JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

*J. Agric. Food Chem.* **2005,** *53,* 3095−3100 **3095**

# The Use of Carbohydrate Profiles and Chemometrics in the Characterization of Natural Honeys of Identical Geographical Origin

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A study of the real possibilities of carbohydrate profiles and chemometrics to characterize the botanical origin of honey from a single geographical area, the Province of Soria (Spain), is presented. To this end, 14 carbohydrates were quantified using high-performance liquid chromatography (HPLC) with pulsed amperometric detection (PAD) in 77 natural honeys, the botanical origins of which were ling, spike lavender, French lavender, thyme, forest, and multifloral. Principal component analysis has been employed as a first approach to characterize the honey samples analyzed, showing similarities between spike lavender and multifloral honeys. The best discrimination among groups is obtained when four canonical discriminant analyses were carried out sequentially, origin by origin, achieving an overall percentage of success of 90% following cross-validation.

**KEYWORDS: Carbohydrate profiles; chemometrics; blossom honey; characterization; pulsed amperometric detection; HPLC**

## INTRODUCTION

Nowadays, consumers are demanding products that must fulfill the criteria of quality that, day by day, are becoming more and more specific. The market for monofloral honeys, indeed, is subject to these same demands, and as such, both their botanical characterization and their geographical origin must be demanded, clearly addressed as a guarantee of quality to the consumer.

Traditionally, honey has been classified by pollen analyses (*1*, *2*), but these methods are time-consuming and require high levels of expertise. Quantitative analysis of the minor components of honey has gained acceptance more recently, given that differences in the minor components are more appropriate for differentiating among various kinds of honey than are variations in major components. In this way, determinations of amino acids (*3*−*7*), flavors (*8*−*11*), organic acids (*12*), mineral content (*13*−*15*), and quality parameters (*16*−*18*) have been used for determining the floral and geographical origin of honey with varying degrees of success.

Oligosaccharides are minor carbohydrates found in honey the distribution of which may be used as an index of honey authenticity and floral origin. Gas chromatography (GC) and high-performance liquid chromatography (HPLC) procedures have been traditionally recommended for analyzing carbohydrates in honey (*19*−*24*). HPLC methods are the most widely used given that derivatization is not normally necessary; among such methods, HPLC coupled to pulsed amperometric detection (PAD) is preferred for evaluating carbohydrates due to the low detection limits that can be achieved (*25*−*31*). On the other hand, some papers describe the use of fructose/glucose (F/G) or maltose/isomaltose (M/I) ratios (*32*) and, more recently, those of sucrose/turanose (S/T) and maltose/turanose (M/T), to evaluate the adulteration of honey with glucose or high-fructose syrups, but these ratios cannot be taken as universal, given that some data published in the literature and attributed to pure honeys are out of range when considering the values commonly accepted for those ratios (*33*).

In light of the above considerations, we have tried to verify whether the use of carbohydrate profiles together with chemometrics treatment allows for differentiating the botanical origin of honey. For this purpose, we have chosen the samples from a characteristic province in Spain, precisely due to the high quality of its honey production. The Soria Province is a sparsely populated region with a very characteristic climate, particularly cold, where only a limited surface area is devoted to crops and large wooded areas are covered primarily with oaks and pines. These features confer on the melliferous production of this stated region certain characteristics allowing for establishing a geographical denomination of honeys from this specific province.

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10.1021/jf0489724 CCC: $30.25 
Published on Web 03/26/2005

