Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis 38 (2005) 133–138
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Application of capillary zone electrophoresis in the separation and determination of the curcuminoids in urine

Kailong Yuan, Qianfeng Weng, Hongying Zhang, Jianhui Xiong, Guowang Xu*

National Chromatographic R.&A. Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116011, PR China

Received 6 August 2004; received in revised form 3 December 2004; accepted 6 December 2004
Available online 12 January 2005

Abstract

The major components of the plant *curcuma longa* are the curcuminoids that include curcumin, demethoxycurcumin and bisdemethoxycurcumin. It has been reported the curcuminoids have some important activities. A new CZE method with diode array detection has been developed for the separation and determination of the curcumin, demethoxycurcumin and bisdemethoxycurcumin. Three curcuminoids could be readily separated within 7 min with a 15 mM sodium tetraborate buffer containing 10% methanol (v/v) at pH 10.8, 25 kV and 30 °C. The method has been validated and shows good performance with respect to selectivity, reproducibility, linearity, limits of detection and recovery. The proposed method was successfully applied to determine the curcuminoids in urine.

Keywords: Curcuminoids; Capillary zone electrophoresis; Urine; *Curcuma longa*

1. Introduction

Turmeric is a widely cultivated tropical plant (Curcuma domestica) of Asia and Central America, having yellow flowers and an aromatic, somewhat fleshy rhizome. The powdered rhizome of this plant, used as a condiment and a yellow dye, has been also very popular in Asian medicine for the treatment of coryza, hepatic disorders and rheumatism [1]. It is valued for its yellow coloring components (curcuminoids) and the main coloring pigment is curcumin (CurI), accompanied by two related minor curcuminoids, demethoxycurcumin (CurII) and bisdemethoxycurcumin (CurIII) that are the major active constituents of turmeric (Fig. 1). Recently, curcuminoids have been reported to have very strong anti-inflammatory, anti-carcinogenic, anti-oxidant, antiallergic, anti-bacterial, and anti-tumor activities [1–4].

Although most of research regarded curcumin as the primary object, the activities of other two curcuminoids have been attracting scientists' interesting. Preclinical studies using cell cultures have shown curcumin has antiproliferative effects on human breast carcinoma cells and to induce apoptosis of the myelogenous leukemia HL-60 tumor cells [5,6].

Ruby compared mature curcuminoids for their cytotoxic, tumor reducing and antioxidant activities and found CurIII was more active than the other two as a cytotoxic agent and in the inhibition of Ehrhlich ascites tumor in mice [7]. The ability of these compounds to suppress the superoxide production by macrophage indicated that all the three curcuminoids inhibited superoxide production and CurIII produced the maximum effect.

The importance of monitoring relevant physiological and psychiatric parameters during all the period of treatment is obvious. Furthermore, it is necessary to check the amount of the drug or of the various drugs that the patients everyday have to ingest. Little is known about the absorption, distribution, and metabolism of curcuminoids in humans. The first step in in vivo physiological and pharmacokinetic studies is to develop a method to measure curcuminoids in urine.

Several methods have been developed to separate the curcuminoids: thin-layer chromatography (TLC) [8],

* Corresponding author. Tel.: +86 411 8369 3403; fax: +86 411 8369 3403.
E-mail address: dicp402@mail.dlptt.ln.cn (G. Xu).

0731-7085/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2004.12.009

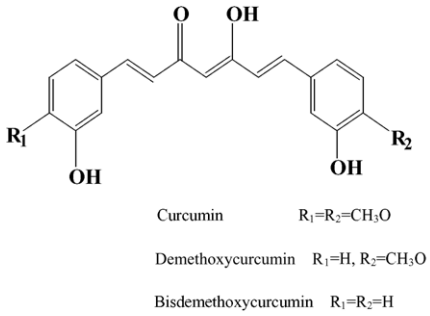

Fig. 1. Chemical structures of curcuminoids.

supercritical fluid chromatography (SFC) [9], gas chromatography (GC) [10], high performance liquid chromatography (HPLC) [11,12], and HPLC–mass spectrometry (LC–MS) [10,13]. Heath [14] developed a simple HPLC method for the analysis of curcumin in plasma and urine, only curcumin was determined and the other two active constituents were not mentioned.

Until now, there is no report on the separation and determination of the curcuminoids by capillary electrophoresis (CE). Nowadays, the application of CE for the separation of analytes in biological samples has become increasingly widespread because of its minimal sample volume requirement, short analysis time and high separation efficiency.

