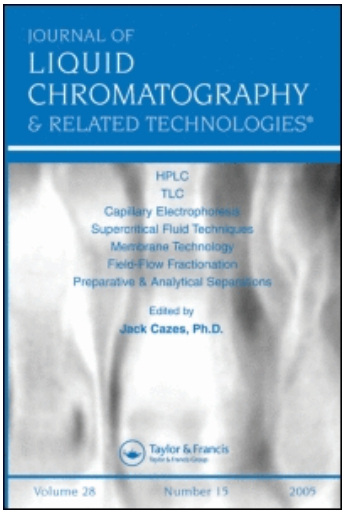

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Application of an HPLC Method for Analysis of Propolis Extract

M. L. Bruschi[a]; S. L. Franco[a]; M. P. D. Gremião[a]
[a] Programa de Pós-Graduação em Ciências Farmacêuticas, UNESP, Araraquara, SP, Brazil

Online publication date: 23 July 2003

To cite this Article Bruschi, M. L. , Franco, S. L. and Gremião, M. P. D.(2003) 'Application of an HPLC Method for Analysis of Propolis Extract', Journal of Liquid Chromatography & Related Technologies, 26: 14, 2399 — 2409
To link to this Article: DOI: 10.1081/JLC-120023254
URL: http://dx.doi.org/10.1081/JLC-120023254

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES®
Vol. 26, No. 14, pp. 2399–2409, 2003

Application of an HPLC Method for Analysis of Propolis Extract

M. L. Bruschi, S. L. Franco, and M. P. D. Gremião*

Programa de Pós-Graduação em Ciências Farmacêuticas,
UNESP, Araraquara, SP, Brazil

ABSTRACT

Propolis obtained from honeybee hives has been widely used in medicine, cosmetics, and industry due to its versatile biological activities (antioxidant, antimicrobial, fungicidal, antiviral, antiulcer, immunostimulating, and cytostatic). These activities are mainly attributed to the presence of flavonoids in propolis, which points out the interest in quantifying these constituents in propolis preparations, as well as validation of analytical methodologies. High-performance liquid chromatography (HPLC) methods have been reported to quantify isolated flavonoids or these compounds in complex biological matrices, such as herbal raw materials and extractive preparations. An efficient, precise, and reliable method was developed for quantification of propolis extractive solution using HPLC with UV detection. The chromatograms were obtained from various gradient elution systems (GES) tested in order to establish the ideal

*Correspondence: M. P. D. Gremião, Programa de Pós-Graduação em Ciências Farmacêuticas, UNESP, Rod, Araraquara-Jaú, km 1, CEP 14802-902, Araraquara, SP, Brazil; E-mail: pgremiao@fcfar.unesp.br.

DOI: 10.1081/JLC-120023254
1082-6076 (Print); 1520-572X (Online)

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

conditions for the analysis of propolis extractive solution, using methanol and water : acetonitrile (97.5 : 2.5, v/v) as mobile phase. Gradient reversed phase chromatography was performed using a stainless steel column (250 × 4.6 mm i.d., 5 μm) filled with Chromsep RP 18 (Varian), column temperature at 30.0 ± 0.1°C and detection at 310 nm. The main validation parameters of the method were also determined. The method showed linearity for chrysin in the range 0.24–2.4 μg mL^{-1} with good correlation coefficients (0.9975). Precision and accuracy were determined. The obtained results demonstrate the efficiency of the proposed method. The analytical procedure is reliable and offers advantages in terms of speed and cost of reagents.

Key Words: Propolis; High-performance liquid chromatography (HPLC); Chrysin; Phytochemicals; Flavonoids.

