

# Extraction of the Protein Components as Amino-Acids Hydrolysates from Chrome Leather Wastes Through Hydrolytic Processes

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*This paper presents results of the investigations concerning the alkaline and enzymatic alkaline hydrolysis of the chrome leather debris coming out from natural leather processing as protein containing solid wastes. Collagen hydrolysates with low average molecular mass, considered chromium-free products, were characterized by atomic absorption, gas chromatography and HPLC, electrophoresis, IR and FT/IR ATR spectral analysis. Chemical composition, rich in essential amino-acids and protein hydrolysates with average molecular mass less than 10 kDa, as well as their physical properties are fully recommending the alkaline and enzymatic alkaline hydrolysis products for applications as bio-growth enhancers in foliar fertilization by environmental safe technologies, and eventually in organic crop production.*

*Keywords: chrome leather, hydrolysis, enzyme, collagen, amino acids, foliar fertilizer*

Leather tanning is one of the oldest human activities and still remains the most important operation in the raw leather processing to marketable products. Turning rough hides into tanned leather involves not only large amounts of water and many chemical and mechanical processing operations, but also the release of large amounts of fluid and solid wastes with particular and variable content in deleterious compounds [1]. Chrome leather (unfinished) composition is made up by 85-87% dermal substance (related to the humidity-free product), 4.5-5.5% chromium oxide (related to the humidity-free product), and up to 100 percent are occurring the mineral salts which were anchored in the deep layers of the skin thoroughly the entire course of preliminary substrate dressing stages. Actually, the salts are originating from the waters, which inevitably are seized by leather during all the wet operations. Between the dressing and finishing operations the raw tanned leather passes through mechanical operations by which the leather is split to achieve the desired thicknesses and tailored to standard shapes by removing the useless edges. Leather debris resulting from mechanical operations ends up as solid chrome leather waste, which may comprise up to 25% of preserved leather used as raw material. Legislative constraints and the responsibility of leather manufacturers resulted in earnest searches for scientific and technical solutions devoted to harmonization of the best available technologies in production and finishing leather with the requirements of high performance environmental management.

The objective of this paper is an investigation on protein components extraction from chrome leather debris as collagen hydrolysates for friendly environmental applications, i.e. hydrolysates use in foliar nutritive fluids formulated as emulsions, and eventually the hydrolysis solid wastes disposal as non hazard wastes [2, 3]. In recent years, methodical studies regarding the treatment of chrome leather wastes have been carried out at laboratory and pilot scale [4-9]. Unlike other solid wastes as raw hides or only limed hides, whose hydrolysis do not assume too

many burdensome or elaborated stages, in the case of tanned wastes, the de-tanning process arises as a laborious stage, less sensitive to the common control parameters.

The dermis as substrate and chromium as modifying agent of the substrate are exhibiting peculiar interlacements in terms of chemical composition, structure and functionality, making the hydrolytically de-tanning process in which they are both involved more complex in its mechanism and kinetics. From the recent researches in this field, it is clear that hydrolysis must be approached as a multi-steps process, each of it with its different mechanism and kinetics, requiring various chemical de-tanning agents that back up each other at least in one stage [10-12].

## Experimental part

### Materials

Debris of chrome leather wastes have been sampled from natural leather processing line production at SC Pielorex S.A. Jilava, Romania. Mean averaged chemical composition of samples was: humidity 54.14%, total ash 9.97%, chromium oxide 4.71%, total nitrogen 15.00%, dermal substance 84.30% and 5.71% other degradable substances up to 800°C. All these values are related to the humidity-free product. Alkaline hydrolysis agent calcium hydroxide has been used as diluted suspension of calcium hydroxide due to its property to precipitate simultaneously most of sulphate ions as calcium sulphate together with chromium hydroxide. The co-precipitation of the two compounds increases the separation yield and rate of finely precipitated chromium hydroxide from the reaction mixtures resulted after entire hydrolysis process end. The appropriate ratios alkaline agent/hydrolyzing material (2 parts of CaO to 1 part of chromium in leather wastes) were carefully chosen, so as the whole amounts of chromium from the hydrolyzing material to be incorporated in the precipitated solid wastes, and the liquid emerging phase to be at large extent freed from the chromium burden. Proteases Oropon ON2 (active at 37°C and pH = 7-9) and

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2.5 L Alcalá (active at 70°C and pH = 7-10) have been used in all the alkaline enzymatic hydrolysis experiments.

### Methods

Experiments have been carried out only to laboratory scale sample dimensions. In all hydrolysis experiments, both alkaline and alkaline enzymatic hydrolysis stages, the bath liquid/solid ratio has been 10/1. Temperature as variable parameter ranged from 70°C to 98°C for the alkaline hydrolysis, and from 37°C to 70°C for the alkaline enzymatic hydrolysis (temperature at which active enzymes are active), respectively. Duration of the hydrolysis process was extended to 3-6 hours for alkaline hydrolyses, and 2-4 h for enzymatic hydrolyses, respectively. All the hydrolysis experiments were carried out under the compulsion of hydrolysis steps number. Accordingly, some particular working schemes (figs. 1-5) were conveniently considered for concluding the significant and representative parameters describing both alkaline and alkaline hydrolysis processes and hydrolysis products.

### Analytical protocol

Collagen hydrolysates obtained by the alkaline and enzymatic alkaline hydrolysis of chrome leather debris have been chemically analyzed in order to find out the content in dermal substance, amine nitrogen and mineral compounds. Based on the amine nitrogen content the average molecular weights of collagen hydrolysates (Sørensen method) have been computed [13]. Also, collagen hydrolysates have been subjected to analytical investigations through instrumental methods: atomic absorption (AAS, Thermo Instruments) for cationic chromium content determination, gel electrophoresis (SDS-PAGE) to determine average hydrolysates molecular weight ranges, IR spectral analysis (FT/IR 7.0 VERTEX and FT/IR-4200 with ATR device equipped with diamond reflection crystal) for the identification and assignment of hydrolysates spectral bands, GPC chromatography (Waters with Waters 410 detector – Differential Refractometer and PL aquagel columns – OH 50 and PL aquagel – OH 30 – Polymer Laboratories) for checking the average molecular weights and HPLC chromatography (Thermo Electron – Finnigen Surveier with DAD detector) for qualitative and quantitative determination of amino acids content.

