SUPERCRITICAL ANTISOLVENT FRACTIONATION OF PROPOLIS TINCTURE

O. J. Catchpole*, **J. B. Grey, K. A. Mitchell, J. S. Lan**

Industrial Research Limited, PO Box 31-310, Lower Hutt, New Zealand
Fax +64 4 5690 132, e-mail o.catchpole@irl.cri.nz

Propolis is used by bees for holding a hive together, and for sterilizing the hive against microbial infections. Propolis contains a high concentration of flavonoids, which are used in a wide range of cosmetic and health food preparations for their antimicrobial properties. Propolis is usually dissolved in ethanol or ethanol/water mixtures to produce a tincture, and to remove insoluble material such as waxes and detritus from the hive. A new supercritical antisolvent/extraction process has been developed for the fractionation of propolis tincture to increase the concentration of flavonoids and remove high molecular mass components and the essential oil. Flavonoids are practically insoluble in pure CO_2, but sufficiently soluble in CO_2 + ethanol to enable their separation from high molecular mass and/or more polar components. In the first step of the process, supercritical CO_2 is used both as an anti-solvent to precipitate high molecular mass components (and water), and as a solvent to extract the ethanol and soluble components of the propolis. This extract is then fractionated in two separation steps to give a concentrated flavonoid fraction as the main product, and an essential oil/ethanol fraction as a secondary product. The effects of pressure, temperature, flow rate ratio, tincture composition and concentration on product quality and yield were determined at a laboratory and pilot scale. The tincture concentration of Propolis, and tincture composition has the greatest effect on the yield and concentration of flavonoids in the product fraction.

INTRODUCTION

Propolis is a substance collected by bees from the resinous exudates from certain trees (poplar, birch, beech and conifer trees in temperate zones [1-3]), and used for the protection of the hive [1,2]. The name propolis comes from the Greek *pro-*, for or in defence, and *polis-*, the city i.e. the defence of the bee city or hive [1-3]. Propolis {also known as bee glue) has been known since ancient times for its medicinal properties. These properties are reputed to be antiseptic, antimycotic, bacteriostatic, astringent, choleric, spasmolytic, anti-inflammatory anaesthetic and antioxidant [2]. Propolis tinctures and extracts have found application in a wide range of nutraceutical and cosmetic applications, such as dental hygiene products, wound healing creams, antibacterial soaps, immune system booster supplements and anti-rheumatic preparations. The active ingredients have been identified as the flavonoids and caffeic acid derivatives [2]. Propolis in its raw state also contains a high concentration of waxes and hive detritus including dead bees, other insect and microbial invaders, and wood. The propolis is dissolved in ethanol or ethanol/water mixtures to remove the insoluble waxes and detritus, and then filtered to remove entrained comtaminants [2]. The composition of the resultant tincture depends on the location of the hives and availability of suitable trees, the country of origin, seasonality, distance from the equator, and the ethanol/water ratio used in the tincture [1-4]. The propolis samples contain flavonoids and cinnamic acids that have biological activity [2].

The flavonoids and cinnamic acid derivatives are polar, and have minimal to zero solubility in supercritical CO_2. Solid propolis was extracted using supercritical CO_2 by Stahl et al [5] at 600 bar and 313 K to extract the wax and leave behind the insoluble flavonoids. The extraction of the flavonoids found in Brazilian and Chinese propolis has been attempted using supercritical CO_2 + ethanol mixtures [6]. A Chinese patent reports that sequential extraction of propolis + ethanol mixtures at increasing pressures can be used to extract different classes of compounds [7]. Supercritical extracts of propolis have been used to test for anti-cancer properties [8], Supercitical antisolvent precipitation using CO_2 has been widely reported for the precipitation of fine particles. It has not been extensively used for the fractionation of natural product mixtures. Here, the process is applied to the fractionation of propolis. The desired flavonoids and cinnamic acid derivatives are to be extracted by the CO_2 + ethanol, while more polar and high molecular mass components are precipitated.

EXPERIMENTAL

I – MATERIALS AND ANALYSIS

Propolis tincture samples were supplied by a local apiary products company (100 % ethanol tincture) and solvent extraction company (70:30 ethanol:water tincture). The tincture samples varied in propolis concentrations from 5 to 40 % by mass. The flavonoids concentration of the total propolis solids dissolved in ethanol was in the range 14.9 to 23.5 % by mass. The total cinnamic acid and derivatives, and caffeic acid and derivatives, was in the range 5-11 % by mass of the total propolis solids. The ratio of individual flavonoids to each other tends to be similar, although the total concentration varied according to the location and time of collection. The propolis also contained fatty acids, essential oil, and high molecular mass components that have yet to be characterized. Analysis of flavonoids, caffeic acid and derivatives, and cinnamic acid and derivatives was carried out by HPLC [4].

