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ALLERGENIC PROPERTIES OF POLISH NECTAR HONEYS

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ABSTRACT

Background. Small amounts of protein can be found in honey, including well known allergen sources, such as plant pollen and honeybee secretions. Despite this, there are few case reports describing allergic reactions following the consumption of honey. The aim of this study was to examine the allergenic properties of nectar honey collected throughout the entire beekeeping season from different provinces in Poland.
Materials and methods. The immunoreactive properties of 20 Polish nectar honeys were analysed using the sera of IgE pollen allergenic patients ($n = 5$). The botanical origins and pollen of the anemophilous plants in the studied honeys were identified through palynological analysis.
Results. The significant differences in the protein content between the five varieties of honey and the differences in protein pattern and pollen profiles were observed. All of the honey samples contained immunoreactive fractions reacting with IgE present in the sera of patients allergenic to different pollens.
Conclusions. Although honey allergies are reported relatively rarely, all the tested samples of Polish nectar honeys contained many protein fractions which reacted with the IgE antibodies of allergenic patients. In all samples, the immunoreactive protein band with a molecular weight around 60 kDa, probably secreted by bees, was present. The results do not allow the immunoreactive fractions characteristic for particular honey varieties to be identified.

Keywords: allergenicity, nectar honeys, SDS-PAGE, immunoblotting

INTRODUCTION

Honey is essentially a highly concentrated water solution consisting of several sugars (which represent 95–99% of the dry mass), where fructose and glucose are the major components. It contains components derived from bees (mainly enzymes from gland secretions and wax), as well as from substances related to their foraging activity (flower nectar and grain pollens). The proteins present in honey are in small amounts ranging from 0.2% to 1.6% in *Apis mellifera* honey (Won et al., 2009). They originate from the nectar and pollen of flowers but also from the salivary secretions of honeybees. Most authors (Baroni et al., 2004; Girolamo et al., 2012; Rossano et al., 2012) have reported a higher contribution of proteins in honey from the bee's glands than from plants, whereas da Silva et al. (2016) identified pollen as the main protein source. In nectar honey, there are also airborne pollens that are sprinkled with nectar and collected by bees, along with pollens from anemophilous plants, which stick to hairs on the body of a bee during flights to search for food, as well as sticky nectar (Wang and Li, 2011). The average

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content of these pollens in polish nectar honey can be up to 6.5% (Kruczek et al., 2015).

Nectar honey proteins originate from allergenic sources, suggesting that honey may cause sensitization. Nevertheless, allergies to honey are reported rather rarely. The actual incidence of allergy in the general population is unknown; it is estimated to be less than <0.001%, and only few cases have been reported (Aguiar et al., 2017; Cifuentes, 2015). This is even more surprising, taking into account that 10–30% of the population in the world are sensitive to aeroallergens (WAO, 2013), whose pollen can also be found in honey. Additionally, about 90% of individuals are also allergic to foods which cross-react with pollen (Osterballe et al., 2005). Cross-reactivity is connected with the reaction of specific IgE-antibodies present in allergic sera that recognize, bind and induce an immune response to structurally similar molecules (homologs), though they are of different origin (Sampson et al., 2014). Among patients suffering from a respiratory allergy, cross-reactivity between aeroallergens and foods is considered to be one of the causes of food allergies, which can range from oral syndrome to life threatening anaphylaxis (Popescu, 2015).

In the presented work, an attempt was made to characterize the pollen content and allergenic properties of mono-floral nectar honey varieties collected throughout the entire beekeeping season in four provinces in Poland.

MATERIALS AND METHODS

Honey samples

The present study was carried out on five different raw nectar *Apis mellifera* honey varieties in liquid or crystallized states collected directly from 4 apiaries located in different provinces in Poland (n = 20; Fig. 1). The selected samples for the experiment were nectar honeys from Rapeseed (*Brassica napus*), Acacia (*Robinia pseudoacacia* L.), Linden (*Tilia europeae* L.), Buckwheat (*Fagopyrum esculentum*) and Heather (*Calluna vulgaris*). The beekeepers supplied additional information on the date the honey was collected and the location of the apiary. The same varieties of nectar honey were collected in different regions of Poland in the same month: May – rapeseed, June – acacia, July – linden, August – buckwheat, September – heather).

