Development of an Automated Complex Mixture Analysis qNMR Method within Mestrelab Mnova: Application to *Aloe vera* Raw Material Analysis and the Beer Brewing Process

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^{1}H qNMR –*Aloe vera* Analysis – wt%

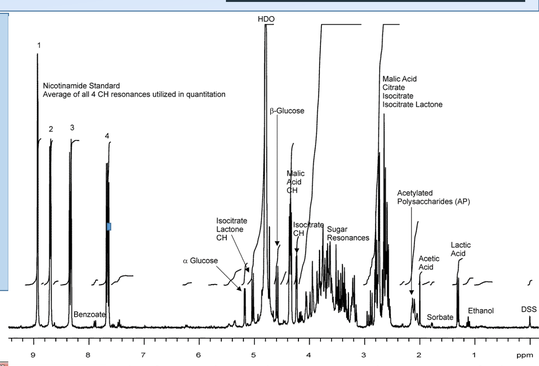
A simple mixture analysis (SMA) functionality is being developed for the Mestrelab Mnova platform. We have utilized this to analyze a number of *Aloe vera* samples. Here we present two different samples:

- *Aloe vera* Whole Leaf Juice Freeze Dried Powder
- *Aloe vera* Whole Leaf Juice Freeze Dried Powder + Maltodextrin

We present the software process by which the quantitative ^{1}H NMR is performed by the analyst. The approach of analyzing one sample at a time is shown for the first sample (Sample 458). However, it is possible to get answers from two different spectra in a single analysis, and we demonstrate that capability in the second sample analysis (Sample 42).

Overview: An automated approach to complex mixture ^{1}H qNMR was developed for the Mnova platform and small molecule quantitation was performed on a series of *Aloe vera* raw materials and *Aloe vera* containing products utilizing nicotinamide as the internal concentration standard. The analysis automatically generates a comprehensive report of the three active components, degradation products, additives, and adulterants of commercial *Aloe vera*. Individual species are quantified using areas derived from Global Spectrum Deconvolution (GSD) analysis, which is insensitive to peak overlap and poor baseline. The known area for a mixture component can be flexibly converted into the desired concentration units using a flexible *formula editor*. The workflow is *automated* to facilitate high-throughput analysis, but still allows visual inspection and editing of each sample to account for peak movements. The quantitative values derived from this rapid, straight-forward automated analysis allows decisions to be made on the purchase of materials by end users as well as implementation of controlled process changes in the manufacture of the materials.

A complete NMR analysis of a number of beer varieties was also performed with a special emphasis on sour beers produced by wild fermentation. In this analysis the same cast of small molecules was analyzed with the Mnova qNMR analysis performed to provide ethanol, butandiol, ethyl acetate, lactic, acetic, succinic, citric, and malic acids concentrations along with glucose. The spectra and the qNMR results are used as the basis for principal component analysis that will allow targeted and non-targeted identification of beer types and variations of intermediates in the brewing process.



The figure above shows a typical spectrum of *Aloe vera* leaf juice powder in D_2O with assignments

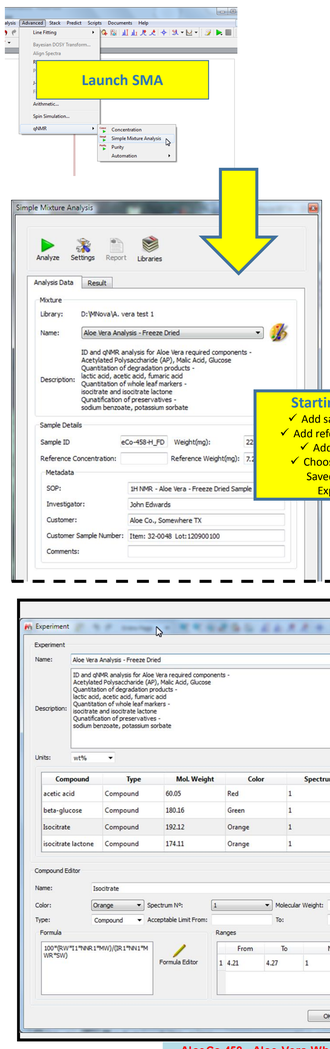
Logo found on aloe products that have passed the quality standards described In the table below.

