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HPLC Analysis of In-Shell Walnuts for the Natural Incidence and Co-Occurrence of Fusarial Toxins - Zearalenone, Zearalenol and Deoxynivalenol

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Abstract: ***An investigation was carried out to determine the natural occurrence of some fusarial toxins viz., zearalenone (ZEA), zearalenol (ZAL) and deoxynivalenol (DON) in commercially important in-shell walnut kernels. HPLC analysis showed contamination of ZEA in 63.33 % samples and that of ZAL in 13.33 % samples. Among the thirty market samples that were investigated, only one sample of in-shell walnuts was found to be contaminated with DON. Co-occurrence of these fusarial toxins was also detected in some of the in-shell samples. Of the samples analysed, 10% showed co-occurrence of ZEA and ZAL, whereas 3.33% samples showed co-occurrence of ZEA and DON. In all these samples, the detected amount of ZAL and DON was much higher than that of ZEA.***

Keywords: *Juglans regia*, zea, zal, don, mycotoxins.

1. Introduction

India has traditionally been one of the world's major producer of edible nuts due to its varied climatic and soil conditions, large domestic markets and relatively simple methods of storage. Among the major edible nuts and dried fruits, walnut (*Juglans regia* L.) is an important tree nut as every part of the plant has utility and as such has carved its special place in socio- religious and economic well being of the people. It belongs to the angiospermic family Juglandaceae and is commonly called as 'akhroot'. Considerable interest has been generated in walnut as they are believed to possess plasma cholesterol-lowering properties (Sabate *et al.*, 2003). Similarly, concentration of omega-3-fatty acids in walnuts have many potential health benefits ranging from cardiovascular protection to the promotion of better cognitive function to anti- inflammatory benefits helpful in asthma, rheumatoid arthritis and inflammatory skin disease such as eczema and psoriasis. Traditionally, walnuts are used to treat cough, stomach ailments and cancer in Asia and European countries (Fakuda *et al.*, 2003). In addition, walnuts are popular ingredients in baked foods and are frequently served in-shell during fall and winter seasons. They are mostly consumed after drying but undried and just harvested walnuts are also popular in the production places.

Being a dry fruit, walnut has an inherent potential for prolonged storage. However, its shelf- life is governed by the physical characteristics of in-shell nuts, moisture content of the kernels and microbial status of the kernels after shelling. Shell morphology with respect to its surface (rough/porous), suture (tight/corky) and seal (split/tight) are also important parameters, which determine the shelf-life potential. Porous suture and loose seal provide an easy entry for microorganisms and acts as a foci of infection in storage. In addition, due to the unscientific methods of harvesting and storage, these dried walnuts are prone to deteriorating effects of storage microorganisms, especially the xerophilic fungal species, which flourish very well on them and may deteriorate their quality. Moreover, the in-shell walnuts are usually sun-dried, and stored in traditional gunny bags or heaped as such on the ground till they are marketed or processed, which makes them more vulnerable to the huge diversity of soil borne opportunistic microbes especially the fusarial species. Some of these fusarial species may be toxigenic and produce mycotoxins during storage. Further, since walnuts contain high concentration of nutrients like fats, proteins, minerals, vitamins and a substantial quantity of dietary fibres, it is anticipated that they may form a very good substrate for the growth of a large number of moulds, which are known to stimulate the mycotoxin production. In view of this, an investigation was initiated to determine the contamination of fusarial toxins viz., zearalenone (ZEA), zearalenol (ZAL) and deoxynivalenol (DON) from the market samples of in-shell walnuts.

2. Materials and Methods

Extraction of fusarial toxins from walnut kernels

Market samples of in-shell walnuts were analysed for zearalenone (ZEA), zearalenol (ZAL) and deoxynivalenol (DON) by using modified multimycotoxin method developed by Roberts and Patterson (1975). In this method, 25g of finely ground in-shell kernels were taken in an Erelenmeyer flask (250 ml capacity) and 100 ml mixture of acetonitrile and 4% potassium chloride (90:10 v/v) was added to it. The flask containing mixture was kept for horizontal shaking on a rotary shaker for 30 minutes. Thereafter, extract was filtered through Whatman no. 41 filter paper and the filtrate was defatted twice with 50 ml iso-octane in a separating funnel (250ml capacity). When the layers separated clearly, upper iso-octane layer was discarded and the lower acetonitrile layer was re-extracted with 50ml iso-octane. Discarded the upper lipid containing layer and added 12.5ml distilled water to the lower acetonitrile layer. This layer was extracted thrice by using 20ml chloroform each time. Lower chloroform – acetonitrile layer was collected in a conical flask and drained through Whatman no. 41 filter paper having a bed of anhydrous sodium sulphate. The extract was collected in a beaker and marked as extract I. The aqueous layer left in the separating

