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Short Communication

Micellar electrokinetic capillary chromatography determination of zinc bacitracin and nystatin in animal feed

An MEKC procedure was developed for the separation of zinc bacitracin (Zn-BC) and nystatin (NYS) in mixtures and in animal feedstuff. The running buffer was 15 mM borate/19 mM phosphate, pH 8.2, containing 20 mM SDS and 10% v/v methanol. Samples were run at 25°C, the applied voltage was 25 kV, and an additional pressure of 5 mbar was applied. Both analytes were detected by UV simultaneously at 215 nm, Zn-BC alone at 192 and 254 nm, and NYS alone at 305 nm. The method was shown to be specific, accurate (recoveries were 100.0 ± 0.6% and 100.1 ± 0.6% for Zn-BC and NYS, respectively), linear over the tested range (correlation coefficients 0.9991 and 0.9994), and precise (RSD below 1.3% for both analytes). The method was applied to determine Zn-BC and NYS as additives in animal feed.

Keywords: Animal feed additives / Bacitracin / Micellar electrokinetic capillary chromatography / Nystatin

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1 Introduction

Bacitracin (BC), a polypeptide antibiotic produced by strains of *Bacillus licheniformis* and *Bacillus subtilis*, is a mixture of many structurally related compounds including BCs A–I [1]. It has been shown that BC A is the main component and has the most potent activity. BC is commonly used against Gram-positive organisms, especially as an animal feed additive [1]. It has frequently been used in association with zinc (Zn-BC complex), because this combination is more stable than BC alone. It has been used as an antibiotic with growth-promoting effects and feed conversion in poultry, pigs, and cattle. Since 1 January, 1999, the European Union has forbidden the use of Zn BC as an additive in animal feed [2]. BC methylene disalicylate is a stable form currently used as an additive in animal diets and in feedstuffs.

Nystatin (NYS), also known as mycostatin, is an antifungal antibiotic used to treat yeast and yeast-like fungal infections. This group of antibiotics is commonly referred to as polyene macrolide antifungal, because they comprise a macrocyclic ring of carbon atoms closed by lactonization and contain a series of conjugated carbon double bonds. NYS contains a diene and hydroxylated tetraene moiety attached to mycozamine. It is prescribed for treatment of superficial oral and intestinal infections caused by *Candida* species, and is used in different pharmaceutical preparations. A liquid chromatographic determination was reported for its analysis [3]. Commercially available NYS can be a mixture of two or more biologically active components, of which NYS A_1 is the most common. NYS is also used as animal feed additive, but to a lesser extent than BC [4]. Both macromolecules (BC, NYS) are unstable at high temperatures and in light [5].

In order to characterize the BC components, several separation methods have been reported. TLC enabled eight to ten components to be separated [6]. Counter-current chromatography and HPLC were used for the separation of Zn-BC [7, 8].

TLC and HPLC [9], LC [3], and derivative spectrometry [10] have been used for the identification and determination of NYS.

CE has become an important liquid separation technique, complementary to LC. One of the commonly used CE modes is MEKC, which is efficient for the separation of both neutral and ionic analytes. In MEKC, success of the separation is based mainly on appropriate selection of the surfactant. Zn-BC and NYS have been determined in pharmaceutical formulations by MEKC, using SDS as a

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Abbreviations: BC, bacitracin; **NYS,** nystatin; **BP,** British Pharmacopoeia

surfactant [11–15]. Kang *et al.* [16] have used MEKC with PAPS and Brij 35 as surfactants to separate BC components.

We studied the influence of running pressure on the separation of Zn-BC and NYS and validated the method. The method was tested with animal feedstuff spiked with the investigated additives.

2 Materials and methods

2.1 Apparatus

MEKC was performed on the HP^{3D} CE system (Hewlett Packard, Waldbronn, Germany) with a diode-array detector, controlled by HP ChemStation software. Compounds were separated on a 48 cm (40 cm to the detector) × 50 μm id fused-silica capillary (with bubble cell, 150 μm) (Agilent, Waldbronn, Germany).

A Consort C-831 pH meter (Turnhout, Belgium) was used for pH measurement.

