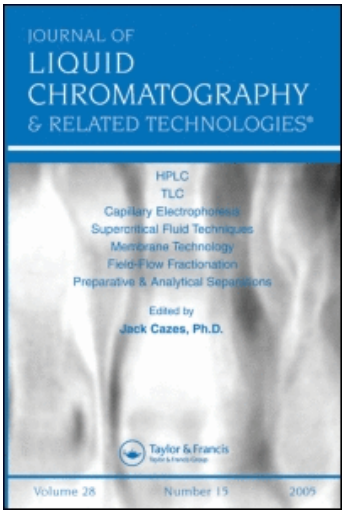

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Automated on-Line Dialysis and Column-Switching HPLC Determination of Flumequine and Oxolinic Acid in Fish Liver

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AUTOMATED ON-LINE DIALYSIS AND COLUMN-SWITCHING HPLC DETERMINATION OF FLUMEQUINE AND OXOLINIC ACID IN FISH LIVER

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ABSTRACT

An automated method for residue analysis of oxolinic acid and flumequine in liver of Atlantic salmon is described. Oxolinic acid and flumequine are extracted from liver with 0.4 M phosphate buffer pH 10 and the extracts are automatically analysed by on-line dialysis and column-switching in an HPLC system. The limit of detection was 4 µg/kg for oxolinic acid and 7 µg/kg for flumequine with fluorescence detection. The on-line combination of dialysis and column-switching HPLC was shown to be a reliable technique for residue control of these drugs in fish liver.

INTRODUCTION

The extensive use of oxolinic acid and flumequine by the fish farming industry for treatment of bacterial infections in fish has created a demand for automated methods for residue control of these drugs in fish. Usually liver samples and eventually muscle samples are collected from Atlantic salmon for drug residue analysis. Because of the

complex nature of the matrix sample preparation is an essential part of a drug residue analysis.

Several methods based on high-performance liquid chromatography (HPLC) have been published for the determination of oxolinic acid and flumequine in fish tissue (1-7). All methods apply extraction of the compounds with organic solvents and the manual work-up procedures include treatment such as liquid-liquid extraction (1-6) or solid-phase extraction (5-7). In principle all these methods can be automated by using modern technology such as robotic systems, but many problems may arise because of the complex matrix.

Recently the on-line combination of dialysis and column-switching high-performance liquid chromatography (HPLC) has been used for automated analysis of drug residues in edible products (8-10). The analytes were extracted from tissue with an aqueous solvent while liquid products such as milk and eggs were decreamed and/or diluted before injection into the dialyser. On-line dialysis removes proteins and other macromolecular compounds from the sample. The dialysate containing the analytes is cleaned and concentrated on a trace enrichment column. Upon column-switching the analytes are eluted from the trace enrichment column into the analytical column of the liquid chromatograph. Dialysis efficiencies of 50-60 % can be achieved in 5-6 min. Nitrofuran residues in edible products such as milk, eggs and meat have been automatically determined by on-line dialysis and column-switching HPLC. The limits of detection ranged from 1 to 10 µg/kg with UV detection (10). Aflatoxin M1 was determined in milk with a limit of detection of 50 ng/kg with fluorimetric detection (9). On-line dialysis and column switching HPLC has also been used for the determination of oxolinic acid and flumequine in edible muscle of Atlantic salmon (11). The drugs were extracted from muscle with 0.05 M phosphate buffer pH 9. The limit of detection was 2-3 µg/kg with fluorimetric detection. This procedure was not found useful for the analysis of oxolinic

acid and flumequine in liver from Atlantic salmon because the extraction recovery was low. This paper describes an optimized procedure for analysis of oxolinic acid and flumequine in salmon liver.

EXPERIMENTAL

Chemicals

Oxolinic acid was supplied by Sigma (St. Louis, MO, U.S.A.) and flumequine was purchased from Solchemn Italiana (Mulazzano, Italy). Sodium dihydrogenphosphate, sodium hydrogencarbonate, disodium tetraborate, sodium hydroxide and orthophosphoric acid were all of analytical-reagent grade from E. Merck (Darmstadt, F.R.G). HPLC-grade acetonitrile and tetrahydrofuran were from Rathburn (Walkerburn, U.K.). HPLC grade water was obtained from a Milli-Q (Millipore, MA, U.S.A.) water purification system.

