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Evaluation of peptide electropherograms by multivariate mathematical–statistical methods

I. Principal component analysis

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Abstract

Depository effects in slowly metabolised proteins, typically glycation or the estimation of products arising from the reaction of unsaturated long-chain-fatty acid metabolites (possessing aldehydic groups) are very difficult to assess owing to their extremely low concentration in the protein matrix. In order to reveal such alterations we applied deep enzymatic fragmentation resulting in a set of small peptides, which, if modified, are likely to change their electrophoretic properties and can be visualised on the resulting profile. Peptide maps of collagen (a mixture of collagen types I and III digested by bacterial collagenase) were applied as the model protein structure for detecting the nonenzymatic posttranslational changes originating during various physiological conditions like high fructose diet and hypertriglyceridemic state. Capillary electrophoresis in acidic media (sodium phosphate buffer, pH 2.5) was used as the separation method capable of (partial) separation of over 60 peptide peaks. Two to 13 changes were revealed in the profiles obtained reflecting the physiological conditions of the animals tested. Combination of peptide profiling with subsequent *t*-test evaluation of individual peak areas and principal component analysis based on cumulative peak areas of individual sections of the electropherograms allowed to determine in which section (part) of the electropherogram the physiological state indicating changes occurred. Simultaneously it was possible to reveal the qualitative differences between the four physiological regimes investigated (i.e., which regime affects the collagen molecules most and which affects them least). The approach can be used as guidance for targeted pre-separation of the very complex peptide mixture. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Principal component analysis (PCA) a versatile and easy-to-use multivariate mathematical–statistical

method has been developed to contribute to the extraction of maximal information from large data matrices containing numerous columns and rows [1]. PCA makes possible the elucidation of the relationship between the columns and rows of any data matrix without having one dependent variable. PCA is a so-called projection method representing the original data in smaller dimensions. It calculates the

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correlations between the columns of the data matrix and classifies the variables according to the coefficients of correlations. PCA has been frequently used in many fields of up-to-date research. Thus, PCA has been employed in quantitative structure–activity relationship (QSAR) studies [2], for the exploration of molecular structure–property relationships [3], for the evaluation of molecular lipophilicity [4,5], for theoretical organic chemistry [6], for quantitative structure–retention studies in chromatography [7], for the elucidation of structure–biodegradation relationships [8], for the clustering of amino acids [9], for the assessment of solvent properties [10], etc. As the resulting matrices of PC loadings and variables are generally multidimensional they cannot be evaluated by visual methods except in the cases when the first two sets of PC loadings and components explains the overwhelming majority of variance. The nonlinear mapping (NLMAP) technique has been developed for the reduction of the dimensionality of complicated multidimensional matrices [11]. Unfortunately, significance probes cannot be employed for the results of the NLMAP technique, that is, the distribution of variables and observations on the two-dimensional NL map does not indicate that the differences between the points are significant or not. To the best of our knowledge only one graphical approximation has been developed to include the standard deviation in the matrices of PC loadings and variables [12].

In protein chemistry peptide mapping, both by chromatographic and electromigration methods, is a widely applied approach [13]. Unfortunately current separation methods do not exhibit sufficient selectivity to offer baseline separations in complex peptide maps, perhaps with the exception of two-dimensional gel electrophoresis which, regrettably, is applicable only to relatively high-molecular-mass entities.

There are a number of situations (typically glycation, see below), in which proteins present in tissues undergo minor frequently nonenzymatic modifications the assessment of which is extremely difficult.

Owing to its slow metabolic turnover collagen is typical in its capability to accumulate reactive metabolites through enzymatically nonregulated reactions. Peptide maps can serve as model mixtures for the PCA studies aimed at revealing the presence or absence of such minor (though biologically quite

important) modifications. Glycation and formation of advanced glycation end products (AGEs) represent typical examples [14]. In spite of numerous efforts the chemical nature of the arising products (except pentosidine) remains unknown [15]. On the other hand the presence of such products can be revealed by the 370/440 nm luminescence of the modified intact collagen molecule [16]. It is anticipated that reactions of this type involve a free aldehydic group (typically that of aldehydic sugars and products of unsaturated fatty acid metabolism).

The main problem of assaying these modifications is based on the fact that the arising compounds (adducts) are unstable under conditions under which collagens can be hydrolysed to constituting amino acids. Luminescence based methods, though they added to the basic information about the arising products, are not selective enough even if the synchronous luminescence approach is applied [15].

