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Fully automated derivatization and quantitation of Glyphosate and AMPA in beer using a standard UHPLC-MS/MS system

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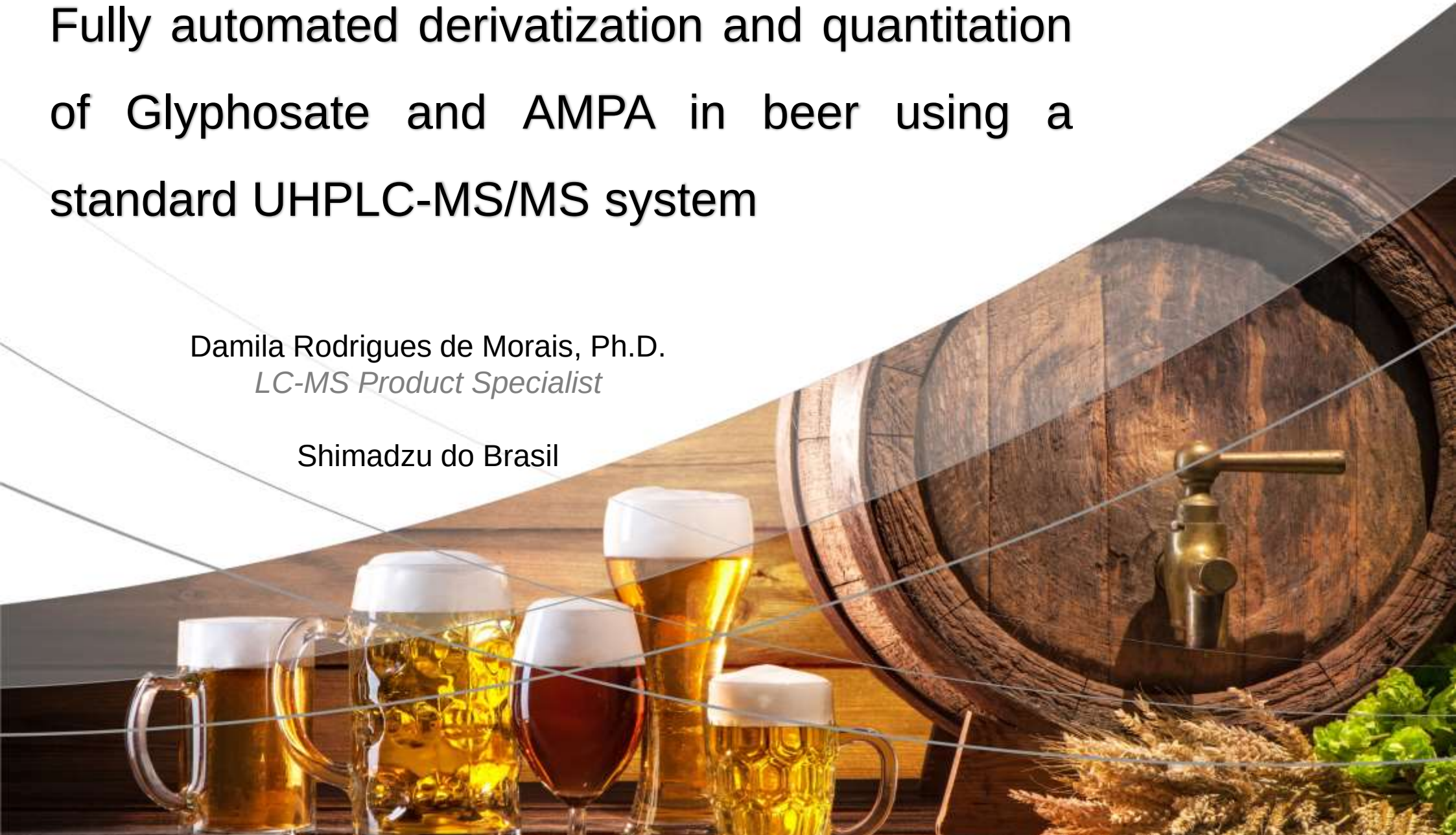

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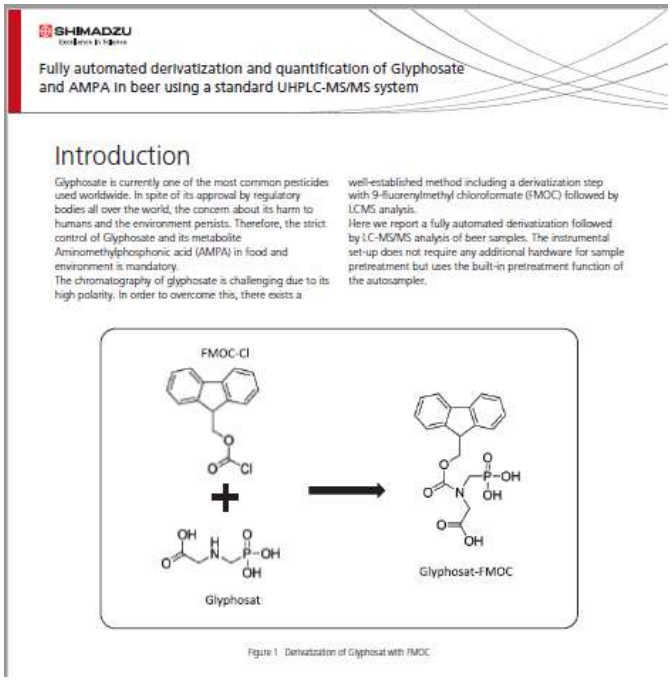

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Fully automated derivatization and quantification of Glyphosate and AMPA in beer using a standard UHPLC-MS/MS system

Introduction

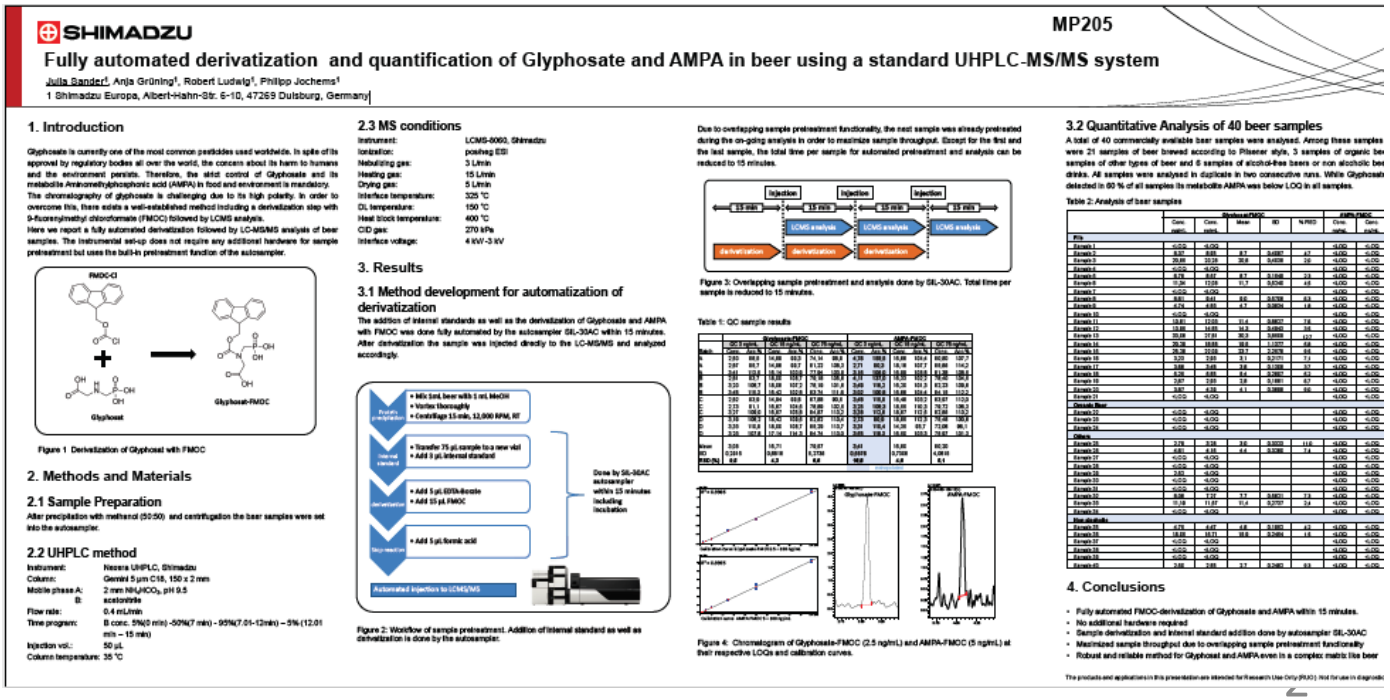
Glyphosate is currently one of the most common pesticides used worldwide. In spite of its approval by regulatory bodies all over the world, the concern about its harm to humans and the environment persists. Therefore, the strict control of Glyphosate and its metabolite Aminomethylphosphonic acid (AMPA) in food and environment is mandatory.
The chromatography of glyphosate is challenging due to its high polarity. In order to overcome this, there exists a well-established method including a derivatization step with 9-fluorenylmethyl chloroformate (FMOC) followed by LCMS analysis.
Here we report a fully automated derivatization 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.

