Rapid near infrared spectroscopic prediction of secondary metabolites in tea drugs and spice plants

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Introduction

Essential oils derived from many plants are known to possess antifungal and antimicrobial activities. Among them are species of the genera *Mentha, Origanum and Salvia,* which are also widely used in the flavouring of food, cosmetics and health care products as well as alcoholic beverages.

During the last years, much interest has been focused on catechins occurring in green tea infusions, not only for their antioxidative activity, but also because of their known antimutagenic and antitumorigenic properties. In this context, also, rooibos tea, a shrub belonging to the Leguminosae family, has been increasingly recognised due to its relatively high content of C-glycosidic dihydrochalcones. Especially fresh, unprocessed rooibos tea contains very high amounts of aspalathin, which, up to now has only been isolated from this species. This is the reason why industry has some interest in using rooibos extracts as an additive for food or cosmetic products to reduce oxidative influences.

Usually, the variation in the chemical composition of different essential oils and phenolic compounds in plant extracts is analysed by GC and HPLC methods, respectively.

During the past two decades, NIR has been successfully used for the analysis of various food and agricultural products.[1–3] Most applications in this section have been developed for quality control purposes but, nowadays, to a larger extent, on-line control of technological processes is also realised. Since previous work in this area predominantly focused on the analyses of main constituents such as fat, water, carbohydrates and proteins, up to now only few studies have addressed the potential to determine minor plant components as, for instance, valuable secondary metabolites.[4–9] Therefore, the objective of this paper was to review some of our own results and also to present new, reliable NIR methods for this special field of application. Additionally, limits of this innovative technique are described and possible reasons for failing measurements are discussed.

Materials and methods

Samples

Different cultivars of peppermint, sage and marjoram were grown in the experimental garden of the Federal Centre of Breeding Research in Quedlinburg. Dried rosemary leaves were received from MAWEA in Aschersleben, Germany. A collection of numerous peppermint and camomile genotypes were kindly supplied by German phytopharmaceutical companies (M. Bauer GmbH in

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Vestenbergsgreuth and P. Müggenburg GmbH in Alveslohe). Unfermented rooibos tea was harvested over a long period from different types in the western districts of the Cape Province of South Africa, particularly in the Cedarberg Mountains around Clanwilliam. Samples of fermented rooibos tea were collected at the local processing facilities in the Clanwilliam area.

Green tea leaves were supplied by local farmers in the northern part of Thailand (Mae Chedi). The freshly harvested plant material was immediately dried in a microwave oven (at 100 W for 40–60 s) in order to inhibit uncontrolled enzymatic reactions.

Reference analyses

In order to determine the volatile terpenoid compounds, the dried and sieved leaves were hydro-distilled according to the standard method described in the *European Pharmacopoeia*[10] and the oils obtained were analysed by GC/FID using a Hewlett-Packard chromatograph, 5890 Series II, fitted with a HP-Innowax 60 m × 0.25 mm fused silica column (film thickness: 0.5 μm). Fresh peppermint and sage leaves were homogenised in isooctane solution and separated from insoluble residues by centrifugation. The resulting clear solution was used to analyse volatile constituents without performing further treatments. GC conditions used to separate the essential oils were the same as those recently published.[8] Determination of the carnosic acid content in dried rosemary leaves was performed as described by Schwarz and Ternes.[11] The individual concentration amounts of five main catechins, as well as caffeine in dried green tea leaves, were assayed by HPLC according to the method developed by Ding *et al.*[12] Based on a recently published paper,[13] a modified HPLC method was developed to quantify aspalathin and nothofagin, the two most valuable phenolic compounds in rooibos tea.[14]

NIR data collection and calibration process

NIR spectra were collected from 1100 to 2500 nm in 2 nm increments using a dispersive NIRSystems 5000 (Foss Analytical GmbH, Rodgau, Germany) running a commercial spectral analysis programme (NIR2, Infrasoft International Inc., Port Matilda, USA) to process the data and to determine the most appropriate chemometric methods.

Dried and freshly sieved plant material of about 2–3 g was scanned in the reflectance mode by using a rectangular cup (dimensions: 51 × 64 mm) placed in a transport unit, moving the sample up and down. Each sample was measured with 32 scans each time and the mean spectrum was taken for the calibration process.

Fresh leaves of peppermint and sage were placed into a quartz cuvette and pressed on from the back-side by a diffuse gold reflector. For the NIR determination of non-volatile substances (phenolic compounds, alkaloids) the dried tea leaves were ground with a laboratory mill in order to get optimally homogenised samples.

