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A New Methodology Based on GC–MS To Detect Honey Adulteration with Commercial Syrups

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Honey adulterations can be carried out by addition of inexpensive sugar syrups, such as high fructose corn syrup (HFCS) and inverted syrup (IS). Carbohydrate composition of 20 honey samples (16 nectar and 4 honeydew honeys) and 6 syrups has been studied by GC and GC−MS in order to detect differences between both sample groups. The presence of difructose anhydrides (DFAs) in these syrups is described for the first time in this paper; their proportions were dependent on the syrup type considered. As these compounds were not detected in any of the 20 honey samples analyzed, their presence in honey is proposed as a marker of adulteration. Detection of honey adulteration with HFCS and IS requires a previous enrichment step to remove major sugars (monosaccharides) and to preconcentrate DFAs. A new methodology based on yeast (*Saccharomyces cerevisiae*) treatment has been developed to allow the detection of DFAs in adulterated honeys in concentrations as low as 5% (w/w).

KEYWORDS: Adulteration; honey; syrup; difructose anhydrides (DFAs); yeast; GC−MS.

INTRODUCTION

Adulteration is a fraudulent modification of foods carried out by adding inert or hazardous material or substances of minor quality, or subtracting those components which confer food properties and value (*1*). Adulteration mostly occurs because of less expensive substances being added. Two approaches are possible for detecting adulteration in food products: (i) to demonstrate that a foreign component (a marker) is present and (ii) to detect significant deviations from expected values in the concentration of naturally occurring components. In the practice, both approaches are commonly used, although the first affords more accuracy.

Being a natural food of a relatively high price, honey has been adulterated with inexpensive products for a long time. Honey is mainly composed of carbohydrates (around 80% of honey); therefore, honey adulterations mainly involve the addition of inexpensive sugar products (*2*, *3*). Molasses, caramels, sugar syrups from corn, sugar cane and sugar beet, inverted (IS) by acids and enzymes, as well as corn syrups (CS) or high fructose corn syrups (HFCS) obtained by isomerization from CS have been detected as adulterants in honey (*4*, *5*).

Recently, there is an increasing interest in the control of honey quality, and the governments encourage researchers to find new, simple, and economical methods to detect honey frauds. However, the detection of honey adulterations can constitute a difficult task, especially due to both the high variability in honey composition which depends on its botanical or geographical origin (*6*, *7*), and the use of syrups with a chemical composition similar to that of natural honey (*3*).

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Table 1. Mono-, Di-, and Trisaccharide Content (mg/g Product) for Selected Honey Samples (H1, Nectar Honey and H18, Honeydew Honey),Syrups (80HFCS and IS), and for Yeast Treated Honey Samples H1 and H18 ((H1 + Yeast) and (H18 + Yeast))[a]

	mg/g product			
	monosaccharides	disaccharides	trisaccharides	total
H1	593.0 (77%)	163.5 (21%)	16.9 (2%)	773.4
H18	462.9 (62%)	241.8 (32%)	41.4 (6%)	746.1
80HFCS	907.1 (97%)	27.3 (3%)	0.0	934.4
IS	703.4 (96%)	33.4 (4%)	0.0	736.8
H1 + yeast	5.5 (7%)	60.1 (83%)	7.0 (10%)	72.6
H18 + yeast	0.7 (1%)	66.2 (89%)	7.2 (10%)	74.1

[a] Figures in parentheses are the percentages over the total of carbohydrates quantified.

The analysis of stable carbon isotope ratio (SCIRA) has been used for the detection of sugar syrups in honey, being especially suitable for the detection of those originated from C4 (sugarcane) plants. Its use has been recently extended to the detection of adulterations up to 7% with syrups from both C3 (sugarbeet) and C4 plants (*8*, *9*); however, the requirement of very expensive instrumentation is the main limitation in the application of this procedure. Other methods based on spectroscopic (FT-IR, *10*; FT-Raman, *11*) or calorimetric (*12*) techniques have also been used; nevertheless, their successfulness has not been yet completely assessed.

