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Characterization of filtered honey by electrophoresis of enzyme fractions*

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Abstract – According to both the European Council's Honey Directive 2001/110/EC and the present Codex Aliminerius Honey Standard, filtration of honey is permitted. After this filtration process, the microscopic determination of the botanical and geographical origin of honeys is no longer possible since all the pollen has been removed. In many honey countries, there is a considerable difference in the price of honey depending on the botanical and geographical origin. There is the risk of fraud if expensive unfiltered honey is mixed with cheap filtered honey. In this research project, a method was developed that allows the detection of mixtures of filtered and unfiltered honey. Comparative tests showed that enzyme activities, mainly sucrase, were influenced by this process. The protein content did not decrease use isolated by gel chromatography and analysed by gel electrophoresis. One of the wordominating protein bands with 40 kDa and 65 kDa decreased significantly after filtration, which led to a shift in the natural ratio between them. The quantitative densitometric analysis of these two protein bands allows the detection of 15% added filtered honey.

honey / filtration / enzyme / protein / electrophoresis

1. INTRODUCTION

In the former German Honey Regulation, it was not permitted to remove natural components. With the EC Honey Directive No. 2001/110/EC, it is now possible to market "filtered honey", meaning honey from which foreign organic and inorganic matter was removed "in such a way as to result in the significant removal of pollen" (Annex I, point 2 (b) (viii)). Thus, it is possible to bring honey on the market from which natural constituents have been removed. However, such a product must then be called "filtered honey".

According to Art. 2, Point 2 (b), the product names of unfiltered honeys "may be supple-

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mented by information referring to (1) floral or vegetable origin if the product comes wholly or mainly from the indicated source and possesses the organoleptic, physico-chemical and microscopic characteristics of the source or (2) regional, territorial or topographical origin if the product comes entirely from the indicated source and (3) specific quality criteria".

In the Preamble to the European Directive, No. 7, it is stated explicitly that honeys, which include the indications named above, "may not have filtered honey added to it".

The reason for honey producers to apply such filtration techniques is to stop the crystallization process and to remove small impurities. Liquid honey has a tendency to crystallize, depending on the honey type. This results in cloudy and unsightly products. Furthermore, the growth of osmotolerant yeasts

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is supported because the crystal water is made available through crystallisation and increases the water activity of honeys. Yeasts can accelerate honey fermentation (Beckh and Luellmann, 1999). The crystallization process depends on the saturation level of glucose: the higher the glucose/water ratio, the faster the honey can crystallize due to the supersaturation of glucose (Bhandari et al., 1999). However, a higher concentration of fructose can reduce crystallization (White, 1978).

In the literature, different methods are described for avoiding the crystallization process to keep honey in a liquid state. The further handling of honey is simplified, too. In 1931, Dyce suggested pasteurizing honeys by warming up the product step by step, initially at 49 °C and then at 66 °C to destroy sugar crystals. Already existing yeast cells are destroyed as well. This process is still used in different forms in many countries (Luellmann and Horn, 2006).

Assil et al. (1991) modified this method. They reported that honeys stay liquid over a period of two years when they are heated up initially to 77 °C, cooled down to room temperature rapidly, and stored for at least five weeks at 0 °C.

Kaloyereas and Oertel (1958) showed that the use of ultrasonic waves (9 kHz/s for 15–30 min) produces similar effects. Honeys treated in this manner remain stable for 15 months.

By means of very fine filtration, it is possible to remove crystallization nuclei such as pollen, other foreign particles, and glucose crystals. The first method to filter honeys was described by Lothrop and Paine (1934). While the procedures and the equipment have since changed, the general process is still used by most of the honey packers in the USA (Townsend, 1976). The technical procedure is that the honey is homogenized at 45 °C and then warmed up initially to 70-80 °C for a few minutes to reduce viscosity. Filtration is carried out with membranes that have a pore size of 20 µm (pressure: 3-5 bar), and diatomaceous earth is added as an active filter aid. Afterwards, the honey is cooled down to bottling temperature.

Microscopic pollen analysis (melissopalynology) is the decisive method for the botanical and geographical authenticity of honey. The pollen spectrum and the pollen percentage of main nectar sources give an unequivocal indication of the honey's origin. Also, frauds can be uncovered when expensive honeys are blended with cheaper honeys. These practices are of high economic interest as honeys of a specific botanical or geographical origin are marketed at higher prices. Thus, melissopalynology serves consumer protection as well as the protection of good manufacturing practices.

