# ISOLATION OF ISOFLAVONES FROM RED CLOVER *(TRIFOLIUM PRATENSE)* BY MEANS OF SFE

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**Abstract :**

The aim of this work was to produce continuously isoflavones from raw ethanolic red clover extract. Batch and counter-current (CC) supercritical fluid extraction (SFE) has been studied in the development of such a process. It has been shown that isoflavones Biochanin A and Formononetin can be obtained at high selectivity from raw material, using a continuous SFE at moderate pressure and temperature with 5 – 7 % EtOH as modifier.
Following steps were involved in this study :
1) Parameter variation in a batch extraction plant of 4L capacity to study the effect of temperature, pressure, modifier and its concentration on the extraction process,
2) Experiments in a 2,2m lab scale CC-SFE plant to study the continuous operation.

## I INTRODUCTION :

Red clover (*Trifolium Pratense*) is a member of the Family Leguminosae. It is one most talked about material as a source of isoflavone.
Red clover contains isoflavones Biochanin A and Formononetin in the concentration range of 0.1%-0.9% in the dry forage. Minor isoflavonoids, below 0.08% in the dry forage, are Genistein, Genistin, Daidzein, Daidzin, Pratensein, Pectolinarigenin, Calycosin, Trifoside, Pseudobaptigenin and Isoquercitrin [1]. Red clover extracts containing total isoflavones of 2.5%, 8%, 18% are available on the market.

## II. STATE OF THE ART

Bibliographic research has revealed that the extraction of isoflavones is practised mainly from two natural substances : soybean and red clover. The extraction of isoflavones from red clover is performed in industry with conventional extraction methods and conventional organic solvents, including ethanol, methanol and chloroforme.
A process of extracting isoflavones from the red clover using water-ethanol as a solvent is described by [2]. In this process red clover plant material is chopped and extracted with a mixture of water-miscible organic solvent and water. Sixty percent ethanol in water is used for this purpose. Extraction is carried out at 90° C for twenty-four hours. The supernatant is separated from non-dissolved plant material, and solvent removed by distillation. The residue comprising water, water-soluble components and non-water soluble components is extracted with a non-water-miscible organic solvent (petroleum ether), followed by removal of the aqueous phase containing water-soluble components. Removal of organic solvent by distillation (or drying under vacuum) gives a tar-like residue, which can be dried to give a powder, or which can be dissolved in organic solvent to give an isoflavone containing extract.

SFE at moderate temperatures could hence be a possible alternative for isoflavone extraction, avoiding the switch of solvents from ethanol-water to petrolether. However, there is no reference of SFE being employed for the commercial production of isoflavones. [3] studied the extractability of isoflavones using supercritical carbon dioxide. In their work they focused on the extraction of synthetically produced Daidzein, Genistein, Formononetin and Biochanin A. The extractability of various isoflavones under different conditions is as summarized below.

***Table 1 :*** *Extractability of selected isoflavones from a model mixture [3]*

| **Experimental condition** | **Isoflavones extracted** |
|---|---|
| A) 400 atm., without modifier | Formononetin, Biochanin A |
| B) 400 atm., 5% Chloroform | Formononetin, Biochanin A |
| C) 400 atm., 5% Methanol | Genistein, Formononetin, Biochanin A |
| D) 600 atm., 20% Methanol | Daidzein, Genistein, Formononetin, Biochanin A |
| E) 600 atm., 20% Ethanol | Daidzein, Genistein, Formononetin, Biochanin A (->93%) |

From above it can also be noted that Biochanin A and Formononetin are the most easily extractable isoflavones, as well as ethanol is the best modifier for isoflavone extraction among the modifiers used above.

