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Title: Novel method based on Ion Mobility Spectroscopy for the quantification of adulterants in honeys

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Keywords: Honey; adulteration; sweeteners; Ion Mobility Spectrometry; Sum Spectrum; chemometrics; quantification

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Abstract: According to European Union Regulation, honey is a food product whose composition cannot be modified. However, high-quality honey is often adulterated by adding sweeteners of other sugar compounds. This paper studies the suitability of Ion Mobility Spectra from generated headspace as a method for the detection and discrimination of honey adulterated by different substances. A Box-Behnken design in conjunction with a response surface methodology was employed to optimize five different variables related to headspace generation (incubation temperature, incubation time, injection volume, weight of the samples and pre-heating time). The resulting model showed a regression coefficient of R2=88.07%, it is therefore suitable for a reliable selection of the experimental variables. Repeatability and intermediate precision were also evaluated, and coefficients of variation below 5% were obtained (CV of 4.6% and 4.2% respectively). The developed method has been applied to different samples resulting for the mixture of honey and other sweeteners at different percentages (10%-50%) in an attempt to mimic the adulterated products that are more commonly found in the market. A thorough and exact classification (100%) with regards to the presence/absence of adulterant as well as the type of adulterant used has been achieved. A Partial Least Squares regression model was completed in order to determine the percentage of the different adulterants. The prediction error was below 4% in all the cases. These results demonstrate the applicability of the developed method for the detection and quantification of adulterated honey with different adulterant contents.

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Cover Letter

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Food Control
Editorial Office

Dear Editor, please find enclosed a manuscript entitled "**Novel method based on IMS Sum Spectrum for the quantification of adulterants in honeys"** by Mª José Aliaño-González, Marta Ferreiro-González, Estrella Espada-Bellido, Gerardo F. Barbero and Miguel Palma, which we would like to be considered for publication in Food Control. This paper is unpublished and has not been submitted for publication elsewhere.

Honey is a pure product, so it is illegal the fact of adding to or removing from it any kind of substances. Although, it is one of the most likely products to be a target for adulteration in the food market. The most common adulterants used are sweeteners with lower prices than honey and some similarities to honey composition, specific hydrates of carbon percentage.
At the first time, Ion Mobility Spectrometry (IMS) has been used for the detection and discrimination of honey adulteration. In this work, first, we have included the optimization of a method based on HS-GC-IMS for the discrimination between pure honey and five different adulterants (Rice Syrup, Invert Sugar, Brown Sugar, Fructose Syrup and High Fructose Corn Syrup). Afterward, the developed method has been applied to adulterated honey with the five adulterants at different percentages (5%-50%). Then, the feasibility of use Ion Mobility Spectra information combined with multivariate analysis has been tested in order to develop a screening method that could be applied in routine laboratories (i) to detect honey adulteration, (ii) to discriminate among different types of adulterants and (iii) to quantify the level of adulteration.

Kindest regards,

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Novel method based on Ion Mobility Spectroscopy for the quantification of adulterants in honeys

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ABSTRACT

According to European Union Regulation, honey is a food product whose composition cannot be modified. However, high-quality honey is often adulterated by adding sweeteners of other sugar compounds. This paper studies the suitability of Ion Mobility Spectra from generated headspace as a method for the detection and discrimination of honey adulterated by different substances. A Box-Behnken design in conjunction with a response surface methodology was employed to optimize five different variables related to headspace generation (incubation temperature, incubation time, injection volume, weight of the samples and pre-heating time). The resulting model showed a regression coefficient of R^2=88.07%, it is therefore suitable for a reliable selection of the experimental variables. Repeatability and intermediate precision were also evaluated, and coefficients of variation below 5% were obtained (CV of 4.6% and 4.2% respectively). The developed method has been applied to different samples resulting for the mixture of honey and other sweeteners at different percentages (10%-50%) in an attempt to mimic the adulterated products that are more commonly found in the market. A thorough and exact classification (100%) with regards to the presence/absence of adulterant as well as the type of adulterant used has been achieved. A Partial Least Squares regression model was completed in order to determine the percentage of the different adulterants. The prediction error was below 4% in all the cases. These results demonstrate the applicability of the developed method for the detection and quantification of adulterated honey with different adulterant contents.

Keywords: Honey; adulteration; sweeteners; Ion Mobility Spectrometry; Sum Spectrum; chemometrics; quantification

1. Introduction

The composition of honey is mainly based on carbohydrates (70%-80%), followed by water (10%-20%), and minor components such as vitamins, pollen or polyphenol compounds (da Silva, Gauche, Gonzaga, Costa, & Fett, 2016; Pascual-Maté et al., 2018). It also contains Volatile Organic Compounds (VOCs) of different chemical families such as monoterpenes, C13-norisoprenoids, sesquiterpenoids, benzene derivatives (including *trans*-linalool oxide, furfural, hotrienol and in minor extent, 1,3-dihydroxy-2-propanone, 5-hydroxymethylfurfural, benzene

acetaldehyde, ethyl decanoate, ethyl dodecanoate, *o*-methoxyacetophenone and 2-ethyl hexanoic acid)(da Costa et al., 2018; Pontes, Marques, & Câmara, 2007). VOCs have been previously used to determine the floral and geographical origin of honey (Devi, Jangir, & K.A., 2018; Patrignani, Fagúndez, Tananaki, Thrasyvoulou, & Lupano, 2018). Recent researches have shown the correlation between honey composition and some really interesting properties for us such as its nutritive, revitalizing, antioxidant, anti-inflammatory or antimicrobial properties (Fan & Roos, 2019; Seraglio et al., 2019; Zhao et al., 2017). For instance, honey has been daily consumed due to its important nutritious and energizing properties. However, novel discoveries on honey properties have meant a notorious increase in honey applications to different fields (Bankova, Popova, & Trusheva, 2018; Han, Lee, & Pak, 2013; Ota et al., 2019).

This increment in honey consumption has also increased consumers' interest in high-quality honey. For this reason, Protected Designation of Origins (P.D.O.) have been established. P.D.O.s is a legal regulation of the European Union (EU) that allows us to know both the geographical and the botanical origin of honey. This should ensure the quality of the honey that is found in the markets. In Spain, in particular, there are only four registered P.D.O.s (Tenerife, Granada, La Alcarria and Villuercas-Ibores).

The increment in honey consumption and the fall in the number of bees over the last years (Seitz, vanEngelsdorp, & Leonhardt, 2019) have resulted in a noticeable increment of high-quality honey price (Amiry, Esmaiili, & Alizadeh, 2017). This has made of honey an attractive and profitable product to be mixed with cheap industrial sweeteners. Nevertheless, according to European Union Regulations (Codex Alimentarius Commission and Council Directive 2001/110/EC of 20 December 2001 relating to honey) honey is a pure product, which means that the addition or removal of any kind of substance to its composition is considered illegal (Food, 2001). Despite this prohibition, honey is one of the most often adulterated products in food markets nowadays. This is considered an economic fraud to consumers and although the most commonly used adulterants, i.e. regular sugars, should not have any serious consequences to human health, safety concerns related to allergens should be carefully considered (Arlorio et al., 2009).

