Rapid Method for the Discrimination of Red Wine Cultivars Based on Mid-Infrared Spectroscopy of Phenolic Wine Extracts

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Mid-infrared spectroscopy and UV−vis spectroscopy combined with multivariate data analysis have been applied for the discrimination of Austrian red wines, including the cultivars Cabernet Sauvignon, Merlot, Pinot Noir, Blaufränkisch (Lemberger), St. Laurent, and Zweigelt. Both authentic wines and their phenolic extracts were investigated by attenuated total reflectance (ATR)−mid-infrared spectroscopy. Phenolic extracts were also investigated by UV−vis spectroscopy. The wine extracts were obtained by solid-phase extraction with C-18 columns and elution by methanol containing 0.01% hydrochloric acid. Hierarchical cluster analysis was performed with mid-infrared spectra of both wines and extracts, as well as with UV−vis spectra of the phenolic extracts. Data processing involved vector normalization and derivation of the spectra. Due to varying concentrations of main components including sugar and organic acids, satisfactory classification of untreated wines was not achieved. However, when using mid-infrared spectra of the phenolic extracts, almost complete discrimination of all cultivars investigated was achieved. The use of UV−vis spectroscopy for cultivar discrimination was found to be limited to the authentication of the Burgundy species Pinot Noir. In addition, soft independent modeling of class analogy was applied to the mid-infrared spectra of the extracts. It was possible to establish class models for five different wine cultivars and to classify test samples correctly.

Keywords: *Wine; polyphenols; anthocyanin; flavonoids; mid-infrared spectroscopy; classification; authentication; cluster analysis; UV−vis spectroscopy; solid-phase extraction; SIMCA; pattern recognition; hierarchical clustering*

INTRODUCTION

A rapid analytical method for the determination of the varietal origin of grapes and wines is of great interest to both the wine industry and the consumer. The individual phenolic fingerprint as reflected in the composition of anthocyanins and flavonoids (*1*), as well as procyanidins, hydroxycinnamic acids, and their derivatives is distinctive for any plant. Mainly the analysis of anthocyanins but also the analysis of flavonoids has therefore been used for distinguishing among grape varieties (*2*−*5*). Commonly, solid-phase extraction of grape skin extracts or wines followed by reversed phase HPLC-UV−vis methods is used to analyze this part of the phenolic fingerprint. Also, HPLC combined with mass spectrometry is gaining increased attention for investigating the anthocyanin and flavonoid composition (*6*, *7*). These chromatography-based methods provide essential information on the composition of wines and allow discrimination of cultivars, but they are time-consuming and cost-intensive. HPLC does enable resolution and accurate measurement of residual monomeric anthocyanins. However, as the wine ages more stable oligomeric and polymeric pigments are formed (*8*, *9*), causing a decrease in the concentration of the monomeric anthocyanins. This might influence the correct classification of mature wines based on anthocyanin analysis (*10*). Although the phenolic composition may be influenced by vinification, maturation, and aging, the differences in the overall phenolic fingerprints might still be characteristic for each cultivar. In earlier studies besides the anthocyanins also other phenolic components, in particular flavonoids, of various cultivars were investigated by liquid chromatography. The most outstanding differences in grape varieties were those occurring in the distribution of flavan-3-ol monomers. Here varieties were found in which the concentration of (+)-catechin is higher than that of (−)-epicatechin. The differences in composition observed can be related to the variety (*11*). In this context wines of the same cultivar but growing in different areas, as was the case in this study, might also vary in the concentration of the flavan-3-ols. In Pinot Noir remarkably high concentrations of (+)-catechin have been found in every region surveyed (*12*). It was found that there is a much higher concentration of catechins in Pinot Noir than in any other cultivar. Moreover, the ratio of (+)-catechin/(−)-epicatechin also differed among cultivars, with wines from Pinot Noir having the highest and those from Shiraz the lowest ratios. In the survey of McDonald et al. (*13*) free and conjugated myricetin and quercetin contents (flavonols) of red wines were studied. High levels up to 41.6 mg/L were found particularly in small berry cultivars, for example, Cabernet Sauvignon. The absence of acylated anthocyanins is a strong characteristic of the Burgundy family (Pinot Noir) and is well-known and widely described in the literature (*14*). In contrast to chromatography, spectroscopic techniques (UV, vis, IR), when applied to mixtures, are less selective. However, spectra contain information about the complete phenolic composition of the wines under investigation. Near-infrared

