IAG section Feed Microscopy

Newsletter 2018

Dear colleagues and relations,

The availability of food is a basal right to every human being. Food safety is prerequisite for effectuation of this right, and in the course of the food production chain safe feed is indispensable. Safety of feed and food products covers a range of issues. A set of four basic safety areas include *a)* hazards in the area of biology, e.g. prions, allergens and unintentional presence of plant toxins and mycotoxins, *b)* in the area of chemical compounds without a direct biological source, e.g. pesticides, antibiotics, growth promotors, heavy metals and process contaminants (e.g. dioxins), *c)* in the area of microbiology such as zoonoses and pathogens, and *d)* in the area of physics, e.g. microparticles and packaging material. These elements of what can be indicated as the BCMP safety cocktail demands for a variety of different monitoring methods. Visible light methods and microscopy can be applied in several of these areas, and might in some cases be the only opportunity.

This Newsletter of IAG section Feed Microscopy provides interesting presentations with an array of topics. The editorial reflects on physical hazards, most notably microplastic particles. Furthermore attention will be given to monitoring for the prevention of prion-based hazards (animal by-products in feed) and the analysis of composition of feed. A specific part of that composition, the mineral fraction, can express itself in a remarkable way. The presence of seeds of a plant species producing saponins and triterpenes, known for their toxic effect, is reported in whole kernel cereals. Together with the detection of *Ambrosia* seeds in bird feed, and the analysis of composition in general serves the need of traceability and label control in the feed production chain. In one contribution the combination of visual inspection and Genetically Modified Organism detection is presented. This example shows moreover that multidisciplinary research is an attractive approach in a range of cases.

The board of IAG section Feed Microscopy wishes you a pleasant time reading this Newsletter.

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Contents

Presidents address

Dear colleagues,

It is with great pleasure that I take the opportunity to address this “End of Year Greeting Letter 2018” to you, and for the first time I will do this only in the frame of the Newsletter. This allows me to skip the detailed summary of our Annual Conference and of the Ring Tests, as other members of the board will summarize them.

In my point of view, the following subjects impacted the year 2018:

- Meat and Bone Meal in fish feed is established and its control as well: PCR is performed for the detection of ruminant DNA, and often the microscopy lab “does not see” the samples any more. We have to get used to it.
- Insect meal in feed becomes more concrete: the control methods are being developed and will involve microscopy. It is an interesting topic, but it includes some questions about the increase of work (double sedimentation yes or no?).
- EURL-AP Revision of the Annex VI, EU/152/2009, light microscopy, was communicated to the NRLs on 30 November 2018, and a technical and scientific review shall be realized by experts (NRLs), for a closure by the end of February 2019. The main changes proposed are: 1) “land animal” particles will be modified into “terrestrial vertebrate” to exclude insects; and 2) the new flowchart includes two determinations for all positive samples regardless of the level of contamination. The detection of insects is currently not included, and the threshold of 5 particles remains. The IAG board may communicate comments as a consultative body.
- Purity, composition, detection of undesired substances are persisting subjects involving microscopy.

Coming back to the activity of our IAG group, I have the pleasure to point out the following aspects:

- Our IAG meeting was a great success; it was very well organized and well attended (> 40 participants) and included 16 scientific presentations, good discussions and memorable excursions.
- Several participants described an increase in activity, introduced new and motivated colleagues, and related the renewing and improvement of their microscopy equipment.
- Numerous problems of purity of raw materials have been disclosed (including fraudulent adjunctions); contamination with packaging materials is less and less negligible and will induce an increase of microscopy analyses.
- IAG ring tests show their importance, -we are very lucky to have RIKILT being able to organize them.
- Our strength is to bring together top specialists of different types of institutes and entities (official and private labs, as well as feed producers), and our strength is the flexibility to do what the majority of the members desires.
- IAG members wish to organize the Annual meetings and are aware of the importance of it.

Our weaknesses may be our informal constitution, the difficulty to raise money for our (small) needs (basically the Website), and the little time we can invest in our tasks and matters.

Hoping that you will read this newsletter with interest and further contribute to the life of our association, I wish you all to enjoy a nice time in coming winter days and a very good start in 2019.

Yours sincerely

Geneviève Frick

Editorial: microplastic

Human activities lead to a lot of overproduction, to by-products and to waste flows. Initiatives are being taken to reduce the volume of products from the food production chain. In 2015, the European Commission launched a comprehensive plan for a Circular Economy. A strategy was presented in 2018 by the EC for reducing plastic in the context of a circular economy. A reuse as valuable as possible of these products implies that food-grade goods which are not sold within date limits such as “best before ...” or “use before ...” has to enter alternative routes for processing. In the vast majority of situation these goods are packed (HACCP rules) and unpacking is a prerequisite for further use. The consequence is that plastic and other particles will enter the feed and food production chain. Another source is the degradation of large plastic items such as bags which finally enter the ocean and which will degrade gradually to smaller particles.

Microplastic is defined as plastic particles in the size range of 1 µm to 5 mm. Findings are reported in beach sand, sea salt, fish, tap water and flying insects. Recently, research of human stool revealed the presence of 20 particles of microplastic on average in 10 grams of material originating from 8 persons from 8 countries (Medical University of Vienna, press release 23 October 2018). These reports indicate that microplastic actually entered the feed and food production chain.

The methods to detect microplastic differ among the type of matrix. Since microplastic particles are relatively inactive in a chemical sense, and in view of absence of DNA markers, visual methods are the primary choices for monitoring. Technical opportunities include ultraviolet fluorescence microscopy, differential staining and specific preparations of materials for separation, e.g. hydrolysis, centrifuging or sedimentation. The application of visual light microscopy, or microscopic techniques based on other wavelength ranges offer fast and relatively cheap opportunities for the detection of microplastic particles. In general, contaminations in the feed and food chain with materials over 1 µm in size are likely to be subjected to approaches with microscopy or visual inspection.

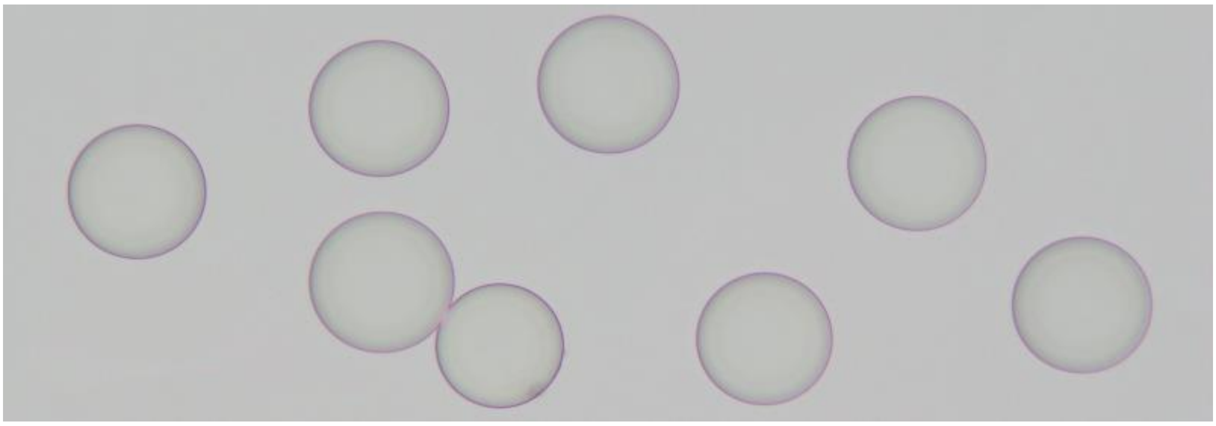
https://www.researchgate.net/publication/328702413_ASSESSMENT_OF_MICROPLASTIC_CONCENTRATIONS_IN_HUMAN_STOOL_-_FINAL_RESULTS_OF_A_PROSPECTIVE_STUDY

