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Disponibilidade, valorização e inovação: uma abordagem multidimensional dos alimentos

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# Botanical authentication of honey by DNA barcoding: application to lavender honey

*Sónia Soares*[a], *Liliana Grazina*[a], *Joana Costa*[a], *Joana Amaral*[a,b,*], *M. Beatriz P. P. Oliveira*[a], *Isabel Mafra*[a,*]

[a]REQUIMTE-LAQV, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal; [2]Instituto Politécnico de Bragança, Bragança, Portugal; * Email: isabel.mafra@ff.up.pt; jamaral@ipb pt

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## ABSTRACT

Nowadays there is a high demand for monofloral honeys, increasing their commercial value and making them prone to adulteration. Therefore, there is a need to assess and authenticate its botanical origin. To overcome the traditional pollen identification, which is time consuming and greatly dependent on the experience of trained analysts, DNA-based approaches have been suggested as efficient alternatives. In this work, the use of DNA mini-barcodes coupled to high resolution melting (HRM) analysis is proposed to identify and differentiate *Lavandula* species in lavender monofloral honey. For this purpose, three DNA barcode loci, namely the plastidial coding genes *rbcL* and *matK* and the noncoding intergenic *trnH-psbA* region, were used to design primers targeting *Lavandula* spp. However, only the primers targeting plastid genes had adequate kinetics to be used for HRM analysis, with primers Lav1-F/Lav1-R showing promising results. The developed methodology allowed the discrimination of *Lavandula* species into distinct clusters with high level of confidence. When applying the methodology to honey samples, lavender honey was classified in the cluster of *Lavandula stoechas* (endemic species in Portugal), thus confirming its botanical origin.

## 1. INTRODUCTION

Honey is a highly consumed product due to a variety of beneficial nutritive and health effects. Its properties are related to its chemical composition, which mostly varies according to botanical origin, making each honey unique. Honey can be classified as monofloral, when arising predominantly from a single botanical origin, or multifloral. The former has limited availability and can attain higher market values due its distinctive flavour and taste, thus being prone to economical frauds. Therefore, assessing the botanical and geographical origin of honey is of utmost importance to guaranty its quality and authenticity. Melissopalynology is the traditional method used for botanical authentication of honey, but it is time-consuming and strongly dependent on the qualification and judgment of the analysts. For its high specificity, sensitivity and speed, DNA-based methods have been suggested as adequate alternatives. In particular, DNA barcoding is an increasingly used technique for taxonomic identification of plants and was recently suggested for the identification of plant species in

honey using the *rbcL* and *trnH-psbA* plastid regions [1]. Sequencing of other regions, such as the nuclear 18S rDNA [2] and the plastid *trn*L gene [3] have been also proposed. Another suggested approach relies on the use of real-time PCR with primers and probes to specifically identify different plant species in honey [4].
The main objective of this study was to develop molecular markers, with special emphasis on DNA mini-barcodes, to authenticate the botanical origin of lavender monofloral honey.

## 2. MATERIALS AND METHODS

### 2.1 Sampling and DNA extraction

Two monofloral lavender (*Lavandula* spp.) honeys and two multifloral honeys were acquired from local producers and commercial stores in Portugal. A total of 16 voucher plant specimens of *Lavandula* spp. were obtained from germplasm banks and botanical gardens. Plants of three wild *Lavandula* spp. were collected in the north and centre regions of Portugal. DNA was extracted from plant material using the kit NucleoSpin® Plant II (Macherey-Nagel, Düren, Germany), while DNA from honeys was extracted as described by Soares et al. [5].

### 2.2 Gene selection and primers

Three DNA barcode loci, namely the plastidial coding genes *rbcL* and *matK* and the noncoding intergenic *trnH-psbA* region, were used to design new primers targeting *Lavandula* spp. (Table 1). The primers were tested using qualitative PCR and real-time PCR with high resolution melting (HRM) analysis.

