



## What is the best spectroscopic method for simultaneous analysis of organic acids and (poly)saccharides in biological matrices: Example of *Aloe vera* extracts?

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### ABSTRACT

Several species of (poly)saccharides and organic acids can be found often simultaneously in various biological matrices, e.g., fruits, plant materials, and biological fluids. The analysis of such matrices sometimes represents a challenging task. Using *Aloe vera* (*A. vera*) plant materials as an example, the performance of several spectroscopic methods (80 MHz benchtop NMR, NIR, ATR-FTIR and UV–vis) for the simultaneous analysis of quality parameters of this plant material was compared. The determined parameters include (poly)saccharides such as aloverose, fructose and glucose as well as organic acids (malic, lactic, citric, isocitric, acetic, fumaric, benzoic and sorbic acids). 500 MHz NMR and high-performance liquid chromatography (HPLC) were used as the reference methods.

UV–vis data can be used only for identification of added preservatives (benzoic and sorbic acids) and drying agent (maltodextrin) and semiquantitative analysis of malic acid. NIR and MIR spectroscopies combined with multivariate regression can deliver more informative overview of *A. vera* extracts being able to additionally quantify glucose, aloverose, citric, isocitric, malic, lactic acids and fructose. Low-field NMR measurements can be used for the quantification of aloverose, glucose, malic, lactic, acetic, and benzoic acids. The benchtop NMR method was successfully validated in terms of robustness, stability, precision, reproducibility and limit of detection (LOD) and quantification (LOQ), respectively.

All spectroscopic techniques are useful for the screening of (poly)saccharides and organic acids in plant extracts and should be applied according to its availability as well as information and confidence required for the specific analytical goal. Benchtop NMR spectroscopy seems to be the most feasible solution for quality control of *A. vera* products.

### 1. Introduction

(Poly)saccharides and organic acids are main compounds of various biological matrices, e.g., fruits, plants, food, and beverages, as well as biological fluids [1–3]. Simultaneous analysis of these classes of substances is important in quality control as well as to understand macro-parameters of biological samples such as pharmacological activity, sensory characteristics and health disorders [4–6].

Different instrumental methods are normally necessary to get a comprehensive overview of such matrices. Simultaneous analysis of organic acids is usually performed by conventional reverse-phase high performance liquid chromatography (RP-HPLC) with UV detection at 210 nm. Since the carboxyl group is a weak chromophore, detection is not very sensitive but sufficient for main acids [7,8]. Due to the lack of chromophore, analysis of monosaccharides requires specialized columns, derivatization and/or refractive index (RI) detection [9,10].

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Unfortunately, polysaccharide species cannot be detected using this procedure. Nowadays, nuclear magnetic resonance spectroscopy (NMR) provides almost an exclusive opportunity for holistic analysis of mono-, oligo and polysaccharides as well as organic acid in a single analytical run. Expensive high-field NMR equipment was successfully used for analysis of plant beverages, extracts, and biological fluids [11].

Increasing customer and regulatory requirements and cost-pressures nowadays force analytical laboratories to replace traditional expensive methods with faster and more economical ones with the ultimate aim to develop a methodology that could control most of the targeted analytes in a single assay. One possibility is the use of inexpensive spectroscopic techniques including low-field NMR, Near-Infrared (NIR), Attenuated Total Reflection - Fourier Transform Infrared (ATR-FTIR) and Ultra-violet-visible (UV-vis) spectroscopies. While being cheap, specialized multivariate techniques is usually required for modeling of conventional UV-vis, IR and NIR data due to overlap of matrix signals [12]. On the contrary, low-field NMR spectrometers based on permanent magnets are only recently entered the market [13]. The analytical potential of this techniques remains largely unexplored. Therefore, this study is aimed to compare traditional (UV-vis, IR, NIR) and new (low-field or so called benchtop NMR) inexpensive spectroscopic techniques in the context of simultaneous analysis of organic acids and (poly)saccharides.

Extracts from *A. vera* plant, which finds extensive biomedical applications, was used as a model biological matrix for such purpose [14, 15]. Additionally, our group is experience in analysis of this matrix by high-field NMR spectroscopy [16].

## 2. Experimental section

### 2.1. Samples and chemicals

In total, fifty-three powdered *A. vera* samples were investigated. The dried powdered samples were provided from Spectral Service (Cologne, Germany). Deuterated water of 99.8% purity containing 0.1% 3-(trimethylsilyl)-2,2,3,3-tetradeutero propanoic acid sodium salt (TSP) was obtained from Eurisotop (Saarbrücken, Germany). Sodium salt of ethylenediaminetetraacetic acid (EDTA) was purchased from AppliChem (Munich, Germany). As reference substances L(-)-malic acid ( $\geq 99\%$ ), citric acid (water free,  $> 99.5\%$ ), acetic acid (100%), sodium benzoate ( $> 99\%$ ), magnesium chloride hexahydrate ( $\geq 99\%$ ) and calcium chloride dihydrate ( $\geq 99\%$ ) from Roth (Karlsruhe, Germany) were utilized. L-(+)-Lactic acid (90% in H<sub>2</sub>O, TECHNICAL, VWR Chemicals, Darmstadt, Germany), succinic acid ( $> 99\%$ , Merck, Hohenbrunn, Germany), D/L-isocitric lactone (97%, Aldrich, Taufkirchen, Germany), D (+)-glucose monohydrate (97%, Diacleanshop, Dortmund, Germany), D-fructose (research grade, Serva, Heidelberg, Germany), and maltodextrin (Dextrin 10 from maize starch, Bio Chemika, New Anarkali, Pakistan) were used. Sorbic acid ( $> 99\%$ ) and nicotinamide (NSA, purity 99%) which is utilized as internal standard (IS) were both purchased from Thermos scientific (Kandel, Germany).

