Rapid Determination of the Attenuation Limit of Beer Using Middle-Infrared (MIR) Spectroscopy and a Multivariate Model

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ABSTRACT: A new approach for the determination of the attenuation limit of beer samples using the specific fingerprint region of middle-infrared (MIR) spectroscopy in combination with multiple regression by partial least-squares (PLS) was developed using an attenuated total reflectance (ATR) module. A specific spectral region between 1200 and 800 cm^{-1} was identified as highly informative for the quantification of the limit of attenuation. The absorptions in this region are induced by vibrational bands of ethanol (1080, 1040, and 880 cm^{-1}) and dissolved extract, in majority maltotriose (1160−1140 and 1040−980 cm^{-1}). The multivariate calibration results in a root mean squared error of calibration (RMSEC) of 0.40% and a validation procedure with independent samples results in a root mean squared error of validation (RMSEV) of 0.50%. A repeatability test, concerning the precision of the developed MIR method as well as the reference method, was analyzed using Student's t test. The test has shown no significant difference between the two random samples.

KEYWORDS: MIR spectroscopy, attenuation limit, PLS, beer, quantification, multivariate calibration

■ INTRODUCTION

Application of Infrared Spectroscopy for Analyzing Foods and Beverages. In past years infrared spectroscopy has gained in importance throughout various scientific and application-oriented branches.[1,2] This scientific importance is based on the high information content of the infrared spectra and the diversity of nondestructive sample preparation techniques. Besides this, the technique of infrared spectroscopy is, compared to other analytical techniques, a very fast and efficient technology, which is important in routine analysis. In recent years, infrared spectroscopy has been widely and successfully applied for quality assurance in the food and beverage industry.[3] Throughout the food and beverage industry, several applications proclaim middle-infrared (MIR) spectroscopy as a powerful tool for qualitative (e.g., cognacs and other distilled drinks,[5] organic and nonorganic wines,[6] wines and brandies,[7] fruit used in jam[8]) as well as quantitative (e.g., sugars in mango juice,[4] sucrose in fruit juices,[9] organic acids and sugars in soft drinks,[10] free fatty acids in cheese[11]) purposes. The specific character of near-infrared (NIR) spectroscopy certainly yields to several quantitative applications in beer analysis (for alcohol and real extract concentration by the majority).[12−16] In a previous contribution we have reviewed investigations concerning the determination of quality parameters of beer using NIR spectroscopy.[17] Nowadays, NIR spectroscopy, due to its many practical advantages (possibility of using quartz cells, availability of light fibers, easy measurement in diffuse reflectance, etc.), is widely used as an analytical tool in the food and beverage industry. Compared to the NIR spectral range, in which molecular overtone and combination vibrations of molecules take place,[18] absorptions in MIR spectroscopy are due to the fundamental vibrations, especially in the so-called fingerprint region,[19] which is much more specific than the NIR spectral range. Several investigations concerning the comparison of NIR and MIR spectroscopy postulate MIR spectroscopy to be the spectroscopic technique with the higher power in quantitative as well as qualitative prediction.[20−24] Table 1 presents an overview of previously published investigations on the quantitative determination of beer constituents using MIR spectroscopy. The major advantage of IR spectroscopy, besides the fact that different quality parameters can be predicted by one and the same system, is the shorter time usually needed for the measurement compared to wet-chemical or chromatographic techniques. Titze et al.[27] present a comparison between a calibrated Fourier transform Infrared (FTIR) spectrometer and an automated beer analyzer in the determination of several quality parameters in beer. The authors summarized that the developed system is, compared to the traditional beer analyzer, easier to operate and considerably faster. Lachenmeier[28] postulates that FTIR spectroscopy offers considerable advantages, when measured against conventional methods of analysis, especially as an efficient high-throughput tool for screening alcoholic beverages (30 samples per hour).

Attenuation of Beer. Due to economical and technological reasons it is important for the brewer to know the amount of extract that can be fermented during the brewing process and how much extract was already fermented during fermentation and storage of the product, respectively.

