IAG ring test visual detection of ergot sclerotia in rye 2015

L.W.D. van Raamsdonk, N. van de Rhee, J.J.M. Vliege, V.G.Z. Pinckaers

WAGENINGEN
UNIVERSITY & RESEARCH

IAG ring test visual detection of ergot sclerotia in rye 2015

L.W.D. van Raamsdonk, N. van de Rhee, J.J.M. Vliege, V.G.Z. Pinckaers

RIKILT Wageningen University & Research
Wageningen, October 2016

RIKILT report 2016.013

WAGENINGEN
UNIVERSITY & RESEARCH

Raamsdonk, L.W.D. van, N. van de Rhee, J.J.M. Vliege, V.G.Z. Pinckaers, 2016. *IAG ring test visual detection of ergot sclerotia in rye 2015.* Wageningen, RIKILT Wageningen University & Research, RIKILT report 2016.013. 24 pp.; 2 fig.; 3 tab.; 24 ref.

Project number: 126.73.351.01
Project title: IAG ringtesten
Project leader: L.W.D. van Raamsdonk

This report can be downloaded for free at http://dx.doi.org/10.18174/393609 or at www.wur.eu/rikilt (under RIKILT publications).

P.O. Box 230, 6700 AA Wageningen, The Netherlands, T +31 (0)317 48 02 56, E info.RIKILT@wur.nl, www.wur.eu/rikilt. RIKILT is part of Wageningen University & Research.

RIKILT report 2016.013

Distribution list:

- International Association for Feeding stuff Analysis (IAG), Section Feeding stuff Microscopy (I. Paradies-Severin, J. Vancutsem, G. Frick, R. Weiss)
- European Commission (EC; F. Verstraete)
- Joint Research Centre, Geel (IRMM-JRC; C. von Holst, A. Boix-Sanfeliu)
- All participants of the ring test
- Netherlands Food and Consumer Product Safety Authority (NVWA; W. Ooms, R. Theelen, R. Herbes)

Contents

Summary

Ergot alkaloids are recognised as seriously toxic compounds, which caused a series of outbreaks in the past. In the EU, enforcement is implemented by visual detection and quantification of ergot sclerotia produced by moulds of the genus *Claviceps*.
On behalf of the IAG section Feedstuff Microscopy, RIKILT organised a ring test for the visual detection of ergot sclerotia in two unground rye samples in September 2015. In this report the results from the ring test for ergot in rye 2015 are presented.
The ring test ergot sclerotia in rye was designed to test the capability to visually detect sclerotia or parts thereof at relatively high levels. One sample was based on a level of approx. 400 ppm, and the second sample contained an amount of approx. 1000 ppm (EU legal limit for feeds and ingredients: 1000 ppm = 1 gram/kg = 0.1%). An amount of approx. 250 grams of rye grains was chosen as sample size. All samples were individually spiked. Thirty participants enrolled for the ring test. Participants were requested to report the number of recovered (fragments of) sclerotia and the total weight per sample. The percentage of recovery for every sample was calculated. A dedicated IAG method as well as other (lab internal) methods were allowed for application. Principally, methods are based on sieving (preferably with a mesh size of 0.5 mm), examination of every particle (grain) in the fraction with full grains or particles larger than 0.5 mm, selection of sclerotia fragments supported by documentation, and weighing the final selection of bodies.
The average recovery for both samples was approx. 97%. All results except one were between the expected recovery limits (80 – 110 % w/w). Supporting data from an intralaboratory validation study of the IAG method showed trueness at different low spike levels between 98 and 105% w/w. Limit of detection was established at 7 ppm.
It can be concluded that examination by visual detection of sclerotia is a valuable indicator of the expected presence of ergot alkaloids. The results of this study provides the data for a partial validation of the method of IAG for the examination of whole kernel cereal samples.

1 Introduction

Some of the first documented cases of food poisoning caused by infested plant ingredients are the intoxications by ergot alkaloids in north Norway early 17th Century (Alm, 2003) and in New England in the late 17th Century (Woolf, 2000), among other cases (Scott, 2009). The intoxication symptoms were already known as Saint Anthony's fire from the Middle Ages (Lee, 2009a). After an outbreak in France (Pont St. Esprit; Lorenz and Hoseney, 1979; Scott, 2009) in 1951 increased attention was given to the analysis of ergot alkaloids.

Sclerotia or ergot bodies are the fruit bearing mycelia of the moulds of the genus *Claviceps*. They start growing before the fruit of cereals is starting to form, and in this way the grain is replaced. The ergot alkaloids are principally produced in the sclerotia, but ergot alkaloids were occasionally detected in visually non-detectable infestations of cereal samples, and sclerotia were found without any detectable level of ergot alkaloids (Mulder *et al.*, 2012). Ergot content in individual sclerotia can vary considerably (Lorenz and Hoseney, 1979; EFSA, 2005; Krska and Crews, 2008; Scott, 2009).