^{1}H NMR is the recognized method for Acemannan and isocitrate quantitation.

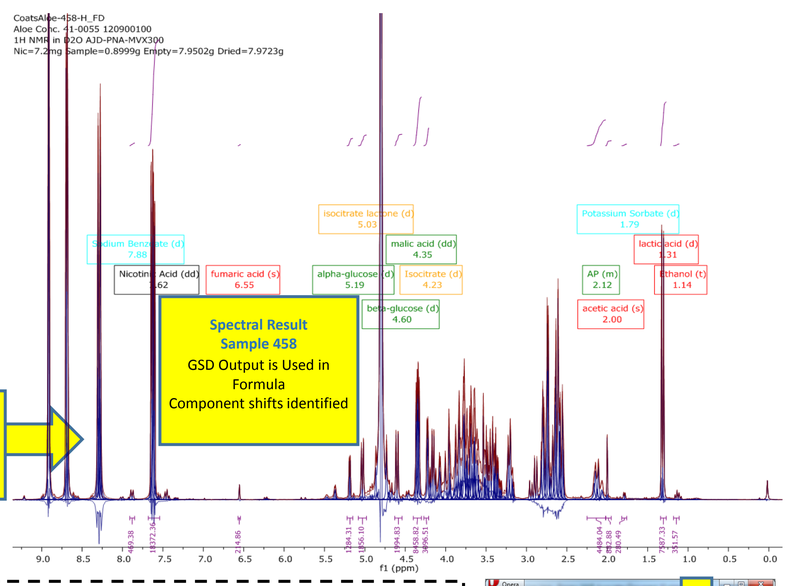
Quality Specifications for *Aloe vera* Raw Materials Enforced by International Aloe Science Council

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%

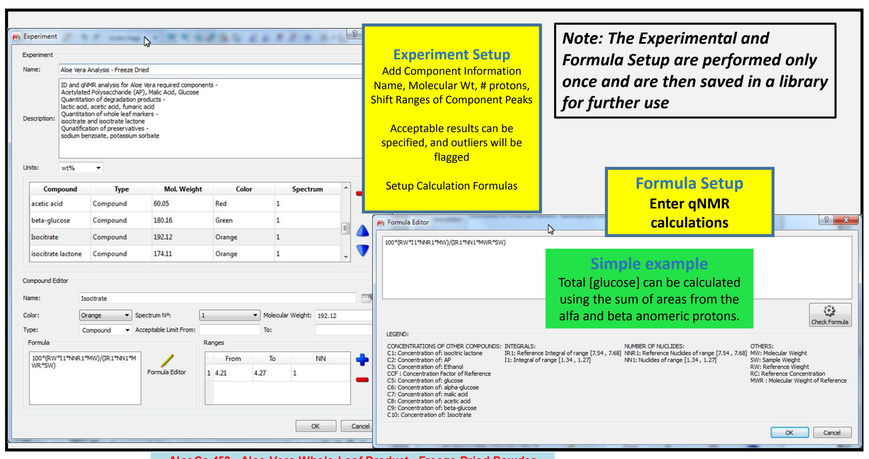
Analysis of Aloe Sample 458 – Whole Leaf Aloe Powder



Starting Screen
✓ Add sample weight
✓ Add reference weights
✓ Add Metadata
✓ Choose Previously SavedLibrary and Experiments



Spectral Result Sample 458
GSD Output is Used in Formula Component shifts identified



Experiment Setup
Add Component Information Name, Molecular Wt, # protons, Shift Ranges of Component Peaks

