A Time-Efficient Method for Pesticide Analysis in Beer

Fully Automated Derivatisation and Quantification of Glyphosate and AMPA using a Standard LC-MS/MS System

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By using a standard LC-MS/MS system set-up, the analysis of glyphosate and AMPA in beer could be simplified, without requiring an additional instrument, e.g. a liquid handling system for sample pretreatment. By staggering the pretreatment and the LC-MS/MS analysis, the method is very time-efficient. Calibration curves showed excellent precision and accuracy, and even in a complex matrix such as beer, Glyphosate and AMPA (metabolite aminomethylphosphonic acid) can be quantified at or below 5 ng/mL, which is below the European Union (EU) maximum residue levels (MRL). 60% of all tested beer samples contained traces of glyphosate, but all of them were far below MRL.

Introduction

Glyphosate is currently one of the most common pesticides used worldwide. In spite of its approval by regulatory bodies all over the world, the concern about its harm to humans and the environment persists [1, 2]. Therefore, the strict control of glyphosate and its metabolite aminomethylphosphonic acid (AMPA) in food and environment is mandatory.

In 2016, the year of the 500th anniversary of the German Beer Purity law, glyphosate has gained dubious fame after being found in many German beers [3]. While there are defined maximum residue levels (MRL) for drinking water and some food products, there is no dedicated MRL for beer [4]. In this case, the MRL of the individual ingredients apply, e.g. the MRL for barley which is used for malt production (20 mg/kg) or for hops (0.1 mg/kg). These values are far above the MRL for drinking water (0.1 µg/L).

Fully automated derivatisation followed by LC-MS/MS analysis

The quantification of glyphosate and AMPA is very challenging. Both molecules are highly polar which results in poor retention on reversed-phase columns. On the other hand, there are not many transitions for LC-MS analysis available for these molecules due to their low molecular weight. Additionally, beer is a complex matrix that requires chromatography of high integrity.

In order to overcome the low retention of glyphosate on reversed-phase columns, there is a well-established method that includes a derivatisation step with 9-fluorenylmethyl chloroformate (FMOC) [4] followed by LC-MS analysis. FMOC derivatisation leads to a decreased polarity of the target compounds, resulting in increased retention on a standard reversed-phase column. The derivatisation requires several pipetting steps. They can be executed manually, which is tedious and prone to errors, or automatically, which requires dedicated additional hardware.

This article reports a fully automated derivatisation followed by LC-MS/MS analysis of beer samples. The instrumental set-up does not require any additional hardware for sample pretreatment, but uses the built-in pretreatment function of the autosampler.

Experimental

Sample preparation

A beer sample of 500 µL was mixed with 500 µL methanol in order to precipitate proteins. After vortexing, the samples were centrifuged (15 minutes, 12,000 g) and placed into the autosampler, which handled all further sample pretreatment steps in a fully automated manner.

Pretreatment steps:

UHPLC method	
Instrument:	Nexera UHPLC, Shimadzu
Column:	Gemini 5 µm C18, 150 mm x 2 mm (L x i.d.)
Mobile phase A:	2 mM NH_4HCO_3, pH 9.5
B:	Acetonitrile
Injection vol.:	50 µL
Column temperature:	35°C

MS conditions	
Instrument:	LCMS-8060, Shimadzu
Ionisation:	pos/neg ESI
Nebulising gas:	3 L/min
Heating gas:	15 L/min
Drying gas:	5 L/min
Interface temperature:	325°C
DL temperature:	150°C
Heat block temperature:	400°C
CID gas:	270 kPa
Interface voltage:	4 kV/ -3 kV

Table 1: UHPLC method

Temps (min)	% B	Flow rate	Valve position
0.00	5	0.4	to waste
2.50			to MS
5.00			to waste
7.00	50	0.4	
7.01	95	0.6	
12.00	95	0.6	
12.01	5	0.6	
14.00	5	0.6	
14.01	5	0.4	
15.00	5	0.4	

