A simple method for the separation and detection of trace levels of buprofezin, flubendiamide and imidacloprid by NP-HPTLC and RP-HPTLC

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A study was undertaken to evaluate the retention (R_F and R_M) and separation (ΔR_F, R_F^{α}, α and R_S) of buprofezin (B), flubendiamide (F) and imidacloprid (I) using *n*-hexane-acetone (6.5 : 3.5 v/v) in the case of NP-HPTLC and methanol-water (8 : 2 v/v) for RP-HPTLC as mobile phase. The study revealed that increasing the acetone content in NP-HPTLC and decreasing the water content in RP-HPTLC resulted in high resolution with increase in R_F values for B, F and I. $\Delta R_F > 0.04$ and $R_S > 1.5$ were achieved for all pairs of compounds ($\Delta R_{F(B–F)} = 0.35$, $\Delta R_{F(F–I)} = 0.19$, $\Delta R_{F(B–I)} = 0.54$, $R_{S(B–F)} = 4.12$, $R_{S(F–I)} = 7.34$, $R_{S(B–I)} = 2.02$ using NP-HPTLC; $\Delta R_{F(F–B)} = 0.23$, $\Delta R_{F(I–F)} = 0.26$, $\Delta R_{F(I–B)} = 0.49$, $R_{S(F–B)} = 2.63$, $R_{S(I–F)} = 2.97$, $R_{S(I–B)} = 5.92$ using RP-HPTLC). Imidacloprid was adsorbed strongly on NP-HPTLC layer and buprofezin on RP-HPTLC layer, as indicated by their high R_M values. The maximum absorption of UV for B, F and I was found to be 252, 242 and 276 nm respectively. Stability analysis indicated that these compounds were stable up to 6 h in methanol and on the plates (NP-HPTLC and RP-HPTLC layers). This protocol is useful for toxicologists to detect a mixture of these insecticides in forensic as well as environmental samples.

Keywords: Detection and separation, human toxicity, insecticides, thin-layer chromatography.

THE widespread use of synthetic insecticides, fungicides and herbicides has increased drastically to improve modern agricultural productivity. Buprofezin (B), flubendiamide (F) and imidacloprid (I) are recently introduced acute insecticides which are known for their high potency and claimed to have low mammalian toxicity and favourable persistence; however they have been released in the market without appropriate data on direct human toxicity[1–3].

Buprofezin (2-tert-butylimino-3-isopropyl-5-phenyl-perhydro-l,3,5-thiadiazin-4-one) is a thiadiazine insect regulator with larvicidal action, inhibiting chitin biosynthesis and also affecting the hormone levels of nymphs[4]. This insecticide is most commonly used against pest homopterans, coleopterans and mites in many crops such as citrus fruits and greenhouse plantations of different vegetables worldwide[5–7]. Flubendiamide (N^2-[1,1-dimethyl-2-methyl sulphonyl ethyl]-3-iodo-N^1-2-methyl-4-{1,2,2,2-tetrafluro-1-(trifluromethyl)ethyl}phenyl), a pthalic acid diamide insecticide belonging to benzene dicarboxamide group of insecticides, acts on the insect ryanodine receptor, including calcium release and leading to uncoordinated muscular contraction[8]. It is effective against a broad spectrum of lepidopteran pests, including those resistant to other classes of insecticides[9–13]. Imidacloprid [1-(6-chloro-3-pyridylmethyl)-*N*-nitroimida-zolidin-2-ylideneamine], a neonicotinoid insecticide acts on the nicotinyl acetylcholine receptors in the nervous system. It has high potency systemic action against homopteran pests and some species of the order Coleoptera, Diptera and Lepidoptera[14–18]. In agriculture, it is used for seed-dressing or directly applied to the soil or foliage to control insect pests of corn, cotton, potato, rice, vegetables and fruits[17,19].

In general, all the three insecticides are commonly used for pest control in vegetables, fruits and cereals, which are most essential for daily living. Soon after spraying any pesticide crops need to go through some waiting period before harvest, which varies for different insecticides and crops. Once the waiting period has lapsed, the food products are safe for consumption. If the vegetables, fruits or cereals are harvested before lapse of the waiting period, they are likely to contain higher levels of insecticide residues, which are hazardous to health. In addition, excessive use of insecticides can contaminate the soil, water and vegetation. Despite restrictions and regulations on insecticide use, India accounts for one-third of pesticide poisoning cases in the world[20]. Hence, studies on development and validation of different analytical methods for B, F and I are of primary interest to toxicologists and to forensic analysts.

Various analytical techniques have been used for the detection of buprofezin, viz. gas chromatography-nitrogen phosphorous detector (GC-NPD), gas chromatography-flame photometric detector (GC-FPD)[6], gas

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chromatography-mass spectrometry (GC-MS)[21,22], high-performance liquid chromatography (HPLC)[9], liquid chromatography-mass spectrometry (LC-MS)[23]; flubendiamide, viz. high-performance liquid chromatography ultraviolet (HPLC-UV)[9,10], high-performance liquid chromatography photodiode array detector (HPLC-PDA)[24], high-performance liquid chromatography-electrospray ionisation-tandem mass spectrometry (HPLC-ESI-MS/MS)[13], high-performance thin-layer liquid chromatography (HPTLC)[25], and LC/MS[25], and imidacloprid, viz. by thin-layer chromatography (TLC)[26], HPLC[27] and GC-MS[28]. The LC/MS instruments are more expensive, whereas HPLC methods are time-consuming and need large volumes of solvents, whose decantation in the open environment is also a concern of bio-safety. Presently, the multi-residue methods are commonly used to determine insecticide levels in food products. For the detection of imidacloprid, GC method is used which is a thermo labile and low volatile, two-step process to convert imidacloprid to imidacloprid–urea, a more volatile form[29]. Among various chromatographic methods, HPTLC is a comparatively simple, rapid and convenient method for identifying drugs, pesticides and other xenobiotics in various samples such as pharmaceutical formulations, pesticide formulations and forensic samples[2,30–34]. An analytical method for the determination of insecticide residues in any complex matrix such as the soil, water bodies, cereals, vegetables and forensic samples requires a simple separation method. Among several chromatographic methods reported for the separation and detection of traces of B, F and I, high-performance HPTLC is found to be a comparatively simple, rapid and convenient method. Organophosphorus insecticides, fungicides[29,35] and drugs[36] from forensic samples have been separated using HPTLC methods. Separation of flubendiamide from soluble concentrate formulations by HPTLC methods has already been reported[33]. However, there are no or limited studies on the simultaneous separation and detection methods of these three insecticides by TLC, HPTLC and/or any other sensitive methods. In order to separate and detect these insecticides from complex matrices such as forensic and environmental samples, a simple, rapid and cost-effective HPTLC method was optimized.

