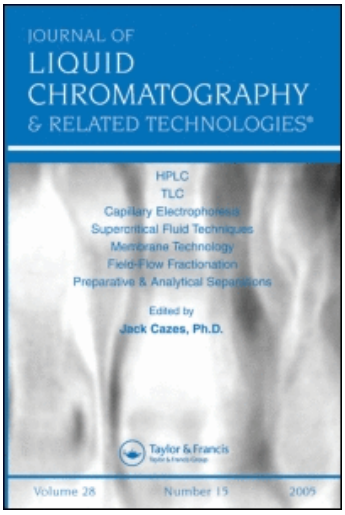

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A Simple and Quick Solid Phase Extraction and Reversed Phase HPLC Analysis of Some Tropane Alkaloids in Feedstuffs and Biological Samples

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A SIMPLE AND QUICK SOLID PHASE EXTRACTION AND REVERSED PHASE HPLC ANALYSIS OF SOME TROPANE ALKALOIDS IN FEEDSTUFFS AND BIOLOGICAL SAMPLES

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ABSTRACT

A simple and rapid reversed - phase high performance liquid chromatographic (HPLC) method using an ultraviolet detector for the analysis of tropane alkaloids in feedstuffs and biological samples is presented.

Hyoscyamine, scopolamine and the internal standard bamifylline are adsorbed on a C_{18} cartridge employing the solid - phase liquid extraction technique for sample clean up and subsequent separation of those compounds and internal standard from endogenous interfering compounds and preconcentration.

Chromatographic analysis is achieved on a Lichrosorb RP-18 10μm reversed phase column and the mobile phase is an isocratic mixture of acetonitrile, methanol and 0.05 M ammonium acetate (20.9 : 27.9 : 51.2) at a flow rate of 1.30 ml/min. The eluted alkaloids are detected at 210 nm. The retention time is 3.990 min for scopolamine, 6.934 min for hyoscyamine and 8.750 min for the internal standard bamifylline. The correlation of the integrated peak area ratios of scopolamine and

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hyoscyamine to internal standard with the concentrations of those compounds showed a linear relationship between 1.20 to 60.0 ppm for scopolamine and 1.25 to 40.0 ppm for hyoscyamine. The linearity, employing the clean up technique, of scopolamine and hyoscyamine is 1.20 to 15.0 ppm and 1.25 to 20.0 ppm respectively per 20 μl injection. The absolute detection limits are 12.05 ng and 13.25 ng for scopolamine and hyoscyamine respectively.

The method outlined in this paper is applied to the simultaneous determination of scopolamine and hyoscyamine in feedstuffs (soya and corn) and biological samples (eggs, blood serum and urine).

INTRODUCTION

The world need for protein is resulting in the development of projects designed to obtain for human and animal consumption. Agriculturally advanced countries, like in Europe and America, have a vital need to use soya beans for human and animal feedstuffs.

Among soya plants there is a weed growing called datura (datura ferox, datura stramonium, datura innoxia, datura metel, datura meteloides, e.t.c.). Datura weeds have been shown to contain several tropane alkaloids, especially hyoscyamine and scopolamine[1-2].

There is a great variation in the amounts and percentages of these alkaloids present, depending on the portion of plant analyzed and the stage of maturation.

Human beings appear most susceptible to poisoning by the datura plants, but some cases have been recorded in pigs, cattle, horses, dogs, sheep, mules and fowls[3]. Animals may be poisoned by eating grain contaminated with the seeds of datura plants, particularly crushed grain but those are more resistant than humans because of the enzyme esterase of atropine they dispose[4-7].

Typical findings in datura poisoning are dryness of the mouth, thirst, flushing, fever, amnesia, urinary retention, decreased salivation, pupillary dilaration, tachycardia, hallucinations, which are often frightening, palpitation, ataxia, delirium leading to coma, cardiac and respiratory arrest and death[8-9].

In the literature there are numerous publications dealing with the isolation and determination of scopolamine, hyoscyamine and related compounds. Scopolamine and hyoscyamine have been separated and determined by chromatographic

techniques such as paper chromatography, thin layer chromatography, gas chromatography and high - performance liquid chromatography[10-17].

The rapid and simultaneous determination of scopolamine and hyoscyamine in feedstuffs and biological samples still is a problem.

This paper describes a simple and quick solid phase extraction and reversed phase HPLC analysis of scopolamine and hyoscyamine in feedstuffs (soya and corn) and biological samples (eggs, blood serum and urine).

EXPERIMENTAL

Apparatus: The high performance liquid chromatograph employed was a ternary gradient pump (Spectra Physics, Model SP 8800 California USA) and a Spectra Physics, Spectra Chrom 100 variable wavelength UV-VIS detector, operated at 210 nm and a sensitivity setting of 0.002 AUFS. The gradient elution was used for optimization of the eluent system which was found to be acetonitrile - methanol - 0.05 M ammonium acetate (20.9 : 27.9 : 51.2). A Rheodyne 7125 (California USA) injection valve was fitted with a 10 µl loop. The pressure was 2150 psi at a flow rate of 1.0 ml/min. The analytical column was a Spherisorb ODS-2 10 µm 250 x 4.6 mm I.D., stainless steel from Spectra Physics.

