

# MOLECULARLY-IMPRINTED MATERIALS FOR POTENTIOMETRIC TRANSDUCTION: APPLICATION TO THE ANTIBIOTIC ENROFLOXACIN

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*Enrofloxacin (ENR) is an antimicrobial used both in humans and in food producing species. Its control is required in farmed species and their surroundings in order to reduce the prevalence of antibiotic resistant bacteria. Thus, a new biomimetic sensor enrofloxacin is presented. An artificial host was imprinted in specific polymers. These were dispersed in 2-nitrophenyloctyl ether and entrapped in a poly(vinyl chloride) matrix. The potentiometric sensors exhibited a near-Nernstian response. Slopes expressing  $mV/\Delta \log([ENR]/M)$  varied within 48–63. The detection limits ranged from 0.28 to  $1.01 \text{ } \mu\text{g mL}^{-1}$ . Sensors were independent from the pH of test solutions within 4–7. Good selectivity was observed toward potassium, calcium, barium, magnesium, glycine, ascorbic acid, creatinine, norfloxacin, ciprofloxacin, and tetracycline. In flowing media, the biomimetic sensors presented good reproducibility (RSD of  $\pm 0.7\%$ ), fast response, good sensitivity ( $47 \text{ mV}/\Delta \log([ENR]/M)$ ), wide linear range ( $1.0 \times 10^{-5}$ – $1.0 \times 10^{-3} \text{ M}$ ), low detection limit ( $0.91 \text{ } \mu\text{g mL}^{-1}$ ), and a stable baseline for a  $5 \times 10^{-2} \text{ M}$  acetate buffer (pH 4.7) carrier. The sensors were used to analyze fish samples. The method offered the advantages of simplicity, accuracy, and automation feasibility. The sensing membrane may contribute to the development of small devices allowing in vivo measurements of enrofloxacin or parent-drugs.*

**Keywords:** Enrofloxacin; Fia; Fish; Molecularly-imprinted sensors; Potentiometry

## INTRODUCTION

Industrial aquaculture is a rapidly growing industry in many developed and developing countries (Cabello 2006). Significant growth of food fish production has been observed over the past decade due to the prevention or elimination of fish diseases. The introduction of veterinary medicines such as antimicrobials in the food production area led to this scenario. However, the wide use of antibiotics in aquaculture led to environmental and food spread of antimicrobials, and may result in the emergence of antibiotic-resistant bacteria in aquaculture environments (Cabello 2006; Maki et al. 2008). For food safety purposes, fish samples must be subject to rigorous and frequent controls that ensure that residues of antimicrobials are below the maximum legal levels (EEC 377/90 2005).

Enrofloxacin (ENR) is a quinolone drug authorized for use in food animal production (EEC 377/90 2005). It is one of the most important antibacterial chemicals against Gram-negative and Gram-positive bacteria (Crumplin 1986), by impairing the bacterial enzyme gyrase that plays a major role in DNA replication, which blocks the synthesis of certain DNA sections and leads to the death of the bacteria (Scheer 1987). Enrofloxacin is one of the several antimicrobials administered to fish in the aquaculture environment; however, its residues are potentially persistent (Grönlund and Bengtsson 2001) and may be found in fish (Xu et al. 2006; Hormazabal et al. 1991), which necessitates that fish meat be subject to routine and rigorous control by means of suitable analytical techniques.

Analytical procedures suggested in literature for ENR utilized microbiological methods (Cabello 2004; Park et al. 2007; Pearson and Inglis 1993); liquid-chromatographic techniques with mass spectrometry (MS) (Dufresne et al. 2007; Hernando et al. 2006; Hatano 2004; Turnipseed et al. 2003; Johnston, Mackay, and Croft 2002; Turnipseed et al. 1998; Scheider et al. 2005); fluorescence (Schneider, Darwish, and Freeman 2007; Verdon et al. 2005; Lucchetti et al. 2004; Ramos et al.

2003; Roybal et al. 2002; Hernandez-Arteseros et al. 2000; Horie 1994); and/or with a combination of UV, diode-array detector (DAD), or MS (Park et al. 2007; Horie 1995; Schneider et al. 2005); or voltammetric assays (El-Maali 2004). The former is unsuitable for routine control procedures because each trial may take several days and laboratories require proper facilities to handle biological compounds safely. Chromatographic techniques are accurate, precise, and robust, but they are not as expeditious as required for routine control purposes. They may also contribute to the emission of effluents of high toxicity. The same comments may apply to the electrophoretic-based procedures reported in literature (Juan-Garcia, Font, and Pico 2007; McCourt, Bordin, and Rodriguez 2003; Wang, Wu, and Xie 2005). Other methods are based on immunoassays (Kato et al. 2007; Huet et al. 2006; Bucknall et al. 2003; Holtzapple, Buckley, and Stanker 1997; Duan and Yuan 2001); although they provide specific responses, the overall procedure is long and too expensive for routine analytical measurements. Alternative and advantageous methods should rely on expeditious and efficient procedures providing highly specific and sensitive measurements, such as those employing ion-selective electrodes (ISEs). They offer high precision and rapidity, low cost of analysis, enhanced selectivity, and sensitivity over a wide range of concentrations (Cosofret and Buck 1993; Bakker et al. 1994; Kisiel, Michalska, and Maksymiuk 2007). To our knowledge, there are no ISEs reported for ENR.