FMOC-Cl

Glyphosat

Glyphosat-FMOC

Figure 1 Derivatization of Glyphosat with FMOC



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MP205

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1. Introduction

2.3 MS conditions

3. Results

3.1 Method development for automatization of derivatization

2. Methods and Materials

2.1 Sample Preparation

2.2 UHPLC method

3.2 Quantitative Analysis of 40 beer samples

4. Conclusions

- Fully automated FMOC-derivatization of Glyphosate and AMPA within 15 minutes.
- No additional hardware required
- Sample derivatization and internal standard addition done by autosampler SIL-30AC
- Maximized sample throughput due to overlapping sample pretreatment functionality
- Robust and reliable method for Glyphosat and AMPA even in a complex matrix like beer

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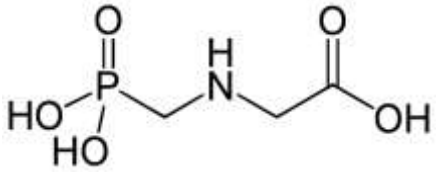
The controversial use of Glyphosate

- One of the most common pesticides worldwide;
- Brought to market in 1974 by Monsanto under the trade name Roundup;
- Harm to human and environment still under discussion;
- In December 2017, European Commission renewed the approval of Glyphosate for 5 years



Glyphosate can be used as an active substance in Plant Protection Products (PPPs), until 15 December 2022, subject to each PPP being authorised by national authorities following an evaluation of their safety.

Source: https://ec.europa.eu/food/plant/pesticides/Glyphosate_en

Why analyze Glyphosate in beer?

- Munich Environmental Institute published a report, showing traces of Glyphosate in all tested beer samples

Maximum residue levels (MRL's):

- drinking water: 0.1 µg/L
- barley : 20 mg/kg
- hops: 0.1 mg/kg
- **beer: not defined**

  →MRL is derived from raw agricultural commodities (barley, hops)

Source: http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=pesticide.residue.CurrentMRL&language=EN

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Glyphosate analysis

extraction

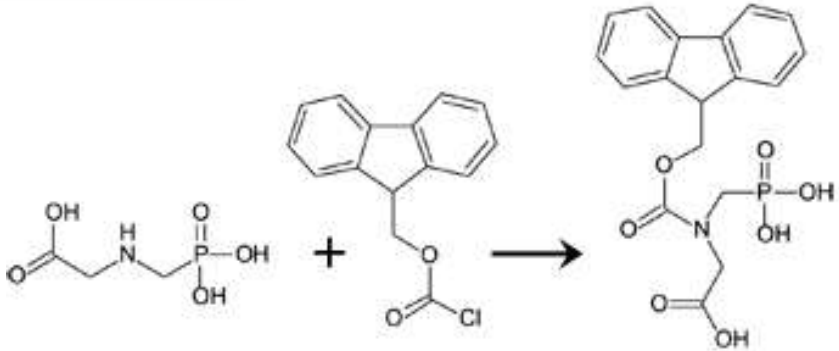
1 ml beer

1 ml MeOH

vortex

Zentrifuge 15 min, RT

Protein precipitation



Without derivatization

LCMS analysis

With derivatization

Add 25 µl EDTA-Borate and 75 µl FMOC

1 h at 50 °C

Add 30 µl phosphoric acid and 120 µl water

LCMS analysis

Sample volume 500 µL

Basic buffer

Derivatization agent

Stop reaction

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Glyphosate analysis

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Application News

LCMS

No. SCA-210-032

Residual highly polar pesticides in beer:

A comparative study about sample pretreatment with and without derivatization

■ 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. The chromatography of glyphosate is challenging due to its high polarity. In order to overcome this, there exists a well-established method including a derivatization step with 9-fluorenylmethyl chloroformate (FMOC) followed by LCMS analysis. However, this derivatization is not only time consuming and more expensive but also susceptible to errors. A further drawback of this method is the fact that not all highly polar pesticides are susceptible to derivatization with FMOC as for example Glufosinate's metabolite 3-methyl-phosphinico propionic acid (MPPA).
Therefore, a sample pretreatment without derivatization is desirable as it will be not only faster and cheaper but also not limited to detection of Glyphosate and allow the simultaneous detection of other highly polar pesticides and their metabolites as well.

Here we compare the quantification of Glyphosate, its metabolite Aminomethyl-phosphonic acid (AMPA) as well as Glufosinate and its respective metabolite MPPA based on derivatization to a method without any derivatization. We tested a total of 24 different kinds of beer. The use of a high sensitivity mass spectrometer allows to skip a sample concentration step by solid phase extraction (SPE) for both methods, which additionally reduces time and cost for the analysis.

■ Materials and methods

Sample Pretreatment

1 ml MeOH was added to 1 ml beer, vortexed and afterwards centrifuged for 15 min at 12000 rpm. 500 µL of the supernatant were used for the underivatised sample. For the derivatisation sample 25 µl EDTA-Borate buffer and 75 µl FMOC were added to another 500 µl of the supernatant. After 60 min incubation at 50 °C we added 30 µl 0.2% phosphoric acid to stop reaction. Finally, 125 µl water were added.

Analytical conditions

As derivatized and underivatized samples required different chromatographic conditions the Nexera MX for multiplex analysis with Dual Stream Technology was used. This allows the use of a multiplexed method with different columns and LC methods in one batch and thus increasing through-put and improving efficiency.

Figure 1: Nexera MX with LCMS-8060 triple quadrupole mass spectrometer

With derivatization:

↑ Better retaining on column (normal C18 column);

↓ Either more manual work or additional instrumental set up required.

Without derivatization:

↑ No manual work;

↓ Good chromatography only with special column.

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Fully automated derivatization UHPLC-MS/MS system

- Various automated pretreatment functions are available

- Reduces the workload for sample pretreatment
- Improves throughput and precision by eliminating the need for manual sample preparation

Automatic Pretreatment Function

- **Dilution**
- **Reagent Addition**
- **Mixing**
- **Co-injection**
- **Overlap Injection**

