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Sugar composition and sugar-related parameters of honeys from the northern Iberian Plateau

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Highlights

- Honeydew and chestnut honeys are rich in isomaltose
- Chestnut honeys are poor in sucrose, trehalose and maltotriose
- Heather honeys are rich in monosaccharides and poor in maltose and erlose
- Lavender honeys are poor in isomaltose and raffinose
- Isomaltose tends to inhibit honey crystallization
Abstract

This research was aimed to study the sugar composition of fifty-four representative artisanal honeys from the northern Iberian plateau. Moisture, specific rotation, and crystallization indexes were also determined. Sugars were analyzed by gas chromatography-flame ionization detector (GC-FID) after Pourtallier’s derivatization procedure. Fourteen carbohydrates were reliably quantified: two monosaccharides, five disaccharides, six trisaccharides and one tetrasaccharide. Honeydew honeys showed the highest disaccharides (6.71%) and trisaccharides (1.81%) averages and the lowest monosaccharides (63.10%) average, in contrast to heather honeys that had the lowest disaccharides (4.93%) and trisaccharides (0.69%) averages and the highest monosaccharides (70.96%). Chestnut honeys possessed low concentrations of monosaccharides, sucrose, trehalose, and trisaccharides. Clover and lavender honeys possessed high monosaccharide and disaccharide percentages. As expected, lavender samples showed the highest sucrose concentrations (0.05–5.18%). Isomaltose contents were particularly high in honeydew (1.17–2.49%) and chestnut (1.34–1.74%) samples, and low in lavender (0.6–1.16%) honeys, the latter also being low in raffinose (0.01–0.05%). Moisture percentages (14.4–18.6%) indicated optimum honey ripeness. Averages for all groups of samples were levorotatory. In contrast to honeydew and chestnut honeys, lavender samples showed the fastest granulation tendency. In the analyzed honeys, the higher the percentage of isomaltose was, the lower the crystallization tendency the honeys exhibited.

Keywords: food analysis; food composition; honey; moisture; specific rotation; sugars; crystallization; GC-FID

OPTIONAL PUBCHEM CHEMICAL COMPOUNDS LIST

Fructose
Glucose
Maltose
Isomaltose
Sucrose
Gentiobiose
Trehalose
Melezitose
Raffinose
Erlose

1. Introduction

Honey is an interesting sweetener with potential health benefits and technological advantages when added to other food commodities, so that its commercialization is currently growing (Honey Market Presentation, 2015). Consumers show an increasing interest in quality foods, appreciating honeys from specific botanical/geographical origins, which achieve high prices on the markets. To guarantee the quality of honey, it is necessary to assess the authenticity of local honeys, carrying out extensive compositional analyses (Estevinho et al., 2012), which can help accomplish a desirable quality scheme (OJEU, 2012), such as a protected designation of origin (PDO), protected geographical indication (PGI), or traditional speciality guaranteed (TSG) that dramatically improve honey commercialization. Thus, honey authentication helps to protect customers from mislabeling, adulterations and fraud (Karabagias et al., 2014), and was initiated by market demand (Mateo and Bosch-Reig, 1998).

Apart from melissopalynology and sensory analyses, honey composition has been widely used for its characterization. Many researchers described profiles for a variety of honey compounds that could represent a “fingerprint” of specific origins, among them, the profile of carbohydrates (Gašić et al., 2015; Biluca et al., 2016; Se et al., 2018; De-Melo et al., 2018). Carbohydrates are the major components of honey (70–80%), followed by water (15–20%). Sugar composition and moisture percentages are considered as ripeness parameters of honey. The main sugars of honey are fructose and glucose, whose sum, together with sucrose content, are used as quality criteria for honeys within the European Union (OJEC, 2002). Honey also contains a complex
mixture of other mono-, di-, tri-, oligo- and polysaccharides (Val et al., 1998; Bogdanov et al., 2008). Sugars contribute to the antibacterial properties of honey and its preservation, because they provide high osmolarity and low water activity, which makes microorganism growth difficult. Honey shows variability in sugar composition, which is related to botanical origin, and to a lesser extent to climatic conditions and geographical origin (Anklam, 1998; Khalil et al., 2011). Honey sugars and sugar isotope ratios are also interesting parameters to detect some honey adulterations (Cabañero et al., 2006; Ruiz-Matute et al., 2007; Efflein and Raezke, 2008; Wu et al., 2017; Cengiz et al., 2018). The sugar composition of honey has been determined by different analytical procedures, discussed and summarized in the reviews of Bogdanov et al. (2004), Pita-Calvo et al. (2017), Siddiqui et al. (2017) and Pascual-Mate et al. (2018).

The northern Iberian plateau is located in the center of the northern half of the Iberian Peninsula (Castile-Leon area). It is the largest region of Spain and the third of the European Union, with an area of 94,200 km². Its climate is mainly Continental-Mediterranean, with Oceanic Climate in the North. The most representative nectar-producing botanical families are Compositae, Cruciferae, Ericaceae, Fagaceae, Labiatae, Leguminosae and Rosaceae. Beekeeping is of paramount economic importance in the northern Iberian plateau, it being the area with the largest number of bee farms in Spain (MAPAMA, 2017).

The European Commission (2018) recommended compilation of a database with reliable data of characteristics of genuine honeys. Accordingly, the purpose of this research was to study the ripeness-related and crystallization parameters of honeys from the northern Iberian plateau, researching their optical rotation and describing in detail their sugar composition, so that it can contribute to European honey authentication.
2. Materials and Methods

2.1. Samples

This study was carried out on 54 artisanal honey samples from the northern Iberian plateau (Spain), harvested in 2011, and directly collected and provided by beekeepers from their hives. In the laboratory, honeys were stored at 4 ºC. All analytical determinations were carried out during the first four months after sampling.

Botanical origin of the samples was determined by melissopalynology (Terradillos et al., 1994; Louveaux et al., 1978; Von der Ohe et al., 2004) and sensory analyses (Piana et al., 2004; Marcazzan et al., 2018). With regard to melissopalynology, specific recommendations for the interpretation of results of chestnut (Seijo et al., 1997; OJEU, 2007; Escuredo et al., 2012) and oak honeydew honeys (Mateo and Bosch-Reig, 1998; Rodríguez-Flores et al., 2015) were taken into account.

