

## Herbicide monitoring

# A global presence: Speaking of Glyphosate

Automated derivatization, cleanup and LC-MS/MS determination of glyphosate and its main metabolite aminomethylphosphonic acid (AMPA).

**G**lyphosate-based broad spectrum herbicides are the most widely used chemical weedkillers in the world. Herbicides containing glyphosate are used by agricultural businesses to clear fields before sowing.

Glyphosate doesn't target particular plants, but rather destroys virtually any plant except those bred to be resistant. Integrated in a formulation that facilitates distribution from root to leaf, glyphosate [N-(phosphonomethyl)glycine] radically targets a plant's metabolism by bonding manganese, an essential element for all organisms. This, in turn, disrupts synthesis of the enzyme 5-enolpyruvyl-shikimate-3-phosphate (EPSP) synthase. The plant in question is subsequently deprived of essential metabolites and dies.

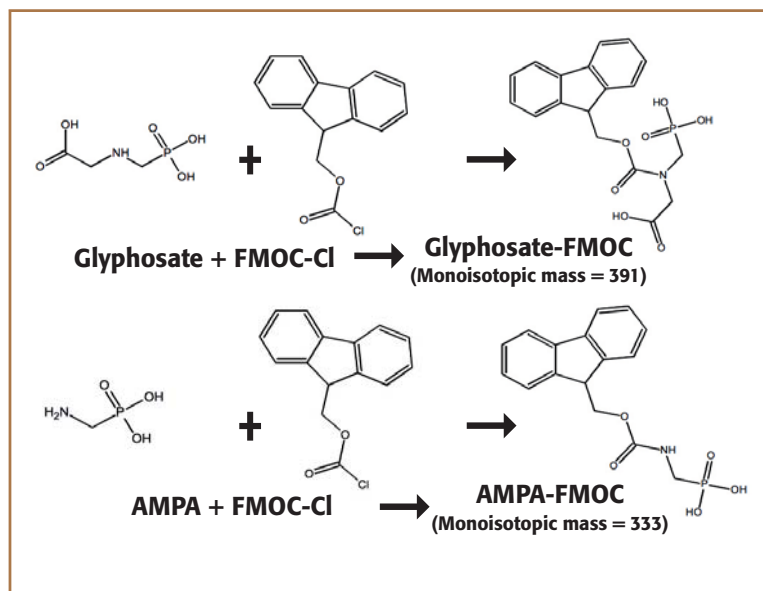
The glyphosate business is booming internationally thanks to transgenic crops such as corn and soy. Typically, the genetic makeup of these plants is altered to render them resistant to glyphosate. Simply put, the more transgenic plants are cultivated, the more glyphosate will be used. In addition, private individuals apply glypho-

sate for gardening purposes in significant quantities. After all, glyphosate is a home gardener's best friend and it eliminates a lot of tedious and back-breaking work such as weeding out flowerbeds. Products that con-

Indeed, the best-known glyphosate product (Roundup®) is sold in 130 countries.