Most of the attempts to analyze the carbohydrate fraction of honey have been done using chromatographic techniques such as high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC−PAD) (*3*, *13*, *14*) which allows the study of high oligosaccharides in honey (from

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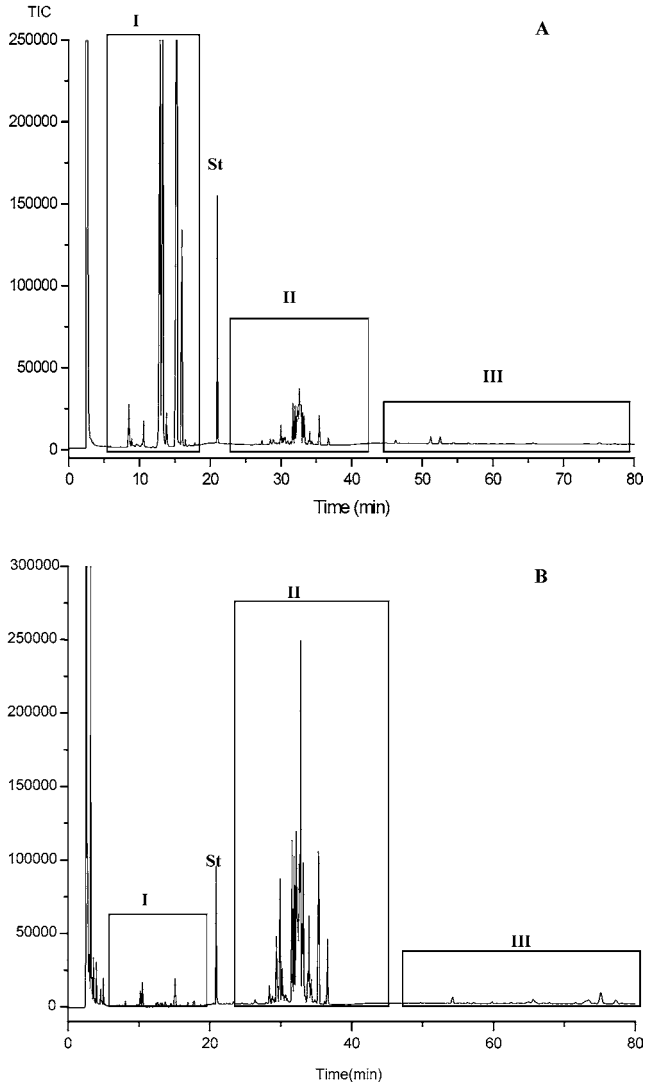

Figure 1. Gas chromatographic profile of *O*-TMS derivatives of carbohydrates in nectar honey H1: (A) before and (B) after 52 h of fermentation with yeast. (I) monosaccharides; (II) disaccharides; (III) trisaccharides; (St) internal standard.

DP2 to DP7 or higher; *15*). Gas chromatography (GC) is an affordable technique to study the mono-, di-, and trisaccharides of honey with a relatively high resolution and sensitivity (*16*) and has been previously described in the literature to detect honey adulterations. Maltose and isomaltose contents and their ratios determined by GC have been used to detect adulterations with HFCS (*17*, *18*). More recently, the GC fingerprint of di- and trisaccharides has been employed to determine adulterations with IS (*19*). In 2003, Cotte et al. (*2*) combined the use of HPAEC−PAD and GC−FID data with a statistical processing by principal component analysis to demonstrate the addition of IS and CS to honey samples.

Despite all these attempts, there is still a need for an appropriate indicator which allows the detection of low levels of adulteration, mainly with HFCS and IS, in honey. The aim of this work was to search for such a marker.

MATERIALS AND METHODS

Standards. Analytical standards of carbohydrates, cellobiose, erlose, fructose, gentiobiose, glucose, isomaltose, isomaltotriose, 1-kestose, kojibiose, laminaribiose, maltose, maltotriose, melezitose, nigerose, panose, β-phenyl-glucoside, raffinose, and sucrose, were obtained from Sigma Chemical Co. (St. Louis, MO). Maltulose was purchased from Aldrich Chem. Co (Milwaukee, WI) and leucrose, melibiose, palatinose, α,α-trehalose, α,β-trehalose, and turanose from Fluka (Madrid, Spain). Trehalulose was provided by Dr. Wach from Südzucker (Mannheim, Germany) and theanderose by Dr. G. R. Côté (USDA, Peoria, IL).

Honey Samples. Twenty Spanish unifloral honeys were acquired in specialized markets or purchased directly from beekeepers: citrus (samples H1−H4), heather (samples H5−H8), eucalyptus (samples H9−H12), rosemary (samples H13−H16), and honeydew from oak (samples H17−H20).

