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Differentiation of bovine and porcine gelatins using principal component analysis

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Abstract

Gelatin is a collagen derivative, which has a large application in the pharmaceutical, food and adhesive industries as well as photography. The large similarity in structure and properties of gelatins from different origins makes their differentiation difficult. Certain chemometric methods, such as principal component analysis (PCA), can help to classify and characterize gelatin components. In this study 14 bovine and 5 porcine gelatins were examined. The analysis procedure involved complete hydrolysis of samples by classic acid hydrolysis in order to release their amino acid residues. Separation and determination of amino acids was achieved by reversed-phase (RP) HPLC following pre-column derivatisation. Orthophtaldialdehyde (OPA) and 4-chloro-7-nitro benzofurazane (NBD-Cl) were used as derivatisation reagents. From the 20 peaks detected by HPLC analysis, one was very typical in bovine gelatin. Peak height, area, area percentage and width were used to make matrixes. Principal component analysis with the MATLAB program was used to differentiate these gelatins. PCA on matrix of height, width and total matrix were resulted in good differentiation between bovine and porcine gelatins.

Keywords: Gelatin; Bovine; Porcine; Principal component analysis; Derivatisation

1. Introduction

Gelatin is a high molecular weight polypeptide derived from collagen, the primary protein component of animal connective tissues. Industrial preparation of gelatin involves the controlled hydrolysis of the organized structure of collagen to obtain soluble gelatin. The most important sources of collagen for gelatin production are bovine hide, bone and pigskin. The source, age and type of collagen, all influence the properties of the gelatins derived from them [1].

There are numerous studies about the gelling and thickening properties of gelatin and its application in the pharmaceutical and food industries and of the analysis of collagen and gelatin structure [1,2]. Gelatins from different sources can be very similar in their physicochemical properties, which makes their differentiation very difficult.

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The traditional approach to amino acid analysis is based on the technology developed by Moore and co-workers [3], in which amino acids with free amino groups are separated by cation-exchange chromatography followed by post-column derivatisation (Ninhydrin or orthophtaldialdehyde) [4].

During the last two decades, interest has been focused on pre-column derivatisation followed by reversed-phase (RP) HPLC which constitutes a much faster, more efficient and more sensitive alternative to former amino acid analytical procedures [4,5].

For amino acid analysis on a routine basis, the ideal derivatisation agent must satisfy several requirements such as reacting rapidly under mild conditions and providing stable derivatives of both primary and secondary amino acids, as well as being easily automated. There should also not be any interference from the reagent, its breakdown products or side reactions, and the response should be linear over the concentration ranges examined. Moreover; the RP-HPLC procedure should be simple, robust and easily reproducible by other laboratories.

Among the numerous compounds used in pre-column derivatisation of amino acids, two reagents seem to be favored, reversed and 4 chloro-7-nitrobenzofurazane (NBD-Cl). Neither of these reagents are completely satisfactory with the major disadvantage of the orthophtaldialdehyde (OPA) procedure the lack of reaction with secondary amino acids. Overcoming this problem is possible by using NBD-Cl because of its selectivity to secondary amino acids [5].

Despite to the wide field of gelatin application, there are limitations with regard to consumption of gelatins from particular sources. These limitations are due to cultural reasons or even because of therapeutic aims. For instance, in Islamic countries porcine gelatin cannot be used and in Chinese traditional medicine a particular source of gelatin, donkey skin gelatin, is used for treatment of some diseases [2]. Since fraud is very common in this area, it is important to establish a method to differentiate the various different kinds of gelatins. In this study the differentiation of 19 gelatin samples, from two different sources, was studied. Analysis was performed by determining the presence of amino acids in the various gelatins structure and some other factors in their chromatograms including height, area, area percentage and width using the principal component analysis (PCA) method.

PCA is an unsupervised data projection method that can be helpful in classification. The central idea of PCA is to reduce the dimensionality of a data set. It computes a few linear combinations of the original variables, which can be used to summarize the data with minimal loss of information. PCA has been applied in many classification studies [6,7].

