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Rapid quantification of constituents in St. John's wort extracts by NIR spectroscopy

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Abstract

A quantitative near-infrared reflectance spectroscopy (NIRS) method was established for the determination of two major constituents (hyperforin and I3,II8-biapigenin) in St. John's wort extracts. Hyperforin was chosen due to the fact that it is found in a concentration range from 1 to 5%, a common one for NIRS determinations. I3,II8-Biapigenin on the other hand was selected as a constituent with very low concentrations (0.1–0.7%) but an extensive chromophore that allows very precise measurements in the ultraviolet (UV) and thus exact reference values that are vital for proper NIRS calibrations. Reference measurements were performed by reversed-phase high performance liquid chromatography (HPLC), determining the constituents' content in 35 pharmaceutical dry extracts of different origins. The reference method was validated according to the ICH guideline Q2B. Using partial-least squares (PLS) regression a multivariate calibration was done for the two ingredients each (PLS1). Satisfactory calibration statistics were obtained for hyperforin with a root mean square error of calibration (RMSEC) of 0.17 and a root mean square error of prediction (RMSEP) of 0.22 at a concentration range from 1 to 6% in the dry extracts. Due to the very low concentrations of I3,II8-biapigenin the accuracy of prediction is somewhat lower. However, it is possible to obtain very good results and reliable prediction by dividing the concentration range at 0.35%. The study emphasizes the potential of NIRS as a rapid and highly effective alternative method to conventional quantitative analysis of plant extracts.

Keywords: Hypericum perforatum; St. John's wort extracts; NIR spectroscopy; RP-HPLC; Quantification

1. Introduction

With the increase of the public's demand for herbal remedies and alternative therapies more and more attention is focused on the efficacy, safety, and cost of these treatments [1]. While the antidepressant effect of St. John's wort extract [2] as well as its superior safety [3] is well documented the problem of standardization remains. A correlation between pharmacological activity and certain characteristic substances in standardized Hypericum perforatum extracts was demonstrated but also a substantial variation in their chemical composition [4]. As the actual method of standardization based on the content of hypericin

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and hyperforin was found to be lacking [4], quality control of St. John's wort preparations must include a robust method allowing the analysis of the whole extract.

Several high performance liquid chromatography (HPLC) methods for the determination for the major constituent, e.g. rutin, hyperoside, isoquercitrin, quercitrin, quercetin, I3,II8-biapigenin, pseudohypericin, hypericin, adhyperforin, and hyperforin have been reported [5–8]. While these methods fulfill the necessary requirements of accuracy, specificity, reproducibility, and the determination of the most important constituents, they are very time-consuming, requiring sophisticated equipment and plenty of solvents, resulting in high costs. In short, they do not readily accommodate today's demand for increased sample volume, reduced sample cost, and reduced analysis time and are unsatisfying in regard to environmental issues.

Although near-infrared reflectance spectroscopy (NIRS) has already been used for quantifying components in plant material of different origins [9–12], it has not yet been established as a valid alternative in routine quality control of plant extracts in the pharmaceutical industry. This is due to a great extent to the relatively high limit of determination and quantitation, respectively, (about 1%) which has so far been a major hindrance to the usage of NIRS as a means of quantifying important constituents in plant material that are found in very low concentrations compared with accompanying substances. Successfully implementing NIRS in the quantitative determination of the constituents in St. John's wort extracts will offer a technique that is simple to use, requires no sample preparation, is non-destructive, rapid, and comparable in accuracy to traditional methods. As NIRS calibration quality depends to a high degree on the reference method, the development and validation of the HPLC method was based on the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guideline Q2B.

With hyperforin and I3,II8-biapigenin, two constituents of Hypericum perforatum that contribute mainly to its antidepressant effect [13], and of which I3,II8-biapigenin is present in unusually low concentrations, the potential of NIRS as an alternative to conventional HPLC methods for quality control purposes is exemplarily shown in this paper.

2. Materials and methods

2.1. Standards and samples

I3,II8-Biapigenin was purchased from Roth (Karlsruhe, Germany). Hyperforin was isolated by Orth et al. [14] and further purified by semi-preparative LC at our laboratory. The purity and structural identity was chemically characterized by HPLC–photodiode array detection (DAD), liquid chromatography–electrospray-ionization–mass spectrometry (LC–ESI–MS), and fast atom bombardment (FAB) MS data (unpublished). Hypericum perforatum dry extracts were kindly provided by different pharmaceutical companies (Finzelberg Ltd. & Co. and Lichtwer Pharma, both Germany). Methanol HPLC gradient grade and trifluoroacetic acid (TFA) Uvasol® grade were purchased from Merck (Darmstadt, Germany).