## III: Materials and Methods

The raw red clover extract has been produced according to the standard of General Extract Products company, Flensburg, Germany. The raw red clover extract is a dark tan color liquid with density of 1250 kg/m$^3$ and is highly viscous. This raw red clover extract is soluble in a mixture of ethanol and water (75:25 v/v) but not soluble in pure ethanol. The carbon dioxide has been furnished from KWD Hydrogas, Neuwied, Germany, the purity of the $CO_2$ used is more than 99.995%. Analytical grade ethanol (from SIGMA) was used for the preparation of samples.
For this work the method given by [1] was used for the analysis, as it exclusively deals with the analysis of isoflavones from the red clover extract. The conditions for HPLC as suggested by the authors are an HP ODS Column, Hypersil, 5 µm, 200 x 2.1 mm, solvent is water/ methanol , linear gradient elution with 0 –100% methanol in 30 min, flow is 0,2 ml/min at 45 °C, detection at UV $\lambda$ = 260 nm. The evaporation of the modifier from the extract was done using flash evaporator.

The effect of various parameters on the extraction of isoflavones was studied in the Batch Supercritical fluid extraction pilot plant.
The Batch Pilot consists of an autoclave (4L) capacity, which is heated by a jacket, a separator of 2L capacity also heated by jacket, two cyclone separators and a compressor. All the parts in contact with process fluid are of stainless steel material. There is a provision to add a modifier stream into the extractor inlet, using an HPLC pump. The extracted components along with the $CO_2$ and modifier leave the extractor and are expanded, due to which the extracted components are collected in the first cyclone, the second cyclone and in the two-liter separator.

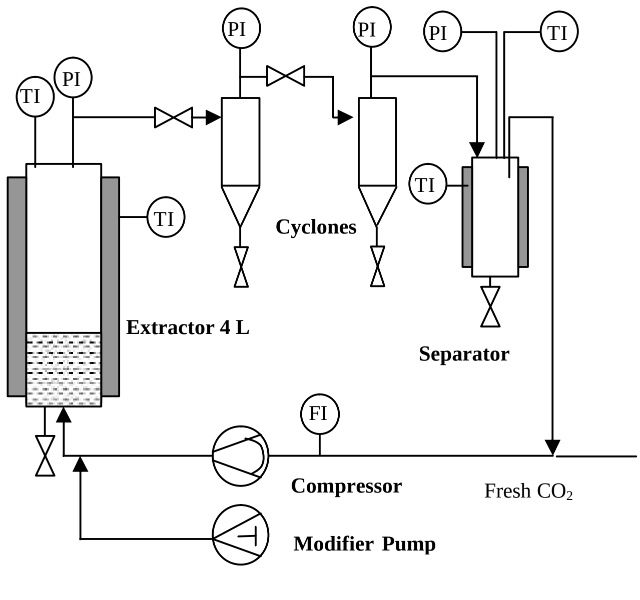


***Figure 1 :*** *Batch SFE Pilot Plant*

The $CO_2$ flows back to the compressor suction and is compressed back to the desired pressure. The temperature required for the extraction is maintained by oil heating through the jacket of the extractor.

The schematic diagram of the CC-SFE plant is as shown in figure 2. It consists of a separation column of 25 mm internal diameter with EX packing of Sulzer make. The column is made of stainless steel. There are two packed sections each of 1.1 m height. The raw red clover extract is mixed with ethanol water mixture of composition 75%:25% by volume and added to the feed reservoir. The feed can enter the column from the top or from the middle. If the feed is added from the top, the total height of the packed section (2.2 m) is utilized for the mass transfer.

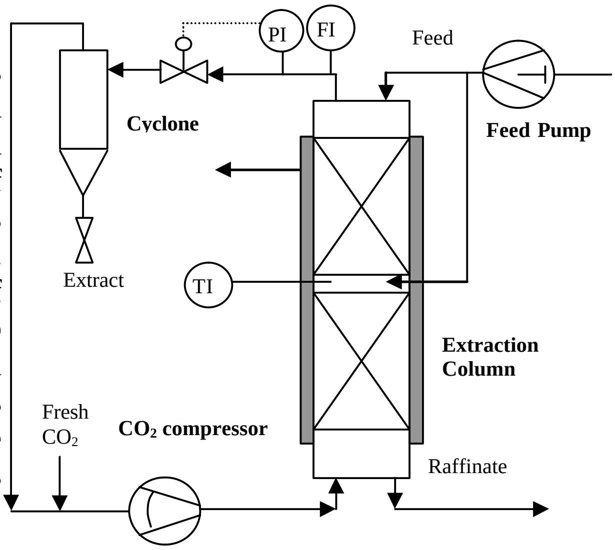


***Figure 2 :*** *CC- SFE Laboratory Plant*

The plant operates in a compressor mode. $CO_2$ is circulated through the column from bottom to top using the $CO_2$ compressor. The extract along with $CO_2$ and modifier is recovered from the top of the column, depressurized and subsequently separated in the cyclone separator. The $CO_2$ is recycled back to the suction of the compressor and recompressed. The PIC device controls the pressure in the column with control valve located at the downstream of the extraction column.

