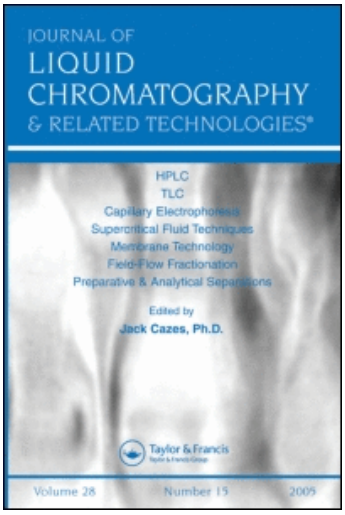

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LIQUID CHROMATOGRAPHIC DETERMINATION OF ASULAM AND AMITROLE WITH PRE-COLUMN DERIVATIZATION

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ABSTRACT

A liquid chromatographic pre-column derivatization method with fluorimetric detection for the simultaneous determination of asulam and amitrole was developed. The separation was accomplished in less than 15 min. R.S.D.'s (n = 10) of 1.13% and 1.6% (concentration) and 1.4% and 0.97% (retention time), were obtained for asulam and amitrole respectively. Recoveries from spiked tap water ranged from 90% to 118%, and detection limits of 0.04 ng for asulam and 7.5 ng for amitrole, were obtained.

INTRODUCTION

The pesticide asulam is a translocation herbicide, absorbed by leaves and roots causing slow chlorosis in susceptible plants. It interferes with cell division and expansion and is used to control the growth of grasses.[1]

The pesticide amitrole is a non - selective herbicide absorbed by roots and leaves, and translocated. It inhibits chlorophyll formation and regrowth from buds. It is used around established apple and pear trees between fall harvest and the following summer. It is also used as a non selective herbicide before planting kale, maize, oilseed rape, potatoes, wheat; on fallow land and in other non crop situations. Its activity is enhanced by the addition of ammonium thiocyanate.[2]

Methods for determination of asulam include liquid chromatography,[3-7] gas chromatography[8] and fluorescence synchronous derivative technique.[9] Amitrole was determined by liquid chromatography[10-15] and gas chromatography.[16] The detection limits of the methods previously reported was ranged from 0.5 mg to 0.1 ng and from 0.5 mg to 0.05 μg for asulam and amitrole, respectively. The proposed method in this work is more sensitive than the methods previously described for asulam and amitrole. The liquid chromatographic method with amperometric detection[15] presents a detection limit of 200 pg for amitrole. This method is more sensitive than the method proposed in this work but its relative standard deviation is 11% while 1.6% is obtained in this work.

Amitrole does not show native fluorescence, thus the technique of spectrofluorimetry has not been previously applied to its determination. Asulam presents a low native fluorescence, thus the derivatization of fluorescamine reagent increases the sensitivity for the spectrofluorimetric determination of asulam.[9]

Udenfriend et al.,[17] introduced fluorescamine as a labeling reagent to determine primary amines; this is superior to dansyl chloride because both the reagent and its hydrolysis products are non-fluorescent and permit homogeneous fluorogenic labelling. Such approach has proved its usefulness in numerous analytical applications for some 30 years.[18-19]

Considerable efforts have been made to develop highly selective and sensitive derivatization reagents for use in liquid chromatography with fluorescence detection. Several excellent reagents are currently available for most functional groups, e.g., hydroxy, amino, thiol, carbonyl and carboxyl groups. The most useful application of fluorescamine in high performance liquid chromatography derivatization is its ability to hydrolyze the excess of reagent after the derivatization process, releasing non-fluorescent products. Thus, the fluorimetric detection is blind to the fluorescamine excess. This is specially useful in pre-column derivatization HPLC methods.

In this work the optimum experimental conditions for the spectrofluorimetric determination of asulam and amitrole based on fluorophore generation by derivatization with fluorescamine (FC) were investigated. Reverse phase LC determination of asulam and amitrole with pre-column derivatization was also carried out and applied to spiked tap water.

