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Nanoscale Affinity Chip Interface for Coupling Inhibition SPR Immunosensor Screening with Nano-LC TOF MS

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The on-line nanoscale coupling of a surface plasmon resonance (SPR)-based inhibition biosensor immunoassay (iBIA) for the screening of low molecular weight molecules with nano-liquid-chromatography electrospray ionization time-of-flight mass spectrometry (nano-LC ESI TOF MS) for identification is described. The interface is based on a reusable recovery chip (RC) that contains a nanoscale biosorbent composed of a hydrogel layer modified with antibodies raised against the analyte featuring the unique possibility of performance characterization using the SPR biosensor. Various hydrogel chemistries were evaluated, and the standard Biacore CM5 chip showed the highest capture capacity in combination with affinity-purified polyclonal antibodies. The procedure has four stages: the samples are prepared (1) and screened using a screening chip (SC) in the iBIA (2). Suspected noncompliant samples as being noncompliant are reinjected over the RC, and the analyte is captured at subnanogram level (3). The captured analyte is released, and the eluate is analyzed with nano-LC ESI TOF MS via a loop-type interface (4). The coupling of the technologies proved effective for screening enrofloxacin, a model compound, in incurred chicken muscle samples followed by identity confirmation in suspected noncompliant samples. Ciprofloxacin, a known metabolite of enrofloxacin, was identified as well in incurred chicken samples. This demonstrates the potential of the technologies coupled by means of a RC for the rapid screening and identification of known as well as unknown compounds. Finally, we demonstrate the feasibility of combining the two biosensor chips (SC and RC) with a robust chip-based nano-LC chip TOF MS system, thus providing a robust alternative triple-chip system.

A wide variety of surface plasmon resonance (SPR)-based biosensor applications have been reported during the past decade.[1,2] These applications exploit biorecognition events of (at least) two binding partners that interact at the sensor chip surface where one of them is immobilized. The binding of the partners increases the total mass and the density of the molecules at the chip surface, hence resulting in a local refractive index change that is proportional to the SPR biosensor signal output. A major advantage is that the interaction can be monitored in real time allowing the determination of the kinetic parameters. The concentrations of interacting biomolecules can be quantitatively estimated either by the direct binding of the analyte to the chip surface (direct binding assay (DBA)) or by constructing an inhibition (competitive binding) assay. Since the signal output depends directly on the mass of the interacting analyte, the DBA format is widely used for fishing high molecular weight biomolecules out of complex biological matrices. Nevertheless, with the proper experimental forethought, this approach has also proven effective for the determination of kinetic parameters of low molecular weight molecules binding to antibodies or receptors.[2,3] Such antibody-based DBAs were developed for the direct detection of low molecular weight drug residues (aminoglycosides with molecular weights of 466 and 583 Da) in milk of dairy cows.[4,5] However, for low molecular weight compounds the inhibition assay format is generally preferred in biosensor immunoassays (BIAs) because of its flexibility, higher response, and robustness.[4,6] In this format, the sample (containing the analyte/s) is mixed with the high molecular weight affinity biomolecule (i.e., antibody, receptor, etc.) and injected over the chip surface previously coated with the analyte. The signal drop due to the inhibition of the binding of the biomolecule to the immobilized analyte on the chip surface will be proportional to the analyte concentration in the sample. The robustness of such a low molecular weight compound coated chip has been proven with a sulfamethazine derivative coated chip which was used for approximately 1100 cycles, and no significant change in performance was observed.[6]

The main drawback of the biosensor screening technology is the lack of information about the identity of the interacting analyte.

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Hence, its combination with mass spectrometry is a powerful approach that already yielded a number of successful attempts in the field of proteomics,[7,8] where the presence of proteins retained during the direct assay interaction analysis can be unambiguously confirmed by coupling the SPR biosensor with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).[9] The SPR-MALDI-TOF MS analysis has evolved from the direct measurement of the interacting biomolecules from the chip surface (yielding the chip unusable after one binding cycle) to the further deposition of the biomolecules on MALDI plates for analysis upon elution[10,11] to a small volume elution using automated commands[12−14] and lately to a bifunctional SPR/MS flow cell with removable MS probes.[15] Alternatively to MALDI-TOF MS analysis, the analysis of the chip eluate using liquid chromatography electrospray ionization MS (LC-ESI-MS) has been reported.[16,17] Although these interfacing concepts have proven well suited for the identification of high molecular weight molecules in conjunction with DBAs, they are not functional for the screening of compounds of low molecular weight.

Within the scope of the EU project BioCop (www.biocop.org), several inhibition biosensor immunoassays (iBIAs) are being developed as screening methods for chemical contaminants in food. Our challenge within this project is to couple the iBIA with MS for confirmation of identity and/or the identification of unknowns having activity in the iBIAs. We have recently reported a method for the parallel coupling of these technologies using a dual wellplate LC fractionator.[18] In the present study we demonstrate the proof of principle, using enrofloxacin (Enro) as the model analyte, for the serial coupling concept between the iBIA and nano-LC-ESI-TOF MS. Enro is an antibiotic widely used in cattle and poultry that should be monitored and is regulated in accordance to EC Council Directive 96/23/EC.[19] The proposed system is composed of the Biacore 3000 SPR biosensor that carries out the screening using a robust iBIA. When a suspected noncompliant sample is found, the system stops the screening procedure and an aliquot of the sample without antibody is reinjected into the accessory surface preparation unit (SPU) that accommodates a recovery chip (RC) containing anti-Enro antibodies. This RC contains a 100−200 nm thick porous polymer (hydrogel) layer modified with antibodies. The antibodies in the biosorbent specifically capture the analyte while the sample matrix flows through. The chip surface is washed, and next, the captured analytes are eluted into a loop from where they enter the nano-LC system and routed toward a precolumn followed by an analytical column and finally electrosprayed in the TOF MS for mass analysis. Both an iBIA and a DBA were developed for Enro (the latter for RC characterization). The hyphenated system was tested with standard solutions and with chicken muscle extracts in a regulatory-relevant concentration range. The detection of unknown compounds was addressed along with the viability of using high-capacity RC surfaces. Finally, a more robust chip−nano-LC TOF MS alternative to the standard nano-LC chip TOF MS set up was briefly studied in combination with the SC and RC to create a triple-chip approach.