2.2. Analytical procedures

In all samples moisture, specific rotation and sugar composition were analysed in triplicate.

2.2.1. Moisture

Water content of a previous liquefied honey was determined by measuring the refractive index at 20 ºC with an Abbe refractometer Officine Galileo, Milan, Italy). Chataway conversion table revised by Wedmore was used to calculate the moisture content (%), according to the Official Methods of Analysis of the Association of
2.2.2. **Specific rotation**

Angular rotation was determined in a solution of 12.00 g of honey diluted to 100 mL with distilled water, after clarification with Carrez reagents (I and II). Specific optical rotation \([\alpha]_D^{20}\) was read at 20 °C the following day after filtration, using a digital polarimeter (Cecchinato MOD D-400; Cecchinato, Marcon, Venice, Italy), equipped with a sodium lamp. Results were read in angular degrees using a 200-mm polarimeter tube (AOAC, 2012).

2.2.3. **Sugar composition**

2.2.3.1. **Reagents.** All standards were chemical grade of the highest purity. Fructose, glucose, sucrose, isomaltose, erlose, panose, isomaltotriose and maltotetraose standards were purchased from Sigma-Aldrich (St. Louis MO). Maltotriose, gentiobiose standard and mannitol (used as internal standard) were purchased from Fluka BioChemika (Buchs, Switzerland). Trehalose, maltose, raffinose and melezitose standards were purchased from Carl Roth KG (Carl Roth GmbH + Co. KG. Karlsruhe, Germany).

2.2.3.2. **Procedure.** Sugars were determined by gas chromatography with flame ionization detection (GC-FID), according to Pierce-Portallier´s method (Bogdanov et al., 2009). A solution of 3.00 g of honey was filled up to 500 mL with distilled water after addition of mannitol internal standard. A volume of 100 μL of the solution was
transferred to a conical bottomed vial and dried under nitrogen at 50 °C. Thereafter, sugars were derivatized to their oximes by adding 200 μL of oxime reagent (12 mg/mL hydroxylamine hydrochloride in pyridine). After heating at 75 °C for 30 min and cooling to room temperature, the sugar oximes were silylated by adding 100 μL of hexamethyldisilazane and 10 μL of trifluoroacetic acid. After centrifugation, the trimethylsilyl derivatives were separated and quantified by gas chromatography in a Carlo Erba 4160 Chromatograph (Fisons Instruments S.p.A., Rodano, Milan, Italy) equipped with a cold on-column injector, a flame ionization detector and a Mega 5 capillary column. A 0.6-μL injection of each sample was analyzed under the following GC conditions: initial oven temperature 70 °C, then programmed at 49 °C/min from 70 to 140 °C and from 140 to 300 °C at 6 °C/min with helium as carrier gas (Sabatini, 2001). Data acquisition and analysis of the chromatographic peak areas were carried out using JASCO Borwin Integrator Software 1.5 (JASCO Corporation Europe, Cremella, Lecco, Italy). For qualitative analysis, retention times relative to mannitol both for standards and sample peaks were used. The concentrations of sugars were calculated by the internal standard method on the basis of the response factors for sugar standards eluted under the same conditions as samples. For compounds with two anomeric forms, total area was calculated as the sum of areas for both anomers. Results were expressed in g of sugars per 100 g of honey. The method had been previously validated by us. Repeatability data were obtained from twelve replicates of the honey samples under the same analytical and instrument conditions. Outliers were removed by normality tests (Shapiro-Wilk and Huber). The relative standard deviations for monosaccharides glucose and fructose were <3% and for minor sugars were around 20%. The limits of quantification (LOQ) were calculated with signal-to-noise ratio (S/N = 10), and evaluated in a 'peak-free' area of the chromatogram where noise was the baseline.
fluctuation without the analytes. LOQ for disaccharides and trisaccharides ranged between 0.01% and 0.03%.

2.2.4. Statistical analyses

One-way analysis of variance (ANOVA) was applied when the assumptions of normality of data, homogeneity of variances and independence of variables were met. Sometimes different transformations were carried out for data normalization, such as $x^2$, $1/x$, $(x + 1)/2$, $\log x$ and $x^{0.5}$, among others. The Student-Newman-Keuls test was used to perform a multiple comparison of means, where group differences were considered statistically significant at the 95.0% confidence level. When homoscedasticity was met but normality of the data was not possible, the nonparametric procedure Kruskal-Wallis ANOVA was done, to compare the medians instead of the means. To determine which medians were significantly different, the median notch in the Box-and-Whisker Plot was used. ANOVA was not performed when data did not meet homoscedasticity conditions. The unequal sample sizes led to heterogeneity of variances. Correlations among variables were measured and multivariate statistical principal component analysis (PCA) was also carried out. The statistical package software Statgraphics Centurion XVI.II (2010) was employed.

3. Results and Discussion

3.1. Melissopalynology

Seventy-one different pollen types, belonging to thirty-six botanical families were identified. The number of pollen types of each honey sample ranged from 8 to 27, with an average of 18 per sample. The average number of pollen was similar to those
reported by other authors (Thrasyvoulou and Manikis, 1995). They constituted the common palynological spectrum of the northern Iberian plateau honeys (Serra-Bonvehí and Ventura-Coll, 1993; Valencia-Barrera et al., 2000; Herrero et al., 2002). All samples contained the pollen types *Trifolium* sp., *Genista* sp. and *Rubus* sp., and 80% of the samples contained *Castanea sativa* and Ericaceae pollens (*Calluna vulgaris* and *Erica vagans* types). Melissopalynology showed that 27.8% samples were multifloral honeys rich in broom, clover, chestnut and/or sunflower. Among the “monofloral honeys” honeydew honey was the highest represented honey type of the studied samples (33.3%), followed by heather (18.5%), chestnut (7.4%), lavender (7.4%) and clover (5.6%).

### 3.2. Analytical determinations

Table 1 shows the mean values, standard deviations and concentrations ranges for moisture, optical rotation and sugar composition.

