ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
16 (1997) 295–302

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Photon correlation spectroscopy applied to characterisation of denaturation and thermal stability of human albumin

P.C. Sontum *, C. Christiansen

Research and Development Section, Nycomed Imaging AS, P.O. Box 4220, Torshov, N-0401 Oslo, Norway

Received 2 September 1996; received in revised form 31 January 1997

Abstract

Photon correlation spectroscopy and light absorption measurements have been applied for characterisation of denaturation kinetics and thermal stability of human albumin in solution. The hydrodynamic size of the molecules has been studied as a function of pH, and the denaturation rate of ten different lots of 5% (w/v) human albumin solution has been measured at various temperatures. In the native (pH 7) state, the hydrodynamic molecular diameter was found to 6.3 nm. The molecular size was relatively stable between pH 10 and 5, but increased with decreasing pH to approximately 20 nm at pH 3. The denaturation rate, measured as change in hydrodynamic diameter per min, was strongly dependent on temperature and increased 3-fold per degree in the 73–75°C range. The investigated lots of albumin solution showed large variations in stability at 74°C, with denaturation rates ranging from 10 to 100 nm min^{-1}. The observed thermal stability for the lots investigated was ranked identically with both the employed techniques. In an effort to explain the observed lot to lot variations in denaturation rate, a broad chemical characterisation including determination of free SH content, fatty acid content and composition and metal content, was performed. However, lot to lot variations in these parameters was not found to fully elucidate the observed variations in thermal stability. © 1997 Elsevier Science B.V.

Keywords: Photon correlation spectroscopy; Human albumin; Thermal stability; Denaturation rate; Fatty acid composition; Metal content

1. Introduction

Infoson® (Molecular Biosystems, San Diego, USA) is a new contrast agent intended for use during medical imaging by sonography. The product consists of microbubbles of air encapsulated by a thin, insoluble, layer of albumin, suspended in a human albumin solution [1]. Infoson® is produced by sonication of a 5% (w/v) solution of human albumin, heated to approximately 70°C. Although the protein is protected by addition of the stabilizers octanoic acid (4 mM) and *N*-acetyltryptophane (4 mM), potential variations in the thermal stability of the raw material may lead to heat denaturation followed by aggregation and precipitation of protein if the heat exposure

* Corresponding author. Fax: +47 22891200.

0731-7085/97/$17.00 © 1997 Elsevier Science B.V. All rights reserved.
PII S0731-7085(97)00032-0

during production is not rigidly controlled. For this reason, a method for relevant characterisation of the thermal stability of the raw materials for the Infoson® production was necessary.

Thermal stability of albumins has earlier been extensively studied both by differential scanning calorimetry (DSC) [2–5] and by gel permeation chromatography (GPC) and other fractionation techniques [6–8]. The DSC technique offers excellent information on the denaturation temperature, but it is not conveniently used to study the kinetics of the denaturation process at a certain temperature. The fractionation techniques may convey quite small changes in the amount of molecular oligomers and aggregates caused by a given heat exposure, but as they are quite time consuming these techniques are also not easily employed to study the kinetics of the process. For conformational studies of proteins, the use of photon correlation spectroscopy (PCS) has also been reported [9,10].

The PCS technique investigates temporal fluctuations in light scattered from a solution of molecules. These fluctuations are a direct function of the Brownian motion of the scatterers and may be used to calculate the hydrodynamic size of the solute. For small scatterers, below approximately 100 nm, the intensity of the scattered light per unit mass is proportional to the third power of the size of the scatterer. The technique is therefore inherently sensitive to aggregates and should be suitable for the investigation of the early stages of a heat denaturation/aggregation process.

The current paper explores the PCS technique for characterisation of denaturation and thermal stability of human albumin by studying the conformation of the protein molecules as a function of pH, as well as the kinetics of heat denaturation at higher temperatures. The results obtained with the PCS technique have also been compared to results from a conventional light absorption measurements. Extensive chemical characterisation including determination of free SH content, fatty acid content and composition and metal content is also performed.

