The influence of extrusion on loss of and racemization of amino acids

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Abstract. The influence of the operation conditions (temperature and residence time) of a thermic treatment on the total amount (free and protein-bound) of amino acid enantiomers of dry fullfat soya was investigated. Total amino acid content was determined using conventional ion-exchange amino acid analysis of total hydrolyzates and chiral amino acid analysis was performed with HPLC after precolumn derivatization with o-phthaldialdehyde and 1-thio-β-D-glucose tetraacetate. Contrary to corn that was investigated previously notable racemization was detected even at lower temperatures. At $140\,^{\circ}\mathrm{C}$ the ratio of the D-enantiomer was 0.87% for glutamic acid, 2.81% for serine and 1.92% for phenylalanine, at $220\,^{\circ}\mathrm{C}$ the ratio of the D-enantiomer in the case of the above amino acids was 1.43%, 4.61% and 4.68%, respectively. The concentration of several L-amino acids decreased. At $220\,^{\circ}\mathrm{C}$ there was 10% less L-glutamic acid,

17% less L-serine, 5% less L-phenylalanine, 6.6% less L-aspartic acid and 21% less L-lysine than in the control and their loss can be assigned to the L – D conversion in a different degree. While transformation of L-phenylalanine nearly completely can be attributed to the racemization, the main cause of the loss of L-lysine is not racemization. The treatments in the same order of magnitude resulted in the formation of more D-amino acids and greater extent of racemization of amino acids in fullfat soya than that of maize.

1   Introduction

Epimerization (partial racemization) of protein-bound and free amino acids may occur during the processing of food if the operation conditions involve application of heating and/or alkaline conditions [11, 12, 32]. Heating in alkaline medium has been shown to yield significant amount of D-amino acids through the mechanism of base-catalyzed racemization [10, 18, 19, 21]. Albeit the medium is usually neutral or weakly acidic during food processing, reducing sugars can induce partial racemization of free L-amino acids in the course of the Maillard reaction [3, 8], and D-amino acids can also be formed via the mechanism of acid-catalyzed racemization [9, 18]. Epimerization of L-aspartic acid can also occur through transpeptidation reactions [14].

The digestibility of the proteins decreases when significant ratio of the protein-bound amino acids is in the D-configuration due to the stereospecificity of the proteinases and peptidases [11, 12, 31]. The rate of absorption can be discriminative to D-amino acids [25, 29] and the bioavailability due to the restricted efficiency of D-amino acid oxidase system can be diminished [22, 24]. The activity of D-amino acid oxidases depends on several factors (species, age, organ, tissue, substrate) and there is a big variation in the efficiency of utilization of the D-amino acids among species [12]. In the case of mammals only small ratio of the D-amino acids were utilized following oral consumption and the D-stereoisomers of the essential amino acids in some cases caused growth inhibition and were mainly excreted in the urine [22]. The value of relative oral bioavailability (RBV) for D-Met is only 30% for humans [1]. From a nutritional standpoint, racemization could result in the loss of protein that is one of the most valuable components of the food.

On the other hand the oral consumption of D-serine, lysinoalanine [4, 15, 16] and D-proline [17] have been claimed to induce histological changes in the rat kidney while others found no sign of organic disorders in the case of D-proline and D-aspartic acid [28]. Recently it has been shown that D-

amino acids that are present in the different organs and tissues of animals and humans have specific biological functions. In the central nervous system, D-serine and D-aspartate occur in considerable concentrations [13]. D-serine is synthesized and metabolized endogenously by human serine racemase [23] and the same might account for D-aspartate. Some part of D-serine in brain can be originated from exogenous source through the blood-to-brain transfer [2].

The food industry is nowadays aware of the potential risk of the treatment of proteinaceous food, and the aim is to define conditions when the aim of the treatment is completed without significant change in the structure of the biological valuable components.