In terms of selectivity and sensitivity, the most vital component of a potentiometric sensor is the ionophore or the ion carrier, as its binding to the target ion is the molecular-level phenomenon sensed by an ISE (Faridbod et al. 2008). Ion exchangers and neutral macrocyclic compounds have been the “vital components” employed over the past decades for polymeric membrane potentiometric transduction, but the design

of sensing materials that are complementary to the size and charge of a particular ion can lead to very selective interactions, thus enhancing the selectivity of the sensing unit.

Molecularly imprinted polymers (MIPs) can be easily tailored with selectivity for a guest molecule (Wulff 2002; Vlatakis et al. 1993; Mayes and Whitcombe 2005; Rimmer 1998; Busi et al. 2004; Katz and Davis 2000). Their stability at extremes of pH and temperature, high mechanical strength, low cost, and reusability have led to the development of various MIP applications, including chromatography (Hosoya et al. 1998; Sellergren 1994; Peter, Schweitz, and Nilsson 2003), artificial antibodies (Lavignac, Allender, and Brain 2004; Nilsson et al. 1994; Ye and Mosbach 2001), chemical sensors (Marx et al. 2004; Hirayama et al. 2002; Kriz, Ramstrom, and Mosbach 1997), and solid-phase extraction (SPE) (Andersson 2000; Lanza and Sellergren 2001; Ariffin et al. 2007). Although MIPs may also be used as sensing materials of ISEs (Kamel et al. 2008), only few works report their use as potentiometric sensors (Kamel et al. 2008; Hutchins and Bachas 1995). The advantage of the association of MIP to ISEs is the avoidance of the need for template extraction from the host-tailored particle. This extraction may leave vacant recognition sites, ready for binding, which is a typical source of uncertainty in the determination or a sensitivity-limiting factor. In addition, there is no size restriction on the template compound because it does not have to diffuse through the membrane.

The essential part of ISE is the ion-selective membrane that is made of plasticized PVC and is hydrophobic and immiscible with water. The potential difference, across the membrane, results from the transfer of the ionized analyte across the interface between the sample and membrane phase. Nernstian responses can be obtained when the primary ion is the only major ion that is selectively transferred across the interface between the two phases (Amemiya 2007). In the proposed electrode, the selectivity was achieved by doping the membranes with MIP particles that, in principal, may act as neutral or charged ionophores that selectively and reversibly form complexes with the analyte.

Typically, MIP-based sensors are fabricated by assembling MIP materials onto the surface of the transducer, and thus the analyte binding is transformed into a measurable signal. In principle, many physical measurements such as electrochemical voltammetry, fluorescence, piezoelectricity, and surface plasmon resonance can be used for signal detection in MIP-based sensors (Guan et al. 2008; Bossi et al. 2007).

The present work describes the development of ENR MIP-based ISEs. The sensor is synthesized with methacrylic acid (MAA) and/or 2-vinyl pyridine (VP)=MAA functional monomers, cross-linked by ethylene glycol dimethacrylic acid (EGDMA) within the template molecule. The sensing materials replace the conventional ionophores in the selective membranes and are dispersed in a PVC matrix plasticized with *o*-nitrophenyl octyl ether (*o*NPOE). The sensors are evaluated in steady-state and flowing media, and applied to the analysis of fish.

## EXPERIMENTAL

### Apparatus and Chemicals

All potential measurements were made by a Crison mpH 2002 decimilivoltammeter ( $\pm 0.1$  mV sensitivity), at room temperature, and under constant stirring, by

means of a Crison, micro ST 2038. The output signal in steady state evaluations was transferred to a commutation unit and reconnected to one of six ways out, enabling the simultaneous reading of six ISEs. The assembly of the potentiometric cell was as follows: conductive graphite | ENR selective membrane | buffered sample solution (acetate,  $5 \times 10^{-2} \text{ mol L}^{-1}$ , pH 4.7) | electrolyte solution, KCl | AgCl(s) | Ag. The reference electrode was an Orion Ag=AgCl double-junction (Orion 90-02-00). The selective electrode was prepared in conventional or tubular configurations (Kamel et al. 2008) for batch and flow mode evaluations, respectively. Both devices had no internal reference solution and epoxy-graphite was used as the solid contact.

The Flow Injection Analysis (FIA) assembly had a Gilson Minipuls 2 peristaltic pump, fitted with PVC tubing (diameter /  $\frac{1}{4}$  0.80, 1.60 and/or 2.00 mm), and a four-way Rheodyne 5020 injection valve holding a loop of variable volume. The several components were joined by PTFE tubing (Omnifit, Teflon, 0.8 mm i.d.), Gilson end-fittings, and connectors. The support devices for tubular and reference electrodes, and the confluence point accessory were constructed in Perspex (Kamel et al. 2009). After reaching a stable baseline, the E=mV was recorded continuously by means of a home-made high-impedance data acquisition eight-channel box connected to a PC through the interface ADC 16 (Pico Tech., UK) and PicoLog for windows (version 5.07) software.

When necessary, the pH was measured by a Crison CWL=S7 combined glass electrode connected to a decimilivoltammeter Crison, pH meter, GLP 22.

