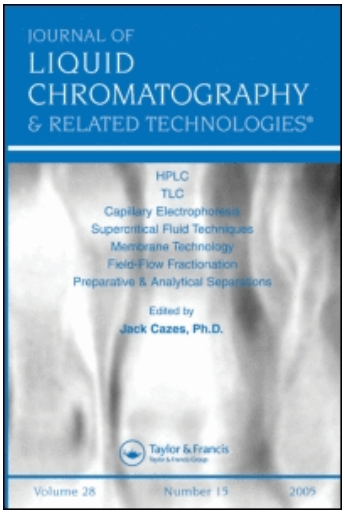

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A STUDY ON THE POSSIBILITY OF USING HPLC FOR THE DETERMINATION OF 2,4-D IN TOMATOES

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ABSTRACT

An HPLC method for the determination of 2,4-D in tomatoes has been studied. Tomato samples were extracted with acetone-dichloromethane (2:1) after hydrolysis. The extract was cleaned up with acid-base partition and furthermore eluting through XAD-2 column. Then, it was analyzed using reverse phase HPLC. Four different mobile phase mixtures, at two different flow rates of 1.0 and 1.5 mL/min, were tried, to choose the best conditions in the final HPLC determination step. The best results were obtained using the mixture of acetonitrile:water, containing 2% of acetic acid (50:50, v/v), at a flow rate of 1.0 mL/min for the separation of 2,4-D standard solutions, but the baseline separation of 2,4-D in the final solution of sample extract could not be achieved due to interferences causing poor resolution as well as low recovery.

INTRODUCTION

Although 2,4-dichlorophenoxyacetic acid (2,4-D) is a herbicide widely used for almost 50 years in modern agriculture,[1-3] it is also known as a plant growth regulator.[4-7] The effect of 2,4-D on fruit setting and development of greenhouse-grown tomatoes is well known for a number of years.[8,9]

2,4-D is allowed to be used only as a herbicide in Turkey, but it is suspected that some local producers use it as a growth regulator to stimulate tomato fruit set in greenhouses during mild winter conditions. The maximum permitted concentration of 2,4-D established by Food and Agricultural Organization/World Health Organization (FAO/WHO) in various foods is in the range of 0.05-0.5 ppm.[10] Because of uncertainty of the carcinogenic effect of 2,4-D, there is a strong pressure towards the abolishment of its use.

For the residual analysis of 2,4-D in plant materials, gas chromatography (GC), with electron capture detection, is mainly used.[11-15] However, because of the high polarity of 2,4-D in its acid form, it has to be derivatized first for GC analysis.[2,16-19] Various derivatization procedures have been developed for the determination of acidic residues to render them volatile. In doing so, additional analysis time, expense and, sometimes, errors due to non-reproducible results are introduced by derivatization, making the method unattractive to many chemists.[20] Methylation has been the method of choice for a number of years, because the reaction is rather simple with few side products.[16] High-performance liquid chromatography (HPLC) can also be used for the separation of 2,4-D in its underivatized free acid form.[16,19,21-29] However, no residue method for the determination of 2,4-D or any other compounds having similar chemical structure in plant materials has appeared in the literature using HPLC.

This study was carried out to investigate the possibility of using HPLC in place of GC for the determination of 2,4-D residues in tomatoes.

EXPERIMENTAL

Material

Tomato samples used throughout the recovery trials were all 2,4-D free and kept in a deep freezer until just prior to analysis.

Reagents

2,4-D, hexadecyltrimethylammonium bromide (cetrimide), K_2HPO_4 and NaH_2PO_4, $NaHCO_3$, NaCl, NaOH, H_2SO_4 and anhydrous Na_2SO_4 were all reagent grade and obtained from Merck. All solvents used were of HPLC grade (Merck) and water was bidistilled.

Cetrimide was dissolved in methanol to a concentration of 0.03 M and $NaHCO_3$ was dissolved in 80% acetonitrile in water to a concentration of 0.1 M. All other solutions used throughout the experiments were prepared in bidistilled water.

