EXTRACTION AND FRACTIONATION OF ANTIMALARIC DRUGS BY SUPERCRITICAL FLUID

Della Porta G.*, Reverchon E.*, A Benakis °
* Dipartimento di Ingegneria Chimica e Alimentare, Università di Salerno, Via Ponte Don Melillo, 84084 Fisciano (SA), Italy.
E-mail: gdellaporta@unisa.it, tel +39 089964104 Fax +39 089964057

° Centre Medical Universitaire, Parmacology University of Geneve, 9 Avenue de Champel, 1211 Geneva 4, Switzerland

Artemisinin and its derivatives are drugs active against chloroquine-resistant strains of *Plasmodium Falciparum* responsible of celebral malaria. Supercritical CO_2 extraction coupled to a fractional separation technique has been used to concentrate the active principles from vegetable matter. Various extraction conditions have been explored and fractional separation technique has been used to recover an artemisinin and its derivatives rich extract. GC-MS analysis of the fractions obtained at different conditions allowed the identification of the best operating conditions.

INTRODUCTION

Artemisia annua L., is native of China, where is know as Q*uingHao or Chinese wormwood*. The plant has a strong antimalaric activity due to the presence in its aerial parts of Artemisinin and its derivative compounds (artemisin, dehydroartemisinin). These compounds are particularly active against drug resistant strains of *Plasmodium Falciparum.*

The chemical synthesis of artemisinin has been achieved in the 1983 with a yield of 30%, but, it is not competitive since it is much more complex and expensive with respect to the extraction and purification of the drug from the vegetable matter. Artemisinin content in the plant is in the range from 0.1 to 0.4% w/w; some clones cultivated especially in Vietnam can contain over than 1% by weight. Artemisinin derivatives can be present in the range from 0.4 to 1.3% w/w [2-4].

Artemisia annua extract is usually obtained by *n*-hexane extraction. The process is not expensive, however, can induce extract degradation and/or contamination and requires some post-processing since it is not selective. It is possible to overcome these problems using supercritical carbon dioxide extraction (SFE). Indeed, SFE can be performed at relatively low pressures and at near room temperature. Moreover, carbon dioxide is completely eliminated from the product at the end of the extraction process [5-6].

Supercritical extraction of artemisinin was performed by Kohler and co-workers [7] using and analytical apparatus (JASCO micro-SFE, I.V. 1 mL). These authors optimized the extraction conditions at 150 bar, 50°C using a CO_2 flow rate of 2 ml/min for 20 min and a 3% of methanol, as a co-solvent. They obtained very low overall yield and the co-extraction of undesired compounds with high molecular weight was

observed. Moreover, the use of co-solvent introduces again the problems of extract contamination and post-processing.

Therefore, the aim of this work is to find the optimum operating conditions to produce an active principles enriched extract from *Artemisia annua L.*, using supercritical CO_2 extraction plus fractional separation. The influence of the extraction pressure and temperature on extract composition will be also evaluated by GC-MS analysis performed on the various fractions recovered at different process conditions.

I – APPARATUS, METHODS AND PROCEDURES

Apparatus

Supercritical CO_2 extraction was performed in a laboratory apparatus equipped with a 400 cm^3 extraction vessel operated in the single-pass mode of passing CO_2 through the fixed bed of vegetable particles. The fractions extracted were recovered using a stagewise depressurization in two separation vessels of 200 cm^3 each. The cooling of the first separator was achieved by using a thermostated bath (Neslab model PBC-75-II; accuracy ± 0.1 °C). The second separator allowed the continuous discharge of the product. The extraction was carried out in semibatch mode: batch charging of vegetable matter and continuous flow of solvent. Carbon dioxide flow was monitored by a calibrated rotameter (Matheson, model 604) located after the last separator. Total CO_2 delivered during an extraction test was measured by a dry test meter (Sim Brunt, model B10). Temperatures and pressures along the extraction apparatus were measured by thermocouples and Bourdon-tube test gauges, respectively. More details on the apparatus were given elsewhere [5-6].

Materials

Dried aerial part of *Artemisia annua L.* were supplied by Prof. A. Benakis, (CMU, Geneve, Switzerland). The Artemisinin (m.w. 282.3 daltons, $C_{15}H_{22}O_5$, purity 90%) and the Dehydroartemisinin (m.w. 284.3 daltons, $C_{15}H_{24}O_5$, purity 95%) were used as external standard in order to calculate the detector response factor and were supplied by SIGMA. CO_2 (purity 99.9%) was supplied by SON (Società Ossigeno, Napoli, Italy).