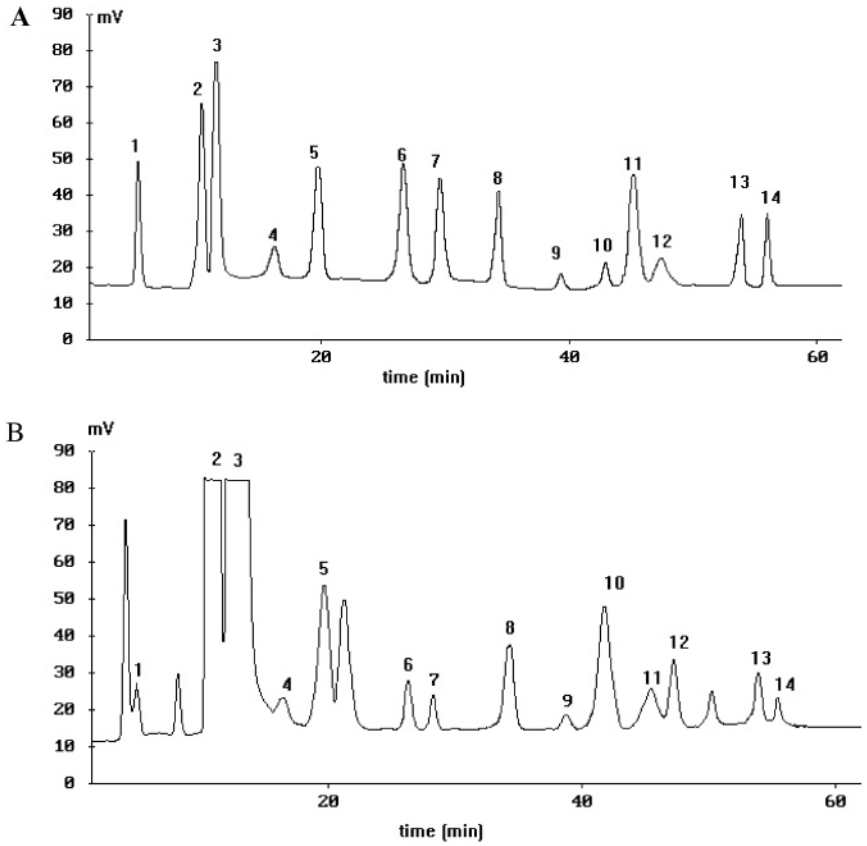


**Figure 1.** Chromatograms obtained from (A) a standards mixture and (B) a spike lavender honey sample. See text for chromatographic conditions. See Table 2 for peak identification.

Additionally, nowadays, the province is the main producer in Spain of ling honeys subject to export to many countries, though there are also numerous other typical productions of lesser economic importance. A total of 14 carbohydrates (including mono- and oligosaccharides) were quantified in 77 samples by using HPLC coupled to PAD. In addition, F/G, M/I, M/T, and S/T ratios were calculated to compare them with data published in the literature for natural honeys.

## MATERIALS AND METHODS

**1. Reagents.** Sugar standards were supplied by Aldrich Química S.A. (Madrid, Spain) and were used to prepare stock solutions by dissolution in water. A stock solution of 5 M NaOH (Panreac, Madrid) was used to prepare daily working solutions by appropriate dilution in water. Water used in the experimentation was obtained in a Milli-RO plus system together with a Milli-Q system from Millipore (Bedford, MA). Cellulose nitrate filters of 0.45 $\mu$m pore diameter were obtained from Waters (Milford, MA).

**2. Instrumentation.** The chromatographic setup used comprised the following elements: A model PU-1580 pump from Jasco (Tokyo, Japan) was used to propel an eluent consisting of water (solvent A) and 0.4 M NaOH (solvent B), according to the gradient shown in Table 1. The eluent was degassed with helium and delivered at a flow-rate of 1 mL/min. A ConstaMetric III pump from Milton Roy (Riviera Beach, FL) was used to propel the 0.3 M NaOH solution at a flow-rate of 0.6 mL/min, which was added to the eluate for boosting the detector signal. A Promis II autoinjector from Spark Holland (Emmen, The Netherlands) furnished with a fixed loop of 20 $\mu$L was used. A 4 mm $\times$ 250 mm Carbo-Pac PA1 column with pore size of 10 $\mu$m was used in conjunction with a 32.5 mm Carbo Pac Guard precolumn, both from Dionex Co. (Sunnyvale, CA). The eluate and the 0.3 M NaOH solution were passed, following merging, through a Teflon tube 50-cm long $\times$ 0.3 mm i.d. for purposes of reducing baseline oscillations. A Coulochem II 5040 pulsed amperometric detector from ESA, Inc. (Bedford, MA) equipped with a gold working electrode was set as follows: measurement potential, $E_1 = 200$ mV; measurement time, $t_1 = 500$ ms; delay time, $t_D = 300$ ms; $E_2 = 700$ mV; $t_2 = 120$ ms; $E_3 = -900$ mV; $t_3 = 160$ ms; range $= 5$ $\mu$A.

