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Correlation between methyl anthranilate level and percentage of pollen in Spanish citrus honey

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Abstract

The common level of methyl anthranilate (MA) in Spanish citrus honey and the correlation between this compound and the percentage of citrus pollen (sometimes underrepresented) is evaluated. The MA analysis methodology was validated before analyzing the honeys (harvested in 2011 and 2012), which were characterized by pollen, MA, hydroxymethylfurfural, electrical conductivity, moisture and colour. Pollen ranged 1-88% and MA 0.5-5.9 mg/kg and there was no quantitative correlation between both. However, significant correlations with moderate Pearson coefficients were observed: MA/electrical conductivity (-0.678); MA/colour (-0.559); pollen/electrical conductivity (-0.553) and pollen/colour (-0.556). 89.2 % of samples from 2011 and 95.4% from 2012 had the required level of citrus pollen (at least 10%), although only 53.5% and 61.4%, respectively, had the commercially required of MA (2 mg/kg,). Only about half of the samples satisfied both parameters. The MA value should be recommended only when the honey has an unexpectedly low percentage of citrus pollen, and after assessing organoleptic and physicochemical parameters.

Keywords: citrus honey, methyl anthranilate, *Citrus* spp pollen, correlation, validation, melissopalynological analysis, electrical conductivity, colour

Introduction

Traditionally, honey is authenticated by identifying and quantifying the percentage of pollen (Bogdanov, 2002). However, in the case of citrus varieties this melissopalynological analysis alone, widely used for other types of monofloral honey, is sometimes not sufficient. The problem lies in the fact that the production of pollen and nectar in citrus blossom is not always simultaneous. Which is to say that citrus trees sometimes produce nectar before the anther produces pollen. Apart from this, bees occasionally collect nectar from sterile hybrid varieties of citrus trees, which are characterized by their small amounts of pollen. For these reasons the percentage of pollen in citrus honey may be lower than expected (Molins, et al., 1995). This clearly implies a difficulty in relation to the minimum percentage of pollen from *Citrus* spp. required for this type of honey: 10% (Persano-Oddo, et al., 1995; Molins et al., 1995; DOGV, 2002); 12% (Gomez-Pajuelo, 2004); 18.6% (Persano-Oddo & Piro, 2004); ≥5 % (Rodriguez et al., 2010). This is a problem for the commercialization of this valuable type of honey since it can be unfairly rejected, even though its organoleptic characteristics are undisputed.

Methyl anthranilate (MA) is a specific compound in citrus blossom nectar; its aroma is characteristic of this type of flower (ISO 5496, 2006) which may vary depending on the citrus cultivars (Jabalpurwala, et al., 2009). The presence of this compound in a honey indicates that the bees have taken nectar from citrus trees (White, 1966; White & Bryant, 1996; Castro-Vazquez, et al., 2007). For this reason, MA has been used for decades as a marker in citrus honey and should be a good tool for the classification of citrus honey when the level of pollen is very low. However, at present, commercial transactions require that citrus honey has a minimum citrus pollen content (between 10 and 20%), together with a methyl anthranilate level of at least 2 mg/kg (Sesta, et al., 2008). According to the data reported by different authors, the quantity of MA in the nectar citrus varies,

53 depending on the country. Citrus honey from Florida has a mean value of 3.10 mg/kg of
54 MA (SD = 0.91) (White & Bryant, 1996). However, Sesta et al., in 2008 suggested that a
55 minimum content of 0.5 mg/kg is sufficient for *Citrus* honey produced in Italy. There are
56 a few old studies related to the level of this aromatic compound in Spanish citrus honey:
57 minimum of 0.5 mg/kg (Serra-Bonvehí, 1988), and average of 2.3 mg/kg (SD=0.5)
58 (Ferreres, et al., 1994; Serra-Bonvehí & Ventura, 1995). In addition to variation due to
59 geographic origin, levels of MA can differ depending on storage conditions (Serra-
60 Bonvehí & Ventura, 1995; White & Bryant, 1996; Sesta et al, 2008). Therefore, it is
61 important to take this into consideration in order to make appropriate comparisons.

62 Clearly, there is a great disagreement about the required level of MA in citrus honey.
63 However, the origin of this discrepancy could in part be in the application of different
64 analytical techniques over the last three decades: HPLC-DAD (Ferreres, et al., 1994;
65 Nozal, et al., 2001; Sesta et al, 2008), Photometry (White & Bryant, 1996), HS-SPME-
66 GC (Bertelli, et al., 2008; Papotti, et al., 2009; Papotti, et al., 2012), P&T/GC-MS-thermal
67 desorption (Escriche, et al., 2011). As there is no official methodology described for the
68 analysis of MA, the only way to ensure the quality of the obtained results is to validate
69 the quantification methodology.

70 For the above mentioned reasons, the present work aims to evaluate the level of MA
71 in Spanish citrus honey and to determine the extent to which this level can be related to
72 the percentage of pollen from citrus genus. In order to ensure the utility of the
73 chromatographic procedure used to quantify this compound, it was validated before
74 analyzing the samples.