Materials and methods

Chemicals

Buprofezin, flubendiamide and imidacloprid were purchased from Sigma-Aldrich (>99% purity). All other chemicals and solvents were of analytical grade. Standard stock solutions (1 mg ml^{-1}) were prepared by dissolving reference standards in methanol and stored at 4°C. Working standard solutions were freshly prepared on the day of analysis by diluting standard stock solutions to the required concentrations in methanol.

Instrumentation

Normal phase HPTLC (NP-HPTLC) was performed on 10 cm × 10 cm silica gel 60 F_{254} and reversed phase-HPTLC (RP-HPTLC) on 10 cm × 10 cm silica gel 60 RP-18 WF_{254} aluminium plates (Merck, Germany). The plates were prewashed with methanol and activated at 120°C for 30 min. Standard solutions were applied to the plates as 6 mm bands, 14 mm apart, 22 mm from the edges and 10 mm from the bottom of the plates using CAMAG (Mutttenz, Switzerland) Linomat 5 automatic sample applicator equipped with a 100 µl syringe and N_2 flow. The injection volume was 1 µl and sample delivery speed was 100 nl s^{-1}. The plates were developed in a CAMAG glass twin-trough chamber previously saturated with mobile-phase vapour. The development distance was 8 cm from the lower edge of the plate. Different ratios (10 : 0, 9.5 : 0.5, 9 : 1, 8.5 : 1.5, 8 : 2, 7.5 : 2.5, 7 : 3, 6.5 : 3.5, 6 : 4, 5.5 : 4.5 and 5 : 5 v/v) of *n*-hexane–acetone and methanol–water were used as mobile phase for NP-HPTLC and RP-HPTLC respectively[37].

Chromatography

Chromatograms were obtained in triplicate. Retardation factor (R_F), R_M, separation factor (ΔR_F), selectivity (α), pair separation constant (R_F^{α}) and peak resolution (R_S) were calculated for all densitograms using equations reported in the literature[37].

In brief, separation factor (ΔR_F), pairs of separation constant (ΔR_F^{α}), and selectivity (α) were calculated using the eqs (1)–(3) respectively[37].

$$\Delta R_{F(1,2)} = R_{F1} - R_{F2} \text{ (where } R_{F1} > R_{F2}), \quad (1)$$

$$\Delta R^{\alpha}_{F(1,2)} = R_{F1}/R_{F2} \text{ (where } R_{F1} > R_{F2}), \quad (2)$$

$$\alpha = (1/R_{F1} - 1)/(1/R_{F1} - 2) \text{ (where } R_{F1} < R_{F2}). \quad (3)$$

R_{F1} and R_{F2} are the R_F values of two adjacent peaks on the densitograms in all cases.

However, because $k = (1 - R_F)/R_F$ and $\log k = R_M$, the retention factor $(R_M) = \log(1 - R_F)/R_F$.

Peak resolution ($R_{s(b)}$) and ($R_{s(h)}$) and average peak resolution ($R_{s(a)}$) were calculated using the following equations:

$$R_{s(b)} = 2d/(W_{b1} + W_{b2}), \quad (4)$$

$$R_{s(h)} = d\sqrt{\ln 4}/(W_{h1} + W_{h2}), \quad (5)$$

$$R_{s(a)} = (R_{s(b)} - R_{s(h)})/2. \quad (6)$$

where W_{b1} and W_{b2} are the peak widths at baseline, W_{h1} and W_{h2} the peak widths at half height and d is the distance between the centres of two adjacent peaks on the densitogram.

Table 1. Retention and separation data obtained for buprofezin (B), flubendiamide (F) and imidacloprid (I) in NP-HPTLC using different concentrations of *n*-hexane (H)–acetone (A) as mobile phase (v/v)

