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Development and Validation of HPLC Method for Quantification of Phytoconstituents in *Phyllanthus emblica*

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ABSTRACT

A high performance liquid chromatography method coupled with diode array detection was developed to simultaneously determine eight different marker compounds in Phyllanthus emblica. These markers are ascorbic acid (1), gallic acid (2), methyl gallate (3), chlorogenic acid (4), ethyl gallate (5), ellagic acid (6), rutin (7) and chebulagic acid (8). HPLC analysis was carried out at wavelength 272nm. The developed method was able to determine the marker compounds with excellent resolution, precision and recovery. The chromatographic separation was performed on Thermo Scientific BDS HYPERSIL Phenyl reversed-phase column (250mm×4.6mm, 5µm). The mobile phase was consisted of 0.1% ortho-phosphoric acid: Methanol (95:05v/v) (A) and acetonitrile (B) at a flow rate of 1.5 ml/min gradient mode. Regression equations showed good linear relationships (R^2 > 0.998) between the peak area of each marker and concentration. The assay was reproducible with overall intra- and inter-day variation of less than 3.4%. The recoveries, measured at three concentration levels, varied from 97.8% to 101.1%. The method was applied to determine the amounts of the marker compounds in dried fruits of Phyllanthus emblica, and significant variations in phytoconstituents were observed.

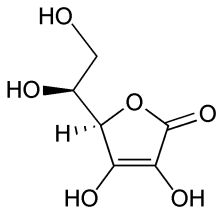
Keywords: *Phyllanthus emblica*, RP-HPLC, Method validation, Ascorbic acid, Gallic acid, Chebulagic acid.

INTRODUCTION

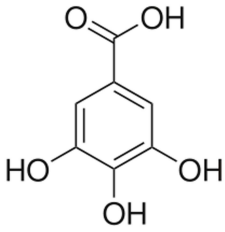
Emblica (*Phyllanthus emblica* L.), an Euphorbiaceous plant, is widely distributed in subtropical and tropical areas of China, India, Indonesia and Malay Peninsula, and used in many traditional medicinal systems, such as Chinese herbal medicine, Tibetan medicine and Ayurvedic medicine (1). Emblica fruits have been used for fever and inflammatory treatments by rural populations in its growing areas. The earlier study have demonstrated potent anti-microbial, anti-oxidant, adaptogenic, hepatoprotective, anti-tumour and anti-ulcerogenic activities (2-4). Phyllanthus emblica fruits possess antimutagenic and anticarcinogenic properties owing to the combined presence of β-carotene, ascorbic acid and chlorophylin and have also been shown to modulate several biochemical events associated with tumor promotion such as alteration of protein kinase C (PKC) activity (5). It is a rich dietary source of vitamin C, minerals, flavonoids and amino acids, and also contains a wide variety of phenolic compounds, such as tannins, phyllembelic acid, phyllemblin, rutin, curcuminoides, mucic acid, and emblicol (6-10). Scartezzini et al. proposed a reliable HPLC-DAD for the identification and quantification of ascorbic acid and further indicated that high antioxidant activity is due to a large percentage of the presence of ascorbic acid (11).

Reversed-phase high-performance liquid chromatography (RP-HPLC) is the commonly used analytical separation technique for either phenolic compounds like flavonols or for various derivatives of benzoic acid. Due to the variability of column filling materials and solvent systems, RP-HPLC exhibits a great potential in separating complex mixtures of phenolic compounds [12–18].

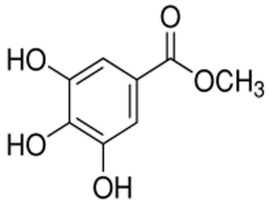
The purpose of this study was to develop a novel high-performance liquid chromatography procedure for simultaneous determination of all eight phytoconstituents in the fruits of *Phyllanthus emblica* Linn.



