CODA-CERVA

VETERINARY AND AGROCHEMICAL RESEARCH CENTRE

OPERATIONAL DIRECTORATE OF CHEMICAL SAFETY OF THE FOOD CHAIN

UNIT OF TOXINS AND NATURAL COMPONENTS

NATIONAL REFERENCE LABORATORY FOR MYCOTOXINS

NRL NATIONAL REFERENCE LABORATORIES

Administrative and experimental evaluation of fast commercial test kits for the determination of deoxynivalenol in cereals

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Foreword & scope of the report

Methods using liquid or gas chromatography and electrophoresis are tedious, laborious, time-consuming, require sophisticated equipment and/or trained personnel, and can not be used *in situ*. Therefore, simple and reliable methods of analysis are needed within the food control authorities as well as in the food industry for the purpose of internal control. A number of methods which are based on antibodies (enzyme linked immunosorbent assays (ELISA), fluorescence-polarization immunoassays (FPIA)), usually in complete test kits, are marketed as fast, reliable, sensitive, specific and relatively easy to perform compared to the the aforementioned methods. The test kits are based solely on materials provided by the manufacturers, which conduct first the validation of the test kits, and state on their performance characteristics. Thereafter, organizations such as GIPSA/USDA, AOAC Research Institute, NordVal and AFNOR offer producers an impartial review of the validation, as a step in the certification of the test kits.

For mycotoxin determination in food and feedstuffs, these rapid methods are applied for the analyses of aflatoxin (AFLA), deoxynivalenol (DON), zearalenone (ZEA), ochratoxin A (OTA), fumonisins (FB_1 & FB_2), T2-toxin that may occur in vegetable products and feedstuffs. As national reference laboratory (NRL), CODA-CERVA agreed for 2008 to appoint DON as a starting mycotoxin in order to review the analysis methods that are currently available on the market for usability, based on currently available knowledge levels regarding accuracy, reproducibility, repeatability, recovery, cost level. For this purpose, various ELISA formats based on antigen-antibody reactions and used as fast, specific and inexpensive screening of DON in cereals (barley, wheat, maize…) and cereals products were firstly inventoried using existing datasets and contacts with the manufacturers during the international meetings. After conducting this inventory task and taking into account the quantitative and qualitative aspects for DON determination kits, an administrative evaluation of the analytical performances of the most representative (well-used) kits was performed using the validation dossiers provided by the manufacturers. This desk-research evaluation was completed by checking in our laboratory some possible drawbacks regarding the cross reactivity, environmental temperature and incubation duration sensitivities as well as the matrix interferences. Furthermore, comparison of DON contamination via ELISA kits against reference values was performed using the datasets collected in the field by ELISA kit users in Belgium.

The present executive summary report presents the overall outcome of the administrative and experimental evaluations after a brief presentation of the inventory. Mainly addressed to the Federal Agency for Safety of Food Chain (FASFC) and to the laboratories (network of authorized labs in Belgium) that intend to implement this rapid method in determining the presence of DON in cereals and feedstuffs, it should be regarded as the compilation of the analytical performances previously obtained during the validation procedures extended to some additional data obtained experimentally at the NRL.. On behalf of the CODA-CERVA, we express the hope that this report can contribute to this objective and that the present methodology could be extended to other kits devoted to other mycotoxins (AFLA, OTA, ZEA, T2-toxin) via an enzyme immunoassay (EIA) or a fluorescence–polarization immunoassay (FPIA) techniques.

1- Inventory of providers of rapid mycotoxin testing kits

The currently available immunochemical methods are based on enzyme immunoassay (EIA) and fluorescence polarization immunoassay (FPIA). Antibodies are used either in micro-well plate or in "strip" technologies, such as lateral flow, in different kinds of "chip" technologies and optical biosensors. As the starting point of the evaluation of rapid methods, an inventory work was performed and dealt with the state-of-art of the commercial mycotoxin kits existing nowadays in the market.

1.1 Specific objectives

- Identify the existing commercial kits devoted to mycotoxin determination in food and feedstuffs in Belgium ;
- Establish a collaboration work with the identified kit providers.

1.2 Overall outcome

From the inventory, it appears that eight manufacturers from the United States of America (Charm, Neogen, Strategic Diagnostica[1]), Germany (Aokin, R-Biopharm), Austria (Romerlabs), Italy (Tecnalab) and The Netherlands (Eurodiagnostica) produced 21 DON kits that are available in the European markets (Figure 1). In Belgium, these test kits are commercialized either by local distributors (Biognost, Forlab, Stonehuis[2]) or provided from Germany (Coring system) and The Netherlands (Kentron Microbiology). MycoChek DON Test Kit from Strategic Diagnostica could be ordered from SDI Europe Ltd at Segensworth East, Hampshire in the United Kingdom whereas AokinMycontrolDON and I'screen DON "GOLD should be directly ordered from their respective manufacturers in Berlin (Germany) and Trieste (Italy). DON kits from Vicam are under validation procedure and were not available on the market during our investigation period. Hence, it was not taken into account in the present evaluation. So far, this kit named DONCHECK™, a qualitative DON kit (with cut off point of 1 ppm), is now available on the market. Furthermore, other distributors (TEPNEL, TRANSIA, INNOVATIVE BIOTECH, NEOGEN EUROPE, ELISA TEK, TOXI TEST and CEREAL TESTER) can worldwide provide the DON kits.

