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Foodstuffs - Sample comminution for mycotoxins analysis - Comparison between dry milling and slurry mixing

Lebensmittel - Probenvorbereitung für die Mycotoxinanalytik - Vergleich zwischen Trockenvermahlung und Aufschlämmung

Produits alimentaires - Préparation d'échantillons gros volume pour l'analyse des mycotoxines - Comparaison entre broyage a sec et broyage par voie humide

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Lebensmittel - Probenvorbereitung für die Mycotoxinanalytik - Vergleich zwischen Trockenvermahlung und Aufschlämmung

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Foreword

This Technical Report (CEN/TR 15298:2006) has been prepared by Technical Committee CEN/TC 275 “Food analysis - Horizontal method”, the secretariat of which is held by DIN.

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Introduction

Since 1999-01-01, EC directives for aflatoxins entered into force, which consisted of sampling plans resulting in sample weights of up to 30 kg. This raised questions on how these relatively big samples could fulfil the requirement to “finely grind and mix thoroughly each laboratory sample using a process that has been demonstrated to achieve complete homogenisation” [1]. Since the analytical sample is taken out of this big sample, the critical step is to take a representative increment out of it. As such this topic has been subject of several studies in the past. Dickens and Satterwhite [2] developed a mill that could handle up to 25 kg peanut samples. They presented results of tests on 5 kg samples from which they withdrew 50 g sub-samples, but gave no data on larger samples. Velasco and Morris [3] considered use of a water slurry to obtain finer particles and a more uniform particle distribution. Another advantage of slurry preparation is the avoidance of clogging of samples that have high oil content. They presented experiments on different matrices with sample weights up to 4,5 kg, whereas they mentioned that slurry preparation is limited only by the capacity of the equipment. Whitaker et al. [4] considered a compromise. They prepared a slurry from a sample, which was first comminuted by another milling process. Due to the regulations of the USDA they limited themselves to an amount of only 1 100 g. Nevertheless this restriction in their method was developed into the alternative best foods method used for aflatoxin in peanuts [5]. Dorner and Cole [6] started all over again from the beginning: the 218 kg sample of raw, shelled peanuts for analysis in official USDA approved laboratories. They compared variability by grinding with four different mills, but only with sub-sample sizes up to 4 kg. So the question how the result would be on 21,8 kg samples remained unanswered. Their statistical data, especially CV values, on the 2 kg and 4 kg sub-samples were less favourable than the ones that can be achieved by applying the slurry method. Scholten and Spanjer [7] published data on slurry preparation for samples up to 10 kg, whereas the laboratory of Wiertz, Eggert and Jörissen had similar experiences, even when applying samples up to 30 kg. Data of the latter are compiled in this report. Worldwide however, sub-sampling mills are in favour because they are easy to apply and fast in comminuting samples into analytical portions. Calori-Domingues et al. [8] demonstrated this with a poster presentation at the Xth International IUPAC symposium on mycotoxins and phycotoxins in May 2000. They tested variability for aflatoxin analysis in peanuts associated with sample preparation by dry milling with a RAS mill. Unfortunately however they only investigated samples up to 5 kg.

So the labs of the Inspectorate for Health Protection, a delivery unit of the Dutch Food and non-food Authority, and of Wiertz, Eggert and Jörissen, a member of the Eurofins Scientific group, decided to perform new experiments with following goals: 1. what CV values are achieved when milling 10 kg samples, and 2. are correct aflatoxin values measured while doing so? The choice of matrices has been discussed at a CEN/TC 275/WG 5 (Comité Européen de Normalisation, Technical Committee 275, Working Group 5, Biotoxins) meeting, considering existing and upcoming legislation for different mycotoxins and food types. Combining both items lead to the conclusion that a lot of matrices, existing as dried, whole or ground raw material are to be considered. Also differences in sample weight, i.e. between nuts and spices, exist. Suggestions for representative commodities were:

— cereals, since for this staple food directives exist on as well as aflatoxins, as ochratoxin A and as DON;

— raisins, because these are included in directives for aflatoxins and ochratoxin A;

— paprika powder as an example of a ground commodity.