75 **Material and Methods**

76 *Honey samples*

77 Ninety eight different samples of Spanish citrus honey were used in this study. The
78 samples were harvested in the only areas (East and South) of Spain where citrus trees
79 grow. The same beekeepers (B) directly supplied twenty eight samples from 2011 and
80 fifty samples in 2012, ensuring that the hives had been located in citrus groves. The
81 remaining twenty samples (10 from 2011 and 10 from 2012) were purchased, in the same
82 period of time, in different retail outlets (R) in the city of Valencia, checking in all cases
83 that they were sold as citrus honey harvested in Spain. A melissopalynological analysis
84 was carried out on all samples to ascertain the percentage of pollen of *Citrus* spp.

85 All samples were analysed as soon as they were received in the laboratory. Samples
86 that came from beekeepers were sent to the laboratory immediately after they were
87 harvested. None of the samples used exhibited signs of crystallization.

88 *Melissopalynological analysis*

89 Pollen analysis was performed using the recommendations of the International
90 Commission for Bee Botany (Ohe, et al., 2004), without an acetolysis solution to preserve
91 all the components. Slides were prepared as follows: 10 g of honey were dissolved in
92 acidulated water (H_2SO_4, 5%) on a heating plate at 40 ºC. Subsequently, it was
93 centrifuged, the supernatant was decanted and the precipitate was suspended in 10 mL of
94 distilled water. A second centrifugation was performed, the supernatant was decanted off
95 and 0.2 mL of water was added to the precipitate. After stirring, 0.2 mL were deposited
96 on a slide and dried. Finally a drop of glycerin was used to seal the coverslip. A light
97 microscope (Zeiss Axio Imager, Göttingen, Germany) with DpxView LE image analysis
98 software attached to a DeltaPix digital camera was used in this analysis. A count of at
99 least 600 pollen grains was performed observing at ×400–1000 magnifications. These
100 grains of pollen were classified according to pollen morphology as in the literature
101 (Carretero, 1989; Saenz-Laín & Gómez-Ferreras, 2000).

Methyl anthranilate analysis

Methodology

A HPLC-DAD (Diode-Array Detection) method, based on Sesta et al. (2008), was used in the present work for MA determination. The method consists of acid hydrolysis, followed by extraction with copolymer cartridges and then chromatographic analysis. An LC Agilent 1120 Compact LC, including a binary pump, a thermostat column compartment, an auto-sampler and a UV detector were used. The chromatographic column and the software system used in the HPLC-UV method was the same as that used for the HMF analysis.

Chromatographic separation was carried out with a mobile phase consisting of water (A) and acetonitrile (B). Binary gradient conditions were used: first an isocratic step from 0 to 3.1 min with 70% A, and then a linear gradient was applied arriving at 42% A in 2 min. After that, a second linear gradient was applied arriving at 10% A, held for 2 min, and re-equilibrates to the initial conditions in 3 min. The flow-rate was 1 mL/min. The injection volume was 20 μL, and the oven column was maintained at 30 ºC. The MA was monitored at 335 nm.

A HPLC Alliance 2695, with a 2996 photodiode array detector (Waters, USA), equipped with the same column, was also used to corroborate the absorbance spectra, necessary for the identification of MA. The UV absorbance spectrum of MA presented an intense absorbance peak at 218 nm and 2 less intense peaks at 245 and 334 nm. As noted by Sesta et al. (2008), it was considered more appropriate to quantify at the absorbance of 334 nm as it presents less interference than the other ones.

Under the specified chromatographic conditions the MA peak was eluted at a retention time of about 6.8 min. Quantification was realized by means of matrix calibration curves obtained from spiked fortified blank samples. In order to ensure the quality of the results

and evaluate the stability of the proposed method, an internal quality control (a spiked blank sample with a final concentration of 2 mg/kg) was injected in the equipment as a first step before each batch of sample.

In all cases a polyfloral honey with absence of MA was used as a honey blank. The absence of citrus pollen in this honey was corroborated previously.

Reagents and standards solutions

HPLC-grade acetonitrile was purchased from VWR and the standard MA (purity > 99%) from Merck (Darmstadt, Germany). Analytical grade sulphuric acid was from Scharlab (Barcelona, Spain). For Solid Phase Extraction, Oasis HLB cartridges (200 mg/6 mL) from Waters were used. De-ionized water of MilliQ quality was used throughout.

The stock standard solution of MA was prepared by weighing the appropriate amount of the pure standard and diluting it with water to obtain a final concentration of 1 mg/mL. The working standard solution was obtained at a concentration of 0.1 mg/mL in H_2SO_4 1M in the same way as the samples,. The stock standard solution was stored at -20ºC and the working standard solution was at +4ºC.

