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INFLUENCE OF DIFFERENT FREEZING PARAMETERS AND STEAM-COOKING ON ANTIOXIDANT PROPERTIES OF GREEN PEA

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The influence of freezing process at two variants (-18°C, water-blanching and -35°C, steam-blanching) as well as steam-cooking process of fresh and freeze-stored material on the antioxidant activity of green pea was investigated. Nitrogen-containing antioxidants were extracted by water and phenolic antioxidants by 70% acetone. The processes applied resulted in loss of total nitrogen, its solubility, non-protein nitrogen, total polyphenols and condensed tannins content, however their influence on antioxidant activity towards $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ free radicals and in linoleic acid oxidation systems catalyzed non-enzymatically by hemoglobin and enzymatically by lipoxygenase was ambiguous – both loss and gain of activity were noted for different processes.

INTRODUCTION

Seeds of leguminous plants are a source of many valuable nutritious substances, mainly easy assimilable proteins. In recent years many research also indicates the content of compounds exhibiting antioxidant activity in legume seeds. Such compound are, among others, albumins and globulins. Our previous studies showed the ability of leguminous seed proteins to deactivate free radicals [Worobiej, 2001] or breaking the autooxidation process [Wolosiak, 2001] and enzymatically catalyzed oxidation reactions [Wolosiak & Klepacka, 2001]. Similar antioxidant activity show also polyphenols present in seeds of leguminous plants. It is a group with a very different structure and features [Simonetti *et al.*, 1997]. Polyphenols act as compounds scavenging free radicals, they create complexes with metals of transition groups and stop the activity of lipoxygenases and other enzymes catalyzing oxidation reactions [Drużyńska & Klepacka, 2005]. Free radicals and products of lipids oxidation pose severe danger to health because they can be metabolized in the body of a human being and attack biologically-active compounds revealing carcinogenic and mutagenic effects and participating in the process of ageing [Halliwell *et al.*, 1992, Stadtman, 1992]. It is very important to provide a relevant amount of antioxidants in the diet. Seeds of leguminous plants are a significant source of these compounds, but as season vegetables, after technological ripeness are subjected to preservation processes (*i.e.* freezing) and consumed mostly after technological or culinary processing. Such processes may significantly influence the effectiveness of natural antioxidants [Nicoli *et al.*, 1999], so it is necessary to conduct examinations of changes in the content and activity of antioxidant compounds of commonly consumed leguminous plant, green pea under the influence of different parameters of the freezing processes and cooking.

MATERIAL AND METHODS

Green pea var. Grand of proper technological maturity was used for the study and it was subjected to different freezing processes with two kinds of initial treatment: blanching in water (96°C, 3 min) and steam-blanching (100°C, 2 min). Blanched samples, after fast cooling and surface drying, were packed in foil bags and frozen (natural convection, temp. - 18°C and -35°C, resp.) in a freezer (Whirlpool, AFF 512). All samples were kept in the freezer until further processing. Investigations were conducted on the raw material, steam-cooked and on frozen seeds stored for one year. Directly before examination, the seeds were unfrozen in water at 20°C using a freezing bag as a membrane or steam-cooked.

The investigations were run on raw and processed material using water and 70% acetone extracts. To this end, fresh or frozen seeds were ground in a laboratory grinder (Retsch Grindomix GM200) and mixed with a solvent at a ratio of 1:10 (w/v) and shaken for 2 h at a room temperature, then centrifuged.

Homogenized preparations were determined for the contents of total protein, dry matter and ash. In turn water extracts

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were examined for the contents of soluble protein and non-protein nitrogen. Acetone extracts were analysed for the content of total polyphenols – the results were expressed as the amount of gallic acid [Singleton & Rossi, 1965] and for condensed tannins – results expressed as (+)catechin [Price, 1978].

To characterise proteins in water extracts, they were separated with a HPLC method using a Supradex 200 HR 10/30 column. Detection was conducted at 280 nm (detector UV SPD-6A Shimadzu). A mobile phase: buffer 50 mmol/L Na-phosphate, pH 7 + 0.15 mol/L NaCl with the flow rate of 0.4 mL/min was applied. Integrator C-R6A Shimadzu was used for peak integration. Models of molecular weight (Pierce): cytochrome (12.5 kDa), chymotrypsynogen (25 kDa), egg albumin (45 kDa), bovine serum albumin (67 kDa), catalase (158 kDa), ferritine (240 kDa) and blue dextran (2000 kDa) were used for column calibration; 100 μL of the extracts were injected onto the column.