The numbers of the quantitative, qualitative or semi-quantitative Kits for DON determination as they can be provided on the market in Belgium.

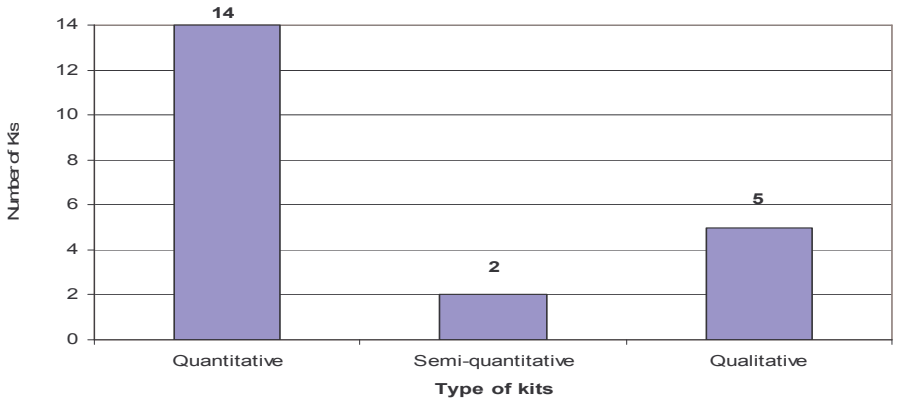

Figure 1: Type and number of available kits for DON determination in cereals and cereal products via immunoassay

[1] : Nowadays EuroProxima B.V.
[2] : Business is nowadays taken over by Coring System

Of the 21 kits available on the market, the most representative DON kits were quantitative kits (14) against 5 qualitative kits.

Data from the manufacturers were gathered for the administrative evaluation of both quantitative and qualitative kits for DON determination in cereals and cereal products

2- Objective and strategy of the administrative evaluation

The administrative evaluation is a desk research, mainly based on the manufacturers' data provided by information exchange through documentation. The aim is to carefully evaluate the performances of the available DON kits regarding relevant analytical criteria:

In order to carry out this evaluation, the working group of CODA-experts invited the manufacturers for a session aimed at presenting their DON kits at CODA-CERVA (Tervuren). Based on the relevant information obtained from these presentations and the available literature as well as the expertise of the panel members with regard to the review of the performances of the commercial DON kit, a checklist information was developed and addressed by post and electronic mailing to the kit manufacturers during May - July 2008 for quantitative DON kits and during July - September 2008 for qualitative DON.
The information provided were checked against the AOAC requirements, USDA/GIPSA/FGIS directives as well as the 2006/401/EC decisions and the Dutch PVD Quality Series n°96 (2004) regarding the procedures for verifying the performance of quantitative and qualitative mycotoxin and biotechnology rapid test kits. The statistical significances of data were tested applying relevant statistical tools.

The evaluation investigation was conducted in accordance with specific requirements of the Grain Inspection, Packers and Stockyards Administration (GIPSA) of the United States Department of Agriculture (USDA) as well as the Association of Official Analytical Chemists (now AOAC International) requirements and intended to meet technical criteria required for an analytical method validation using sound scientific and quality performances for immunoassay. Overall guidelines were used for performing the evaluation of the selected kits. Note that the CEN-CR 13505:1999 and 2002/657/CE decisions indicate the parameters that are relevant for detecting mycotoxin specifically for confirmation methods whilst the European commission regulation (EC) No 401/2006 established the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs.

For mycotoxins, criteria for screening methods are not available, but, according to Commission Decision 2002/657/EC, the requirement for a screening method is as follows: only those analytical techniques, for which it can be demonstrated in a documented traceable manner that they are validated and have a false compliant rate of < 5 % at the level of interest shall be used for screening purposes. In case of a suspected non-compliant outcome, the result shall be verified by a confirmatory method.

According to the Rapid Test Performance Evaluation Program established by GIPSA, the following basic four step process is proposed:

- The rapid test manufacturer submits a data package supporting their claims.
- The GIPSA staff reviews the data submitted by the manufacturer.
- If the data package is complete and the claims of the rapid test are supported by the data, GIPSA conducts an in-house performance verification of the rapid test.
- If the manufacturer's claims are verified by the GIPSA in-house performance testing, a Certificate of Performance is issued to the manufacturer for the rapid test.

The quantitative test kits subjected to the administrative evaluation procedure carried out by the NRL are listed in table 1.

Table 1: list of quantitative test kits involved in the administrative evaluation

Kit name	Providers	Enzyme Linked ImmunoSorbent Assay (ELISA)		Fluorescence Polarization ImmunoAssay (FPIA)
1. AGRAQUANT	Romerlabs	Micro-Well Plate	-	-
2. AGRASTRIP	Romerlabs	-	Lateral Flow device	-
3. DON EIA	Eurodiagnostica	Micro-Well Plate	-	-
4. MYCONTROLDON	Aokin	-	-	Vials
5. ROSA DON	Charm	-	Lateral Flow device	-
6. VERATOX	Neogen	Micro-Well Plate	-	-

3 Objective and strategy of the experimental evaluation

In Belgium, several laboratories are involved in mycotoxin determination in food and feedstuffs by using ELISA or FPIA. These rapid methods are applied to the analyses of aflatoxin (AFLA), deoxynivalenol (DON), zearalenone (ZEA), ochratoxin A (OTA), fumonisins (FB_1 & FB_2), T2-toxin that occurred in vegetable products and feedstuffs. Strategically the CODA group of experts has decided to work on the main problems encountered by the users. We therefore established a collaboration work with a mycotoxin kits users' committee. So far, they are all working with ELISA tests for the determination of DON in cereals and cereal products.

