TITLE: Analysis of multifloral bee pollen pellets by advanced digital imaging applied to functional food ingredients

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ABSTRACT

Bee pollen is a hive product, resulting from floral pollen agglutination by worker bees and it is characterized by its excellent bioactive and nutritional composition. Currently, research is focused on bee pollen applications on food industry, because this product has been considered an excellent source of compounds for human nutrition. It is also important in some industries, where color and particle size are important characteristics for production. Due to the granular nature of bee pollen, conventional colorimetry does not allow describing color correctly; thus, digital image analysis is a better alternative. This technique could also allow classifying bee pollen according to its appearance beyond the color. Consequently, the aim of this work was to develop a novel methodology for image data processing to classify bee pollen as ingredient in food industry. Seven color groups in samples were established regarding harvest month and particle size. It was possible to calculate the percentage of each color group in all samples. This methodology also allowed selecting each fraction for different applications in food industry using colorimetry, granulometry and the

relationship between both of them.

Keywords: Bee pollen; Tristimulus colorimetry; Image analysis; Particle size

INTRODUCTION

Hive products are gaining attention due to the presence of bioactive compounds that are associated to health benefits, like antioxidant activity and protection against lipid oxidation [1-3]. One of its main products is bee pollen, resulting from floral pollen agglutination from different botanical sources; this process is done by worker bees when they collect nectar and pollen [4]. This food product has nutrients like proteins, with contents between 75-400g kg^{-1}, which are characterized by enzyme content and by essential and non-essential amino acids (methionine, lysine, threonine, histidine, leucine, isoleucine, valine, phenylalanine and tryptophan); carbohydrates between 150-600 g kg^{-1}, with the presence of reducing sugars, polysaccharides, starch and soluble and non-soluble fibers; lipids with contents between 5-200g kg^{-1}, standing out sterols, triglycerides and essential fatty acids (oleic, linoleic and linolenic). Myristic, stearic, oleic, arachidonic, caproic, palmitic, caprylic, capric, lauric, tricosanoic, behenic, and lignoceric acids were also quantified in diverse bee pollen samples; as well as minority compounds, such as minerals: macronutrients as Ca, P, K, Na and Mg, and micronutrients as Fe, Cu, Zn, Mn and Se. It also has vitamins (water and fat soluble like provitamin A, E, D, B1, B2, B5, B6, C and H) and some bioactive compounds and antioxidants, such as carotenoids, flavonoids, catechins and phenolics acids [1,4-7]. However, composition and color varies according to several factors such as climatological conditions, botanical origin, time of the year, age and nutritional state of the plant, recollection pollen methods, extraction methods, storage conditions, and treatment processes of fresh pollen [1,8]. One of the main aspects is the botanical origin, because composition and color depend mainly on pollen source, and both are linked to the origin flower. Different studies have shown correlation between composition and botanical origin [9,10] and it is important to take into account that botanical origin is linked to geographical position, because each place houses many species. For this reason, different floral species exist even in the same country, with different composition and color. The latter is one of the main characteristics of consumer's acceptance, because of

that, in food industry, raw materials are chosen according to the needs of the final product. Selection could be done by nutritional composition, physical or microbiological characteristics. Bee pollen main consumption is as a food supplement; therefore, it is marketed after a drying and cleaning process, without any additional treatment. The study of this matrix as raw material in other foods is incipient [11], which gives the possibility of using it in a variety of products. However, it is necessary to know deeper bee pollen as ingredient through methodologies different from those already used; it is also important to select both, pollen and food to be used, in order to avoid off-flavors generation, contamination and ingredients incompatibility. A sample of bee pollen may consist of pellets having different size and color, but conventional Tristimulus Colorimetry cannot give further information than a single measurement of a small area [12]. Novel methodologies like Digital Image Analysis are currently being used to evaluate color in non-uniform matrices, morphological characteristics and general appearance [12]. Using digital cameras and video colorimeters, it is possible to obtain information from each pixel. These systems acquire colorimetric information in Red, Green, Blue (RGB) color space. This device-dependent color space has to be transformed in device-independent color spaces such as CIELAB. One of the most advanced systems is DigiEye®, which allow to obtain CIELAB coordinates in an automated manner through a specialized software [13]. This color space is ideal for color recognition because it allows the separation of chromatic and achromatic information. Additionally, it is a uniform color space, similar to human visual perception, and also one of the most used for food [14]. Image analysis has been used widely on food: wines, grape seeds, gulupa (*Passiflora edulis* f.*edulis*), among others [13,15]. Regarding bee pollen, some studies has been done using image analysis for classifying floral species in some selected samples [16,17]. In [14], Spanish bee pollen was also used for detecting fake bee pollen: using computer vision and bee pollen color, authors could classify some pollen and create an algorithm to detect fraudulent pollen types. So far, the colorimetric characterization and classification of complex samples of bee pollen has not been carried out due to its heterogeneous appearance. Consequently, the aim of this work was to implement a novel methodology for image data processing to classify by color or particle size Colombian Highland bee pollen

