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Simple and Rapid High-Performance Liquid Chromatography Method for Simultaneous Determination of Picloram and 2,4-D in Pesticide Formulations

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Abstract: Picloram and 2,4-D are systemic herbicides used to control a wide range of broad-leaved weeds and post-emergence annual and perennial broad-leaved weeds, respectively. The use of plant protection products containing Picloram and 2,4-D necessitates the development of analytical methods, useful for the laboratories focused on control, for monitoring. In this study, we designed and validated an analytical method for the rapid determination of picloram and 2,4-D by HPLC-DAD. The method involves the extraction of the substances by sonication of the sample with methanol, followed by dilution in acetonitrile, and direct injection on a liquid chromatography system, based on the use of a Gemini C18 column. We used an isocratic mobile elution consisting of acetonitrile and water acidified 1% with H_3PO_4 (50:50, *v/v*). We validated the proposed method, which demonstrated linearity within the concentration range of 0.01–0.028 mg/mL for picloram and 2,4-D, with a correlation coefficient (R^2) of 0.9993 for picloram and 0.9999 for 2,4-D. We considered precision, repeatability and selectivity in the validation. The repeatability of the method expressed as percent of relative standard deviation (%RSD) was lower than 1% for both substances. The proposed method is suitable for the simultaneous determination of picloram and 2,4-D in pesticide formulations.

Keywords: picloram; 2,4-D; HPLC; pesticide; formulation

1. Introduction

The wide use of phytosanitary products in agriculture led to the development of analytical methods that are increasingly rapid and adequate for the environmental needs of laboratories.

In accordance with European Regulation n.1107/2009, the reduction in risks associated with the use of pesticides is fundamental for human health and the environment. To achieve this goal, the quality control and monitoring of plant protection products are two fundamental steps for the protection of human and environmental health.

For this reason, we require adequate analytical methods that can be applied by laboratories focused on control for monitoring.

Additionally, the use of plant protection products containing more than one active substance is common, as the synergy among the substances increases the effectiveness of the product against the pests to be controlled.

Picloram [4-amino-3,5,6-trichloropyridine-2-carboxylic acid] is a selective systemic herbicide used for general woody plant control (Figure 1a).

Picloram is also used to control most annual and perennial broad-leaved weeds (except crucifers), including woody weeds, bracken, ferns, docks on grassland and non-crop areas. However, most grasses are resistant to it. A chlorinated derivative of picolinic acid, picloram is part of the pyridine family of herbicides. Picloram is often sold mixed with 2,4-D (2,4-dichlorophenoxy acetic acid), which is a selective systemic herbicide (Figure 1b).
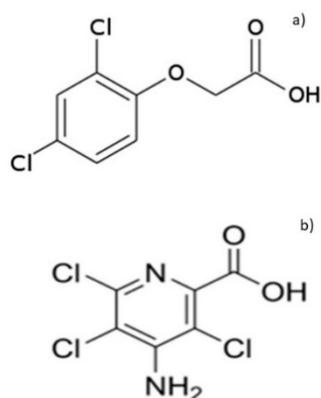
Figure 1. (**a**) Chemical structure of Picloram and 2,4-D (**b**).

A number of analytical methods are available for the determination of picloram residues in food and environmental matrices [1–8]. In the literature, few analytical methods have been reported for the determination of picloram in formulations. AOAC is the only official method for the determination of picloram and 2,4-D in mixtures in pesticide formulations, which applies a liquid chromatographic method [9]. A spectrophotometric method for the determination of picloram is available [10,11]. HPLC-UV detection was used to determine picloram and 2,4-D in mixed formulations [12]. CIPAC reported an analytical method for the simultaneous determination of picloram and 2,4-D in mixtures [13].

Salt is readily adsorbed by the roots of plants, whereas esters are readily adsorbed by the foliage. Translocation occurs with accumulation principally at the meristematic regions of the shoots and roots. Salt acts as a growth inhibitor and is used in post-emergence control of annual and perennial broad-leaved weeds in cereals, maize, sorghum, grassland turf, grass seed crops, orchards, rice, and forestry, and on non-crop land.

