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Effect of germinaton on acrylamide reduction during baking of wheat

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■ **ABSTRACT :** Acrylamide is toxic compound, probable carcinogenic, formed via the browning process by maillard reaction between amino group of free amino acid asparagine and reducing sugar during heating of carbohydrate-rich foods. Wheat contains high level of these precursors. The main objective of this investigation was to study the effect of germination on reduction of acrylamide formation of baked wheat. Wheat soaked for 12 hours and germination at 25 °C for different time period 24, 48, 72 hours and baked at 200 °C for 20 min and un-germinated flour baked was considered as control. Acrylamide content was determined by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS). The results of this study effective on acrylamide, control baked wheat found 0.153 mg/kg of acrylamide and 24 hours germinated wheat flour baked which found 0.026 mg/kg, 48 hour sample found 0.016mg/kg and 72 hours sample found 0.005mg/kg. Acrylamide reduced after 24 hours germination 83.00 per cent and after 48 hours germination acrylamide decreased from baked wheat dough was 89.54 per cent and after 72 hours germination decreased 96.53 per cent. In conclusion, germination was an efficient way to reduce acrylamide content in baked wheat.

■ **KEY WORDS :** Acrylamide, Wheat, Germination process, Baking, Mitigation strategy

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Acrylamide (2-propanamide) is a water soluble compound which is colorless, non-volatile crystalline solid and has molecular weight of 71.08 kDa. Acrylamide has melting point of 84.5±0.3°C and a high boiling point (136°C at 3.3 kPa/25 mmHg; Norris 1967; Ashoor and Zent 1984 and Eriksson 2005). Acrylamide is toxic compound in food that formation was found to occur during the browning process by the Maillard reaction between amino group of free amino acid asparagine and carbonyl group of reducing sugar such as glucose and fructose at temperatures higher than 120°C. The highest amounts of acrylamide were found in French fries and potato chips (Becalski *et al.,* 2003). In April 2002, a group of Swedish Researchers reported that acrylamide found in high levels in high carbohydrate (starch-rich) foods such as cereals, potato, coffee (Surdyk *et al.,* 2004; Tarek *et al.,* 2002 and Svensson *et al.*,2003). Acrylamide has been classified by the International Agency for Research on Cancer (IARC, 1994) as "potentially carcinogenic to humans" and in 2001, the scientific committee on toxicity, ecotoxicity and the environment determined its intrinsic toxic properties such as carcinogenicity, neurotoxicity, reproductive toxicity and genotoxicity. Acrylamide is genotoxic (mutagenic), which increases the incidence of cancer in rats at doses 1-2 mg/kg body weight per day. According

to WHO 2005, the maximum permissible level of acrylamide is 0.3-2 µg/kg body weight for general population. Maillard reaction has been indicated major pathway for the acrylamide formation and amino acid asparagine closely linked to the acrylamide formation. Maillard reaction is non-enzymatic browning reaction formed in foods during baking and frying (Coughlin, 2003, Tareke *et al.*, 2000).

Wheat (Genus Triticum) cereal grain is most important staple food for many people in developed and developing country. Wheat originally from the South West Asia, but now cultivated worldwide. It has been describe as the "King of cereals". India is one of the largest producers of agricultural production in the world. India second largest producer in wheat.Wheat cultivation in India traditionally been dominated by the northern region of India.The northern states Punjab and Haryana plains in India have been prolific wheat producers (Limbore, 2005).

Germination is the process formation of sprouts after the period of dormancy. Germination triggers the enzymatic activity of sprouting seeds, leading to breakdown of protein, carbohydrates and lipids into simpler form. Germination of cereal grains like wheat and sorghum which are rich in carbohydrate (Nout and Ngoddy, 1997). Germination cause decrease the pH and carbohydrate content with increasing sprouting days which effect on reduction of acrylamide.

METHODOLOGY

Chemicals and materials:

Material:

Ajantha variety ofwheat procured from Vasantrao Naik Marathwada Krushi Vidhyapith, Parbhani.

Chemicals:

Chemicals required for this investigation are sulfuric acid, nitric acid, HCL, sodium hydroxide, hexane, copper sulphate, potassium sulphate, mixed indicaters, boric acid and acrylamide standard (> 99%), acetonitrile, magnesium sulphate, NaCl, primary secondary amine, other solvent and reagent were analytical grade used and obtained from Department of Food Engineering, Maharashtra Institute of Technology, Aurangabad and Department of Food Chemistry and Nutrition, MIT College of Food Technology.

