Project Report No. 394 Part 2
Extension

August 2007

EVALUATION OF:

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

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

by

N J Matthews and J M Pratt

Campden and Chorleywood Food Research Association,
Station Road, Chipping Campden, Gloucestershire GL55 6LD

This report is an update of Project No. 3150, completed in July 2007, to incorporate further tests on the ROSA (DON) quantitative test under the existing Project Report number and title.

The Home-Grown Cereals Authority (HGCA) has provided funding for this project but has not conducted the research or written this report. While the authors have worked on the best information available to them, neither HGCA nor the authors shall in any event be liable for any loss, damage or injury howsoever suffered directly or indirectly in relation to the report or the research on which it is based.

CONTENTS

Abstract	**49**
Background	**49**
Evaluation of ROSA® DON (P/N) Test	
Introduction	**50**
Procedure	**51**
Results	**51**
Discussion and Conclusions	**53**
Evaluation of ROSA® DON (Quantitative) Test	
Introduction	**55**
Procedure	**55**
Results	**56**
Discussion and Conclusions	**60**
Figure One	**63**
Figure Two	**64**
Figure Three	**65**
Figure Four	**66**
Figure Five	**67**

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.

Visual interpretation of the analysed test strip is possible by comparison with the analysis of a control (500 parts per billion (ppb) standard, supplied with the test kit). However, subjective visual interpretation can be removed by purchasing the ROSA-M Reader (order code LF-ROSAREADER-M), also available from Charm Sciences Inc. For the evaluation of: ROSA® DON (P/N) and ROSA® DON (Quantitative) Test, diagnostic test kits, the ROSA-M Reader, was used for all analysis.

Evaluation of ROSA® DON (P/N) Test: Qualitative LF Test in grains and feed.

Introduction

ROSA® DON P/N Test is a lateral flow immunoassay, which provides a qualitative estimate of DON extracted in aqueous solution from a ground wheat sample. (NB estimates of DON in barley are also possible using this diagnostic test kit, but the scope of this evaluation is restricted to estimates of DON in wheat.)

The principle of the test is to add an aliquot of dilute, buffered DON extract (the test portion) to the LF device, which is inserted into the incubator. DON present in the sample interacts with coloured beads concentrated in **C** (Control) and **T** (Test) lines. For a test to be valid, the **C** (Control) line must be visible. Following the 3-minute incubation, the test strip is inserted in the ROSA-M-Reader and the result is read from the display. The kit is GIPSA (Grain Inspection, Packers, and Stockyards 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)

