ELSEVIER

Journal of Chromatography A, 943 (2001) 33–38

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Fast analysis of flavonoids in plant extracts by liquid chromatography–ultraviolet absorbance detection on poly(carboxylic acid)-coated silica and electrospray ionization tandem mass spectrometric detection

C.W. Huck*, M.R. Buchmeiser, G.K. Bonn

Institute of Analytical Chemistry and Radiochemistry, Leopold-Franzens University, Innrain 52a, A-6020 Innsbruck, Austria

Received 11 June 2001; received in revised form 18 October 2001; accepted 22 October 2001

Abstract

A highly hydrophilic poly(7-oxonorbornene-5,6-dicarboxylic acid-*block*-norbornene) [=poly-(ONDCA-*b*-NBE)]-coated silica was investigated for the liquid chromatographic (LC) determination of flavonoids in plant extracts of complex biological origin using UV absorbance and mass spectrometric (MS) detection. Compared to the most commonly used octadecyl derivatized silica this sorbent allowed fast separations even at extreme pH values. Furthermore, UV absorbance and MS detection were evaluated. As we found, UV detection at 254 nm allows the determination of flavonoids down to the ng range with a linearity of $R^2>0.9906$. For the more selective characterization the validated LC system was coupled to a quadrupole ion trap mass spectrometer via an electrospray ionization (ESI) interface. MS detection showed high linearity ($R^2>0.9904$) for all investigated flavonoids. Due to the relatively high flow-rate of 1 ml/min the limits of detection were found in the lower-μg range. Collision induced dissociation was applied to obtain characteristic fragmentation fingerprints. Finally, the validated LC–ESI-MS–MS method demonstrated that this poly-(ONDCA-*b*-NBE) stationary phase allows fast characterization and quantitation in onion, elderflower blossom, lime blossom, St. John's Wort and red wine.

Keywords: Flavonoids; Poly(carboxylic acid)-coated silica

1. Introduction

Flavonoids are low-molecular-mass compounds found in all vascular plants. They act as antioxidants or as enzyme inhibitors, are involved in photosynthesis and cellular energy transfer processes, and may serve as precursors of toxic substances [1,2]. Specific flavonoids are known to have pharmacological activity, particularly anti-allergic, anti-inflammatory, anti-viral or anti-carcinogenic effects. Due to these health benefits, analytical techniques for the characterization and quantitation are required in order to support the steady progress of medicinal sciences. Reversed-phase liquid chromatography (RPLC) of flavonoids over a C_{18} derivatised silica stationary phase is most commonly used [3]. Generally, UV absorbance and fluorescence (FL) detection methods are used, with FL offering higher sensitivity

*Corresponding author. Tel.: +43-512-507-5195; fax: +43-512-507-2965.

E-mail address: christian.w.huck@uibk.ac.at (C.W. Huck).

0021-9673/02/$ – see front matter
PII: S0021-9673(01)01428-5

and selectivity. In addition, the utility of LC separation for the identification of flavonoids with increased selectivity compared to UV and FL and quite similar sensitivity compared to FL can be greatly enhanced by mass spectrometric (MS) detection. Therefore, atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) have emerged as highly useful methods which allow the direct conjunction with liquid-phase separation techniques [4,5]. Furthermore, collisionally induced dissociation (CID) is used to obtain fragment ions of structural relevance for identifying target compounds in highly complex mixtures. The major drawbacks of octadecyl silica (ODS) sorbents are the dissolution at elevated pH values and the need for long analysis times. A poly(7-oxonorbornene-5,6-dicarboxylic acid-*block*-norbornene) [=poly-(ONDCA-*b*-NBE)]-coated silica support can be used under such conditions, as it was recently demonstrated for lanthanides and aromatic amines [6–8]. In this paper we describe the fast LC–MS of flavonoids occurring in different plant extracts on this sorbent.

2. Experimental

2.1. Materials and reagents

Acetonitrile (gradient grade), acetic acid (analytical-reagent grade) and trifluoroacetic acid (analytical-reagent grade) were purchased from Merck (Darmstadt, Germany). Water purified by a NanoPure-unit (Barnstead, Boston, MA, USA) was used. Acacetin, kaempferol, myricetin, quercetin and quercitrin were from Sigma (Deisenhofen, Germany).

