Amino Acids (2006) 31: 45–53
DOI 10.1007/s00726-006-0308-z

Amino Acids
Printed in Austria

Near infrared spectroscopy, cluster and multivariate analysis hyphenated to thin layer chromatography for the analysis of amino acids

N. Heigl, C. W. Huck, M. Rainer, M. Najam-ul-Haq, and **G. K. Bonn**

Institute of Analytical Chemistry and Radiochemistry, Leopold-Franzens University, Innsbruck, Austria

Received Februray 28, 2006
Accepted March 8, 2006
Published online June 1, 2006;

Summary. A method based on near-infrared spectroscopy (NIRS) was developed for the rapid and non-destructive determination and quantification of solid and dissolved amino acids. The statistical results obtained after optimisation of measurement conditions were evaluated on the basis of statistical parameters, Q-value (quality of calibrations), R^2, standard error of estimation (SEE), standard error of prediction (SEP), BIAS applying cluster and different multivariate analytical procedures. Experimental optimisation comprised the selection of the highest suitable optical thin-layer (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mm), sample temperature (10–30 °C), measurement option (light fibre, 0.5 mm optical thin-layer; boiling point tube; different types of cuvettes) and sample concentration in the range between 100 and 500 ppm. Applying the optimised conditions and a 115-QS Suprasil® cuvette (V = 400 µl), the established qualitative model enabled to distinguish between different dissolved amino acids with a Q-value of 0.9555. Solid amino acids were investigated in the transflectance mode, allowing to differentiate them with a Q-value of 0.9155. For the qualitative and quantitative analysis of amino acids in complex matrices NIRS was established as a detection system directly onto the plate after prior separation on cellulose based thin-layer chromatography (TLC) sheets employing n-butanol, acetic acid and distilled water at a ratio of 8:4:2 (v/v/v) as an optimised mobile phase. Due to the prior separation step, the established calibration curve was found to be more stable than the one calculated from the dissolved amino acids. The found lower limit of detection was 0.01 mg/ml. Finally, this optimised TLC-NIRS method was successfully applied for the qualitative and quantitative analysis of L-lysine in apple juice. NIRS is shown not only to offer a fast, non-destructive detection tool but also to provide an easy-to-use alternative to more complicated detection methods such as mass spectrometry (MS) for qualitative and quantitative TLC analysis of amino acids in crude samples.

Keywords: Amino acid – Near infrared spectroscopy – Cluster analysis – Multivariate analysis – Thin layer chromatography

Introduction

One of the forerunners of modern near-IR applications, Karl Norris of the U.S. Department of Agriculture, used the near-infrared (NIR) wavelength region in the mid-1960s for the spectral analysis of many substances such as the moisture content of grain and seeds (Norris and Hart, 1996). Today, near-infrared spectroscopy (NIRS) has become an important and widely accepted tool for not only the qualitative but more importantly for quantitative analysis (Ciurczak and Drennen, 2002). The NIR region covers the overtone and combination transitions of the C–H, O–H and N–H groups in a wavenumber range from 4000 to 12800 cm^{-1} (2500–780 nm). NIR absorption bands are much weaker and harder to identify in comparison to mid-IR (MIR, 2500–25000 nm) spectra because of the higher grade of excited bondings. Wavelengths from 190–650 nm are those that most bio molecules absorb fluorescent. The NIR wavelength region provides higher sensitivity for detection of biomolecules because of the lack of high background (auto fluorescence) caused by the molecules themselves (190–650 nm) and thus better signal-to-noise (S/N) ratio (Raghavachari, 2001). Therefore, NIR spectrometry coupled with multivariate methods (e.g., principal component analyses, PCA; partial least squares, PLS; multiple linear regression, MLR; principal component regression, PCR) has been successfully used for the simultaneous analysis of various compounds (Burns and Ciurczak, 1992). That means if a particular component provides an NIR absorption spectrum and its concentration is high enough that the spectrum contributes meaningfully to the NIR absorption profile, then it is in principle possible to perform analysis by using NIR spectroscopy. There are many studies of the feasibility and efficiency of Raman, mid- and near-IR spectroscopic detections of amino acids (István et al., 2003), di-, tri- (Troy and Tran, 2001) and tetra peptides (Tran and Kong, 2000), proteins (Suyrewicz and Mantsch, 1996), different clinical (Jackson and Mantsch,

1996), pharmaceutical (Cogdill and Drennen, 2005), agricultural and food (Siesler et al., 2002) applications. The most important advantages of near-infrared spectroscopy to other spectroscopic techniques are the fast sample preparation, the possibility to use optical thin-layers up to centimetres (Siesler et al., 2002) and the avoidance of fluorescence and laser-induced sample damage. So it is worth to investigate, develop and improve the spectroscopic analysing technique in the NIR wavelength region. Today NIR spectrometers are very user-friendly and highly sophisticated analysing systems, equipped with fast and very sensitive detectors. Light fibres in different length, variety of very precise wavelengths selection modules with great reproducibility and high resolution, a wide range of measurement options and powerful software packages (Stark and Luchter, 2005) are available. In addition to that reliable in- and online monitoring can be performed with well-equipped instruments, what makes NIRS very popular in agricultural, food and clinical science (Murray and Cowe, 1991).

