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Evaluation of rapid test kits for deoxynivalenol (DON)

by

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This report was updated in July 2007 to incorporate further tests on the ROSA (DON) quantitative test under the existing Project Report number and title. The new work carries on from page 47 onwards.

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ABSTRACT

EU limits for Fusarium mycotoxins in grain will come into force on July 1st 2006. The analytical reference method for deoxynivalenol (DON) is time consuming and expensive. Therefore, there is a need for rapid DON detection kits that involve a simpler test protocol yet still provide analytical results upon which decisions can be made. Such test kits have the potential for use at intake points within the grain chain to assure safety of supply into the human or animal feed markets.

This project was initiated to evaluate the suitability of commercially available test kits to screen intake samples for DON and to provide reliable quantitative data rapidly. Specifically, the project set out to provide the cereal processing chain with information on the appropriateness of kits for use in intake situations and, thus, to help cereal processors to meet the requirements of impending legislation.

A set of criteria, against which DON test kits were evaluated, was generated within the project in consultation with the milling industry. These criteria included test sensitivity and reliability, specificity for DON, cost of analysis (including the cost of ancillary equipment) plus speed and ease-of-use of the assay. Thus, each kit was assessed in terms of its practical potential for use as a screening tool at specific points in the cereal chain rather than in terms of a complete, statistically robust validation. Two specific applications within the cereal chain were identified by industry. These were: (i) grain intake where a sample turnaround of < 30 minutes is required and (ii) storage or merchanting facilities where batch analysis prior to delivery is more relevant.

Results from the basic evaluation showed that all test kits were capable of detecting DON in ground wheat samples, i.e. were "fit for purpose," and could be used to screen intake wheat for DON levels.

In order to improve the quality of decision made using any format of rapid test kit, it is recommended that all tests are duplicated. In addition, for quality control purposes a suitable standard (a pure DON chemical or a wheat based check sample of known DON level close to the agreed threshold) should be tested alongside unknown samples to provide a means of monitoring kit and operator performance.

Mycotoxin distribution is not uniform within a sample and therefore careful sampling is essential for reliable DON results.

The best overall performance across the DON concentration range measured was achieved with the microtiterplate format and fully quantitative test kits. These are higher throughput techniques that take longer to produce results and require greater capital investment in instrumentation to measure colour changes and

interpret these in terms of DON concentration. A threshold at between 800 and 1000 parts per billion (ppb) would help to ensure that samples with unacceptably high DON levels (>1250ppb) are screened out of the cereal chain but would also result in rejection of some samples within the limit.

Within this study, semi-quantitative microtiterplate assays were not favoured as they did not meet the speed or ease-of-use requirements of a grain intake situation nor the sensitivity required for either grain intake or storage/merchant application.

Lateral flow devices proved to be very simple to use and require minimal laboratory equipment or technical experience. In order to introduce objectivity into the assessment the inclusion of a low cost reader is a significant advantage. Such kits are particularly suited to rapid sample turnaround situations (e.g. grain receipt point, where they can be used to screen incoming wheat against an agreed threshold), or as a screening tool in the assessment of the extent of immediate risk posed by DON. Problems or disputes would subsequently be confirmed using appropriate fully quantitative measurement. Used as a stand-alone test, an adequate margin of safety would be to operate at one level below that closest to the legal limit. However, such strategy would result in rejection of samples below the limit. This may be compounded by the tendency of this format of test kit to overestimate DON concentration when only a single standard is used.

1. SUMMARY

Fusarium species are important pathogens of cereal crops, but it is the potential to generate trichothecene toxins, particularly deoxynivalenol (DON), that is of specific concern to members of the cereal chain in the UK and worldwide. Concerns were raised within the UK milling and animal feed industries in relation to the increased incidence of *Fusarium* in the 2004 UK wheat harvest. The visible signs of this *Fusarium* infection, i.e. the presence of pink grain, which despite being shown to be a poor predictor of actual mycotoxin levels, was used as a rejection tool by end-users. Processors expressed significant concerns regarding potential mycotoxin levels in this wheat crop and the prospect of raw material purchasing problems in the 2004/2005 season. The specific problems relating to the 2004 wheat crop raised industry awareness of the need to be able to screen incoming raw material quickly and accurately in order to select appropriate parcels of grain for flour milling and animal feed manufacture. In addition, EU limits for *Fusarium* mycotoxins in grain apply from July 1st 2006. For DON in wheat destined for the human food chain, the limits will be 1250 parts per billion (ppb) in wheat, 750ppb in flour and 500ppb in bread. Therefore, from this date cereal producers will be faced with the legal requirement to ensure that raw material entering the marketplace is below the stated legislative limits.

The reference method for measurement of DON is expensive and time-consuming and, therefore, there is a need for suitable kit-based methods that are rapid and simple to use and interpret. Such test kits have the potential for use at intake points within the grain chain to assure safety of supply into the human or animal feed chain. They should provide the required capability to screen processors' raw material in relation to food safety limits quickly and provide the required documentation for traceability purposes.

The majority of rapid test kits for DON detection involve the use of enzyme linked immunosorbent assay (ELISA) technology. Immunoassays are based on the properties of antibodies, produced by the immune systems of animals as a defence response to an invading molecule or micro-organism.

The sample set used in this study was provided by HGCA from their pink grain survey (Hook & Williams, 2004). This valuable resource provided ground sub-samples together with reference trichothecene mycotoxin values, pink grain counts and records of the actual *Fusarium* species found on each sample. This sample set covered a very wide range of DON levels, from 11 to 11,500ppb. The sample which had the highest DON level was deliberately excluded from the test set as this contained significant numbers of pink and mouldy grains and was considered too extreme for measurement of DON analytically. This reduced the DON range to 11-4723ppb, thus providing an excellent test bed for any DON assay. Cross-reactivity with acetylated forms of DON is perceived to be an issue with ELISA kits. Despite the extreme DON levels, the sample set did not contain any material with measurable acetylated DON levels that could be used to test this claim.

This project was initiated to evaluate the suitability of commercially available test kits to screen out intake samples with positive DON levels above the legal limit or to provide reliable quantitative data more quickly. The objectives of the project were: (i) to provide an independent basic evaluation of commercially available test kits for DON measurement in order to provide the cereal processing chain with information on the appropriateness of kits for use in intake situations; and (ii) to help cereal processors to meet the requirements of impending legislation. Legal limits for DON in wheat create a need for due diligence with respect to samples entering the food and feed processing chains from July 2006. The knowledge gained will enable decisions to be taken by suppliers regarding the screening of wheat to provide assurance of safety in this key raw material. The project plan deliberately split the experimental work into two phases. Phase 1, included basic evaluation of all test kits and was carried out to assess "fitness for purpose". Following phase 1 consultation with industry was undertaken. This was key to identifying the kits which most clearly met their specific needs for different applications. In phase 2, the selected kits were evaluated using a larger sample set to investigate repeatability and "between kit batch" variation.

A shortlist of test kits with potential for use at grain intake was produced. Test kits are typically manufactured in one of the following formats:

- Microtiterwell format (commonly 48 or 96 wells) with break-apart microwells that enable the user to test smaller batches.
- Lateral flow devices provided as single strips, similar to pregnancy testing kits, where the extract is simply applied to the test strip and flows through a membrane where it comes into contact with specific antibodies.

The shortlist of test kits included 10 test kits, of which the 8 shown in the following table were evaluated within this project using real samples.

Kit manufacturer	Kit name	Format
R-Biopharm	Ridascreen Fast DON	Competitive ELISA, microtiterwell plate format. Plate or strip reader required for quantification.
	Ridascreen DON Express	Competitive ELISA, microtiterwell plate format. Visual comparison with DON standards. Plate or strip reader required for quantification.
	Ridascreen Quick DON	Immunochromatography in lateral flow device. Visual comparison of test line with photographic image or samples of known DON level to produce semi-quantitative results.
Neogen	Veratox 5/5	Competitive ELISA, microtiterwell plate format. Plate or strip reader required for quantification.
	Agri-Screen for DON	Competitive ELISA, microtiterwell plate format. Visual comparison with DON standards. Plate or strip reader required for quantification.
	Reveal	Immunochromatography in lateral flow device. AccuScan palm reader measures the intensity of the test line to produce semi-quantitative results.
Strategic Diagnostics Inc. (SDI)	MycoChek	Competitive ELISA, microtiterwell plate format. Visual comparison with DON standards. Plate or strip reader required for quantification.
Romer	AgraQuant DON	Competitive ELISA, microtiterwell plate format. Visual comparison with DON standards. Plate or strip reader required for quantification.

A set of criteria against which DON test kits were evaluated was produced by CCFRA in consultation with representatives of the UK milling industry. The criteria used include: sensitivity and reliability; specificity (i.e. no significant cross-reactivity with other trichothecenes); cost per test plus the cost of ancillary equipment; speed of assay; and ease-of-use of the assay. Thus, each kit was assessed in terms of its potential for use as a screening tool at specific points in the cereal chain rather than in true statistical terms. A copy of the actual criteria plus a basic outline of the proposed project was provided to all kit manufacturers. Acceptance of the project conditions was obtained from each manufacturer prior to the evaluation of their test kit.

Results from the basic evaluation carried out in phase 1 of this work showed that all test kits were capable of detecting DON in ground wheat samples, i.e. were "fit for purpose," and could be used to screen intake wheat for DON levels. Kits essentially performed according to the specifications laid down by manufacturers in their advertising information. During the consultation stage with wheat processors , i.e. between phase 1 and 2, the performance of test kits was assessed in relation to the pre-set criteria. However, it was also agreed that rapid test kits should meet the needs of two specific applications within the cereal chain: (i) grain intake where a sample turnaround of < 30 minutes is required and (ii) storage or merchanting facilities where samples can be measured in large batches prior to delivery against customer contract.

The best overall performance across the DON concentration range measured was achieved with fully quantitative test kits. These are higher throughput techniques that take longer to produce results and greater capital investment in instrumentation to measure colour changes and interpret these in terms of DON concentration. Such techniques are more suited to a centralised laboratory environment where the required level of laboratory facilities and technical expertise already resides. The option to use a low cost colorimeter (Biotek EL 301) in a reduced throughput situation was considered to provide the flexibility required to enable these assays to be used more widely in cereal testing facilities. Two low cost strip readers were identified by manufacturers, Biotek EL 301 and Stat-Fax, as meeting this requirement. R-Biopharm provided CCFRA with a Biotek EL 301 instrument for use with their microtiterwell assays and this was used on other fully quantitative immunoassays. This low cost colorimeter met two of the industry's key criteria: cost and ease-of-use. One fully quantitative test kit, the R-Biopharm Ridascreen Fast DON, was selected to progress to phase 2 by members of the **nabim** Technical & Regulatory Affairs Committee (TRAC). The provision of the Biotek EL 301 by R-Biopharm was a contributing factor in this decision.

Semi-quantitative microtiterplate assays were not favoured by either TRAC members or users at CCFRA. Such tests are normally threshold tests, the final result depending on visual comparison with a standard or standards. They were not perceived to provide the benefits of ease-of-use found for the lateral flow devices and, due to limitations of supplied standards, frequently failed to meet the required limit of detection and sensitivity obtainable from the fully quantitative assay.

Lateral flow devices proved to be very simple to use and require minimal laboratory equipment or technical experience. As for the semi-quantitative microtiterplate assays, the standard or standards supplied with the kit were vital to kit performance and the technique tended to result in an overestimation of DON levels. The use of more standards generally improved the ranking of samples. Such kits are particularly suited to rapid sample turnaround, as required at mill intake or any grain receipt point. TRAC members considered that lateral flow technology offered opportunities to screen out high DON levels. Both lateral flow devices tested in phase 1 were considered to offer benefits and to be worthy of further evaluation within phase 2 of this study.

In phase 2, the performance of selected test kits was assessed using a set of 20 samples covering the most commercially sensitive range, i.e. 400 to 1600ppb. The aim was to provide a continuum of DON levels across a limited range and thus challenge kits with respect to their reliability in the critical measurement range where the majority of home-grown samples are likely to occur. In addition, a significant number of samples were selected close to the forthcoming legislative limit of 1250ppb in order to evaluate the suitability of each kit in this critical region. "Within kit lot" variation and the "between kit lot" variation was included in the total test repeatability in order to give potential users a guide to overall kit performance compared with the reference measurement.

Rapid test kits have a role to play in ensuring the safety of raw material (wheat) entering the human or animal food chain. They can be used by the supplier to screen samples in relation to the legal limits set by the EU, but it is important that an element of caution is applied to their use. For fully quantitative tests a threshold at between 800 and 1000ppb would help to ensure that samples with unacceptably high DON levels (1250ppb) are screened out of the cereal chain but would also result in rejection of some samples within the limit.

Lateral flow devices provide a quick and easy way to screen samples at intake. In addition, test strip readers have been developed in order to introduce objectivity into the assessment. As an initial screening tool, lateral flow devices provide the means to assess the extent of the immediate risk posed by DON, with subsequent analysis provided by approved fully quantitative test methods. As a stand-alone test the screening threshold will depend on the level of discrimination possible. Due to the limitations of classification possible with such devices, it is not as simple to apply the same kind of safety margin recommended for the fully quantitative test kits. If a margin of safety is required it may be necessary to operate at one level below that closest to the legal limit, i.e. use a threshold at 500ppb to ensure compliance with a 1250ppb limit. However, such a risk-averse strategy would result in rejection of samples below the limit. This may be compounded by the tendency of this format of test kit to overestimate DON concentration when only a single standard is used.

In order to improve the quality of decision made using a rapid test kit, it is recommended that all tests are duplicated. In addition, for QC purposes a suitable standard (a pure DON chemical or a wheat based check sample of known DON level close to the agreed threshold) should be tested alongside unknown samples to provide a means of monitoring kit and operator performance.

2. INTRODUCTION

Fusarium species are important pathogens of cereal crops, being responsible for the disease known as Fusarium head or ear blight (FHB/FEB). This disease can be manifested as pink and/or deformed grains (‘tombstones’). Concerns were raised within the UK milling and animal feed industries in relation to the high incidence of discoloured (pink) and mouldy grains and, by inference, potential mycotoxin (trichothecene) levels in wheat from the 2004 UK harvest.

Due to serious concerns within the cereals chain regarding the levels of pink grains present and significant rejections of grain at intake by processors, the following initiatives were taken to tackle the issues raised:

- HGCA sponsored a survey of UK feed wheat to investigate whether there was a relationship between pink grain content and DON levels (Hook & Williams,.2004). This survey included 68 samples, all of which had visible pink grains present and thus represented the “worst case scenario” for the 2004 wheat

crop. The study showed that, for UK wheat, there was no relationship between pink grain content and either levels of DON or *Fusarium* species and thus the number of pink grains present in a sample did not permit prediction of mycotoxin levels. Despite this, the absence of pink grains was still used as a preliminary screen at intake by many wheat processors.

- The National Association of British & Irish Millers (**nabim**) sponsored a review by CCFRA in October 2004 to examine the scientific literature in order to identify rapid, robust, relatively simple and inexpensive methods that could be used to screen wheat at intake (Hutton & Salmon, 2004). Potentially suitable rapid methods included: visual inspection and image analysis; immunoassay-based techniques; near-infrared spectroscopy; acoustic screening; electronic nose; and indirect chemical analyses for detection of *Fusarium per se* (by measurement of DNA, exoantigen or ergosterol levels). Within the category of immunoassay-based techniques, Enzyme-Linked Immunosorbent Assay (ELISA) based assays were the most highly developed, although the potential for surface plasmon resonance based, fluorescence polarisation and radio-immunoassay techniques were identified. Of all the methods considered in this survey, immunoassay-based techniques appeared to offer a relatively straightforward laboratory based approach that could be employed to screen wheat at grain receipt for the key trichothecene, namely deoxynivalenol (DON). From this literature survey, a number of test kits appeared to be capable of detecting commercially sensitive quantities of trichothecenes.

This project was initiated to evaluate the suitability of commercially available test kits to screen out intake samples likely to exceed forthcoming EU limits for DON and to provide reliable quantitative data quickly. The reference method for trichothecene measurement (including DON) is expensive and takes ~2 days to complete both the detection and quantification phases. There is, therefore, a need for rapid DON detection methods that are relatively portable and available in kit format, that involve a simple protocol (including both sample handling and kit operation) and that provide rapid results that are simple to interpret. Such test kits have the potential for use at intake points within the grain chain to assure safety of supply into the human or animal feed chain. They provide the capability to control and inspect in-coming raw material, to screen prior to making decisions relating to food safety, to minimize storage and release times and to provide the required documentation for traceability purposes.

