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Evaluation of the botanical origin of commercial dry bee pollen load batche using pollen analysis: a proposal for technical standardization

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ABSTRACT

High quality of bee pollen for commercial purpose is required. In order to attend the consumer with the best identi cation of the botanical and floral origin of the product, 25 bee pollen batches were investigated using two techniqu of pollen grain preparation. The first started to identify pollen loads of different colors in two grams of each well mix batch, and the second to identify pollen grains in a pool made of all the pollen loads comprised in two grams. The be result was obtained by this last technique, when a pollen grain suspension was dropped on a microscope slide and cir 500 pollen grains were counted per sample. This analysis resulted in the recognition of monofloral and bifloral poll batches, while the use of the first technique resulted in all samples receiving a heterofloral diagnosis.

Key words: *Apis*, pollen loads, commercial pollen batches, pollen analysis, botanical origin.

INTRODUCTION

Bee pollen production increased during the last years as a response to commercial demand. *Apis mellifera* L. is the best pollen supplier mainly in tropical countries where entomophilous plant species are dominant. Beekeepers use pollen traps of different types in order to obtain the pollen loads from bees when coming home to hives. This pollen is humid and has to be dried before commercialization. Then, it may be distributed in vials receiving an identification that comprises, in addition, its botanical origin. This procedure is important to avoid missing the accurate scientific definition of the botanical name, since beekeepers report a lot of common names of plants that were visited by the bees.

Pollen grains present a huge variation of morpho- ity and are not influenced by environmental ev changes. Pollen analysis is the tool to recogniz where the pollen grains are coming. Several la rial techniques are used to prepare pollen grains croscope observation and identification. The di remains in the interpretation of the obtained da the evaluation of numerical and personal inform Several aspects are to be considered, the first one ing the bee behavior upon flower visitation for extraction. In this case, single bees are captur both of their pollen loads were analyzed (Carval Marchini 1999, Marques-Souza et al. 2002, Noc 2009). Secondly, when pollen traps are used, pollen loads perform one sample that, for analys to be well mixed; a group of pollen loads, in gen lected by its weight or color (Almeida-Muradian

The main issue to address is the significance or not of the choice by color. An accurate description of the pollen load sample processing and analysis is frequently missed during experiment descriptions (Modro et al. 2007, 2009a). Commonly, a fragment of a pollen load of each color is analyzed; no sample centrifugation is used. In another way (denoted technique 1), ten pollen loads (Barth et al. 2009) or 25 ones of each color batch (Barth, personal information and in the present paper) are mixed using ethanol, and a little amount is analyzed after centrifugation. As pollen grains have different weight, a gradient is obtained, from which pollen grains are removed using a piece of glycerin jelly. The significance of the obtained results is not clear. Another technically very complex preparation of pollen batches identification was recently used comprising several steps of pollen drying, hydrating and acetolysis (Novais et al. 2009) and, thus, missing important information besides pollen morphology.

The proposed technique by this study (technique 2) may help to get quick and accurate information about the botanical and regional origin of commercial bee pollen load products. It neither demands long time to be performed nor is expensive.

MATERIALS AND METHODS

Twenty five samples of commercial batches of bee pollen loads were obtained in apiaries of several states in Brazil and analyzed (Table I). Each sample was analyzed using two techniques. The first technique considers sub samples according to the pollen load colors; each sample was made of well-mixed two grams and each sub sample was made of 25 units or pollen loads of each color. When two or more types of color were mentioned in a same sub sample (Table I, column 2), it indicates that no sufficient pollen loads of a unique color were available to perform n=25, and several poorly represented color batches were mixed. Color was determined according to the Red Green Blue (RGB) color classification system. The color values correspond to: bright = RGB 247/240/183; brown = RGB 186/97/97, caramel = RGB 237/202/29, dark = RGB 117/11/11, green = RGB 98/156/128, orange = RGB 255/106/37, violet = RGB

Pollen grain identification used the available literature, meanly Barth (1989), Roubik and Moreno (1991), and the Palynological Slide Collection of the Laboratory of Palynology. Pollen frequency standard classes (Louveaux et al. 1978) were used, making an additional subdivision of the dominant pollen class (Table I).

TECHNIQUE 1 (TABLE I, COLUMNS 2-4):

Preparation of pollen load samples:

The pollen loads of two grams of each sample were distributed in batches or sub samples according to its color.

