Analytica Chimica Acta, 290 (1994) 159–165
Elsevier Science B.V., Amsterdam

Second derivative synchronous scanning fluorescence spectrometry as a sensitive detection technique in immunoassays. Application to the determination of α-fetoprotein

Evriklia S. Lianidou *, Pinelopi C. Ioannou and Eftichia Sacharidou

University of Athens, Laboratory of Analytical Chemistry, Panepistimiopolis, Zografou, 15771 Athens, Greece

(Received 25th May 1993)

Abstract

Second derivative synchronous (scanning) fluorescence spectrometry (SDSFS) has been used for the first time as an alternative to time-resolved fluorescence for the detection of Tb^{3+} chelates. This approach minimizes the background signal by taking advantage of the large Stokes shift properties of Tb^{3+} chelates and offers high sensitivity by narrowing the spectral bands. The analytical performance of this detection technique has been evaluated by choosing the well-defined enzyme-amplified lanthanide luminescence (EALL) immunoassay of α-fetoprotein (AFP) as a model. Monoclonal "capture" antibodies and monoclonal biotin-labelled antibodies in a "sandwich-type" assay configuration in a microwell format have been used. Alkaline phosphatase (ALP) conjugated to an antibiotin antibody was used as an enzyme label. ALP cleaves phosphate from salicylphosphate to produce salicylic acid which forms a highly fluorescent ternary complex with Tb^{3+} and EDTA, which is monitored by SDSFS. The method allows the measurement of AFP with a limit of detection of 2 pg ml^{-1}, coefficients of variation in the range 4.5–9.9% and mean recovery from four pooled serum samples at two concentration levels equal to $94 \pm 11\%$.

Keywords: Second-derivative synchronous fluorescence spectrometry; Immunoassay; Terbium chelates; α-Fetoprotein; Enzyme amplified lanthanide luminescence immunoassay

1. INTRODUCTION

The development of sensitive immunoassays based on novel non-isotopic labelling systems is of considerable interest [1]. Among the most sensitive non-isotopic immunoassay methodologies available today, time-resolved fluorescence is well established [2,3] as an ultrasensitive detection technique [4] and is widely and successfully applied in immunoassays [5] as well as in DNA probe assays [6]. As is well known, time-resolved fluorescence takes advantage of the unique long fluorescence lifetime properties of rare earth metal chelates (Eu^{3+}, Tb^{3+}, Sm^{3+}), to decrease the background signal and thus to achieve an important gain in sensitivity.

In this work an attempt will be made to evaluate the applicability of another fluorescence detection technique, second derivative synchronous (scanning) fluorescence spectrometry (SDSFS), as an alternative to time-resolved fluorimetry for the detection of lanthanide chelates.

Synchronous (scanning) fluorescence spectrometry (SFS), a technique first suggested by Lloyd [7], involves the simultaneous scan of the excitation and emission monochromators while keeping a constant wavelength interval ($\Delta\lambda$) between them [8]. The synchronous fluorescence spectrum obtained in that way is mainly characterised by narrower spectral bands than by con-

* Corresponding author.

SSDI 0003-2670(93)E0643-L

ventional luminescence spectrofluorimetry and by simplification of emission spectra (by appropriate choice of $\Delta\lambda$) [9]. The band narrowing effect obtained by the synchronous scanning approach if combined with the fluorescence derivative technique [10], can be valuable in increasing sensitivity as has already been reported [11,12].

In this article for the first time the use of SFS and SDSFS for the detection of Tb^{+3} chelates is reported, which have already been used as non-isotopic labels in time-resolved fluorescence immunoassays [13,14]. This approach minimizes the background signal by taking advantage of the large Stokes shift properties of Tb^{3+} chelates and offers high sensitivity by narrowing the spectral bands. The analytical performance of SDSFS as a sensitive detection technique in immunoassays has been evaluated by choosing the well-defined enzyme-amplified lanthanide fluorescence immunoassay [13] for α-fetoprotein (AFP) [14] as a model. SFS and SDSFS are also described.

2. EXPERIMENTAL

2.1. Apparatus

A Model 512-A double beam fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT) equipped with a 150-W xenon arc lamp and a magnetic stirrer under the cell holder was used for measuring Tb^{3+} fluorescence from 96 microtiter wells. The instrument was interfaced to an Amstrad CPC-6128 microcomputer [15,16]. Home made software [15] was used for spectral acquisition, calculations of the spectrum derivatives and automatic evaluation and presentation of the analytical signals. Smoothed and derivative spectra were defined using the Savitzky-Golay method [17].