Prior to calibration, the original reflectance spectra were corrected from interferences of scatter light and they were transferred with second- or third derivative processing. Usually the calibration programme was set up with the full wavelength range; only at measurements performed on fresh plant materials and dried rosemary leaves, the water bands occurring at 1450 and 1940 nm were cut off.

Generally, cross-validation statistics were applied using a modified partial least squares (PLS) algorithm to estimate the predictive ability of the calibration equations developed during this study. Furthermore, in some special questions principle component analysis (PCA) was performed to achieve a qualitative interpretation of spectral data.

Table 1. Range, mean and NIR correlation statistics for the essential oil content (% in the plant material) and composition (% in the oil) occurring in fresh and dried leaves of peppermint. All NIR predictions are based on GC analysis results received from dried leaves.

		Essential oil	Limonene	1,8-cineole	Menthofuran	Menthone	Isomenthone	Menthol	Menthyl acetate
dried leaves	Range	0.63 – 3.63	0.00 – 6.47	1.15– 6.04	0.00 – 4.20	10.10 – 56.90	2.02 – 8.44	15.90 – 58.60	0.77 – 7.89
	Mean	2.13	1.25	3.59	1.01	30.91	4.16	38.13	3.78
	SECV	0.19	0.72	0.47	0.64	4.00	0.98	3.93	1.11
	R^2	0.98	0.93	0.92	0.87	0.95	0.84	0.95	0.74
fresh leaves	Range	0.92 – 2.74	0.30 – 3.47	3.30 – 10.50		9.95 – 71.40	2.13 – 6.59	6.62 – 64.68	
	Mean	1.90	1.43	5.72		43.71	4.30	31.13	
	SECV	0.27	0.36	0.91		7.40	0.72	7.62	
	R^2	0.93	0.92	0.92		0.79	0.83	0.88	

Results and discussions

Essential oil components

Table 1 summarises the results obtained in the development of calibration equations for the individual valuable volatile substances present in fresh and dried peppermint. As can be seen there, the essential oil content and the most relevant terpenoids, such as menthol, menthone, limonene and 1,8-cineole can be reliably predicted by the developed NIR method. For those components which usually occur in smaller amounts (for example, menthofuran, isomenthone, pulegone) at least a semi-quantitative determination is possible.

Relating to the comparably high correlation between NIR-predicted and reference values for the amounts of essential oil, menthol, menthone, isomenthone and 1,8-cineole, a prediction of these parameters can also be performed on living plant material. This is a very useful approach to define the optimal harvest time with respect to as high as possible an oil and menthol content in the plant.

Results with a similarly high prediction quality were received at measurements performed on sage leaves. As presented in Table 2 the individual essential oil and thujone contents can be analysed, not only in the dried herb, but also in the fresh leaves within a few seconds. Since these two parameters have been found to be genetically determined,[15,16] a fast and reliable selection of appropriate sage genotypes within breeding experiments is possible.

In spite of some artefacts occurring, especially during the distillation process of marjoram herb (*cis*-sabinenhydrate acetate and *cis*-sabinenhydrate are partly transferred into α- and γ-terpinen as well as terpinen-4-ol), the amount and composition of marjoram essential oil can be predicted with acceptable accuracy. In order to produce an authentic description of the phytochemical plant status, instead of the usually applied distillation procedure the herb should be carefully extracted with isooctane prior to reference GC analysis.[9] The NIR spectra of marjoram leaves and marjoram essential oil are demonstrated well in Figure 1. Obvi-

Table 2. Range, mean and NIR correlation statistics for the amounts of essential oil (% in the plant material) and thujone (% in the oil) in different sage genotypes. All NIR predictions are based on GC analysis results received from dried leaves.

		Essential oil	Thujone
Dried leaves	Range	0.33–3.46	1.01–27.11
	Mean	1.63	4.75
	SECV	0.17	1.27
	R^2	0.92	0.89
	SEV	0.18	1.21
	Bias	0.02	0.25
Fresh leaves	*SECV*	0.21	2.01
	R^2	0.88	0.79
	SEV	0.24	1.79
	Bias	0.03	2.21

ously, the absorption bands measured in the drug mainly refer to the cellulose components of the plant tissue. Whereas water residues in the air-dried samples (approximately 6–9%) cause strong absorption maxima at 1450 and 1940 nm, the terpenoid substances of the essential oil only have secondary influences on the presented NIR spectrum.

