CHAPTER III

EXPERIMENTAL

3.1 Chemicals and Materials

Standards of abamectin, ivermectin and doramectin were purchased from Dr. Ehrenstorfer (Augsburg, Germany). SDS and ethyl 4-hydroxybenzoate were supplied by Sigma-Aldrich (Steinheim, Germany). 1-Butanol, tert-butanol, 4-methyl-2-pentanol, 1-hexanol and *n*-octane were purchased from Fluka (Buchs, Switzerland). Isoamyl alcohol was obtained from Carlo Erba (Val De Reuil, Italy). 2-Butanol was perchased from Analar BDH Ltd. (Dorset, UK). 85 % w/w Phosphoric acid (density = 1.71 g/ml) and all organic solvents at least HPLC grade were obtained from Merck (Darmstadt, Germany). Dodecylbenzene (DB), used as microemulsion marker, was purchased from Sigma-Aldrich (Steinheim, Germany). Commercial products of avermectins were purchased from a market in Thailand, and the actual brand names cannot be disclosed. Water used in this work was Milli-Q water obtained from Millipore (Massachusetts, USA).

3.2 CE Analysis

3.2.1 CE conditions

All CE separations were performed using a Beckman Coulter MDQ-CE system equipped with a photodiode array detector scanning from 190 to 400 nm and monitoring at 214 nm and 245 nm. An uncoated fused silica capillary used is 40.2 cm in length (30 cm to detector) × 50 μm I.D., and thermostated at 25 °C. Voltage was set at -15 kV. A sample solution was introduced by 0.5 psi pressure injection for 3 s. Prior to MEEKC analysis each day, the capillary was rinsed with ethanol, 0.1 M NaOH and the microemulsion buffer for 15 min each. Between consecutive analyses, the capillary was flushed with ethanol for 2 min and then followed with the microemulsion buffer for 2 min. After analysis each day, the capillary was rinsed with

ethanol for 5 min, water for 5 min, 0.1 M NaOH for 10 min and then water for 10 min.

3.2.2 Preparation of microemulsion buffer

A stock solution of 500 mM phosphate buffer at pH 2.5 was prepared by pipetting a desired amount of phosphoric acid into a 150 ml beaker containing approximately 90 ml of water. The solution was adjusted to pH 2.5 with 1.0 M NaOH, and then transferred to a 100 ml volumetric flask. The final solution was made up to 100 ml with water. The microemulsion buffer was prepared by pipetting the appropriate amount of stock aqueous solutions of 500 mM SDS and 500 mM phosphate buffer at pH 2.5 into a 10 ml volumetric flask, followed by pipetting 110 µl of *n*-octane, and 814 µl 1-butanol. The final solution was made up to 10 ml with water. In case of preparation of the microemulsion buffer containing organic co-solvent such as methanol, ethanol, 2-propanol or acetonitrile, the appropriate volume of organic co-solvent was added before addition of water. All microemulsion buffers were sonicated for 30 min to obtain clear and highly stable microemulsion. The microemulsion buffers were filtered through 0.45 µm membrane filters prior to MEEKC analysis.

3.2.3 Preparation of standard solutions

Stock solutions of 5000 ppm abamectin, ivermectin and doramectin were separately prepared by weighing an appropriate amount of each standard and then dissolving this in ethanol. For CE analysis, the working standard solutions containing abamectin, ivermectin and doramectin in a range of 25 to 400 ppm were prepared by pipetting the appropriate amounts of the stock standard solutions, 100 µl of 5000 ppm dodecylbenzene (DB) in ethanol and 100 µl of 5000 ppm ethyl-4-hydroxybenzoate in ethanol, and then diluting the mixture with the microemulsion to have final volume of 5.0 ml. Therefore, each final working standard solution contained 100 ppm DB as a microemulsion marker and 100 ppm ethyl-4-hydroxybenzoate as internal standard (ISTD). The microemulsion used for diluting working solution contained 180 mM SDS, 890 mM 1-butanol and 1.1 % v/v *n*-octane. For HPLC analysis, the working standard solutions containing abamectin, ivermectin and doramectin in a range of 10

to 100 ppm were prepared by pipetting the appropriate amounts of the stock standard solutions and then diluting the mixture with methanol to have final volume of 5.0 ml.