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funnel was acidified with 1ml of 1.0N HCL and the acidic mycotoxins were extracted from it thrice by using 10 ml chloroform each time. Lower chloroform layers were combined, passed through anhydrous sodium sulphate bed, collected in a beaker and marked as extract II. Extracts I and II were combined and then evaporated to dryness on a water bath. After evaporation, the residue was dissolved in 1ml of chloroform and stored in small screw cap vials for qualitative and quantitative estimation of fusarial toxins.

Detection of fusarial toxins

For detection of ZEA, ZAL and DON, aliquots of sample extract (50µl) were spotted on TLC plates along with the standards and developed in a solvent system consisting of toluene : ethyl acetate : formic acid (6:3:1, v/v/v). After drying, plates were observed under long wave UV light. ZEA spots were located as blue green fluorescent spots; ZAL spots were located as light blue fluorescent spots and that of DON as sky blue spots. Confirmation was done by spraying the plates with freshly prepared saturated solution of aluminium chloride in 95% ethanol and then heating it at 120°C for 10 min. The spots of ZEA, ZAL and DON became brighter in appearance.

Quantitative estimation of fusarial toxins

Quantitative analysis of ZEA, ZAL and DON was done through high performance liquid chromatography. The analytical equipment for HPLC (CLASS-LC10 SHIMADZU) consisted of a liquid chromatographic pump LC-10AT, an auto-injection system SIL-10A with a 50 µl sample loop, and a variable wavelength absorbance UV - VIS detector SPD -10 set at 365 nm. The analytical column was C-18 (250 x 4.6 mm), filled with ODS (M), RP-18 material, 5 µm particle size (Merck).

Analysis of ZEA was done by using modified method of Scudamore and Patel (2000). For this, a variable wavelength absorbance fluorescent detector set at 274nm excitation and 440 nm emission was used. The mobile phase consisted of acetonitrile : water (55:45, v/v) and was used at a flow rate of 0.5 ml/min. Injection volume for extract solution was 10 and 20 µl for different samples. Analysis was performed at room temperature (25-30°C) and quantification of ZEA was done by comparison of the retention time (9.5 min) and peak area observed in the ZEA standard with those observed for samples (Figure 1a).

For analysis of ZAL, method of James *et al*. (1982) was modified and used. In this method, a variable wavelength absorbance UV detector set at 236 nm was used. The mobile phase consisted of methanol : water (75:25 v/v) and was used at a flow rate of 1 ml/min. Injection volume for extract solution was 2, 3 and 5 µl for different samples. Analysis was performed at room temperature (25 - 30°C) and quantification was done by comparison of the retention time (5.8 min) and peak area observed in the ZAL standard with that observed in samples (Figure 1b).

Quantitative analysis of deoxynivalenol (DON) was done by following the method of Golinski *et al*. (1996) after modification. In this case, a variable wavelength absorbance UV detector set at 229 nm was used. The mobile phase consisted of methanol: water (85:15 v/v) at a flow rate of 1 ml/min and the retention time was 2.94 min. Injection volume for extract solution was 5 and 7 µl for different samples. Analysis was performed at room temperature (25-30°C) and quantification of DON was done by comparing the retention time and peak area observed in the DON standard with that observed in samples (Figure1c).

3. Results

Detection of zearalenone

Zearalenone was detected as an important toxic contaminant in some of the investigated samples. From a total of 30 samples of select grade of walnuts that were screened for this fusarial toxin, 63.33% were found to be positive. Quantitative analysis by HPLC showed that the range of contamination in the ZEA positive samples varied between 1.11 and 9.57 µg/g (Table 1). Earlier, Abdel-Hafez and Saber (1993) reported 125µg of ZEA/kg in walnuts from Egypt. But in a survey conducted in Germany, ZEA has been detected in small amounts in various fruits and nuts (Schollenberger *et al.*, 2005).

