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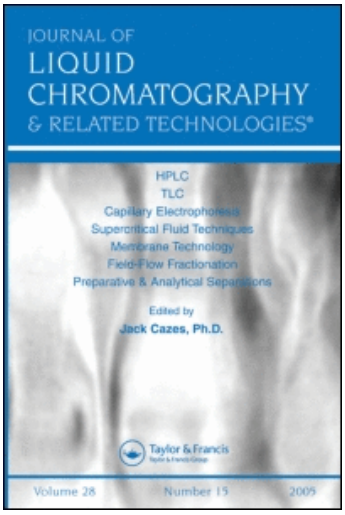

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Method Development and Validation for the HPLC Assay of Hydrolysed Gelatine

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Abstract: This paper describes the development and validation a of reversed-phase high performance liquid chromatography (RP-HPLC) method for the assay of hydrolysed gelatine (Gelita-Sol P, trade name). Key chromatographic parameters were investigated, including short and long alkyl chains of stationary phases (C_4 and C_{18}), column temperatures (30–60°C), and additives of ion-pairing reagents (trifluoroacetic acid and heptafluorobutyric acid) in the mobile phase. Analytical validation parameters such as specificity and selectivity, linearity, accuracy, precision, limit of detection, limit of quantitation, robustness, and system suitability, were evaluated. The calibration curve for hydrolysed gelatine was linear ($r^2 = 0.997$) from 20–200% range of the analytical concentration of 50 mg/mL. The precision of this method calculated as the relative standard deviation (R.S.D.) was 1.22% ($n = 6$). The R.S.D. for the intermediate precision study was 1.77, and recovery of the hydrolysed gelatine ranged between 97.08 and 97.76%. The limits of detection and quantitation were determined to be 5.0 and 10.0 mg/mL, respectively.

Keywords: Hydrolysed gelatine (Gelita-Sol-P), HPLC, Method development, Validation

INTRODUCTION

Gelatine is a collagen derivative, which has many applications in the pharmaceutical, food, and adhesive industries, as well as in photography. In the last decade, many research efforts have been done to develop techniques and

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methods for the separation, purification, and characterisation of hydrolysed gelatine (also called Gelita-Sol P, trade name). Hydrolysed gelatine is a highly purified collagen hydrolysate manufactured from hide. The average molecular weight (MW) is approximately 3 k. Hydrolysed gelatine contains approximately 97% protein based on dry substance. Gelatine is a high molecular weight polypeptide derived from collagen, the primary protein component of animal connective tissues. Industrial preparation of gelatine involves the controlled hydrolysis of the organized structure of collagen to obtain soluble gelatine. The most important sources of collagen for gelatine production are bovine hide, bone, and pigskin. Gelatines from different sources can be very similar in their physiochemical properties, which makes their differentiation very difficult. Collagen hydrolysate is manufactured from animal bones and hides. The material is homogenised and washed, and the bones are demineralised with dilute mineral acid. The resulting product, ossein, is practically pure collagen. After alkaline or acid processing, depending on whether the source is bovine or pigskin, respectively, the raw materials are extracted in several stages with warm water. During this process, the gelatine goes into solution. After concentration, gelatine takes place during the cooling process. Advanced variants of gelatine in the form of gelatine hydrolysate do not gel any further, giving it the advantage of being soluble in cold water.

Pharmaceutical grade collagen hydrolysate (PCH) is a soluble powder obtained by hydrolysis of pharmaceutical gelatine (USP XXII/NF XVIII), by use of an enzymatic process with a US Food and Drug Administration (FDA) approved enzyme. There is a final sterilising step before drying. The average molecular weight of PCH ranges from 2,000 to 6,000 Daltons (2 to 6 kD). Its molecular weight is less than the molecular weight of gelatine, yet more than the average molecular weight of peptones. Unlike gelatine, PCH does not bind significant amounts of water, but it is disbursable and emulsion stabilizing.

