

Solubilization of bromadiolone in humic acid pseudomicellar media

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Abstract. Bromadiolone (BRD), a second generation anticoagulant often applied to the living environment to control rodents, is usually considered to have low environmental toxicity due to its poor solubility in water. In this study of the effect of humic acid (HA) on BRD using electronic absorption spectroscopy, it has been observed that BRD is appreciably solubilized even in low concentrations of aqueous HA solutions. The BRD solubilization efficiency of aqueous HA was found to be 2.39 ± 0.14 ($4.53 \pm 0.26 \mu\text{M ppm}^{-1}$). It was also seen that BRD is reasonably solubilized in aqueous extract of farm soil.

Keywords: bromadiolone; humic acid; absorbance; solubilization; rodenticide

1. Introduction

Bromadiolone (BRD), a substituted 4-hydroxycoumarin derivative, is known to possess anti-coagulant activity with acute toxicity (Saunders 1983, Rao 2005). BRD is a widely known rodenticide used all over the world. Chemical structure of BRD is shown in Fig. 1. It can effectively control the population of rodents in agricultural as well as rural areas (Parshad *et al.* 1987). It reaches the living system by contact (through skin) and by direct consumption (Saunders 1983), and acts as vitamin-K antagonist (Kohn *et al.* 2003). Animals exposed to BRD cause internal toxicity such as nose bleeding, bleeding gums, blood in urine and feces due to ruptured blood vessels and external skin damage (Hui *et al.* 1996).

Moreover, BRD residues remains in the bodies of dead or dying rodents can cause toxic effects to scavengers, predator and indirectly to humans (Berny *et al.* 1997, Dowding *et al.* 2010). Also an unusual increase of BRD toxicity for human keratinocytes in presence of humic acid (HA) was observed (André *et al.* 2004). BRD is usually applied to the site as 'baits' containing very low concentrations ca. 0.005% of BRD (Swann *et al.* 1983, WHO/FAO 1996). Even though the possibility of secondary poisoning and accidental consumption exists, BRD is still considered as a reliable rodenticide in field application throughout the world because of its low solubility in water at 20°C, 18.4 mgL^{-1} or $3.49 \times 10^{-5} \text{ M}$ and high partition coefficient (K_{oc}) of 2.1×10^5 between water and soil organic carbon (Swann *et al.* 1983, Meylan *et al.* 1992). pH studies have already indicated that at pH 7.0, BRD exists in its anionic form (Subbiah *et al.* 2005). Since the BRD has low volatility, vapour pressure of $1.5 \times 10^{-8} \text{ mm of Hg}$ and decomposes before its melting point 200°C (Hartley and Kidd 1983), through-air interaction is expected to be less.

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BRD is almost insoluble in water and it is known to form aggregates in water even at low concentration. It has already been reported that (Subbiah *et al.* 2005) micellization of aqueous medium causes enhanced solubilization of BRD by providing a hydrophobic environment in the micellar pseudo-phase. The critical aggregation concentration (cac) of surfactant is sometimes decreased by two orders of magnitude in presence of another micellar forming system (Wandruszka *et al.* 1998). Humic acids present in soil humus are amphiphilic in nature and are known to form pseudo-micellar aggregates. Hydrophobic nature of HA favours the formation of “micelle”- like structures with hydrophilic groups on the water side and the hydrophobic nucleus helps in inner absorption of organic hydrophobic pollutants (Steinberg *et al.* 1993). ¹³C-NMR and FT-IR spectral studies were carried out on HA show that it has great affinity for binding hydrophobic compounds (De Paolid and Kukkonen 1997). Second generation rodenticide, BRD has longer biological half life and are hydrophobic in nature, which enhances the solubility of these drugs in water or soil containing more humic substances. It is possible that the HA pseudo-micellar aggregates normally present in soil and water of agricultural fields can cause effective solubilization of BRD. The micellization occurs at lower surfactant concentrations due to the presence of the polyelectrolyte, HA. The molecular weight of HA varies from 1 kDa to 2,000 kDa (Richard *et al.* 2011). The distribution of humic components in hydrophilic or hydrophobic environment is a function of its molecular weight. High molecular weight fraction is bound to soil surface and can make a hydrophobic environment over the soil, where BRD normally adsorbs. But the low molecular weight fraction (< 60 kDa) could reach the water in agricultural fields or sub-soil water and forms pseudo-micellar hydrophobic aggregates (Wandruszka *et al.* 1998). At pH values from 5 to 8, the possibility of the humic acid molecules forming pseudo-micelles increases due to a combination of neutralized and dissociated charged sites (De Moraes and Rezende 2005).