II – APPARATUS AND METHOD

A schematic of the pilot scale experimental apparatus is shown in figure 1. CO_2 is supplied from liquid supply cylinders and subcooled in heat exchanger HX1 prior to being compressed by pump RCP1. CO_2 then passes through HX2 before entering the precipitation chamber EX1. Just prior to the EX1, the CO_2 is co-currently mixed with propolis tincture, which is delivered from storage tank 1 pump RCP2. After contacting of the two fluids, the mixture passes into EX1 via a downcomer tube and/or static mixer. The fraction of propolis tincture that is not soluble in supercritical CO_2 + ethanol immediately precipitates and collects at the bottom of the precipitation chamber. Some water may also be precipitated. The precipitated solids (and

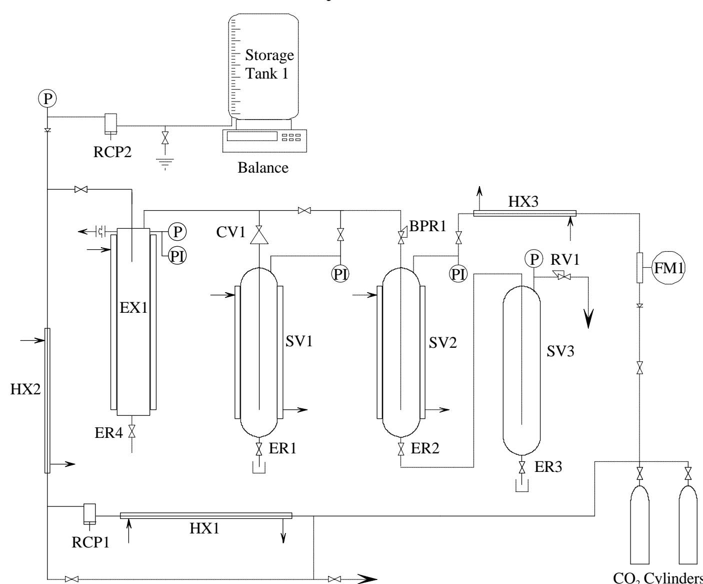

Figure 1 Schematic of Experimental Apparatus

water) are recovered through valve ER4. CO_2, ethanol and the dissolved propolis solids then leave EX1 and pass through a pressure reduction valve, CV1, and into separation vessel, SV1, where the flavonoids are precipitated. A small portion of the ethanol is also precipitated. This extract is recovered through valve ER1. The CO_2, remaining ethanol and propolis essential oil then pass through a back pressure regulating valve, BPR1, before entering separation vessel, SV2. The remaining ethanol and propolis extract is precipitated. The ethanol and propolis extract is further depressurised through valve ER2 and collected in vessel SV3 while previously dissolved CO_2 is flashed off through relief valve RV1. The bulk of the CO_2 is recycled back to the main pump RCP1 via water-cooled heat exchanger HX3, mass flow meter FM1, and condenser/subcooler HX1. The laboratory and demonstration experiments were performed in a similar manner to the pilot plant, except that the CO_2 was not recycled at a laboratory scale. The laboratory and demonstration scale plants in other configurations have been described elsewhere [9,10]. The tincture processing rates were up to 200 ml/hr at a laboratory scale, 2 L/hr at a pilot scale, and 30 L/hr at a demonstration scale.

RESULTS AND DISCUSSION

I - TINCTURE CONCENTRATION (PURE ETHANOL TINCTURE)

The most significant variable in regard to the recovery of desired components was found to be the tincture concentration of propolis solids, and in particular, the concentration of flavonoids and caffeic/cinnamic acids and derivatives. The concentration of flavonoids in the main product and raffinate as a function of the tincture concentration of propolis solids is shown in figure 2 for all the laboratory scale experiments carried out with pure ethanol tinctures. The concentration of flavonoids in the extract decreases, while the raffinate concentration increases as the tincture concentration increases. At high tincture concentrations, most of the propolis solids are precipitated into the extraction vessel, and the raffinate concentration begins to exceed the extract concentration. There is insufficient ethanol for the flavonoids to be completely soluble in the ethanol + CO_2 solution. Extrapolation of the graph to very high solids concentrations, equivalent to the extraction of solid propolis with CO_2 + ethanol as a co-solvent indicates that an almost negligible recovery of flavonoids is likely. At low tincture concentrations, there is very good recovery of flavonoids, but also an increased recovery of undesirable components, which dilute the product. There is sufficient ethanol for the flavonoids to be completely soluble in the ethanol + CO_2 solution. It is desirable to use the highest tincture concentration possible to enable the highest possible product throughput at a production scale whilst also maximising the yield of

