different F values when the cultivars Scout and Lancer were compared at six locations to TR-131 in percent unsaturated fatty acid. TR-131 was also significantly different than Scout and Lancer in flour percent short-chain and odd-chain fatty acids. When the analysis of variance test was performed on the above three cultivars with respect to ratio of percent C_{iso-14} to total C_{14}, significant F values were obtained for both grains and flours whereas in the case of the C_{iso-16} ratio to C_{16}, significant differences were only noted for the grains and not the corresponding flours. Analysis of variance calculations of cultivars TR-385 and TR-386 grown at two locations revealed the same significant differences as above. Also, the same significant differences were noted when least significant intervals were calculated and plotted. In addition, interaction plots were prepared that verified that varietal differences were indeed significant but that location was not.

Generally, it can be concluded that, even though there were slight differences in fatty acid composition between the winter wheat and winter triticale cultivars, the effect of location had a more pronounced influence on total protein and amino acid content than on fatty acid composition.

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Chemical and Nutritional Modifications of Sunflower Proteins Due to Alkaline Processing. Formation of Amino Acid Cross-Links and Isomerization of Lysine Residues

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Treatment of sunflower protein isolates with sodium hydroxide reduces their content of cystine, arginine, threonine, serine, isoleucine, and lysine, in agreement with known data from alkaline hydrolysis of proteins. Unusual amino acid residues are formed during these treatments; using ion-exchange chromatography and high-voltage paper electrophoresis, alloisoleucine, ornithine, lysinoalanine, and lanthionine were identified. The presence of the latter two compounds indicates the formation of cross-links in the protein and may explain observed changes in the in vitro proteolytic digestibility. The formation of ornithine and the decrease in arginine content appear to be the best indicators of the severity of the alkaline processing. Severe treatments with sodium hydroxide (>0.2 *M*, 80°, 1 hr) also provoke a marked degree of isomerization of the L-lysine residues into D-lysine, as demonstrated by both enzymatic and microbiological methods of analysis.

Alkaline processing of proteins is increasingly applied in the technological treatments of foods and feeds by solubilization and purification, to destroy toxic contaminants (Screenwasamurthy, 1967), to obtain functional properties, including the formation of textured vegetable protein fibers (*Nutr. Rev.*, 1967).

It is well known that severe alkaline treatments can cause chemical modifications of amino acid residues: destruction of cystine, arginine, threonine, serine, and lysine residues (Mellet, 1968; Blackburn, 1968; Parisot and Derminot, 1970). Formation of new cross-linked compounds also takes place.

The formation of lysinoalanine (LAL) has been demonstrated in acid hydrolysates of various alkali-treated proteins such as lysozyme, keratin, ribonuclease, wool, silk, and soy protein isolate (Bohak, 1964; Patchornick and Sokolovsky, 1964; Corfield and Wood, 1967; Miró and Garcia-Dominguez, 1967; Mellet, 1968; Asquith and Garcia-Dominguez, 1968; De Groot and Slump, 1969; Asquith et al., 1969). LAL was probably formed by the condensation of an ϵ-amino group of a lysine residue with a dehydroalanyl residue, leading to cross-links within or between polypeptide

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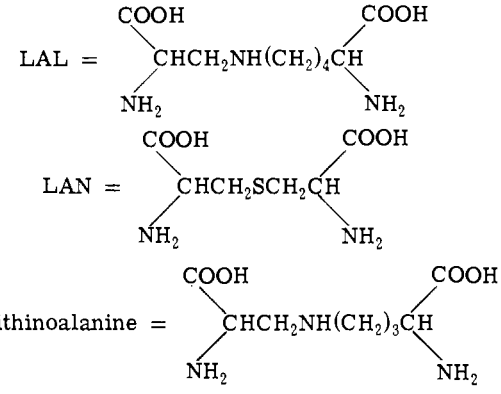
chains (Bohak, 1964; Patchornick and Sokolovsky, 1964; Asquith et al., 1969). It is thought that dehydroalanyl residues mainly derive from cystine residues through β elimination (Bohak, 1964) and probably also from seryl residues (Blackburn and Lee, 1956; Bohak, 1964; Ziegler, 1964; Corfield and Wood, 1967; Mellet, 1968).