### Results and discussions

Both types of experiment, alkaline and enzymatic alkaline hydrolysis, were yielded yellow collagen hydrolysate solutions with variable colour intensity and hue. Opalescence of these solutions was given mostly by mineral burden with some of its components close to their saturation concentration.

### Alkaline hydrolysis

Alkaline hydrolysis process is obviously dependent on temperature and batch residence time for a constant ratio between alkaline agent and chrome leather debris (fig. 6). Measurable parameter expressing this interdependence is the fraction of dermal substance passed into hydrolysate solution or yield of dermal substance extraction. As the figure 6 shows, at a temperature higher than 80°C and extended batch residence time (6 h), the hydrolysis process duration seems to bring about just minor influences on the reactions yields and consequently on fractions of dermal matter converted into hydrolysates. These yields are laying around 90-95%, with little chances to go farther up to some better output, if the batch residence time is pushed over 6

h. Conversely, below 80°C, for example at 70°C, the influence of batch residence time on the dermal substance fraction passed into hydrolysates is significantly positive and a steady increase in yields is easily noticed on figure 6 till a batch residence time of 5 h. Some yield lags in variation of dermal matter fraction converted by hydrolysis against batch residence time has to be considered as decay in the process rates at the end of a particular stage of hydrolysis. Nevertheless, the random extraction yields variation might be brought on either by the non-polar amino acids (valine, phenylalanine, leucine, isoleucine, methionine) and hydroxyl-amino acids (lysine, arginine, histidine) found in protein waste, which are fairly hydrophobic in alkaline conditions and can emerge as particulate shaped hydrophobic areas inaccessible to water [15], or by collagen moieties forming covalent cross-links between compatible functional groups, which resist to hydrolysis [16]. Beyond this yield lag, in the further advanced stages of hydrolysis, the yields are quickly increasing over 95%, overtopping the maximum yield reached at 90°C after 5 h of batch reaction (97%). This peculiar behaviour in mechanism and kinetics of protein hydrolysis is undoubtedly a clue for the idea of running hydrolysis process in many stages and to extend the each stage duration for a better molecular mass control.

The hydrolysis yield as the main parameter describing the hydrolysis process efficiency has to be substantiated by accurate measurements of the amine nitrogen content in hydrolysate solution or ratio amine nitrogen/total protein nitrogen (table 1). Actually, the ratio amine nitrogen/total protein nitrogen reveals not only the conversion yield of protein nitrogen into amine nitrogen, but it might be related to the average molecular mass of collagen hydrolysates. There is a rather more complex correlation between amine nitrogen/total nitrogen ratio and other working parameters including the hydrolysis yield (table 1). For example, table 1 illustrates the obvious dependence of both hydrolysate averaged molecular mass and nitrogen content (respectively, amine nitrogen/total nitrogen ratio) on temperature and batch residence time under all experimental configuration of the hydrolysis process. Thus, the expected correlation between molecular mass of the collagen hydrolysates and temperature or reaction time prove to be very strong, and as a result these two parameters may be boosted for eventual forcing the hydrolysis process under any circumstances to lower molecular mass and increase the content in amine nitrogen. Other way, it seems that simple extraction of dermal matter from waste leather is quite fast, reaching 85-90% from hydrolysate dry matter in the first 3 h of reaction (figure 7). Subsequently, only the extension in bath reaction time may concurrently raise the hydrolysis yield and diminish molecular mass of the hydrolysates. The same conclusion is better emphasized in figure 7. Accordingly, the raise in temperature from 70 to 98°C and the extension of reaction time to 6 h results in a fair drop in averaged molecular mass of hydrolysate compounds to as low as 3000-4000 Da. If the lower molecular mass is the final target of the protein hydrolysis process, as it happens when hydrolysis products containing aminoacids are used as growth enhancers in the foliar nutritive fluids [14], it should be noticed that the maximum yield in protein conversion has to be accomplished with minimal energy consumption. Also, the process productivity might be achieved through some significant shortening in batch residence time. All the above efficiency indicators (molecular mass, hydrolysis yield or amine nitrogen

No.	Hydrolysis type	Temperature, °C	Batch reaction time, hours	Yield of extraction, %	Dermal substance in dry Hydrolysates, %	Amine nitrogen content, %	Averaged molecular mass, Daltons
1	Alkaline - one stage	70	3	26.86	71.54	0.61	24700
2			4	78.46	86.44	0.87	15300
3			5	73.37	89.81	0.89	14900
4			6	94.05	90.20	0.97	13500
5	Alkaline - one stage	80	6	97.25	90.98	1.18	10450
6			5	89.56	87.39	1.03	12600
7			4	81.27	86.55	0.96	13650
8			3	82.64	86.43	0.77	18100
9	Alkaline - one stage	90	3	85.44	88.01	0.95	13800
10			4	89.42	88.96	0.98	13400
11			5	97.02	89.19	1.04	12450
12			6	89.60	89.53	1.30	9100
13	Alkaline - one stage	98	6	87.94	88.90	2.23	3400
14			5	86.31	88.29	1.28	9400
15			4	91.09	87.84	1.20	10300
16			3	87.73	87.28	1.16	10800
17a	Alkaline - stage I	98	4	94.77	83.62	1.24	9600
17b	Alkaline - stage II	98	2	92.40	82.39	2.25	3350
18a	Alkaline - stage I	98	6	85.58	84.41	2.12	3750
18b	Enzymatic - stage II	37	4	87.17	81.83	2.30	3250
19a	Alkaline - stage I	98	4	85.83	86.15	1.16	10800
19b	Enzymatic - stage II	70	2	80.40	75.65	1.35	8550
20a	Alkaline - stage I	80	6	90.03	85.42	1.15	10900
20b	Enzymatic - stage II	70	3	75.27	79.30	1.46	7450
21	Alkaline/Enzymatic - one stage-two steps	98/37	2/3	80.90	78.90	1.23	9900
22	Alkaline/Enzymatic - one stage-two steps	98/37	3/2	82.24	83.23	1.44	7400
23	Alkaline/Enzymatic - one stage-two steps	80/37	2/4	86.88	78.46	1.10	11600
24	Alkaline/Enzymatic - one stage-two steps	70/70	3/3	82.00	78.74	1.06	12250
25	Alkaline/Enzymatic - one stage-two steps	80/70	3/3	66.12	80.53	1.51	6900
26	Alkaline/Enzymatic - one stage-two steps	80/70	3/4	68.93	73.23	1.82	5100

