

Integrated Amperometric Affinity Biosensors Using Co^{2+} –Tetradentate Nitrilotriacetic Acid Modified Disposable Carbon Electrodes: Application to the Determination of β -Lactam Antibiotics

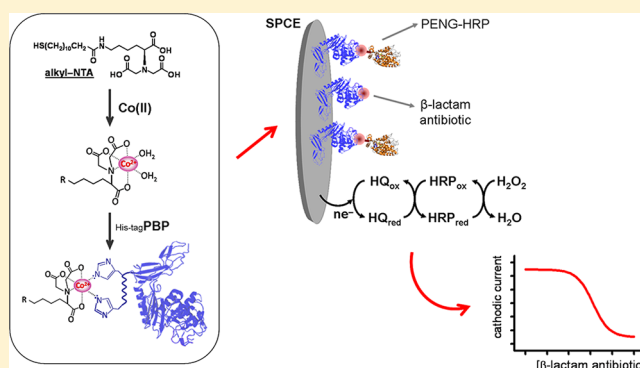
Felipe Conzuelo,[†] María Gamella,[†] Susana Campuzano,[†] Paloma Martínez-Ruiz,[‡] María Esteban-Torres,[§] Blanca de las Rivas,[§] A. Julio Reviejo,[†] Rosario Muñoz,[§] and José M. Pingarrón^{*,†}

[†]Departamento de Química Analítica, and [‡]Departamento de Química Orgánica I, Facultad de CC. Químicas, Universidad Complutense de Madrid, Avenida Complutense s/n, 28040 Madrid, Spain

[§]Laboratorio de Biotecnología Bacteriana, Instituto de Ciencia y Tecnología de Alimentos y Nutrición, C/Juan de la Cierva 3, 28006 Madrid, Spain

S Supporting Information

ABSTRACT: A novel strategy for the construction of disposable amperometric affinity biosensors is described in this work. The approach uses a recombinant bacterial penicillin binding protein (PBP) tagged by an N-terminal hexahistidine tail which was immobilized onto Co^{2+} –tetradentate nitrilotriacetic acid (NTA)-modified screen-printed carbon electrodes (SPCEs). The biosensor was employed for the specific detection and quantification of β -lactam antibiotics residues in milk, which was accomplished by means of a direct competitive assay using a tracer with horseradish peroxidase (HRP) for the enzymatic labeling. The amperometric response measured at -0.20 V versus the Ag pseudoreference electrode of the SPCE upon the addition of H_2O_2 in the presence of hydroquinone (HQ) as redox mediator was used as the transduction signal. The developed affinity sensor allowed limits of detection to be obtained in the low part-per-billion level for the antibiotics tested in untreated milk samples. Moreover, the biosensor exhibited a good selectivity against other antibiotics residues frequently detected in milk and dairy products. The analysis time was of approximately 30 min.



Many efforts have been done in the last years to achieve the immobilization of biological macromolecules on electrode surfaces with a significant retention of their activity. Among the large variety of immobilization strategies described (adsorption, covalent attachment, polymer encapsulation, electrostatic or hydrophobic interactions, etc.), methods based on affinity ligands or biospecific recognitions are particularly attractive because (i) the activity of the immobilized biomolecule is generally preserved, (ii) the spatial distribution can be well-controlled, (iii) the efficiency of the specific immobilization can be high, making it possible to work with low biomolecule loadings, and (iv) the stability of the biomolecule is usually improved.¹ One of the most versatile and powerful affinity ligand methods is based on the use of metal ion chelating agents and their specific affinity for proteins containing histidine residues, which constitutes a standard tool in affinity chromatography (immobilized metal ion affinity chromatography, IMAC). The coordination of appropriate chelating agents such as the tridentate iminodiacetic acid (IDA) or the tetradentate nitrilotriacetic acid (NTA) to bivalent metal cations (e.g., Cu^{2+} , Ni^{2+} , Zn^{2+} , Co^{2+}) allows further ligation of the free coordination sites of the chelator–metal complex to

the imidazole moieties of the histidine tags thus enabling a localized, oriented, and reversible immobilization of proteins.² The histidine tag possesses a small size, and its position at the protein surface can be controlled, thus leading to an oriented immobilization without interfering with structure and function of the protein.³ This latter point is of significant importance in analytical approaches involving heterogeneous biomolecular recognitions because the random attachment of an affinity protein can alter its binding capacity resulting from steric hindrance of adjacent immobilized proteins or with the surface itself.¹ This histidine-tagged protein immobilization chemistry has been widely reported on gold supports using different approaches.^{4–8} However, carbon electrodes have been scarcely used with this purpose due to the laborious and time-consuming multiple chemical steps of functionalization required for their modification.¹

On the other hand, the development of receptor–ligand-based biosensing strategies can result in rapid and sensitive

Received: December 12, 2012

Accepted: February 19, 2013

Published: February 19, 2013

analytical methodologies for a wide range of analytes. In this work we used penicillin-binding proteins (PBPs) as molecular receptors. PBPs are enzymes found in the cytoplasmic membrane of bacteria involved in the growth of the microorganisms by maintaining and determining cellular morphology.⁹ They catalyze transpeptidation reactions resulting in glycan chains cross-linking by short peptides, as well as the elongation of these glycan chains by transglycosylation.¹⁰ β -Lactams bind irreversibly to the active site of PBPs due to their analogy with the 2-peptide D-alanyl-D-alanine, leading to the PBPs inactivation by acylation of the active site serine residue involved in the transpeptidase reaction.¹¹

β -Lactams are frequently prescribed in veterinary medicine for the treatment of mastitis in lactating dairy cows¹² and constitute one of the most commonly identified antimicrobial group found in milk. Misuse of antibiotic drugs relies on public health risks causing allergic reactions in sensitized individuals or affecting the intestinal flora of consumers and financial losses due to the inhibition of growth of starter cultures used for food technological processes.¹³ Moreover, it is also important the fact that low-level doses of antibiotics in foodstuffs during long periods may lead to the spread of drug-resistant microorganisms.¹⁴ Maximum residue limits (MRLs) have been established by the European Union for common β -lactams antibiotics based on their potency. For example, the MRL for amoxicillin, ampicillin, and benzylpenicillin is $4 \mu\text{g kg}^{-1}$ in milk, while for cefapirin it is $60 \mu\text{g kg}^{-1}$.^{15,16} Although the analysis of veterinary drugs residues is generally made by liquid chromatography, there is a high demand for rapid and inexpensive analytical methods for the screening of β -lactam antibiotics in certain samples. Actually, a variety of commercial test kits and strips are available.^{17,18} In this context, immunoanalytical methods show some limitations since many are nonspecific or not sufficiently sensitive because the chemical reactivity of the β -lactam ring avoids the synthesis of specific and sensitive antibodies against β -lactam antibiotics.¹⁹ Moreover, microbial inhibition tests, which are the only tests recognized by the European Union as screening methods for penicillins, require a few hours of incubation and, since they are not selective among antibiotics, sometimes may give false positives.¹⁸ All these considerations support the development of receptor ligand based strategies leading to rapid, specific, and sufficiently sensitive analytical methodologies available to be used by untrained personnel.

