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Molecularly Imprinted Sol–Gels for Nafcillin Determination in Milk-Based Products

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A study has been made of the analytical application of a nafcillin-imprinted sol−gel to the direct determination of the β-lactamic antibiotic in spiked milk-based samples using a room temperature phosphorescent flow-through system. The influence of the sample matrix on the transduction and the recognition processes was statistically determined, and results demonstrated that the imprinted sol−gel optosensing system could be effectively applied to real sample analysis. The analytical performance characteristics were as follows: The detection limit results for aqueous and skimmed milk were 5.8×10^{-6} and 3.3×10^{-5} mol L^{-1}, respectively, and a relative standard deviation less than 5% was found for both matrices. Statistical analysis of variance studies have been shown to have no significant effect on different skimmed milk commercial products over the imprinted material recognition. This fact provides an indicator of the ruggedness/robustness of the proposed analytical system and the possibility to use external real matrix calibration. Application of the method to nafcillin analysis in other milk-based samples is outlined.

KEYWORDS: Molecular imprinting polymers; sol−gel materials; room-temperature phosphorescence; milk-based products; nafcillin

INTRODUCTION

At present, molecular imprinting is a rapidly developing field of research as a means of creating spatial memory of template molecules in highly cross-linked polymeric matrices (*1*, *2*). In the analytical chemistry arena, molecular imprinting is receiving extraordinary attention because this methodology provides an approach to synthesizing highly substrate- and enantioselective polymers with applications as high-performance liquid chromatography (HPLC) stationary phases for chiral resolution, in sensor design, as substitutes for antibodies in immunoassays, and for protein separation (*3*, *4*).

Most reported molecularly imprinted materials are acrylic-based, and recognition is achieved in organic media. The application of these molecularly imprinted materials to develop sensing or solid-phase extraction (SPE) approaches may be limited, to a large extent, when used in aqueous samples such as biofluids or when the target template is water soluble. To address these drawbacks, a current trend based on the sol−gel process is to syntheses molecularly imprinted materials by choosing appropriate metal alkoxide precursors. Different metal oxides (silica and mixed metal oxides) have been imprinted to produce materials with applications as sensing phases, catalysts, and adsorbents (*5*). In a self-assembly imprinting approach, the template may be directly added to the metal alkoxyde solution prior to acid- or base-catalyzed hydrolysis and condensation. By using an adequate polar/nonpolar sol−gel functional precursor and a fairly polar solvent (e.g., ethanol), imprinted sites may be generated by electrostatic, π-stacking, van der Waals, etc. interactions between the template and the sol−gel network: During the cascade of these events, the template molecule organizes itself onto the cavities of the amorphous silica. Following a drying step, the macropores should be extracted with an adequate solvent to remove the template, thus leaving specific receptor sites with a favorable size, shape, and chemical environment to selectively rebind the template.

Intense ongoing research in the food and agricultural area has proven that MIPs can be efficiently used in these fields (*6*). While the majority of interest has been focused upon the analysis of herbicides and pesticides, other analytes have been the object for research as well, such as additives, pathogenic organisms, toxins, trace metals, and pharmaceuticals. A quick overview of published methods using MIPs for the analysis of these compounds in food complex samples reveals that most of them are based on a separation process (HPLC and/or SPE) and that most of the MIPs are acrylic- or methacrylic-based. Recently, we found that the sol−gel route is a very efficient approach for the preparation of nafcillin (β-lactamic antibiotic)-imprinted materials with highly specific receptor sites capable of rebinding the imprint molecules in preference to other similar β-lactamic antibiotics (*7*). Herein, we provide a detailed account of the analytical application of a nafcillin-imprinted sol−gel to the direct determination of the antibiotic in nafcillin-spiked milk and milk-based samples using a room temperature phosphores-