through a harvest year as a potential ingredient in food industry.

MATERIAL AND METHODS

Bee pollen samples were collected monthly throughout 2016 by random sampling. The experimental unit was an apiary in the geographic region of the Colombian high Andean forest (2.800 y 3.200 meters above sea level) in Boyacá. Samples were dried at 60°C for 4 hours by hot-air drying process in a conventional dryer; final moisture obtained was 4.5%. The granulometric analysis was made using a shaker with 7, 10, 12, 16, 18, 30 and bottom sieves, according to a previous study made about bee pollen of this region [18]. The selected fractions were from sieves 10 (2.00mm), 12 (1.68mm), 16 (1.19mm) and bottom (<0.6mm). Fractions from sieves 10, 12 and 16 were selected because the most quantity of bee pollen collected is in those fractions. The fraction from the bottom was selected because of its potential as a raw material for food colorant. The others fractions were rejected because they have several impurities. Samples were stored at vacuum and room temperature until image analysis. For pollen analysis, qualitative analysis was carried out using acetolyzed slides, following Erdtman [19]. At least 400 pollen grains were counted in each pollen load by manual counting in a microscope. Analysis and counting were done by duplicate. The different pollen types were identified using mainly the Melisopalynology Laboratory of LABUN reference collection and the pollen catalogs of Giraldo et al. [20]. For Digital Image Analysis, the DigiEye® system was used. This device consists of a closed illumination box, specially designed (by VeriVide Ltd., Leicester, UK) to illuminate the samples consistently with two fluorescent tubes that emulate the standard illumination D65; a 10.2-megapixel digital camera Nikon® D80 with Nikkor® 35mm f/2D objective; and a computer Pentium IV. Lamps were switched on at least 10 minutes before being used, according to manufacturer conditions, to stabilize them [12,13]. The camera was calibrated by means of a calibration chart included in the equipment (DigiTizer, VeriVide, Leicester, UK). Bee pollen samples were distributed homogeneously on Petri dishes and put on the grey sample tray for acquiring images. The use of these dishes ensures a delimited area that improves segmentation process, avoids shadows and ensures enough thickness to remove the influence of background in samples color.

Establishment of groups

Images were first evaluated through expert visual analysis, from which the most representative color types in all bee pollen images were selected. A maximum of 12 groups were set, and the colorimetric coordinates were determined using DigiFood® software [12]. The average CIELAB color of each group was obtained from DigiEye images according to each possibility of grouping. Pellets with different color were selected to represent most of the color possibilities in all samples, and colorimetric coordinates of all possible pixels were obtained. The selection of the number of groups was done by calculating the percentage of pixels in each group of each image. Then, a comparison between the percentages, the real images and those obtained by the programming in Matlab® was made. The classification analysis allowed identifying 7 groups, as the selection that provides more information for a similar categorization of pixels to the original image (Figure 1). Although the samples may appear overlapped in the figure, they were different in the perpendicular axis (brightness, L*), hence there is no overlap in the three-dimensional space.

Image processing

Using an algorithm made *ad hoc* with MATLAB 2016a (The Mathworks Inc., Natik, USA), images were processed following the following steps sequentially (Figure 2). (1) Load the DigiEye image containing the CIELAB coordinates in each pixel. (2) Apply a k-means clustering for distinguish the Petri dish from the sample tray. Identify the centroid of the dish and crop the image for reducing computing workload; (3) Make a segmentation mask belonging to Petri dish and erode the edge to avoid the influence of background. The influence of shadows among pellets was also removed by selecting pixels having L*>20. This threshold was optimized after observing the histogram of this colorimetric variable in all available images and then included in the segmentation algorithm. (4) Select each pixel of the region of interest and calculate de color difference between the pixel color and the color of each group. (5) Assign the group according to the minimum color difference ΔE^*_{ab} and repeat steps 4 and 5 for all pixels belonging to the region of interest. (6) Once all pixels are assigned, calculate the percentage of each group in the sample and tabulate it. The percentage of each group in each pollen image was calculated considering the number of pixels per group

