PTV *In Situ* Derivatization of Several Acidic Herbicides Using a Newly Developed GC-MS/MS Method

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The uses of herbicides increased during the last decades. Because acid herbicides can be identified in environmental samples, specific analytical methods are required. In this work a new GC-MS/MS method was developed in order to detect some pyridinecarboxylic acids (clopyralid, triclopyr and fluroxypyr) and a methoxybenzoic acid (dicamba) from environmental water samples. Because these polar analytes involve a derivatization step, a Programmed Temperature Vaporizing injector (PTV) was used to shape the derivatization conditions and to minimize the working time. This process is called in port derivatization (IPD), a relative new technique which encourages greener practices in analytical laboratory. MtBSTFA was chosen to be the most suitable silylation agent due to generation of specific derivatized analyte structures, which can be further fragmented in the EI ionization source to produce specific ions for the targeted analytes, easily detectable with high sensitivity.Few studies analyzed the derivatization of above-mentioned herbicides with MtBSTFA, while no other paper work was identified in studying the in port derivatization of these compounds. The results revealed that the developed method is sensitive and robust to obtain quantitation limits below 10 ng/L.

Keywords: acidic herbicides, PTV in situ derivatization, GC-MS/MS, validation, environmental water samples

The risk of finding hazardous organic pollutants in environmental samples is high according with recent researches [1-5]. The pyridine herbicides are used especially in cereal agricultural industry as emergent products. These compounds are also known as synthetic auxins due to plant growth control. Concentrations of indicated herbicides from ng/L to μg/L levels were detected in surface water samples, underground water, and wastewater [6-8].

Several chromatographic methods were developed in order to quantify the compounds from different types of water, soil and biota, without matrix interferences. Due to their high polarity and water solubility, LC and LC-MS techniques are the most used for determining these compounds. LC-MS is usually used with single quadrupole or triple quadrupole MS detectors (MS and MS/MS) [8].

Alternative techniques are represented by GC and GC-MS for their reduced cost and also lower matrix effects (compounds detected by GC need to be volatile and thermally stable) when compared to ESI-LC-MS. Because the discussed auxin pyridines are not characterized by these physical-chemical properties, they cannot be determined directly using GC techniques without derivatization. The presence of the polar -COOH and -NH_2 (fluroxypyr) functional groups is the main reason for which the targeted auxin pyridines are polar molecules with very strong affinity to the GC column active sites and an increased boiling points (low volatility) [9], while other polar analytes as nitrosamines are quantified by GC without derivatization [10]. Volatility can be increased by specific derivatization processes, using specific chemical reactions like alkylation (diazomethane, methanol) [11], silylation (MtBSTFA, BSTFA) and acylation (MBTFA, MBHFBA) [12] because these reactions extract the acidic hydrogen from the polar group. Identified studies quantified these compounds by GC coupled with ECD detector, FID detector, but especially with mass spectrometric detector (using MS or tandem MS detection - MS/MS) [9, 13-15].

Beside the uses of derivatization to reduce the boiling point and to improve fragmentation for better MS detection, it is also used to enhance the compounds separation from the solution or extract [16]. In the literature data there were identified different process moments when derivatization reaction was achieved, as: derivatization in the aqueous sample before extraction, on the surface of the adsorbents (XAD-2, anion exchange resin, C18) in the sample after extraction [17], in GC inlet [9], or in mass spectrometer [17]. The applicability of PTV (programmed temperature vaporization) is well known for the obtained results in combination with large volume injection (LVI) [9]. Anyway, derivatization in PTV inlet does not require high sample volumes, instead it necessitates optimization of specific operational parameters which can lead to a complete reaction of the analytes in a shorter time period and with much less sample preparation steps, reducing thus significantly the error sources and improving method reproducibility.

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In the present study it was developed a new PTV-GC-MS/MS method preceded by a SPE extraction and concentration step to detect several pyridine herbicides from environmental samples with high sensitivity and reduced sample preparation time.