Methods

For each extraction test on vegetable matters, the 400 cm^3 extractor was charged with about 200 g of *Artemisia annua L.* previously grounded. The mean particle size, determined by mechanical sieving, was of about 200 μm. CO_2 flow rate was of 0.8 kg/h.

Analytical Procedures

The gas chromatographic-mass spectrometric (GC-MS) apparatus is a Varian (San Fernando, CA) capillary GC connected to an ion trap detector (Finnigan Mat, San Josè CA, mod. ITS 40 Magnum). Separation is achieved by a fused-silica capillary column (mod. DB-5, J&W, Folsom, CA) 30 m lenght, 0.25 mm of internal diameter, 0.25 μm film thickness. GC conditions used are: oven temperature of 40°C for 5 min, then programmed heating from 40 to 250°C at 2°C/min and subsequent holding at 250°C for 60 min. The injector is maintained at 250°C (splitless 20 cm^3/min) and

helium is used as the carrier gas (1 cm^3/min). Samples are run in dicloromethane with a dilution factor of 0.05 % w/w.

The content of Artemisin and its derivatives in the extracts was calculated from the gas chromatographic area traces converted into an absolute value using the ion trap relative response factors. The response factor was calculated using an external standard. Other extract components were identified by matching their mass spectra and retention times with those of pure compounds whenever possible. NIST (National Institute of Standards and Technologies Mass Spectra Library, version 4.0, for Magnum software on ITS 40 Finnigan) and WILEY5 Mass Spectra Library (version 5.0, for Magnum software on ITS 40 Finnigan) were also used as a reference.

II - RESULTS AND DISCUSSION

The first step in the supercritical fluid extraction is the optimisation of pressure and temperature conditions to obtain an efficient recovery of the desired active principle and to avoid the co-extraction of many undesired compounds (fatty acids and their methyl esters, polimethossiflavones, anthocianes and other colouring matter).

During all the experiments, we used the fractional separation technique to avoid the co-precipitation of cuticular waxes that cover the leaves surface. Indeed, these high molecular weight compounds are extracted by SC-CO_2 when a vegetable matter is used [5]. Particularly, we split the extract into two different separators. Indeed, at temperature around 0°C the solubility of paraffins in liquid CO_2 is near to zero; whereas, artemisinin and its derivatives show a high solubility at these conditions. Therefore, paraffins were precipitated in the first separator and drugs enriched extract was collected in the second separator, where a large pressure reduction induce the passage of CO_2 to the gaseous state [5]. A good fractionation was obtained by cooling the first separator at -10°C and depressurizing the second separator at 15 bar at 10°C.

The first set of experiments was carried out at 80 bar and 55°C (CO_2 density 0. 203 kg/dm^3), but the amount of extract collected in the second separator was negligible. This result can be explained considering that at these process conditions CO_2 shows a very low density; i.e., a very low solvent power.

The second set of experiments was carried out at 90 bar and 50°C (CO_2 density 0.288 kg/dm^3); when an overall asymptotic yield was obtained, we changed the operating conditions into 200 bar and 50°C (CO_2 density 0.784 kg/dm^3). Therefore, a second extraction step was performed on the same charge of material. This procedure was adopted to control if the active principles were completely extracted during the first extraction step. Operating at these conditions, the overall yield in the second separator was of 1.6% during the first extraction step and of 2.7% during the second extraction step.

During the extraction process, the second separator was discharged at fixed time intervals. Therefore, GC-MS analyses of the various fractions collected at different extraction times were used to monitor changes in active principles content as a function of time (see also methods section). Particularly, we revealed the presence in the extract of artemisinin, dehydroartemisinin and artemisin. The overall active drugs yield was calculated from GC traces and was of 0.9% in the first step and of 1.1% in the second step. All yields proposed were calculated by weight on the charged material.

These results are illustrated in the diagrams reported in **Figures 1** and in **Figure 2** that correspond the total extract yield and the active principles yield, respectively.

