IAG section Feed Microscopy

Newsletter 2016

It is a pleasure to share the activities of the IAG section Feed Microscopy with a large audience. A lot has happened. Main topics for the past year were insects and composition analysis.

The microscopic detection of animal proteins is traditionally an important aspect of our daily research. New sources of animal proteins are increasingly important, most notably insect proteins. Two major aspects of food security applies: safety in terms of chemical or microbiological contaminations, and traceability. For the latter aspect, microscopy can play a role. This was addressed during the annual conference in June in Copenhagen, and in this Newsletter an abstract of a submitted paper is included.

Also during the last conference, the analysis of botanic composition was discussed. We have again gained in expertise in this area. The abstract of the report of the annual proficiency test is included in the Newsletter, and a special interlaboratory study is announced.

The board of IAG section Feed Microscopy will invite you to read further in this Newsletter. Interesting information is presented, although important questions remain. The show will go on.

Contents

Presidents address

Dear colleagues und members,

With great pleasure I´ll take the opportunity presenting to you in this 3rd edition of our IAG newsletter some of the engaged activities we performed in the framework of the IAG Section Feedstuff Microscopy in 2016.

For our annual IAG conference 2016 we met in June in Copenhagen by invitation of our colleagues from the Danish Institute of Veterinary and Food Administration.

Several presentations focussed on recent results in the detection of animal constituents in feedingstuff.

Again the presentation of the IAG ring test results belonged to the main topics of the meeting.
The IAG ring tests “Animal Proteins 2016”, “Feed Composition 2016”, “Ergot sclerotia in Rye 2015”, all three organised by RIKILT, NL and IAG ring test “Ambrosia in Bird Feed 2015” organised by Agroscope, CH were discussed.

You´ll find the summaries of the reports in this newsletter.

IAG ring tests are open for all kind of labs performing microscopic analysis of feed material: Official control labs as well as commercial and industrial working labs. They cover a wide range of microscopic aspects including the detection of animal constituents in feed, feed composition and the determination of undesired and/or prohibited substances in feed, considering always actual microscopic questions.
The large number of laboratories from various European countries participating year by year in IAG ring tests demonstrate the importance of the offered ring tests for the users. Ring tests are one of the most important tools to improve proficiency and to gain microscopic experience. They are needed to keep the demands on quality assurance asked from the accreditation bodies and they support method development and revision.
Planned ring test for 2017 are announced in this newsletter.

You´ll also find some information on unusual constituents which were observed in feed by microscopic determinations of our colleagues.

As insects will also be a challenge in future IAG microscopy I´d like to raise your attention to the contribution “Insects as new feed ingredients”.

For the annual IAG meeting 2017 we are invited by our colleagues from the National Veterinary Institute, Uppsala, Sweden. The meeting date is June, 13-15, 2017.

In the name of the IAG board I´m looking forward to another year of interesting and engaged work of the IAG Section Feedstuff Microscopy and I hope you enjoy the reading of our newsletter.

Yours sincerely

I. Paradies-Severin, president

The annual conference of IAG section Feed Microscopy in Copenhagen

The annual conference of IAG section Feed Microscopy was held in Copenhagen, Denmark, from 07.06. – 09.06. 2016, organised by the team of the Danish Veterinary and Food Administration (FVST) of the Ministry of Environment and Food of Denmark. Interesting lectures were accompanied by vivid discussions on a range of topics. These included:

- Opening and Welcome by Dr. N. Ellermann (FVST) and Dr. I. Paradies-Severin (President of IAG section Feed Microscopy)
- Presentation of the participants and activities of 2015/2016
- Presentation of new participants (Irish Equine Center and ALcontrol AS)
- Information on the annual EURL-AP workshop and activities
- Detection and identification of hydrolyzed products
- Identification of insects
- Ergot analysis
- Recognition of milk powder
- Purity of alfalfa pellets
- Microscopy as supplement to chemical methods

The technical lectures will be referred to in the Proceedings of our meeting.

Ring Tests

Results of following IAG Ring tests were presented and discussed during the annual meeting:

- Ring Test 2015: *Ambrosia* seeds in bird feed - Agroscope (CH)
- Ring Test 2015: Ergot sclerotia in unground rye - RIKILT (NL)
- Ring Test 2016: Animal Proteins - RIKILT (NL)
- Ring Test 2016: Composition - RIKILT (NL)

→ summaries of the single ring tests will be found on the next pages.

→ a scheme of planned ring tests 2017 is also included in this newsletter.

Method Reading

It was decided to install the working group of interested colleagues who should work continuously on the actualizing of IAG methods (actualizing every 5 years, pictures in methods, validation etc.). The coordinator of method revision is Roland Weiss. The other members of this group are Betzabe Allain Arbe (Germany), Lotte Houghs (Denmark), Gabriele Russ (Germany), Igor Ujcic-Vrhovnik (Slovenia), Pascal Veys (Belgium) and also the scientific officer Leo van Raamsdonk (Netherlands).

It is planned to start with the adaption of IAG Method A5" Method for the Determination of Ambrosia (*Ambrosia artemisiifolia* L.) in non-pelleted Animal Feedingstuff" also for pelleted feed, for more detailed information and pictures of the seeds, the validation of the method (data from the IAG Ring tests) and including the Tetrazoliumtest with beginning of 2017.

IAG Board matters

- Decision that Inge Paradies-Severin (Germany) will continue to be IAG president for one more year → Election of the new IAG president at the annual meeting 2017.
- Change of Secretary: Genevieve Frick (Switzerland) will also be the secretary besides being the vice-president for one year.
- Installation of the working group for actualizing the IAG-methods: → "Method Reading" .
- Installation of the scientific officer: Leo van Raamsdonk (NL): also responsible for the IAG-Ring tests.
- New Webmaster: Jeroen Vancutsem (Belgium).

It was also decided to make an extension to the current Rules and Regulations. Extra items: mission and scope of the IAG section, tasks of board members, procedure for board elections and activities (e.g. Newsletter, Website, Ring tests, Methods…)

For our annual conference 2017 we are invited to our colleagues from Sweden and the meeting will take place from 13. to 15. 06.2017 in Uppsala.

Foreseen topics: Results of the IAG ring tests 2017

IAG method A5 revision

IAG affairs (Election of new President, board)

and much more…

Many thanks to the organizer team from FVST Copenhagen!!

R. Weiss, AGES, Vienna

Participants of the IAG annual conference in Copenhagen, 2016.

Feed Conference Geel, Belgium, 19 & 20 October 2016

Jeroen Vancutsem, FAVV, Belgium

On 19-20 October the Feed2016 Conference was organised by the JRC-IRMM in Geel, Belgium. Among the very interesting presentations, we choose some topics in relationship with microscopy and animal by-products to present here.

A complete overview of the conference topics can be found on this website: www.feed2016.eu/ .

Light microscopy technique for the discrimination of insect processed animal proteins versus marine arthropods (M. Ottoboni, University of Milano)

This study evaluated the use of light microscopy for the discrimination of insect PAPs against marine arthropods classified as fish meal. A staining method was tested for the detection of chitin consisting from insects as ingredient in animal feed. Four species samples were analysed: *Tenebrio molitor*, *Hermetio illuscens* and 2 marine arthropods, shrimp material and krill. A staining with alizarine red (AR) and chlorazol black was tested. It was seen that the exoskeleton of insects did not stain with alizarine red, but it was stained with chlorazol black, as the exoskeleton of the other arthropods. It is unknown why the exoskeleton of insects did not stain with AR: a lower amount of calcium, another form of calcium (that makes a complex with alizarine red). Is it dependent from the physiological stage? Chlorazol black shows a staining response, but the staining was not specific and not with a same intensity, possibly dependent on the thickness of the particle.

New feed ingredients: the insect opportunity (L. van Raamsdonk, RIKILT)

See article in this newsletter.