Experimental part

Chemicals

The water samples were spiked with high purity (>95%) analytical standards which are purchased individually in solid state from Sigma Aldrich (clopyralid, dicamba, fluroxypyr) and from Dr. Ehrenstorfer (triclopyr). H_2SO_4 98% from Sigma Aldrich was used to acidify the water samples to pH 2. Methanol (J.T. Baker) and deionized water (Merck Millipore) were used in SPE cartridges conditioning, while samples were extracted with ethyl acetate (Sigma Aldrich, GC purity). Two types of cartridges were used for analytes extraction, polymeric Strata-X (1000 mg) and Strata-C18-E (1000 mg) from Phenomenex (USA). Anhydrous magnesium sulphate from Chempur was used to dry the extracts before injection. Final extraction volume and the mass spectrometric response variation were corrected with a syringe internal standard, pentachloronitrobenzene known as quintozene (Fluka, neat, analytical grade). Derivatization was performed with neat MtBSTFA with 1% t-BDMCS, acquired from Cerilliant.

Equipment

The solid phase extractions were realized automatically with Dionex AutoTrace 280 SPE from Thermo Scientific. All analyses were performed using a GC coupled with a triple quadrupole mass spectrometric detector (GC-MS/MS TSQ 8000 Evo, Thermo Scientific). A volume of 1 µL extract was injected in a splitless mode using a PTV inlet, where T1 starts from 150°C to T2 of 200°C (hold 1 min) with 14°C/sec evaporation rate. With a rate of 14°C/sec the sample was transferred into the GC column at 1 mL/min flow-rate (He 6.0 purity). The compounds were separated on a fused-silica capillary column, TG-5SILMS (60 m, 0.25 mm x 0.25 µm). The oven temperature program started from 50°C (2 min), increased to 200°C with 30°C/min rate, then increased with 10°C/min to 290°C (5 min). The transfer line was maintained at 300°C. The analytes were ionized at 250ºC (70 eV, EI). The compounds were identified in SIM mode (single ion monitoring), then quantified in SRM mode (single reaction monitoring) corresponding to ion transitions after fragmentation with N_2 (5.3 purity) at variable collision energies (5-40 eV) applied in the second quadrupole (collision cell).

Samples preparation

The method development was carried out with deionised water. A volume of 100 mL water acidified to pH 2 and spiked with targeted analyted was extracted (loaded) at a 10 mL/min flow-rate on polymeric Strata-X cartridges which were previously conditioned with 10 mL methanol, 10 mL pH 2 deionised water and 10 mL ethyl acetate. The cartriges were dried for 15 min with gentle nitrogen stream and analytes were desorbed using 5 mL ethyl acetate at a 5 mL/min flow-rate. In the final extract an injection internal standard was added (200 µg/L qunitozene), and then extracts were dried with approximate 0.5 g magnesium sulphate and concentrated to 1.0 mL. After adding 10 µL of derivatization agent in the GC injection vial (MtBSTFA), the samples were rapidly injected. The derivatization procedure is performed in the GC inlet (PTV).

Results and discussion

Optimization of PTV conditions for in situ derivatization reaction

As previously mentioned, due to their polarity, the pyridine acidic herbicides cannot be directly detected using GC-MS; for this reason, they require derivatization [17]. Derivatization reaction time and temperature are key factors which affect the derivatization reaction efficiency. The MtBSTFA classical silylation process of pyridine carboxylic herbicides which was realized at 80°C for 60 min was shortened by direct reaction in the GC PTV inlet. Several PTV parameters were optimized in order to increase the GC-MS/MS response for the selected compounds, increasing thus method sensitivity and avoiding formation of secondary products.

The PTV injection mode involves several steps like: injection phase, evaporation phase, analyte transfer phase and GC liner cleaning phase. The optimized parameters are injection port temperature (T1), evaporation temperature (T2), evaporation rate and stationary time before column sample transfer. High T1 values correspond to lower detections which may be a consequence of compounds loosing from the injection port. Anyway, injecting at T1 200°C and transferring into column at 250°C decrease the signal yield with 15%, while the 50-150°C variation slightly improve the esterification reaction.

