Acta Chromatographica 21(2009)4, 659–669
DOI: 10.1556/AChrom.21.2009.4.11

Triterpenes in Traditional and Supercritical-Fluid Extracts of *Morus alba* Leaf and Stem Bark

A. BÖSZÖRMÉNYI[1,*], SZ. SZARKA[1], É. HÉTHELYI[1], I. GYURJÁN[2], M. LÁSZLÓ[2], B. SIMÁNDI[3], É. SZŐKE[1], É. LEMBERKOVICS[1]

[1]Department of Pharmacognosy, Semmelweis University, Üllői út 26, 1085 Budapest, Hungary
[2]Department of Plant Anatomy, Eötvös Loránd University, Pázmány Péter út 1/C, 1117 Budapest, Hungary
[3]Department of Chemical and Environmental Process Engineering, Budapest University of Technology and Economics, Műegyetem rkp. 3., 1111 Budapest, Hungary
E-mail: aboszormenyi@gmail.com

Summary. Our objectives were to establish a GC method capable of quantitative analysis of terpenoids without derivatisation and to examine the amount of β-sitosterol extracted from *Morus alba* L. leaf and stem bark by use of traditional organic solvent extraction and supercritical-fluid extraction (SFE). To measure β-sitosterol content without derivatization, GC–FID was used with 5-α-cholestan-3-one as internal standard. To identify terpenoid constituents, GC–MS was used; β-sitosterol, phytol, lanost-7-en-3-on, α-amyrin, β-amyrin, and lupeol were identified. We established that for *Morus* leaf the best SFE method for β-sitosterol was pilot scale SFE; the β-sitosterol content of this extract was higher than that of the hexane solvent extract. Among analytical SFE conditions, 200 bar for 90 min and 300 bar for 60 min resulted in extraction of the most β-sitosterol. For mulberry stem bark, solvent extraction with hexane and SFE at 400 bar and 40°C for 60 min proved the best methods.

Key Words: *Morus alba* L., mulberry leaf, stem bark, supercritical fluid extraction, gas chromatography–mass spectrometry, β-sitosterol, α and β-amyrin, lupeol, lanost-7-en-3-one

Introduction

Mulberry (*Morus alba* L., *Moraceae*) was originally cultivated in China where the leaves were used as food for silkworms [1]. There is an increasing interest in mulberry since the leaves and root bark were found to have anti-hyperglycaemic effect because of the presence of deoxynorjirimicin, a poly-hydroxylated piperidine alkaloid which is a potent inhibitor of mammalian α-glucosidases [2–5]. The leaves and bark have also been used for treatment of arthritis and rheumatism, as anti-inflammatory agents [6]. Further active

components, flavone glycosides and moracins have antioxidant and scavenger activity [7, 8].

The terpenoids are common components of flora. They are present in many fruits, nuts, seeds, cereals, legumes, and vegetable oils. One of the most important terpenoids is the phytosterol β-sitosterol which has been shown to inhibit 5-α-reductase, which has a key role in steroid biosynthesis. This effect has been studied in human and animal experiments in benign prostatic hyperplasia [9–12]. The ursane triterpenoid α-amyrin, the oleanane β-amyrin, and the lupan lupeol have proved to be potent inhibitors of protein kinase in rat liver cells, resulting in a strong anti-inflammatory effect [13].

Literature data on the terpenoid content of *M. alba* are sparse. From the leaf and bark only β-sitosterol (bonded with caprate and palmitate) and isofucosterol have been identified [14–16]. Our objective was to identify the characteristic terpenoid components of *Morus* leaf and stem bark, and to determine the β-sitosterol content of different extracts.

For analysis of terpenoids, gas chromatographic methods are primarily used, with derivatization of compounds with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide or *n*-butyl ester-trimethylsilyl ether [17, 18]. Gutierrez et al. [19] used a rapid GC method without derivatization for measurement of terpenoids, but they quantified the peaks by area normalization only. Other objectives were to develop a GC method suitable for quantitative analysis of terpenoids, primarily β-sitosterol content, without derivatization, using an internal standard to improve the accuracy of the results, and to study the effectiveness of different extraction methods – laboratory and pilot scale supercritical-fluid extraction (SFE) and traditional (solvent) extraction.

Experimental

Plant Material, Chemicals, and Reagents

Mulberry leaf and stem bark samples were collected from Budapest, South-Buda, during the fruit ripening period. Collected plant material was dried at 50°C in drying cabinet and loss on drying was measured before extraction according to process of Ph. Eur. 5. [20].

