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HPLC ANALYSIS OF *TRIGONELLA FOENUM-GRAECUM S*EEDS TO ASSESS PHYTOESTROGENS

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ABSTRACT

Phytoestrogens are compounds synthesized in plants which mimic steroidal estrogen activity in mammals. *Trigonella foenum-graecum* (L.) (Fabaceae, Fenugreek) is employed in many traditional systems as an antibacterial and antidiabetic agent, gastric stimulant and galactogogue and also shows estrogenic, antidiabetic and anti-invasive activity. The present study report the separation of phytoetrogenic components of three different classes – isoflavonoids (genistein and daidzein), lignans (secoisolaricoresinol, metairesinol) and coumestrol from fenugreek seeds before and after alkaline hydrolysis. For this an HPLC system with a C-18 analytical column (100 x 4.6 mm , 5 micron) and detector of UV @ 254nm were employed. The mobile phases contained water/acetonitrile/acetic acid. The limit of detection for isoflavones is 1 µg/g, and for lignans and coumestrol 2.5 µg/g. The results pointed the presence of isoflavones (daidzein-18.2ppm, genistein-11.8ppm) and lignans (secoisolariceresinol-283.6ppm) unhydrolysed and presence of isoflavones (daidzein-100.9ppm, genistein-56.1ppm), lignans (secoisolariceresinol-1893ppm) and coumestrol (170ppm) in hydrolysed fenugreek seeds. Alkaline hydrolysis helped in better separation and quantification of phytoestrogens. High pressure liquid chromatography (HPLC) has been found to be an efficient and sensitive method for identification and quantification of different classes of phytoestrogens-isoflavonoids, lignans and coumestrol.

Key words: Phytoestrogens, Fenugreek seeds (*Trigonella foenum-graecum)*, High Performance Liquid Chromatography (HPLC), Isoflavones, Lignans, Coumestrol.

INTRODUCTION

Trigonella foenum-graecum (L.) (Fabaceae), commonly known as Fenugreek, has three culinary uses: as a herb (dried or fresh leaves), as a spice (seeds), and as a vegetable (fresh leaves, sprouts, and microgreens). Gujarat is one of largest producer of this herb in India. Most studies on *Trigonella foenum-graecum have focused on* antipyretic, anthelmintic, antileprotic, antibronchitic, carminative and anti diabetic properties (Shailajan *et.al.*, 2011, Mehrafarin *et.al.,* 2011 and Fatoppo *et.al.,* 2009). Phytoestrogens have been reported to have many beneficial properties, especially in hormone-related conditions, cancer and coronary heart disease, osteoporosis, and menopausal symptoms. Exogenous estrogens or hormone replacement therapy (HRT) is in use to improve the menopausal symptoms, but long term side effects of HRT can lead to breast cancer, changes in the intrauterine lining, irregular bleeding etc. The situation thus calls for an increasing demand of herbal approach as an alternative. The phytoestrogens are now gaining interest as an alternative for hormone replacement therapy (Sreeja and Anju, 2010). These plant compounds with estrogenic or antiestrogenic activities comprise three major classes: isoflavones, lignans, and coumestans. The phytoestrogens are ingested in their natural beta-glycosidic forms, which are hydrolyzed to their aglycones in the intestine, absorbed, and then glucuronidated in the intestinal wall. The major circulating forms of the isoflavones are the glucuronidated metabolites (Setchell et.al., 2001). Lignans are as important as isoflavones contributing approximately equal proportions to total phytoestrogen levels (Gunter *et.al.,* 2008).

From the various chromatographic methods available for the analysis of phytoestrogen from food, an HPLC technique is found to be very easy, sensitive and reliable method for the primitive identification and quantification of the compound [(7)]. The present study aims to identify and quantify the phytoestrogens present in *Trigonella foenum-graecum* (fenugreek) and also efforts are made to develop a method for assessment of phytoestrogenic components from all three classes using a single assay approach. The method was the modification

of methods suggested by K. J. Lee *et.al*, 2008; Xin Li *et.al*, 2008 and C Eliasson *et.al*, 2003.

MATERIAL AND METHODS

MATERIALS

All solvents used for HPLC and optical density readings were of analytical or HPLC grade, vacuum-filtered through a 0.45 µm filter. HPLC grade water and acetonitrile were purchased from Fisher Scientific Company. The standard chemicals of daidzein 98%, genistein 100%, secoisolariciresinol 95%, and coumestrol 97.5% were purchased from Sigma Aldrich. Co. (USA).

