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Determination of hyperforin in human plasma using solid-phase extraction and high-performance liquid chromatography with ultraviolet detection

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Abstract

Hyperforin is one of the most important active components in St. John's wort (*Hypericum perforatum*), a botanical dietary supplement used as an alternative treatment modality for mild to moderate depression. A solid-phase extraction (SPE) and an isocratic high-performance liquid chromatography (HPLC) analysis with ultraviolet (UV) detection were developed to determine hyperforin in human plasma samples. Benzo[*k*]fluoranthene was used as an internal standard. The absolute recovery for hyperforin was more than 89% for plasma concentrations ranging from 25 to 500 ng/ml. The linearity of calibration curves, inter-day and intra-day relative standard deviations were investigated. The limit of detection (LOD) of hyperforin was 4 ng/ml in plasma and the limit of quantitation (LOQ) was 10 ng/ml. Hyperforin concentrations in human plasma following St. John's wort administration were analyzed. The result suggests that this method is rapid, sensitive, reproducible and capable of quantitative analysis of hyperforin plasma concentrations.
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Keywords: Hyperforin; St. John's wort

1. Introduction

St. John's wort (*Hypericum perforatum*) is a botanical medicine widely used in Europe and the USA as an alternative treatment for mild to moderate depression. Its growing popularity stems from a safety and efficacy profile that challenges that of many conventional antidepressive agents [1–3]. To date, a number of phytochemicals have been isolated from St. John's wort, including phloroglucinols (hyperforin and adhyperforin), naphthodianthrones (hypericin and pseudohypericin), flavonoids and bioflavonoids [4–6].

Hyperforin (Fig. 1), the most abundant lipophilic component in St. John's wort (*Hypericum perforatum*), appears to be the key constituent responsible for the antidepressant property of St. John's wort preparations. Inhibition of serotonin, norepinephrine

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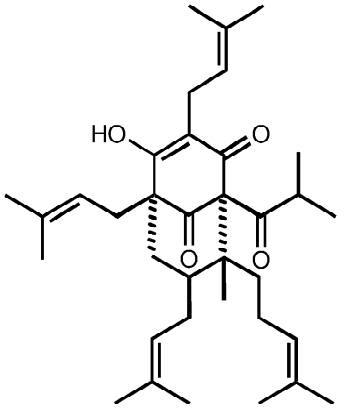

Fig. 1. Structure of hyperforin.

and dopamine re-uptake, as well as down-regulation of cortical β-adrenoceptor expression has been proposed as a mechanism for hyperforin's antidepressive effect. In addition, extracts of St. John's wort standardized for hyperforin content have been correlated in a dose-dependent manner with clinical antidepressive efficacy [7].

Studies in recent years have demonstrated that dietary supplements containing St. John's wort were associated with the increased metabolism of several co-administered drugs [8], including the HIV protease inhibitor, indinavir [9], the immunosuppressant, cyclosporin [10,11] and the synthetic estrogen, ethinylestradiol [12]. These effects were explained in part by the action of hyperforin on the pregnane X receptor system to induce the expression of hepatic and intestinal cytochrome P450 3A4 (CYP3A4) [13] and also in part by the increase of intestinal P-glycoprotein [14]. An in vitro study, however, has shown that hyperforin was a competitive inhibitor of CYP3A4 and a non-competitive inhibitor of CYP2D6 [15].

An understanding of hyperforin's pharmacokinetic profile is important for clinical dosing schedules, and essential for mechanistic and toxicological studies of St. John's wort. A few high-performance liquid chromatography (HPLC) methods have been reported for the determination of hyperforin in human plasma. Biber et al. [16] reported a liquid–liquid extraction using hexane–ethyl acetate (7:3) and HPLC analysis with tandem mass spectrometry (MS–MS) detection that was highly sensitive, but could be cost prohibitive for many laboratories as the required instrumentation is not routinely available. Subsequently, Chi and Franklin [17] developed a method for analyzing hyperforin in human plasma using solid-phase extraction (SPE) and HPLC separation with ultraviolet (UV) detection. The validated assay range was 150–300 ng/ml and the extraction recovery tests for hyperforin were performed at concentrations >300 ng/ml values exceeding those typically found in humans after St. John's wort ingestion.

