Quantitative ^{1}H qNMR Method for Complex Mixture Analysis: Determination of Acetylated Polysaccharides, Glucose, Maltodextrin, Isocitrate, Preservatives, Additives and Degradation Products in Aloe Vera Leaf Juice - Raw Material and Consumer Products

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This ^{1}H Quantitative NMR (qNMR) method was developed and validated by Process NMR Associates (Danbury, CT) and a similar ^{1}H NMR approach has been subjected to independent validation by Spectral Service (Köln, Germany), Unigen Pharmaceuticals, Inc. (Lacey, WA), and the Department of Chemistry, Saint Martin's University (Lacey, WA) (Ref. 1, Jiao et al. 2010). The method can be used for the direct detection and quantitation of the primary components of interest in Aloe Vera juice products and raw materials for compliance with International Aloe Science Council (IASC) certification requirements, specifically, for determination of the content of acetylated polysaccharides (AP), the presence of glucose, the presence and concentration of maltodextrin, and the concentration of isocitrate (see Table I). Additionally, for meeting quality control specifications beyond IASC requirements, the presence and concentration of the following groups of compounds can be determined: degradation products (e.g. lactic acid, succinic acid, fumaric acid, acetic acid, formic acid, and ethanol), preservatives (e.g. potassium sorbate, sodium benzoate, and citric acid/citrate), and other atypical impurities, additives, or adulterants (e.g. methanol, glycine, glycerol, sucrose, maltodextrin, flavorants (propylene glycol/ethanol)). The method provides advantages over separation based test methods in that it is rapid, allows specific recognition of molecular chemistry, minimal sample preparation, and is quantitative.

The method describes a common internal standard qNMR methodology that does not require additional equipment or advanced automation software. There are other quantitative NMR methods that utilize internal, calibrated electronic reference signals, as well as the use of multiple standard calibration solutions that allow direct calculation of the components present in the sample utilizing specialized software automation and spectral deconvolution algorithms.

The method is applicable to a large number of different Aloe Vera raw materials and products, including liquid and dried juices. In aloe vera finished products the method is only applicable when the observable aloe vera constituents are present at a high enough concentration and are not obscured by additional product ingredients with signals in overlapping areas.

Table I: Aloe Vera Inner Leaf Juice constituents and additives that need to be analyzed and reported for IASC certification

Compound	IASC Certification requirement
Acemannan	≥ 5% dry weight
Glucose	Present
Aloin	10 ppm or less in 0.5% aloe vera solids solution, analysed by HPLC or other fit for purpose methodology approved by IASC
Isocitrate	≤ 5% dry weight
Maltodextrin	Must be listed on label and analysis must meet label claims. If undeclared, is considered an adulterant.
Solids	≥ 0.46% in single-strength juice (for example, a 10x concentrate should have ≥ 4.6%)
Ash	≤ 40%

There are three main constituents present in fresh Aloe Vera Inner Leaf Juice produced by processing the inner gel of the aloe leaf. These are acetylated polysacchride, glucose, and malic acid. Fresh Aloe Vera Leaf Juice, produced by processing the entire leaf, contains glucose, malic acid, acetylated polysaccharide along with citric acid cycle components such as citrate, isocitrate, and isocitrate lactone. According to IASC standards, all Aloe Vera Leaf Juice raw material should contain > 5% dry weight acetylated polysaccharide. In addition, IASC-certified raw materials and products labeled as Aloe Vera Inner Leaf Juice must contain ≤ 5% dry weight isocitrate. IASC-certified raw materials and products with isocitrate levels of > 5% dry weight are defined as Aloe Vera Leaf Juice, in accordance with IASC nomenclature.

Some Aloe Vera products and raw materials may also contain high levels of lactic acid and acetic acid due to malolactic bacterial fermentation, hydrolysis, or thermal degradation of the material during production and/or storage. Finished *Aloe vera* products often contain additives such as preservatives and flavourants. This method can readily be adapted to allow analysis of any or all of these constituents.