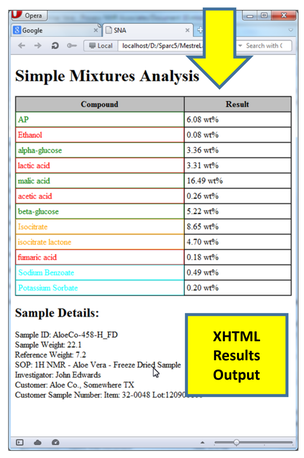
Acceptable results can be specified, and outliers will be flagged

Setup Calculation Formulas

Note: The Experimental and Formula Setup are performed only once and are then saved in a library for further use

Formula Setup
Enter qNMR calculations

Simple example
Total [glucose] can be calculated using the sum of areas from the alfa and beta anomeric protons.



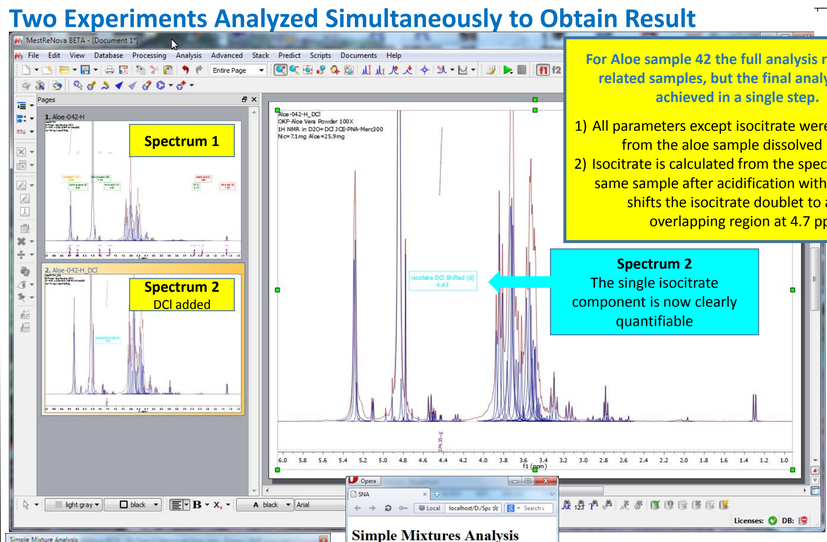
XHTML Results Output

AloeCo-458 - Aloe Vera Whole Leaf Product - Freeze Dried Powder

Component	MNova Mixture Analysis	Manual Integration
AP - Acetylated Polysaccharide	6.08	6.47
Ethanol	0.08	0.08
glucose	8.58	8.72
lactic acid	3.31	3.6
malic acid	16.49	16.52
acetic acid	0.26	0.32
Isocitrate	8.65	8.33
isocitrate lactone	4.70	4.91
fumaric acid	0.18	0.14
Sodium Benzoate	0.49	0.52
Potassium Sorbate	0.20	0.25

Comparison of ^{1}H qNMR calculations of component wt% obtained from 1) Mnova SMA and 2) Manual Integration + Excel Method (validated)

Aloe Sample 42 – Whole Leaf Powder with Maltodextrin Adulterant Two Experiments Analyzed Simultaneously to Obtain Result



For Aloe sample 42 the full analysis required 2 related samples, but the final analysis was achieved in a single step.

1) All parameters except isocitrate were calculated from the aloe sample dissolved in D_2O.
2) Isocitrate is calculated from the spectrum of the same sample after acidification with DCl, which shifts the isocitrate doublet to a non-overlapping region at 4.7 ppm

AloeCo-42 - Aloe Vera Whole Leaf Powder - Mixed with Maltodextrin

Component	MNova Mixture Analysis	Manual Integration
AP - Acetylated Polysaccharide	0.55	0.47
Ethanol	0.00	0.00
glucose	7.66	8.08
lactic acid	0.53	0.50
malic acid	1.40	1.42
acetic acid	0.02	0.02
isocitate DCl Shifted	0.19	0.30
Maltodextrin	71.66	72.37

