Critical Assessment of Methodologies used for the Characterization of Agave Syrups

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EXECUTIVE SUMMARY

As with all natural products there is a need to develop a sampling scheme so that raw materials are checked on a regular basis for safety and quality parameters to protect a supplier's reputation.

For agave syrups there is a Mexican standard that provides some information and gives general compositional guidelines for this material. This standard lays down such parameters as a minimum value for the soluble solids content and fructose and maximum levels for hydroxylmethylfurfural (HMF), glucose and sucrose. Although these parameters are included in the Standard, they are not the most sensitive at detecting the addition of other sugar syrups to agave products. The use of isotopic and other "screening" methods are required as they are more sensitive to adulterations than the conventional parameters.

Some safety parameters which would be useful to include in a screening program are heavy metals (e.g. arsenic, cadmium, mercury and lead), agrochemicals residues (such as pesticides residues) and cleaning agents such quaternary ammonium detergents ("Quats"). Although these items do not impinge directly on the "authenticity" of agave syrups, the FDA has taken the view in the past that if a product is contaminated with another material, such as a heavy metal or mycotoxin, the product is considered adulterated. Therefore if a screening program is being set up, the incorporation of these elements should be considered.

Unlike the case of honey, the use of global delta^{13}C data is inappropriate to check for the addition of cane or corn derived materials to agave syrups, due to their close isotopic values. Presented in this report is reasoning why the use of High Performance Liquid Chromatography linked with Isotope Ratio Mass Spectrometry (HPLC-IRMS) is also insensitive to this type of adulteration.

However, a site specific NMR method (^{13}C-SNIF-NMR) has been shown to be very useful to detect additions of both C_4 and C_3 derived sugars to agave syrups. Capillary Gas Chromatography linked with flame ionization detection (Cap-GC-FID) has also proved to be a very useful screening method to detect the addition of starch derived and invert sugar syrups.

It is our contention that these two methods provide the best approach to detect the extension of agave syrups with other cheaper sugar materials.

INTRODUCTION

The extension of sugar rich products, such as agave syrups, honey and fruit juices, with cheaper sugar sources without labeling such additions has been a problem for many years. It has been found that as control laboratories developed new ways of detecting such additions producers have adapted their mixing strategies in order to better conceal their blending activities.

The production of sugar syrups from Agave plants, Agave tequilana & Agave salmiana, has been carried out for many years, but the majority of this production went into the preparation of alcoholic beverages such as tequila and mescal. However, more recently these syrups have found another outlet. Due to their low glycemic index (GI), and the bad publicity that surrounds high fructose corn syrups, their use as a sugar substitute has increased in popularity.

Agave syrups are produced by the hydrolysis of inulin, a polysaccharide found in Agave plants, which they use as their main sugar storage vehicle. Unlike starch, which is the "normal" molecule that plants use to store their sugar, inulin is a polyfructan (fructose molecules linked together) typically containing terminal glucose residues. During agave syrup production the sugar polymers are either:-

a) hydrolyzed by a combination of the natural enzymes from the plant and heat
or
b) by added enzymes, in a "cold" process, which limits browning during processing and so produces a higher quality syrup.

These hydrolysis processes break the polymers down to typically fructose, glucose, low levels of sucrose and oligofructoses. Although it has been reported that these syrups contain significant levels of oligosaccharides, this was not the finding of Willems and Low's (1) recent paper on this subject.

The extension of agave syrups is possible via a number of different routes. It can involve the addition of simple cane sucrose, which would be available in Mexico as it is grown in hot climates. However, the level of adulteration using this type of material is limited to less than 4% because of the inclusion of the maximum for sucrose given in the Mexican standard (2) for agave syrup.

High fructose corn syrup {HFCS} (55DE or 90DE) has also been used for extending agave syrups. The latter is a very good extender for agave as it shares similar concentrations of the main sugars (fructose and glucose) and so does not distort the proportions of these simple carbohydrates. These corn derived syrups are prepared from starch, the main storage carbohydrate of this plant. They are significantly less expensive than agave syrups and are readily available. In Willems and Low's paper an average price of agave syrups was given as $1.27/lb and that for HFCS was $0.24/lb. This provides a large incentive for processors to extend/blend their products with these lower cost materials without proper label declaration.

Since publication of this paper there has been a sharp price rise for the agave pinia, the raw material for agave syrup, which has more than doubled with a corresponding increase in the cost of the syrups. The value of agave syrup FOB Mexico in 2011 was around $25 million, it is now in excess of $50 million due to this increase in raw material costs.