Although the alkaloids are the primary cause of disease symptoms, the detection of sclerotia is currently the target of the official control. A limit of 1000 mg/kg sclerotia is set in the European Union for feed materials and compound feeds containing unground cereals (EC Directive 2002/32/EC (Consolidated version February 2015: European Commission, 2015), whereas an action limit of 500 mg/kg sclerotia is set for common wheat and durum wheat in intervention procedures (European Commission, 2009a). The same limit applied to rye in intervention procedures according to Regulation 689/92 (European Commission, 1992), but this regulation was repealed in 2000 (European Commission, 2000). No regulatory limits in the EU apply to ergot bodies in grain for human consumption (Egmond and Jonker, 2004). Other relevant limits are 200-500 mg/kg in cereals depending on purpose (Switzerland), 10 mg/kg for feed grains and zero tolerance for other grains (UK), and 100-3300 mg/kg depending on cereal species and grade (Canada). Krska and Crews (2008) and Scott (2009) provided detailed overviews of legal limits. Eight out of 21 member states of the European Union reported in 2007 to have detected ergot sclerotia in samples of cereals, making it the most frequently found undesired substance of botanic origin (van Raamsdonk, 2007). The frequency of occurrence ranged from a few samples to a share of 25-50% positive samples (van Raamsdonk *et al.*, 2009). An increase in occurrence was reported for Germany (Krska and Crews, 2008). Notwithstanding the diverse relationship between amount of sclerotia versus alkaloid content, visual examination prior to chemical analysis, as recommended by EFSA (EFSA, 2005) appeared to be effective (Mulder *et al.*, 2012).
The official method for detection of "besatz" (impurities) in cereals, including sclerotia, according to EC (2009a) is published as official standard (CEN, 2008). In addition, the IAG section Microscopy has developed and published a method for detection of sclerotia in unground cereals or other feeding stuffs (IAG, 2010).

The IAG section Feedstuff Microscopy (http://www.iag-micro.org/) organises annually proficiency tests for a range of topics concerning feed stuff analysis with visual methods. On behalf of this section, RIKILT organised late 2015 a ring test for the visual detection of ergot sclerotia in two unground rye samples. In this report the ring test for ergot in rye 2015 is presented.

2 Methods

2.1 Materials

The ring test ergot sclerotia in rye was designed to test the capability to visually detect sclerotia or parts thereof at relatively high levels. One sample was based on a level of approx. 40% of the legal limit, and the second sample contained an amount at approx. 100% of the legal limit for feed and ingredients (1000 ppm = 1 gram/kg = 0.1%). An amount of approx. 250 grams of rye grains was chosen as sample size.
Rye was obtained from a commercial supplier. The batch was examined for impurities. Specimen of grain weevil (*Sitophilus* sp.) were removed.
Ergot sclerotia were taken from the RIKILT collection. Large sclerotia were fractionated in order to have comparable numbers of sclerotia fragments in all jars of the same sample.

2.2 Procedure for production

Sixty jars (two per participant) were filled with approx. 250 grams of rye grains. Every individually numbered jar was spiked with a number of sclerotia fragments for reaching the necessary weight percentage. Sample size, number and total weight of the sclerotia were stored per jar in order to allow exact evaluation of the results.

2.3 Homogeneity study

Control of homogeneity was not necessary, and principally impossible, for the reason that every jar was individually spiked.

2.4 Organization of the ring trial

Participants were invited early 2015. A total of 30 IAG members enrolled for the ring test. The participants are listed in Annex 1.
The packages with two jars each were distributed late August 2015 and the result were expected to be submitted early October 2015. All results were received on time. Participants were requested to report the number of recovered (fragments of) sclerotia and the total weight per sample.
Details on the method used were requested to be submitted as well. Instructions are presented in Annex 2.
The general outline of the IAG method for detection of ergot sclerotia (IAG, 2010), in the current case as to be found in unground cereals, consists of the following elements. The entire sample (if necessary in fractions of principally 500 grams) is evaluated in order to avoid problems from inhomogeneity. The method involves weighing the sample, sieving using a sieve of 0.5 mm and selecting [1] all sclerotia or fractions thereof. Special attention should be given to grains infested by smut which might mimic sclerotia. Every particle which fits the description of a sclerotium or part of it will be separated. The minimum size of the fragments to be selected is 0.5 mm. Hand books and illustrations will be used to achieve a positive and reliable identification. After a full examination of the sample one or more fractions, ergot sclerotia and other components if selected, remain. The fraction of ergot sclerotia is weighted and the percentage is calculated.

[1] It can be helpful to spread a portion of the grains in a large flat bin. The grains preferably form a layer of one grain thick. Every grain is inspected based on shape and colour and moved to a pile of cereal grains in the corner of the bin.

2.5 Analysis of results

The percentage of recovery for every sample was calculated. Statistics (average, minimum, maximum) were calculated for sample size, number and weight of sclerotia fragments spiked and number and weight of sclerotia fragments recovered are calculated as well.
Recovery percentage was expected to be between 80% and 110%. The background of these limits will be discussed further.