INTRODUCTION

Propolis (bee glue) is a generic name for the resinous hive product collected by bees from various plant sources. The word propolis is derived from the Greek *pro*-, for or in defence, and *polis*-, the city, that is, defence of the city (or the hive). This material is a strongly adhesive, resinous substance collected, transformed, and used by bees to seal holes in their honeycombs, smooth out the internal walls, and protect the entrance against intruders. Honeybees (*Apis mellifera* L.) collect resin from the cracks in the bark of trees and leaf buds. Salivary enzymes are added to this resin and the partially digested material is mixed with beeswax and used in the hive.[1–3]

The beekeepers scrape the hive "supers" (superstructure) with a hive tool, usually in the fall of the year after the honey is extracted. The first step in propolis processing is the evaluation of the material on its arrival. The second step involves dissolving the propolis in ethyl alcohol. Through a proprietary process, the remaining beeswax, as well as bee parts and wood chips, are removed. The final step involves filtration of the propolis extract to remove any remaining small particles of foreign material.[1]

Propolis has wide application in medicine, cosmetics, and food industry due to its versatile biological activities, these including antioxidant, antimicrobial, fungicidal, antiviral, antiulcer, immunostimulating, hypotensive, and cytostatic activities.[1,3–9]

Its chemical composition is complex and typically consists of waxes, resins, water, inorganics, phenolics, and essential oils, the exact composition of which is dependent upon the source plant(s).[2,3,6,10,11] In Europe, North and South America, and western Asia the dominant propolis source is the bud

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

exudate of poplar (*Populus*).[12] Less commonly, in other parts of the world, species such as *Betula*, *Ulmus*, *Pinus*, *Quercus*, *Salix*, and *Acacia* are utilized as propolis sources.[6,12] At least 200 propolis constituents have been identified so far. The most important constituents of propolis seem to be phenolics, which constitute more than 50% of its total weight and are related to a substantial part of its biological activity. For this reason, most of investigations on the chemical composition of propolis have been connected with its phenolic constituents.[10,13] Moreover, the literature points out that some of these activities can be especially related to flavonoids, the main propolis phenolic compounds. Chrysin (5,7-dihydroxyflavone) is one of main propolis flavonoids and it is used as a reference standard.[1,11] Thus, the relationship between the flavonoids and propolis biological effects reveal the interest of quantifying these constituents in propolis preparations.

The high potential of propolis utilization in a wide medicinal field demonstrates the interest in the quality control and standardization of propolis preparations. Gas chromatography, mass spectrometry, and high-performance liquid chromatography (HPLC) were applied in analysis of propolis extracts, but the analytical procedures were always complex, with long analysis time and dependent on several flavonoids, analytical standards, and expensive reagents.[14–17]

In this context, the aim of this work is to develop a reliable, efficient, and with advantages in terms of speed and cost of reagents, HPLC method for separation and quantification of constituents in propolis extracts. The main validation parameters of the proposed method are also determined.

EXPERIMENTAL

Chemicals and Reagents

Methanol (Mallinckrodt; HPLC grade), water filtered through a Milli-Q apparatus (Millipore) and acetonitrile (Mallinckrodt; HPLC grade) were used as mobile phase. Analytical grade standard, chrysin, was purchased from Sigma and it was used as external standard. Acetone (Merck; analytical grade), ethyl acetate (Merck; analytical grade), methanol (Merck; analytical grade), and ethyl alcohol (96°GL; pharmaceutical grade) were also used.

Apparatus

High-performance liquid chromatography analyses were performed using a Varian HPLC equipped with two pumps (Model Prostar/Dynamax 2.4),

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

an automatic controller of flow (Model Prostar), an Ultraviolet-Visible Photo Diode Array spectrophotometric detector module (Model Prostar 330), a column oven, and an integrator system (Model Star). A fixed loop injector (Rheodyne VS 7125, 100 μL) was used to carry the sample onto the column.

Chromatographic separation was accomplished using a stainless steel analytical column Chromsep RP C18 (Varian; 250 × 4.6 mm i.d., 5 μm) with methanol (pump A) and acetonitrile–water (2.5 : 97.5, v/v) (pump B), previously filtered through a 0.45 μm filter (PALL-Gelman) and degassed using an ultrasonic bath. Gradient separation was performed at a flow rate of 1.0 mL min^{-1}, with the column temperature set at $30 \pm 0.1^{\circ}C$. The sensitivity was 0.05 a.u.f.s. and the chart speed was 0.30 cm min^{-1}.