In practice however it turned out that the availability of naturally contaminated lots that could be used for these experiments was the limiting factor. The presented results show what exactly has been examined. After these experiments the detailed work of Schatzki and Toyofuku [9], who measured particle size distributions on pistachio slurries, became available. This lead to a joint presentation at the 2nd World Mycotoxin Forum, February 2003, in The Netherlands [10]. This report is a combined outline of both investigations.

1 Scope

A comparison was made between dry milling and slurry mixing as comminution step preceding mycotoxins analysis. Such in respect to EC legislation that consists of sample schemes up to 30 kg. Cacao, green coffee, almonds and pistachio samples of 10 kg were milled by a RAS mill and all three sub-samples were completely analysed for aflatoxin B_1 or Ochratoxin A. The differences in analytical results are explained by measurements of particle size distributions of both milling types. The obtained data are compared with literature data on coefficients of variation (CV) for various milling procedures. For dry milling CV values were generally not below 20 % for aflatoxin B_1 levels up to 38 µg/kg in peanuts, whereas slurry mixing could achieve CV values below 5 % at aflatoxin B_1 levels down to 4 µg/kg in pistachios. Measurements also showed possible difference in mycotoxin content of a sample between both milling types. This could lead to false positive or negative results when rejecting or accepting a lot, as this is based on the sample result. It was concluded that slurries contain smaller particles than dry milled samples and thus generate the lowest possible CV values which in turn leads to better sample homogenisation.

2 Test methods

2.1 Apparatus

2.1.1 Slurry mixer, Slurry mixer - Silverson type EX mixer ® [1)];

2.1.2 RAS mill, Romer Analytical Sampling mill ® [1)]

Other laboratory equipment and slurry preparation procedures as described before (see [7] and [9]). The RAS mill was applied according to the manual (Release 2, January 1998) of the supplier. Before the dry milling process the pistachio samples were frozen overnight at minus 20 °C.

2.2 Reagents and materials

Aflatoxin measurements were performed as described in EN 14123. Ochratoxin measurements were carried out in cacao and in green coffee beans as described in EN 14132, including quality control. The only difference is that fluorescence detection for ochratoxin A is carried out as published by Zimmerli and Dick [11].

2.3 Procedure

For each commodity, experiments were carried out by the following procedure:

1. sampling according to the EC directive, resulting in 10 kg sample;
2. milling the 10 kg sample by a Romer mill with a split ratio of 10 %;
3. taking a dry sample out of the 10 % part as usual for Romer mill users (sub-sample A);
4. slurry mixing of the remaining part of the 10 % part of the sample (sub-sample B);
5. slurry preparation of the 90 % part by Silverson mixing (sub-sample C);
6. analysing the three sub-samples A, B and C by HPLC methods.

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[1] Silverson type EX mixer is the trade name of a product supplied by Silverson Machines Ltd., Waterside, Chesham, Bucks, England. Romer Analytical Sampling (RAS) mill is the trade name of a product supplied by Coring-System Diagnostic GmbH, Robert-Bunsen-Straβe 4, D-64579 Gernsheim, Germany. This information is given for the convenience of the users of this Technical Report and does not constitute an endorsement by CEN of the product named. Equivalent products may be used if they can be shown to lead to the same results.

Doing so the complete mycotoxins content in the sample can be reconstructed afterwards by calculation.

3 Results and discussion

The results of all experimental values are given in the second, third and fourth column of Table 1. They consist of measurements of ochratoxin A in cacao and green coffee beans and of aflatoxins, of which only aflatoxin B_1 is useful for this purpose, in almonds, pistachios and a sample of mixed spices. All other columns in Table 1 are filled with figures that are calculated from these data. From the weight of each sub-sample and its mycotoxins content, it is possible to calculate what the mycotoxins content would have been in the total sample if it had been measured in one sample as a whole. This calculated value is presented in the column “sample value” in the first row, such as to facilitate several comparisons that will be made in the clause results and discussion. In the last three columns the mathematical mean of the A, B and C sub-sample results, the standard deviation and the coefficient of variation of these three measurements are given, which will be discussed later as well.

