ACTA SCIENTIARUM POLONORUM
Acta Sci. Pol. Technol. Aliment. 14(1) 2015, 29–36
www.food.actapol.net pISSN 1644-0730 eISSN 1889-9594 DOI: 10.17306/J.AFS.2015.1.3

CHANGES OF ANTIOXIDANT POTENTIAL OF PASTA FORTIFIED WITH PARSLEY (*PETROSELINUM CRISPUM* MILL.) LEAVES IN THE LIGHT OF PROTEIN-PHENOLICS INTERACTIONS

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ABSTRACT

Background. Pasta is considered as an effective carrier of prohealth ingredients in food fortification. The aim of this study was to examine the changes of antioxidant potential of wheat pasta affected by fortification with powdered parsley leaves. A special attention was paid to effectiveness of fortification in the light of protein-phenolic interactions.
Material and methods. To improve antioxidant activity of pasta, part of wheat flour was replaced with powdered parsley leaves from 1% to 4% (w/w). The total phenolics content was determined with Folin-Ciocalteau reagent. Antioxidant capacity was evaluated using *in vitro* assays – abilities to scavenge free radicals (ABTS) and to reduce iron (III) (FRAP). Predicted phenolic contents and antioxidant activity were calculated. To determine the protein-phenolics interactions SE-HPLC and SDS-PAGE techniques were used.
Results. Fortification of pasta had a positive effect on its phenolic contents and antioxidant properties. The highest phenolics level and antioxidant activity of pasta were obtained by supplementation with 4% of parsley leaves. However, in most cases experimental values were significantly lower than those predicted. The protein profiles obtained after SDS-PAGE differed significantly among control and enriched pasta. Furthermore, the addition of parsley leaves to pasta resulted in increase of peaks areas obtained by SE-HPLC. Results indicate the occurrence of the protein-phenolics interactions in fortified pasta.
Conclusion. Overall, the effectiveness of fortification and consequently biological effect is limited by many factors including interactions between phenolics and pasta proteins. In the light of this results the study of potential interaction of bioactive supplements with food matrix should be taken into account during designing new functional food products.

Key words: antioxidant activity, parsley, protein-phenolic interactions, food fortification

INTRODUCTION

Pasta is one of the most popular staple food in many countries. Due to a high content of complex carbohydrates it is a valuable source of energy in the human diet. Additionally, pasta is very versatile food, easy to prepare and has a relatively long shelf life. This product is also considered as an effective carrier of prohealthy ingredients in food fortification (Borneo and Aguirre, 2008; Boroski et al., 2011).

Parsley (*Petroselinum crispum* Mill.) is a widely consumed culinary herb due to its characteristic taste and aroma. Fresh and/or dried parsley leaves are used as condiment, garnish, and flavoring ingredients

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(Zhang et al., 2006; Díaz-Maroto et al., 2002). Beside this, bioactive compounds of parsley show a wide range of pharmacological activities such hepatoprotective, brain protective, anti-diabetic, analgesic, spasmolytic, immunosuppressant, anti-platelet, gastroprotective, cytoprotective, laxative, estrogenic, diuretic, hypotensive, antibacterial and antifungal (Farzaei et al., 2013).

It should be noted that parsley leaves are a rich source of natural antioxidants (Kuźma et al., 2014; Elisia and Kitts, 2008), wherein a study of Fejes et al. (1998) shows that antioxidant capacity of this plant is strongly correlated with the level of flavonoids (apiin, luteolin- and apigenin-glycosides). Antioxidants protect some important biomolecules (like DNA, lipids and proteins) against oxidative damage induced by reactive oxygen species (ROS) under pathological conditions. ROS including superoxide (O_2^-), hydroxyl radical ($OH^\bullet$), hydrogenperoxide (H_2O_2) and lipid peroxide have been implicated in the etiology of large number of chronic diseases eg. cancer, inflammatory, cardiovascular, and neurodegenerative diseases (Shahidi and Naczk, 1995). These constituents are dominant phenolics antioxidants of this plant (Farzei et al., 2013). Additionally, between bioactive constituents of parsley such phytochemicals should be mentioned: essential oil (apiol, miristicin), cumarines (bergapten, imperatorin) and vitamin C (Fejes et al., 1998).

