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Optimized methods for capillary electrophoresis of tetracyclines

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Abstract

Optimized methods for the analysis of some tetracyclines by capillary electrophoresis are described. Different buffer systems were employed for the separation of tetracycline, oxytetracycline and demeclocycline from their respective major impurities, including the 2-acetyl-2-decarboxamido derivatives. The influence of buffer pH and buffer concentration was systematically investigated. Non-ionic surfactant Triton X-100 and methyl-β-cyclodextrin were used to obtain improved selectivity in the case of oxytetracycline and demeclocycline. The results are compared with those of previously established liquid chromatography methods. Good correlations were obtained. © 1997 Elsevier Science B.V.

Keywords: Capillary electrophoresis; Tetracycline; Oxytetracycline; Demeclocycline; 2-Acetyl-2-decarboxamido derivatives

1. Introduction

Tetracycline (TC), oxytetracycline (OTC) and demeclocycline (DMCTC) are three members of the tetracyclines (TCs), an important group of broad spectrum antibiotics, which are widely used. Their structures are shown in Fig. 1. Each of them can contain several impurities from fermentation or degradation.

Analysis of TCs and their impurities is mainly performed by liquid chromatography (LC) and has been studied extensively [1,2]. In recent years, capillary electrophoresis (CE) for analysis of TCs has also been reported [3–10]. We have previously reported CE methods for assay and purity control of TC [6], OTC [7], DMCTC [8], doxycycline [9] and minocycline [10]. However, the 2-acetyl-2-decarboxamido (AD) derivatives, which are fermentation impurities of the first three tetracyclines, were not separated by all these CE methods.

This paper concentrates on the separation of AD derivatives. Various modifications were employed, e.g. for 2-acetyl-2-decarboxamidotetracycline (ADTC), a higher pH and buffer concentration were used, for 2-acetyl-2-decarboxamidooxytetracycline (ADOTC) non-ionic surfactant Triton X-100 was added to the buffer system and for 2-acetyl-2-decarboxamidodemeclocycline (ADDMCTC), methyl-β-cyclodextrin (M-β-CD) was also added to the buffer system with Triton X-100. Using these different buffers and additives,

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the separation of ADTC, ADOTC and ADDMCTC from the respective main components was obtained. At the same time, all other impurities remained completely separated. The results show a better separation than with LC and good quantitative correlations between both methods.

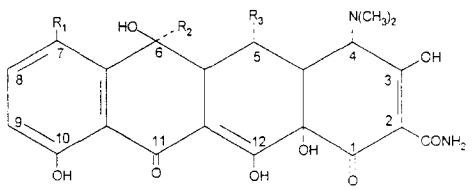
2. Experimental

2.1. Instrumental and operating conditions

Capillary electrophoresis was performed on Spectraphoresis 500 equipment (Thermo Separation Products, Fremont, CA), coupled to a 3396 series II integrator (Hewlett Packard, Avondale, PA). A UV absorption detector was employed with the following wavelengths: TC: 270 nm, OTC: 254 nm and DMCTC: 254 nm. Injection was done hydrodynamically for 4 s. pH Measurements were performed on a Consort pH-meter (Turnhout, Belgium) using calibration buffers constituted according to the European Pharmacopoeia [11]. When necessary, the pH of running buffers was adjusted using 0.1 M NaOH or 0.1 M HCl before making up to volume. Throughout the study, all samples were dissolved in running buffer to obtain better peak symmetry.

2.2. Materials

All reagents were of analytical grade (Merck, Darmstadt, Germany or Acros Chimica, Geel, Belgium). TC, OTC and their related substances were obtained from Acros Chimica. DMCTC and its related substances were available in the laboratory as inhouse standards. Small amounts of ADTC, ADOTC and ADDMCTC were isolated by a thin-layer chromatographic method [12]. The fused silica capillary was from Polymicro Technologies (Phoenix, AZ). Throughout the study, Milli-Q^{50} water was used (Millipore, Milford, MA). All the solutions were filtered through 0.2 μm nylon filters (Euroscientific, Lint, Belgium).



	R_1	R_2	R_3
TC	H	CH_3	H
OTC	H	CH_3	OH
DMCTC	Cl	H	H

Fig. 1. Chemical structures of TC, OTC and DMCTC

3. Results and discussion

All development experiments were performed on an uncoated fused silica capillary of 50 μm diameter and 38 cm effective length. Sodium carbonate was used as the background electrolyte and 1 mM EDTA was added in all cases to prevent interaction of the tetracycline structures with metals through complexation.

