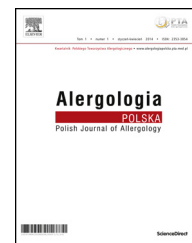


Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/alergo

Original research article/Artykuł oryginalny

Why not evaluate the allergenic potential of bee pollen with a skin testing method?



Claude Nonotte-Varly*

Marie-Jose Treffot Hospital, Hyeres, France

ARTICLE INFO

Article history:

Received: 10.05.2016

Accepted: 11.08.2016

Available online: 24.08.2016

Keywords:

- Allergenic pollen
- Allergenic potency
- Bee pollen
- Melissopalynology
- Skin reactivity

ABSTRACT

Background: Bee pollen (BP) is a dangerous food for allergics because of allergenic pollens (AP). We present a method to evaluate allergenic potentials of AP in BP by using skin prick testing. **Method:** First, it is experimentally characterized relationship between mass of one AP ($Mass_{AP}$) content in BP and its allergenic potential measured by skin reactivity testing. $Mass_{AP}$ is identified in BP mass after having calculated the proportion of AP using the BP pollinic spectrum based on the European standard. Skin reactivity to AP is assessed by measuring wheal diameters (W_{AP}) from skin prick tests using three serial dilutions of BP on allergic patients to AP, in order to calculate equation of the relationship between $Mass_{AP}$ and skin reactivity. Relationship equations are calculated for several AP. Then, for a BP having several AP, allergenic potentials of AP (W_{AP}) are assessed by BP spectrum analysis, AP identifications, $Mass_{AP}$ calculations and W_{AP} calculations using equations. **Results:** Linear functions $\text{Log}(W_{AP}) = A(\text{Log}(Mass_{AP})) + B$ ($R^2 = 0.99$) were established using BP samples rich in only one genus of AP. For a polyfloral BP with different AP, W_{AP} of each AP is proportional to $Mass_{AP}$ included in a stated dose of BP. E.g., for a BP with 0.017 mg of artemisia (A) and 0.367 mg of forage grasses (FG) per mg, W_A and W_{FG} are 7.7 mm and 8.9 mm, respectively, for 1 g of BP and 12.5 mm and 13.1 mm for 5 g. **Conclusion:** This method could help to diagnose of anaphylactic accident after BP ingestion or to prevent anaphylactic risk for allergic consumers.

© 2016 Polish Society of Allergology. Published by Elsevier Sp. z o.o. All rights reserved.

Introduction

Bee pollen (BP) is widely consumed by general public, and is also used as a functional food [1].

Flower pollen grains are assembled by the bee in loads. Each load has a weight of 5–10 mg [2] and has several hundred thousand grains of a single floral species. BP consists of various loads from different plant species.

Bees visit numerous plant flowers [3, 4] and some floral pollens are allergenic pollen (AP). BP could be a dangerous food for allergics. Anaphylactic accidents related to the use of BP are on the increase [5]. All published serious BP allergies have been among pollen allergics. Further, the prevalence of allergy is steadily increasing and half of the next generation in Europe could be allergic. Actual incidence of BP allergy is about 0.50% among Polish and the main BP allergic side effects are gastrointestinal symptoms and

* Correspondence to: Marie-Jose Treffot Hospital, Marechal Juin avenue, BP 50082, 83407 Hyeres cedex, France. Tel.: +33 494086182; fax: +33 494086198.

E-mail address: allergologie@limperialsante.net.

<http://dx.doi.org/10.1016/j.alergo.2016.08.002>

2353-3854/© 2016 Polish Society of Allergology. Published by Elsevier Sp. z o.o. All rights reserved.

rashes [6]. But, the mild allergic accidents to beehive products could be common and could be in the matter under-reported and thus underestimated [7]. Currently the BP allergy impact is not estimated accurately, even for pollinosis patients.

Furthermore, allergy to BP is unconnected from other bee product allergies because of a lack of cross-reactivity between bee products [6]. BP has never appeared to contain *in fact* allergens of bee venom. Skin or serum sensitizations to bee venom were not referred by literature among allergic to BP [5]. The incidence of positive skin tests to bee proteins content in BP with no allergen pollen was zero among sensitive patients to pollens with no history of bee sting reactions and no food allergy [8]. The accident risks could be exacerbated because popular belief dissociates floral pollen collected by bee and airborne floral pollen (anemophilous) with the misconception that bees only use pollen from insect-pollinated plants (entomophilous) [9]. Allergy to BP is considered serious by the health authorities in some countries. The official Food Code is imposing BP statement to warn about potential allergic reactions in Australia and New Zealand, but there is no international standard [9].

In early spring and late summer, plants and trees (e.g., Oleaceae, Asteraceae, Graminaeae) represent allergenic pollen suppliers for honeybees at locations where the anemophilous trees and plants are an important pollen source [10]. Anemophilous pollens collected by bees are botanically closely related to common airborne AP or could cross-react with unrelated allergenic plants. These allergic cross-reactions are caused by proteins sharing important structural homologies with several plant families [11]. Allergenic proteins of AP appear to retain their allergenic properties in BP from the time they enter the beehive to the harvesting by the beekeeper and their use by the end consumer [12]. But sold BP is difficult to characterize or to standardize because it is highly variable.