2.2 Reagents and solutions

All solvents and reagents were of analytical grade unless indicated otherwise. Solutions were prepared with deionized water (Milli-Q-quality). NYS was obtained from SB TRADE (Belgrade, Serbia) and Zn-BC from Krka (Novo mesto, Slovenia); the quality of both was according to BP requirements. Animal diet and feedstuff mixtures for pigs TSV-2 (protein min 14%, crude fat max 10%, ash max 9%, crude fiber max 7%, calcium max 0.8%, phosphorus min 0.45%, sodium min 0.15%, methionine 0.25%, cystine 0.2%, and lysine 0.70%), and cattle KM-19 (protein min 19%, ash max 10%, crude fiber max 15%, calcium max 0.8%, phosphorus min 0.50%, sodium min 0.20%, and NaCl max 1%), were manufactured by the Institute for Hygiene and Pathology of Animal Nutrition (Ljubljana, Slovenia). Vitamins A, D_3, E, C, and B complex, as well as zinc, manganese, sulfur, potassium, magnesium, iodine, cobalt, copper, and iron are also present in these mixtures. Animal feed for pigs TS-2 (protein min 14%, crude fat max 10%, moisture max 13.5%, crude fiber max 7%, calcium max 0.8%, phosphorus min 0.45%, sodium min 0.15%, methionine + cystine 0.45%, lysine 0.70%, vitamins A, D_3, E, C, B complex, zinc, manganese, selenium, iodine, cobalt, copper, iron), and chicken feed TKN-2 (protein min 15%, moisture max 13.5%, crude fiber max 8%, calcium 3–4%, phosphorus 0.5–0.8%, sodium 0.15–0.20%, methionine + cystine 0.55%, vitamins A, D_3, E, K_3, C, B complex, zinc, manganese, selenium, iodine, cobalt, copper, iron) were manufactured by Fooder (Zupanja, Croatia).

Buffer solutions were prepared by dissolving the appropriate amount of NaH_2PO_4 (15 mM) and $Na_2B_4O_7$ (15 mM) in deionized water and the pH was adjusted to 8.2 with H_3PO_4 (10% v/v). All these reagents were p.a. from ZORKA (Sabac, Serbia). SDS was from Acros Organics (Geel, Belgium).

The BGE was 15 mM borate + 19 mM phosphate buffer, pH 8.2, containing 20 mM SDS and 10% methanol.

2.2.1 Preparation of standard stock solutions

Stock solutions of Zn-BC and NYS were prepared by weighing 50 mg of the drugs and dissolving in 50 mL methanol–deionized water (1:1 v/v). The stock solutions were diluted with methanol–deionized water (1:1 v/v) to obtain the concentration ranges required (10–60 mg/L for Zn-BC and 5–85 mg/L for NYS).

2.2.2 Sample preparation and extraction

Spiked feed mixtures were prepared by grinding 1 kg of feedstuff and adding 50 mg of each Zn-BC and NYS. To prepare extracts, blank feed mixture or feed mixture with added Zn-BC and NYS were weighed (100 g) and extracted with methanol (5 × 20 mL), first by shaking, then in an ultrasonic bath for 15 min. The extracts were combined, filtered, transferred to 100 mL volumetric flask, and diluted with methanol. Different known volumes were placed in 10 mL calibrated flasks and diluted to volume with 30:70 v/v methanol–deionized water.

2.3 Operating conditions

The capillary was conditioned prior to its first use by flushing with 0.1 M NaOH for 20 min and then with water for 10 min. In the optimized method, the capillary was conditioned at the beginning of each day with methanol under high pressure for 2 min, then rinsed for 2 min with 0.1 M NaOH and for 3 min with BGE. This was followed by hydrodynamic sample injection at 600 mbar. Separations were performed at 25 kV and 25°C (under a running pressure of 5 mbar) in 15 min; under these conditions the current was 47–48 μA. UV detection was at 192 and 254 nm for Zn-BC and 305 nm for NYS, and 215 nm for the mixture.

3 Results and discussion

Preliminary investigations to optimize the separation of Zn-BC and NYS were carried out. Recently, an MEKC method was published for the determination of hydrocortisone and its most important associated compounds (including Zn-BC and NYS) in topical pharmaceutical preparations [11, 12]. Since we were only concerned with a binary mixture of Zn-BC and NYS in our study, some modifications of the SDS concentration and running pressure were assessed to obtain a shorter run time.