Amino acids possess at least two or more C–H, O–H and N–H groups, so NIR spectroscopy is useful for studying hydration and hydrogen bonds in peptides and proteins (Siesler et al., 2002). For instance the potential of generalised 2-D NIR correlation spectroscopy in protein research has been demonstrated (Wang et al., 1998) for ovalbumin solutions. Heise et al. (1998) described an assay of blood substrates of total protein. Since the 1970s there has been a continued interest in the application and development of combining separation techniques, such as thin-layer chromatography (TLC) and near-infrared spectroscopy (Fong and Hieftje, 1995), because of fast, simple and relatively inexpensive sample analysis (Ciurczac et al., 1991). In situ spectroscopic evaluation of separated zones on TLC plates has been used mainly in the UV, visible and infrared regions (Stahlmann and Kovar, 1997). Several authors have described direct NIR (Fong and Hieftje, 1995) and Raman (Istvan et al., 2003; Everall, 1992) measurements of different TLC layers based upon silica high-performance thin-layer chromatographic (HPTLC) plates, silica gel plates 60 Å, Si 60 F_{254s} Raman silica gel HPTLC-Raman plates and Aluminium Oxide 60 F_{254} alumina sheets. If the materials are not coloured or fluorescent, derivatisation is necessary, and the poor reproducibility of the derivatisation process decreases the accuracy of measurements (Yamamoto et al., 1991). To avoid the need for derivatisation, NIR or MIR spectroscopy can be used. Compared to other analytical techniques such as liquid chromatography (LC; Stecher et al., 2003), capillary electrophoresis (CE; Huck et al., 2003) or on-/off-line mass spectrometry (MS), NIRS enables a very fast and easy analysis (Huck et al., 2005). Because of the quick sample preparation and short scanning-times, samples can be measured within seconds, enabling high-throughput analysis (Siesler et al., 2002). In this work qualitative and quantitative analysis of amino acids are described. The goal is to distinguish between different amino acids, which were measured either in solid or liquid optimised condition. In addition a method for the direct measurement of amino acid spots on TLC sheets using a Fourier-transform NIR is presented. NIR spectroscopy turns out to be a suitable analysing method for investigating amino acids, peptides and proteins and the advantages of rapid, easy and inexpensive measurements makes it a very interesting and important tool in chemical, agricultural, food, pharmaceutical and especially clinical science for e.g. blood analysis (Elwell and Beard, 2005).

Materials and methods

Materials and reagents

L-forms of glutamic acid, glutamine, lysine, isoleucine, phenylalanine, tyrosine, cysteine, aspartic acid, asparagine, arginine, threonine, alanine, methionine, tryptophan, histidine, proline and serine (all with 99.9% purity) were purchased from Serva Biochemica (Heidelberg, Germany). Ammonium acetate (analytical gradient grade) was purchased from Arcos Organics, NJ, USA and water was purified by a Nanopure – unit (Millipore, Barnstead, MA, USA). Samples were measured in three different ways. Dissolved amino acids were placed either into a specially prepared glass stovepipe (V = 15 ml) or into cuvettes made of Suprasil® (Hellma, Jena, Germany) consisting of types 100-QS (V = 3500 µl), 104-QS (V = 1400 µl), 115-QS (V = 400 µl), 105.201-QS (V = 100 µl) and optical glass (Starna, Pfungstadt, Germany) type 7G (V = 3500 µl). Solid samples were measured directly with an optic glass fibre.

Near-infrared spectroscopy

NIR spectra were recorded on a single beam polarisation NIR Fourier-Transform spectrometer (FT-NIR; Büchi, Flawil, Switzerland) equipped with a tungsten-halogen lamp, a temperated lead sulfide detector (30 °C) and a standard 2 m optic glass fibre (silica glass, Infrasil, Bes Optics Inc., Warwick, Great Britain, optical thin-layers 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mm). Transflectance, transmittance and diffuse reflectance modes were employed depending on the measurement method and sample state-of-matter. With Fourier-transform spectroscopy, the interferogram is obtained by scanning the difference in the optical path lengths of two beams of a two-arm interferometer. The optical system of the said spectrometer contains a triangular prism of high refractive index which moves perpendicularly to the optical axis to change the optical distances of the two polarising components of the incident light, that pass through the crystal. Wavenumber ranges from 4000 cm^{-1} to 10000 cm^{-1} (1000–2500 nm). The instrument offers a resolution of 12 cm^{-1}, an absolute wavelenght accuracy of ± 2 cm^{-1} and a relative reproducibility of 0.5 cm^{-1}.

Spectra were recorded in transflectance (solutions; light fibre, optical thin-layers 0.5–3.0 mm), transmittance (solutions; cuvettes, optical thin-layer 10.0 mm and boiling point tube, optical thin-layer 2.0 mm) and reflectance modes (solid samples; light fibre direct on sample). The sample temperature was regulated by a heater (Julabo – model PC/4, Seelbach, Germany) that stabilized the temperature of the water bath with a variation limit of ± 0.1 °C.

Chemometrical software NirCal 4.21 (Büchi) was used for creating a model, i.e., selection of spectra and wavelengths, mathematical pre-treatment and statistical analysis performing cluster analysis, principal component analysis (PCA) and principal component regression (PCR). Spectra were randomly divided into a so called learning-set (67%, C-set), i.e., calibration samples, and a test-set (33%, V-set), i.e., samples for testing the calibration equation. The optimum number of factors used for the individual prediction was determined by cross-validation and quality of cluster analysis was described in Q-value calculated by the NirCal 4.21 software. The selection of the best quantitative regression model is based on the following calculated values:

1) BIAS, i.e., the average deviation between the predicted values (y_n) and the actual values (x_n) in the calibration-set, should be close to zero.

$$\text{BIAS} = \frac{1}{N}\sum(x_n - y_n)$$

2) PRESS, Predicted Residual Error Sum Square is the sum of the square of the deviation between predicted and reference values. The PRESS value of the validation set should be as small as possible and similar to that of the calibration set.

$$\text{PRESS} = \sum(x_n - y_n)^2$$

3) SEE, Standard Error of Estimation, i.e., the standard deviation of the differences between reference values and NIRS-results in the calibration set.

$$\text{SEE} = \sqrt{\frac{1}{N}\sum(x_n - y_n - \text{Bias})^2}$$

4) SEP, Standard Error of Prediction, i.e., the counterpart for the test-set samples. SEE and SEP should be as small as possible.

$$\text{SEP} = \sqrt{\frac{1}{N}\sum(x_n - y_n - \text{Bias})^2}$$

5) The correlation coefficient (R^2) should approach 1.

Preparation of standard solutions

Amino acids were measured at 0.5 mg/ml concentration in ammonium acetic buffer (50 mmol/ml), made up in deionised H_2O. The pH was adjusted to 4.0 by adding appropriate volumes of acetic acid. All samples and buffers were freshly prepared at 20–23 °C and pH was checked with an electronic pH meter (WTW, model multilab 540).

