International Journal of Pharmaceutics, 63 (1990) 57–63
Elsevier

IJP 02133

Preparation of a low-alcohol extract of *Rosmarinus officinalis* using a reverse osmosis membrane

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(Received 19 February 1990)
(Accepted 12 March 1990)

Key words: Low-alcohol extract; *Rosmarinus officinalis*; Tangential filtration; Reverse osmosis membrane

Summary

The alcohol content in a *Rosmarinus officinalis* extract was reduced to 7% by tangential filtration, in association with diafiltration, using a Millipore spiral wound reverse osmosis cartridge equipped with a polyamide membrane characterized by an 85% sodium chloride rejection. The operating conditions were 9 bar and 20–25°C. The permeation rate was inversely proportional to the concentration of alcohol and soluble solids. The concentration of several aromatic compounds and of rosmarinic acid was determined by GC and HPLC, respectively, at several stages in the alcohol reduction process. In the final *R. officinalis* extract, we recovered 74% of the rosmarinic acid, 8–82% of the oxygenated monoterpenoids and traces of the monoterpene hydrocarbon compounds.

Introduction

Over the past 20 years, the potential of developing a number of cross-flow membrane separation techniques has been of interest to the food industry for the clarification, fractionation and concentration of fruit juice and whey and for the production of low-alcohol beer and wine (Fenton-May et al., 1971; Matsuura et al., 1974; Nielsen, 1982; Cuenat et al., 1985). In the pharmaceutical industry, the cross-flow reverse osmosis (RO) membrane system has been applied to the production of pure water, the concentration and purification of low molecular weight species and thermolabile molecules such as antibiotics (Fries, 1985; Orchard, 1986; Bang-Xiao, 1987). The present report describes the first application of this technique to the preparation of medicinal plant extracts. The preparation of a low-alcohol extract of *Rosmarinus officinalis* using a polyamide reverse osmosis membrane is described. The influence of this technique on the composition of the final *R. officinalis* extract was evaluated.

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Materials and Methods

Preparation of feed solutions

Rosmarinus officinalis L. leaves were collected in the south of France. A 70% (v/v) alcohol extract was produced by maceration of leaves for

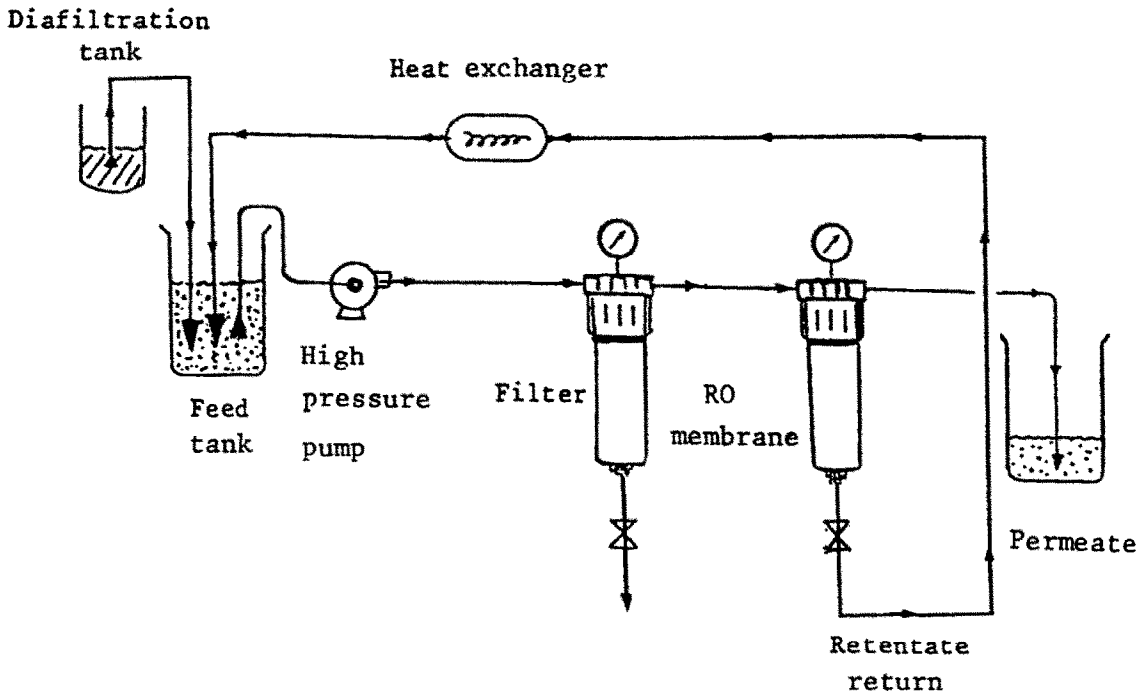

Fig. 1. Reverse osmosis module.

