HARMONISED METHODS OF THE INTERNATIONAL HONEY COMMISSION

IHC responsible for the methods:
Stefan Bogdanov
Swiss Bee Research Centre
FAM, Liebefeld, CH-3003 Bern, Switzerland

send all feed-back to: stefan.bogdanov@fam.admin.ch

INTRODUCTION AND GENERAL COMMENTS ON THE METHODS

Many of the present official honey analysis methods and thus also the regulatory norms based on them, are outdated and need revision (1). A Commission was formed in 1990 to carry out this task. Stefan Bogdanov, Switzerland, is ex-chairman the IHC and responsible for compiling the methods, Werner der Ohe, Germany is present chairman, and Peter Martin, UK is secretary.

The present selection of methods has been made to include all those which at the present state of knowledge are sufficient for the determination of honey quality. It includes some old methods, which are still widely used in routine analysis, as well as some more modern ones. All the methods compiled in this publication have been published previously and with one exception (specific rotation) have been subjected to collaborative study. Most of them have also been through the standardisation procedure of the German Institute for Norms (DIN).

The methods were originally published in 1997 in "Apidologie" (2). As the methods are currently improved, changes to these methods have been made since then, which, however, should improve the methods. Thus, it is the aim of this Internet publication to make the latest version of the IHC methods available to as many people and countries as possible. Everybody concerned is invited to give feed-backs, so that the methods can be improved in the future.

The methods can freely by reached also by other Homepages by making a link to the Homepage of the IHC. No changes can be made without the approval of the IHC.

Additions and remarks to the methods, made after the Apidologie publication:

Determination of humidity by digital refractometry (Method 1) and determination of invertase (Method 10): decided at the Dijon meeting in October 1999.
Determination of HMF by HPLC (Method 5.1): change of the sample preparation.

Collaborative trials and Precision of the methods

The precision data for the methods have been compiled from 3 sources:

a) the original DIN methods, which use the ISO 5725 standard for collaborative studies (3,4)

b) from an U.K. collaborative study (5) according to the ISO norm

c) from the collaborative tests carried out by the International Honey Commission. These have been interpreted by the more modern robust method of statistics(6-8).

The ISO method has the drawback that outlier laboratories are eliminated from the computation of the precision parameters and also has other drawbacks (6). The robust method, introduced recently, does not have the drawbacks of the ISO methods and takes into account the results of all laboratories taking part in the trial. It is gaining increasing acceptance. In the new edition of the guide for conducting collaborative studies (reference 4, currently in revision) the ISO protocols and the robust method are going to be combined.

In the precision results three parameters are given: the average or the range of the determined parameter and the two precision parameters, repeatability r and reproducibility R, as these are the crucial parameters for evaluation of the precision of a method.

Repeatability r

The difference between the results of two determinations, obtained in rapid succession by the same method on identical test material under the same conditions (same operator, same apparatus, same laboratory) shall not exceed the values given in the precision tables

Reproducibility R

The difference between the results of two independent determinations, obtained by the same method on identical test material under different conditions (different operator, different apparatus, different laboratory) shall not exceed the values given in the precision tables.

Both measurements are valid with a probability of 95 %. This means that on average in carrying out 20 determinations, 1 outlier may be expected.

The interlaboratory variation, determined as the coefficient of variation of R or RSD_R % is the quality parameter most often used to compare the precision of analytical methods (8). RSD_R % is calculated as: 100 R/$\bar{x}$ 2.8

Generally RSD % decreases exponentially with increasing concentration of the measured variable. In the methods for analysis of major foodstuff components, which lie between 0.01 g/100 g and 10 g/100 g, the coefficient of variation will mostly lie between 0.1 and 10 % (9). The greater the RSD_R % value, the poorer the reproducibility of the method. In order to compare the performance of the methods we have summarised the extreme RSD_R % values for each method from the collaborative studies (table 1).

Method, parameter	RSD_R % MIN-MAX
1. Moisture, refractometry	1.0 - 2.5
2. Electrical conductivity	3.4 - 4.4
3. Ash content	4.3 - 13.2
4. Free acidity by titration to pH 8.3 (2)	10.3-22.0
5. Free acidity (2) with equivalence point titration	8.7-46.8
6. Hydroxymethylfurfural with HPLC	6.1-10.9
7. Hydroxymethylfurfural after White	3.7-22.0
8. Hydroxymethylfurfural after Winkler	7.9-15.2
9. Diastase activity after Schade	20.5-26.1
10. Diastase activity with Phadebas	11.0-17.9
11. Apparent reducing sugars	no trials
apparent sucrose	
12. Sugars by HPLC	
fructose	1.5-1.9
glucose	1.6-3.2
sucrose	11.4
13. Sugars by GC	
fructose	3.9-8.6
glucose	2.6-7.5
sucrose	7.2
14. Sugars by HPLC with pulsed amperometric detection	
fructose	6.0-7.4
glucose	7.3-7.8
sucrose	6.8-12.5
15. Insoluble matter	26.5-84.4
16. Invertase activity	2.7-9.6
17. Proline	2.3-3.4
18. Specific rotation	no trials

TABLE 1: Harmonised honey analysis methods: comparison of precision

The reproducibility variation coefficient of each method was calculated from collaborative trials of the International Honey Commission (methods 1, 4,5, 6,7,8,9,10,13,14,16) DIN (2,3,12,17) and the Association of Public Analysts (11,15).

Methods and Composition Criteria

The selection of methods for use in routine honey control has been made to include all those which at the present state of knowledge are sufficient for the determination of honey quality. It includes some old methods which are still widely used in routine analysis, as well as some more modern

ones. Analyses for the detection of added syrups, such as the stable carbon isotope ratio, are not included. Most of the methods can be used to determine the quality criteria for honey specified in the European or in the Codex Alimentarius standard. We have proposed amendments to the EU legislation and to the Codex Alimentarius standard (2) for some of these compositional criteria, such as reducing sugars, apparent sucrose, ash content and acidity. Some other criteria, such as individual sugar analysis, electrical conductivity and invertase, which are based on modern methods, have been used increasingly, especially in the more industrialised countries. Thus a more precise characterisation of the honey is achieved. For these criteria we have proposed standards, based on data from long term routine honey control. We have included also a few methods (invertase, proline, specific rotation), which are used in some countries and which could prove to be useful in future honey quality evaluation.

Moisture

Honey moisture is the quality criterion that determines the capability of honey to remain stable and to resist spoilage by yeast fermentation: the higher the moisture, the higher the probability that honey will ferment upon storage. The determination of moisture by refractometry (10) does not yield the true water content and yields lower values than the Carl Fischer method (see 1.1) However, it is a very simple and reproducible method, successfully used up to the present time and thus there is no need for alternative methods. The RSD_R values varied from 0.8 to 2% over the whole determination range. The harmonised method is based on a measurement with an Abbe refractometer. During the past decade digital refractometers have replaced the Abbe in routine measurements of syrups and jams. Recent work has been done to examine the possible use of digital refractometers. The first trials with these instruments are promising, but more routine use will show, if they can successfully serve as an alternative to the Abbe refractometer.

Lower moisture limits (e.g. 19%), ensuring a better shelf-life of honey which would be met by a large majority of the commercial honeys, have been proposed by some countries for the revision of the Codex Alimentarius.

Electrical conductivity

This measurement depends on the ash and acid contents of honey: the higher their content , the higher the resulting conductivity (11). It is a very easy and quick method, needing only inexpensive instrumentation. The RSD_R values varied from 3 to 4% over the whole determination range and have been found lower than the corresponding values of the ash determination method.

The conductivity is a good criterion of the botanical origin of honey and thus is very often used in routine honey control. A lower limit has been proposed for blossom than for honeydew honeys (1). Exceptions have to be made for some blossom honeys, e.g., Tilia, Erica, Calluna, Arbutus, Gossipium, Lavender, Eucalyptus, in which the conductivity shows considerable natural variation (1).

Ash content

This method will probably be replaced by the faster and easier conductivity measurement (see above). The ash content is a quality criterion for honey origin, the blossom honeys having a lower ash content than the honeydew ones. The RSD_R values of one ring trial varied from 4 to 11% over the whole determination range.

pH and acidity

Two methods have been proposed. Titration of the acidity has the major drawback that the endpoint of the titration is not well defined because of lactone hydrolysis, which leads to a constant drift in the endpoint. Theoretically the equivalence point titration is the correct method for determination of honey acidity, as the equivalence point of the titration is fixed for each honey. While the endpoint titration method has been officially used in most countries (14), in France the equivalence point titration with automatic titrators is used (12). For both methods the RSD_R values were very high: 11 to 22 % for the endpoint titration and 8.7-46.8 % for the equivalence point titration. Thus the reproducibility of these methods is very poor and unsatisfactory in both cases. This very high interlaboratory variation throws some doubt on the usefulness of this measurement

to determine the quality of honey. The International Honey Commission has proposed 50 milliequivalents as the maximum permitted acidity in honey. However one should bear in mind the poor precision of the method when interpreting acidity results close to the limit.

Hydroxymethylfurfural (HMF)

Three methods can be used for the determination of HMF (10, 13,14). These methods were tested collaboratively by the International Honey Commission with 3 honey samples to cover the main range of determination (1). The results are summarised in the table below (all values in mg/kg).

	Bisulfite White			HPLC			Winkler		
sample	X	r	R	x	r	R	x	r	R
1	3.8	0.9	2.3	5.2	0.4	1.6	7.5	1.1	3.2
2	22.3	1.2	3.9	22.8	1.2	4.9	22.5	1.7	7.6
3	42.1	2.2	4.4	42.3	2.1	7.3	42.9	2.5	9.5

There were only small differences between the methods and only at very low levels, of no interest for assessing honey quality. In the higher range all three methods yielded comparable results. The repeatability r and the reproducibility R of the White and the HPLC method were better than those of the Winkler method. With the exception of the measurements at the lowest concentration range with RSD_R values above 10 %, the interlaboratory precision of all methods is acceptable.

Note: Because p-toluidine may be carcinogenic, the Winkler method should not be used if one of the other methods are available.

The Codex Alimentarius limit is 80 mg/kg, while the EU limit is 40 mg/kg. The discrepancy is unresolved at the present time.

Diastase

Two different methods are used to determine honey diastase. The traditional Schade method uses starch as a substrate and determines the diastase activity expressed in Schade units. The Phadebas method on the other hand uses an artificial substrate. There is a very good correlation between the diastase activity expressed in Schade units and the absorbance measured with the Phadebas test (see Phadebas method) so that by means of a factor one can calculate the diastase activity in Schade units. The RSD_R values for both methods are above 10 %, but this can be explained by the lower precision of enzymatic methods which measure much lower quantities than chemical methods. The precision of the Phadebas method, as expressed by the RSD_R value, was almost twice as good as the Schade method. A possible explanation might be that the Phadebas method uses a defined substrate, whereas the commercially available starch varies considerably in its quality.

In the last drafts of the Codex Alimentarius a limit of “at least “ 8 diastase units is proposed.

Invertase

The invertase measurement has been widely used in some countries such as Germany, Italy and Switzerland as a freshness indicator, as this enzyme is particularly sensitive to heat and storage damage. The RSD_R values for this method are considerably better than that of the diastase determination methods. The International Honey Commission has proposed minimum invertase activity values for especially carefully treated or fresh honeys.

Up to know the results were expressed in Hadorn units (invertase numbers). However Recent experiments showed, that it would be better to express those in international units.

Sugars

Non-specific methods

The reducing sugars (mainly fructose and glucose), as well as the apparent sucrose content are measured by the old Fehling method (10). While the precision of the reducing sugar measurement is acceptable, that of the apparent sucrose measurement is not satisfactory. This method qualifies as “apparent sucrose“ all non-reducing sugars and is calculated as the difference between the total and the reducing sugars.

Specific methods

All three methods (15-18) presented here measure specifically fructose, glucose and sucrose by chromatographic methods. No honey sugars are known to co-elute with these three sugars in any of the three methods. HPLC and GC analysis of 4 honeys (15) and ion chromatography and gas chromatography (16) also yielded the same results. We have proposed honey quality criteria to consist of: 1. the sum of glucose and fructose and 2. of the sucrose content. Other sugars, e.g. isomaltose, erlose, melezitose, which might be important for specific analyses, can also be determined by these methods, using the necessary standards.

The RSD_R values of all three methods are below 10%, with the exception of measurements of low levels of sucrose. The HPLC method showed the least interlaboratory variation.

Insoluble matter

The measurement of insoluble matter (10) is an important means to detect honey impurities higher than the permitted maximum. However, interlaboratory coefficient of variation, lying between 26 and 85 % is very high. This should be borne in mind when interpreting results.

Proline

The proline content is used as a criterion of honey ripeness and, in some cases, sugar adulteration. The method has a satisfactory interlaboratory variation. The proline content of honey varies greatly from honey to honey. In Germany a honey with less than 180 mg/kg is considered as either non-ripe or adulterated.