	Retention data				Separation data			
H–A	R_F	R_M	ΔR_F	R_F^α	α	$R_{S(b)}$	$R_{S(h)}$	$R_{S(a)}$
10 : 00								
B	0.01	1.99	$0.00_{(B–F)}$	$1.00_{(B–F)}$	$1.00_{(F–B)}$	$0.00_{(B–F)}$	$0.00_{(B–F)}$	$0.00_{(B–F)}$
F	0.01	1.99	$0.00_{(F–I)}$	$1.00_{(F–I)}$	$1.00_{(I–F)}$	$0.00_{(F–I)}$	$0.00_{(F–I)}$	$0.00_{(F–I)}$
I	0.01	1.99	$0.00_{(B–I)}$	$1.00_{(B–I)}$	$1.00_{(I–B)}$	$0.00_{(B–I)}$	$0.00_{(B–I)}$	$0.00_{(B–I)}$
9.5 : 0.5								
B	0.29	0.39	$0.28_{(B–F)}$	$29.00_{(B–F)}$	$40.43_{(F–B)}$	$4.67_{(B–F)}$	$5.50_{(B–F)}$	$5.08_{(B–F)}$
F	0.01	1.99	$0.00_{(F–I)}$	$01.00_{(F–I)}$	$1.00_{(I–F)}$	$0.00_{(F–I)}$	$0.00_{(F–I)}$	$0.00_{(F–I)}$
I	0.01	1.99	$0.28_{(B–I)}$	$29.00_{(B–I)}$	$40.43_{(I–B)}$	$4.67_{(B–I)}$	$7.32_{(B–I)}$	$5.99_{(B–I)}$
9 : 1								
B	0.44	0.10	$0.42_{(B–F)}$	$22.00_{(B–F)}$	$38.50_{(F–B)}$	$7.00_{(B–F)}$	$8.24_{(B–F)}$	$7.62_{(B–F)}$
F	0.02	1.69	$0.01_{(F–I)}$	$02.00_{(F–I)}$	$2.02_{(I–F)}$	$0.33_{(F–I)}$	$0.40_{(F–I)}$	$0.36_{(F–I)}$
I	0.01	1.99	$0.43_{(B–I)}$	$44.00_{(B–I)}$	$77.78_{(I–B)}$	$7.16_{(B–I)}$	$8.43_{(B–I)}$	$7.79_{(B–I)}$
8.5 : 1.5								
B	0.54	–0.07	$0.50_{(B–F)}$	$13.50_{(B–F)}$	$28.17_{(F–B)}$	$6.67_{(B–F)}$	$7.84_{(B–F)}$	$7.25_{(B–F)}$
F	0.04	1.38	$0.03_{(F–I)}$	$04.00_{(F–I)}$	$4.12_{(I–F)}$	$11.15_{(F–I)}$	$13.27_{(F–I)}$	$12.21_{(F–I)}$
I	0.01	1.99	$0.53_{(B–I)}$	$54.00_{(B–I)}$	$116.22_{(I–B)}$	$0.41_{(B–I)}$	$0.50_{(B–I)}$	$0.45_{(B–I)}$
8 : 2								
B	0.60	–0.17	$0.48_{(B–F)}$	$5.00_{(B–F)}$	$11.00_{(F–B)}$	$4.92_{(B–F)}$	$5.82_{(B–F)}$	$5.37_{(B–F)}$
F	0.12	0.86	$0.08_{(F–I)}$	$03.00_{(F–I)}$	$3.27_{(I–F)}$	$9.33_{(F–I)}$	$11.17_{(F–I)}$	$10.25_{(F–I)}$
I	0.04	1.38	$0.56_{(B–I)}$	$15.00_{(B–I)}$	$36.00_{(I–B)}$	$0.86_{(B–I)}$	$1.60_{(B–I)}$	$1.23_{(B–I)}$
7.5 : 2.5								
B	0.63	–0.23	$0.44_{(B–F)}$	$3.31_{(B–F)}$	$7.25_{(F–B)}$	$4.00_{(B–F)}$	$4.70_{(B–F)}$	$4.37_{(B–F)}$
F	0.19	0.62	$0.12_{(F–I)}$	$2.71_{(F–I)}$	$3.11_{(I–F)}$	$7.46_{(F–I)}$	$8.80_{(F–I)}$	$8.13_{(F–I)}$
I	0.07	1.12	$0.56_{(B–I)}$	$9.00_{(B–I)}$	$22.62_{(I–B)}$	$1.14_{(B–I)}$	$0.33_{(B–I)}$	$0.73_{(B–I)}$
7 : 3								
B	0.69	–0.34	$0.39_{(B–F)}$	$2.30_{(B–F)}$	$5.19_{(F–B)}$	$4.21_{(B–F)}$	$4.86_{(B–F)}$	$4.54_{(B–F)}$
F	0.30	0.36	$0.17_{(F–I)}$	$2.30_{(F–I)}$	$2.86_{(I–F)}$	$5.60_{(F–I)}$	$8.30_{(F–I)}$	$6.95_{(F–I)}$
I	0.13	0.82	$0.56_{(B–I)}$	$5.30_{(B–I)}$	$14.89_{(I–B)}$	$1.70_{(B–I)}$	$2.02_{(B–I)}$	$1.86_{(B–I)}$
6.5 : 3.5								
B	0.75	–0.47	$0.35_{(B–F)}$	$1.88_{(B–F)}$	$4.50_{(F–B)}$	$3.78_{(B–F)}$	$4.47_{(B–F)}$	$4.12_{(B–F)}$
F	0.40	0.17	$0.19_{(F–I)}$	$1.90_{(F–I)}$	$2.50_{(I–F)}$	$6.75_{(F–I)}$	$7.94_{(F–I)}$	$7.34_{(F–I)}$
I	0.21	0.57	$0.54_{(B–I)}$	$3.57_{(B–I)}$	$11.28_{(I–B)}$	$1.85_{(B–I)}$	$2.20_{(B–I)}$	$2.02_{(B–I)}$
6 : 4								
B	0.81	–0.62	$0.30_{(B–F)}$	$1.59_{(B–F)}$	$4.10_{(F–B)}$	$3.00_{(B–F)}$	$3.54_{(B–F)}$	$3.27_{(B–F)}$
F	0.51	–0.01	$0.21_{(F–I)}$	$1.70_{(F–I)}$	$2.42_{(I–F)}$	$6.80_{(F–I)}$	$8.06_{(F–I)}$	$7.43_{(F–I)}$
I	0.30	0.36	$0.51_{(B–I)}$	$2.70_{(B–I)}$	$9.95_{(I–B)}$	$1.90_{(B–I)}$	$2.26_{(B–I)}$	$2.08_{(B–I)}$
5.5 : 4.5								
B	0.84	–0.72	$0.21_{(B–F)}$	$1.33_{(B–F)}$	$3.08_{(F–B)}$	$2.27_{(B–F)}$	$2.67_{(B–F)}$	$2.47_{(B–F)}$
F	0.63	–0.23	$0.24_{(F–I)}$	$1.61_{(F–I)}$	$2.66_{(I–F)}$	$5.14_{(F–I)}$	$6.05_{(F–I)}$	$5.59_{(F–I)}$
I	0.39	0.19	$0.45_{(B–I)}$	$2.15_{(B–I)}$	$8.21_{(I–B)}$	$2.08_{(B–I)}$	$2.45_{(B–I)}$	$2.26_{(B–I)}$
5 : 5								
B	0.88	–0.86	$0.15_{(B–F)}$	$1.20_{(B–F)}$	$2.71_{(F–B)}$	$1.36_{(B–F)}$	$1.60_{(B–F)}$	$1.48_{(B–F)}$
F	0.73	–0.431	$0.24_{(F–I)}$	$1.49_{(F–I)}$	$2.81_{(I–F)}$	$4.33_{(F–I)}$	$5.10_{(F–I)}$	$4.71_{(F–I)}$
I	0.49	0.01	$0.39_{(B–I)}$	$1.80_{(B–I)}$	$7.63_{(I–B)}$	$1.84_{(B–I)}$	$2.17_{(B–I)}$	$2.00_{(B–I)}$

