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OPTIMUM YIELD OF PYRIDOSINE AND FUROSINE ORIGINATING FROM MAILLARD REACTIONS MONITORED BY ION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

An improved ion-exchange chromatographic method for rapid separation and quantitative estimation of deoxyfructosyllysines and their hydrolysis products (pyridosine and furosine) is described. The proposed elution procedure has good reproducibility; the optimum hydrolysis conditions for the highest molar ratio of pyridosine and furosine are given. The furosine/pyridosine molar ratios obtained by "hydrolysis" of model fructosyllysine as well as of natural protein matrices (soya beans, tomato powder, barley and malts) are in good agreement and without exception > 1. The smallest amount of pyridosine determined was 0.009% (w/w). The relationship between the compounds pyridosine, furosine, lysine and carbohydrate units present in the parent moiety is discussed.

INTRODUCTION

The reactive lysine content of protein matrices containing reducing sugars decreases mainly through well known Maillard interactions[1,2]. The first biologically inactive, irreversible product originating from the interaction of the saccharide/carbonyl group and of the lysine/ε-amino group is N_ε-deoxyfructosyllysine (ε-DFL). Quantitation of the selective hydrolytic products of ε-DFL (pyridosine and furosine) is an excellent way of following the extent of the early Maillard reactions (Fig. 1).

In the absence of an appropriate analytical method, the molar ratios of the hydrolytic products of ε-DFL (lysine, pyridosine, furosine) given in the literature are contradictory[3,4].

The high-performance liquid chromatographic (HPLC) method published[5] enables quantitation only of the furosine content of the protein hydrolysate. The ion-exchange chromatographic (IEC) methods reported[4,6–8] are (i) time-consuming and (ii) in the course of elution at least four peaks were found between pyridosine

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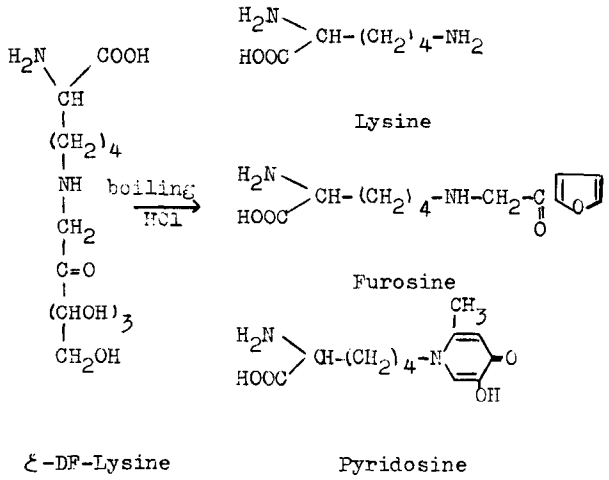

Fig. 1. Compounds obtained by the treatment of ε-DFL with acid. The percentages of lysine, furosine and pyridosine were 50, 20 and 10 (according to Bujard and Finot[3]) and 30, 30 and 30 (according to Steinig and Montag[4]), respectively.

and furosine. A gas–liquid chromatographic (GLC) procedure published recently[9] seems to be a powerful method for the analysis of pyridosine and furosine.

Our work was intended to provide a suitable IEC method for the rapid measurement of lysine, pyridosine and furosine with good reproducibility in order to clarify the optimum conditions for determination of the extent of ε-DFL formation. The aim was then to optimize (i) the time of hydrolysis, (ii) the concentration of hydrochloric acid, resulting in (iii) an analytically utilizable molar ratio of furosine to pyridosine.

MATERIALS AND METHODS

Reagents

All reagents were of analytical purity obtained from E. Merck (Darmstadt, F.R.G.) and from Reanal (Budapest, Hungary).

Preparation of the model deoxyfructosyllysine sample

In the absence of pure standards for pyridosine and furosine, we prepared a model deoxyfructosyllysine according to the literature[10,11]. The deoxyfructosyllysine syrup free from glucose, shown by GLC to be the trimethylsilyloxime derivative, was dissolved in warm, water-free methanol precipitated by the dropwise addition of diethyl ether. The white-brown crystalline precipitate was filtered off, washed with diethyl ether and dried, without delay, using a vacuum desiccator. The dry material, the so-called "crystalline deoxyfructosyllysine" (CDFL), was kept in a closed bottle in a refrigerator and served as a reference sample for our model investigations.