3.1. Separation of TC and its impurities

LC and TLC studies have shown that 4-epitetracycline (ETC), anhydrotetracycline (ATC), 4-epianhydrotetracycline (EATC) and ADTC are the most important impurities present in commercial tetracycline samples [13,14]. Chlortetracycline (CTC) and demethyltetracycline (DMTC) were mentioned as minor impurities [13,14]. A good method for purity control of TC should therefore in the first instance be able to separate TC, ETC, ATC, EATC and ADTC. The mixture for method development is a commercial sample containing TC, ADTC, ETC, EATC, ATC and CTC. The following parameters were optimized consecutively: buffer pH and buffer concentration. The results were as follows.

3.1.1. Influence of buffer pH

pH is one of the most important parameters for improving selectivity in CE and small differences can cause the separation of closely related substances [15]. The investigated range was restricted to the alkaline region to avoid sample adsorption on the capillary wall and epimerization of TC.

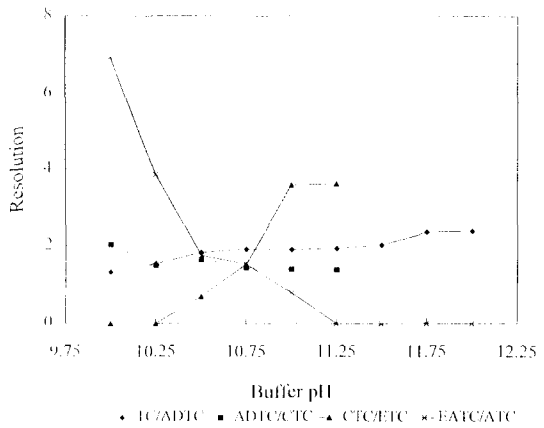


Fig. 2. Influence of buffer pH on the separation of TC and its impurities. Capillary: uncoated fused silica, L = 44 cm, l = 38 cm, i.d. = 50 μm; background electrolyte, sodium carbonate 120 mM including 1 mM EDTA; temperature, 15°C; voltage, 12 kV.

The optimization was performed with a sodium carbonate (120 mM)–EDTA (1 mM) buffer, at a voltage of 12 kV and a temperature of 15°C. The pH was varied between 10.0 and 12.0 with steps of 0.25. The isoelectric point of TC is 4.8 [16] and the isoelectric points of the other compounds are presumably also below the buffer pH. Therefore TC and its impurities are negatively charged and their electrophoretic mobility is opposite to electroosmosis. The resolution is dependent on the sum of electrophoretic mobility of each solute and electroosmotic mobility. For every substance, migration time was longer with increasing pH, which shows that an increase in electrophoretic movement of the substances has overcome the increase in electroosmosis. However, some substances were affected more than others due to structural differences, which result in a selectivity change. Results of resolution are shown in Fig. 2. Since the resolution of ETC/EATC was very high (> 8) in all cases, it is not shown in the figure. It was found that at pH 10.75, the best separation of TC and its main impurities could be obtained. This pH was chosen for further method development. It should be pointed out that, if present in a sample, DMTC migrates between TC and ADTC in this system and approaches TC with a pH increase (data not shown). It can only be separated partly from TC under pH 10.75. CTC is unstable in the alkaline background electrolyte and converts partly into isochlortetracycline (ICTC) [17]. Over pH 11.5, it is converted almost completely during the run so that Fig. 2 can not give resolutions of ADTC/CTC and CTC/ETC over pH 11.5. Because DMTC and CTC are less important, pH 10.75 is chosen.

3.1.2. *Influence of buffer concentration*

Buffer concentration has a big influence on electroosmotic flow and the current produced in the capillary [15]. Keeping other conditions constant (pH 10.75, 15°C, 12 kV and EDTA at 1 mM), buffer concentration was varied from 80 to 130 mM in steps of 10 mM. With a concentration increase, the resolutions of TC/ADTC, ADTC/CTC and CTC/ETC increased continuously, but the pair EATC/ATC was almost not affected. Results are shown in Fig. 3. Under normal conditions, as the buffer concentration is increased, the electrophoretic and electroosmotic mobilities both decrease. The results imply the former is reduced less with increasing buffer concentration, except for the pair EATC/ATC. 120 mM was finally chosen because it gave sufficient resolution for each pair of substances. Comparing the separation of TC and ADTC with the influence of pH where resolution was almost not affected, it can be concluded that changes of buffer concentration