Validation
Comparison of qNMR calculations of component wt% obtained from 1) Automated Mixture Analysis and 2) Manual Integration + Excel Method

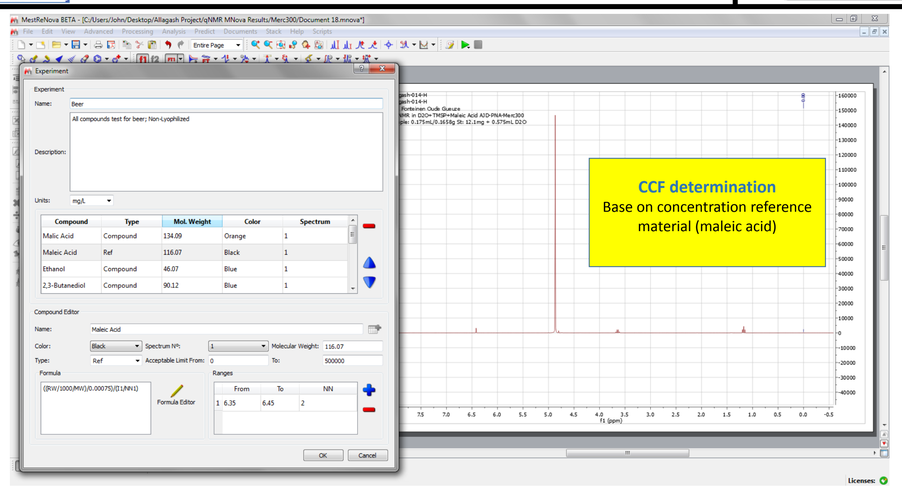
^{1}H qNMR – Beer Analysis – mg/L

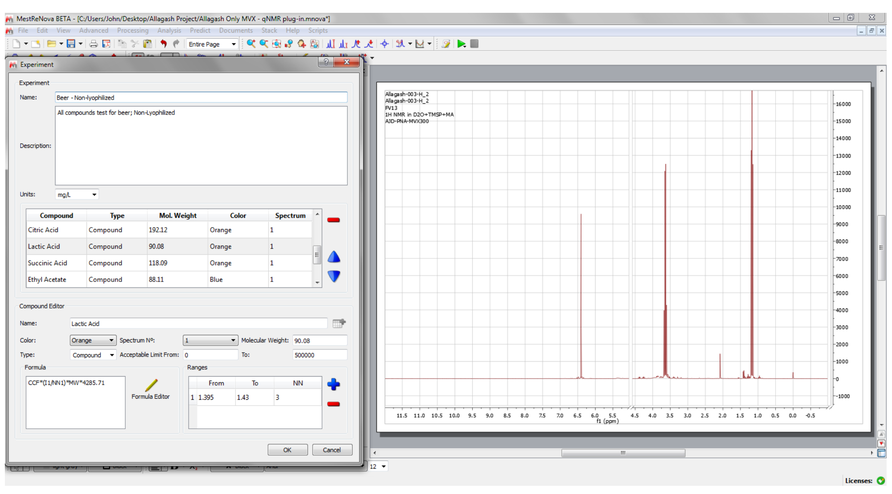
qNMR standard Parameters: concentration reference mass (RW, in grams), sample volume (in L), Molecular Weight (MW), and Hydrogen # (NN1) are entered to determine the *Concentration Conversion Factor* (CCF).