Furthermore, the Fe(II) chelating ability both in water and acetone extracts was determined by the method of Lai *et al.* [2001]. Chelating ability was calculated basing on the decrease of absorbance (λ=562 nm) of iron(II)-ferrozine complex as a result of iron chelation.

The antiradical activity of the extracts was determined towards $ABTS^{\bullet+}$ radical cations [Re *et al.*, 1999] and $DPPH^{\bullet}$ stable radicals [Yen & Chen, 1995] expressing the results as mg of Trolox (antioxidant standard used for calibration curve) per 1 g of sample and 1 g of dry matter. Antioxidative activity was also measured as an ability to inhibit oxidation of linoleic acid emulsion in autoxidation reaction catalyzed by hemoglobin [Kuo *et al.*, 1999] and enzymatic reaction catalyzed by lipoxygenase. In both cases the amount of hydroperoxides was measured applying the spectrophotometric (λ=480 nm) ferric thiocyanate method after the reactions were stopped by 0.5% HCl in ethanol addition. The results were expressed as percent of oxidation's inhibition using control as a marker of the process scope without antioxidative compounds.

RESULTS AND DISCUSSION

Basing upon investigations it was observed that in the case of fresh product, the content of nitrogen amounted to 4.7% d.m. and after green pea was subjected to the freezing process, its amount dropped insignificantly to the same level in both frozen products and amounted to 4.5% of d.m. Insignificant loss of nitrogen in unfrozen seeds can result from a cellular juice leakage. The steam-cooking process resulted in insignificant loss of nitrogen in fresh seeds to the level of 4.4%. Cooking frozen seeds resulted in the drop of nitrogen comparing to fresh seeds but this loss was the same as in the seeds unfrozen in water of a room temperature. In fresh product the amount of soluble nitrogen was found to be at a level of 50% total nitrogen. The application of the freezing process resulted in the decrease of the nitrogen solubility with the level depending upon the method of freezing. Its content was bigger in seeds blanched with water and frozen at a temperature of -18°C (22.6% N) than in those blanched with steam and frozen at -35°C (28.7% N). Cooking both raw and frozen seeds resulted in the drop of nitrogen solubility, which is connected with the denaturation of proteins during thermal processing. The biggest decrease of solubility after cooking was observed in fresh seeds (by approx. 55%), but for frozen seeds this process also effected in the drop of nitrogen solubility but to a smaller extent and depending upon the method of freezing of the initial material. In the material frozen at -35°C the solubility dropped after cooking by 36% and in green pea frozen at -18°C by 13%. The content of non-protein nitrogen in all examined extracts exceeded 50% of soluble nitrogen. The smallest participation of non-protein nitrogen in soluble nitrogen was found in fresh product, *i.e.* 56%. Freezing increased the content of non-protein nitrogen in the extracts obtained which was higher in extracts from seeds frozen at -18°C (75%) than in extracts from seeds frozen at -35°C (61%). The results obtained suggest greater denaturation changes of proteins during freezing of seeds at a higher temperature. Cooking

TABLE 1. Content of total, soluble and non-protein nitrogen, total polyphenols, condensed tannins and Fe(II) chelating ability in water and acetone extracts.

Sample	Total nitrogen (% d.m.)	Soluble nitrogen (g/100g N)	Non-protein nitrogen (g/100g N)	Total polyphenols (mg/100g d.m.)	Condensed tannins (mg/100g d.m.)	Chelating ability of water extract (μmol Fe/g d.m.)	Chelating ability of acetone extract (μmol Fe/g d.m.)
Raw	4.70 (±0.07)	49.85 (± 0.49)	27.76 (±1.45)	141 (± 2)	50 (± 1)	7.88 (± 0.03)	6.72 (± 0.00)
Steam-cooked	4.36 (±0.10)	22.38 (± 2.37)	13.73 (±0.41)	115 (± 1)	44 (± 1)	6.62 (± 0.04)	4.57 (± 0.02)
Frozen (-18°C)	4.52 (±0.09)	22.59 (± 0.41)	16.89 (± 0.43)	98 (± 2)	39 (± 1)	6.55 (± 0.05)	5.42 (± 0.01)
Frozen (-35°C)	4.50 (± 0.07)	28.70 (± 0.00)	17.59 (± 1.31)	95 (± 2)	32 (± 2)	8.10 (± 0.02)	6.11 (± 0.08)
Steam-cooked after freezing (-18°C)	4.32 (± 0.14)	19.57 (± 0.50)	15.00 (± 0.43)	72 (± 2)	40 (± 2)	6.68 (± 0.02)	6.55 (± 0.01)
Steam-cooked after freezing (-35° C)	4.52 (± 0.08)	18.24 (± 0.41)	15.37 (± 1.23)	65 (± 2)	31 (± 2)	6.90 (± 0.02)	6.11 (± 0.07)

