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Real time detection for food integrity – Are we there yet?

I recently wrote about some of the problems involved with testing foods for microbiological hazards. Part of that discussion centred on the fact that you can’t realistically test enough samples to give the necessary assurance that a lot is ‘clean’. But what if it was possible to test a large proportion, or possibly all, of the items being produced by some non-destructive real time means? While such a technology wouldn’t replace HACCP it would be a major boost for the tasks of verification and due diligence.

Multispectral imaging has been used to study the spoilage of beef¹ and image data shown to be correlated with bacterial counts (with some caveats). The real problem lies in the sensitivity. If a particular pathogen is not permitted in a 25g sample is it realistic to expect one cell in 25g to be detected? Not at the moment, but detection of indicators of contamination may be a solution. Near Infrared hyperspectral imaging has also been used to measure fat, water and protein in lamb² and such techniques also look to be promising in detecting fraudulent meat substitution³. A research tool that can identify meat species in less than five seconds is Rapid Evaporative Ionisation Mass Spectrometry (REIMS) when the ‘iknife’ sampling device is used⁴.

Other currently available approaches need a little more time for samples to be taken, although sampling need not be destructive and could be automated. The next level of time to detection is in the 1 hour to 1 day bracket⁵. For example, the LAMP assay mentioned in this issue can take only a few minutes to produce a result. Biosensors can detect bacteria rapidly but only in small volumes of liquid so the sensitivity is not equivalent to current slow conventional tests⁶. In this case it might be possible to introduce a short enrichment of a few hours to increase cell concentration making detection of viable cells more likely. There are some examples of this; for example, a 4.5h enrichment in testing for Salmonella⁷.

Microfluidic, or ‘lab-on-a-chip’ approaches allow detection of chemicals to be made in minutes⁸. The paper provides a table with the range of chemicals that have been detected using this technique and, as an example, bisphenol A was detected at 3.9ng/ml. However, both sample preparation and detection technologies remain key challenges.

Although it is not possible to cover all possible approaches in 600 words, the answer to the question in the title is, sorry but no.

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The gold standards in microbiological testing are the methods published by the International Standards Organisation (ISO) (www.iso.org/iso/home/about.htm). The drawback with the ISO methods is that they tend to rely on culture and can therefore be laborious and time-consuming, although they have the advantage of detecting only cells that are viable and able to multiply. However, for bacteria that might be in the hypothesised viable but non-culturable state\(^1\) and for those that are harmful even if dead at the time of analysis (e.g. by having released toxins into the food when viable), culture-based methods will not be appropriate. Molecular methods targeting nucleic acid sequences overcome some of these drawbacks and offer speed and higher specificity than culture-based methods.

Contamination of food products with pathogenic microorganisms is a major concern for food safety. The issue is exacerbated by modern production systems where high volumes, high speed and global distribution are common features. In order to protect consumers, regulations and procedures are in place to control microbiological hazards in the food chain, and testing food samples for a range of bacterial, fungal and viral pathogens is an essential element.

Loop-mediated isothermal nucleic acid amplification (LAMP) for food microbiology testing

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An extensive range of polymerase chain reaction (PCR)-based nucleic acid detection methods has been developed for the specific detection of numerous foodborne pathogens. A more recent development is the loop-mediated isothermal amplification (LAMP) technique and this is emerging as the basis for rapid testing and in-field applications. LAMP is a rapid DNA amplification method that relies on a DNA polymerase that is able to amplify DNA at constant temperature (typically 60°C-65°C). A minimum set of four primers (short DNA strands that specifically determine which DNA fragment will be amplified) is required which recognise six independent regions in the target DNA sequence, achieving high specificity. The amplification products are a variety of DNA molecules with a characteristic loop structure containing copies of the target sequence. An additional pair of primers (loop primers) complementary to the DNA sequence in the loop structure can be included to make the reaction faster.

Advantages of LAMP over PCR-based techniques

As LAMP amplifies DNA with high efficiency under isothermal conditions, it does not require expensive thermocycler instruments. Assay times are shorter than for PCR, typically 15-60 minutes, depending on the specific polymerase and assay conditions used.

LAMP is highly specific, avoiding both background noise often detected in other nucleic acid amplification techniques and interference by non-target DNA that might be present in the sample.

