Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 854 (2007) 219–223
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Simultaneous determination of *trans*-resveratrol-3-*O*-glucoside and its two metabolites in rat plasma using liquid chromatography with ultraviolet detection

Maojin Zhou [b], Xiaoyan Chen [a], Dafang Zhong [a,b,*]

[a] *Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 646 Songtao Road, Shanghai 201203, China*
[b] *Laboratory of Drug Metabolism and Pharmacokinetics, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110016, China*

Received 22 January 2007; accepted 20 April 2007
Available online 1 May 2007

Abstract

A sensitive and selective high-performance liquid chromatographic method was developed for simultaneous determination of *trans*-resveratrol-3-*O*-glucoside (TRG) and its metabolites, *trans*-resveratrol-3-*O*-glucuronide (TRN) and *trans*-resveratrol (TR) in rat plasma. The plasma proteins were precipitated with acetonitrile and supernatant was evaporated to dryness. The analytes and internal standard baicalin were chromatographed on a C_{18} column. The mobile phase consisted of 25% acetonitrile and 75% H_2O adjusted with formic acid to pH 3.5. The flow-rate was 1.0 ml/min and ultraviolet detection was set at 320 nm. Standard curves were linear over the concentration range of 0.04–40 μg/ml for TRG and TRN, and 0.04–10 μg/ml for TR, respectively. The precision, expressed as the intra-day R.S.D. and inter-day R.S.D., was below 9.3% for TRG, TRN and TR. The accuracy, expressed as the relative error (RE) was within ±7.4% for all analytes. The mean recoveries of TRG, TRN, TR and I.S. were 93.6%, 93.1%, 91.0% and 87.9%, respectively. This method was successfully applied to a pharmacokinetic study of TRG after an oral dose of 150 mg/kg to Wistar rats.

Keywords: *Trans*-resveratrol-3-*O*-glucoside; *Trans*-resveratrol-3-*O*-glucuronide; *Trans*-resveratrol

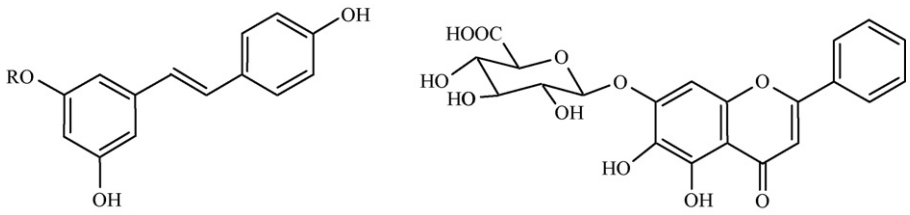
1. Introduction

Trans-resveratrol-3-*O*-glucoside (*trans*-piceid, TRG, Fig. 1), a stilbene compound isolated from the dried roots of *Polygonum cuspidatium*, is the glucosilated form of *trans*-resveratrol (TR) [1,2]. Certain studies showed that TRG exhibited various pharmacological activities, such as preventing thrombosis from arterial endothelial damage, improving microcirculation and increasing survival rate of shocked rats, alleviating tissue-organ damage induced by ischemia-reperfusion, oxygen free radical and endotoxin, decreasing hyperlipemia, suppressing lipid peroxide [3,4], reducing triglyceride synthesis from ^{14}C-palmitation in the liver of mice [5], and anti-platelet aggregation [6]. After TR is administered orally to rats, the plasma concentration of TRN is much higher than that of TR [7–10].

TRG is now in development as a drug candidate. In order to investigate its pharmacokinetics in animals, the first step is to develop a sensitive method to measure TRG in plasma. The reported methods for the determination of only TR in biological fluids were high-performance liquid chromatography with detection of UV [11–15] and gas chromatographic method [16], whereas analytical methods for the simultaneous determination of TR and TRN in biological samples included high-performance liquid chromatography with detection of electrochemical [10] and mass spectrometry [7,8], gas chromatographic method [17,18]. To our knowledge, the method for simultaneous determination of TRG and its metabolites in plasma has not been reported up to the present. We therefore developed and validated a method for the simultaneous quantification of TRG and its metabolites, namely TR and TRN in rat plasma using liquid chromatography with UV detection.