3.3 MEEKC Optimisation

The mixed standard solution of 100 ppm abamectin, ivermectin and doramectin, used for investigation of the suitable MEEKC conditions for separation, was prepared by pipetting the desired amount of the stock standard solutions and then diluting with the microemulsion solution without the phosphate buffer. MEEKC optimization was performed by varying of types and concentrations of organic co-solvents, SDS concentration, types of co-surfactants, temperature and applied voltage. All experiments in this section were performed in duplicate runs.

3.3.1 Effect of types and concentration of organic co-solvents

The microemulsion buffer used in this section contained a 50 mM phosphate buffer at pH 2.5, 180 mM SDS, 890 mM 1-butanol, 1.1 % v/v *n*-octane and appropriate types and amount of organic co-solvent. The organic co-solvent, such as acetonitrile, methanol, ethanol and 2-propanol, at levels of 0 to 35 % v/v were separately added in the microemulsion buffer. Other CE conditions used are previously given in Section 3.2.1. Results are discussed in Section 4.2.1.

3.3.2 Effect of SDS concentration

In this section, the microemulsion buffer used was similar to that in Section 3.3.1, except that 30 % v/v ethanol was added in the buffer, and SDS concentration in a range of 130 to 220 mM were used. Other CE conditions used are previously given in Section 3.2.1. Results are discussed in Section 4.2.2.

3.3.3 Effect of co-surfactant types

MEEKC analysis was carried out using the microemulsion buffer containing a 50 mM phosphate buffer at pH 2.5, 180 mM SDS, 1.1 % v/v *n*-octane, 30 % v/v ethanol and desired types of co-surfactants, such as 1-butanol, 2-butanol, tert-butanol,

isoamylalcohol, 4-methyl-2-pentylalcohol and 1-hexanol. CE conditions used are previously mentioned in Section 3.2.1. Results are discussed in Section 4.2.3

3.3.4 Effect of temperature

The microemulsion buffer used in this section contained a 50 mM phosphate buffer at pH 2.5, 180 mM SDS, 890 mM 1-butanol, 1.1 % v/v *n*-octane and 30 % v/v ethanol. The separation temperatures in a range of 20 to 40 °C were performed. Other CE conditions are shown in Section 3.2.1. Results are given in Section 4.2.4.

3.3.5 Effect of applied voltage

In this section, MEEKC analysis was carried out using the similar microemulsion buffer used in Section 3.3.4. The separation voltages in a range of -15 to -25 kV were applied. Other CE conditions are shown in Section 3.2.1. Results are discussed in Section 4.2.5.

3.3.6 Effect of pressure

Study of effect of pressure applied on MEEKC analysis was carried out using the similar microemulsion buffer used in Section 3.3.4. The pressure in a range of 0.1 to 1.0 psi was applied along the capillary during voltage separation. Other CE conditions are shown in Section 3.2.1. Results are discussed in Section 4.2.6.

3.4 Validation of MEEKC Method

In this section, the microemulsion buffer contained a 50 mM phosphate buffer at pH 2.5, 180 mM SDS, 890 mM 1-butanol, 1.1 % v/v *n*-octane and 30 % v/v ethanol. All MEEKC experiments were carried out using applied voltage of -15 kV, temperature of 25 °C and other conditions as previously mentioned in Section 3.2.1.

3.4.1 Limit of detection (LOD) and limit of quantitation (LOQ)

Various levels of the diluted concentration of avermectins standards were prepared and the ratios of signal-to-noise (*S/N*) were determined using the microemulsion buffer as in Section 3.4 and other conditions as in Section 3.2.1. Limit of detection (LOD) and limit of quantitation (LOQ) for analytes are defined as the analyte concentration giving signal-to-noise of 3 and 10, respectively, and obtained from the electropherograms at 245 nm. Results of LOD and LOQ for avermectins are shown in Section 4.3.1.