In this paper, we describe a novel, extremely simple, versatile, and fast approach for the functionalization of carbon electrodes in a single step with dense monolayers of chelating agents without need for applying any previous harsh pretreatment to the electrode surface. We report herein the first successful adsorption of long alkyl chains-NTA derivatives on disposable carbon electrodes and the use of the resulting film for the subsequent coordination of cobalt ions and the histidiny residues in the tagged PBP, allowing the immobilization of the receptor protein in a reproducible and reversible manner, with a controlled molecular orientation and maintaining its entire biological activity. The resulting integrated amperometric affinity sensor allows the quantification of β -lactam antibiotics residues in milk at the low part-per-billion concentration level through competitive binding between sample β -lactam and a HRP-labeled specific tracer for the binding sites of the immobilized PBP. The electrochemical detection of the enzyme product was carried out using hydroquinone (HQ) as electron-transfer mediator and H_2O_2 as the enzyme substrate.

MATERIALS AND METHODS

Apparatus and Electrodes. Amperometric measurements were performed with a CHI812B potentiostat (CH Instruments, Inc., U.S.A.) controlled by software CHI812B. All measurements were carried out at room temperature (RT). Impedance and voltammetric measurements were accomplished using a μ Autolab Type III FRA 2 instrument. A Bunsen AGT-9 vortex was used for homogenization of the samples.

The transducers employed for the development of the integrated amperometric affinity sensors were screen-printed carbon electrodes (SPCEs) (DRP-C1110, DropSens) consisting of two elliptic carbon working electrodes ($\phi \sim 2 \text{ mm}$), a carbon counter electrode, and a Ag pseudoreference electrode. A specific cable connector (DRP-BICAC also from DropSens) acted as the interface between the SPCE and the potentiostat.

Reagents and Solutions. All the reagents used were of the highest available grade. N -[N_α -Bis(carboxymethyl)-L-lysine]-12-mercaptododecanamide (HS-NTA), N_α -Bis(carboxymethyl)-L-lysine hydrate (ANTA), Tween20, $\text{K}_3\text{Fe}(\text{CN})_6$, $\text{K}_4\text{Fe}(\text{CN})_6$, sodium acetate, hydroquinone (HQ), hydrogen peroxide (30%, w/v), lauroyl chloride, 4-(N,N -dimethylamine)pyridine (DMAP), ampicillin sodium salt (AMP), and tetracycline hydrochloride (TC) were purchased from Sigma-Aldrich. The enzyme tracer, HRP-conjugated β -lactam (PENG-HRP) was acquired from United States Biological. Sulfapyridine (SPY), cefapirin sodium salt (CEF), and enrofloxacin (ENRO) were from Fluka. Sodium dihydrogen phosphate, disodium hydrogen phosphate, potassium chloride, sodium hydrogen carbonate, and sodium chloride were purchased from Scharlau. Sodium carbonate anhydrous, dimethyl sulfoxide (DMSO), and cobalt(II) chloride were acquired from Panreac. Copper(II) chloride was from Merck, A.G. Nickel(II) nitrate and zinc nitrate were acquired from Probus S.A. Imidazole was purchased from Acros. All buffer solutions were prepared with Milli-Q water ($18 \text{ M}\Omega \text{ cm}$ at 25°C). Acetate buffer, pH 4.5, was prepared from 0.1 M sodium acetate and carbonate buffer, pH 9.6 from 0.05 M carbonate buffer solution. Binding and washing buffer (B&W) was 50 mM phosphate buffer solution, pH 8.0, containing 300 mM NaCl and 0.01% Tween20. Pull and down buffer (P&D) was 3.25 mM phosphate buffer solution pH 7.4 containing 70 mM NaCl and 0.01% Tween20. PBST is phosphate-buffered saline (0.01 M phosphate buffer solution containing 137 mM NaCl and 2.7 mM KCl, pH 7.5) with 0.05% Tween20.

The 10 mM stock solutions of the different antibiotics were prepared in DMSO (SPY and ENRO) or in P&D buffer (AMP, CEF, and TC) and stored at 4°C . Antibiotic standard solutions were prepared daily upon dilution of the corresponding stock solutions in P&D buffer or in milk. Hydrolyzed β -lactams solutions were prepared by dissolving 10 mg of the antibiotic (AMP or CEF) in 9.8 mL of methanol (Scharlab) and 0.2 mL of 1.0 M potassium hydroxide (Scharlab) and left to react overnight at 4°C .¹³

Whole UHT milk samples were purchased in a local supermarket, spiked, and used for the determination of β -lactams after 1:1 dilution with P&D buffer.

Synthesis of Lauroyl-NTA. Lauroyl-NTA (N^2,N^2 -bis(carboxymethyl)- N^6 -dodecanoyllysine, LAU-NTA) was synthesized as follows: 93 mg (0.76 mmol) of DMAP and 243 μL (3.05 mmol) of pyridine were successively added to a

suspension of 200 mg (0.76 mmol) of ANTA in 5 mL of DMF at room temperature while vigorously stirring. After 10 min, 220 μL (0.92 mmol) of lauroyl chloride was added and the mixture was stirred for 2 days at room temperature. Then the solvent was evaporated in vacuum, and the crude was dissolved in 2 mL of water. After carefully acidification with 1.0 M hydrochloric acid, the resulting precipitate was filtered and successively washed with water and diethyl ether to give the title compound (56% yield). Experimental data agree with those previously described by Richard et al.²⁰

Preparation of Co^{2+} -NTA-Modified SPCEs. In order to functionalize with the NTA layer, 5 μL of a 1.0 mM HS-NTA or a 3.0 mM LAU-NTA solution (prepared in carbonate buffer) was cast on the working electrode surface and incubated 2.5 h at room temperature in a humidified chamber. After washing with deionized water and drying with nitrogen, the resulting NTA-modified SPCE was activated by complexation of Co^{2+} by treatment with 5 μL of a 7.5 mM CoCl_2 solution (prepared in acetate buffer) for 30 min, followed by thorough washing with deionized water and drying under a nitrogen stream. The Co^{2+} -loaded electrodes showed no damage when they were left on the benchtop for 3 days before use (no longer times were checked).