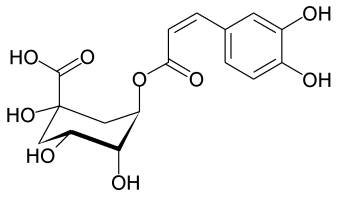
(a) Ascorbic acid



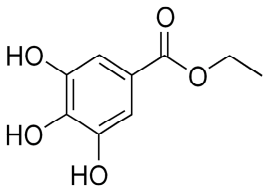
(b) Gallic acid



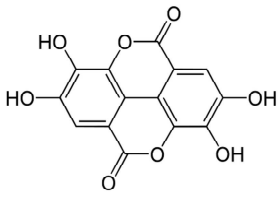
(c) Methyl gallate



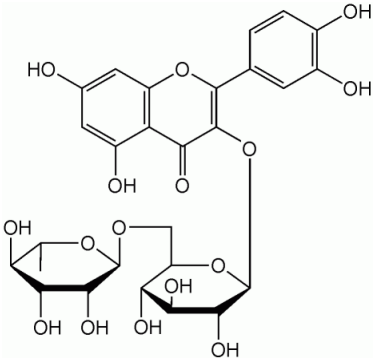
(d) Chlorogenic acid



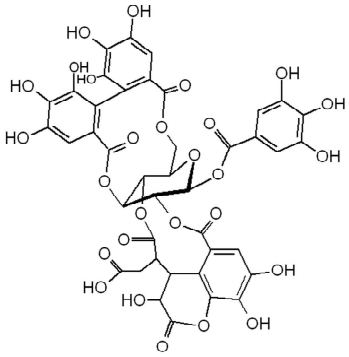
(e) Ethyl gallate



(f) Ellagic acid



(g) Rutin



(h) Chebulagic acid

Fig. 1. Structures of phytoconstituents in *Phyllanthus emblica*

EXPERIMENTAL SECTION

Chemicals and materials:

The chemicals used were analytical or HPLC-grade. HPLC-grade acetonitrile, methanol, ortho-phosphoric acid (AR grade) were purchased from Merck specialty India Pvt. Ltd. Ultra pure water, generated by use of a Milli-Q System (Millipore), was used for sample preparation and preparation of mobile phases for HPLC analysis. Dried and fresh fruits of *Phyllanthus emblica* were procured from local market in Mumbai, India and authenticated at Agharkar Research Institute Pune, India, with a voucher specimen (F-140) which deposited in the herbarium. Standard compounds ascorbic acid (1), gallic acid (2), chlorogenic acid (4), ellagic acid (6), rutin (7) were purchased from Sigma Aldrich. Other standard compounds such as methyl gallate (3), ethyl gallate (5) and chebulagic acid (8) were isolated from the dried fruit of the *Phyllanthus emblica* Linn. (Euphorbiaceae).