2.2. Synthesis of the stationary phase and packing procedure

Synthesis of the poly-(ONDCA-*b*-NBE)-coated silica was carried out following the procedure described by Buchmeiser et al. [8]. Columns were packed using the following high-pressure slurry packing technique: the sorbent (1.5–2 g) was suspended in water–acetonitrile (20:80, v/v), sonicated for 3 min and then packed into a 150×4 mm I.D. polyether ether ketone (PEEK) column (Bischoff, Leonberg, Germany). For driving a solvent consisting of water–acetonitrile (20:80, v/v) at a constant pressure of 30 MPa, a pneumatic LC pump (Knauer, Berlin, Germany) was used.

2.3. High-performance liquid chromatography

The LC system used consisted of a low-pressure gradient pump (Model 616, Waters, Milford, MA, USA), a helium degassing system, an autosampler (Model 717, Waters) with a 200 μl loop and a diode array detector (Model 996, Waters) with a 10 mm pathlength flowcell. Separations were carried out at ambient temperature. Data was recorded on a personal computer-based data system (Millenium Version 32, Waters).

2.4. High-performance liquid chromatography coupled to electrospray ionization mass spectrometry

For LC–MS–MS experiments a low-pressure gradient micropump (Model Rheos 2000, Flux, Karlskoga, Sweden), a degasser (Knauer, Berlin, Germany), a microinjector (Model CC000030, Valco, Houston, TX, USA) with a 5 μl internal loop, a variable-wavelength detector (Model Linear UV–VIS 200, Linear Instruments, Fermont, CA, USA) with a 1.2 μl detector cell connected to a quadrupole ion trap MS (Model LCQ, Finnigan, San Jose, CA, USA) were used. The following parameters were used in all experiments: source voltage, 4.51 kV; source current, 7.18 μA; vaporizer temperature, 450°C; sheath gas flow-rate, 60 (nitrogen); auxiliary gas flow-rate, 10; capillary voltage, 6.58 V; temperature of the heated capillary, 200°C; tube lens offset, −5; second octapole offset, −5; first octapole offset, −2; inter octapole lens, −16.

3. Results and discussion

3.1. LC using UV-absorbance detection

For the present investigations, Nucleosil 50-7 (150×4 mm I.D., 7 μm, 50 Å), coated with poly-(ONDCA-*b*-NBE) was used. Fig. 1 shows the repetitive unit of the polymer, which consists of a succinic acid connected to a tetrahydrofuran ring. The poly-

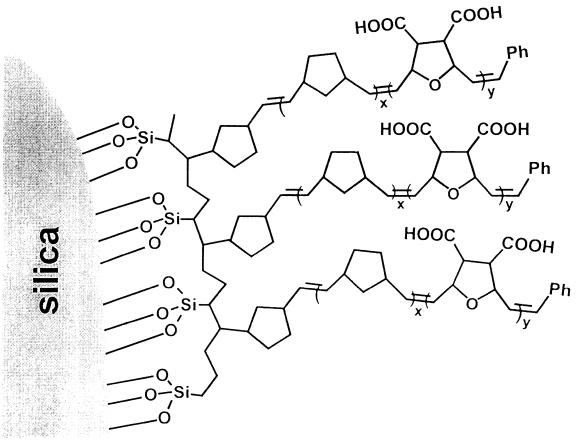

Fig. 1. Structure of poly-(ONDCA-*b*-NBE) coating.

(ONDCA-*b*-NBE)-coated material was found to be entirely stable under acidic conditions (5% nitric acid) as well as up to a pH larger than 12 and the material can be reused after careful clean-up without any loss of performance.

For the analysis of the flavonoids quercitrin, myricetin, quercetin, kaempferol and acacetin in biological samples of complex origin an LC system was assembled that allows shortest possible analysis time with baseline separation and that was also suitable for the coupling to MS. After first separations with a water–acetonitrile gradient and a 250×4 mm I.D. column packed with poly-(ONDCA-*b*-NBE)-coated silica gave promising results, 20 m*M* trifluoroacetic acid was added to the mobile phase to avoid deprotonation of phenolic groups of the flavonoids and to ensure that all carboxylic acids of the stationary phase are present in the protonated form. Furthermore, the column length was shortened to 120 mm to allow a reduction of analysis time to 5 min after optimization of the gradient to give approximately the same efficiency as with the longer column. The elution order quercitrin, myricetin, quercetin, kaempferol and acacetin is governed by the highest polarity of quercitrin (5,7,3′,4′-tetrahydroxy-3-*O*-rhamnoside) mainly influenced by the rhamnoside side chain (Fig. 2). Thereby, the hydrophilic character of flavonoid glycosides is not only marked by the number of carbohydrates in the side chain, but also by their nature. Thus, for a particular flavonoid, retention factors are enhanced in the order glucoside<arabinoside<rhamnoside. Additionally, the carbonyl group of the chromanole ring can be shielded by the carbohydrate side chain. The remaining elution order is determined by the decreasing number of hydroxyl groups: myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone)>quercetin (3,5,7,3′,4′-pentahydroxyflavone)>kaempferol (3,5,7,4′-tetrahydroxyflavone), acacetin (5,7-dihydroxy-4′-methoxyflavone) (Fig. 2). As expected, the flavonoid mixture could not be separated using uncoated silica as stationary phase. Under the optimized conditions using poly-(ONDCA-*b*-NBE)-coated silica the flavonoids are separated within less than 5 min using