Freeze-drying procedures may lead to the underestimation (or even non-observation) of some of the compounds. The freeze-drying process also removes acetic acid, ethanol, methanol, sorbate, benzoate, and formic acid from the sample. If these components are of interest to the manufacturer or marketer of the products being analyzed then the NMR analysis should be performed on the juice sample without freeze-drying. The NMR processing and final calculations for liquid Aloe Vera juice samples are identical to those performed on Aloe Vera juice powders and freeze-dried samples. The calculated concentration values in liquid samples will be much less than the dry weight values suggested by IASC, as the majority component of the sample will be water. Weight values will be 10-200 times less as the dry matter is typically 0.5%-10% dry weight of the sample.

Table IV: Characteristic chemical shift values, peak multiplicity, protonated carbon type and N values used for detection and quantitation of the major natural components of aloe vera leaf juice

Substance	Signal Type and N Parameter	Chemical shift, ppm
Acetylated Polysaccharides	Broad Group of CH_3 Singlets (N=3)	2.0-2.3
Isocitric acid	CH, Doublet (N=1)	4.25
Malic acid	CH, 4 peak multiplet (N=1)	4.45
α-Glucose	CH Doublet (N=1)	4.6
β-Glucose	CH Doublet (N=1)	5.2
Isocitric lactone	CH Doublet (N=1)	5.05

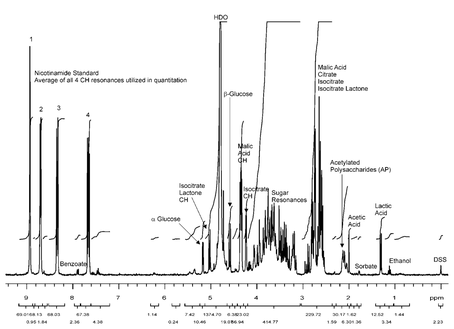

Fig 1: ^{1}H qNMR spectrum: Freeze Dried Aloe Vera 5x Leaf Juice Sample

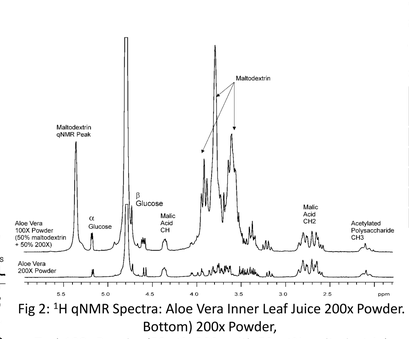

Fig 2: ^{1}H qNMR Spectra: Aloe Vera Inner Leaf Juice 200x Powder. Bottom) 200x Powder, Top) 100x Powder (50wt% 200x with 50 wt% maltodextrin)

1. Jiao, P., Jia, Q., Randel, G., Diehl, B., Weaver, S., Milligan, G., "Quantitative ^{1}H-NMR Spectrometry Method for Quality Control of Aloe Vera Products", J. AOAC Int. 93(3), 842-848, 2010.
2. Manna, S., McAnalley, B.H. 1993. Determination of the position of the O-acetyl group in a b-(1-->4)-mannan (acetylated polysaccharide) from *Aloe barbardensis* Miller. Carb. Res., 241, 317-319, 1993.
3. Chow, J.T-N.,Williamson, D.A.Yates, K.M., and Warren J. Gouxa,W.J., "Chemical characterization of the immunomodulating polysaccharide of Aloe vera L", Carb. Res., 340, 1131–1142, 2005.

This method is in the final stages of review before inclusion in the American Herbal Pharmacopeia Monograph on Aloe Vera to be published in late 2012.

Sample Peparation

Liquid Juice Samples and Aloe Vera-Containing Commercial Products

Dissolve 150-200 mg of liquid aloe vera sample and 5-10 mg of the internal standard (nicotinamide) in ~0.7 mL deuterium oxide (D_2O), transfer to 5mm tube.

