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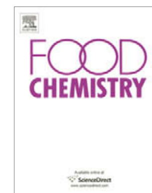

Analytical Methods

Estimation of aroma precursors in radiation processed fenugreek

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Bound volatile compounds of fenugreek were isolated and identified. The glycoside profile was dominated by phenyl glucopyranoside accounting for 90% of the total glycosides. The content of this glycoside as estimated both by high performance liquid chromatography (HPLC) and thin layer chromatography (TLC)-densitometry was found to be approximately 0.7 mg gm^{-1} of fenugreek. Gamma-radiation processing resulted in dose dependent break down of phenyl glucopyranoside with a reduction by almost 30% at a dose of 10 kGy. A corresponding increase in phenol content with dose was also observed in the steam volatile oil of the irradiated spice. Based on pulse radiolytic studies the mechanism of radiolytic cleavage was shown to occur via a carbon centred radical. Estimation of absorbed dose based on phenol released during radiation processing is proposed herein. The method is simple and rapid and could estimate absorbed dose in the range of 2.5–10 kGy within an error of 15%.

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1. Introduction

Bound volatile constituents such as aroma glycosides are an important class of nonvolatile precursors that are ubiquitous in plant kingdom (Sarry & Günata, 2004; Winterhalter & Skouroumounis, 1997). They are currently gaining increased interest and attention for their role in imparting unique aroma to plant derived foods (López, Ezpeleta, Sánchez, Cacho, & Ferreira, 2004; Sarry & Günata, 2004; Sánchez Palomo, Pérez-Coello, Díaz-Maroto, González Viñas, & Cabezudo, 2006; Winterhalter & Skouroumounis, 1997). They exist mainly as mono and diglycoside derivatives (Winterhalter & Skouroumounis, 1997) and rarely as trisaccharide glycoconjugates. These compounds have been isolated and characterised in several food stuffs where their contribution to the characteristic aroma has been established. Most of the studies on this class of compounds relate to the isolation and characterisation of hydrolytically released aglycones. Very few reports exist on the stability of these compounds during post-harvest processing and storage.

Amongst the newer non-thermal methods for post-harvest hygienization of food, radiation processing using gamma radiation/electron beam occupies a unique position (Raso & Barbosa-Canovas, 2003). Extensive studies have established the efficacy of this process as a safe method for preservation of food without producing any organoleptic changes at the recommended doses (Diehl, 1995). With a ban on chemical fumigation world over due to its adverse effects on human health and environment, processing of food by gamma radiation/electron beam has attained greater significance in recent years (Thakur & Singh, 1994).

Amongst the food products that are amenable to radiation processing, spices occupy a prime position. They are an important class of food commodity widely traded for their unique aroma and flavour. Fenugreek is an important commercial spice extensively used for its flavouring and pharmacological properties. The aroma of the spice is mainly contributed by its steam volatile essential oils. Sotolon (3-hydroxy-4, 5-dimethyl-2(5H)-furanone) a hemiterpenoid γ-lactone has been identified as the character impact compound of the spice (Sauvare, Petit, Baissac, & Ribes, 2000). Bound aroma precursors in several spices have been extensively investigated. However, no report exists on the aroma precursors of fenugreek.

Changes induced by ionising radiation at the doses approved for food applications are minute and rarely specific to the treatment. This makes identification of the treatment in the processed product a challenging task. A significant amount of research work in this field has been carried out (Delincee, 1998). Methods proposed, however, cannot be applied for routine field application. Development of a simple and rapid method for detection of radiation processed food is thus an urgent need. We have earlier reported a dose dependent decrease in glycosidic precursors of nutmeg during radiation processing. Monitoring decrease in these constituents was proposed as a method for detection of the irradiated spice (Arul, Variyar, & Sharma, 2006).

The present study thus aims at isolation, identification and estimation of major glycosidic precursors in fenugreek. Effect of radiation processing on the stability of this important class of precursor molecules will be investigated. Possibility of monitoring

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specific compounds released from bound glycosidic conjugates into the steam volatile constituents as an aid in identifying radiation processed fenugreek will be examined.