Food fortification is defined as the addition of one or more components, whether or not it is normally contained in the food, for the purpose of correcting and/or improving a potential biological activity of designed food product (Świeca et al., 2014). Several studies have investigated the effect of functional ingredients incorporation such as dried amaranth leaves (Borneo and Aguirre, 2008), oregano and carrot leaves (Boroski et al., 2011), buckwheat flour and bran (Biney and Beta, 2014), sorghum flour (Khan et al., 2013) and powdered onion (Rajeswari et al., 2013) on pasta quality including consumer acceptance and nutritional, nutraceutical and technological properties.

The improvement of cereal food products with natural phenolic compounds may be limited by many factors such as degradation (oxidation) during processing or binding with food matrix (Sivam et al., 2010; Świeca et al., 2014). A strong affinity of phenolics to bind with proteins may lead to a decrease in their free levels, and consequently to decreasing their bioavailability and subsequent reduction of biological effect (lowering antioxidant capacity). Additionally, protein-phenolic interactions (PPI) may change a digestibility of proteins and functional properties of finial product (Shahidi and Naczk, 1995; Ozdal et al., 2013; Świeca et al., 2013, 2014).

The aim of this study was to examine the changes of antioxidant potential of wheat pasta affected by fortification with powdered parsley leaves. A special attention was paid to effectiveness of fortification in the light of protein-phenolic interactions.

MATERIAL AND METHODS

Chemicals

Folin-Ciocalteau reagent, ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), potassium ferricyanide were purchased in Sigma-Aldrich (St. Louis, MO, USA) company. All others chemicals were of analytical grade.

Supplement preparation

Leaves from parsley were obtained from the Experimental Agricultural Station of the University of Life Sciences in Lublin, Poland. Parsley leaves were washed, rinsed, and air dried at temperature 30°C (SML30, Poland) until constant weight was attained. Residual moisture of 80 $g \cdot kg^{-1}$ was present in the final dried samples. Dried leaves were milled (Labolatory Mill, Saskia, Germany) and sifted (Thyr, Saskia, Germany) to produce parsley leaves flour (flours passed through 0.25 mm sieve). Parsley leaves flours (PL) samples were stored in air-tight dark plastic bottles until needed for pasta production.

Pasta manufacture

Semolina pasta wheat flour (120 $g \cdot kg^{-1}$ moisture, 105 $g \cdot kg^{-1}$ protein) was purchased from a local store. Spaghetti pasta was prepared with pasta flour and different concentrations of parsley flour (0% – C, 1 – 4%, P1-P4, respectively; w/w). For each formulation, wheat flour, parsley flour and water were mixed using a domestic blender (Kitchen Aid, Mod K5SSWH) for 5 min, to obtain homogeneous dough. This dough was formed and cut in a pasta machine (Pasta machine, Kitchen collection, Mod 20171, Chillicothe, OH).

Spaghetti pasta (about 2.5 mm thickness) was dried in a laboratory dryer (SML30, Poland) at 40°C. Residual moisture of 120 $g \cdot kg^{-1}$ was present in the final dried pasta samples.

Extraction procedure

Powdered samples of fortified pasta (1 g of dry weight (DW) were extracted for 1 h with 7.5 ml of PBS buffer (phosphate buffered saline, pH 7.4) (PBS) or 20 mM hydrochloric acid in methanol: acetone: water solution (30:30:40; v/v/v; pH = 2) (CHEM). The extracts were centrifuged (6800 x g, 20 min), extraction procedure was repeated. Extracts were combined and stored in darkness at –20°C until analysis.

Determination of total phenolics content (TPC)

The amount of total phenolics was determined using Folin-Ciocalteau reagent (Singleton and Rossi, 1965). To 0.5 ml of the sample, 0.5 ml H_2O, 2 ml Folin-Ciocalteau reagent (1:5 H_2O) were added, and after 3 min, 10 ml of 10% Na_2CO_3 and the contents were mixed and allowed to stand for 30 min. Absorbance at 725 nm was measured in a UV-Vis spectrophotometer. The amount of total phenolics was calculated as a gallic acid equivalent (GAE) in $mg \cdot g^{-1}$ of dry weight (DW).