Freeze-Dried Juice Samples or Commercially Dried Juice Products

Dissolve 20-50 mg of dried aloe vera leaf or inner leaf juice powder and 5-10 mg of the internal standard (nicotinamide) in ~0.7 mL of D_2O, transfer to 5-mm tube.

Note: The exact amount of sample or standard is not important, but all weights must be recorded to the nearest 0.1 mg. Volume of solvent is also not critical as the final result will be calculated in terms of wt% and does not require a volume to be used as is required for mg/ml calculations.

Reagents

NMR solvents:

D_2O (99.9% D-atom) + 0.01mg/ml DSS (0.7 ml) (Example: Cambridge Isotope Laboratories (Andover, MA) - Catalog No. DLM-6DB-10x0.7, individual glass ampules)*

DCl (20% in D_2O, 99.5% D-atom). (Example: Cambridge Isotope Laboratories (Andover, MA) - Catalog No. DLM-2-50, 50g Ampule)*

* Equivalent deuterated solvents from other manufacturers can be used.

qNMR internal standard: Nicotinamide (> 99.5% purity).

Note: Some automated approaches (not described here) require external standards of glucose, malic acid, lactic acid, and acetic acid, as well as a standard acetylated polysaccharide solution (e.g., Immuno-10, Unigen, Lacey, WA, USA).

All small molecule components can be obtained from commercial chemical companies at purity of > 98%.

Equipment

NMR spectrometer: Varian Mercury-300MVX with ^{1}H-^{19}F/^{15}N-^{31}P 5-mm PFG AutoX DB Probe or 5-mm H/F/P/C 4-nucleus probe. Operating with Varian VNMR-6.1C software. Equivalent NMR systems and software include those from the following manufacturers: Agilent/Varian (VNMR or VNMRJ software), Bruker (Topspin software), JEOL (Delta software). The necessary requirements are ^{1}H Resonance Frequency of 300-500 MHz and a functional ^{1}H probe.

Examples of third party commercial and non-commercial NMR software capable of processing spectral data acquired on commercial NMR spectrometers (as above) include ACD/NMR Processor (ACD/Labs), MNOVA (Mestrelab Research), SpinWorks (freeware), Chenomx NMR Suite (Chenomx).

Weighing equipment: Calibrated weighing balance capable of measuring accurately to 0.1 mg.

Freeze dryer: Virtis BTK Benchtop "K" Manifold (SP Industries) or equivalent.

Analytical Conditions

The typical NMR instrument parameters are shown in Table II. There is some variation of these parameters brought about by differences in field strength and experimental preference. All experiments must be optimally shimmed and the acceptance criteria for acceptable spectral performance is based on the quality of the nicotinamide standard resonance located at 7.65 ppm which should optimally be a well resolved, symmetric, 4 peak multiplet. The water resonance set to 4.8 ppm is utilized as the chemical shift standard in non-acidified samples. Preferentially internal chemical shift standards readily available in NMR deuterated solvents 4,4-Dimethyl-4-silapentane-1-sulfonic acid (DSS) or 3-(trimethylsilyl)-2,2',3,3'-tetradeuteropropionic acid (TMSP-d4) can also be utilized as the reference for 0 ppm. The DSS, TMSP, or small molecule component line-shapes should also be utilized to validate the line-shape and thermal stability of the acquisition. Other resonances in the sample that can be used for confirmation of lineshape are glucose (doublet at 5.2 ppm), lactic acid (if present, doublet at 1.35 ppm).

