

# CHEMICAL COMPOSITION OF PORTUGUESE PROPOLIS BIOACTIVE PROPERTIES

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*Thesis submitted to the Faculty of Sciences of the University of Porto  
in partial fulfillment of the requirements for the  
degree of Doctor in Chemistry*

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***To my Parents and Miguel***



## **Acknowledgments**

To Fundação para a Ciência e a Tecnologia (FCT) for my PhD Grant with reference SFRH/BD/44855/2008 financially supported by program QREN-POPH-Tipology 4.1-Advanced Formation, European Social Fund and MCTES.

To Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade do Porto for accepting me as a PhD student in the Doctoral Program of Chemistry.

To my supervisor, Professor Cristina Freire, for accepting to supervise my work, for her knowledge, the dedication in supervising this work, her friendship, the valuable advices and suggestions and the constant availability for helping me.

To my co-supervisor, Professor Miguel Vilas-Boas, for the excellent work and dedication in supervising, his knowledge and for the fruitful discussions. For always questing me about my results and the encouragement given in pursuing new scientific goals. For all the accomplished goals and above all for his friendship. To have worked under your supervision made me grow up and recognize that professional success is the result of the effort and dedication.

To Professor Ana Cristina Figueiredo from Departamento de Biologia, Faculdade de Ciências, Universidade de Lisboa for welcoming me so well during my research trainings/stay in Lisbon, and for the profitable discussions regarding volatile compounds, for revising my work, for her patience, friendship, sympathy and for the constant availability in the discussing of the results.

To Professor Maria do Rosário Domingues from the Departamento de Química, Universidade de Aveiro for welcoming me during my work of mass spectrometry and for her help in the MS characterization of propolis extracts and availability in the discussion of the chemical structures of propolis composition.

To Professor Susana M. Cardoso from Escola Superior Agrária de Coimbra, for her friendship, patience, availability and fruitful discussions about propolis MS characterization as well as for the help in revising my MS results.

To Professor Paula Gomes, from the Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade do Porto, for welcoming in my LC-MS studies.

To Doctor Nuno Vale, from the Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade do Porto, for his friendship since “ancient times”, for the collaboration in the biological studies and the help in the LC-MS characterization of the propolis extracts.

To the Members of the Scientific Committee of the Doctoral Program of Chemistry and to all the Professors of Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade do Porto, for their enthusiasm in teaching and for sharing their scientific knowledge during my undergraduate formation.

To Federação Nacional de Apicultores de Portugal, for providing the propolis samples.

To Professor Isabel Ferreira, from Escola Superior Agrária de Bragança, for her friendship, sympathy, encouragement and support during this work and for her valuable advices.

To Eng. Sá Morais, from Escola Superior Agrária de Bragança, for his friendship, availability and precious help in the resolution of all kind of chromatographic problems.

To Lillian Barros, from Laboratório de Química e Bioquímica Aplicada, for the help in the laboratory and the talks about MS characterization of phenolic compounds. For all the good moments in the lab and in our travels to scientific congresses. Above all, for her friendship and fellowship during the last 7 years.

To all my colleagues from Laboratório de Química e Bioquímica Aplicada, past and present, for the pleasant moments spent in the Lab and for all the shared laughs, in particular to João, Ricardo, Sandrina, Rafaela, and to the more recent members Filipa,

Ângela, Inês, Márcio, Carla, Eliane. Special thanks for Vânia, Hélder, Andreia, Joana and Sofia with whom I worked more directly, in the bee's world, during these four years.

To Anabela Sousa, Ana Paula Pereira, Ivo Oliveira, Daniela Correia and Sofia Meirinho start companions in the adventure of the PhD, for their friendship and for all the good moments.

To all my friends outside the chemistry world, especially Marta, Humberto (and the little ones Gonçalo and Joana), for always being present in the good and bad moments, for the lifetime friendship, for the support, long talks dinners and weekends.

A special thanks to my family. To my mother, for her unconditional love, friendship, support, encouragement, wise advices, for always help me in my life, believing in me and being by my side in all the moments. To my father, for his love, friendship and for always be present in my life given me encouragement and support. To my grandfather for the friendship and support. To my grandmothers and godmother, that are no longer here but are always present in my heart and who are an inspiration and an example of courage.

Finally, to my husband Miguel, for his love, friendship, for giving me support and motivation in all moments, for always believing in me and being in my side in the good and bad moments. For all the fantastic moments, laughs and happiness in my life. To my unborn baby, who is so little but who I love so much, for giving me strength and courage to finish this work.

**FCT** Fundação para a Ciência e a Tecnologia  
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR Portugal

**POPHOR** QUADRO DE REFERÊNCIA ESTRATÉGICO NACIONAL PORTUGAL 2007-2013  
PROGRAMA OPERACIONAL POTENCIAL HUMANO

  
UNIÃO EUROPEIA  
Fundo Social Europeu



## Abstract

The characterization of Portuguese propolis from different continental and islands regions has as its main target the establishment of quality criteria and biological activity that enable the association of this beehive product with its phytogeographic origin, in an attempt to standardize the product and contribute for its commercial valorization. The results here described are structured according to seven resulting scientific publications: three published, two submitted for publication and two in final conclusion.

To begin, the study focused on the evaluation of the physical and chemical properties of forty propolis samples from six Portuguese regions, particularly in terms of water, ashes, wax contents and colour index. Besides these parameters, the phenolic composition and the antioxidant activity were also assessed using spectrophotometric techniques. The results attested the existence of two different types of propolis within the six regions under study. The first, with an orange colour and a botanical origin in poplar, common in temperate regions, revealed a high content in phenolics which reflects in its high bioactivity. The second, with darker green colour was less rich in phenolics and generally less bioactive.

Propolis is a matrix with high phenolic complexity so that the phenolic identification was achieved through electrospray ionization coupled to tandem mass spectrometry (ESI-MS<sup>n</sup>) and liquid chromatography with diode-array detection coupled to electrospray ionization tandem mass spectrometry (LC/DAD/ESI-MS<sup>n</sup>). The results allowed the identification of seventy six phenolic compounds, which included not only the typical phenolic acids and flavonoids found in propolis from temperate zones but also new compounds which have never been referred to in the literature. Within the new compounds, it was possible to find esterified and/or hydroxylated derivatives of flavonoids and unusual pinocembrin/pinobanksin derivatives containing a phenylpropanoic acid derivative moiety in their structure. In addition to these compounds, it was also possible to identify an unusual group of quercetin and kaempferol glycosides, described for the first time in propolis.

In order to establish a correlation between the propolis samples and its geographical and botanical origin, the study was extended to the quantification of the phenolic fraction using reversed phase high-performance liquid chromatography (HPLC). The resinous extracts of *Populus x canadensis* and *Cistus ladanifer*, two potential floral sources for propolis production, were also considered. Flavonoids were predominant in all samples, with propolis from north, central coast and Azores archipelago presenting a richer composition in phenolic compounds, similar to those observed in poplar buds, while the central interior and south samples, revealed several kaempferol derivatives, resembling the *Cistus ladanifer* exudates. The compound kaempferol-dimethyl-ether can indeed be considered as a marker compound for botanical origin discrimination since it was found only in the last group of samples.

The evaluation of the volatile fraction of propolis by gas chromatography coupled to mass-spectrometry (GC-MS), revealed a well diversify composition, with two hundred and one compounds detected, where terpenoids were predominante. The comparison with the essential oils extracted from the floral sources also allowed the association of *Populus x canadensis* and *Cistus ladanifer* volatiles with Portuguese propolis. Nevertheless, other *Populus* species as well as other plants like *Juniperus* genus may contribute to the resin in specific geographical locations.

The electrochemical techniques, especially cyclic voltammetry and differential pulse voltammetry, are alternatives to the spectrophotometric methods in the evaluation of antioxidant activity, mainly due to its sensitive to the electronic transfer process. Its application in the current work showed the existence of several oxidation processes, some of which were identical between samples within the same region. The similarity between the voltammetric profile of the propolis samples and that for floral sources, give these techniques the ability to discriminate the floral origin of the samples. The results obtained for the electrochemical antioxidant power were in agreement with the results obtained by other analytical methodologies, with a higher total electrochemical antioxidant power found for the coast centre samples, followed by north ~ Azores archipelago > central interior > Madeira island > south.

Finally, the antiprotozoal, antibacterial and antifungal activity of some Portuguese propolis samples and its two floral sources were screened, with the results revealing a low selectivity and moderate activity against pathogenic protozoa. The best bioactivity results were verified against the protozoa *Trypanosoma brucei*. and the fungus *Trichophyton rubrum*.

## Resumo

A caracterização da própolis Portuguesa, proveniente de diversas regiões do continente e ilhas, teve como principal objetivo a definição de critérios de qualidade e de atividade biológica que possam associar este produto da colmeia com a sua origem fitogeográfica, permitindo a padronização da própolis e contribuindo para a sua valorização comercial. Os resultados aqui descritos estão estruturados de acordo com as sete publicações científicas resultantes: três publicadas, duas submetidas para publicação e duas em preparação.

A fase inicial do estudo incidiu sobre a avaliação das propriedades físicas e químicas das quarenta amostras de própolis provenientes de seis regiões Portuguesas, nomeadamente o conteúdo em água, cinzas, ceras e índice de cor. Além destes parâmetros, foi também analisada a composição fenólica através de técnicas espectrofotométricas e a sua atividade antioxidante. Os resultados permitiram a definição de dois tipos distintos de própolis em Portugal, o primeiro de cor laranja, com origem botânica no choupo e comum nas regiões de clima temperado, apresenta um teor elevado de compostos fenólicos que se reflete na sua bioatividade e o segundo tipo de própolis, com uma cor verde escura, apresenta um baixo teor de compostos fenólicos e geralmente menor bioatividade.

A própolis é uma matriz de complexidade fenólica muito elevada pelo que a identificação do perfil fenólico foi efetuada através de espectrometria de massa tandem com ionização por electrospray (ESI-MS<sup>n</sup>) e cromatografia líquida com deteção por matriz de díodos, acoplada a espectrometria de massa tandem com ionização por electrospray (LC/DAD/ESI-MS<sup>n</sup>). Os resultados permitiram identificar setenta e seis compostos fenólicos, incluindo, não só os ácidos fenólicos e flavonóides típicos da própolis de zonas temperadas, mas também novos compostos cuja ocorrência nunca foi referida na literatura. Entre os compostos novos, encontraram-se derivados esterificados e/ou hidroxilados de flavonoides e derivados não usuais de pinocembrina/pinobanksina contendo estruturas derivadas do ácido fenilpropanóico. Para além destes compostos, foi ainda possível encontrar um grupo raro de glicosilados derivados da quercetina e campferol, descritos pela primeira vez na própolis.

Para se procurar estabelecer uma relação das amostras com a sua origem geográfica e botânica estendeu-se o estudo à quantificação da componente fenólica por cromatografia líquida de alta eficiência em fase reversa (HPLC), incluindo-se a avaliação dos extratos resinosos de *Populus x canadensis* e *Cistus ladanifer*, duas fontes potenciais utilizadas pelas abelhas na elaboração da própolis. Os flavonóides foram a classe de compostos predominantes, apresentando as amostras do norte, litoral centro e arquipélago dos Açores uma composição rica em compostos fenólicos e semelhante aos rebentos de *Populus*, enquanto as regiões do interior centro e do sul apresentaram vários derivados do campferol similar aos exsudados de *Cistus*. O composto éter de dimetilcampferol pode ser inclusive considerado como marcador de origem

para discriminação botânica uma vez que apenas foi encontrado neste último grupo de amostras.

A avaliação da componente volátil por cromatografia em fase gasosa acoplada a espectrometria de massa (GC-MS), revelou uma composição bastante diversificada com a presença de duzentos e um compostos nas amostras de própolis e com uma predominância em terpenóides. A comparação com os óleos essenciais extraídos das fontes florais permitiu também a associação dos voláteis de *Populus x canadensis* e *Cistus ladanifer* com a própolis Portuguesa. No entanto, outras espécies de choupo, bem como outras plantas, do género *Juniperus* poderão estar também na origem da resina em localizações geográficas específicas.

As técnicas electroquímicas, particularmente a voltametria cíclica e a voltametria de impulso diferencial, são alternativas à avaliação da atividade antioxidante por métodos espectroscópicos, pois são sensíveis ao processo de transferência eletrónica. A sua aplicação neste estudo mostrou a existência de vários processos de oxidação, alguns dos quais comuns entre amostras de uma mesma região. A semelhança entre o perfil voltamétrico das amostras de própolis e as fontes florais, confere a estas técnicas a habilidade para discriminar a origem floral das amostras. Os resultados obtidos na avaliação do poder antioxidante por eletroquímica estão de acordo com os resultantes de outras técnicas analíticas utilizadas, observando-se uma maior capacidade antioxidante total para as amostras do litoral centro, seguido do norte ~ arquipélago dos Açores > interior centro > ilha da Madeira > sul.

Finalmente, foram avaliadas as atividades antiprotozoária, antibacteriana e antifúngica de algumas amostras de própolis Portuguesa e das suas fontes florais, verificando-se uma baixa seletividade e uma atividade moderada contra protozoários patogênicos. Os melhores resultados de bioatividade foram verificados contra o protozoário *Trypanosoma brucei* e contra o fungo *Trichophyton rubrum*.

## Résumé

La caractérisation de la propolis Portugaise provenant de diverses régions du continent et des îles a eu comme principal objectif la définition de critères de qualité et d'activité biologique qui puissent associer ce produit de la ruche à son origine phytogéographique, permettant la normalisation de la propolis et contribuant à sa valorisation commerciale. Les résultats décrits ici sont structurés selon les sept publications scientifiques qui en résultent, trois publiées, deux soumises à publication et deux en préparation.

La phase initiale de l'étude se concentre sur l'évaluation des propriétés physiques et chimiques de quarante échantillons de propolis provenant de six régions Portugaises, c'est-à-dire le contenu en eau, cendre, cire et indice de couleur. Outre ces paramètres, a été également analysée la composition phénolique grâce à des techniques spectrophotométriques et son activité anti-oxydante. Les résultats ont permis la définition de trois types distincts de propolis au Portugal, le premier de couleur orange, d'origine botanique dans le peuplier et commun dans les régions au climat modéré, présente un contenu élevé de composés phénoliques qui se reflète dans sa bio-activité et le second type de propolis, de couleur vert sombre, présente un faible contenu de composés phénoliques et généralement une moindre bio-activité.

La propolis est une matrice de complexité phénolique très élevée raison pour laquelle l'identification du profil phénolique a été effectuée à travers une spectrométrie de masse tandem avec ionisation par électrospray (ESI-MS<sup>n</sup>) et chromatographie liquide avec détention par matrice de diodes, accouplée à une spectrométrie de masse tandem avec ionisation par électrospray (LC/DAD/ESI-MS<sup>n</sup>). Les résultats ont permis d'identifier soixante-six composés phénoliques, incluant non seulement les acides phénoliques et flavonoïdes typiques de la propolis des zones tempérées, mais aussi de nouveaux composés dont l'occurrence n'a jamais été signalée dans la littérature. Parmi les nouveaux composés on trouve des dérivés estérifiés et/ou hydroxylés de flavonoïdes et des dérivés plus habituels de pinocembrine/pinobanksine contenant des structures dérivées de l'acide phénylpropanoïque. En dehors de ces composés, il a été également possible de trouver un groupe rare de glycosylées dérivées de la quercétine et kaempferol, décrits pour la première fois dans la propolis.

En cherchant à établir une relation des échantillons avec leur origine géographique et botanique, on a élargi l'étude à la quantification de la composante phénolique par chromatographie liquide de haute efficacité en phase inversée (HPLC), incluant l'évaluation des extraits résineux de *Populus x canadensis* et *Cistus ladanifer*, deux sources potentielles utilisées par les abeilles dans l'élaboration de la propolis. Les flavonoïdes ont été la classe de composés prédominants, les échantillons du nord, littoral centre et archipel des Açores présentant une composition riche en composés phénoliques et similaire aux pousses de *Populus*, alors que les régions de l'intérieur centre et du sud ont présentées plusieurs dérivés de kaempferol similaire

aux exsudats de *Cistus*. Le composé éther de diméthylkaempferol peut être également considéré comme le marqueur de l'origine pour la discrimination botanique vu qu'il a été uniquement trouvé dans ce dernier groupe d'échantillons.

L'évaluation de l'élément volatile par chromatographie en phase gazeuse accouplée à la spectrométrie de masse (GC-MS), a révélé une composition assez diversifiée avec la présence de deux cent un composés dans les échantillons de propolis, avec une prédominance en terpénoïdes. La comparaison avec les huiles essentielles extraites des sources florales a permis également l'association des volatiles de *Populus x canadensis* et *Cistus ladanifer* et de la propolis portugaise. Cependant, d'autres espèces de peuplier, ainsi que d'autres plantes, du genre *Juniperus* pourront être aussi à l'origine de la résine dans des localisations géographiques spécifiques.

Les techniques électrochimiques, particulièrement la voltammétrie cyclique et la voltammétrie d'impulsion différentielle, sont des alternatives à l'évaluation de l'activité anti oxydante par méthodes spectroscopiques, car elles sont sensibles au procédé de transfert électronique. Leur application dans cette étude a montré l'existence de différents procédés d'oxydation, certains communs parmi les échantillons d'une même région. La ressemblance entre le profil voltamétrique des échantillons de propolis et les sources florales, confère à ces techniques la compétence de discriminer l'origine florale des échantillons. Les résultats obtenus par électrochimie sont en accord avec ceux résultant d'autres techniques analytiques utilisées, pouvant être observée une plus grande capacité anti oxydante totale pour les échantillons du littoral centre, suivi du nord ~ archipel des Açores > intérieur centre > île de Madère > sud.

Finalement, ont été évaluées les activités anti protozoaire, anti bactérienne et antifongique de quelques échantillons de propolis portugaise et de leur sources florales, se vérifiant une basse sélectivité et une activité modérée contre les protozoaires pathogéniques. Les meilleurs résultats de bio activité ont été vérifiés contre le protozoaire *Trypanosoma brucei* et contre le champignon *Trichophyton rubrum*.

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## List of Abbreviations and Symbols

|                     |                                                             |
|---------------------|-------------------------------------------------------------|
| A                   | Azores archipelago                                          |
| $a^*$               | chromatic component from green (−a) to red (+a) colour      |
| Ac                  | acetyl                                                      |
| AD                  | anno domini                                                 |
| AOAC                | Association of Official Analytical Chemists                 |
| $b^*$               | chromatic component from blue (−b) to yellow (+b) colour    |
| BC                  | before Christ                                               |
| BSTFA               | <i>N,O</i> -bis(trimethylsilyl)trifluoroacetamide           |
| C                   | <i>Cistus ladanifer</i> L.                                  |
| $C^*ab$             | chroma value                                                |
| CAPE                | caffeic acid phenylethyl ester                              |
| CC                  | central coast                                               |
| CI                  | central interior                                            |
| CID                 | chemical induced dissociation                               |
| Coum                | <i>p</i> -coumaroyl                                         |
| CV                  | cyclic voltammetry                                          |
| Da                  | dalton unit                                                 |
| DNP                 | 2,4-dinitrophenylhydrazine                                  |
| DMCA                | 3,4-dimethyl-caffeic acid                                   |
| DPPH                | 2,2-diphenyl-1-picrylhydrazyl                               |
| DPV                 | differential pulse voltammetry                              |
| E                   | potential                                                   |
| EAP                 | electrochemical antioxidant power                           |
| EC <sub>50</sub>    | half maximal effective concentration                        |
| ESI                 | electrospray ionization                                     |
| ESI-MS <sup>n</sup> | electrospray ionization coupled to tandem mass spectrometry |
| ESI-MS              | electrospray ionization coupled to mass spectrometry        |
| E <sub>p</sub>      | peak potential                                              |
| EtOH                | ethanol                                                     |
| FC                  | Folin- Ciocalteu                                            |
| FID                 | flame ionization detector                                   |
| HIV                 | human immunodeficiency virus                                |

|                            |                                                                                                              |
|----------------------------|--------------------------------------------------------------------------------------------------------------|
| GC                         | gas chromatography                                                                                           |
| GC-MS                      | gas chromatography coupled to mass-spectrometry                                                              |
| Glc                        | glucose                                                                                                      |
| GlcUA                      | glucuronic acid                                                                                              |
| HAT                        | hydrogen atom transfer                                                                                       |
| HPLC                       | high-performance liquid chromatography                                                                       |
| HPLC-DAD                   | high-performance liquid chromatography coupled to diode array detection                                      |
| HPLC-UV                    | high-performance liquid chromatography coupled to ultraviolet detection                                      |
| IS                         | internal standard                                                                                            |
| $j$                        | current density                                                                                              |
| LC-MS                      | liquid chromatography couples to mass spectrometry                                                           |
| $L^*$                      | lightness component                                                                                          |
| LC/DAD/ESI-MS <sup>n</sup> | liquid chromatography with diode-array detection coupled to electrospray ionization tandem mass spectrometry |
| LC/MS <sup>n</sup>         | liquid chromatography couples to tandem mass spectrometry                                                    |
| LDA                        | linear discriminant analysis                                                                                 |
| LOD                        | limit of detection                                                                                           |
| LOQ                        | limit of quantification                                                                                      |
| M                          | Madeira Island                                                                                               |
| $m$                        | slope                                                                                                        |
| MS                         | mass spectrometry                                                                                            |
| MS-MS                      | mass spectrometry coupled to mass spectrometry                                                               |
| $m/z$                      | mass/charge ratio                                                                                            |
| MW                         | molecular weight                                                                                             |
| N                          | north                                                                                                        |
| ORAC                       | oxygen radical absorbance capacity assay                                                                     |
| Pent                       | pentose                                                                                                      |
| PF                         | <i>Populus x Canadensis</i> Moenchen, female                                                                 |
| PM                         | <i>Populus x Canadensis</i> Moenchen, male                                                                   |
| ppm                        | parts per million                                                                                            |
| $R^2$                      | correlation coefficient                                                                                      |
| RI                         | retention index                                                                                              |

|                 |                                                         |
|-----------------|---------------------------------------------------------|
| Rham            | rhamnose                                                |
| Rut             | rutin                                                   |
| $S_b$           | standard deviation                                      |
| $S_{corr}$      | correlation coefficient                                 |
| SET             | single electron transfer                                |
| SPSS            | statistical product and service solutions               |
| t               | trace                                                   |
| $t_R$           | retention time                                          |
| TBAP            | tetrabutylammonium perchlorate                          |
| TEAC            | trolox equivalent antioxidant capacity assay            |
| TEAP            | total electrochemical antioxidant power                 |
| TRAP            | total radical-trapping antioxidant parameter assay      |
| UPGMA           | unweighted Pair Group Method with Arithmetical Averages |
| UV-VIS          | ultraviolet-visible                                     |
| UV              | Ultraviolet                                             |
| v/w             | volume/weight                                           |
| $\lambda$       | wavelength                                              |
| $\lambda_{max}$ | maximum wavelength                                      |



## Preface

Over the last years, the consumption of natural products increased significantly, associated with the idea that the use of a product may, in addition to fulfil the basic purpose, add a competitive advantage, whether nutritional, to promote health, improve general well-being, or reduce the risk of developing certain illnesses. In this group are the "functional food", products also known as nutraceuticals, designed foods, therapeutic foods, or medicinal foods, in which the bee products are included.

Bees have been in existence for over 125 millions of years and their evolutionary success has allowed them to become perennial species that can exploit virtually all habitats in the world. This success in the animal kingdom is largely because of the chemistry and applicability of its bee products: honey, beeswax, venom, propolis, pollen and royal jelly. Propolis, being the most important "chemical weapon" of bees against pathogen microorganisms, has been used as a remedy by humans since ancient times. For this reason, propolis has become the subject of intense pharmacological and chemical studies for the last years. There is a global interest in propolis research which is linked to the diverse biological properties and the high added value in the business markets.

In the last decade, beekeeping in Portugal began an important professionalization process, thus reducing the recreational aspect of the activity. Consequently, economic exchanges with the outside increased significantly, but still confined to honey. This fact is due to the reduced scientific information on the quality of other national bee products, especially propolis, in contrast to the amount of international information. Propolis is an extremely complex mixture of natural substances and its quality depends on plants available around the hive, as well on the geography and climatic conditions of the site. Mainly it is constituted by phenolic compounds, which are responsible for many biological properties, attributed to their powerful antioxidant and antiradical activity. Being the product of plants secondary metabolism, they are present in propolis as the result of the interaction with honeybees. Thus, to increase the demand of this product and therefore its production and commercial value, it is important to perform an extensive chemical characterization and promote its potential applications.

This thesis is devoted to the extensive characterization of propolis from different Portuguese regions, a country with botanical diversity, which will allow, for the first

time, the characterization of the product at national level and establish quality criteria parameters for commercial purposes and thus for its valorisation.

The main objectives of this work were:

- (i) to characterize the physicochemical parameters of Portuguese propolis and to quantify, globally, through spectrophotometric methods, the phenolic compounds;
- (ii) to study the phenolic profile of Portuguese propolis and quantify the components through chromatography and mass spectrometry. The results will contribute, with the help of the discovery of markers compounds, to create a national phenolic pattern that will be used to establish geographical authenticity;
- (iii) to characterize the volatile composition of Portuguese propolis;
- (iv) to evaluate the antioxidant properties of propolis samples using chemical and electrochemical methods and correlate these results with their phenolic profile;

The thesis is divided in 10 chapters. Chapter 1, corresponding to the introduction is an revision of different aspects involving propolis, from historical applications, production, manipulation and importance for bees to the chemical composition and plant origin, biological properties and analytical methods applied in its analysis. The chapter is concluded with a general overview of the propolis research in Portugal. Chapter 2 reports the physicochemical characterization, phenolic content and antioxidant activity of Portuguese propolis. Chapter 3 and 4 describes the phenolic profiling of Portuguese propolis by ESI-MS, ESI-MS<sup>n</sup> and LC-MS. Chapter 5 reports the quantification, through HPLC, of the phenolic content of the Portuguese propolis and its potential floral sources, *Populus x Canadensis* Moench buds and *Cistus ladanifer* L. Chapter 6 describes the volatile components, analysed by GC and GC-MS, present in the propolis samples and potential floral sources under study. Chapter 7 reports the establishment of a rapid, easy and low-cost tool, for the evaluation of the antioxidant capacity in propolis from different origins, by using electrochemical methods like cyclic voltammetry and differential pulse voltammetry. Chapter 8 is devoted to the evaluation of some bioactive properties of propolis samples and potential plant sources, including antiprotozoal, antibacterial and antifungal activities. Finally, Chapter 9 describes the main conclusions and future perspectives.

The work reported in Chapters 2 to 8 is based on seven scientific papers, four of which have already been published in refereed Journals, one is accepted and two are in conclusion to be submitted for publication. Some adaptations were performed in those chapters to standardize the thesis formatting. The experimental details of each Chapter are described in the Appendices.

## List of publications

### Chapter 2

Falcão SI, Freire C, Vilas-Boas M. A proposal for quality standards and antioxidant activity of Portuguese propolis. *J. Am. Oil Chem. Soc.* **2013**, Accepted.

### Chapter 3

Falcão S, Vilas-Boas M, Estevinho LM, Barros C, Domingues MRM, Cardoso SM. Phenolic characterization of Northeast Portuguese Propolis: usual and unusual compounds. *Anal. Bioanal. Chem.* **2010**, 396, 887-897.

### Chapter 4

Falcão SI, Vale N, Gomes P, Domingues MRM, Freire C, Cardoso SM, Vilas-Boas M. Phenolic profiling of Portuguese propolis by LC-MS spectrometry: uncommon propolis rich with flavonoid glycosides. *Phytochem. Anal.* **2013**, 24 (4), 309-318.

### Chapter 5

Falcão SI, Tomás A, Vale N, Gomes P, Freire C, Vilas-Boas M. Phenolic quantification and botanical origin of Portuguese propolis. *Ind. Crop. Prod.* **2013**, 49, 805-812.

### Chapter 6

Falcão SI, Freire C, Figueiredo AC, Vilas-Boas M. The volatile composition of Portuguese propolis towards its origin discrimination. To be submitted, **2013**.

### Chapter 7

Falcão SI, Tomás A, Freire C, Vilas-Boas M. Propolis electroactivity: a new analytical/voltametric tool for origin discrimination. To be submitted, **2013**.

### Chapter 8

Falcão SI, Vale N, Cos P, Gomes P, Freire C, Maes L, Vilas-Boas M. In vitro evaluation of Portuguese propolis and floral sources for antiprotozoal, antibacterial and antifungal activity. *Phytoter. Res.* **2013**, DOI: 10.1002/ptr. 5013.



# Chapter 1

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## Introduction



# 1

## Introduction

The food and the pharmaceutical industry has encouraged, in recent years, the emergence of countless research works dealing with beehive products, particularly focusing in propolis composition and its biological properties. The scientific information gathered is been revealing the enormous potentialities of this resin as a source of natural compounds with many nutraceutical applications, from foods and beverages to the everyday products such as soaps and toothpastes. Propolis is a resin obtained from plants which the bees use to protect the honeycomb against the ingress of air currents, predators and microorganisms. The chemical composition of this bee product is highly variable and complex, depending strongly on the plant sources available to the bees at the site of collection as well as with the geographic and climatic conditions of the site. The knowledge of the chemical composition as well as the plant sources is needed for propolis typification and thus for its chemical standardization and for ensuring the quality and safety necessary for its commercialization.

### 1.1 The history of propolis

The word “propolis” originates from the Greek “pro” (for ‘in front of’, ‘at the entrance to’) and “polis” (‘community’ or ‘city’) and means a substance with a protecting role for the bee colony.<sup>1,2</sup> Propolis is an old remedy that has been known for centuries and has been widely used by different cultures for different purposes, among which the use in medicine is included. The history recall its use at least back to 300 BC and remains today in home remedies and personal products.<sup>3</sup> In ancient Egypt, propolis was known for its anti-putrefactive properties and used to embalm cadavers.<sup>4</sup> The

ancient Jews considered *tzori*, the Hebrew word for propolis, as a medicine, mentioned in the Old testament.<sup>4</sup> The Greeks used propolis as the primary ingredient of a perfume called *polyanthus*, which combined propolis, olibanum, styrax, and aromatic herbs.<sup>2</sup> Also, several Greek and Roman physicians, such as Aristoteles, Dioscorides, Hippocrates, Pliny, and Galen reported medicinal preparations and applications of propolis.<sup>5</sup> Although the harvesting methods in the ancient world are unknown, the Roman scholar Pliny (23-79 AD), postulated that propolis was originated in the buds of different trees like willow, poplar, elm, reed and other plants.<sup>2,4</sup> Marcos Terentius Varro wrote that propolis was used by physicians in making poultices and because of that had a higher price than honey on the Via Sacra.<sup>4,5</sup> Roman soldiers carried propolis as an emergency medicine for war wounds.<sup>6</sup> Also, Incas employed propolis as an antipyretic agent and Persians described it as a drug against eczemas, myalgia, and rheumatism.<sup>1,5</sup> In the Middle ages and among Arab physicians, the bee glue was described in medicinal preparations which were used for the treatment of oral and pharyngeal infections, dental caries, as well as an antiseptic and cicatrizing in wound treatment.<sup>1</sup> In Europe, the London pharmacopoeias of the seventeenth century listed propolis as an healing ointment in the treatment of inflammations and wounds, which had the origin in the black poplar tree buds.<sup>2</sup> Between the seventeenth and twentieth century's, the propolis became very popular in Europe on account of its antibacterial activity.<sup>1</sup>

The development of research on propolis was strictly connected with the development of chemistry. The first work indexed by *Chemical Abstracts* on propolis composition was in 1908, and the first patent was described in 1904 (USA—Composition for treating pins and piano strings). One hundred and nine years after the first publication in the *Chemical Abstract*, the number of publications on propolis reached 3,880 in journal and 2,884 in patents.<sup>7,8</sup> Currently, there is a new resurgence in the interest of propolis composition, biological properties and its application as a dietary supplement as well as its use in the pharmaceutical and cosmetic industry.<sup>9,10</sup>

## 1.2 Propolis: the guardian of the hive

Bees gathered resinous exudates from leaf buds, shoots and petioles of leaves from different plants (elm, poplar, willow, birch, hoarse chestnut, pine, fir, oak trees and some herbaceous plants) with their mandibles, which once introduced into the hive are mixed with wax and salivary secretions, in order to produce propolis.<sup>3,9,11</sup>

The product is usually aromatic, with a resinous flavor. The texture is variable, some samples being hard and friable, others moldable and sticky as well as the taste (bitter, slightly spicy or insipid) or the consistency. The colour of propolis ranges from light yellow, to green or dark brown,<sup>6,12</sup> Figure 1.1.



**Figure 1.1** Samples of propolis collected in different Portuguese regions

Bees make use of propolis as a building material and to defend the hive.<sup>13</sup> Within the different functions of propolis in the beehive, bees use it in the construction and reinforcement of the combs for waterproofing walls against sap (if tree-cavity nesting) or external moisture, to polish the interior of the walls of the comb hexagonal cells and thus to facilitate cleaning, to seal crevices and openings in the hive reducing air currents or to reduce size of the entrance avoiding the entrance of intruders, this contributes additionally to maintaining the hive inner temperature at 35°C.<sup>3</sup> On the other hand, is also used for embalming invaders killed by bees inside the hive which are too heavy to be removed to outside, preventing the decomposition process, acting as disinfectant. Propolis has a strong anti-bacterial, anti-fungal and anti-viral activity which is responsible for the low incidence of bacteria and fungi inside the hive compared to the outside.<sup>2,3</sup> It has also been proposed that propolis has a role in the social immunity of honeybees, reducing the risk of disease and parasite transmission through the colony.<sup>14</sup> Propolis extracts are active against honeybee health threats, such as the *Paenibacillus larvae*,<sup>15</sup> the highly damaging mite *Varroa destructor*<sup>16</sup> and the wax moth *Galleria mellonella*.<sup>17</sup> These observations strengthen the value of propolis as a source of biologically active substances.

The resin collection is made only by a few workers, not older than 15 days old which are specialized in propolis foraging.<sup>2</sup> Resin-foragers gather propolis during the warm time of the day, when the glue is soft from droplets appearing on the bark of the

trunks or limbs of trees,<sup>18</sup> from the surfaces of some fruits,<sup>19</sup> or more typically on the vegetative apices (buds and young leaves),<sup>2,20</sup> showing a preference for the last ones, Figure 1.2 (a). In a first step, during the collection process, bees extract the resins from the trichomes and ducts by fragmenting the early leaves using their mandibles,<sup>21</sup> then the material is passed to the forelegs, transferred to the middle legs, and then pressed against the corbícula of the hind leg.<sup>20</sup> This sequence is repeated until there is a full resin load on both corbicula, which takes about seven minutes.<sup>19</sup>

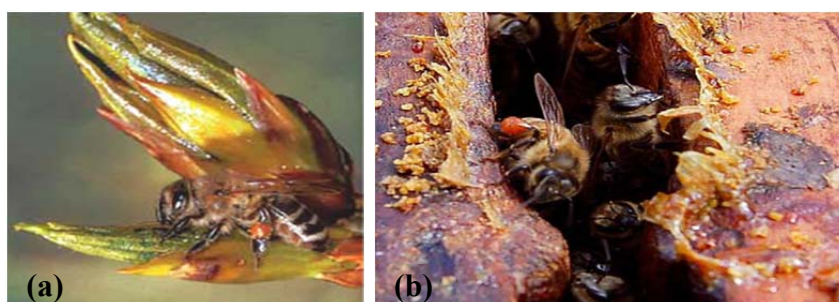


**Figure 1.2** (a) Bee collecting resin from a poplar bud;<sup>2</sup> (b) Resin-forager returns to the hive with a load of resin on her corbicula.<sup>14</sup>

Once returning to the hive, the resin collected by the resin-foragers is bite off from the corbicula by other bees, known as cementing bees, Figure 1.2 (b). They smooth the resin with the mandibles, in a process very similar to that of wax construction.<sup>14</sup> One resin-forager gathers into the hive about 10 mg propolis per flight.<sup>2</sup> Annual production (10-300g/hive) varies in accordance with the variety of bee, the climate, the flora and the collection mechanism used.<sup>12</sup>

There appears to be some seasonality linked to the resin collection, with an increased propolis production in late summer, for temperate regions, through autumn when the honey flow is greatly reduced. Some authors suggested that this behavior pattern is due to the change in foraging behavior rather than climate changes because some bees can be induced to collect resin during any time in the season.<sup>14</sup> Also, the high levels of resin collection in late summer could be related the reduction of pollen and nectar foraging.<sup>14</sup>

Generally, beekeepers collect propolis by scrapping off the bee glue present on the comb frames and in the bee hive box.<sup>9</sup> To obtain a propolis with good quality and low degree of debris beekeepers make use of plastic or grid nets, Figure 1.3, which are placed on top of the beehive, leading bees to seal the gaps with propolis.<sup>2,22</sup> Harvesting

is carried out before the arrival of the winter season in the temperate climates, while in tropical zones, harvesting takes place on the beginning of the rainy season, which is the period when propolis production appears to be more active.<sup>12</sup>



**Figure 1.3** Harvesting of propolis with a plastic net placed on the top of the hive.<sup>2</sup>

The production of propolis is also correlated to the bee's species, some races rely very little on the substance, while others use resins and propolis extensively.<sup>23</sup> Propolis is gathered mostly from *Apis mellifica* bees. Colonies of *Apis dorsata* may use resin occasionally to strengthen the site of comb attachment on a branch, while the bees *Apis florea* and *Apis cerana* do not gather propolis.<sup>2</sup> The tropical stingless bee species also gather propolis for nest construction, incorporating clay, soil and wax to form the nest itself and its supporting structures. When resins are mixed with soil or clay material the resulting mixture is often termed geopropolis or batumen, whereas when it is only mixed with wax it is simply called propolis or cerumen with respect to non-honey bee species.<sup>24</sup>

## 1.3 Propolis: composition and plant origin

### 1.3.1 Resin sources

The variability of propolis chemical composition is strongly dependent on the plant sources available around the hive and on the geographic and climatic conditions, although bees show preference for specific resin sources.<sup>13</sup>

In temperate regions, such as Europe, North America and non-tropical regions of Asia, the main plant sources of resin are poplar trees (*Populus* spp.), namely *Populus nigra*.<sup>9,13</sup> In Russia, especially in the northern parts, birch buds (*Betula verrucosa*, *Betula pubescens*) supply bees with propolis.<sup>2,13</sup> However other species like *Ulmus*,

*Pinus*, *Quercus*, *Salix* e *Acacia*, are adequate resin sources for temperate propolis, particularly when poplars are unavailable.<sup>25</sup> The most probably resin sources in the Mediterranean areas is the Cupressaceae family and the specie *Ferula communis*.<sup>26,27</sup> Also, in Tunisia, leaf exudates of some *Cistus* spp. are described to be potential propolis sources.<sup>28</sup> In tropical regions, bees mostly collected the resin from *Clusia* spp. flowers and *Baccharis dracunculifolia*.<sup>6</sup> Recently a leguminous species (*Dalbergia* spp.) also has been identified as a common source in tropical climates.<sup>29</sup> In Brazil, other species like *Hyptis divaricata*, *Araucaria angustifolia*, *Eucaliptus citriodora* have been suggested for possible sources.<sup>30</sup> In the Pacific islands, *Macaranga Tanarius* fruits are the resin source for propolis.<sup>19</sup>

Although the specificity of local flora is a determinant aspect in the chemical composition of the resin, there is another important factor, the choice made by bees. Currently, the cues that bees rely on to select the resin plant sources are unknown, nevertheless, they show a clear preference for specific sources. Indeed, depending on the availability, plants of a certain species prevail to a higher or lower extent as resin sources. As well as bees choose sticky resinous materials because of their physical properties, they may also select the plant sources by the bioactive composition that fulfill their needs.<sup>6,31</sup>

### 1.3.2 Chemistry of propolis

The chemical composition of propolis is highly variable depending on the phytogeographic conditions of the site of collection. Although propolis is a bee product, during its production and use the resins does not appear to be chemically modified by bees, as occurs with the nectar. While there is some evidence that the general chemical profile can vary slightly from the leaf buds of the plant source, it is likely that this variation could occur due to the volatilization of some chemicals during the collection process rather than to a chemical change processed by bees.<sup>13,14</sup>

Propolis contains approximately 50% of resin and vegetable balsam (phenolic compounds), 30% of wax, 10% of essential and aromatic oils, 5% of pollen, and 5% of other substances (aminoacids, vitamins, mineral salts) including organic debris,<sup>9</sup> Table 1.1.

**Table 1.1** Chemical composition of raw propolis.<sup>2,11,13,22,32</sup>

| Composition                              | Substances                                                                                                         |
|------------------------------------------|--------------------------------------------------------------------------------------------------------------------|
| <b>Balsam (ethanol soluble fraction)</b> |                                                                                                                    |
| Resin<br>40-60%                          | Phenolic acids, flavonoids, lignans, alcohols, aldehydes esters, aliphatic acids and aliphatic esters and ketones. |
| Essential oils<br>3-5%                   | Mono- and sesquiterpenes, aromatic compounds.                                                                      |
| <b>Non-balsam</b>                        |                                                                                                                    |
| Wax<br>20-35%                            | Beeswax and vegetable origin wax (monoesters and hydrocarbons).                                                    |
| Pollen<br>5%                             | Proteins, amino acids (mainly arginine and proline).                                                               |
| Other compounds<br>5%                    | Minerals, vitamins, sugars, lactones, quinones, benzoic acid and esters, steroids.                                 |

More than 300 chemical compounds were identified in propolis from different regions.<sup>13</sup> The main chemical classes and most bioactive compounds found in propolis are the phenolic compounds, including phenolic acids and flavonoids (chalcones, dihydrochalcones, flavones, flavonols, flavanones, dihydroflavonols and isoflavonoids),<sup>11,13</sup> Figure 1.4.

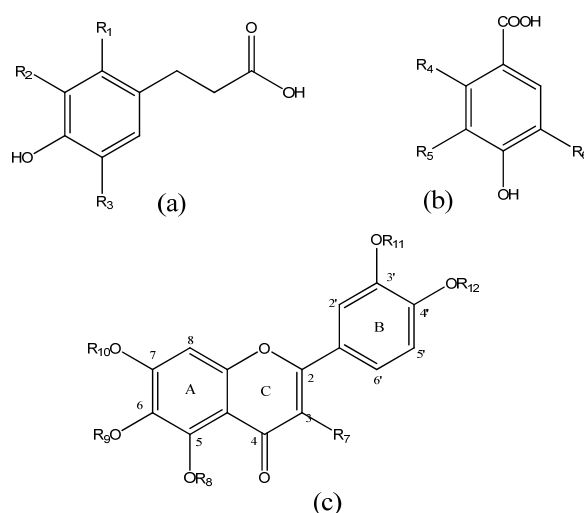


**Figure 1.4** Basic structures of the most common phenolic compounds found in propolis. (a) cinnamic acids; (b) benzoic acids; (c) flavonoids.

Volatile oils are also found in propolis but in much lower concentrations, nevertheless their aroma and biological significance make them also important for characterization. This fraction is mainly composed by mono- and sesquiterpenes, albeit the diversity of volatile compounds present is very high.<sup>13</sup>

As a product strongly linked with the floral origin of the resin, the chemical composition of propolis is for that related with the phytogeographic origin. Table 1.2

summarizes several types of propolis, described in the literature, according to its major plant sources and chemical constituents.

**Table 1.2** Most widespread propolis types.

| Propolis type | Geographic origin                                                                  | Plant source                                | Major compounds                                               | Ref.      |
|---------------|------------------------------------------------------------------------------------|---------------------------------------------|---------------------------------------------------------------|-----------|
| Poplar        | Temperate regions (Europe, North America, non-tropic regions of Asia, New Zealand) | <i>Populus</i> spp. section <i>Aigeiros</i> | Flavones, flavanones, cinnamic acids and their esters         | 13        |
| Birch         | Russia                                                                             | <i>Betula verrucosa</i>                     | Flavones and flavonols (different from poplar type)           | 33        |
| Mediterranean | Sicily, Greece, Malta                                                              | Cupressaceae                                | Diterpenes (mainly acids of labdane type)                     | 26<br>27  |
| Green         | Brazil                                                                             | <i>Baccharis</i> spp.                       | Prenylated phenylpropanoids, caffeoylquinic acids, diterpenes | 6<br>20   |
| Red           | Brazil, Cuba, Mexico                                                               | <i>Dalbergia</i> spp.                       | Isoflavonoids, neoflavonoids, pterocarpanes, lignans          | 34-<br>36 |
| Clusia        | Brazil, Cuba, Venezuela                                                            | <i>Clusia</i> spp.                          | Polyprenylated benzophenones                                  | 37-<br>38 |
| Pacific       | Okinawa, Taiwan, Indonesia                                                         | <i>Macaranga tanarius</i>                   | C-prenyl-flavanones                                           | 19<br>40  |

In temperate regions, where poplars are the main source of propolis resin, the most abundant compounds are flavonoids without B-ring substituents (*e.g.* pinocembrin, pinobanksin, galangin, chrysin) and their esters, together with phenylpropanoids and their esters (*e.g.* caffeic acid phenylethyl ester, CAPE),<sup>11,13</sup> Figure 1.5.

Tropical propolis has a different chemical profile. Prenylated phenylpropanoids (*e.g.* artepillin C, drupanin) and caffeoylquinic acids (*e.g.* dicaffeoylquinic acid) and its derivatives were shown to be very common and abundant in green propolis from south-eastern Brazil, Figure 1.6, with origin in the vegetative buds and apical tissues of *Baccharis dracunculifolia*.<sup>6,13,41</sup> The presence of diterpenoids were also identified in south Brazil propolis, being its probable plant source *Araucaria* spp.<sup>42</sup>

Red propolis is gathered by bees in Brazil, Cuba and Mexico from Leguminosae, namely *Dalbergia* species and contains pterocarpanes, isoflavonoids, lignans and phenylpropanoids.<sup>34-36</sup> Poliprenylated benzophenones, Figure 1.7 (a), are characteristic of propolis from Venezuela, Cuba, and in northeast and Amazon region of Brazil and linked with the flower exudates of *Clusia* spp.<sup>37-38</sup>

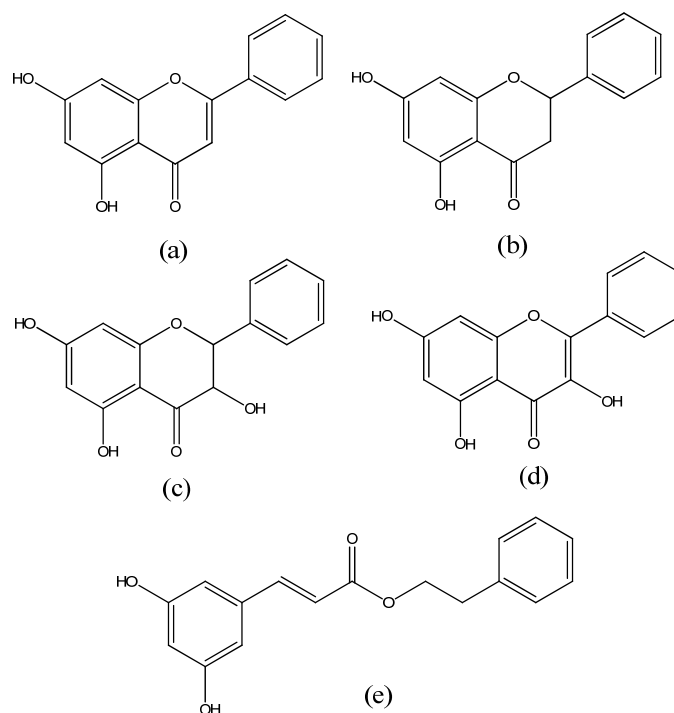


**Figure 1.5** Phenolic compounds found in temperate propolis. (a) chrysin; (b) pinocembrin; (c) pinobanksin; (d) galangin; (e) CAPE.

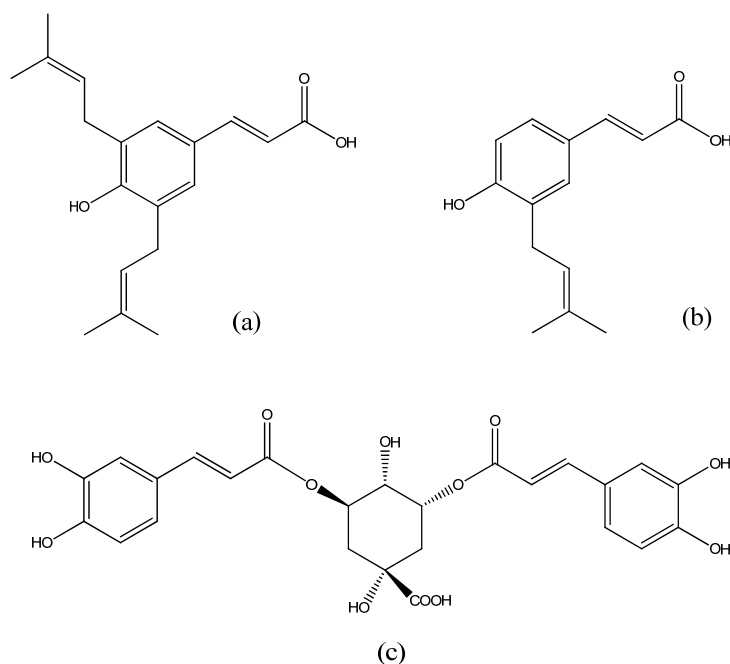


**Figure 1.6** Phenolic compounds found in green propolis. (a) artepillin C; (b) drupanin; (c) dicaffeoylquinic acid.

In the Pacific islands, a new family of compounds were reported in propolis, the C-prenyl flavanones, so called propolins, Figure 1.7 (b). These compounds were associated with the resin from the fruit exudates of the tree *Macaranga tanarius*.<sup>19,40</sup>

Recently, propolis research from Africa regions such as Egypt and Kenya reported the presence of geranyl derivatives very similar to those of Pacific islands.<sup>43,44</sup>

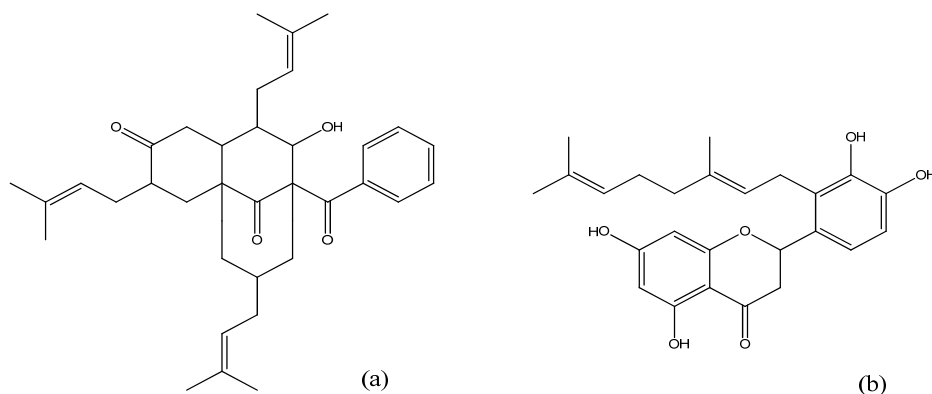


**Figure 1.7** (a) Polyprenylated benzophenone; (b) C-prenyl-flavanone.

Mediterranean propolis from Greece and Malta had also exhibit some specificities with a rich composition in diterpenes<sup>26,27</sup> and anthraquinones, however, while the source of the diterpenes seems to be coniferous species of the Cupressaceae family, nothing has been suggested regarding the plant origins for the anthraquinones found.<sup>45</sup> In propolis from the Canary Islands and Chile the chemical composition revealed the presence of lignans and furofuran type compounds.<sup>46,47</sup>

Propolis from Myanmar has also a unique composition with prenylated flavanones and cycloartane triterpenoids, the latter having been found in no other propolis types.<sup>48</sup> Recently, a new type of propolis from Jeju Islands, with a rich composition in chalcones and coumarins, were characterized, being *Angelica keiskei*, a large biennial-to-perennial plant from the Apiaceae family, the potential plant source.<sup>49</sup>

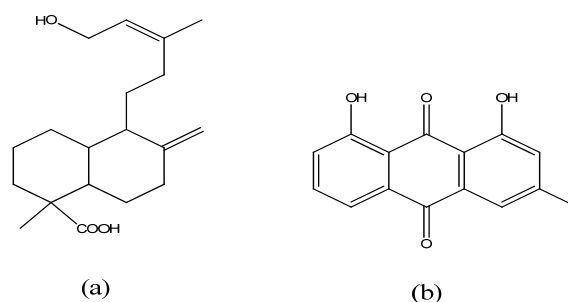


**Figure 1.8** Compounds found in Mediterranean propolis. (a) Diterpene (isocupressic acid); (b) anthraquinones (chrysophanol).

Due to the chemical complexity of propolis and its dependence in the local flora present in the hive neighborhoods, propolis is a source of new compounds and every day new studies are reported.

### **1.3.3 Propolis standards**

The use of propolis as raw material in the food and pharmaceutical industry requires an adequate chemical standardization that enables to process the propolis in an industrial chain, guaranteeing the quality and safety of the final product. As previously stated, propolis composition is highly variable. With propolis commercialization and standardization in mind, chemical variation is a serious constraint.<sup>41,43</sup> Propolis is a bee product derived from plants, whose composition is not changed by bees.<sup>13,14</sup> Consequently, the problem of standardization can be approached in the same manner as it is done for medicinal plants. In order to establish relevant quantitative criteria for quality in medicinal plants and extracts, different concepts have to be followed depending on the available knowledge on the active principle(s).<sup>50</sup> If the active principles are known and accepted, they have to be quantified using an appropriate analytical method. If the active compounds are not known or still under discussion, the total extract is regarded as the “active principle” and in that case marker compounds must be used for quality control.<sup>41</sup> A marker is a substance known to be produced by the plant source and assumed to be abundant and constant in a certain propolis type.<sup>43</sup> Regarding propolis, a lot of knowledge has already been gathered on active components and one of the most important active principles was found to be CAPE.<sup>51</sup> When CAPE is absent, for example in different origin propolis like tropical region, artepillin C has been established as a marker of Brazilian green propolis.<sup>52</sup> The propolis typification on the basis of plant sources knowledge is a useful tool for its chemical standardization and thus for ensuring the quality and safety necessary for its commercialization.

Apart from the establishment of marker compounds other quality criteria have been proposed. In a first approach, propolis can be evaluated by its sensory (consistence, odour, taste and colour) and physical-chemical properties (density, melting point, solubility in ethanol).<sup>2</sup> For the quality of propolis to be considered as high, propolis should be free from contaminants, contain low percentages of wax, insoluble materials and ash, the botanical origin should be known to define the active compounds and should contain a high content of biological active compounds.<sup>53</sup> Standards for

poplar and green propolis have been proposed.<sup>2,54</sup> The poplar and green propolis standards are based on the quantification of the main biologically active substances, balsam, total flavone and flavonol content, total flavanone and dihydroflavonol content, and total phenolic content,<sup>41</sup> Table 1.3.

**Table 1.3** Standard for poplar and green propolis.<sup>2,54</sup>

| <b>Component</b>                      | <b>Poplar propolis<br/>Min values, g/100g</b> | <b>Green propolis<br/>Min values g/100g</b> |
|---------------------------------------|-----------------------------------------------|---------------------------------------------|
| Balsam                                | 45                                            | 35                                          |
| Total phenolics                       | 21                                            | 7                                           |
| Total flavones and flavonols          | 5                                             | -                                           |
| Total flavanones and dihydroflavonols | 4                                             | -                                           |
| Total flavonoids                      | 9                                             | 1                                           |
| Beewax                                | Max 25                                        | Max 25                                      |
| Insoluble matter                      | Max 5                                         | Max 5                                       |
| Ash content                           | Non specified                                 | Max 5                                       |

## 1.4 Biological properties

In the last years, propolis has been described to have a wide spectrum of biological and pharmaceutical properties. Most of these properties are attributed to the powerful antioxidant and antiradical activity demonstrated by phenolic compounds.<sup>55</sup> The chemistry diversity of propolis from different origins leads to the expectation that the biological properties of different propolis types will be dissimilar, however, in most cases, this is not true.<sup>56,57</sup>

Among the many biological and pharmacological properties present by propolis, it can be highlighted the antimicrobial,<sup>9</sup> antioxidative, antihepatotoxic,<sup>51</sup> antitumoral, anti-inflammatory,<sup>58</sup> anti-HIV-1,<sup>59</sup> anti-neurodegenerative<sup>60</sup> and antituberculosis.<sup>61</sup> Table 1.4 summarizes the different biological properties of propolis compounds.