EU limits for *Fusarium* mycotoxins in grain will come into force on July 1st 2006. For DON in wheat destined for the human food chain, the limits will be 1250ppb in wheat, 750ppb in flour and 500ppb in bread. During the development of limits for DON, a value of 1000ppb for wheat had been suggested and this was the threshold used by test kit manufacturers when designing and validating their products. There are currently no EU limits for other *Fusarium* mycotoxins such as Nivalenol, T-2 or HT-2 toxins and therefore the focus of this study has been to evaluate kits which could be used to screen for DON raw material coming into grain storage, being handled by a grain merchant or co-operative or entering a processing facility.

Birzele *et al*. (2000) reported on studies comparing a competitive ELISA test kit produced by R-Biopharm. Cross reactivity with acetylated DON compounds was reported and as a result the output of the test kit is the sum of 3-acetyl DON (3-Ac DON), 15-acetyl DON (15-Ac DON), 3,15-diacetyl DON and 3,7,15-triacetyl DON and DON. A number of kit manufacturers openly quote cross reactivity to 3-Ac DON and 15-Ac DON in their literature. Within the test set obtained for this study, a wide range of DON levels existed (11-11500ppb) and thus the set was considered ideal for evaluating test kits over the proposed legislative limits and commercially sensitive levels. However, measurable 3-Ac DON and 15-Ac DON levels were rare and associated with very high DON levels (>2000ppb) and therefore the magnitude of any cross reactivity is considered insignificant in terms of the test kit repeatability.

A number of key factors need to be addressed. These include a basic requirement that the method be "fit for purpose", be sufficiently sensitive based on the limit of detection (LOD) or limit of quantification (LOQ) and be specific for the analyte in question (i.e. exhibit a lack of cross-reactivity to other trichothecenes). In addition an assessment of kit performance should focus on the robustness of the data produced in terms of accuracy and precision of result plus repeatability in the hands of a single operator and reproducibility between different operators in different locations. Finally, it is important that any rapid test kit should be simple and practical for use within the intended environment (in this case a grain intake facility), be unaffected by matrix and processing effects and produce results that correlate well with established reference values.

Based on the outcome of the review of methods (Hutton & Salmon, 2004) and an internet search, a shortlist of commercially available test kits which appeared to show potential for use at intake points was produced.

The European Mycotoxin Awareness Network (www.eman.org) is an excellent source of information on test kits and provides details of companies and relevant test kit products with information on the format, quantification, sensitivity and approval status. Many organisations across the world carry out validation and certification exercises to evaluate the performance of a range of mycotoxin test kits. Website links to the most commonly quoted are supplied below. The organisations quoted provide information on performance criteria for qualitative and quantitative DON mycotoxin test kits. These are:

- USDA (www.gipsa.usda.gov) The site also provides a list of test kit specified readers
- AOAC (www.aoac.org/testkits/kits-toxins.htm)

The International Association of Cereal Chemists (ICC) has recently created a workforce to bring together knowledge and information on the mycotoxin test kit evaluation see www.icc.or.at/task/index.php for further information).

Test kits with potential for measurement of DON at intake are shown in Table 1. The aim of this project was to review the performance of selected test kits with particular reference to use at mill intake and ease-of-use.

Table 1: Test kits with potential for use at intake to monitor DON levels in wheat

Kit manufacturer	Kit name	Format
R-Biopharm	Ridascreen Fast DON	Competitive ELISA, microtiterplate format. Plate or strip reader required for quantification.
	Ridascreen DON Express	Competitive ELISA, microtiterplate format. Visual comparison with DON standards. Plate or strip reader required for quantification.
	Ridascreen Quick DON	Immunochromatography in lateral flow device. Visual comparison of test line with photographic image or samples of known DON level to produce semi-quantitative results.
Neogen	Veratox 5/5	Competitive ELISA, microtiterplate format. Plate or strip reader required for quantification.
	Agri-Screen for DON	Competitive ELISA, microtiterplate format. Visual comparison with DON standards. Plate or strip reader required for quantification.
	Reveal	Immunochromatography in lateral flow device. AccuScan palm reader measures the intensity of the test line to produce semi-quantitative results.
Strategic Diagnostics Inc. (SDI)	MycoChek	Competitive ELISA, microtiterplate format. Visual comparison with DON standards. Plate or strip reader required for quantification.
Romer	AgraQuant DON	Competitive ELISA, microtiterplate format. Visual comparison with DON standards. Plate or strip reader required for quantification.
	FluoroQuant DON	Fluorimetric assay, test tube format. Fluorimeter required for quantification.
Toxi-Test	*Toxi-Test for Deoxynivalenol*	Lateral flow enzyme immunoassay with visual comparison of test line.

For technical reasons (related to manufacturer requirements to re-validate test kits as a result of changes in the proposed legislative limits from 1000ppb to 1250ppb DON), the test kits shown in italics and underlined were not available for basic evaluation within the tight timeframe of this project.

Table 1 shows that, with the exception of the fluorimetry based test kit, all relevant commercial kits with potential for use at intake involve immunological measurement of DON and are based on specific antibodies produced against this mycotoxin. The Romer FluoroQuant DON, which is a chemical assay based on measurement of fluorescence, was of particular interest due to this difference. The kit was demonstrated to CCFRA by the manufacturer but could not be fully evaluated and therefore only the principles involved in measurement of DON have been described in this report.

3. PROJECT OBJECTIVES

- To provide an independent evaluation of commercially available test kits for DON measurement in order to provide the cereal processing chain with information on the appropriateness of kits for use in intake situations.
- Impending legislation creates a need for due diligence with respect to samples entering the food and feed processing chains from July 2006. The knowledge gained will enable decisions to be taken by suppliers regarding screening of wheat-based raw material to provide assurance of safety in key raw materials.

4. MATERIALS AND METHODS

HGCA kindly provided access to the sample set and analytical data from the pink grain survey (Hook & Williams, 2004). The reference trichothecene measurements within this study were carried out by RHM Technology in 2004 using a UKAS accredited method. This method is acknowledged as the reference method for DON and trichothecenes in the UK. In summary, samples (1-2kg) were ground finely and mixed thoroughly prior to extraction using acetonitrile/water followed by charcoal/alumina clean-up. Sample extracts were derivatised to form the trimethyl silyl derivative of the trichothecene and measured by gas chromatography with mass spectrometry (GC-MS). The analysis of spiked samples was used to validate recoveries and to correct trichothecene results for recovery. The measurement of uncertainty quoted for this GC-MS method is ± 20% of the reported result. Due to the many factors involved in uncertainty of measurement in mycotoxin testing, a value of ±20% is considered typical. The reference DON method quoted and used for comparative purposes by most manufacturers is based on the use of high-performance liquid chromatography (HPLC). The results obtained with this method would not be identical to those obtained using HPLC.

Results of trichothecene mycotoxins and the *Fusarium* species found on each sample are provided in Appendix 1, Table 1.

Test kits generally contain all specific equipment required for use, are always supplied with detailed instructions that should be followed with care and frequently include easy-to-follow schematics to simplify instructions. In contrast to other users of DON test kits, CCFRA was not able to spend large amounts of time in familiarisation of all the kits during phase 1: basic evaluation. In some cases, the manufacturers provided one-to-one training in their kits to ensure that the evaluation was carried out under the optimum conditions. This would be provided to all potential users and was considered to be a significant advantage.

Mycotoxin distribution is not uniform within a sample and therefore careful sampling is essential for accurate DON results. All manufacturers recommend that laboratories follow a clearly defined sampling

protocol to ensure that the sample collected is as representative as possible of the bulk. The sample should be ground finely to facilitate extraction of DON and well-mixed before taking a sample of 20-50g. Larger samples are recommended in order to reduce variation due to heterogeneity.

In addition to the kit contents provided, there is a requirement for certain laboratory equipment/reagents to perform DON assays and the list tends to be common to all test kits. The following lists some of the specifics required (an asterisk indicates that the equipment should be calibrated before use):

- Grinder for grain, capable of producing a finely ground wholemeal is ideal although no specific particle size distribution has been identified or tested within this work.
- Analytical balance*, capable of weighing up to 50g to one decimal place.
- Standard laboratory glassware e.g. beakers, volumetric flasks, measuring cylinders and test tubes.
- Vortex mixer or shaking device
- Micropipettes*, capable of dispensing microlitre quantities of liquid accurately. For microtiter based kits, a multi-channel pipettor improves the speed of sample analysis.
- Instrumentation required for quantitative measurement. This could be a spectrophotometer, fluorimeter, microtiter plate scanner or microwell reader provided by the kit manufacturer.
- In order to minimise the potential for cross-contamination, all reusable glassware should be cleaned using 5% hypochlorite solution. This is considered good laboratory practice in a mycotoxin testing laboratory.
- Most kits include one or more DON standards. Consideration should be given to the use of additional standard DON solutions that may be more appropriate for specific uses, e.g. 700ppb or 1000ppb.

4.1 Principles involved in test kits used for DON detection

Enzyme linked immunosorbent assay (ELISA)

The majority of rapid test kits for DON detection involve the use of enzyme linked immunosorbent assay (ELISA) technology. Immunoassays are based on the properties of antibodies, produced by the immune systems of animals as a defence response to an invading molecule or micro-organism. Antibodies have the advantages of binding tightly to the component that generated their production and having good specificity to this component. In the case of DON, a few manufacturers have identified cross-reactivity to molecules that are very closely related to DON, i.e. the acetylated DON molecules. The ELISA assay is designed with two antibodies (a capture antibody which is typically held in each plastic well within the microtiter plate) that binds to any antigen (DON) present in the sample. A second antibody linked to an enzyme is added to the test well which also binds to the captured DON, thus releasing the enzyme, which catalyses a colour change. Thus if no DON is present, the sandwich does not form, no enzyme is released and no colour change occurs. The colour change can be assessed by visual inspection or quantified by using a spectrophotometer.

Fluorescence

Fluorescence methodology is based on solvent extraction of DON from finely ground material, followed by a simple solid phase column clean-up of the sample prior to the addition of fluorescent reagents. A xenon lamp is used to excite the solution and the fluorescence is measured. A direct read out in parts per million (ppm) of DON is provided.

4.2 Kits not tested by CCFRA

For completeness and ease of reading, details of the individual test kits evaluated have been included in the Results & Discussion section. However, two test kits listed in Table 1 could not be evaluated by CCFRA staff within the timeframe of this project. A summary of these kits is provided below.

ROMER FLUOROQUANT DON

As suggested by the name, this kit utilises a chemical technique (fluorimetry) rather than the more common ELISA technique to measure DON in cereal products. The technique was of considerable interest as it was unlikely to suffer from cross-reactivity problems (3-AcDON or 15-AcDON) associated with ELISA tests. Despite having GIPSA (Grain Inspection, Packers, and Stockyards Administration) approval for measurement of DON in wheat, the test was not available for assessment at CCFRA. However, the manufacturer demonstrated the Fluoroquant technique to CCFRA staff and a summary of the principle of this test kit is included for completeness.

Unlike ELISA techniques, the test employs a solvent/water mix for DON extraction. This means that the test may be more suited to a central laboratory that has more specialised facilities and is more used to solvent storage and disposal. (Pre-prepared mixtures of acetonitrile/water can be purchased from suppliers whilst the disposal of waste solvent can be out-sourced, thus removing these concerns.) A 50g sample of ground grain is extracted with acetonitrile/water (84/16), blended simply by shaking for 2 minutes before filtration. The kit includes a clean-up stage using a simple push down clean-up column that is proprietary to Romer Labs. 5ml of the filtrate is placed in a test tube and forced through the column by simply pushing this gently down into the tube. (This step very effectively clarifies the extract, but it was not possible to ascertain whether there was an impact on DON recovery. This aspect would require investigation should this test kit be utilised for grain intake screening purposes.) An aliquot of the extract (1000µl) was added to the fluorescence developers and incubated at 70^{o}C for 8 minutes. Following cooling of the solution, the fluorescence is read in a proprietary fluorimeter that has been calibrated using reference standard samples. In terms of sample turnaround, the FluoroQuant DON test format is suited for use at grain receipt. Individual samples could be measured on receipt at a mill or storage facility.

TOXI-TEST

This test kit is marketed as an inexpensive and portable technique for screening DON in cereals. The kit uses flow-through technology, claims to complete analysis within 10 minutes (which is compatible with other lateral flow devices) and can be used for single or multiple sample analysis. It is of particular interest as the manufacturer has simplified sample preparation, extraction and analysis in order to meet the requirements of on-farm testing. A simple electric coffee grinder is used to generate a ground sample and extraction is performed by simple hand shaking for 3 minutes. Sample extracts and reagents are added to the test strip using droppers supplied within the kit, thus removing the need to purchase pipettes capable of measuring microlitre quantities. It was not possible to evaluate this technique during the timeframe of this study. This was unfortunate as potentially it appears to offer growers the opportunity of low cost "on site" screening for DON.

4.3 Phase 1: Basic evaluation

This involved assessing the suitability of available kits for use at grain intake using selected samples as shown in Table 2. The sample which had the highest DON level (~11,000ppb) was deliberately avoided as this would be considered a clear reject on visual appearance at intake and thus too extreme for measurement of DON analytically.

Cross-reactivity is perceived to be an issue with ELISA kits and some manufacturers state in their literature that their antibody may cross-react with acetylated forms of DON. However, the sample set did not contain any material with measurable 3Ac- or 15Ac-DON which could be used to substantiate these claims. (Essentially all samples with Ac-DON levels significantly above the limit of detection had DON levels above 1500ppb and therefore cross-reactivity was not considered an issue for this set of UK wheat.)

In order to obtain an impression of the repeatability of test results, each sample was analysed at least twice in two completely separate experiments. During this phase, no attempt was made to examine between batch variability for a particular kit. However, due to the format of some test kits, this sample replication included the use of more than one kit whereas for others a single kit was used.

Table 2: Sample used in basic evaluation of test kits

(Samples are arranged in order of increasing DON levels, as measured by the reference method in ppb or μg/kg)

Sample Number : CM/81246/	Source	DON	3AcDON	15AcDON	NIV
67	NE	11	<10	<10	<10
44	Y&H	177	<10	<10	13
46	EM	451	<10	<10	<10
68	SE	593	<10	<10	<10
2	E	649	<10	<10	28
65	E	655	<10	<10	<10
7	SW	720	<10	<10	36
10	SE	923	<10	<10	36
64	Y&H	1235	<10	10	75
16	E	1276	<10	<10	30
6	E	1839	<10	<10	27
20	EM	1866	<10	14	36
37	E	4079	25	37	82

A set of criteria, shown in Table 3, were produced by CCFRA in consultation with representatives of the UK milling industry [namely the members of the Technical & Regulatory Affairs committee of **nabim**] and used to evaluate kit performance. Thus, each kit was assessed in terms of its potential for a specific application rather than in true statistical terms. A copy of these criteria plus a basic outline of the proposed project was provided to all kit manufacturers and their acceptance of these conditions was obtained prior to the evaluation of their test kit.

Table 3: Criteria for consideration of rapid DON test kits for use at grain receipt

Criteria	Target
Limit of detection	<500ppb
Speed of analysis including sample preparation	<30 minutes
Cost per test (consumables only)	<£5
Cost of ancillary equipment	Minimal investment in specialised lab equipment not normally found in an intake laboratory
Ease-of-use by intake lab staff	Simple to follow instructions with any critical points clearly identified. Good "between operator" agreement.
Error level	Absence of false positives

The project plan included reporting of the results of basic assessment of DON test kits to members of the cereal chain (**nabim** and AIC) in order to select kits for more stringent evaluation. Rapid DON test kits were judged against the above criteria in order to select the kits which best fitted the needs of users in a grain intake situation. It was acknowledged that certain compromises would need to be made, e.g. there may need to be a trade-off between speed of analysis and accuracy. There was also the view that the needs of the milling and grain merchanting industries would differ with respect to sample screening for DON. For example, millers may prefer to monitor selected individual samples on arrival at a mill whereas AIC members would be more likely to have access to large numbers of samples which could be screened at the same time.

4.4 Phase 2: Evaluation of selected kits

Following discussions with a statistician, a set of 20 samples was selected for measurement from the HGCA pink grain survey. The primary selection criterion was the GC-MS reference value for DON. The aim was to remove the extremes of DON content and select samples within the range 400 to 1600ppb. The purpose of this sample selection was to permit evaluation of two factors:

i) by attempting to provide a continuum of DON levels, it should be possible to challenge the selected kits with respect to their reliability in the critical measurement range where the majority of home-grown samples are likely to occur.

ii) by ensuring that a significant number of samples (~40%) traverse the forthcoming legislative limit of 1250ppb, it should be possible to evaluate the suitability of each kit to screen samples for compliance against this limit.