2.2. Reagents

The monoclonal antibodies used for the AFP immunoassay (for coating and detection), as well as alkaline phosphatase labelled streptavidin (SA-ALP) and human AFP (Scripps Labs., San Diego, CA) were kindly donated by Dr. E.P. Diamandis, Clin. Biochem. Dept., University of Toronto. The antibiotin-alkaline phosphatase conjugate was obtained from the Abbott IMx/HBsAg assay (bottle 2). 96-well microtiter plates were obtained from Nunc, Denmark. The phosphate ester of salicylic acid (SAP) was synthesised and purified after continuous recrystallisations according to the literature [18]. Terbium oxide was obtained from Merck. All other chemicals used were from Sigma, St. Louis, MO, unless otherwise stated.

2.3. Solutions

All solutions were prepared in deionised, doubly distilled water (DDW).

Carbonate buffer, 50 mmol l^{-1}, pH 9.60, was used for coating. A 6% (w/v) solution of bovine serum albumin (BSA) in a 50 mmol l^{-1} Tris buffer, pH 7.40, containing 0.5 g l^{-1} of sodium azide was used for blocking, and dilutions of AFP standards, serum samples and biotinylated antibodies.

AFP standards in the range of 0–500 ng ml^{-1} were prepared from human AFP (Scripps) (calibrated with the Sorin AFP/RIA assay) by appropriate dilutions in 6% BSA.

The working solution of monoclonal biotinylated detection antibody for AFP was prepared by 1600 fold dilution of the stock solution (0.66 ng ml^{-1}) in 6% BSA.

The wash solution was prepared by dissolving 87.7 g of NaCl, 5.0 ml of polyoxyethylenesorbitan monolaureate (Tween 20) and 9.75 g of sodium azide in 1000 ml of 0.100 mol l^{-1} Tris buffer, pH 8.00. This solution was diluted 10 fold in DDW before use.

The working solution of the SA-ALP conjugate was prepared by 20000 fold dilution of the stock solution (0.75 mg ml^{-1}) in a 50 mmol l^{-1} Tris buffer, pH 7.40, containing 3% BSA. The working solution of the antibiotin-ALP conjugate was accordingly prepared by 2 fold dilution of the stock solution (0.03 μg ml^{-1}) in the same buffer.

Salicylic acid phosphate ester (SAP) stock solution, 3×10^{-2} mol l^{-1}, was prepared by dissolving 25.9 mg of SAP in 4.00 ml of a 0.1 mol l^{-1} NaOH solution and was kept at 4°C. Working solutions of SAP were prepared just before use by 10 fold dilution of the stock solution in ALPs substrate buffer.

The "developing solution" contained Tb^{3+}–

EDTA complex in a final concentration of 1×10^{-3} mol l^{-1} and 0.5 mol l^{-1} Tris buffer, pH 12.60, and is prepared according to the literature [14].

2.4. Procedure

AFP monoclonal antibodies were coated on 96-well microplates by applying 100 μl/well of antibody coating solution (5 mg l^{-1}) and overnight incubation at room temperature without shaking. After coating, the wells were washed once with wash solution and blocked for 1 h, with 200 μl/well of BSA 6%. After blocking the wells were washed once and were ready for the assay procedure.

Assay procedure

AFP standards at concentrations of 0, 0.5, 5.0, 10, 40, 100, 250, and 500 ng ml^{-1} as well as serum samples were used after 10 fold dilution in 6% BSA. 50 μl of diluted AFP standards or serum samples were pipetted (in triplicate) into the monoclonal antibody coated wells, and 50 μl of working solution of biotinylated monoclonal antibody was added. The plates were incubated at 37°C with mechanical shaking for 90 min and then washed three times. Working solutions of SA-ALP or antibiotin-ALP, 100 μl/well, were added, the plates were incubated another 30 min as above and then washed four times. Working SAP substrate solution, 3×10^{-3} mol l^{-1}, 100 μl/well, was then added, the plates were further incubated for 30 min as above and then Tb-EDTA developing solution, 100 μl/well, was added.