Anhydrous Na_2SO_4 was dried at 550°C for 2 hrs. Filter papers were extracted with dichloromethane before use.

Mobile Phases

In order to optimize the chromatographic conditions for the separation of 2,4-D, its capacity factor on a C_{18} column was determined with three different mobile phase compositions, at two flow rates of 1.0 and 1.5 mL/min. Mobile phase compositions used were as follows:

I. Methanol:water (75:25, v/v), containing 0.001 M PO_4^{-3} and 0.005 M cetrimide.

II. Acetonitrile:water, containing 2% of glacial acetic acid (75:25, v/v).

III. Acetonitrile:water containing 2% of glacial acetic acid (50:50, v/v).

Mobile phase mixtures were passed through a 0.45 μm regenerated cellulose acetate membrane (Sartorius) and degassed ultrasonically.

2,4-D Standard Solutions

Stock solution of 2,4-D, at a concentration of 1000 μg/mL, was prepared, both in methanol and in the mixture of isopropanol:water (75:25, v/v). Working standard solutions of 0.5, 1.0, 2.0, 3.0 and 4.0 μg/mL were prepared, appropriately diluting the stock solutions with methanol for mobile phase I and with the mixture of isopropanol:water (75:25, v/v) for mobile phases II and III to improve the separation. These solutions were kept at 4°C just prior to analysis.

High-Performance Liquid Chromatography

An HPLC system, consisting of Varian 9010 solvent delivery system, Varian 9050 variable wavelength UV-VIS detector, Rheodyne 7161 six way injector, equipped with a 10 μL sample loop was used. The MicroPak® column, made of stainless steel (150 x 4.0 mm I.D.), packed with octadecyl groups (C_{18}, 5μm), was operated at ambient temperature. It was protected with a microparticulate guard column (40 x 4.0 mm I.D.).

UV Spectra

Working standard solutions in the mixture of isopropanol:water (75:25, v/v) were used to record UV spectra for 2,4-D on a Shimadzu 2101 UV-Vis spectrophotometer.

Sample Preparation

Tomatoes were blended in a Waring blender. 15 mL of 1 N NaOH and 60 mL of water were added into 25 g of blended tomato sample and the mixture was hydrolyzed in a water bath at 95°C for 2 hrs. After cooling, the amount of water evaporated during hydrolysis was added and the mixture was acidified, adding 5 mL of 20% H_2SO_4. Then it was homogenized with 200 mL of acetone for 2 min, at medium speed, in a Virtis homogenizer and filtered through a black band filter paper into a graduated cylinder. The filtrate volume was recorded (V_f) and the filtrate was transferred into a 1000 mL separatory funnel.

It was then saturated with V_f/10 g of NaCl, shaking vigorously for 3 min. 100 mL of dichloromethane was added and the filtrate was extracted by shaking for 2 min. After phase separation, the aqueous phase was discarded. The organic phase was quantitatively transferred into a flask containing 25 g of anhydrous Na_2SO_4 and dried for 20 min. The organic phase was then filtered through a black band filter paper into a 1000 mL separatory funnel and extracted with 100 and 50 mL of 0.5 N NaOH. Combined NaOH extracts were acidified with 20% H_2SO_4 (pH≤1.0).

The extract was cleaned up by eluting through Amberlite® XAD-2 column at a flow rate of 5 mL/min, and eluate was discarded. The column was then washed with water to neutralize the eluate. The column was eluted with 0.1 M $NaHCO_3$ in 80% acetonitrile in water at a flow rate of 2.0 mL/min and the eluate was collected in a flask. The eluate was acidified with 20% H_2SO_4 (pH≤ 1.0) and transferred into 250 mL separatory funnel. It was extracted with 2x50 mL of dichloromethane.

Combined dichloromethane extracts were filtered through a black band filter paper, covered with a layer of anhydrous Na_2SO_4 into a 250 mL round bottom flask. It was evaporated to dryness in a rotary evaporator at 40°C. Furthermore, residue was dried under a gentle stream of nitrogen. Dried residue was redissolved immediately in the mixture of isopropanol:water (75:25, v/v). 10 μL of this solution was injected into the HPLC column.

