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Journal of Chromatography A, 706 (1995) 353–359

JOURNAL OF CHROMATOGRAPHY A

Contribution of high-performance liquid chromatographic analysis of carbohydrates to authenticity testing of honey

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Abstract

A high-performance anion-exchange liquid chromatography method with pulsed amperometric detection was developed to investigate the oligosaccharide composition of honey. Quantification was achieved by reference to a maltotetraose internal standard. The oligosaccharide profiles of 91 authentic UK honey samples were obtained and multivariate statistical techniques employed to investigate whether these profiles could be used as a basis for identification of flower source. Canonical Discriminant Analysis proved most successful. Results indicated that honey oligosaccharide profiles have a potentially valuable role to play in the assessment of floral origin of honey though it is unlikely that this procedure alone will allow unambiguous determination of all floral types.

1. Introduction

In the UK the regulatory provisions regarding the composition and labelling of honey are contained in the Honey Regulations 1976 (1976/1832) and the Food Labelling Regulations 1980 Amendment (1980/1849). Honey Regulations 1976 (1976/1832) implement Council Directive 74/409/EEC which sets honey composition criteria, defining a number of parameters including minimum reducing sugar content and maximum moisture, sucrose and water-insoluble solids content. The UK regulatory provisions also account for misrepresentative labelling of honey origin and state: (i) If a type of blossom or plant is indicated, the honey must be wholly or mainly from that source; (ii) If a name of a country, etc. is indicated, the honey must originate wholly from that place.

Hence, the blending of honeys from different floral or geographical origins is permitted in the UK, so long as the labelling does not claim that the honey is from a single source. Since single flower honeys or honeys from a specific geographical origin command premium prices there may be an economic incentive to misrepresent the source of a honey.

The detection of misrepresentative honey labelling has been carried out in the past using melissopalynology [1–4] (pollen analysis), but this technique requires a high level of expertise and is very time consuming. Other techniques have been applied. Determination of geographical origin has been attempted on the basis of free amino acid content [5–8] and chemical composition [9,10], with varying degrees of success. Determination of floral origin has received less attention, although identification on the basis of

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SSDI 0021-9673(94)01074-9

chemical composition [11,12] has shown potential.

In 1988, Swallow and Low [13] published a paper on the analysis and quantification of the carbohydrates in honey. This method employed high-performance anion-exchange liquid chromatography (HPAE) with pulsed amperometric detection (PAD) [14–19]. It was observed that oligosaccharide profiles appeared to be characteristic of floral source. Hence, they could potentially act as "fingerprints" for floral origin identification. This paper details the development of a new method, based on that employed by Swallow and Low, to determine the floral authenticity of honey.

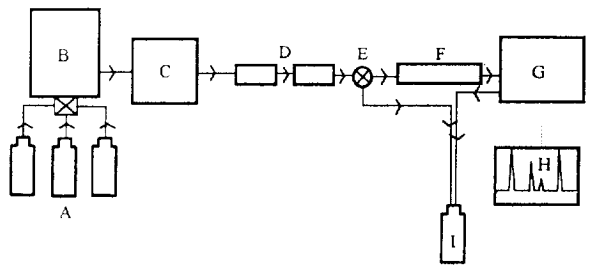

Fig. 1. HPAE–PAD schematic layout. The arrows represent the mobile phase flow path, (A) solvents leading to proportioning valve, (B) Waters 625 LC system and pump, (C) Gilson 231 XL autosampler fitted with a 20-μl injection loop, (D) two Dionex PA1 guard columns (50 × 4.6 mm), (E) Universal valve switching module fitted with a Tefzel rotor seal, (F) Dionex PA100 column (250 × 4.6 mm), (G) Waters 464 electrochemical detector, (H) Waters Millennium 2010 chromatography manager, (I) waste.

2. Experimental

2.1. Instrumentation

The HPAE apparatus comprised of a Waters 625 LC system (Milford, MA, USA), a Gilson 231 XL autosampler (Middleton, WI, USA) fitted with a 20-μl injection loop and a Universal Valve Switching Module (UVSM) fitted with a Tefzel (base resistant) rotor seal which was supplied by Anachem (Luton, Bedfordshire, UK). Separation of the oligosaccharides took place on two Dionex PA1 guard columns (50 × 4.6 mm) and a Dionex PA100 column (250 × 4.6 mm) (Sunnyvale, CA, USA). Detection was carried out by a Waters 464 electrochemical detector, operated in pulse mode, employing a stainless steel cell, a single gold working electrode, and a base resistant reference electrode. The data collection system was a Waters Millennium 2010 Chromatography Manager. Fig. 1 shows a schematic diagram of the apparatus layout.