Humic substances play a crucial role in removal and/or migration of environmental pollutants in natural water, because some of the pollutants are effectively incorporated into the matrix of humic substances through hydrophobic interaction, electrostatic interaction, hydrogen bonding, and coordinate bonding (Simpson *et al.* 2002). Thus HA induced solubilization of BRD in aqueous phase may have important implication towards the bioavailability of BRD. A study of the interaction, BRD with HA assumes significance in this context.

The binding of a series of anticoagulant rodenticides with the main soil organic component, humic acid (HA) and concentration of unbound rodenticide to HAs were studied using frontal analysis approach (André *et al.* 2005). The dissolved BRD can be measured using conventional methods

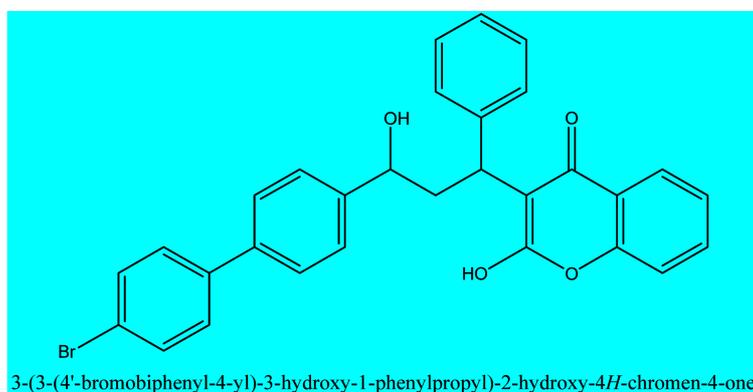


Fig. 1 Chemical structure of bromadiolone (BRD)

such as ultrafiltration and equilibrium dialysis. But these methods have technical difficulties such as undesirable drug adsorption onto the membrane and leakage of bound drug through the membrane (Terakita *et al.* 1993). Techniques based on HPLC and multisyringe flow injection analysis (MSFIA) have introduced for the accurate determination of very low amount of BRD dissolved in water (De Armas *et al.* 2002). But these methods require many preprocessing steps like pre-concentration on a solid-phase, elution with organic solvents etc. Coumarin derivative BRD absorbs in the UV-Visible region (Hunter *et al.* 1988). So the UV-Visible electronic absorption spectroscopy can be used as an analytical tool to study the interaction between HA and BRD. Since the micellar solubilization of the BRD in HA is higher than normal solubility of BRD in water (18 mg L^{-1}), a study of enhanced solubility of BRD in HA is important. Also the enhanced solubility can be monitored using electronic absorption studies and these absorption spectral calibrations could easily predict the amount of BRD in aqueous HA solution. In this work, the BRD solubilization efficiency by HA in aqueous HA has been studied by examining the changes in the absorbance spectra.

2. Experimental

2.1 Materials

Pure sample of BRD was a gift from Forensic Sciences Department, Chennai. HA was purchased from Sigma–Aldrich (CAS Number: 68131-04-4) and was used as received. Methanol was purchased from S.D. Fine Chemicals. NaOH, Na_2CO_3 and HNO_3 were purchased from Sisco Research Laboratories Pvt. Ltd. Triple-distilled water (TDW) was used as solvent for the work.