Cyanogen bromide cleavage which is routinely used to bring connective tissue collagen into solution could also not be exploited as our preliminary experiments revealed that the selectivity of both flat bed gel or capillary electrophoresis (CE) is not capable to reveal the few modifications occurring in the molecule in vivo [17].

Therefore we applied deep cleavage of the parent collagen molecules with *Clostridium histolyticum* collagenase. This leads to a very complex peptide mixture (theoretically about 172 peptides could arise from the naturally occurring collagen type I and III mixture). This set of peptides can be partially separated by CE in a bare-silica capillary at acidic pH with a selectivity sufficient to indicate differences in individual peptide profiles, however, it has to be kept in mind that many of the peaks seen in the well producible profiles do not necessarily represent pure peptides.

The objectives of our study was profiling of peptides in various collagen hydrolysates using capillary zone electrophoresis (CZE) and two different integration methods, the elucidation of the similarities and dissimilarities between the amount and distribution of peptides in the electropherograms by PCA, and the inclusion of standard deviation in PCA. Bacterial collagenase digests of crude tail tendon collagen samples composed of collagen types I and III obtained from controls, genetically hypertri-

glyceridemic animals, genetically hypertriglyceridemic animals kept simultaneously for a period of time on a high fructose diet, and genetically hypertriglyceridemic animals with gemfibrozil (antihyperlipidemic agent) treatment served as model mixtures to reveal the practical applicability of the above described approach.

2. Material and methods

2.1. Chemicals

All chemicals used were either of analytical grade or highest available purity. Calcium chloride was obtained from Lachema (Brno, Czech Republic), collagenase E.C. 3.4.24.3., 0.8 U/mg, from *Clostridium histolyticum*, was from Fluka (Buchs, Switzerland, product number 27676), phosphate buffer 100 mmol/l, pH 2.5, with polymer modifier was purchased from Bio-Rad Labs. (Hercules, CA, USA, c.n. 148-5010). All other chemicals (inclusive gemfibrozil) were obtained from Sigma (St. Louis, MO, USA). All solutions were prepared in Milli-Q water (Millipore, Bedford, MA, USA).

2.2. Tissues and animals used

Tendons were dissected from tails of 6-month-old female Wistar strain rats (six per group).

Four groups of rats were compared:

- (1) Controls (group C).
- (2) Hereditary hypertriglyceridemic (HTG) rats (selected from Wistar strain rats) (group H).
- (3) HTG rats kept on high fructose diet – last 3 weeks before being sacrificed for the experiment the rats were kept on water containing 10% fructose (group F).
- (4) HTG rats cured with gemfibrozil – last 3 weeks before being sacrificed for the experiment the rats were under gemfibrozil treatment (30 mg/kg/day) (group G).

In order to obtain peptide maps collagen was digested by bacterial collagenase (collagen/collagenase ratio, 100:1, w/w). The samples were incubated at 37°C for 48 h in the collagenase activating buffer (200 mmol NH_4HCO_3 , 1 mmol CaCl_2 , pH 7.8).

2.3. Apparatus and operating conditions

A Beckman P/ACE 5000 CE system (Fullerton, CA, USA) was used through this study.

Separations were run at 10 kV in a bare fused-silica capillary [37 cm (30 cm to the detector) × 75 μm I.D., at 20°C]. UV absorbance at 200 nm was used for detection.

Phosphate buffer 100 mmol/l, pH 2.5, with a polymer modifier (Bio-Rad Labs., c.n. 148-5010) was used as background electrolyte.

The samples were diluted with the run buffer (sample–buffer, 1:2, v/v). Injection was done hydrodynamically by overpressure (3.45 kPa, 1 s). Before analysis the capillary was conditioned by the run buffer (1 min). After every run the capillary was fused step-wise with the run buffer (1 min), water (1 min), 1 M NaOH (3 min), water (1 min), 3 M HCl (1 min) and water (1 min).

3. Evaluation of the electropherograms

3.1. Percentual *t*-test evaluation of the electropherograms

Peptide profiles of collagen obtained after bacterial collagenase digestion from controls, HTG, HTG+fructose, HTG+gemfibrozil rats were compared: 65 peptide peaks of the profile were quantitated by the valley-to-valley integration method, and the total peak area was calculated. The total peak area was considered as 100%. The area percentage was calculated for each peak, and the peak area difference (if any) was calculated using a conventional *t*-test [significant change ($P < 0.05$) in comparison to controls].