HPLC ANALYTICAL CONDITIONS

The details of the instrument used were Merck Hitachi HPLC (Lachroma model) L-7100 pump with UV-VIS L-7420 detector having D-7000 interface. The C_{18} column (Chrombudget-Bischoff Chromatography, A German Company) of 0.46x100 cm long having 5 micron was used for the separation. The mobile phases were composed of water, acetonitrile and acetic acid. The adjustable experimental variables were the conditions of gradient modes and mobile phase compositions. The mobile phase A consists of water/acetonitrile/acetic acid (94.9/5/0.1 v/v/v) and mobile phase B consisted of water/acetonitrile/acetic acid (5/94.9/0.1 v/v/v). The flow rate was kept at 1 ml/min, the injection volume was 20 µl with following step wise gradient over 25 minutes- where 100% of mobile phase A ran for 15minutes; from 15 to 20 minutes where mobile phase changes from 0% to 70% and mobile phase A changes from 100% to 30%; and finally during last five minutes mobile phase A again changes to 100%.Phytoestrogens were detected using UV absorbance at 245 nm. Optimisation of HPLC conditions as a standard procedure was carried out prior to analysis.

STANDARD SOLUTIONS

The standards for each phytoestrogen (Daidzein, Gensitein, Secoisolariciresinol, and Coumestrol) were dissolved in 60% ethanol to prepare a stock solution of 1000 ppm. Working standard solutions were prepared by serial dilution of the stock standard.

SAMPLE PREPARATION

The dry seeds of *Trigonella foenum-graecum* were procured from local market of Vadodara (Gujarat, India). The seeds were ground into fine powder using a grinder and resulting sample was used for further analysis. The extraction was carried with the help of 60% ethanol. The resultant mixture was sonicated for 4-5 minutes and allowed to stand for 40 minutes. The supernatant was collected and filtered before injecting into HPLC system.

ALKALINE HYDROLYSIS

For alkaline hydrolysis, 0.5 g powder was treated with 25 ml 2M NaOH, for 1 hour at room temperature, followed by acidification step with 40% H_2SO_4 to adjust for pH 3. The extraction was done with the same procedure as described earlier. All the analysis was carried out in duplicates. The values were expressed in µg/g (ppm).

RESULTS AND DISCUSSION

Different methods are available for analysis of phytoestrogens which have their own advantages and limitations. There are many appropriate methods for identification of unknown polyphenol phytoestrogens using HPLC, GC-MS and LC-MS. HPLC is relatively rapid, easy to perform, cost effective and reliable method for the routine analysis of phytoestrogens (Wilkinsona et.al., 2002). In the current study HPLC was used and conditions were optimized to achieve baseline resolution between all analytes while keeping an adequate run time of 25 minutes Lignans are compounds, derived from plants, possessing a 2,3-dibenzylbutane skeleton and are associated with dietary fibre. The main dietary lignans which was studied in the current context are- secoisolariciresinol and metairesinol which are transformed by human intestinal bacteria to the mammalian lignans- enterodiol and eneterolactone. A dose related inhibition of cell proliferation and cytokine production which in turn resulted in decrease tumor necrosis factor-α was reported when peripheral blood lymphocytes were treated with enterodiol and eneterolactone (Corsini et.al., 2010). The studies reported the degradation of secoisolariciresinol due to acid hydrolysis, though the type of food and hydrolysis time also matters for this degradation. The stability of lignans due to acid hydrolysis has recently been questioned (Toure and Xueming, 2010). Therefore in the present study we used alkaline hydrolysis as an alternative option.

Calibration curves were plotted for all standard solutions of concentration ranges from 0.1µg/g to 100µg/g. The instrument limit of detection was different for each analyte: for isoflavones (daidzein and genistein) 1µg/g and for lignans (secoisolariciresinol) and coumestrol it was 2.5µg/g. The individual concentrations at which the analytes showed the best peak resolution with their respective retention time which are depicted in the Table 1 and Figure 2. Under the same experimental conditions, the chromatograms of sample Trigonema foenum seeds are shown in Figure 3 (a) for non hydrolysed extract and (b) for alkaline hydrolysed extract.

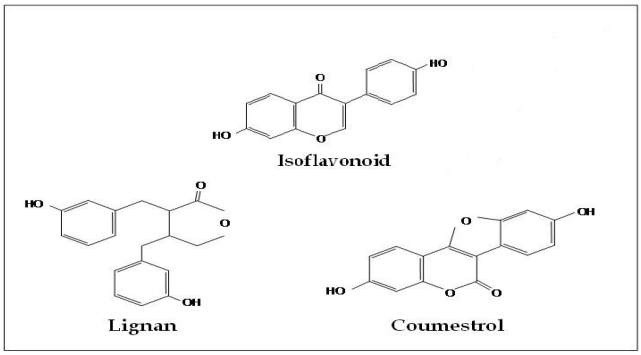

Fig. 1 Structure of phytoestrogens: Isoflavone, Lignans, Coumestrol

The HPLC analysis revealed the presence of isoflavones: daidzein-18.2ppm; genistein-11.8ppm and lignans: secoisolariceresinol-283.6ppm in non hydrolysed extract. The presence of isoflavones: daidzein-100.9ppm; genistein-56.1ppm; lignans: secoisolariceresinol-1893ppm