Lanthionine (LAN) has been found in acid hydrolysates of alkali-treated wool and silk (Horn et al., 1941; Miró and Garcia-Dominguez, 1964; Derminot and Tasdhomme, 1965; Asquith and Carthew, 1972b), and comes from the condensation of a dehydroalanyl residue and a cysteinyl residue.

Ornithinoalanine has also been found in acid hydrolysates of alkali-treated wool and silk (Ziegler et al., 1967).



Treatment of proteins in alkaline solutions has also long been known to cause isomerization of amino acid residues at certain asymmetric carbon atoms (Dakin, 1912–1913; Levene and Bass, 1928; Neuberger, 1948; Hill and Leach, 1964; Pollock and Frommhagen, 1968; Tannenbaum et al., 1970). Isomerization of free amino acids also occurs, although at a slower rate. Changes in optical rotation of acid-treated proteins have also been observed (Schein and Berg, 1946; Blackburn, 1968). However, isomerization occurs less readily in acid than in alkaline solutions and measurable extents of isomerization only occur in concentrated solutions at high temperature (Neuberger, 1948; Goodman et al., 1962).

The present study was undertaken to evaluate chemical and nutritional modifications of alkali-treated sunflower protein isolate and casein. Destruction of amino acid residues was evaluated. New amino acids were identified using ion-exchange chromatography and high-voltage paper electrophoresis. A significant degree of isomerization of L-lysyl residues was observed using enzymatic and microbiological methods of analysis.

Destruction of amino acids residues, formation of new cross-links, and/or isomerization of the lysyl residues were also found to lower the nutritional value of the proteins, as evaluated by an in vitro proteolytic test with Pronase.

MATERIALS AND METHODS

Chemicals. All chemicals were from Merck (E. Merck, Darmstadt, West Germany), unless otherwise indicated. D-Alloisoleucine and L-lysine decarboxylase from *B. cadaveris* were purchased from Fluka (ref. 05705 and 62890, respectively). D,L-Lanthionine and L-ornithine were purchased from Sigma (4.6682 and 0.2375, respectively). The synthetic lysinoalanine was kindly provided by Dr. P. Slump (Central Institute of Nutrition and Food Research TNO, Zeist, Netherland) and the ciliate protozoon *Tetrahymena pyriformis* W by the Laboratoire de Zoologie 2, Université Paris-Sud, 91-Orsay, France). *Leuconostoc mesenteroides* P-60 was purchased from the Institut Pasteur, Paris, France.

Food Proteins. Experiments were carried out on casein (Hammarsten, Merck no. 2242) and two sunflower protein isolates (SPI).

SPI_1 (14.1% N, 94% D.W.) was prepared by an initial solubilization of defatted sunflower seed meal in 0.05 *M* NaOH (55°, 30 min). The proteins were isolated by precipitation with 1 *N* HCl at pH 4.8 and 4°, and then freeze-dried. SPI_1 was kindly provided by Mr. Maubois (Laboratoire de Recherche de Technologie Laitière, I.N.R.A., Rennes, France).

SPI_2 (16.5% N, 96% D.W.) was prepared by an initial solubilization of the defatted and dehulled sunflower seed meal in 1 *M* NaCl and then mixed with 0.02 *M* sodium borate (pH 8.6–8.7) (meal/solvent = 10). The proteins were precipitated by adding an equal volume of saturated ammonium sulfate solution (adjusted to pH 8–9 with NH_4OH). The sediment was dialyzed against water for 5 days at 4° and then freeze-dried. SPI_2 was kindly provided by Mr. Mossé (Laboratoire des Protéines et des Acides Aminés, Station de Technologie, C.N.R.A., Versailles, France).

