Discrimination of honey of different floral origins by a combination of various chemical parameters

Technical Officer: Zora Jandrić

In the previous newsletter, we reported on research into methodology for the classification of honey of various floral and geographical origins using UPLC-QToF MS and MVA.

The research on honey authenticity was expanded using multivariate data analysis of data generated by a number of different analytical techniques to discriminate honeys of different floral origins.

The feasibility of a multivariate approach, including various chemical parameters and multivariate data analysis, for the discrimination of various honeys originating from one region, was explored.

Figure 1. Discrimination of honeys of different floral origins (see text for explanation).

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The chemical analyses used were elemental profiling, stable isotope analysis, metabolomics (UPLC-QToF MS), NIR, FT-IR, and Raman spectroscopy fingerprinting. Partial least squares discriminant analysis (PLS-DA) was used to determine which technique or combination of techniques provides the best classification and prediction ability.

Authentic honey samples with different floral origins (clover vs. manuka, >50%) were used to test this approach. Various chemical parameters (metabolites, stable isotope ratios of carbon ($\delta^{13}C$) and hydrogen ($\delta^{2}H$), concentrations of major, minor and trace elements, and spectroscopic data) were used to investigate possible clustering between honeys.

Variability in the metabolite fingerprint was the single best discriminator (classification success rate 77%) (Fig. 1A). Higher contents of secondary pollen had an effect on sample clustering (red circles, ~26% rewarewa; orange circles, ~30 % manuka; and black circles, ~30% clover). Segregation between the two sample groups was also obtained using the combined spectroscopic data (Fig. 1B) and combined trace elements/isotope ratio data (Fig. 1C) but with lower classification success of 44% and 36%, respectively.

The combination of all chemical parameters analysed did not significantly improve classification success (78%) (Fig. 1D).

The results demonstrate the potential of multivariate analysis for the differentiation and classification of honeys traded between countries. This approach could provide a potential tool to help Member States to protect their own authentic honey products and to detect adulterated or counterfeit/mislabelled honey products on the market.

The results of this research were presented as a poster at the second Food Integrity and Traceability Conference in Belfast, UK, 8–10 April 2014.

**Stable Isotopes Applied to Authenticating Honey**

Technical Officers: Russell Frew and Aiman Abraham

One of the earliest applications of nuclear techniques for food authenticity was the use of carbon isotopes to detect the addition of cheap sugars in honey in the 1970s. The principle is that the cheap sugar (fructose) is derived from corn and has a higher $\delta^{13}C/\delta^{12}C$ than the fructose from honey. This difference is due to the different photosynthetic pathways; corn is a C4 plant and that mechanism does not discriminate against the $\delta^{13}C$ as much as the C3 pathway used by most honey-producing plants. Consequently the two plant types have quite different carbon isotope ratios (see figure). Thus measurements of the carbon isotope ratios can distinguish between the two sources of fructose. However, within each plant population there is natural variability in isotope ratios. This makes detecting the addition of small amounts of corn syrup difficult. The test was further refined by its developers to use $^{13}C/^{12}C$ measurements on protein purified from the honey as an internal reference, based on the fact that if the sugar and protein are from the same plant then they should be closely related in isotopic ratio. This refined method was adopted by the Association of Analytical Chemists as an official method (AOAC 998.12) and is part of the Codex Alimentarius standard for testing authenticity of honey. This test is generally reliable. However, some honey, notably New Zealand manuka, has a frequent fail rate. Manuka is a premium honey valued for its non-peroxide antimicrobial activity (NPA). The NPA is thought to be due to high levels of methyl glyoxal (MGO) and it is the manuka honey with high levels of MGO that fail the C4 sugar adulteration test. Work by FEPL indicates that this is partly due to the beekeeping practice of feeding sugar to bees during the winter. However, that does not explain the late season failures, or that the extent of failure increases as manuka honey ages. The MGO levels in manuka increase with age and it has been shown that high MGO is correlated with high apparent C4 sugar content.

Current research in this field in FEPL is focused on modifying the AOAC method to overcome these false positives in the C4 sugar adulteration. A method has been developed for the removal of MGO prior to the purification of the protein that is measured as internal standard. It is hoped that the removal of the MGO will eliminate the interference in the isotope test. Tests are now underway to establish the optimum conditions for the removal of MGO and to show that the additional procedure does not affect the isotopic composition of the purified protein. Once those tasks are completed the work will move to the validation stage and involve other laboratories to test the procedure.

A consequence of MGO being responsible for the NPA that gives manuka honey its high value is that it provides motivation for unscrupulous operators to attempt to gain higher prices by doping honey with MGO. Work being conducted in parallel with the modification to the C4 sugar test aims to apply stable isotope measurements to see if different sources of MGO may be distinguished, hence providing a test for adulteration with MGO. So far, methods have been established for quantifying MGO in honey by GC and HPLC. We are awaiting the availability of GC-rIRMS facilities in FEPL to complete this work.
The use of analyte protectants in pesticide residue analytical work

Technical Officers: Britt Maestroni and Victoria Ochoa

A common challenge in gas chromatography (GC) is the quantification of certain important pesticides in cases where there are apparent analyte losses and/or peak tailing. For susceptible analytes, significant peak quality improvements can be obtained when matrix components are present because they fill active binding sites in the GC system, thus reducing analyte interactions with those binding sites. This phenomenon is called “matrix-induced chromatographic response enhancement” and has been a subject of many studies in the last decade.

