

## Metabolomics applied to proton nuclear magnetic resonance profile for the identification of seven floral origin of French honeys

FRANCOIS GUYON – ELODIE CHAVEZ DA COSTA –  
ARNAUD MAURIN – LAETITIA GAILLARD – MURIEL LANDURÉ – LOUIS GOUGEON

### Summary

A sample preparation protocol was developed for honey profiling by proton nuclear magnetic resonance ( $^1\text{H}$  NMR). The honey samples (0.5 g each) were solubilized in 5 ml of oxalate buffer (0.25 mol·l<sup>-1</sup>, pH 4.3). This solution (1 ml) supplemented with a lock solution (D<sub>2</sub>O, 0.1 ml) containing 3-(trimethylsilyl) propionic acid D4 sodium salt was used for analysis. The novelty of the proposed method consists in no need for subsequent pH adjustment. The developed method was used for the analysis of 212 French honeys split in seven floral origins (acacia, chestnut tree, scrub, colza, lavender, sunflower and honeydew). A soft independent modelling of class analogy (SIMCA) data treatment was applied to the  $^1\text{H}$  NMR spectra after a range scale pre-processing. The obtained class distance indicated successful discrimination between the seven floral origins, confirmed by a 100% correct classification when a honey test set was used. The model was applied to commercial mono-floral honeys and the results were fully corroborated by sensory and pollen analyses.

### Keywords

oxalate buffer; honey floral origin; chemometric analysis; proton nuclear magnetic resonance

Honey is produced by the *Apis mellifera* bees, from the nectar and secretions of living parts of plant and/or from plant sucking insect excretions followed by bees treatments into the hive. Since honey is a natural product, its chemical profile depends on the floral [1] and the geographical origins of the plant [2] that were involved in the honey production process. Analytical criteria of honey were first described in the Codex Alimentarius [3] and applied by commercial entities, producers and sellers, only on a voluntary basis. Later on, these analytical criteria were incorporated into a European directive [4], ultimately becoming a regulation. The European regulation states that the honeypot labelling should specify the geographical origin. The information on the floral origin can also be added to the label. This information is important to many consumers who not look only for specific taste but often also for

specific floral origin and health properties [5]. The rareness of honey of some floral origins can result in significant price increase, supporting adulteration of honey, i.e. mislabeling, honey mixing, addition or substitution with cheaper ingredients. To address the latter issue, some analytical methods have been developed for assuring the botanical origin of honeys.

The methods for honey floral origin determination are based on various approaches, in particular melissopalynology analysis (pollen characterization) and organoleptic analysis [6, 7]. The common physico-chemical analytical methods involve gas chromatographic methods to characterize honeys using volatile compounds profile [1] and liquid chromatography techniques to characterize various honey fractions, e.g. flavonoids [8], amino acids or oligosaccharides [9]. The analysis of stable isotope ratios analysis (C-13) in honeys,

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**Francois Guyon, Elodie Chavez Da Costa, Arnaud Maurin, Laetitia Gaillard**, Service Commun des Laboratoires, 3 Avenue du Dr. A. Schweitzer, 33680 Pessac, France.

**Muriel Landuré**, Service Commun des Laboratoires, 146 Traverse Charles Susini, 13388 Marseille, France.

**Louis Gougeon**, CEnologie Research Unit, Institut des Sciences de la Vigne et du Vin, Université de Bordeaux, 210 Chemin de Leysotte, F-33882 Villenave d'Ornon, France.

*Correspondence author:*

Francois Guyon, e-mail: guyonfra@gmail.com

of proteins and ethanol resulting from the honey fermentation also showed some potential in the floral origin characterization [10–12]. The discriminating power of these analyses can be supported by measurement of mineral element concentrations [13, 14] and by chemometric methods. In the past decade, nuclear magnetic resonance (NMR) has become a major tool for food authentication. It has been applied to honey for the purposes of floral origin recognition [15–19], characterization of specific honeys (Manuka honey) [20, 21] and possible sugar adulteration [22, 23].

This work reports on proton nuclear magnetic resonance ( $^1\text{H}$  NMR) fingerprinting of 212 French mono-floral honey samples and on the development of a working protocol, which can be easily implemented by control laboratories. The approaches reported in the literature to date have not fulfilled the criteria required for routine analysis in food control laboratories, which require precise pH control in each sample preparation. For this reason, an improved sample preparation protocol was developed here and applied to a set of 212 authentic French honeys analysed by  $^1\text{H}$  NMR. The obtained spectra were subsequently used for multidimensional analysis using an untargeted approach. A soft independent modelling of class analogy (SIMCA) data treatment was used to build a model able to simultaneously discriminate between seven mono-floral honeys. The model was validated using an independent test set of honeys and further applied to commercial mono-floral honeys.