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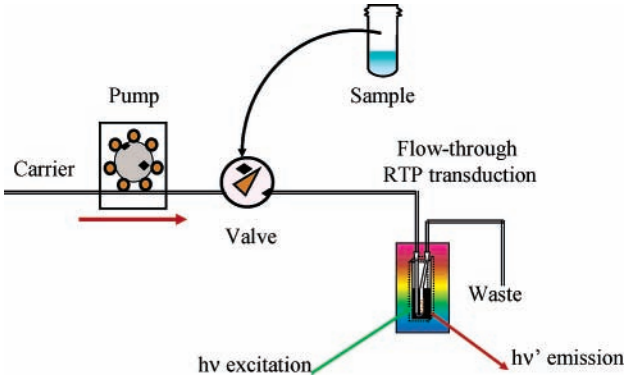

Figure 1. Flow injection setup for room temperature phosphorescence optosensing.

cent flow-through system. For the first time, the influence of the sample matrix on the signal transduction and on the imprinted sol−gel recognition process was statistically determined and results demonstrated that the imprinted sol−gel optosensing system could be effectively applied to real sample analysis.

MATERIALS AND METHODS

Materials. Nafcillin [6-(2-ethoxy-1-naphthamido) penicillin] was purchased from Sigma. Tetramethyl orthosilicate (98%, TMOS), methyltrimethyl orthosilicate (98%, MTMOS), phenyltrimethyl orthosilicate (97%, PhTMOS), tetrabutyl-ammonium fluoride (97%, TBAF), and potassium iodide were obtained from Fluka. Sodium sulfite, potassium hexacyanoferrate, and hydrochloric acid were obtained from Merck. Zinc acetate was purchased from Panreac. All syntheses were carried out using distilled−deionized water (18.0 M mho; Millipore system). All solvents were of analytical-reagent grade and were used without further purification unless stated otherwise. The standard antibiotic solution (1×10^{-3} mol L^{-1}) was prepared in water every 3 weeks and was kept under 4 °C to avoid degradation.

Instrumentation. A Perkin-Elmer LS-50B luminescence spectrophotometer, which has a xenon discharge excitation source (pulse width at peak half-height <10 μs), was used for phosphorescence measurement. Instrumental parameters and processing data were controlled by the Fluorescence Data Manager software. The excitation and emission wavelengths were set at 283 and 505 nm, respectively. A gate time of 3 ms and a delay time of 0.04 ms were used throughout. Excitation and emission slits were set at 15 and 20 nm, respectively.

Synthesis of Molecularly Imprinted Sol−Gel Particles. Sols were made by mixing, in that order, 1140 μL of 1×10^{-3} mol L^{-1} nafcillin, 1550 μL of ethanol, 120 μL of TMOS, 1230 μL of MTMOS, 150 μL of PhTMOS, 760 μL of 0.025 mol L^{-1} TBAF, and 50 μL of 0.1 mol L^{-1} HCl. Control sol−gels were prepared using the same protocol in the absence of the template molecule. Solutions were gently mixed for a period of 2 min to ensure homogeneous mixing. The sols were allowed to gel and dry for 72 h under ambient conditions and then at 45 °C to constant weight for approximately 2 weeks. The resulting sol−gels were mechanically crushed and sieved in fragmented particles of diameters ranging between 0.16 and 0.08 mm. In order to remove the template molecules, the sol−gel particles were Soxhlet rinsed using a 80:20 (v/v) ratio of methanol−acetic acid (24 h, 120 cycles) and methanol (8 h, 40 cycles).

Optosensing Setup. The manifold used in this study is shown in **Figure 1**. A Hellma model 176.52 flow-through cell (25 μL), which contained the imprinted sol−gel, was placed in the sample compartment of the spectrofluorimeter. The flow stream was generated by a Minipuls 2 four-channel peristaltic pump (Gilson, Worthington, OH). The samples were introduced by a type 50 PTFE six-way rotary valve (Omnifit, Cambridge, United Kingdom) provided with a 150 μL sample loop. PTFE tubing (0.8 mm i.d.) and fittings were used for connecting the flow-through cell, the valve, and the carrier reservoir. In the optimized system, the carrier consisted of a solution containing 1.5×10^{-2} mol L^{-1} Na_2SO_3 and 1 mol L^{-1} KI.