Specific rotation

The specific rotation is used in Italy to distinguish between blossom and honeydew honeys (19). The method is very useful for this purpose but limits are yet to be agreed.

Format

Generally, the subheadings of the methods used follow the ISO Format (1), but some subheadings are omitted as unnecessary in a particular case.

Sampling

The sample to be analysed should be representative of the honey lot. All honey samples should be prepared in the following way before analysis. For straining, use a stainless steel sieve, mesh diameter 0.5 mm.

Liquid or crystallised honey free from extraneous matter.

Homogenize the laboratory sample by stirring thoroughly (at least three minutes). Be careful that as little air as possible is stirred into the honey, especially if the sample is to be used for determination of hydroxymethylfurfural. If the honey is crystallised in a hard and compact mass, it can be previously softened by heating it in stove or thermostatic bath at no more than 40°C.

Liquid or crystallized honey containing extraneous matter.

Remove any coarse material, subsequently stir the honey at room temperature and pass through a 0.5 mm sieve. Gently press crystallised honey with a spatula through a 0.5 mm sieve.

Comb honey.

Uncap the comb. Drain the comb through a 0.5 mm sieve without heating in order to separate honey from the comb.

Compositional Criteria and Standards

Presently the Codex and Alimentarius are revising their standards. The Draft of the Codex Alimantrius Honey standard is presently at step 6 of the Codex procedure. The draft will be discussed at the seventh session of the Codex Alimentarius commission in London, 9-11 February 2000. The EU is awaiting the Codex decision on the honey standard, before it can propose a standard of its own. The proposition of the IHC are summarised in a recent publication (8).

After:

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DESCRIPTION OF METHODS

1 Determination of moisture, refractometric method

1. SCOPE

The standard describes a procedure to measure the water content of honey.

2. DEFINITION

The water content is that value determined from the refractive index of the honey by reference to a standard table.

3. PRINCIPLE

The method is based on the principle that refractive index increases with solids content. The table was constructed from a plot of the logarithm of the refractive index minus unity plotted against the water content as determined by vacuum drying, a technique which requires much greater manipulative skill (1 - 7).

4. EQUIPMENT.

Flasks, 50 ml.

Water bath.

Abbé or a digital refractometer, that can be thermostated at 20^0 C, regularly calibrated with distilled water or with another certified reference material. The refractive index for water (n_D) at 20°C is 1.3330.

5. PROCEDURE

Sample preparation.

Carry out according to the section Sampling of INTRODUCTION AND GENERAL COMMENTS ON THE METHODS.

Dissolution

Homogenise the prepared sample again and put in a flask. Close the flask and place in a water bath at 50°C (±0.2) until all the sugar crystals are dissolved. Cool the solution to room temperature and stir again.

Note:

Ensure that the flask is air tight.

Determination

Ensure that the prism of the refractometer is clean and dry. Directly after homogenisation, cover the surface of the prism evenly with the sample. After 2 minutes (Abbe refractometer) read the refractive index. Measure each honey twice and take the average value. Read the corresponding moisture content from the table. Carefully clean the prism after use.

Note:

The method refers only to the use of the Abbé refractometer, not to digital instruments.

6. RELATIONSHIP OF WATER CONTENT OF HONEY TO REFRACTIVE INDEX

Water Content,	**Refractive Index**	**Water Content**	**Refractive Index**
g/100 g	20°C	g/100 g	20°C
13.0	1.5044	19.0	1.4890
13.2	1.5038	19.2	1.4885
13.4	1.5033	19.4	1.4880
13.6	1.5028	19.6	1.4875
13.8	1.5023	19.8	1.4870
14.0	1.5018	20.0	1.4865
14.2	1.5012	20.2	1.4860
14.4	1.5007	20.4	1.4855
14.6	1.5002	20.6	1.4850
14.8	1.4997	20.8	1.4845
15.0	1.4992	21.0	1.4840
15.2	1.4987	21.2	1.4835
15.4	1.4982	21.4	1.4830
15.6	1.4976	21.6	1.4825
15.8	1.4971	21.8	1.4820
16.0	1.4966	22.0	1.4815
16.2	1.4961	22.2	1.4810
16.4	1.4956	22.4	1.4805
16.6	1.4951	22.6	1.4800
16.8	1.4946	22.8	1.4795
17.0	1.4940	23.0	1.4790
17.2	1.4935	23.2	1.4785
17.4	1.4930	23.4	1.4780
17.6	1.4925	23.6	1.4775
17.8	1.4920	23.8	1.4770
18.0	1.4915	24.0	1.4765
18.2	1.4910	24.2	1.4760
18.4	1.4905	24.4	1.4755
18.6	1.4900	24.6	1.4750
18.8	1.4895	24.8	1.4745
		25.0	1.4740

Temperatures above20° C: add 0.00023 per °C.

Temperatures below 20° C: substract 0.00023 per °C.

The table is derived from a formula developed by Wedmore from the data of Chataway and others:-

$$W = \frac{\bar{1}.73190 - \log(R.I.-1)}{0.002243}$$

W is the water content in g per 100 g honey and R.I. is the refractive index

7. PRECISION OF THE METHOD

0) Measurement by Abbe refractometer only (calculation carried according to ISO)

a)According to the DIN Norm :

$$r = \frac{0.093 \text{x} W}{W - 5.97}$$

$$R = \frac{0.067 \text{x} W}{W - 9.20}$$

Where W is the measured water content in g/100g.

b) according to the U.K. collaborative study :

Sample No.	% water content g/100 g (mean value)	r	R
1	15.9	0.28	0.92
2	16.0	0.17	0.55
3	17.3	0.21	0.40
4	17.8	0.53	0.87

c) according to the study of the International Honey Commission

Sample No.	% water content g/100 g (mean value)	r	R
1	16.4	0.13	0.48
2	17.3	0.15	0.52
3	18.1	0.15	0.68
4	20.0	0.11	0.68

In both studies the repeatability and reproducibility have been calculated from the results of four types of honey analysed by all laboratories collaborating in the study.

Note:

Determination of water with digital and Abbe refractometers

Stefan Bogdanov, 1999

Samples

To 5 flower and 2 honeydew honeys different water amounts were added, so that 20 honey samples resulted, with water content from 14 to 21 %.

Instruments

Abbe Carl Zeiss and digital refractometers: Mettler-Toledo RE 40, Bellingham RFM 330 and Atago 5000, numbered in the table R1 to R3 not in the same order.

Procedure

Abbe according to 1.1

Digital refractometer: measurement at 20^{o} C, after waiting for 6 minutes for equilibration.

Results and discussion

The differences between the results, achieved with all refractometers are very small and lie within the r values, achieved in the ring trials (see 1.1). The results presented in the table show, that the digital refractometers can all be used for the determination of water content. However, before they can be recommended for routine use, more comparative measurements with a higher number of honey samples should be made. Please send remarks on this measurement to Stefan Bogdanov.

Table: Measurement of water content with Abbe and three digital refractometers (R1-R3).

Nr.	Abbe	R1	A-R1	R2	A-R2	R3	A-R3
4	14.0	13.9	0.1	13.9	0.1	13.6	0.4
1	14.2	14.5	-0.3	14.6	-0.2	14.2	0
5	14.4	14.4	0.0	14.4	0.0	14.2	0.2
6	14.8	14.9	-0.1	15.0	-0.2	14.6	0.2
2	15.2	15.4	-0.2	15.3	-0.1	15.0	0.2
41	15.6	15.8	-0.2	15.9	-0.3	15.6	0
11	15.8	15.8	0.0	16.0	-0.2	15.7	0.1
51	16.7	16.6	0.1	16.8	-0.1	16.6	0.1
21	16.8	16.9	-0.1	17.0	-0.2	16.7	0.1
12	17.3	17.4	-0.1	17.6	-0.3	17.2	0.1
42	17.4	17.3	0.1	17.5	-0.1	17.2	0.2
7	17.5	17.6	-0.1	17.8	-0.3	17.4	0.1
61	17.7	17.9	-0.2	18.0	-0.3	17.7	0
31	18.1	18.2	-0.1	18.3	-0.2	18.1	0
52	18.5	18.5	0.0	18.6	-0.1	18.4	0.1
71	19.1	19.2	-0.1	19.4	-0.3	19.2	-0.1
62	19.1	19.2	-0.1	19.4	-0.3	19.1	0
32	19.8	20.0	-0.2	20.1	-0.3	19.9	-0.1
22	20.1	20.4	-0.3	20.5	-0.4	20.3	-0.2
72	20.8	21.0	-0.2	21.0	-0.2	20.8	0
Average difference			**- 0.1**		**- 0.2**		**0.1**
R (n=8)							
11	**0.13**	**0.28**		**0.15**		**0.25**	
42	**0.15**	**0.30**		**0.13**		**0.48**	

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2 Determination of electrical conductivity

1. SCOPE

The method is valid for the determination of the electrical conductivity of honey in the range 0.1 - 3 $mS.cm^{-1}$.

2. DEFINITION

The electrical conductivity of honey is defined as that of a 20% weight in volume solution in water at 20^0C, where the 20% refers to honey dry matter. The result is expressed in milliSiemens per centimetre ($mS.cm^{-1}$).

3. PRINCIPLE

The electrical conductivity of a solution of 20 g dry matter of honey in 100 ml distilled water is measured using an electrical conductivity cell. The determination of the electrical conductivity is based on the measurement of the electrical resistance, of which the electrical conductivity is the reciprocal. The method is based on the original work of Vorwohl (1 - 5).

4. REAGENTS

If not otherwise specified, the reagents shall be of recognized analytical quality.

Water shall be freshly distilled or of equal quality.

Potassium chloride solution, 0.1M. Dissolve 7.4557 g of potassium chloride (KCl), dried at 130 °C, in freshly distilled water in a 1000 ml flask and fill to volume with distilled water. Prepare fresh on the day of use.

5. EQUIPMENT

Conductivity meter, lower range 10^{-7} S.

Conductivity cell, platinized double electrode (immersion electrode).

Thermometer with divisions to 0.1^0 C.

Water bath, thermostatically controlled at a temperature of 20°C ± 0.5^oC.

Volumetric flasks, 100 ml and 1000 ml.

Beakers, tall form.

6. PROCEDURE

Determination of the cell constant

If the cell constant of the conductivity cell is not known, proceed as follows:

Transfer 40 ml of the potassium chloride solution to a beaker. Connect the conductivity cell to the conductivity meter, rinse the cell thoroughly with the potassium chloride solution and immerse the cell in the solution, together with a thermometer. Read the electrical conductance of this solution in mS after the temperature has equilibrated to 20^0C.

Note:

Most conductivity meters are direct current. In order to avoid false results due to polarization effects, the measurement time should be as short as possible.

Calculate the cell constant K, using the following formula:

$K = 11.691 \times 1/G$

Where:

K = the cell constant in cm^{-1}.

G = the electrical conductance in mS, measured with the conductivity cell.

11.691= the sum of the mean value of the electrical conductivity of freshly distilled water in $mS.cm^{-1}$ and the electrical conductivity of a 0.1M potassium chloride solution, at 20 °C.

Rinse the electrode thoroughly with distilled water after the determination of the cell constant.

When not in use keep the electrode in distilled water in order to avoid ageing of the platinum electrode.

Sample preparation

Carry out according to the section Sampling of INTRODUCTION AND GENERAL COMMENTS ON THE METHODS.

Preparation of the sample solution

Dissolve an amount of honey, equivalent to 20.0 g anhydrous honey, in distilled water. Transfer the solution quantitatively to a 100 ml volumetric flask and make up to volume with distilled water.

Note:

If necessary, a 1 in 5 w/v dilution of a smaller amount of honey can be used.

Pour 40 ml of the sample solution into a beaker and place the beaker in the thermostated water bath at 20 °C. Rinse the conductivity cell thoroughly with the remaining part of the sample solution. Immerse the conductivity cell in the sample solution. Read the conductance in mS after temperature equilibrium has been reached.

Notes:

1. Most conductivity meters are direct current. In order to avoid false results due to polarization effects, measurement time should be as short as possible.

2. If the determination is carried out at a different temperature, because of lack of thermostated cell, then a correction factor can be used for calculation of the value at 20 °C:

For temperatures above 20 °C : subtract 3.2 % of the value per °C

For temperatures below 20 °C : add 3.2 % of the value per °C

Data from measurements corrected with the above factors values have not bee validated in ring trials. However there were no significant differences between conductivity of 50 honeys, measured at 20 °C and at temperatures varying from 20 to 26 °C after applying the above correction factor (5).

7. CALCULATION AND EXPRESSION OF RESULTS

Calculate the electrical conductivity of the honey solution, using the following formula:

$S_H = K \cdot G$

Where:

S_H = electrical conductivity of the honey solution in $mS.cm^{-1}$

K = cell constant in cm^{-1}

G = conductance in mS

Express the result to the nearest 0.01 $mS.cm^{-1}$.

8. PRECISION

The precision of the method was determined in DIN trials and the data are valid for honeys with a range between 0.1 - 3 $mS.cm^{-1}$ (all data in $mS.cm^{-1}$).