Fluorescence measurement

180 μl of the SA–Tb–EDTA complex fluorescing solution, developed finally in each well, and 700 μl of developing solution were brought into a 1.00-ml cuvette and mixed thoroughly. The synchronous fluorescence spectra were obtained by scanning both monochromators simultaneously at a constant wavelength difference $\Delta\lambda = 226$ nm ($\lambda_{exc} = 280$–380 nm). Hereafter all wavelengths referring to synchronous spectra are taken to be equal to those of the corresponding excitation wavelengths. The SFS signal at $\lambda_{exc} = 320$ nm and the second derivative signal within the spectral range of 316–344 nm were recorded.

2.5. Calculations of the results

Data expressed as SFS or SDSFS analytical signals and the corresponding concentrations of AFP standards were entered in a PC for processing. All data were transformed to log values and a statistical program (Statgraphics) was used to create a calibration curve based on a cubic spline algorithm. Unknown concentrations of serum samples were automatically calculated by a special program from the constructed calibration curves.

3. RESULTS AND DISCUSSION

The analytical performance of SDSFS as a sensitive detection technique in immunoassays has been evaluated by choosing the well defined enzyme amplified lanthanide luminescence (EALL) immunoassay for AFP [13,14] as a model. For that purpose only slight modifications in the proposed procedure [14] were made, so that the determined detection limits by SDSFS could be compared with the ones reported for time resolved fluorimetry.

3.1. Selection of $\Delta\lambda$

In SDSFS the choice of the appropriate scanning interval ($\Delta\lambda$) is mainly dictated by requirements of resolution and sensitivity. For selection of the appropriate $\Delta\lambda$ value various synchronous spectra at different $\Delta\lambda$ have been recorded. The best results have been obtained for $\Delta\lambda = 226$ nm (Stokes shift). This can be easily explained by taking under consideration that terbium chelates are characterised by a strong excitation spectral band, characteristic of the chromophore (320 nm for SA in that case), and by a major emission spectral band of Tb, typically at about 546 nm. A $\Delta\lambda$ of 226 nm was chosen for this study, so as to obtain the maximum signal.

3.2. Other instrumental parameters

Selection of the other instrumental parameters is not critical because the differentiation is attained numerically. A scan speed of 4 nm s^{-1} and a "fast" response time were selected. The sam-

pling rate was defined to be 1 point every 4 nm. For the calculation of the second derivatives of the synchronous spectra by the Savitzky-Golay method a filter size of 11 points was selected.

3.3. Comparison of spectra

The synchronous fluorescence spectrum (SFS) of the SA–Tb–EDTA complex and its corresponding conventional fluorescence spectrum (CFS) ($\lambda_{exc} = 320$ nm) are shown in Fig. 1a. As can be seen, the emission spectrum of the complex shows three maxima corresponding to the emission of SA ($\lambda_{em} = 400$ nm) and to the characteristic emission of Tb^{3+} ion ($\lambda_{em} = 491$ and 546 nm). The synchronous spectrum of the complex is much simpler and consists of one very narrow band with $\lambda_{ex/em} = 320/546$ nm which corresponds to the predominant radiative transition of excited Tb^{3+}. The second derivative of the synchronous spectrum, as shown in Fig. 1b, results in a higher signal compared with the second derivative of the conventional spectrum due to the narrower spectral band of the SFS spectrum.