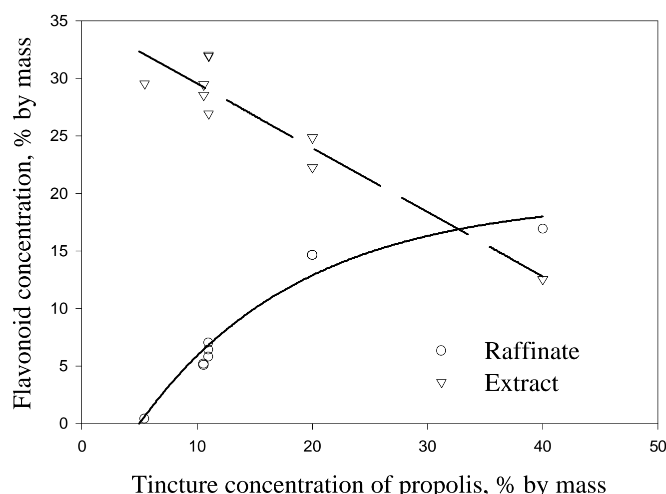

Figure 2 Flavonoid concentration in extract and raffinate as a function of tincture solids concentration (fitted lines for visual aid only)

flavonoids, and minimizing the yield of undesirable components. The optimum tincture concentration appears to be around 10 % by mass, which results in a high yield and concentration of flavonoids in the extract, small losses of flavonoids in the raffinate (but good removal of undesirable high molecular mass components), and reasonable throughput of propolis solids.

II - FLOW RATE RATIO (PURE ETHANOL)

The mass flow rate ratio (tincture to CO_2), at 300 bar, 333 K and 10 % solids tincture concentration, also has a significant effect on the extract and raffinate concentrations of flavonoids, yield of flavonoids in the extract, and enhancement in concentration of flavonoids over the feed concentration. The yield of flavonoids as a percentage of that available from the tincture fed to the experiment, and ratio of the raffinate propolis solids to combined extract solids is shown on the left and right hand y axes respectively as a function of the flow rate ratio of tincture to CO_2 in figure 3. At low ratios, there is a yield loss as there is insufficient ethanol to solubilize the flavonoids. At high ratios, the effective concentration of ethanol in the ethanol/CO_2 mixture increases to the point where the flavonoids are almost completely extracted. However, the undesirable components are also extracted resulting in a low raffinate to extract ratio and thus dilution of the extract flavonoids. This behaviour is in stark contrast to the fractionation of propolis tincture containing water, where this ratio increases with increasing flow rate ratio (the scale on the axes is the same to enable comparison with figure 5). It is desirable to operate at a high tincture to CO_2 ratio to maximise the throughput of tincture at fixed CO_2 flow rate, to maximise the production rate of the flavonoid concentrate. The effects of the flow rate ratio were further investigated at a laboratory scale with ethanol/water tinctures, and then at a pilot scale with pure ethanol tinctures.

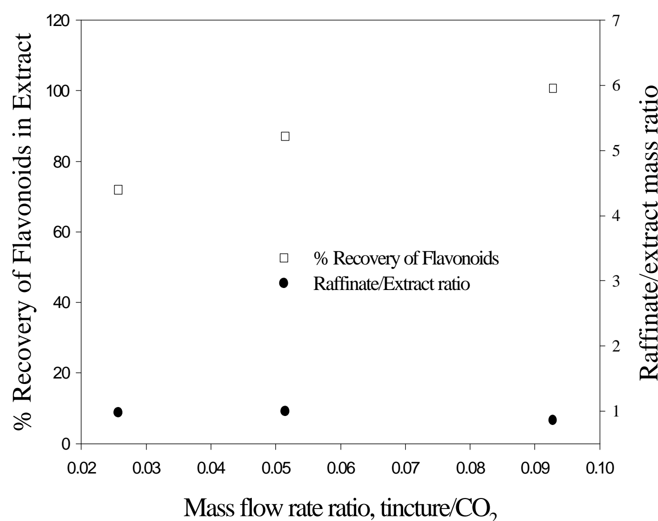

Figure 3 Percentage recovery of flavonoids fed to the experiment in the extract; and ratio of raffinate solids to extract solids (right hand axis) as a function of mass flow