Thin-layer chromatography (TLC)

The cellulose based thin-layer chromatography sheets (TLC, Carl Schleicher & Schüll, Dassel, Germany) were added with the samples (1 µl capillary) of various concentrations, i.e. 100, 150, 200, 250, 300, 350, 400, 450 and 500 ppm, 10 times each ($R_f = 0.44$). Afterwards they were brought into the mobile phase (~3 h), made up of *n*-butanol, acetic acid and distilled water at a ratio of 8:4:2 (v/v/v). The sheets were brought into an oven at 95 °C for 15 minutes. Ninhydrin spray reagent was taken as a contrast agent.

Results and discussion

Optimisation of experimental parameters

Optical thin-layer (OTL)

Measurements with the optical light fibre have been performed by using different optical thin-layers (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mm). Lysine in water has been measured at a concentration of 0.5 mg/ml with regard to the most intense absorption bands in a wavenumber range from 4000–10000 cm^{-1} (2500–1000 nm). A specially prepared glass stovepipe (V = 15 ml) has been used as a vessel to analyse the samples in transflectance mode. The use of different optical thin-layers showed an optimum at 0.5 mm, which resulted in spectra (average of 10 scans at T = 23 °C) of various absorption intensities and quality. Excitations at 5320 cm^{-1}, 5630 cm^{-1}, 7160 cm^{-1}, 7440 cm^{-1} and 8700 cm^{-1} showed clear peaks (Fig. 1; kind of vibration is discussed later on) with all the tested optical thin-layers and on the basis of its 1st Derivative Taylor 3 Points statements concerning the involved molecular bondings

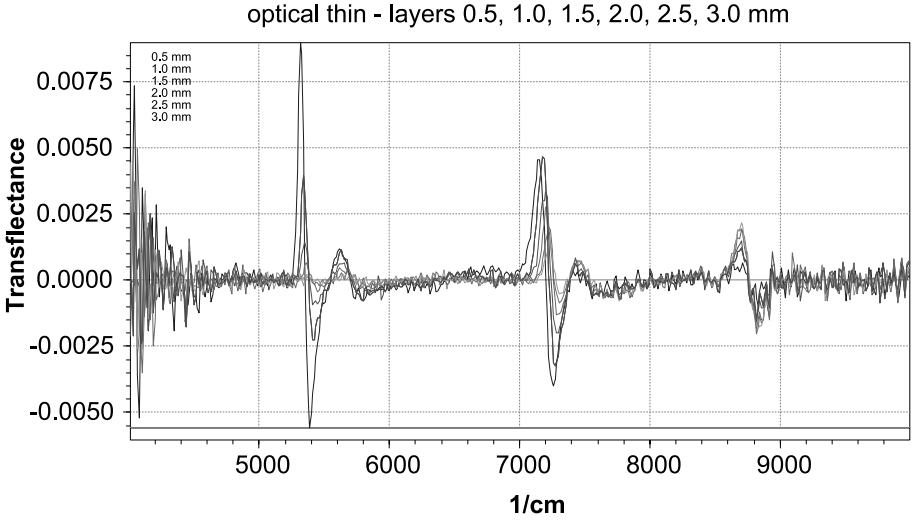

Fig. 1. NIR-absorption spectra of dissolved amino acid lysine in water by applying different optical thin-layers. Conditions: wave number range, 4000–9996 cm^{-1}; optical thin-layers: 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mm; scans, 10; temperature, 23 °C, 1st Derivative Taylor 3 Points

were made. Looking at the original absorption spectra at 4500 cm^{-1} only the optical thin-layer of 0.5 mm showed satisfactorily results in the form of a clear peak allowing to distinguish between different amino acids, while larger OTL did not. Further optimisation was conducted with a standard 2 m optical fibre with an optical thin-layer of 0.5 mm ensuring the highest signal-to-noise ratio (SNR). Amino acid samples were measured in transflectance mode over a wavenumber range from 4500–10000 cm^{-1}. Absorption spectra of asparagine, glutamic acid and alanine showed total reflectance over the wavenumber range mentioned above. That is why an OTL of 0.5 mm is not the best suitable choice for measurements of amino acids in transflectance mode. Another reason for the unsuitability of this analysing mode is, that recorded spectra of dissolved amino acids show clear separation at higher wavenumbers from 5496–6600, 7596–8208 and 8700–9996 cm^{-1}. Figure 2 shows higher sensitivity for the cuvette type 100-QS (V = 3500 µl) at higher wavenumbers in contrast to the optical light fibre (0.5 mm OTL). These facts lead to the deployment of a cuvette with an OTL of 10.0 mm for further amino acid investigations. Because of small sample volume (400 µl) and higher sensitivity at wavenumbers from 7000–10000 cm^{-1}, cuvette type 115-QS made of synthetic glass (Suprasil®) turned out to be the best choice.

Sample temperature

Adjusting the sample temperature also led to spectra with different absorption intensities. Figure 3 shows the effect of sample temperature on the absorption energy, measured between 10 and 30 °C at 2 °C steps. Shifts of the absorption bands were observed at temperature changes within ±0.5 °C. The model depicted in Fig. 3 illustrates the absorption intensities of the two most intense peaks on the basis of its pretreated spectra (1st Derivative Taylor 3 Points) at 5320 cm^{-1} (C–H and C = O stretching, 1880 nm, Peak 1) and 7160 cm^{-1} (C–H stretching first overtone, 1397 nm, Peak 3). Between 10 °C and 20 °C the absorption intensity stayed stable. In a range from 20 to 23 °C the intensity rose slightly and above 23 °C a distinctive fall of the absorption intensity could be observed. That means, the sample temperature has to be monitored precisely otherwise important information could be overlooked. Based upon these results all further measurements of liquid samples have been performed at a temperature range from 20 to 23 °C.

