FOOD CHEMICAL CONTAMINANTS

Determination of Deoxynivalenol in Cereals and Cereal Products by Immunoaffinity Column Cleanup with Liquid Chromatography: Interlaboratory Study

Susan J. MacDonald, Danny Chan, and Paul Brereton
Central Science Laboratory, Sand Hutton, York, YO41 1LZ, United Kingdom
Andrew Damant and Roger Wood
Food Standards Agency, Aviation House, 125 Kingsway, London, WC2B 6NH, United Kingdom

Collaborators: K. Dao Duy; I. Felgueiras; T. Feron; M.-P. Herry; E. Ioannou-Kakouri; P. Koch; R. Krska; P. Majerus; J. Nesbit; M. Reuter; S. Stangroom; P. Symonds; R. Weber

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An interlaboratory study was performed on behalf of the UK Food Standards Agency to evaluate the effectiveness of an immunoaffinity column cleanup liquid chromatographic (LC) method for the determination of deoxynivalenol in a variety of cereals and cereal products at proposed European regulatory limits. The test portion was extracted with water. The sample extract was filtered and applied to an immunoaffinity column. After being washed with water, the deoxynivalenol was eluted with acetonitrile or methanol. Deoxynivalenol was quantitated by reversed-phase LC with UV determination. Samples of artificially contaminated wheat-flour, rice flour, oat flour, polenta, and a wheat based breakfast cereal, naturally contaminated wheat flour, and blank (very low level) samples of each matrix were sent to 13 collaborators in 7 European countries. Participants were asked to spike test portions of all samples at a range of deoxynivalenol concentrations equivalent to 200–2000 ng/g deoxynivalenol. Average recoveries ranged from 78 to 87%. Based on results for 6 artificially contaminated samples (blind duplicates), the relative standard deviation for repeatability (RSD_r) ranged from 3.1 to 14.1%, and the relative standard deviation for reproducibility (RSD_R) ranged from 11.5 to 26.3%. The method showed acceptable within-laboratory and between-laboratory precision for all 5 matrixes, as evidenced by HorRat values <1.3.

Deoxynivalenol (DON), also known as vomitoxin (3α,7α,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one, CAS No. 51481-10-8), is from a family of mycotoxins known as trichothecenes, which are produced by the *Fusarium* mold species. *Fusaria*, common soil fungi, are known to infect a wide variety of crops (e.g., *Fusarium* Ear Blight), including wheat, barley, oats, and maize. DON is the most prevalent of the trichothecenes and has been found in high concentrations in America and Europe (1). Feed refusal and vomiting are the classic symptoms of DON toxicoses in farm animals (2).

There is as yet no legislative control in the European Union (EU) on the maximum permitted level of DON in cereals. However, several EU countries have adopted an industry standard of 500 μg/kg for retail cereal products and 750 μg/kg for raw untreated cereals. A temporary tolerable daily intake (t-TDI) of 1 μg/kg bw/day has been set for DON by both the European Scientific Committee on Food (SCF; 3) and the JOINT FAO/WHO Expert Committee on Food Additives (JEFCA; 4). The report of the EU Scientific Cooperation on Questions Relating to Food (SCOOP) task on the collection of occurrence data of *Fusarium* toxins was recently published (5). A general conclusion was that although dietary intakes of *Fusarium* toxins are generally less than the proposed TDI values, there is a lack of harmonization in terms of sampling and analytical methodology for these toxins which could influence the reliability of results.

Limits for deoxynivalenol are currently under discussion within the European Commission and Member States. Current proposals are a limit of 1000 ng/g for unprocessed cereal, 1500 ng/g for unprocessed durum wheat (and possibly maize), 500 ng/g for foodstuffs for direct human consumption, 750 ng/g for dry pasta, 200 ng/g for breakfast cereals and snacks, and 100 ng/g for cereal-based infant food (6). The European Committee for Standardization (CEN) Working Group on mycotoxins has identified the need for an analytical method for deoxynivalenol that has been validated according to internationally recognized protocols and meets previously agreed minimum performance criteria (7). The acceptable performance characteristics [relative standard deviations for

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Corresponding author's e-mail: s.macdonald@csl.gov.uk.