However, skin reactivity was always called to witness of the allergenic potential of a "cocktail" of allergenic proteins extracted from an allergen raw material such as floral pollen. The *in vivo* skin tests to allergens provide information about the biological potency of the allergenic raw material, but not on its specific composition [13].

To the best of our knowledge, however, there is currently no technical definition of the allergenic potential of AP in BP. Therefore, it is important to have a technical evaluation of allergenicity potential of floral allergenic pollen of BP among allergics, to give a better information to BP consumers and to better prevent accident risks. The purpose of this paper is to present a method to evaluate the allergenic potential of AP in bee pollen among allergics to airborne pollen by using *in vivo* skin prick testing.

Materials and methods

1) Analysis of bee pollen spectrum:

A pollen analysis of bee products is usually performed by a specialist laboratory by analysing the beehive products. In our case, we used Honey Expertise Laboratory – Naturalim France Miel, 39330 Port-Lesney, France.

Such an analysis defines the type and frequency of each botanical genus or family floral pollen and determines the total mass of floral pollen.

BP is thoroughly cleaned when intended for human consumption. Cleaning the pollen separates pollen dust balls, bee body fragments, wax or propolis.

Melissopalynology is based on the European standard without acetolysis, as recommended by the International Commission for Bee Botany [14].

Counting and identifying floral pollen grains are made by examination under a microscope. Identification of pollen types is based on the laboratory pollen reference collection for local plants or on guides specialized in the pollen morphology of floral species.

Ten grams of well-homogenized BP were dissolved and washed in distilled water, centrifuged, then prepared using aqueous glycerine and paraffin for smear preparations. Each smear was studied under a microscope to identify 500 floral pollen grains in order to determine the percentage of each type of floral pollen.

For each of the 1 to n types of floral pollen observed in the BP analysis, $\%_{pn}$ is the percentage of floral pollen pn .

With BP, the floral pollen mass is equated with the BP mass (Mass_{BP}) because it is accepted that the BP pellets only contain kneaded floral pollen grains.

Indeterminate fractions are ignored.

2) Calculation of each AP mass Mass_{AP} in BP:

2.1) Calculate the volume V_{pn} of each of the 1 to n types of floral pollen from the BP spectrum.

It is used the formula $V_{pn} = 4/3\pi r^3$ if the pollen grain is spherical or the formula $V_{pn} = 4/3\pi e^2 l$ if the floral pollen has an ellipsoidal shape.

The values of the radius r and of the mid-equatorial and longitudinal axes e and l are obtained from the literature from observations made on bee product pollen, including BP.

It is important to take into account changes in volumes of floral pollen due to orthodox or recalcitrant pollen qualities when pollen grains come into contact with aqueous bee fluids.

2.2) Calculate the proportion of the volume P_{AP} of each AP

$$P_{AP} = \frac{V_{AP} \times \%_{AP}}{(V_{AP} \times \%_{AP}) + (V_{p2} \times \%_{p2}) + \dots + (V_{pn} \times \%_{pn})}$$

($\%_{pn}$ is the percentage of floral pollen pn in BP analysis).

2.3) Calculate the mass of each AP Mass_{AP}

$$\text{Mass}_{AP} = P_{AP} \times \text{Mass}_{BP}$$

3) Calculation of the equations defining the allergenic potential of the AP existing in the BP:

Before calculating the equation defining the allergenic potential of one AP, it is necessary to:

- Use a BP with only one AP,
- Calculate the mass of AP as indicated above,
- Use a BP without any AP as a "BP negative control" to eliminate a skin sensitization to bee specific allergens.

3.1) Preparation of BP extracts:

Samples were prepared with the two types of BP defined above. Five grams of fresh or frozen BP was well homogenized on a glass plate and 450 mg of BP was diluted in 4.5 ml

of isotonic 0.4% phenol diluent (dilution weight/volume: 100 mg of bee pollen/ml of diluent) and was homogenized with a stirrer at a maximum speed for one minute. Samples were stored at room temperature for 24 hours and homogenized one more time with the stirrer before being removed, then 0.5 ml of the 100 mg/ml diluted solution was diluted in 4.5 ml of isotonic 0.4% phenol diluent to produce a 10 mg/ml diluted solution. These steps were repeated one more time to produce at least three samples of BP, i.e., 100 mg/ml, 10 mg/ml and 1 mg/ml.

The mass of AP contained per millilitre of each sample was deduced using the mass of AP in the BP. Samples were kept at 5 °C and were used within five days.

3.2) Measurement of skin reactivity to AP contained in BP:

Skin prick tests were duplicated on the inner side of the forearms of subjects. Patients had been referred for seasonal symptoms (rhino-conjunctivitis and/or asthma) produced in spring or summer. They were not hyposensitized and were positive skin prick tested with a commercially available allergen pollen extract. They were sensitized to major allergen of allergen pollen by testing for specific IgE-antibodies. Patients might be sensitive to others aeroallergens (e.g., mites, cats) but none had a history of bee sting reactions or food allergy.

Informed consent was obtained from each patient.

Skin reactivity was assessed by geometric measuring of the two largest wheal diameters observed twenty minutes after the pollen sample prick tests, positive (histamine 10 mg/ml) and negative (glycerinated saline) controls and commercial extract tests (Stallergenes allergen pollen 100 IR/ml).