Collagen hydrolysate generally has been regarded as having a low biologic value. It does not contain all of the essential amino acids; tryptophan is not present, and cysteine only in small amounts. However, the protein value of gelatine may relate not only to its amino acid composition, but also to its combined effect with other nutritional proteins. In animal experiments, high value protein (casein with addition of methionine) can be replaced up to one third by gelatine, without animal growth being significantly affected. It is also regarded as a valuable nutritional component because of its excellent digestibility.

Clinical studies have suggested a role for collagen hydrolysate in the management of osteoarthritis, based on the postulate that hydrolysed collagen with its abundant amino acids plays a role in cartilage matrix synthesis.[1,2] Gelatine products, which have been used as foods for a number of centuries, are attractive with respect to safety and overall lack of toxicity.[3] Hydrolysed gelatine products have long been used in pharmaceuticals and foods in the United States and Europe.

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Research on structural and physicochemical properties of proteins has been essential for elucidating their molecular structure responsible for their functionality in food and or pharmaceuticals. In addition, the development of methods for the purification of the proteins has been of utmost interest in biotechnology research. In fact, the purity of a protein is a prerequisite for its structure studies or its application, low degree of purity being requested for industrial application in food and pharmaceutical industries.

If HPLC in different modes is a well established technique in food and pharmaceuticals protein research, the new emerging technique of capillary electrochromatography is expected to have great potential in the separation of proteins.[4]Analytical approaches based on the use of mass spectrometry (MS) are also well established in protein products analysis. A number of review papers on the application of chromatography and MS to proteins have appeared in the literature, attesting to a large increase in related publications and the increasing interest and efforts made in this direction.[5–10] To my knowledge, this is the first report on HPLC based separations of this hydrolysed gelatine compound. Herein, my effort in developing an HPLC assay method for this important compound is described. The purpose of this study is to develop HPLC separation method for hydrolysed gelatine that can be applied to differentiate between good and bad batches of hydrolysed gelatine materials.

In most cases, HPLC method development is carried out with ultraviolet (UV) detection, using either a variable wavelength (spectrophotometric) or a diode array detector (DAD). Therefore, we selected UV detection, which can provide an adequate response for most samples. Alternative detectors can be selected primarily when: Samples have little or no UV absorbance; Analyte concentrations are too low for UV detection; Sample interference is important; Qualitative structural information is required.

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EXPERIMENTAL

Materials

Acetonitrile (HPLC grade) was purchased from Merck (Darmstadt, Germany). Trifluoroacetic acid (99.8%, spectrophotometric grade) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Hydrolysed gelatine was supplied by Gelita Europe (Eberbach, Germany).

Instrumentation

The analytical separations were carried out on a Perkin Elmer (Norwalk, CT) HPLC system, equipped with a model LC 200 UV/Vis detector, series 200 LC pump, series 200 autosampler, and series 200 peltier LC column oven. The

analytical column was a Jupiter C_4 (250 x 4.6 mm) 5 μm, 300Å pore size (Phenomenex, Torrance, CA, USA).

Chromatography

The mobile phase consisted of 0.02% (v/v) trifluoroacetic acid (TFA) in filtered deionised water as solvent A and acetonitrile containing 0.02% TFA (v/v) as solvent B. The mixture was pumped as a gradient, starting at 98% A and 2% B and was maintained for 2 min. Over a 2 min period, the mixture changed to 85% A and 15% B, which was maintained for 5 min and then changed to 40% A and 60% B, which was maintained for 33 min. The system was equilibrated for 15 min for the next injection. The mobile phase was filtered through a 0.45 μm membrane filter and continuously degassed with an on-line degasser. The flow rate was 1.20 mL/min. Column temperature was maintained at 35°C. UV detection was measured at 230 nm and the volume of sample injected was 10 μL. The control of the HPLC system and data collection was by a Dell Pentium III computer, linking with a 600 interface and equipped with Perkin Elmer Totalchrom software.

Sample Preparation

All sample solutions at 50.0 mg/mL were prepared by dissolving hydrolysed gelatine in deionised water.