III - FLOW RATE RATIO (ETHANOL/WATER TINCTURES)

The effect of flow rate ratio on flavonoid recovery at 300 bar and 333 K was determined using a 70:30 ethanol:water mass ratio tincture, with a propolis solids content of ~ 12 % by mass. Ethanol/water extracts a larger amount of polar, high molecular mass compounds from the propolis, as well as glycosylated flavonoids, which are not expected to be soluble in CO_2 + ethanol. Extracts were obtained with flavonoid concentrations in the range 25-30 % by mass, which compares well with the pure ethanol tinctures. Cinnamic acids were in the range 3-4 %. Again, a significant fraction of the extracts were not chemically characterized. However, the raffinate also contained high concentrations of flavonoids, at 15 – 20 % by mass, as compared to around 5-10 % by mass for pure ethanol tinctures.

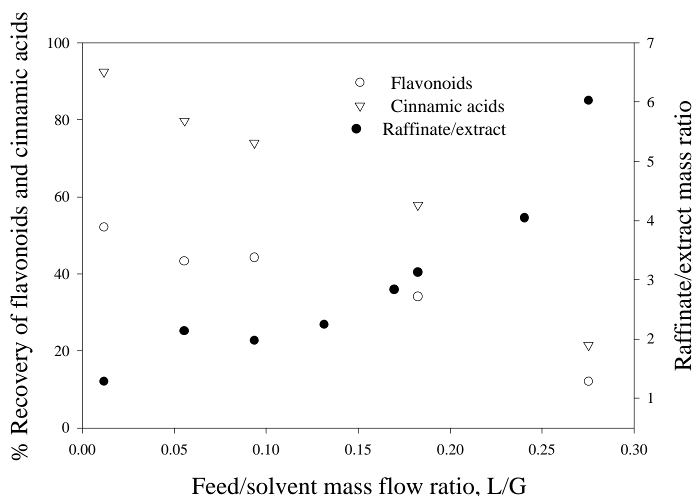

Figure 4 % recovery of flavonoids and cinnamic acids fed to the experiment in the extract; and ratio of raffinate propolis solids to extract solids as a function of feed to solvent mass flow ratio

However, the raffinate also contained high concentrations of flavonoids, at 15 – 20 % by mass, as compared to around 5-10 % by mass for pure ethanol tinctures. The recovery of flavonoids in the extract was substantially lower than that obtained with pure ethanol tinctures. The extraction and fractionation behaviour was quite different to pure ethanol tinctures with respect to the mass flow ratio. There was a strong dependency of total extract yield and flavonoid yield on the flow rate ratio, with both yields decreasing as the ratio of tincture to CO_2 increased. At low flow rate ratios, the yield of flavonoids was approximately 50 % of that fed to the experiment, whilst the yield of cinnamic acids was almost 100 %. Both yields decreased almost linearly with a linear increase in the flow rate ratio, whilst the ratio of raffinate to total extract propolis solids increased in an exponential manner. The recovery of flavonoids and cinnamic acids (% of that fed to the experiment) as a function of the flow rate ratio; and the ratio of the raffinate to extract propolis solids is shown in figure 5. The mass of ethanol in the raffinate increases with increasing flow rate ratio, which results in lower equilibrium coefficients for extraction of flavonoids and cinnamic acids into the CO_2 rich phase. Because of the reduced flavonoid and cinnamic acid yields relative to pure ethanol tinctures, pure ethanol was chosen as the optimum tincture solvent for scale-up trials.

IV - PILOT AND DEMONSTRATION SCALE RESULTS

Pilot scale experiments were carried out at the optimised conditions established from the laboratory scale experiments: tincture concentration 10 % by mass in pure ethanol, pressure 275-300 bar, temperature 333 K. The pilot scale experiments were carried out to demonstrate the stability of the fractionation/extraction process over an extended period, to determine the best separation conditions for minimizing losses in the second separator product, and to confirm the results of the laboratory scale experiments. The flavonoid concentrations in the pilot scale extracts were all in the range 20-35 % by mass, and were dependent on the initial flavonoid concentration in the feed material. Similarly, the flavonoid concentrations in the raffinate were all in the range 5-10 % by mass.