Validation of a selected pig real-time PCR assay for the detection of processed animal proteins in feedingstuffs (O. Fumière, CRA-W)

A method for the detection of pig-DNA in feed was validated. The results were satisfying for specificity (tested against 45 animal and 7 plant species), sensitivity (fitness for the detection of minimum 0,1 % w/w of pig PAP in feed), efficiency (between 80 and 120%), LOD (20 copies with a cut-off set at 5 copies) and robustness. After this a validation study a second interlaboratory study was organised. NRLs indicated an excellent performance confirming what was obtained during the validation study.

Utilising Animal By-Products as a sustainable feed choice for food producing animals (C. van Vuure, Darling Ingredients)

After slaughtering of the animal there is a leftover of around 40% ABPs predominantly as category 3 material. There are several uses of the by-products as from the pharma to fertilizer industry.

For example:

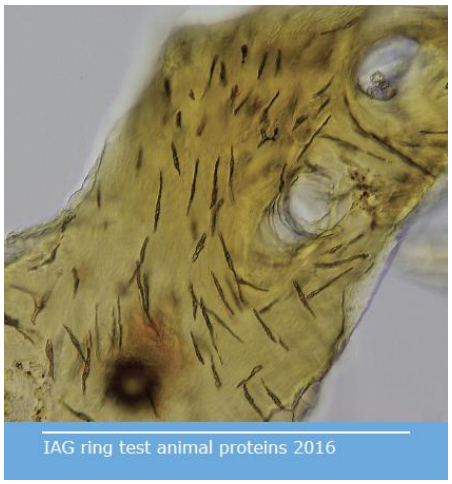
- Nowadays antibiotics are more and more replaced by products as proglobuline, mucopro.
- Organic phosphates can also reduce the use of fish meal in fish feed.
- Fish oil can also be partially replaced by pig oil with no taste difference of the fish.
- Also the petfood industry is growing: nowadays 61% of the ABP are used in petfood.

Annual ring test animal proteins

L.W.D. van Raamsdonk, N. van de Rhee, I.M. Scholtens, T.W. Prins, J.J.M. Vliege, V.G.Z. Pinckaers, 2016. *IAG ring test animal proteins 2016.* Wageningen, RIKILT Wageningen UR (University & Research centre), RIKILT report 2016.016.

Abstract

The annual ring test 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 method for their local quality systems. A further aim was to gather information about the application of the microscopic method. The current 2016 version of the IAG ring test for animal proteins facilitated the full scenario with 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.



All four samples were based on an artificial feed mimicking a formulation for ruminant feed. Two samples were labelled as fish feed (B and D), which was effectuated by adding 2% of a general fish meal. Adulteration was achieved by adding 0.1% pig MBM (B), 0.1% ruminant MBM (D) and a combination of 0.1% ruminant MBM and 0.1% fish meal (C). This combination of different spikes allowed the diverse application of the detection methods.

Forty eight participants enrolled for the ring test, of which 45 submitted microscopic results. Of these, 20 participants applied the combination of microscopic and PCR analysis. Three participants submitted exclusively PCR results.

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 the method used.

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 and as alternative considered as negative. By comparing both ways of analysis it is possible on one hand to compare the results with those from previous ring trials (there these numbers were considered positive based on the legal principle of zero tolerance), and on the other hand to compare it to the official method (where numbers between 1 and 5 are considered negative).

Most of the specificity and sensitivity scores for microscopy were at good or reasonable levels. In the combination of fish meal (0.1%) and ruminant MBM (0.1%) the detection of fish material was sub optimal. Considering numbers of particles below threshold as negative, the sensitivity scores show a considerable drop. The results indicate that the overall performance of the microscopic method is satisfactory, but applicants of the microscopic method could benefit from good and effective training and documentation in order to achieve a higher reliability in identifying particles.

PCR

The specificity (samples A and B) and sensitivity (samples C and D) scores for PCR were between 0.87 and 0.91.

Combined scenarios for microscopy and PCR

Several participants applied incorrect numbers of determination cycles, either too many or too less. The scenarios for correct combination of microscopy and/or PCR are published in an accompanying SOP. Several deviations from these scenarios were applied, such as examination by PCR of the terrestrial animal feeds, reporting the presence of fish in the fish feeds, and the absence of a final conclusion combining the results of both methods where appropriate. It is, however, a very good situation to have all (intermediate) results reported for each of the two methods in order to have a good documentation in the framework of a ring test.

http://dx.doi.org/10.18174/388254

Visit of downtown Copenhagen during the IAG annual conference

Analysis of pine nuts causing PNS (pine nut syndrome)

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Introduction

People are consuming pine nuts more and more. This has several consequences. It can lead to environmental damage in the forests where the nuts are gathered, as is the case in the southeast of Siberia where the economically important *Pinus koraiensis* grows. In addition, the prices of pine nuts have risen from 10€/kg to 40€/kg in 2009 as a result of a poor harvest in this region. At that time non-edible species of pine nuts appeared on the international market, such as pine nuts of the *P.armandii* species (spread, see figure 1).

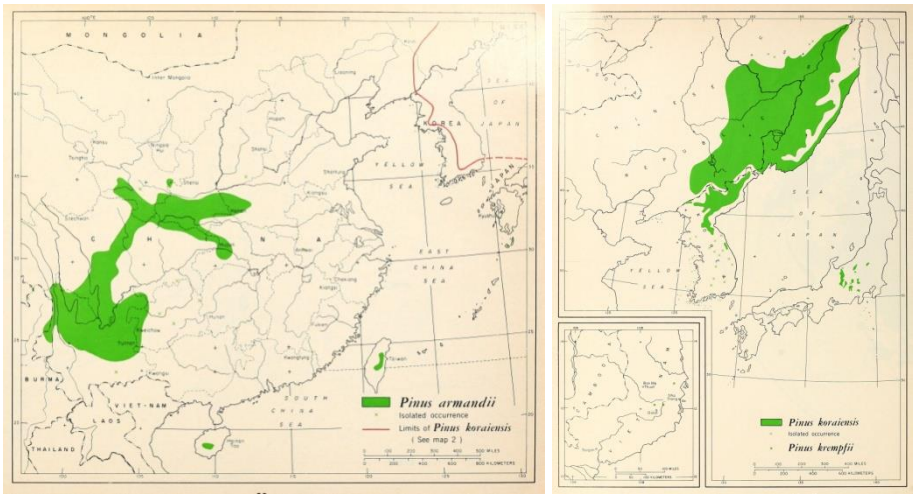


Figure 1 : Spread of P. armandii (left) and P.koraiensis (right)

The FAO has published a list of edible pine nut species (see table 1).

Pine nut syndrome (PNS)

Consuming pine nuts of the *P. armandii* species causes the 'pine nut syndrome' (PNS). One to two days after eating these nuts, a bitter taste is noticed which usually may last several days, but sometimes several weeks. Why these pine nuts cause PNS couldn't be found out as yet. Contrary to other *Pinus*-species such as *P. pinea,* these pine nuts contain a lot of pinolenic acid (see figure 2) that stimulates the enteric-endocrine system to produce

*Table 1: Edible pine nut species with *the most important economically (FAO, 1998; Zonneveld, 2008)*

P. ayacahuite	Mexico, Central America
P. albicaulis	West Canada and the US
P. cembra	Europe (Alps and Carpathians)
P. flexilis	West Canada and the US
*P. gerardiana**	East Afghanistan, Pakistan, North India
*P. koraiensis**	East China, Japan, Korea, SE Siberia
P. lambertiana	W US (California, Oregon)
P. monticola	NW US and adjacent Canada
*Piñon pines**	N Mexico, SW US
P. pumila	E Siberia, E China, Korea, N Japan
*P. sibirica**	Russia (Central Siberia), Mongolia
P. strobifonnis	N Mexico, SW US
P. coulteri	US (California)
*P. pinea**	Mediterranean Europe and Near East
P. ponderosa	West Canada and the US
P. sabiniana	US (California)
P. roxburghii	India
P. torreyana	US (California)

cholecystokinin (CCK) which is an interesting hypothesis for this syndrome.