While the purpose of this paper is to address agave syrup characteristics that are useful in assessing authenticity, it is appropriate to note that the published Mexican mandatory specification for agave syrup [2] is part of the NOM (Official Mexican Standard) where adherence is affirmed by a compliance statement (avadavat) by the processors. The Agave Processors Association ANIJFA (Asociacion Nacional Industrial de Jarabes and Fructanos de Agave, CA) is in communication with the Mexican authorities to have agave syrup characteristics, specifications and product quality included in the NMX (Mexican Standards) (for additional information, see http://economia.gob.mx/standards/mexican-standards-catalog). Parallel to that, Eurofins and the scientific community continues to make progress in assessing product characteristics and testing means to differentiate between 100% agave syrups and those that have been blended with other sugars/syrups.

OBSERVATIONS FROM COMMERCIAL SAMPLE TESTING

Eurofins has been analyzing commercial agave syrups for several years and found a high level of nonconforming materials. The majority of these failures have been due to the presence of maltose and isomaltose in the samples, which would not normally be expected to be present in agave syrup.

In the US, of the syrups examined, 44 % of the products analyzed were seen to contain the HFCS marker compounds by Cap-GC. In Europe a higher failure rate (68 %) was seen but here both Cap-GC and ^{13}C-SNIF-NMR procedures are regularly in use.

These data do not represent a systematic study of either the US or European markets. They represent samples submitted to our laboratories for analysis and may not reflect the whole market. Notwithstanding this rider, they do indicate that purchasers of these materials should develop a routine screening program to protect their reputation as there seems to be a high level of undeclared blending of syrups taking place.

The following section of this report will review the methodologies available for the assessment of agave syrups.

METHODOLOGIES

Sugar analysis

The main sugar components of agave syrups have been reported to be fructose (ca 80%) and glucose (ca 10%). Lower levels of sucrose, two polyols {reduced sugars} mannitol & inositol have also been seen. Although it has been stated that agave syrups contain a range of oligosaccharides, in Willems and Low's paper [1] only a low level of these oligomers were detected.

HPLC can be used to determine the major sugars using either an amino column, a calcium loaded or mixed resin, or polymer columns. These separation methods can be linked with a range of different detection procedures such as: - refractive index, electrochemical (Pulsed Amperometric, PAD) or evaporative light scattering (ELSD). The major sugars (fructose, glucose and sucrose) can also be measured using enzyme linked procedures [17,18], which may offer more selectivity than the HPLC route. The minor sugars and polyols can also be determined by HPLC linked with either PAD or ELSD.

In Willems and Low's paper they reported data on a total of 19 agave syrups (blue and salmiana varieties) from two production seasons. The following mean values were found for the major carbohydrates and polyols:

Table 1: Main sugar/polyol levels taken from Willems and Low's paper (n=19) (1)*

	Fructose (%)	Glucose (%)	Sucrose (%)	Mannitol (%)	Inositol (%)
Mean	84.29	8.33	0.16	0.7	0.38
Standard deviation (SD) among samples	5.58	2.87	0.28	0.64	0.04

** Data as reported in Willems and Low's paper, which are assumed to be on a dry weight basis*

They found the sucrose level to be very variable, with many samples containing no detectable concentrations (limit of detection (LD) = 0.01 %). None of their samples contained a level close to the 4% maximum listed in the Mexican standard. This probably reflects the fact that the method of analysis used at the time of the preparation of the Mexican standard may not have been capable of separating sucrose from other disaccharides in the syrups.

It was noted in Willems and Low's paper that when they used HPLC-PAD they also detected levels of sucrose, up to 4%, but Cap-GC analysis on these samples showed that this was not actually due to sucrose but another co-eluting disaccharide inulobiose (Fruβ(2β1)Fru). These disaccharides were not resolved using HPLC but were separated from each other by Cap-GC. This is not an unusual problem in sugars analysis, where there is sometimes insufficient difference in the molecules (polarities, shape) to allow resolution on the HPLC column.

Eurofins' laboratory in Des Moines, Iowa has observed similar sugar results in the samples that have analyzed and judged to be authentic, data presented in Table 2. For 73 "pure" samples similar mean values for fructose, glucose and inositol were seen to that reported in Willems and Low's paper.