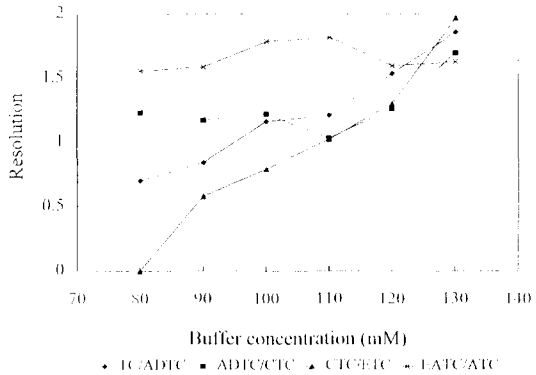


Fig. 3. Influence of buffer concentration on the separation of TC and its impurities. Capillary: uncoated fused silica, L = 44 cm, l = 38 cm, i.d. = 50 μm; background electrolyte, sodium carbonate x mM including 1 mM EDTA; pH 10.75; temperature, 15°C; voltage, 12 kV.

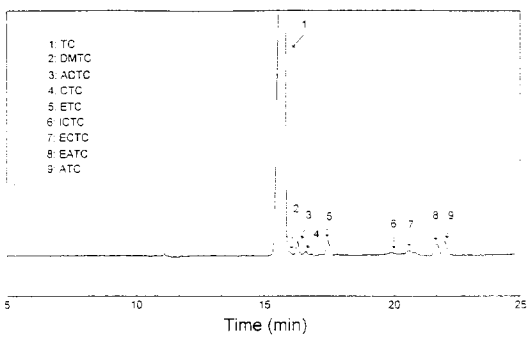


Fig. 4. Electropherogram of TC and its impurities. Capillary: uncoated fused silica, L = 44 cm, l = 38 cm, i.d. = 50 μm; background electrolyte, sodium carbonate 120 mM including 1 mM EDTA; pH 10.75; temperature, 15°C; voltage, 12 kV.

are a more important factor for separation of ADTC from TC.

Influence of capillary temperature and applied voltage on resolution was also investigated. It was found that separation became slightly worse with temperature and voltage increase. 15°C and 12 kV were chosen as the optimal conditions.

Fig. 4 shows a typical electropherogram using a commercial sample. A comparison with the performance of LC [13] shows that the separation of TC/ADTC is better and that the total analysis time is shorter than with LC. The difference with previous CE methods [3,4,6] is that ADTC is well separated.

3.2. Separation of OTC and its impurities

Method development was performed with an artificial mixture containing OTC and its potential impurities anhydrooxytetracycline (AOTC), α-apooxytetracycline (α-APO), β-apooxytetracycline (β-APO), 4-epioxytetracycline (EOTC), TC, ETC and terrinolide (TL). ADOTC was not available in sufficient quantities to include it in the mixture and therefore was only used in the final stage. The following parameters were optimized consecutively: buffer pH, buffer concentration and Triton X-100 concentration. Influence of voltage and capillary temperature were also examined.

3.2.1. Influence of buffer pH, and buffer concentration

Using the same approach as Section 3.1, buffer pH and concentration were varied. Under no circumstance however could ADOTC be separated. When the non-ionic surfactant Triton X-100 was introduced to the buffer system (0.5%, v/v), a clear improvement of separation was observed and the migration order was changed substantially (Fig. 5). Keeping the Triton X-100 concentration at 0.5% (v/v), buffer pH and buffer concentration were optimized again. pH 11 and a concentration of 50 mM sodium carbonate were finally chosen because they gave the best overall resolution (data not shown).