3.2. Evaluation of the electropherograms by PCA

As the migration time of peptides showed slight variations among the samples and the baseline separation was not always achieved the migration times and relative peak areas of the individual peaks cannot be used as the elements of the original data matrix for PCA. Therefore, the electropherograms were divided in seven different sections showed in Fig. 1. Peak areas were calculated by two integration

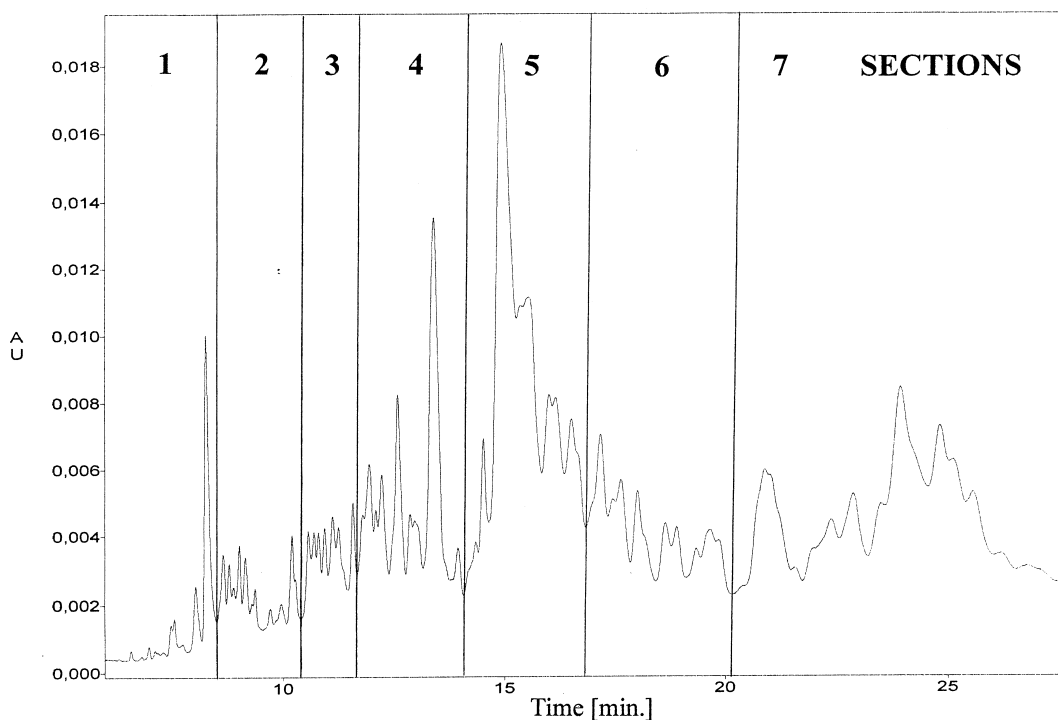


Fig. 1. Peptide map obtained from tail tendons of untreated rats after bacterial collagenase treatment. The seven sections of the profile used for PCA are indicated by numbers.

methods: A=valley-to-valley; B=one baseline for more peaks. The relative peak areas were summarised between the migration times marked in Fig. 1 and then they were summarised for all the seven sections. The relative peak areas (in percent) for each time section were calculated by taking the sum of total peak areas as 100%. The mean and standard deviation of each section for the four different treatment was calculated. Calculations above were carried out twice using separately integration methods A and B.

PCA has been applied four times:

- *PCAI*. Calculation based on integration method A. Input matrix consisted of the average peak areas, average peak areas minus twice standard deviation, average peak areas plus twice standard deviation for treatments C, G, H and F as variables (altogether $3 \times 4 = 12$ variables) and the seven fractions were the observations.
- *PCAI*. Calculation based on integration method A. Input matrix consisted of the average peak areas, average peak areas minus twice standard

deviation, average peak areas plus twice standard deviation for the seven fractions as variables (altogether $3 \times 7 = 21$ variables) and the four treatments were the observations.

- *PCAI*. Calculation based on integration method B. Input matrix consisted of the average peak areas, average peak areas minus twice standard deviation, average peak areas plus twice standard deviation for treatments C, G, H and F as variables (altogether $3 \times 4 = 12$ variables) and the seven fractions were the observations.
- *PCAI*. Calculation based on integration method B. Input matrix consisted of the average peak areas, average peak areas minus twice standard deviation, average peak areas plus twice standard deviation for the seven fractions as variables (altogether $3 \times 7 = 21$ variables) and the four treatments were the observations.