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(NIR) as well as mid-infrared (MIR) spectroscopic techniques combined with multivariate data analysis are very promising in this context. NIR spectroscopy has already found widespread application in quality control and process analysis in the food industry. Applications for the purpose of classification include coffee beans (*15*), wheat flour (*16*), soy sauce (*17*), and olive oil (*18*). However, due to the fact that NIR absorptions reflect overtones and combination bands of fundamental transitions, NIR spectra are much less distinct than MIR spectra. MIR absorption bands are generally well resolved and can be related to defined vibrational transitions. NIR-based applications dominate so far due to the ruggedness and ease of operation of NIR with respect to MIR spectrometers. However, significant improvements in the instrument design and auxiliary optics have made modern MIR spectrometers sufficiently robust for routine applications. In the case of liquids or pastes, MIR spectra are conveniently recorded using the attenuated total reflection (ATR) technique (*19*). Earlier studies show applications in, for example, coffee classification (*20*), fruit identification in purees (*21*), and identification of meat products (*22*), edible oils, and butters and margarines (*23*).

In the present paper UV−vis and MIR spectroscopy, combined with multivariate data analysis, was applied to wine analysis with the aim of discriminating among wines of different cultivars of *Vitis vinifera*. The new approach of this method was to avoid the time-consuming separation and analysis of single compounds and to take a significant spectrum of the whole phenolic fingerprint instead. To our best knowledge this has not been done with wines or wine extracts either in the UV−vis or in the mid-infrared region so far.

MATERIALS AND METHODS

Wine Samples. Thirty-eight red wine samples were collected from collaborating wineries in Lower Austria and Burgenland (Austria). Authentic wines from the grapes Cabernet Sauvignon (8), Blaufränkisch (6), Merlot (3), Pinot Noir (8), St. Laurent (7), and Zweigelt (6) were selected from the 1999 harvest. According to the wineries the wines were individually fermented and aged either in new oak or in stainless steel tanks.

Sample Cleanup by Solid-Phase Extraction (SPE). Bond Elute SPE cartridges containing 1000 mg of sorbent and 6 mL of total volume were obtained from Varian. Methanol (Fluka HPLC gradient grade), hydrochloric acid (Fluka), and distilled water were used as solvents. All wines were filtered before SPE with disposable syringe filters (0.45 μm pore size, Millipore). The cartridges were preconditioned successively with 10 mL of methanol and 10 mL of distilled water. Samples (3 mL) were diluted 1:5 with distilled water and loaded onto the columns. To remove the polar components of the wines, the cartridges were washed with 20 mL of water and dried by means of a vacuum pump (15−20 mbar). The extracts were obtained by eluting with six portions of 0.5 mL of acidic methanol (0.01% HCl). The completeness of elution was checked by measuring the infrared spectra of the eluates.

Data Acquisition. All spectra of the extracts and wines were collected using an FTIR spectrometer (Equinox IFS 55, Bruker), equipped with a horizontal ATR cell (Dura SamplIR, SensIR Technologies) and a highly sensitive narrow-band mercury cadmium telluride (MCT) detector. For the control of the spectrometer the software package OPUS 3.0/IR (Bruker) was used. All spectra were recorded at 4 cm^{-1} resolution from 4000 to 700 cm^{-1} (300 scans, apodization function: Blackman-Harris-3-term). Spectra of the clean and dry ATR crystal against air were used for the background. One microliter of methanolic extract or wine was transferred onto the ATR crystal and allowed to dry. Spectra were recorded after complete drying of the sample. All samples were measured twice, following the above-described method.