Free radical scavenging assay (ABTS)

The experiments were performed using an improved ABTS depolarization assay (Re et al., 1999). $ABTS^{+\bullet}$ was generated by the oxidation of ABTS with potassium persulfate. The ABTS radical cation ($ABTS^{+\bullet}$) was produced by reacting 7 $mmol \cdot dm^{-1}$ stock solution of ABTS with 2.45 $mmol \cdot dm^{-1}$ potassium persulphate (final concentration). The $ABTS^{+\bullet}$ solution was diluted (with distilled water) to an absorbance of 0.7 ±0.05 at 734 nm. Then, 50 µl of samples were added to 1.45 ml of $ABTS^{+\bullet}$solution and the absorbance was measured at the end time of 15 min. The ability of the extracts to quench the ABTS free radical was calculated as a Trolox equivalent (TE) in $mg \cdot g^{-1}$ of dry weight (DW).

Ferric reducing antioxidant power (FRAP)

Reducing power was determined using the method described by Oyaizu (1986). Extracts (0.5 ml) were mixed with phosphate buffer (0.5 ml, 200 $mmol \cdot dm^{-1}$, pH 6.6) and 0.5 ml of 10 $g \cdot dm^{-1}$ aqueous solution of potassium ferricyanide $K_3[Fe(CN_6)]$. The mixture was incubated at 50°C for 20 min. A portion (0.1 ml) of 100 $g \cdot dm^{-1}$ trichloroacetic acid was added to the mixture, which was then centrifuged at 6800 × g for 10 min. The upper layer of solution (0.5 ml) was mixed with distilled water (0.5 ml) and 0.1 ml of 1 $g \cdot dm^{-1}$ $FeCl_3$, and the absorbance was measured at 700 nm. The ability of the extracts to reduce iron (III) was calculated as a Trolox equivalent (TE) in $mg \cdot g^{-1}$ of dry weight (DW).

Predicted phenolic contents and antioxidant activity

Predicted (PV) total phenolic contents (TPC) was calculated as follow (Świeca et al., 2014):

$$PV = \left(TPC_{CP} - \left(TPC_{CP} \cdot \frac{N}{100\%}\right)\right) + \left(\frac{TPC_{PL} \cdot N}{100\%}\right)$$

where: TPC_{CP} – phenolic content of control pasta, TPC_{PL} – phenolic content of parsley leaves, N – percent of parsley leaves supplement.

Predicted (PV) antioxidant activity (AA) was calculated as follow (Świeca et al., 2014):

$$PV = \left(AA_{CP} - \left(AA_{CP} \cdot \frac{N}{100\%}\right)\right) + \left(\frac{AA_{PL} \cdot N}{100\%}\right)$$

where: AA_{CP} – activity of control bread, AA_{PL} – activity of parsley leaves, N – percent of parsley leaves supplement.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were analyzed by SDS-PAGE in 12% (w/v) acrylamide gels, as described by Laemmli (1970). Samples (PBS extracts) were mixed with loading buffer (ratio 3:1, v:v) containing 2% SDS (w/v) and boiled for 2 min, aliquots (20 µl) of protein samples were loaded into each lane. The polypeptides in gels were fixed with 10% (w/v) trichloroacetic acid and stained with Coomassie Brillant Blue G-250. Molecular mass of complexes was carried out with ColorBurst™ Markers (Sigma-Aldrich, St. Louis, MO, USA). Proteins electrophoresis was analyzed with Polydoc, Molecular Imaging System, Vilber Lourmat supplied with software PhotoCapt.

Size exclusion high performance liquid chromatography (SE-HPLC)

The samples were characterized by SE-HPLC using a Varian ProStar HPLC System separation module (Varian, Palo Alto, USA) equipped with a column (COSMOSIL 5Diol-20-II Packed Column 7.5 mm ID × 300 mm) and a ProStar DAD detector (Świeca et al., 2014). The column thermostat was set at 30°C. The amount of 40 µl of each sample solution was loaded on the column, and protein and peptides were eluted using a 20 mM PBS buffer pH 7.4. The flow rate was 0.8 mL·min^{-1}. Ultraviolet detection was performed at a wavelength of 280 nm.

