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Hydrotropic extraction of bioactive limonin from sour orange (*Citrus aurantium* L.) seeds

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Abstract

Limonoids are potential bioactive compounds present only in citrus among fruits and vegetables. A new process for extraction of limonoid aglycones from sour orange (*Citrus aurantium* L.) seeds was investigated using aqueous hydrotropic solutions. The extraction efficiency was dependent on hydrotrope concentration, extraction temperature and percent of raw material loaded. Two hydrotropes such as sodium salicylate (Na-Sal) and sodium cumene sulphonate (Na-CuS) were studied using Box-Behnken experiment design. Response surface analysis (RSA) of data was performed to study the effect of parameters on extraction efficiency. Prominent limonoid aglycone such as limonin was extracted and quantified for process optimization. Both hydrotropes gave maximum limonin yield at 2 M concentration, extraction temperature of 45 °C and 10% solid loading. A maximum limonin yield of 0.65 mg/g seeds was obtained using Na-CuS whereas only 0.46 mg/g seed was obtained using Na-Sal. Using this process, the use of organic solvents can be reduced dramatically to keep the process environmental friendly for the extraction of bioactive compounds.
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Keywords: Sodium salicylate; Sodium cumene sulphonate; Limonin

1. Introduction

Limonoids are a unique class of bioactive compounds present in citrus fruits as a group of highly oxygenated tetracyclic triterpenoids. So far, 37 limonoid aglycones and 17 limonoid glucosides have been isolated from citrus and its closely related genera (Mandadi, Jayaprakasha, Bhat, & Patil, 2007). The aglycones occur as neutral dilactones, acidic monolactones or dicarboxylic acids whereas limonoid glucosides are 17-β-D-glucopyranoside esters of the acidic aglycones. Prominent aglycones are low to medium polarity compounds usually soluble in organic solvents.

Both aglycones and glucosides showed health maintaining properties and hence their isolation, identification and biological activity have been explored (Hasegawa, Berhow, & Manner, 2000; Hasegawa & Miyake, 1996; Rice Evans, Miller, & Paganga, 1997; Poulose, Jayaprakasha, Mayer, Girennavar, & Patil, 2007). Recently, we have observed that limonoid glucosides have the ability to induce caspase 3/7 activity, suggesting that limonoids were capable of inducing apoptosis (Poulose, Harris, & Patil, 2005). Furthermore, our studies also demonstrated that consumption of limonin or grapefruit suppresses colon cancer development (Vanamala et al., 2006). Studies on HepG2 cells indicated that citrus limonoids were partly responsible for lowering LDL cholesterol (Kurowska, Manthey, & Hasegawa, 2000). Furthermore, recent animal studies showed that citrus limonoids significantly decreased the LDL/HDL-cholesterol ratio, and prolonged LDL oxidation susceptibility, which will help reduce the risk of atherosclerosis (McGill & Green, 2001).

Despite increasing demand for limonoids with documented and potential health benefits, lack of well-defined

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environmentally friendly and economical extraction methods have precluded development of these bioactive compounds (Braddock & Cadwallader, 1992). Recently a method was developed for separation and purification of limonoid glucosides from citrus; this is the first method for the purification of limonoids on a multigram scale (Jayaprakasha, Bhat, & Patil, 2007). The major challenge in isolating and purifying limonoids from citrus raw material is their low abundance. Large amounts of raw material need to be processed using equivalent amounts of solvents to obtain sufficient yield of limonoids. Additionally different solvents are required for extraction and purification of limonoids with different polarities (Jayaprakasha, Brodbelt, Bhat, & Patil, 2006).

Limonoid aglycones are medium polarity compounds usually extracted using dichloromethane (DCM), ethyl acetate and acetone. Several methods were developed for the extraction of limonoid aglycones using organic solvents (Bennett & Hasegawa, 1982; Dreyer, 1966; Hasegawa, Bennett, & Verdon, 1980; Mandadi et al., 2007). Recently attempts were made to isolate limonoid aglycones and glucosides using supercritical fluid extraction (SFE) (Miyake et al., 2000; Yu, Dandekar, Toledo, Singh, & Patil, 2006; Yu, Dandekar, Toledo, Singh, & Patil, 2006, 2007; Patil et al., 2006). SFE methods are green environmentally friendly processes. However, very often organic solvents used to modify the supercritical CO_2 to improve yield and selectivity are at hazardous levels. Scaling up of SFE methods is also not easy as high pressure conditions used are difficult and not cost effective.