Preparation of the Extractive Solution

Propolis samples obtained from Maringá city (Paraná State, Brazil) were used to prepare the ethanolic extractive solution (EES), by turbo extraction. The propolis: ethanol ratio of 30 : 70 (w/w) was employed. The EES was filtered through a paper filter and the weight was made up to 500 g with ethanol. An analytical aliquot of 1.0 mL of EES was extracted with 25 mL of ethyl acetate. The acetate fraction was dried using a water bath (40°C) and the residue was dissolved in 10.0 mL of methanol. This solution was filtered through a 0.20 μm membrane filter (Millipore-GTTP) and an aliquot (50 μL) of the filtrate was diluted with 10.0 mL of methanol, obtaining the ES. A 100 μL aliquot of ES was analysed by HPLC.

Chrysin Calibration Curve

The chrysin standard was dissolved in methanol yielding concentrations of 0.08, 0.16, 0.24, 0.4, 0.8, 1.6, 2.4, and 3.2 μg mL^{-1}. The solutions were filtered through a 20 μm membrane filter (Millipore-GTTP). Evaluation of each point was conducted (system E) in triplicate and the calibration curve was fitted by linear regression.

Validation

The linearity was determined for the calibration curve obtained by HPLC analyse of chrysin standard solutions. The range of the appropriate amount of samples was then determined. The slope and the other statistics of the calibration curves were calculated by linear regression.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation (SD) and the slope (S) of the calibration curve.[18] The precision of the method was determined following ICH guidelines.[18] For evaluation of the repeatability, the SD and relative standard deviation (RSD) of five injections were considered.

The accuracy was determined by recovery analyses, adding measured amount of chrysin to simulated sample. The recovery experiments were performed in triplicate. The recovery data were determined by dividing the value obtained for the sample prepared with the added standard, by the amount added and then multiplied by 100%.[18]

Propolis Extractive Solution Quantification

The quantification of the extractive solution (ES) was performed using the external calibration method. The ES and the chrysin standard solutions were analysed by HPLC (system E) in triplicate. The three main peaks (markers) were quantitated in chrysin ($mg\,mL^{-1}$).

RESULTS AND DISCUSSION

In this study, same mobile phase, column, and other chromatographic conditions were always employed. The chromatograms were obtained from various gradient elution systems (GES) tested (Table 1), in order to establish the ideal conditions for the analysis of the propolis extractive solution, using methanol and water : acetonitrile (97.5 : 2.5, v/v) as mobile phase. The other chromatographic conditions always were the same and detection at 310 nm. The GES that showed high performance in the separation of propolis multiple peaks, with a possible less time of analysis, was the system E (Fig. 1). With system A, good separation was not obtained and most of the peaks showed poor definition. With system B, the time of analysis was increased (62 min) and the initial amount of methanol was decreased (47%), which provided a good separation, but did not improve peak definition. Increased times of analysis (systems C and D) did not provide improvement in the separation. The initial concentration of methanol (systems E and F) was varied, showing that an initial amount of 50% of methanol showed better separation and definition of the peaks (system E).

The evaluation of propolis extract by HPLC with photodiode array detection was indispensable to define the quantification parameters. Through this resource, the UV spectra of the three major peaks with retention times of 21, 24, and 43 min could be observed. Comparing the obtained spectra, it was

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

Table 1. Gradient elution systems tested in separation of propolis extract.