2. Experimental

2.1. Materials

Three separate lots of dry fenugreek seeds were procured from a local market. Each lot was divided into two sets. One set was kept as non-irradiated control sample. The other set was subjected to gamma radiation in air in a cobalt-60 Food Package Irradiator, MDS Nordion Int. (Kanata, Ontario, Canada) at a dose rate of 10 Gy min^{-1}. Absorbed doses between 2 and 10 kGy were given to the samples. Dosimetry was performed by a Fricke dosimeter (Fricke & Hart, 1966, Chapter 12). The seeds were powdered before analysis. Both the control and radiation processed samples in each lot were analysed in triplicate for volatile oils and aroma glycosides within one week of storage as described in the following sections. In a separate set of experiment three different samples of fenugreek were subjected to radiation doses (not revealed to the authors) as above and the samples were then analysed in triplicate for essential oil composition. The content of phenol estimated was used for determining absorbed dose. This experiment was designated as blind trial. All solvents (analytical reagent grade) including HPLC grade solvents were redistilled before use. High purity (>99.9%) N_2O was procured from British Oxygen Co. (Mumbai, Maharashtra, India).

2.2. Methods

2.2.1. Isolation and analysis of aroma glycosides of fenugreek

Aroma glycosides were extracted from fenugreek (50 g) according to the procedure reported earlier (Arul et al., 2006). Aroma glycosides present in the extract were then recovered from this solution by passing through a column of Amberlite XAD-16 essentially according to the procedure of Gunata, Bayonove, Baumes, and Cordonnier (1985). The isolate thus obtained was made to 1% solution in methanol.

2.2.2. Thin layer chromatography (TLC) analysis

TLC of the glycosidic conjugates was carried out on silicagel G plates using ethyl acetate:iso-propanol:water (65:30:15) as developing solvent system (Arul et al., 2006). Separated bands were identified either by exposing the plate to iodine vapour or by heating the plate at 180 °C for 30 min after spraying with 50% sulphuric acid. The major band at R_f 0.85 isolated from prep TLC was eluted with methanol, evaporated to dryness and dissolved in the same solvent to make a 1% solution. Quantitative estimation of glycosidic conjugates was performed on a dual wavelength flying spot scanning densitometer, CS-9301PC, Shimadzu, (Kyoto, Japan). The density of the spots of interest was determined in the reflectance mode at a wavelength of 529 nm. Aliquots of suitably diluted samples were spotted on the plate and the concentration of the individual spots in the sample (control as well as irradiated) was obtained from the standard curve (correlation coefficient 0.99) prepared using phenyl-β-D-glucoside (linear in the range 2–25 μg) and expressed as μg g^{-1} of food sample.

2.2.3. High performance liquid chromatography (HPLC) analysis

HPLC analysis was carried out on a Jasco HPLC system, Jasco Corporation (Tokyo, Japan) equipped with a C-18 reverse phase stainless steel column (30 cm × 0.46 cm) and a PDA detector set at a wavelength of 275 nm. Samples of the above total glycosides as well as those isolated from TLC (10 μl, 0.01% solution in methanol each) were injected on to the column and then eluted with water as solvent A and acetonitrile as solvent B. A gradient elution from 0% to 100% B in A over a period of 30 min, at a flow rate of 1.0 ml min^{-1} was carried out (Ly, Yamauchi, Shimoyamada, & Kato, 2002). Peaks were identified by comparing their retention times with that of authentic standards injected under identical conditions. Content of glycosidic conjugates was estimated from a standard curve (correlation coefficient 0.99) prepared using phenyl-β-D-glucopyranoside (linear in the range of 2–20 μg). Content of the phenyl-β-D-glucopyranoside present in each of the samples was expressed as μg g^{-1} of food sample.

