CHAPTER III

Materials and Methods

3.0. MATERIALS AND METHODS

3.1. INSTRUMENTATION

1. High Pressure Liquid Chromatography was performed with an isocratic High Pressure Liquid Chromatography system (Shimadzu HPLC class VP series, Shimadzu Corporation, Kyoto, Japan) with two LC-10 AT, VP pumps, variable wavelength programmable UV/Visible detector SPD-10A, VP, CTO-10AS VP column oven, SCL-10A, VP system controller. A 20 µL Hamilton syringe was used for injecting the samples. Data were analyzed by using PEAK software.
2. Double beam UV-VIS spectrophotometer Model UV-VIS 2301 (Tech-comp limited, Hong Kong, Japan) was used for spectral studies.
3. Degassing of the mobile phase was done by using ultrasonic bath sonicator (Loba Chemie Pvt. Ltd. Mumbai, India)
4. Samples were weighed by using Denver electronic weighing balance (Denver instruments, Colorado, USA.).
5. Kromosil C18 analytical column (250 mm × 4.6 mm I.D., 5 µm particle size), under reversed phase chromatographic conditions, is used for the chromatographic analysis of the following drugs:
    - Chlortetracycline
    - Sulfadimidine
    - Enrofloxacin
    - Fenvalerate
    - Cypermethrin
    - Oxytetracycline
    - Permethrin

6. Kromosil C8 analytical column (150×4.6 mm I.D., 5 μm particle size), under reversed phase chromatographic conditions, is used for the chromatographic analysis of the following drugs:
    - Diclofenac sodium
    - Ampicillin
    - Doxycycline
    - Sulfadimethoxine
    - Chlortetracycline
7. Kromosil C18 analytical column (150 mm × 4.6 mm I.D., 5 μm particle size), under reversed phase chromatographic conditions, is used for the chromatographic analysis of the following drugs:
    - Nalidixic acid
    - Ciprofloxacin
    - Chloramphenicol
    - Methyl parathion
    - Chlorpyrifos

3.2. MOBILE PHASES (MP)

The chemicals and solvents used for the preparation of mobile phases are of HPLC grade. Milli-Q-water was used throughout. Acetonitrile, orthophosphoric acid, methanol, oxalic acid, acetone and cyclohexane were purchased from Merck Specialities Pvt. Ltd., Mumbai, India. Potassium dihydrogen phosphate, disodium hydrogen phosphate, and glacial acetic acid are from Thermo Fisher Scientific India Pvt. Ltd, Mumbai, India

3.2.1. MP1: The MP1 consists of a mixture of acetonitrile and 0.05M phosphate buffer (pH 4) in the ratio of 40:60 *v/v*. The phosphate buffer (0.05M) was prepared by dissolving 6.804 g of potassium dihydrogen phosphate in water and diluted to

1000 mL with water. The pH was adjusted to 4 with orthophosphoric acid. The MP1 is used for the analysis of ampicillin using method M1.

3.2.2. MP2: The MP2 consists of a mixture of water and methanol in the ratio of 30:70 *v/v*. The MP2 is used for the analysis of diclofenac sodium using method M2.

3.2.3. MP3: The MP3 used for the analysis of chlortetracycline using method M3 is a mixture of water and methanol in the ratio of 40:60 *v/v*.

3.2.4. MP4: The analysis of sulfadimidine using method M4 is performed with MP4 consisting of 1% glacial acetic acid in water and acetonitrile in the ratio of 60:40 *v/v*.

3.2.5. MP5: A mixture of acetonitrile and 0.1 M phosphate buffer at a ratio of 60:40 *v/v* was prepared for the analysis of doxycycline by method M5. 0.1M phosphate buffer was prepared by dissolving 13.608 g of potassium dihydrogen phosphate in water and diluted to 1000 mL with water. The pH was adjusted to 4 with orthophosphoric acid.

3.2.6. MP6: The MP6 consists of a mixture of methanol, acetonitrile, water and glacial acetic acid in the ratio of 2:2:9:0.2 *v/v*. The MP6 is used for the analysis of sulfadimethoxine using method M6.

3.2.7. MP7: The analysis of chlortetracycline using method M7 is performed with MP7 consisting of 0.01M oxalic acid buffer (pH 1.6), acetonitrile and methanol (77:18:5 *v/v*).