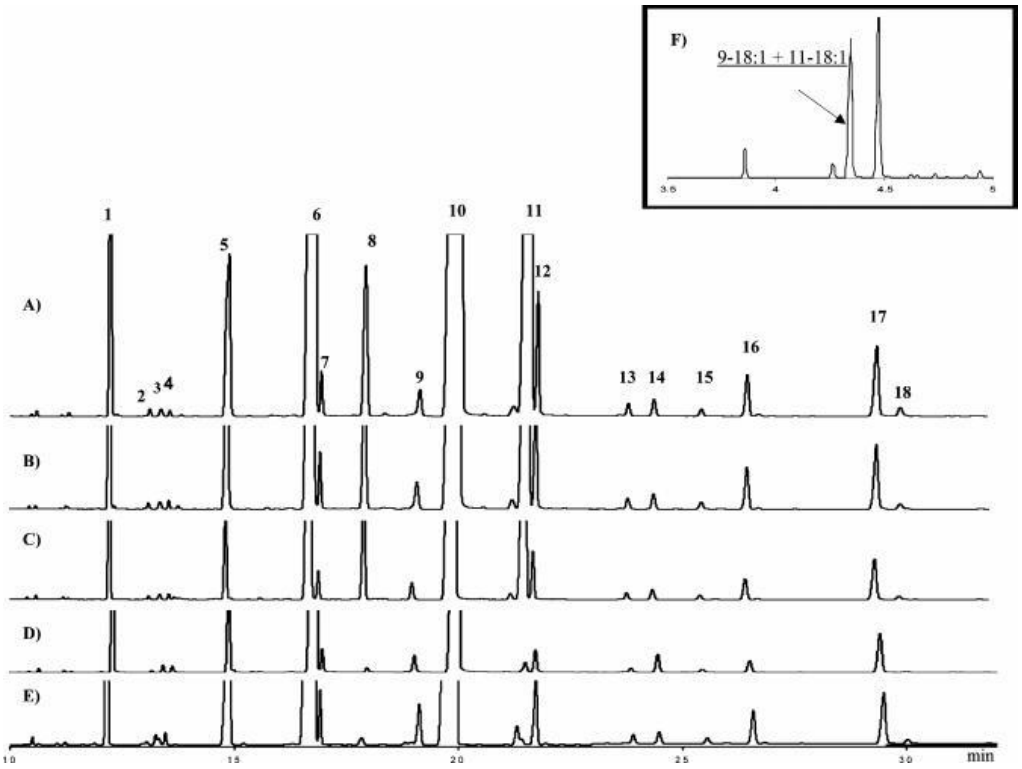


Figure 2 : Typical chromatograms of fatty acid methyl esters of (A) P. koraiensis (B) P. armandii; (C) P. sibirica; (D) P. gerardiana; (E) P. pinea with GC-FID with (11) = pinolenic acid (source: Fardin-kia et al., 2012)

Among other things, CCK provides a higher production of bile in the liver and sees to it that bile is exuded in the gastrointestinal tract. The excessive bile could cause a "cross-wiring" in the brain of the bitter receptors of the tongue and of the gastrointestinal tract causing an aftertaste of bitterness. This hypothesis offers also a solution for the fact that these symptoms remain for a long time, because some metabolites of the liver which are excreted via the bile tract into the duodenum are reabsorbed by way of enterohepatic recirculation.

Analysis methods

Visual method

In 2010, the FLVVT developed a visual analysis method. Based on reference samples (see figure 3) obtained via the anti-poison centre and an operator, an identification method was developed using visual characteristics. The number of pine nuts per 100g leading to identification is one of the characteristics and linked to this the fact that nuts fall through a sieve with a mesh size of 4 mm.

GC-FID

From 2011 till 2013 the LFSAGx has carried out confirmation analyses using GC-FID according to the method developed by Destaillats *et al*. 2010). Pine nuts are identified on the basis of the different ratio of fatty acids in various species - the diagnostic index (DI)

The DI is defined as - $DI = \frac{\text{C + 5,9,12-18:3 + 5,11,14-20:3}}{\text{(18:1 n-9 en n-7)+ 18:2 n-6 + 20:2 n-6}} \times 10$ with the values of the individual fatty acids expressed in % of the total content of fatty acids.

Figure 3 : Pine nuts (A) P. pinea, (B) P. armandii, (C) P. gerardiana, (D) P. koraiensis, (E) P. sibirica, (F) P. massoniana, (G) P. yunnanensis, (H) P. pumila (pictures not in proportion)

Based on the publication by Wolff *et al.* (2000) a reference DI could be determined for the different pine nut species (see table 2).

Table 2 : DI value for various pine nut species (Wolff et al., 2000)

Species	*P. gerardiana*	*P. pinea*	*P. koraiensis*	*P. armandii*	*P. sibirica*	*P. massoniana*	*P. tabuliformis*	*P. yunannensis*
DI	0,17	0,34	2,50	2,92	3,03	3,55	3,82	4,30

This method may be used if the sample does not consist of a mixture of various pine nut species as otherwise an intermediary signal will appear. In case of a mixture, it will be necessary to separate the nuts manually.

RT-PCR

Because of the fact that a visual separation is not always quite simple, as some species are very alike and not every pine nut looks the same, the FLVVT developed in 2013-2014 a new method in the framework of a master's degree dissertation at the KULeuven. In this respect, a recently published RT-PCR method with some modifications has been developed in practice - Handy *et al*. (2013) chose interesting areas in the plastid genome where they searched for single nucleotid polymorphisms (SNPs) that are unique for *P. armandii*. Three candidate-areas were selected and tested. Primers have been developed so as to detect these SNPs with an amplicon length of +/- 150 bp. The primers were optimized during the master's degree. DNA was extracted using the DNeasy Blood and Tissue kit (FLVVT: Wizard® Magnetic DNA Purification

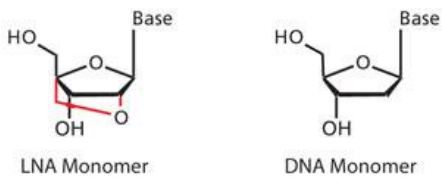


Figure 4 : LNA- and DNA-monomeres

System for Food). In addition, LNA probes (Locked Nucleic Acid) were used. LNA-probes are probes with modified amino acids in which the ribose ring is fixed by a methylene bridge between the 2'-O atom and the 4'-C atom (see figure 4). By fixing the molecules, they are in an ideal confirmation for the Watson-Crick binding which is faster and more stable.

In addition to the FAM-BHQ1-probe a blocker probe (HEX-BHQ1) which differs by one base has been developed and designed to bind with genetically related species among which the most important *P. koraiensis*.

HEX and FAM can be observed in one and the same RT-PCR run and can also be used f.i. as an indication of other pine nut species (see figure 5).