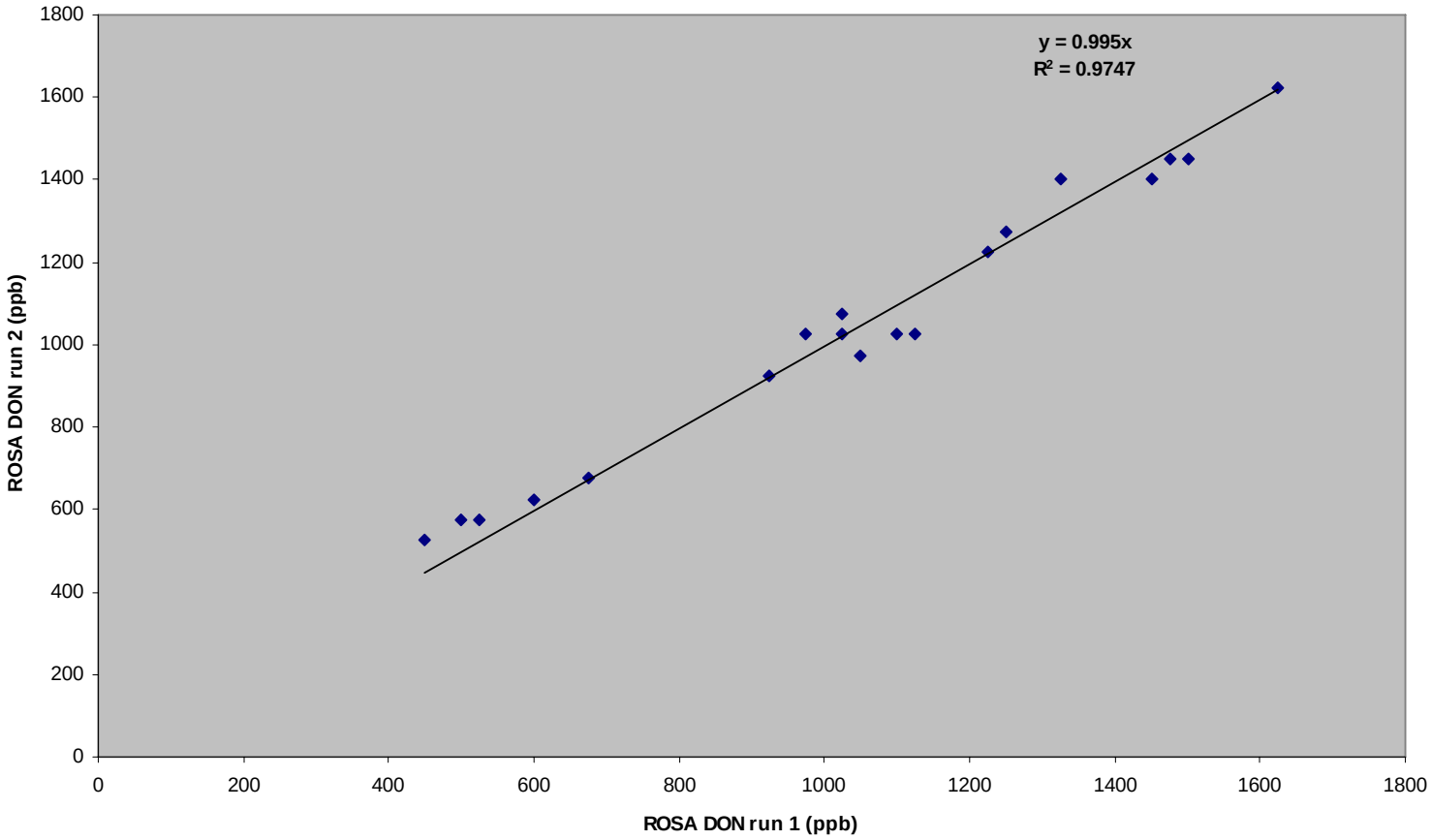

Figure 2:ROSA DON (Quantitative)
Test Run 1 12.04.07 (A002001B-06) vsTest Run 1 16.04.07(B00200122B-08)
(Inter-batch variation)