Analytical methods are available for the determination of 2,4-D pesticide residues on fruits and vegetables [14–18], and some analytical methods, for the determination of 2,4-D in formulations using HPLC, are available [19,20]. The herbicide 2,4-D is sold in different formulations and in mixtures with other substances, such as MCPA, diuron, dichlorprop, mecoprop, picloram and many other herbicides.

A literature review showed that no methods are available for the simultaneous determination of 2,4-D and picloram in formulations. Most of the available methods concern the simultaneous determination of other substances in pesticide formulations. Hafeez [21] described the simultaneous determination of fipronil, chlorfenapyr and pyriproxyfen in insecticide formulation by HPLC. Balayiannis and Karasali [22] described a rapid method for the determination of famoxadone and cymoxanil in pesticide formulation with HPLC. Quintas [23] described the determination of folpet and metalaxyl in pesticide formulation using IR-FTIR spectroscopy. Other authors described the analysis of fosetyl-aluminium [24], metalaxyl and its adjuvant [25], chlorpyrifos and its impurity [26,27] and azoxystrobin and its impurity [28,29] in different types of formulations. The Official Collaborative International Pesticide Analytical Council (CIPAC) proposed a method for the analysis of formulated pesticides containing 2,4-D and picloram [13]. The method recommended the use of an ion exchange column with liquid chromatography and the use of reagents, such as NaOH and $Na_2B_4O_7/NaClO_4$, as eluents. This method is not rapid enough for control-focused laboratories, due to the type of column and eluents.

In this study we sought to develop a rapid and simple HPLC method for the simultaneous determination of 2,4-D and picloram in pesticide formulation using a Gemini C18 column. We developed an analytical method that uses few chemicals reagents and a green solvent, such as acetonitrile and water. We validated the method in terms of accuracy, precision and linearity. One of the advantages of this method is the simultaneous determination of picloram and 2,4-D in pesticide formulation. The developed method could be used for a simple and reliable simultaneous analysis in monitoring laboratories.

2. Materials and Methods

2.1. Chemicals and Reagents

Picloram (99.4% purity), 2,4-D (99.7% purity) and propiophenone (97.5% purity) of certified standards were supplied by ChemService (West Chester, PA, USA). We purchased HPLC-grade methanol and acetonitrile from VWR Chemical International (Randor, PA, USA). We obtained the ultrapure water required for HPLC analysis from a Milli Q system obtained from Merck (Milano, Italy). We purchased phosphoric acid ($\geq$85% purity) from Sigma Aldrich (Milano, Italy). We obtained a 0.45 μm Chromacol Filter from Thermo Scientific (Rockwood, TN, USA). We obtained the plant protection product containing picloram and 2,4-D from the Italian market.

2.2. Instrumentation

We purchased an Elmasonic S 60 H ultrasonic water bath from Elma Schmidbauer GmbH (Singen, Germany). We used a Mettler Toledo AE200 analytical balance from Gibertini elettronica Srl (Milano, Italy), and an STR4 rotator drive from Bibby Stuart Scientific (Staffordshire, UK). The HPLC system used for the determination consisted of a Flexar quaternary pump, a Flexar autosampler, an oven column, and a Flexar PDA detector (Shelton, CT, USA).

2.3. Chromatographic Procedure

The separation was accomplished using a Gemini C18 column with 150 mm $\times$ 3 mm id and a 5 μm particle size (Chemtek analytica Bologna, Italy). The column flow rate was 0.5 mL/min. We held the column oven at 25 °C, the autosampler injected 10 μL, and we performed detection at 235 nm. The mobile phase consisted of acetonitrile and water acidify 1% with H_3PO_4 (50:50, *v/v*) in isocratic mode. The duration of analysis was 20 min.

For chromatographic analysis, we used Chromera software (version 4.1.2.6410).

2.4. Sample Preparation

Before analysis, we mechanically homogenized the sample, which was stored at ambient temperature without further additives. The label contents of picloram and 2,4-D were 32.8 and 109 g/kg, respectively. Due to the difference in the concentration of the substances, we prepared different sample solutions to obtain solution concentrations equivalent to those of the standard solutions.

2.4.1. Standard Solution

We placed 25 mg each of picloram and 2,4-D in a 25 mL flask and filled the remaining volume with methanol. We maintained this solution (S1) at 4 °C, and we used it to prepare the calibration solution for the linearity curve.