The particle size distribution of the ground raw materials was determined by sieving. For evaluation of sieve analysis the Rosin–Rammler–Bennet (RRB) distribution was chosen. The representative particle size (d_0) was 0.335 ± 0.006 mm, and the uniformity coefficient (n) was 2.80 ± 0.06 [21, 22].

Samples were identified macroscopically in the Department of Pharmacognosy, Semmelweis University, Budapest, where the samples and herbarium specimens have been deposited (MA 2007/006-021).

The standard compounds β-sitosterol α and β-amyrin, lupeol, and 5α-cholestan-3-one, were purchased from Sigma–Aldrich (St Louis, MO, USA). Phytol standard was from Merck (Darmstadt, Germany). All solvents were of HP-3D-CE quality (Reanal, Budapest, Hungary, and Merck).

Extraction Methods

All the extractions were performed in triplicate.

Traditional Organic Solvent Extraction

For traditional organic solvent extraction with *n*-hexane, 5 g dried powdered plant was extracted with 100 mL *n*-hexane in a Soxhlet apparatus for 20 h on a water-bath at the boiling point of *n*-hexane.

For traditional organic solvent extraction with 96% ethanol, 5 g dried, powdered plant was extracted with 100 mL ethanol (96%, *v*/*v*) in a Soxhlet apparatus, for 20 h, on a water-bath.

Soxhlet extractions were continued until the extract lost its colour, so we established that the sample collected in the 20th hour did not contain any dry material. Repeatability of Soxhlet extraction was monitored by performing three parallel measurements; relative standard deviation (RSD%) was <1.94%.

Supercritical Fluid Extraction (SFE)

For laboratory-scale (analytical) supercritical extraction, 5 g dried, powdered drug was extracted with liquid carbon dioxide (flow rate 0.280–0.300 mL min^{-1}) in an ISCO2-10 instrument at 40°C and 200, 300, or 400 bar pressure. The extraction time was 60 min (30 min static and 30 min dynamic extraction) or 90 min (30 min static and 60 min dynamic). Repeatability was monitored by performing three parallel measurements; RSD was <9.37%.

For pilot-scale extraction 1000 g dried, powdered plant was extracted with liquid carbon dioxide (flow rate 7 kg h^{-1}) at 450 bar and 40°C; the pressure of the separator was 40 bar. The apparatus was developed by the Technical University, Budapest [23, 24]. The pilot-scale extraction was conducted until the amount of the extract collected in 1 h decreased to below

0.15% of the dried raw material. Total extraction time was 510 min. Repeatability of pilot scale SFE was controlled by performing three parallel measurements; RSD was 1.71%.

Preparation of Samples for Analysis

Organic solvent extracts and supercritical fluid extracts were saponified by the method of Ph. Eur. 5 [25], with modification. The extracts were evaporated to dryness and redissolved in 20 mL ethanol (96%, v/v). The ethanolic solutions were boiled for 1 h with 2 g potassium hydroxide. A solvent–solvent extraction with petroleum ether (3 × 30 mL) was followed by removal of the alkali by washing with water (3 × 30 mL). The collected organic phase was evaporated to dryness at 30°C under reduced pressure, weighed, and redissolved in chloroform. The chloroform solutions, purified extracts containing the terpenoids, were used for GC analysis.

Gas Chromatography

GC–FID

Gas chromatography was performed with an Agilent 6890N instrument equipped with flame ionization detection (FID). Compounds were separated on a 25 m × 0.2 mm i.d. capillary column coated with a 0.33-μm film of DB-5MS. The carrier gas was helium (purity 99.9999%) at a flow rate of 1 mL min^{-1}. Splitless injection of 1 μL was performed at an injector temperature of 280°C. The FID temperature was 330°C. The column temperature was programmed at 10°min^{-1} from 120°C (1 min isothermal) to 300°C (14 min isothermal), then at 10°min^{-1} to 310°C (10 min isothermal).

Peaks were identified by comparison of retention data and by standard addition. Quantitative measurement was performed by internal standard calibration with 5α-cholestan-3-one as internal standard. The percentage evaluation of compounds was performed on the basis of peak area.

