EXTRACTION OF ANTIMICROBIAL COMPOUNDS FROM WOOD USING SUPERCRITICAL CO_2

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A new anti-microbial product, totarol, has been extracted using supercritical CO_2 from the heartwood of Totara, an indigenous tree of New Zealand. Totarol is an effective antimicrobial agent against gram-positive bacteria, and also has preservative and antioxidative properties. It has recently been incorporated into natural product-based cosmeceuticals and nutraceuticals. Extraction trials have been performed at a laboratory through to a production scale, and include measurements of the totarol solubility, purification of the extract, effect of particle size on extraction rate and yield, and solution of scale-up problems. Two extracts are obtained: a totarol rich fraction, and an essential oil/water rich fraction. While extraction of totarol and related compounds from the wood is straightforward, recovery of the extract and operation at a production scale is difficult. Totarol extract is solid, and has a tendency to block pipes and valves. Novel solutions to the blockage problems have been found, which generate an additional product. A method has also been found to generate fine particles of totarol.

INTRODUCTION

Totarol, $C_{20}H_{30}O$, is a potent antimicrobial agent against gram-positive bacteria [1-4]. Totarol is also a natural preservative and antioxidant that inhibits the peroxidation of unsaturated lipids [5]. Totarol is present in high concentrations (approximately 5 % by mass on a dry basis) in the heartwood of Totara (*Podocarpus Totara*), which is found only in New Zealand; and in much lower concentrations (less than 1 % by mass) in other *Podocarpus, Dacrycarpus, Cupressus and Juniperus* species [6]. Totarol is remarkably resistant to oxidation and degradation [6] and coupled with its antimicrobial properties, the level in dead heartwood remains constant despite the age of the timber. The chemistry, synthesis and bioactivity of totarol and totarol derivatives have recently been reported [1, 7-9]. Totarol can be produced synthetically by a number of routes [1], but the economics and suitability of these methods for production on a large scale have not been assessed. Production of totarol for nutraceutical and cosmeceutical applications is thus commercially possible only by extraction of the naturally occurring compound from suitable wood raw material. Totara heartwood that has been previously used for building, fenceposts, bridge pilings, and foundations provides a significant resource, and no living trees need to be harvested. This work investigates the feasibility of extracting totarol from Totara heartwood using supercritical CO_2.

EXPERIMENTAL

I – APPARATUS AND METHODS

Pilot scale extraction experiments were performed in the apparatus shown schematically in figure 1. Totara was ground in a knife mill, and then added to an extraction basket, which was

