POLLEN ANALYSIS OF ROYAL JELLY: CONTRIBUTION TO ANALYTICAL METHODS AND CHARACTERIZATION[1]

M. Lucia PIANA*, Paola BELLIGOLI, Livia PERSANO ODDO, Sara PIPERNO

C.R.A. - Istituto Sperimentale per la Zoologia Agraria, Sezione di Apicoltura

Via L. Rech 36, 00156 Roma (Italy)

Tel: +39 06 86895456; fax: +39 06 86895463

*e-mail luciapiana@libero.it

[1] The research was carried out in the frame of the project BIOGEL, financed by the Italian Ministero delle Politiche Agricole e Forestali.

Abstract

Royal jelly (RJ) contains pollen grains derived from the foraging activity of honeybees that reflect the environmental location of the beehives. Microscopic analysis of the pollen in RJ can be used to determine its geographical origin. This paper describes a new simplified method for determining the total amount of pollen grains and the relative frequencies of the pollen from various plant types in RJ, including an estimation of the precision of the method. As a first application of these methods, the pollen content of 37 commercial RJ samples (imported and domestic) was studied. In addition, an experiment was conducted to study the influence of pollen supplements on RJ sediment.

Keywords: Royal jelly / pollen analysis / geographical origin

1. Introduction

On the European market royal jelly (RJ) originates mostly from China, the world's largest producer and exporter of this product. In recent years, after the European ban on Chinese RJ (2002, 2003 and part of 2004) because of the presence of chloramphenicol, the production of RJ in European countries has been increasing. Therefore the problem of establishing RJ origin is very important for both producer's and consumer's protection.

Even though it is a secretion of bee glands and does not derive directly from plants, RJ contains, similar to honey, pollen grains coming from the foraging activity of the honeybees, thus reflecting the environment where beehives are located. Therefore microscopic analysis of RJ can be used to determine its geographical origin.

Previous studies describe a method for determining the relative frequencies of pollen types in RJ (qualitative pollen analysis), and provide an indication to distinguish RJ from different geographic locations [3, 5, 8, 10]. In this research the method for microscopic analysis of RJ [10] was simplified. In order to reduce some of the variability arising from sample preparation and counting, we

implemented and detailed the procedure, according to the guidelines harmonized by the International Honey Commission for honey pollen analysis [17]. Then a test was performed to verify the method's repeatability.

Moreover, a simple method for determining the total amount of pollen grains/10g of RJ (quantitative pollen analysis) was developed and a repeatability test was performed. This kind of pollen analysis has been rarely applied to RJ [11], so it was considered interesting to investigate the pollen amount in RJ in order to verify possible information that could derive from this parameter. The method is based on spiking the sample with a known number of *Lycopodium* spores. This method is widely used in fossil palynology [14] and is also used in melissopalynology [6]. As a first application of these methods, the pollen content of commercial RJ samples (imported and domestic) was studied. In addition, an experiment was conducted to examine the influence of the pollen supplements given to bee colonies on RJ sediment.

2. Materials and methods

2.1. Description of the methods: qualitative pollen analysis of royal jelly

The method is based on the work of Ricciardelli D'Albore and Battaglini [10] and Von der Ohe *et al.* [17]. During the entire procedure, great care must be taken to prevent any contamination from foreign pollen coming from either previous preparations (disposable supplies are recommended as far as possible) or from airborne grains (close windows and limit the exposure).

2.1.1. Sample preparation

- Weigh 1.0 g of RJ into a pointed centrifuge tube (capacity ca. 50 ml) and add 10 ml of KOH 1%;
- Dissolve the RJ by mixing thoroughly (vortex);
- Centrifuge for 10 minutes at 1000 G;
- Suck up the liquid supernatant by means of a Pasteur pipette connected to a pump;

- Add 10 ml of distilled water and dissolve the RJ by mixing thoroughly (vortex); fill the tube with water up to 45 ml;
- Centrifuge again for 10 minutes at 1000 G;
- Decant the supernatant liquid, allowing any excess liquid to be taken up on absorbent paper;
- Heat a heating plate to 40°C and liquefy the glycerine gelatine (mounting medium);
- Draw a 10 x 10 mm square on the back of the microscope slide and put the microscope slide onto the heating plate;
- Transfer the entire sediment onto the slide with a single use Pasteur pipette and spread it evenly over the marked area of 10 x 10 mm. Leave the slide on the heating plate only for the time strictly necessary to dry the sediment;
- Apply one drop of glycerine gelatine on a cover slip forming a large cross diagonally and place the cover slip on the slide.