In this study, we have demonstrated for the first time that the curcuminoids from standards and urine samples can be simultaneously separated by CE with photodiode array detector (DAD). Hence, a method based on capillary zone electrophoresis (CZE) was envisaged for the separation and determination of three structurally comparable products, the influence of several parameters were investigated.

2. Experimental

2.1. Apparatus

All capillary electrophoresis experiments were carried out with a Beckman P/ACE system MDQ equipped with an on-column photodiode array detector (Beckman Coulter, Fullerton, CA, USA). Fused-silica capillaries (supplied by Hebei Yongnian optical fiber factory, China) of 50 cm (effective length 42 cm) × 50 μm i.d. × 375 μm o.d. were used. Spectroelectropherograms were registered in the range 200–800 nm. Date acquisition and processing were performed with Beckman P/ACE Station software (Version 1.5) running on a personal computer.

2.2. Chemicals and analytes

All other chemicals used for the buffer preparation, such as borate, NaOH, NaH_2PO_4 were of analytical grade. The reference standards of curcuminoids were purchased from Dalian Meiluo Pharmacy (curcumin) and Shichuan Tianyin Company (demethoxycurcumin and bisdemethoxycurcumin). Deionized water from a Milli-Q System (Millipore, Corp, Bedford, MA) had an electric resistance larger than 18 MΩ. HPLC-grade methanol was from Tedia (USA).

2.3. Preparation of standard solution and calibration curve

A curcumin stock solution was prepared by dissolving 10 mg curcumin in 10 ml methanol (other curcuminoids operated as the same). The standard solutions of various concentrations could be obtained by further dilution of the above prepared stock solutions. These solutions were used to obtain the calibration curve for quantitation. The autosampler temperature was maintained at 10 °C.

Appropriate dilutions (from 0.5 to 1000 μg/ml) for calibration curves were prepared. Since curcuminoids are slightly oxidized and photodecomposed, each solution was wrapped with black paper and stored in a freezer at −18 °C before use. The standard solutions are prepared everyday.

2.4. Preparation of the running buffer

Fifteen millimolar borate buffer, containing 10% methanol was prepared, and pH was adjusted to 10.8 by 1.0 M NaOH before dilution to the final volume. The buffer solutions were filtered through a 0.25 μm membrane filter before use.

2.5. Preparation of urine samples

Urine samples from fasted healthy individuals were collected into urine collection tube, centrifuged at 6000 rpm for 10 min and stored in −18 °C.

Two independent urine samples (1 ml) were prepared and one was spiked with varying amounts of curcuminoids from the previously prepared stock solutions. The tubes were mixed for 1 min at medium speed setting by vortex. Seven millilitres methylene chloride was added in each tube. The solutions were mixed by vortex for 2 min. Then tubes were centrifuged at 6000 rpm for 10 min. After centrifugation, the tubes were left in the dark for 20 min and the upper organic layer about 6 ml was carefully removed into a clean microcentrifuge tube. This organic layer was dried under a stream of nitrogen gas using low heat setting. Samples were dissolved in 100 μl of methanol for capillary electrophoresis analysis.

2.6. CE procedure

Each day, the capillary was conditioned by flushing with 0.1 M sodium hydroxide, deionized water and buffer for 10 min each and finally with the running buffer for 10 min each. Prior to each analysis, the capillary was rinsed with the buffer solution for 3 min. Samples were injected hydrodynamically at the anodic and in low pressure mode (0.5 psi) for 5 s. Electrophoresis was carried out at positive power supply

of 25 kV for 10 min and the temperature was kept at 30 °C. The wavelength was 262 nm.

2.7. Animal studies

Curcumin was administered orally to male rats (200–250 g) at 2 g/kg and total urine in the period of 12 h was collected (about 4 ml) after administration. Fresh urine was cooled in −18 °C and treated as described. Animal treatment was in full accordance with the European Community Guidelines for the care and management of laboratory animals.

3. Results and discussion

3.1. Selection of liquid–liquid extraction solvents

Methylene chloride, petroleum ether, aether, hexane, ethyl acetate were first chosen to extract the curcuminoids. In experiments, high emulsification appeared when using petroleum ether, aether or hexane. The selectivity of ethyl acetate is un-ideal and the satisfying electrophoretogram was obtained by using methylene chloride. So methylene chloride was chosen as extracting solvent.