Co-injection is unique to Shimadzu

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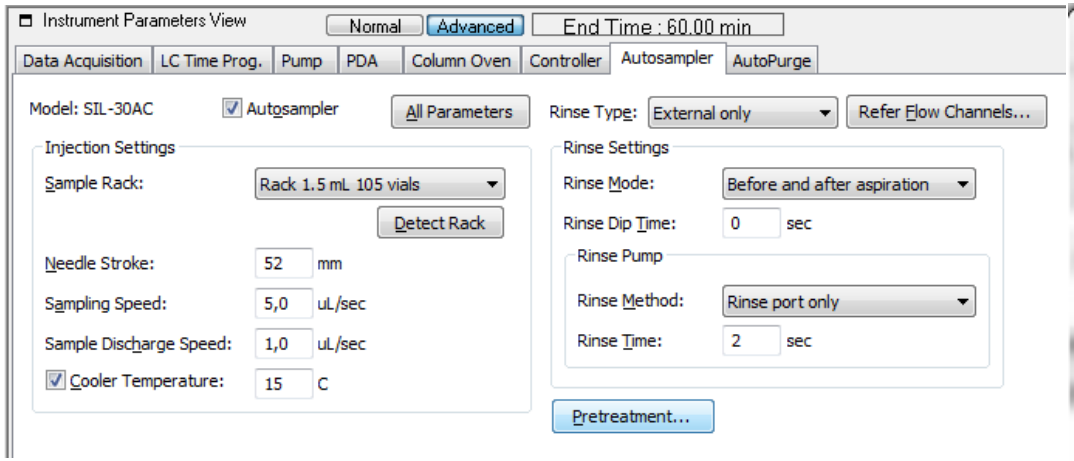
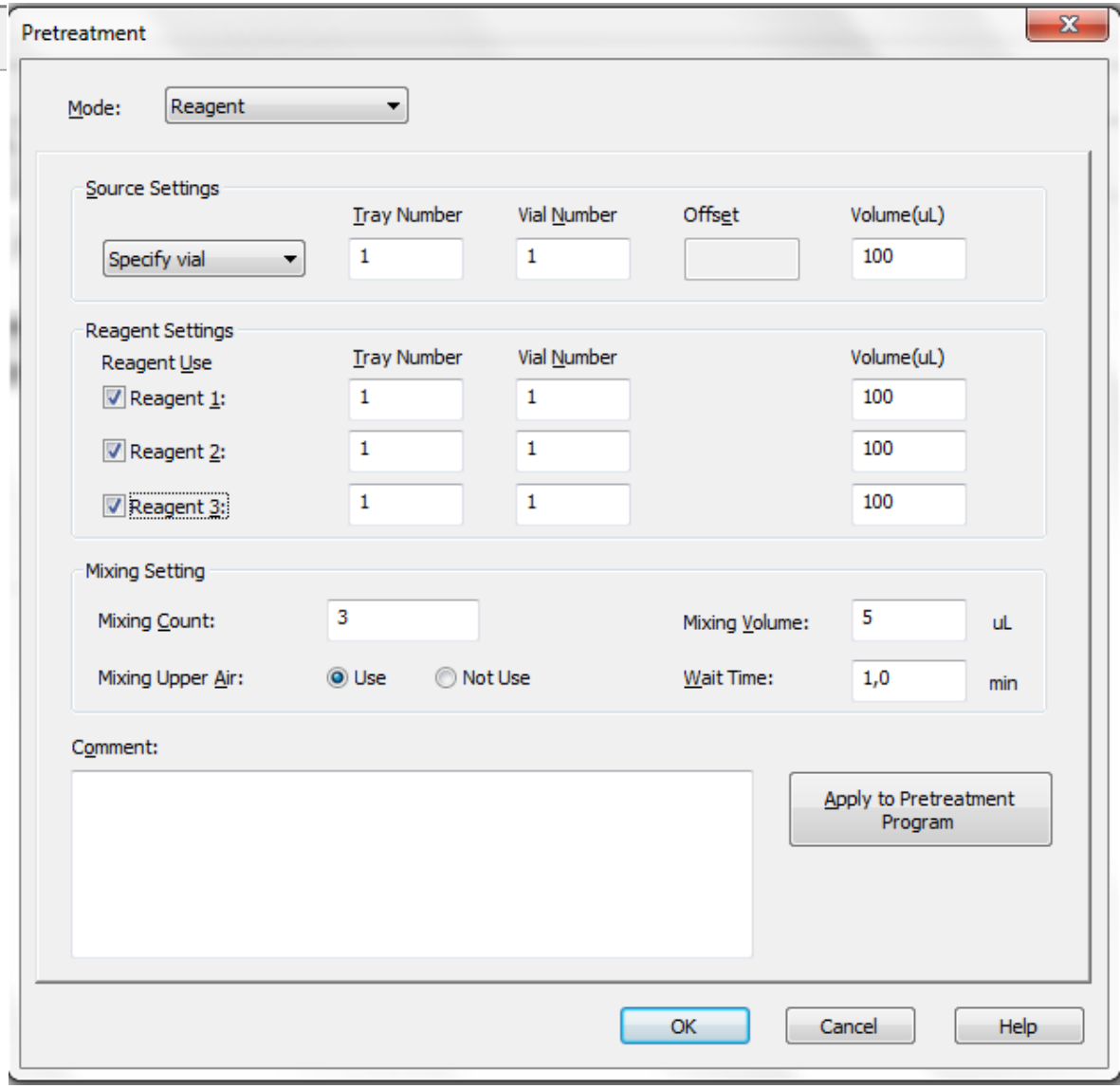
Fully automated derivatization UHPLC-MS/MS system





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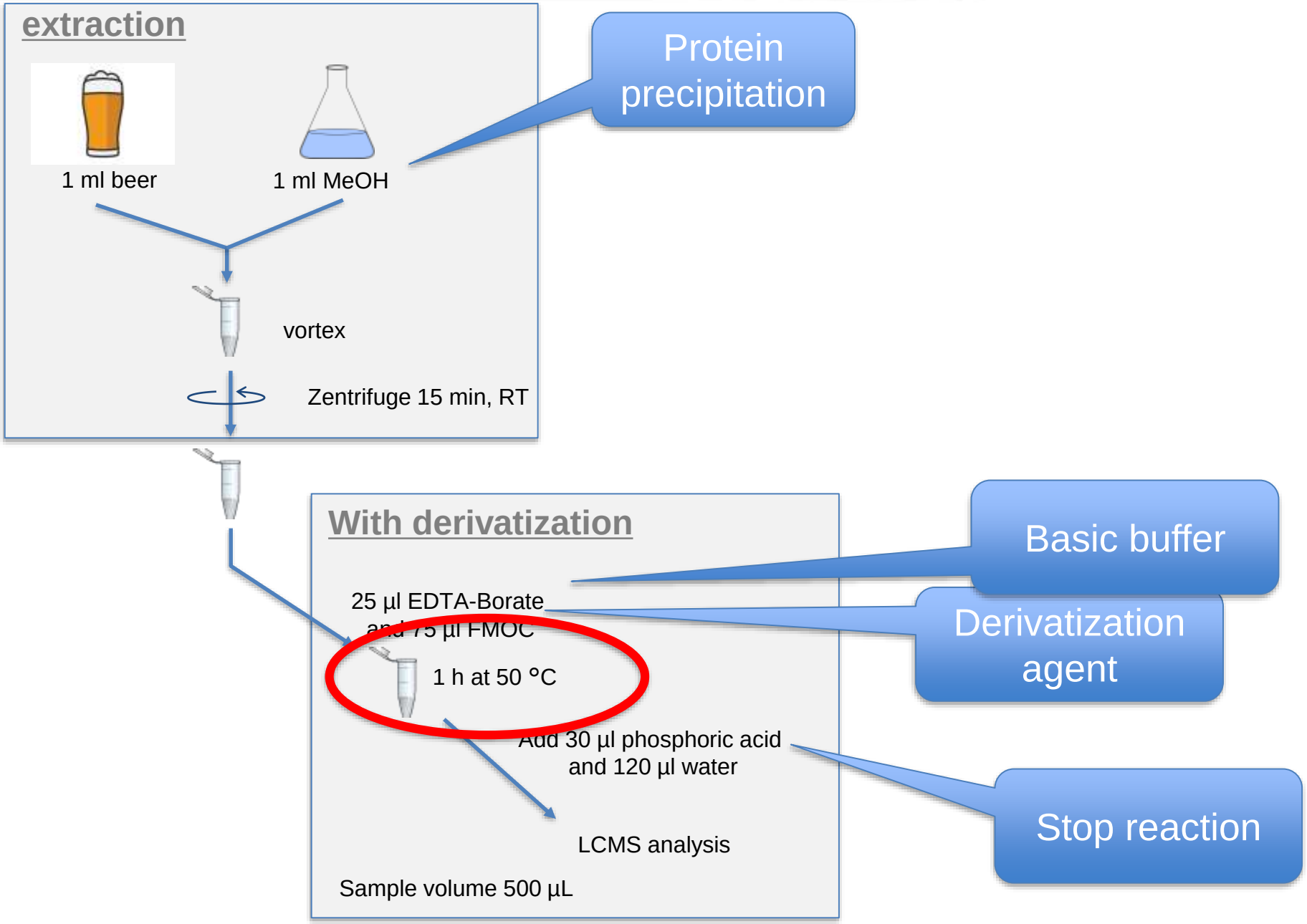
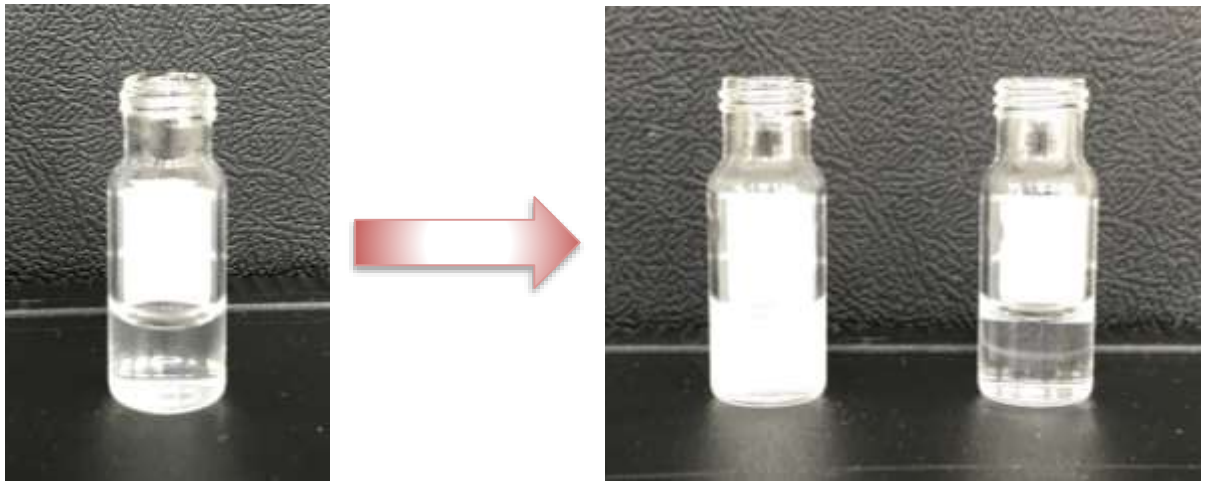
Fully automated method with derivatization using the pretreatment function of SIL-30AC