7 days with intermittent shaking. The ratio of plant material to solvent was 1:5 (w/v). This extract, designated M70, was filtered through gauze and then diluted with distilled water to obtain the diluted macerate (DM) with an alcohol content of 35% (v/v).

Apparatus

A Millipore RO module (Fig. 1) was used to prepare the low-alcohol medicinal plant extract. The module was composed of: (i) a Millipore pump (type HPCF 130 F), (ii) a polysulphone filter of 0.6 μm porosity and (iii) a spiral wound RO polyamide membrane with a molecular weight cut-off of 100–200 and a membrane surface area of 1.39 m^2. The membrane was characterized by an NaCl rejection of 85% and a pure water permeability of 12 l/h per m^2.

Procedure

A DM feed solution (9.126 l) was pumped from the feed tank through the polysulphone filter to obtain a filtered diluted macerate (FDM), which was then passed over the RO membrane. The retentate was recycled over a period of 13 h and diafiltration was performed for 10 h by addition of water to the feed tank at a rate equal to the permeate flux. Water and alcohol that had passed through the RO membrane were collected (permeate). All experiments were carried out at 20–25 °C and 9 bar hydraulic pressure.

The equipment was cleaned in situ with a solution containing 0.1% NaOH and 0.1% EDTA, and then with distilled water. The membrane was stored in 0.05% sodium azide.

To characterize the membrane, the pure water permeability and NaCl rejection of a feed solution containing 250 ppm NaCl were determined at 25 °C. The sodium chloride concentration was determined using a conductivity bridge. This control was performed before and after preparation of the low-alcohol extract.

Analytical methods

Fig. 2 describes the general procedure used to obtain the samples that were analyzed by GC. The volatile oil fraction recovered by distilling *R. officinalis* L. leaves in steam was diluted 10-fold in

pentane for analysis. 40 ml of DM; FDM; R4, R8, RF (the latter refer to retentate collected after 4, 8 and 13 h) and 80 ml of P4, P8, PF (permeate collected after 4, 8 and 13 h) were extracted with dichloromethane (4 × 5 ml) after addition of 1 g of sodium chloride and then dried using anhydrous sodium sulphate. These extracts were transferred to a 25 ml volumetric flask and made up to volume with dichloromethane.

The aromatic compounds in the samples were analyzed by GC on a Delsi 330 gas chromatograph with a flame ionization detector (FID) equipped with an Enica 21 integrator. The compounds were first tentatively identified by peak enrichment and by their GC retention indices on two fused silica capillary columns (25 m × 0.25 mm coated with OV 101 and 25 m × 0.22 mm coated with Carbowax 20 M). Nitrogen was the carrier gas; the oven temperature was programmed from 50 to 200 °C at 5 °C/min.

The aromatic samples were analyzed by GC-MS using a Hewlett-Packard capillary GC-quadrupole MS system (Model 5970) fitted with a 10 m × 0.13 mm i.d. fused silica column coated with OV 101. Operating conditions were as follows: injector temperature, 180 °C; detector temperature, 210 °C; column temperature, 60 °C for 1 min and then increased (5 °C/min) to 180 °C. Helium was used as the carrier gas at a flow rate of 0.9 ml/min. The mass spectrometer was operated at 70 eV. The aromatic compounds were identified by comparing the fragmentation patterns of their mass spectra with those indicated in the literature.

The percent composition of each sample was obtained by peak area normalisation without taking into account the relative response factors. To quantitate the aromatic compounds, the GC internal standard method was used (Recueil de Normes Françaises; Huiles essentielles, 1986) with tridecane as internal standard.