https://www.theguardian.com/environment/2017/sep/06/plastic-fibres-found-tap-water-around-world-study-reveals

https://orbmedia.org/sites/default/files/FinalBottledWaterReport.pdf

https://phys.org/news/2018-09-microplastics-deep-sand-turtles.html

https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:52018XC0416(01)&from=DE



Particles of Poly methyl methacrylate (PMMA) with a diameter of 100 µm.

Image: B. Hedemann, RIKILT, Wageningen.

The annual conference of IAG section Feed Microscopy 2018

Jeroen Vancutsem, FAVV, Belgium.

The 2018 annual conference of the IAG section Feed Microscopy from June 5-7 took place in Hamburg and was organized by SGS. There were 42 participants coming from 25 different organisations and from 9 different countries.

Summary of the main topics:

OPENING SPEECH AND A SUMMARY OF THE LAST YEAR (G. Frick)

INTRODUCTION OF SGS (R. Hörner)

Round table of presentations WHAT HAPPENED IN THE LABS?

During the round table the participants share their experiences and issues of the past year.

ON VALIDATION OF MICROSCOPIC METHODS (R. Krull-Wöhrman)

Some of the RASFF notifications on *Ambrosia* are reported with measurement uncertainty, some without. According to ISO 17025:2017 laboratories need to determine a measurement uncertainty but at the other side EU Regulation 152/2009 mentions that a measurement uncertainty is not possible in case of microscopic analysis. Probably a measurement uncertainty can be calculated for microscopic analyses, e.g. based on the results of interlaboratory studies with $U = 2 \times s_R$.

Also the problem of the definition of 'trace amounts not quantitatively determinable' was discussed.

ADULTERATIONS OF RAW MATERIAL FOR FEED PRODUCTION This year's examples (P. Czajkowski)

Several examples of adulteration of raw materials for feed production were given. As a conclusion it can be stated that microscopy is still important for these cases.

UNDESIRABLE SEEDS ACCORDING TO DIR 2002/32/EC ANN.I SECT.VI.1 (R. Krull-Wöhrman)

The need was raised for a definitive list of undesirable seeds. In EU Directive 2002/32 a maximum content of 3000 mg/kg of weed seeds and unground and uncrushed fruits containing alkaloids, glucosides or other toxic substances is set, but without mentioning plant species. A SCAN-opinion document from 2003 contains some undesirable plant species but is incomplete.

VLOG-INTER LABORATORY TEST FOR DETERMINING THE SOY MASS IN FEED: RESULTS & SUMMARY (G. Russ)

In 2017 VLOG organised a BIPEA coordinated ring trial for the determination of the mass of soy in the framework of botanical impurities for GMOs. Sixteen laboratories participated with PCR, ELISA and microscopy. As a conclusion it can be stated that quantification of soy is possible for soybean contents < 2%.

FOLLOW-UP ON GMO BRASSICA IN BIRD FEED (G. Frick)

A Swiss program was set up around transgenic *Brassica* and *Ambrosia* contamination in bird feed. The presence of GM rapeseed was confirmed and also some samples contained more than 50 mg/kg of *Ambrosia*, the maximum content for bird feed.

SUMMARY OF THE EURL-AP WS 2018 (Pascal Veys)

An overview of the activities of the EURL-AP was given. From the EURL-AP ring trial can be concluded that most labs were not able to detect insect meal. At present research is going on for the analysis of PAPs by LC-MS/MS. Suitable marker peptides will be selected.

ANIMAL PROTEINS RING TEST 2018 (L. van Raamsdonk)

The IAG ring trial of 2018 was based on a sample with 2% fish meal, a blank feed, a sample with 0.1% PAP and 2% fish meal and a sample with 0,1% PAP. 28% of the participants reported one or more wrong results. 31% of the participants reported results containing errors that lead to a lot of extra work. A detailed presentation of the results is reproduced in this Newsletter.

COMPOSITION RING TEST 2018 (L. van Raamsdonk)

The sample for composition determination contained tapioca which was underestimated or not reported by 88% of the participants. More details are given in this Newsletter.

IMPLEMENTATION STUDY OF EXPERT SYSTEM FEED INGREDIENTS 2017 (L. van Raamsdonk)

RIKILT organised an implementation study of an expert system on feed ingredients. The expert system contains at present 29 different types of feed ingredients described with 26 different characteristics. Ten IAG-members participated in the study by analysing 10 different samples for evaluation of the system.

BOTANIC IMPURITIES RING TEST 2017 (L. van Raamsdonk)

RIKILT organised also a ring trial on botanical impurities. Two samples were prepared. One was containing 2% *Brassica nigra*, the other 2% of *Jatropha curcas*. 91% of the participants was able to identify *Brassica* and 57% of the participants identified *Jatropha*. See contribution in this Newsletter.

INSECT PAP FRAGMENTS ISOLATION PROTOCOL FOR MICROSCOPIC ANALYSES (P. Veys)

An isolation protocol for the isolation of insect PAP fragments by using a mixture of 70/30 TCE/PE was presented. For 37/40 samples a higher concentration of the insect PAPs was obtained. This study is published: Veys, P. & V. Baeten (2018). Protocol for the isolation of processed animal proteins from insects in feed and their identification by microscopy. Food Control, 92, 496-504.

PLASTIC RICE: HOAX OR NOT? (J. Vancutsem)

It was discussed whether fake rice made of a synthetic was on the market. Probably this story turns out to be a hoax.

METHOD FOR THE DETERMINATION OF FRUITS AND SEEDS OF AMBROSIA SPP. IN ANIMAL FEEDING STUFF (R. Weiss)

The IAG-method on the detection of *Ambrosia* was discussed.

POSTER PRESENTATIONS:

Verification of the use of a drying step on wet samples for the analysis of feeding stuffs for animal proteins (J. Kinane-Kennedy, J. Darcy, T. Buckley)

Samples with a moisture content of 75-80% were spiked with PAPs from terrestrial animals and fish meal and subsequently dried at 100°C for several hours. After drying the samples were blended and analysed. The drying step of wet feeds had no effect on the detection of terrestrial animal and fish particles.