Table 2: QC sample results

	Glyphosate-FMOC						AMPA-FMOC					
	QC 3 ng/mL		QC 15 ng/mL		QC 75 ng/mL		QC 3 ng/mL		QC 15 ng/mL		QC 75 ng/mL	
Batch	Conc.	Acc.%	Conc.	Acc.%	Conc.	Acc.%	Conc.	Acc.%	Conc.	Acc.%	Conc.	Acc.%
A	2.60	86.5	14.89	99.3	74.14	98.9	4.76	158.5	15.66	104.4	80.80	107.7
A	2.87	95.7	14.96	99.7	81.22	108.3	2.71	90.3	16.16	107.7	85.65	114.2
A	3.41	113.5	15.14	100.9	77.94	103.9	3.15	105.0	15.99	106.6	81.38	108.5
B	2.81	93.7	16.00	106.7	79.18	105.6	4.11	137.0	15.33	102.2	78.40	104.5
B	3.20	106.7	16.08	107.2	76.19	101.6	3.49	116.2	15.20	101.3	82.23	109.6
B	3.46	115.3	15.42	102.8	83.74	111.6	3.02	100.8	15.66	104.4	84.15	112.2
C	2.82	93.9	14.94	99.6	67.88	90.5	3.48	115.9	15.48	103.2	83.97	112.0
C	2.73	91.1	15.67	104.5	76.89	102.5	3.25	108.3	16.55	110.3	79.72	106.3
C	3.27	109.0	15.87	105.8	84.87	113.2	3.38	112.6	16.87	112.5	82.65	110.2
D	3.19	106.2	16.42	109.5	82.82	110.4	2.73	90.9	16.85	112.3	75.46	100.6
D	3.33	110.9	16.00	106.7	85.29	113.7	3.31	110.4	14.35	95.7	72.06	96.1
D	3.23	107.6	17.14	114.3	84.74	113.0	3.55	118.3	15.50	103.3	75.97	101.3
Mean	3.08		15.71		79.57		3.41		15.80		80.20	
SD	0.2915		0.6816		5.2735		0.5676		0.7306		4.0615	
RSD (%)	**9.5**		**4.3**		**6.6**		**16.6**		**4.6**		**5.1**	
							Extrapolated					

Table 3: Analysis of beer samples

	Glyphosat-FMOC					AMPA-FMOC	
	Conc. ng/mL	Conc. ng/mL	Mean	SD	% RSD	Conc. ng/mL	Conc. ng/mL
Pils							
Sample 1	<LOQ	<LOQ				<LOQ	<LOQ
Sample 2	8.37	8.95	8.7	0.4087	4.7	<LOQ	<LOQ
Sample 3	20.85	20.28	20.6	0.4038	2.0	<LOQ	<LOQ
Sample 4	<LOQ	<LOQ				<LOQ	<LOQ
Sample 5	6.78	6.57	6.7	0.1549	2.3	<LOQ	<LOQ
Sample 6	11.34	12.08	11.7	0.5240	4.5	<LOQ	<LOQ
Sample 7	<LOQ	<LOQ				<LOQ	<LOQ
Sample 8	8.61	9.41	9.0	0.5706	6.3	<LOQ	<LOQ
Sample 9	4.74	4.63	4.7	0.0834	1.8	<LOQ	<LOQ
Sample 10	<LOQ	<LOQ				<LOQ	<LOQ
Sample 11	10.81	12.03	11.4	0.8627	7.6	<LOQ	<LOQ
Sample 12	13.95	14.65	14.3	0.4943	3.5	<LOQ	<LOQ
Sample 13	33.06	27.61	30.3	3.8509	12.7	<LOQ	<LOQ
Sample 14	20.29	18.68	19.5	1.1377	5.8	<LOQ	<LOQ
Sample 15	25.28	22.09	23.7	2.2578	9.5	<LOQ	<LOQ
Sample 16	3.23	2.93	3.1	0.2171	7.1	<LOQ	<LOQ
Sample 17	3.66	3.48	3.6	0.1308	3.7	<LOQ	<LOQ
Sample 18	5.25	5.65	5.4	0.2807	5.2	<LOQ	<LOQ
Sample 19	2.67	2.93	2.8	0.1881	6.7	<LOQ	<LOQ
Sample 20	3.87	4.39	4.1	0.3698	9.0	<LOQ	<LOQ
Sample 21	<LOQ	<LOQ				<LOQ	<LOQ
Organic Beer							
Sample 22	<LOQ	<LOQ				<LOQ	<LOQ
Sample 23	<LOQ	<LOQ				<LOQ	<LOQ
Sample 24	<LOQ	<LOQ				<LOQ	<LOQ
Others							
Sample 25	2.79	3.26	3.0	0.3323	11.0	<LOQ	<LOQ
Sample 26	4.61	4.15	4.4	0.3260	7.4	<LOQ	<LOQ
Sample 27	<LOQ	<LOQ				<LOQ	<LOQ
Sample 28	<LOQ	<LOQ				<LOQ	<LOQ
Sample 29	2.52	<LOQ				<LOQ	<LOQ
Sample 30	<LOQ	<LOQ				<LOQ	<LOQ
Sample 31	<LOQ	<LOQ				<LOQ	<LOQ
Sample 32	8.06	7.27	7.7	0.5621	7.3	<LOQ	<LOQ
Sample 33	11.19	11.57	11.4	0.2737	2.4	<LOQ	<LOQ
Sample 34	<LOQ	<LOQ				<LOQ	<LOQ
Non alcoholic							
Sample 35	4.75	4.47	4.6	0.1952	4.2	<LOQ	<LOQ
Sample 36	16.05	15.71	15.9	0.2454	1.5	<LOQ	<LOQ
Sample 37	<LOQ	<LOQ				<LOQ	<LOQ
Sample 38	<LOQ	<LOQ				<LOQ	<LOQ
Sample 39	<LOQ	<LOQ				<LOQ	<LOQ
Sample 40	2.50	2.85	2.7	0.2482	9.3	<LOQ	<LOQ