The routine analyses of the biological samples and feedstuffs were performed on an isocratic system consisted of an SSI 222 D pump (State College, PA 16803 USA), an SSI 500 variable wavelength UV-VIS detector operated at 210 nm and a sensitivity setting of 0.002 Absorbance Units Full Scale. A Rheodyne 9125 injection valve was fitted with a 20 µl loop. The pressure was 1375 psi at a flow rate of 1.30 ml/min. The integrator was a Hewlett Packard 3396 Series II (Avondale, PA 19311, USA). The analytical column was a Lichrosorb RP-18, 10 µm ODS, 250 x 4.0 mm I.D., stainless steel from MZ Analysentechnik. Bond Elut C_{18} cartridges were obtained from Analytichem International, a division of Varian (Harbor City, USA).

Computations were performed using a Vip 312 Computer.

Materials: Scopolamine hydrochloride and Hyoscyamine were from SIGMA Chemical Company (St Louis MO 63178 USA), while bamifylline was supplied from Alfa Wassermann SpA (Bolognia, Italy). All standard solutions of these compounds were prepared by dissolving the appropriate quantities in methanol. The reagents were used as provided without further purification. Methanol and acetonitrile HPLC - grade reagents were from Merck (Darmstadt, Germany). Ammonium acetate, pro analysi reagent was also from Merck. All other reagents used such as: hydrochloric acid, ammonia, potassium sulphate and dichloromethane were HPLC grade from Merck. Glass - distilled water was used throughout.

Chromatographic Conditions: The eluent system used was a mixture of acetonitrile - methanol - 0.05 M ammonium acetate (20.9 : 27.9 : 51.2) and was found through a number of other eluent systems on the basis of their polarities and low absorption at the wavelength used. The flow rate was 1.30 ml/min at a pressure of 1375 psi. The detection was performed at 210 nm with a sensitivity setting of 0.002 Absorbance Units Full Scale (AUFS). The above mentioned optimum chromatographic conditions were selected among several ones examined, as shown in Table 1, since they were found to be the most suitable for a good separation of scopolamine, hyoscyamine and the internal standard of bamifylline. The chromatographic analyses were performed at ambient temperature 22 °C.

System Suitability: The Lichrosorb RP-18, 10 μm reversed phase analytical column was equilibrated with acetonitrile - methanol - 0.05 M ammonium acetate (20.9 : 27.9 : 51.2) the eluting solvent system used, at a flow rate of 1.30 ml/min. After an acceptable stable baseline was achieved, the samples, the internal standard bamifylline and the standards were analyzed. The resolution factors, R_t, were calculated between the three peaks[18] and found to be 4.72 for scopolamine, 3.68 for hyoscyamine and 1.92 for bamifylline. The above given resolution factors signify complete separation between the alkaloids examined. The chromatogram and the molecular types of the alkaloids are given in Figure 1.

Table 1. High-Performance Liquid Chromatographic Conditions Examined in the Present Study.

Eluent System Isocratic (Is), Gradient (Gr)	Wavelength (nm)	Flow Rate (ml/min)	Sensitivity (AUFS)	Retention Time (min)		
				Scopolamine	Hyoscyamine	Bamifylline
(Gr) CH_3OH-CH_3CN-0.5% CH_3COONH_4 (50 : 25 : 25)	228	t=0 1.2 t=3.5 2.2 t=7.1 1.2	0.001	4.33	7.31	7.11
(Gr) CH_3OH-CH_3CN-0.5% CH_3COONH_4 (65 : 20 : 15)	210	t=0 1.0 t=3.0 1.5 t=6.0 1.1	0.001	3.46	6.12	ND
(Gr) CH_3OH-CH_3CN-0.5% CH_3COONH_4 (45 : 25 : 30)	210	t=0 0.8 t=2.5 1.4 t=6.5 1.0	0.002	3.52	6.37	6.61
(Gr) CH_3OH-CH_3CN-0.5% CH_3COONH_4 (40 : 30 : 30)	220	t=0 1.0 t=6.1 1.3 t=8.6 1.5	0.01	4.34	7.71	7.68
(IS) THF-0.4% CH_3COONH_4-CH_3CN-CH_3OH (0.9 : 54.1 : 25 : 20)	228	1.0	0.01	2.50	4.28	4.41
(IS) CH_3OH-CH_3CN-0.5% CH_3COONH_4 (50 : 13 : 37)	228	1.0	0.01	4.89	9.65	9.38
(IS) CH_3OH-CH_3CN-0.5% CH_3COONH_4 (60 : 27 : 13)	228	1.0	0.005	5.35	7.51	7.36
(IS) CH_3OH-0.043% CH_3COONH_4 (24 : 76)	257	1.32	0.001	13.70	14.94	ND
(IS) CH_3OH-0.043% CH_3COONH_4 (24 : 76)	257	1.0	0.001	15.41	18.51	17.83
(IS) CH_3OH-0.053% CH_3COONH_4 (33.4 : 66.6)	257	1.0	0.001	8.92	ND	8.34
(IS) CH_3OH-0.04% CH_3COONH_4 (50 : 50)	257	1.0	0.001	10.21	11.42	12.81
(IS) CH_3OH-0.19% CH_3COONH_4 (44 : 56)	210	1.0	0.001	9.34	23.34	9.67
(IS) CH_3OH-CH_3CN-0.344% CH_3COONH_4 (36 : 9 : 55)	210	1.20	0.002	5.60	10.10	ND
(IS) CH_3OH-CH_3CN-0.344% CH_3COONH_4 (36 : 9 : 55)	210	1.35	0.002	4.93	8.71	ND
(IS) CH_3OH-CH_3CN-0.344% CH_3COONH_4 (34.7 : 14.5 : 50.8)	210	1.35	0.002	4.81	7.84	4.68
(IS) CH_3OH-CH_3CN-Buffer[a] (65 : 17 : 18)	208	1.35	0.001	15.41	14.82	15.69
(IS)*CH_3OH-Buffer-CH_3CN-0.5% CH_3COONH_4 (35.5 : 5.5 : 5 : 54)	210	1.0	0.002	5.71	9.51	ND
(IS) CH_3OH-CH_3CN-0.294% CH_3COONH_4 (33 : 12 : 55)	210	1.30	0.002	5.54	10.13	10.49
(IS) CH_3OH-CH_3CN-0.375% CH_3COONH_4 (42 : 16 :42)	210	1.45	0.02	3.50	5.94	6.18
(IS) CH_3OH-CH_3CN-0.375% CH_3COONH_4 (42 : 16 : 42)	210	1.20	0.002	4.30	7.51	7.90
(IS) CH_3OH-CH_3CN-0.375% CH_3COONH_4 (35 : 18 : 47)	210	1.45	0.002	4.31	6.84	8.91
(IS) CH_3OH-CH_3CN-0.386% CH_3COONH_4 (28,5 : 21.5 : 50)	210	1.45	0.002	3.62	6.13	7.94
(IS) CH_3OH-CH_3CN-0.386% CH_3COONH_4 (27.9 : 20.9 : 51.2)	210	1.30	0.002	3.99	6.93	8.75

