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Optimization of conditions for supercritical fluid extraction of flavonoids from hops (*Humulus lupulus* L.)*

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Abstract: Waste hops are good sources of flavonoids. Extraction of flavonoids from waste hops (SC-CO_2 extracted hops) using supercritical fluids technology was investigated. Various temperatures, pressures and concentrations of ethanol (modifier) and the ratio (*w*/*w*) of solvent to material were tested in this study. The results of single factor and orthogonal experiments showed that at 50 °C, 25 MPa, the ratio of solvent to material (50%), ethanol concentration (80%) resulted in maximum extraction yield flavonoids (7.8 mg/g). HPLC-MS analysis of the extracts indicated that flavonoids obtained were xanthohumol, the principal prenylflavonoid in hops.

Key words: Optimization, Supercritical fluid extraction, Flavonoids, Xanthohumol, Hops (*Humulus lupulus* L.)
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INTRODUCTION

The hop plant (*Humulus lupulus* L., Cannabinaceae) is a dioecious twining perennial widely cultivated throughout the temperate zones of the world. The inflorescences (hops or hop cones) are used in breweries to give beer its characteristics such as flavor and aroma. In this respect, the bitter acids and essential oils have received much attention. Carbon dioxide (CO_2) is currently the most accepted solvent for the manufacture of hops extracts by breweries. Its extracts contain nearly all essential oils in hops, a high ratio of α-acids (humulones) to β-acids (less bitter lupulones), a small amount of hard resins, and minor traces of triglycerides, waxes, chlorophylls and inorganic salts (Laws *et al*., 1977). These features distinguish CO_2 extracts from those made with conventional organic solvents, and consequently enable production of beers with a better balance of hops aroma and bitter flavor. Moreover, CO_2 extracts lack traces of unpleasant solvents. Therefore, supercritical CO_2 (SC-CO_2) extraction has become an industrial process for the production of brewery ingredients.

Nowadays, supercritical CO_2 extraction of hops exists in Australia, Germany, UK, USA and China (Gardner, 1993; Palmer and Ting, 1995). However, SC-CO_2 extraction has its limitation. For example, polar compounds such as flavonoids cannot be extracted by SC-CO_2 alone (Anna *et al*., 2004; Catchpole *et al*., 2004; de Maria *et al*., 1997). The flavonoids remain in the waste product of the hops processing industry. Hops are very rich sources of prenylflavonoids, which are secreted along with bitter acids and essential oils by the lupulin glands of the inflorescences (Stevens *et al*., 1998). Xanthohumol is structurally a simple prenylated chalcone that exists

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only in the hop plant, where it is the main prenylflavonoid of the hop cones (Stevens and Page, 2004). Xanthohumol and other prenylated chalcones are now attracting greater attention in the medical field. To date, some prenylflavonoids examined in vitro present many biological activities: inhibition of the growth of breast cancer (MCF-7) cells in a dose-dependent manner (Miranda *et al*., 1999); inhibition of the cytochrome P450-mediated activation of procarcinogens (Henderson *et al*., 1998); inducing the activity of the carcinogen-detoxifying enzyme, quinone reductase (Miranda *et al*., 2000). Other biological activities include inhibition of bone resorption, inhibition of diacylglycerol acyltransferase and antimicrobial activities (Tobe *et al*., 1997; Tabata *et al*., 1997; Mizobuchi and Sato, 1984). Furthermore, the 8-prenylnaringenin, which is also present in hops, has been recognized as the most potent phytoestrogen isolated to date (Milligan *et al*., 2000). This new and exciting biological activity may lead to biomedical application of xanthohumol and 8-prenylnaringenin in the future.

Considering the continuous decrease of hops market prices, hops growers are now very interested in alternative applications (Stevens and Page, 2004). The comprehensive utilization of hops, especially the waste hops (SC-CO_2 extracted hops) are of utmost importance. The extraction and separation of flavonoids from waste hops with organic solvents have been conducted, but the extraction using supercritical fluid (with ethanol added as modifier) has not been investigated so far. The purpose of the present study was to screen out the optimum conditions of supercritical fluid extraction (SFE) of flavonoids from waste hops.