EXPERIMENTAL SECTION

Chemicals and Materials. Sensor chips (CM5), HBS-EP buffer [pH 7.4, consisting of 10 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid, 150 mM sodium chloride, 3 mM EDTA, 0.005% (v/v) surfactant polysorbate (P20)], an amine coupling kit [containing 0.1 M *N*-hydroxysuccinimide (NHS), 0.4 M *N*-ethyl-*N*-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and 1 M ethanolamine hydrochloride−NaOH (pH 8.5)], CNBr-activated Sepharose 4B and the SPR-based biosensor systems Biacore 3000 and Q, the SPU accessory as well as the AKTA purifier and HI Trap protein G columns (1 mL) were supplied by GE Healthcare (Uppsala, Sweden). Sensor chips featuring other hydrogel chemistries were purchased from XanTec bioanalytics GmbH (Muenster, Germany). Ethylene diamine (EDA) was obtained from VWR International (Amsterdam, The Netherlands). Acetonitrile (ACN) HPLC far-UV grade was from Lab-Scan Ltd. (Dublin, Ireland). Water was purified using a Milli-Q gradient A10 system (Millipore, Bedford, MA). The 0.45 μm Durapore filters and the 30 kDa and 50 kDa Amicon cutoff filters were from Millipore. Ciprofloxacin (Cipro) and enrofloxacin (Enro) were from Fluka (Zwijndrecht, The Netherlands). All other reagents were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) unless otherwise stated. The AKTA purifier and HiTrap protein G columns (1 mL) where provided by Amersham Biosciences (Uppsala, Sweden). Mouse anti-Enro/Cipro monoclonal IgM antibody (clone 72FIG1F7#1) (MAb72F) was obtained from Abcam (Cambridge, U.K.). The preparation and purification of the polyclonal antiserum (PAb MH40) is described in the Supporting Information.

Equipment. The system (Figure 1) consists of the Biacore 3000 SPR biosensor containing both a CM5 chip coated with Enro for the iBIA screening (inset 2) and an SPU accessory containing the RC, a CM5 chip coated with antibodies, for analyte capture (inset 3). The flow cell carrier type 2 (FCT2) on top of the RC features a serpentine-like flow cell (area ≈16 mm^2, height of 50 μm, and volume of 800 nL). The SPU is connected to a 10 μL loop-type interface with a Micro Tight tubing sleeve (i.d. 535 μm) and Peek tubing (i.d. 255 μm, 510 μm o.d., 9.6 cm). The inlet of the loop interface is connected to the Waters (Milford, MA) model Acquity UPLC pump. The outlet of the loop-type interface is connected to the tee of a nano-LC switching system adapted from Meiring et al.[20] using $^1/_{16}$ in. connections having short (0.02 in. i.d.) Peek sleeves and fused-silica capillary tubing (360 μm o.d., 200 μm i.d., 60 cm). The flow is split (≈1/2000), with this switching system, between the restrictor (360 μm o.d., 50 μm i.d.

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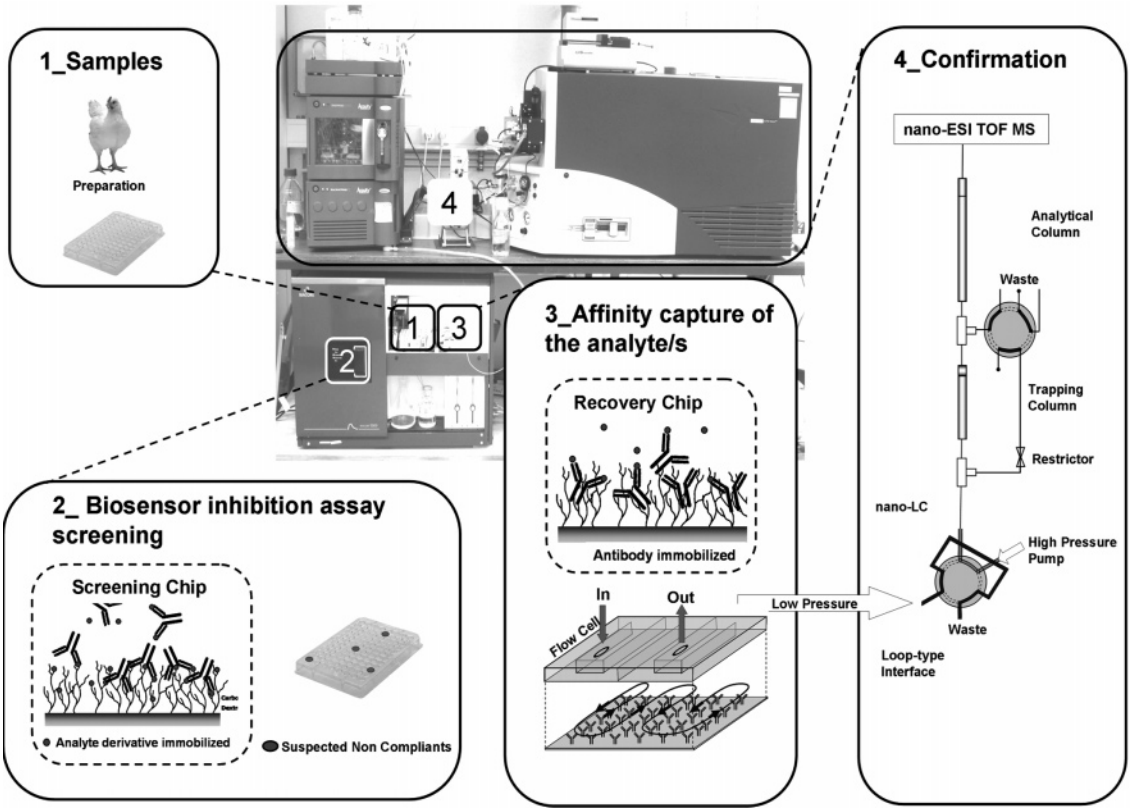

Figure 1. System setup overview with exploded view of key components. Muscle extracts (inset 1) are screened in the SPR biosensor with the Enro iBIA using the screening chip (SC) (inset 2). The analytical efforts are focused only on the suspected noncompliant samples that are reinjected over a recovery chip (RC) with immobilized antibodies (inset 3). Enro is captured on the RC surface and eluted into a loop-type interface (inset 4). The first valve is switched to the high-pressure side, and the analyte is transferred from the loop-type interface to the trapping column. Once the analyte is trapped, the second valve is switched and the nano-LC gradient is started. Dashed frames in insets 2 and 3 depict the mirror-image immunochemistry nature between the SC and RC.

$\times$ 50 cm) and the trapping column (Biosphere C18 120 Å 5 μm 50 μm i.d., 360 μm o.d., 2 cm length) between the first and the second tee. Following the second tee, the analytical column (Biosphere C18 120 Å 3 μm 50 μm i.d., 360 μm o.d., 20 cm length) is directly butt-connected with the Nanoease emitter (Waters) through the universal NanoFlow sprayer mounted on the Waters model QTOF-micro MS system. The nano-LC gradient separation was performed using a mobile phase consisting of (A) water/formic acid (99.8:0.2; v/v) and (B) ACN/water/formic acid (89.8:10:0.2; v/v/v). Sample loading through the trapping column is performed at 10 μL min^{-1} for 5 min with 100% solvent A. Upon valve switch, the precolumn split is activated and the restrictor back pressure allows a flow in the order of 300 nL min^{-1} through the analytical column. Details concerning nano-LC columns, capillaries, connectors, valves, solvent composition and flow gradient, and the MS settings can be found in the Supporting Information.