Validation of the MA analysis method

The guidelines established by Commission Decision (2002), were followed in order to validate the MA analytical methodology. To this end several parameters were studied: linearity, accuracy and precision (repeatability and reproducibility). The accuracy of the method was established through recovery studies and the precision by: repeatability or intraday precision (RSD_r) and reproducibility or interday precision (RSD_R). LODs (limit of detection) and LOQs (limit of quantification) were estimated as the amount of analyte for which signal-to-noise ratios (S/N) were higher than 3 and 10 respectively.

Physicochemical and colour analysis

5-hydroxymethylfurfural (HMF) determined by HPLC-UV methodology and physicochemical parameters (moisture content and electrical conductivity) were analyzed as described in "Harmonized Methods of the European Honey Commission" (Bogdanov, 2002). The chromatographic column used for the analysis of HMF was a ZORBAX Eclipse Plus C18 (4.6 x 150 mm, 5 μm particle size) purchased from Agilent (Agilent Technologies, USA). The mobile phase for this analysis was water-methanol (90:10, v:v), with a flow rate of 1 mL/min. The detector was set to 285 nm. The EZChrom Elite system software was used for HPLC data processing. Colour was determined with a millimeter Pfund scale C 221 Honey Color Analyzer (Hanna Instruments, Spain). All analyses were performed in triplicate

Statistical analysis

The pollen percentage, physicochemical (MA, HMF, electrical conductivity) and colour data were analyzed by a multifactor analysis of variance (ANOVA) (significance level $\alpha = 0.05$) (using Statgraphics Centurion for Windows) to study the influence of the year of harvesting and the type of sample (beekeeper and retail). The method used for multiple comparisons was the LSD test (least significant difference) with a significance level $\alpha = 0.05$. The bivariate Pearson correlations were obtained (significance level $\alpha = 0.05$) in order to measure the strength and direction of the linear relationships between pairs of variables using SPSS 16.0. The contingency table analysis (cross tabulations) was carried out to evaluate the interrelation between pollen and MA, considering these variables as categorical, using the same SPSS 16.0.

Results and Discussion

Validation of Methyl anthranilate analytical methodology

The results from the validation procedure are shown in Table 1. In order to obtain the linearity evaluation an external standard calibration curve was constructed using spiked

fortified blank honeys (honeys without MA) with final concentration levels of: 0.5, 1, 2, 3 and 5 mg of MA/kg honey. These concentrations covered the values of this compound which were expected to be found in the honey samples. Six replicates were carried out for each level. Injections were performed in triplicate. A calibration curve was obtained by plotting the peak area of the compound at each level versus the concentration of MA added to the sample. A good linearity response in the range of the concentration considered was observed, with a correlation coefficient (R^2= 0.995) between peak areas and injected nominal concentrations.

The recovery studies were performed by adding known quantities of MA to the blank honey (0.5, 1, 2, 3 and 5 mg of MA/kg). All spiked fortified sample levels were done in triplicate and analyzed by the HPLC method. The results displayed in Table 1 show that the method used led to recovery of MA between 96 and 105% for the concentration range studied. The relative standard deviation (RSD) corresponding to recovery values ranged from 6.0 to 11.6%. As these values were less than 20%, the accuracy of the analytical method was confirmed (Commission Decision, 2002).

Repeatability (RSD_r) was evaluated by performing the assay on six replicates of fortified honey samples, at the same levels (0.5, 1, 2, 3 and 5 mg of MA/kg), and performed by the same operator on the same day. To evaluate reproducibility (RSD_R) the experiment was carried out on 3 consecutive days, with 2 different operators. The results were expressed as the percentage of relative standard deviation of the measurements. As shown in Table 1, intra-day precision (RSD_r) ranged from 1.40% to 7.20% and inter-day precision (RSD_R) from 4.50% to 13.96%. These RSD values are in complete agreement with Commission Decision (2002), requirements since they were always lower than 20% for all the concentration levels assayed. Therefore it can be concluded that the method used has good precision. The LOQ obtained was 0.1 mg of MA/kg.

The results of the validation demonstrate that the applied analytical procedure guarantees satisfactory the quantitative values of MA obtained in the samples analyzed.

Melissopalynological, physicochemical, colour and methyl anthranilate characterization

Table 2 shows the percentage of *Citrus* spp. pollen, the average values of MA and the physicochemical parameters quantified (HMF, electrical conductivity, and moisture), as well as the colour of each of the samples supplied by beekeepers (B) and purchased in retail outlets (R) in 2011 and in 2012. In addition, this table shows the result (P-value, F-ratio and minimum and maximum LSD values) of the multifactor ANOVA carried out considering the factors: year of collection (2011 and 2012) and "type of sample" of citrus honey (beekeepers and retail). The respective double interactions were not significant in any case (data not shown). Fig.1 shows the box and whisker plots for all the values obtained in order to facilitate the comparison of variability patterns between the four sources of citrus honey (beekeepers 2011; beekeepers 2012; retail 2011 and retail 2012).

The citrus pollen percentage varied significantly among type of samples (beekeepers and retail) but not among years of harvesting. This percentage ranged from 1 to 88 (average=34) and from 1 to 69 (average=33) in beekeeper samples from 2011 and 2012, respectively. With regard to the supermarket samples, the pollen percentage ranged from 7 to 40 (average=18) and from 8 to 42 (average=19), respectively.