Determination of the rosmarinic acid content in M70, FDM, RF and PF was carried out on a Shimadzu high-pressure liquid chromatograph composed of an LC-6A variable-wavelength detector set at 330 nm. An SCL-6B module system controller was used. The reverse-phase system was Spherisorb ODS1 C18 packed in a column (25 cm × 4.6 mm i.d.) with 10-μm particles. The mo-

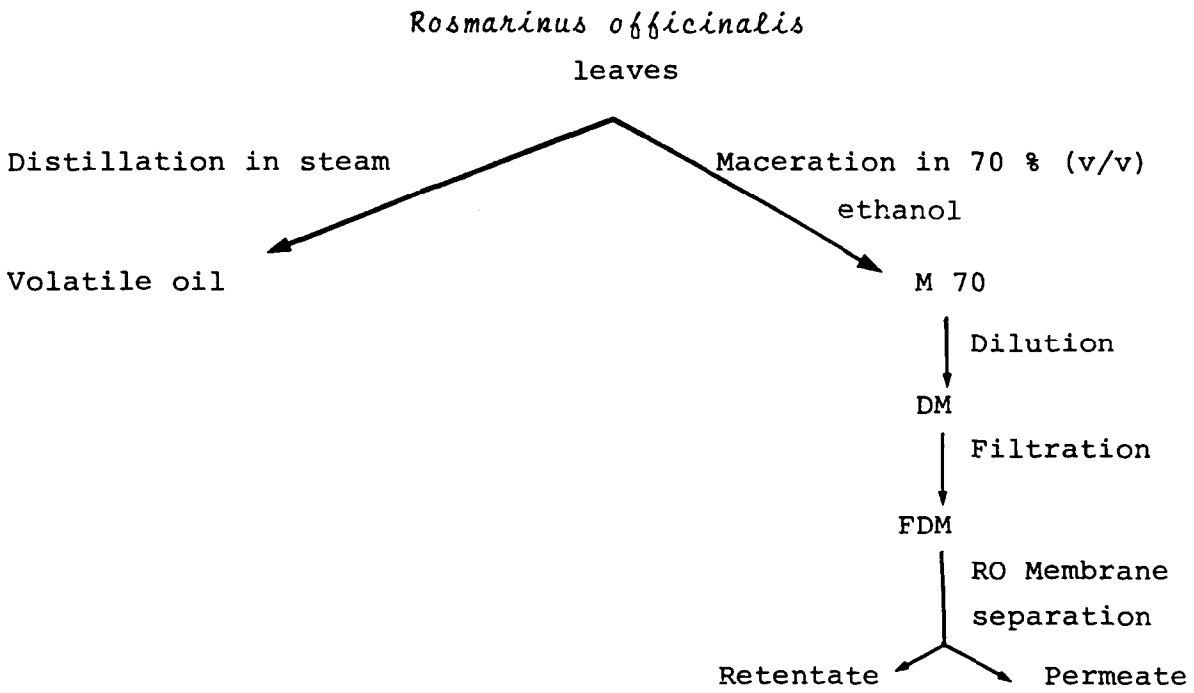

Fig. 2. Flow diagram for the preparation of samples subjected to analytical analysis.

bile phase consisted of a mixture of methanol and 0.16 M phosphoric acid (40 : 60 v/v). Rosmarinic acid obtained from Extrasynthèse (Genay, France) was used as standard. Linearity of the assay was determined using the following solutions of rosmarinic acid in methanol: 40, 60, 100, 120 and 140 μg/ml. Evaluation of each point was repeated 5 times. The calibration curve was fitted by linear regression: $C = 1.643 \times 10^{-4} X + 15.4868$ where C represents the concentration (g/ml) and X the pic surface. The coefficient of correlation was 0.99. M70 (50 ml), FDM (100 ml), RF (50 ml) and PF (100 ml) were evaporated in a water bath to dryness. These residues were dissolved in ethyl acetate. The solutions were filtered and dried on anhydrous sodium sulphate. Next, they were evaporated to dryness and then made up to 10 ml with methanol for HPLC and TLC analysis (described below).

