

# HIGH-RISK BIO-WASTE PROCESSING BY ALKALINE HYDROLYSIS AND ISOLATION OF AMINO ACIDS

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Original scientific paper

Slaughterhouse waste can be very dangerous and potential risk for animal and human health. Brains and spinal cords are deemed high-risk substances and can be infected with prions; their treatment is therefore only possible in strictly controlled conditions. One of the methods which can achieve the necessary reduction in health risk is alkaline hydrolysis. Standard alkaline hydrolysis is at 150 °C, 3-6 hours, and 4 bars. In this investigation focus was on different alkaline conditions: temperature (135 °C, 150 °C, 153 °C), time (2, 3, 6 hours), and base (NaOH and KOH) due to the fact that amino acids are sensitive to variations on temperature, pressure and base media. Analyzed amino acid composition of hydrolyzed material at different testing conditions show successful hydrolysis. The highest value of protein, which is generally relatively low, was achieved with NaOH at a temperature of 135 °C and time of 2 hours, whereas in the reaction with KOH the highest obtained value was achieved at 150 °C and 3 hours. The dry substance was increasing with time, and the highest value was achieved at the temperature of 150 °C and time 6 hours for both bases (NaOH, and KOH). Most of the amino acids went full racemization during the process; especially in D-amino acids like aspartic acid, glutamic acid, tryptophan and isoleucine. Lysine shows higher resistance to alkaline medium than other amino acids. Isolation of certain amino acids, like: asparagine, glutamic acid, glycine, alanine, and leucine, is possible because they show higher concentrations in hydrolysates.

**Keywords:** *alkaline hydrolysis parameters; health risks; slaughterhouse waste; waste management*

## Obrada visokorizičnog bio otpada metodom alkalne hidrolize i izolacija amino kiselina

Izvorni znanstveni članak

Nusproizvod životinjskog podrijetla vrlo je opasan za životinje i ljudsko zdravlje. Mozak životinja i ledna moždina su vrlo rizični te mogu biti zaraženi prionima te je stoga vrlo važno da se zbrinjavanje ovakvog materijala obavlja u strogo kontroliranim uvjetima. Metoda kojom se postiže smanjenje rizika za zdravlje ljudi je metoda alkalne hidrolize. Standardna metoda alkalne hidrolize odvija se pri 150 °C, 3 h do 6 h i tlaku od 4 bara. Rad donosi istraživanje na polju izolacije amino kiselina nakon provedene alkalne hidrolize pri različitim uvjetima temperature (135 °C, 150 °C, 153 °C), vremena (2 h, 3 h, 6 h) i lužinama (NaOH i KOH) uzimajući u obzir činjenicu da su amino kiseline osjetljive na promjenu temperature, lužine i tlaka. Analizirani hidrolizat pokazuje uspješno provedenu alkalnu hidrolizu pri različitim eksperimentalnim uvjetima. Veći sadržaj proteina, koji je generalno u svim uzorcima relativno nizak, postignut je s NaOH pri temperature 135 °C i vremenu 2 h, dok je u reakciji s KOH veća koncentracija proteina postignuta pri 150 °C i 3 h. Suha tvar se povećavala s duljinom hidroliziranja te je najveća vrijednost postignuta pri temperaturi od 150 °C i vremenu od 6 h s obje lužine. Većina aminokiselina je prošla process racemizacije u D-amino kiseline poput: Asparagina, Glutaminske kiseline, Triptofana i IzoLeucina. Lizin je pokazao veću otpornost na alkalni medij u usporedbi s ostalim aminokiselinama. Izolacija određenih amino kiselina poput Asparagina, Glutaminske kiseline, Glicina, Alanina i Leucina moguća je budući iste pokazuju zadovoljavajuću količinu u hidrolizatu.

**Ključne riječi:** *parametric alkaline hidrolize; zdravstveni rizici; nusproizvod životinjskog podrijetla; gospodarenje otpadom*

## 1 Introduction

Increasing waste volumes are a grave burden to modern civilisation and the reduction of their quantity must be set as a priority. The volume of generated slaughterhouse waste is also alarmingly increasing. Each year, millions of tons of high-risk slaughterhouse waste are disposed of or treated worldwide. Greater re-usage and recycling, among other things, requires the application of new technologies.