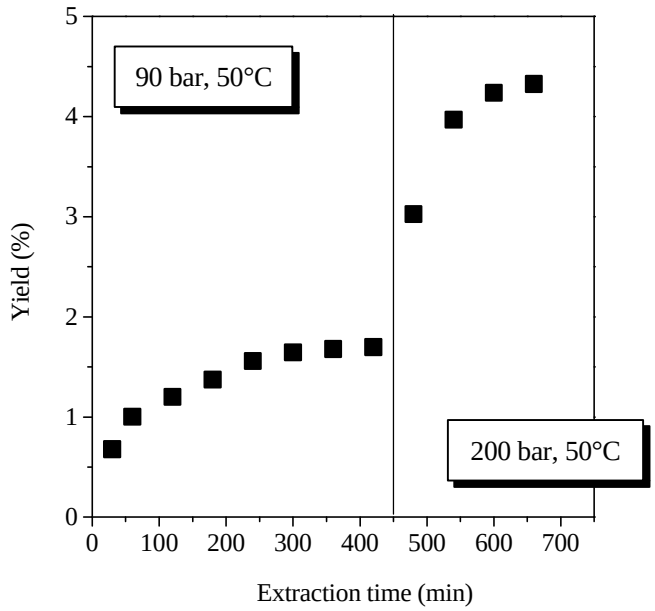

Figure 1. Example of total yield obtained at 90 bar, 50°C (I step) and at 200 bar, 50°C (II step).

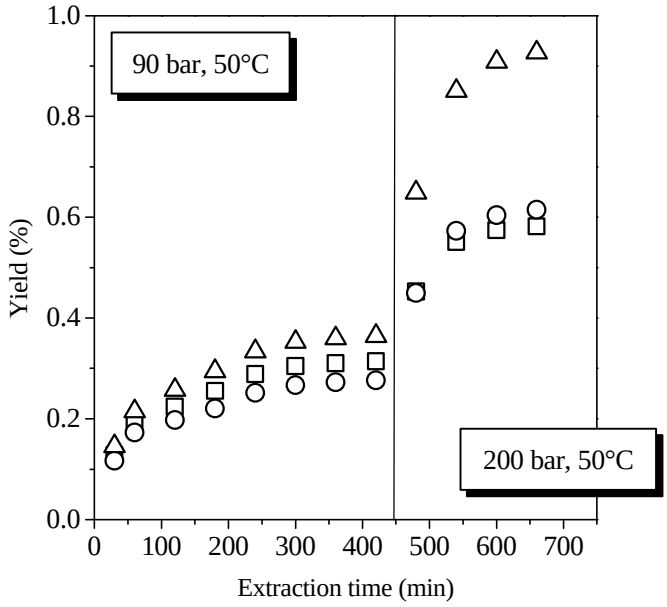

Figure 2. Example of active principles yield calculated by GC-MS and obtained at 90 bar, 50°C (I step) and at 200 bar, 50°C (II step). ? Artemisinin; O Dehydroartemisinin; ? Artemisin.

From **Figures 1 and 2** it is possible to observe that artemisinin and its isomers were not completely extracted during the first extraction step. During the second step drugs were still extracted; but, undesired compounds were also co-extracted. As a consequence, these condition are not suitable for drugs enriched extract production.

To optimise the process conditions, other experiments were performed operating at 100 bar and 40 °C (CO_2 density 0.623 kg/dm^3) in the first extraction step and at 200 bar and 40°C (CO_2 density 0.840 kg/dm^3) in the second extraction step. In this case the total extract yield in the second separator was of 3.5% during the first step and of 1% during the second step, as illustrated in **Figure 3**.

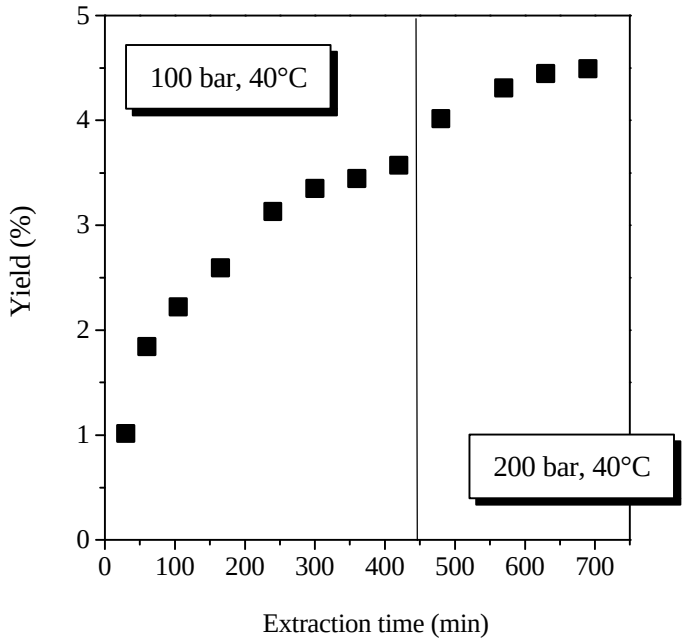

Figure 3. Example of total extract yield obtained at 100 bar, 40°C (I step) and at 200 bar, 40°C (II step).