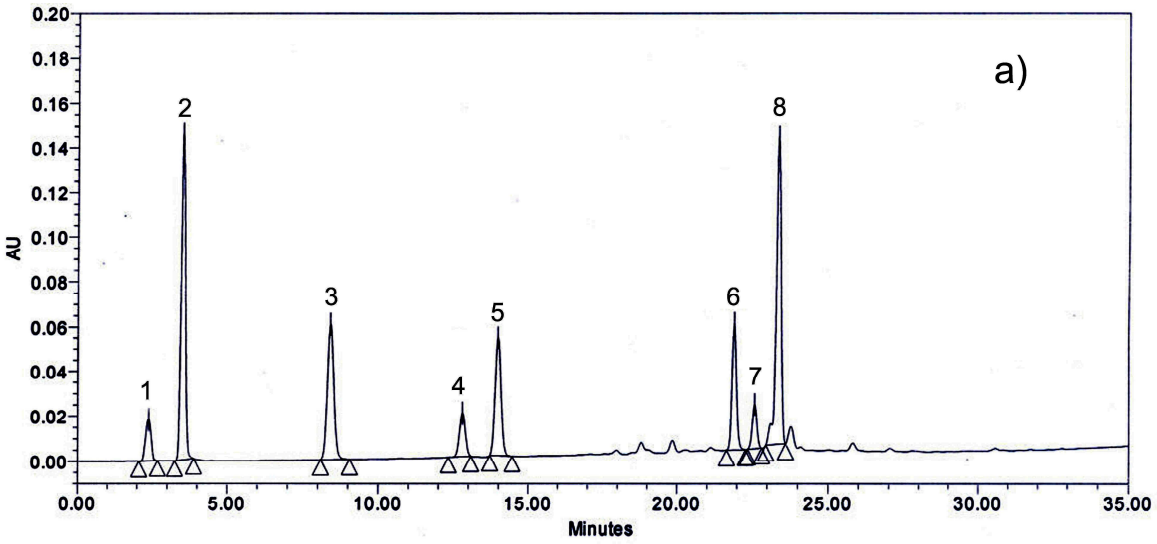
Sample preparation

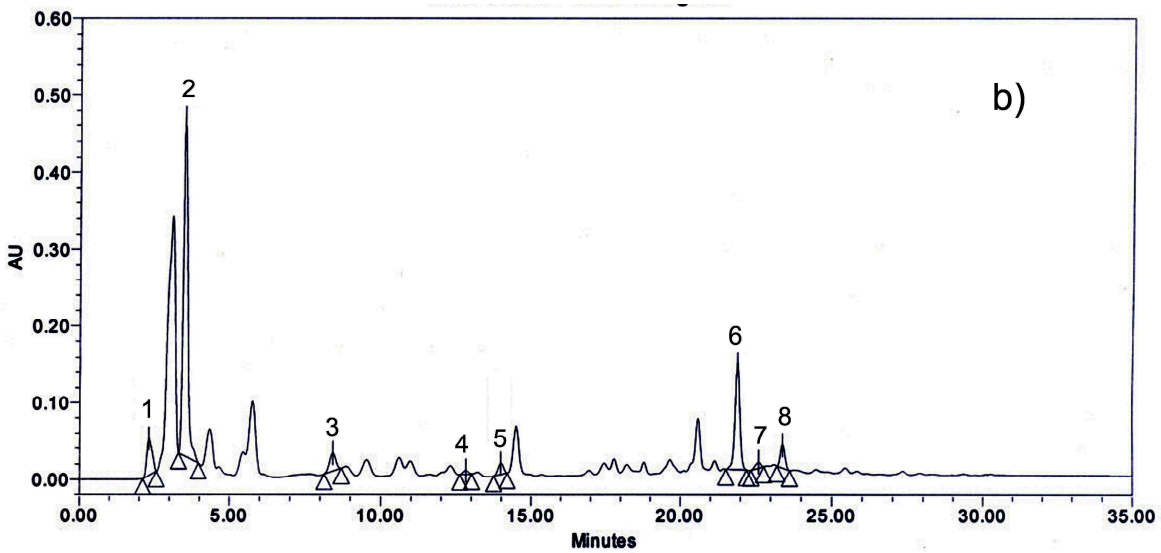
The accurately weighed dried fruits powder (0.2 g) was transferred in 50ml volumetric flask contains 30ml extraction solvent (methanol–water (70:30, v/v) and sonicated for 20 min at 27±3°C in ultra sonicator water bath and diluted up to mark. The fruits (1kg) were cut into pieces, and expressed to get juice at room temperature for 2 h with stirring. The filtered juice was freeze-dried (Freezone 4.5, Labconco, USA) under high vacuum (133 × 104 mBar) at −40 ± 2°C. to get dry powder. The freeze-dried powder (100 mg) was extracted overnight, with 10 ml methanol. The solutions of dried fruits (AM-D) and fresh fruits (AM-L) were filtered through a 0.45-µm membrane prior to injection into the HPLC system.

Chromatographic condition

HPLC Analysis was performed on a Waters chromatographic system consisting Waters 2695 separation module (quaternary pump) equipped with an auto injector and Waters 2998 photodiode array detector. Data acquisition was made with Waters Empower Pro software. Separation was achieved on Thermo Scientific BDS HYPERSIL Phenyl reversed-phase column (250mm×4.6mm, 5µm). The mobile phase was consisted of 0.1% ortho-phosphoric acid: Methanol (95:05v/v) (A) and acetonitrile (B) at a flow rate of 1.5 ml/min (A) and acetonitrile (B). The gradient program was as follows: 0–5 min, linear gradient 0–0% B; 5–20 min, 0–15%

B; 20–30 min 15–25% B; 30–35 min 25-30% B; 35-36 min 30-0% B; 36-45 linear gradient 0–0% B. The flow rate was 1.5 ml/min and aliquots of 10µl were injected. The UV detection wavelength was set at 272 nm. Absorption spectra of compounds were recorded between 200 and 400 nm. The compounds were identified by comparing their retention times and UV spectra with those of the markers.