Different techniques have been used for the detection of adulterated honey, most of them based on DNA analysis methods (El Sheikha, 2018; Sobrino-Gregorio, Vilanova, Prohens, & Escriche, 2019; Utzeri, Ribani, & Fontanesi, 2018), surface plasmon resonance spectra (Zainuddin et al., 2018), rheological analysis (Oroian, Ropciuc, Paduret, & Todosi, 2018; Yilmaz et al., 2014) or liquid chromatography analyses (Wang et al., 2015). These methods are based on analyses for the identification of particular components, which implies two main drawbacks. Firstly, most of the sweeteners used as adulterants in honey simulate its natural carbohydrate profile and consequently, they are not easy to detect (Cordella, Faucon, Cabrol-Bass, & Sbirrazzuoli, 2003). Furthermore, these instrumental methods are expensive, time-consuming, destructive and require a considerable analytical skill level, which would limit their use as routine monitoring.

In the last few years, new general profile methods have been applied to food analysis. Such methods get round any individual compound identification and use changes on signals instead, for example, differences in VOCs intensities. The use of these techniques in combination with chemometric tools allow to determine the characteristic fingerprint of each sample. Fingerprints can be used to detect and discriminate adulterated honey in a rapid and easy way (Naila, Flint, Sulaiman, Ajit, & Weeds, 2018a). Some of the general profile methods used for the detection and discrimination of adulterated honey are visible and near-infrared spectroscopy techniques (Ferreiro-González et al., 2018; Li et al., 2017; Qu et al., 2015; Se, Ghoshal, Wahab, Ibrahim, &

Lani, 2018), the electronic tongue (e-tongue) (Sobrino-Gregorio, Bataller, Soto, & Escriche, 2018) or the electronic nose (e-nose) (Zakaria et al., 2011).

Ion Mobility Spectrometry (IMS) is an analytical technique mainly related to the analysis of VOCs. It is based on gas phase ion separation inside a drift tube under the influence of a constant electric field at atmospheric pressure (Gabelica & Marklund, 2018). The ionization of VOCs can be carried out by means of an electrospray, a laser, an ultraviolet lamp or by chemical means. Chemical ionization is one of the most common methods used because of the stable and reliable operation compared to the use of radioactive sources. This technique has been applied to the detection of food adulteration in recent years due to its numerous advantages (Arroyo-Manzanares et al., 2018; Garrido-Delgado, Eugenia Muñoz-Pérez, & Arce, 2018; Karpas, 2013; Tzschoppe, Haase, Höhnisch, Jaros, & Rohm, 2016). It presents a very low limit of detection, usually in the range of ppb. It does not require any other sample preparation but headspace generation. Furthermore, the methods based on this technique do not usually produce residues as they do not use solvents; therefore, it can be considered environmentally friendly. Lastly, IMS operates at atmospheric pressure, which means that IMS could be used in real-time monitoring analysis and makes it really interesting for routine analysis against fraud (Reinecke & Clowers, 2018; Ridgeway, Lubeck, Jordens, Mann, & Park, 2018).

The aim of this research is to study the feasibility of Ion Mobility Spectra for the detection and discrimination of honey adulterated by different adulterants. For that purpose, a method based on the technique headspace-gas chromatography-ion mobility spectrometry (HS-GC-IMS) has been optimized in order to discriminate between pure honey and honey adulterated by adding five of the most frequently used adulterants in the market. Afterward, a chemometric study has been completed to demonstrate the applicability of the developed method for the detection and discrimination of adulterated honey samples with adulterant content in the range 5%-50%.

2. Materials and Methods

2.1. Samples

2.1.1. Pure Honey

Unadulterated honey was provided by Granada P.D.O. (Lanjaron, Granada, Spain). Specifically, 33 different pure multi-floral honey samples were collected in 2016. Multi-floral honey was selected as pure honey since it is one of the most common types of honey [28] and consequently, one of the most often adulterated. All of the samples were mixed in order to guarantee the representation of unadulterated and adulterated samples, obtaining a final matrix of 2 kilograms. Two replicas of 8 grams each were selected as unadulterated honey samples whereas the rest was used to prepare the adulterated honey samples.

2.1.2. Adulterants

Five different common sweeteners were chosen to be used as adulterants: rice syrup (RS) brown cane sugar (BS) (Biospirit S.L., Gerona, Spain), invert sugar (IS), fructose syrup (FS) (Sosa Ingredients S.L., Moiá, Barcelona, Spain), and high fructose corn syrup (HFCS) (Cargill S.L.U., Martorell, Barcelona, Spain). All of them were purchased from Spanish regular suppliers.

2.1.3. Honey Adulteration

The adulterated samples were prepared by mixing the pure honey with the different adulterants at different ratios: 5%, 10%, 20%, 25%, 30%, 40%, and 50%. These adulteration percentages were chosen because they are the most commonly found in the markets (Ferreiro-González et al., 2018). Two replicas of each adulteration ratio were prepared. A total of 77 samples were finally obtained for the analysis (2 pure honey samples, 5 pure adulterants, and 70 adulterated honey samples).

Pure honey samples were named as PH followed by the replica number (1 or 2) and pure adulterants were identified by their initial letters (RS, IS, BS, FS or HFCS). Finally, adulterated honey samples were named as follow: the initial letters of the adulterants followed by the adulteration ratio and the sample replica number. Each analysis was carried out in duplicate, so each duplicate was named as _A or _B. For example, the first sample for the first analysis of honey adulterated with rice syrup at 25% would be named as RS_25%_1_A. Likewise, the first sample for the second analysis of honey adulterated with rice syrup at 25% concentration rate would be identified as RS_25%_1_B.

Pure honey, adulterants, and adulterated samples were stored in the dark at room temperature prior to analysis.

2.2. HS-GC-IMS Analysis Acquisition

The samples were analyzed by headspace-gas chromatography-ion mobility spectrometry (HS-GC-IMS) Flavour Spec (G.A.S., Dortmund, Germany). The vials with pure honey, pure adulterants or adulterated honey samples were directly placed in the auto sampler oven to be heated and agitated in order to generate the HS. The GC column was multicapillary MCC OV-5 of 20 centimetres (G.A.S., Dortmund, Germany). The drift gas and carrier gas selected was nitrogen at 99.999% purity, obtained from a nitrogen generator (G.A.S., Dortmund, Germany). The ionization method used was 3H Tritium beta radiation. Conditions related to GC-IMS analysis are shown in Table 1.

2.2.1. Box-Behnken Design (BBD) and Statistical Analysis

The objective of the present research is to determine the feasibility of HS-GC-IMS technique for the discrimination between pure honey and pure adulterants. For that, a method was optimized based on the discrimination of these samples, then a Box-Behnken design (BBD) with response surface methodology (RSM) was selected for this purpose. Previously published papers have shown the influence of different variables such as the incubation time, the incubation temperature and the injection volume on Ion Mobility Spectra results (Snow & Slack, 2002). In this study, five variables have been chosen to be optimized: incubation time, incubation temperature, sample weight, injection volume and pre-heating time. Being pre-heating time the time that vials that contain the samples are kept open inside a chamber at 30°C without any kind of treatment to reach a regular temperature. The five variables were optimized at three different levels (level identification codes are as follows: -1 for the low level, 0 for the medium level and 1 for the high level) (Table 2). In order to determine an easy and rapid method, specific levels were determined for each operating variable. For this reason, the incubation time and the pre-heating time were limited to a maximum of 15 and 25 minutes respectively. The injection volume was selected according to the instrument´s options and small samples were selected based on the group's previous experience with IMS applied to other food products. Finally, the incubation temperature range between 30°C and 50°C was selected. The low limit of the range matches the minimum temperature allowed by the equipment, and the maximum limit was based on published literature on the subject that demonstrates that some

honey components may suffer degradation when subjected to higher temperature levels (Naila, Flint, Sulaiman, Ajit, & Weeds, 2018b). In the experimental design,6 central points were added, therefore a total of 46 experiments were run as described in Table S.1 in the Supplementary material.