All chemicals were of analytical grade and de-ionized water (conductivity  $<0.1 \text{ mS cm}^{-1}$ ) was employed. ENR, potassium *tetrakis*(4-chlorophenyl)borate (TpCIPB), oNPOE, poly (vinyl chloride) (PVC) of high molecular weight, EGDMA, VP, and MAA were purchased from Fluka. Benzoyl peroxide (BPO), methanol (MeOH), and tetrahydrofuran (THF) were from Riedel-deHäen.

## Synthesis of Host-Tailored Polymers

The MIPs were prepared by placing the template (ENR, 0.5 mmol) in a glass tube (14.0 mm i.d) with the functional monomer (5.0 mmol MAA), the cross-linker (EGDMA, 24.5 mmol), and the radical initiator (BPO, 0.32 mmol), all dissolved in 3 mL MeOH. A copolymer of VP and MAA was prepared similarly, by using a mixture of 2.5 mol of each monomer (VP and MAA) as functional monomers. The mixture was sonicated, degassed with nitrogen for 5 min, and cured at 70°C for 30 min. Non-imprinted polymers (NIP) were also prepared in a similar way, by excluding the template from the procedure.

The resulting polymers were grounded and sieved to particle sizes ranging 50–150  $\mu\text{m}$ . Extraction of the template molecule and washout of non-reacted species was carried out with methanol=acetic acid (5:1, v=v). The absence of ENR in the MIP particles was confirmed by measuring the 277 nm absorbance of the washout solution; the particles were repeatedly washed until ENR was no longer detected. All polymers (MIP=MAA, NIP=MAA, MIP=VP-MAA, NIP=VP-MAA) were dried after, at 60°C until constant weight.

## Preparation of ENR Sensors

The polymeric material was used to dope PVC-membrane selective electrodes. Thus, the sensing membranes were prepared by mixing 200 mg of PVC, 400 mg of plasticizer oNPOE, and 7 mg of the sensing polymer (Table 1). Some membranes were also added to 2 mg of TpCIPB, acting as anionic additive. The mixture was stirred until the PVC was well moistened, and dispersed in 3.0 mL THF; the uniformity of the dispersion was guaranteed by continuous agitation on a magnetic stirrer. These membranes were placed in conductive supports of conventional or tubular shapes (Kamel et al. 2009). Although “coated-wire” configurations have been correlated to random drifting potentials, this graphite-based solid contact has been used for long (Lima, Montenegro, and Sales 1996) with negligible drift. It has also the advantage of enabling future miniaturization because it uses a solid contact instead of using an internal reference solution. Membranes were let dry for 24 h and conditioned in a  $1 \times 10^{-3}$  M ENR solution. The electrodes were kept in this solution when not in use.

## Potentiometric Procedures

All potentiometric measurements were carried out at room temperature. Emf=mV values of each electrode were measured in solutions of fixed pH and ionic strength. Increasing concentration levels of ENR were obtained by transferring 0.0200–10.0 mL aliquots of  $1.0 \times 10^{-3}$  M ENR aqueous solutions to a 100 mL beaker containing 50.0 mL of  $5.0 \times 10^{-2}$  M of suitable buffer. Potential readings were recorded after stabilization to  $\pm 0.2$  mV and Emf=mV was plotted as a function of logarithm ENR concentration,  $\log ([\text{ENR}]=M)$ . Calibration graphs were used for

Table 1. Membrane composition of ENR PVC membrane sensors and their potentiometric features in  $10^{-2}$  M acetate buffer, pH 4.7

Characteristic	ISE I	ISE II	ISE III	ISE IV	ISE V	ISE VI
<i>Membrane materials</i>						
<i>Sensing polymer</i>	MIP=MAA	MIP=MAA	NIP=MAA	MIP=MAA-VP	MIP=MAA-VP	NIP=MAA-VP
<i>Additive</i>	TpCIPB	—	—	—	TpCIPB	—
<i>Slope=mV=Δlog([E<sub>NR</sub>]=M)</i>	56.8±0.3	63.4±0.5	48.3±0.9	47.5±0.3	65.2±0.7	47.4±0.8
<i>r<sup>2</sup> (n¼ 5)</i>	0.997	0.99	0.998	0.996	0.998	0.998
<i>LOD=M</i>	$2.0 \times 10^{-7}$	$7.9 \times 10^{-7}$	$6.3 \times 10^{-7}$	$2.8 \times 10^{-6}$	$7.9 \times 10^{-7}$	$8.9 \times 10^{-7}$
<i>LLLR=M</i>	$4.0 \times 10^{-7}$	$5.0 \times 10^{-6}$	$1.6 \times 10^{-6}$	$3.0 \times 10^{-6}$	$1.6 \times 10^{-6}$	$1.6 \times 10^{-6}$
<i>pH working range</i>	4–7	4–7	4–7	4–7	4–7	4–7
<i>Response time=s</i>	<15	<15	<15	<15	<15	<15
<i>r=mV</i>	0.6	0.8	0.4	0.9	1.1	0.9
<i>Precision=%</i>	1.1	0.9	1.2	1.1	0.8	1.5
<i>Repeatability=mV</i>	0.3	0.3	0.6	0.5	0.4	0.9

R<sup>2</sup>: correlation coefficient; LOD: limit of detection; LLR: lower limit of linear range; r: standard deviation; MIP: molecularly-imprinted polymer; NIP: non-imprinted polymer; MAA: methacrylic acid; VP: 2-vinyl pyridine; and TpC1PB: potassium *tetrakis*(4-chlorophenyl)borate.

subsequent determination of unknown ENR concentrations.  $mV = D \log([ENR] = M)$  was the slope of the calibration and its theoretical value for ENR (that is a monovalent cation) was 59 mV per unit of  $D \log([ENR] = M)$ ;  $D \log([ENR] = M)$  equals 1 when the concentrations being subtracted vary by 10 times, that is,  $\log(5.0 \times 10^{-3} = M) - \log(5.0 \times 10^{-4} = M) \approx 1$ . In this manuscript the term  $mV = D \log([ENR] = M)$  will be used to address the typical mV=decade used in literature.