Table 2: Sugar/polyol data from 73 agave syrups analyzed at Eurofins Des Moines (IA) judged to be authentic by Cap-GC screening*

	Fructose (%)	Glucose (%)	Sucrose (%)	Mannitol (%)	Inositol (%)
Mean	82.3	9.1	0.30	1.20	0.39
Standard deviation (SD) among samples	7.44	5.32	0.57	1.40	0.18

** Reported on a dry weight basis*

However, in our larger dataset there was more variation in the concentrations of the sugars, as seen from our significantly higher standard deviations (SD). Generally higher concentrations of sucrose and mannitol were also detected in our dataset, with our means around twice the values reported in Willems and Low's paper.

However, we did find that over 50% of our samples also contained no detectable sucrose (<0.05%) which was similar to that reported in Willems and Low's study. Only around 14% of our samples showed concentrations above 1% sucrose. These data suggest that the 4% sucrose level given in the Mexican standard may actually be too high for authentic agave syrups if a "true" sucrose value is measured.

Inositol, measured by HPLC-PAD or HPLC-ELSD, may prove to be a useful marker for authenticity as the concentration seen in syrups, which were judged to be adulterated by Cap-GC, was found to be low or not detectable. The mean concentration of inositol in these "adulterated materials" was found to be slightly less than half (0.18%) that seen in the authentic materials.

Screening samples for simple carbohydrates is a quite useful means of detecting blended syrups. However, the method is not very sensitive due to the natural variability of the levels seen in the syrups. There are also some sugar syrups, e.g. a 90DE HFCS that closely mimic these simple sugar values and so make them indistinguishable from 100% agave syrups by this method.

Oligosaccharide screening

Professor Low's group at the University of Saskatchewan has developed a number of methods to check for sugar syrup addition to fruit juices over the years. The original method, using HPLC-PAD(3), was designed specially to detect the addition of medium invert syrups to sucrose rich juices such as orange and pineapple. However, this method sometimes provided false positive results when analyzing samples which had been subjected to high thermal treatments such as those common in aseptic packaging.

His later method [4, 5] using Capillary-Gas Chromatography (Cap-GC) has proved to be very useful for assuring the authenticity of fruit juices. This single procedure will detect three types of adulterants in juices:

- High fructose syrups (e.g. HFSS, HFCS) and glucose syrups derived from starch
- High fructose syrups from inulin (e.g. HFSI) {not relevant in the agave context}
- Invert syrups derived from either cane or beet sucrose (IS)

The detection of the presence of these syrups in fruit juices relies on the detection of specific marker compounds associated with each type of syrup. This method is also one of the main procedures used by Willems and Low in their agave paper.

The addition of high fructose syrups from starch, such as those derived from corn, potato or rice, can be detected in agave syrups based on the presence of maltose and isomaltose. These two compounds are produced from starch, during its hydrolysis, in the manufacturing process. A section of a typical GC-chromatogram of an agave syrup adulterated with a high fructose corn syrup is given in figure 1 and shows the two markers for isomaltose IsM1 & 2.

