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Food Safety

Herbal warfare

Plant based food and feed can contain toxic pyrrolizidine alkaloids (PAs) due to co-harvesting of weeds that contain PAs. Experts therefore recommend testing crops known to co-exist with PA containing weeds before use in food or feed. The method of choice for direct determination is solid phase extraction (SPE) cleanup followed by LC-MS/MS analysis.

By Guido Deussing

Green and lush pastures and fields present a wonderful sight. A closer look, however, can reveal more than just healthy greens and harmless edible plants. Among the crops we grow for use as food and feed, wild herbs crop up that are a thorn in the flesh of farmers and horse breeders. In temperate zones in Europe, ragwort is a ubiquitous, particularly toxic wildflower, which looks similar to plants of the arugula family. Unlike arugula, however, it is anything but wholesome. Ragwort and related species contain toxic pyrrolizidine alkaloids (PAs), presumably synthesized by the plants as a defense against herbivorous enemies. PAs are natural toxins whose toxicity is not limited to insects: Many are toxic both to humans and to the herbivores we rely on for meat and dairy products. Some PAs cause liver damage and are both genotoxic and carcinogenic. Regular intake of even small amounts can lead to a creeping process of progressive damage to internal organs, as can be observed in horses and cattle. PA poisoning can lead to serious illness and even death.

How big is the threat?

The challenge facing producers and consumers is significant: A total of more than 6,000 plant species produce PAs, equal to about 3 % of the total number of flowering plants world-wide as calculated by BLL, the German Federation for Food Law and Food Science. BLL is the main industry association of the German food sector.

More than 600 PAs and their N-oxides have been found and characterized in more than 350 different plant species Worldwide, according to the Federal German Institute for Risk Assessment and Consumer Safety (BfR) in Berlin. PA toxicity has long been established; a recent scientific publication by US FDA scientists also established the toxicity of a number of PA N-oxides, recorded to induce Hepatic Sinusoidal Obsctruction Syndrome (HSOS) in humans, and further confirming PA N-oxide-induced hepatotoxicity on mice with hepatotoxicity similar to, but potency much lower than, the corresponding PAs.

The FDA scientists conclude that the levels of both PAs and PA N-oxides in herbs and foods should be regulated and controlled [2]. As an example, PAs have been found in herbs used for herbal infusions - also referred to as herbal teas. Examples are fennel-, chamomile-, herbal-, peppermint-, nettle-, and garden balm (Melissa) tea. In 2013, the BfR assessed the situation and concluded that PA levels in some foods (herbal tea, rooibos tea, black and green tea as well as honey) can pose a threat to children and adults alike when these foods are consumed on a regular (chronic) basis and that effort should be made "to minimize the PA contents in herbal teas and teas in order to minimize the putative higher cancer risk of frequent consumers and in particular of children" [1].

Consumer protection

The widespread occurrence of PA-synthesizing plants worldwide means that we as consumers should accept the fact that the risk of having PAs in food and feed cannot be totally eliminated. This may be part of the reason that until now, no country has introduced legally binding maximum concentration levels, relying instead on recommendations. In 2001, the United States Food and Drug Administration (FDA) issued a ban of comfrey based products marketed for internal use, and a warning label for those intended for external use. In an opinion published by the BfR, food producing companies have an obligation to take steps to ensure that PA levels in food are reduced. In preparation for such action, and based on a request from the European Commission, the European Food Safety Authorities (EFSA) in 2016 published a scientific report entitled: Dietary exposure assessment to pyrrolizidine alkaloids in the European population [3]. A further extensive publication in the form of a "Statement" was published by the EFSA in 2017, entitled: Risks for human health related to the presence of pyrrolizidine alkaloids in honey, tea, herbal infusions and food supplements [4]. In summary, the EFSA recommends gathering more toxicological data, expanding the proposed list of 17 PAs to be monitored, and developing more sensitive analysis methods. EFSA currently sees no possibility of establishing a tolerable daily intake (TDI) value. In Germany, the BfR therefore recommends a zero-tolerance approach. While this is commendable, it is highly unlikely to be implemented in practice. According to the BfR, the aim of following this approach is simply to keep PA levels in food and feed as low as possible and to support this effort by monitoring, which requires efficient and highly sensitive chemical analysis including sample preparation.