Natural protein matrices

Soya bean 1 is an untreated, defatted commercial variety, NKS 1346 Glycine

TABLE I

CONDITIONS FOR RAPID ELUTION OF PYRIDOSINE AND FUROSINE

Analytical column: 385 mm × 3.2 mm, BTC Biotronik 2710. Buffer flow-rate: 0.28 cm^3/min. Ninhydrin flow-rate: 0.15 cm^3/min. Coil temperature: 125°C.

Buffer	*[Li^+] (M)*	*Lithium-citrate (M)*	*In the presence of*	*pH*	*Time (min)*	*Temperature (°C)*
A	0.10	0.0685	6% Methanol	2.75	5	31
B	0.12	0.0685	5% Methanol	3.00	5	49
C	0.18	0.0685	–	3.55	5	49
D	0.40	0.0670	1.5 g/l Boric	3.95	8	60
E	1.40	0.0176	4 g/l acid	3.35	47	60
F	0.30	–	–	6	60	
A	0.10	0.0685	6% Methanol	2.75	25	31

max. Soya bean 2 and 3 are processed variants of soya bean 1. Sample 2 was heated to 100°C for 5 min by applying microwave heat treatment. Sample 3 was acidified with hydrochloric acid to pH 2 and was not neutralized.

Tomato powder 404 was obtained from Spreda (Burgdorf, Switzerland).

Barley and malt samples were purchased from Rhein-Ruhr-Malz (Mülheim, F.R.G.). Malt 1 was predried continuously at 56°C, to a water content of 48%. Malt 2 is a finished light malt obtained from malt 1 by drying at 78°C for 4 h. Malt 3 is a typical dark malt heated to 100°C for 4 h. Malt 2 had a water content of 24%, malt 3 one of 3%.

Apparatus

The amino acid analyser used was a Model LC 5001 instrument (Biotronic Wissenschaftliche Geräte, Maintal, F.R.G.). Chromatographic peak area determinations were made with a Data Processor C-R3A (Shimadzu, Europa, Düsseldorf, F.R.G.).

IEC analysis

The operating conditions are detailed in Table I.

Preparation of the samples

Various amounts of samples (see Tables II and III), weighed with analytical accuracy, were transferred to a 20-cm^3 Pyrex screw-cap vial furnished with PTFE-faced septa, and 5 cm^3 hydrochloric acid of different concentrations were added. High-purity nitrogen gas was bubbled through the solution for 5 min. The closed vials were kept at 110°C in a drying oven for different periods of time. At the end of the hydrolysis (after filtering if necessary), 10.00-cm^3 stock solutions were made up in distilled water. A 1.00-cm^3 volume of the stock solution was evaporated to dryness under vacuum using a rotary evaporator and a water-bath kept at <60°C. The residue was dissolved in 1 cm^3 buffer solution pH 3.55 (buffer C, Table I).

RESULTS AND DISCUSSION

Our model investigations performed with the CDFL (Table II, Figs. 2–4) and

TABLE II

COMPOSITION OF CDFL: BEFORE HYDROLYSIS AND IN ITS ACID-TREATED SOLUTIONS PREPARED IN DIFFERENT WAYS

Amount of sample		*Conditions*		*Components (%, w/w)**											*Molar ratio F/P*
Hydrolyzed (mg)	*Injected (μg/50 μl)*	*Hydrochloric acid (M)*	*Time (h)*	X_1	X_2	X_3	*α,ε-DDFL*	*α-DFL*	*ε-DFL*	*Ammonia*	X_4	*Lysine*	*Pyridosine (P)*	*Furosine (F)*	
13.00	13.0	Without hydrolysis		3.2	0	4.8	39.4	13.1	28.8	1.0	4.6	4.8	0	0	–
13.00	65.0			2.6	0	5.2	40.6	13.8	30.6	1.0	5.2	5.2	0	0	–
11.4	11.4	6	0.5	0.3	4.9	5.9	30.8	12.3	27.5	3.0	8.1	6.0	1.1	0.2	0.2
12.9	12.9	6	1	0.2	7.5	4.3	10.2	13.5	25.3	2.8	6.7	17.9	6.4	5.0	0.8
12.7	12.7	6	2*	0.3	5.1	7.1	0.9	6.0	10.0	2.3	10.6	46.4	12.2	16.0	1.3
14.3	71.5	6	2	0.6	0	3.0	0.8	6.5	9.3	0.9	8.8	39.0	13.0	18.3	1.4
13.6	68.5	6	5	0.8	0	3.6	1.2	4.9	2.2	0.8	9.9	40.9	19.6	20.2	1.3
12.5	62.5	6	20	0.5	0	3.9	0	3.2	2.7	0	16.4	40.9	13.9	18.8	1.4
15.2	76.0	1.5	5	0.6	0	2.8	0	5.8	10.0	0.9	10.2	59.2	2.1	8.4	4
12.4	62.0	3.0	5	0.7	0	2.6	0	4.8	2.3	0.9	20.9	50.4	7.1	11.2	1.6
11.4	57.2	8.0	5	1.0	0	3.8	0	4.0	2.3	0	14.0	35.6	14.7	24.5	1.7
12.2	61.0	12.0	5	1.6	0	5.1	0	1.9	2.6	0	112.0	31.1	10.7	33.9	3.2