Analytical instrument :

Shimadzu LC-MS/MS model 8045 system equipped with a SIL-30AC autosampler and two series of LC pump (LC-30AD) were used. The mode of pump binary gradient having total flow 0.3000 ml/ min. LC column was Inertsil ODS (4.6 × 150 mm 5 µm) used. Mobile phase- 97: 2.5 Water: Methanol+ 0.1% Formic acid. Mass spectrometer system was operated by CID gas at 230 kPa in multiple reaction monitoring acquisition mode with positive polarity.Liquid chromatography oven temperature was 35°C and injection volume of sample was 10 µl.

Methods:

Wheat germination:

Wheat grains were cleaned by first physically arranged to evaluate disfigured, little, broken, tidy, sand, stones and other foreign materials. The grains were then washed by water, mixed by hand and screened out of the water. Then wheat grains were steeped in tap water ratio 1:2 (grain to water) for overnight with two changes

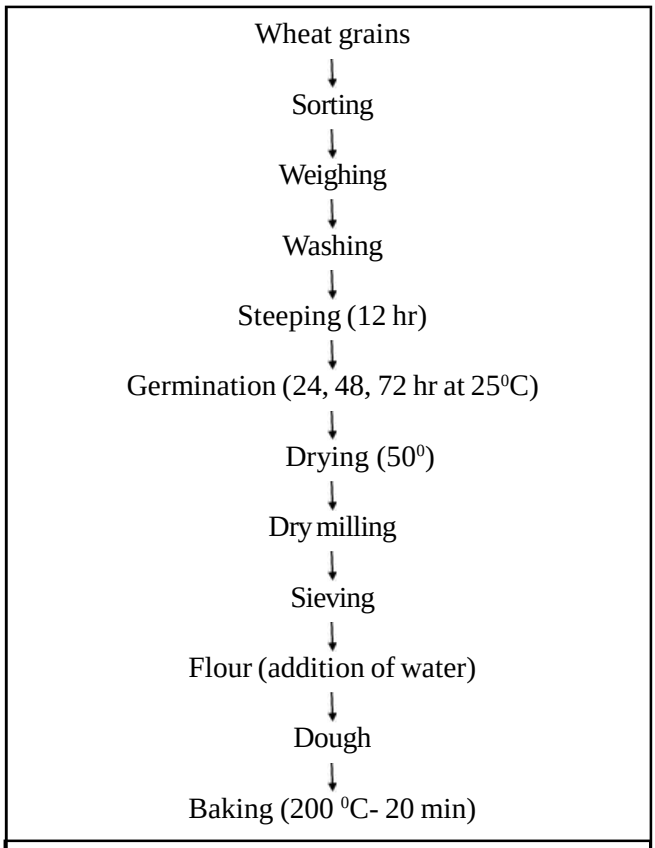

Fig. A : Procedure for germinated wheat flour dough baking

of water to remove dirt, dust and husk. Later, the wet wheat grains spread out thinly on a jute bag saturated with water and covered with another jute bag. Grains were allowed to germinate in dark at controlled temperature (25^0C) and 90 per cent relative humidity (RH) in an incubater. Grains were germinated for 24, 48,72 hours. After germination grains were dried to constant weight at 50^0C in tray dryer. The root portions were manually removed. Then the dried germinated wheat grains where ground in the laboratory mill.

Chemical analysis:

The germinate wheat flour were analyzed for moisture content, protein, fat, Ash and fibre content according to the method described by AOAC 2005. The carbohydrate content of the flour were determined by difference.

Detection of acrylamide:

For the detection of acrylamide after LC separation tamdem mass spectrometry was most used to detect the characteristics ions of acrylamide. Besides of GC-MS for determination of acrylamide, recent studies pay more attention to assay employing LC-MS/MS technique for the routine analysis because this chromatographic technique applied for the quantitative analysis of acrylamide has high selectivity and avoid the derivatization step (Clarke *et al.*,2002 and Leung *et al*., 2003).