UV−vis spectra were recorded by means of a UV−vis diode array spectrometer (HP 8452A, Hewlett-Packard) equipped with an 18 μL quartz cuvette (Hellma) with 1 cm optical path length. Methanolic extracts were diluted such that the absorbances fitted into the linear range of the instrument and were measured against methanol.

Data Analysis. *Hierarchical Cluster Analysis.* All data processing was carried out using OPUS 3.0/IR. Data pretreatment by vector normalization of IR spectra was done in the range of 1640−950 cm^{-1} for the phenolic extracts and between 940 and 1760 cm^{-1} in case of the wine samples. In the case of UV−vis the spectral region 250−600 nm was normalized. No baseline correction was needed. For the data analysis normalized spectra and their first derivatives were used. Derivatives were calculated by using the Savitsky−Golay (*24*) algorithm using nine smoothing points. For the evaluation of the spectra a hierarchical cluster analysis was performed. The distance matrix was calculated using Euclidean distances. From the distance matrices the dendrograms were created using the Wards algorithm.

Soft Independent Modeling of Class Analogy (SIMCA). The PCA and SIMCA analyses were performed using the software packages Unscrambler (Camo ASA, Trondheim, Norway) and PLS_Toolbox (Eigenvector Research, Inc., Manson, WA) for Matlab (The Mathswork, Inc., Natick, MA).

Data treatment for SIMCA included calculation of the first-derivative spectra by applying a first-order Savitsky−Golay filter with nine smoothing points, restriction of the spectra to the wavenumber range 1640−950 cm^{-1}, and normalization.

For validation of the SIMCA classifications a "leave-a-third-out" cross-validation procedure was applied. Two-thirds of the samples were assigned to the training set and the other third of the samples to the test set. This assignment was repeated three times. For the classification into five different classes a "leave-one-out" procedure was used. One sample per class was taken out as a test sample in a randomized manner, and models for the five classes were calculated with the remaining training samples. This procedure was repeated eight times, so that each sample was classified at least once.

Models for the classes were calculated after individually mean-centering the training samples of the respective class. Then the models were applied to the test samples, the classification being based on the sample-to-model distances and the leverage values (sample's distance to the model center).

RESULTS AND DISCUSSION

MIR Spectroscopy and Cluster Analysis of Untreated Wines. In the spectra of untreated wines maximum absorbance values can be found in the region 900−1100 cm^{-1}, with a maximum at 1050 cm^{-1}. These bands can be assigned to C−O valence vibrations of the residual carbohydrates including mainly fructose and glucose in the wine. Additionally, bands corresponding to characteristic vibrations of organic acids also influence the spectra in the region between 1500 and 900 cm^{-1} (*25*). Both carbohydrates and organic acids contribute to some extent to the varietal characteristics, but mainly they reflect the individual style of the wine sought after by the wine-maker. Estimates of total phenolics in numerous red wines from many wine-growing regions have been from 0.8 to 4 g/L (*26*). Because the concentration of sugars and acids in wine is higher, in dry Austrian table wines typically 1−9 g/L of residual sugar and 4−7 g/L of titratable acid are present, they mask the characteristic phenolic composition of the wine. It can therefore be assumed that classification of the cultivars based on the whole wine spectra is difficult.

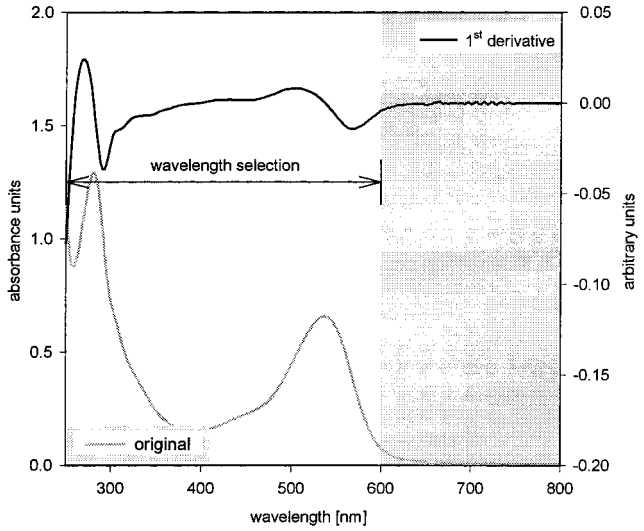

Figure 1. Example of a UV−vis spectrum of a phenolic extract and the corresponding first derivative.