3.2.2. Influence of Triton X-100 concentration

Triton X-100 concentration was varied from 0 to 0.6% (v/v) in steps of 0.1% (v/v). Separation of all eight substances could be obtained only from 0.5% (v/v) on. This concentration was finally retained because resolution between TC and ETC decreased at 0.6% (v/v). The results are shown in Fig. 5 and can be explained by an interaction of the analytes with the alkyl chain of Triton X-100, possibly through hydrophobic interactions. More hydrophobic compounds interact more strongly with the micelle which moves faster than the negatively charged compounds. Therefore, the migration time of the more hydrophobic compounds

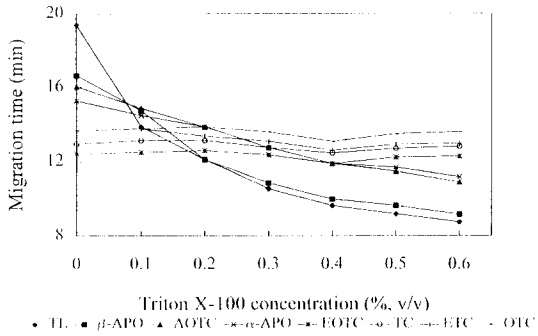


Fig. 5. Influence of Triton X-100 concentration on the separation of OTC and its impurities. Capillary: uncoated fused silica, L = 44 cm, l = 38 cm, i.d. = 50 μm; background electrolyte, sodium carbonate 50 mM including 1 mM EDTA and x% (v/v) Triton X-100; pH 11.0; temperature, 10°C; voltage, 10 kV.

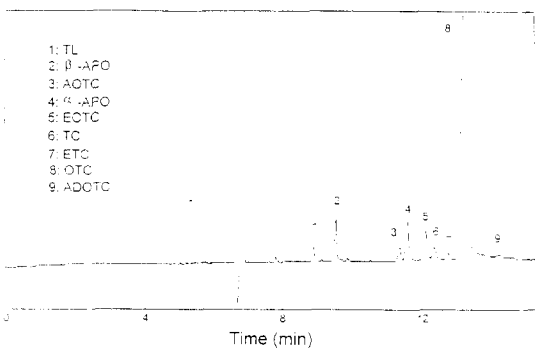


Fig. 6. Electropherogram of OTC and its impurities. Capillary: uncoated fused silica, L = 44 cm, l = 38 cm, i.d. = 50 μm; background electrolyte, sodium carbonate 50 mM including 1 mM EDTA and 0.5% (v/v) Triton X-100; pH 11.0; temperature, 10°C; voltage, 10 kV.

decreases with a rise in Triton X-100 concentration. This explanation can be confirmed by the results of reversed-phase LC (RPLC) [19,20] where the elution order was almost opposite. In RPLC, the more hydrophobic compounds are retained longer. The selectivity of the system was markedly influenced by the Triton X-100 concentration, as the migration order shows substantial changes.

These results show that in this system Triton X-100 concentration affected separation more than pH and buffer concentration but it is difficult to explain this in detail by correlation with their structures.

At this stage, ADOTC was added to the sample. It was found that ADOTC can be separated from OTC. The order of migration under final optimal conditions is TL, β-APO, AOTC, α-APO, EOTC, TC, ETC, OTC and ADOTC. This order is totally different from the CZE method [7], where the peak order was EOTC, TC, ETC, OTC, α-APO, AOTC, β-APO and TL.

Influence of capillary temperature and applied voltage on resolution was also investigated. It was found that separation became worse with increasing temperature and voltage, specifically for the critical pair TC/ETC (data not shown). 10°C and 10 kV were chosen as the optimal conditions.

Fig. 6 shows a typical electropherogram of a sample spiked with ADOTC. A comparison with the performance of LC [18,19] shows that this method is better for separation of OTC/ADOTC and takes less time than LC. The latter analysis took over 30 min compared to 20 min for this method including the washing procedure. Furthermore, the LC method needs a gradient elution.

3.3. Separation of DMCTC and its impurities

The main impurities of DMCTC are demethyltetracycline (DMTC), 4-epidemeclocycline (EDMCTC), 4-epidemethyltetracycline (EDMTC) and ADDMCTC. Method development was undertaken with a DMCTC commercial sample containing the impurities mentioned.

3.3.1. Influence of buffer pH buffer concentration and Triton X-100 concentration

Analogously to Section 3.2, buffer pH and buffer concentration were first optimized. Without Triton X-100 or M-β-CD in the buffer, only two peaks could be obtained for five compounds. With the addition of Triton X-100 or M-β-CD to the buffer system, the separation of every pair of substances was greatly improved and five peaks were present. The peak order was DMCTC, ADDMCTC, DMTC, EDMCTC and EDMTC. Since resolution of ADDMCTC and DMTC was still poor, both Triton X-100 and M-β-CD were introduced to the buffer system. This produced a clear improvement in the ADDMCTC/DMTC separation. Keeping Triton X-100 and M-β-CD in the buffer, pH and buffer concentration were optimized again. pH 12.25 and a concentration of 110 mM sodium carbonate were chosen for further method development (data not shown). Then, keeping 20 mM of M-β-CD in the buffer, Triton X-100 concentration was varied from 0 to 0.4% (v/v) in steps of 0.05%. A concentration of 0.35% was retained (data not shown). It should be pointed out that both Triton X-100 and M-β-CD do not affect peak order in this system.