As a preference, any sample previously measured in phase 1 of the trial would have been avoided in phase 2 in order to ensure that the widest test set was used and that all samples had been stored under the same conditions prior to analysis. Samples were stored at –20^{o}C at CCFRA to minimise the possibility of sample deterioration. However, due to the limitations of the sample set it was necessary to include 4 samples in phase 2 that had been in phase 1 evaluation and hence these samples would have been exposed to several freeze-thaw cycles. On the positive side, in the absence of certified samples of known DON levels, these samples served as reference points enabling comparison with previous measurements.

During phase 2, it was essential to test repeatability by examining both the "within kit lot" variation and the "between kit lot" variation. Manufacturers were asked to supply kits from different manufacturing batches and experimental work was planned carefully to ensure that samples were tested over several days and manufacturing batches. Due to the fact that the assessment was carried out in one laboratory with limited technician input, it was not possible to test true Reproducibility.

5. RESULTS AND DISCUSSION

5.1 Phase 1: Basic evaluation

Basic evaluation of all test kits was performed using the same set of test samples. The samples were selected on the basis of providing good coverage of the range of DON levels observed in UK grain from the 2004 harvest. The published uncertainty of the method used to provide these reference measurements is ±20% of the absolute value quoted. Thus, for a sample with a measured DON level of 1250ppb the actual result could lie between 1000 and 1500ppb. These uncertainties of measurement should be borne in mind when assessing the performance of any rapid test kit and when setting thresholds to use to screen wheat at intake.

The performance of each test kit was assessed with reference to the pre-determined set of criteria shown in Table 3. Since all test kits met the "cost per test" criteria, only the need for investment in capital equipment is considered under the category of "cost of analysis"

5.1.1 R-BIOPHARM

Results of tests carried out on all three R-Biopharm kits are presented in Table 4 against the reference DON levels for each sample. In addition, the uncertainty of the reference result (± 20%) has been taken into account to ensure that comparison between reference and test kit results are fair. A brief description of each test is also included to facilitate comparisons.

Table 4: Results of phase 1 evaluation of R-Biopharm test kits

Sample Number: CM/81246/	DON (ppb by GC-MS)	DON (ppb) Range based on ± 20% uncertainty	Fast DON		DON Express			Quick DON
			EL 301 (ppb)	Ascent (ppb)	EL 301 (ppb)	Ascent (ppb)	Visual (500ppb or 0.5ppm threshold)	Visual (2000ppb or 2ppm threshold)
2	649	519-779	982	1060	564	616	<500	<2000
6	1839	1471-2207	1648	1768	1594	1641	>500	<2000
7	3016	2413-3619	3197	3215	2517	2585	>500	>2000
9	1069	855-1283	924	934	1110	1038	>500	<2000
10	923	738-1108	917	1008	926	890	>0.5	<2.0
16	1276	1021-1531	775	848	1114	1105	>0.5	>2.0
20	1866	1493-2239	2172	2652	1896	1978	>0.5	>2.0
37	4079	3263-4895	4768	4635	4146	4152	>0.5	>2.0
44	177	142-212	381	443	462	508	<0.5	<2.0
46	481	385-577	754	704	487	467	>0.5	<2.0
65	655	524-786	730	669	623	654	>0.5	<2.0
67	11	9-13	17	0	68	94	<0.5	<2.0
68	593	474-712	196	364	510	485	>0.5	<2.0

RIDASCREEN FAST DON

A competitive, quantitative ELISA that has been approved by AOAC and USDA/FGIS and is provided in a 48 or 96 microtiter well format with break-apart microwells. For quantification, a spectrophotometer capable of reading microtiter plates or individual wells is required. DON is detected at a wavelength of 450nm via a colour change from blue to yellow. The incubation time is 8 minutes and as the substrate-chromogen is known to be light sensitive, readings must be taken within 10 minutes of the incubation. 5 standards are supplied with the test kit (0, 222, 666, 2000 and 6000ppb) and these must be run in each batch in order to generate the calibration curve against which any unknown is measured. The limit of detection (LOD) quoted by the manufacturer is 200ppb. Software (RidaR Soft Win) is available from the manufacturer to aid construction of the calibration curve and automate calculation of test results. However, it was not possible to test the performance of the software within this study. Due to the supplied format of the test kit and the need to measure 5 standards for every unknown sample tested, the assay is suited to batch analysis. Analysis time is ~120 minutes for a batch of 36 samples. The performance of a relatively low cost spectrophotometer (Biotek EL 301) was compared with a microtiter plate reader (Thermoskan Ascent).

The Fast DON assay was found to work well across the entire range of DON levels within this part of the study. The measured coefficients of variation between replicates using a single batch of the Fast DON kit were 0.1 to 12.9% for the Biotek EL 301 and 0 to 7.3% for the Thermoskan Ascent. The upper value for the coefficient of variation for the Biotek EL-301 is based on the 11ppb DON sample where a standard deviation of 2.1 was recorded. (Removal of this datapoint would reduce the maximum coefficient of variation to 10.5%). These results suggest that the repeatability of the test is good and within the limits quoted by the manufacturer.

Differences can be seen between the reference values and Fast DON results in Table 4. In two cases, namely the 11 and 177 ppb samples, kit results were outside the range. In general, the Fast DON kit tended to produce higher results than the GC-MS method. This could provide an element of safety if the ELISA kit were to be used as a screening tool. Comparing the Fast DON test results obtained with a low cost colorimeter (Biotek EL 301) and a batch microtiter plate reader (Thermoskan Ascent) showed that, when used to measure the colour change in the Fast DON kit, it tended to produce slightly higher DON results. This work suggests that, where analysis of large batches are not required, there is no need to invest in the more costly microtiter plate reader to screen wheat samples for DON content.

When used in fully quantitative mode, this ELISA technique meets the criteria set in respect to:

- Limit of detection – capable of measuring down to 500ppb with a tendency to generate higher results
- Speed of analysis - when used in strip format with the Biotek EL 301 will meet the 30-minute analysis time, but when used in full batch format of 48 or 96 wells the time constraint was not relevant.
- Cost of analysis – the Biotek EL 301 colorimeter option provided by the manufacturer offers a low cost solution for the smaller, less sophisticated laboratory. For the high throughput situation, significant investment in a microtiter plate, proprietary software and multi-channel pipettor would be required.
- Ease of use – this was acceptable for a fully quantitative test kit.
- The Fast DON kit appeared to be most suited for use in a centralised laboratory due to the requirement for a spectrophotometer to measure Absorbance and the need to convert this to a numerical value by calculation using algorithms held on a computer. The kit is suitable for the measurement of DON levels in a variety of cereal grains.
- Error levels - despite observed differences between the reference and kit results, when used to screen against a 1250ppb threshold, all samples were correctly classified.

RIDASCREEN DON EXPRESS

A competitive ELISA that can be used in semi-quantitative or fully quantitative mode depending on whether the colour change from blue/dark green to yellow is measured by visual inspection or using a spectrophotometer at a wavelength of 650nm. No official approvals for this test kit are quoted by the manufacturer in advertising literature. The incubation time is 5 minutes and as the substrate-chromogen is known to be light sensitive readings must be taken within 10 minutes of the incubation. As for Fast DON,

RidaR Soft Win software is available from the manufacturer but was not tested within this study. The test kit is supplied with up to 4 standards (500, 1000, 2000 and 5000ppb). Only the 500ppb standard was used in the basic evaluation of this test kit and no LOD is quoted by the manufacturer. The kit format and procedure for use mean that this assay is more suited to test intake samples as received. Typically 1 standard is tested against 3 unknown samples.

This assay was evaluated in both fully and semi-quantitative modes. Once again, two systems were used for measuring colour changes quantitatively, namely the Biotek EL 301 colorimeter and the ThermoSkan Ascent microtiter plate reader. Results for the DON EXPRESS test kit are shown in Table 4. The data provided shows that the DON EXPRESS assay produced results that correlated well with the GC-MS reference values across the range of DON levels. Once again, the more costly microtiter plate reader only appeared to provide a benefit over the Biotek EL 301 colorimeter when batch sizes were large. Due to problems relating to the first measurement of samples, no estimate of variation in replicate analyses is provided.

In the absence of any spectrophotometer, the results of the assay can be assessed visually and the results of subjective assessment of the colour change are also presented in Table 4. Technical staff did have some concerns when the grain extract under test was not totally clear and the use of a spectrophotometer significantly improved decision-making. Despite this results show that; with the exception of sample CM/81246/46, visual assessment correctly classified all samples with respect to a threshold of 500ppb. The use of a 500ppb threshold would introduce a safety margin in testing samples for compliance with the proposed legislative limit of 1250ppb. However, the use of a single threshold would result in the rejection of wheat that would comply with the forthcoming legislation. Semi-quantitative microtiterplate assays were not favoured for DON screening at intake by industry representatives.

When used in fully quantitative mode this ELISA technique meets the criteria set in respect to:

- Limit of detection – capable of measuring down to 500ppb with a tendency to generate slightly lower results.
- Speed of analysis - when used in strip format with the Biotek EL 301 will meet the 30 minute analysis time, but when used in full batch format the time constraint was not relevant.
- Cost of analysis – as shown for Ridascreen Fast DON.
- Ease of use – this is acceptable for a fully quantitative test kit, although some technical problems were experienced by CCFRA staff. R-Biopharm did provide additional one-to-one training in an attempt to solve observed problems. However, test repeatability was not tested.
- Error levels – one false negative (CM/81246/16) was observed. With this exception if used to screen against a 1250ppb threshold all other samples were correctly classified.

RIDASCREEN QUICK DON

An immunochromatography based kit in which the antibody-antigen reaction results in a colour change from pink to clear. The strip has two sections: the sample extract is deposited in the first section of the strip and is carried by lateral flow through to the reading section or window. The reaction between the antibody and DON occurs in the result window and creates the colour change from pink to clear. Thus, the presence of a visible pink test line on the lateral flow device indicates the absence of DON in the sample whilst the partial disappearance or total absence of a line shows that measurable DON is present. The test strip contains a second (control) line which also appears in the result window and shows that sample flow through has occurred correctly and that the strip is functioning properly. Visual inspection of the result window on the test strip means that the test is only semi-quantitative. (Subsequent to this evaluation, R-Biopharm introduced the low cost reader RidaXScan for use with the Quick DON test strips.). The kit contains a 2000ppb standard and the claimed LOD is 1000ppb. The lateral flow format means that this kit is ideally suited to single determinations, but for cost effectiveness and for compatibility with incubation times of 5-10 minutes one standard should be run with 9 unknowns. The manufacturer recommends that users of the Quick DON test kit run known positive and negative wheat samples against test material to facilitate decision–making.

In the current format, the pink line generated is compared with a photographic image. This should be performed within 5 minutes of completion of the analysis. However, the bands appeared to be relatively stable and in this study, if protected from the light could be stored for several days.

Figure 1: Results of Quick DON test carried out on wheat extracts with low DON levels (reference values quoted in ppb)

The Quick DON test kit proved to be extremely simple to use. During phase 1 of the evaluation, this test kit was provided by the manufacturer as being validated for use with a 1:10, dilution i.e. 1g of ground wheat to 10ml of water, and a 2000ppb threshold (validation using a 1:5 dilution and a 1000ppb threshold was later claimed but was not tested in this phase of the study). Figure 1 shows examples of the lateral flow devices for samples with DON levels of 11-655ppb. All four samples would be classified as <2000ppb whilst the 11ppb would be classed as negative.

Table 4 shows the results of Quick DON analyses carried out using a single batch of this kit. Each result is the average of 2 tests per sample. The result is based on visual assessment of the test strip against a photographic image and on this occasion there was no observed difference between replicate results. However, comparison between a real strip and a photographic image is not ideal (not least because the photographic image is printed on gloss paper). It is obvious from Table 4 that when this kit is used at a 2000ppb threshold it correctly classifies 11 out of the 13 samples tested. With respect to the 2 apparent false positives, both samples had DON levels above 1250ppb and hence food producing end-users would wish to reject these during screening at intake.

When compared with the criteria set, the Quick DON kit performed as follows:

- Limit of detection – not capable of measuring down to 500ppb and only a 1000ppb threshold was claimed.
- Speed of analysis – easily meets the 30-minute analysis time required
- Cost of analysis – no significant capital investment required for this test kit.
- Ease of use – extremely easy to use, suited to rapid turnaround screening at intake. No problems with visual threshold at 2000ppb threshold.
- Error levels – two false positives observed.

Despite the fact that the kit did not fully meet the requirements of industry during phase 1, it was clear that significant "in-house" development was being undertaken and it was decided that as a result of this there was some value in further evaluation of the Quick DON test kit. The Quick DON assay therefore progressed to phase 2 evaluation.

5.1.2 NEOGEN

Results of tests carried out on all three Neogen assays are presented in Table 5 against the reference DON levels and associated uncertainties for each sample. A brief description of each test is also included to facilitate comparisons.

Table 5: Results of phase 1 evaluation of Neogen test kits

Sample Number CM/81246/	DON (ppb by GC-MS)	DON (ppb) Range based on ± 20% uncertainty	Neogen Veratox 5/5 ppb	Neogen Agiriscreen (1000ppb or 1.0ppm threshold)	Neogen Reveal for DON (upper limit in ppb)
2	649	519-779	696	<1000	1000
6	1839	1471-2207	1803	>1000	2000
7	3016	2413-3619	825	>1000	2000
9	1069	855-1283	909	>1000	1000
10	923	738-1108	1006	<1000	1000
16	1276	1021-1531	1136	>1000	2000
20	1866	1493-2239	1824	>1000	2000
37	4079	3263-4895	4500	>1000	2000
44	177	142-212	185	<1000	500
46	481	385-577	500	<1000	1000
65	655	524-786	761	<1000	1000
67	11	9-13	8	<1000	0
68	593	474-712	527	<1000	1000

VERATOX 5/5

A competitive ELISA available in a 48 microtiterwell format that can be used in semi-quantitative or fully quantitative mode depending on whether the colour change from blue to pale blue is measured by visual inspection or using a microtiter plate spectrophotometer at a wavelength of 650nm. Microtiter plates are supplied in break-apart format which provides greater flexibility with respect to batch analysis. Due to the limitations of the plate reader located at CCFRA, readings were taken at 630nm rather than the recommended wavelength of 650nm. CCFRA would recommend that users who chose this test kit follow the manufacturer's instructions and use 650nm. The kit is USDA-GIPSA approved with an LOD of 100ppb being claimed by the manufacturer. The kit includes 5 standards with the following range of DON levels: 0, 250, 500, 1000 and 3000ppb to produce a calibration curve for quantification of unknowns. The kit is, therefore, more suited to batch analysis (a batch of 19 samples plus 5 standards fit with the assay time of 25 minutes). The manufacturer quotes the fact that cross reactivity with acetylated DON compounds occurs.

Only single measurements were made with this test kit and therefore no standard deviations or coefficients of variation are available. Within the test set used, which did not contain significant levels of acetylated DON compounds, it was not possible to test the manufacturer's claim that cross-reactivity with acetylated DON

occurs. With one major exception this assay generally worked well across the range of DON levels. One sample produced an anomalous result, CM/81246/7, where this kit produced a "false negative" by recording a value 825ppb for a sample where the reference result was recorded as 3016ppb. Whilst no explanation for this could be determined and no other kit exhibited any particular problem with this test sample, operator error must be considered given the close correlation of all other results.

The VERATOX 5/5 technique meets the criteria set in respect to:

- Limit of detection – capable of measuring down to 500ppb, due to the inclusion of suitable standards in the test kit.
- Speed of analysis – recommended for batch analysis by manufacturer and thus the 30 minute time constraint is not relevant. The Biotek EL 301 was advertised on the Neogen website (www.neogeneurope.com) and was used in combination with this test kit. An alternative to the Biotek EL 301 suitable for use in Europe, namely the Stat Fax microstrip reader, is also offered by Neogen. This instrument has the advantage of being able to produce a standard curve and extrapolate values for unknown samples removing the need for a separate PC. These low cost readers increase assay flexibility and enable the kit to meet the needs of the smaller laboratory where smaller batches using 1 –3 strips of microwells may be required and thus make the kit suitable for use of this kit in rapid turnaround intake situations.
- Cost of analysis – as advertised, significant investment would be required in a microtiter plate reader and multi-channel pipettor. Ease of use – some technical difficulties were experienced initially. As the kit is intended for batch analysis with a microtiter plate reader it is accepted that greater technical skill will be required. The use of a multi-channel pipettor significantly improves kit handling and sample throughput.
- Error levels – one false negative (CM/81246/7) was observed, all other samples were correctly classified.

