Whitepaper

Critical Evaluation of Methodologies for Characterization of Agave Syrup

by Quality Services International GmbH

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Introduction

Alternative sweeteners are gaining more and more importance. Among these, agave syrup is the most popular especially for organic markets and vegan nutrition. Due to its low glycemic index it is well appreciated for diet purposes.
Agave syrup is a risk product in terms of economically motivated adulteration based on high production costs due to a long growing period of the agave plant before harvesting (approx. 8-14 years).

The "success" of an adulterated product is based on a sophisticated adulteration method using hardly detectable sugars or syrups. With every improved or new method to determine adulteration the "food fraud business" develops more pure sugar syrups, which cannot be detected with "simple" methods. For instance, you can easily buy syrup without a detectable trace of oligosaccharides – so the analysis of an oligosaccharide profile or the detection of polyglucanes will always be negative and gives a false negative result.
Several methodologies are described in literature and are applied in the laboratories. All the methods are using the differences in the specific properties of agave syrup vs. sugars or syrups from other sources.

Official bodies are focusing more and more on food fraud aside from food safety. Consumers are quickly aware of scandals caused by adulterated food. So it is essential for the trade to guarantee the authenticity of a product by setting strict specifications based on sophisticated methodologies and quality tests. At the moment, several techniques for agave syrup authenticity testing are available from different private laboratories. Also, Mexico as main producer has recently created a governmentally approved paper ("NOM-003-SAGARPA-2016") as an official guideline for the characterisation of pure agave syrup.

Status Quo: SNIF-NMR, δ^{13}C-IRMS and Oligosaccharides (GC-FID)

SNIF-NMR

The SNIF-NMR (site-specific natural isotopic fractionation nuclear magnetic resonance) is an official method for wine and juice testing [1] using ^{2}H-NMR spectroscopy to measure a non-statistical distribution of deuterium in different sites of an ethanol molecule (D/H-ratio). For the agave syrup analysis this method needed to be adapted [2]. Quantitative ^{13}C-NMR was used to determine δ^{13}C values of the CH_2(methylene)- and the CH_3(methyl)-site of ethanol. To detect adulteration with syrup or sugars the agave syrup samples need to be fermented and distilled first. After that the alcohol grade is calculated and the sample is further prepared for the ^{13}C-NMR analysis leading into informative δ^{13}C values specific for agave syrup.

The SNIF-NMR method seems to be a complex method regarding the necessary sample amount of 300 g and the long analysis time due to the full fermentation and distillation and nevertheless the price. For example it can take 10-30 days until the customer receives results. However, the most important part is the reproducibility of the results and the robustness of the method. In case of the SNIF-NMR there are too many steps which can lead to deviating results. How certain is the full fermentation of the product and the necessary distillation afterwards? At the end two δ^{13}C values

are given for the CH_2- and the CH_3-site of the ethanol molecule. How easy is it to manipulate those values with specially produced syrup, which fits exactly the thresholds? Right now, to our knowledge, two laboratories offer SNIF-NMR for agave syrup (Eurofins and Bavarian State Office for Health and Food Safety).

δ^{13}C-IRMS

The analysis of stable isotopes for the authenticity of different goods like food, flavors, wood etc. has been applied for more than 30 years. Especially for testing of sugary foods the distribution between the "normal" Carbon-12 and the stable isotope Carbon-13 is used, because this so called "δ^{13}C-value" is strongly depending on the natural source.
A lot of different methods which focus on the determination of sugar or syrups in products like e.g. fruit juice, wine, honey were published [3, 4, 5]. All these methods are based on the fact that there are three different ways (and kinds of plants) for producing sugar: C3, C4 and CAM-plants. Typical values and species are:

Table 1: Overview about natural distribution of isotopic values in different plant species

	δ^{13}C	examples
C4-plants	-17‰ up to -9‰	Cane, Corn
C3-plants	-32‰ up to -20‰	Rice, Sugar beat
CAM-plants (dryness)	-17‰ up to -9‰	Agave, Pineapple

C3 plants and their syrups are easy to differentiate from agave syrup, but C4 plants are basically in the same isotopic range. That's why the analysis of the total δ^{13}C-value is not very informative. To solve the problem, prior to the analysis of the isotope value the different substances of the syrup are separated online and directly measured ("LC-IRMS").

The sugar fractions are expected to have the same ^{13}C-value as the original Fructans. Adding syrups with different isotope values can be seen in a shift of fractions. Additionally, the difference between the fractions will increase. Comparable methods are used for honey since 1998.

Still, the method is not able to detect an addition of syrup with exactly the same isotopic properties as the agave syrup. Nevertheless, adulterations very difficult and needs a high level of knowledge, if the right isotopic properties are to be taken into account.

Oligosaccharides (GC-FID)

In 2012, a method for analysing an oligosaccharide profile via capillary gaschromatography with flame ionization detection (CGC-FID) was published by Willems et al [6]. Using this application, an adulteration of agave syrup can be detected. Pure agave syrup basically shows a series of oligosaccharides (mainly disaccharides). Agave syrup adulterated with e.g. high fructose corn syrup (HFCS) 90 (90 % fructose) leads to additional peaks due to the presence of alpha- and beta-isomaltose, whereas agave syrup debased with dextrose syrups such as DE 42 shows additional peaks of alpha- and beta-maltose. Both substances do not occur naturally in agave syrup. This method is known to be sensitive, as an addition of about 1 % of HFCS 90 and 0.5 % of DE 42 can be detected. [6]

However, the exact quantification of the adulteration cannot be performed since the concentration of the marker compounds in the specific adulterant has to be known *a priori,* which is usually not the

case. Furthermore, it is not possible to prove the addition of all kinds of syrups, e.g. cane invert does not show the isomaltose and maltose peaks and therefore an adulteration with this syrup cannot be detected using this method. Further markers and methods need to be applied in such cases.