Beyond those properties, propolis also present several medical applications, particularly in stomatology and odontology, otorhinolaryngologic and respiratory diseases, gastroenterology, against cancer and in the treatment of skin lesion, wounds, burns and ulcers.<sup>56</sup>

**Table 1.4** Propolis chemical compounds and its biological properties.

| Propolis type tested | Component                    | Biological activity                                                                                                                                                                                                                                           | Ref.         |
|----------------------|------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------|
| All propolis types   | Polyphenols, flavonoids      | Antibacterial, antiviral, antifungal, antioxidant, antiaging, antiulcer, antitumor, antiallergic, anti-inflammatory, antiosteoporotic, antitrombogenic, antiatherosclerosis, cardioprotective, immunomodulating, hepatoprotective, cicatrizing, ulcer healing | 3, 9, 51, 62 |
| Poplar               | CAPE and other caffeates     | Antioxidant, anti-inflammatory, antitumor, antibacterial, antiviral, fungicide, immunomodulatory, cardioprotective, hepatoprotective, antiosteoporosis                                                                                                        | 31, 63       |
| Poplar, green        | Caffeic acid                 | Antiviral, Antioxidant, antiulcer, antitumor                                                                                                                                                                                                                  | 31           |
| Clusia               | Polyprenylated benzophenones | Antioxidant, anti-inflammatory, antitumor                                                                                                                                                                                                                     | 51           |
| Green                | Artepillin C                 | Antioxidant, anti-inflammatory, antitumor, apoptosis inducing                                                                                                                                                                                                 | 31, 64       |
| Green                | Dicaffeoyiquinic acid        | Hepatoprotective                                                                                                                                                                                                                                              | 65           |
| Pacific              | Prenylated flavanones        | Antioxidant, anticancer, apoptosis inducing                                                                                                                                                                                                                   | 31           |
| Greece, Crete, Malta | Diterpenes                   | antibacterial, antifungal                                                                                                                                                                                                                                     | 26           |
| Canary islands       | Lignans                      | Antibacterial                                                                                                                                                                                                                                                 | 46           |
| Brazil, Poland       | Essential oils               | Antibacterial                                                                                                                                                                                                                                                 | 66           |

## 1.5 Analytical methods applied to propolis analysis

### 1.5.1 Analysis of balsam content, wax, moisture and insoluble matter

The evaluation of the balsam content, waxes, moisture and insoluble matter in raw propolis is often used as an indication of propolis quality. The amount of balsam in propolis is determined by preparing an extract of crude propolis in 70 % ethanol, which is how propolis is used in tradicional medicine. The percentage of balsam in propolis is an important indicator since a high percentage of balsams results in a low percentage of wax and insoluble matter and therefore an higher content of biologically active components.<sup>54</sup> Crude propolis containing amounts of wax and insoluble materials above the usual may have been the result of poor beekeeping practices when removing the propolis from the hive, or mean that it comes from a region where the bees could not obtain a satisfactory amount of resins in neighboring plants.<sup>67</sup> In both cases, the amount of alcohol extractible bioactive resins will be lower. The ash content of crude propolis, determined by the sample incineration at 600 °C and then dessicated until constant

weight,<sup>67</sup> together with the balsam content have been used to evaluate this parameter. The wax contents are usually determined by freezing the ethanolic extracts overnight (-16°C at least) and separating the precipitated wax by filtration at this temperature.<sup>67</sup> A excessive amount of moisture in crude propolis is indicative of inadequate storage or manipulation conditions. Although the amount of water may be determined by different procedures, usually the sample is submitted to a temperature of 100°C until constant weight, with a maximum of 8% humidity considered acceptable.<sup>67</sup>

### 1.5.2 Phenolic compounds extraction

The analysis of phenolic compounds within the propolis, one of the fraction most studied, requires a previous extraction process, which will remove the inert material.<sup>68</sup> There is no standard either for extractive procedure or composition of products that contain propolis extracts. The methodology will depend on type compounds to be isolated, but when the extracts are supposed to be representative of the global composition of the bee glue, the extraction procedures should be standardized.<sup>67</sup>

Classical techniques for extraction of compounds from natural matrixes are based in the use of solvents with stirring and/or heating. Among the most commonly used techniques are soxhlet extraction, maceration, ultrasonic extraction, microwave extraction and the use of supercritical fluids.<sup>69</sup> Maceration extraction with continuous solvent renovation, using ethanol or an ethanol/water mixture is the most commonly method applied in propolis,<sup>68</sup> albeit other solvents like acetone or chloroform can be used.<sup>70</sup> It is an adequate extraction procedure to collect polyphenolic rich propolis extracts with a low wax content.<sup>71</sup> Ethanol at 70% was found to extract most of the active components of propolis.<sup>72</sup> For some applications, however the alcoholic content of the solvent is not desirable, so other alternatives have been tested. Water extractions showed a lower yield, with a extract content mainly composed by phenolic acids and characterized by the absence of the more lipophilic phenolic compounds.<sup>68</sup> Cunha *et al.* (2004)<sup>67</sup> compared the extraction yields using ethanol, with maceration, maceration with solvent renewal and soxhlet extraction (with and without light). The maximum yield was obtained with the soxhlet extraction, although the great amount of total phenolics was obtained by maceration.

### 1.5.3 Qualitative and quantitative analysis of propolis

The increased use of propolis in preparations for therapeutic purposes as well as biological activity studies made the qualitative and quantitative characterization of its constituents a priority.<sup>73</sup>

Chromatographic and spectrophotometric techniques are the most applied in the analysis of propolis extracts, although some analytical procedure can be complex and not always suitable for routine analysis.<sup>73,74</sup> Spectrophotometric methods are preferred when the quantification of compounds by classes with the same characteristics is favoured over individual quantification.<sup>74</sup> Through UV-VIS spectrophotometry it is possible to determine the overall amount of phenolic compounds with the Folin-Ciocalteu method,<sup>75</sup> the flavones and flavonols by the  $\text{AlCl}_3$  method and the flavanones and dihydroflavonols by the 2,4-dinitrophenylhydrazine (DNP) method.<sup>74</sup>

For detailed characterization and individual quantification of propolis compounds, the hyphenated techniques are the most appropriate ones: HPLC-DAD, LC-MS, LC-MS<sup>n</sup>, GC-MS, etc.<sup>71, 76,77</sup> The relatively polar nature of propolis constituents (in general they have several OH groups in their molecules), combined with soft ionization techniques compatible with liquid chromatography, made HPLC-DAD and LC-MS the favourite methods for analysis of propolis constituents.<sup>78</sup> In the identification of new compounds structure both mass spectrometry with electrospray ionization (ESI-MS)<sup>76,77</sup> in the negative or positive mode or nuclear magnetic resonance (NMR)<sup>79</sup> studies are the most adequate. Recently Wu *et al.* (2008) applied infrared spectroscopy with Fourier transform (FT-IR) to discriminate Chinese propolis extracts.<sup>80</sup>

Gas-chromatography (GC) and gas chromatography coupled with mass spectrometry (GC-MS) has been successfully applied to the analysis of essential oils of propolis, since MS allows the acquisition of molecular mass data and structural information together with the identification of compounds.<sup>81</sup> The process of extraction and isolation of the essential oil as well as the subsequent treatments are determinants in the final composition of the oil. Several methods can be used like hydrodistillation through a Clevenger system, distillation/extraction through a Likens-Nicckerson system, Soxhlet and maceration extraction with solvents and vacuum extraction.<sup>82</sup> However, the more polar compounds found in propolis are not volatile enough for direct

GC–MS analysis and so derivatization with BSTFA or through methylation was necessary to make the extracted compounds more volatile.<sup>68,83</sup>

#### 1.5.4 Antioxidant activity

The evaluation of the antioxidant activity is frequently employed during the characterisation of natural extracts in order to measure its potentiality as a source of bioactive compounds. The propolis, like other bee products such as honey, is a rich pool of plant secondary metabolites such as flavonoids and phenolic acids, thus presenting a high antioxidant capacity.<sup>67</sup>

In the literature, several methods can be found to evaluate the antioxidant properties of propolis. These methods, developed in accordance with the properties and mode of action displayed by phenolic compounds, were optimized for the determination of antioxidant capacity in complex matrices, such as food, biological samples and natural extracts.<sup>84,85</sup>

Antioxidant capacity assays can be roughly divided into two categories, depending on the chemical reaction involved: hydrogen atom transfer (HAT) and single electron transfer (SET) reaction based assays. HAT-based assays monitor competitive reaction kinetics (i.e., oxygen radical absorbance capacity assay (ORAC) and the total radical-trapping antioxidant parameter assay (TRAP). The SET-based assays involve one redox reaction with the oxidant (i.e., trolox equivalent antioxidant capacity assay, TEAC), ferric ion reducing antioxidant parameter assay (FRAP), DPPH-based assay, copper (II) reduction capacity, and total phenolics assay by Folin- Ciocalteu (FC).<sup>84</sup>

The ability of phenolic compounds to promote or inhibit lipid oxidation processes led to the development of a number of other methods, enzymatic and non-enzymatic inhibition of lipid peroxidation, using linolenic acid or other fatty acids as a model system.<sup>86</sup>

The limitations of antioxidant activity methodologies, such as high cost and interferences in the results,<sup>87</sup> increased the demand for finding new methods able to identify the profile and antioxidant properties of organic substances in natural matrices. The electrochemical techniques, which includes cyclic voltammetry, the square wave voltammetry and the differential pulse voltammetry are good candidates for such type of analysis and were already been applied in the characterization of various types of antioxidants including phenolic acids and flavonoids.<sup>88</sup> Phenolic compounds due to their

structural characteristics are electroactive substances, easily subject to electron transfer reactions, like oxidation and reduction, and therefore can be studied by electrochemical methods. Hydroxyl groups are oxidized through transference of two electrons, resulting, after the release of two  $H^+$ , in the corresponding quinone form. Due to stabilization of the ring, it is established that the greater the number of hydroxyl substituents on the aromatic ring, the lower the oxidation potential.<sup>89</sup>

The electrochemical techniques allow the evaluation of the antioxidant activity as well as the measurement of the total amount of antioxidant species present in the sample,<sup>88</sup> providing advantages over the other methods due to speed, simplicity and reduced consumption of analytical reagents. The ability to investigate functional characteristics of the samples without using specific reagents, since the redox process is based on the electrochemical behaviour of compounds and thus in their physicochemical properties, can be considered as a fast test for antioxidant capacity.<sup>8</sup> Regarding propolis research, some work have already been done using electrochemical methods, mainly in the evaluation of the antioxidant activity by amperometric flow injection analysis,<sup>90</sup> cyclic voltammetry,<sup>91</sup> and polarography.<sup>92</sup>

## 1.6 Current status of the propolis research in Portugal

In recent years, propolis attracted interest also among the Portuguese researchers. Most of the studies deal with chemical composition and some biological properties of geographically limited propolis samples. Moreira *et al.* (2008)<sup>93</sup> evaluated the phenolic content and the antioxidant potential of Portuguese propolis from two different regions, including the Northeast. Latter on the same research group presented a new study, within the same area, with pollen, sensorial and physicochemical analysis together with the microbiological characterization and antibacterial activity.<sup>94</sup> Also Miguel *et al.* (2010)<sup>95</sup> characterized the total phenols, flavonoids and antioxidant activity of propolis samples from three areas of Algarve. The effect of extractor solvent and propolis collection time was also addressed. The impact of the treatment against the Varroa mite was evaluated through the volatile profile in propolis samples from the south of Portugal, presenting the samples an intense rock-rose aroma supported by the presence of characteristic *Cistus* and labdanum oil volatile components.<sup>96</sup>

Within the biological activities investigated, the phenolic compounds showed strong antioxidant activity and decreased erythrocyte membrane fragility in hereditary

spherocytosis.<sup>97</sup> Propolis extracts also strongly suppress the proliferation of primary renal cancer cells in a concentration-dependent manner<sup>98</sup> and are able to exert moderate neuroprotection through the inhibition of caspase-3 activation.<sup>99</sup> Portuguese propolis extracts were also shown to have antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Candida albicans*, with the Gram-negative bacteria being more sensitive to the bee glue.<sup>100</sup>

The results within this study complement the investigation made from other researches, but most importantly allow systematizing and fulfilling a lack of information on Portuguese propolis, either due to the limit geographical sampling in former works, but mostly due to the increment in the amount of chemical information arising from the use of other technical approaches. Particularly relevant is the phenolic profile, which will enable an association of specific compounds with the bioactivity of propolis. Indeed, recent studies address the importance of this correlation due to the toxicity effects of the entire extracts that could mask future applications of this bee product.<sup>101</sup>

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## Chapter 2

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### **Quality standards and antioxidant activity of Portuguese propolis**





## Quality standards and antioxidant activity of Portuguese propolis<sup>\*,†</sup>

### Abstract

Propolis is a complex resinous product gathered by honeybees whose chemical composition is highly variable, depending strongly on the plant sources available to the bees at the site of collection. Propolis from the six regions of Portugal, including islands, was fully characterized in terms of physicochemical properties, phenolic composition and antioxidant activity, enabling the establishment of quality standards. Colour, frequently used as a commercial parameter, was accessed by the first time in propolis with the CIELAB system, and proposed as a quick and reliable method for quality recognition. The results attested the existence of two different types of propolis within the six regions under study. One, the poplar propolis most common in temperate regions, revealed a high content in phenolics and show high bioactivity. The other, with darker green colour is less rich in phenolics and generally less bioactive.

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\*Adapted from Falcão SI, Freire C, Vilas-Boas M. A proposal for quality standards and antioxidant activity of Portuguese propolis. *J. Am. Oil Chem. Soc.* **2013**, Accepted.

†The experimental details about the propolis sampling sites, phenolic compounds extraction, spectrophotometric determination of phenolic compounds and antioxidant activity are indicated in Appendices A, B, C and D respectively.

## 2.1 Introduction

Propolis is a complex resinous substance manufactured by honeybees (*Apis mellifera*, L.) from the collection of leaf buds and cracks in the bark of various plants that are transformed in the presence of bee enzymes.<sup>1</sup>

The bee glue plays an important role in the hive as building material to seal holes, to repair and strengthen the thin borders of the comb, and for making the entrance of the hive weather tight or easier to defend. Moreover, it is also a defensive substance with a biological action against microorganisms.<sup>2</sup>

This product is widely used in traditional medicine and has become the subject of intense pharmacological and chemical studies for the last 30 years.<sup>1,3</sup> Amongst its biological and pharmacological properties, the antibacterial, antifungal, antiviral, anti-inflammatory, hepatoprotective, antioxidant and antitumor power activities stands out.<sup>4</sup>

Propolis it is a hive product of complex standardization with a diversified chemical composition which is determined by its botanical origin, geography and climatic conditions at the site of collection.<sup>3</sup> It is of common knowledge that in Europe and in other temperate zones of the world, bees collect the resin mainly from poplar buds (*Populus* spp.) whereas in tropical regions, poplars are seldom cultivated and other plants are sources of the bee resin, like *Baccharis* spp. in Brasil and *Clusia* spp. in Venezuela.<sup>2,5</sup> Other species like Birches (*Betula* sp.), elms (*Ulmus* sp.), pine trees (*Pinus* spp.), oaks (*Quercus* spp.), chestnut trees (*Aesculus hippocastanum*) and eucalyptus (*Eucalyptus* spp.) are among the more important resin sources in the northern hemisphere.<sup>6</sup> The identification of plant origin of propolis in a certain area does not exclude the possibility of other plants contribute to the resin, although it is described that bees do not change the source if the species mentioned above are present.<sup>7</sup>

Despite of the possible differences in composition due to the different botanical origin, most propolis samples share considerable similarity in its general chemical nature. Mainly, it's composed by 50% resin (phenolic compounds) and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% of various other substances, including organic debris.<sup>1</sup> It has a pleasant smell and the colour may be cream, yellow, green, red, light or dark brown depending on the plant source and age. Some samples have a friable, hard texture, while other samples may be elastic and gummy.<sup>8</sup> Polyphenols (including flavonoids, phenolic acids and their esters) are the

most abundant and pharmacologically active molecules in propolis from temperate zones.<sup>2</sup>

Recently, some research was performed to study Portuguese propolis. Moreira *et al.* (2008)<sup>9</sup> have described the phenolic content and the antioxidant potential of two Portuguese propolis samples from different regions, northeast and interior center. Also Miguel *et al.* (2010)<sup>10</sup> evaluated total phenols, flavonoids and antioxidant activity of propolis samples of three main areas of Algarve. The effect of extractor solvent and propolis collection time was also evaluated. The phenolic composition of one sample from the Northeast of Portugal was extensively characterized in our previous work.<sup>11</sup> That work allowed the identification of thirty seven phenolic compounds, which included the typical phenolic acids and flavonoids found in propolis from temperate zones, but also several compounds which occurrence have never been referred in the literature. Some biological properties like the protection against free radical induced erythrocyte damage, inhibition of human renal cancer cell growth *in vitro*<sup>12</sup> and protection of the erythrocytes membrane of patients with hereditary spherocytosis were also reported.<sup>13</sup>

Although its geographic location indicates a poplar type propolis common of temperate zones, Portugal is a country of botanical diversity, which is reflected in its propolis chemical variety. The works previously published focused on specific regions and point mainly to the phenolic characterization, leaving the gap on many other country regions and lacking a complete set of data including the physicochemical parameters with significance for a future quality standardization of Portuguese Propolis with commercial purposes. In this context, this work aims to study the physicochemical parameters, including water, ash and waxes content, colour index, spectrophotometric determination of phenolic compounds, and antioxidant capacity of propolis samples from the different regions of continental Portugal and islands in order to establish criteria that allow the association of this beehive product with its geographical origin in an attempt to standardize the product, a key factor for its commercial valorization.

## 2.2 Experimental

### 2.2.1 Materials, reagents and solvents

The phenolic compounds naringenin, quercetin, chrysin were purchased from Sigma Chemical Co (St Louis, MO, USA). Analytical grade reagents like sodium carbonate, potassium hydroxide, Folin-Ciocalteu reagent, acetic acid, sulphuric acid and formic acid, ethanol, methanol, *n*-hexane were obtained from Panreac (Barcelona, Spain). Aluminium chloride, potassium ferricyanide, ferric chloride, trichloroacetic acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were from Sigma Chemical Co (St Louis, MO, USA) and 2,4-dinitrophenylhydrazine (DNP) from Fluka (Buchs, Switzerland). Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

### 2.2.2 Propolis samples

This work was performed in forty propolis samples from different regions of continental Portugal (north - N; central interior - CI; central coast - CC; south - S), Azores archipelago (A) and Madeira Island (M).

### 2.2.3 Water content

The water content was determined by drying a sample of raw propolis, for 2h, to constant weight in a conventional kiln at 105°C.<sup>14</sup>

### 2.2.4 Ash content

The ash content was determined by incineration of the raw propolis samples at  $600 \pm 15$  °C, based on the AOAC procedures (1995).

### 2.2.5 Wax content

The wax content was determined according to the method reported by Woiksy and Salatino (1998) with some modifications, as described below. A raw propolis

sample (500 mg) was treated with *n*-hexane in a Soxhlet for 3 h. The *n*-hexane extract was then evaporated under reduced pressure, and 20 mL of hot methanol was added to the previously weighed dry residue. The mixture was boiled and the methanolic phase was filtered through Whatman n°. 41 filter paper, into a previously weighed flask. The flask was cooled to 0°C and the content filtered again through a previously weighed Whatman n°. 41 filter paper. After drying to constant mass, the flask (with some solid residue on it) and the residue on the paper were weighed and the content of wax expressed as mass percentage.

### 2.2.6 Colour index

The colour of raw propolis was determined by means of a Minolta colourimeter Chroma meter CR-400 (Osaka, Japan) and recorded in  $L^*a^*b^*$  colour system. This system, CIELAB, consists of a lightness component ( $L^*$ ) and two chromatic components, where the  $a^*$  value represents green ( $-a$ ) to red ( $+a$ ) and the  $b^*$  value represents blue ( $-b$ ) to yellow ( $+b$ ) colours. Values of chroma ( $C^*ab$ ) obtained from  $a^*$  and  $b^*$  parameters were also given by the instrument. The colourimeter was calibrated using a standard white plate ( $L^* = 94.56$ ,  $a^* = -0.31$ ,  $b^* = 4.16$ ,  $C^*ab = 4.18$  and  $hab = 94.3$ ). The average values of three measurements were used.

### 2.2.7 Statistics

The statistic analysis was performed using SPSS version 18.0 program. All the analytical parameters were subject to a bivariate correlation test. Due to the deviation from normal distribution of some parameters, the analysis was performed using the Spearman coefficients with a two-tailed test of significance. The variable dependency is judged based on the significance level. A linear discriminant analysis (LDA) was used to assess the classification of the propolis samples from the different Portuguese regions, according to their physicochemical properties. In a first stage six region groups were defined considering the number of samples under analysis and the geographical location: north, central interior, central coast, south, Azores archipelago and Madeira island. For the second stage, only two groups were defined according to the cluster analysis used to explore the results. All independent variables were introduced together. A leaving-one-out cross-validation procedure was carried out to assess the model

performance. The hierarchical cluster analysis was performed with the between-groups linkage method, with standardized variables.

## 2.3 Results and discussion

The evolution of bees over time allowed them to select, from nature, the substances that could fulfil its needs. The same needs, in different locations, require the use of different raw materials, that combined, could guarantee the same results. It is then understandable, that albeit the flora diversity around the apiary, bees were able to find different plant sources that gives to propolis equivalent properties as a construction and defence material; at the same time, it gives rise to a variance in the chemical composition. Although the propolis samples in this study are from a temperate zone which fits in a typical poplar propolis composition,<sup>17</sup> significant differences were found in the various parameters studied, which relates not only with the flora specificity around the hive, but also with the geographic and climatic conditions of the site of collection. The extensive characterization of propolis from different regions of Portugal it is therefore relevant for its future standardization.

### 2.3.1 Physicochemical characterization

The physicochemical parameters for water content, ash, waxes and colour index of the bee glue samples under study are shown in Table 2.1. The propolis water content is affected by the manipulation conditions and time storage and should be considered as a quality parameter, since the high phenolic content of this product is likely to degrade with time. The obtained results were generally below 8% which is in accordance with the specification for raw propolis suggests by Cunha *et al.* (2004). The sample M1 from Madeira Island, however, falls clearly outside this criterion with a content of water above 13%. Indeed, the mean value for Madeira propolis (9%) is statistical different from the other regions using one-way analysis of variance. The islander origin of those samples could be the reason for this high level of humidity, nevertheless the samples from Azores islands, did not confirm the same pattern.

Analyzing the ash content, Table 2.1, the resulting values are in the range of 0.5-16%, with the minimum for the samples from central coast (CC1) and Azores (A3) and the maximum for the sample M1 from Madeira Island. For raw Brazilian *Baccharis*

propolis it is recommended a maximum ash content of 5 %, <sup>18</sup> but for poplar propolis there is no specific value, although the average content described in the literature is around 2%. <sup>19</sup> In general, the analyzed samples present an ash content of 1-2%, which is in accordance with the poplar type propolis, with the exceptions of Madeira Island M1 sample (16.1%) and central interior of Portugal with values around 4-5%. High ash content is frequently correlated to a high mineral salt content. <sup>14</sup>

The presence of waxes and fatty acids in propolis comes not only from beeswax added by bees during propolis manufacturing, but also from plants. <sup>20</sup> The literature refers a percentage of waxes of 30% in raw propolis <sup>1</sup> and more specific an interval of 20-35% in poplar propolis. <sup>19</sup> The collection method, the state of the bee colony, and the production season can also play an important role in the wax content. The demand for closing gaps (plastic screens) or the reduce availability of resinous sources around the apiaries can lead the bees to add more beeswax during propolis manufacturing to fulfill the needs. A high content of waxes, which are biologically inert, indicates low percentage of pharmacologically active compounds <sup>21</sup> what can affect the commercial value of the product. In the samples under study, the comparison between propolis collected by conventional scraping or through plastic screens did not reveal a specific pattern. However, the content of wax in the Portuguese propolis presents a large amplitude, ranging from 4% for sample CC4 (central coast) and 35% for the sample N1 (north), Table 2.1. The maximum limit for waxes is in accordance with the values referred for poplar type propolis. <sup>19</sup> Furthermore, 35% of the samples exhibit a lower wax content than the commonly observed, indicative of a low content in bio-inactive substances and therefore a higher commercial value; the richness and accessibility of resinous sources around the apiaries could be linked with this low wax content.

One of the first properties used to commercially describe propolis is its colour. The propolis from *Baccharis* spp, which have a good market value, is commercially labeled as “green propolis”, but other labels like “red propolis” are also implemented to specifically distinguish the geographical or flora origin. The propolis colours may vary from yellow, red, green, light or dark brown depending on the plant source and age <sup>8</sup> which depends on the chemical composition.

**Table 2.1** Physico-chemical parameters for Portuguese propolis

| Sample   | Water content (%) | Ash content (%) | Wax content (%) | Colour index |            |            |             |
|----------|-------------------|-----------------|-----------------|--------------|------------|------------|-------------|
|          |                   |                 |                 | <i>L</i> *   | <i>a</i> * | <i>b</i> * | <i>C*ab</i> |
| N1       | 3.6±0.0           | 0.7±0.0         | 35±1            | 50±0         | 2.2±0.1    | 27±0       | 27±0        |
| N2       | 6.3±0.0           | 2.1±0.2         | 15±1            | 41±0         | 2.6±0.1    | 16±0       | 16±0        |
| N3       | 3.8±0.1           | 1.5±0.1         | 18±2            | 55±0         | 12.0±0.5   | 36±1       | 38±1        |
| N4       | 2.2±0.4           | 0.8±0.1         | 33±3            | 55±3         | 7.7±0.1    | 38±1       | 39±1        |
| N5       | 2.9±0.2           | 7.0±0.6         | 15±3            | 53±2         | 3.4±0.2    | 33±0       | 33±1        |
| N6       | 4.4±0.1           | 1.1±0.0         | 25±2            | 43±0         | 12.5±0.1   | 29±0       | 31±0        |
| N7       | 4.2±0.2           | 1.6±0.2         | 16±1            | 45±1         | 1.8±0.1    | 26±0       | 26±0        |
| N8       | 2.0±0.0           | 0.8±0.1         | 19±1            | 50±2         | 8.2±0.9    | 30±2       | 31±3        |
| N9       | 3.1±0.1           | 0.9±0.3         | 19±2            | 52±0         | 5.2±0.0    | 37±0       | 37±0        |
| N10      | 5.1±0.5           | 1.7±0.0         | 30±2            | 28±0         | 0.3±0.1    | 3±0        | 3±0         |
| N11      | 3.6±0.3           | 2.5±0.0         | 34±0            | 40±1         | 3.3±0.0    | 22±1       | 22±1        |
| N12      | 4.2±0.1           | 1.7±0.2         | 20±4            | 48±1         | 3.3±0.1    | 23±0       | 23±0        |
| N13      | 3.8±0.0           | 1.3±0.2         | 27±5            | 42±1         | 3.3±0.1    | 17±0       | 17±0        |
| N14      | 9.0±0.0           | 1.0±0.0         | 19±2            | 55±0         | 1.9±0.0    | 28±0       | 28±0        |
| X(N)±SD  | 4.1±0.8           | 1.8±0.8         | 23±9            | 46±4         | 5±1        | 26±3       | 26±3        |
| CI1      | 3.6±0.3           | 1.9±0.02        | 32±1            | 33±0         | 2.3±0.1    | 11±0       | 11±0        |
| CI2      | 4.4±0.4           | 4.0±0.2         | 25±3            | 34±1         | 2.2±0.2    | 12±0       | 12±0        |
| CI3      | 4.5±0.3           | 4.2±0.0         | 13±4            | 32±0         | 1.6±0.0    | 8±0        | 8±0         |
| CI4      | 4.4±0.1           | 1.3±0.2         | 27±4            | 36±1         | -1.2±0.1   | 10±1       | 10±1        |
| X(CI)±SD | 4.2±0.6           | 2.9±0.3         | 24±6            | 33±1         | 2.0±0.2    | 10±1       | 10±1        |
| CC1      | 3.3±0.2           | 0.5±0.1         | 10±4            | 46±1         | 11±2       | 31±2       | 33±2        |
| CC2      | 3.6±0.4           | 0.8±0.2         | 11±1            | 48±0         | 6.5±0.3    | 26±0       | 27±0        |
| CC3      | 5.1±0.0           | 0.9±0.4         | 12±1            | 41±0         | 5.7±0.0    | 24±0       | 24±0        |
| CC4      | 4.2±0.2           | 1.1±0.2         | 4±0             | 47±1         | 7.7±0.0    | 34±1       | 35±1        |
| X(CC)±SD | 4.1±0.5           | 0.8±0.4         | 9±4             | 45±1         | 8±2        | 29±2       | 30±2        |
| S1       | 3.0±0.1           | 2.2±0.4         | 6±4             | 32±0         | 0.8±0.1    | 7±0        | 7±0         |
| S2       | 3.7±0.6           | 5.2±0.1         | 26±5            | 22±0         | 1.3±0.1    | 4±0        | 4±0         |
| S3       | 3.6±0.6           | 3.3±0.2         | 31±4            | 23±0         | 1.2±0.1    | 4±0        | 4±0         |
| S4       | 4.0±0.2           | 3.7±0.6         | 28±1            | 24±0         | 1.9±0.1    | 7±0        | 7±0         |
| X(S)±SD  | 3.5±0.9           | 3.6±0.8         | 23±8            | 25±1         | 1.3±0.2    | 6±0        | 6±0         |
| A1       | 3.4±0.2           | 1.3±0.1         | 29±4            | 40±1         | 6.1±0.2    | 24±1       | 25±1        |
| A2       | 5.6±0.2           | 1.3±0.0         | 10±2            | 40±1         | 2.7±0.3    | 21±1       | 22±1        |
| A3       | 4.9±0.2           | 0.5±0.2         | 10±3            | 45±1         | 6.8±0.2    | 29±1       | 30±2        |
| A4       | 4.1±0.3           | 1.0±0.1         | 22±1            | 42±0         | 3.8±0.2    | 25±0       | 25±0        |
| A5       | 4.6±0.2           | 0.8±0.0         | 26±3            | 42±0         | 6.5±0.5    | 25±0       | 26±0        |
| A6       | 4.2±0.1           | 1.0±0.1         | 20±2            | 42±0         | 5.4±0.1    | 25±0       | 26±0        |
| A7       | 3.8±0.1           | 1.3±0.0         | 22±2            | 38±1         | 4.9±0.3    | 21±1       | 21±1        |
| A8       | 3.9±0.5           | 2.1±0.2         | 6±2             | 42±1         | 3.8±0.1    | 24±0       | 24±0        |
| A9       | 4.4±0.1           | 1.6±0.1         | 14±2            | 41±0         | 3.8±0.1    | 23±0       | 23±1        |
| A10      | 4.2±0.1           | 1.2±0.2         | 16±2            | 44±0         | 4.2±0.2    | 26±0       | 26±0        |
| A11      | 4.1±0.2           | 1.4±0.1         | 12±1            | 41±0         | 4.4±0.2    | 24±0       | 24±1        |
| X(A)±SD  | 4.3±0.8           | 1.2±0.4         | 17±8            | 42±2         | 4.8±0.8    | 24±3       | 25±3        |
| M1       | 13.6±0.3          | 16.1±0.2        | 17±1            | 24±1         | 1.9±0.1    | 5±0        | 6±0         |
| M2       | 7.1±0.3           | 1.0±0.1         | 31±2            | 35±0         | 6.0±0.1    | 16±0       | 18±0        |
| M3       | 5.3±0.2           | 1.8±0.4         | 16±2            | 27±0         | 1.5±0.2    | 8±0        | 8±0         |
| X(M)±SD  | 8.7±0.5           | 6.2±0.5         | 21±2            | 29±0         | 3.1±0.2    | 10±0       | 10±0        |

Each value is the mean ± standard deviation. X- Average; SD – Standard deviation.

Portuguese propolis presented different colours depending on its origin. Through visual observation, the samples from north (excluding N12 and N13), central coast and Azores archipelago show a colour that varies from light orange to dark orange. The samples from central interior, south and Madeira Island were darker, with colour from brown to dark brown. Sample N12, N13 and CI4 present however a distinct dark green colour. Considering that there is no official method for propolis colour identification and, to the best of our knowledge, there are no literature references concerning to this parameter, the CIELAB colour system<sup>22</sup> was applied as an attempt to a better differentiation of this parameter in propolis, independent of the observer. The CIELAB system is an approximately uniform three-dimensional colour scale, extensively used to evaluate food colours. In this scale, the coordinate  $L^*$  measures the degree of lightness, and  $a^*$  e  $b^*$  are the horizontal axes defining green/red (-60/60) and blue/yellow (-60/60) chromaticity, respectively);<sup>23</sup> Table 2.1 shows the results for the colour density,  $C^*ab$ .

In the present study, 93% of the samples from north, 100% from central coast and 90% from Azores Archipelago with a orange colour present high  $L^*$  values, above 40, which means lighter colours, while samples from the other regions have lower values which represents darker colours. For the  $a^*$  coordinate, in general the values were low. Samples N3, N6 and CC1 have the highest values which are correlated to a reddish tone propolis. Propolis with darker colour, like central interior and samples M1 and M2 from Madeira island possess values of  $a^*$  below 3, moving the colour to the green direction. The sample CI4 from central interior even shows a negative value for  $a^*$  parameter, displaying a more greenish tone. Analyzing the  $b^*$  coordinate results, Table 2.1, most of the bee glue from north, central coast and Azores Archipelago have values above 20, what combining with the red tone observed in parameter  $a^*$  will justify the orange colour observed in these samples. Once again, the darker samples from central interior, south and Madeira island, present the lower values of  $b^*$ . The parameter  $C^*$  can be used to quantify the overall colour component, brightness, whose value derive from the  $a^*$  and  $b^*$  coordinates. Once again, and supported by the statistical analysis using one way analysis of variance, the Portuguese propolis can be divided in groups, with samples from north, central coast and Azores archipelago representing the brighter group with  $C^*ab$  values above 20 and samples from central interior, south and Madeira island samples, a fade colour group with lower  $C^*ab$  values, below 10. In general, the colour of Portuguese propolis becomes darker and colourless as the collection sites are southern.

### 2.3.2 Phenolic content

Propolis from temperate regions is well known for its richness in phenolic compounds, especially phenolic acids, flavonoids (flavones, flavanones and dihydroflavonols) and their methylated and ester derivatives, which are responsible for its biological properties.<sup>11</sup>

The composition in total phenols and flavonoids in the propolis samples under study were achieved by several spectrophotometric procedures after the hydro-alcoholic extraction. These rapid, simple and reliable methods allowed the quantification of active compounds within groups with equal or similar chemical structures.<sup>24</sup> While total phenolic content were obtained through the colourimetric Folin–Ciocalteu method, the flavonoid content was achieved by two different spectrophotometric approaches: for flavones and flavonols its carbonyl and hydroxyl groups react with aluminium chloride to form a yellow stable complex that shows an electronic band at  $\lambda = 415$  nm. Although flavanones and dihydroflavonols compounds, which are commonly present in a large amount in temperate propolis, reacts as well with aluminium chloride; the absorbance of the resulting complexes are too low, and consequently another method needs to be applied.<sup>25</sup> The compound 2,4-dinitrophenylhydrazine (DNP) reacts with ketones and aldehydes groups to form stable coloured hydrazones complexes. Because flavonoids with the C2-C3 double bond, like flavones, flavonols and isoflavones, do not react with DNP,<sup>25</sup> it is an excellent method for the quantification of flavanones and dihydroflavonols and thus differentiate these major flavonoid groups.

The definition of quality control parameters for propolis requires the standardization of analytical methodologies combined with an agreement in the standards used for calibration. Total phenolics calculated with the Folin-Ciocalteu method for natural extracts are frequently expressed in equivalent terms of gallic acid, but as noticed by Miguel *et al.* (2010), the corresponding equivalent values are significantly lower when comparing with other standards like pinocembrin. This variation is a consequence of the differences in the extinction coefficient of the complex formed during the spectrophotometric analysis. For example, for a 100 ppm flavonoid solution the absorbance of the  $\text{AlCl}_3$ -galangin complex at  $\lambda = 415$  nm is 0.358, while for the complex  $\text{AlCl}_3$ -quercetin is 0.451.<sup>25</sup> Therefore, the absorbance for a propolis sample expressed in equivalent terms of galangin will be higher than if expressed in terms of quercetin, invalidating any comparison between different published works, if the

standards do not match. Additionally, the use of gallic acid in propolis analysis seems unreasonable since this compound is frequently absent and do not represent the propolis composition, rich in flavonoids with a distinctive chemical structure. A calibration curve should mimic the matrix as close as possible and it seems reasonable to use a mixture of chemical species that represent each phenolic group in propolis, rather than just a phenolic compound.

In this context, for total phenolics analysis, we opted for the use of a mixture of caffeic acid, galangin and pinocembrine as phenolic acid, flavone/flavonol and flavanone/dihydroflavonol representatives, respectively. For the individual flavonoid analysis the same corresponding standard was used: galangin for flavone/flavonol and pinocembrine for flavanone/dihydroflavonol. Additionally, the total phenolics were also calculated using a mixture of galangin/pinocembrin as standard to allow the comparison with previous publish results.<sup>17</sup>

The yields of phenolic compounds extracted from propolis using 80% ethanol/water (balsam), total phenols and flavonoids content are shown in Table 2.2. Balsam content presents some variability between samples, ranging from 37% to 89%, although the majority fits above 60%. Samples N11, N12, CI3 and from Madeira Island, M1 and M2, are the lower exceptions which could means represent less commercial interest.

The different phenolic and flavonoid compounds concentrations varies widely even within the same region suggesting different floral contributions for the resin; this is in accordance with other published works<sup>10</sup> where quantitative differences were found in total phenolic and flavonoids of propolis collect in the same region. The majority of the samples reveal a total phenolic content between 200-400 mg/g of the ethanolic extract which is comparable with the concentrations found in other European and Chinese propolis.<sup>26,27</sup> However, there are 30% of samples that follow below this level, in particular those from central interior, south and Madeira Island, where a minimum value of 7 mg/g extract was found. In general, the ethanolic extracts of propolis from central coast, north and Azores Archipelago are the richest in phenolic content with values around 327 mg/g of extract.

**Table 2.2** Balsam content and phenolic composition in the ethanolic extracts of Portuguese propolis

| Sample   | Balsam content (%)    | Total phenolics (mg/g extract) <sup>a</sup> | Total phenolics (mg/g extract) <sup>b</sup> | Flavones/Flavonols (mg/g extract) <sup>c</sup> | Flavanones/Dihydroflavonols (mg/g extract) <sup>d</sup> | EC <sub>50</sub> DPPH <sup>e</sup> (mg/mL) <sup>e</sup> | Reducing Power (mg/g extract) <sup>a</sup> |
|----------|-----------------------|---------------------------------------------|---------------------------------------------|------------------------------------------------|---------------------------------------------------------|---------------------------------------------------------|--------------------------------------------|
| N1       | 64±2                  | 316.1±0.1                                   | 404.5±0.1                                   | 80.7±0.1                                       | 105±4                                                   | 0.018±0.001                                             | 7(±0)x10 <sup>2</sup>                      |
| N2       | 67±1                  | 233.2±0.1                                   | 266.1±0.2                                   | 97.7±0.2                                       | 109±3                                                   | 0.017±0.000                                             | 6(±0)x10 <sup>2</sup>                      |
| N3       | 80±1                  | 415.5±0.2                                   | 545.8±0.2                                   | 89.3±0.1                                       | 77±2                                                    | 0.015±0.001                                             | 9(±0)x10 <sup>2</sup>                      |
| N4       | 58±1                  | 357.1±0.2                                   | 461.3±0.4                                   | 114.1±0.1                                      | 118±4                                                   | 0.020±0.009                                             | 7(±0)x10 <sup>2</sup>                      |
| N5       | 73±2                  | 277.9±0.1                                   | 342.5±0.1                                   | 85.4±0.1                                       | 90±6                                                    | 0.021±0.000                                             | 9(±0)x10 <sup>2</sup>                      |
| N6       | 73±0                  | 223.9±0.2                                   | 259.5±0.3                                   | 80.3±0.1                                       | 86±1                                                    | 0.017±0.001                                             | 5(±0)x10 <sup>2</sup>                      |
| N7       | 62±1                  | 337.6±0.2                                   | 420.4±0.3                                   | 80.2±0.1                                       | 106±9                                                   | 0.016±0.000                                             | 6(±0)x10 <sup>2</sup>                      |
| N8       | 71±1                  | 320.0±0.1                                   | 394.9±0.1                                   | 114.2±0.1                                      | 114±3                                                   | 0.021±0.006                                             | 6(±0)x10 <sup>2</sup>                      |
| N9       | 67±1                  | 271.9±0.2                                   | 340.5±0.2                                   | 54.5±0.1                                       | 86±2                                                    | 0.019±0.001                                             | 7(±0)x10 <sup>2</sup>                      |
| N10      | 67±7                  | 273.5±0.2                                   | 328.9±0.3                                   | 55.3±0.0                                       | 86±8                                                    | 0.022±0.001                                             | 2(±0)x10 <sup>2</sup>                      |
| N11      | 49±6                  | 357.8±0.1                                   | 462.3±0.1                                   | 37.7±0.6                                       | 111±4                                                   | 0.010±0.000                                             | 13(±1)x10 <sup>2</sup>                     |
| N12      | 37±2                  | 191.2±0.2                                   | 208.3±0.3                                   | 56.5±0.1                                       | 112±4                                                   | 0.013±0.004                                             | 13(±0)x10 <sup>2</sup>                     |
| N13      | 58±4                  | 158.0±0.2                                   | 165.3±0.3                                   | 51.1±0.2                                       | 48±2                                                    | 0.026±0.004                                             | 4(±0)x10 <sup>2</sup>                      |
| N14      | 85±6                  | 202.3±0.1                                   | 229.5±0.1                                   | 26.9±0.1                                       | 84±6                                                    | 0.022±0.002                                             | 5(±1)x10 <sup>2</sup>                      |
| X(N)±SD  | 7(±1)x10 <sup>1</sup> | 281.2±0.6                                   | 345.0±0.9                                   | 73.1±0.8                                       | 10(±2)x10 <sup>1</sup>                                  | 0.018±0.012                                             | 7(±1)x10 <sup>2</sup>                      |
| CI1      | 60±5                  | 291.0±0.1                                   | 364.5±0.1                                   | 59.5±0.1                                       | 57±5                                                    | 0.008±0.000                                             | 13(±1)x10 <sup>2</sup>                     |
| CI2      | 57±2                  | 127.1±0.2                                   | 118.0±0.3                                   | 38.6±0.1                                       | 60±3                                                    | 0.057±0.007                                             | 1(±0)x10 <sup>2</sup>                      |
| CI3      | 47±2                  | 120.0±0.2                                   | 119.4±0.2                                   | 51.2±0.0                                       | 94±3                                                    | 0.065±0.003                                             | 1(±0)x10 <sup>2</sup>                      |
| CI4      | 59±0                  | 165.7±0.2                                   | 183.0±0.2                                   | 44.5±0.4                                       | 59±1                                                    | 0.053±0.001                                             | 1(±0)x10 <sup>2</sup>                      |
| X(CI)±SD | 56±6                  | 176.0±0.3                                   | 196.2±0.5                                   | 48.4±0.5                                       | 67±6                                                    | 0.045±0.007                                             | 4(±1)x10 <sup>2</sup>                      |
| CC1      | 76±1                  | 376.3±0.2                                   | 488.1±0.2                                   | 91.1±0.1                                       | 101±1                                                   | 0.014±0.000                                             | 10(±0)x10 <sup>2</sup>                     |
| CC2      | 67±4                  | 344.3±0.1                                   | 430.1±0.1                                   | 99.0±0.1                                       | 114±5                                                   | 0.018±0.001                                             | 4(±0)x10 <sup>2</sup>                      |
| CC3      | 76±1                  | 268.7±0.4                                   | 323.2±0.6                                   | 84.2±0.3                                       | 85±3                                                    | 0.017±0.000                                             | 7(±0)x10 <sup>2</sup>                      |
| CC4      | 83±0                  | 322.0±0.3                                   | 405.3±0.4                                   | 97.6±0.2                                       | 91±2                                                    | 0.013±0.000                                             | 8(±1)x10 <sup>2</sup>                      |
| X(CC)±SD | 75±4                  | 327.8±0.5                                   | 411.7±0.7                                   | 93.0±0.4                                       | 98±6                                                    | 0.015±0.001                                             | 7(±1)x10 <sup>2</sup>                      |
| S1       | 70±8                  | 337.0±0.1                                   | 428.1±0.1                                   | 95.5±0.1                                       | 109±6                                                   | 0.017±0.000                                             | 7(±0)x10 <sup>2</sup>                      |
| S2       | 54±5                  | 90.6±0.2                                    | 66.4±0.3                                    | 34.5±0.1                                       | 33±5                                                    | 0.064±0.004                                             | 1(±0)x10 <sup>2</sup>                      |
| S3       | 51±1                  | 189.1±0.1                                   | 202.3±0.2                                   | 41.3±0.2                                       | 92±8                                                    | 0.050±0.002                                             | 1(±0)x10 <sup>2</sup>                      |
| S4       | 60±0                  | 157.4±0.3                                   | 166.8±0.5                                   | 26.4±0.2                                       | 63±2                                                    | 0.045±0.001                                             | 2(±0)x10 <sup>2</sup>                      |
| X(S)±SD  | 59±9                  | 193.5±0.3                                   | 215.0±0.6                                   | 49.4±0.3                                       | 8(±1)x10 <sup>1</sup>                                   | 0.044±0.004                                             | 3(±0)x10 <sup>2</sup>                      |
| A1       | 73±2                  | 385.4±0.1                                   | 490.9±0.1                                   | 91.0±0.1                                       | 92±3                                                    | 0.012±0.002                                             | 7(±0)x10 <sup>2</sup>                      |
| A2       | 76±1                  | 468.1±0.2                                   | 610.9±0.3                                   | 72.2±0.1                                       | 104±3                                                   | 0.008±0.000                                             | 8(±0)x10 <sup>2</sup>                      |
| A3       | 80±2                  | 314.5±0.2                                   | 398.5±0.4                                   | 63.3±0.1                                       | 99±5                                                    | 0.009±0.001                                             | 11(±0)x10 <sup>2</sup>                     |
| A4       | 65±3                  | 309.6±0.3                                   | 390.5±0.4                                   | 66.7±0.3                                       | 75±3                                                    | 0.013±0.000                                             | 9(±0)x10 <sup>2</sup>                      |
| A5       | 70±1                  | 322.1±0.4                                   | 400.6±0.6                                   | 56.1±0.1                                       | 94±5                                                    | 0.011±0.001                                             | 9(±0)x10 <sup>2</sup>                      |
| A6       | 71±4                  | 373.7±0.3                                   | 475.4±0.4                                   | 73.5±0.1                                       | 92±5                                                    | 0.021±0.006                                             | 12(±0)x10 <sup>2</sup>                     |
| A7       | 65±1                  | 342.5±0.1                                   | 432.7±0.1                                   | 62.6±0.2                                       | 87±3                                                    | 0.014±0.004                                             | 9(±0)x10 <sup>2</sup>                      |
| A8       | 61±0                  | 296.6±0.4                                   | 367.3±0.5                                   | 57.1±0.3                                       | 83±2                                                    | 0.012±0.001                                             | 9(±1)x10 <sup>2</sup>                      |
| A9       | 69±2                  | 285.6±0.3                                   | 352.5±0.4                                   | 50.3±0.1                                       | 73±2                                                    | 0.013±0.000                                             | 7(±0)x10 <sup>2</sup>                      |
| A10      | 89±4                  | 164.9±0.2                                   | 176.5±0.3                                   | 28.6±0.0                                       | 53±2                                                    | 0.025±0.001                                             | 1(±0)x10 <sup>2</sup>                      |
| A11      | 84±1                  | 271.0±0.2                                   | 325.3±0.3                                   | 33.0±0.0                                       | 80±1                                                    | 0.021±0.001                                             | 3(±0)x10 <sup>2</sup>                      |
| X(A)±SD  | 73±8                  | 321.3±0.8                                   | 401.9±1.2                                   | 59.5±0.5                                       | 8(±1)x10 <sup>1</sup>                                   | 0.014±0.008                                             | 8(±1)x10 <sup>2</sup>                      |
| M1       | 42±3                  | 7.27±0.01                                   | 9.9±0.0                                     | 5.2±0.3                                        | 45±1                                                    | 0.093±0.004                                             | 1(±0)x10 <sup>2</sup>                      |
| M2       | 60±2                  | 170.7±0.5                                   | 181.3±0.7                                   | 89.4±0.2                                       | 56±6                                                    | 0.027±0.001                                             | 5(±0)x10 <sup>2</sup>                      |
| M3       | 44±0                  | 156.9±0.2                                   | 163.8±0.3                                   | 51.1±0.2                                       | 104±5                                                   | 0.032±0.002                                             | 2(±0)x10 <sup>2</sup>                      |
| X(M)±SD  | 49±4                  | 111.6±0.5                                   | 118.3±0.8                                   | 48.6±0.4                                       | 69±8                                                    | 0.051±0.004                                             | 3(±0)x10 <sup>2</sup>                      |

Values expressed as: <sup>a</sup> caffeic acid: galangin: pinocembrine (1:1:1) equivalents; <sup>b</sup> galangin: pinocembrine (1:1); <sup>c</sup> as galangin equivalents; <sup>d</sup> pinocembrine equivalents. Each value is the mean ± standard deviation. X- Average; SD – Standard deviation.

Concerning the flavonoid content, flavones/flavonols and flavanones/dihydroflavonols were present in the range of 5-114 mg/g of extract and 35-118 mg/g of extract respectively, while the total flavonoids range from 50 to 232 mg/g. Flavonoids in a range of 64-158 mg/g extract are reported for some countries of Europe

(Bulgaria and Hungary),<sup>26</sup> while a recent work by Bonvehí and Gutiérrez (2011) described flavones/flavonols and flavanones/dihydroflavonols contents of 26-62 mg/g of extract and 55-100 mg/g of extract in ethanolic propolis extract from northeastern Spain.

Our results are in agreement with those obtained for the Basque country propolis, with a higher contribution of flavanones/dihydroflavonols to the flavonoid content.

**Table 2.3** Phenolic content in ethanolic extracts of Portuguese propolis expressed in g/100g of raw propolis.

| Sample   | Total phenolics <sup>a</sup> | Total phenolics <sup>b</sup> | Flavones/Flavonols <sup>c</sup> | Flavanones/Dihydroflavonols <sup>d</sup> | Total flavonoids |
|----------|------------------------------|------------------------------|---------------------------------|------------------------------------------|------------------|
| N1       | 20.23±0.01                   | 25.89±0.01                   | 5.16±0.01                       | 6.7±0.2                                  | 11.9±0.2         |
| N2       | 15.62±0.01                   | 17.83±0.01                   | 6.55±0.01                       | 7.3±0.2                                  | 13.8±0.2         |
| N3       | 33.24±0.01                   | 43.67±0.02                   | 7.15±0.01                       | 6.2±0.1                                  | 13.3±0.1         |
| N4       | 20.71±0.01                   | 26.76±0.02                   | 6.62±0.01                       | 6.8±0.3                                  | 13.4±0.3         |
| N5       | 20.29±0.01                   | 25.00±0.01                   | 6.24±0.01                       | 6.6±0.4                                  | 12.8±0.4         |
| N6       | 16.27±0.01                   | 18.87±0.02                   | 5.84±0.01                       | 6.2±0.1                                  | 12.1±0.5         |
| N7       | 20.93±0.01                   | 26.06±0.02                   | 4.97±0.01                       | 6.6±0.5                                  | 11.5±0.2         |
| N8       | 22.72±0.01                   | 28.04±0.01                   | 8.11±0.01                       | 8.1±0.2                                  | 16.2±0.2         |
| N9       | 18.22±0.01                   | 22.81±0.02                   | 3.65±0.01                       | 5.7±0.1                                  | 9.4±0.1          |
| N10      | 18.33±0.01                   | 22.04±0.02                   | 3.70±0.00                       | 5.8±0.5                                  | 9.5±0.5          |
| N11      | 17.53±0.00                   | 22.65±0.01                   | 1.85±0.03                       | 5.4±0.2                                  | 7.3±0.2          |
| N12      | 7.07±0.01                    | 7.71±0.01                    | 2.09±0.01                       | 4.1±0.1                                  | 6.2±0.1          |
| N13      | 9.16±0.01                    | 9.59±0.02                    | 2.96±0.01                       | 2.8±0.1                                  | 5.7±0.1          |
| N14      | 17.20±0.01                   | 19.51±0.01                   | 2.29±0.01                       | 7.1±0.5                                  | 9.4±0.5          |
| X(N)±SD  | 18.40±0.04                   | 22.60±0.05                   | 4.80±0.04                       | 6±1                                      | 11±1             |
| CI1      | 17.46±0.01                   | 21.87±0.01                   | 3.57±0.01                       | 3.4±0.3                                  | 7.0±0.3          |
| CI2      | 7.24±0.01                    | 6.73±0.02                    | 2.20±0.01                       | 3.4±0.1                                  | 5.6±0.1          |
| CI3      | 5.64±0.01                    | 5.61±0.01                    | 2.41±0.00                       | 4.4±0.1                                  | 6.8±0.1          |
| CI4      | 9.69±0.01                    | 10.70±0.01                   | 2.60±0.03                       | 3.5±0.1                                  | 6.1±0.1          |
| X(CI)±SD | 10.01±0.02                   | 11.23±0.02                   | 2.69±0.03                       | 3.7±0.4                                  | 6.4±0.4          |
| CC1      | 28.60±0.01                   | 37.09±0.02                   | 6.93±0.01                       | 7.7±0.3                                  | 14.6±0.3         |
| CC2      | 23.07±0.01                   | 28.82±0.01                   | 6.63±0.01                       | 7.6±0.2                                  | 14.3±0.2         |
| CC3      | 20.42±0.03                   | 24.57±0.04                   | 6.40±0.02                       | 6.4±0.2                                  | 12.8±0.2         |
| CC4      | 26.63±0.02                   | 33.52±0.03                   | 8.08±0.02                       | 7.6±0.0                                  | 15.7±0.0         |
| X(CC)±SD | 24.68±0.04                   | 31.00±0.06                   | 7.01±0.03                       | 7.3±0.4                                  | 14.4±0.4         |
| S1       | 23.59±0.01                   | 29.97±0.01                   | 6.68±0.01                       | 7.6±0.4                                  | 14.3±0.4         |
| S2       | 4.89±0.01                    | 3.58±0.01                    | 1.86±0.01                       | 1.9±0.3                                  | 3.7±0.3          |
| S3       | 9.64±0.01                    | 10.32±0.01                   | 2.11±0.01                       | 4.7±0.4                                  | 6.8±0.4          |
| S4       | 9.40±0.02                    | 9.96±0.03                    | 1.58±0.01                       | 3.8±0.1                                  | 5.4±0.1          |
| X(S)±SD  | 11.88±0.02                   | 13.46±0.03                   | 3.06±0.02                       | 4.5±0.7                                  | 7.6±0.7          |
| A1       | 28.13±0.01                   | 35.84±0.01                   | 6.64±0.01                       | 6.7±0.2                                  | 13.4±0.2         |
| A2       | 35.58±0.01                   | 46.42±0.02                   | 5.48±0.01                       | 7.9±0.2                                  | 13.4±0.2         |
| A3       | 25.16±0.02                   | 31.88±0.03                   | 5.07±0.01                       | 7.9±0.4                                  | 13.0±0.4         |
| A4       | 20.13±0.02                   | 25.38±0.02                   | 4.34±0.02                       | 4.9±0.2                                  | 9.2±0.2          |
| A5       | 22.54±0.03                   | 28.04±0.04                   | 3.93±0.01                       | 6.5±0.4                                  | 10.5±0.4         |
| A6       | 26.53±0.02                   | 33.75±0.03                   | 5.22±0.01                       | 6.6±0.4                                  | 11.8±0.4         |
| A7       | 22.26±0.01                   | 28.12±0.01                   | 4.07±0.01                       | 5.7±0.2                                  | 9.7±0.2          |
| A8       | 18.03±0.02                   | 22.33±0.03                   | 3.47±0.02                       | 5.0±0.1                                  | 8.5±0.1          |
| A9       | 19.71±0.02                   | 24.33±0.03                   | 3.47±0.01                       | 5.0±0.1                                  | 8.5±0.1          |
| A10      | 14.68±0.02                   | 15.71±0.02                   | 2.55±0.00                       | 4.7±0.2                                  | 7.2±0.2          |
| A11      | 22.77±0.02                   | 27.32±0.02                   | 2.78±0.00                       | 6.7±0.1                                  | 9.5±0.1          |
| X(A)±SD  | 23.23±0.06                   | 29.01±0.09                   | 4.27±0.03                       | 6.2±0.8                                  | 10.4±0.8         |
| M1       | 4.17±0.00                    | 4.68±0.01                    | 0.22±0.01                       | 1.9±0.1                                  | 2.1±0.1          |
| M2       | 10.24±0.03                   | 10.88±0.04                   | 5.37±0.01                       | 3.4±0.4                                  | 8.7±0.4          |
| M3       | 6.90±0.01                    | 7.21±0.01                    | 2.25±0.01                       | 4.6±0.2                                  | 6.8±0.2          |
| X(M)±SD  | 7.11±0.03                    | 7.59±0.04                    | 2.61±0.02                       | 3.3±0.4                                  | 5.9±0.4          |

Expressed as equivalents of: <sup>a</sup> caffeic acid; galangin: pinocembrin (1:1:1); <sup>b</sup> galangin: pinocembrin (1:1); <sup>c</sup> galangin equivalents; <sup>d</sup> pinocembrine equivalents. Each value is the mean ± standard deviation, *n* = 3. X- Average; SD – Standard deviation.

The sample M2 from Madeira Island shows again a very poor content in total flavonoids with only 50 mg/g of extract.

Popova *et al.* (2007) proposed several minimum values for the quality assessment of the biologically active substances of poplar type propolis, based on the analysis of samples from north and central Europe: 45% for the balsamic content (propolis ethanolic extract), 21% for total phenolics; 4% for total flavones and flavonols; and 4% for total flavanones and dihydroflavonols (all values are expressed for raw propolis).

In terms of balsam content, only three samples under studied are just below this minimum, however, when comparing the other three parameters, and using the data from Table 2.2 to convert the results in g/100g of raw propolis, less than 50% of the samples obey simultaneously to all the criteria, Table 2.3. Again, this deviation is mostly clear in samples from central interior, south and Madeira, suggesting effective differences in the phenolic composition of those Portuguese propolis, when compared to the north and central Europe propolis. Nevertheless, the samples N11 and A11, despite the low flavonoid content, show significant values in the total phenolics, indicating a propolis composition richer in phenolic acids and other similar compounds, rather than flavonoids.

### 2.3.3 Antioxidant activity

Recent studies proved that formation of reactive oxygen and nitrogen species have been implicated in the oxidative deterioration of food products, as well as in several human pathologies caused by oxidative stress processes.<sup>28</sup> Propolis, a rich source of phenolic compounds, can act as antioxidant with high potentialities in scavenging free radicals associated with various diseases.<sup>26</sup> This property can then be used to assess the influence of the chemical composition and phyto-geographic diversity of the site of collection in the bioactive quality of propolis.

In the past years several methods have been used for the determination of antioxidant capacity *in vitro*.<sup>28</sup> In the present work, DPPH scavenging capacity and reducing power were chosen for bioactivity evaluation of propolis. DPPH is a stable nitrogen-centred free radical, the colour of which changes from violet to yellow upon reduction by either the process of hydrogen or electron donation. Substances which are able to perform this

reaction can be considered as antioxidants and therefore radical scavengers.<sup>29</sup> The EC<sub>50</sub> values are shown in Table 2.2 and range between 0.008 mg/mL for propolis samples CI1 and A2, to 0.093 mg/mL for Madeira Island sample M1. A mixture of caffeic acid: galangin: pinocembrine (1:1:1) was tested as reference, resulting in EC<sub>50</sub> of 0.007 mg/mL. This value is close to the strongest effect observed in the present study, confirming the high capacity of propolis to capture free radicals. The samples from north, coast and Azores archipelago show, however, best scavenging effects with 50% inhibition in the interval of 0.01 to 0.03 mg/mL, while the samples from central interior, south and Madeira Island require a higher amount of extract, 0.05 to 0.09 mg/mL, to archive the same result. Samples CI1 from Guarda and S1 from Aljezur appear as outliers within each region, with EC<sub>50</sub> values of 0.008 mg/mL and 0.017 mg/mL, respectively, which indicate a higher antioxidant activity. In fact, and excluding the samples just mentioned, the results for samples CI2 and CI3 are in agreement with the value found by Moreira *et al.* (2008)<sup>9</sup> for a sample of the same region, Fundão (0.052 mg/mL). Furthermore, in Algarve region, samples S2 and S3 from Aljezur and S4 from Moncarapacho fit the same pattern of low scavenging effect as observed by Miguel *et al.* (2010)<sup>10</sup> in samples from central Algarve, collected in the spring.