The feasibility of the triple-chip approach was carried out using the nano-LC chip TOF MS system of Agilent Technologies (Waldbronn, Germany) previously described elsewhere.[21] The chip trapping column had a volume of 40 nL and was packed with Zorbax 300SB C18 5 μm particles. The nano-LC chip column had a 75 $\times$ 50 μm cross section, length of 43 mm, and was packed with Zorbax 300SB C18 3.5 μm. Mobile phases were similar to those used with the capillary nano-LC TOF MS system described above. The electrospray voltage was set to 2400 V at the MS inlet capillary. Nitrogen drying gas at 350 °C and 4 L min^{-1} was used for spray desolvation.

Screening Chip Preparation and iBIA Procedure. Enro was immobilized via its carboxyl group to the carboxymethylated chip surface using EDA as a bifunctional spacer. The SC surface was preconditioned by consecutive duplicate injections of 10 μL of HCl (10 mM), NaOH (50 mM), sodium dodecyl sulfate (0.1% w/v), and water at 100 μL min^{-1} in one flow channel (Fc2) of the integrated μ-fluidic cartridge (IFC) in the biosensor. Then, the surface was activated with 150 μL of a mixture of 0.4 M EDC and 0.1 M NHS (1:1; v/v) and then coated with 100 μL of 2 M EDA in water (pH 10), both at 10 μL min^{-1}. Then, all remaining activated groups were blocked with 100 μL of EA (1 M). The SC surface and the IFC were cleaned by two injections of 10 μL of a mixture of 0.2 M NaOH, ACN, and water (1:1:3; v/v/v) using the "Wash needle" and "Wash IFC" wizards in the biosensor software.

Meanwhile, the carboxyl group of Enro was activated by adding 10 μL of an Enro solution (10 mg mL^{-1} in methanol) to one vial containing 115 μL of 0.4 M EDC and to another vial containing 115 μL of 0.1 M NHS. Both freshly prepared vials were mixed (1:1; v/v) in the biosensor before the injection of 150 μL at 2 μL min^{-1} over the EDA-modified SC surface. Finally, the SC was washed with 0.05 M NaOH and HBS-EP.

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Table 1. Evaluation of Screening Chips with Different Hydrogel Properties in the Inhibition SPR Biosensor Immunoassay (iBIA) for Enro

	hydrogel		inhibition BIA		
chip code	length (nm)	chain density	immobilized Enro (RU)	bound PAb MH40 (RU)	sensitivity IC_{50} (pg μL^{-1})
A[a]	100−200	low	600	11700	7.1
B[b]	150	high	300	3800	4.8
C[b]	200	low	800	10000	5.3
D[b]	200	high	700	4000	3.3
E[c]	500	high	400	6400	5.5
F[c]	1000	medium	1400	6700	6.6

[a] Carboxymethylated dextran chip from Biacore. [b] Carboxymethylated dextran chip from XanTec. [c] Linear carboxylated hydrogel matrix from XanTec.

Table 2. Evaluation of Chips with Different Hydrogel Properties for the Production of Enro Recovery Chips[a]

	DBA biosensor		nano-LC TOF MS
chip code	immobilized antibody (RU)[b]	captured Enro (RU)	captured Enro (pg mm^{-2})
A	19600	12	31
A1	18900	0	3
B	16400	10	*c*
D	4800	4	*c*
E	8200	0	13
F	9200	9	14

[a] The chips having a surface of 16 mm^2 were evaluated using both the direct binding assay format in the SPR biosensor and the nano-LC TOF MS system. [b] PAb SpMH40 immobilized except in chip A1 where monoclonal antibody (MAb72F) was immobilized. [c] Not measured.

For the iBIA procedure, the sample extracts or standard solutions in HBS-EP buffer were mixed (4:1; v/v) with the antibodies (diluted antiserum PAb MH40 or its purified IgG fraction (SpMH40)) in the biosensor, and 50 μL was injected at a flow rate of 25 $\mu L\ min^{-1}$. The SC surface was regenerated with 15 μL of a mixture of 0.2 M NaOH and ACN (4:1; v/v).

Recovery Chip Preparation. Antibodies (SpMH40 or MAb72F) were immobilized onto the entire carboxymethylated surfaces of the sensor chips (Biacore CM5 or XanTec chips assembled in Biacore frames as described by the manufacturer). Preconditioning was the first step, and the second step involved the activation of the sensor chip surface and the immobilization of the antibodies. For the preconditioning step, 100 μL aliquots of the preconditioning solutions (HCl (10 mM), NaOH (50 mM), and sodium dodecyl sulfate (0.1% v/v)) were successively pipetted over the chip surface. The chip surface was thoroughly rinsed with distilled water and activated with 100 μL of a mixture of 0.4 M EDC and 0.1 M NHS (1:1; v/v) for 20 min. Finally, SpMH40 antibody was diluted (1:10; v/v) in acetate buffer (pH 5) and 100 μL was placed onto the activated chip surfaces, left to react for 90 min, and the remaining active groups where deactivated by pipetting 100 μL of 1 M EA. The immobilization levels were evaluated by measuring the relative responses of the chips in the biosensor before and after immobilization. The characteristics of the sensor chip hydrogel chemistries used for preparing the RCs (Table 2) are presented in Table 1.

Samples and Standards. Muscle samples from untreated broilers and broilers treated with Enro were obtained from an animal experiment that is described elsewhere.[22] The chicken breast samples (1 g) were extracted by homogenizing with 10 mL of water. The homogenate was centrifuged, and the supernatant was filtered through a 0.45 μm filter. Two milliliters of the filtrate was passed through a 30 kDa cutoff filter and diluted (1:1; v/v) in HBS-EP buffer for further experiments. Enro and Cipro standard solutions were prepared by dissolving 10 mg in 10 mL of a mixture of methanol and formic acid (99.9:0.1; v/v) and subsequently diluted with HBS-EP buffer to relevant concentrations.

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RESULTS AND DISCUSSION

Screening Chip Performance. Different screening chips (SCs) were prepared by immobilizing Enro on sensor chips having different hydrogel properties (see Table 1). The amount of immobilized Enro is proportional to the increase in response units (RU) measured on the different chip surfaces, and these varied from 300 RU in SC B to 1400 RU in SC F. To assess the maximum binding capacity of the Enro-modified chips, high amounts of antibodies (raw antiserum PAb MH40 diluted in HBS-EP buffer (1:10; v/v)) were injected (50 μL at 25 $\mu L\ min^{-1}$) over each chip, and the maximum response varied from 3800 RU for SC B to 11700 RU for chip A. After regeneration with a mixture of 0.2 M NaOH and ACN (4:1; v/v), the responses returned to baseline and a new sensorgram could be produced with similar binding responses.

