

1 Category by field: Plant Science > Plant biochemistry > Other compound
2 Category by field: Biochemistry > Other compound > Triterpene
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6 Isolation of Triterpenes from Propolis (Bee Glue)

7 Muhammad N. Kardar¹ and Veronique Seidel^{1*}
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9 ¹Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow G4
10 0RE, Scotland, UK

11 *For correspondence: veronique.seidel@strath.ac.uk
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14 **[Abstract]** Propolis (bee glue) is a natural substance produced by bees upon collection of mainly
15 plant resins. Bees use it as antiseptic sealing agent between honeycombs and to preserve the hive
16 from external contamination. Numerous scientific studies have been published on the biological
17 properties of propolis including its anti-inflammatory, anti-oxidant, immunostimulant, antitumour and
18 antimicrobial activity. Different propolis chemotypes have been characterised based on the nature of
19 the plant-derived substances present and the geographical origin of collection. Here, we describe the
20 isolation of nine triterpenes from a sample of propolis originating from North-Western Cameroon. All
21 compounds were identified following analysis of their spectroscopic data and comparison with
22 previously published reports.
23

24 **Materials and Reagents**

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- 26 1. Raw propolis (Nature's Laboratory Ltd, code number: P5)
- 27 2. Ethanol 96% v/v (extra pure, Specified Laboratory Reagent) (Fisher, Scientific, catalog
28 number: 10162252)
- 29 3. Hexanes (for HPLC, 95% *n*-hexane approx.) (Fisher Scientific, catalog number: 10703611)
- 30 4. Ethyl acetate, extra pure, Specified Laboratory Reagent (Fisher Scientific, catalog number:
31 10080130)
- 32 5. Sodium sulfate anhydrous, extra pure (Fisher Scientific, catalog number: 10606082)
- 33 6. TLC silica gel 60 F254 plates (VWR International Ltd, catalog number: 1.05554.0001)
- 34 7. Silica gel 60 (0.063–0.200 mm) (VWR International Ltd, catalog number: 1.07734.1000)
- 35 8. Silica gel 60H (VWR International Ltd, catalog number: 1.07736.1000)
- 36 9. 98% *p*-Anisaldehyde (Sigma-Aldrich, catalog number: A88107)
- 37 10. Sulfuric acid (pure) (Fisher Scientific, catalog number: 10222282)
- 38 11. Acetic acid glacial (pure) (Fisher Scientific, catalog number: 10375020)
- 39 12. Methanol (extra pure, Specified Laboratory Reagent) (Fisher Scientific, catalog number:
40 10214490)

- 41 13. Sephadex® LH-20 (Sigma-Aldrich, catalog number: LH20100)
42 14. α -amyrin (Sigma-Aldrich, catalog number: 53017)
43 15. Cycloartenol (Sigma-Aldrich, catalog number: 08172)
44 16. Lupeol (Sigma-Aldrich, catalog number: S957712)
45 17. Dichloromethane, extra pure, stabilised with amylene, [Specified Laboratory Reagent](#) (Fisher
46 Scientific, catalog number: 10458210)
47 18. Chloroform-d (CDCl₃) (Sigma-Aldrich, catalog number: 10080130)
48 19. Anisaldehyde-sulphuric acid reagent (see Recipes)