Preparation of microscope slides:

Twenty five pollen loads of each sub sample were macerated using 10 mL of 70% ethanol distributed into two 15 mL centrifuge tubes and centrifuged during 3 minutes at 1500 rpm. The sediment was resuspended with 10 mL of 70% ethanol and centrifuged again. In sequence, 5 mL of a water/glycerin 1:1 mixture were added to each tube, stirred, and left for 30 minutes. After centrifugation, the tubes remained with their aperture down on absorbent paper during a few minutes. Then, the pollen sediment was mixed and pollen slides were prepared using a little piece of glycerin jelly to capture the pollen grains, and slides were sealed with paraffin. Counting and identification comprised at least 500 pollen grains.

TECHNIQUE 2 (TABLE I, COLUMNS 5-6):

Preparation of pollen load samples:

Dry pollen loads of two grams of a sample (this is a pool of circa 300 pollen loads) were weighted into a 15 mL Falcon centrifuge tube, mixed using 70% ethanol just to complete 13 mL, and left for 30 minutes. Treatment with ultrasound (if disposable) during 5 minutes can be suitable for particle dispersal. The sediment obtained after centrifugation may be extracted with ethanol, and submitted to ultrasonic treatment again. A solution of distilled water/glycerin 1:1 was added to the sediment just to complete 13 mL, and left for circa 30 minutes.

Preparation of microscope slides:

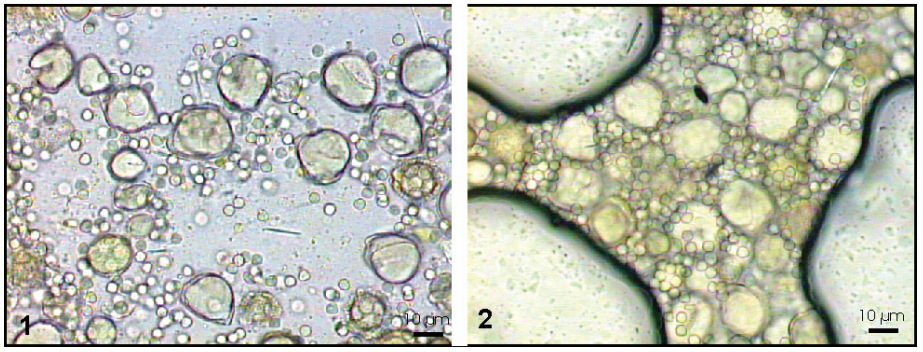

Fig. 1 – A pool of pollen loads prepared using technique 2; pollen grains are well dispersed all over the slide. Fig. 2 – A pool of pol
prepared using technique 2 three weeks later; the slide started to dry, and the pollen grains overlapped and were pressed against the air b

maintained in a horizontal position for circa one month or less, when drying starts, and the pollen grains were selectively pressed against the air bubbles. Then discard the slide (Figs. 1 and 2).

Preparation of stock samples:

One mL of the well mixed pollen grain suspension was put into a 1.5 mL Eppendorf centrifuge tube and centrifuged. A 15 mL Falcon tube can be used as a support for the Eppendorf tube. After discarding the supernatant, one mL of glycerin was added, mixed, and then the tube was closed and identified for additional preparations. It may be kept at room temperature.

Preparation of additional pollen slides:

The content of the Eppendorf tube has to be homogenized. One drop of this pollen grain suspension was mixed with one drop of distilled water in a clean Eppendorf tube. A microscope slide, as formerly described may be prepared after 30 min waiting for water impregnation.

Counting and identification of pollen grains and structured elements:

At least 500 pollen grains have to be counted and identified using a 400× magnification, preferentially within 10 days, before drying starts.

pollen loads, establishing several sub samples. N
ple was single colored. When no sufficient polle
of a unique color were available, two, rarely th
ferent colored pollen loads were joined together i
to make up a group of 25 unities (column three)
never constituted a dominant sub group in a sar
batch.

Fifty two pollen types presenting a frequenc
or higher than 3% of the pollen sum were reco
Technique 1 presented 46 pollen types, 22 being
sives. Technique 2 presented 29 pollen types, si
exclusives.