Critical evaluation of NOM-003-SAGARPA-2016: *HPLC-ECD*

The core part of the method in the NOM [7] (besides the physico-chemical and microbiological testing) is the analysis of the main sugars, 5-hydroxymethylfurfural (HMF) and detection of adulterations using HPLC with an electrochemical detector (HPLC-ECD). Herein, we want to share our experience with this method and evaluate the analysis.

According to the NOM, a sample of agave syrup is diluted with water and analysed before and after enzymatic hydrolysis using a Carbopac PA 1 250 x 4mm column. The enzymes amyloglucosidase (EC: 3.2.1.3) and fructanase (EC: 3.2.1.80) are added and after sample preparation the content of the sugars as well as the content of fructan is calculated.

The standard, consisting of fructose, glucose and sucrose as well as sorbitol, mannitol and HMF, is prepared according to the NOM and measured with HPLC-ECD. Figure 1 shows a chromatogram of this standard in a concentration range of approx. 10 µg/ml to 400 µg/ml, depending on the component (sorbitol, sucrose and mannitol: 10 µg/ml, HMF: 30 µg/ml, glucose: 40 µg/ml, fructose: 400 µg/ml).

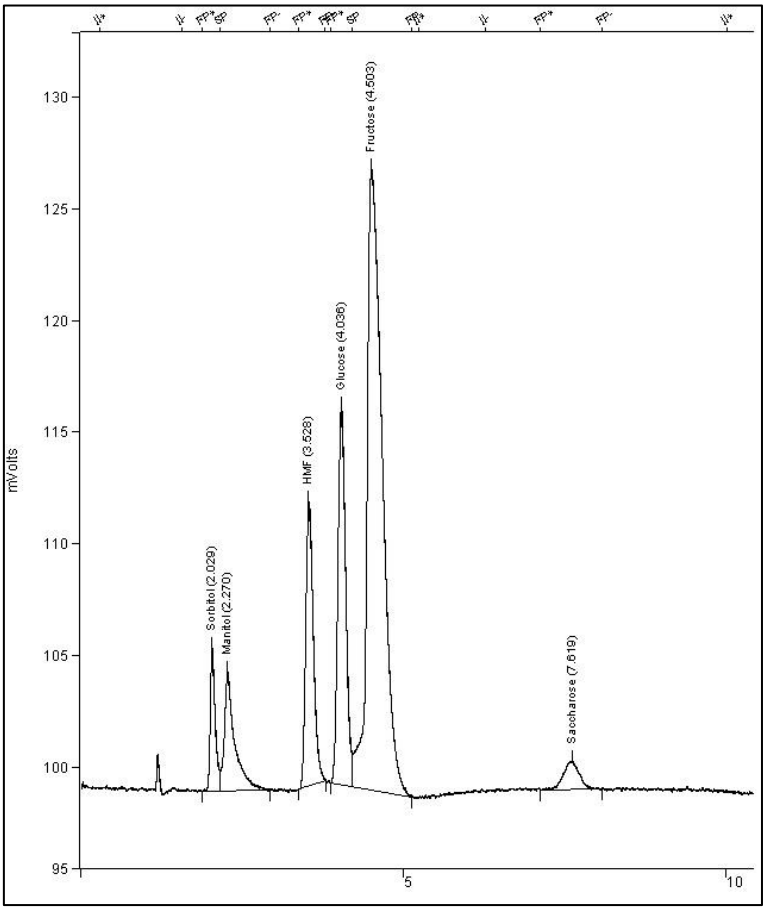

Figure 1: Chromatogram of standard mix. concentration range 10 µg/ml to 400 µg/ml

According to the NOM, the runtime of the analysis should be around 50 minutes. Working in a routine laboratory with a high sample throughput this runtime is barely practicable.

Consequently, the gradient of the mobile phase was optimised by us resulting in a new runtime of 33 minutes. Moreover, for the analysis of one single sample eight different preparation steps have to be measured. In addition, at least three different standard concentrations plus two blank samples containing the diluted enzymes should be analysed. In sum for one single sample 13 chromatograms are recorded which results in 650 minutes (NOM runtime) or 429 minutes (modified runtime) total runtime. Every other sample will take another 400 minutes or 264 minutes, respectively, plus blanks in between the samples for rinsing the system and column, which are not even mentioned in the NOM.

Hence, even when using the modified method, hardly two samples with standards and blanks could be measured within 12 hours which is just not practicable for the agave industry which demands fast and high volume testing capacities.