### 2.2. Sample preparation and measurements

#### 2.2.1. UV-vis

UV-vis measurements were carried out on a DR6000 UV-vis spectrometer (Hach, Loveland, USA) with 1 nm resolution. The layer thickness of the cuvette was 1 cm, the spectra were measured in the 190–400 nm range. For sample preparation, phosphate buffers were prepared at different pH: 2, 3, 3.5, 4.5, 5.5 and 7. The optimal results were obtained at pH=7. Approximately 2 mg of each sample or appropriate amount of reference compounds was dissolved in 4 ml phosphate buffer (pH = 7). UV-vis spectra were measured within two hours after preparation to avoid degradation.

#### 2.2.2. Vibrational spectroscopic techniques (NIR and ATR-FTIR)

ATR-FTIR spectra were recorded on Perkin Elmer Spectrum Three

spectrometer and the respective Spectrum software version 10.7.2 (Waltham, Massachusetts, USA). The dried powdered samples were placed with a spatula on the surface of the attenuated total reflection (ATR) unit. The spectra were measured at least twice with 16 scans from 4000 to 400 cm<sup>-1</sup> and a resolution of 4 cm<sup>-1</sup>.

NIR spectra were measured on a NIR spectrometer Vector 22/N (Bruker, Ettlingen, Germany). The spectral range of the instrument was 3700–12,500 nm and the resolution was 4 cm<sup>-1</sup>. 32 scans were recorded in diffuse reflection by placing a quartz vial with dried sample powder on the integration sphere of spectrometer. Each sample was measured in triplicate.

#### 2.2.3. NMR

Benchtop NMR measurements were performed on a Spinsolve 80 Carbon 80 MHz spectrometer equipped with automatic sample changer for twenty samples (Magritek GmbH, Aachen, Germany). In this study, NMR methodology was transferred from our recent studies on high-field NMR of *A. vera* products [16]. For sample preparation, 30 mg of a sample and 15 mg of internal standard NSA were dissolved in 600  $\mu$ l of Cs-EDTA buffer. Stock Cs-EDTA solution was prepared by weighting of approximately 2.9 g of EDTA and 6 g of Cs<sub>2</sub>CO<sub>3</sub> and dissolving them in 100 ml D<sub>2</sub>O. The pH was adjusted to 9.0. <sup>1</sup>H NMR spectra were recorded with an acquisition time of 3.2 s., repetition time of 10 s., 512 scans, time domain of 16 K and a pulse angle of 90°. All selected signals showed a T1 value between 0.4 and 2.0 s. The data were recorded automatically under the control of Spinsolve software 14.2.1 (Magritek GmbH, Aachen, Germany).

NMR spectra were manually processed using Mestrenova 14.2.3–29,241 (Mestrelab Research S.L., Santiago de Compostela, Spain). In particular, all spectra were referenced to trimethylsilylpropanoic acid (TSP) and then manual phase correction was first performed for the whole spectral region. Baseline was corrected for regions selected for quantification. Line broadening function and zero filling were set to 0.2 Hz and 32 K, respectively. Integration was performed using sum integration (NSA, glucose, aloverose, acetic acid, lactic acid) or using peak integration (malic acid, whole leaf marker (WLM), Mg-EDTA and Ca-EDTA).

Benchtop NMR method was additionally validated. For reproducibility, three representative *A. vera* extracts were prepared, measured, and analyzed five times within one day, on three days and by three operators independently. The samples for intraday precision were additionally measured after two, four and ten days to evaluate method stability. Between stability measurements, the samples were stored at room temperature.

To obtain realistic values of limit of detection (LOD) and quantification (LOQ), “in matrix” approach was used. For calculations, samples with the smallest possible contents of analytes were selected. The SNR was set as SNR = Y / noise, where Y is the amplitude of the peak, which was used for quantification, and noise is the root-mean-square deviation calculated in a signal free area of the spectrum ( $\delta$  –2–0 ppm in our case). The LOD/LOQ were calculated for aloverose (0.9%, SNR=24), malic acid (0.9 w/w%, SNR=25), acetic acid (0.03 w/w%, SNR=18), lactic acid (0.1 w/w%, SNR=7), glucose (1.1 w/w%, SNR=9.4), WLM (1.2 w/w%, SNR=), Ca (2.6 w/w%, SNR=37) and Mg (1.0 w/w%, SNR=30).

Reference NMR measurements were performed on a Bruker Avance III 500 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany) with BBO Prodigy cryo probe equipped with Bruker Automatic Sample Changer (B-ACS 120). Details about these measurements and quantification procedure can be found elsewhere [16].