The solubility of propolis solids (combined first and second separator products) in the mixed solvent CO_2 + ethanol as a function of ethanol mass fraction in CO_2 is shown in figure 5 for laboratory, pilot and demonstration scale trials using 10 % tincture solids and pure ethanol; and laboratory scale trials using 70:30 ethanol/water as the tincture solvent. There is good agreement between the solubilities measured at all scales of operation, and between the different tincture solvents. The latter result is unexpected, due to the different fractionation behaviour observed when water is included in the tincture solvent. Substantially higher mass flow ratios (tincture to CO_2) were used with the 70:30 ethanol:water tincture to achieve the

same ethanol concentration in the supercritical phase. The results suggest that using additional ethanol as a co-solvent may improve the recovery of flavonoids when a 70:30 tincture is used. Scale-up of the fractionation process resulted in improvements to the yield and separation of flavonoids. Flavonoid yields were similar or superior to the laboratory scale experiments. Losses of flavonoids in the non-soluble residue, and especially in the second extract fraction were reduced. At a laboratory scale, the mass ratio of main product S1 to minor product S2 was on average 3:1. At a pilot scale, this ratio increased to 30:1.

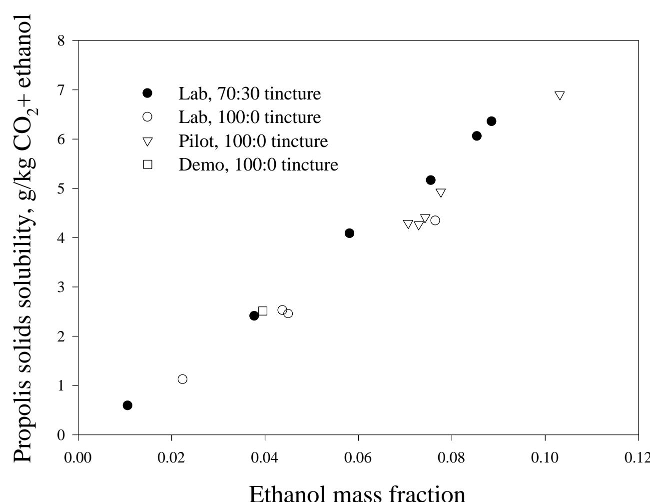

Figure 5 Propolis solids solubility in CO_2 + ethanol as a function of ethanol mass fraction for 70:30 tincture (laboratory scale) and 100:0 tincture (laboratory, pilot and demonstration scale)

CONCLUSIONS

A new supercritical antisolvent fractionation process was developed for producing an extract fraction with a high concentration of bioactive flavonoids (25-30 % by mass) from propolis tincture. The process was optimised at a laboratory and pilot scale, and then demonstrated at a semi-commercial scale. An additional extract fraction was produced which contained essential oil, and a raffinate fraction, that contained high molecular mass and/or polar compounds. The key parameters in the fractionation process are the tincture concentration of propolis, mass flow ratio of tincture to CO_2, propolis concentration of flavonoids, and antisolvent fractionation pressure.

REFERENCES

[1] GHISALBERTI, E. L. Bee World, vol 60, **1979**, p 59-84

[2] BURDOCK, G. A. Food Chem. Technol., vol 36, **1998**, p 347-363

[3] BANKOVA, V. S.; DE CASTRO, S. L.; MARCUCCI, M. C. Apidologie, vol 31, **2000**, p 3-15

[4] MARKHAM, K. R.; MITCHELL, K. A.; WILKINS, A. L.; DALDY, J. A.; LU, Y. Phytochem., vol 42, **1996**, p 205-211

[5] STAHL, E.; GUIREN, K.-W.; GERARD, D. Dense Gases for Extraction and Refining. Springer-Verlag, Berlin, **1988**, p 103

[6] YOU, G.-S., LIN, S.-C., CHEN, C.-R., TSAI, W.-C., CHANG, C. J., HUANG, W.-W. J. Chin. Inst. Chem. Engrs., vol 33, **2002**, p 233-241

[7] WU, J.; LUYU, Q. Chinese Patent CN 1,258,511 **2000**

[8] KIMOTO, N.; HIROSE, M.; KAWABE, M.; SATOH, T.; MIYATAKA, H.; SHIRAI, T. Cancer Letts., vol 147, **1999**, p 221-227

[9] CATCHPOLE, O. J.; GREY, J. B. Proceedings of 2nd International Meeting on High Pressure Chemical Engineering, Hamburg-Harburg, Germany, CD-Rom, **2001**.

[10] CATCHPOLE, O. J.; GREY, J. B.; NOERMARK, K. A. J. Supercritical Fluids, vol 19, **2000**, p 25-37