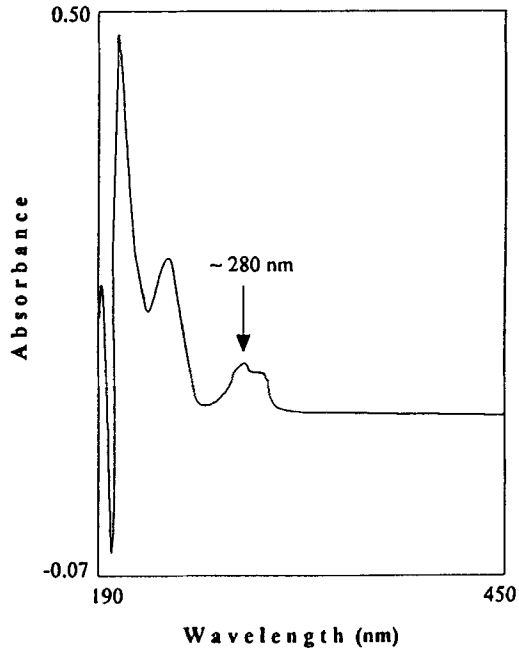

Figure 1. UV spectrum of 2,4-D.

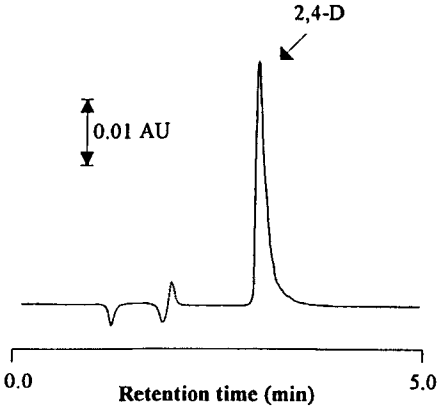

Figure 2. Separation of 2,4-D on C18 column. Chromatographic conditions: mobile phase: mixture of acetonitrile:water containing 2% of glacial acetic acid (50:50, v/v), flow rate: 1.0 mL/min; λ=280 nm.

Calculation of the Results

Corresponding sample amount in the extract was calculated by the following formula:

$$m = \frac{S \times V_f}{T}$$

where,

m = amount of sample in the extract, g
S = initial weight of sample, g
V_f = volume of filtrate, mL
T = total theoretical volume of filtrate, mL (200 mL acetone + 60 mL water + 15 mL 1 N NaOH + 5 mL 20% H_2SO_4 + water in tomato sample)

The volume contraction of acetone and water must be taken into account when determining total theoretical volume of the filtrate.

Concentration of 2,4-D in the final solution was calculated using the calibration curve prepared daily, based on concentration vs. peak height. The amount of 2,4-D in tomato was then calculated as follows:

$$2,4-D \text{ in tomato } (\mu g / g) = \frac{C_f \times V}{m}$$

where,

C_f = concentration of the final solution, μg/mL
V = volume of the final solution, mL
m = amount of sample in the extract, g

RESULTS AND DISCUSSION

Specific wavelength of 2,4-D for maximum absorbance was determined to be 280 nm, in accordance with the result of Roseboom et al. (1982). Figure 1 illustrates the typical spectrum of 2,4-D. Detection wavelength during HPLC analysis of 2,4-D was therefore set at 280 nm to obtain maximum sensitivity.

