

SFE/SFC OF PHOTOSENSIBILIZING FUROCOUMARINS FROM UMBELLIFERAE PLANT

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ABSTRACT

The ability of supercritical fluid chromatography to serve as a tool for both analytical and preparative chromatography method for furocoumarin mixtures from natural source has been explored. It has been shown that normal phase regime suits for such separation and allows to obtain baseline separation of three major furocoumarins from Umbelliferae plant *Ammi majus* - bergapten, xanthotoxin and isopimpinellin. 2-ethylpyridine column has provided the most satisfying selectivity and retention for these substances. The best separation has been achieved when 11% of isopropyl alcohol was added as a cosolvent in a mobile phase. Pressure appeared to cause little influence on retention and selectivity. Found SFC method could be scaled up for preparative chromatography in order to produce pure furocoumarins for pharmaceutical industry.

INTRODUCTION

Furocoumarins are aromatic compounds produced by a variety of plant as natural phytoalexins. These chemical possess several features which allow them to prevent microorganisms' aggression. Firstly, they can penetrate into living cells and intercalate DNA. Secondly, being exposed to light these molecules move into an excited state from which they can take part in [2+2] cycloaddition by two lateral double bonds. Combination of these two qualities gives furocoumarins the ability to bind two spirals of DNA by forming covalent bonds with thymine nucleobases on both of them thus preventing cells from fission and triggering apoptosis.

Modern medicine uses furocoumarins as photoagents in so called PUVA-therapy which combines photosensibilizing chemical administration with UV-A radiation. This treatment is immensely effective against diverse skin diseases such as vitiligo, dermatitis, certain types of non-melanomic skin cancer etc. But furocoumarins possess a number of negative side-effects and should be treated with great caution. Even natural ones can be dangerous for humans and the more so do artificial synthetic ones. Since plenty of common plant products contain furocoumarins it's worthwhile to elaborate an accurate technique for their qualitative and quantitative determination. Furocoumarins can be found in a whole variety of different plant but there are especially widespread in Umbelliferae and Rutaceae family plant. So on the one hand there is a vast quantity of possible natural furocoumarin source for PUVA-therapy drugs, but from the other - one has to establish furocoumarin percentage control in plant raw materials used in food, cosmetics and perfume production. For instance, according to European Cosmetics Directive 76/768/EEC, total level of furocoumarins in any cosmetics production should be less than 1 ppm. Every method of plant raw material processing and further complex mixtures separation requires high efficiency chromatography at some stage to obtain pure individual substances. So when dealing with Umbelliferae or Rutaceae plants one might fancy a precise analytical chromatographic technique to control furocoumarin content of the product as well as a high-throughput preparative chromatography to purify furocoumarins for PUVA-therapy.

So far the main chromatographic method for solving above-mentioned tasks is high performance liquid chromatography (HPLC). Numerous amount of analytical HPLC

techniques, both in normal and reverse phase regimes, are used for furocoumarins determination in natural product species [see, for example, 1 and links thereof]. Preparative HPLC and MPLC are also used to purify individual furocoumarins on pilot and industrial scale [2]. Well-known disadvantages of these approaches are usually attributed to large volume of toxic and expensive solvents used for elution, high viscosity of the latter which doesn't allow to pump mobile phase fast enough thus making purification procedures tedious and solvent-, energy- and time-consuming. Plus by far not all traditional liquid HPLC solvents can be easily removed from gathered substances. Sometimes they can form intermolecular complexes with some furocoumarin molecules active groups thus making total residual solvent removal very difficult or even impossible. It can be easily negligible when biocompatible solvent like ethanol is used for drug production, but it becomes less welcome if the solvent in question is acetonitrile or dichloromethane. The problem of solvent residue is particularly important in the field of children cosmetics, food or drugs manufacturing. But even if solvent traces in a final substance is not a problem, large quantities of solvent wastes needed to be utilised after usage still cause troubles. A greener, less solvent consuming, ecologically and economically friendly approach of natural furocoumarin sources processing could significantly improve current situation and make PUVA-treatment more readily available for people suffering from various skin diseases.