W_{AP} was defined by geometric measuring of skin reactivity to AP contained in BP.

3.3) Analysis of the relationship between skin reactivity to AP in BP (W_{AP}) and mass of AP ($Mass_{AP}$):

If the model curve was a power regression:

$$(W_{AP}) = b(Mass_{AP})^a$$

then the linear function was calculated as follows, where A and B are specific pollen allergen constants:

$$\text{Log}_{10}(W_{AP}) = A \text{Log}_{10}(Mass_{AP}) + B$$

Variances analysis was performed by calculating R^2 , which records the results of the value dispersions associated with regression. The closer R^2 is to 1, the more the total variance is explained by the linear regression.

4) Calculation using the equations of the allergenic potential of each AP in proportion to the mass of BP:

BP may have many AP.

For each AP observed in the pollinic spectrum analysis of BP, the skin reactivity was assessed by the calculation of the wheal diameter as follows:

- Calculate the mass of AP per unit of the mass of BP as indicated above,
- Calculate the mass of AP for a stated dose of BP,
- Calculate the wheal diameter using the equation of the allergenic potential specific to AP where a and b are specific AP constants:

$$(W_{AP}) = b(Mass_{AP})^a$$

Results

To illustrate the method, calculations were done with different BP: one BP rich in *Artemisia* pollen (A), one rich in forage grasses pollen (FG) that belong to the Poaceae and Agrostideae tribes of the Pooideae subfamily, one rich in *Zea* pollen (Z), one rich in *Fraxinus* pollen (F), one rich in a non-allergen pollen *Hedera helix* (H) and one rich in two different AP.

1) Calculation of $Mass_A$ of bee pollen and calculation of the equation defining artemisia allergenic potential:

Our bee pollen has an AP: *Artemisia*. It was collected in August 2012 in Eguisheim (France) at the GPS location: X 48.0428, Y 7.3062.

Its spectrum includes 43.1% *Artemisia*, 25.7% *Mercurialis*, 16.0% *Lythrum salicaria* and 14.7% *Brassicaceae* (<0.5% undetermined).

1.1) Calculations of V_{pn} volumes, of P_A proportion and of $Mass_A$:

The calculations of V_{pn} volumes, of P_A proportion and of $Mass_A$ are shown in Table I.

There was 0.246 mg of *Artemisia* pollen per mg of BP.

1.2) Measurements of skin reactivity to *Artemisia* pollen and analysis of the relationship between W_A and $Mass_A$:

Skin prick tests were duplicated on the inner side of the forearms of 11 subjects. Patients (seven women/four men) aged between 19 and 46 (mean: 29.7), were recruited for seasonal symptoms produced in July and August.

They were positive skin prick tested with a commercially available *Artemisia vulgaris* extract (Stallergenes) and sensitized to Art-v1 by testing for specific IgE-antibodies (>0.27 kui/l). In addition to mugwort, they were sensitive to grasses (n = 5), cats (n = 3), cypress (n = 4), olive (n = 2), mites (n = 7) and fungi mould (n = 1).

Out of the 11 patients sensitized to *Artemisia*, one was excluded because of a positive control test of less than 3 mm.

Skin prick test results with three 10-fold dilutions of bee pollen with 0.246 mg of *Artemisia* pollen per milligram are shown in Table II.

The model dose-response curve of *Artemisia* bee pollen is a power regression:

$$(W_A) = 3.33(Mass_A)^{0.30} \quad R^2 = 0.99$$

The dose-response curve linear function is:

$$\text{Log}_{10}(W_A) = 0.30 \text{Log}_{10}(Mass_A) + 0.52 \quad R = 0.99$$

The dose-response curve linear function is shown in Fig. 1.

2) Calculation of $Mass_{FG}$ of BP and calculation of the equation defining FG allergenic potential:

Our BP has an AP: FG. It was collected in July 2013 in Thezillieu (France) at GPS location: X 45.8833, Y 5.6.

Its spectrum includes 6.1% FG, 46.1% *Robinia*, 22.9% *Brassicaceae*, 19.6% *Clematis*, 3.4% *Trifolium*, 1.5% *Cornus* (<0.1% undetermined).

2.1) Calculations of V_{pn} volumes, of P_{FG} proportion and of $Mass_{FG}$:

The calculations of V_{pn} volumes, of P_{FG} proportion and of $Mass_{FG}$ are shown in Table I.

Table I – Bee pollen spectrums and Mass_{AP} of bee pollens with a single AP

Bee pollen	Pollen ^a	Diameter (μm)	Volume (μm ³)	Percentage	Porportion AP	Mass AP (mg)
1 (artemisia)	Artemisia	20	4187	43.1	0.246	0.246
	Mercurialis	25	8177	25.7		
	Lythrum	26 × 36	17 643	16.0		
	Brassicaceae	20	4187	14.7		
2 (FG)	FG	35	22 438	6.1	0.168	0.168
	Robinia	23	6367	46.1		
	Brassicaceae	20	4187	22.9		
	Clematis	22	5572	19.6		
	Trifolium	25	8177	3.4		
	Cornus	58	102 109	1.5		
3 (zea)	Zea	95	448 693	49.3	0.983	0.983
	Apiaceae	25	8177	24.3		
	Matricaria	23	6367	22.7		
	Trifolium	28	11 488	3.4		
4 (fraxinus)	Fraxinus	23	6367	30.0	0.273	0.273
	Salix	18	3052	31.8		
	Aceraceae	25	8177	13.8		
	Quercus	25	8177	8.8		
	Prunus	35	22 438	7.9		
	Ranunculaceae	26	9198	4.9		
	Brassicaceae	20	4187	1.9		
5 (hedera h)	Hedera helix	25	8177	99	1	1

^a Indeterminate fractions are ignored.