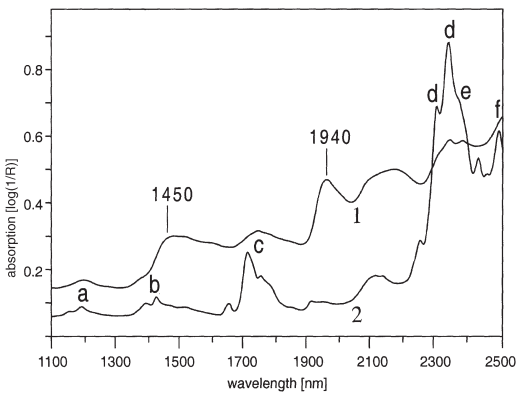

Figure 1. Near infrared spectra obtained from dried leaves (1) and the isolated essential oil (2) of marjoram. (a) 3 × ν(CH); (b) 2 ν CH) + δ(CH); (c) 2 × ν(CH); (d) ν(CH) + δ(CH); (e): 3 × ν(CH); (f) ν(CH) + ν(CC). ν = stretching vibration, δ = deformation vibration.

Phenolic compounds, alkaloids

For 30 years carnosic acid has been well known as a powerful antioxidant; therefore, in recent years, rosemary extracts have been widely used to stabilise fat or fat-containing foodstuffs.[17] In order to optimise the yield of this valuable phenolic diterpene, numerous wild rosemary genotypes have to be screened with special regard to their individual level of carnosic acid in the plant tissue. Usually this evaluation of rosemary samples is performed by solvent or CO_2 extraction of the plant material and subsequent HPLC analysis. It is worth mentioning here that, in this context, the clean-up must be carried out very carefully, because carnosic acid tends to build artefacts with γ- or δ-lactone structure (carnosol, rosmanol, epirosmanol).[18] Applying a newly developed NIR method, the carnosic acid con-

Table 3. Range, mean and NIR correlation statistics for the amounts of carnosic acid (% in the drug), essential oil (% in the drug) and individual terpenoids (in the essential oil) occurring in dried rosemary leaves.

	Carnosic acid	Essential oil	α-pinene	Camphene	Camphor	1,8-cineole	Myrcene	Limonene	Borneol	Bornyl acetate
Range	0.05 –7.8	0.2 – 4.1	1.6 – 37.7	3.2 – 8.0	2.5 – 42.4	3.1–58.8	0.4 – 7.3	1.8 – 5.9	1.6 – 9.9	0.1–7.7
Mean	3.9	1.7	22.6	6.1	14.4	18.7	3.3	3.7	4.0	2.9
SECV	0.38	0.21	2.45	0.59	4.0	4.0	0.91	0.44	0.91	0.91
R^2	0.95	0.95	0.97	0.90	0.95	0.97	0.98	0.94	0.84	0.89

tent can be predicted without performing any pretreatments; furthermore, it is possible to determine simultaneously the essential oil content as well as the amount of major terpenoids such as 1,8-cineole, camphor, α-pinene and camphene (Table 3). For all calibration equations, the resulting R^2-values are > 0.9; the corresponding *SECV* values are in the same scale as the standard errors of the individual reference method.[19] According to first tentative results, rosemary CO_2-extracts containing carnosic acid to an amount of up to 30% can also be analysed very precisely. For applications in the food and pharmaceutical industry, these extracts are adsorbed on silica acid or other carrier materials (for example, sodium chloride or glucose). The calibration equation for carnosic acid in this matrix shows a high correlation between NIR-predicted and HPLC values (R^2 = 0.98, *SECV* = 1.43). However, it must be taken into consideration that there is some need to develop an individual calibration function for each carrier material, because the spectral influences relating to these compounds interfere more or less strongly with the absorption bands caused by the rosemary extract (Figure 2).