R_F, retardation factor; ΔR_F, separation factors; R_F^α, pair separation constant; α, selectivity and R_S, peak resolution. Retention and separation data mentioned are mean values of three determinations. $R_{S(b)}$, peak width at baseline; $R_{S(h)}$, peak width at half height; and $R_{S(a)}$, average of peak resolution.

In situ detection

In situ densitometric scanning was performed with a CAMAG TLC Scanner 3 equipped with winCATS 1.4.2 software, in absorbance mode at 254 nm using the deuterium light source. The slit dimension was 6 mm × 0.45 mm, scanning speed 20 mm s^{-1}, data resolution 100 μm per step and the optical filter was second order[29].

Stability analysis

Stability of B, F and I in the solution (methanol) was evaluated by maintaining the freshly prepared insecticide samples at room temperature as well as at 4°C for 0, 3 and 6 h. Similarly, stability was accessed on the plates by incubating them for 0, 3 and 6 h (ref. 37).

Table 2. Retention and separation data obtained for B, F and I in RP-HPTLC using different concentrations of methanol (M)–water (W) as mobile phase (v/v)

	Retention data				Separation data			
M–W	R_F	R_M	ΔR_F	R_F^{α}	α	$R_{S(b)}$	$R_{S(h)}$	$R_{S(a)}$
10 : 00								
B	0.68	–0.33	$0.13_{(F–B)}$	$1.19_{(F–B)}$	$2.00_{(B–F)}$	$1.13_{(F–B)}$	$1.33_{(F–B)}$	$1.23_{(F–B)}$
F	0.81	–0.63	$0.02_{(I–F)}$	$1.02_{(I–F)}$	$1.13_{(F–I)}$	$0.14_{(I–F)}$	$0.17_{(I–F)}$	$0.15_{(I–F)}$
I	0.79	–0.58	$0.11_{(I–B)}$	$1.16_{(I–B)}$	$1.76_{(B–I)}$	$0.95_{(I–B)}$	$1.07_{(I–B)}$	$1.01_{(I–B)}$
9.5 : 0.5								
B	0.56	–0.10	$0.18_{(F–B)}$	$1.32_{(F–B)}$	$2.23_{(B–F)}$	$1.44_{(F–B)}$	$1.70_{(F–B)}$	$1.57_{(F–B)}$
F	0.74	–0.45	$0.04_{(I–F)}$	$1.05_{(I–F)}$	$1.24_{(B–I)}$	$0.27_{(I–F)}$	$0.32_{(I–F)}$	$0.29_{(I–F)}$
I	0.78	–0.58	$0.22_{(I–B)}$	$1.39_{(I–B)}$	$2.78_{(B–I)}$	$1.83_{(I–B)}$	$2.15_{(I–B)}$	$1.99_{(I–B)}$
9 : 1								
B	0.42	0.14	$0.22_{(F–B)}$	$1.57_{(F–B)}$	$2.68_{(B–F)}$	$2.82_{(F–B)}$	$3.32_{(F–B)}$	$3.07_{(F–B)}$
F	0.66	–0.29	$0.10_{(I–F)}$	$1.16_{(I–F)}$	$1.72_{(B–I)}$	$1.22_{(I–F)}$	$1.43_{(I–F)}$	$1.32_{(I–F)}$
I	0.77	–0.52	$0.32_{(I–B)}$	$1.83_{(I–B)}$	$4.62_{(B–I)}$	$0.37_{(I–B)}$	$4.34_{(I–B)}$	$2.35_{(I–B)}$
8.5 : 1.5								
B	0.29	0.39	$0.24_{(F–B)}$	$1.82_{(F–B)}$	$2.76_{(B–F)}$	$2.66_{(F–B)}$	$3.14_{(F–B)}$	$2.90_{(F–B)}$
F	0.53	–0.05	$0.18_{(I–F)}$	$1.34_{(I–F)}$	$2.17_{(B–I)}$	$1.90_{(I–F)}$	$2.23_{(I–F)}$	$2.06_{(I–F)}$
I	0.71	–0.39	$0.42_{(I–B)}$	$2.45_{(I–B)}$	$5.99_{(B–I)}$	$4.42_{(I–B)}$	$5.20_{(I–B)}$	$4.81_{(I–B)}$
8 : 2								
B	0.18	0.66	$0.23_{(F–B)}$	$2.28_{(F–B)}$	$3.16_{(B–F)}$	$2.42_{(F–B)}$	$2.85_{(F–B)}$	$2.63_{(F–B)}$
F	0.41	0.16	$0.26_{(I–F)}$	$1.63_{(I–F)}$	$2.92_{(B–I)}$	$2.73_{(I–F)}$	$3.22_{(I–F)}$	$2.97_{(I–F)}$
I	0.67	–0.30	$0.49_{(I–B)}$	$3.72_{(I–B)}$	$9.24_{(B–I)}$	$5.44_{(I–B)}$	$6.41_{(I–B)}$	$5.92_{(I–B)}$
7.5 : 2.5								
B	0.11	0.90	$0.16_{(F–B)}$	$2.45_{(F–B)}$	$2.99_{(B–F)}$	$2.13_{(F–B)}$	$2.51_{(F–B)}$	$2.32_{(F–B)}$
F	0.27	0.43	$0.37_{(I–F)}$	$2.37_{(I–F)}$	$4.80_{(B–I)}$	$4.35_{(I–F)}$	$5.12_{(I–F)}$	$4.73_{(I–F)}$
I	0.64	0.25	$0.53_{(I–B)}$	$5.82_{(I–B)}$	$14.38_{(B–I)}$	$6.62_{(I–B)}$	$7.80_{(I–B)}$	$7.21_{(I–B)}$
7 : 3								
B	0.05	1.28	$0.10_{(F–B)}$	$3.00_{(F–B)}$	$3.35_{(B–F)}$	$1.42_{(F–B)}$	$1.68_{(F–B)}$	$1.55_{(F–B)}$
F	0.15	0.75	$0.42_{(I–F)}$	$3.80_{(I–F)}$	$7.51_{(B–I)}$	$5.25_{(I–F)}$	$6.18_{(I–F)}$	$5.71_{(I–F)}$
I	0.57	–0.12	$0.52_{(I–B)}$	$11.40_{(I–B)}$	$25.18_{(B–I)}$	$6.50_{(I–B)}$	$7.65_{(I–B)}$	$7.07_{(I–B)}$
6.5 : 3.5								
B	0.03	1.51	$0.06_{(F–B)}$	$2.45_{(F–B)}$	$3.19_{(B–F)}$	$0.92_{(F–B)}$	$1.08_{(F–B)}$	$1.00_{(F–B)}$
F	0.09	1.00	$0.45_{(I–F)}$	$2.37_{(I–F)}$	$11.86_{(B–I)}$	$5.62_{(I–F)}$	$6.62_{(I–F)}$	$6.12_{(I–F)}$
I	0.54	–0.07	$0.51_{(I–B)}$	$5.81_{(I–B)}$	$37.95_{(B–I)}$	$6.80_{(I–B)}$	$8.01_{(I–B)}$	$7.40_{(I–B)}$
6 : 4								
B	0.02	1.70	$0.03_{(F–B)}$	$3.00_{(F–B)}$	$2.57_{(B–F)}$	$0.50_{(F–B)}$	$0.59_{(F–B)}$	$0.32_{(F–B)}$
F	0.05	1.28	$0.44_{(I–F)}$	$6.00_{(I–F)}$	$18.25_{(B–I)}$	$1.05_{(I–F)}$	$6.10_{(I–F)}$	$3.57_{(I–F)}$
I	0.49	0.01	$0.47_{(I–B)}$	$18.00_{(I–B)}$	$47.07_{(B–I)}$	$0.40_{(I–B)}$	$7.38_{(I–B)}$	$3.89_{(I–B)}$
5.5 : 4.5								
B	0.01	2.00	$0.01_{(F–B)}$	$2.00_{(F–B)}$	$2.02_{(B–F)}$	$0.25_{(F–B)}$	$0.30_{(F–B)}$	$0.27_{(F–B)}$
F	0.02	1.70	$0.40_{(I–F)}$	$21.00_{(I–F)}$	$35.48_{(B–I)}$	$5.00_{(I–F)}$	$5.90_{(I–F)}$	$5.45_{(I–F)}$
I	0.42	0.14	$0.41_{(I–B)}$	$42.00_{(I–B)}$	$71.68_{(B–I)}$	$5.85_{(I–B)}$	$6.90_{(I–B)}$	$6.37_{(I–B)}$
5 : 5								
B	0.01	2.00	$0.00_{(F–B)}$	$1.00_{(F–B)}$	$1.00_{(B–F)}$	$0.00_{(F–B)}$	$0.00_{(F–B)}$	$0.00_{(F–B)}$
F	0.01	2.00	$0.33_{(I–F)}$	$33.00_{(I–F)}$	$48.76_{(B–I)}$	$4.57_{(I–F)}$	$5.38_{(I–F)}$	$4.97_{(I–F)}$
I	0.33	0.30	$0.33_{(I–B)}$	$33.00_{(I–B)}$	$48.76_{(B–I)}$	$4.92_{(I–B)}$	$5.80_{(I–B)}$	$5.36_{(I–B)}$