Filth in pre- and post-milling products (SGS, Institut Fresenius)

Samples were treated with hypochloric acid and paraffin after autoclaving. After several washing steps the sample is filtered through a filter paper. The filter residue is analysed with a microscope for the detection of animal contamination; insect, bird or rodent matter; soil, glass, plastics,…

Annual ring test animal proteins

L.W.D. van Raamsdonk, B. Hedemann, C.P.A.F. Smits, J.J.M. Vliege, 2018. *IAG ring test animal proteins 2018.* Wageningen, RIKILT Wageningen UR (University & Research), RIKILT report 2018.008.

The annual ring test 2018 for the detection of animal proteins in animal feed of the IAG - International Association for Feeding stuff Analysis, Section Feeding stuff Microscopy was organized by RIKILT - Wageningen UR, The Netherlands. The aim of the ring study was to provide the participants information on the performance of the local implementation of the detection methods for their local quality systems. A further aim was to gather information about the current practices in the application of the microscopic method. The current 2018 version of the IAG ring test for animal proteins facilitated the full analytical part of the methods for microscopy and PCR as published in Regulation (EC) 51/2013 amending Annex VI of Regulation (EC) 152/2009 together with accompanying SOPs.

The four samples contain ruminant material at the legally required technical limit (0.1% w/w; Regulation (EC) 152/2009), or fish meal at a spike level of 2% (w/w), or both. A fourth sample was left blank. The samples were based on a pig feed produced in a pilot plant, based on a commercial average composition. The feed contained 3% (w/w) of bakery by-products. None of the samples was labelled as fish feed.

A total of 49 participants subscribed to the ring test animal proteins. Four participants did not submit their results. Of the remaining 45 participants, two applied exclusively PCR, leaving 43 sets of microscopic results, accompanied by PCR results in 20 cases.

Microscopy

All participants were requested to determine the presence or absence of land animal and/or fish, to indicate the type of material found and to describe the method used to achieve these results.

Ten participants (23.3% of 43 participants) applied an incorrect number of determination cycles for one or more samples as required by the EU protocol. In total five participants (12%) included incorrect interpretations of the encountered number of particles (e.g. “below threshold” for zero particles, “present” for 5 particles). In addition, two participants did not submit final conclusion on one or all samples. Therefore, all evaluations were based on the actual number of particles reported by all participants.

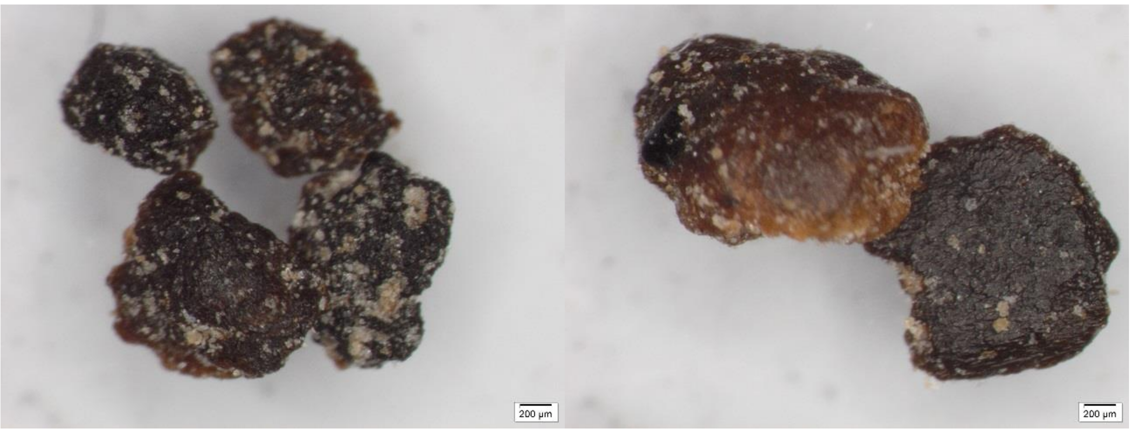
Incorrect positive results (positive deviations) were expressed in a specificity score and incorrect negative results (negative deviations) were expressed in a sensitivity score. An optimal score is 1.0. The results are analysed in two ways: numbers below threshold (between 1 and 5 particles per determination cycle inclusive) have been considered positive (complying to the zero tolerance) and as alternative considered as negative (for matching the official evaluation).

For all samples several participants did not detect terrestrial animal particles when present (sensitivity 0.95 and 1.0 in the presence or absence, respectively, of fish material) or erroneously reported terrestrial animal material when absent (specificity 0.84 and 0.91 in the presence or absence, respectively, of fish material).

The documentation for and training of microscopists for correct identification of particles of animal origin would deserve further attention in order to guard specificity. Evaluation of several aspects of the application of the current microscopic methods would be beneficial for improving harmonization among the laboratories applying the microscopic method.

PCR

Ruminant material was correctly detected in both samples containing 0.1% (w/w) of terrestrial animal material in all 20 cases where PCR was applied. In the two samples without added terrestrial animal material, but still containing the bakery by-products, ruminant DNA was detected as far as analysed, except for one participant who reported negative. The list of recognised sources such as milk and milk products, and ruminant gelatine can be extended with bakery by-products, which is important for the recycling of food by-products.



Examples of assumed blood particles (left) and palm pit expellers (right).

Undesirable substances in compound feed

Leo van Raamsdonk, RIKILT, the Netherlands

Directive 2002/32/EC provide legal limits for a set of biological undesirable substances, partly to be detected exclusively as whole seeds, and partly to be found in ground material as well. This category includes weed seeds and fruits containing toxic compounds (with *Datura* sp. (thorn apple) mentioned as the most notable genus), *Crotalaria* spp. (rattlebox), seeds and husks from *Ricinus communis* (castor oil plant), *Croton tiglium* (rushfoil), and *Abrus precatorius* (rosary pea), *Jatropha curcas* (purghera), *Fagus sylvatica* (beech mast), seeds from *Ambrosia* spp., and mustard seeds (several species of the genus *Brassica*). Late 2017 a proficiency test was organised for monitoring parts of the latter category. Two samples were designed based on a poultry feed with 70% of cereals and lacking any material mimicking the target contaminant. This meant in particular the absence of *Brassica* material for proper recognition of mustard seed material. The two samples of 50 grams each contained 1 gram of ground black mustard for sample A (2% *B. nigra*), and 1 gram of *J. curcas* for sample B (2%). The report file showed all undesirable substances as listed in Directive 2002/32/EC in order to show the scope of the test, and at the same time without revealing any information on the type of material used for spiking. Every participant was requested to give the identity of the spike with an estimation of the spike level. Twenty-eight participants subscribed to the test of which 22 send in their results.

