Identification of Impurities in Technical Anilofos and Their Effect on Transplanted Rice

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Seven impurities, namely, isopropylaniline (**III**), isopropylacetanilide (**V**), isopropylchloroacetanilide (**VII**), oxoanalogue (**VIII**), dithiodimer (**IX**), monothiodimer (**X**), and mercaptoacetanilide (**XI**), have been identified from technical anilofos. The quantification of each impurity was carried out by comparison with authentic samples of known concentration by GC and HPLC. The structure of authentic samples (synthesized and isolated) was established on the basis of NMR, IR, and MS spectral data. Bioassay of all the detected impurities along with three possible contaminants was carried out on transplanted rice under laboratory conditions. The oxo compound (**VIII**) was found to have an inhibitory effect on transplanted rice at 1 μg/g of soil.

Keywords: *Anilofos; degradation products; impurities; contaminants; effect on rice*

INTRODUCTION

The herbicide anilofos [*S*-4-chlorophenyl-*N*-isopropylcarbaniloyl methyl]-*O*,*O*-dimethylphosphorodithioate] (Figure 1) effectively controls grassy and some broad leaved weeds in rice (*Oryza sativa*) crop (*1*), either alone (*2*) or as a mixed herbicide (*3*). It is applied as pre- as well as postemergence (7 days after transplanting) and controls problematic weeds such as *Echinocloa crusgalli* and *Ischaemum rugosum* (*4*). The use of this herbicide has also been extended in other crops such as chicory (*5*) and as a mixed herbicide in wheat (*6*). Effect of the herbicide on RNase and DNase activity in seeds/seedlings of rice and *E. crusgalli* L has also been studied (*7*).

Typical of organophosphorus pesticides, the technical grade anilofos (90%) has shown several impurities in thin-layer chromatography (TLC), gas chromatography (GC), and high performance liquid chromatogarphy (HPLC), although, with the concentration of anilofos in technical materials, these are less visible. To estimate their concentration in technical samples, the method of fractionation and addition of analytical results have been used. These impurities could originate in two ways. Either they originate from the starting material at the stage of synthesis, or the technical material degrades at room temperature to some other products that act as contaminants in the sample. The latter is important in prescribing the shelf life of a pesticide formulation. These degradation products could result from hydrolysis or oxidation of the original compound and might have undesired effects on crop growth. In view of this, the impurities of technical anilofos have been isolated and identified, and their phytotoxicity have been evaluated on transplanted rice under laboratory conditions.

MATERIALS AND METHODS

Materials. Analytical grade anilofos, supplied by Gharda Chemicals Ltd., was recrystallized from hexane, mp 51−52 °C. 4-Chloroaniline (**II**) was purchased from E. Merck and crystallized from hexane, mp 69−70 °C. Six compounds (**III**−**VIII**) were synthesized in the laboratory by the method described in our earlier work (*8*). Dimeric and monomeric compounds (**IX** and **X**) were isolated from the mother liquor left after crystallization of technical anilofos. Mercaptoanilide (**XI**) was obtained from Gharda Chemicals Ltd. Acetone, benzene, chloroform, hexane, methyl alcohol, ethyl alcohol, and acetonitrile were redistilled before use. All other chemicals were of commercial quality. The chemical names and structures of different standard materials are given in Table 1 and Figure 1, respectively. For impurity detection, two brands of technical material were taken and named as brand I and brand II.

Techniques. *Thin Layer Chromatography (TLC).* TLC was carried out on 5 × 20 cm glass plates coated with silica gel G containing 13% of gypsum as binder (E. Merck) with 0.25 mm thickness. For preparative TLC, the plate size used was 20 × 20 cm with 0.5 mm thickness. The TLC was developed in a benzene/acetone (17:3) solvent system. The spots were detected by visualizing in iodine vapors. R_f (retention factor) of each compound is summarized in Table 2.

Gas−Liquid Chromatography (GLC). GLC was carried out on the Hewlett-Packard gas chromatograph model 5890 fitted with a megabore column (10 m × 0.53 mm i.d., fused silica) packed with OV-1 and a nitrogen phosphorus detector (NPD). The temperature of the oven was programmed as 140 °C for 5 min, and then increased at the rate of 10 °C min^{-1} and finally 250 °C for 5 min. Nitrogen was used as carrier gas with a flow rate of 20 mL min^{-1}. R_t (retention time) for each compound is given in Table 2.

High-Performance Liquid Chromatography (HPLC). HPLC was performed on a Waters HPLC instrument using RP-18 column and UV−vis detector at λ_{max} 258 nm. Mobile phase used was acetonitrile and water with gradient elution and a flow rate of 0.5 mL min^{-1}. Each run was for 30 min using the step gradients with 25% acetonitrile for 10 min, 50% acetonitrile for 5 min, and finally 100% acetonitrile for 10 min. After a period of 25 min, the column was flushed with 100% acetonitrile at the rate of 1 mL min^{-1}. The R_t values as obtained for different compounds are given in Table 2.

Mass Spectrometry (MS). Mass spectra were obtained using a JEOL JMS − D300 mass spectrometer at 70 ev using electron impact ionization with the source at ambient temperature.

Proton Magnetic Resonance Spectrometry (^{1}H NMR). Nuclear magnetic resonance spectra (proton) were recorded on a varian EM-360 (60 MHz) instrument using trimethylsilane (TMS) as an internal standard. The compounds were dissolved in deuterated chloroform ($CDCl_3$).