49

50 **Equipment**

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- 52 1. Heated ultrasonic water bath (Fisher Scientific, catalog number: 10215332, [340 W](#))
53 2. Büchi rotary evaporator, Rotavapor® R-210 (VWR International Ltd, catalog number: 531-
54 0850)
55 3. UV viewing cabinet CC10 (Fisher Scientific, catalog number: 11778201)
56 4. UV lamp UVG-11 230V 50/60Hz 4W hand held short wave (Fisher Scientific, catalog number:
57 11718241)
58 5. UV lamp UVL-21 230V 50/60Hz 4W hand held long wave (Fisher Scientific, catalog number:
59 11728241)
60 6. Analytical balance (VWR International Ltd, catalog number: 611-2267)
61 7. Precision balance (VWR International Ltd, catalog number: 611-2695)
62 8. Vacuum water jet pump (VWR International Ltd, catalog number: 181-9100)
63 9. Pear-shaped (separating) funnel, 1000 ml (Scientific Glass Laboratories, catalog number:
64 SFP1L)
65 10. Round bottom flasks, 250mL ([Total number of 12](#)). (Scientific Glass Laboratories, catalog
66 number: FRS250/B19 or FRS250/B24)
67 11. Glass foot measuring cylinder with hexagonal base, borosilicate glass, class "B", 25 mL and
68 250 mL (Scientific Glass Laboratories, catalog number MCB/25 and MCB/250)
69 12. Conical Erlenmeyer flasks, 500 ml (Scientific Glass Laboratories, catalog number:
70 FC500/B24)
71 13. Filter funnels, borosilicate (Scientific Glass Laboratories, catalog number: BFF75 and
72 BFF100)
73 14. Rubber vacuum tubing NW8 (VWR international Ltd, catalog number: 189.3111)
74 15. Glass Pasteur pipettes 150 mm (Fisher Scientific, catalog number: 10006021)
75 16. Glass Pasteur pipettes 230 mm (Fisher Scientific, catalog number: 10209381)
76 17. Glass micropipettes (homemade from glass Pasteur pipettes 230 mm using a Bunsen burner)
77 18. Büchner filter with cone and thread, GL14, for vacuum liquid chromatography (VLC)
78 (Scientific Glass Laboratories, catalog number TBF/4/B24/POR3)
79 19. Twin trough chambers for TLC plates (VWR International Ltd, catalog number:552-0011)
80 20. Glass atomiser reagent sprayer (VWR International Ltd, catalog number: 552-0031)

- 81 21. Wilmad® NMR tubes 5 mm diam., precision (Sigma-Aldrich, catalog number: Z274275)
- 82 22. Glass chromatography columns (CC) with sintered discs (Scientific Glass Laboratories,
- 83 catalog number: R2/40)
- 84 23. Snap-cap vials (VWR International Ltd, catalog number 548-0555)
- 85 24. Duratool DO1600 hot air [heat gun](#) (from www.amazon.co.uk)
- 86 25. Whatman qualitative filter paper, grade 1 (Sigma-Aldrich, catalog number WHA1001125)
- 87 26. Exactive Orbitrap mass spectrometer (MS) operating in a positive and negative electrospray
- 88 ionisation (ESI) switching mode ([Thermo Scientific, UK](#))
- 89 27. JEOL 505HA high resolution electron impact (HREI) mass spectrometer (MS) using direct
- 90 probe at elevated temperature (110~160 °C) at 70 eV
- 91 28. Xcalibur software (version 2.2) for MS data processing ([Thermo Scientific, UK](#))
- 92 29. JEOL Lambda Delta 400 NMR spectrometer (JEOL Ltd)
- 93 30. Mestre Nova (MNova) software (version 8.0.0) for NMR processing (Mestrelab Research SL,
- 94 Spain)

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96 **Procedure**

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- 98 1. The whole procedure described below takes around 6 weeks to complete. All steps are done
- 99 at room temperature (20-25 °C) unless otherwise stated. Weigh propolis (ca. 50 g) in a
- 100 conical Erlenmeyer flask then add 70% ethanol in water (500 mL) to the flask.
- 101 2. Place the flask in the ultrasonic water bath on high for 1 h at 60 °C.
- 102 3. Recover the supernatant into a second conical Erlenmeyer flask and re-extract the raw
- 103 material left-over in the original flask using fresh 70% ethanol (500 mL) as in step 2. Repeat
- 104 this process 10 times and combine all supernatants.
- 105 4. Filter the pooled supernatants into a round-bottom flask using a glass filter funnel overlaid
- 106 with filter paper. Discard the insoluble material (brown solid) remaining on the filter paper. The
- 107 filtered extract is a clear amber solution. Keep this filtered extract in a fridge at 4 °C.
- 108 5. Concentrate the filtered extract in the round-bottom flask to dryness under reduced pressure
- 109 at ≤40 °C using a rotary evaporator. To achieve this, adjust the water bath to 60 °C and apply
- 110 a vacuum of 175mbar. Complete evaporation will occur in around 30 min.
- 111 6. Add distilled water (250 mL) and pure (96%) ethanol (25 mL) to the dried extract to obtain a
- 112 suspension.
- 113 7. Place a separating funnel onto a metallic stand (Fig 1), close its stopcock and slowly pour the
- 114 suspension into the separating funnel.
- 115 8. Add hexane (250 mL) to the separating funnel,
- 116 9. Hand-shake the funnel gently several times. Remove the top stopper, then allow for the
- 117 solvents to settle until into two immiscible phases. Recover the aqueous phase (bottom layer)
- 118 and the hexane phase (top layer) in separate Erlenmeyer flasks. Do this by slowly opening
- 119 the stopcock and closing it just before the curved meniscus between the two liquids reaches
- 120 the stopcock.