2. Materials and methods

2.1. Materials

The samples investigated in this study were from ten different lots of human albumin solution 5% (w/v) purchased from the Swiss Red Cross, Switzerland. The lots were intended for human infusion and therefore prepared according to the pharmacopoeia. For thermal stabilisation, the samples contained 4 mM octanoic acid and 4 mM *N*-acetyltryptophane. The lots were made isotonic by addition of NaCl and were adjusted with NaOH to pH 7. The sample bottles were stored cold and protected from light prior to analysis. For studies of changes in molecular conformation with pH, samples in the pH range 3–10 were prepared by addition of 1 N HCl or 1 N NaOH (Merck) to the original albumin solutions.

2.2. Photon correlation spectroscopy

The albumin samples were characterized by photon correlation spectroscopy (PCS) performed with a Malvern 4700 PS/MW spectrophotometer (Malvern Instruments, UK) set up with a 7032 Multi 8, 128 channel, correlator and a Cyonics 70 mW Argon ion (488 nm) laser (Cyonics Corp., USA). Round bore, glass measuring cells with an internal diameter of 8 mm were used. During measurements the light output power was set to 100 mW, scattering angle was 90° and the aperture opening in front of the PM tube was 200 μm. Solvent and solute refractive indexes were set to 1.330 and 1.450, respectively and the solute imaginary refractive index was set to 0. Solvent viscosity was set to 0.890 and 0.3830 centi-Poise for analysis at 25 and 74°C, respectively. Samples were measured in the manual mode of the Automeasure software (version 1.23, Malvern Instruments, UK). The correlator settings were automatically determined by the software with separate samples prior to each new analysis and the duration of each measurement was 30 s. The analysis was started immediately after introducing the sample into the measuring cell compartment,

and measurements were repeated every 40 s for up to 15 min.

As responses, the Z-average (i.e., intensity weighted average) hydrodynamic diameter of the scatterers was followed as a function of time. The Z-average diameter was calculated by fitting a third order cumulant expression to the measured correlation function. The light intensity and volume size distributions were calculated using the model free, exponential sampling time, algorithm in the software. To obtain a single parameter response for the denaturation rate, linear regressions were performed for the diameter versus time. Regressions were performed on all data obtained from 4 min after start of analysis. The slope (nm min^{-1}) of the regression line was used as a measure of the denaturation rate.

Analyses of samples with variable pH were performed at 25°C while measurements of thermal stability were performed at 74°C. To enable sufficient temperature control, a waterbath was coupled to the external temperature control recirculation system of the measuring cell compartment. The temperature of the water within the cell compartment, monitored by the instrument, was within ± 0.1°C of the nominal analytical temperature. Samples were equilibrated to room temperature prior to use and 1.5 ml was transferred to the sample cell. Each lot was analysed from 2 to 10 times at a nominal analytical temperature of 74°C. In addition, one batch was also analysed at 73 and 75°C. The dynamics of the heating process and the actual, equilibrium, temperature in the sample during analysis, was also evaluated. The temperature within the measuring cell was monitored with a Labotherm Thermometer Caye Digistrip 4S PLUS Datalogger with copper constantan mantel thermosensors calibrated to within ± 0.1°C accuracy. Analysis was performed as described above with a thermosensor inserted through a hole in the cell stopper and placed within the measuring cell, approximately 3 mm above the scattering volume. The temperature was initially measured 60 s after the cell was introduced into the heated cell compartment and then each 20 s for approximately 15 min.

2.3. Light absorption measurements

To compare the results from the PCS measurements with an alternative method, the heat stability of each albumin lot was also characterized with a light absorption technique using a Cary 3 UV-visible spectrophotometer with a 10 mm light path. In this method the increasing light scattering effect during denaturation was recorded as an apparent increase in absorption of light ($\lambda = 600$ nm). The denaturing rate at nominally 74°C, was then measured as an increase in absorption as a function of time. As a single parameter response for the denaturation rate, the maximum slope (absorbance min^{-1}) of the absorbtion versus time curve was used.

