JRC Scientific and Technical Reports

Evaluation of the Effect of Mycotoxin Binders in Animal Feed on the Analytical Performance of Standardised Methods for the Determination of Mycotoxins in Feed

Follow-up study

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EUR 24955 EN - 2011

JRC EUROPEAN COMMISSION

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JRC66712

EUR 24955 EN
ISBN 978-92-79-21401-1 (PDF)
ISBN 978-92-79-21400-4 (print)

ISSN 1831-9424 (online)
ISSN 1018-5593 (print)

doi:10.2787/52525

Luxembourg: Publications Office of the European Union, 2011

Summary

This report deals with the follow-up study on the evaluation of the effect of mycotoxin binders in animal feed on the analytical performance of standardised methods for the determination of mycotoxins in feed. The study covers the following aspects: influence of the addition of mycotoxin binders to animal feeding stuffs with recommended limits for mycotoxins much lower (approximately one order of magnitude) than those tested in the previous study (i.e. DON in complementary and complete feeding stuffs for pigs and ZEA in complementary and complete feeding stuffs for piglets and gilts) and possible effects of binder addition and feed processing (pelletizing) on the analytical performance of the standardised (CEN) method for determination of AFB_1 in feed. With regard to the first aspect, it was shown that, similar to our previous investigation, the tested binders had no effect on the level of DON and ZEA found. As far as the second aspect is concerned, it has been demonstrated that only the addition of the tested binders to the feed in combination with wet pelletizing had a significant effect on the level of AFB_1 found.

Outline of the Study

In 2009, the investigation concerning the effect of mycotoxin binders in animal feed on the analytical performance of official methods for determination of mycotoxins in feed was performed (Kolossova et al., 2009). With respect to the previously discussed issues not covered in this investigation, a follow-up study has been carried out. The study covered the following aspects:

I. Influence of the addition of mycotoxin binders to animal feed for which recommended limits for mycotoxins are much lower than those tested in the previous study (DON and ZEA):
    a) 0.9 mg/kg DON in animal feed (complementary and complete feeding stuffs for pigs)
    b) 0.1 mg/kg ZEA in animal feed (complementary and complete feeding stuffs for piglets and gilts)

    The experiments and data analysis were carried out in the same layout and using the same methodology as previously described (Kolossova et al., 2009). The tested mycotoxin levels were approximately 1.5 times the recommended limits, and the binders were added by groups. All the binders were added to the test sample portions at the maximum recommended level.

II. Combination of binder addition and feed processing (pelletizing): the experiments were performed for inorganic binders and AFB_1, since this was the only mycotoxin for which a possible effect had been mentioned in the literature. Other mycotoxins, such as DON, ZEA, etc., were not included in the study. The feed processing was simulated at small scale, since large scale processing equipment could not be used due to the amount of feed materials needed for such a study.

Methodology

Samples and methodology for binder experiments with DON and ZEA were the same as previously described (Kolossova et al., 2009). Naturally contaminated or spiked feed materials were applied for the investigation. Homogeneity of the materials was verified. Not sufficiently high naturally contaminated feed materials or materials with no corresponding mycotoxin found were spiked to obtain levels approximately 1.5 times higher than the legislative or recommended limits. Mycotoxin binders (Table 1) were added by groups. Group A and group B were aluminosilicate clays. Group A contained binders 2, 3, 4, 12, 13, and group B – binders 15-19. Group C comprised yeast cell walls and included binders 1, 5, 6, 7 and 9. Group D included binders 8, 10, 11, which were mixtures of organic and mineral components. Group E was formed by fibre binders 14 and 20. The binders, which belonged to the same group, were added together to each portion of feed test material. All the binders were added to the test sample portions at the maximum recommended level (Table 1). Feed material without binder was used as a reference. The samples (with and without binders) were prepared as independent triplicates for each experiment and each replicate was injected twice. The samples were analysed according to the method protocols indicated in the previous study (Kolossova et al., 2009).