The first step of the collaboration aimed at identifying of the main drawbacks in using DON kits. All the participants agreed with the following problems:

- matrix effects
- specificity of the antibodies
- false positive results
- Temperature (the room temperature has to be checked out before testing)
- Sample preparation : grinding size (found to give the best reproducibility: 0.5mm – Retsch milling system)

A research protocol has then been elaborated and discussed with the users' committee. Several contacts have been made with the kits providers to obtain kits. Practical exercises started with the demonstration trials in order to be able to use the provided kits.

Checking the identified drawbacks at CODA's Labs is focused in the following drawbacks:

- Cross reactivity
- Environmental temperature sensitivity
- Incubation duration sensitivity
- Matrix interferences

The following kits were checked: AGRAQUANT®, DON EIA, VERATOX® for DON 5/5, ROSA LF-DONQ and AOKINMycontrolDON. Their batch numbers are mentioned in table 2.

Table 2: Experimentally tested DON kits and relevant information given by the providers

Provider	Concerned kit	Batch (number)	Format	Expired date
Aokin	AokinmycontrolDON	002001200708000	20 vials/per box	March 01, 2009
Romerlabs	AGRAQUANT®	400717-0805	96 wells/per box	March 17, 2009
Charm	ROSA LF-DONQ	005 (J005003-12)	100 strips/per can	March 2009
Eurodiagnostica	DON EIA	JN5900	96 wells/per box	August 2009
Neogen	Veratox® for DON 5/5	5329	48 wells/per box	May 01, 2009

Test procedure and reagents contained or recommended by ELISA kits providers were used.

4 Results and scoring of the evaluation

4.1 AGRAQUANT and AGRASTRIP (ROMERLABS)

AGRAQUANT (catalogue reference COKAQ4000) is a competitive ELISA (Microwells Plate) test kit provided by Romerlabs. The kit is used for screening DON contents in wheat, Barley, Corn, Oats, Malted barley, rice, wheat flour, wheat midds.

AGRAQUANT is characterized by:

- a minimum of 8 months shelf life (when stored 2-8°C).
- a limit of detection (LOD) which varies by commodity and ranges from 0.13 – 0.23 ppm and the maximum measured concentrations in the target matrix is 5 ppm.
- the capability to check maximum EU admissible limits without any dilution procedure of the water-extract.
- the capability to perform 16 runs per day with a maximum of 43 samples per run
- good precision with overall recovery that complies with the minimum performance criteria of EC regulation No 401/2006/EC
- very good accuracy with the results being consistent with the HPLC (reference method). Nevertheless, possible overestimation by AGRAQUANT ($DON_{AGRAQUANT}$ > DON_{HPLC}) with a mean discrepancy of 447 ppb has been calculated.
- possible false positive result may also occur around the LOD = 0.23 ppm (for blank samples, caution should be paid to the determination of DON concentrations values < 0.25 ppm)
- low or absence of cross reactivity against mycotoxins such as NIV, 15-AcDON, DAS, T2-toxin, HT2-toxin, VCL and ZEA within the range of the tested concentrations
- mild relative cross reactivity (up to 52 %) against DOM-1 and D3G
- very high relative cross reactivity against 3ACDON (up to 770 %)
- demonstrated validity when used in environmental temperatures ranging from 18 to 25 °C
- demonstrated validity when incubation was performed during 15 min according to manufacturer's claims, protocol in User Manuals
- demonstrated no matrix interferences when wheat, winter barley and oat are analyzed.

In addition, AGRASTRIP (catalogue reference 1002034), a competitive ELISA lateral flow kit from ROMERLABS was only administratively evaluated. A balance scoring of the main characteristics (Table 3) resulted to 83 and 87% as overall score for the administrative evaluation of **AGRASTRIP** and **AGRAQUANT**, respectively.

For **AGRAQUANT,** the experimental evaluation leads to 73% as the overall percent score (Table 4).

Table: 3: Scoring of the administrative evaluation of AGRASTRIP AND AGRAQUANT of ROMERLABS.