3.2. Optimization of CE conditions

3.2.1. Effect of organic modifiers

Methanol, acetonitrile, ethanol and formamide were used to improve the resolution, but we found that only methanol was effective in the separation of the curcuminoids after a series of designed experiments. Fig. 2 shows the effect of methanol concentration on the separation of the three adjacent peaks. It can be seen that the organic modifier played an important role in a successful separation. The baseline separation was obtained when the concentration of methanol was 10% (v/v).

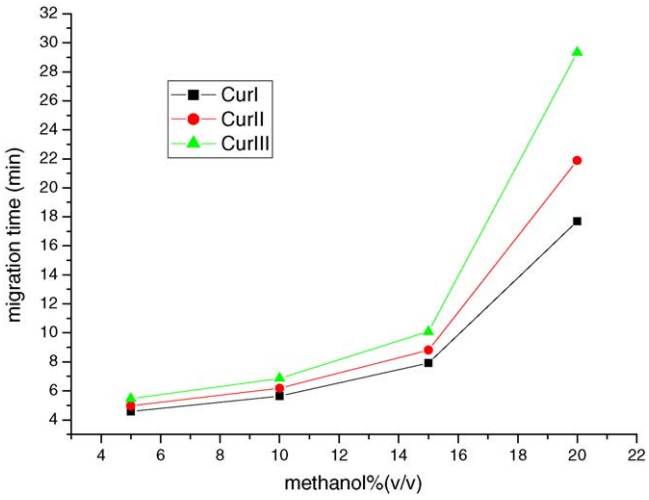

Fig. 2. Effect of methanol (%) on the separation of curcuminoids. The experimental conditions: running buffer 15 mM sodium tetraborate buffer containing 10% methanol (v/v) at pH 10.8, 25 kV and 30 °C.

3.2.2. Influence of electrolyte buffer concentration

The concentration of the $Na_2B_4O_7$ buffer was varied between 5 and 30 mM under the same experimental conditions as above. An increase in the buffer concentration resulted in a decrease in the electroosmotic flow (EOF) due to compression of the double layer and in an increase of migration time of the solutes. Buffer concentration dramatically affected the peak shape due to a stacking effect, increasing the buffer concentration to 15 mM resulted in a better peak shape and

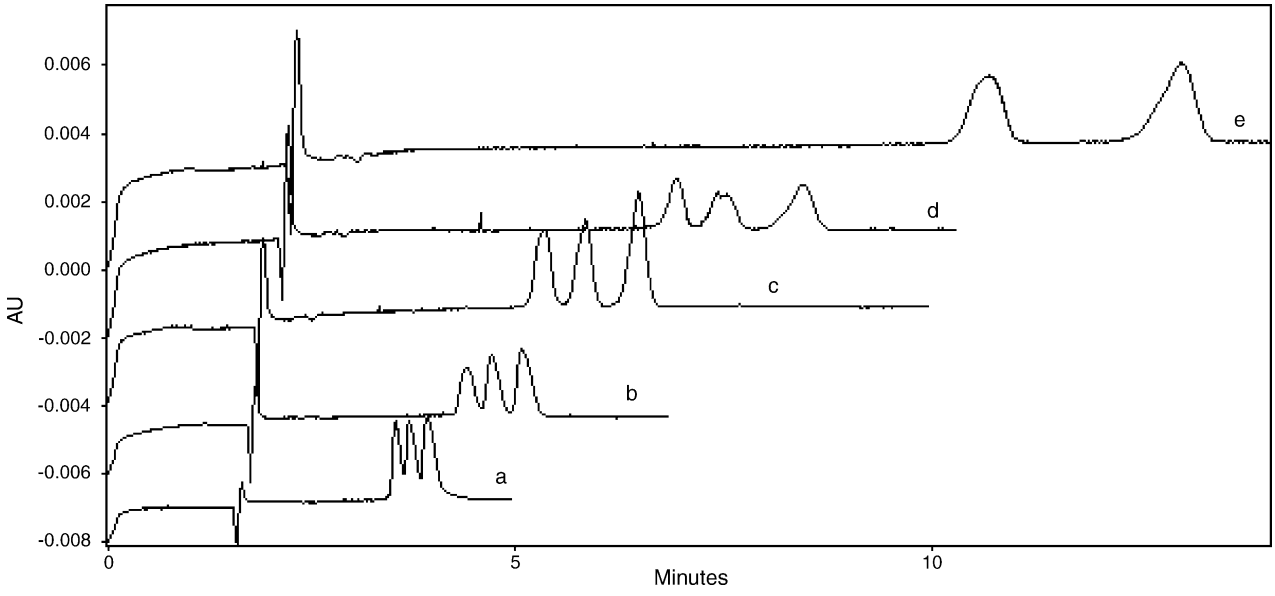

Fig. 3. Effect of the buffer concentration on the separation of curcuminoids. The experimental conditions were the same as in Fig. 2 except for the variation in the buffer concentrations (a: 5 mM; b: 10 mM; c: 15 mM; d: 20 mM; e: 25 mM).