System A					
Time (min)	0	28	25	40	43
Methanol : acetonitrile/ water (%)	50 : 50	80 : 20	100 : 0	100 : 0	50 : 50
System B					
Time (min)	0	47	50	58	62
Methanol : acetonitrile/ water (%)	47 : 53	80 : 20	100 : 0	100 : 0	47 : 53
System C					
Time (min)	0	56	60	70	74
Methanol : acetonitrile/ water (%)	50 : 50	80 : 20	100 : 0	100 : 0	47 : 53
System D					
Time (min)	0	84	90	105	111
Methanol : acetonitrile/ water (%)	50 : 50	80 : 20	100 : 0	100 : 0	47 : 53
System E					
Time (min)	0	47	50	58	62
Methanol : acetonitrile/ water (%)	50 : 50	80 : 20	100 : 0	100 : 0	47 : 53
System F					
Time (min)	0	47	50	58	62
Methanol : acetonitrile/ water (%)	60 : 40	80 : 20	100 : 0	100 : 0	47 : 53

observed that these substances present two bands (A and B) very similar to the flavonoids,[19] with maximum absorption (λ_{max}) in the same range of wavelength, between 305 and 315 nm (Fig. 2). The same profile was found in chrysin (5,7-dihydroxyflavone) (Fig. 3). With the likeness of the spectra, we can infer that these substances are probably flavonoids. Thus, the three major peaks were used as markers and quantified in relation to chrysin.

For validation of an analytical method, the guidelines of the ICH (International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use) recommends the accomplishment of tests of specificity, linearity, accuracy, precision, LOD, and LOQ of method.[18]

To analyse complex biologic matrices, as in the case of propolis extractive solution, the type of method and its respective use determine which parameters should be evaluated. The linearity of the HPLC method was investigated for chrysin in the range 0.08–3.2 $\mu g\,mL^{-1}$ at eight concentration levels. Chrysin

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

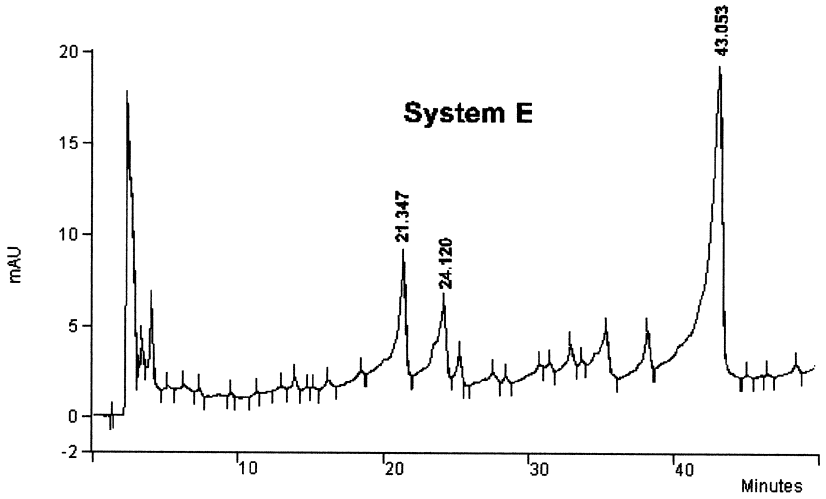

Figure 1. High-performance liquid chromatography chromatogram of propolis extractive solution obtained by E gradient elution system.

presented retention times of 29 min. The calibration curve was linear in the range 0.24–2.40 $\mu g\,mL^{-1}$. The representative linear equation was $Y = 1204781.9550X - 175630.2071$ ($n = 5$; $r = 0.9975$) and the RSD of the slope was 4.06%.

The LOD, taken as the lowest absolute concentration of analyte in a sample, which can be detected but not necessarily quantified as an exact value under the stated experimental condition, was 0.18 $\mu g\,mL^{-1}$ for chrysin. The LOQ, taken as the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy under the stated experimental condition, was 0.55 $\mu g\,mL^{-1}$ for chrysin.

The selectivity of the proposed method was evaluated by analysis of the chromatograms of EES. The chromatograms presented high resolution of the peaks, indicating that the proposed method could be applied for the quantifications and control of propolis preparations.