Parameter	Cumulative rate	Requirements	Max rate*	Agrastrip		Agraquant	
				Result	Rating	Result	Rating
Calibration curves (number of standards according AOAC requirements)	**5**	5 (not zero)	**5**	5	5	4	3
Compliance (with EU Acceptable limits without dilution)	**15**	1.00 µg/g	**5**	OK	5	OK	5
		1.25 µg/g	**5**	OK	5	OK	5
		1.75 µg/g	**5**	OK	5	OK	5
Number of target commodities	**5**	Main cereals (>3)	**5**	OK	5	OK	5
LOD requirements (GIPSA requirement)	**5**	0.25 µg/g	**5**	OK	5	OK	5
Recovery percents (EU requirements)	**5**	60 - 110% (for 0.1 – 0.5 µg/g) 70 -120 % (for >5.0 µg/g)	**5**	OK	5	OK	5
Precision (relative standard deviation, EU requirement)	**5**	RSDr <= 20%	**5**	OK	5	OK	5
Accuracy (GIPSA requirements)	**8**	25% for 0.5 µg/g	**2**	OK	2	OK	2
		20% for 1.0 µg/g	**2**	OK	2	OK	2
		15% for 2.0 µg/g	**2**	OK	2	OK	2
		10% for 5.0 µg/g	**2**	OK	2	OK	2
Confirmation (Cross validation)	**15**	Concordance	**5**	91%	5	98%	5
		Mean discrepancy	**5**	161 ppb (13 %)	3	447 ppb (74%)	3
		Questionable concentration range (> EU limits)	**5**	> 2 µg/g	3	< 0.23 µg/g	3
Cross reactivity of antibody used	**10**	3ACDON (<10%)	**5**	No data	0	>100%	0
		NIV (<10%)	**5**	No data	0	0%	5
Time requirement (GIPSA requirements)	**5**	30 min	**5**	11	5	30	5
Cost /unit of kit		> 6 months		8 euros		6 euros	
Shelf life (2 - 6°C)				8 months		8 months	
Overall 'ease to use'				Yes		Yes	
Scientific support for providers	**5**		**5**		5		5
Total Score					**69**		**72**
Max rate	**83**				**83**		**83**
Score (%)					**83%**		**87%**

*: Max rate was fixed by evaluator

Table 4: Results and scoring of the experimental evaluation of AGRAQUANT of ROMERLABS

Parameter class	Max rate for class	Parameter	Max rate	Measured effect	Rating
Cross-reactivity (in %)	60	NIV	10	0	10
		3AC-DON	10	770	0
		DOM-1	10	35	7
		D3G	10	52	7
		FUSX	5	0.7	4.5
		15Ac-DON	5	1.6	4.5
		DAS	2	0	2
		T2	2	0	2
		HT2	2	2.1	1
		VCL	2	0	2
		ZEA	2	0	2
		18°C	10	130-161%	5
		21°C	10	91-116%	10
		25°C	10	108-156%	5
Effect of incubation time	15	multiplying time by 0.5 and/or 1.5 (at 21°C)	15	109-135%	7,5
Matrix effect	30	Wheat	10	1,4	10
		Winter barley	10	13	10
		Oat	10	6.5	10
Overall "ease to use"	15	CODA users	15		12
Technical support from providers	5		5		4
Total Score					**115.5**
Max rate	**155**				**155**
Total score (%)					**75%**

*: Max rate was fixed by evaluator

4.2 VERATOX for DON 5/5 (NEOGEN)

VERATOX for DON 5/5 (catalogue reference #8331) is a competitive ELISA (Microwells Plate) test kit provided by NEOGEN CORPORATION and used for screening DON contents in wheat, barley, maize, malted barley, oats, rice, distillers grains.

VERATOX has

- a minimum of 10 months shelf life (when stored 2-8°C).
- a limit of detection (LOD) which varies by commodity and ranges from 0.10 – 0.20 ppm and the maximum measured concentrations in the target matrix is 2 ppm
- the capability to check maximum EU admissible limits without any dilution procedure of the water-extract.
- the capability of manually throughput per day of 500 samples in 8 hours (with a maximum of 19 samples per run)
- good precision with overall recovery that complies with the minimum performance criteria of EC regulation No 401/2006/EC
- very good accuracy with the results being consistent with the GC/MS (reference method) and these two methods are interchangeable.
- the expanded combined uncertainties, calculated by including the bias components and applying k=2, being generally below 30% (according to the administrative evaluation).
- low or absence of cross reactivity against mycotoxins such as NIV, FUS X, 15-AcDON, DAS, T2-toxin, HT2-toxin, VCL and ZEA within the range of the tested concentrations
- mild relative cross reactivity (up to 40 %) against 3ACDON

- high relative cross reactivity against DOM-1 and D3G (157 and 212%, respectively)
- demonstrated validity when used in environmental temperatures ranging from 18 to 25 °C
- demonstrated validity when incubation was performed during 5 min according to manufacturer's claims, protocol in User Manuals
- demonstrated no matrix interferences when wheat and oat are analyzed in contrast to winter-barley.

Table 5 and 6 present the detail of the balance scoring of the main characteristics of VERATOX for DON 5/5 which obtained 95 and 77% as the overall scores for the administrative and experimental evaluations, respectively.

Table: 5: Scoring of the administrative evaluation of VERATOX for DON 5/5 of NEOGEN

Parameter	Cumulative rate	Requirements	Max rate	Result	Rating
Calibration curves (number of standards according AOAC requirements)	**5**	5 (not zero)	**5**	4	3
Compliance (with EU Acceptable limits without dilution)	**15**	1.00 µg/g	**5**	OK	5
		1.25 µg/g	**5**	OK	5
		1.75 µg/g	**5**	OK	5
Number of target commodities	**5**	Main cereals (>3)	**5**	OK	5
LOD requirements (GIPSA requirement)	**5**	0.25 µg/g	**5**	OK	5
Recovery percents (EU requirements)	**5**	60 - 110% (0.1 – 0.5 µg/g)	**5**		
		70 -120 % (>5.0 µg/g)		OK	5
Precision (relative standard deviation, EU requirement)	**5**	RSDr <= 20%	**5**	OK	5
Accuracy (GIPSA requirements)	**8**	25% for 0.5 µg/g	**2**	OK	0
		20% for 1.0 µg/g	**2**	OK	2
		15% for 2.0 µg/g	**2**	OK	2
		10% for 5.0 µg/g	**2**	Not applied	
Confirmation (Cross validation)	**15**	Concordance	**5**	97%	5
		Mean discrepancy	**5**	71 ppb (8 %)	5
		Questionable concentration range (> EU limits)	**5**		5
Cross reactivity of antibody used	**10**	3ACDON (<10%)	**5**	105%	0
		NIV (<10%)	**5**	3.80%	5
Time requirement (GIPSA requirements)	**5**	30 min		10	
Cost /unit of kit				8 euros	
Shelf life (2 - 6°C)		> 6 months	**5**	10 months	5
Overall 'ease to use'				Yes	
Scientific support for providers	**5**		**5**		5
Total Score					**72**
Max rate	**83**				**81**
Score (%)					**89%**