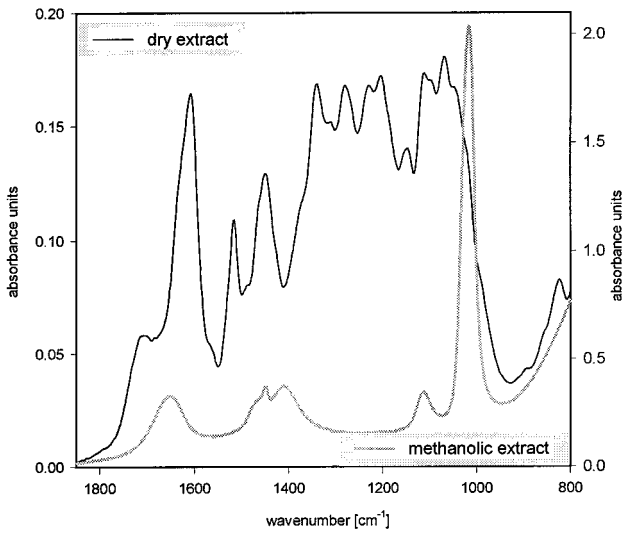

Figure 2. MIR spectra of a methanolic extract in comparison with the corresponding dry extract.

The result of the cluster analysis performed confirmed this conclusion. Some relationships between clustering and origin were found, but the result was not acceptable for quality control purposes. The use of derivatives also did not improve the classification. Therefore, we concluded that for successful classification, the variable main wine components including fructose, glucose, organic acids, and alcohols among others must be removed prior to analysis.

Optimization of Sample Cleanup by SPE. Sample cleanup was performed to remove the major components of wine including carbohydrates and organic acids. In the literature various sorbent materials were studied (*27*). Octadecyl silica sorbents are well described in the literature and proved to combine both excellent retention and elution characteristics for both monomeric and polymeric phenols in wines and musts; therefore, they are widely used for sample cleanup (*28*−*32*).

Different volumes (1−5 mL) of wine samples were loaded onto the columns. The maximum sample tolerated was 4 mL. As recommended in the literature (*28*, *31*) acidic methanol was used for the elution step. Less than 2 mL of eluent was not recommended in this case because of the high sorbent mass in the cartridges. Fractions of 0.5 mL were collected and evaluated by MIR spectroscopy. The last fraction did not contain any phenolic compounds. Therefore, the selected amount of eluent (3 mL) was sufficient to quantitatively elute all phenols from very concentrated and phenol-rich wines.

Characterization of Methanolic Extracts by UV—Vis Spectroscopy. UV−vis spectra contain valuable information on the identity of phenolic substances and are also used for identification purposes (*31*, *33*, *34*). To obtain a general overview on the composition of the extracts, UV−vis analysis was performed. A typical UV−vis spectrum of a wine extract and its first derivative is given in Figure 1. Two major bands with maxima at 538 and 280 nm and shoulders at 520 and 310 nm can be observed. The major anthocyanidins delphidin, malvidin, and petunin exhibit maxima at 538 nm, whereas cyanidin derivates have their absorption maxima at 523−525 nm (*31*). Flavonoids and procyanidins as well as the UV-absorbing parts of the anthocyanin structure show maxima in the 280 nm region (*33*, *35*). Compared to anthocyanin and other flavonoid spectra in the literature (*35*), where spectra of 175 flavonoids and their molar extinction coefficients are published, the intensity of the 280 nm band relative to the 538 nm band indicates remarkable amounts of noncolored phenolics. These findings are in agreement with earlier studies (*36*) in which along with a C-18 cleanup procedure as described above also flavonoids, procyaninidins, and hydroxycinnamates were extracted together with the anthocyanins.

