**Development of carbohydrate functionalized magnetic nanoparticles for
aminoglycosides magnetic solid phase extraction.**

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Abstract

Magnetic nanoparticles decorated with D-galactose and galactitol ($\text{Fe}_3\text{O}_4@\text{SiN}$ -galactose and $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactitol) were synthesized and employed as sorbent in a magnetic solid phase extraction (MSPE) procedure prior the analysis of aminoglycosides (AGs) in honey samples by LC-MS/MS. AGs are broad spectrum antibiotics, characterized by aminosugars, widespread used in therapeutic and veterinary applications. AGs can be found in the environment and food of animal origin. $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactose and $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactitol were synthesized via copper catalyzed alkyne azide cycloaddition and the synthesis was efficiently followed by infrared spectroscopy. They were characterized by electron microscopy, thermo gravimetric analysis and magnetization curves. The nature of the loading (acetonitrile:water, 50:50 v/v) and elution solution (formic acid 190 mM) were studied in order to optimize the MSPE. Quantitative difference between MSPE with $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactose and MSPE with $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactitol in terms of recovery was found. The final optimized method using $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactose and $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactitol was applied in the determination of AGs in honey. The MSPE performance of $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactitol was found to be superior to that of MSPE with $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactose. The limits of quantification were between 2 to 19 $\mu\text{g Kg}^{-1}$ for amikacin, dihydrostreptomycin, tobramycin and gentamycin. A good correlation between predicted and nominal values of AGs in honey was found (trueness from 84 % to 109%). This MSPE procedure not only requires a minimum amount of sorbent (1 mg) and sample (0.2 g), but it can also be accomplish in a rather short time.

1 Introduction

Sample preparation is a critical step of the analytical process. It includes: sample stabilization, removing sample contaminants, sample enrichment, analysis selectivity and avoid fouling of the detector [1].

The inherent disadvantages of liquid-liquid extraction (LLE) such as a tendency to form emulsions and the inability to extract polar compounds, lead to an increasing tendency for methods based on solid phase extraction (SPE) instead of LLE. Meanwhile, SPE has become a well accepted and tested methodology for analyte preconcentration and matrix removal.

Trends in the development of sample preparation methodologies intend to fulfill the requirements of green analytical chemistry, such as reduction of volume, organic solvent consumption and analysis time [2]. The development and improvement of SPE make possible the achievement of microextraction techniques, which intend to overcome SPE drawbacks, and make the analytical procedure greener. Miniaturized sorption-based extraction techniques like solid-phase microextraction (SPME), stir bar sorptive extraction (SBSE) and magnetic solid phase extraction (MSPE) allow lower solvent consumption and low amount of sample handling [3].

MSPE involves magnetic particles as sorbents which are added to the sample solution. Analytes of interest interact with the surface of the magnetic particle, opening possibilities for magnetic separation. Following, the target analytes can be desorbed for determination. In this sense, analytes are transferred from a complex matrix to a simpler one and can also be concentrated [2].

During the last decades the development of hydrophilic sorbents for the SPE extraction of polar compounds has gained interest and great efforts have been concentrated in the

development of new stationary phases [3-5]. In this sense, hydrophilic polymeric sorbents with di-vinyl-benzene (DVB) skeleton are widely commercially available.

Regarding that, in MSPE traditional stationary phases like C-18 [6-8], hydrophilic and hydrophobic hybrid stationary phases [9, 10] and also hydrophilic stationary phases [11-14] were developed and can be found in recent literature.

“Click” reactions such as copper catalyzed alkyne azide cycloaddition (CuAAC), thiol-ene and thiol-para-fluorine “click” reaction are the most versatile and modular approaches for coupling two reagents in a simple, fast, selective and reliable way and also shares the properties of green chemistry reactions [15]. For these reason, “click” reactions were successfully employed for magnetic nanoparticles (MNPs) functionalization. Zheng *et al.* [13], synthesized glucose-functionalized MNPs by CuAAC and used them in the extraction of glycopeptides and glycans. However, examples concerning MNPs modified with carbohydrates for MSPE are scare, so it constitutes a vacancy area for potential development.

The aim of this work is to synthesize and characterize D-galactose or galactitol functionalized MNPs, and to study and compare their use in MSPE based on hydrophilic interactions.

In this study, aminoglycosides (AGs) are selected as the target analytes. AGs are broad spectrum antibiotics, characterized by aminosugars, widespread used in therapeutic and veterinary applications [16]. Accordingly, AGs can be found in the environment and food of animal origin [17]. AGs residues in water sample represent a risk in the development of resistant species and destroy the aquatic environment [16]. In addition, AGs are used illicitly as growth promoters which may lead to high residue level in food of animal origin. Thus, monitoring AGs in food is an important task to ensure food safety.

Due to the lack of a chromophore and their extreme polarity, AGs determination is considered a challenge and needs special sample preparation and analysis. Indeed, special sorbent are reported for AGs SPE like cation exchange and polymeric phase [16].