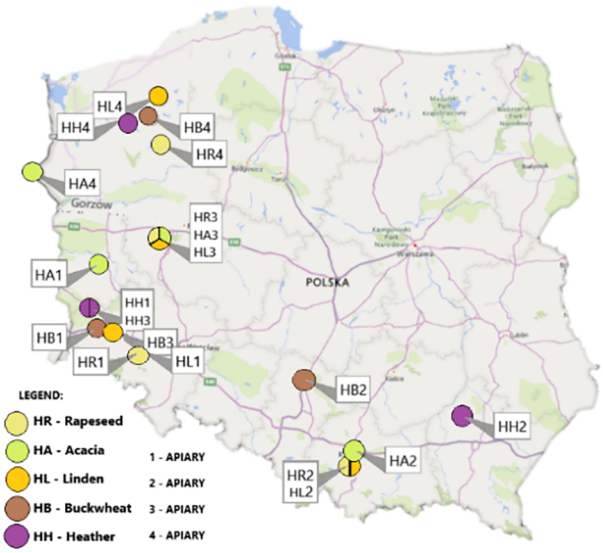


Fig. 1. Locations of the itinerant apiaries from which samples of different varieties of nectar honey were collected

Classification of the honey varieties was done individually by the beekeepers. All the samples were stored in glass jars at 10°C in a dark room until analysis.

Patient sera

The sera of five patients with documented IgE allergies to different pollens were obtained from the SNOZ Alergologia Plus Center for Diagnosis and Treatment of Allergy Therapy in Poznań, Poland under the consent of the Bioethics Commission (no. 670/17). An allergy to honey was not observed by any of the patients. An allergy to inhalants was diagnosed based on skin tests. The acquired sera were divided into two groups in accordance with the calendar pollen of plants in Poland (Table 1): A – early allergy (suffered due to pollen dusting from February to May) and B – late allergy (from June to December). For immunodetection, the same volume of sera was pooled from each of the two groups.

Protein extraction

Proteins from the honey were extracted by 0.01M PBS, pH 7.4; in the proportions of 6 g of sample per 4.5 ml of buffer. The extraction was carried out for 24 h at 4°C with shaking. The samples were then centrifuged for 30 min (4°C; 5500 g), the pellet was discarded,

Table 1. Characteristics of patients' sera with inhalation allergy specified by the RAST test, class/kU/L

Groups of sera	Birch and oak pollen	Alder and hazel pollen	Grass and weeds	Dust mite	*Cladosporium* / *Alternaria*
Early (A)	3/8.5	2/2.8	2/2.8	–	–
	3/6.3	–	2/1.5	–	–
Late (B)	2/2.6	2/1.4	4/31	6/>100	5/50
	2/1.8	1/0.68	1/0.36	–	4/25.0
	3/3.8	3/5.9	4/18.0	4/31.0	–

Class 0 indicates no allergy. Class 5 or 6 indicates high allergy.

and the clear supernatant was used for further analysis. The protein in the extracts was determined using the Bradford method (1976) and calculated per 1 g of honey. Measurements were performed in triplicate and the data were presented as a mean ±standard deviations.

Pollen analysis

The honey samples for pollen analysis were prepared according to the International Commission of Beekeeping Botany (Louveaux et al., 1978), PN-88/A-77626 (1988) and the Regulation of the Polish Ministry of Agriculture and Rural Development from 2009 no. 17 item 94. The honey samples were classified according to their botanical origin using the method described by Von der Ohe et al. (2004) and PN-88/A-77626 (1988; Table 2).