A calibration curve for Enro was measured for each chip (data not shown) as described in the iBIA procedure using diluted raw antiserum PAb MH40 (1:40; v/v). More than 2-fold difference in sensitivity (defined as the concentration at 50% inhibition (IC_{50})) was observed between SC A (IC_{50} = 7.1 pg μL^{-1}) and SC D (IC_{50} = 3.3 pg μL^{-1}). However, the high maximum response obtained for the blank (B_0 = 1950 RU) with SC A allowed a further 3-fold PAb MH40 antiserum dilution with a concomitant maximum response decrease for the blank (670 RU) and a 2-fold increase in sensitivity (IC_{50} = 3.7 pg μL^{-1}). The measurement of a calibration curve (see Figure 2A) using the purified antiserum fraction (SpMH40) (1:50; v/v) in SC A resulted in an additional increase in sensitivity (Enro IC_{50} = 2.2 pg μl^{-1}) with a robust maximum response of 750 RU. All further iBIA experiments were performed using SpMH40 in combination with SC A. The sensitivity of the iBIA for Cipro, a known Enro metabolite, was lower (IC_{50} = 85.4 pg μL^{-1}), and the cross-reactivity (CR) was 3%. The iBIA assay time, including mixing the reagents, sample injection, the regeneration of the surface, and washing steps, was 8.5 min per sample.

Development of the Recovery Chip Procedure. The Biacore 3000 software was modified by GE Healthcare to allow the development of a method for the injection of suspected noncompliant samples over the RC mounted in the SPU, followed by washing steps and analyte forward-elution. The procedure for the recovery of the analyte from samples suspected as being noncompliants in the iBIA started with the injection of 40 μL of sample without antibody over the RC at 10 $\mu L\ min^{-1}$. The RC was washed

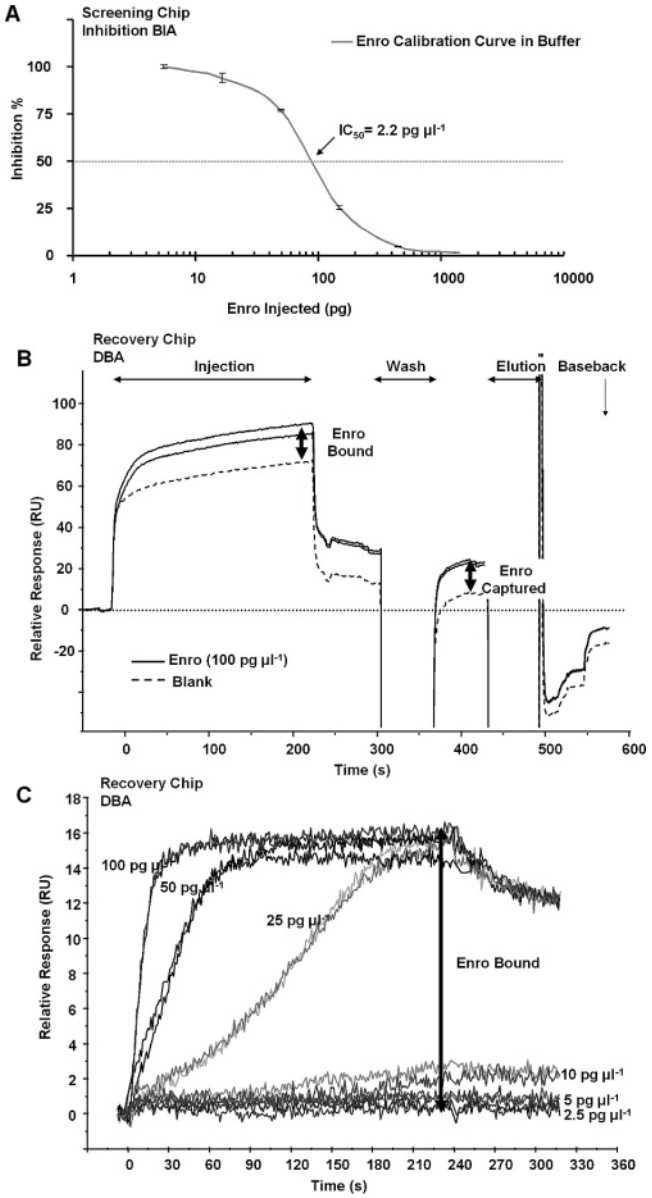

Figure 2. (A) Calibration curve of Enro in the iBIA with SC A (see Table 1 and the Screening Chip Performance section). (B and C) Evaluation of the direct binding of Enro to SpMH40 immobilized on the RC A (see Table 2 and the Recovery Chip Performance section). (B) Overlaid sensorgrams of complete ligand/wash/regeneration cycles obtained in the direct binding assay (DBA) format measured with the Biacore Q. (C) Blank-subtracted (i.e., dashed line in B subtracted from the Enro sensorgrams) sensorgrams of duplicate injections of Enro at various concentrations to evaluate the maximum capacity and saturation time of RC A.

for 2 min with water, then 10 μL of formic acid (1%) was injected to disrupt the interaction of the analyte with the biosorbent followed by the injection of a transfer volume (water) to position the eluate in the loop-type interface leading toward the nano-LC TOF MS. In order to reduce carryover issues, two washing steps were added in the RC controlling software after the elution step. The first one was the elution solvent (i.e., formic acid 1%), and the second one was water to condition the antibodies for the next recovery cycle.

The transfer volume required to position the 10 μL RC eluate plug from the SPU into the loop-type interface was optimized using a glass chip (instead of a functionalized RC) that was fitted in the SPU and connected to the loop-type interface and the nano-LC TOF MS system. Injections of 10 μL of a standard solution of Enro (100 pg μL^{-1}) in 1% formic acid were followed by increasing transfer volumes. A transfer volume of 13 μL showed that the area counts of the Enro peak in the chromatogram matched the area counts of an injection of Enro from the nano-LC TOF MS autosampler. With the same procedure, a calibration curve injected from the SPU and the loop-type interface was constructed by measuring increasing concentrations of Enro standards (0−500 pg) with the nano-LC TOF MS system. The Enro calibration curve was used for all further estimations of the RC capacity.