Table II Typical NMR instrument parameters

Acquisition Time	3-8 Seconds
Relaxation (Recycle) Delay	2-6 Seconds
Frequency, MHz	300-500 MHz
Nucleus	^{1}H
Number of Pulse Accumulations*	16-256
Original FID Points	16384-84000
Zero-filled Points	32768-262144
Pulse sequence	Single pulse
Solvent	D_2O
Sweep width, ppm	16
Temperature	Ambient (25 ºC)
Line Broadening	0.35 Hz
Steady State Pulses	8
Pre-Acquisition Delay	60 seconds

* Number of transients depends on the component concentration present in the sample being analyzed. Signal-to-noise (S/N) must be high (>10:1 for the smallest component signal to be quanitified, >3:1 on smallest component to be detected). The analyst must decide the appropriate number of transients to obtain adequate S/N.

Limits

The limit of detection (LOD) and limit of quantitation (LOQ) values for some of the aloe vera leaf juice components calculated for this method can be seen in Table III. The LOD/LOQ values can vary based on the spectrometer field strength, NMR probe type and configuration, and post-processing procedures such as apodization. For full description of typical LOD, LOQ, linearity, robustness, accuracy and reproducibility results of the method, see Ref 1, Jiao et al. (2010).

Table III Limits of detection (LOD) and quantitation (LOQ) for some of the constituents naturally present in aloe vera leaf juice

Substance	Signal-to-Noise ratio (S/N) > 3	Signal-to-Noise ratio (S/N) > 10
	LOD, mg/mL	LOQ, mg/mL
Acetylated Polysaccharide	< 0.05	< 0.1
Glucose	< 0.05	< 0.05
Malic acid	< 0.05	< 0.05
Lactic acid	< 0.005	< 0.005
Acetic acid	< 0.001	< 0.005

Aloe Vera Leaf Juice Concentrate		
Component	Dry Matter Composition Wt%	Juice Product Composition Wt%
Acemannan Wt%	4.13	0.113
Glucose Wt%	7.72	0.211
Malic Acid Wt%	15.86	0.434
Lactic Acid	0.06	0.002
Citric Acid	4.46	0.122
Pyruvic Acid	0.02	0.001
Sorbate	2.10	0.093
Benzoate	2.19	0.095
Acetoin	0.00	0.000
2,3-butandiol	0.00	0.000
Whole Leaf Marker (WLM)	Observed	Observed
Isocitnte (WLM-1)	5.28	0.145
Isocitnte Lactone (WLM-2)	4.30	0.118
Acetic Acid	0.09	0.004
Succinic Acid	0.00	0.000
Formic Acid	0.00	0.000
Fumaric Acid	0.00	0.000
Ethanol	0.05	0.007
Glycine	0.00	0.000
Propylene Glycol	0.00	0.000
Maltodextrin	0.00	0.000
Dry Matter Weight (%)	2.74	

Aloe Vera Inner Leaf Powder 200X	
Component	Wt%
Acemannan	8.23
Glucose	19.64
Malic Acid	39.62
Lactic Acid	2.21
Citric Acid	1.36
Pyruvic Acid	0.23
Isocitrate (WLM-1)	0.00
Isocitrate Lactone (WLM-2)	0.00
Acetic Acid	0.14
Succinic Acid	0.00
Fumaric Acid	0.16
Maltodextrin	0.00

Typical qNMR results.

Table V Chemical shift values, peak and chemistry descriptions, molar conversion factors that can be used for detection and quantitation of aloe vera leaf juice preservatives, additives, and degradation products

Compound	Type of compound	Signal type	Chemical shift, ppm
Propylene glycol	Additive	CH_3, doublet (N=3)	1.1
Ethanol	Degradation product or additive	CH_3, triplet (N=3)	1.15
Lactic acid	Degradation product	CH_3, doublet (N=3)	1.33
Potassium sorbate	Preservative	CH_3, doublet (N=3)	1.82
Acetic acid	Degradation product	CH_3, singlet (N=3)	1.96
Pyruvic acid	Degradation product	CH_3, singlet (N=3)	2.35
Citric acid	Naturally present or added as pH regulator or preservative	2 x CH_2, Multiplet (N=4)	2.5-3.0
Succinic acid	Degradation product	2 x CH_2, singlet (N=4)	2.6
Glycerol	Additive	CH_2 and CH, multiplet	3.5
Glycine	Additive	CH_2, singlet (N=2)	3.51
Sucrose	Additive	CH, doublet (N=1)	5.4
Fumaric acid	Degradation product	2 x CH, singlet (N=2)	6.5
Sodium benzoate	Preservative	2 x CH, doublet (N=2)	7.95
Formic acid	Degradation product	CH, singlet (N=1)	8.2-8.3