FMOC derivatization tests

- **Low solubility of FMOC in water at room temperature (RT);**
- **Glyphosate is not soluble in organic solvents (ACN, MeOH, IPA, acetone…);**

→ **Standard method: incubation at high temperature;**



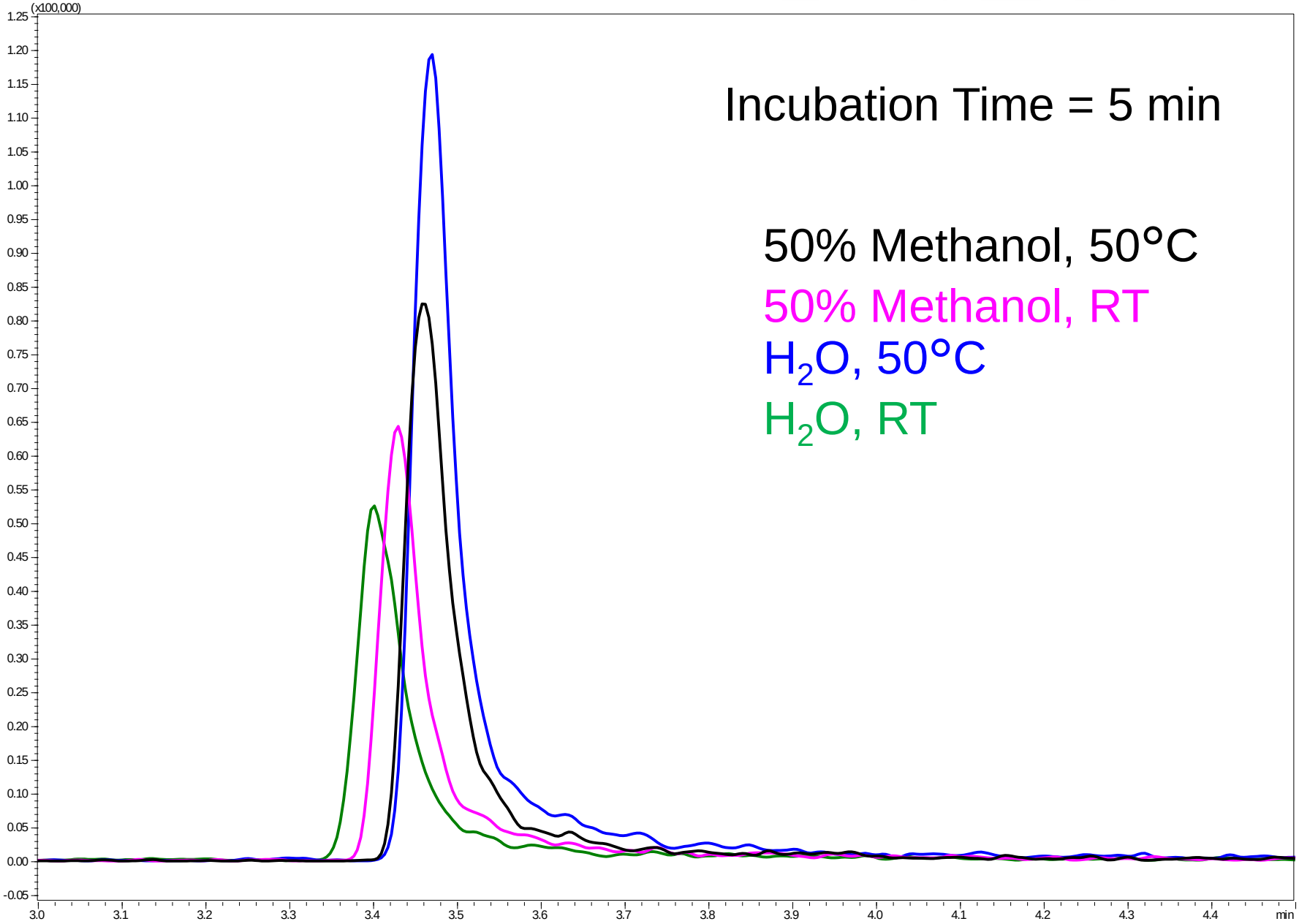
H_2O, 50°C

H_2O, RT

H_2O:MeOH (50:50), RT

→ **FMOC as well as Glyphosate solve well in H_2O and MeOH (50:50 v/v) mixture at RT.**