Retention and separation data mentioned are mean values of three determinations.

Limit of detection

Limit of detection (LOD) which is the lowest analyte concentration likely to be reliably distinguished from the limit of blank (LOB) and at which detection is feasible, was analysed by applying insecticides at the lowest concentration[36]. LOD was established by considering the area of each insecticide on the densitogram, and purity of the spectrum obtained[38].

Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) using Genstat software (14th edition, version

14.1.0.5943, VSN International Ltd, United Kingdom), to judge the significance of differences between the treatments by *F*-test, while the treatment means were compared by least significant difference (LSD) at $P < 0.05$. Ducan's multiple range test (DMRT) was used to determine the differences between treatments.

Results and discussion

Mobile phase optimization

To select an appropriate mobile phase for the simultaneous separation of buprofezin, flubendiamide and imidacloprid tests were performed using mono-component mobile phases on NP-HPTLC silica gel 60 F_{254}. The results revealed that retention of insecticides increased with decreasing eluent strength. The values of $\Delta R_F > 0.04$, $\alpha > 1.00$, $R_S > 1.5$ and R_F between 0.05 and 0.9 are indicative of good separation[29]. Similar R_F values for a given mixture of compounds could be due to the similarity of solvent strength parameters P' of the solvents used for separation. Hence, R_F values between 0.05 and 0.9 are mainly considered to avoid selective solvation of the stationary phase that leads to demixing problems[37]. No solvent has separated all the three insecticides in optimum R_F range (0.2–0.8), except chloroform, with slight variation in R_F values for *B* ($R_F = 0.82$, greater than normal range) and I ($R_F = 0.10$, lesser than the normal range). On observing the values of R_F, R_M, ΔR_F, R_F^{α} and α, chloroform can be suggested as a pure single solvent for simultaneous separation of B, F and I. However, B showed good separation in dichloromethane, toluene and xylene, with R_F values between 0.18 and 0.48; *F* in diethyl ether and tert-butyl methyl ether, with R_F values between 0.65 and 0.69, and *I* in butan-1-ol, ethanol, ethyl acetate, propan-2-ol, methanol, propan-1-ol and tertahydrofuran (R_F = 0.33–0.74). Acetone, acetonitrile and *N*′*N*′-dimethylformamide were comparatively more polar solvents with R_F values more than 0.85, and the three insecticides were found strongly adsorbed on NP-HPTLC layer in hexane, xylene, carbontetrachloride and cyclohexane (0.01–0.19).