3.2.8. MP8: The MP8 consists of a mixture of acetonitrile and 0.05M phosphate buffer (pH 5.1) in the ratio of 30:70 *v/v*. The phosphate buffer (0.05M) was prepared by dissolving 6.804 g of potassium dihydrogen phosphate in water and

diluted to 1000 mL with water. The pH was adjusted to 5.1 with orthophosphoric acid. The MP8 is used for the analysis of nalidixic acid using method M8.

3.2.9. MP9: The MP9 used for the analysis of ciprofloxacin using method M9 is a mixture of acetonitrile and 0.25 M orthophosphoric acid in the ratio of 60:40 *v/v*.

3.2.10. MP10: The analysis of chloramphenicol using method M10 is performed with MP10 consisting of methanol, water and acetonitrile in the ratio of 15:50:35 *v/v*.

3.2.11. MP11: A mixture of acetonitrile, methanol and water at a ratio of 17:13:70 *v/v* was prepared for the analysis of enrofloxacin by method M11.

3.2.12. MP12: The analysis of fenvalerate using method M12 is performed with MP12 consisting of acetonitrile, methanol and orthophosphoric acid (50:40:10 *v/v*).

3.2.13 MP13: The MP13 consists of a mixture of water and methanol (65:35 *v/v*) The MP13 is used for the analysis of methyl parathion using method M13.

3.2.14. MP14: The MP14 consists of a mixture of acetonitrile and 1 mM phosphate buffer (pH 4.5) in the ratio of 85:15 *v/v*. The phosphate buffer (1 mM) was prepared by dissolving 178 mg of disodium hydrogen phosphate in water and diluted to 1000 mL with water. The pH was adjusted to 4.5 with orthophosphoric acid. The MP14 is used for the analysis of chlorpyrifos using method M14.

3.2.15. MP15: The analysis of cypermethrin using method M15 is performed with MP15 consisting of acetone and cyclohexane in the ratio of 5:95 *v/v*.

3.2.16. MP16: The MP16 consists of a mixture of acetonitrile and water in the ratio of 85:15 *v/v*. The MP16 is used for the analysis of oxytetracycline using method M16.

3.2.17. MP17: A mixture of 100 mM ammonium acetate (pH 7.2), water and methanol at a ratio of 10:60:30 *v/v* was prepared for the analysis of chloramphenicol by method M17. The pH of ammonium acetate was adjusted with glacial acetic acid.

3.2.18. MP18: The mobile phase M18 consisted of an acetonitrile, orthophosphoric acid and (70:29.5:0.5 *v/v*). The analysis of permethrin using method M18 is performed with MP18.

Before using, the mobile phases are filtered through a 0.45 μm Millipore membrane filter and degassed with ultrasonic bath sonicator for 15 minutes.

3.3. STOCK STANDARD SOLUTIONS

The bulk samples of the selected drugs were obtained from various companies and were used as received. The mobile phase is used as solvent for the preparation of standard solution of drugs. Stock standard solutions of drugs were prepared by dissolving the accurate weighed amount in a definite volume of respective mobile phases, to get the required concentration (1mg/mL).

- Ampicillin (Medrich Ltd, Bangalore, India)
- Diclofenac sodium (Medrich Ltd, Bangalore, India)
- Chlortetracycline (Natco Pharma Limited, Hyderabad, India)
- Sulfadimidine (Medrich Ltd, Bangalore, India)
- Doxycycline (Natco Pharma Limited, Hyderabad, India)
- Sulfadimethoxine (Natco Pharma Limited, Hyderabad, India)
- Nalidixic (Apex Drugs & Intermediates Limited, Hyderabad, India)
- Ciprofloxacin (Apex Drugs & Intermediates Limited, Hyderabad, India)
- Chloramphenicol (Matrix Laboratories Limited, Hyderabad, India)
- Enrofloxacin (Apex Drugs & Intermediates Limited, Hyderabad, India)
- Fenvalerate (Aimco Pesticides Ltd, Mumbai, India)

- Methyl parathion (Aimco Pesticides Ltd, Mumbai, India)
- Chlorpyrifos (Modern Insecticide Limited, Punjab, India)
- Cypermethrin (Paramount Pesticides Ltd, meerut, India)
- Oxytetracycline (Matrix Laboratories Limited, Hyderabad, India) and
- Promethrin (Luxica Pharma Inc., Bharuch, India)

3.4. SAMPLE COLLECTION

3.4.1. Milk samples

Milk samples were collected from different dairy forms, local milk suppliers in Tenali, Repalle, Bhattiprolu, Indira Nagar, Guntur, Bapatla, Ponnur, Cherukupalli, Vaddeswaram, Mangalagiri, Gudivada, Machilipatnam, Pamaru, Avanigadda and Diviseema in Andhra Pradesh, India. The milk samples were analyzed for the determining the concentration of ampicillin, diclofenac sodium, chlortetracycline and sulfadimidine residues. The samples were packed in polythene bags. The milk packets were labeled, frozen and brought to the laboratory under frozen conditions in foam box containing chiller packs. The samples were stored at -20°C until analysis.