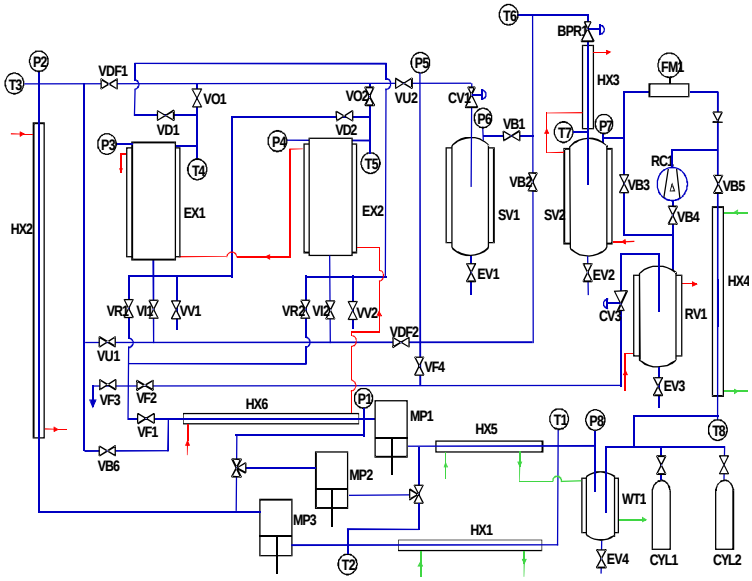

Figure 1 Schematic of pilot scale apparatus

then inserted in extraction vessel EX1 or EX2. The vessel was filled with CO_2 up to the cylinder pressure, and then filled via pump MP3 to the operating pressure. CO_2 then passed through EX1 or EX2. The pressure was then reduced to between 100 and 85 bar through valve CV1. The main totarol fraction was precipitated into separation vessel SV1. CO_2 and remaining extract then passed through back pressure regulator BPR1 and heat exchanger HX3 where the pressure was reduced to cylinder pressure (~ 60 bar) and the temperature raised to around 313 K. Water and totarol "essential oil" was precipitated into vessel SV2. CO_2 was then recycled back to MP3 via heat exchanger HX4, water-trap WT1 and condenser-subcooler HX1. Sampling from vessels SV1 and SV2 was attempted at regular time intervals. The extract was very difficult to remove from SV1 as it solidified in valve EV1. Experiments were performed to determine the solvent loading as a function of extraction temperature and pressure; and the effect of crushing method and pre-drying on extraction yield of totarol and water. Additional experiments were performed where carrier fluids (oil or water) were injected into the flowing stream of CO_2 and dissolved extract to dissolve totarol to prevent blockages in the plant (oil) and produce fine particles (water). Vegetable oil or water was either continuously injected into the flow after back pressure BPR1 but before heat exchanger HX3 (oil only); or immediately beneath valve CV1. An experiment was also performed where a mixed bed of crushed Evening Primrose seeds and Totara was prepared in a ratio to give a desired level of totarol in oil. The solubility of totarol was determined in laboratory scale experiments to establish optimal separation conditions and to ascertain the possibility of purification of the extract using CO_2, by re-extraction to remove lowly and highly soluble contaminants.

II - ANALYSIS AND ANTIMICROBIAL ACTIVITY DETERMINATION

An HPLC analysis method was devised to quantify the totarol content of the extracts, and identify the contaminants that are co-extracted with totarol. The identity of the contaminants was confirmed by ^{1}H NMR, by comparison with published spectra [10]. The National Committee for Clinical Laboratory Standards agar dilution method for microbial susceptibility testing was used [11]. The totarol extract samples were dissolved in ethanol, and then further diluted in water to give final concentrations, in a doubling dilution series from 0.004 to 64.0 mg/L. The agar used was Mueller Hinton Agar. An innoculum of approximately 10^4 organisms of specific gram-positive bacterial strain was replicated onto agar plates containing the totarol extract. The inoculated plates were incubated for 18 hours at 238 K.

RESULTS AND DISCUSSION

I - EXTRACTION EXPERIMENTS

The extraction of totarol™ from Totara has been found to be feasible using supercritical CO_2. The extract is solid and orange/brown in colour, and also contains water, which is co-extracted. Totarol™ extract solubility in CO_2 is relatively low (as shown in III) and so high extraction pressures were used. Two stage pressure reduction results in poorly soluble contaminants being collected in the first separator, totarol™ being split between the two separators, and essential oil and most of the co-extracted water being collected in the second separator. The first separator conditions determine the split between the two products. Totarol™ extract solubility is temperature dependent at low pressures, which causes precipitation after pressure reduction valves. This resulted in blockages in the pipework, especially when liquid CO_2 was used as the solvent. Extraction curves for Totara using supercritical CO_2 at 300 bar and 313 K; and 70 bar, 298 K show the yield of the main extract in g extract per kg of totara wood chip as a function of dimensionless CO_2 usage (kg of solvent used per kilogram of Totara feed to the experiment). The experiment using liquid CO_2 was abandoned before completion, as extract could not be recovered from the separator, and a blockage occurred at the back-pressure regulator. The rate of extraction from fine powder (~ 0.5-1 mm) is more rapid than coarse particles (1-2 mm), although the yield is unaffected. The bulk density of ground Totara is strongly affected by the particle size, increasing by 25 % as the particle size increases from fine to very coarse (~ 3 mm diameter). This decreases the yield of extract by 25 % per basket load of raw material, and thus impacts on the economics of the extraction process. However, the grinding time increase proportionally with a decrease in particle size, and so an optimum must be found.

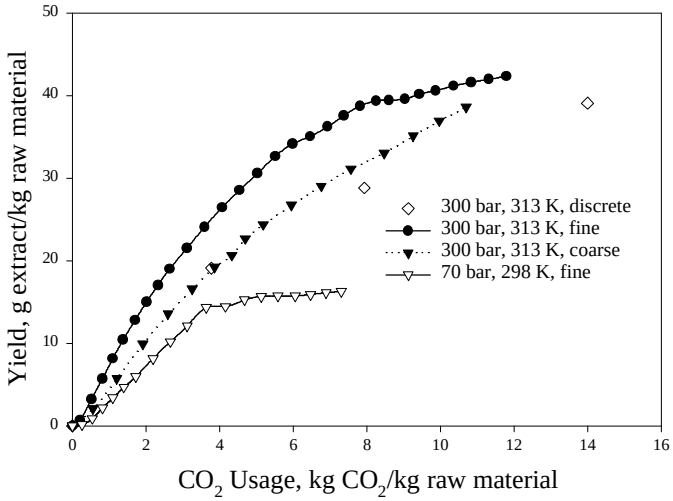

Figure 2 Extraction of totarol from Totara wood chip: influence of extraction conditions and chip size