3 Results

Thirty packages with two samples were sent to all participants. All participants returned their results. The participants originated from 10 countries. The list of participants is presented in Annex 1. Half of all participants (15) originated from Germany.
All results were received by E-mail, in most cases by means of a scan and the original report file. Not in all cases a scan as pdf-file was submitted although clearly requested. All reported results were included. One participant did not submit details on the method used.
The full results are presented in the tables of Annex 3 and 4.

3.1 Application of the method

Eighteen participants applied the IAG method (IAG, 2010). Four participants reported to have applied method VDLUFA[2] 30.2, one participant referred to another method and six participants indicated to have applied their own internal method.
Eight participants reported the presence of grain weevils in one or both samples. Participants' comments are reproduced in Annex 5.

3.2 Recovery

The average recovery was approx. 97% (Tables 1 and 2). All results except one were between the expected limits (80 – 110 %).
Two participants for sample A and six participants for sample B reported a number of detected sclerotia fragments differing from the spiked number. In three cases this number was higher. Participant 37 reported three out of four spiked fragments in sample A (40% spike level). Missing one fragment resulted in a recovery of 76.6% w/w. Participant 44 reported 300 mg for sample B, resulting in a recovery of 109.9% w/w.

Table 1 *Statistics of the results for the 40% spike level (400 ppm). Original data in Annex 3.*

	spiked		recovered		recovery percentage
	# sclerotia	mg	# sclerotia	mg	
average	4.7	114.1	4.7	110.5	96.8
max	6	136	7	140.0	104.3
min	3	99	3	85.8	76.6

Table 2 *Statistics of the results for the 100% spike level (1000 ppm). Original data in Annex 4.*

	spiked		recovered		recovery percentage
	# sclerotia	mg	# sclerotia	mg	
average	10.4	286.4	10.4	278.1	97.1
max	13	314	13	310	109.9
min	6	271	7	250	84.7

[2] Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten.

Participants applied either the IAG method or another (VDLUFA or own lab) method. The results obtained with these different methods are not differing. Most results show a recovery between 95% and 100%, except for four participants at the 40% w/w spike level, and five participants at the 100% w/w level (Figure 1 and 2).

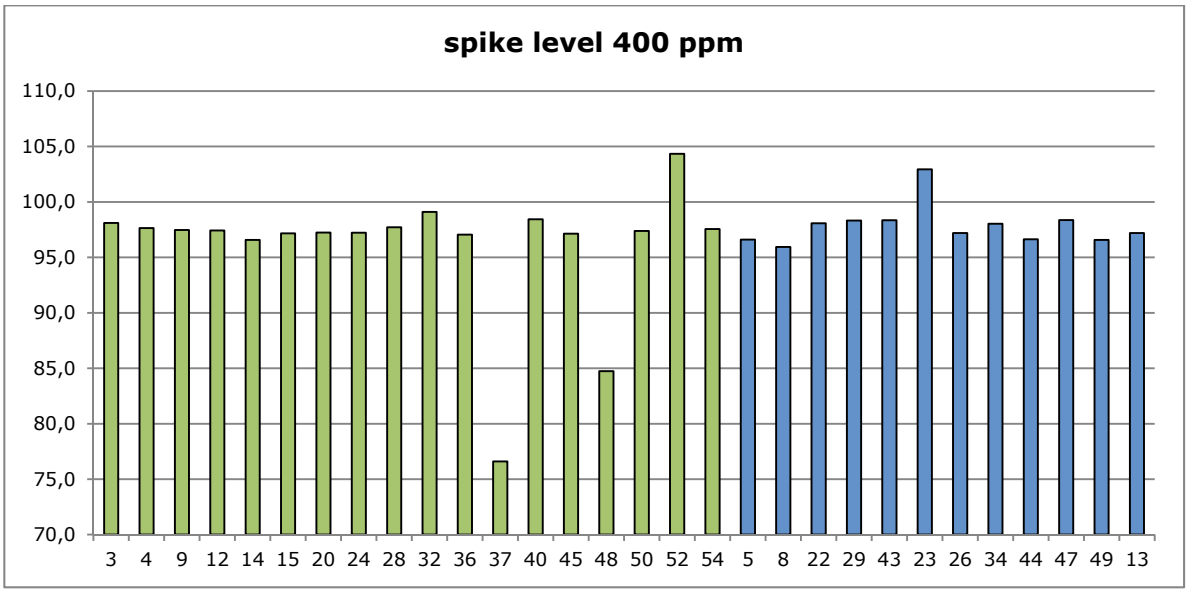

Figure 1 *Individual results of the participants at a spike level of 400 ppm (40% of legal limit). Green: IAG method, blue: other method. Original data in Annex 3. Y-axis: % recovery.*