Applicability of supercritical fluid extraction (SFE) for furocoumarin recovery from plant materials has been rather extensively explored throughout last several decades [3-7]. It has been shown that SFE with CO₂ can be just as effective in furocoumarin retrieval as chloroform Soxhlet extraction. To our knowledge, no economical evaluation of SFE as a method of choice for furocoumarin raw material processing has been published in peer-reviewed journals so far. But by analogy with specialty oils or essential oils one can assume that since furocoumarins are valuable chemicals their production by means of SFE could be meaningful. At the same time information on supercritical fluid chromatography feasibility to separate complex natural mixtures containing furocoumarins is much less available. An excellent paper by Lesselier et al [8] has been published recently providing results of a complete furocoumarin analysis of lemon residue as well as small-scale preparative separation of all major components. Among all tested sorbent types pentafluorophenyl-type ones (PFP) have been found the most suitable for sufficient resolution between this citrus oil furocoumarins which was explained by high contribution of π - π interaction into retention on such columns. Small quantities of ethanol as a cosolvent in a mobile phase (MP) were used to elute all furocoumarins from the stationary phase (SP). Influence of column type, cosolvent percentage, pressure and temperature on resolution has been investigated. An optimized gradient method allowed to separate 16 furocoumarins within 10 minutes [8].

In our previous work we've shown possibility to resolve closely related furocoumarins by normal phase SFC with Silica column and ethanol as a cosolvent [7]. Umbelliferae plant Ammi majus seeds have been used as one of the most common industrial sources of therapeutically attractive furocoumarins - bergapten, xanthotoxin and isopimpinelin . Here we present the results of further research on separation of major furocoumarins from Ammi majus by supercritical fluid chromatography.

MATERIALS AND METHODS

Materials

HPLC-grade solvents - methanol, ethanol and isopropanol - has been purchased from Sigma-Aldrich and used without any further purification. Food grade CO₂ has been purchased from

Linde Gas. Samples of Ammi majus dried seeds were provided by Dr M.V. Moshnin («Psoriasis-center», Moscow, Russia).

The following sorbents were used for SFC separations: Kromasil Silica, Kromasil CN (Akzo Nobel, Bohus, Sweden) and Viridis 2-ethylpyridine (2EP, Waters Corp., Milford, USA). All the exploited columns were of 250x4.6 mm size and were packed with 5 µm sorbent with 10 nm pores in it.

SFE

Extraction from Ammi majus seeds was conducted according to procedure described elsewhere [7]. SFE-1000 extraction system by Thar (Waters Corp., Pittsburgh, USA) was used. Briefly, dried seeds were milled with an electrical grinding machine, sieved through 1 mm Retsch test sieves and put into extraction vessel. Vessel was sealed, all pumps and heaters started to work automatically. Extraction was conducted under the following conditions: solvent - CO₂/EtOH 90/10, pressure 550 bar, temperature 40°C, total flowrate 50 g/min. Collected extract solutions in residual ethanol were degassed overnight and after that used for chromatographic analysis without any additional probe preparation.

SFC

Semi-preparative Thar Investigator (Waters Corp., Pittsburgh, USA) was used to perform analytical SFC separation of Ammi majus SFE extracts. UV detection by Gilson 151-152 UV/VIS detector was held at wavelength of 220 nm. Initial screening as well as modifier percentage rough optimization was conducted at gradient elution condition. Gradient in use was: 2% in 1 minute, 2-20% in 10 minutes, 20% in 2 minutes, 20-2% in 1 minute. Column equilibration after cosolvent change was conducted within 10 minutes. Following steps depended on particular MP-SP combination and were optimized in pursuit for maximum allowable resolution between all three major furocoumarin constituents of Ammi majus extracts.

Pressure influence has been evaluated for each MP-SP system at optimized cosolvent type and percentage. Three points for each system has been tested - 100, 150 and 200 bar.

According to authors preliminary experiments as well as results obtained by Lesellier [8] flowrate has virtually no influence on furocoumarin separation, so all the experiments has been held at a constance flowrate of 4 ml/min. Temperature also was kept constant throughout this work at 35°C.

RESULTS

Column evaluation

All three columns chosen in this study appeared to be able to provide baseline separation for three major furocoumarins from Ammi majus - bergapten (5-methoxypsoralen, 5-MOP), xanthoptoxin (8-methoxypsoralen, 8-MOP) and isopimpinellin (5,8-dimethoxypsoralen, 5,8-diMOP) (Fig. 1).

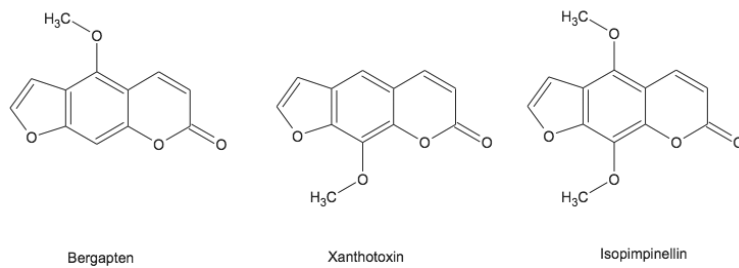


Figure 1: Bergapten, xanthotoxin and isopimpinellin - major furocoumarin constituents of Ammi majus seeds
 Figures 2-4 represent typical separation profile gathered on each column at cosolvent optimized conditions.