Alkaline Treatments. SPI was dissolved (5%, w/v) in 0.05–1 *M* sodium hydroxide solutions and held at 55, 60, or 80° for 1–18 hr.

Amino Acid Analysis. The amino acids were separated in 19 hr on a NC1 Technicon autoanalyzer equipped with a single column (0.6 × 140 cm) which was kept at 60° (Piez and Morris, 1960), the resin used being Chromobeads type A. In some cases a 0.6 × 75 cm column packed with Chromobeads C_2 resin kept at 60° was used according to the technique recommended by Technicon (*Tech. Amino Acid Anal.*, 1966) which was further elaborated by Robin and Robin (1971). This technique permits the separation and analysis of amino acids in 6 hr and is adequate for tryptophan analysis.

Protein hydrolysis was carried out with 6*N* HCl at 110° for 24 hr under nitrogen. Norleucine was used as an internal standard (Gundlach et al., 1959).

In Vitro Proteolysis and Measurement of Amino Acids Released. Samples treated with sodium hydroxide as indicated above were hydrolyzed with Pronase (Calbiochem) at 50° for 8 hr. The pH was kept equal to 8 with NaOH using a Radiometer pH-Stat. Pronase was added in the proportion 1.5 g per 100 g of protein. Free amino acids were determined with the autoanalyzer after treatment of the hydrolysate with sulfosalicylic acid (Hamilton, 1962). Peptides only minimally interfered with the base line. The same determination was also performed on filtrates obtained by ultrafiltration of the hydrolysates with Amicon Diaflo PM 10 membranes at 0°. The results obtained were identical.

Desalting and High-Voltage Paper Electrophoresis. Proteins submitted to alkaline treatments underwent modifications in their amino acid contents as shown below. New peaks appeared on the chromatogram and had to be identified. Each compound corresponding to an unknown peak was first isolated by ion-exchange chromatography (autoanalyzer) and desalted on an Amberlite CG 120 resin (1.2 cm × 25 cm column) using NH_4OH as eluent. It was then identified by high-voltage paper electrophoresis (acetic acid–formic acid–water buffer, 87:25:888; pH 1.9; potential = 25 V/cm for 90 min), according to Atfield and Morris (1961).

Evaluation of the Degree of Isomerization of Lysine Residues. Two different analytical techniques were used.

(a) *L-Lysine Decarboxylase from B. cadaveris.* The activity and specificity of the enzyme preparation were first tested on L- and D-lysine solutions and on a solution containing L-lysine plus 14 other amino acids. The proportions of L- and D-lysine residues in alkaline-processed protein samples were then measured on acid hydrolysates of the samples, as follows: 0.5 ml of the L-lysine decarboxylase preparation (4 mg/ml) was added to sample hydrolysates containing 0.4–6.9 μmol of lysine in 0.5–2 ml of 0.5 *M* sodium acetate buffer (pH 6.0). After 135 min at 80°, the reac-

Table I. Composition of Basal Medium; Microbiological Evaluation of L-Lysine with *Leuconostoc mesenteroides* P-60[a]

Components	Quantity per 100 ml, mg	Components	Quantity per 100 ml
DL-Ala	200	K_2HPO_4	1 g
L-Arg·$2H_2O$	40		mg
L-Asp	100	$MgSO_4 \cdot 7H_2O$	180
L-Cystine	20	$MnSO_4 \cdot H_2O$	30
L-Glu	210		
Gly	20	Thymine	2
L-His	20	Adenine	2
L-Ile	20	Guanine	2
L-Leu	20	Cytidine	2
L-Met	20	Uracil	2
L-Phe	35		μg
L-Pro	20	Thiamine	200
L-Ser	25	Riboflavine	200
L-Thr	20	Biotin	6
L-Trp	20	Pyridoxal·HCl	60
L-Tyr	20	Pyridoxamine·2HCl	60
L-Val	20	Pyridoxine	200
		Folic acid	2
Glc	4	*meso*-Inositol	10
NaOAc	700	Lipic acid	2
NaCitrate·$2H_2O$	4	Nicotinamide	200
NH_4Cl	600	*p*-Aminobenzoic acid	40
$FeSO_4 \cdot 4H_2O$	6	Ca pentothenate	200
NaCl	40		