RESULTS AND DISCUSSION

Method Development

Some chromatographic parameters such as column type, mobile phase, and conditioning time were investigated to obtain a good separation of the hydrolysed gelatine analyte within an acceptable time span. In RP-HPLC method development, the important parameters for choosing a column include the type of bonded phase, column dimensions, particle size, carbon load, and the degree of end capping. For protein analysis, the scope of the RP-HPLC method development has been limited to wide pore, silica based columns of shorter alkyl chain length and pores perfusion resins of highly cross-linked polystyrene-divinylbenzene to minimize recovery losses of hydrophobic species. RP-HPLC applications for large hydrophobic proteins typically employ n-butyl (C_4) silica based columns of 5 μm particle size, $\geq$300Å pore size to obtain adequate loading capacity, recovery, backpressure, and flow rate.[11]

Preliminary experiments were performed to select the column most suitable for our purpose: the separation of the hydrolysed gelatine. Three

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C_{18} and one C_4 column were tried in the following order: Biosuite C_{18}, Hypersil C_{18}, ODS, Hyperclone C_{18}, and Jupiter C_4. The different columns showed different selectivity due to the different degree of silanization and different carbon percentage.

Hypersil and Hyperclone did not give a good separation, even when changing the composition of the mobile phase. Biosuite and Jupiter gave satisfactory results in terms of separation, but the final choice was the Jupiter C_4 column because, under the same experimental conditions (see chromatography section), the retention times were shorter and the peaks were sharper than those obtained on the Biosuite C_{18} column. Under these conditions, all hydrolysed gelatine peaks are eluted in less than 14 min with acceptable separation.

Mobile phase: ion-pairing reagents are often used in RP-HPLC analysis to shield the effective charge of functional groups on a protein. Within the pH range for chromatography on silica based columns, the ionizable functional groups include carboxylates (pK_a of 2 and 4), sulfhydryls (pK_a of 8), amines (pK_a of 6, 9, 10, or 11), and guanidines (pk_a of 12 and 13). Two approaches to neutralizing these functional groups are to lower the pH to about 2 to reduce the carboxylate charge and to use anionic ion-pairing reagents to neutralize the positive charged functional group, thereby, increasing the hydrophobic nature of the separation. Although, standard RP-HPLC methods for protein characterization almost exclusively employ mobile phases containing a default level of 0.1% TFA with a gradient of acetonitrile, the resulting TFA suppression of the mass spectrometric signal makes identification and characterization of low levels of proteins unfeasible. Lower amounts of TFA can be used for good chromatographic peak resolution. Other ion-pairing reagents used less frequently include heptafluorobutyric acid (HFBA) and pentafluoropropionic acid (PFPA).

Initially, we tried both TFA and HFBA of ion-pairing reagents under the same experimental conditions and found good separation with the TFA reagent (Figure 1). The mobile phase consisted of 0.02% TFA (v/v) in filtered deionised water as solvent A and acetonitrile containing 0.02% TFA (v/v) as solvent B (see chromatography section). Peak 1, 4, and 5 are very reproducible in every commercial hydrolysed gelatine batch tested in the present study (see applications of the method section). Peak 5 is the principal hydrolysed gelatine. The differences between good and bad hydrolysed gelatine batches were seen in the region of peak 2 and 3. Some batches gave more than 2 peaks in this region.

The optimal wavelength for hydrolysed gelatine detection was established using two UV absorbance scans over the range of 190 to 400 nm, one scan of the mobile phase, and the second of the analytes in the mobile phase. It was shown that 230 nm is the optimal wavelength to maximize the signal. Method development work demonstrated that column temperature had a major impact on separation and recovery of proteins. A sample of hydrolysed gelatine 50 mg/mL was run on the RP-HPLC using column