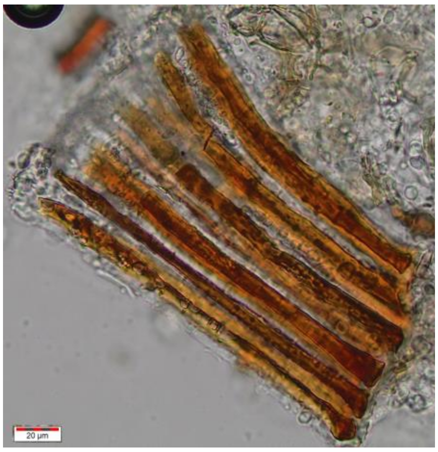
The results were as follows:

Sample A: 20 participants reported *Brassica* in general at levels between 0.1 – 3.43% (average 0.87%). Only one of these participants reported specifically the presence of *B. juncea* and *B. carinata*, two mustard species closely related to *B. nigra*. One participant reported the presence of *Datura* (0.21%), and one participant reported blank.

Sample B: 13 participants reported the presence of *Jatropha* at levels between 0.07 – 4.2% (average 1.7%), and nine participants found *Ricinus* between 0.2 – 1.9% (average 1.3%). Two of these participants reported both substances. In addition, two participants detected *Croton* material at levels between 1.2 – 1.3%.

Documentation from hand books reveals characteristics to discriminate between the oil seeds (*Brassica napus* and *B. rapa*) and the mustard species belonging to the genus *Brassica*. Although the oil seeds are not included in Directive 2002/32/EC, only one participants reported mustard species instead of *Brassica* in general. The genera *Jatropha*, *Ricinus* and *Croton* all belong to the family of Euphorbiaceae. The characteristics of the hull are quite specific and properly recognised at the family level.

Two main conclusions can be drawn. All materials were recognised in a certain sense. In the presence of oil seed hulls or expellers from *Brassica*, it is still necessary to distinguish between authorised and prohibited material of *Brassica* species. It is further recommended to pay attention to the detailed differences between *Jatropha*, *Ricinus* and *Croton.* As second conclusion, the need to prohibit certain undesirable substances at the species level should be evaluated. Especially the genus *Croton* consists of several hundreds of species. Although usually little is known of these species, the possibility exists that these plants are toxic as well. It could be considered to prohibit these plants at the genus level or even at the level of family.



Hull of Jatropha curcas*, magnification 100X.*

Monitoring bird feed for the presence of undesired and possibly viable seeds harmful for the animals or the environment.

Geneviève Frick, Nicolas Pradervand, Heinrich Boschung, Agroscope, Switzerland

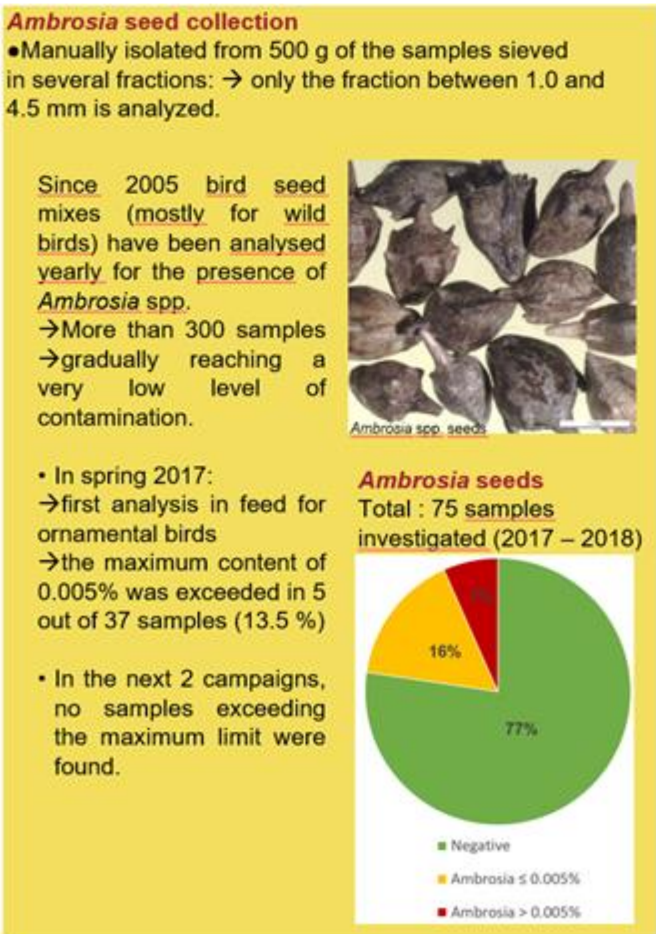
For years, feed control authorities have been well aware of the environmental hazard posed by alien seeds (ex. *Ambrosia* spp.) contaminating bird feeds.

Indeed, those seeds may have retained their ability to germinate and may easily disseminate in the environment (either by the out-door disposal for wild birds or the composting of the dung).These risks applies to all the seeds in a bird feed, including desired component species. Knowing that some of the bird seed mixes or their components are produced in North America, the importance of checking these feeds also for the presence of GMO was admitted.

Among the recurring components found in bird feeds are the Brassicacea seeds (*Brassica napus* as well as *Brassica rapa)* and both have known GMO cultivars produced in North America. As well, small amounts of *Brassica spp.* often contaminate other seed lots, for example wheat. GM *Brassica spp*. are considered to present a high risk of colonization in Switzerland and of crossing with native wild species through pollination.

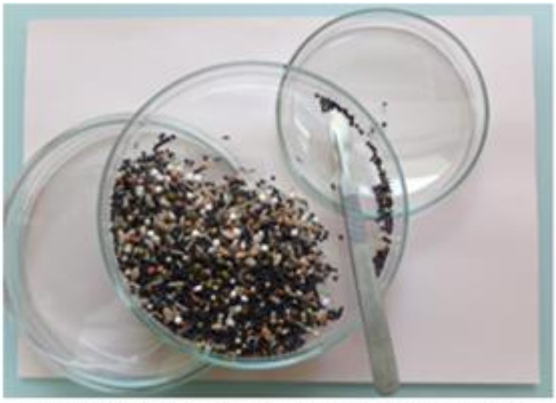
Ambrosia spp. monitoring is performed since more than 10 years, with a set maximum content (0.005%). The analyses performed in 2017 and 2018 show results in line with those of previous years with a few contaminated samples, rarely exceeding the maximum content.

In the course of three campaigns (spring 2017, fall 2017, spring 2018), GM-*Brassica* seeds (all authorized for feeds in the EU) were found in at least one third of the samples at a low percentage. In some cases, more than one GMO event was detected per sample. The percentage of contaminating GM-*Brassica* was more severe when the *Brassica* were found as contaminants and not as components. This campaign was organised in the framework of Swiss legislation (FMV Art. 68 Futtermittel mit Spuren von gentechnisch veränderten Organismen).