*: Max rate was fixed by evaluator

Table 6: Results and scoring of the experimental evaluation of the VERATOX for DON 5/5.

Parameter class	Max rate for class	Parameter	Max rate for parameter	measured effect	Rating
Cross-reactivity (in %)	60	NIV	10	0	10
		3AC-DON	10	40	9
		DOM-1	10	212	5
		D3G	10	157	6
		FUSX	5	0	5
		15Ac-DON	5	0	5
		DAS	2	0	2
		T2	2	0	2
		HT2	2	0	2
		VCL	2	0	2
		ZEA	2	0	2
		18°C	10	70-81%	5
		21°C	10	95-101%	10
		25°C	10	79-100%	10
Effect of incubation time	15	multiplying time by 0.5 and/or 1.5 (at 21°C)	15	85-103%	15
Matrix effect	30	Wheat	10	0,7	10
		Winter barley	10	23	0
		Oat	10	15	5
Overall "ease to use"	15	CODA users	15		13
Technical support from providers	5		5		5
Total Score					**123**
Max rate	**155**				**155**
Total score (%)					**79%**

*: Max rate was fixed by evaluator

4.3 DON EIA (EURODIAGNOSTICA)

DON EIA (catalogue reference 5121DON1p) is a competitive ELISA (Microwells Plate) test kit provided by EURODIAGNOSICA and it is used for screening DON contents in Cereals, food, feed, beer, silage.

DON EIA has:

- a minimum of 14 months shelf life (when stored 2-8°C).
- a limit of detection (LOD) of 0.03 ppm and the maximum measured concentrations in the target matrix is 1.0 ppm.
- the capability to check maximum EU admissible limits up to 1.0 µg DON/g without any dilution procedure of the water-extract. However, dilution procedure of the water-extract is needed before checking the maximum EU admissible limits being 1.25 µg DON/g for unprocessed cereals other than durum wheat, oats and maize and 1.75 µg DON/g for unprocessed durum wheat and oats.
- the capability of manually throughput per day of 120 samples (3 runs of 40 samples/run)
- good precision with overall recovery that complies with the minimum performance criteria of EC regulation No 401/2006/EC
- very good accuracy with the results being consistent with the GC/MS (reference method) and these two methods are interchangeable. Nevertheless, this kit has failed in accurately determining DON concentrations in wheat samples with high DON contents (> 2 ppm) (necessity of confirmation).

- low or absence of cross reactivity against mycotoxins such as 15-AcDON, DAS, T2-toxin, HT2-toxin, VCL and ZEA within the range of the tested concentrations
- mild relative cross reactivity against DUS X (32%)
- high relative cross reactivity against DOM-1 (84%), D3G (115%), NIV (198%) and 3AcDON (230%)
- demonstrated validity when used in environmental temperatures ranging from 18 °C
- demonstrated validity when incubation was performed during 60 minutes at 4-6°C followed by a second incubation during 30 minutes at room temperature according to manufacturer's claims, protocol in User Manuals.

Table 7 and 8 present the detail of the balance scoring of the main characteristics of DON EIA which obtained 69 and 62% as the overall scores for the administrative and experimental evaluations, respectively.

Table: 7: Scoring of the administrative evaluation of DON EIA (EURODIAGNOSTICA)

Parameter	Cumulative rate	Requirements	Max rate	Result	Rating
Calibration curves (number of standards according AOAC requirements)	**5**	5 (not zero)	**5**	7	5
Compliance (with EU Acceptable limits without dilution)	**15**	1.00 µg/g	**5**	OK	5
		1.25 µg/g	**5**	NO	3
		1.75 µg/g	**5**	NO	3
Number of target commodities	**5**	Main cereals (>3)	**5**	OK	5
LOD requirements (GIPSA requirement)	**5**	0.25 µg/g	**5**	OK	5
Recovery percents (EU requirements)	**5**	60 - 110% (0.1 – 0.5 µg/g) 70 -120 % (>5.0 µg/g)	**5**	OK	5
Precision (relative standard deviation, EU requirement)	**5**	RSDr <= 20%	**5**	OK	5
Accuracy (GIPSA requirements)	**8**	25% for 0.5 µg/g	**2**	OK	2
		20% for 1.0 µg/g	**2**	OK	2
		15% for 2.0 µg/g	**2**	NO	0
		10% for 5.0 µg/g	**2**	Not applied	
Confirmation (Cross validation)	**15**	Concordance	**5**	92%	5
		Mean discrepancy	**5**	229 ppb (1.2%)	3
		Questionable concentration range (> EU limits)	**5**	> 5.0 ppm	3
Cross reactivity of antibody used	**10**	3ACDON (<10%)	**5**	96%	0
		NIV (<10%)	**5**	40%	0
Time requirement (GIPSA requirements)	**5**	30 min		120	
Cost /unit of kit				10 euros	
Shelf life (2 - 6°C)		> 6 months	**5**	14 months	5
Overall 'ease to use'				Yes	
Scientific support for providers	**5**		**5**		5
Total Score					**61**
Max rate	**83**				**81**
Score (%)					**75%**

Table 8: Results and scoring of the experimental evaluation of the DON EIA.