Statistic analysis

All experimental results represented mean ±S.D. of three parallel measurements. One-way analysis of variance post-hoc and Tukey's test were used to compare groups. α values < 0.05 were regarded as a significant.

RESULTS AND DISCUSSION

The results presented in Table 1 show that fortification of pasta with PL had a positive effect on its phenolic contents and antioxidant properties. The highest phenolics level and antioxidant activity of pasta were obtained by supplementation with 4% PL. In comparison to control, contents of phenolic compounds in buffer (PBS) and chemical (CHEM) extracts obtained from pasta with 4% PL supplement were higher from 29.1% and 71.1%, respectively. Antiradical potential (ABTS) of PBS extracts was strictly depended on the percentage of PL supplement and was higher (in comparison to control) by 4.3%, 9.9%, 13% and 17.9% for P1-P4, respectively. In chemical extracts (CHEM) ability to quench ABTS radicals was a significantly higher only in case of pasta fortified with 4% of PL (elevation by 10.7% in comparison to control). In comparison to control, fortified pasta was characterized by significantly increased ability to reduce iron (FRAP). For pasta with 4% of PL addition an elevation by 144.2% and 159.4% was found for PBS and CHEM extracts, respectively. To better evaluate the changes in the nutraceutical potential of fortified pasta affected by phenolics-food matrix interactions predicted values (PV) was calculated (Table 1). In most cases experimental values (EV) were

Table 1. Phenolic contents and antioxidant activity and of control and pasta enriched with parsley leaves

Assays	Sample	PBS		CHEM	
		EV	PV	EV	PV
TPC mg GAE·g^{-1} DW	C	0.67 ±0.05^{a}	–	0.63 ±0.06^{a}	–
	P1	0.72 ±0.02^{a}	1.78	0.74 ±0.03^{b}	3.12
	P2	0.76 ±0.01^{b}	2.90	0.87 ±0.05^{c}	5.61
	P3	0.78 ±0.03^{b}	4.01	0.92 ±0.06^{c}	8.09
	P4	0.87 ±0.03^{c}	5.13	1.08 ±0.04^{d}	10.58
ABTS mg TE·g^{-1} DW	C	1.34 ±0.04^{a}	–	0.51 ±0.02^{a}	–
	P1	1.40 ±0.02^{b}	5.38	0.51 ±0.01^{a}	1.48
	P2	1.47 ±0.02^{c}	9.42	0.50 ±0.02^{a}	2.45
	P3	1.51 ±0.01^{d}	13.46	0.54 ±0.02^{ab}	3.43
	P4	1.58 ±0.03^{e}	17.50	0.56 ±0.03^{b}	4.40
FRAP mg TE·g^{-1} DW	C	0.09 ±0.01^{a}	–	0.14 ±0.01^{a}	–
	P1	0.10 ±0.00^{b}	0.71	0.19 ±0.00^{b}	0.17
	P2	0.14 ±0.00^{c}	1.32	0.25 ±0.01^{c}	0.20
	P3	0.15 ±0.00^{d}	1.94	0.32 ±0.01^{d}	0.23
	P4	0.21 ±0.00^{e}	2.56	0.37 ±0.00^{e}	0.26

All values were averages of three measurements and were expressed as mean ±SD (standard deviation). Means followed by different small letters, for selected extracts and features, are significantly different at α < 0.05.

PBS – PBS extracts, CHEM – chemical extracts, EV – experimental value, PV – predicted value, TPC – total phenolic contents, ABTS – ability to quench ABTS radicals, FRAP – ferric reducing antioxidant power, GAE – gallic acid equivalent, TE – trolox equivalent, C – control pasta, P1-P4 – pasta fortified with 1–4% of parsley leaves, respectively.

significantly lower than those predicted (PV) which, may indicate on blocking of reactive groups of polyphenols by bread components. Surprisingly, in case of chemical extracts (CHEM) experimental values for reducing power were significantly higher than those predicted.