## Binding Experiments

Binding experiments were carried out by placing 20.0 mg of MIP particles in contact with 10.0 mL ENR solutions ranging 0.04–2 mM. The mixtures were shaken for 12 h at room temperature and the solid phase separated by centrifugation (3000 rpm, 10 min). The concentration of free ENR in the supernatant was detected by UV spectrophotometry at 277 nm.

## Determination of ENR in Fish

Constant weights of well grinded fish (rv2.0 mg) from aquaculture origin were transferred to 15 mL tubes. A 10 mL portion of 0.05 M acetate buffer pH 4.7 was added and thoroughly mixed with the fish sample. A sonication period of 5 min was allowed to ensure convenient extraction of the analyte. A supernatant liquid was obtained by centrifugation at 1000 rpm and transferred into a 25 mL volumetric flask after filtration. Analytical measurements were conducted over this solution after completing the flask to final volume with buffer.

E=mV measurements in steady state were made by immersing the electrochemical cell in the sample test solution and waiting until equilibrium was reached (10–20 s). For hydrodynamic measurements (FIA), a flow stream of  $5.0 \times 10^{-2}$  M acetate buffer of pH 4.7 carrier solution was allowed to pass through the flow-cell at  $4 \text{ mL min}^{-1}$ . Aliquots of 260  $\mu\text{L}$  of the standard ENR solutions (for calibration) or unknown test sample solutions were injected into the flowing stream. The corresponding potential change was measured and recorded vs. time. The concentration of ENR was calculated using previous calibration data.

## RESULTS AND DISCUSSION

Typical potentiometric sensors have fast and reversible binding, which requires low activation energies. This may be achieved by means of non-covalent binding between template molecule and MIP particles (Dickert and Hayden 1999). For this purpose, VP and MAA are used as monomers to self-assemble the sensors. They allow electrostatic interactions mostly by establishing hydrogen bonds with the template compound. As can be seen in Fig. 1, ENR has both amino and carboxylic acid groups in its structure that may interact with either basic (VP) or acidic (MAA) monomers.

## General Analytical Features of the Sensors

The ENR sensors contained MIP or NIP particles as electroactive materials dispersed in oNPOE plasticizing solvent and PVC. Their main analytical features are shown in Table 1, and were evaluated after IUPAC recommendations (Buck

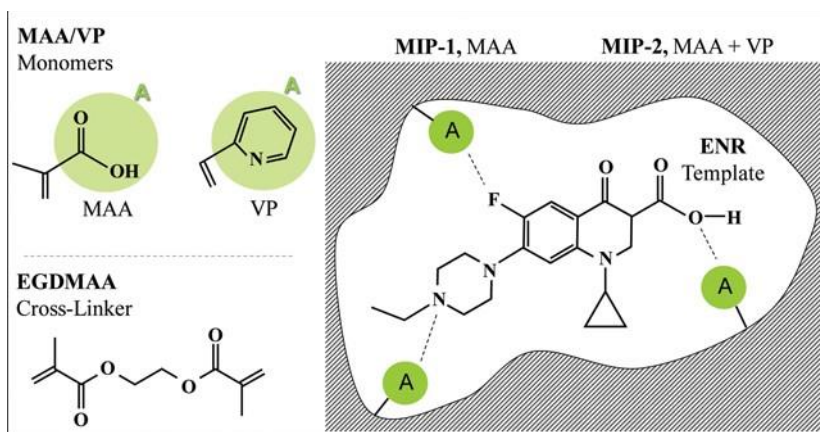


Figure 1. Chemical structure of monomers, cross-linker, and molecularly-imprinted polymers showing non-covalent binding sites to ENR. (Figure available in color online.)

and Lindner 1994). The sensors prepared with MAA and MAA-VP displayed linear behavior after  $5.0 \times 10^{-6}$  and  $3.0 \times 10^{-6}$  M ENR, cationic slope [ $mV = D \log([ENR] = M)$ ] of 63.4 and 47.5 and detection limits of  $0.38$  and  $1.35 \text{ mg mL}^{-1}$ , respectively. The corresponding NIP particles displayed smaller sensitivities, with  $mV = D \log([ENR] = M)$  of 48.3 and  $47.4 \text{ mV} = D \log([ENR] = M)$ . Their linear responses started in  $1.6 \times 10^{-6}$  M ENR and the detection limits were  $0.3$  and  $0.4 \text{ mg mL}^{-1}$ , respectively. In general terms, MIP sensors made with MAA monomers displayed the higher values of slope, with a near-Nernstian behavior, whereas those with NIP particles showed the widest linear response ranges (Fig. 2).