If sediment is particularly abundant, it is possible to spread the sediment over a more suitable surface by drawing a square bigger than 10 x 10 mm.

With respect to the original method [10], among other differences, the second washing with a diluted solution of sulphuric acid solution was eliminated.

2.1.2. Pollen counting

Count pollen grains under the microscope following the matrix represented in Figure1. Pollen grains are counted along 5 parallel equidistant lines uniformly distributed from one edge of the 10 x 10 mm smear to the other (in total at least 500 pollen grains are counted). See Von der Ohe *et al.* [17] for further details. Abortive, irregular or broken pollen grains are counted if they can be identified. Note separately non-identifiable or non-identified grains. For each pollen type calculate the relative frequency as a percentage of the total number of pollen grains counted.

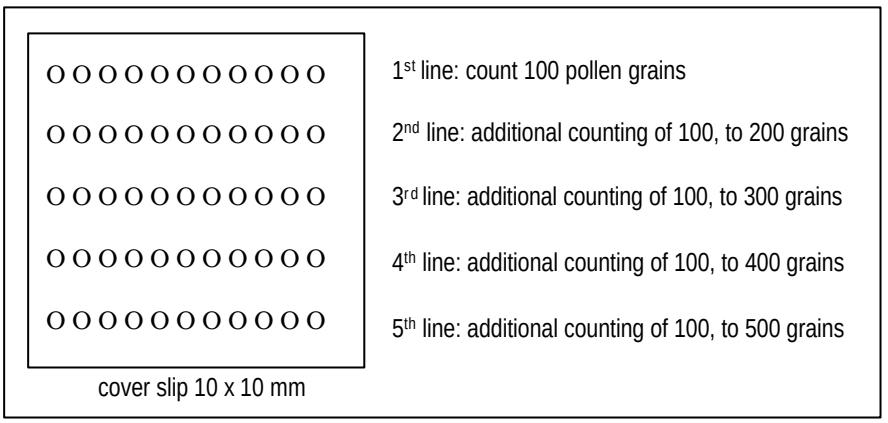


Figure 1. A matrix, used for counting pollen grains, which guarantees representative results (O = a whole microscopic field of view) [17]

2.2. Description of the methods: quantitative pollen analysis of royal jelly

2.2.1. Sample preparation

The preparation is the same as for qualitative analysis, with the addition, after the first point (addition of KOH), of one tablet of *Lycopodium* spores (produced by Lund University, Department of Geology, Sweden) [6, 14]. The spores are acetolysed and one tablet contains a defined number of spores (18,583 ± 764 per tablet, in the batch used in this work) [14, 15].

2.2.2. Pollen counting

Count at least 500 *Lycopodium* spores and the correspondent pollen grains. In order to examine uniformly the entire sediment, look at fields in five equidistant parallel lines from one edge of the 10 x 10 mm smear to the other. To calculate the absolute number of pollen grains in 10g RJ (PG/10g), apply the following formula:

$$PG_{10g} = \frac{PG_{counted} \cdot Lycopodium_{total}}{Lycopodium_{counted}} \cdot \frac{10}{p}$$

where:

PG_{10g} = absolute number of pollen grains in 10 g RJ

$PG_{counted}$ = number of pollen grains counted in the slide

$Lycopodium_{total}$ = average number of *Lycopodium* spores added to the preparation (18,583 for one tablet of the batch used in this work)

$Lycopodium_{counted}$ = number of *Lycopodium* spores counted in the slide

p = weight of RJ in grams

The best results are obtained with a pollen/spore ratio between 0.5 and 2. If sample is poor in pollen (<50,000/10g) or rich (>400,000/10g) it may be convenient to do a second preparation with a bigger quantity of RJ or by adding more tablets.

