analytical chemistry

Article

pubs.acs.org/ac

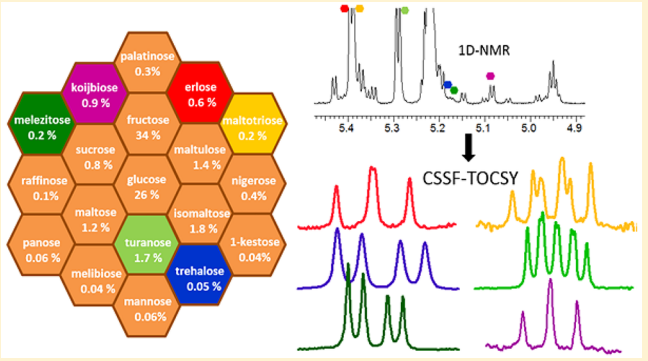
NMR Quantification of Carbohydrates in Complex Mixtures. A Challenge on Honey

Elisabetta Schievano,* Marco Tonoli, and Federico Rastrelli

Dipartimento di Scienze Chimiche, University of Padova, via Marzolo 1, 35131 Padova, Italy

Supporting Information

ABSTRACT: The knowledge of carbohydrate composition is greatly important to determine the properties of natural matrices such as foodstuff and food ingredients. However, because of the structural similarity and the multiple isomeric forms of carbohydrates in solution, their analysis is often a complex task. Here we propose an NMR analytical procedure based on highly selective chemical shift filters followed by TOCSY, which allows us to acquire specific background-free signals for each sugar. The method was tested on raw honey samples dissolved in water with no other pretreatment. In total, 22 sugars typically found in honey were quantified: 4 monosaccharides (glucose, fructose, mannose, rhamnose), 11 disaccharides (sucrose, trehalose, turanose, maltose, maltulose, palatinose, melibiose and melezitose, isomaltose, gentiobiose nigerose, and kojibiose), and 7 trisaccharides (raffinose, isomaltotriose, erlose, melezitose, maltotriose, panose, and 1-kestose). Satisfactory results in terms of limit of quantification (0.03−0.4 g/100g honey), precision (% RSD: 0.99−4.03), trueness (bias % 0.4−4.2), and recovery (97−104%) were obtained. An accurate control of the instrumental temperature and of the sample pH endows an optimal chemical shift reproducibility, making the procedure amenable to automation and suitable to routine analysis. While validated on honey, which is one of the most complex natural matrices in terms of saccharides composition, this innovative approach can be easily transferred to other natural matrices.



Simple carbohydrates are among the most important components of foodstuff and food ingredients, wherein specific mono-, di-, and oligo-saccharides can be naturally present or added to the final product for technological, nutritional, or hedonistic purposes. Indeed, in the human diet, carbohydrates are a major source of calories, as well as the cause of some potentially serious diseases. To ensure important characteristics such as quality, authenticity, and flavor, detailed information regarding the sugar composition in specific foodstuff are mandatory. Not surprisingly, there is a growing interest in the development of analytical methods for the accurate quantification of simple carbohydrates.[1−4]

The currently accepted methods for quantification of sugars and oligosaccharides are mainly based on separation techniques (possibly preceded by derivatization[5]) with different detection schemes. Due to its stable performance in quantitative analysis, high-performance liquid chromatography (HPLC) is certainly the most popular analytical method for this purpose.[6−10]

An improvement over HPLC is represented by high-performance anion exchange chromatography (HPAEC) with pulsed amperometric detector (PAD).[11] Albeit providing a fast analysis of several sugars, this technique is affected by interfering substances such as lipids and proteins, which must be pre-emptively removed.[12]

Carbohydrates can also be determined and quantified using GC, provided they are derivatized either as alditol acetates or as trimethylsilyl derivatives. In both cases, two reactions are needed.[13−15]

Recently, GC-MS[16] and LC-ESI-MS/MS methods have been reported in the separation and quantification of sugars; the performances of these two techniques were compared to HPLC with evaporative light scattering detection (HPLC-ELSD) in the analysis of small-molecule carbohydrates in jujube extracts.[13] While achieving a better sensitivity, it was found that MS can reliably detect most analytes but with lower recoveries than those of HPLC-ELSD.

FT-IR has been also successfully employed to analyze glucose, fructose, and sucrose content composition in fruit juices and honey. In this case, an ATR accessory is commonly employed, and calibration sets containing mixtures of the sugars are necessary to develop a partial least-squares regression model to fit the data.[17,18]

Finally, among other separation techniques that can provide high resolution, capillary electrophoresis (CE), has been occasionally selected for the determination of sugars in food products.[19,20]

In general, however, most of the aforementioned methodologies are either restricted to few sugars, or they do not always deliver a satisfactory resolution.

Received: September 6, 2017
Accepted: November 6, 2017

DOI: 10.1021/acs.analchem.7b03656
Anal. Chem. XXXX, XXX, XXX−XXX

Nuclear magnetic resonance (NMR) is a powerful tool in the analysis of a large number of constituents in complex mixtures,[21] and in the past few years, the use of quantitative NMR (qNMR) as a tool in food analysis has increased considerably because of the availability of high-field NMR systems.[22−25] qNMR is nondestructive, highly reproducible, precise, and accurate. Moreover, it can simultaneously quantify several compounds without the need for chromatographic separation, it requires minimal sample treatment and no derivatization steps. These features have fueled an increasing use of qNMR as an alternative to LC-based quantitation, which is reflected by the rise of new literature on qNMR applications.[24,26,27]

Not surprisingly, research in this field has taken many routes: improvements in the spectral resolution are pursued by switching from 1D to 2D techniques (mostly HSQC[28]), while the intrinsic low sensitivity of NMR is tackled by use of high fields, cryogenic probes, and, more recently, even by hyper-polarization or dynamic-nuclear polarization techniques.[29]

Despite its potential, NMR spectroscopy is seldom used for the profiling of carbohydrates. The principal reasons for this are (1) the severe resonances overlap due to the modest variance of ^{1}H chemical shifts in sugars; (2) the low sensitivity for low-concentration sugars; and (3) the conformational equilibria of different anomeric forms for reducing sugars.[25,30] Nonetheless, qNMR methods based on deconvolution of standard ^{1}H NMR[31−33] or multidimensional NMR[34] are being very strongly supported for routine analysis of fruit juice, wine,[35] and, more recently, also for honey. Although useful for fast screening, these methods generally lack accuracy, are restricted to few sugars, and the limits of quantification are still too high.

In this challenging context, we propose an NMR method based on chemical shift-selective filtration to enhance the selectivity on specific target resonances.[36] When complemented with TOCSY, this method allows to isolate a specific spin system for each analyzed carbohydrate, despite the similar structure of these molecules. An analytical procedure has been developed and tested on honey samples, without any pretreatment and derivatization other than dissolution in D_2O. Eventually, up to 22 oligosaccharides were identified and quantified in genuine honey from several different botanical origins (4 acacia, 3 chestnut, 3 linden, 3 orange, 3 honeydew, 3 cherry, 3 coriander). To the best of our knowledge, this represents one of the largest number of quantified carbohydrates in the currently available literature.