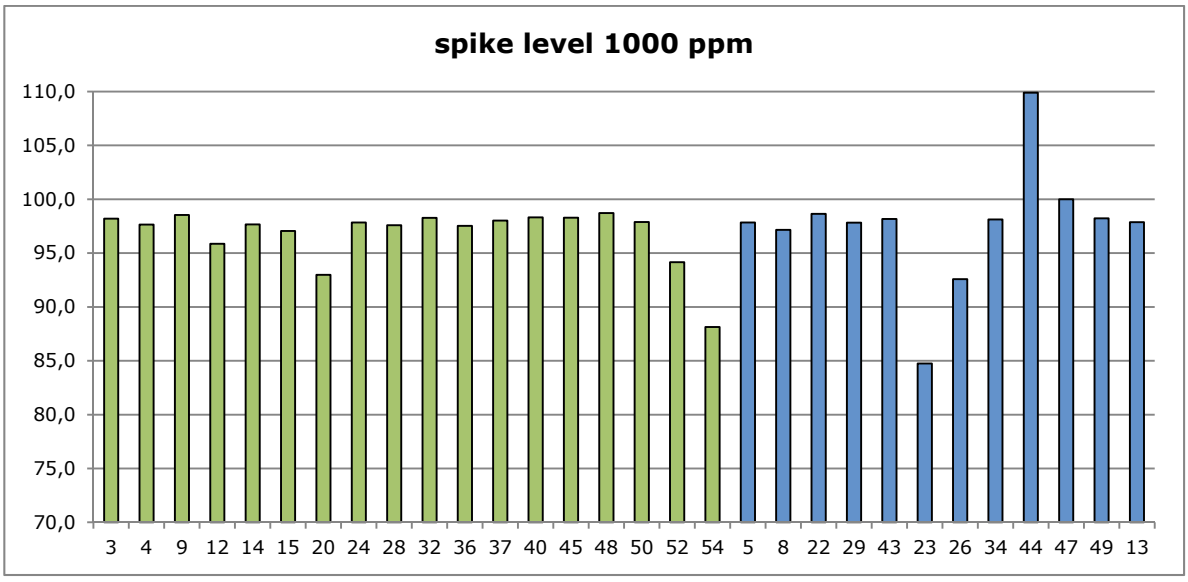

Figure 2 *Individual results of the participants at a spike level of 1000 ppm (100% of legal limit). Green: IAG method, blue: other method. Original data in Annex 4. Y-axis: % recovery.*

4 Discussion

4.1 Criteria for recovery

Regulation (EC) 152/2009 presents in Annex II, part C-6 "Measurement uncertainty and recovery rate in case of analysis of undesirable substances", criteria for recovery rate and measurement uncertainty. It is stated that these would not apply to microscopic analysis. It can be argued whether a visual method should be indicated as "microscopic" in strict sense, and in this particular case quantitative results are available. However, as far as encountered, criteria for trueness in general apply specifically to results of chemical methods.
In order to have any indication for criteria for recovery, some indications can be given. Regulation (EC) 152/2009 in Annex II, part C-6, states that a result should be corrected if it exceeds the range between 90% and 110% (EC, 2009). Directive 2002/657/EC section 2.3.2.1 indicates limits for recovery of 80 – 110 % w/w for spike levels above 10 µg/kg. A note of SANCO (12571/2013) presents a general indication of 70 – 120 % w/w. RIKILT applies limits of 80 – 110 % w/w for intralaboratory studies, based on CEN information.
Besides few individual deviations, the methods applied in this interlaboratory study generally seems to be fit to detect ergot sclerotia within a recovery rate of 85-105% w/w.

4.2 Validation of visual detection of ergot sclerotia

Additional information on the performance of the IAG method was collected in an intralaboratory study by RIKILT Wageningen UR, carried out in 2011 (results unpublished). The study comprised a set of six separate experiments to establish different performance parameters. The summarised results are shown in Table 3.

Table 3 *Performance parameters of the method for quantification of sclerotia bodies in cereals.*

parameter	result	a-priori criterion
intra-lab reproducibility (n=8)	$SR_{w\ ,\ 0.2}$ = 9.62 %	RSD_{wR} = 20 %
accuracy 0.2% (n=8)	J = 99.5 % (98.0 % - 100.8 %), $d_{T,rel}$ = -0.52	80 – 110 % w/w
accuracy 0.01% (n=8)	J = 101.3 % (100.0 % - 104.9 %), $d_{T,rel}$ = 1.30	80 – 110 % w/w
Limit of detection	AG_W = 7 mgr/kg (0.0007 % w/w)	AG_W = 0.01 % w/w
selectivity (niger seeds; n=2)	RA = -0.8 %	5 % *
robustness (smut; n=2)	RA = 1.2 %	5 % *
robustness (time; n=2)	RA = -2.3 %	5 % *

*: the criterion set is based on maximum allowed recovery minus established maximum recovery: 110 – 104.9 > 5%. With a maximum of 5% relative deviation the recovery will be still within the limits. RA: relative deviation.

