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The influence of disposal technology obtained with alkaline treatments on D-amino acid content of slaughterhouse waste

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Abstract. One possibility of disposing of animal proteins is alkaline hydrolysis that can be applied even for waste materials infected with *transmissive encephalopathy* since the alkaline hydrolysis decomposes the prions therefore it proved to be especially effective for the treatment of materials of animal origin of the highest risk. In our experiment the change in D-amino acid content of slaughterhouse waste due to the treatments was examined. The treatments were done with sodium and potassium hydroxide solution, respectively, for 2, 3 and 6 hours at 135, 150 and 153 °C. D-Asp, D-Glu,and D-Trp content was determined using a *Hitachi Merck LaChrom HPLC*, and D-allo-Ile content using an *INGOS* amino acid analyzer. In case of tryptophan the hydrolysis was carried out with 3 M p-toluenesulfonic acid in the presence of 3-indolylpropionic acid, in other cases the 6 M hydrochloric acid hydrolysis was applied. Hydrolysis temperature was in both cases 110±2 °C. Summarized, it can be said that due to the heat and alkali combinations we used aspartic acid, glutamic acid, tryptophan and isoleucine transform in 40-50% into the D-isomer. Even though the hydrolysed product obtained this way met in other parameters the requirements of the modern feeding, one should be expect that most of the amino acids undergo full racemization during this process.

1 Introduction

Waste materials are necessary by-products of the modern civilization and everyday's life. By producing more and more waste materials humans significantly disturb the natural balance. Therefore one of the most important tasks of environmental protection is solving the problem of waste materials. According to the recent standpoints such strategies should be elaborated whose aim is to keep the sources of the nature and protect the environment. As basic target was defined the reduction of the amount of pollutive materials by recycling them with new, modern technologies.

Animal by-products include mostly slaughterhouse waste of ungulated farm animals and poultries since consumption of meat of these animals continuously increased during the last 20 years. Animal by products were processed earlier into meat-flour and industrial fat. Meat- and bone-flour were potential sources of animal protein in feeding. Animal proteins have high biological value, their composition is much more favourable than that of yeast, soybean or sunflower. But after the appearance of *bovine spongiform encephalopathy* (BSE) the use of animal proteins was banned in animal feeding. Vegetable proteins can be of high biological value in animal feeding if well combined with each other.

Recently meat- and bone flour are used for fertilizing, animal proteins are

used for energy production in increasing amounts. Energy is very important nowadays in both economic and social respect. Today's technologies are not suitable yet so that the polluting materials can be used as source of energy without dangering the environment. In order to achieve this, further researches are necessary, among which developments associated with animal proteins are of special importance. Alkaline hydrolysis is a possibility for the treatment of by-products of animal origin which can provide a solution in case of proteins.

In its entirety, waste of animal origin does not represent a significant problem in waste material processing, however, the contamination of soil, water and air that can be caused by improper treatment of animal waste, can be dangerous to our environment (*Vučemilo et al.*, 2003).

According to the data of FAO the world's meat production reached 250 million tons per year in 2004 which is continuously growing. In the Republic of Croatia the number of slaughtered animals hardly changed during the passed 10 years, around 37 million chickens, 100 thousand cattles, 900 thousand pigs per year (*Vrabec*, 2004). In Hungary in 2006 the quantity of slaughtered animals was as follows: beef-cattles 84 thousand tons, hogs 613 thousand tons, poultries 632 thousand tons (*KSH*, 2007). If we take into consideration that around 50-58% of these quantities can be utilized in the human nutrition, it can be established that around 25% of the body mass of slaughtered animals is a slaughterhouse waste.

