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Short communication

Analysis of plant extracts by NIRS: simultaneous determination of kavapyrones and water in dry extracts of *Piper methysticum* Forst.

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Abstract

A near-infrared reflection spectroscopy (NIRS) method was developed to determine the total content of kavapyrones, kavain and water in dry extracts of *Piper methysticum* Forst. (kava kava, Piperaceae). Based on the recorded spectra and the reference data, performed by HPLC and Karl Fischer titration, a chemometrical analysis was calculated using PLS 2 algorithm. In general, good calibration statistics are obtained for the prediction of the different contents presenting high correlation coefficients ($r^2 > 0.9913$) and low root mean square errors of prediction (RMSEP < 0.094%). Usually the main water bands are "cut out" of the spectra to improve the model, however this is associated with the loss of relevant spectroscopic information. Thus, the entire spectrum including the OH bands is used, as these are not only found in water but also in the kavapyrones.

The use of this new strategy succeeds in overcoming the difficulties in NIRS and establishes NIRS as a valid alternative in the routine quality control of plant extracts.

Keywords: NIR spectroscopy; Routine quality control; Partials least squares 2 (PLS 2); *Piper methysticum* Forst.

1. Introduction

Near-infrared spectroscopy (NIRS) has become a widely used analytical method of quality control in the pharmaceutical industry. It is a rapid, cost effective and non-destructive method allowing, besides the identification of drugs or excipients, the simultaneous determination of components in a mixture by multivariate data analysis [1].

Therefore it is not surprising to find an increasing number of publications dealing with NIRS in the field of phytochemical analysis. Several examples exist where NIRS has been used successfully for quantifying single components in plant material of different origin, and this demonstrates the great potential of NIRS for phytopharmaceutical industry [2–7]. Nevertheless, there are still difficulties that have to be overcome in order to establish NIRS as a valid alternative in routine quality control. These difficulties are mainly due to the complexity of the mixture and to the sometimes very low concentrations of the quantifying active compounds compared with the accompanying substances [8]. Another point, which is crucial for a reliable application of the method, is the sensitivity of the NIRS to physical properties. The spectra are influenced significantly by particle size, morphology and particularly the water content. These parameters cause an additional variance within the data, which is not related to the compounds of interest, thus making their determination by NIRS more difficult. Especially water turned out to be a major problem because its effects cannot be removed or minimised like light scattering effects by data pre-treatments (multiplicative scatter correction or standard normal variate). However, the water content plays an important role for the further processing of the extract. Therefore, the determination of residual water in plant extracts is essential and is usually carried out by the time-consuming Karl Fischer titration. NIRS has been proven to be a suitable alternative method for

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the determination of water content in powders and powder blends by NIR spectroscopy [9,10]. The possibility of determining the residual water content in plant extracts by NIRS and to evade the conventional method is demonstrated using the example of *Piper methysticum* Forst. (kava kava; Piperaceae). Preparations of kava kava were used in Europe for the treatment of anxiety, restlessness and nervousness. Kava kava has been used for hundreds of years, and is still used, as an intoxicating beverage in ceremonial rites by the natives of the islands of the South Pacific [11]. The kavapyrones (methysticin, dihydromethysticin, kavain, dihydrokavain, yangonin and desmethoxyyangonin) are known to be the active substances and are therefore used for standardisation. Several HPLC methods for the determination and quantification of these six major components have been published [12–15].

In the present paper, a new strategy was chosen for the compensation of differences in the water content using the example of dry extracts of *P. methysticum* Forst. by implementing the differing values into the calibration. The possibilities and limitations of NIRS combined with multivariate analysis as an alternative method to the conventional methods are investigated by the quantification of kavain, the total content of kavapyrones and, most important, the water content in kava kava dry extracts.

2. Materials and methods

2.1. Standards and samples

Kavain was purchased from Roth (Karlsruhe, Germany).

- The purity and identity was chemically characterised by HPLC-photodiode array detection and liquid chromatography/electrospray-ionisation-mass spectrometry.
- *P. methysticum* dry extracts were kindly provided by Finzelberg Ltd. & Co. (Andernach, Germany).
- Methanol and 2-propanol gradient grade were purchased from Merck (Darmstadt, Germany), acetonitrile was purchased from Roth.

2.2. Sample preparation

The kava kava dry extracts were dissolved in methanol. The extract concentrations were 2 mg/ml and the injection volume was 10.0 μl.

No sample preparation was required for NIRS measurements as the dry extracts were directly measured in glass vials (Fisher Scientific, Ulm, Germany) on three different days. Each day three samples from the same batch were measured three times with a rotation of 120°.