[a] The medium was adjusted to pH 6.0 and sterilized by filtration (Millipore filter HAWPO 1300, 0.22 μ).

tion was stopped by deproteinization with sulfosalicylic acid (Hamilton, 1962). The amino acid contents of both enzyme-treated and untreated samples were then determined using the autoanalyzer.

(b) *Microbiological Method.* The growth of L-lysine auxotrophic protozoa (*Tetrahymena pyriformis* W) and bacteria (*Leuconostoc mesenteroides* P-60) was used to determine the proportion of L isomer according to Stott and Smith (1966) and Itoh et al. (1973), respectively.

Each assay medium for the lysine evaluation with *T. pyriformis* W consisted of: 2 ml of solution E (Stott and Smith, 1966); *n* milliliters of a standard L-lysine solution (corresponding to 0, 45, 90, 180, 270, and 540 μg of a 20 mg/ml of L-lysine solution) or the samples to be tested ($n <$ 3 ml); $(3 - n)$ ml of water.

The alkali-treated or untreated protein samples (100 mg) were first hydrolyzed (6 *N* HCl, 110°, 24 hr). The microbiological evaluation of the L-lysine was carried out on an aliquot of the hydrolysate corresponding to 6.25 mg of initial protein.

After sterilizing the above solutions in screw-capped tubes at 120° for 10 min, 5 ml of sterile solution G (Stott and Smith, 1966) was added aseptically to each tube. These tubes were then inoculated with 50 μl of a 3-days broth culture of *T. pyriformis* W and incubated under agitation at 27° for 4 days. Samples (1 ml) were then transferred into smaller tubes containing 1 ml of preserving fluid (Stott and Smith, 1966). The organisms were counted in a hemocytometer, depth 0.2 mm, Fuchs-Rosenthal cell.

Absorbance of the samples was also measured at 580 nm after appropriate dilution according to Shorrock and Ford (1973).

The L-lysine content was also evaluated with *Leuconostoc mesenteroides* P-60 according to the method of Itoh et al. (1973). Inocula transferred from the stock culture were grown for 18 hr at 37° in 2 ml of the complete medium (1 ml of basal medium as shown in Table I + 1 ml of a 40 mg/100 ml of L-lysine solution). After centrifugation, the cells were resuspended in 10 ml of sterile 0.9% NaCl solution.

The assay medium consisted of 2 ml of basal medium (Table I), 2 ml of the samples to be tested (corresponding to 1.25 mg of protein, pH 6.0), or 2 ml of the lysine standard (L-lysine solution, 40 mg %, or D-lysine solution, 50 mg %, both sterilized at 121° for 20 min). The inocula described above (50 μl) were used to inoculate each assay tube. The assay culture was carried out at 37° for 18 hr in matched 127 × 10 mm cylindric tubes. Growth was estimated by direct turbidimetry on the assay tubes with a Bausch and Lomb spectronic 20 photometer at 660 nm.

RESULTS AND DISCUSSION

Amino Acid Destruction and Formation of Unusual Amino Acids. Sunflower protein isolate no. 1 (SPI_1) and casein were submitted to 0.05, 0.1, 0.2, 0.5, and 1 *M* sodium hydroxide (final pH 9.6, 9.8, 11.5, 12.7, and 12.9, respectively) at 55 and 60° for 1, 5, 15, and 16 hr.