Recently, a method of liquid–liquid extraction using hexane–ethyl acetate (9:1) combined with modified HPLC–UV analysis was reported by Bauer et al. [18]. An advantage of this method was the incorporation of an internal standard; however, a potential drawback was the use of hexane–ethyl acetate (9:1, v/v) in liquid–liquid extraction that might potentially affect hyperforin stability. Our recent data showed that the concentration of hyperforin (500 ng/ml) in the hexane–ethyl acetate (9:1) system decreased to 82% of the original level after 1 h at room temperature (21 °C) in the dark.

A simple and reproducible HPLC method utilizing SPE for the determination of hyperforin in human plasma is described herein. A primary objective of the study was to develop an accurate and precise method (with high absolute recovery values) that precluded the use of nonpolar solvents and inorganic acids, which might impair hyperforin stability. The utility of the method is demonstrated by characterizing the hyperforin concentration–time profile in a human subject following ingestion of a commercially available St. John's wort supplement. Additional data are presented for measuring hyperforin concentrations in human volunteers following 28 days of St. John's wort supplementation.

2. Experimental

2.1. Chemicals and materials

Formic acid (97.8%), triethylamine (99%) and benzo[*k*]fluoranthene were purchased from Sigma (St. Louis, MO, USA). C_8, C_{18} and Oasis cartridges were purchased from Waters (Milford, MA, USA) and Phenomenex (Torrance, CA, USA). Hyperforin was isolated in our laboratory from a St. John's wort

leaf/flower mixture distributed by Frontier (Norway, IA, USA). The purity of hyperforin was determined by NMR, HPLC–MS and HPLC–PDA (>99%) [19]. All other reagents used were purchased from J.T. Baker (Phillipsburg, NJ, USA) and were HPLC grade. Water was distilled, deionized and passed through a Purification Pak (Milli-Q water purification system, Waters) prior to use. Human plasma samples were obtained from the Department of Pharmaceutical Sciences, College of Pharmacy, University of Arkansas for Medical Sciences (Little Rock, AR).

2.2. Apparatus and chromatographic condition

Chromatographic analysis was carried out using a Waters HPLC system composed of a Model 600 pump with a Model 717 autoinjector. Instrument control and data processing were accomplished with Millennium M32 Chromatogram Manager software (Waters). The analysis was performed on a Luna C_{18} column (150×4.6 mm, 3 μm particle size) protected by a C_{18} guard column (4×3 mm) (Phenomenex). To prepare the mobile phase, methanol–acetonitrile (3:2, v/v) was mixed with water (92:8). To 1000 ml of this solution, 2 ml each of formic acid and triethylamine were added to result in a solution with pH of 3.2±0.1. The flow-rate was 1 ml/min. Column effluent was monitored by ultraviolet absorbance detection (287 nm) using a Waters Model 996 photo diode array detector.

2.3. General procedure for sample preparation

Spiked human plasma or hyperforin-containing plasma samples (0.5 ml) were mixed with 0.5 ml of cold acetonitrile in a clean borosilicate glass test tube (5 ml). Tubes were tightly capped, shaken for 5 min using a mechanical shaker (Eberbach, Michigan, IL, USA), and centrifuged at 4500 *g* for 10 min. Each supernatant was mixed with 1.0 ml of water, and loaded onto a preconditioned solid-phase extraction (SPE) column (Oasis HLB, 6 mg, 3cc) (Waters). The preconditioning procedure consisted of passing two volumes of methanol (3 ml) followed by a 3-ml volume of 10% methanol in water. The flow-rate for preconditioning and sample loading was controlled to 3–5 ml/min by a vacuum manifold system (Waters). After sample loading, each SPE cartridge was washed with 3 ml 10% methanol in water and drained to dryness under vacuum. Hyperforin was eluted with 5 ml methanol. The eluent was dried under nitrogen and reconstituted in 200 μl methanol containing benzo[*k*]fluoranthene (200 ng/ml). A 50-μl aliquot was injected onto the analytical column.

Due to the susceptibility of hyperforin to photodegradation, sample preparation and analysis were conducted under yellow light.