Analysis methods

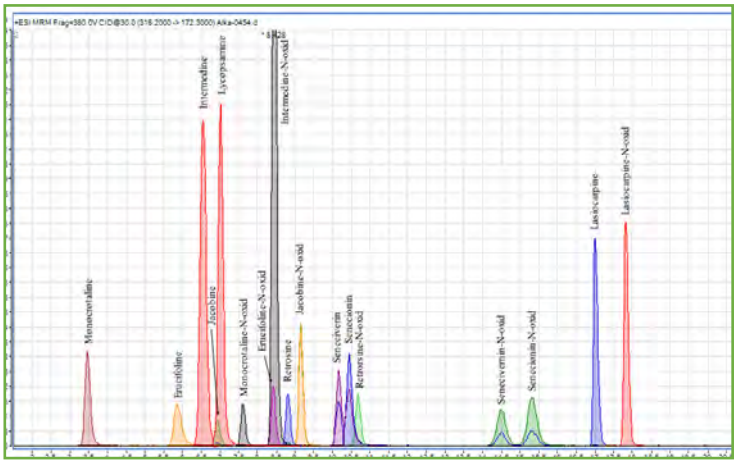
Monitoring PAs in agricultural products, such as food and feed, is not exactly easy: PAs are structurally diverse, they are present in a wide range of products and the analysis poses a real challenge. Over the past years, scientists at the BfR have developed accurate and reliable methods for the determination of PAs. These methods have been validated in round-robin tests. The methods could be implemented in food and feed monitoring performed by individual German states, but currently only a limited number of PA standards are commercially available. This has led the BfR to develop additional analysis methods that can be used to estimate the total amount of PAs in a sample. To determine PAs in plant material, the BfR recommends analyte concentration using solid phase extraction (SPE) followed by LC-MS/MS determination: The PAs are extracted from plant material in an ultrasonic bath using diluted sulfuric acid. The material is extracted twice and the extracts combined and centrifuged. An aliquot of the supernatant is taken for solid phase extraction (SPE) using a C18-phase. Following elution with methanol, the eluate is evaporated to dryness and the residue taken up in a methanol-water mixture. Finally, the sample is injected for chromatographic separation and MS/MS detection [1].

Automation provides added value

A contract laboratory that specializes in the analysis of food and feed requires efficient sample processing in order to be competitive. That also applies to PA, of course. "This is where I see a deficit of the BfR methods", says Franziska Chmelka, Food Technologist at TeLA GmbH, "the sample preparation is quite labor intensive". TeLA is an accredited contract laboratory for food and environmental analysis in Northern Germany. In order to improve the productivity of the BfR analysis method, TeLA automated it, including sample preparation. Franziska Chmelka and her colleagues used the GERSTEL MultiPurpose Sampler (MPS) to automate the required sample preparation steps for the PA determination. A key component of the system is automated SPE in combination with LC-MS/MS (Agilent Technologies 1290 HPLC and 6495 Triple Quadropol LC/MS).

Instrument setup used for the automated determination of pyrrolizidine alkaloids: GERSTEL MultiPurpose Sampler (MPS) configured for automated SPE in combination with an LC-MS/MS system from Agilent Technologies (1290 HPLC and 6495 Triple Quadrupole LC/MS).

