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Molecularly imprinted solid-phase extraction for the selective determination of valnemulin in feeds with high performance liquid chromatography

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ABSTRACT

A simple, sensitive and reproducible high performance liquid chromatographic method was developed for determining valnemulin in feeds. Feed samples were extracted with ethyl acetate under alkaline condition, cleaned up by molecularly imprinted solid-phase extraction, and analyzed by high performance liquid chromatography with ultraviolet detection. The characteristics of the synthesized polymer were evaluated and the loading capacity of the polymer was about 1000 μg analyte/g imprinted polymer. The new procedure for the feed sample cleanup using the prepared polymer cartridge gave higher recoveries and fewer matrix interferences. The assay exhibited a linear dynamic range of 5.0–200 mg kg^{-1} with the correlation coefficient above 0.9993. Recoveries of valnemulin from feed samples spiked at 5.0, 20 and 50 mg kg^{-1} ranged between 76.0% and 94.4% with relative standard deviations of less than 9%. The limit of detection for valnemulin in feeds was 1 mg kg^{-1}.

1. Introduction

Valnemulin (VAL) is a new semisynthetic pleuromutilin derivative related to tiamulin (Fig. 1), which acts by inhibition of bacterial protein synthesis [1–3], and has effective antimicrobial activities against mycoplasma and brachyspira [4–7]. Valnemulin is widely used as a feed additive to promote growth of the food-producing animals, and, however, few methods have been published for the determination of valnemulin in various matrices [8–10]. For food safety concerns, it is necessary to establish an ideal analytical method for monitoring and detecting valnemulin in feeds. Because of the complex feed samples, it is difficult to find a proper cleanup procedure in some cases. Some extracts from the cleanup procedure using conventional solid-phase extraction (SPE) columns, such as C_{18}, MCX, etc., may not be clean enough for chromatographic analysis. In recent years, the cleanup procedure based on molecularly imprinted polymer (MIP) SPE seems to circumvent the drawbacks of traditional SPE techniques [11–16].

A molecularly imprinted polymer is synthesized in the presence of a template molecule and consists of a highly cross-linked polymer network. After the polymerization, the template is removed by washing, leaving a polymer network with strategically positioned functional groups in binding sites that are complementary in size and shape to the template molecule. These binding sites have the potential to rebind with the template molecule or other molecules, which have a similar molecular structure to the template molecule, in a strong and selective way. MIP is highly selective to capture the target analyte as the antibody, more stable in harsh conditions (organic solvents, extreme pH and high temperature, etc.), and can be re-used [17–19].

In this study, a novel MIP was synthesized using an intermediate of valnemulin (Pre-VAL, Fig. 1) as the mimic template, methacrylic acid as the monomer, ethylene glycol dimethacrylic as the cross-linker in acetonitrile solvent with 2,2′-azobisisobutyronitrile as initiator. The imprinted polymer was successfully applied to purify the extracts of feed samples. Finally, a simple, sensitive and reproducible high performance liquid chromatographic (HPLC) method for the selective determination of valnemulin in feeds with molecularly imprinted solid-phase extraction (MISPE) protocol was developed.

2. Experimental

2.1. Reagents and standards

Valnemulin hydrochloride (purity 97%) was supplied by the Guangdong Dahuanong Animal Health Products Co. Ltd. (Guangdong, China). Valnemulin hydrogen tartrate reference standard (European pharmacopoeia, BP907-F67029, purity 98.7%) was obtained from J&K Chemical Ltd. (Beijing, China). Methacrylic