2.2.4. Acid hydrolysis of aroma glycosides

A part of the aroma glycoside (1% (w v^{-1}) solution) from the control and irradiated samples as well as TLC isolated band at R_f 0.85 were subjected to acid hydrolysis (1 M HCl, 1 h, 80 °C). The free aroma was extracted with diethyl ether as reported earlier (Arul et al., 2006) and then subjected to Gas chromatography-Mass spectrometry (GC/MS) analysis. In case of the TLC isolated band the remaining aqueous solution was neutralised with 1 N KOH, dried under vacuum and the residue dissolved in dry methanol. This methanol solution was subjected to TLC in order to identify the sugar residue. The acetylated sugar residue was also analysed by GC/MS to confirm the nature of the carbohydrate moiety. Glucose was the only sugar moiety identified in the isolated band.

2.2.5. Pulse radiolysis

The pulse radiolysis system using 7 MeV electrons, generation and studies on the free radical reactions have been described earlier (Adhikari & Mukherjee, 2001). Dosimetry was carried out using an air-saturated aqueous solution containing 5×10^{-2} M KSCN assuming Gϵ for $(SCN)_2^-$ = 23,889 M^{-1} cm^{-1} per 100 eV at 500 nm (Buxton & Stuart, 1995). The width of the electron pulse was 50 ns and the dose per pulse was 15 Gy. The kinetic spectrophotometric detection system covered the wavelength range from 250 to 800 nm. The bimolecular rate constant was calculated by plotting the pseudo-first order rates of formation of the transient species against the corresponding solute (reactant) concentrations. The uncertainty in the measurement in bimolecular rate constant is less than ±10%.

2.2.6. Isolation of volatile oil

Ground fenugreek (50 g) samples were subjected to steam distillation using simultaneous distillation–extraction technique according to the procedure described earlier (Variyar, Ahmad, Bhat, Niyas, & Sharma, 2003). Peroxide free diethyl ether, S.D. Fine-Chem. Ltd., (Mumbai, Maharashtra, India) was used as extracting solvent. The essential oils thus obtained (2% in ether) were then analysed by gas liquid chromatography (GLC) as described below. Each sample lot as described above was analysed in triplicate making a total of nine repetitions for each food material.

2.2.7. Analysis of essential oil

Analysis of essential oil was carried out using a GC–MS instrument (Shimadzu Corporation, Kyoto, Japan) equipped with a GC-17A gas chromatograph and provided with a DB-5, J&W Scientific (Folsom, CA, USA) capillary column ((5%-phenyl)-methylpolysiloxane, length, 30 m; id., 0.25 mm and film thickness, 0.25 μm). The operating conditions were: column temperature programmed from 60 to 200 °C at the rate of 4 °C min^{-1}, held at initial temperature and at 200 °C for 5 min. and further to 280 °C at the rate of 10 °C min^{-1}, held at final temperature for 20 min; Injector and interface temperatures, 210 and 230 °C, respectively; carrier gas helium (flow rate, 0.9 ml min^{-1}); ionisation voltage, 70 ev; electron multiplier voltage, 1 kV. Samples were analysed in scan mode in the mass range of m z^{-1} 50–600. Peaks were tentatively identified

by comparing its mass fragmentation pattern with that of standard compounds wherever available, from standard spectra available in the spectral library (Wiley/NIST Libraries) of the instrument as well as from literature data. Content of phenol in the essential oil of the non-irradiated and irradiated samples was estimated from a standard curve of concentration versus peak area (correlation coefficient 0.99, linear in the range 2–20 μg) prepared using standard phenol and expressed as μg g^{-1} of fenugreek.

2.2.8. Validation of experimental data

The ability of the method to predict unknown doses was validated according to ICH guidelines on validation of analytical methods followed earlier by Apers, Naessens, Pieters, and Vlietinck (2006).

2.2.8.1. Specificity. Specificity of radiation treatment to release free phenol from its glycosidic precursors was tested by comparing radiation processed samples with boiled (1 h, 100 °C), room temperature stored (28 °C, 12 months) and roasted fenugreek samples. Free phenol was only detected in radiation processed samples indicating its specificity to radiation processing.

