LC-IRMS: Authenticity Control of Honey Using the Thermo Scientific LC IsoLink LC-IRMS

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Key Words
Compound Specific Isotope Analysis, Honey, HPLC, Isotope Ratio MS, LC IsoLink LC-IRMS, Sugar

Goal
Determine of $^{13}C/^{12}C$ isotope ratios of carbohydrates for authentication of honey

Introduction
Sugars are one of the biggest and most common families of molecules in life. They serve as energy resources, the backbone of DNA and RNA, the carrier of biologically active compounds and as structure building material of plants. Sugars can carry information of their origin and processing. If isotopically labeled they tell us about their pathways and metabolism.

Isotope ratio MS applications are based on the analysis of smallest isotope differences in compounds originating from physical and biochemical isotope fractionation in nature. Isotopic tracer experiments with isotope ratio MS tell the pathways and rates of sugar metabolism by using extremely low tracer amounts (<< 0.1 at %). The very high precision of isotope ratio MS even allows the administration of natural tracers like C4 compounds in a C3 based metabolism.[1] C3 and C4 are acronyms for the two pathways of CO_2 fixation in plants, which result in a $^{13}C/^{12}C$ isotope ratio difference of ca. 15 ‰ (δ-notation, ~ 0.015 at %). This is already a wide range in isotope ratio MS in relation to the high precision of better ± 0.2 ‰.

Isotope ratio monitoring-LC/MS (or LC-IRMS) with the Thermo Scientific™ LC IsoLink™ LC-IRMS is the technique of choice for the analysis of sugars. Neither coupling with GC nor with elemental analyzer (EA) can compete.

Figure 1. The Thermo Scientific LC-IRMS system.

Table 1. $\delta^{13}C$ values and reproducibilities of a sugar mixture measured by LC-IRMS.

	$\delta^{13}C$ (‰)	
	Mean	S.D. (1σ)
Sucrose	-10.75	0.15
Glucose	-10.20	0.12
Galactose	-24.48	0.16
Fructose	-11.50	0.05
Mannitol	-11.59	0.32
Sorbitol	-09.70	0.24

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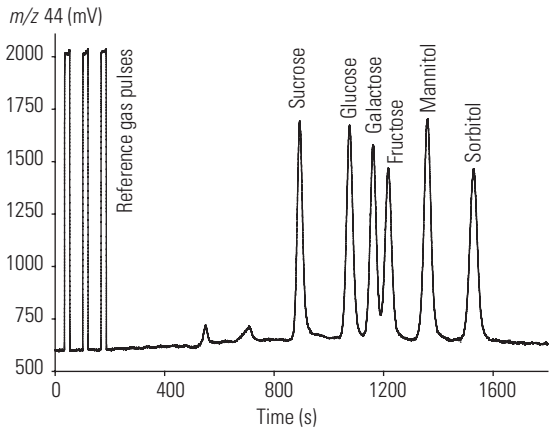

Figure 2. LC-IRMS chromatogram of a sugar mixture.

Table 2. HPLC and LC IsoLink LC-IRMS parameters.

HPLC and Interface Parameters	
HPLC Column	Cation exchange column
Eluent	Water
Flow Rate	300 µL/min
Temperature	90 °C
Loop Size	20 µL
Sample Concentration	20 ng/µL
Oxidation Reagent	0.44 M $Na_2S_2O_8$
Reagent Flow Rate	60 µL/min
Reactor Temperature	99.9 °C
He Flow	1 mL/min

Small amounts in complex mixtures can be analyzed for compound specific isotope analysis without extensive preparation or derivatization. Isotope shifts due to the acetylation of sugars for GC-IRMS are not existent. The analysis of sugars by LC-IRMS results in accurate, precise and fast results because of the absence of derivatization and the reduced number of preparation steps.

A further new method is the bulk isotope analysis of non-separated sub-µg samples in aqueous solution. The ease of sample preparation, speed (< 100 s/sample) and high sensitivity (factor 100 compared to EA) outperforms the classical EA method by far.