**Table 1 –** Oligonucleotide primers used in this study.

| Species | GenBank accession no. | Gene | Name | Sequence 5'- 3' | Amplicon (pb) |
|---|---|---|---|---|---|
| *Lavandula* spp. | KJ196360.1 | *matK* | Lav1-F | AAA GCT TCT TCC GCT TTG CG | 154 |
| | | | Lav1-R | TCG TCT TTT TAC CTC TTC ATC CA | |
| | HQ902822.1 | *psbA-trnH* | Lav2-F | ATT GGA CTT ACC TAG ACT TTT TCC | 172 |
| | | | Lav2-R | ATC CAC TTG GCT ACA TCC GC | |
| | Z37408.1 | *rbcL* | Lav3-F | AGT ACG GTC GTC CTT TGC TG | 101 |
| | | | Lav3-R | AAG TCC ACC GCG AAG ACA TT | |

### 2.3 Qualitative PCR and real-time PCR

Qualitative PCR was carried out in 25 µL of total reaction volume, containing 2 µL of DNA extract (20 ng), 67 mM of Tris-HCl (pH 8.8), 16 mM of $(NH_4)_2SO_4$, 0.01% of Tween 20, 200 µM of each dNTP, 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience, Germany), 1.5 (Lav2-F/Lav2-R) or 3.0 mM of $MgCl_2$ and 200 nM of each primer (Table 1). The reactions were performed in a thermal cycler MJ Mini™ Gradient Thermal Cycler (Bio-Rad Laboratories, USA) using the following programme: 95 ºC for 5 min; 38 or 40 (Lav1-F/Lav1-R) cycles of 95 ºC for 30 s, 55ºC (Lav1-F/Lav1-R) or 58ºC for 30 s, and 72 ºC for 30 s; and a final extension at 72 ºC for 5 min.

The amplifications by real-time PCR were carried out in 20 µL of total reaction volume containing 2 µL of DNA extract (20 ng), 1x of SsoFast™ Evagreen® Supermix (Bio-Rad Laboratories, USA) and 400 nM of each primer targeting the different locus (Table 1). The reactions were performed on a thermal cycler CFX96 Real-Time System (Bio-Rad Laboratories, USA) using the following conditions: 95ºC for 5 min; 50 cycles at 95ºC for 15 s, 55ºC for 15 s and 72ºC for 25 s, with collection of fluorescence signal at the end of each cycle; followed by melting analysis at 95ºC for 1 min, 60ºC for 5 min, 60ºC to 90ºC with 0.2ºC/10 s increments. Data were collected and processed using the software Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories, USA). The fluorescence data were processed using the Precision Melt Analysis 1.2 (Bio-Rad Laboratories, USA) in order to discriminate the different species of *Lavandula*.

## 3. RESULTS AND DISCUSSION

The DNA extracted from each *Lavandula* sp. was amplified with the three proposed set of primers enabling to obtain PCR fragments of 101 bp, 154 bp, and 172 bp for the plastidial coding genes *rbcL* and *matK*, and the noncoding intergenic *trnH-psbA* region, respectively. The results showed that the three loci were positively amplified for the tested *Lavandula* spp. However, only the ones targeting *rbcL* and *matK* genes had adequate kinetics to be further analysed by HRM. From those loci, *matK* proved to be the most efficient at species level identification since the HRM analysis enabled the differentiation of *Lavandula* spp. in different clusters with a high level of confidence (Figure 1A and Table 2). Thus, this approach demonstrated its usefulness at closely related species discrimination.

The specificity of the developed PCR assay targeting *matK* gene was tested using different plant species to evaluate any possible cross-reactivity. From the 49 different plant species investigated, including those frequently reported in lavender honeys, no cross-reactivity was observed, confirming the adequate specificity of the assay for *Lavandula* spp. identification.

When applying the HRM analysis targeting *matK* gene to honey samples, lavender honey was classified on the cluster of *L. stoechas* (endemic species in Portugal) (Figure 1B), confirming its botanical origin.