METHODOLOGY

NMR. In the analysis of complex mixtures, 1D TOCSY experiments featuring the selective excitation of ^{1}H resonances represent a straightforward approach to correlate the signals of each chemical species.[37] However, in the case of honey, the occurrence of overlapping multiplets in the anomeric spectral region often requires a selectivity beyond the reach of standard shaped (RF-modulated) pulses. Chemical shift-selective filters (CSSFs) provide an effective solution to this problem. At difference with shaped pulses, the excitation profile of CSSFs results from the constructive addition of on-resonance signals, while off-resonance magnetization components are eliminated by destructive interference.[36,38] In year 2004, Robinson et al.[39] have substantially improved the performances of CSSFs by complementing the original idea of Hall and Norwood with pulsed field gradients (PFGs). Further refinements of the gradient-enhanced CSSF have been subsequently proposed by Duncan et al.,[40] and more recently, a CSSF-TOCSY-INEPT experiment has been developed for the 1D ^{13}C spectroscopic analysis of isomeric mixtures.[41]

Considering two uncoupled spins I_1 and I_2 separated by a chemical shift difference of ν Hz, the analysis of the pulse sequence proposed by Robinson highlights the following results. When the signal from spin 1 is set on resonance and both spins resonate inside the refocusing band of the soft pulse, the relevant contributions that emerge from the filter are

$$I_{1x} + I_{2x}\cos(2\pi\nu t) \quad (1)$$

where I_{ix} represents the in-phase magnetization of spin i, and the frame of reference has been chosen as rotating at the Larmor frequency of spin 1 (namely, $\omega_1 = 2\pi\nu_1$). All the signal from spins resonating outside the refocusing band of the soft pulse are suppressed by the PFGs, and possible scalar couplings to spins 1 and 2 do not alter the outcome of eq 1. The parameter t represents the duration of the filter, which varies between the values $t = 0$ and $t = t_{max}$ to yield the following expression for the averaged signal:

$$S(\nu) = \frac{1}{t_{max}} \int_{t=0}^{t=t_{max}} I_{2x}\cos(2\pi\nu t)e^{-t/T_2} \quad (2)$$

It is useful to point out that, in the absence of relaxation, eq 2 becomes a $\mathrm{sinc}(2\pi\nu t_{max})$ function. In the experimental practice, the filter duration time is discretized into a series of N time intervals so that $t_{max} = N\,\Delta$ and FIDs collected for each N value are finally coadded. Importantly, smaller chemical shift differences require longer evolution times to provide a good filtration (Figure S-2).

Since only in-phase magnetization emerges from the filter, a CSSF can be conveniently followed by a mixing scheme, leading to highly selective 1D-analogues of 2D experiments like NOESY or TOCSY. In the latter case, the cluster of RF pulses that drives the isotropic mixing also introduces a slight heating of the sample, which may ultimately displace the resonances by a few Hz. Due to the high selectivity of the CSSF, such a slight offset can be detrimental for the experimental output and must be carefully accounted for.

The ^{1}H NMR spectrum of an oligosaccharide can be described in terms of a series of isolated spin systems—one per monomeric unit—separated by the glycosydic bonds. In the case of honey samples, such monosaccharide units are generally limited to glucose, fructose, and galactose. Since the same monomeric unit can be found in several oligosaccharides with similar sequences, the extent of NMR signal overlap is usually very high for sugar mixtures. In practice, when using a simple pulse-acquire experiment on a honey sample, only a few sugars can be determined quantitatively.[31,32] The approach proposed here is based on a highly selective resonance excitation (down to a few Hz) combined with a TOCSY mixing scheme of proper length. For a complex matrix such as honey, this experiment provides a formidable spectral simplification, yielding the subspectra of only a few monosaccharide units, depending on the number of resonances excited by the CSSF pulse scheme.

Application of this technique to the analysis of such a complex mixture requires a careful optimization of the experimental conditions, particularly of the excitation frequency and the selectivity of the CSSF. As the proton resonance frequencies prove to be highly reproducible (within ppb),[30] an optimization of the experimental parameters was carried out on standard solutions of the 22 sugars under investigation.

DOI: 10.1021/acs.analchem.7b03656
Anal. Chem. XXXX, XXX, XXX−XXX

In principle, longer CSSFs perform better in the isolation of signals specific for each sugar. Note however that a possible disadvantage in the use of long duration filters is the partial signal loss from transverse relaxation (about 10% for a 250 ms filter applied to a signal with T_2 = 1 s, see Figure S-3); in addition, the resulting narrow selection band leads to possible offset errors with significant signal loss. For such reasons, filters of shorter duration are always preferable, even in cases where the CSSF excitation profile is semiselective. In this case, indeed, the TOCSY subspectrum must exhibit some well-isolated signals stemming only from the species of interest.

EXPERIMENTAL SECTION

Chemicals. The following sugars were purchased from Sigma-Aldrich: D-Glucose (Glt) ≥ 99.5%, D(+)Mannose (Man), D(−)Fructose (Fru) ≥ 99%, D(+)Turanose (Trn) ≥ 98%, Erlose (Erl) ≥ 94%, Isomaltotriose (Imt), D(+)Melibiose (Mlb) ≥ 99,0%, D(+), Raffinose pentahydrated (Raf) ≥ 98.0%, Palatinose hydrate (Plt) ≥ 99%, L-Rhamnose monohydrate (Rha) min 99%, Sucrose (Scr) ≥ 99.5%, D(+) Maltose (Mlt) monohydrate min 98%, Melezitose (Mlz) ≥ 99.0%, Trealose dihydrate (Trl) (Certified Reference Materials), Maltulose (Mtl) ≥ 98.0%, Nigerose (Ngr) ≥ 94.0%, D-Panose (Pns) ≥ 97%, Maltotriose (Ml3), Isomaltose (Imt) 98%, Gentiobiose (Gnt) ≥ 85%, 1 Kestose (Kst) ≥ 98.0%, Kojibiose (kjb).

The buffer solution was prepared by dissolving 2.55g KH_2PO_4 and 2.45 mg NaN_3 in 50 mL D_2O (*d*- 99.97%) and then adjusting the pD to 4.4 with H_3PO_4.

Spectral Acquisition and Signal Processing. All NMR experiments were performed on a Bruker Avance III spectrometer operating at 500.13 MHz ^{1}H Larmor frequency and equipped with a 5 mm z-gradient broadband inverse (BBI) probe. All NMR samples were thermally equilibrated at 298.1 K for at least 5 min inside the spectrometer. The following acquisition parameters were used for the CSSF-TOCSY experiments (see Figure S-1): 8 scans for minor sugars (2 scans for glucose and fructose) × 14 increments; 6000 Hz spectral width on 8k points (0.7 Hz FID resolution); 1.3 s acquisition time; 2 s relaxation delay; 70 ms DIPSI-2 mixing scheme flanked by zero-quantum filters[42] (see page S4 in the Supporting Information). With these parameters, the overall duration of the CSSF-TOCY experiment was about 7 min for each minor sugar (2 min for glucose and fructose).

All the spectra were processed using a macro programmed in ACDLab v.12.5 (zero filling to 32 k, exponential multiplication with a line broadening of 0.3 Hz). Phase and baseline correction were performed in an automated way.

Automation. An accurate control of pH and temperature endows a high reproducibility (down to parts per billion) of the proton chemical shifts among different samples. Slight alterations can be easily detected (possibly by a software routine) and adjusted, for example, by referencing the H2 resonance of β-D-glucose to one fixed value (3.213 ppm in our case).

^{1}H chemical shifts of carbohydrates are largely insensitive to variations of pH or ionic strength[30] due to the absence of ionizable groups: indeed we have verified that all the sugars signals exhibit constant shifts with respect to the reference.

On this basis, it is possible to keep the acquisition parameters constant for each sample, build a list of excitation frequencies for the CSSF, and automate the acquisition of the spectra (in our case with Icon NMR software and Bruker Sample Jet hardware).

The selected signals for each analyte were automatically integrated by using the same frequency interval centered on the selected resonances, and the absolute integrated signal intensities were produced as output.

Standard Sugars in Buffer Solution. For each reference sample, both a conventional 1D spectrum and CSSF-TOCSY were acquired. The CSSF-TOCSY spectra were obtained by selectively exciting anomeric protons or other isolated protons. The resonance assignment was confirmed by literature data.[30,43]

Honey Samples. Each genuine honey sample was pretreated in a microwave oven for a few seconds until all crystals were dissolved. NMR samples were prepared by dissolving ∼240 mg of honey in the buffer solution and adjusting the ratio honey (mg)/buffer (ml) to exactly 240 mg/mL. The pD was carefully adjusted to 4.40.

