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Sampling for mycotoxins in feed—heterogeneity characterisation

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The presence of mycotoxins, in particular aflatoxin B1, can cause significant health problems as well as severe societal economic losses, and is, therefore, regulated with respect to maximum acceptable concentration in various feed- and foodstuffs. International regulatory authorities have begun to recognise the importance of representative sampling, but sampling guidelines are only partly in compliance with the Theory of Sampling. In particular, practical guidance regarding sampling, including correct design and operation of sampling devices, including explanations on how to develop sufficient sampling protocols are lacking in current guidelines. These are critical practicalities of major importance, especially when dealing with trace concentrations and/or concentrations that are irregularly distributed—as is the case for mycotoxins. Furthermore, heterogeneity characterisation, which is a necessary requirement to be able to develop valid sampling protocols or validation assessments of existing sampling operations, is currently not mentioned in the existing guidelines. The present paper focuses on heterogeneity characterisation with respect to sampling of mycotoxins for 1-D and 3-D feed lots (a full analysis of all critical practicalities in sampling mycotoxins is published elsewhere). Structural guidelines for correctly designing experimental heterogeneity characterisations are presented, allowing evaluation of sampling representativeness and determination of optimal number of increments per composite sample.

Background

Mycotoxins are toxic secondary metabolites of moulds, which can occur during plant growth and during storage and processing. Among various mycotoxin types, aflatoxins are of major concern due to their potential impact on human and animal health. The food and feed industry has set a special focus on aflatoxin B1, which occurs most frequently and is the most toxic aflatoxin, since it has been directly correlated with adverse health affects.[1] Mycotoxins can occur within a concentration range of $\mu g\,kg^{-1}$ to $mg\,kg^{-1}$. The Food and Agriculture Organization of the United Nations (FAO) has estimated that approximately 25 % of the world's agricultural production is contaminated with mycotoxins, resulting in significant economic loss due to their impact on human health, trade and animal productivity.[2] Due to the fact that the presence of mycotoxins in food- and feedstuffs cannot be avoided, valid testing is demanded and, therefore, sampling methods for raw and processed materials are a critical necessity. The US Department of Agriculture (USDA) and its Grain Inspection, Packers & Stockyards Administration (GIPSA) has estimated that non-representative sampling accounts for nearly 90 % of the error associated with aflatoxin detection,[3] mainly due to non-random spatial distribution throughout materials when occurring in the trace concentration range ($mg\,kg^{-1}$ or $\mu g\,kg^{-1}$).

Below, critical practicalities with a focus on the heterogeneity characterisation required for developing sampling protocols for determining mycotoxins in feed (equally applicable to food) are presented. Results are substantiated with data from field trials. The real-world data used here have been redacted and serve specifically to strengthen the general arguments and not to represent specific results of the studied field trials, which are proprietary.

Critical sampling practicalities

The reason for all sampling errors is *lot heterogeneity*, causing material to vary irregularly throughout the lot on spatial but also on compositional dimensions and scales. Increasing the number of correctly extracted increments in a composite sample is the most effective way to decrease primary sampling errors, and will lead to results that are closer to the true lot value. The difficultly is to determine the "optimal number of increments", since this depends on heterogeneity, the analyte concentration level, and the size and lot geometry. In practice, sampling is often a compromise between the desired levels of accuracy/precision and labour/cost deemed necessary. The only criterion that must never be up for negotiation is representativity, which needs to be based on sampling correctness. In particular when dealing with trace concentrations or highly heterogeneous distributions, as is the case for mycotoxins, the sampling variance is by far the dominating source of uncertainty, due to the characteristically skewed, polymodal, highly irregular "distribution" of these analytes.[4,5]

Below, tools for determining optimal number of increments and minimising errors at each sampling and mass reduction step are presented. Examples are based on a real-world field trial performed on various materials used as animal feedstuff for determining aflatoxin B1 levels within each feed component, as well as within the total feed mixture (also termed "total mixed ration", TMR).

Sampling stages

In the present field trials, all total mixed ration components are stored in piles and could only be sampled once unloaded (3-D sampling situation). The feed components are mixed in a predetermined ratio to form the total mixed ration (TMR), which is spread out in elongated feed bunks (1-D sampling situation). For each feed component, as well as the TMR, an individual sampling strategy determining the optimal number of increments has been developed, based on preceding material heterogeneity characterisations. All individual feed components have been analysed for aflatoxin B1 including pre-set control variables (protein, fibre and moisture). Samples collected from the feed mixture (TMR) have also been analysed for the same analytes, allowing a comparison of the TMR results with the analytical results of the individual TMR components.