- 121 10. Place the aqueous phase back inside the separating funnel.
- 122 11. Repeat steps 8 and 10 twice using fresh hexane.
- 123 12. Add ethyl acetate (250 mL) to the funnel and repeat step 9 to 10 (the aqueous phase is the
- 124 bottom layer and the ethyl acetate phase is the top layer).
- 125 13. Repeat step 12 twice using fresh ethyl acetate.
- 126 14. Sprinkle some sodium anhydrous sulfate into the recovered hexane and the ethyl acetate
- 127 phases and gently manually rotate the Erlenmeyer flasks to observe if the cloudiness (i.e.
- 128 residual water droplets) disappears. Continue sprinkling sodium sulphate and rotating the
- 129 flasks until the liquids are completely clear.
- 130 15. Filter each organic phase into a round-bottom flask using a glass filter funnel overlaid with
- 131 filter paper. Discard the insoluble material (sodium sulfate) remaining on the filter paper. The
- 132 filtered extracts are clear solutions; concentrate them to dryness under reduced pressure at
- 133 ≤ 40 °C using a rotary evaporator. To achieve this, adjust the water bath to 60 °C and apply a
- 134 vacuum of 335mbar (hexane) or 240mbar (ethyl acetate). Complete evaporation will occur in
- 135 around 20 min.
- 136 16. Store the hexane and the ethyl acetate extracts at -20 °C.
- 137 17. Dissolve a portion (5 to 10 g) of the hexane extract in 10 mL of a suitable solvent (e.g.
- 138 dichloromethane, ethyl acetate and/or hexane) and mix with silica gel 60 (3 g). Leave the
- 139 mixture to dry on a bench at 20-25°C. A free-flowing and completely dried powder is usually
- 140 obtained overnight.
- 141 18. Connect the Büchner filter for VLC to a vacuum water jet pump and pack it with silica gel 60H.
- 142 Leave a space (ca. 3 cm) above the compacted layer of silica layer so as to accommodate
- 143 the sample and an appropriate volume of solvent. Allow hexane to pass through the column
- 144 under vacuum to check the uniformity of the column.
- 145 19. Slowly and uniformly spread the powder prepared in step 17 as a thin layer (ca, 1cm) directly
- 146 on to the top of the packed VLC column. Carry out the elution with hexane-ethyl acetate
- 147 mixtures of increasing polarity (starting with 100% hexane, hexane/ethyl acetate 95:5, 9:1,
- 148 85:15, 8:2, 7:3, 6:4, 1:1, 4:6, 25:75, 3:7, 2:8, 1:9 and lastly 100% ethyl acetate). Add a defined
- 149 volume of solvent (ca. 300 mL) to the top of the VLC column each time and apply vacuum
- 150 until the column dries up (Fig. 2) (Coll and Bowden, 1986; Pelletier *et al.*, 1986). Collect each
- 151 fraction in a round-bottomed flask and concentrate to dryness under reduced pressure at ≤ 40
- 152 °C using a rotary evaporator. To achieve this, adjust the water bath to 60 °C and apply a
- 153 vacuum of 240 mbar. Complete evaporation will occur in around 20 min.
- 154 20. Dissolve an aliquot of each fraction (ca.5-10 mg) in dichloromethane (ca.5 mL). Use a glass
- 155 micropipette to apply 5-10 spots of each fraction as bands ca. 1 cm above the bottom edge of
- 156 a thin layer chromatography (TLC) silica gel plate (Fig.3A).
- 157 21. Place a filter paper inside a TLC chamber and pour inside the chamber a mixture (20 mL) of
- 158 hexane/ethyl acetate (8:2, v/v). Leave the solvent in the chamber for 30 min at room
- 159 temperature (20°C-25°C) to help reach solvent saturation. Pour a mixture (20 mL) of
- 160 hexane/ethyl acetate (7:3, v/v) in second TLC chamber. Place the spotted TLC plates in the