Also, the determination of a possible adulteration of the agave syrup seems to be rather difficult. According to the NOM, peaks which appear after 10 minutes in the chromatogram should be compared before and after enzymatic hydrolysis. Should peaks in this area vanish after hydrolysis with aminoglucosidase the NOM states that an adulteration with corn syrup is confirmed (figure 2):

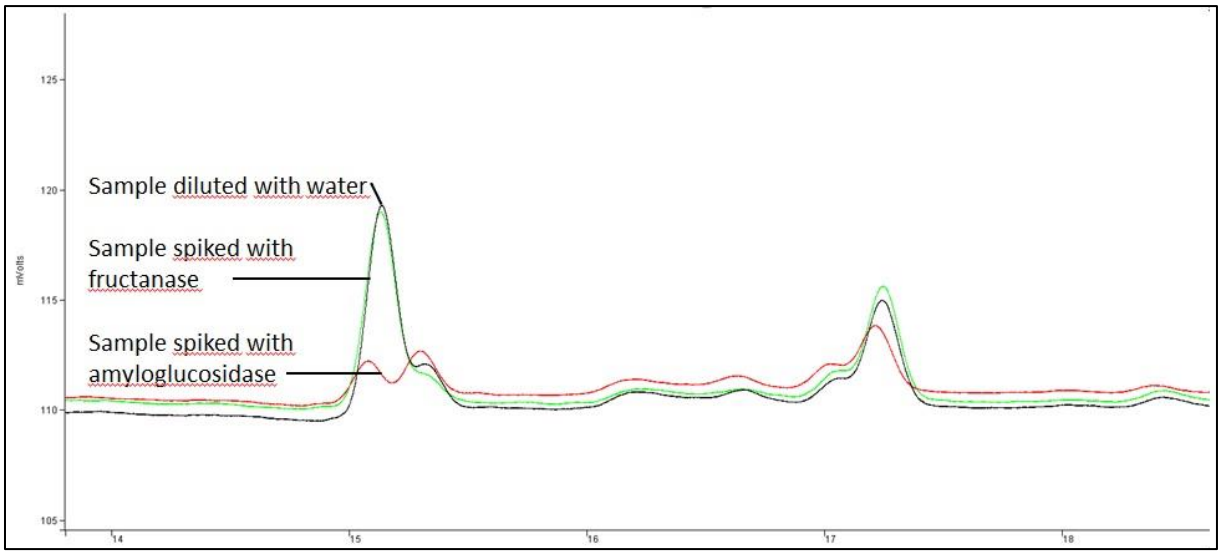

Figure 2: Chromatogram of peaks corresponding with an adulteration, example 1

The first peaks show a significant decrease, when comparing the diluted sample with the aminoglucosidase spiked sample. According to the NOM, an adulteration with corn syrup should now be confirmed. Also, the following peaks at around 17.5 minutes might also show an adulteration, if they decrease just barely. However, those peaks would not always appear as clearly shown in figure 2 and an adulteration is not easy to verify as the following different examples show (figure 3 and 4):