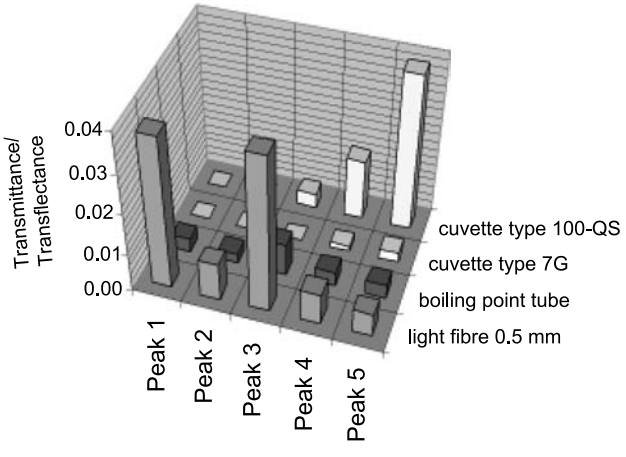

Fig. 2. The five most intense absorption peaks of the pretreated spectra of lysine in water measured with four different options recorded in transmittance or transflectance mode. Conditions: wave number range, 4000–9996 cm^{-1}; optical thin-layer, light fibre 0.5 mm, boiling point tube 2.0 mm, cuvettes 10.0 mm; scans, 10; temperature, 21 °C; 1st Derivative Taylor 3 Points; Peak 1, 5320 cm^{-1}; Peak 2, 5630 cm^{-1}; Peak 3, 7160 cm^{-1}; Peak 4, 7440 cm^{-1}; Peak 5, 8700 cm^{-1}

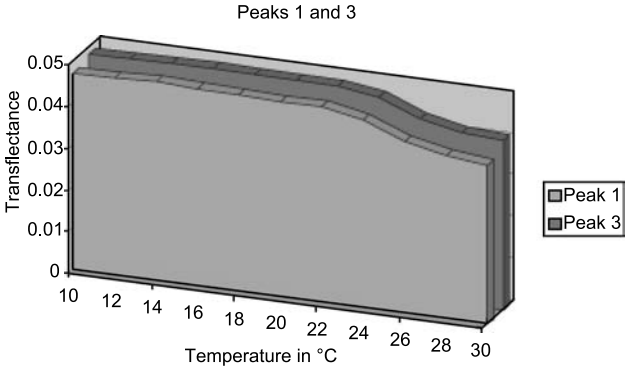

Fig. 3. Effect of temperature on the two most intense peaks out of five of lysine in water on the basis of its pretreated 1st Derivative Taylor 3 Points spectra. Conditions: wave number range, 4000–9996 cm^{-1}; optical thin-layer, 0.5 mm; scans, 10; sample-temperature: 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30 °C

Measurement options

The choice of the measurement options turned out to be a very important basic optimisation, regarding the quality of recorded spectra. A comparison of four completely different measurement options pointed out how various optical thin-layers, sample volumes and detection modes influence the sensitivity and quality of near-infrared absorption spectra. Spectra recorded with an optical light fibre (0.5 mm optical thin-layer), a boiling point tube (2.0 mm optical thin-layer) and two different types of cuvettes (optical thin-layer 10.0 mm) were compared directly. The light fibre offers the most flexible way to perform measurements owing to the possibility to analyse both liquids and solids. Solid samples can be analysed by simply placing the light fibre inside or on the sample and record

spectra in transflectance or reflectance mode. Cuvettes offer the possibility to analyse samples in transmittance mode, therefore cells with different volumes and optical thin-layers up to 10.0 mm can be utilized (Hellma, Starna). Particularly if there is only a small amount of sample volume available, one prefers using cells or cuvettes with small volumes. Figure 2 shows the five main peaks of the pretreated recorded spectra (1st Derivative Taylor 3 Points) of dissolved lysine in water (0.5 mg/ml) at wavenumbers 5320 cm^{-1}, 5630 cm^{-1}, 7160 cm^{-1}, 7440 cm^{-1} and 8700 cm^{-1}. This makes it clear that the light fibre with an optical thin-layer of 0.5 mm is superior to the other measurement options concerning the intensity of recorded absorption spectra over the whole wavenumber range. The boiling point tube only shows weak absorption bands at wavenumbers mentioned above. The worst interpretable spectra showed the cuvette type 7G (V = 3500 µl), made of optical glass, due to an optical thin-layer of 10.0 mm. Hardly any reliable statement could be made affecting the involved molecular bondings, so this cell was not selected for further measurements. The cuvette type 100-QS showed intense peaks at high wavenumbers, especially at 7440 cm^{-1} and 8700 cm^{-1}. So four different cuvettes (types 100-QS, 104-QS, 115-QS, 105.201-QS – all made of Suprasil®) have been investigated concerning their absorption behaviour in NIR wavelength region. Cuvette type 115-QS (V = 400 µl) turned out to be the best suitable cell for measuring solutions in transmittance mode in combination with the NIRVIS FT-NIR spectrometer. It showed the most intense absorbance signals out of the four tested cuvettes over the whole wavenumber range from 4000–10000 cm^{-1} on the basis of the 1st Derivative Taylor 3 Points pretreated spectra. However, compared to the absorption spectra measured in transflectance mode with the light fibre, 0.5 mm optical thin-layer, the signal is much weaker especially in the lower wavenumber region from 4200–4800 cm^{-1} recorded in transmittance mode. It also emerged that ultra-micro cells such as type 105.201-QS are not suitable for measurements in combination with the NIRVIS FT-NIR in transmittance mode because of the slim inner-width of 2 mm. Depending on the light diameter of electromagnetic radiations by the source, light can pass side wall of the cell which can lead to errorneous results. Intensities of the transflectance spectra recorded with a light fibre, 0.5 mm optical thin layer, were always higher in the lower wavenumber regions than those of the spectra recorded in transmission mode, even higher than those measured with the best suitable cuvette (type 115-QS, V = 400 µl). In case of the amino acid analysis, higher wavenumber regions were the most important and best interpretable regions. So further measurements have been performed with the cuvette type 115-QS made of Suprasil®, as also the broader optical thin-layers enhance signal-to-noise ratio (SNR).