LC-MS/MS has high selectivity when working MRM (Multiple Reaction Monitoring) mode, in which transition from precursor ion to a product ion is monitored. MRM means that the transition from ion, which is separated in the first quadrupole, to a product ion, generated by collision with argon in the second quadrupole. As for the acrylamide monomer detection using MRM, injected sample (10µl) from the liquid introduction system enters the ionization source at atmospheric pressure. These ions are sampled through a series of orifice and ion optics into first quadrupole where they filtered according to mass to charge ratio (m/z) of acrylamide. The mass separated ion, then passes into ion tunnel collision cell, with axial field where they either undergo Collision Induced Decomposition (CID), or pass unhindered to the second quadrupole.

The fragment ions of acrylamide after collision using argon as collision gas, then mass analyzed by second quadrupole. Finally, the selected and transmitted ions are detected by a conversion dynode, phosphor and photomultiplier detector system. The output signal is amplified, digitized and presented to the data system. Whole detection procedure of acrylamide (AA) using MRM mode. The transition transitions such as 77>44 and 77>27 have been used analytes confirmation of acrylamide.

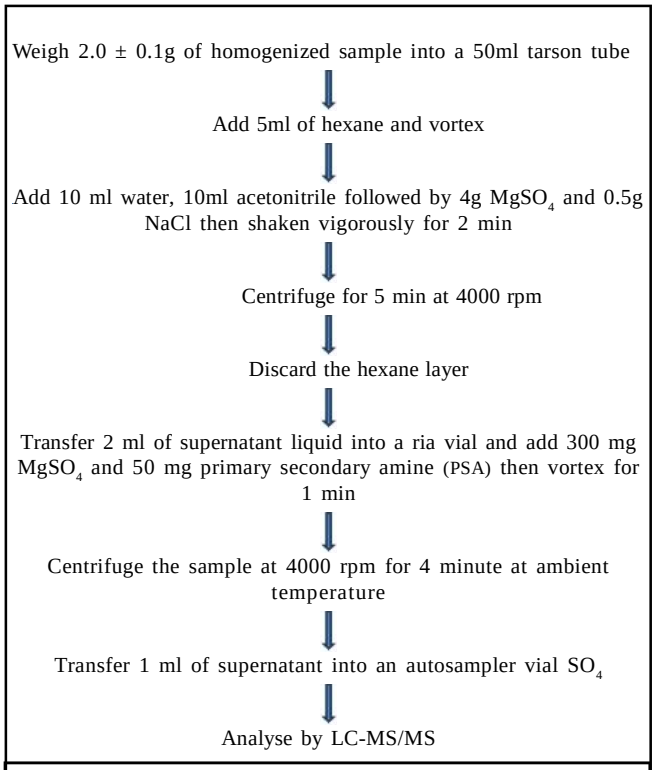

Fig. B: Acrylamide sample extraction procedure (AOAC, 2007)

RESULTS AND DISCUSSION

The results obtained from the present investigation as well as relevant discussion have been summarized under following heads :

Chemical compositions of wheat and germinated wheat flour:

The chemical analysis of wheat and germinated wheat flour are shown in Table 1. The moisture content of wheat flour increased after the germination at 25°C for 24, 48 and 72 hours ranged from 12.00 per cent - 12.52 per cent. The sample D (germinated at 25 °C for 72 hours) having highest moisture content (12.52%), while sample A which is control sample having lowest

moisture content (12.00 %).

The result obtained in this study showed that an increased the protein content of wheat flours after the soaking at 12 hours and germination at 25°C time of 24, 48 and 72 hours. The protein content obtained 10.10 per cent – 18.52 per cent which was calculated by nitrogen × 6.25. Soaking lead changes in the biology of the breakdown of the various components into simpler compounds, however, during germination, protease enzyme increase and is involved in the degradation of peptide component to amino acid and the amount of protein will increases (Suhaidi and Hafiz, 2003). Inyang and Zukari (2008) noted the germination may increase protein content. In cereals protein increases due to presence of protein hydrolysis as well as result of protease enzyme activity during germination the grains (Inyang and Zukari, 2008)

The ranges of fat content in wheat flour analyzed were 1.93 per cent – 1.43 per cent. As the soaking and germination time increases the fat content of samples decreases. The sample A which is control wheat sample having highest fat content (1.93%) and the lowest fat content observed sample D which is germinated at 25 °C for 72 hours at RH 90 per cent-. Inyang and Zukari (2008) noted that germination the seed or grain, decreasing amount of fat is due to increased activity of lipolytic enzyme during germination, which hydrolyzed the fats into fatty acid and glycerol. Sukamto (1992), reported germination process enhance the hydrolysis of complex organic compound which are insoluble in the seeds or grain and form simpler organic compound that are water soluble.