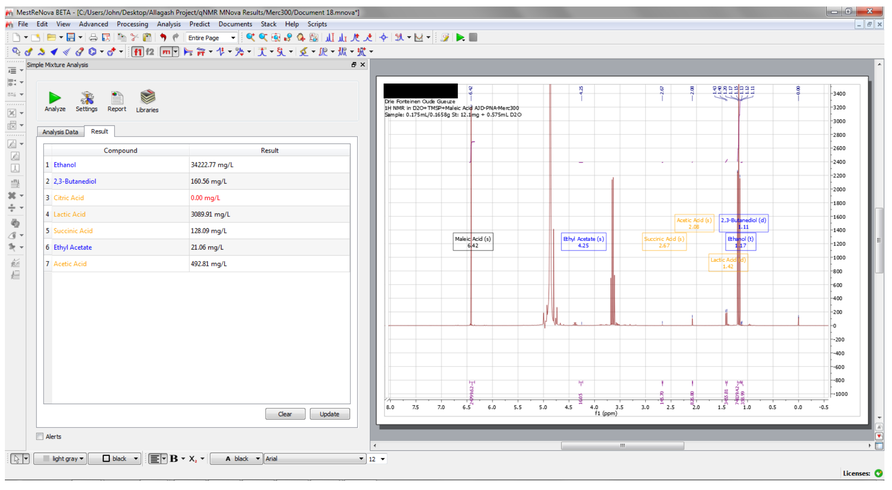
For each component, Integral (I1) is automatically determined from GSD integration based on specified peak ranges, and converted to concentration using the CCF.

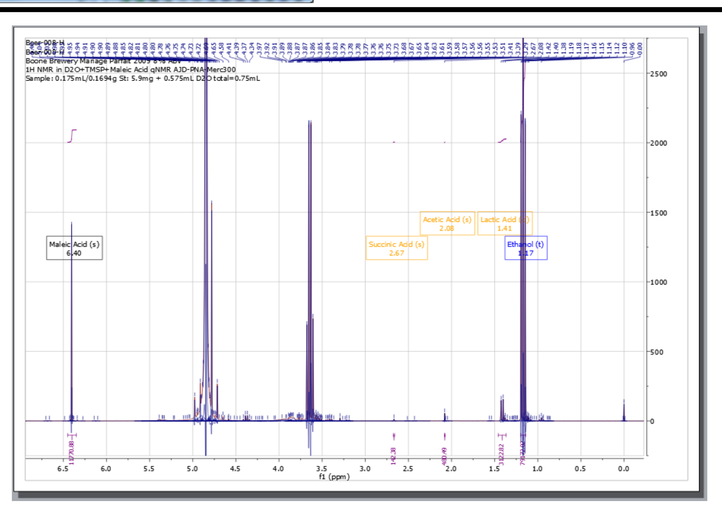
Concentrations of target metabolites are calculated as a function of CCF, NN1, MW & I1. The *terminal coefficient* (4285.71) is a correction factor for sample dilution and conversion to mg/L.

Global Spectral Deconvolution & Peak Picking automatically identifies and integrates peaks based on entered peak ranges. Quantitation results are immediately derived can be exported as XML & XHTML files, copied to clipboard, or pasted onto the spectrum in Mnova.





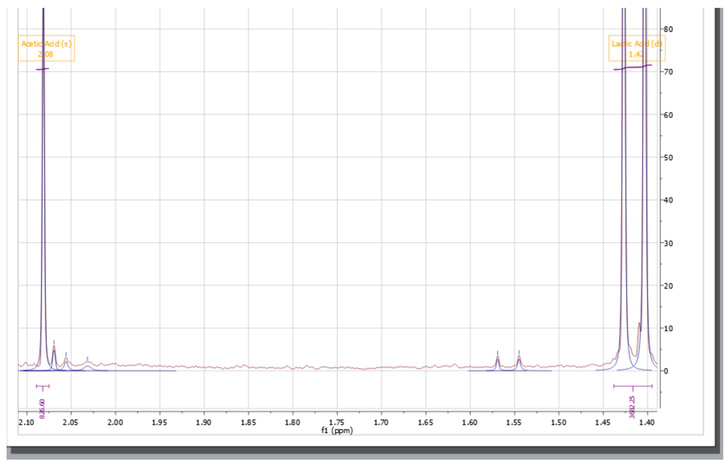




Above: Fully deconvoluted spectrum of analyzed beer, including residuals. Not all quantified species were included in the comparison table (below) as accuracy of qNMR plug-in suffered in high-detail spectral regions.