Preparation of standards

Standard solutions of flumequine and oxolinic acid (1 mg/ml) were prepared in 0.01 M sodium hydroxide. Working standards (2 µg/ml) were prepared by dilution with 0.05 M phosphate buffer pH 10. Spiked liver standards were prepared from the working standards. Liver from Atlantic salmon were kept at - 20°C until analysis.

Extraction of liver

0.4 M phosphate buffer pH 10 was used for extraction of the drugs from the liver of Atlantic salmon. Flumequine was used as internal standard for the determination of oxolinic acid and oxolinic acid as internal standard for flumequine. The internal standard solution (2 µg/ml) was prepared in 0.05 M phosphate buffer pH 10 for determinations in the concentration range of 50-200 µg/kg. To 2 grams of liver were added 200 µl internal standard solution, 20 ml buffer

and 5 ml hexane. The mixture was homogenized for 1 min in an Ultra-Turrax T 25 homogenizer (Ika Werk, Staufen, F.R.G.) and sonicated for 4 min. The mixture was then centrifuged for 5 min (1920 g). The upper hexane phase was discarded. The aqueous extract was filled into the autosampler vials and automatically injected into the dialyser of the sample preparation system.

Automated injection, on-line dialysis and concentration of the dialysate

The sample preparation system was a Gilson ASTED (Automated Sequental Trace Enrichment of Dialysates) unit (Gilson Medical Electronics, Villiers-le Bel, France) consisting of a Model 231 Auto-Sampling Injector, two 401 dilutors equipped with 1 ml syringes and one flat-bed dialyser with a donor channel volume of 370 µl and a recipient channel volume of 650 µl, fitted with a cuprophan membrane, 15 kD molecular weight cut-off. An automated six port Model 7010 valve (Rheodyne, Berkely, CA, USA) connected a trace enrichment column either with the recipient channel of the dialyser or with the mobile phase used in the analytical column of the HPLC system. The trace enrichment column (10mm x 2mm) from Chrompack (Middelburg, The Netherlands) was packed with 36 µm particle size polystyrene-divinylbenzene (Dynospheres,Dyno Particles A.S., Lillestrøm, Norway).

High-performance liquid chromatography

The liquid chromatograph was an LC 6A (Shimadzu, Kyoto, Japan) equipped with a Model RF 535 (Shimadzu) fluorescence detector with a 12 µl flow cell operated at an excitation wavelength of 325 nm and an emission wavelength of 365 nm. Peak heights were recorded on a Chromatopac C-R3A integrator (Shimadzu). The analytical column (150 x 4,6mm I.D.) was packed with 5 µm particle diameter polystyrene-divinylbenzene PLRP-S (Polymer Labs., Churchstretton, U.K.).

ASTED procedure

The ASTED unit was operated in the concurrent mode with automated injections every 14 min. One dilutor was used to inject 370 µl extract into the donor channel of the dialyser. The sample was held static in the donor channel for 6 min 24 s while the other dilutor transported 4 ml of 0.02 M phosphate buffer pH 5 in the pulsed mode through the recipient channel of the dialyser and into the precolumn. The recipient solution was transported through the dialyser at a flow rate of 1,5 ml/min and was divided in 6,15 pulses of 650 µl. After each pulse the recipient solution was held static in the recipient channel for 33 s. Upon switching of the six-port valve the analytes that had been concentrated in the trace enrichment column were brought into the analytical column by elution for 2 min with the HPLC mobile phase. The mobile phase was acetonitrile-tetrahydrofuran-0.02 M orthophosphoric acid (20:14:66, v/v) delivered at a flow rate of 0.7 ml/min. The six-port valve was then switched to bring the precolumn back to the recipient channel of the dialyser. The recipient side of the dialyser and the precolumn was then washed with 2 ml of 0.02 M phosphate buffer pH 5. The donor side of the dialyser was simultaneously washed with 2 ml of 0.02 M phosphate buffer pH 5 containing 100 mg/l Triton X. After the washing procedure the next sample was injected into the dialyser.

Evaluation of extraction efficiency

Phosphate, carbonate and borate buffers having pH values in the range of 7-10 were investigated for extraction of the drugs from liver. 2 g liver spiked with flumequine and oxolinic acid (200 µg/kg) were extracted with 20 ml buffer and 5 ml hexane. 370 µl aqueous extract was injected into the dialysis cell. After dialysis and trace enrichment the analytes were separated on the analytical column. The peak heights were recorded and the recovery and the reproducibility was calculated. The standard curve for measuring the recovery was set up after analysis of

standards according to the same dialysis and trace enrichment procedure. The standards were dissolved in 0.4 M phosphate buffer pH 10.