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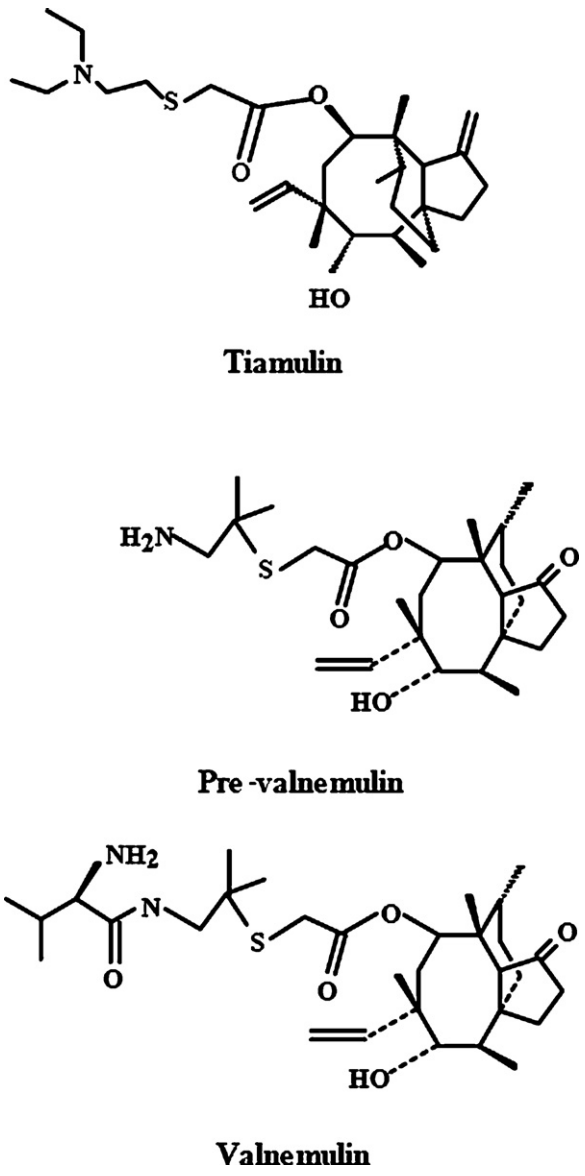

Fig. 1. Chemical structures of valnemulin, pre-valnemulin and tiamulin.

acid (MAA) and acrylamide were purchased from Kermel Chemical Reagents Development Center (Tianjin, China). Ethylene glycol dimethacrylate (EGDMA), 2-hydroxyethyl methacrylate, 2-vinyl pyridine and 4-vinyl pyridine were purchased from Sigma–Aldrich (St. Louis, CA, USA). Active carbon was used to remove inhibitors from all of the monomers and EGDMA before use. 2,2′-azobisisobutyronitrile (AIBN) from Kermel Chemical Reagents Development Center (Tianjin, China) was recrystallized from methanol prior to use. Acetonitrile and methanol were of HPLC grade and purchased from Fisher Scientific (Fair lawn, New Jersey, USA). Ammonium acetate was of HPLC grade and obtained from Tedia (Fairfield, Connecticut, USA). All the other reagents used in the experiment were of analytical grade and were supplied by Guangzhou Chemical Reagent Factory (Guangzhou, China). MCX (60 mg/3 mL) and C_{18} (500 mg/3 mL) solid phase extraction cartridges were purchased from Supelco Inc. (Bellefonte, PA, USA).

Stock solution of VAL was prepared by dissolving 10 mg of VAL in 10 mL methanol and stored in the dark at 4 °C for use within 1 month. The working solutions were prepared by direct dilution of the stock solution with methanol immediately before use.

2.2. Apparatus

Polymerization was carried out in an electric-thermostatic water bath (Changzhou Guohua electrical equipment Company, China). ZD80 Vacuum drying oven was purchased from Kaihong scientific technology Inc. (Beijing, China); KQ-100DE Ultrasonic generator was purchased from Kunshan Instruments Inc. (Kunshan, China); Universal 32R Centrifuge was purchased from Hettich Inc. (Tuttlingen, Germany). Scanning electron micrograph of the MIP was obtained with a FEI XL 30 ESEM (Philips, Netherlands). HPLC analysis was carried out using Waters 2695 Separations Module and Waters 2487 Dual λ Absorbance Detector (Waters, CA, USA). FT-IR spectra in KBr were recorded on a Nicolet 170 SX spectrometer (Nicolet, CA, USA).