In recent years, hydrotropy phenomenon has been investigated for a number of potential process applications (Gaikar & Sharma, 1986; Sadvilkar, Samant, & Gaikar, 1995). Hydrotropes are highly water-soluble organic salts and hydrotropy is the phenomenon of increasing solubility of water insoluble or sparingly water-soluble organic compounds in aqueous solutions in the presence of hydrotropes. The increase in solubility of an organic substance is a function of the hydrotrope concentration and depends not only on the nature of hydrotrope, but also on the nature of solute (Balasubramanian, Srinivas, Gaikar, & Sharma, 1989). Previous research in hydrotropic solubilization was concentrated on its applications in drug and detergent formulations, enhancing the rates of heterogeneous reactions and extractive separations of close boiling substances. Recently Raman and Gaikar (2002) demonstrated that high solubilization capacity and selectivity in solubilization by hydrotropy could be used for extraction of water insoluble bioactive compound such as piperin from complex bio-matrices.

In order to reduce the use of organic solvents for the extraction of limonin, we have investigated the potential of hydrotropic extraction of limonoid aglycones from sour orange seeds. Two hydrotropes have been used for the isolation of citrus limonoids and the process has been successfully optimized for the extraction of limonin.

2. Materials and methods

2.1. Raw material and chemicals

Sour orange (*Citrus aurantium* L.) seeds were obtained from Texas A&M University-Kingsville-Citrus Center, Weslaco, TX. Kernels containing fatty material were manually removed, seed shells were collected and ground. All solvents used were of ACS/HPLC grade and obtained from Fisher Scientific (Atlanta, GA). Sodium salicylate (Na-Sal) was obtained from Aldrich (Milwaukee, WI). Aqueous solution of sodium cumene sulphonate (Na-CuS) (2 M) was obtained from Stepan Company (Romeoville, IL). Limonin was obtained from Aldrich Chemical Co. (Milwaukee, WI).

2.2. Design of experiment (DOE)

Box-Behnken design was used to achieve maximum information about the process from a minimum number of possible experiments. In current process, three variables namely concentration of hydrotrope, temperature of extraction, and solid loading were selected for each set of experiments while keeping the time of extraction (6 h) constant through all the experiments. The Box-Behnken design is an independent quadratic design in which the treatment combinations are at the midpoint of the edge of the process space and the center. This design has limited capability for orthogonal blocking compared to central composite design (CCD) but for three factors, Box-Behnken design requires fewer numbers of runs than CCD.

2.3. Method of extraction

Two hydrotropes of Na-Sal and Na-CuS were used for the extraction of limonoids. The extraction of limonoids from sour orange seed shell powder was conducted in a fully baffled cylindrical vessel of internal diameter 8 cm and height 8 cm, equipped with a four-blade turbine impeller of 3 cm diameter. The variables studied are described in design of experiment above. During the extraction, a known quantity of raw material was suspended in 200 ml of hydrotrope solution of known concentration, maintained at a predetermined temperature (Table 1). Extraction was conducted for 6 h and the extract was separated from the solid residue by filtration.

The 10 ml extract was diluted with 180 ml of deionised water to take hydrotrope concentration below minimum hydrotrope concentration (MHC). The MHC of Na-Sal is 0.65 M and that of Na-CuS is 0.1 M (Balasubramanian et al., 1989). The dilution was completed with or without pH adjustment to get limonoid aglycones as precipitate. The precipitated aglycones were recovered by centrifugation. The residue was washed with water and extracted with dichloromethane (DCM) and quantitatively analyzed for the limonin content.

Table 1
Hydrotropic extraction conditions and yield of limonin from sour orange seeds using Na-Sal and Na-CuS

Run	Hydrotrope concentration (M)	Extraction temperature (°C)	Raw material loading (%)	Yield from Na-Sal (mg/g)	Yield from Na-cus (mg/g)
1	1.5	60	30	0.19 ± 0.002	0.26 ± 0.005
2	2	60	20	0.32 ± 0.013	0.45 ± 0.016
3	1	60	20	0.25 ± 0.022	0.35 ± 0.019
4	1.5	60	10	0.38 ± 0.009	0.53 ± 0.013
5	2	45	30	0.17 ± 0.019	0.24 ± 0.008
6	1	45	30	0.22 ± 0.005	0.31 ± 0.006
7	1.5	45	20	0.35 ± 0.018	0.49 ± 0.015
8	2	45	10	0.46 ± 0.012	0.64 ± 0.013
9	1	45	10	0.38 ± 0.011	0.53 ± 0.015
10	1.5	30	30	0.14 ± 0.006	0.19 ± 0.012
11	2	30	20	0.26 ± 0.005	0.36 ± 0.018
12	1	30	20	0.15 ± 0.005	0.21 ± 0.011
13	1.5	30	10	0.28 ± 0.016	0.39 ± 0.007
14	1.5	45	20	0.36 ± 0.013	0.48 ± 0.010
15	1.5	45	20	0.34 ± 0.004	0.49 ± 0.015

