Exploring the sugar profile of unifloral bee pollen using high performance liquid chromatography

VASILIOS LIOLIOS – CHRYSOULA TANANAKI – MARIA DIMOU – DIMITRIOS KANELIS – MARIA-ANNA RODOPOULOU – ANDREAS THRASYVOULOU

Summary
Sugars constitute the main component of bee pollen, a product that nowadays presents a growing interest as food supplement. The present work describes a simple, rapid and reliable high performance liquid chromatographic method to determine the composition of sugars in bee pollen. Moreover, the effect of bee pollen's botanical origin on the sugars profile along with the significance of the nectar that bees add during bee pollen pellet's formation were investigated. The analysis of 117 samples showed that bee pollen was characterized by a high content of glucose and fructose, constituting almost 90 % of total sugars, which in turn depends on the botanical origin of bee pollen. Fructose and glucose average contents ranged from 155.3 g·kg^{-1} to 334.8 g·kg^{-1} and from 135.9 g·kg^{-1} to 276.9 g·kg^{-1}, respectively. The highest average content of total sugars was found in kiwi bee pollen (635.3 g·kg^{-1}) and the lowest in ivy bee pollen (347.1 g·kg^{-1}). Furthermore, comparative sugars content analysis of *Sisimbrium irio* unifloral bee pollen, collected in different seasons, showed that the added nectar during bee pollen packaging influenced considerably the sugars content. Results of this study may help to consider sugars as an additional parameter in an effort to establish quality standards for bee pollen.

Keywords
unifloral bee pollen; dietary supplement; sugars; botanical origin; seasonal variation

Nowadays, there is a growing consumers' awareness regarding food ingredients. Hence, the study and definition of the chemical composition of each food product is crucial. Bee pollen has been a subject of extensive study not only due to its importance in bee's nutrition but also due to its value as a food supplement in human diet, being a rich source of proteins and amino acids [1, 2]. Moreover, the high contents of reducing sugars, vitamins, unsaturated and saturated fatty acids, the presence of Zn, Cu, Fe and the high K/Na ratio make bee pollen a product of high nutritional value [3, 4]. Thanks to its special beneficial characteristics and due to its gradually increasing consumption, the need for quality standards for bee pollen is widely recognized both for consumer's safety and for the enhancement of beekeeping through added-value products.

So far, legislated quality standards for bee pollen do not exist in the European Union or on international level, and only a few countries such as Brazil [5], Poland [6], Switzerland [7], Argentina [8] and Bulgaria [9] have established bee pollen criteria. However, none of them have set specifications on sugars. Considering that sugars comprise one of the main components of bee pollen's chemical composition, constituting 40 % of its dry matter [10], their determination is fundamental for the establishment of quality standards and authenticity of the product.

Besides that, a great variation of sugar composition can be observed among various bee pollen species. Szczęsna et al. [11] reported a range of sugar content from 281.3 g·kg^{-1} to 479.1 g·kg^{-1}, while Bogdanov [12] from 130.0 g·kg^{-1} to 550.0 g·kg^{-1}. This broad range is significant for consumers that would like to embark on a new food regimen, as they will be able to choose among species of different sugar content. Szczęsna et al. [11] attributed that variation to the sugars content in various plants that bloom in various periods, since the pasture of bee plants

Vasilios Liolios, Chrysoula Tananaki, Maria Dimou, Dimitrios Kanelis, Maria-Anna Rodopoulou, Andreas Thrasyvoulou, Laboratory of Apiculture-Sericulture, Aristotle University of Thessaloniki, Aristotle Farm, 57001 Thermi, Greece.

Correspondence author:
Chrysoula Tananaki, e-mail: tananaki@agro.auth.gr

change with the time. Several other authors also referred to the botanical origin of bee pollen as a significant factor that contributes to variation in the content of sugars [10, 11, 13, 14]. However, a great majority of these studies were based on the analysis of multifloral (mixed) bee pollen and only in few cases the authors examined unifloral bee pollen samples [15–17].

Furthermore, during pollen collection, bees use honey and nectar to form the pollen pellets [11, 15, 18]. Nectar is rich in carbohydrates, so the sugars composition of pollen is influenced significantly by the added nectar during pollen formation [11, 15]. Although the sugars content in nectar is usually characterized also by high concentrations of saccharose [19, 20], the reduction of saccharose and increase of glucose and fructose in bee pollen could be explained by the activity of the enzyme invertase. Invertase, which is present both in bees' secretions and pollen, activates and converts saccharose to monosaccharides glucose and fructose during the addition of nectar for the formation of the pollen pellet [21–23]. Additionally, considering that various types of honey or nectar sources differ in their carbohydrate composition, it is apparent that the sugars content in bee pollen is influenced by the type of nectar that is available to bees during the formation of the pellet. However, the impact of nectar on sugars composition of bee pollen has not been studied in detail and only a few studies discussed its possible effect on bee pollen's sugars profile [11, 24].