2.4. Chemical characterisation

In addition to measurements of heat stability, each lot was analysed for several chemical parameters. For determination of free sulfhydryl (SH) groups in human albumin solution a spectrophotometric method based on the colour forming reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was utilized. The DTNB reagent reacts with free SH groups at neutral pH to yield 1 mol of the coloured anion 2-nitro-*S*-thiobenzoate (NTB) per mol of SH originally present. The quantity of NTB formed is determined by spectrophotometry at 412 NM. The content and composition of long chain fatty acids (LCFA) was measured utilizing reversed phase HPLC. Samples were extracted with chloroform and derivatized before analysis. A C17:0 internal standard was used for quantification of total LCFA and fatty acid composition was determined in % of total area. The content of Fe, Ca, Cu, Mg, Zn and Cr was measured by inductive coupled plasma atomic emission spectroscopy (ICP-AES) while Al, Cd and Mn was determined by graphite furnace atomic adsorption spectroscopy (GF-AAS).

3. Results and discussion

Fig. 1 shows the observed Z-average diameter of the albumin molecules as a function of pH. The

native albumin molecules, in the pH unadjusted samples, were found to be 6.3 ± 0.6 nm which is practically identical to earlier reported values [9,10] for this protein. The precision for the Z-average diameter, expressed as pooled repeatability standard deviation for all samples investigated, was $\pm 8\%$ R.S.D. As seen from Fig. 1, the measurements revealed a relatively stable conformation of the molecules in the range from pH 10 to approximately pH 4.5, followed by the expected acidic expansion in the pH range 3–4, as previously described [11]. Combined, these findings demonstrate the usefulness of PCS for determination of conformational changes in the albumin molecule and the ability of the method to yield precise and accurate results for its molecular size.

After the initial evaluation of the PCS technique with analyses on a stable experimental system at room temperature, the method was further investigated with measurements of denaturation kinetics at high temperatures. Fig. 2 shows typical size distributions for a slow and a fast denaturing sample, at various time points after placing the sample at 74°C. During the first few measurements, i.e. in the pre-denaturated state, the albumin molecules in all investigated lots were contained in a monomodal peak with a volume mean diameter of typically 6–7 nm. As seen from Fig. 2, the intensity distributions of both samples became bimodal after approximately 3 min exposure to 74°C. For the sample from the fast denaturating lot, the initial monomer peak disappeared almost completely after approximately 5 min at 74°C and was replaced by a broad aggregate peak in the 30–100 nm range. For the sample from the more stable lot, the initial peak was still predominant after 5 min, although a small aggregate peak is clearly present. The volume distributions, however, were practically unchanged even after 5 min for the slowly denaturation sample, and only slightly shifted to larger diameters for the more unstable sample. The denaturation process, as observed with PCS, thus starts by the aggregation of a small mass fraction of molecules and the further growth of these oligomers/aggregates. The volume distributions indicate that the bulk of the molecules is unaffected by the heat and remain monomeric for several minutes, even for the fast denaturing lots. The results demonstrate the sensitivity of the intensity distribution towards the strong scattering of the molecular aggregates produced during heat denaturation, and the ability of the technique to detect denaturation at an early stage, long before the bulk of the molecules has been notably affected.

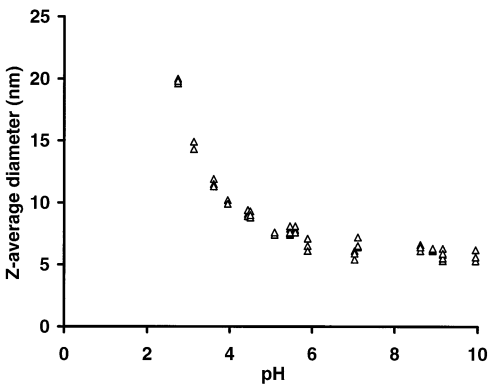

Fig. 1. Conformational changes in the albumin molecules with pH, measured as change in hydrodynamic Z-average molecular diameter by photon correlation spectroscopy.