2.2 Apparatus and methods

A JascoV-650 spectrophotometer equipped with a 1 cm path length quartz cell was used for the absorbance measurements. The pH of the test samples was measured with a Eutech-Cyberscan pH meter. PMT voltage was kept at 700 V and scans speed at $1,000 \text{ nm min}^{-1}$ with the band pass of 2 nm.

3. Preparation of the solutions

3.1 Preparation of aqueous HA solution

A stock solution of HA (50 ppm) was prepared by dissolving 5 mg of material in 10 ml of 1 M NaOH solution followed by the sonication and addition of 10 ml of 1M HNO_3 . The solution was diluted to 90 ml TDW and pH was adjusted to 7 by the drop-wise addition of 0.1 M NaOH. The solution was kept overnight and filtered through the Whatman-4 filter paper which removes the water insoluble component ($< 0.01 \text{ mg}$) in the HA (Divya *et al.* 2009). The pH of the stock solution remained 7.0₃ at 25°C throughout the experiment (Lulakis *et al.* 1995). Since BRD is sparingly soluble in water, an aqueous HA solutions of BRD were prepared from stock solution of BRD ($2 \times 10^{-3} \text{ M}$) in acetone. 0.57₅ mg (1.09 μmol) of BRD (in acetone) was transferred into standard flask and the acetone is evaporated by purging N_2 . After acetone evaporation, various amount of HA solution was added to the flask. The BRD (after acetone evaporation) sticks to the container. The solubility of BRD in added

HA solution was made possible by sonicating the solution in a 20 kHz bath sonicator for 90 minutes. The sonicated solution kept for 2 hrs for attaining equilibrium. It avoids the sonication induced solubility of BRD in HA solution. For absorbance studies the concentration of HA was varied from 1 to 15 ppm with an interval of 1 ppm.

3.2 Soil-extract samples in aqueous media

Farm soil sample was collected from IIT Madras campus. The sample was dried for 24 hours at 80°C. 1 g of the dried and powdered sample was added to 100 mL of TDW and was shaken for 5 hours using shaker. 10 hours after shaking, the suspension was decanted through Whatman-4 filter paper. This aqueous extract was a 1,750 ppm solution, which was further diluted to 175 ppm so as to give a reasonable absorbance of 0.11₅ at 310 nm (the wavelength of BRD absorption maximum). 0.57₅ mg (1.09 μmol) of BRD solid was added to this soil extract and was sonicated for 90 minutes and equilibrated for 2 hours so as to establish equilibrium. The pH of the test HA solution is adjusted to 7 using NaOH base and HNO₃ acid and was kept constant throughout the testing. Soil samples were extracted to triple distilled water (TDW) and the supernatant of the solution was used for the studies. Also pH of the supernatant was found to be 5.8, which is well within the normal range of pH often observed for farm soil (Wandruszka *et al.* 1998). It was decided to carry out the studies at this pH (5.8) since it is already known that the binding properties of BRD with HA are weekly affected in the pH range 5.0 to 8 (De Paolid *et al.* 1997).

4. Results and discussion

4.1 Electronic absorption spectral studies of HA

HA solutions show electronic absorption in the wavelength range 200-450 nm. Since HA have a large number of chromophores with aromatic moieties, the absorbance increases gradually towards shorter wavelengths with a shoulder around 300 nm (Fig. 2). The absorbance of HA was measured at different concentration from 1 ppm to 15 ppm with an interval of 1 ppm. Since, the components other than HA like surfactants, metal complexes are either non-absorbing or absorbs at different wavelengths, the calibration plot (Fig. 2) could predict the HA content of soil samples by making an absorbance calibration plot at any of these two wavelengths 310 or 254 nm.

The absorbance of the HA solution increased linearly with concentration obeying Beer-Lambert's law. Deviation from linearity was observed at higher concentrations of HA possibly because of the formation of higher pseudo-micellar aggregates (Wandruszka *et al.* 1998). The plot of absorbance at 310 nm against concentration (ppm) of HA has good linearity with Pearson's regression coefficient (R^2) is 0.998₇ and standard deviation (S.D.) is 0.005₇ in the analytical range (Fig. 2-inset). This calibration plot can be used for the estimation of the amount of soluble HA fraction (low molecular weight) present in the sub-soil water after proper dilution. Experiments involving BRD solubilization was therefore limited to 15 ppm concentration of HA solution.