* Expressed as a percentage of the air-dried samples, calculated as detailed in the text.

** Hydrolyzed using a reflux condenser; all other hydrolyses were carried out in Pyrex vials wiht PTFE-faced septa.

TABLE III

PYRIDOSINE AND FUROSINE CONTENTS OF SOYA BEAN SAMPLE 1* AS A FUNCTION OF ITS HYDROLYSIS CONDITIONS

Std.E. = Standard error; Std.E.% = relative standard error.

Amount of sample		*Conditions*		*Content (%)***		*Molar ratio*
Hydrolyzed (mg)	*Injected (μg/50 μg)*	*Hydrochloric acid (M)*	*Time (h)*	*Pyridosine*	*Furosine*	*F/P*
100.2	501	6	2	0.09	0.05	0.6
97.3	486	6	5	0.21	0.31	1.5
97.3	486			0.21 ***	0.30 ***	1.4
98.6	493	6	5	0.20	0.29	1.5
99.4	497	6	5	0.16	0.28	1.8
				Mean§ 0.19	0.29	
				Std.E. 0.026	0.01	
				Std.E.% 14	3.4	
98.5	493	6	10	0.17	0.36	2.1
77.7	389	6	22	0.14	0.38	2.7
56.6	283	6	22	0.14§	0.38§	2.7
96.5	483	6	22	0.135	0.36	2.7
				Mean § 0.138	0.373	
				Std.E. 0.003	0.011	
				Std.E.% 2.1	3.1	
100.1	501	6	32	0.13	0.33	2.3
95.4	482	6	50	0.13	0.31	2.4
102.3	511	3	5	0.08	0.10	1.3
99.2	416	3	20	0.10	0.21	2.1
99.4	497	6	5	0.22	0.39	1.8
101.9	506	6	20	0.13	0.37	1.8
102.6	513	8	5	0.14	0.47	3.4
96.4	482	8	20	0.12	0.55	4.6
98.9	495	12	5	0.10	0.77	7.7
106.0	530	12	20	0.09	0.61	6.8

* The saccharide[12,13] and protein compositions[14–16] of this sample were determined by different methods[12–16].

** Calculations based on the response of arginine (t_R 58 min) eluted between the peaks of pyridosine (t_R 54 min) and furosine (t_R 69 min).

*** Data obtained by the parallel elution of the same hydrolysate.

§ Values used for reproducibility measurements originating from different hydrolysis: all data are the means of at least two parallel elutions.

with a characteristic representative of soya bean (sample 2) damaged by Maillard interaction (Table III, Fig. 5), as well as with various protein matrices (Table IV), provided the following results.

(1) In order to confirm the identity of the individual components of CDFL,

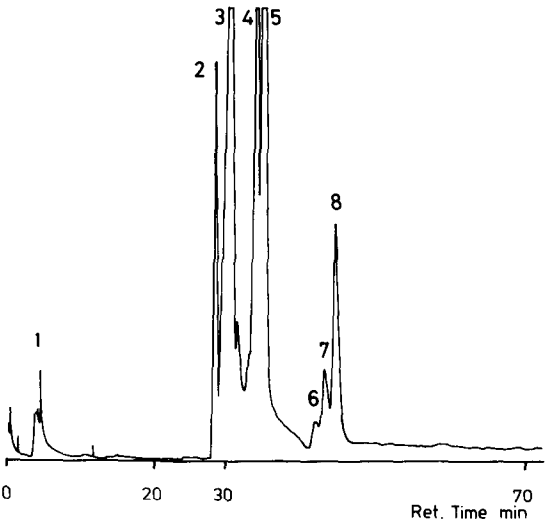

Fig. 2. IEC chromatogram of CDFL. Peaks: 1 = X_1; 2 = X_3; 3 = α,ε-DDFL; 4 = α-DFL; 5 = ε-DFL; 6 = ammonia; 7 = X_4; 8 = lysine. Note: X_1–X_4 are ninhydrin-active components of unknown structure.