3.4.2. Meat samples

Meat samples such as chicken, mutton and pork were collected from different meat processing plants (Erragadda, Eluru, Tirupathi, Guntur, Vizag and Nellore) of Andhra Pradesh, India. The meat was processed on the deboning table where the chilled carcasses were cut, deboned, trimmed and packed. About 100 g of tissue sample was cut at different period of deboning operations and transferred into presterilized colorless selfsealing polyethylene bags. The bags were labeled, frozen and brought to the laboratory under frozen conditions in foam box containing chiller packs. All the samples were stored at -20°C before analysis.

3.4.3. Fish samples

Fish samples (body weight; 250g±10.5g) were collected from different aqua fields at Bhimavaram, Hyderabad and Pittalavanipalem of Andhra Pradesh, India, and few fish samples from local fish markets in Vijayawada and Machilipatnam of Andhra Pradesh, India. The samples were labeled, frozen and brought to the laboratory under frozen conditions in foam box containing chiller packs. They were stocked into the 500 L capacity circular plastic tanks. All of them belong to *Labeo rohita* species.

3.4.4. Shrimp samples

Live samples of shrimp were collected directly from Nellore, Bapatla and Kaikaluru aqua fields of Andhra Pradesh, India. Shrimp samples were collected from different hotels in Gudiwada, Chirala and Guntur of Andhra Pradesh, India. Oxygen was supplied to live shrimps through an oxygen cylinder. The samples are packed in polythene bags. The samples were labeled and brought to the laboratory under frozen conditions in foam box containing chiller packs. The samples were stored at -20°C until analysis

3.4.5. Fruit samples

Fruit samples (mango and grapes) were collected from local market, fruit tree forms and super markets at Vijayawada, Andhra Pradesh, India. The samples are collected randomly from different shops. From each shop on average 6 fruits were collected. Garden fruits samples were collected from different trees randomly and also from different gardens. The samples were labeled and brought to the laboratory. All the samples were stored at −20°C until analysis.

3.4.6. Vegetable samples

Vegetables such as cabbage, cauliflower and capsicum were directly collected from different local vegetable markets at Guntur, Tenali, Ongle and

Vijayawada of Andhra Pradesh, India. The samples were labeled and brought to the laboratory. All the samples were stored at −20°C until analysis.

3.4.7. Honey samples

Seven different brands of honey samples were collected from Tenali local markets. For each 6 honey samples are collected. Raw honey sample was collected from Repalli. The samples were stored at −20°C until analysis.

3.4.8. Paddy samples

Paddy (with husk) samples were directly collected from various paddy fields at Amalapuram, Tenali, Nellore, Gudiwada, Khammam, Miryalaguda and Kurnool. The samples were labeled and brought to the laboratory. All the samples were stored at −20°C until analysis.

3.5. EXTRACTION OF ANTIBIOTICS AND PESTICIDES FROM FOOD STUFF

3.5.1. Extraction of ampicillin, diclofenac sodium, chlortetracycline and sulfadimidine residues from milk samples

The ampicillin, diclofenac sodium, chlortetracycline and sulfadimidine residues were extracted from milk samples by following the optimized extraction procedure (Carol *et al.,* 2009). Milk sample (5 mL) was taken in 10 mL sterilized screw cap centrifuge tubes and vortex mixed with 20 µL of 45% aqueous solution of formic acid (Sdfine-Chem limited, Mumbai, India). The formic acid precipitates the proteins and lipids in the milk sample. The acidified milk was then centrifuged at 3500 rpm for 20 minutes at 4^{o}C. The clear supernatant phase was taken by a disposable syringe and loaded onto Sampli Q SCX cartridge. The cartridge was dried under vacuum for 1 minute. The cartridge was washed with 2 mL of 5% methanol in water (wash 1), 1 mL of 0.5 M HCl (wash 2) and 2 mL of 20% methanol in water (wash 3). The cartridge was again dried under vacuum for 3 minutes. The cartridge was eluted with 2.5 mL of 5% ammonia (Sdfine-Chem

limited, Mumbai, India) in methanol. The eluate was dried under nitrogen and then reconstituted with 1 mL of mobile phase (MP1-amphicillin; MP2-diclofenac sodium; MP3- chlortetracycline; MP4- sulfadimidine).