Investigation of possible effects of binder addition and feed processing (pelletizing) on the analytical performance of the standardised method for determination of AFB_1 in feed

For the experiments, pig feed purchased in a local shop (AVEVE, Geel, Belgium) was used. This feed material was found to be free of any naturally occurring AFB_1. Therefore, 10 kg was spiked at approximately 100 µg/kg. After milling, the material was blended with the blank feed. The obtained material was analysed for AFB_1 content and tested for homogeneity. Apart from that, particle size analysis of the material has been performed at the Reference Materials Unit of IRMM (see Annex). Three mycotoxin binders (No. 17, 18 and 19, Table 1) were added to a portion of the feed material (ca 11 kg). Each binder was added at 0.4% resulting in a total level of 1.2%.

Pelletizing of feed with and without binders was performed at the Laboratory of Feed Technology, University College Ghent (Ghent, Belgium). Pelletizing was done first without and then with steam treatment. Steam treatment was performed at 85 ºC.

After pelletizing, the materials were milled, mixed, and analysed for AFB_1 content using CEN method for determination of AFB_1 in animal feeding stuffs (EN ISO 17375:2006). Each type of the material was analysed in triplicate.

Repeatability tolerance limits at 95% coverage were calculated as

$$1.96 \times (\overline{X} \times RSD_r/100),$$

where

$\overline{X}$ - mean value determined for each type of the feed material, µg kg^{-1}

RSD_r - repeatability relative standard deviation from collaborative trial (%)

Results and Discussion

Analysis of deoxynivalenol

Naturally contaminated feed material was used for these experiments. The amount of DON found in this material was approximately 6000 µg/kg. The material was blended with a blank feed material to dilute the amount of DON to approximately 1300 µg/kg. This is approximately 1.5 times higher than the recommended limit for complementary and complete feeding stuffs for pigs. The mycotoxin values obtained for the feed material without binder was used as a reference and defined as 100 %. The results are given in Figure 1. Statistical analysis indicated that there were no significant differences between binder free material and the material containing different binders under the described mixing conditions. Thus, it could be concluded that the addition of the tested binders had no significant effect on the performance of the analytical procedure used for the detection of DON.

Analysis of zearalenone

The blank feed material used in this experiment was spiked with ZEA at 150 µg/kg, which is 1.5 times higher than the recommended limit for complementary and complete feeding stuffs for piglets and gilts. The experimental design was the same as for DON. The results are presented in Figure 2. Statistical analysis indicated that there were no significant differences between binder free material and the material containing different binders under the described mixing conditions. Thus, it could be concluded that the addition of the tested binders had no significant effect on the performance of the analytical procedure used for the detection of ZEA.

Results of the experiments for DON and ZEA are given in Table 2. Summarising the above part of this study and the previously performed investigation (Kolossova et al., 2009), it can be concluded that applying the repeatability of each method, statistical analysis does not show any significant differences in the methods' capacity to determine mycotoxins in binder free material and materials with mycotoxin binders added.

Table 1. Mycotoxin binders and their amounts used in the study

Code	Type of main product(s)	pH value	Amount per 10 g feed material (mg)
1	Yeast cell wall	2.9	20
2	Clay + organic acid	7.9	10
3	Clay	8.4	10
4	Clay	4.6	25
5	Yeast cell wall	6.2	20
6	Yeast cell wall	6.7	20
7	Yeast cell wall	4.7	25
8	Mixture organic + mineral	6.2	25
9	Yeast cell wall	5.0	20
10	Mixture organic + mineral component	8.6	25
11	Mixture organic + mineral component	6.9	25
12	Clay	9.1	50
13	Clay	8.9	100
14	Fibres	6.7	100
15	Montmorillonite	10.4	40
16	Montmorillonite	10.6	40
17	HSCAS	8.8	50
18	HSCAS	9.8	50
19	HSCAS	9.8	50
20	Fibres (lignocellulose)	5.1	250

