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High-performance liquid chromatographic determination of penicillins by means of automated solid-phase extraction and photochemical degradation with electrochemical detection

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Abstract

An automated precolumn exchange system for on-line solid-phase extraction (OPS-2) coupled to liquid chromatography with photochemical degradation and electrochemical detection was used for the determination of residual amounts of penicillin G, penicillin V, oxacillin, cloxacillin and dicloxacillin. A 5–10-fold increase in sensitivity was obtained when compared with direct UV detection of penicillins at 225 nm. The system is recommended for samples that have received an immunoaffinity clean-up. Analysis of bovine muscle tissue indicated, however, that this procedure could also be suitable for the determination of penicillin G at its maximum residue limit of 50 μg/kg even with conventional solvent partitioning for the first clean-up steps.

Keywords: Solid-phase extraction; Penicillins; Antibiotics

1. Introduction

Penicillins are widely used to prevent and treat bovine mastitis and other diseases, with benzylpenicillin being the most important compound. As a result, residues can occur in milk and edible tissues. Maximum residue limits (MRL) were set for some penicillins by the European Community in 1992 [1]. During the last 2 years, considerable research efforts have been directed to the development of residue analytical procedures for the determination of penicillins. Most approaches used HPLC separation with different modes of detection, i.e., direct UV [2,3], UV and fluorescence after derivatization [4–6], pulsed amperometric detection on gold electrodes [7], particle beam [8] and electrospray mass spectrometry [9–11]. Procedures published earlier are covered by some recent reviews [12–16]. Most of the newer methods are very powerful but require either extensive clean-up steps with laborious, time-consuming sample handling to achieve the necessary sensitivity and specificity or need highly sophisticated instrumentation. In general, only limited interest has been paid to the automation of penicillin residue analysis and the introduction of on-line methods [2,3,5].

Automated procedures for solid-phase extraction (SPE) and on-line sample treatment are known to give better results with respect to accuracy and precision than manual techniques

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and have been successfully applied to the analysis of various drugs [17–20]. In addition, it is noteworthy that sensitive trace-level determination is more easily accomplished by the analysis of total sample extracts rather than aliquots. This approach is followed by combining a highly specific clean-up procedure, i.e., immunoaffinity chromatography (IAC), with an automated procedure for the identification and quantification of penicillin residues. While IAC is still under development, it was the aim of this work to provide an automated procedure for trapping, separating and sensitively determining penicillin residues based on the improvement of a post-column photochemical ($h\nu$) degradation electrochemical detection (ED) technique [21]. A novel automated on-line SPE–HPLC–$h\nu$–ED methodology used for determination of penicillin G along with penicillin V, oxacillin, cloxacillin and dicloxacillin is reported here. Results include minimum detection limits, optimum oxidative working potential and analyses of spiked muscle samples after conventional solvent partitioning clean-up.

2. Experimental

2.1. Chemicals

Penicillin standards (penicillin G potassium, penicillin V potassium, oxacillin sodium, cloxacillin sodium and dicloxacillin sodium) were obtained from Sigma (Deisenhofen, Germany). Standard solutions were prepared by dissolving the compounds in water. Acetonitrile was distilled over K_2CO_3 and water was triply distilled. Chemicals for penicillin extraction [22,23] were dichloromethane, light petroleum (distilled over sodium), 0.5 *M* phosphate buffer (pH 2.2) prepared from 0.5 *M* solutions of potassium dihydrogenphosphate and orthophosphoric acid and 0.2 *M* phosphate buffer (pH 7) prepared from 0.2 *M* solutions of potassium dihydrogenphosphate and disodium hydrogenphosphate. Sodium chloride, dehydrated sodium sulfate. Inorganic salts added to the mobile phase and other chemicals were of analytical-reagent grade and obtained from Merck (Darmstadt, Germany).