Totara woodchip contains between 7 and 10 % moisture as measured by mass loss by drying in an oven. The mass of water extracted is highly dependent on the mass of CO_2 passed through the extraction vessels, the extraction temperature, and to a lesser extent, the extraction pressure. At the typical extraction conditions of 300 bar and 313 K, water solubility is around 3.1 g/kg [12-14], which is around ½ that of totarol™. Over-extraction of Totara chip with too much CO_2 simply results in the extraction of water, and totarol contaminants with low solubility. The water affects the solubility of totarol™ in vegetable oil, which is required for some cosmecuetical uses. An experiment was performed with fully dried wood chips to determine whether water-free extract could be produced. Fully dried chips yielded a similar SV1 extract, and a water-free second separator product. It is likely that some of the more volatile totarol contaminants are lost during oven drying: the aroma of the second separator

product was markedly lower than untreated wood chip. The total yield for fully dried chips at 5.5% was significantly reduced compared to fresh material at around 7-8%, due to the non-extraction of water. Totarol did not appear to be degraded by heating the wood to drive off moisture. Further experiments were performed where the extract was precipitated into a separation vessel containing a "solid" basket or canister (a basket with a solid base plate for collection of the extract), followed by a controlled rate of depressurisation in the separation vessel. This procedure was also used at a production scale. Three separate samples were obtained from one such experiment, corresponding to the points shown for the "discrete" run shown in figure 1. The experiments resulted in a solid extract with a honeycomb-like structure, resulting from the release of carbon dioxide from a liquid phase that exists at high pressure: CO_2 reduces the melting point of the solid. The samples were analyzed for totarol content, which was found to decrease with increasing CO_2 usage.

II – IMPREGNATION/FINE PARTICLES EXPERIMENTS

These series of experiments were carried out to solve blockage problems associated with the extraction process, and to devise a method for obtaining totarol extract from the main separator SV1 in a controlled manner. A further aim was to generate fine particles. When oil is used as a carrier fluid, totarol™ dissolves in the oil, and the oil/totarol mixture can be easily recovered from separation vessels. The oil/totarol™ mixture does not block pipework and valves. Experiments were performed by pumping oil at a known rate into the flowing CO_2 after the backpressure regulator BPR1 but before the second separator. Totarol extract was found to precipitate at the equivalent position in the production scale plant, causing blockage, elevated pressures in the first separation vessel, and the need to shut down the plant. The experiments were successful, and oil impregnated with totarol was successfully recovered from the second separator. Two further experiments were performed where the oil was pumped into the flowing CO_2 immediately after the pressure reduction valve CV1 and before the first separation vessel. Again, impregnated oil was easily recovered from the separation vessel SV1 at high pressure. The oil/totarol™ mixture is potentially a useful product in its own right, as the totarol is usually dissolved in an oil for cosmeceutical applications. High value oils, such as Evening Primrose, Borage, Rose Hip and Kiwifruit Seed can be used directly with minimal heating of the oils and exposure to air. When oil is pumped into the first separation vessel, a totally continuous process can be obtained, as the separation vessel does not need to be opened to remove solid totarol™. An additional experiment was performed where a mixed bed of crushed Evening Primrose seed and Totara chip was extracted. The seed and chip was thoroughly mixed beforehand, and the proportions of each chosen to give an oil extract containing around 5 % by mass totarol. The extract fractions were easily recovered from both separators, and the rate of extraction closely followed that of solely Evening Primrose seeds.

Fine particles of totarol were produced by using water as a carrier fluid. Several methods of contacting water and CO_2/totarol were used, which can be classified as static or dynamic. In the static method, the first separator was partly filled with water, and then the CO_2/totarol mixture was either sprayed above or below the surface of the water. In the dynamic method, water was injected into the flowing CO_2/dissolved totarol immediately after the control valve CV1 but prior to the first separator SV1. In each case, totarol and associated water of crystallisation was obtained as a fine powder simply by depressurization through valve BV1. The powder was recovered *before* water. The powder was free-flowing,

hydrophobic, and less dense than water. This was an unexpected result, as the totarol/water complex must have been denser than water to be recovered from valve BV1 at the base of the separation vessel. The recovery of totarol was incomplete, as some material remained in the separation vessel at the end of the runs.