Table 2. Results of the determination of mycotoxins in feed samples with and without mycotoxin binders

Mycotoxin	Binder(s) added	*t*-value		Amount of mycotoxin, µg/kg		
		critical	calucated	Prep. 1	Prep. 2	Prep. 3
DON	Blank	-	-	1227	1099	1191
	Group A	2.78	0.81	1270	1251	1242
	Group B	2.78	0.23	1246	1229	1112
	Group C	2.78	0.50	1168	1232	1267
	Group D	3.18	0.16	1193	-	1188
	Group E	2.78	1.12	1357	1264	1237
ZEA	Blank	-	-	142.9	143.2	136.4
	Group A	2.78	0.11	138.1	141.1	146.3
	Group B	2.78	0.29	137.0	146.0	147.1
	Group C	2.78	0.07	142.6	141.3	136.6
	Group D	2.78	0.41	129.8	145.3	136.5
	Group E	2.78	0.29	142.2	139.8	132.8

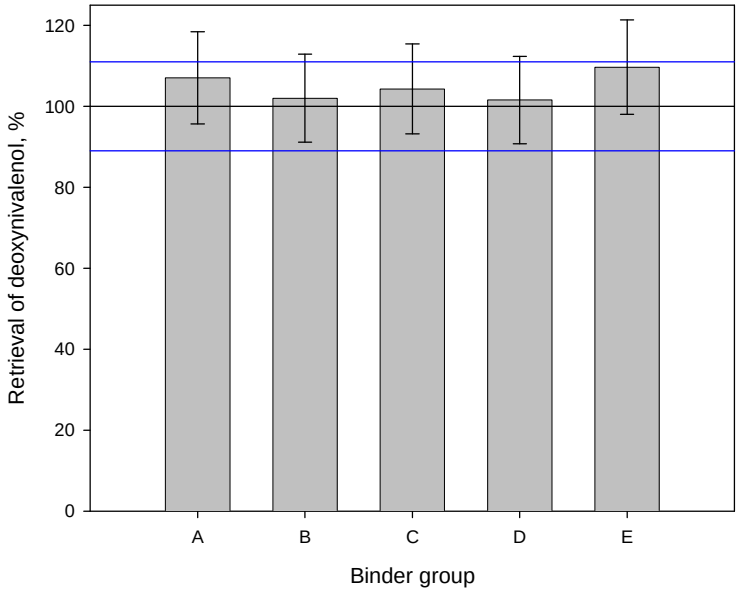

Figure 1. Effect of different binders on the analytical determination of DON by HPLC. The amount of DON determined is related to that in binder free material defined as 100%. Bars are the mean values of triplicates. Error bars correspond to the repeatability (RSD_r) of the method. Blue lines represent the RSDr range for binder free material.