FMOC derivatization tests



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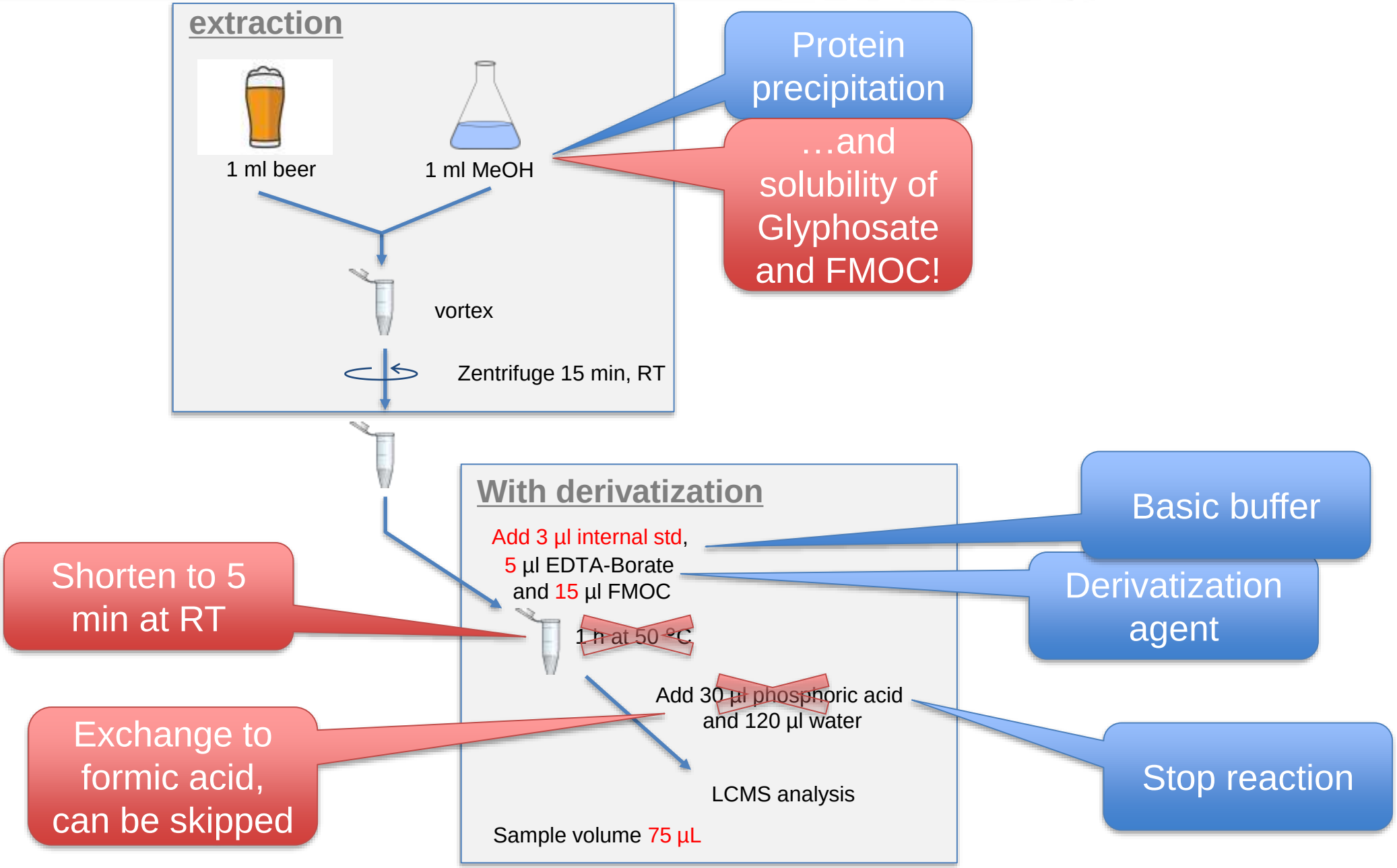
Fully automated method using the pretreatment function of SIL-30 AC



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Analytical conditions - Liquid chromatography

UHPLC	Nexera X2 UHPLC system
Analytical column	Gemini 5 µm C18 110Å, 150 x 2 mm, (Phenomenex)
Mobile phase	A = 2mm NH_4HCO_3, pH 9.5 B = Acetonitrile
Flow rate	0.4mL/min
Time program	B conc. 5% (0 min) – 50% (7 min) – 95% (7.01 – 12min) – 5% (12.01 – 15 min)
Injection volume	50µL
Column temp.	35°C
MS	LCMS-8060 triple quadrupole mass spectrometer, ESI

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Analytical conditions - Mass spectrometry

System: LCMS-8060

- Ionization mode: **positive/negative ESI,**
- Nebulizing Gas Flow: **3 L/min**
- Heating Gas Flow: **15 L/min**
- Interface Temperature: **325 °C**
- DL Temperature: **150 °C**
- Heat Block Temperature: **400 °C**
- Drying Gas Flow: **5 L/min**
- CID Gas: **270 kPa**
- Interface voltage: **4 kV / -3 kV**
- Measurement mode: **MRM**,
- Resolutions: **Unit (Q1)/Unit (Q3)**,
- Transitions :

Compound	+/-	Precursor m/z	Product m/z	Pause	Dwell	DL Bias	Qarray Bias	Q1 Pre Bias	CE	Q3 Pre Bias
Glyphosate	-	390.1	168.05	3	10	-10	0	11	14	15
Glyphosate	-	390.1	150.00	3	10	-10	0	11	24	19
Glyphosate IS	-	393.1	171.05	3	10	-10	0	11	14	15
AMPA	+	334.0	156.10	3	10	10	0	-17	-9	-17
AMPA	+	334.0	178.20	3	10	10	0	-17	-49	-13
AMPA IS	+	336.0	179.20	3	10	10	0	-23	-21	-18

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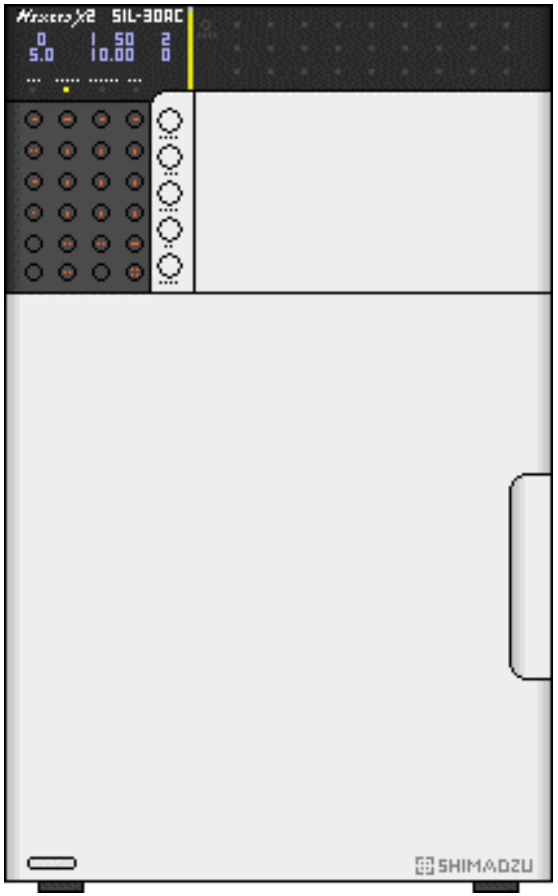
Fully automated method using the pretreatment function of SIL-30AC



SIL-30AC

FMOC-derivatization procedure:

	Manual	Automatic using SIL-30AC
Sample (Beer/MeOH 1/1)*	500 µL	75 µL
Borate / EDTA Buffer	25 µL	5 µL
FMOC	75 µL	15 µL
Acid	30 µL H_3PO_4 (0.2%)	5 µL formic acid (10%)
H_2O	125 µL	--

* if other liquid sample matrix containing mainly water is used the addition of MeOH is mandatory anyway, due to the solubility of the FMOC

In case of this pretreatment file reagents for derivatization and IS are placed in Rack in the following order:

- **Vial 1 – Borat / EDTA Buffer**
- **Vial 2 – FMOC solution**
- **Vial 3 – 10 % formic acid**
- **Vial 4 – ISTD solution (1 ng/µL)**