2.2. Instrumentation and equipment

Fig. 1 illustrates the OSP-2–HPLC–$h\nu$–ED instrumentation and the arrangement of the parts used. The chromatographic system consisted of a Model 655A-11 pump, a Model L5000LC gradient/event controller, a Model 655A-40 autosampler, a Model L6000A pump, an OSP-2 on-line sample preparator, a Model L3500 electrochemical detector with glassy carbon electrochemical cell with stainless-steel auxiliary electrode and Ag–AgCl reference electrode and a Model D-2500 integrator, all from Merck. An ERC-125 column oven was obtained from ERC (Alteglofsheim, Germany), a Beam Boost photoreactor from ICT (Frankfurt, Germany) and an ELV-7000 electric six-port valve from Kranich (Göttingen, Germany). The L5000LC gradient/event controller was programmed according to experimental conditions given in Fig. 2. The Beam Boost photoreactor was equipped with a low-pressure mercury lamp ($\lambda_{max} = 254$ nm) and a 10 m × 0.3 mm I.D. PTFE reaction coil. LiChroCART cartridges (4 mm × 4mm I.D.) from Merck which were packed with LiChrospher 100 RP-18e ($d_p = 5$ μm) were used for on-line solid-phase extraction. The LiChroCART analytical column (250 mm × 4 mm I.D.) packed with LiChrospher 100 RP-18e ($d_p = 5$ μm) was also obtained from Merck.

2.3. Chromatography and detection

Solid-phase extraction (SPE) and elution on to the analytical column were performed by the OSP-2 on-line sample preparator, which was activated by the gradient/event controller. The flow-rates were set as shown in Fig. 2. Data acquisition was started when the precolumn was switched to the analytical column. Solvents for SPE were acetonitrile–water (50:50, v/v) for conditioning, 0.02 *M* phosphate buffer (pH 7.0) for sample application and acetonitrile–0.2 *M* phosphate buffer (pH 3.0) (10:90, v/v) for washing. The mobile phase for analytical HPLC was

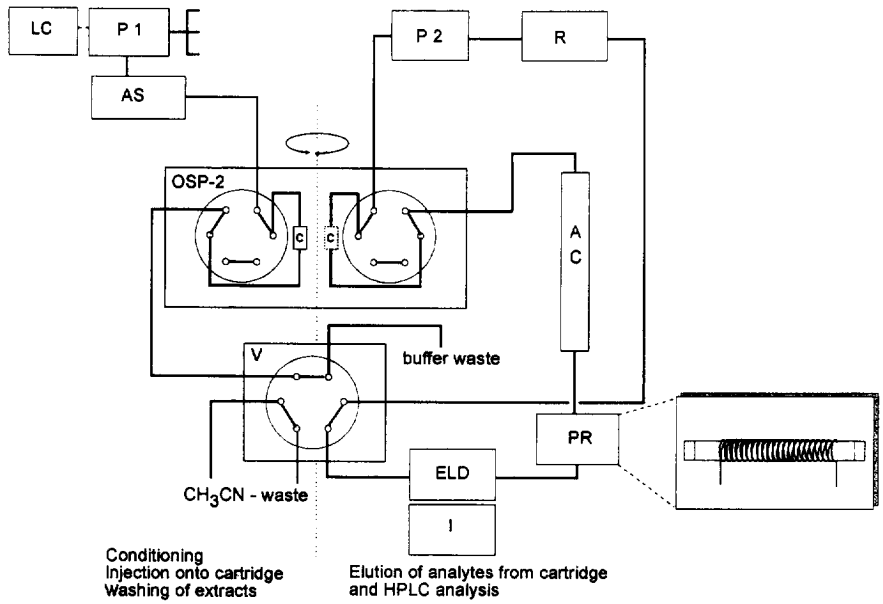

Fig. 1. OSP-2–HPLC–$h\nu$–ED arrangement. AS = 655A-40 autosampler; AC = column oven (T = 35°C) and analytical column (250 × 4 mm I.D. LiChrospher 100 RP-18e, 5 μm); C = a single 4 × 4 mm I.D. LiChroCART LiChrospher RP-18 (5 μm) cartridge; position is changed between enrichment and elution; ELD = L3500 electrochemical detector; LC = L5000LC controller; I = D-2500 Integrator; P1 = 655A-11 pump; P2 = L6000A isocratic pump; PR = Beam-Boost photochemical reactor with low-pressure mercury lamp (λ_{max} = 254 nm) and 10 × 0.3 mm I.D. PTFE reaction coil; R = mobile phase reservoir; V = six-port electric valve.

acetonitrile–0.2 *M* phosphate buffer (pH 3.0) (35:65, v/v) containing 2 m*M* Na_2EDTA. The flow-rate was 1 ml/min. Electrochemical detection was performed at +0.65 V (vs. Ag–AgCl).