To develop an appropriate aflatoxin sampling plan, the following steps have been undertaken:

- Assessment of optimal sampling location (preferentially sampling in a 1-D sampling situation)
- Selection of appropriate sampling devices and mass reduction procedures for each material and lot type
- Design of experiments for characterising material heterogeneity
- Determination of optimal sampling frequency based on empirical experimental outcomes

As stated above, only the total mixed ration can be considered as a 1-D sampling situation, while all individual feed components are piled up in 3-D lots, which were regrettably not able to be sampled during unloading.

Figure 1. TMR "sampling box" covering entire depth and width of target material, which is spread out in the longitudinal (horizontal) direction.

Primary sampling

Before presenting the experimental design for the required material heterogeneity characterisations, the sampling tools used for the elongated TMR, the individual TMR components, as well as applied mass reduction procedures are presented.

The total mixed ration is pre-mixed and spread out in elongated feed bunks. Such a sampling situation (one-dimensional lot) allows extraction of increments covering the entire depth and width of the material, while a fully comprehensive spatial distribution of the increments is covered in the longitudinal direction of the lot (distance in-between increments as well as total number of increment is based on experimental design). In order to correctly delineate and extract the increments, a "sampling box" has been designed, suitable for the relevant lot dimensions and material characteristics, as depicted in Figure 1.

For individual TMR components (three-dimensional lots), the use of sampling spears is claimed to allow the best accessibility for all lot dimensions. Various types of sampling spears exist in the market; but they are seldom designed in compliance with the Theory of Sampling (TOS). The most important aspects with respect to sampling spear design are its length, width, aperture positions and opening width, as well as the closing mechanism. In the optimal case the length of the sampling spear should cover the entire depth of the lot, which allows insertion of the sampling spear vertically at every position within the lot (as indicated by the arrows in Figure 2, left side). However, due to the fact that some of the TMR component piles exceeded the maximum available length of sampling spears, positioning and inserting direction were carefully considered. On the right-hand side of Figure 2, a pile is depicted that exceeds the length of the sampling spear. In order to cover all lot dimensions, i.e. also the lower and bottom parts of the lot at its highest level (row 3), the sampling spear was inserted horizontally in row 2 at the lowest accessible inserting point. It is emphasised that this spear sampling procedure is a result of a compromise based on the actual situation