EU Regulation 1069/2009 divided slaughterhouse waste into three categories: the first category (K1) is high-risk waste suspected of causing infection by transmissible spongiform encephalopathy (TSE); the second category (K2) includes, among others, manure and digestive tract contents; while the third category (K3) encompasses meat returned from stores following expiration, etc. [1].

Suitable slaughterhouse waste can be processed in rendering plants into meat and bone meal and animal fat. Processing involves crushing or grinding of raw material, followed by heat treatment at 133 °C for 20 min [2, 3]. Inedible portions are cooked at high temperatures to remove moisture, kill bacteria, and extract fat and protein. Rendering involves the processing of parts not used for human consumption. These parts include the head, feet, bones, intestines, blood, and various other tissues that we cannot eat or choose not to. During the rendering process,

large amounts of water are removed from animal protein at high temperatures and total live mass is reduced by 60 % [3]. The average composition of non-processed slaughterhouse waste is 15 % protein, 15 % fat and approx. 70 % water.

Processed animal protein contains high concentrations of amino acids. Blood, feather, and fish meals contain at least five essential amino acids. Protein-based feed of animal origin is important in the nutrition of monogastric animals as it contains essential amino acids that these animals cannot produce in their gastrointestinal tract but must ingest through food [4, 5]. However, after the appearance of "mad cow disease" or *bovine spongiform encephalopathy* (BSE), meat and bone meal and all other rendering products were banned in animal nutrition [6], which has left the rendering industry with the difficult task of finding a suitable manner in which to dispose of these products.

The development of new technologies for an efficient use of high-risk materials has therefore become an important aspect for integrated waste management [7, 8].

According to the new Ordinance on Agricultural Land Protection against Pollution [9] and Agriculture act [10] it is very important to test and monitor agricultural soil pollution. Pollution of the soil can be caused by land filled untreated slaughter house waste.

Harmful substance in slaughterhouse waste can be any substance found in waste and after in agricultural soil in a concentration that temporarily or permanently calls into question the basic function of soil as a favourable habitat for cultivated and natural vegetation [9,10]. Harmful substances include heavy metals and potentially toxic elements (Cd, Hg, Mo, As, Co, Ni, Cu, Pb, Cr and Zn) as well as polycyclic aromatic hydrocarbons (PAH). Harmful substances are also substances that are commonly introduced into the agricultural soil but which, due to unprofessional application in inappropriate quantities, at the wrong time or to unsuitable soils may cause damage to the environment [9, 10].

As an alternative method for the management of animal by-products, alkaline hydrolysis offers itself as a solution for the disposal of high-risk biodegradable waste and opens up new horizons regarding highly profitable and environmentally acceptable technologies.

Alkaline hydrolysis is a natural process that occurs, for example, with bodies buried in earth, which are degraded by this process, slowly expedited by the bacteria in the soil. Food in the small intestine is digested to usable nutrients by alkaline hydrolysis expedited by enzymes that operate from pH 7 to pH 8 at body temperature.

Alkaline hydrolysis is particularly applied in the treatment of contaminated K1 and K2 category tissues [11, 12]. Earlier studies have shown that alkaline hydrolysis generates a sterile, neutral solution of amino acids, peptides, sugars, and soap [13, 14]. Proteins are hydrolysed into free amino acids and small peptides by breaking amide bonds; carbohydrates are clipped from glycoproteins; ester bonds between fatty acids and glycerol are hydrolysed yielding completely biodegradable soaps; glycolipids and poly unsaturated fats are destroyed. The hydrolysis of proteins is particularly important because it destroys all pathogens, including prions (responsible for BSE), and converts fixatives such as glutaraldehyde, formaldehyde, and phenol, cytotoxic agents such as chemotherapy drugs, and other toxins to harmless, biodegradable derivatives. The effluent from the process of alkaline hydrolysis is called hydrolysate. This material is a beneficial, highly nutritious solution and an excellent fertilizer. It is also an excellent nutrient source for anaerobic or aerobic sewage treatment plants or biogas plants, often providing micronutrients [11, 12, 6, 15, 16].