NIRS of dissolved amino acids

Figure 4 shows a 3-D graphic of the recorded original absorption spectra of alanine, arginine, asparagine, glutamine, glutamic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, threonine and tryptophan dissolved in ammonium acetate buffer, pH 4.0. The graphic clarifies that differences in NIR absorption spectra only occur in the higher wavenumber regions (7500–10000 cm^{-1}), and therefore it was important to record spectra with a cuvette (transmittance mode) because of higher sensitivity at higher wavenumbers (Fig. 2). Prior to calibration spectra pretreatments were implemented with regard to eliminate disruptive factors like scattering effects. Pretreatments consisted of multiplicative scatter correction (MSC full) and relating to gain better signal-to-noise ratio (SNR). The averaged absorption spectra of 20 measurements and 10 scans for every single amino acid sample were used for a calibration over a wavenumber range from 5496–6600, 7596–8208 and 8700–9996 cm^{-1}. 44 absorption spectra were assigned to the calibration set (C-set, 67%) and 21 spectra to the validation set (V-set, 33%). The two-dimensional factor plot depicted in Fig. 5 shows the correlation between the recorded pretreated spectra based on their loading values. Three primary factors were chosen from

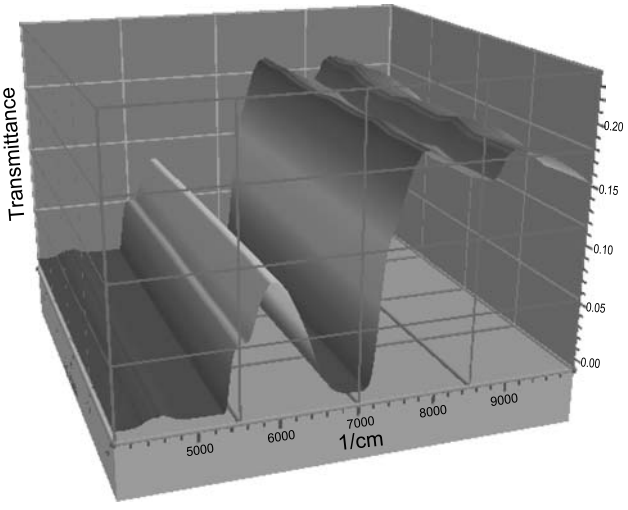

Fig. 4. Three-dimensional view of the original absorption spectra of 13 dissolved amino acids recorded with cuvette type 115-QS, optical thin-layer 10.0 mm. Conditions: wave number range, 5496–6600, 7596–8208, 8700–9996 cm^{-1}; scans, 10; measurements, 20 each amino acid; temperature, 22 °C; 1. MSC full, averaged spectra

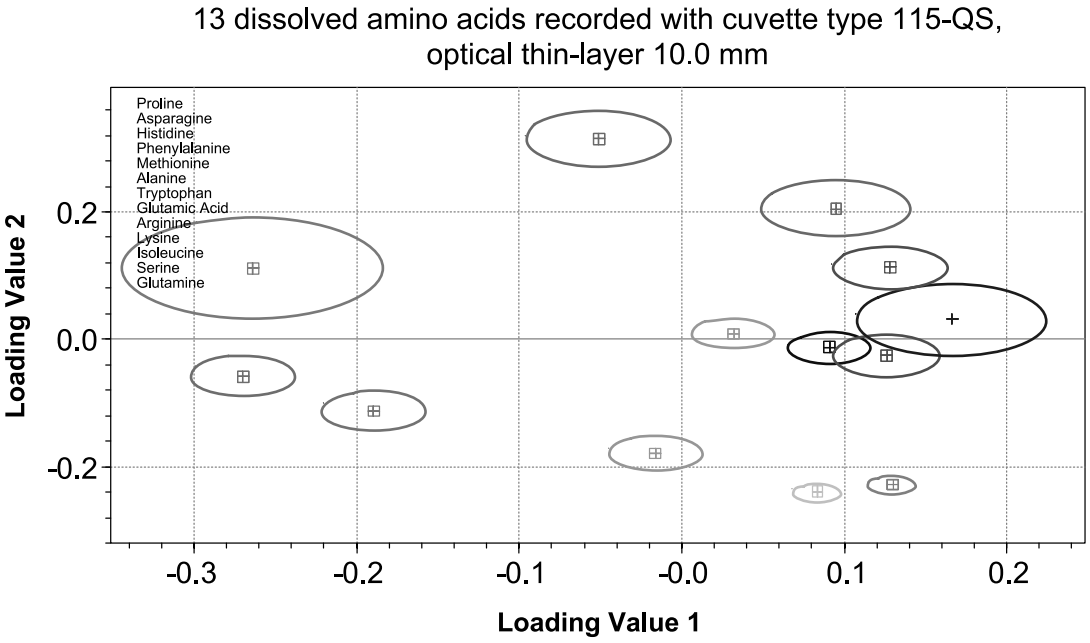

Fig. 5. Two-dimensional factor plot of recorded pretreated absorption spectra of 13 dissolved amio acids. Conditions: wave number range, 5496–6600, 7596–8208, 8700–9996 cm^{-1}; scans, 10; measurements, 20 each amino acid; temperature, 22 °C; 1. MSC full, averaged spectra

the calculated PRESS (Predicted Residual Error Sum Square) function and the loading values of the absorption spectra over the primary factors. Proper wavelengths were chosen by means of the property wavelength regression (PWR) that shows the regression coefficient of spectra over the whole wavenumber range from 4000–10000 cm^{-1}. Also the given spectra residuals fit all within the pre-set values calculated by the NirCal 4.21 Software. The estimated values provide a robust and reliable calibration that confirms a Q-value, quality of calibration, of 0.955473 (1 = perfect calibration). The cluster model depicted in Fig. 6 shows the proof that every amino acid could be classified and assigned to a single cluster. To gain a clean and selective cluster classification, unique spectral features have to exist, therefore Fig. 4 shows the spectral features of each analyte for comparison. Although it is relatively difficult to interpret NIR spectra unambiguously (Tran, 2000), tentative statements of the involved chemical bondings could be assigned on the basis of the first derivative of the recorded spectra. The 8700 cm^{-1} peak can be assigned to a C–H stretching second overtone, bands in the 7440 cm^{-1} region to a 2 × C–H stretching + C–H deforming group, the 7160 cm^{-1} region to a C–H stretching first overtone bonding, the peak at 5630 cm^{-1} may be responsible for the O–H stretching + 2 × C–O stretching group and a peak at 5320 cm^{-1} may show a C–H stretching + C = O stretching group. Differences in the absorptivities enabled the construction of a clear and reliable calibration (Table 1) that shows the classification of each of the 13 amino acids into a single cluster.