There was 0.168 mg of FG pollen per mg of BP.

2.2) Measurements of skin reactivity to FG pollen and analysis of the relationship between W_{FG} and $Mass_{FG}$:

Skin prick tests were duplicated on the inner side of the forearms of 10 subjects. Patients (six women/four men) aged between 18 and 46 (mean: 28.9) were recruited for seasonal symptoms produced in spring and early summer.

They were positive skin prick tested with a commercially available pollen mixture of five grasses extract (Stallergenes). They were sensitized to rPhl p1/rPhl p5b by testing for specific IgE-antibodies (>0.38 kui/l). In addition to Gramineae, they were sensitive to mites ($n = 5$), cats ($n = 2$), cypress ($n = 6$), olive ($n = 2$) and parietaria ($n = 1$).

Skin prick test results with three 10-fold dilutions of BP with 0.168 mg of FG pollen per milligram are shown in Table III.

The model dose-response curve of FG BP is a power regression:

$$W_{FG} = 2.16(Mass_{FG})^{0.24} \quad R^2 = 0.99$$

The dose-response linear function is:

$$\log_{10}(W_{FG}) = 0.24(\log_{10}(Mass_{FG})) + 0.33 \quad R = 0.99$$

The dose-response curve linear function is shown in Fig. 2.

3) Calculation of $Mass_Z$ of bee pollen and calculation of the equation defining zea allergenic potential:

Our BP has an AP: Zea. It was collected in July 2013 in Villers le sec (France) at GPS location: X 47.5994, Y 6.21917.

3.1) Calculations of V_{pn} volumes, of P_Z proportion and of $Mass_Z$:

The calculations of V_{pn} volumes, of P_Z proportion and of $Mass_Z$ are shown in Table I.

There was 0.983 mg of Zea pollen per mg of BP.

Table II – Skin prick test results with three 10-fold dilution of bee pollen with 0.246 mg of artemisia pollen per milligram

Patient	Artemisia				Control	
	Artemisia 24.6 mg/ml	Artemisia 2.46 mg/ml	Artemisia 0.25 mg/ml	Artemisia commercial extract	Positive control	Negative control
P1	22.97	11	8.94	8.48	6.48	0
P2	4.90	2.83	1.73	4.9	7.93	0
P3	6.93	3.87	1	7.93	6.48	0
P4	11.96	9	4.90	9.48	3.46	0
P5	17.97	6	1.41	10.48	6.48	0
P6	6.93	1.73	1	3	4.24	0
P7	8	5.92	3.87	3.87	3.46	0
P8	8.94	6	2.83	9.38	3.87	0
P9	6.93	3.87	1.73	3.87	8.48	0
P10	3	2.83	1	3.87	6.92	0
Mean wheal	8.39	4.61	2.14	5.93	5.49	0

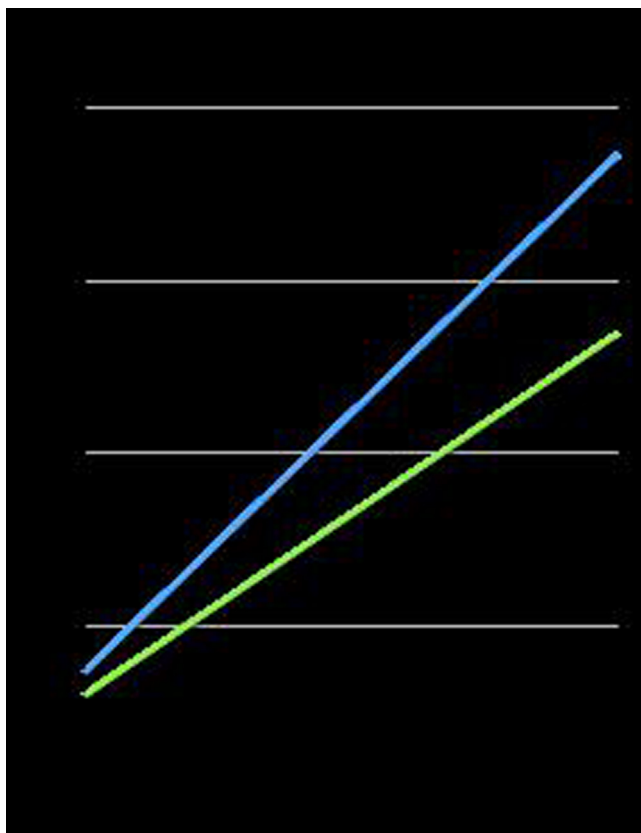


Fig. 1 – Dose-response curve linear regression (blue) between Log Wartemisia and Log Mass artemisia and the dose-response curve linear regression (green) between Log Wfraxinus and Log Mass fraxinus