During alkaline hydrolysis, peptide bonds are cleaved and, depending on the base used, peptides with low molecular weight and potassium or sodium salts of free amino acids are formed [13, 15]. In addition, previous investigations have made it obvious that most amino acids are racemized during alkaline treatment [14, 17, 18, 19]. Taking all of the above stated into consideration, the aim of this paper was to determine the amino acid content in hydrolysed material following the alkaline hydrolysis of slaughterhouse waste in the form of animal remains.

## 2 Materials and methods

### 2.1 Samples

In this study, brain samples from oxen younger than 30 months were used, which was possible because Croatia has had no registered cases of BSE. Prion analysis was not conducted.

### 2.2 Chemicals and reagents

Sodium hydroxide (NaOH) and Potassium hydroxide (KOH), producer: Kemika, Zagreb, Croatia, Hydrochlorid acid (HCl), producer: Merck, Darmstadt, Germany), Ninhydrine (C<sub>9</sub>H<sub>6</sub>O<sub>4</sub>), producer: Renal, Budapest, Hungary, Sodium citrate (C<sub>6</sub>H<sub>5</sub>Na<sub>3</sub>O<sub>7</sub>), producer: Renal, Budapest, Hungary.

### 2.3 Alkaline hydrolysis

The samples were stored at -20 °C. After defrosting, they were homogenized with a hand blender and divided into 400 g. In every sample, 600 cm<sup>3</sup> of distilled water and 44 cm<sup>3</sup> of 45 % KOH or 30 cm<sup>3</sup> of 45 % NaOH solution were added. In the control sample, only water was added.

The laboratory reactor case was made of stainless steel, 175 mm in height and 165 mm in diameter (WR<sup>2</sup>, Indianapolis, USA). The 10 mm wide space between the inner and exterior wall of the case was used for cooling the reactor by circulating plain water at  $\vartheta = 15$  °C at the device's entry point. Hydrolysis was conducted under the following selected testing conditions: hydrolysis duration  $t_1 = 2$  h,  $t_2 = 3$  h, and  $t_3 = 6$  h; temperature  $\vartheta_1 = 135$  °C,  $\vartheta_2 = 150$  °C, and  $\vartheta_3 = 153$  °C; and pressure  $p_1 = 2,75 \times 10^3$  hPa,  $p_2 = 4,78 \times 10^3$  hPa, and  $p_3 = 5,20 \times 10^3$  hPa. These specific conditions were chosen in order to make a comparison between the standard method (temperature  $\vartheta = 150$  °C, pre-pressure  $p = 4 \times 10^5$  Pa bar, and duration  $t = 2$  h) and higher or lower conditions than standardised ones.

After the alkaline hydrolysis treatment, the samples were centrifuged at 5000 rpm for 2 min. The sediment of approx. 0,5 % was removed and the liquid phase was stored in a deep-freeze container. For each treatment, measurements were carried out in three repetitions.

### 2.4 Amino acid hydrolysis

Samples were treated with 6 M HCl acid. Hydrolysis was carried out in closed ampules under nitrogen atmosphere at 110 °C for 24 h. The pH of the hydrolysates was set at 4 M NaOH solution.

Following hydrolysis, the samples were diluted, filtered, and frozen at -24 °C. Immediately before analysis, the sample was filtered through a 0,45 µm hydrophilic membrane filter (Merck Milipore, Darmstadt, Germany).

Analyses of amino acids (asparagine acid, threonine, serine, glutamic acid, proline, glycine, alanine, cysteine, valine, methionine, D-iso leucine, isoleucin, leucin, tyrosine, phenylalanine, lysine, histidine, and arginine) were done by ion alternative chromatography by means of post-colon derivatisation with ninhydrine [14, 20].

Ionic exchange chromatography analyses were carried out on an amino acid analyser (Aminochrom OE-914, LaborMIM, Budapest, Hungary). Amino acids were divided on a cation exchange column (230 mm × 4,5 mm, Kemochrom 9°, Resin, Germany). After passing through a ninhydrine column, amino acid derivatives were detected spectrophotometrically on a photometer at 570 nm (Pyuni-cam solar, Cambridge, Great Britain).

## 2.5 Statistical method

In accordance with the randomly selected experimental design (three repetitions), ANOVA and Student t-test (LSD) were carried out (three repetitions) in order to establish the significance of the differences among the studied factors (temperature, time, and base), and also within their combination (after the proven importance of interaction), using commercial software SAS 9.1® (SAS Institute Inc., Cary, NC, USA). The values are expressed as arithmetic mean  $\pm$  SD of three replications. *P*-values lower than 0,05 obtained by F-test

in ANOVA or LSD were considered statistically significant.