Sample No.	Conductivity value	r	R
1	1.52	0.020	0.120
2	0.44	0.005	0.045
3	0.22	0.002	0.020

The repeatability and reproducibility have been calculated from the results of three types of honey analysed by all laboratories collaborating in the study. The probability level is 95 %. This means that, on average, in carrying out 20 determinations, 1 outlier may be expected.

REFERENCES

1. G. Vorwohl, Messung der elektrischen Leitfähigkeit des Honigs und der Verwendung der Messwerte zur Sortendiagnose und zum Nachweis von Verfälschungen mit Zuckerfütterungshonig. Zeitschr. Bienenforsch. 7, 37-47 (1964).
2. G. Vorwohl, Die Beziehung zwischen der elektrischen Leitfähigkeit der Honige und ihrer trachmässiger Herkunft. In: Ann. de Abeille, 7 (4) 301-309 (1964).
3. Arrête du 15 février 1977 relatif aux méthodes officielles d'analyses du miel. In: Journal officiel de la République Française (1977-04-22).
4. DIN Norm 10 753 Bestimmung der elktrischen Leitfähigkeit von Honig (1991).
5. S. Bogdanov, FAM, Bee Department, 3003 Bern, Liebefeld, Switzerland, personal communication.

3 Determination of ash content

1. SCOPE

This standard prescribes a procedure to determine the ash content in honey. The ash content is used to assess the type of honey.

2. DEFINITION

The ash content of honey means the residue which is obtained by a defined procedure and expressed as a percentage by weight.

3. PRINCIPLE

The honey is ashed at a temperature no higher than 600°C and the residue weighed (1 - 4).

4. REAGENTS

Olive oil, free from ash.

5. EQUIPMENT

Platinum or quartz ash dish of suitable size.

Appliance for preliminary evaporation, such as an infra-red heater, a gas burner or a hotplate.

Electric furnace, adjustable to 600°C (± 25°C).

Desiccator with suitable drying material.

Analytical balance.

6. PROCEDURE

Preparation of the ash dish

Heat the ash dish in the electrical furnace at ashing temperature, subsequently cool in a desiccator to room temperature and weigh to 0.001g (m_2)

Sample preparation

Carry out according to the section Sampling of INTRODUCTION AND GENERAL COMMENTS ON THE METHODS.

Determination

Weigh 5 to 10g of the sample to the nearest 0. 001g into an ash dish that has been prepared as described above (m_0). Add two drops of olive oil. Then remove water and commence ashing without loss at a low heat rising to 350 - 400^0 C by use of one of the appliances.

After the preliminary ashing, place the dish in the preheated furnace and heat for at least 1 hour. Cool the ash dish in the desiccator and weigh. Continue the ashing procedure until constant weight is reached (m_1).

7. CALCULATION AND EXPRESSION OF RESULTS

The proportion of ash W_A in g/100g honey is calculated using the following formula:-

$$W_A = \frac{(m_1 - m_2)}{m_0} 100$$

where

m_0 = weight of honey taken,

m_1 = weight of dish + ash,

m_2 = weight of dish.

Round the result to 2 decimal places.

8. PRECISION

Trial of the DIN:

Table: all values in g/100g

Sample No.	ash value $\bar{x}$	r	R
1	0.065	0.0078	0.0198
2	0.069	0.0064	0.0201
3	0.486	0.0365	0.0512

The repeatability and reproducibility have been calculated from the results of three types of honey analysed by all laboratories collaborating in a DIN study. The probability level is 95 %. This means that on average, in carrying out 20 determinations 1 outlier may be expected.

REFERENCES

1. Codex Alimentarius, Empfohlener Europäischer Regionalstandard für Honig (CAC/RS 12 1969): Methode 6.5 Bestimmung der Asche.
2. Hamburg, Behr´s Verlag, Bd. 3 Standart 1.3.(1988).
3. Williams, S.: Official Methods of Analysis, 14. ed. Arlington AOAC Inc. (1984).
4. DIN Norm Nr. 10755, Determination of honey ash content.

4 pH and free acidity

4.1 Determination of pH and of free acidity by titration to pH 8.3

1. SCOPE

The method can be applied to any sample of honey.

2. DEFINITION

pH is as normally defined. The free acidity of honey is the content of all free acids, expressed in milliequivalents /kg honey, determined by this method.

3. PRINCIPLE

The sample is dissolved in water, the pH measured and the solution titrated with 0.1M sodium hydroxide solution to pH 8.30 (1 - 3).

4. REAGENTS

These must be of analytical quality.

Distilled, carbon dioxide - free water.

Buffer solutions for calibration of the pH meter at pH 3.7 (or 4.0) and 9.0.

0.1M sodium hydroxide solution, accurately standardised (e.g. Titrisol).

5. EQUIPMENT

pH meter, accurate to 0.01 units.

Magnetic stirrer.

Burette 10 ml, 25 ml or automatic titrator.

Beaker, 250 ml.

6. PROCEDURE

Calibration of the pH meter

The meter should be calibrated at pH 3.0, 7.0 and 9.0.

Sample preparation

Carry out according to the section Sampling of INTRODUCTION AND GENERAL COMMENTS ON THE METHODS.

Determination

Ensure the sample is representative. Dissolve 10 g sample in 75 ml of carbon dioxide-free water in a 250 ml beaker. Stir with the magnetic stirrer, immerse the pH electrodes in the solution and record the pH. Titrate with 0.1M NaOH to pH 8.30 (a steady reading should be obtained within 120 sec of starting the titration; in other words, complete the titration within 2 minutes.). Record the reading to the nearest 0.2ml when using a 10ml burette and to 0.01ml if the automatic titrator has sufficient precision.

7. CALCULATION AND EXPRESSION OF RESULTS

pH - Report to two decimal places.

Free acidity, express as milliequivalents or millimoles acid/kg honey

= ml of 0.1M NaOH x 10. Express the result to one place of decimals.

8. PRECISION

The probability level is 95 %. This means that, on average, in carrying out 20 determinations, 1 outlier may be expected.

The precision of the method was determined in

a) Study in the United Kingdom (4)

Sample No.	Acidity meq/kg	r	R
1	7.0	4.7	8.5
2	6.5	2.9	6.2
3	13.5	2.0	7.1
4	13.5	2.6	7.1

The repeatability and reproducibility have been calculated from the results on four types of honey analysed by all laboratories collaborating in the study.

b) Trial of the International Honey Commission

Sample No.	pH	r	R
1	3.6	0.07	0.5
2	3.7	0.07	0.7
3	3.9	0.06	0.5
4	4.0	0.04	0.4
5	4.0	0.06	0.5
6	4.0	0.04	0.2
7	4.3	0.06	0.2

Sample No.	Acidity meq/kg	r	R
1	11.2	0.9	6.23
2	16.6	1.2	10.1
3	17.8	1.5	7.6
4	21.3	0.7	6.7
5	39.5	0.9	11.3
6	42.9	1.0	13.2
7	46.2	1.7	14.6

The repeatability and reproducibility have been calculated from the results on seven types of honey analysed by all laboratories collaborating in the study.

REFERENCES

1. Codex Alimentarius Commission: Recommended European regional standard for honey (CAC/RS 12-1969).
2. AOAC Official Methods of Analysis, Acidity of Honey p.1033, 962.19: (1990).
3. DIN Norm, 10 756, Bestimmung des Gehaltes an freier Säure, (1995).
4. D.W. Lord, M. J.Scotter, A.D.Whittaker and R.Wood, The determination of acidity, apparent reducing sugar and sucrose, hydroxymethylfurfural, mineral, moisture, water-insoluble solids contents in honey; collaborative study, J.Assoc. Publ. Anal.(UK), 26, 51-76 (1988).

4.2 Determination of pH, free acidity, lactones and total acidity: equivalence point titration

1. SCOPE

The method can be applied to all honey samples.

2. DEFINITIONS

a. The free acidity (F.A.) Is the acidity titratable with sodium hydroxide up to the equivalence point (pHe).

b. The lactone acidity (L.A.) corresponds to the combined acidity which is not directly titratable.

c. The total acidity (T.A.) is the sum of the free acidity and the lactone acidity.

3. PRINCIPLE

The pH is measured on a 10% honey solution.

The free acidity is obtained by plotting the neutralization curve with a sodium hydroxide solution and determining the pH of the equivalence point (pHe).

The acidity of the lactones is obtained by adding an excess of sodium hydroxide to the honey solution and plotting the neutralization curve of the excess sodium hydroxide by a back titration with sulphuric acid (1).

4.REAGENTS

0.025M sulphuric acid solution of titrisol quality,

0.05M sodium hydroxide solution (checked daily),

Ultra-pure carbon dioxide-free water,

pH standard buffer solutions (pH 7 and pH 4).

5. EQUIPMENT

pH meter (precision 0.01 unit) and electrodes,

Magnetic stirrer (with bar magnet),

Automatic titrator (with two burettes),

Analytical balance,

50 ml volumetric flask,

25 ml volumetric pipette,

250 ml beaker.

6. PROCEDURE

Calibrations

Calibrate pH meter daily at two pH values with buffer solutions.

Determination of the exact titre [T] of the sodium hydroxide solution.

Sample preparation

If necessary, prepare the honey according to the section Sampling of INTRODUCTION AND GENERAL COMMENTS ON THE METHODS.

Determination of the acidity values.

Accurately weigh about 5 g of honey [M] and dissolve it in a few millilitres of water. Transfer quantitatively to a 50 ml volumetric flask and fill to the mark with water. Mix well. Pipette 25 ml into a 250 ml beaker. Add a bar magnet. Note the initial pH (pHi). Stir gently and titrate first with the sodium hydroxide solution (up to 10 ml), then (into the same beaker) with the sulphuric acid solution (up to the second equivalence point).

7. CALCULATION AND EXPRESSION OF RESULTS

Note from the curve the free acidity neutralization volume in ml [V] and the sodium hydroxide excess neutralization volume (corresponding to pH 7) in ml [V´].

The free acidity is expressed in milliequivalents of sodium hydroxide required to neutralize 1 kg of honey.

F.A.= V x T x (50/25) x (1000/M)

The lactone acidity is expressed in the same units:

L.A. = [(10-V) x T - 0.05 x V´] x (50/25) x (1000/M)

The total acidity is expressed in the same units:

T.A. = F.A. + L.A.

8. PRECISION

The precision of the method was determined in a trial of the International Honey Commission. The repeatability and reproducibility have been calculated from the results on seven types of honey analysed by all laboratories collaborating in the study.

Sample No.	pH	r	R
1	3.6	0.18	0.75
2	3.8	0.24	1.00
3	4.0	0.15	0.79
4	4.0	0.11	0.50
5	4.0	0.11	0.45
6	4.1	0.14	0.67
7	4.3	0.15	0.47

Sample No.	Acidity meq/kg	r	R
1	7.01	1.74	9.09
2	10.64	1.74	3.82
3	10.84	1.79	4.76
4	13.45	0.60	7.43
5	27.00	2.33	7.61
6	28.9	2.13	9.54
7	32.50	2.24	7.84

REFERENCE

1. Arrêté du 15/02/77 relatif aux méthodes officielles d´analyse du miel (Journal Officiel de la République Française - N.C. du 22/04/77).

5 Hydroxymethylfurfural

5.1 Determination of hydroxymethylfurfural by HPLC

1. SCOPE

The method can be applied to all honey samples. Less sample may be necessary if the concentration of HMF is very high.

2. DEFINITION

The method determines the concentration of 5-(hydroxymethyl-)furan-2-carbaldehyde. The result is usually expressed in milligrams per kilogram.

3. PRINCIPLE

Hydroxymethylfurfural (HMF) is determined in a clear, filtered, aqueous honey solution using reverse phase HPLC equipped with UV detection. The signal is compared with those from standards of known concentration. This method is based on the work of Jeuring and Kuppers (1, 2).

4. REAGENTS

Mobile phase: water-methanol (90+10 by volume), both HPLC quality.

Standard solutions: 5-(hydroxymethyl-)furan-2-carbaldehyde (HMF),

(e.g. Merck No. 820 678 or Fluka No. 55690), 1, 2, 5 and 10 mg /L aqueous solution. The solution should be prepared on the day of use.

Determination of standard HMF-content

The absorbance A of the prepared standard solution is determined using an UV spectrophotometer at 285 nm in 1 cm quartz cells with water in the blank cell. The concentration of the standard solutions can be calculated from the literature values for molar absorptivity, $\varepsilon = 16830$ or absorptivity, $a_{1cm}^{1\%} = 133.57$ (3).

Concentration in mg/L = $\frac{\mathbf{A}}{1 \mathbf{x} 133.57} \mathbf{x} 1{,}000$

where A is the absorbance of the standard solution.

The calculated content must correspond to the specifications given by the supplier.

The standard has to be stored at 4 - 8 °C under nitrogen. It is extremely hygroscopic.

5. EQUIPMENT

Liquid chromatograph with UV detector and integrator.