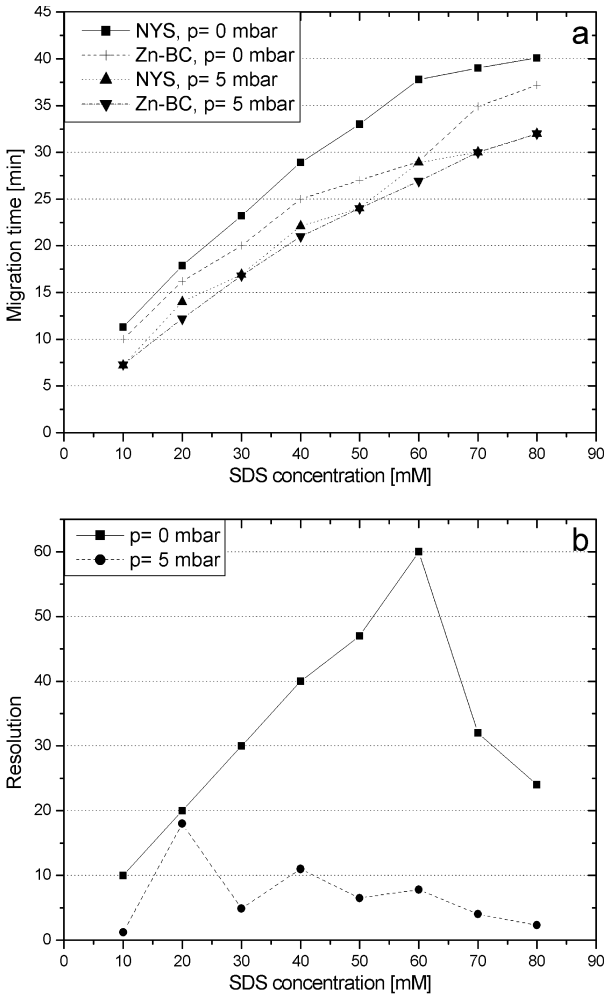

Figure 1. Effect of SDS concentration on (a) migration time (NYS and Zn-BC with and without applied pressure) and (b) resolution with and without applied pressure. Electrolyte solution was 19 mM phosphate/15 mM borate buffer, pH 8.2 containing 10% methanol, and the temperature and voltage were 25°C and 25 kV.

The effect of running voltages in the range of 5–30 kV was tested using a BGE of 15 mM borate/19 mM phosphate buffer, pH 8.2, containing 60 mM SDS and 10% methanol, without running pressure, at 25°C. An acceptable level of baseline noise was achieved by performing experiments at 25°C, and the best results were obtained at 25 kV.

The SDS concentration dramatically affects the migration time of Zn-BC and NYS (Fig. 1a). An SDS concentration of 60 mM was found to be the best compromise (Fig. 1b) for the analysis without applied pressure as it gave the best resolution and symmetrical peaks in all cases, although with a longer analysis time (40 min) (Fig. 1a – curves NYS and Zn-BC at p = 0 mbar).

Running pressures in the range 0–30 mbar were tested, using the above experimental conditions. As expected, decreasing migration times were obtained with increasing applied pressure. A pressure of 5 mbar was selected as optimum because it gave the best resolution and symmetric peaks in all cases, with a shorter run time (15 min). An SDS concentration of 20 mM with an applied running pressure of 5 mbar was selected for analysis as it gave the best resolution (Fig. 1b) without broadened or deformed peaks.

The best results were at 25 kV (with applied pressure of 5 mbar). UV detection was at 192, 215, and 254 nm for Zn-BC and at 215 and 305 nm for NYS.

Under the optimal conditions, both peaks could be obtained within 15 min. The electropherograms for standards and test samples are presented in Fig. 2. The main peaks were identified as Zn-BC and NYS and, from the data obtained at 215 nm, their electrophoretic mobilities were 9.04×10^{-5} and 7.76×10^{-5} cm^2/V.s for standards, and 8.07×10^{-5} and 7.14×10^{-5} cm^2/V.s for test samples, respectively.

3.1 Validation of the method

The procedures and the characteristics used for validation were those described in USP 24 [17], the International Conference of Harmonization (ICH) Guidelines [18, 19], and other literature [20–24].

3.1.1 Selectivity

The selectivity of the method was investigated by observing interfering peaks from matrix present in the feedstuffs. Four different feedstuff mixtures, namely two for pigs and one each for cattle and chicken, were tested for the interferences. There was no interference in MEKC results by the feed's ingredients in any of the tested mixtures, which indicates that the method is selective. An example of a matrix electropherogram for cattle feed is shown in Fig. 2b.

3.1.2 Linearity

Linearity was determined by analyzing a series of standards at five different concentrations that span at least 80–120% of the expected working range of the assay [18, 19].

The linearity of calibration curves (peak area *vs.* concentration) for Zn-BC and NYS over the concentration ranges of 14–52 mg/L for Zn-BC and 8–82 mg/L for NYS gave correlation coefficients of 0.9991 and 0.9994, respectively (Table 1).