EXPERIMENTAL

Instrumentation

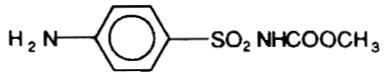
A Merck - Hitachi (Darmstadt, Germany) liquid chromatograph was used consisting of a L-6200 pump, an AS-4000 autosampler, a F-8000 fluorescence detector and a D-6000 interface. Integration was carried out with a PC/AT computer and the instrumental parameters were controlled by Hitachi - Merck HM software. All the derivatization steps are performed automatically by the AS-4000 autosampler.

Fluorescence measurements were made with a Perkin Elmer LS50 Spectrofluorimeter (Beaconsfield U.K) and equipped with a Xenon discharge lamp and two monochromators. Fluorescence Data Manager (FLDM) Software and a RS232C interface were used to send information to an external computer.

Reagents

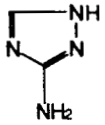
Potassium hydrogen phthalate and borax were obtained from Panreac (Barcelona, Spain), potassium chloride from J. T. Baker Chemicals (B.V. - Deventer - Holland), potassium phosphate dibasic from Codex (Milano, Italy), potassium phosphate monobasic from Probus (Barcelona, Spain), HCl from Merck (Darmstadt, Germany) and Fluorescamine (FC) (98%) from Aldrich (Milwaukee -USA). Asulam (purity 99.9%) and amitrole (98%) were purchased from Dr. S. Ehrenstorfer (Augsburg, Germany). Methanol was of LiChrosolv gradient grade (Merck) and acetone of analytical - reagent grade (Merck). The solvents were previously sonicated for 30 min and filtered through 0.2 μm Nylon membrane filters.

Stock standard solutions of asulam (4.34×10^{-3} M) and amitrole (1.19×10^{-2} M) were prepared by dissolving the compounds in water and stored at 4°C. Working standard solutions were prepared by dilution with water. Fluorescamine (3.59×10^{-3} M) was dissolved in acetone.



ASULAM

Methyl sulphanylcarbamate



AMITROLE

3 - amino - 1 H - 1,2,4 - triazole

Figure 1. Structures of the pesticides.

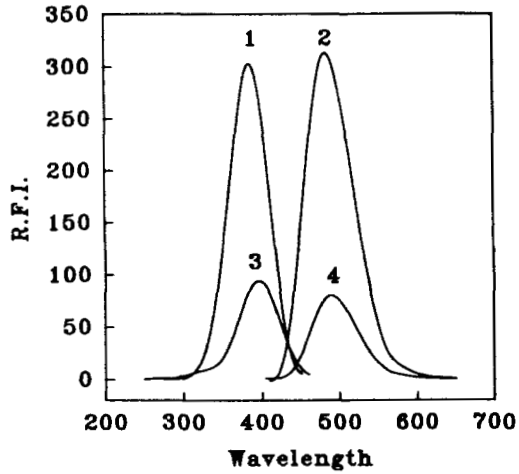


Figure 2. Excitation and emission spectra of FC derivatives of (1,2) amitrole and (3,4) asulam. Asulam 8.7×10^{-7} M and amitrole 2.4×10^{-4} M pH = 3; [FC] = 3.6×10^{-4} M.

Solutions of potassium chloride (0.1 M), potassium hydrogen phthalate (0.05 M), borax (0.1 M) and phosphate buffer (0.1 M) were prepared in doubly deionized water. The solutions were filtered through 0.2 μm Nylon membrane filters.

Derivatization

Aliquots of 100 μL of aqueous standard solutions of asulam (0.04 - 4 μg/mL) and 100 μL amitrole (0.8 - 20 μg/mL) were introduced in a 1.5 mL flask, and then 300 μL of a $3{,}6x10^{-3}$ M acetone solution of fluorescamine and 300 μL of pH buffer solution were added. The mixture was diluted in water up to 1.5 mL. After each reagent addition the mixture was agitated. A volume of 20μL of this solution was injected into the chromatograph and analyzed. All these operations were automatically performed by the autosampler.