Although only small differences are to be seen in the NIR spectra recorded from unfermented and processed rooibos tea, application of principle component analysis (PCA) to the spectral data could be successfully performed to characterise the fermentation process. As presented in Figure 3, the first three PCs account for 85% of all variation in the NIR spectra. So-called "dust-samples", which represent a separated fraction of very fine rooibos tea particles enriched with soil material, are not related to

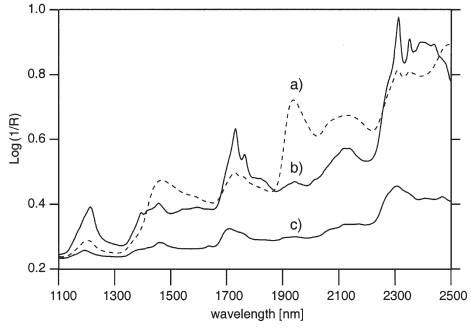

Figure 2. Near infrared spectra measured on dried rosemary leaves (1), rosemary extract on glucose carrier (2) and rosemary extract on silica acid carrier (3).

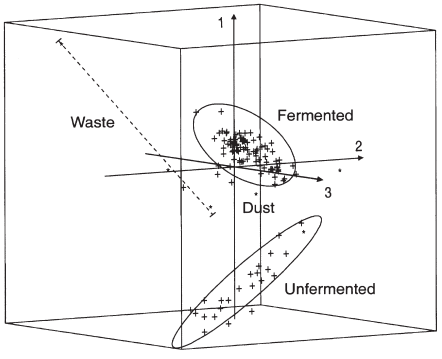

Figure 3. Discrimination of unfermented and fermented rooibos tea samples by principle component analysis based on spectral data (PCA).

Table 4. Calibration and prediction results obtained for 95 green tea samples. DL: drying loss, CAF: caffeine, TB: theobromine, EGC: epigallocatechin, EC: epicatechin, EGCG: epigallocatechin gallate, ECG: epicatechin gallate.

Parameter	Unit	Reference measurements			NIR calibration statistics		
		Range	Mean	*SD*	*SECV*	*SD/SECV*	R^2
DL	%	4.5–7.8	6.1	0.7	0.2	4.12	0.94
CAF	g kg^{-1}	3.3–50.5	34.9	9.1	1.7	5.35	0.97
TB	g kg^{-1}	0.2–4.0	1.4	1.1	0.4	2.75	0.86
EC	g kg^{-1}	4.3–59.6	21.0	16.3	2.6	6.27	0.97
EGC	g kg^{-1}	10.9–45.1	28.7	8.0	3.1	2.58	0.85
EGCG	g kg^{-1}	10.2–122.1	68.1	26.0	6.9	3.77	0.93
ECG	g kg^{-1}	7.8–64.8	32.5	18.9	4.1	4.61	0.95

both factor groups built by unfermented and fermented tea. Waste material (for example, plants damaged by attack of the fungus *Diaporthe phaseolorum*), which was not used for the fermentation process, can also be clearly discriminated from the above mentioned groups. In a similar way NIR measurements of some special unfermented rooibos samples (for example, older shrubs, higher amount of stems in the drug, air-dried plant material) are located, in most cases, separately in the 3-dimensional factor space.

Green tea (*C. sinensis*), which has become very popular in Europe during the last years, contains up to 30% catechins in the dry leaf. Much interest has been focused on these substances, not only for their antioxidative properties, but also because of their known antimutagenic and antitumorigenic activities in man.[20] As demonstrated in Table 4, the NIR technique also permits the estimation of these valuable polyphenols (epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate) and additionally the quantification of caffeine and theobromine, as well as the determination of drying loss with high degrees of accuracy and reliability.[21]

Applying PCA to all measured NIR reflectance spectra, a differentiation of young ("two leaves and the bud") and older tea leaves (third to sixth leaf) is possible. Five PCA axes are required to explain 97.5% of the spectral variation, but only using the first two PCA factors (90.6% of the spectral variation explained) is sufficient to discriminate both population groups. This finding is a consequence of the fact that young tea leaves, representing the better quality, do contain a lower content of epicatechin and significantly higher amounts of epigallocatechin gallate.

In order to receive accurate NIR predictions it is very important to consider the variation in plant composition during different crop years. Therefore, exemplary studies have been performed to approve the reliability of NIR measurements in caraway fruits harvested from 1996 to 1998. Based on common calibration functions, only low differences in the standard error and the R^2 values have been found for the prediction of individual quality parameters determined in the intact fruit (for example, prediction of essential oil content: $SECV = 0.24$; $R^2 = 0.96$).

Generally, application limits of NIR for the quantification of individual secondary metabolites in plants may occur when the analytes (terpenoids or phenolic compounds) are present only in the sub-percent level. Furthermore, interferences with the plant matrix, as for instance superpositions of characteristic absorption bands of the analyte molecule, may be the reason for failing predictions.

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