GC–MS

Analysis was performed with the same instrument coupled to a 5973N mass-selective detector. Compounds were separated on a 30 m × 0.25 mm i.d. capillary column coated with a 0.25-μm film of HP-5MS. The carrier gas was helium (purity 99.9999%) at a flow rate of 1 mL min^{-1}. Splitless injec-

tion of 1 μL (0.7 mg mL^{-1}) was performed at an injector temperature of 280°C. The column temperature was programmed at 10°min^{-1} from 140°C (1 min isothermal) to 270°C (20 min isothermal), and then at 10°min^{-1} to 300°C (6 min isothermal).

Compounds were identified by comparison of retention times and mass spectra obtained from plant extracts with those from authentic standards and with data in the NIST library.

Validation

The linear range for β-sitosterol was studied by chromatography of standard solutions at six different concentrations between 0.1 and 100 μg mL^{-1} (0.1000, 0.2768, 0.6920, 13.8460, 69.2154, and 100.0000 μg mL^{-1}). The concentration of the internal standard, 5α-cholestan-3-one, was constant (12.65 μg mL^{-1}). Calibration plots were obtained by linear fit regression without weighting. The determination coefficient (R^2) was 0.9998. The lower limit of detection (LOD; defined as the amount for which the signal-to-noise ratio, $S/N = 3$) was 0.1 μg mL^{-1} β-sitosterol injected. The limit of quantitation (LOQ; $S/N = 10$) was 0.3 μg mL^{-1} β-sitosterol injected.

Repeatability of GC–FID analysis was assessed by performing three parallel measurements; relative standard deviation of relative peak areas was 1.94%.

Peak purity was confirmed by mass spectrometry. Blank chloroform was analysed to confirm that the solvent did not contain any impurities at the same retention time as the compounds of interest. Blank extracts, without internal standard, were analysed to confirm that no peaks were eluted at the same retention time as the internal standard. Each peak contained only the identified and measured component, except for those of α-amyrin and lupeol, which eluted together under the conditions described.

Statistical Analysis

Analysis of variance (one-way ANOVA) was used to test the significance of differences between means of data groups at $p < 0.05$. Each experiment was performed in triplicate.

Results and Discussion

We examined the yields of different extraction methods and the amount of unsaponifiable matter in the extract and the drug (*Table I*). Of the traditional extraction methods, use of 96% ethanol resulted in higher yields than hexane extraction. Analytical SFE resulted in very low yields. A higher yield was obtained by extraction at 400 bar than at 200 bar ($p < 0.001$), and extraction for 90 min resulted in a higher yield than extraction for 60 min ($p < 0.013$). Use of pilot scale SFE resulted in higher yields than analytical SFE. In extracts obtained from stem bark, yields were always significantly higher than in those from leaves ($p < 0.04$).

Table I. Effect of extraction method on extraction yield, and on unsaponifiable matter and β-sitosterol content

Sample	Extraction yield relative to dry weight of plant (g/100 g)	Unsaponifiable matter content		β-Sitosterol content	
		Relative to dry weight of plant (g/100 g)	Relative to dry weight of raw extract (g/100g)	Relative to dry weight of plant (g/100 g)	Relative to dry weight of raw extract (g/100g)
Mulberry leaf extracts					
Hexane extract	8.86 ± 0.09	3.88 ± 0.08	43.79 ± 0.81	128.82 ± 1.61	3.32 ± 0.24
96% Ethanol extract	16.63 ± 0.11	3.63 ± 0.25	21.82 ± 0.25	27.59 ± 0.99	0.76 ± 0.12
Analytical SFE, 200 bar, 60 min	0.30 ± 0.01	0.18 ± 0.02	58.67 ± 1.22	7.79 ± 0.67	4.33 ± 0.36
Analytical SFE, 200 bar, 90 min	0.28 ± 0.01	0.08 ± 0.01	28.57 ± 0.14	4.54 ± 0.09	5.67 ± 0.16
Analytical SFE, 300 bar, 60 min	0.32 ± 0.03	0.17 ± 0.01	77.22 ± 1.25	9.35 ± 0.09	5.50 ± 0.26
Analytical SFE, 300 bar, 90 min	0.36 ± 0.02	0.03 ± 0.01	83.33 ± 2.13	1.06 ± 0.01	3.53 ± 0.17

Table I. (continued)