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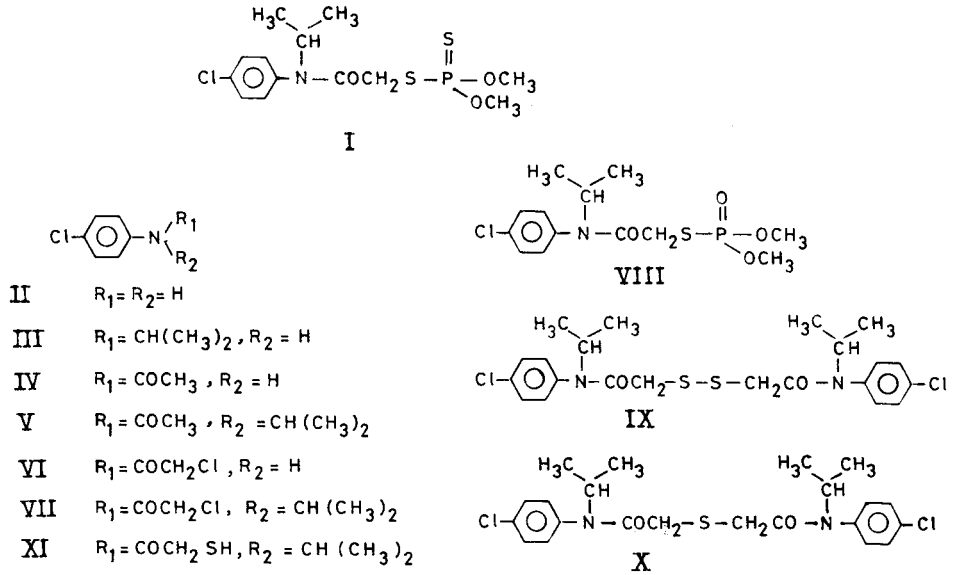

Figure 1. Chemical structures of anilofos and its degradation products or impurities.

Table 1. Chemical Names and Sources of Different Compounds Used in Analysis

compound	chemical name	source	reference
anilofos (**I**)	(*S*-4-chlorophenyl-*N*-isopropylcarbaniloyl methyl)-*O*,*O*-dimethylphosphorodithioate	supplied	Gharda Chem. Ltd.
aniline (**II**)	4-chloroaniline	purchased	E. Merck
isopropylaniline (**III**)	4-chloro-*N*-isopropylaniline	synthesized	(*8*)
acetanilide (**IV**)	4-chloro-*N*-acetanilide	synthesized	(*8*)
isopropyl acetanilide (**V**)	4-chloro-*N*-isopropyl-*N*-acetanilide	synthesized	(*8*)
chloroacetanilide (**VI**)	4-chlorophenyl-*N*-α-chloroacetanilide	synthesized	(*8*)
isopropylchloroacetanilide (**VII**)	4-chlorophenyl-*N*-isopropyl-α-chloroacetanilide	synthesized	(*8*)
oxoanalogue (**VIII**)	(*S*-4-chlorophenyl-*N*-isopropylcarbaniloylmethyl)-*O*,*O*-dimethylphosphorothioate	synthesized	(*8*)
dithiodimer (**IX**)	di-(4-chlorophenyl-*N*-isopropylcarbomoylmethyl) disulfide	isolated from mother liquor	
monothiodimer (**X**)	di-(4-chlorophenyl-*N*-isopropylcarbomoylmethyl) sulfide	isolated from mother liquor	
mercapatoanilide (**XI**)	4-chlorophenyl-*N*-isopropyl-α-mercaptoacetanilide	supplied	Gharda Chem. Ltd.

Table 2. Retention Factor (R_f) and Retention Time (R_t) of Anilofos and Its Impurities/Degradation Products on TLC, GC, and HPLC

compound	R_f (TLC)	R_t (GC) (min)	R_t (HPLC) (min)
I	0.54	13.155	20.50
II	0.27	0.624	20.43
III	0.92	1.324	20.29
IV	0.20	3.181	16.68
V	0.24	3.200	20.19
VI	0.32	4.487	18.28
VII	0.40	6.294	21.93
VIII	0.43	12.233	21.15
IX	0.22	7.184	21.63
X	0.18	7.664	5.41
XI	0.016	1.881	19.55

Column Chromatography. Column chromatography was performed on a silica gel (60−90 mesh) column. Column was eluted with hexane, hexane/benzene (1:1), benzene/acetone (19:1), benzene/acetone (9:1), and finally with acetone. Each fraction was concentrated using a rotary evaporator at 30−35 °C. The residue was dissolved in hexane/acetone (4:1) for injection in GC and in acetonitrile (HPLC grade) for HPLC.

*Isolation of Compounds **IX** and **X** in Pure Form*. The compounds in pure form were isolated from the mother liquor left after the crystallization of technical anilophos. The isolation was done by column chromatogaphy of mother liquor followed by preparative TLC. The mother liquor was chromatographed over a silica gel column (60−90 mesh), and the column was eluted with hexane, hexane/benzene (1:1), benzene/acetone (19:1), benzene/acetone (9:1), and finally with acetone. Fractions eluted with benzene/acetone (19:1) on concentrating gave an oily residue. This was further purified by preparative TLC to give an oil that showed a single spot on TLC. The oil gave a positive test for sulfur (sodium nitroprusside test with sodium extract of the fraction). The compound was identified as a dithiodimer and was assigned the structure of di-(4-chlorophenyl-*N*-isopropylcarbamoylmethyl)-disulfide (**IX**) on the basis of ^{1}H NMR and mass spectral data.