Similarly, for MA the biggest dispersion between maximum and minimum corresponds to beekeeper samples (0.5-4.0 mg/kg in 2011 and 0.8-5.9 mg/kg in 2012) with means of 2.1 and 2.4 mg/kg, respectively; whereas the retail samples ranged from 0.6 to 2.9 mg/kg (mean= 1.5 mg/kg) for 2011 and from 0.7 to 2.9 mg/kg (mean= 1.6 mg/kg) for 2012. In general, although not significant differences were observed for MA related to the type of sample, the lowest values and the lowest dispersion for MA (the same as for % of pollen) were detected, as expected, in retail samples. This is because the

honey packaging industry mixes the raw honeys to produce relatively homogeneous batches to meet the requirements and specifications that companies have for each type of monofloral honey such as citrus honey. After reception, these industries analyse the physicochemical properties and pollen of the raw honey in order to discern the characteristics of the raw batches and be able to mix them appropriately.

The HMF of the beekeeper honeys showed relatively low average values (5.8 mg/kg for 2011 and 4.1 mg/kg for 2012). However, unexpectedly high values of 16.5 and 25 mg/kg were observed in 2011, probably due to sporadic bad practices. Such high values were not observed in 2012 in any case. As commercialized honeys are usually thermally treated (liquefied and pasteurized) by the industry, the highest values were usually and unsurprisingly found in the retail honeys. It should to be pointed out that one sample exceeded the overall permitted limit of 40 mg/kg for HMF (Council Directive 2001/110 relating to honey, 2002).

Electrical conductivity was quite low in the majority of the samples, as expected for the type of honeys under consideration (Persano-Oddo & Piro, 2004; Bogdanov, et al., 2004). The average values were very similar in both types of samples (217 and 230 $\mu S.cm^{-1}$ in the beekeeper samples, and 228 and 224 $mS.cm^{-1}$ in the retail ones) without significant differences neither year nor type of sample.

In the same way no significant differences were found for moisture in relation to both factors, and maximum values did not exceed 19 mg/100g (Cano, et al., 2001), with one exception of 23.3 mg/100g (in beekeeper samples of 2012). Beekeeper samples exhibited lower minimum values, reaching 13.1 mg/100g, whereas the minimum value for retail samples was 15.2 mg/100g. Being spring honeys, higher moisture values than those observed could be expected (Serra-Bonvehí, 1988). Again, a lesser dispersion of moisture values, reflecting greater homogeneity, can be seen for retail honey due to processing practices, as mentioned previously.

252 On the contrary, with regard to colour, significant differences were found both for year
253 and supplier factors. Beekeeper samples had lower values, especially those from 2012.
254 Retail samples reached values of up to 58 mm Pfund. These higher values could be mainly
255 due to the influence of industrial thermal treatments (liquefaction and pasteurization)
256 (Visquert et al., 2014). Although colour level is not a requirement for citrus honey
257 commercialization, if this honey were sold with a specific quality mark, then particular
258 colour requirements would have to be met. This is the case of the Valencian Quality mark
259 (DOGV, 2002) which requires a maximum of 30 mm on the Pfund scale to benefit from
260 this Mark. Considering this level, all of the retail samples and more than half of those
261 from beekeepers in 2011 were above it. However, in the case of beekeeper samples from
262 2012, more than 75% had values lower than 30 mm on the Pfund scale.

263 *Relationship between the analysed parameters*

264 In order to ascertain the possible linear dependence between the analysed variables,
265 Pearson correlation coefficients were calculated for each pair of variables. Only the
266 beekeeper samples were considered in this correlation since the lack of freshness of the
267 retail samples (high HMF values) could have influenced the correlated variables (Serra-
268 Bonvehí, 1988; Sesta et al., 2008).

269 Table 3 shows the correlation matrix obtained; the number in brackets is the P-value
270 which tests the statistical significance of the estimated correlations at the 95.0%
271 confidence level. Although some of the correlations are significant since P-values are
272 below 0.05, the strength of the linear relationship between each pair of variables is far
273 from the value +1 or -1. The best correlations are shown for colour and HMF (0.674 for
274 2011 and 0.706 for 2012) and for colour and electrical conductivity (0.596 for 2011 and
275 0.812 for 2012). The observed correlation between colour and HMF is coherent
276 considering that since from harvesting, honey tends to increase HMF and color naturally
277 as a result of Maillard reactions (Sancho, et al., 1992). The correlation between colour

278 and electrical conductivity is widely accepted. In general terms, the darker the honey the
279 higher electrical conductivity. Since the samples considered for this correlation were only
280 the unprocessed honeys, the mineral content was the main cause of this relationship
281 (Bogdanov et al., 2004).