To improve the operating features of the previous membranes, MIP-based sensors were added to TpCIPB (Table 1), an anionic lipophilic compound. Typically, the addition of an ionic compound of lipophilic nature to potentiometric sensors reduces the anionic interference and lowers the electrical resistance of the membranes (Telting-Diaz and Bakker 2001). In terms of analytical performance, this procedure is expected to widen the linear range with theoretical slope. Sensors based in MIP=MAA and MIP=MAA-VP, showed linear response ranges within  $4.0 \times 10^{-7}$ – $5.0 \times 10^{-4}$  M and  $1.6 \times 10^{-6}$ – $5.0 \times 10^{-4} \text{ mol L}^{-1}$ ,  $0.09$  and  $0.38 \text{ mg mL}^{-1}$  detection limits, and near-Nernstian responses of  $56.8$  and  $65.2 \text{ mV} = D \log([ENR] = M)$ , respectively. When compared to the corresponding sensors without additive, a significant improve in terms of slope was observed for MAA-VP sensors and in terms of lower limit of linear range for MAA sensors (see Fig. 2).

### Response Time and Lifetime

The response time of the potentiometric sensors was the average time required for the ENR selective electrode to reach a potential within  $\pm 1 \text{ mV}$  of the final equilibrium value after immersion in a series of ENF solutions, each having a 10-fold difference in concentration. Stable responses were achieved within 5–15 s for ENR concentrations of  $10^{-6}$ – $10^{-4}$  M. Replicate calibrations for each sensor indicated low potential drift, long-term stability, and negligible change in the response of

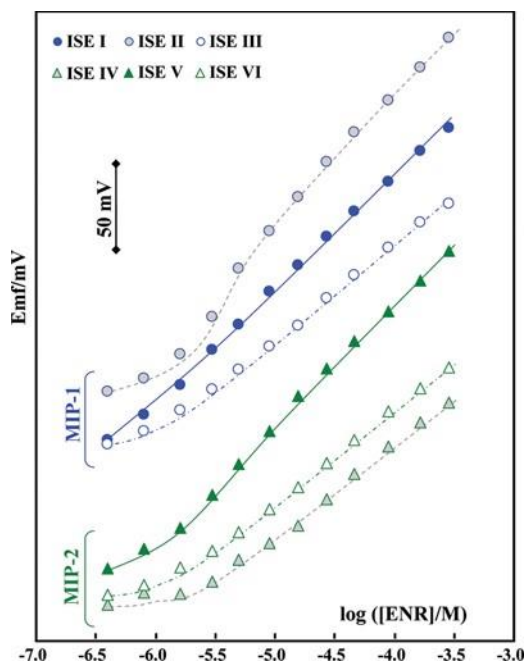


Figure 2. Potentiometric response of ENR PVC membrane sensors ( $E=mV$ ) vs.  $\log ([ENR]/M)$ , under static mode of operation. (Figure available in color online.)

the sensors (Table 1). The sensors were stored and conditioned in  $10^{-3}$  M ENR solution of pH 4.7. With all sensors examined, the detection limits, response times, linear ranges and calibration slopes were reproducible within  $\pm 3\%$  to their original values over a period of at least 7 weeks. During the first 4 weeks the electrodes were calibrated daily and after that once a week. Each calibration allowed an estimated number of 5 determinations.

### Effect of pH

The effect of pH upon the potentiometric response was investigated by measuring the  $E=mV$  of a set of ENR standards in 5 mM acetate or Tris buffers ranging 4–9 pH. The sensitivity of the ENR sensors remained almost unchanged within pHs 4 to 7, but decrease for pH above 7. This was a consequence of the two ionizable functional groups in ENR: a carboxylic group ( $pK_1 \approx 5.94$ ) and a basic piperazinyl group ( $pK_2 \approx 8.70$ ). Depending on the pH of the test solution, ENR existed in different forms (Lizondo et al. 1997) mainly cationic (pH 4, 5 and 6), zwitterionic (pH 7), or anionic (pH 9). Thus, slopes decreased for  $pH > 7.0$  due to the formation of non-cationic species and the decrease of the amount of cationic ENR sensed by the ISE.

### Selectivity of the Sensors

The selectivity of the chemical sensor is one of the most important potentiometric features regarding its analytical application. It was assessed by means of



$K^{POT} = D \log([ENR] = M)$ , the potentiometric selectivity coefficient. It defines the ability of an ion-selective electrode to distinguish between different ions in the same solution. They were calculated by the separate solution method (SSM) (Buck and Lindner 1994) and expressed in  $\log K^{POT}$ .

The following equation was used for this purpose,

$$\log K_{ENR^+, B^{Z+}}^{POT} = \frac{E_2 - E_1}{S} + \left(1 - \frac{1}{Z}\right) \times \log(5.6 \times 10^{-4}) \quad (1)$$

where  $E_1$  is the electrode potential in a  $5.6 \times 10^{-4}$  M ENR solution,  $E_2$  the potential of the electrode facing a  $5.6 \times 10^{-4}$  M concentration in interfering specie  $B^{Z+}$ , and  $S$  is the practical slope calculated after the calibration experiments.