Parameter class	Max rate for class	Parameter	Max rate for parameter	measured effect	Rating
Cross-reactivity (in %)	60	NIV	10	198	2
		3AC-DON	10	230	2
		DOM-1	10	84	6
		D3G	10	115	6
		FUSX	5	32	3
		15Ac-DON	5	0	5
		DAS	2	0	2
		T2	2	0	2
		HT2	2	0	2
		VCL	2	0	2
		ZEA	2	0	2
		18°C	10	94-123%	10
		21°C	10	97-111%	10
		25°C	10	100-115%	10
Effect of incubation time	15	multiplying time by 0.5 and/or 1.5 (at 21°C)	15	43 - 67%	7.5
Matrix effect	30	Wheat	10	28	0
		Winter barley	10	19	5
		Oat	10	10	10
Overall "ease to use"	15	CODA users	15		9
Technical support from providers	5		5		5
Total Score					**100.5**
Max rate	**155**				**155**
Total score (%)					**65%**

*: Max rate was fixed by evaluator

4.4 ROSA DON (CHARM)

ROSA DON is a Lateral Flow Strip test kit provided by CHARM (catalogue reference LF-DONQ) and used for screening DON contents in Wheat, Barley, Corn, Malted Barley, Oats, Rice, Sorghum, Wheat Flour, Wheat Midds. According to BELAC (Belgian Accreditation Body), this kit can not be considered as a confirmation tool.

ROSA DON has

- a minimum of 6 months shelf life (when refrigerated).
- a limit of detection (LOD) which varies by commodity and ranges from 0.037 – 0.13 ppm and the maximum measured concentrations in the target matrix is 6 ppm
- the capability to check maximum EU admissible limits without any dilution procedure of the water-extract.
- the capability of manually throughput per day of 150 samples (with a maximum of 4 per quad incubator)
- good precision with overall recovery that complies with the minimum performance criteria of EC regulation No 401/2006/EC
- very good accuracy with the results being consistent with the GC/MS (reference method) and these two methods are interchangeable.
- the expanded combined uncertainties, calculated by including the bias components and applying k=2, are generally below 40% (according to the administrative evaluation),
- low or absence of cross reactivity against mycotoxins such as NIV, FUS X, DAS, T2-toxin, VCL and ZEA within the range of the tested concentrations
- mild relative cross reactivity against 3AcDON (61%), 15-AcDON (18%) and DOM-1 (14%), HT2-toxin (13%) and D3G (8%)

- demonstrated validity when incubation is made in an oven (incubator) set at 45°C and when reading is performed by the strip reader provided by the manufacturer according to claims, protocol in User Manuals.
- demonstrated no matrix interferences when wheat and oat are analyzed in contrast to winter barley.

A balance scoring of the main characteristics of ROSADON (Table 9 and 10) resulted to 94 and 81% as overall score for the administrative and experimental evaluations of ROSADON.

Table: 9: Scoring of the administrative evaluation of ROSA DON (CHARM)

Parameter	Cumulative rate	Requirements	Max rate	Result	Rating
Calibration curves (number of standards according AOAC requirements)	**5**	5 (not zero)	**5**	6	5
Compliance (with EU Acceptable limits without dilution)	**15**	1.00 µg/g	**5**	OK	5
		1.25 µg/g	**5**	OK	5
		1.75 µg/g	**5**	OK	5
Number of target commodities	**5**	Main cereals (>3)	**5**	OK	5
LOD requirements (GIPSA requirement)	**5**	0.25 µg/g	**5**	OK	5
Recovery percents (EU requirements)	**5**	60 - 110% (0.1 – 0.5 µg/g) 70 -120 % (>5.0 µg/g)	**5**	OK	5
Precision (relative standard deviation, EU requirement)	**5**	RSDr <= 20%	**5**	OK	5
Accuracy (GIPSA requirements)	**8**	25% for 0.5 µg/g	**2**	OK	2
		20% for 1.0 µg/g	**2**	Not applied	
		15% for 2.0 µg/g	**2**	OK	2
		10% for 5.0 µg/g	**2**	OK	2
Confirmation (Cross validation)	**15**	Concordance	**5**	99%	5
		Mean discrepancy	**5**	8.5 ppb (1.1 %)	5
		Questionable concentration range (> EU limits)	**5**		5
Cross reactivity of antibody used	**10**	3ACDON (<10%)	**5**	200%	0
		NIV (<10%)	**5**	0	5
Time requirement (GIPSA requirements)	**5**	30 min		12	
Cost /unit of kit				10 euros	
Shelf life (2 - 6°C)		> 6 months	**5**	> 6 months	5
Overall 'ease to use'				Yes	
Scientific support for providers	**5**		**5**		5
Total Score					**76**
Max rate	**83**				**81**
Score (%)					**94%**