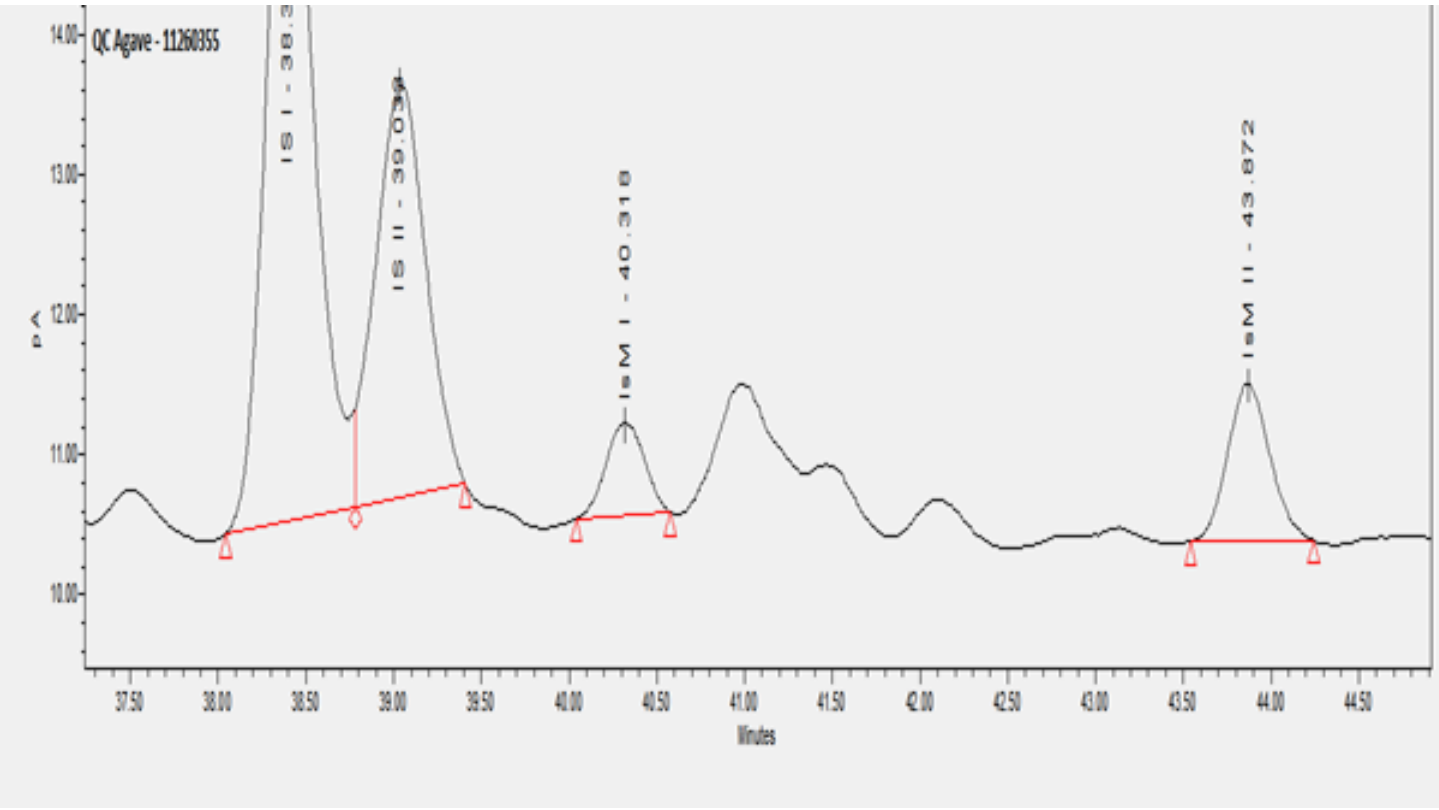

Figure 1: Portion of a chromatogram for adulterated agave syrup showing the HFCS markers (IsM1 and IsM2)

As agave syrups are produced from inulin, a polyfructan, isomaltose and maltose are not naturally seen in these products. The GC method is very sensitive and the addition of as little as 1-2 % of a typical HFCS can be detected using this procedure. The Cap-GC approach also has the added advantage that if an elevated level of sucrose is present this shows up in the middle of the GC chromatogram as a larger than normal peak and so will be detected.

Although quantitative analysis of sugars by GC has been used for many years, there are a number of problems that prohibits this procedure from being able to quantify the level of HFCS addition. The addition level of an adulterant, in an extended syrup, can only be measured if the concentration of the marker compounds in the adulterant are known, which is not generally the case. The concentrations of these marker compounds will vary from one syrup manufacturing process to another and probably between batches so no "accurate" quantification of the level of adulteration is possible.

However, this method does offer a very sensitive qualitative screening procedure for identifying the addition of high fructose syrups from starch (HFSS), glucose syrups from starch or sucrose to agave syrups.