Brassica seed collection

- Manually isolating 500-1000 *Brassica* seeds (approx. 3.5 g) if component
- > 50 seeds if contaminant



Collecting *Brassica* spp. seeds from a bird feed

GM-*Brassica* seeds

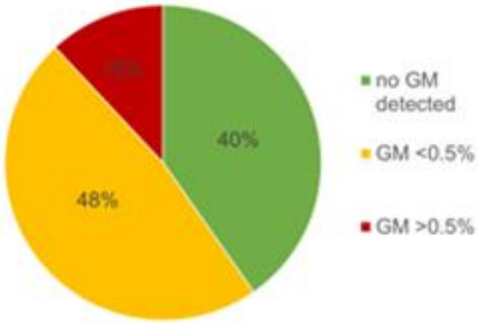
Total : 67 samples investigated (2017 – 2018)

Initial GM element screening:

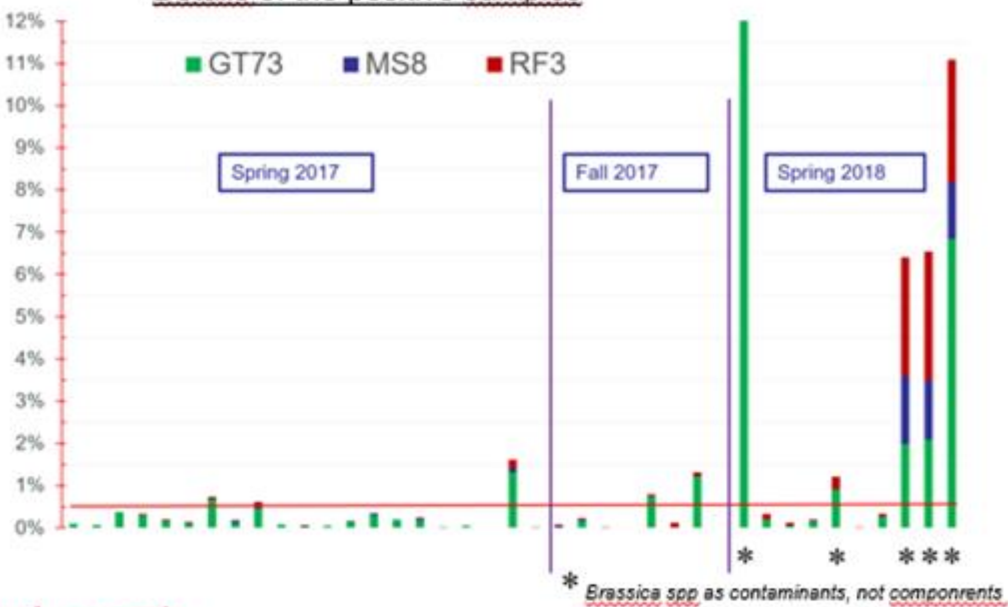
p35S, tNOS, bar, pat, pFMV, ctp2-cp4-epsps, and elementless event DP73496-4

Events found :

GT73, MS8, RF3



Details of the positive samples



Legal aspects

In animal feed, the proportion of viable GM-*Brassica* (authorized in the EU) in the same *Brassica* raw material may not exceed 0.5 %.

The comeback of Corn-cockle (*Agrostemma githago* L.)

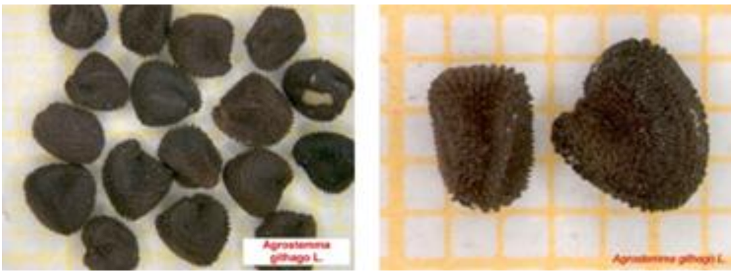
R. Weiss, AGES, Austria

Directive 2002/32/EC provides a list of undesirable substances of botanic origin. In most cases precise indications of species are given. There is one category with a broader circumscription: "*Weed seeds and unground and uncrushed fruits containing alkaloids, glucosides or other toxic substances separately or in combination including* –Datura *sp*.". This is an extremely wide indication. It is estimated that approximately 20% of all plant species produce compounds that might result in hazards for human beings and/or animals. A compendium of hazardous plants in food has been published by EFSA in 2012. A comparable overview for feed is not available, but additional information can be found in Frohne & Pfänder, 2005.

In Spring 2018, some official control samples were tested for botanical impurities at AGES and positive results were obtained for Corn-cockle *(Agrostemma githago* L.). In two cases the legal limit of 3000 mg/kg (ppm) as laid down in Directive 2002/32/EG was exceeded. *Agrostemma* is listed in the mentioned Compendium.

Corn-cockle in barley (left) and wheat (right)

The typical corn-cockle seeds are up to 4,5 mm long, shaped triangular and covered with coarse, pointed tubercles. The seeds are dull and purplish or brownish black in colour. All parts of the plant are poisonous and contain githagin and agrostemmic acid.



Corn-cockle in overlook mag. 6x (left) and in detail mag. 12x (right)

These results were interesting as Corn-cockle was found on the list of endangered species and was hardly found in Europe for the last years.

Background

Corn-cockle was reported as a very common weed in European grain fields during the 19th century and its seeds were inadvertently included in harvested crops and then resown the following season. It is very likely that until the 20th century, most cereals contained some corn-cockle seed.

In parts of Europe, intensive mechanized farming has suppressed the plant and it is now uncommon or locally distributed. This is partly due to changing patterns of agriculture with most wheat now sown in the autumn as winter wheat and then harvested before corn-cockle can develop. The main reason, however, is that the cereal seed is better cleaned. Although the Corn-cockle was nearly extinct as weed in Europe, it is nowadays cultivated as a garden plant. In the United States and parts of Canada, Australia and New Zealand corn-cockle is considered an alien species, probably introduced with wheat imported from European sources.

References

European Food Safety Authority (EFSA) (2012). Compendium of botanicals reported to contain naturally occurring substances of possible concern for human health when used in food and food supplements. EFSA Journal, 10(5), 2663–2722. http://dx.doi.org/10.2903/j.efsa.2012.2663

Frohne, D., & Pfänder, H. J. (2005). Poisonous plants. In A handbook for doctors, pharmacists, toxicologists, biologists and veterinarians. London: Manson Publ. Ltd.

Inhomogeneity in whole kernel feed

Leo van Raamsdonk, RIKILT, the Netherlands

Background

In the process of sampling a contaminated feed or feed material, subsequent subdivision of sampled material, and of the production of a laboratory sample, it is essential to assure that the final analytical result represents the level of contamination in the original sample. Provisions for this process are obligatory as laid down in Regulation (EC) 152/2009 Annex I and Annex II, amended by Regulation (EC) 691/2013. A crucial element is to establish a sufficient homogeneity in every step of this process by a targeted action. Examples of such actions are making a slurry, grinding or quartering the material. Some of these treatments are not applicable for a special type of feeds or feed ingredients: the materials consisting of whole kernels. This type of materials include unground cereals or whole kernel bird and poultry feeds. Therefore, specific requirements are included in the mentioned Annexes I and II of Regulation (EC) 152/2009 for this type of materials. The species listed in Directive 2002/32/EC include mustard seeds, and species of the genera *Ricinus*, *Jatropha*, *Croton*, *Abrus* and *Ambrosia*. The desirability to monitor *Ambrosia* was demonstrated in the first few years after the official control limit. Depending on the member state, between 21% and 75% of the bird feed samples investigated appeared to contain *Ambrosia* (Frick et al., 2011).