Quantitation

After the component peak signals have been properly assigned and identified, the component signal peaks (see Tables IV and V for details) are carefully integrated and the integral values transferred into spreadsheets or utilized in NMR software macros and automation routines. Some advanced software packages might also allow automatic identification and integration of the signals of interest, or completely deconvolute and quantify the components based on spectral deconvolution using pure component spectra as a basis set.

Quantitation of Acetylated Polysaccharides

The multiple NMR peaks associated with acetylation groups are found between 2.0 and 2.3 ppm and these have been chosen as the characteristic peaks for acetylated mannose residues in aloe vera polysaccharides. An expansion of this region shows the expected multi-peak distribution that is a fingerprint of intact acetylation on the polysaccharide backbone (Figure II). Degradation (deacetylation) of the polysaccharides will result in readily observable changes in the relative peak intensities in this region (example shown in Figure I). Quantification of acetylated polysaccharides (AP) by ^{1}H NMR, where the repeat units of the polymer yield superimposed signals in the NMR spectrum is accomplished by multiplication of the molar integrated signal values in the 2.0-2.3 ppm region by the average monomer unit molecular weight. In the case of aloe derived AP it has been demonstrated that the acetylation of the mannose monomer units is at 78% (Ref. 2, Manna et al., 1993) and that the mannose represents 84% of the polysaccharide backbone with the remainder being composed of glucose, galactose, and a few other saccharides (Ref 3., Chow et al., 2005). The acetylation content and the presence of 16% other saccharides must be taken into account so as not to underestimate the AP content.

The molecular weight of AP is calculated under the assumption that 1 water molecule is removed upon condensation of acetyl with mannosyl monomer, and each mannosyl unit shares in the loss of a single water molecule upon condensation form the predominantly mannose based polysaccharide.

Thus, $MW_{mannosyl} = Mw_{mannose} - (MW_{water})/2 = 180.2 - 18 = 162.2$ g/mol

$MW_{AcMann} = Mw_{acetyl\ group} + MW_{mannosyl} - MW_{water} = 60 + 162.2 - 18 = 204.2$ g/mol

Now taking into account the 0.78/1 acetyl/mannosyl residue ratio as well as the presence of ~16% non-mannosysl saccharides in the AP we can calculate the concentration of AP (C_{AP}) by the following equation.

$$C_{AP} = \left(\frac{W_{Nic} * I_{AcMann} * N_{Nic} * MW_{AcMann}}{I_{Nic} * N_{AcMann} * MW_{Nic}} + \frac{W_{Nic} * I_{AcMann} * N_{Nic} * MW_{Mann} * (0.22/0.78)}{I_{Nic} * N_{AcMann} * MW_{Nic}} + \frac{W_{Nic} * I_{AcMann} * N_{Nic} * MW_{Glu} * (0.16/0.84)}{I_{Nic} * N_{AcMann} * MW_{Nic}}\right) * \frac{1}{W_{sample}} * 100\%$$

$$= \frac{W_{Nic} * I_{AcMann}}{I_{Nic}} * \frac{N_{Nic}}{N_{AcMann} * MW_{Nic}} * (MW_{AcMann} + MW_{Mann} * 0.28 + MW_{Glu} * 0.19) * \frac{1}{W_{sample}} * 100\%$$