It is also possible to perform qualitative and quantitative analysis on the same preparation but, in some cases we observed that the addition of the *Lycopodium* tablet leaves some residues on the pollen grains that can make their identification more problematical. Then, in this work, we preferred to do different preparations for quantitative and qualitative analysis.

2.3. Repeatability

For both qualitative and quantitative methods some repeatability tests were performed. For the qualitative analysis, two RJ samples were chosen with different percentages of well recognisable pollen types. Ten slides were prepared with each of the 2 samples and pollen grains were counted according to the described method (2.1.).

For the quantitative analysis, three RJ samples were chosen with different PG/10g. Ten slides were prepared with each of the 3 samples and the absolute number of pollen grains was calculated according to the described method (2.2).

Some statistical parameters were calculated: mean, standard deviation, outliers, repeatability (r) and relative standard deviation (RDS%) [16].

2.4. Samples

In total 59 samples were collected as shown in Table I. From an investigation aimed to evaluate the effect of artificial nutrition on RJ composition [13], 9 RJ samples were taken from bee colonies fed with frozen pollen and 13 from colonies kept without any pollen supplement. Commercial RJ samples were purchased from importers (9 samples) and from Italian producers (28 samples). All the samples were analysed according to the qualitative and quantitative methods described above.

Table I – Samples collected for pollen analysis

Type of samples	**Number of samples**
Experimental samples:	
- From colonies fed with pollen	9
- From colonies without pollen supplement	13
Commercial samples:	
- From importers	9
- From Italian producers (northern regions)	13
- From Italian producers (central regions)	15
TOTAL	**59**

3. Results

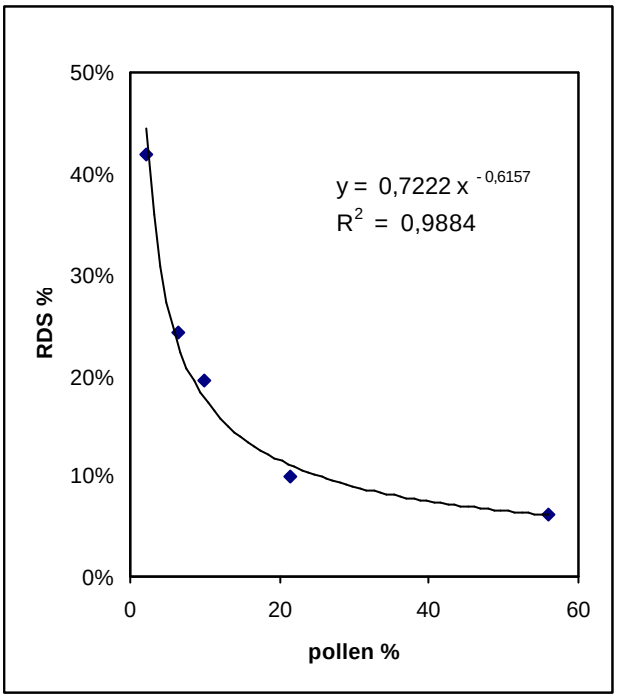
3.1. Repeatability

Results of the repeatability test for qualitative analysis are reported in Table II. They showed that the relationship between the percent of pollen and the relative standard deviation (RSD%) follows a power curve (Figure 2); precision was quite poor for low pollen percentages, while it improved for higher relative frequencies. The repeatability values were quite similar to those found for honey [17].