During thermic processing the integrity of food components is disrupted due to the effect of heat and pressure and a spongoid structure is formed. Heat sensitive antinutritive factors are totally or partially inactivated, and the number of microorganisms is also diminished. The task is to determine the conditions of good manufacturing practice in which the above-mentioned aims are accomplished without significant loss of amino acids. The decrease of the amino acid content in corn grain due to extrusion has been investigated, but in these studies the ratio of the enantiomers was not determined [26, 27]. In our previous experiment the influence of extrusion conditions on the D-amino acid content of corn was investigated [30]. In the present work the thermic treatment of an important vegetal protein source is investigated and the results are compared.

2   Materials and methods

2.1   Extrusion

The raw material of the extrusion was fullfat soya (Glycine max (L.) Merr., 'Borostyán' variety). The basic chemical composition was as follows: the dry matter content was 98.1% and the ash content 4.5% (g/100 g sample). Fullfat soya consisted of 33.7% crude protein, 22.9% crude fat; 3.4% crude fibre and 33.6% nitrogen-free extractable material. The starch content was 5.4% and the total sugar content 8.9%. The amino acid content of fullfat soya and the amino acid composition of its proteins can be seen in *Table 1.*

Table 1: The amino acid content and the amino acid composition of untreated fullfat soybean grain

Amino acid	AA content (g AA/ 100 g sample)	AA composition (g AA/ 100 g protein)
Asp	3.83	11.6
Thr	1.25	3.8
Ser	1.77	5.3
Glu	6.39	19.3
Pro	1.73	5.2
Gly	1.57	4.7
Ala	1.51	4.6
Cys	0.38	1.1
Val	1.43	4.3
Met	0.50	1.5
Ile	0.96	2.9
Leu	2.57	7.8
Tyr	1.36	4.1
Phe	1.64	4.9
Lys	2.26	6.8
His	1.10	3.3
Arg	2.47	7.5
NH_3	0.42	1.3

The raw material was ground with a hammer grinder and the particle size distribution was determined. Due to the high oil content conditioning was not necessary prior to extrusion. Ten kg of material was used for each trial. Extrusion was carried out using a Do-Corder DC 2001 type Brabender machine equipped with a 19 mm i.d. barrel (21:1 length to diameter ratio); a screw with the length of 400 mm with increasing screw diameter from 12 to 17 mm, and a cylindrical die which consists of two parts: a 55 mm long by 8 mm i. d. following a 22 mm long by 5 mm. The barrel and the die were heated by electrically controlled split ring resistance heaters, and the screw speed was also kept under control. The barrel and the die temperatures were monitored by thermocouples mounted in shallow wells. Extrusion trials with the full

cross-classification of the applied nominal temperature and screw speed levels *(Table 2)* were repeated three times on three different days. From the two reported zone temperatures (T_1, T_2), one value was calculated (T) to characterize the effect of temperature. Minimum residence time was determined by introducing a small amount of dye into the feeding port and measuring the time required for the first colored extrudate to exit the die. Prior to sampling, the machine was allowed to equilibrate to the desired temperature, then appr. 200 g sample was collected and allowed to cool down before being homogenized, and sealed in polyethylene bags and stored at $-20\,°C$. Control samples were taken from each batch and treated in the same way as extruded samples.

Table 2: Nominal and measured properties of extrusion of fullfat soya

Levels	T_{nom} (°C)	T_{meas} average±s.d. (°C)(n=12)	Screw speed (s^{-1})	Residence time (s) average±s.d. (n=12)	Throughput (kg/h) average±s.d. (n=12)
1	100	101±4	50	29±0.2	1.6±0.4
2	140	140±3	90	17±0.2	2.8±0.8
3	180	180±3	130	12±0.8	4.1±1.1
4	220	220±3	170	10±1.4	4.8±1.4