AGRISCREEN for DON

For this assay, the extraction kit is supplied separately. A competitive, semi-quantitative ELISA available in a 24 microtiterwell format in order to fit in with assay time of 10 minutes samples would normally be run as 5 unknowns against a single standard. In this test kit the colour change is from blue-green to lilac (positive) and blue (negative) and this colour change is assessed visually. A 1000ppb standard was supplied with the kit.

This assay performed very well in basic evaluation and the results were repeatable. All samples were correctly classified against the threshold of 1000ppb and within this data set the assay appeared to be capable of distinguishing between two samples with DON levels of 923 and 1069ppb. Given the ± 20% uncertainty range associated with the reference measurement, this is perhaps surprising. The use of a 1000ppb threshold provides a safety margin in relation to the legislative limits for wheat of 1250ppb but introduces the risk of rejecting samples below the limit.

- Limit of detection – not capable of measuring down to 500ppb due to the use of a single standard at 1000ppb.
- Speed of analysis – easily meets the 30 minute time constraint set.
- Cost of analysis - no significant capital investment required for this test kit.
- Ease of use – despite analyst difficulties with the colour change involved in this test kit, particularly in the presence of opaque or coloured extracts, all samples were correctly classified, suggesting that this may be an issue of perception rather than reality. The use of a multi-channel pipettor significantly improves kit handling and sample throughput.
- Error levels – no issues raised as all samples were correctly classified.

Thus, results of evaluation show that this assay performs very well and could easily be used to screen wheat at intake. However, semi-quantitative microtiterplate assays such as the AGRISCREEN for DON were not considered ideal for use in the cereal processing chain. They do not offer any significant benefit in terms of ease-of-use over a fully quantitative ELISA and by their very nature are not designed to provide the same level of sensitivity. When compared with a lateral flow device they are also found wanting as they require a much higher level of operator expertise. For these reasons the grain industry did not recommend that any semi-quantitative microtiterplate ELISA assays progressed beyond the basic evaluation.

REVEAL for DON

A lateral flow immunochromatography test kit where a pink test line is generated when no DON is detected in the extract. The kit has a palm-held reader, AccuScan, which permits this lateral flow assay to be used objectively in a semi-quantitative mode. Alternatively, visual assessment of the test strips can be used. No standards are supplied to provide points of reference within the kit. However, each batch of test strips is supplied with a batch specific CD which contains an algorithm to set up the reader, and therefore evaluation including AccuScan was considered to be most appropriate in the context of this study. The kit allows sample results to be classified into four different categories (negative 0, and three positive groups 0-500ppb, 500-1000ppb and 1000-2000ppb). A single test result can be obtained in ~10 minutes. The system is ideally suited to single or small batch analysis (up to 3 samples) but equally can be used to screen a large batch of samples in sequence. The palm-held reader provides a permanent record of the lateral flow test and check lines and allows quantitative results to be downloaded in Excel format for analysis.

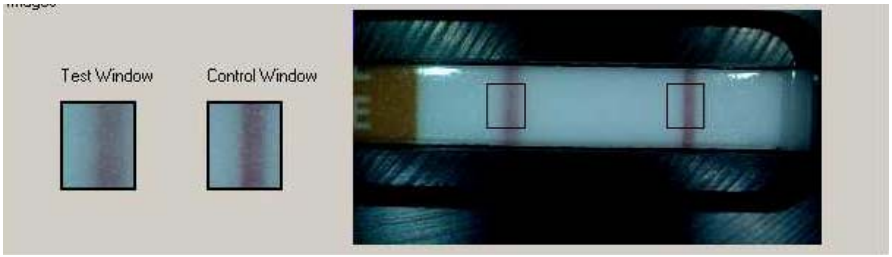


Figure 2: Visual output from palm-held AccuScan reader for Reveal

Results showed that only one sample (CM/81246/67) was identified as being negative with respect to DON. This sample had a reference DON value of 11ppb and therefore was very close to the limit of quantification of the reference method of 10ppb. All other samples in the test set were classified as positive and sorted into one of the following three groups: 0-500ppb, 500-1000ppb and 1000-2000ppb. In each case, the sample would be reported as the upper limit of the group. Thus, this assay has a tendency to overestimate the amount of DON in the sample. In the case of two samples, slight misclassification appeared to occur within the test. For both samples the REVEAL for DON result was shown to be 500-1000ppb. CM/81246/9 had a reference result of 1069±214ppb and CM/81246/46 had a reference result of 485±96ppb, thus the lateral flow assay result is actually within the error of the reference value.

- Limit of detection – using the AccuScan palm-held reader the assay is capable of measuring at and below 500ppb.
- Speed of analysis – easily meets the 30 minute time constraint set.
- Cost of analysis – the palm-held reader option adds significant capital cost to the REVEAL assay, but the advantages provided by this accessory considerably outweigh the cost involved.
- Ease of use – simple to run the assay. Palm-held reader requires some technical expertise in terms of gaining access and loading the correct CD for each kit batch. The AccuScan reader contributes significantly to the performance of this assay in terms of objective assessment of the result and data management.
- Error levels – met the requirements.

The REVEAL for DON assay was considered to provide an excellent combination of ease-of-use and suitability for use at a rapid turnaround grain intake location by representatives of the UK grain industry. The portability of the palm reader was not considered to be a total benefit and users would consider locking this into a testing station to ensure that the reader was available in the right place at the right time.

5.1.3 STRATEGIC DIAGNOSTICS INTERNATIONAL (SDI)

Results of tests carried out to evaluate the SDI MycoChek test kit are presented in Table 6. As for other test kits, the reference DON levels and associated uncertainties for each sample are provided for comparison. A brief description of each test is also included to facilitate comparisons.

Table 6: Results of phase 1 evaluation of Romer Labs and SDI test kits

Sample Number: CM/81246/	DON (ppb by GC-MS	DON (ppb) Range based on ± 20% uncertainty	Romer AgraQuant DON		SDI MycoChek		
			EL 301 (ppb)	Ascent (ppb)	(ppb)	Visual 250,500,1000 & 3000ppb standards	
2	649	519-779	670	717	629	500-1000ppb	500-1000ppb
6	1839	1471-2207	1914	1729	1495	1000-3000ppb	1000-3000ppb
7	3016	2413-3619	2931	2927	3098	>3000ppb	1000-3000ppb
9	1069	855-1283	1136	1227	1010	1000-3000ppb	1000-3000ppb
10	923	738-1108	969	908	940	1000-3000ppb	500-1000ppb
16	1276	1021-1531	1330	1329	1261	1000-3000ppb	1000-3000ppb
20	1866	1493-2239	1843	1530	3448	1000-3000ppb	1000-3000ppb
37	4079	3263-4895	3953	3790	4207	>3000ppb	>3000ppb
44	177	142-212	183	213	245	250-500ppb	250-500ppb
46	481	385-577	494	420	574	250-500ppb	500-1000ppb
65	655	524-786	638	661	577	250-500ppb	500-1000ppb
67	11	9-13	ND	ND	0	250-500ppb	250-500ppb
68	593	474-712	558	550	269	250-500ppb	250-500ppb

MYCOCHEK for DON

A quantitative competitive ELISA that is available in a 48 well format with break-apart microwells. The assay is USDA-GIPSA approved for use with wheat and other cereals and an LOD of 20ppb is claimed for wheat. The colour change from blue to pale blue can be measured visually or using a microtiter plate spectrophotometer/ microwell strip reader at a wavelength of 650nm. The kit contains 5 standards (namely 0, 250, 500, 1000 and 3000ppb of DON) that are used to create the calibration curve for evaluation of unknown samples. As a result, the test format is most suited to batch analysis (maximum – 43 samples plus 5 standards, minimum – 7 samples plus 5 standards). Results can be obtained within the 30 minute deadline, but not for the entire microtiter plate and thus would not represent the most effective use of the test kit. Cross reactivity with acetylated DON compounds has been reported and the chromogen-substrate is light sensitive and therefore it is recommended that measurements are taken within 20 minutes of the completion of incubation.

In the case of two samples, the MycoChek assay measured the DON level in the sample outside the reference range as indicated by the uncertainty column. These were sample CM/81246/20 where the MycoChek result was significantly above the reference value at 3448ppb and CM/81246/68 where the kit result was significantly below the reference result at 269ppb. For CM/81246/20, both the reference test and the MycoChek assay would have classified this sample as above the proposed legislative limit of 1250ppb and therefore this sample was of less concern than the fact that the observed differences were not consistently in one direction. For both the anomalous samples the duplicate results were acceptable (coefficient of variation = 6.5 and 2.6% respectively) and no obvious reason for these results could be found. Otherwise the kit performed well across the range of samples under test.

The MycoChek test kit was also evaluated in the semi-quantitative mode where the ELISA reaction was compared visually with standards of 250, 500, 1000 and 3000ppb. This permitted results to be placed in 4 different categories as follows: 250-500ppb, 500-1000ppb, 1000-3000ppb and greater than 3000ppb. Due to the absence of a 0ppb standard, this system of assessment will always result in overestimation of low values, but as for other kits this may be considered to be an advantage for users. Some differences between replicate results were observed and therefore both sets have been presented. The choice of standards makes the variation look more significant than it actually is. For example, sample CM/81246/10 shows values of either 500-1000ppb or 1000-3000ppb. These are in fact adjacent classes and the uncertainty values quoted indicate that this sample actually straddles the 1000ppb threshold. It is interesting to note that sample CM/81246/20 is classified correctly when the MycoChek assay is used in semi-quantitative format.

Comparing kit performance against the criteria set:

- Limit of detection –capable of measuring to the required DON level in both fully quantitative and semi-quantitative modes.
- Speed of analysis – due to reasons of efficiency and cost effectiveness, the fully quantitative ELISA is most suited to batch analysis and therefore the 30 minute time constraint is not relevant. For the semi-quantitative assay, the above time constraint can be met but batch analysis is still the preferred option for cost effectiveness.
- Cost of analysis - as advertised, significant investment would be required in a microtiter plate reader and multi-channel pipettor. The use of a low cost strip reader option, such as the Biotek EL 301, should be considered in order to increase assay flexibility and meet the needs of the smaller laboratory where smaller batches may be required.
- Ease of use – microtiter assay used in the fully quantitative format is more suited to use in a centralised laboratory due to the requirement for a spectrophotometer. The use of a simple colorimeter such as the Biotek EL 301 would make this kit easier to use in an intake situation. The semi-quantitative option still requires a level of technical expertise that is difficult to justify in terms of any other criteria.
- Error levels – slight concerns regarding inconsistency of errors.

5.1.4 ROMER

Results of tests carried out to evaluate the Romer AgraQuant DON test kit are presented in Table 6.

AGRAQUANT DON

A competitive quantitative ELISA that is available in a 48 or 96 break-apart well format which enables small strips to be tested if required. No official approval is claimed for this assay, but an LOD of 0.2 and an LOQ of 250ppb is claimed by the manufacturer. The colour change from yellow (negative) to pale yellow (positive) appeared to be more subtle than some of the other test kits evaluated and this might be expected to impact on sensitivity and precision, particularly if the sample extract is coloured. A manufacturer's note informs the user of a pH window, pH 6-8, within which the sample extract should fall for optimum performance of the assay. Measurements are made at a wavelength of 450nm using a micotiter plate spectrophotometer or the manufacturer also recommends the use of the Biotek EL301or other strip reader. For each batch of samples, 19 unknowns can be run against a set of 5 standards, namely 0, 250, 1000, 2000 and 5000ppb.

The AgraQuant DON assay performed exceptionally well across the range of DON levels tested. When the Biotek EL 301 was used to measure the colour change, all samples were correctly assigned within the uncertainty bands shown in Table 6. When the microtiter plate reader (Thermoskan Ascent) was used, all samples except CM/81246/44 which had a reference DON level of 177, were correctly assigned. The result for CM/81246/44 was, in fact, just 1ppb outside the uncertainty range for this sample. Once again there appears to be no clear benefit of investment in the more expensive Ascent microtiter plate reader. The standard deviation of differences between replicates ranged from 7.8 to 118.8 across the range of DON levels

from 11 to 4079ppb using the Biotek EL301. These values relate to coefficients of variation between 0.5 and 13.1%. The equivalent values for the more expensive Thermoskan Ascent were: standard deviation ranging from 2.1-231.2 with these values equating to coefficients of variation between 0.2 and 10.1%. No standard deviations or coefficients of variation were quoted for this kit, but the values quoted are comparable with other test kits

Comparing the performance of the AgraQuant DON kit against the criteria set:

- Limit of detection – fully quantitative and therefore capable of measuring to the required DON level. The choice of standards is not ideal for screening samples for compliance with EU regulations and the calibration curve may benefit from replacement of one high DON standard with a sample of 500ppb DON. The manufacturer does supply a standard at 500ppb, but this was not used in this study.
- Speed of analysis – due to reasons of efficiency and cost effectiveness, the fully quantitative ELISA is most suited to batch analysis and therefore the 30 minute time constraint is not relevant. For the semi-quantitative microtiterplate assay, the above time constraint can be met but batch analysis is still the preferred option for cost effectiveness. Sample throughput is increased by using a multi-channel pipettor and is recommended by the manufacturer to meet the samples can be dispensed quickly in order to meet the time requirements of the test.
- Cost of analysis - as advertised, some capital investment would be required in a microtiter plate reader and multi-channel pipettor. The use of a low cost strip reader option, such as the Biotek EL 301 or Stat-Fax (both of which are available from this kit manufacturer), should be considered in order to increase assay flexibility and minimise cost whilst still meeting the needs of the smaller laboratory where smaller batches may be required.
- Ease of use – microtiter assay used in the fully quantitative format is more suited to use in a centralised laboratory due to the requirement for a spectrophotometer. The use of a simple colorimeter such as the Biotek EL 301 would make this kit easier to use in an intake situation.
- Error levels – good performance exhibited across the range when used in fully quantitative format.

The AgraQuant DON test kit showed the required performance to progress to phase 2 of the evaluation. However due to perceived limitations within the DON standards provided and the decision that only one fully quantitative test kit would progress into phase 2, the AgraQuant DON kit was not selected by grain industry representatives. A preference was shown for the test kit that was provided for assessment with a low cost reader option as it was considered that this provided greater flexibility for use within an analytical facility and at a grain intake point.

In summary, all test kits examined within the basic evaluation phase were capable of detecting DON in ground wheat samples, i.e. were “fit for purpose”, and could be used to screen intake wheat for DON levels

at the 1000ppb level or less. Kits essentially performed according to the specifications laid down by manufacturers in their advertising information.

The results of basic test kit evaluation were provided to all kit manufacturers that contacted CCFRA for an update. A presentation of the results of phase 1 was given to the **nabim** Technical & Regulatory Affairs Committee (TRAC) on 6th October 2005. Based on the performance of test kits in relation to the pre-set criteria given in Table 3 and the perceived difference between the requirements of grain merchants and millers in relation to sample turnaround, TRAC agreed that 3 kits should be progressed to phase 2. Due to the tight deadlines of this project it was not possible to schedule a face-to-face meeting with AIC members. However, AIC staff were provided with a copy of the TRAC presentation and recommendations and these were approved prior to moving to phase 2.

Not surprisingly, the best overall performance across the DON concentration range measured was achieved with fully quantitative test kits. These are higher throughput techniques that take longer to produce results (typically operating batches of 32, 48 or 96 including the required standards in around 2 hours). These assays require greater capital investment in ancillary equipment to automate the measurement and provide a direct read-out by calculating the concentration from the absorbance reading and the standard curve and are therefore more suited to a centralised laboratory environment where the required level of laboratory facilities and technical expertise already resides. Such kits can also be used with a lower cost reader (a number of these exist , but only the Biotek EL301 was tested in this study) in a reduced throughput situation.

One fully quantitative test kit, R-Biopharm's Ridascreen Fast DON, was selected to progress to phase 2 by members of TRAC. This fully quantitative ELISA incorporated a clear colour change when DON was present in the sample. The assay format (48 or 96 well microtiter plates) was particularly suited to batch analysis: the envisaged grain merchant use.