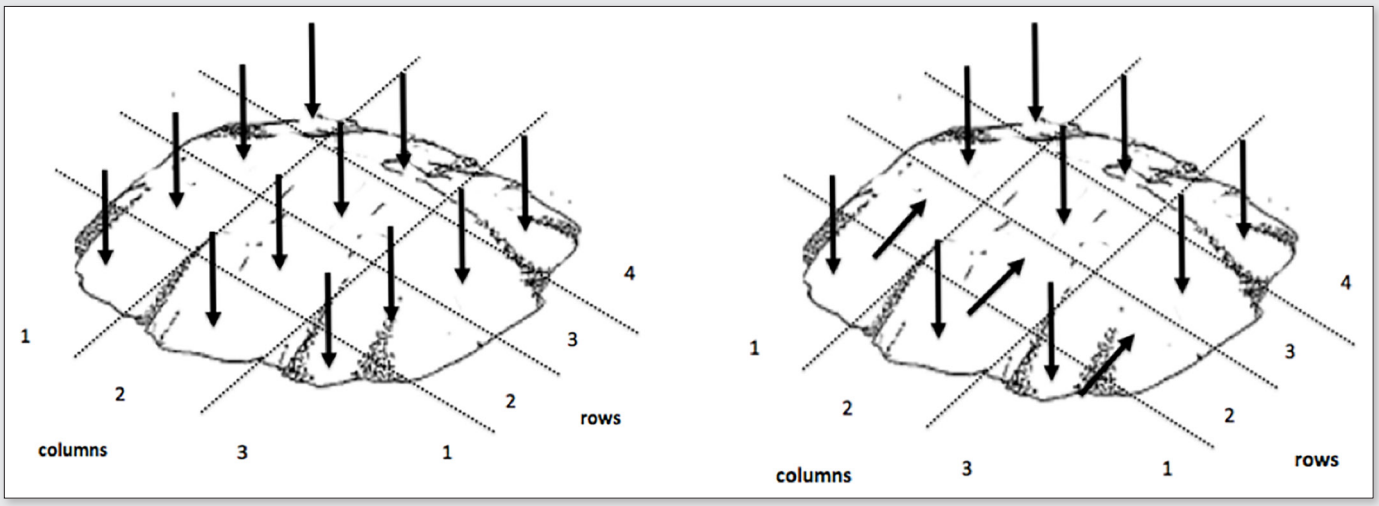

Figure 2. Illustrating stratified composite sampling of non-equal height 3-D storage piles. Sampling spear length versus pile height—spear inserting directions.

that the individual TMR components could not be sampled during unloading (1-D sampling situation). Muzzio *et al.* have published a particularly illuminating exposé of the deficiencies in spear sampling for powders and granular mixtures.[6]

Mass reduction

Correct mass reduction procedures need to be applied or sampling errors will adversely impact the secondary, tertiary etc. sampling stages and inflate the total measurement uncertainty.[7] Petersen *et al.* have performed an extensive study of various available mass reduction procedures and have rated them according to their representativeness, with the conclusion that only riffle splitters and rotational splitters allow correct mass reduction.[8] For the majority of the TMR components, riffle splitters with appropriate chute opening widths have been used, while for some fibrous, very light and wet materials the primary samples have been mass reduced using a circular cutting device, dividing the primary sample in eight equal sectorial cuts (increments). Four of the eight cuts have been used in the secondary sampling stage, while the other four cuts were discarded. All primary samples were mass reduced and further processed in the laboratory, including comminution and mass reduction to analytical sample size. Also in the final analytical mass reduction stage riffle splitters and bed-blending technique have been used to avoid sampling errors, especially important since dealing with a trace concentration range of aflatoxins, *ibid*.

Design of experiments for characterising material heterogeneity

Following the proposed outline for developing an appropriate aflatoxin sampling protocol, the steps are (1) assessment and decision on optimal sampling location (3D vs 1D), (2) selection of appropriate sampling devices and mass reduction procedures, (3) the design of experiments for material heterogeneity characterisation in order to determine (4) the optimal sampling frequency for each material.

Depending on the lot type, the sampling variance associated with the final sampling protocol and the heterogeneity distribution of the targeted analyte (e.g. aflatoxin B1) can be quantified using two different procedures: the replication experiment (stationary 3-D decision units) and variographic analysis (dynamic or stationary 1-D decision units). These assessment methods can also be applied to incorrect sampling procedures, for which the result would reflect the material heterogeneity plus the significantly inflated sampling errors. For the present field trials, sampling errors have been minimised by selection of appropriate increment sampling location and procedures allowing the sampling variability of the heterogeneity of the target analyte in the lot to be characterised; based on that the optimal number of increments for the final composite sample has been determined.

The replication experiment was applied to all TMR components (3-D sampling situations), while a variographic experiment was applied to the sampling variance for the TMR in a 1-D sampling situation.

For the replication experiments, ten primary samples were collected from each TMR component, each time repeating the full lot-to-test portion sampling pathway in completely identical fashion, DS 3077 (2013). Each primary sample consists of 30–40 increments depending on the lot dimensions. The minimum requirement is that the entire spatial geometry of the target material is fully covered by the sampling tool and the selected number of increments. It is important that all sampling operations, particularly at the primary sampling stage, are fully realistic during the replication experiment, meaning for example that the replicates should not be extracted at the exact same locations. In the described experimental field trial, different sampling operators collected the replicate primary samples in order to reflect all possible variation also that caused by individual differences regarding operating the sampling and mass reduction devices. For each replication experiment, the "relative sampling variation (RSV)" and the statistical relative "coefficient of variation ($CV_{\%}$)" were calculated, giving a measure of the specific heterogeneity of the target material (e.g. aflatoxin distribution), as expressed by the specific sampling procedure applied.

Heterogeneity characterisation of the TMR is based on a variographic experiment, for which 60 equally spaced increments have been extracted from the feeding lane using the described TMR sampling procedure. The main objective of the variographic experiment is similar to the replication experiment, meaning to determine the RSV (here called $RSV_{1\text{-}dim}$). Additionally, the influence of different sampling rates (i.e. distance between extracted increments) has been evaluated, allowing determination of the optimal sampling frequency or the optimal sampling interval.