2.2. Sample preparation

St. John's wort extracts were dissolved in methanol and filtered prior to HPLC analysis through polytetrafluoroethylene membrane filtration cartridges (Rezist 30/0.2 PTFE, 0.2 μm, Schleicher & Schuell, Dassel, Germany). Extract concentrations were about 20 mg/ml and injection volume was 30 μl. No sample preparation was necessary for NIRS measurements as the dry extracts were directly measured in glass vials.

2.3. HPLC parameters

The HPLC system consisted of a L7200 LaChrom Autosampler, L6200A Intelligent Pump, and L4250 UV–VIS Detector (Merck Hitachi, Germany). Absorption was measured at 268 nm. The chromatographic data were recorded and processed by the D-7000 Interface Module and

HPLC-Manager software from Merck, Germany. DAD-measurements were performed on a Waters 991 photodiode array detector connected to a Waters 5200 printer plotter, analyzed by Waters 990+ DAD software (Waters, Milford, MA, USA). An API III TAGA 6000E triple-quadrupole mass spectrometer (Sciex, Perkin–Elmer Corp., Toronto, Canada) with an electrospray ion source and an m/z range of 2400 Da was used for ESI–MS measurements.

2.4. Separation conditions

Analyses were carried out on a Grom-Sil 120 ODS-4 HE, column (5 μm, 250 × 4 mm, Grom Analytik + HPLC Ltd., Herrenberg, Germany) using a pre-column (5 μm, 10 × 4 mm). A linear gradient was employed using 0.1% TFA in water as mobile phase A and methanol as B. Initial conditions were 70% A; 0–30 min, changed to 10% A; 30–50 min, to 0% A; kept to 60 min, 60–65 min, went back to 90% A; then equilibrated until 70 min. The flow rate was kept constant at 1.0 ml/min.

2.5. Calibration curves

Hyperforin and I3,II8-biapigenin were dissolved in methanol and diluted to seven equidistant concentrations in the appropriate ranges. The calibration curves were based on at least triple measurements for each concentration level, ten were carried out at the lowest, highest and middle level. Integrated peak areas were plotted against the corresponding amounts of the injected standard.

2.6. NIRS measurements

NIRS reflectance analyses were performed with a dispersive near-infrared NIRSystems 6500 spectrometer fitted with a Direct Contact Analyzer (Foss, NIRSystems, Hamburg, Germany) and equipped with a PbS detector. Each dry extract was measured three times in the range of 1100–2498 nm. The total number of data points was 700 per spectrum. The corresponding spectra were averaged in order to minimize effects resulting from the inhomogeneity of the plant material. A highly reflective ceramic standard served as reference (NIRSystems). All spectra were log rationed against it.

2.7. Spectra treatments and chemometrics

For spectrometer diagnostics and data acquisition Near-Infrared Spectral Analysis Software (NSAS, NIRSYSTEMS) was used. Calculation of derivatives, data pre-treatments, and chemometric calculations were done by means of the multivariate analysis software THE UNSCRAMBLER™ version 7.6 (Camo AS, Norway).

Scatter effects that are caused by physical phenomena, like particle size, interfere with the building of a valid model. Multiplicative scatter correction (MSC) squares the effects by adjusting the spectra based on ranges of wavelengths supposed to carry no specific chemical information [15]. Partial least squares (PLS) regression, which is extensively described in literature [16–18], was employed to extract relevant information from the complex spectra. The optimum number of PLS factors used for prediction was determined by full cross-validation (leave-one-out approach). The accuracy of the calibration models is described by the squared correlation coefficient (R^2), also called coefficient of determination, the root mean square error of calibration (RMSEC), and root mean square error of prediction (RMSEP), which both can be interpreted as the average modeling/prediction error, expressed in the same units as the original response values, i.e. concentration of component in extract. They represent the average difference between predicted and measured response values at calibration/validation stage [17]. Furthermore the bias is given, computed as the average value of the residuals, and showing the systematic difference between predicted and measured values.

