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Simultaneous determination of albumin and immunoglobulin G with fluorescence spectroscopy and multivariate calibration

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Abstract

A method is proposed for the simultaneous determination of albumin and immunoglobulin G (IgG1) with fluorescence spectroscopy and multivariate calibration with partial least squares regression (PLS). The influence of some instrumental parameters were investigated with two experimental designs comprising 19 and 11 experiments, respectively. The investigated parameters were excitation and emission slit, detection voltage and scan rate. When a suitable instrumental setting had been found, a minor calibration and test set were analysed and evaluated. Thereafter, a larger calibration of albumin and IgG1 was made out of 26 samples (0–42 $\mu g\,ml^{-1}$ albumin and 0–12.7 $\mu g\,ml^{-1}$ IgG1). This calibration was validated with a test set consisting of 14 samples in the same concentration range. The precision of the method was estimated by analysing two test set samples for six times each. The scan modes tested were emission scan and synchronous scan $\Delta 60$ nm. The results showed that the method could be used for determination of albumin and IgG1 (albumin, root mean square error of prediction (RMSEP) <2, relative standard error of prediction (RSEP) <6% and IgG1, RMSEP <1, RSEP <8%) in spite of the overlapping fluorescence of the two compounds. The estimated precision was relative standard deviation (R.S.D.) <1.7%. The method was finally applied for the analysis of some sample fractions from an albumin standard used in affinity chromatography.

Keywords: Determination; Albumin; Immunoglobulin G; Fluorescence spectroscopy; Multivariate calibration

1. Introduction

Human serum consists of a number of different proteins, of which albumin and immunoglobulin G (IgG) are found in the highest concentration. The content of albumin in human serum is in the range of 35–60 $mg\,ml^{-1}$ and that of is IgG 8–18 $mg\,ml^{-1}$, and together the two proteins account for about 70% of the total protein content in human serum. Albumin plays an important role in the transportation of, for example, hormones and drugs. Its concentration determines the oncotic pressure of plasma [1] and is also involved in nutrition, etc. The immunoglobulins are antibodies found in human serum and are separated into five major classes on the basis of their physical, chemical and immunological properties: IgG, IgA, IgM, IgD and IgE. Of these five, IgG is the most common circulating antibody.

Various methods of determining albumin have previously been reported in the literature. Examples of methods are, the reaction of albumin with different reagents like bromcresol purple [2], 5,10,15,20-tetrakis(4-sulfophenyl)porphine followed by spectrometric [3] or resonance light-scattering analysis [4], and potassium permanganate followed by chemiluminometric determination [5]. Albumin can also be determined by size-exclusion chromatography [6] and near infrared spectroscopy and partial least squares regression (PLS) [7]. Albumin and IgG have been determined by reaction with tetra-substituted sulphonated aluminium phthalocyanine followed by fluorometric detection [8] as well as by simultaneous determination of albumin and IgG with affinity chromatography [9].

The fluorescence of peptides is caused by the presence of the amino acids tyrosine, tryptophan and phenylalanine [10,11]. Phenylalanine fluorescence is, however only observed in the absence of both tyrosine and tryptophan since its quantum yield is small. Hence the contribution of phenylalanine to the fluorescence of peptides is in most experimental situations negligible. Human serum albumin consists

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of 585 amino acids in one single chain, and in this polypeptide chain there are one tryptophan and 18 tyrosine residues. IgG1 contains 450 amino acids and the molecule consists of two identical halves, each one made up of a heavy and a light chain. In the IgG1 molecule there are 11 tryptophan and 28 tyrosine residues.

Tyrosine is a weaker emitter than tryptophan, but it may still contribute significantly to protein fluorescence because it is usually present in larger numbers. However, the fluorescence from tyrosine can be easily quenched by nearby tryptophan residues because of energy transfer effects. Hence, in proteins where both tyrosine and tryptophan are present, only tryptophan fluorescence is generally detected [11].