- 161 chambers and **leave them** to develop in an ascending direction until the solvent **reaches** ca. 1
162 cm below the top (Fig.3B).
- 163 22. **Take** the developed TLC plates out of the chambers and **observe** under UV light using short
164 ($\lambda = 254$ nm) and long ($\lambda = 366$ nm) wavelengths. **Spray** anisaldehyde-sulphuric acid reagent
165 (see Recipes) over the plates and **heat** the plates to 105-110 °C with a **heat gun** for ca. 1 min
166 until coloured spots **appear** (Fig.3C) (Waldi, 1965). **Calculate** R_f values for individual spots as
167 the ratio of the distance (in cm) from the centre of the spot to the baseline over the distance
168 (in cm) from the solvent line to the baseline (Stahl and Mangold, 1975; Stock and Rice, 1974).
169 Triterpenes typically appear as bright pink spots on TLC plates following spraying with
170 anisaldehyde-sulfuric acid reagent and heating (Fig.3D).
- 171 23. The first pink coloured spot appears in the VLC fraction eluted with 15% ethyl acetate in
172 hexane. **Mix** this fraction with silica gel 60 (3 g) in dichloromethane. **Leave** the mixture to dry
173 on a bench at 20-25°C. A free-flowing and completely dried powder is usually obtained
174 overnight.
- 175 24. In a glass, beaker, mix enough silica gel 60 in hexane to obtain a slurry which has the right
176 consistency so as to be poured to fill a CC glass column. **Leave** the column stopcock open
177 when filling with the slurry to let the latter settle inside the column. **Continue** pouring the slurry
178 inside the column until the silica particles settle and form a horizontal layer about 5 cm from
179 the top of the column. **Ensure** that there is a sufficient volume (around 5 mL) of solvent on
180 top of the silica layer so the packing material does not dry out. **Take** care to prevent the
181 inclusion of any air bubbles (Note 1) (Braithwaite and Smith, 1996; Ravindranath, 1989).
182 **Apply** the powdered fraction to the top of the packed column.
- 183 25. **Carry out** the elution using hexane and ethyl acetate mixtures of increasing polarity (from
184 100% hexane, hexane/ethyl acetate 9:1, 8:2, 7:3, 6:4, 1:1, 4:6, 3:7, 2:8, 1:9 and lastly 100%
185 ethyl acetate) (Fig. 4). **Collect** all eluates in vials, and analyse their contents by TLC (step 20-
186 22).
- 187 26. One of the eluates **appears** as a single pink spot following spraying with anisaldehyde
188 sulphuric acid reagent (R_f = 0.40 in hexane/ethyl acetate 8:2). **Concentrate** this eluate to
189 dryness under reduced pressure at ≤ 40 °C, **dissolve** it in chloroform-d (2.5 mL) and **transfer** it
190 into an NMR tube. **Carry out** ¹H, ¹³C NMR and MS analysis to reveal the presence of four
191 triterpenes, namely α -amyrin (Hernández Vázquez *et al.*, 2012; Basyuni *et al.*, 2006), β -
192 amyrin (Basyuni *et al.*, 2006; Mahato and Kundu, 1994), lupeol (Basyuni *et al.*, 2006;
193 Thanakijcharoenpath and Theanphong, 2007) and cycloartenol (Kamisako *et al.*, 1987; Zhu *et*
194 *al.*, 2012).
- 195 27. **Combine** the VLC fractions eluted with 25% and 30% ethyl acetate in hexane, respectively.
196 **Concentrate** them to dryness under reduced pressure at ≤ 40 °C using the rotary evaporator.
- 197 28. In a glass, beaker, mix enough Sephadex® LH-20 (Note 2) in 5% hexane in dichloromethane
198 to obtain a slurry which has the right consistency so as to be poured to fill a CC glass column.
199 **Leave** the column stopcock open when filling with the slurry to let the latter settle inside the
200 column. **Continue** pouring the slurry inside the column until the Sephadex® LH-20 particles

201 settle and form a horizontal layer about 5 cm from the top of the column. Ensure there is only
202 the minimal volume of solvent remaining on the surface of the packing material so it does not
203 dry out. Take care to prevent the inclusion of any air bubbles (Note 1). (Determann and
204 Brewer, 1975; Kremmer and Boross, 1979).