with respect to the total pixels of the image. (7) In parallel, an RGB image (original one) and the resulting image of the classification is stored for each sample. The algorithm evaluates each pixel individually and automatically generates an image where each pixel is represented according to the color of the assigned group. For this reason, the original image and the generated image have practically the same appearance.

RESULTS AND DISCUSSION

Establishment of groups

Separation of all groups was performed with the pixel colorimetric coordinates selected by each group and can be observed in Figure 1. The formation of individual and non-cross-linked clusters allowed corroborating the correct selection; differences in each group coordinates will avoid incorrect assignment due to proximity or overlap. When multiple clusters have information in common, a wrong value may be assigned to data or pixels assigned to a different group from the expected one. Shenoy et al. [21] evaluated different concentrations of salt-onion mixtures (very low color difference) and found that image analysis is not able to differentiate the ingredients after concentrations of 300 g kg^{-1} salt. In the case of bee pollen, this deficiency generates that similar pollen pixels can be classified erroneously in the same group.

Pollen analysis

Determination of botanical origin was made in the 7 color groups selected (Table 1). It can be observed that *Hypochaeris radicata* and *Brassica* sp. are the main species. There was a relation between botanical origin and color. *Hypochaeris radicata* is in B and E groups, which have orange hues, *Brassica* sp. is in groups C, D and F, which have bright and opaque yellow hues and *Trifolium* sp. is the main specie in groups A and G, which have the darkest color of all groups. Other minor species found were *Eucalyptus globules*, *Viburnum* sp., *Gaiadendron punctatum* and *Bellis* sp. In each group, the main specie content was superior to 75% (except in C), which allows to consider each specie as predominant pollen, according to Louveaux [22]. The pollen types obtained in color groups are similar to those obtained previously in Colombian high Andean forest bee pollen, where the main floral species was *Hypochaeris radicata*, Brassicaceae, *Trifolium* sp., *Eucalytus globulus, Quercus humboldtii* and *Weinmannia* sp. [23].

Image processing

It was found that the best way to classify the pixels was by color difference calculation (ΔE^*_{ab}), since it presented similar results to the discriminant analysis and is a simpler methodology for the computational workload. Figure 3 presents the comparison of real photographs with resulting images obtained by the program. In the image (a) it is possible to observe the detail of the distribution of the pixels in each one of the established groups (black background image); some black spaces appear in representation of spaces and shadows generated by pellets.

It can be observed how selected groups allow obtaining a classification similar to the original images, which verifies the accuracy of the developed program and correct selection of the groups. These results are according to those reported by Chica, where bee pollen was correctly classified using an algorithm and color information [14]. In the same way, Shenoy et al. [21] performed colorimetric characterization between groups of mixtures with similar and different coordinates L*a*b*, finding that image analysis achieves an identification of the ingredients the greater its ΔE^*_{ab}, even with small particle sizes (salt, pepper, paprika, onion). Those findings probe that image analysis is an effective technique to classify diverse sample mixtures.

It is also observed there is no trend in color through the pollen harvest year, however there is a tendency every three and four months: between January-March and April-July, respectively, where hues change from yellow to orange. From September to December, the trend is from orange to yellow. This variation is related to botanical origin. Terrab et al. [10] made a study to observe botanic origin of pollen and its influence in honey color, finding different correlation equations between colorimetric coordinates and flower species. Species as *Raphanus* f, *Ceratonia siliqua* and *Olea europaea* were correlated to yellow pollen, while *Carlina* f, *Quercus* f, *Reseda media* gr and *Citrus* f were related to green pollen. Those results are similar to the ones found in this study, because bee pollen orange and yellow (bright and opaque) have each one a predominant floral specie: 1. Orange (B and E) with *Hypochaeris radicata*, 2. Yellow (C, D and F) with *Brassica* sp., 3. The most achromatic color groups (A and G) with *Trifolium* sp. It is important to notice that

in some cases similar floral species generates differences in color or composition; according to Modro et al. [24], two samples having similar flora specie content (around 88%), vary in its composition and color, one had light brown color, 15.4% of protein and 6.6% of lipids, while the second had light beige color and contents of 20.9% and 3% of protein and lipids, respectively.