Results and discussion of heterogeneity characterisations

The following section explains how results gained from heterogeneity characterisation experiments have been interpreted to correctly determine aflatoxin levels in feed. The results have been redacted, rather serving to explain general features and interpretation possibilities than to present the actual values of the studied field trial, which are proprietary.

Results of individual TMR components

In addition to aflatoxin B1, all materials were also analysed for protein, dietary fibre and moisture content, which serve as control variables to evaluate the applied sampling methods. For TMR components containing no detectable aflatoxin, protein, dietary fibre and moisture are used as control variables to determine required sampling frequency for reflecting inherent material heterogeneity.

The replication experiments used for characterising 3-D lots also allow comparison of the sampling variances originating at different sampling stages (i.e. primary sampling, secondary sampling, tertiary sampling etc.). Figure 3 shows a result of the sampling variances in the different sampling stages for one of the TMR components, protein content. For nearly all materials and analytes in the study, similar results established the primary sampling variance as completely dominating over the secondary and tertiary sampling variance. This also confirmed the correctness of the mass reduction procedures used.

In contrast to Figure 3, Figure 4 shows the sampling variance of dietary fibre for a different TMR component (proprietary), revealing that the sampling variance decreases from primary to secondary sampling stage, but actually increases in magnitude in the tertiary sampling stage. This latter is a clear indication that an incorrect sampling procedure was used at this stage. This example demonstrates how a replication experiment allows detection of "hidden" sampling errors. In this particular case, it was discovered that grab samples were extracted to gain the final test portion (despite the predesigned, correct mass reduction steps),

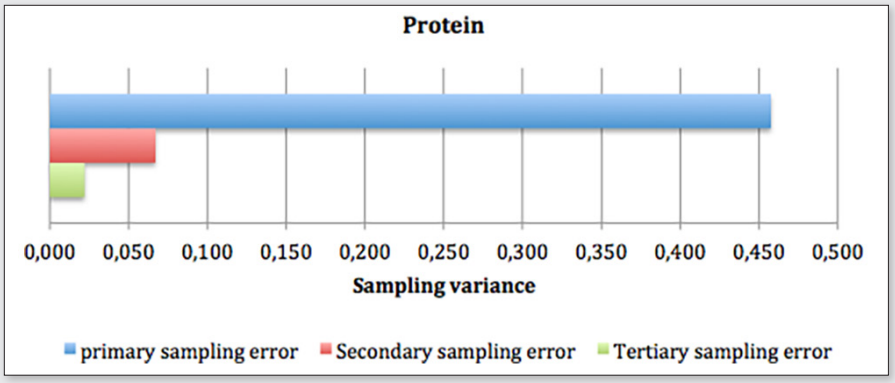

Figure 3. Typical example of comparison of sampling variances from different sampling stages. Dominance of primary sampling variance over secondary and tertiary sampling variance is the typical case.

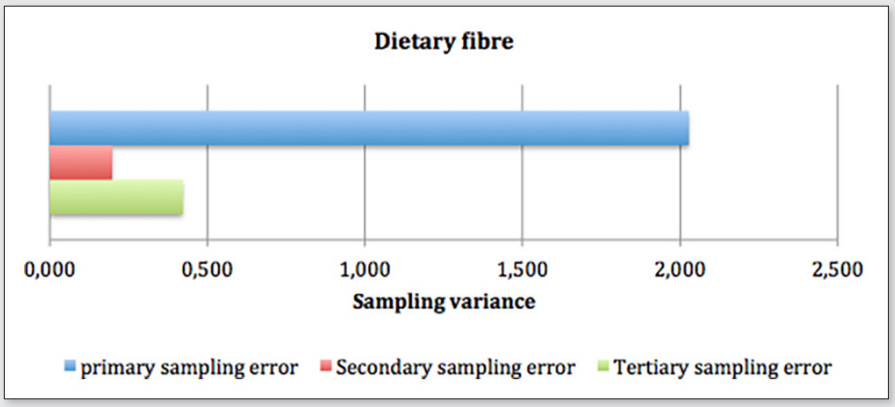

Figure 4. Typical example of comparison of sampling variances from different sampling stages, revealing an incorrect mass reduction procedure in the tertiary sampling stage (see text for details).

disobeying the TOS' principles of sampling correctness. After correction of this incorrect procedure (replacement by a bed-blending technique), the sampling variance of the tertiary sampling stage decreased to a level below the secondary sampling variance, confirming reduction, or elimination of the incorrect mass reduction procedure.