Optimization of ATR Measurements. During the first experiments the methanolic extracts were measured in solution. For this purpose 50 μL of extract was transferred onto the ATR crystal. As can be seen in Figure 2, methanol dominated the spectra and therefore masked the spectral features of the phenolics. To remove the interfering absorbances of the solvent, the samples were allowed to dry on the ATR element. The sample volume was varied between 0.5 and 10 μL; 1 μL of sample provided a homogeneous film on the ATR element. Absorbance values between 0.2 and 0.5 were obtained. Due to different total phenolic contents in the samples, the amount of dry extract formed on the ATR surface varied. However, because the varietal differences in the phenolic composition of the cultivars studied are reflected in the relative intensities of the bands and are independent of the total phenolic content, vector normalization could be used to remove differences in the absolute intensities. The overall procedure, including the SPE step, drying of the extract, and spectra acquisition, was highly reproducible. The standard deviation of the replicates was <1%. This is demonstrated in Figure 3, in which the spectrum of a phenolic extract together with the standard deviation calculated from triplicate analysis is shown.

MIR Spectra of Phenolic Extracts. For illustration of the distinct pattern of the wine cultivars averaged spectra of each cultivar are given in Figure 4. Bands at around 1605, 1520, 1450, 1340, 1280, 1230, 1200, 1145, 1110, and 1060 cm^{-1} are found in all spectra with various relative intensities and minor, but varietal, characteristic shifting in absolute band positions. Thus, for each wine cultivar a unique phenolic fingerprint in the MIR spectral region could be obtained. Most remarkable are the differences at 1145 cm^{-1}, at which Pinot Noir exhibits the highest and St. Laurent the lowest band intensities. Also, the band intensity at 1340

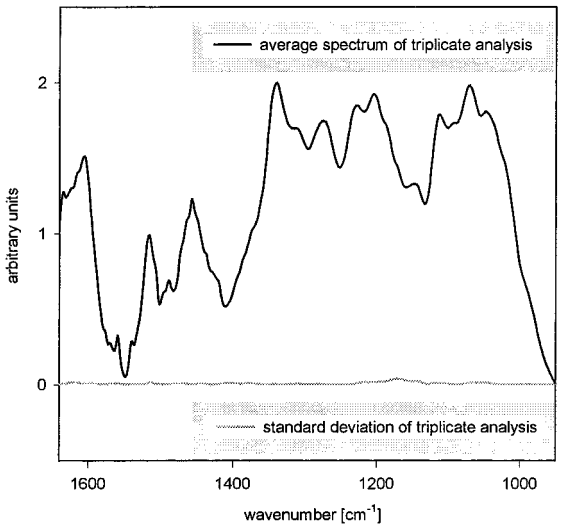

Figure 3. Averaged MIR spectrum compared with the standard deviation of triplicate analysis in the spectral range selected for data analysis.