If a large part of the pollen is removed by filtration, the microscopic identification of a honey's botanical and geographical origin is not longer possible, meaning that the authenticity of the honey cannot be tested. For this reason, the Agricultural Committee of the Federal Assembly of Germany demanded in 2003 that analytical methods needed to be developed for determining blends of filtered with unfiltered honeys.

2. MATERIALS AND METHOD

2.1. Samples

42 honey samples of different botanical and geographical origin were used in this study (see Tab. I). Each sample was available in unfiltered and originally filtered form to compare the honeys directly. Sample extraction was carried out directly before and after the filtration process.

2.2. Comparison of filtered and unfiltered honeys

2.2.1. Melissopalynology

Filtered and unfiltered honeys were compared using the method by Louveaux et al. (1970), where sediments of honey solutions are analysed microscopically.

2.2.2. Enzyme activities

The determination of diastase and sucrase (invertase) activities were carried out photometrically using DIN 10750 and DIN 10759-1. The activity of glucose-oxidase was determined qualitatively with Peroxide-Teststrips purchased from Merck.

Botanical origin	Geographical origin			
Acacia	Romania			
Acacia	South East Europe			
Acacia	South East Europe			
Acacia	South East Europe			
Eucalyptus	Australia			
Eucalyptus	South America			
Eucalyptus	USA			
Clover	Argentina			
Clover	Argentina			
Clover	New Zealand			
Clover	New Zealand			
Clover	USA			
Clover	USA			
Clover	USA			
Lime	Bulgaria			
Lime	Romania			
Lime	South East Europe.			
	South America			
Rape	Germany			
Rape	Austria			
Rape	East Europe			
Rape	Czech Republic			
Sunflower	Argentina			
Sunflower	East Europe			
Sunflower	South East Europe			
Sunflower	Ukraine			
Sunflower	Hungary			
Forest/Honeydew	Italy			
Forest/Honeydew	Italy			
Forest/Honeydew	Spain			
Forest/Honeydew	South America			
Forest/Honeydew	South America			
Polyflora	Argentina			
Polyflora	Brazil			
Polyflora	Bulgaria			
Polyflora	Denmark			
Polyflora	Mexico			
Polyflora	South-, Middle- America			
Polyflora	South East Europe			
Polyflora	USA			
Polyflora	USA			
Heather (Erika)	Spain			
Heather (Calluna)	Germany			

Table I. Botanical and geographical origin of honey samples used in this study.

2.2.3. Further parameters

Filtered and unfiltered honeys were also compared with regard to sugar profiles (method: DIN 10758), HMF (DIN 10751), electrical conductivity (10753), pH, and free acids (DIN 10756). Furthermore, UV- and IR-spectra of honey solutions (1:1 and 1:10 with ultrapure water) were recorded. Flavonoids and phenolic acids were measured using the method of Trautvetter et al. (2009).

2.2.4. Protein content

The protein contents of the honey samples were determined by the Bradford method (1972) using Bradford Protein Assay (BioRad, No. 500-0201). The honey samples were diluted with water 1:40. Then 2 mL of the solution were mixed with 2 ml Bradford's dye reagent, and after 5 min. UV measurement was carried out at 595 nm.

2.3. Gelchromatographic separation of honey enzymes

2.3.1. Equipment

We used a Merck Superperformance 10 column (600 \times 10.0 mm) and a Toyopearl HW-55S gel (Tosoh Bioscience, No. 14686) with a Merck L4250 UV-V is detector.

2.3.2. Sample preparation

4 g of honey were dissolved in 25 mL ultrapure water and filtered. 20 mL of the solution were concentrated using centrifugal concentrators (Vivaspin 20 from Sartorius, No. VS2002) with a molecular weight cut-off of 10 kDa. The samples were centrifuged at 4000 rpm to a final volume of 5 mL.

2.3.3. GPC analysis

2 mL of the rententate were injected into the above Gel Permeation Column (GPC). 0.1 mphosphate buffer (11.66 g KH₂PO₄ and 2.56 g Na_2HPO_4 in 1000 mL water, pH 6.1) was used as eluent at a flow rate of 2.5 mL/min. The detection wavelength was 280 nm and the total analysis time was 90 min.