282 Previous works considered that the MA value could be related to the percentage of
283 pollen and to other specific physicochemical parameters such as moisture and HMF
284 (Serra-Bonvehí, 1988). These authors suggest that MA content decreases with the loss of
285 freshness, with one year old samples showing a lower level of this compound than fresh
286 samples. However no good linear relationship between MA and HMF (-0.375 for 2011
287 and -0.336 for 2012) was observed in this present work. Similarly, there was also no
288 correlation between MA and moisture, despite claims by Serra-Bonvehí (1988) that high
289 moisture content can cause aromatic losses in honey to the point of reducing MA content.
290 It is important to highlight that the range of moistures found in this work was too narrow
291 to draw conclusions about the influence of moisture on this parameter.

292 In relation to the other physicochemical parameters (electrical conductivity and
293 colour), significant correlations, with moderate Pearson coefficients were observed
294 between them and MA, and pollen, especially for 2012 samples, with values of:
295 MA/electrical conductivity=-0.678; MA/colour=-0.559; pollen/electrical conductivity=-
296 0.553 and pollen/colour=-0.556. However, on the contrary to what was expected, there
297 was no correlation between MA and the percentage of pollen, the coefficient being 0.347
298 and 0.253 for samples from 2011 and 2012, respectively.

299 Once the limited quantitative correlation between MA and the percentage of pollen
300 was demonstrated, it seemed interesting to try to correlate them from a qualitative point
301 of view. Therefore both variables were now considered to be categorical. For this purpose,
302 the samples were classified according to whether they fulfilled the criteria for minimum
303 level of pollen [*Citrus* spp. pollen higher than 10%: Molins et al., 1995; Persano-Oddo,

et al, 1995; DOGV, 2002] and minimum level of MA [2 mg/kg: Persano-Oddo & Piro, 2004; Sesta et al., 2008 and commercial criteria according to the Spanish industry]. As a consequence, a contingency table was made (only the beekeeper samples were considered since they were always raw samples and not mixed) (Table 4), which is a double entry constructed by listing the variable "pollen" as rows and the variable "MA" as columns. Each variable has only two levels: comply or not comply. Each cell in the table represents the percentage of samples that satisfy the criterion of the row (% of *Citrus* spp pollen) or the column (MA concentration), both (% pollen and MA) or neither. Of all the observations, 89.2% (in 2011) and 95.4% (in 2012) comply with the pollen requirement (at least 10% citrus pollen) for a Mark of Quality (e.g. the Valencian Quality Mark: DOGV, 2002). In the case of methyl anthranilate the percentage of compliance (at least 2 mg/kg) was 53.5 and 61.4%, respectively. As mentioned above, in the case of citrus varieties, in addition to pollen, the methyl anthranilate content is required for commercial transactions, as was suggested several years ago by some European laboratories (Sesta et al., 2008).

In this work, 53.5% for 2011 and 56.8% for 2012 of the samples fulfilled both % pollen and MA concentration. On the other hand, 35.7 % and 38.6 % of the samples met % pollen but not MA, and 4.6 % of the samples from 2012 complied with MA but not % pollen. Finally, 10.8% of samples from 2011 met neither % pollen nor MA.

It does not seem logical that MA, a parameter that has been proposed to complement the information given by pollen in citrus honeys when pollen is under-represented, is actually an impediment to its classification. The way that MA is being applied does not seem to help this purpose. In fact, if melissopalynological analysis alone were considered in this study, as in other types of monofloral honey, 89.2 % of samples from 2011 and 95.4% from 2012 would be accepted. However, 35.7 % and 38.6% of the samples,

329 respectively, which complied with the pollen requirement would be rejected
330 commercially for not reaching 2 mg/kg for MA.

331 According to the results obtained, the criterion of 2 mg/kg for MA seems to be too
332 demanding, and therefore not suitable for Spanish citrus honeys. Studies carried out by
333 other authors on this type of honey from Spain and Italy (Sesta et al., 2008; Papotti et al.,
334 2009), concluded that MA content was usually lower than the commercial requirement of
335 2 mg/kg. This fact was demonstrated even in honeys which obviously had the sensory
336 characteristics of this type of honey. Maybe, for these Mediterranean countries it would
337 be appropriate to propose a lower value than is expected in other parts of the world (White
338 & Bryant, 1996). According to the results, it seems more appropriate to only demand this
339 value in the case of honeys with an unexpectedly low percentage of citrus pollen. That is,
340 honeys with the typical physicochemical and sensory characteristics of citrus honey (light
341 amber colour, evident notes of acidity and a very unique flavour due to the presence of
342 specific aromatic substances) but without sufficient citrus pollen for this type of honey
343 (Escriche, et al., 2011).

344 **Conclusions**

345 The results showed that there was a very weak linear correlation between the level of
346 methyl anthranilate and the percentage of pollen in the samples analysed. In almost half
347 of the cases the quantity of MA in Spanish citrus honey was lower than the commercial
348 requirement, which was also reported by other authors for Italian citrus honey. This
349 occurs even though the honeys have a more than sufficient commercial level of citrus
350 pollen. This paper proposes reconsidering the level of MA required in Spanish citrus
351 honey and applying a more realistic value. But above all, to only take this parameter into
352 account in the case of honeys with a surprisingly low percentage of citrus pollen, and after
353 evaluating their organoleptic and physicochemical properties.

