или на сајту институције у којој су запослени, а у складу са одредбама лиценце Creative Commons Ауторство-Некомерцијално-Без прерада 3.0 Србија, у било које време након објављивања у часопису. При томе се морају навести основни библиографски подаци о чланку објављеном у часопису (аутори, наслов рада, наслов часописа, волумен, свеска, пагинација), а мора се навести и идентификатор дигиталног објекта – DOI објављеног чланка у форми HTML линка.

Ауторска права

Када је рукопис прихваћен за објављивање, аутори преносе ауторска права на издавача. У случају да рукопис не буде прихваћен за штампу у часопису, аутори задржавају сва права.

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Acta Periodica Technologica се штампа на енглеском језику. Часопис може садржавати и додатке везане за конгресе, научне скупове и симпозијуме.

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Сва коресподенција везана за предају рукописа рада, обавештења о одлукама Уредништва и захтевима за ревизију рукописа врши се електронском поштом са адресе apteff@tf.uns.ac.rs.

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- да је припремљен у складу са овим упутством,
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Ако је рад објављен у целини или само његов део на неком научном скупу, то се мора јасно назначити у фусноти на насловној страни рукописа.

ПРИПРЕМА РУКОПИСА

Језик: Рукопис мора бити написан на еглеском језику.

Припрема: Рукопис мора бити припремљен на максимално 10 страница A4 формата (односи се само на научне радове), у MS Word-у, Times New Roman фонтом са величином слова 10 pt, проредом од 1,5 линије и са свим маргинама од по 2,5 cm. Све странице рукописа морају бити нумерисане. Табеле и слике морају бити постављене на месту појављивања у тексту.

Општи изглед. Рукопис треба да је изложен јасно и да садржи: Насловну страну, Извод рада, Кључне речи, Увод, Експериментални део, Резултате и дискусију, Закључак, Захвалницу и Литературу (све на енглеском језику), као и Извод рада и кључне речи на српском језику.

Насловна страна: На првој страни рукописа треба да стоји наслов рада (без симбола, формула или скраћеница) написан великим болдираним словима. Наслов рада треба да је концизан и јасан и да се односи на садржај рукописа. Испод наслова рада *италик* словима написати пуна имена свих аутора (име, средње слово и презиме), без научних и професионалних звања. Име аутора који је задужен за сву коресподенцију, током свих фаза рецензије и објављивања рада, јасно означити звездицом. Неопходно је навести и његову контакт адресу (електронску и пуну поштанску адресу). Институцију(е) у којој су аутори запослени (или ангажовани) навести испод имена свих аутора. Уколико аутори нису из исте институције иза имена сваког аутора означити бројем у индексу припадност институцији и исти број у индексу написати испред назива одговарајуће институције.

Извод рада (100-250 речи, *италик* слова) написати испод наслова рада и имена аутора. Извод треба да садржи циљ истраживачког рада, методе, резултате и дискусију.

Кључне речи (нормална слова, максимално 5 кључних речи) навести испод Извода рада.

Увод треба да садржи податке везане за претходни истраживачки рад са одговарајућим референцама, као и проблем и циљ истраживања описаних у раду.

Експериментални део. Материјал и методе, који су коришћени у раду, треба да буду јасно и детаљно изложени како би остали научници могли да их понове. Детаљно описати само нове технике и методе, док је за већ познате методе довољно навести одговарајуће референце.

Резултати и дискусија. Резултати морају бити приказани концизно и јасно, у табелама или илустрацијама. Значајност резултата истраживања приказати без понављања материјала изложеног у Уводу. Број и величину табела, илустрација, графика и хемијских формула свести на неопходан минимум.

Закључак треба да покаже значајан допринос проблематике рукописа и могућност њене даље примене.

Захвалница. Текст захвалнице треба да буде што краћи.

Референце у тексту означити по редоследу појављивања арапским бројевима у заградама ( ). Све публикације наведене у рукопису рада навести и у листи референци на посебној страници текста. Скраћени називи часописа треба да буду написани у складу са Chemical Abstracts Service (CASSI Search Tool; http://cassi.cas.org). Списак референци треба написати по редоследу њиховог појављивања у тексту. Навести имена свих аутора (не користити „... и сарадници“), са њиховим иницијалима иза одговарајућег презимена. Иза скраћених назива часописа означити годину издања (**болд**), свеску (*италик*), број (ако постоји), и први и последњи број странице рада.

Примери:

Часописи: Pascual, E.C.; Goodman, B.A; Yeretzian, C. Characterisation of Free Radicals in Solubile Coffee by Electron Paramagnetic Resonance Spectroscopy. *J. Agric. Food Chem.* **2002,** *50* (21), 6114–6122.