## MATERIALS AND METHODS

### Chemicals

Deuterium oxide ( $\text{D}_2\text{O}$ , 99.90% D, CAS 7789-20-0) and 3-(trimethylsilyl) propionic acid D4 sodium salt (TSP, 98% D, CAS 24493-21-8) were obtained from Euriso-Top (Saint Aubin, France). Hydrochloric acid ( $1\text{ mol}\cdot\text{l}^{-1}$ , CAS 7647-01-0) and sodium oxalate (for analysis grade, CAS 62-76-0) came from Carlo Erba (Milan, Italy). Potassium disulphite powder (Normapur quality, CAS 16731-55-8) was obtained from VWR International (Radnor, Pennsylvania, USA).

### Samples

All 212 honey samples used in this study were provided by co-operatives of producers from France, namely ADA Bretagne (Rennes), ADA Poitou Charentes (Limoges), Cooperative France Miel (Paris), Provence Miel et syndicat des miels de Provence et des Alpes du Sud (Aix-en-pro-

vence), Syndicat AOP miel de Corse (Altiani), who guaranteed the floral and the geographical origin of their honeys. Nonetheless, upon receiving of the honeys, their botanical origin was systematically verified by sensory and pollen analysis. The honeys are identified, in this study, by the plant/tree name describing the nectar origin with the number of samples given in parenthesis: acacia (30), chestnut tree (42), scrub (34), honeydew (28), colza (15), lavender (49) and sunflower (14). The honeys were kept at  $4\text{ }^\circ\text{C}$  before analysis for a maximum of one year.

Honey produced by Cooperative France Miel (Paris, France) was used as a quality-control sample. Twenty samples of that honey were prepared and analysed under the experimental conditions that are described in the next section. In each series of analysed honeys, one sample of honey was prepared to control the alignment (chemical shift) and intensity of the  $^1\text{H}$  NMR peaks.

### Sample preparation

Lock solution was prepared by dissolving TSP (5 mg) in  $\text{D}_2\text{O}$  (5 ml). TSP was used for zeroing the chemical shift and detecting any chemical shift variations. A buffer solution was made up as followed, in two steps. In the first step, 3.35 g sodium oxalate ( $\text{pK}_{\text{a}2} = 4.3$  at  $25\text{ }^\circ\text{C}$ ) was dissolved in 180 ml of  $18\text{ M}\Omega$  water (Elga LabWater, Vivendi, Paris, France) in a beaker under stirring. After dissolution, pH of the solution was adjusted to 4.3 with  $1.0\text{ mol}\cdot\text{l}^{-1}$  HCl and another 3.35 g of sodium oxalate was added. The pH value was checked again and adjusted to 4.3, if needed. Then, potassium disulfite (0.02 g) was added and the pH value was verified once more and adjusted if required. Finally, the solution was transferred to a graduated flask and filled up to 200 ml.

The preparation of crystallized honey samples involved preliminary sample heating at  $40\text{ }^\circ\text{C}$  for 2 h followed by homogenization. Then, 0.5 g of honey (average humidity of  $16.6 \pm 0.7\%$ ) was precisely weighed on a precision balance ( $\pm 0.1\text{ mg}$  precision) and dissolved in 5 ml of the buffer solution. After complete dissolution of honey, 1 ml of the solution was poured into a 5 mm NMR tube and 0.1 ml of the lock solution was added. For each series, the commercial honey sample was also prepared using the same protocol for controlling the quality of performed measurements.

### Sample analysis

$^1\text{H}$  NMR measurements were performed using a Bruker Avance III 400 Ultrashield spectrometer (Bruker BioSpin, Billerica, Massachusetts, USA). A broad band inverse (BBI) probe with Z-gradient

coils allowed proton signal recording with a 5 mm width tube without rotation. Temperature was regulated at 300 °K. The sample series were automatically analysed using ICON-NMR (Bruker BioSpin) to control the acquisition parameters and a SampleXpress autosampler (60 positions, Bruker BioSpin). Tuning, matching and shimming were automatically performed on each tube by the NMR routine (atma & topshim). A so-called Honey Profiling Module (Burker Biospin), containing all optimized acquisition and processing parameters, was used to record  $^1\text{H}$  NMR signal: 300 s of temperature equilibrium in the coil, 32 free induction decays (FIDs) of 64 k points with 4 dummy scans were acquired on a scale range of 20 ppm (–5 ppm to +15 ppm) with a receiver gain (RG) of 16, an acquisition time of 3.984 s and relaxation time of 4 s. A NOESY-presaturation pulse sequence (1D noesygppr1d, frequency: 1883.27 Hz, Bruker BioSpin) was used for water signal suppression. All  $^1\text{H}$  NMR FIDs were processed using line broadening of 0.3 Hz and chemical shift calibration was checked using TSP signal (0.0 ppm).

