Tanleque-Alberto, F.; Vargas, M.; Juan-Borras, MDS.; Escriche Roberto, MI. (2021). Thermal properties and hydrocarbon composition of beeswax from Mozambique and other geographical origins. Journal of Apicultural Research. 1-10. https://doi.org/10.1080/00218839.2021.1907086

Thermal properties and hydrocarbon composition of beeswax from Mozambique and other geographical origins

Fernando Tanleque-Alberto[a], María Vargas[b], Marisol Juan-Borrás[b], Isabel Escriche[b*]

[a]Departamento de Ciencias Naturais e Matemática. Universidade Rovuma, Nampula, (Mozambique)

[b]Institute of Food Engineering for Development (IUIAD). Laboratory for Quality Control of honey and bee products. Food Technology Department (DTA). Universitat Politècnica de València. Valencia, (Spain).

*Corresponding author: Isabel Escriche, iescrich@tal.upv.es

Tel.: +34-963877366; fax: +34-963877369

Abstract

The industry demands high quality and standards-compliant beeswax, which is difficult to find in western countries. As a result, it is necessary to explore new geographical sources. This work focuses on the characterization of beeswax from Mozambique in terms of saturated hydrocarbon profile and thermal properties comparing them with others from diverse origins. The hydrocarbons found ($C_{21}H_{44}$-$C_{35}H_{72}$) do not contain enough information for its differentiation. However, melting and cooling enthalpies together with all the temperatures associated with the different lipid polymorphic forms achieved this goal. A higher average melting enthalpy values obtained in Mozambique samples (up to 234 J/g) and Honduras (231 J/g) indicates more energy is required to melt these beeswaxes than those from Spain (193 J/g) and an analytical reactive pure beeswaxes considered as "reference" (168 J/g). This might be linked to the tropical temperatures where the bees produce specific wax. These results are significant in characterising beeswax from tropical climates and for the industry to exploit its peculiar properties.

Keywords: Beeswax, Mozambique, Saturated-Hydrocarbon-Profile, Differential-Scanning-Calorimetry, Thermal-Properties

1. Introduction

Beeswax is a product obtained from worker-bees as a construction material for their combs. It is a natural fatty product highly sought-after in the global market given its multiple applications in the pharmaceutical, cosmetic and food industries, and overall as a foundation beeswax in apiculture (Waś, Szczęsna, & Rybak-Chmielewska, 2014a; Bogdanov, 2016; Haghighat-Kharazi, Milani, Kasaai, & Khajeh, 2018; Reis et al., 2018). These sectors require the highest quality of pure beeswax. Due to the recycling of the empty combs after the extraction of honey (obtaining foundation beeswax), a gradual build-up of foreign chemical substances is found (Svečnjak et al., 2019). For this reason, synthetic paraffins (added as adulterants) or residues from veterinary treatments (e.g. acaricides or antibiotics) are increasingly being detected in commercial beeswax. The process of melting, pressing and the subsequent step of treating with activated carbon, aluminum or magnesium silicates or diatomaceous earth normally used to clean the beeswax, does not eliminate these substances entirely (Serra-Bonvehi & Bermejo, 2012). Although there have been many efforts to develop new techniques for cleaning beeswax, to date, none of them have obtained 100% pure beeswax (Schroeder & Wallner, 2003; Barganska, Slebioda, M., & Namiesnik, 2013; Bogdanov, 2016). Therefore, the great necessity in the market for beeswax free of foreign substances emphasize the importance of exploring new sources from lesser-known geographical origins/bee-breeds, not affected by problems of residues. In this context, African beeswax could be considered for the near future since it is well known that some veterinary residues are not applied to the hive such as acaricides (African bees -*Apis mellifera scutellata*- are not attacked by *Varroa destructor)* (Bogdanov & Gallmann, 2008). Since African beeswax is relatively unknown, it is important to expand the scientific knowledge in terms of its properties.

The evaluation of certain physicochemical parameters such as melting point, saponification number, density, etc. has been widely accepted in establishing the purity of beeswax (The United States Pharmacopeia, 2000*;* European pharmacopoeia, 2008). However, a general consensus of these parameters does not exist and therefore these norms are sometimes considered obsolete (Bernal, Jiménez, del Nozal, Toribio & Martín, 2005); Jiménez, Bernal, del Nozal, Martín, & Bernal, 2006; Jiménez, Bernal, del Nozal, Toribio & Bernal, 2007). In order to be more aware of intrinsic characteristics of pure beeswaxes, the support of other more advanced methodologies are required (Serra-Bonvehi & Bermejo, 2012). Among the possible techniques that allow for a more complete characterization of pure beeswax, those based on the evaluation of hydrocarbons by means of high-temperature gas chromatography with flame ionization detector (HT-GC/FID) and the thermal properties using the Differential Scanning Calorimetry (DSC) can be highlighted (Ritter, Schulte, Schulte & Thier, H.P., 2001; Buchwald, Breed, & Greenberg, 2008; Attama & Müller-Goymann, 2008; Gaillard et al., 2011; Cavallaro, Lazzara, Milioto, Parisi, & Sparacino, 2015).

Hydrocarbons; being one of the main components of beeswax (together with monoesters, free fatty acids, free fatty alcohols, etc.) provide valuable information, both in the scientific world and in the analytical laboratories, for the characterization of beeswax and detection of adulterants. Its analysis is considered the best routine technique since the percentage of specific hydrocarbon compounds in this product could vary with climatic circumstances, type of honey-bees (European, African and Asian) and when other waxes or paraffins have been added (Serra-Bonvehi & Bermejo, 2012).

Differential Scanning Calorimetry (DSC) is a technique that allows for obtaining the thermal fingerprint of different types of waxes through the quantitative characterization of the phase transitions that occur during its heating-cooling (Athukorala & Mazza, 2010). Fusion/crystallization represents a first-order phase transition in materials with a crystalline

75 fraction such as beeswax. DSC allows the measuring of the temperature range over which the
76 phase transition occurs, as well as the associated enthalpy (Turi, 1997; Aboul-Gheit, Abd-el-
77 Moghny & Al-Eseimi., 1997; Buchwald et al., 2008). The melting point of beeswax varies
78 slightly where it comes from, 61-65 °C being the range more frequently reported (FAO, 1992;
79 British Pharmacopeia, 1993; The United States Pharmacopeia, 2000; Code of Federal
80 Regulations, 2004). It is possible to extract more information of the thermal fingerprint of the
81 beeswax by carrying out an in-depth evaluation of the cooling/heating rate of the melting and
82 crystallization peaks that are associated with lipid polymorphisms (Gaillard et al., 2011). As a
83 result, DSC technique could be proposed as an interesting tool to differenciate diverse origins of
84 beeswax. As far as the authors know, there are no previous studies where this technique was
85 used for this purpose.

86 Therefore, the objective of this work was to characterize beeswax from Mozambique regions
87 in terms of hydrocarbon profile and thermal properties, and to compare this African beeswax
88 with others from different origins.