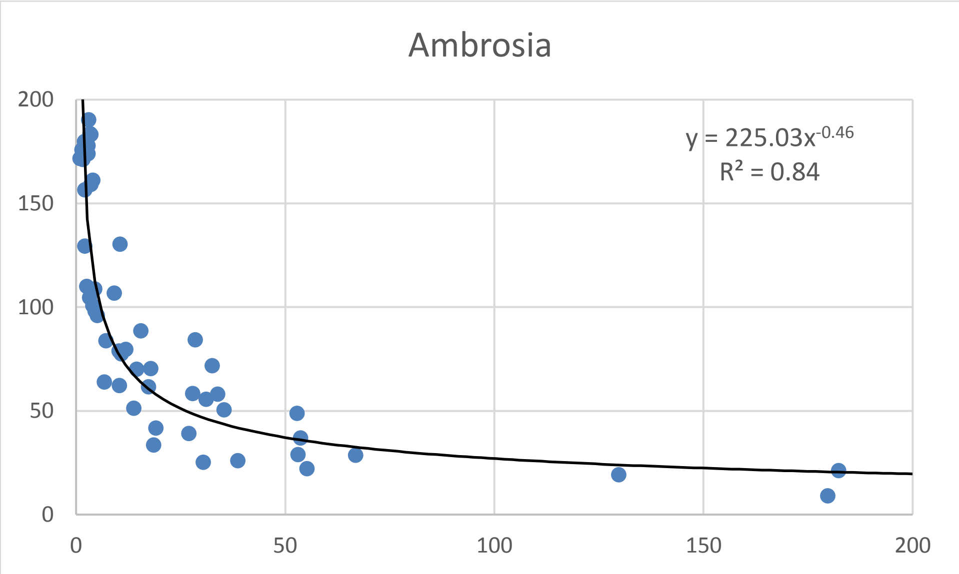
RIKILT started in 2016 a three-year project for the collection and evaluation of the necessary data. The research was intended to find and evaluate the level of inhomogeneity in samples of whole kernel feeds. The presence of *Ambrosia* seeds was investigated as model for establishing the protocol.

Procedure

The procedure for the research was based on the analysis of the entire sample material, ideally 2 kg, and randomly subdivided in four portions. Since January 2016, all samples of bird feed received at RIKILT have been split into four subsamples of approximately 500 grams. This was done by means of a sample divider. The statistical analyses for the distribution of *Ambrosia* seeds in bird feed was based on 116 sets of results. The mean and standard deviation were calculated based on the four contamination levels as established by examination of the four subsamples. The four amounts were pooled to calculate the correct contamination level. The relationship between the relative standard deviation (SD divided by mean) and the correct contamination level was used for evaluation of the inhomogeneity.

Results

In total, 56 out of 116 bird feed samples contained *Ambrosia* seeds (Table 1), of which 11 samples exceeded the statutory limit (50 mg/kg). Two samples contained very high levels of *Ambrosia* seeds, i.e. more than ten times the legal limit. The share of positive samples (48%) fits well in the shares as reported by Frick et al. (2011). At low levels, approximately below 15 mg/kg, the standard deviation is approximately equal to or larger than the calculated mean over the four subsamples. In principle, the difference between counting 1 seed or 2 seeds in a sample is an increase of 100%, resulting in a standard deviation that is larger than the mean (relative standard deviation larger than 100%). At higher seed counts, a difference of 1 seed is a much smaller percentage and the relative standard deviation will be lower. This makes it easier to achieve a lower inhomogeneity at higher seed counts, which appears to be feasible for a future scenario.



Plot of correct mass fractions in mg/kg (x-axis) and relative standard deviations in % (y-axis) for all samples testing positive for Ambrosia*.*

Conclusion

A relationship between the level of contamination of ambrosia seeds in bird feed and the standard deviation (expressed in the relative SD) was established. At contamination levels exceeding approximately 15 mg/kg relative SDs were found (much) lower than 100% (See graph). This level is well below the statutory limit of 50 mg/kg. These results allow to design a procedure for the detection of Ambrosia seed in bird feed. The possibility to extend this procedure to other undesirable substances as mentioned in Directive 2002/32/EC needs to be explored.

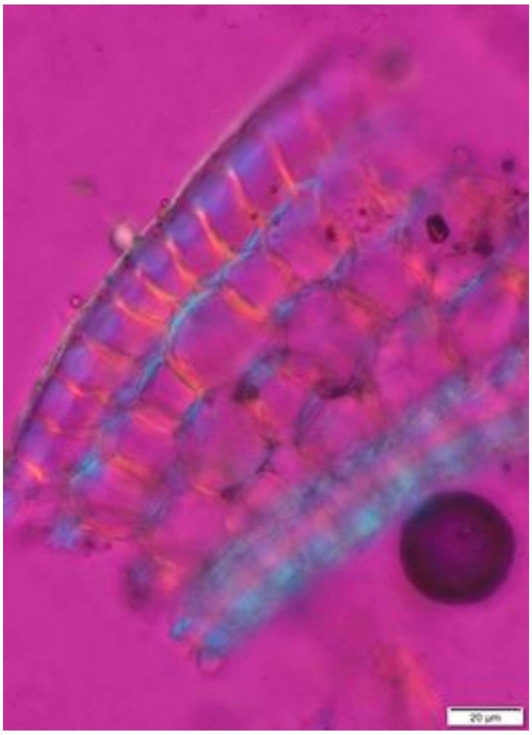
Reference

Frick, G., H. Boschung, G. Schulz-Schroeder, G. Russ, I. Ujčič-Vrhovnik, B. Jakovac-Strajn, D. Angetter, I. John, J.S. Jørgensen, 2011. Ragweed (*Ambrosia* sp.) seeds in bird feed. Biotechnol. Agron. Soc. Environ. 15(S1): 39-44.

Annual ring test composition 2018

Leo van Raamsdonk, RIKILT, the Netherlands

The IAG ring test for composition 2018 was based on a pig feed, produced in 2003 in the framework of the European Union project STRATFEED and based on a regular formulation of pig feed. The sample was spiked with 2% of fish meal, which influenced the shares of each ingredient. The adjusted composition consisted of tapioca (39.2%), soybeanmeal (15.6%), rapeseedmeal (11.8%), wheatglutenfeed and wheat bran (11.8%), palmkernelmeal (5.9%), beetpulp (3.9%), bakery by-products (2.9%), molasse (2%), total fat (2.7%), barley (1%), minerals (1.3%) and fish meal (2%). Since molasse and fat are very difficult or impossible to observe microscopically and are usually ignored in composition analysis, a second adjustment was carried out. The shares of the different ingredients as finally applied for calculation of the lower and upper limit are indicated in the Table.