^{1}H NMR ($CDCl_3$) δ: 1.10 (d, J = 7 Hz, 12H, 2 x -$C(CH_3)_2$), 3.20 (s, 4H, 2 x -$COCH_2$-), 4.80 (quintet, J = 7 Hz, 2H, 2 x -CH), 7.20 and 7.42 (each d, J = 9.5 Hz, each 4H, Ar−H). MS (rel. int.): m/z 485 (55%) M^+; 316 (25%) $M^+ - NCH(CH_3)_2C_6H_4$-Cl; 274 (45%) 316 − $COCH_2$; 242 (50%) 274 − S; 210 (9%) 242 − S; 168(70%) 210 − $COCH_2$, 154 (100%) 168 − CH_3.

Fractions eluted with benzene/acetone (9:1) on evaporation of solvent gave an oily compound that was again purified by preparative TLC. Sodium extract of the compound gave a positive test for sulfur. The compound was assigned the structure of di-(4-chlorophenyl-*N*-isopropylcarbamoylmethyl)-sulfide (**X**) on the basis of ^{1}H NMR and mass spectral data.

^{1}H NMR ($CDCl_3$) δ: 1.02 (d, J = 7 Hz. 12H, 2 x -$C(CH_3)_2$); 3.11 (S, 4H, 2 x -$COCH_2$); 4.83 (quintet, J = 7 Hz, 2H, 2 x -CH); 6.90 and 7.22 (each d, J = 9.5 Hz, each 4H, Ar−H). MS (rel. int.): m/z 453 (15%) M^+; 242 (30%) $M^+ - CH_2CONCH(CH_3)_2C_6H_4Cl$; 210 (100%) 242 − S; 168 (47.5%) 210 − $COCH_2$, 154 (62.5%) 168 − CH_3.

Preparation of Standard Mixture for Analysis. Standard solution of all the compounds were prepared in hexane/acetone (8:2) except *p*-chloro-*N*-isopropyl-α-mercaptoacetanilide (**XI**) which was prepared in acetone. Ten milligrams of each compound was dissolved in a 10-mL volumetric flask with the solvent to produce a 1000 μg mL^{-1} stock solution of each

Table 3. Percentage of Impurities As Found in Two Different Brands of Technical Anilofos

name of the impurity	compound	brand I percentage[a]	brand II percentage[a]
4-chloro-*N*-isopropylaniline	**III**	0.2 ± 0.004	0.2 ± 0.006
4-chloro-*N*-acetanilide	**IV**	1.0 ± 0.002	0.8 ± 0.001
4-chlorophenyl-*N*-isopropyl-α-chloroacetanilide	**VII**	2.11 ± 0.01	1.92 ± 0.009
(*S*-4-chlorophenyl-*N*-isopropyl carbaniloyl methyl)-*O*,*O*-dimethyl phosphorothiomate	**VIII**	0.8 ± 0.003	0.2 ± 0.001
di(4-chlorophenyl-*N*-isopropyl carbomoyl methyl) sulfide	**X**	1.3 ± 0.006	0.5 ± 0.002
di(4-chlorophenyl-*N*-isopropyl carbomoyl methyl) disulfide	**IX**	2.01 ± 0.008	0.8 ± 0.006
4-chlorophenyl-*N*-isopropylmercapto acetanilide	**XI**	1.5 ± 0.009	4.2 ± 0.01
others (unidentified)		1.08	1.38
anilofos	**I**	90.00	90.00

[a] Mean of 20 runs from the same lot.

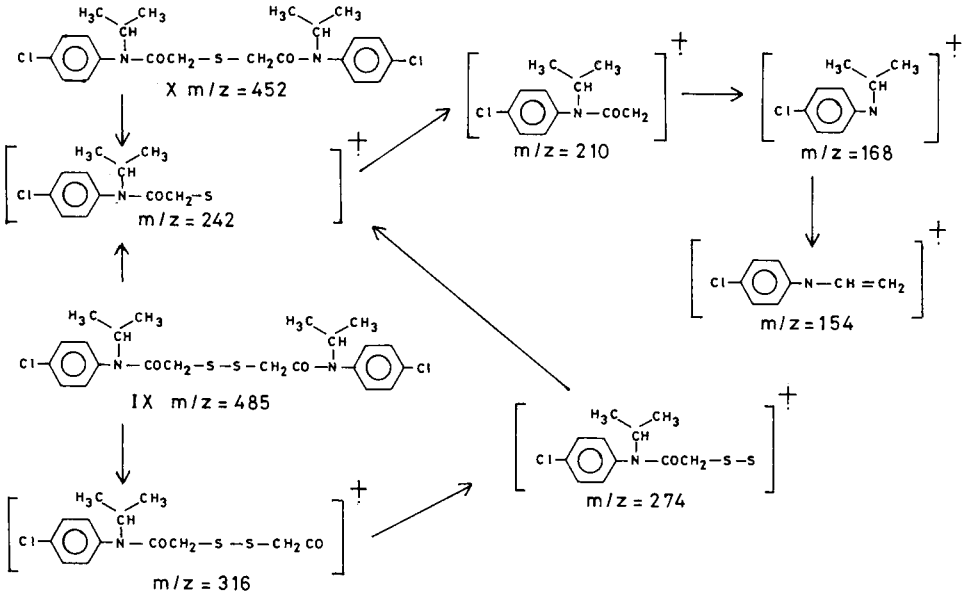

Figure 2. Mass spectral fragmentation of monothiodimer (**X**) and dithiodimer (**IX**) isolated from technical anilofos.

compound. One milliliter of this solution was diluted in a 50-mL volumetric flask with the same solvent mixture to give a standard solution of 20 μg mL^{-1} for every standard compound. For the standard mixture, 2 mL of each of the 11 standard solutions (20 μg mL^{-1}; **I**−**XI**) were mixed together in a 25-mL volumetric flask, and the volume was made up to the mark. This served as a standard solution for GC containing 1.6 μg mL^{-1} of each compound. This was further diluted accordingly. Solutions of individual compounds were also diluted as required.