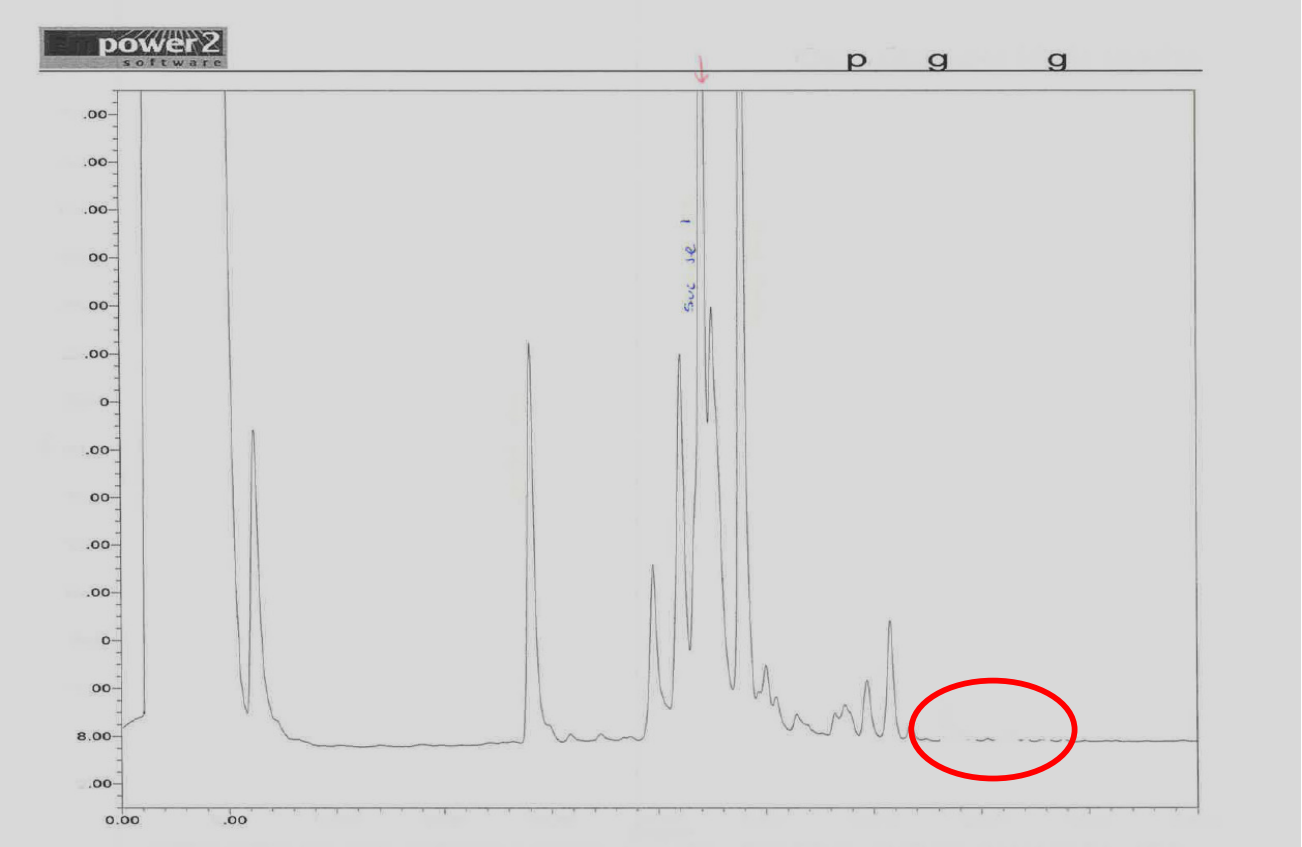

Figure 2:- Typical Cap-GC profile for pure agave syrup

If the addition of HFSS is made less attractive, by the use of the Cap-GC method, it is possible that the suppliers wishing to extend their syrups with cheaper sugar materials may switch to another syrup e.g. cane invert. This type of syrup would not show the isomaltose/maltose markers and so its addition to an agave syrup might be missed.

Cane inverts syrups contain equal levels of glucose and fructose and so do not make ideal adulterants for agave syrups as they distort the concentrations of the simple sugars. However, it would also be possible to further treat these syrups, with a glucose isomerase, to enhance their fructose contents, and allow a higher level of syrup addition to take place before it distorts the normal concentrations of glucose to fructose seen in agave syrups.

Invert syrups are typically prepared by the acid or enzymatic hydrolysis of sucrose at an elevated Brix level. During this process two other markers (IS I & II) are formed that can be used to identify this type of sugar addition. Although these two compounds are naturally seen in agave and other inulin derived syrups (Chicory and Jerusalem artichoke) the ratio of their levels is very different from that seen in invert syrups. This allows a cut off ratio and acceptable size for these peaks in an agave syrup to be defined.

In studies on apple with invert syrups added it was found that internally consistent values can be obtained within a laboratory and externally consistent interpretations of pure or adulterated materials were obtained when collaborative tested [19]. However, there was more variation in the size and ratio of these peaks than would typically be allowed for an analytical procedure. Further studies [5] showed that this variation depended on the specific way the test was carried out (derivatisation and chromatographic systems/conditions). This extensive work on juices showed that a consistent process could be developed and standardized criteria developed, which are detailed in the IFU recommendation #4[19]. It may be that similar normalization work will be required to better define common thresholds for these marker compounds in agave syrups.