Table 1. Statistical analysis of interlaboratory trial results for DON in oat flour, rice flour, wheat flour, polenta, and wheat-based breakfast cereal

Matrix	Avg., ng/g	No. sets of results[a]	r	S_r, ng/g	RSD_r, %	R	S_R, ng/g	RSD_R, %	No. outlier labs	HorRat value (HO_R)	Spike level, ng/g	Recovery[b], %
Oat flour	1768	10	153.8	54.9	3.1	685.3	244.8	13.8	2 (4, 13)	0.9	2000	78
Rice flour (1)	458	11	83.6	29.9	6.5	146.9	52..5	11.5	1 (13)	0.6	500	84
Rice flour (2)	85.4	10	33.7	12.0	14.1	35.5	12.7	14.8	2 (10, 13)	0.7	500	84
Wheat flour	678	12	113.7	40.6	6.0	309.3	110.05	16.3	0	1.0	1000	82
Polenta	123	11	22.1	7.88	6.4	79.6	28.4	23.1	1 (4)	1.1	200	87
Breakfast cereal	217	12	80.6	28.8	13.2	160.0	57.1	26.3	0	1.3	500	74

[a] Participants' results included in the statistical analysis after noncompliant laboratories and outliers were removed.

[b] Recovery values obtained by participants spiking low-level material provided at prescribed levels.

repeatability (RSD_r) and reproducibility (RSD_R)] for DON are $RSD_r \leq 20\%$, $RSD_R \leq 40\%$, and recovery of 70–110% for DON levels >100 ng/g. Although many methods have been published for DON, currently there are no fully validated methods that meet these criteria. A gas chromatographic method was accepted as AOAC final action in 1990; however, RSD_r values ranged from 23 to 48.3%, and RSD_R values from 30.9 to 54.0% for both spiked and naturally contaminated samples, falling outside the acceptable performance characteristics set by CEN (8). RSD_R values outside this range indicate the method is prone to generating false-positive and false-negative results. A thin-layer chromatographic method was also adopted Final Action in 1990; however, in addition to the repeatability and reproducibility not meeting the CEN performance criteria, the method is applicable only at levels >300 ng/g (9). Both methods are applicable only to wheat.

Clearly there is a need for a fully validated method applicable over a wide concentration range and to a variety of matrixes. In addition, for the method to be used for enforcement purposes and in routine control laboratories, it should be simple, not too labor-intensive, and should avoid the use of expensive or highly specialized equipment. One of the main contributing factors to the recent improvements in mycotoxins methodology has been the introduction of monoclonal antibodies used in immunoaffinity columns (IAC; 10). The use of IACs for cleanup, following extraction of matrixes and prior to liquid chromatography (LC), provides a robust methodology that can be automated and offers good specificity. By removing a large number of co-extractives, much improved limits of detection (LODs) can be readily achieved. The candidate method tested in this study uses an IAC cleanup followed by LC with UV detection, targeted at DON levels from 100 to 2000 ng/g in a range of cereal-based matrixes. The LOD of the method, determined by the coordinating laboratory, and based on a signal to noise (S/N) ratio of 3:1, is 30 ng/g.

Interlaboratory Study

Test Materials

Wheat flour, rice flour, polenta, oat flour, and wheat-based breakfast cereal, both naturally contaminated and low level (all ≤50 ng/g), were procured from commercial sources. In all cases the naturally contaminated material was initially milled before being blended. To achieve the target concentrations, a portion of low-level sample was contaminated with a known amount of DON solution. This was left overnight and then tumble mixed with additional low-level material to produce a homogenous sample at the desired concentration. After thorough homogenization about 30 g of the homogenized flour was packed into aluminum foil laminate sachets and heat-sealed. A familiarization exercise was performed using a naturally contaminated homogenized wheat flour. This was packed in 50 g portions in foil sachets. The samples were labeled with 2 series of numbers (not sequential) for each material to provide blind duplicates. Low-level test materials of wheat flour, rice flour, polenta, oat flour, and wheat-based

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breakfast were each thoroughly homogenized before being weighed into foil sachets and sealed. These were labeled "Low Level" material 1–5 to indicate the sample intended for spiking. The test materials were stored at –20°C until dispatch. For distribution the samples were packed into padded bags and mailed to participants. Participants acknowledged receipt of the samples upon their arrival.