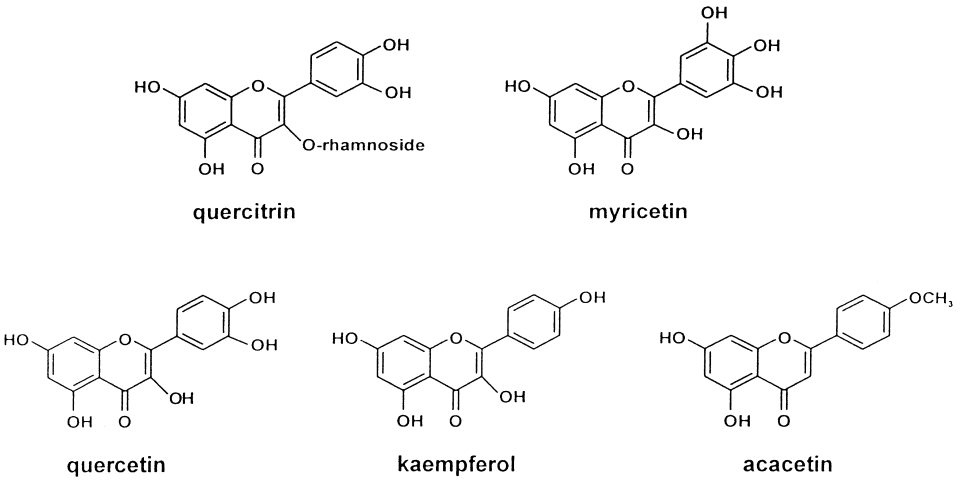

Fig. 2. Chemical structures of investigated flavonoids.

UV-absorbance detection at 254 nm (Fig. 3). This means a dramatic reduction in analysis time compared to separations of similar flavonoid mixtures on ODS phases [9,10].

3.2. LC–ESI-MS–MS

The utility of LC separation using poly-(ONDCA-*b*-NBE)-coated silica as stationary phase for a more specific and selective identification of flavonoids was greatly enhanced by coupling to MS via an ESI interface, especially for the determination in samples with coeluting peaks. To improve the detectability of the trifluoroacetic acid was substituted by more polar and less conductive additive acetic acid. For a more efficient ionization process the flow-rate was reduced to 1 ml/min and the flow was split by a 5 cm×20 μm I.D. capillary 5 cm after the column.