205 29. Apply the combined fractions, re-dissolved in 5% hexane in dichloromethane, onto the top of
206 the column.

207 30. Elute with 5% hexane in dichloromethane to afford an eluate which appears as a single pink
208 spot on TLC following spraying with anisaldehyde-sulfuric acid reagent and heating ($R_f = 0.23$
209 in hexane/ethyl acetate 8:2). Concentrate this eluate to dryness under reduced pressure at \leq
210 40 °C, dissolve it in chloroform-d (2.5 mL) and transfer it into an NMR tube. Carry out ^1H , ^{13}C
211 NMR and MS analysis to reveal the presence of two triterpenes, namely mangiferonic acid
212 (Escobedo-Martinez *et al.*, 2012), and ambonic acid (Da Silva *et al.*, 2005).

213 31. Combine the VLC fractions eluted with 40% and 50% ethyl acetate in hexane, respectively.
214 Concentrate them to dryness under reduced pressure at ≤ 40 °C using a rotary evaporator
215 and subject them to gel filtration (see step 28).

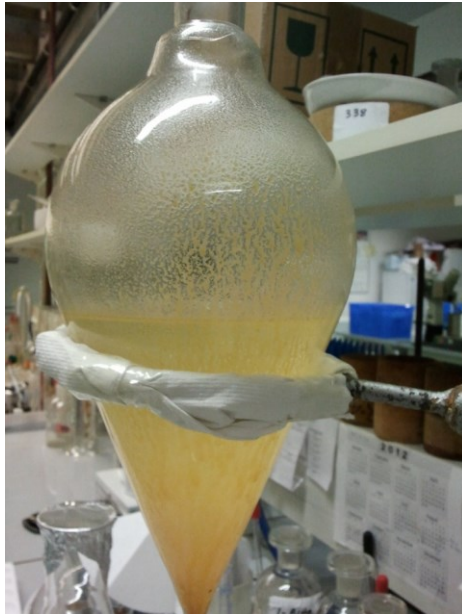
216 32. Elute with 5% hexane in dichloromethane to afford one eluate containing a bright pink spot.

217 33. Fractionate this eluate further by CC (see step 24) using hexane/ethyl acetate mixtures of
218 increasing polarity (from 100% hexane, hexane/ethyl acetate 9:1, 8:2, 7:3, 6:4, 1:1, 4:6, 3:7,
219 2:8, 1:9 and lastly 100% ethyl acetate). One of the eluates appears as a single pink spot on
220 TLC following spraying with anisaldehyde sulphuric acid reagent ($R_f = 0.40$ in hexane/ethyl
221 acetate 7:3). Concentrate this eluate to dryness under reduced pressure at ≤ 40 °C using a
222 rotary evaporator, dissolve it in chloroform-d (2.5 mL) and transfer it into an NMR tube. Carry
223 out ^1H , ^{13}C NMR and MS analysis to reveal the presence of two triterpenes, namely
224 mangiferolic acid (Escobedo-Martinez *et al.*, 2012) and ambolic acid (Escobedo-Martinez *et*
225 *al.*, 2012).

226 34. Subject a portion (5 to 10 g) of the ethyl acetate extract to VLC eluting with hexane: ethyl
227 acetate mixtures of increasing polarity (starting with 100% hexane, hexane/ethyl acetate
228 95:5, 9:1, 85:15, 8:2, 7:3, 6:4, 1:1, 4:6, 3:7, 25:75, 2:8, 1:9 and lastly 100% ethyl acetate)
229 (step 13-15).

230 35. Fractionate the VLC fraction eluted with 40% ethyl acetate in hexane, by gel filtration (step
231 28) eluting with 5% hexane in dichloromethane, followed by dichloromethane, to yield a
232 mixture of ambolic acid and isomangiferolic acid (Escobedo-Martinez *et al.*, 2012) along with
233 mangiferonic and mangiferolic acids as pure compounds.