4.2 Electronic absorption spectral studies of BRD

Electronic absorption spectra of HA solutions contain varying amount of HA (0 ppm to 15 ppm)

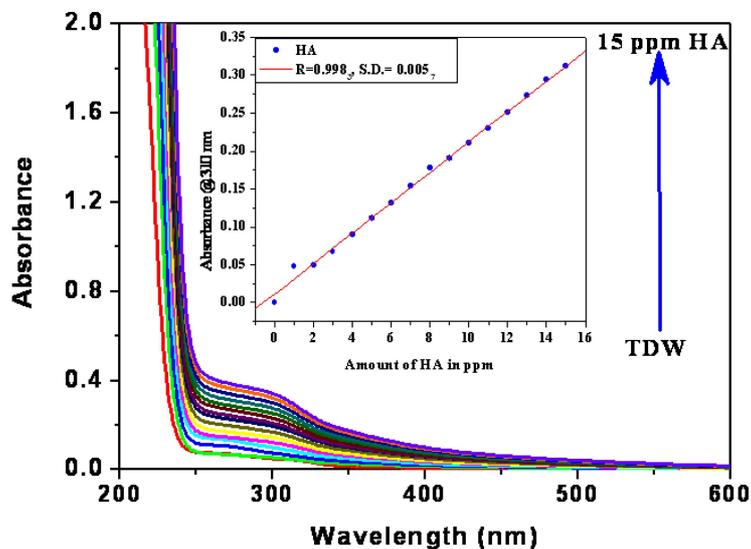


Fig. 2 Electronic absorption spectra of aqueous HA solutions (0 to 15 ppm) is recorded in JascoV-650 spectrophotometer. Inset figure shows the absorption calibration at 310 nm against the concentration of HA at 25°C

and constant amount (0.57₅ mg) of BRD were measured. While recording the electronic absorption spectra of HA solubilized BRD, we have adopted two modes. First, TDW was taken as reference in the double beam UV-VIS spectrophotometer and recorded the sum of the absorbance of BRD and HA. In second mode corresponding concentration of HA in the HA solubilized BRD was taken as the reference and which gives the absorbance of solubilized BRD alone. It is also possible to get the absorbance of BRD by subtracting the absorbance of HA from that of HA solubilized BRD. The combined absorbance of HA and solubilized BRD, measured against TDW as reference are shown in the Figs. 3(a) and (b) gives the net absorbance of BRD by recording the spectra of BRD in HA

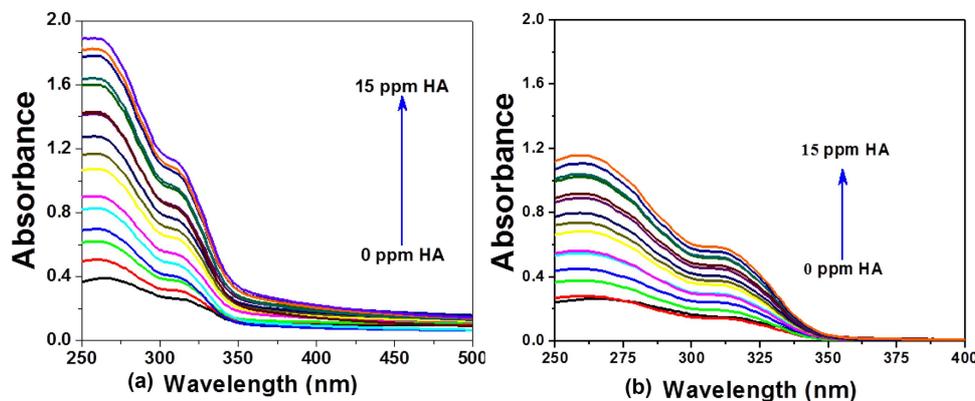


Fig. 3 Electronic absorption spectra of BRD in aqueous HA solutions (0 to 15 ppm) (a) with TDW as reference and (b) with aqueous HA as reference at 25°C