2. Experimental

2.1. Materials

OPA, NBD-Cl, 3-mercaptopropionic acid (MPA) and standard solutions of amino acids for collagen analysis were obtained from Sigma (St. Louis, USA). HPLC-grade methanol and tetrahydrofuran were purchased from Merck (Darmstadt, Germany). Porcine and bovine gelatin samples were obtained from Sigma, Fluka, Merck, BDH and Leiner. All of other reagents were of the highest available purity.

2.2. Standard solutions

Standard solutions of amino acids for collagen hydrolysates that contained 18 amino acids were at 2.5 μmol/ml except for L-proline and hydroxy-L-proline at 12.5 μmol/ml and L-cystine at 1.25 μmol/ml, obtained from Sigma.

2.3. Buffer solutions

Borate buffer was prepared by dissolving 2.85 g sodium tetraborate (water free) in 100 ml distilled water, boiled for 5 min followed by pH adjustment to 9.5 ± 0.05 using concentrated sodium hydroxide solution.

2.4. Reagent solutions

OPA/MPA reagent was obtained by dissolving 1 mg OPA in 900 μl methanol, 100 μl borate buffer and 10 μl MPA solution. NBD-CI reagent was prepared by dissolving 50 mg NBD-Cl in 5 ml methanol [5,8].

2.5. Apparatus

The chromatographic system was a KNAUER HPLC instrument (Knauer, Berlin, Germany) consisting of a Knauer controller Quaternary pump, a Spark

Triathlon autosampler and a fluorescence detector (Knauer, RF-10x1) operating with the Eurochrom 2000 software (version 1.2, 1992–1996). The computations were made with a Pentium III computer. All the programs in the computing process were written in MATLAB version 5.3 for windows.

2.6. Hydrolysis

Acid hydrolysis is the standard method for hydrolysis of proteins and peptides. Standard solutions or samples (100 μl) were added to auto sampler vials [3,9] along with the same volume of 12 M HCl (containing 2% phenol to prevent oxidation of the amino acids). The vials were then sealed with Teflon caps supplied with PTFE septa under a nitrogen atmosphere to remove oxygen. The sealed vials were placed in an oven, which was heated to 110 °C. After a 24 h hydrolysis period, the HCl was removed with a steam of nitrogen (attention: concentrated HCl steam is highly corrosive). The residue was redissolved in 0.1 M HCl which is used for determination of the resulting amino acids by HPLC with pre-column derivatisation.

2.7. Derivatisation

OPA/MPA and OPA/NBD-Cl in methanol were used as derivatisation reagents. Derivatisation for primary amino acids was performed by a Spark autosampler (Triathlon type 900). The auto sampler was programmed to transfer successively sodium borate buffer (pH 9.5, 0.14 M, 100 μl) and OPA/MPA reagent (50 μl) to a sample vial (50 μl hydrolysate + 150 μl HCl 0.1 M). After 1 min 25 μl HCl 0.7 M was added to stop the reactions. Then 200 μl of mobile phase A and 50 μl of sample were transferred to an auto sampler vial and injected on the HPLC column (injection volume = 20 μl).

The OPA/NBD-Cl regent was used for manual derivatisation of secondary amino acids. A 10 μl aliquot of sample was added to 190 μl of sodium borate buffer (to create alkaline conditions, suitable for the NBD-Cl reaction), 10 μl OPA (without MPA to prevent the formation of fluorescent derivatives) to react with primary amino acids and finally 10 μl NBD-Cl in methanol to react with secondary amino acids. After 5 min incubation at 60 °C, the sample was injected on to the HPLC [5].

2.8. Chromatography

Separations were achieved using a 250 mm × 4 mm, reversed-phase column (OPA special, Knauer; Berlin, Germany). For OPA/MPA derivatives the eluent system consisted of two components: eluent (A) was methanol–sodium phosphate (pH 6.5, 12.5 mM) (10:90, v/v), while eluent (B) was methanol–tetrahydrofuran (97:3, v/v). The separation (gradient) conditions were as follows: 15–20% B in 5 min, 20–32% B in 12 min, 32–60% B in 10 min, 60%–90% B in 3 min and 90–15% B in 2 min. The eluent flow rate was 1.0 ml/min. For OPA/NBD-Cl derivatives eluent (A) was methanol–sodium acetate (pH 7.0, 50 mM) (22:78, v/v) and eluent (B) was consisted of methanol–sodium acetate (pH 7.0, 200 mM) (75:25, v/v). A gradient was formed from 0 to 50% B in 10 min, 50–0% B in 2 min followed by 100% A for 33 min with flow rate of 0.3 ml/mm.