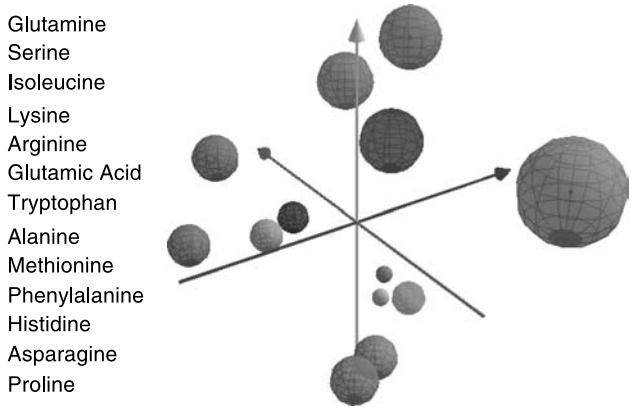

Fig. 6. 3-dimensional cluster plot of recorded pretreated absorption spectra of 13 dissolved amio acids. Conditions: wave number range, 5496–6600, 7596–8208, 8700–9996 cm^{-1}; scans, 10; measurements, 20 each amino acid; temperature, 22 °C; 1. MSC full, averaged spectra

NIRS of solid amino acids

The fifteen L-form amino acids methionine, isoleucine, asparagine, lysine, cystine, tryptophan, arginine, threonine, histidine, phenylalanine, aspartic acid, proline, glutamic acid, alanine and tyrosine were used in this study to classify solid amino acid samples and to create a reliable cluster model on the basis of the recorded NIR absorption spectra.

Table 1. Calibration and validation protocol for the NIRS of dissolved amino acids

Properties in Project	Proline, Asparagine, Histidine, Phenylalanine, Methionine, Alanine, Tryptophan, Glutamic Acid, Arginine, Lysine, Isoleucine, Serine, Glutamine (total 13/13)
Properties in Calibration Set	Proline, Asparagine, Histidine, Phenylalanine, Methionine, Alanine, Tryptophan, Glutamic Acid, Arginine, Lysine, Isoleucine, Serine, Glutamine (total 13/13)
Spectra in Project	65
Spectra in Calibration Set	5, 9–11, 13, 15–18, 20–21, 23–25, 27–31, 34–35, 37–43, 46–47, 49–59, 61, 63–64 (total 44/65)
Spectra in Validation Set	1–4, 6–8, 12, 14, 19, 22, 26, 32–33, 36, 44–45, 48, 60, 62, 65 (total 21/65)
Wavelengths Calibration Set	5496–6600, 7596–8208, 8700–9996.
Data Pretreatment Sequence	1. MSC full, 5496–6600, 7596–8208, 8700–9996 (total 254/500), Mean Vector (500)
Number of Primary Factors	3
Secondary/Calibration Factors	1–3 (total 3/3)
Q-Value	0.955473

Solid samples were measured directly in transflectance mode by placing the samples between the light-fibre end and the teflon reflectance. Eight primary factors were chosen to calculate the cluster calibration. The spectra showed highly overlapping bands over the whole wavenumber region from 4500–9996 cm^{-1}; therefore the mentioned wavenumber range was kept for the calibration. Pretreatment of the spectra (1. Smooth Savitzky–Golay 9 Points) helped to increase the quality of the calculated calibration. Two measurements (10 scans each) were sufficient to achieve good results out of the measurements and to reduce duration of analysis (∼1 min per sample). A Q-value of 0.915536 confirms the clear separation of all amino acids being investigated. Spectra residuals considered all within the pre-set values, calculated by the NirCal 4.21 software and the number of clusters per property showed a value of 1 for all 15 amino acids. Figure 7 depicts the 3-dimensional cluster model of the amino acids measured in solid phase.

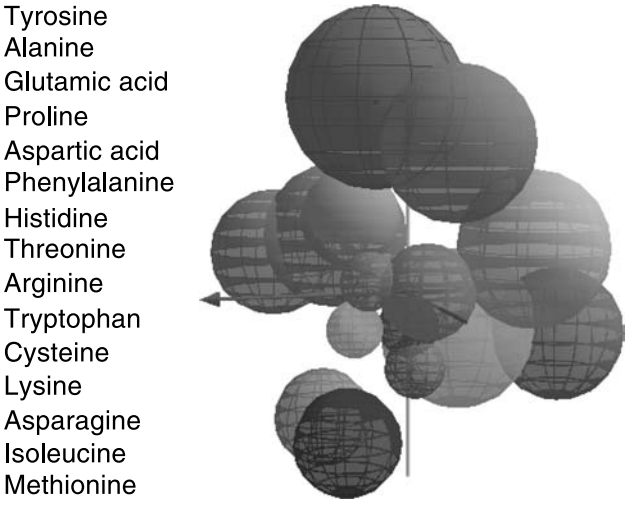

Fig. 7. Three-dimensional cluster view of 15 solid amino acids. Conditions: wave number range, 4500–9996 cm^{-1}; scans, 10; measurements, 2 each amino acid; temperature, 20 °C; 1. Smooth Savitzky–Golay 9 Points

Every sample could be assigned to a single cluster which proves that NIRS offers the possibility to differentiate among different amino acids measured in solid phase (Table 2). Hardly any reliable statement could be made concerning the involved chemical bondings on the basis of the pretreated (1st Derivative Taylor 3 Points) absorption spectra because the strong overlapping absorption bands occurred only in wide wavenumber ranges (4500–5300, 5650–7500, 8100–9100 cm^{-1}). That means no clear single peak could be identified for all the analysed amino acids.