When GC-MS analyses were performed on the extract recovered at fixed time interval during the first process step, a high drug yield was observed and no co-extraction of high molecular weight compounds was monitored. Indeed, artemisinin and its derivatives overall yield of 1.9% w/w was calculated during the first step and the other compounds co-extracted were mainly mono- and sesqui-terpenes responsible of the *Artemisia Annua L*. aromatic flavour. Moreover, only an active drug yield of 0.1% w/w was monitored during the second extraction step performed at 200 bar, 40°C. These results are illustrated in the diagram reported in **Figure 4**.

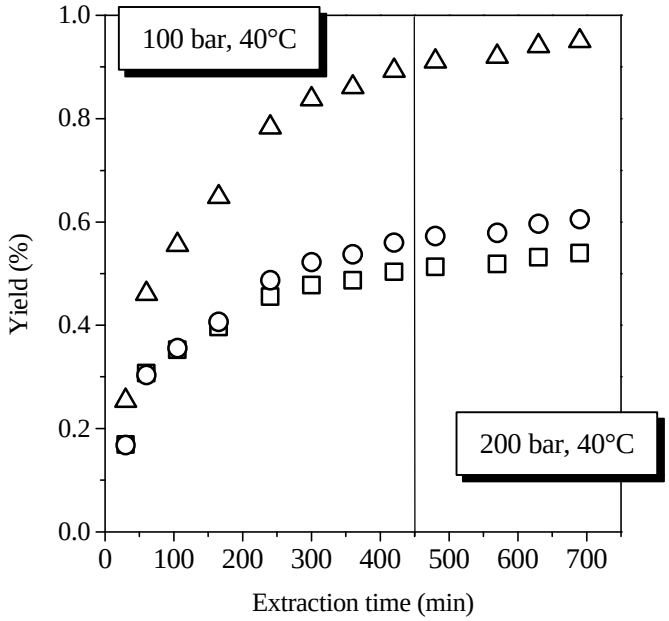

Figure 4. Example of active principles yield calculated by GC-MS and obtained at 100 bar, 40°C (I step) and at 200 bar, 40°C (II step). ? Artemisinin; O Dehydroartemisinin; ? Artemisin.

Artemisinin enriched extract recovered in the second separator was liquid and light yellow, whereas waxes recovered in the first separator were white, solid and colourless. Waxes were mainly formed by *n*-heptacosane , *n*-nonacosane, *n*-hentriacontane, and *n*-tritriacontane.

In conclusion, 100 bar and 40°C seem to be the best operating conditions for the selective extraction of artemisinin and its derivatives.

REFERENCES

[1] SCHMID, G., HOFHEINZ, W., J. Am. Chem. Soc., Vol. 105, **1983**, p. 624.

[2] WOERDEMBERG, H.J., BOS, R., SALOMONS, M.C.; HONDRIKS, H.; PINS, N., MALINGRÈ, T.M., Flavour and Fragrance J., Vol. 8, **1993**, p. 131.

[3] HOLM, Y., LAAKSO, I., HILTUNEN, R., GALAMBOSI, B., Flavour and Fragrance J., Vol. 12, **1997**, p. 241.

[4] MUCCIARELLI, M., CARAMIELLO, R., MAFFEI, M., CHIALVA, F., Flavour and Fragrance J., Vol. 10, **1995**, p. 25.

[5] REVERCHON, E., Journal of Supercritical Fluids, Vol. 10, **1997,** p. 1.

[6] REVERCHON, E., DELLA PORTA, G., D'URSO, E., TADDEO, R., Lebensmitten Wissenshaft und Technologie, Vol. 31, **1998**, p. 454.

[7] KOHLER, M., HAERDI, W., CHRISTEN, P., VEUTHEY, J.L., Journal of Chromatography, Vol. 785, **1997**, p. 353.