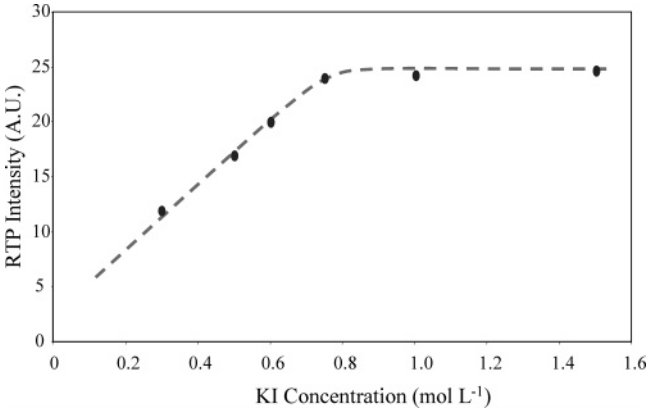

Figure 2. Influence of KI concentration on the flow–injection response of a nafcillin-imprinted sol–gel. Antibiotic, 4×10^{-5} mol L^{-1}; Na_2SO_3, 1×10^{-2} mol L^{-1}.

Preparation of Spiked Milk and Milk-Based Samples. Experiments to detect nafcillin in milk were performed with homogenized and pasteurized skimmed milk, plain milk (fat content, 3.5%), and drinkable skimmed yogurt obtained from a local supermarket. To 1 mL of blank milk samples and milk samples spiked with nafcillin, 200 μL of Carrez I reagent [0.1 mol L^{-1} $K_4Fe(CN)_6$ solution] and 200 μL of Carrez II reagent (0.6 mol L^{-1} zinc acetate solution) were added. After gentle mixing for 1−2 min, the blank and the sample were filtered using a filter paper (pore diameter, 7−11 μm) and washed with water (5×1 mL). The filtrate and washings were quantitatively recovered in a 10 mL flask, where 1 mL of 0.15 mol L^{-1} Na_2SO_3 and 1.66 g of KI were added and then, the blank and sample were brought to volume with distilled water. Finally, these solutions were used for molecular recognition/room temperature phosphorescence analysis using the optosensing setup.

Nafcillin was quantified by means of external calibration curves constructed from six calibration levels. One calibration curve was representative of standard aqueous solutions, and a second curve was representative of a sample matrix. To construct the standard aqueous calibration graph, adequate aliquots of a 1×10^{-3} mol L^{-1} nafcillin standard were transferred into 10 mL volumetric flasks. Then, 1 mL of 0.15 mol L^{-1} Na_2SO_3 and 1.66 g of KI were added. Finally, working solutions were diluted to volume with distilled water. Calibration levels ranged from 0 to 1.3×10^{-5} mol L^{-1} nafcillin. The matrix-based curve was prepared in a similar way, using the blank milk deproteinized matrix instead, and the distilled water and nafcillin levels ranged from 0 to 6×10^{-4} mol L^{-1}.

RESULTS AND DISCUSSION

Optimization of Chemical and Dynamic Variables. Unless otherwise stated, the variables for which the signal of 4×10^{-5} mol L^{-1} nafcillin showed a maximum were selected as optimum. The room temperature phosphorescence, read with excitation at 283 nm and emission at 505 nm, was at its maximum at 1 mol L^{-1} KI and at 1×10^{-2} mol L^{-1} Na_2SO_3 according to the results in **Figures 2** and **3**. The flow rate at which the sample was propelled through the flow system to the imprinted sol−gel recognition zone was studied between 0.15 and 0.35 mL min^{-1}. The signals did not vary up to a flow rate of 0.25 mL min^{-1} and then decreased abruptly in the range of 0.27−0.35 mL min^{-1}. A flow rate of 0.24 mL min^{-1} was found to be suitable as a compromise between sampling frequency and sensitivity. Using these values, a response time of 75 s and a sampling throughput of 23 h^{-1} have been achieved. The response profiles for aqueous standard solution injections were fully reversible in all cases.