No monofloral pollen load batch was reco
by technique 1. Among the 25 analyzed sample
were bifloral presenting two dominant pollen ty
samples were heterofloral comprising three or mo
len types. Considering technique 2, eight polle
batches were monofloral, six bifloral and 11 heter

Nevertheless, technique 1 detected more
types (46) than technique 2 (29), this last pro
showed a better evaluation of the whole pollen
Thus, commercial qualification has to follow th
Consequently, it presented more monofloral batc

Technique 1 was useful in order to teach abo
len loads botanical origin. As explained below, th
of pollen of a plant species may vary (Barth et a
and in the present paper). Therefore, it is not
characteristic to inform about the botanical orig
pollen load batch. While it is not possible to anal
pollen grain composition of each pollen load, a

TABLE I

Comparison of 25 pollen load samples (2g of each batch) from *Apis mellifera* evaluated by its color (sub samples in columns two, three and four) and evaluated by a pool of 2g of each batch (columns five and six). Each sub sample comprised 25 units of pollen loads. Only the pollen types with frequency >3% were considered: very frequent (++++, circa >85%), frequent (+++, circa 45 to 85%), few frequent (++, circa 15 to 45%), rare (+, circa 3 to 15%).

	Technique 1			Technique 2	
Sample register and procedence	Sub samples established by color of pollen loads	Pollen types identified in the sub samples	Evaluation of the sub samples	Pollen types identified in a pool of 2g of the sample	Evaluation of a pool of 2g of the sample
55-Piaui	yellow	*Cyperus* (++++)	monofloral of *Cyperus*	*Cocos nucifera* (+++) *Cyperus* (++) Amaranthaceae (+) Solanaceae (+)	bifloral of *Cocos nucifera* (dominant) and *Cyperus*
	bright and green	*Cocos nucifera* (++++)	monofloral of *Cocos nucifera*		
	dark	*Tapirira* (++++), *Bombax* (+)	monofloral of *Tapirira*		
	brown	*Inga* (++++), *Cocos nucifera* (++)	monofloral of *Inga*		
56-Bahia	bright and orange	*Syagrus* (++++), *Portulaca* (++)	monofloral of *Syagrus*	*Cocos nucifera* (++++)	monofloral of *Cocos nucifera*
	bright and green	*Mimosa scabrella* (++++), Fabaceae (++)	monofloral of *Mimosa scabrella*		
57-Bahia	bright and orange	*Syagrus* (++++), *Portulaca* (++)	monofloral of *Syagrus*	*Mimosa scabrela* (++++) *Syagrus* (+) *Cocos nucifera* (+)	monofloral of *Mimosa scabrella*
	brown and green	several polen types	heterofloral		
58-Minas Gerais	bright	*Brassica* (+++), *Vernonia* (+)	monofloral of *Brassica*	*Eucalyptus* (+++) *Cecropia* (++) *Myrcia* (+) *Syagrus* (+)	heterofloral with dominance of *Eucalyptus*
	orange	*Senecio* (++++)	monofloral of *Senecio*		
	dark	*Vernonia* (++++) and uredospores	monofloral of *Vernonia* and uredospores		
59-São Paulo	caramel	Poaceae (+++) *Anadenanthera* (+)	monofloral of Poaceae	*Eucalyptus* (+++) *Vernonia* (++) *Senecio* (++) *Piper* (+) *Myrcia* (+)	heterofloral
	bright	*Syagrus* (++++)	monofloral of *Syagrus*		
	dark and yellow	*Eucalyptus* (++++)	monofloral of *Eucalyptus*		
	orange	without pollen grains, only uredospores	without pollen grains, only uredospores		
65-Bahia	yellow and brown	*Cyperus* (++++)	monofloral of *Cyperus*	*Mimosa scabrella* (++++)	monofloral of *Mimosa scabrella*
	bright	*Astrocaryum* (++), *Mimosa scabrella* (++), Asteraceae (++)	heterofloral		
	dark	immature pollen	heterofloral		

TABLE I (continuation)