* Corresponding author at: Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 646 Songtao Road, Shanghai 201203, China.
Tel.: +86 21 50800738; fax: +86 21 50800738.
E-mail address: zhongdf@china.com (D. Zhong).

1570-0232/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2007.04.025



I II

Fig. 1. Chemical structures of TRG (I), its metabolites (I) and baicalin (II, internal standard). R: Glucose, *trans*-resveratrol-3-*O*-glucoside, TRG; R: Glucuronic acid, *trans*-resveratrol-3-*O*-glucuronide, TRN; R: H, *trans*-resveratrol, TR.

2. Experimental

2.1. Materials

TRG (99.3% purity) was obtained by courtesy of Liaoning Institute of Pharmaceutical Research (Shenyang, China). TR (99.5% purity) and baicalin (internal standard, 99.0% purity) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile and methanol (Yuwang Chemical, Shandong, China) were of HPLC grade. Other chemicals were of analytical grade. Distilled water, prepared from demineralized water, was used throughout the study.

TRN was isolated and purified from rat urine. A total volume of 1000 ml rat urine was obtained from a study where 20 rats had been dosed with TRG (300 mg/kg). The rat urine was allowed to pass through D101 colophony column and the ethanol extracts were dried by rotary evaporation at 45 °C. The oily residue was applied to ODS extraction cartridges and the fraction of 30% methanol was collected and dried. The residue was dissolved in the mobile phase and analyzed by semi-preparation HPLC with fraction collection. After the fractions were evaporated to dryness, dry material was analyzed by HPLC–UV method. The purity of TRN was more than 98.5%.

The structure of TRN was identified by MS^n (LCQ, Thermo Finnigan, San Jose, CA, USA) and NMR (AV600, Bruker, Faellanden, Switzerland). The two-stage mass spectrum of TRN was the same as the reported by Yu et al. [8], and the ^1H NMR, ^{13}C NMR and HMBC (^1H-detected heteronuclear multiple-bond correlation) data of TRN in DMSO (300 MHz) were the same as the reported by Wenzel et al. [19]. According to the data of MS^n and NMR of TRN, it is confirmed that TRN is *trans*-resveratrol-3-*O*-glucuronide.

2.2. Instrumentation and chromatographic conditions

The chromatographic system (Hewlett-Packard 1100, USA) consisted of a quaternary pump (G1322A), a UV detector (G1314A), a column oven (G1316A), a degasser (G1322A) and a LC-Workstation (Rev.A.06.03). Chromatogaphy was performed on a Diamonsil C_{18} column (200 mm × 4.6 mm i.d., particle 5 μm, Dikma, Beijing, China). The isocratic mobile phase consisted of 25% acetonitrile, 75% H_2O adjusted with formic acid to pH 3.5. The flow-rate was 1.0 ml/min. Ultraviolet detection was set at 320 nm and the column temperature was kept at 25 °C.

2.3. Preparation of standards

The stock standard solutions of TRG, TRN and TR were prepared in acetonitrile to give a final concentration of 400.0 μg/ml for each analyte, respectively. The different volumes of three solutions were then diluted with acetonitrile to achieve standard solutions at concentrations of 0.04/0.04/0.04, 0.10/0.10/0.10, 0.40/0.40/0.40, 1.00/1.00/1.00, 4.00/4.00/2.00, 10.0/10.0/5.00 and 50.0/50.0/10.0 μg/ml for TRG/TRN/TR. A 20.0 μg/ml internal standard (I.S.) working solution was prepared by diluting the 400.0 μg/ml stock solution of baicalin with acetonitrile. All solutions were stored at 4 °C in the dark and were brought to room temperature before use.