Sample	Extraction yield relative to dry weight of plant (g/100 g)	Unsaponifiable matter content		β-Sitosterol content	
		Relative to dry weight of plant (g/100 g)	Relative to dry weight of raw extract (g/100g)	Relative to dry weight of plant (g/100 g)	Relative to dry weight of raw extract (g/100g)
Analytical SFE, 400 bar, 60 min	0.53 ± 0.03	0.33 ± 0.02	62.26 ± 0.21	5.64 ± 0.02	1.71 ± 0.11
Analytical SFE, 400 bar, 90 min	0.65 ± 0.04	0.39 ± 0.02	60.01 ± 0.21	8.67 ± 0.07	2.22 ± 0.10
Pilot-scale SFE	4.67 ± 0.08	0.29 ± 0.03	61.35 ± 0.39	26.10 ± 0.09	9.00 ± 0.15
Mulberry stem bark extracts					
Hexane extract	12.60 ± 0.18	0.99 ± 0.07	7.22 ± 0.31	103.85 ± 1.43	10.49 ± 1.47
96% Ethanol extract	34.38 ± 0.09	1.27 ± 0.09	3.70 ± 0.90	74.93 ± 1.00	5.90 ± 0.88
Analytical SFE, 200 bar, 60 min	0.54 ± 0.04	0.05 ± 0.01	10.51 ± 0.19	–	–
Analytical SFE, 200 bar, 90 min	0.60 ± 0.03	0.21 ± 0.02	34.89 ± 0.25	–	–
Analytical SFE, 300 bar, 60 min	0.46 ± 0.02	0.09 ± 0.01	25.93 ± 0.16	–	–
Analytical SFE, 300 bar, 90 min	0.83 ± 0.06	0.11 ± 0.01	13.27 ± 0.18	10.85 ± 1.29	9.86 ± 0.14
Analytical SFE, 400 bar, 60 min	1.33 ± 0.07	0.31 ± 0.01	23.75 ± 0.45	31.84 ± 1.27	10.27 ± 0.55
Analytical SFE, 400 bar, 90 min	1.97 ± 0.09	0.33 ± 0.02	16.85 ± 0.28	28.88 ± 1.23	8.75 ± 0.02

Extraction with ethanol resulted in the lowest unsaponifiable matter content, which could explain the highest extraction yields–ethanol can dissolve more polar compounds that can be eliminated by saponification. In contrast, ethanol dissolves less apolar material. The ratio of unsaponifiable matter in hexane and supercritical-fluid extracts showed no significant tendencies. The amount of unsaponifiable matter obtained from the stem bark was lower ($p < 0.05$) than that obtained from the leaves, which also correlates with higher yields.

Studying the effect of extraction method on β-sitosterol content, we realized that for *Morus* leaf the best SFE method was the pilot-scale technique–the β-sitosterol content of this extract was higher than that of the hexane solvent extract. Among analytical SFE conditions, 200 bar for 90 min and 300 bar for 60 min resulted in the highest β-sitosterol content. By comparing the β-sitosterol content of mulberry leaf and stem bark in purified hexane and ethanolic, extracts, we established that β-sitosterol was present in higher amounts in the bark than in the leaves ($p < 0.014$). The highest β-sitosterol content was that in the stem bark hexane extract; SFE at 400 bar for 60 min almost reached this value.

Identification of Terpenoid Components in Purified Mulberry Leaf and Stem Bark Extracts

The sterols β-sitosterol (M^+ 414) and lanost-7-en-3-one (M^+ 424), the triterpenes α-amyrin (M^+ 426) and lupeol (M^+ 426), and the open-chain diterpene

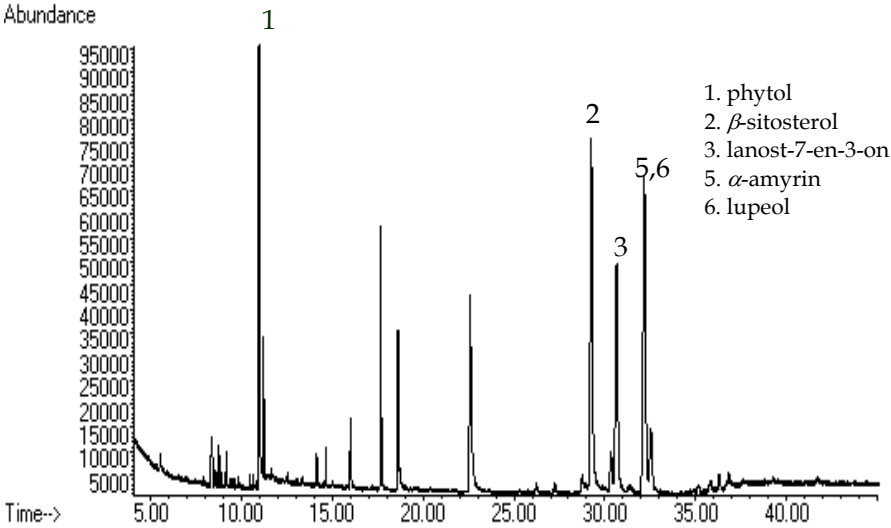

Fig. 1. Total ion chromatograms obtained from purified hexane extracts of *Morus* leaves