### Data processing

Once the spectra were recorded, the  $^1\text{H}$  NMR files were firstly converted from the Bruker format to a specific format so called “Galactic”, readable by the Pirouette software (InfoMetrix, Bothell, Washington, USA) using MestReNova software (Mestrelab Research, Santiago de Compostela, Spain). The model for honey botanical origin was built using SIMCA with a pre-processing “range scale” that constrains all the variables to fall inclusively between 0 and 1. This pre-processing was performed on  $^1\text{H}$  NMR spectra after excluding three areas: two without any signal (from +10 ppm to +15 ppm and from –5 ppm to +0.1 ppm) and a third one corresponding to water-suppressed signal area (4.74–4.84 ppm). All the remaining spectral areas were used to build the model. The class selection corresponded to the mono-floral origin of honey. The maximum factor was fixed at 10, the probability threshold at 95 % and the scope at “local” as recommended by the user guide for SIMCA applications. Data processing was performed with the software Pirouette version 4.5 (InfoMetrix) using two models. The first model included all the samples and its perform-

**Tab. 1.** Leave one-out cross validation of SIMCA model and interclass distance for seven floral origins of honey.

	P_Aca	P_Che	P_Scr	P_HDe	P_Col	P_Lav	P_Sun	No match	Precision [%]
Acacia	30	(8.6)	(7.9)	(3.1)	(1.9)	(2.1)	(4.1)	0	100
Chestnut Tree	0	42	(9.3)	(7.1)	(14.2)	(10.4)	(12.2)	0	100
Scrub	0	0	34	(5.0)	(10.4)	(10.8)	(11.9)	0	100
Honeydew	0	0	0	28	(8.8)	(5.4)	(6.9)	0	100
Colza	0	0	0	0	15	(7.3)	(10.2)	0	100
Lavender	0	0	0	0	0	49	(6.7)	0	100
Sunflower	0	0	0	0	0	0	14	0	100

P\_X – class predicted according to botanical origin (Aca – acacia, Che – chestnut tree, Scr – scrub, HDe – honeydew, Col – colza, Lav – lavender, Sun – sunflower).

Values in parenthesis represent interclass distance between each pair of classes.

**Tab. 2.** SIMCA model prediction on an excluded test-set of honeys.

	P_Aca	P_Che	P_Scr	P_HDe	P_Col	P_Lav	P_Sun	No match	Precision [%]
Acacia	5	0	0	0	0	0	0	0	100
Chestnut Tree	0	10	0	0	0	0	0	0	100
Scrub	0	0	6	0	0	0	0	0	100
Honeydew	0	0	0	5	0	0	0	0	100
Colza	0	0	0	0	0	0	0	0	100
Lavender	0	0	0	0	0	12	0	0	100
Sunflower	0	0	0	0	0	0	0	0	100
Unmodelled	0	0	0	0	0	0	0	0	

P\_X – result of the class prediction of the specified botanical origin (Aca – acacia, Che – chestnut tree, Scr – scrub, HDe – honeydew, Col – colza, Lav – lavender, Sun – sunflower).

ance was evaluated by internal leave-one-out cross validation [24] and by class distance computation (Tab. 1). The second “intermediate” model was obtained after splitting the data into two sets: one for calibration and one, formed by excluded honey samples, for validation (Tab. 2).