2.3. HPLC parameters

The HPLC system consisted of a L7200 LaChrom Autosampler, L6200A Intelligent pump and L7455 DAD (Merck Hitachi, Germany). The UV-absorption was measured 245 nm. The chromatographic data were recorded and processed by D7000 Interface Module and HPLC—Manager Software from Merck, Germany.

2.4. Separation conditions

The determination of the total content of kavapyrones was carried out according to Gracza and Ruff [12]. They varied between 28.8% and 31.3% according to the industrial specifications of Finzelberg Ltd. & Co. The quantification of kavain was done on a Luna C18 RP (Phenomenex, Aschaffenburg, Germany). The mobile phase consisted of 0.1% glacial acetic acid in water, 2-propanol and acetonitrile in a ratio of 65:19:16 (v/v/v). The isocratic flow was kept constant at 0.6 ml/min. The content of kavain of the dry extracts was between 4.5% and 7%.

2.5. Calibration curves

Kavain was dissolved in methanol and diluted to five equidistant concentrations in the appropriate ranges. Each concentration level was measured at least three times; 10 measurements were carried out at the lowest, highest and middle level. Integrated peak areas were plotted against the corresponding concentration of the injected standard.

2.6. Determination of water

The determination of the water content has been carried out by Karl Fischer titration (Ph.Eur.1997) and varied between 1.5% and 4.0% according to the industrial specifications.

2.7. NIRS measurements and data pre-treatment

A Foss NIRSystems 6500 spectrophotometer (NIRSystems, Silver Spring, USA) fitted with a Direct Contact Analyser (DCA) was used for the measurement of all spectra over the wavelength range of 1100–2500 nm. Each spectrum was obtained by averaging 32 scans. All of the spectra were recorded as $\log(1/R)$ with respect to a highly reflective ceramic standard.

2.8. Data analysis

Foss Near-Infrared Spectral Analysis Software (NSAS) 2.21 was used for data acquisition and system diagnostics. Calculation of derivatives, data pre-treatment and PLS 2 models were done by means of The Unscrambler™ 7.6 (Camo A/S, Trondheim, Norway). The spectra were mean-centered, second derivatives and Savitzky-Golay 7 point smoothing of the spectra were calculated in order to minimise spectral variability.

Table 1
Statistical parameters of the HPLC reference method for kavain

Parameters	Kavain
Range (μg/ml)	50–400
Number of standard calibration points	5
Correlation coefficient (r^2)	0.9973
Theoretical limit of detection (μg/ml)	31.80
Theoretical limit of quantitation (μg/ml)	47.35
Relative method standard deviation (%)	1.85

3. Results and discussion

3.1. Reference analysis

The quality of a multivariate calibration of a near infrared spectroscopic method is dependent on the reference method. The means of choice for the identification and quantification of plant extracts is still the HPLC. Therefore, the determination of kavain and the total content of kavapyrones of 12 batches of kava kava dry extracts has been carried out by HPLC. Peak purity and identity were proved by LC/ESI-MS and LC/CIS-MS [16]. The development and validation of the method was completely based on the requirements of the ICH guideline Q2B. The statistical parameters of kavain are given in Table 1; one chromatogram is shown as an example in Fig. 1. They meet the requirements of a reference method.

However, it is possible for NIR spectroscopic predictions to be more accurate than error laden reference values [17] and alike, the NIRS measurements meet the demands of the regulatory authorities.

3.2. NIR spectroscopy

One of the main advantages of NIRS is the complete detection of all organic components. However, the spectra are influenced by different physical properties such as particle size distribution, packing density and crystal structure. In addition, we will find the NIR spectra influenced by both the kavapyrones and the different water content of the dry extracts in contrast to the HPLC method. These differences in water content may reduce the significance of the calibration and validation set because they cause a certain variance in the data set that is not due to the parameter of interest (i.e. the concentrations). Therefore, a quantitative NIR model might predict extracts containing identical percentages of a kavapyrone incorrectly. Hence the spectral data responsible for the OH bands (water absorption maxima are at 1190, 1450 and 1940 nm; the positions of these bands can be slightly shifted by temperature changes or hydrogen bonding between the analyte and the matrix) in the spectrum are usually cut out to improve the model. Since these bands are not only found in water but also in other ingredients of the extract, the exclusion of these bands is accompanied by a loss of relevant spectroscopic information and is a disadvantage for NIRS calibration of complex matrices like plant extracts. In the present study, we chose an alternative strategy to meet this problem. The complete spectrum was used and water content was implemented into the calibration (see multivariate analysis and Fig. 2).