3. Results and discussion

3.1. Reference method

The HPLC chromatogram of Hypericum perfo-

ratum extracts (Fig. 1) shows good separation of both hyperforin and I3,II8-biapigenin from the other constituents. As the absorption coefficient of I3,II8-biapigenin is much higher than of hyperforin, it was possible to achieve similarly good calibration statistics even though the concentration range of I3,II8-biapigenin is much lower than hyperforin. Complying with the ICH guidelines, specificity was achieved by controlling the identity of the peaks by LC–ESI–MS and their purity by frequently recording their DAD spectra and comparing them to the standards'. Accuracy was circumstantiated by comparing the measured concentrations to those of a reference method developed by Finzelberg Ltd. (Germany), where no significant deviation could be noted. Precision was described by the S.D. for hyperforin and I3,II8-biapigenin at 2.05 and 2.01%, respectively, giving the repeatability for ten measurements at maximum test concentration. Linearity was confirmed by performing a Mandel-test and the range was stated. Detection limit and quantitation limit (Table 1) were calculated based on the calibration curve using the dynamic model [19]. The reliability of the reference method was reflected by the good NIRS analysis results.

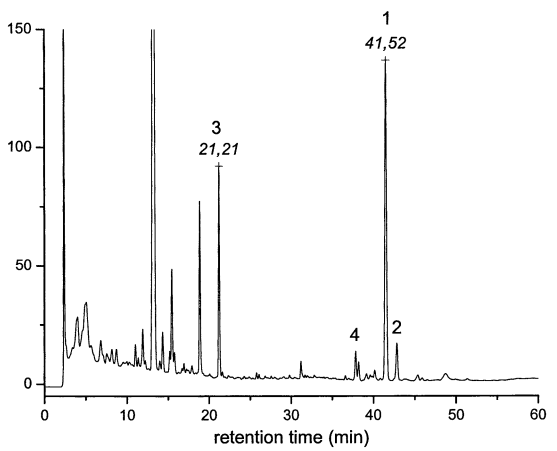

Fig. 1. HPLC separation of St. John's Wort dry extract, (1) hyperforin; (2) adhyperforin; (3) I3,II8-biapigenin; (4) orthoforin and further oxidation products of hyperforin.

Table 1
Statistical parameters of the HPLC reference method ($y = ax + b$, linear model)

Parameters	Hyperforin	I3,II8-Biapigenin
Range (mg/ml)	0.2–6.2	0.1–0.7
Number of calibration standard points	7	7
Slope (a)	816 019	4 746 694
Intercept (b)	−11 698	−71 178
Correlation coefficient (r^2)	0.9999	0.9990
Limit of detection (mg/ml)	0.13	0.07
Limit of quantitation (mg/ml)	0.20	0.10
Relative method S.D. (%)	0.67	2.61

y = Concentration; x = peak area ratio.

3.2. Multivariate analysis

Individual NIRS calibrations for both constituents were developed using 35 factory-made St. John's wort extracts. Table 2 summarizes the statistical parameters of the calibration equations. As full-cross validation is an adequate tool to estimate the predictive ability of the calculation equations, further external validation was made superfluous [17]. Second derivatives of the spectra were calculated using Savitzki–Golay four point smoothing in order to minimize spectral variability due to scattering and enhance spectral resolution [20]. The spectra in Fig. 2 underline effectiveness of this procedure. However, the application of second derivative spectra deteriorates the signal-to-noise ratio by a factor of about two per derivation. Plotting the x-loading weights against the x-variables visualized the wavelengths that contributed to the model to a high degree. By selecting those and omitting the superfluous ones the influence of factors based solely on noise was widely reduced and the aforementioned effects were extenuated.

Good calibration results were achieved for hyperforin in the Hypericum extracts. With a RMSEP of 0.22% at a range of 1.0–6.0% a reliable prediction is possible, particularly considering the demand of the pharmaceutical industry for em-

ploying extraction methods for St. John's wort that yield at least 3% hyperforin in the dry extracts. The content of I3,II8-biapigenin in the studied samples was markedly less, starting at 0.20% and reaching 0.55%. Still, with a RMSEP of 0.024% a surprisingly accurate prediction for the content of I3,II8-biapigenin can be given. However, the visual comparison of the residual calibration and validation variances' plot showed a rather high deviation indicating that the ability of the model to correctly predict further samples may be limited. In order to further improve prediction accuracy an approach well known from analytical regression statistics was employed [19]. The concentration range for the model was divided at 0.35% and a multivariate analysis for the low concentrations was done separately. RMSEP was reduced to 0.007% and the residual variances' plot showed no noticeable deviation. When predicting unknown samples a two-step procedure could be established using first the model for the whole range of usual I3,II8-biapigenin concentrations and, when appropriate, refining the result with the second 0.35%—cutoff model (Fig. 3).