ISOTOPIC TESTING

Global carbon isotope testing

Agave plants are unusual as they use the Crassulacean Acid Metabolism (CAM) to fix carbon dioxide from the atmosphere. This process is quite rare in plants and is mainly reserved to succulents. Plants using this route typically show delta ^{13}C ($\delta^{13}C$) values between -11.0 and -13.5 ‰ relative to PBD (a reference limestone) [6] for their organic compounds. However, in some pineapple juices, such as those from the Ivory Coast, values as low as -15‰ [7] can be seen. The typical $\beta\delta^{13}C$ values reported for agave syrups are between -12.0 and -13.6 ‰ [8, 9].

This range is a long way away from the values seen in most plants that use the photosynthetic fixation process or the so called "C_3 route". Here the rate limiting step is the actual capture of carbon dioxide from the atmosphere to form two three carbon units, hence its name "the C_3 pathway". As this is the rate limiting step there is a large isotope effect and the resultant sugars, acids etc in these types of plant are heavily depleted in the heavy carbon isotope, due to its slower rate of reaction. They hence show $\delta^{13}C$ values around -25 ‰[6].

The third route used to fix CO2 from the atmosphere is used by grasses, such as cane and corn. Here in "the C_4 route", carbon dioxide is fixed from the atmosphere to make a four carbon molecule. The rate limiting step of this reaction is not the fixation of the carbon dioxide, therefore there is a much smaller isotope effect and the sugars/acids are less depleted in the heavy carbon isotope. Here $\beta\delta^{13}C$ values are around -11.5 ‰ for cane derived sugars and -11.2 ‰ for corn starch derived syrups (e.g. HFCS).

Due to the similarity of the carbon isotope ratios seen for cane/corn and agave syrups it has not been possible to differentiate, with any confidence, between them using global ^{13}C values. However, the addition of C_3 derived sugars (such as from beet sucrose, beet invert syrups or syrups derived from a C_3 starch {such as from rice or potato}), can easily be detected from the distortion of the global $\beta\delta^{13}C$ value. Here an addition of around 10% would be detectable.

Internal isotopic testing

In the mid 1990's several teams simultaneously developed the idea to enhance the detection of added cane/corn derived sugars to fruit juices using isotope ratios between the main sugars and or acids [10, 11, 15]. These studies showed that the carbon isotope ratios of the individual sugars (sucrose, glucose and fructose) seen in a range of juices, including orange and apple, were different but related to each other.

If a C_4 sugar, sucrose or cane invert, was added the C_4 derived sugars are concentrated in one of the components (either sucrose or glucose & fructose respectively) and this causes a disturbance in the carbon isotope ratios and means that the detection level is roughly halved over the global IRMS approach.

In work carried out using liquid chromatography linked with elemental analysis and isotope ratio mass spectrometry LC-EA-IRMS on honey [12, 13] and agave syrups other teams have reported that the carbon isotope ratios for glucose and fructose were much closer, within 1.0 ‰ for honey and 0.5 ‰ for agave syrups, than seen in fruit juices. They have also suggested that this route offers an excellent method to detect the adulteration of agave syrups with C_4 derived sugars.

The measurement uncertainty of the LC-EA-IRMS method was given to be between 0.2 and 0.4 ‰, which is similar to or slightly lower than the simple elemental analysis-isotope ratio mass spectrometry (EA-IRMS) procedure, which is unusual. It might have been expected that this hyphenated technique would cause a larger uncertainty than seen with the simpler more straight forward EA-IRMS procedure.

Considering that the difference between the carbon isotope ratios for cane/corn and agave syrups is typically only around 1 to 1.5 ‰, with possible overlaps due to geographical variability, it is our contention this method has limited sensitivity for detecting the adulteration of agave syrups with C_4 sugar sources, which is demonstrated below with a worked example.