With the appearance of BSE Europe faced a huge problem, as processing of waste material that can be brought into connection with BSE is strictly forbidden. By the introduction of the new EU regulations (*EU Directive* 1774/2002) slaughterhouse waste has been categorized into three categories (high, medium and low risk) and conditions of waste treatment were defined. As a result of the restrictions the protein demand of animal breeding considerably increased and also the demand for elaborating new methods for the treatment of waste of animal origin. Such a possiblity is the alkaline hydrolysis of animal proteins which can be applied even in case of waste infected by transmissive encephalopathy. The procedure has been applied in the United States at the Albany Veterninary School since 1993 (*Kaye et al.*, 1998). There are several results showing that alkaline hydrolysis destructs the prions (*Ernst*, 1993; *Tagochi et al.*, 1991; *Taylor et al.*, 1999; *Taylor*, 2000), therefore it proved to be especially effective in the treatment of materials of animal origin of the highest risk.

The alkaline treatment is a procedure during which due the alkaline medium the high molecular proteins disintegrate into smaller peptides, free amino acids. This is a very important step e.g. in the disintegration of prion pro-

teins causing BSE. The alkaline hydrolysis is carried out with NaOH or KOH. The process can be accelerated by heating, where 150°C is considered to be the optimal temperature. The reaction takes place in a steel reactor under continuous stirring at 4 bar pressure in 3-6 hours (*92*/2005/EEZ). During the alkaline hydrolysis the peptide bonds are cleaved and depending on the alkali used peptides with low molecular weight and potassium or sodium salts of free amino acids are forming (*Neyens et al.*, 2003).

It was obtained in earlier investigations that every impact involving high temperature alkaline treatment results in racemization of most of the amino acids (*Imai et al.*, 1996; *Man and Bada*, 1987; *Friedman*, 1991). When treating the proteins with alkali at low temperature or the hydrolysis is carried out at high temperature in neutral or acidic medium, this can also cause racemization, but the combination of alkaline treatment and high temperature results in racemization almost surely. In the course of our research the racemization of amino acids including epimerization of isoleucine was examined in the products of an experiment targeting to render harmless slaughterhouse waste.

2 Materials and methods

Treatment of the samples

In the experiments ox brain samples obtained from Croatia were used. As no cases of BSE were recorded in Croatia and the samples were taken from cattles younger than 30 months old, no prion analysis was conducted.

After sampling the samples were stored at −20°C, then after defrosting they were homogenized and divided into 400 g parts. During treatments to 400 g of sample 600 cm^3 of distilled water and 44 cm^3 of 45% KOH or 30 cm^3 of 45% NaOH solution was added. Accordingly, to the control sample 644 and 630 cm^3 of distilled water was added. The alkaline mixtures were heated at different temperatures and under different pressures (135°C, 2.75 bar; 150°C, 4.78 bar and 153°C, 5.17 bar) and after 2, 3 and 6 hrs sample was taken from the reactor. At the bottom of the reactor a magnetic stirrer ensured the homogenity of the solution. After the treatment the samples were centrifuged at 5,000 rpm for 2 min., the sediment of approx. 0.5% was removed, and the liquid phase was stored in a deep-freeze. Out of each treatments the measurements were carried out in 3 repetitions. Along with the control altogether 19 treatments were carried out. Parameters of the treatments are summarized in *Table 1.*

Table 1: Alkaline treatments applied

Control samples			C
Alkali used	Treatment temperature, °C	Treatment time, hours	Marking
NaOH	135	2	N1
		3	N2
		6	N3
	150	2	N4
		3	N5
		6	N6
	153	2	N7
		3	N8
		6	N9
KOH	135	2	K1
		3	K2
		6	K3
	150	2	K4
		3	K5
		6	K6
	153	2	K7
		3	K8
		6	K9

Hydrolysis conditions

In order to release the amino acids the hydrolysis was carried out using 6 M hydrochloric acid. As tryptophan decomposes under acidic conditions and alkaline conditions lead to racemization, we applied a less rigorous acidic medium during the determination of the Trp enantiomers. The hydrolysis mixture was a 3 M p-toluenesulfonic acid solution containing 3-indolylpropionic acid. The protein/3-indolylpropionic acid ratio was set to the value of 1:1 (*Liu and Chang*, 1971; *Gruen and Nichols*, 1972).