2.4. Analytical methods

All samples were analyzed by HPLC using Perkin Elmer Series 200 pump, Series 200 Autosampler and LC 295 UV/Vis Detector (Salem, MA, USA). Chromatographic separation was achieved by Allsphere Silica (Alltech, Deerfield, IL, USA) column (250 mm × 4.6 mm, 5 μ particle size). The column was eluted at a flow rate of 1.5 mL/min using a linear gradient mixture of cyclohexane (solvent A) and tetrahydrofuran (solvent B) (Manners & Hasegawa, 1999). Initial mobile phase condition was 80% of solvent A and 20% of solvent B. A linear gradient was invoked to 60% of solvent A and 40% of solvent B in 10 min. The final composition was maintained for 13 min for a total run time of 23 min. The limonoid aglycones were detected at 210 nm and quantified using TotalChrom software version 6.2.1 (Perkin, Salem, MA, USA).

2.5. Statistical analysis

The data collected were analyzed using response surface analysis (RSA) procedure (MATLAB 7.0.1). The data from the Box-Behnken Statistical Design was used for calculations and modeling the optimum conditions for the both the hydrotropes.

3. Results and discussion

Hydrotropes are amphiphilic organic substances with a short alkyl chain or an aromatic ring with a short alkyl chain, attached to a strongly polar/ionic group. Alkali metal salts of benzoic and substituted benzoic acids, benzene sulphonic acid and its many derivatives, naphthoic acid, and various hydroaromatic acids are typical hydrotropes. Two hydrotropes such as aromatic carboxylate (Na-Sal) and alkyl benzene sulphonate (Na-CuS) were selected for the extraction studies. The selection of hydrotropes was based on the expected efficiency of solutes. In order to extract aglycones from bio-matrix the hydrotrope solution must penetrate the cellulosic layers of cells and extract the aglycones. In a previous study we have demonstrated that both the hydrotropes are capable of penetrating through cell walls to access and extract the bioactive compounds (Dandekar & Gaikar, 2003).

3.1. Extraction efficiency with respect to time

Initially extraction efficiency for both hydrotropes was studied with respect to time. Extractions were conducted at 2 M concentration of hydrotrope at 60 °C and 10% raw material loading. Samples from the extraction vessel were withdrawn every hour and limonin concentration was quantified. Fig. 1 shows a typical chromatogram of DCM fraction from hydrotropic extraction of sour orange seeds. Prominent compound was found to be limonin and confirmed by HPLC co injection as well as comparing the retention time with corresponding standard. Further, the concentration of limonin was quantified in all the extracts to determine extraction efficiency and the results have been presented in Table 1.

Fig. 2 depicts the extraction time verses limonin concentration. The rate of extraction of limonin in Na-Sal and Na-CuS solutions were same up to 3 h and then the difference in the rate of extraction was increased in Na-CuS dramatically. Initial similarity in extraction rates for both the hydrotropes suggests that the penetration rates through raw material cell structure may be similar and extraction efficiency is mainly dependent on solubilization capacity of each hydrotrope. It was observed that extraction reaches to plateau after 6 h for both the hydrotropes and based on these results; 6 h was established as optimum extraction time and kept constant for all the experiments.

3.2. Recovery of limonoids

Recovery of limonoids from hydrotropic solutions was investigated using three different methods. In first method, extracts were diluted below MHC of hydrotrope with