For HPLC, stock solutions (1000 μg mL^{-1} each) were prepared in acetonitrile. For the standard mixture, 1 mL of each of the 11 standard solutions were mixed together in a 100-mL volumetric flask, and the volume was made up to the mark. This served as a standard solution for HPLC containing 10 μg mL^{-1} of each compound. This was further diluted as required.

Bioassay. Rice seedlings were raised in the laboratory in plastic pots (6 × 6 cm). The pots were filled with soil (50 g). Acetone solution (2 mL) of the individual compounds (**I** to **XI**) was surface applied to the soil in the pots as pre-emergent at the rate of 1 μg/g of soil. Control pots were treated with an equal amount of acetone. Five rice seedlings were transplanted in each pot. The treatments and controls were carried out in triplicate. The plants were watered regularly for 15 days. For harvesting, plants were gently uprooted from wet soil and washed gently with tap water to remove adhered soil. The length, fresh weight, and dry weight of both roots and shoots of each seedling were measured. The results were analyzed statistically using complete randomized block design for computation of standard error (SEM).

Quantitative Estimation of Impurities in Technical Sample. Technical anilofos (100 mg) was chromatographed over a column of silica gel, and a total of 15 fractions (each 200 mL) were collected, by eluting with different solvent systems described in the previous section (5 fractions of hexane, 3 fractions of 1:1 hexane/benzene, 3 fractions of 19:1 benzene/acetone, 3 fractions of 9:1 benzene/acetone, and 1 fraction of acetone). After the eluting solvent was evaporated, the residue obtained for each fraction was dissolved and analyzed by GC to determine the concentrations of different compounds present in the sample. Standard mixture (a standard solution containing all the compounds from **I** to **XI**) of known concentrations was also chromatographed under similar conditions. The response of each peak was obtained in the form of peak area. The concentrations of compounds in each fraction was calculated by comparing the peak area of the particular compound with that of the standard using the equation

$$C = A(\mathrm{RF})$$

where C is the concentration of a compound, A is the area of the peak corresponding to that compound in a particular fraction, and RF (response factor) is the concentration of standard area of the standard. By adding the amount present in each fraction (calculated by each standard peak), the total amount of each compound in 100 mg was estimated. On this basis, the amounts of different impurities present in technical samples were calculated, and the results are given in Table 3.

The results were also confirmed by HPLC. Each fraction was analyzed by HPLC, and the quantity of individual compounds in each fraction was calculated by using the standard mixture made for HPLC analysis. Thus, the presence and concentrations of these impurities in the technical anilofos were confirmed by HPLC. The percentage of impurities was

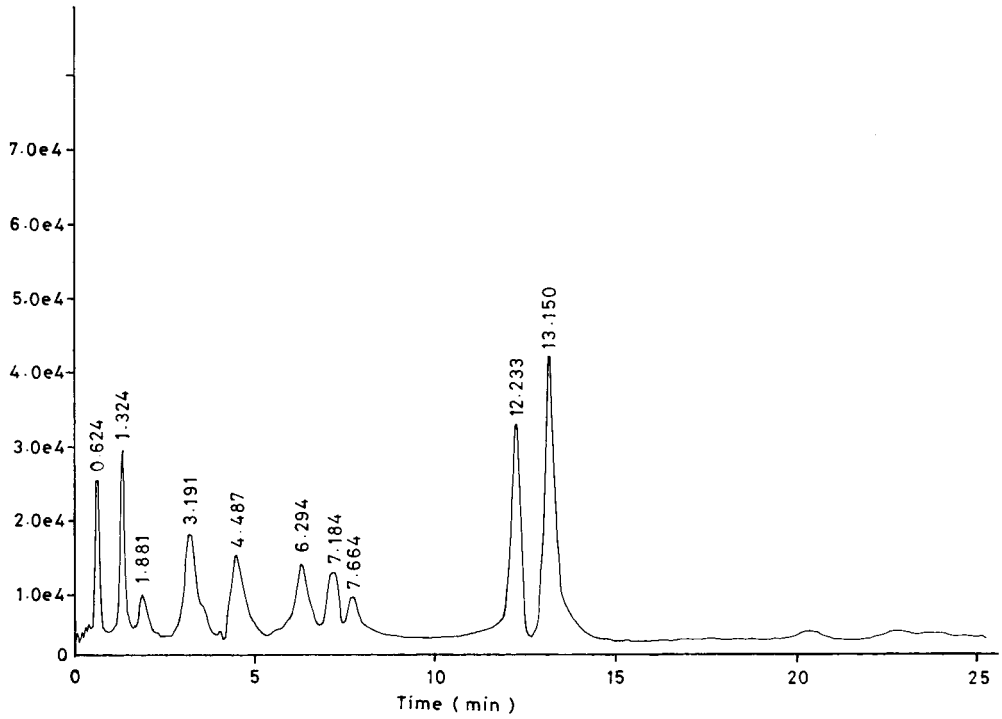

Figure 3. GC chromatogram of standard mixture containing compounds **I** to **XI** (each 1.6 μg/mL).