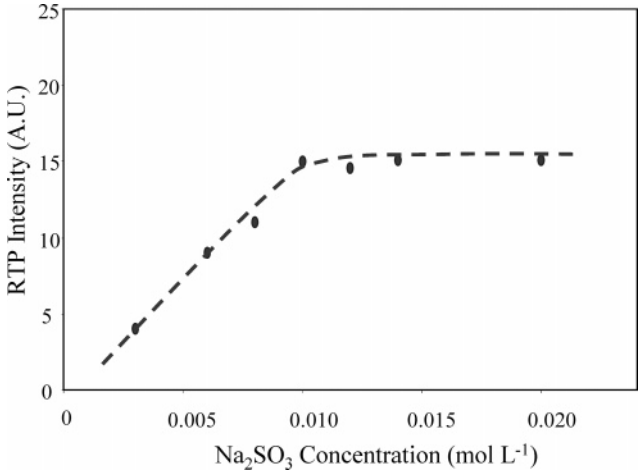

Figure 3. Influence of Na_2SO_3 concentration on the flow–injection response of a nafcillin-imprinted sol–gel. Antibiotic, 4×10^{-5} mol L^{-1}; KI, 1 mol L^{-1}.

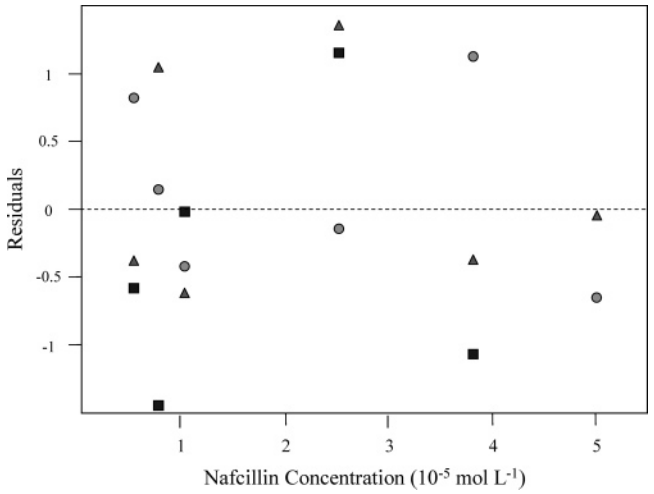

Figure 4. Residuals data set showing random distribution of points.

Features of the Imprinted Sol−Gel Optosensor. The recorded calibration graph for the determination of nafcillin was linear in the range 5×10^{-6}−6×10^{-5} mol L^{-1} and is described, after fitting the experimental data by least-squares regression, by the equation:

$$I_p = (8.1 \pm 0.7) + (3.5 \pm 0.2)10^5 \times [\mathrm{N}]\ (n = 3)$$

where I_p is the room temperature phosphorescence of the antibiotic retained by the imprinted sol−gel and [N] is the molar concentration of nafcillin. A strategy for linearity testing exploits visual examination of the residual errors of a given calibration model in order to get preinformation on the character of the residual errors. Assuming that the straight line calibration model is correct and that the usual calibration and regression assumptions hold, the residuals from the fit should be completely random. If the room temperature phosphorescence signal−concentration relationship is more complex, then a noticeable pattern (parabolic, sinusoidal, etc.) could be observed. According to this, the residual plot (**Figure 4**) shows little evidence of lack-of-fit for the nafcillin determination using the imprinted sol−gel.

The goodness-of-fit was performed using the Fisher test, calculating the ratio of the squares of the standard deviations (*8*). The test was carried out by comparison of the quotient $F_{exp} = (S_{y/x})^2/(S_y)^2$ with the critical value of F given in the form $F_{\alpha,f1=p-2,f2=m-p}$, where α is the 95% confidence interval and the null hypothesis adopted was that there was no difference between F_{exp} and F_{crit}. For $(p - 2)$ and $(m - p)$ degrees of freedom, the experimental value of $|t|$ ($P = 0.05$) is 0.966. Because the critical value of $|t|$ for $P = 0.05$ is 2.617 (*9*), the difference between the two values is significant at the 5% level and the null hypothesis is rejected.