2.4. Stock solutions and method validation

The stock internal standard solution (benzo[*k*]fluoranthene, 0.10 mg/ml) was prepared by dissolving 10.0 mg benzo[*k*]fluoranthene in benzene (100–200 μl). Methanol was added to bring the final volume to 100 ml. This solution is stable at room temperature indefinitely. A working internal standard solution (200 ng/ml) was prepared by diluting 0.5 ml of the stock solution to 250 ml with methanol. The working solution is also stable at room temperature for several months, provided that care is taken to avoid frequent opening of the bottle stopper.

The hyperforin stock solution (20.0 μg/ml) was prepared in methanol and stored at −70 °C. It is stable for 1 year. Appropriate amounts of the stock solution were diluted in methanol to obtain calibration standards in the range of 0.1 to 20.0 μg/ml. Calibration curves in human plasma ranging from 10 to 1000 ng/ml were prepared in triplicate by spiking with the appropriate hyperforin standard in blank plasma. Peak area ratios (hyperforin/benzo[*k*]fluoranthene) were used for quantitative computations. Calibration curves were calculated by linear regression analysis using SigmaPlot-2000. Absolute recoveries were calculated by comparing the hyperforin content found in spiked plasma at 25, 50, 100, 150 and 200 ng/ml after the SPE treatment to those prepared in methanol and analyzed directly by HPLC.

2.5. Human studies

Human study protocols were approved by the University of Arkansas for Medical Sciences Institutional Review Board and all participants provided written informed consent. In the first study, a single

dose of six St. John's wort capsules (300 mg/capsule, standardized to 0.3% hypericin; our test showed hyperforin content at 4.75 mg/g; Vitamer® Laboratories, Lake Forest, CA, USA) containing a total of 8.55 mg hyperforin were ingested by a volunteer and serial blood samples (5 ml) were drawn at 0.0, 0.5, 1.0, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0, 10.25, 12.0, 18.8, and 25.0 h. Plasma hyperforin concentration–time profiles were fitted to select polyexponential equations using a nonlinear least-squares fitting computer program (SAAM II, University of Washington, Seattle, WA, USA) and pharmacokinetic parameters were calculated from computer-generated coefficients and exponents by compartmental analysis. Twelve young adults (six females; age, mean±SD=25±3.9) participated in the second human subject study. St. John's wort capsules (Wild Oats markets, Boulder, CO, USA) were given three times daily for 28 days. Based on a hyperforin content of 13.6 mg/g, each subject ingested ~12.2 mg daily. Blood samples were collected from each subject about 1 h after the last dose.

3. Results

3.1. Solid phase extraction (SPE)

During the assay development, a variety of SPE cartridges was evaluated. The HLB Oasis cartridge (60 mg, Waters) extracted hyperforin from plasma with much lower variations than C_8 or C_{18} columns and was able to accommodate concentrations as high as 1000 ng/ml, making it suitable for both animal and human assays [16]. Interestingly, the napthodianthrones present in St. John's wort, hypericin and pseudohypericin, were adsorbed strongly onto the Oasis cartridges and were not eluted by 10 ml methanol. Absolute recoveries for hyperforin plasma concentrations ranging from 25 to 500 ng/ml were >89% (Table 1).

3.2. HPLC analysis

Using the isocratic HPLC method as developed in this study, the hyperforin and benzo[*k*]fluoranthene peaks were easily resolved with retention times of 12 and 7.5 min, respectively (Fig. 2). No interference from endogenous plasma components, or other phytochemicals present in St. John's wort (e.g. hypericin and pseudohypericin) was noted. Standard curves exhibited excellent linearity as evidenced by the regression equations and correlation coefficients. Within the range of 10 to 200 ng/ml, the calibration curve was $Y = 0.00210X - 0.00400$ (Y=peak area ratio and X=concentration ng/ml) and $r^2 = 0.9998$ (four replicates at each of five concentration levels). The linearity was also very good within the range of 200 to 1000 ng/ml, $Y = 0.00228X - 0.0234$ and $r^2 = 0.9998$ (triplicates at each of four concentration levels).