LC Operating Conditions

The pesticide sample was analyzed using a LiChrospher 100 RP-18 reverse phase column (25 cm x 4 mm I.D.; 10 μm particle size) from Merck. The injection volume was 20 μL for the standard aqueous solutions and samples. The mobile phase composition was 25% methanol aqueous at a 1 $mL.min^{-1}$ flow rate. The peak - area response was measured at the retention times of asulam (5.96 min) and amitrole (10.14 min). A calibration graph was constructed using the responses.

Recovery test

Tap water samples from Antequera (Spain) were used to prepare two samples with known levels of added asulam and amitrole (0.05 ng of asulam + 20 ng of amitrole and 2 ng of asulam + 100 ng of amitrole). The mixture was filtered through a Sep-Pak silica 3 cc cartridges. The solutions were diluted with water to a final volume of 5 mL. These solutions were used for analysis.

RESULTS AND DISCUSSION

Asulam and amitrole (Fig 1) react with FC to form two fluorophores whose spectra are very similar. Figure 2 shows the excitation and emission spectra of the FC derivatives of asulam and amitrole under the final experimental conditions. As expected, the spectral parameters for both compounds are similar. Each compound is characterized by its well resolved excitation maximum (398 nm) and its single emission peak (490 nm) for asulam and amitrole.

The operating parameters for the individual compounds can be optimized to give an analytical method for each. Consequently, after fixing the individual optimum conditions in order to determine isolates asulam and amitrole, a new set of conditions was selected to obtain good emission signals for each compound before carrying out the analysis of mixtures of asulam and amitrole by high performance liquid chromatography.

As a fluorigenic reagent for amino compounds, FC lacks selectivity, which emphasizes the need for more detailed information about the effect of the main reaction conditions so that the fluorescence yield might be improved to permit the selective analysis of mixtures of fluorophores with FC.

Influence of Reaction Variables

The effect of pH on fluorescence intensity was explored by carrying out several assays of solutions in 5 mL volumetric flasks containing 0.2 μg/mL of asulam (or 20 μg/mL of amitrole) and 1 mL of different buffer solutions that covered the pH range 1 - 10, together with 0.5 mL of FC standard solution (1 mL in the case of amitrole), the solution was then diluted with water.[18]

Figure 3 shows that the maximum fluorescence of the asulam fluorophore occurred at pH 2 and that of amitrole at pH 4. In both instances, the narrow range in which the fluorescence intensity was maximum suggests that careful control of the pH solution is required.

On the other hand, to obtain good yields in the labelling reactions of mixtures of both compounds, the pH setting must be a compromise and in this work, pH 3 appeared to be the optimum.

FC reacts very quickly with primary amines ($t_{1/2}$ = 100 - 500 ms), but frequently a great excess of FC is needed to produce good thermodynamic equilibrium conditions, as described previously.[19]

The effect of FC concentration on fluorophore formation was observed by measuring the fluorescence intensity for each compound at different FC concentrations, while all other experimental conditions were kept constant at the optimum values. Figure 4 shows that the maximum response was obtained when the FC concentration was 5.03×10^{-4} M for asulam and 2.16×10^{-4} M for amitrole. For the simultaneous determination of the two compounds a [FC]= 7.20×10^{-4} M was selected.