**Table 1.** Pump Conditions

| time (min) | % solvent A (water) | % solvent B (0.4 M NaOH) |
|---|---|---|
| 0 | 95 | 5 |
| 15 | 95 | 5 |
| 35 | 80 | 20 |
| 45 | 60 | 40 |
| 48 | 0 | 100 |
| 70 | 0 | 100 |
| 71 | 95 | 5 |
| 80 | 95 | 5 |

**3. Sampling and Treatment.** Representative samples were kindly furnished by different beekeepers from the same geographical area (Province of Soria, Spain) during the years 2001−2003. Experts in melissopalynological and sensorial analysis established the predominant floral origin of samples: ling (*Calluna vulgaris* (L.) Hull; 15 samples), spike lavender (*Lavandula latifolia* Med.; 17 samples), French lavender (*Lavandula stoechas* L.; 8 samples), thyme (*Thymus* sp.; 10 samples), multifloral (12 samples), and forest (*Quercus* sp.; 15 samples). Climatology (particularly severe in 2002) and the reduced size of the county where honeys were collected were the main factors for not arranging a larger number of samples to carry out this study.

Samples were prepared by dilution using water (1:50 for oligosaccharide analysis and 1:5000 for glucose and fructose analysis) and passed through a 0.45 $\mu$m filter. Twenty microliters of the final solution was injected into the chromatograph. All samples were analyzed at least three times.

**4. Calibration Graphs.** Stock solutions of the carbohydrates (10 g/L) were prepared in water. Standard solutions were prepared from stock solutions by sequential dilution with water. Concentrations ranged

**Table 2.** Retention Times and Detection Limits for the Carbohydrates Analyzed

| | compound | retention time (min) | LOD[a] (mg/L) |
|---|---|---|---|
| 1 | trehalose | 3.7 | 5 |
| 2 | glucose | 9.1 | 5 |
| 3 | fructose | 11.1 | 5 |
| 4 | sucrose | 14.6 | 5 |
| 5 | isomaltose | 17.1 | 10 |
| 6 | melezitose | 22.3 | 20 |
| 7 | gentibiose | 27.3 | 20 |
| 8 | turanose | 35.7 | 20 |
| 9 | isomaltotriose | 39.5 | 5 |
| 10 | nigerose | 43.4 | 5 |
| 11 | maltose | 45.8 | 15 |
| 12 | erlose | 48.0 | 20 |
| 13 | panose | 54.4 | 10 |
| 14 | maltotriose | 56.7 | 10 |

[a] Experimental detection limit.

from 60 to 600 ppm for maltose and fructose, from 20 to 200 ppm for glucose, sucrose, and trehalose, from 160 to 1600 ppm for turanose, isomaltotriose, nigerose, and erlose, and from 80 to 800 ppm for the other carbohydrates.

**5. Statistical Analysis.** Data were subjected to univariate and multivariate analysis of variance (ANOVA) and discriminant analysis using the program SPSS 11.0 from SPSS Inc. (Chicago, IL). Principal component analysis was carried out using the program MATLAB 6.0 from The MathWorks Inc. (Natick, MA).

## RESULTS AND DISCUSSION

The results obtained have been statistically analyzed. As a first approach, ANOVA has been employed to look for significant statistical differences among botanical origins. The high number of variables studied in this work necessarily required the use of multivariate statistical techniques such as principal component analysis and discriminant analyses.

A discriminant analysis achieved in a single step does not offer good results in the simultaneous discrimination of all kinds of honeys, possibly attributed to monofloral honeys studied in this work having been collected from a small area in July (thyme), August (French lavender), September (spike lavender and forest), and November (ling), which sometimes confers similar characteristics for honeys of different botanical origins, hence making more difficult their characterization. For this reason in the present work, a sequence of four discriminant analyses has been tested, isolating in each step of the sequence a specific type of monofloral honey, choosing those carbohydrates more useful for discriminating every botanical origin being characterized at each stage.

Carbohydrates were separated using the pump program shown in Table 1 optimized for the separation of 14 carbohydrates in less than 60 min, using our experience in their analysis in other matrixes (*28*). The chromatograms obtained for a mixture of standards and a spike lavender honey sample are shown in Figure 1, parts A and B, respectively. Retention times and detection limits are presented in Table 2. Carbohydrate identification was confirmed by adding standards to the sample. Cleaning and conditioning steps (never less than 30 min) before each sample injection are strongly recommended to obtain good reproducibility.

Mean values and 95% confidence intervals for each carbohydrate against the botanical origin are shown in Table 3. From the results of the descriptive statistical analysis, it may be observed that the most abundant disaccharides were turanose, nigerose, maltose, and isomaltose. Among the trisaccharides, erlose was the most abundant.