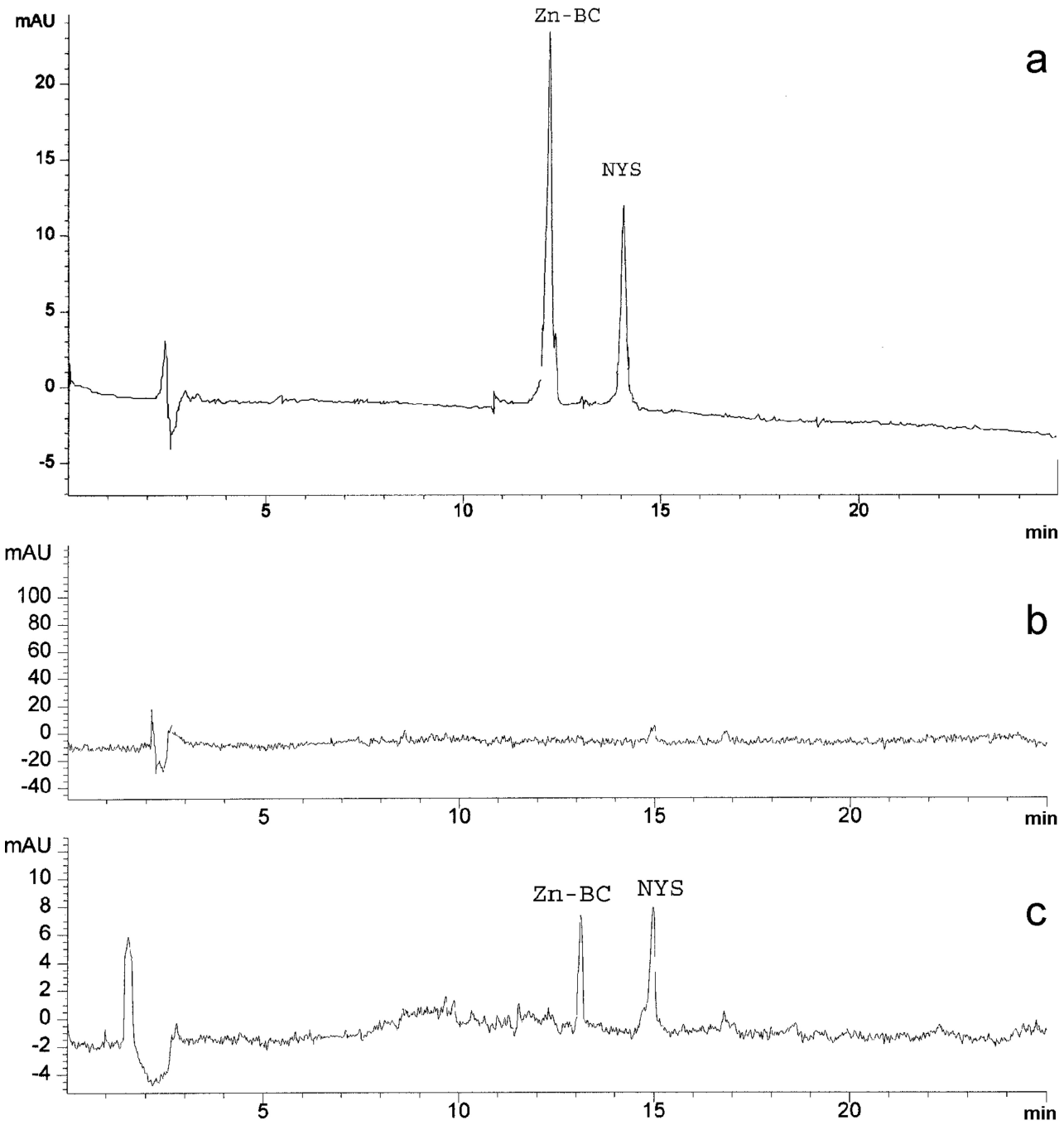

Figure 2. Electropherograms obtained at 215 nm for (a) 1 g/L Zn-BC and NYS standards, (b) placebo formulation (cattle feedstuff KM-19), (c) 50 mg/L Zn-BC and NYS from animal feedstuff under the optimized conditions. BGE was 19 mM phosphate + 15 mM borate buffer, pH 8.2, containing 20 mM SDS and 10% methanol, the running pressure was 5 mbar, the temperature and voltage were 25°C and 25 kV, respectively.