The standard solutions (100 μl) were added to a glass tube and evaporated to dryness at 40 °C under nitrogen. Then 100 μl of blank rat plasma were added and vortex-mixed to obtain calibration solutions or quality control samples in the pre-study validation and during the pharmacokinetic study.

2.4. Sample preparation

To a 100 μl aliquot of rat plasma were added 100 μl of the internal standard, 100 μl of formic acid in water (pH 3.5) and 300 μl of acetonitrile, respectively. The samples were vortexed for 1 min, and then centrifuged at 4000 *g* for 10 min. The supernatant was transferred into a tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 100 μl of the mobile phase and a 50 μl aliquot of the solution was injected onto the HPLC system. The whole experiments, including plasma collecting, sample preparation and analysis, were performed in dim light to avoid photochemical isomerization of TRG and its metabolites to the *cis* forms [12].

2.5. Data acquisition and analysis

Data acquisition was performed with a LC-Workstation (Rev.A.06.03). Peak area ratios of analytes to the internal standard were utilized for the construction of calibration curves, using $1/x^2$ weighted linear least-squares regression analysis of the plasma concentrations versus the measured peak area ratios [20]. Concentrations of analytes in quality control (QC)

and unknown samples were calculated from the calibration curves.

2.6. Method validation

To evaluate linearity, calibration curves were prepared and assayed in triplicate on three consecutive days. Intra- and inter-day precision was assessed from results of QC samples. The mean values and R.S.D. for QC samples were calculated over three validation runs. Six replicates of each QC level were determined in each run. These data were then used to calculate the intra- and inter-day precision (R.S.D.) by using a one-way analysis of variance (ANOVA). The accuracy of the method was determined by calculating the percentage deviation observed in the analysis of QC samples and expressed as the relative error (RE).

The recoveries of analytes and I.S. from plasma were determined as follows: drug-free plasma with added TRG/TRN/TR (0.10/0.10/0.10, 4.00/4.00/2.00, 40.0/40.0/8.00 μg/ml) and I.S. (20.0 μg/ml) was processed by the method described above. Recoveries were calculated by comparing the peak areas so obtained with those obtained by the same procedure of aqueous solutions.

The stability of analytes in rat plasma was investigated under a variety of storage conditions: performing QC samples three cycles of freeze (−20 °C)–thaw (room temperature), placing processed QC samples in mobile phase and unprocessed QC samples at room temperature for 12 h in dim light, and placing QC samples in blank rat plasma for 30 days under −20 °C freezer.

2.7. Application of the analytical method to a pharmacokinetic study

The validated method was used to investigate the profiles of TRG and its metabolites in Wistar rats. TRG was prepared at a concentration of 15 mg/ml in a 40% (v/v) PEG-400 (polyethylene glycol) aqueous solution, and administered orally (150 mg/kg) to intact rats (body weight of 230 ± 15 g). Aliquots of blood (250 μl) were serially collected by puncture of the retro-orbital sinus at 0.17, 0.33, 0.67, 1.0, 2.0, 4.0, 6.0, 8.0, 12.0 and 24.0 h post-dosing, respectively. Three or four time points corresponded to three or four samples of one rat. Plasma was separated by centrifugation at 2000 *g* for 10 min and stored frozen at −20 °C until analysis.

3. Results and discussion

3.1. Pretreatment of samples and optimization of chromatographic conditions

During the method development, a variety of organic solvents, such as ethyl acetate [14], *n*-hexane–dichloromethane–isopropanol (150:300:5, v/v/v), *n*-hexane–ether (2:3, v/v) and ether were investigated as the liquid–liquid extraction solvents, but extraction recoveries were low (25.5% for *n*-hexane–dichloromethane–isopropanol (150:300:5, v/v/v), 30.6% for *n*-hexane–ether (2:3, v/v) and 35.3% for ether) except for ethyl acetate. Though the extraction recovery was high (66.5%) when ethyl acetate was chosen as the extraction solvent, the peaks of TRN and TR were interfered by endogenous substances. Whereas acetonitrile was used as the deproteinization solvent, a good cleanup of the plasma samples and adequate recoveries values (approximately 90%) were obtained. Therefore, deproteinization was selected as the method of pretreatment of samples.