89 **2. Materials and Methods**

90 *2.1. Samples of beeswax*

91 Forty-six beeswax samples from northern (12 from Nampula) and central (10 from Zambezia,
92 12 from Sofala, 12 from Manica) Mozambique with high production of bee products were
93 analysed. Nampula and Zambezia samples were directly obtained from traditional beehives
94 (made with locally available resources) while those from the provinces of Sofala and Manica
95 were commercialized by the Mozambique honey Company (recycled mixes produced by local
96 beekeepers).

97 In parallel, 6 beeswax samples from Spain (ecologically certified and purchased from company
98 Melazahar Cooperativa Valenciana, Spain), 4 from Honduras (wild beehives provided by Lenca

Community of Siguatepeque, Departamento de Comayagua, Honduras) and 4 analytical reactive pure beeswaxes considered as "reference" in this work (Fluka, Buchs, Switzerland) were analysed.

2.2. Hydrocarbon chromatographic analysis

A gas chromatograph Agilent Techologies (7820A, CG System), coupled with flame ionization detector (FID) and capillary column (SGE) (25 m x 0.32 mm ID HT5 x 0.1 micrometer), with helium as the carrier gas (flow of 1 mL/min) were used to analyse the saturated hydrocarbons of the beeswax samples. The temperatures of the injector and detector were 275 ºC and 330 ºC, respectively. The oven temperature was programmed as follows: initial temperature of 70 ºC for 2 minutes, a ramp of 10 ºC/min until 310 ºC and held for 5 minutes, and finally a ramp of 20 ºC/min until 330 ºC and then kept at this temperature for 7.5 min.

Identification of the different hydrocarbons was carried out by comparing the retention times obtained in the sample chromatograms with those of the alkane standards (C21-C40), analytical reactive (Sigma, Aldrich), analysed under the same conditions.

Quantification of each compound in the samples was carried out by comparing peak areas with those of an external standard reference (squalene) as described by Serra-Bonvehi & Bermejo, 2012. The preparation of the samples was as follows: a beeswax sample (0.025g) placed in flask (5 mL) was dissolved (approximately 15 min) in chloroform (Scharlau 99%) (Jimenez et al., 2006). For each sample, 1 μL in splitless mode was injected in triplicate.

2.3. Differential Scanning Calorimetry (DSC)

Thermal properties of beeswax samples were analyzed by means of a Differential Scanning Calorimeter (Mettler Toledo, DSC1, Switzerland) equipped with an intra-cooler. The purge gas used for this analysis was Nitrogen (99.99% purity at 20 mL/min). The equipment was calibrated with indium and zinc. Samples (between 9 and 12 mg) were set into aluminum pans

(40 µL, ME-26763, Al-Crucibles), covered, hermetically sealed and weighed (Mettler Toledo XS205 analytical scale, Switzerland). An empty pan was considered as the reference sample. Based on Buchwald et al. (2008) (from now on referred to as fast condition) to obtain the melting and crystallization enthalpy (ΔH_m and ΔH_c) and melting and crystallization temperature (T_m and T_c), the samples were subjected to the following conditions: cooling from 25 º C to 5 º C at a rate of 5 º C/min and held for 5 min; heating from 5 º C to 65 º C at a rate of 5 º C/min and held for 5 min; heating from 65 º C to 85 º C and cooling from 85 º C to 5ºC at a rate of 5 º C/min.

Based on Gaillard et al. (2011) (from now on referred to as slow condition) with the aim of obtaining all possible melting and crystallization temperatures associated to lipid polymorphic forms of beeswax, samples were also subjected to the following conditions: heating from 20 º C to 75 º C at a rate of 1 º C/min; cooling from 75 º C to 20 º C at a rate of 1 º C/min (slow scan).

The melting temperature together with melting and crystallization enthalpy were obtained using Mettler Toledo DSC STARe SW 12.00 evaluation software. The analysis of each sample was carried out in triplicate.

2.4. Statistical analysis

The influence of beeswax origin on the hydrocarbon profile and thermal properties were evaluated by means of an ANOVA (LSD for multiple comparisons, and α=0.05 as significance level) using the Statgraphics Centurion for Windows XVII. In addition, data were analysed using a principal component analysis (PCA) applying the software Unscrambler X.10.5 CAMO.

3. Results

3.1. Saturated hydrocarbons profile

147 Table 1 shows the mean and standard deviation values of the hydrocarbons quantified in the
148 beeswax from the four provinces of Mozambique (Nampula, Zambezia, Sofala, Manica), as
149 well as from other origins (Spain, Honduras and "reference"). This table also shows the
150 ANOVA results with the statistical significance and the corresponding homogeneous groups.

151 A total of 15 saturated hydrocarbons ranging from $C_{21}H_{44}$ to $C_{35}H_{72}$ were identified with a much
152 higher amount of odd than even carbon number hydrocarbons. Statistically significant
153 differences among origins were found for seven of the fifteen compounds: $C_{21}H_{44}$, $C_{22}H_{46}$,
154 $C_{23}H_{48}$, $C_{26}H_{54}$, $C_{29}H_{60}$, $C_{34}H_{70}$ and $C_{35}H_{72}$ (Table 1). In some cases, for instance $C_{29}H_{60}$, values
155 from specific origins, like Spain, were much higher than others.

156 Occasionally, several hydrocarbons were not identified in specific origins such as Honduran
157 ($C_{22}H_{46}$, $C_{24}H_{50}$, $C_{26}H_{54}$, $C_{28}H_{58}$), "reference" samples ($C_{22}H_{46}$, $C_{24}H_{50}$, $C_{32}H_{66}$, $C_{34}H_{70}$, $C_{35}H_{72}$)
158 or Sofala and Manica ($C_{22}H_{46}$).

159 The greater number of total hydrocarbons (average of 19.70 g/100 g), both even (average of
160 1.54 g/100 g) and odd (average of 18.16 g/100 g) were observed in Spanish samples; whereas
161 the smallest corresponded to the "reference" beeswax (average of 0.84 g/100 g and 15.83 g/100
162 g, for even and odd, respectively). The remaining samples showed intermediate quantities of
163 total hydrocarbons, being the samples from Honduras that showed the lowest amount of total
164 odd hydrocarbons (15.72 g/100 g), but the highest of total even hydrocarbons (1.35 g/100 g),
165 except for Spanish samples. Despite the differences observed in the mean values of the total
166 odd hydrocarbons, the ANOVA analysis does not exhibit any differences. This is due to the
167 ample range of data variability of some origins, similar to the the Spanish samples.

168 *3.2 Thermal properties*

169 The melting and crystallization enthalpy (ΔH_m and ΔH_c) and the melting and crystallization
170 temperature (T_m and T_c), were obtained applying the quick conditions at a rate of 5 ºC/min.

Figure 1 shows examples of DSC thermograms obtained applying these conditions to beeswax samples from the different geographical origins. This figure highlights (see Sofala example) the melting enthalpy as a shaded area below the curve and the melting temperature as the lower peak of the line (indicated as an x). This is applicable to all analyzed samples.

The application of the slow conditions (at 1 °C/min) heating-cooling scan allowed for the observation of all possible melting and crystallization temperatures associated to lipid polymorphic forms of beeswax. Examples of DSC thermograms obtained under these conditions are shown in Figure 2 (4 provinces of Mozambique) and Figure 3 (Honduras, Spain and "reference").