3.5.2. Extraction of doxycycline, sulfadimethoxine and chlortetracycline residues from meat samples

The doxycycline, sulfadimethoxine and chlortetracycline residues were extracted from meat samples by following the optimized extraction procedure (; Gentili *et al.,* 2004; Biswas *et al.,* 2007). Frozen tissue samples were thawed and finely diced with scissors. The finely cut samples were blended in a high speed (15,000 rpm) tissue blender for 2 min. A representative portion of this sample (10 g) was weighed into a polypropylene tube and homogenized with 10 mL of Milli-Q water for 1.5 min using Ultra-Turrex T25 tissues homogenizer. An aliquot (0.5 g) of homogenized sample was transferred into a glass test tube and 3 mL of McIIvaine buffer was added. The mixture was vortexed at high speed, incubated for 5 min at room temperature and centrifuged at 3,500 rpm for 10 min in a refrigerated centrifuge. The extraction was repeated by adding 2 mL of McIIvaine buffer [prepared by mixing 139 mL of 0.1 M citric acid (Sdfine-Chem limited, Mumbai, India) solution in 111 mL of 0.2 M Na_2HPO_4 (Thermo Fisher Scientific India Pvt. Ltd, Mumbai, India) solution and the pH was adjusted to 3.85 with citric acid solution] and the supernatant was pooled. The supernatant was filtered and loaded on an Oasis HLB 6 cm^3 polymeric cartridge previously conditioned with 3 mL of methanol and 2 mL of water. The cartridge containing the sample was washed with 5 mL of water. The target drugs (doxycycline, sulfadimethoxine and chlortetracycline) were eluted with 4.5 mL of 0.01 M methanolic oxalic acid (pH 1.8). One milliliter of eluent was filtered through 0.22 μm nylon filter. The sample was subjected to analysis by the proposed methods M5, M6 and M7 for the determination of doxycycline, sulfadimethoxine and chlortetracycline, respectively.

3.5.3. Extraction of nalidixic acid and ciprofloxacin residues from fish samples

The methanol extract of fish protein was prepared with optimized method as described by Hellio *et al.* (2002). The targeted antibiotics (nalidixic acid and ciprofloxacin) from the fish protein were extracted by following the optimized extraction procedure (Ong *et al.,* 2010). 50 g of fish protein was mixed with 50 mL of methanol and homogenized using apolytron homogenizer. The mixture was then centrifuged at 10,000 rpm for 30 minutes. Supernatant was then collected and filtered through Whatman no.1 filter paper. The filtrate was collected and purified using a syringe with 0.22 µm filter. Elutes were collected and stored in refrigerator at 4°C. This sample was used for quantitative and qualitative analysis of target antibiotics, nalidixic acid and ciprofloxacin, in fish samples by the proposed methods M8 and M9, respectively.

3.5.4. Extraction of chloramphenicol and enrofloxacin residues from shrimp samples

The chloramphenicol and enrofloxacin residues from shrimp samples were extracted with optimized extraction method as described by Brisa *et al.* (2012) and Kirbiš and Flajs (2005). Shrimp samples were decapitated and the cuticle was removed manually. The muscle was placed in properly labeled containers. 2.5 g of shrimp muscle was taken and 1.5 mL of Trizma Base solution (pH 9) was added. The tissue was homogenized with a glass rod followed by one min of vortexing. The mixture was allowed to stand for 15 min. and then 4 mL of reagent grade acetonitrile (Merck Specialities Pvt. Ltd., Mumbai, India) was added. The mixture was vortexed. The samples were sonicated for 15 min and centrifuged at 5000 rpm for 30 min. The supernatant was removed with a Pasteur pipette and placed in a 15 mL centrifuge tube. The extraction was repeated with an additional 4 mL of acetonitrile to remove the supernatant and collect the extract. The supernatant was evaporated in a water bath at 45–50°C to a total volume of 2.5 mL. Fat was

removed from the extract by adding 1.5 mL of reagent grade hexane (Merck Specialities Pvt. Ltd., Mumbai, India). The extract was then centrifuged at 5000 rpm for 30 min at 15°C. The upper layer was discarded, and the process was repeated. The remaining aqueous phase was filtered using a using a syringe with 0.22 µm filter. The extract was used for quantitative and qualitative analysis of chloramphenicol and enrofloxacin by the proposed methods M10 and M11, respectively.