Table 2. The minimum percentage of predominant pollen in nectar honey (Regulation of the Polish Ministry of Agriculture and Rural Development from 2009 no. 17 item 94 and PN-88/A-77626, 1988)

Variety of honey	Minimum percentage of predominant pollen
Rape (*Brassica napus*)	45
Acacia (*Robinia pseudoacacia*)	30
Linden (*Tilia* sp.)	20
Buckwheat (*Fagopyrum esculentum*)	45
Heather (*Calluna vulgaris*)	45
Multifloral	without predominant pollen

SDS PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the Laemmli procedure (1970) on 14% acrylamide gels. The Pre-stained Protein Molecular Weight Marker (26612, Thermo Fisher) and honey protein extracts (about 4.5 µg or maximum extract volume – 15 µl) mixed with the sample buffer (4:1, v/v) and heated for 5 min at 95°C were applied to the gels. Electrophoresis was run at 90V in stacking gel and at 180V in separating gel. Next, the gels were stained in Coomassie Brillant Blue solution R250 and analysed using Totallab CLIQS image analysis software version 1.0.

Western blotting

The proteins separated by SDS-PAGE were transferred onto 0.45 µm polyvinylidene membranes (PVDF; Merck Millipore) by semi-dry transfer (V10 Semi-Dry Blotters, Scie-Plas Ltd, 4.5 mA/cm^2 30 min, and 0.36 mA/cm^2 60 min). Membranes were blocked with 1% bovine serum albumin in Tris-buffered saline, at pH 7.4 (TBS-BSA). After washing three times with TBS Tween 20 buffer (TBST), the membranes were incubated with early and late sera diluted 1:20 in blocking buffer for 16 h at 4°C. They were then washed 5 times with TBST and incubated for 1 h with diluted 1:1000 mouse anti-human IgE monoclonal antibody conjugated with alkaline phosphatase (A3076; Sigma). Visualization was performed using Calbiochem BCIP/NBT (5-bromo-4-chloro-3'-indolyphosphate and nitro-blue tetrazolium). The reaction was stopped with water. Then the membranes were dried and analysed using Totallab CLIQS image analysis software version 1.0.

Slot-blot

The crude protein honey extracts (50 µl) were applied directly onto PVDF membranes (Merck Millipore) in SLOT-Blot apparatus (BCD48 48-well Dot Blot Manifold, Biocomdirect). Then TBS-BSA was used as the blocking solution (1 h incubation). For the detection of the early and late sera of the allergenic patients, diluted 1:20 in blocking buffer were used. The procedure of visualization was the same as in the western blotting described above.

Statistical methods

The data were presented as a mean ±standard deviations. An analysis of one-way variance (ANOVA) with the Tuckey post-test was used to compare quantified variables in the honey samples. $P < 0.05$ was considered statistically significant. Statistica software version 13.3 was used for analysis.

RESULTS AND DISCUSSION

Protein content of honeys

The soluble protein content of honey samples was determined using the Bradford method (1976), as was recommended for honey by Azeredo et al. (2003). In all the tested samples from the complete beekeeping season, the soluble protein content varied depending on the variety and time the honey was collected (Table 3). Honeys from the beginning of the beekeeping season (spring: rapeseed and acacia) had around a five times lower soluble protein content compared to those from the end of the season (late summer: buckwheat and heather) with 0.258 mg/g and 1.309 mg/g respectively ($p < 0.05$). A similar observation was made by Rocco Rossano et al. (2012) who found the highest soluble protein content in eucalyptus honey harvested at the end of the beekeeping season, and the lowest in the extracts obtained from orange (*Citrus*) and chestnut honey (*Castanea sativa*) collected at the beginning. The number of protein compounds in bee honey is probably related to the development cycle of *Apis mellifera* and the protein requirements of bee families during the season (Bogdanov, 2004). In our research, surprisingly, a very low protein content was noted in the samples: HA3, HA4, HB3.

Pollen analysis

In Poland, the variety of honey is declared by the beekeepers themselves. The requirements for each variety are specific in the Regulations of the Ministry of Agriculture and Rural Development 2009 no. 17 item 94 and PN-88/A-77626 (1988) – the authenticity is checked using laboratory tools, among other things, by palynological analysis.

Tables 4 and 5 present the results of the palynological analysis. From twenty tested honey samples, only twelve varieties were correctly classified: all rapeseed with *Brassica napus* pollen above 45%, one acacia with *Robinia pseudoacacia* pollen 30%, three linden with *Tilia* pollen over 20%, two buckwheat with *Fagopyrum* pollen, and two heather honeys with *Calluna* pollen above 45%. In addition, samples with a surprisingly low soluble protein content (HA3, HA4 and HB3) did not contain a predominant amount of pollen.