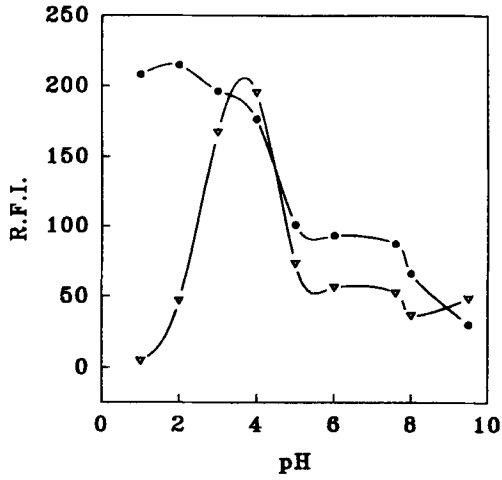


Figure 3. Influence of pH on the relative fluorescence intensity of (●) asulam and (▼) amitrole. Asulam 8.7×10^{-7} M and amitrole 2.4×10^{-4} M [FC] = 3.6×10^{-4} M.

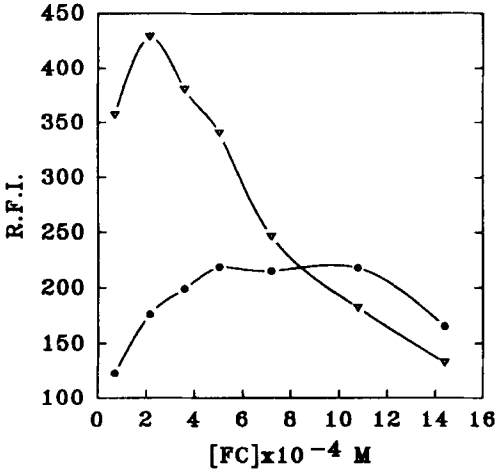


Figure 4. Influence of the [FC] on the relative fluorescence intensity of (●) asulam (pH = 2) and (▼) amitrole (pH = 4).

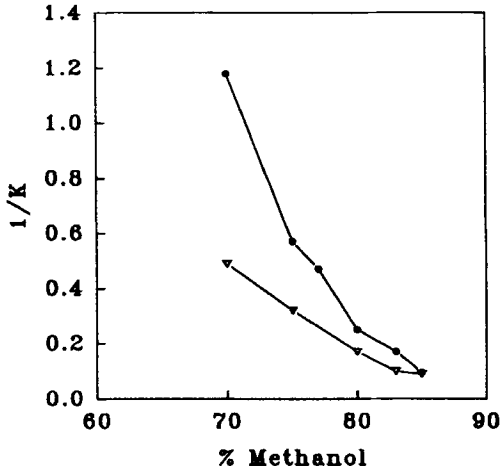


Figure 5. Capacity factor of the asulam (●) and amitrole (▼) vs methanol percentage.

Optimization of the Chromatographic Conditions

The detection was accomplished to the wavelength obtained by the emission and excitation spectra (λ_{exc} = 398 nm, λ_{ems} = 490 nm). The column used was a LiChrospher 100 RP - 18 and the solvents for the mobile phase water - methanol. A form of choosing the better composition of the mobile phase for the resolution of the mixture of pesticides is plotting capacity factor, K, against the percentage of methanol. Taking into account results shown in Fig. 5, while the methanol proportion in the mobile phase increases the capacity factor also increases, therefore the pesticides are more retained by the stationary phase and the retention times are greater.

It can be concluded that a 25% methanol percentage gives the best separation of the pesticides and the overall chromatographic time is not very high (15 min).

Calibration Graphs

The calibration graphs are linear between 0.04 ng - 4 ng for asulam and between 16 ng - 400 ng for amitrole. The lower limit of the linear dynamic range is determined by the quantification (C_Q) limit. Typical relative standard deviations (R.D.S.s) are between 1.13% - 1.6%. Linear regression analysis gave the following fit.

