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Comparing Determination of Deoxynivalenol in Cereals by HPLC Using Two Different Clean-Up Methods

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Abstract: Two different clean-up methods for determination of Deoxynivalenol (DON) in cereals by liquid chromatography with DAD detection were compared. The efficiencies of two clean-up principles for rice extracts were compared: solid-phase and immunochemical extraction. There are advantages and disadvantages with both methods. In aspect of recovery, Immunoaffinity Columns (IAC) seemed to be more efficient but Solid Phase Extraction (SPE) column are more precise. Although both methods meet the requirements for reliability, for unknown matrices SPE method is somehow unreliable and has false positives. In terms of price SPE is less expensive because of self-made columns but IAC clean-up method is a specific method.

Key words: Mycotoxins, deoxynivalenol, clean-up methods, immunoaffinity columns, solid-phase extraction columns

INTRODUCTION

The determination of mycotoxins in food and animal feed is of great importance because of their variety of toxic effects on humans and animals. Since, the 1960s analytical methodology for mycotoxins has been developed considerably due to the death of 100 000 turkey poults in Great Britain as a result of aflatoxin-contaminated feed (Liu *et al*., 1985).

Deoxynivalenol or vomitoxin is a member of the trichothecene family of mycotoxins The occurrence of DON is associated primarily with *Fusarium graminearum* and *Fusarium culmorum*, both of which are important plant pathogens commonly found in cereal and other crops (JECFA, 2001). This mycotoxin occurred worldwide in wheat, barley, corn, rice and oats (Bhat *et al*., 1989). The consumption of contaminated feeds by livestock has been associated with a variety of adverse health effects including feed refusal and vomiting (IARC/WHO, 1993/1994; Kuiper-Goodman, 2002).

DON is one of the polar trichothecenes with a molecular weight of 296.32. It contains one primary and two secondary hydroxyl groups and is soluble in water and polar solvents such as methanol and acetonitrile. Unlike many of the other trichothecenes the molecule contains a conjugated carbonyl system and these results in some UV absorbance that assists its detection by TLC or HPLC methods.

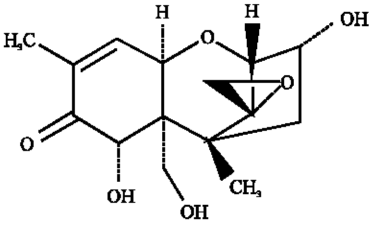

Fig. 1: Deoxynivalenol(12,13-epoxy-3α,7α,15-trihydroxy-trichothec-9-ene-8-one)

For the determination of DON, several methods are applied, which are preceded by a sequence of operations including sampling, sample preparation, extraction and clean-up. The results of the most sophisticated chromatographic procedure will be determined by the efficiency of these steps. As a large number of interfering compounds originally present in samples contaminate the primary sample extracts, these components must be removed as completely as possible.

Many analytical procedures have been developed for determining DON in foods and feeds, including several liquid chromatography techniques (LC), gas chromatography, thin layer chromatography and enzyme-linked immunosorbent assays (Kotal and Radova, 2002; Scott *et al*., 1981; Fernandez *et al*., 1994; Usleber *et al*., 1992). The most frequently used techniques today are liquid and gas chromatography. Different authors,

Corresponding Author: Zohreh Pouretedal, Institute of Standard and Industrial Research of Iran, P.O. Box 15855-484, Tehran, Iran however, present different data in respect to the determination procedure using the same method, as well to the accuracy and precision of the method.

For any method to be used for enforcement purposes and in routine control laboratories, it should be reliable, simple, not too labor intensive and not too expensive.

In the present research, the advantages and disadvantages of both clean-up methods under identical instrumental condition will be discussed in terms of accuracy, precision, reliability, selectivity/specificity and complexity. This comparison has been done to choose the best method for a food quality laboratory in which all methods should be not only reliable but also economical. It will be shown how both SPE and IAC clean-up methods can be useful in routine analysis.

