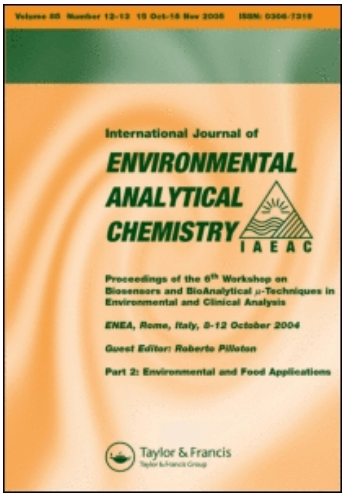

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Direct Determination of Glyphosate in Environmental Waters Using Capillary Electrophoresis with Electrospray Condensation Nucleation Light Scattering Detection

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DIRECT DETERMINATION OF GLYPHOSATE IN ENVIRONMENTAL WATERS USING CAPILLARY ELECTROPHORESIS WITH ELECTROSPRAY CONDENSATION NUCLEATION LIGHT SCATTERING DETECTION

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An electrospray condensation nucleation light scattering detector (ESI-CNLSD) was coupled with capillary electrophoresis (CE) for analysis of glyphosate, a chemical of agricultural interest, which is otherwise difficult to detect owing to its lack of chromophores or fluorophores. To reduce the absorption of glyphosate on the CE capillary and to reduce the separation time, an *N*-cetyltrimethylammonium bromide (CTAB) pre-rinsing CE method was developed here. The protocol consisted of 15 min pre-rinsing of the capillary before analysis with CTAB solution and 5 min with ammonium acetate buffer at pH 2.8. The capillary inner wall coating established by this treatment lasted up to 10 h without bleeding to interfere with CNLSD signal. Calibration data were linear over two orders of magnitude, the instrument detection limit was 0.06 μg/mL and the method detection limit was 0.2 μg/mL. The method was applied to the analysis of local (rural area) lake water and commercial herbicide samples.

Keywords: Glyphosate; Electrospray condensation nucleation light scattering detection; CTAB-pre-rinsing capillary electrophoresis

INTRODUCTION

Condensation nucleation light scattering detection (CNLSD) is an improved technique of evaporative light scattering detection. CNLSD is a universal detection method since light scattering is not compound selective and has detection limits comparable to that of fluorescence detection [1,2]. The operation of the detector has been thoroughly described in several publications [3,4] and only a brief outline is given here. In CNLSD the effluent from the capillary outlet is nebulized by electrospray to an aerosol from which the mobile phase is evaporated, leaving behind particles of analytes.

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Desolvated particles serve as nuclei for heterogeneous nucleation when exposed to saturated butanol vapor, where they can grow to large droplets. The number of particles is then measured using a light scattering technique. This detection method is very universal and in comparison with evaporative LSD has 100–200 times higher sensitivity [5]. Although the excellent performance of ESI-CNLSD has been demonstrated in the analysis of a wide variety of compounds [5], it has not yet received wide acceptance.

From the description of the detector, it follows that its response is universal and independent of the nature of the analytes other than their volatility. It is thus valuable for the detection of compounds that lack chromo- or fluorophores or are electrochemically inactive. One such 'difficult' analyte is glyphosate [*N*-(phosphoronomethyl) glycine], a popular herbicide widely used in agricultural practice, and commonly called Roundup®. Hundreds of tonnes of glyphosate are applied to fields every year, making analytical determination of this substance in environmental samples of great interest. Different analytical methods for glyphosate and other herbicides have been reviewed by Stalikas and Kaniden [6] (see also [7]). The difficulties in establishing analytical methods for the determination of this analyte include its relatively high solubility in water, insolubility in organic solvents and favored complexing behaviors. Since glyphosate does not have chromophores or fluorophores in its structure (Fig. 1), it is not easy to detect with UV-vis detection except at low UV wavelengths, where the detection limits are not favorable. Lengthy extraction, clean-up and derivatization procedures are always required for the analysis of real samples.