[a]Buffer = CH_3COOH - CH_3COONa, pH = 4.6 ND = Not Detected
* Plus triethylamine

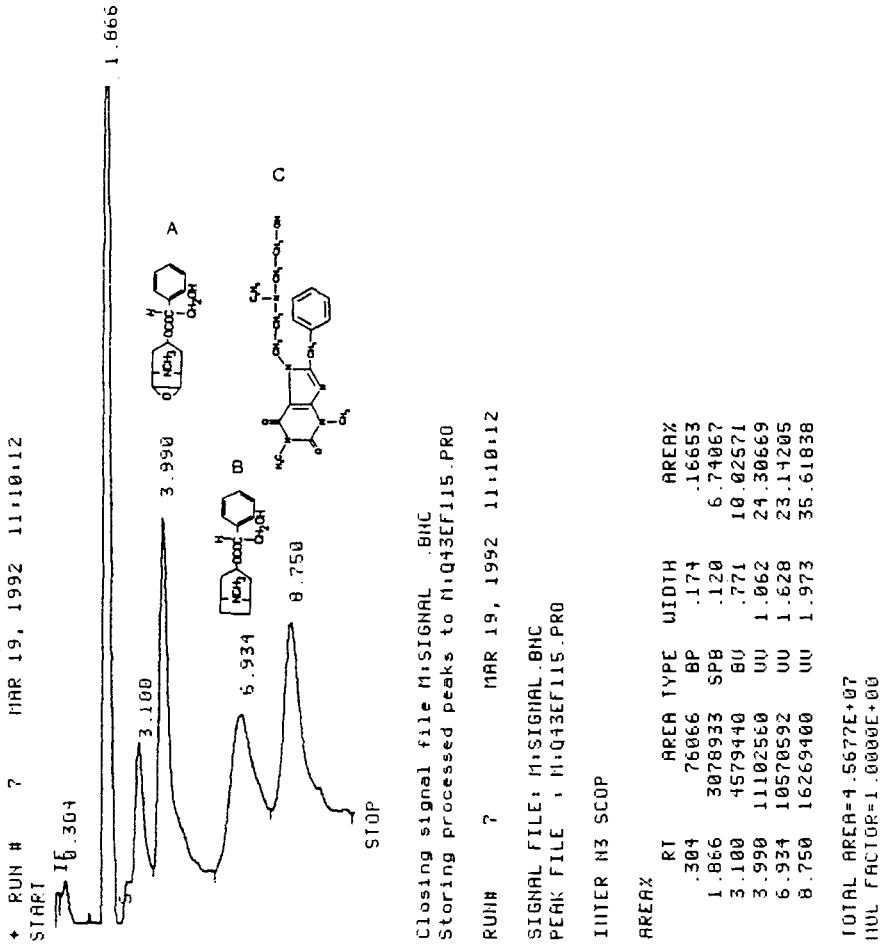

Figure 1. High - Performance Liquid Chromatogram of Scopolamine and Hyoscyamine using Bamifylline as Internal Standard.
Peaks: (3.990) = Scopolamine [7.23 ppm], (6.937) = Hyoscyamine (7.95 ppm) and (8.750) = Bamifylline (2.0 ppm). Chromatographic conditions as given in text.
A = Scopolamine, B = Hyoscyamine and C = Bamifylline.