The environment of each tryptophan residue in a protein such as IgG1 is generally different and there is an overlap of the emissions of all residues. Hence, one cannot easily separate the spectral contribution of each tryptophan in a multi-tryptophan protein [11]. It is therefore difficult to draw direct conclusions from the number of tryptophans in a protein about the actual fluorescence obtained experimentally. The proportion of tryptophan residues in albumin is 1:585 and in IgG1 11:450, although the different fluorescence intensity seen between albumin and IgG1 does not directly follow these proportions.

The high sensitivity and the selectivity of fluorescence spectroscopy could make it an attractive method for the determination of albumin and IgG1. Albumin has previously been determined with fluorescence spectroscopy and univariate calibration with ordinary linear regression with the help of selective reagents [5,8]. But with multivariate techniques like partial least squares regression (PLS) it should be possible to determine albumin and IgG1 simultaneously, without the need for reagents. The aim of the following study was to investigate whether it is possible to determine albumin and IgG1 by means of fluorescence spectroscopy and multivariate calibration, something which to our knowledge has not previously been reported in the literature.

2. Theory

For very dilute solutions the relationship between the fluorescence intensity and concentration is linear. But at high concentrations the fluorescence intensity decreases because of self-quenching and the inner filter effect. Self-quenching is caused by the collision of excited molecules creating radiation less transfer of energy. The inner filter can be explained by the fact that the solution at the front of the cell is exposed to a higher light intensity than the sample near the opposite side, because of the absorbance of the intervening solution [12]. Furthermore, at high concentrations light-scattering is also more pronounced. For dilute solutions with $\varepsilon bc < 0.05$ AU, however, the linearity of fluorescence holds over a wide range of concentrations.

A scanning fluorescence spectrophotometer can measure in three different scan modes: excitation, emission and synchronous scan. In excitation scan the emission wavelength is fixed while the excitation wavelength is changed, hence recording an excitation spectrum. Emission scan measures the emission at different wavelengths with the excitation wavelength fixed, which result in an emission spectrum. The emission always occurs at longer wavelengths than the excitation, a fact caused by small energy losses in the brief period before emission. By changing both the excitation and the emission wavelength in a stepwise manner, a synchronous scan can be made. The interval between excitation and emission wavelengths is designated by the symbol delta (Δ). Measuring synchronously with excitation at 250 nm and with $\Delta\lambda = 60$ nm will then give the emission at 310 nm as the first point in the synchronous spectrum.

Multivariate calibration is the process of constructing a mathematical model that relates a property, such as content to the absorbance or fluorescence intensity of a set of known reference samples at more than one wavelength. From this calibration set of samples a calibration model is determined using methods like PLS or principal component regression (PCR). In this study PLS was used and the theories behind it have been described elsewhere [13,14].

If ordinary PLS regression is used for the analysis of fluorescence spectra, three different types of scanning modes can be used: excitation spectrum, emission spectrum or synchronous spectrum. If a whole emission spectrum is collected at each excitation wavelength, an excitation-emission matrix (EEM) can be constructed. The EEM can be used to decide which scan modes should be applied for the multivariate calibration.

For the evaluation of the predictive ability of a multivariate calibration model, the root mean square error of prediction (RMSEP) [15] and relative standard error of prediction (RSEP) [16] can be used

$$\mathrm{RMSEP} = \sqrt{\frac{\sum_{i=1}^{n}(y_{\mathrm{pred}} - y_{\mathrm{obs}})^2}{n}} \quad (1)$$

$$\mathrm{RSEP}\ (\%) = 100 \times \sqrt{\frac{\sum_{i=1}^{n}(y_{\mathrm{pred}} - y_{\mathrm{obs}})^2}{\sum (y_{\mathrm{obs}})^2}} \quad (2)$$

where y_{pred} is the predicted concentration in the sample, y_{obs} is the observed or reference value of the concentration in the sample and n is the number of samples in the test set.