## IV. Results and Discussion

### IV.1. Batch Extraction

The parameter variation is done to study the effect of various parameters like pressure, temperature, modifier and its concentration on the extraction process. The following diagrams (Figure 3) illustrate the influence of process parameters on total yield. The standard parameters are : P = 180 bar, T = 60 °C, Solvent/Feed ratio 0,27g./min.g, Modifier Content ist 5%.

From figure 3 a) it can be seen that the increase in pressure from 130 bar to 210 bar causes an increase in the solubility of the extractable components. This effect is due to the increase in the density of supercritical fluid solvent. From figure 3b) it can be seen that the increase in the temperature of extraction causes a decrease in the extraction yield. The pressure under consideration is 180 bar and the temperature is in the range of 45 to 70° C. Comparison of overall yield at the specific solvent consumption of 80 g $CO_2$/g feed input reveals that the cumulative yield increases from 2.4% to 3.3% as the extraction temperature is lowered from 70 to 45°C. It can be deduced that the influence of lower density overlaps the effects of higher vapor pressures at increasing temperature.

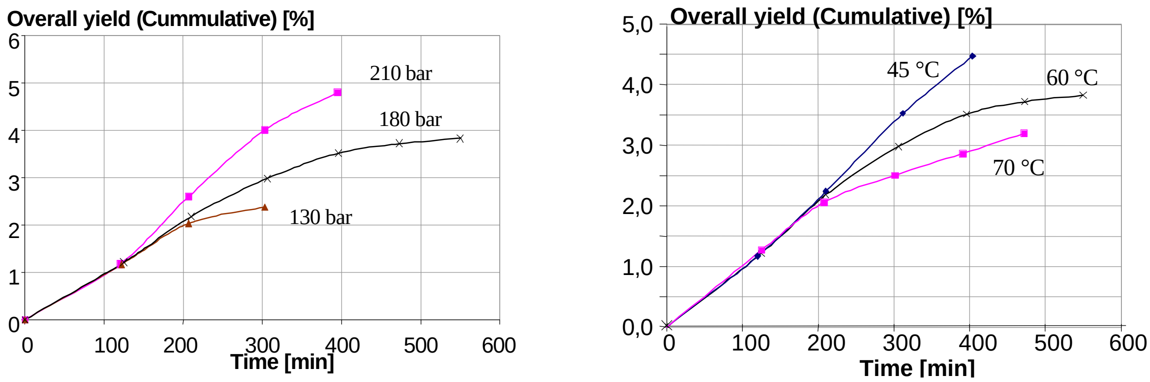


***Figure 3 a)b):*** *Influence of Pressure and Temperature on cumulative overall yield (5%EtOH, S/F = 0,27 g/min.g)*

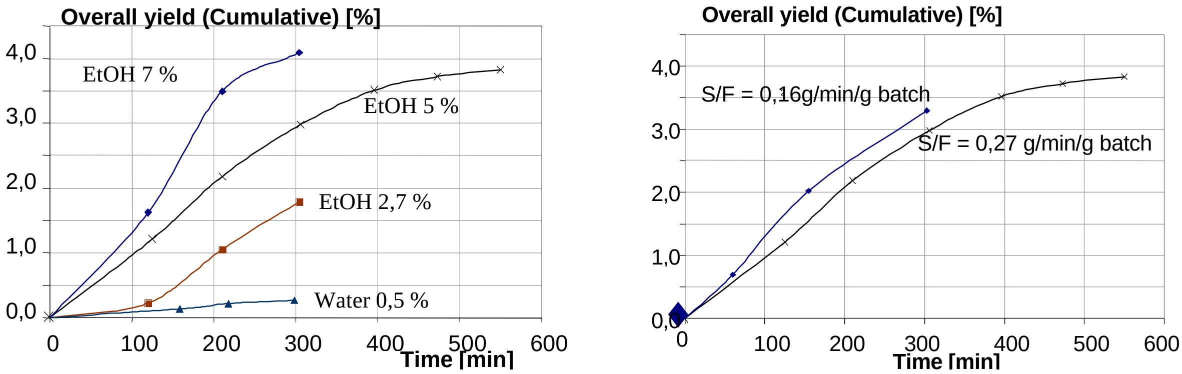


***Figure 3 c) d):*** *Influence of modifier and Solvent/Feed ratio on cumulative overall yield (P= 180 bar, T = 60 °C)*

From figure 3 c) it can be seen that the increase in the concentration of modifier (Ethanol) from 2.7% to 7.2% causes an increase in the solubility of the extractable components. The extraction yield without the modifier is very low (not shown in this report). In general, a modifier that has a lower critical temperature than the supercritical gas causes decrease in the solubility of a low volatile component in the gas whereas, a modifier with higher critical temperature causes an increase in the solubility.

The solvent/feed ratio shows only marginal influence on the overall extraction curve. A lowe S/F gives higer yields in a defined run time, this means that at given pressure and temperature the extraction seems to be a diffusion controlled one.

The raw red clover extract and the extracts obtained from SFE were analyzed for their isoflavone content . The results are in line with the findings in the literature mentioned under [3]. A systematic comparison of extract diagrams leads to the result that the enrichment of Biochanin A is in the range of 10 to 25, whereas higher pressure, lower temperature and higher modifier content favor the enrichment. The same behavior is given for Formononetin with a range of enrichment between 5 and 20.

Biochanin A and Formononetin are the most easily extractable isoflavones as cited in the above literature.

The other iso-flavones of aglycone form e.g. Daidzein, Genistein contains an additional polar –OH group in their structure as compared to methyl group in Formononetin and Biochanin A.

Their isolation would require pressure more than 400 bar and modifier conc. of more than 20% as cited in the literature [3].