Synthetic Honey Solutions. Synthetic solutions containing selected sugars among those mostly represented in honey were prepared to build the calibration curves. The range of sugars concentration was matched with the values reported in the literature for honey,[44] to reproduce the natural matrices as closely as possible. The synthetic solutions contained a constant amount of glucose (74.00 mg/mL) and fructose (98.28 mg/mL) and variable concentrations of minor sugars, which were chosen in such a way as to provide the same total amounts of sugars. The pD was adjusted to 4.40. Eight concentration intervals were considered in the range of 0.80−12.7 mg/mL. One calibration curve, for each sugar, was constructed by linear regression of the integrated signal intensities of the selected peaks against the concentrations.

In the case of glucose and fructose, the calibration curves were built by using solutions wherein the concentration of only one of the two sugars was incremented, keeping the same total concentration to about 172 mg/mL.

The calibration curves were employed to estimate the concentration of the sugars via the absolute integrated intensities of the signals selected in CSSF-TOCSY experiments. This value was converted to g/100g of honey for consistency with the literature.

Instrumental Stability. To check the stability of the instrument, the spectra of synthetic honey solution (see next paragraph) were acquired weekly for one year and the integrated signal intensities of a reference signal were compared among the spectra. The variations were found to be in the range of 0.2% − 1%.

Analytical Performance of the Method. The linearity and accuracy (precision and trueness)[45] along with the limit of detection (LOD), limit of quantitation (LOQ), and recovery were evaluated as follows.

Linearity. The linearity was tested for all 22 carbohydrates by regression analysis on the absolute integrals of the peaks of the analytes with respect to their corresponding concentrations in standard solutions.

Limits of Detection and Quantitation. The detection (LOD) and quantitation (LOQ) limits are defined as the analyte concentrations whose signal responses are 3 and 10 times the average noise level, respectively. For each sugar, the LOD and LOQ limits were determined by plotting S/N ratio of CSSF-TOCSY selected peaks (extracted by the spectra of synthetic honeys) vs concentrations.

Accuracy. The accuracy of the method was determined on synthetic honey solutions. For each sugar the precision of the method was determined by calculating the relative standard deviations (RSD, %) of an integrated signal for nine repeated measurements (three different preparations of the synthetic honey solutions and three different data acquisitions). The relative error

DOI: 10.1021/acs.analchem.7b03656
Anal. Chem. XXXX, XXX, XXX−XXX

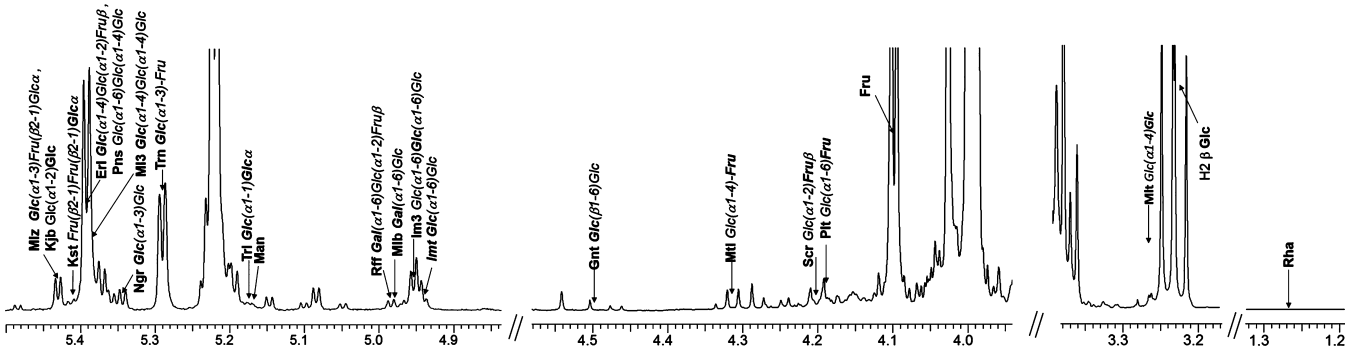


Figure 1. Selected regions of the ^{1}H spectrum of a honey sample diluted in the NMR buffer (pD = 4.40). The arrows indicate the selected excitation frequency for each of the 22 sugars under investigation. In the condensed name, the sugar moiety containing the anomeric proton excited by the CSSF is highlighted in bold on top of the corresponding arrow.

in concentration was derived by error propagation on the model used for linear least-squares regression analysis.

The trueness of the method was expressed by assessing the agreement between the measured (mc) and nominal (nc) concentrations of the sugar under investigation, as $(mc - nc) \times 100/nc$ (bias).

Analytical Recovery. Albeit the synthetic honey solutions are prepared to reproduce as closely as possible the genuine honey solutions, a recovery test was performed to evaluate possible elusive matrix effects. The most detrimental matrix effects include an alteration of the anomeric equilibria for reducing sugars[46] and/or a shortening of the relaxation times due to possible paramagnetic impurities.[47,48] The achievement of the same anomeric equilibrium between synthetic and genuine honey solutions can be tested by comparing the ratio between selected TOCSY signals from α- and β-glucose in both genuine and synthetic honey. For all the samples tested in our lab, this ratio was found to be invariant. Potential sources of paramagnetic relaxation can be tested by comparing the relative intensities of TOCSY subspectra from α- or β-glucose in genuine and synthetichoney. When the (properly scaled) traces are coincident, the relaxation effects for the two samples are similar in the adopted conditions.

After these preliminary tests, the percentage of recovery was determined for several sugars by using the gravimetric standard-addition method.[49] The relative error on concentration in standard addition experiments was calculated through propagation of uncertainty.[50]

The measurements were performed for two different types of honey (acacia and chestnut) on the following minor sugars: sucrose, maltose, maltulose, palatinose, turanose, and mannose. In addition, raffinose and melezitose recoveries were tested on honeydew honey. The stock solution contained the eight minor sugars under investigation (6 mg/mL) dissolved in the NMR buffer. A typical standard addition series consisted of seven different concentration levels of each sugar. The seven solutions were prepared by dissolving exactly 240 mg of honey in different volumes of stock solution to reach increasing concentrations of sugar and adjusting the final volume to 1 mL with NMR buffer. The sugar concentration levels were varied in the range of 0.6−3.0 g/100 g of honey. All the honey samples (genuine and synthetic) were equilibrated at room temperature for at least 24 h. The results obtained by standard additions were compared with those obtained by the calibration curves, and the percentage of recovery was calculated.

RESULTS AND DISCUSSION

The unambiguous identification and the accurate quantification of sugars in natural matrices is hampered by their similar structure and similar polarity, their lack of chromophores and the presence of many structural isomers. So far, NMR quantification of sugars in natural samples has mostly focused on conventional 1D proton NMR spectra, (without any previous separation or preconcentration steps), and the spectral overlap is dealt with by line shape deconvolution. This approach however can only be applied to those signals that are at least partially resolved (a requirement hardly met in 1D spectra of honey samples) and when all the mixture components are already known.[29]

As demonstrated in previous studies, frequency-selective 1D TOCSY experiments can largely improve the discriminatory power,[37] yet they still fail when severe spectral overlap occurs. On this premise, chemical shift-selective filters deliver a dramatic selectivity improvement, and in combination with TOCSY, they yield highly resolved subspectra of carbohydrates.[39]

Because TOCSY propagates the magnetization only within a spin system (namely, an ensemble of spins connected by network of nonvanishing J-couplings), a monosaccharide will generally provide a 1D CSSF TOCSY spectrum containing the same resonances (yet with different intensities) found in its 1D spectrum. In the case of oligosaccharides, where each sugar unit provides a separate spin system, CSSF-TOCSY experiments can be tailored to highlight the resonances of each sugar unit.