Homogeneity Testing of Packaged Material

For each test material (contaminated and low level) at least 7 foil sachets (maximum of 10) from each batch of packaged material were removed for homogeneity testing. Sixty sachets of the naturally contaminated test material containing DON and 100 sachets of the low-level material were prepared for the familiarization exercise; about 50 g was packed into each sachet. Sixty sachets each of contaminated and low-level materials were prepared for the main trial; about 30 g test material was packed into each sachet. All samples were kept frozen at –20°C prior to homogeneity testing. The contents of each sachet were analyzed in duplicate at the author's laboratory using the initial proposed interlaboratory trial method, but taking only half the sample size.

Data produced from the homogeneity testing were evaluated by means of analysis of variance (ANOVA; 11). From the results of the statistical evaluation, all the test materials were demonstrated to be homogenous; as in each case the calculated *F*-value was less than the *F*-critical value. Thus, the results of the ANOVA without any outlier exclusion showed that the difference of the "Between-Group Variance" and the "Within-Group Variance" regarding all materials was not significant. Therefore, the Between-Sample Standard Deviation was negligible. It was concluded that the test materials could be considered homogenous at a minimum sample intake of 10 g for main trial samples and 20 g for the familiarization sample.

Organization of Interlaboratory Study

Thirteen collaborators from 7 different European countries representing a cross section of government, food control, and contract laboratories took part in the interlaboratory study. The study was planned to be performed in 2 stages: (*1*) a familiarization study initially with only wheat flour material would be distributed; and (*2*) after these results were processed a second stage would distribute a second wheat flour, 2 rice flours, polenta, oat flour, and a wheat-based breakfast cereal.

For the first stage each collaborator received one test material of wheat flour to be analyzed in duplicate by the prescribed method. Participants were also sent 2 low-level wheat flour test materials to be used for spiking and blank correction purposes. Each participant was also supplied with a set of instructions, a copy of the method to be followed, and a results proforma. Following analysis of the results of this exercise it was clear the method needed modification. The extraction solvent was changed and the familiarization exercise was repeated for the modified method using the same test material and participants.

For the second stage of the study the same set of participants received (*a*) 12 coded samples of flour (blind duplicates at 6 content levels) plus 10 labeled low-level samples for spiking and blank correction purposes; (*b*) a copy of the method; (*c*) a set of detailed instructions; (*d*) a report form for analytical data, criticisms, and suggestions; (*e*) an interlaboratory study materials receipt form. Each participant was required to prepare one extract from each material, perform the cleanup, and analyze the extracts by LC. Participants were asked to spike one portion of each of the low-level test materials 1–5 at levels from 200 to 2000 ng/g DON following the instructions given. After adding the spike solution, participants were instructed to let the samples stand for at least 40 min to allow the solvent to evaporate before extraction. Unspiked portions of low-level samples were also analyzed to facilitate recovery correction calculation for the test materials.

Statistical Analysis of Results

The results of the 2 stages of the study were examined for evidence of individual systematic error ($P < 0.025$) using Cochran's and Grubbs tests progressively, by procedures described in the internationally agreed Protocol for the Design, Conduct and Interpretation of Method-Performance Studies (12). Calculations for repeatability (r) and reproducibility (R) as defined by that protocol (12) were made on those results remaining after removal of outliers. When assessing a new method there is often no validated reference or statutory method with which to compare precision criteria; hence, it is useful to compare the precision data obtained from a collaborative trial with predicted levels of precision. These predicted levels are calculated from the Horwitz equation. Comparison of the trial results and the predicted levels indicates whether the method is sufficiently precise for the level of analyte being measured (13). Historically the Horwitz predicted value has been calculated from the Horwitz equation (13):

$$RSD_R = 2C^{-0.15}$$

where C = measured concentration of analyte expressed as a decimal (e.g., 1 g/100 g = 0.01).