Table 3. Soluble protein content of different nectar honey varieties, mg/g

Apiary	Honey variety				
	HR	HA	HL	HB	HH
1	$0.279^{a} \pm 0024$	$0.348^{c} \pm 0.028$	$0.252^{a} \pm 0.019$	$1.752^{c} \pm 0.101$	$0.844^{a} \pm 0.084$
2	$0.324^{bc} \pm 0.016$	$0.401^{d} \pm 0.032$	$0.395^{b} \pm 0.037$	$1.913^{d} \pm 0.011$	$0.926^{a} \pm 0.037$
3	$0.292^{b} \pm 0.009$	$0.090^{b} \pm 0.020$	$0.309^{b} \pm 0.064$	$0.408^{a} \pm 0.020$	$1.462^{b} \pm 0.059$
4	$0.310^{bc} \pm 0.025$	$0.014^{a} \pm 0.038$	$0.573^{c} \pm 0.015$	$1.346^{b} \pm 0.034$	$1.818^{c} \pm 0.019$
Average	$0.302^{A} \pm 0.017$	$0.213^{A} \pm 0.165$	$0.382^{A} \pm 0.121$	$1.355^{B} \pm 0.584$	$1.262^{B} \pm 0399$

[a–d]The same letter means no statistical differences between apiaries (in column; $P < 0.05$).
[AB]The same letter means no statistical differences between honey varieties (in raw; $P < 0.05$).

Table 4. Classification of analysed honey sample type based on PN-88/A-77626 (1988)

Sample	Variety according to the label	Predominant pollen, %		Secondary pollen, %		Type of honey according to analysis
HR1	rapeseed	*Brassica napus*	59			monofloral
HR2	rapeseed	*Brassica napus*	56			monofloral
HR3	rapeseed	*Brassica napus*	57			monofloral
HR4	rapeseed	*Brassica napus*	46			monofloral
HA1	acacia	*Robinia pseudoacacia*	30			monofloral
		Phacelia	53			
HA2	acacia			*Rubus type*	26	multifloral
				Robinia pseudoacacia	22	
HA3	acacia	*Robinia pseudoacacia*	22	*Rubus type*	16	multifloral
HA4	acacia			*Robinia pseudoacacia*	16	multifloral
HL1	linden	*Tilia*	45	*Asteraceae*	11	monofloral
HL2	linden	*Tilia*	23	*Anthriscus*	30	monofloral
				Centaurea cyanus	23	
HL3	linden			*Asteraceae*	17	multifloral
				Anthriscus type	17	
HL4	linden	*Tilia*	26	*Phacelia 18*		monofloral
HB1	buckwheat	*Fagopyrum*	46	*Phacelia*	16	monofloral
HB2	buckwheat	*Fagopyrum*	46			monofloral
HB3	buckwheat			*Fagopyrum*	28	multifloral
				Brassicaceae	16	
HB4	buckwheat			*Fagopyrum*	26	multifloral
HH1	heather	*Calluna*	57	*Fagopyrum*	16	monofloral
HH2	heather	*Calluna*	46	*Fagopyrum*	16	monofloral
HH3	heather			*Calluna*	30	multifloral
				Brassicaceae	16	
HH4	heather			*Calluna*	35	multifloral
				Brassicaceae	18	

According to microscope analysis, they were classified as multi-floral.

The average content of Anemophilous plant pollen grains in relation to the total number of pollens present in the honey samples constituted 8.5%. In early rapeseed and acacia honey, there were pine, willow and birch pollens, whereas in summer (linden) and late honeys (buckwheat and heather), mainly pollen from