The analytical sensitivity of the method (γ), defined as the ratio between the slope of the calibration line (A) and the standard deviation of measurements ($\gamma = a/s_y$), does not depend on measurement units nor amplification factors. Its reverse is usually employed, and a value of 2.4×10^{-6} mol L^{-1} nafcillin was found for γ^{-1}. The limit of detection has been defined by both IUPAC (*10*) and ISO (*11*) using the expression:

$$\mathrm{LOD} = 2 \cdot t_{0.05,} m_{-2} \cdot s_0$$

for the situation where the variance is constant. Calculation of s_0 by measuring ten blanks based on the equation

$$s_0 = \frac{S_{y/x}}{A}\sqrt{\left(\frac{1}{n} + \frac{1}{m} + \frac{\bar{x}^2}{\sum_{i=1}^{m}(x_i - \bar{x})^2}\right)}$$

was carried out, where A is the calibration slope, n is the number of measurements performed for each nafcillin concentration ($n = 3$), m is the total number of calibration points, x_i is the concentration of each calibration point, and $\bar{x}$ is the mean calibration concentration. In our system, an estimate of t based on 18 degrees of freedom results in $t_{0.05,16} = 2.12$ (*12*), thus resulting in a LOD = 5.8×10^{-6} mol L^{-1} nafcillin. Finally, the quantification limit, expressed as

$$\mathrm{LOQ} = 10 \cdot s_0$$

resulted in 1.7×10^{-5} M nafcillin. The calibration plot was linear from the LOD up to 6×10^{-5} mol L^{-1} nafcillin ($R^2 = 0.996$).

The repeatibility of the analytical method (expressed as relative standard deviation) was assessed for injections of a 4×10^{-5} mol L^{-1} nafcillin solution. The assays were carried out with six replicate nafcillin samples along 1 day of work ($n = 6$) and with three replicate nafcillin samples over 10 days on the same instrumental setup by one operator ($n = 30$). The RSD values obtained were 4 and 16%, respectively. In the latter case, the analytical signal change did not show time dependence as there was a random variation around the mean value. The batch-to-batch reproducibility of the imprinted sol−gel molecular recognition properties was evaluated by preparing five different sol−gel replicates and using these materials as the recognition element in the flow injection setup. By measuring the repeatibility of the results obtained for triplicate injections of a nafcilin solution 4×10^{-5} M, a %RSD of 16% was found. In order to investigate the long storage stability of the response of MISGs, the sol−gel was tested over a period of 10 days, during which three injection assays/day were performed. The reproducibility of the relative intensity of the signal was less than 5% without time dependence. The same sol−gel was used 3 months later, and the response remained reasonably constant (5% reproducibility).

Recognition Ability. The selectivity of the nafcillin-imprinted sol−gel was determined by competitive binding using the flow injection system and by injecting mixtures of a constant nafcillin

concentration (4×10^{-5} mol L^{-1}) and the competing molecule under study at different concentration levels. β-Lactamic- and anthracycline-based antibiotics were used as subject compounds in order to evaluate the influence of the chemical structure (β-lactamics) and that on the optical transduction method (anthracyclines) on the whole sensing process. The results of this study demonstrated (*6*) that most of the assayed antibiotics did not interfere at the concentration levels tested. Only ampicillin at an ampicillin/nafcillin molar ratio $\geq$ 5:1 may produce a noticeable interference. This ampicillin/nafcillin molar ratio is much higher than that expected for real samples, so no interference from foreign antibiotics in real sample analysis is to be expected.