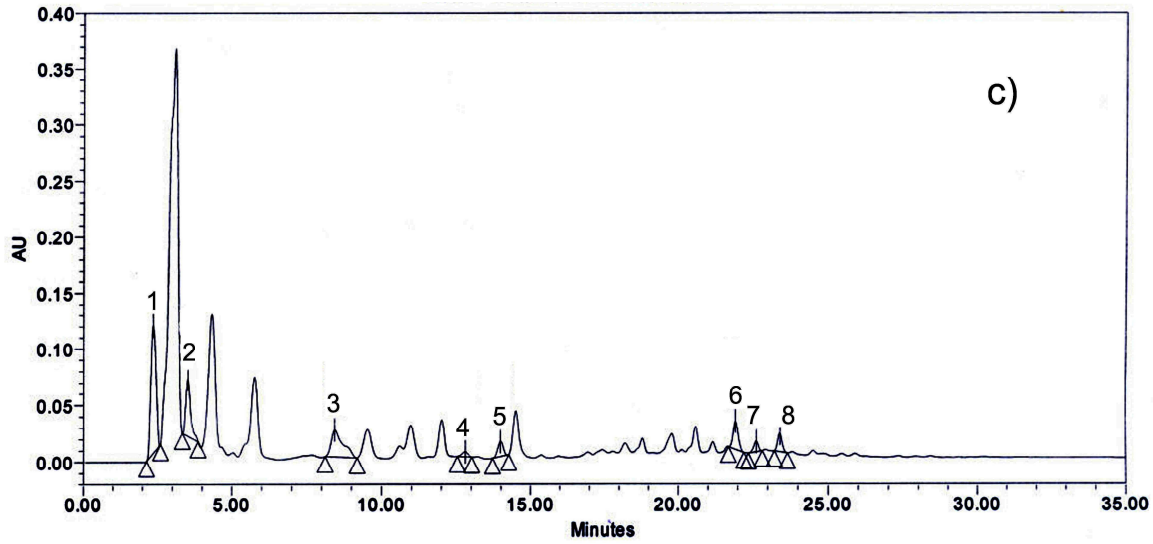


Fig.2. HPLC chromatograms of (a) standard mixture: ascorbic acid (1), gallic acid (2), methyl gallate (3), chlorogenic acid (4), ethyl gallate (5), ellagic acid (6), rutin (7) and chebulagic acid (8). (b) *Phyllanthus emblica* dried fruits (AM-D) (c) *Phyllanthus emblica* fresh fruits (AM-L).

Table.1 Method validation data (Linearity, LOD & LOQ)

Phytoconstituent	RT (min)	R^2	Linear range (µg/ mL)	LOD (µg/ mL)	LOQ (µg/ mL)
Ascorbic acid	2.38	0.999	90-240	0.7	2.331
Gallic acid	3.51	0.998	40-140	0.6	1.998
Methyl gallate	8.41	0.999	5-30	0.5	1.665
Chlorogenic acid	12.80	0.999	20-45	0.4	1.332
Ethyl gallate	13.98	0.998	5-30	0.5	1.665
Ellagic acid	21.90	0.999	30-80	0.6	1.998
Rutin	22.58	0.999	20-45	0.4	1.332
Chebulagic acid	23.36	0.999	100-350	0.5	1.665

RESULTS AND DISCUSSION

Extraction procedure

Various extraction methods, solvents and times were evaluated to obtain the best extraction efficiency. The results revealed that ultrasonic bath extraction was better than other extraction methods, so the further experiments were carried out with ultrasonic bath extraction. Various solvents including water, methanol–water (50:50 v/v; 60:40 v/v; 70:30 v/v; 80:20 v/v) and methanol were screened. Methanol–water (70:30 v/v) exhibited complete extraction of all the major constituents. No second extraction step was found necessary.

Optimization of chromatographic conditions

During the optimization of method different columns of different selectivity like C18, Cyno and Phenyl columns were tried. When acetonitrile used as organic modifier achieved better separation with short retention time and sharp peak shape than methanol. After a series of screening experiments, it was concluded that 0.1% ortho-phosphoric acid: Methanol (95:05v/v) (A) and acetonitrile (B) at a flow rate of 1.5 ml/min (A) and acetonitrile (B) in gradient mode

gave better separation with good resolution of adjacent peaks with acceptable tailing factors. The chromatographic separation was achieved on a Thermo Scientific BDS HYPERSIL Phenyl reversed-phase column (250mm×4.6mm, 5µm) The mobile phase was consisted of 0.1% ortho-phosphoric acid: Methanol (95:05v/v) (A) and acetonitrile (B) at a flow rate of 1.5 ml/min (A) and acetonitrile (B) in the gradient mode to keep short run time 35 min. As the maximum types of phytoconstituents show UV maxima between 270-278nm UV detector was set at 272nm to provide sufficient sensitivity for each analyte.