In this work we have applied this method for the quantification of 22 oligosaccharides generally present in honey samples: 4 monosaccharides (glucose, fructose, mannose, ramnose), 11 disaccharides (sucrose, trehalose, turanose, maltose, maltulose, palatinose, melibiose, isomaltose, gentiobiose, nigerose, koijbiose), and 7 trisaccharides (raffinose, isomaltotriose, erlose, melezitose, maltotriose, panose, and 1-kestose). The acquisition of a selective 1D CSSF-TOCSY for a given sugar unit requires the knowledge of the exact resonance frequency of the proton that will provide the source magnetization. In the case of honey, visual inspection of the 1D conventional spectrum (see Figure 1 for the anomeric region of honeys) is not a viable approach because of the extensive signal crowding.

The optimal frequencies for selective excitation were therefore determined on standard solutions of each individual sugar dissolved in the buffer solution (Figure S-4) rather than directly on the honey sample under investigation. More precisely, the 1D standard spectrum and different CSSF-TOCSY spectra with selective excitation of the most isolated signals (typically those

DOI: 10.1021/acs.analchem.7b03656
Anal. Chem. XXXX, XXX, XXX−XXX

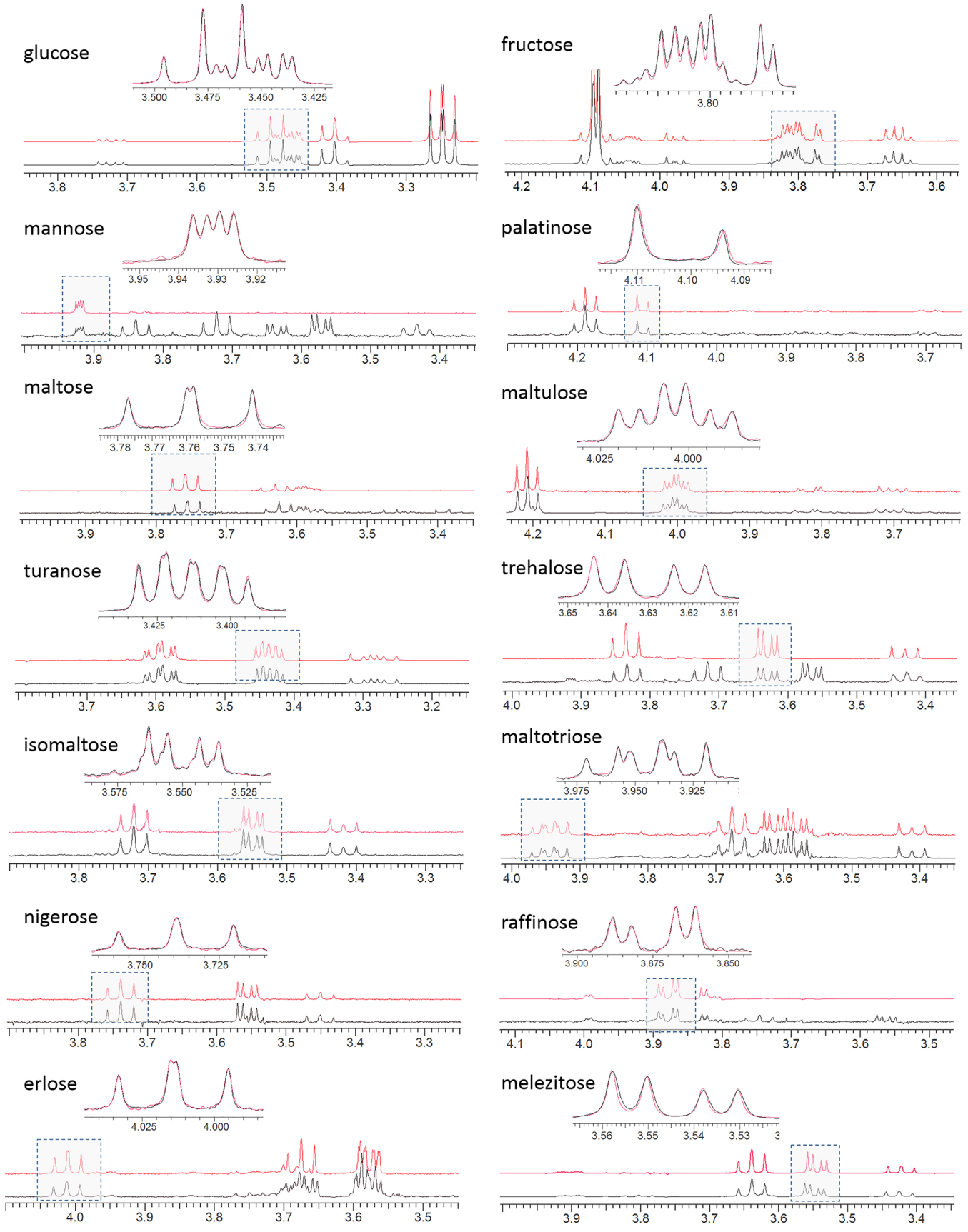


Figure 2. continued

DOI: 10.1021/acs.analchem.7b03656
Anal. Chem. XXXX, XXX, XXX−XXX

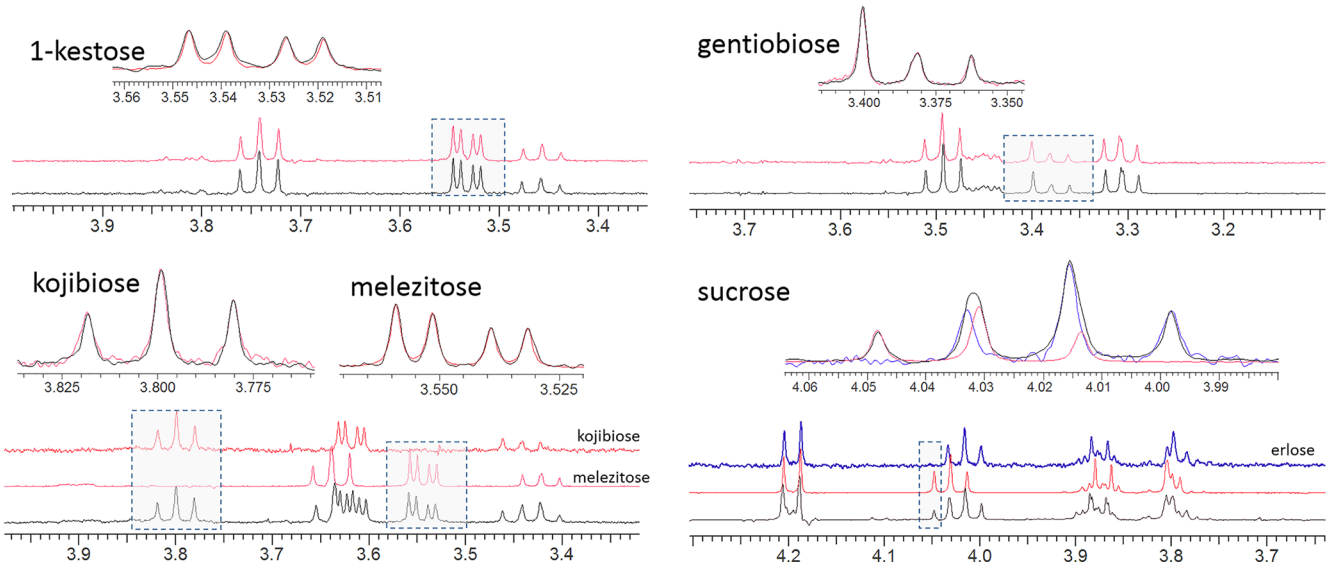


Figure 2. CSSF-TOCSY spectra of sugars in standard solution (red or blue traces) and in honey solution (black traces), respectively. The dashed area highlights the signal chosen for quantification. On top of the CSSF-TOCY traces, the overlap between honey and standard signals inside the dashed area is reported.

from the anomeric protons) were acquired for each standard solution.The comparative analysis of these spectra allowed the identification of the optimal excitation frequencies for each sugar. The same approach was used to set up the optimal filter duration time and to identify the resonances specific for each sugar, so to ensure the accuracy of the quantification by integration. Experiments on spiked honey samples were performed to confirm the excitation frequencies and to exclude overlap with other unidentified sugars. These experiments confirmed that ^{1}H chemical shifts of sugars in honey are reproducible with a precision of ppb. Consequently, the excitation frequency can be easily calculated with reference to the H2 signal of internal β-glucose resonating at 3.213 ppm. In this way, all the analytical procedures are accurate and also become automatable.