3. Experimental

3.1. Instrumentation

The fluorescence spectrophotometer used was a Varian Cary Eclipse with the software Cary Eclipse, with the scan application. Five matched Quartz Suprasil 10 nm quartz cells for fluorescence spectroscopy were used. The quartz cells were cleaned by rinsing with Milli-Q water and acetone and

than drying with air. The UV-Vis spectrophotometer used was a Varian Cary 100 scanning spectrophotometer.

For the creation of the experimental designs and their evaluation by means of multiple linear regression (MLR), Umetrics software MODDE 5.0 was used. For the multivariate calibration with PLS, Umetrics software Simca-P 8.1 was used.

3.2. Reagents

Human serum albumin (Sigma), and IgG1, human (Sigma), were used. Phosphate-buffered saline (PBS) 50 mmol l^{-1} (pH 6.7 ± 0.3) consisted of 3.45 g $NaH_2PO_4 \cdot H_2O$, 4.35 g $Na_2HPO_4 \cdot 2H_2O$, 5.84 g NaCl and water. The buffer was filtered through a 0.45 μm filter before use. Albumin and IgG1 were weighed and dissolved in PBS, after which the solutions were further diluted with the same solvent with a digital dilutor, Hamilton Microlab 1000, to the concentration range used. The water used in the study was provided by a Millipore Milli-Q filtration/purification system.

In order to investigate which concentration range and scan settings to be used in the study, some UV spectra were recorded on test solutions. The appropriate maximum concentrations of albumin and IgG1 were decided from the UV spectrum having an λ_{max} of 0.05 AU. This corresponded to albumin and IgG1 concentrations of 50 and 20 μg ml^{-1}, respectively. The wavelength of maximum UV absorbance was around 278 nm for both albumin and IgG1.

3.3. Experimental design

The influence of some of the instrumental parameters was investigated with the help of a full factorial design comprising 19 experiments. The parameters investigated were excitation slit (1.5–20 nm), emission slit (1.5–20 nm), detection voltage (400–800 V) and scan rate (30–9600 nm min^{-1}). The experimental design is shown in Table 1. In each experiment a sample containing 50 μg ml^{-1} human serum albumin in Milli-Q water was analysed with excitation at 280 nm recording the emission 290–550 nm. This excitation wavelength was chosen since the maximum UV absorbance was around 278 nm. The fluorescence intensity at 322 nm was used in the experimental design since the peak of albumin fluorescence is at 300–350 nm (Fig. 1a).

When the results of the first experimental design had been evaluated, a second design was made consisting of the important factors found, excitation and emission slits as well as detector voltage. This second experimental design was a full factorial design with 11 experiments and each factor was at two levels (Table 2). The interval of the slit width was narrowed to 2.5–10 nm while the detection voltage was in the same range as in the first experimental design, 400–800 V. The scan rate was set to 600 nm min^{-1}.

Both experimental designs were evaluated with MLR since the designs used were orthogonal (condition number 1.14 in the first design and 1.17 in the second design), only one single response was used and no missing values were present in the worksheet.

Table 1
Experimental design used to investigate the effect of instrumental parameters on fluorescence intensity

Sample number	Excitation slit (nm)	Emission slit (nm)	Scan rate (nm min^{-1})	Detection voltage (V)
1	1.5	1.5	30	400
2	20	1.5	30	400
3	1.5	20	30	400
4	20	20	30	400
5	1.5	1.5	9600	400
6	20	1.5	9600	400
7	1.5	20	9600	400
8	20	20	9600	400
9	1.5	1.5	30	800
10	20	1.5	30	800
11	1.5	20	30	800
12	20	20	30	800
13	1.5	1.5	9600	800
14	20	1.5	9600	800
15	1.5	20	9600	800
16	20	20	9600	800
17	10	10	1200	600
18	10	10	1200	600
19	10	10	1200	600