and coumestrol -170ppm in hydrolysed extract of fenugreek seeds. Details are provided in Table 2. Various studies reported for the isoflavones estimation, used non hydrolysed sample [13, 14] or used acid hydrolysis [15, 16] of the sample for the isoflavones estimation, but the current study reported the improvement in quantification of isoflavones following alkaline hydrolysis. The reason could be in plants the phytoestrogens exist along with their glycosidic conjugates and complex forms, where alcoholic hydrolysis helps to convert them into aglycon forms. The study showed that from total phytoestrogen content of Trigonella *foenum-graecum* , the lignans was high in concentration.

Table 1: Standards' LOD (limit of detection) and retention time for HPLC analysis

Sr. No.	Compound	Concentration (µg/g) showed best resolution	Mean Retention Time (minutes)	Standard Errorr of mean for Retention Time	Area	Limit of detection (µg/g)
1	Daidzein	10	14.17	0.33	1262279	1
2	Genistein	25	16.06	o.35	2393157	1
3	Coumestrol	25	16.71	0.33	1978038	2.5
4	Secoisolariciresinol	25	12.53	0.37	77341	2.5

Fig. 2 HPLC chromatograms for standards: Daidzein, Genistein, Secoisolariciresinol and Coumestrol

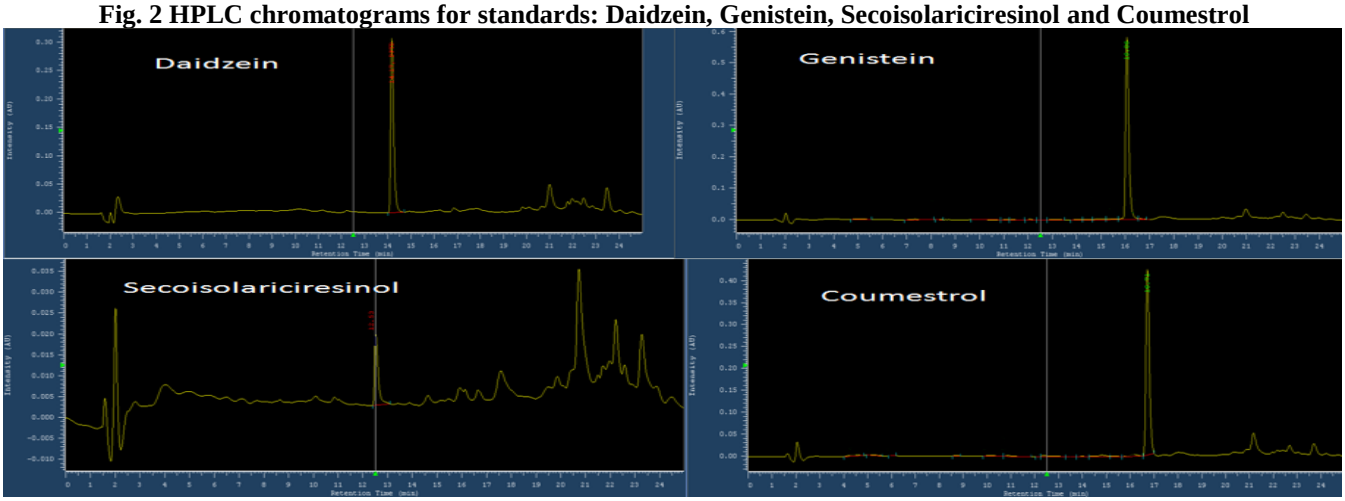

Table 2: Concentration of various phytoestrogen components of sample *Trigonella foenum-graecum*

Sr. No.	Sample condition	Retention time (minutes)	Area	Identified Component	Concentration (µg/g)
1.	Non hydrolysed extract	13.94	50685	Daidzein	18.17
		16.18	23029	Genistein	11.79
		12.39	18865	Secoisolariciresinol	283.66
2.	Alkaline Hydrolyzed extract	13.99	272359	Daidzein	100.96
		15.95	109554	Genistein	56.07
		12.65	125827	Secoisolariciresinol	1893
		16.83	281924	Coumestrol	170.12

CONCLUSION

The phytoestrogens were extracted effectively by alkaline hydrolysis followed by HPLC-UV determination. The proposed method is found to be a rapid, efficient method which requires a simple sample preparation for the quantification of phytoestrogens. The *Trigonella foenum-graecum* seeds contain considerable amount of isoflavones, lignans and coumestrol. These results can serve as a basis for estimating the amount of phytoestrogens in *Trigonella foenum-graecum (fenugreek)* so as to quantify the exact amount of phytoestrogens consumed.

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