The degree of fermentation or the attenuation A describes the amount of extract already fermented until the point of time and is defined as

$$A = \frac{E_0 - E_t}{E_0} \times 100\%$$

Table 1. Overview of Investigations Concerning Quality Parameters Using MIR Spectroscopy

	parameter	accuracy
Llario et al.[25]	real extract	RMSEP 0.10% w/w
	original extract	RMSEP 0.20% w/w
	alcohol	RMSEP 0.12% v/v
Inon et al.[26] [a]	real extract	RMSEP 0.10% w/w
	original extract	RMSEP 0.19% w/w
	alcohol	RMSEP 0.12% v/v
Titze et al.[27]	original wort	SECV 0.0473% w/w
	alcohol	SECV 0.027% v/v
	apparent extract	SECV 0.0206% w/w
	real extract	SECV 0.021% w/w
	specific gravity	SECV 0.0001 g/L
	apparent fermentation	SECV 0.4578%
	real fermentation	SECV 0.392%
	caloric value	SECV 0.6995 kJ/100 mL
	refraction index	SECV 0.1141
	pH value	SECV 0.0418
Lachenmeier[28] [b]	relative density	SEP 0.0006
	alcohol	SEP 0.21% v/v
	original gravity	SEP 0.44% w/w
	pH value	SEP 0.11
	lactic acid	SEP 78.52 mg/L
	bitterness unit	SEP 5.18
	EBC color	SEP 19.10
Gallignani[29]	ethanol	not specified

[a]Combined NIR and MIR spectroscopy. [b]Test set validation using full spectrum.

where E is the amount of extract given in weight percentage or degrees Plato, E_0 refers to the amount of extract at the starting product of the fermentation process ($t = 0$, original gravity), and E_t is the amount of extract still available (i.e., not yet fermented) after a certain time t of fermentation or storage, respectively. Depending on the time, three specific attenuations are of industrial importance: (a) cellar attenuation (A_c) is the attenuation determined in the fermentation cellar at the end of the main fermentation step; (b) final product attenuation (A_f) is the attenuation of the final product as delivered to the customer after fermentation and storage; and (c) attenuation limit (A_l) is a laboratory analysis and is determined in the final product to determine the degree of extract that can be fermented by the yeast at ideal conditions.[29] In the case of the reference method an enforced fermentation is performed to ferment all fermentable sugars. Only a small nonfermentable part of the extract (E_{nf}) is left in the beer sample. The attenuation limit is then calculated as

$$A_l = \frac{E_0 - E_{nf}}{E_0} \times 100\%$$

If in the final product the difference between A_f and A_l is too high, the residual fermentable extract can be utilized as carbon source by yeasts and other microorganisms, leading to haze and formation of undesired flavor components. Otherwise, the dissolved extract has a significant impact on the overall beer taste.[30] Therefore, the attenuation limit is an important quality criterion and is determined in the brewing industry as an everyday routine analysis according to the method of the

European Brewery Convention (EBC).[31,32] The EBC procedure is a time-consuming method (almost 24 h per sample) and has a restricted potential for automation. On this account, the aim of this investigation is to use MIR spectroscopy coupled with a multivariate model as a specific, fast, reliable, and robust method to determine the attenuation limit of beer. According to this and the aforementioned definitions, the combination of the MIR method has to be sensitive and selective to separate between (i) extract, already fermented during fermentation and storage; (ii) fermentable extract, not yet fermented during fermentation and storage; and (iii) nonfermentable extract (e.g., amino acids, nucleotides, proteinsm and polysaccharides). Besides the possibility of using this method as a routine laboratory analysis, in situ IR spectroscopy would enable an online measurement of the attenuation limit during the brewing process.

To the best of our knowledge, there are no previous investigations published using the MIR spectroscopy for the determination of the attenuation limit.

■ MATERIALS AND METHODS

Samples. The sample set, conducted for this investigation, consists of 50 samples of regular beer (pilsner and export beer) from different German breweries, from which 30 samples were used for model building (calibration set) and 10 samples were used for testing the obtained model (validation set). An additional 10 samples were used for testing the repeatability of the calibration. All samples in the validation and the calibration set were selected to cover the whole range of the reference value. An overview of the samples together with their reference values for the attenuation limit and some statistical parameters is given in Table 2.