Brewery	Beer	Lactic Acid	Acetic Acid	Succinic Acid	Citric Acid	Lactic Acid	Acetic Acid	Succinic Acid	Citric Acid
Allagash	Confluence	850.5	402.4	167.4	248.2	901.3	437.6	184.3	353.2
Budweiser	Bud Light	93.9	38.1	35.8	82.7	117.1	17.7	34.2	62.6
Crooked Stave	Surette	4699.2	564.9	195.7	265.5	4247.9	549.2	185.6	262.4
Drie Fonteinen	Oude Gueuze	5137.6	865.9	234.6	0.0	4935.2	787.1	204.6	0.0
Drie Fonteinen	Oude Gueuze	5389.9	917.7	228.9	0.0	5180.0	775.2	194.4	0.0
LoverBeer	D'Uva Beer	3071.4	781.5	876.0	358.0	3302.8	759.9	842.4	377.3
Boone	Mariage Parfait 2009	4506.5	488.1	216.9	0.0	4591.8	403.2	193.1	0.0

Above: Comparison of results obtained by manual integration (solid border, left) and by automatic Mnova SMA (dotted border, right). The general trend indicates lower concentrations achieved by using the qNMR plug-in. This is most likely due to the use of global spectral deconvolution (GSD) with the plug-in , which eliminates contribution of stacked signals, e.g. amino acids, when integrating. The use of a "hard" baseline with GSD negates tangential contributions to signals, as can be seen to the sides of each peak of interest (below). The GSD-derived integrals are therefore more accurate.



Overview

- Mestrenova SMA is an effective tool for metabolite quantification in complex mixtures such as *A. vera* and beer.
- Manual integral range dragging & peak picking allows the user to correct any errors made by Mnova, or peak movements.
- Concentrations can be calculated in terms of multiple units (wt%, mass per unit volume, M)
- IA single calculation file can be used to process all similar samples.
- f coupled with batch processing (not currently available for Simple Mixture Analysis (SMA)), quantification of hundreds of spectra simultaneously is possible.

Experimental:

For the beer process analysis in our laboratory the metabolite quantifications were calculated on a concentration (mg/L) basis: samples were prepared by diluting 175 µL of de-gassed beer with 575 µL D_2O + 0.03% TMSP. A known mass of Maleic Acid standard was then added to the sample. Experiments were performed in a quantitative manner on a Varian Mercury-300 MVX spectrometer operating at 299.67 MHz equipped with an ATB probe.

Aloe vera analyses were performed on a Varian Mercury-300 spectrometer operating at 299.94 MHz and equipped with a 4-Nucleus probe. Samples and the Nicotinamide internal standard were weighed to the nearest 0.1 mg on a Sartorius GD-503-NTEP microbalance. qNMR calculations were obtained on a wt% basis.

Spectrum analysis used Mnova v 8.2.0, and the SMA plug-in.

References:

"Quantitative Proton Nuclear Magnetic Resonance Spectrometry (^{1}H NMR) for Determination of Acetylated Polysaccharides, Glucose, Maltodextrin, and Isocitrate in Aloe vera Leaf Juice" John C. Edwards, **American Herbal Pharmacopoeia -** Monograph on **Aloe vera Leaf, Leaf Juice & Inner Leaf Juice, Analytical NMR Method, 2013.**

"Application of Quantitative Nuclear Magnetic Resonance Spectroscopy to Biological Acidification of Barley Mashes", Adam Dicaprio, John C. Edwards, Submitted to **J. Inst. Brew.**

"Composition of Beer by ^{1}H NMR Spectroscopy: Effects of Brewing Site and Date of Production", C.Almeida, I.F.Duarte, A.Barros, J.Rodrigues, M.Spraul, A.M.Gil, J.Agric.Food Chem., 2006, 54(3), 700–706.