3.4. Scan modes

When using fluorescence spectroscopy, it is essential to decide an appropriate scan mode where the compound of interest fluoresces. In order to investigate this, the EEMs of albumin and IgG1 were analysed at similar concentrations and the result is shown in Fig. 1. In Fig. 1a the EEM matrix of albumin is shown. The sample contained 20 μg ml^{-1} albumin in PBS and the analysis was made with excitation 250–400 nm and emission 300–450 nm. As can be seen, the fluorescence of albumin is in the lower left part of Fig. 1a at excitation about 250–300 nm and emission 300–440 nm. Rayleigh and Raman scattering causes the diagonal band shown. The horizontal dotted line shows the emission scan

Table 2
Second experimental design used to investigate the effect of the most important instrumental parameters on fluorescence intensity

Sample number	Excitation slit (nm)	Emission slit (nm)	Detection voltage (V)
1	2.5	2.5	400
2	10	2.5	400
3	2.5	10	400
4	10	10	400
5	2.5	2.5	800
6	10	2.5	800
7	2.5	10	800
8	10	10	800
9	5	5	600
10	5	5	600
11	5	5	600

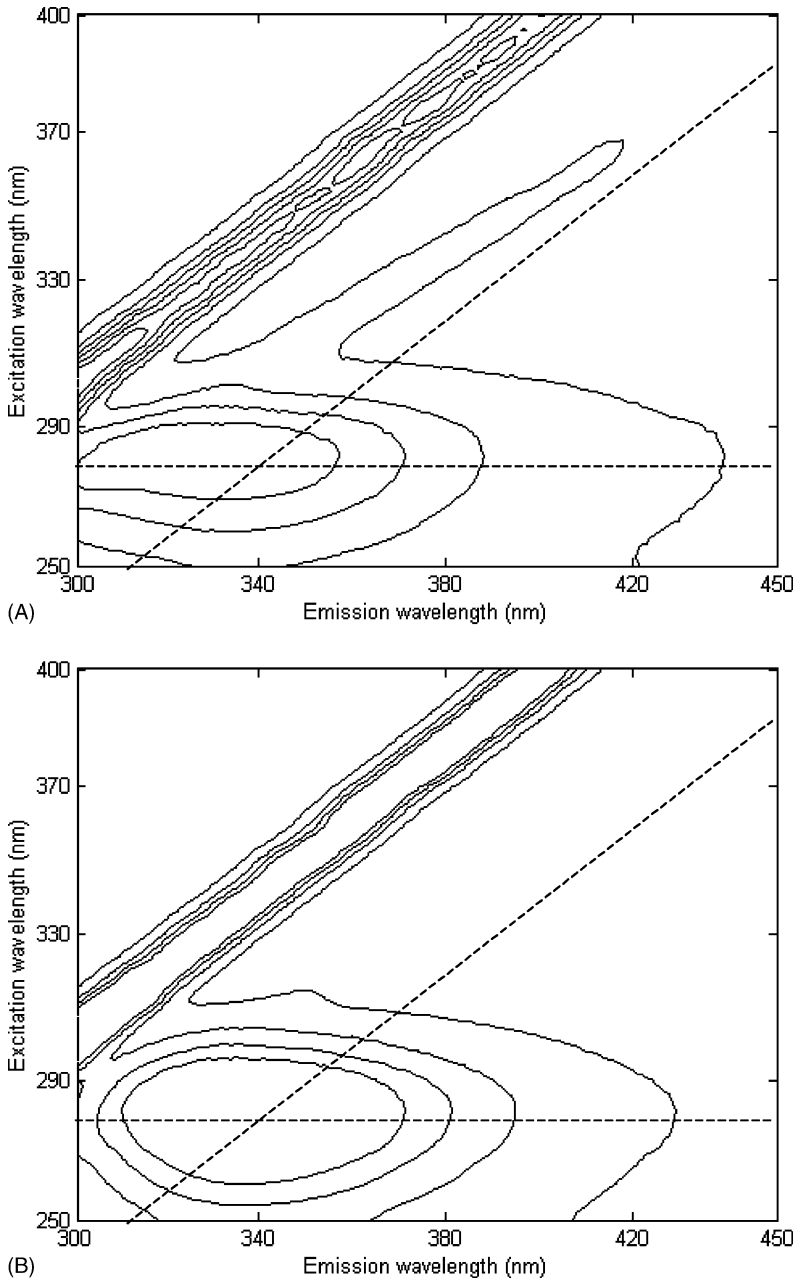

Fig. 1. EEM, excitation 250–400 nm and emission 300–450 nm, (a) albumin (20 μg ml^{-1}), (b) IgG1 (20 μg ml^{-1}).