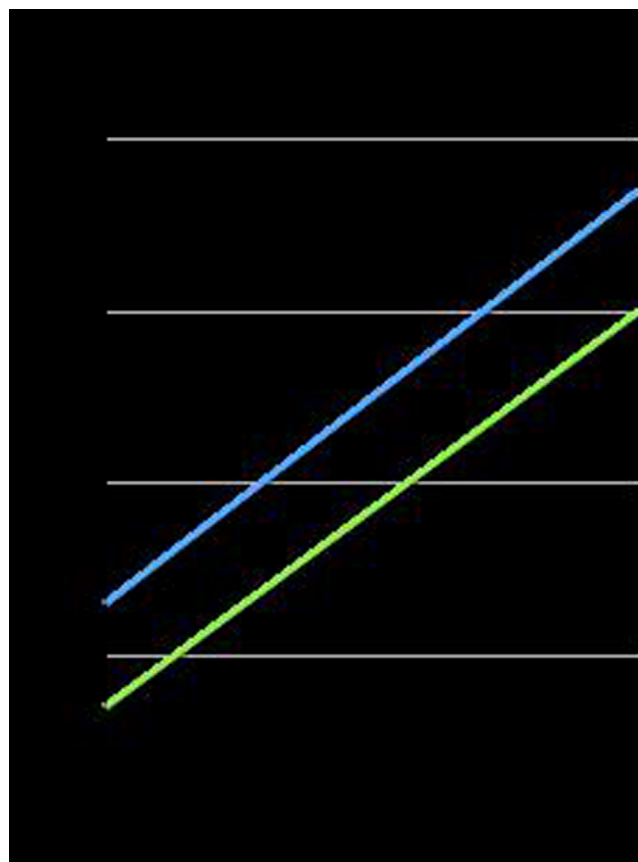


Fig. 2 – Dose-response curve linear regression (blue) between Log Wfg and Log Mass fg and the dose-response curve linear regression (green) between Log Wzea and Log Mass zea

3.2) Measurements of skin reactivity to Zea pollen and analysis of the relationship between W_z and $Mass_z$:

Patients are the same as FG.

Skin prick test results with three 10-fold dilutions of BP with 0.983 mg of Zea pollen per milligram are shown in Table III.

The model dose-response curve of Zea BP is a power regression:

$$W_z = 1.4(Mass_z)^{0.23} \quad R^2 = 0.99$$

The dose-response linear function is:

$$\log_{10}(W_z) = 0.23 \log_{10}(Mass_z) + 0.14 \quad R = 0.99$$

The dose-response curve linear function is shown in Fig. 2.

Table III – Skin prick test results with three 10-fold dilution of bee pollen with 0.168 mg forage grasses pollen per milligram or with 0.983 mg Zea pollen per milligram

Patient	Forage grasses			Zea			Grasses Commercial extract	Control	
	16.8 g/ml	1.68 mg/ml	0.17 mg/ml	98.3 mg/ml	9.83 mg/ml	0.98 mg/ml		Positive	Negative
P1	3.87	2	1	2.45	1.41	1	6.48	6.48	0
P2	4	1.73	1	4	3.46	1.73	5.48	4.24	0
P3	4.47	3	1.41	3.87	1.73	1	5	4.24	0
P4	7.48	4.47	2.83	8.49	6	3	4	6.92	0
P5	6.48	3	1.73	3.46	2	1	3.46	8.48	0
P6	2.45	1	1	2.45	1.73	1	6	7.93	0
P7	4.90	3	1.41	5	2.83	1.73	5.48	3.87	0
P8	4.47	3.87	2.45	5	4	2	5	6.92	0
P9	5.92	2.83	1.41	3.87	2	1.41	5.48	8.48	0
P10	2	1.41	1	4	1.73	1	5.58	6.92	0
Mean wheal	4.29	2.40	1.42	4.00	2.42	1.38	5.12	6.45	0

4) Calculation of $Mass_F$ of bee pollen and calculation of the equation defining Fraxinus allergenic potential:

Our BP has an AP: Fraxinus. It was collected in April 2013 in Contault (France) at GPS location: X 48.91094, Y 4.78943.

4.1) Calculations of V_{pn} volumes, of P_F proportion and of $Mass_F$:

The calculations of V_{pn} volumes, of P_F proportion and of $Mass_F$ are shown in Table I.

There was 0.273 mg of Fraxinus pollen per mg of BP.

4.2) Measurements of skin reactivity to Fraxinus pollen and analysis of the relationship between W_F and $Mass_F$:

Skin prick tests were duplicated on the inner side of the forearms of 10 subjects. Patients (three women/seven men) aged between 19 and 33 (mean: 25.3) were recruited for seasonal symptoms produced in spring.

They were positive skin prick tested with a commercially available pollen mixture of three Oleaceae extract (Ligustrum-Olea-Fraxinus Stallergenes). They were sensitized to Ole e1 by testing for specific IgE-antibodies (>0.53 kui/l). In addition to Oleaceae, they were sensitive to mites ($n=5$), cats ($n=3$) and cypress ($n=5$).

Skin prick test results with three 10-fold dilutions of BP with 0.273 mg of Fraxinus pollen per milligram are shown in Table IV.

The model dose-response curve of Fraxinus BP is a power regression:

$$W_F = 2.46(Mass_F)^{0.21} \quad R^2 = 0.99$$

The dose-response linear function is:

$$\log_{10}(W_F) = 0.21 \log_{10}(Mass_F) + 0.39 \quad R = 0.99$$

The dose-response curve linear function is shown in Fig. 1.