#### 3.2.1. Moisture percentage

Water content of honey is related to different factors, such as botanical origin of nectar, climatic circumstances, harvesting season and degree of maturity, among others (Finola et al., 2007). Current regulations (OJEC, 2002) establish a maximum moisture value of 20%, 23% being the maximum value for ling heather (*Calluna vulgaris*) honey as an exception. In our study, moisture values ranged from 14.4% (honeydew honey) to 18.6% (clover honey), indicating optimum harvesting practices and a good degree of ripeness (Downey et al., 2005). In general, our honeys exhibited lower moisture percentages than others from the same botanical origins reported in the literature (Alves
et al., 2013; Moise et al., 2013), heather honeys being particularly conspicuous because their water content was lower than 17.4%. Table 1 shows that clover and chestnut honeys possessed significantly higher moisture values (16.7% and 16.5%, respectively) than lavender samples (15.2%). With regard to clover honeys, literature references report them as having high moisture contents (Codex Alimentarius Standard for Honey, 2001; Bogdanov et al., 1999; Nanda et al., 2003). However, the water content of our clover samples was only slightly higher than the moisture percentage of honeys from other botanical origins, and lower than the water values reported by Malacalza et al., (2005) for clover honeys. Regarding lavender samples, our average was lower than the mean value obtained by Estevinho et al., (2013) in Portuguese lavender honeys. Finally, for chestnut and honeydew honeys, variable moisture values were reported in the literature (Mateo and Bosch-Reig, 1998; Golob and Plestenjak, 1999; Terrab et al., 2003b; Küçük et al., 2007), agreeing with our data.

3.2.2. Specific rotation

Optical rotation of honey depends on sugars composition and concentration (Bogdanov et al., 1999; Dinkov, 2003). In some countries, such as Greece, Italy and UK, optical rotation has been used to help distinguish blossom (levorotatory) and honeydew (dextrorotatory) honeys. The positive optical rotation (dextrorotatory) of honeydew honeys (Persano-Oddo et al., 1995; Bogdanov et al., 2004; Bertoncelj et al., 2011) was attributed to their lower fructose content and their higher oligosaccharide mass fraction, mainly melezitose ([α]D20 = +88.2°) and erlose ([α]D20 = +121.8°) (Persano-Oddo et al., 1995; Bogdanov and Martin, 2002). Nevertheless, in this study, all samples showed negative optical rotation (laevorotatory) averages, including honeydew honeys. Can et al. (2015) reported very low positive optical rotation for oak honeys (0.74°), while higher positive specific rotation values were found in metcalfa (17.00°) or
fir (12.60º) honeydew samples (Persano-Oddo et al., 1995; Bertoncelj et al., 2011). In the case of our honeydew honeys, the negative mean value was the lowest in absolute value (‒1.81º), having exhibited 6 out of the 18 honeydew honeys positive optical rotation data. In absolute values, both the highest negative optical rotation average (‒13.45º) and the highest negative result (‒21.00º) corresponded to heather honeys. The maximum value of ‒2.50º in heather honeys was found in a ling heather sample, with a low percentage of Calluna vulgaris pollen and a few honeydew elements. For chestnut honeys, variable data were described in literature, being all of them negative values. Regarding clover and heather honeys, our values were similar to those reported by Nanda et al. (2003) for Indian Trifolium alexandrinum honeys, and by Marini et al. (2004) and Persano-Oddo et al. (2004) for Italian Erica sp. honeys. With regard to lavender honeys, Persano-Oddo et al. (2004) reported mean values similar to ours for European lavender samples. Conversely, lower negative values for spike lavender (Lavandula latifolia) and French lavender (Lavandula stoechas) from Spain were found by Pérez-Arquillué et al. (1995). Since specific rotation is a potentially useful parameter for honey characterization and its measurement is simple, rapid and low cost, it would be interesting an international collaborative research to set up and establish a harmonized procedure for the analysis of honey’s optical rotation.

3.2.3. Sugar composition

In this study, 14 sugars were identified and quantified, including two monosaccharides (fructose and glucose), five disaccharides (sucrose, trehalose, maltose, gentiobiose and isomaltose), six trisaccharides (raffinose, erlose, melezitose, maltotriose, panose and isomaltotriose) and one tetrasaccharide (maltotetraose). Figure 1 displays a typical GC sugar profile.
3.2.3.1. Monosaccharides. As expected, fructose followed by glucose, were the main carbohydrates of the analysed honeys.

Fructose. Its content varied between 32.69% and 43.38% (both in chestnut honeys). Heather honeys possessed the maximum mean value (39.93%) and honeydew honeys the lowest (36.24%).

Glucose. The minimum value was 24.14% (multifloral honey rich in chestnut), and the maximum was 35.31% (multifloral honey rich in clover). Lavender honeys, with the highest average (31.38%) together with heather samples, presented significantly higher glucose values than honeydew (average 26.86%), chestnut (average 27.30%), and multifloral honeys rich in chestnut from the North Western area (average 24.9%). These latter honeys showed a glucose mean value considerably lower than the glucose average for the rest of multifloral samples (31.4%). There were also differences in glucose contents for heather honeys, mean values for Calluna vulgaris (28.00%) being lower than results obtained for Erica sp. honeys (33.05%). In general, our values of monosaccharides were in agreement with those reported in the literature for honeys from the same botanical origins as our samples (Persano-Oddo et al., 1995; Mateo and Bosch-Reig, 1997; Persano-Oddo and Piro, 2004; Pérez et al., 2007; Feás et al., 2010; Ouchemoukh et al., 2010; De la Fuente et al., 2011; Waś et al., 2011; Escuredo et al., 2013).

As expected and in contrast to heather honeys (70.96% monosaccharides), honeydew honeys showed the minimum average for the sum of fructose and glucose (63.10%). In respect of current regulations (OJEC, 2002), one sample that had been classified as chestnut, whose sediment contained 70% Castanea sativa Miller pollen, and about 30% honeydew elements, showed a result of 57.43% for the sum of fructose and glucose, thus being under the limit established for blossom honeys (“not less than 60 g/100 g for blossom honeys”). Nevertheless, for the mentioned sum of
monosaccharides, this honey fulfilled the requirement corresponding to honeydew blends ("not less than 45 g/100 g for honeydew honey and blends of honeydew honey with blossom honey"). Therefore, the 70% minimum percentage of chestnut pollen proposed by Seijo et al. (1997), OJEU (2007) and Escuredo et al. (2012) seems to be excessively low to be considered as a minimum limit for the authentication of actual unifloral Castanea sativa Miller honeys, because this pollen is extremely overrepresented in honeys (Louveauix et al., 1978; von der Ohe et al., 2004; von der Ohe and von der Ohe, 2007). This research brings to light that the sediment of unifloral chestnut honeys should contain at least 85% Castanea sativa Miller pollen, which is the minimum percentage required in several European Countries (Thrasyvoulou et al., 2018).