2.2. Chemicals

Sodium hydroxide solution (46–48%), analytical reagent grade, was purchased from Fisons Scientific Equipment (Loughborough, Leicestershire, UK). Sodium acetate 3-hydrate, HPLC grade, was purchased from Merck (Lutterworth, Leicestershire, UK). Maltotetraose, HPLC grade, was from Sigma (Poole, Dorset, UK). Anion-exchange resin, AG 1-X8 100–200 mesh formate form, was from Bio-Rad (Richmond, CA, USA). Water was purified using a Milli-Q water purification system (Millipore, Milford, MA, USA). 0.22-μm filters were from Millipore.

2.3. Samples

Samples were obtained from various hive sites in the UK during 1992. Standard pollen analysis techniques were employed to determine the floral pollen percentages present in each honey sample. 600 Pollen grains per sample were analysed to obtain this data. The conversion from pollen percentages to floral nectar percentages was achieved by the use of pollen coefficients which take into account the over or under representation of pollen from a particular nectar source in the honey. The data referring to the floral nectar percentages was used to determine the description of the honey sample in terms of floral origin and purity. Unifloral samples were classified as those where one flower type constituted more than 50% of the nectar source.

The variety of unifloral samples was limited by the collection scheme. Six distinct unifloral types were provided by 91 samples. These were: bramble (70 samples), ling [heather] (5 samples), oil

seed rape (8 samples), white clover (5 samples), hawthorn (2 samples), willow-herb (1 sample).

2.4. Method

Sample preparation

Amounts of 0.80 (±0.15) g of each honey sample were dissolved in water. Then 400 μl of a 20 mg/ml aqueous maltotetraose (internal standard) solution was added before making up to 20 ml in a volumetric flask with water. The sample solution was cleaned-up by passing it through 8 ml of anion-exchange resin loaded as a water slurry into a disposable 10-ml plastic syringe plugged with glass wool. The sample solution was not collected until a viscosity change was observed as droplets from the syringe entered a container. Once this change had been observed ≈3 ml were allowed to pass through the resin into a measuring cylinder prior to the collection of the remaining eluent. Each solution was filtered (0.22 μm) before chromatographic analysis.

Enzymes in the honey sample solutions were shown to reduce the concentration of the added maltotetraose internal standard over a period of time. By keeping the time between the addition of internal standard to the honey sample solutions and the clean-up stage to below 15 min any enzyme effect was made negligible.

Preparation of eluents

Three solutions were employed in a gradient elution program, 0.1 *M* sodium hydroxide, 0.1 *M* sodium hydroxide–0.1 *M* sodium acetate 3-hydrate and 0.3 *M* sodium hydroxide. Sodium hydroxide solutions were prepared by diluting known weights of 46–48% sodium hydroxide. All weights were accurate to within 0.01 g. 46–48% sodium hydroxide solution is used for mobile phase preparation because it is low in sodium carbonate (<0.1%) which can cause variations in chromatographic retention times by competing with the carbohydrates for stationary phase sites. Mobile phase solutions were prepared as quickly as possible to minimise any absorption of carbon dioxide from the atmosphere and sparged for at least 20 min with helium (100 ml/min) before use.

Chromatographic conditions

The injection volume of sample solution was 20 μl. The composition and flow-rate of the mobile phase throughout each HPAE sample analysis is detailed in Table 1. For the first 3 min after sample injection the eluent flow passes through the guard columns and is diverted to waste by the switching valve. For the remainder of the run the eluent passes from the switching valve to the PA100 column. The two PA1 guards provided sufficient retention of the oligosaccha-

Table 1
HPAE gradient system for the separation of honey oligosaccharides

Line	Time after injection (min)	Flow (ml min)	%A 0.1 *M* sodium hydroxide	%B 0.1 *M* sodium hydroxide and 0.1 *M* sodium acetate	%C 0.3 *M* sodium hydroxide
1	0.0	0.75	100	0	0
2	10.0	0.75	100	0	0
3	45.0	0.75	0	100	0
4	46.0	0.75	0	100	0
5	47.5	1.50	0	0	100
6	55.5	1.50	0	0	100
7	57.0	1.50	100	0	0
8	65.0	1.50	100	0	0
9	70.0	0.75	100	0	0
10	75.0	0.75	100	0	0

Mobile phase composition and flow-rate changes between adjacent lines were linear with respect to time.