The capability for reducing Fe(III) is another bioactivity evaluation method, working as an indicator of electron- donating activity, which is an important mechanism of phenolic antioxidant action.<sup>30</sup> These results, express again as caffeic acid: galangin: pinocembrine (1:1:1) equivalents, are shown in Table 2.2 for the Portuguese propolis ethanolic extracts under study. The reducing power varies widely according to the origin of the samples, with 30% of the samples presenting a reducing power above 400 mg/g of extract. The maximum activity is observed for sample N11 with a reducing power of 757 mg/g of extract while the minimum of 110 mg/g of extract is observed for the sample M1 from Madeira Island. As observed for the radical scavenging effect, the higher reducing power is also found for the samples of north, coast and Azores archipelago, with samples CI1 and S1 revealing high values when compared with those from the same regions, suggesting a different phenolic composition.

The antioxidant activity and the phenolic composition are in fact closely related, as demonstrated on Table 2.4. The Spearman's correlation coefficients indicate a significant relationship between these parameters, in particular with the total phenolics content. Although in less extent, the presence of flavonoids can justify some of the bioactive potentialities of propolis, with no differences found between flavones or

flavanones, unlike what was observed in the work of Miguel *et al.* (2010). This disagreement with our results could be explained by the higher number of samples and their diversity. Quality parameters like ash, wax and water contents show to be independent of the antioxidant properties, and therefore must be considered individually when defining quality parameters of propolis. On the other hand, the correlation observed between colour and the phenolic composition or antioxidant activity can be an important tool to explore for a quick analysis in propolis quality.

**Table 2.4** Spearman's correlations between the propolis individual parameters and the phenolic composition/bioactivity

|                             | <b>Total phenolics</b> | <b>Total Flavonoids</b> | <b>Radical scavenging</b> | <b>Reducing power</b> |
|-----------------------------|------------------------|-------------------------|---------------------------|-----------------------|
| Ash                         | -0.542 <sup>a</sup>    | -0.557 <sup>a</sup>     | 0.321 <sup>b</sup>        | -0.340 <sup>b</sup>   |
| Wax                         | -0.360 <sup>b</sup>    | -0.403 <sup>b</sup>     | 0.205 NS                  | -0.073 NS             |
| Water                       | -0.299 NS              | -0.257 NS               | 0.142 NS                  | -0.275 NS             |
| C*ab                        | 0.592 <sup>a</sup>     | 0.630 <sup>a</sup>      | -0.399 <sup>b</sup>       | 0.479 <sup>b</sup>    |
| Balsam contente             | 0.720 <sup>a</sup>     | 0.669 <sup>a</sup>      | -0.366 <sup>b</sup>       | 0.287 NS              |
| Total phenolics             | -                      | 0.848 <sup>a</sup>      | -0.625 <sup>a</sup>       | 0.593 <sup>a</sup>    |
| Flavones/flavonols          | 0.781 <sup>a</sup>     | 0.930 <sup>a</sup>      | -0.419 <sup>a</sup>       | 0.408 <sup>a</sup>    |
| Flavanones/dihydroflavonols | 0.811 <sup>a</sup>     | 0.918 <sup>a</sup>      | -0.452 <sup>a</sup>       | 0.372 <sup>b</sup>    |

Spearman's correlation significance levels: NS not significant. <sup>a</sup>Correlation is significant at  $P < 0.01$ .

<sup>b</sup>Correlation is significant at  $P < 0.05$ .

### 2.3.4 Propolis standards

The differences discussed on the physicochemical properties described above clearly demonstrate the existence of diversity in the Portuguese propolis quality. Although the number of samples under study for each Portuguese region is reduced, we applied the discriminant analysis to identify a correlation between the geographical origin of propolis and its quality. The results for classification with the obtained discrimination model are presented in Figure 2.1, where two discriminant functions with acceptable prevision were established.

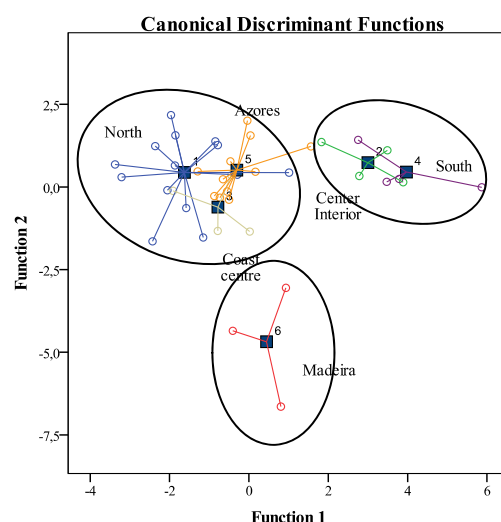


**Figure 2.1** Two-dimensional plot for the first and second discriminant canonical functions considering the geographical origin of Portuguese propolis.

The Wilks' Lambda test showed that both the canonical discriminant functions were significant ( $p < 0.001$ ). The second function, associated mainly with ash and water contents, enables the differentiation of the samples from Madeira Islands, whereas the first function, described by the colour and bioactivity parameters, is more significant for the differentiation of the other regions. However, the model sets distinguish just three groups, not allowing a correct separation of samples from central interior and south, with positive scores of function 1, and from the north, central coast and Azores, all with negative or close to zero scores. These results indicate that the groups set for discrimination cannot be based on the geographical origin, since some of those regions present similar propolis characteristics. To define how different or similar are the samples, without considering the geographical condition, we applied a hierarchical cluster analysis using the between-groups linkage method. These reveal that the Portuguese propolis could be separated in two major groups: one with samples from north, central coast and Azores (excluding samples N10 to N13) and the other with samples from central interior, south and Madeira (excluding samples S1 and M1). Sample M1 is clearly an outlier, not fitting in any of the two former groups. The presence of samples from the north in the second group and a sample from the south in the first group confirm the observation of other authors, where within the same region it is possible to find significant differences in the propolis quality).<sup>10</sup> To validate this observation we redone the linear discriminant analysis addressing each sample to one of the two type of propolis, which result in just one canonical function able to clearly

separate the two groups, Figure 2.2. All the original grouped and cross-validation grouped samples were correctly classified.

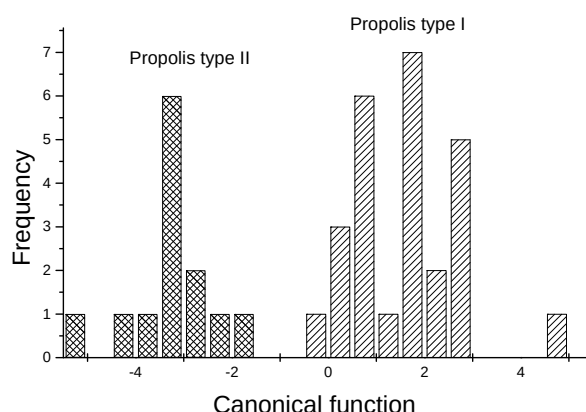


**Figure 2.2** Histogram for the discriminant canonical function considering the type of Portuguese propolis.

These results lead us to propose two separate standards of quality parameters for each type of propolis from Portugal. The minimum and maximum values proposed in Table 2.5 were determined using the same statistical procedures of Popova *et al.* (2007)<sup>17</sup>, where the minimum values were set using the 20% empiric quantiles and the maximum values were set using the 80% empiric quantiles.

**Table 2.5** Standard proposal for Portuguese propolis

|                               | Propolis Type I | Propolis Type II | Literature              |
|-------------------------------|-----------------|------------------|-------------------------|
| Ash content (%)               | Max. 2          | Max. 4           | -                       |
| Wax content (%)               | Max 25          | Max. 31          | Max. 25 <sup>(19)</sup> |
| Water Content (%)             | Max. 5          | Max 5            | -                       |
| $L^*$                         | Min. 40         | Min.23           | -                       |
| $a^*$                         | Min. 2          | Min. 1           | -                       |
| $b^*$                         | Min. 23         | Min. 4           | -                       |
| $C^*ab$                       | Min. 23         | Min 4            | -                       |
| Balsam content (%)            | Min. 65         | Min. 45          | Min. 45 <sup>(27)</sup> |
| Total phenolics               | Min. 18         | Min. 6           | Min. 21 <sup>(17)</sup> |
| (% in raw propolis)           |                 |                  |                         |
| Flavones/flavonols            | Min. 3          | Min. 2           | Min. 4 <sup>(17)</sup>  |
| (% in raw propolis)           |                 |                  |                         |
| Flavanones/ihydrofavonols     | Min. 5          | Min. 3           | Min. 4 <sup>(17)</sup>  |
| (% in raw propolis)           |                 |                  |                         |
| EC <sub>50</sub> DPPH (mg/mL) | Max. 0.02       | Max. 0.06        | -                       |
| Reducing power (mg/g extract) | Min. 600        | Min. 96          | -                       |

In both cases and for each variable, the outliers were removed before statistical treatment. Portuguese propolis type I presents similar maximum and minimum values of poplar propolis of north and central Europe confirmed by the phenolic profile described in our previous work.<sup>11</sup> However, the Portuguese propolis shows a higher minimum for the balsam content, which does not reflect in the phenolic composition. In fact, the minimum value for total phenolics and flavones/flavonol contents are slightly lower than those observed in the north and central Europe. The smaller value of total phenolics, as described above, is artificial due to the different standards used in our calibration curve, but the lower amount of flavones/flavonols seems to be a characteristic of Portuguese poplar propolis. Portuguese type II propolis is significantly different from poplar type propolis, with distinct chromatic properties and a higher amount of wax. In regions where bees found limitations in resin sources to prepare propolis, for instance the inexistence of poplar trees, it is reasonable to expect an increment in the wax content, which can explain this observation.<sup>1</sup> The lower content in phenolic compounds is also significant, both in the total phenolics and in the flavonoid content, with high impact in the bioactive properties of propolis. If no specific bioactivity were found, these characteristics will reduce the commercial value of this type of propolis. The floral origin of this propolis is still unknown and cannot be assessed based only on the physicochemical parameters, but requires the evaluation of the other components such as the phenolic profile; this work is now under investigation.

## **2.4 Concluding remarks**

This study reports a description of propolis from the different regions of Portugal in terms of its physicochemical properties, total phenolic composition and bioactive properties. A clear identification of two different types of propolis was established: the one typical of temperate zones from poplar and another type with different colour and with lower phenolic content. A full set of quality standards is proposed for both types, including some parameters never defined before in propolis from temperate climates. CIELAB colour system is applied by the first time in propolis and is proposed as a quick and reliable technique for quality evaluation of propolis, due to the relation between colour and phenolic composition or bioactivity. The evaluation of total phenolic composition in propolis must follow the same standardized methods, but there is also a need to express the results using the same chemical standards to

enable comparison of results. For that, specific mixtures of phenolics, which resemble the typical composition of propolis, were proposed for total phenolics, flavones/flavonols and flavanones/dihydroflavonols assays.

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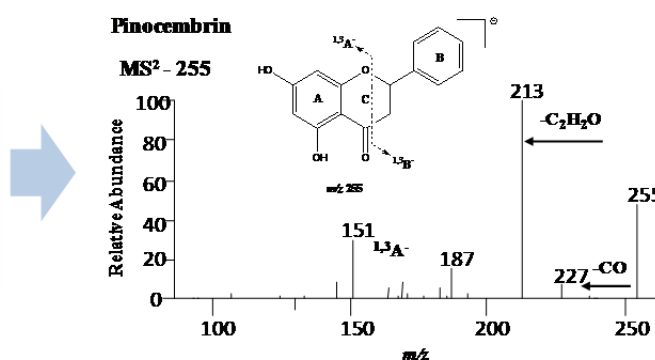
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## Chapter 3

### Phenolic characterization of Northeast Portuguese propolis: usual and unusual compounds





## Phenolic characterization of Northeast Portuguese propolis: usual and unusual compounds<sup>\*,†</sup>

### Abstract

In this study, an ethanolic extract from Portuguese propolis was prepared, fractionated by high-performance liquid chromatography, and the identification of the phenolic compounds was done by electrospray mass spectrometry in the negative mode. This technical approach allowed the identification of 37 phenolic compounds, which included not only the typical phenolic acids and flavonoids found in propolis from temperate zones but also several compounds in which its occurrence have never been referred to in the literature. Four of the novel phenolic compounds were methylated and/or esterified or hydroxylated derivatives of common poplar flavonoids, although six peculiar derivatives of pinocembrin/pinobanksin, containing a phenylpropanoic acid derivative moiety in their structure, were also identified. Furthermore, the Portuguese propolis sample was shown to contain a p-coumaric ester derivative dimer.

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\*Adapted from Falcão S, Vilas-Boas M, Estevinho LM, Barros C, Domingues MRM, Cardoso SM. Phenolic characterization of Northeast Portuguese Propolis: usual and unusual compounds. *Anal. Bioanal. Chem.* **2010**, 396, 887-897.

†The experimental details about the phenolic compounds extraction and the HPLC analysis are indicated in Appendices B and E respectively.

### 3.1 Introduction

Propolis or bee glue is a sticky dark-coloured substance produced from the collected buds or exudates of plants (resin) by bees (*Apis mellifera*, L.). The resin is masticated, salivary enzymes are added and the partially digested material is mixed with beeswax and used in the hive to seal the walls, strengthen the borders of combs and embalm dead invaders. Propolis is a building material, but it is also the most important “chemical weapon” of bees against pathogen microorganisms.<sup>1</sup>

Propolis has been used as remedy by humans since ancient times.<sup>2</sup> In the last years, this product has been the subject of intensive studies, highlighting its biological and pharmacological properties, such as the antimicrobial,<sup>3</sup> antioxidative, antihepatotoxic,<sup>4</sup> antitumoral, anti-inflammatory,<sup>5</sup> anti-HIV-1,<sup>6</sup> anti-neurodegenerative<sup>7</sup> and antituberculosis.<sup>8</sup> Propolis was also tested as food preserver due to its bactericidal and bacteriostatic properties.<sup>9</sup> Furthermore, most of its components are natural constituents of food and recognized as safe substances.<sup>9</sup>

The successful medical applications of propolis led to an increased interest in its chemical composition.<sup>10</sup> In general, resin comprising flavonoids and related phenolic acids represent approximately half of the propolis constituents, while beeswax, volatiles and pollen represent approximately 30%, 10% and 5%, respectively.<sup>11</sup> Still, the chemical composition of the bee glue is extremely dependent on the plants found around the hive, as well on the geographic and climatic characteristics of the site. Buds from *Populus* species are the main source of resins in Europe and North America propolis (“poplar type” propolis).<sup>2</sup> Alternatively, in regions where these plants are not native, other species such as *Clusia* in Cuba and *Baccharis* in Brazil are used as resin sources, increasing its diversity and complexity.<sup>12</sup> Less commonly, species such as *Betula*, *Ulmus*, *Pinus*, *Quercus*, *Salix* and *Acacia* are also used.<sup>13</sup>

More than 300 constituents have been identified in different types of propolis,<sup>11</sup> being the phenolics the most abundant compounds. In propolis from temperate zones, the most frequently reported phenolic components include the flavonoids pinocembrin, galangin and chrysin and the phenolic acids caffeic acid, ferulic acid and the cinnamic acid.<sup>2</sup> Instead, the propolis from tropical zones, in particular those from the Southeastern region of Brazil, were shown to be rich in prenylated phenylpropanoids,<sup>11</sup> although non-typical flavonoids from ‘poplar type’ propolis, such as kaempferide and isosakuranetin, have been found.<sup>14,15</sup> Moreover, Cuban propolis has recently caught the

attention of scientists because its peculiar enrichment in polyisoprenylated benzophenones turns it chemically distinct from both the European and the Brazilian bee glue.<sup>16</sup>

A large number of analytical methods have been used for the analysis of phenolic compounds in propolis, including spectrophotometry,<sup>17</sup> thin-layer chromatography,<sup>18</sup> gas chromatography-mass spectrometry (GC-MS),<sup>19</sup> high-performance liquid chromatography (HPLC),<sup>20</sup> liquid chromatography-mass spectrometry (LC-MS),<sup>21-23</sup> electrospray ionization-mass spectrometry (ESI-MS) and electrospray ionization-tandem mass spectrometry (ESI-MS<sup>n</sup>)<sup>2</sup> capillary electrophoresis<sup>25</sup> and nuclear magnetic resonance (NMR).<sup>26</sup> Among these methods, mass spectrometry (MS) with electrospray ionization (ESI) is one of the most used techniques in the research of natural products, as it is able to analyze complex mixtures because of its high selectivity. This method also provides adequate structural information and thus, it facilitates the structural identification of unknown compounds.

The chemical composition of European propolis was determined by GC-MS,<sup>11,19</sup> LC-MS, ESI-MS.<sup>21-24</sup> Also Moreira *et al.* (2008)<sup>27</sup> have recently described the phenolic content and the antioxidant potential of Portuguese propolis from two different regions, including the Northeast, but still, their phenolic components were not elucidated. In this context, this paper aims to characterize the phenolic composition of Northeast Portuguese propolis by ESI-MS. For that, the ethanolic extract was fractionated by reversed-phase HPLC and the major phenolic compounds of each fraction were identified by detailed analysis of their fragmentation pathways. New compounds were found in propolis for the first time and their structure will be assigned in the present study.

## 3.2 Experimental

### 3.2.1 Materials, reagents and solvents

The phenolic standards naringenin, quercetin, kaempferol, pinocembrin, chrysin, caffeic acid, ferulic acid, cinnamic acid and *p*-coumaric acid were purchased from Sigma Chemical Co (St Louis, MO, USA). Genkwanin and luteolin were obtained from Lancaster Synthesis (Morecambe, England). The analytical grade reagents formic acid and ethanol were obtained from Panreac (Barcelona, Spain) and methanol and

acetonitrile with HPLC purity were purchased from Lab-Scan (Lisbon, Portugal). Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

### 3.2.2 Propolis samples

Three distinct propolis samples were collected in the fall of 2007 from *Apis mellifera* hives located in different apiaries in the Northeast of Portugal. They were obtained after the honey extraction, by scratching the hive walls and frames, following by the removal of debris of wood and bees. These propolis samples were then stored at -20 °C until analysis.

### 3.2.3 Mass spectrometry analysis by ESI-MS and ESI-MS<sup>n</sup>

The freeze-dried HPLC fractions were dissolved in methanol and directly injected into the ESI source by means of a syringe pump, at flow rate of 8 µL/min. Studies were performed in the negative mode using a Linear Ion trap LXQ (ThermoFinnigan, San Jose, CA, USA). Typical ESI conditions were: nitrogen sheath gas 30 psi, spray voltage 4.7 kV, capillary temperature 350°C, capillary voltage -7.0 V and tube lens voltage -71.8 V. CID-MS/MS and MS<sup>n</sup> experiments were performed on mass-selected precursor ions using standard isolation and excitation configuration. The collision energy used was between 15 and 40 (arbitrary units). Data acquisition was carried out with Xcalibur<sup>®</sup> data system (ThermoFinnigan, San Jose, CA, USA).

## 3.3 Results and discussion

### 3.3.1 HPLC fractionation

The chromatographic profiles at 280 nm were similar for all the ethanolic extracts studied, independent of the sample location, consistent with the relationship between the phenolic profile and the surrounding apiary flora.<sup>2</sup> The representative chromatogram is shown in Figure 3.1. As also represented in this figure, the HPLC analysis allowed the collection of thirty five fractions, suggesting that the propolis samples contained a large variety of phenolic compounds. Due to this high diversity, the identification of the compounds in each HPLC fraction was made by electrospray

ionization mass spectrometry (ESI-MS and MS<sup>n</sup>), even when its retention time matched with that of an available reference compound.

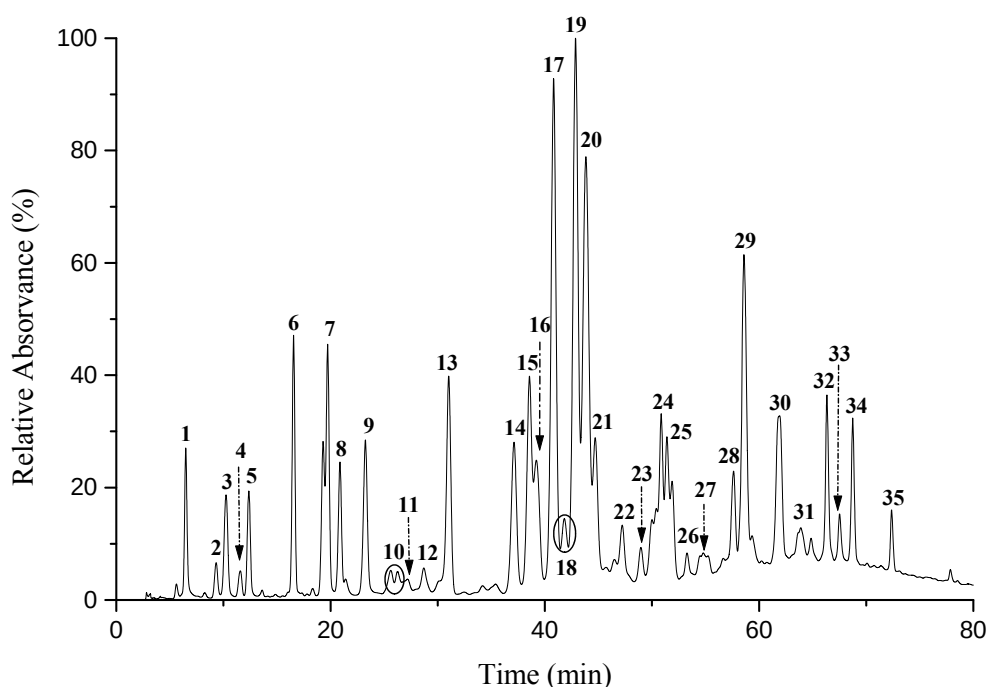


**Figure 3.1** Chromatographic profile at 280 nm of the ethanolic extract obtained from the Portuguese propolis samples. The numbers in the figure correspond to the fractions collected for further analysis by ESI-MS<sup>n</sup>.

### 3.3.2 ESI-MS and MS<sup>n</sup>

These analyses were carried out in the negative ion mode because of its higher sensitivity in the detection of the distinct classes of phenolic compounds.<sup>28</sup> Table 3.1 summarizes the data obtained for each of the analyzed fractions.

In general, the identification of the corresponding compound was based on the search of the [M-H]<sup>-</sup> deprotonated molecule together with the interpretation of its MS<sup>n</sup> fragmentations. Still, when standards were available, the identification of phenolic compounds was determined by comparison of the ESI-MS<sup>n</sup> data to that of the standards. The structure of other phenolic compounds was also confirmed by the MS-MS data published in the literature, as indicated in Table 3.1. With respect to these latter compounds, it should be noted that their fragmentation pathways will not be discussed

in detail in the present work, as these were previously reported.<sup>21-24</sup> The chemical structures of these compounds are shown in Figure 3.2.

**Table 3.1** Identification of HPLC-eluting fractions from propolis extract and correspondence with results obtained by mass spectrometry analysis.

| Fraction number | Retention time (min) | [M-H] <sup>-</sup> | MS <sup>2</sup> (% base peak)                              | Compound                                                                  |
|-----------------|----------------------|--------------------|------------------------------------------------------------|---------------------------------------------------------------------------|
| 1               | 6.48                 | 179                | 135                                                        | Caffeic acid*                                                             |
| 2               | 9.32                 | 135                | 107 (100), 91 (25)                                         | 3,4 – Dihydroxy vinylbenzene <sup>++</sup>                                |
| 3               | 10.2                 | 163                | 119                                                        | <i>p</i> -Coumaric acid*                                                  |
| 4               | 11.6                 | 193                | 177 (16), 149 (47), 133 (100)                              | Ferulic acid*                                                             |
| 5               | 12.4                 | 193                | 177 (16), 149 (47), 133 (100)                              | Isoferulic acid <sup>+</sup> (23)                                         |
| 6               | 16.5                 | 207                | 163 (60), 102 (100)                                        | 3,4-Dimethyl-caffeic acid (DMCA) <sup>+</sup> (23)                        |
| 7               | 19.5                 | 285                | 267 (100), 252 (13), 239 (29), 165 (1)                     | Pinobanksin-5-methyl-ether <sup>+</sup> (23)                              |
| 8               | 20.9                 | 177                | 163 (100), 119 (15)                                        | <i>p</i> -Coumaric acid methyl ester <sup>+</sup> (23)                    |
| 9               | 23.2                 | 271                | 253 (100), 225 (19), 151 (10)                              | Pinobanksin <sup>+</sup> (23)                                             |
| 10              | 25.9                 | 269                | 254 (94), 227 (100), 165 (48)                              | Pinocembrin-5-methyl-ether <sup>+</sup> (23)                              |
| 11              | 27.2                 | 269                | 225 (100), 151 (17)                                        | Apigenin* (22)                                                            |
| 12              | 28.7                 | 267                | 252 (100), 223 (12)                                        | Chrysin-5-methyl-ether <sup>+</sup> (23)                                  |
| 13              | 31                   | 299                | 284                                                        | Unknown <sup>+++</sup>                                                    |
|                 |                      | 173                | 129                                                        | Cinnamylidenacetic acid <sup>+</sup> (23)                                 |
|                 |                      | 329                | 314 (100), 299 (30), 285 (3)                               | Unknown <sup>+++</sup>                                                    |
| 14              | 37.1                 | 247                | 179 (100), 135 (13)                                        | Caffeic acid isoprenyl ester <sup>+</sup> (22, 23)                        |
| 15              | 38.6                 | 247                | 179 (100), 135 (13)                                        | Caffeic acid isoprenyl ester (isomer) <sup>+</sup> (22, 23)               |
| 16              | 39.2                 | 269                | 179 (100), 135 (60)                                        | Caffeic acid benzyl ester <sup>+</sup> (22, 23)                           |
| 17              | 40.8                 | 255                | 213 (100), 211 (55), 151 (35)                              | Pinocembrin*                                                              |
| 18              | 41.8                 | 285                | 179 (8), 145 (40), 139 (100)                               | Unknown                                                                   |
| 19              | 42.7                 | 253                | 209 (100), 151 (5)                                         | Chrysin*                                                                  |
| 20              | 43.8                 | 313                | 271 (20), 253 (100)                                        | Pinobanksin-3- <i>O</i> -acetate <sup>+</sup> (23)                        |
| 21              | 44.7                 | 283                | 179 (100), 135 (24)                                        | Caffeic acid phenylethyl ester (CAPE) <sup>+</sup> (22, 23)               |
| 22              | 47.2                 | 283                | 268                                                        | Chrysin-6-methyl-ether <sup>+</sup> (23, 29)                              |
| 23              | 48.9                 | 231                | 163 (100), 119 (12)                                        | <i>p</i> -Coumaric acid isoprenyl ester <sup>+</sup> (23)                 |
| 24              | 50.4                 | 295                | 179 (100), 135 (80)                                        | Caffeic acid cinnamyl ester <sup>+</sup> (23)                             |
| 25              | 51.6                 | 327                | 271 (10), 253 (100)                                        | Pinobanksin-3- <i>O</i> -propionate <sup>+</sup> (23)                     |
|                 |                      | 599                | 555 (40), 437 (100), 255 (<1)                              | Unknown                                                                   |
|                 |                      | 403                | 385 (57), 293 (100), 281 (25), 267 (31), 255 (61)          | Unknown <sup>+++</sup>                                                    |
|                 |                      | 433                | 415 (48), 323 (13), 309 (100), 255 (3)                     | Unknown <sup>+++</sup>                                                    |
|                 |                      | 461                | 443 (74), 419 (16), 401 (65), 351 (100), 291 (43), 253 (2) | Unknown <sup>+++</sup>                                                    |
|                 |                      | 266                | 285 (92), 267 (70), 252 (<1), 239 (100), 165 (<1)          | Unknown <sup>+++</sup>                                                    |
| 26              | 53.3                 | 369                | 254 (100), 251 (57), 165 (30)                              | Unknown <sup>+++</sup>                                                    |
| 27              | 54.8                 | 269                | 271 (4), 253 (100)                                         | Pinobanksin-3- <i>O</i> -butyrate or isobutyrate <sup>+</sup> (23)        |
| 28              | 57.6                 | 341                | 402 (16), 385 (8), 281 (89), 267 (100)                     | Unknown <sup>+++</sup>                                                    |
| 29              | 58.6                 | 377                | 359 (35), 335 (21), 333 (44), 317 (60)                     | Unknown                                                                   |
|                 |                      | 475                | 433 (31), 415 (100), 400 (10), 253 (2)                     | Unknown <sup>+++</sup>                                                    |
|                 |                      | 355                | 271 (3), 253 (100)                                         | Pinobanksin-3- <i>O</i> -pentanoate or 2-methylbutyrate <sup>+</sup> (23) |
| 30              | 61.9                 | 355                | 271 (3), 253 (100)                                         | Unknown <sup>+++</sup>                                                    |
| 31              | 64.3                 | 403                | 297 (15), 283 (19), 271 (17), 253 (100)                    | Unknown <sup>+++</sup>                                                    |
|                 |                      | 565                | 455 (8), 417 (17), 283 (100), 269 (25)                     | Unknown <sup>+++</sup>                                                    |
| 32              | 66.3                 | 293                | 275 (16), 249 (8), 209 (7), 197 (39), 185 (100)            | Fatty acid <sup>+</sup> (31)                                              |
|                 |                      | 297                | 295 (70), 279 (100), 237 (24), 183 (21), 171 (36)          | Fatty acid <sup>+</sup> (31)                                              |
| 33              | 67.5                 | 471                | 423 (100), 405 (30), 393 (53)                              | Unknown                                                                   |
| 34              | 68.7                 | 471                | 423 (100), 405 (30), 393 (53)                              | Unknown                                                                   |
| 35              | 72.4                 | 339                | 183                                                        | Unknown                                                                   |

\* Confirmed with standard; <sup>+</sup> Confirmed with references; <sup>++</sup> Confirmed with MS<sup>n</sup> fragmentation, <sup>+++</sup> Compounds to be elucidated in the present study.

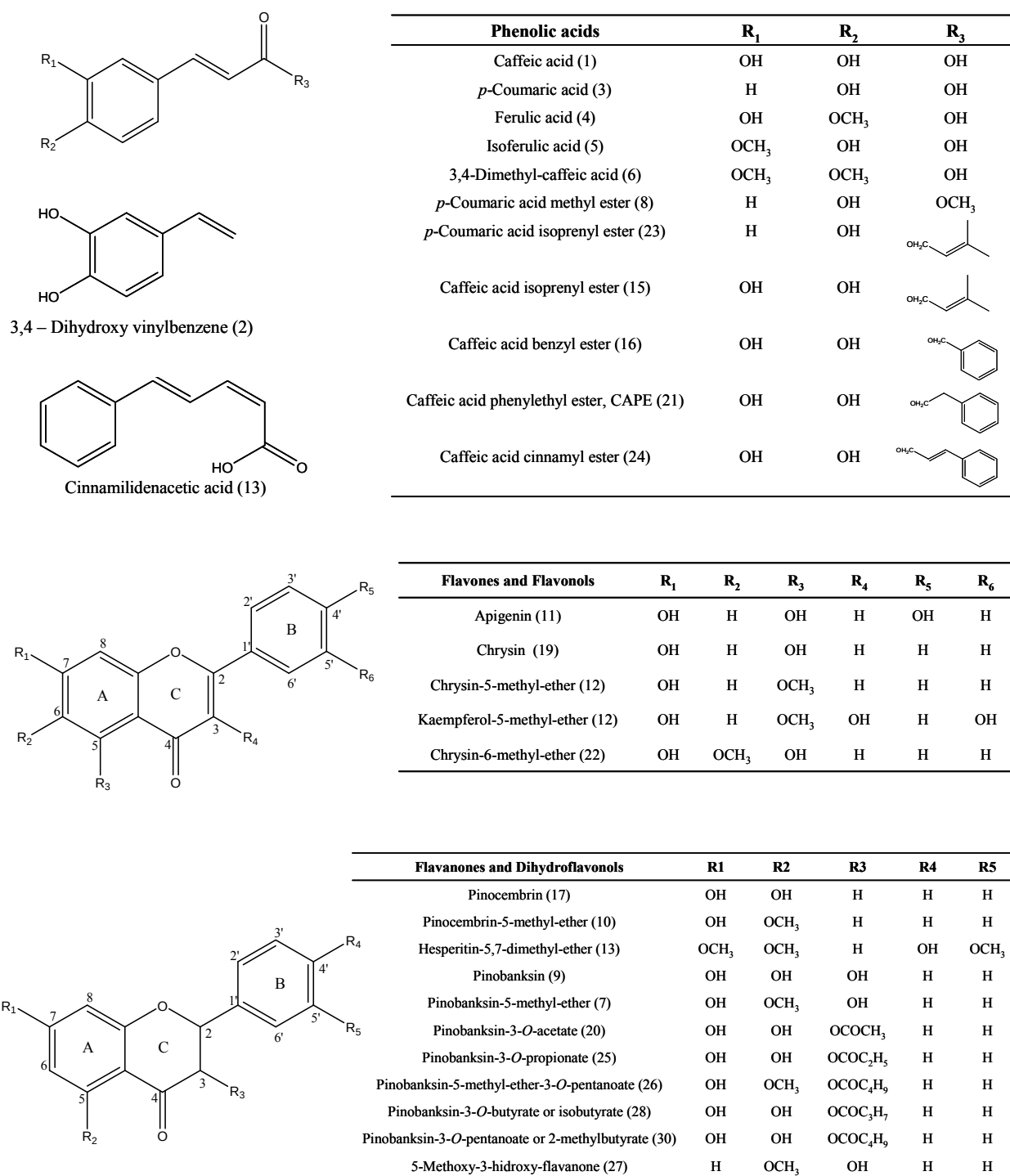


**Figure 3.2** Structures of several phenolic acids and flavonoids isolated from the Portuguese propolis. The numbers in parentheses denote the number of the fraction according to Figure 3.1. Compounds corresponding to the fractions 12, 13, 26 and 27 were elucidated for the first time in this study.

Overall, the mass spectrometry analysis of the HPLC fractions allowed the identification of thirty seven phenolic compounds, Table 3.1, which included phenolic acids and flavonoids already reported in propolis, but also new compounds. Due to the

high number of phenolic components, their identification will be discussed according to the mentioned classes.

### 3.3.2.1 Phenolic acids

Simple phenolic acids eluted in the first thirteen minutes of the HPLC program, corresponding to fractions 1 to 5, Table 3.1 and Figure 3.2. The comparison of the ESI-MS/MS data with that of standards and/or that described in the literature allowed the identification of caffeic acid ( $m/z$  179, fraction 1), 3,4 – dihydroxy vinylbenzene ( $m/z$  135, fraction 2), *p*-coumaric acid ( $m/z$  163, fraction 3), ferulic and isoferulic acids ( $m/z$  193, fractions 4 and 5). These phenolic compounds demonstrated a common fragmentation pattern, with a loss of the carboxyl group ( $\text{CO}_2$ , -44 Da). Besides the simple phenolic acids, some esterified and/or methylated derivatives of these compounds were also found in the Portuguese propolis sample. These included five esters of caffeic acid, which are commonly found in high amounts in the phenolic extracts of propolis from temperate zones.<sup>2,23</sup> Namely, the caffeic acid isoprenyl ester and its isomer ( $m/z$  247, fractions 14 and 15, respectively), the caffeic acid benzyl ester ( $m/z$  269, fraction 16), the caffeic acid phenylethyl ester ( $m/z$  283, fraction 21) and the caffeic acid cinnamyl ester ( $m/z$  295, fraction 24). All the caffeic acid esters had a major fragment at  $m/z$  179, that corresponds to the phenolic acid, and another product ion at  $m/z$  135, resulting from the loss of  $\text{CO}_2$  from the phenolic acid. This data is in accordance with the fragmentation pathways reported for caffeic acid esters derivatives.<sup>22</sup> Besides the esters derivatives, a methylated derivative of this phenolic acid, the 3,4-dimethyl-caffeic acid (DMCA), was identified in fraction 6 (ion at  $m/z$  207). The  $\text{MS}^2$  spectrum of this ion showed a fragment at  $m/z$  163 and a main product ion at  $m/z$  102 (-105 Da), resulting from the loss of  $\text{CO}_2$  and of the two methoxy substituents together with the  $\text{CO}_2$  group, respectively.

*p*-Coumaric acid esters were identified by comparison of the MS-MS data to that described in the literature.<sup>23</sup> This approach allowed detecting *p*-coumaric acid methyl ester ( $m/z$  177, fraction 8) and *p*-coumaric acid isoprenyl ester ( $m/z$  231, fraction 23). As for the caffeic acid esters discussed above, the MS-MS spectra of these ions had a fragment corresponding to the phenolic acid (ion at  $m/z$  163) and other resulting from the loss of a  $\text{CO}_2$  group from the phenolic acid (ion at  $m/z$  119).

### 3.3.2.2 Flavonoids

The flavonoids that have been previously described in propolis and were also detected in this Portuguese extract were identified by comparison of the MS-MS data of the compound to that of the standards and/or by comparison to literature data. It is worthy to note that as the distinct classes of flavonoids differ in their pattern of substitution, which strongly influences the fragment pathway, the interpretation of MS/MS data provides specific structural information about the type of molecules. In this context, the analysis of the phenolic extract allowed the detection of dihydroflavonols, flavones, flavanones and flavonols, either as free form, or their methylated /esterified forms, Figure 3.2. In particular, it was possible to identify the aglycones forms of apigenin, ( $m/z$  269, fraction 11), pinobanksin ( $m/z$  271, fraction 9), pinocembrin ( $m/z$  255, fraction 17) and chrysin ( $m/z$  253 fraction 19), the esterified derivatives pinobanksin-3-*O*-acetate ( $m/z$  313, fraction 20), pinobanksin-3-*O*-propionate ( $m/z$  327, fraction 25), pinobanksin-2-*O*-butyrate or isobutyrate ( $m/z$  341, fraction 28) and pinobanksin-3-*O*-pentanoate or 2-methylbutyrate ( $m/z$  355, fraction 30) and the methylated derivatives pinobanksin-5-methyl-ether ( $m/z$  285, fraction 7), pinocembrin-5-methyl-ether ( $m/z$  269, fraction 10), chrysin-5-methyl-ether ( $m/z$  267, fraction 12) and chrysin-6-methyl-ether ( $m/z$  283, fraction 22). Still, it should be noted that the two peaks collected as fraction 10 were distinct, and no differences were noted in their MS analysis. It is possible that isomers can be present, although further NMR analysis must be performed.

The MS<sup>2</sup> spectrum of many of these flavonoids, Table 3.1, revealed the fragments at  $m/z$  151 or at  $m/z$  165, which are resultant from the retro Diels-Alder mechanism.<sup>28</sup> Also, neutral losses commonly described to occur in these compounds,<sup>28</sup> such as the small molecules CO (-28 Da), CO<sub>2</sub> (-44 Da), C<sub>2</sub>H<sub>2</sub>O (-42 Da), as well as the successive losses of these molecules, were also observed. In accordance with Cuyckens *et al.* (2004), methylated flavonoids presented a significant [M-H-CH<sub>3</sub>]<sup>-</sup> product ion.

Attending to the HPLC-UV profile showed in Figure 3.1, it is possible to suggest that this Portuguese propolis samples are mostly rich in pinocembrin (fraction 17), chrysin (fraction 19) and pinobanksin-3-*O*-acetate (fraction 20), and thus, this fits well with the phenolic profile of propolis from temperate zones.<sup>2</sup> Still, it should be noted that the typical flavone galangin was not found in this sample, which may suggest a peculiar characteristic of the Northeast Portuguese propolis.

### 3.3.2.3 New compounds

The phenolic compounds that were identified for the first time in propolis could be considered in two main groups: a) methylated and/or esterified or hydroxylated derivatives of flavonoids already described in propolis, and thus denoted as derivatives of common flavonoids, and b) those containing a much peculiar structure, derived from phenolic compounds never found in propolis and thus were denoted as rare phenolic compounds. The elucidation of the structural characteristics of these phenolic components was based on the detailed analysis of their MS/MS and MS<sup>n</sup> data, together with some information on the fragmentation of reference compounds.

#### 3.3.2.3.1 Derivatives of common flavonoids

The phenolic compounds related to previously reported flavonoids corresponded to the [M-H]<sup>-</sup> at *m/z* 299 (fraction 12), at *m/z* 329 (fraction 13), at *m/z* 369 (fraction 26) and at *m/z* 269 (fraction 27), Table 3.1. The MS<sup>2</sup> spectrum of the [M-H]<sup>-</sup> at *m/z* 299 showed an ion at *m/z* 284 (-15 Da), which can be attributed to the loss of a methyl group. This latter fragment was ascribed to kaempferol, since its MS<sup>3</sup> spectrum demonstrated a similar fragmentation pathway to that of the reference compound (ions at *m/z* 257 and *m/z* 151). Thus, the results suggested that the ion at *m/z* 299 is a methylated derivative of kaempferol. Most commonly, these substituents are linked to C-5 or C-7 of A-ring,<sup>29</sup> but its exact position cannot be discriminate by MS analysis. Still, in accordance with Gardana *et al.* (2007), the 5-derivatives flavonoids tend to elute before the aglycones in HPLC reversed phase conditions and that was the reason why this position is considered in the present study, Figure 3.2.

The ESI-MS spectrum of fraction 13 showed a [M-H]<sup>-</sup> ion at *m/z* 329, Table 3.1, and its MS<sup>2</sup> fragmentation indicated the loss of three methyl groups (product ions at *m/z* 314, 299 and 285). As the MS<sup>3</sup> spectrum of the ion at *m/z* 299 showed product ions at *m/z* 285 (-CH<sub>3</sub>) and *m/z* 242 (CH<sub>3</sub>-CO<sub>2</sub>-), that are consistent with the fragmentation pathway of the reference compound hesperitin, the results suggested that the phenolic compound in fraction 13 is a dimethylated derivative of hesperitin, probably linked to C-5 and C-7, as proposed in Figure 3.2.

The major [M-H]<sup>-</sup> ion in fraction 26 appeared at *m/z* 369 and its MS<sup>2</sup> spectrum demonstrated a main peak at *m/z* 285, Table 3.1), which corresponded to the loss of a pentanoate group (-84 Da) and an ion at *m/z* 267, that is in accordance with the structure

of a methylated chrysin. As described by Gardana *et al.* (2007), and also observable in Table 3.1 for fractions 20, 25, 28 and 30, chrysin is the main fragment ion of the esterified pinobanksins. Thus, these data suggested that the phenolic compound eluted in fraction 26 is pinobanksin-5-methyl-ether-3-pentanoate. This structure was reinforced by the MS<sup>3</sup> spectrum analysis of the ion at  $m/z$  285, since this was similar to that of pinobanksin-5-methyl-ether (fraction 7).

Fraction 27 showed an ESI-MS spectrum with a main  $[M-H]^-$  ion at  $m/z$  269. The MS<sup>2</sup> spectrum of this ion had some similarities to that of pinocembrin-5-methyl-ether ( $m/z$  269, fraction 10), but still, the ion eluted in peak 27 showed a fragment resultant from the loss of water (-18 Da, fragment at  $m/z$  251), indicating that this compound must have an OH group that can be lost very easily. Considering the high intensity of the ion at  $m/z$  254 (-15 Da, generated from the methoxy group fragmentation), as also observed for the pinocembrin-5-methyl-ether, the methoxy group is proposed to be linked to position C-5. More, the high abundance of the ion  $m/z$  251 (-18 Da) may be explained considering OH in position 3. In fact, the loss of H<sub>2</sub>O leads to the formation of a double bond that is conjugated with the aromatic ring, promoting this fragmentation pathway and generating an abundant fragment.

### 3.3.2.3.2 Rare phenolic compounds

These comprised three of the co-eluted compounds in fraction 25 ( $[M-H]^-$  ions at  $m/z$  403,  $m/z$  433 and  $m/z$  461), one co-eluted compound in fractions 28 and 29 ( $[M-H]^-$  ions at  $m/z$  461 and  $m/z$  475, respectively) and two co-eluted compounds in fraction 31 ( $[M-H]^-$  ions at  $m/z$  403 and  $m/z$  565). It should be noted that, in general, the molecular weight of these compounds are considerably higher than the phenolic compounds commonly found in temperate propolis.<sup>23</sup>

The analysis of the fragmentation pattern of the mentioned  $[M-H]^-$  ions revealed that, with the exception of the ion at  $m/z$  565 in fraction 31, all the remaining compounds belong to the flavonoid family. These latter compounds embraced pinocembrin derivatives, since their MS/MS spectra showed an ion at  $m/z$  255, correspondent to the  $[M-H]^-$  ion of pinocembrin, and pinobanksin derivatives, as these yielded a product ion at  $m/z$  253, which is the  $[M-H]^-$  ion of chrysin. As already described in the present study, chrysin is the main fragment ion of the esterified pinobanksins. Also, as discussed in detail in the following sections, the high molecular

weight of these compounds, together with their fragmentation pathways under ESI-MS/MS, supported the hypothesis that these flavonoids are linked with the basic structures of phenolic acids, such as the phenylpropanoic acid moiety. To our knowledge, these structural characteristics were not previously described to occur in phenolic extracts from propolis or related products, although the flavanone derivative pinobanksin 3-cinnamate has been reported to occur in *Cheilantheid ferns*.<sup>30</sup>

The MS analysis of the  $[M-H]^-$  signals at  $m/z$  403 and  $m/z$  433 of fraction 25 demonstrated a similar fragmentation pattern, suggesting that they have related structures. The MS<sup>2</sup> spectrum of the ion at  $m/z$  403 showed an ion at  $m/z$  255, Table 3.1 and Figure 3.3, the latter attributed to pinocembrin, as confirmed by its MS<sup>3</sup> fragmentation (ions at  $m/z$  213 and  $m/z$  151).

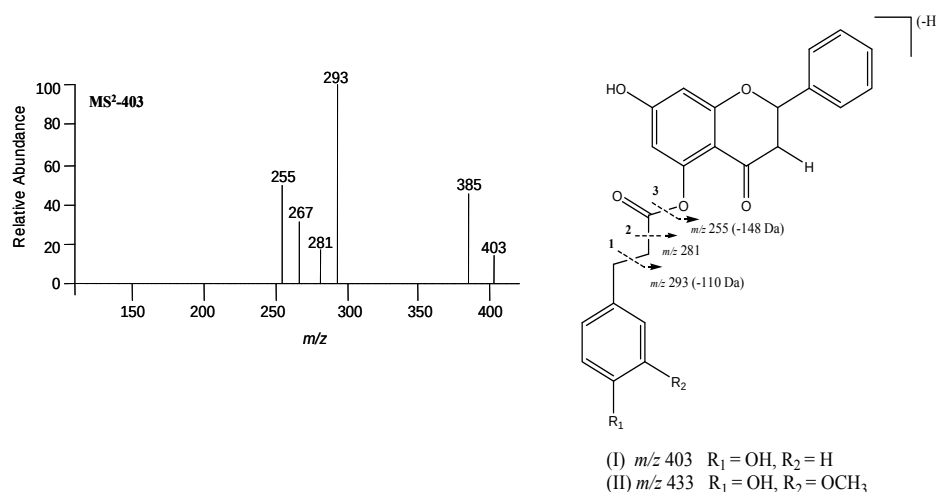


**Figure 3.3** ESI-MS<sup>2</sup> spectrum of the  $[M-H]^-$  at  $m/z$  403 detected in fraction 25. The tentative structure for this ion (I) and for ion at  $m/z$  433 (II) is also shown. Some relevant fragments for the ion at  $m/z$  403 are also represented in the figure.

These results indicated that the phenolic compound of the  $[M-H]^-$  ion at  $m/z$  403 is a pinocembrin derivative. As the ion corresponding to pinocembrin (at  $m/z$  255) was formed by the loss of 148 Da ( $-C_9H_8O_2$ ) from the  $[M-H]^-$  (cleavage at position 3 denoted on the structure I of Figure 3.3), this result suggested that a 4-hydroxyphenylpropanoid group is present in the molecule. This hypothesis was also supported by the presence in the MS<sup>2</sup> spectrum of a main product ion at  $m/z$  293, formed by the loss of 110 Da. This latter ion can result from the cleavage of the hydroxyphenylpropanoid in the  $\beta$ -position relative to the carboxylic group, as

represented in the structure I of Figure 3.3 (position 1). It should be noted that, although the substituent is represented in the position C-5 of the flavanone structure, this can be arbitrarily located in C-5 or C-7. The elucidation of its precise position was not achieved through the MS technique and would require the NMR analysis of the compound.

The MS<sup>2</sup> spectrum of the ion at  $m/z$  433 also showed a fragment at  $m/z$  255 and a major product ion at  $m/z$  309, Table 3.1. As described above, the former fragment corresponded to pinocembrin, and the latter, which resulted from the loss of 124 Da, can be justified by the cleavage of a 3-methoxy-4-hydroxyphenylpropanoid substituent in the  $\beta$ -position relative to the carboxylic group, like in the ion at  $m/z$  403. The presence of a methoxy group in the molecule was also confirmed by the ion peak at  $m/z$  401 (-32 Da). The tentative structure of this compound is also represented in Figure 3.3.

Fraction 25 showed other unknown phenolic compound, with a molecular ion at  $m/z$  461, Table 3.1. As for the  $[M-H]^-$  ion at  $m/z$  403 discussed above, the MS<sup>2</sup> spectrum of this ion, Figure 3.4 (a) showed a fragment corresponding to the loss of 110 Da (ion at  $m/z$  351), which can be justified by the cleavage at position 1 (structure I in Figure 3.4), and thus suggesting that a phenylpropanoid group is linked to C-5 or C-7. The fragmentation pattern of the signal at  $m/z$  461 also demonstrated a strong ion at  $m/z$  401 (-60 Da), which is consistent with the loss of an acetate group. Considering that the loss of a C-3 substituent usually originates the base peak in a spectrum (as discussed above), it is probable that the acetate group of this molecule is linked at that position. The presence of an acetate group in the C-3 was also supported by the analysis of the MS<sup>3</sup> spectrum of the ion at  $m/z$  351, Figure 3.4 (b), that showed a main loss of the acetate group (-60 Da, ion at  $m/z$  291). The general structure of this compound was also supported by the MS<sup>3</sup> spectrum of the ion at  $m/z$  401, Figure 3.4 (c), which demonstrated fragments resulting from the losses of 110 Da (ion at  $m/z$  401) and 148 Da (ion at  $m/z$  253), consistent with cleavages at positions 1 and 3, as denoted in Figure 3.4. Moreover, the MS<sup>3</sup> data of the ion at  $m/z$  253 was in agreement with the structure of chrysin (fragments at  $m/z$  209 and  $m/z$  151), and thus, this compound can be considered a derivative of pinobanksin.

The MS<sup>2</sup> data of the ion  $[M-H]^-$  at  $m/z$  475 eluted in fraction 29, Table 3.1, was consistent with a methylated derivative of the ion at  $m/z$  461, Figure 3.4. The ion showed fragments at  $m/z$  433 (-42 Da), a main ion fragment at  $m/z$  415 (-60 Da) corresponding to the loss of the acetate group and a fragment at  $m/z$  400 (-75 Da), which

can be justified to the loss of the acetate and the methyl groups. In opposition to the methylated compound discussed above (ion at  $m/z$  433), the methyl group of this molecule was considered to be linked to C-7, because there was no evidence of a methoxylated hydroxyphenylpropanoid substituent in the molecule (fragment resulting from the loss of 124 Da). This was also supported by the MS<sup>3</sup> data of the ion at  $m/z$  415, which showed a major fragment at  $m/z$  400 (-15 Da).

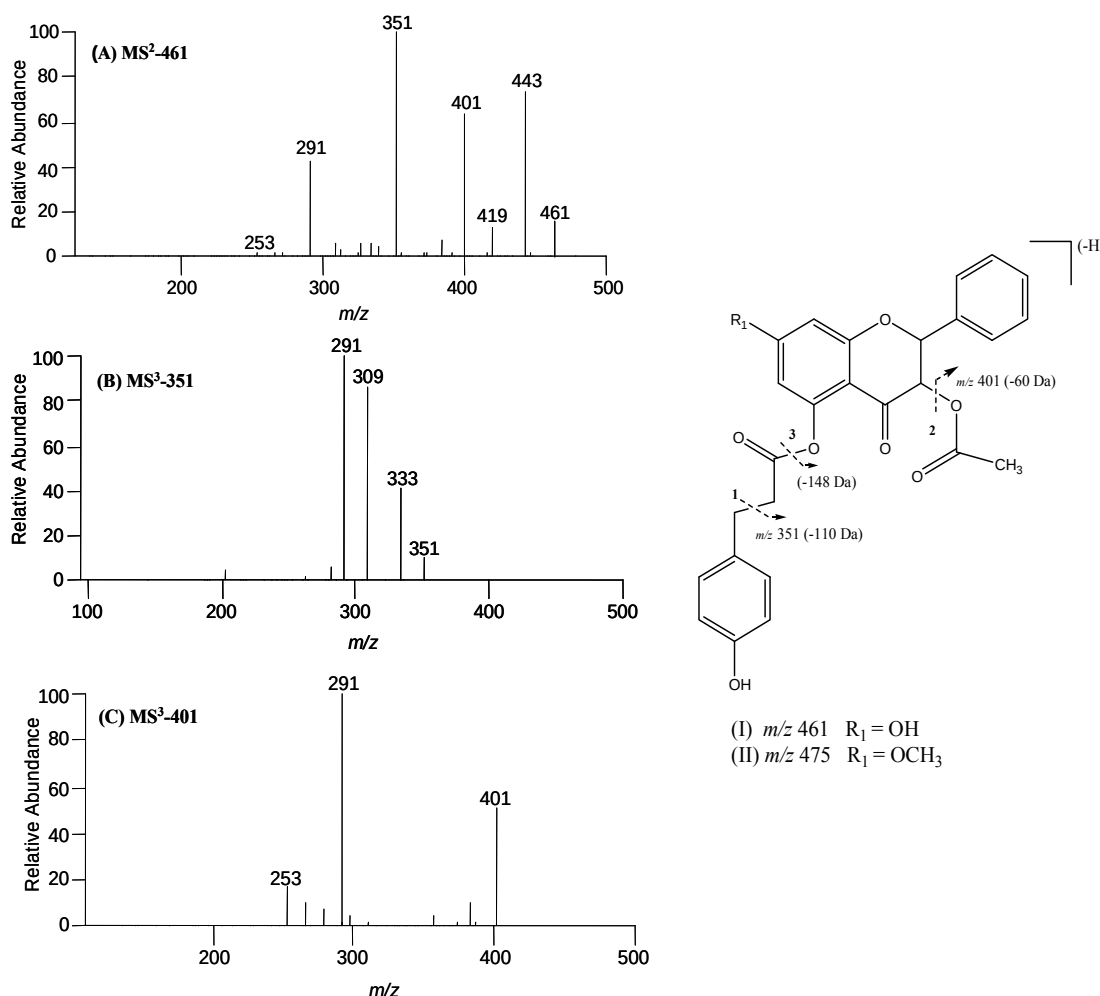


**Figure 3.4** ESI-MS<sup>n</sup> spectra of the  $[M-H]^-$  at  $m/z$  461 detected in fraction 25. (A) ESI-MS<sup>2</sup> spectrum; (B) ESI-MS<sup>3</sup> spectrum of the ion at  $m/z$  351; (C) ESI-MS<sup>3</sup> spectrum of the ion at  $m/z$  401. The tentative structure for this ion (I) and for ion at  $m/z$  475 (II) is also shown. Some relevant fragments for the ion at  $m/z$  461 are also marked in the figure.

The fragmentation pattern of the ion at  $m/z$  403 eluted in fraction 31 was different from that of the ion at  $m/z$  403 in fraction 25. In particular, the MS/MS spectrum of the former phenolic compound showed a major fragment ion at  $m/z$  253, Table 3.1, ascribed to chrysin according to its MS<sup>3</sup> data. Additionally, the fragment ion

at  $m/z$  253 (-150 Da), may result from the loss of a phenylpropanoic acid, as shown in Figure 3.5.

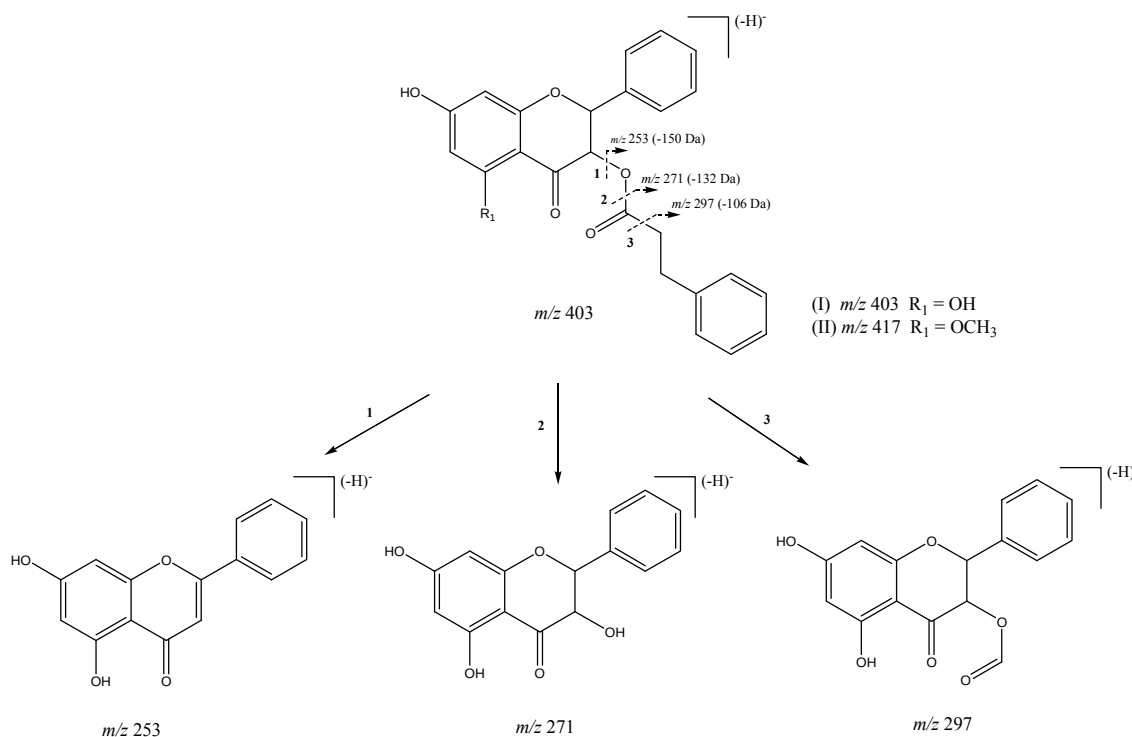


**Figure 3.5** Tentative structure for the ions  $[M-H]^-$  at  $m/z$  403 (I) and 417 (II), detected in fractions 28 and 31, respectively. Proposed scheme for fragmentation of the  $[M-H]^-$  at  $m/z$  403 (I).