#### 2.2.4. HPLC reference analysis

HPLC analysis was performed on a high-performance liquid chromatograph LC-2010A equipped with an autosampler and UV detector (Shimadzu GmbH, Duisburg, Germany). A special column (300  $\times$  8 mm) for organic acid analysis was used based on a polystyrene-divinylbenzene copolymer from CS-Chromatographie Service GmbH

(Langerwehe, Germany). The mobile phase consisted on deionized water and diluted phosphoric acid. For 1000 mL, ca. 6 mL of 25% phosphoric acid were added to deionized water for a pH of 2.1. *A. vera* extracts were prepared in 10 mL volumetric flasks in mobile phase to yield concentrations of 1.5 to 7.0 g L<sup>-1</sup>. 10 µL are then injected with a flow rate of 0.8 mL/min at a column temperature of 60 °C. Most analytes reached the detector within 15 min and were detected at 190 nm. The method was validated for the quantification of glucose, fructose, malic acid, citric acid, isocitric acid as whole leaf marker (WLM), lactic acid, acetic acid, succinic acid, sorbic acid, and sodium benzoate in *A. vera* extracts.

### 2.2.5. Preprocessing of spectroscopic data for multivariate modeling

First of all, the raw data were examined to select the most informative regions for each particular compound. Principal component analysis (PCA) was used to investigate outliers in the UV, NIR, and FTIR datasets. For vibrational spectroscopic methods, the transmittance was first converted into absorbance. Several preprocessing techniques were tested: raw data, mean-centering, first and second derivatives using Savitzky-Golay algorithm, standard normal variate (SNV), and multiplicative scatter correction (MSC). Moreover, the combination of above-mentioned routines was investigated. Multivariate modeling was also performed on raw data for comparison.

### 2.2.6. Multivariate regression of spectroscopic data

SAISIR package for MATLAB R2019B (The Math Works, Natick, MA, USA) was used for statistical calculations [17]. In this study, partial least squares regression (PLS) was used as a multivariate regression method [18]. Validation of the models was performed using splitting the data set into calibration (75%) and validation (25%) sets. The splitting was performed randomly 10 times. The results were investigated using box plots for graphical demonstration. The simplest models regarding the number of latent variables (LVs) with the minimum value of root-mean-square error of validation (RMSEV) were chosen. The results were evaluated based on coefficient of correlation (R<sup>2</sup>) for calibration and validation as well as root-mean-square-error of calibration (RMSEC) and validation (RMSEV).

## 3. Results and discussion

### 3.1. Reference analysis (HPLC and high-field NMR) of investigated samples

A total of fifty-three *A. vera* samples were analyzed using high-field NMR spectroscopy and HPLC. These two methods can be regarded complementary to each other as only fructose and aloverose (a D-mucopolysaccharid, also called acemannan) content can be quantified only using HPLC or high-field NMR, respectively. The results for the other investigated compounds (glucose, WLM, malic, citric, lactic and acetic acids) were comparable with each other ( $p < 0.0001$ ,  $R^2 = 0.99$ ) and, therefore, were averaged. Isocitric acid was quantified as WLM.

The quantitative reference results were summarized in Fig. 1, where for each compound the central mark is the median, the edges of the boxes correspond to the 25th and 75th percentiles, the whiskers extend to the most extreme data points not considered outliers. Regarding the distribution of (poly)saccharides, the data showed broad variations in aloverose (0.9–14.5%, median 10.4%), glucose (1.2 - 26.6%, median 18.6%) and fructose (2.7 - 10.4%, median 7.6%) contents. The median values for organic acids were found to be 22.5% (malic acid), 0.85% (citric acid), 0.60% (lactic acid) and 0.2% (acetic acid). Added preservatives, sodium benzoate and potassium sorbate, were observed in one sample with the content of 2% and 1%, respectively. The compounds related to the WLM were detected in 33 samples and the quantitative values expressed as isocitric acid varied between 0.2% and 23.3%. Fumaric acid - the product of enzymatic hydrolysis - was contained in trace amounts in a few samples.

Generally, three outliers were identified: two samples (S12 and S13) showed low aloverose and glucose, but high fructose contents. Moreover, three samples S12, S13, and S15 contained small amounts of malic acid (up to 3%) and glucose (up to 4%). These samples were excluded from reference data set for multivariate modeling of NIR and UV-vis data.

### 3.2. UV-vis spectroscopy

First, UV-vis spectra of all reference substances were recorded at different pH values. The spectra of the most reference compounds showed pronounced bathochromic shifts by increasing pH values. For example, glucose has its maxima at 192 nm and 198 nm at pH2 and pH7, respectively. Fig.2 showed the UV-vis spectra of all reference compounds at pH7. The spectra of acids (citric, malic, lactic, acetic, succinic