If a sample of agave syrup (with a $\delta^{13}C$ value near the mean of -12.5 ‰ and typical glucose to fructose levels of 1.5:8.5) is adulterated at the 10% level with a DE55 HFCS (with a $\delta^{13}C$ value of -11.2 ‰ and glucose to fructose levels of 4.0:5.5) the carbon isotope ratios can be calculated in the extended product as follows:

Fructose

{(% carbon due to fructose in agave syrup x $\delta^{13}C$ value) + (% carbon due to fructose in HFCS x $\delta^{13}C$ value for HFCS)}/ total carbon in sample due to fructose

=> {[(8.5 x 90/100) x -12.5] + [(5.5 x 10/100) x -11.2]}/8.2 = -12.4 ‰

Glucose

{(% carbon due to glucose in agave syrup x $\delta^{13}C$ value) + (% carbon due to glucose in HFCS x $\delta^{13}C$ value for HFCS)}/ total carbon in sample due to glucose

= > {[(1.5 x 90/100) x -12.5] + [(4.0 x 10/100) x -11.2]}/1.75 = - 12.2 ‰

Using these calculations there would only be a negligible shift in the carbon isotope value seen in the fructose and only a small shift in the glucose value, which is far too small to call significant if the measurement uncertainty was a minimum of 0.2 ‰ and the maximum difference between the carbon isotope values for glucose and fructose $\pm$ 0.5 ‰.

However, this product would actually be readily detectable in the blend by the high levels of maltose (ca 4%) and isomaltose that this standard type of HFC syrup would contain.

If a DE90 HFCS was added, the shift in the carbon isotope ratio would be even smaller as the adulterant and starting materials have similar proportions of glucose and fructose so there would be an even smaller shift in the $\delta^{13}C$ value for glucose.

Taking the measurement uncertainty and difference in $\delta^{13}C$ values into account a difference between glucose and fructose would have to be in the region of 0.7 to 0.9 ‰ before a product could be identified as a mixture allowing for the between molecule values of 0.5 ‰ plus the measurement uncertainty of 0.2 to 0.4 ‰. This value and the calculation above means that the minimum level of detection of a C_4 derived syrup to an agave product is at least as high as 50%! In conclusion this method is not very useful for identifying agave syrup with added C_4 sugars.

Quantitative ^{13}C-SNIF-NMR

Eurofins pioneered the use of Deuterium-NMR (SNIF-NMR®) and other isotopic techniques to verify the authenticity of a wide range of products e.g. fruit juice, wine, spirits, flavors, honey etc.

SNIF-NMR® was developed in the early 1980's as a means to detect the addition of exogenous sugars to wine [16]. In 2010 the technique was extended to cover the analysis of the ethanols derived from CAM plants (pineapple and agave) [14]. The authentication of these materials was not possible using the global ^{13}C-IRMS measurement, as discussed above, or Deuterium-NMR due to the similarities in the isotope ratios obtained for the ethanols derived from agave syrups and the main adulterants cane and corn. However, using quantitative ^{13}C-SNIF-NMR it was possible to differentiate between ethanols derived from the sugars from cane, corn and CAM plants, such as pineapple and agave. This differentiation is possible because the ethanol derived from CAM plants is both depleted in the heavy carbon isotope at its methyl site and simultaneously enriched at its methylene site, relative to that seen for ethanols derived from cane and corn sugars. These features allow the differentiation between these three sugar sources, see figure 3.

As with SNIR-NMR® this ^{13}C-method involves the careful and complete fermentation of all the simple sugars into ethanol. The ethanol is then carefully recovered by automatic spinning band distillation and analysed by quantitative ^{13}C-NMR using a 400 or 500 MHz Bruker Spectrometer.

This approach has successfully been applied to pineapple juices, agave syrups and tequilas[14]. The detection limit for the addition of C_4 derived sugars to pineapple juices and agave syrups is in the region of 15%. It should be noted that this is the only method able to detect the addition of C_4 derived sugars syrups, that do not show the any significant levels of oligosaccharide marker peaks, to an agave syrup.