Fig. 3 shows the results from the evaluation of the actual, equilibrium temperature in the sample during analysis. As seen from this figure, the temperature increases rapidly during the first 3 min after placing the sample in the heated environment, and then equilibrates at a reasonably well defined level. In the six samples investigated with a thermosensor within the measuring cell, the average temperature in the time range from 5 to 15 min was 72.8 ± 0.3°C and thus, somewhat less than the nominal analytical temperature of 74°C, measured in the surrounding water. The temperature dependence of the denaturation rate of an albumin sample is visualized in Fig. 4, showing the Z-average diameter of the molecules as a function of time, at three different temperatures. As seen from this figure, the heat stability was strongly affected by relatively small temperature changes in the range investigated. The denaturation rate, measured as change in diameter per minute, was 1.4, 5.3 and 15.3 nm min^{-1} at 73, 74 and 75°C, respectively. Hence, a 3-fold increase in the denaturation rate per degree increase in temperature is indicated. These observations under-

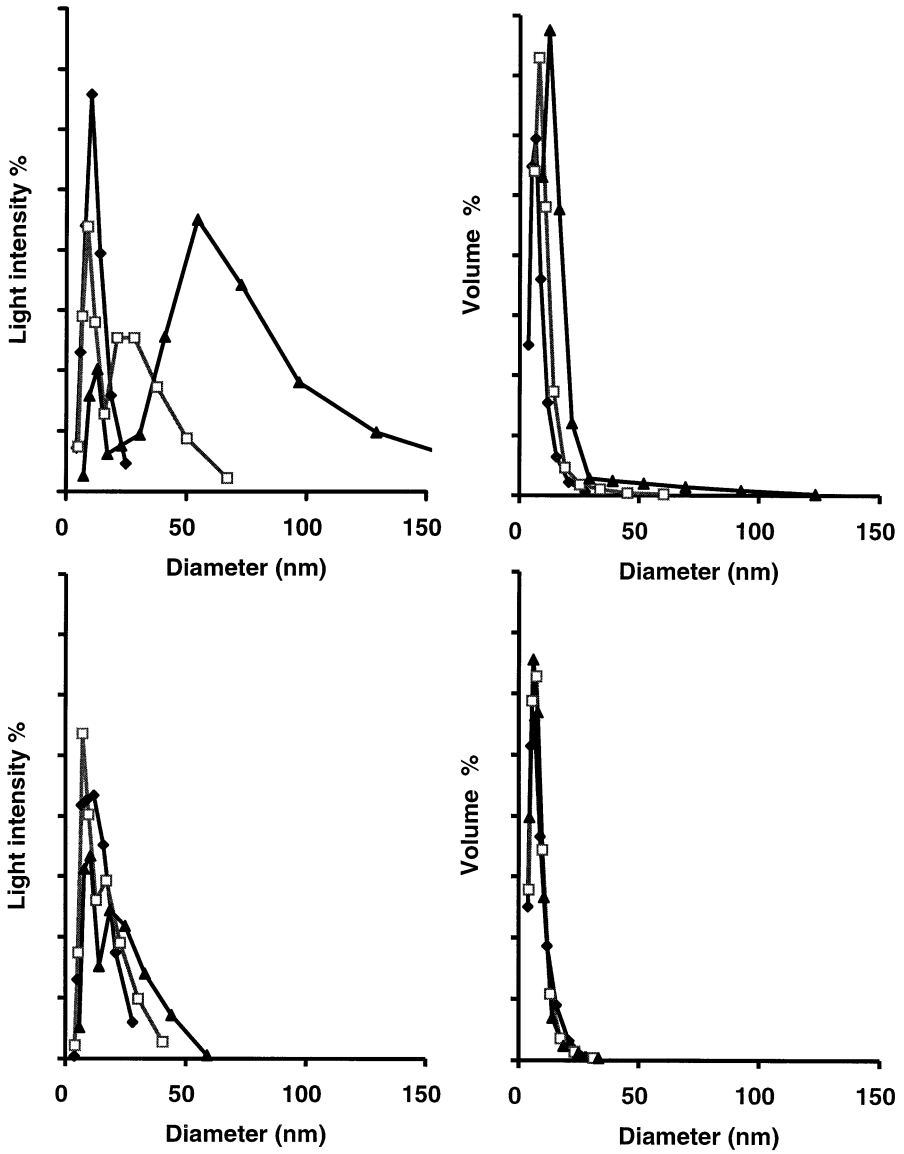

Fig. 2. Typical intensity (left) and volume (right) size distributions of a slow (upper) and a fast (lower) denaturating sample of albumin at 120 s (◆), 200 s (□) and 280 s (▲) after exposure to nominally 74°C.