According to previous studies fortification of pasta with phenolic rich ingredients was a successful technique, which allows increasing phenolic contents and/or antioxidant potential (Boroski et al., 2011; Khan et al., 2013; Biney and Beta, 2014). Antioxidant potential of foods may be created by such important factors as phenolic-proteins (Shahidi and Naczk, 1995; Świeca et al., 2013, 2014; Ozdal et al., 2013) phenolic-phenolic (Gawlik-Dziki, 2012; Durak et al., 2014) and phenolic-starch interactions (Zhang et al., 2011; Chai et al., 2013). Additionally, antioxidant level of pasta does not depend only on the type of supplement, but also on parameters of pasta processing. Thus, it is well known, that the presence of oxygen, water and heat treatment during processing may induce the oxidative degradation of antioxidants (Fares et al., 2010). Furthermore, it should be kept in mind that supplementation with functional components rich in antioxidants (phenolics) significantly changes the relationships between pasta components affecting on nutrients and bioactive ingredients bioavailability, microstructure, cooking, rheological and sensory characteristics of pasta (Boroski et al., 2011; Khan et al., 2013; Rajeswari et al., 2013; Biney and Beta, 2014).

Electrophoretic patterns of PBS extractable proteins from control and fortified pasta are shown in Figure 1. The protein profiles differed significantly among control and enriched pasta. Obtained results of SDS-PAGE showed 13 protein bands for control and 12 bands for P1-P4 fortified pasta. All bands were located between 100 kDa and 6.5 kDa. For fortified pasta (P1-P4) the band correlated with protein with molecular mass about 29 kDa was not observed (Fig. 1). Model studies described by Rawel et al. (2002a, 2002b) demonstrated that in some cases PPI, may negatively affect on the solubility of proteins. Additionally, the same study indicates the presence of intra- and intermolecular protein cross-linking caused by PPI and showed that electrophoretic patterns of protein derivates may be changed by intermolecular protein cross-linking (Rawel et al., 2002b).

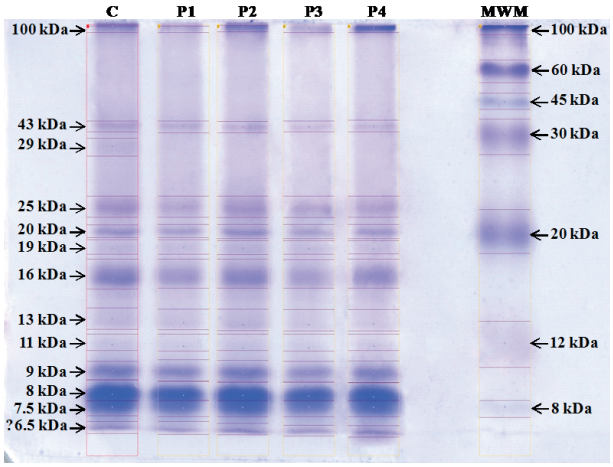
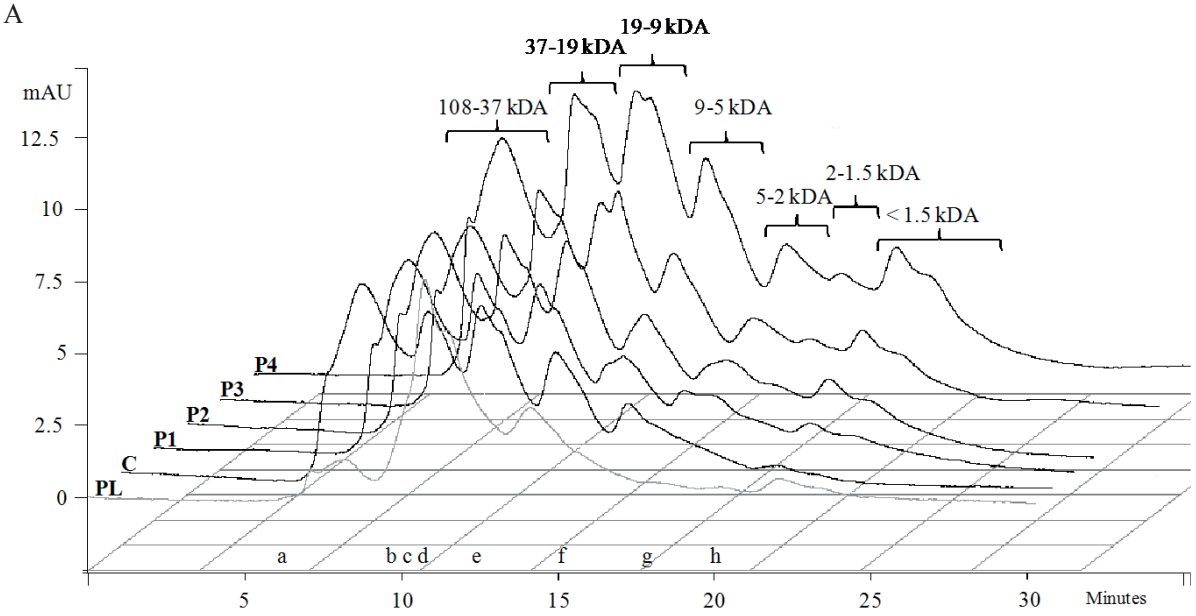