all seeds resulted in an increase of the non-protein nitrogen content which in fresh seeds increased up 61%, but in frozen products depended upon freezing parameters. Cooking seeds frozen at -18°C caused an insignificant increase of the non-protein nitrogen content up to 77% but freezing at -35°C resulted in a significant increase of its content up to 84%, which indicates the greatest denaturation changes in proteins after the application of these two processes.

Total polyphenols content dropped after cooking (from 141 mg to 115 mg/100 g d.m.) and after both freezing processes (from 141 mg to 98 mg/100 g d.m. at -18°C and to 95 mg/100 g d.m. at -35°C). It was noticed that in extracts from cooked products, the one from fresh seeds contained the highest amount of polyphenols. This amount dropped in extracts from both frozen cooked products (to 72 mg after the freezing process at -18°C and up to 65 mg/100g d.m. after freezing at -35°C).

Testing of extracts showed also the drop of the condensed tannins contents both after cooking and freezing processes, while cooking of fresh product resulted in a smaller drop of the tannins content than the freezing processes. It shall be noted that the content of condensed tannins in extracts obtained directly from frozen products and frozen products being subjected to cooking, did not differ. It may also be noted that the content of condensed tannins determined with chemical methods can frequently be burdened with an error which depends upon a level of polymerization, method of extraction and kind of seeds [Shahidi & Naczk, 1989, Kahkonen *et al*., 1999]. A statistically significant relationship was found between polyphenols content (PP) and condensed tannins content (T) at 95% confidence level (PP = -26.3 + 3.1*T), however quite low correlation coefficient (0.812) indicates that this group of polyphenols undergoes similar, but not fully proportional changes upon the processes applied.

Substances extracted by water from the raw material were more potent in chelating Fe(II) ions than those obtained by acetone (Table 1). Water extracts from the processed material exhibited lower chelating ability and at a similar level, except for the one derived from material frozen at -35°C which had the best chelating ability in the study. Among all the acetone extract, the highest ability to Fe(II) chelate exhibited extract from the raw material (6.7 μmol/L Fe/g d.m.). It has been stated that extracts obtained from cooking and freezing green pea show an insignificantly lower chelating ability. Cooking after freezing had no significant influence on the ability of preparations to Fe(II) chelate.

TABLE 2. Antioxidant activity of the extracts derived from raw, frozen and cooked material investigated.

Sample	Activity against $ABTS^{\bullet+}$ (mg Trolox/g sample)	Activity against $DPPH^{\bullet}$ (mg Trolox/g sample)	Activity against peroxides in hemoglobin-catalysed process (%)	Activity against peroxides in lipoxigenase-catalysed process (%)
Water extract of raw material	2.07 (±0.08)	0.16 (±0.00)	22.9 (±1.4)	11.8 (±3.8)
Acetone extract of raw material	1.05 (±0.02)	0.14 (±0.00)	13.7 (±1.2)	3.5 (±1.4)
Water extract after cooking	1.74 (±0.04)	0.11 (±0.00)	9.2 (±1.0)	3.1 (±0.6)
Acetone extract after cooking	0.82 (±0.03)	0.17 (±0.00)	6.8 (±1.1)	13.0 (±3.3)
Water extract of frozen (-18°C) material	1.65 (±0.08)	0.11 (±0.01)	12.7 (±1.6)	1.7 (±4.3)
Acetone extract of frozen (-18°C) material	0.52 (±0.08)	0.09 (±0.01)	5.2 (±1.6)	-11.1 (±1.4)
Water extract after freezing (-18°C) and cooking	1.42 (±0.02)	0.08 (±0.00)	11.9 (±1.1)	10.5 (±1.4)
Acetone extract after freezing (-18°C) and cooking	0.57 (±0.13)	0.10 (±0.00)	4.3 (±1.7)	1.1 (±3.2)
Water extract of frozen (-35°C) material	1.56 (±0.05)	0.13 (±0.00)	5.4 (±2.6)	6.1 (±2.5)
Acetone extract of frozen (-35°C) material	0.45 (±0.02)	0.08 (±0.00)	4.5 (±0.83)	4.9 (±3.6)
Water extract after freezing (-35°C) and cooking	1.85 (±0.04)	0.09 (±0.00)	5.0 (±1.7)	16.1 (±5.6)
Acetone extract after freezing (-35°C) and cooking	0.57 (±0.04)	0.12 (±0.00)	9.4 (±1.2)	2.3 (±1.10)