Among the non-aqueous poly-component mobile phases, *n*-hexane–acetone remained the best choice for chromatographic separation of many insecticides on NP-HPTLC layers and methanol–water solvent mixture was widely used as a first try solvent for C_8 or C_{18} layers[29,39]. In NP-HPTLC, retention of compounds decreased with increasing mobile phase polarity (Table 1). F and I showed gradual increase in R_F value with the addition of 0.5 ml of acetone, whereas B showed drastic increase in R_F value from 0.01 to 0.29. In RP-HPTLC, addition of 0.5 ml water resulted in decrease in R_F values for all three insecticides (Table 2). Figures 1 and 2 show plots of R_F values against volume composition of mobile phases for B, F and I using NP-HPTLC and RP-HPTLC respectively.

On observing the values of R_F, ΔR_F, R_F^{α} and α, it was affirmed that B, F and I were best separated from each other on NP-HPTLC layers by the mobile phase comprising *n*-hexane–acetone in the ratio 6.5 : 3.5 v/v, with $\Delta R_{F(\text{B–F})} = 0.35$, $\Delta R_{F(\text{F–I})} = 0.19$, $\Delta R_{F(\text{B–I})} = 0.54$; $R^{\alpha}_{F(\text{B–F})} = 1.88$, $R^{\alpha}_{F(\text{F–I})} = 1.90$, $R^{\alpha}_{F(\text{B–I})} = 3.57$; and $\alpha_{(\text{F–B})} = 4.50$, $\alpha_{(\text{I–F})} = 2.50$, $\alpha_{(\text{I–B})} = 11.28$. In RP-HPTLC, best separation of B, F and I was observed in mobile phase of methanol–water at 8 : 2 v/v ratio ($\Delta R_{F(\text{F–B})} = 0.23$, $\Delta R_{F(\text{I–F})} = 0.26$, $\Delta R_{F(\text{I–B})} = 0.49$; $R^{\alpha}_{F(\text{F–B})} = 2.28$, $R^{\alpha}_{F(\text{I–F})} = 1.63$, $R^{\alpha}_{F(\text{I–B})} = 3.72$ and $\alpha_{(\text{F–B})} = 4.50$ and $\alpha_{(\text{B–F})} = 3.16$, $\alpha_{(\text{F–I})} = 2.92$, $\alpha_{(\text{B–I})} = 9.24$). Since densitometric methods are more precise compared to visual methods, R_S was calculated by densitometric method. Peak resolutions based on peak width at baseline ($R_{S(\text{b})}$), peak width at half height ($R_{S(\text{h})}$)

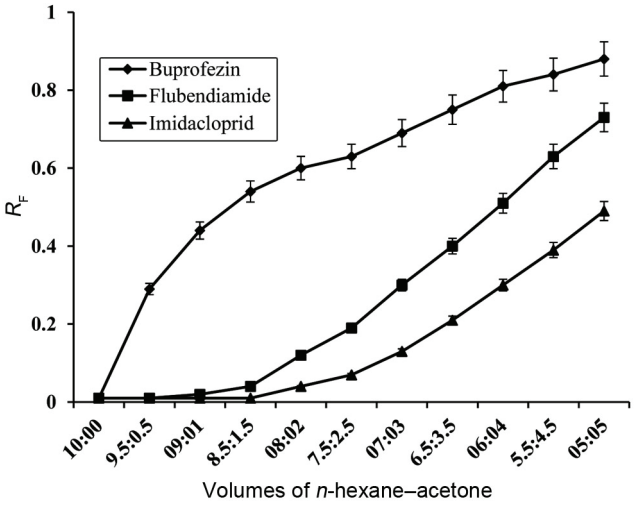

Figure 1. R_F values of three insecticides in NP-HPTLC using *n*-hexane–acetone as mobile phase.

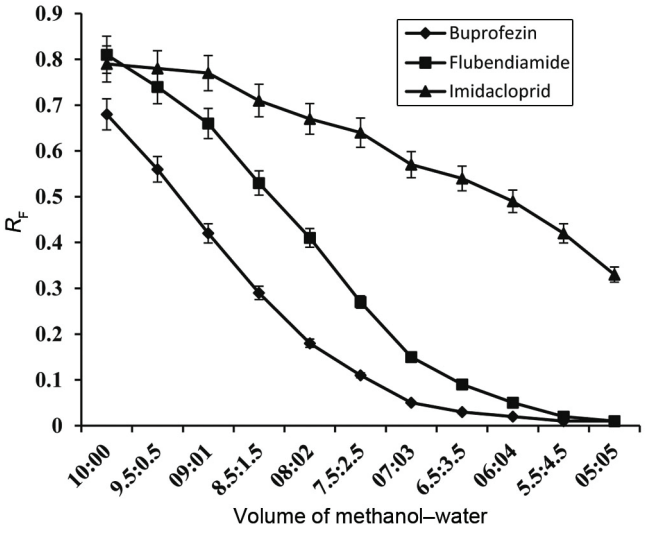

Figure 2. R_F values of three insecticides in RP-HPTLC using methanol–water as mobile phase.