Figure 1 reports selected regions of the ^{1}H spectrum of a honey sample, along with the excitation frequencies appropriately chosen for all the 22 carbohydrates under investigation.

In some cases, the severe crowding in the anomeric region does not allow for a clean selection of the resonances of a single saccharide, and the duration of the filter must be optimized. A higher selectivity is easily reached by using a longer chemical shift filtration time, yet at the price of signal loss by relaxation (Figure S-2 and S-3). As such, semiselective CSSF-TOCSY experiments are used whenever it is possible to find a well isolated signal stemming only from the sugar of interest. On the basis of such a criterion, a shorter filtration time (50 ms) was employed for 17 out 22 investigated carbohydrates. The remaining five carbohydrates (palatinose, melibiose, raffinose, isomaltose, isomaltotriose), because of their structural similarity, display severe overlaps and require a longer filtration time (250 ms).

Eventually, we have been able to identify isolated signals that could be safely integrated and used for a quantitative analysis in all the 22 carbohydrates (see Table S-1 for summarized data). Figure 2 shows the specific TOCSY subspectra isolated from a solution of honey (black trace) and from the standard solutions (red trace).

Spectral intervals employed for quantification are also shown (Figure 2). Evidently, an entire multiplet is integrated for almost all sugars with the exception of sucrose and panose. The disaccharide sucrose, (Glc(α1−2β)Fru) has a spectrum whose resonances are significantly overlapped with those of the trisaccharide erlose (Glc(α1−4)Glc(α1−2β)Fru) (Figure 2). However, whereas in the spectrum of erlose it is possible to isolate a quantifiable signal (belonging to the first glucose of the sequence, panel “erlose” in Figure 2), the sucrose spin systems are always partially overlapped with those of erlose, and its quantification is obtained by integration of the isolated portion of the triplet at 4.04 ppm (belonging to the fructose moiety) as evident in the relative panel of Figure 2. A similar case is found also for panose (Glc(α1−6)-Glc(α1−4)-Glu), whose signals are partially overlapped with sucrose resonances.

The most complicated case of maltose (Glc(α1−4)-Glc) is discussed in the Supporting Information (Figures S-5 and S-6).

Figure 2 reports the expansions of the integrated multiplets of 18 out of 22 sugars. There is an excellent match between the traces of the analyzed sugars in honey and in the standards, despite the complexity of this matrix. In the analyzed honey samples, the four sugars rhamnose, melibiose, panose, and isomaltotriose are always found to be under the limit of quantification. Samples spiked with standards of such sugars, however, reveal the possibility to detect them in much the same way as all the other saccharides (see Figure S-7). This result highlights the specificity of the experiment: because of the large dependence of the chemical shift on the molecular structure, the resulting pattern is virtually unique.[51] Moreover, any possible overlap of signals from other sugars can be tracked as an alteration of the aforementioned traces either in the number of signals or in the signals shape (see Figure S-5 panel a). The case of raffinose in chestnut honey further substantiates the specificity of our procedure. About the resonance frequency of one anomeric proton of raffinose, the 1D spectrum of chestnut honey shows a doublet which is comfortably assigned to this sugar (Figure 3, top). However, when the CSSF is set on resonance with such doublet, the resulting TOCSY spectrum looks rather different from that of honey spiked with raffinose (Figure 3, bottom), where additional signals characteristic of this sugar are observed (red trace). Indeed, the CSSF-TOCSY prevents the occurrence of a false positive that may have

DOI: 10.1021/acs.analchem.7b03656
Anal. Chem. XXXX, XXX, XXX−XXX

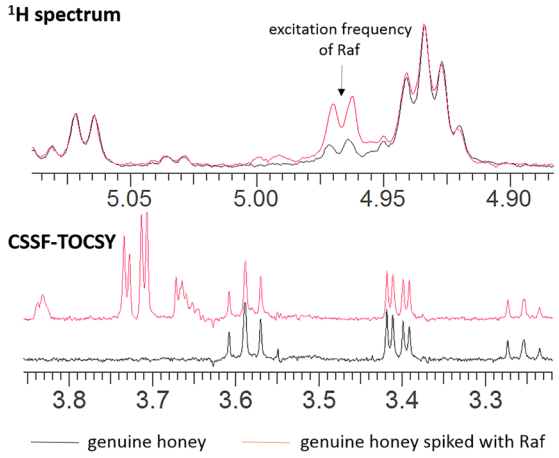


Figure 3. Top: conventional ^{1}H spectra of chestnut honey. Bottom: CSSF-TOCSY spectra of chestnut honey resulting from excitation at the frequency indicated. The black and the red traces refer to the genuine and the raffinose-spiked samples, respectively.

resulted by use of deconvolution on the conventional ^{1}H spectrum.

After the setup of the experimental parameters, the analytical performance of the method was tested. Linear responses were observed, as demonstrated by the correlation coefficients (R^2) larger than 0.995 for all analytes (Table 1 and Table S-2).

Table 1. Correlation Coefficient (R^2) and Limit of Detection (LoD) and of Quantification (LoQ) Expressed As g/100g

	sugar	R^2	LoQ[a]	LoD[a]
monosaccharide	glucose	0.9957	0.09	0.028
	fructose	0.9968	0.17	0.05
	rhamnose	0.9997	0.03	0.009
	mannose	0.9995	0.06	0.018
disaccharide	sucrose	0.9995	0.05	0.016
	palatinose	0.9985	0.05	0.015
	maltose[b]	0.9995	0.14	0.04
	maltulose	0.9991	0.10	0.03
	turanose	0.9997	0.17	0.05
	trehalose	0.9978	0.05	0.015
	melibiose	0.9931	0.13	0.04
	isomaltose	0.9978	0.39	0.11
	gentiobiose	0.9991	0.09	0.03
	nigerose	0.9983	0.09	0.03
	koijbiose	0.9965	0.18	0.05
trisaccharide	isomaltotriose	0.9979	0.18	0.05
	raffinose	0.9996	0.21	0.06
	erlose	0.9981	0.06	0.018
	melezitose	0.9993	0.13	0.04
	maltotriose	0.9993	0.30	0.09
	1-kestose	0.9986	0.14	0.04
	panose	0.9999	0.20	0.06

[a]For the proposed methodology, these values may be lowered using different dilution or/and acquisition parameters. [b]For maltose only one of the regression lines is reported (see SI).

The LoQ values, reported in Table 1, are all in the range of 0.03−0.2 g/100 g of honey except for isomaltose and maltotriose, whose values are 0.39% and 0.30% respectively. These LoQs values for most abundant sugars are similar to those obtained with HPLC-PAD[18] and lower than the values obtained from fast capillary electrophoresis[18] and from conventional ^{1}H NMR-based methods.[31,32]

The accuracy was examined on solutions of synthetic honey by performing nine measurements (three different preparations and three acquisitions) for each sugar under investigation (Table 2). All the determinations were carried out with the same experimental conditions in different days. A satisfactory precision is demonstrated by the RSD % on concentration always lower than 4%. The low bias values (ranging from 0.43 for turanose to 4.2% for panose) demonstrate the correct quantification of all the saccharides.