The separation of extracted protein fractions by gel chromatography showed that the most differentiated in regard to the participation of protein fractions is the one obtained from raw seeds (Figure 1). In the extracts there can be found a fraction of 168 kDa in significant amounts (participation of approx. 8%) corresponding to the 7S globulin [Gueguen, 1991] and a fraction of 93 kDa with the participation of 9% corresponding to an albumin, the presence of which was not noted in other extracts. No fraction corresponding to legumin (320 kDa) was found in the extract from fresh seeds, but it was present in insignificant amounts (up to 2.0%) in other samples. All extracts showed a high content of a fraction below 10 kDa. Its amount was the lowest in extracts from raw seeds (44%) and the highest in the extract from green pea frozen at -18°C (62%). Differences were observed in fractions exceeding 500 kDa; they were mostly observed in extracts from fresh seeds (21%) but in extracts from frozen products their amount depended upon freezing conditions and was the lowest in the extract from green pea frozen at -18°C (3.8%) but higher in extracts from a green pea frozen at -35°C (20.2%).

The cooking process caused an increase of the high molecular weight fractions content in fresh seeds and frozen at -18°C while after freezing at -35°C their amount has dropped. The increase of the high molecular weight fractions content after cooking of fresh seeds was also observed by Carbonaro *et al.* [1997] in water extracts from bean and chickpea.

Fresh pea water extracts exhibited two times higher antiradical activity towards $ABTS^{\bullet+}$ (2.07 mg Trolox/g sample) than acetone extracts – 1.05 mg Trolox/g sample (Table 2). The results obtained for $DPPH^{\bullet}$ radicals deactivation ability were lower and very close when comparing constituents extracted with water and acetone. Fresh material cooking