Column: any column with C_{18}-reversed phase material.

e.g Hypersil ODS 5 µm, 125 x 4 mm or 250 x 4 mm.

Membrane filter, 0.45 µm (e.g. Dynagard).

6.PROCEDURE

Preparation of samples.

Carry out according to the section Sampling of INTRODUCTION AND GENERAL COMMENTS ON THE METHODS.

Determination

Note from 01.07.2002

After the sample preparation, originally proposed by Jeuring and Kuppers, losses, due to HMF break-down during the storage of the sample are encountered (Känzig et al., 2001) see on-line report on the IHC website http://www.apis.admin.ch/english/host/pdf/honey/HMF_abstract.pdf.

For that purpose, the original sample preparation should only be used, if the HPLC determination is carried out within half an hour after honey dissolution. Otherwise, the sample preparation should be carried out according to the White method, in which a Carrez solution is added for honey clarification. The addition of a Carrez solution will stop this break-down. HPLC analysis will be carried out with the same solution, used for spectrometry (see 5.2).

Accurately weigh about 10 g of prepared honey sample into a 50 ml beaker. Dissolve the sample in approx. 25 ml of water and transfer quantitatively to a 50 ml volumetric flask. Dilute to 50 ml with water. Filter through a 0.45 μm membrane filter to provide a sample solution ready for chromatography.

Conditions for chromatography

flow rate	1.0 ml/ minute
quantity injected	20 μL of sample or standard solution.
detection	UV 285 nm; range: 0.2 AUFS.

7. CALCULATION AND EXPRESSION OF RESULTS

The HMF content of the sample is calculated by comparing the corresponding peak areas of the sample and those of the standard solutions, taking into account the dilution. There is a linear relationship between the concentration and the area of the HMF peak. Results are expressed in mg/kg, to 1 decimal place.

8. PRECISION

The precision of the method was determined in a trial of the International Honey Commission. The repeatability and reproducibility have been calculated from the results on three types of honey analysed by all laboratories collaborating in the study.

Sample No.	HMF mg/kg	r	R
1	5.2	0.4	1.6
2	22.8	1.2	4.9
3	42.3	2.1	7.3

9. COMPARISON WITH THE OTHER METHODS

At low HMF levels (about 5 mg/kg) the values obtained with this method are comparable to those obtained with the White method, but are lower than those obtained with the p-toluidine method (4). At higher HMF levels (20 and 40 mg/kg) the values with all three methods are not significantly different from each other.

Note:

Furfural, which is found only in very small quantities compared with HMF, can be determined by the same method. Furfural elutes about 1.5 minutes after HMF.

REFERENCES

2. J. Jeuring and F. Kuppers, High Performance Liquid Chromatography of Furfural and Hydroxymethylfurfural in Spirits and Honey. J.Ass. Off. Anal. Chem. 63, 1215 (1980).
3. Determination of Hydroxymethylfurfural by HPLC, Swiss Food Manual, Kapitel Honig, Eidg. Druck und Materialzentrale (1995).
4. J. White, Spectrophotometric Method for Hydroxymethylfurfural in Honey, J. Ass Off. Anal. Chem. 62, 509 (1979).
5. V. Figueiredo, Report on the HMF interlaboratory trial of the International Honey Commission, Basel, (1991).

5.2 Determination of hydroxymethylfurfural after White

1. SCOPE

The method can be applied to all honey samples, but a double-beam spectrophotometer may be necessary in some cases (see clause 10).

2. DEFINITION

The method determines the concentration of 5-(hydroxymethyl-)furan-2-carbaldehyde. The result is usually expressed in milligrams per kilogram.

3. PRINCIPLE

The determination of the hydroxymethylfurfural (HMF) content is based on the determination of UV absorbance of HMF at 284 nm. In order to avoid the interference of other components at this wavelength the difference between the absorbances of a clear aqueous honey solution and the same solution after addition of bisulphite is determined. The HMF content is calculated after subtraction of the background absorbance at 336 nm. This method is based on the original work of White (1, 2).

4. REAGENTS

Carrez solution I: dissolve 15 g of potassium hexacyanoferrate(II), $K_4Fe(CN)_6 \bullet 3H_2O$ in water and make up to 100 ml.

Carrez solution II: dilute 30 g of zinc acetate, $Zn(CH_3.COO)_2.2H_2O$ and make up to 100 ml.

Sodium bisulphite solution 0.20 g/100 g: dissolve 0.20 g of solid sodium hydrogen sulphite $NaHSO_3$, (metabisulphite, $Na_2S_2O_5$), in water and dilute to 100 ml. Prepare fresh daily.

5. EQUIPMENT

Spectrophotometer operating in a wavelength range including 284 and 336 nm.

1 cm quartz cells

Vortex mixer.

Filter paper (general purpose).

6. PROCEDURE

Sample preparation.

Carry out according to the section Sampling of INTRODUCTION AND GENERAL COMMENTS ON THE METHODS.

Determination.

Accurately weigh approximately 5g of honey into a 50 ml beaker. Dissolve the sample in approximately 25 ml of water and transfer quantitatively into a 50 ml volumetric flask. Add 0.5 ml of Carrez solution I and mix. Add 0.5 ml of Carrez solution II, mix and make up to the mark with water(a drop of ethanol may be added to suppress foam). Filter through paper; rejecting the first 10 ml of the filtrate. Pipette 5.0 ml in each of two 2 test tubes (18 x 150 mm). Add 5.0 ml of water to one of the test tubes and mix well (the sample solution). Add 5.0 ml of sodium bisulphite solution 0.2% to the second test tube and mix well (the reference solution).

Dilution of sample and reference solutions is carried out as follows:

Additions to test-tube	Sample solution	Reference solution
Initial honey solution	5.0 ml	5.0 ml
Water	5.0 ml	-
0.2% sodium bisulphite solution	-	5.0 ml

Determine the absorbance of the sample solution against the reference solution at 284 and 336 nm in 10 mm quartz cells within one hour. If the absorbance at 284 nm exceeds a value of about 0.6, dilute the sample solution with water and the reference solution with sodium bisulphite solution to the same extent in order to obtain a sample absorbance low enough for accuracy. If dilution is necessary,

$$\text{The Dilution , } D = \frac{\text{Final volume of sample solution}}{10}$$

7. CALCULATION AND EXPRESSION OF RESULTS

$$\text{HMF in mg/kg} = (A_{284} - A_{336}) \times 149.7 \times 5 \times D/W$$

Where:-

A_{284} = absorbance at 284 nm

A_{336} = absorbance at 336 nm

$$149.7 = \frac{126 \text{x} 1000 \text{x} 1000}{16830 \text{x} 10 \text{x} 5} = \text{Constant}$$

126 = molecular weight of HMF

16830 = molar absorptivity ε of HMF at λ= 284 nm

1000 = conversion g into mg

10 = conversion 5 into 50 ml

1000 = conversion g of honey into kg

5 = theoretical nominal sample weight

D = dilution factor, in case dilution is necessary

W = Weight in g of the honey sample

Express results in mg/kg to 1 decimal place.

8. PRECISION

a) trials according to DIN (3)

Sample No.	HMF mg/kg	r	R
1	15-21	1.5	4.5
2	48-64	1.7	5.2

The repeatability and reproducibility have been calculated from the results on two types of honey analysed by all laboratories collaborating in the study.

b) trial conducted by the International Honey Commission

Sample No.	HMF mg/kg	r	R
1	3.8	0.9	2.3
2	22.3	1.2	3.9
3	42.1	2.2	4.4

9. COMPARISON WITH THE OTHER METHODS

At low HMF levels (about 5 mg/kg) the values obtained with this method are comparable to those obtained with the HPLC method, but are lower than those obtained with the Winkler method (4). At higher HMF levels (20 and 40 mg/kg) the values with all three methods are not significantly different from each other.

10. NOTE

Some honeys, such as lime, may show strong absorbance at 284nm due to interfering substances. If these have an absorbance at 336nm different to that at 284nm, the result will be in error. This difficulty is normally overcome by using a double-beam spectrophotometer. In the absence of such an instrument, sample dilution may be tried. In this case the dilution factor must be taken into account when calculating the result. If dilution is too great to give adequate accuracy, an alternative method should be used.

REFERENCES

1. J.W. White: Spectrophotometric Method for Hydroxymethylfurfural in Honey, J. Ass. Off. Anal. Chem. 62, 509 (1979).
2. Official Methods of Analysis AOAC, No. 980.23, edition 15 (1990).
3. DIN Norm 10751, Bestimmung des Gehaltes an Hydroxymethylfurfural, Teil 2 (1990).
4. V. Figueiredo, HMF Interlaboratory Trial of the International Honey Commission, Report for the participants, Basel canton chemist laboratory, (1991).

5.3 Determination of hydroxymethylfurfural after Winkler

1. SCOPE

The method can be applied to all honey samples.

2. DEFINITION

The method determines the concentration of 5-(hydroxymethyl-)furan-2-carbaldehyde, defined as the constituents of honey which are capable of combining with barbituric acid and p-toluidine under the conditions of the test.

3. PRINCIPLE

This method describes the determination of hydroxymethylfurfural in honey and is based on the original method of Winkler (1).

To aliquot parts of a honey solution, solutions of p-toluidine and barbituric acid are added and the resultant colour is measured against a blank in 1-cm cuvettes at 550nm.

4. REAGENTS

p-toluidine-solution.

NOTE: p-toluidine is carcinogenic and presents a risk to health. Contact with the reagent should be avoided. If possible, use one of the other two methods for HMF determination.

Dissolve 10.0 g p-toluidine in 50 ml 2-propanol by gently warming on a water bath. Transfer with a few ml of 2-propanol to a 100 ml volumetric flask and mix with 10.0 ml glacial acetic acid. After cooling to ambient temperature, fill to volume with 2-propanol.

Store in the dark for at least 24 hours before use. Discard after three days or if there is undue coloration.

Barbituric acid solution.

Transfer 500 mg barbituric acid as quickly as possible to a 100 ml volumetric flask with about 70 ml water. Dissolve by warming the stoppered flask gently on a water bath. Cool to ambient temperature and dilute to volume.

Carrez solution I: dissolve 15 g of potassium hexacyanoferrate(II), $K_4Fe(CN)_6 \bullet 3H_2O$ in water and make up to 100 ml.

Carrez solution II: dissolve 30 g of zinc acetate, $Zn(CH_3.COO)_2.2H_2O$ in water and dilute to 100 ml with water.

5. EQUIPMENT

Spectrophotometer or filter photometer for measuring absorbance at 550 nm.

1 cm cells.

Volumetric flasks, 50 and 100 ml.

Test tubes.

Beaker.

Filter paper, analytical grade.

6. PROCEDURE

Sample preparation

Carry out according to the section Sampling of INTRODUCTION AND GENERAL COMMENTS ON THE METHODS.

Preparation of the sample solution

Weigh about 10g of honey to the nearest mg. Dissolve in about 20 ml water and quantitatively transfer to a 50 ml volumetric flask. Add 1 ml of Carrez I, shake well, add 1 ml of Carrez II, shake

once more, dilute to volume with water and mix once more. A drop of ethanol prevents possible foaming. Filter the solution through filter paper. Discard the first 10 ml of the filtrate. Complete the rest of the analysis immediately.

In the case of very clear samples, clarification with Carrez' reagents is not necessary.

Determination

Pipette 2.0 ml of the sample solution to each of two tubes and add 5.0 ml p-toluidine solution to both.

Add 1.0 ml of water to one tube (blank value) and 1.0 ml of barbituric acid solution to the other with gentle shaking. Carry out without delay and complete in 1 - 2 minutes. Measure the absorbance of the sample against the blank as soon as the colour intensity has reached a maximum (3 - 4 minutes after adding the barbituric acid solution), using 1cm cells at 550nm.

7. CALCULATION AND EXPRESSION OF RESULTS

The content of HMF is calculated as follows:

$$\mathrm{HMF} = \frac{192 \mathrm{x} A \mathrm{x} 10}{\mathrm{Weight} \cdot \mathrm{of} \cdot \mathrm{honey} \cdot \mathrm{in} \cdot \mathrm{grams}}$$

Where

A = Absorbance,

192 = Factor for dilution and extinction coefficient

Express results in mg/kg to 1 decimal place

8. PRECISION

The precision of the method was determined in collaborative studies of:

a) The International Honey Commission (2)

Sample No.	HMF mg/kg	r	R
1	7.6	1.1	3.2
2	22.5	1.7	7.6
3	42.9	2.6	9.6

The repeatability and reproducibility have been calculated from the results on three types of honey analysed by all laboratories collaborating in the study.

b) the U.K. study (3)

Sample No.	HMF mg/kg	r	R
1	11.4	9.4	11.0
2	21.2	2.5	7.0
3	41.8	8.2	27.9
4	54.8	4.6	20.2

The repeatability and reproducibility have been calculated from the results on four types of honey analysed by all laboratories collaborating in the study.

c) the DIN Norm , where r and R can be calculated in the range 1 to 85 mg/kg with the following formulas:

$$r = 0.36 \ln HMF + 0.40$$

$$R = 1.76 HMF^{0.44}$$

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6 Diastase

6.1 Determination of diastase activity after Schade

1. SCOPE

The method can be applied to all honey samples.