The chromatographic conditions were investigated to optimize sensitivity, resolution and peak shape. Several variations to the mobile phase were undertaken: changes in nature and percentages of organic solvent (methanol or acetonitrile) and in the apparent pH of the mobile phase. The high resolution among analytes and symmetric peaks shape were obtained when mobile phase consisted of 25% acetonitrile, 75% H_2O adjusted with formic acid to pH 3.5 (Fig. 2).

A number of compounds, such as salicylic acid, baicalin, baicalein, genistein, daidzein and scutellarin were tested for potential use as internal standard for this assay. Among these,

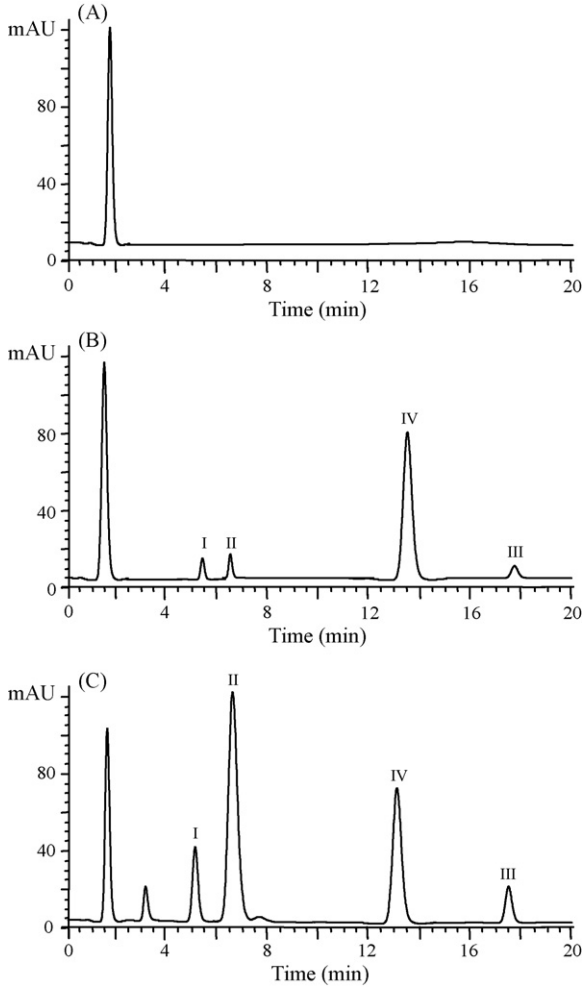


Fig. 2. Representative chromatograms of TRG and its metabolites. (A) A blank rat plasma sample; (B) a blank rat plasma sample spiked with 0.04 μg/ml TRG, 0.04 μg/ml TRN, 0.04 μg/ml TR and 20.0 μg/ml I.S., respectively; (C) a rat plasma sample 0.17 h after an oral administration of 150 mg/kg TRG. Peaks I, II, III and IV refer to TRG, TRN, TR and I.S., respectively.

Table 1
Summary of precision and accuracy from QC samples of rat plasma ($n = 3$ day, 6 replicates per day)

Analytes	Added C (μg/ml)	Found C (μg/ml)	Intra-run R.S.D. (%)	Inter-run R.S.D. (%)	Relative error (%)
TRG	0.10	0.10	8.2	9.3	−1.9
	4.00	4.15	4.4	8.4	3.8
	40.0	42.9	5.7	9.3	7.3
TRN	0.10	0.10	7.2	8.3	−2.9
	4.00	4.25	3.4	6.5	6.6
	40.0	41.5	4.7	7.4	3.8
TR	0.10	0.10	8.6	7.3	1.4
	2.00	2.15	4.3	6.2	7.4
	8.00	8.58	3.9	6.1	7.3

baicalin has chemical structure similar to those of the analytes and its retention time was 13.5 min. Therefore, baicalin was chosen as internal standard.