Reference Analysis. The attenuation limits of the beer samples were determined according to the method of the EBC.[31,32] Approximately 300 mL of the sample is decarbonized, and the original gravity (E_0) is determined according to the Balling equation by measuring the real extract and alcohol content of the sample. The real extract and the alcohol concentration are usually determined either by

Table 2. Beer Samples (Calibration and Validation Set) Used in This Investigation Including Reference Values for the Attenuation Limit (A_l)

| calibration set				validation set	
no.	A_l (%)	no.	A_l (%)	no.	A_l (%)
1	82.25	16	80.57	31	80.12
2	85.14	17	82.80	32	86.18
3	84.54	18	83.29	33	83.58
4	84.57	19	83.48	34	81.01
5	84.51	20	80.13	35	83.11
6	82.95	21	84.57	36	86.03
7	82.25	22	86.29	37	79.98
8	79.76	23	85.20	38	82.76
9	84.45	24	83.45	39	82.30
10	81.48	25	79.32	40	83.19
11	78.74	26	83.46		
12	79.65	27	82.75		
13	80.53	28	83.16		
14	83.24	29	82.82		
15	86.11	30	81.47		
min	78.74			min	79.98
max	86.29			max	86.18
mean	82.76			mean	82.83
median	83.06			median	82.94
SD	2.04			SD	2.14
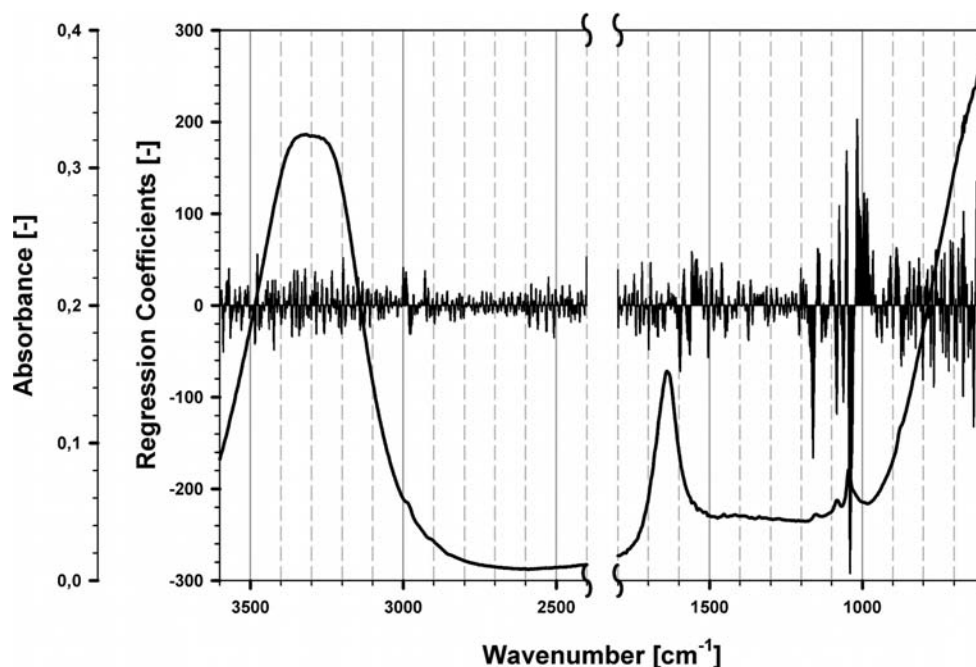
Figure 1. Regression coefficients of multivariate calibration with five PLS factors (bar chart) and MIR spectra of a beer sample (line chart) of spectral region between 3600 and 600 cm^{-1}.

automated analyzing systems (e.g., densitometer in combination with NIR spectroscopy) or by a distillation method. However, approximately 200 mL of the sample is afterward transferred into a 500 mL flask and 15 g of pressed brewers' yeast (*Saccharomyces* sp.) is added. For at least 24 h the sample is stirred at 20 °C before an aliquot of the sample is filtered and the specific gravity is measured. From this point the specific gravity is determined every 3 h to verify the fermentation is complete. If the fermentation is complete, the specific gravity will increase due to autolysis of the yeast resulting from carbon source deficiency. The lowest specific gravity is used to calculate the nonfermentable extract (E_{nf}) and the attenuation limit (A_l), respectively.