LC-IRMS Technology

The LC IsoLink LC-IRMS is the first high sensitivity interface connecting high performance liquid chromatography (HPLC) with Isotope Ratio MS for the reproducible and accurate on-line determination of $^{13}C/^{12}C$ isotope ratios. All organic compounds eluting from an HPLC column are analyzed while maintaining the chromatographic resolution. In the LC IsoLink LC-IRMS the sample is oxidized within the aqueous solvent eluting from the HPLC, afterwards the generated CO_2 is separated from the liquid phase. This process is quantitative and fractionation-free.

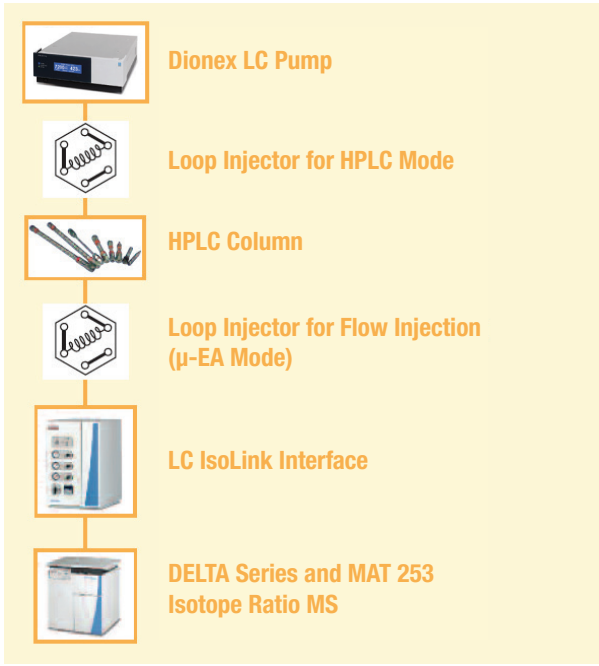

Figure 3. Scheme of the Thermo Scientific LC-IRMS workflow with the LC IsoLink LC-IRMS.

The oxidation reagent consists of two solutions, the oxidizing agent and phosphoric acid. Both are pumped separately and added to the mobile LC phase. Within this mixture all individual organic compounds eluting from the HPLC column are oxidized quantitatively into CO_2 when passing through a heated reactor. In a downstream separation unit the CO_2 is removed from the liquid phase and entrenched into a stream of He. The individual CO_2 peaks in He are subsequently dried in an on-line gas drying unit and then admitted to the Isotope Ratio MS via an open split interface.

The $\delta^{13}C$ value is the $^{13}C/^{12}C$ ratio of the sample related to the $^{13}C/^{12}C$ ratio of a reference material to ensure international compatibility of data sets.

$$\delta^{13}C = (({}^{13}C/{}^{12}C)_{Sample} / ({}^{13}C/{}^{12}C)_{Reference} - 1) \times 1000$$

For a rough estimation $\delta^{13}C$ relates to atom% divided by 1000.

Detection of Honey Adulteration

Honey is a high quality natural sweetener, which is produced by bees from flower nectar or from honeydew. Floral honey is composed mainly of glucose and fructose with sucrose, the disaccharide of glucose and fructose, as a minor compound. Such mixtures or compounds can be added from other sources, like from high fructose corn syrup (C4 based sugars), to adulterate honey.

For the detection of honey adulteration with C4 sugars up to now the $\delta^{13}C$ value of the honey and its protein fraction is compared by EA.[2] But the limit of reliable detection of adulteration with this method is ca. 7% of C4 sugar addition. Another technique used is the pattern recognition of sugars by HPLC.[3] But low levels of C4 adulteration and especially addition of C3 sugars is very difficult to detect by bulk analysis.

Table 3. $\delta^{13}C$ values of eight honey samples analyzed by LC-IRMS and by EA.