line the necessity of a precise temperature control to achieve reproducible results. A precision for the denaturation rate, expressed as pooled reproducibility R.S.D., of $\pm 15\%$ was achieved with the PCS method. In view of the observed strong temperature dependency discussed above, the lack of precision can be easily attributed to variations in analytical temperature, and may probably be improved by a more rigid temperature control.

For cross evaluation of the results for the denaturation rate from the PCS measurements, a conventional light absorption technique was also investigated. This technique measures changes in the apparent light absorption of a protein solu-

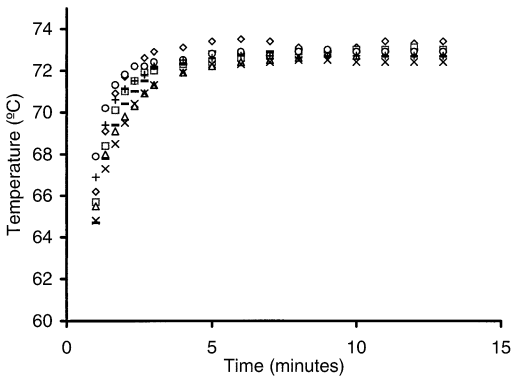

Fig. 3. Actual temperature development within six samples of albumin after exposure to nominally 74°C in the Malvern 4700 measuring cell.

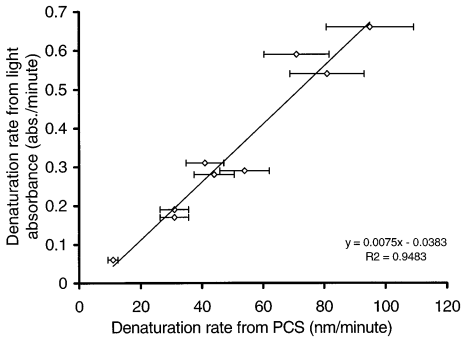

Fig. 5. Correlation between denaturation rates for various lots of albumin measured with PCS and light absorbance techniques. Line from linear regression included. Error bars reflect a pooled R.S.D. of ±15%.

tion as aggregates are formed during heat denaturation, and the method only detects denaturation at a late stage, were the bulk of the molecules have aggregated to form a turbid suspension of precipitating matter. The denaturation rates of the various lots investigated are visualized in Fig. 5. As seen from these results, the investigated lots displayed strong variations in heat stability, but the two methods rank the stability of the various batches identically to within the precision of the comparison. These results indicate that the kinetics during heat induced aggregation are similar at the very early and very late stage of the process,