3.5.5. Extraction of fenvalerate and methyl parathion residues from fruit samples

The fenvalerate and methyl parathion residues from fruit samples were extracted with optimized extraction method as described by Toth *et al.* (1992). Twenty grams of fruit was collected from the sample and were kept into a conical flask. The sample was thoroughly mixed with 30 mL of dichloromethane (Merck Specialities Pvt. Ltd., Mumbai, India) and 15 g of sodium carbonate (Sdfine-Chem limited, Mumbai, India). The mixture was kept undisturbed for 12 hrs. After that the mixture was filtered through whatmann no.1 filter paper. The tundish was washed with dichloromethane. The filtered liquid phase was taken in open watch glass. Dichloromethane was dried out and 5 mL of methanol was added to extract the target pesticides (fenvalerate and methyl parathion). The extraction was repeated twice with 2 mL of methanol. These extractions were mixed and diluted with methanol to 10 mL, filtered and then subjected to analysis by the proposed methods M12 and M13 for the estimation of fenvalerate and methyl parathion, respectively.

3.5.6. Extraction of chlorpyrifos and cypermethrin residues from vegetable samples

The pesticides such as chlorpyrifos and cypermethrin residues were extracted from vegetable samples with optimized extraction method as described by Hussain and Samia (2010). Twenty grams of vegetable sample was taken and

20 mL of distilled water was added. The mixture was left undisturbed for 15 minutes, after which 50 mL of acetonitrile was added and the sample was homogenized by crushing in a pestle and mortar. The sample was filtered by suction. To the remaining residue on the filter, 20 mL of acetonitrile was added and again the sample was homogenized and filtered by suction. Both filtrates were combined together and the volume was increased to 100 mL by adding acetonitrile. From this solution 20 mL of sample was taken and 10 g of NaCl and 20 mL of 0.5 M phosphate buffer was added and shaken. The solution was left undistributed for removal of the aqueous layer. The organic layer was dried over anhydrous sodium sulphate (Sdfine-Chem limited, Mumbai, India). The dried sample was reconstituted with mobile phases MP14 and M15 for the analysis of chlorpyrifos and cypermethrin by using the proposed methods M14 and M15, respectively.

3.5.7. Extraction of oxytetracycline and chloramphenicol residues from honey samples

The oxytetracycline and chloramphenicol residues from honey samples were extracted with optimized extraction method as described by Pagliuca *et al.* (2002). Five gram honey sample was takena and dissolved in 20 mL of 0.1 M Na_2EDTA-Mcllvaine buffer at pH 4. The Solution was vortexed for 5 minutes, filtered and made ready for solid phase extraction (SPE) clean-up procedure. After extraction, 60 mg of sample was loaded on a SampliQ OPT 3 mL cartridge previously conditioned with 1 mL methanol and 1 mL water. The SPE cartridge was then washed with 10 mL water. Finally, the sample was eluted with 1 mL ethyl acetate (Sdfine-Chem limited, Mumbai, India) directly in sample tube. After evaporating the solvent at 40ºC under nitrogen stream, the residues were reconstituted with 1 mL of mobile phases MP16 and MP17 for the analysis of oxytetracylcine and chloramphenicol with the proposed methods M16 and M17, respectively.

3.5.8. Extraction of permethrin residues from paddy samples

The permethrin residues were extracted from the paddy samples by following the optimized method (Shishovska, *et al.,* 2010; Paranthaman *et al.,* 2012). 50 g of paddy husk was homogenized with 100 mL of acetonitrile. 10 g of sodium chloride (Sdfine-Chem limited, Mumbai, India) was added and again homogenized for 5 minutes. From this 13 mL of supernatant solution was collected and transfered the supernant to 15 mL graduated centrifuge tube and 3 g of sodium sulphate (Sdfine-Chem limited, Mumbai, India) was added. The mixture was mixed well and centrifuged for 5 minutes at high speed. After centrifugation 10 mL of supernatant was collected and nitrogen was added and allowed to evaporate till 5 mL. The target pesticides were eluted with 20 mL of acetonitrile:toluene (3:1) mixture. Later sample was concentrated to 2 mL using a rotary evaporator by adding 2 mL of acetone. The resultant solution was analyzed by the proposed method M18 for the quantification of permethrin.

3.6. GENERAL ASSAY PROCEDURE

After systematic and detailed study of the various parameters involved, as described under results and discussion chapter, the following procedures were recommended for the determination of selected drugs in bulk and selected food stuffs.