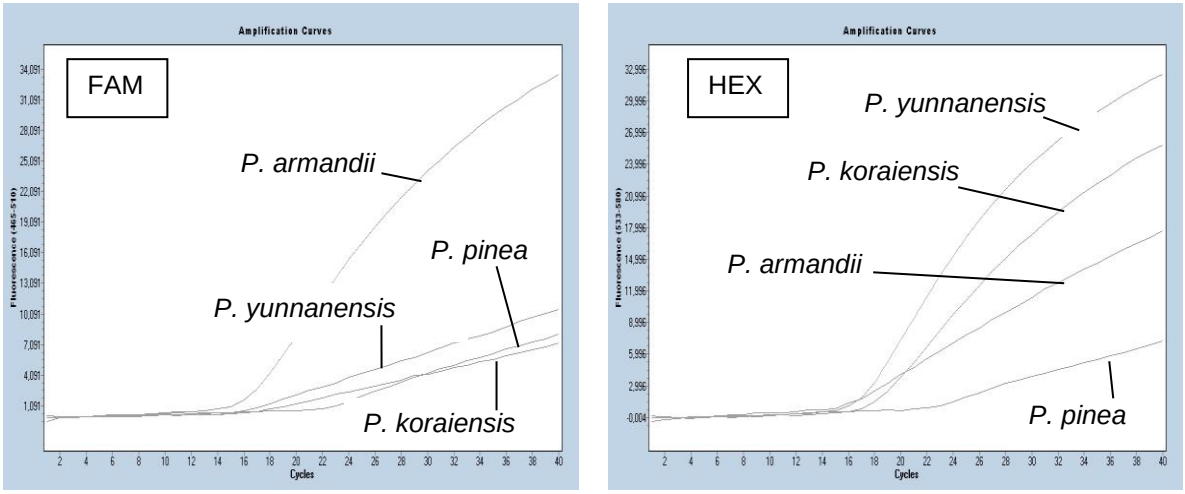


Figure 5: Amplification curb of some pine nut species with FAM (left) and HEX (right) signals.

Several matrices have been analyzed using this method and so far no cross reactivity has been observed. One of the next steps is to search for *P. armandii* in mixtures, in which the *P. armandii* nuts can be revealed at a concentration of 1% according to scientific literature.

Literature

Destaillats, F., Cruz-Hernandez, C., Giuffrida, F., & Dionisi, F. (2010). Identification of the botanical origin of pine nuts found in food products by gas-liquid chromatography analysis of fatty acid profile. *Journal of agricultural and food chemistry*, *58*(4): 2082–2087.

Doms, K. (2014). Identificatie van pijnboompitten van *Pinus armandii* door middel van qPCR (71p).

FAO (1998). Seeds, fruits, and cones. In: Non-wood forest products from Conifers. Food and Agriculture Organization of the United Nations, Rome.

Fardin-kia, A. R., Handy, S. M., & Rader, J. I. (2012). Characterization of Pine Nuts in the U.S. Market, Including Those Associated with " Pine Mouth " , by GC-FID. Journal of Agricultural and Food Chemistry, 60: 2701–2711.

FAVV (2011). Pijnboompitten met bittere nasmaak…? Nieuwsbrief van het federaal voedselagentschap 9e jaargang, Nr 41.

FAVV (2014). http://www.afsca.be/tips/pijnboompitten.asp

Flesch, F. (2010). Pignons de pin et dysgueusie retardée (19p).

Handy, S. M., Timme, R. E., Jacob, S. M., & Deeds, J. R. (2013). Development of a locked nucleic acid real-time polymerase chain reaction assay for the detection of Pinus armandii in mixed species pine nut samples associated with dysgeusia. *Journal of agricultural and food chemistry*, *61*(5): 1060–1066.

Köbler, H., Monakhova, Y.B., Kuballa, T., Tschiersch, C., Vancutsem, J., Thielert, G., Mohrning, A. and, & Lachenmeier, D. W. (2011). Nuclear magnetic resonance spectroscopy and chemometrics to identify pine nuts that cause taste disturbance. *Journal of Agricultural and Food Chemistry.*, *59*: 6877–6881.

Möller, G. (2010). The curious case of the epicurean nut. Food Technology Magazine 64(5): 1–6 .

Pasman, W.J., Heimerikx, J., Rubingh, C.M., van den Berg, R., O'Shea, M., Gambelli, L., H., & H.F.J., Einerhand, A.W.C., Scott, C., Keizer, H.G. and Mennen, L. I. (2008). The effect of Korean pine nut oil on in vitro CCK release, on appetite sensations and on gut hormones in post-menopausal overweight women. *Lipids in Health and Disease*, *7*: 10.

www.pinenut.com (2010): Why the price of pine nuts increased - the imported ones - and how to save $10 - $15 per pound 23/06/2010

Poeder, S. (2015). Bedreigt onze pestoconsumptie de Siberische tijger? National Geographic 29/12/2015 .

Slaght, J.C. (2015). Making Pesto? Hold the Pine Nuts. New York Times 19/10/2015.

Wang, X.R., Tsumura, Y., Yoshimaru, H., Nagasaka, K., Szmidt, A.E (1999). Phylogenetic relationships of Eurasian pines (Pinus, Pinaceae) based on chloroplast rbcL, MATK, RPL20-RPS18 spacer, and TRNV intron sequences. American Journal of Botany; 86(12):1742-53.

Wolff, R. L.; Pédrono, F.; Pasquier, E.; Marpeau, A. M. General characteristics of Pinus spp. seed fatty acid compositions, and importance of Δ5-olefinic acids in the taxonomy and phylogeny of the genus. Lipids 2000, 35, 1–22.

Zonneveld, B. J. M. (2011). Pine nut syndrome : a simple test for genome size of 12 pine nut – producing trees links the bitter aftertaste to nuts of *P. armandii* Zucc . ex End.l Plan*t Systematics and Evolution*. 297: 201–206.

Note from the editor

The pine nut research is a nice example of multidisciplinary research: it combines (descriptive) biology, analytical chemistry and molecular biology. A challenge here is the proper identification of mixtures of different species, especially in the case of edible species with one of those few that cause PNS. Analytical chemistry is targeted at trillions of copies of the same chemical compound, and the presence of some copies of an undesirable type can be mimicked. This is a largely different situation from biological identification. Here the number of items is limited (i.e. the individual nuts) and manual discrimination of different types can be the basis for further characterization of subsamples, either chemically or biologically; provided the right set of descriptors is at hand. In this way progress in food reliability can be achieved.

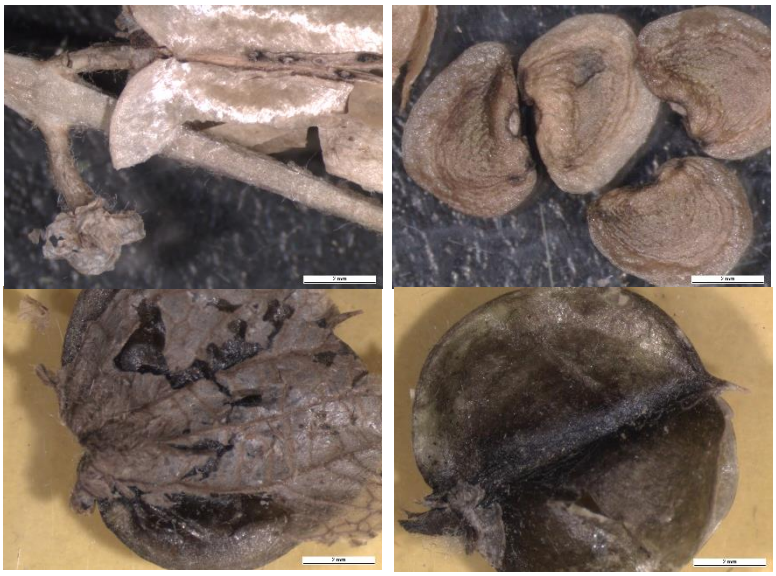
Request to IAG members to identify a toxic plant in hay (cf. *Rhinanthus*)

Geneviève Frick, Agroscope Posieux, Switzerland

A sample of hay which was suspected to be responsible for health problems when fed to horses, was analysed by microscopy. One unknown plant was found in relatively high proportion (see pictures below). With help of several IAG colleagues who sent pictures and references, the plant was determined as *Rhinanthus sp.* which seeds and vegetative parts have been described as being slightly toxic, at least when fresh.

This is a good example of how our IAG network can be useful and helpful for its members and their clients.

Overview of the selected material



Details of flower parts and fruits

Annual ring test composition

L.W.D. van Raamsdonk, N. van de Rhee, V. Pinckaers, J.J.M. Vliege, 2016. *IAG ring test composition 2016.* Wageningen, RIKILT Wageningen UR (University & Research centre), RIKILT report 2016.014.