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436 **FIGURE CAPTIONS**

437 **Fig. 1.** Box & whisker plots for pollen, MA, HMF, electrical conductivity, moisture and
438 colour for honeys from the beekeepers (B) and retail outlets (R). Samples harvested in
439 2011 and in 2012.
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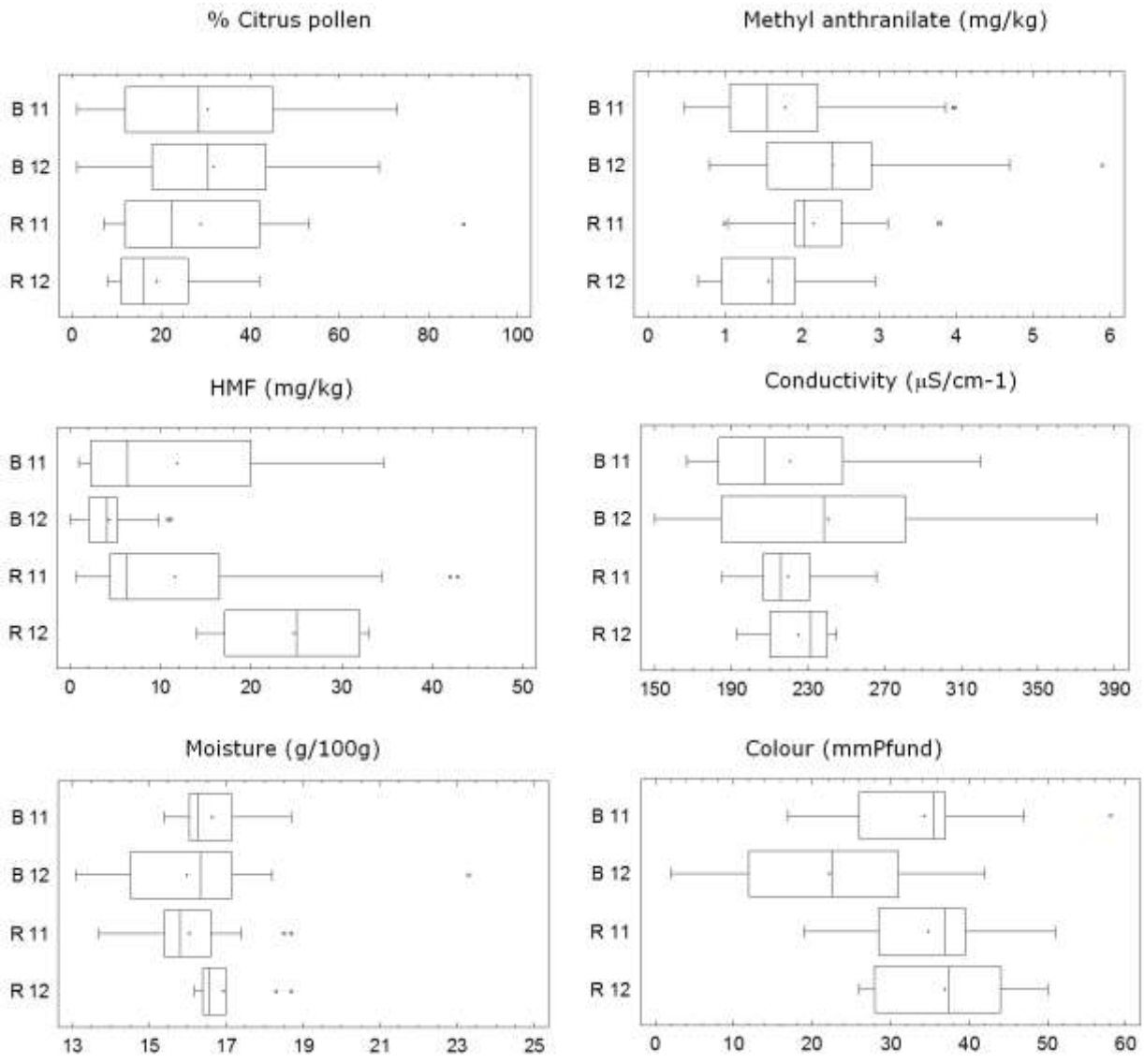

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Table 1. Validation parameters (accuracy and precision) of methyl anthranilate (MA) methodology. The numbers in brackets are the relative standard deviation. Six replicates were carried out for each level.