$\log K^{Pot}$  data were plotted in Fig. 3, and indicated the degree of preferential interaction for ENR over different organic and inorganic species that are common in biological and food samples. The former group included the antibiotic tetracycline ( $TC^P$ ), other fluoroquinolones used in aquaculture, namely norfloxacin (NF) and ciprofloxacin (CF), as well as glycine ( $Gly^P$ ), ascorbic acid (AA), and creatinine ( $Ct^P$ ). In general, their interference was considered negligible, with most  $\log K^{Pot}$  ranging  $-5$  to  $-2$ , and with an anti-Hofmeister pattern. Only antibiotics (with

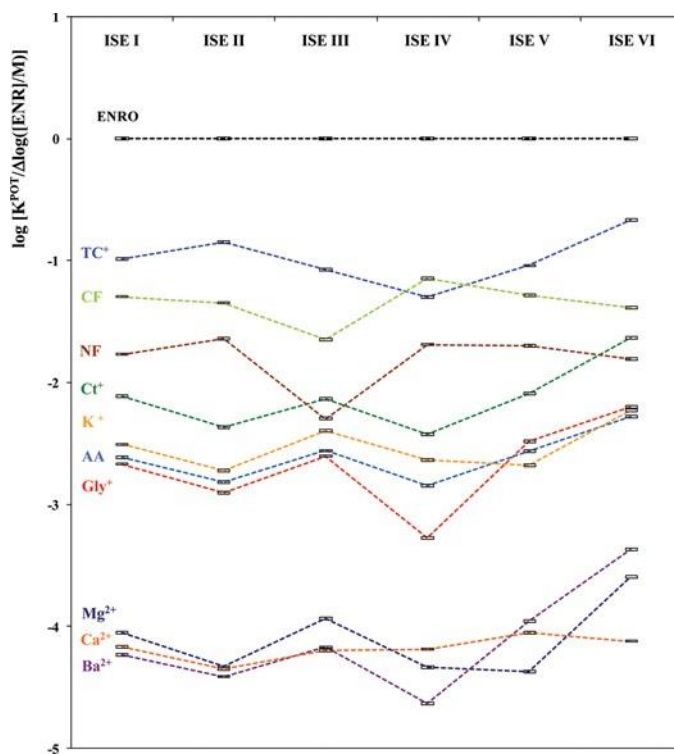


Figure 3. Potentiometric selectivity coefficients ( $\log K^{POT}$ ) of all ENR sensors toward several interfering species in 0.05 M acetate buffer of pH 4.7. (Figure available in color online.)

similar structure to the analyte) had selectivity coefficients within  $-2$  and  $-1$ , but these will never play an interfering role as they are not associated to ENR to treat veterinary infections.

The ISEs bearing different electroactive material displayed different selectivity patterns. This was already expected because this is the component of the selective membrane that exerts greater influence upon this property, as the mechanism of selectivity is mainly governed by stereospecific and electrostatic aspects. The ISEs with MIP=MAA differed from the MIP=MAA-VP in potassium, glycine, and divalent ions. Although their magnitude of interference was similar, the  $\log K^{\text{POT}}$  order was changed, inferring the influence of the electroactive material. Greater differences were not recorded most probably because both MIP ISEs used MAA monomers.

The ISEs with NIP sensors displayed a slightly higher interference than those with the corresponding MIP (see Fig. 3 and Table 1). This behavior suggested the existence of stereospecific interactions between analyte and host sensing material, although electrostatic interactions seem to have a relevant role in this process. The anionic additive was also responsible for a slight increase in  $\log K^{\text{POT}}$ , therefore contributing to worsen the selectivity of the potentiometric sensors. This was observed for both sensing materials (Fig. 3).

Combining the selectivity data with the analytical performance observed for all electrodes, further experiments were carried out only with ISEs II. They displayed the best sensitivity and selectivity.

### Binding Properties of MIPs

Binding mode and site distribution between the polymeric sensing material and ENR were evaluated after carrying out binding experiments. For this purpose, fixed amounts of MIP=MAA were incubated with different concentrations of ENR until equilibrium was reached. The resulting binding capacity of MIP ( $Q$ ) was calculated according to following equation:

$$Q = \frac{\mu\text{mol}(\text{boundENR})}{\text{g}(\text{MIP})} \quad (2)$$

which divides the amount of bound ENR by the amount to polymeric material (MIP) used as absorbent surface. For this purpose, varying concentrations of ENR were let stand with the synthesized particles under continuous stirring until equilibrium was reached. Bound ENR was calculated by subtracting the free ENR in solution to the initial ENR amount placed in contact with the solid MIP=MAA. The resulting binding capacities were plotted against the initial ENR concentration (Fig. 4A). The adsorption data showed that the binding capacity of imprinted polymer increased with the increasing of the initial concentration of ENR and had a tendency to saturation for higher analyte concentrations.

The corresponding experimental data was used to carry out the Scatchard analysis and estimate further binding parameters. The Scatchard equation:

$$Q/C_{\text{free}} = (Q_{\text{max}} - Q)/K_d \quad (3)$$

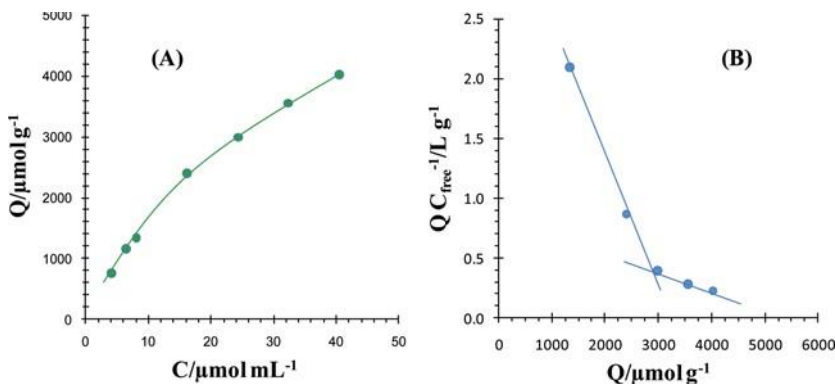


Figure 4. Binding isotherm (A,  $Q=\mu\text{mol g}^{-1}$  is plotted as a function of ENR concentration,  $C=\mu\text{mol mL}^{-1}$ ) and Scatchard plot (B,  $Q C_{\text{free}}^{-1} = \text{L g}^{-1}$  is plotted as a function of  $Q=\mu\text{mol g}^{-1}$ ) for ENR=MAA imprinted polymer.  $Q$  is the amount of ENR bond per g of polymer;  $t$  ¼ 25°C;  $V$  ¼ 10.00 mL; binding time: 20 h. (Figure available in color online.)

was applied for this purpose, where  $Q$  is the binding capacity;  $C_{\text{free}}$  the free analytical concentration in equilibrium ( $\text{mmol}=\text{L}$ );  $Q_{\text{max}}$  is the maximum apparent binding capacity; and  $K_d$  is the dissociation constant in binding site. The equilibrium dissociation constant was calculated from the slopes and the apparent maximum number of binding sites from the  $y$ -intercepts in the linear plot of  $Q=C_{\text{free}}$  vs.  $Q$ .

The Scatchard plot (Fig. 4B) showed non-linear behavior, inferring binding site heterogeneity in the polymer matrix. This is a typical behavior for non-covalent imprinting processes. The ENR may also have established hydrogen bonds to the imprinted cavity by means of two functional groups, the carboxylic and the amine, which may have contributed as well to the observed heterogeneity. The two distinct straight lines in Fig. 4B suggested the existence of two classes of binding sites, corresponding to high and low affinity populations. The  $K_{d1}$  and  $Q_{\text{max}1}$  were, respectively, 873  $\text{mmol}=\text{L}$  and 3.16  $\text{mmol}=\text{g}$  for the high affinity binding sites, and 6036  $\text{mmol}=\text{L}$  and 5.34  $\text{mmol}=\text{g}$  for the low affinity binding sites.

### Optimization of Flow Injection System

For the routine control of an analyte, the continuous mode of operation is of regular selection. This may be achieved by means of flow injection analysis (FIA) systems. These are particularly attractive in view of their versatility, simplicity, and suitability for large-scale analyses. The flow assembly was double-channel, allowing the on-line adjustment of pH and ionic strength. A flow cell of tubular configuration was used to accommodate the potentiometric device. This cell was of simple fabrication and allowed full membrane=sample contact, maintaining the general features of conventional configuration ISEs in terms of homogeneity, thickness, and fixed area. To take full advantages of this FIA system, flow-rate and injection volume were optimized in terms of sensitivity, sampling-rate, reagent consumption, and wastewater generation.

The sample loop was varied within 30 and 500 mL. For each injection volume, a set of ENR standards ranging  $1.0 \times 10^{-6}$  to  $1.0 \times 10^{-3}$  M was injected into the buffer carrier stream. The sensitivity of the response increased markedly with the injection volume up to 260 mL. For higher volumes, the slope of the potentiometric response remained almost constant (less than 5% increase). This observation was coupled to decreased sampling-rates, sample consumption and waste generation. Therefore, a sample volume of 260 mL was selected for further experiments.

The effect of flow-rate was examined from  $1.5$  to  $6.0 \text{ mL min}^{-1}$  for ENR solutions ranging  $1.0 \times 10^{-5}$  to  $1.0 \times 10^{-3} \text{ mol L}^{-1}$ . No significant changes in slope were observed, but the peak width and peak height decreased with increasing flow-rates. This was accompanied by increasing sampling-rates and wastewater generation. As a compromise, a flow-rate of  $4 \text{ mL min}^{-1}$  was selected after these results.

The main analytical features of the flow sensor were recorded under the previously selected conditions. Variation or fluctuation of the base line did not exceed  $\pm 5\%$  of the peak height. The potentiometric sensor showed linear behavior ( $r > 0.9991$ ) with slopes of  $46.6 \pm 1.1 \text{ mV} = D \log([E \text{ NR}] = M)$  with detection limits of  $0.91 \text{ mg mL}^{-1}$  and lower limits of linear range of  $1.0 \times 10^{-5} \text{ M}$  (Fig. 5). The relative standard deviation of the transient signals was  $\pm 1.5\%$  for  $10^{-6} - 10^{-3} \text{ M}$  ENR. The sampling-rate was rv35 runs per hour.