Considering the results have to be done from the starting point of the experiments: milling the 10 kg sample by a Romer mill, which creates a division of the original sample in two sub-samples of different weight. When RAS milling is used in daily routine analysis this step is followed by taking an incremental sample out of the smallest sub-sample for further clean up and chemical analysis. This situation is comparable with the results for sub-sample A in this experiment with the crucial difference that data as presented for sub-samples B and C are never measured in daily practice. In case of sample preparation by means of slurry, the whole sample is dealt with. A portion of the slurry is taken for further analysis. Regarding the methods in detail reveals that it will never be possible to do an experiment by applying both preparations towards one sample. Therefore the best estimate of a measurement of these samples, as if they were handled by preparing a slurry, can only be made by calculating the amount of mycotoxins from the individual A, B and C sub-sample values. This calculated value is presented as “sample value” in Table 1.

Table 1 — Results of sampling, milling and mixing experiments as described in 2.3

Ochratoxin A	Sample	Sub	Sub	Sub	A,B,C		
Matrix [a]	Value (µg/kg)	A (µg/kg)	B (µg/kg)	C (µg/kg)	Mean (µg/kg)	STD (µg/kg)	CV (%)
Cacao	0,4	0,5	0,4	0,4	0,4	0,1	13,3
Cacao	0,6	0,4	0,5	0,6	0,5	0,1	20,0
Cacao	1,0	0,9	1,7	0,9	1,2	0,5	39,6
Cacao	1,1	0,8	0,4	1,2	0,8	0,4	50,0
Cacao	1,2	1,5	0,7	1,2	1,1	0,4	35,7
Cacao	1,2	2,6	1,5	1,2	1,8	0,7	41,7
Cacao	1,7	1,1	3	1,6	1,9	1,0	51,8
Cacao	1,7	1,5	1,5	1,7	1,6	0,1	7,4
Cacao	2,2	0,8	2,1	2,2	1,7	0,8	45,9
Cacao	3,5	5,2	1,5	3,7	3,5	1,9	53,7
Cacao	11,9	1,3	1,8	13	5,4	6,6	123,3
Green coffee	1,5	8,1	0,4	1,6	3,4	4,2	123,2
Green coffee	1,9	1,8	2,3	1,8	2,0	0,3	13,4
Green coffee	2,0	2,7	2,6	2,0	2,4	0,4	16,1
Green coffee	2,0	1,5	2,0	2,0	1,8	0,3	14,4
Aflatoxin B_1	Sample	Sub	Sub	Sub	A,B,C		
Matrix	Value (µg/kg)	A (µg/kg)	B (µg/kg)	C (µg/kg)	Mean (µg/kg)	STD (µg/kg)	CV (%)
Almonds	2,0	1,0	0,2	2,2	1,1	1,0	88,8
Almonds	2,4	1,0	4,2	2,2	2,5	1,6	65,5
Almonds	3,1	0	0	3,4	1,1	2,0	173,2
Almonds	4,1	0,5	6,7	3,8	3,7	3,1	84,6
Mixed spices	7,8	4,2	8,1	7,75	6,7	2,2	32,6
Pistachio in shell	33,8	88,2	38	33	53,1	30,5	57,5
Pistachio in shell	44,1	51,4	42,4	44,2	46,0	4,8	10,4
Pistachio kernels	114,1	250	108	114	157,3	80,3	51,0
Pistachio kernels	126,0	204	122	126	150,7	46,2	30,7

[a] Measurements carried out according to the schedule as mentioned above, by Kastrup, WEJ Hamburg, Germany and Scholten, Inspectorate for Health Protection, Amsterdam, the Netherlands.