We placed 25 mg of propiophenone in a 25 mL flask and filled it to volume with methanol (IS1). To determine linearity, we prepared six solutions.

We diluted a quantity of picloram, ranging from 0.25–0.80 mL of S1 solution, in a 25 mL flask and added 0.5 mL of internal standard (IS1), which we brought up to volume with methanol.

For quantitative analyses, we prepared calibration solutions with about 25 mg of picloram and of 2,4-D in a 25 mL flask, which we brought to volume with methanol. We diluted 1 mL of this solution in a 50 mL volumetric flask and added 1 mL of internal standard solution (IS1), which we brought up to volume with acetonitrile.

2.4.2. Samples Solutions

For picloram, we weighed a quantity of the product to contain 25 mg of picloram, which we added to 20 mL of methanol sonicate for 5 min, which we left to reach ambient temperature and, then, brought up to volume at 25 mL with methanol (Sol A). We diluted 1 mL of Sol A, added 1 mL of IS1, and filled up to a volume of 40 mL with methanol.

For 2,4-D, we weighed a quantity of the product to contain 25 mg of 2,4-D, to which we added 20 mL of methanol. We then sonicated the mixture for 5 min, left it to reach ambient temperature, and filled it up to a volume of 25 mL with methanol (Sol A). We diluted 1 mL of Sol A, added 1 mL of IS1, and filled up to a volume of 40 mL with methanol.

The typical chromatograms for picloram and 2,4-D samples are shown in Figure 2, respectively.
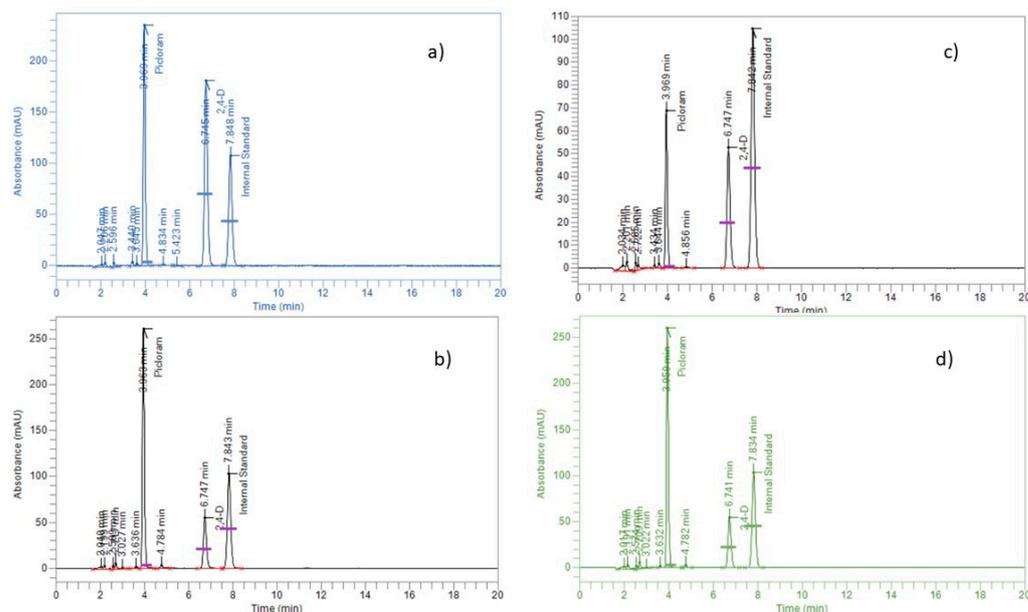

Figure 2. Chromatograms of picloram and 2,4-D determination at 235 nm: (**a**) picloram sample at concentration of 0.7 mg/mL of product corresponding to 0.025 mg/mL of picloram with propiophenone (IS) 0.025 mg/mL; (**b**) standard of picloram solution at concentration of 0.025 mg/mL with propiophenone (IS) 0.025 mg/mL; (**c**) 2,4-D sample at concentration of 0.2 mg/mL of product corresponding to 0.025 mg/mL of 2,4-D with propiophenone (IS) 0.025 mg/mL; (**d**) standard of 2,4-D solution at concentration of 0.025 mg/mL with propiophenone (IS) 0.025 mg/mL.