Although many methods for measuring TR and TRN in biological samples have been presented [7,8,10,17,18], TRN concentration was calculated indirectly by enzymatic hydrolysis in these methods because the reference substance of TRN was not available. Therefore, this is the first study quantifying simultaneously TRG, TR and TRN in rat plasma.

3.2. Method validation

3.2.1. Selectivity

The selectivity of the method was assessed by comparing chromatograms of six independent plasma samples from rats, each as a blank and a spiked sample. Fig. 2 shows the typical chromatograms of a blank plasma sample, a blank plasma sample spiked with TRG/TRN/TR at the LLOQ and internal standard, and a plasma sample from a rat 0.17 h after an oral administration. No significant interferences with analytes or internal standard from endogenous substances were detected. The retention times for TRG, TRN, I.S. and TR were 5.4, 6.6, 13.5 and 17.8 min, respectively.

3.2.2. Linearity and lower limit of quantification

Calibration standards were prepared by spiking 100 μl of the standard solutions of TRG/TRN/TR to 100 μl of blank rat plasma to achieve the final standard plasma concentrations for TRG/TRN/TR. The linear regression curves were obtained over the concentration range of 0.04–50.0 μg/ml for TRG and TRN, and 0.04–10.0 μg/ml for TR in rat plasma, respectively. Typical equations of the calibration curves were as follows:

$$\text{TRG}: \quad Y = 1.39 \times 10^{-1}\ X + 7.40 \times 10^{-3} \quad (r = 0.9985);$$

$$\text{TRN}: \quad Y = 9.64 \times 10^{-2}\ X + 2.51 \times 10^{-3} \quad (r = 0.9992);$$

$$\text{TR}: \quad Y = 1.64 \times 10^{-1}\ X + 2.44 \times 10^{-3} \quad (r = 0.9963).$$

where Y is the peak area ratios of analytes to internal standard, and X is the concentrations of analytes.

Lower limit of quantification (LLOQ) was defined as the lowest concentration of an analyte in a sample that can be quantitatively determined with suitable precision ≤15% and accuracy within ±15% [21]. LLOQs of TRG, TRN and TR in 100 μl rat plasma were at 0.04 μg/ml. These LLOQs are sufficient for pharmacokinetic studies of TRG, TRN and TR in rat.

3.2.3. Precision and accuracy

The precision was evaluated by the intra-day R.S.D. and inter-day R.S.D., and the accuracy was evaluated as the relative error [(concentration found − concentration spiked)/(concentration spiked) × 100%]. Table 1 summarizes the intra- and inter-day precision and accuracy for TRG, TRN and TR from QC samples. In this assay, the intra- and inter-day precisions ranged from 4.4% to 8.2% and from 8.4% to 9.3% for each QC level of TRG, from 3.4% to 7.2% and from 6.5% to 8.3% for each QC

Table 2
Stability of TRG, TRN and TR from QC samples in rat plasma ($n = 3$)

	TRG (μg/ml)			TRN (μg/ml)			TR (μg/ml)		
	0.10	4.00	40.0	0.10	4.00	40.0	0.10	2.00	8.00
Freeze–thaw stability (relative error, RE, %)									
1 cycle	3.2	3.5	6.3	4.2	−2.2	−1.4	−4.3	3.2	−2.3
3 cycle	−6.8	2.0	3.8	6.3	3.3	4.8	−5.9	6.8	0.2
Storage stability (−20 °C, RE, %)									
30 day	−6.3	1.3	−5.2	1.0	−3.9	−5.7	−3	3.1	−2.6
Processed plasma samples in mobile phase at room temperature (RE, %)									
12 h	2.9	−2.8	−0.9	−2.8	4.4	−1.0	−3.9	2.4	1.1
Unprocessed plasma samples at room temperature (RE, %)									
12 h	0.0	−3.2	−2.8	−1.0	2.6	−3.6	3.9	−0.1	2.4