Table 2. Peak Area Ratios of Scopolamine to Bamifylline over the concentration range (4.82 - 12.05 ppm) in methanolic solutions, equivalent quantities injected (96.4 - 241 ng)

Concentration of Scopolamine (ppm)	Equivalent Quantity Injected (ng)	Peak Area Ratios of Scopolamine to Internal Standard Bamifylline								X	SD	RSD (%)
		1	2	3	4	5	6	7	8			
4.82	96.4	0.9838	0.9708	0.8945	0.9436	0.9938	1.0094	1.0576	0.9946	0.9810	0.0478	4.88
7.23	144.6	1.5008	1.4523	1.6745	1.6214	1.5391	1.5523	1.5576	1.5612	1.5574	0.0681	4.37
9.64	192.8	2.0792	1.9434	2.0048	1.9730	2.2946	2.1342	2.1687	2.2336	2.1039	0.1263	6.00
12.05	241	2.3409	2.5158	2.5191	2.4659	2.6514	2.4802	2.3763	2.6336	2.4979	0.1092	4.37

Table 3. Peak Area Ratios of Hyoscyamine to Bamifylline over the concentration range (5.30 - 13.25 ppm) in methanolic solutions, equivalent quantities injected (106 - 265 ng)

Concentration of Hyoscyamine (ppm)	Equivalent Quality Injected (ng)	Peak Area Ratios of Hyoscyamine to Internal Standard Bamifylline								X	SD	RSD (%)
		1	2	3	4	5	6	7	8			
5.30	106	0.7232	0.7307	0.6680	0.7124	0.7383	0.6941	0.7544	0.7038	0.7156	0.0272	3.80
7.95	159	1.0281	1.0486	1.0948	1.0149	1.0571	1.0227	1.0926	1.0159	1.0468	0.0325	3.11
10.6	212	1.3777	1.4239	1.3797	1.3407	1.3215	1.3352	1.3639	1.3630	1.3632	0.0321	2.35
13.25	265	1.6814	1.7268	1.6421	1.6964	1.7704	1.7112	1.7267	1.7187	1.7092	0.0376	2.20

The relative standard deviations of eight replicate analyses of four standards 4.82, 7.23, 9.64 and 12.05 ppm for scopolamine and 5.30, 7.95, 10.60 and 13.25 for hyoscyamine were found to be at the range 4.37 to 6.00% for scopolamine and 2.20 to 3.80% for hyoscyamine respectively.

These results are given in Tables 2 and 3.

Selectivity: The selectivity of HPLC elution method was investigated at the retention times of scopolamine, hyoscyamine and bamifylline employing either isocratic or gradient elution. No interferences from endogenous compounds were found in chromatograms of samples of biological interest cleaned up by using solid phase liquid extraction. Therefore, the proposed method can be used in the analyses

of scopolamine and hyoscyamine in these samples using bamifylline as internal standard.

Detection and Quantitation Limits: The detection limits of scopolamine and hyoscyamine were assessed in the presence of the internal standard, bamifylline, and were considered to be the quantities producing a signal of a peak height twice the size of background noise. The minimum detectable quantities, expressed in ng injected on the column, were found to be 12.05 ng for scopolamine and 13.25 ng for hyoscyamine.

The quantitation limits of these two compounds were assessed in the presence of the internal standard and were considered to be the quantity producing a signal five to ten times the peak height the detection limit quantity produces. These limits are 37.98 ng for scopolamine and 38.18 ng for hyoscyamine.

Calibration Curves for the Simultaneous Determination of Scopolamine and Hyoscyamine, in Methanol, in the Presence of Bamifylline: Calibration curves for the determination of scopolamine and hyoscyamine were constructed in the presence of internal standard, bamifylline. Scopolamine, hyoscyamine and bamifylline were accurately weighed and dissolved in methanol to give stock solutions of 96.4 ppm for scopolamine, 106 ppm for hyoscyamine and 100 ppm for bamifylline. Standard solutions of 0.6025, 1.205, 2.410, 3.615, 4.820, 6.025, 7.230, 9.640, 12.050 and 24.10 ng/μl for scopolamine were prepared with bamifylline, in 20 ml volumetric flasks by serially diluting the stock solutions. The standard solutions of hyoscyamine were 0.6625, 1.325, 2.650, 3.975, 5.30, 6.625, 7.950, 10.60, 13.250 and 26.50 ng/μl.

In both cases the concentration of bamifylline was 2.0 ng/μl. All dilutions were made with methanol. Aliquots of 20 μl of each solution were injected onto the analytical column through the injection valve. The peak area ratios of scopolamine and hyoscyamine to bamifylline were recorded and plotted as functions of scopolamine and hyoscyamine concentrations. All determinations were repeated eight times and the results were treated statistically.

Determination of Scopolamine and Hyoscyamine in Feedstuffs and Biological Samples

Datura Seeds: 0.5 g of ground datura seeds were quantitatively transferred in a glass beaker with 30 ml of n-hexane and stirred for 2 h. The organic layer was removed and 25 ml of concentrated ammonia were added. The mixture was stirred and sonicated for 30 min, then 15 ml of dichloromethane were added and the mixture was stirred and sonicated again for 10 min. The two layers were separated and the organic phase was diluted by 15 ml of deionized water. The mixture was centrifuged at 3500 rpm for 10 min. The upper aqueous phase was combined with the aqueous ammonia layer and the mixture was extracted with 15 ml of dichloromethane and centrifuged at 2500 rpm for 15 min. The yellow aqueous phase was removed and the organic layer was kept. The intermediate white gel was extracted with 10 ml of dichloromethane and centrifuged at 5000 rpm for 10 min. The organic layer was combined with the previous one and the mixture was dried by potassium sulphate. Then the organic phase was evaporated to dryness under stream of nitrogen. The residue was redissolved with 3 ml of methanol and cleaned up with 1 ml of bamifylline 2.0 ppm. The cartridge was washed by 3 ml of deionized water and the alkaloids were eluted by 3 ml of methanol 0.01 M in HCl. The acidic methanol was evaporated to dryness under stream of nitrogen at 45 °C. The residue was dissolved in 1 ml of methanol and aliquots of 20 µl were injected on to the HPLC column.