T_{nom} = Nominal temperature
T_{meas} = Measured temperature
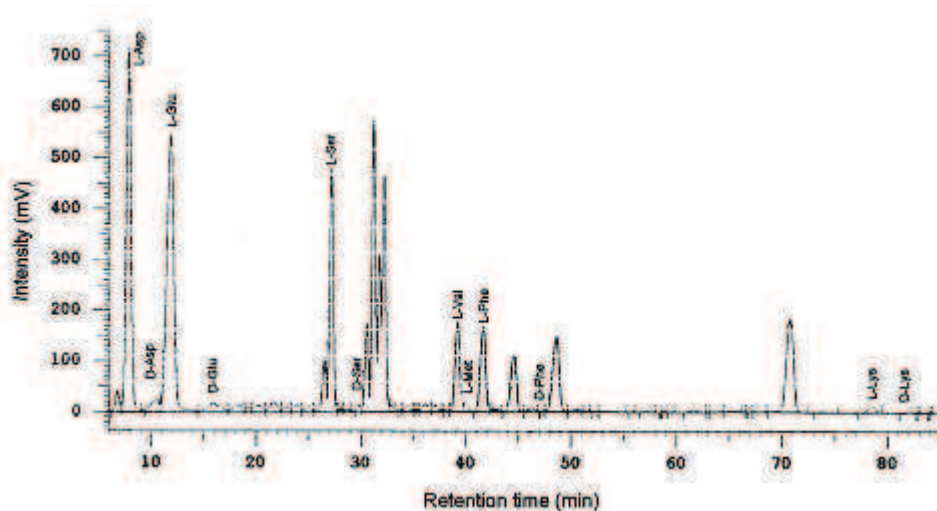
2.2   Chemical analysis

The moisture content was determined with the standard procedure of MSZ ISO 1442, the crude protein content measurement based on the basic method of Kjeldahl (MSZ EN ISO 5983-1:2005). Crude fat (MSZ 6369-15:1982), total ash (MSZ ISO 749:1992), crude fibre (MSZ ISO 6865:2000), starch (MSZ 6830-18:1988) and total sugar content examinations (MSZ 6830-26:1987) were carried out with the use of standard procedures approved by the Hungarian Standards Institution.

Prior to amino acid analysis the samples were dissolved in hydrochloric acid (6 M; 5 cm^3) and proteins were hydrolyzed at $105\pm1\,°C$ for 24 h. The amino acid content and composition was determined with an INGOS AAA 400 amino acid analyzer (INGOS, Praha, Czech Republic) equipped with a 35×0.37 cm

column packed with OSTION Lg ANB.

The concentration of the amino acid enantiomers was also determined from the total hydrolizate of the samples. After cooling, the pH was set to pH = 7 with sodium hydroxide solution, then diastereomers were produced with OPA (*o*-phthaldialdehyde) and TATG (1-thio-β-D-glucose tetraacetate) (Sigma, St. Louis, MO, USA) during precolumn derivatization and separated with HPLC following detection with a fluorescence detector as described elsewhere [5, 7, 30]. A typical chromatogram of the derivatives of the examined L- and D-amino acids of soybean can be seen in *Figure 1*. Before the analysis of soybean samples standard solutions of D- and L-amino acids were derivatized and analyzed, and calibration curves were established and response factors were calculated for each analyzed component. The amount of the amino acid enantiomers of the samples was calculated based on these calibration curves. Moreover, the concentration of the enantiomers was determined after the hydrolysis of the total amount of samples thus the sum of free and protein-bound amino acids was determined.

Figure 1: The chromatogram of the OPA/TATG derivatives of the amino acid enantiomers obtained from hydrolyzed fullfat soya proteins

See conditions in the text.