Application of the Nafcillin-Imprinted Sol−Gel. Milk is an emulsion of fat droplets in complex aqueous milk plasma, consisting of a mixture of water, lipids, protein, enzymes, minerals, phosphatides, and other compounds (*13*). Because of this matrix complexity, antibiotic residues may remain bound to proteins in milk, even after acidification or skimming. In particular, nafcillin is known to be tightly bound (>90% bound nafcillin) to proteins (*14*, *15*). For highly protein-bound drugs, conventional SPE sorbents rarely give recoveries above 40%. Because of this difficulty, conventional extraction procedures such as liquid−liquid extraction and chemical curdling (precipitation and removal of protein) are the primary sample preparation step. In our work, different protein precipitation chemicals were tested in naficillin-spiked milk samples in order to precipitate any protein and solubilize bound nafcillin. Acetonitrile, trichloroacetic acid, methanol, and ammonium sulfate were found not to be adequate for removing milk proteins or, in turn, to extract nafcillin. Protein precipitation by means of Carrez reagents, as described in the Materials and Methods, resulted in a quantitative recovery of bound nafcillin (95−105%) as assessed by nonprotected room temperature phosphorescence measurements (*16*) on the filtrate.

The applicability of the imprinted sol−gel as a recognition material in a flow−injection approach was checked by analyzing the nafcillin-spiked milk samples, according to the procedure described in the Materials and Methods. Blank solutions, consisting of milk samples without added antibiotic, did not produce any room temperature phosphorescence signal when injected into the flow-through optosensing system. As shown in **Figure 5a**, the flow−injection response profile for nafcillin in skimmed milk samples is in compliance with the standard nafcillin solution.

The negative peak could be due to the different refraction index of the milk sample with respect to that of the carrier solution. Because important decisions are often made on the basis of analytical results, the true analytical performance characteristics of nafcillin determination in skimmed milk samples using the proposed method were statistically quantified. The critical response characteristics of the sol−gel-imprinted optosensing method using a calibration curve of nafcillin constructed in a matrix blank spiked in the $0-6 \times 10^{-4}$ mol L^{-1} nafcillin are summarized in **Table 1**.

As can be seen, there is a depressive matrix effect that contributed to an increase in the LOD of the method. Although the true detection limit was ca. 5-fold higher than that of the standard matrix method, the contents of nafcillin in milk samples could be determined by the optosensing system. Analysis of variance tests were performed to compare the results; the null hypothesis adopted that there is no difference in reliability. Assuming that the milk matrix effect was the same for all samples, we would expect, from the null hypothesis, that sample means do not vary from one sample to the next. In **Table 2**, the analytical results, expressed as the room temperature phosphorescence intensity, for the determination of nafcillin in four spiked (3×10^{-4} mol L^{-1}) skimmed milk samples and the within-sample variation (8 degrees of freedom) are shown. The variance of the means of the samples gives an estimate of between-sample variation (σ_0^2/n) of $\sigma_0^2/4 = 0.68$ (3 degrees of freedom). If the null hypothesis is correct, the two estimates of σ_0^2 should not differ significantly. To test whether the between-