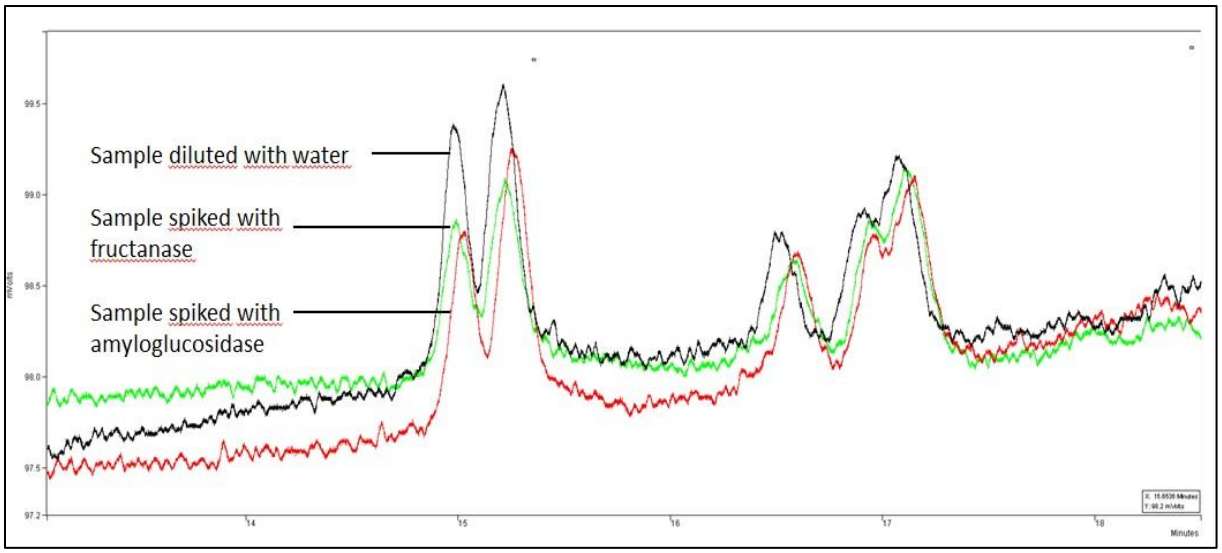

Figure 2: Chromatogram of peaks corresponding with an adulteration, example 2

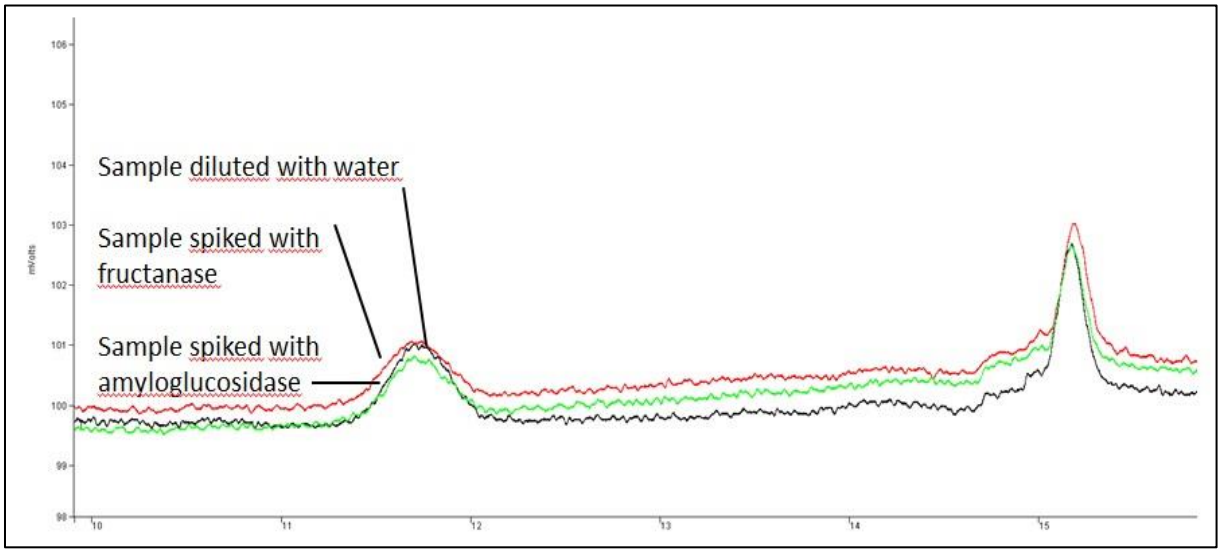

Figure 3: Chromatogram of peaks corresponding with an adulteration, example 3

As shown in the chromatograms above the appearance and shape of the target peaks seem to depend on the type of agave syrup. Considering a sample as adulterated or not adulterated only based on this kind of analysis does not seem sufficient to us. Further tests, such as ^{1}H-NMR or isotopic testing, are therefore strongly recommended.

Taking the rather instable measurement of the peaks corresponding with adulteration and the long runtime into account, the HPLC-ECD method does not seem applicable for analysing a larger quantity of agave syrups, when other methods like ^{1}H-NMR or isotope analysis will do it in a faster and even more reliable way (while also detecting a bigger range of different syrups, even "high purified" ones without any minor carbohydrates).

In our opinion, the NOM in its present form is not suitable for a reliable testing. A lot of problems during the implementation of the NOM due to the partially inconclusive and inaccurate method description were observed. It was hard to follow the method in terms of traceability and comprehensibility. Especially due to the mix of quantification of mayor components like fructose and glucose as well as some minor components like HMF and taking further the chemical properties of the inulin and the analysis of adulteration markers into account the methods were difficult to implement without knowledge of the ideas behind the combination of the different tests. Other substances like sorbitol are also to be analysed, but the NOM nowhere explains the reason and judgement behind positive findings.

Our methodologies

LC-IRMS

In this analysis the 3 main sugar fractions of the agave syrup (fructose, glucose and fructans) are separated and the ^{13}C-isotopic value is measured for each fraction. The ^{13}C-isotope value depends on the photosynthesis pathway of the plant source of the agave syrup. The sugar fractions are expected to have the same ^{13}C-value as the original fructans. Adding syrups with different isotope values can be seen in a shift of fractions and difference between the fractions isotope values will increase. Comparable methods are used for honey since 1998 [8].

The following ^{13}C isotopic acceptance values for agave syrups (table 2) were empirically derived and are (unofficially) harmonized between some laboratories:

Table 2: Acceptance δ^{13}C –values for agave syrup

δ^{13}C total sample	-10.8 to -13.5 ‰
δ^{13}C Fructose and d ^{13}C Glucose	-10.8 to -13.5 ‰
Difference δ^{13}C Fructose – δ^{13}C Glucose:	+0.8 and -1.0 ‰

The measurement uncertainty in the LC-IRMS is taken into account for the acceptance criteria. The day to day differences between fructose and glucose are varying in the range of ±0.4‰, so that the combined uncertainty of the difference is ±0.6‰.

By analysis of samples directly bought from the Mexican market and retailers it could be clearly demonstrated (see table 3) that in 13 out of 20 samples both techniques the LC-IRMS and also the NMR showed signs of adulteration. Some samples were not even showing traces of glucose, so that the interpretation (see table 2) of the isotopic LC-IRMS values could not be applied.

The following table 3 shows some examples of agave syrup samples from the Mexican market and retailers and the results of ^{13}C-IRMS and ^{1}H-NMR analyses.

Table 3: Overview of the ^{13}C- IRMS and ^{1}H-NMR results of Mexican market and retailer agave syrup samples

Sample No.	$\delta^{13}C$ Fructose	$\delta^{13}C$ Glucose	$\delta^{13}C$ Disaccharides	$\delta^{13}C$ Inulin	$\delta^{13}C_{Fruc}$-$\delta^{13}C_{Glc}$	Evaluation ^{13}C-IRMS	Evaluation ^{1}H-NMR
1	-11.47	-12.77	-10.44	n.b.	1.3	not complying	untypical
2	-11.52	-11.95	-11.68	-13.06	0.43	complying	typical
3	-11.67	-13.43	-12	n.b.	1.76	not complying	untypical
4	-10.02	-14.58	-12.1	n.b.	4.56	not complying	untypical
5	-11.69	-11.91	-10.98	-12.79	0.22	complying	untypical
6	-11.2	-12.61	-10.4	-10.32	1.41	not complying	untypical
7	-11.74	-14.09	-10.37	n.b.	2.35	not complying	untypical
8	-11.17	-9.36	-11.96	n.b.	-1.81	not complying	untypical
9	-11.58	-10.35	-12.08	-10.87	-1.23	not complying	untypical
10	-11.23	-11.16	-11.42	-12.61	-0.07	complying	typical
11	-11.6	-13.15	n.b.	n.b.	1.55	not complying	untypical
12	-11.96	-12.57	-12.25	n.b.	0.61	complying	untypical
13	-11.9	-11.81	-12.17	-11.86	-0.09	complying	untypical
14	-11.29	-13.15	-12.78	n.b.	1.86	complying	untypical
15	-11.39	-10.52	-11.43	-11.62	-0.87	not complying	untypical
16	-11.22	-9.52	-11.75	n.b.	-1.7	not complying	untypical
17	-11.44	-11.89	-10.95	n.b.	0.45	complying	typical
18	-11.1	-10.11	-11.75	n.b.	-0.99	not complying	untypical
20	-11.67	-12.67	-10.98	-12.08	1	not complying	untypical
21	-11.24	-12.95	-10.15	n.b.	1.71	not complying	untypical