at 280 nm and the diagonal line shows the synchronous scan with Δ60 nm used in the study.

The EEM matrix for a sample containing 20 μg ml^{-1} IgG1 is shown in Fig. 1b. For IgG1 the fluorescence is also seen in the lower left part of Fig. 1b at excitation about 250–300 nm and emission 300–440 nm. The Rayleigh scatter is clearly shown as the broad diagonal band, while the Raman scatter is less obvious due to somewhat different scaling than in Fig. 1b. This is caused by the higher quantum yield of IgG1 compared to albumin. As in Fig. 1a, the horizontal and diagonal dotted lines in Fig. 1b shows the used emission scan and synchronous scan.

3.5. Calibration model

When the appropriate concentration range, scan mode and instrumental settings had been found, a screening multivariate calibration was performed. This consisted of a smaller calibration and test set in order to determine a linear relationship between albumin, IgG1 concentration and fluorescence intensity. The concentration range of albumin was 0–35 μg ml^{-1}, that of IgG1 0–10 μg ml^{-1} and the calibration set contained eight samples and the test set three samples. Four scan modes were used for evaluation: excitation spectrum (250–310 nm) recording the emission at 335 nm,

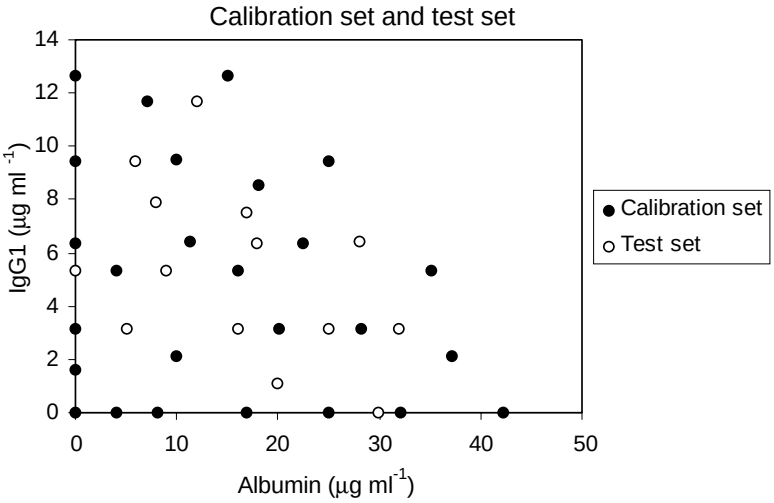

Fig. 2. Design of calibration and test set for binary mixtures of albumin and IgG1. Concentration range of albumin: 0–37.1 μg ml^{-1} and IgG1: 0–12.7 μg ml^{-1}.

emission spectrum (300–500 nm) with excitation at 280 nm and synchronous scan Δλ 45 and 60 nm.

When the screening multivariate calibration had been evaluated, a larger calibration and test set was constructed with 26 calibration samples and 14 test set samples in the concentration range of albumin 0–37.1 and IgG1 0–12.7 μg ml^{-1}. The design of the calibration set and test set are presented in Fig. 2. This design was created manually in order to cover the concentration domain well and avoid overlap of calibration and test set samples. The lack of samples in the upper right quadrant of Fig. 2 is caused by the fact that the fluorescence intensity in this region becomes over-ranged (fluorescence intensity >1000). All the samples were analysed in randomised fashion in order to minimise the experimental error. The precision of the instrument was estimated by analysing two calibration samples for six times each. One of these samples contained only buffer solution and the other one contained 22 and 6.33 μg ml^{-1} albumin and IgG1, respectively. Two scan modes were used for the analysis of the larger calibration and test set samples: synchronous scan Δ60 nm and emission scan recording the emission spectrum (300–500 nm) with excitation at 280 nm. The fluorescence spectrum of albumin and IgG1 for these two scan modes are shown in Fig. 3. All spectra were mean-centered before the PLS models were made.