**Table 1**  
CHROME LEATHER HYDROLYSIS  
PARAMETERS AND YIELDS

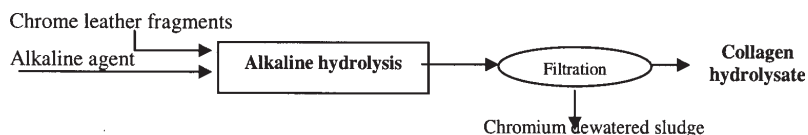


Fig. 1. Flowchart of the alkaline hydrolysis experiments in one stage

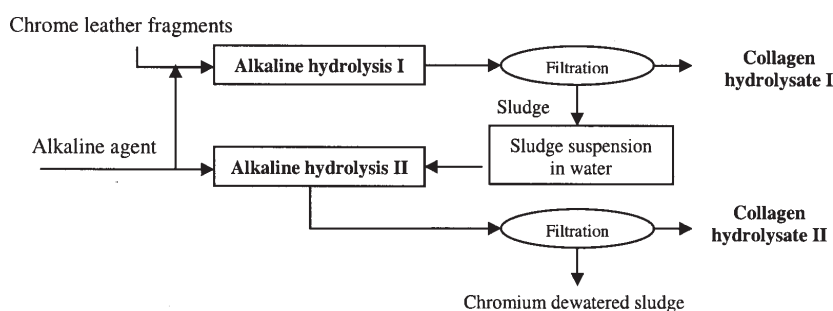


Fig. 2. Flowchart of the alkaline hydrolysis experiments in two stages

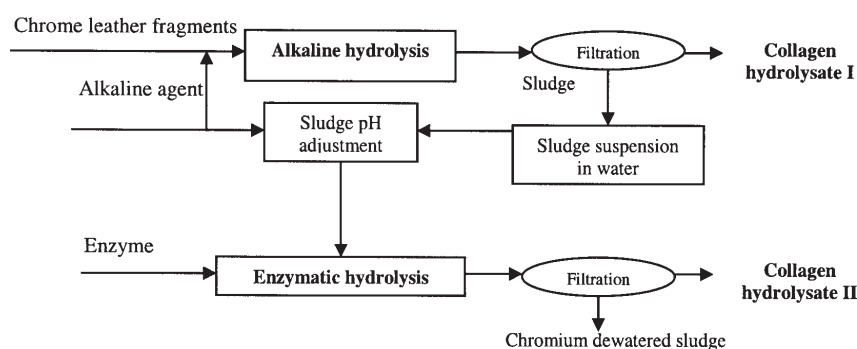


Fig. 3. Flowchart of the enzymatic-alkaline hydrolysis experiments in two stages

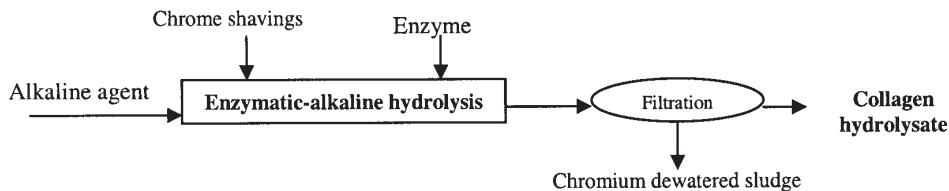


Fig. 4. Flowchart of the enzymatic-alkaline hydrolysis experiments in one stage

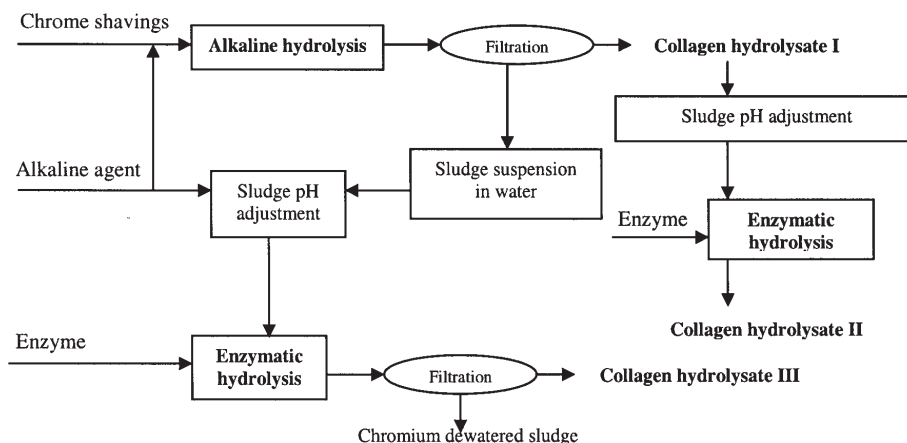


Fig. 5. Flowchart of the enzymatic-alkaline hydrolysis experiments in three stages

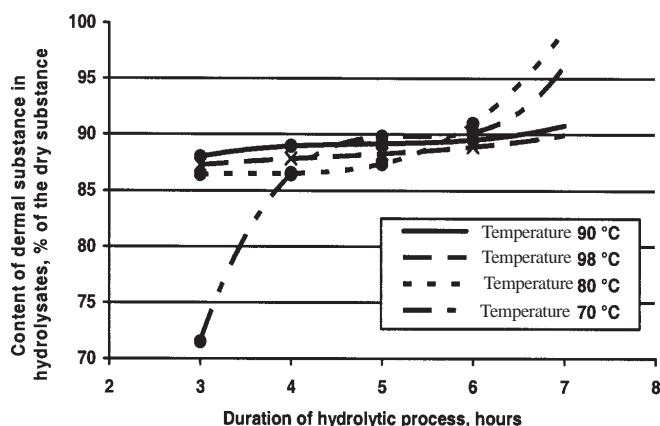


Fig. 6. Influence of the temperature and duration of alkaline hydrolysis on the content of dermal substance in hydrolysates