LAMP is less sensitive to inhibitory substances than PCR, and this can reduce the need to carry out expensive and time-consuming DNA purification procedures prior to testing.

The assay itself is simple and easy to perform, and does not per se require specialist instruments or a laboratory set up. A simple water bath or heating block is sufficient, which is

Figure 1: Results of a S. Typhimurium LAMP assay using fluorescence readout. The amplification graph shows the incorporation of the fluorescent dye into the reaction products. The anneal derivative graph shows the characteristic curves of the target and internal amplification control (IAC) amplification products. Red line: results obtained with a sample contaminated with S. Typhimurium; Blue line: results obtained with a sample not containing S. Typhimurium.

One LAMP-based method, for detection of Salmonella in soya meal, has been validated.
an advantage for deployment of the technology in remote areas or in low income regions. Detection of amplification products can be by the naked eye or via photometry for turbidity caused by the accumulation of magnesium pyrophosphate precipitate as a by-product of the reaction\textsuperscript{4}. Alternatively, the reaction can be followed in real-time by measuring fluorescence using dyes which interact with DNA or fluorescent probes (Figure 1, page 5), and relatively inexpensive portable instruments are now available for this.

**Appropriate controls for LAMP-based methods**

In order to avoid any false positive results an internal amplification control (IAC) can be incorporated into the reaction. This control may be a short sequence of DNA with recognition sequences for the same primers as the target but with a different nucleotide sequence elsewhere\textsuperscript{5,6}. Target DNA and IAC have different physico-chemical properties and therefore, following amplification, they can be easily distinguished from one another by monitoring their behaviour via fluorescence reading after a quick heating step. The amount of IAC for a given assay is optimised to minimise competition with the target DNA. If the sample contains no target DNA, the IAC will be amplified demonstrating that the assay ‘worked’ and no target was present. This control is essential in samples that may contain inhibitors of DNA polymerases. Although LAMP polymerases are very robust, when using crude samples of variable composition, e.g. poultry litter, a certain level of inhibition might occur. The presence of the IAC-specific signal will then serve as confirmation of the negative status of the sample, rather than a failed test which without an IAC would be indistinguishable from a negative result.

Future prospects of LAMP in food microbiology testing

LAMP technology offers great potential for food microbiology and indeed for biological diagnostics in general due to its sensitivity, specificity, speed and practical applicability (Figure 2 illustrates a rapid procedure for carcass swab testing using LAMP). The technology is clearly emerging as a valuable tool for applications where a rapid answer is required regarding the

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*Figure 2: Workflow of a carcass swab testing procedure using LAMP. Results are obtained within one hour*
presence/absence of a microorganism to inform decision making and possible further analysis. Most of the applications described thus far have the limitation of not being able to discriminate living from dead, or viable but non-culturable cells. Nonetheless, recent reports have described developments in the use of chemicals such as propidium monoazide to block the amplification of DNA from dead cells prior to LAMP. An alternative option is the detection of mRNA as target since this is only found in viable cells.

Many other improvements to the LAMP technologies are progressing concerning multiplexing, integration with sample preparation, equipment-free procedures, etc. However, an important barrier to acceptance and implementation of molecular-based methods for routine food safety testing is lack of validation. A novel method should be shown to be as effective as the corresponding standard method in order to be fully acceptable as an alternative to the standard, but this has only been done for very few non-proprietary molecular methods for foodborne pathogen detection. Only one LAMP-based method, that for detection of Salmonella spp. in soya meal, has been validated against the corresponding standard ISO 6579. However, the high utility of LAMP warrants consideration of investment to develop efficient modern methods for rapid and sensitive foodborne pathogen detection.

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About the Authors

Rosario Romero has a long track record in cell biology and protein biochemistry in biomedical research. Since joining Fera in 2011, Rosario has been leading work on applications of protein tools and technologies to food research, with a particular interest in developing proteomics methods for food safety.

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Best practice for crystallisation kinetics measurements via NMR

Low resolution or pulsed NMR (nuclear magnetic resonance) is an easy and fast way to determine solid fat content (SFC) of cocoa butter and other fats. It can also be used to observe the crystallisation process. However, in literature several ways of sample preparation and handling, especially for crystallisation kinetics measurement, are reported. These differences in preparation might not be critical when comparing results from one laboratory, but could be crucial when interlaboratory results are compared. The latter one is the case when it comes to quality control of raw materials. In this study different sample preparation and handling methods were used and their impact on the final results was investigated.