The presence of phenylpropanoic acid in the molecule was also supported by the fragment ions at  $m/z$  271 (-132 Da) and at  $m/z$  297 (-106 Da), since they can correspond to a cleavage at position 2 and 3, respectively. In this case, the substitution is proposed to be at C-3 due to the high intensity of the fragment at  $m/z$  253. This product ion was also the base peak in the  $MS^3$  spectrum of the ion at  $m/z$  271 (data not shown).

As observed in Table 3.1, the major fragment of the ion at  $m/z$  417 eluted in fraction 28 also resulted from the loss of 150 Da (ion at  $m/z$  267), suggesting the presence of a phenylpropanoic acid moiety on the C-3 position of the flavonoid. Moreover, the molecule should contain a methoxy group at C-5 or C-7, as inferred by the fragment ions at  $m/z$  385 (-32 Da). In conclusion, these results indicated that the phenolic compound correspondent to the molecular ion at  $m/z$  417 is a methylated derivative at C-5 or C-7 position of the ion at  $m/z$  403 (fraction 31), as represented in Figure 3.5.

The MS/MS analysis of the other compound eluted in fraction 31 (ion at  $m/z$  565) showed a main fragment at  $m/z$  283 (-282 Da), suggesting that this compound is a dimeric specie, Figure 3.6 (a).

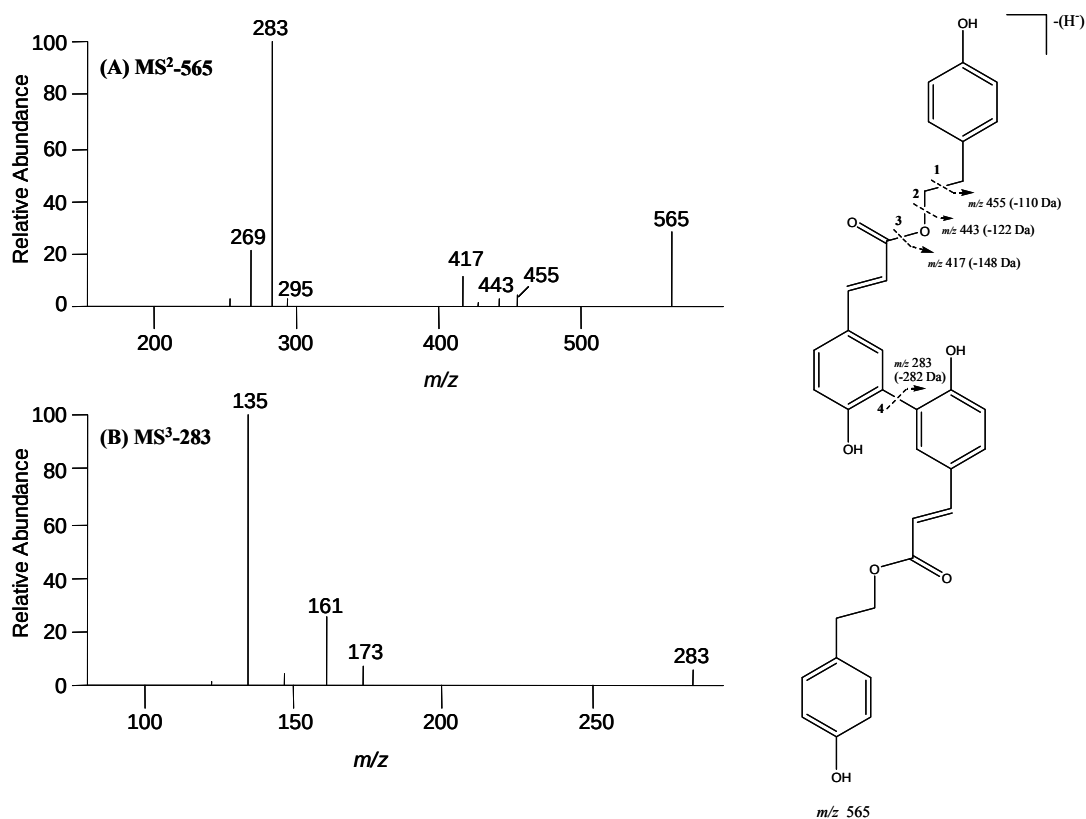


**Figure 3.6** ESI-MS<sup>n</sup> spectra of molecular ion at  $m/z$  565 detected in fraction 31. (a) ESI-MS<sup>2</sup> spectrum; (b) ESI-MS<sup>3</sup> spectrum of the ion at  $m/z$  283. The tentative structure of the compound is also shown.

Also, the MS/MS product ions at  $m/z$  455 (-110 Da), at  $m/z$  443 (-122 Da) and  $m/z$  417 (-148 Da) indicated the presence of 4-hydroxyphenyl propanoic acid in the structure, as already described in this study (cleavages at positions 1, 2 and 3 denoted in Figure 3.6). The presence of this acid in the dimeric structure was also supported by the fragment at  $m/z$  269 (correspondent to the loss of two fragments of 148 Da) and by the analysis of MS<sup>3</sup> spectrum of the ion at  $m/z$  283, Figure 3.6 (b). This latter showed, once again, fragments resultant from the losses of 110 Da (ion at  $m/z$  173), 122 Da (ion at  $m/z$  161) and 148 (ion at  $m/z$  135). Thus, the analysis of the fragmentation data of the  $[M-H]^-$  at  $m/z$  565 suggested that the compound is a *p*-coumaric ester derivative dimer.

### 3.4 Concluding remarks

The present work allowed to characterize the phenolic compounds of Portuguese propolis for the first time. The most abundant compounds in this matrix were those commonly found in propolis of the temperate zones. Still, the Portuguese propolis also contained several new phenolic components, namely, some methylated and/or sterified or hydroxylated derivatives of flavonoids already described in propolis and also rare pinocembrin or pinobanksin derivatives that contain basic structures of phenolic acids. It is probable that these groups are linked to the positions C-5, C-7, or C-3 of the flavonoid skeleton, although their precise position would only be elucidated by NMR analysis. Moreover, it was possible to detect the presence of a p-coumaric ester derivative dimer (MW 566 Da) in this sample.

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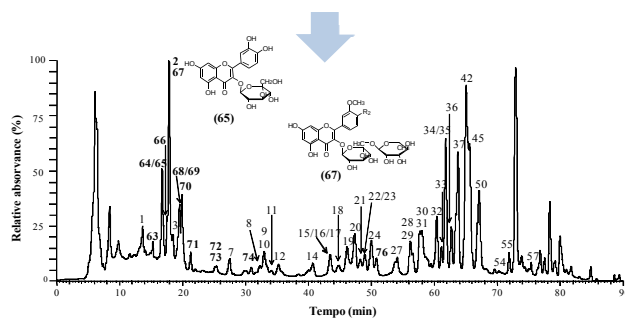
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## Chapter 4

### Phenolic profiling of Portuguese propolis by LC-MS spectrometry: uncommon propolis rich with flavonoid glycosides





## Phenolic profiling of Portuguese propolis by LC-MS spectrometry: uncommon propolis rich with flavonoid glycosides<sup>\*,†</sup>

### Abstract

Propolis is a chemically complex resinous substance collected by honeybees (*Apis mellifera*) from tree buds, comprising plant exudates, secreted substances from bee metabolism, pollen and waxes. Its chemical composition depends strongly on the plant sources available around the beehive with direct impact in the quality and bioactivity of the propolis. Being Portugal a country of botanical diversity, the phenolic characterization of propolis from the different regions is a priority. This study outlines an extensive characterization of the phenolic composition of Portuguese propolis. For that, 40 propolis samples from different regions of continental Portugal and islands were extracted and analyzed by liquid chromatography with diode-array detection coupled to electrospray ionization tandem mass spectrometry (LC/DAD/ESI-MS<sup>n</sup>). Seventy six polyphenols were detected in the samples and two groups of propolis types were established: the common temperate propolis, which contained the typical poplar phenolic compounds such as flavonoids and their methylated/esterified forms, phenylpropanoid acids and their esters and an uncommon propolis type with an unusual composition in quercetin and kaempferol glycosides some of them never described in propolis.

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\*Adapted from Falcão SI, Vale N, Gomes P, Domingues MRM, Freire C, Cardoso SM, Vilas-Boas M. Phenolic profiling of Portuguese propolis by LC-MS spectrometry: uncommon propolis rich with flavonoid glycosides. *Phytochem. Anal.* **2013**, 24(4), 309-318.

†The experimental details about the propolis samples, phenolic compounds extraction and LC/DAD/ESI-MS<sup>n</sup> analysis are indicated in Appendices A, B and E respectively.

## 4.1 Introduction

Amongst all bee products, propolis is one of the most complex and, at the same time, very fascinating. Propolis is the name given to an extremely sticky, resinous substance, collected from various floral sources, transformed and used by the honeybees (*Apis mellifera* L.) to seal holes in their honeycombs, smooth out the internal walls and protect the entrance against intruders. The origin of the word propolis comes from the Greek: *pro-* standing for “in defense”, and *polis-* meaning “the city”, that is, defense of the city (or the hive).<sup>1</sup>

Propolis acts as a strong biocide within the hive, being responsible for the low incidence of bacteria and fungi.<sup>2</sup> This bee product is widely used in folk medicine since ancient times and recently gained popularity all over the world as an important ingredient of healthy foods and cosmetics. Propolis is thought to improve human health and to prevent diseases such as inflammation, heart disease, diabetes and even cancer.<sup>3</sup>

Frequently named as “bee glue”, is mainly composed by resin (phenolic compounds) but also contains significant amounts of vegetable balsam, wax, essential oils and pollen. Other organic compounds such as amino acids, vitamins, mineral salts and insoluble debris are found as residual components.<sup>1,2</sup> The variability of propolis chemical composition is strongly dependent on the plant sources available around the hive and on the geographic and climatic conditions, although bees show preference for specific resin sources.<sup>4</sup> In Europe, North America and other temperate zones the main resin source are the exudates of apical buds of *Populus* species, in particular those from *Populus nigra*.<sup>2</sup> Propolis from these regions shows similar phenolic composition, with the main compounds being flavonoids (pinocembrin, pinobanksin, chrysin and galangin), phenolic acids and their esters.<sup>4,5</sup> Different phenolic profiles were found in propolis from Canary Islands, in which furofuran lignans were the main compounds.<sup>4</sup> Moreover, terpenes were the major components in propolis from Mediterranean Sea areas which origin was most probably the Cupressaceae family and the specie *Ferula communis*.<sup>6,7</sup> In tropical areas, particularly in Brazil, propolis originating from *Baccharis dracunculifolia* (green propolis) presented a rich composition in prenylated phenylpropanoids and in caffeoyl quinic acids, whereas propolis from Cuba, Venezuela and some Brazil areas, arising from flowers of *Clusia* spp., were shown to be rich in prenylated benzophenone derivatives.<sup>4</sup> Chen et al. (2003)<sup>8</sup> have also identified a new

family of compounds in propolis samples from Taiwan, the C-prenylflavonoids (or propolins).

In recent years, different analytical approaches have been used for phenolic characterization of propolis from all over the world<sup>9-11</sup> and of these, the technique of choice is by far the liquid chromatography hyphenated with mass spectrometry (LC/MS).<sup>5,12-14</sup> LC/MS is a powerful tool for the analysis of natural products. The high sensitivity of MS analytical approach provides the discovery of new minor constituents, which are difficult to obtain by conventional means. More detailed structural information can be obtained by resorting to tandem mass spectrometry (MS/MS) which allows the characterization of unknown compounds even without the reference standards.<sup>15</sup>

In our previous work,<sup>5</sup> the phenolic compounds of northeast Portuguese propolis were studied by the combined off-line analysis of HPLC and electrospray mass spectrometry in the negative ion mode. This approach allowed to characterize thirty seven phenolic compounds, which included the typical phenolic acids and flavonoids found in propolis from temperate zones, but also new methylated, esterified and/or hydroxylated derivatives of common poplar flavonoids and pinocembrin/pinobanksin derivatives containing a phenylpropanoic acid derivative moiety in their structure.

More recent works in Portuguese propolis from other regions of Portugal (unpublished results)<sup>16</sup> revealed however the presence of propolis diversity, observed through differences in the physicochemical parameters and in total phenolic content. This is consistent with the botanical diversity of the country, and the need that bees have for search alternative resin sources in regions where poplar is not present. Our recent work in the study of propolis from distinct Portuguese continental regions and islands clearly identified the existence of different types of propolis, based on the distinct profile of physicochemical parameters identified (unpublished results). The phenolic characterization of propolis from the different regions is therefore a priority.

In this paper we present the results of an extensive study on the phenolic profile of distinct Portuguese continental regions and islands, performed by liquid chromatography with diode-array detection coupled to electrospray ionization tandem mass spectrometry (LC/DAD/ESI-MS<sup>n</sup>), in an attempt to establish the Portuguese propolis phenolic profile. A propolis type with an unusual composition of flavonoid glycosides is herein described.

## 4.2 Experimental

### 4.2.1 Materials, reagents and solvents

Chrysin, galangin, quercetin, pinocembrin, naringenin, hesperetin, ellagic acid, benzoic acid, cinnamic acid, caffeic acid, ferulic acid, isoferulic acid, *p*-coumaric acid, caffeic acid isoprenyl ester, caffeic acid phenylethyl ester (CAPE) and quercetin-3-*O*-rhamnoside were purchased from Sigma Chemical Co (St Louis, MO, USA). Apigenin, acacetin, kaempferol, chrysoeriol, chrysin-7-methylether, chrysin-5,7-dimethylether, pinocembrin-7-methylether, pinocembrin-5,7-dimethylether, 3,4-dimethyl-caffeic acid, quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-neohesperidoside, isorhamnetin-3-*O*-rutinoside, luteolin-7-*O*-glucoside were from Extrasynthese (Genay, France). Isorhamnetin and *p*-coumaric acid methyl ether were from Phytolab (Vestenbergsgreuth, Germany). Genkwanin and luteolin were obtained from Lancaster Synthesis (Morecambe, England). Analytical grade formic acid and HPLC grade ethanol were obtained from Panreac (Barcelona, Spain). HPLC grade methanol and acetonitrile were purchased from Lab-Scan (Lisbon, Portugal). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

### 4.2.2 Propolis samples

This work was performed in forty propolis samples from different regions of continental Portugal (north - N; central interior - CI; central coast - CC; south - S), Azores archipelago (A) and Madeira Island (M).

## 4.3 Results and discussion

The LC/DAD/ESI-MS<sup>n</sup> study of the forty Portuguese propolis samples allowed the elucidation of phenolic compounds by comparison of their chromatographic behaviour, UV spectra and MS information, to those of reference compounds. When standards were not available, the structural information was confirmed with UV data combined with MS fragmentation patterns previously reported in the literature. This

study was carried out using LC-MS in the negative ion mode because of its higher sensitivity in the analysis of the different polyphenols classes.<sup>15</sup>

Overall, the Portuguese propolis samples could be arranged in two distinct groups, Table 4.1: (A) common temperate propolis type, e.g., which contained the typical poplar phenolic compounds as the main phenolic components and (B) uncommon temperate propolis type which, besides the typical poplar flavonoids, also contained significant amounts of unusual flavonoid glycosides in their composition.

**Table 4.1** Portuguese propolis types according with the phenolic profile.

| Geographical location | Code | Propolis type | Geographical location | Code | Propolis type |
|-----------------------|------|---------------|-----------------------|------|---------------|
| North                 | N1   | Common        | Central coast         | CC3  | Common        |
|                       | N2   | Common        |                       | CC4  | Common        |
|                       | N3   | Common        | South                 | S1   | Common        |
|                       | N4   | Common        |                       | S2   | Common        |
|                       | N5   | Common        |                       | S3   | Common        |
|                       | N6   | Common        |                       | S4   | Uncommon      |
|                       | N7   | Common        | Azores<br>Archipelago | A1   | Common        |
|                       | N8   | Common        |                       | A2   | Common        |
|                       | N9   | Common        |                       | A3   | Common        |
|                       | N10  | Common        |                       | A4   | Common        |
|                       | N11  | Uncommon      |                       | A5   | Common        |
|                       | N12  | Uncommon      |                       | A6   | Common        |
|                       | N13  | Uncommon      |                       | A7   | Common        |
|                       | N14  | Common        |                       | A8   | Common        |
| Central interior      | CI1  | Uncommon      |                       | A9   | Common        |
|                       | CI2  | Common        |                       | A10  | Common        |
|                       | CI3  | Common        |                       | A11  | Common        |
|                       | CI4  | Common        | Madeira island        | M1   | Common        |
| Central coast         | CC1  | Common        |                       | M2   | Uncommon      |
|                       | CC2  | Common        |                       | M3   | Common        |

Figure 4.1 shows the representative chromatographic profile at 280 nm for the two types of propolis found. Following, the phenolic composition of the two groups is detailed.

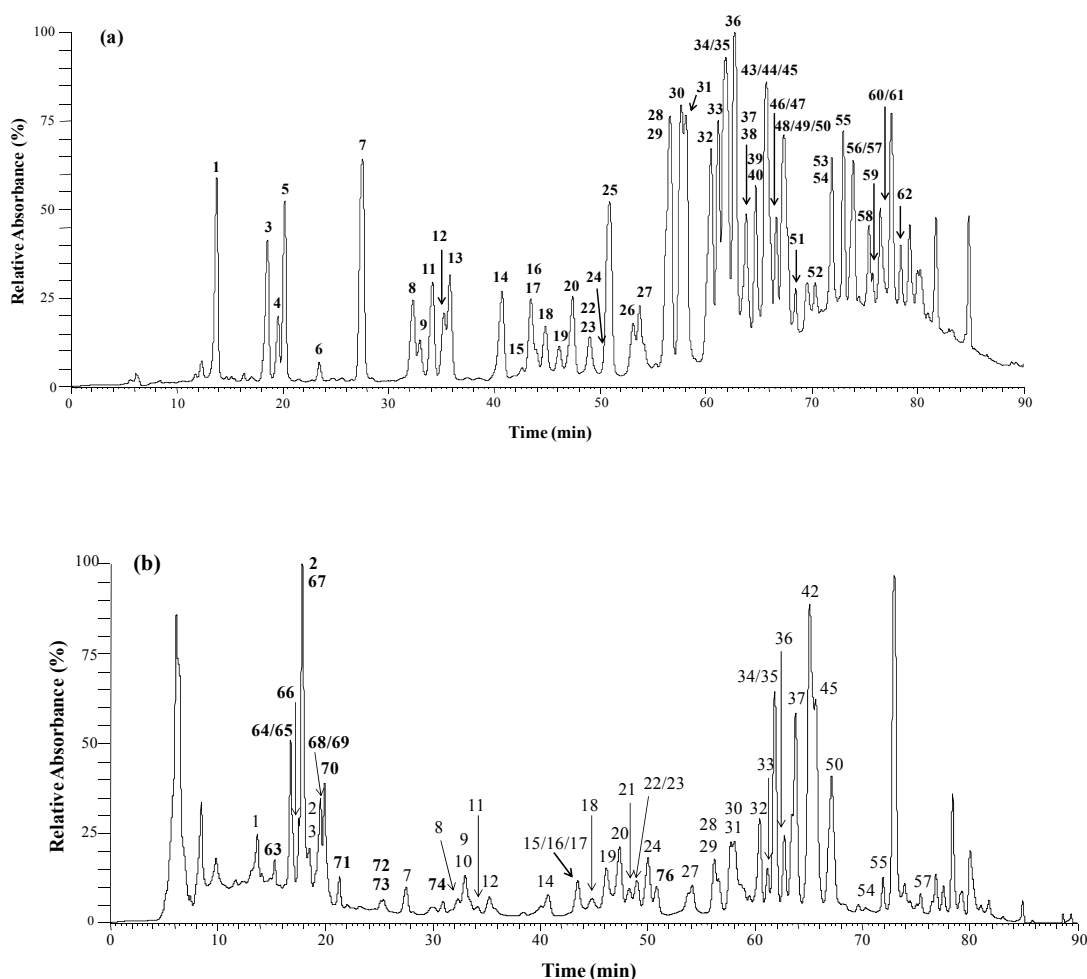


**Figure 4.1** Representative chromatographic profile at 280 nm of ethanolic propolis extracts: **a** common temperate propolis type; **b** uncommon propolis type.

#### 4.3.1 Common temperate propolis type

Overall, more than 85% of the Portuguese propolis samples under study were identified as common temperate propolis with a common phenolic matrix.<sup>5</sup> This group included all the samples from central coast and Azores archipelago, as well as the majority of samples from north, and some from the central interior, south and Madeira Island. Still, it is important to note that some of the samples included in this group showed singular deviations to the typical phenolic profile of temperate regions. In particular, all samples from coast center, samples N11-N13 from the north, samples S2-S3 from the south and sample M3 from Madeira Island contained an additional flavonol, the kaempferide. Moreover, samples CI2-CI4 from center interior and S2-S4 (south) were poor in pinobanskin derivatives but contained a kaempferol-dimethyl-

ether, which is not typically observed in the temperate propolis profile. Sample M1 from Madeira exhibits a small number of phenolic compounds.

In general, the analytical approach used in the present study allowed the identification of sixty two compounds in common temperate propolis type samples (Table 4.2). These included the thirty seven phenolic compounds previously reported<sup>5</sup> plus: a) nineteen new confirmed phenolic compounds (6, 9, 11, 12, 18, 19, 22-24, 26-28, 36-38, 44, 47, 54, 62) and b) six new compounds which the structure will be herein partially elucidated (46, 49, 55, 56, 59, 60).

In addition to our previous results, benzoic acid (MW 122 Da) was now detected as a minor peak in all samples (compound 6 at 23.5 min), while cinnamic acid ( $m/z$  147, compound 11) was found in most of the samples. Note that in this study, these phenolic acids (as well as the remaining simple acids in the propolis extracts) were mainly detected in the LC-MS both with formation of the  $[M+HCOOH]^-$  adduct ( $[M+46]^-$ ). Formation of adduct with formic acid was observed before in the characterization of this type of phenolic compounds.<sup>17</sup> Besides those two acids, one ferulic acid derivative and three other caffeic acid derivatives were herein detected. Indeed, although the structure of these compounds was not totally elucidated, it was possible to observe an UV spectrum equivalent to that of caffeic acid (292, 322 nm) in compounds 55, 56 and 60. Moreover, the MS<sup>2</sup> spectrum of the  $[M-H]^-$  at  $m/z$  399 (isomeric compounds 55 and 56) showed a base peak ion at  $m/z$  179, which corresponds to caffeic acid and a ion at  $m/z$  135 resulting from the loss of CO<sub>2</sub> from the phenolic acid. In a similar manner, the  $[M-H]^-$  at  $m/z$  419 in compound 49 was assigned to a ferulic acid derivative, as it presented  $\lambda_{max}$  at 298 and 322 nm and its MS<sup>3</sup> spectrum showed an ion at  $m/z$  193, both consistent to ferulic acid.

It is worth noting the large number of methoxylated flavonols in these Portuguese propolis samples, namely quercetin and kaempferol. These polyphenols were not detected in our previously work due to the methodological approach used, where the major eluted peaks were collected as fractions and directly injected into the ESI source and so analyzed.<sup>5</sup> These compounds include several monomethyl-ether and polymethyl-ether quercetin derivatives, namely quercetin-3-methyl-ether (12), isorhamnetin (19), quercetin-dimethyl-ether (22), quercetin-tetramethyl-ether (24), rhamnetin (27) and quercetin-dimethyl-ether (28), which have previously been detected as major components of plant exudates, resins and waxes.<sup>18</sup>

**Table 4.2** Characterization of the phenolic compounds by LC/DAD/ESI-MS<sup>n</sup> from Portuguese propolis

| Nr | t <sub>R</sub> (min) | λ <sub>max</sub> (nm) | [M-H] <sup>-</sup> m/z         | LC/ESI-MS <sup>n</sup> m/z (% base peak)                                                           | Compound                                                            |
|----|----------------------|-----------------------|--------------------------------|----------------------------------------------------------------------------------------------------|---------------------------------------------------------------------|
| 1  | 13.7                 | 292, 322              | 179, [M+46] <sup>-</sup> : 225 | MS <sup>2</sup> [301]: 301 (100), 257 (77), 229 (96)                                               | Caffeic acid <sup>a,b</sup>                                         |
| 2  | 18.1                 | 253, 367              | 301                            |                                                                                                    | Ellagic acid <sup>a,b</sup>                                         |
| 3  | 18.5                 | 310                   | 163, [M+46] <sup>-</sup> : 208 |                                                                                                    | <i>p</i> -Coumaric acid <sup>a,b</sup>                              |
| 4  | 19.5                 | 295sh, 322            | 193, [M+46] <sup>-</sup> : 238 | MS <sup>2</sup> [285]: 267 (100), 252 (13), 239 (27)                                               | Ferulic Acid <sup>a,b</sup>                                         |
| 5  | 20.2                 | 298, 319              | 193, [M+46] <sup>-</sup> : 238 |                                                                                                    | Isoferulic acid <sup>a,b</sup>                                      |
| 6  | 23.5                 | 229                   | 121, [M+46] <sup>-</sup> : 166 |                                                                                                    | Benzoic acid <sup>a,b</sup>                                         |
| 7  | 27.5                 | 295sh, 322            | 207, [M+46] <sup>-</sup> : 253 | MS <sup>2</sup> [301]: 179 (100), 151 (60)                                                         | 3,4-Dimethyl-caffeic acid <sup>a,b</sup>                            |
| 8  | 32.3                 | 286                   | 285                            |                                                                                                    | Pinobanksin-5-methyl-ether <sup>b,c</sup>                           |
| 9  | 32.9                 | 256, 370              | 301                            |                                                                                                    | Quercetin <sup>a,b</sup>                                            |
| 10 | 33.0                 | 253, 268sh, 349       | 285                            | MS <sup>2</sup> [285]: 285 (100), 267 (54), 241 (63), 175 (52)                                     | Luteolin <sup>a,b</sup>                                             |
| 11 | 34.1                 | 277                   | 147, [M+46] <sup>-</sup> : 193 | MS <sup>2</sup> [147]: 103                                                                         | Cinnamic acid <sup>a,b</sup>                                        |
| 12 | 35.2                 | 256, 355              | 315                            | MS <sup>2</sup> [315]: 300; MS <sup>3</sup> [300]: 271 (100), 255 (59), 151 (<1)                   | Quercetin-3-methyl-ether <sup>b,d (13)</sup>                        |
| 13 | 35.7                 | 307                   | 177, [M+46] <sup>-</sup> : 223 | MS <sup>2</sup> [271]: 253 (100), 225 (26), 151 (10)                                               | <i>p</i> -Coumaric acid methyl ester <sup>a,b</sup>                 |
| 14 | 40.6                 | 292                   | 271                            |                                                                                                    | Pinobanksin <sup>b,c (5)</sup>                                      |
| 15 | 42.5                 | 286                   | 269                            |                                                                                                    | Pinocembrin-5-methyl-ether <sup>b,c (5)</sup>                       |
| 16 | 43.3                 | 268, 337              | 269                            | MS <sup>2</sup> [269]: 225 (100), 151 (29)                                                         | Apigenin <sup>a,b</sup>                                             |
| 17 | 43.3                 | 268, 313              | 267                            | MS <sup>2</sup> [267]: 253 (100), 224 (25)                                                         | Chrysin-5-methyl-ether <sup>b,c (5)</sup>                           |
| 18 | 44.6                 | 265, 364              | 285                            | MS <sup>2</sup> [267]: 285 (100), 257 (13), 151 (20)                                               | Kaempferol <sup>a,b</sup>                                           |
| 19 | 45.9                 | 253, 370              | 315                            | MS <sup>2</sup> [315]: 300                                                                         | Isorhamnetin <sup>a,b</sup>                                         |
| 20 | 47.2                 | 265, 352              | 299                            | MS <sup>2</sup> [299]: 284                                                                         | Kaempferol-methyl-ether <sup>b,c (5)</sup>                          |
| 21 | 48.4                 | 265, 340              | 329                            | MS <sup>2</sup> [329]: 314; MS <sup>3</sup> [314]: 299 (100)                                       | Kaempferol-methoxy-methyl-ether <sup>b</sup>                        |
| 22 | 48.9                 | 253, 355              | 329                            | MS <sup>2</sup> [329]: 314; MS <sup>3</sup> [314]: 299 (48), 285 (100), 271 (78), 243 (28)         | Quercetin-dimethyl-ether <sup>b,c (14)</sup>                        |
| 23 | 48.9                 | 289                   | 327                            | MS <sup>2</sup> [327]: 285 (100), 267 (18), 239 (31)                                               | Pinobanksin-5-methyl-ether-3- <i>O</i> -acetate <sup>b,d (13)</sup> |
| 24 | 49.9                 | 256, 349              | 359                            | MS <sup>2</sup> [359]: 344; MS <sup>3</sup> [344]: 329 (100), 301 (35), 179 (<1), 151 (<1)         | Quercetin-tetramethyl-ether <sup>b</sup>                            |
| 25 | 50.7                 | 310                   | 173, [M+46] <sup>-</sup> : 219 | MS <sup>2</sup> [283]: 268 (100), 239 (60), 211 (10)                                               | Cinnamylidenacetic acid <sup>b,c (5)</sup>                          |
| 26 | 53.0                 | 265, 300sh, 352       | 283                            |                                                                                                    | Galangin-5-methyl-ether <sup>b,e (14)</sup>                         |
| 27 | 53.5                 | 256, 367              | 315                            |                                                                                                    | Rhamnetin <sup>b,f (19)</sup>                                       |
| 28 | 56.1                 | 256, 355              | 329                            | MS <sup>2</sup> [329]: 314; MS <sup>3</sup> [314]: 299; MS <sup>4</sup> [299]: 271 (100), 151 (<1) | Quercetin-dimethyl-ether <sup>b,e (14)</sup>                        |
| 29 | 56.5                 | 298, 325              | 247                            | MS <sup>2</sup> [247]: 179 (100), 135 (15)                                                         | Caffeic acid isoprenyl ester <sup>a,b</sup>                         |
| 30 | 57.5                 | 298, 325              | 247                            | MS <sup>2</sup> [247]: 179 (100), 135 (15)                                                         | Caffeic acid isoprenyl ester (isomer) <sup>b,c (5)</sup>            |
| 31 | 58.1                 | 298, 325              | 269                            | MS <sup>2</sup> [269]: 178 (100), 134 (32), 161 (12)                                               | Caffeic acid benzyl ester <sup>b,c (5)</sup>                        |
| 32 | 60.3                 | 289                   | 255                            | MS <sup>2</sup> [255]: 213 (100), 211 (32), 151 (48)                                               | Pinocembrin <sup>a,b</sup>                                          |

<sup>a</sup> Confirmed with standard; <sup>b</sup> Confirmed with MS<sup>n</sup> fragmentation, <sup>c</sup> Confirmed with references.

Table 4.2 (continued)

|    |      |                   |     |                                                                                                                                                      |                                                                                                     |
|----|------|-------------------|-----|------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------|
| 33 | 61.1 | 295, 325          | 283 | MS <sup>2</sup> [283]: 179 (100), 135 (28)                                                                                                           | Caffeic acid phenylethyl ester <sup>a,b</sup>                                                       |
| 34 | 61.7 | 268, 313          | 253 | MS <sup>2</sup> [253]: 225 (17), 209 (100), 151 (5)                                                                                                  | Chrysin <sup>a,b</sup>                                                                              |
| 35 | 61.7 | 292               | 313 | MS <sup>2</sup> [253]: 271 (18), 253 (100)                                                                                                           | Pinobanksin-3- <i>O</i> -acetate <sup>b,c (5)</sup>                                                 |
| 36 | 62.5 | 265, 300sh, 358   | 269 | MS <sup>2</sup> [269]: 269 (100), 241 (61), 227 (20), 197 (22), 151 (20)                                                                             | Galangin <sup>a,b</sup>                                                                             |
| 37 | 63.3 | 268, 331          | 283 | MS <sup>2</sup> [283]: 269                                                                                                                           | Acacetin <sup>a,b</sup>                                                                             |
| 38 | 63.9 | 265, 364          | 299 | MS <sup>2</sup> [299]: 284, 151 (<1); MS <sup>3</sup> [284]: 151                                                                                     | Kaempferide <sup>b,d (13)</sup>                                                                     |
| 39 | 64.6 | 265, 300sh, 350sh | 283 | MS <sup>2</sup> [283]: 269                                                                                                                           | 6-Methoxychrysin <sup>b,c (5)</sup>                                                                 |
| 40 | 64.8 | 294, 310          | 231 | MS <sup>2</sup> [231]: 163 (100), 119(12)                                                                                                            | <i>p</i> -Coumaric acid isoprenyl ester <sup>b,c (5)</sup>                                          |
| 41 | 64.6 | 292               | 461 | MS <sup>2</sup> [461]: 443 (68), 401 (75), 351 (100), 291 (55), 253 (2)                                                                              | Pinobanksin-3- <i>O</i> -acetate-5- <i>O</i> - <i>p</i> -hydroxyphenylpropionate <sup>b,c (5)</sup> |
| 42 | 65.1 | 250, 268sh, 343   | 313 | MS <sup>2</sup> [313]: 298; MS <sup>3</sup> [298]: 283 (100), 270 (20), 255 (7)<br>MS <sup>4</sup> [283]: 255 (100), 227 (4)                         | Chrysoeriol-methyl-ether <sup>b</sup>                                                               |
| 43 | 65.6 | 295, 324          | 295 | MS <sup>2</sup> [295]: 178 (100), 134 (24)                                                                                                           | Caffeic acid cinnamyl ester <sup>b,c (5)</sup>                                                      |
| 44 | 65.6 | 294, 310          | 231 | MS <sup>2</sup> [231]: 163 (100), 119(12)                                                                                                            | <i>p</i> -Coumaric acid isoprenyl ester (isomer) <sup>b,e (14)</sup>                                |
| 45 | 65.6 | 295               | 433 | MS <sup>2</sup> [433]: 415 (3), 401 (31), 323 (15), 309 (100)                                                                                        | Pinocembrin-5- <i>O</i> -3-hydroxy-4-methoxyphenylpropionate <sup>b,c (5)</sup>                     |
| 46 | 65.6 | 265, 346          | 313 | MS <sup>2</sup> [313]: 299 (10), 298 (100); MS <sup>3</sup> [298]: 283 (100), 269 (8), 255 (41), 241 (2); MS <sup>4</sup> [283]: 255 (100), 151 (<1) | Kaempferol-dimethyl-ether <sup>b</sup>                                                              |
| 47 | 66.5 | 295, 324          | 295 | MS <sup>2</sup> [295]: 178 (100), 134 (24)                                                                                                           | Caffeic acid cinnamyl ester (isomer) <sup>b</sup>                                                   |
| 48 | 67.2 | 289               | 327 | MS <sup>2</sup> [327]: 271 (9), 253 (100)                                                                                                            | Pinobanksin-3- <i>O</i> -propionate <sup>b,c (5)</sup>                                              |
| 49 | 67.2 | 298, 322          | 419 | MS <sup>2</sup> [419]: 313 (33), 299 (100), 193 (66); MS <sup>3</sup> [299]: 193                                                                     | Ferulic acid derivative <sup>b</sup>                                                                |
| 50 | 67.2 | 289               | 369 | MS <sup>2</sup> [369]: 285 (53), 267 (65), 239 (100)                                                                                                 | Pinobanksin-5-methyl-ether-3- <i>O</i> -pentanoate <sup>b,c (5)</sup>                               |
| 51 | 68.4 | 292               | 475 | MS <sup>2</sup> [475]: 433 (9), 415 (100), 400 (8), 253 (<1)                                                                                         | Pinobanksin-7-methyl-ether-5- <i>O</i> - <i>p</i> -hydroxyphenylpropionate <sup>b,c (5)</sup>       |
| 52 | 70.2 | 289               | 269 | MS <sup>2</sup> [269]: 254 (100), 251 (54), 165 (22)                                                                                                 | 3-Hydroxy-5-methoxyflavanone <sup>b,c (5)</sup>                                                     |
| 53 | 71.9 | 292               | 341 | MS <sup>2</sup> [341]: 271 (5), 253 (100)                                                                                                            | Pinobanksin-3- <i>O</i> -butyrate or isobutyrate <sup>b,c (5)</sup>                                 |
| 54 | 71.9 | 292               | 353 | MS <sup>2</sup> [353]: 271 (7), 253 (100)                                                                                                            | Pinobanksin-3- <i>O</i> -pentenoate <sup>b,c (5)</sup>                                              |
| 55 | 72.9 | 292, 322          | 399 | MS <sup>2</sup> [399]: 355 (39), 179 (100), 135 (50)                                                                                                 | Caffeic acid derivative <sup>b</sup>                                                                |
| 56 | 73.9 | 292, 322          | 399 | MS <sup>2</sup> [399]: 355 (39), 179 (100), 135 (50)                                                                                                 | Caffeic acid derivative (isomer) <sup>b</sup>                                                       |
| 57 | 73.9 | 289, 345          | 565 | MS <sup>2</sup> [565]: 455 (10), 417 (36), 283 (100), 269 (43)                                                                                       | <i>p</i> -Coumaric acid-4-hydroxyphenylethyl ester dimer <sup>b,c (5)</sup>                         |
| 58 | 75.3 | 292               | 355 | MS <sup>2</sup> [355]: 271 (5), 253 (100)                                                                                                            | Pinobanksin-3- <i>O</i> -pentanoate or 2-methylbutyrate <sup>b,c (5)</sup>                          |
| 59 | 76.2 | 292               | 367 | MS <sup>2</sup> [367]: 271 (100), 253 (45);<br>MS <sup>3</sup> [271]: 253 (100), 225 (27), 151 (12)                                                  | Pinobanksin- <i>O</i> -hexenoate <sup>b</sup>                                                       |
| 60 | 76.4 | 292, 322          | 315 | MS <sup>2</sup> [315]: 179 (100), 135 (8)                                                                                                            | Caffeic acid derivative <sup>b</sup>                                                                |
| 61 | 76.4 | 292               | 403 | MS <sup>2</sup> [403]: 271 (16), 253 (100)                                                                                                           | Pinobanksin-3- <i>O</i> -phenylpropionate <sup>b,c (5)</sup>                                        |
| 62 | 78.3 | 292               | 369 | MS <sup>2</sup> [369]: 271 (14), 253 (100)                                                                                                           | Pinobanksin-3- <i>O</i> -hexanoate <sup>b,d (13)</sup>                                              |

<sup>a</sup> Confirmed with standard; <sup>b</sup> Confirmed with MS<sup>n</sup> fragmentation; Confirmed with reference.

Within the kaempferol derivatives, it was possible to detect kaempferol-methyl-ether (20), kaempferide (38) and kaempferol-dimethyl-ether (46). Also, galangin-5-methyl-ether (26), with a precursor ion at  $m/z$  283 was identified in the majority of the propolis samples. A flavone derivative was also found in samples of Portuguese common temperate propolis type identified as acacetin (37).

Besides the dihydroflavonols already described in Portuguese propolis pinobanksin-3-*O*-pentenoate (54) and pinobanksin-3-*O*-hexanoate (62), were herein assigned by comparison of its UV spectrum and fragmentation pattern to that of reported literature and also with the pinobanksin derivative (59) eluted at 76.2 min. The latter presented an absorption maximum peak at 292 nm, which is consistent with pinobanksin.<sup>13</sup> Moreover, its ESI-MS showed a  $[M-H]^-$  ion at  $m/z$  367 (Table 4.2), and the main product ion ( $m/z$  271) in its MS<sup>2</sup> spectrum resulted from the loss of an hexenoate group (-96 Da),<sup>20</sup> suggesting an isomer of pinobanksin-*O*-hexenoate.

#### 4.3.2 Uncommon propolis type

Six of the forty Portuguese propolis samples had a particular phenolic profile different from that of the common temperate propolis samples, Table 4.1. As shown in Figure 4.1, their chromatograms had a group of peaks at early retention times, associated with flavonoid glycosides. Due to their rarity in propolis matrices, we will describe these compounds in detail in the following subsection. Moreover, these six propolis samples contained other four phenolic compounds which were not detected in common propolis samples, Table 4.2: ellagic acid (2), luteolin (10), a dimethoxylated flavonol (21) and a dihydroxy-dimethoxyflavone (42).

The UV spectrum of the dimethoxylated flavonol peak was equivalent to that of kaempferol and its full MS spectrum had a  $[M-H]^-$  at  $m/z$  329. Moreover, MS<sup>2</sup> and MS<sup>3</sup> experiments indicated the successive loss of methyl groups (-15 Da), with the formation of the product ions at  $m/z$  314 and  $m/z$  299, respectively. Overall, the data pointed to the presence of kaempferol-methoxy-methyl-ether with the methoxy group linked to C-6 position, as previously described.<sup>21</sup>

Experimental data of the dihydroxy-dimethoxyflavone (42) suggested the presence of chrysoeriol-methyl-ether. In fact, its UV spectrum was equivalent to that of chrysoeriol (250, 268sh and 343 nm) and the MS<sup>2</sup> spectrum of the molecular ion ( $m/z$  313) showed a loss of a methyl group, which resulted in the formation of a base peak

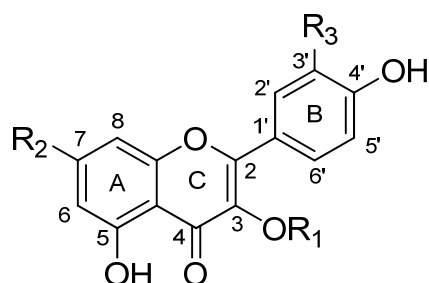
product ion ( $m/z$  298), the latter with a similar fragmentation pattern to that of chrysoeriol.

#### 4.3.3 Flavonoid glycosides

As already mentioned, uncommon propolis type samples typically contained flavonoid glycosides in their composition. We must highlight that phenolic glycosides are rare in propolis because of the hydrophobic nature of plant sources of the resin and due to the presence of  $\beta$ -glucosidase enzymes during propolis collection and processing. This last point has been under debated, as recent studies<sup>21</sup> demonstrated the inefficiency of the enzyme to hydrolyse  $\beta$ -diglycosides. Also, Bankova *et al.* (2000) suggested the possibility of no chemical changes in plant material during propolis collection. Anyway, to our knowledge, the only flavonoids glycosides previously reported in propolis matrices were the isorhamnetin-3-*O*-rutinoside, isolated from Cretan propolis,<sup>6</sup> and rutin (quercetin 3-*O*-rutinoside), that has been identified in European, Asian, and South American propolis.<sup>23</sup>

The LC/DAD/ESI-MS<sup>n</sup> data of the fourteen flavonoid glycosides of the uncommon propolis type samples showed that these were quercetin and kaempferol derivatives. In this study, five of those compounds were identified by comparison of UV and LC/MS data to those of the corresponding reference product: quercetin-3-*O*-rutinoside (63), quercetin-3-*O*-glucoside (65), kaempferol-3-*O*-rutinoside (66), isorhamnetin-3-*O*-rutinoside (67) and quercetin-3-*O*-rhamnoside (69). The structure of the nine remaining compounds was assigned based on their UV spectra and interpretation of their fragmentation pathways observed in MS<sup>n</sup> spectra, as will be discussed in detail. Sugar moieties in flavonoids were assigned as glucosides, rutinosides and glucuronides since those are most common and frequent in nature and now confirmed in propolis, although the presence of galactoside moieties seems also consistent, particularly due to its resistance to bee enzymes hydrolysis. Note that glycosylation position of these polyphenols will not be ascribed in the present work, but these are commonly C-3 or C-7.<sup>24</sup> Representative structures of the flavonoids glycosides found in uncommon temperate propolis type samples are shown in Figure 4.2, where the most probably linkage positions are considered. Thus, the structures proposed in Figure 4.2 should only be regarded as an example.

Seven of those compounds, of which, 64, 68, 70, 72-75, corresponded to quercetin glycosides. The UV spectrum of compound 64 (similar to that of quercetin) and the identification of the  $[M-H]^-$  ion at  $m/z$  477 in the correspondent MS spectrum, suggested that the compound with MW of 478 Da is a glucuronic derivative of quercetin.



| Compound | Name                                                 | R <sub>1</sub> | R <sub>2</sub>   | R <sub>3</sub>   |
|----------|------------------------------------------------------|----------------|------------------|------------------|
| 63       | Quercetin-3- <i>O</i> -rutinoside                    | Rut            | OH               | OH               |
| 64       | Quercetin-3- <i>O</i> -glucuronide                   | GlcUA          | OH               | OH               |
| 65       | Quercetin-3- <i>O</i> -glucoside                     | Glc            | OH               | OH               |
| 66       | Kaempferol-3- <i>O</i> -rutinoside                   | Rut            | OH               | H                |
| 67       | Isorhamnetin-3- <i>O</i> -rutinoside                 | Rut            | OH               | OCH <sub>3</sub> |
| 68       | Isorhamnetin- <i>O</i> -pentoside                    | Pent           | OH               | OCH <sub>3</sub> |
| 69       | Quercetin-3- <i>O</i> -rhamnoside                    | Rham           | OH               | OH               |
| 70       | Isorhamnetin- <i>O</i> -glucuronide                  | GlcUA          | OH               | OCH <sub>3</sub> |
| 71       | Kaempferol-methyl-ether- <i>O</i> -glucoside         | Glc            | OCH <sub>3</sub> | H                |
| 72       | Isorhamnetin- <i>O</i> -acetyl-rutinoside            | Rut-Ac         | OH               | OCH <sub>3</sub> |
| 73       | Rhamnetin- <i>O</i> -glucuronide                     | GlcUA          | OCH <sub>3</sub> | OH               |
| 74       | Quercetin-dimethyl-ether- <i>O</i> -rutinoside       | Rut            | OCH <sub>3</sub> | OCH <sub>3</sub> |
| 75       | Quercetin-dimethylether- <i>O</i> -glucuronide       | GlcUA          | OCH <sub>3</sub> | OCH <sub>3</sub> |
| 76       | Kaempferol- <i>O</i> - <i>p</i> -coumaroylrhamnoside | Rham-Coum      | OH               | H                |

Rut – Rutin; GlcUA – Glucuronic acid; Glc – Glucose; Pent – Pentose; Rham – Rhamnose; Ac – Acetyl; Coum – *p*-coumaroyl.

**Figure 4.2** Proposed structures for the flavonoid glycosides identified in Portuguese propolis.

In fact, the product ion at  $m/z$  301 (quercetin) was formed by the loss of 176 Da, which is indicative of a glucuronyl unit.<sup>15</sup> Besides the aglycone fragment, the MS<sup>2</sup> spectrum of this quercetin-*O*-glucuronide also showed a major product ion at  $m/z$  300, formed by the homolytic cleavage of the *O*-glycosidic bond and has been proposed as indicative for quercetin glycosides.<sup>25</sup> The ion at  $m/z$  300 was also observed in the MS<sup>2</sup> spectrum of the remaining quercetin glycosides. Compounds 68, 70, 72-75 were identified as methylated derivatives of quercetin glycosides, and all presented an UV spectrum equivalent to that of isorhamnetin-3-*O*-rutinoside (Table 4.3). Compound 68 showed an ESI-MS<sup>2</sup> spectrum with a base peak ion at  $m/z$  315 (-132 Da) and the fragmentation pattern of the latter ion corresponded to that of isorhamnetin.

**Table 4.3** List of flavonoid glycosides identified by LC/DAD/ESI-MS<sup>n</sup> in uncommon propolis type

| Nr | t <sub>R</sub><br>(min) | λ <sub>max</sub> (nm) | [M-H] <sup>-</sup><br>m/z | LC/ESI-MS <sup>n</sup> m/z (% base peak)                                                                                               | Compound                                                          |
|----|-------------------------|-----------------------|---------------------------|----------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------|
| 63 | 15.3                    | 256, 352              | 609                       | MS <sup>2</sup> [609]: 301(100), 300 (87)                                                                                              | Quercetin-3- <i>O</i> -rutinoside <sup>a,b</sup>                  |
| 64 | 16.8                    | 256, 355              | 477                       | MS <sup>2</sup> [477]: 301; MS <sup>3</sup> [301]: 179 (100), 151 (39)                                                                 | Quercetin-3- <i>O</i> -glucuronide <sup>b</sup>                   |
| 65 |                         | 256, 355              | 463                       | MS <sup>2</sup> [463]: 301(100), 300 (64)                                                                                              | Quercetin-3- <i>O</i> -glucoside <sup>a,b</sup>                   |
| 66 | 17.6                    | 265, 349              | 593                       | MS <sup>2</sup> [593]: 285                                                                                                             | Kaempferol-3- <i>O</i> -rutinoside <sup>a,b</sup>                 |
| 67 | 17.9                    | 253, 355              | 623                       | MS <sup>2</sup> [623]: 315 (100), 300 (22)                                                                                             | Isorhamnetin-3- <i>O</i> -rutinoside <sup>a,b</sup>               |
| 68 | 19.6                    | 253, 346              | 447                       | MS <sup>2</sup> [447]: 315 (100), 300 (8); MS <sup>3</sup> [315]: 300; MS <sup>4</sup> [300]: 300 (100), 271 (47), 243 (47), 151 (<1)  | Isorhamnetin- <i>O</i> -pentoside <sup>b</sup>                    |
| 69 | 19.9                    | 256, 349              | 447                       | MS <sup>2</sup> [447]: 301(100), 300 (47)                                                                                              | Quercetin-3- <i>O</i> -rhamnoside <sup>a,b</sup>                  |
| 70 | 19.9                    | 253, 346              | 491                       | MS <sup>2</sup> [491]: 315; MS <sup>3</sup> [315]:300; MS <sup>4</sup> [300]: 271 (100), 255 (68), 151 (<1)                            | Isorhamnetin- <i>O</i> -glucuronide <sup>b</sup>                  |
| 71 | 20.1                    | 265, 343              | 461                       | MS <sup>2</sup> [461]: 446 (91), 299 (100), 284 (11); MS <sup>3</sup> [299]: 284; MS <sup>4</sup> [284]: 284 (100), 256 (60), 255 (52) | Kaempferol-methyl-ether- <i>O</i> -glucoside <sup>b</sup>         |
| 72 | 21.3                    | 253, 352              | 665                       | MS <sup>2</sup> [665]: 623 (18), 315 (100), 300 (14); MS <sup>3</sup> [315]: 300; MS <sup>4</sup> [315]:271(100), 255(57), 151 (<1)    | Isorhamnetin- <i>O</i> -acetylrutinoside <sup>b</sup>             |
| 73 | 25.2                    | 256, 349              | 491                       | MS <sup>2</sup> [491]: 315; MS <sup>3</sup> [315]: 300 (40), 193 (69), 165 (100)                                                       | Rhamnetin- <i>O</i> -glucuronide <sup>b</sup>                     |
| 74 | 25.4                    | 253, 349              | 637                       | MS <sup>2</sup> [637]: 329 (100), 314 (18); MS <sup>3</sup> [329]: 314; MS <sup>4</sup> [314]: 299 (100), 285 (25), 271 (26), 243 (10) | Quercetin-dimethyl-ether- <i>O</i> -rutinoside <sup>b</sup>       |
| 75 | 30.9                    | 253, 349              | 505                       | MS <sup>2</sup> [505]: 329 (100), 314 (18); MS <sup>3</sup> [329]: 314; MS <sup>4</sup> [314]: 299 (100), 285 (24), 271 (24), 243 (3)  | Quercetin-dimethyl-ether- <i>O</i> -glucuronide <sup>b</sup>      |
| 76 | 50.1                    | 265, 322              | 577                       | MS <sup>2</sup> [577]: 431 (6), 285 (100); MS <sup>3</sup> [285]: 257 (100), 151 (41)                                                  | Kaempferol- <i>O</i> - <i>p</i> -coumaroylrhamnoside <sup>b</sup> |

<sup>a</sup> Confirmed with standard; <sup>b</sup>Confirmed with MS<sup>n</sup> fragmentation.

Thus, overall, data indicated that the phenolic compound eluting at 19.6 min corresponded to an isorhamnetin pentoside derivative. Most probably, the pentoside residue moiety could be addressed to xylose or arabinose, since these two are the most commonly found in polyphenols.<sup>15</sup> On the other hand, compounds 70 and 73 both presented a  $[M-H]^-$  ion at  $m/z$  491 and the base peak in its  $MS^2$  spectrum ( $m/z$  315) was formed due to the loss of a glucuronide residue (-176 Da). Moreover, the fragmentation pathway (observed in the  $MS^3$  spectrum) of the ion at  $m/z$  315 in compound 70 was equivalent to that of isorhamnetin (methyl group in C-3' position), while that of compound 73 was equivalent to that of rhamnetin (methyl group in C-7 position). In accordance to these results, compounds 70 and 73 (MW 492 Da) were respectively assigned to isorhamnetin-*O*-glucuronide and rhamnetin-*O*-glucuronide. The MS analysis of compound 72 show the  $[M-H]^-$  at  $m/z$  665, and the  $MS^2$  spectrum showed a main product ion at  $m/z$  315 (-350 Da, a combined loss of rutinose and one acetyl residue), which corresponded to isorhamnetin, and an ion at  $m/z$  623 (-42 Da, loss of acetyl group). Both these product ions are coherent with the presence of an acetyldisaccharide moiety in the molecule. Note that flavonoid glycosides with an acylated glycosyl moiety part can be identified by the presence, in their  $MS^2$  spectra, of the product ions  $[acylsugar-H]^-$  and  $[M-H-acyl]^-$ ,<sup>26</sup> which are reported here. Thus, the discussed data led us to propose the compound 72 as isorhamnetin-*O*-acetylrutinoside. The linkage positions of the acyl group on the glycosidic part of the molecule and that of the acylsugar group on the aglycone could not be established on the basis of the UV spectra neither on the MS data as also found in the literature.<sup>26</sup> Compounds 74 and 75 showed a similar base peak ion in their ESI- $MS^2$  spectrum ( $m/z$  329) (Table 3), that was obtained by the loss of a rutinoside (-308 Da) and a glucuronide unit (-176), respectively. More, as the fragmentation pattern of that ion ( $m/z$  329) was consistent with that described earlier for quercetin-dimethyl-ether, we propose to assign the compounds 74 and 75 to quercetin-dimethyl-ether-*O*-rutinoside and quercetin-dimethyl-ether-*O*-glucuronide, respectively.

Kaempferol glycosides were present in compounds 71 ( $m/z$  461) and 76 ( $m/z$  577). In the first case, the  $MS^2$  data of the ion  $[M-H]^-$  at  $m/z$  461 was consistent with kaempferol-methyl-ether-*O*-hexoside: it showed a product ion at  $m/z$  446 (-15 Da) and a base peak product ion at  $m/z$  299 (-162 Da, loss of a hexoside, most probably glucose). For compound 76 the ESI- $MS^2$  data obtained for its  $[M-H]^-$  ion at  $m/z$  577 presented a base peak product ion at  $m/z$  285 with a fragmentation pattern similar to that of

kaempferol, and a mass loss of 146 and 292 Da. The elution time for this compound is significantly greater than all the other flavonoid glycosides and the UV spectrum shape show deviations from the flavonoid derivatives, with maximums at 265 and 319 nm (data not shown), where the band I is shifted to lower wavelengths with an increase in intensity. This UV behaviour was observed when an acylation by an aromatic acid occurs.<sup>24</sup> The results for this compound are consistent with a kaempferol linked with an acylated glycoside, most probably a *p*-coumaroylrhamnose,<sup>15,24</sup> however, further structural studies by NMR are necessary to determine the precise location of the groups in the flavonoid.

Overall, LC-MS data analysis of glycoside propolis type samples suggest the existence of one or more plant sources of propolis resin around the respective apiary, besides *Populus* species. The diversity of resin sources available for honeybees makes difficult the identification of the exact botanical origin of the flavonoid glycosides found in those propolis samples. Still, as these collection regions are rich in conifer plants, it is possible that these can be the main source of the glycoside flavonoids<sup>6</sup> herein described.

#### 4.4 Concluding remarks

This study allowed the establishment of the phenolic profile of Portuguese propolis from different geographical locations, with the detection of seventy six polyphenols and the recognition of two groups of propolis types: the common temperate propolis, which contained the typical poplar phenolic compounds such as flavonoids and their methylated/esterified forms, phenylpropanoid acids and their esters and an uncommon propolis type with an unusual composition in quercetin and kaempferol glycosides some of them never described in propolis. The existence of specific phenolic compounds in some propolis samples, such as kaempferol-dimethyl-ether, could be assigned as geographical markers. Data suggest that other botanical species besides poplar trees can be important sources of resins for Portuguese propolis.

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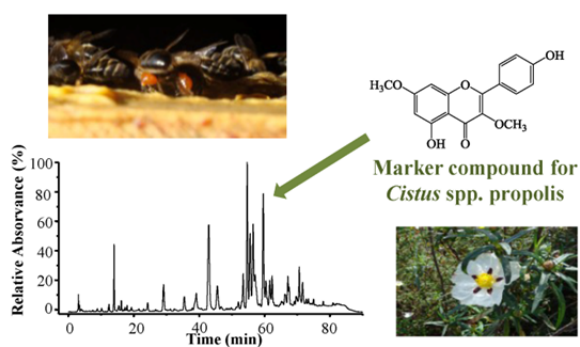
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# Chapter 5

## Phenolic quantification and botanical origin of Portuguese Propolis





## Phenolic quantification and botanical origin of Portuguese Propolis<sup>\*,†</sup>

### Abstract

The production of propolis by honeybee's results from a selective collection of exudates from leaf buds and plants present in the hive neighborhood leading to a resin with many potentialities in the pharmaceutical industry. This study aims to quantify the phenolic content in propolis from different Portuguese regions and in the potential floral sources, *Populus x Canadensis* Moench buds and *Cistus ladanifer* L., in order to establish links with geographical and botanical origin. The Portuguese propolis revealed a phenolic profile with marked differences in concentrations: the richness in flavonoids is common in all regions, but more evident in propolis from central interior, south and Madeira. The composition of poplar type propolis common in temperate zones was observed in the north, central coast and Azores, while the central interior and south samples, with a composition rich in kaempferol derivatives, resemble the *Cistus ladanifer* exudates, a spontaneous bush widespread in the Mediterranean. The compound kaempferol-3,7-dimethyl-ether, absent in the poplar type propolis, can be regarded as marker for this botanical origin discrimination.