In some cases the evaluation of both analysis techniques are not consistent with each other, like for sample no. 12. The NMR profile shows deviations in the aliphatic and aromatic region of the ^{1}H-spectrum which lead to an untypical result in comparison with the agave syrup database. But such deviations are known to be a result of different processing and not an indication for adulteration. Therefore we always recommend both analyses in combination to reveal us much information about the sample as possible.

^{1}H-NMR

The NMR spectroscopy is used since the 1960s for structural analysis of molecules and is now implemented in food authenticity testing, e.g. for juice, wine or honey.

In an ^{1}H-NMR spectrum of a product (like e.g. honey) a so called fingerprint profile can be observed depending on the product's geographical and botanical origin. The same applies for agave syrup. However, in this case it is limited in the geographical origin (only Mexico) and also fewer botanical origins (compared to honey) are available like for example the agave tequilana weber and salmiana.
A single measurement leads to the quantification of several agave components like sugars (fructose, glucose, sucrose, etc.), organic and amino acids and processing parameters (HMF). The focus on these single parameters (including their quantification) is called a targeted analysis. The quantification for both high and low concentrated substances, like fructose (RSD: 1.9 %), glucose (RSD: 2.0 %) and sucrose (RSD: 2.4 %), is highly reproducible.
Additionally untargeted analysis reveals deviations in the fingerprint profile of the NMR spectrum in comparison with a database.

More than 400 agave syrups from different botanical origins, harvest years and different producers were collected and measured by means of ^{1}H-NMR and δ^{13}C -IRMS in order to build up the database. Right now approx. 200 of those agave syrup samples show no deviations in the NMR and comply with authentic agave syrup according to the δ^{13}C-IRMS. Therefore, these 200 samples were used for the database.

The following tables show the specification of the NOM-003-SAGARPA-2016 (table 4) and the distribution of fructose, glucose and sucrose of all measured agave syrups including the database samples (table 5).

Table 4: Specification of the NOM-003-SAGARPA-2016

Parámetro	Valor Mínimo	Valor Máximo	Expresado en unidades	Método de Prueba
Humedad	20	28	(g/100g)	Ver apéndice B "Método de prueba para la Determinación de Humedad en el Jarabe de Agave" (Apéndice B)
pH	4.0	6.0	unidades de pH	NMX-F-317-NORMEX-2013 (Ver 3 "Referencias")
Cenizas	--	0.60	(g/100g)	NMX-F-607-NORMEX-2013 (Ver 3 "Referencias")
Sacarosa/difructosa	0.015	1.00	(g/100g)	Ver apéndice A "Método de prueba para el análisis del perfil de carbohidratos del jarabe de Agave " (Apéndice A)
Glucosa	3.00	12.0	(g/100g)	Ver apéndice A
Fructosa	60	75	(g/100g)	Ver apéndice A
Fructanos del agave	[*] (valor positivo)	5	(g/100g)	Ver apéndice A
Manitol	0.005	1	(g/100g)	Ver apéndice A
Otros Azúcares propios del Agave	--	0.1	%	Ver apéndice A
Carbohidratos (incluyendo azúcares) no propios del agave	No se permite	No se permite	%	Ver apéndice A
Hidroximetil Furfural	--	0.7	(g/100g)	Ver apéndice A

Table 5: Overview of the main sugars (fructose, glucose and sucrose) of 485 measured agave syrup samples

Total measured sample numbers	**Fructose** 60-75 g/100g	**Glucose** 3-12 g/100g	**Sucrose** 0.015-1.00 g/100g
n = 485	131	193	396
fit NOM in %	27%	40%	82%

Only 27% of all measured agave syrup samples comply with the NOM-003-SAGARPA-2016 specification for fructose. In contrast, the remaining 73% are below 60 g/100g. That means in particular that the low fructose products especially demanded by the European market would hardly fit the criteria of the NOM. 40% of the agave syrup samples were within the NOM regarding their glucose content, while 53% showed higher glucose concentrations than 12 g/100g. The sucrose range is fulfilled by 82% of the 485 agave syrups. However, more than 15% of them have a sucrose concentration higher than 1 g/100g.

Figure 5 shows a so called quantilplot of an authentic agave syrup sample.