In addition, use of different methods of analysis contributed further to substantial variation of published sugars profiles of bee pollen. Day et al. [15] used a method based on colorimetric reaction with carbohydrates, while Yang et al. [17] calculated the sugars content by subtracting the sum of protein, lipid and ash contents from the total components of bee pollen. Serra–Bonvehi and Jorda [25] as well as Szczęsna et al. [11] used gas chromatography (GC), whereas Szczęsna [10], Dominguez-Valhondo et al. [16] and Martins et al. [26] used high performance liquid chromatography attached to refractive index detector (HPLC-RID).

Among the mentioned methods, HPLC is widely used thanks to its accuracy and convenience. In this study, we developed and validated a liquid chromatographic method to quantify the major and minor sugars of bee pollen and we analysed several unifloral bee pollen samples. Also, we analysed bee pollen from a single taxon throughout the whole apicultural period and from the same area, in an effort to shed some light on the impact of the nectar and surrounding vegetation on sugars composition of bee pollen pellets. The study of the impact of botanical origin on bee pollen's sugar composition would be useful for the proposal of national or international quality standards of the product.

MATERIALS AND METHODS

Sampling

Bee pollen samples were harvested from the apiary of Apiculture-Sericulture Laboratory of Aristotle University of Thessaloniki, located in the University Farm at the area of Eastern Thessaloniki (North Greece). The farm is extended on over 180 ha and consists of the university orchard and mixed cultivations of grains, vegetables and forage plants. The collection of bee pollen lasted from March to October.

Pollen traps (n = 3) were fitted in the entrance of each beehive and the pollen pellets were harvested every 1–2 days. The pollen was cleaned of debris and kept in glass jars at –21 °C until its analysis. Pollen pellets were classified according to their colour, as well as to their shape and texture. Each examined pollen pellet was placed on a slide with a drop of a glucose solution and stained with alcoholic fuchsine [27]. The slide was then dried by slight warming (not above 40 °C) and mounted with a rapid medium for microscopy Entellan (Entelan Microscopy, Karlsruhe, Germany). In each slide, we counted at least 300 pollen grains to verify the homogenity of each colour fraction. For microscopic identification of pollen types, the collection of reference slides from the Laboratory of Apiculture of the Aristotle University of Thessaloniki was used [28]. At least two representative pellets of each colour fraction were used to identify the botanical origin of each pellet, in accordance with the International Commission of Bee Botany of International Union of Biological Sciences [27]. Totally, 90 bee pollen samples, three for each of the 30 identified taxa, were analysed. Each of these three samples was collected from different bee colony at the same period.

In addition, to determine the impact of added nectar on the sugars content during bee pollen load formation by the bees, we studied the sugars composition of *Sisymbrium irio* bee pollen during a whole apicultural period. Totally, 27 samples were collected and analysed. *S. irio* is a very important bee-value taxon, which is abundant in the area of the study and, to our knowledge, it is the only plant that combines high visitation from the bees and a very long flowering season (March–June and August–November) in Greece [28].

Chemicals and reagents

Acetonitrile (HPLC-grade) and methanol (99 %) were purchased from Chem-Lab (Zedelgem, Belgium). The purified water used in all experiments was produced by a Millipore Simplicity 185 system (Merck, Darmstadt, Germany). The standards of fructose (99 %), glucose (99.5 %), saccharose (99.5 %), turanose (99 %), maltose (99 %), trehalose (99.5 %) and the reagents potassium hexacyanoferrate (II) trihydrate (99.5 %) and zinc acetate dihydrate (99.5 %) were from Fluka (Buchs, Switzerland). The sugars melibiose (99 %) and melezitose (99 %) were purchased from Sigma (St. Louis, Missouri, USA).

Sample preparation

For the sample preparation, four procedures were examined:

A – filtration,
B – sonication for 15 min at 40 °C and filtration,
C – addition of potassium hexacyanoferrate (II) trihydrate and zinc acetate solutions and filtration,
D – addition of potassium hexacyanoferrate (II) trihydrate and zinc acetate solutions, centrifugation and filtration.

The basic procedure in all cases was the following: An amount of 1 g of each sample was accurately weighed and diluted with 5 ml of water/methanol solution (3:1). Then, the diluted samples were transferred to a 10-ml volumetric flask. The solution was mixed by a vortex mixer (TopMix, FB15024; Thermo Fisher Scientific, Waltham, Massachusetts, USA), the flask was filled to 10 ml volume with the water/methanol solution and the mixture was homogenized. The liquid was filtered through a disposable polyvinylidene difluoride syringe filter (pore size 0.22 μm; Merck) and the filtrate was collected into 2 ml glass vials (Chromacol, Herts, United Kingdom).