In GC analysis [8], sample derivatization is necessary to enhance the volatility of glyphosate. Typical derivatization agents used include trifluoroacetic anhydride in conjunction with trifluoroethanol [9–12] and heptafluorobutanol [13]. High-performance liquid chromatography (HPLC) methods have also been used to achieve the separation [14,15]. HPLC techniques with pre- or post-column derivatization offer more variability. 9-Fluorenylmethyl chloroformate [16,17], *p*-toluenesulphonyl chloride [18] and *o*-phthalaldehyde-2-mercaptoethanol [19–22] have been used as derivatization agents with fluorescence detection. Post-column indirect fluorescence detection [23] was also reported where an Al^{3+}-morin (3,5,7,2′,4′-pentahydroxylflavone) complex provides the background fluorescence. In addition, LC-electrospray mass spectrometry [24,25] has been reported for the determination of glyphosate and its metabolite aminomethylphosphonic acid (AMPA).

In recent years, capillary separations have gained popularity owing to their high resolving power and speed. *p*-Toluenesulfonyl chloride [26] was used to derivatize glyphosate and AMPA prior to separation by CE, followed by detection with a UV absorbance

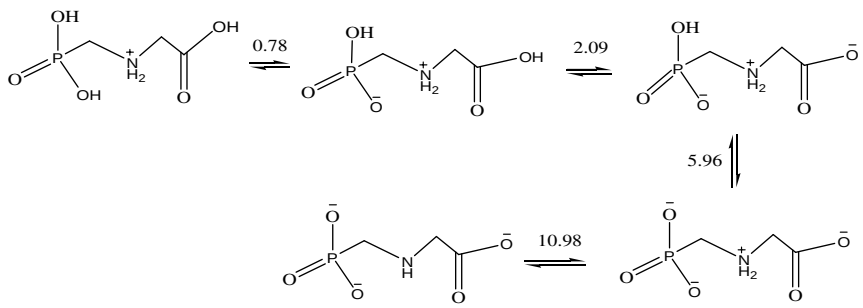

FIGURE 1 Structure of glyphosate.

detector, with detection limits (LODs) of 0.1 and 0.2 μg/mL for glyphosate and AMPA in spiked serum, respectively. Ribonucleotides [27] were used for indirect photometric detection of glyphosate and AMPA at the same level of LODs. Phthalate [28] was also employed to provide the background signal for indirect UV detection in CE analysis of glyphosate and AMPA. Two on-column concentration methods were applied here to increase the method sensitivity. With stacking, the LODs are 0.8 and 0.6 μg/mL for glyphosate and AMPA, respectively, and field amplified sample injection can tremendously decrease the LOD for glyphosate to 2 ng/mL. A scheme for the separation and detection of glyphosate and some other herbicides using CE coupled with indirect fluorescence detection was described by Chang and Liao [7]. In this work fluorescein was employed as the buffer fluorophore, an argon-ion laser was used to induce the fluorescence background and the concentration LODs were at the micromolar level. The potential of isotachophoresis-contactless conductivity detection for two phosphonic and amino acid group-containing herbicides (glyphosate and glufosinate) was investigated by Goodwin *et al.* [29]. Here a LOD of 0.025 μg/mL for glyphosate was reported. Safarpour and Katz [30] also presented their work with CE-ESI-MS for the determination of glyphosate in degradation-grade-formulated pesticide products.

Due to the highly ionic nature of glyphosate, CE should be a preferred method for its separation and analysis. On the other hand, as follows from the discussion above, its detection is a challenging task, involving either sophisticated derivatization or indirect detection procedures. In this study, we provide another approach for determination of glyphosate using direct CE separation of the sample with detection by ESI-CNLSD. Since operation of this detector requires using volatile buffer, a specific method for capillary preprocessing (rinsing with CTAB before analysis) is provided to facilitate this task. Reproducibility, LODs and calibration linear range are measured, and applications for spiked analysis of real field samples and a diluted commercial herbicide sample, Roundup®, are demonstrated.

EXPERIMENTAL

Sampler

The sample was introduced into the column using a home-built pneumatic autosampler. The dimensions of the autosampler were 2 × 2 × 1 cm and it was made of PEEK plastic material. Its construction is given in several papers [31–33], so only a brief description is given here. The sampler is based on the principle of a rapid interchange of the flow of different liquids (such as buffer, sample or other necessary solutions) in a narrow input channel (1-mm I.D., 80-μL volume) into which the capillary and high-voltage electrodes are inserted. The flow of the liquids is controlled by a PC that activates solenoid valves connected to the gas displacement pumps, thus providing the pressure pulses necessary for activating the liquid flow in the pumps. The sampling mechanism is believed to be a mixed mode of both electrokinetics and hydrodynamics [33]. Exhaustive description of the sampler construction and performance together with some applications in flow injection-CE type measurements is given in our publications [34,35].