3.2.3.2. **Disaccharides.** With regard to disaccharide content, all samples contained all disaccharides determined in this study.

*Maltose.* It was the major disaccharide (67% of total disaccharides) and the third main sugar in our honeys with values ranging from 2.32% (heather) to 6.56% (honeydew), agreeing with the literature (Serra-Bonvehí and Ventura-Coll, 1993; Bentabol-Manzanares et al., 2014). In our study, clover samples showed the highest maltose average (5.24%), ranging between the values reported by Shin and Ustunol (2005) for samples of the same botanical origin (10.20%), and the results found by Ouchemoukh et al. (2010) for *Melilotus* sp. (1.45%), and *Trifolium* sp. (1.65%) honeys. In respect of our chestnut honeys’ maltose data, they were higher than those described by Primorac et al. (2011) and Escuredo et al. (2013) for European chestnut honeys. Our maltose averages were similar to those reported for Spanish heather (Bentabol-Manzanares et al., 2014), honeydew (Mateo and Bosch-Reig, 1998; Bentabol-
Manzanares et al., 2011) and lavender (Serra-Bonvehí and Ventura-Coll, 1993; Mateo and Bosch-Reig, 1997) honeys. Our values of maltose were the expected ones for honeys that had not been adulterated with sugar syrup or starch hydrolysate (Horvath and Molnar-Perl, 1997; Cotte et al., 2003).

Isomaltose. In our study, isomaltose was the second most abundant disaccharide, with the exception of one ling heather honey, whose major disaccharide was isomaltose. Honeydew honeys showed the highest isomaltose average (1.75%), followed by chestnut samples (1.61%). In contrast, lavender honeys had the lowest isomaltose average (0.88%). Values of isomaltose were in agreement with the results found in the literature for European chestnut (Persano-Oddo et al., 1995; Cotte et al., 2003), Spanish oak honeydew (Mateo and Bosch-Reig, 1998; De la Fuente et al., 2011) and lavender (Serra-Bonvehí and Ventura-Coll, 1993; Mateo and Bosch-Reig, 1997) honeys. Some researchers reported higher isomaltose concentration in honeydew honeys than in blossom ones (Serra-Bonvehí et al., 1987; Mateo and Bosch-Reig, 1997; Bentabol-Manzanares et al., 2011). In the literature, isomaltose averages for Spanish heather honeys widely varied from 0.38% (Terrab et al., 2003a) to 3.60% (Serra-Bonvehí and Granados-Tarrés, 1993). It is very likely that the contrasting values of maltose and isomaltose in samples from the same origins (geographical and/or botanical), and different harvests bring to light a strong influence of the harvesting year and climatic conditions on the composition of both sugars. Nozal et al. (2005) reported isomaltose averages considerably lower than ours for heather, lavender and forest honeys from the same geographical origin as our samples. On the other hand, Martins et al. (2008) showed higher averages for isomaltose than for maltose in heather honeys from “Serra da Lousa” (Portugal) harvested in 1992 and 1993, in contrast to the higher maltose averages found in samples that had been harvested in 1991.
Maltose/isomaltose ratio (M/I) was proposed as a possible marker of honey adulteration with cheap sweet products, such as invert syrup, corn syrup and high fructose corn syrup (Horvath and Molnar-Perl, 1997). These syrups possess high amounts of glucose or high quantities of different di- and oligosaccharides, such as maltose, maltotriose or sucrose (Anklam, 1998). Horvath and Molnar-Perl (1997) suggested that high M/I values might indicate adulteration with starch hydrolysate, whereas low M/I values might indicate adulteration with high fructose syrups. These researchers described the ranges of M/I for authentic honeys between 0.50 and 21.80. However, further studies demonstrated that this ratio should only be used for guidance, because some genuine honeys with natural M/I high index could be erroneously rejected (Nozal et al., 2005). Bentabol-Manzanares et al. (2011), whose data also fulfilled the proposed interval (Horvath and Molnar-Perl, 1997), showed a higher M/I average for honeydew honeys (7.04) than for blossom ones (5.53). In our research, the M/I ratios ranged between 0.92 and 11.45 (both values in heather honeys), all our results being within the interval proposed for authentic honeys (Horvath and Molnar-Perl, 1997). Chestnut honeys, followed by honeydew samples, showed the lowest averages (2.68 and 2.77 respectively), and lavender the highest (5.23).

Sucrose. It is an important sugar from a legislative point of view, with a general maximum of 5% (OJEC, 2002). As exceptions, some honeys can have a maximum of 10% (such as clover honeys belonging to Medicago sativa genus) or 15% (such as lavender honeys). None of our honeys reached these limits, showing low concentrations for this sugar. The maximum value was found in one lavender sample (5.18%). Chestnut honeys had the lowest sucrose average (0.10%) and lavender the highest (1.50%), in accordance with those reported by Cotte et al. (2003). Ling heather honeys had lower average than the other heather samples (0.04% vs 0.40%). These low sucrose
values suggest an advanced stage of honey ripening, due to conversion of sucrose into glucose and fructose (Terrab et al., 2001; Pasini et al., 2013), as well as the absence of artificial feeding of bees with sucrose syrups (Escuredo et al., 2013). With regard to sucrose content, in the literature higher mean values than those found in our study were reported for chestnut (Golob and Plestenjak, 1999; Šarić et al., 2008) and clover honeys (Shin and Ustunol, 2005; Ouchemoukh et al., 2010). Meanwhile, the sucrose distribution for oak honeydew, heather and lavender honeys was broad, ranging between ND (not detected)–3.36%, 0.02–4.12% and 0.24–8.01%, respectively (Terrab et al., 2003a; Nozal et al., 2005; Nozal-Nalda et al., 2005; Moise et al., 2013). Our lavender samples had lower sucrose than maltose averages, in contrast to the results of Cotte et al. (2003).