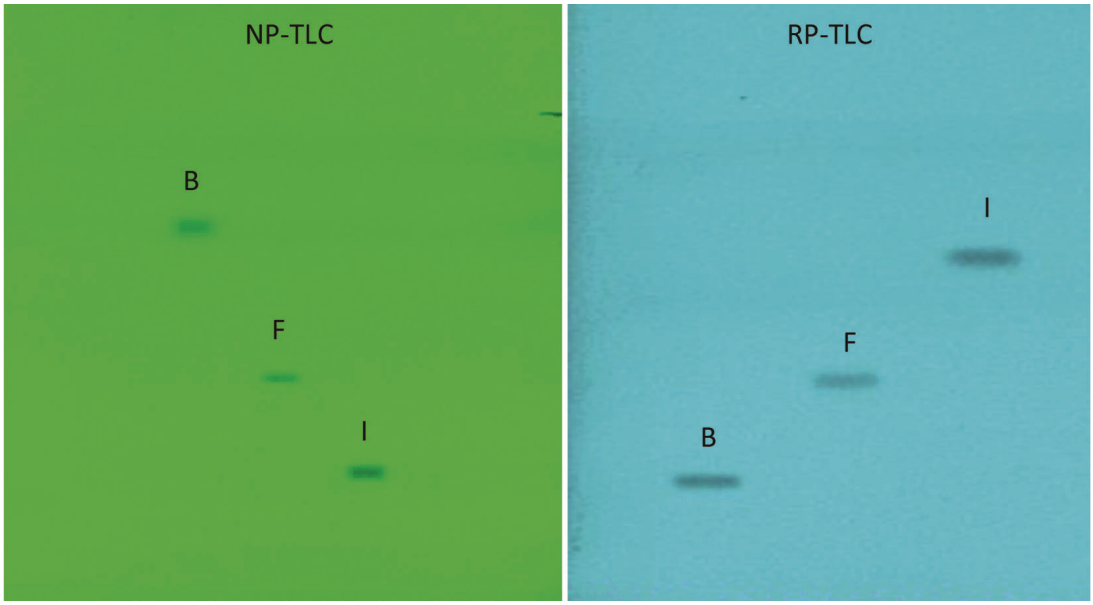

Figure 3. Chromatograms obtained for buprofezin (B), flubendiamide (F) and imidacloprid (I) by NP-TLC using *n*-hexane–acetone (6.5 : 3.5) and by RP-TLC using methanol water (8 : 2 v/v) as mobile phase.

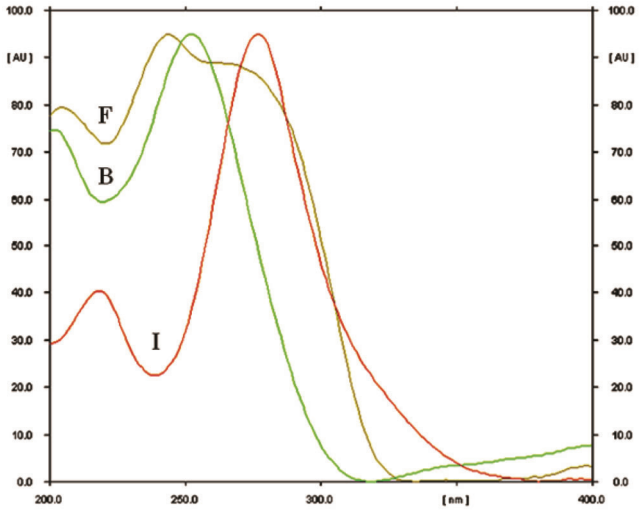

Figure 4. UV spectrum of buprofezin (B), flubendiamide (F) and imidacloprid (I).

and average peak resolutions ($R_{S(a)}$) were calculated by use of eqs (4)–(6) respectively. In NP-HPTLC, *n*-hexane–acetone in the ratio 6.5 : 3.5 v/v yielded better resolution values ($R_{S(B–F)} = 4.12$, $R_{S(F–I)} = 7.34$, $R_{S(B–F)} = 2.02$) while in RP-HPTLC methanol–water in the ratio 8 : 2 v/v yielded better resolution values ($R_{S(F–B)} = 2.63$, $R_{S(I–F)} = 2.97$, $R_{S(I–B)} = 5.92$). Figure 3 shows the representative chromatograms obtained for B, F and I in the mobile phases of *n*-hexane-acetone 6.5 : 3.5 v/v (NP-HPTLC) and methanol–water 8 : 2 v/v (RP-HPTLC).

As an alternative to the *n*-hexane–acetone mobile system, dichloromethane (DCM)-tert-butyl methyl ether (TBME) mixture with different compositions was used for the analysis of B, F and I. The data obtained in mobile phase composition 8.5 : 1.5 v/v affirm good separation of all the three insecticides ($R_{F(B)} = 0.92$, $R_{F(F)} = 0.51$, $R_{F(I)} = 0.22$; $\Delta R_{F(B–F)} = 0.41$, $\Delta R_{F(F–I)} = 0.29$, $\Delta R_{F(B–I)} = 0.70$, $R^{\alpha}_{F(B–F)} = 1.80$, $R^{\alpha}_{F(F–I)} = 2.31$, $R^{\alpha}_{F(B–I)} = 4.18$, $\alpha_{(F–B)} = 11.05$, $\alpha_{(I–F)} = 1.59$, $\alpha_{(I–B)} = 40.79$, $R_{S(B–F)} = 3.30$, $R_{S(F–I)} = 1.70$, $R_{S(I–B)} = 5.86$). The only drawback while using this mobile phase composition is the high R_F value obtained for *B* ($R_F = 0.92$). Combination of dichloromethane with acetone and ethyl acetate was also used for separation of these insecticides along with other mixtures such as benzene–chloroform, cyclohexane–acetone, toluene–acetone, xylene–acetone, carbontetrachloride-acetone, carbontetrachloride–tetrahydrofuran and toluene–tetrahydofuran. However, none of these combinations could separate these insecticides in the optimum range.

In situ detection

UV apex of maximum absorption for all the three insecticides was determined by performing multi-wavelength scan in the range 200–300 nm, with an increment of 10 nm wavelength per step. The plates developed in *n*-hexane–acetone 6.5 : 3.5 v/v and methanol–water 8 : 2 v/v were used for this purpose. The heights and areas of the chromatographic bands obtained were maximum at 252 nm for B, 242 nm for F and 276 nm for I. These values are in conformity with the UV apex of maximum absorption obtained with *in situ* UV spectrum of each insecticide. Figure 4 shows the UV spectrum of each insecticide.