The sampler used in this work employed four solenoid valves (Brükkert, Denmark), three of which controlled the flow of buffer, sample and CTAB rinsing liquid, respectively. The fourth solenoid allowed the input channel to be closed when a particular gas

displacement pump was pressurized, thus directing the pressurized liquid flow through the capillary. All the programming and timing of the liquid flows were controlled by a Toshiba Satellite laptop computer with software written in Labview environment (National Instruments, Austin, TX, USA) via an 'Adam-6030' interface board (Advantech, Taiwan, Taipei, ROC).

CE–Electrospray Interface and CNLSD System

The CNLSD system included a TSI (St. Paul, MN, USA) Model 3025A Ultrafine condensation particle counter (CPC) operated in low-flow mode (aerosol uptake at 300 mL/min), an electrospray aerosol generator, a spray chamber and a neutralizer, as described previously for use with CE [1]. The CPC provides output of the number of detected particles per unit gas-phase volume (mL), here represented as No./mL. A high-voltage power supply (Series 230, Bertan, Hicksville, NY, USA) was used to power the electrospray. A laboratory-written Basic program was used to transfer data (No./mL) from the CPC to an IBM386 computer at 1 Hz. Electropherograms were edited using in-house-written software in a Matlab environment (MathWorks, Natick, MA, USA).

To fabricate the electrospray emitter, 2–3 cm of the outlet capillary was painted with a gold paint (OG 805 Premium Gold, Duncan Enterprises, Fresno, CA, USA). The gold paint was then heated using a heat gun to form a smooth coating on the capillary surface, which is required for stable electrospray operation. The end of the capillary was cut with a capillary cutter (Supelco, Bellefonte, PA, USA) to yield a flat cross-section surface. An additional 5 cm (from the gold painted part) of the capillary was painted with copper paint (Quick Grid Repair Resin, Loctite, Cleveland, OH, USA) for connection to the power supply. The outlet of the transfer capillary with a fabricated electrospray tip at its terminus was placed in the cylindrical, glass spray chamber (1.5-cm I.D., 5 cm long) through a length of stainless steel tubing, which was used to position the capillary within the spray chamber. The aerosol was carried with a 0.9 L/min flow of carbon dioxide regulated with a rotameter. A cylindrical flow-through neutralizer (model P-2021SS Nuclecel in-line ionizer, NRD, Grand Island, NY, USA) was placed directly at the end of spray chamber. The high negative voltage necessary for the electrospray process was directly applied to the neutralizer. The neutralizer contains polonium-210 (alpha emitter) of 10-mCi activity whose decay creates a weak bipolar plasma. The electrons from the plasma neutralize the charges from the highly charged droplets resulting from the electrospray process. Figure 2 represents the experimental setup.

Chemicals

Glyphosate was purchased from Sigma (St. Louis, MO, USA), 2,4- dichlorophenoxyacetic acid (2,4-D) was from ULTRA Scientific (North Kingstown, RI, USA), and ammonium acetate (NH_4Ac, 99.999%) was from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). Analytical grade *n*-cetyltrimethyl-ammonium bromide (CTAB), acetic acid (HAc), ammonium hydroxide (NH_4OH), and butanol were from Fisher Scientific (Fair Lawn, NJ, USA). Roundup®, a commercial herbicide containing glyphosate and produced by Monsanto, was bought locally. All the solutions were prepared using Barnstead NANOpure water (Dubuque, IA, USA).