Table 1
The absolute recovery of hyperforin extracted from plasma by solid-phase extraction ($n=3$)

Plasma concentration (ng/ml)	Absolute recovery (%)	RSD (%)
25	90.1±8.4	8.40
50	88.9±5.4	8.43
100	91.5±3.0	3.31
500	91.1±1.4	1.54

3.3. Limit of detection (LOD) and limit of quantitation (LOQ)

By using a Waters 996 PDA detector in this study, the LOD of hyperforin was 4 ng/ml corresponding to a signal-to-noise ratio of 3:1. The LOQ of hyperforin, 10 ng/ml, was determined by the lowest concentration of the standard curve. Four replicates were analyzed and the relative standard deviation (RSD) was 13.2%.

3.4. Intra-day and inter-day variation

The intra-day and inter-day variations were investigated at three concentration levels. The results are shown in Table 2. The RSD for intra-day variations ranged from 5.28 to 9.79 and for inter-day variations ranged from 6.12 to 16.10. The accuracy was between 99.61% and 108.2%.

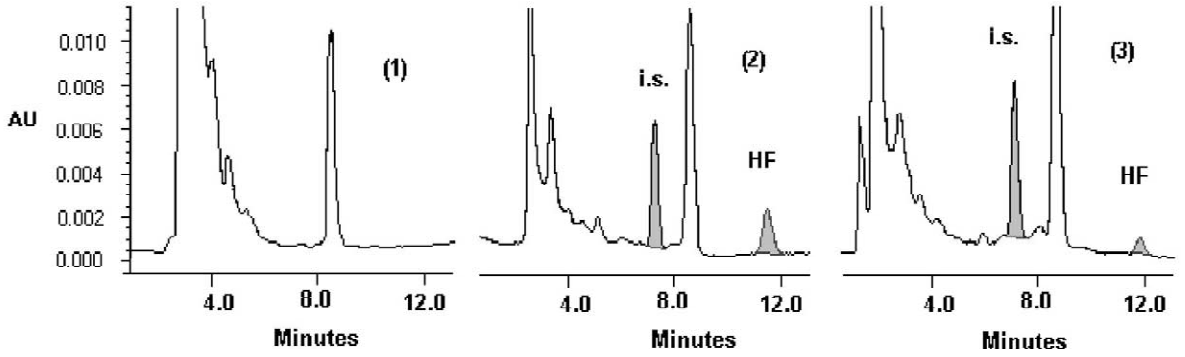

Fig. 2. HPLC analysis of hyperforin in human plasma. Chromatograms of (1) blank plasma, (2) blank plasma spiked with hyperforin (concentration at 150 ng/ml) and internal standard, (3) hyperforin in plasma (concentration 23.9 ng/ml) from human 3 h after administration of St. John's wort capsules (containing 8.55 mg hyperforin).

Table 2
Intra-day and inter-day variations for the assay of hyperforin

Hyperforin (ng/ml) Spiked	Intra-day variation ($n=6$)			Inter-day variation ($n=6$)		
	Detected Mean±SD	RSD (%)[a]	Accuracy (%)[b]	Detected Mean±SD	RSD (%)	Accuracy (%)
25	27.16±2.66	9.79	108.2	25.10±4.26	16.10	100.4
50	51.15±2.99	5.84	101.9	51.15±4.26	8.32	102.3
100	99.61±5.26	5.28	99.2	98.23±6.01	6.12	98.2

[a] RSD (%) (relative standard deviation)=(SD/mean)×100.
[b] Accuracy (%)=(mean concentration detected/concentration spiked)×100.

3.5. Applications in clinical studies

Using the described analytical method, a hyperforin plasma concentration–time profile was generated after the administration of a single dose of St. John's wort (900 mg) containing 8.55 mg hyperforin (first study; Fig. 3). The results of a pharmacokinetic analysis revealed a maximum concentration (C_{max}) of 27.6 ng/ml, a time to maximum concentration (t_{max}) of 4.4 h, an elimination half-life of 3.5 h, and apparent clearance of 33.7 l/h. The hyperforin plasma concentrations were also determined for the 12 subjects (second study) after injection of the supplements for 28 days. The means of triplicate analyses of each subject ranged from <10 ng/ml to 82.78 ng/ml. The analytical relative standard deviations (%RSD) of the triplicate analyses ranged from 1.80 to 7.25% with an average of 4.05%. The mean