2.3. Preparation of the imprinted polymer

The mimic template (Pre-VAL, 1.0 mmol), was dissolved with 6 mL acetonitrile in a 15 mL polypropylene tube fitted with a screw cap. The functional monomer (MAA, 4 mmol) was then added. The tube was sonicated for 5 min and pre-polymerized at 4 °C for 2 h to facilitate template-monomer complex formation. Thereafter, the cross-linking monomer (EGDMA, 20 mmol) and the initiator (AIBN, 20 mg) were added, and the resultant solution was cooled on an ice bath and degassed with oxygen-free nitrogen for 5 min before being sealed under nitrogen. The polymerization was carried out at 60 °C in a thermostatic water bath for 24 h. After polymerization, the polymer monolith was crushed, mechanically ground and sieved to obtain the imprinted particles in the size range of 38–75 μm, which were then suspended by methanol until the upper solution became clear. Finally, the particles were packed into a 15 mm × 200 mm glass column with a piston and washed with 200 mL methanol–acetic acid (90:10, v/v) at a flow rate of less than 1 mL min^{-1} to remove the template and conditioned with 100 mL methanol. The particles were dried under vacuum at 60 °C for 24 h and stored at desiccators for further use. In order to verify that retention of template was due to molecular recognition and not to non-specific binding, a control, non-imprinted polymer (NIP) was prepared as the same procedure, including washing, but with the omission of the template (Pre-VAL).

2.4. HPLC analysis

Chromatographic separation was performed using Agilent Extend-C_{18} column (4.6 mm × 250 mm, 5 μm). The mobile phase consisted of phosphate buffer solution (0. 94 g L^{-1} disodium hydrogen phosphate and 8.7 g L^{-1} potassium dihydrogen phosphate, pH 2.5) (A) and acetonitrile (B). The equal elution program was 62% B with a flow rate of 1.0 mL min^{-1}, and the detection wavelength was 210 nm.

2.5. Optimization of the MISPE

The MIP cartridge was conditioned sequentially with 3 mL of methanol, 3 mL of water purified with Milli-Q system (Millipore, MAT, USA) and 3 mL of 2% formic acid aqueous solution. The common organic solvent, such as methanol, acetonitrile, ethyl acetate or chloroform, deionized water containing 50%, 60%, 70%, and 80% (v/v) of methanol (or acetonitrile), and formic acid (0.5%, 1.0%, 2.0% and 3.0%, v/v) aqueous solution was selected as loading solution, respectively. 3 mL of the corresponding tested solution containing 20 μg of VAL was loaded onto MIP and NIP cartridge, respectively. After loading, the cartridge was washed with 3 mL of water, then 5 mL of acetonitrile (methanol) and a full vacuum was applied through the cartridge for 5 min to remove residual solution. Finally, the elution was performed by passing 5 mL of acetic acid/methanol

(1:19, v/v) or methanol containing 3%, 4%, 5%, 6% and 8% (v/v) of ammonium hydroxide. The fractions collected after the loading, washing, and elution steps were evaporated to dryness at 50 °C using a stream of nitrogen gas and reconstituted with methanol prior to analysis for VAL by HPLC.

2.6. Sample pretreatment

2.6.1. Extraction

An amount of 1.0 g spiked feed sample was accurately weighed into a 50 mL polypropylene centrifuge tube. The sample was incubated at room temperature for about 30 min before proceeding. 5 mL of 1% ammonium hydroxide solution and 5 mL of ethyl acetate were added into the tube. After vortexing for 1 min, the tube was shaken vigorously on the vibrator for 10 min, and then centrifuged at 4 °C, 7500 rpm for 10 min. The upper organic layer was then transferred to another glass tube. The lower aqueous layer was extracted once again with 5 mL of ethyl acetate. The extracts were combined, evaporated to dryness in vacuum at 50 °C. The residue was recovered with 3 mL of 2% formic acid aqueous solution for cleanup.

2.6.2. Cleanup

MISPE (NISPE) cartridge was conditioned with 3 mL of methanol, 3 mL of deionized water and 3 mL of 2% formic acid aqueous solution. The sample extracts were transferred to the cartridge. Then, the cartridge was washed sequentially with 3 mL of water and 5 mL of acetonitrile. Air was passed through the cartridge for 5 min. Finally, the analyte was eluted with 5 mL of methanol containing 5% (v/v) of ammonia hydroxide. The elution was evaporated to dryness under a gentle nitrogen stream at 50 °C. The residue was dissolved with 1 mL of methanol and filtered through a 0.45 μm syringe filter for further HPLC analysis.