5) Calculation of $Mass_H$ of bee pollen and calculation of the equation defining Hedera helix allergenic potential:

Our BP is a pure, unique, floral pollen, Hedera helix (99%; indeterminate percentage $<0.9\%$). It was collected in September 2013 in Thezillieu (France) at GPS location: X 45.8833, Y 5.6.

5.1) Calculations of V_{pn} volumes, of proportion P_H and of $Mass_H$:

The calculations of V_{pn} volumes, of proportion P_H and of $Mass_H$ are shown in Table I.

There was 1 mg of Hedera helix pollen per mg of BP.

5.2) Measurements of skin reactivity to Hedera helix pollen and analysis of the relationship between W_H and $Mass_H$:

Patients are the same as all patients as above.

The model dose-response curve of Hedera helix BP is not a power regression:

$$W_H = 0.26(Mass_H)^{-0.6} \quad R^2 = 0.054.$$

Skin reactivity (data no show) of the Hedera helix BP is zero to three bee pollen concentrations and is not significantly different from negative control (Z test, $\alpha > 0.05$). No correlation relationship is established between skin reactivity and BP concentrations.

Hedera helix BP is a BP without any AP. It can be use as a BP negative control to eliminate a skin sensitization to bee specific allergens.

6) Calculation using the equations of the allergenic potential of each AP of a BP:

Our BP has two AP: Forage Grasses and Artemisia. It was collected in July 2012 in Eguisheim (France) at the GPS location: X 48.0428, Y 7.3062.

Its spectrum includes, 34.9% Mercurialis, 18.4% Trifolium, 17.2% FG, 15.9% Matricaria, 4.3% Artemisia, 3.9% Hypericum and 3.6% Melilotus ($<1.8\%$ undetermined).

6.1) Calculations of V_{pn} volumes, of P_A and of P_{FG} proportions and of $Mass_A$ and $Mass_{FG}$:

The calculations of V_{pn} volumes, of P_A and of P_{FG} proportions and of $Mass_A$ and $Mass_{FG}$ are shown in Table V.

There were 0.017 mg of Artemisia pollen and 0.367 mg Forage Grasses pollen per mg of BP.

6.2) Calculation of skin reactivity to Artemisia pollen and to Forage Grasses pollen in proportion to mass of BP:

For 1 mg of BP, there were 0.017 mg of Artemisia pollen and 0.367 mg Forage Grasses pollen:

$$W_A = 3.33(Mass_A)^{0.30} = 3.33(0.017)^{0.30} = 1 \text{ mm}$$

$$W_{FG} = 2.16(Mass_{FG})^{0.24} = 2.16(0.367)^{0.24} = 1.7 \text{ mm}$$

For 1 g of BP, there were 17 mg of Artemisia pollen and 367 mg Forage Grasses pollen:

$$W_A = 3.33(Mass_A)^{0.30} = 3.33(17)^{0.30} = 7.7 \text{ mm}$$

$$W_{FG} = 2.16(Mass_{FG})^{0.24} = 2.16(367)^{0.24} = 8.9 \text{ mm}$$

Table IV – Skin prick test results with three 10-fold dilution of bee pollen with 0.273 mg of fraxinus pollen per milligram

Patient	Fraxinus				Control	
	Fraxinus 27.3 mg/ml	Fraxinus 2.73 mg/ml	Fraxinus 0.27 mg/ml	Oleaceae commercial extract	Positive control	Negative control
P1	5	4	3.97	14.49	8.48	0
P2	4.90	2.45	2	8	3.46	0
P3	4.47	4	1.73	8.94	6.48	0
P4	3.87	2.45	1.73	7.93	6	0
P5	3.87	1.73	1	3.87	5.47	0
P6	4.90	3	1.73	9.16	8.48	0
P7	4.90	1.73	1	5.91	4	0
P8	5	2	1.73	7	6.32	0
P9	9.49	6.92	5.91	10.39	6.70	0
P10	6.92	3.87	1.41	11.83	5.47	0
Mean wheal	5.14	2.93	1.89	8.26	5.87	0

Table V – Bee pollen spectrum and Mass_{AP} of a bee pollen with several AP

Bee pollen	Pollen ^a	Diameter (μm)	Volume (μm ³)	Percentage	Porportion AP	Mass AP (mg)
BP with several AP (artemisia and FG)	Mercurialis	25	8177	34.9	0.367	0.367
	FG	35	22 438	17.2		
	Trifolium	28	11 488	18.4		
	Matricaria	23	6367	15.9	0.017	0.017
	Hypericum	20	4187	3.9		
	Artemisia	20	4187	4.3		
	Melilotus	27	10 301	3.6		

^a Indeterminate fractions are ignored.