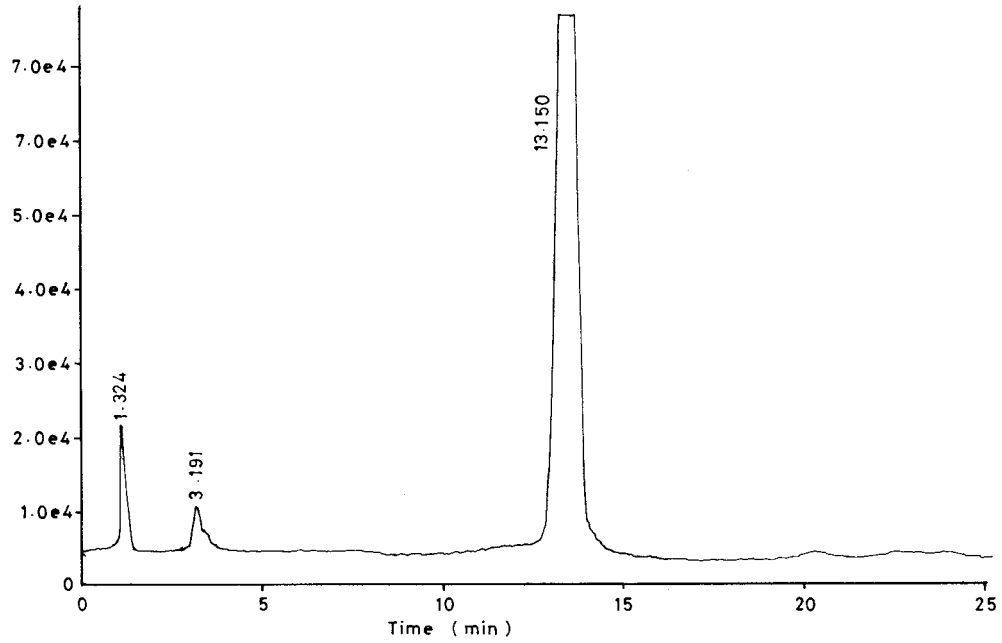

Figure 4. GC chromatogram of technical anilofos (20 μg/mL) brand I.

calculated in two analytical samples, and the result as found is given in Table 3.

RESULTS AND DISCUSSION

The quantification of the impurities in the technical sample was done by GC and HPLC comparison with standard mixture of authentic compounds. We analyzed 10 compounds besides anilofos, out of which six (**II** to **VIII**) were synthesized in laboratory by our own method (*8*). Two dimeric compounds **IX** and **X** were isolated from the mother liquor left after crystallization of anilofos. Both the compounds showed a positive test for sulfur. The structures of these compounds were assigned on the basis of ^{1}H NMR and mass spectral data. ^{1}H NMR spectra of these compounds were similar as both the compounds showed isopropyl groups, aromatic protons, and -$COCH_2$ linkage. Using ^{1}H NMR, it was rather difficult to distinguish the compounds, but they showed different R_f and R_t values on TLC, and GC, HPLC, respectively. Finally, the compounds were identified on the basis of mass spectra that showed molecular ions 485 and 453 for **IX** and **X**, respectively. Fragments showing *m/z* 274 and *m/z* 316 in the mass spectrum of compound **IX** confirmed the presence of a disulfide linkage, and thus its structure was assigned as a dithiodimer. Compound **X** was identified as a monothiodimer. The mass spectral fragmentation of compounds **IX** and **X** is depicted in Figure 2.

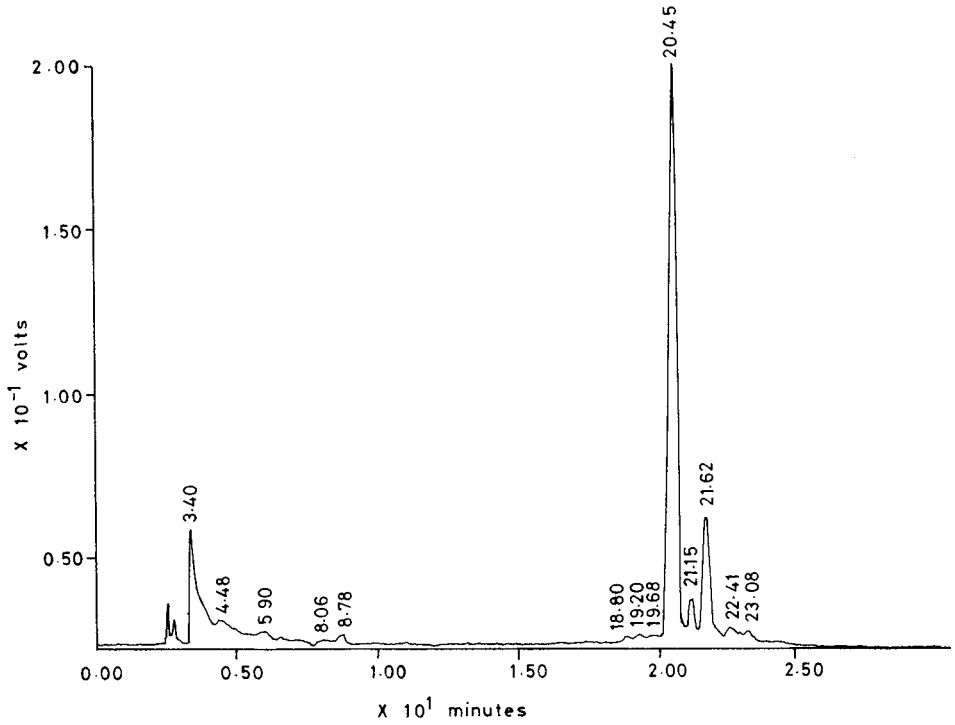

Figure 5. HPLC chromatogram of technical anilofos (brand I), 1000 μg/mL.