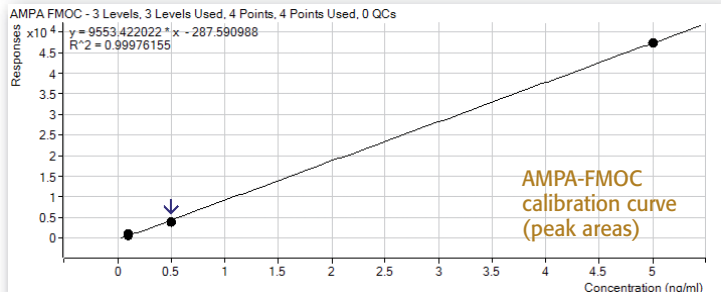
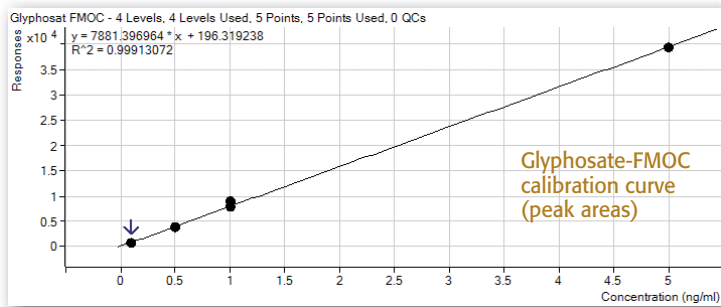
The success of glyphosate was long attributed to its purported environmental compatibility including its limited half-life in the environment. According to the US-EPA, glyphosate readily and completely biodegrades even under low temperature conditions with an average half-life in soil of about 60 days and in water of just a few days, part of which can probably be attributed to adsorption on surfaces [1]. However, recent studies have indicated that glyphosate may harm the agricultural ecosystem. Furthermore, the contention is that the absence of wild weeds and herbs after glyphosate use reduces biodiversity in the food chain [2] and is suspected of making plants more susceptible to diseases and of reducing the availability of nutrients for plants. This, in turn, triggers an increased reliance on pesticides and fertilizers. According to the

same studies, more herbicide will ultimately be used in the medium term, as ever more weeds are developing resistance to glyphosate. For these reasons, environmentalists



Structural representations of glyphosate, AMPA and their FMOC-derivatives: Efficiently automated derivatization of AMPA and glyphosate with FMOC-Cl enables accurate and sensitive determination with excellent throughput.

tain glyphosate are readily available in garden centers, hardware stores, and online. If you search for glyphosate on eBay, you will find no shortage of offers of inexpensive products.



Calibration curves for glyphosate-FMOCC (top) and AMPA-FMOCC (bottom) in water.  $R^2$  for both curves were  $>0.999$  from 0.1 – 5 ng/mL.

are demanding that the widespread herbicidal use of glyphosate be addressed with renewed urgency, pointing to plans for cultivating glyphosate-resistant, genetically modified plants in Europe.

Conversely, Germany's influential Federal Institute for Risk Assessment (BfR) has concluded that there is no risk to human reproduction as alleged by environmentalists. According to the BfR: "Not only the EU, but also the WHO and the US-EPA have come to the conclusion that glyphosate is detrimental neither to the reproduction nor the development of mammals including humans" [3]

Whether there is cause for alarm or not, the increased awareness has led to increased demand for reliable and sensitive determination of glyphosate. Monitoring would apply both to agricultural products, for food safety reasons, and to environmental samples such as soil and water.

## Analysis Method

Glyphosate and its most important and main metabolite, aminomethylphosphonic acid (AMPA) are generally determined using HPLC, coupled with multidimensional mass selective detection (MS/MS). When we developed our method, we wanted to achieve LC-MS/MS trace-level determination of glyphosate and AMPA in water using direct injection. The focus was not on copying existing methods, but rather on developing them further and automating them for higher throughput, lower limits of detection (LODs) and improved accuracy while using standard systems. In particular, we wanted to improve method sensitivity using a concentration step. Due to their high polarity, glyphosate and AMPA are difficult to analyze directly using HPLC. However, the compounds can be derivatized using 9-fluorenylmethyl chloroformate (FMOC-Cl) as described in method DIN/ISO 214587 [4], which has been used for this analysis for many years and is based

on HPLC fluorescence detection. Derivatization in our case was performed using a combined autosampler and liquid handling robot (GERSTEL MultiPurpose Sampler).

While derivatization combined with direct injection to the analysis system is used successfully for water samples, food samples will require additional SPE cleanup. Commonly used direct large volume injection methods without sample clean-up are severely influenced by matrix, making accurate and reliable determination in food very difficult. The method presented here combines derivatization using FMOC-Cl with cleanup on an on-line high pressure SPE system (GERSTEL SPE<sup>xos</sup>) positioned between the autosampler and the HPLC system (1290 UHPLC, Agilent Technologies).