Blood Serum: Standard scopolamine and hyoscyamine methanolic solutions 100 µl of 4.820, 7.230, 9.640, 12.050 and 24.10 ng/µl for scopolamine and 2.40, 5.80, 10.60, 13.00 and 26.00 ng/µl for hyoscyamine with bamifylline as internal standard and methanol 1 ml for protein precipitation were added to blood sample 200 µl. After vortex mixing for 1 min and centrifugation at 3600 rpm for 20 min the supernatant was passed through C_{18} Bond Elut cartridges preconditioned with 3 ml of methanol and 3 ml of water. The cartridges were fitted in a vacuum system (Vac Elut) and washed with 3 ml of water and the tropane alkaloids were isolated by elution with 3 ml of methanol 0.01 M in HCl. These acidic methanolic solutions were evaporated to dryness on a water bath under a stream of nitrogen at 45 °C. The residues were redissolved in 100 µl of methanol and aliquots of 20 µl were injected onto the analytical column.

Urine Samples: For urine assay a 100 µl volume of scopolamine and hyoscyamine, stock methanolic solutions 2.41, 4.82, 7.23, 9.64 and 13.05 ng/µl, and 2.65, 5.30, 7.95, 10.60 and 13.25 ng/µl respectively and 200 µl methanol were added to 100 µl urine sample. After vortex mixing for 2 min and centrifugation at 3500 rpm for 15 min, the supernatants were treated by solid - phase liquid extration using C_{18} cartridges (Bond Elut). These phases were slowly forced through the cartridges which were previously conditioned, by passing 2 ml of methanol and washed with 2 ml of water. The cartridges were fitted in a vacuum system (Vac Elut) and the tropane alkaloids were eluted with 2.5 ml of methanol 0.01 M in HCl. These methanolic solutions were evaporated to dryness on a water bath under a nitrogen stream at 45 °C. The residues were redissolved in 100 µl of methanol and aliquots of 20 µl were injected into the chromatograph.

Egg Samples: 5.0 g of yolk or white of the egg were accurately weighed and quantitatively transferred with 7 ml of acetonitrile into a centrifuge tube, vortexed for 1 min and then centrifuged at 4000 rpm for 20 min. The upper liquid phase was transferred into another centrifuge tube and 7 ml of n-hexane was added. The tube was vortexed for 1 min and centrifuged at 4000 rpm for 20 min. The upper layer of n-hexane was wasted and 15 ml of dichloromethane were added. The mixture was vortexed for 1 min and centrifuged at 3000 rpm for 20 min. The dichloromethane layer was filtered through sodium sulphate and evaporated to dryness. The residue was reconstituted with 500 µl of methanol and analyzed.

RESULTS AND DISCUSSION

The retention times of scopolamine, hyoscyamine and bamifylline were found to be reproducible under the experimental conditions used. The average relative standard deviations of the retention times were found to be less than 0.3%. The mobile phase used enables a good column performance for long periods of time.

The area ratios of the chromatographic peaks for both compounds, scopolamine and hyoscyamine, to those of the internal standard bamifylline, were linearly related to the concentration of the tropane alkaloids. The linear regression equations and correlation coefficients were found to be:

STATISTICAL EVALUATION

Samples of Scopolamine	Regression Equation	Correlation Coefficient
Peak Area Ratio to Bamifylline	$Y = (-0.1842825 \pm 0.2465734) + (1.20 \cdot 10^{-2} \pm 1.16 \cdot 10^{-3})$ QL = 1.90 ppm, DL = 0.6025 ppm Linearity up to 40.0 ppm	0.99953
Peak Area	$Y = (-1099881 \pm 2368948.07) + (72064.52 \pm 11035.75)X$ QL = 3.36 ppm, DL = 0.6025 ppm Linearity up to 35.0 ppm	0.98564
Peak Area Ratio to Bamifylline after Solid Phase Extraction	$Y = (0.2205164 \pm 0.464810) \pm (1.11 \cdot 10^{-2} \pm 3.25 \cdot 10^{-3})X$ QL=3.31 ppm, DL=0.6025 ppm Linearity up to 15.0 ppm	0.99693
Samples of Hyoscyamine		
Peak Area Ratio to Bamifylline	$Y = (4.02 \cdot 10^{-2} \pm 0.1104) + (6.30 \cdot 10^{-3} \pm 4.43 \cdot 10^{-4})X$ QL = 1.91 ppm, Dl = 0.6250 Linearity up to 40.0 ppm	0.99753
Peak Area	$Y = (1176612 \pm 1326686.94) + (20606.17 \pm 5248.09)X$ QL = 5.37 ppm, DL = 0.6250 ppm Linearity up to 38.0 ppm	0.98358
Peak Area Ratio to Bamifylline after Solid Phase Extraction	$Y = (0.1525226 \pm 0.1885408) + (8.02 \cdot 10^{-3} \pm 1.20 \cdot 10^{-3})X$ QL = 1.89 ppm, DL = 0.6250 ppm Linearity up to 20.0 ppm	0.99165

Where Y = Peak area ratio of Scopolamine or Hyoscyamine to Bamifylline,
X = Concentration of Scolopamine or Hyoscyamine in ng/µl.

Solid phase liquid extraction of scolopamine and hyoscyamine was performed on C_{18} Bond Elut cartridges which were found to be the most suitable for this purpose. The recovery results of different Bond Elut cartridges are given in Table 4.

Experimental results for the determination of scopolamine and hyoscyamine using bamifylline as internal standard are laid out in Table 5.