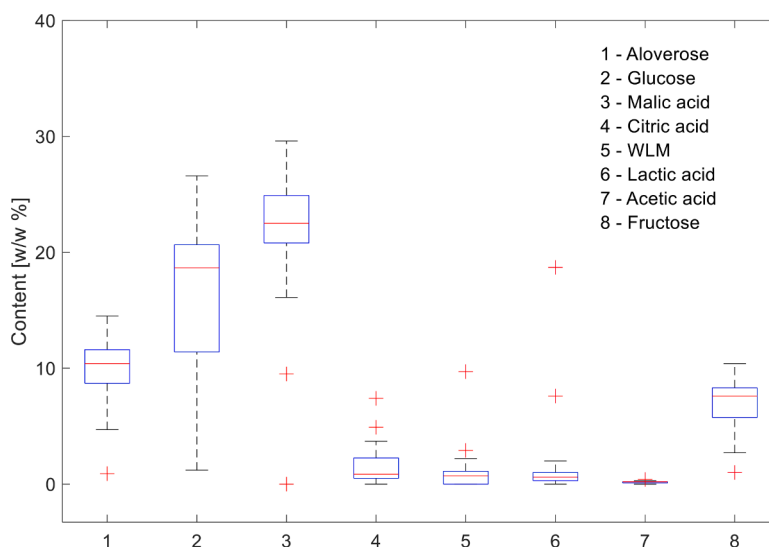
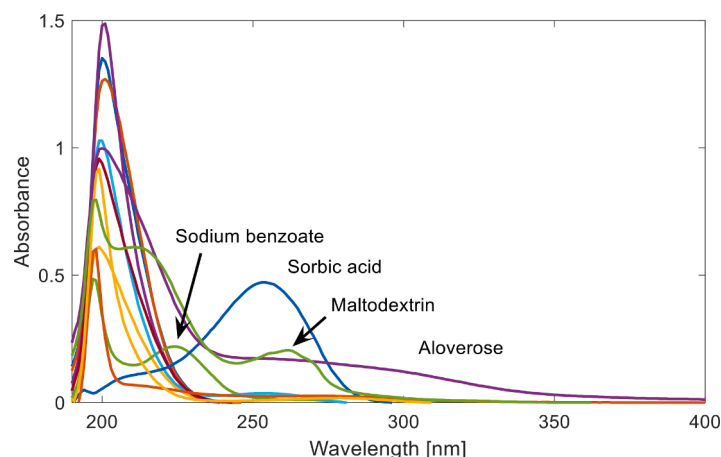


Fig. 1. Reference results (NMR and HPLC) for the investigated samples.



**Fig. 2.** UV–vis spectra of reference compounds at pH 7. The spectra of acids (citric, malic, lactic, acetic, succinic and isocitric) and monosaccharides (glucose and fructose) showed almost identical profiles between 190 and 220 nm.

and isocitric) and monosaccharides (glucose and fructose) showed almost identical profiles between 190 and 220 nm. Only preservatives (sorbic acid and sodium benzoate) and polysaccharides (maltodextrin and aloverose) showed specific UV–vis spectra.

The UV–vis spectra of all samples at pH=7 are plotted in Fig.3. As UV–vis a non-specific spectroscopic technique, it can be only used to qualitatively identify clear outliers in the dataset. The structure of particular compounds that were responsible for deviation can be elucidated using other spectroscopic techniques. In this study reference high-field NMR spectroscopy proved that samples S12 and S13 contained sorbic acid and sample S15 contained sodium benzoate (S15) (Fig.3). These samples also showed distinguishable organic acid and monosaccharide composition (see previous section).

Most of other samples showed absorption mainly between 190 nm and 220 nm mostly due to the presence of different acids. Because of highly overlapping UV–vis spectra quantitative determination of organic acids in this region is not possible. An attempt to use PLS regression to correlate UV–vis spectra with the data of reference analysis was failed. Satisfactory model ( $R^2$  for validation 0.80, RMSEV 2.8%) was obtained only for malic acid – predominant organic acid in *A. vera* matrix. As for sugars (fructose, glucose, aloverose and maltodextrin), their extinction coefficients in this region is much lower than for organic acids, therefore, their quantification was not possible using UV–vis spectroscopy.

UV–vis spectroscopy was previously utilized mainly for the

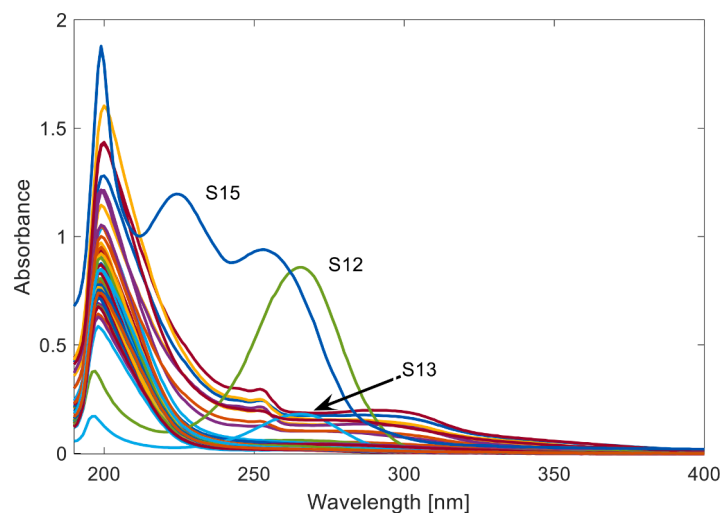
determination of antioxidant properties of the plant extracts such as bearberry leaves, hemp and hop extracts [19–21]. However, to achieve an excellent predictive ability fusion with chromatographic data was necessary [19,20]. In another study UV–vis spectroscopy was used for the characterization of extracts from *Justicia* (family *Acanthaceae*) plant leaves [22]. There is no study available for the simultaneous UV–vis determination of organic acids.

To conclude, although UV–vis spectroscopy is a time- and labor-efficient method, it can be only used for detection of preservatives (sorbic acid and sodium benzoate). For this reason, other spectroscopic methods – vibrational and low-field NMR spectroscopy – were tested for the simultaneous quantification of organic acids and sugars in *A. vera* samples.