In procedures C and D, 0.1 ml of the solutions potassium hexacyanoferrate (II) trihydrate (0.15 g·ml^{-1}) and zinc acetate (0.3 g·ml^{-1}) were added before mixing. Eventually, in the case D, centrifugation was used to support filtration of the bee pollen solution. Also, the addition of potassium hexacyanoferrate (II) trihydrate and zinc acetate eliminated the turbidity of the samples.

For centrifugation of the samples, an International Equipment Company (IEC) centrifuge model Centra CL 2 (Thermo Fisher Scientific) was used and the rotation was 1 500 ×*g*. Finally, to express the results as grams per kilogram of dry matter of bee pollen, the water content in samples was determined by using the gravimetric method [29].

Preparation of standard solutions

For the creation of the calibration curve for each sugar, the following stock solutions were prepared: 0.40 g·ml^{-1} for fructose and glucose, 0.03 g·ml^{-1} for turanose, and 0.05 g·ml^{-1} for the other five sugars (saccharose, maltose, melebiose, melezitose, trehalose). These solutions were kept in the freezer. Using the stock solutions, the following working solutions were made: 20 mg·ml^{-1} for fructose and glucose and 3 mg·ml^{-1} for the other sugars.

HPLC analysis

Sugars analysis of bee pollen was performed by HPLC-RID using Agilent Technologies 1200 series system (Agilent Technologies, Santa Clara, California, USA). Elution was performed using acetonitrile/water (80 : 20) as the mobile phase, at a flow rate of 1.3 ml·min^{-1}. The sugars were separated on a Zorbax column for carbohydrate analysis (150 mm × 4.6 mm, particle size 5 μm; Agilent Technologies). The column and the refractive index detector were maintained at 30 °C. The injection volume was 10 μl.

Calibration curves

For each sugar, a five point calibration curve was created using the following solutions: 2.0, 4.0, 8.0, 15.0 and 40.0 mg·ml^{-1} for fructose and glucose, and 0.1, 1.0, 2.0, 3.5 and 5.0 mg·ml^{-1} for saccharose, turanose, trehalose, maltose, melibiose and melezitose. Each mixture of standards was analysed for five times.

Method validation

For the determination of the sugars, the HPLC method was validated for linearity, limit of detection (*LOD*), limit of quantification (*LOQ*), precision and accuracy. Linearity was calculated by least squares linear regression analysis of calibration curve. The *LOD* and *LOQ* values were calculated from the calibration curves using the slope and the standard deviation of the curve, according to the equations:

$$LOD = 3.3 \times \frac{\sigma}{S} \quad (1)$$

$$LOQ = 10 \times \frac{\sigma}{S} \quad (2)$$

where σ represents the standard deviation of the intercept and S is the slope of the calibration curve.

The intra-day precision (based on five repetitions on the same day) and inter-day precision (based on five repetitions over three different days), relative standard deviation (*RSD*)

and accuracy (recovery) of the method expressed in percent were assessed, using three different levels of concentration of each sugar (2.5 mg·ml^{-1}, 12.0 mg·ml^{-1}, 18.0 mg·ml^{-1} for fructose and glucose, 2.5 mg·ml^{-1}, 3.0 mg·ml^{-1}, 4.0 mg·ml^{-1} for the rest of sugars). Finally, in order to determine the repeatability of the method, the same sample of bee pollen was analysed five times and, for reproducibility calculation, the same sample of bee pollen was analysed for five different days.

Statistical analysis

The principal component analysis (PCA) was performed to identify variability and to reduce the dimensions of the dataset of the 30 different bee pollen types. The one-way analysis of variance (ANOVA) and the Tukey's multiple range test were used to compare the sugars profile of bee pollen collected in different time periods. The significance level of the statistical tests was set at $\alpha \leq 0.05$. The analyses were carried out using the Minitab v.17.1.0 software (Minitab, Coventry, United Kingdom).

RESULTS

Method validation

Linearity

The linearity of the method for each sugar assayed was examined. The concentrations of fructose and glucose for the calibration curves ranged from 2.0 mg·ml^{-1} to 40 mg·ml^{-1} and the concentrations of sugars saccharose, turanose, maltose, trehalose, melibiose and melezitose ranged from 0.1 mg·ml^{-1} to 5.0 mg·ml^{-1}. Linear least squares regression was used to calculate the slope and intercept, with their correlation coefficient. The regression equations for the examined sugars are given in Tab. 1. The correlation coefficients ranged from 0.9998 to 0.9999 for all the analysed sugars.

Tab. 1. Regression equations of examined sugars.