$N_{\alpha\varepsilon}$-deoxydifructosyllysine (α,ε-DDFL), N_α-deoxyfructosyllysine (α-DFL) and N_ε-deoxyfructosyllysine (ε-DFL) were associated with the respective compounds identified in earlier work[10,11].

As to the identification of the pyridosine and furosine peaks, since pure standards are not available, we first assumed that the two new peaks in the acid-treated solutions of CDFL are pyridosine and furosine (Figs. 2–4). This assumption was verified by a cake-hydrolysate containing pyridosine and furosine identified by Erbersdobler *et al.*[8] and indirectly also by GLC–mass spectrometry (MS)[8,9].

(2) The percentage product distributions of CDFL and those of its "hydrolysates" were expressed as a percentage of the total ninhydrin-active components eluted by IEC (Table II).

Since the standard CDFL was free from saccharides and inorganic materials[10,11] and contains only "ninhydrin-active" components, we assumed (in the absence of standards) that the ninhydrin activities (responses) of the individual fructosyllysins are probably the same. Thus in the case of CDFL the percentages shown in Table II represent peak area percentages.

For protein matrices the basis of the calculation of the pyridosine and furosine contents was the molar response of arginine (t_R 58 min) eluted between pyridosine (t_R 54 min) and furosine (t_R 69 min) (Tables III and IV).

(3) Our elution program is advantageous since: (i) in the case of protein hydrolysates the only amino acid eluting between pyridosine and furosine is arginine serving as the basis of the calculations and (ii) both peaks eluted as well resolved, except in the case of partial hydrolysis (Fig. 5, chromatogram A).

(4) For the model investigations of the hydrolysis procedure, two standard samples were used: (i) CDFL which can be weighed reproducibly with analytical accuracy and (ii) soya bean sample 2, a natural protein matrix with a relatively high deoxyfructosyllysine content[12–16].

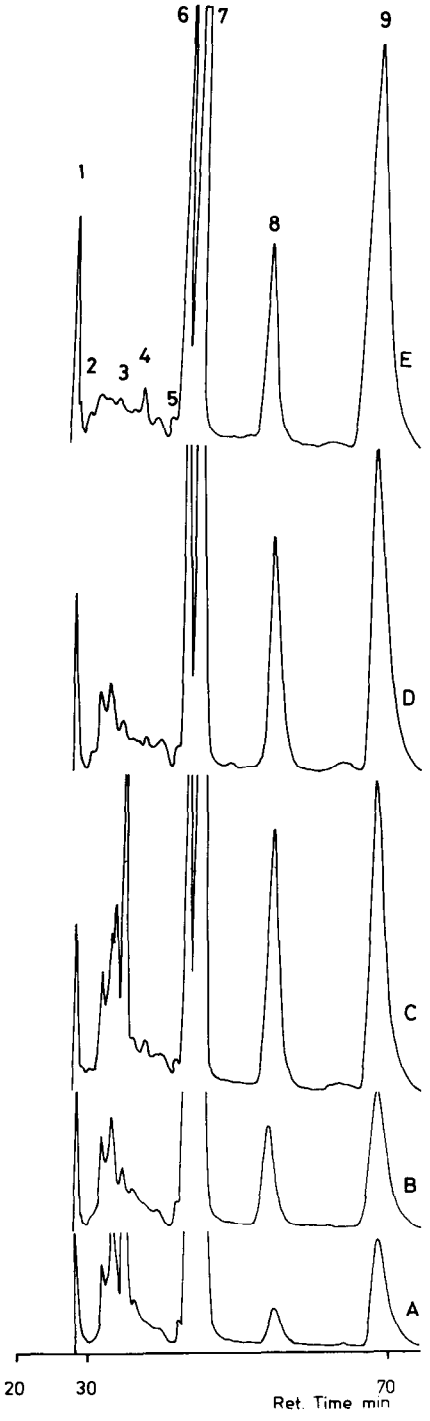

Fig. 3. IEC chromatograms of CDFL after treatments with hydrochloric acid of different concentrations at 110°C, for 5 h. Hydrochloric acid concentrations in A, B, C, D and E were 1.5, 3, 6, 8 and 12 *M* respectively. Peaks: 1 = X_3; 2 = α,ε-DDFL; 3 = α-DFL; 4 = ε-DFL; 5 = ammonia; 6 = X_4; 7 = lysine; 8 = pyridosine; 9 = furosine.