Standard substances of diastase (Fluka, No. 09962), sucrase (Fluka, No. 57629) and glucose-oxidase (Sigma-Aldrich, No. G7141) were used to identify honey enzymes. Furthermore, the peaks were fractionated, and each solution was analysed with regard to enzyme activities, using the methods described in Section 2.2.

2.4. Electrophoresis of sucrase fractions

2.4.1. Equipment

We used a Bio-Rad Mini-Protean III electrophoresis system with a Ready Gel 4–20% Tris/HCl (BioRad, No. 161-1105).

2.4.2. Sample preparation

The GPC sucrase fraction was concentrated with Vivaspin 20 to approximately 0.6 mL. 100 μ L of the concentrate were mixed with 50 μ L Roti-Load 1 containing Laemmli-buffer and β -mercaptoethanol (Roth, No. K929.1) and homogenized. The mixture was heated at 95 °C for 5 min., and 20 μ L of the solution were used for electrophoresis. 10 μ L of Roti-Mark protein standard (Roth, No. T851.1) were used as a marker for the determination of the molecular weight of the proteins in the samples.

2.4.3. Electrophoresis

30 mL of electrophoresis buffer $(10 \times \text{Tris/Glycine/SDS}, \text{Bio-Rad}, \text{No. 161-0732})$ were dissolved in 270 mL ultrapure water. The electrophoresis was carried out at 200 V for 45 min, then the gel was added to a fixation solution (40 mL methanol + 10 mL acetic acid + 50 mL ultrapure water) for 15 min, and afterwards dyed with a Coomassie solution (Rotiphorese Blue R from Roth) for 2 h. Lastly, colour removal was carried out with fixation solution for 3 h.

2.4.4. Evaluation

A photograph of the freshly prepared gel was taken with a CCD camera (Power Shot G7 from Canon). The analysis of the gel pattern was carried out densitometrically with the software Gelscan 5.1 for Windows (purchased from BioSciTec GmbH, Hanauer Landstr. 521, D-60386 Frankfurt/Main). In this process, the colour intensity and thus the concentration of the proteins bands was measured. The molecular weights of the proteins were calculated by means of the protein standard. **Table II.** Loss of diastase and sucrase activities and protein contents after filtration.

	Loss after filtration			
Diastase activity	$10\% \pm 5\%$			
Sucrase activity	$90\% \pm 8\%$			
Protein content	$8\% \pm 3\%$			

3. RESULTS AND DISCUSSION

The first aim of our study was to compare filtered and unfiltered honeys (84 samples) in regard to their pollen spectrum, enzyme activities of diastase and sucrase, protein content, HMF, sugar profiles, electrical conductivity, pH, free acids, UV- and IR-absorption, flavonoids, and phenolic acids. It was shown that except for the pollen spectra, enzyme activities, and HMF content, no differences could be detected between unfiltered and its filtered honeys.

Using melissopalynology, no pollen was found in filtered honeys, as expected.

After filtration, the amounts of HMF were higher, resulting from the honey being heated prior to the filtration process.

Diastase activities decreased in all the filtered honey samples (see Tab. II), but the reduction was insufficient for distinguishing between filtered and unfiltered honeys due to the large natural variation of this parameter.

The decrease of sucrase activities in filtered honeys was much higher than that of diastase (see Tab. II). In this case, a differentiation between filtered and unfiltered honeys is difficult too since sucrase is more heat-sensitive than diastase. The sucrase activity can be influenced by many external factors, such as the heating processes during honey bottling or transport.

Enzymes consist mostly of proteins. Since different changes in the activities of diastase and sucrase were noticed after filtration, the amount of honey proteins was analysed in the next step. The question to be answered was if the enzymes and proteins were denatured or if they were completely removed by the filtration process.

Comparative tests showed that only less than 10% of the protein content was lost after filtration, as can be seen in Table II. Since



Figure 1. GPC chromatogram of unfiltered clover honey (G glucose oxidase; S sucrase; D diastase; Z sugar fraction).

a large portion of proteins is still left in filtered honeys, the separation of honey enzymes took place in the next step.

Gel chromatography (GPC) was used to separate honey enzymes in order to find out if the ratios of defined enzyme peaks showed clear changes after filtration. The studies by Bergner and Diemair (1975) and Bergner and Sabir (1977) were the basis for the development of this method.