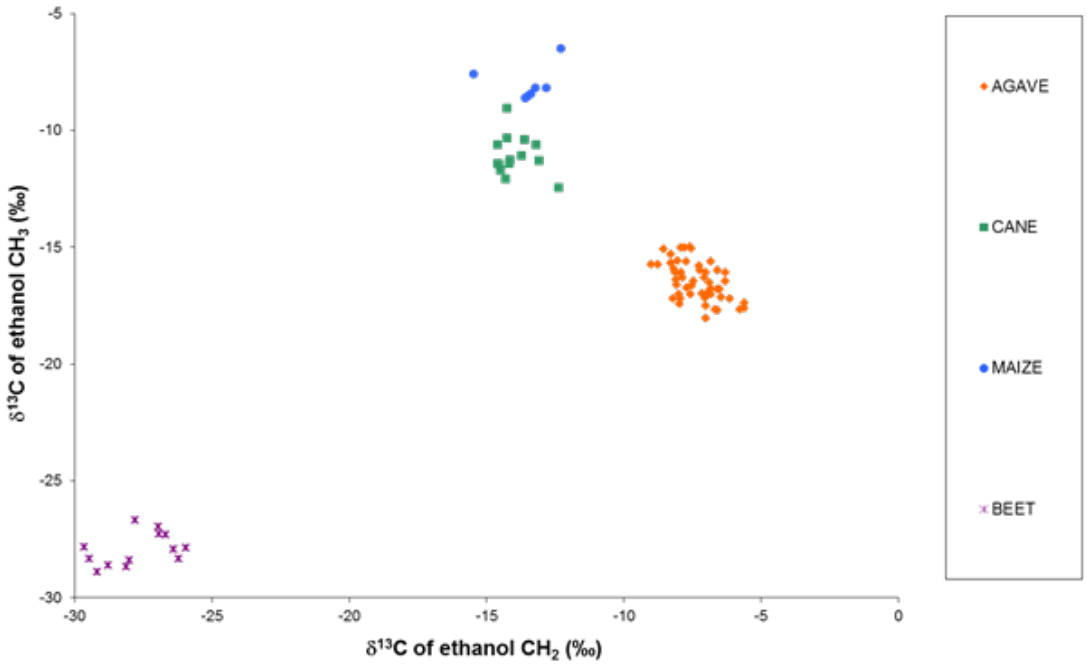

Figure 3: Plot of site specific δ^{13} values for ethanols from a range of origins, agave (orange diamonds), cane (green squares), corn (blue circles) and beet (purple crosses).

Finally, many of the tests performed are also useful in assessing product stability. If the moisture content, as reflected in a low Brix value or sum of sugars, is too high the syrup becomes susceptible to microbial growth. Other quality parameters to consider include residue testing covering pesticides, cleaning solutions, process contaminants (e.g. 5-hydroxymethylfurfural (5-HMF) and toxic metals as well as plant related components such as saponins.

CONCLUSIONS

There are many ways to assess the authenticity of agave syrups. The more methods that are used the better, as this limits the options open to suppliers to extend their products without labeling such additions.

Using conventional sugar analysis the addition of sucrose is limited by the maximum set out in the Mexican standard. Here a maximum of only 4% extension is possible. It is possible that when the Mexican standard was drawn up a method was used to set this level that could not separate sucrose from other disaccharides that are present in the syrups. This may mean that a lower sucrose level may actually be more appropriate for pure agave syrups, as shown by Willems and Low's paper[1] and in our data discussed above.

Screening of agave syrups using the Cap-GC methodology, offers a fast and cost effective qualitative method to assess for extended syrups. Currently it is somewhat difficult and expensive to fully remove the HFSS/HFCS marker compounds in the adulterant, which means that this remains a useful method. However, it should be remembered that it is not impossible to produce syrups with none of these markers.

If the use of HFSS, as an extender, is made less attractive by implementation of the Cap-GC method, it is possible that suppliers may switch to use cane invert syrups instead. Such an adulteration is also detectable using the Cap-GC procedure. However, additional normalization work is suggested to better standardize the "cut-off" values that should be used by laboratories.

Global ^{13}C measurement by IRMS is insensitive to the addition of C_4 derived syrups to agave, but will detect the presence of low levels of any C_3 derived sugars.

The internal isotope ratios method is also good at detecting the addition of C_3 derived sugars to agave syrups. However, as demonstrated above in this report, it appears to have a limited sensitivity for detecting the addition of C_4 derived sugars to agave syrups. This is due to the very close isotopic values seen for cane, corn and CAM sugars. The measurement uncertainty of the LC-EA-IRMS procedure and the variability of the isotope ratios seen for the sugars within a CAM syrup is of a similar size to the difference is ratios seen between adulterant and agave syrup.

The ^{13}C-SNIF-NMR method is a very good confirmatory procedure for verifying the addition of C_4 derived syrup or cane sucrose addition at levels above the LOD and would of course also easily detect the addition of C_3 derived sugars. This is the only method available to detect the addition of a C_4 derived sugar syrup which contains very low or no detectable level of marker compounds.

In short, there are many methods available for determining the composition and quality of agave syrups, all with their strengths and shortcomings. While no single test or combination of tests can guarantee that a product originates purely from agave, the optimal selection of tests can provide additional confidence that a product is accurately represented by its label.

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