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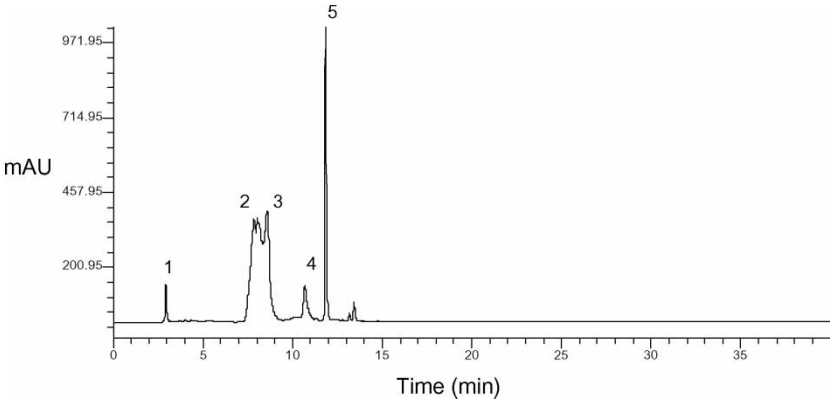

Figure 1. HPLC chromatogram of hydrolysed gelatine.

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temperatures ranging from 30 to 60°C (Figure 2). The recovery and peak shape was optimised at approximately 35°C, while at lower temperatures excessive peak tailing, poor recovery, and column fouling were observed. With an optimised column temperature of 35°C, good separation of the main component was achieved using a Jupiter C_4 column of 5 μm particle size and 300Å pore size, described in the chromatography section.

To evaluate the quantitative nature of the method, a series of samples were run to test the linearity, range, and recovery. Using a Jupiter C_4 column, linearity was assessed by injecting eight reference standards that ranged in concentration from 5 to 200 mg/mL. The integrated peak areas were plotted versus amount injected. The calibration curve was found to be linear from concentration range 10–100 mg/mL with a correlation coefficient of 0.996. On the bases of these data, the best concentration was chosen as a working concentration for the assay. The linearity study also showed as hydrolysed gelatine concentration increases, the column performance decreased due

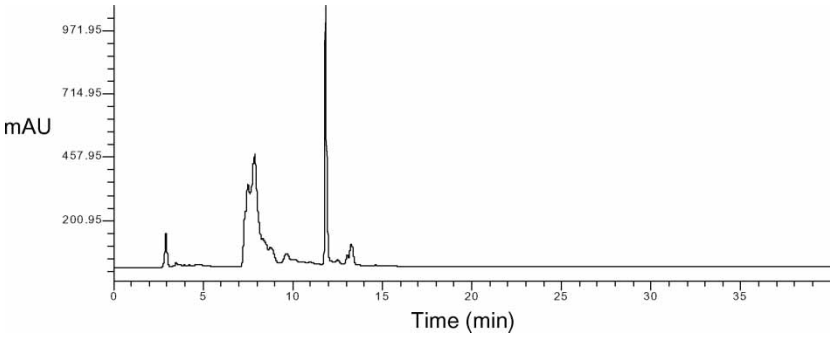

Figure 2. HPLC chromatogram of hydrolysed gelatine at 60°C.

to sample/column overloaded. At a high concentration of compound hydrolysed gelatine in the mobile phase (>100 in Figure 3), linear isotherm behavior is no longer observed, with predictable effects on the separation. Additional analysis was performed to assess the injection reproducibility and robustness of the chromatography.

A system suitability test was developed for the routine application of the assay method. Prior to each analysis, the chromatographic system must satisfy suitability test requirements (resolution and repeatability). Peak-to-peak resolution, between each peak measured on a reference solution, must be above 1.0. The percent relative standard deviation (%RSD) of the response factor (area-mass ratio) for hydrolysed gelatine sample peaks was determined from seven replicate injections of the reference solutions and is required to be less than 2.0%.

System suitability testing was performed to determine the accuracy and precision of the system by making seven injections of a solution containing 50 mg of hydrolysed gelatine/mL. All peaks were well resolved, and the precision of injections for all peaks was acceptable. The percent relative standard deviation (%RSD) of the peak area response was measured. The %RSD of peak areas averaged 1.92% ($n = 7$); the tailing factor (T) for each peak of the hydrolysed gelatine was 1.08, theoretical plate number (N) was 101624.98, and resolution was >5.14 for the main hydrolysed gelatine peak. The retention time variation %RSD was 0.20 for seven injections.