The content of aspartic and glutamic acids, alanine, leucine, methionine + methionine sulfoxide, phenylalanine, proline, and tyrosine was not found to be significantly modified by the various treatments. Progressive and marked losses in arginine, cystine, threonine, serine, lysine, and isoleucine were found to occur (Table II). Arginine was the amino acid most affected with a 100% loss for the most severe treatment. The content of ornithine increased proportionately to the arginine loss in agreement with its proposed formation from arginine (Geshwind and Li, 1964; Parisot and Derminot, 1970). The cystine content, initially very low, was decreased below analytical sensitivity by relatively mild alkaline treatments. The losses in serine and threonine amounted to 55 and 82%, respectively, for the most severe treatment. These results correspond to well-known data concerning the effects of alkaline hydrolysis of proteins (Blackburn, 1968).

Unusual amino acids residues were formed as a result of these alkaline treatments of SPI_1 and casein; using ion-exchange chromatography and high-voltage paper electrophoresis after acid hydrolysis it was possible to identify lysinoalanine (just before lysine on the autoanalyzer), lanthionine (in the peak of glycine), alloisoleucine (just before isoleucine), and ornithine (between ammonia and lysine). In wool alkali-treated samples, Ziegler et al. (1967) found ornithinoalanine which we could not identify in our samples with the methods of analysis employed. Synthetic ornithinoalanine could not be purchased for reference. The formation of alloisoleucine was inversely proportional to, and most probably resulted from, the destruction of isoleucine.

Lysinoalanine was found to be present in the initial SPI_1, which had been mildly alkali treated during its preparation. The lysinoalanine content appeared to increase and then decrease as the severity of the alkaline treatment was increased. The reason for the apparent loss in lysinoalanine (and recovery of lysine) upon severe treatment with 1 *N* NaOH at 80° for 16 hr has not been elucidated. However, it was qualitatively found that pure lysinoalanine was partly destroyed when submitted to 1 *N* NaOH (0.626 μ*M*/ml) at 80° for 16 hr.

The cysteine–cystine content of SPI_1 is too low to account for both the lysinoalanine and lanthionine formed. In the present experiments, it is likely that dehydroalanine residues came partly from the decomposition of serine (and possibly threonine) in line with the results of other investigators obtained with various proteins (Bohak, 1964; Corfield and Wood, 1967; Mellet, 1968; Asquith et al., 1969; De

Table II. Modifications of the Amino Acid Composition of a Sunflower Protein Isolate and of Casein Due to Alkaline Treatments

Amino acid (mmol/100 g sample)	Sample and alkaline treatment: NaOH (M); T (°C); time (hr)										
	SPI_1	SPI_1: 0.05; 55; 1	SPI_1: 0.1; 55; 1	SPI_1: 0.2; 55; 1	SPI_1: 0.2; 80; 1	SPI_1: 0.2; 80; 5	SPI_1: 0.2; 80; 16	SPI_1: 0.1; 80; 16	SPI_1: 0.5; 80; 16	SPI_1: 1; 80; 16	Casein	Casein: 0.2; 80; 15
Asp	53	52	54	53	53	53	52	53	52	52	53.5	53.5
Glu	106	105	106	104	108	105	106	109	106	105	131	131
Pro	28	28	29	28	29	28	30	29	30	29	84	71
Ala	35	35	37	36	35	35	36	35	34	35	31	34
Val	33	33	33	33	30	33	31	35	33	34	49	48
Met	8.5	10	10	10	11.1	2.5	11	12	11	6.5	15	18
MetSO[b]	2	0.9	1	1	0.5	7	0.5	0	1	4	3.5	0.5
Leu	36	34	38	35	36	36	36	36	36	36	64	62
Tyr	11	11	12	11	12	10	11	11	11	10	28	27.5
Phe	24	23	25	23	24	25	25	25	25	22.5	28	29
Arg	38	36	35	33	31	20	12	30	1	Traces	20	5.5
Thr	22	22	22	21	18	13	10	18	4	4	32	11
Ser	27	27	28	27	23	15	14	22	12	11	46	15
Gly[a]	45	46	50	49	51	52	57	52	59	58	21	39
Half-Cys	2.5	2.5	2	Traces	0	0	0	0	0	0	0	0
*a*Ile	0	0	0	0.5	1.5	3.5	4	1.5	11	12	0	++
Ile	25	26	26	24	24	23	21	25	16	14	37	31
Orn[b]	1	1	1	1.5	5	16	21	4.5	33	31	0	13
LAL[b]	2.8	3	3.5	3	5.5	4	3	3.2	2	1.3	0	+++
Lys	11	10	10	10	8.5	9.5	10	7.5	11	12	48	45
His	13	10	13	12	12	11	10	13	9	6	18	16