Validation of the procedure

The standard curves, accuracy and precision of the method were evaluated by analysis of spiked liver samples. The calibration curves were based on measuring the peak height ratios relative to the internal standard.

RESULTS AND DISCUSSION

Extraction procedure

Flumequine and oxolinic acid are acids with low solubility in water. Their solubility increases in alkaline solutions. To obtain a high extraction recovery of these drugs from tissue an alkaline buffer must be selected as extraction solvent. Hexane is used for removal of lipids. The buffer should have a favourable pH for dissolution of the drugs and the buffer capacity must be high enough to maintain the desired pH after extraction of the tissue. To reduce handling time a single extraction is desirable. 0.05 M phosphate buffer pH 9 has previously been used for extraction of oxolinic acid and flumequine from the edible muscle tissue of Atlantic salmon. The pH of the supernatant of 8.7 gave a reproducible recovery of 85% for both oxolinic acid and flumequine (11). The liver from Atlantic salmon has a high buffer capacity and a high buffer strength is necessary to maintain the pH. Table 1 shows the pH measured in the supernatant after extraction of liver with 0.025 - 0.8 M phosphate, carbonate and borate buffers pH 10. Of these buffers borate had the highest buffer capacity for extraction of liver. However, 0.13 M is the solubility limit for preparation of borate buffers (12) and by storing 0.1 M buffers at 4° C precipitation may easily occur. Borate buffers were therefore omitted from

TABLE 1
pH IN EXTRACTS AFTER EXTRACTION OF FISH LIVER WITH PHOSPHATE, CARBONATE AND BORATE BUFFER pH=10.

Concentration (M)	Phosphate pH	Carbonate pH	Borate pH
0.025			9.53
0.05	7.90	9.36	9.76
0.10	8.22	9.62	9.86
0.20	9.10	9.79	
0.40	9.80	9.98	
0.60	9.82	10.09	
0.80	10.04	10.01	

further investigation. Table 1 shows that a buffer strength equal to or larger than 0.4 M maintains the desired pH in the extract. The extraction recovery increases by increasing the pH of the extract as shown in Fig. 1 for phosphate buffer. A single extraction with 0.4 M buffer pH 10 gives an extraction recovery larger than 80%. The recovery and reproducibility of the extraction from liver with 0.4 M phosphate and carbonate buffers pH 10 are shown in Table 2. Both buffers gave reproducible extractions for both oxolinic acid and flumequine with a recovery of 83% for phosphate buffer and 80% for carbonate buffer. Phosphate buffer was selected for further studies allowing only one buffer system to be used through the whole analytical procedure i. e. extraction, on-line clean-up and HPLC analysis.

On-line dialysis

Schematic representations of the ASTED-HPLC system has earlier been shown in several publications (9,10,13). In this procedure the ASTED was operated in the concurrent mode

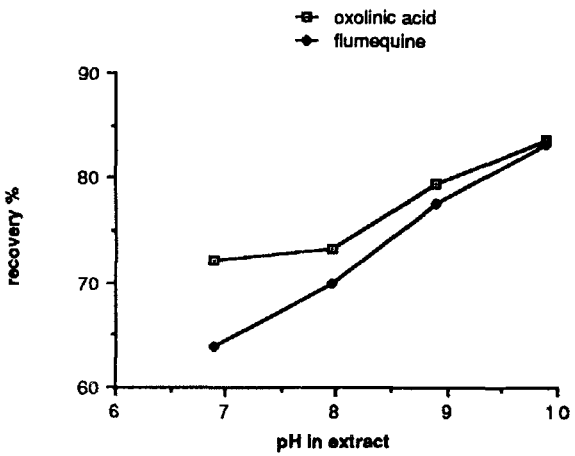

Fig. 1.
Recovery of oxolinic acid and flumequine extracted from fish liver with 0.4 M phosphate buffer pH 7-10.