$$= \frac{W_{Nic} * I_{AcMann}}{I_{Nic} * W_{sample}} * \frac{4}{3 * 122.1} * (204.2 + (162.2 * 0.47)) * 100\%$$

$$= \frac{W_{Nic} * I_{AcMann}}{I_{Nic} * W_{sample}} * 3.06 * 100\%$$

AcMann = acetylmannosyl, *Mann*=Mannosyl, *Glu*=glucosyl
N = number of protons in the group - molar conversion factor - N_{Nic}=4, N_{AP}=3
C_{AP} = content of acetylated polysaccharides (AP) in the sample, wt%
W_{Nic} = weight of added internal standard (mg), W_{Sample} = weight of sample (mg)
I_{AcMann} = integration area of acetylation methyls (multiple peaks in 2.0-2.3 ppm region)
I_{Nic} = sum of integration areas of the 4 aromatic CH peaks of the nicotinamide standard
MW_{AcMann} = Molecular Weight of acetylated polysaccharides (204.2 g/mol)
MW_{Nic} = molecular weight of nicotinamide standard (122.1 g/mol)
MW_{Mann}=MW_{Glu}=162.2

Quantitation of Isocitrate

In Aloe Vera Leaf Juice, obtained by processing the entire leaf, the 2.5-3.0 ppm region of the spectrum is further complicated by the presence of compounds that occur in the green outer leaf of the aloe plant. Their presence leads to overlapping of the signals of the CH_2 protons of malic acid and citric acid with those of isocitrate and isocitrate lactone. This means that the 2.5-3.0 ppm region cannot be used directly for the quantitation of these components. Instead quantitation of is performed in the region of the spectrum where the CH resonances isocitrate be found (doublet at 4.25 ppm). The chemical shift value of the typical CH signal peak used for quantitation of isocitrate is indicated in Table IV and indicated on the Fig. 3 below.

The lactic acid CH quartet and isocitrate CH doublet signals may overlap in the 3.9-4.2 ppm region. This will not be determined until after the initial ^{1}H-NMR analysis has been performed and the concentration of lactic acid ascertained. If it is found that there is overlap, the isocitrate concentration must be calculated from an observation of the ^{1}H-NMR spectrum after pH adjustment with a single drop of concentrated DCl (or any other deuterated mineral acid). The addition of the mineral acid shifts the isocitrate CH signal into an area of the spectrum (~ 4.45 ppm) where it is free from interference and can be properly integrated. The pH of the final solution is not important if the analysis is to be performed by an NMR analyst. However, the pH must be known and controlled if automated spectral deconvolution methods are to be used.

C_{ICA} (wt%) = 100% * (W_{Nic} * I_{ICA} * N_{Nic} * MW_{ICA}) / (I_{Nic} * N_{ICA} * MW_{Nic} * W_{Sample})

= 100% * (W_{Nic} * I_{ICA} * 4 * 192.1) / (I_{Nic} * 1 * 122.1 * W_{Sample}) = 100% * (W_{Nic}*I_{ICA}/I_{Nic}*W_{Sample}) * 6.29

C_{ICA} = content of isocitrate in the sample, weight%
W_{Nic} = weight of added nicotinamide internal standard (mg), W_{Sample} = weight of sample (mg)
I_{ICA} = integration area of CH proton resonance of isocitrate (doublet at 4.45 ppm (dissolved in D_2O and acidified with DCl), or 4.2 ppm (dissolved in D_2O))
I_{Nic} = sum of integration areas of the 4 aromatic CH peaks of the nicotinamide standard
MW_{ICA} = Molecular Weight of Isocitrate (192.1 g/mol), MW_{Nic} = molecular weight of nicotinamide standard (122.1 g/mol)
N = number of protons in the group - molar conversion factor, N_{Nic}=4, N_{ICA}=1