4. Conclusion

In this study NIRS was successfully employed for the quantification of hyperforin and I3,II8-biapigenin in dry extracts of St. John's Wort with precision similar to HPLC. The demand for an overall analysis of plant extracts rather than the standardization to a few components is emphasized in today's phytochemical preparations, as safety and efficacy of these medications obviously depend on the exact dosage of the constituents. In comparison to HPLC procedures, NIRS has the distinct advantages of being much faster—once initial calibration is done—requiring less or no chemicals at all and no sample preparation. Thus, not only cost of analysis is considerably reduced, but also environmental and safety concerns are met. Yet in spite of its advantages, this calibration model can only be applied to the two tested constituents and not to unknown compounds and not at all to their elucidation or identification. However, expansion of the model with the quantitation of at least ten components of Hypericum perforatum extracts of varying origins will be done in the future, allowing for an overall implementation of this method. Although NIRS is suitable for quality control purposes and monitoring of extract content in pharmaceutical routine analysis, regular reviewing and, in case of need, extension of the PLS model is still necessary. Thus, HPLC as a reference method will not be replaced entirely, but reduced to a great extent.

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Table 2
Calibration and validation (prediction) results obtained for 35 St. John's Wort dry extract samples by NIRS

Parameters	Hyperforin		Biapigenin		Biapigenin cut-off	
	Calibration	Prediction	Calibration	Prediction	Calibration	Prediction
Slope	0.983	0.969	0.963	0.912	0.985	0.996
Offset	0.053	0.104	0.012	0.030	0.004	0.010
Correlation coefficient (r^2)	0.992	0.986	0.982	0.961	0.993	0.984
Root mean square error	0.173	0.222	0.018	0.026	0.005	0.007
S.E.	0.174	0.223	0.018	0.026	0.005	0.007
Bias	$-1.987e-08$	0.007406	$-1.407e-08$	-0.000681	$5.795e-09$	$-9.584e-06$