From the 46 experiments, six types of samples were analyzed by BBD-RSM (pure honey and 5 pure adulterants). So, firstly, the total area of the Ion Mobility Sum Spectrum (IMSS) obtained from each sample under specific conditions was analyzed by BBD (46 different conditions). The differences between the six samples were calculated for any of the specific conditions, that is, the differences between pure honey samples and pure adulterants and the differences between the five adulterants. The sum of all these differences was selected as the response variable. Its value for each one of the 46 experiments can be found in Table A.1.

2.3. Data Analysis.

2.3.1. IM Sum Spectrum Acquisition

In the present study, the use of IMSS as a novel alternative is proposed. IMSS is the spectrum obtained by adding the total intensities at the different drift times regardless of the retention time, it means that no chromatographic information is used (Fig. 1). IMSS included total intensity data from different drift times (from 0.000 ms to 4.283 ms). IMSS for all the samples were obtained by Laboratory Analytical Viewer software (LAV) (G.A.S., Dortmund, Germany). Any drift time was automatically normalized to the signal of the Reaction Ion Peak (RIP) by LAV software. RIP represents the total available ions generated by the source, so that it indicates the water content in the nitrogen ionized by ^{3}H radiation. This value is used as a reference indicator. The spectroscopic range between 1.050 and 1.600 (RIP relative), which comprises all the compounds of interest, was used to calculate the differences between pure honey and pure adulterants, using a total of 578 drift times. Each sample was normalized by assigning one unit to the maximum intensity.

2.3.2. Chemometric Tools

The BBD with RSM for the analysis of the optimum conditions was applied using the software Statgraphic Centurion XVII (Statgraphics Technologies, The Plains, VA, USA). Once the optimal conditions had been determined, the feasibility of the method to detect and discriminate adulterated honey was studied. For that, different chemometric tools such as hierarchical cluster analysis (HCA), linear discriminant analysis (LDA) and partial least squares regression (PLS) were used. HCA and LDA were performed by means of IBM SPSS Statistics 22 (Armonk, NY, USA). PLS analysis was applied using Unscrambler (version 10.1, Camo Software AS, Oslo, Norway).

3. Results and Discussion

3.1. Optimization Study

The main purpose of this research is to determine the feasibility of IMSS to detect adulterated honey with different adulterant contents. For that purpose, it was necessary to determine if IMSS could produce different indicators for pure honey and for each one of the five adulterants used in the experiments (RS, IS, BS, FS, and HFCS). It must be noted that only volatile compounds would influence the IMSS results.

An optimization study based on BBD-RSM has been applied to maximize the sum of the differences on intensities between pure honey and adulterants and between adulterants. Five variables were chosen to be optimized: incubation time, incubation temperature, sample weight, injection volume and pre-heating time.

The six different samples (pure honey, pure RS, pure IS, pure BS, pure FS, and pure HFCS) were analyzed at the 46 working conditions obtained from the experimental design. A total of 276 IMSS (46 experiments x 6 samples) were obtained, and the spectroscopic range from 1.050 to 1.600 (RIP relative) was selected. Each sample was normalized by assigning one unit to the maximum intensity level.

The response variable for each of the 46 experiments was obtained (Section 2.2.1.) and the total intensity differences among the samples were calculated. The experimental values for the discrimination were fitted with the predicted values by a polynomial function model (Equation 1).

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_5X_5 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{14}X_1X_4 + \beta_{15}X_1X_5 + \beta_{23}X_2X_3 + \beta_{24}X_2X_4 + \beta_{25}X_2X_5 + \beta_{34}X_3X_4 + \beta_{35}X_3X_5 + \beta_{45}X_4X_5 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{44}X_4^2 + \beta_{55}X_5^2.$$

(**Equation 1)**

In this equation Y is the predicted response, that is, the difference between the sum of the differences between the six samples at all the drift times. β_0 is the ordinate at the origin; X_1 (incubation time), X_2 (incubation temperature), X_3 (injection volume), X_4 (sample weight), and X_5 (pre-heating time) are the independent variables. β_i are the linear coefficients; β_{ij} are the cross-product coefficients, and β_{ii} are the quadratic coefficients. The suitability of the model was validated by ANOVA. Coefficients for the different parameters of the quadratic equation and their significance (*p*-values) are represented in Table 3. Factors with a *p*-value below 0.05 were considered as significant factors.

The significant variables affecting the responses (with *p*-values lower than 0.05) were: injection volume (*p*-value = 0.0000), sample weight (*p*-value = 0.0000), incubation temperature (*p*-value = 0.0001), quadratic interaction of injection volume (*p*-value = 0.0001), quadratic interaction of sample weight (*p*-value = 0.0086), the interaction between injection volume and sample weight (*p*-value = 0.0409) and the interaction between incubation time and incubation temperature (*p*-value = 0.0431).

Incubation temperature showed a coefficient of b= 0.294, the injection volume showed a coefficient of b=103.234 and the sample weight of b=66.921. For these three variables, the effect was positive, which means that the higher their values, the higher the differences between the samples. The squared correlation coefficient (R^2) of the model obtained was R^2=88.07%, which indicates a statistically significant agreement between the measured and the estimated responses.

Injection volume and sample weight as influential factors were recorded in three-dimensional surface plots obtained by using a polynomial equation (Fig.2). Fig. 2 illustrates the combined effects of the injection volume and the sample weight on the difference between pure honey samples and adulterants and between adulterants.

As it could be seen the maximum difference between the samples was achieved for injection volumes between 0.93 mL and 1.00 mL and at sample weights between 0.3 grams and 0.7 grams.

3.2. Optimized Conditions

The optimum conditions for the developed method were 0.45 grams of sample pre-heated for 25 minutes, incubated for 15 minutes at 50°C and 0.83 mL of volume of injection used for the analysis by GC-IMS. On the one hand, it was detected that optimal incubation time and pre-heating time were reached at the top limits of the experimental range. Thus, a clear difference between pure honey and between adulterants was possible when 25 minutes of pre-heating and 15 minutes of incubation were applied. Therefore, in order to maintain a short analysis time, no longer time values were applied. Also, the best incubation temperature to create headspace was determined at 50°C, which is also the top limit of the experimental temperature range. No higher values were applied since temperature levels higher than 50°C could degrade some of the carbohydrates in the samples (Naila et al., 2018a).

3.3. Method Repeatability and Intermediate Precision

In order to study the repeatability and intermediate precision of the optimized method for the discrimination of the samples, a total of 12 analyses were carried out at optimal conditions. Six analyses on the same day and the other six in two different days (three per day). For each of these analyses, the six samples (pure honey and the five adulterants) were analyzed (12 samples of pure honey and 12 samples of each adulterant), which makes a total of 72 samples.

IMSS was obtained from each analysis and the sum of their intensity differences was calculated as above explained. The Coefficient of Variation (CV) was calculated to determine the analysis precision. Their Repeatability CV was 4.6%, while their Intermediate Precision CV was 4.2%.