With regards to fibre content of the wheat flour, the highest was 2.45 per cent found wheat flour soaking 12 hour and germination 25°C for time of 72 hours and 1.70 per cent observed in control wheat sample A. Dicko *et al.* (2006) noted that germination process increased activity of β-amylase enzyme. Also evaluation of ash content carried out and it is present in a range of 2.01 per cent - 1.43 per cent. The lowest content of ash found in germination time of 72 hours.

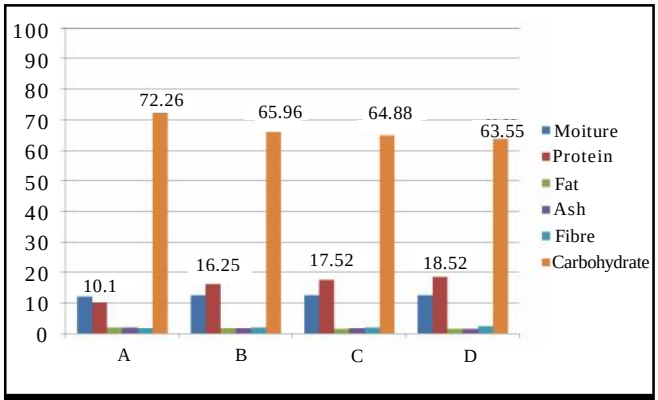

Fig. 1 : Graphical representation of chemical composition of germinated wheat flour

The carbohydrate content carried out by mean difference method. Carbohydrate contents of wheat decreased by increasing time period of germination. Carbohydrate contents of wheat flour range of 72.26 per cent - 63.55 per cent. Carbohydrate content in sample A which is control sample having highest and after germination time period of 72 hour having lowest. After germination at 25°C for 72 hours wheat decreased carbohydrate content 8.71 per cent.

Effect of germination acrylamide:

The effect of germination on acrylamide determined by the liquid chromatography tandem mass spectrometry (LC-MS/MS). In this study the germination pretreatment for reduction level of acrylamide in baked wheat. The

Table 1 : Chemical composition of wheat and germinated what flour

Sample	Parameter (%)					
	Moisture	Protein	Fat	Ash	Fibre	Carbohydrate
A	12.00	10.10	1.93	2.01	1.70	72.26
B	12.49	16.25	1.65	1.72	1.93	65.96
C	12.50	17.52	1.50	1.59	1.01	64.88
D	12.52	18.52	1.43	1.43	2.45	63.55
C.D. (P=0.05)	0.038	0.088	0.602	0.305	1.025	0.086
S.E.±	0.11	0.026	0.182	0.092	0.309	0.026
CV%	0.096	0.198	4.303	2.128	6.547	0.082

*Each value represents the value of three determinations. Sample A- Control wheat flour, B- Germination at 25°C for 24 hr., C-Germination at 25°C for 48 hr., D- Germination at 25°C for 72 hr.