MATERIALS AND METHODS

All experiments were performed in Marjaan Khatam laboratory located in Tehran.

All the solvents used for the DON extraction from samples was of analytical grade, all the solvents used for elution as well as for the preparation of the mobile phase were of LC grade.

The solutions were prepared in doubly deionized water except when stated otherwise. Deoxynivalenol was purchased from Biopure (Tulln, Austria). Stock standard solution was prepared in MeCN and the concentration of solution was determined using spectroscopic method (Usleber *et al.*, 1992).

DON spiking solution (100 μg mL^{-1})- was prepared by diluting the above solution in a 5 mL volumetric flask. It was stored in freezer when not in use.

DON working standard (10 μg mL^{-1})-100 μL^{-1} of spiking solution is dried in a 40°C water bath under stream of air and then 10 mL of mobile phase was added to have a solution of 10 μg mL^{-1}. Working Calibrants-were prepared according to Table 1.

The standard solutions were stored at 4°C. Activated charcoal (Darco G-60, Sigma-Aldrich, Steinheim), Alumina (70-230 mesh, Merck, Darmstadt) and Celite (545, Merck, Darmstadt), were used as received.

Apparatus: The equipment consisted of a LC system-Waters and a Chromolit column (100 _ 4.6 mm i.d., particle size 5 μm, Merck KGaA, Darmstadt).

SPE column was prepared in the following way: A plug of glass wool was inserted into the tapered end of a 3 mL plastic syringe; then 2 g mixture of activated charcoal, alumina and Celite (7:5:3) were added, then a piece of glass wool was entered and was pushed.

Table 1: Preparation of working calibrants

Working calibrant	C (ng mL^{-1})	Working standard (μL)	Mobile phase (μL)
1	5000	1000	1000
2	2500	500	1500
3	2000	400	1600
4	1000	200	1800
5	500	100	1900
6	100	20	1980

DONPREP Immunoaffinity column was purchased from R-Biopharm Rhone. High speed blenders were purchased from Warring, USA.

Procedure

Preparation of spiked rice sample: One kilogram rice sample (blank material, DON at <0.1 μg g^{-1}) was prepared by grinding in a laboratory mill to pass a 1.0 mm sieve and subsequently well mixed. The rice sample prepared in this way was then stored at 5°C. Prior to analysis, 25.0 g of the sample was measured into a blender then; the rice sample was spiked with a known volume of stock standard solution of DON, kept at room temperature for 120 min and then extracted.

Extraction and clean-up

IAC: Twenty five gram test portion of ground laboratory sample was weighed into a high speed blender and DON was extracted from sample with 200 mL water after 2 min. The extract was filtered through a glass fiber filter paper. Two mililiter of filtered extract was then passed through the IAC. Using a positive manifold, the extract passed through the column at a rate of 1 drop sec^{-1}. before the IAC become dry, it was washed with 5 mL water. Using air, it was dried. Then the column was eluted into a vial with 1.5 mL of methanol at a rate of 1drop sec^{-1} (MacDonald *et al.*, 2005).

SPE: Twenty five gram test portion of ground laboratory sample was weighed into a high speed blender and DON was extracted from sample with 100 mL MeCN 84% after 3 min. The extract was filtered through a glass fiber filter study. Five mililiter of filtered extract was then passed through the SPE column. Using a positive manifold, the extract passed through the column at a rate of 1 drop sec^{-1} and collected in a vial. Then 2.5 mL of MeCN 84% was passed and collected (Abramovic *et al.*, 2005).

After filtration, regardless of which column was used, the eluate was evaporated just to dryness, the residue was redissolved in 1 mL mobile phase and analyzed by reversed-phase LC with DAD detection.

The optimal mobile phase was found in literature that a mixture of acetonitrile-water, was the most frequently used mobile phase (Cahill *et al.*, 1999; Sano *et al.*, 1987; Mateo *et al.*, 2001).