Selectivity was also studied over extended time using several columns and many different batches of mobile phase. Relative RT ranges (RT of peak of interest/RT of hydrolysed gelatine) were as follows: peak 1 = 7.72; peak 2 = 8.66; peak 3 = 9.75; peak 4 = 10.92; peak 5 = 11.88. This data indicate that the RT windows for each impurity/amino acid are unique and do not overlap. Overall selectivity was established through determination of purity for each peak using the PDA UV detector.

For assessment of method robustness within a laboratory, a number of chromatographic parameters were varied, which included flow rate, temperature,

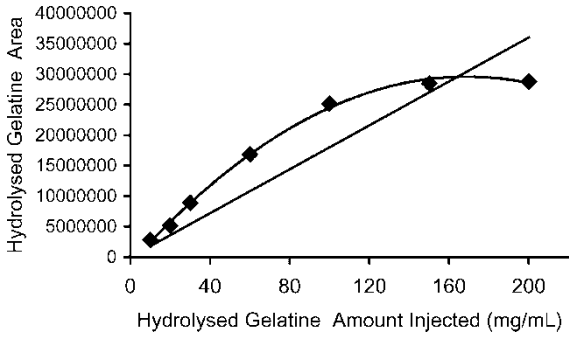

Figure 3. Typical calibration graph obtained after analysis of hydrolysed gelatine to demonstrate column overloaded with high hydrolysed gelatine concentration.

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mobile phase composition, and columns from different lots. In all cases, good separations were always achieved, indicating that the method remained selective for hydrolysed gelatine components under the tested conditions.

The stability study of hydrolysed gelatine solutions was also investigated. The solutions were stable during the investigated eight days, and the %RSD was in between 0.06 and 0.10% for retention time for the main hydrolysed gelatine peak 5 at 11.87 min. Standard solutions stored in a capped volumetric flask on a laboratory bench under normal lighting conditions for eight days, were shown to be stable with no significant change in hydrolysed gelatine concentration over this period. Based on these data that show quantitative recovery through eight days, solutions of hydrolysed gelatine should be assayed within eight days after preparation.

Validation of the Method

Validating analytical methods is a crucial component of successful product development, testing, and quality. All product types require some level of evaluation and testing either at the raw material, intermediates, or final product level. Critical decisions may be made based on these results, making it imperative that pharmaceutical and diagnostic companies ensure their accuracy and reproducibility to remain compliant with regulatory guidelines[12,13] in the current climate of increased enforcement. The step by step written and approved protocol for test method validation should be followed.[14]

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Linearity

The linearity of the method should be tested in order to demonstrate a proportional relationship of response versus analyte concentration over the working range. It is usual practice to perform linearity experiments over a wide range of analytes. This gives confidence that the response and concentration are proportional and, consequently, ensures that calculations can be performed using a single reference standard, rather than the equation of a calibration line. In this study, linearity was studied using five solutions in the concentration range 10–100 mg/mL ($n = 3$). The regression equation was found by plotting the peak area (y) versus the hydrolysed gelatine concentration (x) expressed in mg/mL. The correlation coefficient ($r^2 = 0.997$) obtained for the regression line demonstrates that there is a strong linear relationship between peak area and concentration of hydrolysed gelatine (Table 1).

Precision (Repeatability and Intermediate Precision)

The precision of the chromatographic method, reported as %RSD, was estimated by measuring repeatability (intra-day assay precision) on six

Table 1. Linearity study of the HPLC method for the assay of hydrolysed gelatine

Concentration of hydrolysed gelatine (mg/mL)	Concentration as percent of 50 mg/mL of hydrolysed gelatine	Hydrolysed gelatine peak area as mean of two injections (μV s)
10	20	2795601
20	40	5157972
30	60	8918107
60	120	16837051
100	200	25162025

Correlation coefficient: 0.997; Intercept (%): −73; Equation for regression line: $y = 250897x + 734692$.