2.9. Detection

Detection was performed using the fluorescence (Fl) (Knauer; RF − 10 × L) detector. Blank tests, calibration tests and gelatins were evaluated at 330 nm (OPA/MPA amino acids) as well as at the optimum excitation (λ_{ex})/emission (λ_{em}) wavelengths: at $\lambda_{ex}/\lambda_{em} = 330/450$. For OPA/NBD-Cl derivatives $\lambda_{ex}/\lambda_{em}$ was 470/530.

2.10. Data analysis

Principal component analysis was performed using singular value decomposition method with the MATLAB program. All of data matrices were mean centered and autoscaled before doing PCA.

3. Results and discussion

3.1. Basic statistics

The bovine and porcine gelatin samples were analyzed by pre-column derivatisation with reversed-phase HPLC. For the bovine gelatin, 14 samples were used and 20 peaks were obtained, while for porcine gelatin 5 samples and 20 peaks were analyzed. Fig. 1 shows chromatograms and Table 1

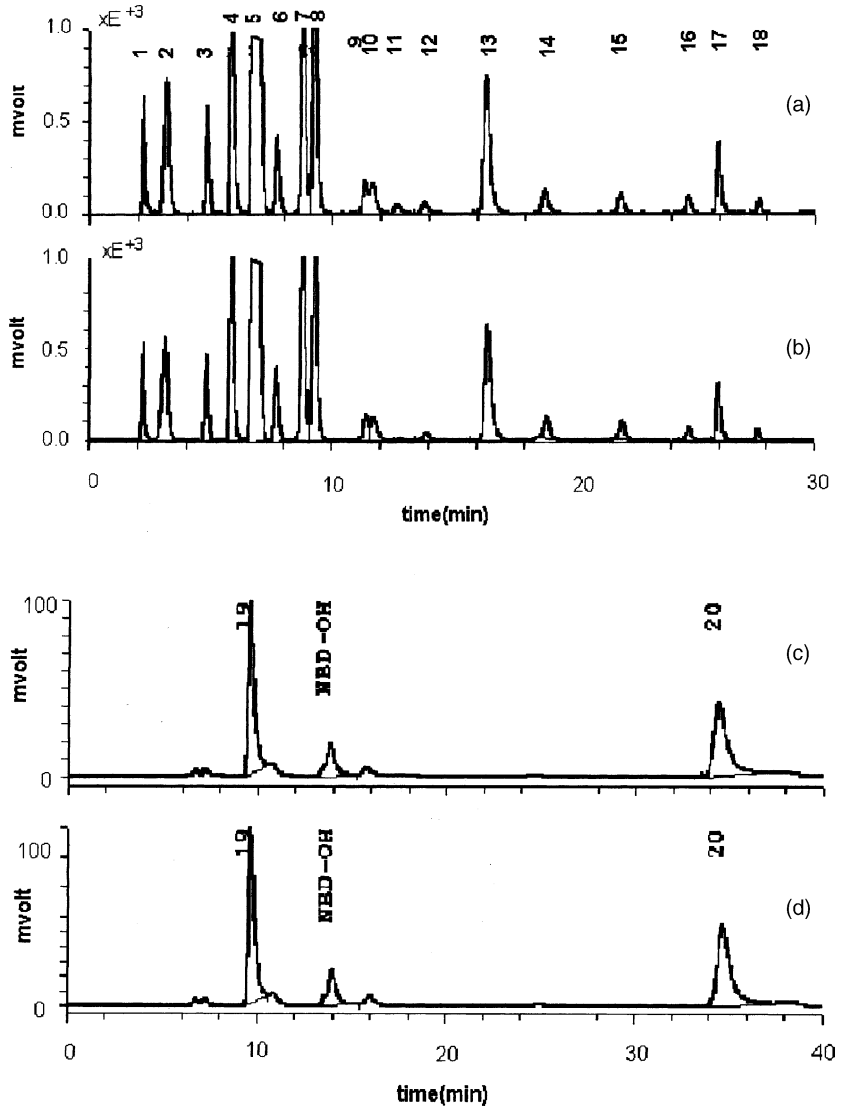

Fig. 1. Chromatograms of Sigma bovine and porcine gelatins after hydrolysis and pre-column derivatisation: (a) bovine and (b) porcine with OPA; (c) bovine and (d) porcine with NBD-Cl.