Today, in most cases NMR is used for standardised solid fat content (SFC) measurement⁴. However, measuring crystallisation and melting processes of cocoa butter was already described in 1980⁵. Ever since NMR was used to obtain crystallisation kinetics, but sample preparation and measuring conditions varied considerably⁴. In this study we compare proceedings described in literature and hand out advice on best practice. To facilitate the evaluation of influencing factors, standardised SFC measurements were used. Each measurement consisted of three scans with 2s recycle delay. Temperature was recorded using a thermologer Almemo 8590-9. For temperature measurement inside the NMR probe head fiber optics were used.

For SFC measurements a direct⁶ or an indirect⁷ method can be used⁴. The direct method is used for pure fat samples without particles, such as cocoa butter, while the indirect method is suitable for samples containing particles, such as chocolate or cocoa nibs, liquor and powder⁴.

Sample preparation
A difference between the direct and the indirect method, which will be in focus at the end of the article, is the filling height of the samples. Samples are placed in glass tubes with 10mm diameter. For measurements using the direct method, filling height is 30-40mm; for indirect method it is 8-10mm⁴. This means that the volume of samples that needs to be cooled is four times smaller in case of the indirect method compared to the volume used for direct method measurements. For the smaller filling height the spacer was used. The heat release of cocoa butter and vegetable oil samples with eight and 32mm filling height in a tempered NMR probe head can be seen in Figure 1 (page 9).

Due to the lesser warming of the sample with smaller filling height, we suggest the indirect method for crystallisation kinetic measurements.

Sample handling
Before each measurement samples were fully melted and homogenised before placing the respective amount in an NMR tube. Prior to the measurement, the sample was heated to 70°C for 30min to erase thermal history. Afterwards crystallisation is initiated by putting the sample into an aluminum block, water bath or in the tempered NMR-probe head.
In doing so, the sample might be rocked. Rocking might disturb the arrangement of the molecules or it might initiate the crystallisation especially in pure cocoa butter samples. Keeping your sample steady is recommended.

Choosing a crystallisation temperature
The temperature of the probe-head, which is the measuring temperature, is often chosen in accordance to DSC measurements. However, it could also be in the range of MultiTherm or Shukoff measurements, which would be at 17.6°C and 0°C or 8°C, respectively. Therefore the temperature progression in an NMR tube with cocoa butter at a filling height of 8mm was measured for different given temperatures of an aluminum block in a water bath, shown in Figure 2.

All curves show a temperature decrease in the first 5-10min. The release of latent crystallisation heat starts earlier for lower given temperatures. At 19, 18 and 17 °C the temperatures decrease in the beginning and the increase during crystallisation are baseline separated. This is not the case for lower temperatures.

Sample tempering
Sample tempering can be done in a water bath, in an external aluminum block or inside the NMR. The heat release in a water bath is better due to improved heat transfer in water compared to air. However, care has to be taken when wiping off the water. To investigate if the remaining water influences the measurement 20 samples were prepared as described for standardised SFC measurement. The samples were divided in two groups in which the last tempering step of 1h at 20°C varied. For one group the last step was water free in an aluminum block tempered in a water bath. The second group was placed directly in the water bath and the water was wiped off the tubes just before the measurement.

A t-test showed, that the two groups are statistically significant different with $\alpha=0.05$ and $P<0.001$.

However, heat transfer in an aluminum block and inside the NMR probe head also differs. Therefore the temperature progression in a cocoa butter sample (filling height: 8mm) in an aluminum block and in the NMR probe head (with NMR turned off) at 19°C was compared, as can be seen in Figure 3.

The temperature equilibrates faster in the aluminum block than in the NMR probe head. There is hardly a baseline separation in the sample cooled in the NMR probe head.

Wiping off the water from a sample tempered indirectly in a water bath is individually different and depends on the operator. Therefore tempering in an external aluminum block or directly in the NMR probe-head is recommended. External tempering allows performing several measurements at the same time by hand or by using an auto sampler. However, when the sample is moved by hand, rocking and shaking should be avoided. Internal tempering can be used with automated measurement, but for only one sample at a time.