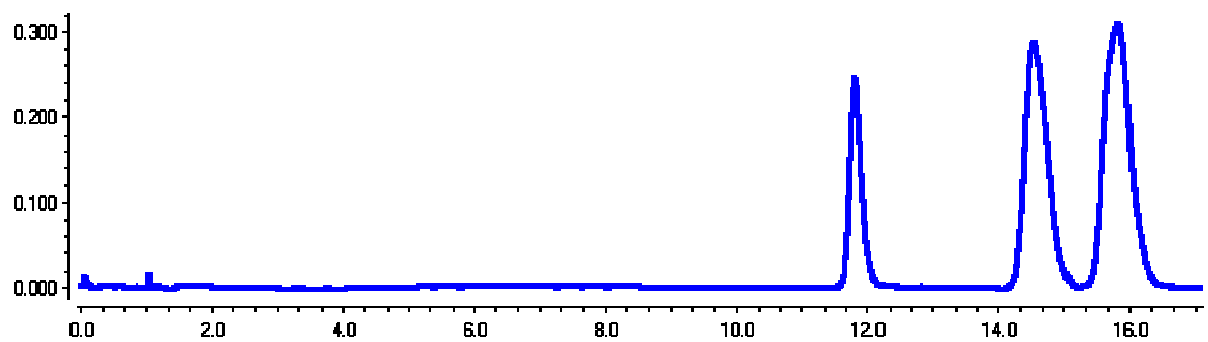


Figure 2: Separation of Ammi majus extract on Kromasil Silica

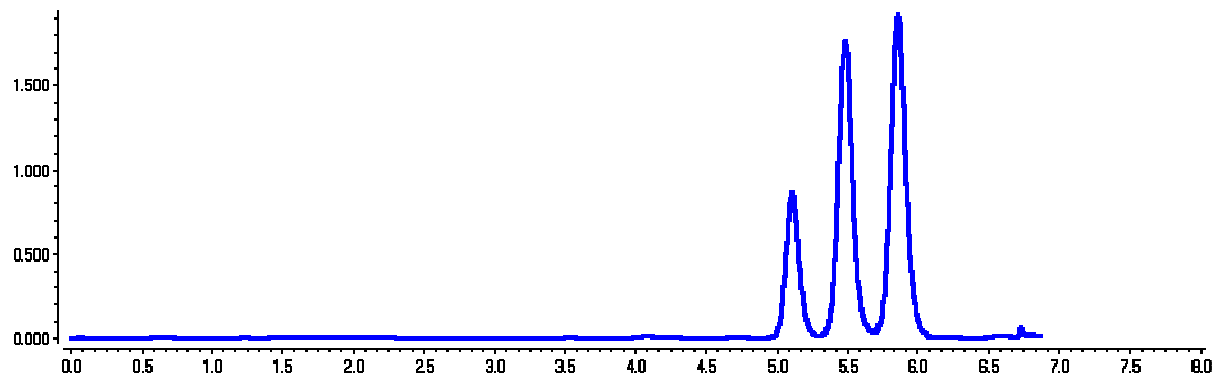


Figure 3: Separation of Ammi majus extract on Kromasil CN

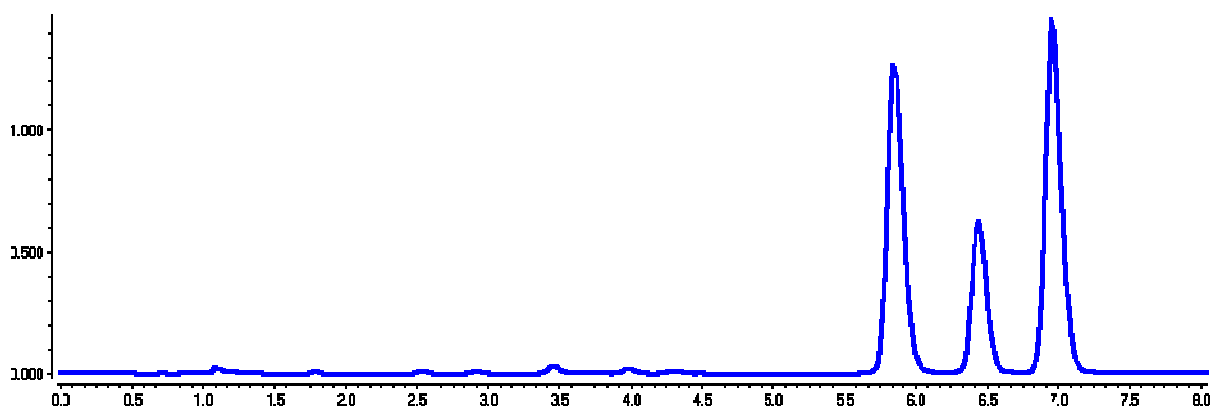


Figure 4: Separation of Ammi majus extract on Viridis 2EP

The smallest area peak on all chromatograms corresponds to bergapten, the medium one - to xanthotoxin and the largest - to isopimpinellin.