		Intra-day-precision	Inter-day-precision
MA Added (mg/kg)	Mean Recovery RSD (%)	Mean value (mg/kg) RSD_r (%)	Mean value (mg/kg) RSD_R (%)
0.5	96.0 (6.0)	0.50 (4.10)	0.49 (12.00)
1.0	105.0 (6.5)	1.08 (7.20)	1.03 (5. 80)
2.0	104.0 (10.8)	2.10 (4.11)	2.06 (13.96)
3.0	99.0 (11.6)	2.63 (4.02)	2.36 (13.70)
5.0	100.0 (6.4)	5.06 (1.40)	5.05 (4.50)

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Table 2. Percentage of *Citrus* spp. pollen, average values (n=3) of methyl anthranilate (MA) and physicochemical parameters (HMF, electrical conductivity, moisture) and the colour of each of the samples supplied by beekeepers (B) (28 from 2011 and 50 from 2012) and purchased in retail outlets (R) (10 from 2011 and 10 from 2012). Average and standard deviation (in brackets) for each parameter. Multifactor ANOVA results (P-value, F-ratio, minimum and maximum LSD values) obtained for the factors: year (2011 and 2012) and sample (beekeepers and retail).

	%Pollen *Citrus* spp		MA (mg/kg)		HMF (mg/kg)		Electrical conductivity ($\mu S.cm^{-1}$)		Moisture (mg/100g)		Colour mm Pfund	
Samples	**2011**	**2012**	**2011**	**2012**	**2011**	**2012**	**2011**	**2012**	**2011**	**2012**	**2011**	**2012**
B01	48	35	1.9	1.4	5.1	1.2	264	167	15.4	15.4	36	2
B02	42	48	3.2	2.5	1.1	0.5	183	150	15.5	16.7	28	3
B03	73	47	2.1	3.6	4.0	1.4	241	161	16.2	14.5	37	5
B04	48	40	1.2	3.3	3.7	1.7	243	157	17.9	17.8	27	6
B05	36	68	1.3	3.5	1.7	1.6	167	163	15.5	17.2	17	6
B06	1	47	0.5	4.5	11.8	1.6	283	164	16.8	16.4	37	7
B07	5	46	1.3	3.3	7.6	1.5	248	167	16.2	18.0	32	9
B08	30	69	0.9	3.5	9.4	2.0	271	169	15.8	17.4	43	11
B09	27	4	1.5	4.7	3.8	1.5	208	187	16.3	14.5	35	11
B10	63	48	3.8	5.9	1.7	1.1	167	161	18.6	18.2	20	11
B11	57	60	4.0	2.5	1.5	2.6	172	170	17.8	16.3	20	11
B12	33	37	2.2	1.1	1.5	0.7	194	233	17.2	15.9	22	19
B13	42	37	2.2	2.9	2.4	4.3	198	183	16.7	18.0	26	13
B14	46	23	2.5	2.2	3.2	2.6	189	210	16.6	14.3	19	18
B15	12	48	1.9	2.9	16.5	4.6	205	188	16.6	16.9	33	14
B16	36	1	2.1	3.6	7.9	2.2	202	254	16.5	14.8	25	16
B17	43	37	2.2	2.6	5.4	3.8	185	207	17.3	14.0	22	18
B18	88	30	1.3	1.6	25.0	3.0	212	290	16.3	13.2	35	20
B19	53	65	3.8	2.4	3.8	3.9	208	233	15.4	16.1	24	20
B20	8	12	1.1	1.5	5.6	7.1	215	238	16.0	17.1	33	21
B21	19	42	1.9	2.3	8.7	4.5	210	246	14.8	23.3	39	21
B22	11	35	2.9	2.3	7.6	5.2	228	222	15.8	16.7	37	22
B23	35	10	2.1	1.9	6.8	3.9	209	288	13.8	13.3	37	23
B24	17	20	1.9	2.9	4.6	4.9	266	239	16.6	14.5	40	24
B25	10	45	2.4	1.6	0.7	4.2	169	259	17.8	13.3	20	24
B26	42	26	1.9	1.7	4.9	2.8	233	273	18.6	14.7	32	25
B27	14	20	2.2	2.7	4.8	7.9	255	258	15.4	16.8	40	27
B28	12	28	2.7	0.8	0.9	5.3	220	312	15.6	14.4	37	27
B29		30		2.5		11.1		241		17.2		27
B30		19		1.4		4.1		332		13.3		29
B31		27		2.0		5.2		273		13.8		29
B32		34		1.3		4.3		278		13.6		29