**Table 3.** Mean and 95% Confidence Interval, in g/kg, Obtained for the Carbohydrates in the Honey Samples Analyzed

| | mean ± CI[a] | | | | | |
|---|---|---|---|---|---|---|
| | ling ($n = 15$) | spike lavender ($n = 17$) | french lavender ($n = 8$) | thyme ($n = 10$) | multifloral ($n = 12$) | forest ($n = 15$) |
| fructose | 366 ± 46 | 374 ± 32 | 370 ± 85 | 316 ± 96 | 363 ± 48 | 381 ± 50 |
| glucose | 314 ± 58 | 361 ± 39 | 356 ± 69 | 290 ± 88 | 355 ± 47 | 336 ± 49 |
| turanose | 18.0 ± 6.8 | 17.4 ± 4.0 | 18.3 ± 6.5 | 21.5 ± 7.9 | 22.0 ± 4.4 | 20.0 ± 5.4 |
| erlose | 19.6 ± 4.3 | 6.7 ± 2.7 | 11.2 ± 3.6 | 8.4 ± 5.0 | 7.8 ± 3.4 | 10.8 ± 4.7 |
| nigerose | 14.1 ± 5.1 | 12.7 ± 3.9 | 17.8 ± 6.9 | 19.2 ± 5.3 | 12.2 ± 3.7 | 16.8 ± 4.1 |
| isomaltose | 14.9 ± 5.2 | 11.4 ± 3.5 | 14.4 ± 4.5 | 18.1 ± 5.8 | 13.6 ± 3.2 | 16.4 ± 3.9 |
| maltose | 8.0 ± 3.1 | 9.0 ± 3.1 | 11.2 ± 3.6 | 6.3 ± 4.4 | 10.4 ± 4.1 | 13.3 ± 5.2 |
| panose | 3.7 ± 1.7 | 2.7 ± 1.2 | 4.1 ± 1.8 | 3.3 ± 1.1 | 3.5 ± 1.1 | 4.2 ± 1.2 |
| trehalose | 3.8 ± 2.0 | 1.4 ± 0.6 | 1.2 ± 0.5 | 2.1 ± 1.9 | 2.5 ± 1.6 | 7.3 ± 2.9 |
| melezitose | 2.0 ± 1.1 | 1.8 ± 0.9 | 2.5 ± 1.3 | 2.5 ± 1.6 | 1.8 ± 1.2 | 24.7 ± 6.0 |
| maltotriose | 2.1 ± 1.1 | 2.0 ± 1.1 | 6.6 ± 3.2 | 1.5 ± 1.0 | 2.5 ± 1.4 | 3.2 ± 1.3 |
| gentibiose | 2.2 ± 1.6 | 0.8 ± 0.3 | 1.4 ± 1.0 | 0.9 ± 1.1 | 1.2 ± 0.8 | 2.0 ± 0.9 |
| sucrose | 2.1 ± 0.7 | 4.7 ± 1.2 | 2.4 ± 0.9 | 2.3 ± 1.2 | 2.4 ± 1.8 | 3.5 ± 1.6 |
| isomaltotriose | 2.8 ± 1.3 | 2.4 ± 1.4 | 3.6 ± 1.3 | 2.8 ± 1.4 | 3.7 ± 1.2 | 2.1 ± 1.3 |

[a] Confidence interval for mean honey samples analysed.

**Table 4.** Mean and 95% Confidence Interval Obtained for Some Carbohydrate Ratios

| | mean ± CI[a] | | | | | |
|---|---|---|---|---|---|---|
| | ling ($n = 15$) | spike lavender ($n = 17$) | french lavender ($n = 8$) | thyme ($n = 10$) | multi-floral ($n = 12$) | forest ($n = 15$) |
| fructose/ glucose | 1.20 ± 0.13 | 1.05 ± 0.08 | 1.06 ± 0.21 | 1.14 ± 0.17 | 1.03 ± 0.08 | 1.15 ± 0.12 |
| maltose/ isomaltose | 0.57 ± 0.22 | 0.95 ± 0.35 | 0.92 ± 0.89 | 0.31 ± 0.15 | 0.84 ± 0.34 | 1.06 ± 0.82 |
| maltose/ turanose | 0.48 ± 0.15 | 0.53 ± 0.17 | 0.89 ± 1.17 | 0.31 ± 0.22 | 0.52 ± 0.26 | 0.66 ± 0.33 |
| sucrose/ turanose | 0.14 ± 0.05 | 0.34 ± 0.30 | 0.13 ± 0.07 | 0.15 ± 0.10 | 0.12 ± 0.08 | 0.21 ± 0.12 |