## 3 Results and discussion

As a result of the treatments, the concentration of individual amino acids in samples increased compared to the initial materials. The reason for this lay in the fact that, during the treatments, the samples lost a certain amount of solvent thus becoming more concentrated. The results obtained are presented in Tabs. 1, 2 and 3, forming the mean values and interactions of parameters on the contents of the proteins, dry substance, and all of the amino acids.

**Table 1** The effects of time, temperature, and base processing of high-risk bio-waste using alkaline hydrolysis on average values of protein, dry matter, and amino acid content

Parameters	Protein g/100 g of sample	DM g/100 g of protein	Asp g/100 g of protein	Thr g/100 g of protein	Ser g/100 g of protein	Glu g/100 g of protein
Temperature	$P < 0,0001$	$P < 0,0001$	$P < 0,0001$	$P = 0,0003$	$P < 0,0001$	$P = 0,0001$
135 °C	$5,40^a \pm 0,29$	$11,5^b \pm 1,35$	$9,86^a \pm 0,64$	$0,074^a \pm 0,069$	$0,567^a \pm 0,059$	$18,25^c \pm 1,66$
150 °C	$5,27^a \pm 0,13$	$12,8^a \pm 1,28$	$8,31^b \pm 0,87$	$0,027^b \pm 0,012$	$0,359^c \pm 0,084$	$18,43^b \pm 1,69$
153 °C	$4,97^b \pm 0,44$	$12,9^a \pm 0,92$	$7,96^c \pm 0,97$	$0,019^b \pm 0,005$	$0,389^b \pm 0,090$	$18,62^a \pm 1,84$
Time	$P = 0,4043$	$P < 0,0001$	$P < 0,0001$	$P = 0,2191$	$P < 0,0001$	$P < 0,0001$
2 h	$5,27^a \pm 0,34$	$12,3^b \pm 1,08$	$8,93^b \pm 0,66$	$0,041^a \pm 0,043$	$0,448^b \pm 0,116$	$18,42^b \pm 1,74$
3 h	$5,21^a \pm 0,35$	$11,6^c \pm 1,21$	$9,33^a \pm 0,53$	$0,051^a \pm 0,065$	$0,500^a \pm 0,097$	$18,24^c \pm 1,67$
6 h	$5,15^a \pm 0,38$	$13,3^a \pm 1,21$	$7,86^c \pm 1,51$	$0,028^a \pm 0,020$	$0,367^c \pm 0,114$	$18,63^a \pm 1,78$
Base	$P = 0,2414$	$P = 0,6988$	$P = 0,2594$	$P = 0,2344$	$P < 0,0001$	$P = 0,0074$
NaOH	$5,25^a \pm 0,47$	$12,4^a \pm 1,55$	$8,77^a \pm 1,17$	$0,046^a \pm 0,064$	$0,474^a \pm 0,102$	$18,34^b \pm 1,76$
KOH	$5,17^a \pm 0,18$	$12,4^a \pm 1,10$	$8,64^a \pm 1,19$	$0,033^a \pm 0,019$	$0,402^b \pm 0,128$	$18,52^a \pm 1,68$
Interactions between factors						
$T \times t$	$P = 0,8991$	$P = 0,8174$	$P < 0,0001$	$P = 0,4308$	$P = 0,7495$	$P = 0,1599$
$T \times B$	$P < 0,0001$	$P < 0,0001$	$P = 0,9588$	$P = 0,0619$	$P = 0,0044$	$P = 0,0009$
$t \times B$	$P = 0,3046$	$P = 0,0333$	$P = 0,1196$	$P = 0,9100$	$P = 0,7797$	$P = 0,7024$
$T \times t \times B$	$P = 0,9997$	$P = 0,2486$	$P = 0,7385$	$P = 0,9908$	$P = 0,8043$	$P = 0,9877$

*B* = base; *T* = temperature; *t* = time; *DM* = dry matter; *Asp* = asparagine acid; *Thr* = threonine; *Ser* = serine; *Glu* = glutamic acid;  $T \times t$  = interaction temperature  $\times$  time;  $T \times B$  = interaction temperature  $\times$  base;  $t \times B$  = interaction time  $\times$  base;  $T \times t \times B$  = interaction temperature  $\times$  time  $\times$  base. Different letters in superscript added to average values indicate statistically significant differences at  $P \leq 0,05$ .