3.3.2. Influence of M-β-CD concentration

When introducing only Triton X-100, ADDMCTC/DMTC can not be well separated, so the combination of α-, M-β- and γ-CD with Triton X-100 was tested for further enhancement of se-

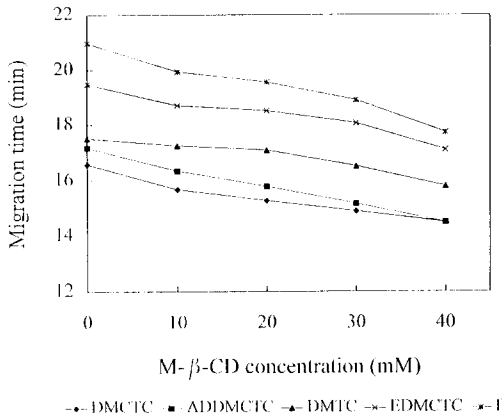


Fig. 7. Influence of M-β-CD concentration on the separation of DMCTC and its main impurities. Capillary: uncoated fused silica, L = 44 cm, l = 38 cm, i.d. = 50 μm; background electrolyte, sodium carbonate 110 mM including 1 mM EDTA, 0.35% (v/v) Triton X-100 and x mM M-β-CD; pH 12.25; temperature, 15°C; voltage, 12 kV.

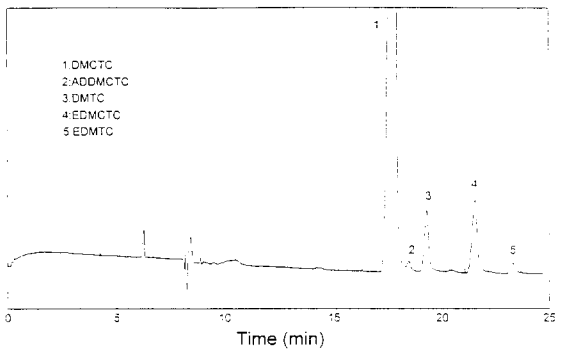


Fig. 8. Electropherogram of DMCTC and its main impurities. Capillary: uncoated fused silica, L = 44 cm, l = 38 cm, i.d. = 50 μm; background electrolyte, sodium carbonate 110 mM including 1 mM EDTA, 0.35% (v/v) Triton X-100 and 10 mM M-β-CD; pH 12.25; temperature, 15°C; voltage, 12 kV.

lectivity. α- And γ-CD did not improve separation. Fig. 7 shows a graph of migration time versus M-β-CD concentration. The latter was varied from 0 to 40 mM with steps of 10 mM. Migration times decrease with increasing M-β-CD concentration because each substance interacts more with M-β-CD, which moves faster than the negatively charged tetracyclines. This decrease is different for the separate substances, because their degree of inclusion complexation most probably differs due to variations in hydrophobicity and size. This results in a change in selectivity. 10 mM was finally chosen as the optimal condition because it gave a suitable resolution for all substances. 12 kV and 15°C were chosen as temperature and voltage.

Fig. 8 shows a typical electropherogram using a commercial sample. A comparison with the performance of LC [20] shows that a better separation for DMCTC/ADDMCTC was obtained with CE, but analysis time is slightly longer.

3.4. Quantitative analysis

The quantitative features of these analytical CE methods for TC, OTC and DMCTC were examined (using corrected peak area) and the results