MATERIALS AND METHODS

Plant material

Commercial pellets of hops (Qingdao big flower, produced in Nov. 2003) were purchased from Xinjiang (China). Samples were pulverized in a high-speed mixer-grinder and sieved by 40-mesh screen ($Dp \leq 0.42$ mm). Sieved powders were first extracted by supercritical carbon dioxide at 200 bar, 40 °C for 3 h (Jose *et al*., 2003). The remainder (SC-CO_2 extracted hops) was kept in sealed plastic bags in a refrigerator (4 °C) until use for extraction of flavonoids by supercritical fluid.

Apparatus for supercritical fluid extraction

The system consists of solvent and feed pumps, refrigeration module, 5 L/50 MPa and 1 L/50 MPa extraction vessels, and 2 L/30 MPa and 1 L/30 MPa absorbent vessels. Light-phase fluid (carbon dioxide) was supplied from the CO_2 cylinder by a high-pressure metering pump. Heavy-phase fluid (aqueous ethanol) was supplied to the system by means of a duplex high-pressure pump. CO_2 flow was controlled by pump displacement and was monitored with high-pressure mass-flow meter. Operating temperature was regulated in the extractor and separators by means of three thermo-static baths. A series of valves regulated the pressure in the extractor and separators.

Assays

Flavonoids content was estimated with Zhuang and Yu (1992) method. The constituents of flavonoids were determined by HPLC-MS methods. HPLC separations were achieved on a Zorbax Eclipse RP C_{18} column (4.6 mm×150 mm, 5 μm) at 1 ml/min using a linear solvent gradient from 40% to 80% B (acetonitrile) in A (1% aqueous acetic acid) over 40 min, followed by 80% B for 20 min. Detection was routinely accomplished by monitoring the absorbance signals at 370 nm (Stevens *et al*., 1997).

For the mass spectrometry, Agilent 1100 LC/MSD SL mass spectrometer was operated using atmospheric pressure chemical ionization (APCI) source in the positive ion mode. Mass spectrometry conditions were as follows: drying gas temperature 350 °C, drying gas flow 13.0 L/min, nebulizer pressure 60 psig, vaporizer temperature 325 °C, capillary voltage 4 000 V, corona current 4 μA, mass range 200~450, fragmentor 100 V.

Experimental design and statistical analysis

1. Single factor experiments design

Four factors were tested respectively for the relation between their variation and the yields of flavonoids. The extraction time, temperature, pressure, ethanol concentration and the ratio of solvent to material (the amount of modifier) were fixed at 90 min, 50 °C, 200 bar, 70%, 40% (with each of them as a

variation factor).

2. Orthogonal experiment design

The following four variable factors: temperature, pressure, modifier (ethanol) concentration and the amount of modifier were used to optimize the extraction process according to the design in Table 1.

3. Statistical analysis

Every experiment was replicated three times. Data of interest were analyzed with LSD (least significant data test) method. DPS (data processing software) was used to design and process experiment in this study.

RESULTS AND DISCUSSION

Effect of the amount of ethanol added on the yield of flavonoids

Considering one of the aims of this work is to propose a suitable quality control method for extracting flavonoids from hops in industrial processes, ethanol was selected as a modifier because it is environmentally benign and relatively safe to human health. The amounts of ethanol added were 30%, 40%, 50% and 60% of the total sample (*w*/*w*), respectively.

Fig.1 shows an increase of ethanol percentage up to 50% resulted in an increase of flavonoids recovery. This was consistent with the results of Lin *et al*.(1999) that under certain temperature and pressure, the higher the percentage of modifiers, the higher was the extraction yield achieved. However, ethanol above 50% exhibited a tremendous drop in flavonoids yield. It should be noted that the addition of large amounts of modifier would considerably change the critical parameters of the mixture and that the fluids were no longer supercritical above this value (Tong and Imagawa, 1995; van der Velde *et al*., 1994). Therefore, appropriate amount of ethanol in supercritical fluid is very crucial for flavonoids extraction.

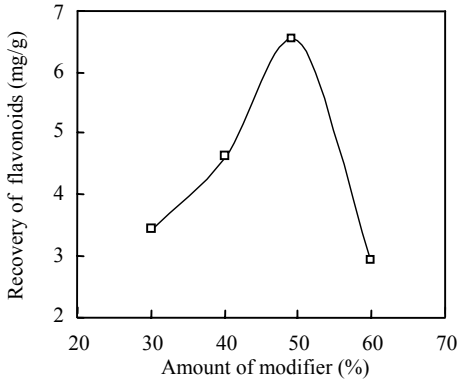

Fig.1 Effect of amount of ethanol added on the yield of flavonoids

Effect of ethanol concentration on the yield of flavonoids

The results of ethanol concentration on the recovery of the flavonoids are shown in Fig.2. The yield of flavonoids increases with an increase in ethanol concentration until 80%. Ethanol concentration beyond 80% resulted in a reduction of flavonoids recovery.