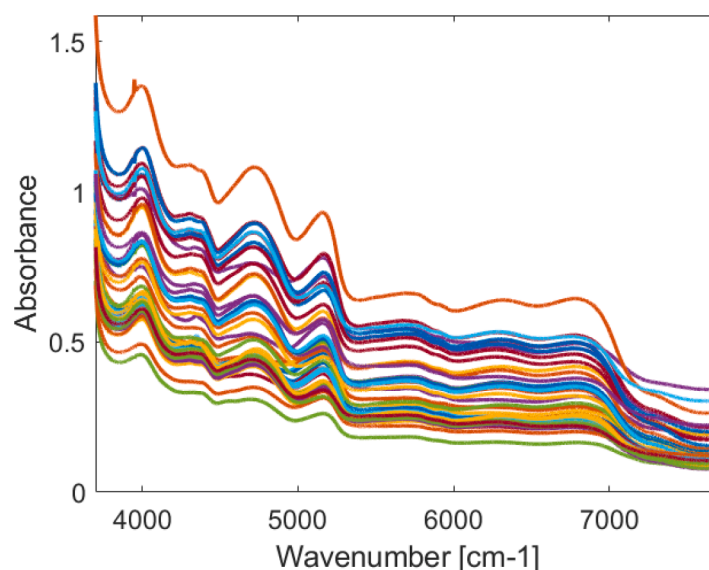
### 3.3. Near-infrared spectroscopy (NIR)

The first vibrational spectroscopic method, which was tested for multicomponent analysis of *A. vera* samples, was NIR spectroscopy. As for main ingredients, aloverose showed the maxima at 3988, 4717, 5151 and 6806  $\text{cm}^{-1}$ ; glucose at 3988, 4717, 5728, 6306  $\text{cm}^{-1}$ ; malic acid at 4312 and 4717  $\text{cm}^{-1}$ . Fig. 4 showed raw NIR spectra collected from investigated *A. vera* samples. As UV–vis spectra, NIR data display overlapping signals of targeted compounds, which makes quantification by univariate calibration impossible.

As the solution, multivariate regression (PLS) was used to correlate



**Fig. 3.** UV–vis spectra of investigated *A. vera* samples at pH 7. Three samples S12, S13, and S15 showed the presence of sorbic acid and/or sodium benzoate.



**Fig. 4.** Raw NIR spectra of investigated *A. vera* samples. The average of three measurements has been calculated for each spectrum. Exact assignment of each band to a specific functional group was not possible due to spectral overlap.

NIR data to the data of reference analysis. Parameters of the best-fitting PLS models are listed in Table 1. Quantitative information can be obtained for glucose, lactic and malic acids (values of  $R^2$  for validation were above 0.80). The correlation for citric acid and aloverose were also adequate for a screening procedure. Regression model for fructose ( $R^2$  for calibration 0.71) can be only used for rough screening. The calibration for WLM and acetic acid was difficult (correlation coefficient below 0.5) because of the low contents or complete absence of these compounds in most samples.

### 3.4. ATR-FTIR spectroscopy

The most prominent IR absorption bands in the FT-IR spectra of *A. vera* were assigned as hydroxy and carboxy groups (Fig. 5). The absorption for these groups in different compounds depends on the molecular structure and varies in position, shape, and intensity. Spectral overlap of main matrix compounds – malic acid, glucose and aloverose – makes it difficult to unambiguously assign substances to the absorption bands within a sample. Moreover, the absorption bands of the main components cover up the signals of minor components.

Different preprocessing techniques were tried for the PLS regression of *A. vera* spectral data. The best results were summarized in Table 2. While the main components had broad reference range, the distribution of the minor components is tinier. Aloverose model had a regression coefficient of 0.86. In case of glucose, the constructed model enabled a good prediction with averaged regression coefficient of 0.93 (Table 2). Among other compounds, the model for malic acid showed the best

correlation coefficients and RMSE values. Citric acid content can be well described with IR data. The model for lactic acid showed acceptable results with averaged regression coefficients of 0.97 and 0.88 for calibration and validation, respectively.

As for other compounds, fructose cannot be predicted due to its small absorption. Even though the regression coefficients for WLM were good ( $> 0.82$  and  $> 0.63$ ), extracts with no WLM were predicted to have contents of 0.5–2.0%. The absorption of acetic acid is one of the highest, but the actual contents in the investigated samples were too small to get linear dependence. To conclude, aloverose, glucose, malic acid, citric acid and lactic acid content can be determined using IR spectroscopy in combination with PLS regression.

Similar to UV–vis spectroscopy, ATR-FTIR spectroscopy was previously mainly used to determine phenolic compounds and flavonoids in plants such as olive leaves extracts and in extracts of medicinal plants [23,24]. Moreover, NIR and FTIR spectroscopy were used for analysis of terpenes in Yaobitong capsules and crude soapnuts extracts, respectively [25,26]. Artificial neural networks (ANN) models were used for prediction of total dissolved solids, polyphenol content and antioxidant capacity of dried root vegetable extracts in relation to the recorded NIR spectra [27]. The errors below 6% were established for the determination of hyperforin ((1*R*,5*S*,6*R*,7*S*)-4-Hydroxy-6-methyl-1,3,7-tris(3-methyl-but-2-enyl)-6-(4-methylpent-3-enyl)-5-(2-methylpropanoyl)-bicyclo [3.3.1]non-3-en-2,9-dion) and I3,II8-biapigenin (5,5',7,7'-tetrahydroxy-2,2'-bis-(4-hydroxyphenyl)-[3,8'-bi-4*H*-1-benzopyran]-4,4'-dion) in St. John's wort extracts based on NIR data [28]. In the contexts of

**Table 1**

PLS modeling of NIR data. The results are median values for 10 random splitting into calibration (75%) and validation (25%) sets.