MIR Spectroscopy. A FTIR spectrometer (ALPHA, Bruker Optik GmbH, Ettlingen, Germany) coupled to a thermostatic (20 °C) attenuated total reflection (ATR) module (one reflection) was used for measuring the MIR spectra of the samples. The ATR module uses the physical principal of total internal reflection of electromagnetic waves at the interphase of the ATR crystal and the surrounding. This internal reflectance creates an evanescent wave that extends beyond the surface of the crystal and penetrates the sample at the surface of the crystal only 0.5–5 μm. The energy of each evanescent wave is absorbed according to the specific absorption of the molecules in the sample and passed back into the crystal, generating the specific infrared spectrum. All spectra were recorded in the spectral range of 4000–600 cm^{-1}, by accumulating 64 scans with a resolution of 4 cm^{-1} and air as background. For each sample, a total of three spectra were recorded (determination in triplicate). The acquisition of each averaged spectrum requires approximately 60 s.

Before measuring, the samples had to be decarbonized by shaking approximately 200 mL in a 500 mL Erlenmeyer flask. Afterward, the samples were filtered through a folded filter (Sartorius Stedim Biotech S.A., Aubagne Cedex, France). To avoid loss of alcohol, the funnel is covered with a Petri dish.[33]

All three recorded spectra of each sample were averaged (mean), converted to JCAMP-DX (Joint Committee on Atomic and Molecular Physical data — Data Exchange) format, and transferred to The Unscrambler software (version v9.1, CAMO Software AS, Oslo, Norway) for multivariate analysis.

Multivariate Calibration. The most common statistical methods for solving multivariate problems are (i) multiple linear regression (MLR); (ii) principal component regression (PCR); and (iii) partial

least-squares (PLS) regression.[34,35] In this investigation, a PLS analysis was used for solving the problem and fitting the model, respectively.

For the characterization of the obtained calibration models, various parameters are computable. In this work, the root mean squared error of calibration (RMSEC), the relative root mean squared error of calibration (RRMSEC), and the standard error of calibration (SEC) are characteristics to quantify the difference between the values predicted by a model and the values obtained by the reference method. To further compare the obtained calibration model against the reference, additional quality indicators, the coefficient of correlation (r_{xy}) and the systematic error (bias), are given.

Validation. To validate the obtained model, the root mean squared error of validation (RMSEV), the relative root mean squared error of validation (RRMSEV), and the standard error of validation (SEV) were calculated for a validation sample set, different from the calibration used for model building. Further information about validation procedures can be found in ref 36.

Statistics. In a regression model the residuals should be distributed normally and their mean should not deviate significantly from zero. This is checked statistically by a Shapiro–Wilk test and a Student's t test (one-sample test). Student's t test is also used in this investigation to analyze the repeatability (two-sample test) compared to the reference.