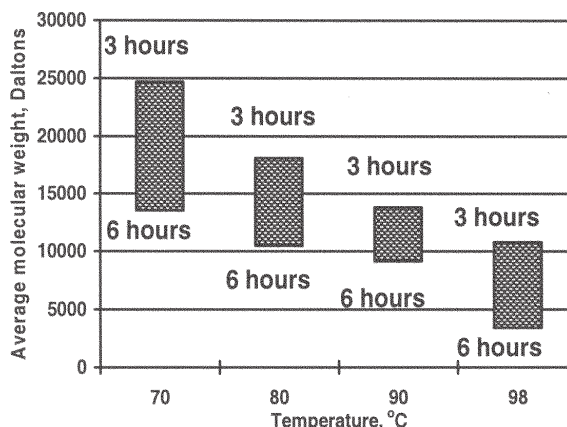


Fig.7. Variation of the average molecular weight of collagen hydrolysates related to the temperature at which alkaline hydrolysis is carried out

content and specific energy consumption) could be brought to reasonable values only by meaningful choice stages and their rational arrangements in the full process flowchart (figs. 1-5). Alternative enzymatic hydrolysis stages may certainly bring in major improvements and efficiency gains. In the case of alkaline hydrolysis at atmospheric pressure, the temperature variation in 70-98°C range and the matching batch residence time to the best choice of temperature as prevailing parameter related to quality of protein waste raw materials, may successfully lead to averaged molecular mass of the protein hydrolysates as low as 5000 Da. Introducing the second stage of alkaline hydrolysis process (fig. 2) with the defined purpose of advanced protein recovery from the sediments separated after the first stage of hydrolysis carried out at 98°C for 4 h (bath liquid/solid ratio 10/1, dermal substance in dry hydrolysate 83.62%, amine nitrogen content 1.24, table 1, fig. 7) and concluding the conversion with a second stage at the same temperature for 2 h (bath liquid/solid ratio 10/1), the dermal substance in dry hydrolysate was diminishes to 82.39%, but amine nitrogen content in hydrolysate solution raised to 2.25%. What is of not less

importance for this process configuration is the very low molecular mass of the hydrolysis products (3350 Da, table 1), close to 3400 Da attained in one through alkaline stage after 6 h reaction time at the same temperature of 98°C (fig. 8). But the two stage process requires less energy and the extraction yield is 2% higher.

#### Alkaline enzymatic hydrolysis

Since alkaline processing at high temperatures certainly involves high energy consumption, enzymatic hydrolytic stages were considered in the more elaborated process configurations (figs. 3-5). These stages were approached, taking advantage of the lower temperature ranges of enzymatic conversion process. Firstly, the enzymatic hydrolysis stage was set out on advanced recovery of protein component from sediments resulting from alkaline hydrolysis (fig. 3). Further, the hydrolysis process was carried out in one trough enzymatic experiments (fig.4) and by a more interlaced schema with two sediments separated from the first alkaline hydrolysis stage and first collagen hydrolysate, which were processed by enzymatic hydrolysis (fig. 5). In all cases, the enzymatic hydrolysis is

**Table 2**  
AVERAGE MOLECULAR MASS AND AMINE NITROGEN CONCENTRATION AFTER THE ENZYMATIC HYDROLYSIS SECOND STAGE

No.	Enzymatic units of Alcalase 2,5L / g total amine nitrogen	Experiment 1- sample 4, table 1		Experiment 2- sample 5, table 1		Experiment 3- sample 20a, table 1	
		Amine nitrogen, %	Averaged molecular mass, Da	Amine nitrogen, %	Averaged molecular mass, Da	Amine nitrogen, %	Averaged molecular mass, Da
1.	0	0.97	13500	1.03	12600	1.15	10900
2.	1.0	1.10	11600	-	-	1.30	9100
3.	1.5	1.14	10800	1.52	6800	1.35	8550
4.	3.0	1.25	9650	1.59	6300	1.40	8000
5.	4.5	1.37	8250	1.63	6000	1.54	6700
6.	6.0	1.40	8000	1.66	5800	1.54	6700
7.	7.5	-	-	1.66	5800	-	-
8.	9.0	-	-	1.66	5800	-	-

**Table 3**  
IR SPECTRAL BANDS ATTRIBUTED TO COMPOUNDS IDENTIFIED IN COLLAGEN HYDROLYSATES

No.	Figure	Spectral band, cm <sup>-1</sup>	Spectral attribution		Possible compounds
			Band, cm <sup>-1</sup>	Vibration type	
1	12	3302.98	3300-3500 3300-3400	ν <sub>OH</sub> ν <sub>NH</sub>	Intermolecular H bonds in polypeptides, histidine, arginine
3	13	3310.70			
4	12	3071.53	3030-3130	ν <sub>NH</sub>	Amino acid
6	13	3070.57			
7	12	1652.94	1610-1660	δ <sub>NH</sub>	Amino acid
9	13	1652.94, 1647.15			
10	12	1554.57 1558.43	1550-1610	ν <sub>C=O</sub>	Aspartic acid Glutamic acid
11	5.19	1559.39			
12	12	1449.45	1430-1470	δ <sub>CH<sub>2</sub></sub>	Proline
14	13	1450.42			
15	12	1160.14	1140-1230	ν <sub>C-OH</sub> (phenol)	Tyrosine
17	13	1109.03	1100-1120	ν <sub>C-OH</sub> (secondary alcohol)	Threonine
18	12	1036.85	1010-1075	ν <sub>C-OH</sub> (primary alcohol)	Serine
19	12	656.74 875.65	500-1000	δ <sub>CH</sub>	Glycine
21	13	676.03			

actually an enzymatic alkaline process, because both employed enzymes exhibit maximum activity at pH greater than 7.0. But, in any enzymatic stage (figs. 3-5) the adjustment of alkalinity is mandatory demanded for enzymes protection.

In terms of average molecular mass of collagen hydrolysates, enzymatic hydrolysis as second step of the full hydrolysis process largely depends on the batch residence time and phase advancement of reaction at the end of first alkaline hydrolysis stage. Due the enzymes sensitivity, the temperature could not be exploited in handling either conversion rate or molecular mass. Since protein conversion yields in the first alkaline stage are conveniently increased through balanced rises in temperature and reaction time, averaged molecular mass of the hydrolysates is usually staggering in the best circumstances around 10.000 Da. Thus, the enzymatic stage rather infers the averaged molecular mass diminishing then the upsurge in conversion yields. But some progress in hydrolysis still takes place, even if it is accounting just for a meager protein conversion increment. Consequently, the ratio enzymatic units/total protein nitrogen turns out as the prevailing parameter in hydrolysate molecular mass control. The factual data about these experiments run under figure 3 constrictions are given in table 2. All the experiments were initiated by cooling the reaction mixture after the first alkaline hydrolysis stage