For MCX cartridge, the SPE steps are the same as the MISPE steps except for using methanol instead of acetonitrile in washing step. For C_{18} cartridge, the SPE steps are also the same as the MISPE steps except for no acetonitrile washing and eluting analyte with methanol.

3. Results and discussion

3.1. Synthesis and characteristics of the MIP

The application of MIPs as sorbents in SPE is limited because the possibility of leaking of residual template molecules that remains trapped in the polymers after they have been washed extensively. If the MIPs are not completely template free, then their use might compromise quantitation if trace level analysis is required [20]. To date, the most successful way to overcome this problem is the use of an analogue of the target molecule during MIP design and production, known as mimic molecularly imprinted polymer [21–23]. In the current study, in order to circumvent the problem of template bleeding, Pre-VAL, a structural analogue of VAL (its intermediate), was selected as template molecule in the synthesis of MIP.

For obtaining imprinted material capable of binding VAL selectively from hydrophilic matrices, MIP using MAA, 2-hydroxyethyl methacrylate, acrylamide, 2-vinyl pyridine and 4-vinyl pyridine as monomer was synthesized in the porogens including methanol, acetonitrile, acetone, ethyl acetate, chloroform and dimethyl formamide, respectively. The results revealed that the MIP prepared only using MAA as monomer in acetonitrile showed good molecular recognition ability to VAL. However, The MIP prepared in other polymerization systems showed poor specific recognition to VAL. Thus, the carboxyl group of MAA in the polymer is proposed to play an important role in the specific recognition step. The optimized formula of the MIP prepared was 1:4:20:20:6 (mmol/mmol/mmol/mg/mL, Pre-VAL/MAA/EGDMA/AIBN/acetonitrile).

The MIP particles prepared with the optimized formula were tested with scanning electron microscopy. A porous surface in the imprinted polymer indicated that the MIP was a potential sorbent of SPE for separation and enrichment of target analytes from sample matrix.

The infra-red (IR) spectrum of the synthesized polymer was tested. Characteristic bands of carboxyl group clearly appeared in IR spectra of the polymer at 3444 cm^{-1}, 1728 cm^{-1}, and 1260 cm^{-1}. But the specific sharp peak at 1620 cm^{-1} (stretching vibration of vinylic C=C bonds) was very weak, so it suggested that the C=C bond should be disappeared after polymerization.

3.2. Binding capacity of the MIP

Firstly, the amount of MIP using for a MISPE cartridge was optimized. The experiment showed that similar recoveries above 80% were obtained when both of 150 mg and 200 mg MIP were packed into cartridges, while low recovery was obtained when 100 mg MIP was packed. The recovery below 40% was obtained when 50 mg MIP was packed. So the cartridge packed 150 mg MIP was used for the following experiments.

The cartridge binding capacity was calculated as reported in the literature [24]. It is expressed as the absolute amount of analyte loaded into the cartridge in conditions in which more than 1% (w/w) of the sample is not retained by the stationary phase. Operatively, this was attained by loading increased volumes (an aliquot of 1 mL) of 10 μg mL^{-1} VAL solution (2% formic acid aqueous solution) and measuring the analyte not retained by the cartridge. During the loading step some leaching was observed when 120 μg (12 mL × 1 mL) of analyte were introduced into the cartridge, while the 1% of the total was reached when up to 150 μg (15 mL × 1 mL) of analyte were added. As loading capacity is about 1000 μg analyte/g imprinted polymer, the imprinted polymer can be considered suitable for retaining large amounts of analyte without any loss when a cartridge is loaded with aqueous samples.