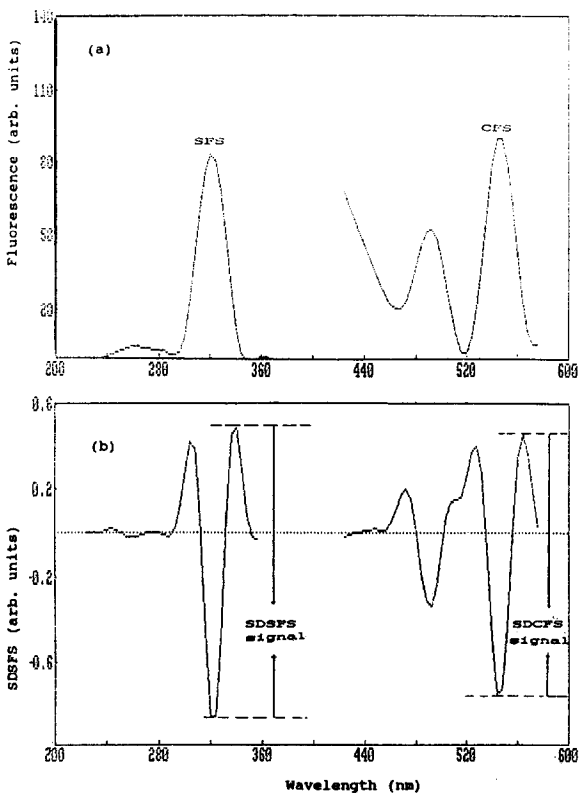

Fig. 1. (a) Synchronous (scanning) fluorescence spectrum (SFS) ($\Delta\lambda = 226$ nm) and conventional emission fluorescence spectrum (CFS) ($\lambda_{em} = 320$ nm) of the SA–Tb–EDTA complex, (b) second derivative synchronous (scanning) fluorescence spectrum (SDSFS) and second derivative conventional fluorescence spectrum (SDCFS) of the SA–Tb–EDTA complex. SA, 1×10^{-7} mol l^{-1}; Tb–EDTA, 1×10^{-3} mol l^{-1} (final concentrations in Tris buffer, 0.50 mol l^{-1}, pH 12.60).

3.4. Stability of the fluorescence of the SA–Tb–EDTA complex

The stability of the fluorescence of the SA–Tb–EDTA complex solutions with time was studied. The wells were tightly covered with a nylon membrane during this study, so that a gradual drop in pH which is due to CO_2 absorption and results in a significant decrease in fluorescence [14] would be avoided. As shown in Fig. 2, after an initial decrease of about 20% in the first 60 min, the fluorescent signal remained almost constant for at least 24 h. It was decided that the plates should be tightly covered during the measurements and that the fluorescence spectra should be taken after stabilisation of the fluorescence signal.

3.5. SAP concentration study

Kinetic parameters for SAP as an alkaline phosphatase substrate were determined. Kinetic measurements were taken at 25 ± 1°C, for 1 U ml^{-1} ALP, in ALP's substrate buffer. K_m and V_{max} values for SAP were determined to be 0.469×10^{-3} mol l^{-1} and 0.615 μmol min^{-1} U^{-1} respectively. As is well known, the substrate concentration must be significantly higher than its K_m value ($S \gg K_m$) so that the signal taken would be proportional to the catalytic activity of the enzyme to be determined [19]. For this reason, four different SAP concentrations were studied (1×10^{-3} mol l^{-1}, 3×10^{-3} mol l^{-1}, 6×10^{-3} mol l^{-1}, and 8×10^{-3} mol l^{-1}), corresponding to

2, 6, 12, and 17 K_m values, respectively. The whole procedure for the AFP-EALL immunoassay was followed and the optimum concentration for SAP was found to be 3×10^{-3} mol l^{-1} while SAP concentrations greater than this showed a significantly higher background leading to a tremendous decrease in the analytical signal. The optimum concentration found for SAP is quite different from the one proposed in the literature [13,14] for fluorosalicylic acid phosphate ester (FSAP) as ALP substrate. This can be easily explained by the different K_m values reported for FSAP under the same experimental conditions [13].

3.6. SAP hydrolysis study

The spontaneous hydrolysis of SAP was studied by incubation at room temperature of the working substrate solution for 0, 30, 60 and 90 min, followed by addition of Tb–EDTA developing solution and measurement of the fluorescence intensity. A 30 min incubation time for SAP substrate was chosen so that its spontaneous hydrolysis (26%) would not be significant.

3.7. Selection of alkaline-phosphatase conjugate

The performances of an antibiotin-ALP and a SA-ALP conjugate (proposed in the AFP-EALL immunoassay [14]) were compared. Both conjugates were used at their optimum dilutions found to be 1:20 000 (37.5 ng ml^{-1}) for SA-ALP and 1:1 (15 ng ml^{-1}) for antibiotin-ALP. The results are shown in Fig. 3. The antibiotin-ALP conjugate at a final concentration of 15 ng ml^{-1} was chosen to be used for all subsequent assays.

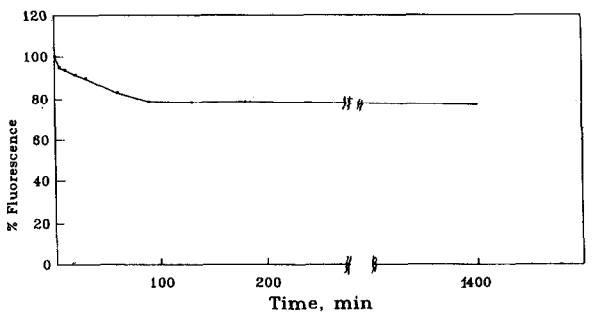

Fig. 2. Stability of the fluorescence of SA–Tb–EDTA solutions vs. time (SA concentration = 1×10^{-6} mol l^{-1}).