Sample register and procedence	Technique 1			Technique 2	
	Sub samples established by color of pollen loads	Pollen types identified in the sub samples	Evaluation of the sub samples	Pollen types identified in a pool of 2g of the sample	Evaluat a pool o of the sa
65-Bahia	green	*Borreria densiflora* (++++)	monofloral of *Borreria densiflora*		
72-Espírito Santo	bright and brown	*Euterpe edulis* (+++), *Vernonia* (++)	bifloral of *Euterpe edulis* and *Vernonia*	*Vernonia* (++) *Cecropia* (++) *Euterpe edulis* (+) *Myrcia* (+) *Eupatorium* (+) uredospores (+)	heterofl
	orange and yellow	*Persea* (+++) and several pollen types	heterofloral with dominance of *Persea*		
	red and violet	without pollen grains, only uredospres	without pollen grains, only uredospores		
74-Bahia	yellow	*Syagrus* (+++)	without pollen grains, only uredospores	*Cecropia* (++) *Caesalpinia peltophoroides* (++) Asteraceae (++) *Cyperus* (+) *Syagrus* (+) *Cocos nucifera* (+) *Myrcia* (+)	heterofl
	bright	*Cocos nucifera* (+++), *Astrocaryum aculeatissimum* (+++)	bifloral of *Cocos nucifera* and *Astrocaryum aculeatissimum*		
	dark	*Mangifera indica* (++++)	without pollen grains, only uredospores *Mangifera indica*		
	orange and green	*Borreria* verticillata (+++), *Bidens* (++)	bifloral of *Borreria* verticillata and *Bidens*		
	brown and orange	*Cocos nucifera* (+++), *Cyperus* (+++)	bifloral of *Cocos nucifera* and *Cyperus*		
77-Sergipe	bright and orange	*Cocos* (++++), *Montanoa* (+)	monofloral of *Cocos*	*Mimosa scabrella* (+++) *Cocos nucifera* (++) *Eupatorium* (+)	bifloral *Mimosa scabrell* (domina and *Coc nucifera*
	brown	*Cocos nucifera* (+++), *Mimosa caesalpiniaefolia* (+++)	bifloral of *Cocos nucifera* and *Mimosa caesalpiniaefolia*		
80-Sergipe	bright	*Cocos nucifera* (++++), *Richardia* (+)	monofloral of *Cocos nucifera*	*Mimosa scabrella* (+++) *Cocos nucifera* (++) *Myrcia* (+)	bifloral *Mimosa scabrell* (domina and *Coc nucifera*
	dark and caramel	several pollen types	heterofloral		
	orange	*Caesalpinia*	monofloral of		

TABLE I (continuation)

Sample register and procedence	Technique 1			Technique 2	
	Sub samples established by color of pollen loads	Pollen types identified in the sub samples	Evaluation of the sub samples	Pollen types identified in a pool of 2g of the sample	Evaluation of a pool of 2g of the sample
83-São Paulo	bright, green and brown	*Schinus* (+++), *Cecropia* (+++) and others	bifloral of *Schinus* and *Cecropia*	*Cecropia* (+++) *Senecio* (+) *Trema* (+) *Myrcia* (+) *Eupatorium* (+) *Vernonia* (+)	monofloral of *Cecropia*
	orange	*Senecio* (++++)	monofloral of *Senecio*		
84-Minas Gerais	yellow	*Antigonon leptopus* (++++)	monofloral of *Antigonon leptopus*	*Antigonon leptopus* (+++) *Baccharis* (++) *Mimosa scabrella* (+), *Mimosa caesalpiniaefolia* (+), unknown (+)	monofloral of *Antigonon leptopus*
	bright	*Baccharis* (+++) and others	monofloral of *Baccharis*		
	orange and brown	several pollen types	heterofloral		
85-Minas Gerais	yellow	*Antigonon leptopus* (+++), *Cyperus* (+)	monofloral of *Antigonon leptopus*	*Cecropia* (+++) *Myrcia* (++) *Vernonia* (+)	bifloral of *Cecropia* (dominant) and *Myrcia*
	bright and brown	*Anadenanthera* (++), Asteraceae (++)	heterofloral		
	dark	*Myrcia* (++++)	monofloral of *Myrcia*		
86-São Paulo	yellow	*Eucalyptus* (++++)	monofloral of *Eucalyptus*	*Eucalyptus* (+++)	monofloral of *Eucalyptus*
	orange	*Eucalyptus* (+++), Euphorbiaceae (+++)	bifloral of *Eucalyptus* and Euphorbiaceae		
	brown	*Eucalyptus* (++++)	monofloral of *Eucalyptus*		
87-Minas Gerais	bright and brown	*Eucalyptus* (++), *Vernonia* (++), *Croton* (+)	bifloral of *Eucalyptus* and *Vernonia*	*Eucalyptus* (+++) *Vernonia* (+)	monofloral of *Eucalyptus*
	violet, orange and yellow	*Eucalyptus* (+++), *Senecio* (+++)	bifloral of *Eucalyptus* and *Senecio*		
88-São Paulo	yellow	*Baccharis* (++++)	monofloral of *Baccharis*	*Eucalyptus* (+++) Poaceae (+)	monofloral of *Eucalyptus*
	bright	*Baccharis* (++), *Syagrus* (++)	bifloral of *Baccharis* and *Syagrus*		
	brown	*Eucalyptus* (++++)	monofloral of *Eucalyptus*		
97-Santa Catarina	yellow	*Ilex* (++++), Onagraceae (+)	monofloral of *Ilex*	*Eupatorium* (++), Melastomataceae	heterofloral