[a] Interference with lanthionine. [b] Orn = ornithine; LAL = lysinoalanine; MetSO = methionine sulfoxide.

Table III. Release of Essential Amino Acids from Alkali-Treated Sunflower Proteins by in Vitro Proteolysis

Sample and treatment	Thr[a]	Val[a]	Met[a]	Ile[a]	Leu[a]	Phe[a]	Lys[a]	Σ(free AA released)/ Σ (AA content of alkali-treated SPI)
SPI_2	20	50	75	55	55	65	45	25.5
SPI_2, NaOH, 0.1 M, 30°, 5 hr	18.5	40	45	45	35	55	20	17
SPI_2, NaOH, 0.1 M, 50°, 5 hr		35	45	40	35	35	26	15
SPI_2, NaOH, 0.1 M, 50°, 15 hr		25	30	30	30	25	20	12
SPI_1	25	50	50	60	47	55	30	22.4
SPI_1, NaOH, 0.1 M, 60°, 18 hr	6.5	25	15	25	20	15	13.5	8.6
SPI_1, NaOH, 0.2 M, 80°, 15 hr	15	7.5	11	11	9.5	9.5	6.5	8.6

[a] Millimoles of free amino acid released (Pronase)/millimoles amino acid (acid hydrolysis) × 100. For the alkali-treated samples, acid hydrolysis was performed after the alkaline treatment.

Groot and Slump, 1969; Parisot and Derminot, 1970; Asquith and Carthew, 1972a,b).

The formation of ornithine and the decrease in arginine content appeared as the most sensitive indicators of the severity of the alkaline treatment to which the sunflower protein had been submitted.

Some of the alkaline treatments used in this study were similar to those used for the purification and spinning of sunflower proteins (0.05 to 0.1 M NaOH at 55° for a few hours). The data from more severe treatments could be relevant to cases of overprocessing. Damage to lysine must be considered as particularly detrimental since lysine is the limiting amino acid of SPI.

In Vitro Proteolysis of Alkali-Treated Sunflower Proteins. The "overall in vitro digestibility" of the treated SPI and an expression of the "in vitro digestibility" of essential amino acids were evaluated by the measurement of free amino acids released by in vitro proteolysis with Pronase and calculation of the two following ratios: total free amino acid released/total amino acid content of alkali-treated SPI, and free essential amino acid released/treated protein content of the same amino acid. These ratios markedly decreased as a function of the severity of the alkaline treatments (Table III). The "in vitro availability" of all the essential amino acids was simultaneously and similarly reduced.

For better validity from a nutritional standpoint, these results should be compared with those of in vivo experi-

Table IV. Isomerization of the L-Lysine Residues of Sunflower Protein Isolate No. 1 Due to Alkaline Treatments

	Alkaline treatment: NaOH (M); T (°C); time (hr)								
		0.05; 55; 1	0.1; 55; 1	0.2; 55; 1	0.2; 80; 1	0.2; 80; 5	0.2; 80; 16	0.1; 80; 16	0.5; 80; 16	1; 80; 16
Total Lys[a]	11	10	10	10	8.5	9.5	10	7.5	11	12
D-Lys[b]	0.4	0.5	0.5	0.75	1.3	2.8	3.7	2.8	6	6.4
(D-Lys × 100)/ total Lys	4	5	5	8	15	30	40	35	50	50
Total Lys[a]	11						9.6	9	11.5	12.3
L-Lys[c]	11						5.5	6.2	6.2	6.2
(D-Lys × 100)/ total Lys	0						43	31	47	50