2.4. Sample preparation

Bovine muscle samples were prepared by spiking drug-free muscle tissue with standard solutions prior to extraction. Penicillins were extracted from the matrix with acetonitrile and transferred to phosphate buffer according to the literature [5,22,23]: weigh 25.0 ± 0.2 g into 100-ml centrifuge tubes (add standard solutions at this point for spiked samples, mix and allow to stand for 10 min at room temperature). Hold the tube into an ice-bath, add 25 ml of acetonitrile, start homogenizing with the Ultra-Turrax for 1 min, add 5 ml of 0.5 *M* phosphate buffer (pH 2.2) while the Ultra-Turrax is still running, add an additional 65 ml of acetonitrile and homogenize for a further 1 min. Centrifuge for 10 min at 4000 *g* and decant the supernatant into a 250-ml separating funnel containing 7 g of sodium chloride and 50 ml of dichloromethane. Shake the separating funnel for 2 min and allow to stand for ca. 30 min until the phases have separated. Discard the aqueous (lower) layer and decant the organic layer into a 250-ml erlenmeyer flask with a ground-glass joint and stopper containing ca. 5 g of anhydrous sodium sulfate (do not run off residual drops of aqueous layer). Shake for 30 s, filter the organic layer through a cotton-wool plug into a 250-ml round-bottomed flask and rotary evaporate the extract at 30°C to ca. 10 ml. Transfer the residue into a 25-ml pear-shaped flask and continue rotary evaporation. When the extract has been evaporated to ca. 4 ml, add 3 ml of dichloromethane, repeat the addition and evaporation of dichloromethane, add 3 ml of light petroleum and evaporate until a residue of ca. 0.5 ml is left. Suspend the residue aided by ultrasonication with 3 × 3 ml of light petroleum and transfer into a 13-ml centrifuge tube. Rinse