Table II – Repeatability test on qualitative pollen analysis of RJ: 2 samples (A and B), 10 repetitions for each sample [16]

Pollen type	**Mean (%)**	**St. dev.**	**r**	**RSD%**
Hedera [B]	2.2	0.9	2.5	41.8%
Trifolium pratense gr. [A]	6.5	1.6	4.4	24.3%
Umbelliferae [B]	9.9	1.9	5.3	19.3%
Chenopodiaceae [B]	21.4	2.1	5.9	9.9%
Echium [A]	56.0	3.4	9.5	6.1%
[A] and [B] indicate RJ samples used in this test				

Figure 2. Relationship between pollen percent and relative standard deviation (RSD%)



Results of the repeatability test for quantitative analysis are reported in Table III, and showed a RSD% from about 10% to 14%, similar to those reported for honey [17], but apparently unrelated to the value of PG/10g.

Table III – Repeatability test on quantitative pollen analysis of RJ (PG/10g x 10^3): 3 samples (level 1, 2, 3), 10 repetitions for each sample [16]

Level	Mean	St. dev.	r	RSD%
1	58.3	5.9	16.3	10.1%
2	94.5	9.0	24.9	9.5%
3	520.4	71.5	198.1	13.7%
Average RSD%				**11.1%**

3.2. Results of qualitative pollen analysis of commercial RJ samples

In the Italian samples 119 pollen types were identified. In Figures 3 and 4 the pollen types more frequent in northern and central regions respectively are reported. The pollen spectra reflected the respective plant environments where cultivated and crop associated species prevailed with a characteristic middle

European in northern Italy samples (*Castanea*) and more Mediterranean in Central Italy samples (*Eucalyptus* and *Olea*). Compared to honey from the same areas [7, 12], RJ samples show similar species associations, but non-nectariferous species are more represented in number and frequency. In northern Italy RJ samples some late blossoming species are more common than in corresponding honey [12], most likely because RJ samples were produced later (mainly in July and August).

With imported samples, different patterns were identified (Table IV). Seven of nine of them showed the typical Chinese association, with *Brassica*, *Astragalus sinicus* and *Vicia faba*, but only in three samples *Brassica* and *Astragalus sinicus* were over 50% of the total pollen grains, as reported [10]. The other 4 samples showed only very small amounts of these two species and a more complex spectrum with an important percentage (between 20% and 45%) of a *Castanea*-like pollen grain; in these samples the Chinese origin was less clear. Some other species, typically reported in Chinese honey [2, 9] were occasionally found (*Fagopyrum*, *Thalictrum*, *Tilia*, *Rhamnaceae*, *Eleagnus*, *Citrullus*, *Sanguisorba major*). In all 7 Chinese samples it is interesting to note the constant presence of an unidentified tricolporate form that sometimes reached 15% of the total.

*Table IV – Main pollen types in imported RJ samples: pollen types present in all samples analysed are indicated in bold; * indicates pollen types uncommon or absent in Italian RJ*

Chinese Royal Jelly	**Vietnamese Royal Jelly**
Astragalus sinicus *	*Mimosa pudica* gr. *
Cruciferae	*Mimosa pigra* gr. *
Tricolporate unknown type *	Compositae H f.
Vicia faba *	*Eucalyptus*
Caryophyllaceae *	Other Mimosoideae *
Compositae T f.	
Rosaceae	
Castanea	
Graminaceae	
Quercus robur gr. *	
Salix	
Umbelliferae	