Semi-quantitative microtiterplate assays were not favoured by either TRAC members or users at CCFRA. They were not perceived to provide the benefits of ease-of-use found for the lateral flow assays and frequently failed to meet the required limit of detection and sensitivity obtainable from the fully quantitative assay. As such tests are normally threshold tests, the final result depends on visual comparison with a standard or standards. As a result the value quoted will always be at the top end of the comparison, i.e. the test tends to overestimate the DON result. *Per se* this is not a disadvantage as it should provide a margin of safety with respect to compliance with legislation being built into the sample screening process. However, in a high DON year such as 2004, this approach would lead to a significant decrease in available grain for use in the UK. The sensitivity of semi-quantitative kits is totally dependent on the standard or standards included in kit. A single standard is very common and these frequently did not provide the sensitivity required by industry. This project did not provide technical staff with a long familiarisation period for each test. The

technician involved was frequently testing Kit A and Kit B on adjacent days and, therefore, kits with very clear discrimination between positive and negative results tended to be favoured. These tended to be kits where a spectrophotometer or reader was used to generate a numerical value rather than rely on visual assessment which tended to be influenced by bias relating to colour vision or subjectivity.

Lateral flow devices proved to be very simple to use and require minimal laboratory equipment or technical experience. As for the semi-quantitative microtiterplate assays, the standard or standards supplied with the kit were vital to kit performance and the technique tends to result in an overestimation of DON levels. The use of more standards generally improved the ranking of samples. Such kits are suited to rapid sample turnaround as required at mill intake or any grain receipt point. TRAC members considered that lateral flow based technology offered opportunities to screen out high DON levels, but that it may be necessary to use quantitative methods (reference or established ELISA techniques) to make decisions on borderline cases. Of the two lateral flow devices tested in phase 1, Quick DON offered benefits in terms of ease-of-use and had undergone some slight modification in terms of sensitivity that TRAC considered warranted further evaluation. Subjectivity of assessment was removed in the Reveal for DON kit by the use of a simple palm reader. The capital investment required for this did not preclude its retention within phase 2.

5.2. Phase 2: Evaluation of selected kits

During this phase a strict testing regime was adhered to as shown in Table 7. Kits had been ordered specifically to obtain material from more than one batch of production and thus attempt to measure the impact of any between batch variations. This is particularly important for ELISA type assays where the lot numbers shown actually relate to the antibody source. This is illustrated for the two different R-Biopharm kits evaluated in phase 2, Fast DON and Quick DON, that have exactly the same lot numbers. The user must also exercise great care to ensure that all reagents have lot numbers identical to those stated in the kit enclosure.

Table 7: Testing scheme for phase 2 evaluation

	Day 1	Day 2	Day 3	Day 4	Day 5
Morning		Fast DON Lot 02354	REVEAL Lot 82007	Fast DON Lot 05135	Fast DON Lot 05135
Afternoon	Quick DON Lot 05135	REVEAL Lot 82003	Quick DON Lot 02354	REVEAL Lot 82007	Quick DON Lot 02354

Fast DON

In addition to the experimental details recorded in Section 4.2, for every sample an absorbance reading was taken using the Thermoskan Ascent (microtiter plate spectrophotometer) and the Biotek EL 301 strip-reader.

As a result of double measurement of each sample and the use of a full 48-well plate, it should be noted that the second measurement, i.e. on the Biotek EL 301, could not be made within 10 minutes of adding the stop solution. Thus, this measurement did not strictly comply with the manufacturer's test protocol. Despite this deviation, the correlation between measurements on the two instruments was excellent. Tabulated results are provided in Table 8.

Table 8: Results of phase 2 evaluation of Fast DON test kit

Sample ID	Reference data by GC-MS		Ridascreen Fast DON					
	Reported result ppb	Min-Max (Result ± 20% ppb	Lot:02354 ppb)	Lot:05135 ppb	Lot:05135 ppb	Average ppb	StDev ppb	% CV
CM/81246/1	743	594 - 892	463	601	599	554	79	14.3
CM/81246/9	1069	855 - 1283	963	1090	1000	1018	65	6.4
CM/81246/10	923	738-1108	1064	876	832	924	123	13.3
CM/81246/14	1001	801-1201	1338	1329	1291	1319	25	1.9
CM/81246/15	1122	898-1346	699	758	718	725	30	4.2
CM/81246/16	1276	1021-1531	1362	1342	1354	1353	10	0.7
CM/81246/17	1334	1067-1601	597	577	626	600	25	4.1
CM/81246/21	1058	846-1270	1008	1122	1183	1104	89	8.0
CM/81246/23	1490	1192-1788	1216	1298	1291	1268	45	3.6
CM/81246/30	1010	808-1212	1547	1518	1564	1543	23	1.5
CM/81246/33	601	481-721	678	738	720	712	31	4.3
CM/81246/36	1257	1006-1508	1262	1243	1291	1265	24	1.9
CM/81246/42	1405	1124-1686	1458	1473	1507	1479	25	1.7
CM/81246/43	407	326-488	798	840	850	829	28	3.3
CM/81246/46	451	361-541	380	464	461	435	48	11.0
CM/81246/47	1334	1067-1601	1317	1290	1319	1309	16	1.2
CM/81246/48	1397	1118-1676	997	1038	1050	1028	28	2.7
CM/81246/51	586	469-703	1219	1262	1282	1254	32	2.6
CM/81246/56	945	756-1134	1372	1398	1397	1389	15	1.1
CM/81246/62	1589	1271-1907	1642	1682	1640	1655	24	1.4

Of the 20 samples tested, the Fast DON results lay outside the range of the reference measurements in 9 cases (shaded average results). For one sample, CM/81246/17, the rapid assay recorded a value of less than half the reference result and would have resulted in acceptance of a sample that was above the legal limit. However, this sample has been shown to be mis-classified in all three assays, casting some doubt on the reference data or suggesting that the water extraction used in the test kits is not extracting DON effectively. Of the remaining 8 samples that lie outside the measured range, 4 would have resulted in a sample being rejected which was acceptable for use (i.e. read high) and one gave low readings (leading to acceptance of a sample where the DON level was slightly above the legal limit). There would have been no impact on acceptance for the remaining two samples.

The test set used was designed to challenge the assay and the results suggest that in order to provide an adequate safety margin with respect to DON measurement, the user may have to set a threshold of less than

1000ppb. With the exception of the anomalous sample CM/81246/ 17, the use of this threshold would have rejected all grain with DON levels above 1250ppb.

Across the 20 samples tested, the Repeatability of results within a batch (Lot No 05135) for Fast DON was good and comparable with that observed in phase 1. Results are presented graphically in Figure 3. The correlation coefficient (R^2) obtained was in line with that quoted by R-Biopharm of R^2=0.98.

Figure 3: Within batch performance of Fast DON

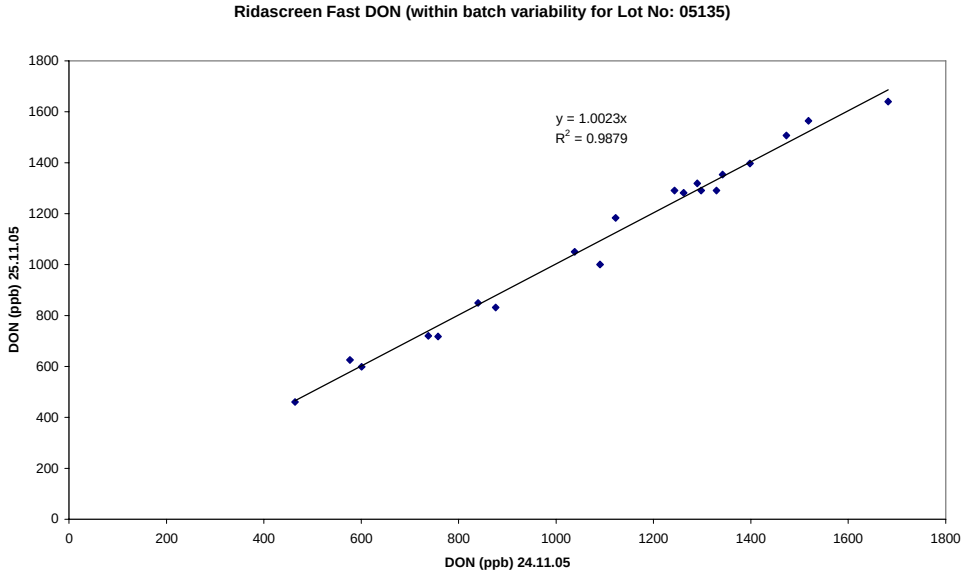
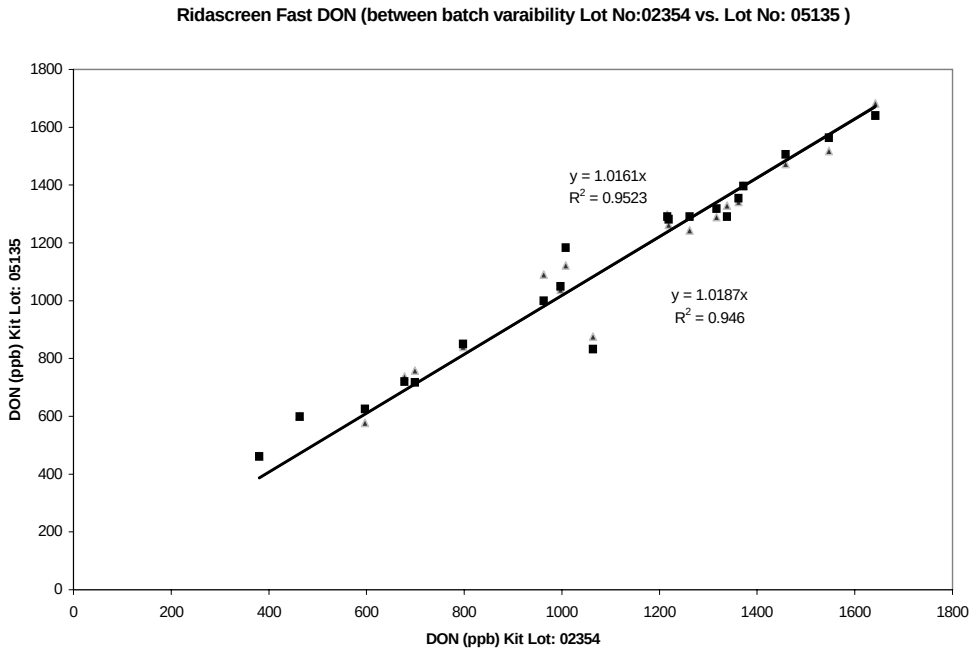


Figure 4: Between batch performance of Fast DON test kit



When the results obtained for two different batches of Fast DON (i.e. between batch variation for Lot Nos. 05135 and 02354) were compared, the following relationships were obtained: R^2 =0.95, intercept y=1.0161and R^2 =0.95, intercept y=1.0187 as shown in Figure 4. These values are slightly better than that quoted by R-Biopharm at R^2 =0.90.

When results for 4 common samples were compared between phase 1 and phase 2 (Table 9), two agreed very well but samples CM/81246/16 and CM/81246/46 showed very poor repeatability. It was not possible to investigate the reason for these anomalies and hence draw any conclusions. However, the data does suggest that the user should include a real wheat check sample of known DON level in every test batch in order to monitor kit performance.

Table 9: Results of test carried out using Fast DON kits on common samples in phase 1 and phase 2

	CM/81246/9	CM/81246/10	CM/81246/16	CM81246/46
Average DON in phase 1	926	962	791	723
Average DON in phase 2	1018	924	1353	435

During phase 2 evaluation, some doubts were cast relating to the lack of CE marking on the the Biotek EL-301 strip reader and the potential for it to be discontinued. As far as CCFRA is aware this has not happened and alternative strip readers are available, albeit at slightly higher cost.

QUICK DON

This semi-quantitative lateral flow device was considered to be the simplest of all to use and had the potential for use at a weighbridge or in a grain storage facility. At the time of evaluation, the lateral flow device employed simple visual comparison between test line formation for an unknown sample and photographic images of samples of positive and negative test results. These images were intended to allow the unknown sample to be placed into a series of categories. Private communication with the manufacturer suggests that work is in progress to produce a reader for this lateral flow device to eliminate subjectivity in measuring DON level and remove the need to rely on photographic images.

Two major changes had occurred in this test kit between phase 1 and phase 2 of the evaluation. In phase 1 the kit was only validated at 2000ppb of DON and the manufacturer recommended that the test result must be read after 5 minutes. The latter constraint had created some difficulty for some extracts as the view pane appeared to be smeared with coloured dye, i.e. the dye did not appear to have fully migrated to its final position within the view pane. By phase 2 of the evaluation, the kit was validated and could be tested at 1000ppb and the new kit instructions stated that "the immuno-chromatographic strip should be read after a period of 10 minutes". The first change makes the test kit suitable for use in screening against a legal limit of 1250ppb; whilst the second change was not explained by the manufacturer, it could be expected to be a requirement of the increased sensitivity and a response to difficulties in measurement. This increase in incubation time would extend the analysis time to ~ 20 minutes.

Table 10: Results of test carried out on 2 different Quick DON kits over three different days

Sample ID	Reference data by GC-MS		RIDASCREEN QUICK DON		
	Reported result ppb	Min-Max Result ± 20% ppb	Lot:05135	Lot:02354 (1ppm Cut-off)	Lot:02354
CM/81246/1	743	594 - 892	Negative	Negative	Negative
CM/81246/9	1069	855 - 1283	Positive	Positive	Positive
CM/81246/10	923	738-1108	Positive	Positive	Positive
CM/81246/14	1001	801-1201	Positive	Positive	Positive
CM/81246/15	1122	898-1346	Positive	Positive	Positive
CM/81246/16	1276	1021-1531	Positive	Positive	Positive
CM/81246/17	1334	1067-1601	Negative	Negative	Negative
CM/81246/21	1058	846-1270	Positive	Positive	Positive
CM/81246/23	1490	1192-1788	Positive	Positive	Positive
CM/81246/30	1010	808-1212	Positive	Positive	Positive
CM/81246/33	601	481-721	Positive	Positive	Positive
CM/81246/36	1257	1006-1508	Positive	Positive	Positive
CM/81246/42	1405	1124-1686	Positive	Positive	Positive
CM/81246/43	407	326-488	Positive	Positive	Positive
CM/81246/46	451	361-541	Negative	Negative	Negative
CM/81246/47	1334	1067-1601	Positive	Positive	Positive
CM/81246/48	1397	1118-1676	Positive	Positive	Positive
CM/81246/51	586	469-703	Positive	Positive	Positive
CM/81246/56	945	756-1134	Positive	Positive	Positive
CM/81246/62	1589	1271-1907	Positive	Positive	Positive

Lateral flow formats are prone to an overestimation of DON levels, since the development of the test band relies on reaching a threshold or limit value. In this respect, the test is qualitative. The kit is capable of use at 2 sensitivity levels, 2000ppb and 1000ppb. Our initial evaluation at 2000ppb was more clear-cut, and is probably representative of the initially intended use of the kit.

Operating at a 1000ppb threshold the Quick DON test kit produced consistent results across different batches of kit and within a single test kit. However, 2 samples out of the 20 were consistently mis-classified as positives despite being significantly lower than the 1000ppb threshold (these are shown as shaded in Table 9). In terms of food safety, false positives are less serious in screening than false negatives and the 1000ppb threshold would provide a safety margin with respect to the proposed legal limit for wheat of 1250ppb. However, it also introduces the risk of rejecting samples below limit. One sample with a reference DON level of >1250ppb was recorded incorrectly as negative. However, this sample was found to give anomalous results in all three assays.

Comparison of the results of the fully quantitative microtiterplate based Fast DON ELISA and the lateral flow technology based Quick DON showed that agreement was generally good apart from samples with DON values in the region 600-1000ppb. Failure of the Quick DON kit to estimate correctly leads to 2 false positives as highlighted in grey in Table 10. An additional difference is indicated with respect to sample CM/81246/15. This produces a low DON level with the Fast DON test kit, but is correctly assigned as above

1000ppb by the Quick DON lateral flow assay. These differences in test results appear slightly odd given that the same antibody is used in both formats.

Despite the good results obtained, technical staff found the visual assessment of test lines quite difficult at times. This format does not provide any lasting evidence that the test was carried out. (The test band fades over time). The "Evaluation Card" is not fully representative of the colour bands produced in real time and hence interpretation can be difficult and in its current format the kit can really only be used to screen at the 1000ppb level. The development of a test strip reader to remove the subjective element of this test should be a priority for R-Biopharm to increase the suitability of this test for use at grain intake facilities.

REVEAL for DON

As in previous work, subjectivity in the reading was removed by the use of the AccuScan palm-held reader. In this format this lateral flow device combines the advantages of a DON screening tool that could be used in a rapid turnaround situation at mill intake. For sample traceability the provision of a permanent record of the DON classification of the sample and an image of the view panel on the lateral flow device were considered significant advantages.