Table 3. Stability of buprofezin, flubendiamide and imidacloprid on NP-HPTLC and RP-HPTLC layers

	NP-TLC		RP-TLC	
Time (h)	Average area (AU)	RSD (%)*	Average area (AU)	RSD (%)*
Buprofezin				
0	7783.35 ± 38.67[a]	0.00	6773.6 ± 45.30[a]	0.00
3	7861.75 ± 33.75[a]	0.77	6816.8 ± 66.25[a]	0.45
6	7775.20 ± 26.20[a]	0.07	6811.6 ± 47.20[a]	0.40
Flubendiamide				
0	4565.35 ± 69.85[a]	0.00	6129.4 ± 17.5[a]	0.00
3	4784.80 ± 81.10[a]	1.90	6215.9 ± 6.05[a]	1.00
6	4859.65 ± 53.75[a]	2.96	6331.1 ± 18.35[a]	2.25
Imidacloprid				
0	7957.25 ± 56.65[a]	0.00	8695.7 ± 63.2[a]	0.00
3	7943.60 ± 62.3[a]	0.12	8835.2 ± 20.15[a]	1.12
6	7962.95 ± 54.95[a]	0.05	7660.3 ± 113.5[a]	9.56

*RSD (relative standard deviation data) and results shown as the mean ± SE values are of five determinations. Column values followed by the same letters are not significantly different from each other at $P < 0.05$.

Table 4. Stability of buprofezin, flubendiamide and imidacloprid in the solution (methanol)

	NP-TLC		RP-TLC	
Time (h)	Average area (AU)	RSD (%)	Average area (AU)	RSD (%)*
At room temperature				
Buprofezin				
0	7783.35 ± 38.65[a]	0.00	6773.60 ± 45.3[a]	0.00
3	7395.18 ± 73.73[a]	3.71	6552.90 ± 38.47[a]	2.38
6	7530.70 ± 67.31[a]	2.40	6613.00 ± 41.81[a]	1.72
Flubendiamide				
0	4656.35 ± 69.85[a]	0.00	6129.40 ± 17.5[a]	0.00
3	4524.68 ± 48.95[a]	2.06	5784.25 ± 48.63[a]	4.22
6	4404.28 ± 89.67[a]	4.04	6015.20 ± 81.25[a]	1.34
Imidacloprid				
0	7957.25 ± 56.65[a]	0.00	8695.60 ± 63.2[a]	0.00
3	7235.63 ± 67.96[a]	7.05	8672.00 ± 37.31[a]	0.19
6	7092.13 ± 118.96[a]	8.62	9626.20 ± 58.28[a]	6.84
At 4°C temperature				
Buprofezin				
0	7783.35 ± 38.65[a]	0.00	6773.60 ± 45.3[a]	0.00
3	7635.17 ± 93.90[a]	1.38	6253.30 ± 95.64[a]	5.90
6	7837.88 ± 69.74[a]	0.50	6653.00 ± 89.98[a]	1.28
Flubendiamide				
0	4656.35 ± 69.85[a]	0.00	6129.40 ± 17.5[a]	0.00
3	4672.33 ± 42.05[a]	0.24	5913.80 ± 88.09[a]	2.58
6	4671.55 ± 25.45[a]	0.23	6241.77 ± 56.03[a]	1.27
Imidacloprid				
0	7957.25 ± 56.65[a]	0.00	8695.60 ± 63.2[a]	0.00
3	7085.05 ± 48.25[a]	8.70	8789.35 ± 23.07[a]	0.75
6	7133.07 ± 74.38[a]	8.17	9547.00 ± 43.97[a]	6.48

*RSD data and results shown as the mean ±SE values are of five determinations. Column values followed by the same letters are not significantly different from each other at $P < 0.05$.

Stability analysis

Evaluation of stability of B, F and I indicated that the standard insecticide solutions were found to be stable at room temperature and at 4°C in methanol. The relative standard deviation (RSD, %) for peak height and peak area was within 10%. Compared with B and F, I showed higher RSD values (7.05%, 8.62% at 3 h intervals and 8.70%, 8.17% at 6 h intervals), indicating dissipation of I in methanol solution. The samples were also evaluated for

persistence behaviour on NP-HPTLC and RP-HPTLC layers soon after development (3 and 6 h) by performing densitometric scan. The results obtained showed that the insecticides were stable on both layers and RSD values less than 10% indicated that the samples were stable on the plate and in solution at room temperature as well as at 4°C (Tables 3 and 4). On RP-HPTLC layer, *I* showed higher RSD value (9.56%) when the plate was scanned after 6 h.

Limit of detection

LOD values for *B*, *F* and *I* on NP-HPTLC layer were 200, 150 and 20 ng respectively; and 50, 100 and 10 ng on RP-HPTLC layer respectively.

Conclusion

Buprofezin, flubendiamide and imidacloprid were best separated from each other on NP-HPTLC with *n*-hexane–acetone (6.5 : 3.5 v/v) as mobile phase and on RP-HPTLC using methanol–water (8 : 2 v/v) as mobile phase. Though mono-component mobile phase, chloroform (10 : 00 v/v) and mixture solvent system, dichloromethane-tert-butyl methyl ether (8.5 : 1.5 v/v), did not provide optimum conditions for separation of B and I, they are still suitable for separation of these three insecticides on NP-HPTLC layer. The UV apex of maximum absorption for individual compounds was 252 (B), 242 (F) and 276 nm (I). The stability analysis, with relative standard deviation, <10%, showed that the compounds were stable up to 6 h at room temperature and for a long time at 4°C. The LOD values obtained for the three insecticides on NP-HPTLC and RP-HPTLC layers were within acceptable range. This work is useful for toxicologists to detect a mixture of these insecticides in forensic as well as environmental samples, where they are present in high concentration.

Conflict of interest: The authors declare that they have no competing interests.

Ethical statement: The present work does not include any studies with human participants or animals performed by any of the authors.

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