For the enforcement of a directive the analytical results are important at the point of accepting or rejecting a lot. Aflatoxin B_1 is regulated in EC directives: 2 µg/kg for nuts and 5 µg/kg for spices. For ochratoxin A only values from a working document [12] can be used: 2 µg/kg for cacao and 3 µg/kg for coffee beans. The latter values are under discussion and are only used in this report to evaluate the presented measurements. With these figures, without adding measurement uncertainties, the differences between judgements of a lot based on dry milling (sub-sample A data) are compared with the data that would have been obtained after slurry preparation

of the sample as a whole (sample value data). Doing so for cacao 2 out of 11 lots would be rejected after a dry milling procedure and 3 out of these 11 lots after slurry preparation. Only in 1 of these cases the lot would be rejected by both procedures. In 2 out of 3 cases the dry milling procedure would accept the lot that is rejected according to the sample value. In 1 case the dry milling procedure would reject a lot, which is accepted by the slurry preparation method. The latter happens also with 1 of the 4 coffee samples. The other 3 commodities are judged likewise for both methods. The results for the measurements on aflatoxin B_1 are worse. In 5 out of 9 cases the dry milling procedure would lead to acceptance of the lot, whereas the slurry preparation would reject 8 out of 9. A striking detail in this respect is the fact that in all 5 cases the dry milling leads to acceptance of a lot, this happens at low levels, i.e. around the limit of the directive. The aflatoxin levels in the pistachios are so high that the measurements lead to rejection in any case. If the overall results of Table 1 were considered the dry milling procedure would reject 7 out of 24 lots, whereas the preparation of slurry would reject 11 out of the same 24 lots. Table 2 gives an overview. It also reveals that dry milling lead to 2 false positive results, one with cacao and one with coffee beans. This is an interesting detail, since both commodities are rather expensive, so from this point of view even false positive results are not desirable.

Table 2 — Overview on rejection of lots and false positive and negative decisions

Mycotoxin		Ochratoxin A	Aflatoxin	Both
Sample	n =	15	9	24
Rejected by	Dry	3	4	7
	Slurry	3	8	11
	Both	1	4	5
False negative		2	4	6
False positive		2	0	2

Apart from compliance of samples to a directive, another point can be learned from Table 1. The data of sub-samples A, B and C reveal that the dry milling process resulted in different mycotoxins content in both samples in which the sample is divided by dry milling. Due to applying slurry preparation to sub-samples B and C, all 3 sub-samples have been analysed exactly. From Table 1 the differences can be seen easily by comparing the columns of A versus B and A versus C. The data on B and C are not available when dry milling is applied in daily routine analysis. For this investigation these values were measured to be able to reconstitute the “sample value”. But since these data are available it is also possible to calculate the standard deviation and the coefficient of variation of the dry milling process on these 10 kg samples. The results are given in the last 3 columns of Table 1. They show that CV values are only once less than 10 % and can be more than 50 %, whether the mycotoxins level is low (< 10 µg/kg) or high (> 100 µg/kg). In this particular case it has to be kept in mind that on a mathematical-statistical basis these values are not of very much significant value. They are based on only three data per calculation and these values are on their turn originating from three different types of processing. So the CV data that are added to Table 1 are just given because they are the only way to express some CV level for the experiments that are performed.

These CV values can be compared with several sets from literature on milling experiments. To be accurate, like in the cited references, these CV values are composed of sub-sampling and analytical variance [13]. If we assume the analytical error to be far less than any other error in any mycotoxins study, we can focus on the published CV’s as being caused by sub-sampling. In all cases we neglect the variance of sampling error. We only focus on the sub-sampling error in all studies. In chronological order we start with the results obtained with the sub-sampling mill of Dickens and Satterwhite for peanut kernels [2]. Their data are given in Table 3. They show that CV varies from 9 % to 43 % in 5 kg samples with aflatoxin B_1 content of 15 µg/kg to 233 µg/kg. It is remarkable that CV is not decreasing when the aflatoxins content increases, as should be expected. Their data on 500 g samples show that 11 out of 18 have CV values below 10 %, also without correlation between CV and aflatoxin B_1 content.