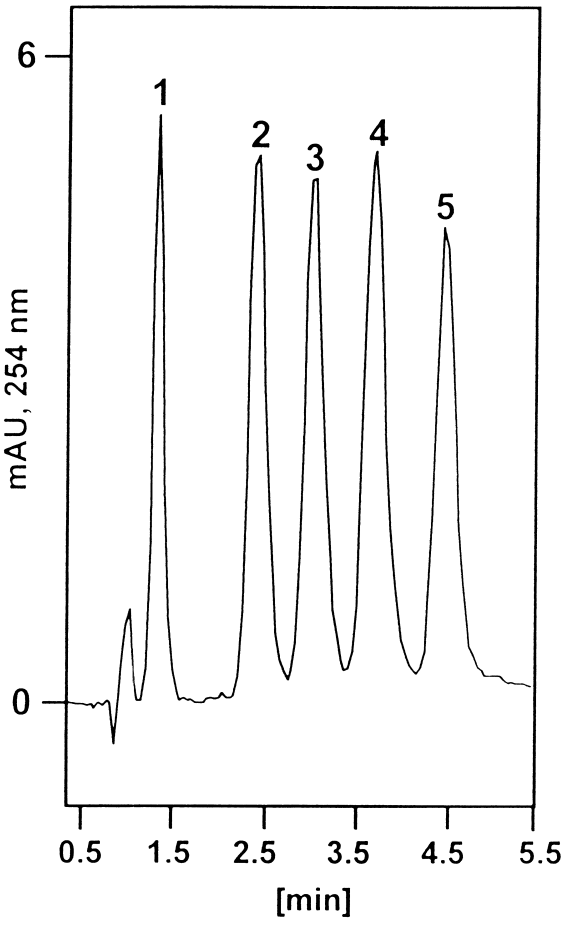

Fig. 3. LC of a flavone standard mixture. Stationary phase, poly-(ONDCA-*b*-NBE)-coated Nucleosil 50-7 (150×4 mm I.D., 7 μm, 50 Å);. Mobile phase, water–CH_3CN, 20 mmol/l TFA; gradient, 78:22–70:30 (v/v) in 3 min; flow-rate, 2 ml/min; sample, 1: quercitrin, 2: myricetin, 3: quercetin, 4: kaempferol, 5: acacetin; injection volume, 5 μl.

Under these ionization conditions all investigated compounds could efficiently be transformed into protonated molecules $[M+H]^+$. Fig. 4a depicts the MS detection after LC of the flavonoid standard mixture, showing the highest intensity for acacetin and the smallest for myricetin. Quercitrin, myricetin, quercetin, kaempferol and acacetin were tracked in the total ion current from selected ion traces at m/z 448–450 (Fig. 4b), m/z 318–320 (Fig. 4c), m/z 302–304 (Fig. 4d), m/z 287.2–288.2 (Fig. 4e) and m/z 284–286 (Fig. 4f). The smaller peak in Fig. 4d corresponds to quercetin, which results from fission of the carbohydrate chain post column. A comparison of the LC–ESI-MS–MS spectra of the raw plant extracts with that of the pure standards facilitated further specific identification and confirmed that the investigated compounds were no artefacts. Tandem mass spectra of the pure quercetin and of quercetin in St. John's Wort are compared in Fig. 5. The fragmentation pathway was characterized by the loss of H_2O (m/z 285.2) and double loss of CO (m/z 257.2 and 229.2). Further assignments of the ions can be made as follows: m/z 165 (cleavage b), m/z 153.2 and 137.2 (cleavage a) [4]. From Table 1 the molecular masses and the main fragments for all investigated flavonoids can be gathered. In the following, this LC–MS–MS method was not only used for the characterization but also for the quantitation of these flavonoids in real samples.

3.3. Calibration, limit of detection, reproducibility, recovery and quantitation

The optimized LC system and the employment of UV-absorbance and MS detection were evaluated for the quantitative determination of flavonoids in complex biological systems. Calibration plots of peak area versus concentration for all analytes were obtained by linear regression analysis of at least three data points per concentration in a concentration range of 30 ng–8 μg using UV-absorbance at 254 nm and 0.3–3 μg using MS detection. Both LC–UV