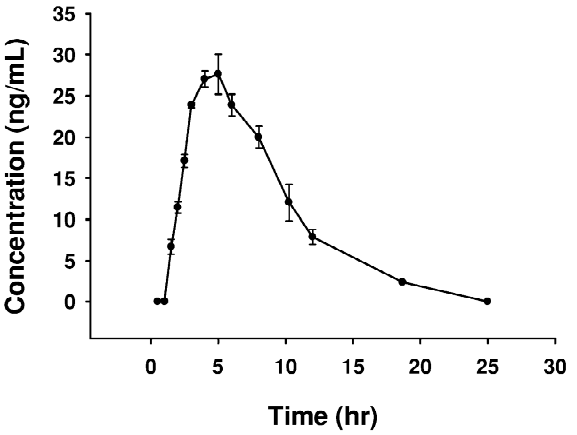

Fig. 3. Plasma concentration–time curve of hyperforin after administration of hypericum extract to healthy volunteers (triplicate analysis of one sample). The values are means and the error bars represent standard deviation (SD).

of the 12 subjects was 43.8±20.6 ng/ml. There was no significant difference between the males and females.

4. Discussion

Hyperforin is highly lipophilic, temperature sensitive, susceptible to photodegradation, and decomposes quickly in non-polar reagents, such as hexane [20,21]. Therefore, analytical methodologies should strive to avoid conditions that may adversely affect the stability of hyperforin. Other investigators have utilized hexane as an extraction solvent [16,18], but our attempts to duplicate these methods resulted in hyperforin absolute recoveries <80% with intra-day relative standard deviations >15%. We suspect that the low recovery and high variability may be due to the hexane-induced instability. Our previous investigation on the isolation of hyperforin from flower and leaf mixtures faced a serious stable problem by using hexane as the major extraction reagent. Recently, we observed that the standard hyperforin at concentration 500 ng/ml in hexane–ethyl acetate (90:10) decreased to 82.1±2.9% within 1 h and only 63.5±7.7% was retained after 2 h at room temperature, even under dark conditions. In order to analyze a large number of plasma samples routinely, we found that the SPE method was satisfactory with reproducible results.

Chi and Franklin developed an SPE method to extract hyperforin directly from plasma by using a 50-mg C_8 sorbent column. The absolute extraction recovery for hyperforin was reported to be 97.8% with good reproducibility [17]. Using the method of Chi and Franklin, we evaluated different brands of C_8 and C_{18} cartridges that contained various amounts of adsorbent (25 to 500 mg) for hyperforin recovery at 100 and 200 ng/ml. Despite careful adherence to the protocol, the absolute recoveries were consistently <60%. In considering the possibility of hyperforin bonds with protein in plasma, we used an equal amount of acetonitrile to mix with plasma sample to precipitate proteins before SPE extraction and the absolute recoveries were improved significantly (>89%) within the assay range.

Hyperforin is unstable and very expensive. It is cost effective to use an internal standard, such as benzo[*k*]fluoranthene, for the analysis of hyperforin plasma samples, although the structure of the internal standard is not similar to hyperforin. In the isocratic HPLC analysis, a mobile phase system of acetonitrile–aqueous 0.1 *M* triethylammonium acetate (8:2) was reported by Gray et al. [22]. In the presence of triethylamine, the maximum of UV absorption of hyperforin and adhyperforin were shifted from 270 to 290 nm and the four important highly lipophilic components in St. John's wort (pseudohypericin, hyperforin, adhyperforin and hypericin) were separated satisfactorily within 8 min. However this system could not be used in analysis of plasma samples, because of the interference of endogenous impurities.

In our previous in vitro metabolic study, the mobile phase system was improved by adding an equal amount of formic acid (2 ml) and triethylamine (2 ml) to the system (1000 ml) of 92% methanol–acetonitrile (3:2) and 8% aqueous phase (pH 3.1±0.1). Both hyperforin and the internal standard, benzo[*k*]fluoranthene, showed perfect peaks and they were not interfered with by the five major hyperforin metabolite peaks. For the plasma analysis, hyperforin and internal standard were also separated from the major endogenous impurities by this mobile phase system.

By using the developed SPE extraction and isocratic HPLC analysis method, good linearity of hyperforin was obtained from 10 to 200 ng/ml and from 200 to 1000 ng/ml. Thus the method can be used for pharmacokinetic studies of both human and rat models. As the concentration of hyperforin in the plasma samples were all <100 ng/ml after single-dose or multi-dose of St. John's wort in this study, we only validated the assay range <100 ng/ml.