Penicillin Binding Protein Production, Overexpression, and Purification. Protocols used for recombinant His-tagged PBP protein production, overexpression, purification and storage were the same than those employed in an earlier paper.²¹

Affinity Biosensor Preparation. Recombinant PBP was immobilized on the Co^{2+} -NTA-modified electrodes through the protein His-tag. In order to do that, a 5 μL aliquot of the PBP solution (880 $\mu\text{g mL}^{-1}$) was dropped on the modified electrode surface and incubated at room temperature for 20 min. Thereafter, the electrode was washed with PBST to remove any unbound protein and dried under a nitrogen stream.

Electrochemical Measurements. Impedimetric and cyclic voltammetric measurements were carried out upon deposition of 50 μL of a $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$, 5.0 mM in each component, solution in 0.1 M phosphate buffer pH 7.0 on the modified electrode surface. Electrochemical impedance measurements were performed at the equilibrium potential of the $\text{Fe}(\text{CN})_6^{4-}/\text{Fe}(\text{CN})_6^{3-}$ pair, with a 10 mV (rms) sinusoidal excitation amplitude and an automatic analyzer integration time (0.001% SD of $-I(j\omega)$ correlator output) with a 100 s cutoff time. Measurements were made at 20 steps per decade in the appropriate frequency range five times at each frequency and averaged during each run. Cyclic voltammograms were recorded from -0.40 to 0.60 V (vs Ag pseudoreference electrode, which shows a potential difference with a SCE of -256 ± 19 mV) at a scan rate of 50 mV s^{-1} .

Amperometric measurements in stirred solutions were performed by applying the desired potential and allowing the steady-state current to be reached. The sensors were transferred to an electrochemical cell containing 10 mL of a 0.1 M phosphate buffer pH 6.0 and 1.0 mM HQ (prepared daily just before the electrochemical measurement), and the amperometric responses at $E_{\text{app}} = -0.20$ V versus Ag pseudoreference electrode were recorded after the addition of 50 μL of 0.1 M H_2O_2 . The time required to reach the steady-state current was approximately 1.5 min.

Affinity Assay Procedure. A calibration curve for AMP was constructed in the assay buffer (1.0 M NaCl-enriched P&D

buffer) containing the optimized enzymatic tracer concentration and different concentrations of the β -lactam antibiotic. The solutions were placed in a vortex to ensure adequate mixing of the components. The competitive assay was performed by casting of a 50 μL aliquot of the corresponding mixture solution on the biosensor surface and incubation at RT for 30 min. Thereafter, the electrode was thoroughly washed with PBST and the electrochemical measurement was carried out.

The recorded amperometric signals were the difference between the steady-state and the background currents. They were plotted as a function of the analyte concentration, and the experimental data were fitted to a four-parameter logistic equation (sigmoidal) according to the equation

$$y = i_1 + \frac{i_2 - i_1}{1 + 10^{(\log \text{IC}_{50} - x)p}} \quad (1)$$

where i_2 and i_1 are the maximum and minimum current values in the plotted calibration graph, IC_{50} is the analyte concentration producing 50% of the maximal amperometric signal, and p is the Hill slope at the inflection point of the sigmoid curve. The limit of detection (LOD) was calculated as the analyte concentration for which the enzymatic tracer binding to the PBP was inhibited by 10%. The dynamic range (DR) was defined as the analyte concentration interval for which the inhibition of the enzymatic tracer binding was between 20% and 80%. Unless otherwise indicated, the presented data corresponded to the average of at least three replicates and the confidence intervals calculated for $\alpha = 0.05$.

Analysis of AMP in Milk. Commercial milk samples purchased in a local supermarket were appropriately diluted with P&D buffer solution and spiked with the β -lactam antibiotic and the enzymatic tracer. Aliquots (50 μL) of these milk solutions were analyzed following the same methodology described above for standard solutions. Nonspiked samples were also checked to exclude the presence of naturally contaminating antibiotics.

RESULTS AND DISCUSSION

The strong adsorption of long alkyl chains leading to the formation of ordered physisorbed monolayers onto carbon electrode surfaces²² was profited to perform the modification of SPCEs with long alkyl-NTA derivatives.^{23–25} Thermodynamic studies demonstrated that the hydrocarbon molecules adsorb with high affinity to graphite surfaces and that the heat of adsorption increases with chain length suggesting that the molecules are adsorbed with their long axis parallel to the graphite surface as confirmed by scanning tunneling microscopy (STM), X-ray, and neutron diffraction studies.²⁶ In order to verify this, we evaluated by electrochemical impedance spectroscopy (EIS) the adsorption of two long alkyl chain NTA derivatives, a thiolated HS-NTA and the nonthiolated analogue (LAU-NTA) on carbon electrodes (see Figure S1, Supporting Information). Coverage percentages were calculated from the charge-transfer resistance values at the modified and unmodified electrodes.²⁷ The obtained values ($74\% \pm 3\%$ and $70\% \pm 7\%$, for HS-NTA and LAU-NTA, respectively) suggested that no significant differences in the coverage were found for both NTA derivatives and demonstrated the successful adsorption of both compounds on SPCEs generating dense layers in only 2.5 h. This fact confirmed that the thiol