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235 **Representative data**



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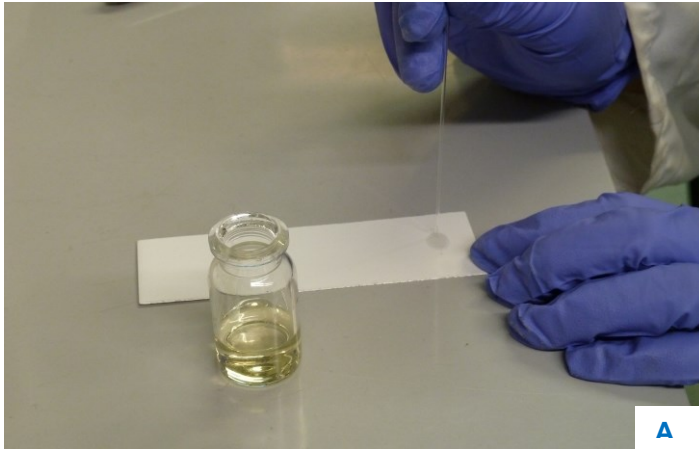
Fig.1. Typical set up for liquid-liquid partition using a separating funnel.



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Fig.2. Typical set up for vacuum liquid chromatography (VLC)

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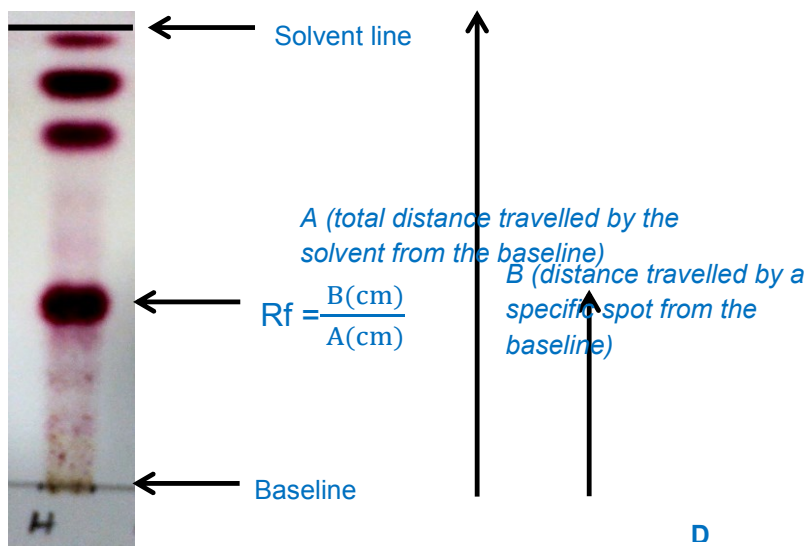


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Fig.3. Thin layer chromatography (TLC) analysis
A) Spotting of sample on TLC plate
B) Spotted plate placed in the TLC tank for elution.
C) Heating of plate
D) Calculation of Rf values for triterpene (pink) spots



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Fig.4. Typical set up for column chromatography

265 **Notes**

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267 1. To prevent the formation of air bubbles, the slurry should be poured slowly onto the inside
268 walls of the column and the packed column should not be allowed to dry out. Any air bubbles
269 can be removed by gently finger tapping the column.

270 2. It is also possible to use some recycled Sephadex® LH-20. The latter is obtained by soaking
271 any used Sephadex® LH-20 slurry in a mixture of dichloromethane and methanol in a Büchner
272 filter connected to a vacuum water jet pump. Once vacuum has been applied, the Sephadex®
273 LH-20 is left to air-dry on the bench at 20-25°C.

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275 **Recipes**

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277 1. Anisaldehyde-sulphuric acid reagent (Waldi, 1965)
278 Mix 0.5 ml of *p*-anisaldehyde with 10 mL glacial acetic acid and 85 mL methanol
279 Slowly add sulphuric acid (5 mL) to that mixture
280 Mix well, place in a glass atomiser (amber) bottle and store at 2-8 °C

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282 **Acknowledgments**

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286 Zhang for technical assistance and Nature's Laboratory Ltd for the supply of propolis.

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