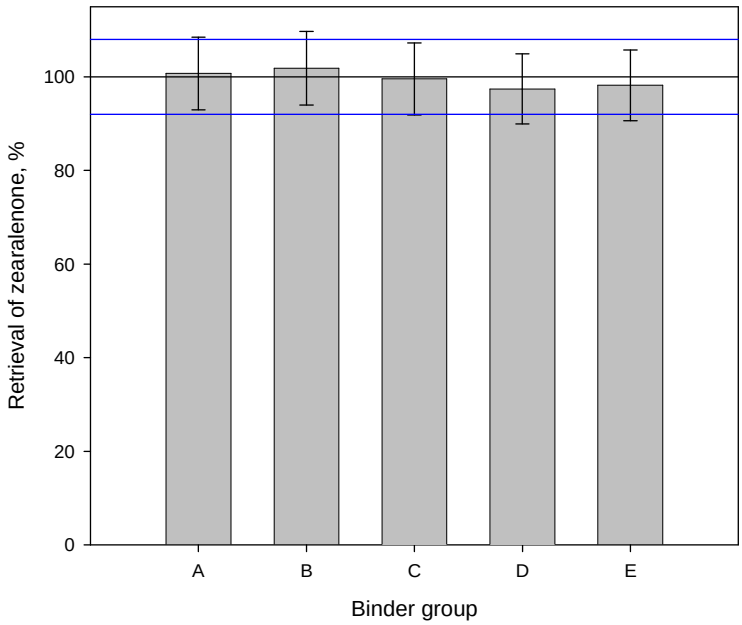

Figure 2. Effect of different binders on the analytical determination of ZEA by HPLC. The amount of ZEA determined is related to that in binder free material defined as 100%. Bars are the mean values of triplicates. Error bars correspond to the repeatability (RSD_r) of the method. Blue lines represent the RSDr range for binder free material.

Combination of binder addition and feed processing (pelletizing)

In a study of Masoero *et al*. (2009), it has been concluded that the way of addition of a mycotoxin binder (commercial magnesium smectite clay) to feed had a significant effect on its ability to reduce levels of AFM_1 in milk when cows were fed with AFB_1 contaminated feed. According to this study, physical processing of feed (here - pelletizing) changed the amount of AFB_1 sequestered by the binder, and the reduction of AFM_1 in milk was not due to any degradation of AFB_1 during the processing. The authors pointed out in their discussion that a partial degradation of AFB_1 by heat treatments had been reported by others, but this effect did not occur in their study. However, the data leading to this conclusion were not discussed. Moreover, the authors supposed that moisture increase during pelletizing could be another enhancing factor for the interaction between AFB_1 and the mycotoxin binder during feed processing. The present research focuses on the possible effects of binder addition and feed processing (pelletizing) on the analytical performance of the standardised (CEN) method for determination of AFB_1 in feed.

Three commercial mycotoxin binders containing a hydrated sodium calcium aluminosilicate (HSCAS) as the main component were chosen for this study. HSCAS (which is also part of the smectite clay group) is perhaps the most extensively investigated mycotoxin binder and is known as one of the most efficient binders of aflatoxin. This clay has been shown to act as an enterosorbent that tightly and selectively binds aflatoxins in the gastrointestinal tract of animals decreasing their bioavailability and associated toxicity (Phillips, 1999). It should be mentioned that according to near infra-red measurements performed in the previous study, the selected binders (coded as No. 17, 18 and 19, Table 1) have slightly different spectral profiles indicating possible inclusion of other components. On the basis of the information of the company which provided those mycotoxin binders, the additional components may include biopolymers and/or mycotoxin-detoxifying enzymes.

The amount of AFB_1 in spiked feed material used in our study was ca. 27 µg/kg, which is approximately 1.5 times higher than the maximum level set by EU legislation for all feed materials. Three mycotoxin binders (HSCAS) were added to a portion of the feed material. Each binder was added at 0.4% (maximum recommended addition level – 0.5%) resulting in a total level of 1.2%. Feed samples with and without binders were pelletized. Pelletizing was done without and then with steam treatment resulting in two types of pellets further referred as dry and wet pellets, respectively. After pelletizing followed by milling and mixing, all the materials were submitted to AFB_1 analysis. Thus, six groups of the feed material (in triplicates) were analysed: non-processed material, dry and wet pellets; each material - with and without mycotoxin binders. Results of the analysis are presented in Table 3.

The average repeatability relative standard deviation (RSD_r) from the collaborative trial as given in the CEN standard is 6.9%. Applying this value to the obtained data (mean values), repeatability tolerance limits with 95% coverage can be calculated (Figure 3). From this figure, it can be seen that only the combination of wet pelletizing and addition of binders to the feed has a significant effect on within-laboratory method performance, in particular on recovery.

Reduction of AFB_1 content observed in our experiments for the wet pellets with the binders might be due to enhanced interaction between aflatoxin and the binders during pelletizing with steam treatment. This also supports the assumption of Masoero *et al*. (2009) that steam addition during pelletizing could be another enhancing factor for the interaction between AFB_1 and the binder during feed processing.