Detection of zearalenol

Zearalenol is an important secondary metabolite produced by various *Fusarium* species. HPLC analysis of in-shell walnut kernels showed that only 13.33% of samples were contaminated with ZAL and the amount of contamination varied from 5.42-11.49 µg/g of the sample. So far, there is no report of ZAL contamination from walnuts and other nuts but it has been frequently reported from other food products (Richardson *et al.* 1984).

Detection of deoxynivalenol

Deoxynivalenol, also known as vomitoxin or DON, is produced by various *Fusarium* species (Golinski *et al.*, 1996; Hossein and Bagheri, 2012). DON belongs to the class trichothecenes of the type B group. It is most widely distributed fusarial toxin and is associated with nervous system disturbance, irritation of the gastrointestinal tract and haemorrhage in the consumers (Rotter *et al*., 1996). As depicted in table 1, only one sample of in-shell walnut kernels was detected to be positive for this contaminant. However, through HPLC analysis, this sample was detected to possess high level of DON contamination (Table 1). Occurrence of DON in food and feed is also potential marker of the occurrence of other mycotoxins (Sobrova *et al.*, 2010).

Co-occurrence of fusarial toxins

Perusal of data given in table 1 also shows that three samples (IS-7, IS-14 and IS-23) of in-shell walnut kernels showed co-occurrence of zearalenol and zearalenone. Among these two fusarial toxins, detected amount of ZAL contamination was more than that of ZEA (Figure 2). ZAL is known to co-occur naturally in dried commodities with other fusarial toxins and is about three to four times more oestrogenic than ZEA and may contribute to hyper-estrogenism (Hagler *et al*.,1979). In the present investigation, only one sample (IS-5) of in-shell walnuts showed co-occurrence of DON and zearalenone (Figure 2). Similar combination of fusarial toxins has been observed in different plant products by various workers (Fazekas *et al*., 1996; Gonzalez *et al*., 1999; Sardjono *et al*., 1998).

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4. Discussion

During the present investigation, very high levels of ZEA were detected from the samples of in- shell walnut kernels (1.11-9.57µg/g), which are popularly consumed in all parts of India. In view of the oestrogenic activities of ZEA (Herrman and Trigo-Stockii, 2002), its detection in walnut kernels may prove dangerous to the consumers. From J&K State, ZEA contamination has not been so far reported from walnuts but it has been reported from some dried herbals (Koul and Sumbali,2008) and dried vegetables (Sodhi and Sumbali, 2012). ZEA has been shown to be hepatotoxic, immunotoxic, genotoxic and haemotoxic (Lioi *et al.*, 2004; Maaroufi *et al.*, 1996; Zinedine *et al.*, 2007). Although ZEA is ubiquitous and toxic, it is dangerous for human and animal health, only when it is absorbed in high amounts or over long time of exposures (Zinedine *et al.*, 2007). Gray *et al*. (2004) discovered oestrogenic effects caused in some post-menopausal woman due to ZEA contamination in ginseng products. Some studies have even demonstrated that ZEA has the potential to stimulate growth of human breast cancer cells containing estrogen response receptors (Ahamed *et al.*, 2001, Yu *et al.*, 2005). The International Agency for Research on Cancer (International Agency for Research on Cancer, 2002) has classified zearalenone as group 3 carcinogen. The Joint FAO/WHO Exert Committee on Food Additives (JECFA) has estabilished a provisional maximum tolerable daily intake for ZEA to be 0.5 µg /kg body weight (The Joint FAO/WHO Expert Committee on Food Additives, 2000). In view of the reported toxicity, such high amounts of ZEA from walnut kernels may prove a health hazard if preventive steps are not taken into consideration.

Detection of very high amount of ZAL in some samples suggests that walnut kernels are good substrate for its production. Some of the kernel samples even showed co-occurrence of zearalenol and zearalenone (Figure 2). ZAL is known to co-occur naturally in dried commodities with other fusarial toxins and is about three to four times more oestrogenic than ZEA and may contribute to hyper-estrogenism (Hagler *et al*.,1979). Zearalenol metabolites cause cytotoxicity by inhibiting cell viability, protein and DNA synthesis and inducing oxidative damage and expression of stress proteins (Othmen *et al.*, 2008). Just like ZEA, the total intake for this metabolite should not exceed 0.5 µg/kg of body weight (Codex Committee on Food Additives and Contaminants, 2000).