Table.2 Method validation data (Recovery and precision)

Phytoconstituent	Recovery [a]	Precision (RSD%)	
	(%)	Intra-day [b]	inter-day [c]
Ascorbic acid	100.48	1.3	2.2
Gallic acid	99.63	1.6	1.4
Methyl gallate	101.47	2.4	2.2
Chlorogenic acid	99.24	2.3	2.6
Ethyl gallate	98.96	2.5	2.1
Ellagic acid	100.40	1.5	2.6
Rutin	99.78	2.2	2.7
Chebulagic acid	100.45	2.1	2.8

[b] *mean of samples were analysed on same day n=6*

Table.3 Quantification of Phytoconstituents in *Phyllanthus emblica*

Phytoconstituent	AM -D (%)	AM-L (%)
Ascorbic acid	0.90	2.15
Gallic acid	3.60	0.44
Methyl gallate	0.24	0.47
Chlorogenic acid	0.12	0.12
Ethyl gallate	0.14	0.14
Ellagic acid	0.80	0.15
Rutin	0.21	0.21
Chebulagic acid	0.71	0.29

Sample analysis

The method was applied to simultaneous determination of phytoconstituents (1-8) in dried fruits of *Phyllanthus emblica* collected from market. Representative chromatograms are shown in Figure. 2. The contents of the eight compounds in the samples were quantified and the results are shown in Table 3 with the mean values of three replicate injections. Variations of the eight compounds content in the fruits of *Phyllanthus emblica* are shown in histogram Figure 3

Accuracy

Recovery tests were carried out to further investigate the accuracy of the method by adding three concentration levels of the mixed standard solutions to known amounts of *Phyllanthus emblica* samples prior to extraction. The resultant samples were then extracted and analyzed with the described method. The average percentage recoveries were evaluated by calculating the ratio of detected amount *versus* added amount. The recovery of the method was in the range of 97.8–101.1%, as shown in Table 2. Considering the results, the method was deemed to be accurate.

Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The intra and inter-day precisions were determined by analyzing known concentrations of the seven analytes in six replicates during a single day and by duplicating the experiments on two successive days. In order to confirm the repeatability, six different working solutions prepared from the same sample obtained from different manufacturers were analyzed. The relative standard deviation (R.S.D.) was taken as a measure of precision and repeatability. The results are shown in Table 2, indicating that the intra-, inter-day and repeatability R.S.D. values of the eight compounds were all less than 3.5%, which showed good reproducibility of the developed method.

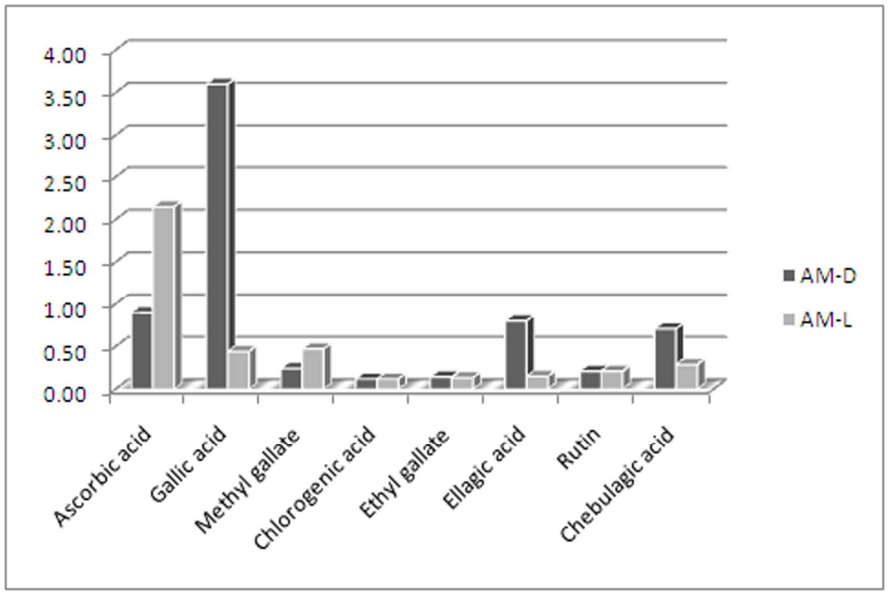


Fig.3. Histogram presenting phytoconstituents contents in *Phyllanthus emblica* samples.