Sugar	Regression equation
Fructose	$Y = (55\,430.77 \pm 278.71)X - (3\,646.06 \pm 5\,445.96)$
Glucose	$Y = (72\,926.96 \pm 437.93)X - (18\,559.90 \pm 8\,557.06)$
Saccharose	$Y = (29\,135.87 \pm 63.95)X + (13\,903.14 \pm 185.93)$
Turanose	$Y = (59\,568.09 \pm 136.76)X - (236.21 \pm 397.61)$
Maltose	$Y = (62\,112.79 \pm 170.70)X - (4\,043.63 \pm 496.28)$
Trehalose	$Y = (55\,366.77 \pm 289.82)X - (6\,097.30 \pm 842.58)$
Melibiose	$Y = (47\,827.48 \pm 154.62)X - (5\,021.5 \pm 449.51)$
Melezitose	$Y = (52\,824.83 \pm 79.70)X - (2\,432.05 \pm 231.70)$

Limits of detection and quantification

The *LOD* values were found to be 0.51 mg·ml^{-1} and 0.61 mg·ml^{-1} for fructose and glucose, respectively, while for saccharose, turanose, maltose, trehalose, melibiose and melezitose, they ranged from 0.02 mg·ml^{-1} to 0.04 mg·ml^{-1}.

The *LOQ* values were determined at 1.55 mg·ml^{-1} and 1.86 mg·ml^{-1} for fructose and glucose, respectively, while for saccharose, turanose, maltose, trehalose, melibiose and melezitose, the range was from 0.06 mg·ml^{-1} to 0.10 mg·ml^{-1} (Tab. 2).

Accuracy and precision of the chromatographic analysis

The intra-day and inter-day precision and accuracy were assessed by analysing each sugar five times at three concentrations (2.5 mg·ml^{-1}, 12.0 mg·ml^{-1}, 18.0 mg·ml^{-1} for fructose and glucose, 2.5 mg·ml^{-1}, 3.0 mg·ml^{-1}, 4.0 mg·ml^{-1} for the rest of sugars). A high degree of accuracy was achieved, as estimated by the recovery values, which ranged from 85.7 % to 108.9 % and from 89.7 % to 105.2 % for the intra- and inter-day calibration, respectively (Tab. 2). The precision was satisfactory, as the *RSD* values ranged from 0.4 % to 5.7 % for the intra-day and from 2.4 % to 7.3% for the inter-day calibration (data not shown).

Repeatability and reproducibility of the method

The repeatability of the method was determined using the same mixed bee pollen sample, containing all the examined sugars, which was analysed for five times the same day. For fructose, the average was (180.4 ± 7.2) g·kg^{-1}, for glucose (164.7 ± 10.3) g·kg^{-1}, for saccharose (26.6 ± 1.9) g·kg^{-1}, for turanose (1.3 ± 0.1) g·kg^{-1}, for maltose (5.8 ± 0.3) g·kg^{-1}, for trehalose (20.6 ± 1.2) g·kg^{-1}, for melibiose (4.3 ± 0.4) g·kg^{-1} and for melezitose (0.8 ± 0.1) g·kg^{-1}. *RSD* for fructose, glucose, saccharose, turanose, maltose, trehalose, melibiose and melezitose was 3.9 %, 6.1 %, 7.2 %, 7.7 %, 5.2 %, 5.8 %, 8.7 % and 5.7 %, respectively, demonstrating good repeatability of the method.

The reproducibility of the method was determined using the same sample of mixed bee pollen that was analysed for five different days. The average fructose content was (184.8 ± 5.2) g·kg^{-1}, glucose (160.6 ± 7.6) g·kg^{-1}, saccharose (270.1 ± 2.1) g·kg^{-1}, turanose (1.32 ± 0.1) g·kg^{-1}, maltose (2.6 ± 0.5) g·kg^{-1}, trehalose (20.8 ± 2.8) g·kg^{-1}, melibiose (5.3 ± 0.4) g·kg^{-1} and melezitose (1.4 ± 0.4) g·kg^{-1}. *RSD* for fructose, glucose, saccharose, turanose, maltose, trehalose, melibiose and melezitose was 2.7 %, 4.4 %, 8.0 %,

Tab. 2. Validation of the analytical method.