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\*Adapted from Falcão SI, Tomás A, Vale N, Gomes P, Freire C, Vilas-Boas M. Phenolic quantification and botanical origin of Portuguese propolis. *Ind. Crop. Prod.* **2013**, *49*, 805-812.

†The experimental details about the propolis samples, phenolic compounds extraction, plant sources LC/DAD/ESI-MS<sup>n</sup> analysis and HPLC analysis are indicated in Appendices A, B and E respectively.

## 5.1 Introduction

The honeybee exploits nature as a store to fulfill its nutritional needs, collecting nectar and pollen, but also as a source for substances with other goals such as construction material or to maintain the antiseptic environment in the hive.<sup>1,2</sup> With the time evolution, bees were able to find in the surroundings of their nest the best source of materials for the desired purposes. Thus, it is not surprising the potentialities exhibit by propolis, a complex natural product gathered by the honeybees from resinous exudates of buds, leaves, branches and barks present in the vicinity of the beehive. Also named as the “bee glue” it plays an important role to guarantee the bee colony health.

Since ancient times, propolis is used in traditional medicine and now is gaining popularity in health foods as well in cosmetic products.<sup>2</sup> A great number of research studies focused on the pharmacological and biological properties present by propolis, including antihepatotoxic, antitumor, antioxidative, antimicrobial, anti-inflammatory and immunomodulatory among others.<sup>3,4</sup> These bioactivities are closely linked with the chemical composition, particularly with the richness in phenolic compounds, which accounts for approximately half of the resin content, while beeswax, volatiles and pollen represent 30%, 10% and 5%, respectively.<sup>1</sup> The propolis chemical composition varies greatly with the plant origin of the resin and thus with the geographic and climatic characteristics of the site.<sup>5</sup> The specificity of local flora is very important, not all plants are resin providers and bees have a marked preference for one or a few sticky resin sources, which are, at the same time sources of biologically active phytochemicals.<sup>6</sup> Based on this knowledge, propolis was typified according to their plant origin and its main chemical constituents.<sup>5</sup> In temperate zones of the world, poplar buds (*Populus* spp.) are the main sources of the bee glue with flavones, flavanones, phenolic acids and their esters as major compounds. Exceptions can be found, for example, the birch propolis type found in Russia, which has its origin in species like *Betula verrucosa*, where the main compounds are flavones and flavonols different from those found in poplar propolis.<sup>3</sup> Also a Mediterranean propolis type was found in Sicily, Crete and Malta, whose main compounds are diterpenes most probably originated in coniferous plant of the genus Cupressaceae.<sup>7</sup> Tropical propolis has a totally different compositional pattern: the green propolis type, found in Brazil, has its main plant source on the leaves of *Baccharis* spp. and mainly contains prenylated phenylpropanoids.<sup>3</sup> In Venezuela and Cuba, the main plant sources are the flower exudates of *Clusia* species, originating a

propolis rich in prenylated benzophenones.<sup>3</sup> C-prenylflavonoids (or propolins) have been described in propolis from Pacific islands, where the resin sources are the fruit exudates of the tree *Macaranga tanarius*.<sup>8</sup> The propolis typification on the basis of plant sources knowledge is a useful tool for its chemical standardization and thus for ensuring the quality and safety necessary for its commercialization.<sup>5,6</sup>

Recently, the phenolic profile of Portuguese propolis was characterized by liquid chromatography with diode-array detection coupled to electrospray ionization tandem mass spectrometry (LC/DAD/ESI-MS<sup>n</sup>).<sup>9</sup> Forty samples from different continental regions and islands were analyzed allowing the detection of seventy six polyphenols and the establishment of two different propolis groups: the common temperate propolis, which contained the typical poplar phenolic compounds such as flavonoids and their methylated/esterified forms, phenylpropanoid acids and their esters and an uncommon propolis type with an unusual composition in quercetin and kaempferol glycosides, some of them never described in propolis. The data suggest a diversified botanical origin for the Portuguese propolis besides poplar buds.<sup>9</sup>

Following those finds, we now present the phenolic quantification of propolis from the different Portuguese continental regions and islands. The results assort the compounds with major contribution to the propolis composition and allow the establishment of links with the geographical origin of this beehive product, a key factor for propolis commercial valorization. The inclusion of two potential floral sources of Portuguese propolis in this study, the buds exudates and surface material present on the leaves and stems of *Populus x Canadensis*, male and female specimens and *Cistus ladanifer* L. enable the correlation between the phenolic profile and the plant source of the resin, and the proposal of kaempferol-3,7-dimethyl-ether as a marker compound for *Cistus ladanifer* propolis.

## 5.2 Experimental

### 5.2.1 Materials, reagents and solvents

Chrysin, quercetin, pinocembrin, caffeic acid, ferulic acid, caffeic acid phenylethyl ester (CAPE), salicylic acid were purchased from Sigma Chemical Co (St Louis, MO, USA). Apigenin, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutoside, acacetin were from Extrasynthese (Genay, France). Analytical grade formic acid and HPLC grade

ethanol were obtained from Panreac (Barcelona, Spain). HPLC grade methanol and acetonitrile were purchased from Lab-Scan (Lisbon, Portugal). Water was treated in a Milli-Q water purification system (Topway Global Inc., Houston, TX, USA).

### 5.2.2 Plant sources samples

For the floral sources of the bee glue we collect, in Bragança region, northeast Portugal, in the spring of 2009, the leaf-buds of *Populus x Canadensis* Moenchen, male (PM) and female (PF) specimens and the leaves and stems of, and *Cistus ladanifer* (C). The voucher specimens are deposited at the herbarium of Escola Superior Agrária of Instituto Politécnico de Bragança with the reference number BRESA 5174, BRESA 5355 and BRESA 5356 for C, PF and PM, respectively. For the phenolic characterization we used the bud exudates of PM and PF specimens, while for *Cistus* the resin were scraped from the stems and leaves. All samples were stored at -20°C until analysis.

### 5.2.3 HPLC quantification

Quantification was achieved using calibration curves for caffeic acid, ferulic acid, quercetin, pinocembrin, chrysin, and caffeic acid phenylethyl ester. When the standard was not available, the compound quantification was expressed in equivalent of the structurally closest phenolic compound. The calibration parameters are shown in Table 5.1. The linearity was investigated by calculation of the regression plots by the least squares method and expressed by the correlation coefficient ( $R^2$ ). Concentrations of all compounds in propolis samples were calculated based on the peak area ratio. The limit of detection (LOD) and quantification (LOQ) were obtained from the y-intercept standard deviation ( $S_b$ ) and the slope ( $m$ ) of the calibration curve [12], thus  $LOD = 3 \times S_b/m$  and  $LOQ = 10 \times S_b/m$ .

### 5.2.4 Statistical Analysis

The statistic analysis was performed using SPSS version 18.0 program, and the hierarchical cluster was obtained with the Ward linkage method, using standardized variables.

**Table 5.1** Calibration parameters for the phenolic acids and flavonoids used as standards (mg/mL). The compound class to be quantified by each standard is also represented.

| Compound     | Linearity range | Slope | Intercept | $R^2$  | LOD (mg/mL) | LOQ (mg/mL) | Group to be quantified       |
|--------------|-----------------|-------|-----------|--------|-------------|-------------|------------------------------|
| Caffeic acid | 0.05-0.6        | 22.3  | -0.2      | 0.9990 | 0.01        | 0.05        | Phenolic acids               |
| Ferulic acid | 0.04-0.6        | 18.8  | -0.1      | 0.9993 | 0.01        | 0.04        | Methylated phenolic acids    |
| Quercetin    | 0.05-2.0        | 8.0   | -0.8      | 0.9999 | 0.02        | 0.05        | Flavonols                    |
| Pinocembrin  | 0.08-1.0        | 16.2  | -0.2      | 0.9991 | 0.02        | 0.08        | Flavanones; dihydroflavonols |
| Chrysin      | 0.03-1.0        | 23.0  | -1.0      | 0.9999 | 0.01        | 0.03        | Flavones                     |
| CAPE         | 0.06-1.0        | 13.8  | -0.1      | 0.9996 | 0.02        | 0.06        | Phenolic acids esters        |

LOD = limit of detection; LOQ = limit of quantification. CAPE = caffeic acid phenethyl ester

## 5.3 Results and discussion

### 5.3.1 LC/DAD/ESI-MS<sup>n</sup> analysis of the plant sources

The chemical composition of the plant source determines the chemical profile of propolis, therefore the profile comparison is the best indicator for the evaluation of propolis origin. In the present work we explore the phenolic composition of two potential plant sources of Portuguese propolis. They were chosen due to its great abundance in the hive neighborhoods and to the empirical knowledge of local beekeepers, which frequently associate these plants as the resin source.

Poplar buds are described as the main source of propolis in temperate zones.<sup>3</sup> The analysis of poplar bud exudates from *Populus nigra* and *Populus balsamifera* showed in their composition terpenoids, phenolic acids and their esters, flavonoid aglycons and their chalcones<sup>13, 14</sup> with a different degree of complexity depending on the specie. *Populus x Canadensis* is a hybrid poplar very common in Portugal and consequently a potential source of resin for honeybee, to our knowledge, not yet described.

For elucidation of its phenolic profile we collected the buds of male (PM) and female specimens (PF) in the neighbourhood of the hives and analysed by LC/DAD/ESI-MS<sup>n</sup>. Both PM and PF ethanolic extracts presented a phenolic profile similar to the one observed in Portuguese common temperate propolis type earlier described.<sup>9</sup> The composition is rich in phenolic acids and their derivatives, mainly caffeic acid, 3,4-dimethyl-caffeic acid, caffeic acid isoprenyl ester and its isomer, caffeic acid benzyl ester and caffeic acid phenylethyl ester. Also flavonoids and its

derivatives were found in the PM and PF extracts, with pinocembrin, chrysin, pinobanksin-3-*O*-acetate and galangin as major compounds. Comparing the two genders, some differences were found: the female poplar presented the compounds pinobanksin-5-methyl-ether-3-*O*-pentanoate, 3-hydroxy-5-methoxyflavanone, pinobanksin-3-*O*-butyrate, pinobanksin-3-*O*-pentenoate, pinobanksin-3-*O*-pentanoate, pinobanksin-3-*O*-hexanoate, which were previously described in Portuguese propolis<sup>13, 14</sup> and were absent from the male poplar phenolic profile.

*Cistus ladanifer* is a spontaneous shrubby plant widespread in the Mediterranean region.<sup>15</sup> Local beekeepers associate this material with propolis due to its abundance near the hives but also based on the typical odor of *Cistus* spp. that can be identified in some samples. The secretions on the surface of the leaves and stems of *Cistus ladanifer* were collected, extracted and analyzed by LC/DAD/ESI-MS<sup>n</sup>. The representative chromatogram at 280 nm is shown in Figure 5.1.

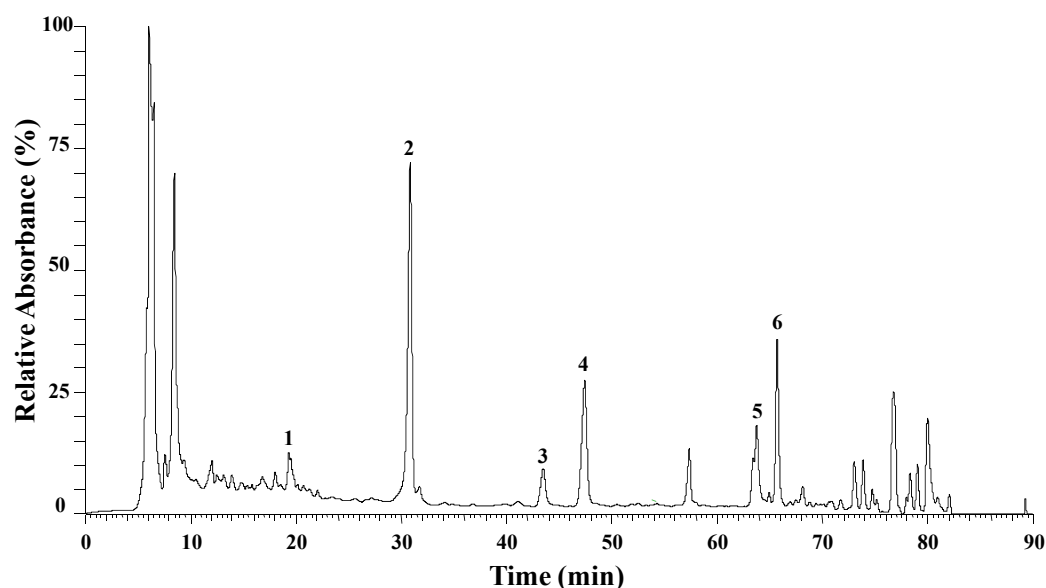


**Figure 5.1** Chromatographic profile at 280 nm for *Cistus ladanifer* exudates ethanolic extract: 1 – kaempferol-3-*O*-glucoside; 2 – kaempferol derivative; 3 – apigenin; 4 – kaempferol-methyl-ether; 5 – acacetin; 6 – kaempferol-3,7-dimethyl-ether.

This procedure allowed the detection of six flavonoids, mainly kaempferol derivatives, Table 5.2. These included kaempferol-3-*O*-glucoside (1), kaempferol-methyl-ether (4) and kaempferol-dimethyl-ether (6), of which the last two were recently described in propolis.<sup>9</sup> For a better assignment of the methyl positions on the flavonoid skeleton, a

deeper look was made on the UV and MS data of the kaempferol-dimethyl-ether. The spectrum of this compound present maximum absorption bands II and I at 265 and 346 nm, respectively. Comparing with the spectral data of kaempferol (265, 364 nm), the introduction of methyl ethers on the free hydroxyls groups undergoes a hypsochromic shift of 18 nm in band I accompanied with a relative drop in the intensity, indicating a 3-*O*-methylation. This remarkable difference is generally used as a diagnostic tool in the identification of free hydroxyls at the C-3 position of the flavonoid molecule, since the methylation in other positions has little effect on the absorption spectrum.<sup>16</sup> The other methyl ether group may be in C-7, C-5 or C-4' position. The introduction of more methyl ethers on the hydroxyls of the kaempferol increases lipophilicity and thus the retention time. Depending on the position of the methyl ether, the effect on the retention time is different, being the introduction on the C-7 and C-4' position less polar than the C-5, since the internal hydrogen bond between the hydroxyl and the carbonyl at C-4 position is no longer possible and thus retention time decreases.<sup>16</sup> For the compound under discussion the retention time is higher than kaempferol, therefore the C-5 position for the second methyl group can be disregarded. The fragmentation pattern of the product ion  $m/z$  313 produced the ion at  $m/z$  298 arising from the loss of methyl radical from the deprotonated molecular ion, as the most prominent fragment. A minor fragment of  $m/z$  165 was also identified which was resultant from the retro Diels-Alder mechanism, indicating the presence of the methyl group in C-7 position.<sup>16</sup> So, the compound was tentatively identified as kaempferol-3,7-dimethyl-ether, nevertheless for an unequivocal determination of the group location, further structural studies with NMR are necessary. The flavonoid 5,3'-dihydroxy-3,7,4',5'-tetramethoxyflavone observed in the composition of Tunisian propolis and assigned by Martos *et al.* (1997)<sup>10</sup> to the leaf exudates of *Cistus* spp. was not here identified. We cannot judge, however, if this difference arises from the geographical origin, or to the botanical specie, since no reference is given concerning the *Cistus* specie evaluated on that work.

**Table 5.2** Flavonoids identified by LC/DAD/ESI-MS<sup>n</sup> in *Cistus ladanifer* exudates.

| Nr | t <sub>R</sub><br>(min) | λ <sub>max</sub><br>(nm) | [M-H] <sup>-</sup><br>m/z | MS <sup>2</sup> (% base peak) | Compound                                       |
|----|-------------------------|--------------------------|---------------------------|-------------------------------|------------------------------------------------|
| 1  | 19.3                    | 265, 331                 | 447                       | 284 (100), 285 (66)           | Kaempferol-3- <i>O</i> -glucoside <sup>a</sup> |
| 2  | 30.9                    | 265, 313                 | 593                       | 285                           | Kaempferol derivative <sup>b</sup>             |
| 3  | 43.4                    | 268, 337                 | 269                       | 225 (100), 151 (69)           | Apigenin <sup>a</sup>                          |
| 4  | 47.4                    | 265, 352                 | 299                       | 284                           | Kaempferol-methyl-ether <sup>b,c</sup>         |
| 5  | 63.7                    | 268,331                  | 283                       | 269                           | Acacetin <sup>a</sup>                          |
| 6  | 65.7                    | 265, 346                 | 313                       | 299 (10), 298 (100)           | Kaempferol-3,7-dimethyl-ether <sup>b,c</sup>   |

<sup>a</sup>Confirmed with standard; <sup>b</sup>Confirmed with MS<sup>n</sup> fragmentation; <sup>c</sup>Confirmed with reference<sup>11</sup>

Besides these compounds, a kaempferol derivative (2) was identified without the total elucidation of its structure. The ESI-MS<sup>2</sup> data obtained for its [M-H]<sup>-</sup> ion at  $m/z$  593 presented a base peak product ion at  $m/z$  285 with a fragmentation pattern similar to that of kaempferol, and a mass loss of 308 Da. Although this loss could correspond to a rutinoside, the elution time is greater than for the commercial standard and the UV spectrum show deviations from that of kaempferol aglycone, with the band I enhanced in intensity and shifted back to 313, Table 5.2. These results could indicate an acylation by an aromatic acid in the molecule.<sup>16</sup> For the elucidation of the structure, further structural studies by NMR are required. Additionally, two flavones were identified, namely apigenin (3) and acacetin (5), which were present in the propolis samples.<sup>9</sup> This flavonoid pattern in the *Cistus ladanifer* exudates is consistent to the one described by Chaves *et al.* (1998)<sup>15</sup> with a composition rich in apigenin and kaempferol derivatives.

#### 5.4. Phenolic quantification in propolis – geographical origin

The phenolic complexity of propolis is linked with the phenolic diversity within a plant resin but also due to the combination of many different plants visited by the honeybees, particularly in sites with phyto-geographic diversity. Not all the resin sources or phenolic compounds within a resin are, however, in the propolis composition at significant amounts. In fact, some compounds are sometimes detected only as trace elements, which mean that the plant is scarce around the hive or it is not the honeybee preferred plant. For a better understanding of the phyto-diversity impact in the Portuguese propolis, we quantified the phenolic composition of forty samples, from different Portuguese geographical locations, using HPLC. The former evaluation of the phenolic profile of the samples<sup>9,11</sup> allowed the detection of seventy six phenolic compounds, including an uncommon group of flavonoid glycosides rarely described in this type of bee product.<sup>9</sup> From the overall list, only forty one compounds were herein detected above the limit of quantification. Figure 5.2a and 5.2b show the experimental chromatograms for the commercial standards used in the quantification and for a typical propolis sample, respectively.

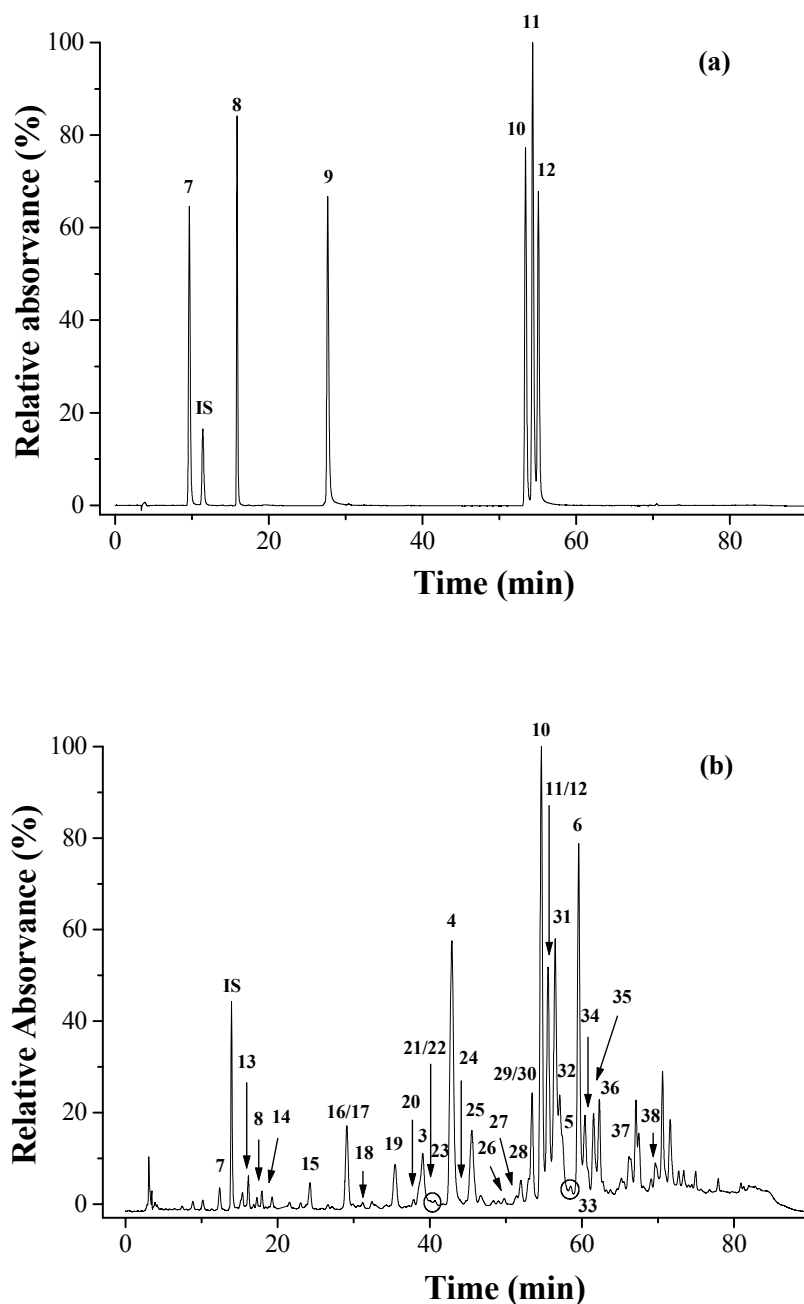


**Figure 5.2** Chromatographic profiles at 280 nm. **(a)** Standards compounds: 7 – caffeic acid (0.2 mg/mL); IS – internal standard (salicylic acid; 0.2 mg/mL); 8 – ferulic acid (0.2 mg/mL); 9 – quercetin (0.6 mg/mL); 10 – pinocembrin (0.3 mg/mL); 11 – chrysin (0.3 mg/mL); 12 – caffeic acid phenylethyl ester (0.3 mg/mL). **(b)** Propolis ethanolic extract of sample CI3 (10 mg/mL): 7 – caffeic acid; IS – internal standard (salicylic acid); 13 – *p*-coumaric acid; 8 – ferulic acid; 14 – isoferulic acid; 15 – 3,4-dimethyl-caffeic acid; 16 – pinobanksin-5-methyl-ether; 17 – cinnamic acid; 18 – *p*-coumaric acid methyl ester; 19 – pinobanksin; 20 – pinocembrin-5-methyl-ether; 3 – apigenin; 21 – chrysin-5-methyl-ether; 22 – kaempferol; 23 – isorhamnetin; 4 – kaempferol-methyl-ether; 24 – quercetin-dimethyl-ether; 25 – cinnamylidenacetic acid; 26 – rhamnetin; 27 –

quercetin-dimethyl-ether (isomer); 28 – caffeic acid isoprenyl ester; 29 – caffeic acid isoprenyl ester (isomer); 30 – caffeic acid benzyl ester; 10 – pinocembrin; 11 – chrysin; 12 – caffeic acid phenylethyl ester; 31 – pinobanksin-3-*O*-acetate; 32 – galangin; 5 – acacetin; 33 – chrysin-6-methyl-ether; 6 – kaempferol-dimethyl-ether; 34 – *p*-coumaric acid isoprenyl ester; 35 – caffeic acid cinnamyl ester; 36 – pinobanksin-3-*O*-propionate; 37 – pinobanksin-3-*O*-butyrate or isobutyrate; 38 – pinobanksin-3-*O*-pentanoate or 2-methylbutyrate.

Due to the number of compounds in the propolis chromatographic profile we decided to use in the quantification procedure a reduced number of phenolic compounds, representative of each class: caffeic acid, ferulic acid, caffeic acid phenylethyl ester, quercetin, pinocembrin and chrysin. For those compounds that do not fit under the chosen standards, the quantification was made in equivalents terms using the phenolic compound of the same chemical class, Table 5.1. Salicylic acid was chosen as internal standard, considering the detector response and the retention time, which did not interfere with the compounds under investigation. The analytical method exhibits a good linear response for all compounds, with correlation coefficients ( $R^2$ ) above 0.9990, and good sensitivity with the LOD varying between 0.01 and 0.02 mg/mL and LOQ varying from 0.03 and 0.08 mg/mL, Table 5.1.

**Table 5.3** Composition of phenolic classes present in Portuguese propolis and its plant sources (mg/g of extract).

| Sample               | Phenolic acids | Phenolic esters | Total phenolics | Flavonols | Flavones | Dihydroflavonols | Flavanones | Flavonoid esters | Flavonoid glycosides | Total Flavonoids |
|----------------------|----------------|-----------------|-----------------|-----------|----------|------------------|------------|------------------|----------------------|------------------|
| <b>Propolis</b>      |                |                 |                 |           |          |                  |            |                  |                      |                  |
| N1                   | 95.5           | 161.0           | 256.5           | 102.9     | 94.83    | 35.9             | 74.6       | 150.2            | nd                   | 458.4            |
| N2                   | 39.6           | 115.1           | 154.7           | 109.5     | 98.41    | 36.3             | 73.3       | 102.2            | nd                   | 419.7            |
| N3                   | 53.6           | 157.2           | 210.8           | 92.2      | 70.99    | 23.5             | 45.9       | 67.7             | nd                   | 300.2            |
| N4                   | 72.1           | 118.2           | 190.3           | 114.7     | 86.45    | 47.3             | 64.0       | 147.7            | nd                   | 460.0            |
| N5                   | 44.3           | 100.7           | 145.0           | 81.1      | 63.41    | 26.9             | 53.8       | 80.9             | nd                   | 306.2            |
| N6                   | 35.3           | 84.7            | 120.1           | 45.4      | 38.36    | 18.2             | 43.4       | 76.3             | nd                   | 221.6            |
| N7                   | 47.1           | 98.1            | 145.2           | 90.6      | 66.01    | 22.3             | 46.4       | 71.3             | nd                   | 296.6            |
| N8                   | 40.9           | 93.2            | 134.1           | 112.2     | 84.37    | 39.0             | 93.4       | 128.3            | nd                   | 457.3            |
| N9                   | 83.5           | 32.3            | 115.9           | 68.7      | 36.39    | 47.8             | 25.4       | 25.1             | nd                   | 203.4            |
| N10                  | nq             | 76.8            | 76.8            | 62.5      | 60.26    | 6.8              | 27.7       | 55.1             | nd                   | 212.3            |
| N11                  | 12.9           | 28.1            | 41.1            | 49.1      | 35.65    | 3.6              | 8.3        | 12.4             | 23.2                 | 132.1            |
| N12                  | 1.5            | 6.4             | 8.0             | 47.1      | 30.16    | 2.7              | 7.8        | 9.6              | 12.6                 | 109.9            |
| N13                  | 1.0            | nq              | 1.0             | 45.5      | 19.49    | 1.9              | nq         | 1.5              | 19.4                 | 87.8             |
| N14                  | 89.9           | 171.4           | 261.3           | 55.9      | 74.97    | 61.2             | 55.5       | 42.6             | nd                   | 290.2            |
| CI1                  | 43.6           | nq              | 43.6            | 167.8     | 96.22    | 10.6             | 17.9       | 14.4             | 227.7                | 534.6            |
| CI2                  | nq             | nq              | nq              | 50.2      | 20.59    | 2.0              | nq         | nq               | nd                   | 72.8             |
| CI3                  | 4.9            | 19.8            | 24.7            | 123.3     | 35.19    | 4.5              | 24.9       | 16.0             | nd                   | 203.9            |
| CI4                  | 15.5           | 27.1            | 42.6            | 99.9      | 33.12    | 5.5              | 15.9       | 25.7             | nd                   | 180.1            |
| CC1                  | 58.4           | 91.8            | 150.1           | 79.3      | 53.39    | 50.1             | 53.4       | 90.4             | nd                   | 326.6            |
| CC2                  | 30.4           | 36.4            | 66.9            | 100.8     | 50.85    | 75.2             | 60.1       | 96.6             | nd                   | 383.5            |
| CC3                  | 59.7           | 97.7            | 157.4           | 85.2      | 62.70    | 24.4             | 52.5       | 72.8             | nd                   | 297.6            |
| CC4                  | 24.9           | 103.1           | 128.0           | 100.7     | 71.55    | 23.3             | 60.5       | 94.3             | nd                   | 350.4            |
| S1                   | 42.5           | 88.3            | 130.8           | 99.6      | 74.48    | 38.4             | 61.4       | 91.9             | nd                   | 365.8            |
| S2                   | nq             | nq              | nq              | 90.4      | 22.21    | 4.0              | nq         | nq               | nd                   | 116.6            |
| S3                   | nq             | nq              | nq              | 121.1     | 24.56    | 6.2              | nq         | nq               | nd                   | 151.9            |
| S4                   | 36.8           | 6.8             | 43.6            | 82.3      | 22.41    | 2.7              | nq         | nq               | 11.5                 | 118.9            |
| A1                   | 51.8           | 129.8           | 181.6           | 99.1      | 84.48    | 33.2             | 64.0       | 88.5             | nd                   | 369.3            |
| A2                   | 67.5           | 171.7           | 239.3           | 56.2      | 67.73    | 11.9             | 36.4       | 65.1             | nd                   | 237.4            |
| A3                   | 56.4           | 130.8           | 187.2           | 41.4      | 63.43    | 11.4             | 45.0       | 50.2             | nd                   | 211.5            |
| A4                   | 47.9           | 110.0           | 157.9           | 39.8      | 52.87    | 10.3             | 38.9       | 41.4             | nd                   | 183.2            |
| A5                   | 55.0           | 98.4            | 153.4           | 40.6      | 47.66    | 10.8             | 32.9       | 43.2             | nd                   | 175.1            |
| A6                   | 63.5           | 142.6           | 206.1           | 47.7      | 59.54    | 10.7             | 39.2       | 63.0             | nd                   | 220.1            |
| A7                   | 60.9           | 139.9           | 200.8           | 55.4      | 53.08    | 10.3             | 31.8       | 56.1             | nd                   | 206.6            |
| A8                   | 35.8           | 85.4            | 121.2           | 50.7      | 45.44    | 7.8              | 25.3       | 44.1             | nd                   | 173.3            |
| A9                   | 38.5           | 73.7            | 112.3           | 52.1      | 48.84    | 12.5             | 30.2       | 47.2             | nd                   | 190.9            |
| A10                  | 52.7           | 92.2            | 144.8           | 56.7      | 47.70    | 13.1             | 25.9       | 20.9             | nd                   | 164.3            |
| A11                  | 60.0           | 123.2           | 183.2           | 82.6      | 69.36    | 23.6             | 40.5       | 31.7             | nd                   | 247.7            |
| M1                   | nq             | nq              | nq              | nd        | 14.15    | nd               | nq         | nq               | nd                   | 14.1             |
| M2                   | nq             | nq              | nq              | 91.4      | 4.64     | 8.4              | nq         | nd               | 14.7                 | 119.1            |
| M3                   | 64.2           | 28.4            | 92.6            | 61.0      | 25.22    | 8.6              | 32.1       | 34.1             | nd                   | 160.9            |
| <b>Plant sources</b> |                |                 |                 |           |          |                  |            |                  |                      |                  |
| PM                   | 29.1           | 59.1            | 88.2            | 41.8      | 35.38    | 6.0              | 21.5       | 23.4             | nd                   | 128.1            |
| PF                   | 29.4           | 64.5            | 93.8            | 30.9      | 46.95    | 3.8              | 36.8       | 41.7             | nd                   | 160.1            |
| C                    | nd             | nd              | nd              | 26.9      | 10.57    | nd               | nd         | nd               | 27.4                 | 64.8             |

nq – not quantified; nd – not detected

The majority of the propolis samples presented a similar phenolic profile, but with marked differences in their concentrations. For an easier interpretation of the results, the individual compounds were aggregated in phenolic classes, as shown in Table 5.3, however the individual phenolic concentration can be found in Tables 5.4-5.7. In all the regions, flavonoids were more abundant than simple phenolics, Figure 5.3a, with pinocembrin, chrysin, pinobanksin-3-*O*-acetate and galangin as major compounds. The difference between these two groups is even more evident in samples from central interior, south and Madeira Island, where flavonoids represented more than 85% of the total phenolic content.

The contribution of the individual chemical classes was not identical for each region: for the simple phenolics, the esters derivatives were more abundant than the corresponding acid compounds, with the propolis from north of Portugal and Azores Archipelago revealing the highest content, with 89 and 117 mg/g, respectively, Figure 5.3b. Samples from the central interior region, south and Madeira Island were exceptions, with an evident low content in simple phenolics, where phenolic acids were prevailing.

The flavonoid content was also distinct between regions and highly abundant in propolis from the north and central coast, Figure 5.3c, although some samples can be seen as outliers in their region, particularly those from central interior CI1 with an abnormal high content, 534.6 mg/g and the samples N11-N13 from north, CI2 from central interior, S2 and S4 from south and M1-M2 from Madeira island, with values below 132 mg/g of extract, Table 5.3. Looking at each individual flavonoid sub-classes, the concentrations profile changed between regions, which must stem from the different vegetation sources around the hive: in the north, central coast and Azores, propolis was richer in flavonols, flavones and flavonoid esters, while on the south and in Madeira the propolis flavonoid composition was mainly flavonols (above 50%). The propolis from the central interior fitted on a different level due to the huge presence of flavonoid glycosides, observed also in the north, south and Madeira but only at very low concentration, Figure 5.3c.

The flavonols concentration varied from the highest average value of 110 mg/g found in the central interior to 50 mg/g in Madeira propolis. With exception of propolis from Azores, this class was the most relevant for the total flavonoid content (25% in Azores to 52% in the south).

**Table 5.4** Phenolic content in Portuguese propolis samples N1-N12 (mg/g of extract)

| Compound                                | t <sub>R</sub> (min) | N1       | N2       | N3         | N4        | N5       | N6       | N7       | N8        | N9        | N10        | N11      | N12      |
|-----------------------------------------|----------------------|----------|----------|------------|-----------|----------|----------|----------|-----------|-----------|------------|----------|----------|
| Caffeic acid                            | 12.4                 | 11.6±0.6 | 8.7±0.3  | 7.39±0.02  | 14.9±0.1  | 7.8±0.0  | nq       | 10.7±0.3 | 8.4±0.0   | 14.3±0.0  | nq         | nq       | nq       |
| <i>p</i> -Coumaric acid                 | 16.2                 | 11.1±0.7 | 4.9±0.1  | 8.08±0.01  | 12.3±0.1  | 8.5±0.0  | 9.6±0.1  | 10.6±0.3 | 8.9±0.0   | 7.9±0.2   | nq         | 4.0±0.8  | nq       |
| Ferulic acid                            | 17.3                 | nq       | nq       | nq         | 6.1±0.1   | nq       | nq       | nq       | nq        | 5.7±0.1   | nq         | nq       | nq       |
| Ellagic acid                            | 17.5                 | nd       | nd       | nd         | nd        | nd       | nd       | nd       | nd        | nd        | nd         | 5.0±0.9  | nq       |
| Isoferulic acid                         | 17.9                 | 10.7±0.4 | 4.4±0.1  | 9.2±0.1    | 8.2±0.1   | 7.9±0.1  | 4.1±0.4  | 9.2±0.3  | 5.1±0.0   | 11.4±0.2  | nq         | 3.9±0.8  | 1.6±0.0  |
| 3,4-Dimethyl-caffeic acid               | 24.2                 | 18.3±0.3 | 7.1±0.1  | 9.45±0.02  | 11.9±0.1  | 8.0±0.0  | 5.3±0.0  | 10.0±0.3 | 9.3±0.1   | 14.7±0.1  | nq         | nq       | nq       |
| Pinobanksin-5-methyl-ether              | 29.1                 | 15.2±0.6 | 13.4±0.4 | 12.7±0.1   | 26.8±0.2  | 14.5±0.1 | 9.4±0.0  | 11.7±0.4 | 23.7±0.1  | 38.9±0.9  | nq         | nq       | nq       |
| Cinnamic acid                           | 29.1                 | 22.0±0.3 | 4.5±0.0  | 5.3±0.1    | nq        | nq       | 7.8±0.1  | nq       | nq        | nq        | nq         | nq       | nq       |
| Luteolin                                | 30.4                 | nd       | nd       | nd         | nd        | nd       | nd       | nd       | nd        | nd        | nd         | nd       | nd       |
| <i>p</i> -Coumaric acid methyl ester    | 31.2                 | 11.5±0.0 | nq       | 11.36±0.02 | 4.29±0.01 | 6.7±0.1  | 4.1±0.0  | 7.1±0.2  | 5.06±0.04 | nq        | nq         | 4.5±0.6  | nq       |
| Pinobanksin                             | 35.4                 | 20.7±0.9 | 22.9±0.7 | 10.79±0.01 | 20.4±0.1  | 12.4±0.0 | 8.8±0.0  | 10.6±0.3 | 15.3±0.0  | 8.9±0.1   | 6.8±0.1    | 3.6±0.4  | 2.7±0.1  |
| Pinocembrin-5-methyl-ether              | 38.0                 | nq       | nq       | nq         | nq        | nq       | nq       | nq       | nq        | nq        | nq         | nq       | nq       |
| Apigenin                                | 39.1                 | 8.4±0.5  | 10.8±0.3 | 9.68±0.01  | 6.7±0.1   | 5.9±0.1  | 5.2±0.0  | 6.7±0.2  | 11.0±0.0  | 5.9±0.6   | 5.2±0.0    | 6.6±0.2  | 5.9±0.1  |
| chrysin-5-methyl-ether                  | 40.8                 | 4.4±0.0  | nq       | nq         | 5.2±0.3   | nq       | nq       | 5.0±0.1  | nq        | 4.6±0.0   | nd         | nd       | nd       |
| Kaempferol                              | 41.0                 | 10.5±0.4 | 10.6±0.1 | 10.4±0.0   | 11.9±0.2  | 9.8±0.1  | 11.4±0.1 | 9.7±0.1  | 12.9±0.4  | 10.4±0.1  | 9.7±0.1    | 12.8±0.4 | 12.2±0.1 |
| Isorhamnetin                            | 42.0                 | 9.6±0.0  | 10.5±0.1 | 9.46±0.04  | 11.2±0.1  | 9.5±0.1  | nd       | 9.6±0.1  | 9.9±0.2   | 9.8±0.1   | nd         | 11.0±0.0 | 10.5±0.1 |
| Kaempferol-methyl-ether                 | 43.0                 | 15.2±0.6 | 20.3±0.5 | 13.1±0.0   | 15.8±0.2  | 16.7±0.2 | 11.6±0.2 | 17.3±0.3 | 19.3±0.3  | 12.1±0.4  | 16.1±0.1   | nd       | nd       |
| Kaempferol-methoxy-methyl-ether         | 44.0                 | nd       | nd       | nd         | nd        | nd       | nd       | nd       | nd        | nd        | nd         | nd       | nd       |
| Quercetin-dimethyl-ether                | 44.8                 | 10.8±0.2 | 12.0±0.2 | 10.1±0.3   | 12.5±0.1  | nd       | nd       | 10.6±0.0 | 11.5±0.1  | nd        | 9.9±0.03   | nd       | nd       |
| Quercetin-tetramethyl-ether             | 45.2                 | nd       | nd       | nd         | nd        | nd       | nd       | nd       | nd        | nd        | nd         | nd       | nd       |
| Cinnamylidenacetic acid                 | 45.5                 | 21.9±0.4 | 10.1±0.3 | 14±1       | 18.6±0.2  | 12.1±0.0 | 8.5±0.0  | 6.6±0.2  | 9.2±0.1   | 29.5±0.1  | nq         | nq       | 21.9±0.0 |
| Rhamnetin                               | 49.8                 | 9.9±0.0  | 11.6±0.1 | 10.2±0.1   | 9.4±0.0   | 9.7±0.4  | nq       | 10.0±0.1 | 10.3±0.2  | 9.42±0.01 | nq         | nq       | nq       |
| Quercetin-dimethyl-ether (isomer)       | 51.4                 | 12.9±0.5 | 11.2±0.1 | 11.0±0.2   | 13.9±0.2  | 9.8±0.6  | nq       | 11.5±0.2 | 14±2      | 9.72±0.04 | 11.31±0.01 | nq       | nq       |
| Apigenin-6,4'-dimethyl-ether            | 51.8                 | nd       | nd       | nd         | nd        | nd       | nd       | nd       | nd        | nd        | nd         | nd       | nd       |
| Caffeic acid isoprenyl ester            | 52.1                 | 26±1     | 15.4±0.5 | 31±3       | 16.7±0.2  | 15.5±0.8 | 13.3±0.2 | 15.5±0.3 | 9±1       | 5.6±0.0   | 10.2±0.2   | 6.1±0.4  | nq       |
| Caffeic acid isoprenyl ester (isomer)   | 53.2                 | 37±1     | 24.4±0.6 | 43±4       | 17.4±0.2  | 21.1±0.4 | 13±1     | 18.8±0.6 | 12.1±0.1  | nq        | 10.3±0.1   | 8.5±0.4  | nq       |
| Caffeic acid benzyl ester               | 53.6                 | 29±2     | 18.1±0.3 | 22±2       | 28.1±0.4  | 20.4±0.0 | 18±3     | 23.7±0.5 | 21.6±0.1  | 11.3±0.0  | 19.0±0.4   | nq       | nq       |
| Pinocembrin                             | 54.7                 | 75±2     | 73±2     | 45.9±0.1   | 64.0±0.8  | 53.8±0.7 | 43.4±0.6 | 46±2     | 93±16     | 25.4±0.1  | 27.7±0.6   | 8.3±0.8  | 7.8±0.0  |
| Chrysin                                 | 55.6                 | 68±2     | 73±2     | 47.9±0.2   | 59.8±0.5  | 43.2±0.2 | 27.6±0.3 | 42±1     | 59±9      | 16.6±0.2  | 38±1       | 12±1     | 9.0±0.1  |
| Caffeic acid phenylethyl ester          | 55.6                 | 20±3     | 22.7±0.7 | 14.7±0.1   | 18.5±0.2  | 13.2±0.1 | 8.3±0.1  | 12.7±0.4 | 18±3      | 4.8±0.1   | 13.4±0.8   | 9±1      | 6.4±0.2  |
| Pinobanksin-3- <i>O</i> -acetate        | 56.5                 | 77±4     | 75±2     | 57.4±0.0   | 94±2      | 51.4±0.9 | 40.5±0.8 | 40±1     | 78.4±0.4  | 25.1±0.2  | 20.0±0.1   | 12±2     | 9.6±0.2  |
| Galangin                                | 57.3                 | 34±2     | 33.4±0.6 | 27.8±0.0   | 39.9±0.6  | 25.6±0.3 | 22.4±0.3 | 22.0±0.5 | 34.6±0.1  | 17.1±0.1  | 15.6±0.1   | 12.2±0.0 | 12.0±0.2 |
| Acacetin                                | 57.5                 | 4.8±0.2  | 7.1±0.0  | 6.2±0.1    | 6.4±0.3   | 8±1      | nq       | 6.1±0.2  | 5.7±0.1   | 7.4±0.1   | 6.2±0.1    | 9.8±0.9  | 8.9±0.2  |
| Kaempferide                             | 58.0                 | nd       | nd       | nd         | nd        | nd       | nd       | nd       | nd        | nd        | nd         | 13.0±0.7 | 12.5±0.2 |
| Chrysoeriol-methyl-ether                | 58.3                 | nd       | nd       | nd         | nd        | nd       | nd       | nd       | nd        | nd        | nd         | nd       | nd       |
| Chrysin-6-methyl-ether                  | 58.6                 | 8.9±0.2  | 7.3±0.0  | 7.1±0.0    | 8.4±0.0   | 5.8±0.0  | 5.6±0.1  | 6.6±0.2  | 9.0±0.21  | 6.4±0.0   | 5.9±0.0    | 7±1      | 6.5±0.3  |
| kaempferol-3,7-dimethyl-ether           | 59.7                 | nd       | nd       | nd         | nd        | nd       | nd       | nd       | nd        | nd        | nd         | nd       | nd       |
| <i>p</i> -Coumaric acid isoprenyl ester | 60.6                 | 6±1      | 19.9±0.4 | 22.1±0.7   | nq        | 5.2±0.2  | nq       | 5.9±0.4  | 6.4±0.3   | nq        | 7.2±0.5    | nq       | nq       |
| Caffeic acid cinnamyl ester             | 61.7                 | 33±10    | 14.5±0.5 | 12.4±0.1   | 33.3±0.1  | 18.6±0.5 | 28.3±0.0 | 14.4±0.9 | 21.4±0.7  | 10.7±0.6  | 17±1       | nq       | nq       |
| Pinobanksin-3- <i>O</i> -propionate     | 62.4                 | 11±3     | nq       | nq         | 25.4±0.2  | 15±2     | 16.2±0.1 | 19±1     | 22.3±0.0  | nq        | 16±1       | nq       | nq       |
| Pinobanksin-3- <i>O</i> -butyrate       | 66.3                 | 39±10    | 8.6±0.2  | nq         | 6.5±0.4   | nq       | 19.5±0.3 | nq       | 11.5±0.9  | nq        | nq         | nq       | nq       |
| Pinobanksin-3- <i>O</i> -pentanoate     | 69.1                 | 23±7     | 19.0±0.4 | 10.2±0.2   | 21.3±0.1  | 14.6±0.2 | nq       | 12.2±0.1 | 16.0±0.6  | nq        | 18.8±0.5   | nq       | nq       |

nq – not quantified; nd – not detected

**Table 5.5** Phenolic content in Portuguese propolis samples N13-14, CI1-4, CC1-4 and S1-2 (mg/g of extract).

| Compound                                | t <sub>R</sub> (min) | N1       | N2       | N3         | N4        | N5       | N6       | N7       | N8        | N9        | N10        | N11      | N12      |
|-----------------------------------------|----------------------|----------|----------|------------|-----------|----------|----------|----------|-----------|-----------|------------|----------|----------|
| Caffeic acid                            | 12.4                 | 11.6±0.6 | 8.7±0.3  | 7.39±0.02  | 14.9±0.1  | 7.8±0.0  | nq       | 10.7±0.3 | 8.4±0.0   | 14.3±0.0  | nq         | nq       | nq       |
| <i>p</i> -Coumaric acid                 | 16.2                 | 11.1±0.7 | 4.9±0.1  | 8.08±0.01  | 12.3±0.1  | 8.5±0.0  | 9.6±0.1  | 10.6±0.3 | 8.9±0.0   | 7.9±0.2   | nq         | 4.0±0.8  | nq       |
| Ferulic acid                            | 17.3                 | nq       | nq       | nq         | 6.1±0.1   | nq       | nq       | nq       | nq        | 5.7±0.1   | nq         | nq       | nq       |
| Ellagic acid                            | 17.5                 | nd       | nd       | nd         | nd        | nd       | nd       | nd       | nd        | nd        | nd         | 5.0±0.9  | nq       |
| Isoferulic acid                         | 17.9                 | 10.7±0.4 | 4.4±0.1  | 9.2±0.1    | 8.2±0.1   | 7.9±0.1  | 4.1±0.4  | 9.2±0.3  | 5.1±0.0   | 11.4±0.2  | nq         | 3.9±0.8  | 1.6±0.0  |
| 3,4-Dimethyl-caffeic acid               | 24.2                 | 18.3±0.3 | 7.1±0.1  | 9.45±0.02  | 11.9±0.1  | 8.0±0.0  | 5.3±0.0  | 10.0±0.3 | 9.3±0.1   | 14.7±0.1  | nq         | nq       | nq       |
| Pinobanksin-5-methyl-ether              | 29.1                 | 15.2±0.6 | 13.4±0.4 | 12.7±0.1   | 26.8±0.2  | 14.5±0.1 | 9.4±0.0  | 11.7±0.4 | 23.7±0.1  | 38.9±0.9  | nq         | nq       | nq       |
| Cinnamic acid                           | 29.1                 | 22.0±0.3 | 4.5±0.0  | 5.3±0.1    | nq        | nq       | 7.8±0.1  | nq       | nq        | nq        | nq         | nq       | nq       |
| Luteolin                                | 30.4                 | nd       | nd       | nd         | nd        | nd       | nd       | nd       | nd        | nd        | nd         | nd       | nd       |
| <i>p</i> -Coumaric acid methyl ester    | 31.2                 | 11.5±0.0 | nq       | 11.36±0.02 | 4.29±0.01 | 6.7±0.1  | 4.1±0.0  | 7.1±0.2  | 5.06±0.04 | nq        | nq         | 4.5±0.6  | nq       |
| Pinobanksin                             | 35.4                 | 20.7±0.9 | 22.9±0.7 | 10.79±0.01 | 20.4±0.1  | 12.4±0.0 | 8.8±0.0  | 10.6±0.3 | 15.3±0.0  | 8.9±0.1   | 6.8±0.1    | 3.6±0.4  | 2.7±0.1  |
| Pinocembrin-5-methyl-ether              | 38.0                 | nq       | nq       | nq         | nq        | nq       | nq       | nq       | nq        | nq        | nq         | nq       | nq       |
| Apigenin                                | 39.1                 | 8.4±0.5  | 10.8±0.3 | 9.68±0.01  | 6.7±0.1   | 5.9±0.1  | 5.2±0.0  | 6.7±0.2  | 11.0±0.0  | 5.9±0.6   | 5.2±0.0    | 6.6±0.2  | 5.9±0.1  |
| chrysin-5-methyl-ether                  | 40.8                 | 4.4±0.0  | nq       | nq         | 5.2±0.3   | nq       | nq       | 5.0±0.1  | nq        | 4.6±0.0   | nq         | nd       | nd       |
| Kaempferol                              | 41.0                 | 10.5±0.4 | 10.6±0.1 | 10.4±0.0   | 11.9±0.2  | 9.8±0.1  | 11.4±0.1 | 9.7±0.1  | 12.9±0.4  | 10.4±0.1  | 9.7±0.1    | 12.8±0.4 | 12.2±0.1 |
| Isorhamnetin                            | 42.0                 | 9.6±0.0  | 10.5±0.1 | 9.46±0.04  | 11.2±0.1  | 9.5±0.1  | nd       | 9.6±0.1  | 9.9±0.2   | 9.8±0.1   | nd         | 11.0±0.0 | 10.5±0.1 |
| Kaempferol-methyl-ether                 | 43.0                 | 15.2±0.6 | 20.3±0.5 | 13.1±0.0   | 15.8±0.2  | 16.7±0.2 | 11.6±0.2 | 17.3±0.3 | 19.3±0.3  | 12.1±0.4  | 16.1±0.1   | nd       | nd       |
| Kaempferol-methoxy-methyl-ether         | 44.0                 | nd       | nd       | nd         | nd        | nd       | nd       | nd       | nd        | nd        | nd         | nd       | nd       |
| Quercetin-dimethyl-ether                | 44.8                 | 10.8±0.2 | 12.0±0.2 | 10.1±0.3   | 12.5±0.1  | nd       | nd       | 10.6±0.0 | 11.5±0.1  | nd        | 9.9±0.03   | nd       | nd       |
| Quercetin-tetramethyl-ether             | 45.2                 | nd       | nd       | nd         | nd        | nd       | nd       | nd       | nd        | nd        | nd         | nd       | nd       |
| Cinnamylidenacetic acid                 | 45.5                 | 21.9±0.4 | 10.1±0.3 | 14±1       | 18.6±0.2  | 12.1±0.0 | 8.5±0.0  | 6.6±0.2  | 9.2±0.1   | 29.5±0.1  | nq         | nq       | 21.9±0.0 |
| Rhamnetin                               | 49.8                 | 9.9±0.0  | 11.6±0.1 | 10.2±0.1   | 9.4±0.0   | 9.7±0.4  | nq       | 10.0±0.1 | 10.3±0.2  | 9.42±0.01 | nq         | nq       | nq       |
| Quercetin-dimethyl-ether (isomer)       | 51.4                 | 12.9±0.5 | 11.2±0.1 | 11.0±0.2   | 13.9±0.2  | 9.8±0.6  | nq       | 11.5±0.2 | 14±2      | 9.72±0.04 | 11.31±0.01 | nq       | nq       |
| Apigenin-6,4'-dimethyl-ether            | 51.8                 | nd       | nd       | nd         | nd        | nd       | nd       | nd       | nd        | nd        | nd         | nd       | nd       |
| Caffeic acid isoprenyl ester            | 52.1                 | 26±1     | 15.4±0.5 | 31±3       | 16.7±0.2  | 15.5±0.8 | 13.3±0.2 | 15.5±0.3 | 9±1       | 5.6±0.0   | 10.2±0.2   | 6.1±0.4  | nq       |
| Caffeic acid isoprenyl ester (isomer)   | 53.2                 | 37±1     | 24.4±0.6 | 43±4       | 17.4±0.2  | 21.1±0.4 | 13±1     | 18.8±0.6 | 12.1±0.1  | nq        | 10.3±0.1   | 8.5±0.4  | nq       |
| Caffeic acid benzyl ester               | 53.6                 | 29±2     | 18.1±0.3 | 22±2       | 28.1±0.4  | 20.4±0.0 | 18±3     | 23.7±0.5 | 21.6±0.1  | 11.3±0.0  | 19.0±0.4   | nq       | nq       |
| Pinocembrin                             | 54.7                 | 75±2     | 73±2     | 45.9±0.1   | 64.0±0.8  | 53.8±0.7 | 43.4±0.6 | 46±2     | 93±16     | 25.4±0.1  | 27.7±0.6   | 8.3±0.8  | 7.8±0.0  |
| Chrysin                                 | 55.6                 | 68±2     | 73±2     | 47.9±0.2   | 59.8±0.5  | 43.2±0.2 | 27.6±0.3 | 42±1     | 59±9      | 16.6±0.2  | 38±1       | 12±1     | 9.0±0.1  |
| Caffeic acid phenylethyl ester          | 55.6                 | 20±3     | 22.7±0.7 | 14.7±0.1   | 18.5±0.2  | 13.2±0.1 | 8.3±0.1  | 12.7±0.4 | 18±3      | 4.8±0.1   | 13.4±0.8   | 9±1      | 6.4±0.2  |
| Pinobanksin-3- <i>O</i> -acetate        | 56.5                 | 77±4     | 75±2     | 57.4±0.0   | 94±2      | 51.4±0.9 | 40.5±0.8 | 40±1     | 78.4±0.4  | 25.1±0.2  | 20.0±0.1   | 12±2     | 9.6±0.2  |
| Galangin                                | 57.3                 | 34±2     | 33.4±0.6 | 27.8±0.0   | 39.9±0.6  | 25.6±0.3 | 22.4±0.3 | 22.0±0.5 | 34.6±0.1  | 17.1±0.1  | 15.6±0.1   | 12.2±0.0 | 12.0±0.2 |
| Acacetin                                | 57.5                 | 4.8±0.2  | 7.1±0.0  | 6.2±0.1    | 6.4±0.3   | 8±1      | nq       | 6.1±0.2  | 5.7±0.1   | 7.4±0.1   | 6.2±0.1    | 9.8±0.9  | 8.9±0.2  |
| Kaempferide                             | 58.0                 | nd       | nd       | nd         | nd        | nd       | nd       | nd       | nd        | nd        | nd         | 13.0±0.7 | 12.5±0.2 |
| Chrysoeriol-methyl-ether                | 58.3                 | nd       | nd       | nd         | nd        | nd       | nd       | nd       | nd        | nd        | nd         | nd       | nd       |
| Chrysin-6-methyl-ether                  | 58.6                 | 8.9±0.2  | 7.3±0.0  | 7.1±0.0    | 8.4±0.0   | 5.8±0.0  | 5.6±0.1  | 6.6±0.2  | 9.0±0.21  | 6.4±0.0   | 5.9±0.0    | 7±1      | 6.5±0.3  |
| kaempferol-3,7-dimethyl-ether           | 59.7                 | nd       | nd       | nd         | nd        | nd       | nd       | nd       | nd        | nd        | nd         | nd       | nd       |
| <i>p</i> -Coumaric acid isoprenyl ester | 60.6                 | 6±1      | 19.9±0.4 | 22.1±0.7   | nq        | 5.2±0.2  | nq       | 5.9±0.4  | 6.4±0.3   | nq        | 7.2±0.5    | nq       | nq       |
| Caffeic acid cinnamyl ester             | 61.7                 | 33±10    | 14.5±0.5 | 12.4±0.1   | 33.3±0.1  | 18.6±0.5 | 28.3±0.0 | 14.4±0.9 | 21.4±0.7  | 10.7±0.6  | 17±1       | nq       | nq       |
| Pinobanksin-3- <i>O</i> -propionate     | 62.4                 | 11±3     | nq       | nq         | 25.4±0.2  | 15±2     | 16.2±0.1 | 19±1     | 22.3±0.0  | nq        | 16±1       | nq       | nq       |
| Pinobanksin-3- <i>O</i> -butyrate       | 66.3                 | 39±10    | 8.6±0.2  | nq         | 6.5±0.4   | nq       | 19.5±0.3 | nq       | 11.5±0.9  | nq        | nq         | nq       | nq       |
| Pinobanksin-3- <i>O</i> -pentanoate     | 69.1                 | 23±7     | 19.0±0.4 | 10.2±0.2   | 21.3±0.1  | 14.6±0.2 | nq       | 12.2±0.1 | 16.0±0.6  | nq        | 18.8±0.5   | nq       | nq       |