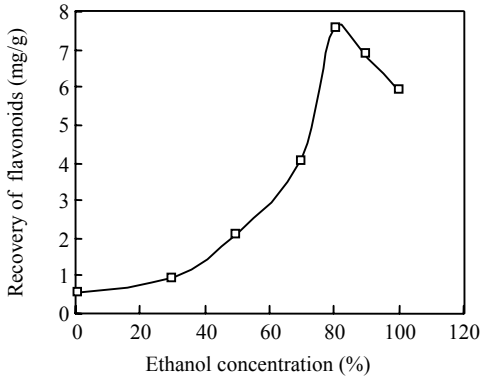

Fig.2 Effect of ethanol concentration on flavonoids recovery

Ethanol added to the supercritical CO_2 increases the polarity of the fluid. The modifier exerts its effect mainly in two basic ways: by interacting with the analyte complex to promote rapid desorption into the supercritical fluid, and by enhancing the solubility properties of supercritical CO_2 (Luque de Castro and Tena, 1996). Various concentration of ethanol used exhibited different effect in changing the fluid polarity and thus had diverse effect on the solubility enhancement of the flavonoids. The optimal extraction yield may be fulfilled when the polarity of the fluid and its flavonoids are coincident. In this study, the results indicated that the optimal ethanol concentration for extraction flavonoids of hops was 80%.

Effect of pressure on the yield of flavonoids

Fig.3 shows that the flavonoids yield was pressure dependent because higher pressure in all cases favors higher recovery of flavonoids. Higher pressure

can increase the bulk density of the fluid mixture that would contribute to solubility enhancement. However, as pressures increase further, the fluid is less compressible, the increase in bulk density is not expected to be very significant (Li *et al*., 2003). Based on the above findings and the feasibility in industrial processes, pressure over 35 MPa was not considered in this study.

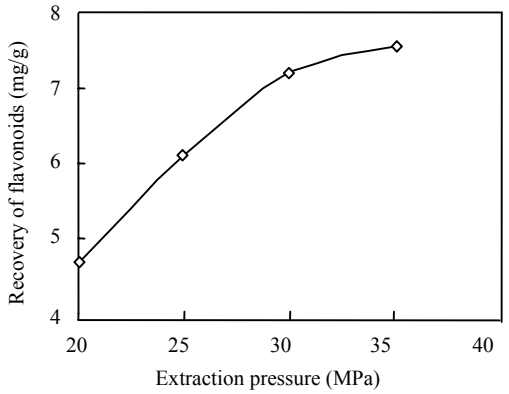

Fig.3 Effect of the pressure on the yield of flavonoids

Effect of temperature on the yield of flavonoids

Fig.4 shows that temperature of 40 °C to 50 °C resulted increased yield. The extraction yield had decreasing trend when temperature increased from 50 °C to 60 °C. Further increase from 60 °C to 70 °C led again increased yield but the recovery rate was lower than that at 50 °C.

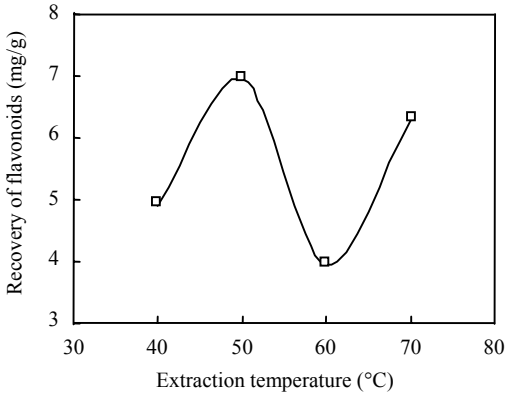

Fig.4 Effect of temperature on the yield of flavonoids

Temperature's effect on extraction is dual. On one hand, higher temperature can accelerate fluid flow and thus increase the solute of compounds; on the other hand, higher temperature can decrease fluid density and thus reduce extraction efficiency. The overall extraction effect of supercritical fluids usually follows the competition between the increasing in solute of compounds and the reduction in SC-CO_2 density due to the rise in temperature (Chiu *et al*., 2002).