Conventional SPE systems rely on cartridges with 100 to 1000 mg of sorbent. SPE<sup>xos</sup> uses small cartridges with only 50 mg of sorbent, allowing elution to be carried out with significantly smaller volumes of solvent. The cartridge can be inserted into the mobile phase used for the HPLC separation and the eluate transferred quantitatively to the separation column, resulting in improved recovery, a higher concentration factor, and low LODs.

Automated sample preparation takes only 20 minutes and is performed simultaneously with the HPLC analysis run of the preceding sample. This means that overall analysis time per sample in a batch of samples will amount to only 20 min. The timing of the sample preparation can be controlled to ensure that samples are injected exactly at the point in time when the HPLC run of the previous sample has finished. A 6460 TripleQuad mass spectrometer (Agilent Technologies) was used as MS/MS detector. The FMOC derivatives of Glyphosate and AMPA were detected in negative ion mode (ESI). The mass transitions monitored were 390 to 168 and 390 to 150 for glyphosate; for AMPA 332 to 136 and 332 to 110 were monitored.



MPS-SPE<sup>xos</sup>-HPLC-MS/MS-System used for glyphosate/AMPA analysis.



## Sample Preparation

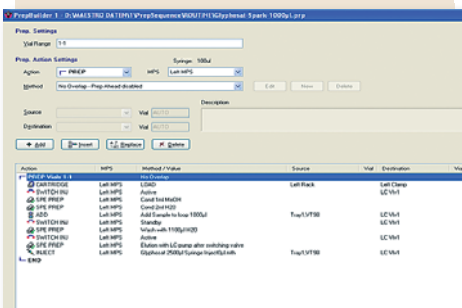
### Extraction

Blended wheat was extracted with acidified water, neutralized and diluted with water. Honey was dissolved in water at approximately 60 °C. Soil samples were extracted using water. Blended tea leaves were extracted with acidified water, neutralized and diluted with water.

### Derivatization

Following extraction, extracts were placed in the MPS autosampler, which added derivatization reagent to each extract and finally injected a 1000 µL aliquot of the extract into the online SPE system for cleanup (see box).

### Solid phase extraction and on-line sample introduction



Listed below are the individual sample preparation steps that are performed synchronizing automated derivatization and SPE<sup>XOS</sup> cleanup with the LC-MS/MS analysis. During LC-MS/MS analysis of a sample, the next sample is prepared just in time to be ready for injection when the LC-MS/MS system becomes ready:

Load the online SPE<sup>XOS</sup> cartridge

Condition the cartridge with 1 mL of Methanol

Rinse the cartridge with 2 mL of water

Introduce 1 mL of sample

Wash the cartridge adsorbent bed with 1.1 mL of water

Switch SPE<sup>XOS</sup> from MPS mode, i.e. sample preparation- and extraction mode, to injection mode for transfer of the eluate to the HPLC system

Elution and derivatization of the next Sample

All steps were fully automated. The sample preparation was completed in 20 minutes. The HPLC-MS/MS analysis cycle time was around 20 minutes.

## Results

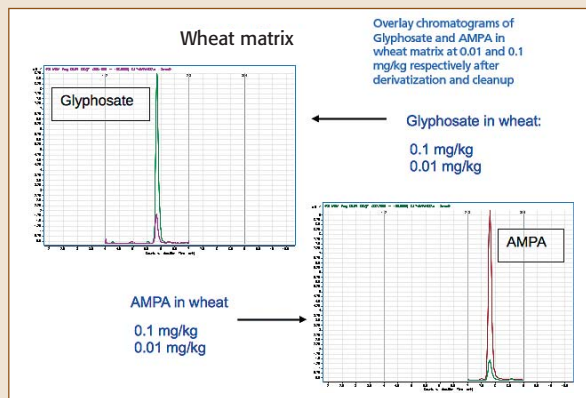
### The limit of quantification (LOQ) and limit of detection (LOD) of glyphosate and AMPA in wheat, water, tea leaves, and honey samples.