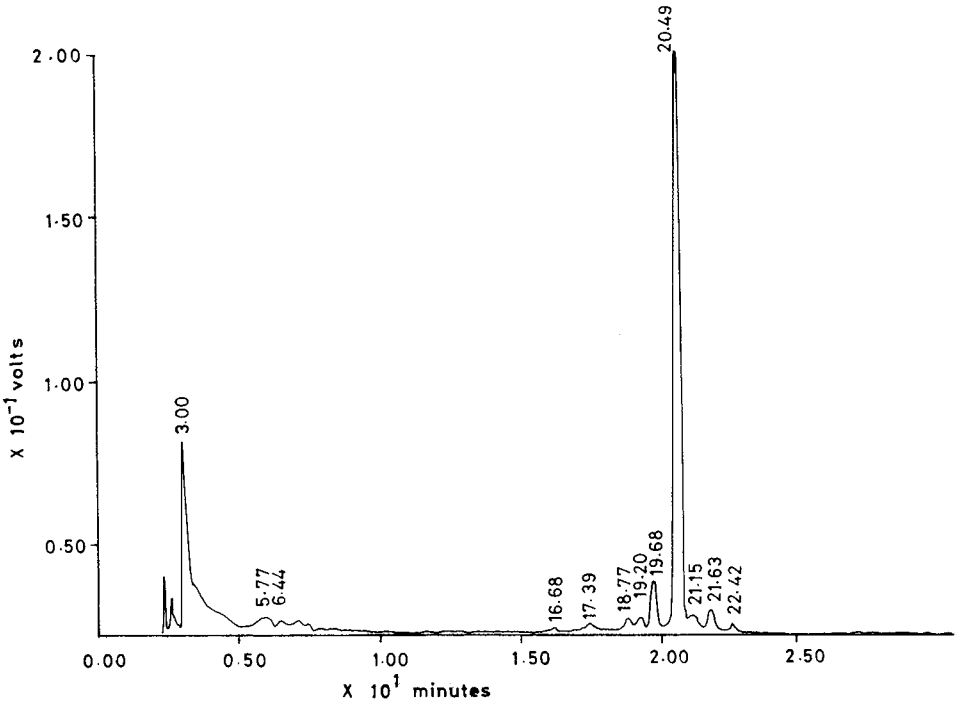

Figure 6. HPLC chromatogram of technical anilofos (brand II), 1000 μg/mL.

The analysis of anilofos and the analogous compounds was standardized by TLC, GC, and HPLC. The R_f and R_t values of the herbicide and other products as obtained on TLC and GC, HPLC are presented in Table 2. Any of the single techniques was insufficient for identification of all the compounds. While qualitative analysis was performed using TLC, quantitative analysis was performed by GC. The results obtained were confirmed by HPLC.

Two acetanilides **IV** and **V** had R_t values of 3.181 and 3.200 min, respectively, when a standard solution of each acetanilide was chromatographed separately on GLC (Table 2). However, whenever both compounds were mixed together and injected the mixture never showed two separate peaks. It always merged to give a single peak at R_t 3.191 min (Figure 3). In HPLC, the R_t values of these compounds were far apart, i.e., 16.68 and 20.19 min. Similarly, in HPLC, aniline (**II**) and anilophos (**I**) had R_t values of 20.43 and 20.50 min, respectively, and thus the possibility of merging of these two peaks was always present, but GC of these two compounds showed R_t values of 0.624 and 13.155 min, and so the compounds were easily separable and recognizable as different compounds. Similar was the case with acetanilide **V** and isopropyl aniline **III**, which showed very close R_t values of 20.19 and 20.29 min,