Thompson (14) recently described the use of a modified Horwitz function to predict levels of precision at ng/g and sub-ng/g levels. The use of this function gives an improved statistical representation at these levels. Therefore, for the purposes of this trial the Horwitz predicted value was calculated from the modified Horwitz function $RSD_R = \sigma_R = 0.22C$. The HorRat value (15) compares the actual precision measured with predicted by the Horwitz equation for a method measuring at that particular level of analyte. It is calculated as follows:

$$H_{oR} = RSD_R \text{ (measured)}/RSD_R \text{ (Horwitz)}$$

In the case of this trial $H_{oR} = RSD_R \text{ (measured)}/\sigma_R = RSD_R \text{ (measured)}/0.22C$.

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A HorRat value (Ho^R) of 1 usually indicates satisfactory interlaboratory precision, whereas a value of >2 usually indicates unsatisfactory precision, i.e., one that is too variable for most analytical purposes or where the variation obtained is greater than that expected for the type of method used. Ho_r is also calculated and used to assess intralaboratory precision, using the following approximation:

$$RSD_r \text{ (Horwitz)} = 0.66\ RSD_R \text{ (Horwitz)}$$

(this assumes the approximation r = 0.66R).

For this trial RSD_r (Horwitz) = 0.66 σ_R.

METHOD

(Applicable to determination of DON at 100–2000 ng/g in cereals and cereal products).

Table 1 shows the results of the interlaboratory study for DON in wheat flour, polenta, rice flour, oat flour- and wheat-based breakfast cereal.

Caution: DON is highly toxic. Protective clothing, gloves, and safety glasses should be worn at all times, and all standard and sample preparation stages should be performed in a fume hood.

Principle

Test portion is extracted with water [with or without polyethylene glycol (PEG)]. The extract is filtered and applied to an immunoaffinity column containing antibodies specific to DON. DON is removed from the immunoaffinity column with acetonitrile or methanol and is separated by reversed-phase LC with UV measurement.

Performance Standard for Affinity Column

The immunoaffinity column should contain antibodies raised against DON. The saturation capacity of the columns should be >2500 ng DON. More than 70% DON must be recovered when 25 ng DON is applied in 1–2 mL water (depending on manufacturer's instructions).

Apparatus

(**a**) *Vortex mixer, or equivalent.*

(**b**) *Blender or homogenizer.*

(**c**) *Displacement pipets*.—10, 5, and 1 mL, and 250, 50, and 25 μL capacity with appropriate pipet tips.

(**d**) *Vacuum manifold*.—To accommodate immunoaffinity columns.

(**e**) *Reservoirs and attachments*.—To fit to immunoaffinity columns.

(**f**) *Vacuum pump*.—Capable of pulling a vacuum of 10 mBar and pumping 18 L/min.

(**g**) *Filter paper*.—Pore size 1.6 μm (Whatman GF/A or similar).

(**h**) *LC apparatus*.—Variable injection system, valve injection system with, for example a 1000 μL injection loop (for partial loop injection). Pump, isocratic, pulse free, capable of maintaining a volume flow rate of 1 mL/min. A janitor switching module or second LC apparatus for washing the column. Column oven, (optional) capable of maintaining a constant temperature above any variability caused by fluctuations in the room temperature (e.g., 25° ± 1°C, or ± 0.5°C temperature repeatability and stability).

(**i**) *LC column*.—C18 reversed-phase ODS, which ensures resolution of DON from all other peaks. The maximum overlapping of peaks must be <10% (it might be necessary to adjust the mobile phase for a sufficient baseline resolution). A suitable pre-column of the same composition as the main column should be used.

(**j**) *UV detector*.—Fitted with an analytical flow cell and set at 220 nm; AUFS set at 0.005.

(**k**) *UV spectrophotometer*.

(**l**) *Top pan balance*.—Accurate to 10 mg.

(**m**) *Centrifuge*.—Capable of centrifuging a volume of 250 mL at 4000 rpm.

(**n**) *Centrifuge tube*.—250 mL or equivalent.

(**o**) *Heating block*.—Reacti Therm, or equivalent, set at 40°C with nitrogen gas line.

Reagents

All reagents are analytical grade unless otherwise stated.

(**a**) *Acetonitrile*.