Table 2. Precision (RSD %) and Trueness (bias %) in Synthetic Honey

	concentration (g/100 g of honey)			
sugars	nominal	measured	RSD %	bias %
glucose	30.000	29.818	1.85	0.61
fructose	38.400	38.018	1.68	0.99
rhamnose	1.223	1.199	1.71	1.96
mannose	1.612	1.623	1.24	0.69
sucrose	1.916	1.939	1.84	1.20
palatinose	1.203	1.167	3.15	3.01
maltose	0.894	0.903	3.38	1.02
maltulose	1.233	1.265	2.18	2.59
turanose	1.241	1.235	1.52	0.43
trehalose	2.071	2.044	2.08	1.30
melibiose	2.506	2.443	3.05	2.51
isomaltose	1.305	1.339	2.40	2.60
nigerose	0.654	0.662	2.18	1.28
gentiobiose	2.025	2.026	1.30	1.39
kojibiose	0.287	0.295	3.10	2.97
isomaltotriose	0.652	0.668	4.03	2.46
raffinose	1.669	1.660	1.02	0.56
erlose	0.916	0.909	2.48	0.78
melezitose	2.191	2.145	0.99	2.10
maltotriose	0.498	0.501	1.38	0.56
1-kestose	0.532	0.508	2.79	4.09
panose	1.341	1.284	3.14	4.23

The reliability of the synthetic honey matrix in the setup of the calibration curves was proven by the results of recovery experiments carried out on three honey samples. The concentration values obtained by the calibration curves were compared with those obtained by the standard addition method (Figures S-8). In this latter case, the value of each sugar concentration can be extracted from the abscissa intercept of the correponding linear standard-addition curve.

An uncertainty of 1−2% in the concentration is obtained from error propagation.

The absence of matrix effects (and consequently the validity of calibration curves) was proven by the satisfactory recovery values (Table 3-S): the recovery values ranged from 97.5% to 103.7% for all the sugars (with the exception of mannose, whose concentration is very close to the limit of quantification).

Table 3 shows the composition of the 22 carbohydrates in 22 honey samples of 7 different botanical origins. The data were compared with those previously reported in literature, where the most frequently quantified sugars are glucose, fructose, sucrose, maltose, turanose, threalose, isomaltose, and melezitose. The content of the remaining sugars, detected by GC, is reported in a few papers.[14,15]

DOI: 10.1021/acs.analchem.7b03656
Anal. Chem. XXXX, XXX, XXX−XXX

Table 3. Composition of the 22 Carbohydrates in the 22 Honey Samples of 7 Different Botanical Origins: Ac = Acacia, Ch = Chestnut, Li = Linden, Hd = Honeydew, Ci = Citrus, Co = Coriander, Cy = Cherry[a]

	Ac-1	Ac-2	Ac-3	Ac-4	Ch-1	Ch-2	Ch-3	Li-1	Li-2	Li-3	Hd-1	Hd-2	Hd-3	Ci-1	Ci-2	Ci-3	Co-1	Co-2	Co-3	Cy-1	Cy-2	Cy-3
Glucose	24.6 ± 0.4	24.3 ± 0.4	29.3 ± 0.5	26.6 ± 0.5	19.5 ± 0.4	19.2 ± 0.4	19.0 ± 0.4	27.4 ± 0.5	30.4 ± 0.6	25.9 ± 0.5	18.7 ± 0.3	19.5 ± 0.4	22.9 ± 0.4	31.0 ± 0.6	32.2 ± 0.6	32.0 ± 0.6	31.4 ± 0.6	24.3 ± 0.4	25.4 ± 0.5	27.4 ± 0.5	28.4 ± 0.5	27.9 ± 0.5
Fructose	40.6 ± 0.5	40.7 ± 0.5	35.6 ± 0.4	38.0 ± 0.5	36.1 ± 0.5	33.7 ± 0.4	35.8 ± 0.5	32.3 ± 0.4	33.6 ± 0.4	31.1 ± 0.4	27.5 ± 0.4	23.8 ± 0.3	25.2 ± 0.3	36.3 ± 0.5	37.0 ± 0.5	36.0 ± 0.5	34.7 ± 0.4	32.3 ± 0.4	31.3 ± 0.4	36.0 ± 0.5	33.7 ± 0.4	31.2 ± 0.4
Rhamnose	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Mannose	<LOD	<LOD	<LOD	<LOD	0.246 ± 0.013	0.55 ± 0.03	0.085 ± 0.005	<0.06	0.142 ± 0.008	0.135 ± 0.008	<0.06	0.253 ± 0.014	0.146 ± 0.018	<LOD	<LOD	<LOD	<LOD	<LOD	<0.06	<0.06	<0.06	0.125 ± 0.007
Sucrose	<0.05	1.95 ± 0.03	1.320 ± 0.025	<0.05	<0.05	<0.05	<0.05	0.058 ± 0.001	<0.05	<0.05	<0.05	<0.05	0.118 ± 0.002	0.251 ± 0.005	1.212 ± 0.022	1.582 ± 0.029	<0.05	<0.05	<0.05	0.075 ± 0.001	<0.05	<0.05
Palatinose	0.15 ± 0.01	0.27 ± 0.02	0.44 ± 0.03	<0.05	0.48 ± 0.04	0.47 ± 0.04	1.09 ± 0.08	0.283 ± 0.022	0.222 ± 0.017	0.221 ± 0.017	0.25 ± 0.02	0.47 ± 0.04	0.353 ± 0.027	0.145 ± 0.011	0.191 ± 0.015	0.218 ± 0.017	0.129 ± 0.010	0.163 ± 0.013	0.299 ± 0.023	0.375 ± 0.029	0.262 ± 0.020	0.184 ± 0.014
Maltose	1.41 ± 0.04	1.35 ± 0.04	1.90 ± 0.06	1.81 ± 0.06	0.77 ± 0.02	0.58 ± 0.02	0.67 ± 0.02	0.541 ± 0.017	1.68 ± 0.05	1.39 ± 0.04	1.89 ± 0.06	2.08 ± 0.06	1.18 ± 0.04	1.29 ± 0.04	2.38 ± 0.07	1.82 ± 0.06	0.738 ± 0.023	0.647 ± 0.020	0.693 ± 0.022	0.761 ± 0.024	0.724 ± 0.023	0.745 ± 0.023
Maltulose	0.99 ± 0.02	1.19 ± 0.03	0.86 ± 0.02	0.61 ± 0.01	2.43 ± 0.05	2.55 ± 0.06	3.26 ± 0.07	1.39 ± 0.03	1.217 ± 0.027	1.17 ± 0.03	1.07 ± 0.02	2.10 ± 0.04	1.17 ± 0.02	0.837 ± 0.018	0.91 ± 0.02	0.789 ± 0.017	1.51 ± 0.03	1.44 ± 0.03	1.48 ± 0.03	1.294 ± 0.028	1.306 ± 0.028	1.352 ± 0.029
Turanose	2.17 ± 0.03	2.19 ± 0.03	2.01 ± 0.02	1.38 ± 0.02	1.58 ± 0.02	1.19 ± 0.02	1.990.03	1.556 ± 0.024	1.593 ± 0.024	1.904 ± 0.029	1.24 ± 0.02	1.460 ± 0.022	1.314 ± 0.020	1.620 ± 0.025	1.916 ± 0.029	1.537 ± 0.023	1.741 ± 0.027	1.766 ± 0.027	1.727 ± 0.026	1.771 ± 0.027	1.733 ± 0.026	1.579 ± 0.024
Trehalose	<LOD	<LOD	<LOD	<LOD	<0.05	<LOD	0.074 ± 0.01	<0.05	<0.05	<0.05	1.17 ± 0.02	<0.05	1.12 ± 0.02	<LOD	<LOD	<LOD	<LOD	<LOD	<0.05	<0.05	<LOD	<LOD
Melibiose	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<0.13	<0.13	<0.13	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<0.13	<LOD
Isomaltose	0.806 ± 0.02	1.135 ± 0.03	0.842 ± 0.02	0.851 ± 0.02	3.19 ± 0.08	3.07 ± 0.07	2.61 ± 0.06	1.65 ± 0.04	2.10 ± 0.05	2.77 ± 0.07	0.89 ± 0.02	4.71 ± 0.11	1.85 ± 0.04	0.823 ± 0.020	1.041 ± 0.025	0.878 ± 0.021	1.96 ± 0.05	1.77 ± 0.04	1.87 ± 0.04	2.26 ± 0.05	2.09 ± 0.05	1.83 ± 0.04
Gentiobiose	<LOD	<LOD	<0.09	<LOD	<LOD	<LOD	<0.09	0.355 ± 0.005	0.341 ± 0.004	0.217 ± 0.003	<0.09	<0.09	<LOD	<LOD	<LOD	<LOD	<0.09	<0.09	<0.09	<0.09	<0.09	<0.09
Nigerose	0.33 ± 0.01	0.35 ± 0.01	0.34 ± 0.01	0.245 ± 0.005	0.511 ± 0.011	0.459 ± 0.010	0.539 ± 0.012	0.480 ± 0.011	0.475 ± 0.010	0.587 ± 0.013	0.485 ± 0.011	0.608 ± 0.013	0.418 ± 0.009	0.282 ± 0.006	0.253 ± 0.005	0.234 ± 0.005	0.430 ± 0.009	0.474 ± 0.010	0.473 ± 0.010	0.410 ± 0.009	0.422 ± 0.009	0.462 ± 0.010
Koijbiose	0.82 ± 0.02	0.75 ± 0.02	0.68 ± 0.02	0.55 ± 0.02	0.88 ± 0.03	0.93 ± 0.03	1.00 ± 0.03	0.865 ± 0.027	1.15 ± 0.04	1.41 ± 0.04	0.97 ± 0.03	1.16 ± 0.04	0.921 ± 0.029	0.597 ± 0.018	0.549 ± 0.017	0.479 ± 0.015	0.839 ± 0.026	0.98 ± 0.03	0.921 ± 0.029	1.03 ± 0.03	1.00 ± 0.03	1.01 ± 0.03
Isomaltotriose	<0.18	<LOD	<0.18	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<0.18	<LOD	<LOD	<0.18	<LOD	<0.18	<0.18	<LOD	<LOD	<LOD	<LOD	<0.18
Raffinose	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.313 ± 0.003	<0.21	<0.21	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Erlose	1.41 ± 0.03	1.30 ± 0.03	1.01 ± 0.02	0.811 ± 0.02	<0.06	<0.06	<0.06	0.324 ± 0.008	0.313 ± 0.008	0.310 ± 0.008	1.26 ± 0.03	2.29 ± 0.06	0.503 ± 0.013	0.75 ± 0.02	0.732 ± 0.02	0.575 ± 0.014	<LOD	<0.06	<0.06	<0.06	<0.06	<0.06
Melezitose	<LOD	<LOD	<0.13	<LOD	<LOD	<0.13	0.77 ± 0.01	0.355 ± 0.003	<LOD	0.576 ± 0.006	5.64 ± 0.06	0.164 ± 0.002	4.03 ± 0.04	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Maltotriose	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.62 ± 0.02	0.563 ± 0.008	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
1-Kestose	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<0.14	<0.14	0.243 ± 0.007	<0.14	<LOD	1.24 ± 0.03	<0.14	<0.14	0.166 ± 0.005	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Panose	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