With the method described above, it was possible to separate the main honey enzymes diastase, sucrase, and glucose oxidase from each other and from the honey sugars. The peak fractionation complied with the enzyme activities of each fraction. In Figures 1 and 2, GPC chromatograms of unfiltered and of filtered Argentinian clover honey are shown. Filtered and unfiltered honeys can only be distinguished when the chromatograms are compared directly, but it is not possible to detect a honey filtration with the GPC analysis only.

The decrease of the sucrase signals through the filtration process was less than that of the corresponding sucrase activities. After the isolation and the concentration of the sucrase peak, this fraction was analysed by electrophoresis as specified in 2.4.

The sucrase fractions of unfiltered honeys showed two dominant protein bands of 40 and 65 kDa. Further bands were also found, but the protein spectra of different tested honey types hardly differed. The reason is that sucrase has a



Figure 2. GPC chromatogram of filtered clover honey (G glucose oxidase; S sucrase; D diastase; Z sugar fraction).



Figure 3. Electrophoresis of unfiltered clover honey (A), filtered polyflora honey (B) and mixtures of filtered honey into unfiltered honey in amounts of 25, 50 and 75%.

bee origin and not a plant origin. Furthermore, there was a relationship between the sucrase activities in the honey and the colour intensity values of both main protein bands: the higher the activity, the higher the intensity values.

In filtered honeys, the large protein band (65 kDa) almost vanished whereas the 40 kDa band changed very little. There is a selective influence on the honey sucrase protein spectra through the filtration process. Filtered and unfiltered honeys can be distinguished unequivocally through large differences in the ratio of the colour intensity values of the two main protein bands.

Electrophoretic investigations of blends of filtered honeys and unfiltered honeys at different mixing ratios showed that the intensity of the 65 kDa-band decreased with increasing amounts of filtered honey. Figure 3 shows an



Figure 4. Scatterplot of absolute colour intensity values of selected filtered and unfiltered honeys.

electrophoresis scan of unfiltered Argentinian clover honey, filtered polyfloral honey from South Eastern Europe, and admixtures of filtered honey into this clover honey in proportions of 25, 50 and 75%. The change of the colour intensity of the 65 kDa-band can be observed visually.

In all 42 *unfiltered* honey samples, the ratios of the colour intensity values of the protein bands 40 kDa and 65 kDa generally were about 3. In all *filtered* honeys, the ratio rose to at least 30. Figure 4 shows a scatterplot of absolute colour intensity values of some selected filtered and unfiltered mono- and polyfloral honeys.

By mixing filtered honey into an unfiltered one, the colour intensities of the 65 kDa-band decreases while that of the 40 kDa-band remains constant. The ratio moved from 3 to at least 7–10 by an addition of 25% filtered honey. The results revealed that a ratio of 6 generally shows an addition of filtered honey. In Table III, an evaluation of protein band ratios is displayed as an example. With an addition of 25% filtered honey, the ratio increased from 2.9 to 9.4. The detection limit can be lowered when discriminating between honey types. When an unfiltered acacia honey with a low sucrase activity is mixed with a filtered honey that had a high activity before filtration (e.g., honey-dew honey), the ratio of the colour intensity values changes already with an addition of 15%. The reason is that the intensity of the 40 kDa-band is strengthened through the addition, whereby the 65 kDa-band decreases somewhat. The same happens when a filtered honey is added to an unfiltered honey with a high sucrase activity; with an addition of 15% filtered honey, a distinct change of ratios can be observed.

However, it is not possible to determine a general detection limit yet. The limit may be lowered if statistical measurements verify that a band ratio of 6 definitely shows an addition of filtered honey.

With the possibility to detect mixtures of filtered and unfiltered honeys, the honey packers and importers are able to purchase faultless products and consumer protection is guaranteed.

	Clover honey	Filtered	Admixture		
	(unfiltered)	Honey	75%	50%	25%
Colour intensity					
Values					
40 kDa	3280	3012	3072	3465	3408
65 kDa	1120	85	125	254	362
Ratio					
40/65	2.9	35.4	24.6	13.6	9.4

Table III. Example of an evaluation of colour intensity values of admixtures of filtered honeys into unfiltered honey.

3.1. Method validation

3.1.1. Precision

Two unfiltered honeys with different sucrase activities (acacia and honeydew honey) were analysed 4 times each. The standard deviation was 8%, hence, the method shows good precision.