[a] Millimoles per 100 grams of sunflower protein isolate (ion exchange chromatography after acid hydrolysis). [b] Enzymatic evaluation. [c] Evaluation with *Leuconostoc mesenteroides* P-60.

ments. It might well be that the formation of lysinoalanine and lanthionine intra- and/or interchain cross-links restricts the accessibility of the protein to proteolytic enzymes and reduces the activity of the latter. Since Pronase has a tryptic activity, it is also likely that this activity is reduced by substitution of the ϵ-amino group of lysine residues. It can be noted in this context that a slight proportion of lysinoalanine residues (3%) is released by Pronase action. (This is also of interest in view of a recent report on the possible toxicity of lysinoalanine; Woodard and Short, 1973.)

Various investigators have demonstrated that alkaline treatments of fish or soybean proteins lower the nutritional value of these proteins for chicks, rats, or lambs (Carpenter and Duckworth, 1950; De Groot and Slump, 1969; Gorril and Nicholson, 1972).

Isomerization of L-Lysine Residues. It was first checked that L-lysine decarboxylase effectively decarboxylates 98% of L-lysine while other amino acids present in the solution including D-lysine and lysinoalanine were not modified.

The relative proportions of L- and D-lysine in several acid hydrolysates of alkali-treated SPI were then determined using the L-lysine decarboxylase method.

Acid hydrolysis and moderate alkaline treatments of SPI were found not to cause any noticeable isomerization of the L-lysine residues. Similarly, free L- and D-lysine were not found to be affected by acid hydrolysis. Treatments with strong acids have been reported to cause isomerization of some amino acids (cf. introductory statement; Neuberger, 1948; Hill and Leach, 1964; Manning, 1970).

Jacobson et al. (1974) have recently shown that 6 N HCl hydrolysis of proteins at 110° for 20 hr causes a 50% isomerization of cystine residues but little or no isomerization of other amino acids; no isomerization of lysine occurred.

With respect to alkaline processing, the ratio of isomerization became significant for a 0.2 M NaOH, 80°, 1-hr treatment and markedly increased with the severity of the alkaline processings, reaching 40% after 16 hr in 0.2 M NaOH at 80° (Table IV). It appeared to reach a theoretical maximum corresponding to complete racemization, but the degree of analytical precision did not permit a conclusion on this point.

Similar results were obtained using the growth of L-lysine auxotrophic microorganisms, *Tetrahymena pyriformis* W protozoa or *Leuconostoc mesenteroides* P-60 bacteria. It was noted that *T. pyriformis* growth overestimated the lysine content of the samples. Therefore, only results from *Leuconostoc mesenteroides* growth were taken into account (Table IV). These last data were obtained from duplicate experiments and although very close to those from determination with L-lysine decarboxylase cannot be considered as statistically valid. The enzymatic method for assessment of the L-lysine isomerization is therefore preferable, in addition to being easier and more rapid to perform.

Isomerization of L-lysine residues was also observed with NaOH-treated casein.

From a nutritional standpoint, isomerization of L-lysine residues is likely to reduce the action of trypsin and the availability of lysine. It is known that D-lysine and many other D-amino acids are not biologically available to the organism (Berg, 1959).

Isomerization of amino acids other than lysine might well have also occurred in the present experiments, and probably contributed to the previously demonstrated decrease in the in vitro digestibility of the treated proteins. Isomerization of aspartic and glutamic acids, alanine, phenylalanine, serine, cystine, methionine, and tyrosine has previously been shown to occur through alkaline treatments (Hill and Leach, 1964; Pollock and Frommhagen, 1968).

In conclusion, although lysine isomerization only occurs under severe conditions, alkaline treatments of food proteins should be kept to a minimum since they readily bring about chemical modifications of cystine, threonine, and lysine with detrimental effects on the nutritional value.