**Gentiobiose and Trehalose.** They were the minor disaccharides found in our samples. Their quantities were lower than 0.35%, with mean values lower than 0.19% for gentiobiose and 0.11% for trehalose. The literature references showed very low concentrations for these sugars (Cotte et al., 2003; Terrab et al., 2003a; Nozal et al., 2005; Martins et al., 2008; De la Fuente et al., 2011; Rybak-Chmielewska et al., 2013; Escuredo et al., 2014; Can et al., 2015), with quantities of trehalose higher than those of gentiobiose. Bentabol-Manzanares et al. (2011) observed higher concentration of trehalose in honeydew honeys (average of 1.89%) than in blossom honeys (average of 1.67%), although in a later study the same authors reported a similar mean concentration (1.81%) in heather honeys from *Erica arborea* (Bentabol-Manzanares et al., 2014).

Honeydew honeys showed the highest average for total disaccharides (6.71%), unlike heather samples, whose percentages for total disaccharides (4.93%) were significantly lower than those of chestnut, honeydew and multifloral honeys.
3.2.3.3. *Trisaccharides*. Erlose was the most abundant and the only trisaccharide detected in every sample analyzed in this study. Melezitose, raffinose, maltotriose and panose were detected in all the samples except in one heather honey; and isomaltotriose was absent in five multifloral honeys, two heather honeys, one honeydew honey and one lavender honey.

*Melezitose, erlose and raffinose*. Melezitose was also proposed to differentiate honeydew and blossom honeys (Persano-Oddo and Piro, 2004), being considered an indicator of the presence of honeydew (Ouchemoukh et al., 2010), together with other trisaccharides, such as erlose or raffinose (Weston and Brocklebank, 1999; Bogdanov, 2011). In our research, the maximum averages for melezitose, erlose and raffinose were found in honeydew honeys (0.50%, 0.84% and 0.08%, respectively). These sugars were also detected in samples from all botanical origins. On the one hand, no statistically significantly differences were found for raffinose among the different botanical origins. On the other hand, clover and honeydew possessed significantly higher values of erlose than heather honeys, and honeydew had significantly higher values of melezitose than clover, heather and lavender samples. Erlose is an intermediate trisaccharide in the metabolism of nectar sugars by honeybees (White and Maher, 1953; Kolayli et al., 2012). In general, the presence of melezitose in blossom honeys is considered to be a result of their mixture with honeydew (Da Costa Leite et al., 2000); meanwhile the origin of raffinose in blossom honey is not clear, being suggested that it could be in the nectar composition or it could also come from honeydew contamination (White et al., 1986; Kaškonienė et al., 2010). Our data were in agreement with the values reported by other researchers, where these three sugars were found in similar concentrations as those of our study (Mateo and Bosch-Reig, 1997; Cotte et al., 2003; Nozal et al., 2005;
De la Fuente et al., 2007; De la Fuente et al., 2011). Maximum averages in the literature ranged from 0.52% (lavender honeys) to 2.47% (oak honeydew honeys) for melezitose (Nozal et al., 2005; Can et al., 2015); 0.24% (chestnut honeys) to 1.96% (heather honeys) for erlose (Cotte et al., 2003; Nozal et al., 2005); and 0.22% (chestnut honeys) to 2.03% (heather honeys) for raffinose (Devillers et al., 2004; Martins et al., 2008). The melezitose average reported in Spanish oak honeydew honeys by Escuredo et al. (2014) was lower than the mean value found in our research (0.21%). Other authors found higher melezitose concentrations in Spanish (Mateo and Bosch-Reig, 1997; Terrab et al., 2003b; Nozal et al., 2005) and European (Can et al., 2015) oak honeydew honeys, and proposed melezitose content as a marker for honeydew honey. In European fir honeydew honeys melezitose averages were higher than in oak honeydew samples, so that melezitose could be a marker of honeydew honeys from different botanical origins (Golob and Plestenjak, 1999; Devillers et al., 2004; Rybak-Chmielewska et al., 2013).

**Maltotriose, Panose and Isomaltotriose.** They were found in very low concentrations (always below 0.41%), in agreement with the averages reported in the literature (less than 0.66%) by different authors (Cotte et al., 2003; De la Fuente et al., 2007; De la Fuente et al., 2011). High concentration of trisaccharides such as maltotriose could indicate an adulteration with different syrups (Cotte et al., 2003).
3.2.3.4. Tetrasaccharides. Maltotetraose, the only tetrasaccharide analyzed, was present at very low concentrations in 14 out of the 54 samples: six honeydew honeys, five multifloral samples, two heather honeys and one lavender sample. Both maximum value (0.28%) and maximum average (0.03%) belonged to heather honeys. No data were found in the literature for maltotetraose in unifloral honeys of the same botanical origins as those of our research.

Honeydew honeys exhibited the highest oligosaccharides average (1.81%), in contrast to heather samples that showed the lowest mean value (0.69%). De la Fuente et al. (2011) also found low concentrations of trisaccharides in Spanish heather honeys, although the values of these researchers were higher than those found in our study.

The total sugar averages varied between 71.62% (honeydew samples) and 77.78% (lavender samples). These data agree with the research of Escuredo et al. (2013), who reported lower total sugar mean contents for oak honeydew and chestnut honeys than for heather samples from the North of Spain. Our data of total sugar were within the wide intervals reported in the literature references for honeys from the same botanical origins as our samples (Persano-Oddo and Piro, 2004; Feás et al., 2010b; De la Fuente et al., 2011; Waś et al., 2011).

Sugar composition was proposed to help differentiate floral and honeydew honeys. According to Bogdanov et al. (2008), sucrose, maltose, trehalose or panose, among others, characterize blossom honeys. In our research, honeydew honeys were characterized by lower values of monosaccharides and higher data of di- and oligosaccharides, mainly isomaltose, melezitose, erlose and raffinose, but differences with blossom honeys in relation to sugar composition were not significant. Our carbohydrate data for honeydew samples agreed with literature (Mateo and Bosch-Reig, 1998; Weston and Brocklebank, 1999; Bogdanov et al., 2008). The numerous di- and
trisaccharides in honeydew honeys could be produced by microbial activity and enzymatic reactions in the intestinal tract of the aphids and during honey ripening (Kolayli et al., 2012). Cotte et al. (2003) characterized chestnut honeys by low trisaccharides quantity. In our study, chestnut samples were also poor in oligosaccharides.