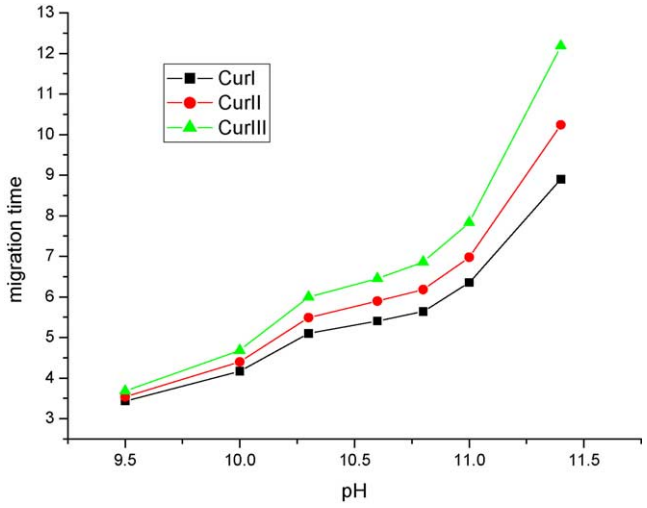

Fig. 4. Effect of pH on the separation of curcuminoids. The experimental conditions were the same as in Fig. 2 except for the variation in pH.

efficiency, whereas higher concentrations resulted in a peak broadening due to Joule heating (Fig. 3).

3.2.3. Influence of pH

The effect of buffer pH on peak resolution was evaluated by adjusting the buffer pH in the following values: 9.5, 10.0, 10.3, 10.6, 10.8, 11.0 and 11.4. An increase in pH values caused higher migration times (Fig. 4). It is well known that the buffer pH always plays an important role in the separation of ionizable analytes because it determines the ionization extent of each individual analyte and also influences the surface of the capillary wall.

3.2.4. Influence of the temperature and the voltage

The effect of the temperature on separation was investigated between 15 and 40 °C. Temperature regulation of the instrument is efficient only until 10 °C below room temperature. An increase in the temperature caused a decrease in the EOF due to lower electrolyte viscosity. The increased migration times of solutes were due to both changes in the viscosity of the medium and partition coefficients. A temperature of 30 °C was selected as it gave the best compromise between resolution and running time with an acceptable level of baseline noise.

The voltage from 5 to 30 kV was investigated using the same experimental conditions as above. A potential of 25 kV yielded the best compromise in terms of run time, current generated and efficiency of separation.

Base on the conditions, the CE electrophoretogram of a mixture of curcuminoids is shown in Fig. 5(a).

3.3. Method validation

3.3.1. Reproducibility

Five repeated injections of different concentrations of curcumoids gave the data presented in Table 1. The CZE-technique used gives reproducible results. The precision and accuracy data of intra- and inter-assays at three different concentrations (low, medium and high) are satisfactory.

3.3.2. Linearity and sensitivity

The linearity of the CE method was evaluated by analyzing standard solutions containing the three curcuminoids. Three solutions diluted to various concentrations ranging from 0.5 to 1000 μg/ml were prepared in order to determine the relationship between the peak area and the concentration of the analytes. The results, given in Table 2, show that linear responses were obtained over the investigated concentration range for all compounds with correlation coefficients (r) greater than 0.999. The limit of detection (LOD) of concentration and the linear range are also showed in Table 2.