and inoculation of the enzyme suitable amounts. Samples 4, 5 and 20a were considered representative trial assays for first alkaline hydrolysis stage in order to set up the enzymatic hydrolysis investigations. Table 2 as well as the figure 9 illustrates the significant diminishing in hydrolysate molecular mass, after 3 h batch residence time at 70°C. Unexpectedly, the enzyme effect is phasing out beyond the ratios enzyme/ protein nitrogen of 6.0 U/g. Also, the amine nitrogen content in hydrolysates moieties is far back the best result accomplished by the full alkaline hydrolysis. If the enzyme is active at a higher temperature than 30-37°C, this aspect has to be taken as a promising alternative for the augmentations in amine nitrogen content or for reducing the average molecular weight and substantial cut back in batch residence time. Thus, the amine nitrogen survey in samples 18b, 19b and 20b was revealing that the rise of enzymatic hydrolysis stage temperature from 37°C to 70°C has recorded substantial gains of 8.5, 16.5 and 26.9%, respectively in amine nitrogen concentration. However, the average molecular mass lessening and poor savings in energy consumption are of worthless use for a competitive hydrolysis process design. Actually, the choice for alkaline hydrolysis at high temperature and long residence time batches, which chiefly implies top conversion yields and rates in protein molecule bonds breaking, could thoroughly support the enzymatic-alkaline

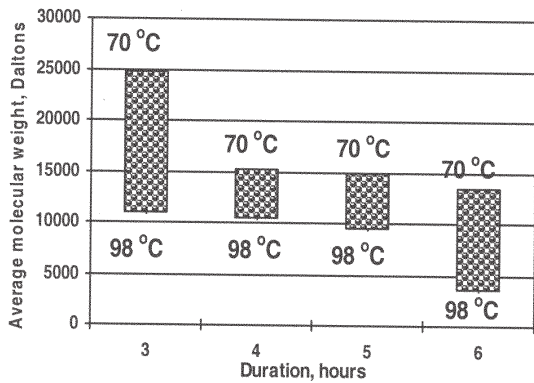


Fig.8. Variation of the average molecular weight of collagen hydrolysates related to the duration of alkaline hydrolysis process

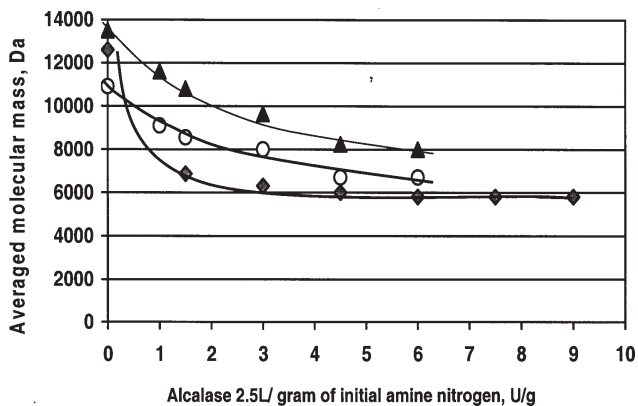


Fig.9. Influence of the enzyme concentration on the hydrolysates averaged molecular mass; ▲ Stage 1 molecular mass 13600, ◆ Stage 1 molecular mass 12600; o Stage 1 molecular mass 10900

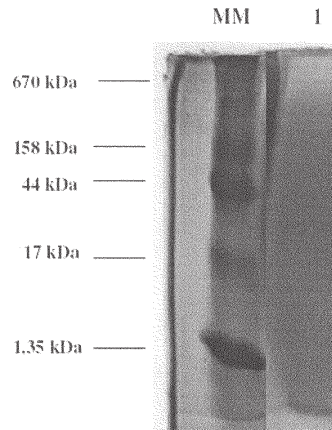


Fig.10. Electrophoresis of a collagen hydrolysate samples with average molecular weight of 24.70 kDa

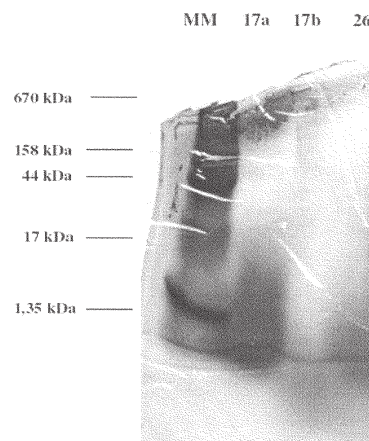


Fig.11. Electrophoresis of a collagen hydrolysate sample with average molecular weight of 10.30-3.35 kDa

**Table 4**  
AVERAGE MOLECULAR MASS OF THE PROTEIN HYDROLYSATES MEASURED BY CHROMATOGRAPHY AND SORENSEN METHODS

No.	Sample number in table 1	Average molecular weight, Daltons	
		Chromatographic method	Sørensen method
1	13	3358	3400
2	17b	3303	3350
3	18b	3196	3250

**Table 5**  
AMINO ACIDS CONCENTRATION IN THE COLLAGEN HYDROLYSATES

No.	Amino acids	Content in hydrolysate samples from table 1, mg/100ml							
		4	5	19a	19b	20a	20b	22	25
1	Aspartic acid	10.45	2.72	0.91	7.29	1.35	13.29	10.10	19.88
2	Glutamic acid	25.09	6.35	0.82	32.80	1.49	10.84	11.17	16.54
3	Serine	46.15	4.33	1.73	66.30	1.97	9.85	5.64	99.95
4	Histidine	14.15	2.95	-	51.83	0.78	-	20.17	29.66
5	Glycine	10.21	6.74	1.42	8.14	11.11	3.54	1.37	17.53
6	Threonine	-	-	-	-	-	-	15.12	-
7	Alanine	-	13.63	-	-	-	-	7.39	-
8	Tyrosine	-	-	5.42	-	8.26	44.49	-	75.31
9	Valine	-	-	-	-	-	-	7.45	53.56
10	Methionine	-	-	1.09	-	-	-	-	-
11	Phenylalanine	-	-	-	-	-	-	-	32.96
12	Isoleucine	-	-	-	-	-	7.91	1.24	49.14
13	Leucine	-	-	-	-	-	3.38	4.49	139.45
14	Proline	44.57	36.07	63.90	44.98	61.14	46.80	0.21	35.75
Total		150.62	72.79	75.29	211.34	86.10	140.1	84.35	569.73

hydrolysis at moderate temperatures as secondary stage, where the dominant change consist in the amine nitrogen content growth at expense of the averaged molecular mass decline (fig. 3). On the other hand, the enzymatic hydrolysis

by one through stage with two temperature reaction steps (fig. 4) can be fully use only under extended batch residence time at 70-80°C with adequate high temperature enzymes (samples 21-26 in table 1).