(**b**) *PEG*.—Molecular weight ca 8000.

(**c**) *Deoxynivalenol (DON)*.

(**d**) *Methanol*.

(**e**) *Injection solvent for LC analysis*.—Water–methanol solution (90.5 + 9.5, v/v).

(**f**) *Mobile phase*.—Water–methanol solution (85 + 15, v/v). Degas before use, for example, with helium.

(**g**) *Helium-purified compressed gas*.

(**h**) *Wash solvent*.—Water–methanol solution (1 + 1, v/v).

(**i**) *DON stock solution (ca 250 μg/mL)*.—Add 4.0 mL acetonitrile to accurately weighed 5 mg DON to obtain a 1.25 mg/mL solution. Dilute 800 μL of the 1.25 mg/mL solution to 4 mL with acetonitrile to obtain a standard concentration of ca 250 μg/mL. Add 200 μL of the 250 μg/mL standard to 1.8 mL acetonitrile to obtain a standard concentration of ca 25 μg/mL.

Calibrate the 250 μg/mL solution by recording the UV spectrum of the 25 μg/mL solution from 200 to 270 nm against solvent used for solution in the reference cell. Determine the concentration of the DON solution by measuring A at wavelength of maximum absorption close to 217 nm. Concentration of the 250 μg/mL solution will be:

$$\text{Concn, } \mu g/mL = \frac{1000 \times A \times MW \times 10}{\varepsilon \times \delta}$$

where A = the absorbance of the 25 μg/mL solution; MW = molecular weight of DON (296.3 for DON); ε = molar absorptivity (6400 for DON in acetonitrile) (16); δ = path length of quartz cell (cm).

(**j**) *DON spiking solution (100 μg/mL)*.—Pipet an aliquot of the calibrated 250 μg/mL DON solution equivalent to 500 μg DON in a 5 mL volumetric flask. Dilute to 5 mL total

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Table 2. Preparation of working calibration solutions

Working calibrant	Standard mass concn, ng/mL	Working standard, μL	Equivalent sample concn, ng/g
1	1000	200	2000
2	750	150	1500
3	500	100	1000
4	250	50	500
5	50	10	100

volume with acetonitrile. Store this solution in a freezer or refrigerator when not in use. Allow to reach room temperature before opening. This solution is stable for at least 4 weeks.

(**k**) *DON working solution (10 μg/mL).*—Pipet 500 μL **(j)** in a 5 mL volumetric flask. Dilute to 5 mL total volume with acetonitrile. Store this solution in a freezer or refrigerator when not in use. Allow to reach room temperature before opening. This solution is stable for at least 4 weeks.

(**l**) *Immunoaffinity columns.*—(*See Performance Standard for Affinity Column*.) For example, columns from Vicam (Watertown, MA) and R-Biopharm Rhone (Glasgow, UK) have been found to meet these criteria.

Extraction

Weigh 20 ± 0.1 g test portion of ground laboratory sample into a large centrifuge tube. Add 4 g PEG and 80 mL water, and homogenize at high speed for 3–4 min. Centrifuge the homogenized slurry at 4000 rpm for 15 min, and filter the supernatant through a glass fiber filter paper.

Note: The above procedure was found suitable for Vicam immunoaffinity columns. For R-Biopharm Rhône columns use 20 g test portion and 160 mL water for extraction without addition of PEG.

Immunoaffinity Column Cleanup

The cleanup may be performed by using a vacuum, by positive pressure, or by allowing the specified volumes to pass through the column under gravity. Do not exceed the maximum specified flow rates. Prepare the IAC according to the manufacturer's instructions. Accurately measure 1 mL (or 2 mL following column manufacturer's instructions) of the filtrate and add to the reservoir. Pass the extract completely through the IAC at a rate of ca 1 drop/s. Do not permit the IAC to run dry. Wash the IAC with 5 mL water. Dry the column by pushing ca 5 mL air through it. Place a vial under the IAC. Elute the DON into a vial with 2 mL acetonitrile at a rate of 1 drop/s, or with a suitable solvent following the column manufacturer's instructions. Place the vial in a heating block at 40°C, and evaporate the affinity column eluate to dryness, under nitrogen. Redissolve in the residue in 0.5 mL injection solvent. Mix well on a Vortex mixer to ensure the residue is totally dissolved. Transfer to an LC vial for analysis (V_3).