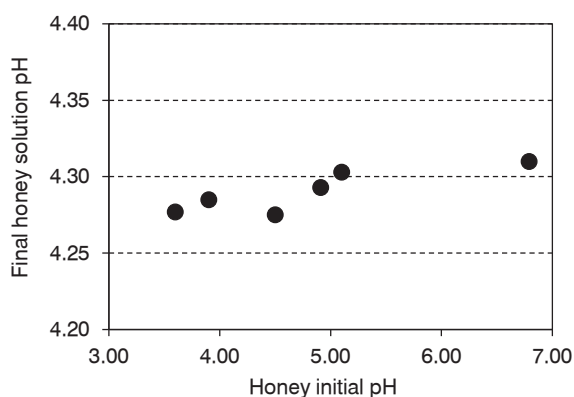
## RESULTS AND DISCUSSION

### <sup>1</sup>H-NMR sample preparation methodology

Building a statistical model using <sup>1</sup>H NMR signals requires an analysis that follows a very strict protocol capable of assuring similar experimental conditions in the measurements of all samples. This is a challenge, as the proposed approach is based on proton chemical shifts and signal intensities that are very sensitive to differences in the experimental conditions during the sample preparation steps. In order to minimize the effect of experimental conditions, several preliminary requirements need to be met. First, the <sup>1</sup>H NMR probe temperature should be rigorously controlled as the proton chemical shift is temperature-dependent [25]. This can be achieved by using of a temperature controller that allows for fixing the probe temperature and allows enough time (300 s) for equilibration of the tube temperature in the coil before each measurement. The signal intensity depends on the number of protons and on the amount of honey used in the preparation. As chemical composition of all honeys is similar, the observed <sup>1</sup>H NMR profiles are also similar (with a few exceptions [20]) with different peak intensities depending on concentration. Since the amount of honey used in the measurements is critical, the amount of 0.50 g of honey was weighed precisely. Then, the buffer and <sup>1</sup>H NMR lock solutions were added using pipettes (5 ml, 1 ml and 0.1 ml) that

were under the quality control system. Besides the sample quantity, the pH value also strongly affects chemical shifts [26], as does, to some extent, the mineral elements content [27]. While the concentration of metals is hardly adjustable, the pH value can be fixed by using a buffer. For the honeys studied here, the pH values were found to be in a range between 3.5 and 6.9 (Fig. 1), which results in a chemical shift of 0.1 ppm for similar molecules and, consequently, in an overlap of some peaks. In order to avoid such a chemical shift, a buffer solution based on phosphates ( $pK_{a1} = 2.15$ ,  $pK_{a2} = 7.20$ ,  $pK_{a3} = 12.42$ ) was proposed by many authors for controlling pH [17, 21]. The preparation of the pH 4.5 phosphate buffer solution typically involves addition of sodium azide, an antimicrobial preservative that has a major disadvantage of being environmentally hazardous. The reported protocol involves dissolution of honey in water to which is added a mixture of the buffer and lock solutions at a ratio of 7:3:1 (by volume). Nonetheless, a pH adjustment is further required to reach the target value. The lack of pH buffering strength of the added solution results from its pH value outside the phosphate buffer range, its limited phosphate concentration and its low added volume [17].

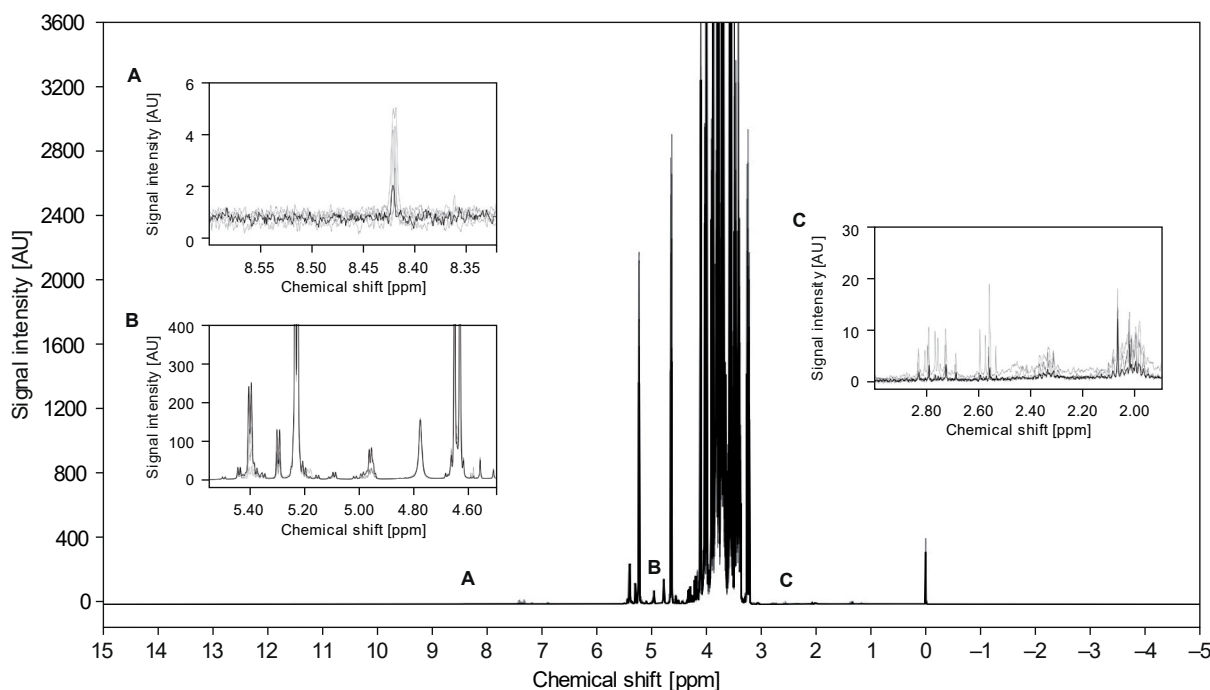
In order to overcome the above shortcomings, the protocol was significantly modified in this work. First, potassium disulphite, a common food preservative, was utilized to prevent microbial growth, second, a pH 4.3 buffer solution with no <sup>1</sup>H NMR signature was prepared using sodium oxalate ( $0.25 \text{ mol} \cdot \text{l}^{-1}$ ,  $pK_{a2} = 4.3$ ). In the third and final modification, honey was dissolved directly in the buffer solution, which helped maintain the constant pH of 4.3 throughout the entire sample preparation process. The initial pH of honeys and final pH values for the solutions of six honey are summarized in Fig. 1. Within the experimental error of the pH-meter ( $\pm 0.05$ ), the final pH value was found to be the same for all six honeys, regardless of what it was initially, and no further pH adjustment was necessary.