Table 4. Percentage Recovery Data of Scopolamine and Hyoscyamine in the Presence of Bamifylline (IS) on Different Cartridges after Solid Phase Liquid Extraction

Cartridge	Scopolamine 8.0 ppm	Hyoscyamine 8.0 ppm	Bamifylline (IS) 2.0 ppm
Si	24.14	0	72.07
NH_2	39.74	54.76	75.71
PSA	70.56	93.30	83.50
2OH	92.72	25.84	82.75
PH (A)	45.44	47.77	61.00
(B)	24.69	18.71	90.42
C_8 (A)	50.97	66.07	43.10
(B)	34.52	37.40	86.81
C_{18} (A)	86.81	102.93	86.43
(B)	74.33	32.13	67.05

(A) = Elution with MeOH - HCl (0.01 M)

(B) = Elution with MeOH - CH_3CN - HCl (0.01 M)

Table 5. Experimental Results for the Determination of Scopolamine and Hyoscyamine in Methanolic Solutions using Bamifylline as Internal Standard by HPLC

Compound	Retention Time (min)	Added (ng)	Found[α] (ng)
Scopolamine	3.990	96.40	97.03 ± 4.74
		144.60	145.02 ± 6.34
		192.80	190.53 ± 5.43
		241.0	223.34 ± 4.76
Hyoscyamine	6.934	106.0	107.09 ± 4.07
		159.0	159.60 ± 4.96
		212.0	209.80 ± 4.93
		265.0	264.60 ± 5.82

[α] Average Value of Eight Determinations ± Standard Deviation

The intra - day precision and accuracy of the method were assessed by the repeated analyses of methanolic solutions in the presence of the internal standard bamifylline. The relative standard deviation is under 5% for twelve replicate determinations of four different samples of scolopamine and hyoscyamine. The results taken are given in Table 6.

The between - day precision and accuracy of the method were assessed by the repeated analyses of methanolic solutions in the presence of the internal standard over twenty days. The concentrations of scopolamine and hyoscyamine ranged from 4.82 ng/μl to 12.05 ng/μl and 5.30 ng/μl to 13.25 ng/μl respectively. Twelve replicate samples at each of the four concentrations were used in the assessment of the between - day variability. The results are presented in Table 7.

The mean percentage recovery of scopolamine and hyoscyamine was measured by comparing the peak areas obtained from the injection of known

Table 6. Intra - Day Precision and Accuracy of Scopolamine and Hyoscyamine Assays in the Presence of Bamifylline as Internal Standard (n = 12)

Quantity Injected (ng)	Mean ± SD (ng)	RSD (%)
Scopolamine		
96.40	97.03 ± 3.98	4.10
144.60	145.02 ± 5.67	3.91
192.80	190.53 ± 9.52	4.99
241.0	223.34 ± 9.09	4.07
Hyoscyamine		
106.0	107.10 ± 4.32	4.03
159.0	159.62 ± 5.16	3.23
212.0	209.78 ± 5.08	2.42
265.0	264.65 ± 5.96	2.25

Table 7. Between - Day Precision and Accuracy of Scopolamine and Hyoscyamine Assays in the Presence of Bamifylline as Internal Standard (n = 12)

Quantity Injected (ng)	Mean ± SD (ng)	RSD (%)
Scopolamine		
96.40	98.40 ± 6.53	6.64
144.60	138.10 ± 6.40	4.63
192.80	182.10 ± 16.40	9.01
241.0	222.10 ± 19.70	8.88
Hyoscyamine		
106.0	108.40 ± 4.35	4.01
159.0	162.30 ± 4.90	3.02
212.0	207.20 ± 6.53	3.15
265.0	257.70 ± 12.11	4.70

Table 8. Mean Recovery Data for Scopolamine and Hyoscyamine Assay in Seed Samples (n=6)

Seed Samples Scopolamine Quantity (ng)	Found ± SD (ng)	Recovery (%)
72.3	70.14 ± 2.3	97.0
144.6	142.24 ± 3.7	98.4
192.8	193.17 ± 6.4	100.2
Seed Samples Hyoscyamine Quantity (ng)		
53.0	50.27 ± 3.1	94.8
106.0	101.84 ± 4.6	96.0
159.0	152.13 ± 6.8	95.7

quantities of the pure tropane alkaloids, with those obtained from the direct injection of extracted feedstuffs, blood serum, urine samples and eggs spiked with three different concentrations of scopolamine and hyoscyamine.

The mean percentage recovery of scopolamine and hyoscyamine assay in seed samples is 98.5% and 95.5% respectively. These results are given in Table 8.

The mean percentage recovery of scopolamine and hyoscyamine, in blood serum and urine, at various concentrations averaged 90.2%, 96.5% and 91.7%, 98.3% respectively. There results are given in Tables 9 and 10.

The peaks on the chromatogram are identified by their retention time. Quantitation was done by comparison of the peak area ratio of scopolamine and hyoscyamine to bamifylline in the unknown samples with those of the standards containing known quantities of scopolamine and hyoscyamine, extracted by solid phase liquid extraction and chromatographed in exactly the same way. Peak area ratios were verified to be linearly related to the concentrations of scopolamine and

Table 9. Mean Recovery Data for Scopolamine and Hyoscyamine Assay in Blood Serum Samples (n = 6)

Plasma Scopolamine Quantity (ng)	Found ± SD (ng)	Recovery (%)
96.40	88.68 ± 2.6	91.9
192.80	176.89 ± 5.2	91.7
482.0	419.96 ± 18.9	87.1
Plasma Hyoscyamine Quantity (ng)		
48.0	44.85 ± 3.2	93.4
212.0	203.12 ± 6.8	95.8
530.0	533.02 ± 26.5	100.5

Mean Recovery = $x \pm (t.SD/\sqrt{n})$ / amount added x 100 where x = mean value for n = 6 determinations at 95% confidence level.