The results (average and standard deviation) for all thermal properties, obtained from the thermograms carried out in all the samples analyzed are shown in Table 2. This table also indicates the ANOVA results (F-ratio and significant differences) for the factor origin. To a different extent, all these parameters were significantly affected by beeswax origin depending on the parameter considered. As expected, the beeswax of the Mozambique regions showed ~~quite~~ similar thermal behavior since 10 of the 11 parameters studied were not significantly different among the 4 provinces (same homogeneous group of the ANOVA).

A Principal Component Analysis (PCA) was carried out using the information obtained from all thermal properties of beeswax samples and using the average values of the three repetitions for each sample. Figure 4 shows the PCA biplot (scores and loadings) of this analysis. Two components explained 62% of the total variance (PC1=43% and PC2=19%). An acceptable difference between types of beeswax can be observed. PC1 promotes the discrimination of samples from Mozambique. However, samples from the different provinces of this country did not show any noticeable separation among them. The widest separation was observed between beeswax from samples from Mozambique and the other origins. Taking into account the PC2,

there is a bigger separation of the Spanish samples, from the rest, although this axis explains less than PC1.

4. Discussion

The 15 saturated hydrocarbons found in all the samples (with a much higher amount of odd than even carbon number hydrocarbons) are considered as typical components of pure beeswax (Aichholz & Lorbeer, 1999; Aichholz & Lorbeer, 2000; Jimenez et al., 2004; Maia & Nunes, 2013; Waś, Szczęsna, & Rybak-Chmielewska, 2014b). The presence of alkanes containing over 35 atoms of carbon is not a characteristic of pure beeswax (Waś et al., 2014a).

The three most abundant hydrocarbon observed: $C_{27}H_{56}$ (average values from 4.18 to 5.25 g/100 g) followed by $C_{29}H_{60}$ (from 2.15 to 4.10 g/100 g) and $C_{31}H_{64}$ (from 2.69 to 3.42 g/100 g), represent approximately 60% of the total amount of hydrocarbons. This is a common pattern in pure beeswax regardless of the geographical origin (Serra-Bonvehi & Bermejo, 2012; Waś et al., 2014a). Serra-Bonvehi & Bermejo, (2012) in pure Spanish beeswax reported: 3.10-5.42 g/100 g; 1.42-3.78 g/100 g and 1.97-3.37 g/100 g, for $C_{27}H_{56,}$ $C_{29}H_{60}$ and $C_{31}H_{64,}$ respectively; these values representing almost 70% of the total of hydrocarbons. Similar compositions were described in Polish beeswax (Waś et al., 2014a; Waś et al., 2014b), where for these three compounds it was found: 3.23, 2.23 and 1.93 as well as 3.03, 2.30 and 1.88 g/100 g in light and dark combs, respectively. In the same way, in Portuguese virgin beeswax (Maia, Barros, & Nunes, 2013) the relative abundance of these three main even hydrocarbons was in accordance with the values found in the samples herein analysed.

Serra-Bonvehi & Bermejo, (2012) in Spanish beeswax reported a total hydrocarbon value close to 18%, whereas Koster-Keunen, (2010) 15-18% in African beeswax. In both studies, the reported data are comparable with the results found in the present study. However, it seems that these values are not in line with the International Honey Commission (IHC) that has set a limit of 14.5% for the total hydrocarbons in beeswax and 13.8% in the case of beeswax produced by

220 African or Africanized bees (Bogdanov, 2016). It is evident that the beeswax composition may
221 vary depending on the bee melliferous subspecies, from the time it was collected and/or the
222 weather conditions during production, among others. However, all these considerations do not
223 justify the difference of values suggested by IHC (Koster-Keunen, 2010; Serra-Bonvehi &
224 Bermejo, 2012). These discrepancies might be caused by the different analytical methodology
225 used to calculate the percentages: gravimetry by IHC and chromatography in the present and
226 the above mentioned studies.

227 Numerous authors have agreed that pure beeswax is characterised by a number of hydrocarbons
228 balanced between even and odd. This ratio, widely accepted as quality criteria, is expressed as
229 "Carbon Preference Index" (CPI=Σ even hydrocarbons/Σ odd hydrocarbons) (Jiménez, Bernal,
230 del Nozal, Martín, & Toribio, 2009; Serra-Bonvehi & Bermejo, 2012; Maia & Nunes, 2013;
231 Waś et al., 2014a; Waś et al., 2014b). In this sense, a CPI value between 0.02 and 0.09 indicates
232 beeswax of good quality (without foreign substances); and a CPI between 0.10 and 0.16 could
233 be a sign of adulteration higher than 5%. According to this criterion, with the only exception of
234 two samples from Spain, those remaining comply with this quality standard. These two samples
235 could contain small quantities of external elements such as paraffins since these provoke the
236 increase of even hydrocarbons and therefore a higher CPI value. It is worth mentioning the
237 lowest CPI values of the samples from Honduras (average=0.031) and the similarity of all CPI
238 values of the samples from Mozambique (average=0.05-0.06) to the "reference"
239 (average=0.05).

240 The global effect of the beeswax origin on the saturated hydrocarbons profile (performed with
241 a principal component analysis -PCA- not shown in this work), reflects that there is no
242 spontaneous classification of the samples whatsoever, which demonstrates that the saturated
243 hydrocarbons do not contain usable information to differentiate the studied beeswax according
244 to their origin.

With respect to the thermal properties, the thermograms show a typical pure beeswax behavior, similar to what was observed in other African beeswax (Basson & Reynhardt, 1988) and from other origins such as USA, Indonesia, China and the Philippines, and Costa Rica, among others (Buchwald et al., 2008).

The complete melting ranged between 63.1 º C and 64.4 ºC (shown in Figures 2 and 3), which is coherent with the values reported in other works (66.2 ºC) (Gaillard et al., 2011). It can be observed that in all cases the heating and cooling thermograms are quite similar, although some differences have been detected. In general, during the heating scan (lower part of the figures), three polymorphic transitions can be confirmed since three melting temperatures were detected (T_{m1}, T_{m2}, T_{m3}). However, in the cooling scan (upper part of the figures) four melting temperatures appeared (T_{c1}, T_{c2}, T_{c3}, T_{c4}). The last peak is not detected on the heating scan or might be masked by the width of the melting peak which is much wider than the correspondent to crystallization. This same performance was also observed by Gaillard et al. (2011) in beeswax samples from France.

Taking into account all samples, T_m varied from 63.80 ºC to 64.49 ºC being within the range of the values reported in previous studies in beeswax produced by *Apis Mellifera* specie. Knuutinen & Norrman (2000) reported 64.4 °C as average values of T_m, and Buchwald, et al., (2008) 64.6 °C±0.67. In both cases, beeswax was from the same bee specie and T_m was obtained also by DSC.

Considering that beeswax is not a single-phase material; it is not appropriate to base its characterization almost exclusively in terms of T_m which provides imprecise information. In addition, the procedure proposed to determine T_m in the European legislation and pharmacopoeia is obsolete since it presents the inconvenience of the subjective visual observation of the analyst, which is the main cause for its wide range of variability. Applying

this capillary classical procedure, the values found were: 62-65 °C (Utermark & Schicke, 1963); 60-65 °C (Tulloch, 1980); 61-65 °C, (Bogdanov, 2016).