[a]Experimental uncertainties were derived by error propagation.

DOI: 10.1021/acs.analchem.7b03656
Anal. Chem. XXXX, XXX, XXX−XXX

The main sugars, fructose (23.8−40.6%) and glucose (19.0−32.2%), show a high variability. Honeys with the lowest percentages of fructose and glucose were those of nonfloral origin (i.e., honeydew honeys[52]) and chestnut, whereas citrus honey contains the highest amount of these two monosaccharides.[18,52,53] Among disaccharides, maltose, turanose, maltulose, and isomaltose are the most abundant in the majority of honey samples, followed by nigerose, koijbiose, sucrose, and palatinose; melibiose is the lowest concentrated disaccharide. Among the identified and quantified trisaccharides, erlose is the most represented,[15] while panose and isomaltotriose are always present in concentrations lower than the LoD and LoQ.

CONCLUSIONS

We have presented a new NMR approach based on CSSF-TOCSY that allows the identification and quantification of carbohydrates directly in aqueous solutions without any pretreatment of the sample. As shown in the present application, the selectivity of the technique combined with the specificity of chemical shifts relative to the molecular structure allows a straightforward discrimination in honey of as many as 22 sugars, despite their structural similarity. The entire analytical procedure also allows an accurate quantitative determination, even at low quantification limits, for each of the 22 sugars investigated. The instrumental stability observed over about one year, along with the optimum chemical shift reproducibility, make the procedure amenable to automation and suitable to routine analysis.

The technical advantages of the method over the corresponding 2D TOCSY mainly stem from the much faster acquisition and the higher digital resolution of 1D spectra with respect to 2D maps. Notably, a 2D TOCSY experiment requires the acquisition of hundreds of transients (typically 256), wheras the 1D CSSF-TOCSY only requires as many acquisitions as the species to be quantified (22 in our case). In addition, possible t_1 noise in 2D TOCSY may preclude a correct integration of the signals along the F2 dimension, where the resolution is highest. Other multidimensional NMR methods such as HSQC have been proposed for enhancing the resolution and reducing the overlaps. Not surprisingly, for the case of carbohydrates in honey, Petersen et al. showed that as many as 3072 transients are required to provide a sufficient resolution in HSQC maps acquired at 18.7 T (800 MHz).[30] In summary, the proposed method proves to be a valid alternative to traditional methods for carbohydrates identification and quantification. We have chosen honey to demonstrate our approach for saccharides determination since this natural matrix proves to be particularly challenging. Just as clearly, however, the same approach can be easily transferred to other food matrices such as fruit juices, milk, and also to biofluids or even to new classes of molecules other than carbohydrates.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b03656.

Additional figures and tables as noted in text and experimental details on the method, including the case of maltose (PDF)

AUTHOR INFORMATION

Corresponding Author

*E-mail: elisabetta.schievano@unipd.it.

ORCID

Elisabetta Schievano: 0000-0002-4781-9932

Notes

The authors declare the following competing financial interest(s): Part of the present methodology is filed under the Italian patent application: E. Schievano, F. Rastrelli, 102017000071682 (27/06/2017).

ACKNOWLEDGMENTS

This work was supported by the Università degli Studi di Padova (CPDA150718/15 granted to E.S.). We thank prof. Fabrizio Mancin for providing generous access to the NMR instrumentation.

REFERENCES

(1) Martínez Montero, C.; Rodríguez Dodero, M. C.; Guillén Sánchez, D. A.; Barroso, C. G. *Chromatographia* **2004**, *59*, 15−30.

(2) Magwaza, L. S.; Opara, U. L. *Sci. Hortic.* **2015**, *184*, 179−192.

(3) Pereira da Costa, M.; Conte-Junior, C. A. *Compr. Rev. Food Sci. Food Saf.* **2015**, *14*, 586−600.

(4) Wu, L.; Du, B.; Vander Heyden, Y.; Chen, L.; Zhao, L.; Wang, M.; Xue, X. *TrAC, Trends Anal. Chem.* **2017**, *86*, 25−38.

(5) Sanz, M. L.; Martinez-Castro, I. *Journal of chromatography. A* **2007**, *1153*, 74−89.

(6) Márquez-Sillero, I.; Cárdenas, S.; Valcárcel, M. *Microchem. J.* **2013**, *110*, 629−635.

(7) Rentschler, E.; Kuschel, B.; Krewinkel, M.; Claaßen, W.; Glück, C.; Jiang, B.; Mu, W.; Stressler, T.; Fischer, L. *Food Analytical Methods* **2016**, *9*, 2210−2222.

(8) Corradini, C.; Cavazza, A.; Bignardi, C. *Int. J. Carbohydr. Chem.* **2012**, *2012*, 1−13.