The variation explained was set to 99% for each PCA. We are well aware that the numbers of observations are fairly low in *PCAI* and *IV*, therefore, the results obtained by these PCAs have been

treated cautiously. When necessary, the dimensionality of the matrix of PC loadings was reduced to two by the NLMAP technique. The iteration was carried out to the point where the difference between the last two iterations was lower than 10^{-8} . NLMAP was carried out on both the original values of PC loadings and on their absolute values. The inclusion of the $\pm 2 \times$ standard deviations in the input matrices was motivated by the supposition that this method facilitates the evaluation of the significant differences between the variables distributed on the two-dimensional nonlinear maps.

4. Results and discussion

Fig. 1 demonstrates the total profile of the collagenous peptides obtained by bacterial collagenase cleavage. The whole electropherogram was divided into seven sections every one of which was subjected to the evaluation by PCA as described in Section 3.2.

Analogous profiles of collagen digests obtained from rat tendons of HTG, HTG+fructose and HTG+gemfibrozil treated rats are shown in Figs. 2–4. The differences in the profiles revealed by the valley-to-valley integration relatively to controls are indicated by arrows. Because it is visualised that not many (if any at all) of the peaks separated represent a pure peptide, the results can be considered as qualitative differences only (the differences were evaluated by simple *t*-test of the compared peak areas). Increased peaks (indicated by full arrows) and decreased peaks (indicated by open arrows) were observed. All profiles were normalised with the respect to dominant peaks, which were considered as internal standards in order to abolish migration time differences in between individual runs. Table 1 shows the summary of the results: the peptide map of the preparation obtained from HTG+fructose treated rats differed most from the control preparation, HTG being the second and HTG+gemfibrozil showing the smallest difference.

Because this approach may be not persuasive

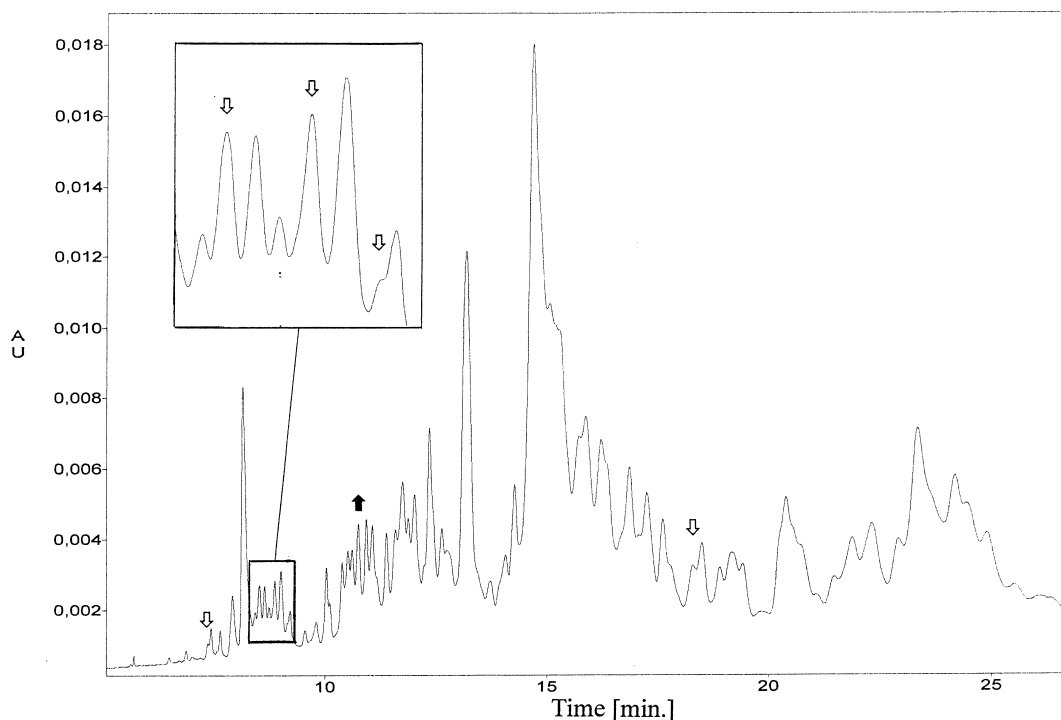


Fig. 2. Peptide map obtained from the tails of genetically hypertriglyceridemic rats after bacterial collagenase treatment. Sectioning of the electropherogram corresponds to that used in Fig. 1. Increased and decreased peaks are indicated by black and open arrows, respectively.