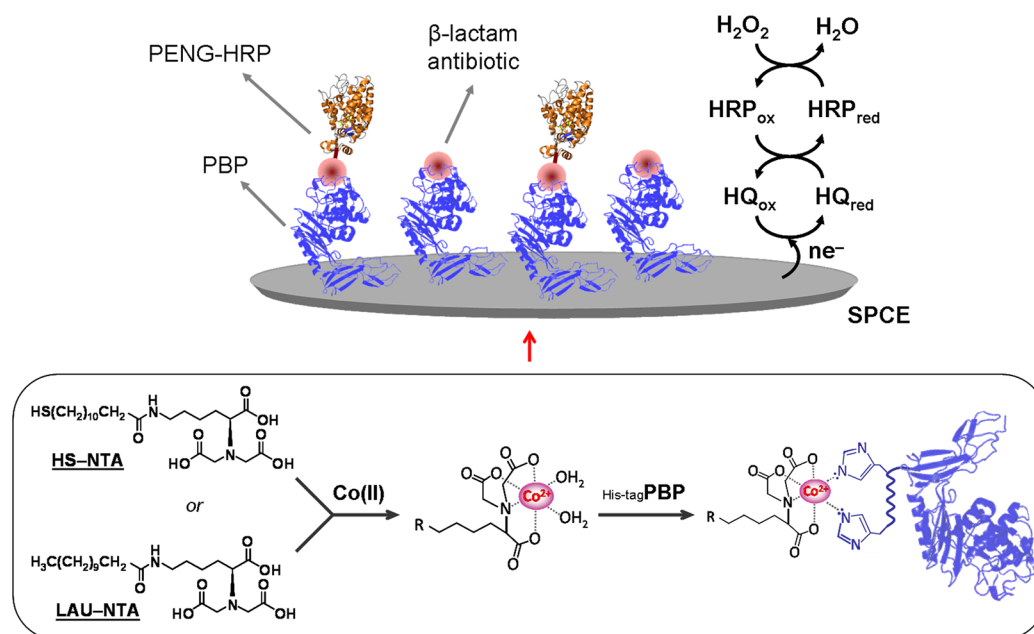


Figure 1. Schematic display of steps involved in the development of the affinity biosensor. Bottom inset: details of the surface chemistry involved on the immobilization of the recombinant histidine-tagged PBP by using Co^{2+} -NTA-modified SPCEs.

moiety did not play a major role in the adsorption of long alkyl chain NTA derivatives on a carbon surface.

As commented above, recombinant PBP immobilization was accomplished through the interactions between the protein His-tag and the Co^{2+} -NTA functionality existing at the modified electrode surfaces. The employed scheme involved the competition of the unlabeled analyte and a fixed concentration of the enzymatic tracer (PENG-HRP) for the limited number of binding sites of the recombinant binding protein immobilized on the SPCE surface using the Co^{2+} /NTA chemistry (see Figure 1).

First, the successful modification of the electrode surface with the Co^{2+} -NTA moiety and the subsequent immobilization of the PBP were verified by monitoring the stepwise assembly using cyclic voltammetry (CV) and EIS. Figure 2 shows the CVs recorded for the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox pair at a bare SPCE, and after the successive modifications with HS-NTA,

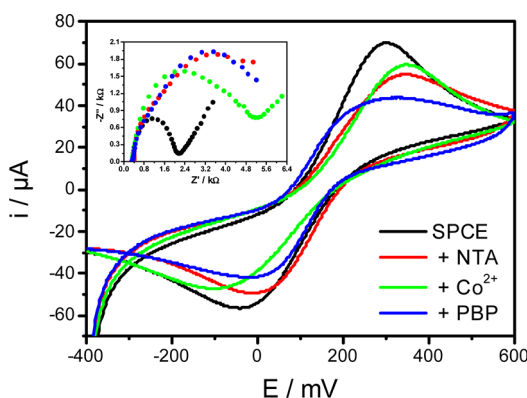


Figure 2. Cyclic voltammograms and Nyquist plots ($-Z''$ vs Z') obtained at a bare SPCE, NTA-SPCE, Co^{2+} -NTA-SPCE, and PBP- Co^{2+} -NTA-SPCE for 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (1:1) in a 0.1 M phosphate buffer pH 7.0 solution. EIS parameters: 0.01–10 000 Hz frequency range with a 0.01 V rms signal at +0.25 V (vs Ag pseudoreference electrode); CV, $\nu = 50 \text{ mV s}^{-1}$.

Co^{2+} , and PBP, whereas the inset of Figure 2 displays the corresponding EIS Nyquist plots. As expected, the quasi-reversible voltammetric response observed at the unmodified SPCE was strongly attenuated at the HS-NTA-modified SPCE, reflecting the electrostatic repulsion between the redox pair and the $-\text{COO}^-$ groups at neutral pH. This repulsion provoked the anodic shift of the formal electrode potential upon the electrode modification with the HS-NTA moiety. This effect disappears as the carboxylates groups in the ligand are chelated to the metal ion.¹ Also, a slight increase in the peak current was observed after Co^{2+} complexation which can be attributed to the partial shielding of the negative surface charges by complexed Co^{2+} ions.²⁸ The PBP immobilization on the Co^{2+} -NTA-SPCE caused an additional barrier for the access of the redox probe to the electrode thus leading to a further decrease in the voltammetric signal showing the ability of the Co^{2+} -NTA-SPCEs to bind recombinant PBP via N-terminal hexahistidine tail. These results are also consistent with the changes observed in the electron-transfer resistance (Figure 2 inset).

On the other hand, similar amperometric responses were obtained for solutions containing no β -lactam antibiotic with biosensors constructed using both HS-NTA- and LAU-NTA-modified SPCEs (Figure S2, Supporting Information). Therefore, taking into account that the adsorption of both compounds takes place by the long alkyl group and not by the terminal functional group, we decided to continue working only with the commercial HS-NTA derivative.

Optimization of Experimental Variables. The optimum experimental conditions for achieving the best affinity assay performance were sought out. All the experimental variables involved in the affinity sensor preparation and functioning were, therefore, optimized by taking as the selection criterion the maximum current measured at -0.20 V in the absence of β -lactam antibiotics.