Microscopic cross section of cork cells in tapioca in polarised light, magnification 200x.

Results

Table 1 and Figure 1 show the summarised results of 24 participants. In general the estimations as made by the participants were good. In the view of an ingredient (tapioca) which is largely underestimated, the slight overestimations of the other five major ingredients (Figure 1) are acceptable, since the final declaration has to sum up to 100%. Bakery by-products are primarily underestimated as well.

Discussion

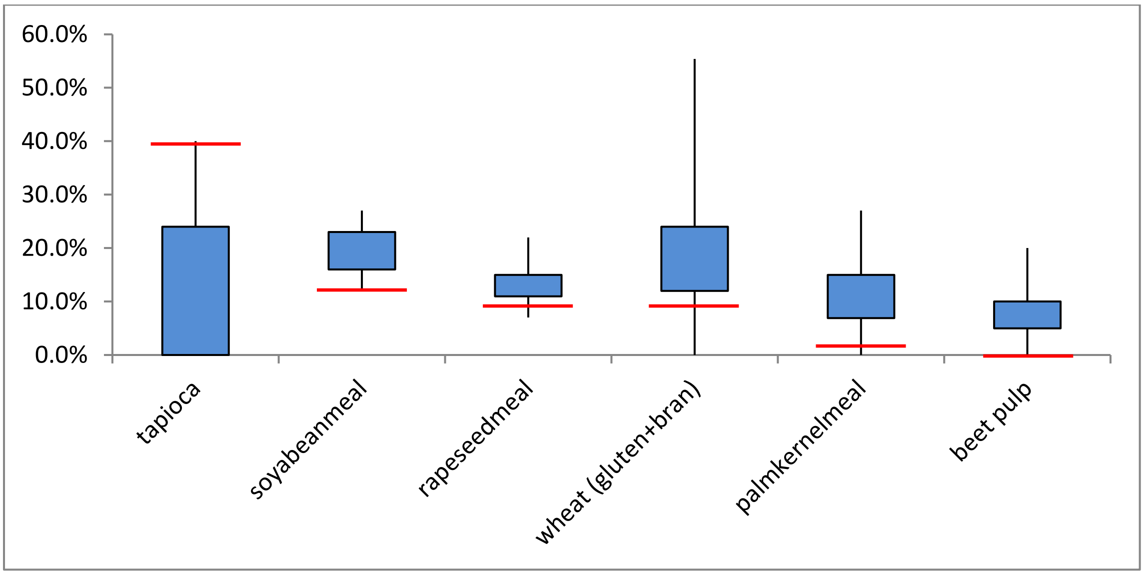
There are still two remaining issues. A majority of the participants underestimated or overlooked the presence of tapioca. Further evaluation of this result revealed that tapioca is not a regular feed ingredient in certain member states or is completely absent in the formulation. It is obvious in the situation where microscopists are primarily educated in their own institute that expertise can be gained only for available ingredients. Notwithstanding this, feed trade and production are very internationally orientated and exchange of expertise among institutes, preferably in an international framework might be profitable.

Another issue is the general underestimation in the detection of bakery by-products. It is foreseeable that this type of products, i.e. food-grade material either non-sold or not consumed for another economic reason, will have an increasing share in feed production. More background can be found in the Editorial on microplastic. It is therefore necessary that this category of putative ingredients in the feed production chain will get more attention.

Overview of over- and underestimations of the 10 ingredients of pig feed.

N=24	correct	range *	overestimations	underestimations
tapioca	41.1%	31.1-51.1%	0	21 (88%)
soyabeanmeal	16.4%	8.2-24.6%	4 (17%)	0
rapeseedmeal	12.3%	6.2-18.5%	2 (8%)	0
wheat (gluten+bran)	12.3%	6.2-18.5%	11 (46%)	3 (13%)
palmkernelmeal	6.2%	1.2-11.2%	11 (46%)	1 (4%)
beet pulp	4.1%	0.0-8.2%	10 (42%)	1 (4%) **
bakery by-products	3.1%	0.0-6.2%	5 (21%)	17 (71%) **
barley	1.0%	0.0-2.0%	1 (4%)	12 (50%) **
minerals	1.3%	0.0-2.6%	3 (13%)	4 (17%) **
fish meal	2.1%	0.0-4.2%	2 (8%)	6 (25%) **

*: according to the IAG uncertainty model, Rostock, 2006.
**: number and percentage of participants which did not find or report the ingredient.



Box plots of six ingredients. Vertical line: min-max range; box: P25-P75 range; red horizontal line: actual share in % of ingredient.

Recommendation

The establishment of the composition of a feed can be carried out for several objectives: label control (Regulation (EC) 767/2009), traceability (Regulation (EC) 178/2002) or detection of fraud (Regulation (EC) 882/2004; Decision (EU) 2015/1918). An expert system for identification of major feed ingredients running on the platform Determinator was developed by RIKILT. This system provides information on 26 groups of ingredients covering over 80 entries of the Feed Catalogue. It was tested by six European laboratories in 2017. The results of this test will be the basis for improvements to be established in 2019. It is recommended to use this or comparable sources of information as standard tool for composition analysis of feeds, in order to comply to the legal requirements as set in the best way possible.

Growing crystals in the flotate

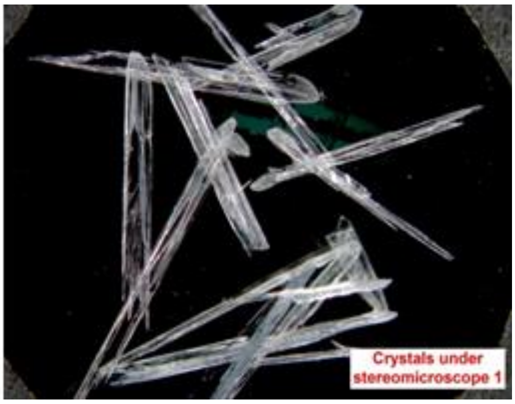
Roland Weiss, AGES, Austria

The analysis of a sample for official control revealed a special case during the drying of the flotate prior to the analysis for animal proteins and packaging materials.

While after sedimentation of the complementary feed for pigs everything looked like as usual, after the drying process of the flotate on a heating plate overnight crystals were formed as shown in the picture below.