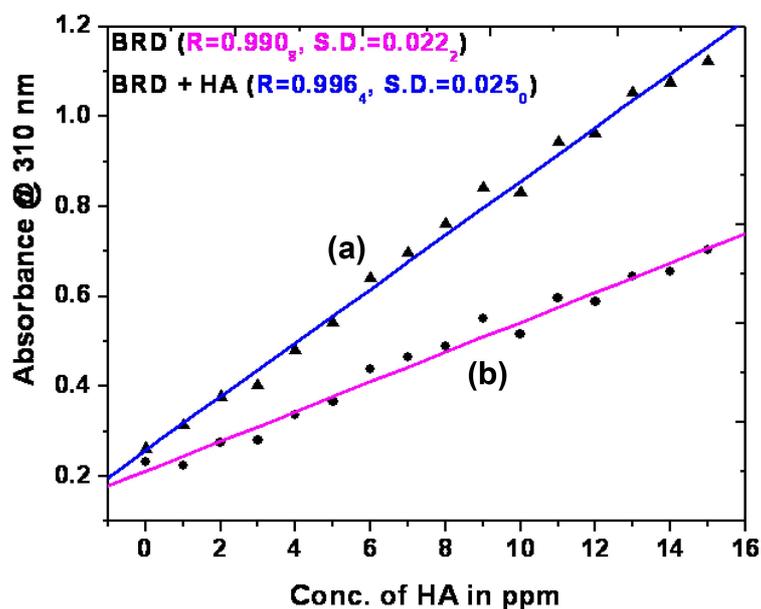


Fig. 4 Plots of absorbance at 310 nm against concentration of HA for the samples of (a) HA solution of BRD with TDW as reference and (b) HA solution of BRD with HA as reference at 25°C

solution with HA as reference. It is clearly seen that there is progressive enhancement of absorbance of BRD in solutions of increasing HA concentration.

The enhancement of BRD absorbance comes only from the solubilization of solid BRD the amount of which was kept constant (0.575 mg) throughout the experiment. It is seen that BRD absorbance increases even at fairly low concentration of HA, well below the critical micellar concentration (cmc) of HA (De Moraes and Rezende 2005). The pre-micellar pseudo-aggregates of HA appear to provide sufficient hydrophobicity for effective BRD solubilization.

BRD has an absorption maximum at 310 nm for the singlet electronic transition from S_0 to S_1 . So the calibration plots of absorbance of BRD in aqueous HA at 310 nm against concentration of HA were recorded and are shown in Fig. 4. The calibration plots (4(a) and (b)) of the absorbance of BRD and HA corresponding to Fig. 4(a) and that of solubilized BRD corresponding to Fig. 4(b) show good linearity with R values of 0.996₄ and 0.990₈, and S.D. values of 0.025₀ and 0.022₂ respectively. The slope of the plot 4(b) is $0.03_3 \pm 0.001$ ppm⁻¹. This gives the net enhancement of BRD solubilization with increasing HA concentration, which can be used to define the BRD solubilization efficiency of HA.

Direct proportionality of absorbance with concentration as indicated by the linear plot (plot (b) in Fig. 4) can in principle enable estimating BRD concentration in sub-soil water containing low concentration of HA (section 4.3). It is to be noted, however, that soil would have different solubilities and the HA used in this study may not be fully representative of HA component in soil water. The electronic absorption calibration of BRD in aqueous HA solution (Fig. 4(a)) loses its linearity above 15 ppm of HA due to the pseudo-micellar aggregation of HA (De Moraes and Rezende 2005, Guetzloff and Rice 1994). At higher concentrations, HA forms 'micelle' like aggregates (Steinberg *et al.* 1993), and the hydrophobic core of the micelles can trap hydrophobic molecule like BRD. Significantly enhanced scattering of these aggregates can lower the exposure of

BRD to the incident photons. As the absorption spectra are recorded with the same concentration of HA as reference, the BRD absorbance shows a decrease at higher concentration of HA.