Detection of DON in one of the samples of walnut kernels again suggests its susceptibility as a substrate for fusarial toxins. Presence of DON in walnut kernels is quite alarming as it can affect the immune system of consumers and make them susceptible to microbial diseases. So far, there is very little information on the contaminant DON in nuts and dried fruits (Boutrif and Caner, 1998, Council for Agricultural Science and Technology, 2003; Food and Agriculture Organisation, 1997) but it is the most widely distributed fusarial toxin reported from other dried agricultural commodities (Bhat *et al*., 2010; Gouze *et al.*, 2006; Koul and Sumbali, 2008; Sobrova *et al.*, 2010; Sodhi and Sumbali, 2012). In the present investigation, walnut sample showing contamination of DON also showed co-occurrence of ZEA (Figure 2).Thuvander *et al*.(1999) studied the combined exposure of trichothecenes and concluded that it resulted mainly in additive, antagonistic and synergistic effects. Canady *et al.* (2001) reported that the World Health Organization Joint Expert Committee on Food Additives has evaluated the safety of DON for human, and it has established a provisional maximum tolerable daily intake (PMTDI) for DON to be 1- µg/kg body weight.

5. Conclusion

Detection of three most important fusarial toxins (ZEA, ZAL and DON) from some of the investigated samples suggests that in-shell walnut kernels are quite susceptible to their formation. In view of these observations, the in-shell walnut kernels are not completely safe for human consumption. Although very few samples showed co-occurrence of fusarial toxins, yet their accumulation is expected to create adverse health problems to the consumers. Therefore, levels of these toxins in walnuts should be kept below the permissible limits by using post-harvest management strategies that could restrain fungal growth and formation of their toxic metabolites. In addition, frequent analytical surveillance programme by food control agencies is highly recommended to control the incidence of mycotoxin contamination in walnut kernels.

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Table 1: Zearalenone (ZEA), Zearalenol (ZAL) and Deoxynivalenol (DON) contamination detected from the in-shell kernels of walnuts

In-shell kernels	*FUSARIAL TOXINS (µg/g)*		
	Zearalenone (ZEA)	*Zearalenol (ZAL)*	*Deoxynivalenol(DON)*
IS-1	-	-	-
IS-2	-	-	-
IS-3	-	-	-
IS-4	-	-	-
IS-5	5.890	-	29.342
IS-6	-	7.326	-
IS-7	3.269	11.124	-
IS-8	-	-	-
IS-9	-	-	-
IS-10	2.201	-	-
IS-11	9.572	-	-
IS-12	9.396	-	-
IS-13	-	-	-
IS-14	4.107	5.421	-
IS-15	4.660	-	-
IS-16	2.943	-	-
IS-17	6.161	-	-
IS-18	3.316	-	-
IS-19	4.220	-	-
IS-20	5.080	-	-
IS-21	3.863	-	-
IS-22	-	-	-
IS-23	3.919	11.490	-
IS-24	4.536	-	-
IS-25	1.110	-	-
IS-26	-	-	-
IS-27	-	-	-
IS-28	-	-	-
IS-29	-	-	-
IS-30	-	-	-
Positive samples (%)	63.33	13.33	3.33