The nonvolatile compounds were analyzed by thin-layer chromatography (TLC) using silica gel plates (Merck 60 F_{254}, thickness 0.2 mm). The solvent system was toluene/ethyl formiate/formic acid (5 : 4 : 1, v/v). 25 μl of the samples in methanol was deposited. A rosmarinic acid solution was used as standard.

The soluble solids were determined according to an established procedure (Pharmacopee Francaise, 1965) using samples of 10 g; each analysis was repeated 3 times.

The percent alcohol in the retentate was determined using an alcoholometer.

The percent solute recovery ($R\%$) was estimated by the following formula:

$$R\% = \frac{V_R \times C_R}{V_f \times C_f} \times 100$$

where V_R represents the volume of DM, FDM, R4, R8, RF or P4, P8, PF; C_R is the solute concentration in DM, FDM, R4, R8, RF or P4, P8, PF; V_f denotes the volume of feed solution, and C_f is the solute concentration in feed solution.

The percent solute separation (Sep % or rejection %) obtained in this experiment was calculated from the equation:

$$\text{Sep}\% = \frac{C_f - C_p}{C_f} \times 100$$

where C_f and C_p represent the solute concentra-

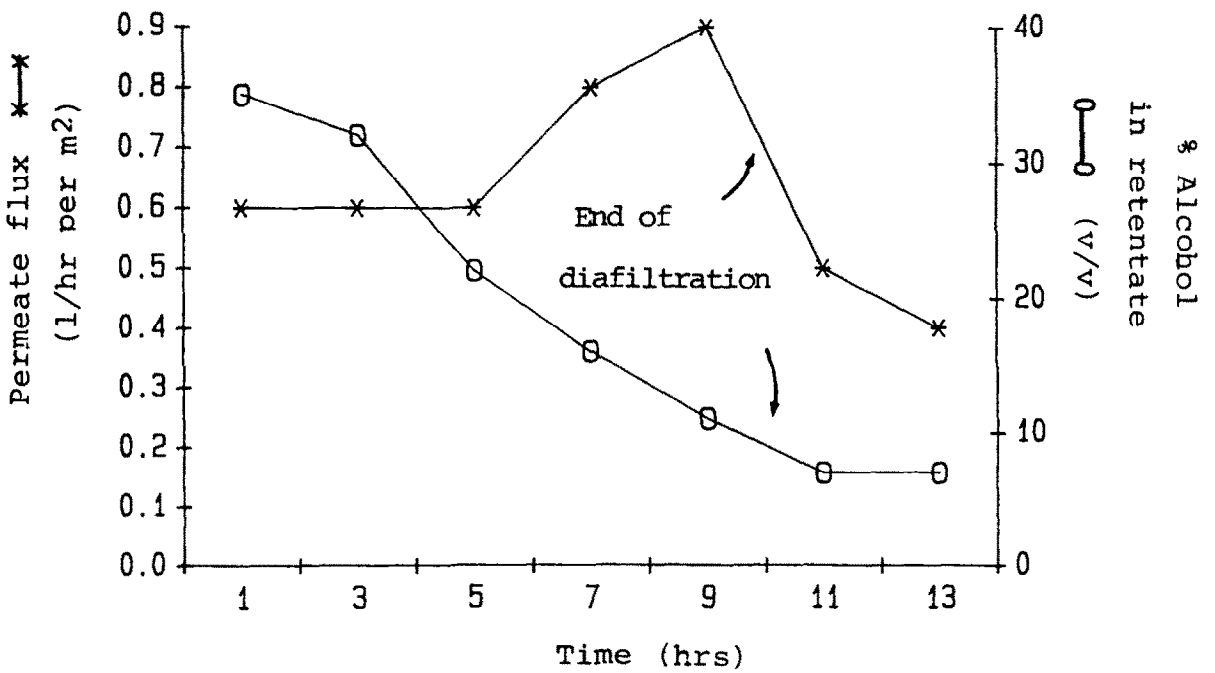

Fig. 3. Permeate flux and rate of alcohol decrease in a *Rosmarinus officinalis* extract using an RO membrane.

tion in feed solution and in the permeate, respectively.