Table 10. Mean Recovery Data for Scopolamine and Hyoscyamine Assay in Urine Samples (n = 6)

Urine Scopolamine Quantity (ng)	Found ± SD (ng)	Recovery (%)
48.20	46.40 ± 4.6	96.2
96.40	84.21 ± 0.9	87.4
144.60	132.62 ± 8.4	91.7
Urine Hyoscyamine Quantity (ng)		
53.0	56.41 ± 2.2	106.4
106.0	93.70 ± 7.8	88.4
212.0	212.14 ± 11.4	100.1

hyoscyamine within the ranges investigated 1.205 - 15.0 ng/μl and 1.25 - 20.0 ng /μl respectively.

According to the analytical procedure described above the detection limit of scopolamine and hyoscyamine, taken as a signal - to - baseline noise ratios of two at a sensitivity setting of 0.002 AUFS, were estimated to be 0.6025 ppm and 0.6250 ppm respectively.

The mean percentage recovery of scopolamine and hyoscyamine assay in the white part of the eggs is 97.75% and 90.80% respectively. These results are given in Table 11.

The chromatographic profile of tropane alkaloids in the presence of the internal standard bamifylline after solid phase liquid extraction is given in Figure 2.

The developed method was applied to the analysis of tropane alkaloids in biological fluids, blood serum and urine. Employing the standard addition technique it is possible to determine scopolamine and hyoscyamine in 200 μl of blood serum

Table 11. Mean Recovery Data for Scopolamine and Hyoscyamine Assay in the White Part of the Eggs (n=6)

White Egg Scopolamine Quantity (ng)	Found ± SD (ng)	Recovery (%)
17.85	19.24 ± 2.4	107.7
44.63	47.33 ± 4.6	106.0
89.26	80.98 ± 6.3	90.7
178.52	154.73 ± 7.4	86.6
White Egg Hyoscyamine Quantity (ng)		
100.0	87.24 ± 3.9	87.2
150.0	139.76 ± 8.6	93.1
200.0	184.32 ± 7.8	92.1

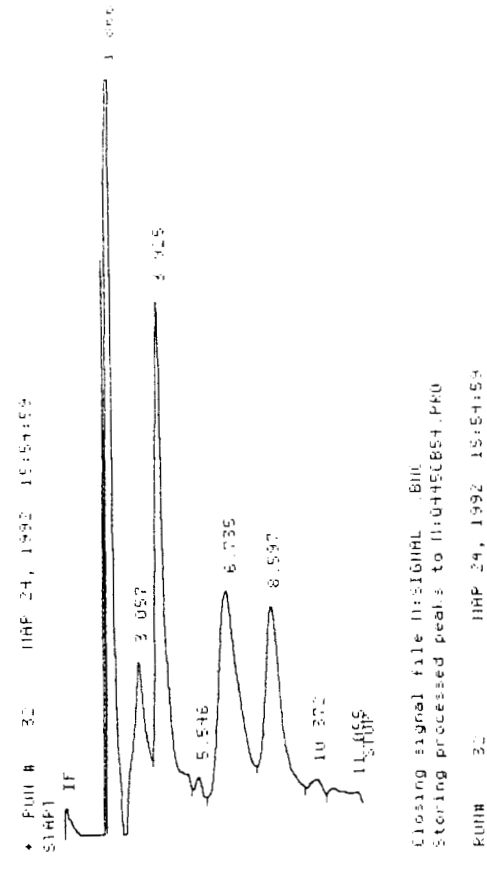

Figure 2. High - Performance Liquid Chromatographic profile of Scopolamine (3.925) and Hyoscyamine (6.735) in the presence of Bamifylline (8.597) after solid phase liquid extraction.
Scopolamine [12.05 ppm], Hyoscyamine [13.25 ppm] and Bamifylline [2.0ppm]

and 100 μl of urine samples in under ten minutes time. In both cases scopolamine and hyoscyamine were successfuly determined, employing the procedures described using bamifylline as internal standard. The chromatograms are given in Figure 3.

The developed method was applied to the analysis of the tropane alkaloids in seed samples. The results taken are given in Table 12.

The proposed procedure was applied to the determination of scopolamine and hyoscyamine in pig blood serum samples. The results taken are given in Table 13.

The method was also applied to the analysis of scopolamine and hyoscyamine in pig urine samples. The results obtained are illustrated in Table 14.

The recovery of scopolamine and hyoscyamine from faeces samples and liver tissues is not high and reproducible. This is due to the chemical bonds between the alkaloids and the endogenous compounds, mainly proteins, of the faeces samples. Hydrolysis using acids or bases did not solve the problem. To this purpose we continue our research trying different extraction procedures, liquid-liquid or solid phase liquid extraction.