The standard deviation of the **intra-laboratory reproducibility** was $SR_{w,0.2}$ = 9.62 % w/w. This value complies with the a-priori set criterion. The relative deviation of the **accuracy** for the eight samples at the contamination level of 0.2 % w/w was $d_{T,rel}$ = -0.52, indicating that near to 100 % w/w of the contaminated material was recovered. The variation in recovery at the contamination level of 0.01 % is higher. Notwithstanding this result, the recovery is well within the set limits.
The calculation of the **limit of detection** was based on the analysis of eight samples contaminated at a level of 0.01 % w/w. The standard deviation of the eight results was S_{AG} = 1.17, resulting in a detection limit of $AG_W = 3 * S_{AG}$ = 3.5 mg (calculation according to NEN 7777). As shown, the accuracy at the 0.01 % level is within the set limits, which allows the conclusion that a reliable quantification is sufficiently reached at this level. Since the method was applied to samples of approx.

500 grams, this detection limit represents a relative amount of 0.0007 % w/w. The limit of detection is well below the criterion of 0.01 % w/w (see Table 3).
The relative deviations after adding confusing materials (niger seeds) for determining **selectivity** and after changing some method parameters (smut infested grains and shorter examination time) for determining **robustness** are low in all cases (Table 3). Because of the exclusive nature of the presented method parameters, setting criteria is difficult. A deviation of 5 % w/w was set as criterion, since this value limits the results including the deviations between the range for recovery of 80 - 110 % w/w. The results for selectivity and robustness (smut) are well within this criterion. As alternative, two samples were investigated in duplicate by quick, random examination of the sample as spread in the examination bin for a maximum of 10 minutes or 15 minutes, respectively. The relative deviation (RA) for this alternative examination is within the limit, but higher than for the other parameters (Table 3).

4.3 Relevance

The ultimate goal of ergot sclerotia detection is the establishment of the level of the toxic components, the ergot alkaloids, and the effectiveness of a visual examination method should be evaluated in the framework of the performance of the final (e.g. confirmation or identification) analysis. The relationship between visual detection of sclerotia and chemical analysis ergot alkaloids can be compared to the situation for animal proteins. In that case the target of the screening method (microscopy: bone fragments) is not identical to that of the identification method (PCR: DNA) or confirmation method (MS/MS or immunoassay: protein). See for details van Raamsdonk *et al.* (2007), Liu *et al.* (2011), Bremer *et al.* (2012). In both situations the first step in the monitoring procedure is targeting the (possible) vector of the compound at stake. This is principally different from other situations where the target of screening and confirmation is identical (e.g. dioxins screening: CALUX, confirmation: MS; Hoogenboom, 2002; van der Dungen *et al.*, 2016). In those cases the screening method performance can be precisely adjusted to the performance limits of the confirmation method. Therefore, the performance of the currently validated method for visual detection of ergot sclerotia need to be evaluated in the view of chemical results.
Considering the situation that sclerotia show a large range of different levels of alkaloid contents (Lorenz and Hoseney, 1979; EFSA, 2005), a firm statistical relationship between sclerotia weight and ergot alkaloid content cannot be expected. Data for sclerotia weight and ergot alkaloid content in rye, triticale and wheat samples collected in the framework of the Dutch monitoring program (2007-2010) was presented by Mulder *et al.* (2012). In 82 out of 116 examined samples the visual examination for sclerotia appeared to be a correct, although qualitative, predictor for the presence or absence of ergot alkaloids. In 24 samples, approx. one fifth of all samples, the presence of sclerotia was connected to absence of any ergot alkaloids. The main parameter for comparing the visual and chemical detection was the number of samples which turned out positive for ergot alkaloids in the absence of sclerotia. Less than 9% (10 samples) was not marked as suspect after visual examination. These samples without sclerotia showed nonetheless ergot alkaloid levels ranging from 5 – 124 µg/kg, with one higher level of 297 µg/kg. It is known that cereal grains, showing no infection by moulds of the genus *Claviceps*, still can contain ergot alkaloids in rare occasions (Mulder *et al.*, 2012).
An alternative to visual examination was studied. The examination based on the detection of sclerotia by means of near-infrared (NIR) was shown to have a limit of detection of 145 mg/kg, and a limit of quantification of 341 mg/kg (Vermeulen *et al.*, 2012). This is much higher than the LOD of the visual method, but, as stated by Vermeulen *et al.* (2012) well below the limit as set for feed ingredients in the European Union (EC, 2012: 1000 mg/kg). The LOD of the NIR detection method might influence the predictability of the presence of ergot alkaloids.

5 Conclusions and recommendations

It can be concluded that examination by visual detection of sclerotia is a valuable indicator of the presence of ergot alkaloids. The application of visual detection will comply with the requirements as published in EC (2009a). The results of this study provides the data for a partial validation of the method of IAG (2010) for the examination of whole kernel cereal samples.