### 3.1 Protein content

Protein content in the hydrolysate (Tab. 1) was relatively low, as compared to meat and bone meal and soy where it usually ranges around 50 %, [1, 3, 4, 15]. The obtained values of temperature and time interaction were not significant. The highest measured protein content was achieved at 135 °C and 2 h. The temperature and base interaction showed a significant correlation ( $P < 0,0001$ ). When NaOH was applied, the highest protein content was achieved at 135 °C, whereas the highest when applying KOH was at 150 °C. The time and base interaction was not significant and the most favourable results with both bases were achieved at 2 h. Comparing the values obtained by the joint activity of all the factors: temperature, alkali, and time the protein content was not significant and in reaction with NaOH the highest value of protein was achieved at 135 °C and 2 h, whereas with KOH the highest obtained value was achieved at 150 °C and 3 h.

### 3.2 Dry matter content

Dry matter amino acid content in the analysed samples was generally very low (Tab. 1). This was expected, since

it refers to a reaction in alkali medium [17]. The obtained values of temperature and time interaction were not significant. The amount of dry matter increased as the temperature increased and the highest results were achieved with the longest treatment time. The highest measured value was at 150 °C and 6 h. The temperature and base interaction showed significant correlation. The time and base interaction was also significant. With both bases, the most favourable results were achieved in 6 h. Comparing the values obtained by the joint activity of all factors, the dry matter content was not significant.

### 3.3 Amino acid contents

The results of this study suggest an identical effect even at lower temperatures than the standard one (150 °C), which is visible from the values of the Amino acids such as asparagine, glutamic acid, glycine, alanine and leucine achieved higher concentrations. Regarding the contents of other amino acids; arginine has experienced certain degradation; threonine appears only in traces, whereas serine was present in a very low concentration. The concentrations of cysteine, methionine and tyrosine were also low. Regarding interactions between the duration of hydrolysis and temperature the

presented results suggest also the achievement of the same effect even at lower temperatures than the standard one. It should be noted that the isolation of some amino

acids was possible because they showed higher concentrations in the hydrolysates.

**Table 2** The effects of time, temperature, and base processing of high-risk bio-waste using alkaline hydrolysis on average values of amino acid content