3.1.2. Linearity

It was proved that the ratios of the two main protein bands rise linearly with increasing amounts of filtered honey. In this test, two different filtered honeys were added to five unfiltered honeys (mono- and poly-floral) in amounts of 25, 50, and 75%. An average correlation coefficient of more than 0.98 between the admixture level and the ratio of the colour intensity was found. In Figure 5, the linear correlation between the two parameters of a mixture of filtered polyfloral honey into Argentinian clover honey is displayed.

3.1.3. Influence of heat

We tested whether the method indicates filtration and is not due to honey heating during the filtration process. Two unfiltered honeys were warmed up to 80 °C for a few minutes (using a drying chamber) according to the filtration conditions as described above (pressure 3-5 bar, filtration through membranes with a pore size of 20 µm with diatomaceous earth as filter aid). The results revealed that there were no differences between the gel pictures before



Figure 5. Linearity of protein band ratios of admixtures (filtered polyflora honey into unfiltered clover honey).

and after heating. Thus, heating during filtration does not influence the protein spectra.

Our results show that now there is a promising method for the detection of filtered honey. However, before this method can be accepted as an official diagnostic, more honeys should be validated with a larger number of samples and should be tested in a collaborative trial.

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Caractérisation du miel filtré par électrophorèse d'enzymes fractionnés.

miel / filtrage / enzyme / protéine / électrophorèse

Zusammenfassung – Charakterisierung von gefiltertem Honig mittels Elektrophorese Enzymfraktionen. Mit von der Richtlinie 2001/110/EG wurde erstmals die Vermarktung von "Gefiltertem Honig" zugelassen. Diesen Erzeugnissen werden Kristallisationskeime und damit die Pollen entzogen. Die botanische und geographische Herkunft eines Honigs mittels mikroskopischer Pollenanalyse ist nach der Filtration nicht mehr feststellbar, und Beimischungen von billigen, filtrierten Honigen zu Sortenhonigen können nicht nachgewiesen werden. In dieser Arbeit wurde eine analytische Methode erarbeitet, um in Honigmischungen einen illegalen Zusatz von filtrierten Honigen festzustellen.

Vergleichende Versuche von gefilterten und ungefilterten Honigen zeigten zunächst, dass die Enzymaktivitäten, vor allem die der Saccharase, durch einen derartigen Prozess verringert wurden. Der Gesamtproteingehalt der Honige nahm hingegen kaum ab.

Daraufhin wurde eine Methode zur gelchromatographischen Trennung der Honigenzyme entwickelt. Dazu wurden die Honigproteine nach Aufkonzentrierung an *Toyopearl HW-55S*-Gel chromatographiert. In den Chromatogrammen konnten signifikante Veränderungen der Enzymbzw. Proteinfraktionen durch den Filtrationsvorgang beobachtet werden. Vor allem der Peak, der der Saccharase zugeordnet werden konnte, nahm deutlich ab.

Im nächsten Schritt wurden die Proteine der Saccharase mittels SDS-PAGE aufgetrennt. Dazu wurde die Saccharasefraktion der Gelchromatographie isoliert und angereichert. Anschließend wurde das Konzentrat mit Lämmli-Puffer und 2-Mercaptoethanol denaturiert und diese Lösung zur SDS-PAGE eingesetzt. Die Banden wurden mit der Coomassie-Färbetechnik sichtbar gemacht.

Für ungefilterte Honigproben konnten in den Saccharasefraktionen jeweils zwei starke Banden mit den Massen 40 kDa und 65 kDa detektiert werden. In filtrierten Honigen war die Intensität der Bande für das Protein mit der Masse 65 kDa hingegen nur äußerst schwach, so dass filtrierte und unfiltrierte Honige über die Gelbilder differenziert werden konnten. Eine densitometrische Auswertung der einzelnen Banden ergab, dass der Quotient der Farbdichtewerte der beiden Hauptbanden bei sämtlichen ungefilterten Honigen konstant zwischen 2 und 3 lag, der der gefilterten Honige hingegen bei mindestens 30.

Zumischversuche von filtrierten zu unfiltrierten Honigen zeigten, dass Bandenverhältnisse um 6 immer auf einen Zusatz von gefilterten Honigen schließen lassen. Dies bedeutet, dass Zusätze von gefiltertem Honig ab ca. 15% in Abhängigkeit von den Honigsorten erkannt werden können.

Honig / Filtrierung / Enzym / Protein / Elektrophorese

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