4.3 BRD solubilization efficiency of HA

The electronic absorption spectra of BRD in 1:1 methanol solution are shown in Fig. 5(a) and its calibration plot at absorption maximum at 310 nm (linear fit $R = 0.994_6$, $S.D. = 0.024_1$) is shown in Fig. 5(b). Since methanol does not affect the absorption spectral property of BRD and has high BRD solubility, this calibration plot was made in 1:1 aqueous methanol solution. The amount of BRD in aqueous HA was calculated from a calibration plot of absorbance of BRD in 1:1 methanol solution at 310 nm against concentration of BRD. The absorbance of BRD in TDW was 0.22_4 , which corresponds to a solubility value of 3.31×10^{-5} mol of BRD in TDW, as obtained from the calibration plot. This is close to the previous reported value of 3.49×10^{-5} mol (WHO/FAO 1996). The absorbance of BRD in the aqueous HA solution was calculated from the calibration plot of absorbance of BRD in aqueous HA with HA as reference (Fig. 4(b)).

The obtained absorbance value and the calibration plot of BRD absorbance at 310 nm against concentration of BRD (Fig. 5(b)) were used for the determination of BRD in aqueous HA, assuming that the molar extinction coefficient (ϵ) of BRD is the same in aqueous and aqueous methanolic media. The value of ϵ estimated for BRD is $7300 \pm 200 \text{ M}^{-1}\text{cm}^{-1}$. Using this value, the slope of Fig. 4(b) can be converted to a $4.53 \pm 0.26 \mu\text{M ppm}^{-1}$, which gives the BRD solubilization efficiency of HA.

The interaction of BRD with HA has already been studied using an HPLC based method and a binding constant of BRD with HA has been reported, with the amount of HA expressed in molar units (André *et al.* 2005). Since the molar mass and components of HA are poly-dispersive in nature, we have preferred to express the BRD solubilization efficiency of HA by the amount of BRD solubilized in unit amount (in ppm units) of HA. This efficiency factor can also be expressed as a unit-less quantity if the BRD concentration is also expressed in ppm units. Expressed this way, the BRD solubilization efficiency by HA is given by 2.39 ± 0.14 .

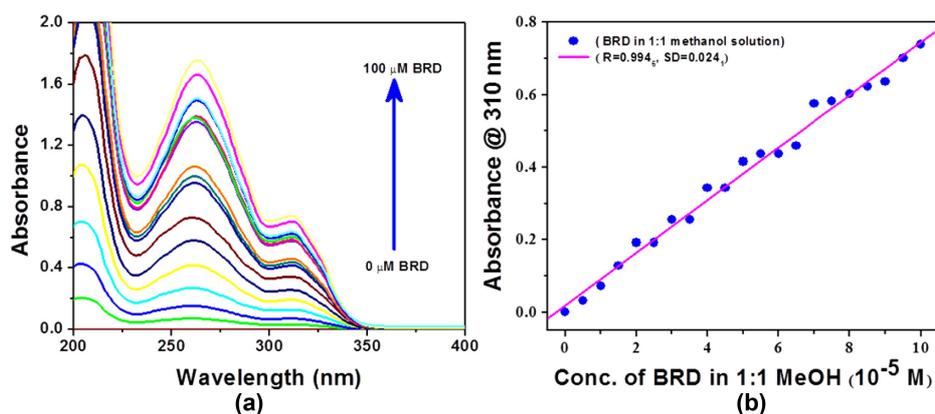


Fig. 5 (a) The electronic absorption spectra of 0 to 100 μM (interval 5 μM) of BRD in 1:1 aqueous methanol solution and (b) its calibration plot of absorbance at 310 nm against the concentration of BRD in 1:1 aqueous methanol solution at 25°C