Total time is reduced to 15 min

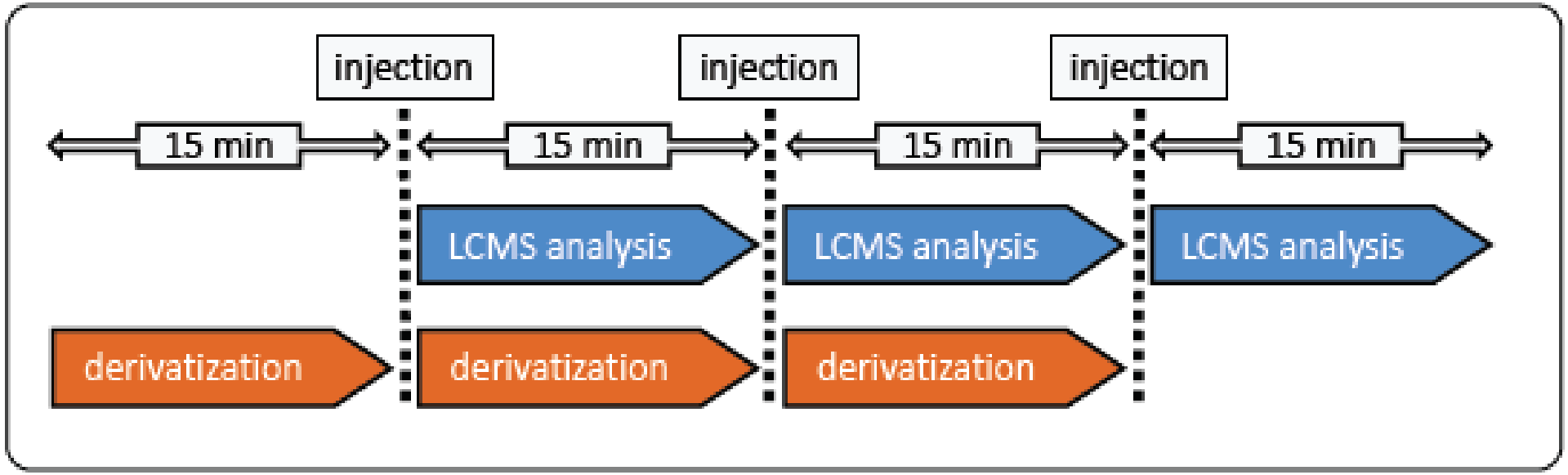
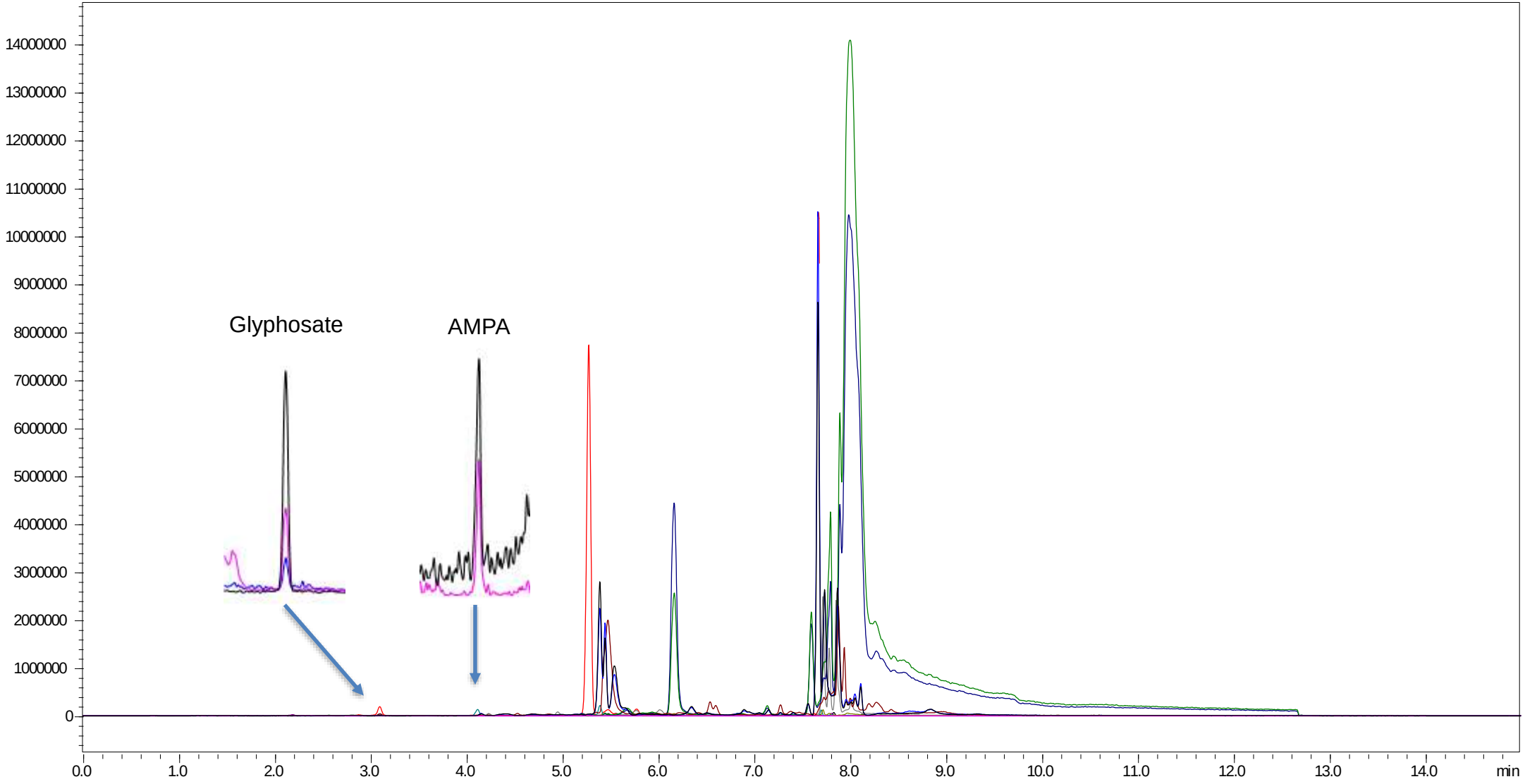


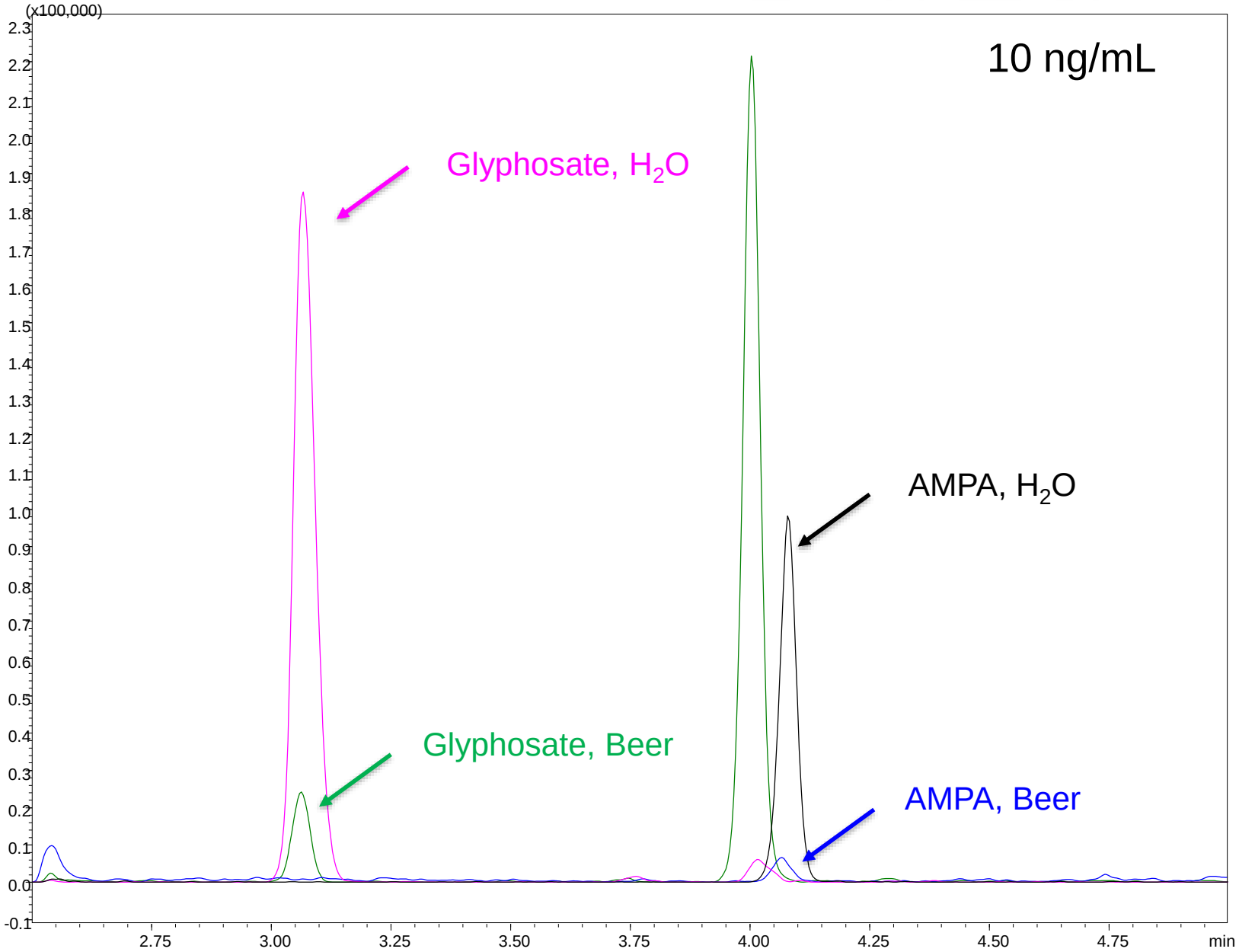
Figure 3: Overlapping sample pretreatment and analysis done by SIL-30AC. Total time per sample is reduced to 15 minutes.