Acknowledgements

The board of IAG section Feeding Stuff Microscopy (dr. I. Paradies-Severin (LUFA, Hameln), dr. G. Frick (ALP, Posieux), ir. J. Vancutsem (FAVV, Tervuren) and dr. R. Weiss (AGES, Vienna)) supported this study as advisory board for communication with the scientists and laboratories working in this research field, and in the final report activities. Their contributions are greatly acknowledged. Thanks to colleagues dr. L. van der Geest, dr. J. de Jong and dr. A. Peijnenburg for the internal RIKILT review procedure.

References

Alm, T., 2003. The witch trials of Finnmark, northern Norway, during the 17th century: evidence for ergotism as a contributing factor. Economic Botany 57: 403-416.

Bremer, M., O. Fumière, W. Hekman, A. Marien, A. Kemmers-Vonken, J. A. Fernández Pierna, J. Vliege, V. Baeten, L. van Raamsdonk and G. Berben, 2012. Combination methods for PAP detection and species determination of animal particles. In: Methods for the detection of processed animal proteins, chapter 13, J.S. Jørgenson and V. Baeten, eds.

CEN, 2008. EN 15587 - Cereals and cereal products - Determination of Besatz in wheat (*Triticum aestivum* L.), durum wheat (*Triticum durum* Desf.), rye (*Secale cereale* L.) and feed barley (*Hordeum vulgare* L.).

Dungen, M.W. van den, Kok, D.E., Polder, A., Hoogenboom, L.A.P., Leeuwen, S.P.J. van, Steegenga, W.T., Kampman, E., Murk, A.J., 2016. Accumulation of persistent organic pollutants in consumers of eel from polluted rivers compared to marketable eel. Environmental Pollution, 219: 80-88.

EFSA, 2005. Opinion of the scientific panel on contaminants in food chain on a request from the commission related to ergot as undersirable substance in animal feed. The EFSA Journal 225: 1-27. http://www.efsa.europa.eu/en/scdocs/doc/contam_op_ej225_ergot_en1.pdf.

Egmond, H.P., Jonker, M., 2004. Worldwide regulations for mycotoxins in food and feed in 2003. Food and Nutrition Paper No. 81. Rome (Italy): Food and Agriculture Organisation of the United Nations.

European Commission, 1992. Regulation (EEC) No. 689/92 of 19 March 1992 fixing the procedure and conditions for the taking-over of cereals by intervention agencies. Official Journal L 074: 18–22.

European Commission, 2000. Commission Regulation (EC) No 824/2000 of 19 April 2000 establishing procedures for the taking-over of cereals by intervention agencies and laying down methods of analysis for determining the quality of cereals. Official Journal L 100, 20.4.2000, p. 31–50.

European Commission, 2002a. Directive 2002/32/EC of the European parliament and of the council of 7 May 2002 on undesirable substances in animal feed. Official Journal L 140: 10–21.

European Commission, 2002b. Commission Decision 2002/657/EC of 14 August 2002, implementing Council Directive 96/25/EC concerning the performance of analytical methods and the interpretation of results. Official Journal L 221, 17.8.2002, p. 8-36.

European Commission, 2009a. Commission Regulation (EU) No 1272/2009 of 11 December 2009 laying down common detailed rules for the implementation of Council Regulation (EC) No 1234/2007 as regards buying-in and selling of agricultural products under public intervention. Official Journal L 349, 29.12.2009, p. 1–68.

European Commission, 2012. Commission Regulation (EU) No 744/2012 of 16 August 2012 amending Annexes I and II to Directive 2002/32/EC of the European Parliament and of the Council as regards maximum levels for arsenic, fluorine, lead, mercury, endosulfan, dioxins, Ambrosia spp., diclazuril and lasalocid A sodium and action thresholds for dioxins. Official Journal L 219, 17.8.2012, p. 5–12.

Hoogenboom, L.A.P., 2002. The combined use of the CALUX Bioassay and the HRGC/HRMS method for the detection of novel dioxin sources and new dioxin-like compounds. Environmental Science and Pollution Research Vol.9 (5): 304-306.

IAG (International Association of Feedingstuff Analysis) – Section Feedingstuff Microscopy. 2011. Method for the determination of ergot (*Claviceps purpurea* Tul.) in Animal Feedingstuff. IAG-Method A4. [Internet]. January 2013. Hameln, Germany: IAG; http://www.iag-micro.org/files/iag-a4_ergot.pdf

Krska, R. & C. Crews, 2008. Significance, chemistry and determination of ergot alkaloids: A review. Food Additives & Contaminants: Part A, 25 (6): 722-731.

Lee, M.R., 2009a. The history of ergot of rye (*Claviceps purpurea*) I: From antiquity to 1900. J R Coll Physicians Edinb 39: 179–184.

Liu, X., Han, L., Veys, P., Baeten, V., Jiang, X., Dardenne, P., 2011. An overview of the legislation and light microscopy for detection of processed animal proteins in feeds. Microsc. Res. Tech. 74: 735-43.