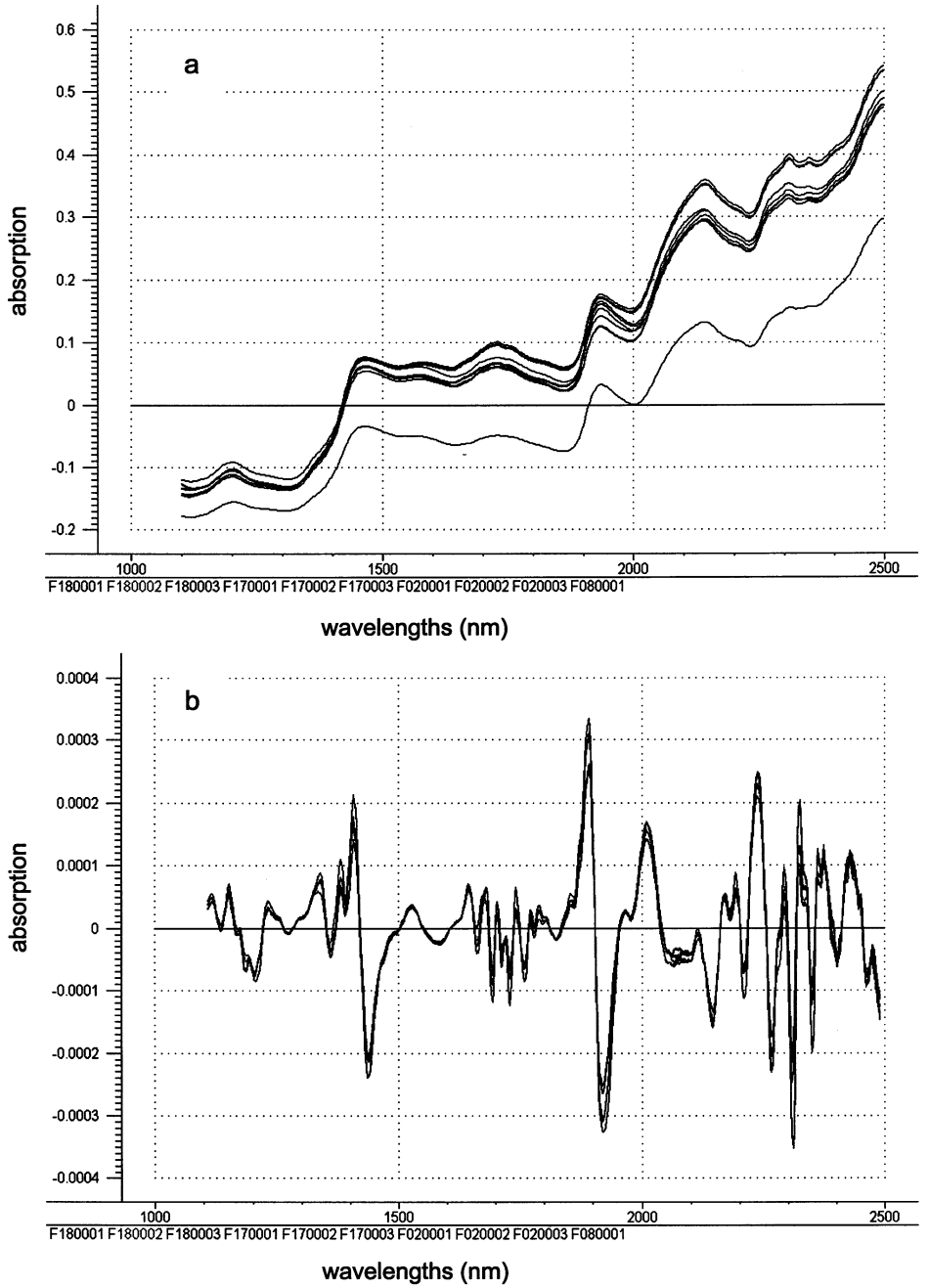

Fig. 2. Raw spectra (a) and second derivative spectra treated with MSC (b) of ten exemplary St. John's Wort NIR measurements.

ESI–MS measurements; the DFG (Deutsche Forschungsgesellschaft) for funding this work as part of the Graduiertenkolleg fuer Analytische Chemie (Tuebingen); Foss GmbH (Hamburg, Germany) for donating the spectrometer applied in this work.