4. Results and discussion

The experimental designs were evaluated with MLR and before evaluation the variables were scaled to unit variance and the response factor was logarithmic, since it varied in a large interval from 0.05 to 1000. Initially the experimental designs were evaluated with interaction models but since no interaction terms were significant linear models were used instead. The evaluation of the first experimental design is presented in Table 3, where the value of each coefficient (scaled and centered), the standard error of the coefficient as well as the probability and 95% confidence interval are given. The standard error is a measure of the experimental error as it affects each coefficient and it hence shows the uncertainty of the coefficients. The *P*-value shows the probability of obtaining the obtained coefficient value if its true value was zero. Hence a small *P*-value indicates a significant coefficient. The model created had a goodness of fit (R^2) of 0.68 and a goodness of prediction (Q^2) of 0.50 [17]. The analysis of variance (ANOVA) of the experimental matrix showed that the model obtained was significant, as was also indicated by the goodness of fit measures. As can be seen in Table 3, the scan rate is not significant in the experimental domain investigated, as shown by the high *P*-value and the fact that the confidence interval is larger than the coefficient value. The significant factors are the excitation

Table 3
Statistical analysis of the first experimental design

Terms included in the model	Constant	Excitation slit (nm)	Emission slit (nm)	Scan rate (nm min$^{-1)}$	Detection voltage (V)
P-value	0.0027	0.0011	0.0044	0.506	0.078
Coefficient	0.872	0.756	0.880	−0.169	0.806
Standard error	0.241	0.260	0.260	0.249	0.260
Confidence interval	±0.516	±0.557	±0.557	±0.534	±0.557