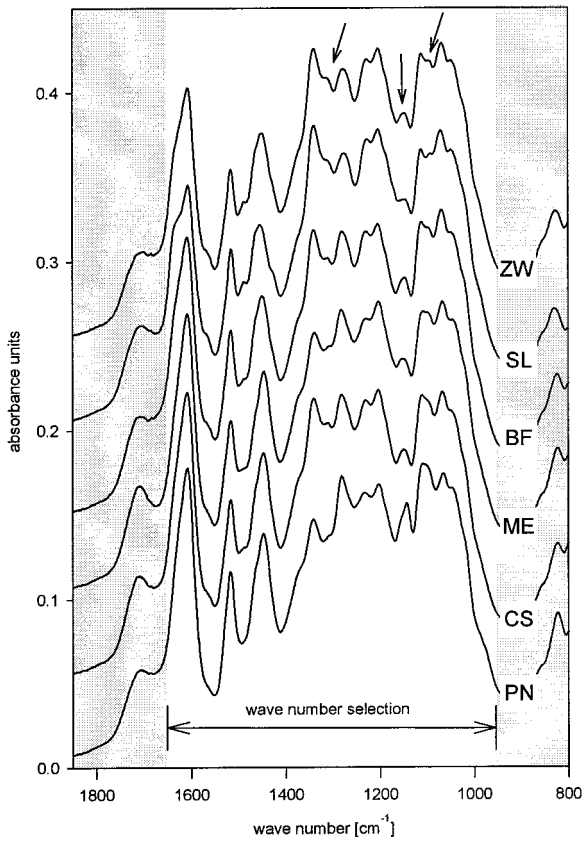

Figure 4. Averaged MIR spectra of phenolic extracts of the cultivars Blaufränkisch (BF), Cabernet Sauvignon (CS), Merlot (ME), Pinot Noir (PN), St. Laurent (SL), and Zweigelt (ZW).

cm^{-1} can be considered as characteristic for each cultivar. Wines are complex mixtures of various phenolic compounds, so a full assignment of the spectral bands is a challenging task and will not be attempted in this work. Generally in the region 1680−900 cm^{-1} numerous bands originating from wine phenols can be found. According to Hesse et al. (*37*) the bands at 1600 and 1520 cm^{-1} can be assigned to C=C bond vibrations, which are typical for aromatic systems. A strong contribution of OH deformation vibrations can be found in the region 1410−1260 cm^{-1}. Strong C−O valence vibrations between 1150 and 1040 cm^{-1} overlap with aromatic fingerprint bands at 1225−950 cm^{-1}. CH_3 symmetric deformation vibrations occur in the region 1190−1370 cm^{-1}.

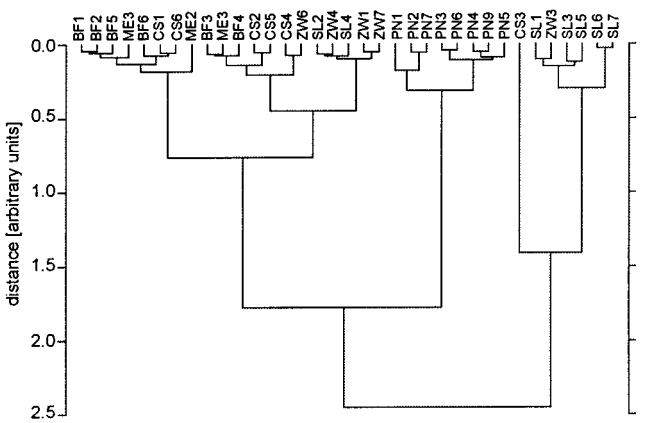

Figure 5. Dendrogram calculated from normalized first derivatives of UV−vis spectra collected from phenolic wine extracts.

Data Analysis. *Cluster Analysis of UV−Vis Spectra.* Identification of various anthocyanins (*31*, *35*), flavonoids (*35*), and procyanidins (*33*) has previously been achieved by UV−vis spectroscopy. We therefore also investigated the potential of UV−vis spectroscopy on the phenolic extracts for cultivar identification. For cluster analysis the wavelength region between 250 and 600 nm was selected. The clustering of vector-normalized UV−vis spectra resulted in three major groups. All Blaufränkisch can be found in the first group, St. Laurent mixed with Zweigelt in the second, and Pinot Noir in the third group (data not shown here). The cultivars Cabernet Sauvignon and Merlot were randomly distributed among these groups, whereas two Merlot samples were related to the Pinot Noir group. The first derivatives of the spectra were selected to successfully refine the method. As shown in Figure 5 the variety Pinot Noir was clearly separated. Five of seven St. Laurent wines formed a group with Zweigelt 3 (see also IR spectroscopy), whereas the others were mixed with the remaining Zweigelts. Wines of the varieties Blaufränkisch, Merlot, and Cabernet Sauvignon could not be separated. Although the separation seemed to be promising to some extent, UV−vis spectroscopy was not capable of clearly separating all investigated cultivars with the exception of Pinot Noir.