In countries with seasons, bee pollen can be classified as unifloral, while, in tropical countries this denomination is scarce because of the high floral diversity. In Brazil and Colombia, 33 and 126 different botanical species have been found [1,23], whereas in countries such Serbia and Portugal the species found were 26 and 8 respectively [8,25]. Colombian high Andean forest has a great variability in its flora through the year, so bee pollen from that region can be considered multifloral and given the close relationship between botanical origin and color, highland Cundiboyacense bee pollen will have a diversity of colors, which can be verified in Figure 3.

Classification applications by color groups

Bee pollen classification in various color groups allows knowing the colorimetric coordinates and to carry out analyses for the pollen selection according to the subsequent process to be submitted. Equally allows establishing the relationships between particle size and colorimetric characteristics.

Bee pollen selection for industrial food processes

Image analysis is a technology widely used in food analysis. which in this case can be used as a starting point for bee pollen selection, since it allows a classification by tristimulus values or particle size. Also, there are few studies where this technology has been applied to bee pollen. Table 2 shows the average colorimetric coordinates for each group obtained experimentally for bee pollen samples classification.

Chroma (C^*_{ab}) and hue (h_{ab}) identify the color groups, which will allow to make a selection of the fractions or months according the use that will have bee pollen in the industry. C^*_{ab} and h_{ab} values of E group indicates orangish color and their L* value presents it as the darker after the group G. A, C and F have higher value in the tone (h_{ab}), so they have yellow coloration; C is the clearest (highest L* value). Group C is the one with the highest value of C^*_{ab}, so it will be the most vivid color of all the yellow ones present in bee pollen.

Groups A and G are the ones with the lowest C^*_{ab} values, they are the most achromatic colors.

Since bee pollen has colorimetric coordinates in yellow-orange range, the industries could use it are bakery, confectionery, ice creams and beverages. These last two only can use bee pollen if the final product has the mentioned colorations. It is important to take into account sampling time, because different months present different hues (Figure 3), so to obtain groups E and B the main months would be March, June and September, while for D and F would be January, April, May, August, November and December. February, July and October are months that present the highest chromatic variety depending on particle size, which makes granulometry an important technique for select the harvest season.

In addition to colorimetric coordinates, particle size in food is an important factor in some processes, because they require a specific particle size in their products. Bee pollen fractions with average diameters between 1.19mm and 2.00mm can be used in foods, which the presence of pollen pellets is appreciated, such as whole grain breads, multigrain breads, crackers, cereal bars, granola and chocolates, among others. Bee pollen with diameters less than 0.6mm can be used as an ingredient in preparation: replacement of wheat flour, powder colorant and functional component where granular characteristic is not desired, such as sauces, juices or ice cream.

According to the information previously mentioned, bee pollen samples can be selected for different processes depending on the colorimetric coordinates or particle size, however the most complete way to select bee pollen is to relate both variables. Initial pollen has a mixture of several colors that can be used as a functional ingredient, however for some foods is important the final color obtained, for that reason sieving generates a range of colors that can be more effectively used by food industries. In addition to the variables mentioned above, each group (A-G) quantity through the year should be considered as another decision variable. With these three parameters can be determined the harvest time, as has been done evaluating the ripening in grapes and gulupa [12,15].

Figure 4 shows group percentages in each fraction. The majority group is D, which has percentages above 20% in most samples, followed by groups E, F, and B. Groups A, C and G may be considered minority at

some times of the year. Information could be analyzed according to industries requirements: a specific group or particle size is needed.

Regarding the group, it is possible to observe the great variability in hues according to particle size over time, except in sieving 4 (size <0.6mm) where color is more homogeneous (Figure 3). It is very important to consider this variation, because depending on bee pollen purpose, it will require some specific months and/or fractions.