Table 2
Pulsed amperometric detection program

Pulse potential (mV)	Duration (s)	Comments
E^a	0.2	Measurement pulse
$E + 650$	0.2	Cleaning pulse
$E - 650$	0.5	Conditioning pulse

[a] The definition of E is given in the text.

rides such that >95% of the high concentration monosaccharides (glucose and fructose) could be sent to waste whilst the oligosaccharides were retained. The remaining monosaccharides and oligosaccharides could then be separated on the PA100 column without any overloading. The column switching technique also reduced any column and detector deterioration. All oligosaccharides are eluted within 45 min. Column washing and re-equilibration extends the run time to 75 min.

Pulsed amperometric detection

The electrochemical detector was operated in pulsed mode, using the three pulse repeated cycle given in Table 2. E, the optimum measurement potential [20], was determined by use of a cyclic voltammogram (CV). When running a CV the pulsed electrochemical detector was operated in the scan mode and the current and electrode potential responses were monitored whilst eluent (0.1 *M* sodium hydroxide, 0.75 ml/min) was passed through the electrode cell. The electrode potential was cyclically varied from −700 mV to 700 mV and back to −700 mV at a rate of 10 mV/s. As the potential became more negative a peak in the current response was observed, corresponding to the reduction of gold oxide formed on the electrode. The potential corresponding to this peak was E.

Data analysis

Up to 40 characteristic oligosaccharide peaks (see Fig. 2) were identified in each of the honey samples according to retention time. The peak heights of these oligosaccharides were measured for each sample and ratioed to the peak height of the internal standard, maltotetraose, eluted at approximately 45 min. Peak height measurement was performed by the Millennium 2010 Chromatography Manager. SPSS 5.0 for Windows (SPSS, Chicago, IL, USA) was used to perform multivariate statistical analysis on the peak height ratios.

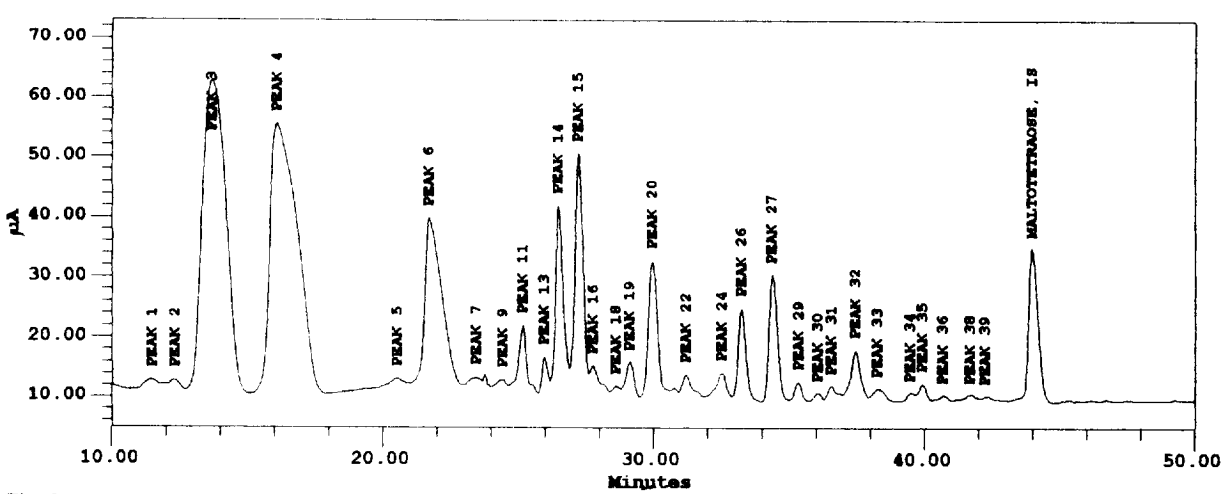

Fig. 2. A typical honey chromatogram with a number of labelled peaks. Standard compounds were not available for many of the oligosaccharides of interest so all peaks were quantified against a maltotetraose internal standard. Maltotetraose elutes in a clear area of the chromatogram at approximately 45 min.

3. Results

All 40 measured oligosaccharide peak height ratios for the honey samples were employed in the multivariate statistical analyses. Hierarchical Cluster Analysis (HCA), Principal Component Analysis (PCA) and Canonical Discriminant Analysis (CDA) were all evaluated for their ability to identify unifloral honeys. The success of the statistical analyses was judged against the floral classification by melissopalynology. PCA and HCA both identified an outlier bramble sample and this was removed from the data set. The use of CDA proved most valuable in differentiating the sample groups.