The RSD values of the chrysin areas obtained by HPLC were 3.12% (0.24 $\mu g\,mL^{-1}$), 3.33% (0.4 $\mu g\,mL^{-1}$), 2.88% (0.8 $\mu g\,mL^{-1}$), 2.61% (1.6 $\mu g\,mL^{-1}$), and 1.22% (2.4 $\mu g\,mL^{-1}$). These results demonstrated the reproducibility. The repeatability of the HPLC analysis of EES (Table 2) was demonstrated with a RSD range of 2.65–6.15%. This result could be considered satisfactory since the majority of phytochemicals present a range from 3% to 6%.[20]

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

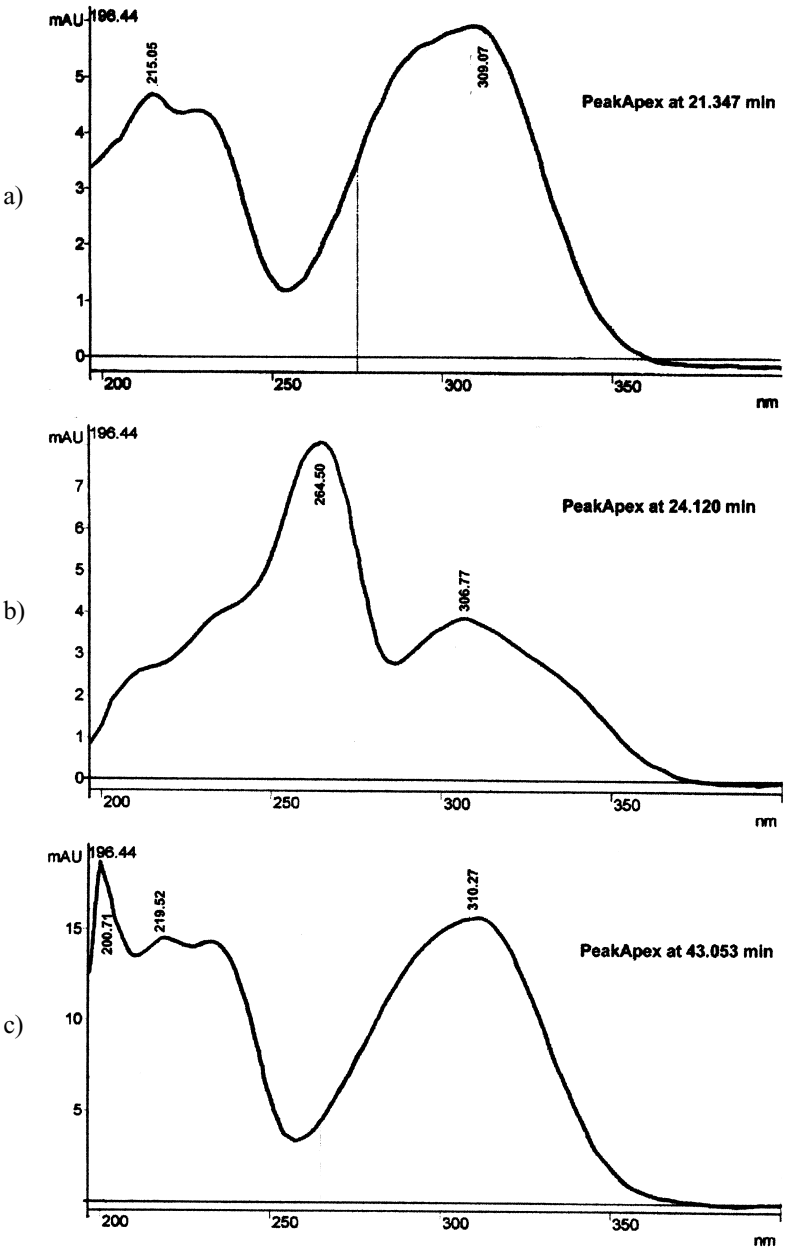

Figure 2. Diode array spectrum, 200–400 nm, of propolis; three major peaks were obtained: (a) 21 min; (b) 24 min; (c) 43 min.