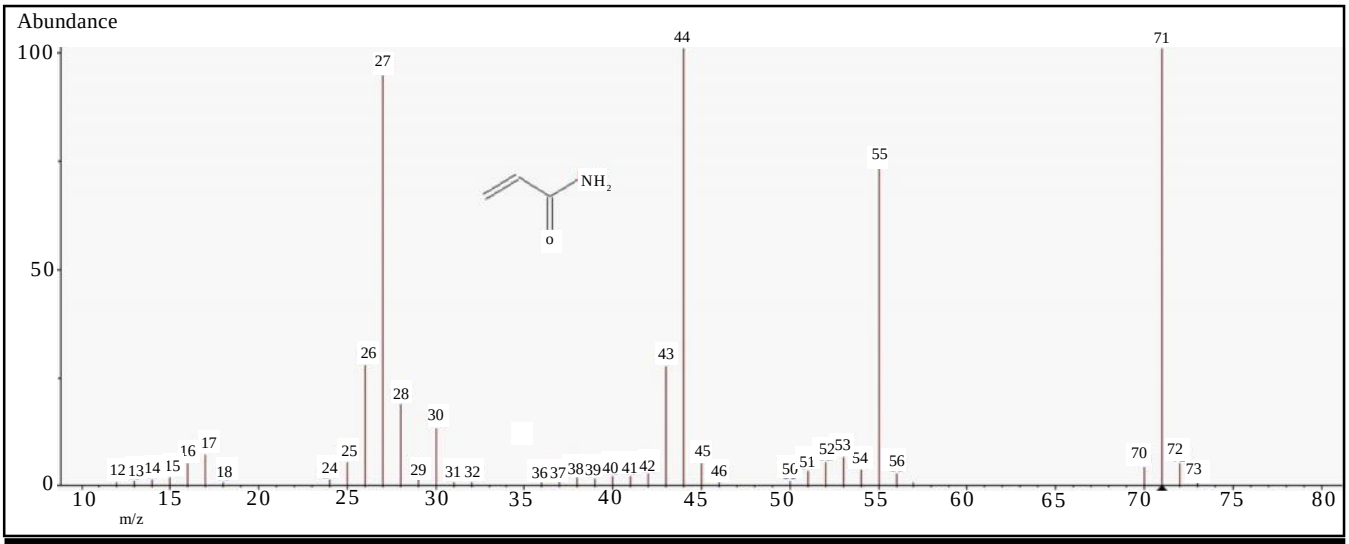

Fig. 2 : Mass spectra of acrylamide

germination was carried out at 25°C for different times-24hr, 48hr, 72hr.

The samples were analyzed by Liquid chromatography tandem mass spectrometry (LC-MS/MS) with positive electrospray ionization (ESI) using column inertsil ODS, using deionized water and methanol as mobile phase. LC-MS/MS with electrospray ionization (ESI) is a powerful tool for detection of acrylamide in foods at low level (Reidiker, Stadler 2003; Rosen and Hellenas, 2002 and Tarek *et al.*, 2002).

The electrospray has following setting (with nitrogen), interface voltage 4.00 kV, drying gas (N_2) flow of 10.00 L/min. acrylamide was identified by multiple reaction monitoring (MRM). The precursor ion $(M+H)^+$= 72.20 was fragmented and production 55.20 (Collision Energy 16eV), 27.20 (Collision Energy 26 eV) and 44.10 (Collision Energy 25 eV). The yield of specific daughter ion (72>27.20 and 72>44.10) allows MS/MS method to analyse complex sample extract with high specificity and sensitivity.

The standard curve for LC-MS/MS analyse were produced by linear regression using the peak area ratios of the analytes at mass to charge ratio m/z 72>27.20 collision energy (CE) 26.0 and linearity covered five orders of magnitude in the concentration ranging from 0.001 to 0.1 mg/kg with high linearity (r^2= 0.9975). The limit of detection (LOD) and limit of quantification (LOQ) were calculated as three times of SD.

Mass to charge ratio m/z : 72>27.20

Rr^1= 0.9987527, Rr^2= 0.9975070
Mean RF: 9.3752229e+006, RF SD: 1.969044e+006, RF% RSD: 21.002620

Sr. No.	Conc. (Ratio)	Mean area	Area
1.	0.001	12785	12785
2.	0.005	44338	44338
3.	0.01	89643	89643
4.	0.05	427362	427362
5.	0.1	771163	771163

Concentration of acrylamide decreased by germination treatment in which three samples were baked germinated wheat dough and one was control sample *i.e.* baked wheat dough. The sample A is control wheat baked dough, sample B is of wheat soaking in water for 12 hours ratio 1:2 (wheat: water) by changing water after 8 hours intervals and germinated at 25°C for 24 hours. Sample C is of wheat soaking for 12 hour and germinated 25°C for 48 hours and last one sample D is of wheat soaking for 12 hour and germinated 25°C for 72 hours.

Table 2 : Concentration of acrylamide in baked wheat samples with its retention time and area

Sample	Retention time	AA conc. (mg/kg)	Area
A	2.542	0.153	249116
B	2.541	0.026	47051
C	2.534	0.016	30968
D	2.541	0.005	13281

Acrylamide decreased after 24 hours germination 83.00 per cent and after 48 hours germination acrylamide decreased from baked wheat dough was 89.54 per cent and after 72 hours germination decreased 96.53 per cent as shown in Fig. 3.