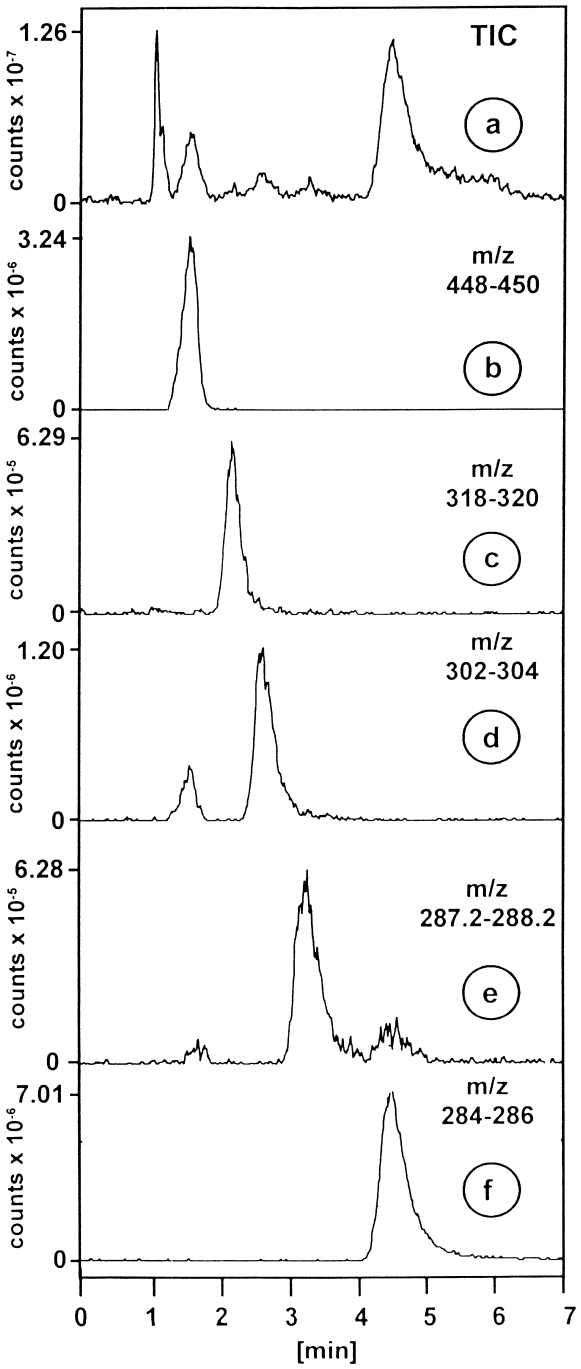

Fig. 4. LC–ESI-MS of a flavone standard mixture. Stationary phase, poly-(ONDCA-*b*-NBE)-coated Nucleosil 50-7 (150×4 mm I.D., 7 μm, 50 Å); mobile phase, 25% acetonitrile in 1% (v/v) aqueous HOAc; flow-rate, 1 ml/min, split, capillary (5 cm×20 μm I.D.); detection, ESI, full scan 80–500; temperature, 22°C; sample volume, 5 μl; (a) total ion current (TIC), (b) quericitrin, (c) myricetin, (d) quercetin, (e) kaempferol, (f) acacetin.

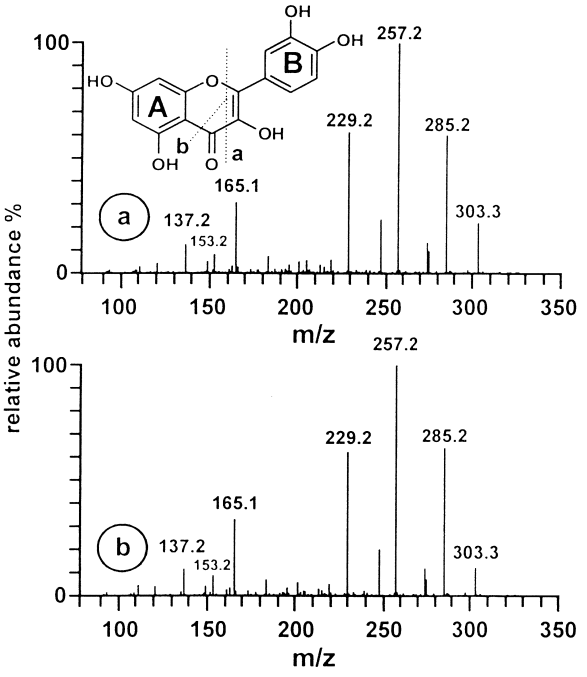

Fig. 5. Tandem mass spectra of (a) quercetin standard, (b) quercetin in St. John's Wort. Stationary phase, poly-(ONDCA-*b*-NBE)-coated Nucleosil 50-7 (150×4 mm I.D., 7 μm, 50 Å); mobile phase, 25% acetonitrile in 1% (v/v) aqueous HOAc; flow-rate, 1 ml/min, split, capillary (5 cm×20 μm I.D.); detection, ESI, scan 85–350; relative collision energy, 30%; temperature, 22°C; sample volume, 5 μl.