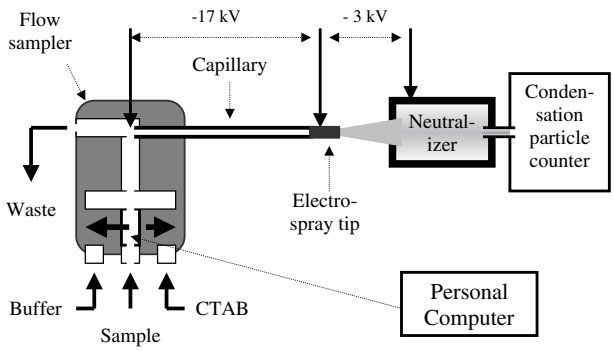

FIGURE 2 Experimental set-up of CE-ES-CNLSD system.

Real samples were collected from the rural area of Southern Illinois, USA in October 2002. The first set of samples was from Cedar Lake, near Carbondale, IL and the second set of samples were collected from the lake located at the experimental field of the College of Agriculture, Southern Illinois University at Carbondale (situated 10 km west of the university campus). It was known that the field around the latter site was treated with glyphosate in July 2002. The samples were refrigerated immediately after collection.

CE Separation Conditions

The analytes were separated in a fused-silica capillary column (Polymicro Technologies, LLC, Phoenix, AZ, USA) of 45-cm length, 150-μm O.D. and 50-μm I.D. The high voltage was provided by an ISCO model 3850 electropherograph (ISCO, Inc. Lincoln, NE, USA). The separation voltage was −17.21 kV relative to the grounded capillary end. The separation electrolyte was 5 mM ammonium acetate (NH_4Ac) buffer whose pH was adjusted to 2.8 with acetic acid.

RESULTS AND DISCUSSION

Method Development

Glyphosate is well known to be absorbed by soil [36], which indicates the possibility of its adsorption on the separation capillary as well. Indeed, we observed that above pH 4 (positive voltage was applied at the capillary inlet) no glyphosate peak was observed. Reduction of the pH below 3 neutralizes the capillary inner wall, thus preventing the absorption of the glyphosate, but it also stops the electroosmotic flow (EOF) necessary for feeding the electrospray. Then the decision was made to rinse the capillary before analysis with CTAB for 15 min and then with NH_4Ac buffer whose pH was adjusted to 2.8 with acetic acid for 5 min. This treatment forms a positively charged coating inside the capillary inner wall. Applying a negative voltage at the inlet end of the capillary will direct the EOF towards the capillary outlet, thus ensuring the necessary liquid feed for electrospray. At pH 2.8, glyphosate has one negative charge and migrates before neutral compounds. However, the main metabolite of glyphosate, AMPA,

remains neutral at this pH, and it will migrate together with other neutral compounds, which makes its quantification difficult under the given separation protocol.

This coating lasted over 10 h, so in general it was sufficient to apply the CTAB-rinsing procedure at the beginning of every working day. It should be noted here that the common approach of reversing EOF for CE separation involves CTAB as one of the buffer components, so in fact the inner wall of the capillary is kept dynamically coated. This approach is not applicable in the present case however, because CTAB as a buffer component will generate a rather high CNLSD background signal as it is nonvolatile. The problem was first encountered in ESI-MS and a capillary pre-rinse with EOF modifier was proposed [37]. This solution was found feasible for our purpose as well.

The capillary emitter lifetime was about the same order as that of the capillary inner wall coating by CTAB. The gold coating degenerated during the working hours of the capillary and this was indicated by visual observation of the spray using a microscope, as well as by the occasional appearance of spikes in the signal. The spikes were easily recognized from the signal and removed by software means. This observation contradicted our previous (unpublished) experience, which suggested that painting the (polyimide coated) outer wall at the end of the capillary with gold paint resulted in an electrospray tip that had long-term stability. The present behavior could be attributed to the low pH of the separation buffer. The emitter performance was restored every morning by simply cutting the capillary shorter by a couple of millimeters.

Performance Data

The developed method was assessed in terms of precision, calibration linearity and LODs. The repeatability was assessed by ten replicate injections. Peak area repeatability was found to be equal to 7.1%. Assesment of linearity and calculation of the calibration curve must take into account that environmental samples are analytes in an aqueous matrix. The conductivity of such samples is much lower than the buffer conductivity and sensitivity will be enhanced by analyte stacking. Local variability of conductivities of the sample sources suggests introducing some standardization of conductivities of different samples by diluting them in a buffer solution whose concentration is equal to or less than that used for the separation. Further, since the inherent dynamic range of the CNLSD is about two orders of magnitude, stacking of a low conductivity sample might overload this detector. As a result, it was decided to collect calibration lines for two different sample conductivities in order to facilitate measurement of both low and high concentration samples and thus extend the dynamic range of the developed method. These calibration data are represented in Table I. It follows from Table I that calibration is linear over about three orders of magnitude when using two different calibration lines and the lowest detected amount (peak height three times higher than base line noise standard deviation values, or the instrumental detection limit) is about 60 ppb.