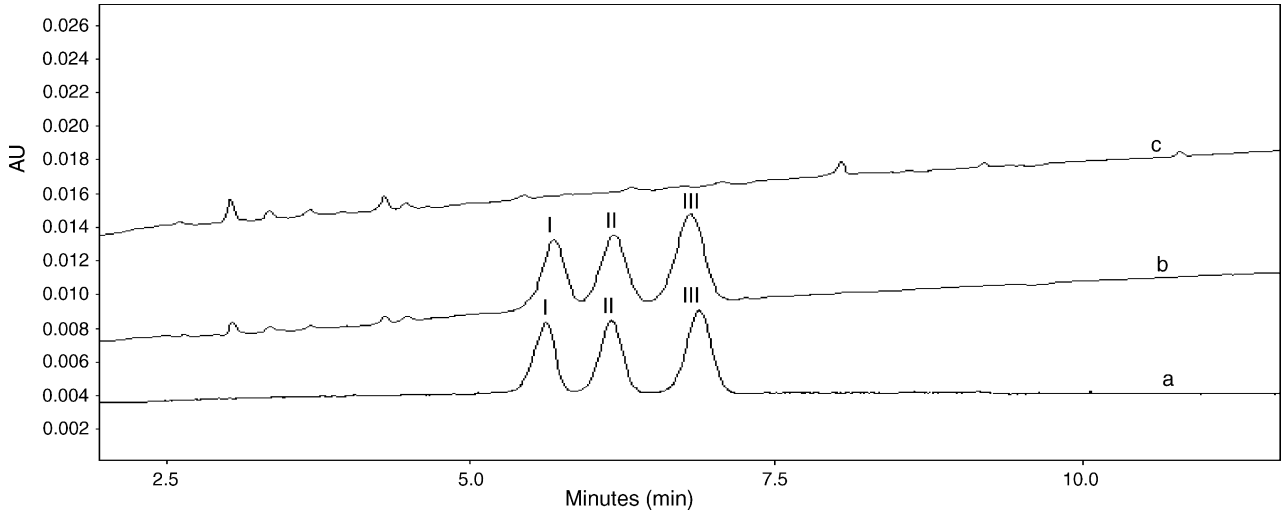

Fig. 5. Electrophoretogram of curcuminoids standard (a) (I: CurI; II: CurII; III: CurIII) and the urine spiked with curcuminoids (b) and blank urine (c). The experimental conditions were the same as in Fig. 2.

Table 1
Reproducibility of the intra- and inter-assay of analytes ($n = 5$)

Compound	Calculated concentration (μg/ml)	Intra-day concentration (μg/ml)		Inter-day concentration (μg/ml)	
		Mean	R.S.D (%)	Mean	R.S.D (%)
CurI	2.00	1.97	5.80	1.95	6.49
	10.0	9.91	5.12	9.93	5.46
	50.0	50.0	3.01	49.7	4.52
CurII	2.00	1.94	7.12	1.92	8.43
	10.0	9.90	6.56	9.84	7.27
	50.0	50.0	6.70	49.8	7.82
CurIII	2.00	1.96	3.23	1.94	5.17
	10.0	9.94	3.12	9.88	4.24
	50.0	50.7	1.42	50.6	3.02

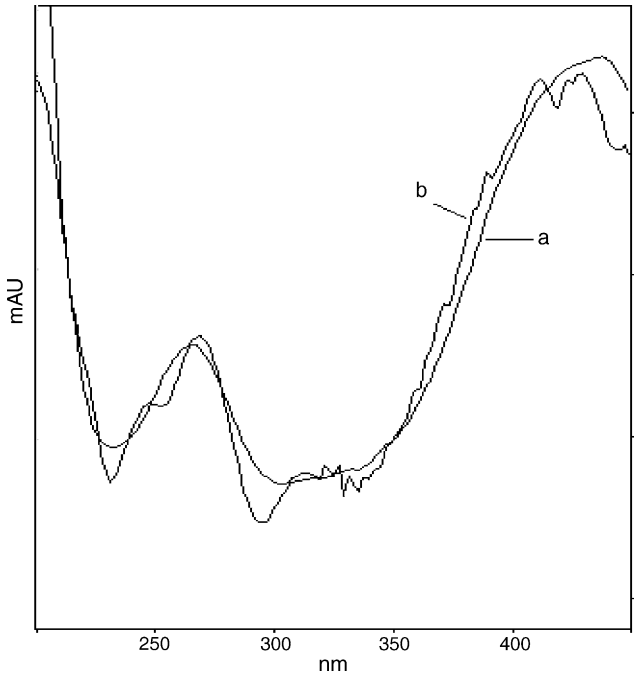

Fig. 6. UV spectra of curcumin standard (a) and curcumin in urine (b) after purification obtained by diode array detection.

3.3.3. Selectivity

To improve selectivity, curcumin was quantified at its maximum absorbance at 262 nm. The UV spectrum shown in Fig. 6 indicated the curcumin is well resolved and free from interference peaks. The use of a diode array detector allowed the confirmation of the chromatographic peak not only by its migration time but also its spectrum.