■ RESULTS AND DISCUSSION

Preprocessing of Spectral Data. The effect of spectral shift in NIR or MIR spectroscopy is a common problem in quantification and is discussed in several investigations.[17,15,25,37−41] Prior to every investigation, concerning spectroscopic calibration, a certain time has to be spent on the study of an adequate preprocessing technique, which fits the specification. This can only be achieved by an ite, on procedure of preprocessing the data and evaluating the results. In this investigation the linear baseline correction, the EMSC, the MSC, the SNV, and the first- and second-order derivations as well as the combination of the techniques were studied. The best results were obtained by the first-order derivation and the EMSC. According to this, the results presented in this investigation were obtained using the first-order derivation.
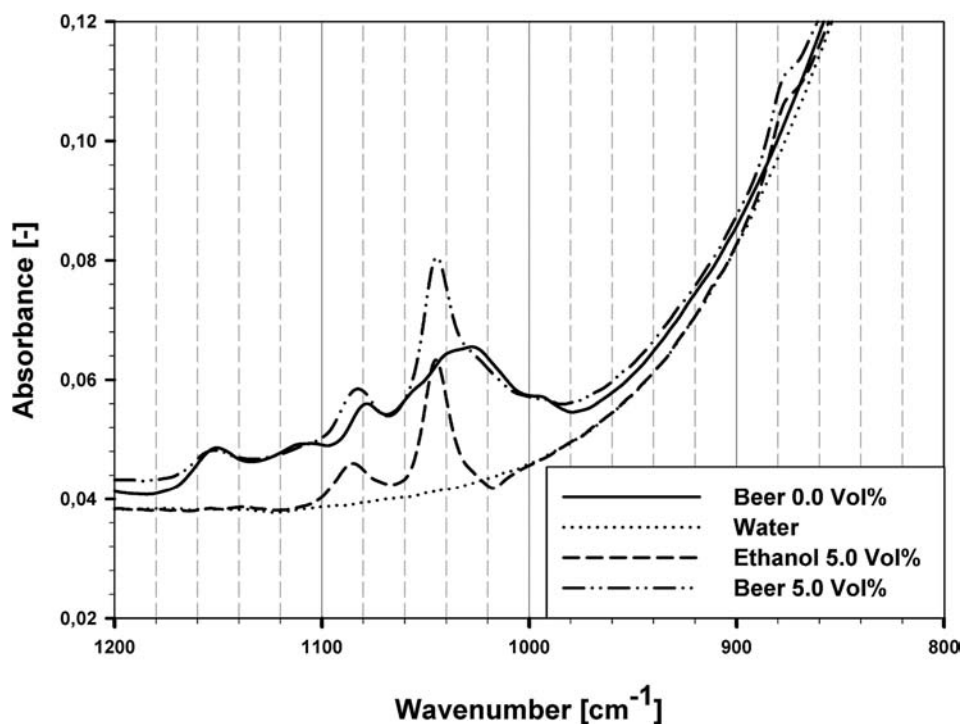
Figure 2. Comparison of MIR spectra (fingerprint region 1200 to 800 cm^{-1}) of beer, alcohol-free beer (beer 0.0 vol %), water, and aqueous ethanol solution (5 vol %).
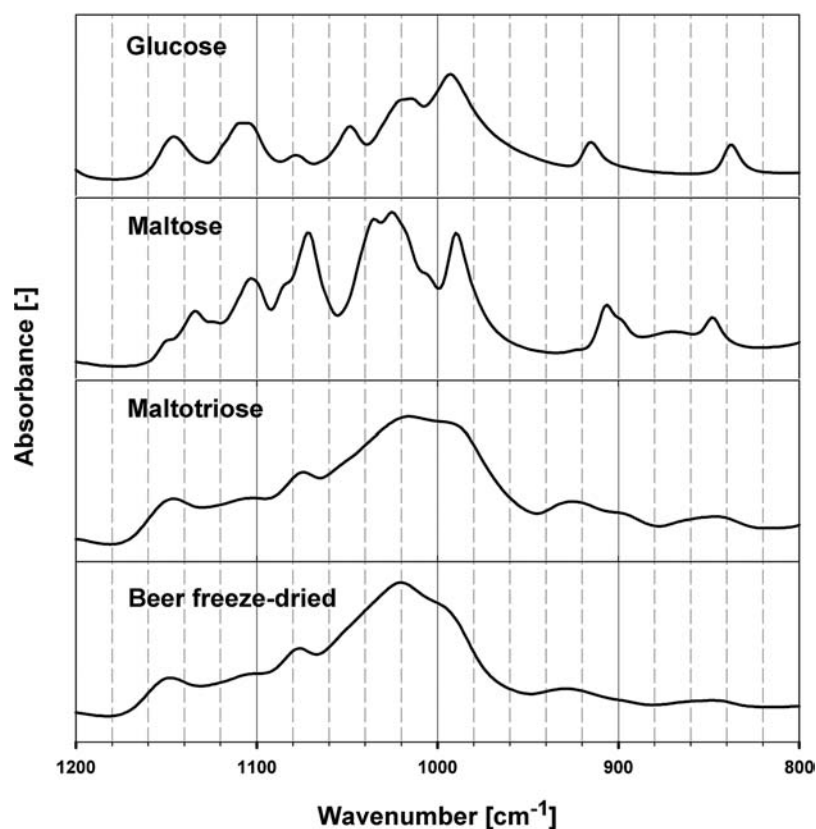

Figure 3. Comparison of MIR spectra of glucose, maltose, maltotriose, and freeze-dried beer in the spectral region between 1200 and 800 cm^{-1}.