3.3. Crystallization indexes

Honey granulation is a natural process of paramount importance because improper crystallization could lead to problems in handling and processing (Özbalci et al., 2013). It also affects honey texture, making this foodstuff less appealing to consumers (Cavia et al., 2002). The tendency to honey granulation was predicted on the basis of different crystallization indexes (Table 2). This foodstuff generally crystallizes faster with a fructose/glucose ratio (F/G) lower than 1.11; and a glucose percentage, a glucose/water ratio (G/W), a (glucose – moisture)/fructose ratio ((G – W)/F) and a melezitose percentage higher than 35%, 2.16, 0.49 and 10%, respectively. On the contrary, honeys with F/G higher than 1.33, and glucose content, G/W and (G – W)/F lower than 28%, 1.70 and 0.30 respectively, generally remain liquid for longer periods (Serra-Bonvehí, 1989; Sancho et al., 1991; Lupano, 1997; Manikis and Thrasyvoulou, 2001; Cavia et al., 2002; Smanalieva and Senge, 2009). Data of these granulation indexes should be considered only as illustrative figures, because some studies have demonstrated that they are not accurate for all honeys. Tabouret (1979) indicated that G/W was not a proper index for honeys with low moisture percentages. Manikis and Thrasyvoulou (2001) observed that glucose was not a useful granulation index in samples that had medium crystallization tendency. They also observed that melezitose
was a poor index because Greek fir honeys had more than 10% melezitose and possessed a slow crystallization tendency.

Depending on the crystallization index, the granulation tendency of each of our five unifloral groups was different. Nevertheless, in line with the calculated crystallization indexes, lavender was always the honey that possessed the fastest granulation tendency monitored by simple observation in the mass of the product, while honeydew and chestnut honeys presented the slowest one. According to \((G - W)/F\), proposed by Serra Bonvehí (1989) as a quite reliable granulation index, crystallization speed of our samples increased in the following order: chestnut < honeydew < clover < heather < lavender.

The vast majority of the researchers reported that both chestnut and honeydew were the honeys that remained liquid for longest (Mateo and Bosch-Reig, 1997; Smanalieva and Senge, 2009; Primorac et al., 2011). Moderate crystallization tendency rates were found for heather honeys, in comparison with other honey types (Smanalieva and Senge, 2009; Escuredo et al., 2014), agreeing with our data. In comparison with literature data, we found higher \(F/G\) values for chestnut samples and lower \(G/W\) results for chestnut and lavender honeys (Persano-Oddo et al., 1995; Mateo and Bosch-Reig, 1998; Cotte et al., 2003; Persano-Oddo and Piro, 2004; Can et al., 2015), whereas for the other monofloral honeys, our averages were within the intervals proposed by other researchers.

It is interesting to highlight the fact that lavender honeys, which showed the highest granulation tendency, were also the samples with the lowest averages of isomaltose. Conversely, chestnut and honeydew honeys, with the lowest granulation tendency were the samples with the highest isomaltose contents. Thus, isomaltose might be likely a disaccharide that helps inhibit honey crystallization.
3.4. **Statistical analyses**

3.4.1. **Correlations**

Pearson test ($p < 0.05$) was applied to the results to check possible significant correlations among parameters. Rotary power was correlated with total sugars ($r = -0.6661$), fructose ($r = -0.7628$), glucose ($r = -0.7468$), isomaltose ($r = 0.7275$), panose ($r = 0.6777$), and total oligosaccharide content ($r = 0.6023$). Total sugar content was correlated with fructose ($r = 0.8730$), glucose ($r = 0.8973$), isomaltose ($r = -0.7260$), panose ($r = -0.6384$) and gentiobiose ($r = -0.6153$). Fructose and glucose were strongly correlated ($r = 0.7971$). Other sugars correlations were found, the most important being glucose and isomaltose ($r = -0.8197$), fructose and panose ($r = -0.7200$), gentiobiose and isomaltose ($r = 0.8154$), panose and isomaltose ($r = 0.8357$), and isomaltose and isomaltotriose ($r = 0.7386$).

3.4.2. **Principal component Analysis (PCA)**

PCA was applied to the results, in order to check the influence of the analysed parameters on the botanical origins of honeys, from a descriptive perspective. First, a PCA was carried out with all parameters except sugar composition: moisture, specific rotation, and crystallization indexes. The best results regarding sample characterization were obtained with the following parameters: specific rotation, fructose/glucose, glucose/water and (glucose – water)/fructose (Figure 2). Two components with eigenvalues greater than or equal to 1.0, explained 93.76% of the variance. PC1 comprised the information about the crystallization indexes (fructose/glucose, glucose-water/fructose and glucose/water), while PC2 showed the differences in respect of
specific rotation. Four separate groups of honeys were observed. Multifloral honeys were not properly separated from the other groups. Lavender honeys exhibited a high crystallization tendency. Clover honeys presented intermediate crystallization tendencies, as well low specific rotation results. Honeydew honeys showed high specific rotation values. Heather honeys exhibited the lowest specific rotation values. Chestnut honeys, presented a low crystallization tendency, all samples being grouped on the positive PC1 axis.

PCA was also applied to sugar composition. Agreeing with literature (De la Fuente et al., 2011), unifloral honeys could not be properly differentiated on the basis of sugar composition. With regard to classification, the best results were obtained with fructose, glucose, maltose, isomaltose, sucrose, gentiobiose, trehalose, melezitose and raffinose. Four components, with eigenvalues greater than or equal to 1.0, explained 82.07% of the variance. The first two components explained 59.38% of the variance (Figure 3). This PCA showed two groups of samples. The first group consisted of lavender, clover and the vast majority of heather honeys, because of their high concentrations of monosaccharides. The second group was formed by honeydew honeys, with high values of isomaltose, melezitose, maltose, gentiobiose and raffinose. Chestnut and multifloral honeys were spread within both groups. PCA carried out with both specific rotation, crystallization indexes and sugar composition did not improve the separation of samples.

