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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***

*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. 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.*
(Keywords: slaughterhouse waste, waste treatment, alkaline hydrolysis, racemization of amino acids, D-amino acids)

## INTRODUCTION

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. The alkaline hydrolysis of animal proteins has been applied in the United States at the Albany Veterinary School since 1993 (*Kaye et al.*, 1998). There are several results showing that alkaline hydrolysis destructs the prions (*Tagochi et al.,* 1991; *Ernst,* 1993; *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 proteins causing BSE.

It was obtained in earlier investigations that every impact involving high temperature alkaline treatment results in racemization of most of the amino acids (*Man and Bada*, 1987; *Friedman*, 1991; *Imai et al.*, 1996). 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.

## MATERIALS AND METHODS

### Treatment of the samples

In the experiments ox brain samples obtained from Croatia were used. 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. Out of each treatments the measurements were carried out in 3 repetitions. Along with the control altogether 19 treatments were carried out. (The samples: N1, N2, N3, N4, N5, N6, N7, N8, N9 and K1, K2, K3, K4, K5, K6, K7, K8, K9; N = hydrolysis with NaOH, K = hydrolysis with KOH).

### Protein hydrolysis conditions for amino acid analysis

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 3 M p-toluenesulfonic acid solution containing 3-indolyl-propionic acid during the determination of the Trp. 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 of amino acid enantiomers

From the amino acid enantiomers during precolumn derivatization with OPA (o-phtaldialdehyde) and TATG (1-thio-β-D-glucose-tetraacetate) diastereoisomers were formed (*Einarsson et al.,* 1987; *Csapó et al.,* 1995). The derivatization and analysis were carried out using a MERCK-Hitachi HPLC apparatus. Diastereomer derivatives of Asp and Glu were separated on a 125 mm×4 mm i.d. Superspher 60 RP-8e column. For the analysis of the Trp enantiomers a 125 mm×4 mm i.d. Purospher RP-18e column was used. The derivatives were detected by fluorescence detector (ex.: 325 nm, em.: 420 nm). L-isoleucine and D-allo-isoleucine content was determined using an INGOS AAA400 amino acid analyzer. Separation took place on a 350×3.7 mm, OSTION Lg ANB cation-exchange column.

## RESULTS AND DISCUSSION

During our research ox brain samples obtained from Croatia and treated with NaOH and KOH, respectively, at different temperatures for different durations, were analyzed. 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.

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. 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. 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.

**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. Proportion of the D-amino acids is expressed by the formula of D/(D+L)×100 (*Table 1.* and *Table 2*). 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.

**Table 1**

**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% |

**Table 2**

**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. The extent of racemization for Trp is given in *Table 3*. 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.

**Table 3**

**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**

It was found that the control sample did not contain D-allo-isoleucine even in traces. The extent of the racemization is shown in *Table 4*. 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.

**Table 4**

**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% |

## CONCLUSIONS

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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