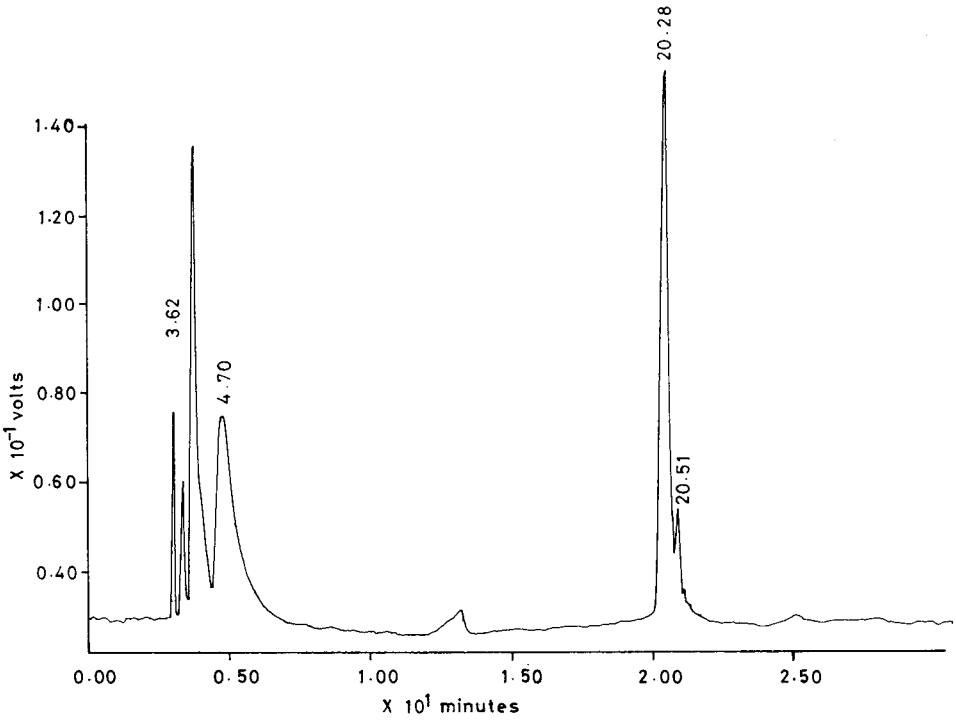

Figure 7. HPLC chromatogram of fraction 2 eluted with hexane.

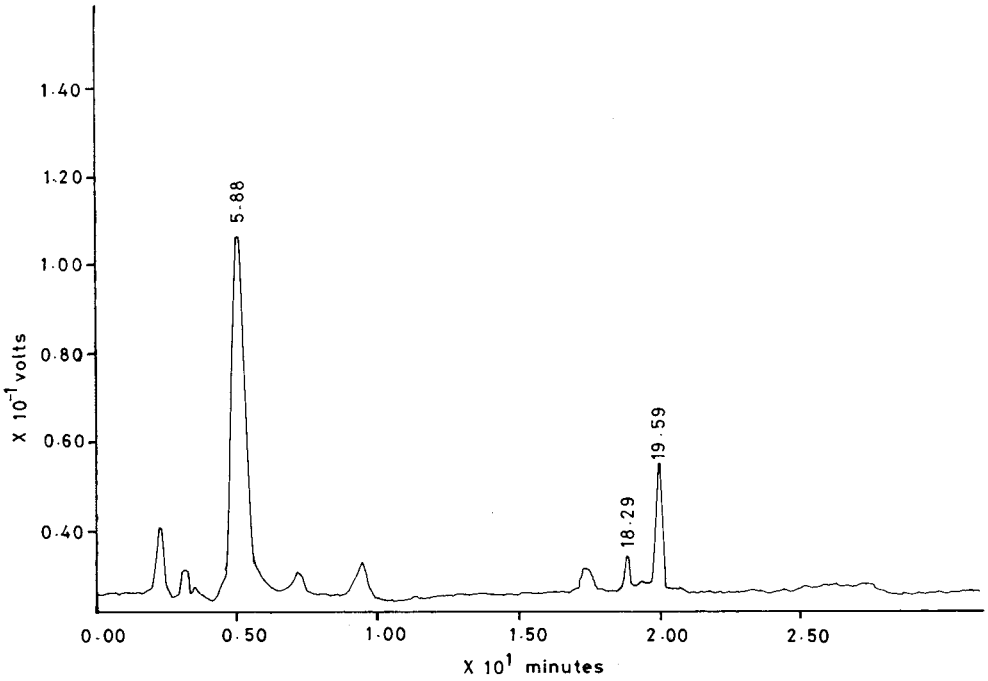

Figure 8. HPLC chromatogram of fraction 14 eluted with benzene−acetone (9:1).

respectively, in HPLC, but very well separated in GC at 3.200 and 1.324 min, respectively. In HPLC, dithiodimer **IX** and chloroacetanilide **VII** eluted at 21.63 and 21.93 min, respectively, while in GC they eluted at 7.184 and 6.294 min, respectively. Thus, using both techniques confirmation became more authentic.

GC was more helpful than the other techniques, as almost all the compounds except acetanilides **IV** and **V** showed good separation. HPLC was helpful as the sensitivity of most of the compounds was quite high.

Direct TLC of the analytical compound showed only two spots besides anilofos. Similarly, GC of the technical sample (Figure 4) showed three peaks out of which two corresponded to isopropyl aniline (**III**) and chloroacetanilide (**VII**) by comparison with authentic samples, while one was unidentified. HPLC of the technical sample showed six peaks besides anilofos, and only three could be identified by comparison and the rest were unidentified. Figures 5 and 6 show the HPLC chromatograms of technical anilophos of two different brands, namely, brand I and brand II, respectively. However, we found that this was not the complete picture of impurities. With GC, the problem was that anilophos itself was too sensitive to NPD and gave a very big peak, and in a solution of very low concentration for GC, the impurity peaks were not visible.