B33		32		3.0		4.1		278		16.4		30
B34		12		1.9		7.2		315		16.5		32
B35		36		1.0		4.3		313		14.9		33
B36		31		1.1		6.0		288		16.9		33
B37		15		2.6		9.7		242		17.2		34
B38		16		1.1		2.9		381		13.1		36
B39		24		2.6		7.8		236		17.4		36
B40		16		2.4		9.5		224		18.1		36
B41		17		1.7		4.5		357		16.4		37
B42		15		1.4		5.8		373		15.9		40
B43		13		2.5		3.1		217		16.0		38
B44		30		1.2		10.8		284		17.1		40
B45		35		1.4		1.2		167		15.4		2
B46		48		2.5		0		150		16.7		3
B47		47		3.6		1.4		161		14.5		5
B48		40		3.3		1.7		157		17.8		6
B49		68		3.5		1.6		163		17.2		6
B50		47		4.5		1.6		164		16.4		7
Average	**34(21)**	**33(17)**	**2.1(0.9)**	**2.4 (1.8)**	**5.8(5.2)**	**4.1(2.6)**	**217(7)**	**230 (63)**	**16.3(0.2)**	**16.0(1.7)**	**31(2)**	**20(11)**
R01	15	8	0.7	2.0	34.3	25.2	175	240	16.6	16.6	50	44
R02	14	42	1.5	1.9	17.0	33.1	255	220	17.9	16.4	39	50
R03	8	9	0.8	1.8	32.0	32.2	171	210	16.3	18.7	47	26
R04	25	11	0.6	0.7	17.7	25.3	208	229	18.3	18.3	58	39
R05	21	28	1.7	1.7	21.8	30.4	320	193	16.3	16.4	58	26
R06	9	26	2.9	1.5	33.8	14.1	191	245	16.2	16.5	37	45
R07	10	11	1.2	1.4	24.3	16.0	240	244	16.9	16.3	37	40
R08	7	22	2.4	1.0	34.3	17.1	238	198	17.5	16.2	48	28
R09	26	16	1.4	2.9	42.4	22.0	228	235	15.2	17.0	51	35
R10	40	16	1.9	0.8	24.8	33.1	229	234	15.6	16.9	37	36
Average	**18 (10)**	**19(11)**	**1.5(0.7)**	**1.6(0.7)**	**28.2(8.1)**	**25.2(7.5)**	**228 (12)**	**224 (19)**	**16.5(0.3)**	**16.9(0.8)**	**46(3)**	**37(8)**
Year factor												
P-value	**0.679**		**0.038**		**0.518**		**0.353**		**0.846**		**0.006**	
F-ratio	**0.17**		**4.41**		**0.42**		**0.87**		**0.04**		**7.72**	
LSD												
2011 (min/max)	**22.50/31.26**		**1.60/2.12**		**13.85/16.78**		**202.11/239.31**		**16.02/16.78**		**33.06/40.61**	
2012 (min/max)	**19.08/31.51**		**1.97/2.71**		**12.40/16.57**		**215.33/249.95**		**15.96/16.96**		**26.01/33.04**	
Sample factor												
P-value	**0.0004**		**0.203**		**0.0000**		**0.605**		**0.079**		**0.0000**	
F-ratio	**13.01**		**1.64**		**242.52**		**0.27**		**3.13**		**25.24**	
LSD												
B (min/max)	**29.38/36.65**		**2.03/2.46**		**3.64/6.08**		**217.45/242.53**		**15.85/16.45**		**24.12/29.21**	
R (min/max)	**12.48/25.84**		**1.56/2.35**		**22.70/27.19**		**201.25/245.46**		**16.16/17.26**		**35.21/44.19**	

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Table 3. Correlation matrix (Pearson correlation coefficients) between percentage of pollen (*Citrus* spp), methyl anthranilate (MA), HMF, moisture, electrical conductivity and colour. Samples harvested in 2011 and 2012.

	Pollen		**MA**		**HMF**		**Moisture**		**Electrical conductivity**	
	2011	**2012**	**2011**	**2012**	**2011**	**2012**	**2011**	**2012**	**2011**	**2012**
MA (mg/kg)	0.347	0.253								
	(0.062)	(0.098)								
HMF(mg/kg)	-0.292	-0.380	-0.375	-0.336						
	(0.011)	(0.011)	(0.001)	(0.026)						
Moisture (g/100g)	0.090	0.285	0.037	0.303	-0.069	0.200				
	(0.437)	(0.061)	(0.754)	(0.046)	(0.556)	(0.194)				
Electrical conductivity	-0.213	-0.553	-0.334	-0.678	0.101	0.413	-0.132	-0.352		
($\mu S/cm^{-1}$)	(0.065)	(0.000)	(0.003)	(0.000)	(0.386)	(0.005)	(0.255)	(0.019)		
Pfund colour (mm)	-0.401	-0.556	-0.519	-0.559	0.674	0.706	-0.189	-0.107	0.596	0.812
	(0.003)	(0.000)	(0.000)	(0.000)	(0.000)	(0.000)	(0.102)	(0.491)	(0.000)	(0.000)

Numbers in brackets = P-value

Table 4. Contingency table for pollen (comply with 10% of *Citrus* spp pollen) and MA (comply with 2 mg/kg). Samples harvested in 2011 and 2012.

	Comply with MA≥2mg/kg		**Not comply MA≥2mg/kg**		**Total**	
	2011	**2012**	**2011**	**2012**	**2011**	**2012**
Comply with pollen ≥10% *Citrus* spp	53.5%	56.8%	35.7%	38.6%	89.2%	95.4%
Not comply with pollen ≥10% *Citrus* spp	0%	4.6%	10.8%	0%	10.8%	4.6%
Total	53.5%	61.4%	46.5%	38.6%	100.0%	100.0%