Table 3
Summary of results for recovery of added curcuminoids[a]

Compounds	Calculated concentration (μg/ml)	Recovered concentration (μg/ml)	R.S.D. (%)	Recovery (%)
CurI	1.00	0.95	5.10	95.0
	5.00	4.68	3.30	93.6
CurII	1.00	0.92	1.50	92.0
	5.00	4.44	2.30	88.8
CurIII	1.00	0.94	2.20	94.0
	5.00	4.51	1.30	90.2

[a] Number of assays: 5.

3.4. Quantification of the curcuminoids in urine

Fig. 5 curve b and c depict the electropherogram of a treated urine sample spiked with the curcuminoids standard and a blank urine sample. To evaluate the recovery, curcuminoids were added to urine in the concentration 1.0 and 5.0 μg/ml. The samples were deproteinized and prepared for CE analysis as described in Section 2. The results obtained from urine samples are listed in Table 3.

3.5. Application

To assess the validity of the method, Urine taken from rat administering curcumin (2 g/kg) was analyzed. It was found that the concentration of urinary curcumin was 3.0 μg/ml and the method developed in this work is useful. As being short of enough standard, the other two curcuminoids were not studied by animal experiments.

Table 2
Linearity and detection limit of the CE method

Compound	Regression equation, $y = a + bx$[a]	Correlation coefficient	Linear range (μg/ml)	Detection limit (μg/ml)[b]
CurI	$y = 690x - 7.498$	0.999	0.5–1000	0.247
CurII	$y = 683x - 5.124$	0.999	0.5–1000	0.157
CurIII	$y = 910x - 4.589$	0.999	1.0–1000	0.426

[a] Where the y and x are the peak area and concentration of the analytes (μg/ml), respectively.
[b] The detection limits corresponding to concentrations giving signal-to-noise ratio of 3.

4. Conclusion

In the paper, a CZE method for the determination of the curcuminoids in urine has been successfully developed. The results demonstrate that the CE is simple and rapid technique for assaying these compounds in biological samples and appears to offer some advantages over HPLC in terms of analysis time and resolution. Our further work will be focused on the application of the method in clinical phase I trials.

Acknowledgement

This study has been supported by the National Natural Science Foundation of China (No. 90209048), National Basic Research Program (2003CB716003) of State Ministry of Science and Technology of China, and Knowledge Innovation Program of DICP, Chinese Academy of Sciences (K2002A11).

References

[1] H.P.T. Ammon, M.A. Wahl, Planta Med. 57 (1991) 1–7.
[2] K.K. Soudamini, R. Kuttan, J. Ethno Pharmacol. 27 (1989) 227–233.
[3] T. Masuda, A. Jitoe, Phytochemistry 32 (1993) 1557–1560.
[4] R. Motterlini, R. Foresti, R. Bassi, C.J. Gree, Free. Radic. Biol. Med. 15 (2000) 1303–1312.
[5] Z.M. Shao, Z.Z. Shen, C.H. Liu, M.R. Sartippour, V.L. Go, D. Heber, M. Nguyen, Int. J. Cancer. 98 (2002) 234–240.
[6] R.J. Anto, A. Mukhopadhyay, K. Denning, B.B. Aggarwal, Carcinogenesis 23 (2002) 143–150.
[7] A.J. Ruby, G. Kuttan, K.D. Babu, K.N. Rajasekharan, R. Kuttan, Cancer Lett. 94 (1995) 79–83.
[8] A. Janßen, Th. Gole, Chromatographia 18 (1984) 546–549.
[9] M.M. Sanagi, U.K. Ahmad, R.M. Smith, J. Chromatogr. Sci. 31 (1993) 20–25.
[10] R. Hiserodt, T.G. Hartman, C.T. Ho, R.T. Rosen, J. Chromatogr. A 740 (1996) 51–63.
[11] H.H. Tonnesen, J. karlsen, J. Chromatogr. A 259 (1983) 367–371.
[12] H. Khurana, J. Liq. Chromatogr. 11 (1988) 2296–2304.
[13] X.G. He, L.Z. Lin, L.Z. Lian, M. Lindenmaier, J. Chromatogr. A 818 (1998) 127–132.
[14] D.D. Heath, M.A. Pruitt, D.E. Brenner, C.L. Rock, J. Chromatogr. B 783 (2003) 287–295.