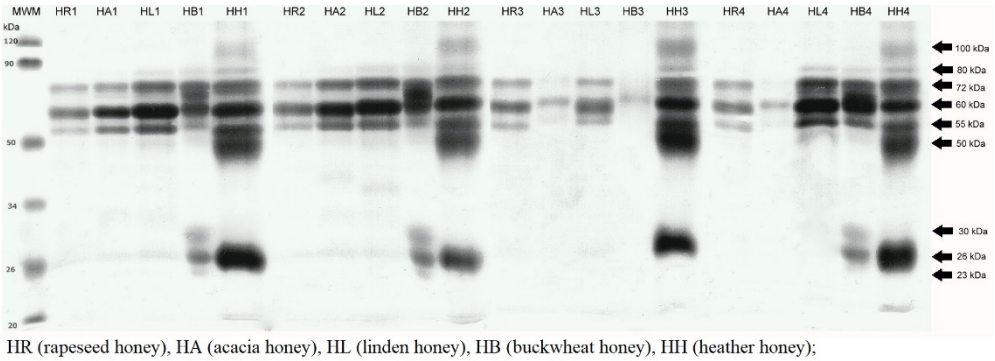
Table 5. Average pollen contribution of anemophilous plants in particular honey varieties

Collection month	Samples	Main anemophilous taxa	Range, %	Average, %	Contribution of anemophilous taxa and honeydew elements in honey, %
05/2018	HR1-4	*Salix*	2–13	4.5	8.25
		Pinus	2–8	4.25	
06/2018	HA1-4	*Betula*	2–3	2	8.25
07/2018	HL1-4	*Chenopodium*	1	3.25	9.5
08/2018	HB1-4	*Artemisia*	1–12	4	10.25
09/2018	HH1-4	*Artemisia*	2	2	6.35
		Poaceae	2–4	2	

grass, mugwort, sorrel, plantain, quinoa and spores of *Alternaria* and *Cladosporium* were found. The non-nectariferous pollens found in the tested honeys were typical of the pollen season in Poland, hence they can be the source of inhalation allergens. Therefore, this can be expected to increase allergic symptoms from people consuming honey and can be dangerous, especially for individuals with oral allergy syndrome (OAS). Other authors have also reported the frequent pollination of Polish honeys by the pollen of anemophilous plants, e.g. oak, elm, poplar, plantain, mugwort, grasses, sedge, walnut, as well as birch and quinoa (Stawiarz, 2009; Teper, 2011). A palynological analysis of the frequency of spring honey pollens carried out by Ceglińska (2008) showed almost 70% grass, over 50% oak and sorrel, 10% cereal, hazel and hops, with the lowest percentage (less than 3%) for pine and mugwort. A similar outcome was discovered by Šaulienė et al. (2015), who analysed Lithuanian honey samples and found 10 allergenic morphotypes and reported that anemophilous allergenic pollen constituted 44% of all the pollen detected in the honey. Considering the above, it can be assumed that the consumption of honey can be dangerous for extremely sensitive people, as this may result in an immediate allergic reaction (Denisow and Weryszko-Chmielewska, 2015).

Protein profiles of honey

The SDS-PAGE pattern of the proteins extracted from the tested honeys showed that almost all of them contain characteristic cluster band fractions with molecular weights of around 72, 60 and 55 kDa (Fig. 2). Spring and summer honeys (rapeseed, acacia and linden) have



HR (rapeseed honey), HA (acacia honey), HL (linden honey), HB (buckwheat honey), HH (heather honey); 1 - Apiary 1, 2 - Apiary 2, 3 - Apiary 3, 4 - Apiary 4. MW molecular weight marker

Fig. 2. SDS-PAGE pattern of honey extracts. The arrowhead indicates molecular weight of separated protein fractions

a similar protein profile with a dominant fraction of 60 kDa. The protein profiles of late summer honeys (buckwheat and heather) were different compared to samples from the beginning of the beekeeping season. They were noted to have more bands and a larger contribution of fraction with a molecular weight of around 26 kDa, especially in the heather honey samples. Characteristic for this variety were also proteins with a high-molecular weight of 50, 55, 60, 72, 80 and 100 kDa, whereas the buckwheat honey samples (HB1, HB2, HB4) contained cluster band fractions of around 30, 26 and 23 kDa.

The protein patterns of HA3, HA4 and HB3 were less visible, which was due to the extremely low protein content (Table 3). The results of the protein content, pollen analysis and the protein profiles, indicated that these honey samples had a reduced quality and were not mono-floral as was declared on the label by the producer (Table 4).