In both cases the hydrolyses were carried out in closed ampoules under nitrogen atmosphere at 110°C for 24 hrs. Th pH of the hydrolysates was set with 4 M NaOH solution.

Derivatization and analysis

Before the analysis the samples were filtrated on a 0.45 μm membrane filter, then from the amino acid enantiomers during precolumn derivatization

with OPA (o-phtaldialdehyde) and TATG (1-thio-β-D-glucose-tetraacetate) diastereomers were formed (*Einarsson et al.*, 1987; *Csapó et al.* 1995).

The derivatization and analysis were carried out using a MERCK-Hitachi HPLC apparatus consisting of the following units: L-7250 programmable autosampler, L-7100 pump, L-7350 column thermostat, L-7480 fluorescence detector, AIA data processing unit for D-7000 HPLC system manager.

Diastereomer derivatives of Asp and Glu were separated on a 125mm×4 mm i.d. Superspher 60 RP-8e column. Composition of the mobile phase was at start 28% (v/v) methanol, 72% (v/v) phosphate buffer (50 mM, pH=7). After 10 min the proportion of acetonitrile was increased from 0% to 17% and at the same time that of the phosphate buffer was decreased to 55%. From the 40. min the proportion of acetonitrile was increased to 40%, whereas that of the phosphate buffer decreased to 36%, and that of methanol to 24%.

For the analysis of the Trp enantiomers a 125 mm×4 mm i.d. Purospher RP-18e column was used. Composition of the mobile phase was as follows: at the start of the measurement 20% (v/v) methanol (remained unchanged during the analysis), 64% (v/v) phosphate buffer (50 mM, pH=7), and 16% (v/v) acetonitrile. After 38 min isocratic elution the proportion of phosphate buffer was decreased to 53% and that of acetonitrile was increased to 27%. From the 50. min the proportion of the phosphate buffer decreased further to 45% and at the same time that of acetonitrile increased to 35%. From the 65. min the starting composition was used again.

For both HPLC separations the columns were thermostated at 40 °C. Flow rates were 1 cm^3/min. The derivatives were detected by fluorescence detector (ex.: 325 nm, em.: 420 nm).

D-allo-isoleucine content was determined using an *INGOS* AAA 400 amino acid analyzer. Separation took place on a 350×3.7 mm, OSTION Lg ANB cation-exchange column. In the amino acid analyzer working on the principle of the ion-exchange chromatography D-allo-isoleucine eluted between methionine and isoleucine and was detected via postcolumn derivatization with ninhydrin.

3 Results

As a result of the treatments the concentration of the individual amino acids in the samples increased compared to the starting materials. The reason for this was that during the treatments the samples lost some amount of solvent and by this they become more concentrated.

Racemization of aspartic acid and glutamic acid

It was established that even during the hydrolysis carried out at the lowest temperature and for the shortest time (135 °C, 2 hrs) more than 40% of both amino acids racemized (Fig. 1. and 2.). Proportion of the D-amino acids is expressed by the formula of D/(D+L)×100 (Table 2. and 3.). After analysis of the control, not heat-treated sample for D-aspartic acid 3.9% and for D-glutamic acid 1.6% was obtained which could be attributed to the intervention prior to the heat treatment and to the racemization occurred during sample preparation and protein hydrolysis, respectively.

It was observed that at the two higher temperatures, during longer treatment the concentration of the amino acids decreased despite the solutions becoming more concentrated the concentration of the amino acids decreased, reason for which was presumably the decomposition of Asp and Glu.