Probe-head tempering during the measurement
During measurements with external tempering, the NMR probe-head can optionally be tempered to the crystallising temperature too. The use of an external tempering unit without tempering the probe head was simulated by manually putting a molten sample (cocoa butter with filling height of 8mm) in an aluminum block cooled to 19°C for 2min followed by 6s in an aluminum block at 40°C. This procedure was repeated until the latent heat release during crystallisation could no longer be observed. The temperature progression of a cocoa butter sample kept in the aluminum block at 19°C and a sample with simulated measurement for 6s at 40°C is shown in Figure 3.
The results show, that even during the short measuring time, the sample warms up. Temperature increases up to 2°C and thus increases the effective crystallisation temperature and extends the heat release of the sample. Therefore tempering the NMR probe-head to crystallisation temperature is always recommended.

Choosing your method: direct vs. indirect

For the direct method only one measurement is needed. The ratio of the signal in the beginning, including the solid and the liquid phase, and the second signal, which includes the signal of the liquid phase is used to calculate the SFC. Due to the dead time, the first signal of the solid and liquid phase cannot be detected directly after the high frequency impulse. An extrapolation factor \( f \) is used to calculate the signal at time \( t=0 \). Therefore the direct method results in an approximated value because of the \( f \)-factor for several reasons. First, a linear extrapolation is assumed to calculate the signal at \( t=0 \), but the signal shows a non-linear decrease. Additionally, the \( f \)-factor depends on the molecular mobility, e.g. temperature, polymorphism and crystal size\(^{10,11}\). Additionally its temperature dependency is also due to the dilatation of the liquid phase. Since the \( f \)-factor depends on the polymorphic state, it changes during the crystallisation process, which can be seen in Figure 4. From the recorded signals, the direct SFC, the indirect SFC and the \( f \)-factor were calculated as described in\(^6\).

Another influencing value is the solid phase in samples with non-fat solids. Therefore two measurements of a sample, one at the solidified and one at melted state are necessary to distinguish between protons in the crystallised fat and those in the solid non-fat material of chocolate (non-fat cocoa solids, sugar). Ziegleder et al. (1998) described this problem and developed a correction function to calculate the SFC of chocolate\(^8\).

To avoid these insecurities, we recommend the indirect method. For the indirect method, only the signal of the liquid phase is used\(^6\). The signal of the molten and the crystallised sample, as well as the signal of a reference at the same temperature is measured. The values of the reference are needed for temperature correction. As reference oils, which are liquid at the measuring temperature range, e.g. sunflower, canola or hazelnut oil can be used. Additionally, online recording of the temperature throughout the measurement would allow considering the temperature increase during crystallisation.

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6. Solid fat content (SFC) by low-resolution nuclear magnetic resonance—the indirect method.
Dr Tarun Anumol, LC-MS Applications Scientist, Market Development Group, Agilent Technologies, was the speaker for this webinar. Tarun obtained his PhD in Environmental Engineering at the University of Arizona working with Dr Shane Snyder in 2014. He also has a Master’s in Civil & Environmental Engineering from Carnegie Mellon University and a Bachelor’s degree in Chemical Engineering from India. His work focuses on developing sensitive analytical techniques using LC-MS technologies for detection of emerging organic contaminants in the Environment and Food.

In his presentation Tarun discussed sample extraction procedures, specifically the effectiveness of the Enhanced Matrix Removal (EMR)-Lipid for >100 veterinary drugs in bovine meat. In addition to this extraction procedure, and others more traditionally used, he also talked about complementary methods such as a rapid and sensitive method for veterinary drugs analysis using the UHPLC-MS/MS with low ng/g reporting limits.

The webinar is now available on demand on the New Food website.

On the 17th January 2017 New Food presented this webinar on veterinary drugs in meat, sponsored by Agilent Technologies. This type of analysis is challenging due to the vast number of disparate analytes and complex matrix. This webinar provided a complete overview of potential solutions to the issues faced with analysis of veterinary drugs in meat, including sample extraction using the Enhanced matrix removal (EMR)-Lipid along with a rapid and robust quantitative method using UHPLC-MS/MS for >100 veterinary drugs targeted by the USDA in meat.