Results and discussion

Method development for automated derivatisation

The highly polar glyphosate and its degradation product AMPA are highly soluble in water, but not soluble in many organic solvents such as methanol, isopropanol or acetonitrile. In contrast, the non-polar FMOC is not soluble in water but is very miscible in organic solvents. Bringing together the analyte and the derivatisation agent in an environment that allows solubility for both of them is crucial for the whole analysis.

One simple, widely employed technique to achieve this is incubation at a higher temperature which increases the solubility of FMOC in water [5]. For an automated approach, this requires a dedicated instrumental setup enabling heated incubation.

In this report, an acceptable solubility could be achieved for FMOC, glyphosate and AMPA in a solution with 50% methanol (data not shown) without the need for heating the sample during incubation. This allowed for a fully automated pretreatment of the samples by using the pretreatment function of the autosampler. The only manual pretreatment required was adding 0.5 mL methanol to 0.5 mL of beer, vortexing the sample and centrifugation.

The supernatant was placed into the autosampler, which executed all following steps (adding FMOC and internal standard, stopping the reaction by addition of 10% formic acid) automatically (Figure 1). After derivatisation the sample was injected directly to the LC-MS/MS and analysed accordingly. The time required for all derivatisation steps is only 15 minutes.

The following chromatography also requires 15 minutes. Due to the overlapping sample pretreatment functionality, the next sample was already pretreated during the on-going analysis in order to maximise sample throughput (Figure 2). Except for the first and the last sample, the total time per sample for automated pretreatment and analysis can be reduced to 15 minutes.

Figure 3 shows typical chromatograms for LOQs of glyphosate-FMOC (2.5 ng/mL) and AMPA-FMOC (5 ng/mL) and their calibration curves (glyphosate 2.5 – 100 ng/mL; AMPA 5 – 100 ng/mL). The method shows excellent linearity for glyphosate-FMOC (R^2=0.9986) and AMPA-FMOC (R^2=0.9995) using linear weighted regression (1/concentration). By analysing QC samples in 3 different concentrations (3 ng/mL, 15 ng/mL and 75 ng/mL), the accuracy of the method was proven to be very high. The relative standard deviation was below 10% for all QC except the smallest QC for AMPA-FMOC, which was extrapolated as it was below LOQ (Table 2).

Quantitative analysis of 40 beer samples

After the successful development of a fully automated method, a total of 40 commercially available beer samples were analysed. Among these samples, there were 21 samples of beer brewed according to

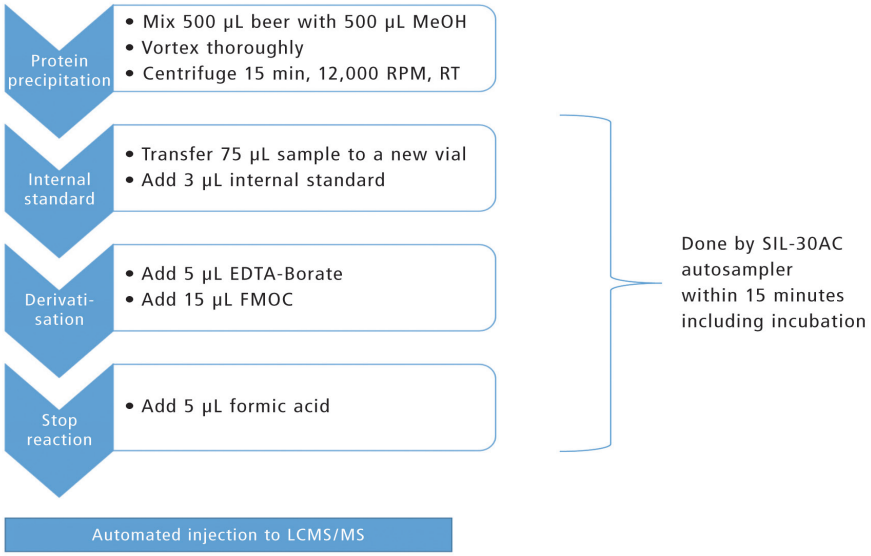

Figure 1: Workflow of sample pretreatment. Addition of internal standard as well as all remaining derivatisation steps are done by the autosampler.