TABLE 2
RECOVERY AND REPRODUCIBILITY AFTER EXTRACTION OF 2 g LIVER WITH 20ml 0.4 M PHOSPHATE AND CARBONATE BUFFER pH=10

Compound	Recovery ± C.V. (%), n=6 Phosphate	Carbonate
oxolinic acid	83.6 ± 1.9	78.9 ± 1.6
flumequine	83.2 ± 2,8	81.1 ± 2.0

with static-pulsed dialysis i.e. the sample is held static in the donor channel of the dialyser while the recipient solvent is delivered as pulses through the recipient channel of the dialyser. This mode gives a high dialysis efficiency and is preferred for the analysis of very dilute solutions such as extracts of drugs from tissue. Static-pulsed dialysis was also used for the analysis of oxolinic acid and flumequine in extracts from muscle of Atlantic salmon (11). The donor channel of the dialyser (370 µl) is filled with the extract and the volume of recipient solvent aspirated at each pulse is equal to the acceptor channel volume (650 µl). When the ASTED is operated in the concurrent mode the fastest process will always wait for the slowest process. In the present method, the HPLC separation of the analytes is the slowest process. The dialysis time being 6 min 24 s the ASTED cycle (dialysis of the sample, trace enrichment of the dialysate, elution of the concentrated analytes from the trace enrichemt column into the analytical column and regeneration of the system) was finished within each chromatogram allowing continous injections of new samples every 14 min.

Fig. 2 shows the dialysis efficiencies of oxolinic acid and flumequine when they are dialysed from liver extracts in the pulsed mode and the extract was held static in the donor channel for 6 min 24 s while 1-8 ml recipient solution was pulsed through the recipient channel. The experiment was carried out by spiking drug-free liver extracts with oxolinic acid and flumequine (200 µg/kg) and the efficiency was determined by peak height measurements from a calibration graph set-up after direct injections of the drugs into the analytical column. 1 ml aspirated as one pulse of 650 µl and one pulse of 350 µl with a residence time of 2 min 55 s for each pulse volume in the recipient channel of the dialyser gave a dialysis efficiency of 37% for both analytes. The dialysis efficiency increases when the volume of recipient solution is increased. 4 ml

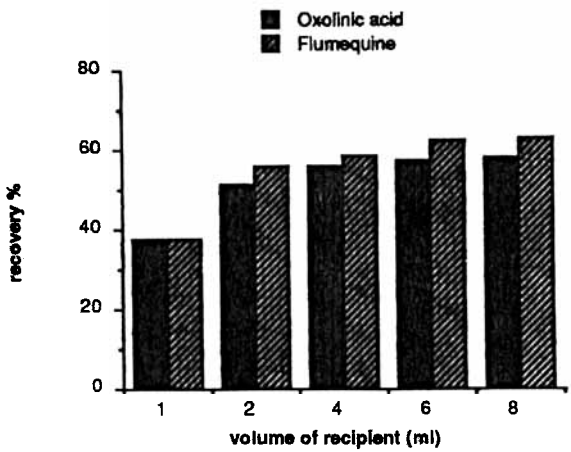

Fig. 2
Dialysis efficiency of oxolinic acid and flumequine plotted against the volume of recipient solvent aspirated in the pulsed mode through the recipient channel of the dialyser. For experimental conditions see text.

recipient solution aspirated as 6 pulses of 650 µl and one pulse of 100 µl with a residence time of 33 s for each pulse volume gave an extraction efficiency of 56% for oxolinic acid and 58% for flumequine. Only a slight increase in efficiency was observed when the samples were dialysed against larger resipient volumes. Dialysis was therefore carried out with 4 ml of recipient solution.

The matrix did not influence dialysis efficiency. The same peak heights were obtained after dialysis of standards dissolved in buffer as in standards dissolved in drug-free liver extracts. Accordingly, the recoveries calculated for evaluation of the extraction procedure could be determined by peak height measurements from a calibration graph set-up after dialysis of the drugs in buffer.