The replication experiments of the field trial have also been used to quantify the heterogeneity of each TMR component, in particular with respect to the aflatoxin concentration. For all TMR components containing aflatoxin, the pertinent distributions are significantly skewed to the right; a characteristic of aflatoxin which has also been confirmed by various other studies.[9,10] The relative sampling variation (RSV) confirms this observation, ranging from around 50 % to above 300 % for the analysed materials. Since sampling errors have been minimised by means of the experimental design, the determined RSV values measure the total empirical sampling variance influenced by the aflatoxin heterogeneity of the target material. The RSV values for the control variables for all TMR components ranges between 2 % and 15 %, confirming that the comparatively high RSV values for materials containing aflatoxin is dominantly caused by the irregular, non-normal distribution of aflatoxin, rather than by incorrect sampling procedures. In order to lower the sampling variance for aflatoxin (if required by quality specifications), the number of increments per composite sample would need to be increased.

Results of TMR mixture

The total mixed ration (mixture of all individual feed components) is the last point at which aflatoxins can be detected before being fed to the animals and potentially causing dangerous health effects. The high RSV values determined for the various TMR components with respect to aflatoxin B1 indicate that despite elimination of potential incorrect sampling errors, the overall uncertainty on aflatoxin concentration is still uncomfortably high. For the field trials, a specific uncertainty level on aflatoxin level in the TMR was pre-set, requiring that the sampling method and sampling frequency guarantee this uncertainty level. A variographic analysis also allows determining the influence of different sampling rates on the overall uncertainty, which has also been assessed for the present field trial.

Figure 5 shows the variographic results of the control variables for the TMR, comparing the number of increments used for final composite sample with the corresponding relative uncertainty incurred. The exact numerical values of the corresponding uncertainty are again not shown here due to confidentiality reasons.

Adding the variographic results for aflatoxin B1 to the same graph (see Figure 6), it is obvious that the corresponding uncertainty for aflatoxin is dramatically higher (~10× higher) compared to the control analytes, as also concluded from the assessment of the RSV values of the individual TMR components. The steepest decrease of uncertainty can be observed increasing the number of increments from one to two and from six to ten for the final composite sample. For this field trial the pre-set acceptable uncertainty level has been reached combining 10 increments to a final composite sample. In case a lower uncertainty level is required in the future, the appropriate number of increments can be selected directly from these variographic results, allowing full detection and uncertainty control of the aflatoxin concentration present in the TMR.

Conclusions

Critical practicalities in feed sampling for mycotoxins have been presented, which are currently not considered in the relevant sampling guidelines. The main problem for detection of mycotoxins, and especially aflatoxin in feed, is their decidedly irregular, non-normal distribution in the target feed/food materials. "Hot spot" characteristics and low trace concentration ranges and distributions make representative sampling critical for valid mycotoxins concentration control. Assessment of optimal sampling locations as well as selection of the appropriate sampling and mass reduction devices forms the basis for representative sampling. A primary consideration is to determine the optimal number of increments, since practical sampling is a trade-off between labour/economic efforts and sample quality. When the empirical effect from increasing the number of increments is known, an