Due to overlapping sample pretreatment functionality, the next sample was already pretreated during the on-going analysis in order to maximize sample through put. Except for the first and the last sample,the total time per sample for automated pre treatment and analysis can be reduced to 15minutes.

Results in beer sample

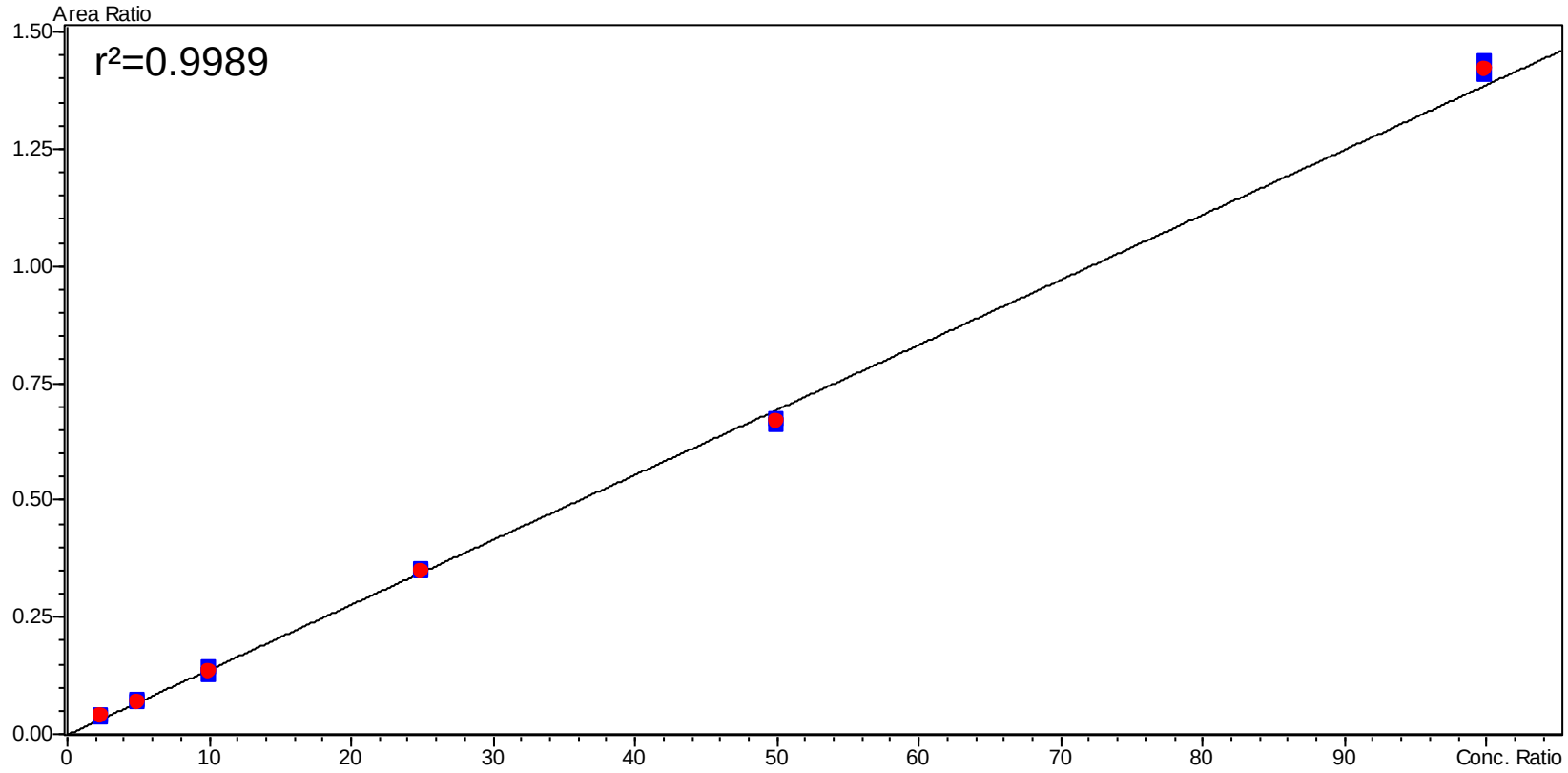


Results in beer sample

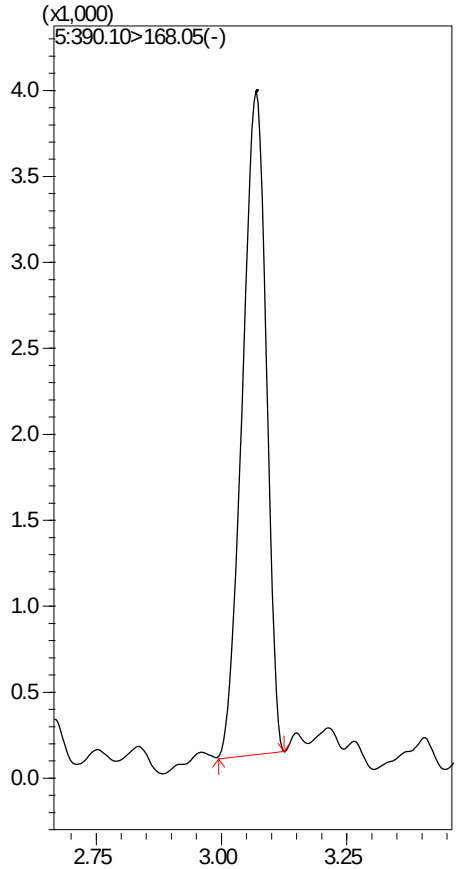


Glyphosate - Calibration Curve and LOQ Chromatogram

Glyphosate-FMOC
m/z 390>168 (-)
Calibration curve 2.5 – 100 ng/mL

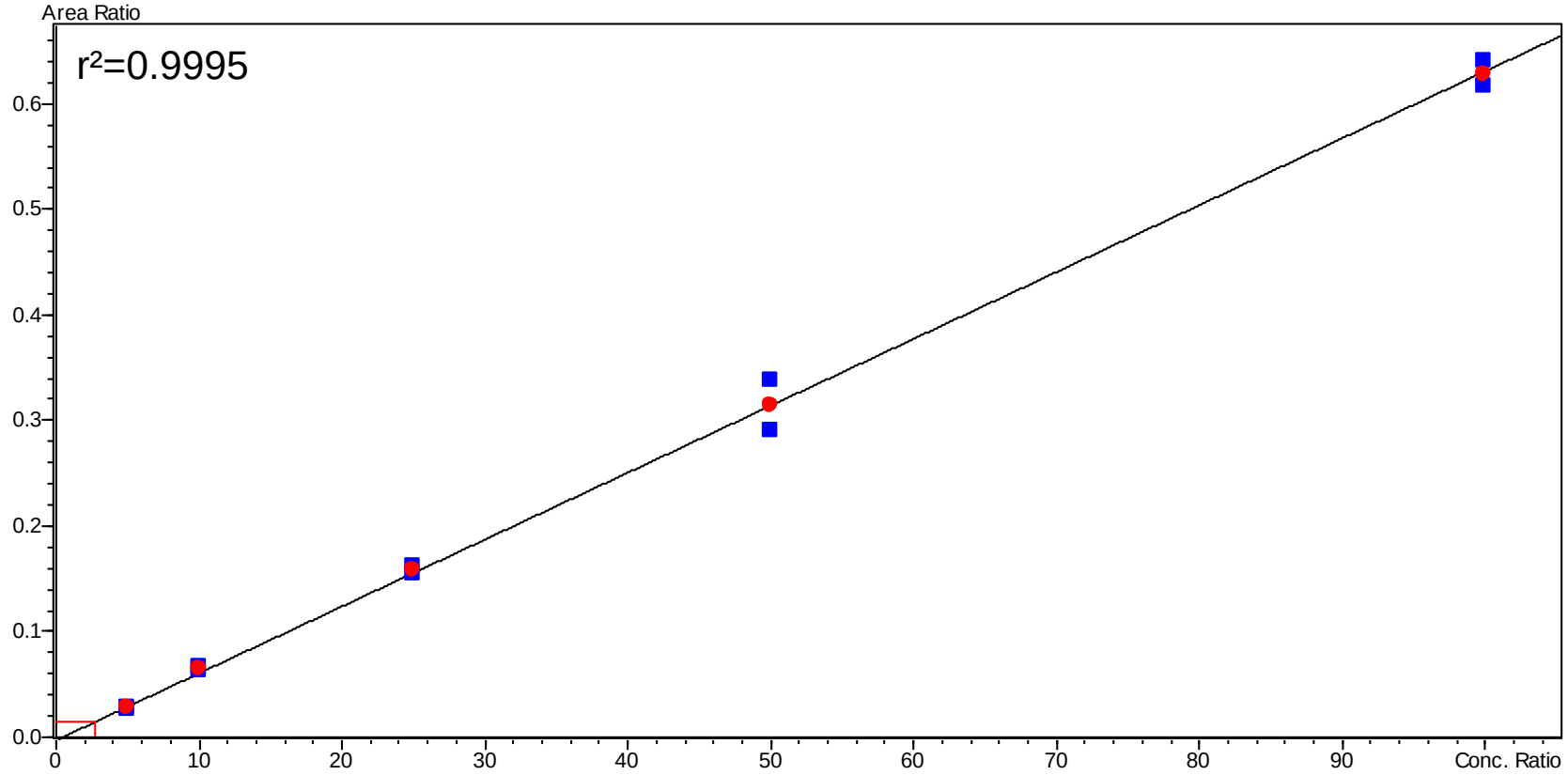


Glyphosate-FMOC
m/z 390>168 (-)
2.5 ng/mL

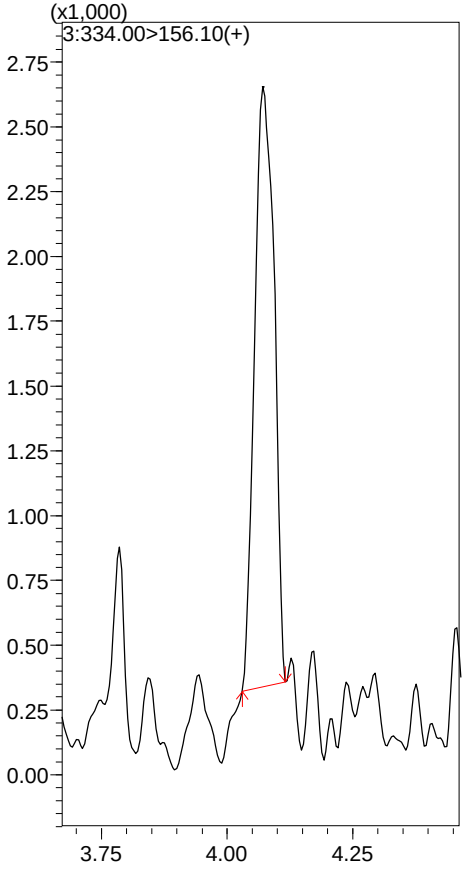


AMPA - Calibration Curve and LOQ Chromatogram

AMPA-FMOC
m/z 334>156 (+)
Calibration curve 5 – 100 ng/mL



AMPA-FMOC
m/z 334>156 (+)
5 ng/mL



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Quantitative Analysis of 40 beer samples

A total of 40 commercially available beer samples were analyzed:

- ✓ 21 samples of beer brewed according to Pilsener style,
- ✓ 3 samples of organic beer,
- ✓ 10 samples of other types of beer,
- ✓ 6 samples of alcohol-free beers or non alcoholic beer mix drinks.