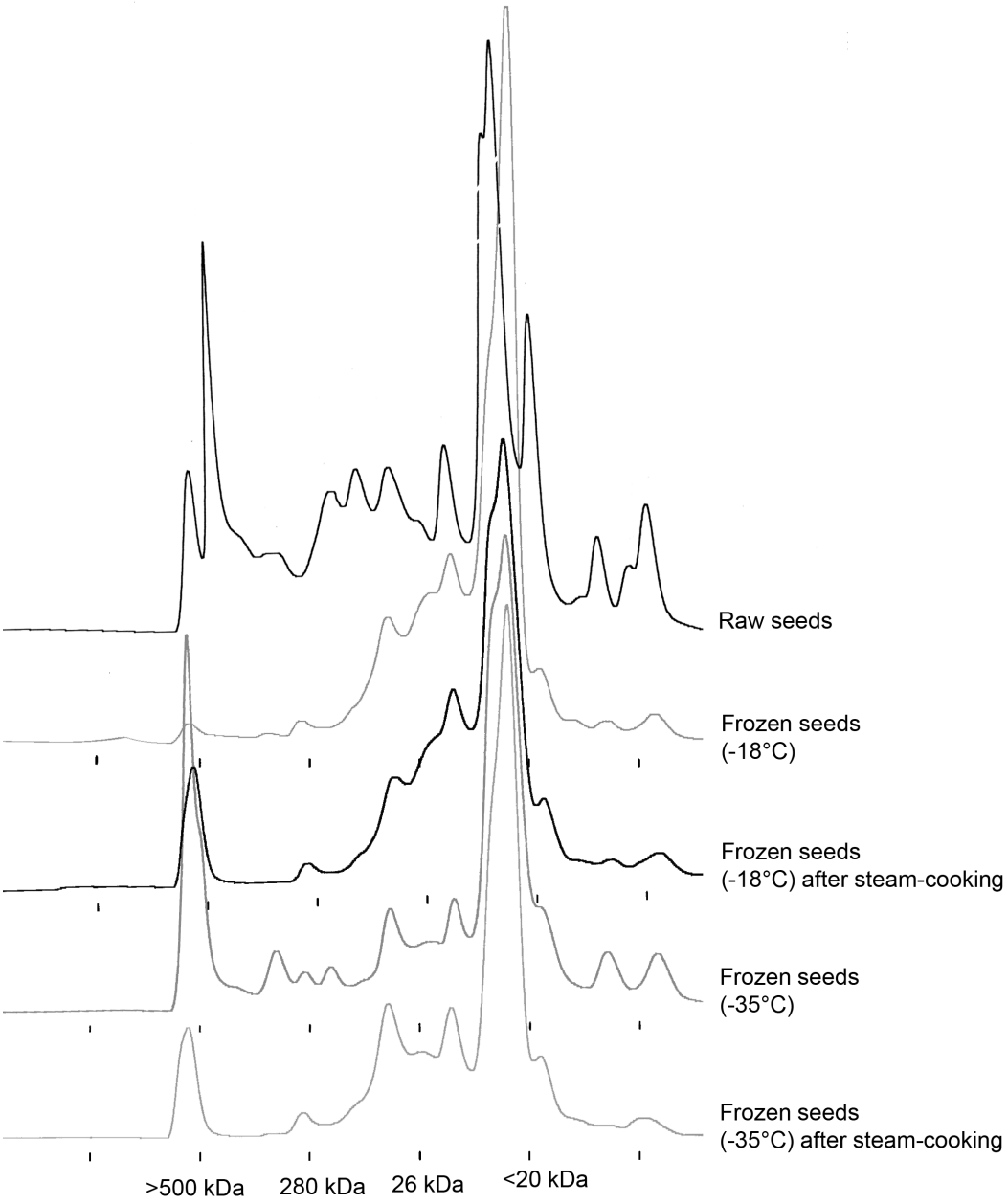

FIGURE 1. Size exclusion chromatograms of proteins in water extracts of green pea: raw and frozen (-18°C, -35°C) and after cooking process.

process caused a decrease of antiradical activity (by 15-27%) of all the extracts investigated but the acetone one towards DPPH$^{\bullet}$. Low activity (approx. 20%) of pea towards these radicals relating to other green vegetables (broccoli – 78%, spinach – 67%) was stated also by Turkmen *et al.* [2005], who proved also its slight drop after the cooking process.

Both variants of green pea freezing process influenced more the decrease of antiradical activity in the case of acetone extracts (for -18°C and -35°C: by 50 and 57% towards ABTS$^{\bullet+}$; by 34 and 43% towards DPPH$^{\bullet}$, respectively) than in water extracts (by 20 and 24% towards ABTS$^{\bullet+}$; by 30 and 18% towards DPPH$^{\bullet}$, respectively). Basing on the introduced results it was also stated that the application of the first variant of the freezing process (blanching in water, final temperature -18°C) caused in most cases a little lower decrease of the activity than after freezing according to the second variant (blanching with steam, final temperature -35°C). Similar decrease of antioxidant activity after freezing at -30°C (water-blanching) for water soluble pea (by 30%) and spinach (by 50%) constituents was observed by Hunter & Fletcher [2002].

After steam-cooking of the frozen peas the antiradical activity of the extracts was lower by approx. 20% comparing to the results obtained for the cooking of fresh pea. A slight rise of the activity (by 6%) was observed only for water extracts of the cooked material after freezing at -35°C against ABTS$^{\bullet+}$ radical cations.

The activity of fresh pea in case of emulgated linoleic acid oxidation was running at a low level (from 3 to 23%) and in the reaction catalyzed non-enzymatically as well as enzymatically more active were substances extracted by water than acetone (two and three times, respectively). Moreover, both fresh material extracts investigated more efficiently slowed down the linoleic acid oxidation process in the system catalyzed by hemoglobin than lipoxygenase.