Silica appears to be the most retaining column of three tested, total elution in the optimized conditions occurs on it in more than 16 minutes against 6 min at CN and 7 min at 2EP (Fig. 2). That is not due to its truly strong retaining but due to its poor selectivity for the substances in question. Optimized conditions allowing to achieve full mixture separation on this column required a very small cosolvent percentage - only 2% of isopropanol - thus causing large retention times. Silica is also causing furocoumarin peak tailing in SFC condition. And this effect could only partly be attributed to low cosolvent volume, because tailing wasn't observed on other columns while using similar amount of modifier. This is rather intriguing because tailing of non-metal containing organic in SFC is usually attributed to their acidic or basic nature. Easy ionization of such analytes in a mobile phase leads to interaction of ionized substance with sorbent causing non Henri-like sorption and thus tailing. In case of furocoumarin which are neither organic acids nor bases we can only speculate on some kind of condensed aromatic system electron basicity as a reason for tailing. Perhaps peak tailing could be caused by simultaneous interaction of two close aromatic ring π -electron systems with two geminal silanol groups on silica. Tailing effect significantly reduced when some sufficient amount of cosolvent (above 5-10% depending on solvent type) was applied but didn't vanish. It's well established that cosolvent not only increases solvating power of mobile phase in SFC but also dynamically modifies sorbent surface, partially masking column active sites etc. [9]. So this partial shielding of geminal silanol groups by cosolvent sorption can provide an explanation for tailing decrease in larger modifier amount conditions. Further experiments with non-alcoholic cosolvent and BEH-type columns could shed a light on actual mechanisms of observed furocoumarin tailing.

CN column provides baseline separation at 6% of EtOH as a cosolvent (Fig. 3). Peak shape is symmetrical under these conditions. Although such a system look attractive on analytical scale in comparison with silica it still doesn't fit the requirements for preparative separation because selectivity is rather small even at this scale. Such a system will be difficult to scale up for preparative volume.

Separation observed when using Viridis 2EP columns as SP with 11% isopropanol as a cosolvent in MP looks the most promising of all tested SP-MP systems (Fig. 3). Unexpectedly, peak elution order on 2EP differs from CN and silica columns - isopimpinellin elutes first instead of bergapten. That order does not vary depending on cosolvent used. Detailed explanation of this phenomenon may require some additional experiments but to date we can say that 2EP column seems to provide several retention mechanisms for furocoumarin possibly involving π - π interaction. 5,8-dimethoxypsoralen possesses more sterically hindered aromatic ring system than 5- and 8-methoxypsoralens so close «flat» approach to pyridine ring of 2-ethylpyridine groups may be more difficult for it.

Cosolvent screening

Figures 5-7 represent the results of cosolvent screening on three columns.