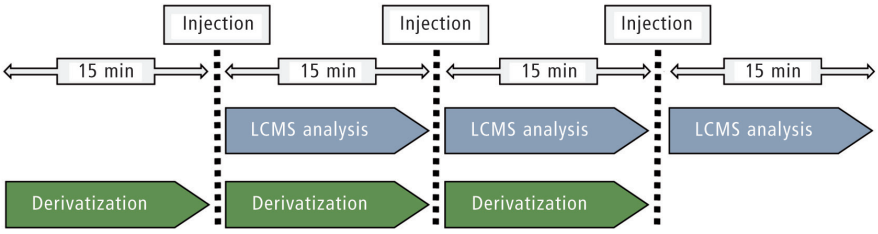

Figure 2: Overlapping sample pretreatment and analysis done by SIL-30AC. Total time per sample is reduced to 15 minutes.

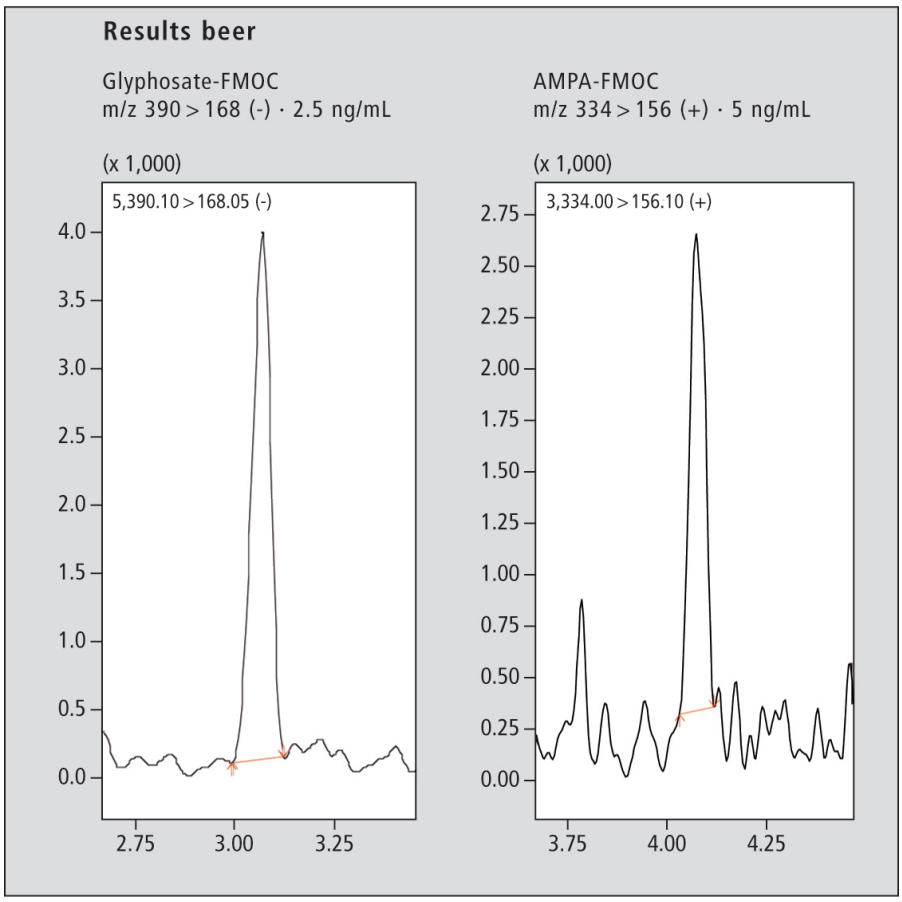

Figure 3: Chromatograms of Glyphosate-FMOC (2.5 ng/mL) and AMPA-FMOC (5 ng/mL)

Pilsner (Pilsener) style, 3 samples of organic beer, 10 samples of other types of beer, and 6 samples of alcohol-free beers or mixed non-alcoholic beer drinks.

All samples were analysed in duplicate in two consecutive runs. While glyphosate was detected in 60% of all samples, its metabolite AMPA was below LOQ in all samples (Table 2).

There is no correlation between the kind of beers (Pilsener style, alcohol-free and others like wheat beer) and the detection of glyphosate, as there were samples containing glyphosate among all kinds of beers. Only the 3 organic beers tested were completely free of glyphosate. But even for the beers which tested positively, the amount was far below the MRL so none of the beers presents a health hazard – at least with regard to glyphosate levels.

Conclusion

The reported method is able to derivatise glyphosate and AMPA with FMOC fully automatically within 15 minutes. The only manual pretreatment required is protein precipitation. No additional hardware is required as all pretreatment steps are performed by the autosampler in a standard LC configuration. The method is robust and reliable for samples even in a complex matrix such as beer, which makes it suitable for high-throughput analysis. Additionally, the throughput was doubled by using the overlapping sample pretreatment functionality. This allows sample pretreatment while the previous sample is analysed.

References

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