Abstract

The analysis of composition in terms of ingredients is important for detecting economic fraud and for monitoring feed safety. Composition analysis and label control of feed is regulated in Regulation (EC) 767/2009. In a broader view, composition analysis in the entire food chain can improve the effect of monitoring actions. The new legislation on food labelling (Regulation (EC) 1169/2011), effective from December 13th 2014, obliges to provide more detailed information to customers on composition and related topics.


IAG ring test feed composition 2016

A ring test was organized for the microscopic determination of botanic composition in animal feed in the framework of the annual ring tests of the IAG - International Association for Feeding stuff Analysis, Section Feeding stuff Microscopy. The organizer of the ring test was 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 method for composition analysis of feed.

The sample was based on an artificially produced feed mimicking a ruminant feed, and distributed without label information. The participants were requested to produce a correct declaration of the ingredients of the sample. The results were analysed using the IAG model for uncertainty limits. Shares of ingredients in the feed formulation outside the limits of the model were indicated as under- or over-estimations.

A total of 25 sets of results was returned. The percentage of under- or over-estimations was 28.6% for the seven main ingredients. In the overview of results, the two declared wheat ingredients and the two declared corn products were pooled to one ingredient each. This was necessary since some participants declared a general ingredient (“wheat” and “corn”) and others a specific type (gluten or bran). The use of the original declarations would result in an extra number of non-matching estimations without precise justification. The share of the citrus pulp, in the presence of an equal amount of beet pulp, was underestimated or not detected in 44% of the results. Citrus pulp as such is recognisable as feed ingredient. Still almost three quarter of all estimations appeared to be correct in the ranges of the uncertainty model. This means that visual inspection of the composition of a sample can be used for label control and this method can support traceability of ingredients in case of an incidence.

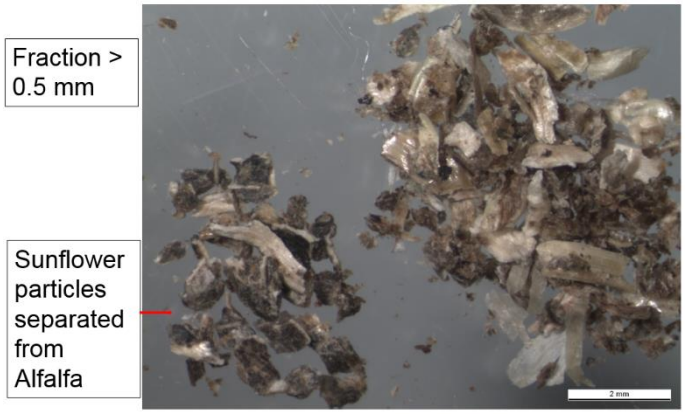
The current results indicate that specific formulations can influence the precision of the estimation of the composition of the feed. The current lack of a complementary system for (chemical) proximate analysis could be a drawback for the overall approach of supporting traceability, necessary for fighting food fraud and for supporting feed safety. Besides a proper method description and up-to-date descriptions of ingredients, well developed skills of technicians are vital for a good performance.

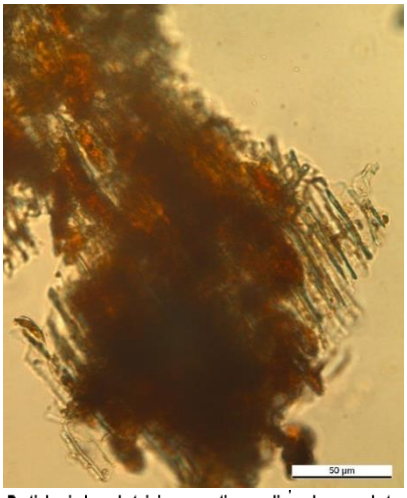
http://dx.doi.org/10.18174/393610

Sunflower cake in organic Alfalfa pellets

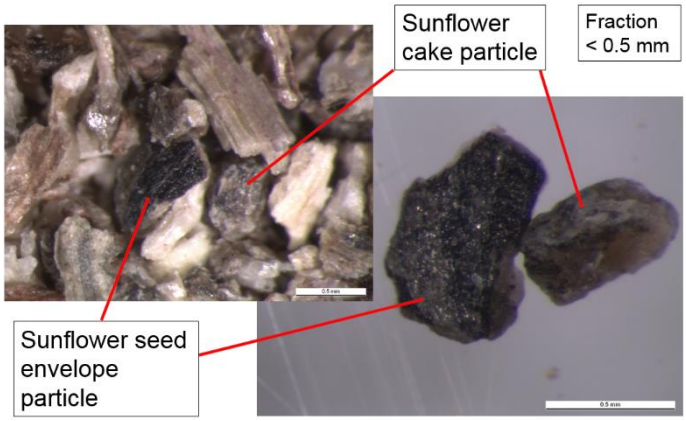
Genevieve Frick, Anton Vonlanthen, Agroscope Posieux, Switzerland

In the frame of the official Control of organic Feedingstuff, Glyphosate (a broad-spectrum systemic herbicide) was detected in 4 batches of organic Alfalfa pellets. By microscopy analysis, low amounts of sunflower cake particles were observed in these samples and sorted. The estimated percentages of sunflower in the different samples corresponded roughly with the Glyphosate contamination level. These observations helped asserting the explanation for the origin of the pesticide contamination. The fact that sunflower cake (a protein rich by-product of oil production) was present in the alfalfa could be a sign that it was intentionally added to increase the protein content of the material. Furthermore, this finding can indicate a possible pesticide contamination of the oil which was produced with the same sunflower seed batch. The explanation could have been a different one if only seed envelopes had been found. This because various seed hulls were mentioned by the producer to be used as fuel for the Alfalfa dryer and a contamination at this time-point would have been possible.





Particles in Lugol staining mounting medium show seed structures and protein rich alleurone cells.



Ring test Ambrosia in bird feed 2015

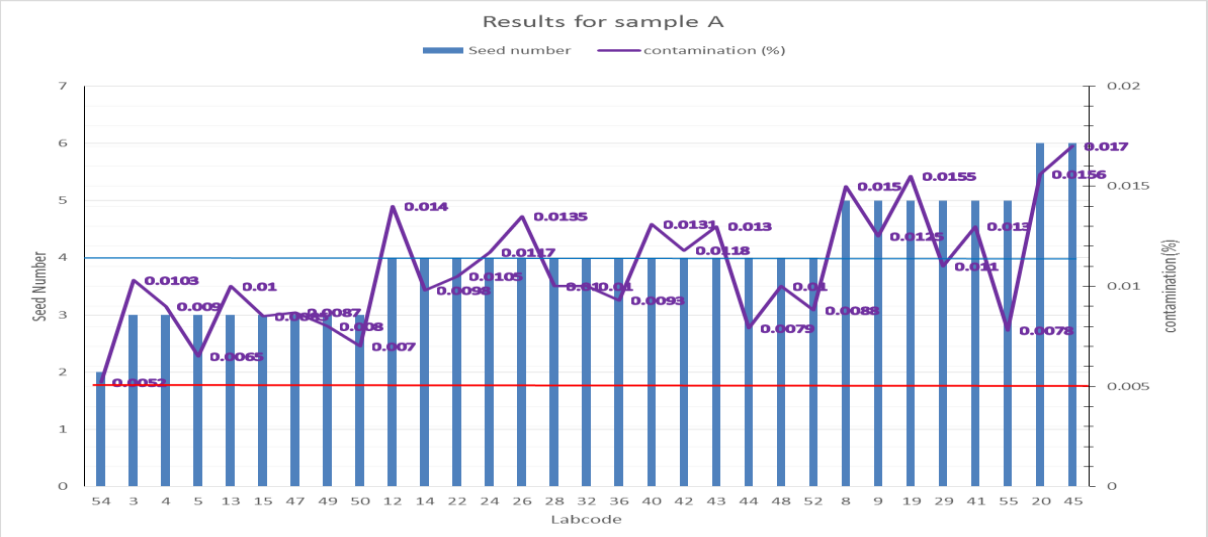
Geneviève Frick, Agroscope Posieux, Switzerland

The ring test Ambrosia in bird feed was aimed at exercising the detection of *Ambrosia* whole seeds (Directive 2002/32/EC). The test was organized in Autumn 2015 and comprised two samples of 200 g of bird feed spiked with a known number of *Ambrosia* seeds.