3.6.1. Method 1 (M1: Assay of ampicillin)

Working standard solutions equivalent to 2 to 14 µg/mL of ampicillin were prepared by appropriate dilution of the stock standard solution with the mobile phase MP1. Twenty µL aliquot of each solution was injected into the column in triplicate. The mobile phase was pumped from the solvent reservoir to the column at a flow rate of 1 mL/min. The peaks were determined at 254 nm (Figure 18b).

3.6.2. Method 2 (M2: Assay of diclofenac sodium)

Working standard solutions equivalent to 25 to 150 µg/mL of diclofenac sodium were prepared by appropriate dilution of the stock standard solution with the mobile phase MP2. Twenty µL aliquot of each solution was injected into the column in triplicate. The mobile phase was pumped from the solvent reservoir to the column at a flow rate of 1 mL/min and the chromatograms were recorded at 258 nm (Figure 19b).

3.6.3. Method 3 (M3: Assay of chlortetracycline)

Working standard solutions in the range of 10 to 60 µg/mL of chlortetracycline were prepared by appropriate dilution of the stock standard solution with the mobile phase MP3. Twenty µL aliquot of each solution was injected into the column in triplicate. The mobile phase was pumped from the solvent reservoir to the column at a flow rate of 1 mL/min. The peaks were recorded at 254 nm (Figure 20b).

3.6.4. Method 4 (M4: Assay of sulfadimidine)

Working standard solutions equivalent to 5 to 35 µg/mL of sulfadimidine were prepared by appropriate dilution of the stock standard solution with the mobile phase MP4. Twenty µL aliquot of each solution was injected into the column in triplicate. The mobile phase was pumped from the solvent reservoir to the column at a flow rate of 1 mL/min. The peaks were recorded at 260 nm (Figure 21b).

3.6.5. Method 5 (M5: Assay of doxycycline)

The stock standard solution was diluted in the stepwise manner with the mobile phase MP5 to get the working standard solutions equivalent to 5 to 30 µg/mL of doxycycline. Twenty µL aliquot of each solution was injected into the column in triplicate. The mobile phase was pumped from the solvent reservoir to

the column at a flow rate of 1 mL/min. The peaks were recorded at 325 nm (Figure 22b).

3.6.6. Method 6 (M6: Assay of sulfadimethoxine)

Working standard solutions equivalent to 10 to 60 µg/mL of sulfadimethoxine were prepared by appropriate dilution of the stock standard solution with the mobile phase MP6. Twenty µL aliquot of each solution was injected into the column in triplicate. The mobile phase was pumped from the solvent reservoir to the column at a flow rate of 1 mL/min. The peaks were recorded at 332 nm (Figure 23b).

3.6.7. Method 7 (M7: Assay of chlortetracycline)

Working standard solutions equivalent to 2 to 12 µg/mL of chlortetracycline were prepared by appropriate dilution of the stock standard solution with the mobile phase MP7. Twenty µL aliquot of each solution was injected into the column in triplicate. The mobile phase was pumped from the solvent reservoir to the column at a flow rate of 1 mL/min. The peaks were recorded at 354 nm (Figure 24b).

3.6.8. Method 8 (M8: Assay of nalidixic acid)

Working standard solutions equivalent to 0.5 to 3 µg/mL of nalidixic acid were prepared by appropriate dilution of the stock standard solution with the mobile phase MP8. Twenty µL aliquot of each solution was injected into the column in triplicate. The mobile phase was pumped from the solvent reservoir to the column at a flow rate of 1 mL/min. The peaks were recorded at 266 nm (Figure 25b).

3.6.9. Method 9 (M9: Assay of ciprofloxacin)

The stock standard solution was appropriately diluted with the mobile phase M9 to produce working standard solutions in the range 1 to 7 µg/mL of ciprofloxacin. Twenty µL aliquot of each solution was injected into the column in

triplicate. The mobile phase was pumped from the solvent reservoir to the column at a flow rate of 1 mL/min and the chromatograms were recorded at a wavelength of 282 nm (Figure 26b).

3.6.10. Method 10 (M10: Assay of chloramphenicol)

Working standard solutions equivalent to 0.2 to 1.2 µg/mL of chloramphenicol were prepared by appropriate dilution of the stock standard solution with the mobile phase MP10. Twenty µL aliquot of each solution was injected into the column in triplicate. The mobile phase was pumped from the solvent reservoir to the column at a flow rate of 1 mL/min. The peaks were recorded at 246 nm (Figure 27b).