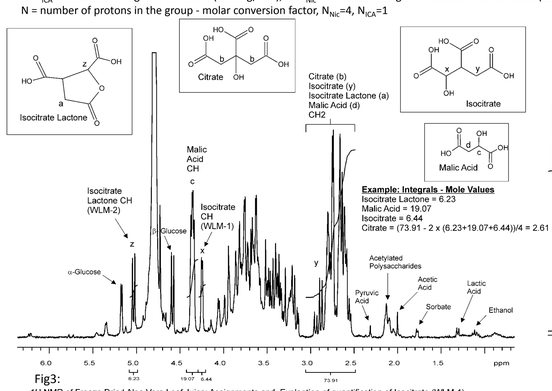

Fig3:
1H NMR of Freeze Dried Aloe Vera Leaf Juice: Assignments and Explantion of quantification of Isocitrate (WLM-1), Isocitrate Lactone (WLM-2), Citrate, and Malic Acid.
Note: peaks marked z, x, and y do not appear in aloe vera inner leaf materials.

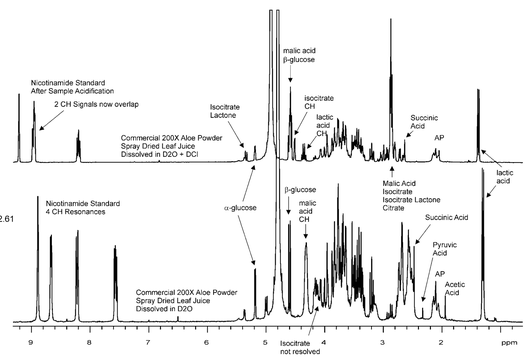

Fig 4: ^{1}H NMR spectra showing the effect of sample acidification on the component signals. Note especially the well resolved final shift of the isocitrate CH resonance at 4.55 ppm.

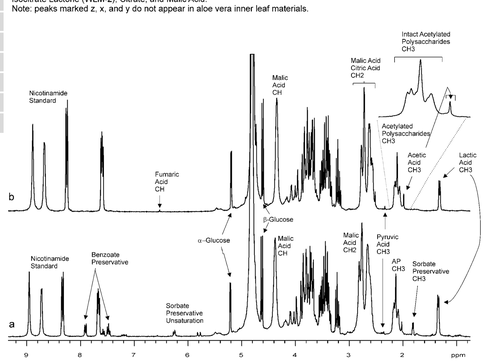

Fig 5: Aloe Vera inner leaf powder – with and without preservatives

Beyond the two examples of acetylated polysaccharides and isocitrate it is possible to utilize qNMR to quantify other aloe vera components such as glucose, citrate, isocitrate lactone. Degradation products such as acetic acid, formic acid, succinic acid, lactic acid, fumaric acid, pyruvic acid, ethanol can also be quantified along with preservatives (potassium sorbate, citrate, sodium benzoate). In commercial products fructose, sucrose, maltodextrin, glycerol, ethanol, propylene glycol, glycine and other additives can be identified and quantified. The general calculation formula to calculate the wt% of any molecular component is:

C_X (wt%) = 100 * (W_{Nic} * I_X * N_{Nic} * MW_X) / (I_{Nic} * N_X * N_{Nic} * W_{Sample})

Where:
C_X = concentration of component
W_{Nic} = weight of added nicotinamide internal standard (mg)
W_{Sample} = weight of sample (mg)
I_X = integration area of unique proton resonance from spectrum of component
I_{Nic} = sum of integration areas of 4 aromatic CH peaks of the nicotinamide standard
MW_X = Molecular Weight of Component
MW_{Nic} = molecular weight of nicotinamide standard (122.1 g/mol)
N = number of protons in peak group - molar conversion factor, (CH=1, CH_2=2, CH_3=3), for nicotinamide N = 4 (4 aromatic CH resonances) – or 3 if one CH resonance is overlapped by other components such as benzoate or formic acid and is therefore not utilized in the qNMR calculation.