Cluster Analysis of MIR Spectra. For cluster analysis of the normalized absorbance spectra different wavelength regions were investigated. Using a spectral region from 1640 to 950 cm^{-1}, optimal results were obtained in terms of separation of the different cultivars (Figure 6). The cultivar Pinot Noir could be clearly distinguished. All St. Laurent were grouped together; however, this group also included most of the Zweigelts. Blaufränkisch were mixed with Cabernet Sauvignon, the remaining Zweigelts, and Merlot, although the cultivar Merlot formed a subgroup. To achieve separation of all cultivars, further data preprocessing was investigated. First derivatives of the normalized absorbance spectra were calculated and subjected to cluster analysis. The result of clustering first derivatives is illustrated in Figure 7. As can be seen, the separation of the varieties could be significantly improved, with all varieties being separated. To be able to compare the results of cluster analysis with SIMCA classification, a

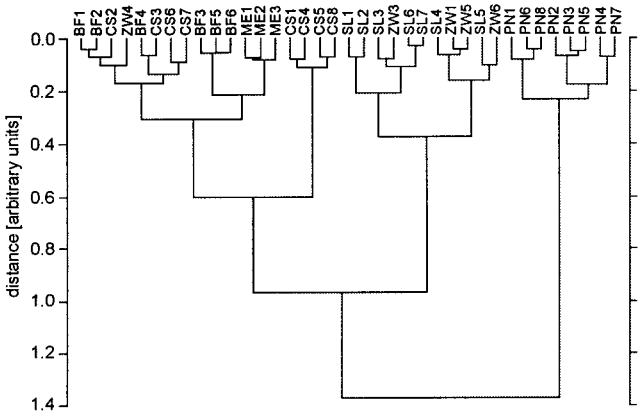

Figure 6. Dendrogram of the normalized MIR spectra obtained from the phenolic wine extracts.

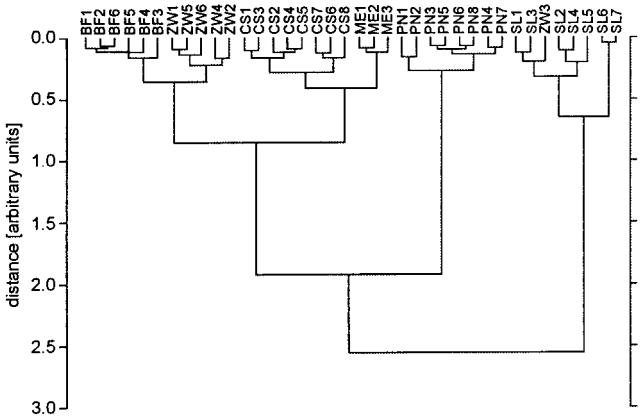

Figure 7. Dendrogram of normalized and derived (first derivative) MIR spectra of the phenolic wine extracts.

principal component analysis (PCA) was performed on the first-derivative spectra of the 38 samples, too, and hierarchical cluster analysis was performed on the obtained PC scores. The use of the first five (or more) PC scores gave the same results as clustering with the full spectra.

In particular, the cultivars Pinot Noir and St. Laurent could be clearly differentiated. However, one sample of Zweigelt was found in the group of St. Laurent. This same sample was also found in the St. Laurent group when cluster analysis based on the UV−vis spectra of the extracts was performed. In the 1920s Zweigelt was hybridized in Klosterneuburg, Austria, of St. Laurent and Blaufränkisch. This relationship may explain the clustering behavior of Blaufränkisch and Zweigelt. Although they could be separated, their similarity is reflected by the short distance of the two groups in the dendrogram. The Bordeaux cultivars Cabernet Sauvignon and Merlot could also be distinguished from each other. Their similarity to one another, in comparison to the other cultivars, can be seen from the distances in the dendrogram. Anthocyanin analysis of Cabernet Sauvignon and Merlot (*3*) showed similar anthocyanin patterns of the two varieties, and their flavonoid contents are also comparable (*12*).

SIMCA. Because of the very promising results of hierarchical clustering, SIMCA, a well-known method for supervised classification that is widely used in food analysis (*38*), was applied to the MIR spectra of the wine extracts. SIMCA (*39*, *40*) is based on PCA. Examples for application of SIMCA in food analysis are classification of olive oils (*41*), fruits (*42*), meat products (*22*), and wines (*43*). However, to our best knowledge SIMCA has not been applied to MIR spectra of wines or wine extracts.