Syrups. Six commercial syrups were used. One partially inverted syrup (PIS; feed syrup with fructose) from Südzucker (Mannheim, Germany), one inverted syrup from sugar cane (IS), and three high fructose corn syrups with different percentage of glucose isomerization: 80HFCS (80%), 40HFCS (40%), and 20HFCS (20%), provided by Mariano Dólera S.L. (Murcia, Spain).

Adulterated Samples. Syrups 80HFCS and IS were added in different proportions (5, 10, and 20%, w/w) to honey samples H1 (nectar) and H18 (honeydew) to intentionally simulate honey adulteration.

Thermal Treatment and Storage. The honeydew honey sample (H18) was submitted to two different heating treatments: (i) 120 °C for 0.5 and 1 h; (ii) 80 °C for 1, 5, and 8 h.

Five honeys (H4, H8, H12, H16, and H20) were stored for 3 years at room temperature.

Carbohydrate Enrichment. Carbohydrate enrichment was carried out using a yeast treatment according to Yoon et al. (*20*) and Sanz et al. (*21*) slightly modified. A 20% (w/v) solution of honey, syrup, and adulterated honey in deionized water was treated with a 1% (w/v) aqueous solution of *Saccharomyces cerevisiae* (Maizena, Unilever) at 30 °C. Samples (1 mL) were taken at 0, 24, 52, and 72 h, centrifuged at 7000*g* for 5 min, and filtered through 0.22 μm filters (Sartorius, Germany) to remove the yeast. All the experiments were carried out in duplicate.

Carbohydrate Analysis. GC carbohydrate analysis was carried out according to Sanz et al. (*16*), using a two-step derivatization procedure (oximation and trimethylsilylation). An amount of 0.5 mL of a solution prepared by dissolving 1 g of sample in 25 mL of 70% ethanol/water was mixed with 0.5 mL of a 70% ethanolic solution of phenyl-β-D-glucoside (1 mg mL^{-1}) employed as an internal standard. After evaporation of ethanol under vacuum at 38−40 °C, derivatives were formed by addition of 350 μL of a solution of 2.5% hydroxylamine chloride in pyridine after 30 min at 75 °C. The oximes obtained in this step were silylated with hexamethyldisilazane (350 μL) and trifluoroacetic acid (35 μL) at 45 °C for 30 min. After reaction, samples were centrifuged at 7000*g* for 5 min at 5 °C, and 1 μL of supernatant was injected into the GC injection port.

GC analyses were carried out on a gas chromatograph equipped with a flame ionization detector (FID) (HP 5890, Palo Alto, CA) using nitrogen as the carrier gas. A 25 m × 0.25 mm i.d. × 0.25 μm film thickness fused silica column coated with SPB-1 (crosslinked methyl silicone) from Supelco (Bellefonte, PA) was used. The oven temperature was held at 200 °C for 20 min, then programmed to 270 °C at a heating rate of 15 °C min^{-1}, then programmed to 290 °C at 1 °C min^{-1}, and finally programmed to 300 °C at 15 °C min^{-1} and held for 40 min. Injector and detector temperature were 300 °C. Injections were made in the split mode, with a split ratio of 1:40. Chromatographic peaks were measured using a Chrom-Card 1.20 acquisition system (CE Instruments, Milan, Italy).

GC−MS analyses were carried out using a Hewlett-Packard 6890 gas chromatograph coupled to a 5973 quadrupole mass detector operating in electronic impact (EI) mode at 70 eV (both from Agilent, Palo Alto, CA). Operating conditions other than carrier gas (He at 1 mL min^{-1}) were identical to those previously described for GC analysis. Acquisition was done using a HPChem Station software (Hewlett-Packard, Palo Alto, CA).