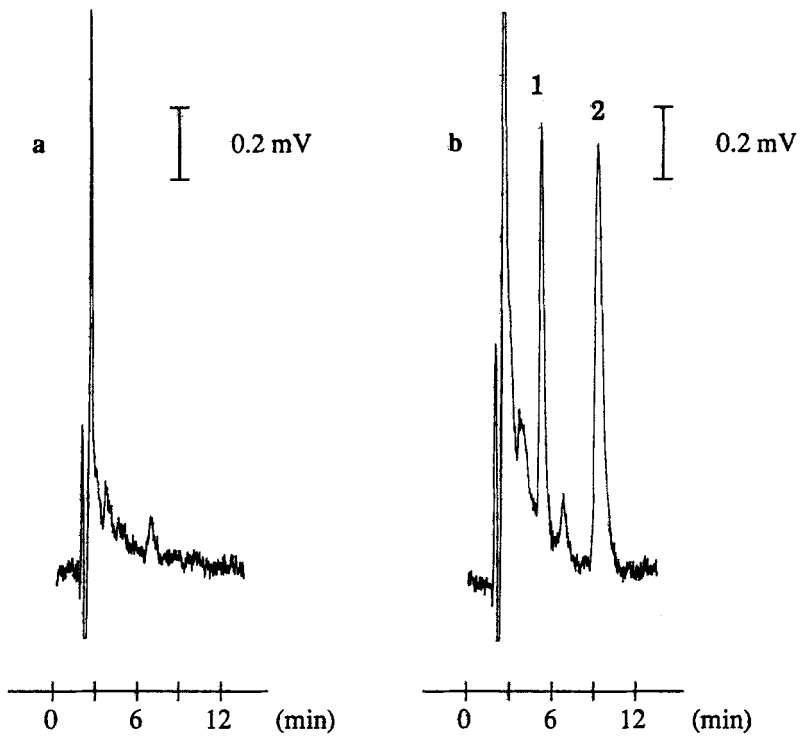

Fig. 3
Chromatograms of (a) an extract of drug-free liver and (b) an extract of liver spiked with 50 µg/kg of oxolinic acid and 100 µg/kg of flumequine after on-line dialysis and concentration of the dialysate on the precolumn. Detection: fluorescence with exitation at 325 nm and emission at 365 nm. Peaks: 1 = oxolinic acid; 2 = flumequine. For chromatographic conditions see text.

TABLE 3
ACCURACY AND PRECISION OF THE METHOD AFTER EXTACTION OF FISH LIVER WITH 0.4 M PHOSPHATE-BUFFER pH=10 AND ON-LINE DIALYSIS.

Compond	Concentration added ng/g	Concentration found mean ± C.V., n=5
oxolinic acid	50	48.2 ± 1.5
	200	191.9 ± 3.4
flumequine	50	53.5 ± 1.9
	200	206.3 ± 4.7

Trace enrichment of the dialysate

A trace enrichment column packed with polystyrene-divinylbenzene was used for trace enrichment of the dialysate. This precolumn has earlier been shown to retain the analytes strongly when used in column-switching systems with acidic aqueous eluents (14). Elution with alkaline eluents may result in break-through of the analytes. In the present procedure an alkaline sample solution with pH 10 was dialysed. However, 0.02 M phosphate buffer pH 5 as recipient solution brings the analytes under acidic condition into the trace enrichment column and no break-through of the analytes was observed. Fig. 3 shows chromatograms of extracts of a drug-free liver and of a liver spiked with 50 µg/kg oxolinic acid and 100 µg/kg flumequine after on-line dialysis and concentration of the dialysate on the precolumn. As can be seen from the chromatograms a narrow front and no impurities interfering with the analytes is obtained. This shows that the on-line combination of dialysis and trace enrichment effectively removes macromolecules and smaller compounds which could have interfered with the fluorescence detection.