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Introduction – the mycotoxin problem

The mycotoxin contamination of crops is a major concern for stakeholders along the food and feed chain. The European Commission pointed out that 5–10% of global crop production is lost annually due to mycotoxin contamination. The total costs of losses due to mycotoxin contamination, such as reduced yields; food and feed losses; increased costs for inspection and analyses; as well as others, may easily reach billions of Euros annually, as estimated by Mitchell and colleagues.

Mycotoxin-contaminated cereals and derived products, such as dried distillers’ grain solubles (DDGS) in animal feed, impact livestock production as well. For example, the decreased weight gain in pigs in the USA, due to including 20% DDGS contaminated with fumonisins in the feed, is estimated to result in losses of up to US$147 million annually.

The social impact of mycotoxin contamination is also of growing concern since recent reports suggest possible links between aflatoxin exposure and the stunting of children in Africa. Furthermore, extreme weather events due to climate change impact the mycotoxin map worldwide, thus challenging existing forecasting and detection methods of mycotoxins in food and feed. Despite previous achievements, the risk of mycotoxin exposure is still high: 81% of globally collected feed samples were contaminated with at least one mycotoxin and 45% contained more than one secondary metabolite of fungi. Furthermore, most notifications registered in the EU’s Rapid Alert System for Food and Feed (RASFF) between 2004 and 2015 were related to mycotoxin contamination exceeding the maximum regulatory limits (see Figure 1, page 13). Thus, despite previous achievements in tackling mycotoxin exposure from food and feed, continuous and collaborative research is needed.

MyToolBox – the smart way to tackle mycotoxins

To tackle these issues, existing knowledge must be combined with novel findings to bridge gaps on mycotoxin reduction along the food and feed chain. By using mainstream information and communication technology (ICT), losses and waste along the food and feed chain can be prevented and traceable information to the supply chain and consumers can be provided. This is the mission of MyToolBox (www.mytoolbox.eu) (see Figure 2, page 13), a four year project, which is funded by the European Commission (EC) and was launched in March 2016. The project applies a multi-actor and multi-disciplinary approach throughout the food and feed chain with 40% industry participation including five end-users and three well-known institutions from China, who will collaborate closely with
farmers and stakeholders from the industry. The end-user engagement, which extends to the food and feed industry, farming communities, agronomists, manufacturers, SMEs and academia from the EU and beyond, will ensure the usability and applicability of the MyToolBox decision support system.

The overall objective of the MyToolBox project is to develop a series of integrated measures that reduce different kinds of losses that result from mycotoxin contamination. MyToolBox specifically addresses the most prevalent Fusarium mycotoxins (Deoxynivalenol [DON], T-2 toxin, HT-2 toxin, Zearalenon [ZEA] and fumonisins) in wheat, oats, maize and animal feed, ochratoxin A (OTA) in wheat and aflatoxins in maize, peanuts and dried figs. Besides a field-to-fork approach, MyToolBox also considers safe use options of mycotoxin-contaminated batches to efficiently produce biogas and bioethanol, thus considering alternative use options of otherwise wasted cereal badges. Legislative implications are also foreseen, for which advice is sought from institutions experienced in establishing regulatory limits for contaminants in foods and feeds, such as from representatives from the European Food Safety Authority (EFSA) and the EC’s Directorate-General DG Santé. Eventually, a sound scientific basis for setting authorisation standards of mycotoxin detoxifying feed additives in China will complement the project’s goals.

Expected impacts along the food and feed chain
The pre- and post-harvest measures should lead to significant economic and food safety benefits and improvements in mycotoxin management along the food and feed chains, leading to a reduction of contamination incidents and more transparent communication in the supply chains. Furthermore, farmers and other stakeholders along the food and feed chain will benefit financially by saving resources (e.g. fertilisers, seeds, etc.) and by lowering the risk of rejection of their products due to unexpected contamination. As such, mycotoxin-related border rejections noted by RASFF are expected to decrease further. Overall, the integrated measures are expected to lead to a 20-90% reduction of mycotoxin contamination in food and feed, thus contributing to improved consumer trust in imported food and feed.