Sample A contained Sunflower, Wheat, Milocorn and Peanuts and 4 *Ambrosia artemisiifolia* seeds (approximately 0.01 %). Sample B contained Sunflower, Hemp and Linseed and 1 *A. artemisiifolia* seed (approximately 0.0025%). 31 participants out of 12 European countries delivered their results.

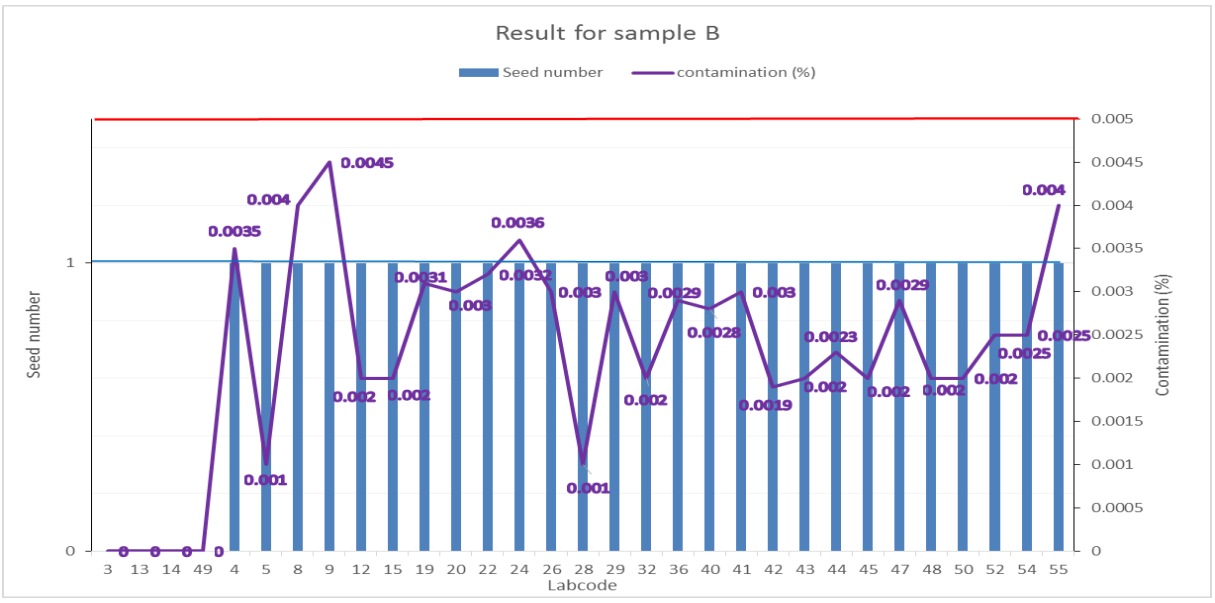
Sample A results

Nine participants (29 %) found 2 or 3 seeds only, whereas eight participants (26 %) found more than 4 seeds (showing that the matrix was probably not free of Ambrosia seeds). All reported results were above the tolerance limit (0.005 %).



Sample B results

Four participants (13 %) found no *Ambrosia* seed, while all other participants found just the 1 spiked seed. All results for the contamination percentage were below the tolerance limit (0.005 %).



Sieving

According to the IAG-Method A5, the sieve fractions between 1,5 –4,0 mm have to be analysed.

From the 10 participants who found less Ambrosia seeds than expected in one or two samples, five (50 %) used the recommended sieves, one (10%) used sieves with not recommended mesh size and four (40 %) did not use sieves.

From the 21 participants who found at least the right seed number in both samples, nine (43 %) used the recommended sieves, four (19%) used sieves with not recommended mesh size, and eight (38 %) did not use sieves.

Conclusions

At the chosen contamination levels (approximately 0.01% for sample A and 0.0025% for sample B), the results were consistent throughout the 31 participants in the perspective of the tolerance limit (above or below the tolerance limit of 0.005%). This is a good result especially in the view of an inadequate sample size (the IAG-Method A5 indicates to analyse at least 500 g).

Using sieves did not seem to influence the performance of the labs.

Seeds of A. artemisiifolia

A. artemisiifolia *in the experimental garden of Wageningen UR*.

Latest news on insects

On Tuesday December 13, 2016, the European Commission voted for the use of insects as ingredient in feed for aquaculture animals. This decision was expected early 2017, but was already taken now. This opening was effectuated by removing the last barrier, the need to have animals killed in official registered slaughterhouses, for processing insects. Regulation (EC) 999/2001 will be amended accordingly.

At the same time, insects for non-ruminant land animals remains prohibited. A spokesman of Wageningen UR assumes to have this ban lifted not earlier than 2020.

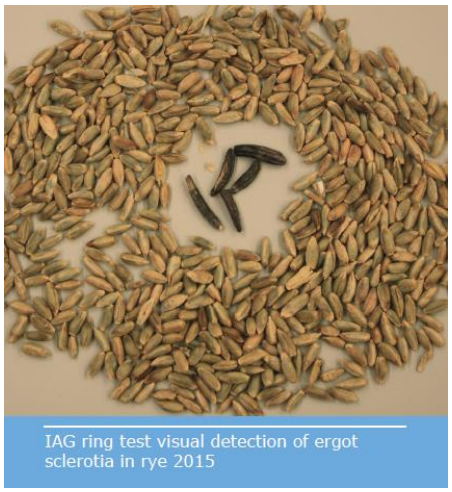
http://www.feednavigator.com/Regulation/Green-light-for-insect-protein-in-fish-feed-in-EU

http://ec.europa.eu/food/sites/food/files/safety/docs/reg-com_biosec_20161213_agenda.pdf

Ring test ergot sclerotia in unground rye 2015

L.W.D. van Raamsdonk, N. van de Rhee, J.J.M. Vliege, V.G.Z. Pinckaers, 2016. *IAG ring test visual detection of ergot sclerotia in rye 2015.* Wageningen, RIKILT Wageningen UR (University & Research centre), RIKILT report 2016.013.

Abstract



Ergot alkaloids are recognised as seriously toxic compounds, which caused a series of outbreaks in the past. In the EU, enforcement is implemented by visual detection and quantification of ergot sclerotia produced by moulds of the genus *Claviceps*.

On behalf of the IAG section Feedstuff Microscopy, RIKILT organised a ring test for the visual detection of ergot sclerotia in two unground rye samples in September 2015. In this report the results from the ring test for ergot in rye 2015 are presented.

The ring test ergot sclerotia in rye was designed to test the capability to visually detect sclerotia or parts thereof at relatively high levels. One sample was based on a level of approx. 400 ppm, and the second sample contained an amount of approx. 1000 ppm (EU legal limit for feeds and ingredients: 1000 ppm = 1 gram/kg = 0.1%). An amount of approx. 250 grams of rye grains was chosen as sample size. All samples were individually spiked. Thirty participants enrolled for the ring test. Participants were requested to report the number of recovered (fragments of) sclerotia and the total weight per sample. The percentage of recovery for every sample was calculated. A dedicated IAG method as well as other (lab internal) methods were allowed for application. Principally, methods are based on sieving (preferably with a mesh size of 0.5 mm), examination of every particle (grain) in the fraction with full grains or particles larger than 0.5 mm, selection of sclerotia fragments supported by documentation, and weighing the final selection of bodies.