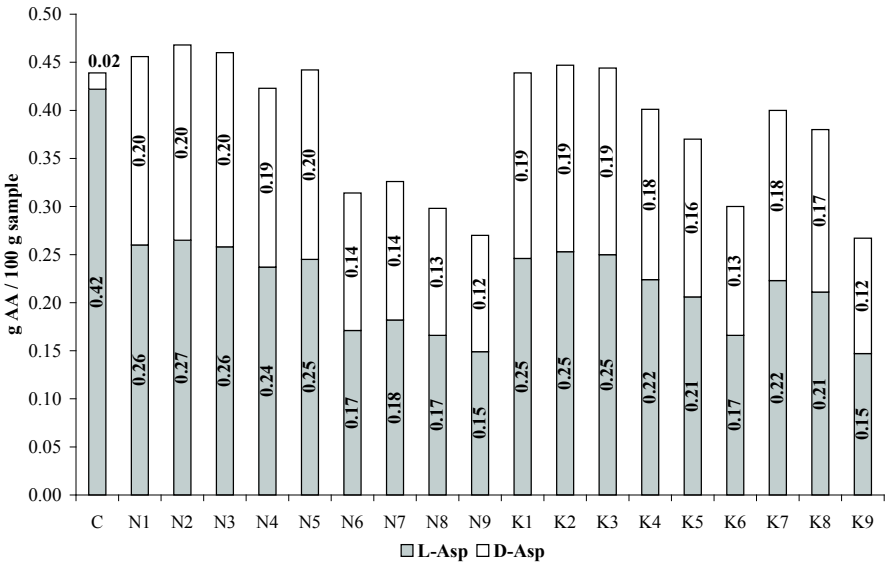

Figure 1: Change in the amount of aspartic acid enantiomers due to alkaline treatment

Table 2: Extent of racemization of aspartic acid due to alkaline treatments

Marking	N1	N2	N3	N4	N5	N6	N7	N8	N9
$\frac{D}{D+L} \times 100$	43.0%	43.4%	43.9%	44.0%	44.6%	45.5%	44.2%	44.3%	44.8%
Marking	K1	K2	K3	K4	K5	K6	K7	K8	K9
$\frac{D}{D+L} \times 100$	43.2%	43.4%	43.7%	44.1%	44.3%	44.7%	44.3%	44.5%	44.9%

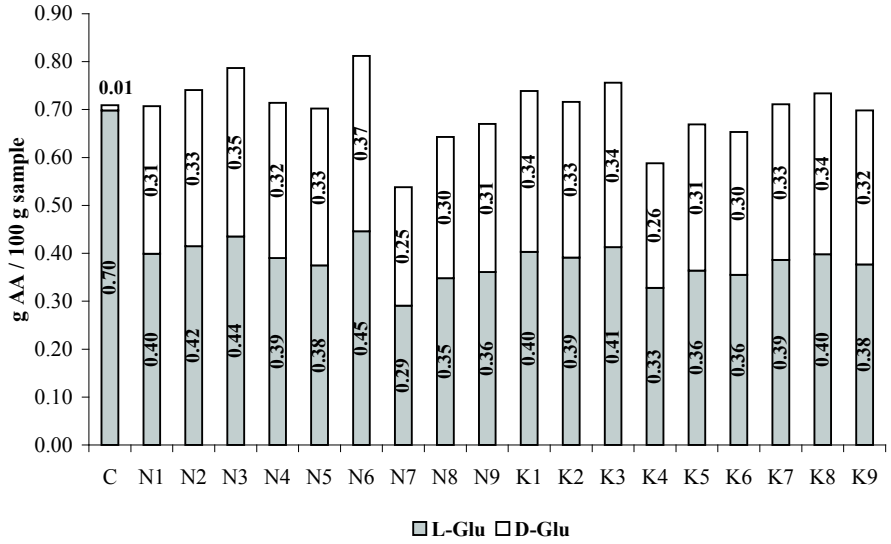

Figure 2: Change in the amount of glutamic acid enantiomers due to alkaline treatment

Table 3: Extent of racemization of glutamic acid due to alkaline treatments

Marking	N1	N2	N3	N4	N5	N6	N7	N8	N9
$\frac{D}{D+L} \times 100$	43.6%	44.0%	44.7%	45.4%	46.6%	45.1%	45.9%	45.9%	46.1%
Marking	K1	K2	K3	K4	K5	K6	K7	K8	K9
$\frac{D}{D+L} \times 100$	45.5%	45.4%	45.4%	44.2%	45.6%	45.6%	45.7%	45.8%	46.0%

Racemization of tryptophan

In case of tryptophan it was necessary to employ another hydrolysis method because tryptophan completely decomposes during the 6 M hydrochloric acidic hydrolysis for 24 hrs due to cleavage of the indole group. During the acidic hydrolysis we applied, using the protecting agent more than 80% of tryptophan

could be recovered from the protein.