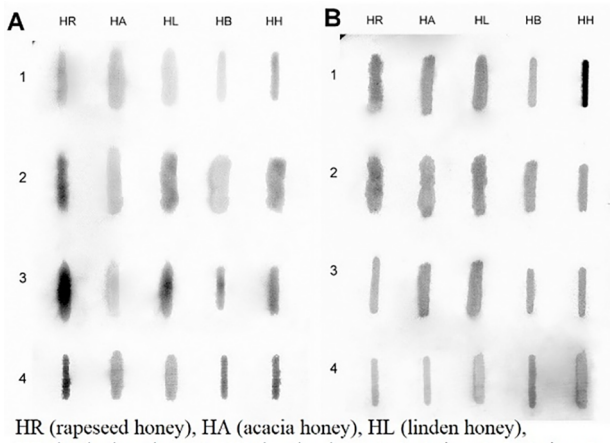


Fig. 3. Slot blot images of protein extracts in early sera patients (A) and late sera patients (B)

Immunoreactivity of honey protein

The allergenic properties of the honeys were examined by slot-blot and western-blot analysis using the sera of early and late allergenic patients.

In all analysed honey samples slot-blot analysis confirmed presence of immunoreactive native proteins beside variety, apiary, harvesting date as well as used sera (Fig. 3). Grey and black spots on the figure indicate positive samples. The antibodies present in the sera in an allergenic person recognized the antigenic determinants which were on the surface of the protein extracted from all the honey samples. The darkest spots on the slot-blot images were obtained for honey extracts: HR2, HR3, HR4 and HL3 with early sera patients (Fig. 3a) and HR1, HH1, HR2, HL2 with late sera patients (Fig. 3b). These honey samples were the most immunoreactive. Differences in the immunoreactivity of the same honey varieties from different apiaries could be due to the different anemophilous plant pollen profiles.

Identification of the protein fractions present in the honey recognized by the sera of allergenic patients was performed using the western blot method. The results indicated the presence of immunoreactive protein fractions in all the honey samples. Regardless of the place and time the honey was collected, antibodies present in the sera of allergic patients recognized the antigenic determinants found on the surface of the proteins extracted from each honey sample. Membranes incubated with the sera of early and late allergenic patients (Fig. 4) recognized almost the same proteins; fractions with molecular weights around 60, 50 and 55 kDa and 30, 26 and 23 kDa. Nevertheless, differences between the varieties were observed. The main immunoreactive fraction in the spring and summer honey varieties was 60 kDa, whereas in late summer buckwheat it was 25 kDa, and in heather 50 and 25 kDa. The protein profiles observed on the membrane incubated with two sera was the same but a little weaker than the immunoreactivity (lighter bands) which was observed on the membranes with late sera.

Other authors have also identified a lot of immunoreactive fractions in honey using the sera of IgE allergic patients. The recognized proteins were: 138, 116, 110, 60 and 54 kDa (Hayashi et al., 2011), 54 and 60 kDa (Yadzir et al., 2011), 57 and 29 kDa (Ibero et al., 2002), 54, 46, 17 and 16 kDa (de la Torre et al., 1997), 72, 60, 54, 33 and 30 kDa (Bauer et al., 1996), 54, 60, 72 and 30 kDa (Florida-Lopez et al., 1995).

The fact that such a large diversity in the molecular weight of the protein fractions recognized as allergenic in honey is related to the fact that the results refer to honey varieties that were collected in different parts of the world. It should be expected that, in addition to proteins derived from different nectar pollens and