Parameters	<i>Pro</i> g/100 g of protein	<i>Gly</i> g/100 g of protein	<i>Ala</i> g/100 g of protein	<i>Cys</i> g/100 g of protein	<i>Val</i> g/100 g of protein	<i>Met</i> g/100 g of protein	<i>D-isoLeu</i> g/100 g of protein
Temperature	$P = 0,0005$	$P = 0,1463$	$P < 0,0001$	$P < 0,0001$	$P = 0,5633$	$P = 0,0484$	$P = 0,0190$
135 °C	$6,04^b \pm 0,34$	$10,59^a \pm 0,26$	$8,74^c \pm 0,45$	$0,267^c \pm 0,069$	$5,74^a \pm 0,25$	$2,71^{ba} \pm 0,06$	$2,48^b \pm 0,28$
150 °C	$6,35^a \pm 0,38$	$10,53^a \pm 0,24$	$9,07^b \pm 0,53$	$0,339^b \pm 0,078$	$5,69^a \pm 0,26$	$2,69^b \pm 0,12$	$2,61^a \pm 0,23$
153 °C	$6,34^a \pm 0,17$	$10,66^a \pm 0,14$	$9,28^a \pm 0,45$	$0,383^a \pm 0,092$	$5,74^a \pm 0,24$	$2,77^a \pm 0,10$	$2,58^a \pm 0,15$
Time	$P = 0,0168$	$P = 0,7118$	$P < 0,0001$	$P < 0,0001$	$P = 0,0204$	$P = 0,3695$	$P = 0,0779$
2 h	$6,17^b \pm 0,31$	$10,60^a \pm 0,18$	$8,96^b \pm 0,42$	$0,311^b \pm 0,076$	$5,73^{ba} \pm 0,25$	$2,74^a \pm 0,13$	$2,56^{ba} \pm 0,23$
3 h	$6,17^b \pm 0,32$	$10,56^a \pm 0,21$	$8,74^c \pm 0,39$	$0,272^c \pm 0,057$	$5,65^b \pm 0,21$	$2,70^a \pm 0,07$	$2,50^b \pm 0,21$
6 h	$6,38^a \pm 0,35$	$10,61^a \pm 0,28$	$9,38^a \pm 0,54$	$0,406^a \pm 0,087$	$5,80^a \pm 0,27$	$2,73^a \pm 0,09$	$2,61^a \pm 0,24$
Base	$P = 0,0125$	$P = 0,9433$	$P = 0,0018$	$P = 0,0147$	$P = 0,1167$	$P = 0,0267$	$P = 0,0957$
NaOH	$6,33^a \pm 0,39$	$10,59^a \pm 0,28$	$9,11^a \pm 0,55$	$0,348^a \pm 0,094$	$5,69^a \pm 0,18$	$2,75^a \pm 0,12$	$2,52^a \pm 0,24$
KOH	$6,16^b \pm 0,25$	$10,59^a \pm 0,15$	$8,94^a \pm 0,47$	$0,311^b \pm 0,089$	$5,76^a \pm 0,30$	$2,70^b \pm 0,07$	$2,59^a \pm 0,22$
Interactions between factors							
$T \times t$	$P = 0,9976$	$P = 0,5498$	$P = 0,0529$	$P = 0,0842$	$P = 0,8289$	$P = 0,7183$	$P = 0,8027$
$T \times B$	$P = 0,0025$	$P = 0,0079$	$P = 0,6081$	$P = 0,02064$	$P = 0,0406$	$P = 0,5316$	$P = 0,0005$
$t \times B$	$P = 0,8520$	$P = 0,2558$	$P = 0,3998$	$P = 0,4322$	$P = 0,0843$	$P = 0,6105$	$P = 0,8767$
$T \times t \times B$	$P = 0,6051$	$P = 0,3464$	$P = 0,9564$	$P = 0,9916$	$P = 0,6821$	$P = 0,5873$	$P = 0,7810$

$B$  = base;  $T$  = temperature;  $t$  = time; *Pro* = proline; *Gly* = glycine; *Ala* = alanine; *Cys* = cysteine; *Val* = valine; *Met* = methionine; *D-isoLeu* = D-iso leucine;  $T \times t$  = interaction temperature  $\times$  time;  $T \times B$  = interaction temperature  $\times$  base;  $t \times B$  = interaction time  $\times$  base;  $T \times t \times B$  = interaction temperature  $\times$  time  $\times$  base. Different letters in superscript added to average values indicate statistically significant differences at  $P \leq 0,05$ .

**Table 3** The effects of time, temperature, and base processing of high-risk bio-waste using alkaline hydrolysis on average values of amino acid content