2.2.8.2. Linearity. Range at which concentration of phenol released was directly proportional to dose was tested. Known doses were given to fenugreek and concentration of phenol was estimated. Plot of dose versus concentration obtained in both cases was tested for linearity by calculating least squares line and correlation coefficient. They were further tested for lack of fit and residuals were graphically examined.

2.2.8.3. Accuracy. Accuracy was determined by the ability of the method to identify the actual dose given to a sample. For this blind trials were conducted. Fenugreek samples were irradiated to unknown doses and the dose delivered was found from a calibration curve. Close agreement between actual and calculated doses were found.

2.2.8.4. Precision. Blind trials were carried out with each sample analysed in triplicate. Results were subjected to ANOVA to determine whether the data obtained from three different analyses were not significantly different. There was no significant difference ($p \leqslant 0.05$) between triplicates proving the precision of method.

3. Results and discussion

Effectiveness of radiation processing as a post-harvest method to reduce food losses and to increase food safety has resulted in an increased interest in this technology amongst food processors, traders and consumers in recent years. The process has been successfully applied for decontamination of dried products such as spices. Breakdown of glycosidic conjugates during radiation processing resulting in enhanced colour of saffron and enhanced antioxidant activity of soybean has been reported (Variyar, Limaye, & Sharma, 2004; Zareena, Variyar, Gholap, & Bongirwar, 2001). Formation or increase in content of specific volatile compounds released from its precursors may thus possibly influence the nature and content of the volatile oil which in turn can modify/enhance the aroma of the treated spice. Estimation of bound glycosidic precursors thus assumes importance.

3.1. Isolation and identification of aroma glycosides

Fig. 1 depicts a representative TLC profile of the aroma glycosides isolated from non-irradiated fenugreek. The major band at R_f 0.85 accounting for 90% of the chromatogram was tentatively identified as phenol β-D-glucopyranoside by comparison of its R_f value with that of authentic standard. The nature of this compound was further confirmed by acid hydrolysis of the isolated band. Phenol was the only aglycone detected by GC/MS whilst glucose was the sole sugar residue identified both by TLC as well GC/MS analysis of its acetylated derivative. Occurrence of glycosidic conjugates of phenol has been reported in celery (Tang, Zhang, Hartman, Rosen, & Ho, 1990) and grapes (Bureau, Baumes, & Razungles, 2000) where their content was reported to be 0.477 ppb and 24 ppm, respectively. In contrast, a high content of phenol glucoside (0.7 mg g^{-1}) was noted in the present study. Changes in the content of this precursor during post-harvest processing may thus have a significant impact on the essential oil quality of spice. Besides phenol other constituents identified include, 6-methyl-5-heptene-2-ol, 4-hydroxy benzene propanoic acid, hexadecanol and heptadecanol that were present at concentrations less than 0.1 mg g^{-1}. Amongst these 6-methyl-5-heptene-2-ol, reported to possess a fruity odour is an important volatile constituent of tomato contributing to the fresh ripe note of this vegetable (Yilmaz, 2001). The remaining compounds have no significant aroma and thus play a limited role in contributing to the fenugreek odour. Since the aroma glycoside profile of fenugreek was dominated by phenyl glucopyranoside, break down of this compound during radiation processing was only taken up for detailed study.

3.2. Changes in phenyl β-D-glucopyranoside during radiation processing

TLC chromatogram of glycosidic fraction isolated from fenugreek subjected to a radiation dose of 10 kGy is illustrated in Fig. 1. Absence of the band corresponding to phenyl glucopyranoside at R_f 0.85 could be clearly noted in the irradiated fenugreek sample (Fig. 1). A breakdown of phenol glucopyranoside could thus be assumed. Changes in the above glycoside during radiation processing were further quantified by HPLC and TLC-densitometry. Table 1 provides a quantitative distribution of the phenol glucopyranoside in both the control and radiation processed samples as estimated by both HPLC and TLC-densitometry. The content of the glucosidic precursor as estimated by HPLC and densitometry are comparable. A decrease in content of phenol glucopyranoside with increase in absorbed dose is noted. At a dose of 10 kGy a