-, not detected

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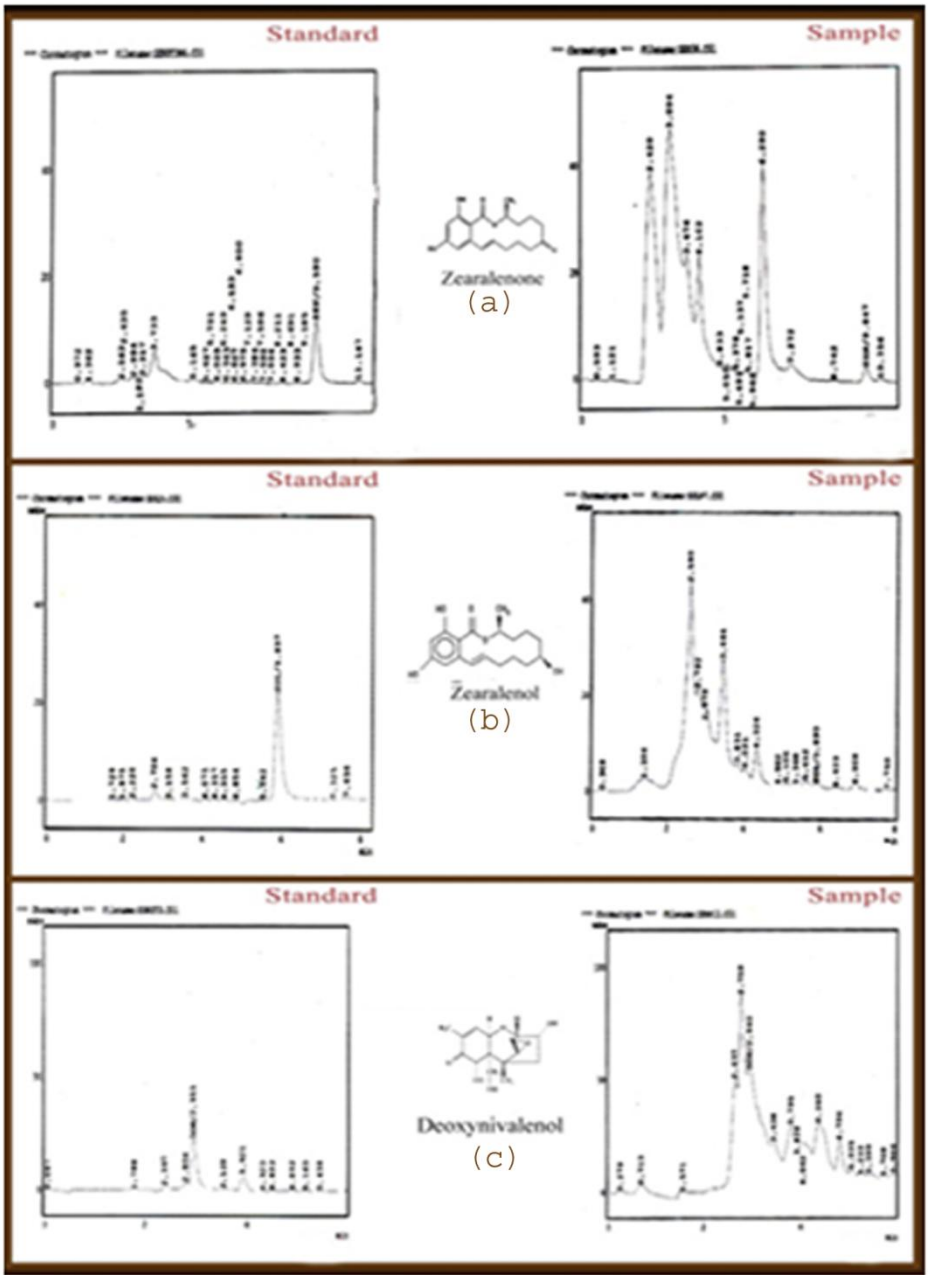

Figure 1: HPLC chromatograms of ZEA, ZAL and DON.

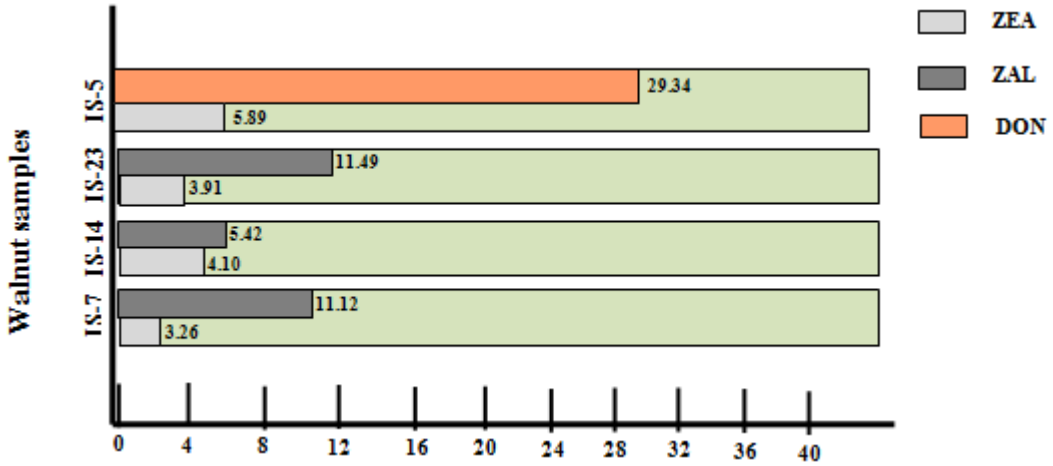

Figure 2: Co-occurrence of zearalenone (ZEA) and zearalenol (ZAL) and deoxynivalenol (DON) detected in the in-shell walnut kernels.

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