The complexity of this multi-component material; due to its chemical composition (hydrocarbons, esters and fatty acids, among others) is qualitatively almost always the same. However, from a quantitative point of view it varies depending on its geographical origin, among other factors (Aichholz & Lorbeer, 1999, Bogdanov, 2016). Therefore, the measurement of all temperatures associated to the different lipid polymorphic forms provides a more robust basis for comparisons of beeswax from different origin (Buchwald, et al., 2008). This is the main reason ~~why~~ the present study is providing the information about the different melting (T_{m1}, T_{m2}, T_{m3}) and crystallization (T_{c1}, T_{c2}, T_{c3}, T_{c4}) temperatures detected during the slow heating and cooling steps. All these parameters varied significantly considering the origin although these differences were not found among the samples of the provinces of Mozambique. Only T_{c1} from Nampula was different from the other provinces.

Regarding melting enthalpy (ΔH_m), samples from Mozambique (average values from 203 to 234 J/g) were not significantly different from those of Honduras (231±16 J/g). However, the differences were more relevant compared to Spain (193±21 J/g) and "reference" (168±8 J/g). These last melting enthalpy values are comparable with those reported by Ritter et al., (2001) in white beeswax (165 J/g); Buchwald, et al., (2008) in beeswax from USA (170.7±4.48 J/g); Gaillard et al. (2011); in beeswax from France (155 J/g) and Attama & Müller-Goymann (2008) in *cera alba* (189 J/g).

Higher melting enthalpy values indicates that more energy is required to melt the beeswax. This fact could be linked to the higher temperatures found in tropical countries. As a result, the different *Apis mellifera* species that live in those countries behave differently producing a wax with specific characteristics because of these higher ambient temperatures in Mozambique and some areas of Honduras (Tulloch, 1980; Serra-Bonvehi & Bermejo, 2012; Bogdanov, 2016).

5. Conclusion

This work has revealed that the DSC technique is suitable for differentiation of beeswax according to their geographical origin. In contrast, saturated hydrocarbons do not contain enough useful information for this goal, despite being a well-consolidated procedure for the detection of adulterants in this product.

The application of the heating-cooling scan both in fast and slow conditions, permits a more complete characterization of the thermal behavior of beeswax, allowing to obtain the melting and cooling enthalpies together with all the temperatures associated to the different lipid polymorphic forms.

Under these conditions, the beeswax samples from the four provinces of Mozambique (Nampula, Zambezia, Sofala and Manica) were correctly differentiated from the other origins considered (Spain Honduras and "reference"). More energy is required to melt Mozambiquean beeswax, as a consequence of the higher melting enthalpy values found, and likely due to the tropical temperatures and the diverse *Apis mellifera* species. This approach is an important tool in helping to differentiate beeswax from hot countries and to have a better insight into its particular characteristics, which could be of interest for the industry.

Acknowledgements

The research was funded by the 2016 ADSIDEO-Cooperación Programa of the Centre for Development Cooperation of the Universitat Politècnica de València (Spain). The authors would also like to thank the Universitat Politècnica de València for the support provided for contracting Marisol Juan Borrás in the frame of PAID-10-17 (Ayuda para la contratación de acceso al Sistema Español de Ciencia, Tecnología e Innovación en Estructuras de Investigación).

317 The authors thank the Ministério de Ciência e Tecnologia Ensino Superior e Técnico
318 Profissional de Moçambique (Project: HEST “Ensino Superior, Ciência e Tecnologia”) and
319 Universidade Rovuma, Nampula (Mozambique) for the grant awarded to Fernando Tanleque
320 Alberto.

321 **References**

322 Aboul-Gheit, A.K., Abd-el-Moghny, T., & Al-Eseimi, M.M., 1997. Characterization of oils by
323 differential scanning calorimetry. Thermochemica Acta. 306, 127-130.

324 Aichholz R., & Lorbeer E., 1999. Investigation of combwax of honeybees with high-
325 temperature gas chromatography and high–temperature gas chromatography–chemical
326 ionization mass spectrometry. I- High-temperature gas chromatography. J. Chromatogr. A.
327 855, 601-615.

328 Aichholz R., & Lorbeer E., 2000. Investigation of combwax of honeybees with high-
329 temperature gas chromatography and high–temperature gas chromatography–chemical
330 ionization mass spectrometry. II–High-temperature gas chromatography-chemical ionization
331 mass spectrometry. J. Chromatogr. A. 883, 75-88.

332 Athukorala, Y. & Mazza, G., 2010. Supercritical carbon dioxide and hexane extraction of wax
333 from triticale straw: Content, composition and thermal properties. Ind Crops Prod. 31, 550-
334 556.

335 Attama, A.A., & Müller-Goymann, C.C., 2008. Effect of beeswax modification on the lipid
336 matrix and solid lipid nanoparticle crystallinity. Colloids Surf A Physicochem Eng Asp. 315,
337 189-195.

338 Barganska, Z., Slebioda, M., & Namiesnik, J., 2013. Pesticide residues levels in honey from
339 apiaries located of Northern Poland. Food Control. 31, 196-201.

340 Basson, I., & Reynhardt, E.C., 1988. An investigation of the structures and molecular dynamics
341 of natural waxes. I. Beeswax. J Phys D Appl Phys. *21*, 14-21.

342 Bernal, J.L., & Jiménez, J.J., del Nozal, M.J., Toribio, L., & Martín, M.T., 2005. Physico-
343 chemical parameters for the characterization of pure beeswax and detection of adulterations.
344 Eur J Lipid Sci Technol. 107, 158-166.

345 Bogdanov, S., & Gallmann, P., 2008. Authenticity of honey and other bee products state of the
346 art. Bern (Switzerland), Forschungsanstalt Agroscope Liebefeld-Posieux ALP.

347 Bogdanov, S., 2016. Beeswax: Production, Properties Composition and Control. Switzerland
348 (Chapter 1). Bee Product Science, www.bee-hexagon.net. Accessed 30.01.2019.

349 British Pharmacopeia, 1993. The British Pharmacopeia Commission Secretariat. London,
350 HMSO Publications Centre.

351 Buchwald, R., Breed, M.D., & Greenberg, A.R., 2008. The thermal properties of beeswaxes:
352 unexpected findings. J Exp Biol. 211, 121-127.

353 Cavallaro, G., Lazzara, G., Milioto, S., Parisi, F., & Sparacino, V., 2015. Thermal and dynamic
354 mechanical properties of beeswax-halloysite nanocomposites for consolidating waterlogged
355 archaeological woods. Polym Degrad Stab. 120, 220-225.

356 Code of Federal Regulations, 2004. Beeswax (yellow and white). Title 21, United States Code
357 of Federal Regulations, Section 184.1973. U.S. Government Printing Office, Washington,
358 DC.

359 European Pharmacopeia, 2008. Council of Europe Strassbourg (6. edition).

360 FAO, 1992. Expert Committee on Food Additives. Beeswax. In Compendium of Food Additive
361 Specifications: Combined Specifications from 1st Through the 37th Meetings, 1956-1990.
362 Rome: FAO. http://www.who.int/iris/handle/10665/37961, Accessed 30.01.2019.