3.8. Recovery

AFP-supplemented serum samples were prepared by adding known concentrations of exogenous AFP to four pooled serum samples. Analytical recovery was assessed by analysing serum samples before and after the additions (Table 1). The mean ± S.D.% analytical recoveries were 92 ± 11% and 94 ± 11% for SFS and SDSFS respectively.

3.9. Precision

Samples at three concentrations of AFP were analysed ten times. The results obtained by SFS and SDSFS are shown in Table 2. It is curious that the relative standard deviations (R.S.D.) are higher for higher concentrations of AFP, because the opposite was expected. As can be seen from Table 2, the R.S.D. values for both detection techniques are almost the same. Precision is good, when taken into account that the whole procedure, as even the measuring step was completely manual.

3.10. Sensitivity

A typical calibration curve used for the determination of AFP by the one-step EALL immunoassay (modified as above) with SDSFS detection is shown in Fig. 4. The detection limit, defined as the analyte concentration that produces a signal twice the standard deviation of the background signal [4], was expressed as the final concentration of AFP in the reaction mixture after taking into account all the dilutions carried out before measurement. The detection limit was found to be of the same order for the two detection techniques, being 1.4 pg ml^{-1} for SDSFS and 1.7 pg ml^{-1} for SFS respectively. These detection limits are nearly comparable with the ones reported (1 pg ml^{-1}) for a similar AFP

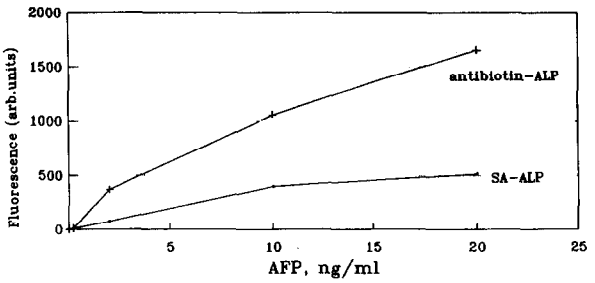

Fig. 3. AFP calibration curves with: (a) SA-ALP (38 ng ml^{-1}), (b) antibiotin-ALP conjugate (15 ng ml^{-1}). For details see Procedure

one-step EALL immunoassay with time-resolved detection [14]. These detection limits correspond to an initial concentration of AFP in serum sample of 0.14 ng ml^{-1} and 0.17 ng ml^{-1} for SDSFS and SFS respectively while for time-resolved the corresponding detection limit is only 0.02 ng ml^{-1} [14]. This difference can be explained by the different sample dilutions carried out before measurement in that case. As can be seen from Fig. 4 the dynamic range extends to 250 ng ml^{-1} (initial concentration of AFP in serum sample).

Table 1
Analytical recovery of α-fetoprotein added to serum samples

Pool	α-Fetoprotein (ng ml^{-1})				
	added	found			
		SFS	Recovery (%)	SDSFS	Recovery (%)
1	–	3.8		3.9	
	5.0	8.9	102	9.3	108
	10.0	14.8	110	15.1	112
2	–	2.2		2.1	
	5.0	6.4	84	6.4	86
	10.0	12.1	99	12.3	102
3	–	2.0		1.9	
	5.0	6.4	88	6.3	89
	10.0	11.5	95	11.7	98
4	–	1.8		1.7	
	5.0	5.8	80	5.9	84
	10.0	10.0	80	10.4	88
Mean recovery (% ± S.D.)		92 ± 11		94 ± 11	

Table 2
Within-run precision of the AFP assay

Mean AFP [a] (ng ml^{-1})	R.S.D. (%) [a] SFS	R.S.D. (%) [a] SDSFS
0	5.4	5.1
0.90	7.4	4.5
5.00	7.0	7.0
100	9.7	9.9

[a] $n = 10$.

3.11. Correlation studies

The accuracy of the method was assessed by analysing 10 serum samples with AFP concentrations in the range of 1.0–16 ng ml^{-1}, by this and another widely used AFP procedure (SORIN, AFP-RIA immunoassay). Although the number of samples was too small and the range was very narrow (limited to the normal range for AFP), the linear regression equation was satisfactory: y(proposed method) = 0.894 + 1.130x(RIA) with a correlation coefficient $r = 0.918$.