The method detection limit (MDL) is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the analyte concentration is greater than zero. Following the Code of Federal Regulations (CFR) [38], the practical protocol to determine MDL specifies taking a minimum of seven replicates of a given spiking concentration in a range of one to five times that of the projected lowest concentration that the detector in the analytical method can measure.

TABLE I Performance data

	Buffer concentration[a] (mM)	
	5.0	0.5
Measured concentration range (μg/mL)	0.204 ... 5.10	0.061 ... 1.02
Slope of calibration line	167501	125700
Intercept of calibration line	−44704	22132
Correlation coefficient of calibration line	0.995	0.994
Lower 95% confidence limit of MDL (μg/mL)	0.77	0.44
Method detection limit (μg/mL)	0.35	0.20
Upper 95% confidence limit of MDL (μg/mL)	0.22	0.13

[a]Buffer concentration in sample.

Then, the MDL is calculated as follows: $\text{MDL} = st_{(0.99,n-1)}$ where s is the standard deviation of the seven replicate measurements and $t_{(0.99,n-1)} = 3.14$ is a t-distribution value taken at a confidence level of 0.99 and degrees of freedom $\text{df} = n - 1 = 6$. The 95% confidence interval estimates for the MDL are computed according to the following equations derived from percentiles of the *chi*-square distribution LCL = 0.64 MDL and UCL = 2.20 MDL, where LCL and UCL are the lower and upper 95% confidence limits respectively, based on seven aliquots.

Keeping in mind that the EPA regulation for glyphosate is 0.7 μg/mL, it follows from Table I that the LODs meet those requirements. The European Union has no specific regulation for glyphosate alone but its regulations for all herbicides are 0.1 ng/mL [39] which is difficult to meet with any direct methods and typically require sample preconcentration.

One might expect a smaller standard deviation of the peak's area measurements than was obtained in the present work (7%) given the use of computerized sampling whose reproducibility was estimated to be about 1 to 2% [31]. The standard deviation obtained could be associated with possible oscillations in the long-term response of the particular CPC model used in this work, which required occasional maintenance during the working day. However, this action should not significantly affect the conclusions of the present work about accuracy and MDL obtained for the separation protocol and the performance of the electrospray-CNLSD-CE as a nonspecific method of detection of glyphosate.

Applications

The developed method in this work was applied to the analysis of a commercial herbicide product, Roundup® produced by Monsanto. The samples were diluted 5×10^4 times with running buffer and filtered through a 0.45-μm membrane filter followed by direct injection into the electropherograph. The product label reports the concentration of the glyphosate to be 12.5%, which, taking the dilution factor into account, would result in a sample concentration value equal to 2.50 μg/mL. The glyphosate concentration at the 95% confidence interval was found to be 2.58 ± 0.14 μg/mL ($n = 9$). This result should be in good agreement with our measurement at the 95% confidence level.

Before the analysis of real samples, the necessary amount of buffer solution was diluted in the sample so that its concentration in the sample could match that of the

calibration solution for the lower glyphosate concentrations (see Table I). However, analysis of both lakes' water samples revealed no traces of glyphosate. This is not surprising because of the soil's ability to absorb large amounts of glyphosate, and the long interval between the application and our sample collection (about three months).