Table 3 — Dickens mill testing 50 g out of 5 kg (n=4) and 25 g out of 500 g (n=2) peanuts

Sub 1 (µg/kg)	**Sub 2** (µg/kg)	**Sub 3** (µg/kg)	**Sub 4** (µg/kg)	**Mean** (µg/kg)	**STD** (µg/kg)	**CV** (%)	**Sub 1** (µg/kg)	**Sub 2** (µg/kg)	**Mean** (µg/kg)	**STD** (µg/kg)	**CV** (%)
14	14	17	14	14,8	1,5	10,2	7,2	7,4	7,3	0,1	1,9
17	17	17	14	16,3	1,5	9,2	7,4	7,3	7,4	0,1	1,0
51	40	51	40	45,5	6,4	14,0	15,9	24,3	20,1	5,9	29,6
57	40	40	51	47,0	8,4	18,0	18,2	18,2	18,2	0,0	0,0
57	69	127	90	85,8	30,7	35,8	21,7	29,6	25,7	5,6	21,8
70	63	63	113	77,3	24,1	31,1	22,6	17	19,8	4,0	20,0
257	114	171	129	167,8	64,2	38,3	22,8	24,8	23,8	1,4	5,9
257	257	171	103	197,0	74,6	37,9	23,7	15,7	19,7	5,7	28,7
257	257	228	343	271,3	49,7	18,3	33,8	35,4	34,6	1,1	3,3
343	257	228	103	232,8	99,3	42,7	37,3	40,1	38,7	2,0	5,1
							39,2	39,2	39,2	0,0	0,0
							44,9	49,2	47,1	3,0	6,5
							57,9	44,5	51,2	9,5	18,5
							58,1	89	73,6	21,8	29,7
							64,8	65,9	65,4	0,8	1,2
							75,3	31,9	53,6	30,7	57,3
							89	90	89,5	0,7	0,8
							126	136,1	130,9	7,4	5,7

In 1976 Velasco and Morris presented the application of water slurries for aflatoxins analysis [3]. As can be seen in Table 4, their results show lower CV values for all matrices. For peanuts they also demonstrate that CV is lower when aflatoxin B_1 content is higher. They conclude that the use of water slurry reduces the variability because distribution of particles is more uniformly achieved with slurry than with dry ground product. This conclusion is confirmed by Whitaker et al. [4], who determined the particle distribution by measuring the percentage of material that passed different sieves. Their second conclusion is that seeds of high oil content are readily reduced to a fine particle size, whereas only a coarse grind is possible with conventional mills because of clogging. Their last conclusion was that the quantity of water slurry that can be prepared is limited only by the capacity of the available blending or homogenizing equipment. Unfortunately they did not prove the latter conclusion by any experiment. Their results as presented in Table 4 were achieved with 1 kg samples.

Table 4 — Comparison between slurry preparation and dry milling of several matrices (n=5)

Milling type	Mean (µg/kg)		STD (µg/kg)		CV (%)	
Matrix	Slurry	Dry	Slurry	Dry	Slurry	Dry
Corn	49,8	49,6	1,3	3,8	2,6	7,6
Cottonseed	66,4	65,2	3	9,6	4,5	14,8
Cottonseed meal	75,3	71,9	3,4	4,1	4,5	5,7
Peanuts (n=8)	13,2		1,03		7,8	
Peanuts	48	40,9	2,5	8,5	5,2	20,8
Peanut butter	51,6	51,9	1,5	2,8	2,8	5,4
Peanut meal	63,6	52,6	2,8	5,5	4,4	10,5
Copra	49,8	53,4	2,2	4	4,4	7,5