Књиге: Morris, R. *The Last Sorcerers: The Path from Alchemy to the Periodic Table;* Joseph Henry Press: Washington, DC, 2003; pp 145–158.

Књиге са више поглавља: Puls, J.; Saake, B. Industrially Isolated Hemicelluloses. In *Hemicelluloses: Science and Technology;* Gatenholm, P., Tenkanen, M., Eds.; ACS Symposium Series 864; American Chemical Society: Washington, DC, 2004; pp 24–37.

Књиге извода радова: Noe, W.; Howaldt, M.; Ulber, R.; Scheper, T. Immunobase elution assay for process control, 8th European Congress on Biotechnology, Budapest, 17–21 August 1997, Book of Abstracts WE 163, p. 246.

Тезе: Linstead, J.B. Effects of adding natural antioxidants on colour stability of paprika. Ph.D. (or M.S.) Thesis, University of Glasgow, november 2006.

Патенти: Lenssen, K. C.; Jantscheff, P.; Kiedrowski, G.; Massing, U. Cationic Lipids with Serine Backbone for Transfecting Biological Molecules. Eur. Pat. Appl. 1457483, 2004.

Необјављени (непубликовани) подаци: Треба да буду цитирани уз коментар „у штампи“, „необјављени резултати“ „личне белешке“.

Подаци преузети са интернета: Треба да садрже аутора, наслов, интернет адресу и датум приступа подацима: Пример: Wright, N.A. The Standing of UK Histopathology Research 1997-2002. http://pathsoc.org.uk (accessed 7 October 2004).

Извод и кључне речи на српском језику треба написати ћириличним писмом на крају рукописа рада (после списка литературних података) и у проширеном облику (највише 1 страница). Наслов рада написати нормалним, великим словима, а испод њега *италик* словима написати имена аутора (име, средње слово и презиме) као и назив институције у којој раде.

Хемијска номенклатура и јединице. Аутори су обавезни да користе SI систем јединица и хемијску номенклатуру која је у складу са правилима Chemical Abstract-a где год је то могуће.

Табеле. Свака табела треба да је нумерисана арапским бројем иза којег следи назив табеле (**Table 1.** Result...). Ширина табеле не сме бити већа од 12,5 цм.

Графици и слике. Сваки график или слику такође треба нумерисати арапским бројем иза којег следи назив (**Figure 1.** Chromatogram of...). Графици морају бити припремљени помоћу програма Microsoft Excel, Origin или Statistica. Шеме морају бити припремљене помоћу програма Microsoft Visio или Corel Draw. Све графике и шеме неопходно је доставити као посебне фајлове у оригиналној екстензији (нпр. xls, xlsx, vdr, cdr). Скениране црно-беле шеме доставити као фајлове са tif, wmf или bmp екстензијом. Фотографије у црно-белој техници доставити као посебне фајлове са jpg екстензијом.

Хемијске формуле и математичке једначине. Написати хемијске формуле и математичке једначине јасно и прецизно и тачно поставити индексе на своја места.

Једначине у тексту означити арапским бројевима у угластим заградама [ ]. Значења коришћених скраћеница и симбола треба детаљно објаснити приликом њиховог првог појављивања у тексту или дати посебан списак на посебној страници на крају рукописа.

Рецензија. Сви радови достављени уредништву часописа биће послати на рецензију код најмање два независна рецензента који ће бити замољени да рецензију достављеног им рукописа изврше у року од 2-4 недеље. Коначну одлуку о публиковању рукописа доноси Уређивачки одбор часописа. Рукопис може бити враћен ауторима на исправку и допуну, уколико је то неопходно. Исправљен и допуњен рукопис треба вратити уредништву часописа што је пре могуће (најдаље за 2 недеље) након достављања примедби и коментара рецензената ауторима.

Рад припремљен за штампу: У последњој фази припреме рукописа аутору задуженом за коресподенцију електронском поштом биће достављен рад припремљен за штампу на корекцију искључиво техничке природе и сагласност за штампање. Све корекције аутори достављају електронском поштом у року од 48 сати од пријема рада припремљеног за штампу.

Додатне информације: Сва питања везана за објављивање радова у часопису слати електронском поштом Уредништву часописа на адресу apteff@tf.uns.ac.rs, или на поштанску адресу: Уредништво часописа *Acta Periodica Technologica*, Булевар цара Лазара 1, 21000 Нови Сад.

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Editorial:

University of Novi Sad, Faculty of Technology,
Bulevar cara Lazara 1, 21000 Novi Sad, Serbia

Phone: +381 21 485 3652
Fax:+381 21 450 413
e-mail: apteff@tf.uns.ac.rs

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