It should be mentioned that to our knowledge, only a few studies regarding the influence of mycotoxin binders on the analytical characteristics of the methods used for determination of mycotoxins were performed so far. In our investigation, methods already available or currently under consideration as CEN standards have been applied for the analysis of mycotoxins in feed materials. In the CEN method for AFB_1 analysis, extraction with a mixture of acetone-water, 85:15 (v/v), is used. Results of our previous experiments with AFB_1 (Kolossova et al., 2009) are generally in agreement with the findings of a recent study of Gallo et al. (2009). It investigates the effect of the inclusion of mycotoxin binders (adsorbents) on AFB_1 quantification in animal feedstuffs by HPLC. Two extraction solvents composed of methanol/water, 80:20 (v/v), and acetone/ water, 85:15 (v/v), were compared. Nine adsorbents were used in the investigation: four clay minerals, one yeast cell wall-based product, one activated carbon and three commercial adsorbents. The study was designed using two samples of AFB_1-contaminated feedstuff (low and high contamination levels of 7.57 and 15.33 mg/kg, respectively) and at two levels of inclusion of each adsorbent (10 and 20 g/kg). With the higher adsorbent level, lower recoveries were observed for both feedstuffs with low and high AFB_1 content. Higher AFB_1 recoveries were obtained using acetone extraction compared with methanol extraction, for both high contaminated (75.0% versus 12.0%, respectively) and low contaminated (84.0% versus 22.8%, respectively) adsorbent-containing feedstuffs. However, when activated carbon and sodium bentonite were included in the feeds, lower AFB_1 concentrations with respect to control values were obtained also using acetone. It has been concluded that routine use of methanol extraction for AFB_1 analysis of unknown feedstuffs can produce misleading results if the feeds contain mycotoxin binders. These considerations are of particular interest for immunochemical methods (e.g. ELISA) which predominantly make use of aqueous methanol for the extraction of mycotoxins including AFB_1.

When evaluating the effect of a mycotoxin binder in animal feed on the analytical performance of a particular method, several factors should be taken into consideration. These include the nature of the organic solvent used for the extraction and solvent-to-water ratio, as well as the nature and amount of a binder. For example, if strong nonspecific adsorbents, such as activated carbon, are used at high levels in feed, they are most likely to have an effect on method performance. However, since such binders are also known to adsorb essential nutrients, they should not be used in high amounts. Apart from that, some mycotoxin binding products contain mycotoxin degrading enzymes which can affect some reagents used in ELISA kits. Furthermore, the method principle should also be taken into account, e.g. immunochemical (ELISA, etc.) vs. physicochemical (chromatography).

Table 3. Results of the determination of AFB_1 in the different types of feed material.

Group[1]	Amount of aflatoxin B_1 found, µg/kg		
	Preparation 1	Preparation 2	Preparation 3
A(-)	27.9	27.9	28.5
A(+)	26.2	26.0	25.6
B(-)	27.0	27.6	27.9
B(+)	24.5	25.4	24.0
C(-)	-	23.9	22.7
C(+)	16.7	16.7	16.3

[1]A – non-processed feed material; B – dry pellets; C – wet pellets;
(-) - without binders (+) – with the binders added