Sugar	Tested concentration [mg·ml^{-1}]	Recovery [%]		Precision [%]		*LOD* [mg·ml^{-1}]	*LOQ* [mg·ml^{-1}]
		Intra-day	Inter-day	Intra-day	Inter-day		
Fructose	2.5	99.2 ± 4.1	105.2 ± 2.1	4.8	7.3	0.51	1.55
	12.0	98.8 ± 4.3	99.6 ± 1.6	4.6	5.8		
	18.0	95.9 ± 0.7	97.3 ± 0.9	3.8	5.2		
Glucose	2.5	100.3 ± 13.4	96.8 ± 1.5	3.4	3.5	0.61	1.86
	12.0	102.2 ± 1.5	99.9 ± 0.6	5.7	4.8		
	18.0	96.8 ± 7.3	97.2 ± 1.8	3.0	2.4		
Saccharose	2.5	106.5 ± 0.7	89.7 ± 6.6	1.1	6.5	0.02	0.08
	12.0	103.1 ± 3.0	85.7 ± 5.4	3.5	5.6		
	18.0	95.9 ± 1.1	97.8 ± 3.6	1.5	4.1		
Turanose	2.5	98.1 ± 5.2	95.9 ± 4.3	4.6	3.6	0.03	0.09
	12.0	94.7 ± 6.1	99.3 ± 2.7	5.1	2.8		
	18.0	98.7 ± 0.7	94.7 ± 1.9	3.9	4.3		
Maltose	2.5	103.9 ± 5.1	97.1 ± 1.6	4.9	4.0	0.03	0.10
	12.0	95.1 ± 4.5	97.9 ± 1.8	2.8	2.5		
	18.0	91.5 ± 3.1	94.1 ± 2.8	4.2	2.4		
Trehalose	2.5	104.3 ± 3.9	93.4 ± 5.0	3.6	5.2	0.03	0.10
	12.0	100.4 ± 7.5	98.0 ± 4.5	1.5	5.0		
	18.0	85.7 ± 2.3	90.7 ± 4.0	2.9	2.7		
Melibiose	2.5	99.4 ± 2.4	94.8 ± 3.7	0.4	3.3	0.04	0.10
	12.0	98.8 ± 3.9	93.5 ± 5.8	1.2	2.5		
	18.0	94.5 ± 4.4	98.6 ± 2.5	2.8	3.2		
Melezitose	2.5	100.6 ± 0.7	97.1 ± 1.7	3.3	3.8	0.02	0.06
	12.0	108.9 ± 1.2	98.4 ± 3.9	2.0	6.5		
	18.0	97.6 ± 2.6	100.9 ± 2.6	1.2	2.5		

LOD – limit of detection, *LOQ* – limit of quantification.

7.1 %, 3.8 %, 7.7 %, 8.5 % and 6.4%, respectively, demonstrating good reproducibility of the method.

Sugars composition of unifloral bee pollen

The results of sugars analysis showed that the levels in unifloral bee pollen varied among the species (Tab. 3).

The average content of total sugars was 421.0 g·kg^{-1} and ranged from 347.1 g·kg^{-1} for *Hedera helix to* 635.3 g·kg^{-1} for *Actinidia chinensis* bee pollen. The most important sugars in all unifloral bee pollen samples were the monosaccharides fructose and glucose, constituting 94.0 % of the total sugars content. The average content of fructose ranged from 155.3 g·kg^{-1} (*Lamium amplexicaule*) to 334.8 g·kg^{-1} (*A. chinensis*). In turn, the average content of glucose fluctuated from 135.9 g·kg^{-1} (*H. helix*) to 276.9 g·kg^{-1} (*A. chinensis*). In general, fructose was by its higher content comparable to glucose in all bee pollen taxa, except for *Phacelia tanacetifolia* and *L. amplexicaule* (Tab. 3).

The disaccharides saccharose, turanose, maltose, trehalose, melibiose and melezitose were found in much lower contents. In particular, saccharose was found in 17 out of 30 taxa and its highest average content (82.4 g·kg^{-1}) was recorded for *Inula viscosa* (Tab. 3). Turanose was detected in 19 samples, in the average content lower than 10 g·kg^{-1}, with the exception of *Chenopodium album* (34.7 g·kg^{-1}). In the rest of the bee pollen samples, average maltose content ranged between 2.6 g·kg^{-1} and 36.6 g·kg^{-1} and the highest value was observed in *H. helix* bee pollen. Melibiose and trehalose were only detected in seven and three bee pollen species, respectively. The average highest content of melibiose was found in bee pollen of *Papaver rhoeas* (8.4 g·kg^{-1}), while the average highest content of trehalose was detected in *Tamarix parviflora* (9.3 g·kg^{-1}). The trisaccharide melezitose was detected only in bee pollen of *Cichorium intybus* at a minor content (0.9 g·kg^{-1}) (Tab. 3).

PCA was conducted to evaluate, from a descriptive point of view, the effect of bee pollen's botanical origin on the sugar composition applying

the covariance matrix. PCA makes achievable the automatic discrimination of the dataset in relation to the analysed parameters. Fig. 1 demonstrates the score plot of 30 different bee pollen taxa after the performed PCA. The portion of 86.1 % of the variations in the dataset could be explained by two factors (F1 explained 68.2 % and F2 explained 17.9 %). In the score plot, proximity between samples shows similar sugar profile. It is evident that the majority of samples showed a good covariance, whereas some common taxa collected by bees such as *A. chinensis*, *I. viscosa*, *Tilia intermedia*, *Erica manipuliflora*, *Cistus creticus*, *C. album* and *P. rhoeas* diverged from the whole.