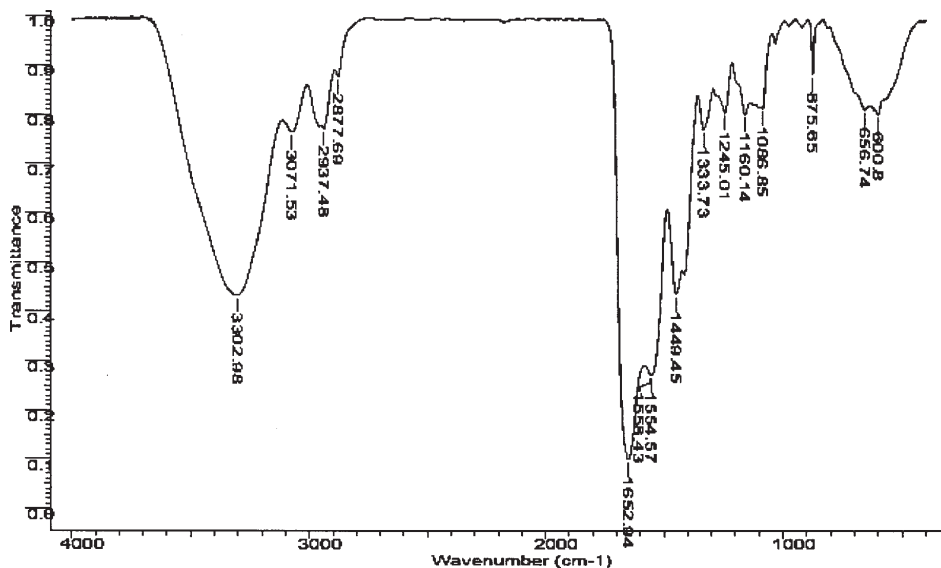


Fig.12. FT/IR spectral analysis of the collagen hydrolysate resulted from alkaline hydrolysis of chrome leather (sample 20a in table 1)

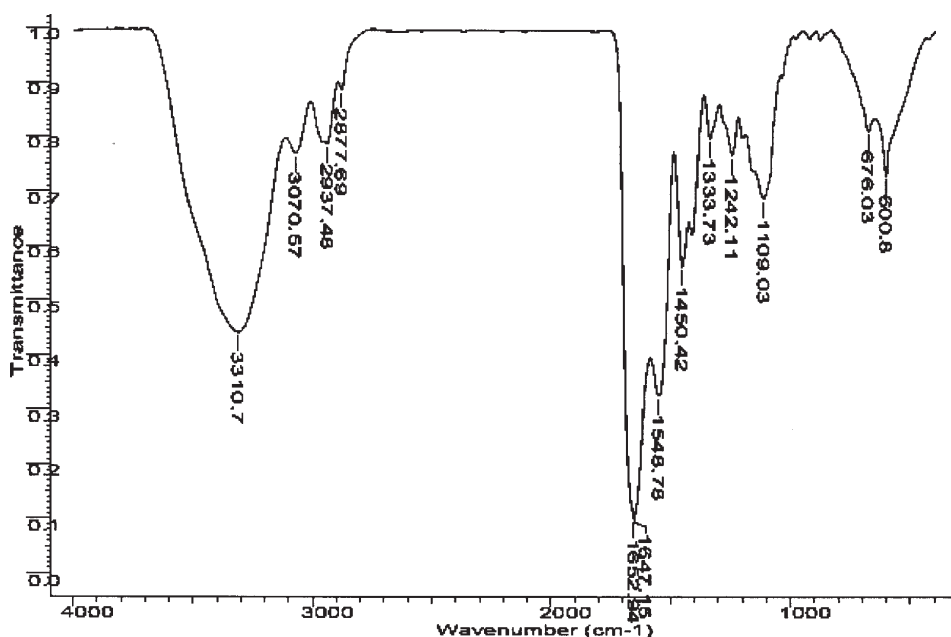


Fig.13. FT/IR spectral analysis of the collagen hydrolysate resulted from enzymatic-alkaline hydrolysis of chrome leather (sample 22 in table 1)

Chrome leather debris hydrolysis assumes a statistical distribution of the breaking events in the protein macromolecules. In turns, the molecular mass distribution in hydrolysate moieties is highly dependent on the basic leather technical history and provenance. To avoid the inconveniency of variable source and quality of chrome leather wastes the flowchart in figure 5 provides many opportunities to control not only the crude molecular mass and amine nitrogen concentration, but also the individual amino-acids distribution in the outcome hydrolysate products. Changing collagen hydrolysate I/precipitated sediment mass ratio and the enzymatic processing of collagen hydrolysate I fraction for advanced splitting in hydrolysate moieties provide the means to secure for the lowest molecular mass amino-acids demanded by their use as crop growth stimulators [2, 14].

#### Hydrolysate products analysis

Collagen hydrolysates encompass a large number of organic and inorganic compounds originating from the chrome leather production and its finishing operations as well as from the hydrolysis process designed to break the

polypeptide molecules to less complex structured compounds. Characterization of these elaborate mixtures as collagen hydrolysates requires a large number of advanced methods of analysis.

#### Analysis by atomic absorption spectroscopy

Analytical investigations by atomic absorption spectroscopy of collagen hydrolysates sample from table 1 have shown variable very low chromium concentrations ranging between 18 and 54 ppb, below the values of about 200 ppb, reported by other authors [17]. Accordingly, these findings eliminate the additional collagen hydrolysate refining before their use as growth stimulators [2, 14]. This simplifies the process flowcharts and diminishes the production costs.

#### Analysis by electrophoresis

Hydrolysis process, both alkaline and enzymatic stages, assumes variable distributions of the molecular mass compounds in collagen hydrolysates. Such particularly investigated distributions were emphasized by electrophoresis on polyacrylamide gel, in discontinuous

system and in the presence of a molecular marker (MM), using sodium dodecyl sulphate as denaturant agent. Figures 10 and 11 present the results of some electrophoresis data concerning the collagen hydrolysate samples originating from different hydrolysis batches. Sample 1 from table 1 shown in figure 10 exhibits advanced degrees of hydrolysis and predominant molecular mass 158 - 0.1 kDa in the outcome hydrolysates. The samples 17a and 17b (table 1) display in figure 11 higher hydrolysis ranges than sample 1 in figure 10, with predominant molecular mass in the interval 10-0.1 kDa. Conversely, the predominant molecular mass in sample 26 (table 1) is laying in the range of 0,5-0,1 kDa.