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replicate injections at 100% test concentration (50 mg/mL), and intermediate precision (inter-day variation) was studied for two days using three solutions in the concentration range 30, 50, and 80 mg/mL ($n = 3$). The %RSD values for the t_R (min) and peak area were found to be less than 2.0% (Table 2) in all cases and illustrated good precision for the analytical method. In addition, the regression equation was found by plotting the peak area (y) versus the hydrolysed gelatine concentration (x) expressed in mg/mL. The correlation coefficient ($r^2 = 0.9999$) obtained for the regression line demonstrates that there is strong linear relationship between peak area and concentration of hydrolysed gelatine (Figures 4–5).

Accuracy/Recovery Studies

The accuracy of an analytical method is determined by how close the test results obtained by that method come to the true value. It can be determined

Table 2. Repeatability and intermediate precision studies

Validation step	Parameter	Concentration (mg/mL)	Results
Repeatability ($n = 6$)	t_R (min) %RSD	50	0.20
	Peak area %RSD		1.22
Intermediate precision ($n = 3$)	Day 1	30	0.20, 1.77
	t_R (min) %RSD	50	0.55, 1.30
	Peak area %RSD	80	0.13, 0.55
	Day 2	30	0.08, 0.43
	t_R (min) %RSD,	50	0.29, 1.53
	Peak area %RSD	80	0.28, 0.49

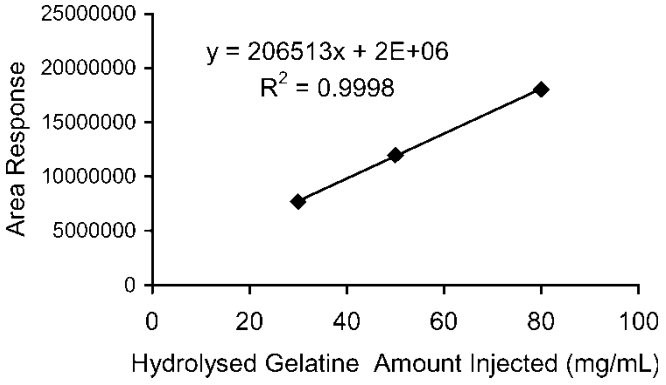

Figure 4. Typical calibration graph obtained after analysis of hydrolysed gelatine to demonstrate intermediate precision variation, day 1.

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by application of the analytical procedure to an analyte of known purity (for the drug substance) or by recovery studies, where a known amount of standard is spiked in the placebo (for the drug product). In this study, a number of different solutions were prepared with a known added amount of hydrolysed gelatine and injected in triplicate. Percent recoveries of response factor (area and concentration) were calculated as shown in Table 3, and it is evident that the method is accurate within the desired range.

Specificity and Selectivity

Forced degradation studies were performed to evaluate the specificity of hydrolysed gelatine and its impurities under four stress conditions (heat,

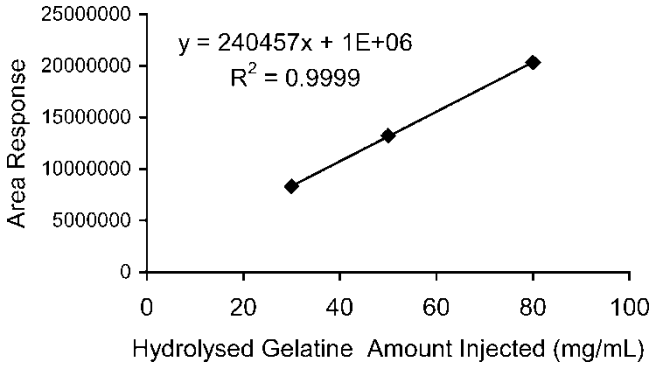

Figure 5. Typical calibration graph obtained after analysis of hydrolysed gelatine to demonstrate intermediate precision variation, day 2.