phytol (M^+ 278; a side chain of chlorophyll) were identified by GC–MS analysis of unsaponifiable extracts from mulberry leaves. In the stem bark, in addition to the compounds mentioned, β-amyrin (M^+ 426) was also identified. Gas chromatograms obtained from extracts of *Morus* leaves and stem bark, and the mass spectra and the chemical structures, are presented in *Figs 1–3*.

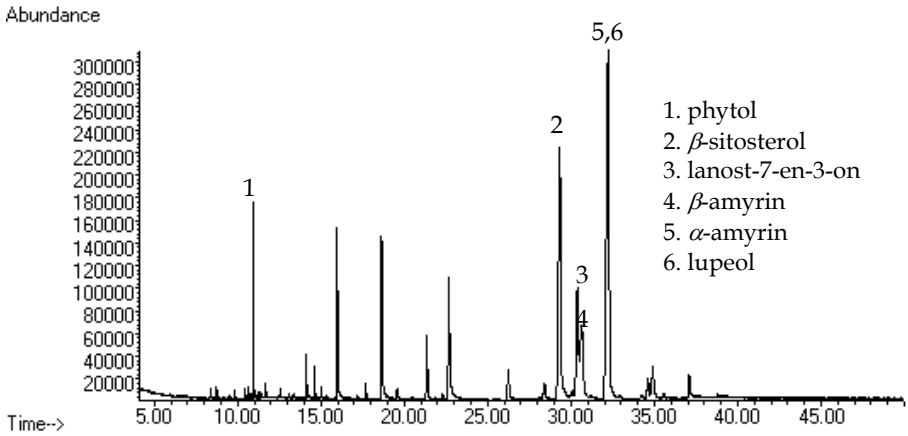

Fig. 2. Total ion chromatograms obtained from purified hexane extracts of *Morus* stem bark

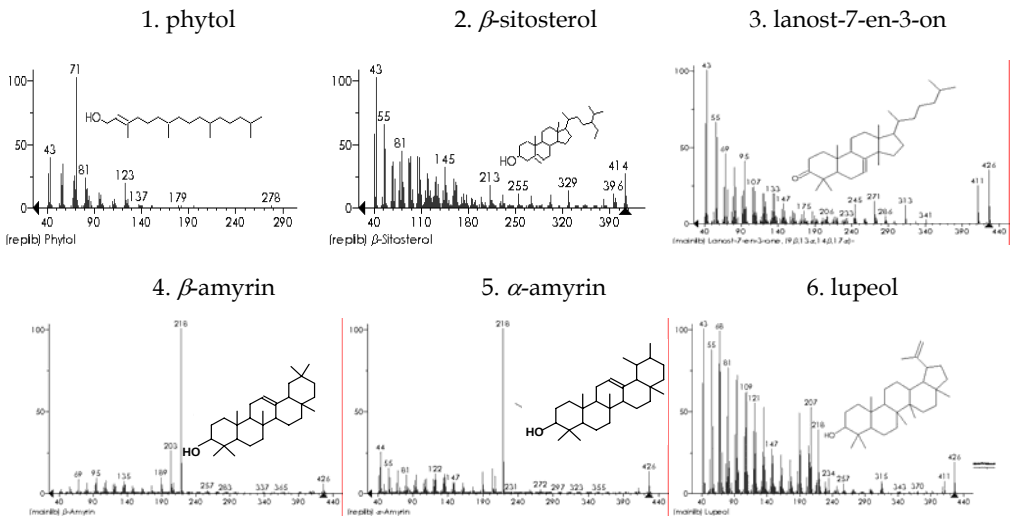

Fig. 3. Mass spectra and chemical structures of the compounds identified in mulberry leaves and stem bark

Conclusions

GC proved an effective method for quantitative measurement of the β-sitosterol content of white mulberry leaves and bark without derivatization. GC–FID performed with a DB-5MS column, with 5-α-cholestan-3-one as internal standard, enabled quantitative analysis of β-sitosterol content. The repeatability of GC–FID analysis was monitored by performing three parallel measurements; relative standard deviation of relative peak areas was 1.94%. Peak purity was confirmed by mass spectrometric detection. In leaves, β-sitosterol, phytol, lanost-7-en-3-one, α-amyrin, and lupeol were identified. In the bark, these compounds and β-amyrin were identified.