TABLE I (continuation)

Sample register and procedence	Technique 1			Technique 2	
	Sub samples established by color of pollen loads	Pollen types identified in the sub samples	Evaluation of the sub samples	Pollen types identified in a pool of 2g of the sample	Evaluation o a pool of 2g of the sample
97-Santa Catarina	orange	*Senecio* (++++)	monofloral of *Senecio*	*Montanoa* (+)	
	brown	*Vernonia* (++++)	monofloral of *Vernonia*		
100-Paraná	yellow	*Alchornea* (+++)	monofloral of *Alchornea*	*Sebastiania* (+++), *Brassica* (++), *Eucalyptus* (++)	heterofloral
	bright	several pollen types	heterofloral		
	orange and brown	several pollen types	heterofloral		
	green	Rosaceae (++++)	monofloral of fruits (plum, apple, pear)		
	red and brown	*Eucalyptus* (++++) and uredosporos	monofloral of *Eucalyptus* and uredospores		
111-Santa Catarina	bright	*Eupatorium* (+++) *Cocos nucifera* (++) *Vernonia* (+)	bifloral of *Eupatorium* and *Cocos nucifera*	*Vernonia* (++) *Myrcia* (++) *Montanoa* (++) *Syagrus* (+) Poaceae (+) Rubiaceae (+) *Crotalaria* (+)	heterofloral
	orange	*Montanoa* (++++)	monofloral of *Montanoa*		
	brown	*Vernonia* (++++)	monofloral of *Vernonia*		
	red	*Baccharis* (+++) *Sebastiania* (++) *Montanoa* (++)	trifloral		
115-Sergipe	bright	*Cocos nucifera* (++++)	monofloral of *Cocos nucifera*	*Mimosa scabrella* (+++), *Cocos nucifera* (++)	bifloral of *Mimosa scabrella* (dominant) and *Cocos nucifera*
	orange	*Mimosa scabrella* (+++), Asteraceae (several pollen types)	monofloral of *Mimosa scabrella*		
	brown	*Mimosa scabrella* (++++), *Cocos nucifera* (++), *Commelina* (++)	monofloral of *Mimosa scabrella*		
116-Sergipe	yellow	*Syagrus* (++++)	monofloral of *Syagrus*	*Mimosa caesalpiniaefolia* (+++), *Cocos nucifera* (++)	bifloral of *Mimosa caesalpiniae* and *Cocos nucifera*
	bright	*Cocos nucifera* (++++)	monofloral of *Cocos nucifera*		
	orange	several pollen	heterofloral		

TABLE I (continuation)

Sample register and procedence	Technique 1			Technique 2	
	Sub samples established by color of pollen loads	Pollen types identified in the sub samples	Evaluation of the sub samples	Pollen types identified in a pool of 2g of the sample	Evaluation of a pool of 2g of the sample
116-Sergipe	brown	*Mimosa caesalpiniaefolia* (++++), Mimosaceae Mv (++)	monofloral of *Mimosa caesalpiniaefolia*		
144-Bahia	yellow	*Syagrus* (+++), *Cocos nucifera* (+), *Eupatorium* (+)	monofloral of *Syagrus*	*Baccharis* (+++), *Cocos nucifera* (++) *Syagrus* (+)	heterofloral
	bright	*Cocos nucifera* (++++)	monofloral of *Cocos nucifera*		
	several colors	several pollen types	heterofloral		
145-Bahia	caramel	*Cocos nucifera* (+++), Poaceae (+++)	bifloral of *Cocos nucifera* and Poaceae	*Mimosa scabrella* (++), *Schinus* (++), Poaceae (+), *Cocos nucifera* (+), *Mimosa caesalpiniaefolia* (+), *Eupatorium* (+) *Syagrus* (+)	heterofloral
	bright	*Cocos nucifera* (+++), *Astrocaryum aculeatissimum* (+++)	bifloral of *Cocos nucifera* and *Astrocaryum aculeatissimum*		
	several colors	*Mimosa caesalpiniaefolia* (++), *Trema* (++), and others	heterofloral		
146-Bahia	caramel	Poaceae (++++) *Triumfetta* (+)	monofloral of Poaceae	*Cocos nucifera* (++) *Schinus* (++) Poaceae (++) *Mimosa caesalpiniaefolia* (++) *Mimosa scabrella* (+)	heterofloral
	bright	*Cocos nucifera* (++++)	monofloral of *Cocos*		
	dark and yellow	several pollen types	heterofloral		
	orange	*Portulaca* (+++) *Commelina* (++)	bifloral of *Portulaca* and *Commelina*		
150-Espírito Santo	yellow	*Alchornea* (+++), Arecaceae (+) and others	monofloral of *Alchornea*	*Eucalyptus* (++) *Cecropia* (++) *Myrcia* (++) Asteraceae (+) *Cocos nucifera* (+) Poaceae (+) *Alchornea* (+)	heterofloral
	caramel	Poaceae (+++), *Croton* (+)	monofloral of Poaceae		
	bright and brown	*Cocos nucifera* (+++), *Syagrus*	bifloral of *Cocos nucifera* and		
	dark and red	several pollen types	heterofloral		
	orange and	*Montanoa*	monofloral of		