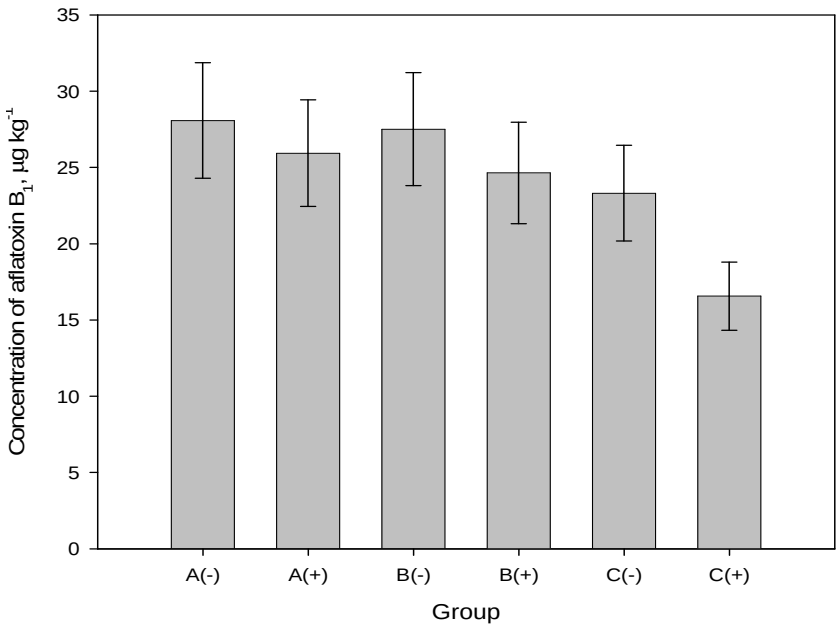

Figure 3. Effects of feed processing (pelletizing) and binder addition on the analytical performance of the CEN method for determination of AFB_1 in feed. Bars are the mean values of triplicates. Error bars correspond to the tolerance limits based on the repeatability (RSD_r).
A – non-processed feed material; B – dry pellets; C – wet pellets; (-) - without binders (+) – with the binders added

Conclusions

It was shown that the tested binders had no effect on the level of DON and ZEA found. As a result, the conclusion of the previous report can be expanded to animal feeding stuffs with recommended limits for mycotoxins much lower (approximately one order of magnitude) than those tested in the previous study (i.e. DON in complementary and complete feeding stuffs for pigs and ZEA in complementary and complete feeding stuffs for piglets and gilts).

With regard to the effects of binder addition and feed processing (pelletizing), it has been demonstrated that only the addition of the tested binders to the feed in combination with wet pelletizing had a significant effect on the level of AFB_1 found.

Acknowledgement

We are very grateful to Prof. Mia Eeckhout (University College Ghent) for her valuable help with the pelletizing experiments.
We also wish to thank Mr. J. Charoud-Got (JRC-IRMM) for the particle size analysis of the animal feed.

References

Gallo A, Masoero F, Bertuzzi T, Piva G, Pietri A. 2010. Effect of the inclusion of adsorbents on aflatoxin B_1 quantification in animal feedstuffs. Food Addit Contam A. 27:54-63.

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Masoero, F., Gallo, A., Diaz, D., Piva, G., Moschini, M., 2009. Effects of the procedure of inclusion of a sequestering agent in the total mixed ration on proportional aflatoxin M_1 excretion into milk of lactating dairy cows. Animal Feed Science and Technology 150: 34–45.

Phillips, T.D., 1999. Dietary clay in the chemoprevention of aflatoxin-induced disease. Toxicological Sciences 52 (Supplement): 118-126.

Annex

A copy of the report on particle size analysis of the feed material used for pelletizing

RM Unit Report of Analysis # 1988

SIEVE ANALYSIS

using Hosokawa Alpine Sieve Analyser 200LS-N (Registration number 96 00008)