HONEY	SUCROSE ‰	GLUCOSE ‰	FRUCTOSE ‰	FRU/GLU RATIO OF AREAS	EA HONEY(4) ‰	EA PROT.(4) ‰	ADULT.(4) %	
1	-23.3	**-23.2**	**-22.9**	1.07	**-21.8**	-24.2	**16.7**	**adulterated**
2	-11.3	**-11.2**	**-13.9**	**0.65**	**-11.9**	n.a.	n.a.	**adulterated**
3	-25.3	-24.9	-24.9	1.42	-24.8	-24.8	0.0	
4	-26.4	-26.5	-26.4	0.97	-25.4	-21.6	0.0	
5	n.d.	-26.1	-26.0	**4.53**	-25.8	-26.1	1.9	**adulterated**
6	-26.1	-25.0	-25.3	1.62	-24.3	-24.3	0.0	
7	-25.0	-25.2	-25.1	1.16	-24.2	-24.7	3.4	
8	n.d.	**-25.1**	**-26.4**	2.17	-24.8	-25.1	1.5	**adulterated**

With the combination of HPLC and isotope ratio MS this gap can now be closed. The $\delta^{13}C$ value of every individual sugar in honey can be analyzed. The comparison of the $\delta^{13}C$ of fructose and glucose, the detection of other unusual sugars as well as the determination of the sugar pattern can be determined within a single HPLC run.

The precision and reliability of the LC-IRMS method was proven by four replicates of honey No. 3 in 2 batches at 2 different days. The mean $\delta^{13}C$ value for glucose is -24.86 ‰ ± 0.05 ‰. The mean value of fructose is -24.86 ‰ ± 0.15 ‰. The sucrose co-elutes with another minor compound and is not used for precision determination.

Table 3 shows 8 honey samples which have been analyzed by LC-IRMS and by EA to show the different cases of natural and adulterated honey. The EA method was able to detect 2 adulterated honeys, the LC-IRMS method detected 4 adulterated products.

Honey 1 shows a normal Fru/Glu pattern but was suspect due to the less negative δ-value of higher than -23.5 ‰. The comparison of bulk honey and protein showed a 16.7% adulteration.

Honey 2 is obvious to be 100 % C4 based. The protein method fails as the protein can not be precipitated. In LC-IRMS a fourth sugar can be detected in front of the sucrose. The Fru/Glu ratio of the areas is 0.65, the sucrose is as abundant as the glucose. The $\delta^{13}C$ value of fructose is 2.7 ‰ more negative than the glucose, which is the final proof that this honey was artificially composed.

Honey 5 is adulterated but can not be detected by isotope ratios. Nevertheless the Fru/Glu ratio is 4.53 stating an artificial honey.

Honey 8 was not detected by the EA method as a difference of 0.3 ‰ (1.5% C4 adulteration) is within the uncertainty of the method. The difference of 1.3 ‰ in $\delta^{13}C$ of glucose and fructose clearly shows that this honey is adulterated. In combination with the Fru/Glu ratio of areas of 2.17 it can even be shown

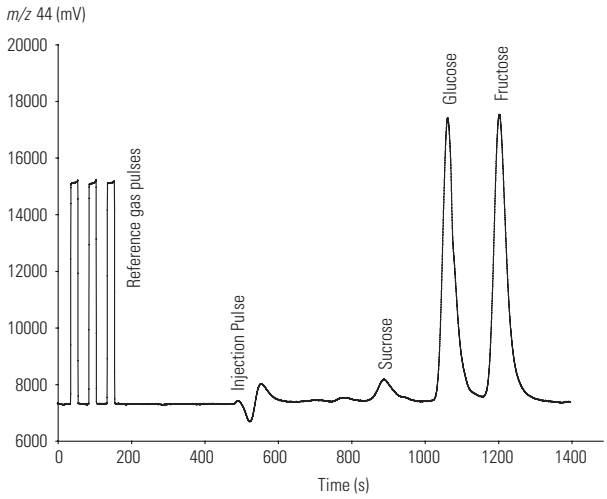

Figure 4. Honey 1.

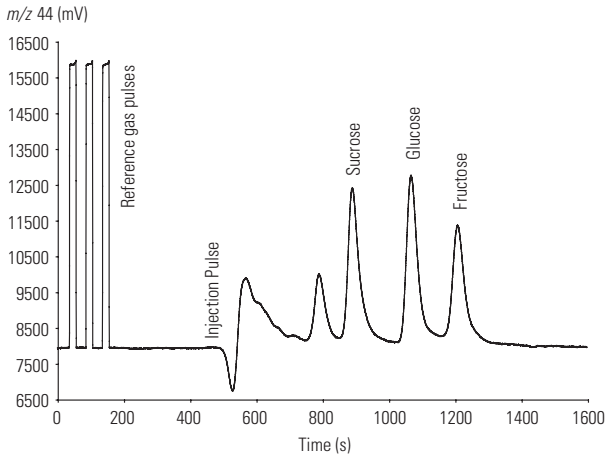

Figure 5. Honey 2.