Change in the Trp enantiomer content of the samples is shown in Fig. 3, the extent of racemization is given in Table 4. It was found that 39-45% of tryptophan racemized during the heat treatment. Like in case of aspartic acid and glutamic acid, treatment at higher temperature and longer treatment time, respectively, led to decomposition of tryptophan.

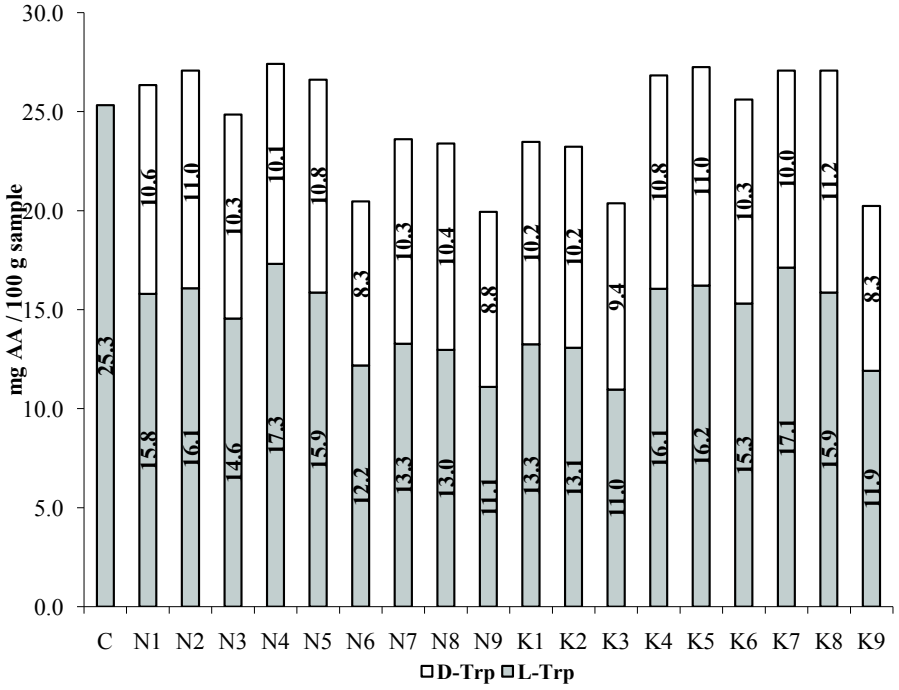

Figure 3: Change in the amount of tryptophan enantiomers due to alkaline treatment

Table 4: Extent of racemization of tryptophan due to alkaline treatments

Marking	N1	N2	N3	N4	N5	N6	N7	N8	N9
$\frac{D}{D+L} \times 100$	40.0%	40.6%	41.5%	36.8%	40.4%	40.4%	43.8%	44.6%	44.3%
Marking	K1	K2	K3	K4	K5	K6	K7	K8	K9
$\frac{D}{D+L} \times 100$	43.5%	43.7%	46.2%	40.2%	40.5%	40.2%	36.8%	41.4%	41.2%

Epimerization of isoleucine

In the last phase of our experiments the epimerization of isoleucine was examined. It was found that the control sample did not contain D-allo-isoleucine even in traces. The results of the treatments are shown in Fig. 4, the extent of the racemization is shown in Table 5. Based on the results it can be said that in the treated samples the total amount of the isomers practically does not change. In contrast with the other three examined amino acids, in case of isoleucine we do not have to reckon with the decomposition of the amino acid.

Examining the extent of the racemization it was established that carrying out the treatment at 135°C with NaOH, the extent of the epimerization of Ile is less than in the other treatments, although it is above 40% also in this case. In the other treatments the racemization can be considered as complete.