**Fig. 1.** Effect of oxalate buffer on honey solution as a function of the initial pH value of honey.

### Multidimensional analysis of <sup>1</sup>H NMR spectra

All honey samples were prepared according to the modified protocol described above and analysed, using the so-called “honey profiling module” of the NMR FoodScreener (Bruker BioSpin), which allows for fixing all the NMR system working parameters. Fig. 2 shows an example of the <sup>1</sup>H NMR spectra of the honeys in the entire spectral range from  $-5 \text{ ppm}$  to  $15 \text{ ppm}$ . The high-intensity bands between  $3.1 \text{ ppm}$  and  $4.5 \text{ ppm}$  correspond to glucose and fructose sugars, two



**Fig. 2.**  $^1\text{H}$  NMR spectra of five different botanical origin honeys after dissolution in the oxalate buffer solution.

A, B, C – insets represent zooms of three spectral regions.

main honey constituents. Data presented in Fig. 2 demonstrate excellent superposition of the  $^1\text{H}$  NMR spectra for five honeys with different initial pH values, illustrating the impact of the buffer solution on the chemical shift and also on the differences in intensities for different honeys, representative of their floral origins. The commercial honey was used as a reference for each honey series to assure quality control of sample preparation and  $^1\text{H}$  NMR measurements. A quality control chart was also established, with chemical shift and intensity data for selected  $^1\text{H}$  NMR peaks. Moreover, the chemical shifts of the commercial honey were used to verify peak positions for studied honeys. Another honey sample was prepared every time a shift greater than 0.03 ppm was observed for the reference peaks. This helped perform a reliable statistical analysis of the  $^1\text{H}$  NMR data for all honeys.

The ability of the model based on  $^1\text{H}$  NMR spectra to identify botanical origin of the honeys was verified using 212 batches of seven monofloral honeys: acacia, chestnut tree, scrub, honeydew, colza, lavender and sunflower. They were all authentic samples, from around France. Once the spectra were recorded, the  $^1\text{H}$  NMR files were firstly converted in a format readable by the data treatment software. The entire  $^1\text{H}$  NMR spectrum of each of the 212 honey samples was automatically included to elaborate the matrix.

Nonetheless, three areas were excluded for data treatment as described in the experimental section: the one corresponding to water peak residual signal and the two ones free of  $^1\text{H}$  NMR signal. The final set of points for each sample contained as many as 31 256 variables, which were chemical shifts, all subject to multidimensional treatment. A class variable was attributed to each sample according to its floral origin. A “range scale” pre-treatment was applied to all signals to focus them to a range from 0 to 1 in order to make the classification independent of the relative intensity. SIMCA was run considering a maximum factor of 10. A 95% confidence interval was considered to build the misclassification matrix, which is an illustration of the success of floral type classification of honey using internal leave one-out cross validation [24].