In summary, specific rotation and crystallization indexes were more efficient for honey characterization than sugar composition. Sugar composition separated floral honeys from honeydew honeys and samples rich in honeydew elements. Nevertheless, sugar composition and sugar-related parameters were not, in themselves, capable of
properly distinguishing unifloral honeys, so further compositional analyses would still be necessary to achieve this challenging goal.

4. Conclusions

Moisture values indicated optimum harvesting practices. Honeydew honeys showed the highest optical rotation, in contrast to heather samples, that presented the lowest average for this parameter. Both honeydew and chestnut honeys exhibited the lowest averages for fructose and glucose, and the highest averages for isomaltose. Honeydew honeys presented the highest disaccharides and trisaccharides averages, and, as expected, the highest mean concentration of the main trisaccharides considered indicators of the presence of honeydew (erlose, melezitose and raffinose). Chestnut samples showed the lowest sucrose, trehalose and maltotriose mean values and a low trisaccharides concentration. Heather, lavender and clover honeys exhibited the highest monosaccharide averages. Heather honeys showed the lowest disaccharides and trisaccharides mean values, as well as the lowest maltose and erlose averages. Conversely, clover and lavender samples presented high disaccharide and erlose concentrations, the highest maltose and the lowest melezitose averages. Lavender samples were characterized for the highest sucrose and the lowest isomaltose and raffinose mean contents.

In respect of crystallization tendencies, lavender honeys showed the fastest granulation tendency, whereas chestnut and honeydew honeys exhibited the slowest one. Isomaltose seems to help inhibit honey crystallization.
Acknowledgements

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References


Biological activities and chemical composition of three honeys of different types from Anatolia. *Food Chemistry*, **100**(2), 526-534.


Figure captions:

Figure 1. GC sugar profile of a honey sample from the northern Iberian Plateau.
   A) Gas chromatogram. Peak attribution (as trimethyl silyl ethers derivatives) of sugars: 1, mannitol; 2, fructose; 3, glucose; 4, sucrose; 5, trehalose; 6, maltose; 7,9, gentiobiose; 10, isomaltose; 11, raffinose; 12, erlose; 13, melezitose; 14, maltotriose; 15, panose; 16,17, isomaltotriose; 18, maltotetraose.
   B) Expanded disaccharides zone of the chromatogram of a honey sample.

Figure 2. Principal component analysis of specific rotation, fructose/glucose, glucose/water and (glucose-water)/fructose in northern Iberian plateau honeys from different botanical origins: (H, honeydew; M, multifloral; C, chestnut; E, heather; L, lavender; T, clover).

Figure 3. Principal component analysis of fructose, glucose, maltose, isomaltose, sucrose, gentiobiose, trehalose, melezitose and raffinose in northern Iberian plateau honeys from different botanical origins: (H, honeydew; M, multifloral; C, chestnut; E, heather; L, lavender; T, clover).

----- Honeydew honeys and honeys rich in honeydew elements.
---- Blossom honeys.
Figure 1