First, in order to evaluate the extent of enzymatic tracer nonspecific binding to the Co^{2+} -NTA-SPCE surface, we compared the responses measured with and without the PBP

immobilized on the modified SPCE in a solution of the enzymatic tracer in P&D buffer. Nonspecific adsorption of PENG–HRP occurred at the PBP-free Co^{2+} –NTA–SPCE surface, leading to an amperometric signal 19% lower than that measured at the PBP– Co^{2+} –NTA–SPCE. Therefore, we performed the competitive reaction in different working media with the aim of minimizing the nonspecific signal. The ratios between the specific and the unspecific responses were calculated for P&D buffer solution supplemented with NaCl (1.0 M) and for P&D buffer 1:1 diluted UHT whole milk. As it can be seen in Figure 3, that ratio increased notably in both

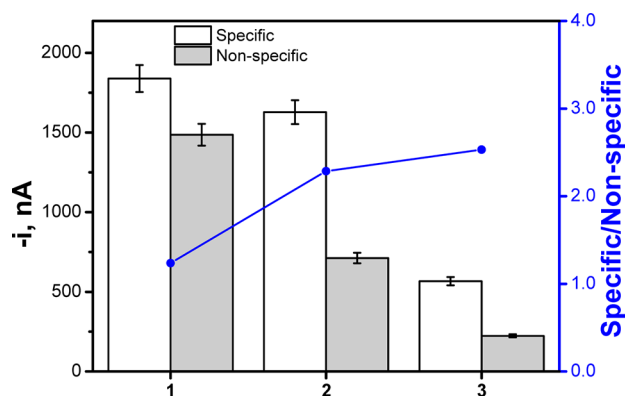


Figure 3. Amperometric responses measured at -0.20 V for solutions containing PENG–HRP in the absence of β -lactam antibiotics with the Co^{2+} –NTA–SPCE (nonspecific signals) and with the PBP– Co^{2+} –NTA–SPCE (specific signal) as well as their corresponding ratios in P&D buffer (1), 1.0 M NaCl-enriched P&D buffer (2), and 1:1 P&D buffer-diluted UHT whole milk (3). Error bars were estimated as a triple of the standard deviation ($n = 3$).

media as a consequence of a remarkable decrease in the nonspecific binding of the enzymatic tracer due to the presence of the high ionic salt or milk matrix components.¹⁵ Accordingly, a P&D buffer solution containing 1.0 M NaCl was chosen as the working medium to evaluate the analytical performance of the affinity biosensor.

Thereafter, the variables involved in the affinity biosensor preparation and functioning were optimized according to the selection criterion mentioned above. The ranges tested for all these variables as well as the selected values are collected in Table 1. It is worthwhile to remark that the functionalization of the SPCE surface with the NTA derivative provided a stable monolayer with the thiol tether not attached to the electrode thus implying a larger accessible potential window when compared with thiolated chain-modified gold electrodes.

Table 1. Optimization of Experimental Variables Involved in the Preparation and Functioning of the PBP– Co^{2+} –NTA–SPCE Affinity Biosensor

exptl variable	checked range	selected value
[NTA], mM	0.0–25	1.0 (HS-NTA)/3.0 (LAU-NTA)
t_{NTA} , h	0.5–o/n ^a	2.5
$t_{\text{Co(II)}}$, min	0–60	30
[Co(II)], mM	0.0–10.0	7.5
t_{PBP} , min	0–60	20
[PBP], $\mu\text{g mL}^{-1}$	440–1330	880
PENG–HRP, dilution	1/25–1/200	1/50

^ao/n: overnight.

Moreover, this modification took only 2.5 h which is a significant advantage with respect to the modification of gold electrodes with NTA-terminated thiolated self-assembled monolayers (SAMs) which requires 12–24 h of incubation in the thiol solutions.^{8,29}

Cu^{2+} , Ni^{2+} , Co^{2+} , and Zn^{2+} were checked as metal ions to be coordinated with the NTA derivative attached to the electrode surface (Figure S3, Supporting Information). Similar specific-to-unspecific responses ratios were obtained using Cu^{2+} , Ni^{2+} , and Co^{2+} . However, the use of Co^{2+} allowed the regeneration of the affinity sensor as discussed below. This was attributed to the lower stability constant of the corresponding complex ($\text{p}K_{\text{a}} = 10.4$).³⁰ Furthermore, the influence of the Co^{2+} concentration used for the coordination with the immobilized NTA derivative on the amperometric response obtained in the absence of free β -lactam antibiotic was evaluated (Figure S4, Supporting Information). It can be observed as the measured current increased with the Co^{2+} concentration which confirmed that the metal ion played an essential role in the PBP binding on the modified SPCE. As expected, the amperometric response obtained when no metal ion was present was only slightly higher than the nonspecific signal resulting in the absence of immobilized His-PBP.

Analytical Characteristics of the Affinity Sensor. The performance of the integrated affinity biosensor was tested using AMP as a model target penicillin. The calibration graph constructed for AMP in P&D buffer solution containing 1.0 M NaCl is displayed in Figure 4. The DR extended between $1.3 \pm$

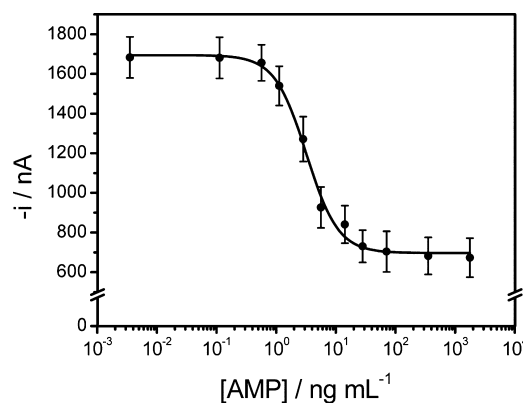


Figure 4. Calibration curve constructed with the PBP– Co^{2+} –NTA–SPCE affinity sensor for AMP in P&D buffer containing 1.0 M NaCl. The experimental points were fitted to eq 1. Error bars were estimated as a triple of the standard deviation ($n = 3$).

0.2 and 9.9 ± 0.9 ng mL^{-1} with an IC_{50} value of 3.6 ± 0.4 ng mL^{-1} and a LOD of 0.7 ± 0.1 ng mL^{-1} , which is approximately 6 times lower than the limit regulated by the EU ($4 \mu\text{g kg}^{-1}$ AMP content in milk samples). Therefore, the obtained results are promising and encouraging for the further application of the disposable amperometric affinity sensor in the analysis of food samples such as milk.