Note: The cleanup, preparation, and LC steps of this method may be performed by an automated system such as an ASPEC™ (Gilson, Luton, UK), provided that the conditions described in this method, e.g., volumes and flow rates, are adhered to.

LC Determination with UV Detection

(**a**) *LC operating conditions*.—When the column specified in apparatus (**i**), with the dimensions 4.6 × 150 mm with 5 μm particle size and the mobile phase specified in reagents (**f**) were used, the following settings were found to be appropriate: flow rate mobile phase (column), 1.0 mL/min; UV detection wavelength, 220 nm; UV detection, 0.005 AUFS; injection volume, 50–300 μL.

(**b**) *Calibration curve.*—Prepare 5 LC standard solutions in separate 2 mL volumetric flasks according to Table 2. Evaporate the stated amount of working solution (10 μg/mL) just to dryness and dilute each standard to volume (2 mL) with injection solvent. Prepare a calibration graph at the beginning of every day of the analysis. Plot the mass of DON in the aliquot injected against peak area (or height) response.

Calculations

Determine from the calibration graph, the masses in ng of the DON in the aliquot of test solution injected onto the LC column. Calculate the mass fraction of DON, w_{DON}, in μg/kg using the equation:

$$w_{DON} = m_a \times \frac{V_3}{V_4} \times \frac{V_1}{V_2} \times \frac{1000}{m_s} \times \frac{1}{1000}$$

where m_a = the mass of DON in the aliquot of test solution injected onto the column, in ng; V_4 = volume of the aliquot of test solution injected onto the column, in mL; V_3 = volume of the test solution, in mL (V_3 = 1.0 mL); V_2 is the volume of sample filtrate used in cleanup, in mL (V_2 = 1 or 2 mL); V_1 = volume of the extraction solvent, in mL (V_1 = 80 or 160 mL); m_s = the mass of the sample extracted, in g (20 g). Express the final result in ng/g.

Results and Discussion

Interlaboratory Trial Results

Of the 13 laboratories that received the test samples, 12 successfully completed the study. All data submitted for the study are presented in Tables 3 and 4. The data are given as individual pairs of results for each laboratory (identified as 1–13). Participants spiked low-level samples for each matrix with different DON levels. Corresponding low-level samples were also analyzed unspiked. Participants were asked to report a single result each (in ng/g) for the spiked and unspiked samples. The coordinating laboratory calculated recovery values for each participating laboratory, and the spike levels and corresponding recovery values (as means) are reported in Table 1.

Statistical Analysis of Results

Precision estimates were obtained using the one-way ANOVA approach according to the IUPAC Harmonized Protocol (11). Details of the cereal matrix, the average analyte

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Table 3. Collaborative trial results of LC determination of DON in oat flour, rice flour, wheat flour, polenta, and wheat-based breakfast cereal[a]

	DON concentration, ng/g[b]											
Lab ID	Oat	Oat	Rice (1)	Rice (1)	Rice (2)	Rice (2)	Whe	Whe	Pol	Pol	Bkcer	Bkcer
1	1662.0	1664.0	433.5	440.0	91.1	84.9	511.0	572.5	145.0	128.0	286.0	240.5
2	1675.0	1645.0	388.7	451.2	83.4	50.5	822.7	755.0	137.1	124.2	191.5	276.8
3	1939.6	1868.1	532.9	464.7	110.4	89.4	731.4	747.7	153.3	153.1	270.5	301.8
4	1569.6[b]	2014.3[b]	450.5	473.6	92.8	87.2	665.2	657.8	228.0[b]	324.4[b]	173.5	204.4
5	1855.0	1930.0	430.0	417.0	75.0	77.0	652.0	649.0	101.0	97.0	161.0	173.0
6	[c]	[c]	[c]	[c]	[c]	[c]	[c]	[c]	[c]	[c]	[c]	[c]
7	2158.0	2219.9	485.4	536.6	88.3	87.0	756.7	744.5	146.2	148.9	257.3	190.0
8	1652.0	1566.0	476.0	488.0	94.0	86.0	682.0	652.0	126.0	138.0	232.0	204.0
9	2040.0	2100.0	540.0	560.0	100.0	86.0	820.0	860.0	160.0	155.0	300.0	310.0
10	1503.3	1334.3	425.6	358.1	31.1[b]	38.4[b]	810.5	734.7	108.8	89.5	214.7	199.7
11	1540.0	1538.0	418.0	464.0	74.0	86.0	580.0	674.0	120.0	110.0	204.0	174.0
12	1702.0	1774.8	404.6	427.0	97.2	67.2	602.4	658.2	125.4	115.4	215.2	220.4
13	62.8[c]	682.2[c]	171.4[c]	301.0[c]	51.5[c]	54.4[c]	416.9	519.6	57.6	69.6	84.9	128.3
Mean	1768.4		457.5		85.4		678.2		123.1		217.2	