For products which small particle size (<0.6mm) is important and it wants to avoid modifying the color radically, it would be ideal to use sieving 4 of month 4 (April), which has higher contents of group F (56.6%) and its color is more achromatic than B (lower C^*_{ab} values) (Table 1). In the case of raw material for a natural orange colorant, pollen fractions with an amount greater than 30% of group E would be needed, such as sieving 1 (2.00mm) of June and September, sieving 2 (1.68mm) of March, June, July and September, and sieving 3 (1.19mm) of February, March, June, July, September and October. If colorant is desired for direct addition, the ideal would be the smaller and more easily dissolving fractions of sieving 3. Bee pollen obtained in June and September is the most indicated, since all the fractions can be used; followed by March and July, which at least two fractions may be used. Fractions having the highest content of group B (most of sieving 4 and some of sieving 3) can also be used in the colorant industry, however a yellow one would be obtained, since hue value is greater than E group.

To obtain group F (greater than 30%) it is necessary to have sieving 1 of January, November and December, sieving 2 of December and sieving 4 of April, July, September and October. Group D is present in most fractions through the year. Group C can be used in yellow foods, since its color is the most saturated of all groups with that hue. Groups A and G have colorations near the grey area and the smallest contents in the various fractions through the year, for that reason could be considered as impurities and fractions with contents greater than 20% should undergo an additional sieving process to take advantage of the other groups presents in these samples.

Respect to particle size, if industry is not interested in the final color of the food, the fractions can be

classified as mentioned previously. However, if the color is taken into account, the fractions depend on harvest time. For that reason, the contents of the different groups are varied; Figure 4 shows as no granulometric fraction has a single group of pollen and assign a single type of industry to each fraction would be unwise. However, if raw material with more homogeneous colorimetric characteristics is needed, it would be advisable to use more sieves to obtain a greater separation.

Granulometric analysis is an effective tool, because different sieves allow to obtain multiple results according to particle size.

CONCLUSIONS

By means of image analysis it was possible to know, classify and evaluate different bee pollen individual samples regarding their color and appearance. This technique allows establishing bee pollen color taking into account the matrix heterogeneity, harvest time and particle size. The proposed algorithm was able to identify the sample in the whole image, to recognize the pixels avoiding interstices and then, to classify each one into seven well-distinguished groups and created for this study. This methodology can be an easier tool to analyze and select bee pollen as ingredient in different processes in food industry, where color and appearance are essential characteristics in raw material.

This first approach to colorimetric characterization may be a starting point for future studies where other characteristics can be related to appearance, such as origin and composition.

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FIGURES:

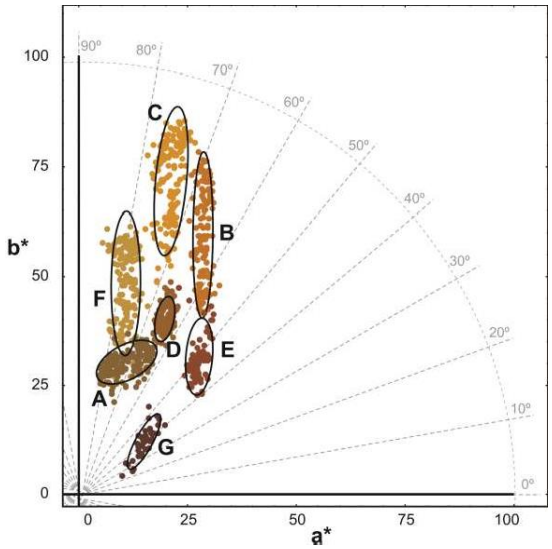

Fig. 1 Colorimetric coordinates in *a*b** plane representation of 7 selected groups

