SEPARATION OF SELECTED PESTICIDES BY AN HPLC TECHNIQUE; PERFORMANCE PARAMETERS AND VALIDATION

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Abstract: *The aim of this work is to get the performance parameters investigated by hight performance liquid chromatography (HPLC), for the separation method of 2,4-Dichlorophenoxiacetic acid, 3,6-Dichloro-2-methoxybenzoic acid, an organic mixture with herbicide action. The chromatographic separation with better peak shape was achieved. The retention times (t_R), peak resolutions (R_S), separation factors (α), column efficiency (N_{eff}), height of theoretical plates (HETP), indicate that the mobile phases in gradient, containing acetonitrile and water with 1% acetic acid are the best for the separation of investigated components on cromatographic column C18. Also it has been shown that in data conditions the methode is sensitive, precise and reproductible.*

Keywords: *HPLC, environment, performance, validation*

1. Introduction

Pesticides are chemical protection tools for plants. They are obtained from one ore more biological compounds efficient.With few exception like growing regulators, the biological active ingredients are toxic. Due to this toxicity are dictate good practice in dose, distribution and in use of pesticides. Also pesticides pass from ground water to vegetables [1], plants and foods and finally they are accumulate in animal fat. Pesticides affect the structure and immune efficiency and reduce the immunity at infection. The farmers that use the pesticides must take into acount the parameters, as follows: the configuration of marketing, the technology of conditioning, the technology to apply, the maximum limit of waste.The mixture of these two components with herbicide action are used in disproof of weed from beating cereals. 2,4- Dichlorophenol is the toxic component born in the of 2,4-dichloro-phenoxiacetic acid manufacturing process and retrieved in the end of the mix in acceptable limit.The performance parameters of this separation method have a great importance, they reflect the correct and the exactely dosage of the components in the mixture and on ground. The presence of pesticides in environment induces the modification of the quality environment components- ground, underground and surface water, the optimization of these quntities meaning an important factor in environmental quality protection.

2. Experimental

Chemicals and reagents

The components of mobile phases: acetonitrile and water (LABOSI), HPLC grade. Acetic acid, glacial degree, 2,4-diclorphenoxiacetic acid 99,9 % purity (named 2,4D acid), 3,6-dichloro-2-metoxibenzoic acid 99,9% purity (named dicamba), 2,4 dichlorophenol 99% purity (named DCF) from Merck.

Instrumentation and conditions

The cromatographic investigations was carry out on a VARIAN PROSTAR liquid cromatograph system equipped with: quaternary pump (model 9100), autosampler (model 9010), UV detector (model 9065). The data were aquired via Prostar data aquisition workstation. Mobile phase consists of water and acetonitrile HPLC grade, injection volume: 20 µl, flow rate : 1ml/minute, λ: 280nm. Reversed phase analysis was performed at 22^0C using an Bondesil C18 column, 5µ (25cm, 4mm ID) [2]. Table 1 shows the gradient elution used.

Table 1
Gradient of mobile phase

Time(minutes)	B (%)	C (%)
0	95	5
9	95	5
17	50	50
30	50	50

B = 1% acetic acide in HPLC water; C = 1% acetic acid in HPLC acetonitrile. Elution order: dicamba, 2,4D acid, 2,4DCF

Standard preparation

To get the separation parameters was used synthetic standard solution named stock solution: 0.07 g 2,4D acid, 0.025g Dicamba, 5ml DCF standard solution, completed to 25 ml with (alkaline) HPLC grade water. Syntetic standard solution keeps the same report between the components like in the mixture with herbicide action. DCF standard solution was prepared from 0,1gDCF diluted to 25ml with (alkaline) HPLC water.

Sample preparation

A representative quantity of sample is weighed and the active ingredients are extracted with selective solvents. Follow the evaporation of solvent and than active ingredients are solved and diluted to 25 ml with HPLC grade water.