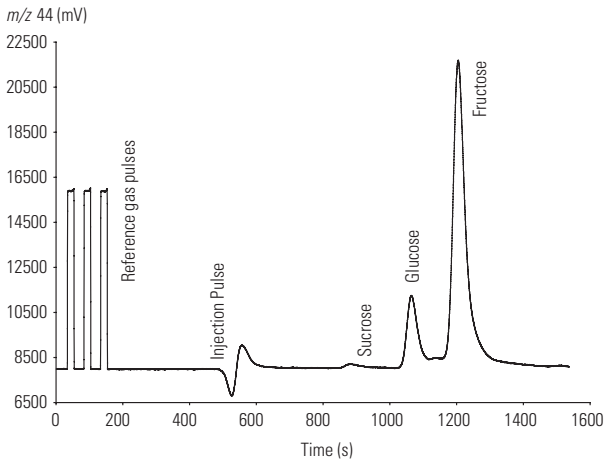

Figure 6. Honey 5.

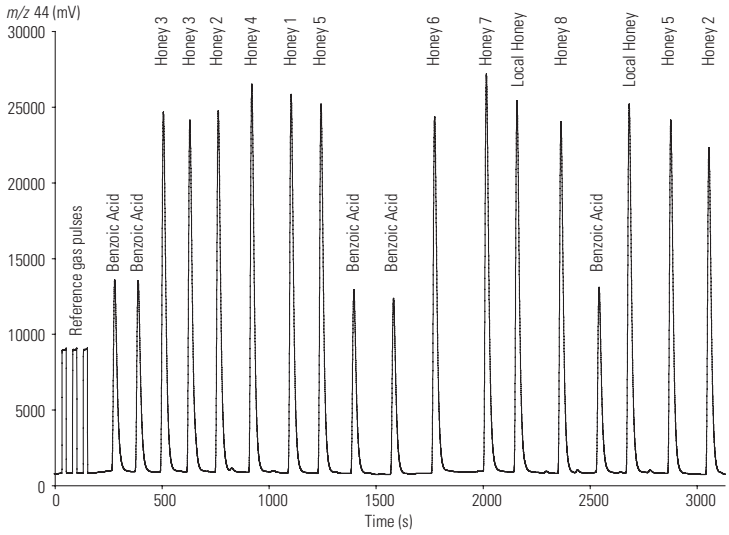

Figure 7. µ-EA: Direct loop injection of bulk honey.

µ-EA by Flow Injection

The LC IsoLink LC-IRMS offers the fast analysis of bulk samples using its direct loop injector positioned immediately after the HPLC column.

The bulk samples are processed exactly the same way as the HPLC separated compounds.

This µ-EA method can be used for very fast bulk analysis of all soluble materials as shown in the chromatogram below. The advantage of this method with an analysis cycle time of < 100 seconds is the direct comparison with reference samples of a certified $\delta^{13}C$ value.

The same technique can also be used within every HPLC run. At the beginning or at the end the bulk sample and/or some reference material can be injected with almost no loss in analysis time.

The results in table 4 were analyzed versus benzoic acid of a known $\delta^{13}C$ value of -29.9 ‰. The mean difference of 0.5 ‰ within the EA (Table 3) and the µ-EA (Table 4) results can originate from differences in referencing as well as from the fact that the µ-EA method does only analyze the compounds soluble in water.

Table 4. $\delta^{13}C$ values of eight honey samples by µ-EA.

$\delta^{13}C_{Bulk}$ (‰)		
Honey	**µ-EA**	**2nd Injection**
1	-22.66	
2	-12.66	-12.70
3	-25.44	-25.63
4	-26.30	
5	-26.04	-26.15
6	-25.16	
7	-23.71	
8	-24.94	

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