**Table 5.** ANOVA Results

| | *p* value[a] |
|---|---|
| fructose | 0.498 |
| glucose | 0.404 |
| turanose | 0.886 |
| erlose | 0.000 |
| nigerose | 0.226 |
| isomaltose | 0.177 |
| maltose | 0.231 |
| panose | 0.416 |
| trehalose | 0.000 |
| melezitose | 0.000 |
| maltotriose | 0.049 |
| gentibiose | 0.070 |
| sucrose | 0.545 |
| isomaltotriose | 0.944 |

[a] Variables were different between kinds of honey with *p* value less than 0.05.

Occasionally, some carbohydrate ratios are used to ascertain honey authenticity. These ratios have been calculated for the 77 samples analyzed. Estimations of means and 95% confidence intervals obtained are presented in Table 4. By comparison of the results obtained with those proposed for natural honeys (*16*, *22*, *32*), it may be observed that for F/G ratios intervals are quite similar, though it must be noticed that for some honeys data obtained is beyond the intervals proposed. This occurs for some spike lavender samples, as well as for certain multifloral and ling samples. Moreover, it can be observed that the ratios M/I and M/T found for many honey samples of the various origins studied are lower than those proposed. Consequently, these intervals should be used only as orientation because it could happen that a natural honey would falsely be rejected. In

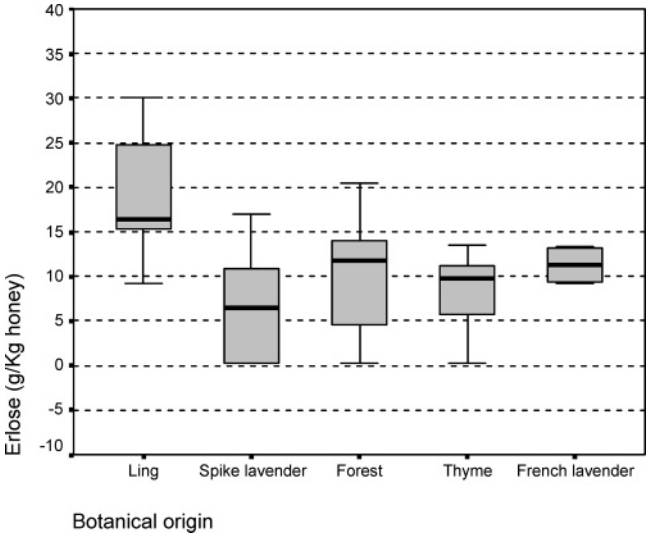


**Figure 2.** Box plot with median, minimum, and maximum values and interquartile range for erlose content (g/kg) in the honeys analyzed.

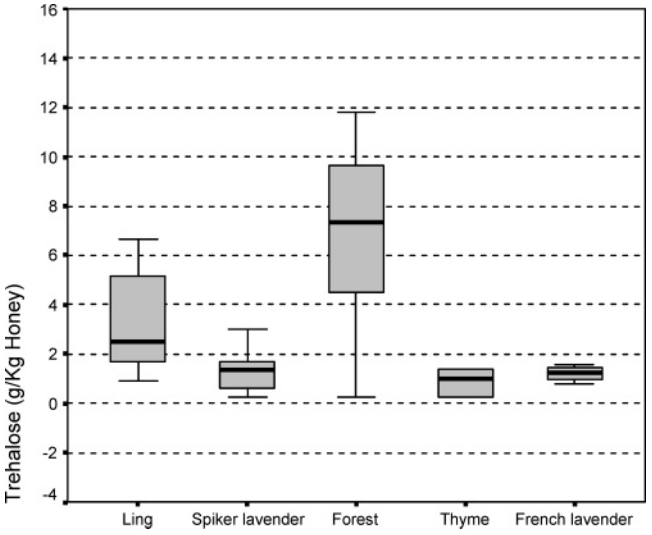


**Figure 3.** Box plot with median, minimum, and maximum values and interquartile range for trehalose content (g/kg) in the honeys analyzed.

the case of the S/T ratio, the intervals found are usually in agreement with those proposed in the bibliography. In general, it can be affirmed that the enormous diversity of natural honeys should be considered before making use of carbohydrate ratio intervals for testing natural honeys.