Analytical Characteristics of the Affinity Sensor in Milk Samples. The usefulness of the affinity biosensor for the determination of β -lactam antibiotics in milk was evaluated selecting a penicillin (AMP) and a cephalosporin (CEF) as target analytes. Calibration curves were obtained for both compounds in whole UHT milk with only a 1:1 dilution with P&D buffer as sample treatment. It is important to remark that the addition of 1.0 M NaCl to the buffer solution was not

needed now because, as commented before, the milk matrix components acted as an effective in situ blocking agent able to minimize the nonspecific signals.¹⁵ The obtained calibration graphs are shown in Figure 5, and the corresponding analytical

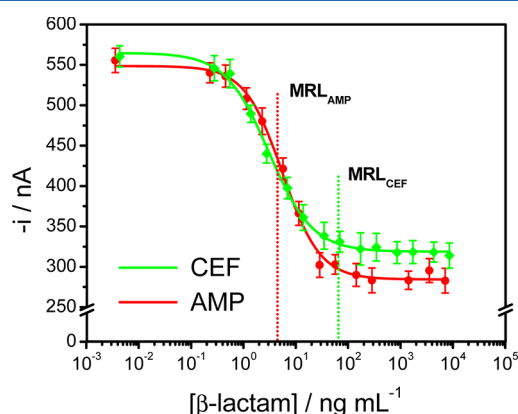


Figure 5. Calibration graphs obtained with the PBP-Co²⁺-NTA-SPCE affinity sensor for AMP and CEF in 1:1 P&D-diluted UHT whole milk. The experimental points were fitted to eq 1. Error bars were estimated as a triple of the standard deviation ($n = 3$).

characteristics are summarized in Table 2. As it can be observed in both cases the LODs are well below the MRLs established by current legislation.

Table 2. Analytical Characteristics for the Determination of AMP and CEF in 1:1 P&D Buffer-Diluted UHT Whole Milk with the PBP-Co²⁺-NTA-SPCE Affinity Biosensor

	AMP	CEF
r^2	0.996	0.997
IC ₅₀ /nM	15.6 ± 0.4	7.1 ± 0.2
IC ₅₀ /ng mL ⁻¹	5.5 ± 0.1	3.0 ± 0.1
LOD/nM	2.5 ± 0.1	0.87 ± 0.03
LOD/ng mL ⁻¹	0.89 ± 0.02	0.37 ± 0.01
MRL/ng mL ⁻¹	4.0	60.0
DR/nM	5.0 ± 0.1 to 49 ± 1	1.89 ± 0.06 to 26.8 ± 0.8
DR/ng mL ⁻¹	1.73 ± 0.04 to 17.2 ± 0.4	0.80 ± 0.02 to 11.3 ± 0.3

The reproducibility of the responses obtained with different sensors prepared in the same manner was evaluated both within and between days by performing measurements in 1:1 P&D buffer-diluted UHT whole milk spiked with the enzymatic tracer and in the absence of β -lactam antibiotics. Six different sensors prepared the same day yielded a RSD value for the steady-state current of 4.6%. Moreover, the RSD value was 5.7% for five sensors prepared in different days. These results demonstrated a good reproducibility of the affinity sensor fabrication procedure. The storage stability of the integrated affinity sensors was evaluated by preparing on the same day different PBP-Co²⁺-HS-NTA premodified SPCEs which were stored at 4 °C under dry conditions. The sensors were tested periodically by evaluating their response in a fresh solution of PENG-HRP. A control chart was constructed by taking the mean value of five measurements obtained the first working day as the central value and setting the upper and lower control limits at $\pm 3SD$ of this initial value. The responses obtained remained within the control limits for 8 days without a noticeable decrease in their original response and kept the 91.2% of it after 11 days.

Furthermore, the reversibility of the oriented immobilization of the histidine-tagged protein onto the Co²⁺-NTA-modified electrode and hence the reusability of the developed affinity sensor was also verified. In order to do that, we tested the amperometric responses obtained with four different electrodes after incubation of the PENG-HRP-PBP-Co²⁺-NTA-SPCEs during 3 h in a 50 mM phosphate solution pH 8.0 containing 300 mM NaCl, 300 mM imidazole, and 0.01% Tween20 under continuous stirring and after 40 min in deionized water. This buffer is the one recommended for reuse of His-tag-MBs. The measured currents were in all cases similar to that obtained with no enzymatic tracer which was attributed to the replacement of the histidine ligand by imidazole and, consequently, to the release of the anchored PENG-HRP. When the Co²⁺ coordination, PBP immobilization, and PENG-HRP recognition protocols were repeated with these regenerated sensors, the initial amperometric response was restored thus evidencing the specificity and reversibility of this binding approach, in contrast with the commonly used biotin-avidin immobilization which leads to irreversible anchoring of biotinylated biomolecules. Therefore, the reusability of the affinity sensors was demonstrated.

Affinity Sensor Selectivity. The selectivity of the developed affinity sensor was tested against three nontarget antibiotics (TC, SPY, and ENRO) which may be present in milk and dairy products. The amperometric signals obtained with the affinity sensor in the absence and in the presence of these potentially interfering antibiotics at two different concentration levels (0.5 and 10 μ M) in 1:1 P&D-diluted UHT whole milk were compared. The obtained results (data not shown) demonstrated that no significant cross-reactivity occurred for the three nontarget antibiotics tested even in the presence of a high excess (10 μ M). In fact, the percentages of cross-reactivity calculated from the corresponding IC₅₀ values³¹ were <1% in all cases indicating that no significant competition was produced and the remarkable group specificity of the PBP used to prepare the affinity sensor.

An important practical aspect is that MRLs are defined for β -lactams with a nonhydrolyzed β -lactam ring. Therefore, the specificity of PBP for active β -lactams should be also tested. In order to do that, we spiked milk samples with hydrolyzed AMP or CEF (according to the procedure described in Reagents and Solutions section) at two concentration levels, 10² and 10³ ng mL⁻¹. The amperometric responses obtained with the affinity sensor showed that no detectable active antibiotic residues were found in these samples demonstrating that no cross-reactivity occurred with hydrolyzed β -lactams (open-ring metabolites or degradation products).

Application to the Analysis of Spiked Milk Samples.