#### FT/IR spectral analysis

Figures 12 and 13 show FT/IR spectra for collagen hydrolysate solutions experimentally obtained by alkaline and alkaline-enzymatic hydrolyses. In these spectra, the bands of  $\nu$  stretching vibrations and  $\delta$  vibration distortion occur at wavelengths attributed to the chemical bonds in polypeptides, peptides, as well as at wavelengths attributed to the chemical bonds in amino acids [18, 19]. Table 3 details the identified IR spectral band and their allocations to the potential compounds may occur in chrome leather waste hydrolysates. FT/IR ATR spectra of the collagen hydrolysates, samples 4 and 5 (table 1), are shown in the figure 14. The range 3450-3300  $\text{cm}^{-1}$  ( $\nu_{\text{OH}}$  +  $\nu_{\text{NH associated}}$ ) with the main absorption band at  $3330 \pm 20 \text{ cm}^{-1}$  indicates the presence of partially free asymmetric -OH groups. It

was found in all analyzed hydrolysates. The range 2500-2000  $\text{cm}^{-1}$  indicates the presence of stretching vibrations given by  $X \equiv Y$ ,  $X=Y=Z$  groups (where X, Y, Z may be C, N, O, S). It is possible that this spectral range, found in all samples, to feature the organic matter accumulated by leather wastes during their initial processing. The range 1650-1630  $\text{cm}^{-1}$  ( $\nu_{\text{C=O}}$  of -CONH-) found in all samples is attributed to amide I. This absorption band attributed to stretching vibrations of carbonyl groups refers to the secondary structure of proteins and is sensitive to conformation changes. The asymmetric shape of this band is due to the fine structures that make up the protein structure. Another contribution to the asymmetry of the band is that of hydrogen bonds vibrations. Bands at 1650 and 1550  $\text{cm}^{-1}$ , through their position and intensity, give information regarding the relative advancement in degradation of the basic peptides. In the range 680-450  $\text{cm}^{-1}$  there are several absorption bands of low intensity, which are attributed to the deformation vibrations of the groups: CH, OH, NH. These bands are present in all collagen hydrolysate spectra.

#### Chromatographic analysis

Gel chromatography was used to verify average molecular weights of experimentally obtained collagen hydrolysates. Figure 15 presents the chromatogram of the collagen hydrolysate sample 13 (table 1). Averaged molecular masses of hydrolysates in three different samples, determined by gel chromatography, were

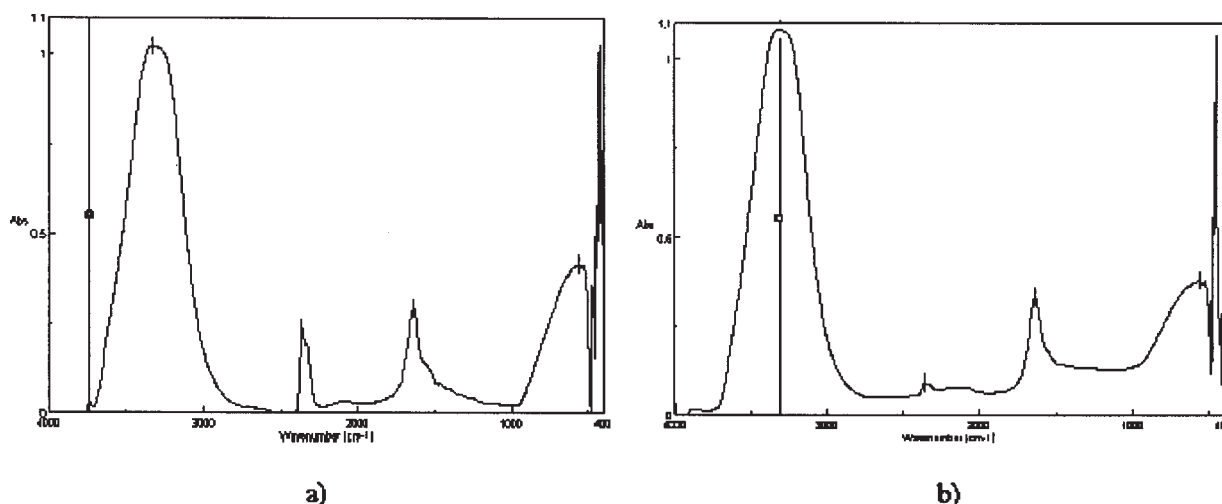


Fig.14. FT/IR ATR spectral analysis of the collagen hydrolysates resulted from alkaline hydrolysis of chrome leather a) sample 4 in table 1), b) sample 5 in table 1

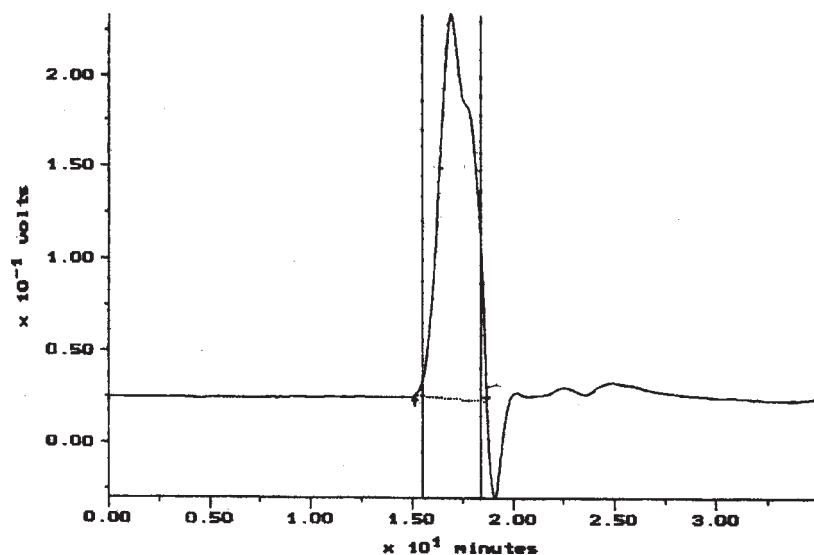


Fig.15. Chromatogram of a collagen hydrolysate with the average molecular weight of 3358

compared with molecular masses measured by Sørensen method for the same samples. From the table 4, it can be seen the difference between the two methods results is less than 2%. This finding attested as satisfactory all the measurements made by Sørensen method (table 1). For the qualitative and quantitative determination of the amino acid content in collagen hydrolysates, the high performance liquid chromatography technique was used (HPLC). The results in table 5 assign the particular composition in amino-acids for both samples collected from alkaline hydrolysis process and enzymatic hydrolysis process, respectively. All the results are in good agreement with recorded FTIR data. Certain essential amino-acids enumerated in table 5, such as threonine, valine, phenylalanine, leucine, isoleucine occur only in the composition of hydrolysates obtained by enzymatic hydrolysis. This is an important point in selection collagen hydrolysates charges for preparation active formulations to be used as plant growth bio-simulators.