Figure 3. Main pollen types identified in RJ samples from northern Italy

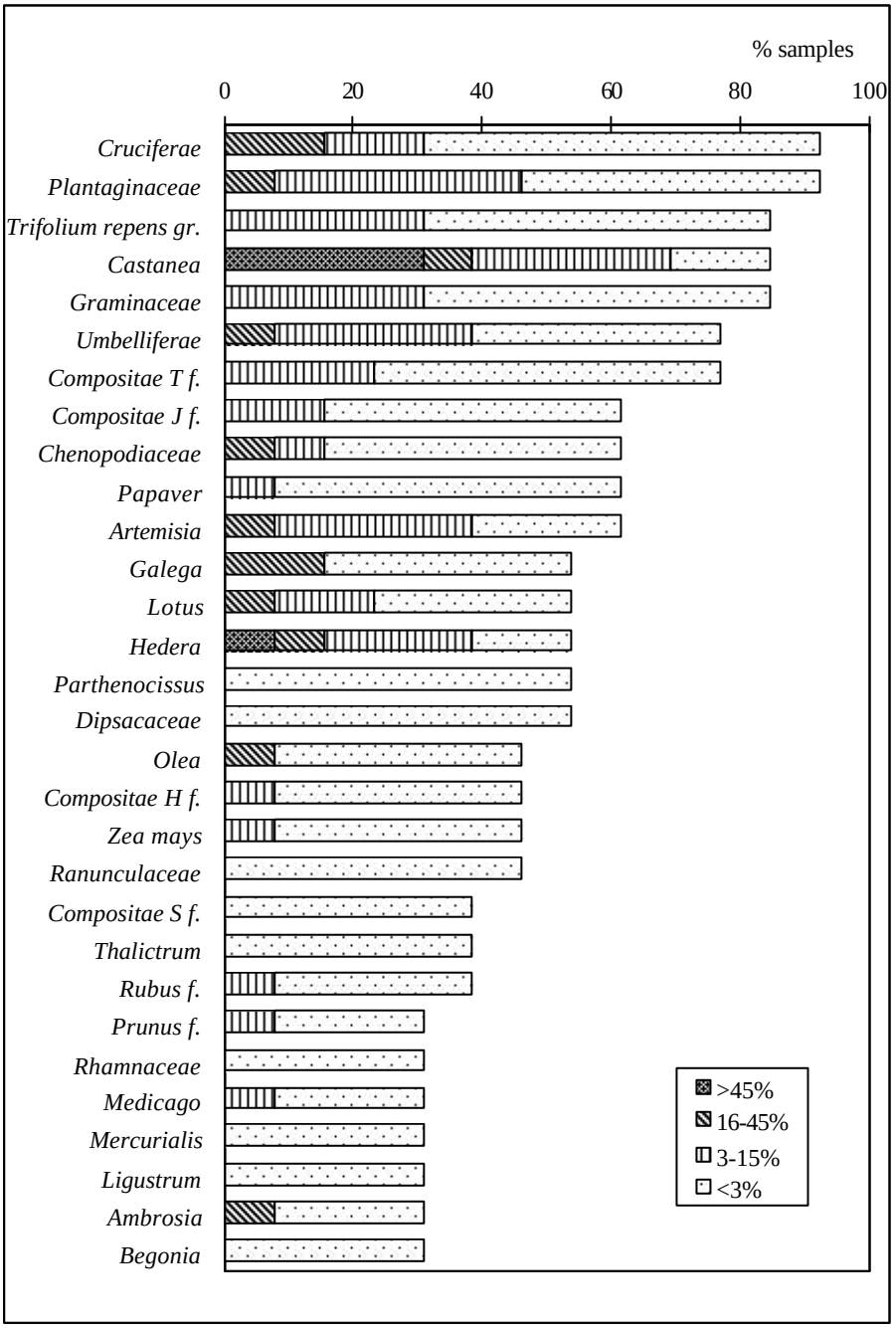
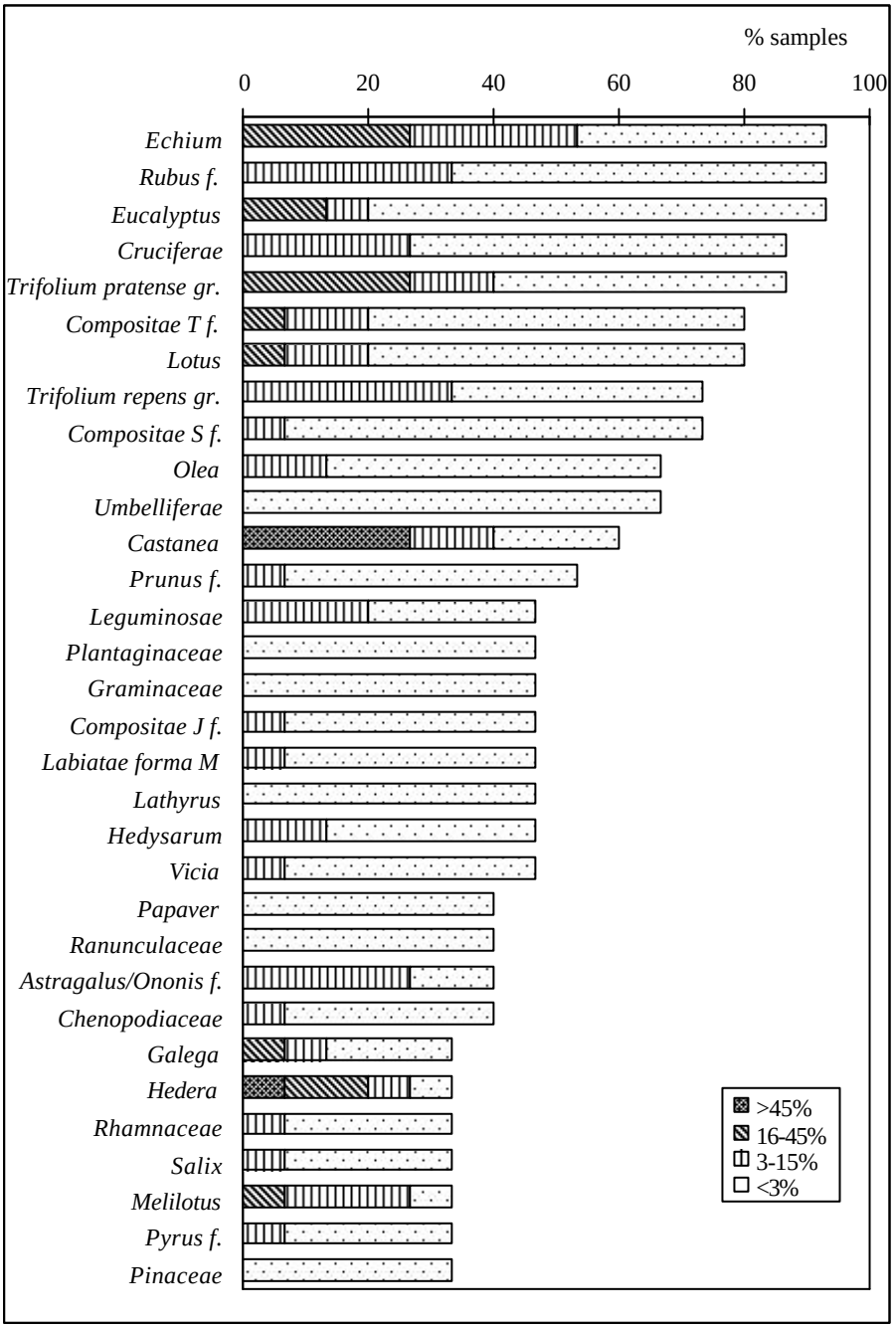