The amperometric affinity sensor was applied for the analysis of UHT whole milk samples (antibiotic-free) spiked with AMP at a 30 nM (~ 10.5 ng mL⁻¹) concentration level. The quantification of AMP was accomplished by interpolation of the measured current values into the calibration plot constructed for the antibiotic in the same matrix sample (Figure 5). The analysis of 10 milk samples yielded an AMP mean concentration of 10.8 ± 0.8 ng mL⁻¹ with a mean recovery of $103 \pm 8\%$ (RSD = 9.9%), the confidence interval being calculated for $\alpha = 0.05$. Taking into account the type of analysis carried out and the straightforwardness of the experimental protocol, these good results demonstrated the usefulness of the affinity sensor for the determination of β -

Table 3. Comparison of the Analytical Characteristics of the Developed Affinity Sensor with Those Reported for Other Assays for β -Lactam Antibiotics Determination^a

assay protocol	detection type	DR/ng mL ⁻¹	LOD/ng mL ⁻¹	sample	assay time (min)	ref
sample was mixed and incubated with PBP2X [*] ; after, DIG-AMPI was added and the mixture was incubated; it was injected over a sensor chip modified surface with anti-DIG	SPR		5 (PENG), 7 (AMP), 8 (AMOX), 2 (PENG)	milk (previously defatted) and raw milk (previously heated)	40	10
PBP2X is immobilized by adsorption into a microplate; after adding the sample, a bifunctional reagent is added (DIG-AMPI); the detection step uses antidigoxigenin HRP labeled	spectrophotometry			milk, meat, honey, egg	125	13
immobilization of PBP by adsorption over SPE-RC surface; the assay consists of a competitive binding assay format using PENG and 7-ACA-GOD	amperometry (+350 mV vs Ag/AgCl)		5 (PENG)	milk (50% diluted and undiluted)	6	15
competitive affinity assay using PENG-HRP on PBP-modified MBs	amperometry ($E_{app} = -0.20$ V vs Ag pseudoreference electrode)	62.0–238.1 (AMP)	14.0 (AMP)	milk (1:1 P&D diluted)	30	21
2-peptide was covalently bound to the sensor surface; the sample was mixed and incubated with 3-peptide and R39; after, 2-peptide antibody was added to the sample and the mixture was injected over a sensor surface	SPR		2.6 (buffer) 5.2 (defatted milk)	milk (previously defatted)	9	32
sample was mixed and incubated with 3-peptide and R39	SPR			milk	7	33
(a) 2-peptide antibody was added to the sample and the mixture was injected over a 2-peptide-modified sensor surface		(a) 1.5–5.0	(a) 1.2 (PENG)			
(b) 3-peptide antibody was added to the sample, and the mixture was injected over a 3-peptide-modified sensor surface			(b) 1.5 (PENG)			
sample was mixed and incubated with 3-peptide and R39	SPR			milk	7	34
(a) 2-peptide antibody was added to the sample and the mixture was injected over a 2-peptide-modified sensor surface			(a) 1.2 (PENG)			
(b) 3-peptide antibody was added to the sample and the mixture was injected over a 3-peptide-modified sensor surface			(b) 1.5 (PENG)			
competitive affinity assay using PENG-HRP on PBP-Co ²⁺ -HS-NTA-modified SPCEs	amperometry ($E_{app} = -0.20$ V vs Ag pseudoreference electrode)	1.7–17.2 (AMP) 0.8–11.3 (CEF)	0.9 (AMP) 0.4 (CEF)	milk (1:1 P&D diluted)	30	this work

^aAbbreviations: 7-ACA-GOD, 7-aminoccephalosporanic acid modified with glucose oxidase; AMOX, amoxicillin; AMP, ampicillin; DIG-AMPI, digoxigenin-labeled ampicillin; HRP, peroxidase; PBP2X^{*}, penicillin binding protein from *Streptococcus pneumoniae*; PENG, penicillin G; 3-peptide, (Ac-L-Lys-D-Ala-D-Ala); 2-peptide, (Ac-L-Lys-D-Ala); R39, D,D-carboxypeptidase from *Actinomyces* R39; R39^{*}, D,D-carboxypeptidase from *Streptomyces* R39; SPE-RC, screen-printed electrode with a rhodized-carbon working electrode; SPR, surface plasmon resonance.

lactam antibiotics, in a total time for the assay of approximately 30 min (once the sensor is prepared).

Comparison with Other Reported Sensors and Methodologies. The performance of the developed amperometric affinity sensor was compared with that provided by other approaches reported in the literature (Table 3). As can be seen, most of the previously developed methodologies used surface plasmon resonance (SPR) detection, whereas only two involved electrochemical transduction.^{15,21} While offering similar analytical performance some important advantages can be claimed for the approach described here against non-electrochemical methodologies. These include the inherent simplicity and much easier potential automation and miniaturization thus making the developed methodology ideal as a field-based screening tool for β -lactam detection in milk. When compared with the other available electrochemical sensors, it is important to remark that, conversely to that occurred with the only integrated affinity sensor reported until now, the nonspecific binding of the PENG–HRP conjugate was independent of the free β -lactam concentration in solution.¹⁵ Moreover, the LOD achieved is 15 times lower than that reported for an amperometric affinity PBP magnetosensor.²¹ It is worth noting also that this is the first time that an integrated amperometric sensor has been developed by immobilization of a recombinant PBP using the His-tag/M²⁺/NTA chemistry.

CONCLUSIONS

This work showed for the first time the successful, simple, and one-step modification of SPCEs with a dense monolayer of long alkyl chain NTA ligands offering an attractive platform for the development of affinity biosensors by reproducible, stable, reversible, and oriented immobilization of His-tagged recombinant proteins. Using this novel platform, an integrated amperometric affinity sensor for the specific and sensitive detection of β -lactam antibiotics in milk was developed by immobilization of a recombinant PBP on the surface of the modified SPCEs using His-tag/Co²⁺/NTA chemistry. The obtained results demonstrated the ability of the developed sensor to detect and quantify low levels of β -lactam antibiotics residues, either penicillins or cephalosporins, in milk samples in a reliable, specific, and rapid manner (30 min) with no need of complex sample treatment steps. On the other hand, it is anticipated that the simple, versatile, efficient, and fast immobilization approach developed, based exclusively on commercially available chemicals, should be useful in the reversible immobilization, with a controlled molecular orientation, of a wide variety of histidine-tagged proteins on carbon electrode surfaces without significant loss of their biological activity finding application in the development of other bioanalytical strategies of interest.

ASSOCIATED CONTENT

Supporting Information

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

AUTHOR INFORMATION

Corresponding Author

*E-mail: pingarro@quim.ucm.es. Phone: +34 913944315. Fax: +34 913944329.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The financial support of the Spanish Ministerio de Economía y Competitividad Research Projects, CTQ2012-34238, and the AVANSENS Program from the Comunidad de Madrid (S2009PPQ-1642) are gratefully acknowledged. Felipe Conzuelo acknowledges an FPU fellowship from the Spanish Ministry of Education.