2. DEFINITION

The unit of Diastase Activity, the Gothe unit, is defined as that amount of enzyme which will convert 0.01 gram of starch to the prescribed end-point in one hour at 40^0C under the conditions of test. Results are expressed in Gothe units (or Schade units) per gram of honey.

3. PRINCIPLE

A standard solution of starch, capable of developing, with iodine, a colour in a defined range of intensity, is acted upon by the enzyme in the sample under standard conditions. The diminution in the blue colour is measured at intervals. A plot of absorbance against time, or a regression equation, is used to determine the time t_x required to reach the specified absorbance, 0.235*. The Diastase Number is calculated as 300 divided by t_x, provided the method is followed precisely. This method is based on the original work of Schade et al (1), modified by Hadorn and Zürcher (2) and White and Pairent (3) as presented by the Codex Alimentarius method.

* - Note: the German DIN method uses 0.301 as an absorbance factor.

4. REAGENTS

Sodium chloride solution: dissolve 2.9 g of sodium chloride in water and dilute to 100 ml.

Acetate buffer solution (pH 5.3): dissolve 43.5g of sodium acetate

(CH_3. COONa. 3 H_2O) in water, adjust the pH of the solution to 5.3 with about 5 ml of glacial acetic acid and dilute to 250 ml with water.

Starch solution:

a). Determination of starch dry weight :

Spread approximately 2 g of air dry soluble starch in a thin layer over the bottom of a weighing bottle (diameter 5 cm, height 3 cm) with a lid.

Weigh accurately (± 0.1 mg) and dry for 90 minutes at 130°C.

Allow the closed weighing bottle to cool for about 1 hour in a desiccator and re-weigh accurately.

b). Preparation of starch solution:

Weigh into a 250 ml conical flask the amount of starch which is equivalent to 2.000 g of anhydrous starch. Add 90 ml of water and mix by swirling. Bring the suspension rapidly to the boil, swirl the flask constantly and boil gently for 3 minutes. Immediately transfer the hot solution to a 100 ml volumetric flask. Cool down rapidly to room temperature in running water, make up to volume with water and mix thoroughly.

Note:

The solution must be made on the day of use.

Use only soluble starch yielding a clear blue solution (see paragraph "Calibration of the Starch Solution").

Iodine stock solution: dissolve 11.0 g of twice sublimated iodine and 22.0 g of potassium iodide in 30 to 40 ml of water and dilute to 500 ml. The stock solution can be kept for approximately 1 year in a closed, dark bottle.

Iodine solution, dilute: dissolve 20.0 g of potassium iodide in water, add 2 ml of iodine stock solution and dilute to 500 ml. This dilute iodine solution must be made on the day of use and should be protected from air as much as possible, immediately closing the flask after use.

5. EQUIPMENT

All equipment must be free from detergents!

Thermostated water bath (40.0 ± 0.2 °C).

1 cm spectrometer cells.

Filter or spectrophotometer with small band interference filter set at 660 nm.

Timer.

6. PROCEDURE

Preparation of test samples

If necessary, prepare the honey according to the section Sampling of INTRODUCTION AND GENERAL COMMENTS ON THE METHODS.

Determination

Weigh 10.0 g of honey prepared for sampling into a beaker and dissolve completely in approximately 15 ml of water and 5 ml of acetate buffer without heating. Transfer the solution quantitatively to a 50 ml volumetric flask containing 3 ml of sodium chloride solution and adjust the volume to the mark with water (sample solution).

Note:

It is essential that the honey should be buffered before coming into contact with sodium chloride, as pH values below 4.0 in the presence of sodium chloride rapidly reduce the diastase activity.

The test sample can be kept for only a few hours. Therefore prepare immediately before the determination.

Calibration of the Starch Solution / Adjustment of Blue Value

This procedure is carried out to determine the amount of water that has to be added to the reaction mixture so that the absorbance range of the iodine starch solution is 0.745 to 0.770.

Pipette into 6 suitable glass flasks or test tubes 20, 21, 22, 23, 24 and 25 ml of water and 5 ml of dilute iodine solution.

Starting with the first test tube, add 0.5 ml of a mixture containing 10 ml of water and 5 ml of starch solution, mix well by agitating and immediately read the absorbance at 660 nm against a water blank in a 1 cm cell.

Proceed in the same way with the other test tubes, until an absorbance in the range 0.770 to 0.745 is obtained.

The amount of water determined in this way is the standard dilution for every determination carried out with the starch solution.

Note:

The time from the addition of the diluted starch solution to the determination of the absorbance should be as constant as possible in the calibration as well as in determining the diastase activity because the colour intensity is time dependent. If an absorbance of less than 0.745 in the first dilution step (20 ml) or a higher absorbance than 0.770 in the last dilution step (25 ml) is obtained, the starch is unsuitable for this method of determination of diastase activity.

Determination in the honey sample solution

Pipette 10 ml of honey solution into a 50 ml flask and place it in the 40°C water bath with a second flask containing about 10 ml of starch solution. After 15 minutes, pipette 5 ml starch solution into the honey solution, mix and start the timer. At periodic intervals, for the first time after 5 minutes, remove 0.5 ml aliquots and add rapidly to 5 ml of diluted iodine solution. Add the amount of water, (as determined in "Calibration of the starch solution"), mix well and immediately read the absorbance of each separate solution at 660 nm against a water blank in a 1 cm cell.

Note:

The intervals after the first removal from the reaction flask must be timed in a way that 3 to 4 values are obtained in a range of the two absorbances 0.456 and 0.155 (linear range).

The following table gives the values of time intervals:

Absorbance at t = 5 min	**Time interval**
A > 0.658	10 min or more
0.658 > A >0.523	5 to 10 min
0.523 > A >0.456	2 to 5 min

If the absorbance at t = 5 min is lower than 0.350 it is recommended that the reaction time for the first determination is reduced appropriately.

Reaction blank

Add 10 ml of sample solution prepared according to "Preparation of Test Samples" to 5 ml of water and mix thoroughly. Remove 0.5 ml of this solution and add to 5 ml of dilute iodine solution. Add the amount of water determined in "Calibration of the Starch Solution", mix well and read the absorbance at 660 nm against a water blank in a 1 cm cell. If there is an absorbance this blank value must be subtracted from the values obtained under "Determination of Test Solution".

7. CALCULATION AND EXPRESSION OF RESULTS

The diastase activity is calculated as diastase number (DN) as follows:

$$DN = \frac{60\text{minutes}}{t_x} \times \frac{0.10}{0.01} \times \frac{1.0}{2.0} = \frac{300}{t_x}$$

t_X = reaction time in minutes obtained as follows:-

If necessary the absorbance values of the test sample solutions are plotted against the corresponding reaction times in minutes on rectilinear paper after subtracting the absorbance of the blank value (see "Blank Value Control"). The regression line is drawn through the measuring points in the range of A = 0.155 to 0.456 in order to determine the time t_X for A = 0.235*. There should be at least three points in the absorbance range 0.155 to 0.456. The time for A = 0.235 can also be calculated from the regression equation and this is generally considered preferable to graphical interpolation.

* - Note: the German DIN method uses 0.301 as an absorbance factor.

8. PRECISION

The values for repeatability (r) and reproducibility (R) have been obtained by two interlaboratory trials:

a) According to the DIN-norm 10750 (4) from the results on nine types of honey analysed by all laboratories collaborating in the study.

They are valid with a probability of 95%. This means that, on average, in carrying out 20 determinations one outlier may be expected.

$$r = -1.4244 \times 10^{-5} DN^2 + 0.0241 \times DN + 0.17999$$

$$R = 0.00682 \times DN^2 - 0.237 \times DN + 3.4906$$

b) An interlaboratory trial was carried out in 1992 in 14 EC laboratories (5,6) with the Codex Alimentarius Schade method. The following r and R values were calculated for 9 honeys with DN in the range between 7.7 and 37.7:

DN	**8,7**	**14,2**	**16,4**	**19,5**	**23,6**	**24,2**	**25,5**	**29,8**	**37,7**
r	0,64	2,15	1,48	0,92	1,20	3,07	1,87	2,06	5,35
R	5,45	9,01	10,40	10,22	12,33	13,93	15,82	15,79	23,68

The following correlation equations were calculated from this data:

$$r = - 0{,}721 + 0{,}126\ DN$$

$$R = - 0{,}0571 + 0{,}587\ DN$$

REFERENCES

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6.2 Determination of diastase activity with Phadebas

1. SCOPE

The method can be applied to all honey samples.

2. DEFINITION

The unit of Diastase Activity, the Gothe unit, is defined as that amount of enzyme which will convert 0.01 gram of starch to the prescribed end-point in one hour at 40^0C under the conditions of test. Results are expressed in Gothe units (or Schade units) per gram of honey

3. PRINCIPLE

Determination of the diastatic activity of honey is by a photometric method in which an insoluble blue dyed cross-linked type of starch is used as the substrate. This is hydrolysed by the enzyme, yielding blue water-soluble fragments, determined photometrically at 620 nm. The absorbance of the solution is directly proportional to the diastatic activity of the sample. The method is based on that originally published by Siegenthaler (1) and modified by Bogdanov (2).

4.REAGENTS

Phadebas tablets, Pharmacia Diagnostics.

Sodium hydroxide 0.5M.

Acetate buffer (0.1M, pH 5.2): Dissolve 13.6 g of sodium acetate trihydrate in water. Adjust the pH of the solution to 5.2 with glacial acetic acid (1 - 2 ml) and dilute to 1L with water.

5. EQUIPMENT

Photometer

Reagent mixer

Thermostated water bath

Timer

6. PROCEDURE

Preparation of test samples

If necessary, prepare the honey according to the section Sampling of INTRODUCTION AND GENERAL COMMENTS ON THE METHODS.

Determination

Weigh 1.00 g of honey into a 100 ml volumetric flask, dissolve in the acetate buffer solution and fill to the mark. Complete the procedure within an hour. Transfer 5.0 ml of the solution to a test tube and place it in the water bath at 40^o C.

Prepare a blank by placing a 5.0 ml aliquot of the acetate buffer in another test tube which is treated exactly as the sample solution.

To both solutions add a Phadebas tablet, using tweezers, and start the timer. Stir the solutions in the reagent mixer until the tablets disintegrate (ca. 10 seconds) and return them to the water bath. Terminate the reaction after exactly 15 minutes by adding 1 ml sodium hydroxide solution. Stir the mixture again in the reagent mixer for approximately 5 seconds. Immediately filter the solutions through filter papers and measure the absorbance in 1 cm cuvettes at 620 nm using water as reference. The absorbance of the blank is subtracted from that of the sample solution (ΔA_{620}). If the absorbance is higher than 1.0, dilute the sample with water. Take into consideration the dilution factor when calculating the results.

7. CALCULATION AND EXPRESSION OF RESULTS

The classical method for the determination of the honey diastase activity is the method of Schade (3,4). The diastase activity is expressed as the diastase number (DN) in Schade units and is

defined as follows: one diastase unit corresponds to the enzyme activity of 1 g of honey , which can hydrolyse 0.01 g of starch in one hour at 40^{o}C.

A simultaneous measurement with the Phadebas and the Schade method (3,4) of 57 different commercial honey samples covering the range of diastase activity from 8 to 40 was carried out. There was a very good correlation (r=0.987) between the two measurements. Linear regression of y (diastase number) against x (ΔA_{620}) yielded the following relation:

$$DN = 28.2 \times \Delta A_{620} + 2.64$$

where 28.2 and 2.64 are respectively the slope and the intercept of the best straight line obtained by linear regression of ΔA_{620} (x axis) on DN (y axis).

For low diastase values (between 0 and 6 DN) a very good correlation (R^2 = 0,927) with the following linear regression of y (diastase number) against x (ΔA_{620}) yielded the following relation:

$$DN = 35.2 \times \Delta A_{620} - 0.46$$

where 35.2 and 0.46 are respectively the slope and the intercept of the best straight line obtained by linear regression of ΔA_{620} (x axis) on DN (y axis).

This equation should be used for the determination of the activity of diastase up to 8 Diastase units.

8. PRECISION

a) Precision data determined in a Swiss data trial (5):

1. Three different types of honey were tested by three laboratories. The maximum deviation (range) of the diastatic activity determined with tablets of the same batch, between the laboratories,was found to be 3.7 %

2. The standard deviation of the diastatic activity determined with tablets of two different batches with the same honey, within one laboratory, was 3.7 % (for n=24, n being the number of analyses per batch).