The results in Tab. 1 demonstrate the model capability of extracting discriminant parameters from  $^1\text{H}$  NMR profiles to identify botanical origin of the studied honeys. The model allowed for full identification of seven different botanical origins with 100% accuracy. This was possible because most of the interclass distances were greater than 3, attesting an obvious class separation [28]. An interclass distance around 2 was found between the acacia honeys and honeys produced from lavender or colza. The smaller distances in these cases can be linked to the lack of



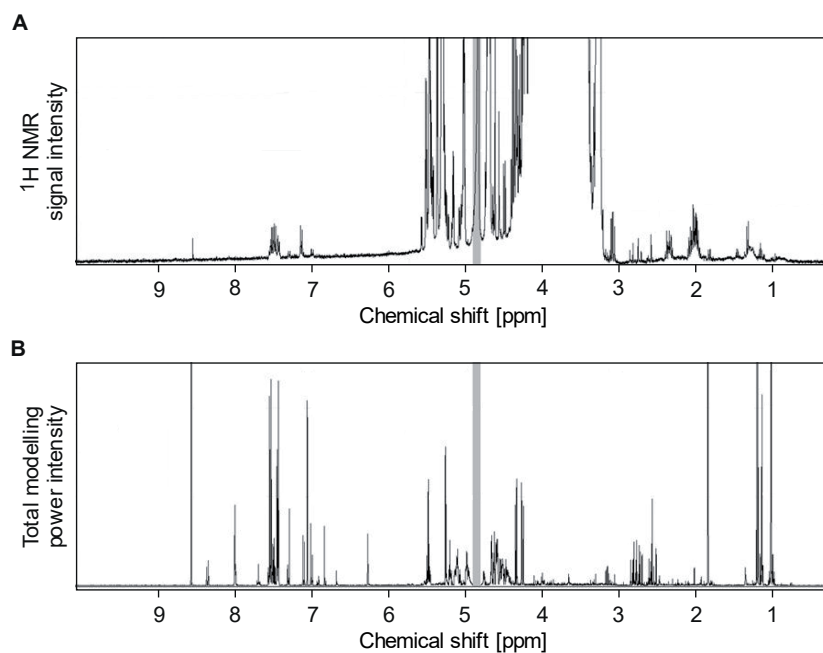
intense colour of these three honeys, possibly indicative of a lower concentration of specific floral origin markers. Fig. 3A shows a  $^1\text{H}$  NMR proton spectrum of a single honey sample within the parts per million limits used in the model construction, with the grey band indicating the excluded area of the water-suppressed signal. Fig. 3B summarizes the discriminating power of each proton signal, as provided by the data processing software.

In order to tentatively assign the types of compounds at the origin of classification, chemical shifts of the honey components were extracted from the literature and, in addition, a set of sugars present in honey at low concentration were analysed to characterize their chemical shifts. The non-exhaustive list of chemical shifts is given in Tab. 3. In Fig 3B, it can be seen that the main zone, characterized by the most intense signal area from 3.50 ppm to 4.15 ppm, was due to glucose and fructose, and did not present any discriminating power in the botanical origin classification. All other spectral areas with proton signal had a significant discriminating power. Among them, two areas presented a higher discriminating power in honey classification. The first one, in the range 6.5–8.5 ppm, was the resonance area of flavonoids, organic acids, the family of phenolic compounds that were also found in melliferous plants [29, 30]. The second area, in the range 0.8–2 ppm, was attributed to the compound family of amino acids

and some organic acids, which were previously used at varietal recognition [31]. The third area, in the range 4.1–5.7 ppm, corresponded to the resonance area of some saccharides. According to data in Tab. 3 and Fig. 3, demonstrating the lack of discriminating power of fructose and glucose, it can be estimated that the discriminating signals could be attributed to saccharides in low concentration as stated in other studies [32]. Finally, the fourth one, in 2.5–3 ppm range, is an area of organic acid resonance, which was previously used at botanical origin discrimination of other products [31].

#### Model validation and application to the control of commercial honeys

The model described above was also applied to a set of 38 honeys excluded from the original set of 212 authentic honeys. Five acacia, 10 chestnut-tree, 6 scrub, 5 honeydew and 12 lavender samples were selected to create a test set. No colza or sunflower samples were selected as the original sample set contained only a small number of samples of these honey types, and the selection of samples from these categories would be critical for the temporary model. The temporary model containing the seven botanical origins was tested using these 38 selected samples, with the classification results listed in Tab. 2. The classification was 100% successful, underscoring model's ability to simultaneously identify all seven botanical origins.



**Fig. 3.** Link between  $^1\text{H}$  NMR signal and SIMCA model discriminating power.

A –  $^1\text{H}$  NMR spectral area used for the model construction, B – discriminating power of each resonance signal. Grey zone represents exclusion area of the irradiated water signal.