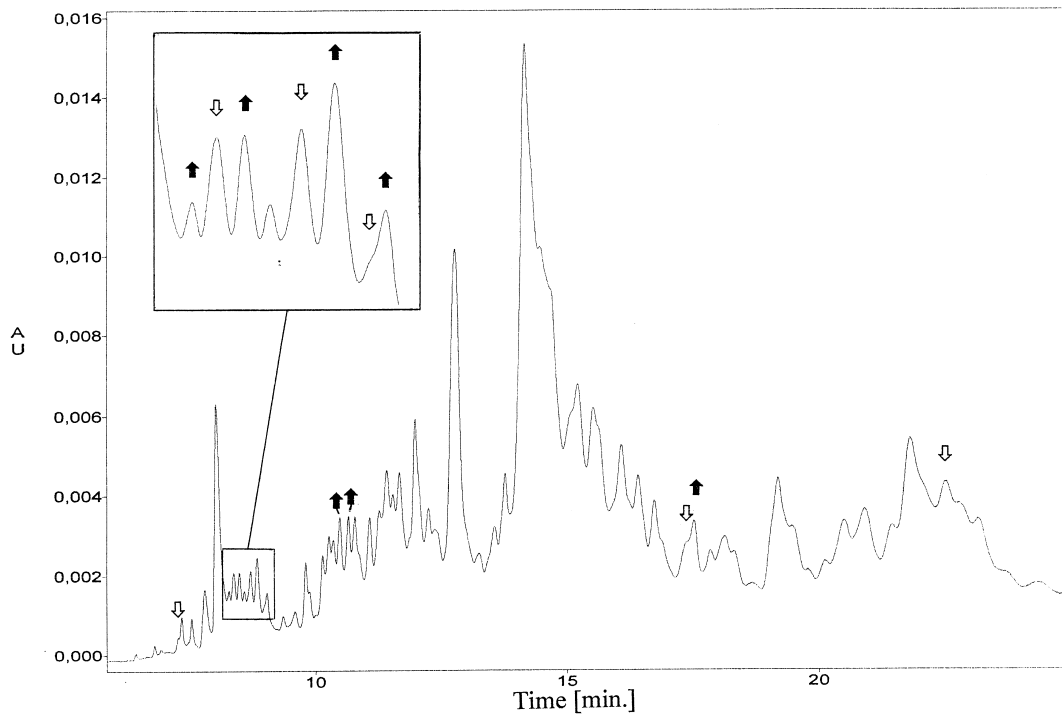


Fig. 3. Analogous peptide map as shown in Fig. 2 obtained from hypertriglyceridemic+high fructose treated rat tail tendons.

enough owing to the complexity of the profile, the seven sections were subjected next to the PCA.

The points depicting the average \pm twice standard deviation are not represented on the two-dimensional maps, because they form overlapping surface indicating the small differences between the peptide profiles and treatments.

PCA I. The first PC component explained 98.72% of the total variance (only one valid dimension), indicating the strong similarity between the peptide profiles. Treatments do not form separate clusters on the map of the first PC loading vs. the second PC loading, however, treatments H and G are near to each other and nearer to the control than treatment F (Fig. 5). This finding indicates that the peptide profile of treatment F differs more markedly from the peptide profiles of other treatments and that of the control.

PCA II. The results of PCA II concerning the similarities and dissimilarities of peptide sections taking into consideration simultaneously each treatment are compiled in Table 2. Eigenvalues indicate the relative importance of the individual principal

components. It is generally accepted that only PC components with eigenvalues higher than 1 should be retained, PC components with eigenvalues lower than 1 include only random values due to the standard deviations of the data in the original matrix. Variance explained refers to the ratio of total variance (present in the original data matrix) explained by the individual PC components. Total variance explained is the sum of the variances explained by the PC components. Principal component loadings show the similarities among the peptide sections. Peptide sections having high loadings in same principal component are similar to each other. Three principal components explain the total variance indicating that the seven original variables can be substituted by three background (abstract) variables with small loss of information. Unfortunately, PCA does not prove the existence of such background variables as concrete physicochemical entities, but only indicates their mathematical existence. The fact that the majority of sections (Fig. 6) have a high loading in the first PC indicates their basic similarity. The distribution of peptide sections on the two-