shows some characteristics of peaks of a bovine and porcine gelatin, respectively, purchased from Sigma. As clearly can be seen there is only one difference between bovine and porcine chromatograms (peak no. 11). It seems that the height of eleventh peak can be used as a simple criterion to discriminate the bovine and porcine samples. However; employing only one peak for differentiation may not provide enough confidence due to the immense similarity of these proteins in structure and properties. Therefore, application of a multivariate statistical method could be helpful since it deals with the overall peaks (variables) and the proportion between them to establish the differences [10,11]. Table 2 shows heights of 20 peaks for 19 gelatin samples. Also Table 3 shows some basic statistical measurements of gelatin samples.

Table 1
Peak report table for Sigma bovine and porcine gelatin

Peak no.	Bovine				Porcine			
	Height	Area	Area (%)	Width	Height	Area	Area (%)	Width
1	628.745	72.36	3.219	0.533	523.363	58.257	2.798	0.5
2	734.319	185.293	8.243	1.017	560.564	155.717	7.48	1.217
3	582.142	98.922	4.401	0.45	467.867	81.254	3.903	0.467
4	986.384	254.349	11.315	0.633	984.839	232.04	11.146	0.212
5	967.21	493.094	21.935	1.05	979.32	488.082	23.445	0.9
6	422.233	80.005	3.559	0.6	399.321	78.361	3.764	0.667
7	996.384	229.17	10.195	0.583	998.351	223.091	10.716	0.6
8	997.698	288.892	12.851	1.067	999.16	267.691	12.859	1
9	172.72	39.052	1.737	0.433	134.534	32.027	1.538	0.45
10	162.841	50.024	2.225	0.867	123.082	41.573	1.997	0.9
11	48.12	14.339	0.638	1.784	8.197	2.759	0.133	0.314
12	50.182	17.522	0.78	0.917	45.829	13.761	0.661	1.267
13	736.933	234.86	10.448	1.784	625.907	203.816	9.79	1.783
14	123.602	34.978	1.556	0.917	117.068	33.895	1.628	0.933
15	104.935	29.288	1.303	0.85	100.615	29.062	1.396	0.883
16	100.854	25.839	1.149	0.767	71.727	18.154	0.872	0.767
17	383.179	86.943	3.868	1.05	316.843	73.239	3.518	1.067
18	39.362	13.042	0.58	0.867	64.574	12.589	0.605	0.883
19	134.957	48.161	44.963	1.216	127.889	46.892	44.598	1.300
20	55.914	46.126	43.063	3.383	54.159	45.836	43.593	3.666

3.2. Principal component analysis

Principal component analysis is a projection method that reduces the dimensionality in a data matrix while retaining the most significant information. PCA was used for processing the peak parameters to extract the principal components (significant variables) and to establish the classification of bovine and porcine gelatins. PCA is an unsupervised method. Samples having *p*-variables may be represented as points in *p*-dimensional space. PCA manipulates the data in the way that these points can be displayed on an *x*, *y*-coordinate system. It does this by computing principal component 1, which is the linear combination of variables that explains the most variation among the samples, and principal component 2, which is orthogonal to PC1 and explains the second largest amount of variation. To display the points in two principal components dimensions, the first two principal components are chosen to represent the information. These are vectors PC1 and PC2 (first and second principal components) [12]. For these gelatins, 20 variables (peaks) were reduced to two new variables. Because the metrics of variables were widely different, the variables were autoscaled. Autoscaling reveals data with zero mean and unit variance [10]. Firstly, 12 samples (9 bovine and 3 porcine) were processed by PCA and were presented in a two-dimensional graph. Then seven samples (five bovine and two porcine) were used as a prediction set and were added to the training set, then all the samples were analyzed by PCA. The localization of 19 samples (14 for bovine and 5 for porcine gelatins), according to their score values, on the plane defined by the new variables factor 1 and 2 are shown in Fig. 2. By comparing the two-dimensional presentation graphs before and after adding the seven prediction samples, it was found that the localization of the training set slightly changed and all the prediction samples fell into the right groups, respectively. All gelatin samples of bovine and porcine origin shown in Fig. 2. can be distinguished by a line. According to the loading values of the stated components, it can be deduced that the variables (peaks) 1, 2, 3, 9, 14, 16 and 17 are the most significant factors in the classification. Besides processing of total matrix (consists of height and width), resulted from peak report, by PCA, this technique was used on some further parameters of the peak report table (such as height, area, width