Separation of the analytes was performed using a Standard-RP-Phase column (Nucleodur C18 HTec 250 x 2 mm x 5 µm, Macherey-Nagel) and gradient elution with 5 mM formic acid (Eluent A) and methanol (Eluent B): 0 min (5 % B) – 3 min (5 % B) – 7 min (20 % B) – 13 min



Analysis of a standard mixture of 17 pyrrolizidine alkaloids resulted in a clean chromatogram with well resolved peaks. Good separation of the individual PA compounds is extremely important. Many exhibit very similar mass transitions and can only be distinguished and identified unequivocally when peaks are fully resolved. (Source: TeLA GmbH)

(20 % B) – 16 min (65 % B) – 17 min (95 % B) – 20,1 min (5 % B). The flow rate was 0.25 mL/min and the column temperature 28 °C. A 5 µL aliquot of the eluate was injected. Target analytes were detected in Multiple Reaction Monitoring (MRM) mode, ESI positive.

For method development, an aqueous standard solution was used, containing 17 different PAs: Monocrotaline, erucifoline, intermedine, jacobine, lycopsamin, monocrotaline N-oxide, erucifoline N-oxide, intermedine N-oxide, retrorsine, jacobine N-oxide, senecivernine, senecionine, retrorsine N-oxide, senecivernine N-oxide, senecionin N-oxide, lasiocarpine and lasiocarpine N-oxide.

Automated SPE to the rescue

"Our focus when developing the method was on automating the most labor intensive step in the process, which is without a doubt the solid phase extraction (SPE)", said Franziska Chmelka. After completing their initial experiments, the application experts from TeLA decided to use a C18-RP sorbent (Macherey-Nagel C18 ec 3 mL/500 mg).

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Chopped Chamomile (left), spiked with 0.1 % ragwort (right).

All SPE steps were successfully automated: Conditioning the sorbent with 5 mL of methanol and 5 mL of water; injecting 5 mL sample onto the sorbent; as well as eluting the analytes with 5 mL methanol. "We also succeeded", says Norbert Helle, Ph.D., President and Owner of TeLA GmbH and established LC/MS expert, "in automating the remainder of the process: Evaporating the eluate to dryness, taking up the residue in 1 mL of a 10 % methanol solution, and injecting 5 µL of the resulting extract into the LC-MS/MS-System".

Analysis of the standard mixture resulted in a clean chromatogram with well resolved peaks. Franziska Chmelka explains the focus: "Good separation of individual peaks is essential. Many of the compounds have similar mass transitions and we must be able to differentiate between the different analytes. That is only possible if we have retention time differences." The application experts from TeLA then put both the method and the instrument setup to the test by running real samples, in this case, ragwort leaves. "All statistically relevant parameters confirmed that our automated SPE-LC-MS/MS method is delivering accurate results", says Franziska Chmelka. Good linearity was achieved across a wide concentration range, going as low as 1 ng/mL, and recoveries ranged from 85 to 98 percent for all analytes. The overall method reproducibility, including sample preparation, was in the range from 1.3 to 4.8 percent for all analytes. Franziska Chmelka: "It was especially important to us to achieve good retention time stability as this is a key indicator of method ruggedness. Using our method, retention times for the analytes only varied between 0.063 and 0.35 percent over a period of several days. Finally, the limits of determination we can reach are between 0.05 and 0.5 µg/kg".

In a further step, the TeLA team tested how many PAs are actually transferred to the herbal infusions we brew and drink and how much PA is transferred in total: Dry Chamomile material was spiked with different levels of Ragwort; the amounts added were 10.1 and 0.1 percent w/w relative to the chamomile. As Franziska Chmelka reports: "Just adding 0.1 percent ragwort resulted in our finding a significant amount of PAs in the brewed herbal infusion".

Conclusion

The automated SPE-LC-MS/MS analysis method was successfully used to determine PAs in various food and feed samples. In all cases, the method worked well and provided good results. Looking forward, the method needs to be extended. A larger number of PAs will need to be monitored and the sensitivity of the method must be improved. In its current form, however, the method provides highly satisfactory results, as Franziska Chmelka reports.

Suggested bedside reading (292 pages):
US Food and Drug Administration, FDA. Bad Bug Book, Foodborne Pathogenic Microorganisms and Natural Toxins. Second Edition.
https://www.fda.gov/downloads/Food/FoodborneIllnessContaminants/UCM297627.pdf

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