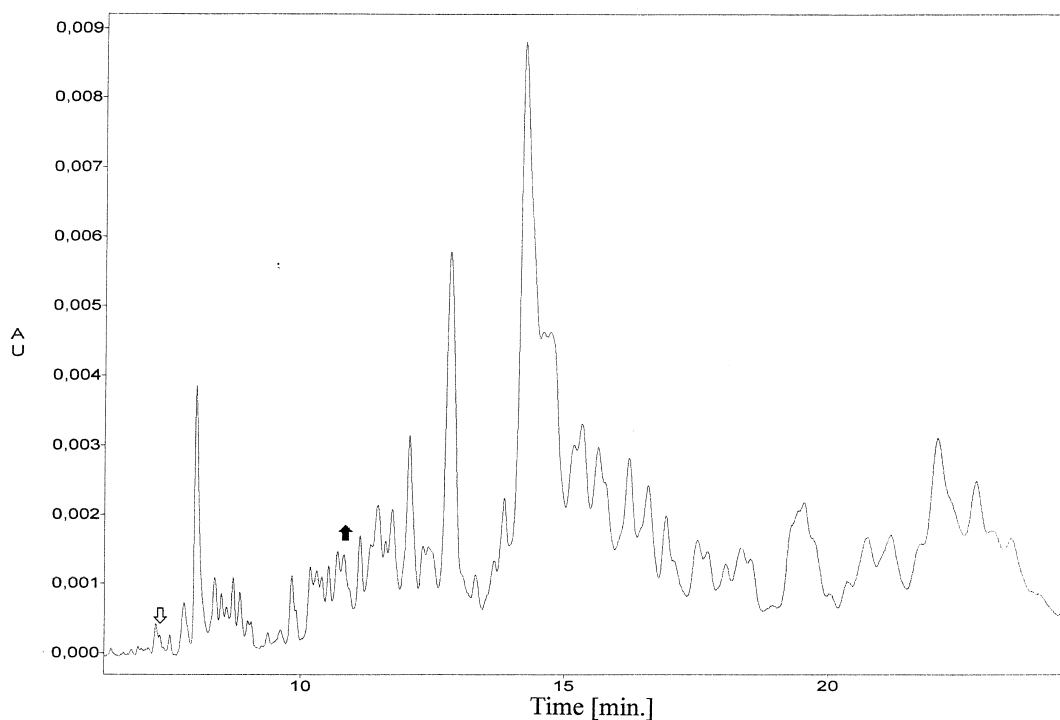


Fig. 4. Analogous peptide map as shown in Fig. 2 obtained from hypertriglyceridemic treated with gemfibrozil rat tail tendons.

dimensional nonlinear map of principal component loadings calculated from the data in Table 2 is shown in Fig. 6. Axes F_1 and F_2 in Fig. 6 have no concrete physical or physicochemical meaning, they only indicate the distance between the points representing peptide sections on a two-dimensional plane. Sections 3, 4, 5 and 7 form a clear-cut cluster on the

two-dimensional nonlinear map indicating that the information drawn from these sections are similar. Sections 1, 2 and 6 are widely scattered on the map. This result suggests that the information content of sections 1, 2 and 6 is different, therefore, these

Table 1

Differences in collagen peptide maps (after bacterial collagenase treatment, see the Materials and methods section for details)

	No. of changed peak areas	
	Increased peak areas	Decreased peak areas
HTG	1	5
HTG+fructose	7	6
HTG+gemfibrozil	1	1

Separations obtained from controls, hypertriglyceridemic rats, hypertiglyceridemic+high fructose treated rats and hypertiglyceridemic+gemfibrozil animals are compared. Notice: appropriate counterparts to the peaks found to decrease/increase in HTG (H-group) are changed in the same direction in HTG animals kept on fructose (group F).

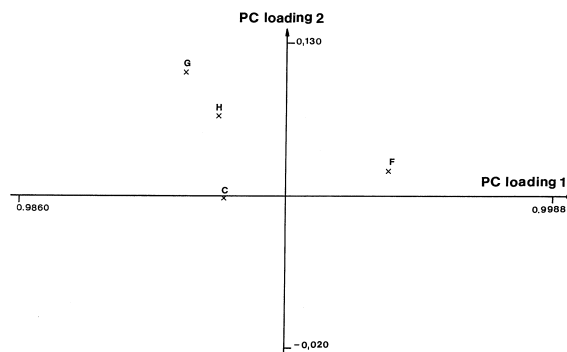


Fig. 5. Similarities and dissimilarities between treatments according to the peptide sections. PC loading 1 vs. PC loading 2 (valley-to-valley integration). Results of PCAI. Letters refer to treatments defined in Section 3.2.