These single-dose study results were similar to those of Biber et al. [16] who administered *Hypericum* extracts containing 14.8 mg of hyperforin to healthy volunteers. In that study, hyperforin T_{max}, elimination half-life, and apparent oral clearance were 3.9 h, 9.1 h, and 19.6 l/h, respectively. Intersubject variability in hyperforin distribution or apparent clearance may account for discrepancies in the pharmacokinetic parameters between the two studies. The results of the multi-dose study showed that a wide range of plasma hyperforin concentrations were observed among the 12 subjects and the analytical

method as developed in this study was applicable for these plasma samples.

5. Conclusions

An SPE extraction with HPLC–UV analysis was developed to determine hyperforin in human plasma. The method is rapid, sensitive, reproducible, and capable of quantitative analysis of hyperforin plasma concentrations following orally administered *Hypericum* extracts.

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References

[1] K. Linde, G. Ramirez, C.D. Mulrow, A. Pauls, W. Weidenhammer, D. Melchart, Br. Med. J. 313 (1996) 253.
[2] H.L. Kim, J. Streltzer, D. Goebert, J. Nerv. Ment. Dis. 187 (1999) 532.
[3] G. Di Carlo, F. Borrelli, A.A. Izzo, E. Ernst, Trends Pharmacol. Sci. 22 (2001) 292.
[4] M. Brolis, B. Gabetta, N. Fuzzati, R. Pace, F. Panzeri, F. Peterlongo, J. Chromatogr. A 825 (1998) 9.
[5] H. Kurth, R. Spreemann, Adv. Ther. 15 (2) (1998) 117.
[6] P. Mauri, P. Pietta, Rapid Commun. Mass Spectrom. 14 (2000) 95.
[7] J.M. Greeson, B. Sanford, D.A. Monti, Psychopharmacology 153 (2001) 402.
[8] E. Ernst, Lancet 354 (1999) 2014.
[9] S.C. Piscitelli, A.H. Burstein, D. Chaitt, R.M. Alfaro, J. Falloon, Lancet 355 (2000) 547.
[10] F. Ruschitzka, P.J. Meier, M. Turina, T.F. Luscher, G. Noll, Lancet 355 (2000) 548.
[11] G.W. Barone, B.J. Gurley, B.L. Ketel, M.L. Lightfoot, S.R. Abul-Ezz, Ann. Pharmacother. 34 (2000) 1013.
[12] A. Fugh-Berman, Lancet 355 (2000) 134.
[13] L.B. Moore, B. Goodwin, S.A. Jones, G.B. Wisely, C.J. Serabjit-Singh, T.M. Willson, J.L. Collins, S.A. Kliewer, Proc. Natl. Acad. Sci. USA 97 (2000) 7500.
[14] D. Durr, B. Stieger, G.A. Kullak-Ublick, K.M. Rentsch, H.C. Steinert, P.J. Meier, K. Fattinger, Clin. Pharmacol. Ther. 68 (2000) 598.
[15] R.S. Obach, J. Pharmacol. Exp. Ther. 294 (2000) 88.
[16] A. Biber, H. Fisher, A. Römer, S.S. Chatterjee, Pharmacopsychiatry 31 (1998) S36.
[17] J.D. Chi, M. Franklin, J. Chromatogr. B 735 (1999) 285.
[18] S. Bauer, E. Strömer, H.J. Graubaum, I. Roots, J. Chromatogr. B 765 (2001) 29.
[19] F.F. Liu, C.Y.W. Ang, T.M. Heinze, J.D. Rankin, R.D. Beger, J.D. Freeman, J.O. Lay Jr., J. Chromatogr. A 888 (2000) 85.
[20] H.C.J. Orth, P.C. Schmidt, Pharm. Ind. 62 (2000) 60.
[21] H.C.J. Orth, C. Rentel, P.C. Schmidt, J. Pharm. Pharmacol. 51 (1999) 193.
[22] D.E. Gray, G.E. Rottinghaus, H.E. Garrett, S.G. Pallardy, J. AOAC Int. 83 (2000) 944.