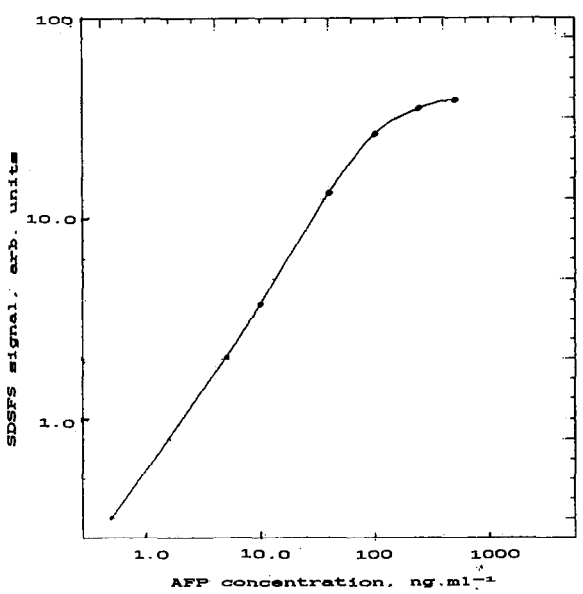

Fig. 4. Typical calibration curve for AFP with SDSFS detection.

4. CONCLUSIONS

This technique is suitable for measuring lanthanide chelates at very low concentrations. However many steps still have to be taken in order to improve the sensitivity, steps like using high energy xenon flash lamps as an excitation source and automated direct fluorescence measurement into the wells, so that the final sample dilution step would be avoided.

ACKNOWLEDGEMENTS

We gratefully thank Dr. E.P. Diamandis, Clin. Biochem. Dept., University of Toronto, for the generous supply of the AFP monoclonal antibodies, AFP standards as well as SA-ALP conjugate. We also thank Mr. J.P. Kyriakopoulos, M.Sc., for providing the software used for the calculation of the results, as well as Dr. V. Theodorou, Lab. of Organic Chemistry, Chem. Dept., University of Athens, for her valuable help in the organic synthesis of SAP.

REFERENCES

[1] W.P. Collins (Ed.), Alternative Immunoassays, Wiley, New York, 1985.
[2] I.A. Hemmila, Applications of Fluorescence in Immunoassays. Wiley, New York, 1991.
[3] E.P. Diamandis, Clin. Chim. Acta, 194 (1990) 19.
[4] T.K. Christopoulos, E.S. Lianidou and E.P. Diamandis, Clin. Chem., 36 (1990) 1497.
[5] E.P. Diamandis, Clin. Biochem., 21 (1988) 139.
[6] L.J. Kricka (Ed.), Nonisotopic DNA probe techniques, Academic Press, San Diego, CA, 1992.
[7] J.B.F. Lloyd, Nature, 231 (1971) 64.
[8] J.B.F. Lloyd and I.W. Evett, Anal. Chem., 49 (1977) 1710.
[9] T. Vo-Dinh, Anal. Chem., 50 (1978) 396.
[10] C.C. Blanco, F.G. Sanchez, Anal. Chem., 56 (1984) 2035
[11] S. Rubio, A. Gomez-Henz and M. Valcarcel, Anal. Chem., 57 (1985) 1101.
[12] F.G. Sanchez, A. Navas and M. Santiago, Anal. Chim. Acta, 167 (1985) 217.
[13] R.A. Evagelista, A. Pollak and E.F.G. Templeton, Anal. Biochem., 197 (1991) 213.
[14] T.K. Christopoulos and E.P. Diamandis, Anal. Chem., 64 (1992) 342.
[15] D.G. Konstantianos, P.C. Ioannou and C.E. Efstathiou, Analyst, 116 (1991) 373.
[16] D.G. Konstantianos and P.C. Ioannou, Analyst, 117 (1992) 877.
[17] A. Savitzky and M.J.E. Golay, Anal. Chem., 36 (1964) 1627.
[18] J.D. Chanley E.M. Gindler and H. Sobotka, J. Am. Chem. Soc., 74 (1952) 4347.
[19] H.U. Bergmeyer (Ed.), Principles of enzymatic analysis, Verlag Chemie, Weinheim, 2nd edn., 1978.