Due to the need to test lateral flow devices from more than one manufacturing batch, some between batch differences were noted in terms of expiry date. For example, the expiry date for the most recent Reveal test kit (Lot 82007) was much shorter than the previous kit (Lot 82003). Communication with Neogen provided no real explanation for this but CCFRA was assured that kit performance was not affected by age of the kit, providing it is used within the expiry date.

A problem occurred in the initial loading of the software algorithm for kit Lot 82003. Due to lack of use, the power to the AccuScan unit had been lost, resulting in the loss of the "AccuScan Data Manager" and "Hot-Sync Connection", a conduit linking the AccuScan to an external PC to transfer data files. These were recoverable but the instruction manual may benefit from including additional information to cover such eventualities as this assay may be used infrequently at some locations.

Table 11: Results of tests carried out on two different REVEAL for DON kits over 3 different days

Sample ID	Reference data by GC-MS		Reveal for DON		
	Reported result ppb	Min-Max (Result ± 20% ppb	Lot:82003 ppm	Lot:82007 ppm	Lot:82007 ppm
CM/81246/1	743	594 - 892	0.5	0.5	0.5
CM/81246/9	1069	855 - 1283	1.0	1.0	1.0
CM/81246/10	923	738-1108	1.0	1.0	1.0
CM/81246/14	1001	801-1201	2.0	2.0	2.0
CM/81246/15	1122	898-1346	1.0	0.5	0.5
CM/81246/16	1276	1021-1531	2.0	1.0	1.0
CM/81246/17	1334	1067-1601	0.0	0.0	0.0
CM/81246/21	1058	846-1270	1.0	1.0	1.0
CM/81246/23	1490	1192-1788	2.0	1.0	1.0
CM/81246/30	1010	808-1212	2.0	2.0	2.0
CM/81246/33	601	481-721	1.0	0.5	0.5
CM/81246/36	1257	1006-1508	2.0	1.0	1.0
CM/81246/42	1405	1124-1686	2.0	2.0	2.0
CM/81246/43	407	326-488	1.0	1.0	1.0
CM/81246/46	451	361-541	0.5	0.5	0.5
CM/81246/47	1334	1067-1601	1.0	1.0	1.0
CM/81246/48	1397	1118-1676	1.0	1.0	1.0
CM/81246/51	586	469-703	2.0	2.0	2.0
CM/81246/56	945	756-1134	1.0	2.0	2.0
CM/81246/62	1589	1271-1907	2.0	2.0	2.0

The quantification of the test line on the lateral flow device using AccuScan is a significant benefit with the following limit values; 0 (negative), 500 (positive), 1000 (positive) and 2000 (positive) ppb (actually shown on screen as 0, 0.5, 1.0 and 2.0ppm) punctuating the algorithm. An element of overestimating the DON level is expected with a system such as this. The instrument places samples into classes based on 500 and 1000ppb and thus appears to provide the required level of quantification for screening purposes. The AccuScan is easy to use, and is suitable for remote operation. A docking console allows data to be transferred to the "AccuScan Data Manager", automatically uploading results. The "on-screen" instructions are straightforward, pointing to the "Data Window", which resembles an Excel spreadsheet with all accumulated results.

A number of anomalous results were obtained. As in previous assays, the most significant variation was shown for the anomalous sample CM/81246/17 which was measured as acceptable by the Reveal kit but has a DON level above the legal limit. Some inconsistency in classification of samples within the commercially sensitive area was also observed. For example, of the other 11 samples with DON levels above 1000ppb, 8 were classified as 1000ppb and only 3 as 2000ppb, suggesting that the expected overestimation of DON levels did not occur. The test set used in phase 2 was very challenging for any DON assay as it contained a high proportion of samples between 1000 and 1500ppb. It is perhaps expecting too much that a simple lateral flow assay should be able to correctly rank all samples into the correct group. Certainly a "corridor of

uncertainty" appears to exist and kit performance may be improved by using a pure reference chemical or wheat check sample with DON levels between 700ppb and 1000ppb.

Some inconsistency between kit lots was also observed for Reveal. For example, Lot No 82007 tended to produce lower values than Lot 82003 for samples CM/81246/15, 16, 23, 33 and but higher for CM/81246/56.

6. CONCLUSIONS

- Errors associated with the reference DON measurement, typically ±20%, make it difficult to make direct comparisons with DON test kits across a range of samples. This is particularly true where the samples are clustered around the commercially sensitive area. In order to make fair comparisons, it has therefore been necessary to use the quoted range (result ±20%) to compare with rapid test kit results. A perfect relationship between the reference method and the test kit procedure, which has a very different chemical basis, cannot be expected.
- Correlations between "reference" and test kit results, provided by manufacturers, frequently use an HPLC reference method rather than the GC-MS method used in this study. There is no guarantee that the same relationship exists with the GC-MS method used here.
- Some of the error associated with the "reference" value is inherent in the sampling due to uneven distribution of mycotoxins in grain, but a significant proportion is due to the analytical procedure that corrects for sample recovery. However, acceptance/rejection decisions are generally made on the basis of an absolute value.
- Given the above statement, there is an expectation that any rapid test kit should generate a number that can be used to make the same absolute accept/reject decisions. This cannot be the case for test kits that are not fully quantitative.
- It should be remembered that the sample set used in this work included some extreme DON levels. HGCA report 354 shows that DON levels of >1250ppb have traditionally been very rare in the UK milling wheat marketplace. In addition, the project only permitted limited evaluation of DON test kits in deliberately challenging conditions. This does not constitute a full validation of any method for which a significantly larger sample set would be required.
- Rapid test kits have a role to play in ensuring the safety of raw material (wheat) entering the human or animal food chain. They could be used by the supplier to screen samples in relation to the legal limits set by the EU, but it is important that an element of caution is applied to their use. In order to take account of the uncertainty of the measurement it may be necessary to consider a threshold below the legal limit. For fully quantitative tests a threshold at between 800 and 1000ppb would help to ensure that samples with unacceptably high DON levels are not allowed into these food chains but would result in rejection of some samples within the 1250ppb limit.

- Lateral flow technology is a fast moving area for kit manufacturers. Firstly, they have been forced to adapt kit thresholds to meet changes in the proposed legal limits for DON from 1000 ppb to 1250ppb (official from July 1st 2006). Secondly, there is a need to develop appropriate test strip readers which can remove the subjectivity from the assessment and allow lateral flow technology to compete in this marketplace. Such developments must improve the quality and consistency of decisions made using lateral flow technology. However, the lateral flow assay cannot compete fully in the quantitative arena. It can only ever be used for screening purposes and the threshold used will depend on the level of discrimination possible. As this is more limited it is not as simple to apply the same kind of safety margin recommended for the fully quantitative test kits. If this is required it will be necessary to operate with a threshold at 500ppb, which is likely to result in numerous 'false positives' i.e. many samples below the 1250ppb limit being rejected. An alternative solution may be to use the 1000ppb threshold and check a proportion of samples that lie in the 500-1000ppb "grey area" by the reference method. This would be expected to reduce the incidence of false positives.
- In order to improve the quality of decision made using a rapid test kit, it is recommended that all tests are duplicated and that a suitable standard (a pure DON chemical or a wheat based check sample of known DON level close to the agreed threshold) should be tested alongside unknown samples.
- A number of challenges still exist for rapid DON test kits:
- Due to the limited life span of antibodies, which are often produced from a single source (animal), such kits are prone to re-issue and this raises the question of the validity of the validation data and the need for re-evaluation when kit contents change significantly.
- A key factor in the sensitivity of test kits is focused on the availability of appropriate reference samples
- As analytical techniques become more and more sophisticated, the speed and sensitivity of standard methods is likely to improve. As new "reference" methods are created there is a need to make comparisons with rapid test kit methods and thus the pressure on rapid kit methods changes. This is already evident in much of the kit advertising material where comparisons have been made with HPLC reference data rather than the GC-MS data used in this study.

7. ACKNOWLEDGEMENTS

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8. REFERENCES

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APPENDIX 1

Table 1: Fusarium mycotoxin levels and species for food wheat samples ex HGCA pink grain survey[1]

CCFRA Code	Region	No of pink grains	Mycotoxin levels (ppb)				*Fusarium* species			
			Deoxy-nivalenol (DON)	3Ac-DON	15Ac-DON	Nivalenol	*F. graminearum*	*F. culmorum*	*F. poae*	*F.* spp
CM/81246/1	E	50	743	<10	<10	29	0	Light	0	Medium
CM/81246/2	E	60	649	<10	<10	28	0	0	0	Light
CM/81246/3	E	TMTC	11500	31	77	93	0	Very Heavy	0	0
CM/81246/4	SW	3	183	<10	<10	17	0	0	0	0
CM/81246/5	Scot	10	20	<10	<10	<10	0	0	0	0
CM/81246/6	E	TMTC	1839	<10	<10	27	0	0	0	0
CM/81246/7	SW	100	720	<10	<10	36	0	Medium	0	0
CM/81246/8	E	35	3016	22	18	50	0	Light	0	Medium
CM/81246/9	SW	7	1069	<10	<10	11	0	Light	0	Medium
CM/81246/10	SE	22	923	<10	<10	36	0	Medium	0	Medium
CM/81246/11	SW	3	58	<10	<10	<10	0	Light	0	Medium
CM/81246/12	SE	5	39	<10	<10	<10	0	0	0	0
CM/81246/13	SE	3	22	<10	<10	<10	0	Light	0	0
CM/81246/14	E	10	1001	<10	<10	139	Medium	Medium	Light	Light
CM/81246/15	E	42	1122	<10	<10	89	0	Light	0	Light
CM/81246/16	E Mids	45	1276	<10	10	30	0	Medium	0	Light
CM/81246/17	Y & H	30	1334	<10	10	31	0	0	0	Light
CM/81246/18	E Mids	11	179	<10	<10	38	0	Light	0	Medium
CM/81246/19	E Mids		261	<10	<10	26	0	Light	0	Light

CCFRA Code	Region	No of pink grains	Mycotoxin levels (ppb)				*Fusarium* species			
			Deoxy-nivalenol (DON)	3Ac-DON	15Ac-DON	Nivalenol	*F. graminearum*	*F. culmorum*	*F. poae*	*F.* spp
CM/81246/20	E Mids		1866	<10	14	36	Light	Light	0	Very Heavy
CM/81246/21	E		1058	<10	<10	28	0	0	0	Heavy
CM/81246/22	Y & H		57	<10	<10	20	0	0	0	Light
CM/81246/23	E Mids	40	1490	<10	<10	36	0	Heavy	0	Medium
CM/81246/24	E Mids	45	2472	11	16	34	0	Medium	0	Heavy
CM/81246/25	E Mids	20	344	<10	<10	<10	Light	0	0	Light
CM/81246/26	E Mids	15	1016	<10	<10	20	0	0	0	0
CM/81246/27	E Mids	20	2380	<10	14	25	0	0	0	Light
CM/81246/28	E	15	538	<10	<10	<10	0	Light	0	Light
CM/81246/29	E	20	241	<10	<10	<10	0	0	0	Light
CM/81246/30	E	30	1010	<10	<10	16	0	Light	0	Medium
CM/81246/31	E	35	791	<10	<10	15	Light	Medium	0	Light
CM/81246/32	E	48	4723	14	20	44	0	Very Heavy	0	Light
CM/81246/33	Y & H		601	<10	<10	46	0	Heavy	Light	Medium
CM/81246/34	E Mids		2644	<10	15	58	0	Heavy	0	Medium
CM/81246/35	E Mids		3088	<10	29	36	0	Very Heavy	0	Medium
CM/81246/36	E		1257	<10	10	37	0	Heavy	0	Medium
CM/81246/37	E		4079	25	37	82	Very Heavy	0	0	Light
CM/81246/38	E	20	341	<10	<10	28	0	Medium	0	0
CM/81246/39	E Mids		33	<10	<10	27	0	Light	0	Light
CM/81246/40	E	20	331	<10	<10	77	Light	0	0	Light
CM/81246/41	E	25	3722	26	46	142	Light	Medium	0	Medium

CCFRA Code	Region	No of pink grains	Mycotoxin levels (ppb)				*Fusarium* species			
			Deoxy-nivalenol (DON)	3Ac-DON	15Ac-DON	Nivalenol	*F. graminearum*	*F. culmorum*	*F. poae*	*F.* spp
CM/81246/42	E	400	1405	<10	13	30	0	Very Heavy	0	Medium
CM/81246/43	Y & H	10	407	<10	<10	61	0	Medium	0	Medium
CM/81246/44	Y & H	20	177	<10	<10	13	0	Medium	0	Medium
CM/81246/45	Y & H	25	294	<10	<10	10	0	Light	0	0
CM/81246/46	E Mids	20	451	<10	<10	<10	Medium	Light	0	Light
CM/81246/47	E	20	1334	<10	<10	36	0	Very Heavy	0	Medium
CM/81246/48	E	10	1397	<10	<10	33	0	0	0	0
CM/81246/49	E	50	3775	20	25	75	0	Very Heavy	0	0
CM/81246/50	SW	20	1800	<10	<10	76	Very Heavy	Light	0	Medium
CM/81246/51	E	8	586	<10	<10	52	Light	Light	0	Medium
CM/81246/52	E	8	438	<10	<10	23	Medium	Light	0	Light
CM/81246/53	E	8	1076	<10	<10	52	0	Light	0	Heavy
CM/81246/54	E	8	416	<10	<10	34	Light	Light	0	Medium
CM/81246/55	E	100	3777	10	15	28	Very Heavy	Medium	0	Light
CM/81246/56	E	40	945	<10	13	23	0	Heavy	0	Medium
CM/81246/57	E	30	1978	<10	17	53	Medium	Light	0	Very Heavy
CM/81246/58	E	40	371	<10	<10	24	0	0	0	Light
CM/81246/59	E	40	1351	<10	<10	61	Heavy	Light	0	Light
CM/81246/60	E	12	1819	10	11	27	0	Very Heavy	0	Light
CM/81246/61	SE	9	243	<10	11	<10	Medium	0	0	Medium
CM/81246/62	SE	50	1589	<10	11	13	Medium	Medium	0	Very Heavy
CM/81246/63	NI	40	148	<10	<10	28	0	0	0	Light

CCFRA Code	Region	No of pink grains	Mycotoxin levels (ppb)				*Fusarium* species			
			Deoxy-nivalenol (DON)	3Ac-DON	15Ac-DON	Nivalenol	*F. graminearum*	*F. culmorum*	*F. poae*	*F.* spp
CM/81246/64	Y & H	50	1235	<10	<10	75	0	Heavy	0	Medium
CM/81246/65	E	11	655	<10	<10	60	Heavy	Light	0	Light
CM/81246/66	E Mids	25	2300	<10	<10	<10				
CM/81246/67	N E	7	11	<10	<10	<10				
CM/81246/68	SE	0	593	<10	<10	<10				

EXTENSION TO HGCA PROJECT REPORT NO. 394:

EVALUATION OF RAPID TEST KITS FOR DEOXYNIVALENOL (DON)

EVALUATION OF:

ROSA® DON P/N Test, Charm Sciences Inc.

ROSA® DON (QUANTITATIVE) Test, Charm Sciences Inc.

N.J. MATTHEWS & J.M. PRATT

CCFRA

CONTENTS

Abstract

This annex to HGCA project report 394 was initiated to evaluate rapid test kits produced by Charm Sciences Inc. that were not available at the time of the initial project. The brief remained to evaluate the suitability of commercially available test kits to screen intake samples for DON and to provide reliable quantitative data rapidly. Specifically, the project set out to provide the cereal processing chain with information on the appropriateness of kits for use in intake situations and, thus, to help the industry meet the requirements of mycotoxin legislation.

ROSA® (Rapid One Step Assay) from Charm Sciences Inc. is a lateral flow (LF) immunoassay available as a positive/negative (P/N) screening test and as a fully quantitative test. Both formats of the kit were evaluated.

Overall, on the basis of the work reported in PR394, the ROSA® DON (P/N) screening test and the ROSA® DON (Quantitative) test kits provide the performance and flexibility required for use as a surveillance tool in the risk management of DON in ground wheat.

ROSA® DON Qualitative P/N and Quantitative Tests Charm Sciences Inc.

Background

This annex to HGCA project report 394 was initiated to evaluate rapid test kits not available at the time of the initial project. The brief remains to evaluate test kits as suitable screening tools and to provide reliable quantitative data, rapidly. ROSA® (Rapid One Step Assay) from Charm Sciences Inc. is a lateral flow (LF) immunoassay available as a positive/negative (P/N) screening test and as a fully quantitative test. Both formats of the kit were evaluated.