For 5 g of BP, there were 85 mg of Artemisia pollen and 1835 mg Forage Grasses pollen:

$$W_A = 3.30(\text{Mass}_A)^{0.30} = 3.30(85)^{0.30} = 12.5 \text{ mm}$$

$$W_{FG} = 2.16(\text{Mass}_{FG})^{0.24} = 2.16(1835)^{0.24} = 13.1 \text{ mm}.$$

Discussion

In the literature, a strong correlation has been noted between cutaneous reactivity to BP containing an AP (e.g., Gramineae, Artemisia, Oleaceae) and the cutaneous reactivity in patients with a positive skin prick test to this pollen commercial extract [12]. Pitsios et al. found that approximately 70% of patients were sensitized to both BP and airborne AP. They considered that it might be due to AP in their samples, but not all melissopalynology analyses of their BP samples have shown AP. This might be due to the qualitative and quantitative methods used to analyse BP.

Only five spherules of different tinges were chosen from each BP sample. But tinge loads are subjective. Colours change with time, if the loads are dry or are exposed to sunlight [15]. Many plant species have pollen loads with very similar colours and sometimes up to three colours are observed for a single genus [3]. Also, anemophilous pollens are often a minor BP and choosing five pellets can raise the risk of non-homogenized samples.

On the contrary, BP analysis using the standard European melissopalynological method recommended by the International Commission for Bee Botany [14] is more suitable. This procedure is based on the study of 10 grams of well-homogenized bee pollen and 10 grams composed of more than 1000 pellets.

Quantifying the absolute mass of AP per gram of BP requires knowing the pollen spectrum of BP and measuring pollen grain sizes. More particularly, this requires knowing pollen sizes when in contact with aqueous fluids. In contact with water, the pollen grain is in osmotic shock. Hydrated grain results in a change of its volume and opens pores and fissures [16] depending on the recalcitrance and orthodoxy of the pollen [17].

Furthermore, two pollens of the same genus can have different reactions, for example, *Helianthus annuus* pollen is orthodox and swells in contact with water, whereas *Helianthus tuberosus* pollen maintains the same volume [17].

Strong relationships were established between the absolute mass of pollen of different allergic plant families in BP

and skin reactivity of sensitive patients to this pollen (results 1–4). This has been shown with an Asteraceae genus: Artemisia, an Oleaceae genus: Fraxinus and two genera of Gramineae pollen: forages grasses and Zea mays [18–20]. It has been shown after removing skin sensitization to bee specific allergens (result 5) and despite patient groups including a small number of sensitive individuals to allergen pollen and to its major allergens.

For all AP studied, dose-response curves were power regression curves from which we were able to deduce the linear curves:

$$\text{Log}_{10}(W_{AP}) = A \text{Log}_{10}(\text{Mass}_{AP}) + B$$

AP in BP appears to be a little or not altered by bee secretions and those allergens retain their allergenic capacity.

Furthermore, allergenicity of pollens of the same family depends of the genus in Gramineae [20] and the family cross reactivity is well-preserved in Oleaceae [19].

According to the present results, it is experimentally established and characterized a relationship between the mass of the AP content in a BP and the allergenic potential of this pollen measured by skin test reactivity.

Bees use taste to assess the macronutrient content of pollen and bees gather pollen grains with an optimal protein content [21] (e.g., pollen grains that had a contact with water are neglected).

Moreover, the literature has shown that the protein content of allergenic pollen is almost constant over time and depends over all of pollen varieties [22].

Thereby, this method might help to diagnose the floral origin of bee pollen in case of an anaphylactic accident after BP ingestion. Indeed, each AP in a BP should be well identified using the BP analysis and the allergenic potential of each AP should be well noted using equations for calculations (result 6). The results might be compared to the patient's sensitization profile to diagnose the responsible AP, without having to extract allergenic proteins contained in BP.

More studies are needed to calculate equations for other AP (e.g., birch, ambrosia, etc.) to get a large panel of equations before testing this method in a cohort of patients with symptoms after ingestion of BP.

This present work provides a method for estimating the allergenic potential of a BP or of a composition thereof, which defines so more simple the types of the AP and the degree of allergen risk of each AP.

But these information should be given with respect to the amount of BP consumed because allergenic potential is

proportional to the mass of AP. Mild accidents occur frequently since they could affect almost half of individuals consuming airborne AP extracts [23]. Mild accidents could occur with very low doses of allergen and gastrointestinal allergies could be allergen dependent-dose [24]. A mixture of allergens or an allergen quantity too big and/or too quickly eaten are risk factors for allergic accidents [25].

The stated dose could be defined as the weight of the portion prescribed or the BP weight of a composition thereof.

This method makes it easy to appoint AP of BP and to quantify their allergenicity. It could be very helpful to give good information to the BP allergic consumer to prevent a risk of allergic reaction. We might imagine a potential allergen index to assess the allergenic potential of an AP from a BP or from a composition thereof. It might as well define for each AP its type and its allergenic potential from the weight of the each AP included in a stated dose of BP.

Conclusion

The allergenicity of AP contained in BP could be deduced of their mass because their mass can be calculated easily from the BP spectrum and because their allergenic potential measured by skin reactivity is proportional to their mass. This method could help to diagnose of anaphylactic accident after BP ingestion or to prevent anaphylactic risk for allergic consumers.

Authors' contributions/Wkład autorów

According to order.

Conflict of interest/Konflikt interesu

None declared.

Financial support/Finansowanie

None declared.

Ethics/Etyka

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments; Uniform Requirements for manuscripts submitted to Biomedical journals.

Acknowledgment/Podziękowania

The author thanks Claudine Guinet for her contribution to the bee pollen collection.