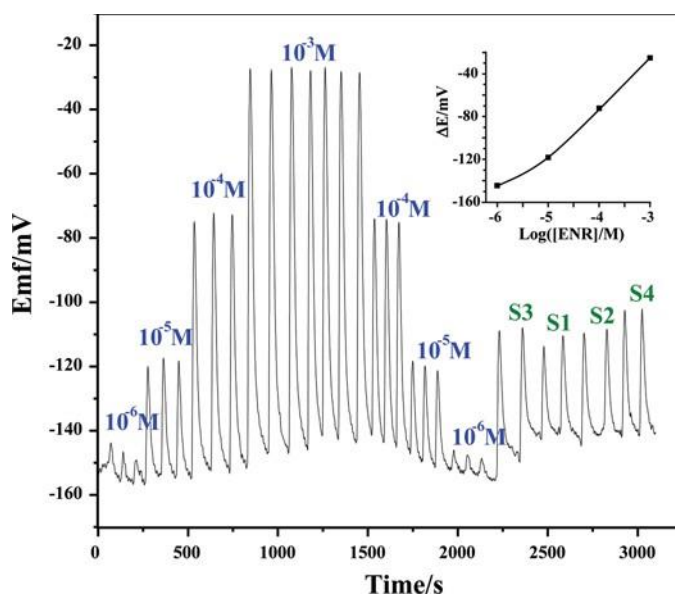


Figure 5. FIA potentiometric signals,  $\text{Emf}=\text{mV}$ , for ENR membrane based sensor are presented as a function of  $\text{time}=\text{s}$ . Inset: corresponding calibration plotting  $\text{Emf}=\text{mV}$  against  $\log [ENR=M]$ . Conditions: carrier solution,  $0.05 \text{ mM}$  acetate buffer pH 4.7; flow rate  $4 \text{ mL min}^{-1}$ ; loop  $260 \text{ mL}$ . (Figure available in color online.)

## Analytical Application

The sensor was successfully used as an indicator electrode in the potentiometric titration of 5.0 and 10.0 mL of 0.1–1.0 mM ENR solution with a 1.0–10.0 mM sodium tetraphenyl borate solution. Figure 6 shows the change in potential after gradual addition of sodium tetraphenyl borate. Sharp inflection breaks of  $\Delta E = 150$  mV at the equivalence point were obtained. The ENR solutions containing 35–350  $\text{mg mL}^{-1}$  ENR were determined and the results obtained showed an average recovery of 97.8% and a standard deviation of 0.9% ( $n = 5$ ).

The method was also used to determine ENR in fish samples from aquaculture source. Fish meat was ground and spiked to 5–7  $\text{mg mL}^{-1}$  ENR. Salmon and trout species from aquaculture origin were used for this purpose. A good agreement was found between added and found amounts of ENR. Mean values of four independent determinations were 5.2, 5.9, 6.1, and 6.9  $\text{mg mL}^{-1}$ . Results of the potentiometric analysis conducted in steady state showed recoveries ranging 97–107% with an average relative standard deviation of 0.8%. In hydrodynamic mode of operation recoveries ranged 98–112%, with relative standard deviations of 1.4%. The *t*-student and *F* tests showed no significant differences between the means and variances of static and hydrodynamic potentiometric sets of results.

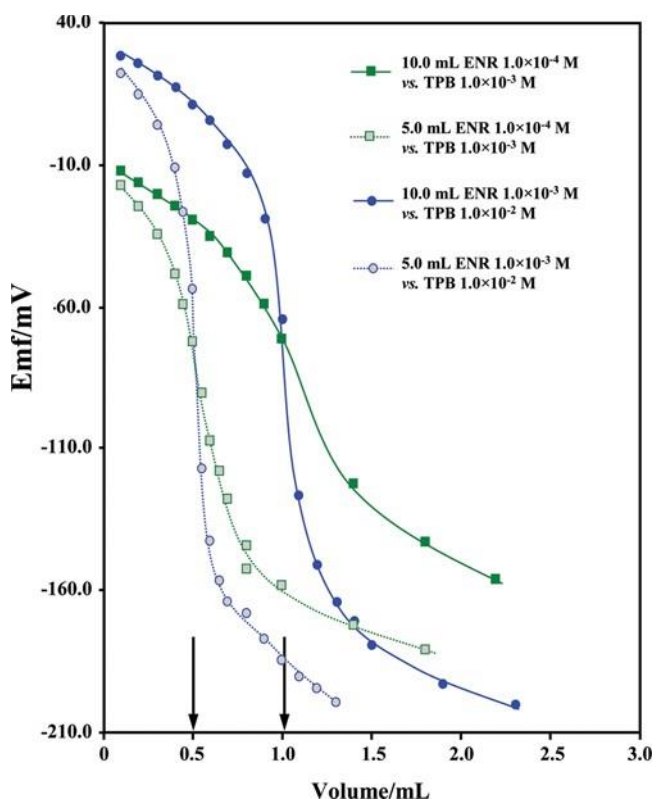


Figure 6. Typical potentiometric titration plots of ENR with TPB. Emf=mV is presented as a function of the volume=mL of titrant (TPB). (Figure available in color online.)

## CONCLUSIONS

Molecular imprinting technique was employed to produce ENR host-tailored sensors for potentiometric transduction. The MAA and/or VP were used as monomers to produce different MIP materials. Both MAA and MAA-VP based sensors offered good potentiometric analytical features capable of discriminating ENR from other fluoroquinolones in aqueous media. Advantages of these sensors included the simplicity in designing, short measurement time, good precision, high accuracy, high analytical throughput, low limit of detection, and good selectivity.

The MIP=MAA sensors were successfully applied to the analysis of food samples, both in steady state and in flowing media. The proposed method was simple, of low cost, precise, accurate, and inexpensive regarding reagent consumption and equipment involved. Wastewaters discharged were of small concern to the environment regarding volume and composition.

The tubular devices are particularly suitable for the routine screening control of ENR in fish meat. They produce quicker responses for ENR than those provided by microbiological methods and are much less expensive than the chromatographic methods that are used for routine purposes.

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