Calculations

Capacity factor (K') [3] was calculated using equation (1):

$$K' = \frac{t_R - t_O}{t_O} = \frac{t'_R}{t_O} \quad (1)$$

where: t_R is the retention time of the solute t_0 is the time for an unretained solute; t'_R is the adjusted retention time of the solute The condition of strong separation from technical book of Varian instrument is K'≥1 [3].

Column selectivity (α). The separation factor (α) [3] was calculated using equation (2):

$$\alpha = \frac{t'_{R2}}{t'_{R1}} \quad (2)$$

where: t'_{R2} and t'_{R1} are adjusted retention times for two adjacent peaks.
The selectivity condition is $\alpha \geq 1$.

Peak resolution (R_S). The peak resolution (R_S) [4] was calculating using the equation (3):

$$R_S = \frac{1.18 \times \Delta t_R}{w_1 + w_2} \quad (3)$$

where: Δt_R is the difference in retention times between the two peaks; w_1 and w_2 are widths of the two peaks at half of their height. The condition of separation is:
$R_S = 1$ means 98% separation; $R_S = 1.5$ means 99.7% separation.

Column efficiency (N_{eff}). The column efficiency [4] was calculated as number of theoretical plates using equation (4):

$$N_{eff} = 5.54 \cdot \left(\frac{t'_R}{w} \right)^2 \quad (4)$$

From technical book of Varian instrument the efficiency condition is $N_{eff} > 400$.

Height of theoretical plates (HETP) [4] was calculated using equation (5):

$$HEPT = \frac{L}{N_{eff}} \quad (5)$$

where: L is the length of the column (cm); N_{eff} is the effective number of theoretical plates. Also, from technical book the accepted value is HETP = 0.001 ÷ 0.002 mm.

Standard deviation (Sr) [5, 6] was calculated using equation (6):

$$S_r = \sqrt{\frac{\sum_{k=1}^{n} (x_k - \bar{x})^2}{n-1}} \quad (6)$$

Repeatability limit (r) [9] was calculated using equation (7):

$$r = t \cdot \sqrt{2} \cdot S_r \quad (7)$$

where t = 1.96, student coefficient for 95% confidence interval.

3. Results and discussions

For cromatographic separation of 2,4D acid, Dicamba and DCF on C18 stationary phase (4,6mm, 5µm) with varying column lengths from 150 to 250 was attempted. Different mobile phase composition containing water and acetonitrile with 1% acetic acid were tried. The column 250mm x 4,6mm, 5 µm showed higher elution times and good resolutions for the components of interest, respectively 21,89 seconds for dicamba, 24,60 seconds for 2,4D acid and 26,20 seconds for DCF. System suitability is shown in Table 2. Using equations from ,,calculations" capter and the chromatogram obtained, was calculated the performance parameters that shows the efficiency of separation in the conditios of the method.

Performance parameters are shown in Table 3. We can see strong values for performance parameters in the conditions of the method: peak resolution, column efficiency and height of theoretical plates. The results show very good performance parameters of this separation methode.This HPLC separation method of the organic mixture with herbicide action is selective, fact demonstrated by the selectivity (specificity) of the instrument/equipment and the separation conditions on chromatographic column, C18.

Table 2
System suitability

Component	dicamba	24D acid	DCF
t_R (minutes)	21,89	24,60	26,20

Table 3
Efficiency of separation

Performance parameter	**Accepted value** [4]	**Obtained value**
Capacity factor (K')	≥ 1	$K'_{Dicamba} = 16.6$ $K'_{2,4DAcid} = 17.6$ $K'_{DCF} = 18.3$
Column selectivity (α)	≥ 1	$\alpha_{Dicamba} = 1.04$ $\alpha_{2,4Dacid} = 1.06$ $\alpha_{DCF} = 1.05$
Peak rezolution R_S	≥ 1 for 98% separation ≥1.5 for 99.7% separation	$R_{Dicamba} = 3.8$ $R_{2,4DAcid} = 6.5$ $R_{DCF} = 4.6$
Column efficiency N_{eff} (number of theoretical plates)	≥ 400	$N_{effDicamba} = 258475$ $Neff_{2,4Dacid} = 171600$ $N_{effDCF} = 307000$
Height of theoretical plate (HETP)	0.001÷0.002mm	$HETP_{Dicamba} = 0.001$mm $HETP_{2,4Dacid} = 0.002$mm $HETP_{DCF} = 0.001$mm

Method validation

The proposed method was validated with respect to linearity, accuracy, precision, specificity, following the HP Guide for HPLC, CE and UV-Vis spectroscopy [7].