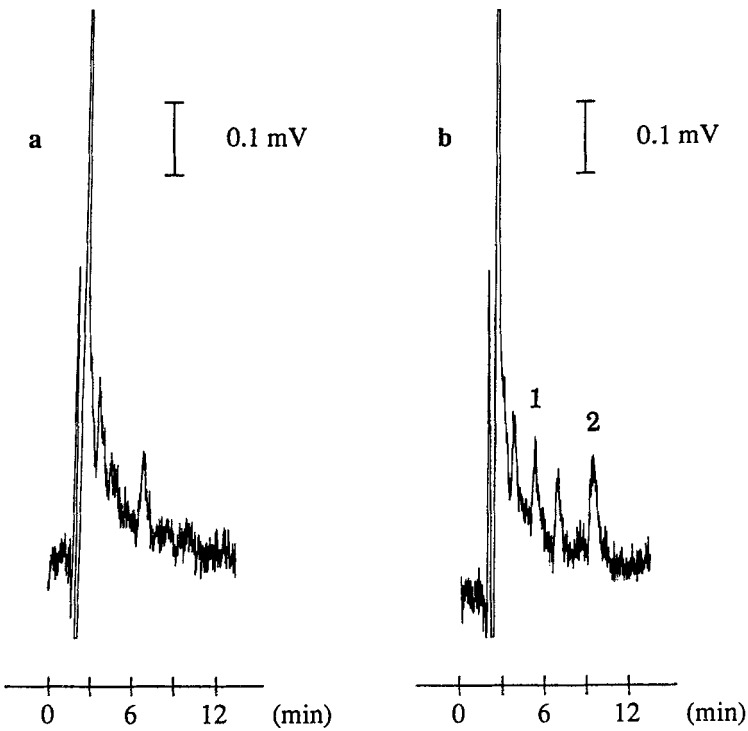

Fig. 4
Chromatograms of (a) an extract of drug-free liver and (b) an extract of liver spiked with 5 µg/kg of oxolinic acid and 10 µg/kg of flumequine after on-line dialysis and concentration of the dialysate on the precolumn. Detection: fluorescence with exitation at 325 nm and emission at 365 nm. Peaks: 1 = oxolinic acid; 2 = flumequine. For chromatographic conditions see text.

The polystyrene-divinylbenzene analytical column and the mobile phase are the same as has been described in previous publications for the analysis of oxolinic acid and flumequine (11,14).

Validation of the method

The internal standard added to the sample before extraction with phosphate buffer corrects for variation in recovery during extraction and dialysis. The calibration graphs obtained by plotting peak height ratios versus concentration in muscle were linear in the concentration range 50-1000 µg/kg with r=0.9999. Results from validation of the procedure are shown in Table 3. The coefficients of variation varied between 1.5-4.7% for samples spiked with 50 and 200 µg/kg of oxolinic acid and flumequine.

Fig. 4 shows chromatograms of an extract of drug-free liver and of an extract of liver spiked with 5 µg/kg oxolinic acid and 10 µg/kg flumequine where the detector was operated at the highest sensitivity. At a signal-to-noise ratio of 3 the limit of detection was estimated to 4 µg/kg for oxolinic acid and 7 µg/kg for flumequine.

CONCLUSION

The results presented in this paper show that oxolinic acid and flumequine can be extracted from the liver of Atlantic salmon with 0.4 M phosphate buffer pH 10 and that sample preparation and analysis of the extract can be fully automated by HPLC in a on-line dialysis and column-switching system. The on-line combination of dialysis and concentration of the dialysate on a trace enrichment column is a highly reliable technique for fully automated clean-up of fish liver extracts of oxolinic acid and flumequine.

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REFERENCES

1 N. Nose, Y. Hoshino, Y. Kikuchi, M. Horie, K. Saitoh, T. Kawachi and H. Nakazawa, J. Assoc. Off. Anal. Chem., 70 (1987) 714

2 S. Horii, C. Yasuoka and M. Matsumoto., J. Chromastogr., 338 (1987) 459

3 L. Ellerbroek and M. Bruhn, J. Chromatogr., 495 (1989) 314

4 O. B. Samuelsen, J. Chromatogr., 497 (1989) 355

5 A. Rogstad, V. Hormazabal and M. Yndestad, J. Liquid Chromatogr., 12 (1989) 3073

6 M. Horie, K. Saito, Y. Hoshino, N. Nose, E. Mochizuki, H. Nakazawa, J. Chromatogr., 402 (1987) 301

7 Y. Ikai, H. Oka, N. Kawamura, M. Yamada, K.-I. Harada, M. Suzuki and H. Nakazawa, J. Chromatogr., 477 (1989) 397

8 M. M. L. Aerts, W. M. J. Beek and U. A. Th. Brinkman, J. Chromatogr., 435 (1988) 97

9 L. G. M. Th. Tuinstra, P. G. M. Kienhuis, W. A. Traag, M. M. L. Aerts and W. M. J. Beek, J. High Resolut. Chromatogr., 12 (1989) 709

10 M. M. L. Aerts, W. M. J. Beek and U. A. Th. Brinkman, J. Chromatogr., 500 (1990) 453

11 T. H. Hoang, A. T. Andresen, T. Agasøster and K. E. Rasmussen, J. Chromatogr., in press

12 Martindale, The Extra Pharmacopeia, Pharmaceutical Press, London,28th ed.,1982, p.337

13 D. C. Turnell and J. D. H. Cooper, J. Chromatogr., 395 (1987) 613

14 K. E. Rasmussen, F. Tønnesen, T.H. Hoang, A. Rogstad and A. Aanesrud, J. Chromatogr., 496 (1989) 355