Gaillard, Y., Mija, A., Burr, A., Darque-Ceretti, E., Felder, E., & Sbirrazzuoli, N., 2011. Green material composites from renewable resources: Polymorphic transitions and phase diagram of beeswax/rosin resin. Thermochim. Acta. 521, 90-97.

Haghighat-Kharazi, S., Milani, J. M., Kasaai, M. R., & Khajeh, K., 2018. Microencapsulation of α-amylase in beeswax and its application in gluten-free bread as an anti-staling agent. LWT-Food Sci Technol. 92, 73-79.

Jiménez, J.J., Bernal, J.L., del Nozal, M.J., Martín, M.T., & Bernal, J., 2006. Sample preparation methods for beeswax characterization by gas chromatography with flame ionization detection. J. Chromatogr. A. 1129, 262-272.

Jiménez, J.J., Bernal, J.L., del Nozal, M.J., Toribio, L., & Bernal, J., 2007. Detection of beeswax adulterations using concentration guide-values. Eur. J. Lipid Sci. Technol. 109, 682-690.

Jiménez, J.J., Bernal, J.L., del Nozal, M.J., Martín, M.T., & Toribio, L., 2009. Identification of adulterants added to beeswax: Estimation of detectable minimum percentages. Eur J Lipid Sci Tech. 111, 902-911.

Knuutinen, U., & Norrman, A., 2000. Wax analysis in conservation objects by solubility studies, FTIR and DSC. In Proceedings of the 15th World Conference on Nondestructive Testing.

Koster-Keunen, L.L.C., 2010. Waxes, beeswax, natural wax. Bladel. Netherlands, Koster Keunen Holland BV.

Maia, M., & Nunes, F.M., 2013. Authentication of beeswax (Apis mellifera) by high-temperature gas chromatography and chemometric analysis. Food Chem. 136, 961-968.

Maia, M., Barros, A.I., & Nunes, F.M., 2013. A novel, direct, reagent-free method for the detection of beeswax adulteration by single-reflection attenuated total reflectance mid-infrared spectroscopy. Talanta 107, 74-80.

Reis, M.O., Olivato, J.B., Bilck, A.P., Zanela, J., Grossmann, M.V.E., & Yamashita, F., 2018. Biodegradable trays of thermoplastic starch/poly (lactic acid) coated with beeswax. Ind Crops Prod. 112, 481-487.

Ritter, B., Schulte, J., Schulte, E., & Thier, H.P., 2001. Detection of coating waxes on apples by differential scanning calorimetry. Eur Food Res Technol. 212, 603-607.

Schroeder, A., & Wallner, K., 2003. The actual situation of varroacides in beeswax: An international comparison. Apidologie. 34, 1-3.

Serra-Bonvehi, J., & Bermejo, F.O., 2012. Detection of adulterated commercial Spanish beeswax. Food Chem. 132, 642-648.

Svečnjak, L., Chesson, L.A., Gallina, A., Maia, M., Martinello, M., Mutinelli, F., Muz, M., N., Nunes, F.M., Saucy, F., Tipple, B.J., Wallner, K., Waś, E., & Waters, T.A., 2019. Standard methods for Apis mellifera beeswax research. J. Apic. Res. 58, 2, 1-108- DOI:10.1080/00218839.2019.1571556

The United States Pharmacopeia. The National Formulary (USP 24-NF 19), 2000. Rockville, Maryland, United States Pharmacopeial Convention c1999.

Tulloch, A.P., 1980. Beeswax: composition and analysis. Bee World. 61, 47-62.

Turi, E.A., 1997. Thermal Characterization of Polymeric Materials (2nd edn). San Diego, Academic Press.

Utermark, W. & Schicke, W., 1963. Melting Point Tables of Organic Compounds. New York Interscience.

Waś, E., Szczęsna, T., & Rybak-Chmielewska, H., 2014a. Hydrocarbon composition of beeswax (*Apis mellifera*) collected from light and dark coloured combs. J Apicult Res. 58, 99-106.

Waś, E., Szczęsna, T., & Rybak-Chmielewska, H., 2014b. Determination of beeswax hydrocarbons by gas chromatography with a mass detector (GC-MS) technique. J Apic Sci. 58, 145-157.

413 **Table 1**. Mean and standard deviation values of the saturated hydrocarbons profile (g/100 g) found in beeswax from Mozambique (Nampula, Sofala, Manica
414 and Zambezia), Spain, Honduras and "reference" (analytical reagent of pure beeswax) and ANOVA results (F-ratio and significant differences).

Saturated hydrocarbons	**MOZAMBIQUE**				**OTHER ORIGINS**			**ANOVA** F-ratio
	Nampula	**Zambezia**	**Sofala**	**Manica**	**Spain**	**Honduras**	**"Reference"**	
$C_{21}H_{44}$	0.44(0.35)^{ab}	0.29(0.27)^{a}	0.77(0.71)^{b}	0.32(0.35)^{a}	0.22(0.25)^{a}	0.20(0.42)^{a}	0.65(0.12)^{ab}	2.6*
$C_{22}H_{46}$	0.13(0.13)^{b}	0.03(0.08)^{a}	<0.001^{a}	<0.001^{a}	0.47(0.12)^{a}	<0.001^{a}	<0.001^{a}	4.3*
$C_{23}H_{48}$	0.47(0.19)^{ab}	0.73(0.28)^{bc}	0.71(0.42)^{a}	0.53(0.22)^{ab}	1.02(0.31)^{c}	0.73(0.32)^{bc}	0.31(0.12)^{a}	4.1*
$C_{24}H_{50}$	0.06(0.14)	0.06(0.10)	0.05(0.06)	0.06(0.14)	0.03(0.07)	<0.001	<0.001	n.s.
$C_{25}H_{52}$	1.63(0.58)	2.13(0.32)	2.12(0.42)	1.97(0.18)	2.06(0.63)	1.89(1.35)	1.46(0.08)	n.s.
$C_{26}H_{54}$	0.11(0.09)^{b}	0.10(0.07)^{bc}	0.02(0.04)^{ab}	0.06(0.08)^{bc}	0.27(0.09)^{b}	<0.001^{a}	0.25(0.01)^{b}	12.6***
$C_{27}H_{56}$	5.24(0.66)	4.72(1.10)	4.18(1.26)	4.75(0.45)	5.20(1.40)	4.41(1.24)	5.25(0.33)	n.s.
$C_{28}H_{58}$	0.23(0.22)	0.13(0.14)	0.28(0.26)	0.29(0.38)	0.24(0.13)	<0.001	0.21(0.02)	n.s.
$C_{29}H_{60}$	2.70(0.40)^{a}	2.55(0.29)^{a}	2.88(0.36)^{a}	2.15(0.66)^{a}	4.10(1.38)^{b}	2.41(0.61)^{a}	2.92(0.22)^{a}	8.1***
$C_{30}H_{62}$	0.25(0.20)	0.27(0.14)	0.15(0.18)	0.16(0.13)	0.24(0.13)	0.30(0.11)	0.38(0.11)	n.s.
$C_{31}H_{64}$	3.40(0.77)	3.14(0.55)	2.71(0.70)	3.08(0.37)	3.42(1.02)	3.00(1.06)	2.69(0.34)	n.s.