Mobile phase III, at a flow rate of 1.0 mL/min was found to be more efficient for the separation of 2,4-D on the C_{18} column. Therefore, it was chosen as the mobile phase for the residual analysis of 2,4-D in tomato. Symmetrical peaks and reproducible results were obtained for 2,4-D on the C_{18} column when mobile phase III at flow rate of 1.0 mL/min was used (Fig. 2). Correlation coefficient ® for the calibration graph of 2,4-D, based on

Table 1

Capacity Factors (k') Obtained on a C_{18} Column

Mobile phase No.	Capacity factor (k') 1.0 mL/min	1.5 mL/min
I	2.88	2.81
II	0.63	0.65
III	2.38	4.32

concentration vs. peak height for mobile phase III at flow rate of 1.0 mL/min, was 0.998 (n=5). Band broadening was observed for mobile phase I, while separation of 2,4-D was not good for mobile phase II. The capacity factors (k'), obtained for three mobile phase compositions at two flow rates of 1.0 and 1.5 mL/min, are given in Table 1.

The extraction procedure applied in this study uses acetone/ dichloromethane extraction after alkaline hydrolysis. Alkaline hydrolysis is thought to be necessary to convert the bound and conjugated residues of 2,4-D present in tomato to free acid residue.[13,17] Percent recoveries obtained were 92.1%, 71.4% and 68.8% for the corresponding spiking levels of 0.5, 2 and 4 μg/g of 2,4-D, respectively. These recoveries are lower than the recoveries recorded in many GC methods.[3,13,14] Although 2,4-D could be sensitively detected as the standard solution by HPLC, it could not be possible to analyze the residues of 2,4-D in tomato samples sensitively due to interferences.

Many of the colored compounds co-extracted from tomato could be removed by acid-base partition. However, the final extract still contained colored interferences after clean-up by eluting through an XAD-2 column. These co-extractives from the tomato matrix adversely affected the separation of 2,4-D on the C_{18} column. Baseline separation could not be achieved, thus preventing proper integration of the resulting chromatograms. Figure 3 illustrates the chromatogram of tomato extract spiked with 0.5 μg/g of 2,4-D.

CONCLUSION

It was thought, in the beginning of this study, that an HPLC method for the residual analysis of 2,4-D in tomato might be useful with some advantages over GC methods, such as ease of operation, economy and rapidity. However, sensitivity achieved was not found to be sufficient from the residual analysis considerations. Co-extractives from tomato matrix were the main problem preventing the separation of 2,4-D on a C_{18} column.

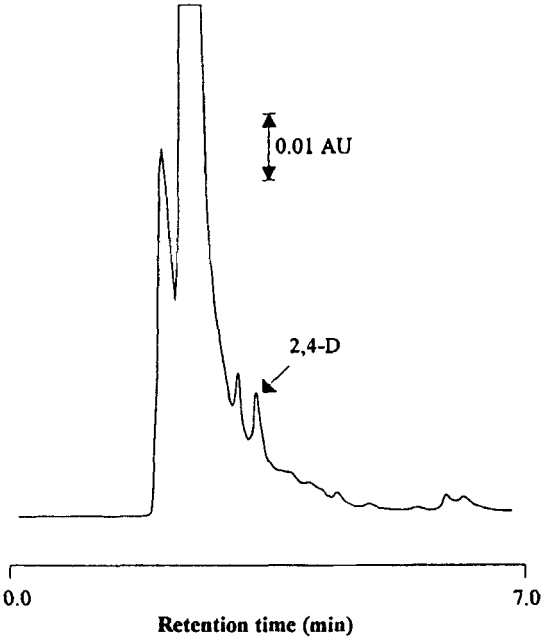

Figure 3. Chromatogram of tomato extract (spiking level=0.5 μg/g). (Chromatographic conditions are same as given in Fig. 2).

Clean-up, using acid-base partition and elution through the XAD-2 column, proven to be efficient in many GC methods appearing in the literature, was not found applicable in this HPLC method. It is thought that a clean-up procedure enabling removal of the interferences causing problems for the separation of 2,4-D in a C_{18} column must be improved.

The chromatographic conditions, proven to be efficient for the separation of 2,4-D as standard solutions in this study, may be used for monitoring the purity of 2,4-D formulations. Also, it may be possible to detect the residues of 2,4-D by HPLC in uncomplicated sample matrices such as water from agricultural areas where 2,4-D is widely used.

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