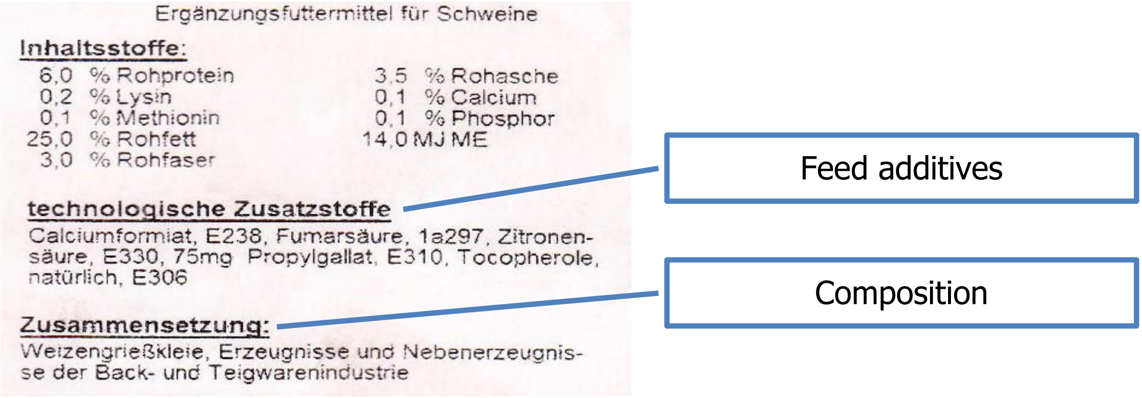
Flotate with crystals

The very first “optimistic” idea was that there were particles of glass or plastic in the sample. But after a first investigation by microscope it turned out that there were a lot of crystals growing on the flotate. These crystals looked like thin sharp needles with a length up to 1,5 cm, coloured like glass and growing slowly by time.



Crystals in the flotate (left) and under the stereomicroscope (right)

Looking at the declaration the main constituents were wheat middlings and bakery products and its byproducts. Furthermore, it contained following feed additives: calcium formate, fumaric acid, citric acid, propyl gallate and tocopherol-rich extracts.



Ergänzungsfuttermittel für Schweine

Inhaltsstoffe:

6,0	% Rohprotein	3,5	% Rohasche
0,2	% Lysin	0,1	% Calcium
0,1	% Methionin	0,1	% Phosphor
25,0	% Rohfett	14,0 MJ ME	
3,0	% Rohfaser		

technologische Zusatzstoffe

Calciumformiat, E238, Fumarsäure, 1a297, Zitronensäure, E330, 75mg Propylgallat, E310, Tocopherole, natürlich, E306

Zusammensetzung:

Weizengrießkleie, Erzeugnisse und Nebenerzeugnisse der Back- und Teigwarenindustrie

Part of the declaration of the sample

After consultation of some analytical chemists of AGES it was soon clear, that these needles originated from calcium formate (official IUPAC indication: methanoate), which found the convenient conditions for precipitation on the heating plate.

Reference:

Pictures made by AGES

Editors note.

The identification of the mineral fraction in a compound feed is interesting, but more importantly, an essential element in composition analysis. There is a variety of chemical compounds, such as vitamins, precursors and spore elements, which are necessary for a balanced physiology of farmed animals. There are opportunities for manipulating, such as the addition of urea for raising the nitrogen content of the feed. Another issue is the situation that certain undesirable substances such as heavy metals might get unintentionally introduced in a feed primarily via minerals. The analysis of the mixture of minerals provide opportunities for tracking and tracing of excess of legal limits.

A range of analytical chemical methods are applied in daily routine for the detection of a range of compounds. The toolbox of the discipline of visual research and microscopy includes several procedures for the separation of fractions of a compound feed, including the mineral fraction. Separate analysis of the vegetal and mineral fractions could reveal the origin of certain restricted chemical compounds. Another part of the mentioned toolbox is a wide array of so-called spot tests. Minor amounts of mineral material can be subjected to specified reagents for the identification of a range of mineral materials. This opportunity can be used as a fast and cheap screening procedure with a follow-up of (targeted) chemical analysis.

Scheme of ring tests 2019

The IAG section Feeding stuff Microscopy organizes annually several ring tests for the evaluation of composition or detection of prohibited constituents in animal feed. The board of the IAG section Feeding stuff Microscopy and RIKILT have agreed to organize together the 2019 ring test for the following situations:

- Test IAG-2019-A. Detection of the presence of animal proteins in a set of four samples. This test was already organised by RIKILT in previous years (see abstract in this Newsletter). Targeted protocol: Regulation (EC) 152/2009, consolidated version of February 12, 2013. Cost for participation: € 250.
- Test IAG-2019-B. Declaration of the composition of a compound feed (one sample). This test was organised from 2014 on by RIKILT as well (see abstract in this Newsletter). Targeted protocol: IAG method A2. Cost for participation: € 60.
- Test IAG-2019-C. Material for the detection of packaging material in former food products (two samples of bakery products). Targeted protocol: RIKILT report 2012.007: "Examination of packaging materials in bakery products". Cost for participation: € 120.

The single sample for the composition test will be part of the animal protein test. On behalf of the IAG section Feeding stuff Microscopy, RIKILT will invite you for participation in these ring tests. RIKILT will encourage you to subscribe to all four tests, although this is not mandatory. Participation in all three test would cost € 430; in this case a discount of 10% will be granted, resulting in a total cost of € 387 for the total set of three tests.

The samples for test IAG-2019-A and IAG-2019-B will be sent around late February or early March 2019. Also a questionnaire will be sent by E-mail, together with instructions and relevant documentation on protocols. A time slot of four weeks is planned for the analyses of the samples by every participant. This means that late March or early April all results are expected to be returned to RIKILT. The samples of test IAG-2019-C will be send late August and results needs to be reported in October. All results are intended to be reported at the annual meeting of the IAG working group Microscopy in Stade (Germany) in June 2019 (tests A and B) or in 2020 (test C). The final reports will be published later in either 2019 or 2020. All communications of the evaluation will be fully anonymous.

If you are interested to participate in one or more ring tests, please return the application form, which accompanies this newsletter, to leo.vanraamsdonk@wur.nl. Subscription closes Thursday February 23rd, 2019. You are requested to make a payment after receiving the invoice from RIKILT. Make sure that the reference number, your name and your institute's name are mentioned upon payment. This information is necessary to avoid loss of payments that cannot be linked to participating institutes.

Closing remark.

Dear reader,

An interesting display of topics was addressed in our work during the year 2018. Besides our regular activities in the known niches of animal proteins, Ambrosia and ergot sclerotia, two messages can be extracted from the topics presented in this newsletter. The first one is that multidisciplinary research can be profitable in a range of cases. As for animal proteins, where the twin of microscopy and PCR is regularly applied, other combinations such as microscopy and analytical chemistry will certainly enhance the effect of monitoring feed and food safety. A second message is the area of physical hazards. This is expected to increase in importance in the framework of circular economy (packaging materials !), and presumably visual techniques will be the primary choice to target the majority of emerging risks in this respect.

We will wish you a prosperous New Year and we are looking forward to have a fruitful 2019 with new challenges and with a variety of colleagues!

Save the date!

The next meeting of IAG section Feed Microscopy will be held on 12 to 14 June, 2019 in Stade. This city is located close to Hamburg, which provide convenient travelling. We are invited by LAVES-Futtermittelinstitut Stade, and the first preparations are being made. As board we will cordially invite you to join us in Stade, and we will also promote the opportunity to participate in exchange of information and discussion of an important area of monitoring.

Old Hanseatic League city centre of Stade. Source:

https://www.h-hotels.com/de/hplus/hotels/hplus-hotel-stade

Board of IAG section Feed Microscopy.