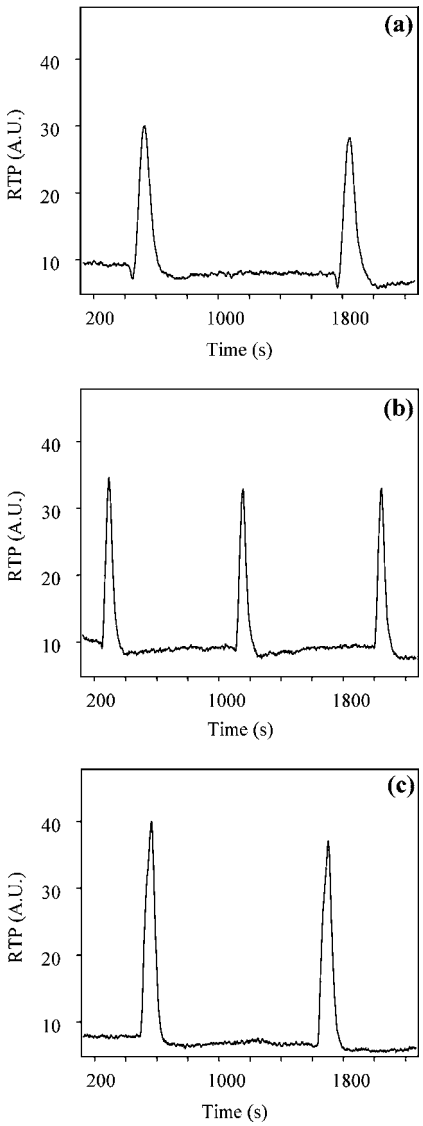

Figure 5. FIA recordings for injections of nafcillin-spiked skimmed milk (**a**), plain milk (**b**), and skimmed drinkable lemon yogurt (**c**) samples. Nafcillin, 3×10^{-4} mol L^{-1}.

Table 1. Critical Response Characteristics of the Imprinted Sol–Gel Optosensing System in a Real Sample Matrix

analytical parameter	value
slope (I_{RTP} mol^{-1} L)	$1.8 \times 10^4 \pm 0.2 \times 10^4$
intercept (I_{RTP})	11.6 ± 0.7
correlation coefficient	0.995
linearity test, P_F	0.524
$F_t = 2.388$; $F_{exp} = 1.494$	
analytical sensitivity (mol L^{-1})	6.1×10^{-5}
detection limit (mol L^{-1})	3.3×10^{-5}
quantification limit (mol L^{-1})	1×10^{-4}
linear range (mol L^{-1})	LOD – 6×10^{-4}

Table 2. Optosensing Signals from Nafcillin-Spiked Skimmed Milk Samples of Equal Nafcillin Concentration (3×10^{-4} mol L^{-1}) (Comparison of Means)

skimmed milk sample	replicate measurements (RTP, A.U.)			mean (RTP, A.U.)	within-sample variation, σ_0^2
Rio	13.7	13.9	15.3	14.3	0.76
Central Lechera Asturiana	14.8	15.5	14.1	14.8	0.49
Pascual	14.5	13.1	12.7	13.4	0.90
Día	14.2	12.1	12.6	13.0	1.21
		overall mean		13.9	0.84

sample estimate is significantly greater, a one-tailed *F*-test was used as follows: $F_{3,8} = 0.68/0.84 = 0.81$. As the calculated value of *F* is lower than critical value of *F* (4.066; $P = 0.05$), the null hypothesis is correct and the sample means do not differ significantly.

Given these results, the method was also used to determine nafcillin in other milk-based matrices spiked with nafcillin (3×10^{-4} mol L^{-1}) and treated according to the described sample treatment. The samples were plain milk and skimmed drinkable lemon yogurt. The different matrices gave a typical room temperature phosphorescence nafcillin response (**Figure 5b**,**c**), demonstrating that the recognition process taking place in the imprinted sol−gel binding sites was not affected by the sample matrix.

To compare the behavior of different milk-based matrices, skimmed milk was also considered in this experience. Three replicate measurements were made on each sample. The results of the analysis, expressed as room temperature phosphorescence intensity, gave a mean σ_0^2 of 0.97 (three replicate measurements, 6 degrees of freedom) and a $\sigma_0^2/3 = 96.93$ (2 degrees of freedom). With these values, an $F_{2,6} = 96.93/0.97 = 99.923$ was calculated. As the calculated value of *F* is higher than the critical value of *F* (5.143; $P = 0.05$), the null hypothesis is rejected and the sample means differ significantly. This information is very important for making decisions about the preparation of standard calibration solutions and means that calibration must be effected directly in a matrix blank spiked with a known concentration of nafcillin.

In conclusion, these results suggest that an endogenous contribution from blank matrix affects the room temperature phosphorescence measurements; however, its influence on the performance of the recognition process of the imprinted sol−gel seems to be negligible.

The effect of different skimmed milk commercial products over the imprinting materials response was statistically studied and provides an indicator of the ruggedness/robustness of the proposed analytical system and the possibility to use external real matrix calibration. Although the tolerance level set by the European Commission was not yet achieved due at the moment, using the present flow-through system, work directed toward an on-line sample preconcentration approach is currently in progress.

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