Recovery Chip Performance. If compared with traditional affinity extraction columns, the RC concept features the unique advantage of being able to characterize the affinity sorbent using the SPR biosensor. Using the DBA format we can study the performance of the RC in detail and obtain useful information to select the biomolecule best suited for each application. Robust biomolecules, with high affinity, low dissociation rate, and an interaction with the analyte that can be disrupted with MS-compatible buffer, are desired. The optimal density of immobilized biomolecules, capacity, affinity constants, and saturation time can also be evaluated. Additionally, the nonspecific binding of matrix components to the RC and the wash and elution steps can be scouted without compromising the nano-LC TOF MS system. As a model, the interaction between Enro and the antibodies (SpMH40) immobilized in RC A (see Table 2) was evaluated using a Biacore Q SPR biosensor. This biosensor features a flow cell height of 50 μm, comparable to the FCT2 flow cell used with the RC in the SPU. Figure 2B depicts the representative sensorgrams obtained with the injection of 40 μL of (100 pg μL^{-1}) Enro standard solution. The bulk response jump upon injection caused by the blank and the Enro standard solution are due to the lack of a reference channel in the Biacore Q for subtraction. The maximum response of Enro binding to the immobilized antibodies was 16 RU as observed from the difference between the Enro and the blank buffer injection. The Enro response decays when the dissociation of the lower strength interactions occurs after the injection ends. About 12 RU of Enro remain captured on the RC A surface after the dissociation and the 1 min washing step. The interaction can be disrupted for elution purposes using a 1 min injection of MS-compatible solvent (formic acid 1%, pH 2). The response returns close to its baseline values with a loss of <20 RU (<0.1%) of immobilized SpMH40 after each cycle. Figure 2C depicts sensorgrams of duplicate injections of standards solutions with increasing concentration of Enro after the subtraction of sensorgrams of blank injections (at least one for each concentration). The SpMH40-based biosorbent in RC A showed to be robust and preserved its binding properties for more than 50 cycles under these wash−elution conditions.

In Biacore SPR measurements an equivalence of 1 RU to 1 pg mm^{-2} is assumed for proteins.[23] Hence, the 19 000 RU of SpMH40 immobilized in RC A are roughly equivalent to 2 pmol on the complete surface of 16 mm^2.

The maximum theoretical response of a ligand ($RU_{LMaxPred}$) binding to immobilized macromolecules (RU_M, response of the macromolecule immobilized) is predicted for proteins using $RU_{LMaxPred} = RU_M n(Mw_L/Mw_M)$, with n being the number of

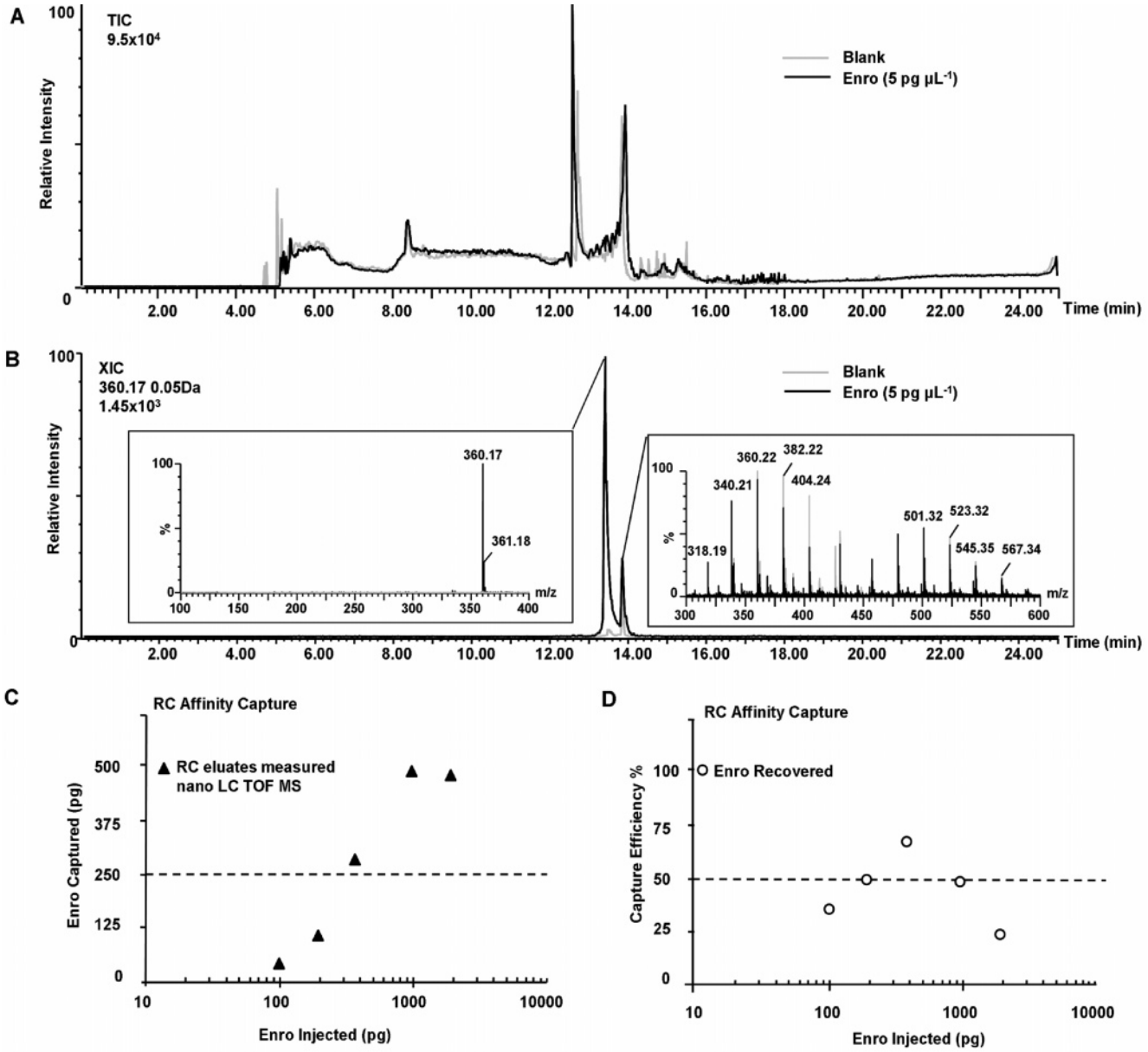

Figure 3. Overlaid chromatograms and mass spectra (see insets) of relevant peaks of RC eluates analyzed on-line by nano-LC TOF MS. Reagent blank (gray) and Enro standard solution (5 pg μL^{-1}) (black) in HBS-EP buffer were injected (40 μL, 10 μL min^{-1}) over RC A and eluted with 10 μL of formic acid (1%). (A) Total ion current (TIC) chromatogram; (B) extracted ion chromatogram (0.05 Da mass window). Enro (360.17) elutes in the first peak. The second peak corresponds to a buffer interference (360.22) also present in the blank. (C) Nano-LC TOF MS analysis of RC eluates after injections with increasing Enro concentrations. (D) Recovery efficiency of the RC A at different concentrations of Enro injected over the RC.