Sugars composition of *Sisymbrium irio* bee pollen collected in different periods

The sugars composition of *S. irio* bee pollen collected from April to October was varied regarding the season of collection (spring, summer, autumn), and the major sugars (fructose, glucose, saccharose) showed statistically significant differences among the periods of collection throughout the study period ($P = 0.032$) (Tab. 4). The differ-

Tab. 3. Sugar composition of 30 unifloral bee pollen species.

Taxa	Content [g·kg^{-1}]								
	Fructose	Glucose	Saccharose	Turanose	Maltose	Trehalose	Melibiose	Melezitose	Total sugars
Actinidia chinensis	334.8	276.9	15.3	ND	8.3	ND	ND	ND	635.3
Castanea sativa	256.1	182.9	4.6	2.7	3.4	ND	ND	ND	449.7
Chenopodium album	204.2	168.5	34.9	34.7	ND	ND	ND	ND	442.3
Cichorium intybus	185.0	153.9	1.9	ND	8.6	ND	ND	0.9	350.3
Cistus creticus	199.4	191.1	35.5	3.5	6.1	ND	ND	ND	435.6
Convolvulus arvensis	221.5	186.0	ND	2.1	10.3	3.5	ND	ND	423.4
Ecballium elaterium	212.0	183.8	2.3	1.3	7.4	ND	ND	ND	406.8
Erica manipuliflora	196.1	195.8	37.3	ND	6.3	ND	3.1	ND	438.6
Inula viscosa	187.6	159.6	82.4	ND	10.9	ND	ND	ND	429.6
Hedera helix	174.6	135.9	ND	ND	36.6	ND	ND	ND	347.1
Lamium amplexicaule	155.3	191.8	ND	9.2	13.8	ND	ND	ND	370.1
Marticaria chamomilla	216.2	158.9	ND	1.4	6.2	ND	1.8	ND	384.5
Oryza sativa	193.6	168.3	ND	ND	21.4	ND	ND	ND	383.3
Papaver rhoeas	236.4	226.5	29.0	1.8	5.8	ND	8.4	ND	507.9
Parthenocissus inserta	188.9	163.2	1.0	ND	4.6	ND	ND	ND	357.7
Phacelia tanacetifolia	206.9	214.8	ND	ND	5.9	ND	5.2	ND	432.8
Pinus halepensis	216.7	205.9	6.9	7.3	6.7	ND	ND	ND	443.5
Polygonum aviculare	242.4	196.9	5.0	2.3	4.3	ND	ND	ND	450.9
Portulaca oleracea	175.1	172.6	ND	0.8	5.2	ND	4.4	ND	358.1
Ranunculus arvensis	232.3	194.9	16.7	1.8	3.3	ND	ND	ND	449.0
Robinia pseudoacacia	232.8	217.0	13.7	1.6	10.8	ND	ND	ND	475.9
Rubus ulmifolius	212.2	209.8	ND	ND	5.6	ND	1.5	ND	429.1
Salvia verbenaca	196.6	164.5	ND	1.4	2.6	ND	ND	ND	365.1
Silybum marianum	215.0	175.5	ND	1.7	4.5	ND	ND	ND	396.7
Sisymbrium irio	197.4	175.9	ND	ND	3.6	2.8	7.3	ND	387.0
Sonchus asper	215.5	183.1	ND	3.5	16.7	ND	ND	ND	418.8
Tamarix parviflora	203.2	177.7	7.7	4.1	8.1	9.3	ND	ND	410.1
Taraxacum officinalis	208.9	186.8	ND	ND	20.5	ND	ND	ND	416.2
Tilia intermedia	206.0	177.4	57.3	3.1	7.7	ND	ND	ND	451.5
Tribulus terrestris	199.9	156.9	12.4	2.5	10.6	ND	ND	ND	382.3
Maximal value	334.8	276.9	82.4	34.7	36.6	9.3	8.4	–	635.3
Minimal value	155.3	135.9	1.0	0.8	2.6	2.8	1.5	–	347.1

Values representing mean are expressed as grams per kilogram of dry weight.
ND – not detected.