(9) Dvorackova, E.; Snoblova, M.; Hrdlicka, P. *J. Sep Sci.* **2014**, *37*, 323−337.

(10) Nozal, M. J.; Bernal, J. L.; Toribio, L.; Alamo, M.; Diego, J. C.; Tapia, J. *J. Agric. Food Chem.* **2005**, *53*, 3095−3100.

(11) Pico, J.; Martinez, M. M.; Martin, M. T.; Gomez, M. *Food Chem.* **2015**, *173*, 674−681.

(12) Brummer, Y.; Cui, S. W. *Food Carbohydrates: Chemistry, Physical Properties, and Applications*; CRC Press: Boca Raton, FL, 2005; p 89.

(13) Sun, S.; Wang, H.; Xie, J.; Su, Y. *Chem. Cent. J.* **2016**, *10*, 25.

(14) de la Fuente, E.; Ruiz-Matute, A. I.; Valencia-Barrera, R. M.; Sanz, J.; Martínez Castro, I. *Food Chem.* **2011**, *129*, 1483−1489.

(15) Cotte, J. F.; Casabianca, H.; Chardon, S.; Lheritier, J.; Grenier-Loustalot, M. F. *Journal of Chromatography A* **2003**, *1021*, 145−155.

(16) Wang, H.; Geppert, H.; Fischer, T.; Wieprecht, W.; Moller, D. *J. Chromatogr. Sci.* **2015**, *53*, 1427−1431.

(17) Duarte, I. F.; Barros, A.; Delgadillo, I.; Almeida, C.; Gil, A. M. *J. Agric. Food Chem.* **2002**, *50*, 3104−3111.

(18) Anjos, O.; Campos, M. G.; Ruiz, P. C.; Antunes, P. *Food Chem.* **2015**, *169*, 218−223.

(19) Rizelio, V. M.; Tenfen, L.; da Silveira, R.; Gonzaga, L. V.; Costa, A. C.; Fett, R. *Talanta* **2012**, *93*, 62−66.

(20) Liang, P.; Sun, M.; He, P.; Zhang, L.; Chen, G. *Food Chem.* **2016**, *190*, 64−70.

(21) Mannina, L.; Sobolev, A. P.; Viel, S. *Prog. Nucl. Magn. Reson. Spectrosc.* **2012**, *66*, 1−39.

(22) Pauli, G. F.; Chen, S. N.; Simmler, C.; Lankin, D. C.; Godecke, T.; Jaki, B. U.; Friesen, J. B.; McAlpine, J. B.; Napolitano, J. G. *J. Med. Chem.* **2014**, *57*, 9220−9231.

(23) Pauli, G. F.; Godecke, T.; Jaki, B. U.; Lankin, D. C. *J. Nat. Prod.* **2012**, *75*, 834−851.

(24) Nord, L. I.; Vaag, P.; Duus, J. O. *Anal. Chem.* **2004**, *76*, 4790−4798.

DOI: 10.1021/acs.analchem.7b03656
Anal. Chem. XXXX, XXX, XXX−XXX

(25) Buda, S.; Nawoj, M.; Mlynarski, J. *Annu. Rep. NMR Spectrosc.* **2016**, *89*, 185−223.
(26) Schievano, E.; Finotello, C.; Navarini, L.; Mammi, S. *Talanta* **2015**, *140*, 36−41.
(27) Malz, F.; Jancke, H. *J. Pharm. Biomed. Anal.* **2005**, *38*, 813−823.
(28) Hu, K.; Westler, W. M.; Markley, J. L. *J. Am. Chem. Soc.* **2011**, *133*, 1662−1665.
(29) Giraudeau, P. *Magn. Reson. Chem.* **2017**, *55*, 61−69.
(30) Petersen, B. O.; Hindsgaul, O.; Meier, S. *Analyst* **2014**, *139*, 401−406.
(31) Spiteri, M.; Jamin, E.; Thomas, F.; Rebours, A.; Lees, M.; Rogers, K. M.; Rutledge, D. N. *Food Chem.* **2015**, *189*, 60−66.
(32) del Campo, G.; Zuriarrain, J.; Zuriarrain, A.; Berregi, I. *Food Chem.* **2016**, *196*, 1031−1039.
(33) Monakhova, Y. B.; Tsikin, A. M.; Kuballa, T.; Lachenmeier, D. W.; Mushtakova, S. P. *Magn. Reson. Chem.* **2014**, *52*, 231−240.
(34) Monakhova, Y. B.; Kuballa, T.; Leitz, J.; Andlauer, C.; Lachenmeier, D. W. *Dairy Sci. Technol.* **2012**, *92*, 109−120.
(35) Minoja, A. P.; Napoli, C. *Food Res. Int.* **2014**, *63*, 126−131.
(36) Hall, L. D.; Norwood, T. J. *J. Magn. Reson.* **1988**, *76*, 548−554.
(37) Sandusky, P.; Raftery, D. *Anal. Chem.* **2005**, *77*, 2455−2463.
(38) Hall, L. D.; Norwood, T. J. *J. Magn. Reson.* **1988**, *78*, 582−587.
(39) Robinson, P. T.; Pham, T. N.; Uhrin, D. *J. Magn. Reson.* **2004**, *170*, 97−103.
(40) Duncan, S. J.; Lewis, R.; Bernstein, M. A.; Sandor, P. *Magn. Reson. Chem.* **2007**, *45*, 283−288.
(41) Yang, L.; Moreno, A.; Fieber, W.; Brauchli, R.; Sommer, H. *Magn. Reson. Chem.* **2015**, *53*, 304−308.
(42) Thrippleton, M. J.; Keeler, J. *Angew. Chem., Int. Ed.* **2003**, *42*, 3938−3941.
(43) Consonni, R.; Cagliani, L. R.; Cogliati, C. *J. Agric. Food Chem.* **2012**, *60*, 4526−4534.
(44) Belitz, H.-D.; Grosch, W.; Schieberle, P. *Food Chemistry*, 4th ed.; Springer: Berlin, 2009; pp 885−887.
(45) Eurachem Method Validation Working Group. *Eurachem Guide: The Fitness for Purpose of Analytical Methods − A Laboratory Guide to Method Validation and Related Topics*, 2nd ed.; Magnusson, B., Ornemark, U., Eds.; Eurachem, 2014.
(46) Wang, C.; Ying, F.; Wu, W.; Mo, Y. *J. Org. Chem.* **2014**, *79*, 1571−1581.
(47) Pohl, P.; Bielawska-Pohl, A.; Dzimitrowicz, A.; Jamroz, P.; Welna, M.; Lesniewicz, A.; Szymczycha-Madeja, A. *TrAC, Trends Anal. Chem.* **2017**, *93*, 67−77.
(48) Altundag, H.; Bina, E.; Altintig, E. *Biol. Trace Elem. Res.* **2016**, *170*, 508−514.
(49) Hauswaldt, A.-L.; Rienitz, O.; Jährling, R.; Fischer, N.; Schiel, D.; Labarraque, G.; Magnusson, B. *Accredit. Qual. Assur.* **2012**, *17*, 129−138.
(50) Eshuis, N.; van Weerdenburg, B. J.; Feiters, M. C.; Rutjes, F. P.; Wijmenga, S. S.; Tessari, M. *Angew. Chem., Int. Ed.* **2015**, *54*, 1481−1484.
(51) Saielli, G.; Bagno, A. *Org. Lett.* **2009**, *11*, 1409−1412.
(52) Lazaridou, A.; Biliaderis, C. G.; Bacandritsos, N.; Sabatini, A. G. *J. Food Eng.* **2004**, *64*, 9−21.
(53) Ouchemoukh, S.; Schweitzer, P.; Bachir Bey, M.; Djoudad-Kadji, H.; Louaileche, H. *Food Chem.* **2010**, *121*, 561−568.

DOI: 10.1021/acs.analchem.7b03656
Anal. Chem. XXXX, XXX, XXX−XXX