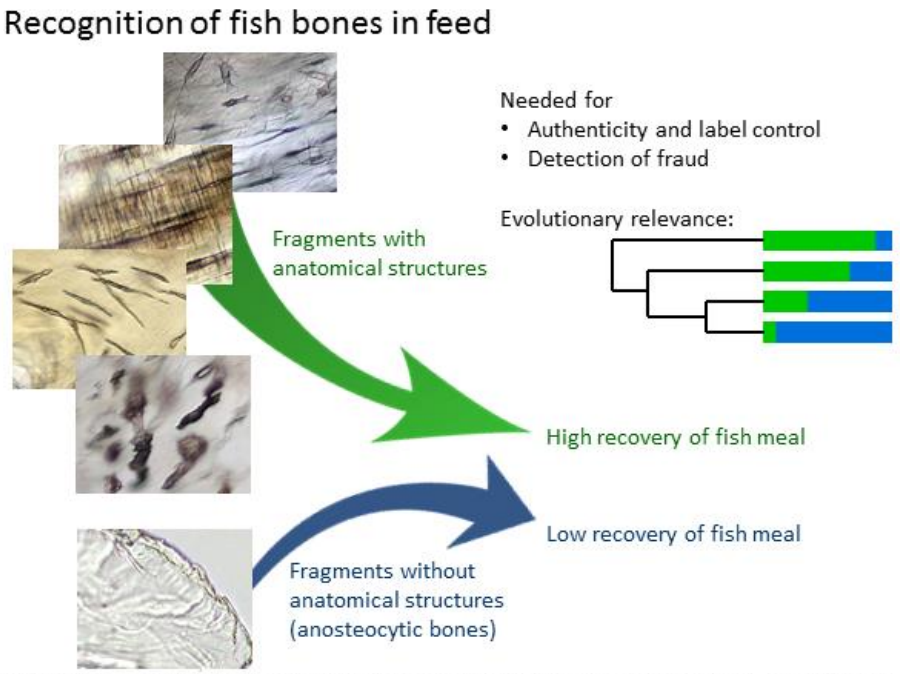
The average recovery for both samples was approx. 97%. All results except one were between the expected recovery limits (80 – 110 % w/w). Supporting data from a RIKILT intralaboratory validation study of the IAG method showed trueness at different low spike levels between 98 and 105% w/w. Limit of detection was established at 7 ppm.

It can be concluded that examination by visual detection of sclerotia is a valuable indicator of the expected presence of ergot alkaloids. The results of this study provides the data for a partial validation of the method of IAG for the examination of whole kernel cereal samples.

http://dx.doi.org/10.18174/393609

Recognition and identification of fish meal in compound feeds

L.W.D. van Raamsdonk, T.W. Prins, N. van de Rhee, J.J.M. Vliege, V.G.Z. Pinckaers. Paper submitted to special issue of the Feed conference of Food Additives and Contaminants, series A.



L.W.D. van Raamsdonk et al. Recognition and identification of fish meal in compound feeds. Food Add. Cont.

Fish meal is an accepted ingredient in compound feeds. Unauthorised application is primarily enforced by visual inspection, i.e. microscopy. In order to document the visually available diversity, fragments of bones and scales of 17 teleost fish species belonging to seven different orders have been investigated for their diversity in the presence of structural elements: lacunae and canaliculae in bone fragments and type of growth rings and teeth of scale fragments. Despite the classical division in cellular bones and acellular bones of teleost fish, i.e. whether or not possessing osteocytes, the current examinations revealed patterns of lacunae, in some types accompanied with canaliculae, in all 17 species investigated. In total seven types of bone structures have been defined, and six types of scale structures. Profiles with the relative frequency of each bone type per species were established. The share of acellular bone fragments appeared to be related to the evolutionary position of the species.

Results of proficiency tests for the detection of fish meal reveal that in most cases the sensitivity and specificity for the detection of fish meal is sufficient to perfect. Only some specified circumstances can hamper proper recognition and identification, most notably salmon bone fragments mimicking bone fragments from terrestrial animals, and pieces of hydrolysed proteins or minerals mimicking acellular fish bone fragments. The expertise gained in this study would help to improve the distinction between fish meal and terrestrial animal material in compound feed, and it supports the application of the species-to-species ban with respect to the valorisation of by-products from fish farms. A high share of

Alizarin staining of a fishmeal from Norway (left) and of an animal protein free mineral mix (right)

acellular bone fragments in commercial fish meals resulted in recovery rates below 65%. Alizarine staining of sediments did not result in reliably higher recovery rates. Two control counts of the mineral mix used to prepare the a-posteriori samples revealed that approximately 1% of the mineral particles appeared to be stained. It is recommended to document the Alizarine staining further before the staining procedure can be validated as part of official control.

In a broader perspective, the current expertise might be helpful to detect fraud throughout the feed and food production chain. The matrix of characteristics versus species will be implemented in a datamodel running in the expert system Determinator in order to facilitate identification.

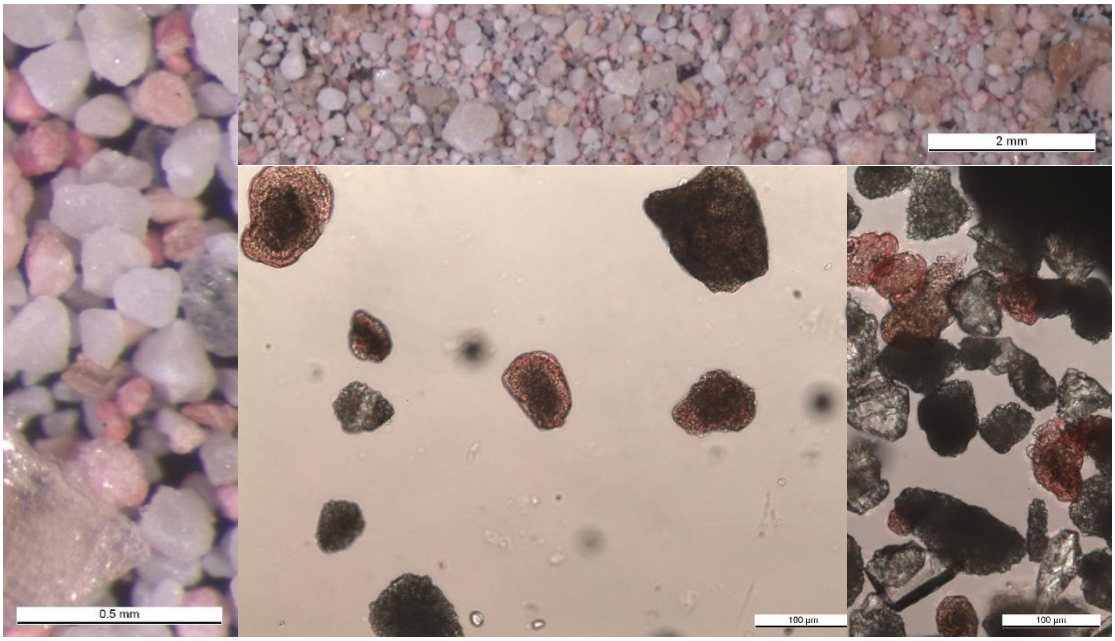
Some particles in sediment which are coloured by Alizarin red

Geneviève Frick, Agroscope Posieux, Switzerland

Photographic report of some particles being coloured by Alizarin Red (AR).

AR staining is described in EU 51/2013 and recommended in “EURL-AP Standard Operating Procedure, Use of staining reagents” to facilitate the screening of bones inside sediments.

The stain is not specific for the bone but it colours the bone major mineral constituent, hydroxyapatite. It is reported also to react with calcium phosphates (e.g. tricalcium phosphate). Therefore, structural features typical of bones (lacunae, canaliculi) must be considered too for determining a stained particle as from bone origin.