3. The weight range, for a sample of 20 tablets, was found to be 5 %, with a standard deviation of 2 %.

c) An interlaboratory trial of the International Honey Commission with the Phadebas method was carried out with 7 honeys having A_{620} values varying from 0.31 to 1.29 in 1992 with 14 EC and 21 Swiss laboratories (6). The batches of the Phadebas reagent were not specified. The following r and R values were obtained:

A-620	0,212	0,314	0,414	0,588	0,704	0,705	0,734	0,970	1,294
r	0,034	0,032	0,032	0,042	0,049	0,043	0,050	0,065	0,060
R	0,107	0,134	0,161	0,202	0,273	0,311	0,250	0,336	0,428

where A-620 is the absorbance value.

The repeatability and reproducibility have been calculated from the results on nine types of honey analysed by all laboratories collaborating in the study.

The following correlation equations were calculated from this data:

$$r = 0.02 + 0.03 \times A_{620}$$

$$R = 0.04 + 0.32 \times A_{620}$$

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7 Sugars

7.1 Determination of apparent reducing sugars and apparent sucrose

1. SCOPE

The method can be applied to all honey samples.

2. DEFINITION

'Apparent reducing sugars' are defined as those sugars which reduce a Fehling's reagent under the conditions specified. 'Apparent sucrose' is defined as 0.95 of the difference in 'apparent reducing sugars' before and after the prescribed hydrolysis procedure.

3. PRINCIPLE

This method is a modification of the Lane and Eynon procedure, involving the reduction of Soxhlet's modification of Fehling's solution by titration at boiling point against a solution of reducing sugars in honey using methylene blue as an internal indicator. The difference in concentrations of invert sugar is multiplied by 0.95 to give the apparent sucrose content. This method is based on the original method of Lane and Eynon and is also used in the Codex Alimentarius standard.

4. REAGENTS

a) Soxhlet's modification of Fehling's solution:

Solution A: dissolve 69.28 g of Copper sulphate pentahydrate ($CuSO_4 \bullet 5H_2O$ in water, MW = 249.71) and make up to 1000 ml. Keep one day before titration.

Solution B: dissolve 346 g sodium potassium tartrate ($C_4H_4NaO_5 \bullet 4H_2O$, MW 282.23) and 100 g sodium hydroxide (NaOH) with distilled water to 1000 ml. Filter through prepared asbestos.

b) Standard invert sugar solution (10 g/L)

Weigh 9.5 g pure sucrose, add 5 ml hydrochloric acid (ca. 36.5 % w/w pure HCl) and dilute with water to about 100 ml. Store this acidified solution for several days at room temperature (ca. 7 days at 12 ^{o}C to 15 ^{o}C or 3 days at to 20 ^{o}C to 25 ^{o}C) and then dilute to 1000 ml. (NB acidified 1 % invert sugar remains stable for several months). Neutralize a suitable volume of this solution with 1 M sodium hydroxide solution (40 g/L) immediately before use and dilute to the required concentration (2 g/L) for the standardization.

c) Methylene blue solution

Dissolve 2 g in distilled water and dilute to 1 litre.

d) Alumina cream

Prepare cold satured solution of alum ($K_2SO_4Al_2(SO_4)_3 \bullet 24H_2O$ in water. Add ammonium hydroxide with constant stirring until solution is alkaline to litmus, let precipitate settle and wash by decantation with water until wash-water gives only slight test for sulphate with barium chloride solution. Pour off excess water and store residual cream in stoppered bottle.

e) Hydrochloric acid (6.34 M, aqueous), for apparent sucrose only

f) Sodium hydroxide (5 M aqueous), for apparent sucrose only

5. PROCEDURE FOR REDUCING SUGARS

5.1 Preparation of test sample, first procedure

applicable to honeys which may contain sediment)

1. Transfer an accurately weighed sample of approximately 25 g (W_1) from the homogenized honey to a 100 ml volumetric flask, add 5 ml alumina cream, dilute to volume with water at 20 ^{o} C and filter.
2. Dilute 10 ml of this solution to 500 ml with distilled water (diluted honey solution).

5.2 Preparation of test sample, second procedure

(honeys with no sediment)

1. Weigh accurately a representative quantity of about 2 g (W_2) of the homogenous honey sample, dissolve in distilled water and dilute to 100 ml in a calibration flask (honey solution)
2. Dilute 50 ml of the honey solution to 100 ml using distilled water (diluted honey solution)

5.3 Standardization of the modified Fehling's Solution

Standardise the modified Fehling's solution A so that exactly 5 ml (pipette), when mixed with approximately 5 ml of Fehling's solution will react completely with 0.050 g invert sugar as 25 ml dilute invert sugar solution (2 g/L).

5.4 Preliminary titration

The total volume of the added reactants at the completion of the reduction titration must be 35 ml. This is made up by the addition of a suitable volume of water before the titration commences. Since the compositional criteria specify that there should be no more than 60 % reducing sugars (calculated as invert sugar), a preliminary titration is necessary to establish the volume of water to be added to a give sample to ensure the reduction is carried out at constant volume. The volume of water to be added is calculated by subtracting the volume of diluted honey solution consumed in the preliminary titration (X ml) from 25 ml.

Pipette 5 ml Fehling's solution AS into a 250 ml Erlenmeyer flask and add approximately 5 ml Fehling's solution B. Add 7 ml distilled water, a little powdered pumice or other suitable antibumping agent, followed by about 15 ml diluted honey solution from burette. Heat the cold mixture to boiling over a wire gauze, and maintain moderate ebullition for 2 minutes. Add 0.2 % aqueous methylene blue solution whilst still boiling and complete the titration within a total boiling time of 3 minutes, by repeated small additions of diluted honey solution used (X ml).

5.5. Titration

Calculate the amount of added water necessary to bring the total volume of the reactants at the completion of the titration to 35 ml by subtracting the preliminary titration (X ml) from 25 ml.

Pipette 5 ml Fehling's solution A into 250 ml Erlenmeyer flask and add approximately 5 ml Fehling's solution B.

Add (25-X) ml distilled water, a little powdered pumice or other suitable antibumping agent and, from a burette, all but 1.5 ml of the diluted honey solution volume determined in the preliminary titration. Heat the cold mixture to boiling over a wire gauze and maintain moderate ebullition for 2 minutes. Add 1 ml 0.2 % methylene blue solution whilst still boiling and complete the titration within a total boiling time of 3 minutes by repeated small additions of diluted honey solution until the indicator is decolourized. Note the total volume (Y ml). Duplicate titration should agree within 0.1 ml.

6. PROCEDURE FOR APPARENT SUCROSE

6.1 Sample preparation

Prepare honey sample as in 5.1: dilute 10 ml of this solution to 250 ml distilled water or according to 5.2.

Hydrolysis

The honey solution (50 ml) is placed in a graduated flask, together with 25 ml distilled water, heat the test sample to 65 ^{o}C over a boiling water bath. The flask is then removed from the eater bath and 10 ml of hydrochloric acid is added. The solution is allowed to cool naturally for 15 minutes, and then brought to 20 ^{o}C and neutralized with sodium hydroxide, using litmus paper as indicator, cooled again, and the volume adjusted to 100 ml (diluted honey solution)

Titration

As in Sections 5.4 and 5.5.

6. CALCULATION AND EXPRESSION OF RESULTS

6.1. Reducing sugars

Where the first procedure has been used:

$$C = \frac{25}{W1} x \frac{1000}{Y1}$$

Where the second procedure has been used:

$$C = \frac{2}{W2} x \frac{1000}{Y2}$$

Where C = g invert sugar per 100 g honey

$W_{1,,}$ weight (g) of honey sample according to 5.1

W_2 weight (g) of honey sample according to 5.2

Y_1 volume (ml) of diluted honey solution consumed in the determination carried out according to 5.1

Y_2 volume (ml) of diluted honey solution consumed in the determination carried out according to 5.2

Notes on the procedure

It is essential to the accuracy and repeatability of the determination that the volume of water necessary to bring the reactant mixture to a total volume of 35 ml be determined for each individual sample; the following table gives typical volumes which may be encountered at the preliminary titration stage for the increment contents of invert sugar shown, assuming the test sample (first procedure) weighs about 25 g or test sample (second procedure) weighs about 2 g.

Invert Sugar content (%)	**Volume of distilled water to be added**
60	8.3
65	9.6
70	10.7
75	11.6

6.2. Apparent sucrose

Calculate percent invert sugar (g invert sugar per 100 g) after inversion using the appropriate formula as for percent invert sugar before inversion in section 6.1:

Apparent sucrose content a (invert sugar per 100 g honey) after inversion minus (sugar content before inversion) x 0.95

The result is expressed as g apparent sucrose/100 g honey

REFERENCES

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7.2 Determination of sugars by HPLC

1. SCOPE

The method determines fructose, glucose, sucrose, turanose and maltose in honey, for which precision data were acquired. It can also be used for the quantitation of other saccharides such as melezitose, erlose, isomaltose, raffinose and others as described in the originally published method by Bogdanov and Baumann (1).

2. DEFINITION

The proportion of each sugar is defined as that calculated from the formula given in the method.

3. PRINCIPLE

This method is based on the originally published method by Bogdanov and Baumann (1). After filtration of the solution, the sugar content is determined by HPLC (High Pressure Liquid Chromatography) with RI-detection. Peaks are identified on the basis of their retention times. Quantitation is performed according to the external standard method on peak areas or peak heights.

4. REAGENTS

If not stated otherwise, chemicals of analytical purity grade should be used.

The water must be distilled or should be of at least equivalent purity.

Methanol for HPLC

Acetonitrile for HPLC

Warning: Acetonitrile is a dangerous substance. Laboratory safety guidelines on dangerous substances at work should be consulted.

Eluent solution for the HPLC. Mix 80 volumes of acetonitrile with 20 volumes of water. Degass prior to use.

The standard substances, fructose, glucose, sucrose, turanose and maltose can be purchased from the usual suppliers, as can melezitose, raffinose and isomaltose. Turanose can be obtained from Senn Chemicals of Dielsdorf, Switzerland, among others. See Reference (1) for retention times and separation of all honey sugars.

Pipette 25ml methanol into a 100 ml calibrated flask. Depending on the sugars to be analysed, dissolve the amounts detailed below in approximately 40ml water and transfer quantitatively to the flask and fill to the mark with water.

fructose: 2.000 g

glucose: 1.500 g

sucrose: 0.250 g

turanose: 0.150 g

maltose: 0.150 g

Use a syringe and a pre-mounted membrane filter to transfer the solution to sample vials.

The standard solutions are stable for 4 weeks in the refrigerator at 4^0 C and for six months at -18^0 C.

5.EQUIPMENT

Sample vials.

Ultrasonic bath.

Calibrated flasks, volume 100 ml.

25-ml-pipette.

Membrane filter for aqueous solutions, pore size 0.45 µm.

Filter holder for membrane filters with suitable syringe.

High Performance Liquid Chromatograph consisting of pump, sample applicator, temperature-regulated RI-detector thermostated at 30^{o} C*, temperature regulated column oven at 30^{0}C, integrator.

Analytical stainless-steel column, e.g. 4.6 mm in diameter, 250 mm length, containing amine-modified silica gel with 5-7 µm particle size. Before use, carry out a system suitability test to ensure all the sugars can be separated.

* Note: the chromatography can be carried out at room temperature without influence on the results of the sugars, determined by the present method. However under these conditions no separation of erlose and melezitose is possible (1).

6. PROCEDURE

Preparation of samples

If necessary, prepare the honey according to the section Sampling of INTRODUCTION AND GENERAL COMMENTS ON THE METHODS.

Preparation of the sample solution

Weigh 5g of honey into a beaker and dissolve in 40 ml water. Pipette 25ml of methanol into a 100ml volumetric flask and transfer the honey solution quantitatively to the flask. Fill to the mark with water. Pour through a membrane filter and collect in sample vials. Store as for the standard solution.

High Performance Liquid Chromatography (HPLC)

If a column of the type described above is used, the following conditions have been found to give satisfactory separation.

Flow rate: 1.3 ml/min

mobile phase: Acetonitrile:water (80:20, v/v)

column and detector temperature : 30^{0}C

sample volume: 10 µl

Note:

If it is not possible to carry out the analysis at 30^{0}C and if the detector cannot be thermostated at 30^{o}C, carry out the analysis at ambient temperature. In this case it is not possible to separate melezitose and erlose.

Note: Identical volumes of sample and standard solution should be injected.

7. Calculation and Expression of Results

The honey sugars are identified and quantified by comparison of the retention times and the peak area of the honey sugars with those of the standard sugars.

The mass percentage of the sugars, W, to be determined of fructose, glucose, etc.. and maltose in g/100g is calculated according to the following formula (external standard procedure):

$$W = A_1 \times V_1 \times m_1 \times 100 / A_2 \times V_2 \times m_0$$

Where

A_1 = Peak areas or peak heights of the given sugar compound in the sample solution, expressed as units of area, length or integration.

A_2 = Peak heights of the given sugar compound in the standard solution, expressed as units of area, length or integration.

V_1 = Total volume of the sample solution in ml

V_2 = Total volume of the standard solution in ml

m_1 = Mass amount of the sugar in grams in the total volume of the standard (V_2)

m_0 = sample weight in g

The result is rounded to one decimal place.