Table 2. Calibration and validation protocol for the NIRS of solid amino acids

Properties in Project	Methionine, Isoleucine, Asparagine, Lysine, Cystine, Tryptophan, Arginine, Threonine, Histidine, Phenylalanine, Aspartic acid, Proline, Glutamic acid, Alanine, Tyrosine (total 15/15)
Properties in Calibration Set	Methionine, Isoleucine, Asparagine, Lysine, Cystine, Tryptophan, Arginine, Threonine, Histidine, Phenylalanine, Aspartic acid, Proline, Glutamic acid, Alanine, Tyrosine (total 15/15)
Spectra in Project	30
Spectra in Calibration Set	1, 3, 5–10, 12–15, 17–21, 24–25, 27, 29 (total 21/30)
Spectra in Validation Set	2, 4, 11, 16, 22–23, 26, 28, 30 (total 9/30)
Wavelengths Calibration Set	4500–9996.
Data Pretreatment Sequence	1. Smooth Savitzky–Golay 9 Points
Number of Primary Factors	8
Secondary/Calibration Factors	1–8 (total 8/8)
Q-Value	0.915536

NIRS hyphenated to thin-layer chromatography (TLC)

A comparative study of the feasibility and efficiency of NIRS combined with thin-layer chromatography (TLC) has been implemented. The aim was to detect amino acid spots on cellulose based TLC sheets in order to classify and identify them in the right manner. Lysine in water at different concentrations (500, 450, 400, 350, 300, 250, 200, 150, 100 ppm) was taken as a sample for this study. Drops of the test solution were added onto the TLC sheet with the help of a 1 µl capillary (10 times each). As a mobile phase, a mixture of *n*-butanol, acetic acid and distilled water at a ratio of 8:4:2 (v/v/v) was taken. The sheets were kept for about 3 h into the mobile phase (saturated chamber), and afterwards the TLC sheets were dried in oven for 15 min at 95 °C. Ninhydrin reagent was sprayed onto the surface for fluorescence. The developed cellulose sheets were analysed directly in reflectance mode with the NIR optical fibre by simply putting the fibre end onto the amino acid spots. The property wavelenght selection function showed that unique spectral features occurred at a wavenumber range from 4500–7308 cm^{-1}. Qualitative identification was achieved with high accuracy. The calibration was calculated by principal component regression (PCR) algorithm on the basis of 6 primary factors (C-set regression coefficient 0.9947, V-set regression coefficient 0.9952). A small deviation between the standard error of estimation (SEE) and the standard error of prediction (SEP), confirms the consistency of this calibration (97.922). A Q-value of 0.859136 (Table 3) gives promise to a highly accurate and reliable calibration. Every spot was measured 3 times (30 scans) and the calibration curve depicted in Fig. 8 shows the coherence between the predicted property values and the true property values calculated by the analysis software. In the NIR region, absorption is rather weak, because it consists of overtones and combination bands of fundamental bands in the mid-infrared (MIR) region. Additional measurements pointed out a detection limit of 0.01 mg/ml sample volume added on the TLC sheet. To test the reliability of the system for the analysis of real samples, L-lysine in apple juice was determined following the established protocol. The found

Table 3. Calibration and validation protocol for the NIRS hyphenated to TLC

Properties in Project	ppm (total 1/1)
Properties in Calibration Set	ppm (total 1/1)
Spectra in Project	27
Spectra in Calibration Set	1, 3, 5–9, 11–13, 16–19, 21–23, 25–26 (total 19/27)
Spectra in Validation Set	2, 4, 10, 14–15, 20, 24, 27 (total 8/27)
Wavelengths Calibration Set	4500–7308.
Number of Primary Factors	6
Secondary/Calibration Factors	1–6 (total 6/6)
C-Set SEE	13.5461
V-Set SEE (SEP)	13.8336
Consistency	97.922
Q-Value	0.859136

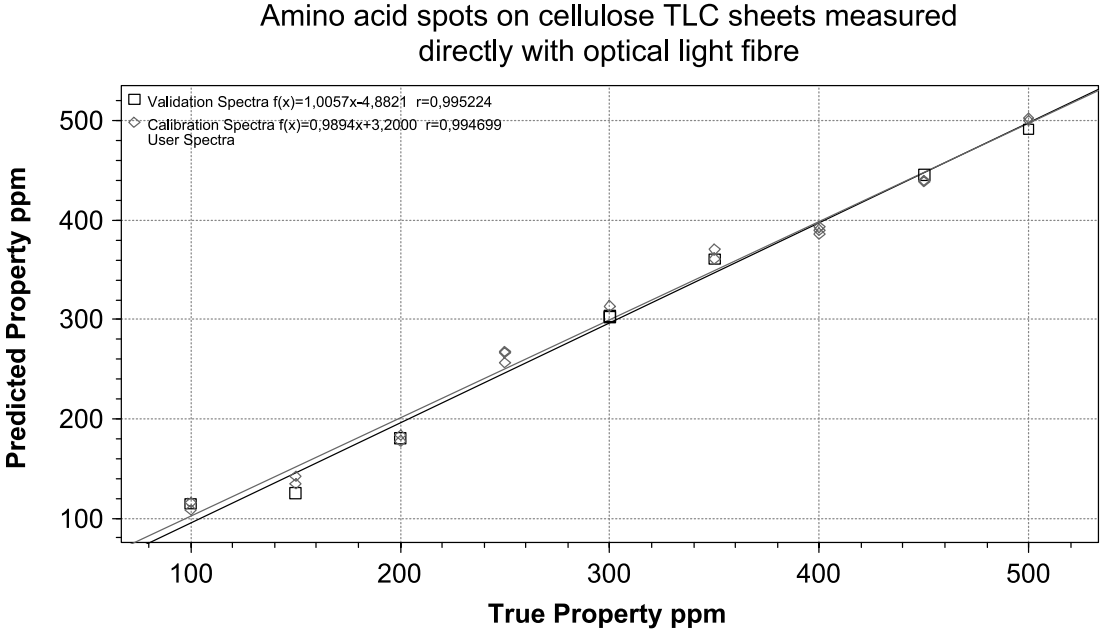

Fig. 8. Predicted vs. actual property of dissolved lysine in water, concentration values directly measured on cellulose based thin-layer chromatographic sheet. Conditions: wave number range, 4500–7308 cm^{-1}; scans, 30/measurements, 3 each concentration level; sample concentration: 100, 150, 200, 250, 300, 350, 400, 450, 500 ppm; temperature, 20 °C

value of 4.7 mg/100 g agreed well to that published in the literature (Souci et al., 2003). The results demonstrate the good compatibility of these two cheap, rapid and relatively simple analysing methods related to the investigated amino acid spots. Thereby the linearity of the calibration curve of the measured amino acid spots on TLC sheets points out to be much more stable (Q-value = 0.859136) than the one calculated from the amino acid solutions only (Q-value = 0.109757). The sensitivity of the established NIRS-model in case of the solid amino acids turned out to be three times higher compared to that of the dissolved, which might be related to the presence of buffer in the solutions.