3.4. Detection/Discrimination of Adulterated Honey Samples

A method has been optimized based on Ion Mobility Spectra to discriminate pure honey and pure adulterants by providing obviously different results between them. Noticeable differences in the volatile compounds of pure honey and the adulterants has been detected in IMSS. The method also intended to identify each one of the five most commonly used adulterants. The feasibility of using these differences to detect and discriminate each adulterant by means of chemometrics was tested. Adulterated honey samples containing five different adulterants (IS, RS, BS, FS, and HFCS) at different percentages ranging from 5% until 50% were analyzed at the previously determined optimum conditions. Pure honey was also analyzed. However pure adulterants were not analyzed at this stage since the main goal was to discriminate between pure and adulterated honey.

A total of 144 analyses were carried out (2 pure honey samples and 2 replicas of adulterated honey samples each one containing one of the five adulterants at different percentages, all in duplicate). The IMSS of all of them were obtained for the range 1.050 - 1.600 (RIP relative) with a total of 578 drift times.

First, a non-supervised method was employed to determine the tendency of the sample to form clusters depending on the intensity differences. For that, an HCA was carried out using Ward's method with squared Euclidean distance. The dendrogram obtained from the HCA is displayed in Fig. 3. The average intensities measured from all the samples replicates were represented in the dendrogram for better visualization. It can be observed that two clusters had formed (A and B). Cluster B comprised both the samples of honey adulterated with IS and also the samples that had been adulterated with FS at percentages of 30% or higher. Cluster A was formed by two subclusters (A_1 and A_2). Within subcluster A_1 the samples of honey adulterated with RS at percentages ≥ 25% formed one group, while the samples that had been adulterated with FS at percentages of 25% or lower from another group. The honey samples that had been

adulterated with BS were remained inside a subcluster and showed a tendency to group together according to their adulterant percentage content.

Subcluster A_2 was formed by samples of pure honey. A separated subcluster was formed by the adulterated honey samples with at low content of RS (5% - 25%). Finally, the adulterated honey samples with HFCS formed two separate groups according to the percentage of adulterant content (≤ 25% and ≥ 30%).

It was first noticed that pure honey samples tended to be grouped together and separate from the adulterated honey samples. It was also noticed that adulterated samples tended to form clusters according to the type of adulterant used and on the percentage of adulterant content. However, some misclassification occurred with samples containing different adulterants. For this reason, Linear Discriminant Analysis (LDA) was used as a supervised method to try and classify by the type of adulterant used.

The same data matrix (144 IMSS) was subjected to LDA. Six groups were considered a priori: pure honey and honey adulterated with RS, IS, BS, FS, and HFCS. The method selected was cross-validation with the stepwise method. Perfect discrimination (100%) between groups was achieved. Three first discriminant functions of the discriminant analysis have been represented in Supplementary Material as Fig. S.1. It can be seen that fully separated groups appeared based on the first three discriminant functions. There were not overlapped areas between those groups. However, the three discriminant functions were required, since not clear discrimination could be established by applying only one of them.

A total of 22 relevant drift times were obtained to discriminate from Fisher's linear discriminant functions. In order to obtain a characteristic fingerprint for each adulterant, an HCA from variables of the 22 relevant drift times was obtained. A trend to form four groups could be observed as follows: Group 1 (1.071,1.074, 1.085, 1.105, 1.129, 1.215, 1.226, 1.283, 1.373, 1.407, 1.410, 1.488, 1.559, 1.568, and 1.571 (RIP relative)), Group 2 (1.146, and 1.157 (RIP relative)), Group 3 (1.346, and 1.349 (RIP relative)) and Group 4 (1.358, and 1.360 (RIP relative)). The results were graphically represented in a dendrogram included in the Supplementary Material as Fig. S.2. The intensity of one of the characteristic drift times in each group (1.085,1.146, 1.346, and 1.358(RIP relative)) was obtained and normalized to the maximum (Fig.4).

Pure honey samples showed a maximum value for 1.146 (RIP relative) whilst the other signals were 40% below the maximum score. There are no other samples with this kind of fingerprint. Adulterated honey with RS also showed the maximum value for 1.146 (RIP relative), however other signals (1.346 and 1.358 (RIP relative)) are over 75% this value. Adulterated honeys with both IS and BS showed the maximum value for 1.346 with the rest of values below 50% for BS and above 50% for IS (1.146 and 1.358 (RIP relative)). Honey samples adulterated with FS and HFCS showed maximum values for 1.358 (RIP relative). For the honeys adulterated with HFCS, the other signals are below 50% whilst FS shows values above 50% for 1.146 and 1.346 (RIP relative). Therefore, clearly different sample fingerprints were observed depending on the adulterant used.

The feasibility of IMSS for the detection and discrimination of adulterated honey with five different adulterants at percentages between 5% and 50% has been demonstrated. Furthermore, a characteristic fingerprint of each sample has been developed using only four drift times, which means that the adulterant can be characterized easy and rapidly.

3.5. Quantification of Adulterant Content

Finally, a Partial Least Squares regression (PLS) with cross-validation was employed in order to develop a multivariate calibration model to correlate adulteration level and IMSS results. One model was created for each type of adulterant according to their adulterant percentage content: 0% (Pure honey), 5%, 10%, 20%, 30%, 40%, and 50%. For external validation, a set of 25% adulterated samples (not included in the model calibration process) was used.

The results are summarized in Table 4. Models prediction capabilities were tested by checking both the root-mean-square error of calibration (RMSEC) and the root-mean-square error of prediction (RMSEP). It was observed that errors were under 4% in both cases. The coefficients of regression were higher than 0.95 in all the cases. Additionally, an external validation was carried out. For that purpose, the multivariate regression that had been developed for the model calibration was applied to the 25% adulterated samples and the difference between the values predicted by the model and the actual values (25%) were calculated. The error of prediction was below 5% in all the cases. These results demonstrate the accuracy and robustness of the calibration model applied to the samples containing each one of the different adulterant percentage contents.

Moreover, a global model comprising samples with all the different adulterant percentage contents was tested. However, the results were not as accurate as the ones previously obtained from the adulterant-percentage specific models. The coefficient of regression was 0.71 and the errors of prediction and calibration were higher than those of the adulterant-percentage specific models. Finally, the validation error was 9.25%. In view of these results, the use of fingerprint to identify the adulterant used was suggested, while the individual model (adulterant-percentage specific models) would be used to determine the adulteration percentage content. Very similar error of prediction was observed regardless of the adulterant used.

4. Conclusions

By employed an optimized method HS-GC-IMS can be used to discriminate between pure honey and pure adulterants. With regards to IMSS results, injection volume, sample weight, incubation temperature, quadratic interaction of injection, quadratic interaction of sample weight, the interaction between injection volume and sample weight and the interaction between incubation time and incubation temperature, were the most influential variables to successfully complete a total discrimination between adulterants and honey. This method has demonstrated to be repeatable and with adequate intermediate precision thanks to its CV below 5%.

IMSS has proven to be a rapid and reliable method to detect and quantify different adulterating substances in honey.

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Conflict of interest: The authors declare that they have no conflict of interest.

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Figure Captions

Fig. 1. IMSS obtained from the HS-GC-IMS analysis used for the discrimination between pure honey and pure adulterants.

Fig. 2. 3D surface plots for the graphical representation of the influence of volume of injection-sample weight.