Parameters	<i>IsoLeu</i> g/100 g of protein	<i>Leu</i> g/100 g of protein	<i>Tyr</i> g/100 g of protein	<i>Phe</i> g/100 g of protein	<i>Lys</i> g/100 g of protein	<i>His</i> g/100 g of protein	<i>Arg</i> g/100 g of protein
Temperature	$P = 0,0303$	$P = 0,0002$	$P = 0,3702$	$P < 0,0001$	$P < 0,0001$	$P < 0,0001$	$P = 0,2658$
135 °C	$2,48^a \pm 0,12$	$8,92^b \pm 0,21$	$3,95^a \pm 0,19$	$5,08^c \pm 0,19$	$7,79^a \pm 0,31$	$2,76^b \pm 0,22$	$0,622^a \pm 0,322$
150 °C	$2,41^{ba} \pm 0,08$	$8,89^b \pm 0,17$	$3,91^a \pm 0,24$	$5,19^b \pm 0,20$	$7,44^b \pm 0,27$	$2,61^b \pm 0,11$	$0,717^a \pm 0,186$
153 °C	$2,39^b \pm 0,08$	$9,06^a \pm 0,19$	$3,97^a \pm 0,16$	$5,30^a \pm 0,16$	$7,54^b \pm 0,39$	$2,58^b \pm 0,16$	$0,689^a \pm 0,118$
Time	$P = 0,9815$	$P = 0,0250$	$P = 0,7949$	$P = 0,0001$	$P < 0,0001$	$P < 0,0001$	$P = 0,4902$
2 h	$2,43^a \pm 0,10$	$8,97^{ba} \pm 0,18$	$3,93^a \pm 0,22$	$5,19^b \pm 0,18$	$7,62^b \pm 0,29$	$2,65^b \pm 0,19$	$0,656^a \pm 0,236$
3 h	$2,42^a \pm 0,10$	$8,89^b \pm 0,20$	$3,94^a \pm 0,19$	$5,11^c \pm 0,17$	$7,81^a \pm 0,24$	$2,74^a \pm 0,17$	$0,656^a \pm 0,255$
6 h	$2,43^a \pm 0,09$	$9,01^a \pm 0,23$	$3,96^a \pm 0,19$	$5,28^a \pm 0,22$	$7,34^c \pm 0,37$	$2,56^c \pm 0,16$	$0,717^a \pm 0,186$
Base	$P = 0,7863$	$P = 0,0158$	$P < 0,0001$	$P = 0,0100$	$P = 0,2712$	$P = 0,0003$	$P = 0,0070$
NaOH	$2,42^a \pm 0,10$	$9,00^a \pm 0,23$	$4,03^a \pm 0,18$	$5,23^a \pm 0,24$	$7,61^a \pm 0,29$	$2,60^b \pm 0,21$	$0,744^a \pm 0,249$
KOH	$2,43^a \pm 0,09$	$8,91^b \pm 0,17$	$3,86^b \pm 0,16$	$5,15^b \pm 0,15$	$7,56^a \pm 0,41$	$2,70^b \pm 0,14$	$0,177^b \pm 0,607$
Interactions between factors							
$T \times t$	$P = 0,9894$	$P = 0,9662$	$P = 0,9285$	$P = 0,3815$	$P = 0,3947$	$P = 0,8994$	$P = 0,6452$
$T \times B$	$P = 0,3892$	$P < 0,0001$	$P = 0,0006$	$P = 0,0028$	$P < 0,0001$	$P = 0,0911$	$P = 0,0257$
$t \times B$	$P = 0,7859$	$P = 0,5267$	$P = 0,5069$	$P = 0,8596$	$P = 0,3515$	$P = 0,6834$	$P = 0,7465$
$T \times t \times B$	$P = 0,8768$	$P = 0,4673$	$P = 0,9952$	$P = 0,9352$	$P = 0,1231$	$P = 0,9352$	$P = 0,9186$

$B$  = base;  $T$  = temperature;  $t$  = time; *IsoLeu* = Isoleucine; *Leu* = leucine; *Tyr* = tyrosine; *Phe* = phenylalanine; *Lys* = lysine; *His* = histidine; *Arg* = arginine;  $T \times t$  = interaction temperature  $\times$  time;  $T \times B$  = interaction temperature  $\times$  base;  $t \times B$  = interaction time  $\times$  base;  $T \times t \times B$  = interaction temperature  $\times$  time  $\times$  base. Different letters in superscript added to the average values indicate statistically significant differences at  $P \leq 0,05$ .

Threonine is an element of the protein of soya or meat-bone meal and ranges usually in the percentage share from 3,96 for soya to 3,27 for meat-bone meal [21, 22]. It is very sensitive to all the forms of chemical reactions, especially the alkali medium, and appears in the analysed samples only in traces. Serine is a component of animal protein and ranges usually in the percentage share of 2,2 % for meat-bone meal of cattle up to 8,5 % for feather meal [21, 22, 23, 24]. It is also very sensitive to all forms of chemical reactions, especially alkali medium, and appears in analysed samples in very small quantities. The glutamic acid shows relatively high values for the alkali medium. Glycine is a component of animal protein

and ranges usually in the percentage share of 6,7 % for meat-bone meal [1, 22]. As with all amino acids it is sensitive to all the forms of chemical reactions, especially alkaline medium, but in analysed samples the quantity is relatively high. The analysed amino acids, all of which are commonly found as proteins in meat and bone meal and soy feed at percentages ranging from 1 % to 9 % [21, 25], exhibited two distinct trends (Tabs. 1, 2, and 3). Threonine, serine, cysteine, methionine, histidine, and arginine are found usually in the protein of animal origin in the quantity of about 1 %, whereas soya as source of protein contains 1,5 % [22]. Cysteine is very sensitive to alkaline medium indicated very low concentrations in the