Table 12. Experimental Results for the Determination of Scopolamine and Hyoscyamine in Seed Samples by Reversed - Phase HPLC

Samples	Found$^{\alpha}$ (ppm)	
	Scopolamine	Hyoscyamine
White Bean	ND	21.110
Datura Ferox (I)	22.490	2.310
Lin Seed	ND	7.940
Sunflower Meal	ND	3.610
Datura Ferox (II)	17.010	1.670
Diet (Corn, Soya, Meat Meal)	ND	3.400

α = Mean Value of Five Determinations, ND = Not Detected

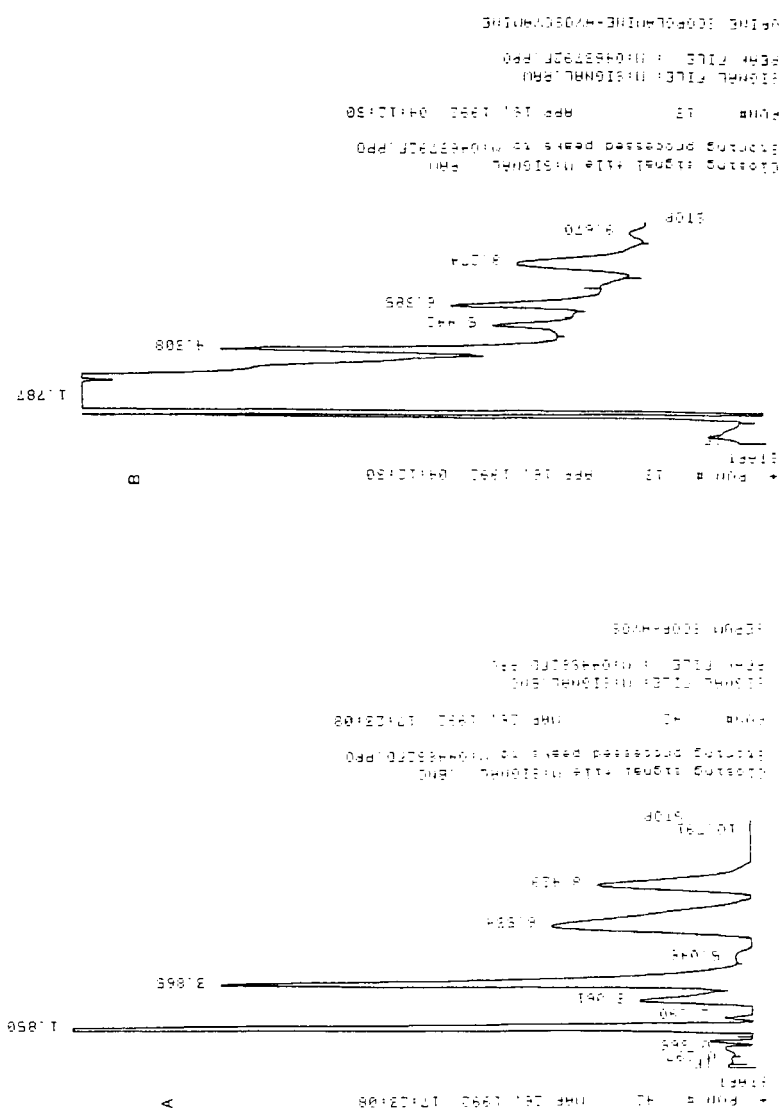

Figure 3. High - Performance Liquid Chromatograms of Scopolamine and Hyoscyamine extracted by solid phase cartridges from Blood Serum (A) and Urine (B) samples in the presence of Bamifylline as internal standard. Chromatographic conditions as described in text.

Table 13. Experimental Results for the Determination of Scopolamine and Hyoscyamine in Pig Blood Serum Samples by Reversed - Phase HPLC, in the Presence of Bamifylline as Internal Standard.

Sample	Found $^{\alpha}$ (ppm)	
	Scopolamine	Hyoscyamine
P_0	ND	ND
P_1	0.640	ND
P_2	ND	0.530
P_3	0.280	0.970
P_4	0.140	0.480
P_5	ND	0.100
P_6	Traces	0.700
P_7	0.510	0.440
P_8	Traces	0.360
P_9	Traces	ND
P_{10}	ND	0.440
P_{11}	0.870	2.990

α = Mean value of five determinations, ND = Not Detected

CONCLUSION

The present simple and rapid reversed - phase HPLC assay provides a reliable and reproducible method for the identification and simultaneous determination of scopolamine and hyoscyamine in the presence of bamifylline as internal standard. The technique is applicable to the determination of the tropane alkaloids in feedstuffs: soya and corn and biological samples: eggs, blood serum and urine. Because of its high specificity, accurary, precision and save of time the described solid phase extraction and simultaneous reversed phase HPLC procedure appears to be very useful for the routine analyses of those tropane alkaloids.

Additionaly the fact that no special HPLC equipment is required - an isocratic pump and a UV detector, common in all analytical laboratories suffices for the determination - render the technique to be widely applicable.

Table 14. Experimental Results for the Determination of Scopolamine and Hyoscyamine in Pig Urine Samples by Reversed - Phase HPLC after Solid Phase Extraction.

Sample	Found[α] (ppm)	
	Scopolamine	Hyoscyamine
U_0	4.787	10.225
U_1	2.280	5.099
U_2	3.074	6.552
U_3	1.735	1.762
U_4	1.317	1.077
U_5	1.364	1.195
U_6	1.447	5.811
U_7	4.136	13.844
U_8	1.515	2.338
U_9	1.451	1.412
U_{10}	1.578	0.959
U_{11}	ND	0.170
U_{12}	ND	0.178
U_{13}	2.179	1.566

α = Mean value of five determinations, ND = Not Detected

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