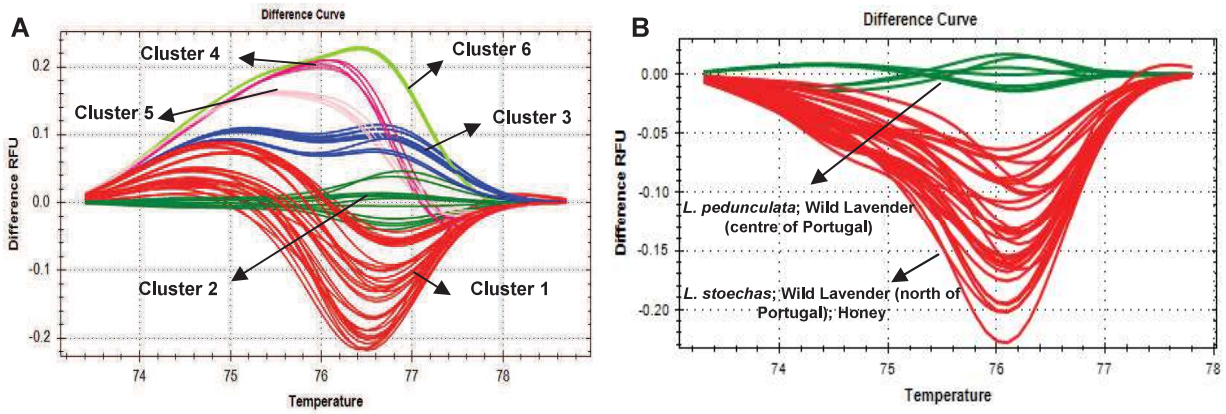

**Figure 1**. HRM analysis of *Lavandula* spp. with primers Lav1-F/Lav1-R. Difference curves obtained by HRM analysis of *Lavandula* spp. (A); difference curves obtained by HRM analysis of *Lavandula* spp. and honey samples (B). Cluster 1 – *L. stoechas*, *L. pedunculata*, *L. viridis*, *Lavandula* spp. (wild); cluster 2 – *L. dentata*, *L. hybrid*; cluster 3 – *L. angustifolia*, *L. latifolia*; cluster 4 – *L. multifida* (Porto); cluster 5 - *L. multifida* (Coimbra); cluster 6 – *L. pinnata*.

**Table 2.** *Lavandula* spp. tested in the present work and results of HRM analysis with clusters and respective levels of confidence corresponding to Figure 1A.

| Species | Origin | HRM | |
|---|---|---|---|
| *Lavandula stoechas* | Jardim Serralves (Porto, Portugal) | Cluster 1 | 98.2% |
| *Lavandula stoechas* | Jardim Botânico de Coimbra (Portugal) | Cluster 1 | 97.8% |
| *Lavandula pedunculata* | Jardim Botânico da Madeira (Portugal) | Cluster 1 | 97.6% |
| *Lavandula pedunculata* | Jardim Botânico de Coimbra (Portugal) | Cluster 1 | 97.7% |
| *Lavandula viridis* | Real Jardín Botánico Juan Carlos I (Madrid, Spain) | Cluster 1 | 97.6% |
| *Lavandula* spp. | Wild (Serra Montesinho, Bragança, Portugal) | Cluster 1 | 98.0% |
| *Lavandula* spp. | Wild (M. Canaveses, Portugal) | Cluster 1 | 98.0% |
| *Lavandula* spp. | Wild (Coimbra, Portugal) | Cluster 1 | 98.0% |
| *Lavandula dentata* | Jardim Serralves (Porto, Portugal) | Cluster 2 | 98.7% |
| *Lavandula dentata* | Jardim Botânico de Coimbra (Portugal) | Cluster 2 | 99.5% |
| *Lavandula hybrid* | JBUTAD (Vila Real, Portugal) | Cluster 2 | 98.8% |
| *Lavandula angustifolia* | JBUTAD (Vila Real, Portugal) | Cluster 3 | 97.2% |
| *Lavandula latifolia* | Jardin Botánique Strasbourg | Cluster 3 | 98.7% |
| *Lavandula multifida* | Jardim Serralves (Porto, Portugal) | Cluster 4 | 99.2% |
| *Lavandula multifida* | Jardim Botânico de Coimbra (Portugal) | Cluster 5 | 99.3% |
| *Lavandula pinnata* | Jardim Botânico da Madeira (Portugal) | Cluster 6 | 99.4% |

## 4. CONCLUSION

This study shows the high potential of the combination of DNA barcode with HRM analysis to successfully identify and distinguish closely related plant species. Hereby, we present a reliable and fast DNA-based methodology to assess the botanical origin of monofloral lavender honey, which can be a suitable alternative to the currently used traditional methods. To our knowledge, this is the first study using HRM analysis for the rapid discrimination of plant species in honey.

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