From the clustering results and preliminary PCA on the whole data (data not shown) set, it was observed that the Burgundy cultivar Pinot Noir wines were most distinct from all other samples, followed by the St. Laurent wines, whereas Blaufränkisch and Zweigelt as well as Cabernet Sauvignon and Merlot were more similar to each other. Therefore, the SIMCA classification of the samples into Pinot Noirs and others was investigated first.

The eight Pinot Noir wines formed the Pinot Noir class, all other varieties (eight Cabernet Sauvignon, seven St. Laurent, six Blaufränkisch, five Zweigelt, and three Merlot) the non-Pinot Noir class. All in all, 37 samples were classified and 100% of the test samples were classified correctly. The numbers of PCs used for the class models were one (Pinot Noir) and three (non-Pinot Noir).

The next step was to find a class on their own for the St. Laurent wines, besides the class of the Pinot Noir and the class of the remaining samples. Again, a total number of 37 samples of the three different test sets were projected to the corresponding class models for Pinot Noir, St. Laurent, and the rest. The percentage of correct classification was 100% as in the case with only two classes; one PC each was necessary to model Pinot Noir and St. Laurent, and the other samples required three PCs for a satisfying model.

The last approach was to model a class for each variety of wine in the data set. The three Merlot wines were eliminated from the data set, as their number was not enough to model a class. The first PC was sufficient to model each class. From the 34 samples 33 were classified correctly and only 1 St. Laurent was misclassified as Zweigelt, which corresponds to a classification rate of 97%. There were also a few cases of double classification of samples. One St. Laurent was assigned to the Zweigelt class in addition to the correct St. Laurent class; three of the Blaufränkisch samples were also assigned to the Zweigelt class in addition to the Blaufränkisch class, but with the samples closer to the Blaufränkisch than to the Zweigelt model. This result shows that the models for Blaufränkisch and Zweigelt were overlapping to some extent. This finding is supported by the fact that Zweigelt and Blaufränkisch cultivars are close relatives (see above), which makes the correct classification of these two cultivars difficult. Whereas not all of the classifications were significant according to the SIMCA criterion within the 5% significance level, in all cases the attributed class was the one with the smallest sample-to-model distance.

Despite the small sample number, the promising results of these SIMCA classifications showed that it is possible to differentiate well among the five cultivars of the study, as confirmed by the validation procedures performed.

Conclusions. MIR spectroscopy and UV−vis spectroscopy combined with multivariate data analysis have been applied for the discrimination of Austrian red wines, including the cultivars Cabernet Sauvignon, Merlot, Pinot Noir, Blaufränkisch (Lemberger), St. Laurent, and Zweigelt. Subjecting the untreated wine samples to MIR analysis proved the need of an SPE step to remove spectral interferences from nonspecific carbohydrates and organic acids. Thus, an SPE step for

sample cleanup of polyphenols was used to obtain phenolic extracts of the wines. UV−vis spectra of the wine extracts allowed the identification of only the Burgundy cultivar Pinot Noir. However, with the use of MIR spectroscopy almost complete discrimination of all cultivars investigated was achieved, which demonstrates that the high information content of MIR spectra can be advantageously used for the fast classification of wine cultivars. Further research activities in this field will be undertaken to investigate more closely the inter- and intraclass variances on a bigger data set. However, on the basis of the results obtained so far, we conclude that MIR spectroscopy of the characteristic phenolic compounds of wines together with modern techniques of data analysis is a very powerful tool to gain information on the varietal origin of wines.

ABBREVIATIONS USED

SIMCA, soft independent modeling of class analogy; PCA, principal component analysis; PC, principal component; MIR, mid-infrared; NIR, near-infrared; UV−vis, ultraviolet−visible; ATR, attenuated total reflectance; SPE, solid-phase extraction; ZW, Zweigelt; BF, Blaufränkisch; PN, Pinot Noir; ME, Merlot; SL, St. Laurent; CS, Cabernet Sauvignon.

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