The FEPL is currently carrying out a study on method validation for the detection of several pesticides in potato samples. The extraction and clean-up method used is known as the Quick, Easy, Cheap, Effective Rugged and Safe (QuEchERS) for pesticide residue determination, and uses a gas chromatograph coupled to a mass selective detector (GC-MSD) for analyte separation and detection. According to the SANCO document (SANCO/12571/2013), matrix effects should be assessed at the initial method validation stage. Therefore as part of the calibration strategies for our method both matrix-matched and solvent calibrators were prepared. It was noticed that in the case of potato samples matrix effects were present for several pesticides, as shown in Figure 1.

Figure 1: Matrix effects in potato samples.
Since the effective elimination of the sources of the matrix-induced response enhancement is usually not feasible in practice, some means of compensating for the effect is necessary. The current compensation approaches include the use of matrix-matched standards and standard addition methods. These techniques require extra labour and costs; moreover, they may still lead to quantitation inaccuracies because the extent of the effect depends on analyte concentration and matrix composition. Another way to compensate for the matrix effect problem is the use of analyte protectants (AP). Analyte protectants prevent/minimize pesticide interactions with the active sites in the GC system and protect pesticides from degradative interactions.

The matrix-matched calibration involved the preparation of calibration standards in blank extracts in an attempt to provide the same amount of matrix-induced enhancement as in the sample extracts. This procedure works reasonably well. However, it does have some disadvantages: the availability of “blank” matrix in a reasonable amount, extra time, labour, and expense for preparing the blank extracts for calibration standards; a greater amount of matrix material injected onto the column which leads to greater GC maintenance; and greater potential for analyte degradation in the matrix solution. In addition, while matrix matched calibration is easy for validation studies, it is difficult to implement in routine testing since there may be differences in the matrix between individual samples, or various different matrices may be included in an analytical sequence.

According to the literature, the best AP coverage along the whole run time was given by a combination of ethylglycol, gulonlactone, and sorbitol. In our experiments we adopted the same composition, and to avoid the use of water, we prepared the AP mixture in ethylacetate: DMSO (80:20). One set of samples was spiked at 0.01 mg/kg and we assessed the validity of using APs at a concentration of 0.48 mg/mL (gulonlactone and sorbitol) and 4.8 mg/mL (ethylglycol). Different calibration strategies were employed: calibration in solvent, calibration in solvent with APs added, matrix matched calibration and matrix matched calibration with APs. All calibration curves had correlation coefficients higher than 0.999. The samples were also injected with and without analyte protectants. The results obtained for some of the pesticides can be found in Figures 2 and 3.

![Figure 2: Recovery values of pesticides from potato samples spiked at 0.01 mg/kg and injected with Analyte Protectants (AP), calibrated using matrix matched (MM), matrix matched with AP, solvent, and solvent with AP.](image1)

![Figure 3: Recovery values of pesticides from potato samples spiked at 0.01 mg/kg and injected without AP, calibrated using matrix matched (MM), matrix matched with AP, solvent, and solvent with AP.](image2)
As can be seen from Figure 2, the quantification of residues using calibrators prepared in solvent is not recommended for chlorpyrifos methyl, parathion methyl, vinclozolin, pirimiphos methyl, chlorpyrifos and the permethryns. In our experiments, and for the selected compounds shown in the figures, the addition of analyte protectants to solvent calibrators did not improve the results; high recovery values were obtained. The best calibration was observed using matrix matched strategies either with or without APs. This is also the case for chloropyrifos which, in previous experiments, did not show any matrix effects. When the samples were injected without the addition of AP acceptable recoveries for all of the compounds discussed above were obtained by matrix matched calibration.

Practical aspects of using analyte protectants should also be mentioned. In our experience the injector port and the source became “dirty” very quickly, after only 18–20 injections (see photo 1) in contrast to reports in the literature of at least 1000 injections without the need for ion source cleaning.

![Photo 1: Liners became dirty very quickly in the injection port.](image)

However, it is recognized that this is the first experience of the use of analyte protectants in FEPL and further studies are needed to provide better information on their usefulness and applicability. For example, the use of DMSO as dissolution agent for the APs needs to be thoroughly evaluated, as well as the effect of AP concentration on our chromatographic system. Further experiments are needed to better assess the applicability of analyte protectants in the routine analysis of fresh fruits and vegetables for pesticide residue analysis.

**FEP Laboratory Staff**

During the first half of 2014, FEPL said goodbye to one intern and hello to another.

In January, Ms Laura Natalia Fernandez Cedi completed a 4-month period as an intern in the laboratory, having accepted a position as a consultant in UNIDO. During her time in FEPL, Natalia worked with staff in a number of areas, principally on methodology for the authentication of foods using metabolomics. She also provided support in activities related to the RALACA laboratory network and the set-up of its web site. We wish Natalia all the best in her new position.

In April, we welcomed Ms Victoria Ochoa, who commenced an internship in the laboratory. Victoria is currently working with FEPL colleagues on the development and validation of a multi-residue method for pesticide residues in potato, and also using her native Spanish language skills in the translation and preparation of technical documents.