Canonical Discriminant Analysis (CDA) was used to find functions based on the peak height ratios which maximised separation between honey types. Fig. 3 shows how the analysis has split the six unifloral groups. The ling samples have been completely isolated from the remaining samples using CDA. Similarly the oil seed rape samples show a good separation from the rest of the honey samples. Whilst hawthorn and willow samples appear separated from the other samples, the number of determinations for these samples would have to be increased before any statistical conclusions are drawn. It appears that the white clover samples cannot be separated from bramble.

In CDA, sample classification into floral group can be performed by the software by application of Fisher's classification coefficients [21]. In order to test the discrimination functions, the analysis was repeated omitting one of the ling/oil seed rape/bramble/white clover samples at a time, then classifying this "unknown" with respect to floral origin. 100% of the ling samples were classified correctly. 62.5% of the oil seed rape samples were classified correctly. 70% of the bramble samples were classified correctly. None of the white clover samples were classified correctly.

The white clover samples were all misclassified as bramble. The three misclassified oil seed rape samples were all classified as bramble. In two cases (samples A and B, see Fig. 3) this can be explained by the quantity of bramble in the sample (sample A was 21% bramble; sample B was 25% bramble) and it can be concluded that a

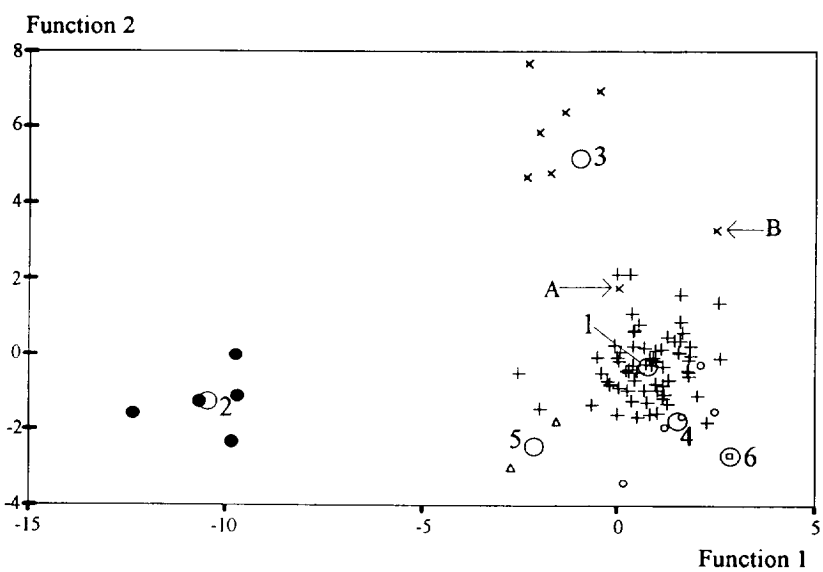

Fig. 3. Separation of the six unifloral honey groups using Canonical Discriminant Analysis. (Numbered ○) Group centroids; (+) Group 1: Bramble, 69 samples; (●) Group 2: Ling, 5 samples; (x) Group 3: Oil seed rape, 8 samples; (○) Group 4: White clover, 5 samples; (△) Group 5: Hawthorn, 2 samples; (□) Group 6: Willow-herb, 1 sample. Samples A and B are referred to in the text.