Calibration. As a result of the multivariate calibration, the regression coefficients of the first five PLS factors according to the attenuation limit are presented in Figure 1. The regression coefficients summarize the influence of each variable (wave-number) on the calibration model, which means the higher the regression coefficient of a certain variable differs from zero, the higher is the influence of this variable on the multivariate model. Figure 1 also presents a MIR spectrum of a beer sample.

Atmospheric CO_2 and H_2O induce bands in the region of 2400–1800 cm^{-1}. This region is not presented in Figure 1 to avoid misinterpretation. However, according to the regression coefficients, the spectral region between 3600 and 1200 cm^{-1} has obviously, compared to the region between 1200 and 800 cm^{-1}, only a relatively low influence on the calibration. To eliminate nonchemical information in the spectral data and to enhance the performance of the calibration, only the region between 1200 and 800 cm^{-1} is used for further investigation.

Figure 2 presents the same MIR spectra as in Figure 1, but in a closer view on the spectral region between 1200 and 800 cm^{-1}. For comparison purposes, the MIR spectra of water, an alcohol-free beer sample, and an aqueous ethanol solution (5 vol % in water) are also presented in Figure 2. The MIR spectrum of the ethanol solution is very similar to the MIR spectrum of the beer sample, besides small differences in the regions between 1160 and 1140 cm^{-1} and between 1040 and 980 cm^{-1}. The two bands at 1080 and 1040 cm^{-1} are due to the stretching vibrations of C−O in the molecule of ethanol.[42] Another weak band located around 880 cm^{-1} corresponds to the stretching vibration of C−C−O of the ethanol molecule.[25,43,44] Especially this band enables a selective quantification of the ethanol concentration, because no band occurs at this wavenumber in the MIR spectra of the alcohol-free beer sample and the water sample. Inon et al.[26] located bands at 1100, 1050, and 875 cm^{-1}, which correspond to the presence of ethanol in different beer samples and can be used for quantification. According to Figures 1 and 2, the content of ethanol has a significant influence on the calibration. This can be explained by the fact that ethanol is metabolized during the fermentation of the extract and is therefore indirectly a measure of the attenuation. As mentioned above, the attenuation limit is defined as the extract, which can be fermented by the yeast at ideal conditions. To determine the attenuation limit of beer, the extract, not fermented during the brewing process, has to be taken into account, too. The bands between 1160 and 1140 cm^{-1} as well as between 1040 and 980 cm^{-1} in the MIR spectrum of regular beer are also present in the MIR spectrum of alcohol-free beer. Therefore, the above-mentioned spectral difference between the ethanol solution and beer in the regions of 1160−1140 and 1040−980 cm^{-1} are probably induced by extract dissolved in the beer. For a detailed view on the main components of the extract in the beer sample, which are responsible for the above-mentioned bands, different beer samples were freeze-dried to gently reduce the water and ethanol content.