### Conclusions

The main purpose of this paper was the investigation of protein components extraction as low molecular mass collagen hydrolysates from chrome leather wastes. Alkaline and alkaline enzymatic hydrolysis processes were carried out under mild temperature conditions at atmospheric pressure. The reaction mixture was processed to recover liquid phase and to use it as plant growth stimulator, respectively, to remove completely the solid phase incorporating the chromium from chrome leather as a disposable non hazard solid waste. The hydrolysis yield as the main parameter describing the hydrolysis process efficiency has been substantiated by additional measurements of the amine nitrogen content and ratio amine nitrogen/total protein nitrogen, and averaged molecular mass of the individual compounds in hydrolysate solution, respectively. Five chart configurations of the chrome leather debris processing were designed and followed by experimental runs and data collecting. One through alkaline hydrolysis process turned out high conversion waste yields to hydrolysates, but poor averaged molecular mass of the reaction end compounds and low amine nitrogen in the hydrolysates. Two stage alkaline hydrolysis process, with second stage framed for full protein recovery from precipitated solid phase waste incorporating chromium compounds, helped improvements in the molecular mass diminishing bellow 5.000 Da and concluding raises in amine nitrogen content in the hydrolysates. Significant energy saving were predicted from data concerning the two stage alkaline hydrolysis process, with the second stage run by enzymatic alkaline hydrolysis processing. High conversion waste yields to hydrolysates and low molecular mass of the hydrolyzed compounds have been achieved, without thrust neither temperature parameters nor the batch residence time in the both stages. It was proved that the enzymatic process is prevalent in advanced splitting of the hydrolysate moieties, providing

the means to secure for the lowest molecular mass amino-acids demanded by their use as plant growth stimulators.

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### References

1. KANAGARAJ, J., VELAPPAN, K. C., CHANDRA BABU, N. K., SADULLA, S., *J. sci. ind. res.*, **65**, no. 7, 2006, p. 541
2. GAIDAU, C., STEPAN, E., TALOI, D., NICULESCU, M., FILIPESCU, L., Additives and advanced biofertilizers obtained from leather industry by-products, *Rev. Chim. (Bucuresti)*, **60**, no. 5, 2009, p. 501
3. NICULESCU, M., IONITA, A.D., RADOVICI, C., FILIPESCU, L., Etude de la sequestration du chrome des boues d'epuration et des eaux residuaires: utilisation de mineraux a multiples fonctions, Premier Colloque Francophone sur La Materiaux, Le procedes et L'environnement, Volume des Papiers (Editura Printech, Bucuresti, 2009, p. 89
4. BROWN, E. M., TAYLOR, M., MARMER, W. N., *J. Am. Leather Chem. Assoc.*, **91**, no. 10, 1996, p. 270
5. TAYLOR, M. M., CABEZA, L. F., MARMER, W. N., BROWN, E. M., KOLOMAZNIK, K., *J. Am. Leather Chem. Assoc.*, **93**, no. 2, 1998 a, p. 40
6. TAYLOR, M. M., CABEZA, L. F., DIMAIO, G. L., K., BROWN, E. M., MARMER, W. N., CARRIO, R., CELMA, P. J., COLT, J., *J. Am. Leather Chem. Assoc.*, **93**, no. 1998 b, p. 61
7. JIAN, S., WENYI, T., Wuyong, C., *Prod.*, 16, no. 5, 2008, p. 591
8. COT, J., *J. Soc. Leather Technol. Chem.*, 99, no. 8, 2004, pp. 322
9. GUO, Z.R., ZHANG, G., FANG, J., DOU, X., *J. Clean. Prod.*, 14, 2006, p. 75
10. HRNCIRIK, J., DVORACKOVA, M., HRUSKA, F., KUPEC, J., KOLOMAZNIK, K., *J. Am. Leather Chem. Assoc.*, **100**, no. 1, 2005, p. 1
11. MU, C., LIN, W., ZHANG, M., ZHU, Q., *Waste manag. : (Elmsford)*, **23**, no. 9, 2003, p. 835 – 843.
12. KUPEC, J., RUDLOVA, M., DVORACKOVA, S., RUICKA, J., KOLOMAZNIK, K., *J. Am. Leather Chem. Assoc.*, **97**, no. 9, 2002, p. 349
13. TRANDAFIR, V., POPESCU, G., ALBU, M. G., IOVU, H., GEORGESCU, M., in "Bioproduse pe bază de collagen" (Bio-products based on collagen), Ed. Ars Docendi, 2007
14. CIRJALIU-MURGEA, M., IONITA, A.D., CHITU, E., CHITU, V., FILIPESCU, L., Emulsified nutritive fluids and their proprieties control, *Acta Hort. (ISHS)*, 2010 (in press)
15. RENZI, D., CARLEVARO, C. M., STOICO, C., VERICAT, F. *Molecular Physics*, 99(11), 2001, p. 913
16. CASTANEDA, L., VALLE, J., YANG, N., PLUSKAT, S., SLOWINSKA, K., *Biomacromolecules*, 2008, 9 (12), p 3383
17. KOLOMAZNIK, K., JANAEOVA, D., LANGMAIER, F., MLADEK, M., 1<sup>st</sup> International Fur Congress, Kastoria-Greece, 1998, p. 134
18. PARAMAKALYANI, M., Multidimensional instrumental analysis of wet-blue leather hydrolysate, M.S thesis, Texas Tech University, 2002, [etd.lib.ttu.edu/theses/available/etd.../31295017082131.pdf](http://etd.lib.ttu.edu/theses/available/etd.../31295017082131.pdf)
19. BALABAN, A. T., BANCUIU, M., POGANY, I., in "Aplicații ale metodelor fizice în chimia organică" (Applications of physical methods in organic chemistry), Ed. Științifică și Enciclopedică, București, 1983

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