$C_{32}H_{66}$	0.14(0.11)	0.14(0.16)	0.16(0.12)	0.27(0.28)	0.23(0.25)	0.11(0.09)	<0.001	n.s.
$C_{33}H_{68}$	3.04(0.40)	2.89(0.49)	2.42(0.89)	2.51(0.79)	2.13(1.02)	2.14(0.77)	2.56(0.20)	n.s.
$C_{34}H_{70}$	0.24(0.19)^{b}	0.13(0.16)^{ab}	0.22(0.13)^{ab}	0.09(0.16)^{ab}	0.06(0.14)^{a}	0.94(0.12)^{ab}	<0.001^{a}	2.3*
$C_{35}H_{72}$	0.75(0.33)^{ab}	1.01(0.87)^{ab}	1.23(0.91)^{b}	1.42(1.58)^{b}	0.01(0.03)^{a}	0.94(0.33)^{ab}	<0.001^{a}	2.8*
Σ of even carbons	1.16(0.23)^{bc}	0.86(0.36)^{ab}	0.88(0.24)^{ab}	0.93(0.31)^{ab}	1.54(0.99)^{c}	1.35(0.14)^{b}	0.84(0.11)^{a}	2.3*
Σ of odd carbons	17.67(1.90)	17.46(1.32)	16.52(1.56)	16.73(1.00)	18.16(5.40)	15.72(1.60)	15.83(1.18)	n.s.
Σ of even and odd	18.83(2.02)^{ab}	18.32(1.30)^{ab}	17.40(1.56)^{ab}	17.66(1.19)^{ab}	19.70(4.70)^{b}	17.70(1.28)^{ab}	16.67(1.27)^{a}	3.5*
Carbon Preference Index (CPI=Σ of even/Σ of odd)	0.06(0.01)	0.05(0.02)	0.05(0.01)	0.05(0.01)	0.08(0.07)	0.031(0.006)	0.052(0.003)	n.s.

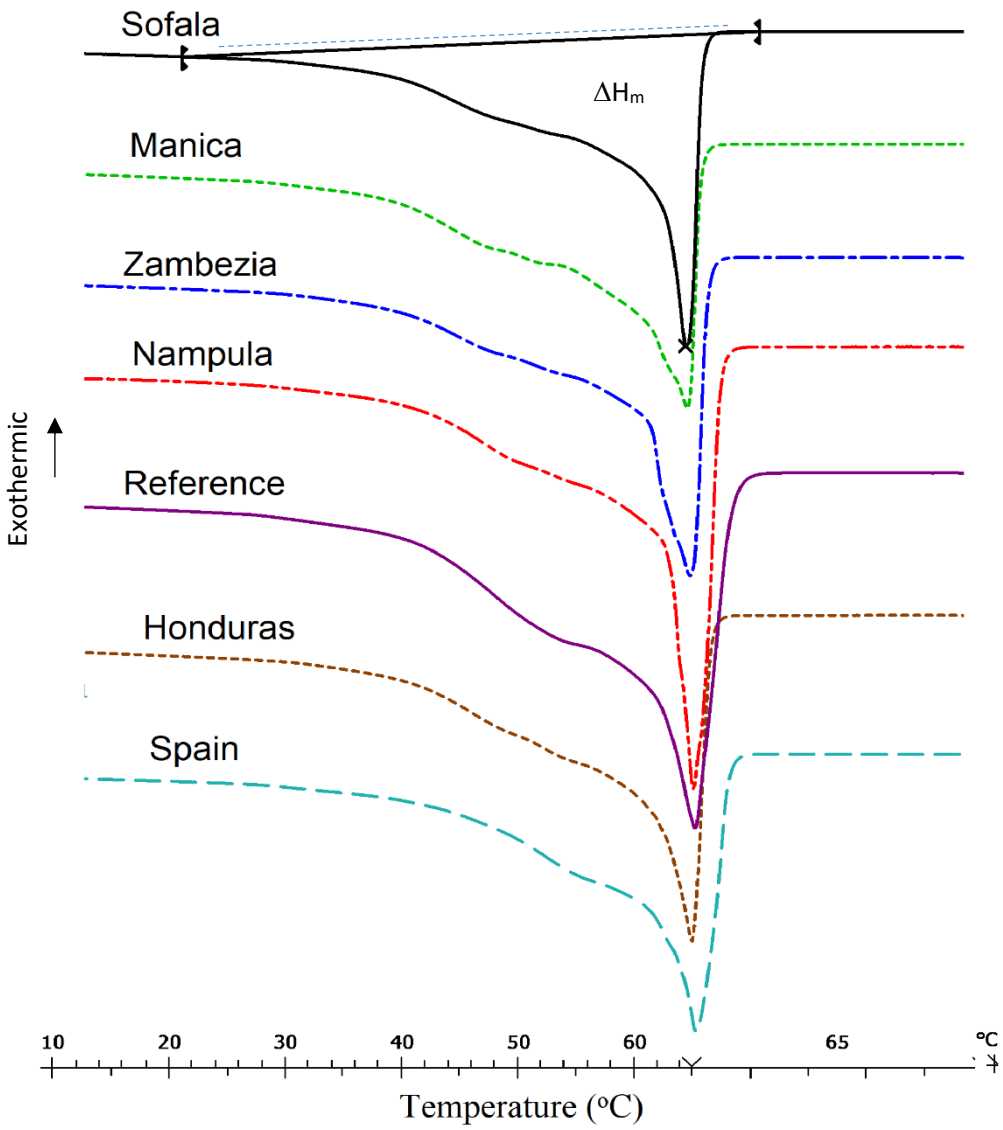
Different letters in the same row indicate significant differences at 95% confidence level as obtained by the LSD test. *p < 0.05; **p < 0.01; ***p < 0.001

n.s: non-significant differences

Table 2. Thermal properties of beeswax samples from four provinces of Mozambique (Nampula, Sofala, Manica and Zambezia), Spain, Honduras and "Reference" samples. Mean values and standard deviation, in brackets.