Preparing slides in five folds, the same pollen spectrum was obtained.

Detailed discussion of the data obtained by the use of the two techniques of pollen load batches analysis (Table I).

Pollen grain color of a plant taxon (third column in Table I) can change from bright to dark as it may be observed in *Cocos* (samples 55, 74, 77, 115, 145, 146), *Vernonia* (samples 58, 111), *Eucalyptus* (samples 86, 87) and *Baccharis* (sample 88) pollen grains, and in the *Mimosa scabrella* pollen type (sample 115). Time to air exposition of pollen grains resulting in exine and cytoplasm oxidation could be responsible for different colored pollen loads of a plant species.

Using technique 1, all pollen batches were heterofloral. Technique 2 revealed monofloral (samples 56, 57, 65, 83, 84, 86, 87, 88) and bifloral (samples 55, 77, 80, 85, 115, 116) samples besides the heterofloral ones.

The monofloral samples presented five dominant pollen types: *Cocos nucifera* and *Mimosa scabrella* (= *M. sensitiva*) pollen types from the state of Bahia, *Cecropia* and *Eucalyptus* from São Paulo State, *Antigonon leptopus* (a garden species) and *Eucalyptus* from the state of Minas Gerais. These commercial batches have obtained the best evaluation.

The bifloral samples presented six important pollen types: *Cocos nucifera* and *Cyperus* from the state of Piaui, *Mimosa scabrella* and *Cocos nucifera* in three samples from the state of Sergipe, *Mimosa caesalpiniaefolia* and *Cocos nucifera* also from the state of Sergipe, and *Cecropia* and *Myrcia* from the state of Minas Gerais. These commercial batches may receive a good evaluation.

The heterofloral pollen load batches analyzed by techniques 1 and 2 do not have palynological definition of any dominance. Their botanical origin is variable depending upon several factors and their reproduction must not be effective.

A curious composition of some pollen loads was made of uredospores of fungi detected by technique 1 (in samples 59, 72, 74, 100). Uredospores never appeared using technique 2. As so, their contribution to any of the pollen batches was not significant. *Cladosporium*

In conclusion, using technique 1 based upo
analysis, more pollen types were identified, but n
inance of pollen type or plant species in a comm
pollen batch was reported. When using the tec
2, besides the monofloral batches, more bifloral a
heterofloral batches were recognized. This result
that a better characterization of a large pollen loa
composition of commercial interest was obtained
using the last technique.

RESUMO

É exigida alta qualidade para a comercialização d
apícola. A fim de atender o consumidor com a melh
tificação da origem botânica e floral do produto, 25
de pólen apícola foram investigadas usando duas di
técnicas na preparação dos grãos de pólen. A prime
tiu da identificação das cargas polínicas contidas em d
mas de cada partida bem misturada segundo suas core
gunda visava identificar os grãos de pólen de um agrup
("pool") de todas as cargas polínicas contidas em dois
de cada amostra. O melhor resultado foi obtido pela
técnica, quando uma suspensão de grãos de pólen e
jada sobre uma lâmina de microscopia e cerca de 500 g
pólen eram contados por amostra. Esta análise resulto
conhecimento de partidas monoflorais e biflorais de pó
cola, enquanto que usando a primeira técnica, todas as a
receberam a diagnose heterofloral.

Palavras-chave: *Apis*, cargas de pólen, partidas comer
pólen, análise polínica, origem botânica.

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