REFERENCES/PIŚMIENNICTWO

- [1] Linskens HF, Jorde W. Pollen as food and medicine – a review. *Econ Bot* 1997;51:78–87.
- [2] Mauricio A. Weitere Untersuchungen an Pollenhoschen. *Beih Schweiz Bienen-Ztg* 1953;2:486–556.
- [3] Kirk W. A colour guide to pollen loads of the honey bee, 2nd ed., Cardiff, UK: International Bee Research Association; 2006, 54 pp.
- [4] Ricciardelli D'Albore G, Intoppa F. Fiori e Api: La flora visitata dalle api e dagli altri apoidei in Europa. Bologna, Italy: Calderini Edagricole; 2000.
- [5] Choi JH, Jang YS, Oh JW, Kim CH, Hyun IG. Bee pollen-induced anaphylaxis: a case report and literature review. *Allergy Asthma Immunol Res* 2014;7(5):513–517.
- [6] Basista K, Filipek B, Sodzawiczny K. Bee pollen allergy in Polish beekeepers and their families. *Postep Derm Alergol* 2012;XXIX(5):343–347.
- [7] Dutau G, Rance F. Allergie au miel et aux produits de la ruche. *Rev Fr d'Allergol* 2009;49S:16–22.
- [8] Nonotte-Varly C. Sensibilisation aux protéines contenues dans un pollen de lierre (*Hedera helix*) récolté par l'abeille chez l'allergique respiratoire aux pollens aéroportés? Une étude pilote in vivo. *Rev Fr d'Allergol* 2015;56(2):100–101.
- [9] Shahali Y. Allergy after ingestion of bee-gathered pollen: influence of botanical origins. *Ann Allergy Asthma Immunol* 2015;114(3):250–251.
- [10] Richardson RT, Lin CH, Sponsler DB, Quijia JO, Goodell K, Johnson RM. Application of ITS2 metabarcoding to determine the provenance of pollen collected by honey bees in an agroecosystem. *Appl Plant Sci* 2015;3(1):1400066.
- [11] Vieths S, Scheurer S, Ballmer-Weber B. Current understanding of cross-reactivity of food allergens and pollen. *Ann N Y Acad Sci* 2002;964:47–68.
- [12] Pitsios C, Chliva C, Mikos N, Kompoti E, Nowak-Wegrzyn A, Kontou-Fili K. Bee pollen sensitivity in airborne pollen allergic individuals. *Ann Allergy Asthma Immunol* 2006;97:703–706.
- [13] Cox L. Standardized allergen extracts: past, present and future. *Expert Rev Clin Immunol* 2005;1(4):579–588.
- [14] Louveaux J, Maurizio A, Vorwohl G. Methods of melissopalynology. *Bee World* 1978;59:139–153.
- [15] Hodges D. The pollen loads of the honeybee: a guide to their identification by color and form. London, UK: Bee Research Association; 1952, 120 pp..
- [16] Human H, Nicolson SW. Nutritional content of fresh, bee-collected and stored pollen of *Aloe greatheadii* var. *davyana* (Asphodelaceae). *Phytochemistry* 2006;67:1486–1492.
- [17] Franchi GG, Piotto B, Nepi M, Baskin CC, Baskin JM, Pacini E. Pollen and seed desiccation tolerance in relation to degree of developmental arrest, dispersal, and survival. *J Exp Bot* 2011;62(15):5267–5281.
- [18] Nonotte-Varly C. Allergenicity of *Artemisia* contained in bee pollen is proportional to its mass. *Eur Ann Allergy Clin Immunol* 2015;47(6):218–224.
- [19] Nonotte-Varly C. In vivo biological potency of *Fraxinus* bee-collected pollen on patients allergic to oleaceae. *Alergol Pol – Pol J Allergol* 2015;2(2):82–88.
- [20] Nonotte-Varly C. Allergenicity of Gramineae bee-collected pollen is proportional to its mass but is highly variable and depends on the members of the Gramineae family. *Allergol Immunopathol (Madr)* 2016;44(3):232–240.
- [21] Ruedenauer FA, Spaethe J, Leonhardt SD. How to know which food is good for you: bumblebees use taste to discriminate between different concentrations of food differing in nutrient content. *J Exp Biol* 2015;218(14):2233–2240.

-
- [22] Fernández-Caldas E, Carnés J, Iraola V, Casanovas M. Comparison of the allergenicity and Ole e 1 content of 6 varieties of *Olea europaea* pollen collected during 5 consecutive years. *Ann Allergy Asthma Immunol* 2007;98(5):464-470.
- [23] Rodríguez-Pérez N, Ambriz-Moreno Mde J, Canonica GW, Penagos M. Frequency of acute systemic reactions in patients with allergic rhinitis and asthma treated with sublingual immunotherapy. *Ann Allergy Asthma Immunol* 2008;101(3):304-310.
- [24] Gidaro GB, Marcucci F, Sensi L, Incorvaia C, Frati F, Ciprandi G. The safety of sublingual-swallow immunotherapy: an analysis of published studies. *Clin Exp Allergy* 2005;35(5):565-571.
- [25] Calderón MA, Simons FE, Malling HJ, Lockey RF, Moingeon P, Demoly P. Sublingual allergen immunotherapy: mode of action and its relationship with the safety profile. *Allergy* 2012;67(3):302-311.