Figure 4. Main pollen types identified in RJ samples from central Italy



Another two samples showed a completely different spectrum, with only two major pollen types, both of the group of the Mimosoideae (*Mimosa pudica* gr. and *Mimosa pigra* gr. [1]) and very few other pollen grains. This spectrum clearly indicated a tropical origin (Table IV). The importer stated they originated from Vietnam, but other tropical origins could possibly give the same pollen composition.

The pollen analysis of RJ samples confirmed some characteristic features already described by other authors [4, 5, 8, 10, 11], in particular the presence of a high percentage (10% to 40%) of broken and/or digested grains. They could have come from the digestive system of the larvae or nurse bees [4, 5, 11], but this is not completely clear. Frequently, larval exuviae, yeasts, fungal hyphae and starch particles were found.

3.3. Results of quantitative pollen analysis of commercial and experimental RJ samples

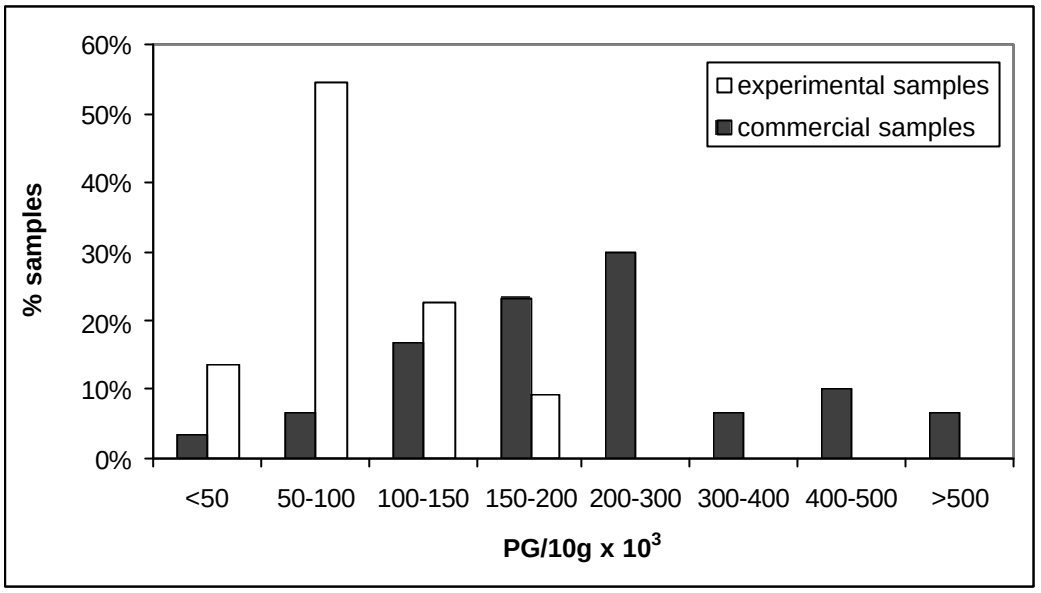
The results of the total pollen counts are reported in Table V and Figure 5. In experimental samples, no differences were found between RJ produced by colonies fed with or without pollen supplement in absolute quantity of pollen grains, or in other microscopic characteristics (pollen types, their diversity and relative frequencies).

In general, compared to honey, RJ showed quite high values of PG/10g, in agreement with previous observations [11]. Commercial RJ samples showed a wider variability and, on average, much higher values than experimental samples (253,500 ± 186,900 versus 89,800 ± 37,300).

Table V – Results of quantitative pollen analysis of RJ samples (PG/10g x 10^3)

	Mean	**St. dev.**	**Min**	**Max**
Experimental samples				
- From colonies fed with pollen	89.3	39.5	34.3	163.0
- From colonies without pollen supplement	90.1	37.3	41.5	177.8
Total	**89.8**	**37.3**		
Commercial samples				
- National	238.2	129.5	48.3	561.0
- Imported	290.9	291.1	131.0	1,022.8
Total	**253.5**	**186.9**		

Figure 5. Absolute pollen content in commercial and experimental RJ samples.



The elevated values found in commercial samples could be partly accounted for by the high percentages of over-represented pollen types, such as chestnut (in 4 Italian samples with more than 300,000 PG/10 g) or *Mimosa pudica* gr. (in one imported sample with 1,022,800 PG/10 g). However the reasons for these great variations among commercial samples, as well as between experimental and commercial groups remain unexplained.

4. Conclusions

The method we implemented and applied for pollen analysis of RJ is simple and precise enough to fit with the need for routine quality control. Some new information about microscopy of Italian RJ is given. In most cases the distinction between Italian and imported RJ does not seem to present any problems. Some foreign RJ spectra may have important overlapping with Italian samples and attention has to be given to all pollen types in order to achieve the right conclusion. In general microscopic analysis is a useful tool to recognize the geographical origin of bee products.

From our experimentation, feeding hives with pollen supplements does not seem to change the total pollen counts in RJ samples. Therefore this analysis cannot be used to identify the nutrition given to colonies.