2.3   Statistical analysis

Data analysis was carried out with the use of SPSS for Windows 10.0 (1999) statistical program (Statistical Package for Social Sciences). There were four levels of temperature factor and four levels of screw speed factor. The number of replication was three; sampling was repeated on three different days with the full cross-classification of the applied levels of factors. The extent of racemization was defined as the percentage of the D-enantiomer within the total (D+L) amino acid content (D/(D+L)×100) [12]. In the above formula 'D' and 'L' means the concentration of the D- and L-enantiomer of the given amino acid calculated with the use of the relevant response factors. The influence of temperature and residence time on the D-amino acid content and the degree of racemization were evaluated with multiple analysis of variance. The equation of the used linear model was the following:

$$Y_{ijk} = \mu + T_i + F_j + TF_{ij} + e_{ijk}$$

Where:

Y_{ijk}	=	the kth observation in the ij^{th} treatment combination,
μ	=	the least squares mean,
T_i	=	the effect of the ith class of factor T (temperature) expressed as a deviation from μ,
F_j	=	the effect of the jth class of factor F (screw speed) expressed as a deviation from μ,
TF_{ij}	=	the interaction effect of the ith class of factor T and the jth class of factor F expressed as a deviation from $\mu + T_i + F_j$ and
e_{ijk}	=	the random error associated with the kth observation in the ij^{th} treatment combination.

If the treatment means differed significantly ($P<0.05$), the comparison of that was accomplished with the Student-Newman-Keuls test.

3   Results

3.1   The influence of extrusion on the D-amino acid content of fullfat soya

Similarly to extrusion of corn residence time did not change when the same screw speed was used at different temperatures [30]. Thus residence time and screw speed could be regarded as traits substituting each other without con-

founding with temperature when their influence on the D-amino acid formation was analyzed *(Table 2)*.

In order to determine the D-amino acid producing capacity of extrusion, the measured D-amino acid values of treated samples have to be corrected with the D-amino acid content of control samples [31] because some formation of D-amino acids during acidic hydrolysis of proteins occurs [6, 21]. There were significant differences in the D-glutamic acid, D-serine and D-phenylalanine content of soybean treated at different temperatures (P<0.05). The amount of these amino acids showed a notable increase when the extrusion temperature was increased from 101 to 140 °C *(Table 3)*, and their concentration was higher in the products extruded at 140 °C than in control samples without extrusion. The variance of D-aspartic acid content was higher than that of the other amino acids and thus significant differences cannot be detected. In the case of corn samples the extrusion on the same instrument below 144°C of 28–72 s did not induce significant (P<0.05) racemization [30].

Table 3: Influence of the extrusion temperature on the total (free and protein-bound) D-amino acid content of fullfat soya (mg/100 g dry matter)

Average ± s.d.[1,2] (n=12)	Temperature (T)			
	101 °C	**140 °C**	**180 °C**	**220 °C**
D-Glu	40[a]±9	57[b]±13	65[b]±24	89[c]±20
D-Ser	29[a]±27	49[b]±20	67[c]±12	74[c]±12
D-Phe	5[a]±23	27[b]±16	42[b]±18	66[c]±10
D-Asp	29[a]±17	20[a]±56	47[a]±92	83[a]±106

[abc] Averages in one row with common superscript do not differ (P≥0.05).
[1] Corrected with control values obtained from untreated fullfat soya.
[2] Averages and standard deviations of samples extruded at the same temperature with different residence times.

The influence of the screw speed (residence time) on the D-amino acid content was not significant at the investigated range.

High-temperature treatments related to the low-temperature treatments resulted in more significant increase of the extent of racemization (D/(D+L)×100, *Table 4)* than that of the amount of D-amino acids *(Table 3)* because the L-amino acid concentration decrease *(Table 5)* exceeded the D-amino acid concentration increase. Namely besides izomerization the intensity of other pro-

cesses that alter the structure of the amino acids was also significant. In the case of corn the loss of aspartic acid and lysine was reported during high temperature extrusion [26, 27, 30].