*: Max rate was fixed by evaluator

Table 10: Results and scoring of the experimental evaluation of the ROSADON (CHARM)

Parameter class	Max rate for class	Parameter	Max rate for parameter	measured effect	Rating
				M2	
Cross-reactivity (%)	60	NIV	10	0	10
		3AC-DON	10	61	8
		DOM-1	10	14	7
		D3G	10	8	7
		FUSX	5	0	5
		15Ac-DON	5	18	3
		DAS	2	0	2
		T2	2	0	1.5
		HT2	2	13	0.5
		VCL	2	1,8	2
		ZEA	2	0	2
Effect of temperature	30	18°C	10	Incubation is made in an oven provided with the system	30
		21°C	10		
		25°C	10		
Effect of incubation time	15	multiplying time by 0.5 and/or 1.5 (at 21°C)	15	Incubation time can not be changed with this system	15
Matrix effect	30	Wheat	10	6	10
		Winter barley	10	33	0
		Oat	10	17	5
Overall "ease to use"	15	CODA users	15		15
Technical support from providers	5		5		5
Total Score					**128**
Max rate	**155**				**155**
Total score (%)					**83%**

*: Max rate was fixed by evaluator

4.5 MYCONTROLDON (AOKIN)

MYCONTROLDON provided is a fluorescence polarization Immunoassay FPIA test kit (tube device) provided by AOKIN AG (catalogue reference MY-0708) and used for screening DON contents in Wheat, Barley, Maize, feedstuffs, bran, oat. According to BELAC (Belgian Accreditation Body), this kit can not be considered as a confirmation tool.

MYCONTROL DON has

- shelf life (when refrigerated of 3 to 6 months
- a limit of detection (LOD) which varies by commodity and ranges from 0.1 ppm in wheat and the maximum measured concentrations in the target matrix is 1.4 ppm
- the capability to check maximum EU admissible limits without any dilution procedure of the water-extract. However, dilution procedure of the water-extract is needed before checking the maximum EU admissible limit being 1.75 µg DON/g for unprocessed durum wheat and oats.
- good precision with overall recovery that complies with the minimum performance criteria of EC regulation No 401/2006/EC
- very good accuracy with the results being consistent with the HPLC (reference method) and these two methods are interchangeable.
- low or absence of cross reactivity against mycotoxins such as 15-AcDON, NIV, FUS X, DAS, T2-toxin, HT2-toxin, VCL and ZEA within the range of the tested concentrations
- mild relative cross reactivity against DOM-1 (10%), and D3G (23%)
- high relative cross reactivity against 3AcDON (148%)
- demonstrated validity when incubation and measurements are made with provided with the system. The kinetic pattern should not be changed with the system. Assays should be

performed as recommended by the manufacturer according to claims, protocol in User Manuals.

- demonstrated no matrix interferences when wheat and oat are analyzed in contrast to winter barley.

Table 11 and 12 present the detail of the balance scoring of the main characteristics of MYCONTROLDON which obtained 86 and 68% as the overall scores for the administrative and experimental evaluations, respectively.

Table: 11: Scoring of the administrative evaluation MYCONTROLDON (AOKIN)

Parameter	Cumulative rate	Requirements	Max rate	Result	Rating
Calibration curves (number of standards according AOAC requirements)	**5**	5 (not zero)	**5**	15	5
Compliance (with EU Acceptable limits without dilution)	**15**	1.00 µg/g	**5**	OK	5
		1.25 µg/g	**5**	OK	5
		1.75 µg/g	**5**	NO	3
Number of target commodities	**5**	Main cereals (>3)	**5**	OK	5
LOD requirements (GIPSA requirement)	**5**	0.25 µg/g	**5**	OK	5
Recovery percents (EU requirements)	**5**	60 - 110% (0.1 – 0.5 µg/g) 70 -120 % (>5.0 µg/g)	**5**	OK	5
Precision (relative standard deviation, EU requirement)	**5**	RSDr <= 20%	**5**	OK	5
Accuracy (GIPSA requirements)	**8**	25% for 0.5 µg/g	**2**	OK	2
		20% for 1.0 µg/g	**2**	OK	2
		15% for 2.0 µg/g	**2**	NO	0
		10% for 5.0 µg/g	**2**	Not applied	
Confirmation (Cross validation)	**15**	Concordance	**5**	99%	5
		Mean discrepancy	**5**	0,8 ppb (0.1%)	5
		Questionable concentration range (> EU limits)	**5**		5
Cross reactivity of antibody used	**10**	3ACDON (<10%)	**5**	300	0
		NIV (<10%)	**5**	3	5
Time requirement (GIPSA requirements)		30 min		15	
Cost /unit of kit				10 euros	
Shelf life (2 - 6°C)	**5**	> 6 months	**5**	3 - 6 months	3
Overall 'ease to use'				Yes	
Scientific support for providers	**5**		**5**		3
Total Score					**68**
Max rate	**83**				**81**
Score (%)					**84%**

*: Max rate was fixed by evaluator

Table 12 : Results and scoring of the experimental evaluation of the MYCONTROLDON (AOKIN)