Linearity

Standard stock solutions containing ascorbic acid, gallic acid, methyl gallate, chlorogenic acid, ethyl gallate, ellagic acid, rutin and chebulagic acid were prepared and diluted to appropriate concentrations for plotting the calibration curves. At least six concentrations of the analyte solutions were analyzed in triplicate, and then the calibration curves were constructed by plotting the mean peak areas *versus* the concentration of each analyte. The calculated results are given in Table 1. All the analytes showed good linearity ($R^2 > 0.998$) in a relatively wide concentration range.

Limits of detection and quantification

The working solutions of the analytes were further diluted with methanol to yield a series of appropriate concentrations. Limit of detection (LOD) and limit of quantification (LOQ) of the developed method were determined by injecting progressively low concentrations of the standard solutions using the developed RP-HPLC method .The LOD and LOQ for each investigated compounds were calculated at signal to noise ratio of 3:1 and 10:1 respectively as shown in Table.1.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness of the method was investigated under a variety of conditions including changes of pH of the mobile phase, flow rate and gradient variation. The degree of reproducibility of the results obtained as a result of small deliberate variations in the method parameters has proven that the method is robust. The ruggedness of the method was determined by repeating the experiments on Jasco HPLC system by different operators in addition to Waters HPLC system.

CONCLUSION

In this study, an HPLC–DAD method for the qualification and quantification of phtyoconstituents in *Phyllanthus emblica* has been developed. The possible reasons for variations are the how the materials processed, polarity of the solvents used for extraction and thermal stability of phytoconstituents. This method is validated for good accuracy, repeatability and precision, and can be used to evaluate the quality of the drug. This multi-phytoconstituents assay method will be helpful to quality control and stability studies of *Phyllanthus emblica*.

REFERENCES

[1] YJ Zhang; T Tanaka; Y Iwamoto; CR Yang; I Kouno. *Tetrahedron Lett*., **2000**, 41, 1781–1784.
[2] J Ahmad; Z Mehmood; F Mohammad. *J. Ethnopharmacol*., **1998**, 62, 183–193.
[3] JB Calixto, ARS Santos, Filho VC, RA Tunes. *J. Med. Biol*., **1998**, 31, 225-228.
[4] JK Jose, Y Kuttan, R Kutan. *J. Ethnopharmacol*., **2001**, 75, 65–69.
[5] L Anila and NR Vijayalakshmi. *Food Chem.*, **2003**, 83, 569–574.
[6] KJ Jeena, G Kuttan, J George, R Kuttan. *J. Clin. Biochem*. Nutr., **1996**. 22, 171–176.
[7] YJ Zhang, T Abe, T Tanaka, CR Yang. I Kouno. *J. Nat. Prod*., **2001**, 64, 1527–1532.
[8] YJ Zhang, T Tanaka, CR Yang, I Kouno. *Pharm. Bull*., **2001**, 49, 537–540.
[9] M Majeed, B Bhat, A Jadhav, J Srivastava, K Nagabhushanam. *J. Agric. Food Chem*., **2009**, 57, 220–225.
[10] S Ghosal, VK Tripathi, S Chauhan. *Indian J. Chem*., **1996**, 35B, 941–948.
[11] P Scartezzini, F Antognoni, MA Raggi, F Poli, C Sabbioni. *J. Ethnopharm*., **2006**, 104, 113–118.
[12] XG He, LZ Lian, LZ Lin. *J. Chromatogr. A.*, **1997**, 757, 81–87.
[13] U Justesen, P Knuthsen, T Leth. *J. Chromatogr. A*., **1998**, 799, 101–110.
[14] A Crozier, E Jensen, MEJ Lean, MS McDonald. *J. Chromatogr. A*., **1997**, 761, 315–321.
[15] M Keina¨nen, R Julkunen-Tiitto. *J. Chromatogr. A.*, **1998**, 793, 370–377.
[16] A Escarpa, MC Gonza´les. *J. Chromatogr. A*., **1998**, 823, 331–337.
[17] M Brolis, B Gabetta, N Fuzzati, R Pace, F Panzeri, F Peterlongo. *J. Chromatogr. A*., **1998**, 825, 9–16.
[18] A Rehwald, B Meier, O Sticher. *J. Chromatogr. A*., **1994**, 677, 25–33.