Fig. 3. Dendrogram obtained from HCA of average pure honey and adulterated honey samples (n = 72). (PH: Pure honey samples, H-IS: honey adulterated with IS, H-RS: honey adulterated with RS, H-BS: honey adulterated with BS, H-FS: honey adulterated with FS, and H-HFCS: honey adulterated with HFCS).

Fig. 4. Characteristic fingerprint of each group obtained for four of the relevant drift times from LDA. (PH: Pure honey, H-IS: honey adulterated with IS, H-RS: honey adulterated with RS, H-BS: honey adulterated with BS, H-FS: honey adulterated with FS, and H-HFCS: honey adulterated with HFCS).

Fig. S1. Score plot obtained for the samples according to F1, F2 and F3 (n = 144).

Fig. S2. Dendrogram from HCA of the variables with the 22 relevant drift times in Fisher's discriminant function (n = 22).

Highlights

- Optimization study for the discrimination between pure honey and five adulterants.
- Application to adulterated samples with five different adulterants (5%-50%).
- Full discrimination among different adulterants has been achieved.
- Individual PLS models for a good prediction of the percentage of adulteration in honey.
- Ion Mobility Spectra with chemometrics can be used for honey quality control.

Figure 1

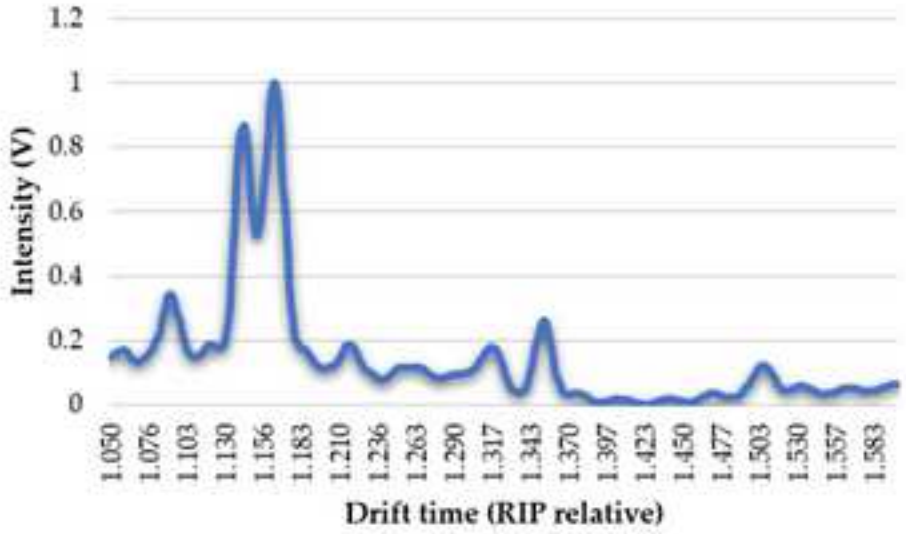

1.2
1
0.8
0.6
0.4
0.2
0
Intensity (V)
1.050
1.076
1.103
1.130
1.156
1.183
1.210
1.236
1.263
1.290
1.317
1.343
1.370
1.397
1.423
1.450
1.477
1.503
1.530
1.557
1.583
Drift time (RIP relative)

Figure 2

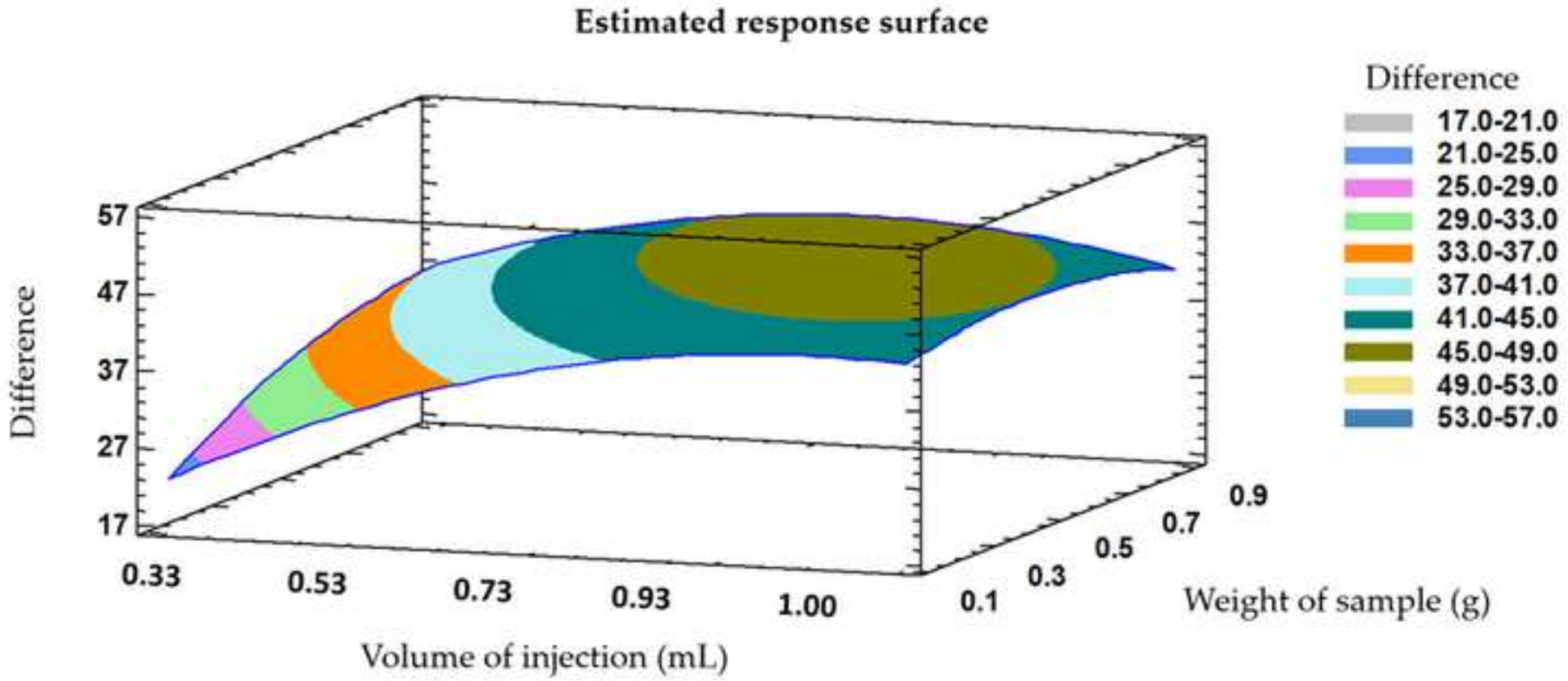

Estimated response surface
Difference
17.0-21.0
21.0-25.0
25.0-29.0
29.0-33.0
33.0-37.0
37.0-41.0
41.0-45.0
45.0-49.0
49.0-53.0
53.0-57.0
Difference
57
47
37
27
17
0.33
0.53
0.73
0.93
1.00
Volume of injection (mL)
0.1
0.3
0.5
0.7
0.9
Weight of sample (g)