**Tab. 3.** Non-exhaustive list of chemical shifts of compounds found in honey.

Compound	Chemical shift [ppm]	Reference
<b>Di-, tri-, tetra-saccharides</b>		
Glucose	5.20–5.27 (d); 4.60–4.69 (d); 3.66–3.94 (m); 3.36–3.57 (m); 3.20–3.28 (m)	[16, 17, 19, 20, 33]
Fructose	3.50–4.15 (m)	[17, 19, 22, 33]
Saccharose	5.45–5.37 (d); 4.18–4.25 (d); 4.01–4.09 (t); 3.62–3.93 (m); 3.52–3.59 (m); 3.43–3.51 (m); 3.34–3.37 (s)	[17, 22, 33]
Arabinose	4.52–4.54 (s)	[17, 33]
Erllose	5.35; 4.05 (t); 3.55–3.72 (m)	[33, 34]
Gentiobiose	5.17; 4.59; 4.45; 3.5 (t); 3.45 (m); 3.38 (t); 3.31 (m)	[33, 34]
Isomaltose	5.18; 4.9; 4.62; 3.71 (m); 3.55 (m); 3.42 (m)	[33, 34]
Isomaltotriose	5.19; 4.90	[33]
Kojibiose	5.38; 5.33; 5.04	[33, 34]
Lactose	4.47 (d)	[19, 34]
Maltose	5.39; 5.17; 4.59	[23, 33, 34]
Maltotetraose	5.36; 5.17; 4.59	[33, 34]
Maltotriose	5.37–5.41 (d); 5.22–5.25 (d); 4.63–4.68 (d); 3.50–4.02 (m); 3.37–3.46 (m); 3.23–3.31 (m)	
Maltulose	5.19; 5.15; 5.10	[33, 34]
Mannose	3.93 (q); 3.84 (t); 3.72 (t); 3.64 (2d); 3.57 (2d); 3.43 (t)	[33]
Melezitose	5.06–5.49 (d); 5.42–5.46 (d); 4.24–4.34 (m); 3.52–3.96 (m); 3.40–3.48 (m)	[19, 33, 34]
Melibiose	5.17; 4.93; 4.62	[33, 34]
Nigerose	5.32; 5.30; 5.17; 4.61	[33, 34]
Panose	5.37–5.43 (d); 5.20–5.26 (d); 4.93–4.99 (d); 4.63–4.68 (d); 3.39–4.03 (m); 3.25–3.31 (m)	
Palatinose	4.94–4.99 (m); 4.16–4.24 (t); 4.09–4.14 (m); 3.50–4.00 (m); 3.37–3.47 (m)	
Raffinose	5.40–5.44; 4.97–5.01 (d); 4.19–4.25 (d); 3.62–4.10 (m); 3.51–3.61 (m)	
L(+)-Rhamnose	5.09–5.13 (d); 1.23 (m)	[17, 19]
Trehalose	5.16–5.22 (d); 3.72–3.89 (m); 3.62–3.68 (m); 3.41–3.49 (t)	
Turanose	5.32–5.28 (d); 5.22–5.19 (d); 5.14–5.17 (d); 3.52–4.4 (m); 3.38–3.50 (m); 3.34–3.36 (s)	
<b>Organic acids</b>		
Citric acid	2.68–2.69 (s)	[17, 19]
Formic acid	8.44–8.47 (s)	[16–19, 35]
Fumaric acid	6.53–6.55 (s)	[17]
Isobutyric acid	1.14 (d)	[19]
Lactic acid	1.33 (d)	[19]
Malic acid	2.70–2.73 (s)	[19]
Phenylacetic acid	7.37; 7.29–7.30; 3.37	[33]
Phtalic acid	7.48–7.53 (m)	[17]
Pyruvic acid	6.42–6.45 (s)	[17]
Succinic acid	2.50–2.52 (s)	[17]
L(+)-Tartaric acid	4.32–4.41 (s)	[17, 19]
<b>Amino acids</b>		
Alanine	3.75–3.85 (m); 1.46 (d)	[16]
Lysine	1.73 (m)	[19]
Glutamine	2.14 (m)	[19]
Glutamic acid	2.34 (m)	[19]
Phenylalanine	7.42 (dd); 7.37 (t); 7.33 (d)	[16, 18, 19, 35]
Proline	4.11 (dd); 3.42 (dt); 3.32 (dt); 2.33 (m); 2.02 (m); 1.99 (m)	[31]
Threonine	4.24 (m)	[19]
Tyrosine	7.19–7.04 (d); 6.90–6.74 (d); 2.62	[16, 18, 19, 35]