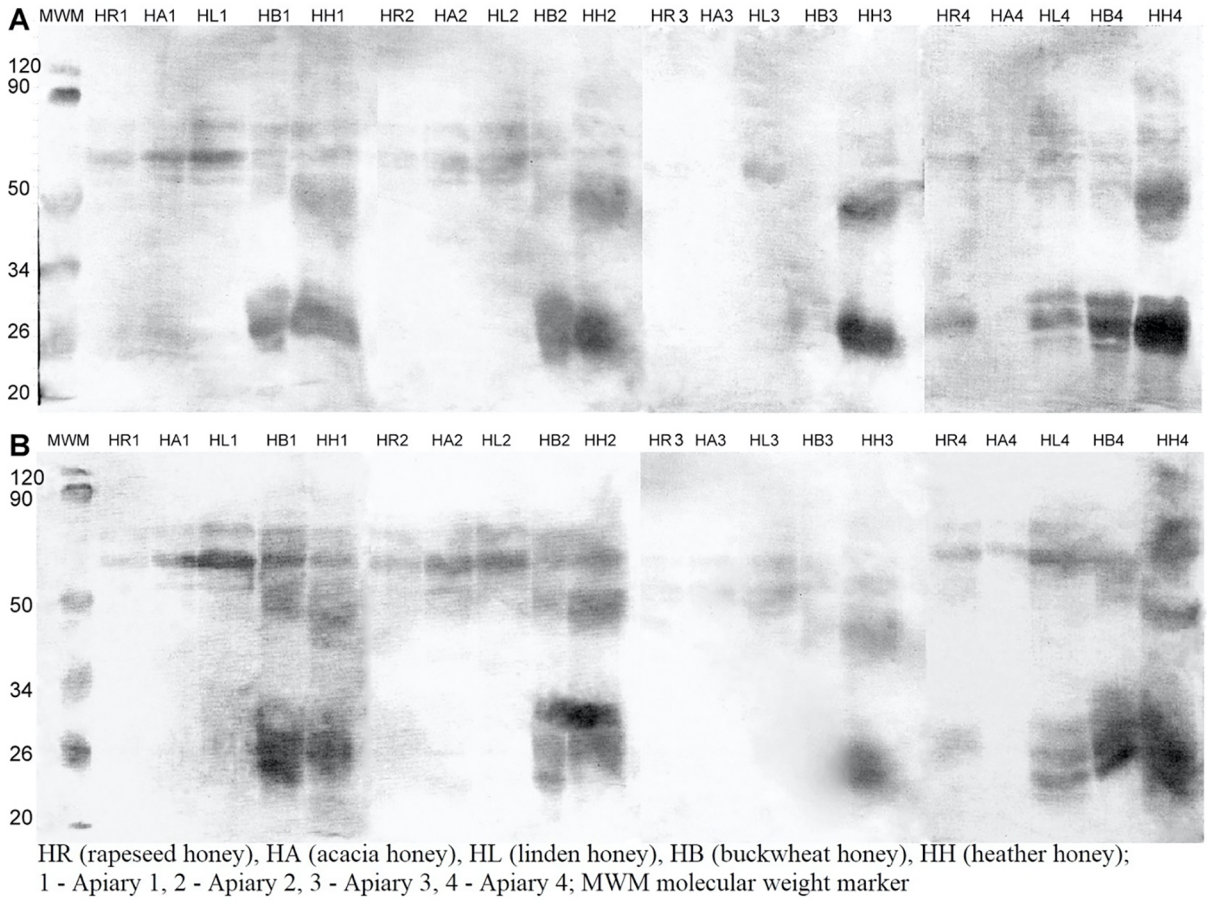


Fig. 4. Immunoblots of honey proteins: A – early sera patients, B – late sera patients

honeybees, honey also contains pollen from locally occurring wind pollinating plants, which may also affect their immunoreactivity (Denisow and Weryszko-Chmielewska, 2015).

CONCLUSION

The analysed samples of different nectar honey varieties from the whole beekeeping season varied in profile and soluble protein content. The results confirmed the presence of allergenic protein fractions in all the analysed samples. Nearly all the proteins present in the honey extracts were also recognized by IgE from the sera of pollen allergenic patients. Despite the differences between the samples in all the analysed honeys, protein bands with a molecular weight of around 60 kDa were recognized by both early and late sera. The molecular weight of these fractions suggests that they can be contained in granular bee secretions so can be dangerous for people who are sensitive to major royal jelly proteins, but this statement should be checked in further research.

The results were not so evident as to indicate the particular protein fractions that can be responsible for the allergic reactions of each nectar honey variety.

The results have shown that nectar honey containing pollens from nectariferous plants, and also anemophilous plants, should be a very allergenic food, particularly dangerous for individuals with a pollen allergy. On the other hand, allergy symptoms after the consumption of honey are not very common, even in an allergy to non-nectariferous plants. Such ambiguous results indicate the need of further, more detailed studies on the immunoreactive properties of honey.

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