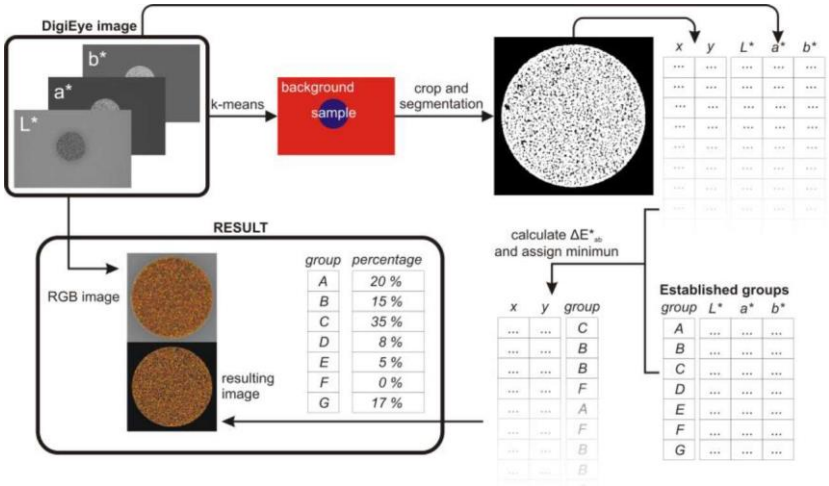

Fig. 2 Image processing flowchart

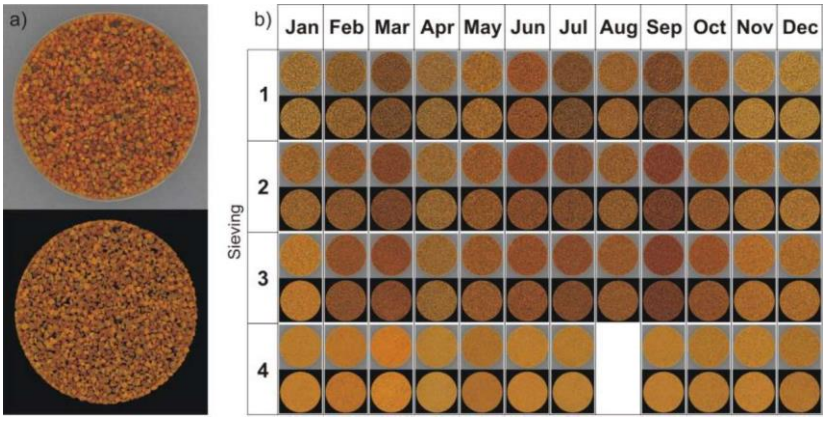

Fig. 3 Comparison between resulting images obtained by the algorithm (black background) and real ones (grey background). (a) Sample image, (b) samples images through the year. Particle size: sieving 1: 2.00mm; sieving 2: 1.68mm; sieving 3: 1.19mm; sieving 4: < 0.6mm

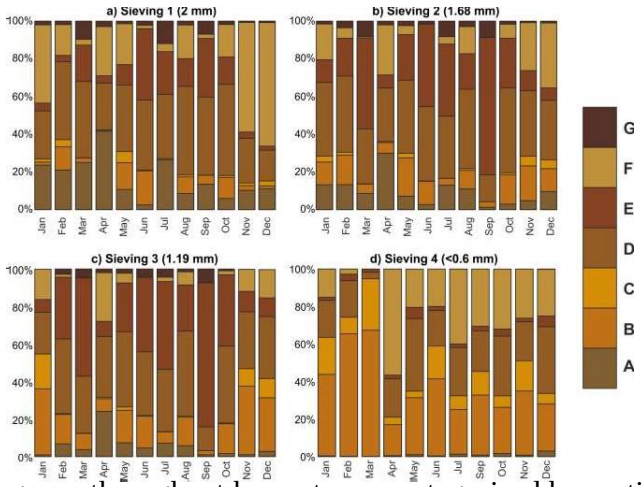

Fig. 4 Percentages of each group throughout harvest year, categorized by particle size. (a) sieving 1, (b) sieving, 2 (c) sieving 3, (d) sieving 4

TABLES:

Table 1. Palynological identification of each color group

Group	Family	Pollen type	Percentage
A	**Fabaceae**	*Trifolium* sp.	88.9
B	**Asteraceae**	*Hypochaeris radicata*	97,8
C	**Brassicaceae**	*Brassica* sp.	67.4
	Myrtaceae	*Eucalyptus globulus*	29.7
D	**Brassicaceae**	*Brassica* sp.	75.5
	Adoxaceae	*Viburnum* sp.	24
E	**Asteraceae**	*Hypochaeris radicata*	97.7
F	**Brassicaceae**	*Brassica* sp.	89.5
G	**Fabaceae**	*Trifolium* sp.	98.2

Table 2. Colorimetric coordinates of the 7 selected groups.

Group	L*	a*	b*	C^*_{ab}	h_{ab}
A	41.87	11.01	30.36	32.3	70.08
B	57.58	28.53	59.47	65.96	64.37
C	66.01	21.21	71.83	74.9	73.55
D	44.5	19.76	40.24	44.83	63.85
E	36.99	27.67	31.64	42.03	48.83
F	60.85	10.82	48.37	49.56	77.4
G	23.87	15	12.11	19.28	38.92