By studying the effect of evaporation temperature noted as T2 ranged in the domain of 150-260°C, while maintaining the injection temperature (T1) constant at lower values, 150°C. The optimum peak areas were obtained at transition temperatures of 150-200°C, higher with 31-97% than the other data, as it is shown in figure 1. Usually the TBDMS derivatives of these compounds are injected at 270°C, in a glass liner [9], but the results at the constant temperature of 250°C were lower than for gradient temperature which suggests that the in port derivatization is strongly influenced by temperature program.

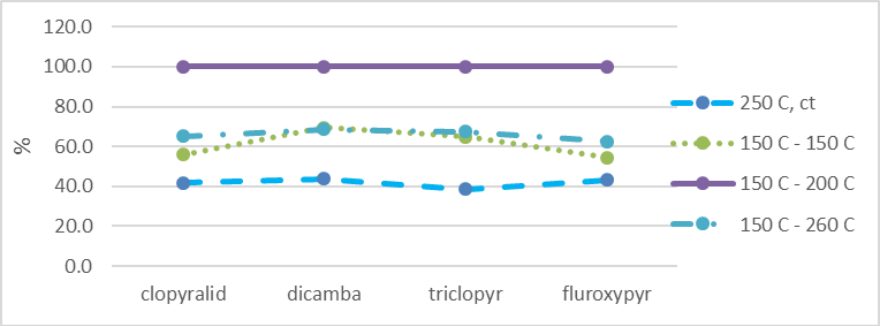


Fig. 1 Percentage compound area reported to the highest response which corresponds to 150-200ºC, with ramp rate 14.5 ºC/sec, inlet residence time 1 min

Variations of the evaporation rate from 2-14.5ºC/sec have insignificant influence which is indicated by small values of relative standard deviation, 3.3-6.4%. The stationary time in liner after evaporation step was varied for 0.5, 1, 2 and 3 min. It was shown that for 1-2 minutes the compounds are stable in liner. The volume of derivatization agent influences the reaction. Thus, for the addition of 2 - 20 µL MtBSTFA, the analytical signal of each compound increases up to 57-66% (Fig. 2).

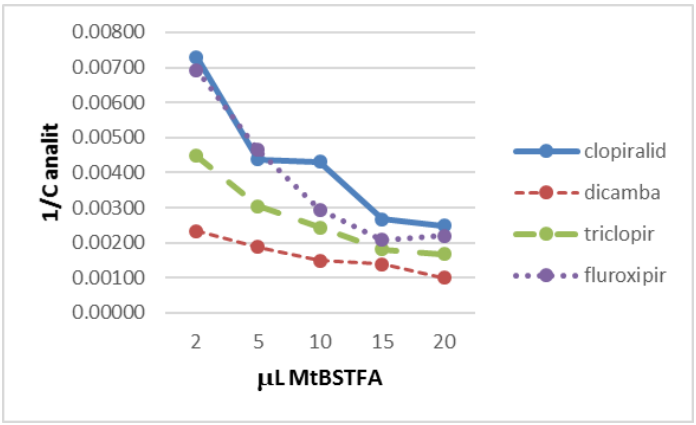


Fig. 2 The representation of MtBSTFA added volume reported to the analyte concentration

MS/MS optimization

After optimization of PTV injection mode parameters for *in situ* derivatization, the following step in method development was optimization of the MS/MS detection parameters. Due to MtBSTFA structure, it was easy to fragment the TBDMS derivatives into specific ions. The abundant mass spectra were obtained in full scan mode, in 50-500 m/z range. The highest ions intensity corresponds to a specific fragmentation of molecular ion $[M]^+$ after electronic impact as the result of the cleavage of:

-$[M-57]^+$ - $^+$t-Bu group for clopyralid and dicamba;

-$[M-115]^+$ - $^+Si(CH_3)_2$-t-Bu group for fluroxpyr and triclopyr.