It has been shown that more MeCN percentage in mobile phase results in less retention time but at the same time the reduction of resolution. So, it was decided to use MeCN 10% as mobile phase.

HPLC operating condition- with a chromolith column (4.6*150 mm with 5 μm particle size)and MeCN (10%) as mobile phase, flow rate of 2 mL min^{-1} found to be appropriate. The HPLC condition was fixed for both methods.

The linearity of the method was assessed by standards ranging in concentration from 100-5000 ng mL^{-1} of DON (6 points in total). A typical calibration equation was as follows:

$$\text{AUC} = 1129\ C_{\text{DON}}\text{-}1844 \qquad r = 0.9999$$

where, C_{DON} is the concentration of DON in ng mL^{-1}.

It should be stated that the calibration standards were injected daily, as the goal of this research was to evaluate both methods for routine analysis.

The within-assay precision was evaluated by 6 repeated for 200 μL injection, separate measurements of a standard solution of DON (1000 ng mL^{-1}). The Relative Standard Deviation (RSD) of the peak areas was less than 1%. The detection limit, measured as the signal-to-noise ratio (3:1), was 100 ng mL^{-1} for DON in final injection solution.

RESULTS AND DISCUSSION

The principle of solid-phase extraction is based on adsorption and ion-exchange process whereby nearly all-interfering substances are retained on the column, while the cleaned-up extract passes through. Solid-phase extraction columns were self prepared column containing activated charcoal, alumina and celite. Figure 2a as can be seen, this column proved to be efficient in the elimination of interfering substances from the matrix. Recoveries were found to be within 69-115% for different spiking levels.

The principle of IAC column clean-up is based on the specific bonding of mycotoxins (in this case of DON) to monoclonal antibodies bonded to an appropriate substrate (Scott and Trucksess, 1997).

As can be shown (Fig. 2b), the efficiency of the removal of interfering matrix substances removal was somewhat better in this case, since the base line was lower and a chromatogram more appropriate for DON quantification was obtained. Recoveries were found to be within 77-105% for different spiking levels.

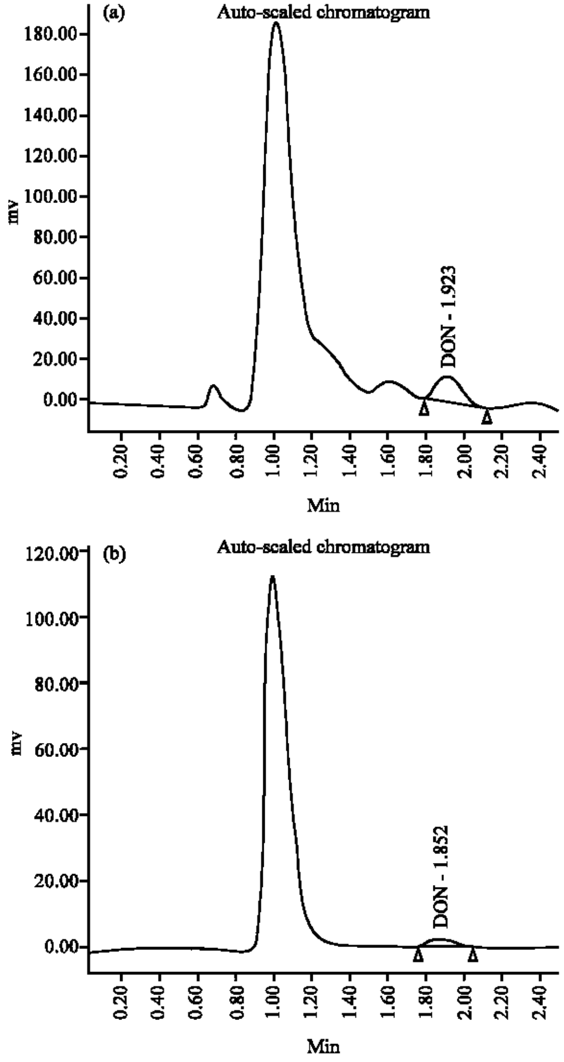

Fig. 2: Chromatograms for 1000 ng g^{-1} spiked sample (a) cleaned up with SPE column and (b) with IAC