Results and Discussion

A *Rosmarinus officinalis* extract (4.38 l) containing 7% (v/v) alcohol and 1.3% soluble solids was produced from 9.126 l of starting solution that contained 70% (v/v) alcohol. After the initial reduction in alcohol content (70 to 35% v/v) some compounds of low polarity became insoluble and were removed by the polysulphone filter; this was shown by a decrease in total soluble solids of 35.5%. When the alcohol content of the FDM was reduced to 7% (v/v), a decrease in total soluble solids of 12.7% was observed. The final low-alcohol *R. officinalis* extract contained 57 g of soluble solids.

The relationship between permeate flux and alcohol reduction is shown in Fig. 3. During the first 9 h of diafiltration, the permeation rate increased from 0.6 to 0.9 l/h per m^2, while the alcohol content in the retentate decreased from 35% to 11% (v/v). An increase in soluble solid concentration from 7.81 g/l in the FDM to 13.00 g/l in the RF and the phenomenon of concentration polarisation probably explain the decrease in the permeation rate after 9 h (Choudhury and Ghosh, 1986; Kuo, 1987).

The values for pure water permeability and NaCl separation before and after preparation of low-alcohol *R. officinalis* were not different, suggesting that the membrane performance was not altered by contact with the alcohol solution.

Table 1 shows that the aromatic composition of the volatile oil fraction was practically identical to that of the DM. However, a slight variability in the relative proportion of the individual compounds was observed. Both fractions showed the presence of the classical *R. officinalis* components (Paris and Moyse, 1972), namely, (i) a high percentage of oxygenated monoterpenes (about 64%) composed of 1,8-cineole and camphor predominantly, but also verbenone, borneol, 3-octanone, α-terpineol, linalol, terpinen-4-ol and bornyl acetate; (ii) a relatively high percentage of hydrocarbonated monoterpenes (about 35%), including

TABLE 1

Aromatic composition (%) of fractions obtained by distillation of Rosmarinus officinalis leaves in steam and by maceration in alcohol

Aromatic compound	Volatile oil	DM [a]	FDM [b]
Camphene	6.1	6.8	0.7
p-Cymene	1.6	1.7	0.4
Limonene	2.6	2.9	Trace
Myrcene	2.8	2.4	0
β-Ocimene (cis)	0.1	0	0
α-Phellandrene	0.8	0.4	0
α-Pinene	15.9	17.6	1.2
β-Pinene	2.0	2.3	Trace
α-Terpinene	0.4	0	0
γ-Terpinene	0.7	0.6	Trace
Terpinolene	0.4	Trace	0
α-Thujene	0.2	Trace	Trace
Tricyclene	0.1	0	0
β-Caryophyllene	0.5	0.9	0
Caryophyllene oxide	0.2	0	0
Borneol	3.4	2.8	4.2
Bornyl acetate	0.4	0.3	Trace
Camphor	25.5	24.8	36.4
1,8-Cineole	25.9	28.2	42.7
Linalol	1.1	0.6	1.1
3-Octanone	2.4	3.4	5.3
α-Terpineol	1.7	0.6	1.1
Terpinen-4-ol	0.7	0.4	0.6
Verbenone	3.7	2.8	5.2
Peak A	0.2	0	0
Peak B	0.3	0.3	0.3
Peak C	0.3	0.2	0.3
Total	100	100	99.5

[a] Obtained by dilution of M70 2-fold with distilled water.
[b] Obtained by filtering DM through a polysulphone filter.

acyclic monoterpene compounds (myrcene and β-ocimene), monocyclic monoterpene compounds (limonene, *p*-cymene, α-phellandrene, α-terpinene, γ-terpinene and terpinolene), bicyclic monoterpene compounds (α-pinene, camphene, β-pinene and α-thujene) and (iii) a low concentration of sesquiterpenes (1%) including β-caryophyllene and the oxygenated compounds such as the caryophyllene oxide. Verbenone is not usually considered to be a common constituent of *R. officinalis*; nevertheless, verbenone has been found in the volatile oil fraction of *R. officinalis* of Mediterranean origin (Algeria, Corse), in amounts up to 37% (Arbousset, 1972).