Identification of *O*-TMS derivatives of carbohydrates present in honey samples was carried out by comparison of their retention times with those of standard compounds; mass spectral data were used to confirm peak identities. Quantitative data for carbohydrates were

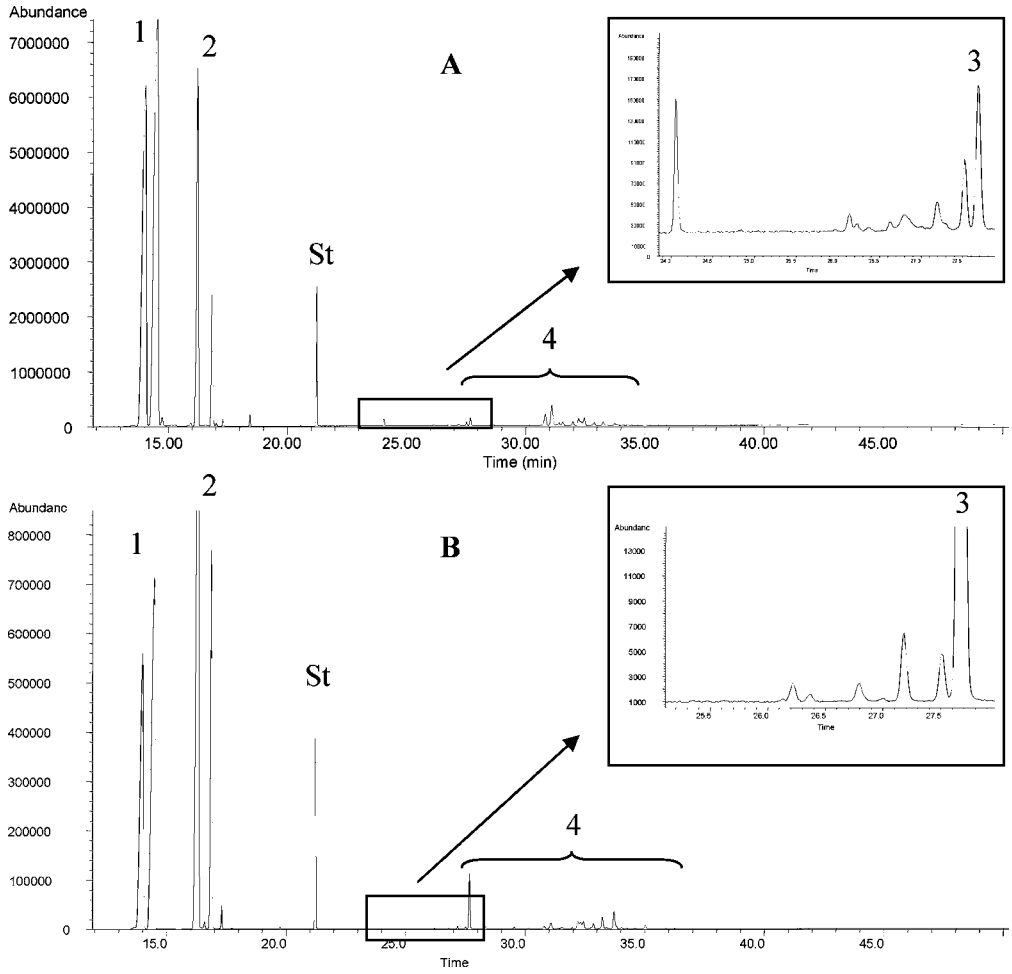

Figure 2. TIC profile for *O*-TMS derivatives of carbohydrates in (A) syrup 80HFCS and (B) syrup IS. 1, fructose; 2, glucose; 3, sucrose; 4, disaccharides; (St), internal standard.

Table 2. Concentration (mg/g Product) of DFAs Selected as Markers for Honey Adulteration with Syrups and Honeys Adulterated with Different Percentages (5–20%, w/w) of 80HFCS and IS

	H1 adulterated						H18 adulterated					
	HFCS			IS			HFCS			IS		
DFAs	5%	10%	20%	5%	10%	20%	5%	10%	20%	5%	10%	20%
DFA 1 (peak 1)[a]	0.10	0.17	0.38	tr[b]	0.01	0.02	0.08	0.19	0.33	tr[b]	tr[b]	tr[b]
DFA 9 (peak 6)	0.02	0.04	0.09	0.04	0.08	0.13	tr[b]	0.02	0.10	0.07	0.09	0.17
DFA 10 (peak 7)	0.15	0.19	0.31	0.05	0.10	0.12	tr[b]	0.19	0.41	0.21	0.18	0.29

[a] Peaks assigned in **Figure 3**. [b]tr: traces.

calculated from FID peak areas according to the method proposed by de la Fuente et al. (*22*). Standard solutions of carbohydrates over the expected concentration range in honey were prepared to calculate the response factor (RF) relative to phenyl-β-D-glucoside (internal standard). Because of the lack of standards, the concentration of difructose anhydrides (DFAs) was estimated assuming a response factor equal to 1. All analyses were carried out in duplicate.