Sample	LOQ	LOD
Wheat	< 1.0 µg/kg	< 0.3 µg/kg
Water	< 10.0 ng/L	< 3.0 ng/L
Tea leaves	< 1.0 µg/kg	< 0.3 µg/kg
Honey	< 1.0 µg/kg	< 0.3 µg/kg

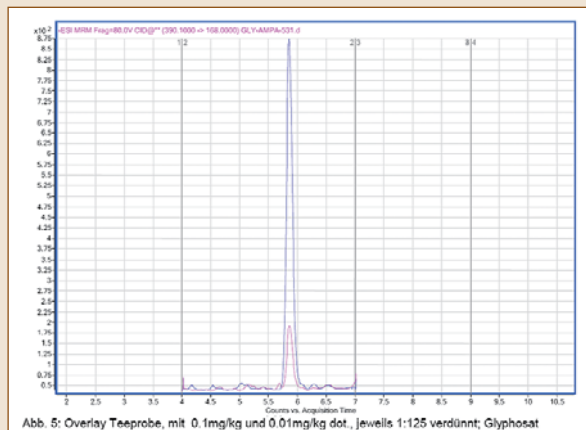
Note: Maximum Residue Level (MRL) in the EU for glyphosate in wheat: 10 mg/kg (US: 30 mg/kg). The LOQ achieved here is substantially lower. EU regulations require glyphosate levels in water to be below 100 ng/L (US: 700 µg/L\*). The LOQ achieved using the presented method is substantially lower.

Maximum Residue Level (MRL) in the EU for glyphosate in tea leaves: 2 mg/kg (US: 1 mg/kg). The LOQ achieved here is substantially lower. The MRL in honey according to EU regulations is 0.05 mg/kg. As can be seen, the LOQ achieved using the presented method is substantially lower.

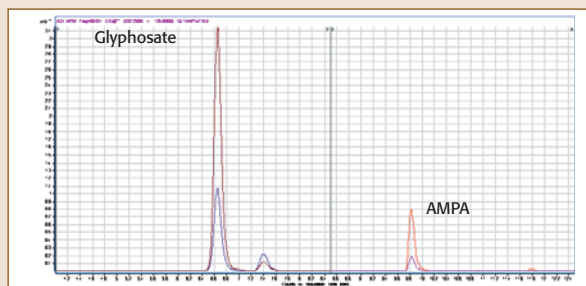
\* water.epa.gov/drink/contaminants/index.cfm US EPA: Drinking Water Standards Maximum Contaminant Level (MCL): 0.7 mg/L, USDA: MRL Wheat: 30 ppm (EU: 10 ppm)



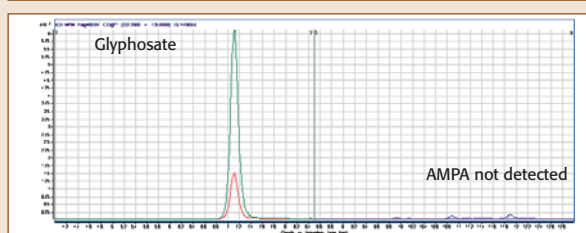
Chromatograms resulting from the extraction of spiked wheat samples. Glyphosate concentrations 0.01 mg/kg (red trace) and 0.1 mg/kg (green trace) respectively. AMPA concentrations 0.01 mg/kg (green trace) and 0.1 mg/kg (red trace) respectively. Mass transitions monitored: 390 → 168 and 390 → 150 for glyphosate; 332 → 136 and 332 → 110 for AMPA.



Overlay chromatograms showing glyphosate in tea spiked at 0.01 mg/kg and 0.1 mg/kg respectively.



Chromatogram resulting from the extraction of a soil sample from Tenerife. Glyphosate concentration found: Approximately 740 µg/kg; AMPA concentration approximately 17 µg/kg. The two traces shown for each compound reflect the two transitions monitored for each compound.