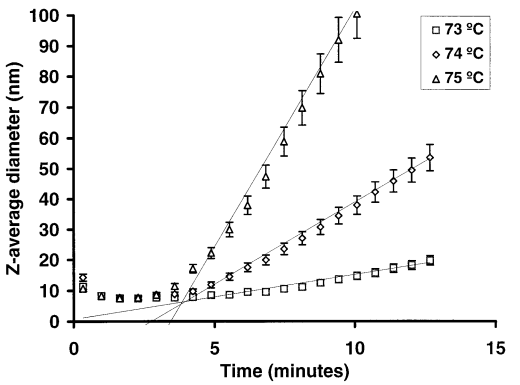

Fig. 4. Denaturation of albumin measured a change in Z-average diameter versus time at nominally 73°C (□), 74°C (◇) and 75°C (△). Lines from linear regression to data points from 4 min are included. Error bars reflect a pooled R.S.D. of ±8%.

and that the less complex and normally easier available light adsorption technique may be used for evaluation of the denaturation kinetics. To compare the sensitivity of the PCS analysis with the absorption method, several samples were investigated with the absorption method, immediately after ended PCS analysis. Even though the Z-average diameter in some of these samples was as high as 50 nm and they thus, had been strongly affected by the heat exposure during the PCS analysis, none of the samples showed detectable increase in light absorption when compared to non-exposed controls. This observation again demonstrate the sensitivity of the PCS technique and its ability to detect and evaluate the denaturation of proteins at a very early stage.

As observed from Fig. 5, the denaturation rate displayed strong variations at 74°C, with values ranging from 11 to 95 nm min^{-1}. In an effort to explain these lot to lot differences in heat stability, several chemical parameters were investigated and the results from these measurements are stated in Table 1 and Table 2. The presence of free SH groups in the albumin molecules is known to influence their thermal stability [12]. Due to the ability of these groups to form intermolecular disulphide bridges, the formation of aggregates during heat induced denaturation is expected to increase with increasing amount of free SH. However, as seen from Table 1, the investigated lots all contained very small amounts of free SH and no

Table 1
Free sulfydryl content and fatty acid content and composition for various lots of 5% (w/v) human albumin solution

Lot no.	SH (% mol mol^{-1})	C 16:0 (%)	C 16:1 (%)	C 18:0 (%)	C 18:1 (%)	C 18:2 (%)	C 18:3 (%)	C 20:4 (%)	Total LCFA (μM)
9.231.045.0	1.1	29.2	3.2	3.2	24.9	35.8	1.0	2.9	778
0.231.050.0	0.8	30.1	3.7	4.5	28.3	28.2	1.8	3.3	754
0.231.051.0	0.8	32.3	3.6	5.5	28.3	25.2	1.9	3.1	720
0.231.059.0	0.6	29.0	3.6	3.8	26.6	31.3	1.9	3.7	798
0.231.071.0	0.6	29.9	3.8	2.8	25.9	30.7	2.1	4.9	799
1.231.006.0	1.3	32.6	3.8	3.6	28.2	25.6	2.2	4.0	854
1.231.024.0	0.5	33.9	4.1	4.2	27.4	24.3	2.5	3.5	893
1.231.039.0	1.0	34.7	3.4	4.6	28.0	24.2	1.5	3.7	1005
2.230.007.0	0.5	33.6	3.6	5.0	29.2	23.7	1.8	3.2	894
2.230.009.0	0.4	32.9	3.8	5.2	26.0	25.8	2.4	3.9	827

Table 2
Content of metals in various lots of 5% (w/v) human albumin solution

Lot no.	Fe (mg l^{-1})	Ca (mg l^{-1})	Cu (mg l^{-1})	Mg (mg l^{-1})	Zn (mg l^{-1})	Mn (μg l^{-1})	Al (μg l^{-1})	Cd (μg l^{-1})	Cr (μg l^{-1})
9.231.045.0	0.76	8.5	0.18	—	—	8.0	130	2.8	45
0.231.050.0	0.76	9.2	0.13	0.47	0.48	9.4	151	1.2	50
0.231.051.0	0.77	9.1	0.13	0.50	0.52	9.4	132	1.3	50
0.231.059.0	0.09	—	—	—	—	—	—	—	—
0.231.071.0	0.66	12.1	0.34	0.66	0.83	14.3	141	1.1	42
1.231.006.0	0.72	10.4	0.20	0.79	0.65	15.9	176	0.9	52
1.231.024.0	0.74	9.4	0.13	0.67	0.66	11.3	132	1.1	51
1.231.039.0	0.76	9.9	0.15	0.38	0.73	13.1	137	1.1	32
2.230.007.0	0.66	10.4	0.15	0.37	0.61	12.7	116	1.2	47
2.230.009.0	0.68	15.5	0.22	0.87	1.04	13.7	174	1.3	58