We established that for *Morus* leaves the best SFE method for β-sitosterol was the pilot scale technique; the β-sitosterol content of this extract was higher than that of hexane solvent extract. Among analytical SFE conditions 200 bar for 90 min and 300 bar for 60 min resulted in the highest extraction yields of β-sitosterol. For mulberry stem bark, solvent extraction with hexane and SFE at 400 bar for 60 min proved to be the best methods.

Acknowledgement

This work was supported by GVOP, grant number: 3.11.-2004-05-0397/3.0.

References

[1] N. Asano, T. Yamashita, K. Yasuda, K. Ikeda, H. Kizu, and Y. Kameda, J. Agric. Food. Chem., **4**, 4208 (2001)
[2] K. Szendrei, K. Csedő, and A. Hunyadi, Gyógyszerészet, **50**, 243 (2006)
[3] C. Miyahara, M. Miyazawa, S. Satoh, A. Sakai, and S. Mizusaki, J. Nutr. Sci. Vitaminol., **50**, 161 (2004)
[4] C. Hansawasdi and J. Kawabata, Fitoterapia, **77**, 568 (2006)
[5] N. Asano, K. Oseki, E. Tomioka, H. Kizu, and K. Matsui, Carbohydr. Res., **259**, 243 (1994)
[6] J.M. Rollinger, A. Bodensieck, C. Seger, E.P. Ellmerer, R. Bauer, T. Langer, and H. Stuppner, Planta Med., **71**, 399 (2005)
[7] T. Katsube, N. Imawaka, Y. Kawano, Y. Yamazaki, K. Shiwaku, and Y. Yamane, Food Chem., **97**, 25 (2006)
[8] S.Y. Kim, J.J. Gao, and W.C. Lee, Arch. Pharm. Res., **22**, 81 (1999)
[9] R.R. Berges, J. Windeler, H.J. Trampisch, and T.H. Senge, Lancet, **3451**, 592 (1995)
[10] A.B. Awad, M.S. Hartati, and C.S. Fink, J. Nutr. Biochem., **9**, 712 (1998)
[11] D. Li, M. Dong, W.J. Shim, and N. Kannan, J. Chromatogr. A, **1160**, 64 (2007)
[12] N. Souchet and S. Lamplante, Biochem. Mol. Biol., **147**, 378 (2007)

[13] M. Hasmeda, G. Kweifio-Okai, T. Macrides, and G.M. Polya, Planta Med., **65**, 14 (1999)
[14] P.H. List and L. Hörhammer, Hagers Handbuch der Pharmazeutischen Praxis, Vol. 5, Springer, Berlin, 1976, pp. 899–900
[15] T. Okuzumi, Y. Kaji, H. Hamada, and Y. Fujimoto, Tetrahedron Lett., **41**, 3623 (2000)
[16] D.D. Kulkarni, Indian J. Exp. Biol., **8**, 347 (1970)
[17] C. Domeno, B. Ruiz, and C. Nerín, Anal. Bioanal. Chem., **381**, 1576 (2005)
[18] A.K. Batta, G. Salen, P. Batta, G.S. Tint, and D.S. Alberts, J. Chromatogr. B, **75**, 153 (2002)
[19] A. Gutierrez, J.C. del Rio, F.J. Gonzalez-Vila, and F. Martin, J. Chromatogr. A, **823**, 449 (1998)
[20] European Pharmacopoeia, Supplement 5.0, EDQM, Strasbourg, France, 2005, p. 222
[21] T. Allen, Particle Size Measurement, Chapman and Hall, London, 1981
[22] B. Nagy, B. Simándi, and Cs.D. Andras, J. Food Eng., **88**, 104 (2008)
[23] E. Rónyai, B. Simándi, A. Kéry, E. Lemberkovics, M. Then, and A. Csordás, Olaj, Szappan, Kozmetika, **45**, 94 (1996)
[24] M. Oszagyán, B. Simándi, J. Sawinsky, A. Kéry, E. Lemberkovics, and J. Fekete, Flavour Fragr. J. **11**, 157 (1996)
[25] European Pharmacopoeia Supplement 5.0, EDQM, Strasbourg, France, 2005, p. 129

Accepted by MWH