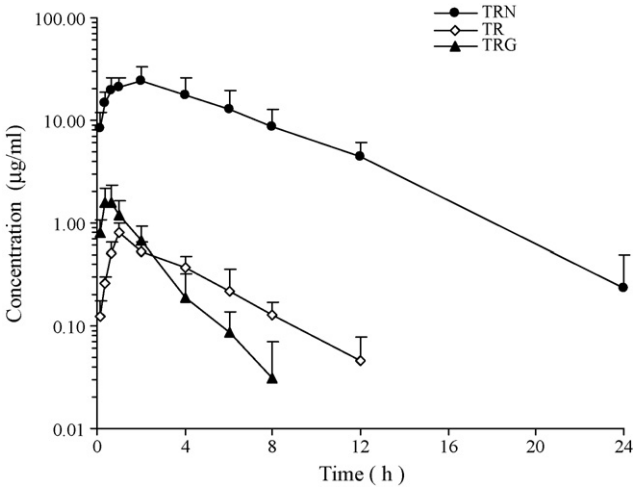


Fig. 3. Mean plasma concentration–time curves of TRG, TR and TRN after an oral administration of 150 mg/kg TRG to Wistar rats ($n = 8$).

level of TRN, from 3.9% to 8.6% and from 6.1% to 7.3% for each QC level of TR, respectively. The accuracies determined from the QC samples were within ±7.3% for each QC level of TRG, ±6.6% for each QC level of TRN and ±7.4% for each QC level of TR. The results indicated that the values were within the acceptable range and the method was accurate and precise [21].

3.2.4. Recovery and stability

The recoveries of TRG were 91.2%, 95.1% and 94.5% ($n = 6$) at concentrations of 0.10, 4.00 and 40.0 μg/ml; those of TRN were 92.7%, 90.8% and 95.9% ($n = 6$) at concentrations of 0.10, 4.00 and 40.0 μg/ml; those of TR were 89.1%, 91.3% and 92.6% ($n = 6$) at concentrations of 0.10, 2.00 and 8.00 μg/ml ($n = 6$), respectively. The recovery of I.S. was 87.9% ($n = 6$) at concentration of 20.0 μg/ml.

Table 2 shows the stability for TRG, TRN and TR from QC samples. As can be seen that analytes were stable (RE% from −6.8% to 6.8%) in rat plasma after three cycles of freeze (−20 °C)–thaw (room temperature); the analytes were stable (RE% from −6.3% to 3.1%) in rat plasma for 30 days under −20 °C freezer; processed QC samples in mobile phase and unprocessed QC samples at room temperature were also stable (RE% from −3.6% to 4.4%) for 12 h in dim light.

3.3. Pharmacokinetic study

After an oral administration of 150 mg/kg TRG to Wistar rats, plasma concentrations of TRG, TRN and TR were determined by the described HPLC method. Fig. 3 shows the mean plasma concentration–time curves of TRG, TRN and TR in rat ($n = 8$).

In rats, the parent drug TRG and the metabolites TR and TRN were detected for at least 8, 12 and 24 h after the oral dose, respectively. Mean plasma concentrations of TRG, TR and TRN were 1.70, 0.81and 26.2 μg/ml, respectively. The elimination of TRG, TR and TRN from plasma was mono-phasic, with mean half-lives of about 1, 2 and 4 h, respectively. In the rat plasma, TRN was the dominant substance with the highest concentration and a relatively long elimination half-life.

4. Conclusions

An HPLC–UV method has been developed and validated for the simultaneous determination of TRG, and its metabolites, TRN and TR in rat plasma. The method has a lower limit of quantification of 0.04 μg/ml for TRG, TRN and TR, using a 100 μl plasma sample, has been proven to be sensitive, selective and reproducible. It was successfully applied in a pharmacokinetic study of TRG and its metabolites in rats.