Compound	Median [%]	Reference range [%]	Preprocessing of raw data	Number of LVs <sup>c</sup>	$R^b$		RMSE [%]	RMSEC <sup>d</sup>	RMSEV <sup>d</sup>
					Calibration	Validation			
Aloverose	10.5	4.7–14.5	S-G <sup>a</sup>	10	0.94	0.66	0.56	1.45	
Glucose	19.5	1.2–26.6	SNV <sup>b</sup>	5	0.85	0.80	2.3	2.8	
Malic acid	22.6	0–32.4	S-G	8	0.91	0.86	1.5	2.4	
Fructose	7.5	0–9.2	SNV	6	0.71	0.52	1.38	2.03	
Lactic acid	0.6	0.–0.4	S-G	10	0.98	0.98	0.55	0.64	
Citric acid	0.8	0.–23.3	S-G	9	0.92	0.72	0.50	1.23	

<sup>a</sup> S-G: the first derivative using a polynomial of power 1 as model and a window size of 9 - 15 was computed;

<sup>b</sup> SNV -Standard normal variate;

<sup>c</sup> LV – latent variable;

<sup>d</sup> RMSEC and RMSEV – root mean square of calibration and validation, respectively.

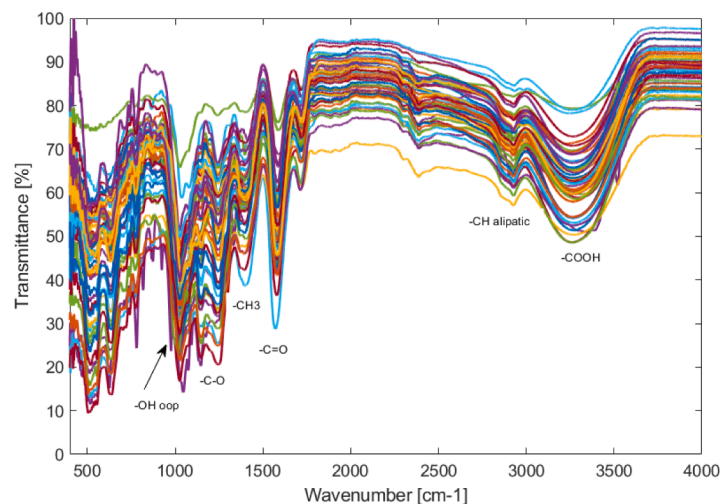


Fig. 5. Raw ATR-FTIR spectra of investigated *A. vera* samples with the assignments of some bands.

Table 2

PLS modeling of FTIR data. The results are median values for 10 random splitting into calibration (75%) and validation (25%) sets.

Compound	Median [%]	Reference range [%]	Preprocessing of raw data	Number of LVs <sup>b</sup>	R <sup>b</sup>		RMSE [%]	RMSEC <sup>c</sup>	RMSEV <sup>c</sup>
					Calibration	Validation			
Aloverse	10.5	4.7–14.5	MSC <sup>a</sup>	9	0.86	0.40	1.1	3.5	
Glucose	19.5	1.2–26.6		8	0.93	0.57	1.8	5.4	
Malic acid	22.6	0–32.4		11	0.97	0.84	1.3	2.6	
Fructose	7.5	0–9.2	Centering	3	0.62	0.48	1.7	2.1	
Citric acid	0.8	0.–23.3		9	0.85	0.54	0.9	1.9	
WLM	0.9	0–18.7		8	0.89	0.62	1.6	3.1	
Lactic acid	0.6	0.–0.4		7	0.88	0.79	1.3	2.1	

<sup>a</sup> MSC – multiple scatter correction;

<sup>b</sup> LV – latent variable;

<sup>c</sup> RMSEC and RMSEV – root mean square of calibration and validation, respectively.

polysaccharide analysis, ATR-FTIR spectroscopy was able to differentiate cell wall polysaccharides (CWPs) according to the degree of methylation [29]. Simultaneous analysis of organic acids in wines, grape berries and Ginkgo biloba leaf extracts by vibrational spectroscopy was described in [30–32]. Again, simultaneous analysis of organic acids and sugars was not described before.

Our results suggested that vibrational spectroscopic techniques clearly provided more qualitative and quantitative information about

*A. vera* extracts than UV–vis. Specifically, apart from malic acid, the contents (at least semi-quantitative) of glucose, aloverse, lactic, malic and citric acids, as well as WLM can be obtained using PLS regression. However, the parameters of multivariate models (R<sup>2</sup> and RMSE) were still not satisfactory. As the next spectroscopic technique, benchtop NMR at 80 MHz was tested for the same sample set.