TABLE 2

Recovery (%) of the principal aromatic compounds at different stages in the alcohol reduction process of the Rosmarinus officinalis extract

Compound	DM	FDM	R4	R8	RF	P4	P8	PF
Camphene	100	8	0	0	0	0	0	0
α-Pinene	100	5.8	0	0	0	0	0	0
Borneol	100	85	76	64	46	0.8	0.4	1.6
Camphor	100	83	69	53	42	0.8	0.5	1.7
1,8-Cineole	100	87	55	35	32	0.6	0.5	1.4
3-Octanone	100	88	32	17	8	7.5	4.7	9.6
α-Terpineol	100	69	71	60	40	0	2.1	0
Verbenone	100	100	100	100	82	1.4	0	3

The results in Table 1 also indicate that after filtration (FDM) through the polysulphone filter, the diluted macerate contained a very low percentage of monoterpene hydrocarbons: only 2.3% of camphene and α-pinene and traces of *p*-cymene. As shown in Table 2, the principal aromatic hydrocarbons of *R. officinalis*, i.e., camphene and α-pinene, disappeared after 4 h (R4) of membrane separation; however, analysis of the oxygenated aromatic compounds showed that 32% and 8% of 3-octanone was recovered after 4 h and 13 h, respectively; this compound was the most membrane permeable of all the aromatic constituents in the FDM; α-terpineol, cineol, camphor showed degressive recovery and reached about 40% after 13 h; verbenone showed a higher final recovery of 82%. Table 2 also indicates that the compounds not recovered in the retentate were not, however, found in the permeate. This loss is probably due to the fact that the increase in solvent polarity led to the formation of insoluble material; we were able to show that aromatic hydrocarbons were associated with this insoluble fraction by analyzing the precipitate and supernatant obtained by centrifuging an aliquot of DM. Whereas the aromatic hydrocarbon content in the supernatant was only 5%, 54% of hydrocarbons was in the precipitate.

Rosmarinic acid is the main substance responsible for the antioxidant properties of *R. officinalis* (Bruneton, 1987). This acid was found in the M70 at a concentration of 0.29 mg/ml. The recovery of rosmarinic acid in the FDM and RF was 75% and 74%, respectively. It was not found in the permeate. This high recovery can probably be explained by the solubility of rosmarinic acid in the RF and by the selectivity of the polyamide RO membrane.

TLC revealed the presence of 11 spots after exposure to light at 366 nm and then spraying with anisaldehyde/sulphuric acid reagent in daylight. A substance with the same R_f as rosmarinic acid was found in M70, FDM and RF. All the spots in the M70 sample were found in FDM and RF except for two spots with higher R_f values found only in M70; the latter were probably carotenoid substances which precipitated as the alcohol content was reduced.

The solute rejection showing the relationship between the solute concentration in the feed and solute concentration in the permeate reflects the solute membrane selectivity. Thus the 99% rejection obtained by our procedure for the aromatics and for rosmarinic acid indicates excellent membrane selectivity for these compounds even though solute recovery was variable: about 74% for rosmarinic acid, 8–82% for the oxygenated aromatic constituents and loss of hydrocarbon aromatics.

In conclusion, we were able to prepare a low-alcohol (7% *v*/v) *R. officinalis* extract using a reverse osmosis polyamide membrane. Although we did not recover any of the aromatic hydrocarbons in the final retentate, we recovered 8% of 3-octanone, 32% of 1,8-cineole, 40% of α-terpineol, 42% of camphor, 46% of borneol and 82% of verbenone. Furthermore, we recovered 74% of rosmarinic acid in the retentate. The reduction in alcohol content is probably the explanation for the loss of certain constituents. This loss could be explained by their insolubilization during the alcohol reduction process and their retention by the polysulphone filter. As for the reverse osmosis polyamide membrane, it showed excellent solute selectivity.

Taken together, the results suggest that the procedure described herein can be used to prepare low-alcohol plant extracts that contain a relatively high percentage of the major oxygenated aromatic compounds and the non-volatile polar compounds.

Acknowledgements

We thank Dr S.L. Salhi for critical comments and editorial assistance. We also thank Millipore for the RO membrane used in this investigation. One of us (V.B.) received a post-graduate research studentship from the Coordenaçao de Aperfeiçoamento de Pessoal de Nivel Superior, Brazil.

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