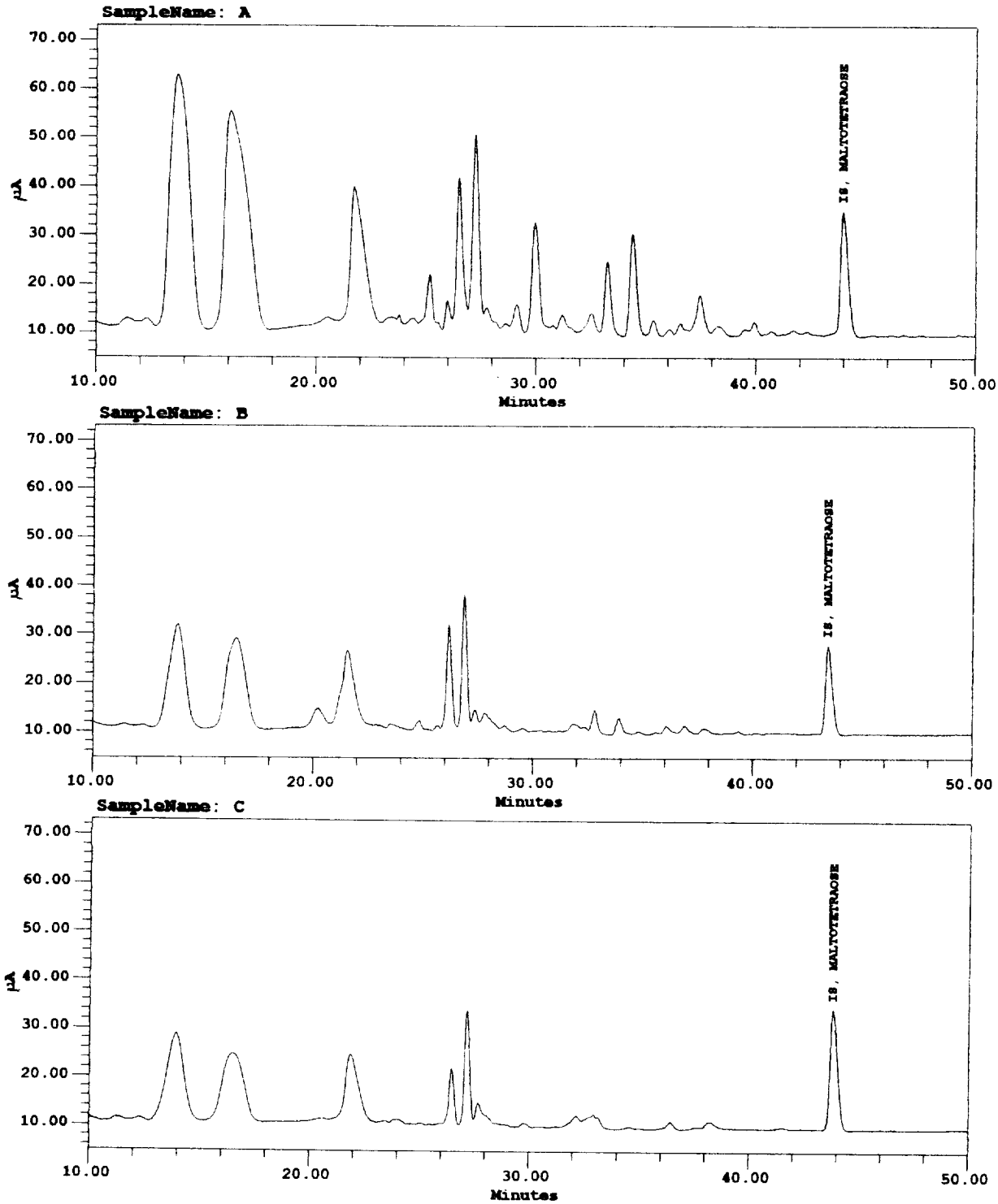

Fig. 4. Oligosaccharide profiles of three typical unifloral honeys: (A) bramble, (B) ling and (C) oil seed rape.

small bramble component has a significant effect on the oligosaccharide profile. For the third sample the reasons for the misclassification are unclear, but may be due to the small data set. The successful classification of five oil seed rape honeys demonstrates that there is a definite region within which a honey can be correctly classified as oil seed rape.

The 100% success rate in classifying the ling samples demonstrates the ability of the derived discriminant functions in differentiating between ling and other floral honeys. Fig. 4 consists of three chromatograms illustrating a typical bramble, a typical ling and a typical oil seed rape sample. These chromatographic patterns are highly repeatable for the ling and rape samples although more variable for the bramble.

4. Conclusions

The high classification rate for ling, bramble and rape samples shows that oligosaccharide profiles are a potentially valuable tool in floral identification. Analysis is quicker than melissopalynology (which can account for at least a day's work in the laboratory per sample) and requires less expertise. It may also be anticipated that the method will be unaffected by filtration during honey processing which can result in the loss of pollen. The misclassification of clover, however, shows the method is not applicable to all floral types.

While the floral species identification is limited at present, a more comprehensive database of unifloral samples may improve the statistical separations seen so far. A possible extension of the technique could be its application to the determination of geographical origin, which is difficult to ascertain by melissopalynology since some floral species which were once native to particular countries are now widespread throughout the world.

Acknowledgements

The authors thank Peter Roberts and the University of Aberystwyth for collecting the honey samples and performing the melissopalynology.

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