nq – not quantified; nd – not detected

**Table 5.6** Phenolic content in Portuguese propolis samples S3-4 and A1-10 (mg/g of extract).

| Compound                                | t <sub>R</sub> (min) | S3        | S4       | A1       | A2        | A3       | A4        | A5       | A6        | A7       | A8       | A9        | A10      |
|-----------------------------------------|----------------------|-----------|----------|----------|-----------|----------|-----------|----------|-----------|----------|----------|-----------|----------|
| Caffeic acid                            | 12.4                 | nq        | nq       | 10.4±0.1 | 18.3±0.1  | 5.8±0.0  | 7.0±0.0   | 9.5±0.3  | 8.7±0.0   | 7.8±0.0  | 6.4±0.0  | 9.6±0.1   | nq       |
| <i>p</i> -Coumaric acid                 | 16.2                 | nq        | nq       | 11.7±0.1 | 11.9±0.1  | 8.3±0.1  | 9.7±0.0   | 10.8±0.3 | 11.9±0.0  | 14.8±0.1 | 6.5±0.0  | 6.6±0.0   | 12±1     |
| Ferulic acid                            | 17.3                 | nq        | nq       | nq       | nq        | nq       | nd        | nd       | nq        | nq       | nq       | nq        | nq       |
| Ellagic acid                            | 17.5                 | nd        | nd       | nd       | nd        | nd       | nd        | nd       | nd        | nd       | nd       | nd        | nd       |
| Isoferulic acid                         | 17.9                 | nq        | 36.8±0.7 | 10.4±0.2 | 17.2±0.0  | 10.1±0.1 | 9.9±0.1   | 12.5±0.5 | 15.4±0.0  | 11.1±0.1 | 7.1±0.0  | 7.2±0.0   | 10.2±0.9 |
| 3,4-Dimethyl-caffeic acid               | 24.2                 | nq        | nq       | 13.5±0.2 | 12.1±0.0  | 14.5±0.1 | 12.1±0.1  | 14.1±0.4 | 17.2±0.0  | 15.7±0.1 | 10.3±0.1 | 9.0±0.1   | 14±1     |
| Pinobanksin-5-methyl-ether              | 29.1                 | nq        | nq       | 12.8±0.0 | nq        | nq       | nq        | nd       | nq        | nq       | nd       | nd        | nq       |
| Cinnamic acid                           | 29.1                 | nq        | nq       | nq       | nq        | 5.7±0.0  | nq        | nq       | nq        | 4.9±0.0  | nq       | nq        | 6.9±0.9  |
| Luteolin                                | 30.4                 | nd        | nd       | nd       | nd        | nd       | nd        | nd       | nd        | nd       | nd       | nd        | nd       |
| <i>p</i> -Coumaric acid methyl ester    | 31.2                 | nq        | nq       | 16.7±0.2 | 15.7±0.1  | 19.2±0.2 | 16.5±0.0  | 15.1±0.6 | 19.9±0.0  | 22.8±0.2 | 9.8±0.0  | 6.2±0.1   | 17±2     |
| Pinobanksin                             | 35.4                 | 6.2±0.0   | 2.7±0.1  | 20.4±0.2 | 11.9±0.1  | 11.4±0.5 | 10.3±0.1  | 10.8±0.4 | 10.7±0.0  | 10.3±0.1 | 7.8±0.0  | 12.5±0.1  | 13.1±0.8 |
| Pinocembrin-5-methyl-ether              | 38.0                 | nq        | nd       | nq       | nq        | nq       | nq        | nq       | nq        | nq       | nq       | nq        | nq       |
| Apigenin                                | 39.1                 | 5.9±0.1   | 6.4±0.2  | 7.5±0.4  | 7.5±0.0   | 6.2±0.0  | 6.0±20.03 | 5.9±0.0  | 6.6±0.0   | 6.3±0.0  | 5.9±0.1  | 6.5±0.0   | 6.8±0.3  |
| chrysin-5-methyl-ether                  | 40.8                 | nq        | nd       | nq       | nq        | nq       | nq        | nq       | nq        | nq       | nq       | nq        | nq       |
| Kaempferol                              | 41.0                 | 10.3±0.1  | nd       | 14±3     | nq        | 9.8±0.2  | 10.0±0.2  | 9.9±0.0  | 10.9±0.3  | 10.0±0.0 | 10.1±0.0 | 10.1±0.1  | 9.6±0.1  |
| Isorhamnetin                            | 42.0                 | nq        | nd       | 9.8±0.5  | 10.5±0.0  | nq       | nq        | nd       | nq        | nq       | nq       | nq        | 12±2     |
| Kaempferol-methyl-ether                 | 43.0                 | 40.8±0.5  | 54±2     | 13.9±0.2 | 10.1±0.1  | 10.4±0.1 | 10.6±0.1  | 11.0±0.2 | 11.6±0.1  | 11.8±0.0 | 11.0±0.1 | 11.2±0.0  | 10.4±0.2 |
| Kaempferol-methoxy-methyl-ether         | 44.0                 | nd        | nd       | nd       | nd        | nd       | nd        | nd       | nd        | nd       | nd       | nd        | nd       |
| Quercetin-dimethyl-ether                | 44.8                 | 9.4±0.3   | nd       | 11.0±0.6 | nd        | nd       | nd        | nd       | nd        | 9.7±0.0  | 9.7±0.1  | 9.9±0.1   | nd       |
| Quercetin-tetramethyl-ether             | 45.2                 | nd        | nd       | nd       | nd        | nd       | nd        | nd       | nd        | nd       | nd       | nd        | nd       |
| Cinnamylidenacetic acid                 | 45.5                 | nq        | nq       | 5.8±0.1  | 8.1±0.1   | 12±1     | 9.2±0.0   | 8.1±0.3  | 10.4±0.0  | 6.8±0.0  | 5.5±0.1  | 6.15±0.04 | 8.7±0.5  |
| Rhamnetin                               | 49.8                 | 9.4±0.3   | nd       | 11.0±0.2 | nq        | nq       | nq        | nq       | nq        | nq       | nq       | nq        | nq       |
| Quercetin-dimethyl-ether (isomer)       | 51.4                 | 9.6±0.4   | nq       | 12±1     | 12.8±0.0  | nq       | nq        | nq       | nq        | nq       | nq       | nq        | nq       |
| Apigenin-6,4'-dimethyl-ether            | 51.8                 | nd        | nd       | nd       | nd        | nd       | nd        | nd       | nd        | nd       | nd       | nd        | nd       |
| Caffeic acid isoprenyl ester            | 52.1                 | nq        | nq       | 25.4±0.2 | 46.3±0.0  | 24.3±0.3 | 22.4±0.0  | 20.2±0.6 | 26.8±0.1  | 25.5±0.1 | 17.2±0.1 | 14.1±0.3  | 18±2     |
| Caffeic acid isoprenyl ester (isomer)   | 53.2                 | nq        | 6.8±0.4  | 33.4±0.5 | 70.1±0.9  | 36.4±0.3 | 32±3      | 32±1     | 48±12     | 40.0±0.2 | 33±7     | 21.1±0.2  | 29±2     |
| Caffeic acid benzyl ester               | 53.6                 | nq        | nq       | 23.9±0.6 | 15.3±0.1  | 18.9±0.1 | 17.6±0.1  | 12.8±0.6 | 16.6±0.4  | 20.0±0.3 | 10.8±0.2 | 14.0±0.3  | 11±2     |
| Pinocembrin                             | 54.7                 | nq        | nq       | 64±1     | 36.4±0.3  | 45.0±0.5 | 38.9±0.0  | 33±1     | 39.2±0.1  | 31.8±0.1 | 25.3±0.0 | 30.2±0.0  | 26±2     |
| Chrysin                                 | 55.6                 | 8.3±0.4   | 5.8±0.1  | 68±1     | 47.6±0.03 | 45±2     | 34±1      | 31±2     | 38.7±0.8  | 32.4±0.2 | 27.2±0.1 | 30.1±0.2  | 29±3     |
| Caffeic acid phenylethyl ester          | 55.6                 | nq        | nq       | 13±3     | 10.0±0.2  | 17±2     | 10.4±0.4  | 9.4±0.7  | 11.8±0.3  | 9.8±0.1  | 8.1±0.0  | 9.1±0.1   | 9±1      |
| Pinobanksin-3- <i>O</i> -acetate        | 56.5                 | nq        | nq       | 58±3     | 46.6±0.3  | 37.7±0.5 | 32±2      | 32.8±0.3 | 50.1±0.8  | 45.8±0.4 | 33.7±0.1 | 36.2±0.5  | 13.9±0.9 |
| Galangin                                | 57.3                 | 14.5±0.3  | nd       | 28.1±0.9 | 22.9±0.1  | 21.2±0.2 | 19.3±0.7  | 19.7±0.1 | 25.2±0.2  | 23.8±0.1 | 19.9±0.0 | 20.9±0.2  | 24±1     |
| Acacetin                                | 57.5                 | 5.6±0.1   | 10.2±0.5 | 9.0±0.4  | 6.1±0.1   | 5.4±0.1  | 5.5±0.4   | 5.0±0.0  | 8.2±0.1   | 7.8±0.0  | 6.5±0.1  | 6.4±0.1   | 5.6±0.3  |
| Kaempferide                             | 58.0                 | 10.0±0.0  | nd       | nd       | nd        | nd       | nd        | nd       | nd        | nd       | nd       | nd        | nd       |
| Chrysoeriol-methyl-ether                | 58.3                 | nd        | nd       | nd       | nd        | nd       | nd        | nd       | nd        | nd       | nd       | nd        | nd       |
| Chrysin-6-methyl-ether                  | 58.6                 | 4.8±0.0   | nd       | 6.3±0.6  | 6.4±0.0   | 6.7±0.1  | 7.0±0.0   | 5.8±0.0  | 6.0±0.1   | 6.6±0.0  | 5.8±0.0  | 5.86±0.01 | 6.3±0.4  |
| kaempferol-3,7-dimethyl-ether           | 59.7                 | 17.07±0.3 | 28±1     | nd       | nd        | nd       | nd        | nd       | nd        | nd       | nd       | nd        | nd       |
| <i>p</i> -Coumaric acid isoprenyl ester | 60.6                 | nq        | nd       | 5.8±0.1  | 7.2±0.0   | nq       | nq        | nq       | 5.79±0.03 | 8±1      | nq       | nq        | nq       |
| Caffeic acid cinnamyl ester             | 61.7                 | nq        | nd       | 11.3±0.1 | 7.1±0.1   | 15.1±0.2 | 11.2±0.4  | 9.3±0.6  | 13.3±0.6  | 13.9±0.1 | 6.7±0.1  | 9.2±0.1   | 8±1      |
| Pinobanksin-3- <i>O</i> -propionate     | 62.4                 | nq        | nd       | 11.8±0.2 | nq        | nq       | nq        | nq       | nq        | nq       | nq       | nq        | nq       |
| Pinobanksin-3- <i>O</i> -butyrate       | 66.3                 | nq        | nd       | 8.0±0.1  | nq        | nq       | nq        | nq       | nq        | nq       | nq       | nq        | nq       |
| Pinobanksin-3- <i>O</i> -pentanoate     | 69.1                 | nq        | nq       | 11±2     | 18.5±0.2  | 12.5±0.1 | 9.8±0.3   | 10.4±0.2 | 12.9±0.4  | 10.2±0.1 | 10.3±0.1 | 10.9±0.4  | 7.0±0.5  |

nq – not quantified; nd – not detected

**Table 5.7** Phenolic content in Portuguese propolis samples A11, M1-3 and plant extracts PM, PF and C (mg/g of extract).

| Compound                                | <i>t<sub>R</sub></i> (min) | A11      | M1      | M2       | M3       | PM        | PF       | C        |
|-----------------------------------------|----------------------------|----------|---------|----------|----------|-----------|----------|----------|
| Caffeic acid                            | 12.4                       | 7.9±0.6  | nd      | nd       | 5.0±0.0  | nq        | 6±2      | nd       |
| <i>p</i> -Coumaric acid                 | 16.2                       | 12±1     | nd      | nd       | 46.0±0.2 | 6.2±0.1   | 4±1      | nd       |
| Ferulic acid                            | 17.3                       | nq       | nd      | nd       | nq       | 8.0±0.1   | nq       | nd       |
| Ellagic acid                            | 17.5                       | nd       | nd      | nd       | nd       | nd        | nd       | nd       |
| Isoferulic acid                         | 17.9                       | 12±2     | nd      | nd       | nq       | nq        | 6±4      | nd       |
| 3,4-Dimethyl-caffeic acid               | 24.2                       | 16±2     | nd      | nd       | 3.6±0.1  | 11.4±0.0  | 6±2      | nd       |
| Pinobanksin-5-methyl-ether              | 29.1                       | nq       | nd      | nd       | nd       | nq        | nq       | nd       |
| Cinnamic acid                           | 29.1                       | 4.7±0.6  | nq      | nd       | 8.1±0.1  | nq        | nq       | nd       |
| Luteolin                                | 30.4                       | nd       | nd      | nd       | nd       | nd        | nd       | nd       |
| <i>p</i> -Coumaric acid methyl ester    | 31.2                       | 16±2     | nq      | nd       | 4.1±0.0  | 7.8±0.2   | 5.3±0.5  | nd       |
| Pinobanksin                             | 35.4                       | 24±2     | nd      | 8.4±0.1  | 8.6±0.0  | 6.0±0.1   | 3.8±0.2  | nd       |
| Pinocembrin-5-methyl-ether              | 38.0                       | nq       | nd      | nd       | nd       | nd        | nd       | nd       |
| Apigenin                                | 39.1                       | 7.4±0.2  | nd      | nd       | 5.4±0.1  | 4.7±0.0   | 5.8±0.9  | 5.0±0.1  |
| chrysin-5-methyl-ether                  | 40.8                       | 4.5±0.1  | nd      | nd       | nd       | nd        | 4.7±0.4  | nd       |
| Kaempferol                              | 41.0                       | 10.1±0.5 | nd      | nd       | 14.1±0.3 | 10.5±0.1  | 12±2     | nd       |
| Isorhamnetin                            | 42.0                       | 11.7±0.4 | nd      | 19.8±0.8 | nq       | nd        | nq       | nd       |
| Kaempferol-methyl-ether                 | 43.0                       | 10.0±0.1 | nd      | nd       | 11.3±0.0 | 10.6±0.0  | nq       | 13.5±0.0 |
| Kaempferol-methoxy-methyl-ether         | 44.0                       | nd       | nd      | nd       | nd       | nd        | nd       | nd       |
| Quercetin-dimethyl-ether                | 44.8                       | 10.0±0.2 | nd      | 13.1±0.3 | nd       | nd        | nq       | nd       |
| Quercetin-tetramethyl-ether             | 45.2                       | nd       | nd      | nd       | nd       | nd        | nd       | nd       |
| Cinnamylidenacetic acid                 | 45.5                       | 7.8±0.8  | nd      | nd       | 1.6±0.0  | 11.4±0.2  | nq       | nd       |
| Rhamnetin                               | 49.8                       | nq       | nd      | nd       | nd       | nq        | nq       | nd       |
| Quercetin-dimethyl-ether (isomer)       | 51.4                       | nq       | nd      | nd       | 10.1±0.1 | nq        | nd       | nd       |
| Apigenin-6,4'-dimethyl-ether            | 51.8                       | nd       | 4.9±0.0 | nd       | nd       | nd        | nd       | nd       |
| Caffeic acid isoprenyl ester            | 52.1                       | 25±3     | nd      | nd       | nq       | 10.6±0.0  | 10.7±0.8 | nd       |
| Caffeic acid isoprenyl ester (isomer)   | 53.2                       | 41±5     | nq      | nd       | nq       | 16.8±0.3  | 19±2     | nd       |
| Caffeic acid benzyl ester               | 53.6                       | 17±2     | nq      | nd       | 8±2      | 13.0±0.1  | 15.0±0.8 | nd       |
| Pinocembrin                             | 54.7                       | 41±5     | nq      | nq       | 32±9     | 21.5±0.3  | 37±4     | nd       |
| Chrysin                                 | 55.6                       | 45±2     | 4.6±0.0 | 4.6±0.0  | 10±2     | 18.2±0.1  | 25±4     | nd       |
| Caffeic acid phenylethyl ester          | 55.6                       | 13.6±0.7 | nq      | nq       | nq       | 5.24±0.02 | 8±1      | nd       |
| Pinobanksin-3- <i>O</i> -acetate        | 56.5                       | 11.7±0.4 | nq      | nd       | 18.4±0.3 | 23.4±0.1  | 19.0±0.3 | nd       |
| Galangin                                | 57.3                       | 41±5     | nd      | nd       | 15.7±0.3 | 20.7±0.0  | 19.1±0.4 | nd       |
| Acacetin                                | 57.5                       | 5.8±0.1  | 4.7±0.0 | nd       | 4.9±0.0  | 6.9±0.1   | 5.4±0.2  | 5.5±0.0  |
| Kaempferide                             | 58.0                       | nd       | nd      | nd       | 9.7±0.01 | nd        | nd       | nd       |
| Chrysoeriol-methyl-ether                | 58.3                       | nd       | nd      | nd       | nd       | nd        | nd       | nd       |
| Chrysin-6-methyl-ether                  | 58.6                       | 7.1±0.2  | nd      | nd       | 5.0±0.0  | 5.8±0.4   | 6.3±0.2  | nd       |
| kaempferol-3,7-dimethyl-ether           | 59.7                       | nd       | nd      | nd       | nd       | nd        | nd       | 13.4±0.1 |
| <i>p</i> -Coumaric acid isoprenyl ester | 60.6                       | nq       | nd      | nd       | nq       | nq        | nq       | nd       |
| Caffeic acid cinnamyl ester             | 61.7                       | 11±1     | nd      | nd       | 16.4±0.0 | 5.7±0.1   | 7±3      | nd       |
| Pinobanksin-3- <i>O</i> -propionate     | 62.4                       | nq       | nd      | nd       | nq       | nq        | 23±2     | nd       |
| Pinobanksin-3- <i>O</i> -butyrate       | 66.3                       | nq       | nd      | nd       | 16±1     | nd        | nq       | nd       |
| Pinobanksin-3- <i>O</i> -pentanoate     | 69.1                       | 20±1     | nd      | nd       | nq       | nd        | nq       | nd       |

nq – not quantified; nd – not detected

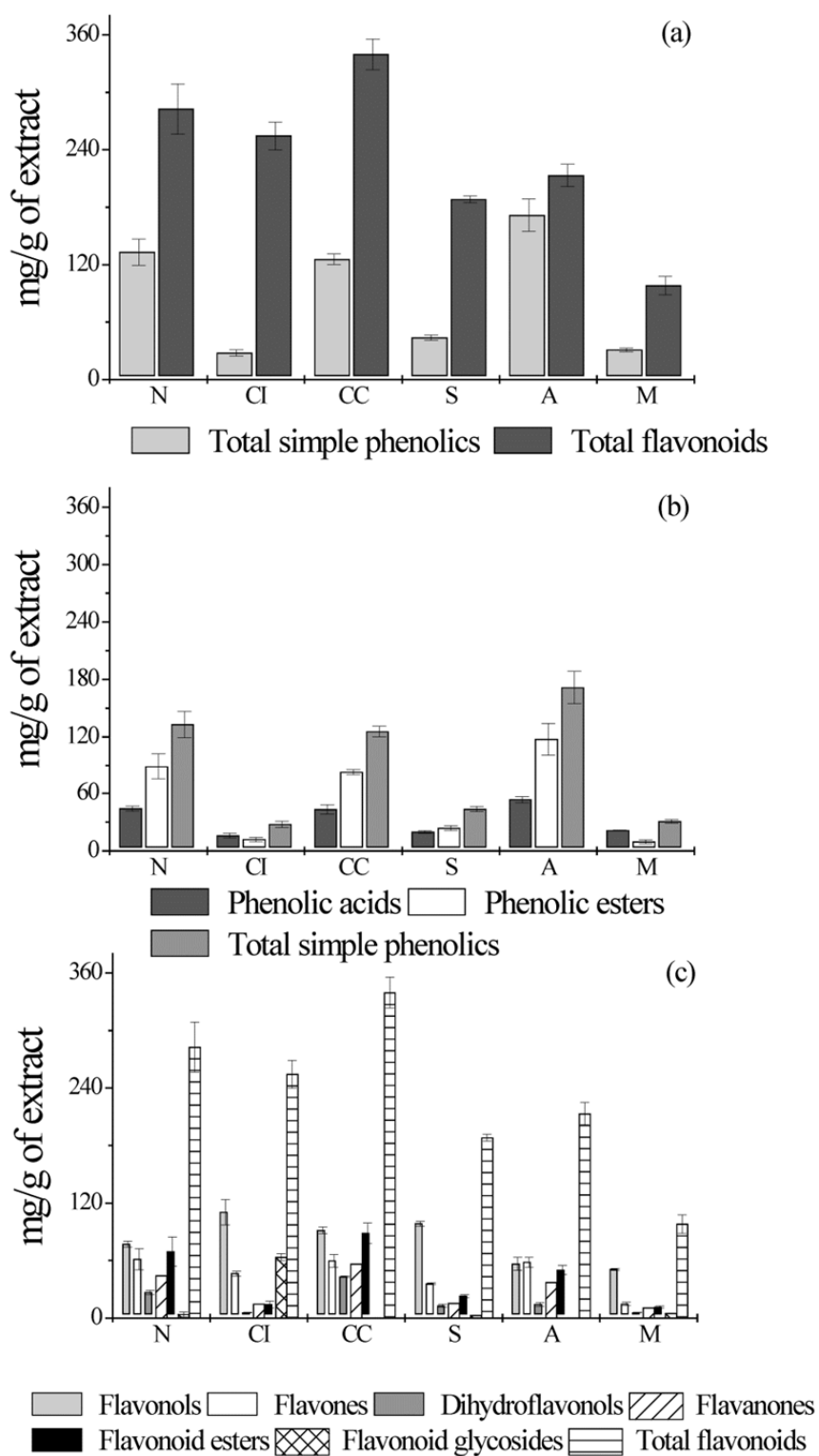


**Figure 5.3** Composition for the main classes of phenolic compound present in propolis samples from: north (N), central interior (CI), central coast (CC), south (S), Azores Archipelago (A) and Madeira Island (M) regions. (a) Total simple phenolics and total flavonoids. (b) Phenolic acids, phenolic esters and total simple phenolics. (c) Flavonols, flavones, dihydroflavonols, flavanones, flavonoid esters, flavonoid glycosides and total flavonoids.

The contribution of flavones was also very significant in the north, central coast and Azores propolis ranging from a maximum of 61 mg/g in the north (22%) down to a minimum of 15 mg/g in Madeira propolis (15%), Table 5.3. In Azores, the flavones content in propolis was even higher than flavonols, Figure 5.3c.

Dihydroflavonols did not played the same role in the propolis flavonoid composition and its input can be down to 2% in samples from the central interior. The maximum amount was observed in the samples from the north and coast centre, but never above 13% (43 mg/g).

In this study, only two flavanones were detected, pinocembrin-5-methyl-ether (20) and pinocembrin (6), and only the last one were quantifiable. The generality of samples from north and coast center showed a high content in pinocembrin, 25 to 93 mg/g and 52 to 61 mg/g of extract, respectively, Table 5.3. Exceptions within the north region were found in samples N11-N12 presenting low values, around 8 mg/g, or sample N13 where pinocembrin was even below the quantification limit. Other samples such as CI1-CI3, S2-S4 or MI-M2 revealed also a poor content in flavanones.

The second class of flavonoids observed in high quantities in the north, central coast and in Azores propolis were the flavonoid esters, representing approximately 25% of the total flavonoid content. Some propolis samples in the north reveal amounts of the pinobanksin esters up to 150 mg/g of extract. For other side, in the south and in Madeira the content of these ester derivatives in propolis was low and sometimes even absent, Table 5.3.

Portuguese propolis has been recently described to present an uncommon composition rich in flavonoid glycosides.<sup>9</sup> This was detected in samples N11-N13 from north, CI1 from central interior, S4 from south and M2 from Madeira Island. On those, fourteen flavonoid glycosides, mainly quercetin and kaempferol glycosides were identified. Due to the complexity of the resulting chromatograms and the proximity in the retention time, the flavonoid glycosides were quantified as one. Sample CI1 from the central interior (Guarda) is clearly different from all the others with almost 228 mg/g, representing 43% of the flavonoids in this sample and contribute to the highest value observed in all the samples under study (535 mg/g). The other propolis samples with flavonoid glycosides had a much lower content, between 12 to 23 mg/g, Table 5.3.

As described, not all the samples within a region present the same phenolic profile or content. The statistic analysis with hierarchical cluster, Figure 5.4, clearly

identified three groups and an outlier sample (CI1): the first group contain most of the samples from Azores, five samples from the north, one sample from Madeira and other from center coast; the second group is mainly composed by the north and central coast samples; the third group contains most of the samples from central interior, south and Madeira, and include samples N11-13 from the north. This later group is distinctive from the above due to the low content on simple phenolics and the presence of flavonoid glycosides. The distinction between the first two groups is not as evident and relies in the total amount of flavonoids: group 2 is richer, particularly in respect to flavonols and dihydroflavonols. These differences can only be explained by the phyto-diversity provided within those Portuguese regions.

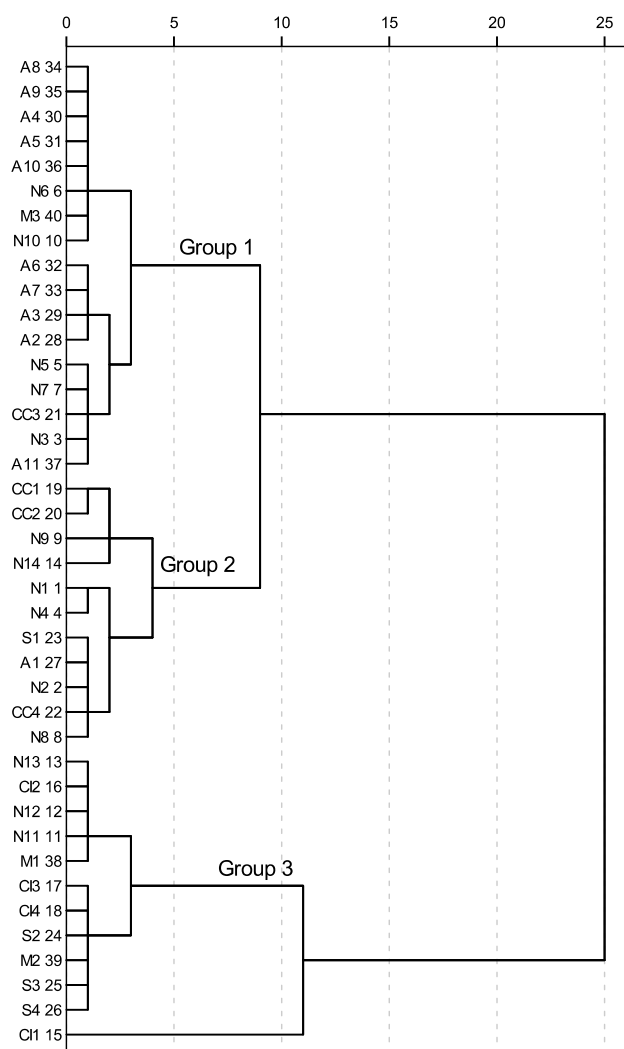


**Figure 5.4** Dendrogram obtained by hierarchical cluster analysis of the propolis phenolic content from: north (N), central interior (CI), central coast (CC), south (S), Azores Archipelago (A) and Madeira island (M).

### 5.5 Phenolic quantification in the plant sources – botanical origin

The ethanolic extracts of *Populus x canadensis*, male (PF) and female (PM), showed a comparable composition of simple phenolics, with higher values for the esters derivatives, up to 65 mg/g (approximately 70% of the total phenolics), Table 5.3. Nevertheless, the total phenolic content is lower in the poplar buds than in propolis, indicating that bees collect resin from other floral sources, thus enriching the phenolic composition of the bee glue. For *Cistus ladanifer* exudates, phenolic acids and esters were not detected at all using this experimental approach. This class of compounds is unusual in *C. ladanifer*, and in the literature only vanillic acid has been described at low concentrations in the methanol: water (80:20) extract of fresh leaves.<sup>17</sup>

The amount of flavonoids in the plant sources under study is higher in comparison with the simple phenolics. The exudates of PM were richer in flavonols (42 mg/g) and flavones (35 mg/g of extract) whereas PF had more flavones (47 mg/g) and flavonoid esters (42 mg/g). Both poplars extracts presented a low content in dihydroflavonols, 4 to 6 mg/g, Table 5.3. These patterns are consistent with the general observation found in the propolis samples, particularly those from the north, central cost and Azores, however, in the latter region, the high quantity of flavones in comparison to flavonols reveal some deviation. It is interesting to notice that the exudates of female poplar reveal the same behavior, what let us consider that the variability in the poplar species around the hive could contribute to those minor differences in quantities within the phenolic composition. In fact, poplar bud exudates were reported to contain a great variety of phenolic compounds, which were dependent on the species studied.<sup>18</sup>

Through the analysis of the exudates of *Cistus ladanifer*, only flavonols, flavones and flavonoid glycosides were detected in a concentration of 27, 11 and 27 mg/g of extract, respectively. The rich composition in kaempferol derivatives present in this plant source was also found in samples CI2-CI4 from central interior and samples S2-S4 from the south, standing out the compound kaempferol-dimethyl-ether (6) which was absent in the other propolis samples with a poplar type composition.

### 5.6 Concluding remarks

In conclusion, the Portuguese propolis samples presented a similar phenolic composition, with significant differences found in their concentrations. The samples

from north (N), central coast (CC), Azores Archipelago (A) and sample S1, from south, revealed the higher phenolic content, up to 261 mg/g in simple phenolics and 460 mg/g in flavonoids, with a profile similar to the one observed in the bud exudates extracts of *Populus x canadensis*. The other samples, which include propolis N11-N13 from north regions, CI2-CI3 from central interior, S2-S4 from south and samples M1-M2 from Madeira Island presented a poor composition in the generality of the phenolic compounds, with less than 44 mg/g of simple phenolics and 204 mg/g of flavonoids. Moreover, the phenolic profile for this propolis type is clearly different, suggesting other floral contributions for the resin rather than poplar. The phenolic constituents found in samples CI2-CI4 and S2-S4, rich in kaempferol derivatives, particularly kaempferol-3,7-dimethyl-ether, resembles the *Cistus ladanifer* exudates. This plant source, very common in those regions, is probably the origin of the resin and the compound kaempferol-3,7-dimethyl-ether, absent in the poplar type propolis, can be regarded as a marker substance for floral origin discrimination between these two types of propolis. Central interior sample, CI1, is clearly an outsider: the exclusive presence of quercetin-tetramethyl-ether, luteolin and chrysoeriol-methyl-ether, all described before in *Labiatae*, particularly in *Origanum* spp.,<sup>19,20</sup> and its high content in flavonoid glycosides, with a probable origin in the conifer plants,<sup>9</sup> reveal the difficulty to unequivocally assign the floral composition of each propolis sample.

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## Chapter 6

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### **The volatile composition of Portuguese propolis towards its origin discrimination**





## The volatile composition of Portuguese propolis towards its origin discrimination\*

### Abstract

The volatiles from thirty six propolis samples collected from six different geographical locations in Portugal (mainland, Azores archipelago and Madeira island) were evaluated. *Populus x canadensis* Moench leaf-buds and *Cistus ladanifer* L. branches essential oils were comparatively analysed. The essential oils were isolated by hydrodistillation and analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS). Cluster analysis based on propolis samples volatiles chemical composition defined three main clusters, not related to sample site collection. Cluster I presented a rich composition in oxygen-containing sesquiterpenes, while cluster II was richer in oxygen-containing monoterpenes and cluster III in monoterpene hydrocarbons. Although *Populus x canadensis* and *Cistus ladanifer* were associated as resin sources of Portuguese propolis, other *Populus* species as well as plants like *Juniperus* genus may contribute to the resin in specific geographical locations.

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\*Adapted from Falcão SI, Freire C, Figueiredo AC, Vilas-Boas M. The volatile composition of Portuguese propolis towards its origin discrimination. To be submitted, 2013.

## 6.1 Introduction

Over the last thirty years, propolis has been attracting researcher's interest all over the world. This fascinating bee product is the result of the collection of resins, bud exudates and part of plants by bees.<sup>1</sup> Propolis is a multifunctional material used by bees in the construction and defense of their hives.<sup>2</sup> It has been proposed that it has a role in the immunity of honeybees, reducing the risk of disease and parasite transmission through the colony.<sup>3</sup> Nowadays propolis is extensively used in food, cosmetic and pharmaceutical industry due to the wide range of biological properties presented.<sup>4,5</sup>

This is an extremely complex mixture of natural substances, with several hundred of different chemical compounds and highly dependent on the plants available around the hive, and therefore on the geography and climatic conditions of the site.<sup>3,6</sup> Overall, the so called bee glue is composed by a balsamic part derived from the collected plant parts which contains 40-70% of resin (mainly phenolic compounds) and 3-5% of essential oils and a non balsamic part which is added by bees and contains 20-35% of wax, 5% of pollen and 5% of other compounds (minerals, polysaccharides, proteins, etc.).<sup>1</sup> According to the botanical origin and consequently its composition, propolis was typified. In temperate zones the bud exudates of *Populus* species and their hybrids are the main source of the bee glue.<sup>2</sup> The typical components of poplar propolis are the phenolics: flavonoid aglycones, (flavones and flavanones), phenolic acids and their esters.<sup>2,7</sup> On the other hand, in tropical regions of the world, where poplars are not native, plant sources are much diversified. The most commercialized propolis type is known as "green propolis", which predominates in the southeast of Brazil, it has its origin in the leaves of *Baccharis dracunculifolia* and is mainly composed by prenylated *p*-coumaric acids and caffeoyl quinic acids.<sup>2</sup>

Even though volatile compounds are found in smaller concentrations, they play an important role in propolis characterization and can enhance the potential uses due to their aroma and significant biological activity. Also, their composition can give valuable information about plant sources in the origin of propolis. Mono- and sesquiterpenes were identified as the major components of propolis although the diversity of volatile compounds present is very high.<sup>2</sup> Research studies concerning European propolis volatile composition revealed a predominance of sesquiterpenes in Bulgarian propolis,<sup>8</sup> monoterpenes in Dalmatian<sup>9</sup> and Greek propolis<sup>10</sup> and organic compounds like benzyl alcohol, benzoic acid and benzyl benzoate in Slavonia propolis.<sup>9</sup> Recently the volatile

profile of propolis samples from acaricide-treated and–untreated beehives in the south Portugal was evaluated, presenting the samples an intense rock-rose aroma supported by the presence of characteristic *Cistus* and labdanium oil volatile components.<sup>11</sup>

Portugal is a country of botanical diversity which is reflected in propolis different compositions. Our previous work allowed the establishment of two distinct groups of propolis based on the phenolic profile.<sup>12</sup> In this work, the volatile composition of propolis samples from the different regions of Portugal, mainland and islands, were characterized with the purpose of creating a pattern for origin discrimination of the samples, and contributing to standardize this bee product. Additionally, two potential floral sources volatiles, *Populus x canadensis* buds and *Cistus ladanifer*, were evaluated.

## 6.2 Experimental

### 6.2.1 Propolis samples

Thirty six propolis samples were collected from six different geographical locations in Portugal. Collection sites were located in continental north (N1-6, Bragança; N8, Mirandela; N9-10, Chaves; N11, Montalegre; N12-13, Boticas; N14, Barcelos), central interior (CI4, Nisa), central coast (CC1, Figueira da Foz; CC2, Leiria; CC3, Coruche; CC4, Ramada), south (S1-3, Aljezur; S4, Moncarapacho), Azores archipelago (A1, Terceira island; A2-11, S. Miguel island) and from Madeira island (M1-3, Funchal) (Appendix A). All the samples were obtained between 2007 and 2009 after the honey harvesting season (July/September), by conventional scraping or through plastic screens.

### 6.2.2 Plant material

*Populus x canadensis* Moenchen (P) leaf-buds and *Cistus ladanifer* L. (C) branches, in the floral stage, were collected from wild growing plants in the Bragança region, northeast Portugal, in the spring of 2009. Voucher specimens were deposited at the herbarium of Escola Superior Agrária of Instituto Politécnico de Bragança under voucher number BRESA 5174 and BRESA 5355 for C and P respectively. All samples were stored at -20°C until analysis.

### 6.2.3 Volatiles extraction

Propolis and the plant volatiles were isolated by hydrodistillation for 3h using a Clevenger-type apparatus according to the European Pharmacopoeia method.<sup>13</sup> The isolation procedure was run at a distillation rate of 3 mL/min, the volatiles were recovered in distilled *n*-pentane, concentrated at room temperature under a slight nitrogen flux, and stored at -20°C in the dark until analysis.

### 6.2.4 Volatiles analysis

#### 6.2.4.1 Gas Chromatography (GC)

Gas chromatographic analyses were performed using a Perkin Elmer Autosystem XL gas chromatograph (Perkin Elmer, Shelton, CT) equipped with two flame ionization detectors (FIDs), a data handling system, and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (polydimethylsiloxane, 30 m x 0.25 mm i.d., film thickness 0.25 µm; J & W Scientific Inc., Rancho Cordova, CA, USA) and a DB-17HT fused-silica column [(50% phenyl)-methylpolysiloxane, 30m x 0.25mm i.d., film thickness 0.15 µm; J & W Scientific Inc., Rancho Cordova, CA, USA]. Oven temperature was programmed, 45-175°C, at 3°C/min, subsequently at 15°C/min up to 300°C, and then held isothermal for 10 min; injector and detector temperatures, 280°C and 300°C, respectively; carrier gas, hydrogen, adjusted to a linear velocity of 30 cm/s. The samples were injected using a split sampling technique, ratio 1:50. The volume of injection was 0.1 µl of a distilled *n*-pentane-oil solution (1:1). The percentage composition of the volatiles was computed by the normalization method from the GC peak areas, calculated as a mean value of two injections from each sample, without response factors.

#### 6.2.4.2 Gas Chromatography-Mass Spectrometry (GC-MS)

The GC-MS unit consisted on a Perkin Elmer Autosystem XL gas chromatograph, equipped with DB-1 fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 µm; J & W Scientific, Inc., Rancho Cordova, CA, USA), and interfaced with Perkin-Elmer Turbomass mass spectrometer (software version 4.1, Perkin Elmer,

Shelton, CT). Injector and oven temperatures were as above; transfer line temperature, 280°C; ion source temperature, 220°C; carrier gas, helium, adjusted to a linear velocity of 30 cm/s; split ratio, 1:40; ionization energy, 70 eV; scan range, 40-300 u; scan time, 1 s. The identity of the components was assigned by comparison of their retention indices, relative to C<sub>9</sub>-C<sub>25</sub> *n*-alkane indices and GC-MS spectra from a home-made library, based upon the analyses of reference oils, laboratory-synthesized components, and commercial available standards.

### 6.2.5 Statistical analysis

The percentage composition of the isolated volatiles was used to determine the relationship between the different samples by a cluster analysis using Numerical Taxonomy Multivariate Analysis System (NTSYS-pc software, version 2.2, Exeter Software, Setauket, New York).<sup>14</sup> For cluster analysis, the correlation coefficient was selected as a measure of similarity among all accessions, and the Unweighted Pair Group Method with Arithmetical Averages (UPGMA) was used for cluster definition. The degree of correlation was evaluated according to Pestana and Gageiro (2000) and classified as very high (0.9-1), high (0.7-0.89), moderate (0.4-0.69), low (0.2-0.39) and very low (<0.2).

## 6.3 Results and discussion

### 6.3.1 Portuguese propolis volatile composition

All propolis volatiles were obtained in a yield <0.05% (v/w). The volatile components isolated from each individual propolis sample was a complex mixture in which two hundred and one components were identified. The identified volatile components are listed in Table 6.1 in order of their elution on the DB-1 column, arranged according to the total three types of volatile oils obtained by agglomerative cluster analysis, with the lowest and the highest percentages found for each component in each volatile oil type.

Despite the major chemical variability, the sesquiterpene fraction was dominant in all thirty six samples analysed (17-83%), while the monoterpene fraction ranged from 1 to 66%, Table 6.1. A fraction named as others, since components were

neither terpenes nor phenylpropanoids or fatty acids, comprising aliphatic and aromatic alcohols, carbonyl compounds and hydrocarbons, ranged from 4 to 36%.

**Table 6.1** Minimum (Min), maximum (Max) range and average (Aver) percentage of components identified in the volatiles isolated by hydrodistillation from 36 propolis samples collected from beehives maintained at different locations in the mainland Portugal, in Azores and Madeira archipelagos, and percentage composition of the essential oils isolated from the plant sources *Populus x canadensis* Moenchen (P) and *Cistus ladanifer* L (C). For samples grouped on each of the clusters I, I and III see Figure 6.1.

| Components                      | RI   | Propolis  |     |      |            |     |      | Plant Sources |         |
|---------------------------------|------|-----------|-----|------|------------|-----|------|---------------|---------|
|                                 |      | Cluster I |     |      | Cluster II |     |      | III           | P C     |
|                                 |      | Min       | Max | Aver | Min        | Max | Aver |               |         |
| <i>n</i> -Heptanal              | 897  |           |     |      |            | t   |      |               |         |
| <i>n</i> -Nonane                | 900  |           |     |      |            | t   |      |               |         |
| Tricyclene                      | 921  |           |     |      |            | 0.1 |      |               | 0.2     |
| $\alpha$ -Thujene               | 924  |           | t   | t    |            | 0.1 |      |               |         |
| Benzaldehyde                    | 927  |           | 0.4 | t    |            | 0.2 |      |               | t       |
| $\alpha$ -Pinene                | 930  |           | 4.7 | 0.5  | t          | 1.3 | 0.3  | 18.8          | 1.6     |
| Camphene                        | 938  |           | 0.2 | t    |            | 0.7 | 0.1  | 0.4           | 1.3     |
| Thuja-2,4(10)-diene*            | 940  |           | 0.6 | t    |            | 1.2 | 0.2  | 0.5           | 0.4     |
| <i>n</i> -Heptanol              | 952  |           |     |      |            | t   | t    |               |         |
| <i>tert</i> -Butyl valerate     | 956  |           | t   | t    |            |     |      |               |         |
| Sabinene                        | 958  |           | t   | t    |            | 0.1 | t    |               |         |
| 6-Methyl-5-hepten-2-one         | 960  |           | 0.3 | t    |            | 0.1 | t    |               | 0.4     |
| 1-Octen-3-ol                    | 961  |           |     |      |            | t   | t    |               |         |
| $\beta$ -Pinene                 | 963  |           | 0.5 | t    |            | 0.1 | t    | 5.5           |         |
| Hexanoic acid (= Caproic acid)  | 968  |           | t   | t    |            |     |      |               |         |
| 2-Pentyl furan                  | 973  |           | t   | t    |            | t   | t    |               | 0.2     |
| <i>n</i> -Octanal               | 973  |           | 1.0 | 0.2  | t          | 0.8 | 0.3  |               |         |
| $\beta$ -Myrcene                | 975  |           | 0.2 | 0.1  |            |     |      |               |         |
| 1,2,4-Trimethylbenzene          | 978  |           |     |      |            | t   | t    |               |         |
| <i>p</i> -Cresol methyl ether   | 987  |           | 0.2 | t    |            |     |      |               | 0.3     |
| $\alpha$ -Phellandrene          | 995  |           | t   | t    |            | t   | t    |               |         |
| Verbenene*                      | 998  |           | t   | t    |            | t   | t    |               |         |
| Benzyl alcohol                  | 1000 |           | 0.1 | t    |            |     |      |               |         |
| Salicylaldehyde*                | 1000 |           | 1.9 | 0.1  |            |     |      |               |         |
| Benzene acetaldehyde            | 1002 |           | t   | t    |            |     |      |               |         |
| $\alpha$ -Terpinene             | 1002 |           | 0.1 | t    |            | 0.6 | 0.1  |               | 0.4     |
| <i>p</i> -Cimene                | 1003 |           | t   | t    |            | 0.5 | 0.1  | 0.1           |         |
| 2,6,6-Trimethyl cyclohexanone   | 1003 |           | 2.0 | 0.2  |            | 5.4 | 0.9  |               | t 29.8  |
| 1,8-Cineole                     | 1005 |           | t   | t    |            |     |      |               |         |
| $\beta$ -Phellandrene           | 1005 |           | 0.2 | t    |            | 0.1 | t    | t             | 0.1     |
| Limonene                        | 1009 |           | 1.1 | 0.1  |            | 0.1 | t    | 0.2           | 0.1     |
| <i>cis</i> - $\beta$ -Ocimene   | 1017 |           | t   | t    |            |     |      |               |         |
| Acetophenone                    | 1017 |           | 0.8 | 0.1  |            | 0.1 | t    |               | 0.1 0.8 |
| <i>trans</i> - $\beta$ -Ocimene | 1027 |           | t   | t    |            |     |      |               |         |
| $\gamma$ -Terpinene             | 1035 |           | 0.2 | t    |            | 0.9 | 0.1  |               | 0.2     |
| <i>cis</i> -Linalool oxide      | 1045 |           | t   | t    |            |     |      |               | 0.2     |
| <i>n</i> -Octanol               | 1045 |           | t   | t    |            |     |      |               |         |
| Fenchone                        | 1050 |           |     |      |            | 0.4 | 0.1  |               |         |
| Methyl benzoate                 | 1053 |           | t   | t    |            |     |      |               |         |
| 2-Nonanone                      | 1058 |           | t   | t    |            |     |      |               |         |
| 2,5-Dimethyl styrene            | 1059 |           | 0.2 | t    |            | 0.3 | 0.1  |               | 0.3     |
| Terpinolene                     | 1064 |           | t   | t    |            | 0.2 | t    |               |         |
| 6-Methyl-3,5-heptadien-2-one    | 1064 |           | 0.1 | t    |            | 0.3 | t    |               | 0.2     |
| Phenyl ethyl alcohol            | 1064 |           | 0.1 | t    |            |     |      |               |         |
| <i>n</i> -Nonanal               | 1073 |           | 2.0 | 0.6  | t          | 1.2 | 0.5  | 0.3           | 0.7     |
| Linalool                        | 1074 |           | 2.7 | 0.7  | t          | 1.5 | 0.6  | 0.3           | 0.3 0.6 |

| Components                                      | RI   | Propolis  |     |      |            |      |      | Plant   |         |     |
|-------------------------------------------------|------|-----------|-----|------|------------|------|------|---------|---------|-----|
|                                                 |      | Cluster I |     |      | Cluster II |      |      | Cluster | Sources |     |
|                                                 |      | Min       | Max | Aver | Min        | Max  | Aver | III     | P       | C   |
| Chrysanthenone                                  | 1081 |           |     |      |            |      |      | t       |         |     |
| cis-Rose oxide                                  | 1083 |           | t   | t    |            | 0.2  | t    |         |         |     |
| $\alpha$ -Fenchol (= <i>Endo</i> -fenchol)      | 1085 |           | 0.1 | t    |            | 0.1  | t    | t       |         |     |
| $\alpha$ -Campholenal                           | 1088 |           | t   | t    |            | 0.8  | 0.1  | 0.6     |         | 0.9 |
| trans- <i>p</i> -2-menthen-1-ol                 | 1095 |           |     |      |            | t    | t    |         |         |     |
| trans-Rose oxide                                | 1100 |           | 0.3 | t    |            |      |      |         |         |     |
| Camphor                                         | 1102 |           | t   | t    |            | 0.2  | t    |         |         | 0.5 |
| trans-Pinocarveol                               | 1106 |           | 0.5 | 0.1  |            | 1.1  | 0.3  | 2.7     |         | 1.9 |
| cis-Verbenol                                    | 1110 |           | t   | t    |            | 0.4  | 0.1  | 0.6     |         | 0.4 |
| trans-Verbenol                                  | 1114 |           |     |      |            | 1.2  | 0.3  | 1.1     |         | 1.5 |
| trans-Tagetone                                  | 1116 |           | t   | t    |            |      |      |         |         |     |
| trans-Pinocamphone                              | 1121 |           |     |      |            |      | t    | 0.2     |         | 0.5 |
| Pinocarvone                                     | 1121 |           |     |      |            | 0.8  | 0.1  | 0.2     |         | 0.5 |
| cis-Tagetone                                    | 1123 |           | t   | t    |            |      |      |         |         |     |
| Benzyl acetate                                  | 1123 |           | 1.0 | 0.1  |            | 0.5  | 0.1  |         | t       |     |
| 2-trans-Nonen-2-al                              | 1124 |           | 0.1 | t    |            |      |      |         |         |     |
| Nerol oxide                                     | 1127 |           | 0.4 | t    |            | 0.3  | t    |         |         |     |
| $\alpha$ -Phellandrol                           | 1134 |           |     |      |            | 0.6  | 0.1  |         |         |     |
| $\beta$ -Mentha-1,5-dien-8-ol*                  | 1134 |           | 0.4 | t    |            | 1.7  | 0.2  |         |         | 3.4 |
| Borneol                                         | 1134 |           | 0.5 | 0.1  |            | 1.7  | 0.3  | 1.1     | t       |     |
| <i>p</i> -Methylacetophenone                    | 1143 |           |     | t    |            | t    | t    |         |         | t   |
| Terpinen-4-ol*                                  | 1148 |           | 1.2 | 0.2  |            | 1.0  | 0.2  | 0.6     |         | 0.9 |
| Octanoic acid                                   | 1152 |           | 0.4 | t    |            | t    | t    |         | t       |     |
| Myrtenal                                        | 1153 |           |     |      |            | 0.6  | 0.1  | 0.4     |         | 0.4 |
| Methyl salicylate                               | 1159 |           | 0.2 | t    |            |      |      |         |         |     |
| $\alpha$ -Terpineol                             | 1159 |           | 0.6 | 0.1  |            | 0.2  | 0.1  | 2.5     | t       | 0.1 |
| Safranal                                        | 1160 |           | t   | t    |            |      |      |         |         |     |
| Verbenone                                       | 1164 |           |     |      |            | 0.3  | t    |         |         |     |
| 2-Decanone                                      | 1166 |           | 0.1 | t    |            |      |      |         |         | 1.6 |
| Myrtenol                                        | 1168 |           | 0.6 | t    |            | 1.0  | 0.2  | 1.9     |         |     |
| cis- <i>p</i> -Menthan-2-one*                   | 1172 |           | 0.1 | t    |            |      |      |         |         |     |
| <i>n</i> -Decanal                               | 1180 |           | 1.2 | 0.2  | t          | 1.0  | 0.6  |         |         |     |
| trans-Carveol                                   | 1189 |           |     |      |            | 0.3  | 0.1  | 0.5     |         | 0.7 |
| cis-Ocimenone                                   | 1200 |           | 0.4 | t    |            | 0.2  | 0.1  |         |         | 0.9 |
| Carvone                                         | 1206 |           |     |      |            | t    | 0.0  |         |         | 0.2 |
| trans-Ocimenone                                 | 1207 |           | 0.1 | t    |            | t    | t    |         |         | 0.4 |
| Carvacrol methyl ether                          | 1224 |           | t   | t    |            |      |      |         |         |     |
| 2-Decenal                                       | 1224 |           | 0.2 | t    |            | 0.1  | t    |         |         |     |
| 2-Phenyl ethyl acetate                          | 1228 |           | 0.7 | 0.1  |            | 0.3  | t    |         | t       | 0.2 |
| Geraniol                                        | 1236 |           | 0.7 | 0.2  |            | 0.2  | 0.1  |         | 0.1     | t   |
| Ethyl guaiacol*                                 | 1242 |           |     |      |            | t    | t    |         |         |     |
| Nonanoic acid (= pelargonic acid)               | 1263 |           | 0.8 | 0.1  |            | 0.4  | 0.1  |         |         | t   |
| Bornyl acetate                                  | 1265 |           | 0.3 | 0.1  |            | 0.7  | 0.1  | t       |         | 1.8 |
| trans-Cinnamyl alcohol                          | 1268 |           | 0.2 | t    |            |      |      |         |         |     |
| Thymol                                          | 1275 |           | 6.5 | 0.6  | 8.2        | 64.3 | 34.6 | t       | 0.5     |     |
| Carvacrol                                       | 1286 |           | 0.4 | 0.1  |            | 0.4  | 0.1  |         |         | t   |
| Dihydrolinalyl acetate*                         | 1217 |           |     |      |            | 0.3  | t    |         |         | 0.2 |
| 2-Phenyl ethyl propionate                       | 1321 |           | 0.5 | t    |            |      |      |         |         |     |
| Eugenol                                         | 1327 |           | 0.3 | t    |            | t    | t    |         | 1.0     | 0.1 |
| Octyl isobutyrate                               | 1338 |           |     |      |            |      |      | t       |         |     |
| $\alpha$ -Cubebene                              | 1345 |           | 0.6 | t    |            | 0.5  | 0.1  | 0.1     |         |     |
| 7-Acetyl-2,6,6-trimethylbicyclo [4.2.0] octane* | 1346 |           |     |      |            | 0.2  | t    |         |         | 0.4 |
| Decanoic acid (= capric acid)                   | 1350 |           | 2.7 | 0.4  |            | 0.3  | 0.2  |         |         | t   |
| Cyclosativene                                   | 1363 |           |     |      |            |      |      |         |         | 0.2 |
| Geranyl acetone                                 | 1370 |           |     |      |            | t    | t    |         |         |     |
| $\alpha$ -Ylangene                              | 1371 |           | 0.6 | 0.1  |            | 0.1  | t    |         | 0.1     |     |
| $\alpha$ -Copaene                               | 1375 |           | 3.9 | 0.2  |            | 1.0  | 0.1  |         | 0.9     |     |
| $\beta$ -Bourbonene                             | 1379 |           | 2.5 | 0.1  |            | 1.3  | 0.3  | 0.5     |         |     |
| Ylang-2,4(15)-diene*                            | 1398 |           | 0.3 | t    |            | 0.2  | t    |         |         |     |
| Longifolene                                     | 1399 |           | 0.5 | t    |            | 0.2  | t    | 1.5     |         |     |
| $\alpha$ -Ionone                                | 1399 |           |     |      |            | t    | t    |         |         |     |
| $\alpha$ -Cedrene                               | 1400 |           | 0.8 | 0.1  |            |      |      |         |         |     |
| trans-Cinnamyl acetate                          | 1414 |           | t   | t    |            | t    | t    |         |         |     |

| Components                              | RI   | Propolis  |      |      |            |      |      | Plant Sources |      |
|-----------------------------------------|------|-----------|------|------|------------|------|------|---------------|------|
|                                         |      | Cluster I |      |      | Cluster II |      |      | P             | C    |
|                                         |      | Min       | Max  | Aver | Min        | Max  | Aver |               |      |
| $\beta$ -Cedrene                        | 1414 |           | 0.2  | t    |            |      |      |               |      |
| $\beta$ -Caryophyllene                  | 1414 |           | 1.2  | 0.2  | 0.5        | 0.2  | 1.5  | 0.1           |      |
| $\beta$ -Copaene                        | 1426 |           | 0.2  | t    | 0.3        | 0.1  |      |               |      |
| <i>trans</i> - $\alpha$ -Bergamotene    | 1434 |           | 0.5  | 0.1  | t          | t    |      |               |      |
| $\beta$ -Ylangene                       | 1435 |           | 0.3  | t    |            |      |      |               |      |
| Guaia-6,9-diene                         | 1447 |           | 0.8  | t    | t          | t    |      |               |      |
| $\alpha$ -Humulene                      | 1447 |           | 1.2  | 0.1  | 0.3        | 0.1  | t    | t             |      |
| <i>allo</i> -Aromadendrene              | 1456 |           | 2.6  | 0.4  | 1.5        | 0.4  |      | 1.5           | t    |
| Geranyl propionate                      | 1461 |           |      |      |            |      |      |               | t    |
| <i>trans</i> -Cadin-1(6),4-diene        | 1469 |           | 2.1  | 0.2  | 0.2        | 0.1  |      | 0.2           |      |
| $\gamma$ -Murolene                      | 1469 |           | 2.3  | 0.4  | 1.7        | 0.6  |      | 1.3           |      |
| $\alpha$ -Amorphene                     | 1469 |           | 0.5  | t    |            |      |      |               |      |
| (10,11)-Epoxycalamenene*                | 1469 |           |      |      | t          | t    |      | 0.7           |      |
| Germacrene D                            | 1474 |           | t    | t    | 0.4        | 0.1  |      |               |      |
| <i>ar</i> -Curcumene                    | 1475 |           | 1.3  | 0.1  |            |      |      |               |      |
| $\gamma$ -Curcumene                     | 1475 |           | 1.7  | 0.1  |            |      |      |               |      |
| $\beta$ -Selinene                       | 1476 |           | 0.2  | t    |            |      |      | 0.3           |      |
| Valencene                               | 1484 |           | 0.9  | 0.2  | 0.4        | 0.1  |      |               |      |
| Viridiflorene                           | 1487 |           |      |      | 1.1        | 0.2  |      |               | 0.3  |
| $\alpha$ -Murolene                      | 1494 |           | 1.7  | 0.3  | 0.7        | 0.2  |      | 1.9           |      |
| $\gamma$ -Cadinene                      | 1500 |           | 3.5  | 1.1  | 2.2        | 0.8  |      | 4.9           |      |
| <i>n</i> -Pentadecane                   | 1500 |           | 1.5  | 0.2  | t          | t    | t    |               |      |
| $\beta$ -Bisabolene                     | 1500 |           | 1.4  | 0.1  |            |      |      |               |      |
| <i>trans</i> -Calamenene                | 1505 |           | 1.6  | 0.4  | 0.7        | 0.2  | t    | 1.7           | t    |
| $\delta$ -Cadinene                      | 1505 | 0.3       | 7.6  | 1.8  | 0.4        | 3.5  | 1.6  | 1.1           | 6.8  |
| $\alpha$ -Calacorene                    | 1525 |           | 2.4  | 0.3  |            | 0.8  | 0.3  |               | 0.7  |
| Isocaryophyllene                        | 1528 |           | 0.3  | t    | t          | t    |      |               | 0.2  |
| $\alpha$ -Cadinene                      | 1529 |           | 0.5  | 0.1  | 0.1        | t    |      |               | 0.8  |
| Elemol                                  | 1530 |           | t    | t    |            |      |      |               |      |
| 1- <i>epi</i> -3,4-Dehydroviridiflorol* | 1533 |           |      |      | 1t         | 2.7  |      |               |      |
| Hexenyl benzoate*                       | 1533 |           | 0.1  | t    |            |      |      |               |      |
| <i>trans</i> - $\alpha$ -Bisabolene     | 1536 |           | 0.2  | t    |            |      |      |               |      |
| <i>trans</i> -Nerolidol                 | 1549 |           | 8.8  | 0.4  | 0.5        | 0.1  | 2.0  |               |      |
| $\beta$ -Calacorene*                    | 1550 |           | t    | t    |            |      |      |               |      |
| $\beta$ -Caryophyllene alcohol          | 1550 |           |      |      | 0.3        | t    |      |               | 0.1  |
| Dodecanoic acid                         | 1551 |           | 2.0  | 0.1  | t          | t    |      |               |      |
| Spathulenol                             | 1551 |           | 1.2  | 0.2  | 0.2        | 1.3  | 0.9  | 0.1           | 0.2  |
| $\beta$ -Caryophyllene oxide            | 1561 |           |      |      |            | 0.9  | 0.2  | 6.3           | 0.1  |
| Globulol                                | 1566 |           |      |      |            | 2.5  | 0.7  |               |      |
| Viridiflorol                            | 1569 |           | 8.1  | 1.3  | t          | 10.2 | 2.1  | 0.1           | 7.0  |
| Cedrol                                  | 1574 |           | 9.6  | 0.6  |            | t    | t    | 2.4           |      |
| Guaiol                                  | 1575 |           | 3.4  | 0.6  |            | t    | t    |               |      |
| Anhydrooplopanone                       | 1576 |           |      |      | 2.1        | 0.6  |      |               |      |
| $\beta$ -Oplopenone                     | 1576 |           | 3.7  | 0.6  |            | 1.2  | 0.2  | 0.9           |      |
| Ledol                                   | 1580 |           | 3.0  | 0.3  |            | 4.7  | 1.5  |               | 2.4  |
| Humulene epoxide II                     | 1580 |           |      |      |            |      | 1.0  |               |      |
| 1,2-Dehydroviridiflorol*                | 1582 |           |      |      | 3.5        | 0.9  |      |               |      |
| <i>n</i> -Tetradecanal                  | 1596 |           | t    | t    | t          | t    |      |               |      |
| <i>n</i> -Hexadecane                    | 1600 |           | 1.1  | t    |            |      |      |               |      |
| 1- <i>epi</i> -Cubenol                  | 1600 |           | 1.1  | 0.2  | 0.2        | 0.1  |      | 0.5           |      |
| $\gamma$ -Eudesmol                      | 1609 | 3.1       | 18.0 | 8.4  | 2.1        | 9.0  | 3.8  | 7.6           | 0.1  |
| T-Cadinol                               | 1616 | 1.0       | 8.8  | 4.0  | 1.5        | 7.2  | 3.2  | 8.9           | 0.4  |
| $\delta$ -Cadinol                       | 1618 |           | 3.4  | 0.1  |            |      |      |               |      |
| $\alpha$ -Murolol                       | 1618 |           | t    | t    | 5.1        | 1.3  |      |               |      |
| $\beta$ -Eudesmol                       | 1620 | 2.1       | 25.5 | 11.2 | 1.0        | 12.7 | 4.4  | 1.3           | 20.4 |
| 1,2-Dehydroglobulol*                    | 1623 |           |      |      |            | 2.6  | 0.7  |               |      |
| Valerianol                              | 1623 |           | 13.5 | 1.2  | 2.1        | 0.3  |      | t             | 0.3  |
| $\alpha$ -Cadinol                       | 1626 |           |      |      | 8.9        | 2.5  |      |               |      |
| $\alpha$ -Bisabolol oxide B*            | 1630 |           | 9.5  | 0.4  |            |      |      |               |      |
| $\alpha$ -Eudesmol                      | 1634 |           | 24.5 | 12.7 | 12.6       | 4.1  | 0.9  | 20.6          | 0.3  |
| Cadalene*                               | 1640 |           | 0.5  | t    | 0.3        | t    |      | 0.1           | 0.1  |
| <i>cis,trans</i> -Farnesol              | 1648 |           | 0.9  | 0.2  |            |      |      |               |      |
| $\alpha$ -Bisabolol                     | 1656 |           | 44.7 | 2.1  |            |      |      |               |      |
| <i>epi</i> - $\alpha$ -bisabolol        | 1658 |           | 0.8  | 0.1  | t          | t    |      |               |      |