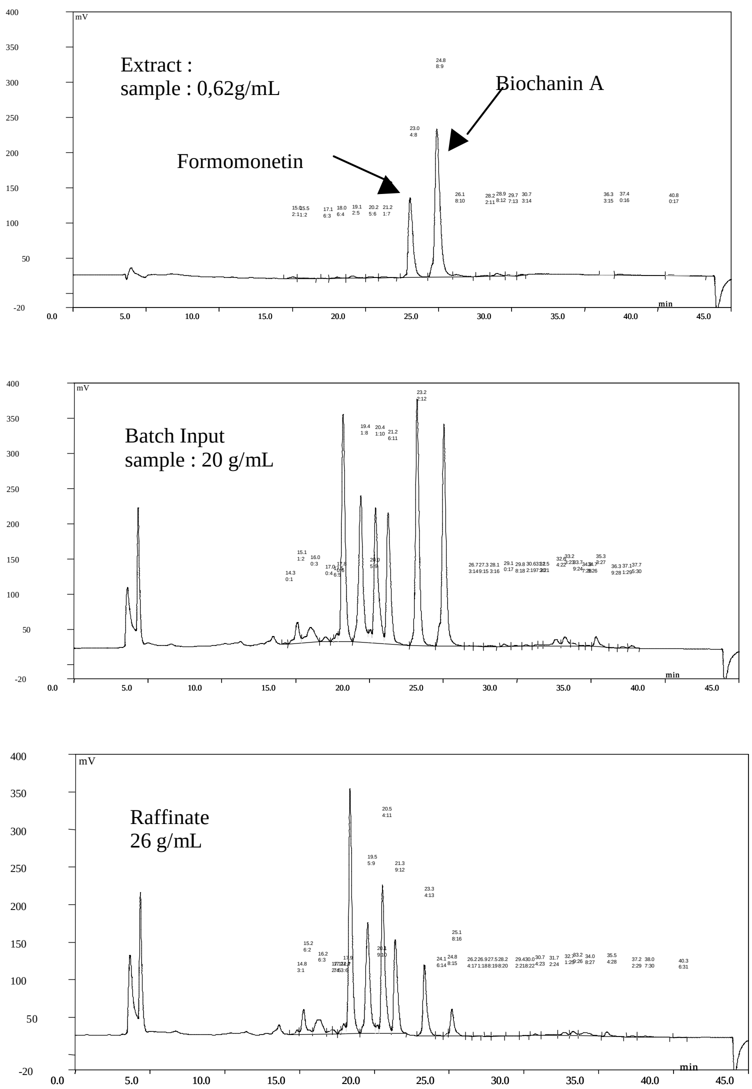


***Figure 5 :*** *Chromatograms of Red Clover (extract, feed, raffinate)*

In order to obtain isoflavone rich extracts continuously, the experiments were performed on Lab Scale CC-SFE. The reference conditions selected for extraction were 180 bar and 60°C.

The experiments were performed with variation of the gas flow rate and liquid (feed) flow rate respectively thereby changing the solvent to feed ratio (S/F). Also the effect of increase in the height of stripping section on the extraction was studied. Enrichment was calculated with the use of the chromatograms of the process output. The process parameters gas flow, the liquid (feed) flow rates and the height of stripping section used are concluded in table 2.

***Table 2:*** *Enrichment of isoflavones in the extract for various experimental conditions*

| Height of Stripping section [m] | Liquid flow [ml/min] | Gas flow [g/s] | Overall yield | S/F ratio [-] | Enrichment in extract | |
|---|---|---|---|---|---|---|
| | | | | | Biochanin A | Formononetin |
| 1.1 | 6.4 | 2 | 2,3 | 19 | 15 | 10 |
| 1.1 | 6.4 | 2.24 | 2,6 | 21 | 18 | 13 |
| 1.1 | 2.5 | 2.24 | 2,4 | 55 | 10 | 7 |
| 2.2 | 2.5 | 2.24 | 2,5 | 55 | 12 | 9 |

The results confirm that the isoflavones Biochanin A and Formononetin can be continuously extracted from an ethanolic red clover solution via CC- SFE.

## V. Conclusion

In this work the isolation of isoflavones from the liquid red clover ethanol extract using supercritical fluid technology was studied. From the experiments done on the Batch SFE plant it can be concluded that the enrichment of isoflavones Biochanin A and Formononetin can be obtained in the extract using supercritical $CO_2$ with ethanol as a modifier. The yield of