Lorenz, K. & Hoseney, R.C., 1979. Ergot on cereal grains, C R C Critical Reviews in Food Science and Nutrition, 11: 311-354.

Mulder, P.P.J., L. van Raamsdonk, H. van Egmond, T. van der Horst and J. de Jong, 2012. Ergot alkaloids in animal feed. Results of a survey in The Netherlands. Report 2012.005, RIKILT, Wageningen, pp. 50.

Raamsdonk, L.W.D. van, 2007. A survey for the presence of botanic undesirable substances in feed. Report 2007.004, RIKILT, Wageningen. P. 13 with 10 tables. Available from: http://edepot.wur.nl/39987.

Raamsdonk, L.W.D. van, C. von Holst, V. Baeten, G. Berben, Ana Boix and J. de Jong, 2007. New developments in the detection of animal proteins in feeds. Feed Science and Technology 133: 63-83.

Raamsdonk, L.W.D. van, Vancutsem, J., Jorgensen, J.S., 2009. A survey on the presence of undesirable botanical substances in feed in the European Union. Biotechnol Agron Soc Environ. 13(S): 33–38.

Scott, P.M., 2009. Ergot alkaloids: extent of human and animal exposure. World Mycotoxin Journal, 2: 141-149.

Woolf, A., 2000. Witchcraft or mycotoxin? The Salem witch trials. J Toxicol Clin Toxicol. 38: 457-60.

Annex 1 List of participants

Austrian Agency for Health and Food Safety-AGES	Austria
CRA-W	Belgium
FLVVT	Belgium
Danish Veterinary and Food Administration	Denmark
S.C.L. Laboratoire de Rennes	France
Bayerisches Landesamt fur Gesundheit und Lebensmittelsicherheit	Germany
CVUA-RRW	Germany
Futtermittelinstitut Stade (LAVES)	Germany
Landesbetrieb Hessisches Landeslabor, Landwirtschaft und Umwelt	Germany
Landeslabor Berlin-Brandenburg	Germany
LLFG Landesanstalt für Landwirtschaft	Germany
LTZ Augustenberg	Germany
LUFA Nord-West	Germany
LUFA Rostock	Germany
LUFA-Speyer	Germany
SGS Germany GmbH	Germany
Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft, GB6-Labore Landwirtschaft / LUFA, FB62	Germany
Thüringer Landesanstalt für Landwirtschaft	Germany
Universität Hohenheim, LA Chemie (710)	Germany
WESSLING GmbH	Germany
Department of Agriculture, Fisheries and Food, Backweston Agri Laboratories	Ireland
Inst. Zooprofilattico Sperimentale della Sardegna	Italy
Istituto Zooprofilattico della Sicilia	Italy
MIPAAF – ICQRF – LABORATORIO DI MODENA	Italy
Eurofins Food Testing Rotterdam BV	Netherlands
Nutreco Nederland BV - Masterlab	Netherlands
Nutrilab BV	Netherlands
TLR	Netherlands
University of Ljubljana, Veterinary Faculty, Natl. Veterinary Institute, Unit for Pathology of Animal Nutrition and Environmental Hygiene	Slovenia
Agroscope (ALP), Swiss Research Station	Switzerland

Annex 2 Instructions

Instructions

Test 2015-C: ergot sclerotia in unground cereals
The package consists of two bags with 250 gram of unground rye. The purpose of the ring test is to detect any piece of sclerotia of Ergot as listed in Directive 2002/32/EC, consolidated version of December 2013.
For each of the two samples the **number of particles** and their **weight** in mg needs to be reported on the report sheet.
The report file will be send to you by mail. The report files consists of:

- A tab with specific instructions.
- A tab with an inquiry for a specification of the procedure.
- A tab for entering the results.

All pink cells have to be filled.
After completing the two forms "Procedure" and "Results", they have to be saved as Excel file by using "Save as …", add your unique lab code to the end of name (replace the ## signs with your lab number). The forms have to be send to the organisers in two ways. Every form has to be sent by E-mail as Excel file and as a scan (preferably *.PDF). and send the two files to Nastasja.vanderhee@wur.nl and to Leo.vanraamsdonk@wur.nl. Results will be included in the final analyses and report only if the forms are send in by electronic mail, and after the **proper receipt of the requested fee**.
Closing date is Tuesday October 5th, 2015.