Table 4: Influence of the extrusion temperature on the degree of partial racemization of the examined amino acids in corn (D/(D+L)×100)

Average ± s.d.[1,2] (n=12)	Temperature (T)			
	101 °C	140 °C	180 °C	220 °C
Glu	$0.57^a \pm 0.13$	$0.87^b \pm 0.18$	$0.99^b \pm 0.38$	$1.43^c \pm 0.36$
Ser	$1.51^a \pm 1.41$	$2.81^b \pm 1.04$	$3.90^c \pm 0.70$	$4.61^c \pm 0.69$
Phe	$0.23^a \pm 1.56$	$1.92^b \pm 1.18$	$2.88^b \pm 1.17$	$4.68^c \pm 0.72$
Asp	$0.69^a \pm 0.40$	$0.44^a \pm 1.35$	$1.06^a \pm 2.15$	$1.93^a \pm 2.46$

[abc] Averages in one row with common superscript do not differ (P≥0.05).
[1] Corrected with control values obtained from untreated fullfat soya.
[2] Averages and standard deviations of samples extruded at the same temperature with different residence times.

Table 5: The total (free and protein-bound) L-amino acid content of soybean treated at different temperatures (g/100 g dry matter)

Average ± s.d.[1] (n=12)	Control	Temperature (T)			
		101 °C	140 °C	180 °C	220 °C
L-Asp	$3.70^b \pm 0.17$	$3.77^b \pm 0.39$	$3.59^{ab} \pm 0.21$	$3.56^{bc} \pm 0.15$	$3.38^a \pm 0.28$
L-Glu	$6.74^b \pm 0.26$	$6.73^b \pm 0.52$	$6.41^{bc} \pm 0.42$	$6.43^{bc} \pm 0.35$	$6.05^a \pm 0.53$
L-Ser	$1.83^c \pm 0.06$	$1.82^c \pm 0.15$	$1.69^b \pm 0.10$	$1.66^b \pm 0.06$	$1.52^a \pm 0.10$
L-Val	$1.53^{bc} \pm 0.06$	$1.57^c \pm 0.08$	$1.50^{bc} \pm 0.09$	$1.45^{bc} \pm 0.07$	$1.38^a \pm 0.13$
L-Met	$0.54^a \pm 0.01$	$0.55^a \pm 0.07$	$0.55^a \pm 0.05$	$0.56^a \pm 0.03$	$0.55^a \pm 0.04$
L-Phe	$1.40^{bc} \pm 0.04$	$1.46^c \pm 0.10$	$1.37^{bc} \pm 0.16$	$1.41^{bc} \pm 0.06$	$1.34^{bc} \pm 0.08$
L-Lys	$2.39^b \pm 0.39$	$2.10^b \pm 0.30$	$1.96^a \pm 0.19$	$1.99^a \pm 0.22$	$1.87^a \pm 0.23$

[abc] Averages in one row with common superscript do not differ (P≥0.05).
[1] Averages and standard deviations of samples extruded at the same temperature with different residence times.

The concentration of most of the L-amino acids under the scope of the study decreased when the temperature of the heat treatment increased *(Table 5)*. The sample extruded at the highest temperature contained 6.6% less L-aspartic acid than the control. The amount of the formed D-enantiomer

(0.08 g/100 g) was account for 25% of the difference (0.32 g/100 g). In the case of L-glutamic acid and L-serine there was a concentration decrease of 10% and 17%, respectively, and 13% (L-serine: 24%) of the loss can be attributed to formation of the D-enantiomers. The degree of the decomposition of L-phenylalanine (5%) was almost the same as the extent of the formation of the D-enantiomer. The highest concentration decrease was detected in the case of L-lysine (21%). Since the degree of racemization of lysine was less than 2%, it can be account for not more than 8% of the concentration decrease of L-lysine (0.52 mg/100 g). Similarly like in the case of corn the main cause of the loss of L-lysine is not racemization.