3.6.11. Method 11 (M11: Assay of enrofloxacin)

Working standard solutions equivalent to 0.1 to 0.6 µg/mL of enrofloxacin were prepared by appropriate dilution of the stock standard solution with the mobile phase MP11. Twenty µL aliquot of each solution was injected into the column in triplicate. The mobile phase was pumped from the solvent reservoir to the column at a flow rate of 1 mL/min. The peaks were recorded at 270 nm (Figure 28b).

3.6.12. Method 12 (M12: Assay of fenvalerate)

The series of working standard solutions (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 µg/mL) were prepared by appropriate dilution of the stock standard solution using mobile phase MP12. From each working standard solution 20 µL was injected into the column in triplicate and the peaks were determined at 239 nm (Figure 29b). The flow rate of mobile phase was maintained at 1 mL/min.

3.6.13. Method 13 (M13: Assay of methyl parathion)

Working standard solutions equivalent to 0.2 to 1.2 µg/mL of methyl parathion were prepared by appropriate dilution of the stock standard solution with the mobile phase MP13. Twenty µL aliquot of each solution was injected

into the column in triplicate. The mobile phase was pumped from the solvent reservoir to the column at a flow rate of 1 mL/min. The peaks were recorded at 225 nm (Figure 30b).

3.6.14. Method 14 (M14: Assay of chlorpyrifos)

Working standard solutions equivalent to 2 to 12 µg/mL of chlorpyrifos were prepared by appropriate dilution of the stock standard solution with the mobile phase MP14. Twenty µL aliquot of each solution was injected into the column in triplicate. The mobile phase was pumped from the solvent reservoir to the column at a flow rate of 1 mL/min. The peaks were recorded at 230 nm (Figure 31b).

3.6.15. Method 15 (M15: Assay of cypermethrin)

Working standard solutions equivalent to 0.5 to 3.0 µg/mL of cypermethrin were prepared by appropriate dilution of the stock standard solution with the mobile phase MP15. Twenty µL aliquot of each solution was injected into the column in triplicate. The mobile phase was pumped from the solvent reservoir to the column at a flow rate of 1 mL/min. The peaks were recorded at 254 nm (Figure 32b).

3.6.16. Method 16 (M16: Assay of oxytetracycline)

Working standard solutions equivalent to 1 to 6 µg/mL of oxytetracycline were prepared by appropriate dilution of the stock standard solution with the mobile phase MP16. Twenty µL aliquot of each solution was injected into the column in triplicate. The mobile phase was pumped from the solvent reservoir to the column at a flow rate of 1.5 mL/min. The peaks were recorded at 360 nm (Figure 33b).

3.6.17. Method 17 (M17: Assay of chloramphenicol)

A series of working standard solutions having concentration of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 µg/mL were obtained by diluting the stock standard solution with

the mobile phase MP17. Twenty µL aliquot of each solution was injected into the column in triplicate. The mobile phase was pumped from the solvent reservoir to the column at a flow rate of 0.8 mL/min. The peaks were recorded at 249 nm (Figure 34b).

3.6.18. Method 18 (M18: Assay of permethrin)

Working standard solutions equivalent to 0.25 to 1.5 µg/mL of permethrin were prepared by appropriate dilution of the stock standard solution with the mobile phase MP18. Twenty µL aliquot of each solution was injected into the column in triplicate. The mobile phase was pumped from the solvent reservoir to the column at a flow rate of 0.8 mL/min. The elute was monitored at 215 nm (Figure 35b).

In all the above methods, calibration graph was prepared by plotting the mean peak areas versus nominal concentration of the drug. The concentration of the unknown was read from the calibration graph or computed from the regression equation derived using the mean peak area-concentration data.

3.7. ASSAY OF ANTIBIOTICS AND PESTICIDES IN FOOD STUFFS

3.7.1. Assay of ampicillin, diclofenac sodium, chlortetracycline and sulfadimidine in milk samples

The extract prepared from milk samples, as described in section "Extraction of amphicillin, diclofenac sodium, chlortetracycline and sulfadimidine residues from milk samples", was further diluted appropriately with the respective mobile phases for the analyses of ampicillin, diclofenac sodium, chlortetracycline and sulfadimidine residues by methods M1, M2, M3 and M4, respectively. The milk sample solution was injected into HPLC system in duplicate. The chromatograms were recorded (Figures 18c, 18d, 19c, 19d, 20c, 20d, 21c and 21d). The area under the peak was calculated. The ampicillin, diclofenac sodium, chlortetracycline and sulfadimidine residues content in the milk samples were

calculated using the corresponding calibration curve or corresponding regression equation.