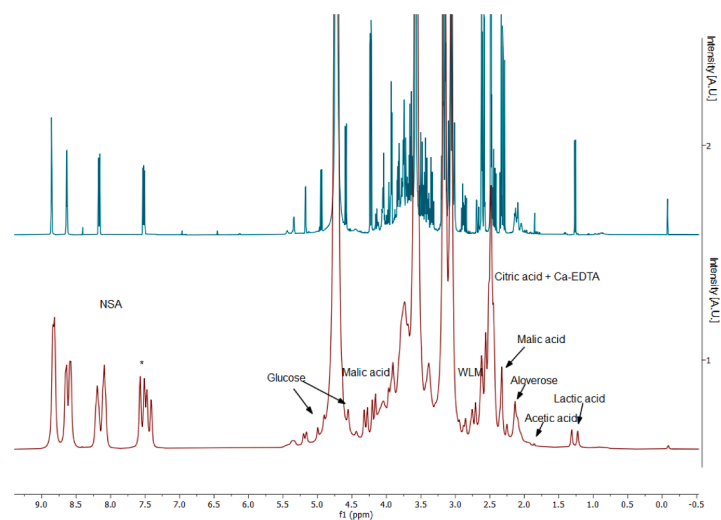


Fig. 6. <sup>1</sup>H NMR spectra of an *A. vera* sample recorded at 80 MHz (lower plot) and 500 MHz (upper plot) NMR spectrometers. The NSA signal used for integration is marked with asterisks.

### 3.5. 80 MHz benchtop NMR study of *A. vera* extracts

High-field NMR already provided an excellent opportunity for automated holistic control of authentic *A. vera* products including isocitric acid as the main whole leaf marker (WLM) compound, carbohydrate profile, microbial and enzymatic degradation markers and inorganic cations [16]. However, there were no studies, which investigated applicability of cheaper benchtop NMR spectroscopy for analysis of this matrix.

NMR spectra of a representative *A. vera* sample measured at the various spectrometers are shown in Fig. 6. Significant differences in resolution can be seen among the low-field and high-field devices. The lines of the high-field devices are narrower and show a better resolution of the overlapping signals. On both spectrometers, however, the NMR resonances of glucose (at  $\delta$  5.23 ppm), malic acid (at  $\delta$  4.30 ppm), the sum of acetylated polysaccharides (at  $\delta$  2.10 ppm), lactic acid (at  $\delta$  1.44 ppm), and acetic acid (at  $\delta$  1.90 ppm) were well separated and these compounds can be directly quantified after proper baseline correction (Fig. 6) [16].

The decrease in resolution is particularly apparent in the region between  $\delta$  2.0 and 3.0 ppm, where special data treatment to resolve the signals of citric acid, malic acid, and Ca(Mg)-EDTA complexes was required even for high-field NMR data [16]. Assignment of signals in this crowded range was also not trivial for 80 MHz NMR device (Fig. 7). First, Mg-EDTA complex can always be identified and quantified. For quantification of Ca-EDTA complex, which partially overlaps with malic acid, the sum of three peaks (at  $\delta$  2.60, 2.56, 2.53 ppm) was integrated. qNMR results for glucose, aloverose, acetic acid, malic acid, lactic acid, magnesium, and calcium on benchtop NMR device were comparable with the reference data (maximum deviation of 10% was observed). For lactic acid, deviations up to 20% were observed for the samples with low content of this compound.

The greatest difficulties were encountered in the quantification of isocitric acid (WLM) and citric acid. In contrast to the high-field spectra, WLM (at  $\delta$  2.90 ppm) could be detected but not quantified in most samples due to strong overlap at 80 MHz. Citric acid as well as succinic acid could not be reliably quantified due to overlap with malic acid (Fig. 7). The same applied for fructose which overlays with matrix signals between 3.0 and 4.0 ppm.

Despite these difficulties, benchtop NMR method was successfully validated for aloverose, glucose, malic acid, lactic acid, acetic acid, magnesium, and calcium. The repeatability of sample measurements achieved standard deviations of < 0.8% for intraday precision, < 0.6%

for interday and < 1.0% for operator precision (Table 3). As expected, the lower the content of a substance, the higher is the relative standard deviation. Stability measurements showed that in EDTA buffer, the samples were stable only one day due to deacetylation of aloverose.

The limit of detection (LOD) and limit of quantification (LOQ) were defined as the concentration at which the SNR exceeds 3 for LOD and 10 for LOQ in the matrix. The LOD and LOQ were 1.0% and 1.5% for both aloverose and malic acid, respectively. For glucose, a SNR of 10 was reached at 1.2% and it was detectable at 1.0%. Acetic and lactic acids can be quantified starting from 0.1%. These were higher than those obtained by routine high-field NMR run (LOQ for aloverose were calculated as 0.01%, for glucose and malic acid as 0.005% and for lactic and acetic acid as 0.0005% [32]), but still sufficient quantify these compounds in authentic samples. The LOQs for magnesium and calcium were 0.1% and 0.7%, respectively. Sorbic acid and maltodextrin were also detectable in *A. vera* matrix.

### 3.6. Comparison of spectroscopic techniques for analysis of *A. vera* extracts

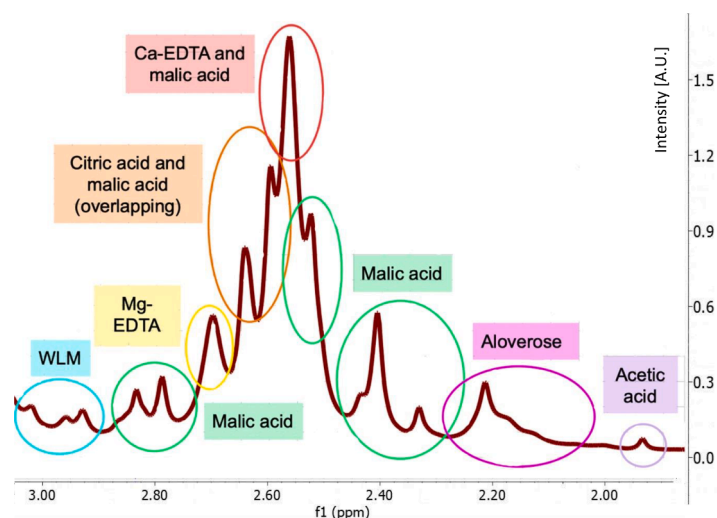
To compare the performance of each spectroscopic technique, the values of regression coefficients for each spectroscopic technique used for analyzing Aloe Vera plant extract were summarized in Table 4. From vibrational techniques used, FTIR showed better  $R^2$  values for glucose, malic acid and WLM. On the contrary, NIR results outperformed FTIR in cases of Aloverose, lactic and citric acid. Both techniques could provide semi-quantitative results for main *A. vera* ingredients.