In order to increase the detection selectivity and method sensitivity, there were chosen the most intense MS/MS transitions (SRMs). For this, it was optimized the collision energy values applied in the collision cell (second quadrupole). The chosen SRMs and collision energies correspond to highest analyte detection intensities and are presented in table 1. The compounds show different fragmentation pathway as a result of more easily removal of possible several groups:

-$[M-102]^+$ - $^+COO\text{-}Si(CH_3)_2$ (clopyralid)

-$[M-44]^+$ - ^+COO (clopyralid)

-$[M-15]^+$ - $^+CH_3$ (dicamba)

-$[M-74]^+$ - $^+OSi(CH_3)_2$ (dicamba)

-$[M-58]^+$ - $^+CH_2COO$ (fluroxypyr, triclopyr)

Table 1

THE HERBICIDES DETERMINATION CHARACTERISTICS IN GC-MS/MS ANALYSIS

TBDMS derivative	tR	Molecular mass	SIM*	SRM	Collision energy
Clopyralid	13.23	306	**248/250**, 204/206, 146/148, 175/177, 290/294	248-> 146 248-> 204	35 eV 15 eV
Dicamba	13.90	334	**277/279**, 203/205, 233/235, 262/264, 188/189	277-> 203 277->262	20 eV 10 eV

Triclopyr	15.19	369	314/312, **256/254**, 316, 258, 75/73, 93, 315	312->254 314 -> 256	15 eV 20 eV
Fluroxypyr	16.39	368	**253**/255, **311**/313	311-> 253 313-> 255	20 eV 20 eV

* The bolded values have the highest intensity

Derivatization reaction

The MtBSTFA reacts with carboxylic group (-COOH) attached to the selected compounds (figure 3**)** by generating t-butyldimethylsilyls (TBDMS) derivatives according to eq. 1.

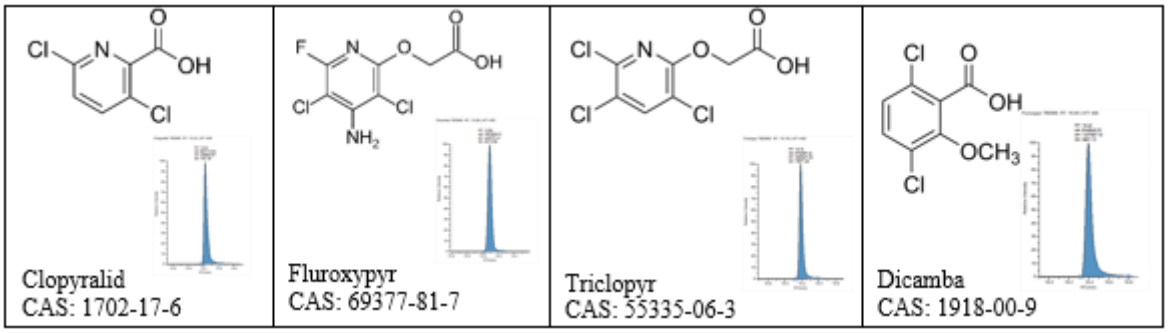
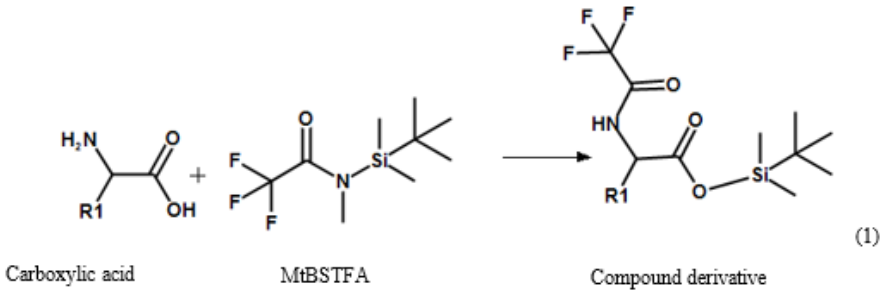


Fig. 3 Herbicides molecular structure



These herbicides are classified as amphoteric compounds due to presence of –COOH group as an acid and pyridine ring which acts as a base. The silylation reaction with MtBSTFA is catalyzed by 1% tert-butyldimethylchlorosilane (t-BDMCS) but pyridine can also be used as a catalyst [16]. It is possible that the pyridine group from high amount of pyridinecarboxylic acid herbicides to influence and enhance the silylation reaction, which may be the reason for the overestimated response obtained for higher concentrations (200-400 ng/L). The calibration in this range fits a quadratic regression, characterized by R^2>0.995.