3.7.2. Assay of doxycycline, sulfadimethoxin and chlortetracycline in meat samples

The extract prepared from meat samples, as described in section "Extraction of doxycycline, sulfadimethoxin and chlortetracycline residues from meat samples", was further diluted appropriately with the respective mobile phases for the analyses of doxycycline, sulfadimethoxine and chlortetracycline residues in chicken, mutton and pork by methods M5, M6 and M7, respectively. Twenty µL of the aliquot was injected into the HPLC system after trimming off external fat and fascia in duplicate. The chromatograms were recorded (Figure 22c, 23c & 24c). The area under the peak was calculated. The doxycycline, sulfadimethoxine and chlortetracycline residues in chicken, mutton and pork, respectively were calculated using the corresponding calibration curve or corresponding regression equation.

3.7.3. Assay of nalidixic acid and ciprofloxacin in fish samples

The extract prepared from fish samples, as described in section "Extraction of nalidixic acid and ciprofloxacin residues from fish samples", was further diluted appropriately with the respective mobile phases for the analyses of nalidixic acid and ciprofloxacin residues by methods M8 and M9, respectively. The fish sample solution was injected into HPLC system in duplicate. The chromatograms were recorded (Figure 25c & 26c). The area under the peak was calculated. The concentration of nalidixic acid and ciprofloxacin in the fish samples were calculated using the corresponding calibration curve or corresponding regression equation.

3.7.4. Assay of chloramphenicol and enrofloxacin in shrimp samples

The extract prepared from shrimp samples, as described in section "Extraction of chloramphenicol and enrofloxacin residues from shrimp samples", was further diluted appropriately with the respective mobile phases for the analyses of chloramphenicol and enrofloxacin residues by methods M10 and M11, respectively. The shrimp sample solution was injected into HPLC system in duplicate. The chromatograms were recorded (Figure 27c & 28c). The area under the peak was calculated. The concentration of chloramphenicol and enrofloxacin in the shrimp samples were calculated using the corresponding calibration curve or corresponding regression equation.

3.7.5. Assay of fenvalerate and methyl parathion in fruit samples

The extract prepared from fruit samples, as described in section "Extraction of fenvalerate and methyl parathion residues from fruit samples", was further diluted appropriately with the mobile phases MP12 and MP13 for the analyses of fenvalerate and methyl parathion residues by methods M12 and M13, respectively. The fruit sample solution was injected into HPLC system in duplicate. The chromatograms were recorded (Figure 29c, 29d, 30c & 30d). The area under the peak was calculated. The concentration of fenvalerate and methyl parathion in the fruit samples were calculated using the corresponding calibration curve or corresponding regression equation.

3.7.6. Assay chlorpyrifos and cypermethrin residues in vegetable samples

The extract prepared from vegetable samples, as described in section "Extraction of chlorpyrifos and cypermethrin residues from vegetable samples", was further diluted appropriately with the respective mobile phases for the analyses of chlorpyrifos and cypermethrin residues by methods M14 and M15, respectively. The vegetable sample solution was injected into HPLC system in duplicate. The chromatograms were recorded (Figure 31c, 31d, 31e, 32c, 32d & 32e). The area under the peak was calculated. The concentration of chlorpyrifos

and cypermethrin in the vegetable samples were calculated using the corresponding calibration curve or corresponding regression equation.

3.7.7. Assay of oxytetracycline and chloramphenicol in honey samples

The extract prepared from honey samples, as described in section "Extraction of oxytetracycline and chloramphenicol residues from honey samples", was further diluted appropriately with the mobile phases MP16 and MP17 for the analyses of oxytetracycline and chloramphenicol residues by methods M16 and M17, respectively. The honey sample solution was injected into HPLC system in duplicate. The chromatograms were recorded (Figure 33c & 34c). The area under the peak was calculated. The concentration of oxytetracycline and chloramphenicol in the honey samples were calculated using the corresponding calibration curve or corresponding regression equation.

3.7.8. Assay of permethrin in paddy samples

The extract prepared from paddy samples, as described in section "Extraction of permethrin from paddy samples", was further diluted appropriately with the mobile phases MP18 for the analyses of permethrin residues by method M18. The extract was injected into HPLC system in duplicate. The chromatograms were recorded (Figure 35c & 35d). The area under the peak was calculated. The concentration of permethrin in the paddy samples were calculated using the corresponding calibration curve or corresponding regression equation.