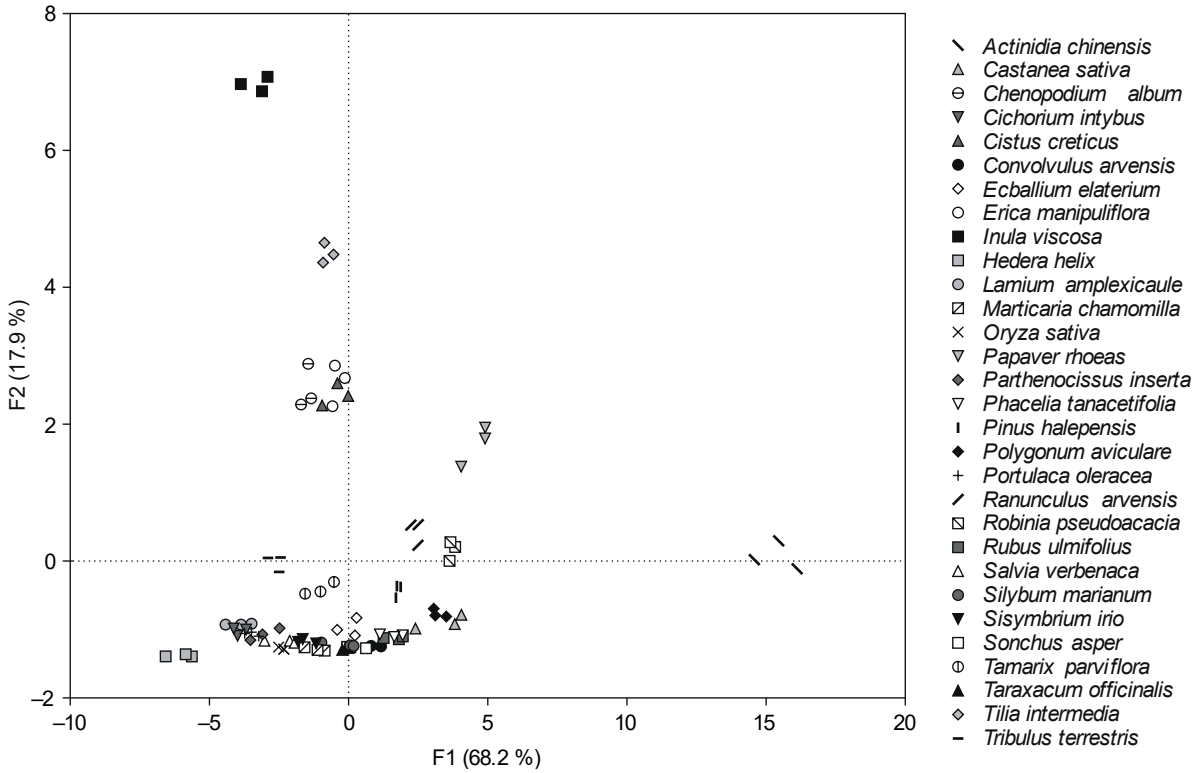

Fig. 1. Principal component analysis of sugar profile of 30 bee pollen taxa.

ences between the minimum and the maximum values for fructose were 32.9 g·kg^{-1}, for glucose 59.0 g·kg^{-1} and for saccharose 16.9 g·kg^{-1}. Saccharose, maltose, trehalose and melibiose were not always detected. Regarding the total sugars, the range among the different collecting periods was 101.6 g·kg^{-1}.

DISCUSSION

Bee pollen, besides being a highly nutritional product for the growth and survival of honeybees, it is also a well-balanced food supplement for human diet, greatly appreciated by the consumers. As carbohydrates constitute a main component of bee pollen, the knowledge of bee pollen's sugars profile is of great importance for both the beekeepers and the consumers. Although some countries have established or suggested their own national legislated quality standards for bee pollen, none of them have included the sugars composition. A global quality criterion was proposed for the first time regarding the bee pollen composition, suggesting that the lower limit of total sugars content of bee pollen be at 400 g·kg^{-1} [30]. Previous studies regarding multifloral samples already showed that the total sugars content of bee pollen can have a great range [10–12, 25]. Indeed, according to the results of the present study, over one-third of the analysed bee pollen species had total sugars content lower than 400 g·kg^{-1}, including some of the most commonly bee pollen sources, such as *S. irio, H. helix, Oryza sativa, Tribulus terrestris* and *C. intybus*, setting them out of the already proposed limits [30]. Taking into account that collection of unifloral bee pollen samples is not uncommon [17, 28, 31–33], the above species may constitute in some cases over 80 % of the multifloral bee pollen sample [28, 34] causing, in this way, possible problems in the trade of the product.

With respect to the sugar profile of the 30 bee pollen species, PCA analysis showed a deviation of some bee pollen taxa (Fig. 1). Indeed, it was found that *A. chinensis* pollen had much higher fructose and glucose contents, *H. helix* pollen had fructose and glucose content lower than the average, while *I. viscosa* pollen had the largest saccharose content (Tab. 3). As commercial bee pollen may come from different taxa as well as from monoculture, the knowledge of the sugars content of a wide spectrum of unifloral bee pollen samples is important during the establishment of national or international quality standards.

So far, studies regarding the sugars content of unifloral bee pollen are very limited and the

Tab. 4. Sugar composition and total sugar content of *Sisymbrium irio* bee pollen in different collecting periods.