Table 1
Quantitative data of TC, OTC and DMCTC by CE

	TC	OTC	DMCTC
LOD (S/N = 3)	5×10^{-4} mg ml^{-1} (0.1%)	5×10^{-4} mg ml^{-1} (0.05%)	5×10^{-4} mg ml^{-1} (0.1%)
LOQ (R.S.D., n)	1.5×10^{-3} mg ml^{-1} (14%, 7)	1×10^{-3} mg/ml (17%, 7)	1.5×10^{-3} mg ml^{-1} (12%, 7)
Calibration curve (Y = corrected area, X = concentration of the analysed solution in mg ml^{-1})	$Y=-5303+190\,665X$, $r=0.9983$, Sy, $x=5180$, range = 0.25–1.5 mg ml^{-1}, 6 points ($n=2$)	$Y=1396+140\,107X$, $r=0.9994$, Sy, $x=2702$, range = 0.25–1.75 mg ml^{-1}, 7 points ($n=2$)	$Y=5069+343\,882X$, $r=0.9971$, Sy, $x=12\,193$, range = 0.25–1.5 mg ml^{-1}, 6 points ($n=2$)
AD derivatives amount (%, w/w), (LC result is given between brackets)	0.8 by peak area normalisation (0.6 expressed as ETC)	1.5 by peak area normalisation (1.3 expressed as EOTC)	0.4 by peak area normalisation (0.4 expressed as DMCTC)

are summarized in Table 1, which also has a comparison with the previous LC method. Good quantitative correlations with LC were obtained.

4. Conclusion

The complete separation of TC, OTC and DMCTC from their respective impurities including ADTC, ADOTC and ADDMCTC was obtained. It is shown that various modifications were necessary to improve CE selectivity. Each tetracycline has to be studied separately. The levels of small amounts of impurities determined by CE were found to be comparable to those found by LC. There is great potential in the use of CE as an alternative tool for the analysis of tetracyclines and their impurities.

Acknowledgements

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References

[1] R.K. Gilpin and L.A. Pachla, Anal. Chem., 65 (1993) 117R–132R.
[2] R.K. Gilpin and L.A. Pachla, Anal. Chem., 67 (1995) 295R–313R.
[3] C.X. Zhang, Z.P. Sun, D.K. Ling and Y.J. Zhang, J. Chromatogr., 627 (1992) 281–286.
[4] S. Croubels, W. Baeyens, C. Dewaele and C. Van Peteghem, J. Chromatogr. A, 673 (1994) 267–274.
[5] M.F.M. Tavares and V.L. McGuffin, J. Chromatogr. A, 686 (1994) 129–142.
[6] A. Van Schepdael, J. Saevels, X. Lepoudre et al., J. High Resol. Chromatogr., 18 (1995) 695–698.
[7] A. Van Schepdael, I. Van den Bergh, E. Roets and J. Hoogmartens, J. Chromatogr. A, 730 (1996) 305–311.
[8] Y. M. Li, A. Van Schepdael, E. Roets and J. Hoogmartens, J. Chromatogr. A, 740 (1996) 119–123.
[9] A. Van Schepdael, R. Kibaya, E. Roets and J. Hoogmartens, Chromatographia, 41 (1995) 367–369.
[10] Y. M. Li, A. Van Schepdael, E. Roets and J. Hoogmartens, J. Pharm. Biomed. Anal., 14 (1996) 1095–1099.
[11] European Pharmacopoeia, 2nd edn., V.6.3.1. Maisonneuve, Sainte-Ruffine, France, 1980.
[12] W. Naidong, S. Hua, E. Roets and J. Hoogmartens, J. Planar Chromatogr., 7 (1994) 297–300.
[13] N. H. Khan, P. Wera, E. Roets and J. Hoogmartens, J. Liq. Chromatogr., 13 (1990) 1351–1374.
[14] W. Naidong, S. Hua, E. Roets and J. Hoogmartens, J. Planar Chromatogr., 5 (1992) 152–156.
[15] S.F.Y. Li, in Capillary Electrophoresis, Principles, Practice and Applications, J. Chromatogr. Library, Vol. 52, Elsevier Science, Amsterdam, 1992, pp. 201–226.
[16] C.R. Stephens, K. Murai, K.J. Brunings and R.B. Woodward, J. Am. Chem. Soc., 78 (1956) 4155–4158.
[17] C.R. Stephens, L.H. Conover, R. Pasternack et al., J. Am. Chem. Soc., 76 (1954) 3568–3575.
[18] N.H. Khan, E. Roets, J. Hoogmartens and H. Vanderhaeghe, J. Chromatogr., 405 (1987) 229–245.
[19] J. Hoogmartens, W. Naidong, N.H. Khan et al., Pharmeuropa, 2 (1990) 77–85.
[20] W. Naidong, E. Roets and J. Hoogmartens, J. Pharm. Biomed. Anal., 7 (1989) 1691–1703.