Figure 3

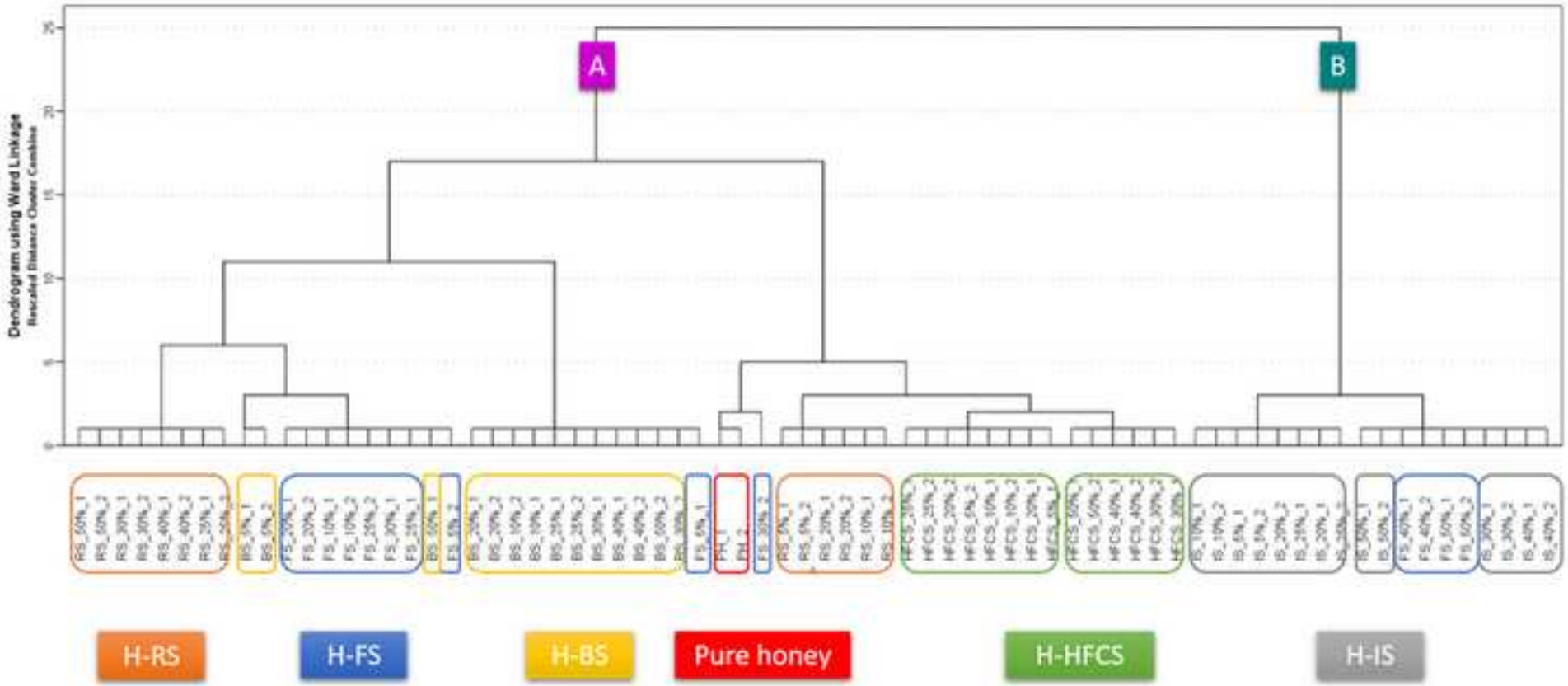

Dendrogram using Ward Linkage
Rescaled Distance Cluster Combine
A
B
H-RS
H-FS
H-BS
Pure honey
H-HFCS
H-IS

Figure 4

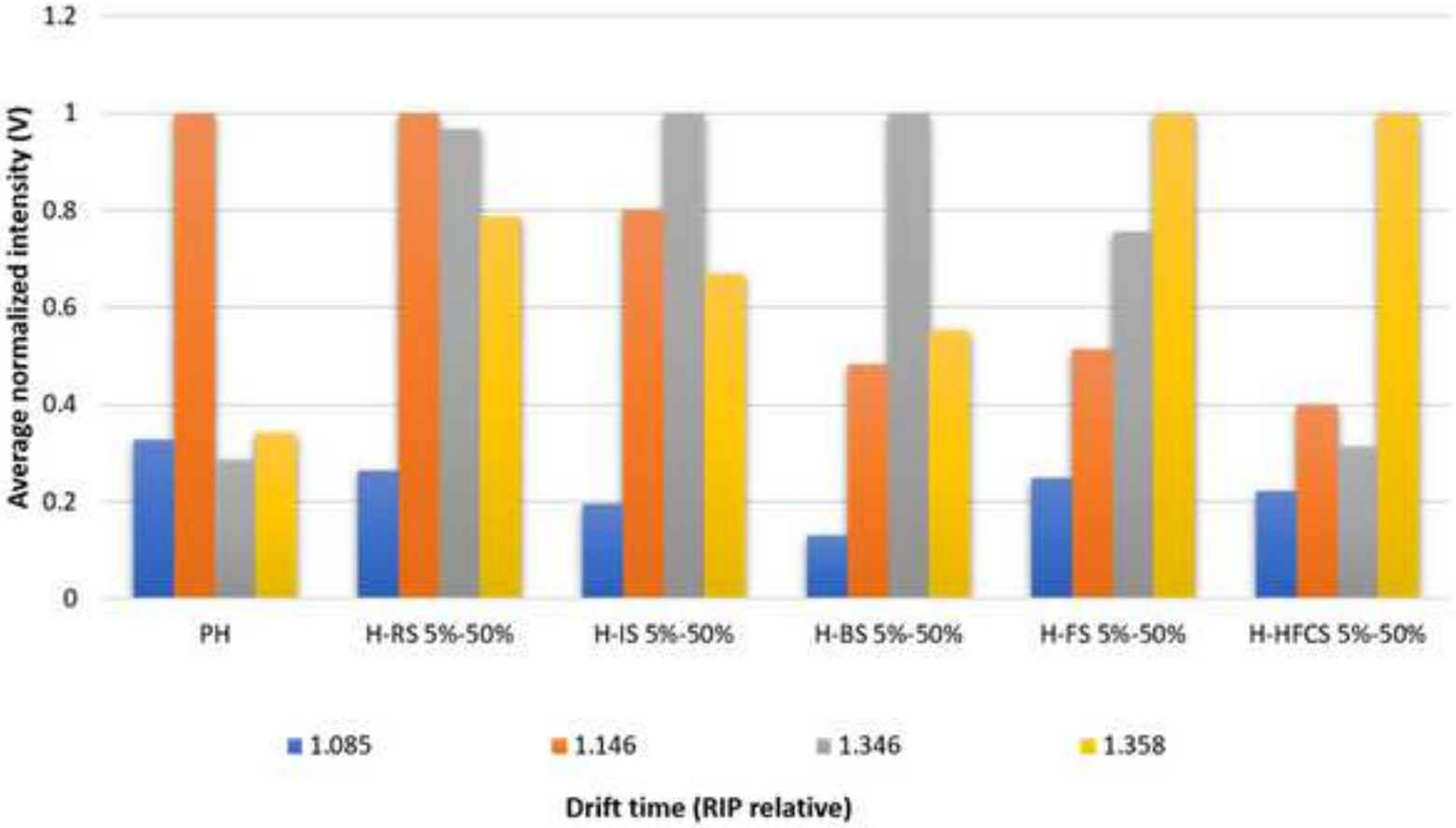

1.2
1
0.8
0.6
0.4
0.2
0
Average normalized intensity (V)
PH
H-RS 5%-50%
H-IS 5%-50%
H-BS 5%-50%
H-FS 5%-50%
H-HFCS 5%-50%
1.085
1.146
1.346
1.358
Drift time (RIP relative)

Table 1. Conditions GC-IMS analysis.