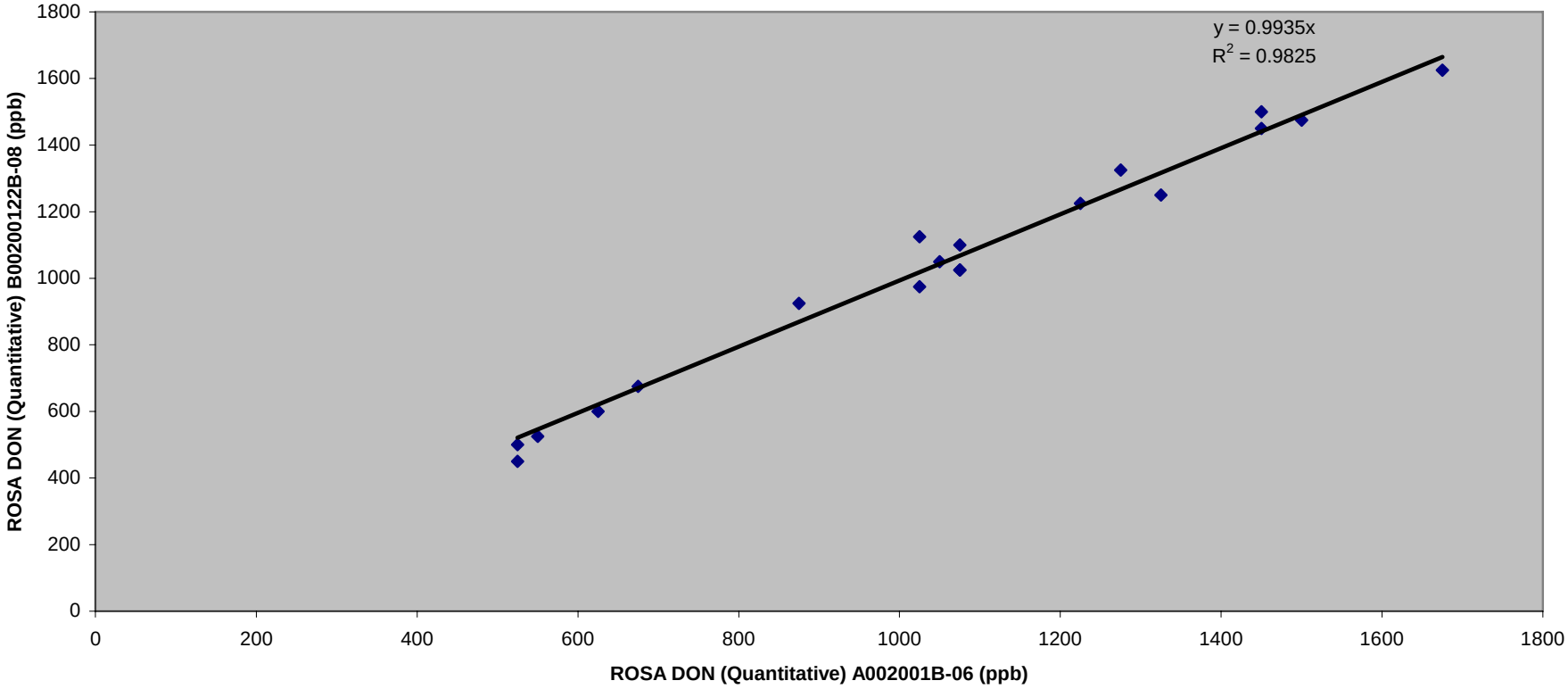

Figure 3:ROSA DON (Quantitative)
Test Run 1 12.04.07 (A002001B-06) vs Test Run 17.04.07 (B00200122B-08)
(Inter-batch variation)

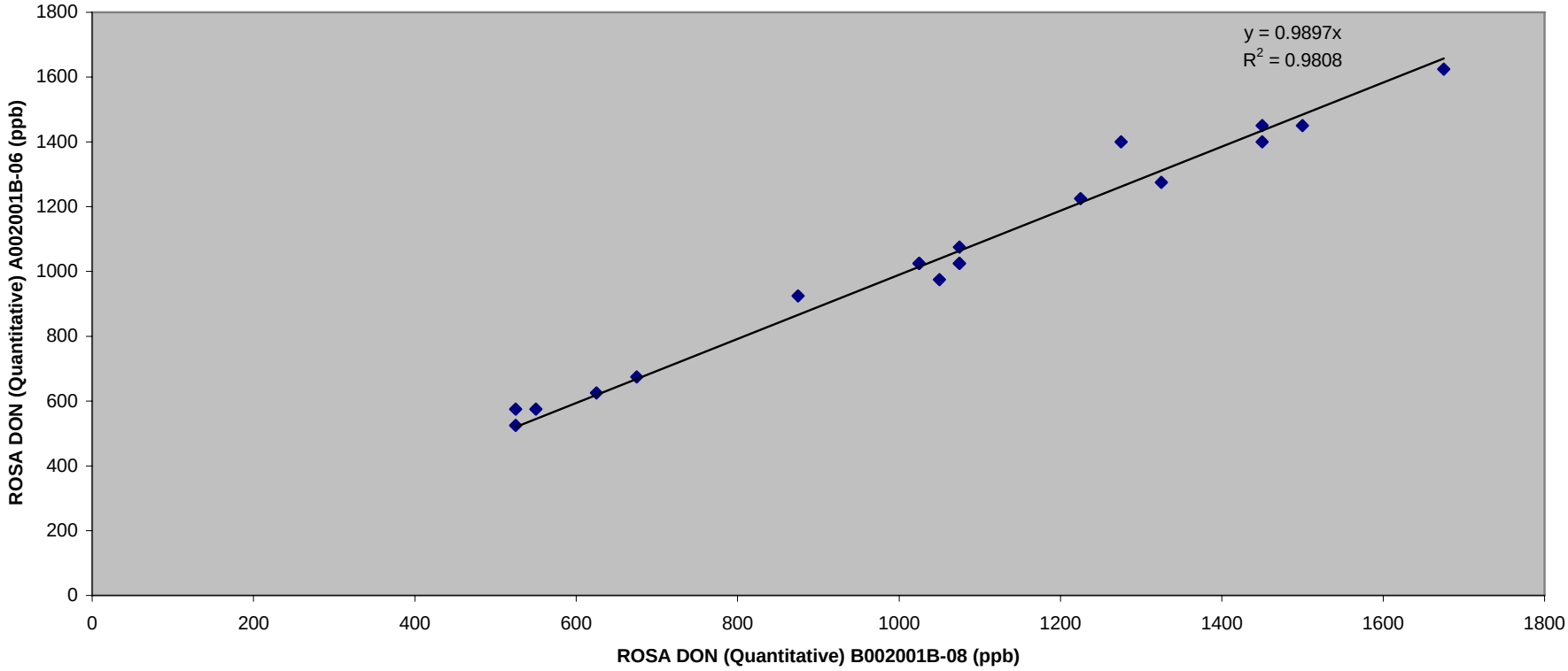

Figure 4:Correlation Between Confirmatory Test (GC/MS) vs ROSA DON (Quantitative) A002001B-06