2.5. Method Validation

2.5.1. Trueness and Precision

As no blank formulation was available, we calculated the trueness of the method as the percentage recovery of the picloram and 2,4-D, by referring to the label contents. We performed five replicates and we calculated the mean recovery. We determined the precision in the case of repeatability (relative standard deviation repeatability (RSDr)) by analyzing five replicate samples on the same day.

2.5.2. Linearity

We drew calibration curves by plotting the mean peak area versus concentration, for each substance. We evaluated the linearity by calculating the correlation coefficient (R^2), intercept, and slope of the regression line at six concentration levels. We prepared each calibration solution by diluting the stock solution for each substance. We added an appropriate internal standard quantity to each calibration solution.

2.5.3. LoD and LoQ

The concentration levels of picloram and 2,4-D in commercial formulations reduce the importance of these analytical figures in the case of pesticide formulation analysis. As such, we calculated these parameters by following ICH guidelines, based on the SD

of the responses (σ) and their respective slopes (S). We determined the SDs based on the calibration curves. We used the following equations:

$$LoD = 3.3\ \sigma/S$$

$$LoQ = 10\ \sigma/S$$

3. Results and Discussion

A system suitability test is a crucial tool in liquid chromatography, which is performed to enhance the resolution of a chromatographic system. Prior to the development of our method, we performed a system suitability test using a standard solution containing picloram, 2,4-D, and prophiophenone (IS). We determined the system suitability parameters, including repeatability of the retention times and peak areas, tailing factor, and resolution.

We determined the repeatability of retention time and area by injecting the standard solution five times and calculating the mean value, standard deviation, and %RSD. The relative standard deviation (%RSD) of the acceptance criteria peak areas and retention times was <1%. The %RSD values obtained for all compounds were less than 1%, which we considered acceptable, as shown in Table 1.

Table 1. System suitability parameters.

Repeatability Retention Time (RSD%)	Repeatability Area (RSD%)	Tailing Factor	Resolution
2,4-D			
0.045	0.401	1.164 ± 0.005; RSD% = 0.465	9.84 ± 0.04 RSD% = 0.38
Picloram			
0.098	0.191	1.227 ± 0.002; RSD% = 0.148	5.33 ± 0.03 RSD% = 0.51
Prophiophenone			
0.04	0.249	1.071 ± 0.002; RSD% = 0.241	10.86 ± 0.03 RSD% = 0.28

The tailing factor (S) is a coefficient that shows the degree of peak symmetry. A new column is acceptable if the tailing factor is 0.9–1.2. In practical terms, an S value below 1.5 is usually suitable, and a value up to 2.0 may be acceptable, depending on the separation and resolution of the peaks. In our study, the tailing factors for each compound were in the range 1.07–1.22, as reported in Table 1, indicating the high-quality performance of the column.

Resolution is an important HPLC performance indicator, usually assessed by how quickly and how completely target compounds in a sample are separated as they pass through the column. A resolution of 1.0 or higher represents adequate separation. The resolution obtained using the Gemini column was higher than 1.0, as reported in Table 1.

On the basis of the results assessing the suitability of the system, we found that the Gemini column separated the compounds well when working with acetonitrile and water acidified with 1% H_3PO_4 mobile phase.

We used specificity, linearity, precision, accuracy, LoD and LoQ to evaluate the performance of the method.

3.1. Specificity

Specificity is a measure of the degree of interference in the analysis of pesticide formulations. As a blank formulation was not available, we evaluated the specificity using a blank extraction and compared the presence of peaks at the same retention times of picloram, 2,4-D and prophiophenone. The blank extraction simulated the extraction of a sample that did not contain the formulation. The comparison showed that no interferences occurred at the same

retention times of 4.05 min for picloram, 7.21 min for 2,4-D and 8.31 min for IS. As such, we found that the proposed method was specific for the studied compounds.

3.2. Linearity

We measured the linearity of the method by establishing calibration curves. We prepared a series of working solutions, as described in the Section 2.4. We used triplicate injections and the mean value for each solution to obtain the linearity curve.

For picloram, the linearity was in the concentration range of 0.01–0.032 mg/mL, with the internal standard at a concentration of 0.02 mg/mL. The regression curve was linear (y = 1.096x + 0.022) with R^2 = 0.9993.