In the antioxidative action of the samples subjected to freezing in both variants of the process bigger differences in activity were found towards linoleic acid peroxides than in the case of the activity towards ABTS$^{\bullet+}$ and DPPH$^{\bullet}$. Better ability to inhibit the reaction of linoleic acid emulsion autoxidation maintained the extracts derived from the pea blanched in water and frozen at -18°C, however after this it was decreased by over 40 and 60% (for water and acetone extracts, respectively). A reverse relation was observed in the extracts action towards linoleic acid peroxides formed in the reaction catalyzed by lipoxygenase. Freezing at -18°C caused a complete change of acetone extracted compounds effective in this system (prooxidativ e action) and a decrease of the water extract activity by 85%. Some growth of activity appeared for acetone extracts obtained for the pea frozen at -35°C and its lower loss for water extracts after this type of process comparing with freezing at -18°C was stated.

Cooking of pea after preservation by freezing and storage brought in both variants no change or an increase of antioxidant activity of water extracts towards linoleic acid peroxides on the contrary to acetone extracts, among which only the one obtained after freezing at -35°C exhibited a growth of activity in the reaction catalyzed non-enzymatically.

The activity of pea seeds constituents towards DPPH$^{\bullet}$ radicals is mostly related to nitrogen compounds extracted by water, for correlations between the activity of water extracts (DPPH) and the solubility of nitrogen (SN), non-protein nitrogen content (NPN) and water extract chelating ability (C) were found (DPPH = 0.206 – 2.227/SN, α=0.01, r=-0.974; DPPH = 0.028 + 0.005*NPN, α=0.05, r=0.855; DPPH = -0.114 + 0.032*C, α=0.1, r=0.762) while no dependency was found between acetone extracts activity and total polyphenols content or condensed tannins content on the contrary to ABTS$^{\bullet+}$ radicals, where only correlation between the activity of acetone extract (ABTS) and total polyphenols content (PP) was proved (ABTS = 0.027 + 0.006*PP, α=0.1, r=0.797). Among the activities towards linoleic acid peroxides, a correlation was found between the activity of water extract in the system catalyzed by hemoglobin (LOOH) and non-protein nitrogen content (NPN): LOOH = -7.529 + 1.056*NPN, α=0.05, r=0.821, which may be a bit surprising while proteins seem to be more important antioxidants in emulsified systems because of their surface properties, in this case probably deactivation of the prooxidative factors in the water phase was more important.

CONCLUSIONS

1. The processes of freezing and cooking caused a decrease of the investigated antioxidative compounds extractability and a subsequent loss of activity of the extracts, however some cases of the activity growth were noted as well.

2. Freezing at -18°C combined with water-blanching seems to provide slightly better antiradical activity, while freezing at -35°C with steam-blanching – the activity against linoleic acid peroxides.

3. The final (steam-cooked) product best maintained the activity when cooking fresh material (which in the case of green pea is not quite possible for the consumer) and then in the case of cooking steam-blanched and frozen at -35°C pea.

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WPŁYW RÓŻNYCH PARAMETRÓW ZAMRAŻANIA ORAZ GOTOWANIA NA PARZE NA PRZECIWUTLENIAJĄCE WŁAŚCIWOŚCI ZIELONEGO GROSZKU

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W pracy badano wpływ procesu zamrażania prowadzonego w dwu wariantach (-18°C, blanszowanie wodne i -35°C, blanszowanie parowe) oraz procesu gotowania na parze materiału świeżego i przechowywanego w stanie zamrożonym na aktywność przeciwutleniającą groszku zielonego. Przeciwutleniacze aminowe ekstrahowano wodą, zaś fenolowe 70% acetonem. Zastosowane procesy spowodowały utratę zawartości azotu ogółem, jego rozpuszczalności, zawartości azotu niebiałkowego, polifenoli ogółem i tanin skondensowanych, jednakże ich wpływ na aktywność przeciwutleniającą wobec rodników $ABTS^{\bullet+}$ i $DPPH^{\bullet}$ oraz wobec kwasu linolowego poddanego utlenianiu nieenzymatycznemu przez hemoglobinę i enzymatycznemu przez lipooksygenazę był niejednoznaczny – odnotowano zarówno spadek, jak i wzrost aktywności w przypadku różnych procesów.