8. PRECISION OF THE PROCEDURE

The parameters r and R were determined in a DIN ring trial (2).

Sample No.	Fructose g/100 g	r	R
1	31.2	0.8	1.6
2	42.4	0.9	2.3
3	37.9	1.0	1.6

Sample No	Glucose g/100 g	r	R
1	23.0	09	2.1
2	28.5	0.8	1.8
3	32.0	1.1	1.4

Sample No	Sucrose g/100 g	r	R
1	-	-	-
2	-	-	-
3	2.8	0.4	0.9

Sample No	Turanose g/100 g	r	R
1	2.1	0.4	0.8
2	1.7	0.3	0.5
3	1.3	0.3	0.8

Sample No	Maltose g/100 g	r	R
1	4.8	0.5	2.5
2	2.0	0.6	1.3
3	2.3	0.5	0.7

The repeatability and reproducibility have been calculated from the results on three types of honey analysed by all laboratories collaborating in the study.

REFERENCES

1. S. Bogdanov, S. E. Baumann, Bestimmung von Honigzucker mit HPLC. Mitt.Gebiete Lebensm.Hyg., 79, 198-206. (1988).
2. DIN Norm 10758 , Bestimmung des Gehaltes an Sacchariden. HPLC Verfahren. (1992).

7.3 Determination of sugars by GC

1. SCOPE

The method determines the predominant sugars in honey.

2. DEFINITION

The proportion of each sugar is defined as that calculated from the formula given in the method.

3. PRINCIPLE I.N.A. AND PIERCE - POURTALLIER DERIVATISATION

In the Pierce-Portallier method any sugars with free aldehyde or ketone groups such as glucose and fructose are converted to their oximes (1). In the I.N.A. method this step is omitted (2).

The sugars alone or with their oximes are silylated and the derivatives separated and quantified by gas chromatography using mannitol as the internal standard.

4. REAGENTS

Mannitol (internal standard), from Carlo Erba or Fluka or equivalent quality.

Sugar standards.

Pipette 25ml methanol into a 100 ml calibrated flask. Depending on the sugars to be analysed, dissolve the amounts detailed below in approximately 40ml water and transfer quantitatively to the flask and fill to the mark with water.

fructose: 2.000 g

glucose: 1.500 g

sucrose: 0.250 g

turanose: 0.150 g

maltose: 0.150 g

Use a syringe and a pre-mounted membrane filter to transfer the solution to sample vials.

The standard solutions are stable for 4 weeks in the refrigerator at 4^0 C and for six months at -18^0 C.

Silanizing agent (5 parts of pyridine + 2 parts of hexamethyldisilazane + 1 part of trimethylchlorosilane). The commercially available Silon, in proprtions 9:3:1 can also be used).

Hexamethyldisilazane,

Trifluoracetic acid,

Oxime reagent (pyridine solution containing 12 mg/ml hydroxylamine hydrochloride).

Hexane.

5.EQUIPMENT

Gas chromatograph fitted with an SE 52 capillary column or equivalent and a flame ionization detector. The fused silica capillary column is 25m, 0.32 mm i.d., 0.1 - 0.15 μ film thickness. Use a carrier gas flow of 4ml/min of hydrogen.

Table-top centrifuge.

Analytical balance with a precision of 0.001 g,

Laboratory oven.

6.PROCEDURE

Preparation of samples

If necessary, prepare the honey according to the section Sampling of INTRODUCTION AND GENERAL COMMENTS ON THE METHODS.Determination

Dissolve 3 grams of honey in distilled water and transfer to a 500 ml volumetric flask. Add a known concentration of internal standard solution (e.g. 5.0 ml of a 10% w/v mannitol solution) and fill to the mark with distilled water. Mix well and transfer 100 µL of the solution to a conical bottomed test tube and allow it to dry out in a current of nitrogen at a temperature of 50^0C.

Pierce-Portallier method.

Add 200 µL of oxime reagent and then seal with a screw-on plug. Mix well and heat at 70 - 75 °C for 30 minutes. Cool sample to room temperature. Add 100 µL hexamethyldisilazane and mix. Add 10 µL trifluoroacetic acid, seal the test tube and let stand for 30 minutes. Centrifuge at 6000 rpm for several seconds. Inject 0.6 µL on to the column with the injection port at 70^0C. Programme the column at 49^0C/min to 140^0C and then at 6^0C to 300^0C.

I.N.A. Method.

Add 500 µL of silanizing solution and then seal with Parafilm® or, alternatively, with a screw-on plug. Mix well (preferably using an ultrasonic bath) and allow to stand overnight in a desiccator or better, in a shaker. The following day allow to dry in a current of nitrogen at a temperature of about 75 °C. Add hexane, stir and then centrifuge at about 5000 rpm for several seconds. Inject 0.6 µL and proceed as above.

7. CALCULATION AND EXPRESSION OF RESULTS

Concentration values are calculated by the internal standard method on the basis of the response factors obtained by introducing a mixture of sugar standards at known concentrations prepared according to the above-described procedure. The results are expressed in g sugar per 100 g honey.

8. PRECISION

In a ring trial of the International Honey Commission with 9 laboratories in total the following precision parameters were obtained. The values are in g/100 g

I.N.A. METHOD (6 Labs)

MULTIFLORAL HONEY

	$\bar{x}$	**r**	**R**
Fructose	39.4	0.751	7.591
Glucose	31.4	0.221	6.556
Sucrose	0.26	0.028	0.596

P.P.METHOD (3 Labs)

MULTIFLORAL HONEY

	$\bar{x}$	**r**	**R**
Fructose	38.2	3.335	5.012
Glucose	31.3	1.365	2.878
Sucrose	0.45	0.100	1.494

ACACIA HONEY

	$\bar{x}$	r	R
Fructose	38.5	0.942	4.222
Glucose	25.7	1.260	1.891
Sucrose	7.69	0.492	1.560

ACACIA HONEY

	$\bar{x}$	r	R
Fructose	36.6	0.872	3.804
Glucose	25.2	0.874	0.874
Sucrose	8.60	0.335	1.751

HONEYDEW HONEY

	$\bar{x}$	r	R
Fructose	31.4	0.660	7.559
Glucose	23.8	0.893	5.522
Sucrose	0.25	0.069	0.414

HONEYDEW HONEY

	$\bar{x}$	r	R
Fructose	30.0	0.540	2.838
Glucose	23.8	0.447	0.555
Sucrose	0.14	0.068	0.195

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7.4 Determination of honey sugars by H.P.LC. with pulsed amperometric detection

1. SCOPE

The method determines the predominant sugars in honey.

2. DEFINITION

The proportion of each sugar is defined as that calculated from the formula given in the method.

3. PRINCIPLE

Sugars (pKa between 12 and 13) behave as very weak acids at high pH (12-14) and are partially or totally ionized. They can therefore be separated by an ion-exchange mechanism.

It is necessary to use a non porous pellicular resin column (strong anion exchange) with the following properties: fast mass transfer and diffusion, high stability to pH variations (0-14) and good mechanical stability (4000 psi).

The sugars are eluted with a sodium hydroxide solution and detected by a pulsed amperometric system with a stable and sensitive response due to the continuous elimination of the sugar oxidation products.

4. REAGENTS

Ultra-pure and carbon dioxide free water (18 MΩ)

50% sodium hydroxide solution

It is very important to avoid sodium hydroxide pellets which are always carbonated. Carbonate has a very high eluting power inducing an important decrease in efficiency and resolving power of the column. (Sodium carbonate is insoluble in 50% sodium hydroxide solution).

Sugars samples of high purity.

5. EQUIPMENT

H.P.L.C. Chromatograph (e.g. Dionex Bio LC 40001) equipped with:

degassing module (Helium)

column : Carbopac-AS6 (Dionex)

precolumn: Carbopac guard P.A.

injection loop: 25 µL

pulsed amperometric detector with:

- gold measuring electrode

- Ag/AgCl reference electrode

- vitreous carbon counter electrode

- detection cell volume: 9 µL

Integrator (e.g. Shimadzu C-R5A Chromatopac).

Analytical balance.

25 ml beakers.

100 ml volumetric flasks.

5 ml tubes with stoppers.

Freezer (-18°C).

1000 ml volumetric flask.

20 ml pipette, graduated to 0.1 ml.

1 ml disposable LUER syringe.

0.02 µm disposable syringe filters (Anotop 10 e.g.).

6. PROCEDURE

Preparation of samples

If necessary, prepare the honey according to the section Sampling of INTRODUCTION AND GENERAL COMMENTS ON THE METHODS.

Preparation of the eluting solution B

Fill the 1L flask with about a half litre of ultra-pure water. Transfer 16 ml of the sodium hydroxide solution by graduated pipette quantitatively to the flask. After cooling complete to the mark with ultra-pure water. Mix well.

Preparation of the standard solution

Weigh exactly the corresponding mass of the first sugar (see table below) and dissolve it in a 25 ml beaker with a few millilitres of ultra-pure water. Transfer quantitatively to a 100 ml flask. Repeat the operation with the other sugars, transferring them quantitatively into the same 100 ml flask. Complete to the mark with ultra-pure water. Mix well. Divide the solution into 5 ml stoppered tubes and immediately place in the freezer. Discard after 3 months, and refrigerated tubes after one week.

An example of the composition of the standard solution is given in the table below.

Sugar	**mass (mg)**	**Sugar**	**mass (mg)**
Trehalose	13,0	Turanose	21,2
Glucose	34,5	Melezitose	13,9
Fructose	41,3	Raffinose	14,4
Isomaltose	13,2	Maltose	11,2
Sucrose	23,8	Erlose	8,4

Calibration (external)

Pipette 1 ml of the standard solution with a syringe. Insert a 0.02µm filter and inject the solution into the loop of the chromatograph. Run the chromatograph with the following conditions:

composition of eluting gradient:

- solution A, water: 45%

- solution B: 55%

flow : 0.5 ml/min for 16 min, then gradient flow at +0.1 ml/min for 5 min, then 1 ml/min for 16 min

detector conditions:

Oxidation potential	**E1 = +0.18 V**	**t1 = 300 ms**
cleaning potential	E2 = +1.00 V	t2 = 240 ms
reducing potential (desorption of oxidised products	E3 = -0.95 V	t3 = 300 ms

After the separation (40 min) introduce the values of sugar concentrations so that the integrator calculates the response factor of each sugar.

Sample analysis

Weigh exactly about 200 mg of honey and dissolve it in a 25 ml beaker with a few millilitres water. Transfer quantitatively to a 100 ml volumetric flask. Complete to the mark with water. Mix well.

Pipette 1 ml into a Luer syringe. Insert a 0.02 μm filter and inject into the loop of the chromatograph. Run the chromatograph with the same conditions as for the standard solution.

7. CALCULATION AND EXPRESSION OF RESULTS

These are calculated by the external standard method and are given with one decimal directly by the integrator after introduction of the sample weight.

It has been established that the detector gives a linear response in the following ranges:

Glucose	0 - 70 μg (injected)
Fructose	0 - 90 μg (injected)
Sucrose	0 - 90 μg (injected)

Therefore the concentration of the honey solution injected must be lower than 6 g/L.

8. Precision

In a ring trial of the International Honey Commission with 7 laboratories in total the following precision parameters were obtained. The values are in g/100 g.

Honey A

	$\bar{x}$	**r**	**R**
Fructose	38.9	1.18	7.83
Glucose	26.9	0.92	5.88
Sucrose	2.36	0.38	0.47

Honey B

	$\bar{x}$	**r**	**R**
Fructose	36.9	1.74	7.66
Glucose	28.2	0.56	6.21
Sucrose	-		

Honey C

	$\bar{x}$	**r**	**R**
Fructose	36.3	2.13	6.08
Glucose	29.6	1.57	6.01
Sucrose	1.23	0.19	0.43

REFERENCE

1. J. Pourtallier, C. Rognone and R. Davico, Une nouvelle technique d'analyse des sucres des miels par chromatographie liquide à haute performance, L'Abeille de France, n°754, 448-451, (1990).
2. K. W. Swallow, and N. H. Low, Determination of honey authenticity by anion-exchange liquid chromatography J. AOAC Internat. 77, 3: 695-702. (1994)

8 Determination of insoluble matter

1. SCOPE

The method can be applied to all honey samples.

2. DEFINITION

Insoluble matter is defined as that material found by the procedure to be insoluble in water. The result is expressed as a percentage by weight.

3. PRINCIPLE

The insoluble matter is collected on a crucible of specified pore size and the dried residue is weighed after being washed free of soluble material.

4. EQUIPMENT

Analytical balance, to 0.1mg.

Sintered glass crucible, pore size 15 to 40 microns.

Drying oven at 135 ± 1^0C.

5. PROCEDURE

Accurately weigh approximately 20 grams of honey and dissolve in about 200ml of water at about 80^0C. Mix well.

Dry a crucible in the oven and leave to obtain ambient temperature in a desiccator containing an efficient desiccant such as silica gel. Weigh the crucible.