[a] Outliers and noncompliant results were not included in statistical analysis.

[b] Outlier results; Oat, rice, whe, pol, and bkcer = samples of oat flour, rice flour, wheat flour, polenta, and wheat-based breakfast cereal, respectively.

[c] Noncompliant results.

concentration, the RSD_r and RSD_R values, the number of statistical outlier and noncompliant laboratories, the HorRat values, and the percent recovery are presented in Table 1. The interlaboratory trial results were examined for evidence of individual systematic error ($P < 0.025$) using Cochran's, and Grubbs tests progressively (11). Noncompliant results were identified as those where no statistics were possible, such as single results instead of pairs of results, or less than values instead of numerical results being reported. For the results given in Table 3, the maximum numbers of outliers identified were 2 laboratories, giving acceptable data ranging from 10 to 12 laboratories. Results for low level samples 1–5 are given in Table 4.

Comments from Collaborative Trial Participants

The study was performed in 2 stages. Initially, only wheat flour (naturally contaminated and spiked blanks) was analyzed, and comments and results were received. Subsequently, the second part of the study involved other cereals and a cereal-based food (oat flour, rice flour, polenta, breakfast cereal) in addition to another wheat flour, and again comments were invited. The method that was distributed for the first familiarization study used an acetonitrile–water extraction solvent and charcoal–alumina column cleanup prior to immunoaffinity column cleanup. The results of this exercise were unsatisfactory (HorRat value 2.8). This was thought to be due to incompatibility of some batches of immunoaffinity column with residual organic solvent in the extracts applied to the IAC. The method was substantially modified and water only extraction with IAC cleanup was adopted.

As the method had changed, a second familiarization exercise was performed, using the same test material, this time resulting in satisfactory results (HorRat value 1.5). In general, the participants found the method clear and easy to perform. Six laboratories reported problems with the breakfast cereal sample absorbing the extraction liquid, which required them to increase the volume used and take account of this in the final calculation. Despite this, the results for the breakfast cereal were satisfactory (HorRat value 1.3). There was no general pattern to the other comments or observations made by the participants.

The immunoaffinity column brand was not specified for the study. Participants could use the IAC of their choice, provided they met the performance characteristics detailed in the method, and they followed any specific operating instructions for that column. Chromatography column and conditions were not prescribed, but an example of conditions that produced satisfactory separation was given. From information supplied on the results proforma it was found that participants used 2 different types of commercial immunoaffinity columns and a range of LC columns. However, most participants used the same chromatography conditions, although some modified conditions slightly to achieve the desired performance. All participants were asked to include their chromatograms when returning their trial

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Table 4. Collaborative trial results for low-level samples 1 to 5

Lab No.	Low-level 1, polenta	Low-level 2, wheat	Low-level 3, oats	Low-level 4, rice	Low-level 5, Bk cereal[a]
1	<20	<20	<20	<20	<20
2	<50	<50	<50	<50	<50
3	<50	<50	<50	<50	<50
4	63.3	ND[b]	ND	ND	ND
5	<30	<30	63	<30	<30
6	NR[c]	NR	NR	NR	NR
7	<40	40	<40	<40	<40
8	<40	<40	44	<40	<40
9	27	30	<25	<25	<25
10	8.2	26.8	ND	ND	NR
11	<60	<60	<60	<60	<60
12	<33	33.4	<33	<33	<33
13	<30	<30	<30	<30	<30

[a] Bk cereal = Breakfast cereal.
[b] ND = Not detected.
[c] NR = No results.

results. The coordinating laboratory assessed all the chromatograms provided as satisfactory.