It is known that primary amino group (-NH_2) reacts slowly with silyl agents than –COOH, but rather with acyl reagents. MtBSTFA contains both sylil and acyl group which makes possible sylilation reaction with –COOH and acylation with –NH_2 (Eq. 1). Aminopyralid have similar chemical structure with fluroxypyr, but in some conditions –NH_2 from aminopyralid is acylated while the amino group from fluroxypyr is less reactive. No other derivatization reactions of aminopyralid with MtBSTFA was recorded from the literature data.

Validation of the developed SPE-PTV-GC-MS/MS method

The newly developed SPE-PTV-GC-MS/MS method for the determination of acidic pyridine herbicides was successfully validated for the determination of acidic pyridine herbicides from environmental water samples. The tested validation parameters were specificity, linearity, precision, accuracy recovery, limit of detection (LOD) and limit of quantitation (LOQ).

For all target analytes the method calibration was linear in the range of 1-100 µg/L. The optimized method is sensitive enough to obtain low limits of quantitation down to ng/L (table 2) which are similar to other reported literature studies for water matrices (table 3). Limits of detection and quantitation were calculated using standard deviation (σ) obtained for 6 samples of 1 µg/L by following equations: LOD=3σ, LOQ=10σ.

The method accuracy (recovery) and precision reflect the errors propagation in the analytical sample preparation and detection steps which in our method's case were: SPE extraction, sample purification and concentration, instrumental GC-MS variability (injection precision, in situ PTV derivatization, column separation, and MS/MS detection). The data assessed in terms of precision (RSD%) and accuracy or recovery (bias - deviation from spiked nominal concentration value) are presented in table 2.

Table 2
VALIDATION DATA FOR THE NEWLY DEVELOPED SPE-PTV-GC-MS/MS METHOD

	LOQ µg/L	Precision Repeatability, %	Precision Reproducibility, %	Recovery, % (50 µg/L)	Recovery, % (100 µg/L)	Recovery, % (200 µg/L)
Clopyralid	0.010	8.10	15.6	102.5	82.7	105.0
Dicamba	0.011	7.95	16.6	94.8	88.6	91.3
Triclopyr	0.008	8.25	20.4	95.0	80.0	96.2
Fluroxypyr	0.010	8.26	17.3	97.9	102.3	119.9

Table 3
SEVERAL GC STUDIES CONCERNING DETERMINATION OF SELECTED HERBICIDES

Method	Derivatization agent	Analytes	Matrix	LOQ	MS mode	Reference
GC/MS	MtBSTFA	clopyralid, dicamba, fluroxypyr, triclopyr	surface water	4-90 µg/L*	SIM	[12]
GC-HRMS	diazomethane	dicamba, triclopyr	surface water	0.17 ng/L	SIM	[13]
GC-CI-MS	pentafluorobenzyl bromide	clopyralid, dicamba, triclopyr	drinking water	1.97-2.4 ng/L	SIM	[14]
GC-ECD	methanol	triclopyr, aminopyralid	pasture, soil	0.02-0.05 mg/kg	-	[11]

*These values are not reported to water volume.

Conclusions

The present study was focused to develop a rapid, accurate and sensitive SPE-PTV-GC-MS/MS method for the determination of several carboxylic herbicides from environmental water samples. The method was successfully validated with respect to specificity, linearity, precision, accuracy, detection and quantitation limits and provided good results.

The chromatographic conditions obtained are effective and sensitive for the determination of ng/L herbicide residues down to ng/L levels (8-11 ng/L).

Choosing the direct derivatization on the injection port minimized significantly the analysis time and number of analytical steps, while MtBSTFA reacted non-selectively with the target analytes. It was assessed that PTV *in situ* derivatization is sensitive to the variation of the evaporation temperature and less influenced by the injection port temperature, evaporation rate and stationary time before column sample transfer. The MtBSTFA volume is also critical in derivatization reaction.

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