3.4.2 Calibration plots

Six levels of the working standard solutions containing abamectin, ivermectin and doramectin in a range of 25 to 400 ppm were prepared by pipetting the appropriate amounts of the stock standard solutions, 100 µl of 5000 ppm dodecylbenzene (DB) in ethanol and 100 µl of 5000 ppm ethyl-4-hydroxybenzoate in ethanol, and then diluting the mixture with the microemulsion to have final volume of 5.0 ml. Therefore, each final working standard solution contained 100 ppm DB as a microemulsion marker and 100 ppm ethyl-4-hydroxybenzoate as internal standard (ISTD). The microemulsion used for diluting working solution contained 180 mM SDS, 890 mM 1-butanol and 1.1 % v/v *n*-octane. Calibration plots were established from the ratio of corrected peak area of the analyte to that of internal standard, $A_{corr,ratio}$, against the analyte concentration for six levels in a range of 25 to 400 mM, where corrected peak area is defined as the peak area divided by migration time. Results are shown in Section 4.3.2.

3.4.3 Accuracy and precision

The effect of sample matrix on accuracy of the method was investigated by spiking a mixture of avermectin standards with known amounts, approximately 2.5-fold and 10-fold of LOQ, in the microemulsion and separately spiking each avermectin with known amounts in the diluted solution of samples containing an interested avermectin. The microemulsion solution used for dilution is similar to Section 3.4.2.

MEEKC analysis was carried out using the similar microemulsion buffer used in Section 3.4 and performed for five runs. Accuracy of this method was reported in terms of recovery (%) of determined amount of analytes spiked in both microemulsion solution and diluted sample solution. Precision was expressed by percentage of relative standard deviation (RSD) of migration time and $A_{corr,ratio}$ obtained. Results are discussed in Section 4.3.3.

3.5 HPLC Analysis

3.5.1 HPLC conditions

All HPLC experiments were carried out on a Waters^{TM} HPLC system equipped with a 600 controller pump, a 996 photodiode array detector scanning from 200-400 nm and monitoring at 245 nm, and a 717plus autosampler. The injection volume was 10 µl. The analytical column was 250 × 4.6 mm I.D., 5 µm Hypersil BDS C_{18} from Thermo Finnigan. Mobile phase and elution conditions used in this work were carried out according to the HPLC analysis of abamectin formulation reported by the Department of Agriculture, the Ministry of Agricultural and Cooperatives, Thailand. Solvents A, B and C are acetonitrile, methanol and water, respectively. The gradient elution conditions with flow rate 1 ml/min and room temperature were initially A:B:C with 60:0:40, programming to 60:30:10 over 40 min, programming to 90:0:10 over 10 min, and programming to 60:0:40 over 1 min and holding for 9 min. The total analysis time is 60 min each run. Before HPLC analysis each day, HPLC column equilibration was accomplished by using the initial mobile phase condition for at least an hour, and the HPLC column was flushed with methanol for an hour after HPLC analysis each day.

3.5.2 Calibration plots

The working standard solutions of avermectins in a range of 10 to 100 ppm were prepared by pipetting the appropriate amounts of the stock standard solution and then diluting the mixed solution with the methanol to have final volume of 5.0 ml. Each of standard concentration was run in duplicate. Results are discussed in Section 4.4.

3.6 Applications to Real Samples

Contents of avermectins in five commercial formulations were determined by MEEKC and HPLC. For MEEKC analysis, stock sample solutions for MEEKC analysis were prepared by weighting the appropriate amounts of samples and then dissolving these with ethanol in a 25.0 ml volumetric flask. The appropriate amounts of stock sample solutions were then diluted, typically 10 fold, with the microemulsion without organic co-solvent in a 5.0 ml volumetric flask, to give the diluted sample solutions containing 100 ppm DB and 100 ppm ISTD. For HPLC analysis, the diluted sample solutions were obtained by diluting stock sample solutions with methanol. All solutions were filtered through 0.45 μm membrane filters prior to MEEKC or HPLC analysis. Table 3.1 shows sampling weight and dilution factor used for MEEKC and HPLC analysis. Results are discussed in Section 4.4.

Table 3.1 Sampling weight of commercial avermectins and dilution factor for analysis by MEEKC and HPLC.

Sample	Sampling weight (g)	Dilution factor for analysis by	
		MEEKC	HPLC
A1	3.0508	10	25
A2	3.1321	10	25
A3	2.6982	10	25
D1	3.6368	10	25
I1	3.4472	10	20