binding sites in the macromolecule (2 for antibodies), Mw_L being the molecular weight of the ligand (359 Da for Enro), and Mw_M being the molecular weight of the macromolecule (150 kDa for antibodies). Hence, given the immobilization level of SpMH40 in RC A (see Table 2), one would expect to measure a response of about 90 RU upon saturation of all binding sites with Enro (4 pmol on the 16 mm^2). Nevertheless, Figure 2, parts B and C, shows that about 16 RU of Enro are bound and 12 RU remain captured after the washing step. Nano-LC TOF MS measurements of Enro actually eluted from RC A (Table 2 and Figure 3) revealed that the maximum Enro capture capacity of the chip after dissociation and wash step is approximately 500 pg (≈1.4 pmol of Enro), which is 34% of the theoretical maximum. This lower capacity is partly due to the nonspecific immobilization method used, that renders many binding sites inactive because it couples primary amines (N-terminal and possibly lysine groups) of the macromolecule with the dextran surface.[24] The actual maximum capacity of the RC as measured with nano-LC TOF MS is approximately 500 pg (30 RU), higher than the 190 pg (12 RU) expected from the SPR measurement. This underestimation of the maximum capacity arises possibly from two erroneous presumptions. The first is the correlation of 1 RU = 1 pg mm^{-2}. This correlation cannot be applied to small molecules because the SPR response depends on the molecular structure as well as the molecular weight.[25]

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Second, the distribution of the antibodies in the hydrogel is homogeneous. Instead, the antibodies in this highly packed affinity biosorbent probably form a gradient perpendicular to the sensor surface, as previously suggested.[26,27] This influences the SPR response because the evanescent wave intensity has a strong exponential decay within a few hundred nanometers next to the surface.[27] Hence, highly concentrated antibodies binding Enro in the distal regions of the hydrogel would contribute little to the final Enro SPR response and also limit the transport to the lower density layers under a higher evanescent wave intensity. The linear binding curves of Enro to the antibodies in the sensorgrams of Figure 2C show that the reaction is strongly limited by mass transport under the conditions of low flow rate (10 μL min^{-1}) and a high flow cell height (50 μm), in accordance with expectation.[28] The sensorgrams also suggest a further diffusion limitation at Enro concentrations below 25 pg μL^{-1} where very low responses are obtained indicating low capture levels. An attempt to obtain the kinetic constants by fitting the Enro sensorgrams of Figure 2C to a simple 1:1 bimolecular model with mass transport, using CLAMP software,[29] resulted as inconclusive, indicating a complex kinetic system influenced by additional diffusion limitations and a heterogeneous kinetic behavior of the polyclonal antibodies. SPR experiments using a lower capacity RC (8900 RU of immobilized antibodies) showed an Enro binding response of 15 RU. This binding response is comparable to the response obtained with the high-capacity RC A and suggests either that a higher proportion of binding sites are active or that they are distributed more homogeneously at lower densities.

The nano-LC TOF MS analysis of RC A eluates from injections of Enro below 25 pg μL^{-1} shown in Figure 3 demonstrates that Enro is indeed captured at levels that should be measurable with the biosensor. An increase in the concentration of Enro injected over RC A correlates with increasing amounts of Enro captured on the RC as shown in Figure 3C yielding a curve that mirrors the calibration curve obtained in the iBIA (Figure 2A). The analysis of the RC capture efficiencies shown in Figure 3D clearly shows that there is an optimal concentration of Enro that maximizes the capture.

Knowing the kinetic constants could have an additional analytical value in the characterization of the biosorbent since they predict the RC behavior and would correlate the estimated analyte concentration values of the suspected noncompliants selected with the iBIA screening with a binding rate and capture efficiency when injected in the affinity chip interface. This would facilitate an automated adjustment of the sample concentration and volume needed in the RC to maximize the capture efficiency. The results presented show the potential insight to which the RC affinity sorbent can be studied using the present system. Last but not least, the predicted RC performance from the SPR measurements represents just a worst case (i.e., SPR can be exploited as a QA/QC check). More considerations about RC hydrogel immobilization capacity can be found in the Supporting Information. Future research will focus first on optimizing the immobilization chemistry and using recombinant antibodies that allow an oriented immobilization, a higher efficiency, and a simpler estimation of the kinetic constants. The nano-LC TOF MS analyses presented in Figure 3 demonstrate that the amount of Enro captured onto the RC is more than sufficient, even when Enro is injected onto the RC at low concentrations of 5 pg μL^{-1}.

The Enro capture capacity of sensor chips of different hydrogel lengths and branching densities (see Table 2) were studied. Again, the SPR biosensor and the on-line biosensor−RC−nano-LC TOF MS system were used for measuring the amount of captured Enro. The standard Biacore chip, RC A, showed the highest antibody immobilization capacity (19.6 ng mm^{-2}) and Enro capture capacity (12 RU). A shorter hydrogel of higher density (RC B) lowered 16% the immobilization capacity and proportionally decreased the Enro capture capacity. However, a small increase in length of this high-density hydrogel (RC D) dramatically lowered the antibody immobilization capacity by 75%. RC E and RC F had a linear polymer hydrogel (undisclosed polymer chemistry by the manufacturer) and showed 59% and 53% lower immobilization capacities, respectively. Nevertheless, these hydrogels exceeded the evanescent wave sensing region, and more antibodies than probed by SPR measurement were expected. The Enro capture capacities of RC E (high-density hydrogel) and RC F (lower density hydrogel) were 0 and 9 RU, respectively, according to the biosensor. In contrast, the nano-LC TOF MS eluate analysis showed that Enro could be captured in RC E and RC F at a similar level of 13 and 14 pg mm^{-2}, respectively. This suggests that the diffusion of Enro in chip E was limited by the high-density hydrogel and that the SpMH40 interaction took place too far from the chip surface. Given the potential of a 5−10-fold higher hydrogel volume featured in RC F, this seems as a suitable candidate for a high-capacity chip still allowing characterization using the biosensor. However, RC A was used throughout this study due to its high capacity and robustness. Further research will focus in optimizing the antibody immobilization procedure for this chip combined with recombinant antibodies currently under development.

Multianalyte Capture. In order to test the feasibility of capturing more than one analyte on the RC surface, a monoclonal antibody IgM (MAb72F) with high CR for Enro and Cipro was immobilized on a chip (RC A1). The level of immobilization (Table 2) was similar to that of RC A with the SpMH40 although the IgM molecules have a molecular weight of 900 kDa. The SPR measurements resulted in a lack of Enro response. The nano-LC TOF MS analysis of eluates from RC A1 after the injection of a mixture of Enro and Cipro (100 pg μL^{-1}) showed that about 3 pg mm^{-2} of Enro (CR 100%) and 1.6 pg mm^{-2} of Cipro (CR 53%) were captured. Comparatively, 30.6 and 1.8 pg mm^{-2}, respectively, were obtained (CR 6%) when the same experiment was repeated using the PAb SpMH40-based RC A described above. These results prove that more than one analyte can be captured and measured with the RC nano-LC TOF MS system, even with an RC A1 based on MAb72F that is an unstable MAb isotype where few binding sites remained active after immobilization.