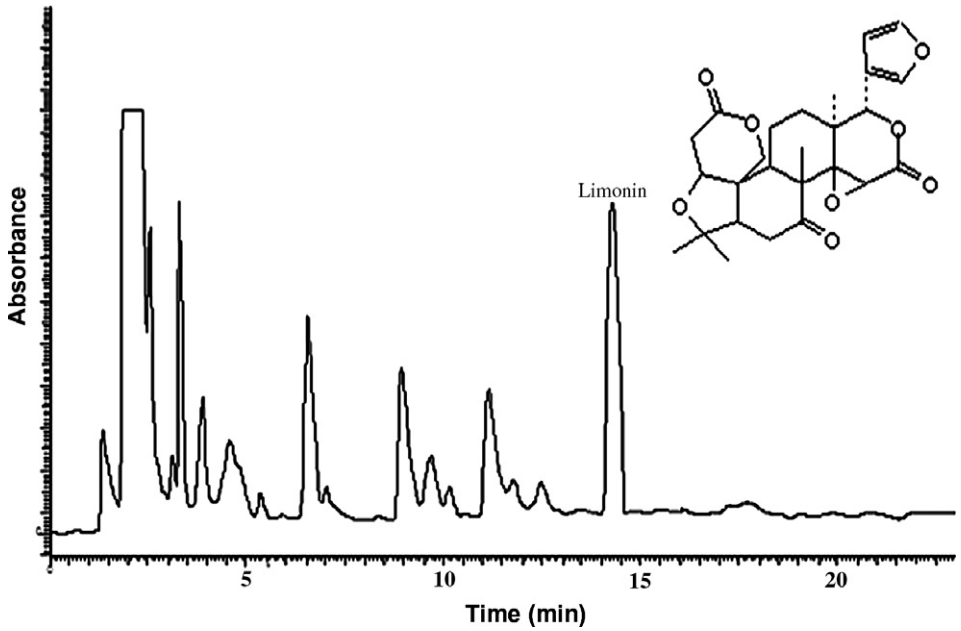

Fig. 1. Typical HPLC chromatogram of DCM fraction obtained from sour orange seeds using hydrotropic extraction and structure of limonin.

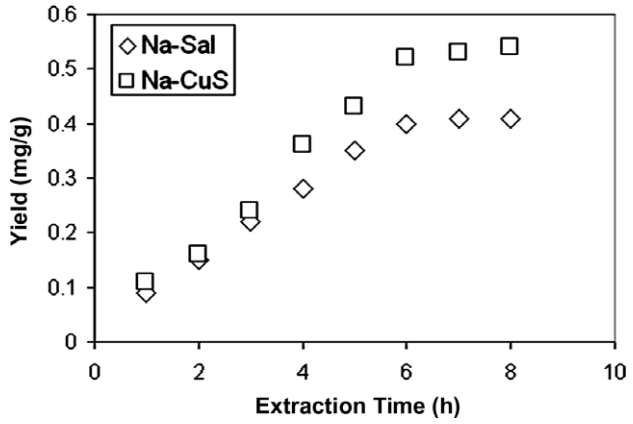

Fig. 2. Extraction of limonin using sodium salicylate (Na-Sal) and sodium cumene sulphonate (Na-CuS) at different time.

water. Easy recovery of aglycones was achieved by this method in case of Na-Sal; however, precipitation took a longer time (24 h) at 4 °C. In case of Na-CuS extraction, dilution of extract using water below MHC did not provide precipitate even after 48 h at 4 °C. It is possible that Na-CuS has greater affinity towards aglycones than Na-Sal. In a previous study Dandekar and Gaikar (2003) reported a greater affinity of alkyl benzene sulphonates towards curcuminoids and recovery of curcuminoids by acidic water. In the second method, extracts were diluted below MHC of hydrotrope using acidic water (pH 3.0). In case of Na-Sal, dilution using acidic water was ineffective since large amount of Na-Sal was converted to salicylic acid. On the other hand, recovery of aglycones from Na-CuS extracts was achieved by this method provided aglycones in the form of white precipitate. Third method used was partitioning the Na-Sal hydrotrope extract with DCM for 5–10 min. Two times partitioning with DCM (1:1) was sufficient to achieve complete extraction of limonoids from aqueous Na-Sal extracts. On the other hand, concentrated Na-CuS extract was partitioned with DCM, no aglycones were moved to organic phase. Even dilution of extract below MHC with water gave very slow recovery of aglycones in DCM and complete recovery of the aglycones was not achieved even after five extractions of dilute sample with DCM in a ratio (1:1).

First and third methods are efficient for the recovery of aglycones from Na-Sal extracts. Recovery of aglycones on dilution using water below MHC is slow but easy to handle even with large volumes of extracts. Partitioning the Na-Sal extract with DCM is fast. In the case of Na-CuS the second method for the recovery of agylcones is efficient and fast recovery.