3.2 Comparison of the effect of extrusion on soya and corn with respect to the formation of D-amino acids

Thermic treatment of fullfat soya resulted in higher amount of D-amino acids related to dry matter than in the case of corn. It can be attributed to the fact that the protein content of fullfat soya is about four-fold higher than that of corn. Contrary to D-amino acid content, the D/(D+L)×100 ratio does not depend on the absolute amount of protein. As the ratio of the amino acids that are most susceptible to racemization (that is serine, glutamic and aspartic acid) is similar in soya and in corn proteins, their common transformation can be investigated. The degree of partial racemization of these amino acids in soya extruded at 180 °C was slightly higher than that of corn at 200 °C. Thus similar heat treatment seems to cause a higher extent of L–D amino acid conversion in the soya proteins than in corn proteins.

The influence of the screw speed (residence time) on the D-amino acid content and racemization was not significant in the case of either of the raw material. It can be attributed to the fact that in the function of screw speed there was only three-fold change of the residence time. 10°C temperature increase resulted in 2.2–5.5-fold increase in the first order reaction rate constant (k) of amino acid racemization [12]. Due to the relationship of reaction time (t) and 'k' in the first order reaction kinetic equitation of racemization, three-fold residence time increase exert about the same effect on the D-amino acid content than 10 °C temperature increase. Therefore within the examined temperature and time intervals, the change in the treatment temperature has only visible effect on the racemization stage of the proteins and the D-amino acid content of products.

3.3 Comparison of the effect of extrusion on soya and corn with respect to the loss of L-amino acids

The rate of contribution of racemization and the other processes to the loss of L-amino acids seems to depend both on the sort of the amino acid and the type of the protein source. In the case of soya the 'non-racemization loss' of L-aspartic acid, L-glutamic acid, L-serine and L-lysine related to the whole concentration decrease was 75, 87, 76 and 92%, respectively. In the case of corn 22% of the loss of L-Asp and 98% of the concentration decrease of L-Lys was not related with racemization only with other processes. Due to heat treatment alteration of side chains of the amino acids and crosslink formation can occur, e.g. serine (after β-elimination as dehydroalanine) and lysine can form lysinoalanine, the side chain of asparagine and glutamine can form imide-type crosslink with lysine, the carboxyl group of acidic amino acids esterify the hydroxyl group of serine. Furthermore, the loss of lysine can also be attributed to the reaction of the ϵ-amino group with reducing sugars in the Maillard reaction. In contrast with the above four amino acids the degree of the concentration decrease of L-phenylalanine was practically the same as the amount of D-enantiomer formed, namely there was no significant concentration decrease due to other processes than racemization.

Both in soya and in corn the decomposition of L-lysine was the highest among amino acids. The loss of L-amino acids was more significant in case of soya than that of corn. High temperature ($200\,^\circ$C) extrusion of corn reduced the amount of L-lysine and L-aspartic acid, while in soya significant decrease of the following other amino acids were also detected such as L-serine, L-glutamic acid and L-phenylalanine. The ratio of lysine within the soya protein (6.8%) was almost three-fold higher than that of corn protein (2.5%) and the main cause of the loss of L-lysine was not the racemization but other processes. Supposedly the greater amount of lysine in the soya protein can form more crosslinks with serine and the acidic amino acids than in corn protein and thus the L-amino acid loss could be higher in the case of soya. This hypothesis is supported by the fact that contrary to corn when racemization is the main cause of the loss of L-aspartic acid (78%), the concentration decrease detected in soya can be attributed to a lesser extent to racemization (25%) than other reactions (75%).

In sum dry extrusion of fullfat soybeans can result in significant loss of the amino acids. Within the decomposition the ratio of racemization and that of the other processes was evaluated. Among essential amino acids the concentration decrease of lysine was the most significant (21%). In nutritional point

of view one can avoid drawing considerable consequences because analytical results only gave gross values and utilization of amino acids depend on several factors. This study pointed out the need of conducting biological tests in order to estimate the possible loss of the bioavailability of amino acids of fullfat soybean due to dry extrusion at different species.

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