Screening, Recovery, and Identification of Enro in Chicken Samples. The applicability of this iBIA for the screening of Enro in chicken muscle extracts was further evaluated by measuring a calibration curve of Enro in chicken muscle extracts diluted (1:1;

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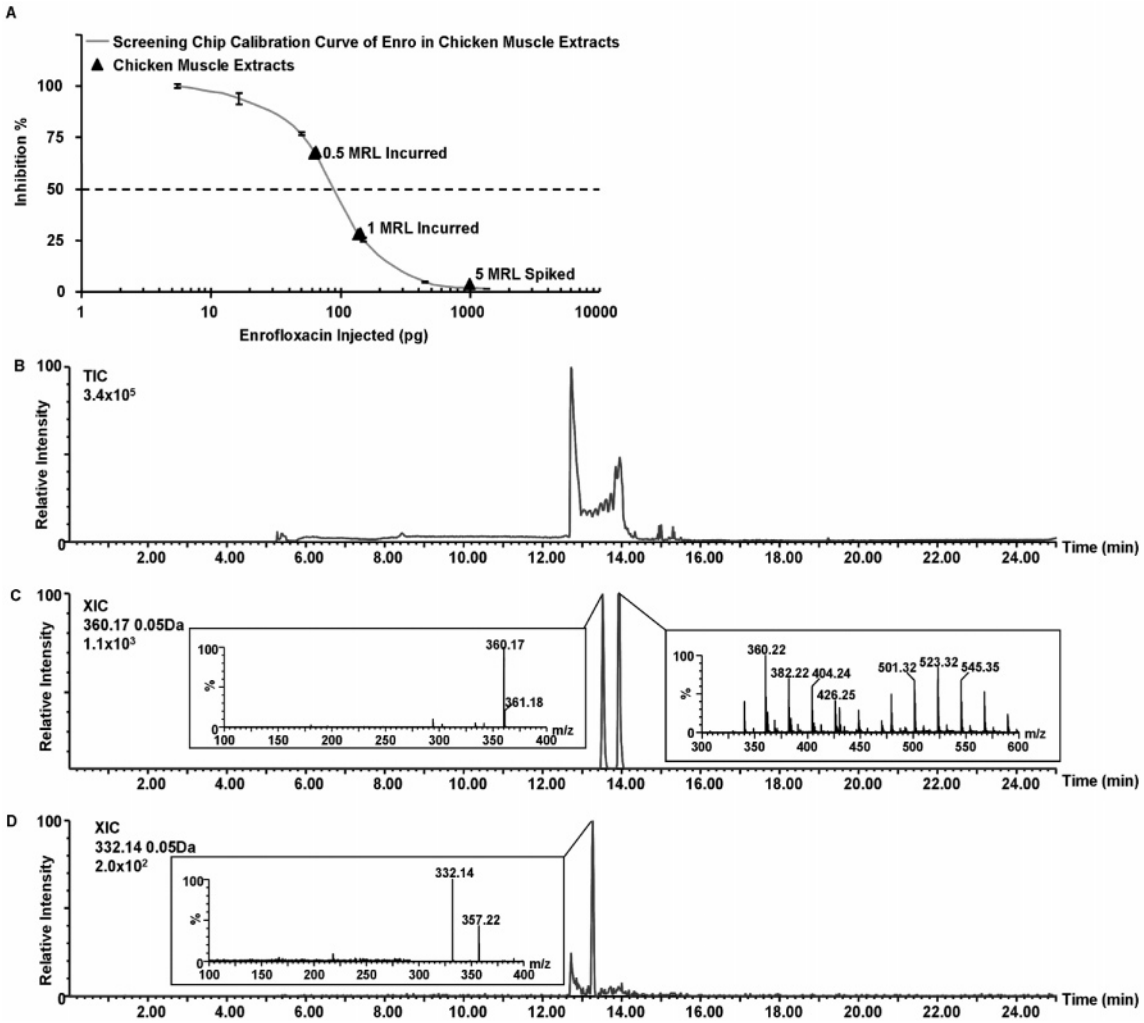

Figure 4. (A) iBIA calibration curve obtained with chicken muscle extract spiked with Enro using SC A. Black triangles show the muscle extract samples from chicken samples either incurred or spiked with Enro. Following the iBIA screening, 40 μL of muscle extract was reinjected into the RC A for affinity extraction and the eluate is used for nano-LC TOF MS analysis. (B−D) Chromatograms and mass spectra (see insets) of relevant peaks of an RC A eluate obtained from muscle extract incurred with Enro at 1 MRL (5 pg μL^{-1} in the extract). (B) TIC and (C) XIC (0.05 Da mass window) of Enro (360.17). The second peak corresponds to a buffer interference (360.22) also present in the blank. (D) XIC (0.05 Da mass window) of Cipro (332.14), a metabolite of Enro.

v/v) in HBS-EP buffer (Figure 4A). Similar sensitivity (Enro IC_{50} = 2.4 pg μL^{-1}) was obtained compared to the calibration curve in buffer (Figure 2A), and no nonspecific binding of the sample matrix to the SC surface was observed. Extracts of chicken samples incurred or spiked with Enro at regulatory-relevant levels (maximum residue level (MRL) 100 μg kg^{-1} for the sum of Enro and Cipro) were measured with the iBIA in conjunction with SC A. Although, due to dilution during the extraction procedure of the chicken muscle samples, the concentration of the sample extract is lowered 20 times, all the chicken muscle extracts (at 0.5, 1, and 5 MRL) could be measured with the iBIA. These samples were considered as suspected noncompliants and were reinjected into the SPU containing RC A for analyte capture and further analyzed with nano-LC TOF MS. Figure 4, parts B and C, shows the total ion current (TIC) and extracted ion current (XIC) chromatogram obtained during the analysis of the incurred chicken sample at 1 MRL. The mass spectra of some peaks between 12.6 to 14.2 min (see the second inset in Figures 3B and 4B), display peaks separated by 44 and 22 Da. These correspond to single- and double-charged ethylene oxide residues of the polyethylene glycol used as surfactant in the HBS-EP buffer used to dilute the extracts, suggesting that the washing steps implemented after the injection of the sample trough the RC surface were not sufficient to completely remove the surfactant. Surfactant removal from the buffer would invariably increase nonspecific binding issues. However, ongoing efforts will further optimize the buffer conditions. Nevertheless, even with these surfactant interferences, the XIC chromatogram clearly displays a peak corresponding to the mass of the $[M + H]^+$ ion of Enro (360.17) at 13.4 min. The second peak at 14 min corresponds to the elution of one of the surfactant isomers having a similar m/z (360.22). We considered the possibility that Cipro might be captured in this sample using RC A with PAb SpMH40. The XIC chromatogram in Figure 4D shows a peak with a mass that corresponds to the $[M + H]^+$ ion of Cipro (332.14) that elutes shortly (13.3 min) before Enro. This finding implies that Cipro, an Enro metabolite, is present at a low concentration in the incurred chicken muscle sample. This result was independently confirmed by an LC/MS/MS confirmatory analysis method (data not shown). These results strongly suggest the potential use of the serial SPR RC nano-LC