	MOZAMBIQUE				OTHER ORIGINS			
	Nampula	**Zambezia**	**Sofala**	**Manica**	**Spain**	**Honduras**	**"Reference"**	**ANOVA** F-ratio
Thermal properties								
ΔH_m (J/g)	203(30)^{bc}	216(22)^{bc}	234(27)^{c}	230(21)^{c}	193(21)^{ab}	231(16)^{c}	168(8)^{a}	6.47***
T_m (ºC)	64.26(0.19)^{a}	64.1(0.3)^{a}	64.21(0.17)^{a}	63.8(0.3)^{a}	64.1(0.7)^{a}	64.43(0.06)^{b}	64.49(0.12)^{b}	4.6*
T_{m1} (ºC)	46.0(0.5)^{b}	45.6(0.2)^{b}	45.7(0.3)^{b}	46(0.3)^{b}	44.08(1.09)^{a}	45.0(1.6)^{b}	45.88(0.18)^{b}	13.86***
T_{m2} (ºC)	50.49(0.13)^{a}	50.42(0.15)^{a}	50.55(0.06)^{a}	50.6(0.2)^{a}	51.3(1.2)^{b}	51.9(0.1)^{c}	51.84(0.12)^{c}	19.04***
T_{m3} (ºC)	62.8(0.4)^{b}	62.6(0.2)^{b}	62.6(0.3)^{b}	62.6(0.4)^{b}	61.9(0.6)^{a}	63.5(0.3)^{c}	62.98(0.15)^{b}	9.8***
ΔH_c (J/g)	151(15)^{b}	152(14)^{b}	160 (5)^{b}	156(9)^{b}	150(4)^{b}	163(12)^{b}	132(17)^{a}	7.1**
T_c	58.9(0.3)^{b}	58.94(0.16)^{b}	58.77(0.16)^{b}	58.9(0.2)^{b}	54(4)^{a}	59.4(0.5)^{b}	54.4(0.9)^{a}	20.82***
T_{c1} (ºC)	44.6(1.2)^{b}	43.7(0.2)^{a}	43.5(0.2)^{a}	43.66(0.17)^{a}	46.2(0.3)^{c}	45.8(0.9)^{c}	45.1(0.9)^{bc}	18.28***
T_{c2} (ºC)	49.6(0.7)^{a}	49.84(0.12)^{a}	49.71(0.06)^{a}	49.89(0.19)^{a}	51.1(0.6)^{b}	51.59(0.15)^{b}	51.2(0.1)^{b}	32.08***
T_{c3} (ºC)	55.19(0.09)^{a}	55.19(0.08)^{a}	55.18(0.03)^{a}	55.31(0.19)^{a}	55.4(1.3)^{a}	55.59(0.08)^{a}	56.5(1.5)^{b}	7.41***
T_{c4} (ºC)	60.3(0.3)^{a}	60.18(0.12)^{a}	60.17(0.13)^{a}	60.4(0.2)^{a}	60.9(0.6)^{ab}	61.38(0.12)^{c}	61.78 (0.12)^{c}	39.1***

Different letters in the same row indicate significant differences at 95% confidence level as obtained by the LSD test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.



426

427

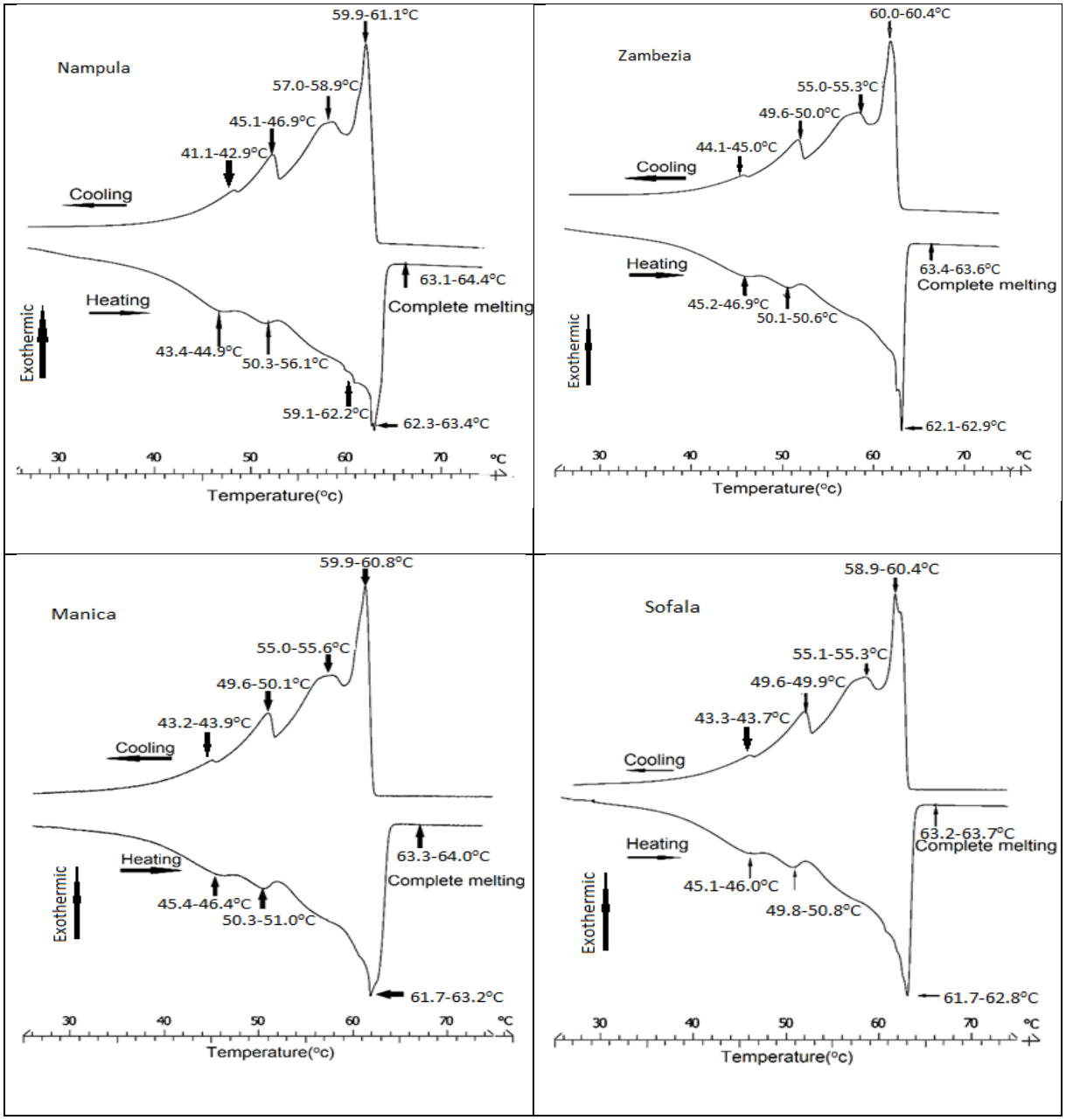
428 **Figure 1.** DSC thermograms (5° C/min with 5 min isotherm step at 65° C) of beeswax samples

429 from four provinces of Mozambique (Nampula, Zambezia, Sofala and Manica), Spain,