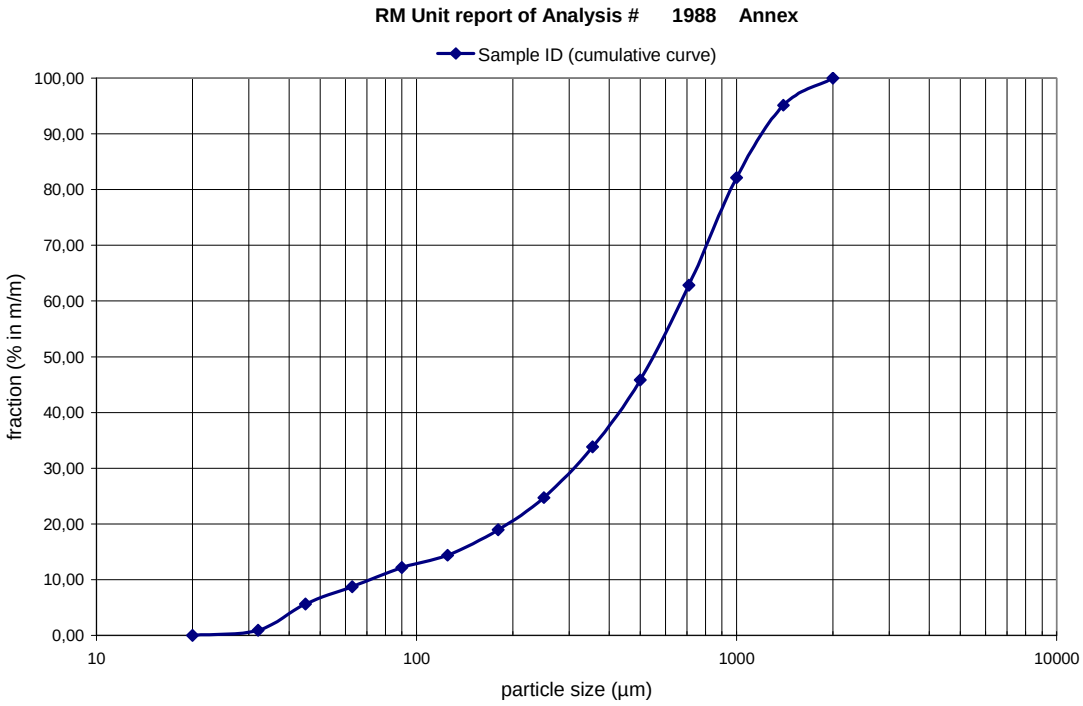
PRODUCT: **Pig feed**
Sample ID: **13411**

Operator: MF Tumba
Date: 21/09/2010
Balance used: (registration number) **9500528**

Powder weight: **31,925** **g**
Vacuum: 4500 Pa

SIEVE		WEIGHT		FRACTION in %			
in use (yes:1)	size in µm	Empty Sieve in g	Sieve after 2 mn sieving in g				
1	20	291,316	323,241	**0,00**	<	20	µm
1	32	292,456	324,089	**0,91**	<	32	µm
1	45	293,533	323,657	**5,64**	<	45	µm
1	63	286,053	315,183	**8,75**	<	63	µm
1	90	291,410	319,450	**12,17**	<	90	µm
1	125	294,677	322,008	**14,39**	<	125	µm
1	180	300,633	326,516	**18,93**	<	180	µm
1	250	316,760	340,795	**24,71**	<	250	µm
1	355	324,443	345,566	**33,84**	<	355	µm
1	500	338,286	355,576	**45,84**	<	500	µm
1	710	354,715	366,580	**62,83**	<	710	µm
1	1000	373,476	379,185	**82,12**	<	1000	µm
1	1400	386,624	388,182	**95,12**	<	1400	µm
1	2000	407,156	407,156	**100,00**	<	2000	µm

Note: No feedback within 4 weeks is seen as acceptance of the report. Potential rests of the samples will be destroyed after that period.


RM Unit report of Analysis # 1988 Annex
Sample ID (cumulative curve)
100,00
90,00
80,00
70,00
60,00
50,00
40,00
30,00
20,00
10,00
0,00
fraction (% in m/m)
10
100
1000
10000
particle size (µm)

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EUR 24955 EN – Joint Research Centre – Institute for Reference Materials and Measurements
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Luxembourg: Publications Office of the European Union
2011 – 16 pp. – 21.0 x 29.7 cm
EUR – Scientific and Technical Research series – ISSN 1831-9424 (online), 1018-5593 (print)

ISBN 978-92-79-21401-1 (PDF)
ISBN 978-92-79-21400-4 (print)

doi:10.2787/52525

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LA-NA-24955-EN-N