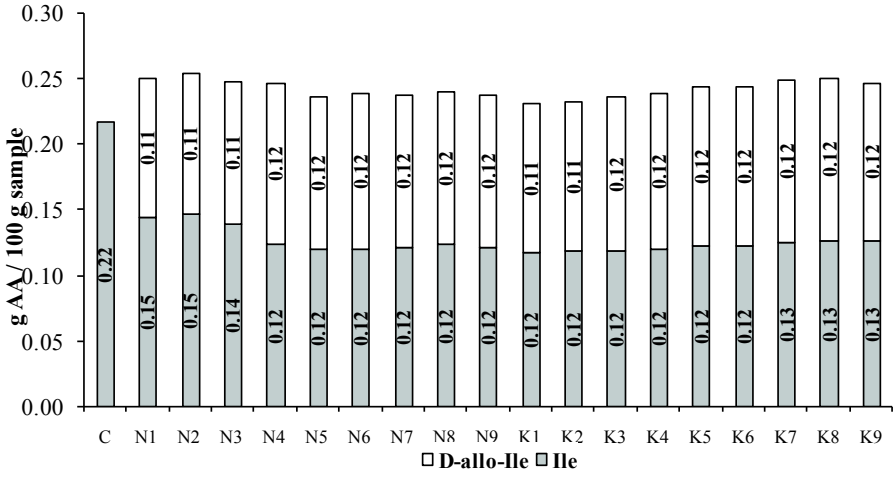

Figure 4: The amount of the isoleucine enantiomers due to alkaline treatment

Table 5: Extent of epimerization of isoleucine due to alkaline treatments

Marking	N1	N2	N3	N4	N5	N6	N7	N8	N9
$\frac{D}{D+L} \times 100$	42.2%	42.4%	43.5%	49.8%	49.4%	49.8%	48.7%	48.3%	48.7%
Marking	K1	K2	K3	K4	K5	K6	K7	K8	K9
$\frac{D}{D+L} \times 100$	48.9%	48.9%	49.6%	49.8%	49.6%	49.6%	49.4%	49.4%	48.6%

4 Conclusions

During our research ox brain samples obtained from Croatia and treated with NaOH and KOH, respectively, at different temperatures for different durations, were analyzed. Aspartic acid, glutamic acid, isoleucine and tryptophan enantiomers of the hydrolysate were determined.

Aspartic acid and glutamic acid were chosen because proteins used in animal feeding contain relatively much of these two amino acids, in some cases their total amount can reach even 30-40% of the crude protein content. Furthermore, these two amino acids were chosen as according to our previous experiences they belong to amino acids racemizing relatively easily, hence they can be used as markers for the estimating the racemization of the other proteinous amino acids. Third reason why we chose these two amino acids was that during the high-performance liquid chromatographic analysis we applied these two amino acids appear at the very beginning of the chromatogram therefore they provide quick information as to whether it is worth to determine the D- and L- enantiomers of the other amino acids as well. If no D-aspartic acid or D-glutamic acid can be detected in the sample, it is not worth continuing with the chromatographic analysis as it is almost for sure that the analyzed material does not contain the D-enantiomers of the other proteinous amino acids, either.

Isoleucine was chosen because the determination of D-allo-isoleucine formed due to the alkaline treatment does not require special analytical procedure, it can be easily analyzed by an automatic amino acid analyzer working on the principle of ion-exchange column chromatography, that can be found in most of the food and feedstuff laboratories, its peak between methionine and isoleucine can be well separated from that of the other proteinous amino acids. On the other hand, this amino acid was chosen because isoleucine belongs – along with leucine and valine – to the amino acids racemizing the hardest, that is, if we can detect D-allo-isoleucine in the sample even in traces, that means that also the other proteinous amino acids underwent racemization with high probability.