these isoflavones increases with the increase in pressure, increase in ethanol concentration and decrease in temperature. The increase is more appreciable at a pressure greater than 180 bar, ethanol concentration greater than 5% and temperature less than 60°C. Water as a modifier is unable to effect the separation/enrichment of isoflavone from the raw red clover extract.

Out of many different types of isoflavones identified in the red clover, only isoflavones Biochanin A and Formononetin could be enriched with the pressure and modifier concentration used. The isolation of other isoflavone aglycone like Daidzein, Genistein etc would require use of higher pressures and modifier concentrations.

The isoflavones glycosides like Daidzin, Genistin and Sissotrin etc. are not extractable using Supercritical $CO_2$ or with modifier. To effect their separation using supercritical extraction, their conversion to aglycone form is necessary.

The experiments done on the CC-SFE plant demonstrated the possibility of obtaining isoflavones Biochanin A and Formononetin continuously from the raw red clover extract. These isoflavones can be obtained in a strongly enriched concentration using a short and simple stripping column, or even in a highly pure form in a countercurrent column provided with reflux.

## References :

[1] Xian-guo He, Long-Ze Lin, Li-Zhi Lian, Analysis of flavonoids from red clover by liquid chromatography- electrospray mass spectrometry. J Chromatography A, 755, 127, 1996.

[2] Thurn M J, Huang Li Jiu. Methods for treating cancer with legume plant extracts. USP 6004558, 1999.

[3] Chandra A, Nair Muraleedharan G. Supercritical carbon dioxide extraction of daidzein & genistein from soybean products. Phytochemical analysis, vol 7, 259, 1996.