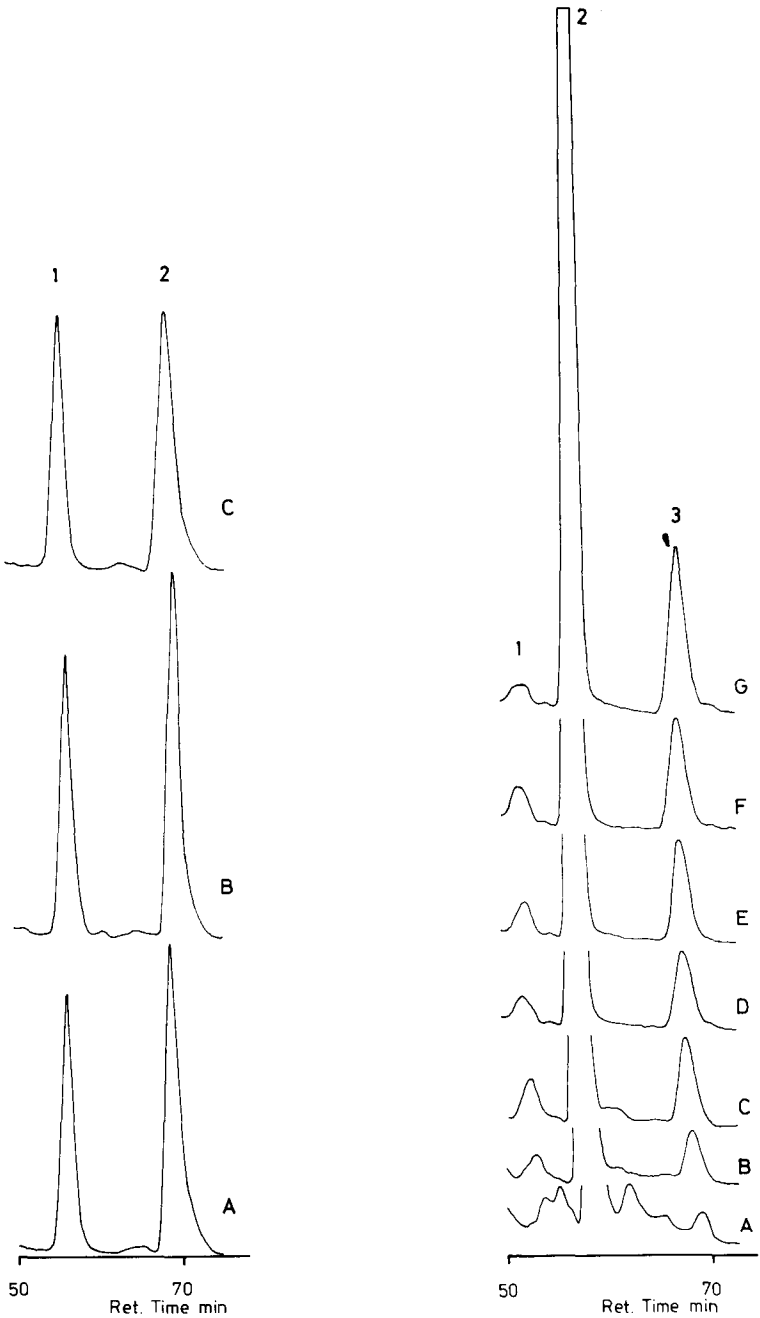

Fig. 4. IEC chromatograms of CDFL after acid treatment ([HCl] = 6 *M*) for 2, 5 and 20 h (A, B and C, respectively). Peaks: 1 = pyridosine; 2 = furosine.

Fig. 5. IEC chromatograms of soya bean 2 hydrolysates after 5 h of hydrolysis (chromatograms A, C, E and G) and after 20 h of hydrolysis (chromatograms B, D and F) with various hydrochloric acid concentrations: A and B, 3 *M*; C and D, 6 *M*; E and F, 8 *M*; G, 12 *M*. Peaks: 1 = pyridosine; 2 = arginine; 3 = furosine.

TABLE IV

PYRIDOSINE AND FUROSINE CONTENTS IN THE HYDROLYSATES OF VARIOUS PROTEIN MATRICES

Conditions of hydrolysis: 6 *M* hydrochloric acid; 20 h; 110°C.