Annex 3 Results sample A (spike level 400 ppm)

Participant	Jar nr	Sample size	spiked		recovered		+/- %
		gram	#	mg	#	mg	
3	276	251.2	5	111	5	108.9	98.1
4	281	251.0	5	119	5	116.2	97.6
9	296	253.1	5	111	5	108.2	97.5
12	301	250.9	5	109	5	106.2	97.4
14	311	250.0	4	105	4	101.4	96.6
15	316	250.9	5	106	5	103	97.2
20	321	256.0	5	109	5	106	97.2
24	336	251.6	5	108	5	105	97.2
28	346	251.1	4	114	4	111.4	97.7
32	356	252.5	5	112	5	111	99.1
36	282	252.9	4	119	4	115.5	97.1
37	287	*251.5*	4	112	3	85.8	76.6
40	292	252.7	4	102	4	100.4	98.4
45	307	250.0	3	105	3	102	97.1
48	317	250.9	5	118	5	100	84.7
50	327	252.0	5	115	5	112	97.4
52	332	248.0	5	115	5	120	104.3
54	337	250.0	5	123	5	120	97.6
5	286	250.0	4	118	4	114	96.6
8	291	251.0	5	123	7	118	95.9
22	326	250.0	4	99	4	97.1	98.1
29	351	251.6	5	119	5	117	98.3
43	297	251.2	5	121	5	119	98.3
23	331	250.0	6	136	6	140	102.9
26	341	250.0	5	107	5	104	97.2
34	277	252.3	4	107	4	104.9	98.0
44	302	251.0	4	119	4	115	96.6
47	312	253.2	5	122	5	120	98.4
49	322	250.0	5	108	5	104.3	96.6
13	306	251.3	5	131	5	127.33	97.2

Annex 4 Results sample B (spike level 1000 ppm)

Participant	Jar nr	Sample size	spiked		recovered		+/- %
		gram	#	mg	#	mg	
3	278	251.5	11	271	11	266.1	98.2
4	283	251.0	9	285	9	278.3	97.6
9	298	255.2	11	295	11	290.7	98.5
12	303	250.6	11	276	10	264.6	95.9
14	313	250.0	8	283	8	276.4	97.7
15	318	251.3	6	271	7	263	97.0
20	323	248.0	11	285	10	265	93.0
24	338	253.0	11	278	12	272	97.8
28	348	252.8	9	287	9	280.1	97.6
32	358	252.2	13	289	13	284	98.3
36	284	250.9	10	295	10	287.7	97.5
37	289	250.4	8	288	8	282.3	98.0
40	294	252.9	13	297	13	292	98.3
45	309	250.0	12	291	12	286	98.3
48	319	254.1	10	314	10	310	98.7
50	329	252.0	10	283	10	277	97.9
52	334	247.0	7	308	7	290	94.2
54	339	247.0	12	295	11	260	88.1
5	288	250.0	11	279	11	273	97.8
8	293	252.0	12	281	12	273	97.2
22	328	250.0	11	296	11	292	98.6
29	353	253.8	13	277	13	271	97.8
43	299	252.2	13	273	13	268	98.2
23	333	251.0	12	295	10	250	84.7
26	343	250.0	9	283	8	262	92.6
34	279	252.2	9	283	9	277.7	98.1
44	304	254.0	10	273	11	300	109.9
47	314	253.9	11	290	11	290	100.0
49	324	250.0	9	294	9	288.8	98.2
13	308	250.6	11	278	12	272.1	97.9

Annex 5 Overview of participants' comments

Lab nr	Comments by participant
3	none
4	none
9	none
12	none
14	all fragments counted
15	Both samples showed feeding traces. Sample 318 contains living grain weevils.
20	none
24	Both samples show nutricional traces and living beetles
28	We found in both samples living corn weevil
32	none
36	none
37	samples contained beetles
40	none
45	none
48	none
50	none
52	none
54	none
5	method: VDLUFA 30.2
8	method: VDLUFA MBIII 30.2
22	method: VDLUFA 30.2
29	used method: VDLufa Bd.III 30.2 - Sample 351: 14 live Sitophilus spp. & Sample 353: 5 live and 2 dead Sitophilus spp.
43	used method: DM 13/04/1994 SO GU n°123 28/05/1994
23	Whole sample analysed using the binocular at magnification 6 x
26	macroscopic & microscopic determination
34	none
44	presence of wheat weevil (Sitophilus granarius)
47	
49	Mijt in beide monsters aangetroffen!!!
13	1 live insect found

RIKILT Wageningen University & Research
P.O. Box 230
6700 AE Wageningen
The Netherlands
T +31 (0)317 48 02 56
www.wur.eu/rikilt

RIKILT report 2016.013

To explore the potential of nature to improve the quality of life

RIKILT Wageningen University & Research
P.O. Box 230
6700 AE Wageningen
The Netherlands
T +31 (0)317 48 02 56
www.wur.eu/rikilt

Report 2016.013

The mission of Wageningen University and Research is "To explore the potential of nature to improve the quality of life". Under the banner Wageningen University & Research, Wageningen University and the specialised research institutes of the Wageningen Research Foundation have joined forces in contributing to finding solutions to important questions in the domain of healthy food and living environment. With its roughly 30 branches, 5,000 employees and 10,000 students, Wageningen University & Research is one of the leading organisations in its domain. The unique Wageningen approach lies in its integrated approach to issues and the collaboration between different disciplines.