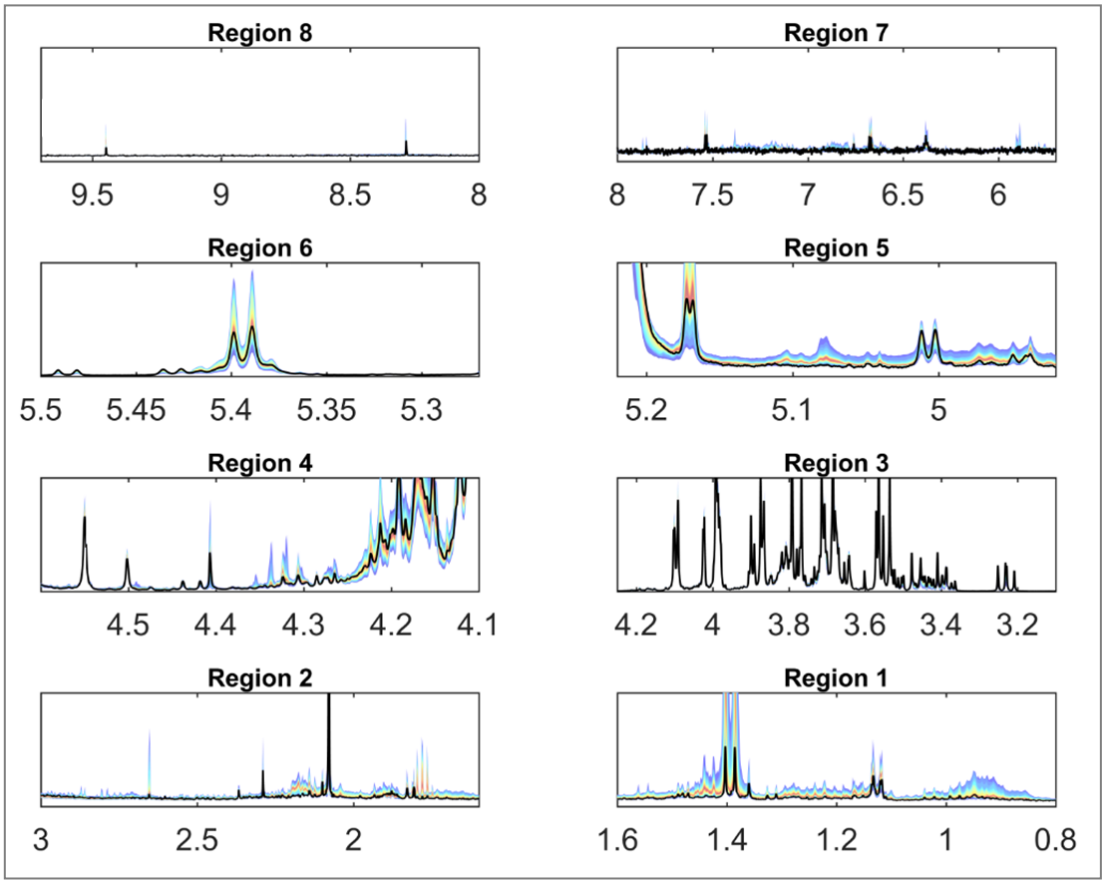

Figure 4: Authentic agave syrup ^{1}H-NMR spectrum areas [ppm] in comparison with the database divided in 8 regions

The black line shows the current measured sample and the coloured background represents the agave syrup database. The ^{1}H-NMR spectrum is divided into 8 regions. Region 1 and 2 show aliphatic compounds like organic acids. In regions 3 to 6 sugars, mainly fructose, glucose and sucrose and in regions 7 and 8 aromatic compounds (like HMF) can be observed. If the investigated sample lies within the database profile (coloured background) and is close to (or even covering) the red line the agave syrup is evaluated as authentic and therefore not adulterated. The red line is representing the average of all authentic agave syrups in the database, while the other colours represent less common areas.

Deviations in the NMR spectrum can be caused by adulteration (see figures 6 and 7) or different processing steps. Therefore, we are also able to distinguish between different agave syrup producers.

One way to adulterate agave syrup is for example to blend it with other cheap syrups, like high fructose corn syrup (HFCS). This syrup consists mainly of fructose and is therefore perfectly suitable for adulteration.

Figure 6 shows a quantilplot of a blend of 60% agave syrup and 40% HFCS.