III - SOLUBILITY/PURIFICATION

The solubility of totarol extract in supercritical carbon dioxide has been measured at 313 K and pressures of 80-300 bar to establish the ideal first separator conditions and to determine whether re-extraction can improve the totarol purity. The separator temperature and pressure have a strong influence on the split between the two extract fractions. The first separator pressure was varied between 85 and 100 bar. Operating with a high SV1 pressure results in a significant proportion of totarol™ being recovered in the second separator. Totarol™ solubility at low pressures appears to be very temperature sensitive. The solubility of extract is shown in figure 3 as a function of density. The solubility data can be fitted to a simple Chrastil-type solubility correlation [15] as shown. The measured solubility is for total extract, and includes totarol™ and contaminants, but not water. The solubility is very low at 80 bar (density of ~ 200 kg m^{-3}) and increases rapidly with a relatively small increase in pressure. To obtain the bulk of the totarol™ yield in the first separator it is recommended that separator conditions of 80-85 bar are used. The solubility at 300 bar and 313 K is approximately 6.5 g/kg CO_2. At liquid CO_2 conditions, the solubility is approximately 2.8 g/kg. However, these figures are uncertain due to the difficulties in recovering the extract from the separators, especially at liquid CO_2 conditions.

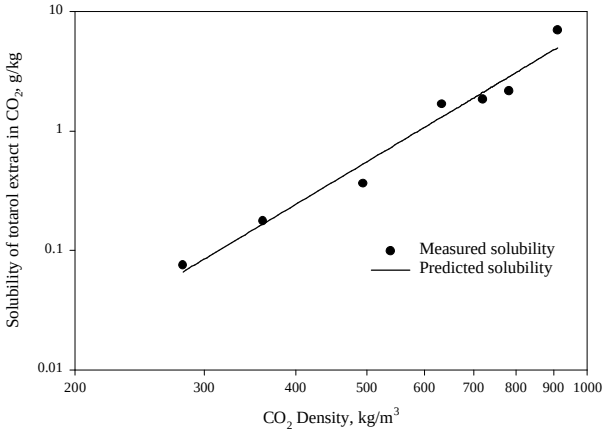

Figure 3 Solubility of totarol extract in CO2 as a function of density

The purity of the main extract (SV1) fraction is around 40-50 % by mass totarol. The extract can be reprocessed if required to increase the totarol concentration. Re-extraction of totarol™ with CO_2 at pressures of 80-100 bar results in an extract with at least 70 % totarol. The lowly soluble contaminants are concentrated in the residue.

IV - ANTIMICROBIAL ACTIVITY

The antimicrobial activity of totarol™ produced at a production scale was evaluated against a range of gram-positive *Staphylococcus* and *Enterococcus* bacteria, ranging from susceptible to penicillin, to highly resistant to penicillin strains. The highly resistant *Staphylococcus* and *Enterococcus* bacteria cause problems in hospitals, where both patients and medical staff become liable to infection. It was necessary to show that the antimicrobial activity of totarol™ product obtained by supercritical extraction was not destroyed by the extraction process, or significantly reduced due to the presence of impurities. The minimum inhibitory concentrations for totarol™ produced by supercritical extraction at a production scale are given in table 1. The MIC levels are very low, and around double the values for pure totarol. The totarol purity in the extracts is around 40 % by mass. Kubo et al [2] have previously shown that totarol and 16 hydroxytotarol are the only active antimicrobial compounds of

those commonly found in *Podocarpus* species, and that the activity of totarol is around 100 times greater than 16 hydroxytotarol.

Table 1: Minimum inhibitory concentration (MIC), µg/ml

Bacterial Strain	Susceptibility	MIC
Staphylococcus aureus Acc2243	susceptible	8
Enterococcus faecalis Acc2244	susceptible	8
Staphylococcus aureus MRS02/2215	epidemic MRSA (EMRSA-15 strain)	4
Staphylococcus aureus MRS02/2214	multiresistant MRSA (Akh4 strain)	4
Staphylococcus aureus MRS02/2249	community MRSA (WSPP1 strain)	8
Enterococcus faecalis Acc3178	high-level gentamicin resistant	8
Enterococcus faecalis Acc3602	vancomycin resistant	4
Staphylococcus pyogenes ARL02/752	erythromycin resistant	4

CONCLUSIONS

A process for the supercritical CO_2 extraction of totarol™ from ground Totara has been established. The process was developed at a pilot scale, and is now undergoing commercial trials. Problems relating to the removal of extract from separation vessels, and blockages in the pipework have been solved with an oil impregnation process. High value oils can be simultaneously impregnated with totarol to a desired concentration for cosmeceutical use. Totarol acts as a natural preservative and antimicrobial agent. The antimicrobial activity (MIC) of the totarol extract was determined against a range of *Staphylococcus* and *Enterococcus* bacteria, and found to be around ½ of that obtained for pure totarol.

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