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

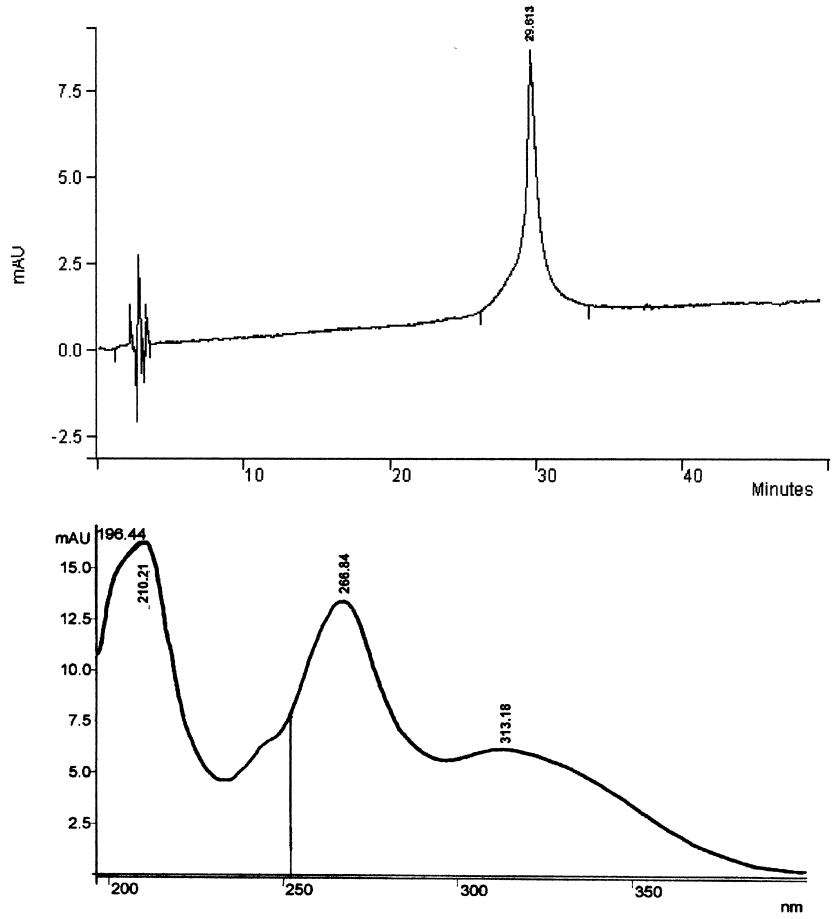

Figure 3. HPLC profile at 310 nm and diode array spectrum, 200–400 nm, of chrysin standard.

Table 2. High-performance liquid chromatography results of propolis EES.

Peak	Chrysin concentration (μmL^{-1})	SD	RSD (%)
A	2444.11	64.6975	2.65
B	1800.81	114.2071	6.34
C	9742.99	598.9640	6.15

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

Preparing a simulated sample containing a known quantity of chrysin determined the accuracy of the HPLC method for the assay analysis of recovery. The recovery of an added standard flavonoid was $92.6 \pm 3.5\%$. These results referred to the average of three assays and they are in good agreement with the results (80–120%) required.[18]

CONCLUSIONS

The three major peaks, with retention times of 21, 24, and 43 min, can be used as markers to propolis preparations characterization and quantifying in relation to chrysin.

The developed method allows efficient and rapid analysis of propolis preparations.

ACKNOWLEDGMENT

The authors thank the CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for their financial support.