significant ($P = 0.1$) correlation between this parameter and the denaturation rate was observed. The presence of fatty acids tends to stabilize proteins against temperature induced aggregation and the content and composition of fatty acids are important regulators of the thermal stability of albumin solutions [3,4,7,8,13]. As shown in Table 1, both the content and the composition of long chain fatty acids varied between the lots investigated. However, no negative correlation between denaturation rate and content of total long chain fatty acids or any other specified acids were observed. More interesting, a possible increase in denaturation rate with increasing content of octadecanoate (stearate) was observed ($P = 0.1$). The reason for such behaviour is currently unexplained. Metal ions may to a variable degree bind to the albumin molecules and also influence the thermal stability of a protein solution [5,11]. As seen from Table 2, significant variations in metal content between the investigated lots were observed, but no correlations between these properties and the denaturation rate were apparent.

In conclusion, photon correlation spectroscopy and light absorption measurements have been sucessfully applied for characterisation of denaturation kinetics and thermal stability of human albumin in solution, and the results demonstrate the excellent sensitivity of the PCS technique to detect small changes in the molecular comformation of the protein. An almost 10-fold difference between the heat stability of various lots of HSA was found, but variations in chemical parameters such as free SH content, fatty acid content and composition and metal content was not found to elucidate the observed variations in stability.

Acknowledgements

The authors would like to thank H. Nicolaysen for performing the light adsorption analyses, O. Møller and G. Hjellum for assistance with the PCS analyses, I. Oulie for performing the HPLC analyses and K.M. Christiansen for performing the SH analyses. The metal analyses were carried out by Center of Industrial Research (Oslo, Norway).

References

[1] C. Christiansen, H. Kryvi, P.C. Sontum, T. Skotland, Biotechnol. Appl. Biochem. 19 (1994) 307–320.
[2] P.D. Ross, J.S. Finlayson, A. Shrake, Vox. Sang. 47 (1984) 19–27.
[3] A. Shrake, J.S. Finlayson, P.D. Ross, Vox. Sang. 47 (1984) 7–18.
[4] A. Shrake, P.D. Ross, J. Biol. Chem. 263 (30) (1988) 15392–15399.
[5] P.-O. Hegg, H. Martens, B. Løfqvist, J. Sci. Food Agric. 30 (1979) 981–993.
[6] B.M. van Liedekerke, H.J. Nelis, J.A. Kint, F.W. Vanneste, A.P. De Leenheer, J. Pharm. Sci. 80 (1) (1991) 11–16.
[7] M.W. Yu, J.S. Finlayson, Vox. Sang. 47 (1984) 28–40.
[8] J. Brandt, L.-O. Anderson, Int. J. Peptide Protein Res. 8 (1976) 33–37.
[9] J.D. Harvey, R. Geddes, P.R. Wills, Biopolymers 18 (1979) 2249–2260.
[10] D.F. Nicoli, D.C. McKenzie, J.S. Wu, Am. Lab. 11 (1991).
[11] T. Peters, Adv. Clin. Chem. 13 (1970) 37–111.
[12] R. Wetzel, M. Becker, J. Behlke, H. Billwitz, S. Bøhm, B. Ebert, H. Hamann, J. Krumbiegel, G. Lassmann, Eur. J. Biochem. 104 (1980) 469–478.
[13] P.D. Boyer, F.G. Lum, G.A. Ballou, J.M. Luck, R.G. Rice, J. Biol.Chem. 162 (1946) 181–198.