Table 1
$[M+H]^+$ precursor ion mass and observed main fragments from protonated flavones

Flavone	$[M+H]^+$	*m/z*
Quercitrin	449.4	431, 413, 303
Myricetin	319.2	301, 273, 245, 153, 137
Quercetin	303.3	285, 257, 229, 153, 137
Kaempferol	287.4	269, 241, 213, 153, 121
Acacetin	285.3	270, 242, 213, 153, 121

and –MS gave calibration plots with correlation coefficients $R^2>0.99$. Changing from LC–UV to MS detection resulted in an average 214% increase of slope values for quercitrin, myricetin, quercetin, kaempferol and acacetin (Table 2). The lower limits of detection (LOD) were determined at a signal-to-noise ratio of 3 being 0.3–1.5 ng (0.66–4.9 pmol) using UV-absorbance. Employing MS gave lower detection limits in the μg range, which has to be lead

Table 2
Calibration curves, linear inhomogeneity (d), regression coefficient (R^2), lower limit of detection (LOD)[a] for flavonoids using UV and MS detection

Compound	Ascent, k	Linear inhomogeneity, d	R^2	LOD
UV, 254 nm				
Quercitrin	$1 \cdot 10^6$	198 418	0.9985	0.3 ng
Myricetin	$1 \cdot 10^6$	−887 797	0.9989	1.3 ng
Quercetin	$2 \cdot 10^6$	−74 385	0.9906	1.5 ng
Kaempferol	$2 \cdot 10^6$	$-1 \cdot 10^6$	0.9999	0.6 ng
Acacetin	725 427	239 639	0.9966	0.6 ng
MS				
Quercitrin	$2 \cdot 10^7$	$2 \cdot 10^6$	0.9904	0.18 μg
Myricetin	$3 \cdot 10^6$	461 045	0.9910	1.3 μg
Quercetin	$6 \cdot 10^6$	$2 \cdot 10^6$	0.9992	1.3 μg
Kaempferol	$4 \cdot 10^6$	$1 \cdot 10^6$	0.9938	1.5 μg
Acacetin	$4 \cdot 10^7$	$-7 \cdot 10^6$	0.9984	0.3 μg

[a] Determined at a signal-to-noise ratio of 3.

back to the split relatively high flow-rate of 1 ml/min. According to this fact, the lower limits of quantitation (LOQs), which were determined at a signal-to-noise ratio of 9 using UV detection were found between 1.2 and 2.5 ng and between 0.4 and 3.1 μg using MS detection. The reproducibility of peak areas was checked by four consecutive injections of a standard mixture. Peak areas of flavonoids showed a relative standard deviation of 4.2% using UV, 5.1% using MS detection. To demonstrate the effectiveness of the LC–MS system for the analysis in food and plant extracts quercitrin and its aglycone quercetin, the main flavonoids in our diet, were determined in onion, elderflower blossom, lime blossom and St. John's Wort. Values found for quercitrin in elderflower blossom, lime blossom and onion were 13, 65 and 62.3 μg/g, for quercetin and quercitrin in St. John's Wort 164 and 46 μg/g. Finally, this system was also used for the analysis of myricetin and kaempferol in red wine (Cabernet Sauvignon). Found values were 9 and 2 μg/ml.

4. Conclusion

The potential of poly-(ONDCA-*b*-NBE)-coated silica has brought about important reduction of analysis time for the quantification of flavonoids in food and phytopharmaceutical preparations by LC and LC–ESI-MS–MS. The material itself is stable within a pH range of 0 and >12, may rapidly be recycled and reconditioned and therefore represents an attractive material for the rapid and quantitative analysis of flavonoids. Due to time and cost reduction this material offers an interesting alternative to silica-based sorbents especially for the phytopharmaceutical industry.

References

[1] J.B. Harborne, T.J. Mabry, The Flavonoids: Advances in Research, Chapman and Hall, New York, 1982.
[2] J.W. McClure, in: Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological and Structure Activity Relationships, Alan R. Liss, New York, 1986, p. 77.
[3] J.B. Harborne, Phytochemical Analysis, Chapman and Hall, London, 1988.
[4] Y.Y. Lin, K.J. Ng, S. Yang, J. Chromatogr. 629 (1993) 389.
[5] C.W. Huck, G.K. Bonn, Phytochem. Anal. 11 (2000) 6.
[6] G. Seeber, P. Brunner, M.R. Buchmeiser, G.K. Bonn, J. Chromatogr. A 848 (1999) 193.
[7] M.R. Buchmeiser, R. Tessadri, G. Seeber, G.K. Bonn, Anal. Chem. 70 (1998) 2130.
[8] M.R. Buchmeiser, G. Seeber, M. Mupa, G.K. Bonn, Chem. Mater. 11 (1999) 1533.
[9] A.M. Pupin, M.J. Dennis, M.F.C. Toledo, Food Chem. 63 (1998) 513.
[10] P.G. Pietta, P.L. Mauri, C. Gardana, A. Bruno, J. Chromatogr. 547 (1991) 439.