Parameter class	Max rate for class	Parameter	Max rate	measured effect	Rating
Cross-reactivity (in %)	60	NIV	10	5	8
		3AC-DON	10	175	2
		DOM-1	10	10	7
		D3G	10	23	6
		FUSX	5	4	4
		15Ac-DON	5	0,8	4.5
		DAS	2	8	1.5
		T2	2	12	1.5
		HT2	2	8	1.5
		VCL	2	5.5	1.5
		ZEA	2	0.2	1.5
		18°C	10	Incubation is made in an oven provided with the system	30
		21°C	10		
		25°C	10		
Effect of incubation time	15	multiplying time by 0.5 and/or 1.5 (at 21°C)	15	Incubation time can not be changed with this system	15
Matrix effect	30	Wheat	10	-14	5
		Winter barley	10	20	5
		Oat	10	24	0
Overall "ease to use"	15	CODA users	15		10
Technical support from providers	5		5		5
Total Score					**109**
Max rate	**155**				**155**
Total score (%)					**70%**

*: Max rate was fixed by evaluator

4.6 Overall evaluation of the DON kits

The final ranking depicted in figure 2 was based on the above overall scoring and must be taken with caution also because a given kit can allow obtaining higher or lower values according to the specific conditions related to specific matrix, temperature and conditions of use (see section 4).

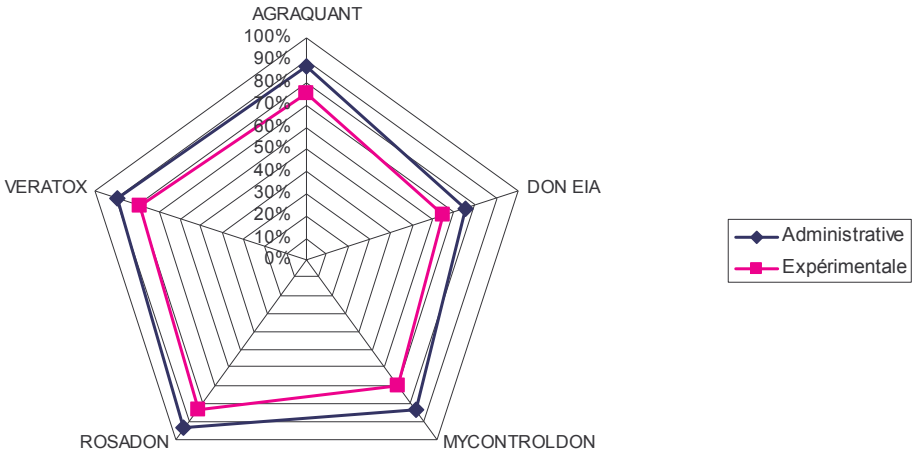

Figure 2: Overall administrative and experimental evaluation of selected kits for DON determination in cereals

More attention has to be paid to the experimental assessment because it is not always easy to check the quality of the data provided for the administrative evaluation.

Cross reactivity do show a particular importance in the present experimental evaluation. High cross reactivity is considered as a drawback because it does not allow checking the compliance with the legal norms. Nevertheless, high cross reactivity may also give some information about the presence of the parent compound as well as of other detectable analogues. Note that it was not possible to carry out the B/Bo calculation method for the measurement of cross reactivity for the LFD and FPIA based kits (i.e. RosaDON and MycocontrolDON). Moreover such measurements of the cross reactivity should be performed in matrix whilst in the present evaluation it was run in water

Additional recommendations to the users of test kits

Based on the results of cross reactivity, we suggested to combine ELISA or FPIA with more accurate methods (HPLC, LC/MS, GC/MS…) to determine the status of compliance, specially for the values around the maximum EU levels. Assays should be performed according to manufacturer's claims, protocol and User Manuals, so far it is important to check the room temperature when starting an ELISA analysis. If the temperature is 18°C, then it is suggested to use an incubator to increase the temperature up to 21 °C, otherwise we recommend to avoid in performing the test.

Some labs in Belgium are well-experienced in using rapid methods for DON determination in cereals but it is quite obvious that some experience has to be gained within the lab and that all test kits do not show the same performances. Nevertheless, networking of several labs (using ELISA, HPLC, GC, LCMS, GCMS, UPLC/MS) in Belgium is recommended (participation to PT, confirmation results).

Each lab must perform its own validation within its specific laboratory conditions as well as its own control chart.

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Appendix : Group of experts involved in the evaluation

The following persons were members of the group of experts for the evaluation of the DON test kits**.**

CODA-CERVA group of experts

- Dr Callebaut Fons (Tervuren, **Chairman**)
- Dr Tangni Emmanuel (Tervuren)
- Dr Pussemier Luc (Tervuren)
- Dr Motte Jean-Claude (Tervuren)
- Dr Debongnie Philippe (Tervuren)
- Dr Van der Stede Yves (Uccle)

Experts from other Belgian institution

- Audenaert Kris (Hogeschool Gent)
- Chandelier Anne (CRA-W, Gembloux)
- Cuignet Marc (Carah, Ath)
- Daeseleire Els (ILVO, Merelbeke)
- De Saeger Sarah (University of Ghent)
- Gillard Nathalie (CER, Marloie)
- Gouwy Patrick (Vanden Avenne, Ooigem),
- Haesaert Geert (Hogeschool Gent)
- De Schrijver,Marnix (FFQ Laboratorium, Merksem)
- Sinnaeve Goerges (CRA-W Gembloux)
- Vancutsem Jeroen (FLVVT, Tervuren)
- Van Hooteghem Kristof (Versele Laga, Deinze)
- Van Loco Joris (WIV, Brussels)