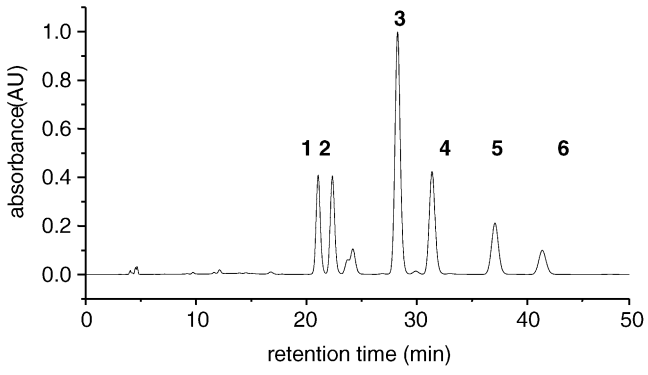

Fig. 1. HPLC separation of the kavapyrones in kava kava dry extracts. (1) Methysticin; (2) dihydromethysticin; (3) kavain; (4) dihydrokavain; (5) yangonin and (6) desmethoxyyangonin; mobile phase: 0.1% CH_3COOH/2-propanol/acetonitrile (65:19:16); flow rate 0.6 ml/min; injection volume 10.0 μl; UV detection at 245 nm.

Additionally, light scattering effects due to the physical parameters mentioned above were corrected by multiplicative scatter correction (MSC), a common method squaring the effects by adjusting the spectra based on ranges of wavelengths supposed to carry no specific chemical information [18]. According to custom, the data is mean-centered, which means that the average spectrum is calculated from all of the calibration spectra and then subtracted from every calibration spectrum. Mean centering has the effect of enhancing the subtle differences between the spectra. Second derivatives of the spectra were calculated using Savitzky-Golay 7 point smoothing in order to minimise spectral variability due to scattering and enhance spectral resolution [19].

3.3. Multivariate analysis

Individual NIRS calibrations were developed for kavain, total content of kavapyrones and water content using 12 batches of kava kava dry extracts. In order to consider the influence of the six major kavapyrones and water on the NIR spectra of the kava kava extracts PLS 2 was applied.

Partial least squares (PLS) regression is a quantitative spectral deconvolution technique to extract relevant information from complex spectra. In contrast to the PLS 1 algorithm, PLS 2 calibrates for all constituents simultaneously. Thus, the results of the spectral deconvolution give one set of scores and one set of PLS factors for calibration, which means that the PLS factors are directly related to constituents of interest rather than larger common spectral variations. Therefore, the calculated vectors are not optimised for each individual constituent like in PLS 1, which might cause a loss in accuracy in the predictions of the constituent concentrations, particularly for complex mixtures, but it could be used nevertheless since only knowledge of the constituents of interest (kavapyrones, kavain, water) in a complex extract is required. The main idea of PLS regression is to get as much concentration informa-