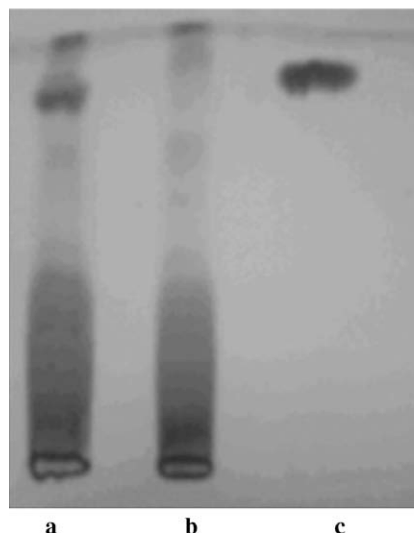

Fig. 1. A representative TLC profile of the bound aroma precursors isolated from both the control and irradiated fenugreek. (a) Non-irradiated, (b) irradiated and (c) standard phenyl-β-D-glucopyranoside.

Table 1
Content of phenyl-β-D-glycopyranoside and free phenol in fenugreek samples as estimated by HPLC, TLC-densitometry and GLC methods.

Dose (kGy)	Phenyl glucopyranoside ($\mu g\ g^{-1}$)[a]		Phenol ($\mu g\ g^{-1}$)[a]
	HPLC	TLC-densitometry	GLC
0	696.77 ± 0.62	687.985 ± 0.20	–
2.5	574.906 ± 0.28	575.946 ± 0.31	44.05 ± 0.21
5	534.29 ± 0.31	530.753 ± 0.69	54.67 ± 0.08
7.5	518.42 ± 0.30	517.417 ± 0.31	61.30 ± 1.05
10	482.03 ± 0.59	–	68.90 ± 3.53

[a] Values are expressed as mean ± SE, $n = 9$.

reduction in glycoside content by nearly 30% could be noted. This decrease reflects the instability of the glycosidic bond during radiation processing. It may however be noted that this compound is highly stable to treatments such as boiling or roasting as no phenol could be detected in the essential oil isolated from the spice subjected to such processing conditions. Specificity of break down of this compound towards radiation processing is thus evident. Mechanism of radiolysis of aroma glycosides was therefore further investigated by pulse radiolytic studies to confirm the release of free aglycone from its glycosidic precursors during radiation processing.

3.2.1. Mechanism of radiolysis of phenyl β-D-glucopyranoside

Using standard phenyl β-D-glucopyranoside steady state γ-radiolysis and pulse radiolytic studies was carried out. In γ-radiolysis experiments hydroxyl radicals upon reacting with β-D-glucopyranoside produced phenol that was confirmed by HPLC measurements. To understand the reaction pathway and elucidate a probable mechanism for the hydroxyl radical induced breakdown of parent glycoside to phenol, pulse radiolysis measurements were carried out. In an N_2O-saturated aqueous solution the glycoside molecule upon reacting with the hydroxyl (OH) radical produced by passage of electron pulses gave a transient absorption with a peak at 320 nm (Fig. 2a). The bimolecular rate constant for the reaction as determined by following the pseudo-first order rate of formation at 320 nm was $5.5 \times 10^9\ LM^{-1}\ s^{-1}$. The resulting absorption decays with time without evolving any other new absorption band up to 400 μs. The decay follows first order rate constant as evident from an experiment carried out at different doses. At a dose of 32 Gy per pulse i.e., with higher radical concentration, no effect on the decay rate constant was observed (Figure not shown). As a general rule second order reactions are manifested by radical–radical interactions that are altered at higher radical concentrations.