Table 1

Spectrofluorimetric Interference Study

Interfer.	Asulam:Interfer.	Recovery(%)	Amitrole:Interfer.	Recovery(%)
Carbaryl	1:500	109	1:5	103.8
Warfarin	1:500	96.4	1:5	91.4
			1:2.5	101.17
Fuberidazol	1:500	100	1:5	88.9
			1:2.5	94.74
MCPA	1:500	103	1:5	98.3
Bentazone	1:500	69.17	1:5	60.13
	1:250	95.8	1:2.500	70.80
			1:1.875	73.46
			1:1.250	90.06
			1:1	96.79

Asulam: $Y = 2436813.6\ X - 17230.5$ $r = 0.999$ $(n= 8)$
Amitrole: $Y = 22514.1\ X - 117219.7$ $r = 0.999$ $(n= 7)$

where Y is area under peak, in arbitrary units, and X is injected quantities in ng.

Application

Prior to the application in real samples, the method was evaluated with synthetic mixtures of the most commonly used pesticides in pre- or post-harvest treatment. Five potential interferents were selected among insecticides, fungicides, and herbicides usually found in cereals, fruits, vegetables and other types of crops. The synthetic mixtures were prepared using a fixed concentration of the pesticide to be recovered and adding the potential interferents at several levels. The pesticides carbaryl, warfarin, fuberidazol, MCPA and bentazone, were added separately. Recoveries from these synthetic mixtures ranged from 94 to 104% for the non-interferent pesticides and from 60 to 94% for those pesticides causing interference. The tolerance criterion was a deviation of ± 5% in the signal, referred to the blank (pesticide alone). Table 1 shows the results obtained.

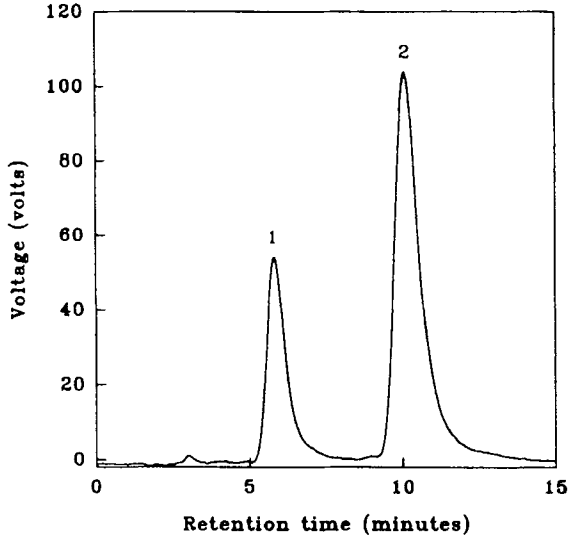


Figure 6. Chromatogram of spiked tap water. (1) 1 ng asulam and (2) 200 ng amitrole.

Table 2

Recovery of Pesticides from Spiked Tap Water

Compound	Taken (ng)	D_l[a] (ng)	C_q[b] (ng)	Recovery %	R.S.D.[c] %
Asulam		0.04	0.13		
	00.050			118	1.7
	2			90	2.2
Amitrole		7.5	25		
	20			108.5	6.9
	104.8			104.8	6.2

[a] detection limit for a signal-to-noise ratio = 3
[b] quantification limit for signal -to-noise ratio = 10
[c] n = 3

The application of herbicides in agriculture may cause the contamination of ground water and subsequently drinking water. The 1989 European Community Water Act states that the maximum admissible concentration of all pesticides in drinking water should be below 0.5 μg/L and the maximum individual pesticides concentration is 0.1 $\mu g.L^{-1}$.[20] The high water solubility of the pesticides studied (30 mg/L for asulam and 280 g/L for amitrole) facilitates their mobilization in water streams and localization at high levels in water.

Two different water samples were spiked, prior to the extraction, with a mixture of the pesticides prepared in doubly deionized water, after checking for the absence of the pesticides under study. After extraction, the samples were subjected to the LC procedure. The chromatogram of water extracts are reported in Fig. 6.

Table 2 presents the results obtained in the determination of asulam and amitrole in water by applying the LC method. As can be seen, recoveries are within 90 - 118%. The results obtained demonstrate the effectiveness of the proposed methods in determining the analytes assayed in these types of samples.

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