Table 3. Recovery studies

Concentration range (mg/mL)	Recovery (%) ($n = 3$)	RSD (%)
10	97.76	1.00
30	97.74	1.37
50	97.15	1.47
80	97.08	1.33

UV light, acid, base). A summary of the stress results is shown in Table 4 and chromatograms in Figure 6. It is evident from Figure 1, that the method has been able to separate the peaks due to the degraded products from that of the hydrolysed gelatine. This was further confirmed by peak purity analysis on a PDA UV detector.

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Limits of Detection and Quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) tests for the procedure were performed on samples containing very low concentrations of analyte. LOD is defined as the lowest concentration of analyte that can be detected above baseline noise. Typically, this is three times the noise level. LOQ is defined as the lowest concentration of analyte that can be reproducibly quantitated above the baseline noise with a signal to noise ratio of 10. In this study, the LOD was 5 mg/mL and the LOQ was 10 mg/mL and %RSD 1.89% ($n = 3$).

Application of the Method

The developed method was applied to the assay of nine different commercial batches of hydrolysed gelatine raw material. Different peaks were eluted in

Table 4. Assay (%) of hydrolysed gelatine under stress conditions

Stress conditions	Sample treatment	t_R (min)	Assay (%)	Peak area (μV s)
Reference	Fresh solution	11.85	98.13	11365875
Acid	1 N HCL for 24 h	11.83	96.53	8819136
Base	1 N NaOH for 4 h	11.91	94.37	9952468
Heat	60°C for 1 h	11.87	98.25	11294417
Light	UV Light for 24 h	11.85	99.04	10725275

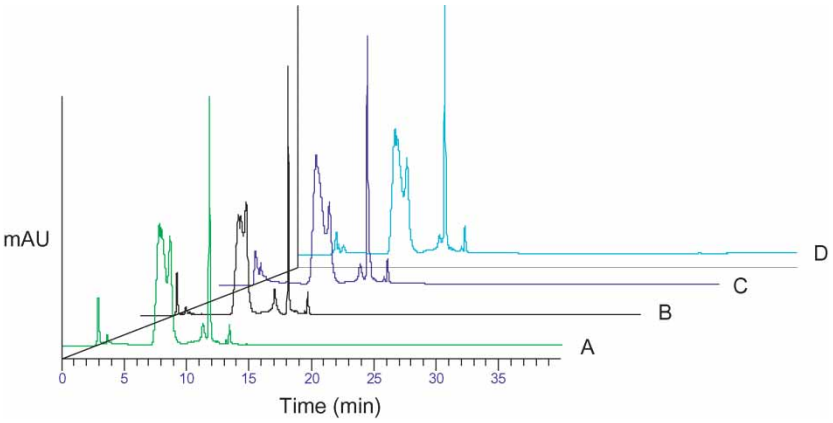

Figure 6. HPLC chromatograms of hydrolysed gelatine under stress conditions (A) UV light; (B) heat at 60°C; (C) base; (D) acid.

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different good and bad hydrolysed gelatine batches (Figure 7). Table 5 shows the retention times, the area percentages, and the precision of each batch calculated from the principal hydrolysed gelatine peak 5.

CONCLUSION

A reversed-phase HPLC method for the separation of complex hydrolysed gelatine is developed and validated that can be reliably applied to differentiate between good and bad hydrolysed gelatine batches. The developed method

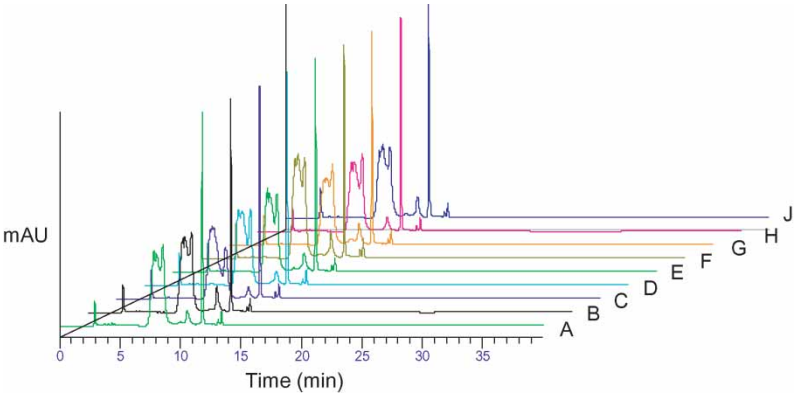

Figure 7. HPLC chromatograms of different nine hydrolysed gelatine batches.