REFERENCES

- (1) Blankespoor, R.; Limoges, B.; Schöllhorn, B.; Syssa-Magalé, J.-L.; Yazidi, D. *Langmuir* **2005**, *21*, 3362–3375.
- (2) Haddour, N.; Cosnier, S.; Gondran, C. *J. Am. Chem. Soc.* **2005**, *127*, 5752–5753.
- (3) Ley, C.; Holtmann, D.; Mangold, K.-M.; Schrader, J. *Colloids Surf., B* **2011**, *88*, 539–551.
- (4) Sigal, G. B.; Bamdad, C.; Barberis, A.; Strominger, J.; Whitesides, G. M. *Anal. Chem.* **1996**, *68*, 490–497.
- (5) Madoz-Gurpide, J.; Abad, J. M.; Fernández-Recio, J.; Velez, M.; Vázquez, L.; Gómez-Moreno, C.; Fernández, V. M. *J. Am. Chem. Soc.* **2000**, *122*, 9808–9817.
- (6) Benson, D. E.; Conrad, D. W.; Lorimier, R. M.; Trammel, S. A.; Hellinga, H. W. *Science* **2001**, *293*, 1641–1644.
- (7) Gizeli, E.; Glad, J. *Anal. Chem.* **2004**, *76*, 3995–4001.
- (8) Valiokas, R.; Klenkar, G.; Tinazli, A.; Reichel, A.; Tampé, R.; Piehler, J.; Liedberg, B. *Langmuir* **2008**, *24*, 4959–4967.
- (9) Harris, A. G.; Hazell, S. L.; Netting, A. G. *J. Antimicrob. Chemother.* **2000**, *45*, 591–598.
- (10) Cacciatore, G.; Petz, M.; Rachid, S.; Hakenbeck, R.; Bergwerff, A. A. *Anal. Chim. Acta* **2004**, *520*, 105–115.
- (11) Jamin, M.; Hakenbeck, R.; Frere, J.-M. *FEBS Lett.* **1993**, *331*, 101–104.
- (12) Fernández, F.; Hegnerová, K.; Pilarik, M.; Sanchez-Baeza, F.; Homola, J.; Marco, M.-P. *Biosens. Bioelectron.* **2010**, *26*, 1231–1238.
- (13) Lamar, J.; Petz, M. *Anal. Chim. Acta* **2007**, *586*, 296–303.
- (14) Liu, W.; Zhang, Z.; Liu, Z. *Anal. Chim. Acta* **2007**, *592*, 187–192.
- (15) Setford, S. J.; Van Es, R. M.; Blankwater, Y. J.; Kröger, S. *Anal. Chim. Acta* **1999**, *398*, 13–22.
- (16) Commission Regulation (EU) No. 37/2010 of 22 December 2009. *Off. J. Eur. Communities: Legis.* **2010**, *15*/1.
- (17) Kantiani, L.; Farré, M.; Barceló, D. *Trends Anal. Chem.* **2009**, *28*, 729–743.
- (18) Babington, R.; Matas, S.; Marco, M.-P.; Galve, R. *Anal. Bioanal. Chem.* **2012**, *403*, 1549–1566.
- (19) Grubelnik, A.; Padeste, C.; Tiefenauer, L. *Food Agric. Immunol.* **2001**, *13*, 161–169.
- (20) Richard, C.; Balavoine, F.; Schultz, P.; Ebbesen, T. E.; Mioskowski, C. *Science* **2003**, *300*, 775–778.
- (21) Gamella, M.; Campuzano, S.; Conzuelo, F.; Esteban-Torres, M.; de las Rivas, B.; Reviejo, A. J.; Muñoz, R.; Pingarrón, J. M. *Analyst* [Online early access]. DOI: 10.1039/C3AN36727D. Published Online: Feb 1, 2013. <http://pubs.rsc.org/en/content/articlelanding/2013/an/c3an36727d>.
- (22) Bickerstaffe, A. K.; Cheah, N. P.; Clarke, S. M.; Parker, J. E.; Perdigon, A.; Messe, L.; Inaba, A. *J. Phys. Chem. B* **2006**, *110*, 5570–5575.
- (23) Martin, D. S.; Weightman, P.; Gauntlett, J. T. *Surf. Sci.* **1999**, *424*, 187–198.
- (24) Enevoldsen, A. D.; Hansen, F. Y.; Dama, A.; Criswell, L.; Taub, H. *J. Chem. Phys.* **2007**, *126* (104703), 1–10.
- (25) Zhao, M.; Jiang, P.; Deng, K.; Yu, A.-F.; Hao, Y.-Z.; Xie, S.-S.; Sun, J.-L. *Appl. Surf. Sci.* **2011**, *257*, 3243–3247.
- (26) Bhide, T.; Clarke, S. M.; Phillips, T. K.; Arnold, T.; Parker, J. E. *Langmuir* **2010**, *26*, 8201–8206.

- (27) Janek, R. P.; Fawcett, W. R.; Ulman, A. *Langmuir* **1998**, *14*, 3011–3018.
- (28) Giess, F.; Friedrich, M. G.; Heberle, J.; Naumann, R. L.; Knoll, W. *Biophys. J.* **2004**, *87*, 3213–3320.
- (29) Lee, J. K.; Kim, Y.-G.; Chi, Y. S.; Yun, W. S.; Choi, I. S. *J. Phys. Chem. B* **2004**, *108*, 7665–7673.
- (30) Matheus, P. R.; Abad, J. M.; Fernández, V. M. *Rev. Tec. Fac. Ing., Univ. Zulia* **2007**, *30*, 225–235.
- (31) Conzuelo, F.; Gamella, G.; Campuzano, S.; Reviejo, A. J.; Pingarrón, J. M. *Anal. Chim. Acta* **2012**, *737*, 29–36.
- (32) Gustavsson, E.; Bjurling, P.; Sternesjö, Å. *Anal. Chim. Acta* **2002**, *468*, 153–159.
- (33) Gustavsson, E.; Degelaen, J.; Bjurling, P.; Sternesjö, Å. *J. Agric. Food Chem.* **2004**, *52*, 2791–2796.
- (34) Sternesjö, Å.; Gustavsson, E. *J. AOAC Int.* **2006**, *89*, 832–837.