Linearity (sensitivity) and range

Linearity test solutions were prepared by diluting stock solution at five concentration levels of analytes concentration. The solutions were injected in triplicate and following regresion equations were found by plotting peak area versus concentration. The response is linear on area of concentration chosen if the results dont't have a signifiant deviation from linearity, this means, an corelation coefficient bigger than 0,997 for all components. The obtained equations for regression lines are: $Y_{Dicamba}$ = 9770,32x + 15685,5; $Y_{2,4Dacid}$ = 79022x - 77878,5; Y_{DCF} = 4441,75x-8114,5. The coefficient of determination (R^2) obtained for regression line demonstrates the excellent relationship between peak area and components concentrations. The results are shown in Table 4.

Precision [7] in retention times and peak area (or height) are major criterion of separation systems. The precision of the chromatographic method reported as percent of relative standard deviation (S_r) was estimated by measuring repeatability on five replicate cromatograms [8].

Table.4.
Linearity results for LC method

Compo-nent	Concen-tration	Equation for regression line	R^2
Dicamba	0,25 - 2,25 mg/mL	Y = 9770,32 · x + + 15685,5	0,998
2,4D acid	0,7-3,5 mg/mL	Y = 79022 · x – – 77878,5	0,999
DCF	5-25 µg/mL	Y = 4441,75 · x – – 8114,5	0,999

The relative standard deviation values (Sr) and the repeatability limit (r) for retention times and areas are shown in Table 5.

$X_k - X_{k-1}$ is the difference between two individual results that must be smaller than repeability limit.The condition $X_k - X_{k-1} \leq r$, is accomplished.

Precision in analisys and accuracy

Accuracy was estimated by spinking the sample matrix of interest with a known concentration of reference material: $C_{2,4DAcid}$ = 28.5%; $C_{Dicamba}$ = 9.5%; $C_{2,4DCF}$ = 0.1% the same as the concentration of formulated herbicides. It was compared the response obtained after the extraction of analyte from the sample and injection in the column with the response of the reference material added to the pure solvent (Table 6).

Table 5.
Repeatability for retention times and areas

Component	Dicamba		2,4D acid		DCF	
Parameter/RUN	Area (counts)	r_t (minutes)	Area (counts)	r_t (minutes)	Area (counts)	r_t (minutes)
Run 1	424827	21.89	2017948	24.56	147776	26.18
Run 2	392906	22.01	1864533	24.49	133477	26.02
Run 3	418781	22	1934687	24.53	141834	26.06
Run 4	403003	22.05	1893372	24.6	156284	26.18
Run 5	383215	21.97	1854117	24.56	1325	26.24

					40	
S_r	17369	**0.06**	66494	**0.05**	9985	**0.18**
r	48003	0.16	183762	0.13	27595	0.49
$X_k - X_{k-1} \leq r$	OK	OK	OK	OK	OK	OK

Table 6
Precision and accuracy of measurement

RUN	2,4D acid (%)	Dicamba (%)	DCF (%)
1	28.50	9.50	0.093
2	28.00	9.60	0.100
3	28.51	9.56	0.075
4	28.36	9.90	0.103
5	28.78	9.47	0.09
Accuracy-Student variable (≤1)	0.148	0.086	0.016
Precision as S_r, (%)	0.28	0.17	0.03

4. Conclusions

The resuls show that HPLC separation method of the organic mixture with herbicide action is selective, fact demonstrated by the selectivity of the instrument/equipment and the separation conditions on chromatographic column, C18. To note, the performance parameters with strong values in the conditions of the methode: the resolution of separation, column efficiency and height of theoretical plate. Also, it has been shown that in data conditions the methode is sensitive, precise and reproductible.

5. References

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