Matrix	*TLC**	*Pyridosine (P) (%)***	*Furosine (F) (%)***	*Molar ratio F/P*	*Blocked lysine (%)***
Soya bean 1	2.73	0.012	0.028	2.3	1.4 (1)
Sya bean 2	2.06	0.14	0.38	2.7	20.3 (25)
Soya bean 3	2.11	0.032	0.047	1.5	2.9 (15)
Tomato powder	0.53	0.050	0.053	1.1	26.3 (87)
Barley	0.52	0.0	0.0	–	0 (67)
Malt 1	0.59	0.010	0.020	2.0	9.9 (53)
Malt 2	0.52	0.009	0.013	1.4	7.4 (72)
Malt 3	0.62	0.017	0.036	2.1	16.2 (72)

* Total lysine value: measured by GLC as their N-trifluoracetyl *n*-butyl esters[17]

** Expresses as in Table II.

*** Calculated according to the formula of Bujard and Finot[3]:

$$\text{Blocked lysine (\%)} = \frac{3.1 \times F \times 100}{TLV + 1.8 \times F}$$

Data in parentheses represent the values obtained by our dye-binding procedure[14].

(5) Comparing the pyridosine and furosine yields in the hydrolysates from both standard samples as a function of the hydrolysis time and hydrochloric acid concentration, it is clear that a reasonable relation of parameters is necessary as follows:

Whereas the amount of furosine increases continuously with the hydrochloric acid concentration (Tables II, III; Figs. 3–5), the optimum amount of pyridosine is obtained at a hydrochloric acid concentration of 6 *M*.

The optimum reaction times for the formation of pyridosine and furosine with the two model samples are different (CDFL, 5 h, Table II, fig. 4; soya bean 2, 20–22 h, Table III, Fig. 5), but interpretable on the basis that in the case of protein matrices the pyridosine and furosine formation is a parallel and/or subsequent reaction to protein hydrolysis.

Thus, the conditions for optimum formation of pyridosine and furosine are as follows: for model systems and for proteins, reaction times of 5 and 20 h respectively; hydrochloric acid concentration of 6 *m*.

(6) To our knowledge, this is the first method providing reproducible data for the pyridosine and furosine analysis (Table III).

(7) As a result of the reproducibility of our method, the earlier contradictory literature data[3,4] can be clarified: the molar ratios of furosine/pyridosine formed on the interaction of deoxyfructosyllysine and hydrochloric acid are $\gg 1$ in most cases investigated (the only exceptions being for tomato powder).

(8) This work in addition to our earlier studies forms part of our comprehensive research concerning the development of new and fast analytical methods for monitoring Maillard reactions[12–16]. Applying our methods for the measurement of soluble saccharides[12,13,16] and "available lysine"[14–16], we used the large saccharide- and protein-containing soya beans, as excellent matrices for Maillard reactions.

In order to be able to evaluate the results of all of our investigations, the same soya bean samples (1–3) were tested. Thus, it has been found that the amounts of pyridosine and furosine found in the hydrolysates of soya bean samples 1–3 are in accord with the transformation of the active components in the Maillard reaction, *i.e.*, reducing saccharides and the free ε-amino group containing lysine of proteins. Namely, as a result of the various treatments of the soya beans: (1) the amount of the main components (sucrose, raffinose, stachyose) of soy saccharides decreases from 9.9% (w/w) (soya bean 1) to 3.4% (w/w) (soya bean 2) and to 0.2% (w/w) (soya bean 3), respectively; (ii) the amount of reducing saccharides, capable of Maillard reaction, expressed in terms of glucose, increases from 0.9% (w/w) (soya bean 1) to 2.6% (w/w) (soya bean 2) and to 3.7% (w/w) (soya bean 3), respectively[12]; (iii) the available lysine content of various samples (obtained by our dye-binding procedure[14,15]) also decreases significantly if compared both to the total lysine content of the same sample, and to the available lysine content of the corresponding untreated sample (Table IV).

Knowing[1,2] that the available lysine content decreases through the formation of ε-DFL mainly with heat-processed matrices, the amounts of blocked lysines obtained are in good agreement with expectations and are very informative: the total decrease in "lysine availability" (Table IV, data in parentheses) in all cases investigated is greater than lysine blocking through ε-DFL formation (Table IV, amounts of blocked lysine calculated from the furosine contents using the formula of Bujard and Finot)[3].

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