3.3. Optimization of the MISPE

Due to the ability of MIP to work in aqueous and organic solutions, the main solvents, which were applied to extract VAL from biological and feed samples, were used as the loading solvents of MISPE. It was experimentally determined that poor recoveries (less than 40%) were obtained from both of MIP and NIP cartridges when methanol, ethyl acetate or chloroform was employed, respectively. The mean recoveries of MIP and NIP were also not significantly different. However, when acetonitrile was employed, recoveries obtained from MIP were more than 80%, while those obtained from NIP were less than 40%. The other hydrophilic solutions designed were also investigated. As shown in Fig. 2, all the experimental solutions containing methanol were not ideal as loading solvents, and recoveries of VAL were lower than 40%. All the aqueous solutions containing acetonitrile obtained good recoveries as well as 0.5% and 1.0% formic acid aqueous solutions. The 2.0% formic acid aqueous solution was the best loading solvent. Under this condition, recoveries of VAL on MIP cartridge were above 85%, and however, recoveries on NIP cartridge were below 45%. Retention on NIP cartridge was much lower than that on MIP cartridge, which suggested that the polymers have been successfully imprinted.

In order to obtain the best specificity and complete recovery, different washing solvents including methanol, acetonitrile and acetone were investigated. It was found that methanol was not only able to wash out the interfering compounds presented in samples, but also able to wash out most of VAL bound to both MIP and NIP. Acetone was able to wash out hydrophobic interfering substances,

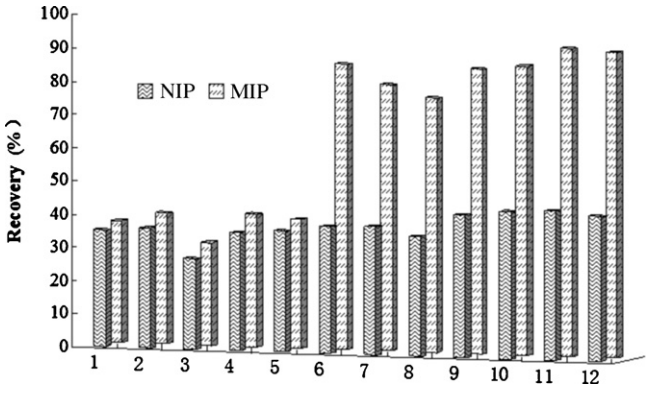

Fig. 2. Recoveries of VAL on MIP and NIP cartridges for different loading solutions (1, 2, 3, 4 and 5 represent methanol, ethyl acetate, chloroform, 50% and 80% methanol in water, respectively; 6, 7 and 8 represent pure acetonitrile, 50% and 80% acetonitrile aqueous solution, respectively; 9, 10 11 and 12 represent 0.5%, 1.0%, 2.0% and 3.0% formic acid in water, respectively).

but unable to wash out VAL bound to both MIP and NIP indicating that the interactions between VAL and the polymers were non-specific under these conditions. However, most of VAL bound to NIP could be removed by acetonitrile and without significant loss of VAL on MIP. It was suggested that acetonitrile increases the specificity of the binding between VAL and MIP and, at the same time, disrupt non-specific interactions with NIP. So acetonitrile was selected as the washing solvent because it resulted in the best selectivity and good retention of VAL on the MIP cartridge.

On the basis of the experimental results of the loading and washing steps, the optimization of the elution step was performed using methanol containing acetic acid (5%, v/v) or ammonia (3%, 4%, 5%, 6% and 8%, v/v) as the main eluents. The results showed that the mean recoveries more than 70% were obtained for all the upper eluents, and the highest recovery (more than 85%) was obtained when using 5 mL of 5% (v/v) ammonia in methanol solution as eluting solvent.

Table 1
Extract recoveries of valnemulin spiked at 20 mg kg^{-1} in feed samples (n = 3).

Extraction solvents	Mean recovery ± SD (%)		
	Feed A	Feed B	Feed C
Methanol	40.0 ± 3.2	32.85 ± 3.4	30.5 ± 6.9
Acetonitrile	37.3 ± 5.4	40.5 ± 2.5	36.3 ± 4.6
Chloroform + 1% ammonia	87.8 ± 2.6	85.2 ± 4.0	75.0 ± 3.5
Ethyl acetate + 1% ammonia	91.9 ± 4.4	88.6 ± 2.8	76.8 ± 2.5

Note: SD represents standard deviation.