Filter the sample solution through the crucible. Wash carefully and extensively with warm water until free from sugars. Check by adding to some filtrate in a test tube some 1% phloroglucinol in ethanol. Mix and run a few drops of concentrated sulphuric acid down the sides of the tube. Sugars produce a colour at the interface.

Dry the crucible at 135^0C for an hour, cool in the desiccator and weigh. Return to the oven for 30 minute intervals until constant weight is obtained.

6. CALCULATION AND EXPRESSION OF RESULTS

$$\% \text{ Insoluble Matter in g/100 g} = \frac{m}{m_1} x100$$

where m = mass of dried insoluble matter and

m_1 = mass of honey taken

7. PRECISION

The precision of the method was determined in the UK collaborative study. The values are in g/100 g.

Mean, $\bar{x}$	0.021	0.009	0.031	0.011
Repeatability (r)	0.016	0.016	0.023	0.010
Reproducibility (R)	0.021	0.016	0.023	0.026

REFERENCES

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9 Determination of invertase activity

1. SCOPE

The method can be applied to all honey samples.

2. DEFINITION

The invertase activity is expressed in units, where one unit is defined as the number of micromoles of substrate destroyed per minute and expressed per kilogram of honey. The activity can also be expressed as the Invertase Number, calculated as described under CALCULATIONS AND EXPRESSION OF RESULTS.

3. PRINCIPLE

p-Nitrophenyl-α-D-glucopyranoside (pNPG) is used as a substrate for the determination of the sucrase number in honey. pNPG is split into glucose and p-nitrophenol by α-glucosidase (invertase, sucrase). By adjusting the pH-value to 9.5 the enzymatic reaction is stopped and at the same time nitrophenol is transformed into the nitrophenolate anion, which corresponds to the amount of converted substrate and is determined photometrically at 400 nm. (2).

4. REAGENTS

Buffer solution (0.1 M; pH = 6.0) : Dissolve 11.66 g of potassium hydrogen phosphate KH_2PO_4 and 2.56 g of disodium hydrogen phosphate $Na_2HPO_4.2H_2O$ in water and dilute to 1L.

Substrate p-nitrophenyl-α-D-glucopyranoside (pNPG) solution, (0.02 M). Dissolve 6.0252 g of pNPG (e.g. Fluka) in buffer solution and make up to 1L. pNPG is sparingly water soluble but the solution is not very stable. Dissolve by heating the buffer solution not above 60^{o}C and cool immediately solution is complete. The solution can be stored in a dark bottle in the refrigerator for up to one month.

Reaction-terminating solution (3 M, pH = 9.5). Dissolve 363.42 g of tris- (hydroxymethyl) aminomethane in water and dilute to 1L. Adjust to a pH-value of 9.5 with 3M hydrochloric acid.

5. EQUIPMENT

Photometer (set at 400 nm)

Thermostated water bath (40 ± 0.5 °C).

Vortex mixer or similar.

pH-meter.

6. PROCEDURE

Preparation of samples

If necessary, prepare the honey according to the section Sampling of INTRODUCTION AND GENERAL COMMENTS ON THE METHODS.

Determination

Honey solution: transfer 5.00 g of honey with buffer solution quantitatively into a 25- ml flask and fill to the mark. This solution can be kept in the refrigerator for 1 day.

Place 5.0 ml of substrate solution in a test tube or a plastic tube in the water bath at 40°C for 5 minutes before adding the honey solution.

Add 0.50 ml of honey solution (starting time).

Mix the contents briefly in a mixer and incubate at 40 °C.

After exactly 20 minutes add 0.50 ml of the reaction-terminating solution and mix again in a mixer (sample solution).

For the blank, incubate 5.0 ml of substrate solution at 40 °C at the same time. After five minutes add 0.50 ml of reaction-terminating solution, stopper the tube, mix well and then add 0.50 ml of honey solution. Prepare a separate blank for each honey tested.

Cool the solutions to room temperature as quickly as possible and measure the absorbances of the sample solutions and the blank in 1-cm cells at 400 nm. The readings should be taken after about 15 minutes and in any case within one hour. Substract the absorbance of the blank from that of the sample solution (=ΔA_{400}).

7. CALCULATIONS AND EXPRESSION OF RESULTS

The amount of p-nitrophenol in µM produced during the test corresponds exactly to the amount of substrate in µM utilised. Therefore, the honey invertase activity can be calculated from the absorbance measured at 400 nm and is indicated in units/kg (U/kg):

$$1U / kg = \frac{1\mu mol \ \ p - NPG}{minutes \ x \ kg \ honey}$$

Invertase in U/kg = 6 x 0.05 x 0.05298 x 10^4 x ΔA_{400}

= 158.94 x ΔA_{400}

Where

U = 1 international unit with a defined utilisation of 1 µM per minute

6 = factor for the ml of sample solution used (total volume).

0.05 = converts reaction time from 20 minutes to 1 minute.

10^4 = converts the amount of honey taken (0.1 g in 0.5 ml) to 1 kg.

0.05298 = 7.37/139.11; conversion factor for µg into µM per ml,

where 7.37 = factor for p-nitrophenol from the corresponding graph

139.11 = molecular weight of p-nitrophenol.

Invertase activity is expressed as the invertase number:

It is common usage to express the invertase activity as invertase number (IN). The IN indicates the amount of sucrose per g hydrolysed in 1 hour by the enzymes contained in 100 g of honey under test conditions (see Hadorn (2)). If the invertase activity is determined simultaneously by the method described above and by the polarimetric method according to Hadorn et al.(1966), the following relation between IN and ΔA_{400} results:

IN = 21.64 x ΔA_{400}

21.64 = slope of linear regression of IN (y axis) on ΔA_{400} (x axis). Report the result to one decimal place.

8. PRECISION

An interlaboratory trial of the International Honey Commission was carried out with 19 laboratories. With four different honeys (acacia honey, blossom honey, honeydew honey, yucatan honey) and triplicate determinations the following results were obtained, using the robust statistic method.

Invertase number (average)	**r**	**R**
6.5	0.26	1.8
7	0.33	1.2
9.4	0.48	1.8
17.7	0.58	1.3

REFERENCES

1. U. Siegenthaler, Eine einfache und rasche Methode zur Bestimung der α-Glucosidase (Saccharase) im Honig. Mitt. Geb. Lebensmittelunters. Hyg. 68, 251-258 (1977).
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3. S. Bogdanov and P Lischer, International Honey Commission Interlaboratory trials: diastase activity, Phadebas and Schade methods, Invertase activity after Siegenthaler and Humidity by refractometry. Report for the participants, 1993.

Note

Recent comparative studies, carried out in Germany (Von der Ohe, Nds. Landesinstitut für Bienenkunde) showed, that the correlation between the Hadorn invertase number (2) and the measurement with the above method depends very much on the honey type. Due to the presence of other enzymes in honey side reactions occur during the determination, and make comparisons of the results difficult. That is why it is proposed, that the results be expressed in international units (Siegenthaler method).

W. von der Ohe et al., Comparison of methods for determination of saccharase

of invertase activity, Apidologie 30 (5) 1999: 412-413

10 Determination of proline

1. SCOPE

The method can be applied to all honey samples. The content of proline is an indication of quality in honeys and an indication of adulteration when it falls below a certain value.

2. DEFINITION

The content of proline is defined as the colour developed with ninhydrin compared with a proline standard and expressed as a proportion of the mass of honey in mg/kg.

3. PRINCIPLE

Proline and ninhydrin form a coloured complex. After adding 2-propanol, the extinction of the sample solution and a reference solution at a wavelength maximum is determined. The proline content is determined from the ratio. The method is based on the original method of Ough (1).

4. REAGENTS

Analytical grade pure chemicals must be used.

Water should be distilled or of corresponding purity.

Formic acid (H.COOH), 98 to 100 %.

Solution of ninhydrin in ethylene glycol monomethylether (methyl-cellosolve), 3% by volume.

Proline reference solution. Prepare an aqueous proline stock solution containing 40mg/50ml. Dilute 1ml to 25ml with water to give a solution containing 0.8mg/25ml

2-propanol, 50 % by volume in water.

5. EQUIPMENT

Spectrophotometer (recording, if possible) measuring in the range of 500 to 520 nm.

Cuvettes, 1 cm.

Tubes with screw cap or stopper, nominal volume 20 ml

Measuring flask, nominal volume 100 ml

Water bath.

6. PROCEDURE

Sample preparation.

If necessary, prepare the honey according to the section Sampling of INTRODUCTION AND GENERAL COMMENTS ON THE METHODS.

Preparation of the sample solution.

Weigh to the nearest mg about 5g honey into a beaker and dissolve in 50 ml water, quantitatively transferring to a 100 ml volumetric flask. Dilute to volume with water and shake well.

Determination.

Note that the coefficient of extinction is not constant. Therefore, for each series of measurements the average of the extinction coefficient of the proline standard solution must be determined at least in triplicate.

Pipette by means of an accurate syringe 0.5 ml of the sample solution in one tube, 0.5 ml of water (blank test) into a second tube and 0.5 ml of proline standard solution into three other tubes. Add 1ml of formic acid and 1ml of ninhydrin solution to each tube. Cap the tubes carefully and shake vigorously for 15 minutes. Place in a boiling water bath for 15 minutes, immersing the tubes below the level of the solution. Transfer to a water bath at 70^0C for 10 minutes. Add 5ml of the 2-propanol-water-solution to each tube and cap immediately. Leave to cool and determine the absorbance 45 minutes after removing from the 70^0 water bath at the maximum near 510 nm, using 1cm cells.

Note: Adherence to the above times is critical.

7. CALCULATION

Proline in mg/kg honey at one decimal place is calculated according to following equation:

$$\text{Proline (mg/kg)} = \frac{E_S}{E_a} \text{ x } \frac{E_1}{E_2} \text{ x} 80$$

Where

E_S = Absorbance of the sample solution

E_a = Absorbance of the proline standard solution (average of two readings),

E_1 = mg proline taken for the standard solution

E_2 = Weight of honey in grams.

80 = Dilution factor

8. PRECISION

The values for repeatability (r) and reproducibilty (R) have been obtained in ring trials according to the DIN norm (2)

Proline (average) mg/kg	**r**	**R**
171	6.6	16.3
289	12.7	18.4
762	24.4	58.4

REFERENCES

1. C.Ough, Rapid determination of proline in grapes and wines, J.Food Science, 34, 228-230 (1969) DIN Norm 10754 (Entwurf 1991) Bestimmung des Prolingehalts von Honig

11 Determination of specific rotation

1. SCOPE

The method can be applied to all honey samples. In particular, most of the honeydew honeys have positive values of specific rotation whereas nectar honeys have negative values.

2. DEFINITION

The specific optical rotation, $[\alpha]_D^{20}$ is the angle of rotation of polarised light at the wavelength of the sodium D line at 20^0C of an aqueous solution of 1 dm depth and containing 1g/ml of the substance.

3. PRINCIPLE

The angular rotation of a clear, filtered aeqous solution is measured by means of a polarimeter. The value is related to the carbohydrate composition. This procedure is based on published methods (1,2)

4. REAGENTS

Carrez I solution. Dissolve 10.6 g potassium hexacyanoferrate(II), ($K_4Fe(CN)_6$ $3H_2O$) in distilled water and dilute to 100 ml.

Carrez II solution: dissolve 24 g zinc acetate ($Zn(CH_3COO)_2 \cdot$ $2H_2O$) in distilled water, add 3 g of glacial acetic acid and dilute to 100 ml with distilled water.

5. EQUIPMENT

Polarimeter capable of angular rotation measurements accurate to within 0.05 circular degrees, equipped with sodium lamp and 2-dm tube.

6. PROCEDURE

Sample preparation.

If necessary, prepare the honey according to the section Sampling of INTRODUCTION AND GENERAL COMMENTS ON THE METHODS.

Determination.

Weigh 12 g honey (corresponding to about 10 g dry substance), dissolve it in distilled water, add 10 ml of Carrez I solution and mix thoroughly for 30 seconds. Add 10 ml Carrez II solution, mix again for 30 seconds and make up to volume in a 100 ml volumetric flask with distilled water. The next day, filter the solution, rinse and fill a clean 2-dm polarimeter tube with the solution, place the tube in the polarimeter and read the angular rotation (α). Measurements must be taken at a temperature of 20^0C.

7. CALCULATION AND EXPRESSION OF RESULTS

Specific angular rotation $[\alpha]_D^{20} = \dfrac{\alpha \cdot x \cdot 100}{l \cdot x \cdot p}$

where α = angular rotation found,

l = length in decimetres of the polarimeter tube

p = grams of dry matter taken

Results are at one decimal place

REFERENCES

1. W.R., Junk and H.M., Pancoast - Handbook of sugars. Avi Publ. Co. Inc., Westport, Connecticut, USA: 295-296; (1973).
2. M. Battaglini and G.Bosi - Caratterizzazione chimico-fisica dei mieli monoflora sulla base dello spettro glucidico e del potere rotatorio specifico. - Scienza e tecnologia degli Alimenti, 3, (4): 217-221) (1973).

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