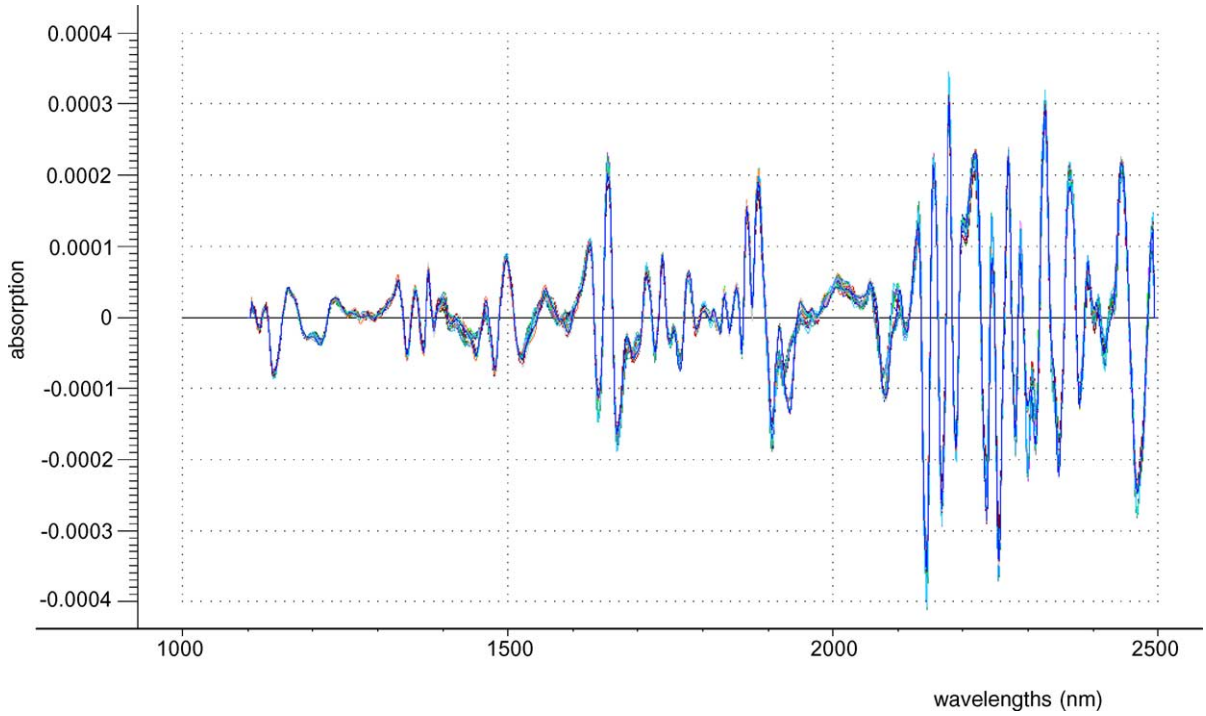

Fig. 2. Second derivative spectra of kava kava dry extracts over the complete wavelength range of 1100–2500 nm. Nine spectra were overlaid.

tion as possible into the first few PLS factors. The optimum number of PLS factors used for prediction was determined by full cross-validation [20].

Full cross-validation is conceptually easy to understand, but it is a method with additional high calculational expenditure to optimise a model. Full cross-validation is also called the leave-one-out method. From the whole training set a sample is removed with the complete spectrum and corresponding concentration data. A PLS regression is calculated again with the remaining test set and the left out sample is predicted. This operation is repeated until all samples have been left out and predicted once [21].

Primarily, the calibration is done to develop the model, predicting later the unknown concentrations with spectral data from new samples. The accuracy of the model is described by the value of the root mean square error of calibration (RMSEC). The accuracy in the model used to predict unknown samples is expressed by the value of the root mean square error of prediction (RMSEP). The root mean square errors have the same units as the original response values, i.e. concentration of kavain, kavapyrones and water in percent. They represent the average difference between measured and predicted response values at the calibration and validation stage [21]. Table 2 shows the NIRS calibration results obtained for the PLS 2 model for the kava kava dry extracts. Good calibration results were achieved, especially for the water content in the dry extracts: RMSEC water: 0.0263%; RMSEC kavain: 0.0543%; RMSEC total content of kavapyrones: 0.0811%. As examples three regression plots of the prediction for kavain, total content of kavapyrones and water are shown in Fig. 3. Reliable prediction results were obtained for the kavain content at a range of 4.5–7.0% (RMSEP = 0.0599%) for the total content of kavapyrones a range between 28.8% and 31.3% (RMSEP = 0.094%) and for the water content at a range of 1.5–4.0% (RMSEP = 0.0294%). The inclusion of the OH regions not only improves the model but also reduces the time-consuming determination of the water content by Karl Fischer titration.

It is necessary that a few more parameters are mentioned to describe the quality of the model: the correlation coefficients (r^2) for calibration/prediction of the water content were 0.9993/0.9992 and 0.9979/0.99749 for kavain, respectively. The correlation coefficients for the total content of kavapyrones were 0.99359 and 0.9914. In addition, the bias is given, computed as the average value of the residuals, and showing the systematic difference between predicted and measured values; for normal distributed values it should be zero. Additionally, the offset in an optimal linear correlation should also be zero and the slope one.

As demonstrated, the PLS 2 regression including the OH bands is a suitable tool for quality control and monitoring of extract content in the pharmaceutical routine.

Table 2
NIRS calibration results obtained from the PLS 2 model of kava kava dry extracts

	Kavain	Kavapyrones	Water
Slope	0.995782	0.987076	0.998655
Offset	0.024507	0.386993	0.003936
Correlation	0.997889	0.993517	0.999327
RMSEC	0.054252	0.081116	0.026306
SEC	0.054590	0.081622	0.026470
Bias	−1.766e−08	4.474e−07	4.268e−08