Table 5. Assay (%) from nine different commercial hydrolysed gelatine batches ($n = 2$)

Batch #	t_R (min) ± RSD (%)	Area (%) ± RSD (%)
A	11.85 ± 0.35	97.27 ± 0.02
B	11.86 ± 0.00	98.13 ± 0.25
C	11.88 ± 0.05	96.70 ± 0.03
D	11.86 ± 0.35	98.72 ± 0.05
E	11.85 ± 0.00	96.09 ± 0.02
F	11.86 ± 0.17	98.88 ± 0.11
G	11.88 ± 0.35	98.09 ± 0.06
H	11.87 ± 0.17	98.75 ± 0.01
J	11.86 ± 0.11	96.11 ± 0.04

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was applied to nine different commercial hydrolysed gelatine batches, and results showed significant differences between good and bad batches. The validation study shows good linearity, accuracy, and precision. The suggested technique can be used in quality control for release for hydrolysed gelatine materials.

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REFERENCES

1. Oberschelp, U. Individual arthrosis therapy is possible. Therapiewoche **1985**, *35*, 5094–5097.
2. Seeligmuller, K.; Happel, H.K. Can a mixture of gelatine and l-cystine stimulate proteoglycan sysnthesis? Therapiewoche **1989**, *39*, 3153–3157.
3. Brook, M.J.V.; Lyster, S.C.; Graham, B.E. Intravenously administered gelatine: a toxicity study. J. Lab. Clin. Med. **1947**, *32* (9), 1115–1120.
4. Xiang, R.; Horvath, Cs. Fundamentals of capillary electrochromatography: migration behavior of ionized sample components. Anal. Chem. **2002**, *74*, 762–770.
5. Leonil, J.; Gagnaire, V.; Molle, D.; Pezennec, S.; Bouhallab, S. Application of chromatography and mass spectrometry to the characterization of food proteins and derived peptides. J. Chromatogr. A **2000**, *881* (1), 1–21.
6. Alomirah, H.F.; Alli, I.; Konishi, Y. Applications of mass spectrometry to food proteins and peptides. J. Chromatogr. A **2000**, *893*, 1–21.
7. Bayard, C.; Lottspeich, F. Bioanalytical characterization of proteins. J. Chromatogr. B **2001**, *756*, 113–122.

8. Moreno-Arribas, M.V.; Pueyo, E.; Polo, M.C. Analytical methods for the characterization of proteins and peptides in wines. Anal. Chem. Acta **2002**, *458*, 63–75.
9. Careri, M.; Bianchi, F.; Corradini, C. Recent advances in the application of mass spectrometry in food-related analysis. J. Chromatogr. A **2002**, *970*, 3–64.
10. Kvasnicka, F. Proteomics: general strategies and application to nutritionally relevant proteins. J. Chromatogr. B **2003**, *787*, 77–89.
11. Wan, H.Z.; Kaneshiro, S.; Frenz, J.; Cacia, J. Rapid method for monitoring galactosylation levels during recombinant antibody production by electrospray mass spectrometry with selective-ion monitoring. J. Chromatogr. A **2001**, *913*, 437–446.
12. International Conference on Harmonisation (ICH). Q2A: Text on Validation of Analytical Procedures, U.S. FDA Federal Register. **1995**, *60*, 11260.
13. International Conference on Harmonisation (ICH). Q2B: Validation of Analytical Procedures: Methodology FDA Federal Register. **1997**, *62*, 27463.
14. Shabir, G.A. Step-by-step analytical methods validation and protocol in the quality system compliance industry. J. Valid. Technol. **2004**, *10*, 314–324.

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