Tab. 3. continued

Compound	Chemical shift [ppm]	Reference
<b>Other compounds</b>		
Caffeine	7.55; 4.01; 3.61; 3.42	[17, 19]
Chrysin	12.81; 7.89; 7.54; 6.68; 6.48; 6.31	[19]
4-HMCHD	6.90; 6.10; 2.30; 1.20	[18]
Dehydrovomifoliol	6.84; 6.47; 5.97; 2.51; 2.34; 2.31; 1.89; 1.10; 1.03	[18]
Ethanol	1.17–1.18 (t)	[18, 19]
HMF	9.43–9.47 (d); 7.54 (d); 6.69 (d)	[17– 19]
8-Hydroxylnalol	8.09; 5.93; 5.43; 5.24; 4.00; 2.08; 1.68; 1.61; 1.30	[18]
Methylglyoxal	2.31; 1.38	[20]
Pinocembrin	5.36; 5.21; 4.35; 4.18; 3.56; 3.45; 2.33; 2.30; 2.04; 1.64; 1.56; 1.30	[20, 23]

Signal multiplicity: s – singlet, d – doublet, t – triplet, m – multiplet, q – quadruplet, dd – doublet of doublets, dt – doublet of triplets, 2d – two doublets.

4-HMCHD – 4-(1-hydroxy-1-methyl)cyclohexa-1,3-diene carboxylic acid, HMF – 5-(hydroxymethyl)furfural.

Tab. 4. Comparative results of commercial honeys controlled by sensory and pollen analysis, and by  $^1\text{H}$  NMR profile analysis along with the multidimensional result.

Sample	Claimed floral origin	Claimed geographical origin	Sensory and pollen analysis	Model
S1	Lavender	France	No	No
S2	Lavender	France	No	No
S3	Acacia	France	Yes	Yes
S4	Chestnut tree	France	No	No
S5	Chestnut tree	France	No	No
S6	Acacia	France	Yes	Yes
S7	Sunflower	France	Yes	Yes

The full model elaborated with the 212 honeys was applied to seven commercial honeys in order to verify their floral origin as stated on the label. The results obtained by  $^1\text{H}$  NMR profile analysis were compared to the outcomes of the sensory and pollen analysis. These seven commercial honeys were analysed according to the established protocol by  $^1\text{H}$  NMR and their spectra compared with the model. The results summarized in Tab. 4 attest to the perfect agreement between the results of the sensory and pollen analysis, and those obtained from the model.

## CONCLUSIONS

A modified protocol for  $^1\text{H}$  NMR analysis of honey, based on dissolving honey in a sodium oxalate buffer solution of pH 4.3, was developed. The buffer solution does not contain any environmentally hazardous chemicals and can be prepared in a laboratory in a cost-effective and rapid manner. This simple method allows for the preparation and  $^1\text{H}$  NMR analysis of a high number of

samples. In this study, a series of 50 honey samples were prepared in a day. The method applicability to classification of a batch consisting of 212 French honeys of various floral origin (acacia, chestnut tree, scrub, honeydew, colza, lavender and sunflower) was verified. A multidimensional analysis was applied to the  $^1\text{H}$  NMR spectra, after excluding three spectral areas not containing characteristic signals. The result of SIMCA data treatment, with a range scale pre-processing, allowed for full identification of the honey botanical origin by the model. It demonstrated the potential of untargeted analysis of  $^1\text{H}$  NMR profiles as a means to classify French honeys according to their botanical origin. The interclass distance was high enough to assert the floral origin of commercial honeys based on the model predictions.

The developed method can be easily implemented in food control laboratories equipped with a  $^1\text{H}$  NMR system. As the statistical data treatment can be directly performed on the spectra, no chemical compounds identification skills are required.

Future research objectives include implementation of the model to the origin of other mono-



floral honeys, application of the model to new production honeys in order to detect any “vintage” influence, and model testing with foreign honeys to detect the influence of the geographical origin on mono-floral honey composition. These plans notwithstanding, the presented first results obtained using commercial honeys already attest to the model potential in predicting the botanical origin of French honeys.

#### Acknowledgements

The authors greatly thank Monica Moertter and Lea Heintz (Bruker BioSpin) for their training on  $^1\text{H}$  NMR screener applications, and Sylvain Thomé (Bruker BioSpin) for his valuable technical assistance. Alina Magdas and Piotr Zelenay are gratefully thanked for their constructive remarks. Honey cooperatives are warmly thanked for providing honey samples for this study.

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Received 28 February 2020; 1st revised 28 April 2020; 2nd revised 12 May 2020; accepted 18 May 2020; published online 8 June 2020.