Variable	Value
EPC1 (Electronic Pressure Control of drift gas)	250 mL/min
EPC2 (Electronic Pressure Control of carried gas)	Ramp of 5 mL/min (t = 0 min), 10 mL/min (t = 5 min), 25 mL/min (t = 10 min)
Total time of analysis	15 minutes
T1 (System temperature)	45 °C
T2 (Column temperature)	+ 5 °C higher than HS temperature
T3 and T4 (Temperatures of the system)	80 °C

Table 2. Experimental variables and levels in the experimental design to develop the method to discriminate among pure honey and the five adulterants.

Variable	-1	0	1
Incubation time (min)	5	10	15
Incubation temperature (°C)	30	40	50
Injection volume (mL)	0.33	0.66	1.00
Weight of sample (g)	0.1	0.5	0.9
Pre-heating time (min)	5	15	25

Table 3

Table 3. ANOVA of the quadratic model adjusted to the discrimination of pure honey and pure adulterant samples.

Variable	Model	Coefficient	Sum of Squares	Degrees of freedom	Mean Square	F-value	*p*-Value
Incubation time	X_1	-2.830	16.926	1.000	16.926	1.890	0.182
Incubation temperature	X_2	0.294	201.012	1.000	201.012	22.420	0.000
Injection volume	X_3	103.234	762.286	1.000	762.286	85.020	0.000
Weight	X_4	66.921	238.504	1.000	238.504	26.600	0.000
Pre-heating time	X_5	-0.789	2.750	1.000	2.750	0.310	0.585
Incubation Time: Incubation Time	X_1^2	0.015	1.172	1.000	1.172	0.130	0.721
Incubation Time: Incubation Temperature	X_1X_2	0.064	40.722	1.000	40.722	4.540	0.043
Incubation Time: Injection Volume	X_1X_3	-0.585	3.847	1.000	3.847	0.430	0.518
Incubation Time: Weight	X_1X_4	0.322	1.654	1.000	1.654	0.180	0.671
Incubation Time: Pre-heating time	X_1X_5	0.028	7.778	1.000	7.778	0.870	0.361
Incubation Temperature: Incubation Temperature	X_2^2	-0.007	4.486	1.000	4.486	0.500	0.486
Incubation Temperature: Injection Volume	X_2X_3	-0.091	0.376	1.000	0.376	0.040	0.839
Incubation Temperature: Weight	X_2X_4	-0.510	16.675	1.000	16.675	1.860	0.185
Incubation Temperature: Pre-heating time	X_2X_5	0.021	17.352	1.000	17.352	1.940	0.176
Injection Volume: Injection Volume	X_3^2	-42.076	194.485	1.000	194.485	21.690	0.000
Injection Volume: Weight	X_3X_4	-24.087	41.676	1.000	41.676	4.650	0.041

Injection Volume: Pre-heating time	X_3X_5	-0.341	5.214	1.000	5.214	0.580	0.453
Weight: Weight	X_4^2	-18.072	72.968	1.000	72.968	8.140	0.009
Weight: Pre-heating time	X_4X_5	-0.398	10.153	1.000	10.153	1.130	0.297
Pre-heating time: Pre-heating time	X_5^2	0.005	2.011	1.000	2.011	0.220	0.640
Pure error			224.147	25.000	8.966		
Total			1878.250	45.000			

Table 4

Table 4. Mathematical models based on PLS for the prediction of level of adulteration.

Model	R^2	REC	REP	External error
GLOBAL	0.72	8.57	9.79	9.25
IS	0.98	2.24	2.54	4.58
RS	0.99	0.94	1.77	0.97
BS	0.97	3.01	3.67	2.01
FS	0.99	1.58	2.52	3.24
HFCS	0.97	2.94	3.56	1.87

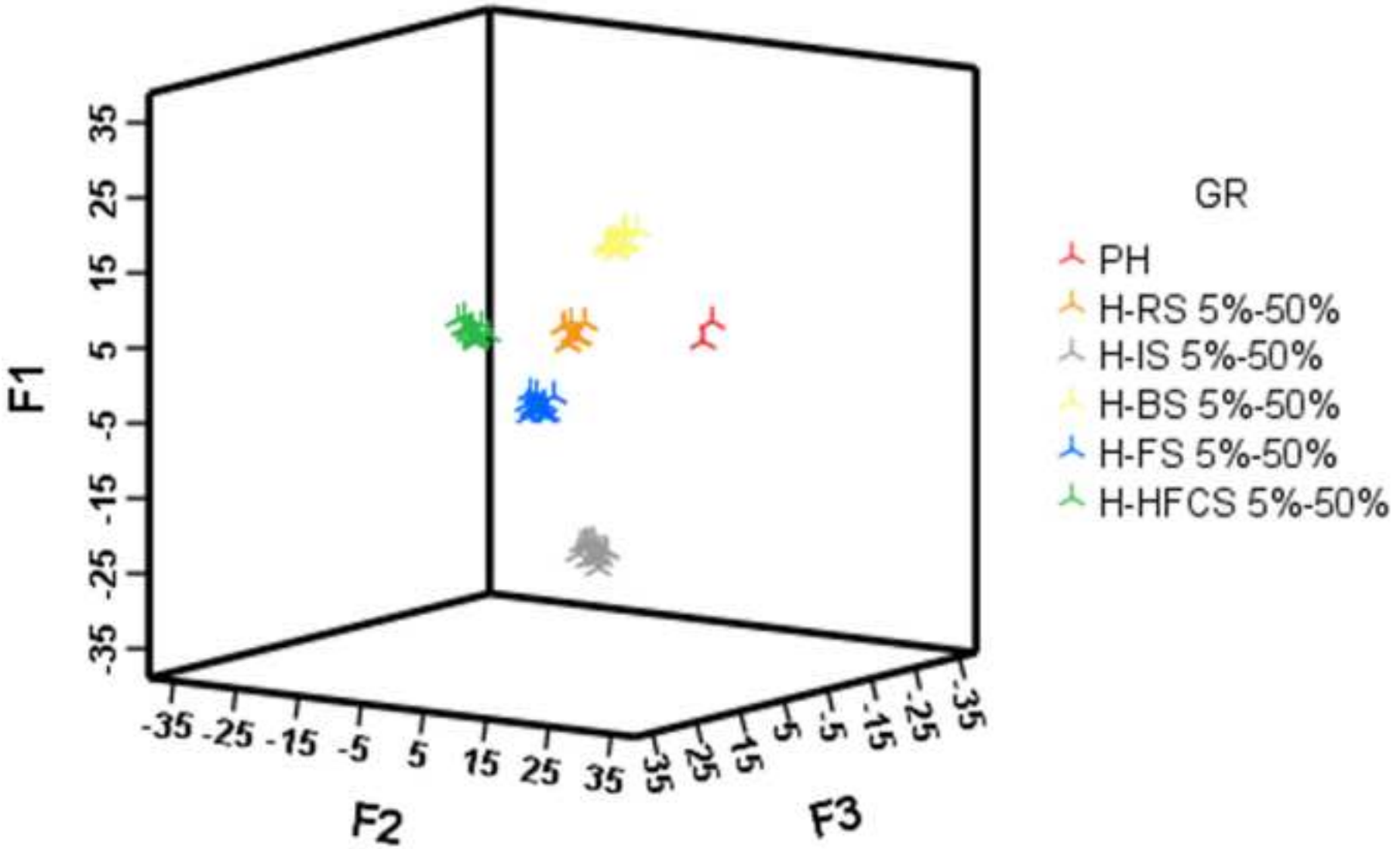
Table S1

Table S.1. BBD-RSM Design.