RESULTS AND DISCUSSION

Mono-, di-, and trisaccharide contents of the 20 honey samples and the 6 syrups under study were determined by GC in order to find differences which allowed one to discriminate between both types of products. Among analyzed honeys, one nectar honey (H1) (see **Figure 1A**) and one honeydew honey (H18) were selected as representative samples. **Table 1** summarizes the carbohydrate composition of the selected samples. Monosaccharides corresponded to 77% of the total carbohydrates quantified in H1, whereas they represented 62% in H18. In both cases, fructose (43% for H1 and 36% for H18) was higher than glucose (34% and 26% for H1 and H18, respectively), as generally described in the literature (*23*, *24*). Di- and trisaccharide content was higher in honeydew honey than in nectar honey, with melezitose and erlose being the major trisaccharides, according to data reported by other authors (*25*−*27*).

Samples 80HFCS and IS were also chosen as representative syrup samples, their sugar composition being summarized in **Table 1**. 80HFCS was constituted by 79% of fructose and 18% of glucose, while IS presented similar quantities of both fructose

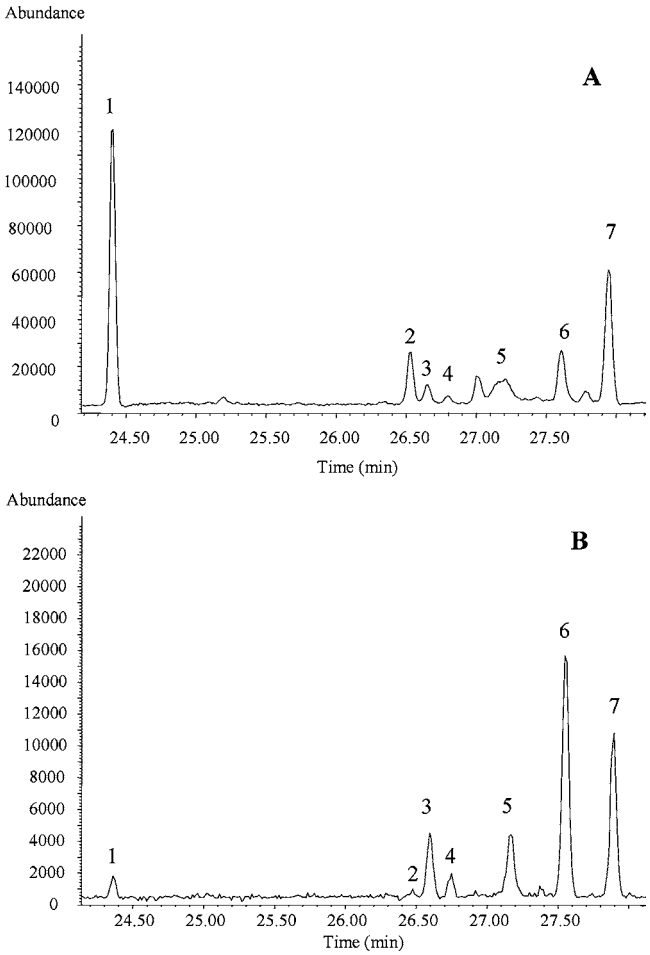

Figure 3. Characteristic gas chromatographic profile obtained for DFAs region in syrups (A) 80HFCS and (B) IS after yeast treatment: 1, DFA1; 2, DFA4; 3, DFA5; 4, DFA6; 5, DFA7; 6, DFA9; and 7, DFA10.