Table 2
Similarities and dissimilarities between the peptide sections

No. of PC	Eigenvalue	Variance explained (%)	Total variance explained (%)
1	10.61	50.51	50.51
2	6.07	28.90	79.42
3	4.32	20.58	100.00

Principal component loadings

Peptide section	No. of principal components		
	1	2	3
1	-0.12	-0.99	0.10
2	0.58	-0.30	0.75
3	-0.88	0.43	-0.14
4	0.91	-0.35	-0.20
5	0.90	0.35	-0.27
6	-0.15	0.78	0.61
7	-0.87	-0.37	0.32

Results of principal component analysis (valley-to-valley integration).

sections are characteristic for the treatments. It can be further concluded from the distribution of peptide sections on the map that sections 1, 2, 6 and one of sections 3, 4, 5 and 7 is sufficient for the differentiation between treatments and the inclusion of more

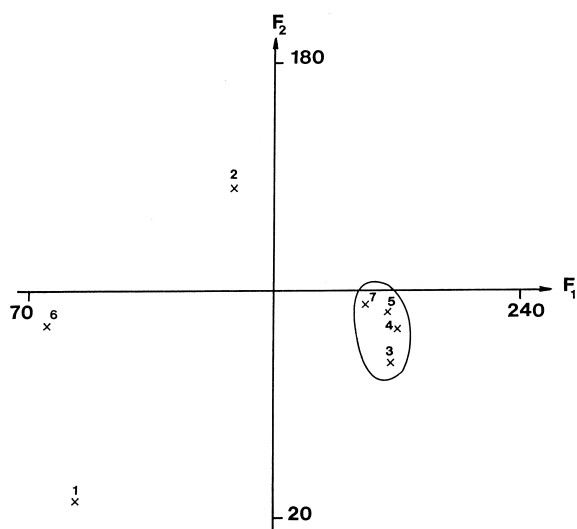


Fig. 6. Similarities and dissimilarities between peptide sections taking into consideration simultaneously each treatment. Two-dimensional nonlinear map of absolute value of PC loadings. Results of PCAII. No. of iterations: 80; maximal error: $2.98 \cdot 10^{-4}$ (valley-to-valley integration). Numbers refer to peptide sections in defined in Section 3.2.

peptide sections in the calculation does not increase the differentiating power of PCA.

PCAIII. The first PC component explained 99.51% of the total variance. This finding indicates that PCA is not suitable to distinguish among the treatments when the peptide profiles are evaluated by using baseline integration.

PCAIV. The results of PCAIV concerning the similarities and dissimilarities of peptide sections taking into consideration simultaneously each treatment are compiled in Fig. 7 and Table 3. Symbols and their meanings are the same as in Table 2. The results are similar to that of PCAII, three underlying variables explain the total variance distributed in the seven original variables. The majority of sections have a high loading in the first PC suggesting again their basic similarity. The distribution of peptide sections on the two-dimensional nonlinear map of principal component loadings calculated from the data in Table 3 is shown in Fig. 7. The distribution of peptide sections is highly different from that of the other map. This somewhat unexpected result indicates that the method of integration exert a considerable influence of the quantitative evaluation of peptide electropherograms.

Comparison of the *t*-test evaluation of individual peptide profiles and their alignment to the PCA analysis deserves some comments.

First, one would expect that the number of in-

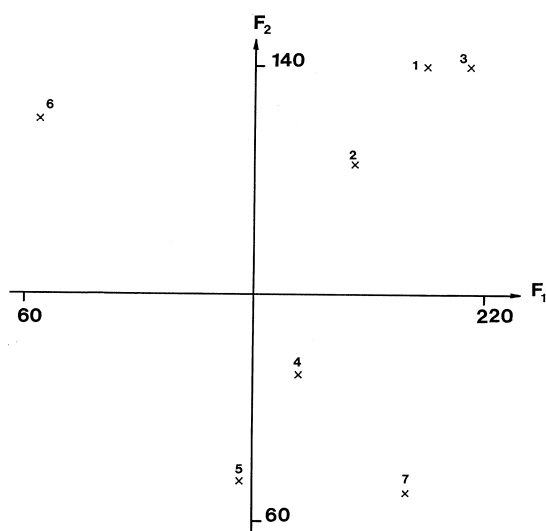


Fig. 7. Similarities and dissimilarities between peptide sections taking into consideration simultaneously each treatment. Two-dimensional nonlinear map of absolute value of PC loadings. Results of PCAIV. No. of iterations: 95; maximal error: $1.99 \cdot 10^{-4}$ (baseline integration). Numbers refer to peptide sections in defined in Section 3.2.

creased peaks should equal the number of decreased peaks; a brief look at Table 1 makes it clear that it is not so. The easiest explanation of this fact is that the runs were stopped routinely after 30 min running time and we know that the presented runs are incomplete. Consequently some decrease/increase in

peak areas were likely lost at the end of the electropherogram. However the peak broadening and migration time shifts after thirty min running time were much too large to allow reasonable evaluation.