Both test formats require incubation of the analysis test strip, using: LF-INC4-3-45D Quad (four-lane) Incubator, or, LF-INC2-3-45D, Dual (two-lane) incubator, for the ROSA® DON (P/N) Test, and LF-INC4-10-45D Quad (four-lane) or LF-INC2-10-45D Dual (two-lane) incubator, for the ROSA® DON (Quantitative) Test. (Available from Charm Sciences Inc.) The incubator is set at a constant temperature of 45°±1°C, and has an in-built timer (pre-set to 3 minutes for the ROSA® DON (P/N) Test, and 10 minutes for the ROSA® DON (Quantitative) Test), to ensure consistency of incubation. A visual indicator strip reveals the actual temperature of the device.

Administration) certified (Certificate of Performance #2006-003), at 500ppb and 1000ppb, for DON in wheat. The kit is available in: 20 Test Strips, 100 Test Strips and 500 Test Strips. The kit contains test strips, a lyophilised standard (500ppb DON) and DON Dilution Buffer. A Certificate of Quality is supplied with each test kit, clearly stating the expiry date for the test strips and the DON Dilution Buffer (NB the expiry date for the lyophilised and reconstituted 500ppb Control, are stated on the vial). Storage conditions for the kit are clearly stated in the enclosed procedure. The ROSA-M-Reader is supplied with calibration strips to assess performance. Users should ensure that the lot number on the canister, containing the calibration strips, is identical to that printed on a self-adhesive label on the underside of the ROSA-M-Reader.

Procedure

The extraction and sample preparation stated in the manufacturer's procedure was followed. A simplified annotated version can be downloaded, free-of-charge from www.charm.com. For this evaluation, the Quad Incubator was used. All analysis was conducted on duplicate extractions. Inter-batch variability was assessed for the analysis of test kits from consecutive production runs. Two analysis protocols can be followed:

- GIPSA recommended procedure requiring 50g of ground sample, extracted using 250ml of distilled or deionised water.
- 10-50g ground sample extracted using 5 times sample mass (in ml) of deionised or distilled water.

The ROSA® DON P/N Test is intended for use at two screening levels: 500ppb and 1000ppb.

For the evaluation, samples were ground on a Perten LM3100 mill attached with an 800µm screen. All analysis followed the recommended GIPSA protocol. All samples were extracted and analysed in duplicate.

Results

The ROSA-M-Reader displays the following:

1. A "NEGATIVE" sample containing less than 500ppb or 1000ppb DON, dependent on the screening level selected.
2. A "POSITIVE" sample contains DON at a level greater than 500ppb or 1000ppb DON, dependent on the screening level selected.

For this evaluation, 19 samples, used in Phase II of the main study (HGCA Project Report 394), were extracted and analysed at the 500ppb and 1000ppb threshold levels, in duplicate and measured using kit lots from consecutive batches. The results are presented in Table 1.

Table 1: Analysis of Selected Samples of Ground Wheat for DON using ROSA DON (P/N)

	Ref data by GC/MS			ROSA DON (P/N)							
Sample ID	Min	Max	Mean	LOT	LOT	LOT	LOT	LOT	LOT	LOT	LOT
	(Mean –20%)	(Mean +20%)	Target	(002-B)	(002-B)	(002-B)	(002-B)	(003-B)	(003-B)	(003-B)	(003-B)
	ppb	ppb	ppb	500ppb	1000ppb	500ppb	1000ppb	500ppb	1000ppb	500ppb	1000ppb
CM/81246/1	594	892	743	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)
CM/81246/9	855	1283	1069	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
CM/81246/10	738	1108	923	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
CM/81246/14	801	1201	1001	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
CM/81246/15	898	1346	1122	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
CM/81246/16	1021	1531	1276	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
CM/81246/21	846	1270	1058	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
CM/81246/23	1192	1788	1490	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
CM/81246/30	808	1212	1010	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
CM/81246/33	481	721	601	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)
CM/81246/36	1006	1508	1257	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
CM/81246/42	1124	1686	1405	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
CM/81246/43	326	488	407	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
CM/81246/46	361	541	451	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)
CM/81246/47	1067	1601	1334	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
CM/81246/48	1118	1676	1397	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
CM/81246/51	469	703	586	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)
CM/81246/56	756	1134	945	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
CM/81246/62	1271	1907	1589	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)

Key

Incorrectly classified analysis.

N.B. Incorrect classifications are based on the Target mean, however, they are all correct within +/-20% range.

Discussion and conclusions

ROSA® DON P/N Test LF immunoassay provides qualitative, positive (+) or negative (-) estimations of DON in aqueous extracts of ground wheat. Screening is possible at two concentration levels (500ppb and 1000ppb), thus widening the scope of potential users to cereal processors. For the purpose of this evaluation, the ability of the test to discriminate between samples above or below the screening threshold level and above or below the EU legislative limit of 1250ppb, for DON in unprocessed grain, and at 500ppb for processed grain was assessed.

One hundred and fifty-two tests were conducted on nineteen samples from 2 kit lots. (Lot 002-B and 003-B). Seventy-six tests were conducted at each screening level. Of the tests conducted at the 500ppb screening level, seventy-two (95%) correlated positively with the confirmatory test (GC-MS) mean reference values. Of the tests conducted at the 1000ppb screening level, sixty-eight (90%) correlated positively with the confirmatory test. The remaining twelve tests, from three samples, did not give confirmatory test values at each screening level (four errors were recorded at the 500ppb screening level, eight errors were recorded at the 1000ppb screening level.) All errors recorded were for samples having GC/MS values within 100ppb of the measured confirmatory test mean (451, 923 and 945ppb). All four errors recorded at the 500ppb level, on the sample with a GC/MS value of 451ppb, were "false positives" (violatives). For data obtained from sample analysis this close to the screening threshold, it would be advisable to recommend further testing, i.e. providing an appropriate "risk averse" strategy to testing. For errors recorded, in comparison to reference values, at the 1000ppb screening level, all 8 results were correctly identified as "positive" at the 500ppb screening level. The high level of agreement between the ROSA® DON P/N Test analysis and the confirmatory test is encouraging.

Those that did not agree were few in number and fell within the Uncertainty of Measurement (UoM) for the confirmatory test method i.e. application of an appropriate "risk averse" strategy would advocate further testing, as stated for the situation at 500ppb.

The most important judgement of assay performance is the correlation with confirmatory test values at or around EU limits, (for the purpose of this evaluation, freedom from "false positives" and "false negatives" at the EU limit for unprocessed grain, i.e. 1250ppb, was considered). From the sample set selected for the evaluation,

eleven samples were selected with confirmatory test values within ± 250ppb of the 1250ppb limit i.e. ± 20% of the threshold. (five samples<1250ppb and six samples >1250ppb), representing forty-four analyses. This part of the evaluation was designed to test assay sensitivity at the EU limit of 1250ppb, for unprocessed grain. The fact that all eleven samples were correctly classified as having a DON level of >1000ppb represents exceptional performance for LF devices of the qualitative/ semi-quantitative type.

Overall, the low incidence of incorrectly categorised samples is very encouraging. All false positive results obtained were recorded on samples within 100ppb of the screening threshold (in comparison to the confirmatory technique). This is a qualitative expression of assay at the screening level, rather than poor performance. The results compare favourably to other LF based test kits evaluated in Project Report 394.

Evaluation of ROSA® DON (Quantitative) Test

The ROSA® DON (Quantitative) Test is a fully quantitative LF immunoassay for the detection of DON, extracted in aqueous solution from a sample of ground wheat, barley, corn or rice.

Assay performance characteristics are quoted as:

Sensitivity: 0-5000ppb DON.

Limit of Detection (LOD): <100ppb

Accuracy:

± 50% of mean ppb concentration at 500ppb

± 40% of mean ppb concentration at 1100ppb

± 30% of mean ppb concentration at 1900ppb

± 20% of mean ppb concentration at 5100ppb

Introduction

The assay kit is available in three sizes: 20 test strips 100 test strips and 500 test strips

The assay kit contains: test strips, lyophilised 1000ppb DON positive control and DONQ Dilution Buffer. When required, the lyophilised 1000ppb DON positive control is reconstituted in 3ml of DONQ Dilution Buffer. Before commencing analysis, a performance check of the ROSA-M-Reader should be conducted using the Calibration Strips provided. The ROSA-M-Reader outputs must be within the limits stated on each Calibration Strip. As described previously, the order number printed on the canister must be identical to that printed on the base of the ROSA-M-Reader.

Procedure

The extraction and sample preparation is clearly stated in the procedure, (a simplified annotated version can be downloaded, free-of-charge from www.charm.com). For this evaluation, the Quad Incubator (INC4-10-45D) was used. The incubation temperature for the assay was 45°±1°C and the in-built timer was pre-set for 10 minutes. All analysis was conducted on duplicate extractions. Inter-batch variability was assessed

from the analysis of test kits from consecutive production runs. Two analysis protocols can be followed:

- GIPSA recommended procedure requiring 50g of ground sample, extracted using 250ml of distilled or deionised water.
- 10-50g ground sample extracted using 5 times sample mass (in ml) of deionised or distilled water.

For the evaluation, samples were ground on a Perten LM3100 mill attached with an 800µm screen. All analysis followed the recommended GIPSA protocol. All samples were extracted and analysed in duplicate.

The ROSA-M-Reader displays results from the ROSA® DON (Quantitative) Test, to the nearest 50ppb. The ROSA-M-Reader stores results automatically in memory, which can be recalled or downloaded into proprietary MYCOsoft software (www.charm.com).

Results

For the purpose of the evaluation, 19 samples selected from Phase II of HGCA Project Report 394 were selected for analysis (Table 2).

All sample analysis was conducted in duplicate, i.e. the average result quoted from two extractions of the same sample. Within and between batch evaluations were conducted for repeatability and reproducibility. Additionally, each batch was correlated with quantitative data obtained using the confirmatory test (GC/MS) procedure.

Table 2: Analysis of Selected Samples of Ground Wheat for DON using ROSA® DON (Quantitative) Test

Sample ID	Reference data by GC/MS			ROSA DON (Quantitative)								
	Min	Max	Mean	A002001B-06			B00200122B-08					
	(Mean-20%)	(Mean+20%)	Target	12.04.07	12.04.07	Mean	16.04.07	16.04.07	Mean	17.04.07	17.04.07	Mean
	ppb	ppb	ppb	R1	R2		R1	R2		R3	R4	
CM/81246/1	594	892	743	700	650	**675**	600	750	**675**	600	750	**675**
CM/81246/9	855	1283	1069	1150	950	**1050**	1000	1100	**1050**	1050	900	**975**
CM/81246/10	738	1108	923	950	800	**875**	850	1000	**925**	950	900	**925**
CM/81246/14	801	1201	1001	950	1100	**1025**	1050	900	**975**	950	1100	**1025**
CM/81246/15	898	1346	1122	1000	1150	**1075**	1200	1000	**1100**	1100	950	**1025**
CM/81246/16	1021	1531	1276	1350	1100	**1225**	1150	1300	**1225**	1350	1100	**1225**
CM/81246/21	846	1270	1058	900	1150	**1025**	1200	1050	**1125**	1000	1050	**1025**
CM/81246/23	1192	1788	1490	1550	1350	**1450**	1450	1550	**1500**	1500	1400	**1450**
CM/81246/30	808	1212	1010	950	1200	**1075**	1050	1000	**1025**	1100	950	**1025**
CM/81246/33	481	721	601	550	500	**525**	450	550	**500**	600	550	**575**
CM/81246/36	1006	1508	1257	1200	1350	**1275**	1400	1250	**1325**	1450	1350	**1400**
CM/81246/42	1124	1686	1405	1350	1550	**1450**	1500	1400	**1450**	1450	1350	**1400**
CM/81246/43	326	488	407	500	550	**525**	500	400	**450**	550	500	**525**
CM/81246/46	361	541	451	500	600	**550**	600	450	**525**	550	600	**575**
CM/81246/47	1067	1601	1334	1250	1400	**1325**	1200	1300	**1250**	1150	1400	**1275**
CM/81246/48	1118	1676	1397	1450	1550	**1500**	1450	1500	**1475**	1550	1350	**1450**
CM/81246/51	469	703	586	700	550	**625**	650	550	**600**	550	700	**625**
CM/81246/56	756	1134	945	1050	1100	**1075**	950	1100	**1025**	1100	1050	**1075**
CM/81246/62	1271	1907	1589	1750	1600	**1675**	1600	1650	**1625**	1750	1500	**1625**

Key

Analysis outside ± 20% of the mean value obtained using the confirmatory (GC/MS) test.

For all rapid test methods based on antibody-antigen reactions, repeatability and reproducibility of results, both in the short-term (intra-batch variation) and long-term (inter-batch variation), are essential measures of consistency of analysis. For the evaluation of ROSA® DON (Quantitative) Test, 3 test kits (1 from Lot A002001B-06 and two from Lot B00200122B-08), were obtained from test kit production runs. Based on duplicate analysis from 2 extractions of each of 19 samples, selected from the HGCA Pink Grain Study (used in Phase II of the main study), the major outcomes were as follows.

ROSA® DON (Quantitative) Test: Intra-batch variation.

From analysis of 19 duplicate extractions, each sample pair (from consecutive Test Runs), was plotted and a linear correlation derived (see Figure 1). The linear correlation derived provided an indication of inter-batch repeatability.

ROSA® DON (Quantitative) Test: Inter-batch variation.

From analysis of 19 duplicate extractions. Sample pairs from consecutive kit lots were plotted graphically (see Figure 2 and 3), and the linear correlation derived provided an indication of inter-batch repeatability.

ROSA® DON (Quantitative) Test: Comparison with confirmatory test method.

The agreement between the ROSA® DON (Quantitative) Test and the results obtained by GC-MS for each batch are given in Figures 4 and 5.

Discussion and Conclusions

The ROSA® DON (Quantitative) Test is an LF immunoassay, which provides fully quantitative results from 1-4 samples of ground wheat, within 20 minutes. From the data collated in Table 2, the intra-batch repeatability was assessed using data from paired extractions from the same kit lot (A002001B-06), extracted on consecutive days. The linear correlation, Figure 1, (R^2=0.97), suggests acceptable short-term repeatability. The inter-batch assay repeatability was assessed using data from paired extractions from different kit lots (A002001B-06 and B00200122B-08) conducted on different days. The linear correlation graphs, Figure 2 and Figure 3, produced correlations of: R^2=0.98 and R^2=0.98, suggesting acceptable long-term repeatability. In terms of performance, the ROSA® DON (Quantitative) LF test kit compares favourably with microtiter plate-based ELISA test kits evaluated in the main report. One of the most important observations made in this evaluation is the high linear correlation between ROSA® DON (Quantitative) and the confirmatory (GC/MS) procedure (see Figure 4 and 5), for both kit lots (A0020001B-06, R^2=0.96 and B00200122B-08, R^2=0.97). Although errors associated with measurement was not specifically investigated the high correlation observed, over the range of samples tested, suggests comparable method performance for a given sample of ground material. This will inevitably have been a factor in ROSA® DON (Quantitative) achieving USDA/GIPSA third-party accreditation status (Certificate No. FGIS 2007-104).

The most critical judgement of assay performance is sensitivity at or around EU limits, when correlated with data obtained from the confirmatory test. Quantitative immunoassay test kits are subject to cross-reactivity i.e. non-specific to DON, leading to an overestimation of results. This has been described elsewhere in the main study as a positive aspect to analysis using rapid test kits, i.e. building a measure of safety into the analysis. However, samples at or around the EU limit of 1250ppb could potentially be subject to processing delays, pending further testing. As a result, UK

cereal processors require rapid tests with greater analytical accuracy and precision, ensuring that consumers are not placed at risk and that processing is not unduly delayed. A simple calculation has been used to provide an estimate of the bias obtained from the ROSA® DON (Quantitative) Test in relation to the confirmatory test, for the selected sample sub-set.

This provides an indication of the sensitivity of the test at or around the EU limit for unprocessed grain. From the sample set selected for the evaluation, a sub-set of 11 samples were selected with confirmatory test values within ± 250ppb of the 1250ppb limit (5 samples<1250ppb and 6 samples >1250ppb). This represents 66 analyses (30 below the1250ppb threshold and 36 above the 1250ppb threshold.) See Table 3.