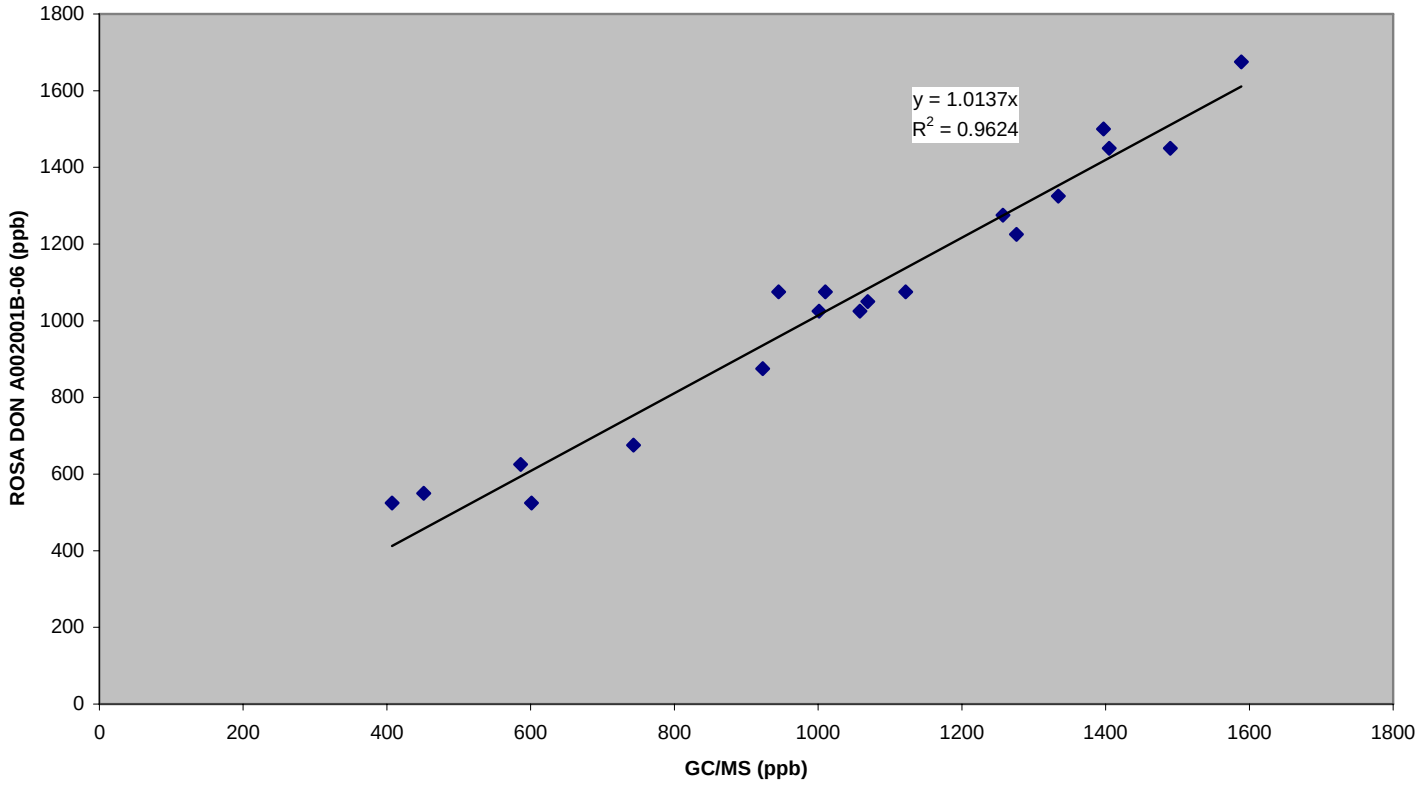

Figure 5: Correlation between Confirmatory test (GC/MS)vsROSA DON (Quantitative) B00200122B-08