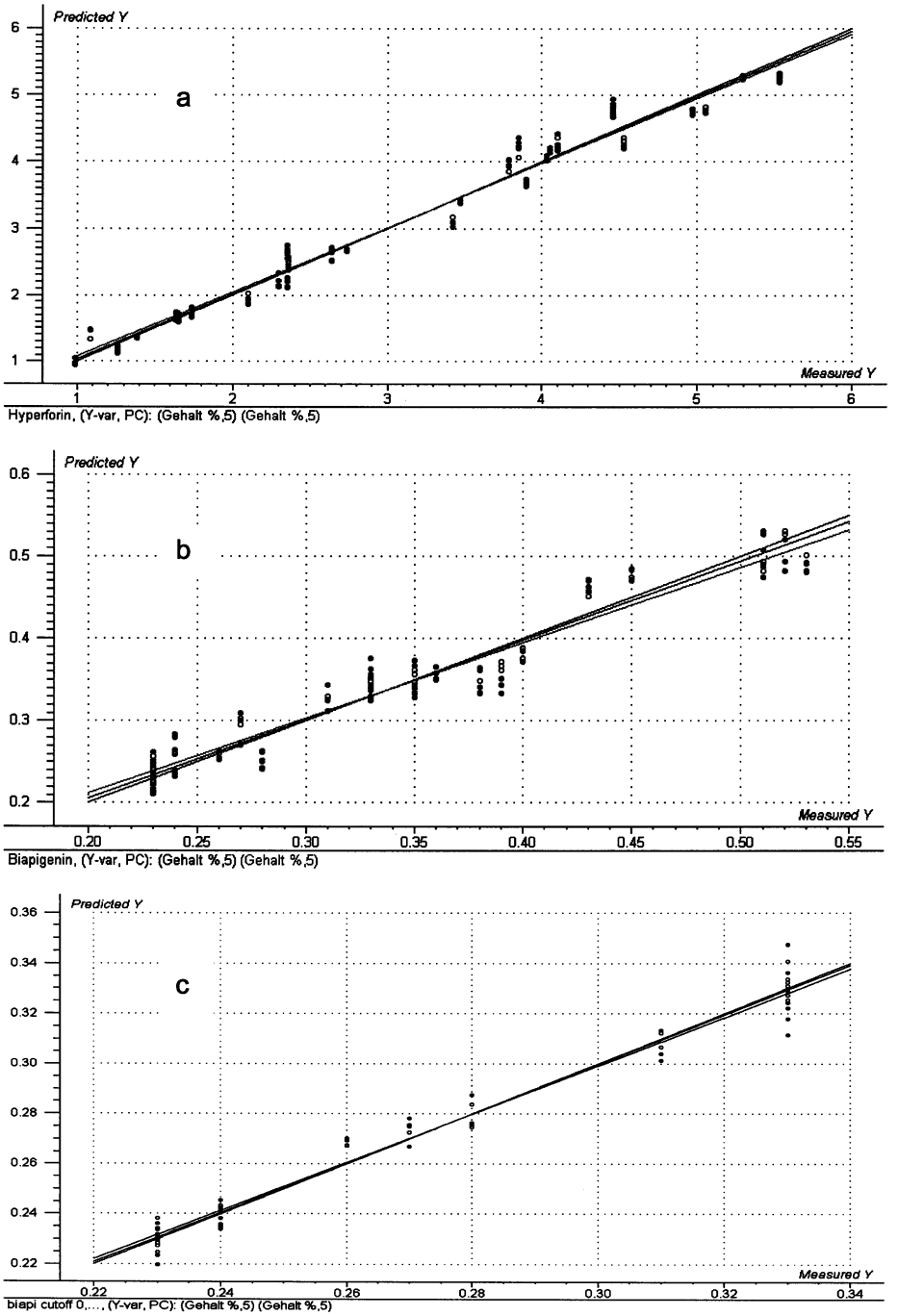

Fig. 3. Regression plots for the multivariate analyses of hyperforin (a); I3,II8-biapigenin full range (b); and I3,II8-biapigenin with reduced range from 0.1 to 0.35% (c).

References

[1] D.M. Ashcroft, A.L.W. Po, Pharmacoeconomics 16 (1999) 321–328.
[2] H.L. Kim, J. Streltzer, D. Goebert, J. Nerv. Ment. Dis. 187 (1999) 532–538.
[3] E. Schrader, Int. Clin. Psychopharmacol. 15 (2000) 61–68.
[4] B.D. Sloley, L.J. Urichuk, L. Ling, L.D. Gu, R.T. Coutts, P.K.T. Pang, J.J. Shan, Acta Pharm. Sin. 21 (2000) 1145–1152.
[5] P. Maisenbacher, K.-A. Kovar, Planta Med. 58 (1992) 291–293.
[6] S.H. Hansen, A.G. Jensen, C. Cornett, I. Bjornsdottir, S. Taylor, B. Wright, I.D. Wilson, Anal. Chem. 71 (1999) 5235–5341.
[7] F.F. Liu, C.Y.W. Ang, T.M. Heinze, J.D. Rankin, R.D. Berger, J.P. Freeman, J.O. Lay, J. Chromatogr. A 888 (2000) 85–92.
[8] M. Brolis, B. Gabretta, N. Fuzzati, R. Pace, F. Panzeri, F. Peterlongo, J. Chromatogr. A 825 (1998) 9–16.
[9] K. Molt, F. Zeyen, E. Podpetschnig-Fopp, Pharmazie 52 (1997) 931–937.
[10] H. Schulz, U.H. Engelhardt, A. Wegent, H.-H. Drews, S. Lapczynski, J. Argric. Food Chem. 47 (1999) 5064–5067.
[11] H. Schulz, H.-H. Drews, H. Krüger, J. Essent. Oil Res. 11 (1999) 185–190.
[12] N.D. Wilson, R.A. Watt, A.C. Moffat, J. Pharm. Pharmacol. 53 (2000) 95–102.
[13] S.S. Chatterjee, S.K. Bhattacharya, M. Wonnemann, A. Singer, W.E. Muller, Life Sci. 63 (1998) 499–510.
[14] H.J.C. Orth, C. Rentel, P.C. Schmidt, J. Pharm. Pharmacol. 51 (1999) 193–200.
[15] A. Candolfi, R. De Maesschalck, D. Jouan-Rimbaud, P.A. Hailey, D.L. Massart, J. Pharm. Biomed. Anal. 21 (1999) 115–132.
[16] D.M. Haaland, E.V. Thomas, Anal. Chem. 60 (1988) 1193–1202.
[17] H. Martens, T. Naes, Partial least squares regression, in: Multivariate Calibration, Wiley, Chichester/New York, 1989.
[18] P. Geladi, B.R. Kowalski, Anal. Chim. Acta 185 (1986) 1–17.
[19] W. Funk, V. Dammann, C. Vonderheid, G. Oehlmann, Statistische Methoden in Der Wasseranalytik, first ed., VCH, Weinheim, Germany, 1985.
[20] M. Blanco, J. Coello, H. Iturriaga, S. Maspoch, C. de la Pezuela, Appl. Spectrosc. 51 (1997) 240–246.