3.4. Selection of sample extraction solvents

VAL is extracted generally using the solvents, such as ethyl acetate, methanol, and acetonitrile. Considering the loading solvent recommendation of MISPE cartridge, methanol, acetonitrile, chloroform, and ethyl acetate were examined for the extraction efficiency. Different modes of extraction with different solvent systems were used to extract VAL from feed samples spiked at 20 mg kg^{-1}. Table 1 depicts the obtained recoveries of VAL for three kinds of feed samples (one premix, two formulated feeds). When methanol and acetonitrile were used as the extraction solvents, low recoveries of VAL were obtained. Higher recoveries from three feed samples were obtained if using ethyl acetate or chloroform as the extraction solvent in alkaline condition. Because ethyl acetate is less toxic than chloroform, therefore, ethyl acetate was chosen as extraction solvent in this study.

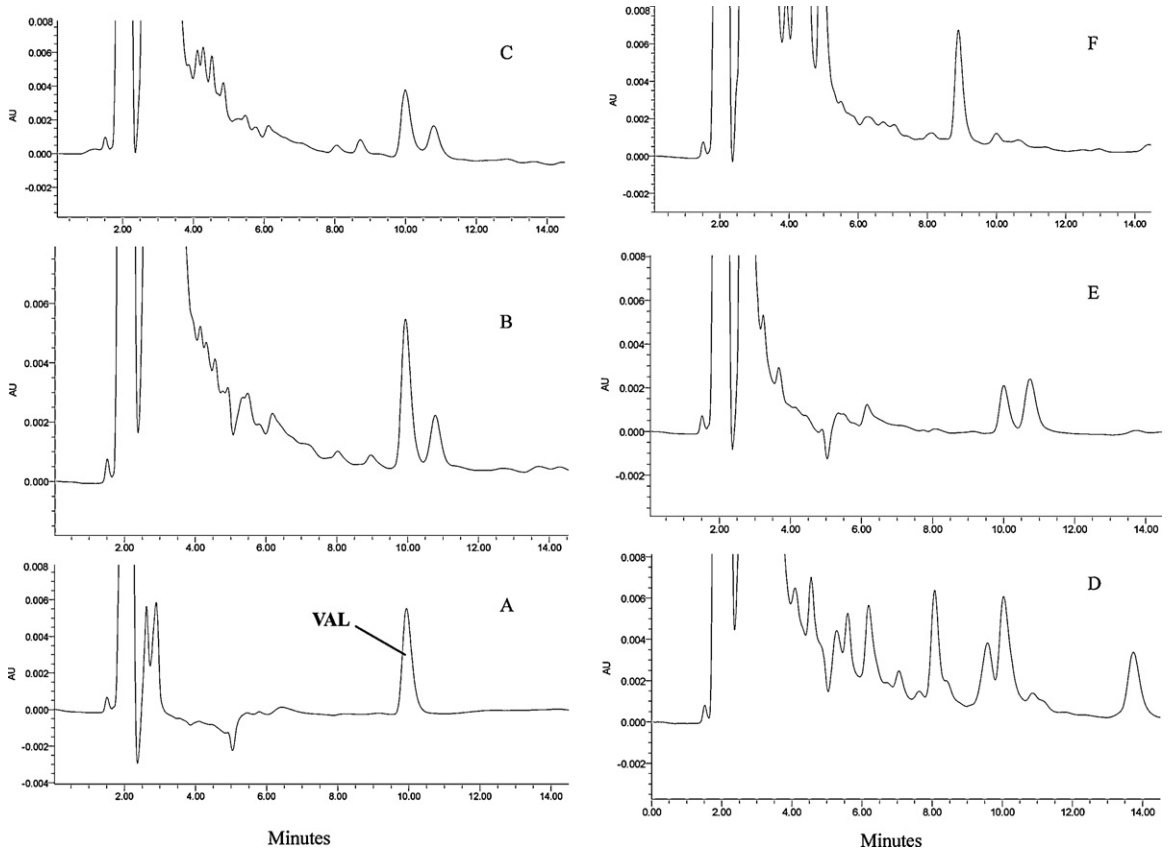

Fig. 3. Typical chromatograms of valnemulin spiked at 20 mg kg^{-1} in feed samples (A: 20 μg mL^{-1} valnemulin, B: MIP, C: NIP, D: MCX, E: C_{18}, F: feed blank).