To get a better understanding about the nature of the resultant radical experiments in presence of low concentration of oxygen (20%) was carried out. An aliphatic carbon centred radical is generally assigned by its strong interaction with oxygen molecule. It was observed in our experiment that the decay becomes considerably faster in presence of oxygen (Fig. 2b), confirming the nature of the transient species to be an aliphatic carbon radical.

Ionising radiation interacts with matter resulting in excitation and ionisation. In the case of dry spices, there is a possibility of formation of more excited states rather than the free radicals because of its lower water content. We performed a pulse radiolytic experiment with phenyl glucopyranoside dissolved in a N_2- purged benzene solution in presence of anthracene, a triplet sensitizer. We could also observe formation of phenol as a product by the Prussian blue assay (Deshpande, Cheryan, & Salunkhe, 1986).

Consequently, from the results of γ-radiolysis and pulse radiolysis experiments it can be inferred that hydroxyl radical attacks the side chain of the phenol glycoside generating a carbon centred radical, which eventually leads to the breaking of the link and formation of the phenol.

3.3. Estimation of phenol by GLC

Release of phenol from its precursors during radiation processing may possibly modify the volatile oil profile of fenugreek. It was therefore of interest to ascertain the distribution of volatile constituents in the essential oil of control and irradiated spice. Fig. 3 provides a representative GLC profile of the essential oil obtained from both the non-irradiated and gamma irradiated (10 kGy) fenugreek. Appearance of a new peak at R_t 9.27 characterised the essential oil isolated from the irradiated samples in both cases. The peak at R_t 9.27 (Kovats index, 972.13) was identified as phenol from its mass spectra as well as comparison with authentic standard. Table 1 shows the content of phenol in the different samples of fenugreek as estimated by GLC. Absence of phenol in the control sample is clearly noted. A correlation between a decrease in glycoside ($r^2 = -0.97$, $p \leqslant 0.01$) and an increase in phenol ($r^2 = 0.98$, $p \leqslant 0.01$) could be established. This confirms release of phenol from its glycosidic precursors during radiation processing. A dose dependent increase in phenol with radiation dose (Table 1) suggests its quantification in irradiated fenugreek as a means of estimating absorbed dose. Results of blind trial studies (Table 2) clearly established such a possibility. The method was found to

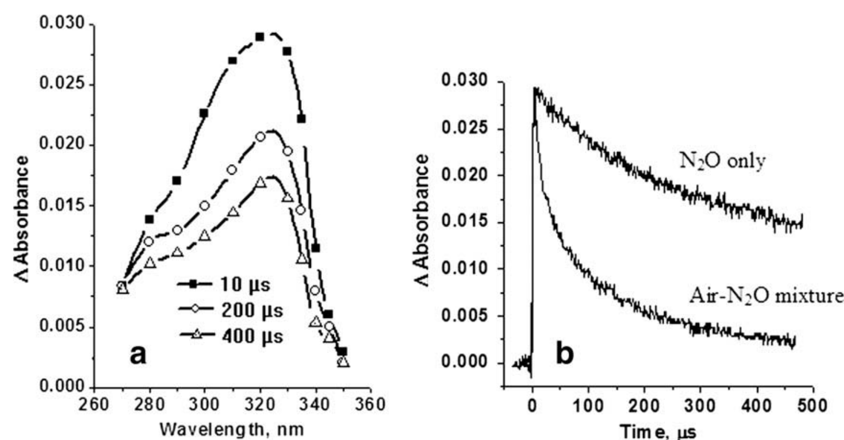

Fig. 2. (a) Transient absorption spectra obtained from an N_2O-saturated aqueous solution (natural pH) containing 2×10^{-4} M phenyl-β-D-glycopyranoside after electron pulse and (b) kinetic traces recorded at 320 nm from the same solution as of Fig. 2a; without oxygen and in presence of 20% oxygen.