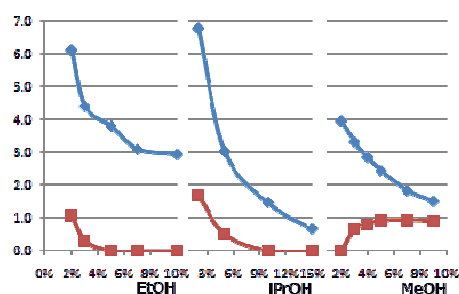


Figure 5: Cosolvent screening on Kromasil Silica

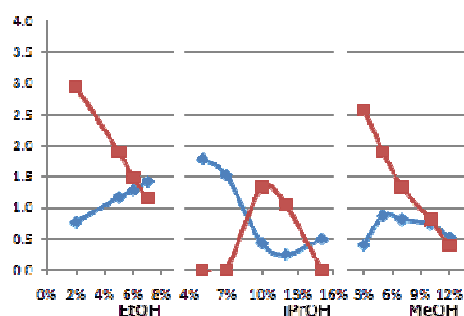


Figure 6: Cosolvent screening on Kromasil CN

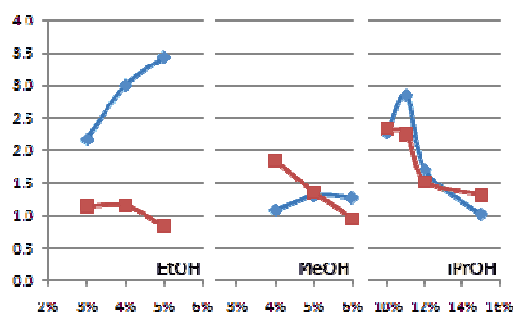


Figure 7: Cosolvent screening on Viridis 2EP

Blue diamonds on all charts on figures 5-10 represent resolution coefficients between first and second eluting peaks on the chromatogram, red squares - between second and third. Evidently cosolvents have complicated influence on furocoumarin separation process. Resolution behaviour on silica column using EtOH and iPrOH as cosolvents can only be called monotonous and predictable. In every other system change in modifier percentage causes differently directed changes in retention and resolution of three main components. Sometimes the modifier effect on retention isn't even monotonous (CN-iPrOH, CN-MeOH, 2EP-iPrOH). It could be explained by removal of modifier layer at the surface of SP in case of CN-MeOH because the graph extremum corresponds to very low cosolvent percentage in MP. Thus if under these conditions the amount of MeOH adsorbed on CN column isn't the same as at higher concentrations - basically we deal with different stationary phases which can retain substances by different mechanisms. But this does not apply to CN-iPrOH and 2EP-iPrOH because cosolvent concentration at extremum points is sufficient enough to effectively adsorb on the column. These effects of type and concentration of modifier aren't huge but they are clearly visible and reproducible. Further examination possibly involving not only SP-MP but also MP-analyte interaction evaluation is required to provide explanation of the observed phenomena.

Pressure influence

Figures 8-10 represent the results of pressure influence evaluation on each SP-MP system at optimized conditions.

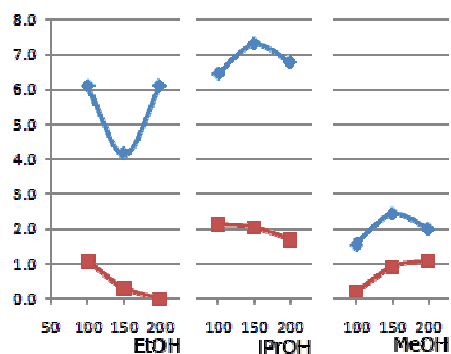


Figure 8: Pressure influence on separation on Kromasil Silica

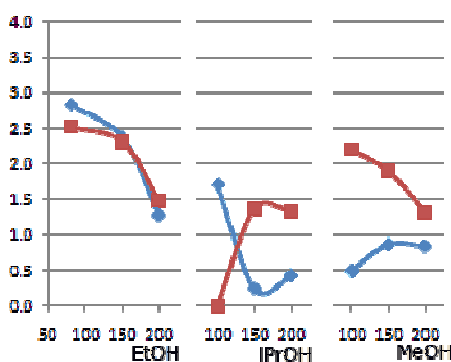


Figure 9: Pressure influence on separation on Kromasil CN

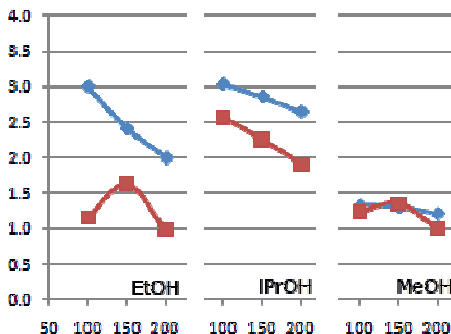


Figure 10: Pressure influence on separation on Kromasil Silica

Generally influence of pressure on resolution of three major furocoumarins from *Ammi majus* is smaller than influence of cosolvent type and concentration. Although in some cases the apparent effect seems to be significant (Silica-EtOH, CN-iPrOH), pressure mostly affects separation efficiency (peak width) than selectivity. Nevertheless it was shown that in most cases 100 bar was the optimum pressure value for getting the best possible separation.

CONCLUSION

Feasibility of normal phase supercritical fluid chromatography to separate three main furocoumarin constituents of Umbelliferae plant *Ammi majus* has been shown using three different columns and traditional alcohols as cosolvents in mobile phase. Retention mechanisms seem to be complicated and not fully understood on all three columns in use.

Observed peak tailing on silica column could be partially explained by aromatic π -electron interaction with acid protons of geminal silanol groups. 2-ethylpyridine phase showed the most promising results while using 11% of isopropyl alcohol as a cosolvent. Fractionation obtained with this SP-MP system should be easily scalable to the preparative scale and used for furocoumarin retrieval from natural source.

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