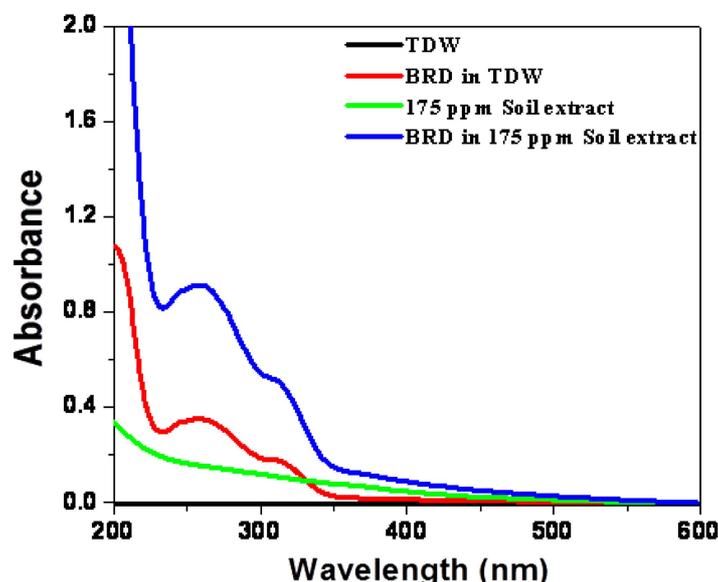


Fig. 6 Electronic absorption spectra of soil extract and TDW with and without BRD at 25°C

4.4 BRD solubilization by soil extract

Since the humus content in the soil varies in a wide range, from very low 1.5% to high 75%, a study of real soil sample was thought necessary. This preliminary study was done for a soil sample obtained from fertile agricultural farm. A comparison of electronic absorption intensity/spectrum of BRD in TDW and the soil extract (175 ppm) is given in Fig. 6. The enhancement of absorbance is clearly due to the enhanced solubility of BRD in humic substance rich-fertile soil/solution.

At 310 nm the absorbance of soil extract is 0.11₂ where as the absorbance of BRD containing soil extract is 0.51₇, which is equivalent to 27.8₆ mg of BRD (calibration plot 5(b)). A 0.11₂ absorbance sample of HA would be equivalent to 5.2 ppm which can solubilize a maximum of 27.2₁ mg of BRD. Thus it appears that fertile soil extract has even higher efficiency of solubilizing BRD, as compared to a HA solution of the same concentration. This could be due to the presence of other soil ingredients like hydrocarbons, surfactants, metal complexes, peptides etc.

4.5 Solubility of BRD in soil sample of different concentration

Electronic absorption spectra of the soil samples of different concentration are recorded with respect to the pure solvent, TDW (Fig. 7). The recorded spectra look similar to the absorption spectra of HA in Fig. 2, which reminds the higher percentage of HA in the soil sample. A calibration plot of absorbance at 310 nm against the concentration of soil with good linearity of $R^2 = 0.989_5$ and is shown in Fig. 7 (inset).

The calibration plot could predict approximate amount of HA present in the soil extract. Due to the presence of HA in soil sample, it can solubilize more amount of BRD rodenticide. An electronic absorption spectra of soil solubilized BRD with respect to soil sample (as the reference) is recorded and which gives the absorbance of BRD solubilized in soil extract. The enhanced solubility of BRD