3.1.3 LOD and LOQ

LOD and LOQ were estimated by the baseline noise method. Baseline noise was evaluated by recording the detector response over a period of ten times the peak width. LOD and LOQ, respectively, were defined as the analyte concentrations resulting in peaks of height three and ten times the baseline noise level [25]. For Zn-BC, LOD and LOQ were 4.72 and 14.27 mg/L, and for NYS 2.96 and 8.31 mg/L, respectively (Table 1).

Table 1. Statistical parameters of the calibration curve (linear regression) for each compound, with LOD and LOQ values

	Zn-BC	NYS
Intercept	−5.4 ± 5.7	1.5 ± 2.5
Slope	2102 ± 9.2	2047 ± 6.8
R	0.9991	0.9994
Linear range (mg/L)	14.1–52.4	8.2–81.5
LOD (mg/L)	4.72	2.96
LOQ (mg/L)	14.27	8.31

3.1.4 Accuracy

Accuracy was determined by analyzing a solution of known concentration (working standard solution) and comparing the measured and known values. The mean recovery was 100.0 ± 0.6% and 100.1 ± 0.6% for Zn-BC and NYS, respectively, proving a good accuracy of the method (Table 2).

3.1.5 Precision

Precision can be measured as repeatability, reproducibility, and intermediate precision. In this work, only repeatability and intermediate precision were studied – reproducibility refers to the results of the same analytical procedure in different laboratories.

3.1.5.1 Repeatability

Standard solutions of concentrations 10, 30, and 50 mg/L were analyzed (six replicates each) (Table 3). The RSD values for migration times (1.76% for Zn-BC and 2.16% for NYS) and for peak areas (1.05–5.70% for Zn-BC and 1.10–2.06% for NYS) indicate that the repeatability of the method is acceptable (Table 3).

3.1.5.2 Intermediate precision

Working solutions of concentrations 10–85 mg/L were injected on each day for 3 days under the same conditions and the results were used for the repeatability study. When stored in the dark under refrigeration, the recovery ranged from 100.5 to 98.5% over 3 days. The RSD values (0.42–0.61% for Zn-BC and 0.33–0.75% for NYS) indicate that the intermediate precision of the proposed MEKC method is acceptable. Decreasing recovery values from 100.37 to 93.31% for Zn-BC and from 100.11 to 93.88% for NYS were observed for the standards in methanolic solutions when they were stored in sunlight at room temperature.

3.1.6 Robustness

The parameters of the optimum MEKC conditions were slightly modified in order to evaluate the robustness. The effects of different concentrations of organic modifier (10 ± 0.5% methanol) and SDS (20 ± 1 mM) in the mobile phase, as well as the effects of buffer pH (8.2 ± 0.06), capillary temperature (25 ± 5°C), running voltage (25 ± 1 kV), and detection wavelength (±3 nm), were determined. The design applied was the fractional factorial design [24]. No significant variations in specificity, accuracy, and precision were found over the tested ranges, which indicated good reproducibility of the method.

Table 2. Determination of accuracy in samples of known concentration

Theoretical concentration (mg/L)	Experimental concentration (mg/L)		Recovery (%)	
	Zn-BC	NYS	Zn-BC	NYS
20	20.3	20.1	101.50	100.50
35	34.8	35.2	99.43	100.57
50	49.7	49.5	99.40	99.00
65	65.2	65.0	100.31	100.00
80	79.8	80.2	99.75	100.25
	Mean ± SD		100.03 ± 0.55	100.06 ± 0.58

Table 3. Determination of repeatability

Theoretical concentration (mg/L)	Zn-BC		NYS	
	Migration time [a)]	Peak area[a)]	Migration time[a)]	Peak area[a)]
10	11.671 ± 0.004	15.59 ± 0.89	13.614 ± 0.005	21.66 ± 0.45
30	12.058 ± 0.002	57.21 ± 0.93	13.754 ± 0.368	62.31 ± 0.72
50	12.130 ± 0.066	99.23 ± 1.04	14.122 ± 0.039	103.08 ± 1.13

a) Mean ± SD (N = 6).

3.1.7 Stability of BC and NYS

According to the literature [5] Zn-BC and NYS are unstable in acidic and basic solutions, their stability being highest in the pH range 6–10. The stability of Zn-BC and NYS in acidic (pH <6) and basic (pH >10) solutions was checked in test samples at room temperature for 24 and 48 h and the recoveries were, respectively, 97.5 and 98.2% at 24 h and 95.8 and 97.1% at 48 h.