In our experience in Switzerland, some feed produce a sediment with nearly 50 % of red stained particles when treated with AR, which could not be classified as bones. We did not determine the nature of the particles, and the declaration did not mention tricalcium phosphate. These findings question the interest of performing AR staining to facilitate the detection of bone particles.

Insects as new feed ingredient

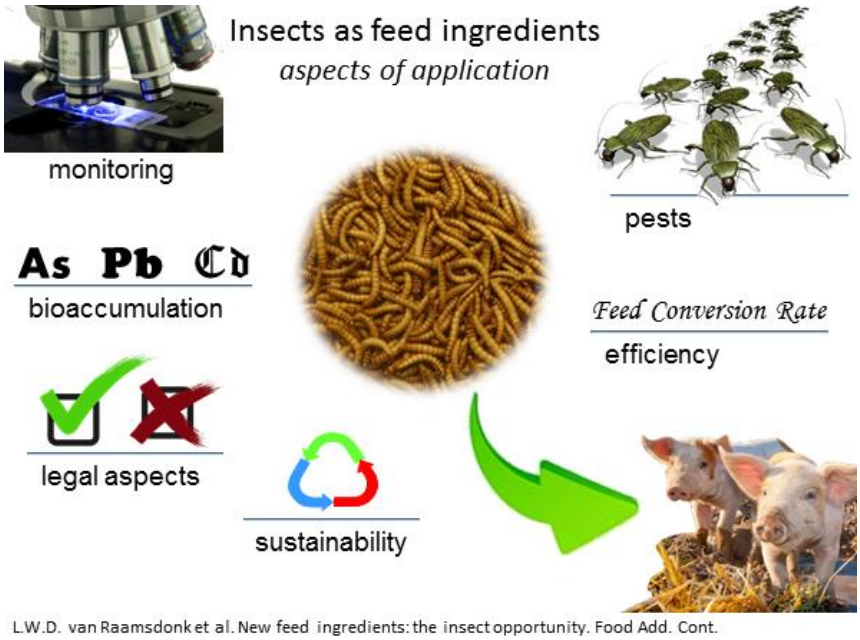
L.W.D. van Raamsdonk, H.J. van der Fels-Klerx and J. de Jong. Paper submitted to special issue of the Feed conference of Food Additives and Contaminants, series A.

In the framework of sustainability and a circular economy, new ingredients for feed are desired and, to this end, initiatives for implementing such novel ingredients have been started. The initiatives include a range of different sources, of which insects get particularly interest. Within the European Union, generally, a new feed ingredient should comply with legal constraints in terms of “yes, provided that” its safety commits to a range of legal limits for heavy metals, mycotoxins, pesticides, contaminants, pathogens etc. In the case of animal proteins, however, a second legal framework applies which is based on the principle “no, unless ....”. This legislation for eradicating Transmissible Spongiform Encephalopathy consists of prohibitions with a set of derogations applying to specific situations. Insects are currently considered animal proteins.

The use of insect proteins is a good case to illustrate this difference between a positive, although restricted, modus, and a negative modus for allowing animal proteins. This overview presents aspects in the areas of legislation, feed safety, environmental issues, efficiency and detection of the identity of insects.

Detection of insects as part of the composition of a feed can be carried out for several objectives: label control (Regulation (EC) 767/2009), traceability (Regulation (EC) 178/2002) and detection of fraud (Regulation (EC) 882/2004; Decision (EU) 2015/1918). The major recent reviews of the use of insects as feed material pay a limited attention to monitoring. However, it could be argued that a risk is higher when monitoring is not (fully) achievable.

Use of insects as extra step in the feed production chain cost extra energy and this results in a higher footprint. An Energy Conversion Rate is proposed to facilitate the comparison between production systems based on cold blooded versus warm blooded animals. Added value can be found by applying new commodities for rearing, including but not limited to category 2 animal by-products, catering and household waste including meat, and manure. Furthermore, monitoring of a correct use of insects is one possible approach for label control, traceability and prevention of fraud. The link between legislation and enforcement is strong. A principle called WISE (Witfull, Indicative, Societal demands, Enforcable) is launched for governing the relationship between the above mentioned aspects.



L.W.D. van Raamsdonk et al. New feed ingredients: the insect opportunity. Food Add. Cont.

Scheme of ring tests 2017

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 2017 ring test for the following situations:

- Test IAG-2017-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: € 230.
- Test IAG-2017-B. Declaration of the composition of a compound feed (one sample). This test was organised in 2014 by RIKILT as well (see abstract in this Newsletter). Targeted protocol: IAG method A2. Cost for participation: € 50.
- Test IAG-2017-C. Detection of botanic impurities (Directive 2002/32/EC) in two samples of ground compound feed. Targeted protocol: IAG methods A3 and A6. 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 € 400; in this case a discount of 10% will be granted, resulting in a total cost of € 360 for the total set of three tests.

The samples for test IAG-2017-A and IAG-2017-B will be sent around late February or early March 2017. 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-2017-C will be send in September 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 Uppsala (Sweden) in June 2017 (tests A and B) or in 2018 (test C). The final reports will be published later in either 2017 or 2018. 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 and Bruno.Hedemann@wur.nl . Subscription closes Thursday February 23rd, 2017. 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.

New colleagues at RIKILT

We can inform you that Nastasja van de Rhee has left RIKILT at 1 November. For 2017 we welcome two new colleagues. Corina Smits will start at 1 January. Bruno Hedemann will join our team per 1 February and he will assist in the organisation of the IAG ring tests.

Interlaboratory study analysis of botanic composition of feed

The analysis of the botanic composition is an important yet delicate process. The results of the 2016 proficiency test, as presented in this Newsletter, show that substantial expertise is needed. During the last few years RIKILT has invested in an expertise system supporting the identification of a range of different types of ingredients. Focus was on cereal by-products and on by-products of oil seeds. A total 26 ingredients are currently included, representing over 80 types of feed ingredients as specified in the Feed Catalogue (Regulation (EC) 68/2013).

The value of such a system needs to be assessed by validation studies. A preliminary in house validation was carried out at RIKILT. The next step is to collect information from external testing. RIKILT is planning the production of a set of samples (approx. 5-8) with either single ingredient as well as compound feeds, which should be identified by other laboratories. The intention is to base the identity solely on the information of the system.

Laboratories are invited to consider to participate in this test. Details will be distributed soon in 2017. Please reply to leo.vanraamsdonk@wur.nl . This initiative is not part of the annual ring test scheme of IAG section Feed Microscopy.

Thanks in advance.

L. van Raamsdonk and team, RIKILT, Wageningen

Closing remark

The topics in this Newsletter show some interesting highlights. At first it appears that microscopic inspection can support chemical analysis and can be used for the traceability of ingredients than might cause problems. This is documented by the cases of a toxic plant in hay, by the sunflower cake in alfalfa pellets and by the pine nut story. In general, the establishment of botanic composition, besides the legal basis of label control according to Regulation (EC) 767/2009, is necessary for monitoring authenticity. The combination of chemistry and biology is the key to gain in effectivity of feed and food safety monitoring. The announced interlaboratory study for the expert system on identification of feed ingredients is also an interesting activity.

The overview of research on pine nuts also reveals a second message. Besides feed analysis, food ingredients can be investigated by microscopic methods as well. In a range of cases the same methods or strategies can be applied to feed as well as to food topics.

The detection and identification of animal proteins is also a key topic. Recent information revealed that the Alizarin staining deserves further attention and is in need to be better documented. Insects are now getting full attention. It is important to had attention to microscopy, primarily for animal proteins, at the Feed Conference in Geel, last October.

Together with all the members of IAG section Feed Microscopy we will continue to contribute to efficient and effective feed and food safety monitoring. The board wishes you all Merry Christmas and a happy New Year.

Board of IAG section Feed Microscopy.