Table 3: Estimation of bias for ROSA® DON (Quantitative) Test against GC/MS

Sample CM81246/	GC/MS (ppb)	A002001B-06 (ppb)	Bias* (ppb)	B00200122B-08 Run 1 (ppb)	Bias* (ppb)	B00200122B-08 (ppb)	Bias* (ppb)
9	1069	1050	-19	1050	+50	975	+94
14	1001	1025	-24	975	+26	1025	-24
15	1122	1075	+47	1100	+22	1025	+97
16	1276	1225	+51	1225	+51	1225	+51
21	1058	1025	+33	1125	-67	1025	+33
23	1490	1450	+40	1500	-10	1450	-10
30	1010	1075	-65	1025	-15	1025	-15
36	1257	1275	-18	1325	-18	1400	-143
42	1405	1450	-45	1450	-45	1400	+5
47	1334	1325	+9	1250	+84	1275	+59
48	1397	1500	-103	1475	-78	1450	-53
Mean bias			-8.5		0		8.5

*Calculation = GC/MS (ppb) - ROSA® DON (Quantitative) Test (ppb)

From Table 3 the overall bias observed was small in relation to the legislative limits and are in all cases < ±20% of the measured value. The mean bias for each kit lot on each analysis date is close to zero indicating both consistent and comparable performance to the confirmatory technique at the EU limit for unprocessed grain. This is a very positive aspect of the ROSA® DON (Quantitative) test kit, especially when compared to anomalies registered against microtiter plate-based ELISA kits

evaluated in HGCA Project Report 394. The manufacturers have invested considerable effort to control the assay protocol e.g. temperature controlled and timed incubation, and by closure of the test sticks, protection from temperature and humidity which would otherwise lead to assay failure. Additionally, no assay failures were recorded from a total number of 114 and 152 test sticks used for the ROSA® DON (P/N) and ROSA® DON (Quantitative) kits, respectively. This is in accordance with UK industry criteria stated on page 16 of HGCA Project Report 394. Overall, on the basis of the work reported here, the ROSA® DON (Quantitative) and ROSA® DON (P/N) test kits, provide the performance and flexibility required for use as a surveillance tool in the risk management of DON in ground wheat.

Figure 1:ROSA DON (Test Run 1 16.04.07) vs ROSA DON (Test Run 2 17.04.07)
(intra-batch B00200122B-08B variation)

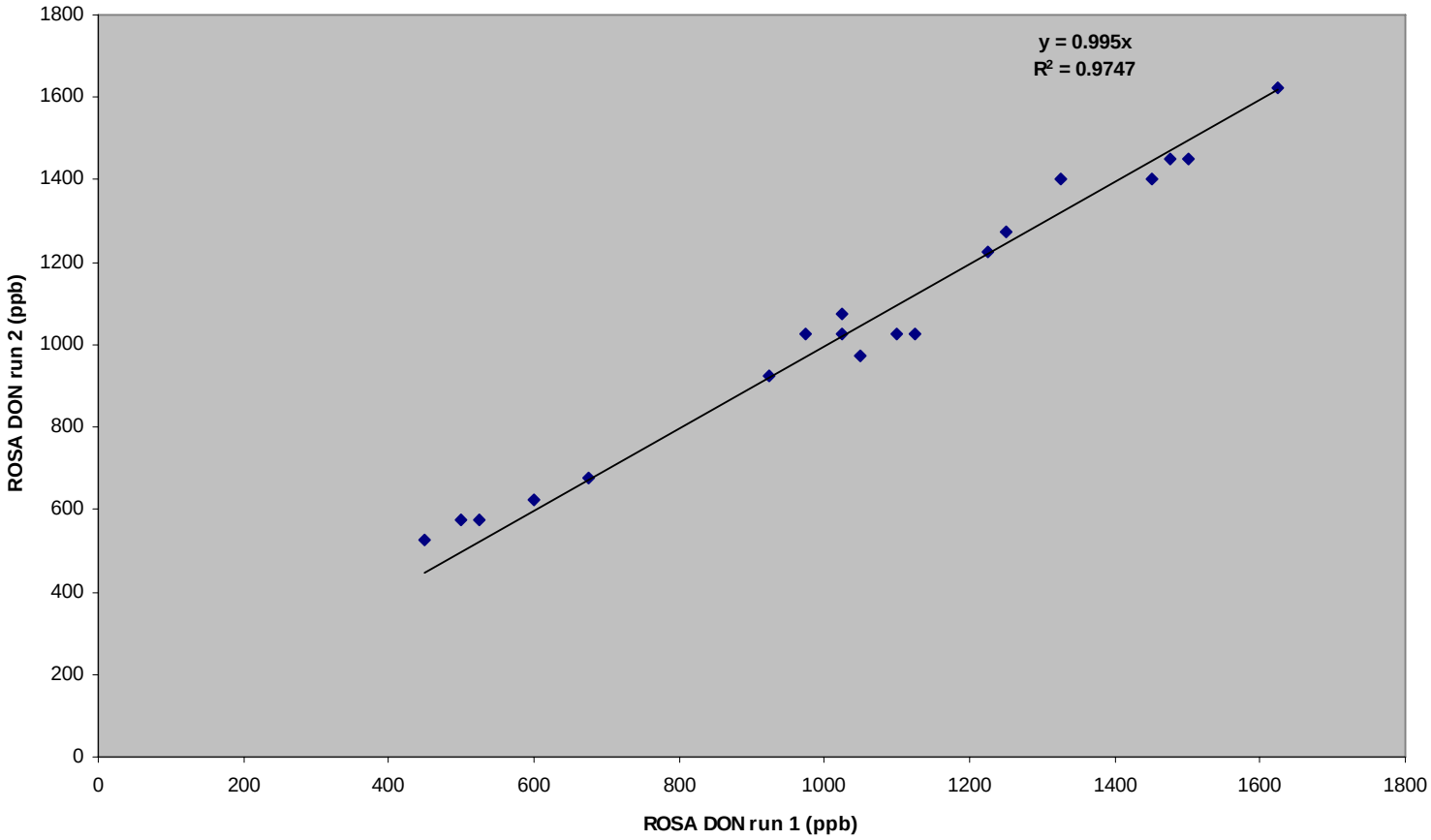

Figure 2:ROSA DON (Quantitative)
Test Run 1 12.04.07 (A002001B-06) vsTest Run 1 16.04.07(B00200122B-08)
(Inter-batch variation)

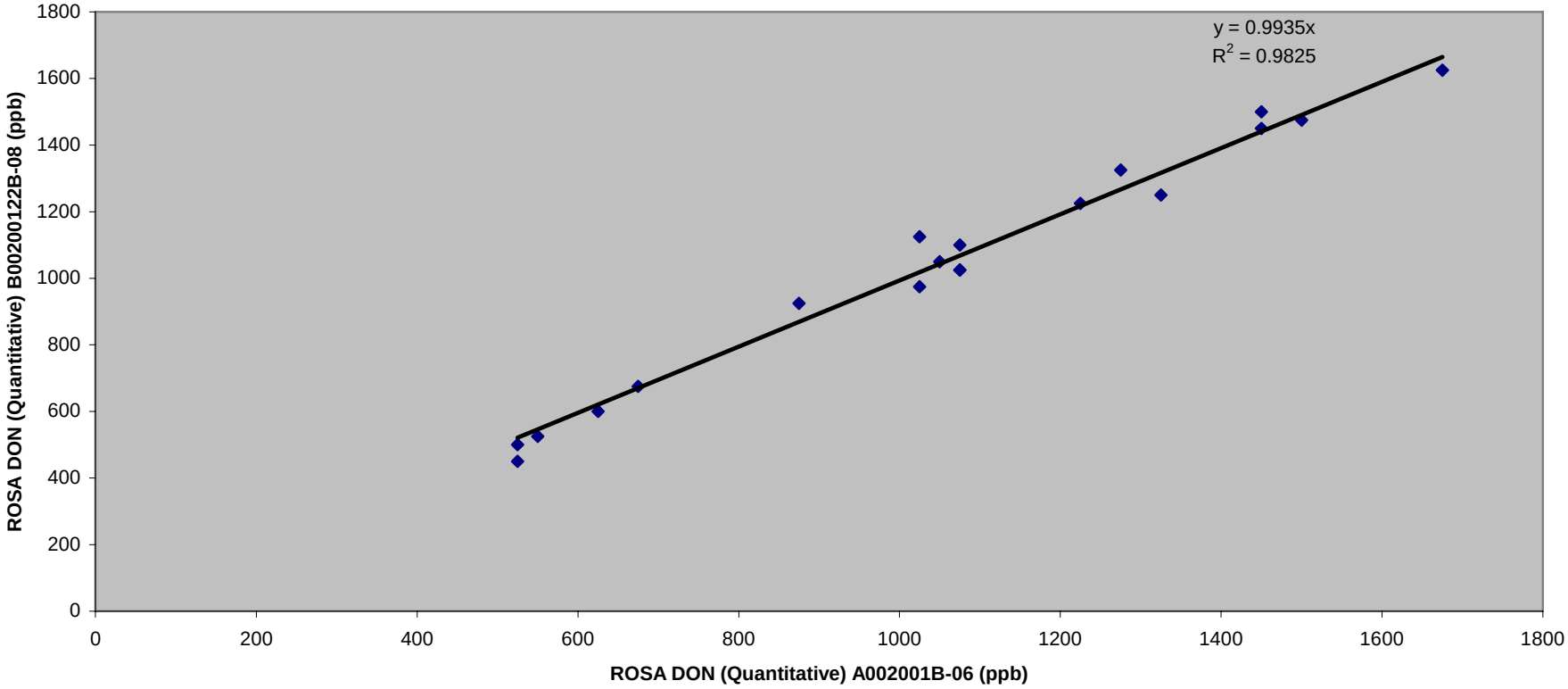

Figure 3:ROSA DON (Quantitative)
Test Run 1 12.04.07 (A002001B-06) vs Test Run 17.04.07 (B00200122B-08)
(Inter-batch variation)

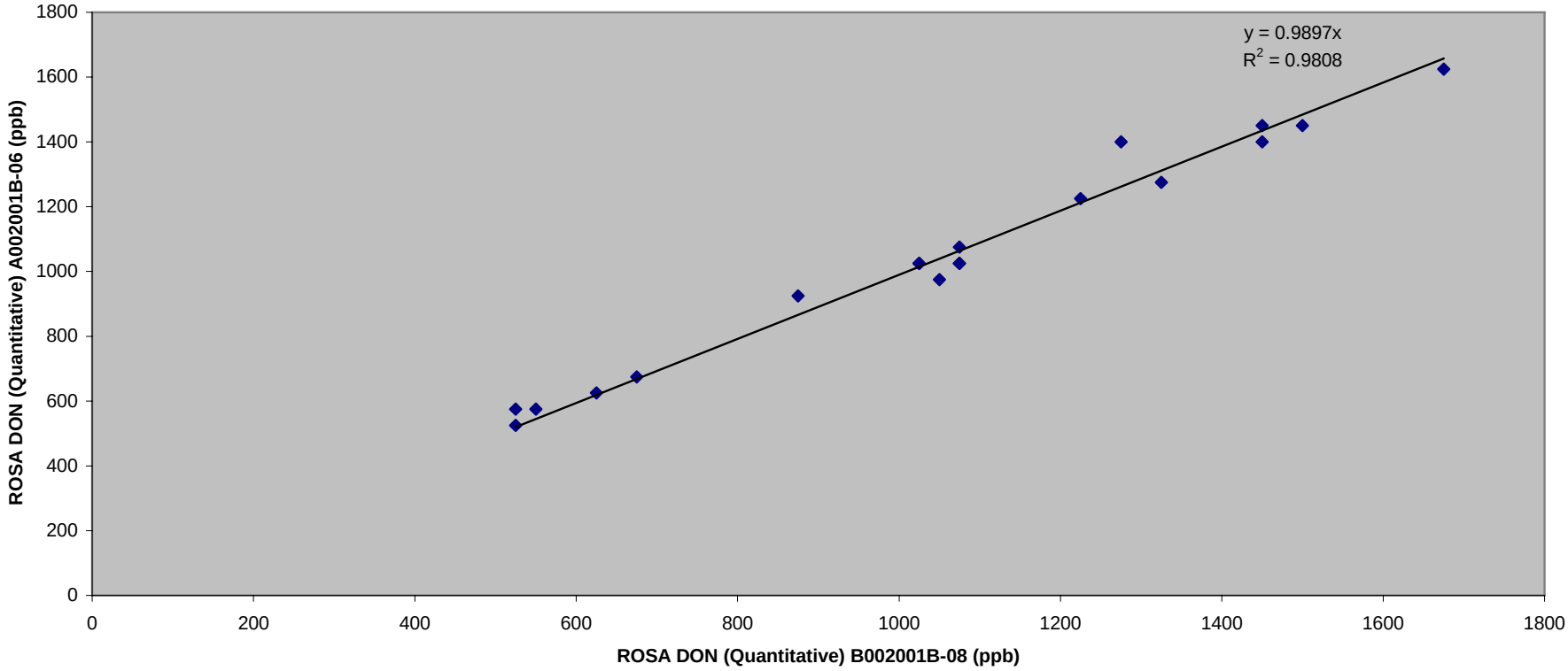

Figure 4:Correlation Between Confirmatory Test (GC/MS) vs ROSA DON (Quantitative) A002001B-06

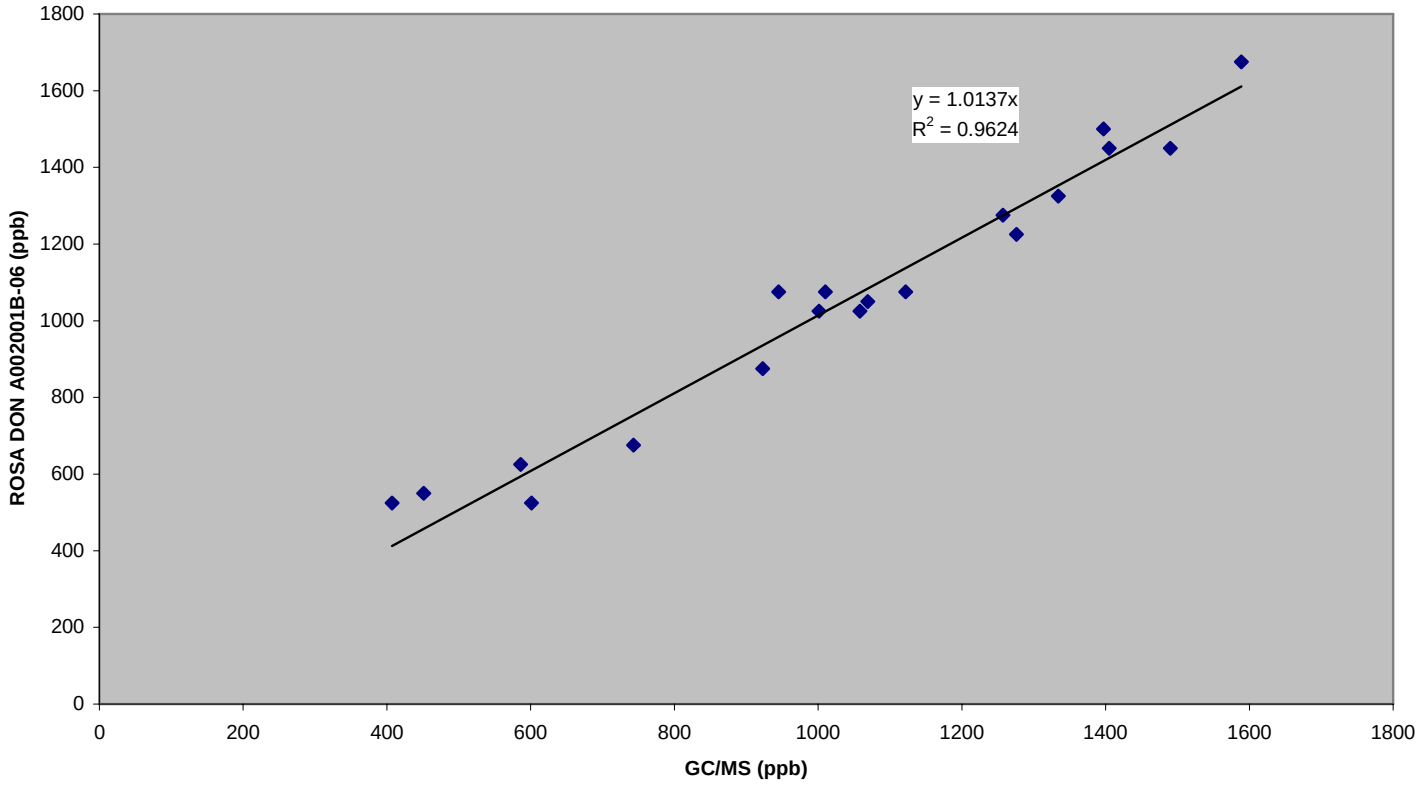

Figure 5: Correlation between Confirmatory test (GC/MS)vsROSA DON (Quantitative) B00200122B-08