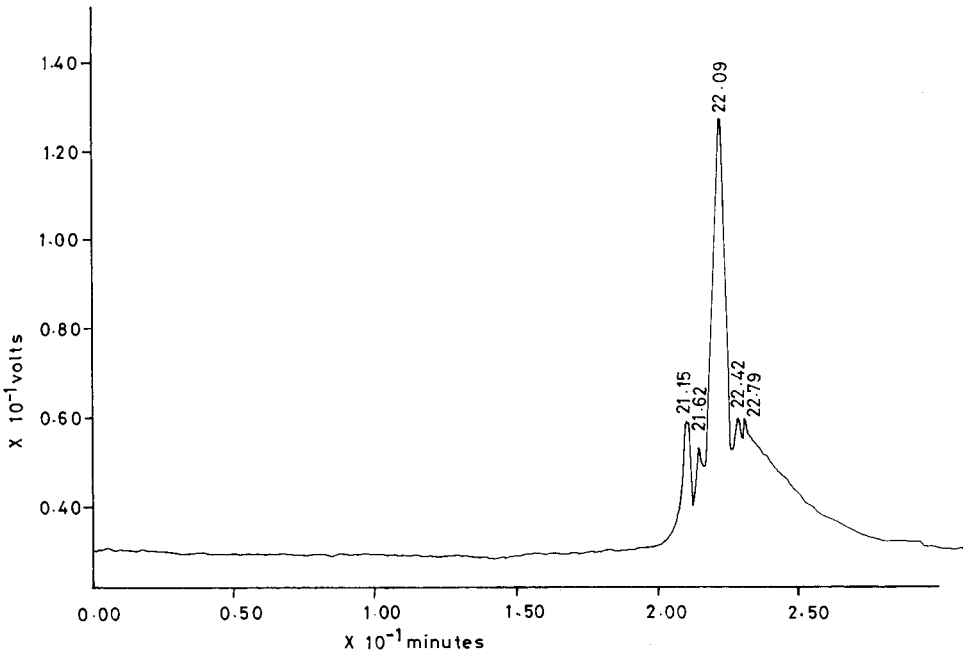

Figure 9. HPLC chromatogram of fraction 11 eluted with benzene−acetone (19:1).

Table 4. Effects of Anilofos and Impurities/Degradation Products on Root and Shoot Length of Rice Plant

	effect on root growth[a]			effect on shoot growth[a]		
compound	root length (cm)	fresh weight (mg/plant)	dry weight (mg/plant)	shoot length (cm)	fresh weight (mg/plant)	dry weight (mg/plant)
II	8.0	21.13	5.02	8.8	24.02	5.42
III	5.8	17.02	4.12	6.0	19.15	4.92
IV	7.0	20.13	5.09	7.8	23.05	5.32
V	6.9	20.14	5.02	7.9	23.13	5.30
VI	6.5	18.90	4.38	7.9	22.99	5.19
VII	5.8	16.96	3.81	8.1	22.92	5.24
VIII	4.7	11.83	2.35	5.0	12.41	2.68
IX	7.7	20.21	5.01	7.6	20.98	5.11
X	7.4	20.10	5.00	8.0	23.12	5.40
XI	6.6	18.59	4.25	7.8	20.23	5.15
control	8.1	21.35	5.10	8.8	24.22	5.43
SEM ±	0.002	0.352	0.187	0.008	0.217	0.315

[a] Indicates average of 15 plants.

However, when we injected a solution of 20 μg mL^{-1}, only two peaks corresponding to **II** and **VII** were visible. In HPLC, as we could inject a 1000 μg g^{-1} solution of anilofos (Figures 5 and 6), many peaks of impurities were visible. However, to have a complete picture, we first fractionated the compound into different fractions and then analyzed these fractions with GC and finally by HPLC. Fractionation of the material simplified the chromatograms and helped to identify different impurities quantitatively. Comparison of Figure 5 or 6 with Figure 7 showed that isopropyl aniline (**III**), which was not visible in the chromatograms 5 or 6, was well separated from anilofos in second fraction. Similarly, in the 14th fraction, monothiodimer appeared in quite high concentration (Figure 8). Figure 9 shows the chromatogram of the 11th fraction containing compound **VII** in higher concentration.

The results showed that technical anilofos had seven impurities, namely, isopropyl aniline (**III**), chloroacetanilide (**VI**), isopropylchloroacetanilide (**VII**), oxo analogue of anilofos (**VIII**), dithiodimer (**IX**), monothiodimer (**X**), and isopropyl mercaptoacetanilide (**XI**). The percentage of each impurity as quantified by GC and HPLC in two different brands of technical anilofos is mentioned in Table 3.

All the impurities and the compounds **II**, **IV**, and **V** being possible contaminants or degradation products of anilofos in the technical product were screened for their phytotoxicity to transplanted rice. After 15 days, the length, fresh weight, and dry weight of the roots and shoots of plants were measured, and the data are presented in Table 4. Only oxo analogue (**VIII**) was phytotoxic to rice at 1 μg g^{-1} soil. The compound reduced root and shoot length to 4.7 and 5.0 cm, respectively, in comparison to the control plant (7.8 and 8.5 cm, respectively). Fresh and dry weights of roots and shoots also decreased up to 50% showing the phytotoxic effect.

CONCLUSION

The direct analysis of the technical anilofos by different techniques showed only a few spots (TLC) and peaks (GC, HPLC) giving an incomplete picture of the contaminants.

The presence and quantification of different compounds was done by first dividing the total eluate of the column into different fractions on the basis of adsorption and polarity and finally adding the concentration of each individual compound found in each fraction obtained by column chromatography.

It was concluded that the impurities of technical anilofos and other contaminants except oxo derivative (**VIII**) do not have a deleterious effect on rice. The reported phytotoxic symptoms of oxo compound **VIII** were observed at a level of 1 μg g^{-1} of soil. Considering the concentration of contaminant found in two different brands of technical sample, i.e., 0.8 and 0.2%, and the rate of application of anilofos (1 kg ha^{-1}), it may not have a significant economic impact on the yield of rice under field conditions. However, since there is a possibility of formation of oxo analogue during storage, which may cause phytotoxicity to crops, the presence of this impurity in the technical anilofos must be checked as it directly affects its shelf life.

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