The data obtained from the monofloral honey samples was analyzed in a one-way ANOVA, and the results obtained are displayed in Table 5. Before ANOVA, Shapiro−Wilk, and Levene's tests were performed for verifying the normality and homoschedasticity, respectively, of data distributions. As shown in Table 5, some di- and trisaccharides, particularly trehalose, erlose, melezitose, and maltotriose, showed significant differences in mean content among monofloral origins. This fact led us to observe that some of these carbohydrates could play a relevant role in the characterization of the botanical origins considered.

Figures 2 and 3 show the box-plot obtained for erlose and trehalose. Differences are observed for erlose between ling and other origins. Regarding trehalose content, the highest concentrations have been found for forest samples. The visualization of the data distributions for these two variables allowed us to confirm the results obtained by means of ANOVA.

**Table 6.** Eigenvalues, Variance Explained, and Cumulative Variance Explained for Principal Components[a]

| | initial eigenvalues | | | extractions sums of squared loadings | | |
|---|---|---|---|---|---|---|
| component | total | % of variance | cumulative % | total | % of variance | cumulative % |
| 1 | 3.786 | 27.042 | 27.042 | 2.778 | 19.845 | 19.845 |
| 2 | 2.490 | 17.787 | 44.829 | 2.252 | 16.086 | 35.931 |
| 3 | 1.621 | 11.578 | 56.407 | 2.099 | 14.991 | 50.922 |
| 4 | 1.320 | 9.432 | 65.838 | 1.617 | 11.550 | 62.473 |
| 5 | 1.145 | 8.180 | 74.019 | 1.616 | 11.546 | 74.019 |
| 6 | 0.866 | 6.184 | 80.203 | | | |
| 7 | 0.700 | 4.996 | 85.199 | | | |
| 8 | 0.605 | 4.324 | 89.523 | | | |
| 9 | 0.522 | 3.730 | 93.254 | | | |
| 10 | 0.307 | 2.193 | 95.446 | | | |
| 11 | 0.219 | 1.563 | 97.010 | | | |
| 12 | 0.185 | 1.323 | 98.332 | | | |
| 13 | 0.157 | 1.121 | 99.454 | | | |
| 14 | $7.647 \times 10^{-2}$ | 0.546 | 100.000 | | | |

[a] Extraction method: principal components.

**Table 7.** Loadings for Each Variable on the Components after Rotation[a]

| | component | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| fructose | 0.058 | 0.911 | –0.028 | 0.116 | –0.009 |
| glucose | –0.066 | 0.893 | –0.059 | 0.092 | 0.298 |
| turanose | 0.772 | 0.299 | 0.272 | –0.197 | 0.213 |
| erlose | 0.054 | –0.140 | 0.731 | 0.003 | –0.201 |
| nigerose | 0.843 | –0.258 | –0.189 | 0.047 | –0.037 |
| isomaltose | 0.737 | 0.163 | 0.221 | 0.385 | –0.094 |
| maltose | 0.314 | 0.271 | 0.093 | –0.058 | 0.584 |
| panose | 0.287 | 0.314 | 0.127 | 0.672 | –0.143 |
| trehalose | 0.094 | –0.336 | 0.528 | 0.434 | –0.094 |
| melezitose | 0.795 | –0.050 | 0.350 | 0.273 | 0.089 |
| maltotriose | 0.031 | 0.027 | –0.094 | 0.787 | 0.189 |
| gentibiose | 0.187 | 0.136 | 0.826 | –0.016 | 0.193 |
| sucrose | –0.164 | –0.021 | –0.167 | 0.184 | 0.832 |
| isomaltotriose | 0.178 | 0.339 | 0.505 | –0.189 | 0.536 |

[a] Extraction method: principal components. Rotation method: normalization Varimax with Kaiser.