Acknowledgement

This paper was supported in part by the 863 Program of China (Grant 2003AA2Z347C).

References

[1] C. Dominguez, D.A. Guillen, C.G. Barroso, J. Chromatogr. A 918 (2001) 303.
[2] G. Regev-Shoshani, O. Shoseyov, I. Bilkis, Z. Kerem, Biochem. J. 374 (2003) 157.
[3] S.F. Luo, X.Z. Jin, J.F. Ye, P.W. Zang, Chin. J. Pharmacol. Toxico. 13 (1999) 1.
[4] Y. Kimura, H. Ohminami, H. Okuda, K. Baba, M. Kozawa, S. Arichi, Hippokrates Verlag. Gmbh. 49 (1983) 51.
[5] H. Arichi, Y. Kimura, H. Okuda, K. Baba, M. Kozawa, S. Arichi, Chem. Pharm. Bull. 30 (1982) 1766.
[6] P.W. Zhang, C.L. Yu, Y.Z. Wang, L.S. Lou, R.S. Li, Acta Pharmacol. Sin. 16 (1995) 265.
[7] J.F. Marier, P. Vachon, A. Gritsas, J. Zhang, J.P. Moreau, M.P. Ducharme, J. Pharmacol. Exp. Ther. 302 (2002) 369.
[8] C. Yu, Y.G. Shin, A. Chow, Y. Li, J.W. Kosmeder, Y.S. Lee, W.H. Hirschelman, J.M. Pezzuto, R.G. Mehta, R.B. van Breemen, Pharm. Res. 19 (2002) 1907.
[9] G. Kuhnle, J.P.E. Spencer, G. Chowrimootoo, H. Schroerter, E.S. Debnam, S.K. Srai, Biochem. Biophys. Res. Commun. 272 (2000) 212.
[10] W. Andlauer, J. Kolb, K. Siebert, P. Fürst, Drugs Exp. Clin. Res. 26 (2000) 47.
[11] Z. Zhu, G. Klironomos, A. Vachereau, L. Neirinck, D.W. Goodman, J. Chromatogr. B 724 (1999) 389.
[12] M.E. Juan, R.M. Lamuela-Raventós, M.C. de la Torre-Boronat, J.M. Planas, Anal. Chem. 71 (1999) 74.
[13] H. He, X. Chen, G. Wang, J. Wang, A.K. Davey, J. Chromatogr. B 832 (2006) 177.
[14] D.J. Boocock, K.R. Patel, G.E. Faust, D.P. Normolle, T.H. Marczylo, J.A. Crowell, D.E. Brenner, T.D. Booth, A. Gescher, W.P. Steward, J. Chromatogr. B 848 (2007) 182.
[15] X. Chen, H. He, G. Wang, B. Yang, W. Ren, L. Ma, Q. Yu, Biomed. Chromatogr. 21 (2007) 257.
[16] D. Blache, I. Rustand, G. Lesgards, G.N. Loreau, J. Chromatogr. B 702 (1997) 103.
[17] D.M. Goldberg, J. Yan, G.J. Soleas, Clin. Biochem. 36 (2003) 79.
[18] G.J. Soleas, J. Yan, D. Goldberg, J. Chromatogr. B 757 (2001) 161.
[19] E. Wenzel, T. Soldo, H. Erbersdobler, V. Somoza, Mol. Nutr. Food Res. 49 (2005) 482.
[20] A.M. Almeida, M.M. Castel-Branco, A.C. Falcao, J. Chromatogr. B 774 (2002) 215.
[21] V.P. Shah, K.K. Midha, J.W.A. Findlay, H.M. Hill, J.D. Hulse, I.J. McGilveray, G. McKay, K.J. Miller, R.N. Patnaik, M.L. Powell, A. Tonelli, C.T. Viswanathan, A. Yacobi, Pharm. Res. 17 (2000) 1551.