Precision Characteristics of the Method

Four participating laboratories were found to be noncompliant by the coordinating laboratory in the familiarization exercise. Failure to return results, procedural errors, and failure to report duplicate results constituted noncompliant data. These results, if reported, were excluded from the calculation of performance criteria. One laboratory did not continue to participate in the main trial, but for the 3 others who did there were no further noncompliant results.

The precision data for all cereal samples in the main trial are summarized in Table 1. Based on results for naturally contaminated wheat flour samples (blind pairs) used in the familiarization exercise, the RSD_r was 3.3%, and the RSD_R was 25.5%. Based on results for fortified samples (blind pairs) used in the main trial, the RSD_r ranged from 3.1 to 14.1%. The RSD_R ranged from 11.5 to 26.3%. The average recovery of DON derived from the cereal samples spiked by participants ranged from 74 to 84%.

Interpretation of Results

The acceptability of the precision characteristics of the method were assessed on the basis of the HorRat values (15), which compare RSD_R at the various levels with those predicted from collaborative trial studies taken from the published literature. The precision values for all the matrixes were well within the satisfactory limits derived by the Horwitz equation (i.e., <2.0). The HorRat values (Ho^R) ranged from 0.6 to 1.3, with 4 out of 6 being ≤1.0. The matrixes with the highest Ho^R were a polenta and a breakfast cereal.

Sensitivity

The LOD of the method determined by the coordinating laboratory and based on S/N ratio of 3:1 was 30 ng/g. Participants reported LODs in the range of 6 to <100 ng/g, but many did not supply information on how the value had been derived. The satisfactory Ho_R value of 1.1 obtained for the polenta sample, which contained a mean level of 123.1 ng/g demonstrates that the method is applicable and reliable at this level.

The method has the advantage of being quick and easy to use and is easily transferable to routine and control laboratories as it avoids the use of specialized equipment. It also has the additional benefit of being environmentally friendly; it does not use large volumes of organic solvents for extraction, thus avoiding the problem and expense of their disposal. It has proved to be robust under the conditions of the interlaboratory study and applicable across a range of cereal matrixes that are likely to be contaminated with DON. The method has improved performance characteristics as demonstrated by the satisfactory Ho_R values, derived from the more stringent modified Horwitz equation (14), and a broader range of applicability than current AOAC Official Methods for DON (8, 9).

Conclusions

This paper reports the first interlaboratory study of an immunoaffinity column method for DON in cereals and cereal products. The method involving IAC cleanup and determination by reversed-phase LC has been successfully validated at ≥100 to ≥2000 ng/g for a variety of cereal matrixes. The method is suitable for enforcement purposes to test compliance with proposed European Directives. It has been shown to have performance characteristics that fulfill European requirements (7) and justify putting the method forward for consideration as a CEN Standard and as a candidate for an AOAC INTERNATIONAL Official Method.

This study forms part of the Food Standards Agency Collaborative Trial Program. In addition to producing validated methods that can be used in the United Kingdom and by the European Commission for enforcement purposes, the Program also addresses wider measurement issues. Further data from the present trial (not included here) examining the issue of recovery will be published elsewhere.

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Illidia Felgueiras and Luísa Calhau, INETI-DTIA, Lisbon, Portugal

Thierry Feron, Contaminants Laboratory Resp., Lestrem, France

Marie-Paule Herry (Nicole Lenetayer), DGCCRF, Rennes, France

Eleni Ioannou-Kakouri and Maria Aletrari (Artemisia Hadjioannou Ralli), State General Laboratory, Cyprus

Priska Koch (P. Good), SQTS, Zurich, Switzerland

Rudolf Krska (Alexandra Parich), IFA Tulln, Tulln, Austria

Paul Majerus (Marita Max), Institut für Lebensmittelchemie, Trier, Germany

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