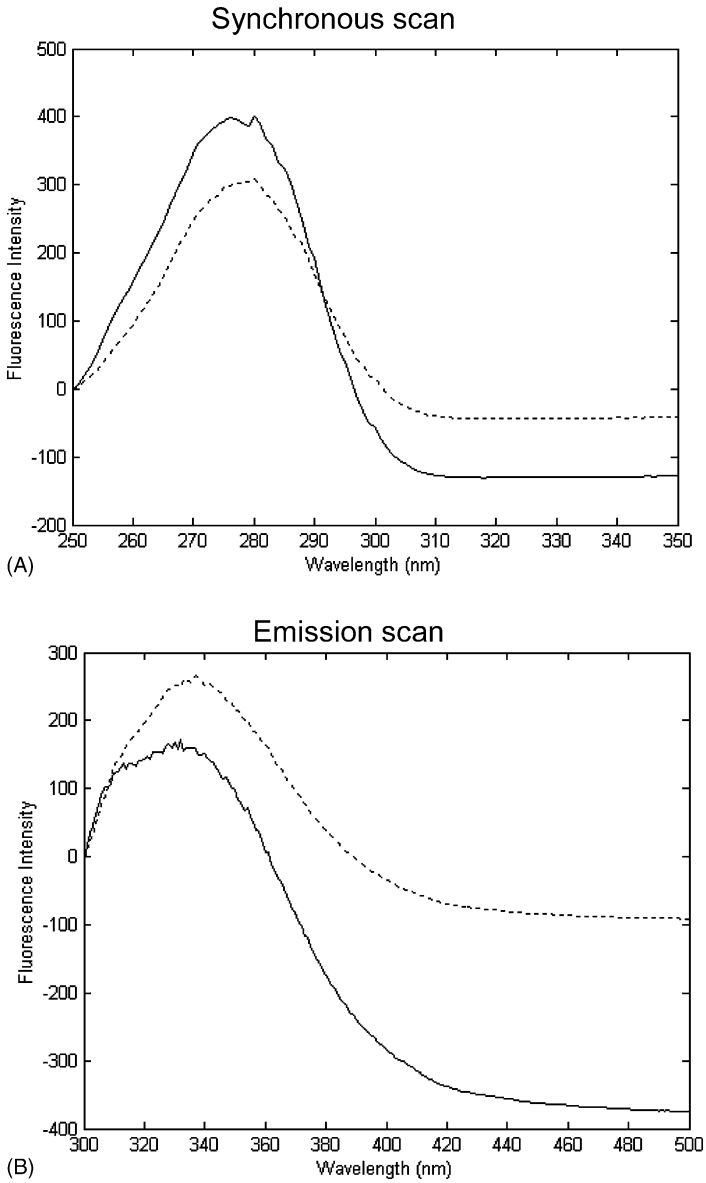

Fig. 3. Fluorescence spectra of albumin (32 μg ml^{-1}) and IgG1 (6.3 μg ml^{-1}). The albumin spectrum is shown with solid line and the IgG1 spectrum with dotted line. (a) Synchronous scan Δ60 nm, (b) Emission scan 300–500 nm with excitation at 280 nm.

and emission slits and the detection voltage, all of which should be set to a high level in order to get a high intensity of the fluorescence. The slit widths could not be increased very much, however, since a slit width of 20 nm caused the detector signal to be over-ranged, resulting in an arbitrary fluorescence intensity above 1000.

In the second experimental design the same response was used logarithmically since the fluorescence intensity in the experiments carried out varied from 0.038 to 1000. The statistical analysis is presented in Table 4 ($R^2 = 0.96$, $Q^2 = 0.92$). The ANOVA of this experimental matrix showed that the model was significant and no lack of fit was present. The values of the coefficients are presented in scaled and centered form. As can be seen, the detector voltage is the most important factor. All the three factors should be set to their higher level to get an intensity of fluorescence as high as possible. Hence the factor setting chosen for the rest of the study was excitation and emission slits 10 nm, detector voltage 800 V and scan rate 600 nm min^{-1}.

The result of the screening PLS calibration showed a linear relationship between the concentrations of albumin,

Table 4
Statistical analysis of the second experimental design

Terms included in the model	Constant	Excitation slit (nm)	Emission slit (nm)	Detection voltage (V)
P-value	1.4E−5	0.0023	0.0013	8.22E−6
Coefficient	0.992	0.508	0.565	1.259
Standard error	0.093	0.109	0.109	0.109
Confidence interval	±0.219	±0.258	±0.258	±0.258

Table 5
Predictions obtained for albumin and IgG1 for the samples in the test set and synchronous scan Δ60 nm.

Calibration sample number	Accepted reference value albumin (μg ml^{-1})	Predicted concentration albumin (μg ml^{-1})	Accepted reference value IgG1 (μg ml^{-1})	Predicted concentration IgG1 (μg ml^{-1})
1	0.00	1.40	5.35	5.03
2	5.00	5.98	3.17	3.23
3	6.00	5.58	9.42	9.67
4	7.99	8.90	7.92	8.22
5	8.99	11.01	5.35	5.34
6	11.99	11.82	11.72	12.27
7	15.99	16.79	3.17	3.67
8	16.82	17.37	7.49	7.99
9	17.99	19.07	6.33	6.50
10	19.98	19.94	1.07	1.38
11	24.98	27.02	3.17	2.77
12	28.03	28.07	6.42	6.74
13	29.98	28.59	0.00	0.23
14	31.97	32.10	3.17	3.39

IgG1 and the fluorescence intensity, as expected. The prediction results obtained with the scan modes tested were RMSEP <3 and RSEP <12% for albumin and RMSEP <0.6 and RSEP <10% for IgG1. Only minor differences in the predictive ability of the different scan modes was obtained. Furthermore the presence of Rayleigh and Raman scatter in the spectra did not seem to affect the predictive ability.