Table 2: Recovry tests for SPE and IAC clean-up methods in different spiked samples

Spiking level (ng g^{-1})	Recover for SPE (%)	Recovery for IAC (%)	Requirements (%)
2000	88	102	70-110
1000	76	95	70-110
350	69	76	70-110

Table 3: Trueness comparison of Food Analysis Performance Assessment Scheme (FAPAS) certified reference material for DON

CRM	Certified value (ng g^{-1})	SPE method (ng g^{-1})	IAC method (ng g^{-1})
FAPAS T2214	898.5	917.5	925.3

Accuracy: To compare accuracy of two methods, two different ways were applied. Identical samples were spiked in different levels and the resuls are tabulated in Table 2.

Table 4: Precision assessment of two clean-up methods at different spiking levels

Spiking level	RSD for 500 (ng g^{-1})		RSD for 1000 (ng g^{-1})		RSD for 2000 (ng g^{-1})			
Method	SPE	IAC	SPE	IAC	SPE	IAC	n	*Regulations (%)
Within day	0.6	4.7			0.65	4.1	3	20
Between days			4.8	11.7			3	40

* Usleber *et al.* (1992)

The recovery was determined at 3 levels, 350, 1000 and 2000 ng g^{-1}. The results were reliable for both methods. It means that the trueness is sufficient for both methods, although IAC results seem to be slightly better. This is probably caused by the retention of some DON in SPE column.

The second step to evaluate the trueness of methods was, using CRM. The results are shown in Table 3.

The matrix of CRM was Wheat and both methods showed excellent performances.

Precision: The precision of method was evaluated in terms of repeatability (within day) and reproducibility (between days). The results are tabulated in Table 4. for each level 3 different sample were analyzed. For between days analysis each of these sample were analyze in a day, all in 3 successive days.

Both Methods meet the criteria. Although, the data is not enough for precise judgment, but it seems that SPE method is more precise in both situation of repeatability and reproducibility. It may be because of more dilution factor which IAC clean-up method is encountered.

Cost: In terms of organic solvent consumption, water is used with IAC instead of a MeCN-water (84:16) mixture, as a consequence, it is a green way and also it should be considered that the price of extraction is lower in case of IAC than SPE column, although the price of column itself is the main determining factor. IAC is more expensive than SPE. It means that the total cost of IAC is higher than SPE and if there is no significant difference between accuracies of 2 methods, use of SPE is preferable.

Selectivity/specificity: There is a failure with SPE that SPE columns are not specific and situation may happen that a matrix component that is not retained in the column be co-eluted with DON. This case just happened when a sample of sweat corn was analyzed with both methods. A large peak was observed in the DON retention time but no meaningful recovery was found. The same sample was analyzed with IAC and recovery was 77% and the sample was DON free. It showed that SPE is a false positive method that can not be relied on for some matrices.

Labor and time consumption: There is no difference in extraction step but the solvents water is safer for operator. According to clean-up process IAC column is sightly better than SPE, because the solvents which must be dried, are different in type and volume. With SPE 7.5 mL MeCN 84% must be dried, but with IAC 1.5 mL MeOH must be evaporated. It takes more time for drying 7.5 mL MeCN 84% than 1.5 mL MeOH.

CONCLUSION

The efficiencies of 2 clean-up principles for rice extracts were investigated: solid-phase and immunochemical extraction. Both have positive and negative aspects. It was found that there is no significant difference in efficiencies, except for some false positives of SPE. It can be concluded that SPE method is appropriate for routine analysis because of costs, but always when there is a positive response (more than limits), IAC clean-up method should be applied.

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