Diverse methodologies for AGs determination were reported mostly employed LC-MS/MS. Those which include an extraction step are based on SPE [16] and some methodologies need an evaporation step after SPE elution and reconstitution in a small volume [18-28]. Recently, colorimetric sensors were also proposed for screening of AGs in milk [29].

Diez *et al.* [26] compared different commercially available SPE cartridges to optimize AGs recoveries from food samples, and recently, polyvinyl alcohol –coated MNPs were employed for AGs extraction [28].

Taking into account the importance and the challenge of AGs determination, galactose or galactitol functionalized MNPs are proposed to serve as sorbent to extract AGs from a complex matrix like honey. Due to some similarity between AGs and superficial galactose or galactitol residues over MNPs in terms of structure and hydrophilicity it is feasible to hypothesize an interaction among them.

To the best of our knowledge, this is the first work describing D-galactose or galactitol decorated MNPs and its performance in a MSPE procedure prior to the analysis of AGs in honey samples by LC-MS/MS.

2. Experimental

2.1. Chemicals and reagents

Iron (III) chloride hexahydrate, 3-chloropropyltriethoxysilane, dimethylformamide (DMF), ascorbic acid and sodium bicarbonate were obtained from Sigma Aldrich (St. Louis, MO, USA). Acetonitrile (ACN) (HPLC grade) and formic acid were obtained from J.T.Baker (New Jersey, USA). Iron (II) sulfate, sodium azide, ammonium hydroxide, copper chloride dihydrate and hydrochloric acid were purchased from Fluka (Switzerland). Tetrahydrofurane (HPLC grade) (THF) was purchased from Sintorgan (Argentina).

Standards of amikacin sulfate (AMI) dihydrostreptomycin (DSTP), tobramycin (TOB) and gentamycin sulfate (GEN) were kindly donated from HLB Lab of Dr. Catalini. GEN consists of a mixture of four components closely related, GEN C1, C1a, C2, and C2a (GEN C1, C2, and C2a being the major components). GEN C2 and C2a are stereoisomers. Figure 1 depicts AGs structure.

3-azidopropyltriethoxysilane, propargyl-galactose (6-*O*-propargyl-D-galactopyranose) and propargyl-galactitol (1-*O*-propargyl-L-galactitol) were synthesized in our laboratory [30, 31] (see supplementary information).

Ultrapure water (conductivity of 0.055 $\mu\text{S}/\text{cm}$) was obtained from an EASY pure™ RF equipment (Barnstead, Dudubuque, IA, USA).

2.2 Apparatus

Transmission electron microscopy (TEM) analysis was performed with a JEOL 2200FS TEM operated at an acceleration voltage of 50 kV. Before analysis, one drop of MNPs suspension was placed onto a carbon coated microscopy grid (75 mesh) and dried at room temperature.

Thermo gravimetric analysis (TGA) was conducted with a Perkin Elmer TGA7 at a heating rate of 20 $^{\circ}\text{C min}^{-1}$ under a helium gas flow.

Magnetization curves were performed in a Lakeshore 7,400 vibrating sample magnetometer (VSM) at room temperature (298 K). Samples were prepared by packing them with Teflon tape (10-15 mg).

FT-IR spectra were recorded on a Bruker Tensor 27 ATR FT-IR. The number of scanning was 32.

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analyses were performed in an Ultimate 3000 System high performance liquid chromatography (HPLC) system (Thermo Fisher Scientific, San Jose, CA, USA).

An Acclaim™ Mixed-Mode HILIC-1 120 Å (5 μm , 4.6 $\times$ 150 mm) column and a guard column (4.6 $\times$ 10 mm) containing the same packing material were employed in all determination (Thermo

Fisher Scientific). The mobile phase consisted of a mixture of water : acetonitrile (80:20 v/v) both containing 0.1% of formic acid. The flow rate was set at 0.5 mL min⁻¹; the injected volume was 10 µL and temperature was set at 30°C. A divert valve was employed to avoid endogenous compounds of honey into the MS system. The divert valve was set as follow: 0-1.8 min column elution to waste, 1.8-2.8 min column elution to MS and finally 2.8-4.0 min column elution to waste.

The LC was coupled to a Quantum Access Max mass spectrometer (Thermo Fisher Scientific, Inc, San Jose, CA, USA), equipped with an ESI interface and triple quadrupole MS analyzer.

The optimized conditions resulted from infusion experiments of individuals AGs standards (1µg mL⁻¹) in flow infusion analysis under varied parameters.

Capillary voltage was set to 3 kV. Vaporizer temperature and capillary temperature was set at 280 °C and 297 °C respectively. Sheath and auxiliary gas (nitrogen) pressure was set at 30 psi and 35 psi respectively.

Argon was employed as collision gas with a pressure of 1 psi in the collision cell. ESI in positive mode was performed for ionization and multiple reaction monitoring (MRM) was employed for acquisition. Taking into account that GEN consist of a mixture of isomers, an additional transition including all precursors of GEN and a common fragment was added in order to quantify total GEN.