A
Figure 3
Table 1. Mean, standard deviation (SD), minimum and maximum values of the parameters analyzed.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Chestnut (n = 4)</th>
<th>Clover (n = 3)</th>
<th>Heather (n = 10)</th>
<th>Honeydew (n = 18)</th>
<th>Lavender (n = 4)</th>
<th>Multifloral (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>moisture (%)</strong></td>
<td>16.5±0.4a</td>
<td>16.7±0.7a</td>
<td>16.2±1.4b</td>
<td>15.6±0.6b</td>
<td>15.2±0.3b</td>
<td>15.5±0.6b</td>
</tr>
<tr>
<td><strong>specific rotation (º)</strong></td>
<td>-5.4±1.57a-b</td>
<td>-9.96±2.00c</td>
<td>-13.45±6.60a</td>
<td>-1.81±7.32a</td>
<td>-7.69±4.59a</td>
<td>-7.37±4.03a</td>
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<tr>
<td><strong>fructose (%)</strong></td>
<td>37.3±4.40</td>
<td>38.08±1.03</td>
<td>39.93±2.27</td>
<td>36.24±1.24</td>
<td>38.52±0.68</td>
<td>39.07±1.76</td>
</tr>
<tr>
<td><strong>glucose (%)</strong></td>
<td>27.30±2.84b</td>
<td>30.24±1.89b</td>
<td>31.03±3.02a</td>
<td>26.86±1.17b</td>
<td>31.38±1.38a</td>
<td>30.07±3.35a</td>
</tr>
<tr>
<td><strong>sucrose (%)</strong></td>
<td>0.10±0.07</td>
<td>0.23±0.05</td>
<td>0.26±0.24</td>
<td>0.37±0.28</td>
<td>1.50±2.47</td>
<td>0.37±0.33</td>
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<tr>
<td><strong>trehalose (%)</strong></td>
<td>0.06±0.05*</td>
<td>0.07±0.01*</td>
<td>0.11±0.09*</td>
<td>0.08±0.04*</td>
<td>0.08±0.01*</td>
<td>0.11±0.06*</td>
</tr>
<tr>
<td><strong>maltose (%)</strong></td>
<td>4.26±1.15ab</td>
<td>5.14±1.14a</td>
<td>3.34±1.03b</td>
<td>4.32±1.17ab</td>
<td>4.04±0.89ab</td>
<td>4.23±0.73ab</td>
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<tr>
<td><strong>gentiobiose (%)</strong></td>
<td>0.19±0.02</td>
<td>0.11±0.03</td>
<td>0.17±0.11</td>
<td>0.19±0.05</td>
<td>0.11±0.04</td>
<td>0.16±0.06</td>
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<tr>
<td><strong>isomaltose (%)</strong></td>
<td>1.61±0.19</td>
<td>1.06±0.41</td>
<td>1.06±0.85</td>
<td>1.75±0.33</td>
<td>0.88±0.29</td>
<td>1.26±0.42</td>
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<tr>
<td><strong>raffinose (%)</strong></td>
<td>0.07±0.09</td>
<td>0.06±0.04*</td>
<td>0.05±0.05*</td>
<td>0.08±0.06*</td>
<td>0.03±0.02*</td>
<td>0.08±0.06*</td>
</tr>
<tr>
<td><strong>erlose (%)</strong></td>
<td>0.41±0.29ab</td>
<td>0.76±0.08a</td>
<td>0.24±0.26b</td>
<td>0.84±0.34a</td>
<td>0.81±0.54ab</td>
<td>0.69±0.41ab</td>
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<tr>
<td><strong>melezitose (%)</strong></td>
<td>0.15±0.14ab</td>
<td>0.08±0.02b</td>
<td>0.16±0.23b</td>
<td>0.50±0.49a</td>
<td>0.07±0.03b</td>
<td>0.25±0.29ab</td>
</tr>
<tr>
<td><strong>maltotriose (%)</strong></td>
<td>0.05±0.02</td>
<td>0.09±0.01</td>
<td>0.07±0.08</td>
<td>0.09±0.02</td>
<td>0.11±0.05</td>
<td>0.09±0.05</td>
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<tr>
<td><strong>panose (%)</strong></td>
<td>0.20±0.06ab</td>
<td>0.25±0.12abcd</td>
<td>0.13±0.12b</td>
<td>0.25±0.05a</td>
<td>0.15±0.02cd</td>
<td>0.17±0.06ce</td>
</tr>
<tr>
<td><strong>isomaltotriose (%)</strong></td>
<td>0.04±0.02</td>
<td>0.01±0.00</td>
<td>0.04±0.07</td>
<td>0.04±0.02</td>
<td>0.01±0.01</td>
<td>0.02±0.02</td>
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<tr>
<td><strong>maltotetraose (%)</strong></td>
<td>0.00±0.01</td>
<td>ND</td>
<td>0.03±0.09</td>
<td>0.01±0.01</td>
<td>0.00±0.01</td>
<td>0.03±0.05</td>
</tr>
<tr>
<td><strong>total sugars (%)</strong></td>
<td>71.95±6.67</td>
<td>76.20±3.1a</td>
<td>76.59±4.69</td>
<td>71.62±2.63</td>
<td>77.68±1.99</td>
<td>76.59±4.14</td>
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<tr>
<td><strong>fructose+glucose (%)</strong></td>
<td>64.83±7.22</td>
<td>68.32±2.90</td>
<td>70.96±5.00</td>
<td>63.10±2.22</td>
<td>69.89±2.04</td>
<td>69.14±7.72</td>
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<tr>
<td><strong>total disaccharides (DS) (%)</strong></td>
<td>6.22±1.12a</td>
<td>6.62±1.57ab</td>
<td>4.93±1.32a</td>
<td>6.71±1.22a</td>
<td>6.60±3.12ab</td>
<td>6.13±0.82a</td>
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<tr>
<td><strong>total tri- and tetrasaccharides (TS) (%)</strong></td>
<td>0.92±0.53ab</td>
<td>1.25±0.24ab</td>
<td>0.69±0.56b</td>
<td>1.81±0.67b</td>
<td>1.17±0.56ab</td>
<td>1.35±0.58ab</td>
</tr>
<tr>
<td><strong>total DS + TS (%)</strong></td>
<td>7.14±1.14</td>
<td>7.87±1.76</td>
<td>5.63±1.74</td>
<td>8.52±1.29</td>
<td>7.77±3.61</td>
<td>7.46±1.06</td>
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<tr>
<td><strong>maltose/somaltose</strong></td>
<td>2.68±0.86</td>
<td>5.16±1.28</td>
<td>5.17±3.67</td>
<td>2.77±1.22</td>
<td>5.23±2.57</td>
<td>3.80±1.73</td>
</tr>
</tbody>
</table>

Mean values within the same row having different lower-case letter are significantly different by Student-Newman-Keuls multiple range test (p < 0.05). Median values within the same row having different capital letter are significantly different by the median nocht in the Box-and-Whisker Plot (p < 0.05). *There are no significant
differences between the means or medians at the 95.0% confidence level ($p \geq 0.05$). Neither letters nor * mean that ANOVA could not be used. ND: not detected.
Table 2. Mean, standard deviation (SD), minimum and maximum values of the sugars ratios of artisanal honeys from Castilla y León.

<table>
<thead>
<tr>
<th></th>
<th>chestnut</th>
<th>clover</th>
<th>heather</th>
<th>honeydew</th>
<th>lavender</th>
<th>multifloral</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 4)</td>
<td>(n = 3)</td>
<td>(n = 10)</td>
<td>(n = 18)</td>
<td>(n = 4)</td>
<td>(n = 15)</td>
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<tr>
<td>mean±SD (min; max)</td>
<td>1.37±0.04</td>
<td>1.26±0.05</td>
<td>1.29±0.08</td>
<td>1.34±0.05</td>
<td>1.23±0.03</td>
<td>1.31±0.13</td>
</tr>
<tr>
<td></td>
<td>(1.32; 1.40)</td>
<td>(1.21; 1.29)</td>
<td>(1.19; 1.40)</td>
<td>(1.25; 1.43)</td>
<td>(1.20; 1.28)</td>
<td>(1.18; 1.59)</td>
</tr>
<tr>
<td>glucose/moisture</td>
<td>1.66±0.15</td>
<td>1.81±0.08</td>
<td>1.92±0.17</td>
<td>1.74±0.11</td>
<td>2.07±0.09</td>
<td>1.94±0.26</td>
</tr>
<tr>
<td></td>
<td>(1.50; 1.86)</td>
<td>(1.74; 1.90)</td>
<td>(1.65; 2.14)</td>
<td>(1.56; 1.98)</td>
<td>(2.00; 2.18)</td>
<td>(1.52; 2.37)</td>
</tr>
<tr>
<td>(glucose – moisture)/fructose</td>
<td>0.29±0.04</td>
<td>0.35±0.01</td>
<td>0.37±0.05</td>
<td>0.31±0.03</td>
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<tr>
<td></td>
<td>(0.25; 0.33)</td>
<td>(0.34; 0.37)</td>
<td>(0.28; 0.43)</td>
<td>(0.26; 0.36)</td>
<td>(0.39; 0.45)</td>
<td>(0.21; 0.49)</td>
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