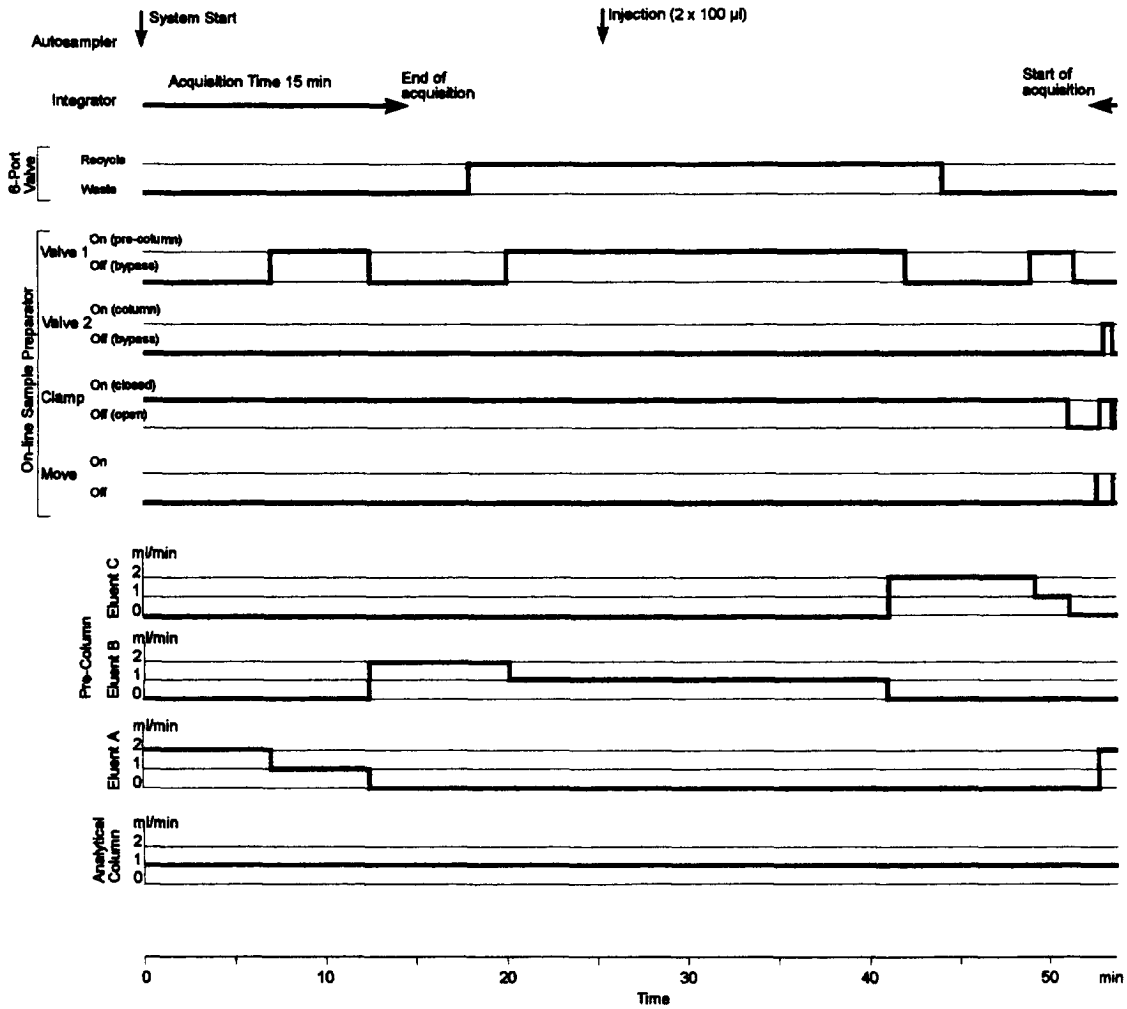

Fig. 2. Time scheme for column- and valve-switching steps and elution conditions for the SPE cartridge (precolumn) and the analytical column. Eluent A, CH_3CN–water (50:50, v/v); eluent B 0.02 *M* phosphate buffer (pH 7); eluent C, CH_3CN–0.2 *M* phosphate buffer (pH 3) (10:90, v/v).

the pear-shaped flask with 2 ml of phosphate buffer (pH 7). Add rinsing solution to the tube, vortex mix for 30 s and separate the layers by centrifuging. Transfer the lower (aqueous) layer into a second centrifuge tube. Repeat this partitioning with 2 ml and twice with 1.5 ml of phosphate buffer (pH 7) and centrifuge the combined aqueous phases. A 1.5-ml portion of phosphate buffer layer is transferred to an autosampler vial to perform on-line SPE.

3. Results and discussion

3.1. Solid-phase extraction

SPE of the penicillins was performed on a short C_{18} reversed-phase precolumn (LiChroCART 4 mm × 4 mm I.D. cartridge) using 0.02 *M* phosphate buffer (pH 7) to apply the penicillins, which were mostly immobile under these conditions. The recoveries of penicillin standards

were found to be nearly 100%. We could use one cartridge for at least 25 analyses with identical analytical performance. The washing solution was optimized to remove most of the matrix interferences from bovine muscle tissue without eluting the penicillins. A six-port switching valve could be used to recycle mobile phase when no analysis was being performed and to separate the waste solvent of the SPE procedure.

3.2. Chromatography and detection

Penicillins were desorbed from the cartridge and eluted (0.6 min) on to a reversed-phase analytical column by isocratic elution with the mobile phase. The retention times of the penicillins were 5.2 min (Pen G), 6.1 min (Pen V), 7.1 min (Oxa), 8.9 min (Clox) and 13.0 (Dclox) (Fig. 3). No drift of retention was observed. Native penicillins with the exception of amoxicillin cannot be detected electrochemically on glassy carbon electrodes without prior photolysis. Recently, it was shown that native penicillins can be oxidized on gold electrodes with a sensitivity comparable to that of direct UV measurement [7]. After photochemical degradation and using a glassy carbon electrode, we were able to detect the penicillin photolysis products with a 5–10-fold increase in sensitivity when compared with direct UV detection of penicillins at 225 nm. The photoreaction was mediated using a transparent PTFE reaction coil. The PTFE tubing was woven to reduce band-broadening effects [24]. On comparing the UV signals (225 nm) of penicillin standards, we observed no significant difference in peak height or peak symmetry with the reactor either in or out of the system. The ideal residence time was 60 s, which means optimum photolytic formation of the desired electrochemically active derivatives with minimum destruction of them prior to detection.

Little information is available that would delineate the reaction mechanism under these particular mobile phase conditions. One can consider cleavage of the C–N bond of the β-lactam ring with the formation of intermediates such as ketene, azomethine and isocyanate derivatives. The reaction products formed can be detected with a high degree of reproducibility and with detection limits between 1 and 5 ng (Table 1).