The pre-harvest measure focus on in-field strategies – to determine the most promising available novel cultural biocontrol strategies targeting the Fusarium inoculum within crop debris, Brassica biofumigation, as well as accelerated biodegradation with an extract or competitive fungi – are being explored. To increase the climate change resilience of EU-maize farmers and thus reduce susceptibility of maize to aflatoxin contamination, MyToolBox is testing two measures: (1) The selection of appropriate genotypes of maize with various traits, and (2) The application of atoxigenic Aspergillus strains to out-compete toxigenic Aspergillus strains in the field. In addition available forecasting models regarding DON contamination in wheat and maize are being validated, combined and extended to cover the most important wheat and maize growing areas in Europe.

Post-harvest strategies span from the sorting of crops, over storage and further processing; up to alternative uses of contaminated batches. In order to achieve significant improvements in the sorting of dried figs (and dramatically improve the work environment) MyToolBox is developing a non-invasive system for the real-time sorting of contaminated dried figs, based on visible and near infrared (NIR) spectral imaging and appropriate algorithms. Innovative pre-milling and milling strategies are combined and up-scaled to provide more accurate separation of grain tissues that have characterised (different) mycotoxin contamination levels. Milling strategies, such as debranning and micronisation, are efficient, but individual tissues of the grains are often inhomogeneous and not well confined. The synergistic potential of debranning, micronisation and dry- or wet-turbo separation will minimise the mycotoxin concentration in raw materials and the final wheat products destined for the consumer. Within MyToolBox the fate of mycotoxins, as well as their modified forms and co-occurrences in naturally contaminated raw materials (with simultaneous co-occurrence of different mycotoxins), will be monitored during thermal food processing at an industrial scale with Barilla (Parma, Italy), one of the main Italian pasta manufacturers. The potential of monitoring mycotoxin concentrations and modifications during food processing will be further explored and adapted in other relevant industries.
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The MyToolBox project does not only pursue a field-to-fork approach to mitigate and reduce mycotoxin contamination in crops, feed and food. This will lead to the increased trust of consumers in the high quality of EU food and feed, which will strengthen the economic position of the EU food producers.

Conclusion

The MyToolBox studies also positively contribute to the current food vs. fuel debate, due to our intention to utilise mycotoxin contaminated batches to produce biofuels on the one hand, and high quality DDGS on the other. In DDGS, a major protein source in feeding stuffs derived from bioethanol production, the mycotoxin concentration can be increased by a factor of three compared to the starting maize. To reduce the mycotoxin contamination of DDGS, the MyToolBox industry partners Südzucker (Mannheim, Germany) and BIOMIN (Getzersdorf, Austria), in cooperation with BOKU/IFA-Tulln, will study the effects of specific mycotoxin-degrading microorganism strains and novel enzymes on the reduction of DON and fumonisins in DDGS.

The EU-China partnership within MyToolBox aims to contribute to the standard setting for authorisation of mycotoxin-detoxifying feed additives in China, wherein current EU guidelines for the registration shall serve as examples for possible adoptions of Chinese legislation. This shall include standardised procedures to verify the safety and in vivo efficacy of detoxifying feed additives. Currently in China, up to 24% of complete feed for pigs have exceeded the maximum limits of deoxynivalenol set by the Chinese authorities. Since approximately half of the world’s total pig population is farmed in China, such legislation is expected to also improve the efficiency of Chinese pork-meat production.

Finally, the web-based MyToolBox platform will represent a one-stop-shop for farmers and other decision makers in the food and feed supply chain. Consisting of information and advisory modules, as well as management tools and technologies, such individuals will be equipped with systematic, cost effective and affordable approaches for the effective monitoring and reduction of mycotoxin contamination in crops, food and feed.

The authors are grateful to all partners’ contributions, in particular Simon Edwards from Harper Adams University, Michele Suman from Barilla, Naresh Magan and Angel Medina Vaya from Cranfield University, Ine van der Fels-Klerx from RIKILT Wageningen University & Research, Vittorio Rossi from HORTA S.R.I., John Gilbert from FoodLife International, Edurme Gaston Estanga from IRIS and Michaela Pichler from ICC.

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Birgit Poschmaier is the Project Manager of MyToolBox, with experience in safeguards management in World Bank financed projects. As a PhD student at the BOKU she will take a closer look at the social and economic impacts of integrated mitigation and reduction strategies against mycotoxin contamination.

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Simultaneous identification and absolute quantification of a multitude of relevant parameters with reference to NMR distribution

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