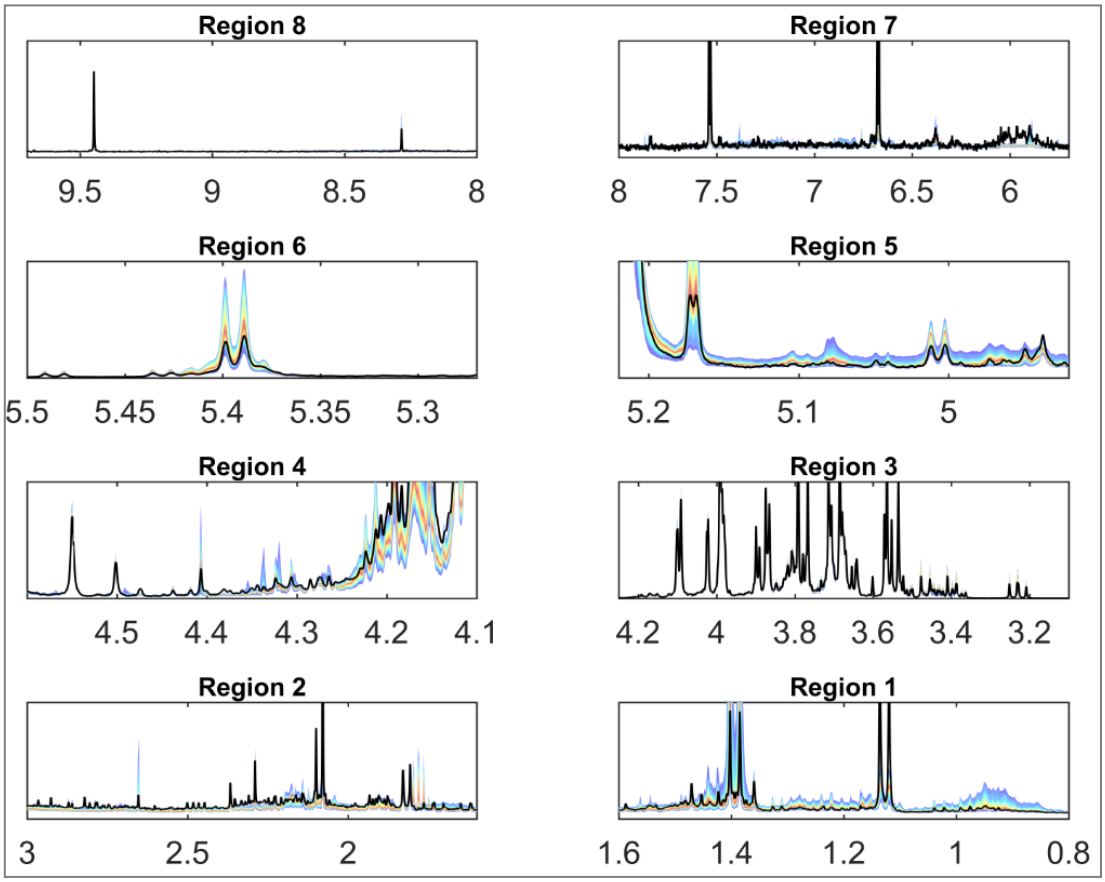

Figure 5: ^{1}H-NMR Spectrum areas [ppm] of agave syrup adulterated with HFCS (60:40) in comparison with the database divided in 8 regions

Region 3 shows a lower glucose concentration and regions 3 and 4 simultaneously a higher fructose quantity. Additionally in region 7 at 6 ppm a not authentic multiplet (in comparison with the database) can be observed. If more syrup is added to the agave syrup the ratio between fructose and glucose (F/G) is increasing even more compared to authentic values, thus indicating adulteration.

Figure 7 shows a quantilplot of an agave syrup sample adulterated with a different type of syrup. It can clearly be observed that the entire spectrum (aliphatic, sugar and aromatic region) shows major deviations in comparison to the database. Furthermore, the sample is slightly fermented and additional sugars like glucose and maltose appear in high amounts in contrast to the low sucrose and fructose concentrations.

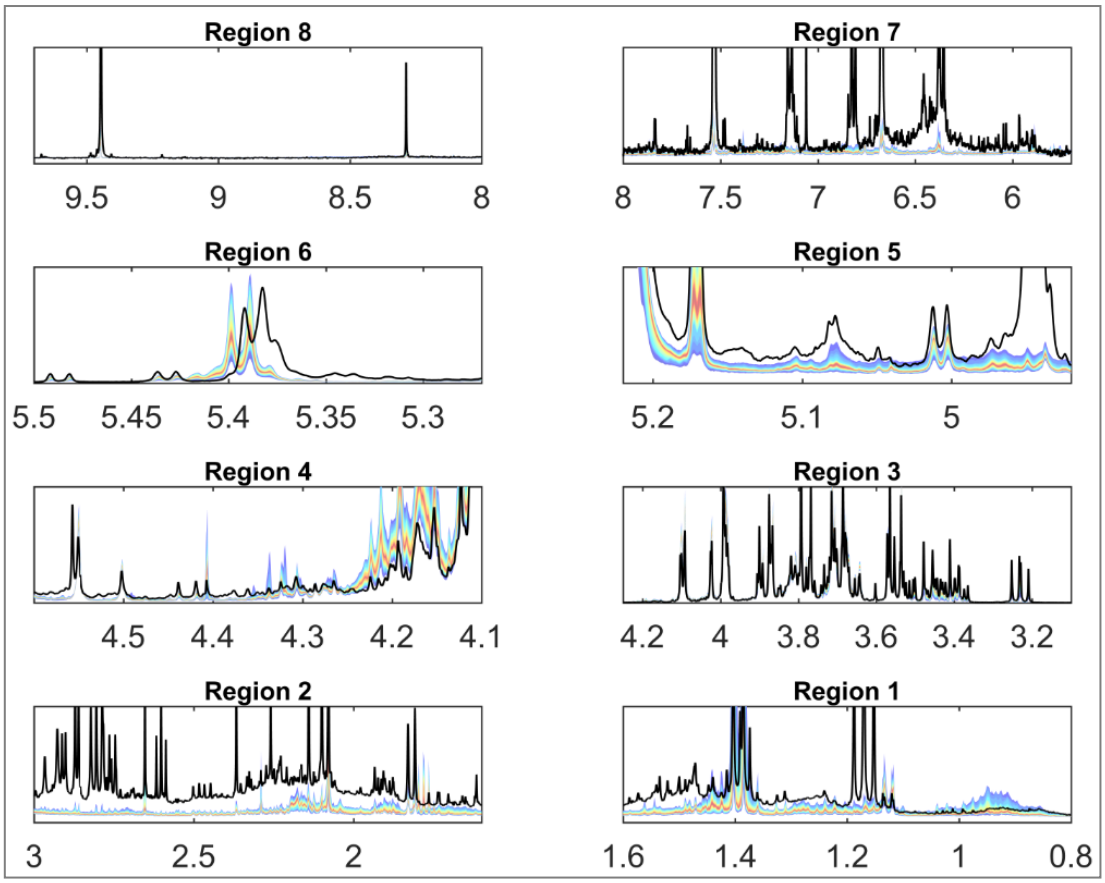

Figure 6: ^{1}H-NMR Spectrum areas [ppm] of adulterated agave syrup sample no. 8 (table 3) in comparison with the database divided in 8 regions

The profile (fingerprint) of the adulterated agave syrup indicates a blending with C4-cane sugar syrup.