Figure 3 presents the MIR spectra of glucose, maltose, maltotriose, and one representative freeze-dried beer sample. According to Figure 3 the MIR spectrum of the freeze-dried beer is very similar to the MIR spectrum of maltotriose, except for the band between 1700 and 1600 cm^{-1}. This band is probably induced by the OH deformation band of water remaining in the sample. However, maltotriose is obviously the main component of freeze-dried beer and therefore the major residual fermentable extract. Wort is mostly composed of the saccharides sucrose, glucose, fructose, maltose, and maltotriose. The brewers' yeast utilizes these saccharides in this approximate sequence, although some overlaps and metabolic repressions occur.[45,46] Gallignani et al.[12] reported that the matrix of alcohol-free beer samples has a NIR spectrum that basically corresponds to the NIR spectrum of maltose solutions. This is probably due to the fact that alcohol-free beer is produced either by vacuum distillation, vacuum evaporation, dialysis, and reverse osmosis, and control of mashing or by stopped fermentation.[47] Maltose is typically the most predominant saccharide in wort and, according to this, the predominant saccharide in alcohol-free beer brewed in a stopped fermentation. This might explain the results of Gallignani et al. However, the small difference between the ethanol spectrum and the beer spectrum in the region between 1160 and 1140 cm^{-1} as well as in the region between 1040 and 980 cm^{-1} in Figure 2 is, according to Figure 3, obviously in the majority induced by maltotriose. Nevertheless, some small differences in the spectra are present when the MIR spectra of freeze-dried beer and maltotriose are compared, in particular in the region between 1040 and 980 cm^{-1}. These differences might be explained by the presence of other fermentable substances and nonfermentable extract. Otter and Taylor presented investigations concerning the sugar composition of different beer samples.[48] In this study, maltotriose is in most of the beer samples the saccharide with the highest concentration, compared to the other saccharides in the samples. Similar results are presented in the study of Gjertsen[49] and in the study of Silbereisen and Bielig.[50] However, according to the results from the freeze-drying and the regression coefficients in Figure 1, the content of maltotriose and ethanol in beer has a significant influence on the determination of the attenuation limit of beer using MIR spectroscopy.

From the literature it is well-known that the elimination of spectral variables containing information not useable for calibration results in most cases in a more robust multivariate model than those models containing the whole spectral information.[25] Investigations, not presented here, have shown an increase of the precision up to 10% of a multivariate model based on the spectral region between 1200 and 860 cm^{-1}, compared to a multivariate model based on the whole spectral region. The selection of the most informative parameters was performed through iterative trial and error processes, taking the RMSEC, the SEC, and the coefficient of correlation as measures of performance into account. An overview of the performance of the obtained multivariate calibration is listed in Table 3. Five PLS

Table 3. Characteristics of the Multivariate Calibration and Validation Regarding the Attenuation Limit

	calibration	validation
n	30	10
RMSE	0.40	0.50
RRMSE	0.49%	0.58%
SE	0.41	0.48
r_{xy}	0.979	0.949
bias	<0.001	<0.001

factors are necessary to determine the attenuation limit with a RMSEC of 0.40%, a RRMSEC of 0.49%, and a SEC of 0.41. Figure 4 presents the prediction plot for this calibration and also for the validation. A PLS regression with more than five factors results in an overfit of the multivariate model. The coefficient of correlation (0.979) indicates a high linear regression between the reference method and the spectral data, in which, according to the bias (<0.001), no systematic error occurs. To study the residuals of the obtained multivariate calibration, different statistic techniques were applied on the residuals. A one-sample Student's t test was applied on the residuals to test if the mean of the residuals significantly deviates from zero (null hypothesis: $\mu = \mu_0 = 0$), and a Shapiro−Wilk test was applied to test if the residuals are normally distributed. The results of the Shapiro−Wilk test and the t test as well as the mean, the median, and the standard deviation of the residuals are presented in Table 4.
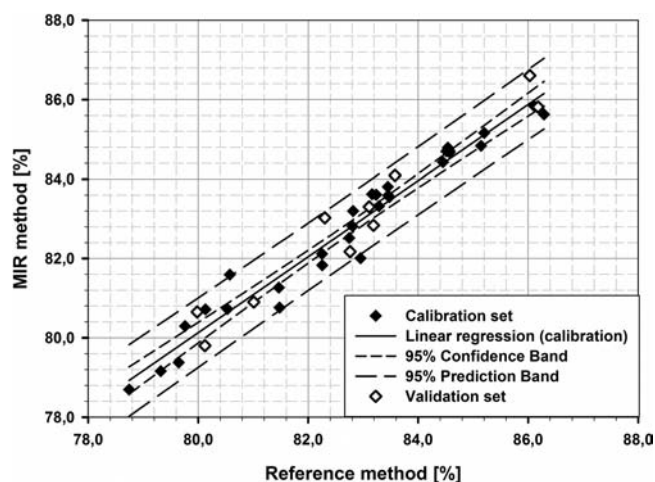
Figure 4. Predicted plot of the multivariate calibration of the attenuation limit (A_l) by MIR spectroscopy and a PLS model.