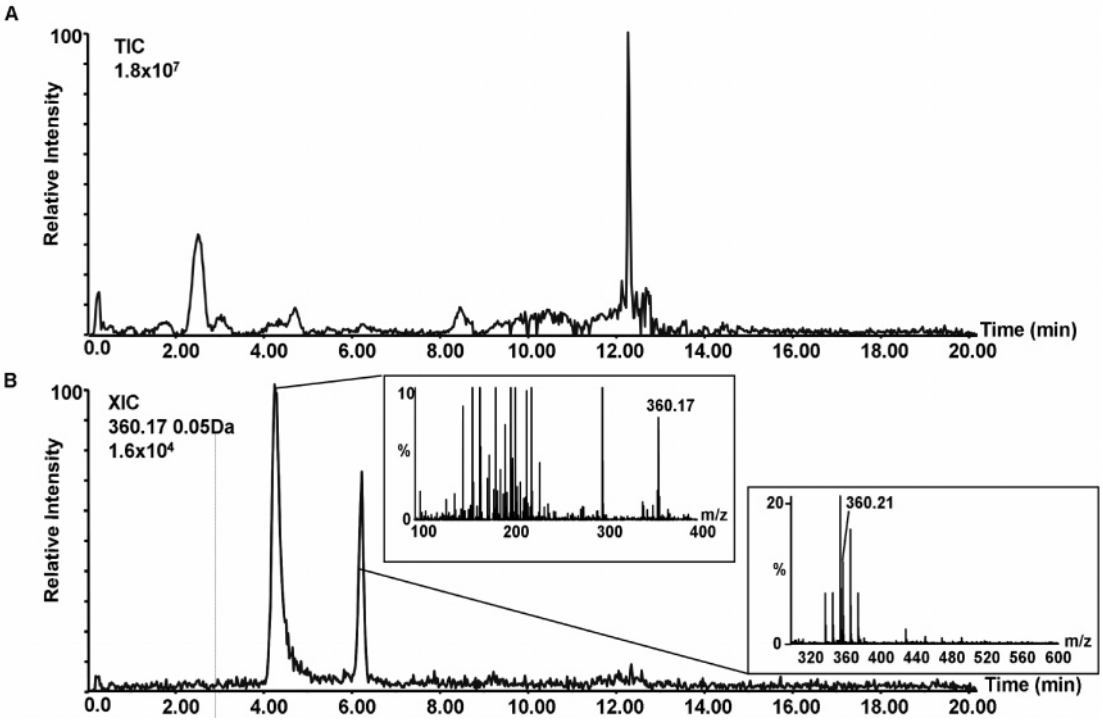

Figure 5. Triple-chip system. Chromatograms and mass spectra (see insets) of an RC A eluate obtained from the injection of 10 μL of muscle extract from chicken incurred with 0.5 MRL level of Enro (2.5 pg μL^{-1} in the extract) and analyzed off-line by chip-LC TOF MS. (A) TIC and (B) XIC (0.05 Da mass window) of Enro (360.17). The second peak in (B) corresponds to a buffer interference (360.21) also present in the blank.

TOF MS coupling concept for the identification of biologically relevant, but yet unknown, analytes in the samples. Optimization of the buffer surfactant interferences and the use of accurate masses will allow an untargeted analysis of relevant peaks, facilitated by the biosorbent featured on the RC that provides a selective cleanup, greatly reducing the number of irrelevant peaks in the mass spectra and minimizing ion suppression.

Triple-Chip Feasibility. The common drawbacks of the classical capillary nano-LC setup described above are nano-ESI spray positioning, blocking of capillaries, dead volumes, etc. Due to these issues, specific skills of the operator are required for troubleshooting. Recently a microfluidic nano-LC chip TOF MS system was introduced which overcomes these traditional nano-LC limitations.[21] We briefly evaluated the feasibility of introducing such a third chip into the system aiming for a more robust overall setup. Chicken muscle samples spiked and incurred with Enro at levels as low as 0.5 MRL were injected over the RC A, and the eluates were analyzed (off-line) with the chip LC TOF MS system as described in the Experimental Section. As a typical example, Figure 5 shows the TIC (Figure 5A) and XIC (Figure 5B) of the SpMH40 RC eluate from a chicken muscle sample incurred with Enro at 0.5 MRL. The XIC clearly displays two peaks, the first one (Rt = 4.3 min) corresponding to the $[M + H]^+$ ion of Enro (m/z 360.1723), and the second one of lower intensity corresponding to the buffer impurity discussed above. The high mass accuracy of TOF MS allowed elemental composition calculation of the analyte of interest. Within the restrictions of $m/z \pm 5$ mDa, ring plus double-bond equivalent number 5−20, atoms $C_{0-30}H_{0-40}N_{0-5}F_{0-5}$ nine elemental composition options remain, $C_{19}H_{23}N_3O_3F$ being on top of the list having the smallest difference between the theoretical and experimentally determined accurate mass. Subsequent data mining for $C_{19}H_{22}N_3O_3F$ using the comprehensive SciFinder database confirmed the presence of Enro in the incurred sample. Such a robust chip format nano-LC ESI system would be probably the only realistic option for routine food analysis laboratories. It should be emphasized that no theoretical limitation would limit the integration of the RC and the nano-LC ESI chip.

CONCLUSIONS

A new concept for coupling an iBIA screening of low molecular weight analytes with nano-LC ESI TOF MS for confirmation of identity using an affinity chip interface has been presented. The interface features the unique advantage of allowing an in-depth characterization of the biosorbent and its interaction with the analyte and sample matrix components. This interface provides a highly selective sample cleanup which greatly reduces the number of irrelevant peaks in the subsequent nano-LC TOF MS analysis. For the application selected, the optimal hydrogel thickness for the RC interface was 100−200 nM. With this RC the interfaced system was able to detect our model analyte in noncompliant samples below the regulatory limits during the screening in incurred chicken muscle samples. Reinjection of relevant samples in the affinity chip interface provided a good recovery and a concentration step of the analyte to be identified in the nano-LC MS analysis, even with a minor biosorbent cross-reactivity for a bioactive metabolite of Enro present in the noncompliant sample. The metabolite was captured in the RC at a suitable level for nano-LC TOF MS analysis.

This is a promising outcome that shows the potential of designing multianalyte affinity chips for multianalyte inhibition BIAs followed by multianalyte recovery. Finally, a more robust

triple-chip approach seems feasible. The concept presented can be easily extended to other applications requiring both a fast bioactivity-based screening and accurate mass assessment with subsequent elemental composition calculation, for example, in the fields of natural toxin analysis and security applications.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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