REFERENCES

1. Burdock, G.A. Review of the biological properties and toxicity of bee propolis (Propolis). Food Chem. Toxicol. **1998**, *36*, 347–363.
2. Ghisalberti, E.L. Propolis: a review. Bee World **1979**, *60*, 59–80.
3. Marcucci, M.C. Propolis: chemical composition, biological properties and therapeutic activity. Apidologie **1995**, *26*, 83–99.
4. Bankova, V.S.; Christov, R.; Kujumgiev, A.; Marcucci, M.C.; Popov, S.S. Chemical composition and antibacterial activity of Brazilian propolis. Z. Naturforcsh **1995**, *50*, 1–6.
5. Bankova, V.S.; Christov, R.; Tejera, A.D. Lignans and other constituents of propolis from the Canary Islands. Phytochemistry **1999**, *49* (5), 1414–1415.
6. Markham, K.R.; Mitchell, K.A.; Wilkins, A.L.; Daldy, J.A.; Lu, Y. HPLC and GC-MS identification of the major organic constituents in New Zealand propolis. Phytochemistry **1996**, *42* (1), 205–211.
7. Nagai, T.; Sakai, M.; Inoue, R.; Inoue, H.; Suzuki, N. Antioxidative activities of some commercially honeys, royal, jelly, and propolis. Food Chem. **2001**, *75*, 237–240.

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

8. Santos, F.A.; Bastos, E.M.A.; Uzeda, M.; Carvalho, M.A.R.; Farias, L.M.; Moreira, E.S.A.; Braga, F.C. Antibacterial activity of Brazilian propolis and fractions against oral anaerobic bacteria. J. Ethnopharmacol. **2002**, *80*, 1–7.
9. Sforcin, J.M.; Fernandes, A., Jr.; Lopes, C.A.M.; Bankova, V.; Funari, S.R.C. Seasonal effect on Brazilian propolis antibacterial activity. J. Ethnopharmacol. **2000**, *73*, 243–249.
10. Bankova, V.S.; Dyulgerov, A.; Popov, S.S.; Evstatieva, L.; Kuleva, L.; Pureb, O.; Zanjansan, Z. Propolis produced in Bulgaria and Mongolia: phenolic compounds and plant origin. Apidologie **1992**, *23*, 79–85.
11. Vanhaelen, M.; Vanhaelen-Fastré, R. Propolis.—I. Origine, micrographie, composition chimique et activité thérapeutique. J. Pharm. Belg. **1979**, *35* (5), 253–259.
12. König, B. Plant sources of propolis. Bee World **1985**, *66* (136), 136–139.
13. Bankova, V.S.; Christov, R.; Popov, S.S. Volatile constituents of propolis. Z. Naturforcsh **1994**, *49* (c), 6–10.
14. Bankova, V.S.; Popov, S.S.; Marekov, N.L. High-performance liquid-chromatographic analysis of flavonoids from propolis. J. Chromatogr. **1982**, *242* (1), 135–143.
15. Christov, R.; Bankova, V. Gas-chromatographic analysis of underivatized phenolic constituents from propolis using an electron-capture detector. J. Chromatogr. **1992**, *623* (1), 182–185.
16. Pereira, A.S.; Pinto, A.C.; Cardoso, N.J.; de Aquino Neto, F.R.; de Souza Ramos, M.F.; Dellamora-Ortiz, G.M.; Pereira dos Santos, E. Application of high-temperature high-resolution gas chromatography to crude extracts of propolis. J. High Resolut. Chromatogr. **1998**, *21* (7), 396–400.
17. Maciejewicz, W.; Daniewski, M.; Bal, K.; Markowski, W. GC-MS identification of the flavonoid aglycones from propolis. Chromatogr. **2001**, *53* (5–6), 343–346.
18. International Conference on the Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Q2B. Validation of analytical procedures. Methodology **1996**.
19. Mabry, T.J.; Markham, K.R.; Thomas, M.B. *The Systematic Identification of Flavonoids*; Springer-Verlag: Berlin. 1970; 353 pp.
20. Andlauer, W.; Martena, M.J.; Fürst, P. Determination of selected phytochemicals by reversed-phase high-performance liquid chromatography combined with ultraviolet and mass spectrometric detection. J. Chromatogr. A **1999**, *849*, 341–348.

Received February 11, 2003
Accepted March 7, 2003
Manuscript 6100

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016