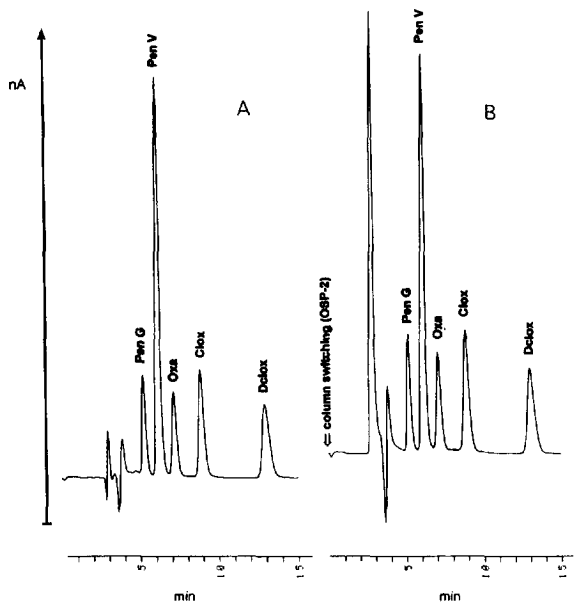

Fig. 3. HPLC–$h\nu$–ED of penicillin standard. Pretreatment by means of OSP-2 SPE and enrichment of 200 μl (B) is compared with direct injection of 10 μl (A) with absolute amounts of 100 ng of each penicillin. Pen G = benzylpenicillin; Pen V = phenoxymethylpenicillin; Oxa = oxacillin; Clox = cloxacillin; Dclox = dicloxacillin.

Table 1
Detection limits[a] and relative standard deviations[b] for HPLC of penicillins after photoreaction and electrochemical detection

Compound	Detection limit (ng)	R.S.D. (%)
Benzylpenicillin	1.2	6.7
Phenoxymethylpenicillin	1.4	8.4
Oxacillin	2.7	7.2
Cloxacillin	2.5	1.4
Dicloxacillin	4.6	6.0

[a] Detection limits were determined according to the DIN 32645 procedure [25] using 200-μl injections with absolute amounts between 10 and 50 ng of each penicillin.
[b] Relative standard deviations (R.S.D.) were determined for on-line SPE extraction with electrochemical detection for absolute amounts of 30 ng for each penicillin ($n = 3$).

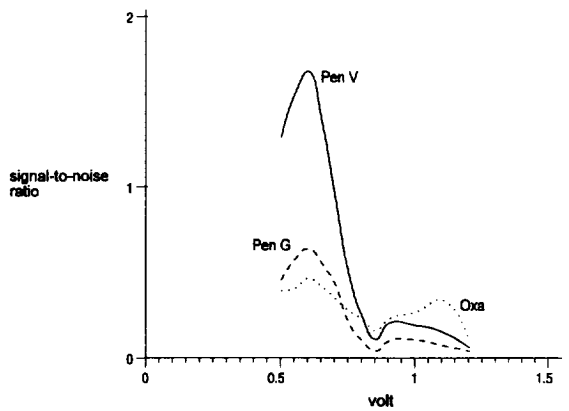

Fig. 4. Signal-to-noise ratio [response (nA)/background current (nA)] in the range 0.5–1.2 V (vs. Ag–AgCl) indicating optimum working potential at +0.65 V. Pen G = benzylpenicillin; Pen V = phenoxymethylpenicillin; Oxa = oxacillin (example for isoxazolyl penicillins).

The optimum detection potential was determined by recording hydrodynamic voltammograms for each penicillin. The best signal-to-noise ratio (Fig. 4) was obtained at +0.65 V (vs. Ag–AgCl). It was important to add EDTA to the mobile phase as it reduced the baseline noise of the electrochemical detector significantly. Column switching by the OSP-2 did not contribute to the background noise level and no loss of sensitivity was observed within 1 week.

It was shown earlier that UV detection is sufficient for the on-line determination of isoxazolyl penicillins [5] in meat at their maximum residue limits (MRLs) of 0.3 mg/kg but lacks sensitivity for benzylpenicillin with its lower MRL of 0.05 mg/kg. The chromatograms of bovine muscle tissue obtained with the described on-line photodegradation technique (Fig. 5) indicate that this procedure could be suitable for the determination of benzylpenicillin (Pen G) at its MRL even with conventional partitioning for the first clean-up steps.