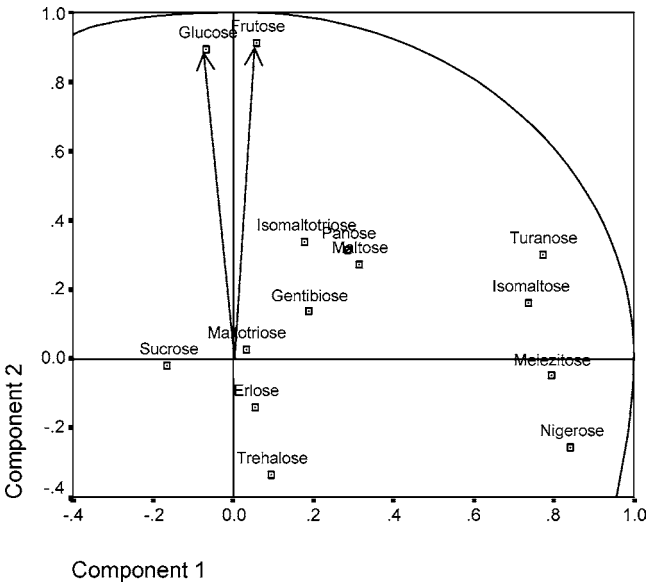


**Figure 4.** Representation of carbohydrates as a function of the two first principal components.

Standardized principal component analysis can explain the 35% of total variance using the two first components, reaching 74% through five components. Table 6 gives eigenvalues,

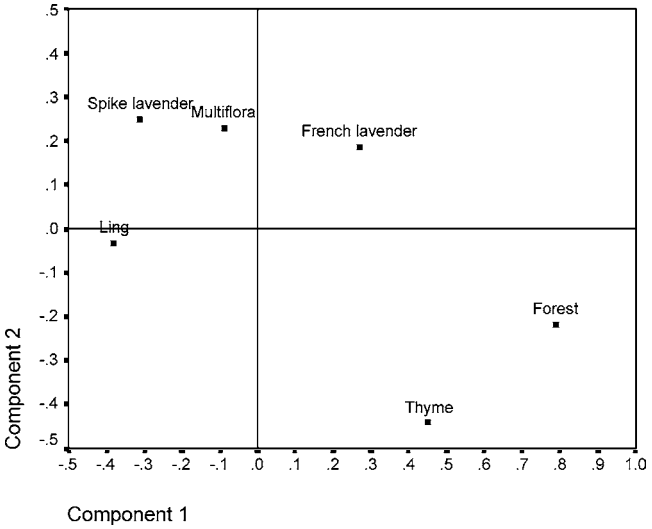


**Figure 5.** Representation of botanical origins as function of the two first principal components.

variance explained, and cumulative variance explained for the component solution. Table 7 shows the rotated component matrix. It can be observed that the first principal component is basically a function of nigerose, melezitose, turanose, and isomaltose. The second principal component is a function of fructose and glucose. As may be observed in Figure 4, these carbohydrates with the highest loadings in both components are best defined by the extracted principal components, because they are close to the edge of a unit radius circle.

Correlations between variables would be determined by means of cosines of angles obtained by joining the coordinates of variables to the origin of coordinates. Thus, when those variables better explained by the two first principal components are involved, a high correlation between glucose and fructose contents is observed (see Figure 4).

Finally, because the most important relative weights in the first component are positive, this may be interpreted as a general index of the statistical size of each honey. Honeys with large values of the first component tend to indicate high values of nigerose, melezitose, turanose, and isomaltose.

In Figure 5, botanical origins are represented according to the two first components, obtaining the botanical origin coordinates, known as centroid, using the scores obtained for honeys against their botanical origin. It may be seen that forest samples obtain the highest scores for the first component, while ling and spike lavender honeys obtain the lowest. As such, the group of carbohydrates formed by melezitose, turanose, isomaltose, and nigerose may play an important role in distinguishing among samples of different botanical origins. Spike lavender and French lavender honeys obtain higher scores in the second principal component than the remainder of botanical origins, above all relative to thyme and forest samples. This fact could allow us to identify these kinds of honeys, relative to glucose and fructose content. Centroid corresponding to multifloral honeys is close to centroid of spike lavender honeys. This could be explained by the fact that honeys generically classified as multifloral are usually collected from beehives where a singular extraction is made, once a year, following the lavender flowering period.

Canonical discriminant analysis was used to find functions based on the content in di- and trisaccharides, which characterize the differences among monofloral honeys. In a first approach, a canonical discriminant analysis was made in a single step, but the results obtained were not successful, because low percentages of honeys correctly classified following cross-validation were obtained for some types.