analysed samples. However, glutamic acid, glycine, D-iso leucine, iso leucine, leucine, tyrosine, phenylalanine, valin and lysine demonstrated resistance to hydrolysis and were found in noticeably higher concentrations. The occurrence of D-iso leucine in the analysed samples indicated that the largest amount of amino acids was most likely racemized during alkaline hydrolysis. Results showed that lysine had higher resistance to the alkali medium than other amino acids. Future isolations of methionine and lysine should inevitably also consider the economic feasibility of applying alkaline hydrolysis. Furthermore, one should also have in mind that, in the isolation of certain amino acids, those that achieve higher concentrations such as asparagine, glutamic acid, glycine, alanine and leucine, often exhibit such behaviour due to the fact that the alkali medium is an aggravating factor in filtering and isolation methods.

Metionin is one of the most significant amino acids when cattle feeding is considered. It appears in animal protein usually in a share of 1,40 %, a similar amount of share in soya which is usually 1,44 % [22, 25, 26]. With alkaline hydrolysis of the studied samples the metionin experienced slight degradation and its concentration, taking into consideration the alkaline medium, is satisfactory. Occurrence of D-iso leucine in the analysed samples indicates the fact that most likely the biggest number of amino acids during alkaline hydrolysis has been racemized [17, 19, 14]. The content of D-iso leucine as well as Iso leucine in animal protein is found usually in the share of 2,7 %, whereas in soya it is found in a share of 4,5 % [19, 22]. With alkaline hydrolysis of high-risk bio-waste relatively high concentrations of iso leucine have been obtained. Leucine is found in animal protein in an amount of 6,3 % whereas soya as protein source contains 7,8 % [19]. In comparison to other amino acids leucine has shown relative resistance to alkali medium and it is found also in higher concentrations. Tyrosine is found in animal protein usually in a share of 2 % [19]. In comparison with other amino acids it experienced, like metionin, smaller degradation which indicates its higher resistance to alkaline medium. Phenylalanine is found in animal protein usually in a share of 3,4 %, whereas it is found in soya in a share of as much as 5,3 % [4, 19]. In comparison to other amino acids it is relatively resistant to alkaline medium and can be found in higher amounts. Lysine is, along with metionin, the most important amino acid component of forage. It is usually found in soya protein in a share of 6 % whereas animal protein is less rich and usually this value amounts to 5 % [22, 24]. Based on the research results one can notice higher resistance of lysine to alkaline medium than in case of other amino acids. Hystidine is found in the soya protein in a share of 3 %, whereas animal protein is less rich with hystidine, usually in a value of 2 % [22]. Based on the research results one can notice the degradation of hystidine and its lower resistance to alkaline medium than in case of other amino acids. With the process of alkaline hydrolysis arginine is almost completely degraded. Although it is usually found in the soya protein and animal protein of up to 7 % [22, 27] the analysed samples indicate very low concentrations.

Advantages of alkaline hydrolysis include the following: combination of sterilization and digestion into one operation, reduction of waste volume and weight, complete destruction of pathogens, including prions, production of limited odour or public nuisances, and production of usable hydrolysates for amino acid analyses.

Disadvantages of alkaline hydrolysis process include the following: at present, limited capacity for destruction of slaughterhouse waste in Croatia and Europe, potential issues regarding disposal of hydrolysate and lack of regulation.

With these investigations we can open new possibilities for using high risk material for amino acid production after the alkaline process. It will eliminate one of disadvantages of the method and open alternative for disposal of hydrolysates.

#### 4 Conclusion

Investigate environmentally suitable and publicly acceptable options for hydrolysates disposal is one of biggest problems for alkaline hydrolysis treatment. This investigation gives as an answer that it is possible to use this material for amino acid isolation. Based on the present investigation we can conclude that the analysed amino acid composition of the hydrolysed material at different testing conditions showed successful hydrolysis for all of the samples. Amino acids appear in small amounts and they are mostly racemized but still we can focus on some specific one. These results will also help in terms of how to use alkaline hydrolysis technology to accommodate large amounts of hydrolysate. It is also important regarding regulation issues, because with clear picture of the complete process and results, this could be proposed like alternative method in EU regulation for treating the slaughterhouse waste.

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