Chromatogram resulting from the extraction of a one year old honey sample from an area where glyphosate is being used. Glyphosate concentration: Approximately 4 mg/kg, approximately 80 times the allowable concentration; AMPA concentration: Not detected.

## Results and discussion

The analysis method enables the determination of glyphosate and AMPA in wheat, water, tea leaves, honey, and soil at sufficiently low limits of detection and limits of quantitation to meet EU guideline requirements as shown in the table on page 8. On page 7 the calibration curves for glyphosate and AMPA in water are shown. For both compounds,  $R^2$ -values of  $>0.999$  are reached.

## Conclusion

The presented automated method for derivatization, online SPE and HPLC-MS/MS determination of glyphosate and its most important metabolite, aminomethylphosphonic acid (AMPA) was successfully implemented for a variety of samples, including spiked water samples, tea leaves, wheat, honey and soil. Analysis of a dilution series produced excellent linearity in the order of 0.999 and low limits of quantitation (LOQ) of 10 ng/L for both glyphosate and AMPA in water. Recovery of glyphosate and AMPA in water, wheat and tea leaves was found to be in the range from 90 to 105 %.

The automated method resulted in LOQs below 1 µg/kg for both glyphosate and AMPA in wheat, tea, and honey, easily meeting EU



Glyphosate is one of the most widely used herbicides in agriculture.

requirements. The same LOQs were reached for soil samples. Variation coefficients reached using the automated system were lower than those typically achieved using manual procedures since the timing of each individual step is accurately and uniformly controlled for all samples such that any decomposition of derivatized analytes would not impact accuracy and precision.

We would like to emphasize, that the AMPA concentrations found in non-spiked real samples of soil and honey were surprisingly low relative to the glyphosate concentra-

tions. This indicates that glyphosate could be more stable than is widely assumed. The same was found to be the case in real-world samples of wheat that we have analyzed.

We have found the presented method to be sensitive, repeatable and robust. Since quite small SPE cartridges are used, the amount of solvent used is significantly lower compared with standard SPE methods.

In our future projects, we will pursue the automation of further methods that up to now require manual liquid sample preparation and SPE clean-up.

## Literature

- [1] US EPA "Technical Fact Sheet on: GLYPHOSATE"; [www.epa.gov/ogwdw/pdfs/factsheets/soc/tech/glyphosa.pdf](http://www.epa.gov/ogwdw/pdfs/factsheets/soc/tech/glyphosa.pdf)
- [2] Greenpeace-Report „Herbicide tolerance and GM crops“; [www.greenpeace.de/fileadmin/gpd/user\\_upload/themen/gentechnik/Herbicide\\_tolerance\\_and\\_GM\\_crops\\_lo\\_res.pdf](http://www.greenpeace.de/fileadmin/gpd/user_upload/themen/gentechnik/Herbicide_tolerance_and_GM_crops_lo_res.pdf)
- [3] Frequently Asked Questions on the Health Risk Assessment of Glyphosate, BfR FAQs, November 11, 2011; [www.bfr.bund.de/cm/349/frequently-asked-questions-on-the-health-risk-assessment-of-glyphosate.pdf](http://www.bfr.bund.de/cm/349/frequently-asked-questions-on-the-health-risk-assessment-of-glyphosate.pdf)
- [4] International Organization for Standardization (ISO) Technical Committee ISO/TC 147, Water quality, subcommittee SC2, Physical, chemical and biochemical methods: Determination of Glyphosate and AMPA using high performance liquid chromatography (HPLC) with fluorescence detection.

## Authors

**Norbert Helle**, PhD, is the owner and general manager of TeLA GmbH, a contract laboratory in the field of Food Safety Analysis. Norbert Helle has more than 15 years experience working in food safety and environmental analysis for various German Federal and State agencies, mainly in the field of HPLC-MS. He chairs two working groups under the German § 64 Foodstuffs and Commodities Act charged with developing food safety analysis methods for phycotoxins and biogenic amines.

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