Conclusion

Near-infrared spectroscopy was used to classify L-form amino acids either in dissolved or solid condition. Regarding the differentiation of amino acid concentrations of dissolved samples, it could be shown clearly, that the combination of thin-layer chromatography (TLC) with near-infrared spectroscopy (NIRS) leads to the best results. The Q-value of the calibration was increased significantly through the combination of these methods. All measured amino acids could be specifically attributed into single clusters. The general task of this feasibility study was to improve the measurement conditions for using NIRS as an analysing tool in laboratory applications. This led to improved performances and quality of calculated calibrations for all measured samples. The technique presented here allows a fast and easy method for classifying and differentiating biological samples in a comprehensive way and comments itself as a promising analysing tool in bioanalysis, e.g. the analysis of amino acids in blood samples.

Acknowledgements

This work was supported by the Austrian Genome Program (Gen-AU), Vienna, Austria and by the special research program "Cell Proliferation and Cell Death in Tumors" (SFB021, Austrian Science Fund), FWF, Vienna, Austria and MVIT-WINN (West Austrian Initiative for Nano Networking), Austria.

References

Burns DA, Ciurczak EW (1992) Handbook of near-infrared analysis. Marcel Dekker, New York

Ciurczak EW, Drennen JK (2002) Pharmaceutical and medical applications of near-infrared spectroscopy. Marcel Dekker, New York

Ciurczac EW, Murphy WR, Mustillo DM (1991) The use of near-infrared spectroscopy in thin-layer chromatography, part 2: Qualitative applications. Spectroscopy 6: 36–40

Cogdill RP, Drennen JK (2005) Near infrared spectroscopy in the pharmaceutical industry. NIR News 16: 23–26

Elwell C, Beard P (2005) NIR in medicine – shedding light on the brain. NIR News 16: 28–30

Everall NJ, Chalmers JM, Newton ID (1992) In situ identification of thin-layer chromatography fractions by FT Raman spectrosc. Appl Spectrosc 46(4): 597–601

Fong A, Hieftje GM (1995) Near-infrared measurement of relative and absolute humidity through detection of water absorbed on a silica gel layer. Anal Chem 67: 1139–1146

Heise HM, Marbach R, Bittner A (1998) Clinical chemistry and near infrared spectroscopy: multicomponent assay for human plasma and its evaluation for the determination of blood substrates. J. Near Infrared Spectrosc 6: 361–374

Hochleitner EO, Bakry R, Huck CW, Flores F, Stöggl WM, Stecher G, Bonn GK (2003) Analysis of isolecteins on non-porous particles and monolithic polystyrene-divinylbenzene based sorbents and electrospray ionisation mass spectrometry. Int J Mass Spectrom 223–224: 519–526

Huck CW, Guggenbichler W, Bonn GK (2005) Analysis of caffeine, threobromine and theophylline in coffee by near infrared spectroscopy (NIRS) compared to high-performance liquid chromatography (HPLC) coupled to mass spectrometry. Anal Chim Acta 538: 195–203

Huck CW, Stecher G, Bakry R, Bonn GK (2003) Recent progress in high-performance capillary bioseperations. Electrophoresis 24: 3977–3997

István K, Keresztury G, Szép A (2003) Normal Raman and surface enhanced Raman spectroscopic experiments with thin layer chromatography spots of essential amino acids using different laser excitation sources. Spectrochim Acta Part A 59: 1709–1723

Jackson M, Mantsch HH (1996) FTIR spectroscopy in clinical sciences. Adv Spectrosc 25: 185–215

Murray I, Cowe AI (1991) Making light work: advances in near infrared spectroscopy. VCH, New York, pp 367–376

Norris HK, Hart JR (1996) Direct spectrophotometric determination of moisture content of grain and seeds. J Near Infrared Spectrosc 4: 23–30

Raghavachari R (2001) Near-infrared applications in biotechnology. Marcel Dekker, New York

Siesler HW, Ozaki Y, Kawata S, Heise HM (2002) Near-infrared spectroscopy principles, instruments, applications. Wiley-VCH, Weinheim

Souci SW, Fachmann W, Kraut H (2003) Lebensmitteltabelle für die Praxis. Wissenschaftliche Verlagsgesellschaft, Stuttgart

Stahlmann S, Kovar KA (1997) Analysis of impurities by high-performance thin-layer chromatography with Fourier transform infrared spectroscopy and UV absorbance detection in situ measurement: chlordiazepoxide in bulk powder and in tablets. J Chromatogr A 813: 145–152

Stark E, Luchter K (2005) NIR instrumentation technology. NIR News 16: 13–16

Stecher G, Huck CW, Stöggl WM, Bonn GK (2003) Phytoanalysis: a challenge in phytomics. TrAC, Trends Anal Chem 22: 1–14

Suyrewicz W, Mantsch HH (1996) Infrared absorption methods for examining protein structure. Spectroscopic methods for determining protein structure in solution. VCH, New York, pp 135–162

Tran CD, Kong X (2000) Near-Infrared spectrophotometric determination of tri- and tetrapeptides. Anal Biochem 286: 67–74

Troy A, Tran CD (2001) Near-infrared spectrometric determination of di- and tripeptides synthesized by a combinatorial solid-phase method. Anal Chem 73: 1062–1067

Wang Y, Murayama K, Myojo Y, Tsenkova R, Hayashi N, Ozaki Y (1998) Two dimensional fourier transform near infrared spectroscopy study of heat denaturation of ovalbumin in aquerous solutions. J Phys Chem 102: 6655–6662

Yamamoto H, Yoshikawa O, Nakatani M, Tsuji F, Maeda T (1991) Densitometry on thin layer chromatographic plates by fourier transform near infrared spectrometry. Appl Spectrosc 45: 1166–1170

Authors' address: Dr. Christian W. Huck, Institute of Analytical Chemistry and Radiochemistry, Leopold-Franzens University, Innrain 52a, 6020 Innsbruck, Austria,

Fax: +43 512 507 2965, E-mail: Christian.W.Huck@uibk.ac.at