3.3. Optimization of extraction parameters

After initial study, parameters were optimized using Box-Behnken experiment design. Three variables were fixed for the extraction experiment, i.e. concentration of hydrotrope (1 M, 1.5 M and 2 M), extraction temperature (30 °C, 45 °C and 60 °C) and percent solid loading (10%, 20% and 30%). All extractions were carried out for 6 h and the extract was analyzed to determine limonin content.

3.3.1. Extraction of limonin using Na-Sal and Na-CuS

Table 1 gives details of each set of experimental design and limonin yield. Response surface analysis (RSA) of data in Table 1 demonstrated that the relationship between limonin yield and experiment parameters is quadratic with very good regression coefficient ($R^2 = 0.99$). Eqs. (1) and (2) show the relationship between limonin yields with

extraction parameters using Na-Sal and Na-Cus, respectively,

$$Y = -1.035 + 0.5125X_1 + 0.0395X_2 + 0.0118X_3 - 0.0013X_1X_2 - 0.0065X_1X_3 - 0.0001X_2X_3 - 0.09X_1^2 - 0.0004X_2^2 - 0.0002X_3^2 \quad (1)$$

$$Y = -1.4463 + 0.6875X_1 + 0.0555X_2 + 0.0171X_3 - 0.0017X_1X_2 - 0.009X_1X_3 - 0.0001X_2X_3 - 0.12X_1^2 - 0.0005X_2^2 - 0.0003X_3^2 \quad (2)$$

where Y is the extraction yield, X_1 is the concentration of Na-CuS and Na-CuS, X_2 is the temperature of extraction and X_3 is the percent solid loading.

Eqs. (1) and (2) demonstrated that limonin yield depends more on hydrotrope concentration followed by extraction temperature while solid loading has the least effect. Figs. 3 and 4 depict the relation between RSA generated limonin yield, hydrotrope concentration and extraction temperature. The yield sharply improved with increase in hydrotrope concentration whereas the effect of extraction temperature on the yield is less. From the experimental data and RSA, the extraction parameters to obtain maximum yield of limonin can be optimized as hydrotrope concentration of 2 M, extraction temperature of 45 °C and 10% solid loading.

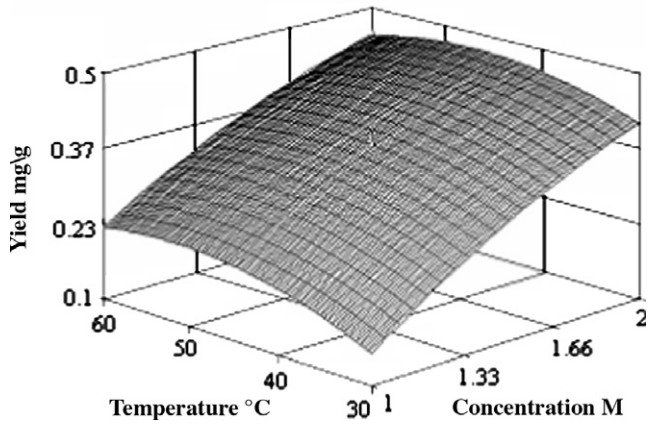

Fig. 3. The response surface analysis for the extraction of limonin content with respect to temperature and concentration of Na-Sal.

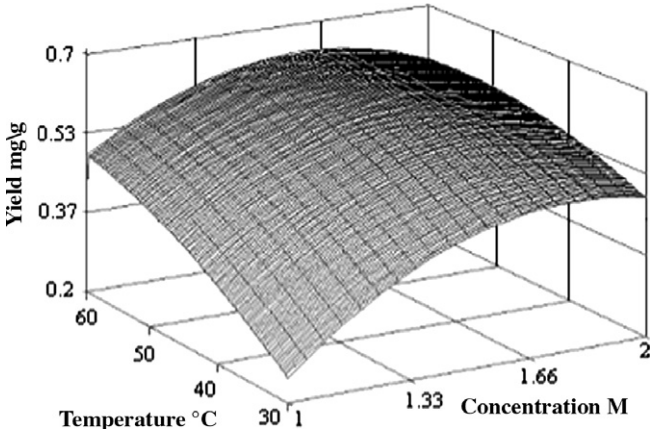

Fig. 4. The response surface plot for the extraction of limonin with respect to temperature and concentration of Na-CuS.

4. Conclusion

Limonin can be extracted successfully from sour orange seeds using hydrotropic solutions. The extraction process is water based and limonin was recovered by dilution of extract using water or acidic water. The yield of limonin was comparable in both the hydrotropes. The method is environmental friendly, inexpensive and easy to handle.

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