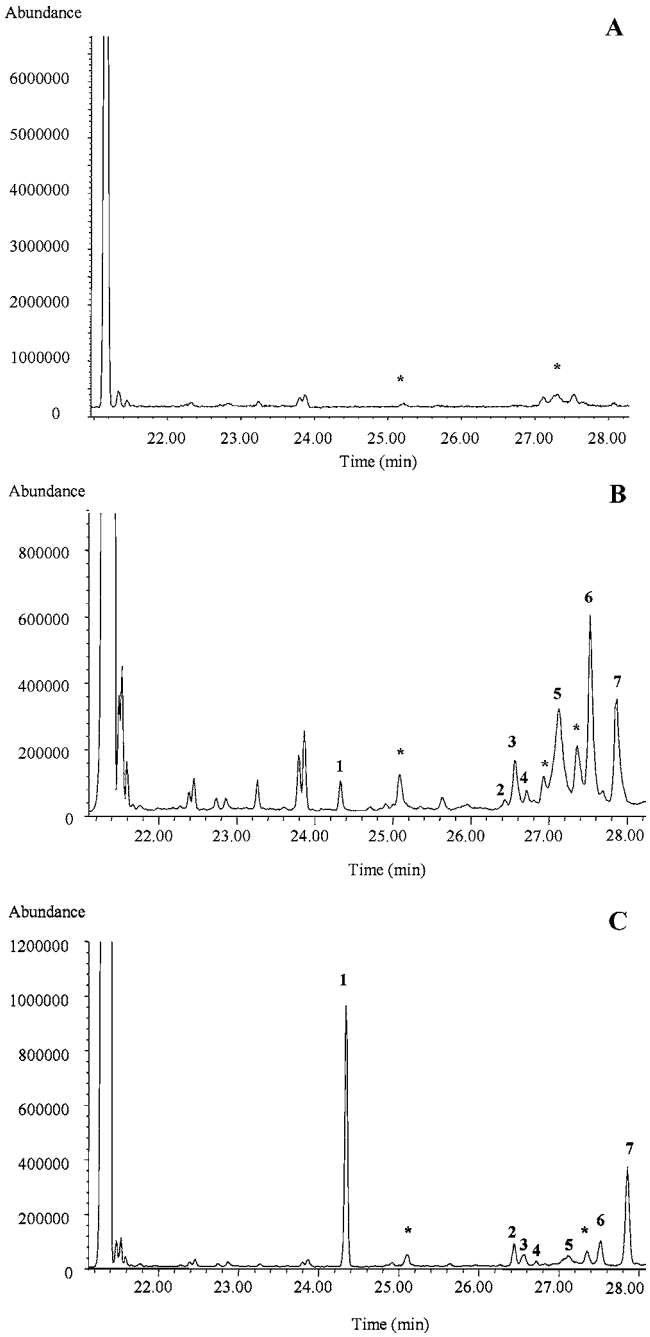

Figure 4. Gas chromatographic profile of DFAs region in (A) nectar honey (H1) and honey adulterated with (B) 20% of IS and (C) 20% of HFCS after yeast treatment. 1, DFA1; 2, DFA3; 3, DFA5; 4, DFA6; 5, DFA7; 6, DFA9; and 7, DFA10 ; ∗, unknown.

and glucose (46% and 50%, respectively) and a smaller proportion of sucrose (1%). **Figure 2** shows the chromatographic profile obtained for *O*-TMS derivatives of carbohydrates in samples 80HFCS and IS. It is worth noting the presence in both chromatograms of several minor peaks eluting before disaccharides with retention times within the range 23.0−28.0 min. Although the TIC profile in this region changes with the sample considered (see close-ups of **Figures 2A,B**), the presence of these peaks was found to be characteristic not only of samples 80HFCS and IS but also of all syrups analyzed (PIS, 20HFCS, and 40HFCS). In strong comparison, these compounds were not detected in any of the 20 honey samples under study.

As previously indicated, both samples H1 (nectar honey) and H18 (honeydew honey) were intentionally adulterated with different proportions (5, 10, and 20%) of both syrups. Carbohydrate composition (mono-, di-, and trisaccharides) of the resultant samples was compared with that of nonadulterated honeys. As the presence of the peaks previously observed in sugar syrups (**Figure 2**) could not be detected in samples adulterated at these levels, an enrichment procedure based on the elimination of the most abundant carbohydrates (mainly monosaccharides) was applied to make the study of these minor compounds possible.

Yeast Treatment. Honey sample (H1) was incubated with *S. cerevisiae* at 30 °C, for 0, 24, 52, and 72 h. The percentage of monosaccharides in the sample decreased with the incubation time, whereas the relative proportions of di- and trisaccharides to the total carbohydrates quantified increased. After 52 h of incubation, the decrease of monosaccharide was very low and di- and trisaccharide contents were scarcely enriched; therefore, 52 h were selected as the best incubation time. In order to evaluate the reproducibility of the enrichment method here proposed, honey sample H1 was submitted 5 times to incubation for 52 h: relative standard deviations (RSD%) around 10% for di- and trisaccharides were obtained.