Exp.	Variables					
	Incubation Time (min)	Incubation Temperature (°C)	Injection Volume (mL)	Weight of sample (g)	Pre-heating time (min)	Response variable
1	-1	-1	0	0	0	47.100
2	0	1	0	1	0	44.889
3	0	-1	0	-1	0	30.103
4	0	0	0	0	0	44.350
5	0	-1	1	0	0	43.582
6	1	0	-1	0	0	32.837
7	0	1	0	0	-1	45.445
8	0	-1	0	0	1	37.406
9	0	0	1	-1	0	38.799
10	-1	0	0	0	-1	43.179
11	0	0	-1	1	0	39.889
12	0	0	0	1	-1	45.642
13	1	0	0	-1	0	42.205
14	0	1	-1	0	0	35.945
15	0	0	0	0	0	43.896
16	0	0	1	0	-1	47.456
17	0	0	0	1	1	45.357
18	-1	0	0	1	0	43.016
19	0	0	0	-1	1	41.935
20	0	0	0	0	0	44.350
21	-1	1	0	0	0	46.459
22	0	0	1	1	0	47.881
23	1	0	0	1	0	48.120
24	1	0	0	0	1	47.246
25	0	-1	0	1	0	38.478
26	-1	0	0	0	1	39.002
27	0	1	1	0	0	47.852

28	-1	0	1	0	0	46.527
29	0	1	0	0	1	48.878
30	0	0	-1	-1	0	17.660
31	0	0	-1	0	1	38.127
32	1	-1	0	0	0	37.257
33	0	0	-1	0	-1	33.176
34	-1	0	-1	0	0	28.362
35	-1	0	0	-1	0	39.674
36	1	0	1	0	0	47.067
37	0	-1	0	0	-1	42.304
38	0	0	0	0	0	44.350
39	1	0	0	0	-1	45.846
40	0	1	0	-1	0	44.680
41	0	0	0	0	0	44.350
42	0	0	0	0	0	44.350
43	0	0	0	-1	-1	35.848
44	0	-1	-1	0	0	30.515
45	0	0	1	0	1	47.786
46	1	1	0	0	0	49.378

Figure S1


F1
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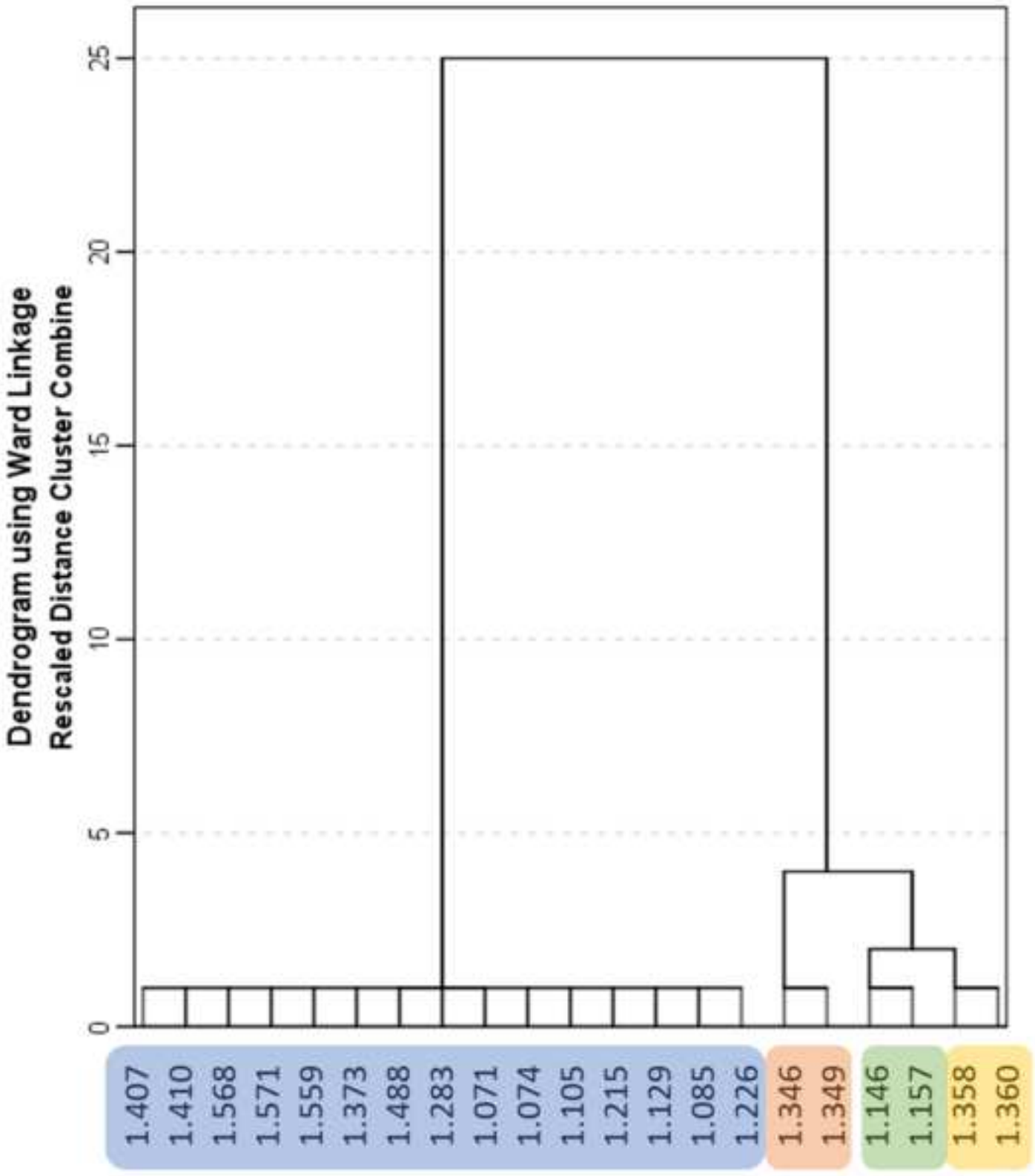
5
-5
-15
-25
-35
F2
-35 -25 -15 -5 5 15 25 35
F3
35 25 15 5 -5 -15 -25 -35
GR
PH
H-RS 5%-50%
H-IS 5%-50%
H-BS 5%-50%
H-FS 5%-50%
H-HFCS 5%-50%

Figure S2


Dendrogram using Ward Linkage
Rescaled Distance Cluster Combine
0
5
10
15
20
25
1.407
1.410
1.568
1.571
1.559
1.373
1.488
1.283
1.071
1.074
1.105
1.215
1.129
1.085
1.226
1.346
1.349
1.146
1.157
1.358
1.360

***Conflict of Interest Form**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Title: Novel method based on IMS Sum Spectrum for the detection and discrimination of adulterated honey"
Authors: Mª José Aliaño-González, Marta Ferreiro-González, Estrella Espada-Bellido, Gerardo F. Barbero and Miguel Palma
Journal receiving the manuscript for evaluation: Food Control

On behalf of all authors

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