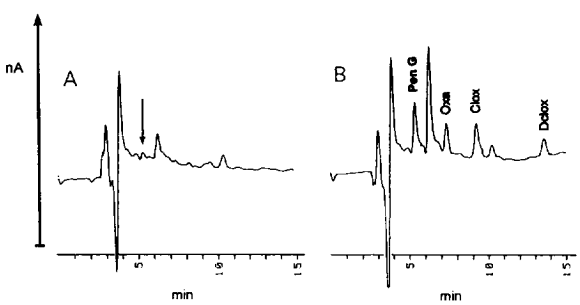

Fig. 5. Chromatogram of penicillins in bovine muscle tissue. (A) blank; (B) spiked tissue: 0.05 mg/kg for each penicillin (=MRL for benzylpenicillin). Concentrations of oxacillin, cloxacillin and dicloxacillin are one sixth of the MRLs (0.3 mg/kg). Arrow indicates the retention time of benzylpenicillin (Pen G). For conditions, see Experimental.

4. Conclusion

The on-line SPE–HPLC–$h\nu$ method coupled with electrochemical detection provides improved ease and sensitivity of analysis for a number of penicillins widely used in veterinary medicine. Further improvements mainly in sample handling and selectivity, can be expected from the use of group-specific, immobilized antibodies for on-line immunoaffinity chromatography combined with the described technique. In current and very promising experiments, the immunaffinity column is placed between the injector and the C_{18} SPE column of the OSP-2 to trap penicillin residues from aqueous sample extracts.

Acknowledgements

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References

[1] Commission Regulation 92/675/EEC, Off. J. Eur. Comm., (1992) L23/8.
[2] W.A. Moats and R. Harik-Khan, J. AOAC Int., 78 (1995) 49.
[3] W.A. Moats, J. AOAC Int., 77(1) (1994) 41.
[4] J.O.K. Boison, L.J.Y. Keng and J.D. MacNeil, J. AOAC Int., 77 (1994) 565.
[5] S. Lihl and M. Petz, Z. Lebensm.-Unters.-Forsch., 199 (1994) 229.
[6] C.C. Hong, C.L. Lin, C.E. Tsai and F. Kondo, Am. J. Vet. Res., 56 (1995) 297.
[7] E. Kirchmann, R.L. Earley and L.E. Welch, J. Liq. Chromatogr., 17 (1994) 1755.

[8] D. Hurtaud, B. Delepine and P. Sanders, Analyst, 119 (1994) 2731.
[9] W.J. Blanchflower, S.A. Hewitt and D.G. Kennedy, Analyst, 119 (1994) 2595.
[10] K.L. Tyczkowska, R.D. Voyksner, R.F. Straub and A.L. Aronson, J. AOAC Int., 77 (1994) 1122.
[11] R. Straub, M. Linder and R.D. Voyksner, Anal. Chem., 66 (1994) 3651.
[12] M. Petz, in V.K. Agarwal (Editor), Analysis of Antibiotic/Drug Residues in Food Products of Animal Origin, Plenum Press, New York, 1992, pp. 147–163.
[13] J.O. Boison, J. Chromatogr., 624 (1992) 171.
[14] B. Shaikh and W.A. Moats, J. Chromatogr., 643 (1993) 369.
[15] N.A. Botsoglou and D.C. Kufidis, in L.M.L. Nollet (Editor), Food Analysis by HPLC, Marcel Dekker, New York, 1992, pp. 457–505.
[16] D.R. Bobbitt and K.W. Ng, J.A Chromatogr., 624 (1992) 153.
[17] U.A.Th. Brinkman, J. Chromatogr. A, 665 (1994) 217.
[18] W.J.J. Krauwinkel, N.J. Volkers-Kamermans and J. van Zijtveld, J. Chromatogr., 617 (1993) 334.
[19] E. Brandsteterova, D. Romanova, D. Kralikova, L. Bozekova and M. Kriska, J. Chromatogr., 665 (1994) 101.
[20] Ph. Hubert, P. Chiap, M. Moors, B. Bourguignon, D.L. Massart and J. Crommen, J. Chromatogr. A, 665 (1994) 87.
[21] C.M. Selavka, I.S. Krull and K. Bratin, J. Pharm. Biomed. Anal., 4 (1986) 83.
[22] U. Meetschen and M. Petz, J. Assoc. Off. Anal. Chem., 73 (1990) 373.
[23] U. Meetschen and M. Petz, Z. Lebensm.-Unters.-Forsch., 193 (1991) 337.
[24] U.A.Th. Brinkman, R.W. Frei and H. Lingeman, J. Chromatogr., 492 (1989) 251.
[25] J. Vogelgesang, Kalibo (Statistics Software), Brussels.