Table 2
Height of 20 peaks for 19 gelatin sample

Peak no.	Sample																		
1	0.3878	0.3559	0.3061	0.2787	0.4216	0.4185	0.5546	0.2645	0.3701	0.3611	0.4162	0.3551	0.2895	0.3900	0.2946	0.2968	0.3005	0.3099	0.2699
2	0.6139	0.6557	0.5743	0.5557	0.7534	0.6955	0.9223	0.5649	0.6936	0.6879	0.7717	0.6907	0.5709	0.6316	0.6444	0.6110	0.5563	0.6118	0.5670
3	0.3169	0.3426	0.2770	0.2584	0.4270	0.3892	0.6009	0.2474	0.3249	0.3568	0.4217	0.3581	0.2911	0.4046	0.2957	0.2994	0.2889	0.3044	0.2885
4	0.9861	0.9815	0.9876	0.9830	0.9853	0.9771	0.9778	0.9677	0.9889	0.9800	0.9882	0.9850	0.9771	0.9866	0.9902	0.9886	0.8986	0.9843	0.9568
5	0.9621	0.9661	0.9609	0.9599	0.9677	0.9683	0.9593	0.9595	0.9768	0.9532	0.9481	0.9571	0.9601	0.9735	0.9831	0.9817	0.9825	0.9739	0.9748
6	0.3115	0.3079	0.2640	0.2517	0.2911	0.3080	0.4651	0.2488	0.3060	0.2348	0.2175	0.2489	0.2360	0.3026	0.3232	0.2725	0.2335	0.2337	0.2474
7	0.7898	0.6782	0.6071	0.5596	0.7323	0.7865	0.9971	0.5648	0.7164	0.6374	0.6896	0.6248	0.5795	0.7221	0.7282	0.6459	0.5374	0.6327	0.5787
8	0.9987	0.9519	0.9291	0.9037	0.9852	0.9984	0.9978	0.8616	1.0011	0.9607	0.9968	1.0003	0.8617	0.9079	0.9192	0.8704	0.7819	0.8603	0.8198
9	0.1029	0.1070	0.0965	0.0947	0.1499	0.1225	0.1854	0.0884	0.1202	0.1109	0.1296	0.1268	0.0891	0.1282	0.1100	0.1023	0.1048	0.0978	0.0957
10	0.1013	0.1264	0.0978	0.1004	0.1302	0.1129	0.1590	0.1008	0.1372	0.1206	0.1362	0.1386	0.0980	0.1287	0.1231	0.1242	0.1257	0.1191	0.1117
11	0.0889	0.0282	0.0785	0.0965	0.1282	0.1177	0.1236	0.0423	0.0397	0.1213	0.1499	0.0991	0.0637	0.0335	0.0046	0.0068	0.0057	0.0088	0.0088
12	0.0390	0.0523	0.0408	0.0447	0.0412	0.0439	0.0556	0.0517	0.0564	0.0485	0.0445	0.0488	0.0496	0.0447	0.0421	0.0531	0.0363	0.0473	0.0500
13	0.4435	0.4843	0.4503	0.4303	0.7331	0.6088	0.7285	0.4181	0.5795	0.5327	0.6600	0.6133	0.3582	0.5264	0.4461	0.4699	0.4747	0.4976	0.4262
14	0.0982	0.0753	0.0701	0.0673	0.0985	0.1025	0.1329	0.0649	0.0747	0.0783	0.0910	0.0741	0.0843	0.0817	0.0760	0.0695	0.0559	0.0743	0.0653
15	0.0728	0.0724	0.0555	0.0506	0.0709	0.0743	0.1076	0.0545	0.0700	0.0633	0.0668	0.0614	0.0584	0.0740	0.0744	0.0626	0.0550	0.0640	0.0592
16	0.0639	0.0501	0.0439	0.0433	0.0653	0.0653	0.1062	0.0421	0.0480	0.0558	0.0597	0.0546	0.0474	0.0653	0.0465	0.0402	0.0396	0.0392	0.0374
17	0.2338	0.1951	0.1688	0.1703	0.2879	0.2662	0.3790	0.1620	0.2035	0.1948	0.2199	0.1981	0.1658	0.2421	0.1815	0.1686	0.1607	0.1734	0.1659
18	0.0304	0.0206	0.0202	0.0152	0.0474	0.0481	0.0619	0.0177	0.0287	0.0339	0.0549	0.0384	0.0142	0.0333	0.0160	0.0140	0.0209	0.0296	0.0210
19	0.1352	0.1332	0.1336	0.1372	0.1319	0.1336	0.1365	0.1371	0.1336	0.1353	0.1333	0.1362	0.1322	0.1349	0.1278	0.1276	0.1263	0.1256	0.1243
20	0.0570	0.0540	0.0520	0.0580	0.0540	0.0550	0.0560	0.0550	0.0540	0.0560	0.0550	0.0570	0.0530	0.0550	0.0540	0.0530	0.0540	0.0520	0.0560