Since this paper was submitted for publication, a complementary determination of the release of free amino acids during alkaline treatment has been made. Less than 0.5% of the amino acid residues of SPI_1 is released by 0.1 N NaOH, 80°, 16 hr, indicating that isomerization effectively takes place on lysyl residues and not on free lysine.

In a recent study with rats, Van Beek et al. found NaOH-treated, lysinoalanine-containing soybean proteins not to provoke the renal cytomegalic toxicity described by Woodard and Short (1973). Only nephrocalcinosis was observed, and could be inhibited by addition of calcium to the diet.

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Analysis of Buchu Leaf Oil

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In addition to the previously described stereoisomers of 8-mercapto-*p*-menthan-3-one about 120 further constituents have been identified in Buchu leaf oil of commercial origin. Some new sulfurated terpenoid ketones as well as *p*-menthan-3-one derivatives oxygenated in the 2 or 4 position found for the first time to occur in nature are discussed in more detail.

Creation of a well-accepted black currant flavor needs the essential oil of Buchu leaves originating from South Africa. Its scarcity during the last few years prompted us to analyze the oil in order to determine its flavorwise important components.

Only three constituents were known in the chemical literature (Gildemeister and Treibs, 1959; Guenther, 1964) until Fluck and coworkers (1961) succeeded in identifying the levorotatory pulegone and the so-called ψ-diosphenol. In 1967 older data were corrected in the sense that not (−)-menthone, but (−)-isomenthone was found to be the major constituent accompanied by (+)-menthone (Klein and Rojahn, 1967).

From our own investigation on Buchu leaf oil of commercial origin, we have been able to verify the occurrence of six main and ten minor constituents cited in the literature. However, we did not succeed in finding any piperitone epoxide also cited to occur in Buchu leaf oil (Klein and Rojahn, 1967), neither in commercial samples of different suppliers nor in samples of specific botanical origin. Being aware of the fact that piperitone epoxide very easily rearranges to diosphenol (Reitsema and Varnis, 1956) which thermally converts to an equilibrium mixture including about 30% ψ-diosphenol (Fluck et al., 1961), both found in Buchu leaf oil, we decided to check our negative findings using synthetic piperitone epoxide as reference material. This was prepared starting from piperitenone via the piperitenone epoxide (Reitsema, 1957) followed by catalytic hydrogenation (Figure 1). The spectra (ir, NMR, MS) thus obtained differ from those of the isolated components (ψ-diosphenol and diosphenol).

The suspicion that the diosphenols of Buchu leaf oil might be artefacts formed from piperitone epoxide during oil production (Klein and Rojahn, 1967) could be dissipated by the fact that even a diethyl ether extract of dry leaves showed the presence of ψ-diosphenol as well as diosphenol in the same ratio as found in the steam-distilled essential oil (Figure 2).

EXPERIMENTAL SECTION

Procedures. Commercial Buchu leaf oil (660 g) (supplier: White, Tomkins and Courage, Reigate, England) was fractionated by distillation, crystallization, and chromatographic procedures. Heat-sensitive constituents were transformed to more stable derivatives (e.g. by mild acetylation) before being separated or purified by preparative GLC. Trace components detected through GLC–MS coupling experiments were identified by correlation with authentic synthetic specimens.

Apparatus. *GLC.* Carlo Erba Model GI with FID and Model Fractovap GV were used. (a) Packed glass columns were used for analytical measurements: length 3 m; 3 mm i.d.; 2% Carbowax 20M on Chromosorb G (AW DMCS, 60–80 mesh); temperature programmed from 120 to 240° with a heating rate of 5°/min or isothermal at 160 or 180°; carrier gas flow, 50 ml of He/min. (b) Packed glass columns were used for preparative purposes: length 3 m; 15 mm i.d.; 10 to 5% Carbowax 20M (loading with stationary phase stepwise decreasing with increasing distance from column entrance) on Chromosorb G (AW DMCS, 80–100 mesh); isothermal at 160 or 180°; carrier gas flow, 200 ml of N_2/

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