Honeys H1 and H18 were submitted to yeast treatment under optimal experimental conditions above-described. **Figure 1A,B** shows the GC profile of the TMS-oximes of carbohydrates obtained before and after yeast treatment of sample H1. As it can be observed, monosaccharides were practically eliminated after incubation with yeast and a fraction richer in di- and trisaccharides was obtained. It is necessary to point out that

trehalose, kestoses, and other sugars are produced by yeast during the incubation time (*28*, *29*). **Table 1** summarizes the changes produced in the different fractions after yeast treatment. Although absolute concentration of di- and trisaccharides after the enrichment process decreased, their relative proportions were higher than those for untreated honey. Disaccharides were the most abundant (>80%) fraction in both samples, followed by trisaccharides.

Syrup samples (IS and 80HFCS) were also submitted to yeast treatment under the experimental conditions optimized for honey samples. The peaks within 23.0 and 28.0 min mentioned above could be easily observed after yeast treatment in both syrups (**Figure 3**). Peaks eluted at 24.3 min (peak 1) and at 28.1 min (peak 7) were the main peaks in 80HFCS while peaks eluting at 27.6 min (peak 6) and at 28.1 min (peak 7) were present in higher amounts in IS. In order to characterize the compounds detected by GC−FID, samples were analyzed by GC−MS to obtain their mass spectra. The compounds were identified as difructose anhydrides (DFAs). These compounds are nonfermentable pseudodisaccharides which consist of two fructose residues and present different structural varieties according to their linkage type (*30*). Seven DFA peaks, numbered according to Ratsimba et al. (*31*) in **Figure 3**, were identified in samples 80HFCS and IS. Peak 1 was assigned as DFA 1 (α-D-Fru*f*-1,2′/2,3′-*β*-D-Fru*f*); peak 6 as DFA 9 (α-D-Fru*f*-1,2′/2,1′-*β*-D-Fru*p*), and peak 7 as DFA 10 (α-D-Fru*f*-1,2′/2,1′-*β*-D-Fru*f*).

DFAs, which are described to be formed during heating of sugars or sugar-rich food (*32*, *33*), have been described as good markers of caramelization reaction in food and food additives (*31*, *34*). As fructose corn syrup and invert syrups are relatively high in fructose, formation of DFAs during heat treatment could be expected. However, to the best of our knowledge, they have never been detected in these syrups.

Detection of Adulterations. Honey samples adulterated by addition of 5, 10, and 20% of 80HFCS and IS were submitted to the optimized yeast treatment and analyzed by GC after derivatization. **Figure 4** shows the chromatographic profile of DFAs region in H1 honey (**Figure 4A**), and those obtained after the addition of 20% of inverted syrup (**Figure 4B**) and 20% of 80HFCS (**Figure 4C**). DFA1 and DFA10 appeared in higher amounts in samples adulterated with 80HFCS, while peaks DFA9 and DFA10 were the major peaks in the adulterations with IS. Quantitative results are summarized in **Table 2**. Selected peaks (1, 6, and 7) were easily quantified even at the 5% adulteration level.

To discard the presence of DFAs in honeys, three approaches were used: first, the remaining 18 honeys of different sources were treated with *S. cerevisiae* following the described methodology and analyzed by GC and GC−MS; DFAs were not detected in any of the samples. Second, in order to assess if these products could be formed in honey by thermal treatment, two samples (H1 and H18) were heated at 80 °C (for 1, 5, and 8 h) and at 120 °C (for 0.5 and 1 h); although these treatments are stronger than those used in the industry, no DFAs were observed in these two samples. Third, no DFAs could be detected in five honeys which have been stored for 3 years at room temperature either.

Therefore, DFAs could be considered good quality indicators of honey adulteration with HFCS and IS, allowing the detection of values down to 5%. The use of markers which are not present in genuine samples is advantageous compared to methods based on relationships of carbohydrates where the natural variability has to be taken into account. More experiments should be done with a higher number of samples from both honeys and syrups in order to accurately characterize the adulteration. Moreover, yeast treatment is an appropriate method to obtain an enriched fraction in DFAs which are not hydrolyzed during the incubation.

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