Table 3
Basic statistics for measurement of gelatin samples

Peak no.	Bovine ($n = 14$)				Porcine ($n = 5$)			
	Height		Width		Height		Width	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
1	0.3693	0.0747	0.5813	0.183	0.2953	0.0162	0.5268	0.1535
2	0.6702	0.1003	0.8552	0.1996	0.6008	0.0379	0.6995	0.1951
3	0.3583	0.0908	0.3895	0.1596	0.29	0.0084	0.2722	0.1847
4	0.9823	0.0059	0.2938	0.1638	0.9644	0.0394	0.2921	0.1695
5	0.9623	0.0076	0.5824	0.1277	0.9812	0.0037	0.6666	0.1524
6	0.2853	0.0612	0.5982	0.2249	0.2727	0.0351	0.6921	0.1943
7	0.6918	0.1163	0.5024	0.0927	0.6352	0.0776	0.4835	0.1188
8	0.9539	0.0522	0.8914	0.1361	0.864	0.0632	0.9372	0.2463
9	0.118	0.0263	0.4163	0.0313	0.1017	0.0061	0.3734	0.0489
10	0.1206	0.0192	0.8502	0.0471	0.1211	0.0056	0.898	0.109
11	0.0865	0.0397	0.9665	0.2091	0.0062	0.001	0.2943	0.0842
12	0.0473	0.0055	1.1208	0.1148	0.0468	0.0073	1.3145	0.0917
13	0.5405	0.1175	1.372	0.2961	0.4538	0.0195	1.2633	0.2742
14	0.0838	0.019	1.0174	0.2759	0.0683	0.0082	1.06	0.1926
15	3.068	0.0139	1.1338	0.1357	0.064	0.0077	1.2317	0.074
16	3.0579	0.0164	1.2277	0.2814	0.0412	0.0034	1.3456	0.1007
17	3.2205	0.0596	1.1066	0.1944	0.1699	0.0078	1.1233	0.1512
18	3.0332	0.0153	0.7335	0.1062	0.0193	0.0042	0.757	0.0866
19	3.1349	0.011	1.21667	0.123	1.1278	0.0015	1.30007	0.0532
20	0.0559	0.0124	3.38334	0.114	3.0541	0.0026	3.66687	0.0423