From the larger calibration set a PLS model of two PLS components (R^2 >0.99 and Q^2 >0.97) was obtained. This calibration model was validated with the test set containing 14 binary mixtures of albumin and IgG1 (Fig. 2). The predicted results for synchronous scan Δ60 nm are shown in Table 5. As can be seen, the determination of albumin and IgG1 can be carried out with reasonable accuracy. The squared product-moment correlation coefficient (r^2) was >0.99 between accepted and predicted albumin and IgG1 levels in Table 5. In Table 6 the predictive ability of the larger calibration model with the two scan modes is summarised. The predicted results for the determination of albumin and IgG1 are presented as RMSEP and RSEP calculated from the predictions of the 14 test set samples. As can be seen, the two scan modes gave results of the same magnitude, although the synchronous scan gave a slightly better result. The precision of the method was investigated by the analysis of two of the samples in the test set six times each. The result showed that the relative standard deviation (R.S.D.) obtained was <1.7% for both albumin and IgG1.

Table 6
Overview of prediction results for emission scan (excitation wavelength 280 nm) and synchronous scan Δ60 nm

Scan mode	Prediction results of albumin (%)		Prediction results of IgG1 (%)	
	RMSEP	RSEP	RMSEP	RSEP
Emission	1.1	5.8	0.5	7.5
Synchronous Δ60 nm	1.1	5.7	0.3	5.4

4.1. Analysis of an albumin standard used in affinity chromatography

In many types of biotechnical applications it is a matter of concern to remove albumin and IgG1 from serum samples in order to be able to analyse proteins in much lower concentrations in these samples. This can be done with affinity chromatography, where albumin or IgG1 can be selectively bound and separated. In order to test the method proposed, determinations took place of albumin in some sample fractions from affinity chromatography. An anti-HSA column was used that selectively bound the albumin, making it possible to elute it later by washing with hydrochloric acid. As for sample an albumin standard of 400 μg was injected and during the chromatographic analysis fractions were collected from the non-bound, i.e. excess, albumin not captured by the column. Thereafter the column was washed with acid and the eluting bound fractions were also collected. The albumin concentrations in two sample fractions containing the majority of the albumin in non-bound and bound phases were then determined with the fluorescence method. In the non-bound phase these were fractions numbers 1 and 2 and in the bound phase fractions numbers 2 and 3. The results of the determination of albumin with the fluorescence method for these four fractions are shown in Table 7. As can be seen, the albumin content in the two first non-bound fractions was predicted to be 124 μg and in the bound phase 278 μg. This means a total of 402 μg albumin, which corresponds to the

Table 7
Results of the determination of albumin in some sample fractions from an albumin standard sample analysed with affinity chromatography

Fraction	Predicted conc. albumin (μg ml^{-1})	Sum of predicted amount albumin
Non-bound no. 1	113	
Non-bound no. 2	11	124
Bound no. 2	204	
Bound no. 3	74	278

Results are presented for fractions 1, 2 non-bound and 2, 3 bound.

total amount injected of 400 μg. It may therefore be concluded that the affinity column used was not able to bind all albumin injected since the non-bound fractions actually contained albumin. The fluorescence method could hence be used to verify the albumin binding capacity of the affinity chromatographic analysis.

5. Conclusion

As reported by Li et al. [8], the determination of albumin and globulin in serum traditionally requires two steps with at least two reagents. The present study, however, shows that albumin and IgG can be determined simultaneously with fluorescence spectroscopy and multivariate calibration despite the similarity of the fluorescence spectra of the two compounds. The method proposed is fast and requires a minimum of sample pre-treatment. Some further development of the method proposed might make it possible to determine albumin and IgG directly in serum samples. It is also possible that the calibration could be extended with other serum proteins, enabling the determination of several proteins simultaneously.

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