**Table 3**

Precision results of three *A. vera* extracts prepared and analyzed five times within one day, three days and by three operators independently (benchtop NMR data).

Substance	Intraday repeatability		Interday repeatability		Operator repeatability	
	Std [%]	Rstd [%]	Std [%]	Rstd [%]	Std [%]	Rstd [%]
Glucose	0.31	1.8	0.21	1.2	0.24	1.4
Malic acid	0.79	3.6	0.56	2.5	0.89	4.0
Magnesium	0.02	6.0	0.04	12.5	0.03	6.3
Calcium	0.13	8.4	0.04	2.9	0.11	6.6
Aloverose	0.19	2.7	0.14	1.8	0.30	2.8
Acetic acid	0.01	5.2	0.03	28.8	0.01	5.7
Lactic acid	0.02	5.8	0.02	4.6	0.02	5.6

Std – standard deviation, Rstd – relative standard deviation.



**Fig. 7.**  $^1\text{H}$  NMR spectrum of a representative *A. vera* sample in the range of  $\delta$  2–3 ppm measured on 80 MHz spectrometer. Y-axis represents intensity in arbitrary units.

Table 4

Regression coefficients ( $R^2$ ) for each spectroscopic technique used for analysed A. Vera plant extract.

Compound	UV <sup>b</sup>	NIR <sup>b</sup>	FTIR <sup>b</sup>	80 MHz NMR <sup>c</sup>
Aloverose	- <sup>a</sup>	0.94	0.86	0.98
Glucose	- <sup>a</sup>	0.85	0.93	0.95
Malic acid	0.80	0.91	0.97	0.96
Lactic acid	- <sup>a</sup>	0.98	0.88	1.05
Citric acid	- <sup>a</sup>	0.92	0.85	- <sup>d</sup>
Acetic acid	- <sup>a</sup>	- <sup>a</sup>	- <sup>a</sup>	0.83
WLM	- <sup>a</sup>	- <sup>a</sup>	0.89	- <sup>d</sup>

<sup>a</sup> no adequate model was constructed.

<sup>b</sup> based on PLS regression.

<sup>c</sup> based on the univariate qNMR approach.

<sup>d</sup> univariate quantification was not possible due to spectral overlap.

On the contrary, low-field NMR spectroscopy provided satisfactory quantitative results. The results from the Benchtop NMR were compared with the data from high-resolution NMR for aloverose, glucose, malic acid, lactic acid, acetic acid, sodium benzoate, magnesium, and calcium. The regression coefficients based on this comparison in all cases outperformed NIR and FTIR (Table 4). The only drawback of benchtop NMR method in comparison with FTIR is impossibility of WLM quantification, which are of importance for the quality of *A. vera* extracts. Multivariate methods are required for quantification of this compound due to signal overlap.

Thus, in comparison to other spectroscopic techniques, low-field NMR is applicable for direct quantification of more organic compounds than UV-vis and IR/NIR spectroscopy (Table 4). Moreover, direct quantification of calcium and magnesium is possible. Having a larger sample set, PLS models could be additionally constructed to quantify WLM and citric acid in *A. vera* extracts, which is part of our ongoing studies.

#### 4. Conclusions

The results demonstrated that spectroscopic techniques can ensure cheap analytical control of authentic *A. vera* products including WLM compounds, carbohydrate profile, microbial and enzymatic degradation markers and two important inorganic cations ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) with the goal to certify commercial products regarding their quality and authenticity. From inexpensive spectroscopic techniques, benchtop NMR spectroscopy seems to be the most feasible solution to control both organic acids and (poly)saccharides in one analytical run. NMR is also advantageous in comparison with IR/NIR/UV-vis since the spectra are more specific and qNMR approach can be used for quantification.

In the past, the strongest argument against application of NMR in plant analysis was the (running) cost of the equipment. While acknowledging the higher instrumental costs compared to other spectroscopic techniques (UV-vis, IR, NIR), benchtop NMR spectrometers are now more affordable for analytical laboratories than high-resolution NMR machines.

This study was focused on only one plant matrix *A. vera*. In the past other individual matrices such as medicinal plant *Maytenus ilicifolia* and blueberries were characterized by the low-field NMR [33,34]. Clearly, the performance of Benchtop NMR spectroscopy should be investigated regarding other vegetable matrices in the future.

It should be noted that low-field NMR can never achieve the same performance as high-resolution alternative. The disadvantages of the Benchtop NMR technology clear lower resolution and sensitivity. Moreover, it is not competitive with HPLC (apart for the possibility of the analysis of aloverose in this case). Still, quantitative information can be obtained for a number of compounds using univariate techniques even for complex matrix such as plant extracts.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data availability

Data will be made available on request.

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