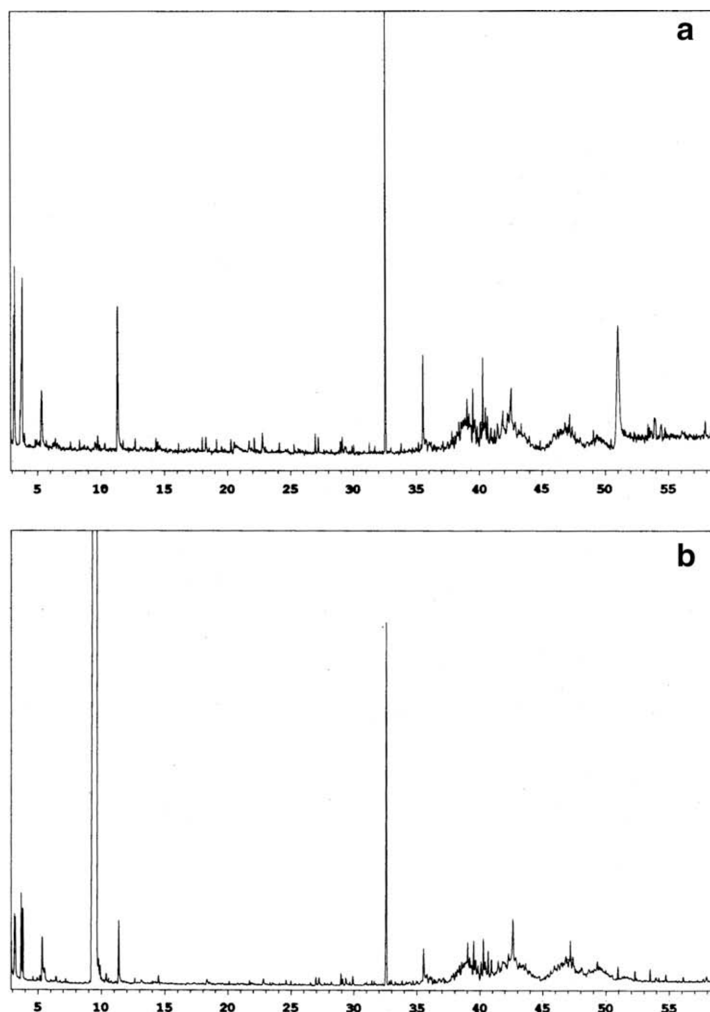

Fig. 3. A representative GLC profile of the essential oil obtained from (a) non-irradiated and (b) gamma irradiated (10 kGy) fenugreek samples (peak at R_t 9.27 corresponds to phenol in the chromatogram).

Table 2
Comparison of experimentally estimated versus actual absorbed dose (calculated by Fricke dosimeter) in fenugreek.

Actual dose (kGy)	Calculated dose[a] (kGy)	Error (%)
4.0	3.4	15.00
6.5	5.8	10.76
8.0	7.22	9.75
13.5	12.76	5.48

[a] Data represented are average values of 9 replications.

Table 3
Linearity data for estimation of phenol by GLC.

Correlation coefficient	0.9895
Slope ± standard error	1.804 ± 0.131
Intercept ± standard error	46.547 ± 0.901
$F_{(LOF)}$	187.76 ($F_{(crit)}$ = 0.005)
Range (dose in kGy)	2.5–10

accurately estimate unknown doses within an error of 15% (Table 2). It was found to be linear in the dose range of 2.5–10 kGy. Quantification limits were established based on the minimum concentration at which peak area could be quantified by the instrument. Below a dose of 2.5 kGy no phenol could be detected under the current GLC conditions. The lowest limit, thus detected was found to be 44 μg gm^{-1} of sample. The method was validated according to International Conference on Harmonization (ICH) guidelines and shown to be specific (no phenols released from its precursors during boiling/heating, storage), linear and accurate (Table 3) within established ranges.

This study has demonstrated the role of glycosidic precursors in modifying aroma profile of fenugreek during radiation processing. Quantification of phenol released in a dose dependent manner from its precursor by GLC resulted in both detection of radiation processing and in estimating absorbed dose. Phenolic precursors are ubiquitous in spices, fruits and vegetable. The present method thus shows considerable promise for extension to other food products.

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