| Components                                            | RI   | Propolis  |      |      |            |      |      |             | Plant Sources |      |
|-------------------------------------------------------|------|-----------|------|------|------------|------|------|-------------|---------------|------|
|                                                       |      | Cluster I |      |      | Cluster II |      |      | Cluster III | P             | C    |
|                                                       |      | Min       | Max  | Aver | Min        | Max  | Aver |             |               |      |
| <i>n</i> -Heptadecane                                 | 1700 | 0.2       | 4.4  | 1.2  | 0.2        | 3.1  | 1.1  | 1.1         | t             | 0.1  |
| Benzyl benzoate                                       | 1701 |           | 4.3  | 0.2  |            |      |      | 1.4         |               | 0.1  |
| $\alpha$ -Bisabolol oxide A*                          | 1702 |           | t    | t    |            |      |      |             |               |      |
| Tetradecanoic acid                                    | 1723 |           |      |      |            |      |      |             |               | t    |
| Phenylethyl octanoate *                               | 1764 |           | 4.4  | 0.2  |            | t    | t    |             |               | t    |
| <i>n</i> -Hexadecanal                                 | 1776 |           | t    | t    |            |      |      |             |               |      |
| Benzyl salicylate                                     | 1790 |           | 9.5  | 0.4  |            |      |      |             |               |      |
| <i>n</i> -Octadecane                                  | 1800 |           | 0.8  | t    |            | 0.4  | 0.1  |             |               |      |
| <i>n</i> -Hexadecanol                                 | 1821 |           | 7.6  | 0.4  |            |      |      |             |               |      |
| Labdanes NI 1                                         | 1821 |           | 8.2  | 0.4  |            |      |      |             |               | 0.3  |
| Labdanes NI2                                          | 1829 |           | 1.1  | t    |            |      |      |             |               |      |
| <i>n</i> -Heptadecanal                                | 1894 |           | 1.1  | 0.1  |            |      |      |             |               |      |
| <i>n</i> -Nonadecane                                  | 1900 |           | 8.9  | 2.1  | 0.7        | 5.1  | 2.0  | 2.9         |               | 0.3  |
| Hexadecanoic acid                                     | 1908 |           | 2.9  | 0.2  |            | t    | t    |             | 1.1           |      |
| Ethyl hexadecanoate                                   | 1936 |           |      |      |            |      |      |             | 0.1           |      |
| 8- <i>epi</i> -13- <i>nor</i> -Ambreinolide* (cistus) | 1965 |           | 1.0  | 0.1  |            |      |      |             |               |      |
| <i>n</i> -Eicosane                                    | 2000 |           | 0.8  | 0.1  |            | 0.4  | 0.1  | 0.8         | t             |      |
| 2-Phenyl ethyl phenyl acetate*                        | 2017 |           |      |      |            |      |      |             |               | 3.6  |
| Abietatriene                                          | 2027 |           | t    | t    |            |      |      |             |               |      |
| Phytol acetate                                        | 2047 |           | 1.1  | 0.2  |            |      |      |             |               |      |
| Manool*                                               | 2047 |           | 7.6  | 0.3  |            |      |      | 1.6         |               |      |
| Abietadiene                                           | 2060 |           | 0.9  | t    |            |      |      |             |               |      |
| Musk ambrette*                                        | 2061 |           | 0.3  | t    |            | 0.2  | 0.1  |             |               |      |
| <i>n</i> -Octadecanol (= Steryl alcohol)              | 2071 |           | 0.9  | t    |            | t    | t    |             |               |      |
| <i>cis,trans</i> -Farnesol tiglic ester               | 2098 |           |      |      |            | t    | t    |             |               |      |
| <i>n</i> -Heneicosane                                 | 2100 | 0.2       | 7.1  | 1.8  | 0.5        | 1.5  | 1.0  | 2.2         | 0.7           |      |
| Linoleic acid                                         | 2125 |           |      |      |            |      |      |             | 0.2           |      |
| Ethyl linoleate                                       | 2137 |           |      |      |            |      |      |             | 0.5           |      |
| <i>n</i> -Docosane                                    | 2200 |           | 1.7  | 0.5  |            | 0.9  | 0.4  | 1.1         | 0.1           |      |
| Labd-7-en-15-ol                                       | 2201 |           | 5.1  | 0.3  |            |      |      |             |               |      |
| Sandaracopimarinol*                                   | 2219 |           | 0.3  | t    |            |      |      |             |               |      |
| <i>trans</i> -Totarol                                 | 2259 |           | 6.9  | 0.3  |            |      |      |             |               |      |
| <i>n</i> -Eicosanol                                   | 2265 |           | 0.8  | 0.1  |            | 0.4  | 0.1  |             |               |      |
| <i>n</i> -Tricosane                                   | 2300 |           | 6.1  | 1.5  |            | 2.3  | 0.7  | 3.0         |               |      |
| <i>n</i> -Tetracosane                                 | 2400 |           | 8.3  | 0.7  |            |      |      | 2.0         |               |      |
| <i>n</i> -Pentacosane                                 | 2500 |           | 2.9  | 0.9  |            | 1.2  | 0.5  | 1.6         | 0.3           |      |
| Grouped Components                                    |      |           |      |      |            |      |      |             |               |      |
| Monoterpene hydrocarbons                              |      |           | 5.8  | 0.8  | t          | 5.5  | 1.1  | 25.5        |               | 4.3  |
| Oxygen-containing monoterpenes                        | 0.3  |           | 8.4  | 2.2  | 8.6        | 65.2 | 38.0 | 12.7        | 0.9           | 16.0 |
| Sesquiterpene hydrocarbons                            | 0.6  |           | 35.0 | 6.5  | 1.0        | 13.5 | 5.6  | 4.7         | 21.5          | 0.8  |
| Oxygen-containing sesquiterpenes                      | 20.4 |           | 76.9 | 44.6 | 16.3       | 49.3 | 30.5 | 13.9        | 59.8          | 11.3 |
| Diterpene hydrocarbons                                |      |           | 9.2  | 0.5  |            |      | 0.0  |             |               | 0.3  |
| Oxygen-containing diterpenes                          |      |           | 14.8 | 0.9  |            |      | 0.0  | 1.6         |               |      |
| Phenylpropanoids                                      |      |           | 0.3  | 0.0  |            | t    | 0.0  |             | 1.0           | 0.1  |
| Fatty acids                                           |      |           | 4.0  | 0.8  |            | 0.6  | 0.2  |             | 1.3           | t    |
| Others                                                |      | 3.6       | 36.2 | 12.2 | 4.8        | 13.8 | 8.7  | 16.4        | 1.8           | 39.0 |

RI - Retention Index relative to C<sub>8</sub>-C<sub>25</sub> *n*-alkanes on the DB-1 column; t - trace (<0.05%); \* Identification based on mass spectra only; unless otherwise stated, an empty cell means that the compound was not detected in the sample.

Cluster analysis based on the volatiles chemical composition grouped the 36 samples in three main clusters (Figure 6.1, Table 6.1). Cluster I was poorly correlated with cluster II ( $S_{\text{corr}} < 0.4$ ), and both showed a very low correlation with cluster III ( $S_{\text{corr}} < 0.2$ ) (Fig. 1). With 28 of the samples, cluster I represented 78% of the sampling performed, including samples from the Azores Archipelago, the central coast and the

majority of the samples from north, south and Madeira Island (Figure 6.1). Cluster I samples showed high relative amounts of oxygen-containing sesquiterpenes (20-77%), sesquiterpene hydrocarbons (1-35%) and of the group of others (4-36%). Monoterpenes, phenylpropanoids and fatty acids were found in relative amounts <10%.  $\gamma$ -Eudesmol (3-18%),  $\beta$ -eudesmol (2-26%),  $\alpha$ -eudesmol (24%) and  $\alpha$ -bisabolol (48%) were the most abundant oxygen-containing sesquiterpenes, Figure 6.2. Nevertheless, not all these compounds were found in every sample, in fact  $\alpha$ -bisabolol was detected in just four out of the twenty eight samples of this cluster, A1 from Azores Archipelago, CC1 and CC2 from central coast and M3 from Madeira Island.

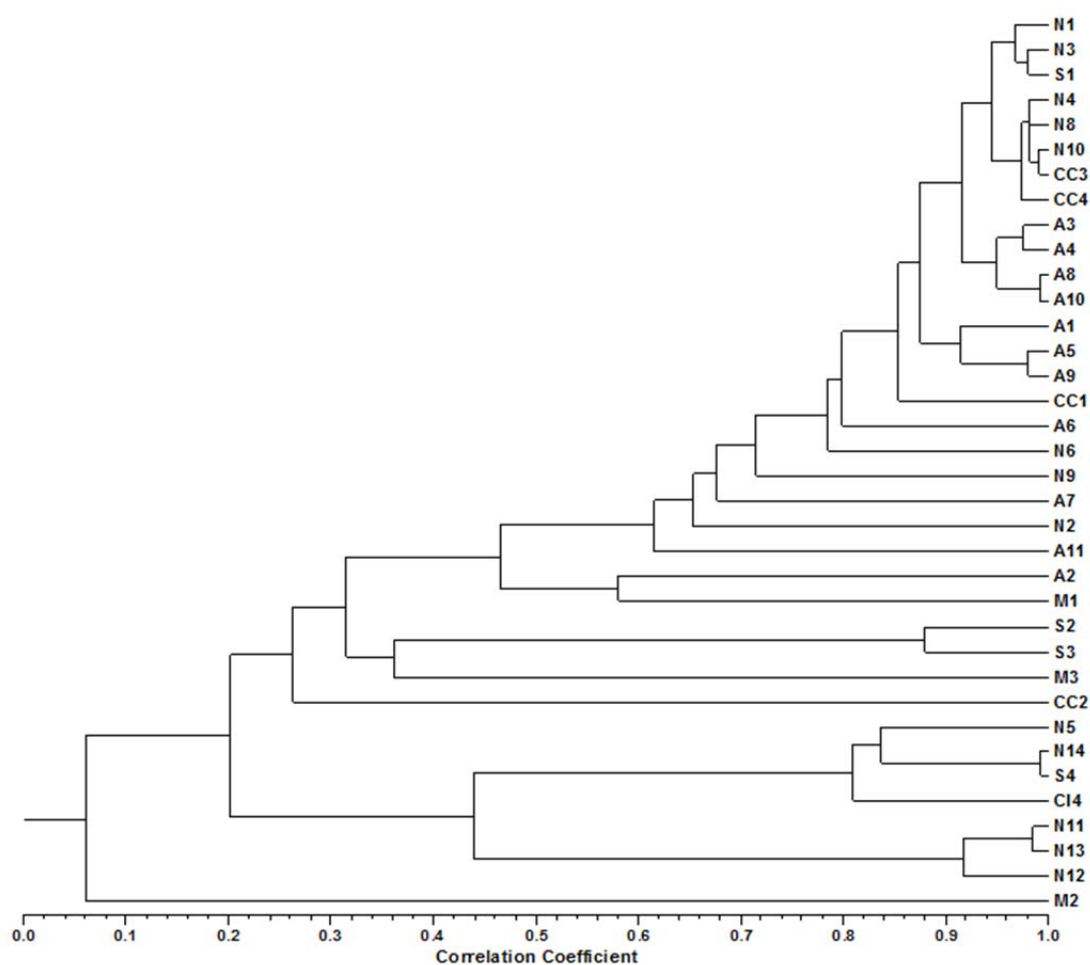


**Figure 6.1** Dendrogram obtained by cluster analysis of the percentage composition of volatiles isolated from propolis based on correlation and using unweighted pair-group method with arithmetic average (UPGMA). Twenty-eight samples constituted cluster I, seven cluster II and one cluster III. For abbreviations see Table 6.1.

Cluster II, with seven samples, comprised samples from the north (N5, N11-14), one from the south (S4) and the sample CI4 from central interior (Figure 6.1). Cluster II was mainly characterized by high oxygen-containing monoterpenes content (9-65%), oxygen-containing sesquiterpenes (16-49%) and the fraction designated as others (5-14%) (Table 6.1, Figure 6.2). Cluster II was sub-divided in two moderately correlated sub-clusters ( $S_{\text{corr}} = 0.44$ ). The sub-cluster comprising samples N5 and N14 (North), S4 (South) and CI4 (central interior) was richer in oxygen-containing monoterpenes while N11, N12 and N13 (North) showed a higher content in oxygen-containing sesquiterpenes. Thymol (8-64%), 1-*epi*-3,4-dehydroviridiflorol (10%), viridiflorol (t-10%),  $\beta$ -eudesmol (1-13%),  $\alpha$ -eudesmol (13%) were the main compounds present in this cluster samples volatiles.

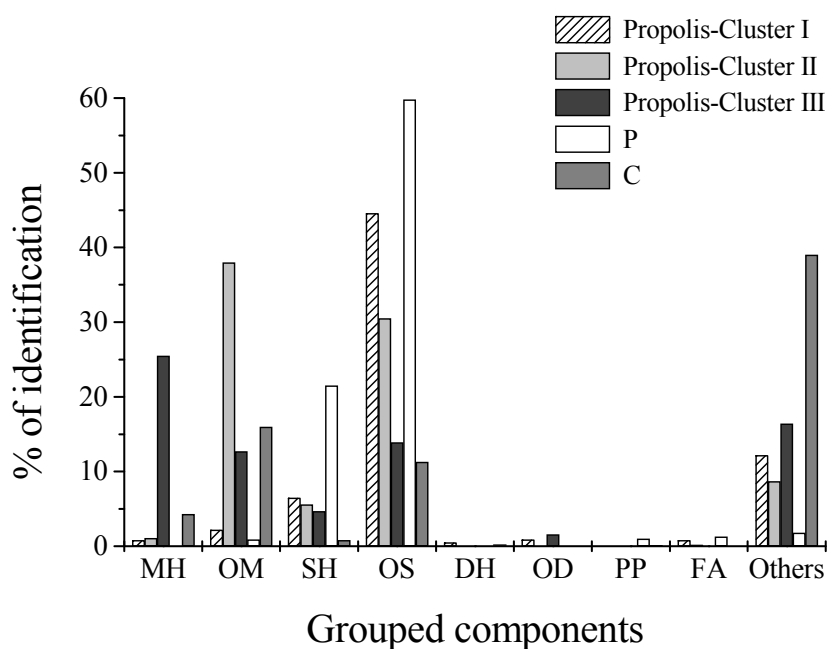


**Figure 6.2** Composition for the main classes of volatile compounds present in the grouped propolis clusters and potential plant sources samples, represented as mean values. MH – monoterpene hydrocarbons; OM – oxygen-containing monoterpenes; SH – sesquiterpene hydrocarbons; OS – oxygen-containing sesquiterpenes; DH – diterpene hydrocarbons; OD – oxygen-containing diterpenes; PP – phenylpropanoids; FA – fatty acids.

The high thymol content in most of these samples is probably linked with the long-term use of this compound as acaricide in the treatment against the ectoparasitic mite *Varroa destructor*, as pointed out by Miguel *et al.* (2012)<sup>11</sup>. These authors found a

predominance of viridiflorol, *n*-tricosane and *n*-nonadecane in the majority of propolis samples volatiles from the Algarve region. Comparing Miguel *et al.* (2012)<sup>11</sup> data with the present results, from samples obtained within the same region, but from different geographical locations, all samples volatiles had in common a high content in viridiflorol (5-8%) and *n*-nonadecane (3-7%), with exception for sample S1, from the east coast in which  $\beta$ -eudesmol (14%) and  $\alpha$ -eudesmol (18%) were the main components.

Cluster III, with only one sample from Madeira Island (M2), was mainly composed by monoterpene hydrocarbons (26%), the others fraction (16%), oxygen-containing sesquiterpenes (14%) and oxygen-containing monoterpenes (13%) (Table 6.1, Figure 6.2). Unlike the other propolis clusters, cluster III was  $\alpha$ -pinene rich (19%) followed by  $\beta$ -caryophyllene oxide (6%) and  $\beta$ -pinene (6%), and showed lower relative amounts of  $\alpha$ -eudesmol and  $\beta$ -eudesmol.  $\alpha$ -Pinene dominance was also detected in propolis from Greek regions.<sup>10</sup> The abundance of the monoterpene fraction, with a high  $\alpha$ -pinene content, was described in the species of the genus *Juniperus*, particularly *Juniperus cedrus*,<sup>16,17</sup> which is endemic to Madeira Island and could therefore be a possible contributor for this propolis sample volatiles.

### 6.3.2 Essential oil composition of plant sources

The odour of propolis is empirically used by beekeepers to assess its floral origin, which reflects the floral sources present around the hive. To address the floral origin of propolis, the evaluation of the volatile composition of the leaf-buds of the hybrid *Populus x canadensis* and branches *Cistus ladanifer* was included in the study, as two potential floral sources referred by propolis producers. *Populus x canadensis* is a hybrid species very common in Portugal and therefore a potential source of resin for the honeybees. *Cistus ladanifer* (rock rose), an wild odorous shrub widespread in the mediterranean region,<sup>18</sup> was also studied as potential plant source due to its great abundance in some hive neighborhoods and to the rock rose aroma presented by some of the propolis samples.

The essential oil isolated from the poplar buds was clear and colourless attained a yield <0.05% (v/w). Forty eight components were identified, representing 86% of the overall essential oil, Table 6.1. Oxygen-containing sesquiterpenes were the main group of constituents (60%), followed by sesquiterpene hydrocarbons (22%).

Monoterpenes, diterpenes and others were found only in very low percentage ( $\leq 2\%$ ), Table 6.1, Figure 6.2. Among the main groups, the sesquiterpene alcohols  $\alpha$ -eudesmol (21%),  $\beta$ -eudesmol (20%),  $\gamma$ -eudesmol (8%), T-cadinol (9%), the sesquiterpene  $\delta$ -cadinene (7%) and  $\gamma$ -cadinene (5%) were predominant. The high level of eudesmol isomers found in the present study was also described in the volatile composition of other poplar species leaf-buds, particularly *Populus nigra* and *Populus balsamifera*.<sup>19,20</sup>

*Populus* species are described to be the main source of propolis in temperate regions of the world.<sup>2</sup> *Populus x canadensis* essential oil composition was comparable with the one obtained for cluster I samples, with a dominance of oxygen-containing sesquiterpenes, especially  $\alpha$ - and  $\beta$ -eudesmol. The presence of high levels of bisabolol in some propolis samples within this cluster is not consistent with the profile observed for the poplar species under study, however, we must consider that other poplar species may show different volatile profiles. Indeed, the presence of bisabolol was previously described in bud exudates of *Populus generosa* and *Populus candicans*.<sup>21,22</sup> Thus, besides the contribution of *Populus x canadensis* as a source of resin for propolis samples of cluster I, other poplar species can be as well potential contributors and so, creating the diversity found in the samples. Also, in cluster II,  $\alpha$ - and  $\beta$ -eudesmol are within the main compounds (at less extent), which is indicative of some contribution of the poplar resin to this type of propolis.

Seventy three components were identified on rock rose essential oil, representing 79% of the overall volatiles (Table 6.1). The fraction named as others (39%), oxygen-containing monoterpenes (16%) and oxygen-containing sesquiterpenes (11%) were the most abundant groups (Table 6.1, Figure 6.2). The main components identified in *Cistus ladanifer* volatiles were the aromatic ketone 2,6,6-trimethylcyclohexanone (20%), viridiflorol (7%), 2-phenyl ethyl acetate (4%) and  $\beta$ -mentha-1,5-dien-8-ol (3%). Other compounds like  $\alpha$ -pinene, *trans*-pinocarveol, *trans*-verbenol, 2-decanone, bornyl acetate and ledol, common in *Cistus ladanifer* composition, were found in lower percentages.<sup>18,23</sup> Although the observed content do not match exactly with other previous studies,<sup>18,23,24</sup> the characteristic compounds of *Cistus ladanifer* and labdanum oil were presented, namely: 2,6,6-trimethylcyclohexanone, acetophenone, viridiflorol, ledol and labdane. These differences are probably linked to the diverse geographical origin of the plants analyzed, to different plant parts analyzed, or to the fact that under the common name rock rose reported by beekeepers, different *Cistus* species can be included.

Comparing the rock rose volatile profile and that from propolis samples, only samples CI4 from central interior and S2-4 from the south showed a predominance of viridiflorol (10% and 5-8% respectively). Additionally, these samples also revealed the presence of ledol (2 to 5%) and other components associated with the typical *Cistus* aroma, like, 2,6,6-trimethyl cyclohexanone, acetophenone and labdane compounds.<sup>25</sup> Thus, *Cistus ladanifer* is likely a strong contributor for Portuguese propolis volatiles, particularly in central interior and south regions.

## 6.4 Concluding remarks

Volatiles composition, isolated by hydrodistillation from 36 propolis samples from different provenances in Portugal (mainland and islands), were evaluated. Cluster analysis showed a major chemical variability in the volatile profile, displaying three poorly correlated main clusters. *Populus x canadensis* and *Cistus ladanifer* essentials oils chemical composition was compared to those of propolis samples and some correlation was found among them. The propolis samples volatiles diversity seems thus dependent on the variability, as well as on the availability, of the flora sources at the site of bee collection.

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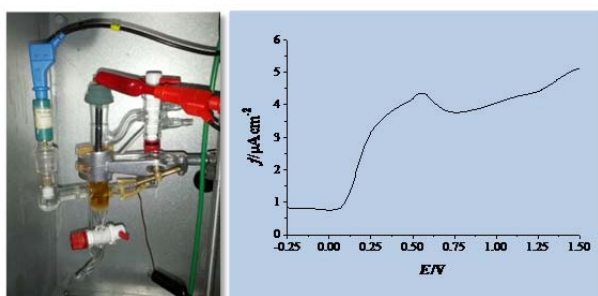
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## Chapter 7

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### **A voltammetric tool for the evaluation of propolis antioxidant capacity**





## A voltammetric tool for the evaluation of propolis antioxidant capacity<sup>\*,†</sup>

### Abstract

Propolis (bee glue) is a sticky dark-coloured material that honeybees collected from parts of plants, buds and exudates, and used as construction and defense material in the hive. This resinous substance is a rich source of phenolic compounds, with its chemical composition strongly influenced by its phytogeographic origin.

This research outlines the evaluation of the redox profile and the quantification of the total antioxidant capacity in Portuguese propolis from different origins and potential floral *Populus x Canadensis* Moench and *Cistus ladanifer* L through cyclic voltammetry and differential pulse voltammetry. Although several oxidation processes were showed by propolis samples, which were dependent of the resin origin, common oxidation potentials within the majority of samples of a region were detected, suggesting an analogous chemical composition in the respect of electroactive species. Also, a botanical discrimination was made, because plant sources electrochemical profile were similar to some of the propolis samples, which was in accordance with previous results obtained by other analytical tools. Based on the quantification of the total electroactive antioxidant power it was found that propolis with higher antioxidant activity was from coast center, followed by north ~ Azores archipelago > central interior > Madeira island > south.

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\*Adapted from Falcão SI, Tomás A, Freire C, Vilas-Boas M. A voltammetric tool for the evaluation of propolis antioxidant capacity. To be submitted, **2013**.

†The experimental details about the propolis samples, phenolic compounds extraction are indicated in Appendices A and B respectively.

## 7.1 Introduction

Natural antioxidants are primarily polyphenols that may occur in many food products of vegetable origin, comprising an extensive and complex group of secondary metabolites that are synthesized during plant growth and in response to stress. They present diverse properties as they can act as reducing agents (free radical terminators), metal chelators and singlet oxygen quenchers. Mainly, they are constituted by phenolic acids and flavonoids, which have a common structure comprising an aromatic ring with one or more hydroxyl substituents. They play an important role in plants, food and humans presenting many biological properties, including cardio-protective, anti-inflammatory, anti-allergenic, anti-carcinogenic and antimicrobial.<sup>1,2</sup> These properties are attributed to their powerful antioxidant and antiradical activity, which is related to the redox properties of phenolic compounds.<sup>3</sup>

Bee products, especially propolis, proved to be a major source of phenolic compounds.<sup>4</sup> More than three hundred substances were identified in propolis including flavonoid glycosides and aglycones, phenolic acids and their esters, phenolic aldehydes, alcohols and ketones, sesquiterpenes, quinones, coumarins, steroids, aminoacids, and inorganic compounds.<sup>5</sup> The complex chemical composition of propolis is highly variable, depending strongly on the phytogeographic characteristics around the hive. In Europe, North America and other temperate regions, the predominance *Populus* species makes them the main source of the bee glue. Generally, temperate propolis is composed by flavonoids without B-ring substituents, such as pinocembrin, pinobanksin, galangin and chrysin and phenylpropanoid acids and their esters (e.g. caffeic acid, caffeic acid phenethyl ester).<sup>6,7</sup> In the bee glue, the phenolic fraction is responsible for almost the biological and health enhancing properties like the antioxidant activity.<sup>8</sup> Of late, several protocols have been proposed for measuring the antioxidant properties in different propolis.<sup>9,10</sup> Antioxidant capacity assays can be roughly divided into two categories, depending on the chemical reaction involved: hydrogen atom transfer (HAT) and single electron transfer (SET) reaction based assays. HAT-based assays monitor competitive reaction kinetics (i.e., oxygen radical absorbance capacity assay (ORAC) and the total radical-trapping antioxidant parameter assay (TRAP)). The SET-based assays involve one redox reaction with the oxidant (i.e., Trolox equivalent antioxidant capacity assay (TEAC), ferric ion reducing antioxidant parameter assay (FRAP), DPPH-based assay, copper (II) reduction capacity, and total phenolics assay by Folin- Ciocalteu (FC)).<sup>11</sup>

Most of these methods are based on spectrophotometric techniques and present several drawbacks like the relatively high cost. Moreover, many antioxidant assays suffer from interference when working with colourful or turbid samples or when interfering compounds (i.e. vitamin C, sugars) are abundantly present in the samples.<sup>12</sup>

Taking advantage of the electrochemical activity present by the phenolic compounds, resulting from the electronic delocalisation on the aromatic nucleus, they can be easily oxidized at inert electrodes and therefore be subject of study by electrochemical methods. Its overall reducing power can be evaluated within food and biological samples without the use of specific reagents.<sup>11</sup> It was already established an important relationship between electrochemical behavior and antioxidant power: a low oxidation potential is associated with a high antioxidant power.<sup>13</sup> On the other hand, the intensity of the anodic current is related to the total content of the reducing species present in the sample.<sup>14</sup> Some authors have already applied electrochemical approaches regarding to propolis analysis, in particular evaluation of the antioxidant activity by amperometric flow injection analysis,<sup>15</sup> cyclic voltammetry<sup>16</sup> and polarography.<sup>17</sup> Also, the redox properties of specific isolated compounds from propolis were investigated by cyclic voltammetry.<sup>18</sup> To our knowledge the use of differential pulse voltammetry, common in the evaluation of other food<sup>19,20</sup> and non-food matrix<sup>21</sup> have never been explored in propolis.

The aim of this work was to establish a rapid, easy and low-cost tool, for the evaluation of the antioxidant capacity in propolis from different origins, by using electrochemical methods like cyclic voltammetry and differential pulse voltammetry. In addition, this new approach allowed the geographical discrimination of samples, and the correlation with the potential floral sources by analysing buds exudates and surface material present on the leaves and stems of *Populus x Canadensis* Moench, male and female specimens and *Cistus ladanifer* L..

## 7.2 Experimental

### 7.2.1 Standards and reagents

Standard compounds such as galangin, pinocembrin and caffeic acid were purchased from Sigma Chemical Co (St. Louis, MO, USA). Tetrabutylammonium

perchlorate (TBAP), electrochemical grade, was from Fluka (Sigma Chemical Co., St. Louis, MO, USA), and kept at 30°C before use. All other chemicals were obtained from Panreac (Barcelona, Spain). Water was treated in a Milli-Q water purification system (Topway Global Inc., Houston, TX, USA).

### 7.2.2 Samples origin and preparation

For propolis floral sources, the buds exudates and surface material present on the leaves and stems of *Populus x Canadensis* Moenchen (an hybrid specie of *Populus*) male (PM) and female specimens (PF) and *Cistus ladanifer* L. (C) were collected during spring in Bragança county. All samples were stored at -20°C until analysis. The voucher specimens are deposited at the herbarium of Escola Superior Agrária of Instituto Politécnico de Bragança with the reference number BRESA 5174, BRESA 5355 and BRESA 5356 for C, PF and PM, respectively.

### 7.2.3 Electrochemical characterization

#### 7.2.3.1 Instrumentation

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) measurements were performed on an Autolab PGSTAT 302 potentiostat/galvanostat using a closed standard three electrode cell. A glassy carbon ( $\phi = 0.314 \text{ cm}^2$ ) was use as the working electrode and a Pt foil as the counter electrode. All potentials are refer to an Ag/AgCl 3 mol/L KCl reference electrode. Prior to use, the working electrode was polished in an aqueous suspension of 0.3  $\mu\text{m}$  alumina (Beuhler) on a Master-Tex (Beuhler) polishing pad, than rinsed with water. Subsequently, in a chemical treatment, the electrode was sonicated in HCl 6 mol/L during 1 minute, and then in methanol. This cleaning procedure was applied always before any electrochemical measurements.

#### 7.2.3.2 Procedure

Propolis, plants ethanolic extracts and standard compounds were studied in ethanol/ Britton-Robinson buffer /TBAP (78:20:2) at pH 4 and 7. The extract solutions were prepared in a concentration of 1 mg/mL. All solutions were analysed immediately

after preparation and the electrochemical response was recorded after the immersion of the glassy carbon electrode, to minimize adsorption of species onto the electrode surface prior to the run. The working electrode surface state was previously checked with the analysis of the ethanol/ Britton-Robinson buffer /TBAP (78:20:2) solution alone. Every experiment was performed in triplicate, and the results are shown as an average.

The characterization of the electrochemical response process was achieved using cyclic voltammetry between 0 and 1.5 V, at 0.1 V/s as scan rate. For the evaluation of antioxidant capability the operating conditions for DPV were set at 0.06 V pulse amplitude and 0.030 V/s as scan rate, between -0.25 and 1.5 V.

### 7.2.3.3 Quantification of the total electrochemical antioxidant power

The quantification procedure was performed at pH=7 considering the intensity of the electrochemical response. For that, a calibration curve ( $y = 75.4590 - 0.2569x$ ;  $R^2 = 0.997$ ) was prepared by plotting the concentration of a standards mixture with caffeic acid: galangin: pinocembrin (1:1:1) against the current density of the respective DPV signals, using several solutions in the range 0.01 to 0.10 mg/mL. The analytical signal of the samples (current intensity) was measured between peak maxima and the baseline, defined as the tangent between the lowest and the highest potential valley. This current signal was expressed in terms of equivalents of standard solution (caffeic acid: galangin: pinocembrin (1:1:1)) per gram of propolis extract (mg/g). The sum of the values calculated at peak maxima for each electrochemical process was used to express the total electrochemical antioxidant power (TEAP) of propolis samples.

## 7.3 Results and discussion

Two electrochemical techniques were applied in the study of the propolis ethanolic extracts from different Portuguese regions: the cyclic voltammetry was used for the evaluation of the redox profile of the samples, while the differential pulse voltammetry, allowed the quantification of the electroactive species present in the different ethanolic extracts. The electrochemical response of two potential floral sources of propolis were also analysed in an attempt to establish the plant origin of the samples.

The electrochemical behaviour of the samples was evaluated at two different pH levels, 4 and 7, Figure 7.1. Oxidation reactions of flavonoids are more susceptible to

occur at a neutral pH, while phenolic acids are more active at acidic pH.<sup>22</sup> The observed pH change are mostly reflected in a shift of the oxidation peaks towards lower potentials at neutral pH,<sup>22</sup> enabling an easier assessment to the peak potentials. Because the oxidation of polyphenols at neutral pH mimics physiological conditions,<sup>23</sup> the results are presented at pH 7.

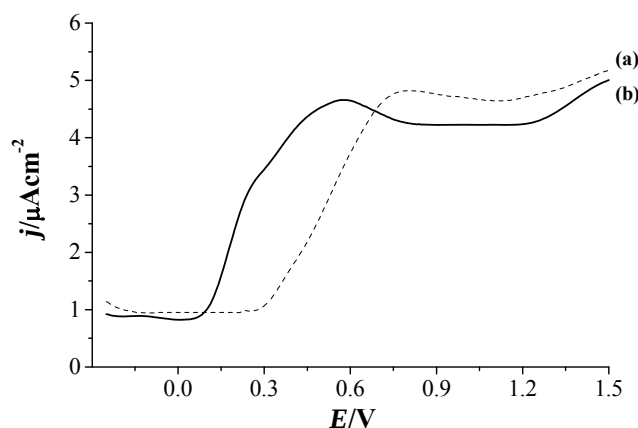


**Figure 7.1** Differential pulse voltammograms of 1 mg/mL propolis extract (sample CC1) in EtOH/ Britton-Robinson buffer /TBAP (78:28:2) solutions at (a) pH 4 and (b) pH 7 obtained with a glassy carbon electrode between -0.25 and 1.5 V, with 0.06 V pulse amplitude at 0.03 V/s.

### 7.3.1 Cyclic voltammetry

The electrochemical response for the extracts obtained with CV at pH 7 exhibited a similar voltammetric profile, Figure 7.2 (left side), with an irreversible oxidation process characterized by a broad intense anodic wave. This wave broadening may be due to the electron removal from a mixture of components, within the sample, with the same hability for oxidation, rather than to just an individual component. The location where this anodic process appears in the potential window, and the observation of other less intense peaks, suggest the existence of samples with different chemical composition, table 7.1. For the majority of the samples the higher intensity anodic wave was observed around 0.6-0.7 V, with the exception of samples A10 and A11 from Azores Archipelago and M2 from Madeira Island which reveal oxidation processes at lower potentials, 0.3-0.4 V. This could be due to the presence of more oxidisable species in these extracts and so a higher reducing power.<sup>24</sup> Some samples reveal one or two additional oxidation processes at more positive potentials (0.8 to 1.1 V). This

behavior was found in some north samples (N10-N14), center interior (CI-1-CI4) south (S4) and Madeira Island (M2-3). The appearance of oxidations processes at high potentials can have a limited contribution to the antioxidant activity, since the substances that should be “protected” may have a higher ability to be oxidized before the antioxidant specie.

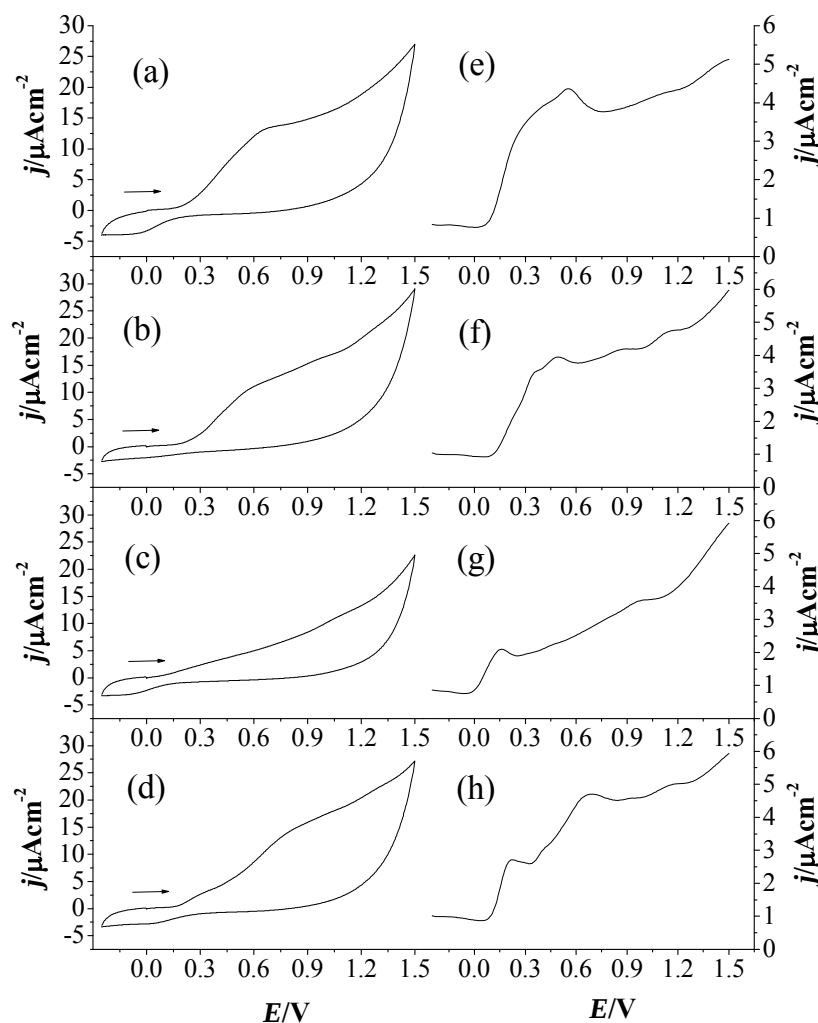


**Figure 7.2** Electrochemical responses for 1 mg/mL propolis extracts of sample in EtOH/ Britton-Robinson buffer /TBAP (78:28:2) solution at pH 7, obtained with a glassy carbon electrode: (left) cyclic voltammogram at 0.1 V/s between -0.25 and 1.5 V. (a) N8, (b) N11, (c) A10 and (d) M2; (right) differential pulse voltammograms obtained with 0.06 V pulse amplitude at 0.03 V.s<sup>-1</sup>, between -0.25 and 1.5 V. (e) N8, (f) N11, (g) A10 and (h) M2.

The potential plant sources, *Cistus ladanifer* and *Populus x Canadensis* showed only one anodic irreversible process near 0.6 V, which was similar to the majority of the samples.

### 7.3.2 Differential pulse voltammetry

Although the cyclic voltammetry appears in the literature as an electrochemical method used when analyzing antioxidants,<sup>14,24</sup> the capacity current associated with the electrochemical system reduces the ability to detect low intense oxidation processes. In the present study, we explore the use of differential pulse voltammetry (DPV) to evaluate the antioxidant capacity rather than CV. DPV is more selective and sensitive technique, because the measurement of the current signal occurs before and after the potential pulse application allowing the discrimination of effects, like absorption on the electrode by organic substances. Those effects are approximately constants in a limited potential interval enabling an easier interpretation of the results with better resolved peaks and overcoming the difficulties of accessing a correct baseline.<sup>25</sup>

The differential pulse voltammograms of representative propolis samples are showed in Figure 7.2 (right side). Through this technique, the number of potential peaks observed is much higher than the ones found by cyclic voltammetry, and range between one in the sample A10 from Azores, to the more complex electrochemical profile of sample CI1 from Central Interior, with seven distinct anodic processes, Table 7.1. However, some of those oxidation processes had only a low current density and were seen as small inflections in the voltammogram.

Generally, the propolis samples showed two common oxidation processes around 0.2 V and 0.6 V, Table 7.1, Figure 7.2 (e). Beside those, some propolis samples from Azores Archipelago, namely A1, A4, A7, A8 and A10, presented a lower peak potential around 0.15 V, well observed in sample A10 due to the inexistence, in this sample, of the oxidation process at 0.2 V, Figure 7.2 (g). Despite the less positive potential, there was no correspondence with a higher reducing power (Chapter 2) as found in other works,<sup>19</sup> maybe to the low intensity of these peaks which could limit the contribution of the oxidation process in the overall activity of the extracts. Most of the samples also present an oxidation peak above 1 V which was also found in the extract of poplar. Within the common peak potentials, the samples from the north, coast center and Azores Archipelago presented the highest current densities (data not showed), meaning a higher

content in oxidizable compounds. This observation is in agreement with the higher content in total phenolics and flavonoids for these samples (Chapter 2).

The chemical composition of the propolis is dependent on the floral diversity around the hive, and although the electrochemical response obtain in this study does not reflect the individual phenolic components of propolis, the distinct electrochemical profiles observed in some samples must reflects the different plant sources available to the bees for the resin production. *Populus x canadensis* male (PM) and female (PF) extracts presented the common oxidation processes at 0.2 and 0.6 V, which is in agreement with the expectable contribution of this plant to the Portuguese propolis as described in the previous chapters, Table 7.1. For the *Cistus ladanifer* (C) resin other oxidation processes were detected at 0.35, 0.85 and 1.05 V. These peaks were also found in propolis samples N11-N13 from the north, CI1-CI4 from central interior and M1 from Madeira Island, while samples S2-S4 from the south presented the last two potentials. Once again, these results are in agreement with the phenolic profile described on Chapter 5, being *Cistus resins* a potential contributor for the propolis from these regions.

**Table 7.1** Peak potentials obtained, at pH 7, for the electrochemical oxidation processes of propolis and floral sources extracts (mean  $\pm$  SD,  $n = 3$ ).

| Sample | Cyclic voltammetry (V) |                 |                 | Differential pulse voltammetry (V) |                 |                 |                 |                 |                 |                 |
|--------|------------------------|-----------------|-----------------|------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|        | Ep2                    | Ep3             | Ep4             | Ep1                                | Ep2             | Ep3             | Ep4             | Ep5             | Ep6             | Ep7             |
| N1     | 0.65 $\pm$ 0.00        | -               | -               | -                                  | 0.25 $\pm$ 0.00 | -               | 0.55 $\pm$ 0.00 | -               | -               | -               |
| N2     | 0.65 $\pm$ 0.01        | -               | -               | -                                  | 0.25 $\pm$ 0.00 | -               | 0.57 $\pm$ 0.00 | -               | -               | -               |
| N3     | 0.66 $\pm$ 0.06        | -               | -               | -                                  | 0.25 $\pm$ 0.00 | -               | 0.57 $\pm$ 0.00 | -               | -               | -               |
| N4     | 0.67 $\pm$ 0.02        | -               | -               | -                                  | 0.23 $\pm$ 0.00 | -               | 0.59 $\pm$ 0.00 | -               | -               | -               |
| N5     | 0.65 $\pm$ 0.01        | -               | -               | -                                  | 0.23 $\pm$ 0.00 | -               | 0.57 $\pm$ 0.00 | -               | -               | -               |
| N6     | 0.63 $\pm$ 0.00        | -               | -               | -                                  | 0.24 $\pm$ 0.00 | -               | 0.56 $\pm$ 0.00 | -               | -               | -               |
| N7     | 0.64 $\pm$ 0.01        | -               | -               | -                                  | 0.24 $\pm$ 0.00 | 0.42 $\pm$ 0.00 | 0.56 $\pm$ 0.01 | -               | -               | -               |
| N8     | 0.63 $\pm$ 0.00        | -               | -               | -                                  | 0.28 $\pm$ 0.00 | -               | 0.55 $\pm$ 0.00 | -               | -               | 1.10 $\pm$ 0.02 |
| N9     | 0.60 $\pm$ 0.01        | -               | -               | -                                  | 0.23 $\pm$ 0.00 | -               | 0.51 $\pm$ 0.00 | -               | -               | 1.06 $\pm$ 0.01 |
| N10    | 0.58 $\pm$ 0.00        | -               | 1.13 $\pm$ 0.00 | -                                  | 0.21 $\pm$ 0.00 | -               | 0.51 $\pm$ 0.00 | -               | -               | 1.02 $\pm$ 0.01 |
| N11    | 0.60 $\pm$ 0.02        | 0.96 $\pm$ 0.00 | -               | -                                  | 0.23 $\pm$ 0.00 | 0.35 $\pm$ 0.00 | 0.49 $\pm$ 0.00 | -               | 0.86 $\pm$ 0.00 | 1.14 $\pm$ 0.00 |
| N12    | 0.57 $\pm$ 0.01        | 0.93 $\pm$ 0.00 | -               | -                                  | 0.23 $\pm$ 0.00 | 0.33 $\pm$ 0.00 | 0.47 $\pm$ 0.01 | -               | 0.85 $\pm$ 0.00 | 1.10 $\pm$ 0.01 |
| N13    | 0.49 $\pm$ 0.01        | 0.86 $\pm$ 0.01 | 1.14 $\pm$ 0.00 | -                                  | 0.20 $\pm$ 0.00 | 0.29 $\pm$ 0.00 | 0.41 $\pm$ 0.01 | -               | 0.80 $\pm$ 0.00 | 1.04 $\pm$ 0.00 |
| N14    | 0.57 $\pm$ 0.04        | -               | 1.11 $\pm$ 0.00 | -                                  | 0.20 $\pm$ 0.00 | 0.37 $\pm$ 0.00 | 0.51 $\pm$ 0.00 | -               | -               | 1.01 $\pm$ 0.00 |
| CI1    | 0.69 $\pm$ 0.00        | 0.94 $\pm$ 0.00 | 1.18 $\pm$ 0.00 | -                                  | 0.23 $\pm$ 0.00 | 0.33 $\pm$ 0.00 | 0.48 $\pm$ 0.00 | 0.65 $\pm$ 0.00 | 0.85 $\pm$ 0.00 | 1.09 $\pm$ 0.01 |
| CI3    | 0.57 $\pm$ 0.00        | 0.84 $\pm$ 0.01 | 1.15 $\pm$ 0.00 | -                                  | 0.23 $\pm$ 0.00 | 0.31 $\pm$ 0.00 | 0.50 $\pm$ 0.00 | -               | 0.77 $\pm$ 0.01 | 1.04 $\pm$ 0.00 |
| CI4    | 0.61 $\pm$ 0.02        | 0.88 $\pm$ 0.00 | -               | -                                  | 0.23 $\pm$ 0.00 | 0.41 $\pm$ 0.02 | 0.52 $\pm$ 0.01 | -               | 0.79 $\pm$ 0.00 | 1.05 $\pm$ 0.00 |
| CC1    | 0.68 $\pm$ 0.01        | -               | -               | -                                  | 0.25 $\pm$ 0.00 | -               | 0.57 $\pm$ 0.00 | -               | -               | -               |
| CC2    | 0.72 $\pm$ 0.00        | -               | -               | -                                  | 0.22 $\pm$ 0.01 | -               | 0.57 $\pm$ 0.00 | -               | 0.82 $\pm$ 0.00 | -               |
| CC3    | 0.60 $\pm$ 0.01        | -               | -               | -                                  | 0.22 $\pm$ 0.00 | -               | 0.52 $\pm$ 0.00 | -               | -               | 1.02 $\pm$ 0.01 |
| CC4    | 0.64 $\pm$ 0.00        | -               | -               | -                                  | 0.26 $\pm$ 0.00 | -               | 0.56 $\pm$ 0.02 | -               | -               | 1.06 $\pm$ 0.04 |
| S1     | 0.70 $\pm$ 0.02        | -               | -               | -                                  | 0.23 $\pm$ 0.00 | -               | 0.58 $\pm$ 0.00 | -               | -               | 1.11 $\pm$ 0.02 |
| S2     | 0.59 $\pm$ 0.00        | -               | -               | -                                  | 0.23 $\pm$ 0.00 | -               | 0.50 $\pm$ 0.00 | -               | 0.79 $\pm$ 0.00 | 1.01 $\pm$ 0.00 |
| S3     | 0.58 $\pm$ 0.01        | -               | -               | -                                  | 0.25 $\pm$ 0.00 | -               | 0.52 $\pm$ 0.00 | -               | 0.80 $\pm$ 0.00 | 1.03 $\pm$ 0.00 |
| S4     | 0.57 $\pm$ 0.00        | 0.84 $\pm$ 0.01 | -               | -                                  | 0.20 $\pm$ 0.01 | -               | 0.47 $\pm$ 0.00 | -               | 0.77 $\pm$ 0.01 | 0.97 $\pm$ 0.00 |
| A1     | 0.67 $\pm$ 0.01        | -               | -               | 0.14 $\pm$ 0.00                    | 0.24 $\pm$ 0.01 | -               | 0.55 $\pm$ 0.00 | -               | -               | 1.07 $\pm$ 0.04 |
| A2     | 0.74 $\pm$ 0.00        | -               | -               | -                                  | 0.23 $\pm$ 0.00 | -               | 0.58 $\pm$ 0.00 | -               | -               | -               |
| A3     | 0.77 $\pm$ 0.00        | -               | -               | -                                  | 0.23 $\pm$ 0.00 | -               | 0.56 $\pm$ 0.00 | -               | -               | -               |
| A4     | 0.67 $\pm$ 0.06        | -               | -               | 0.15 $\pm$ 0.00                    | 0.24 $\pm$ 0.00 | -               | 0.59 $\pm$ 0.00 | -               | -               | -               |
| A5     | 0.66 $\pm$ 0.02        | -               | -               | -                                  | 0.23 $\pm$ 0.01 | -               | 0.55 $\pm$ 0.00 | -               | -               | -               |
| A6     | 0.72 $\pm$ 0.00        | -               | -               | -                                  | 0.26 $\pm$ 0.00 | -               | 0.59 $\pm$ 0.00 | -               | -               | -               |
| A7     | 0.71 $\pm$ 0.02        | -               | -               | 0.16 $\pm$ 0.00                    | -               | -               | 0.55 $\pm$ 0.00 | -               | -               | -               |
| A8     | 0.65 $\pm$ 0.00        | -               | -               | 0.16 $\pm$ 0.00                    | 0.26 $\pm$ 0.00 | -               | 0.55 $\pm$ 0.00 | -               | -               | 0.90 $\pm$ 0.00 |
| A9     | 0.68 $\pm$ 0.00        | -               | -               | -                                  | 0.26 $\pm$ 0.00 | -               | 0.57 $\pm$ 0.00 | -               | -               | 1.10 $\pm$ 0.00 |
| A10    | 0.39 $\pm$ 0.01        | -               | -               | 0.14 $\pm$ 0.00                    | -               | -               | -               | -               | -               | 0.96 $\pm$ 0.01 |
| A11    | 0.43 $\pm$ 0.02        | -               | -               | 0.19 $\pm$ 0.00                    | -               | -               | -               | -               | -               | 1.02 $\pm$ 0.00 |
| M1     | 0.52 $\pm$ 0.01        | -               | -               | -                                  | 0.21 $\pm$ 0.00 | 0.44 $\pm$ 0.01 | -               | -               | 0.74 $\pm$ 0.00 | 1.02 $\pm$ 0.00 |
| M2     | 0.31 $\pm$ 0.01        | 0.79 $\pm$ 0.01 | -               | -                                  | 0.21 $\pm$ 0.01 | 0.41 $\pm$ 0.00 | -               | 0.64 $\pm$ 0.02 | -               | 1.15 $\pm$ 0.01 |
| M3     | 0.59 $\pm$ 0.00        | 0.81 $\pm$ 0.02 | -               | -                                  | -               | -               | 0.53 $\pm$ 0.01 | -               | 0.75 $\pm$ 0.00 | -               |
| PM     | 0.63 $\pm$ 0.03        | -               | -               | -                                  | 0.25 $\pm$ 0.00 | -               | 0.55 $\pm$ 0.00 | -               | -               | 1.11 $\pm$ 0.02 |
| PF     | 0.62 $\pm$ 0.01        | -               | -               | -                                  | 0.24 $\pm$ 0.00 | -               | 0.52 $\pm$ 0.00 | -               | -               | 1.09 $\pm$ 0.01 |
| C      | 0.56 $\pm$ 0.01        | -               | -               | -                                  | 0.17 $\pm$ 0.00 | 0.33 $\pm$ 0.00 | -               | -               | 0.80 $\pm$ 0.00 | 1.07 $\pm$ 0.00 |

To express the antioxidant capacity of each propolis samples the electrochemical current density were quantify in comparison with the electrochemical signal of a standard mixture solution of caffeic acid: galangin: pinocembrin (1:1:1), which mimics this complex matrix constituted by different phenolic groups. The electrochemical response of the standard mixture increase in current density with the concentration of the phenolic compounds, as can be observed in the voltammograms of Figure 7.3 (a), leading to a linear correlation between the two parameters, in the range of 0.01-0.06 mg/mL, Figure 7.3 (b).

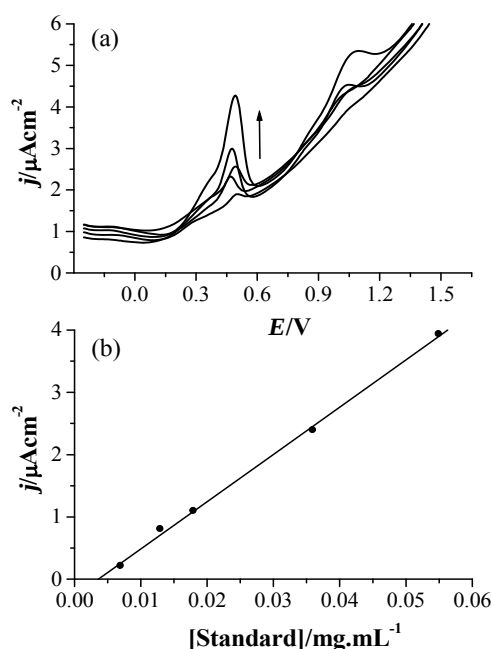


**Figure 7.3** (a) Differential pulse voltammograms obtained for 0.007-0.06 mg/mL of the mixture caffeic acid: galangin: pinocembrin (1:1:1) used as standard, in in EtOH/ Britton-Robinson buffer /TBAP (78:28:2) solutions at pH 7, with glassy carbon electrode between -0.25 and 1.5 V, with 0.06 V pulse amplitude at 0.03 V/s. (b) Dependence of the oxidation current density, obtained by DPV, with the concentration of the standard mixture.

Table 7.2 shows the electrochemical antioxidant power (EAP) expressed as equivalents of caffeic acid: galangin: pinocembrin (1:1:1) calculated for each resolved oxidation peak but also the total electrochemical antioxidant power (TEAP) which resulted from the sum of the different EAP for each sample. It is clear from the table

that the oxidation processes with higher contribution for the TEAP are those with lower potentials.