Collecting period	Content [g·kg^{-1}]							
	Fructose	Glucose	Saccharose	Turanose	Maltose	Trehalose	Melibiose	Total sugars
9–10 April 2016	190.7^{a}	157.3^{a}	2.2^{ab}	3.0^{a}	3.3^{a}	ND	11.3^{abc}	367.9^{a}
22–23 April 2016	201.7^{ab}	176.5^{ab}	7.0^{b}	4.0^{a}	0.9^{a}	11.3^{b}	13.2^{bc}	414.7^{bc}
15–16 May 2016	190.5^{a}	161.8^{a}	3.2^{ab}	1.5^{a}	3.3^{a}	5.3^{ab}	10.9^{abc}	376.6^{ab}
4–5 June 2016	219.3^{c}	195.6^{bcd}	7.3^{b}	4.2^{a}	2.7^{a}	6.2^{ab}	18.7^{c}	454.2^{cd}
21–22 June 2016	223.4^{c}	210.4^{d}	2.5^{ab}	2.6^{a}	1.2^{a}	ND	11.7^{abc}	451.9^{cd}
22–23 August 2016	222.1^{c}	216.3^{d}	16.9^{c}	12.3^{b}	ND	ND	ND	467.6^{d}
7–8 September 2016	193.5^{a}	171.8^{ab}	ND	0.6^{a}	ND	ND	ND	366.0^{a}
23–24 September 2016	199.7^{ab}	182.6^{abc}	ND	1.5^{a}	0.7^{a}	ND	3.4^{ab}	383.1^{ab}
10–11 October 2016	212.3^{bc}	208.6^{cd}	2.5^{ab}	4.3^{a}	ND	ND	18.1^{c}	445.9^{cd}

Values representing mean are expressed as grams per kilogram of dry weight. Different letters in superscript indicate significant differences within the column (Tukey's test; $\alpha = 0.05$).
ND – not detected.

comparison among them is difficult and the results varied. For example, the results on *A. chinensis* bee pollen in the present work were close to those presented by Day et al. [15], who also recorded a high content of sugars regarding kiwi bee pollen. On the contrary, there was a significant difference concerning the total sugars content of *P. rhoeas* bee pollen between the present study and the study of Yang et al. [17]. Regarding the sugars profile found previously in studies analysing mixed bee pollen samples, the average contents of fructose and glucose detected in the present research were slightly higher than the average contents found by Serra-Bonvehi and Jorda [25] and Martins et al. [24]. The average saccharose content was similar [10] or lower [16, 25, 35] compared to the previously published data on multifloral bee pollen samples. Our results on turanose, maltose, trehalose and melezitose contents were in agreement with previous works [10, 25].

A main reason for the varied results among the studies can be attributed to the different methods of analysis. As mentioned above, previous works on sugars content of bee pollen used different methodologies, making the comparison among the studies difficult and the results ambiguous. So far, other authors used modified HPLC methods, which were originally applied to analysis of other products [10, 26] and they lacked validation. In this study, we developed and validated a liquid chromatographic method for the determination of sugars in bee pollen, presenting a very good accuracy and precision. Moreover, we minimized the quantity of required bee pollen (1 g) for the analysis compared to previous studies (e.g. Szczęsna [10] and Dominguez-Valhondo et al. [16] used 2 g and 5 g of bee pollen, respectively). Especially in case of the study of unifloral pollen samples, the determination of the minimum required quantity is crucial, considering that the pellets separation according to colour and shape is tedious and time-consuming.

The differences among the studies may also be attributed to the nectar added by the bees during the bee pollen pellet formation. Specifically, as the sugars content of the nectar used during the formation of bee pollen loads depends strongly on its botanical origin [19, 36, 37], the surrounding vegetation may also affect considerably the sugars content of pollen collected from bees despite its botanical origin. In our study, the variation of sugars content of *S. irio* bee pollen during the year confirmed the significance of the added nectar for the final results discussed in previous studies. [11, 24, 38, 39]. However, the results of this study showed that the botanical origin of bee pollen is also a significant factor regarding the sugars content. In particular, even though we recorded a wide range regarding the contents of sugars of *S. irio* bee pollen during the seasons, this range was more narrow compared to that found in different taxa. Moreover, bee pollen from plants that were co-flowering at the same period in the study area (e.g. *A. chinensis, P. rhoeas, Silybum marianum* and *S. irio* in May) had different sugars content, indicating that there was an additional strong influence of different botanical origin on the sugars profile of bee pollen as well.

CONCLUSIONS

Bee pollen is characterized by a high content of monosaccharides glucose and fructose, which

in turn depends on the botanical origin of the bee pollen. Additionally, the nectar and honey added by the bees during bee pollen pellet formation affects the sugars profile and increases the total sugars content of the collected bee pollen. Further research on the sugars content of unifloral and multifloral bee pollen from different geographical origins would be necessary to understand better the parameters that influence the sugars profile of bee pollen. Finally, adoption of a standard validated method for determination of the sugars content, such as the one proposed in the present study, could minimize the bias among the laboratories and provide comparative and reliable results. Such action should be taken seriously into consideration during the establishment of the quality standards of bee pollen.

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