Tryptophan was chosen because it is an essential amino acid on the one hand, and the alkaline hydrolysis conditions are favourable for avoiding the decomposition of tryptophan, since the indole group of tryptophan completely decomposes under acidic circumstances. Based on the literature, we can be sure that during the alkaline treatment we applied a considerable amount of tryptophan remains unchanged, it is highly probable, however, that its considerable part racemizes during the alkaline treatment.

Based on the analyses it appears that on the racemization in the temperature range of 135-153 °C as well as between the treatment times of 2-6 hrs neither the temperature nor the treatment time have any considerable effect. This is shown by the fact that between the sample treated with NaOH for 2 hrs and the one treated for 6 hrs at 153 °C only a difference of 1.8% in the ratio of D-aspartic acid was obtained which is a negligible difference compared to the average racemization of 43-45%. The same tendency appears in case of the hydrolysis with KOH where between the hydrolysis carried out at the lowest temperature for the shortest time and the hydrolysis at the highest temperature for the longest time a difference of 1.7% was obtained which is negligible compared to the total racemization of 43-45%. In case of aspartic acid, comparing the treatments with NaOH and those with KOH, no significant difference could be found, that is, in the respect of racemization the two alkaline hydrolyses can be considered as equal. It appears that the hydrolysis carried out at 153 °C for 6 hrs in case of both KOH and NaOH leads to a considerable decomposition of aspartic acid. In case of glutamic acid almost fully similar tendency was obtained as described for aspartic acid. This is not surprising because racemization of these two acidic amino acids - based on our earlier experiences - occurs in a similar way and ratio due to the different technological interventions. In case of the treatment with NaOH including the total temperature and time combinations, the proportion of D-glutamic acid varied between 43.6 and 46.1%, while in case of the treatment with KOH between 44.2 and 46.0%. No difference could be found between the two kinds of alkaline treatment regarding racemization of glutamic acid. Although the difference is not so definite than in case of aspartic acid, it can be established that the hydrolysis carried out at higher temperature for a longer time results not only in racemization but also decomposition of glutamic acid.

In case of tryptophan the situation was more complicated as not the usual protein hydrolysis was applied but the protein treated with alkali was hydrolyzed with 3 M p-toluenesulfonic acid in the presence of protecting agent (3-indolylpropionic acid). Regarding the racemization of tryptophan practically the same can be said as already discussed in case of aspartic acid and glutamic acid. Comparing both hydrolyzing media and all the three time and temperature combinations it was found that the extent of racemization of tryptophan varied between 43.6 and 46.1%. Considerable loss of tryptophan was experienced only in case of the six-hour hydrolysis. Due to the alkaline treatment the amount of tryptophan practically did no change in comparison to the control sample.

Evaluating the results for D-allo-isoleucine it was found that the alkaline treatment carried out at 135°C with NaOH resulted in around by 5-6% less racemization than at 150 and 153°C, respectively. At 135°C the extent of the racemization varied between 42.2 and 43.3%, while at the two higher temperatures between 48.3 and 49.8%. In case of treatment with KOH no such a difference was experienced, the racemization ranged between 48.6 and 49.8%.

As it was expected, isoleucine did not decompose practically in any of the treatments since it is known that the aliphatic side chain amino acids resist almost every technological interventions.

Evaluating the results for the four amino acids it was established that during the alkaline treatment each amino acid racemized in 42-46%, hence in practical respect the obtained material can be considered as racemic mixture of the amino acids. The amino acid content of the samples decreased only in the treatment carried out at the highest temperature for the longest time. As the racemization can be considered as complete even in the treatment carried out at the lowest temperature and for the shortest time and since racemization is a result of the roughest technological interventions, we suppose that a treatment at 135°C for 2 hrs with NaOH or KOH is sufficient for the entire destruction of the protein structure.

Based on the above the almost complete racemization makes the obtained material unfit for being used as animal feedstuff since the higher animals – with the exceptions of ruminants – can utilize only L-amino acids, D-amino acids act as growth inhibitor. Nothing seems to be against, however, that the product of the hydrolysis with KOH, after neutralization, is used as nitrogen fertilizer in the soil.

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