^{1}H-NMR profiling vs. SNIF-NMR

In our experience the ^{1}H-NMR profiling of agave syrup is more precise, efficient and faster than the above described ^{13}C-SNIF-NMR method. Only a low sample amount and a comparably quick sample preparation are necessary.

5 g sample is diluted in a buffer and a pH of 3.1 needs to be adjusted. After this step an internal standard is added and the sample is measured by ^{1}H-NMR at 400 MHz. Overall, this results in a total analysis time, including database comparison and the corresponding evaluation, of approx. one hour.

The following sample was analysed by SNIF-NMR and was evaluated as not adulterated. In contrast to this, the ^{1}H-NMR profiling shows major deviations in comparison to the database (see figure 8). The fructose is with 66.9 g/100g on a normal level, but glucose could not be detected because of the low amount. In addition, the sucrose concentration is low as well. Those deviations in sugar content do not comply with authentic agave syrup.

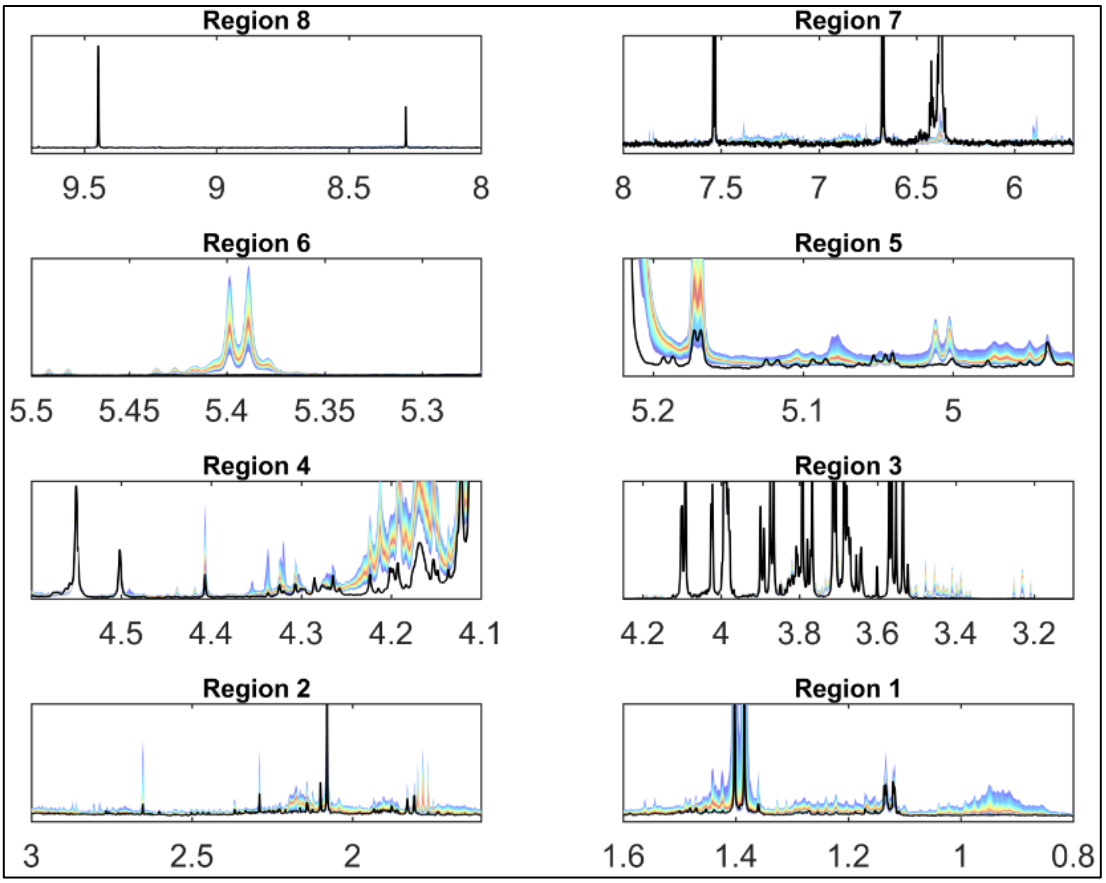

Figure 7: Deviating agave syrup ^{1}H-NMR spectrum areas [ppm] in comparison with the database divided in 8 regions

Conclusion

As we know due to our expertise regarding honey adulteration, nowadays sugar syrups are produced tailored to mimic authentic samples and are nearly free of any marker substances or traces from the production or cleaning process. For this reason we developed further methods which are more sophisticated in detecting possible adulteration which cannot be seen via HPLC-ECD as described in the NOM or other methods like oligosaccharide profiling or even SNIF-NMR.

The Mexican NOM is a good attempt at standardisation of the agave syrup on a very high quality level, but the described techniques are difficult to implement, cost intensive and can lead to false negative results. In our opinion the NOM in its present form is not suitable for a reliable testing. In addition we observed a lot of problems during the implementation of the NOM due to partially inconclusive and defective writing of the method descriptions. From our point of view it is difficult to apply the methods according to NOM without mayor changes.

We recommend for adequate analysis of agave syrup adulteration the combination of ^{1}H-NMR profiling together with LC-IRMS. These two methods are state of art for adulteration detection techniques and are even able to discover highly purified syrups. Additionally, these two methods are fast routine techniques with little sample preparation. The use of a low amount of sample material is furthermore very economical, since it leads to less wasted samples and consequently to lower shipping costs. Moreover, these methods are highly reproducible and within a reasonable pricing.

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