Many palynological characteristics of RJ have possibly some interesting causes. Differences in total amount of pollen, quantity of damaged pollen grains, presence of other microscopic elements like fungal hyphae, yeasts, starch, could be accounted for by physiological and technical aspects. For instance larval age, physiology and behaviour of nurse bees, pollen hoarding instinct, use of selected strains and all techniques of production may play a role. Also it is possible that some of these microscopic features are related to the quality of the product. However currently, all of these aspects remain without any satisfactory explanation and need further investigation.

Literature

[1]. Barth O. M., O polem no mel brasileiro, Rio de Janeiro, Luxor, 1989 pp151

[2]. Battaglini M., Ricciardelli D'Albore G., Differenziazione dei mieli italiani e stranieri in base allo spettro pollinico, Atti Simposio Internazionale Apicoltura, Torino,1972, 96-111

[3]. Biondi C., Bedini, G. Felicioli A., Gelatina reale: metodologia proposta per la determinazione dell'origine geografica e della qualità, Apitalia, 2003, (6) 32-37

[4]. Chauvin R., Action physiologique et thérapeutique des produits de la ruche. La gelée royale. Action physiologique, In: Chauvin R. (ed.), Traité de Biologie de l'Abeille, vol. III. Masson et C., Paris, 1968, 137-139

[5]. Chauvin R., Louveaux J., Etude macroscopique et microscopique de la gelée royale, L'apiculteur, 1956, 33-43

[6]. Moar N. T., Pollen analysis of New Zealand honey, New Zealand Journal of Agricultural Research, 28 (1985), 39-70

[7]. Persano Oddo L., Belligoli P., Carini A., Morgia C., Piazza M.G., Pulcini P., Studi di caratterizzazione geografica: i mieli del Lazio, In: Persano Oddo L., Piana M. L. (ed.), Miele e territorio. Guida alla valorizzazione del miele

attraverso le denominazioni di origine, Ministero delle Politiche Agricole, Istituto Sperimentale per la Zoologia Agraria, Roma, 2001, 157-170

[8]. Ricciardelli D'Albore G., La gelatina reale D.O.C., L'Ape nostra amica, 1991, (4) 4-6

[9]. Ricciardelli D'Albore G., Textbook of melissopalynology, Apimondia, Bucharest, 1997, pp 138

[10]. Ricciardelli D'Albore G., Battaglini M., Origine géographique de la gelée royale. Apidologie, 9 (1978), 1-17

[11]. Ricciardelli D'Albore G., Battaglini M., D'Ambrosio M., Considerazioni sui pollini della gelatina reale, Annali dell'Istituto Sperimentale per la Zoologia Agraria, Firenze, 5 (1977), 203-213

[12]. Sabatini, A. G., Piana, M. L., Grillenzoni, F. V. (ed.), I mieli dell'Emilia-Romagna - Studio di caratterizzazione, Istituto Nazionale di Apicoltura, Bologna, 2000, pp 92

[13]. Sesta G., Persano Oddo L., Nisi F., Ricci L., Effects of artificial sugar feeding on sugar composition of royal jelly, 39th Apimondia International Beekeeping Congress, Dublin, 2005

[14]. Stockmarr J., Tablets with spores used in absolute pollen analysis, Pollen et Spores, 13 (1971), 615-621

[15]. Stockmarr J., Determination of spore concentration with an electronic particle counter, Geological Survey of Denmark Yearbook, 1972, 87-89

[16]. UNICHIM 179/1, Linee guida per la validazione di metodi analitici nei laboratori chimici - valutazione della precisione (ripetibilità stretta) di un metodo analitico eseguito in un unico laboratorio da un solo operatore su di un unico strumento in un breve intervallo di tempo, Milano, 2001, pp 78

[17]. Von Der Ohe W., Persano Oddo L., Piana M. L., Morlot M., Martin P., Harmonized methods of melissopalynology, Apidologie 35 (2004), suppl. 1, 18-25