Fig. 1. SDS-PAGE pattern from control and fortified pasta: C – control pasta, P1-P4 – pasta fortified with 1–4% of parsley leaves addition, respectively, MWM – molecular weight markers

Additionally, to determine the protein-phenolics interaction SE-HPLC techniques were used. Figure 2 A showed the absorbance profiles of the eluates for the buffer extracts of control and fortified pasta. Chromatograms obtained for fortified pasta contain the peaks characteristic for both control pasta (C) and parsley leaves (PL). For control pasta 5 main peaks were found (corresponding with buffer extractable proteins with molecular masses: 108–37, 37–19, 19–9, 9–5 kDA). The addition of PL resulted in increase of peaks areas obtained for fortified pasta (Fig. 2 B). In respect to control, the areas of chromatogram obtained for fortified pasta with 1%, 2%, 3% and 4% PL, were bigger by 10.8%, 22.3%, 33.5%, 79.2%, respectively. This results indicate the occurrence of interactions between wheat protein and phenolic compounds derived from parsley leaves. Similar results (a significant increase in peak areas) were obtained in our previous study considering the interactions between onion skin or quinoa leaves phenolics and wheat proteins in fortified bread (Świeca et al., 2013, 2014). Whereas, model study of Rawel et al. (2002a) indicate that the addition of different phenolic acids and flavonoids to soy glycinin (protein) resulted in significant increase in optical density (UV/VIS spectra (200–500 nm)) of soy glycinin derivates. It may be distinguished four potential types of interactions of phenolic and proteins: hydrogen bonding,

A



B

Sample	Protein MW, kDa							Total peak areas
	108–37	37–19	19–9	9–5	5–2	2–1.5	<1.5	
PL	1 557 923	9 892 847	–	4 897 398	615 657	453 577	1 237 982	18 655 384
C	9 475 953	5 007 514	6 368 855	4 481 635	4 483 294		1 035 817	30 853 068
P1	9 813 275	5 546 817	6 582 442	4 322 808	2 558 274	1 978 190	3 380 132	34 181 938
P2	9 402 208	7 137 069	6 970 516	5 124 393	3 235 551	2 038 440	3 828 896	37 737 073
P3	8 150 732	7 606 992	8 540 653	5 670 236	3 404 125	2 371 997	5 440 014	41 184 749
P4	10 489 201	10 677 394	11 307 022	7 747 422	4 647 422	3 035 925	7 378 705	55 283 091

Fig. 2. Absorbance profiles of the eluates for the PBS extracts of control and fortified pasta obtained after size-exclusion chromatography (A), (B) peak areas: PL – parsley leaves (4%), C – control pasta, P1-P4 – pasta fortified with 1–4% of PL addition, respectively; a-h – molecular mass markers, kDa: a – 102, b – 42, c – 35, d – 22, e – 18, f – 6.5, g – 3, h – 1.5

hydrophobic, ionic, and covalent. Main parameters that affect protein-phenolic interactions are temperature, pH, types of proteins, protein concentration, types and structures of phenolic compounds (Ozdal et al., 2013). The derivates formed by PPI may change some important functional parameters of proteins such as solubility, thermal stability and digestibility of food proteins (Kroll et al., 2003; Ozdal et al., 2013) and decrease a desirable effect linked with enhance antioxidant capacity of product (Shahidi and Naczk, 1995; Ozdal et al., 2013; Świeca et al., 2013, 2014).