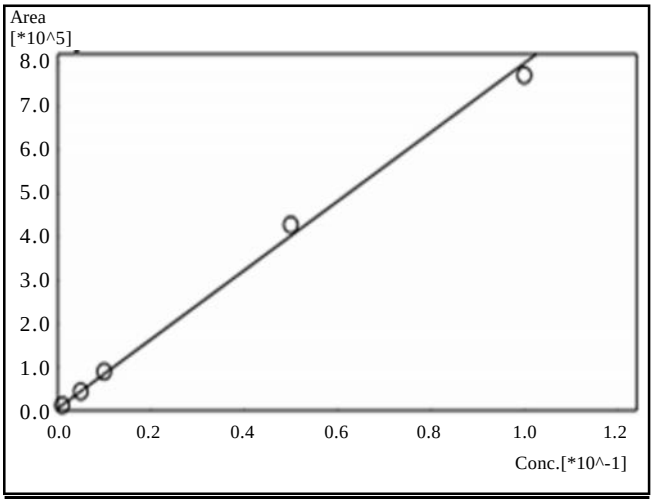

Fig. 3 : Acrylamide linearity curve (linearity) using m/z72 >27.20 and 72>44.10

The results of this study effective on acrylamide control baked wheat having 0.153 mg/kg (153µg/kg) acrylamide and 24 hours germinated wheat flour baked which found 0.026 mg/kg (26 µg/kg) acrylamide. Which decreased 83 per cent of control sample as well as sample B means germinated for 48 hour found 0.016 mg/kg which decreased 89.54 per cent and germinated 72 hour found 0.005 mg/kg which decreased 96.53 per cent. So germination pretreatment was effective mitigation strategy on carbohydrate rich cereal *i.e.* wheat.

Conclusion:

The results showed that the germination process was confirmed to be one of the successful ways of acrylamide mitigation in baking of carbohydrate rich cereal (wheat). The control sample found 153 µg/kg and the wheat soaked 12 hr and germinated at 25°C for 24, 48 and 72 hours and relative humidity 90 per cent were reduced acrylamide content ranges 26µg/kg, 16µg/kg and 72 hours germinated sample observed 5µg/kg.

Germination pretreatment reduce the acrylamide content from baked wheat 96.54 per cent which wheat was soaked for 12 hours and germinated at 25°C for 72 hours (RH-90%) and baked at 220°C for 20 min.

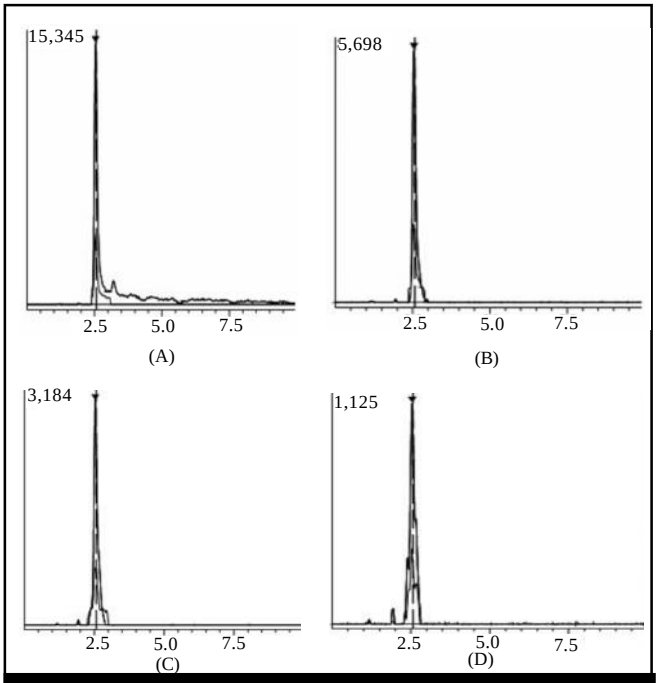

Fig. 4 : LC-MS/MS chromatograms (m/z: 72.20>27.20 and 72.20>44.10) of baked wheat sample (A) control wheat, sample (B) germinated wheat at 25 °C for 24hr., sample (C) germinated wheat at 25 °C for 48hr., sample (D) germinated wheat at 25 °C for 72hr.

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