For 2,4-D, the linearity was in the concentration range of 0.01–0.032 mg/mL, with the internal standard at a concentration of 0.02 mg/mL. The regression curve was linear (y = 0.3927x + 0.0093) with R^2 = 0.9999.

Even if we had not used the curves for quantification, we verified the linearity applying the Lack of Fit test (LoF). We obtained Fobs = 3.19 and Ftab (α = 0.05) = 3.26, for picloram. For 2,4-D, we obtained Fobs = 0.32 and Ftab (α = 0.05) = 3.26. Since Fobs < Ftab the test was passed and the curves were linear for both substances.

3.3. Precision (Repeatability)

We calculated precision in terms of repeatability within one day.

For picloram, the relative standard deviation was 1.26% and the Horwits RSDr was 2.25 at a concentration of 3.2%. Since the relative standard deviation was less than the Horwitz RSDr, the repeatability test result was acceptable for picloram. We calculated a value of 1.17% for the precision as 3 times the SD, which we considered acceptable for picloram with a declared nominal content of 3.28%.

For 2,4-D, the relative standard deviation was 1.54% and the Horwits RSDr was 1.87 at a concentration of 10.9%. Since the relative standard deviation was less than the Horwitz RSDr, the repeatability test result was acceptable for 2,4-D. The value of 5.28% for the precision, calculated as 3 times the SD, could be considered acceptable for 2,4-D with a declared nominal content of 10.9%.

3.4. Reproducibility

We determined the reproducibility by analyzing samples on two different days. The analyses were conducted by two different analysts. We calculated the contents of picloram and 2,4-D, standard deviation and relative standard deviation for each analysis. For picloram, the reproducibility was acceptable, as the RSD was lower than the results of the modified Horwitz equation 2.25 < 3.36, with Horrat equal to 0.68. The mean average content for the two reproducibility tests was 31.0 g/kg with an RSD% of 1.4. For 2,4-D, the reproducibility was acceptable, as the RSD was lower than the results of the modified Horwitz equation 1.87 < 2.79, with Horrat equal to 0.76. The mean average content for the two reproducibility tests was 113.9 g/kg with an RSD% of 1.48. All the RSD% values were <3 (Table 2). This confirmed the reproducibility of the method.

Table 2. Reproducibility for picloram and 2,4-D.

	Picloram		2,4-D	
	1st Day	**2nd Day**	**1st Day**	**2nd Day**
Mean value (*n* = 5)	31.0	31.0	114.0	113.8
Standard deviation (SD)	0.39	0.48	1.76	1.62
Relative Standard Deviation (RSD%)	1.26	1.54	1.54	1.42
Horwitz RSDr	2.25	2.25	1.87	1.87
Horwitz RSDR	3.36	3.36	2.79	2.79
Horwitz ratio (Horrat)	0.56	0.68	0.76	0.76

3.5. Accuracy

As no blank formulation was available, we calculated the recovery rates by referring to the mean quantity of active substances found in each sample under investigation, compared with the label content. We evaluated the accuracy, expressed as mean recoveries, on five samples, as shown in Table 3.

Table 3. Accuracy for picloram and 2,4-D.

Analyte	Label Content (g/kg)	Amount Measured (g/kg; *n* = 5)	Intraday Accuracy (% *n* = 5)	Inter Day Accuracy (% *n* = 10)
Picloram	32.8	31.03	94.63	94.60
2,4-D	109	114.04	104.62	104.53

3.6. LoD and LoQ

The LoD for picloram and 2,4-D were 0.045 and 0.53 µg/mL, respectively. The LoQ were 0.14 and 1.6 µg/mL, respectively.

4. Conclusions

In this study, we developed and validated a green analytical method for the determination of picloram and 2,4-D in commercially available plant protection products by HPLC-DAD. The method used green solvents, such as acetonitrile and water, and used less reagent, making the method useful for routine analysis in monitoring laboratories. The method provided appropriate separation and resolution of chromatographic peaks. The statistical parameter and recovery data revealed the high accuracy and precision of the method. For the linearity curves an $R^2 > 0.997$ demonstrated the high sensitivity of the method. Chromatograms were free from interference, demonstrating the specificity of the method. We concluded that the proposed method is simple, accurate, precise, and reproducible, and is recommended for routine quality control analysis.

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