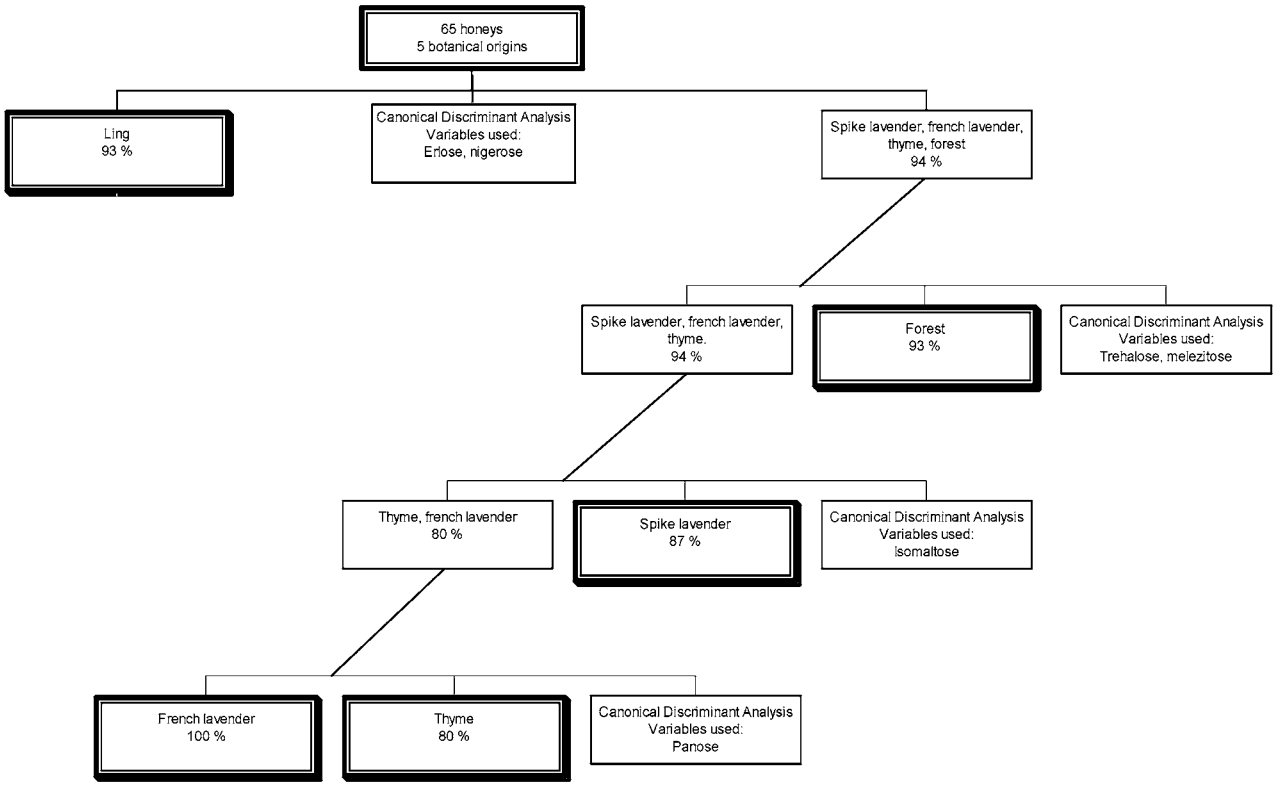


**Figure 6.** Discrimination scheme for differentiation of five types of honey developed in four steps. Variables used and percentages of honey samples correctly classified after cross-validation are shown in each step.

For purposes of improving the percentages of classification, a canonical discriminant analysis in four steps has been carried out, selecting at each stage those variables that discriminate more effectively depending on the origin considered and employing a stepwise method based on the lowest value of $\lambda$ of Wilks. As may be seen in Figure 6, by means of the discriminating scheme proposed, higher percentages for samples correctly classified have been obtained, achieving successful percentages following crossvalidation for French lavender and ling, 100% and 93%, respectively. On the other hand, the percentages of spike lavender and forest honey samples correctly classified are between 87% and 93%. A low percentage of success is obtained for thyme honeys of 80%; consequently, for discriminating this kind of honey, the use of another class of variables is necessarily required, which together with carbohydrate content would allow us to characterize thyme honeys with higher percentages of success. Otherwise, it may be concluded that by means of the use of only the content of six carbohydrates [i.e., erlose, nigerose, trehalose, melezitose, isomaltose, and panose (see Figure 6)], a discrimination of ling, spike lavender, French lavender, and forest honeys can be achieved with a global percentage of success of more than 90% following cross-validation.

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**Received for review June 24, 2004. Revised manuscript received February 24, 2005. Accepted February 25, 2005. Financial support of this project (Grant CAL01-C66-C7-1) by MAPA-INIA (Spain) is gratefully acknowledged.**

JF0489724