430 Honduras and "Reference" sample.

431

432



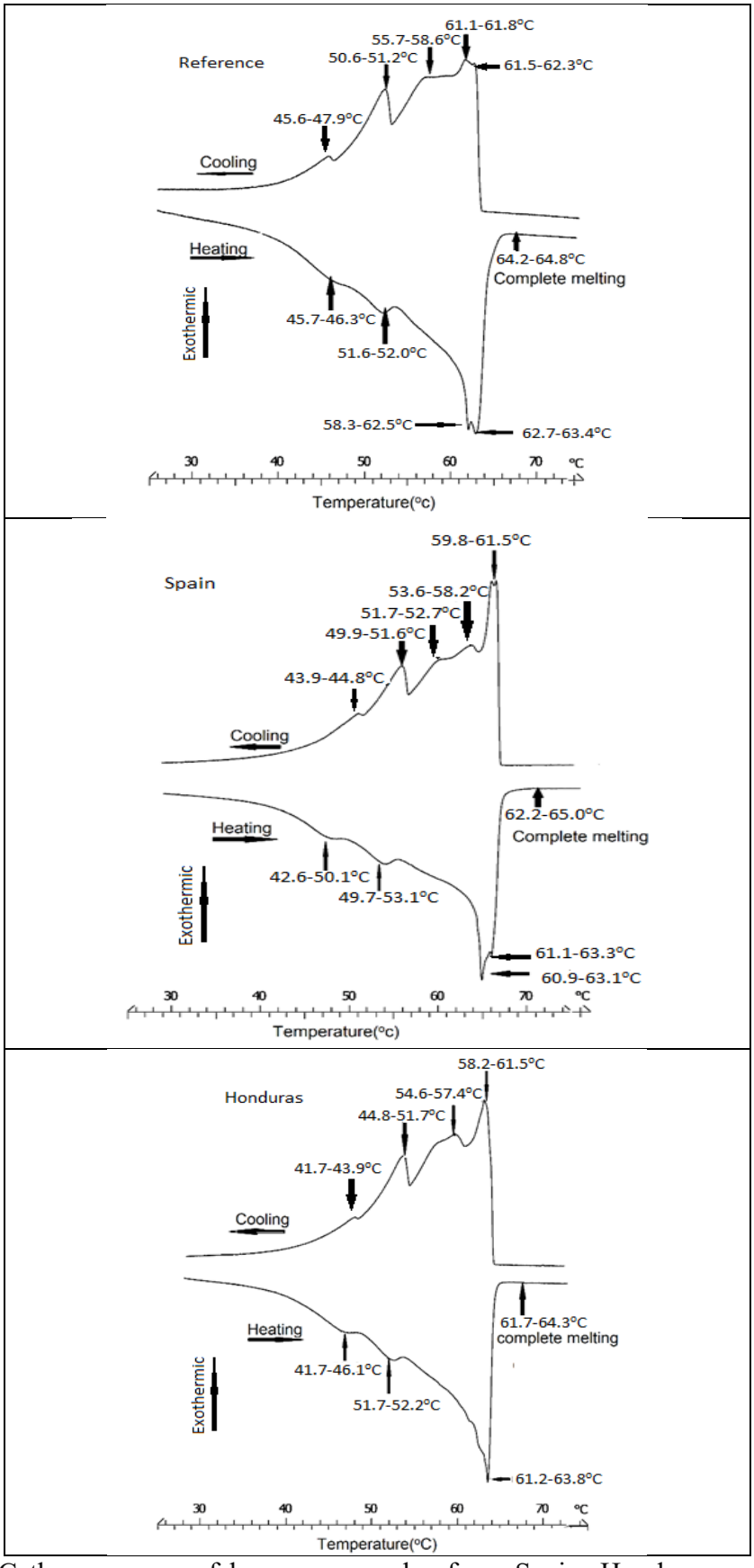
433

434 **Figure 2.** DSC thermograms of beeswax samples from four provinces of Mozambique

435 (Nampula, Zambezia, Sofala and Manica) at a rate of 1 ° C min^{-1}.

436

437



438 **Figure 3.** DSC thermograms of beeswax samples from Spain, Honduras and "Reference"

439 sample at a rate of 1 ° C min^{-1}.

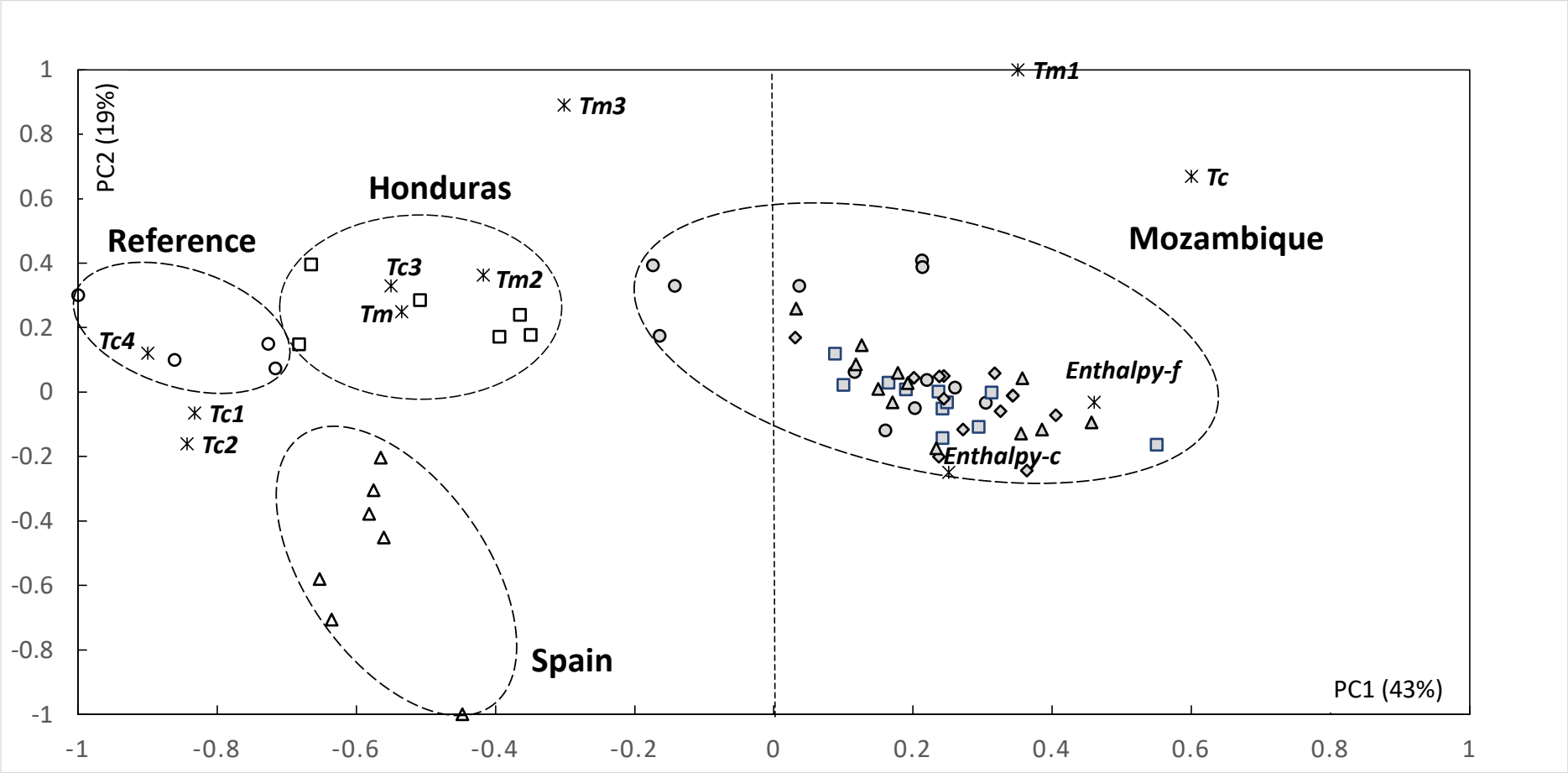


Figure 4. PCA biplot (scores and loadings) of the two principal components from the thermal properties of beeswax samples from four provinces of Mozambique [Nampula (●), Sofala (◆), Manica (▲) and Zambezia (■)], Spain (△), Honduras (□) and Reference sample (○).