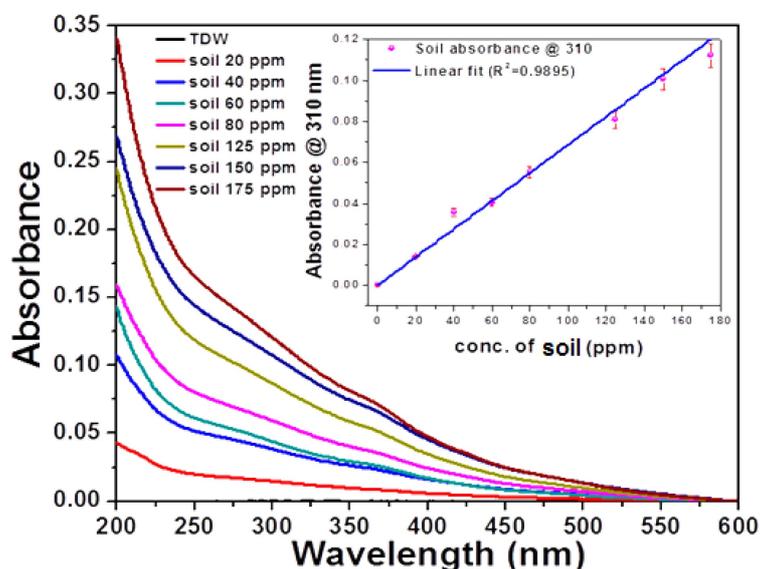


Fig. 7 Electronic absorption spectra of soil sample of different concentrations. Inset figure show a calibration plot of absorbance at 310 nm against the concentration of soil (ppm) with good linearity of $R^2 = 0.9895$

soil sample is clearly visible in the absorption spectra of soil solubilized BRD as well as in the calibration plot of BRD absorbance at 310 nm against the concentration of soil sample and are shown in Fig. 8.

The calibration plot of BRD absorbance at 310 nm against the concentration of soil sample shows

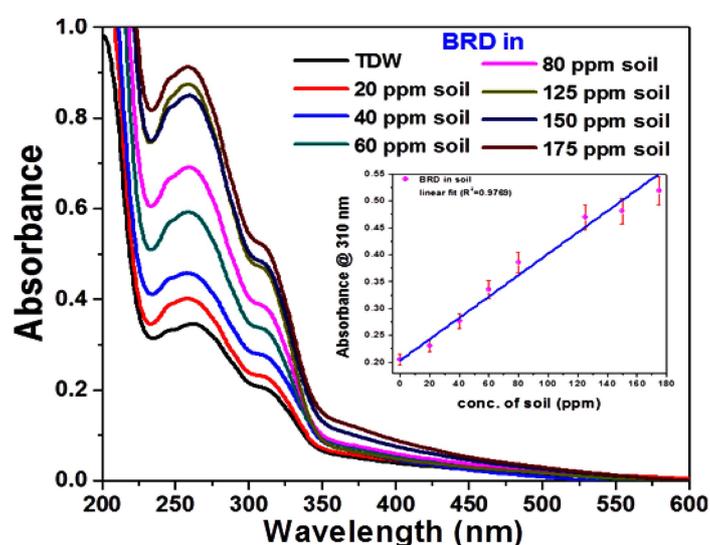


Fig. 8 Electronic absorption spectra of soil solubilized BRD sample of different concentrations. Inset figure show a calibration plot of absorbance at 310 nm against the concentration of soil (ppm) with good linearity of $R^2 = 0.9769$

good linearity of $R^2 = 0.9769$. It proves the solubility of the BRD increases with the soil fraction or amount of HA present in the aqueous media.

5. Conclusions

This work clearly shows that water soluble HA causes an appreciable increase in solubilization of BRD. The BRD solubilization efficiency of the Sigma–Aldrich (CAS Number: 68131-04-4) sample of HA was estimated to be $4.53 \pm 0.26 \mu\text{M ppm}^{-1}$. Thus it is possible that BRD could be extracted from the solid phase by dissolved humic substances thereby increasing its availability in aquatic systems. It would be reasonable to estimate that the BRD solubilization efficiency will be of comparable magnitude for water soluble HA from other sources. The lethal dosages of BRD for aquatic and terrestrial animals are at 1-25 mg/kg (WHO/FAO 1996). Moreover, even at sub-lethal dosages, prolonged exposure to significant amounts of solubilized BRD could pose health risks. The HA induced enhanced solubility of BRD can thus have important implications towards enhanced bioavailability, in terms of being a part of food chain or bioaccumulation (Hui *et al.* 1996, USEPA 1998). A very preliminary study with fertile soil extract also indicates that there is a significant solubilization of BRD by soil extract sample.

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