CONCLUSION

In conclusion, this study demonstrated that fortification of pasta with parsley leaves effectively enhanced phenolic level and antioxidant potential of the final product. Phenolic contents and antioxidant activities of fortified pasta were strongly correlated with enrichment level, however, in most cases experimental values were significantly lower than those predicted. Results of SDS-PAGE and SE-HPLC clearly indicate the occurrence the protein-phenolics interactions

in fortified pasta. Overall, the effectiveness of fortification and consequently biological effect is limited by many factors including interactions between phenolics and pasta proteins. In the light of these results the study of potential interaction of bioactive supplements with food matrix should be taken into account during designing new functional food products.

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ZMIANY POTENCJAŁU ANTYOKSYDACYJNEGO MAKARONU WZBOGACANEGO W LIŚCIE PIETRUSZKI (*PETROSELINUM CRISPUM* MILL.) W KONTEKŚCIE INTERAKCJI BIAŁKO-FENOL

STRESZCZENIE

Wstęp. Makaron jest uważany za skuteczny nośnik składników o prozdrowotnym działaniu w produkcji żywności fortyfikowanej. Celem pracy było określenie zmian potencjału antyoksydacyjnego makaronu pszennego wzbogaconego w liście pietruszki. Szczególną uwagę zwrócono na efektywność wzbogacania w kontekście interakcji białkowo-fenolowych.

Materiał i metody. W celu zwiększenia aktywności przeciwutleniającej makaronu część mąki pszennej zastąpiono sproszkowanymi liśćmi pietruszki w ilości od 1 do 4% (w/w). Całkowitą zawartość związków fenolowych oznaczano z użyciem odczynnika Folina-Ciocalteau. Aktywność przeciwutleniającą określono z zastosowaniem testów *in vitro* – zdolności do neutralizacji wolnych rodników (ABTS) oraz do redukcji jonów żelaza (III) (FRAP). Oszacowano przewidywaną zawartość związków fenolowych i aktywność przeciwutleniającą w produkcie finalnym. W celu określenia interakcji białkowo-fenolowych wykorzystano techniki SE-HPLC i SDS-PAGE.

Wyniki. Fortyfikacja efektywnie zwiększyła zawartość związków fenolowych i potencjał przeciwutleniający otrzymanych makaronów. Najwyższy poziom związków fenolowych oraz aktywnością przeciwutleniającą charakteryzował się makron z 4-procentowym dodatkiem liści pietruszki. Należy podkreślić, że otrzymane wartości eksperymentalne (w większości) były znacznie mniejsze niż przewidywane. Profile białkowe makaronu kontrolnego i wzbogacanego, uzyskane z wykorzystaniem techniki SDS-PAGE, wykazywały istotne różnice. Obecność interakcji potwierdzono z zastosowaniem SE-HPLC, gdzie w próbach otrzymanych z makaronów z dodatkiem liści pietruszki zaobserwowano istotny wzrost powierzchni chromatogramów. Uzyskane wyniki wskazują na występowanie interakcji białkowo-fenolowych w makaronie wzbogacanym.

Podsumowanie. Skuteczność wzbogacania makaronu, a w konsekwencji efekt biologiczny, jest uzależniona od wielu czynników, wśród których należy wymienić interakcje związków fenolowych z białkami. Dlatego określenie potencjalnych interakcji pomiędzy bioaktywnymi składnikami dodatków a matrycą żywności powinno być brane pod uwagę podczas projektowania nowych, funkcjonalnych produktów spożywczych.

Słowa kluczowe: aktywność antyoksydacyjna, pietruszka, interakcje białko-fenol, fortyfikacja żywności

Received – Przyjęto: 9.05.2014 Accepted for print – Zaakceptowano do druku: 3.09.2014

For citation – Do cytowania

Sęczyk, Ł., Świeca, M., Gawlik-Dziki, U. (2015). Changes of antioxidant potential of pasta fortified with parsley (*Petroselinum crispum* Mill.) leaves in the light of protein-phenolics interactions. Acta Sci. Pol. Technol. Aliment., 14(1), 29–36. DOI: 10.17306/J.AFS.2015.1.3