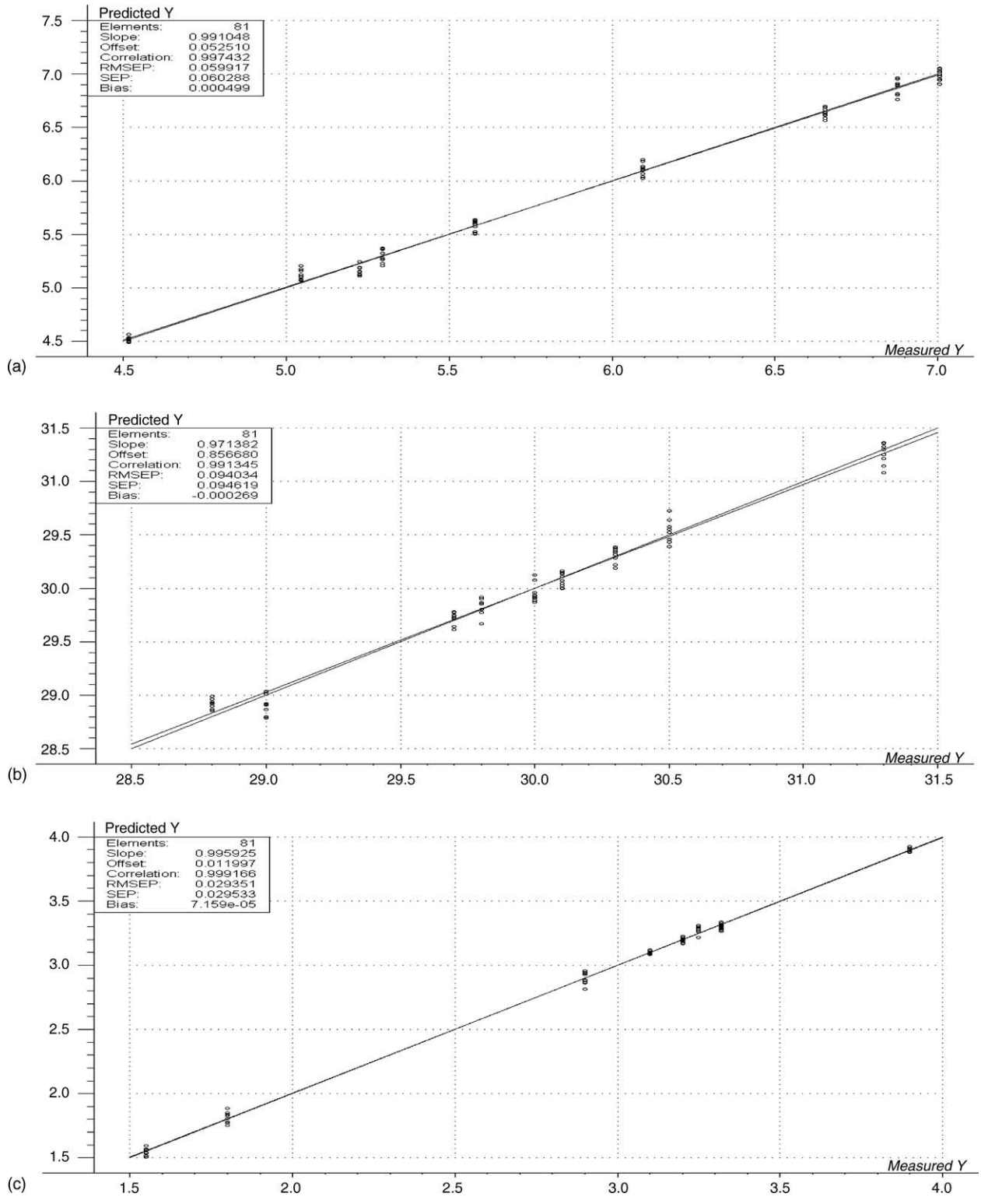

Fig. 3. PLS 2 regression plots for (a) kavain; prediction; $y = 0.991048a + 0.052510$; (b) the total content of kavapyrones, prediction; $y = 0.971382a + 0.856680$; (c) the water content; prediction; $y = 0.995925a + 0.11997$. Elements: number of spectra; slope: slope of the regression between abscissa (X) and ordinate (Y); offset: intercept of the regression line; correlation: correlation between X and Y; varies between −1 and +1; RMSEP: root mean square error of prediction; RMSEC: root mean square error of calibration; SEP: standard error of prediction = standard error of the prediction residuals; SEC: standard error of calibration = standard error of the calibration residuals; bias: average difference between Y and X.

4. Conclusion

This study emphasises the potential of NIRS as a rapid and highly versatile alternative method to the conventional quantitative analysis of plant extracts. NIRS was successfully employed for the quantification of kavain, the total content of kavapyrones and the water content in kava kava dry extracts. The inclusion of the water content significantly improved both the calibration and prediction of the PLS 2 model. Once the time consuming calibration is done NIR spectroscopy offers several distinct advantages compared to the HPLC: it is faster and non-destructive, and it requires no sample preparation and no solvents.

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