Thus, for testing the method and to detect possible interferences, the real samples were spiked with different amounts of glyphosate. Also, to make the sample even more realistic two more chemicals were spiked into the sample to test for their possible interference with glyphosate. One was the widely used herbicide, 2,4-D, and the other was a metabolite of glyphosate, AMPA. The choice of 2,4-D by the authors to simulate real-life samples is rather arbitrary because there could be a multitude of ionic compounds as candidates for interference. 2,4-D was of special interest to us because its previous detection with CE-CNLSD (using different separation protocols) has been problematic. Also, using a particular separation protocol, the AMPA zone in the capillary overlaps severely with the peak of neutral compounds – a disadvantage that suggests the need for further studies for a more advanced method where both compounds, glyphosate and AMPA, are separated from interferences. Corresponding electropherograms for the sample from the lake situated in the College of Agriculture experimental field are shown in Fig. 3. Electropherograms (not included) for the Cedar Lake water samples appeared to be very similar. Several features can be recognized from Fig. 3. First, there is a large peak (4 min) of unknown compounds, probably due to the small anions found in such waters. Second there is a large 'humic hump' extending all over the electropherogram (from 4 to 12 min) and which can probably be attributed to the dissolved organic compounds usually found in natural surface-water samples. Two well-resolved peaks are situated on the hump corresponding to glyphosate and 2,4-D. The last peak in the electropherogram (12 min) is due to the neutral compounds that migrate with EOF. It was also found that AMPA (because it remains neutral under the present separation protocol) migrates out of the column together with water. It follows from the figure that despite the expected complexity of the lake water sample, there are virtually no interferences that could overlap with the glyphosate using the given separation protocol. Although the humic hump overlaps with the peak of the analyte it is not difficult to subtract its contribution to the analyte peak area using suitable reduction software.

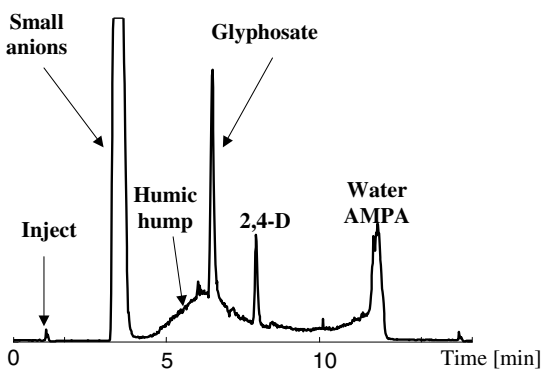

FIGURE 3 Electropherogram of lake water sample. Concentration of glyphosate 2 μg/mL and 2,4-D 2 μg/mL. Separation conditions: buffer 5 mM NH_4Ac. Pre-rinse of 15 min with 0.2-mM CTAB, –17.2-kV separation voltage.

CONCLUSIONS

It follows from the discussion above that a relatively simple separation protocol, consisting of pre-rinse of the capillary with CTAB and using NH_4Ac buffer at pH 2.8, can be implemented for CE analysis of glyphosate, a polar herbicide without chromophores and fluorophores, which could be sensitively detected using CNLSD. The selectivity of the method is good because no interferences are evident from the real environmental water sample components.

Considering the published reports of the CE separation of the glyphosate with different sample preparation/detection modes, the reported (instrumental) LODs are in the order of a few parts per billion, similar to the instrumental LOD obtained in this work. This LOD is far lower than the requirements of EPA regulations (700 ng/mL) but does not meet the much more severe regulations of the European Commission for herbicides in general (0.1 ng/mL). However, taking into account the fact that the toxicity of glyphosate is very low for humans because the shikimic acid pathway does not exist in animals [36], the result should be considered satisfactory. Detection limits can be reduced significantly by implementing head column field amplified stacking (FASS) [40,41] as proposed for glyphosate elsewhere [28]. Owing to the variations of ionic contents in different environmental waters this approach would need certain standardization of samples with predefined conductivity, and FASS was not 'pushed' to the possible limit in this work.

The result is facilitated by the use of universal detector based on CNLSD, which reportedly will soon be commercially available [42,43] meaning that rigorous validation of the method for everyday practice should be forthcoming.

Acknowledgements

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Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
AMPA	aminomethylphosphonic acid
CE	capillary electrophoresis
CNLSD	condensation nucleation light scattering detector
CTAB	*n*-cetyltrimethylammonium bromide
EOF	electroosmotic flow
ESI	electrospray ionization
HPLC	high-performance liquid chromatography
LCL	lower concentration limit
LOD	detection limit
MDL	method detection limit
NH_4Ac	ammonium acetate
UCL	upper concentration limit

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