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Quantitative Analysis of 40 beer samples – Results Table Part I

	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

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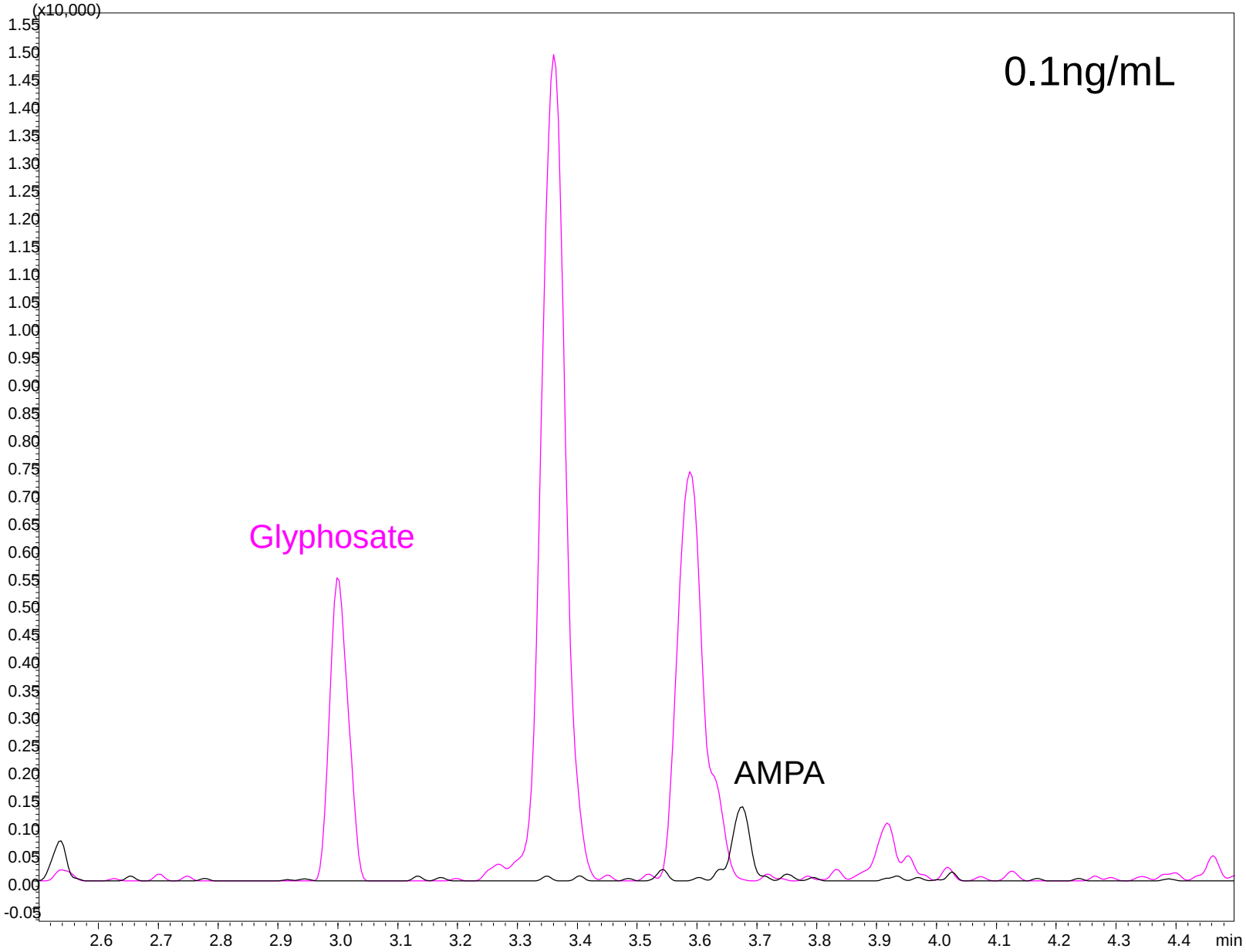
Quantitative Analysis of 40 beer samples – Results Table Part II

	Glyphosat-FMOC					AMPA-FMOC	
	Conc. ng/mL	Conc. ng/mL	Mean	SD	% RSD	Conc. ng/mL	Conc. ng/mL
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

Preliminary results tap water



Conclusion

- Automated derivatization of Glyphosate and its metabolite AMPA was successfully established by use of SIL-30 AC autosampler with automatic pre-treatment function;
- Protein precipitation with MeOH is necessary for complex matrices like beer. Additionally, it is helpful for solubility of FMOC;
- Robust and reliable method for Glyphosate and AMPA even in a complex matrix like beer;
- Glyphosate was detected in the majority of beers. Concentrations are above MRL for drinking water, but far below MRL for commodities.

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Thank you for your attention…

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