Orthogonal experiments

The optimum conditions for the extraction were obtained by using orthogonal design ($L_9 3^4$) based on single factor experiments. Table 1 lists the coded levels of the orthogonal test factors. Table 2 shows factors at different levels in nine experiments conducted and the statistical analysis results. The order of the effect of factors on flavonoids extraction was found to be: B>A>C>D. The optimum extraction conditions obtained from the statistical analysis were $A_2B_2C_3D_2$. It means that 50 °C, 25 MPa, 50% (amount of modifier), 80% (ethanol concentration) were the optimum conditions for flavonoids recovery by supercritical fluid extraction. These conditions were tested later to ascertain the dependability of our results; the recovery of flavonoids was 7.8 mg/g. So the hypothesis of the orthogonal experiment was valid.

Table 1 The coded levels and real levels of orthogonal test factors

Factor/level	1	2	3
A: extraction temperature (°C)	40	50	60
B: extraction pressure (MPa)	20	25	30
C: the amount of modifier (%)	30	40	50
D: ethanol concentration (%)	70	80	90

Table 2 The experimental designs and the orthogonal test results

Run	A	B	C	D	Content of flavonoids (mg/g)
1	1	1	1	1	3.74
2	1	2	2	2	6.19
3	1	3	3	3	6.42
4	2	1	2	3	4.50
5	2	2	3	1	6.67
6	2	3	1	2	5.82
7	3	1	3	2	4.82
8	3	2	1	3	4.48
9	3	3	2	1	3.76
K_1	16.35	13.05	14.04	14.17	
K_2	16.99	17.34	14.45	16.83	
K_3	13.06	16.01	17.90	15.40	
R	1.18	1.28	1.16	0.80	

HPLC-MS results

The conditions of HPLC-MS were described above. A total ion chromatogram of SFE extract is shown in Fig.5. Since the standards of flavonoids of hops were not available, the results compared with those reported in Stevens *et al*.(1997; 1999) based on polarity (retention time), m/z, and relative abundance. Three compounds were proposed. Some flavonoids such as 8-prenylnaringenin, 6-prenylnaringenin, desmethylxanthohumol and so on could not be identified in this study; while other compounds such as peak 4 and peak 5 have not been reported up to now (Table 3). Different varieties of hops and extraction methods could have led to the diversity between our study and other related research (Stevens *et al*., 1997; 1999). Those unknown compounds will be investigated and identified in the future study.

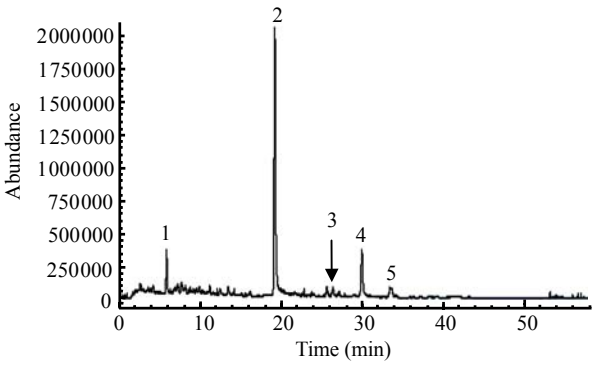

Fig.5 LC-APCI-MS total ion chromatogram of SFE extract

Table 3 LC-APCI-MS characteristics of SFE extract flavonoids

Peak number	*RT* (min)*	$[M+H]^+$ (m/z)	Proposed compound
1	5.864	355	Isoxanthohumol
2	19.239	355	Xanthohumol
3	26.389	353	Dehydrocycloxanthohumol
4	29.902	369	Unknown
5	33.445	385	Unknown

**RT*: retention time

CONCLUSION

The results presented in this work indicated that SFE was feasible for extraction of flavonoids from waste hops, especially for the extraction of xanthohumol, which was reported to have multiple biological activities. Extraction conditions was optimized to find that extraction temperature 50 °C, 25 MPa, the ratio of solvent to material (50%), ethanol concentration 80% were the optimal conditions. HPLC-MS analysis results revealed three purported to exist compounds and two unknown compounds. Considering other parameters such as particle size of samples, fluid flow rate of fluid, etc., simultaneously may require further optimization. More research on flavonoids biological activity should be done in the future research.

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