**Table 7.2** Electrochemical antioxidant power (EAP), obtained by differential pulse voltammetry in EtOH/ Britton-Robinson buffer /TBAP (78:28:2) solution at pH 7.

| Sample | EAP <sup>a</sup> |        |        |        |       |        |        | TEAP <sup>b</sup> |
|--------|------------------|--------|--------|--------|-------|--------|--------|-------------------|
|        | 0.15 V           | 0.25 V | 0.35 V | 0.55 V | 0.7 V | 0.85 V | 1.05 V |                   |
| N1     | -                | 24±2   | -      | 26±1   | -     | -      | -      | 49±2              |
| N2     | -                | 15±1   | -      | 20±1   | -     | -      | -      | 35±2              |
| N3     | -                | 18±2   | -      | 23±0   | -     | -      | -      | 42±2              |
| N4     | -                | 11±2   | -      | 22±1   | -     | -      | -      | 33±3              |
| N5     | -                | 16±2   | -      | 20±1   | -     | -      | -      | 36±2              |
| N6     | -                | 12±0   | -      | 14±1   | -     | -      | -      | 26±1              |
| N7     | -                | 16±1   | 23±2   | 21±3   | -     | -      | -      | 60±4              |
| N8     | -                | 25±1   | -      | 24±1   | -     | -      | 4±0    | 53±2              |
| N9     | -                | 17±0   | -      | 21±2   | -     | -      | 4±0    | 42±2              |
| N10    | -                | 14±1   | -      | 15±1   | -     | -      | 4±0    | 33±1              |
| N11    | -                | 11±2   | 21±2   | 18±1   | -     | 5±0    | 5±0    | 60±3              |
| N12    | -                | 7±1    | 11±1   | 13±0   | -     | 4±0    | 5±0    | 40±2              |
| N13    | -                | 5±0    | 6±1    | 10±0   | -     | 6±0    | 9±0    | 35±1              |
| N14    | -                | 16±1   | 13±1   | 9±0    | -     | -      | 6±0    | 44±1              |
| CI1    | -                | 10±1   | 15±1   | 8±1    | 5±0   | 5±0    | 5±0    | 47±2              |
| CI3    | -                | 8±0    | 8±1    | 8±1    | -     | 7±1    | 8±1    | 39±1              |
| CI4    | -                | 6±2    | 8±3    | 8±2    | -     | 5±0    | 4±0    | 30±4              |
| CC1    | -                | 24±0   | -      | 25±0   | -     | -      | -      | 49±0              |
| CC2    | -                | 12±3   | -      | 32±2   | -     | 20±0   | -      | 64±4              |
| CC3    | -                | 19±1   | -      | 21±1   | -     | -      | 5±0    | 45±1              |
| CC4    | -                | 27±3   | -      | 23±2   | -     | -      | 4±0    | 53±4              |
| S1     | -                | 9±1    | -      | 17±1   | -     | -      | 3±0    | 29±2              |
| S2     | -                | 5±0    | -      | 6±0    | -     | 10±1   | 7±0    | 28±1              |
| S3     | -                | 7±1    | -      | 7±0    | -     | 7±0    | 5±0    | 26±1              |
| S4     | -                | 4±1    | -      | 6±1    | -     | 5±0    | 5±0    | 20±1              |
| A1     | 21±1             | 24±2   | -      | 25±1   | -     | -      | 4±0    | 73±3              |
| A2     | -                | 17±0   | -      | 33±1   | -     | -      | -      | 50±1              |
| A3     | -                | 13±1   | -      | 26±1   | -     | -      | -      | 39±1              |
| A4     | 9±0              | 14±0   | -      | 26±0   | -     | -      | -      | 49±1              |
| A5     | -                | 18±2   | -      | 29±1   | -     | -      | -      | 47±2              |
| A6     | -                | 19±2   | -      | 27±1   | -     | -      | -      | 46±2              |
| A7     | 12±3             | -      | -      | 30±4   | -     | -      | -      | 41±5              |
| A8     | 11±0             | 17±0   | -      | 24±1   | -     | 11±1   | -      | 63±1              |
| A9     | -                | 11±0   | -      | 18±1   | -     | -      | -      | 30±1              |
| A10    | 11±2             | -      | -      | -      | -     | -      | -      | 11±2              |
| A11    | -                | 9±1    | -      | -      | -     | -      | -      | 9±1               |
| M1     | -                | 8±1    | 4±0    | -      | -     | 6±0    | -      | 17±1              |
| M2     | -                | 19±4   | 14±2   | -      | 22±0  | -      | -      | 56±4              |
| M3     | -                | -      | -      | 15±4   | -     | 13±2   | -      | 28±4              |
| PM     | -                | 15±0   | -      | 15±1   | -     | -      | -      | 30±1              |
| PF     | -                | 6±0    | -      | 9±0    | -     | -      | -      | 15±0              |
| C      | -                | 12±0   | 18±1   | -      | -     | 5±0    | -      | 35±1              |

<sup>a</sup>Electrochemical antioxidant power; <sup>b</sup>total electrochemical antioxidant power, at pH 7, expressed in mg/g of caffeic acid: galangin: pinocembrin (1:1:1) at the different potentials presented by the propolis and plant sources extracts (mean ± SD; *n* = 3).

Overall, the highest values of TEAP were found for samples A1, A8 from Azores archipelago and CC2, CC4 from coast center, while samples A10, A11 from Azores archipelago and all the south samples presented the lowest values. These electrochemical results follow the same profile of the other antioxidant methods used in Chapter 2 for the evaluation of the antioxidant activity, with the north, coast center and Azores samples showing the best scavenging effects, while south, CI2-CI4 from central

interior and Madeira Island presented the lowest activities. Concerning the TEAP values presented by plant sources PM, PF and C, the antioxidant activity is significantly lower than the propolis samples, with values of 30, 15 and 35 mg/g respectively. This could be explained by the fact that propolis itself is a product of a selection and concentration of different resins undertaken by bees and so its richness in electroactive species is higher than the plant sources.

## 7.4 Concluding remarks

Electrochemical methods like cyclic voltammetry and differential pulse voltammetry were applied as a fast and easy tool for the evaluation of the redox profile and the quantification of the total antioxidant capacity in Portuguese propolis from different origins. Both techniques showed the existence of several oxidation processes which vary with the origin of the resin, but reveal common oxidation potentials within the majority of samples of a region suggesting an analogous chemical composition in the respect of electroactive species. The electrochemical profile of the plant sources *Populus x canadensis* and *Cistus lanadifer* were similar to some of propolis samples and confirm the botanical discrimination already observed for these samples with other analytical tools. Therefore, the electrochemical profile can be used as a quick method to discriminate between *Populus* and *Cistus* Portuguese propolis. With the quantification of the total electroactive antioxidant power it was possible to attribute the higher antioxidant activity to propolis from coast centre, followed by north ~ Azores Archipelago > central interior > Madeira Island > south.

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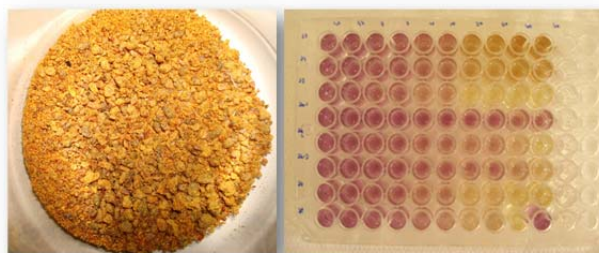
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## Chapter 8

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### **In vitro evaluation of Portuguese propolis and floral sources for antiprotozoal, antibacterial and antifungal activity**





## In vitro evaluation of Portuguese propolis and floral sources for antiprotozoal, antibacterial and antifungal activity<sup>\*,†</sup>

### Abstract

Propolis is a beehive product with a very complex chemical composition, used since ancient times in several therapeutic treatments. As a contribution to the improvement of drugs against several tropical diseases caused by protozoa, we screened Portuguese propolis and its potential floral sources *Populus x Canadensis* and *Cistus ladanifer* against *Plasmodium falciparum*, *Leishmania infantum*, *Trypanosoma brucei* and *Trypanosoma cruzi*. The toxicity against MRC-5 fibroblast cells was evaluated to assess selectivity. The *in vitro* assays were performed following the recommendations of WHO Special Programme for Research & Training in Tropical Diseases (TDR) and revealed moderate activity, with the propolis extracts presenting the relatively highest inhibitory effect against *T. brucei*. Additionally, the antimicrobial activity against *Staphylococcus aureus*, *Candida albicans*, *Trichophyton rubrum* and *Aspergillus fumigatus* was also verified with the better results observed against *T. rubrum*. The quality of the extracts was controlled by evaluating the phenolic content and antioxidant activity. The observed biological activity variations are associated with the variable chemical composition of the propolis and the potential floral sources under study.

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\*Adapted from Falcão SI, Vale N, Cos P, Comes P, Freire C, Maes L, Vilas-Boas M. In vitro evaluation of Portuguese propolis and floral sources for antiprotozoal, antibacterial and antifungal activity. *Phytoter. Res.* **2013**, DOI: 10.1002/ptr. 5013.

†The experimental details about the phenolic compounds extraction spectrophotometric determination of phenolic compounds and antioxidant activity are indicated in Appendices B, C and D respectively.

## 8.1 Introduction

Propolis is a resinous bee product collected by honeybees (*Apis mellifera* L.) from parts of plants, buds and exudates on the vegetation around the hive and enriched with wax, salivary and enzymatic secretions.<sup>1</sup> This natural resinous substance plays an important role as construction and defence material against infections in the hive<sup>2</sup> and preserves some of the medicinal properties of certain plants. For instance, *Cistus* species secrete large amounts of a strong aromatic resin on the surface of leaves and stems, rich in polyphenols and used since ancient times to treat diarrhea, dysentery and menstruation problems.<sup>3</sup> The *Populus* bud exudates have long been used in popular medicine for treating wounds and ulcers. Their antiseptic, anti-inflammatory and antimicrobial properties have been documented.<sup>4,5</sup>

During the last decades, propolis has become the subject of increased scientific interest for its wide variety of pharmacological and biological properties, such as antibacterial,<sup>6</sup> antifungal,<sup>7</sup> antiviral,<sup>8</sup> antiprotozoal,<sup>9</sup> antioxidant, hepatoprotective, antitumor and anti-inflammatory activities.<sup>10</sup> Approximately half of the propolis content corresponds to phenolic compounds, while beeswax, volatiles and pollen account for respectively 30%, 10% and 5%.<sup>11</sup> The chemical composition of propolis is highly variable and complex, depending strongly on the plant sources available at the site of collection and thus on the geographic and climatic characteristics of the apiary location. In regions of temperate climate, bees obtain resins from the buds of *Populus* spp. and derived propolis is mainly composed by flavonoids, phenolic acids and their esters. In tropical areas, species of *Baccharis* spp. in Brazil and *Clusia* spp. in Cuba and Venezuela are the main sources of propolis, with prenylated *p*-coumaric acids and polyisoprenylated benzophenones as the main compounds in *Baccharis* and *Clusia*-derived propolis, respectively.<sup>2</sup> Despite the compositional differences between propolis types, their biological properties are very similar, *e.g.*, all possess antimicrobial activity.

Propolis from North-eastern Portugal was recently characterized, providing identification of thirty-seven phenolic compounds, with pinocembrin, chrysin and pinobanksin-3-*O*-acetate being the most abundant.<sup>12</sup> The phenolic compounds show strong antioxidant activity and decrease erythrocyte membrane fragility in hereditary spherocytosis.<sup>13</sup> Propolis extracts also strongly suppress the proliferation of primary renal cancer cells in a concentration-dependent manner<sup>14</sup> and are able to exert moderate neuroprotection through the inhibition of caspase-3 activation.<sup>15</sup> Portuguese propolis

extracts were shown to have antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Candida albicans*,<sup>16</sup> with the Gram-negative bacteria being more sensitive to the bee glue. To the best of our knowledge, there are yet no studies on the activity of Portuguese propolis against pathogenic protozoa.

In this study, Portuguese propolis phenolic extracts and their potential plant sources were screened *in vitro* for their activity against the pathogenic protozoa *Plasmodium falciparum*, *Leishmania infantum*, *Trypanosoma cruzi* and *Trypanosoma brucei*. To assess selectivity of action, cytotoxicity against MRC-5 fibroblasts and antibacterial and antifungal activities were evaluated in parallel. The quality of the extracts was assessed by evaluating their phenolic content and antioxidant activity.

## 8.2 Experimental

### 8.2.1 Materials, reagents and solvents

Standard compounds such as galangin, pinocembrin, caffeic acid, chloroquine, melarsoprol, benznidazole, miltefosine, tamoxifen, as well as other chemicals, such as aluminium chloride, potassium ferricyanide, ferric chloride, trichloroacetic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and dimethyl sulfoxide (DMSO) were all purchased from Sigma Chemical Co (St. Louis, MO, USA). Analytical grade reagents like sodium carbonate, potassium hydroxide, Folin-Ciocalteu reagent, acetic acid, sulphuric acid, formic acid, ethanol and methanol were obtained from Panreac (Barcelona, Spain). 2,4-dinitrophenylhydrazine (DNP), ampicillin and miconazole were from Fluka Chemical Co (St. Louis, MO, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

### 8.2.2 Sample origin and preparation

The study was performed on propolis and plant samples present in the hive neighborhood that were reported<sup>2</sup> as propolis floral sources. Two different propolis samples were collected from beekeepers after the honey harvesting season by scratching the hive walls and frames, followed by the removal of debris of wood and bees. Sample A1 was collected in North-eastern Portugal (Bragança county), while sample A2 was

collected from the centre of Portugal (Leiria county). For the floral sources of the bee glue, the buds exudates and surface material present on the leaves and stems of *Populus x Canadensis* Moench (an hybrid species of *Populus*) male (PM) and female specimens (PF) and *Cistus ladanifer* L. (C) were collected during spring. All samples were stored at -20°C until analysis. The voucher specimens are deposited at the herbarium of Escola Superior Agrária of Instituto Politécnico de Bragança with the reference number BRESA 5174, BRESA 5355 and BRESA 5356 for C, PF and PM respectively.

For biological studies, stock solutions of the extracts were prepared in 13% DMSO/Water (propolis) or 100% DMSO (floral sources) at 20 mg/mL.

### 8.2.3 Test plate production for antimicrobial evaluation

The experiments were performed in 96-well plates (Greiner) at four-fold dilutions in a dose-titration range of 64 µg/mL to 0.25 µg/mL. Dilutions were carried out by a programmable precision robotic station (BIOMEK 2000, Beckman, USA). Each plate also contained medium-controls (blanks: 0% growth), infected untreated controls (negative control: 100% growth) and reference controls (positive control). Tests were run in duplicate in two independent experiments.

### 8.2.4 Biological screening tests

The integrated panel of microbial screens for the present study and the standard screening methodologies were adopted as described before.<sup>17</sup> Extracts with high cytotoxicity and/or non-selective activity against the different protozoa were not titrated down to their exact IC<sub>50</sub>.

### 8.2.5 Antiplasmodial activity

The chloroquine-susceptible *P. falciparum* GHA-strain was used. Parasites were cultured in human erythrocytes A<sup>+</sup> at 37°C under a low oxygen atmosphere (3% O<sub>2</sub>, 4% CO<sub>2</sub>, and 93% N<sub>2</sub>) in a modular incubation chamber.<sup>18</sup> The culture medium was RPMI-1640 supplemented with 10% human serum. Two hundred microliters of infected human red blood cells suspension (1% parasitemia, 2% hematocrit) were added to each

well of the plates with test compounds and incubated for 72h. After incubation, test plates were frozen at -20°C. Parasite multiplication was measured by the Malstat method.<sup>19</sup> One hundred microliters of Malstat reagent were transferred in a new plate and mixed with 20 µL of the hemolysed parasite suspension for 15 minutes at room temperature. After addition of 20 µL NBT/PES solution (1.6 mg of Nitroblue Tetrazolium- NBT and 0.1 mg of Phenazine ethosulphate-PES) and 2h incubation in the dark, the optical density was spectrophotometrically read at 655 nm (Biorad 3550-UV microplate reader). Percentage of growth inhibition was compared to the negative blanks. Chloroquine was used as reference drug.

#### 8.2.6 Antitrypanosomal activity

Trypomastigotes of *T. b. brucei* Squib-427 strain (suramin-sensitive) were cultured at 37°C and 5% CO<sub>2</sub> in Hirumi-9 medium,<sup>20</sup> supplemented with 10% fetal calf serum (FCS). Assays were performed by adding  $1.5 \times 10^4$  trypomastigotes/well. After 72 h incubation, parasite growth was assessed fluorimetrically by adding resazurin<sup>21</sup> for 24h at 37°C. Fluorescence was measured using a GENios Tecan fluorimeter (excitation 530 nm, emission 590 nm). Melarsoprol was used as reference drug.

Amastigotes of *T. cruzi* (Tulahuen CL2 strain, nifurtimox-sensitive) were maintained on MRC-5 cells in minimal essential medium (MEM) supplemented with 20 mM L-glutamine, 16.5 mM sodium hydrogen carbonate and 5% FCS at 37°C and 5% CO<sub>2</sub>. To determine *in vitro* activity,  $4 \times 10^3$  MRC-5 cells and  $4 \times 10^4$  parasites were added to each well of the test plate with compound. After incubation at 37°C for 7 days, parasite growth was assessed by adding  $\beta$ -galactosidase substrate chlorophenol red  $\beta$ -D-galactopyranoside<sup>22</sup> for 4h at 37°C. The colour reaction was read at 540 nm and optical density values were expressed as a percentage of the blank controls. Benznidazole was used as reference drug.

#### 8.2.7 Antileishmanial activity

Amastigotes of *L. infantum* (MHOM/ET 67) were collected from the spleen of an infected donor hamster and used to infect primary peritoneal mouse macrophages. To determine *in vitro* antileishmanial activity,  $3 \times 10^4$  macrophages were seeded in each well of a 96-well plate. After 48h outgrowth,  $5 \times 10^4$  amastigotes/well were added and

incubated for 2h at 37°C. Pre-diluted compounds were subsequently added and the plates were further incubated for 120h at 37°C and 5% CO<sub>2</sub>. Parasite burdens were determined microscopically after Giemsa staining and expressed as a percentage of the blank controls without propolis sample. Miltefosine was used as reference drug.

### 8.2.8 Cytotoxicity assay

MRC-5<sub>SV2</sub> human foetal lung fibroblasts were cultivated in MEM, supplemented with L-glutamine (20 mM), 16.5 mM sodium hydrogen carbonate and 5% FCS at 37°C and 5% CO<sub>2</sub>. For the assay, 10<sup>4</sup> MRC-5 cells/well were seeded onto the test plates containing the pre-diluted compounds and incubated at 37°C and 5% CO<sub>2</sub> for 72h. Cell viability was determined after addition of resazurin. Tamoxifen was used as reference drug.

### 8.2.9 Antibacterial and antifungal assays

These assays have been also performed at the Laboratory of Microbiology, Parasitology and Hygiene (LMPH), Antwerp University, Belgium<sup>17</sup> against *Staphylococcus aureus*, *Candida albicans*, *Trichophyton rubrum* and *Aspergillus fumigatus*. IC<sub>50</sub> values were determined from five 4-fold dilutions, starting from a maximum concentration of 64 µg/mL. Ampicillin was used as reference for *S. aureus*, while miconazole was used as reference for *Candida*, *Trichophyton* and *Aspergillus*. The impact of toxicity was determined by analyzing the selectivity index (SI), the ratios between the MRC-5<sub>SV2</sub> cytotoxic and the antimicrobial IC<sub>50</sub> values.

### 8.2.10 Statistical Analysis

For the phenolic composition and antioxidant activity, the assays were carried out in triplicate and presented in the figures as mean values with standard deviation. The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD test with  $\alpha = 0.05$ . The analysis was performed using SPSS v. 18.0 program. The result of this analysis can be found in the figures: samples with the same letter are statistically no different.

For the antiprotozoal, antibacterial and antifungal activity, in each experiment, the 50% of microbial growth ( $IC_{50}$ ) and human cell growth ( $CC_{50}$ ) inhibition value was determined from the concentration-response curves and the results were expressed as the mean  $\pm$  standard deviation of two independent experiments.

### 8.3 Results and discussion

Propolis is a bee product with a complex chemical composition which is largely dependent on its plant origin. *Populus* bud exudates and *Cistus ladanifer* L. leaf exudates are documented to be potential resin sources for propolis in Europe.<sup>2</sup> In the present study, these plants were abundant in the hive neighborhood and their phenolic content, antioxidant and antimicrobial activities were evaluated and compared to propolis.

Figure 8.1 shows the content of total phenolic compounds, flavones/flavonols and flavanones/dihydroflavonols in propolis and in the *Cistus* and *Populus* ethanolic extracts.

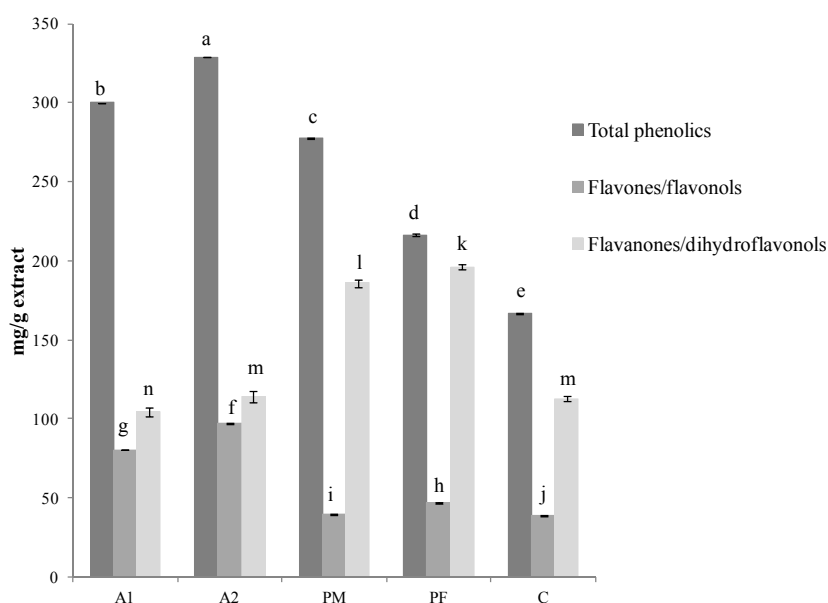


**Figure 8.1** Phenolic composition of propolis and their ethanolic extracts. A1, North-eastern propolis; A2, Central propolis; PM, *Populus x canadensis* male; PF, *Populus x canadensis* female; C, *Cistus ladanifer*. Total phenolics were expressed as caffeic acid: galangin: pinocembrin (1:1:1) equivalents. Flavones/flavonols were expressed as galangin equivalents. Flavanones/dihydroflavonols were expressed as pinocembrin equivalents. In each column, different letters (a–n) mean significant differences between samples ( $p < 0.05$ ).

All values are statistically different. The highest total phenolic content was found in the propolis samples, but there is no great difference between the two samples of distinct geographical origin. The total phenolic content of 329 mg/g for the central propolis (A2) is in line with other studies on Portuguese propolis.<sup>23</sup> For the plant extracts, the values ranged from 167 to 278 mg/g with the male *Populus* sample revealing a higher value than *Cistus ladanifer*.

For the flavones and flavonols quantified by the aluminium chloride method, a higher content was also found in propolis compared to the plant extracts. North-eastern and central propolis samples showed values ranging from 81 to 97 mg/g, while the plant extracts contained 39 to 47 mg/g (Figure 8.1). The flavanones and dihydroflavonols quantified by the DNP method were present in smaller quantities in the *Cistus* extract (113 mg/g) compared with propolis and *Populus* ethanolic extracts (Figure 8.1).

Phenolic compounds, due to their hydroxyl groups, are known to act as antioxidants.<sup>24</sup> Therefore, the activity of the extracts was evaluated with the DPPH free radical scavenging method.<sup>25</sup> The EC<sub>50</sub> values are shown in Figure 8.2A with all propolis and plant extracts possessing significant free radical scavenging activity. The extracts of male and female *Populus x canadensis* bud exudates exhibited the highest activity with EC<sub>50</sub> values of 0.014 and 0.015 mg/mL respectively. These values were close to the standard mixture of pure compounds used in this study (caffeic acid: galangin: pinocembrin, 1:1:1), pointing out the significant activity of these natural extracts. The propolis samples showed an EC<sub>50</sub> value around 0.018 mg/mL, while *Cistus ladanifer* resin presented the lowest scavenging activity (Figure 8.2A).

Fe(III) reduction was used as an indicator of electron-donating activity, which is an important mechanism for phenolic antioxidant action.<sup>26</sup> The reducing power was expressed as caffeic acid: galangin: pinocembrin (1:1:1) equivalents (Figure 8.2B). Once again, the best activity was found for the male *Populus x canadensis* followed by the propolis sample A1 with values of 807 mg/g and 710 mg/g respectively. Sample A2 revealed a very low reduction power, even lower than the plant extracts.

Considering the above, the highest radical scavenging ability or reduction power was not found for the samples with the richer phenolic content, meaning that the observed bioactivity cannot be judge solely on the basis of the overall phenolic content. In fact, some of the individual phenolic compounds present in the extract can play a more important role in the activity than others.

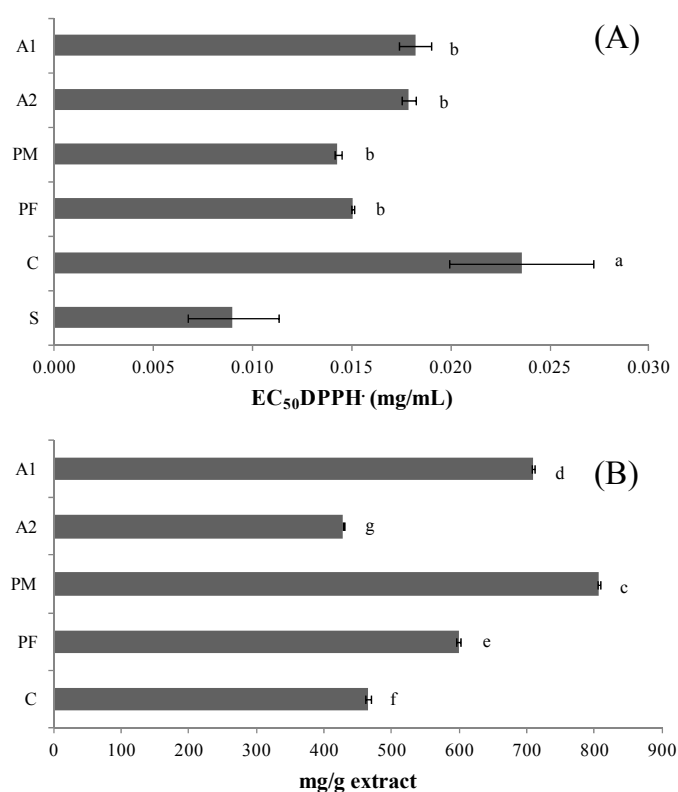


**Figure 8.2** Antioxidant activity of propolis and ethanolic plant extracts (A) against DPPH and (B) reducing power. A1, North-eastern propolis; A2, Central propolis; PM, *Populus x canadensis* male; PF, *Populus x canadensis* female; C, *Cistus ladanifer*; S, caffeic acid: galangin: pinocembrin (1:1:1) used as standard. Reducing power was expressed as caffeic acid: galangin: pinocembrin (1:1:1) equivalents. In each column, different letters (a–g) mean significant differences between samples ( $p < 0.05$ ).

Portuguese propolis phenolic extracts and their potential plant sources were evaluated for *in vitro* activity against the pathogenic protozoa *P. falciparum*, *L. infantum*, *T. cruzi* and *T. brucei* (Table 8.1). To assess selectivity of action, cytotoxicity against MRC-5 fibroblasts was included.

Activity against *P. falciparum* was found for the propolis sample from the central region of Portugal with an IC<sub>50</sub> of 8.8 µg/mL, while the phenolic extract of male *Populus* showed the highest selectivity index (SI=4). Filho *et al.* (2009) reported an IC<sub>50</sub> of 20 µg/mL (SI>2.4) for Brazilian green propolis and of 25 µg/mL (SI>1.9) for the extract of *Baccharis dracunculifolia*, the floral origin of green propolis. These values are actually in the same range as our results and in fact reveal that our samples A2 and PM show a marginally higher antiplasmodial potential, which may be associated with

the richer composition in flavonoids for propolis from *Populus*.<sup>28</sup> For an antimalarial ‘hit’, the WHO Special Programme for Research & Training in Tropical Diseases defines an activity criterion to be  $IC_{50} < 0.2 \mu\text{g/mL}$  with  $SI > 20$ . Therefore, the relevance of the antimalarial action of our samples requires further research. Propolis toxicity was referred by Marcucci (1995) and Burdock (1998) to be associated with some of the propolis components, namely caffeic acid esters present in the European propolis with origin in the poplar buds. The synergistic effect of all compounds in the entire extract can be responsible for a real loss of activity, while the isolated compounds or fractions of it can indeed reveal a higher activity.<sup>27</sup>

**Table 8.1** Antiprotozoal activity against *P. falciparum*, *L. infantum*, *T. cruzi*, *T. brucei*, and cytotoxicity in MRC-5 fibroblast cells.

| Sample <sup>a</sup> | $IC_{50} \mu\text{g/mL}$ (mean $\pm$ sd) - duplicate testing |                    |                 |                   |                |
|---------------------|--------------------------------------------------------------|--------------------|-----------------|-------------------|----------------|
|                     | <i>P. falciparum</i>                                         | <i>L. infantum</i> | <i>T. cruzi</i> | <i>T. brucei</i>  | MRC-5          |
| A1                  | 30.1 $\pm$ 4.1                                               | 8.1 $\pm$ 1.0      | 6.2 $\pm$ 1.9   | 1.7 $\pm$ 0.5     | 12.0 $\pm$ 4.3 |
| A2                  | 8.8 $\pm$ 1.8                                                | 8.1 $\pm$ 0.9      | 7.7 $\pm$ 2.1   | 3.8 $\pm$ 1.2     | 9.7 $\pm$ 3.5  |
| PM                  | 10.9 $\pm$ 2.1                                               | 8.1 $\pm$ 1.1      | 7.8 $\pm$ 1.8   | 5.7 $\pm$ 1.3     | 38.4 $\pm$ 5.8 |
| PF                  | 28.5 $\pm$ 3.8                                               | 8.1 $\pm$ 1.3      | 7.6 $\pm$ 1.6   | 5.3 $\pm$ 1.9     | 33.7 $\pm$ 6.2 |
| C                   | 17.0 $\pm$ 2.5                                               | 32.5 $\pm$ 2.7     | 8.6 $\pm$ 2.0   | 2.0 $\pm$ 0.4     | 32.2 $\pm$ 4.5 |
| Chloroquine         | 0.04 $\pm$ 0.01                                              | -                  | -               | -                 | -              |
| Miltefosine         | -                                                            | 2.4 $\pm$ 0.8      | -               | -                 | -              |
| Benznidazole        | -                                                            | -                  | 2.5 $\pm$ 0.6   | -                 | -              |
| Melarsoprol         | -                                                            | -                  | -               | 0.005 $\pm$ 0.001 | -              |
| Tamoxifen           |                                                              |                    |                 |                   | 10.5 $\pm$ 2.5 |

<sup>a</sup> Samples: A1: North-eastern propolis; A2: Central propolis; PM: *Populus x canadensis* male; PF: *Populus x canadensis* female; C: *Cistus ladanifer*.

For *L. infantum*, propolis samples and male and female *Populus* showed a similar  $IC_{50}$  value of 8.1  $\mu\text{g/mL}$ . *Cistus ladanifer* showed the lowest activity with an  $IC_{50}$  value of 32.46  $\mu\text{g/mL}$ . Duran *et al.* (2011)<sup>29</sup> reported antileishmanial activity on two different Turkish propolis samples with  $IC_{50}$  values ranging between 125-325  $\mu\text{g/mL}$ . Filho *et al.* (2009)<sup>27</sup> presented  $IC_{50}$  values of 49 and 45  $\mu\text{g/mL}$  for a green propolis sample and its plant source *B. dracunculifolia* against *L. donovani*.

Our results for the activity against *T. cruzi* were very similar between all the samples tested, ranging from 6.2 to 8.6  $\mu\text{g/mL}$  (Table 1). Prytyk *et al.* (2003)<sup>30</sup> and Cunha *et al.* (2004)<sup>31</sup> tested the activity against *T. cruzi* in Bulgarian and Brazilian propolis whereby all the extracts showed a lower activity than the Portuguese propolis, with values ranging between 108.8 and 1065  $\mu\text{g/mL}$  for Bulgarian propolis and between 421 and 1437  $\mu\text{g/mL}$  for Brazilian propolis. These biological activity variations are

likely associated with different chemical compositions presented by the different propolis types. No cytotoxicity tests were performed in that study.

For all antiprotozoal assays, the relatively highest activities were found against *T. brucei*. Sample A1 was the most active with an  $IC_{50}$  value of 1.7  $\mu\text{g/mL}$  (Table 8.1) which is in the same range as the antitrypanosomal reference drug suramin.<sup>32</sup> Isolation of phenolic compounds from propolis can significantly increase the activity:  $\beta$ -phenylethyl caffeate showed a high activity ( $IC_{50}$  = 0.013  $\mu\text{g/mL}$ ; SI = 150), while 2,2-dimethylallyl caffeate exhibited a reduced antitrypanosomal activity ( $IC_{50}$  = 12.5  $\mu\text{g/mL}$ ), demonstrating the potential loss of activity when testing the entire extract.<sup>32</sup>

The activity of the propolis and plant ethanolic extracts was also evaluated against bacteria and fungi, Table 8.2.

**Table 8.2** Antimicrobial activity against *Staphylococcus aureus*, *Candida albicans*, *Trichophyton rubrum* and *Aspergillus fumigatus*.

| Sample <sup>a</sup> | $IC_{50}$ $\mu\text{g/mL}$ (mean $\pm$ sd) -- duplicate testing |                    |                  |                     |
|---------------------|-----------------------------------------------------------------|--------------------|------------------|---------------------|
|                     | <i>S. aureus</i>                                                | <i>C. albicans</i> | <i>T. rubrum</i> | <i>A. fumigatus</i> |
| A1                  | 24.6 $\pm$ 3.8                                                  | 32.0 $\pm$ 3.2     | 14.5 $\pm$ 1.2   | >64.0               |
| A2                  | 25.7 $\pm$ 2.9                                                  | 43.1 $\pm$ 4.1     | 11.0 $\pm$ 0.9   | >64.0               |
| PM                  | >64.0                                                           | 33.6 $\pm$ 3.5     | 38.9 $\pm$ 2.3   | >64.0               |
| PF                  | >64.0                                                           | >64.0              | 24.2 $\pm$ 2.1   | >64.0               |
| C                   | >64.0                                                           | >64.0              | 20.8 $\pm$ 1.8   | >64.0               |
| Ampicillin          | 1.1 $\pm$ 0.3                                                   |                    |                  |                     |
| Miconazole          |                                                                 | 4.0 $\pm$ 1.4      | 0.5 $\pm$ 0.1    | 1.5 $\pm$ 0.8       |

<sup>a</sup> Samples: A1: North-eastern propolis; A2: Central propolis; PM: *Populus x canadensis* male; PF: *Populus x canadensis* female; C: *Cistus ladanifer*

The results reveal that the plant extracts do not exhibit relevant antimicrobial activity compared to propolis ethanolic extracts, with exception of *T. rubrum*. In general, both propolis samples revealed a similar antimicrobial effect, with the highest activity found against *T. rubrum* and the lowest against *A. fumigatus*. Recently, verified that propolis from the North and Centre of Portugal was more active against *S. aureus* than against *C. albicans*, which agrees with our results.<sup>16</sup> In fact, considering that the  $IC_{50}$  value for *T. rubrum* is even lower than for *S. aureus*, it can be considered a promising result. However, one should also consider the cytotoxicity, which was rather high in our study.

## 8.4 Concluding remarks

In summary, the present study focused on the screening of Portuguese propolis samples and two potential plant sources against pathogenic protozoa, revealing quite

moderate activity and low selectivity. Since propolis has a complex chemical composition, an extract fractionation will be needed to identify putative active components. Also, these results were evaluated according to criteria set up by the WHO Special Programme for Research & Training in Tropical Diseases, what should be considered by future studies with others propolis samples to enable effective comparisons between scientific findings.

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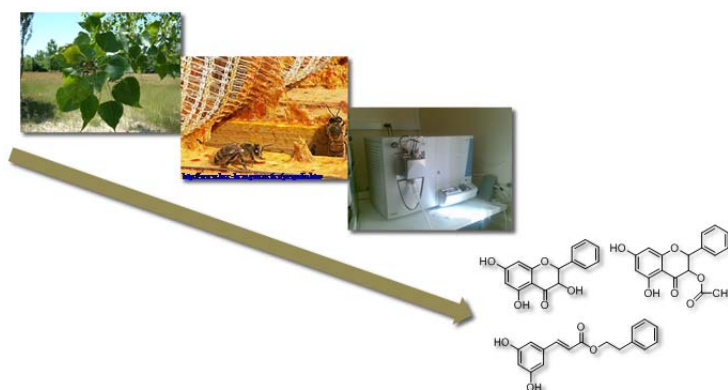
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## Chapter 9

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### Conclusions and future perspectives





## Conclusions and future perspectives

The final chapter of this thesis provides a general overview of the main conclusions and summarizes the most important achievements. Furthermore, new perspectives for future work are proposed.

### 9.1 Conclusions

Through this work it was intended to perform an extensive characterization of propolis from six different Portuguese regions and define quality and biological activity criteria, allowing the association of this beehive product with its phytogeographic origin and thus contributing for its standardization and commercial valorization.

The evaluation of the physicochemical parameters, total phenolic composition and bioactive properties enabled the definition of two distinct types of propolis in Portugal, which are linked to its geographical origin. The first, typical of temperate zones from poplar, included the majority of the north, central coast and Azores archipelago samples, and reveal high phenolic content and bioactivity. The other, comprising the majority of the central interior, south and Madeira Island samples, presented a different colour, a higher amount of wax, lower phenolic content and less bioactivity.

The quality parameters, which were compared with other information available worldwide for propolis, lead to the proposal of standards for both types, which include ash, balsam, water and wax content, colour index, phenolic and flavonoids content and two antioxidant measures for the bioactivity of the sample. The colour index, obtained through the CIELAB colour system, was applied for the first time in this bee product,

and reveal to be quick and reliable technique for quality evaluation of propolis, due to the relation between colour and phenolic composition or bioactivity. The definition of quality parameters for propolis relies on the comparability of the results between different operators and laboratories, and thus it requires the standardization of analytical methodologies. Although most of the literature information was obtained with the same analytical technique, the chemical standards use to calibrate and express the results are not always the same. Thus, it was suggested that the phenolic composition evaluation, which includes the total phenolics, flavones/flavonols and flavanones/dihydroflavonols assays, should be expressed with specific mixtures of phenolics which resemble the typical composition of propolis.

The phenolic profile identification, performed through electrospray ionization coupled to mass spectrometry (ESI-MS), electrospray ionization coupled to tandem mass spectrometry (ESI-MS<sup>n</sup>) and liquid chromatography with diode-array detection coupled to electrospray ionization tandem mass spectrometry (LC/DAD/ESI-MS<sup>n</sup>), allowed the identification of seventy six phenolic compounds within the propolis samples. Although the most abundant compounds in this matrix were those commonly observed in propolis of the temperate zones, there were several new phenolic compounds identified including, some methylated and/or esterified or hydroxylated derivatives of flavonoids already described in propolis and also unusual pinocembrin or pinobanksin derivatives containing a phenylpropanoic acid derivative moiety in their structure. These groups seem to be linked to the positions C-5, C-7, or C-3 of the flavonoid skeleton, although their precise position would only be elucidated by NMR analysis. In addition to these compounds, a rare composition of quercetin and kaempferol glycosides was described for the first time in propolis N11-N13 from north, CI1 from central interior, S4 from south and M2 from Madeira Island.

Overall, it was possible to recognize two groups of propolis types: the common temperate propolis, which contained the typical poplar phenolic compounds such as flavonoids and their methylated/esterified forms, phenylpropanoid acids and their esters and an uncommon propolis type with an unusual composition in quercetin and kaempferol glycosides some of them never described in propolis. The phenolic composition of the bud exudates of *Populus x canadensis* and the resins of *Cistus ladanifer*, two potential propolis sources in Portugal, were also evaluated and used as an indicator for propolis botanical origin. The phenolic profile of Poplar was comparable to the one observed in Portuguese common temperate propolis type earlier described.

Through the analysis of the exudates of *Cistus ladanifer*, only flavonoids were detected, mainly kaempferol derivatives. These rich composition was also present in some central interior and south samples, standing out the compound kaempferol-3,7-dimethyl-ether which was absent in the other propolis samples with a poplar type composition and therefore could be considered as a marker compound for *Cistus ladanifer* propolis.

Quantitatively, propolis from north (excluding samples N11-N13), central coast, Azores Archipelago and sample S1, from south, revealed high values in the phenolic content with a profile similar to the one observed in the bud exudates extracts of *Populus x canadensis*. Thereafter, in these regions poplar type propolis is predominance, which is in accordance with high phenolic content and bioactivity found by spectrophotometric methods. The other samples, which include propolis N11-N13 from north regions, CI2-CI3 from central interior, S2-S4 from south and samples M1-M2 from Madeira Island presented a poor composition in the generality of the phenolic compounds, confirming the spectrophotometric results obtained for these samples, with a lower phenolic content and a lower bioactivity. The phenolic substances found in samples CI2-CI4 and S2-S4, resembles the *Cistus ladanifer* exudates, presenting the marker compound kaempferol-3,7-dimethyl-ether. The data suggest, however, that other botanical species may contribute as well as propolis resin sources: the central interior sample, CI1, presents an exclusive composition in quercetin-tetramethyl-ether, luteolin and chrysoeriol-methyl-ether, which are compounds described in the composition of *Labiatae*, particularly in *Origanum* spp., and a high content in flavonoid glycosides, with a probable origin in the conifers plants. Those findings revealed the difficulty to unequivocally assign the floral composition of each propolis samples, which is a consequence of the flora diversity available around the hive for propolis production.

The volatiles isolated by hydrodistillation from the propolis samples, showed a great variability in the composition with two hundred and one compounds identified, aggregated in three poorly correlated main clusters. Cluster I presented a rich composition in oxygen-containing sesquiterpenes, while cluster II was richer in oxygen-containing monoterpenes and cluster III in monoterpene hydrocarbons. Furthermore, the volatiles composition of *Populus x canadensis* and *Cistus ladanifer* were linked as well with the profile of propolis. The high levels of eudesmol isomers was a characteristic of Poplar propolis, while samples with a *Cistus* contribution showed a rich composition in 2,6,6-trimethylcyclohexanone and viridiflorol. Nevertheless, other *Populus* species as

well as plants like *Juniperus* genus may also contribute to the resin in specific geographical locations.

The electrochemical methods demonstrated to be a potential tool for the evaluation of the antioxidant activity of propolis extracts, particularly the application of differential pulse voltammetry. Although cyclic voltammetry can be important in regard to the identification of the redox processes and their reversibility, this technique has some limitation associated with the capacity current caused by the adsorption of organic molecules on the electrode surface. In this way, cyclic voltammetry is more adequate for qualitative description of propolis extract. For quantitative analysis the differential pulse voltammetry is a more selective and sensitive technique, because the measurement of the current signal occurs before and after the potential pulse application allowing the discrimination of effects. Those effects are approximately constants in a limited potential interval enabling an easier interpretation of the results with better resolved peaks. The electrochemical results showed the existence of several oxidation processes which vary with the origin of the resin. Common oxidation potentials were detected within the majority of samples of a region suggesting an analogous chemical composition in the respect of electroactive species. The electrochemical profile of the plant sources *Populus x canadensis* and *Cistus lanadifer* were similar to some of propolis samples and confirm the botanical discrimination already observed for these samples with other analytical tools. Ordering the results according to its total electroactive antioxidant power it was possible to attribute the higher antioxidant activity to propolis from coast centre, followed by north ~ Azores archipelago > central interior > Madeira island > south. Therefore, the electrochemical methods can be explored as a fast and easy tool for the geographical and botanical origin.

The Portuguese propolis phenolic extracts and their potential plant sources were screened *in vitro* for their activity against the pathogenic protozoa *Plasmodium falciparum*, *Leishmania infantum*, *Trypanosoma cruzi* and *Trypanosoma brucei*, revealing quite moderate activity and low selectivity, with better results against *T. brucei*. Since propolis has a complex chemical composition, it seems that fractionation will be needed to identify putative active components. Additionally, the antimicrobial activity against *Staphylococcus aureus*, *Candida albicans*, *Trichophyton rubrum* and *Aspergillus fumigatus* was also verified with the better results observed against *T. rubrum*. The evaluation of these results, which were performed according to the criteria set up by the WHO Special Programme for Research & Training in Tropical Diseases,

are recommended to be followed in order that future studies with others propolis samples can be effectively compared.

## 9.2 Future perspectives

Propolis is one of the most fascinating bee products. The standardization of propolis it is a challenge due to the nature of the resin, but quality parameters are necessary to guarantee its quality, safety, and efficacy in the diversified biological and pharmaceutical applications of the bee glue. The results from this study represents a strong background that can be used to explore future applications of this bee product in the food and pharmaceutical industry, but also in any other field that could recall specificities of the chemical composition of propolis. Additionally the results it can be used for the definition of quality criteria for the national and European regulations, which are now under debate in the international forum such as the International Honey Commission.

As described along the work, the Portuguese propolis presents a high chemical diversity which is the result of the botanical diversity present in the country. This diversity made the floral classification of propolis a difficult and sensitive task and did not allow in some samples to clearly identify the botanical source. Therefore, some of the future research in the chemistry of propolis should be oriented to the evaluation of the resin extracts from different plants, such as chestnut tree, eucalyptus, cherry tree, and other potential sources sometimes described by beekeepers as resin sources.

A diversified number of phenolic compounds from different classes have been identified in Portuguese propolis, some of which never described in the bee glue. The full recognition of its structural characteristics by nuclear magnetic resonance, together with the evaluation of its bioactivity can be also a field of research to explore. This requires the isolation of the compounds for further screening of different biological activities. Considering the moderated bioactivity observed in this study against protozoa, this screening of individual compounds could clarify the synergistic effect of the extract and highlights some activity of specific compounds.

Concerning the electrochemical methods explored in this study for the evaluation of the electrochemical antioxidant activity, it would be important to obtain additional information on the relation between the electrochemical signals and other antioxidant methodologies to confirm it as an alternative tool. The application of this

method in the evaluation of isolated compounds can also input some important information for a rapid assessment of the antioxidant activity.

## Appendices

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## Appendix A – Propolis samples

### A.1 Propolis samples origin

This work was performed with forty Portuguese propolis samples. As represented in Figure A.1, those were collected from continental north (N), central interior (CI), central coast (CC), south (S), Azores archipelago (A) and from Madeira island (M).

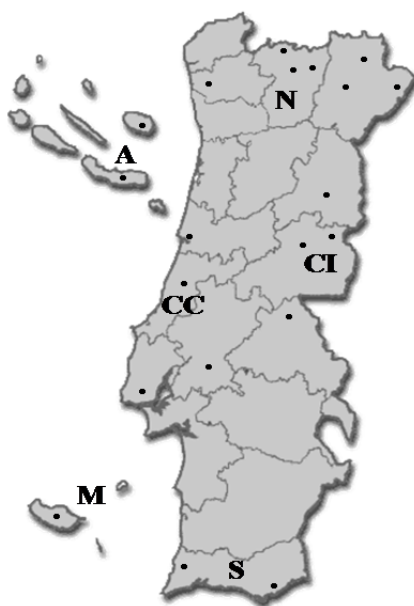


**Figure A.1** Propolis sampling sites

### A.2 Propolis samples description

Table A1 shows the general profile of propolis samples: year, geographical collection sites and collection method. They were obtained after the honey extraction, by conventional scraping or through plastic screens. These propolis samples were then stored at -20 °C until analysis.

**Table A1** Propolis samples

| Region             | Code | Geographical location | Year | Collection method | Visual colour |
|--------------------|------|-----------------------|------|-------------------|---------------|
| North              | N1   | Bragança              | 2007 | 1                 | dark orange   |
|                    | N2   | Bragança              | 2007 | 2                 | dark orange   |
|                    | N3   | Bragança              | 2008 | 2                 | orange        |
|                    | N4   | Bragança              | 2009 | 1                 | dark orange   |
|                    | N5   | Bragança              | 2009 | 1                 | dark orange   |
|                    | N6   | Bragança              | 2009 | 1                 | orange        |
|                    | N7   | Miranda do Douro      | 2009 | 1                 | dark orange   |
|                    | N8   | Mirandela             | 2009 | 2                 | dark orange   |
|                    | N9   | Chaves                | 2009 | 2                 | dark orange   |
|                    | N10  | Chaves                | 2009 | 2                 | dark orange   |
|                    | N11  | Montalegre            | 2009 | 1                 | dark green    |
|                    | N12  | Boticas               | 2009 | 1                 | dark green    |
|                    | N13  | Boticas               | 2009 | 1                 | dark green    |
|                    | N14  | Barcelos              | 2010 | 1                 | dark orange   |
| Central interior   | CI1  | Guarda                | 2009 | 1                 | dark brown    |
|                    | CI2  | Penamacor             | 2009 | 1                 | dark brown    |
|                    | CI3  | Fundão                | 2009 | 1                 | dark brown    |
|                    | CI4  | Nisa                  | 2009 | 2                 | dark green    |
| Central coast      | CC1  | Figueira da Foz       | 2009 | 2                 | orange        |
|                    | CC2  | Leiria                | 2009 | 1                 | dark orange   |
|                    | CC3  | Coruche               | 2009 | 2                 | dark orange   |
|                    | CC4  | Ramada                | 2009 | 1                 | dark orange   |
| South              | S1   | Aljezur               | 2009 | 1                 | dark orange   |
|                    | S2   | Aljezur               | 2009 | 1                 | dark brown    |
|                    | S3   | Aljezur               | 2009 | 1                 | dark brown    |
|                    | S4   | Moncarapacho          | 2009 | 1                 | brown         |
| Azores Archipelago | A1   | Terceira Island       | 2009 | 1                 | dark orange   |
|                    | A2   | S. Miguel Island      | 2009 | 1                 | dark orange   |
|                    | A3   | S. Miguel Island      | 2009 | 1                 | orange        |
|                    | A4   | S. Miguel Island      | 2009 | 1                 | dark orange   |
|                    | A5   | S. Miguel Island      | 2009 | 1                 | orange        |
|                    | A6   | S. Miguel Island      | 2009 | 1                 | orange        |
|                    | A7   | S. Miguel Island      | 2009 | 1                 | dark orange   |
|                    | A8   | S. Miguel Island      | 2009 | 1                 | orange        |
|                    | A9   | S. Miguel Island      | 2009 | 1                 | orange        |
|                    | A10  | S. Miguel Island      | 2009 | 1                 | dark orange   |
| Madeira Island     | A11  | S. Miguel Island      | 2009 | 1                 | dark orange   |
|                    | M1   | Funchal               | 2009 | 1                 | dark brown    |
|                    | M2   | Funchal               | 2009 | 1                 | dark brown    |
|                    | M3   | Funchal               | 2009 | 1                 | dark brown    |

1-Conventional scraping; 2-Plastic screen

## Appendix B – Phenolic compounds extraction procedure

The extraction procedure, which is highly dependent on the experimental conditions such as temperature and solvent polarity, was optimized considering the overall amount of phenolic compounds obtained within the extraction and followed other previous works.<sup>1</sup> Prior to the extraction, the samples were grounded, homogenized and then 1 g mixed with 10 mL of 80% of ethanol/water and kept at 70°C for 1h. The resulting mixture was filtered and the residue was re-extracted in the same conditions. After the second extraction, the filtrated solution was combined, concentrated, frozen at -20°C and freeze-dried. The extraction procedure was run in duplicate.

## References

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## **Appendix C - Spectrophotometric determination of phenolic compounds**

### **C.1 Total phenolic content**

The total phenolic content was determined by a modification of the Folin–Ciocalteu method.<sup>1</sup> An aliquot (0.5 mL) of the ethanolic extracts (0.05 mg/mL) was mixed with of Folin-Ciocalteu's reagent (0.25 mL). After 3 min, a saturated sodium carbonate solution (1 mL) was added to the mixture and the volume adjusted to 5 mL with distilled water. The solution was then heated at 70 °C for 10 min, cooled in the dark for 30 min, and measured the absorbance at 760 nm (Analytikijena 200-2004 spectrophotometer, Analytik Jena, Jena, Germany). For the quantification, two calibrations curves were used, one with a mixture of caffeic acid: galangin: pinocembrin (1:1:1) (0.004-0.06 mg/mL;  $y = 14.441x - 0.0238$ ;  $R^2 = 0.999$ ) and other with galangin: pinocembrin (1:1), (0.005-0.08 mg/mL;  $y = 9.9664x + 0.0098$ ;  $R^2 = 0.9999$ ). The latter calibration curve was used to allow the comparison with previous published results from other authors.

### **C.2 Flavone and flavonol content**

The content in flavone and flavonol was determined based on the method previously described<sup>2</sup> with minor modifications. An aliquot (2 mL) of the ethanolic extract (0.1 mg/mL) was mixed with 0.2 mL of aluminium chloride solution (2% aluminium chloride in 5% acetic acid/methanol) and the volume adjusted to 5 mL with 5% acetic acid/methanol. The mixture was left for 30 min, in the dark at room temperature, and the absorbance was measured at  $\lambda = 415$  nm. Galangin was used to calculate the standard curve (0.002-0.04 mg/mL;  $y = 19.177x + 0.0098$ ;  $R^2 = 0.9999$ ) and the results were expressed as mg of galangin equivalents per g of extract.

### **C.3 Flavanone and dihydroflavonol content**

Flavanones and dihydroflavonols were determined using a previously described method.<sup>3</sup> Briefly, 1 mL of the propolis ethanolic extract (8-17 mg/mL) was mixed with 2 mL of 2,4- dinitrophenylhydrazine (DNP) solution (1 g of DNP was dissolved in 2 mL of 96 % sulphuric acid and the volume adjusted to 100 mL with methanol). The mixture was heated at 50 °C for 50 min with shaking. After cooling to room temperature, the

mixture was diluted to 10 mL with 10% potassium hydroxide in methanol (w/v). An aliquot (1 mL) of the resulting solution was added to 10 mL of methanol and diluted to 50 mL with methanol (volumetric flask). Finally, the absorbance was measured at  $\lambda = 495$  nm. A solution of pinocembrin was used for the calculation of the standard curve ( $0.2\text{--}2$  mg/mL;  $y = 0.2596x - 0.0319$ ;  $R^2 = 0.98$ ), and the results are expressed in equivalents of pinocembrin per g of extract.

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## Appendix D - Antioxidant activity

### D.1 DPPH free radical-scavenging activity

The effect of each propolis extract on DPPH radical was measured according to the procedure described by with modifications.<sup>1</sup> The reaction was performed in a 96-wells microplate where an aliquot of propolis extract solution (0.08 mL) in 80% ethanol/water containing different extract concentrations (0.0025-0.04 mg/mL) was added to 0.292 mL of DPPH 0.025 g/L in 80% ethanol/water, prepared daily. The absorbance was measured at  $\lambda = 515$  nm using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc.). A mixture of phenolic compounds (caffeic acid: galangin: pinocembrin, 1:1:1) was used as standard. The percentage of radical inhibition was calculated as follow:

$$\% \text{Inibition} = [(A_{\text{DPPH}} - A_{\text{sample}}) / A_{\text{DPPH}}] \times 100$$

This percentage was plotted against the extract concentration to obtain the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% ( $\text{EC}_{50}$ ).

### D.2 Reducing power

The reducing power of propolis extract was determined according to the method of.<sup>2</sup> A volume 2.5 mL of the propolis ethanolic extract (0.01-2 mg/mL) was mixed with 2.5 mL phosphate buffer (0.2 mol/L, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. An aliquot (2.5 mL) of 10% trichloroacetic acid was added to the mixture, which was then centrifuged at 3000 rpm (Centurion K<sub>2</sub>R series) for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and FeCl<sub>3</sub> (0.5 mL, 0.1%), and the absorbance was measured at 700 nm. A mixture of caffeic acid: galangin: pinocembrin (1:1:1) was used calculate the standard curve (0.01-0.1 mg/mL;  $y = 4.293x + 0.0781$ ;  $R^2 = 0.999$ ) and the results were expressed as mg of caffeic acid: galangin: pinocembrin (1:1:1) equivalents per g of extract.. Increased absorbance of the reaction mixture indicates increased reducing power.

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## Appendix E – Chromatographic determination of phenolic compounds

### E.1 HPLC analysis

The phenolic profile of the propolis extracts was analyzed by reversed-phase high-performance liquid chromatography (HPLC), based on the method of Gardana *et al.* (2007)<sup>1</sup> with some modifications, as described below. The HPLC analysis was performed on a Knauer Smartline separation module equipped with a Knauer smartline autosampler 3800, a cooling system set to 4°C and a Knauer UV detector 2500. Data acquisition and remote control of the HPLC system was done by ClarityChrom<sup>®</sup> software (Knauer, Berlin, Germany). The column was a 250 mm× 4 mm id, 5µm particle diameter, end-capped Nucleosil C18 (Macherey-Nagel) and its temperature was maintained at 30°C. The mobile phase comprised (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile, which were previously degassed and filtrated. The solvent gradient started with 80% A and 20% B, reaching 30% B at 10 min, 40% B at 40 min, 60% B at 60 min, 90% B at 80 min, followed by the return to the initial conditions. For the HPLC analysis, the ethanolic extract (10 mg) was dissolved in 1mL of 80% of ethanol. All samples were filtered through a 0.2 µm Nylon membrane (Whatman) and 10 µl of each solution was injected. Chromatographic data was acquired at 280 nm. The eluted fractions were manually collected according to the visualization of the UV profile (Chapter 3).

### E.2 LC/DAD/ESI-MS<sup>n</sup> analysis

The LC/DAD/ESI-MS<sup>n</sup> analyses were performed on a Finnigan Surveyor Plus HPLC instrument equipped with a diode-array detector and coupled to a mass detector. The chromatographic system consisted of a quaternary pump, an autosampler, a degasser, a photodiode-array detector, an automatic thermostatic column compartment. HPLC were run on a Macherey-Nagel Nucleosil C18 column (250 mm×4 mm i.d.; 5 µm particle diameter, end-capped) and its temperature was maintained at 25°C. The mobile phase was composed of (A) 0.1% (v/v) formic acid in water and (B) acetonitrile, which were previously degassed and filtered. The solvent gradient started with 80% A and 20% B, reaching 30% B at 10 min, 40% B at 40 min, 60% B at 60 min, 90% B at 80 min and return to the initial conditions. For the HPLC analysis, the freeze-dried extract

(10 mg) was dissolved in 1 mL of 80% of ethanol. All samples were filtered through a 0.2  $\mu\text{m}$  Nylon membrane (Whatman). The flow rate was 1 mL/min and split out 200  $\mu\text{L}/\text{min}$  to MS. Spectral data for all peaks were acquired in the range 200–600 nm.

The mass spectrometer used was a Finnigan Surveyor LCQ XP MAX quadrupole ion trap mass spectrometer equipped with an ESI source. Control and data acquisition was carried out with Xcalibur<sup>®</sup> data system (ThermoFinnigan, San Jose, CA, USA). Nitrogen above 99% purity was used and the gas pressure was 520 kPa (75 psi). The instrument was operated in negative-ion mode with electrospray ionization (ESI) needle voltage set at 5.00 kV and the ESI capillary temperature at 325 °C. The full scan covered the mass range from  $m/z$  50 to 1000. MS<sup>n</sup> data were simultaneously acquired for the selected precursor ion. CID-MS/MS and MS<sup>n</sup> experiments were performed using helium as the collision gas with collision energy of 25-40 eV.

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