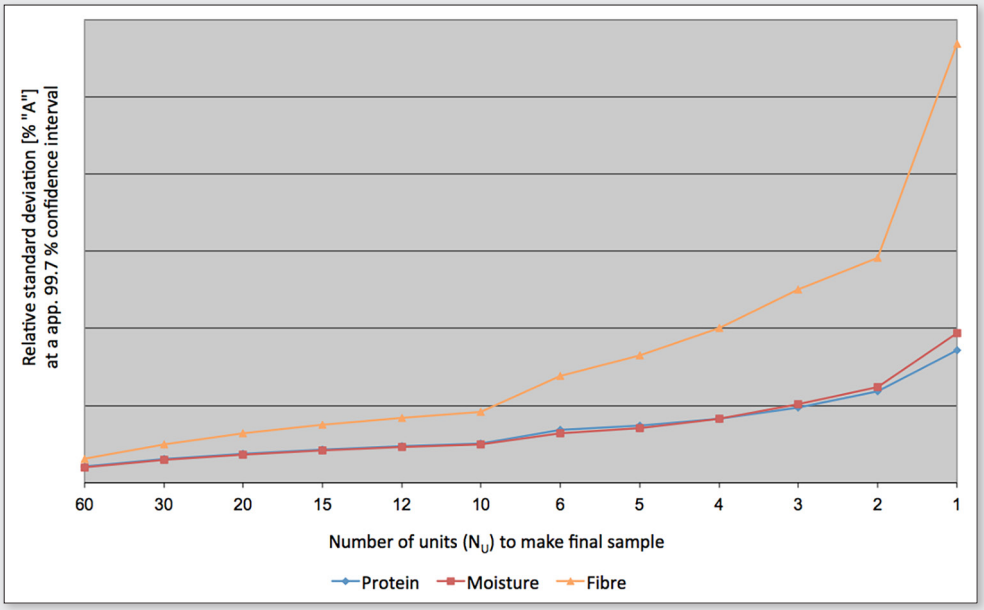

Figure 5. Variographic result for TMR (excluding aflatoxin) for a varying number of increments in a composite sample and the corresponding rel. total sampling-plus-analysis uncertainty. Values of the y-axis have been removed due to confidentiality reasons without any loss of generality. Results are calculated for a systematic sampling mode.

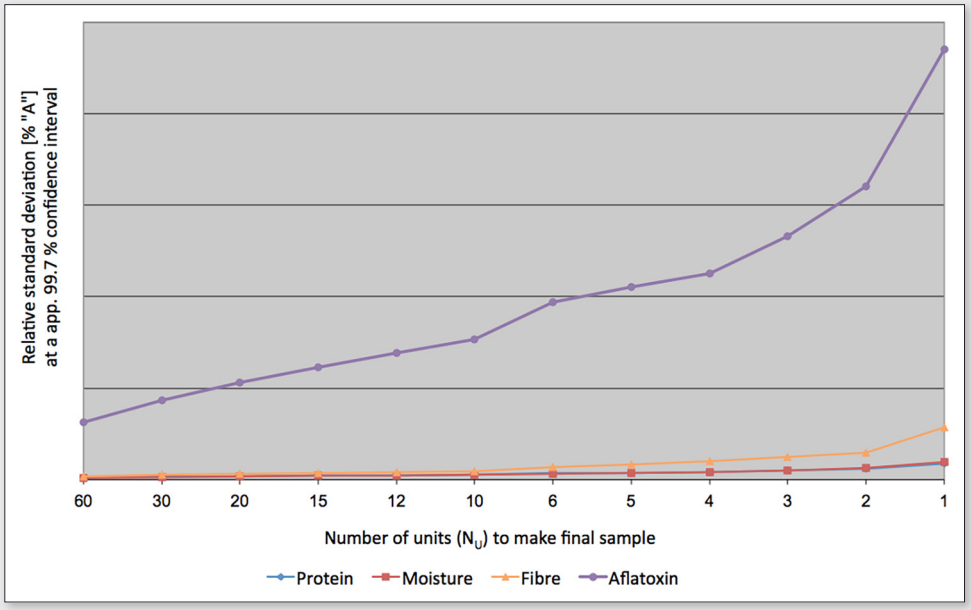

Figure 6. Variographic result for TMR (including aflatoxin) for a number of increments in a composite sample and their corresponding rel. uncertainty. Values of the y-axis have been removed due to confidentiality without any loss of generality. Results are calculated for systematic sampling mode.

educated decision can be made. Replication experiments for 3-D decision units and variographic analysis for 1-D decision units serve as a basis for the mandatory initial material heterogeneity characterisation; and can be used to derive an optimal number of increments. Examples of an industrial field trial were presented including heterogeneity characterisations for various total mixed ration components, as well as for mixed feed itself. Interpretation guidelines were given on how to assess applied sampling methods on the basis of these experimental designs and how to determine an optimal increment number and location. It was highlighted how variography can be used to compare various sampling strategies based on their corresponding total uncertainty levels. The developed criteria regarding sampling practicalities can be transferred to many other feed- and foodstuffs and other commodities with similar characteristics regarding trace concentrations or concentrations which are irregularly distributed throughout the target material.

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