Table 4. Residual Analysis of the Multivariate Calibration and Validation Regarding the Attenuation Limit, Probability Value 95%

	calibration	validation
n	30	10
mean	>−0.001	0.095
median	−0.011	0.044
SD	0.41	0.50
$p_{\text{Shapiro−Wilk test}}$	0.95	0.16
$p_{\text{Student's } t \text{ test}}$	0.86	0.56

According to the results of the significant level ($p > 0.05$), the residuals are normally distributed and the mean of the residuals does not significantly differ from zero. The null hypotheses have to be accepted.

Validation. To test the obtained multivariate calibration with independent samples (samples not used for calibration), 10 samples (Table 1), ranging between 79.98 and 86.18% according to the attenuation limit, were measured. Table 3 presents the RMSEV, the RRMSEV, the SEV, and the coefficient of correlation as well as the results of the statistical analysis. According to the statistical analysis the residuals of the validation do not significantly deviate from a normal distribution, and the mean of the residuals does not significantly deviate from zero (see Table 4). The null hypotheses have to be accepted.

Repeatability. Table 5 presents the results of the repeatability analysis of the reference method and the MIR method concerning the mean, the median, and the standard deviation. Similar to the residual analysis, the results of the

Table 5. Repeatability Analysis of the Reference Method and the MIR Method Regarding the Attenuation Limit

	reference method	MIR method
n	9^a	10
mean	78.68	78.94
median	78.54	78.89
SD	0.30	0.57
$p_{\text{Shapiro−Wilk test}}$	0.05	0.27
$p_{F \text{ test}}$	0.09	
$p_{\text{Student's } t \text{ test}}$	0.25	

^{a}One sample was identified as an outlier according Grubbs' test.

repeatability study were also analyzed regarding the normal distribution (Shapiro−Wilk test) and the Student's t test (unpaired two-sample t test). Both random samples, reference method and MIR method, are normally distributed, and the homogeneity of the variances is given (calculated by Hartley's F_{max} test). On the basis of the results (Table 5), the null hypothesis $\mu = \mu_1 = \mu_2$ has to be accepted. This test gives a first impression about the repeatability of the method, but further investigations have to be performed to study the repeatability, for example, in interlaboratory tests.

In summary, a multivariate PLS calibration was used to identify spectral regions in MIR spectra of different beer samples to correlate the spectral data with the attenuation limit of the samples. Especially the spectral region between 1200 and 800 cm^{-1} has a high influence on model building. This so-called fingerprint region of the MIR spectra locates the stretching vibrations of C−O at 1080 and 1040 cm^{-1} [42] as well as the stretching vibration of C−C−O at 880 cm^{-1} [25,43,44] of ethanol. Furthermore, the spectral regions between 1160 and 1140 cm^{-1} as well as between 1040 and 980 cm^{-1} have been identified as the spectral region of dissolved extract, mostly maltotriose and nonfermentable extract. On this basis, a multivariate model was developed to determine the limit of attenuation of 30 different beer samples. A RMSEC, with respect to the reference method, of 0.40% was obtained. Ten independent samples were used for validation from which a RMSEV of 0.50% resulted. In both cases the residuals do not deviate significantly from a normal distribution and the means do not significantly deviate from zero. An additional sample set was used to determine the repeatability of the developed model and to compare the results with the repeatability of the reference method using Student's t test. No significant difference between the mean value of the reference and the developed MIR method ($\mu = \mu_1 = \mu_2$) was detected concerning the limit of attenuation. This investigation has proved the ability of MIR spectroscopy to be an applicable method to determine the limit of attenuation as a routine laboratory analysis in quality assurance. Compared to the many hours or even days that are needed for a determination of the attenuation limit using the conventional method, the MIR method enables the determination within about a minute. MIR spectroscopy in combination with multivariate models is used as an online measurement technique in various applications of the chemical, biotechnological, food, and pharmaceutical industries to improve the process. However, further investigations are needed to transfer the obtained MIR method from the laboratory to the process, to determine the limit of attenuation online during the brewing process, to enhance the process control of fermentation, and to achieve acceptance of this technique in the field of brewing.

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Notes
The authors declare no competing financial interest.

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