Table 2
Recoveries of valnemulin from spiked feed samples ($n = 5$).

Feed sample	Spiked level (mg kg^{-1})	Mean recovery (RSD) %			
		SPE cartridge			
		MISPE	NISPE	MCX	C_{18}
Feed A	5.00	87.9 (3.5)	32.5 (7.8)	70.0 (9.2)	61.8 (5.4)
	20.0	88.6 (2.8)	40.2 (5.6)	66.4 (7.0)	62.7 (4.2)
	50.0	94.4 (2.3)	37.9 (4.4)	68.7 (6.7)	69.5 (5.0)
Feed B	5.00	82.0 (2.8)	32.7 (6.3)	57.9 (8.0)	56.2 (4.9)
	20.0	83.3 (3.4)	30.6 (3.5)	58.1 (5.9)	58.0 (6.2)
	50.0	86.8 (2.0)	28.3 (4.0)	59.6 (7.2)	54.4 (6.5)
Feed C	5.00	76.0 (8.2)	30.7 (8.7)	57.4 (6.3)	59.0 (4.2)
	20.0	78.4 (5.3)	34.5 (7.4)	58.0 (5.2)	56.0 (6.3)
	50.0	81.9 (4.7)	24.6 (5.5)	56.8 (7.0)	58.5 (6.8)

Note: RSD represents relative standard deviation.

It should be noted that not all of the optimized loading solvents above-mentioned are suitable for real sample. Good recoveries (see Fig. 2) could be obtained if VAL was dissolved in pure acetonitrile or its aqueous solution (as loading solvent). However, for real feed samples, when the extracts of ethyl acetate were evaporated and then the residues were dissolved with acetonitrile or its aqueous solution (as loading solvent), low recoveries (less than 50%) of VAL were obtained from both MIP and NIP cartridges. When the residues were dissolved in 2% formic acid aqueous solution (as loading solvent), high recoveries (more than 80%) were still obtained from MIP cartridge, while low recoveries obtained from NIP cartridge. There are significant differences in recoveries between MIP and NIP cartridges.

3.5. Validation of the method

The validation of specificity, linearity, limit of detection, recovery and precision for the proposed method were determined. Specificity was checked by analyzing twenty blank feed samples. No interfering peaks could be detected at the retention time of VAL (Fig. 3). The linearity and regression study were performed for VAL standard graph to generate calibration curve. The high correlation coefficient ($r = 0.9993$) indicated good linearity over the concentrations ranged from 5.0 to 200 mg kg^{-1} for VAL in feeds. For the recovery study, each feed samples spiked at 5.0, 20 and 50 mg kg^{-1} were used for validation of the extraction procedure and MISPE cleanup. The peak areas for spiked samples were compared with those of standards to determine the recovery. Table 2 shows the results of recovery and repeatability of the method at spiked levels. Recoveries of VAL on MIP cartridge from three kinds of feed samples were between 76.0% and 94.4% with relative standard deviations of less than 9%. However, all the recoveries on NIP, MCX and C_{18} cartridges were no more than 70%. The limit of detection (LOD) was calculated in blank extracts as the lowest analyte concentration that yielded a signal-to-noise (S/N) ratio of 3. The LOD was 1.0 mg kg^{-1}. The data reported indicate that the above method for the analysis of VAL in feed samples can achieve good recovery and repeatability.

4. Conclusions

In this paper, a novel MIP of valnemulin was synthesized by bulk polymerization using an intermediate of valnemulin as the mimic template molecule. Through evaluated in a series of SPE experiments, the polymer exhibited good specific cross-reactivity and selective ability to VAL. An optimized procedure for the cleanup and enrichment of VAL from feed samples using the MIP particles as new adsorbents in SPE was successfully investigated. A sensitive and reproducible SPE-HPLC method based on MIP was developed for routine analysis of VAL in complex feeds

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