The second point to be thought about is the comparison between the PCA analysis and simple peak integration (both refer to the valley-to-valley integration). As mentioned, the PCA analysis says that electropherogram sections in which the differences should be seen are sections 1, 2 and 6. Indeed when comparing this result with individual peak integration the most of the differences are seen indeed in sections mentioned. However, there are a number of differences in section 3 as revealed by *t*-test of the integrated individual peaks. PCA results predict that in addition one of the sections 3, 4, 5 and 7 should be sufficient to reveal the differences in peptide profiles after the different treatments. As demonstrated by the *t*-test electropherogram evaluation it is possible to conclude that it is section 3, which it is most likely to complete the information as additional profile differences can be visualised here.

It is possible to conclude that the differences in the peptide peaks profiles obtained from the tail tendons of the three categories of experimental animals revealed by valley-to-valley integration of individual peaks with subsequent *t*-test calculation are in good agreement with the PCA analysis based on the valley-to-valley integration. Collagen preparations

Table 3
Similarities and dissimilarities between the peptide sections

No. of PC	Eigenvalue	Variance explained (%)	Total variance explained (%)
1	12.08	57.51	57.51
2	5.34	25.44	82.95
3	3.58	17.05	100.00

Principal component loadings

Peptide section	No. of principal component		
	1	2	3
1	-0.97	0.08	0.21
2	-0.91	0.24	-0.34
3	0.99	0.06	-0.11
4	0.77	0.51	0.40
5	0.62	0.63	0.46
6	0.41	0.14	-0.90
7	-0.79	-0.60	0.11

Results of principal component analysis (baseline integration).

obtained from HTG+fructose diet test animals differ most from the controls, which can be ascribed to the presence of two categories of reactive metabolites, namely the aldehydic moiety of the (uncyclised) fructose and the aldehydic components stemming from the metabolism of unsaturated lipids. The former type of reactive entity (fructose) is absent in genetically hypertriglyceridemic rats resulting consequently in a smaller difference in comparison to controls. Gemfibrozil as an antihypertriglyceridemic agent obviously decreases the level of reactive lipidic metabolites bringing thus the profiles observed closer to controls.

Because it is feasible to expect that ϵ -amino groups of lysine are the target of both categories of aldehydic moiety possessing compounds (aldehydes originating from lipid metabolism and fructose) it can be suggested that the differences in the profile observed are due to modified lysine containing peptides. On the other hand the selectivity of the method used is not capable of separating individual peptides (in the collagenase digest of a mixture of collagen types I and III we are dealing with a mixture which contains theoretically 172 peptides), making direct assay difficult (if possible at all). On the other hand less deep fragmentation of the collagen preparations, e.g., by CNBr did not yield usable results as the resulting peptides are much too large to reveal delicate differences caused by the interaction of the protein with both types of aldehydic moiety possessing metabolites. Tryptic cleavage is limited to terminal regions only (telopeptides) being therefore not acceptable either [18].

The failure of revealing the differences between the four categories of sample by PCA analysis based on integration to the common baseline (Table 3) is probably due to the heavy overlaps of incompletely resolved peaks and stresses in importance of the way by which the peak areas are evaluated.

5. Conclusions

Peptide mapping after specific enzymatic cleavage followed by two types of statistical evaluation was found a reliable method for assaying nonenzymatic posttranslational modifications of collagen. It is feasible to assume that this strategy is applicable for

the determination of posttranslational nonenzymatic modifications in a wide spectrum of proteins provided that a suitable fragmentation method is applied.

It was revealed that collagen of hypertriglyceridemic rats is posttranslationally modified in six target loci of the structure; posttranslational modifications of collagen of hypertriglyceridemic rats kept for a period of time on high fructose diet revealed 13 significant changes in the electropherogram. Hypertriglyceridemic animals, which were simultaneously treated with gemfibrozil, revealed only two changes in the profile.

Evaluation of the whole complex electropherograms to visualise the differences between different physiological conditions of the source animals is very tedious indeed. By sectioning the electropherogram and evaluating the individual sections by PCA analysis it is possible to estimate in which regions (sections) of the electropherogram the differences can be seen and get a preliminary idea about qualitative differences between individual treatments, i.e., to reveal which treatment affects the proteins (collagens) involved most and which affects them least. Regarding further characterisation of the modified peptides, the analyst is directed towards analysing the indicative sections only.

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