The stability in the BGE and methanol–deionized water (30:70 v/v) was also checked at 24 h. Recoveries of both compounds were 99.5%, indicating good stability. The possible photodegradation of Zn-BC and NYS in methanolic solution and BGE was also studied for samples of Zn-BC and NYS exposed to direct sunlight, UV light and darkness for 7 days. The samples kept in the dark at room temperature showed full recovery without significant degradation. However, Zn-BC and NYS sample solutions when exposed to sun and UV light showed photodegradation of about 7 and 6%, respectively.

4 Concluding remarks

The results show that MEKC is a useful technique for rapid separation (15 min) of Zn-BC and NYS at low concentration of surfactant (SDS 20 mM) and pH 8.2 (phosphate-borate buffer), when a running pressure of 5 mbar was applied. This system was also applied successfully to their identification and quantitative determination in animal feedstuff spiked with Zn-BC and NYS.

5 References

[1] Brelver, G. A., *Anal. Profiles Drug Subst.* 1980, *9*, 1 – 69.

[2] Molterer, W., *Off. J. Eur. Commun.: Legis.* 1998, *41*, L351/4 – L351/8.

[3] Wilson, P., Stewart, A., Flournoy, V., Zito, S. W., Vancura, A., *J. AOAC Int.* 2001, *84*, 1050 – 1055.

[4] Botsoglou, N. A., Fletouris, D. J., *J. Agric. Food Chem.* 1996, *44*, 1271 – 1274.

[5] Parfitt, K. (Ed.), *Martindale: The Complete Drug Reference,* 32nd Edn., Pharmaceutical Press, London 1999.

[6] Oka, H., Ikai, Y., Kawamura, N., Yamada, M. *et al.*, *J. Chromatogr.* 1988, *449*, 448 – 454.

[7] Oka, H., Harada, K.–I., Ito, Y., Ito, Y., *J. Chromatogr. A* 1998, *812*, 35 – 52.

[8] Capitan-Vallvey, L. F., Titos, A., Checa, R., Navas, N., *J. Chromatogr. A* 2002, *943*, 227 – 234.

[9] Thomas, A. H., Newland, P., Quinlan, G. J., *J. Chromatogr.* 1981, *216*, 367 – 373.

[10] Korany, M. A., Mahgoub, H., *Pharmazie* 1991, *46*, 883 – 885.

[11] Gallego, J. M. L., Arroyo, J. P., *Chromatographia* 2002, *56*, 455 – 462.

[12] Gallego, J. M. L., Arroyo, J. P., *J. Chromatogr. B* 2003, *784*, 39 – 47.

[13] Gallego, J. M. L., Arroyo, J. P., *Chromatographia* 2003, *58*, 277 – 281.

[14] Gallego, J. M. L., Arroyo, J. P., *Anal. Bioanal. Chem.* 2003, *375*, 617 – 622.

[15] Gallego, J. M. L., Arroyo, J. P., *J. Liq. Chromatogr. Relat. Technol.* 2003, *26*, 1011 – 1025.

[16] Kang, J. W., De Reymaeker, G., Van Schepdael, A., Roets, E., Hoogmartens, J., *Electrophoresis* 2001, *22*, 1356 – 1362.

[17] *United States Pharmacopoeia 24 – National Formulary 19*, United States Pharmacopoeial Convention, Rockville, MD 2000, pp 185 – 186.

[18] International Conference on Harmonization, guideline Q2A, *Federal Register* 1995, *60*, 11260.

[19] International Conference on Harmonization, guideline Q2B, *Federal Register* 1997, *62*, 27463 – 27467.

[20] Vander Heyden, Y., Jimidar, M., Hund, E., Niemeijer, N. *et al.*, *J. Chromatogr. A* 1999, *845*, 145 – 154.

[21] Shabir, G. A., *J. Chromatogr. A* 2003, *987*, 57 – 66.

[22] Brown, R., Caphart, M., Faustino, P., Frankewich, R. *et al.*, *LC-GC* 2001, *19*, 74 – 79.

[23] Toro, I., Dulsat, J. F., Fabregas, J. L., Claramunt, J., *J. Chromatogr. A* 2004, *1043*, 303 – 315.

[24] Altria, K. D., Chanter, Y. L., *J. Chromatogr.* 1993, *652*, 459 – 463.

[25] Heyden, Y. V., Nijhuis, A., Smeyers-Verbeke, J., Vandeginste, B. G. M., Massart, D. L., *J. Pharm. Biomed. Anal.* 2001, *24*, 723 – 753.