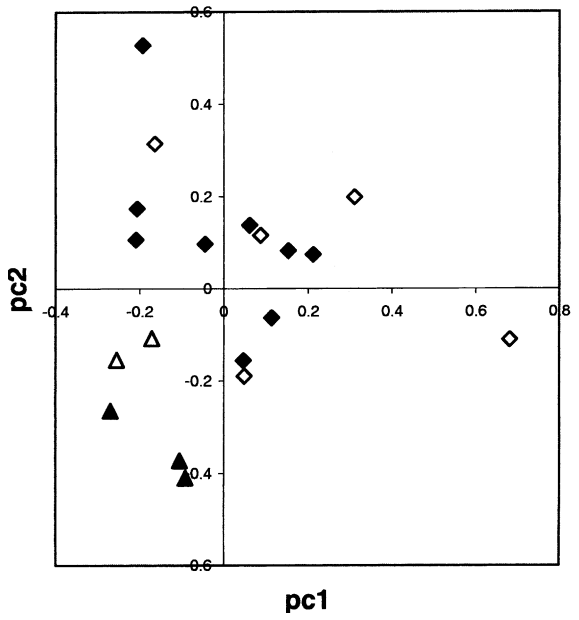

Fig. 2. Principal component analysis plot (two dimensional) from HPLC data for bovine and porcine gelatins. (◆) Bovine, (◇) prediction set for bovine, (▲) porcine, (△) prediction set for porcine.

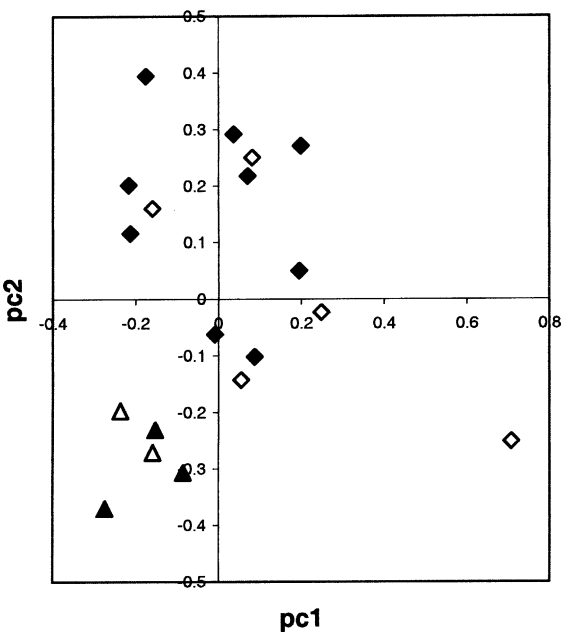

Fig. 3. Principal component analysis plot (two dimensional) of peak height for bovine and porcine gelatins. (◆) Bovine, (◇) prediction set for bovine, (▲) porcine, (△) prediction set for porcine.

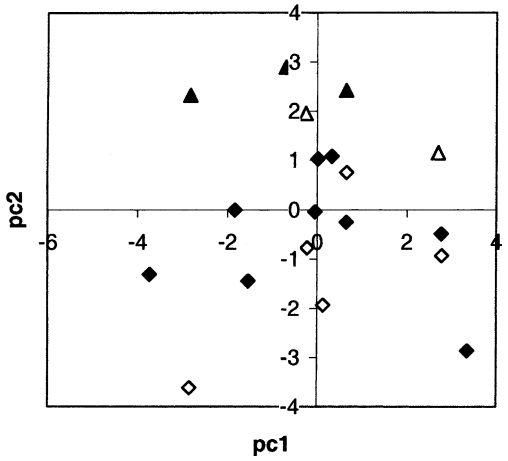

Fig. 4. Principal component analysis plot (two dimensional) of peak width for bovine and porcine gelatins. (◆) Bovine, (◇) prediction set for bovine, (▲) porcine, (△) prediction set for porcine.

and area percentage) individually, leading to good differentiation for height and width (Figs. 3 and 4, respectively). Similar to Fig. 2, all the gelatin samples of bovine and porcine origin shown in Figs. 3 and 4 can be distinguished by a line.

4. Conclusions

Our findings suggest that amino acid analysis of gelatins can be used for the differentiation of bovine and porcine gelatins. Multivariate statistical methods such as PCA can also be employed as a tool for classification of gelatins according to their amino acid composition. The main advantage of principal component analysis is the fact that it is an unsupervised method and does not require any prior information about the classification, and the significant information can be extracted during the statistical treatment. Studies on differentiation between similar proteins are currently very rare. More work needs to be done to establish the performance of this technique to classify other proteins and subjects with very high degree of similarity.

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