Data were recorded with Xcalibur software (Thermo Fisher Scientific, San Jose, CA, USA).

One precursor and two products ions were selected for each compound (Table 1).

Additional instrumentation employed includes an ultrasonic bath (Transonic Digital, ELMA, Kolpingstr) and a shaker (Minither-Shaker, Adolf Kuhner AG Schweiz).

2.3. Preparation of D-galactose or galactitol functionalized Fe₃O₄ magnetic nanoparticles (MNPs)

Magnetite (Fe_3O_4) nanoparticles were synthesized via co-precipitation of Fe^{2+} and Fe^{3+} ions in basic solution. Briefly $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (5.406 g, 20 mmol) and FeSO_4 (1.51 g, 10 mmol) were dissolved in water (80 mL) in a three neck round bottom flask at 70°C under a constant flow of argon and constant stirring. Then a 28-30% solution of NH_4OH (30 mL) was added drop wise and Fe_3O_4 was spontaneously produced. The reaction mixture was kept stirring for 2 more hours [32]. After the reaction, the product was collected by centrifugation at 13500 rpm, 30 min, 20°C , washed with water until a neutral pH in supernatant is reached and finally dried in vacuum at room temperature.

Fe_3O_4 (3.5 g) were dispersed in ethanol (300 mL) by exhaustive stirring and sonication. NH_4OH (11.7 mL) and of 3-azidopropyltriethoxysilane [$\text{N}_3(\text{CH}_2)_3\text{Si}(\text{OCH}_2\text{CH}_3)_3$](10%) (23 mL) were added and the reaction mixture was stirred 24 hours at room temperature, yielding $\text{Fe}_3\text{O}_4@\text{SiN}_3$. The product was collected by centrifugation and washed with ethanol and dried with vacuum at room temperature.

A copper catalyzed alkyne azide cycloaddition (CuAAC) was employed to attach the D-galactose or galactitol moiety to the MNPs. To $\text{Fe}_3\text{O}_4@\text{SiN}_3$ (1.20 g) dispersed in water, 1-*O*-propargyl-L-galactitol or 6-*O*-propargyl-D-galactopyranose (0.53 g) were added. Following sodium ascorbate (0.6 mol per alkyne group) and $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.3 mol per alkyne group) were added. The mixture was stirred at a temperature of 60°C during 48 hours yielding $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactose or $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactitol. The product was collected by centrifugation, washed with ethanol and dried in vacuum at room temperature. Fig. 2 represents the schematic preparation of MNPs.

2.4. Binding and MSPE recovering

The effect of the composition and volume of the interaction solvent, as well as the composition and volume of the elution solvent over recovery of aminoglycosides was evaluated. Either $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactose or $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactitol (1 mg), was added to a

standard solution of AMI, GEN, TOB and DSTP containing 1 µg each and the mixture was vortex and shaken at room temperature. After that, MNPs were recovered employing a magnet and the supernatant (unbound analyte) was analyzed by LC-MS/MS. The retained quantity was calculated by subtracting the unbound analyte concentration from the initial concentration. After removing the supernatant the elution solvent was added in order to elute AGs from MNPs and subsequently analyzed by LC-MS/MS.

2.5 Real samples

Honey samples were purchased from local producers and kept frozen at -20°C before use. An aliquot of 0.2 g of sample was introduced into a polypropylene tube and dissolved in 2 ml of water by vortexing an ultrasound. After that 2 ml of ACN were added and the mixture was vortexed for 2 min and centrifuged 20 min at 15°C. The supernatant was loaded onto 1 mg of MNPs ($\text{Fe}_3\text{O}_4@\text{SiN}$ -galactose or $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactitol) in a 5 mL polypropylene tube. The mixture was shaken for 10 min at room temperature. Then MNPs were recovered employing a magnet as previously described and the supernatant was discharged. After that the elution solvent was added and released AGs were analyzed by LC-MS/MS.

Blank samples were spiked with different known standard solution to prepare fortified samples.

3. Results and discussion

3.1. Synthesis and characterization of MNPs

Figure 2 shows the synthesis of Fe_3O_4 and their surface functionalization. The $\text{Fe}_3\text{O}_4@\text{SiN}_3$ nanoparticles were dispersible in toluene but not in the water phase. The opposite is observed for $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactose and $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactitol where both type of MNPs are suspended in water and cannot be incorporated to the hydrophobic phase of toluene. Due to the enhanced hydrophilicity associated with the presence of multiple hydroxy groups in the grafted galactose and galactitol molecules, respectively. This observation is in accordance with the surface modification of nanoparticles via CuAAC.

Representative images from TEM (Fig. 3A) of $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactitol show a rectangular-square and round shape. A similar morphology was observed for Fe_3O_4 , $\text{Fe}_3\text{O}_4@\text{SiN}_3$ and $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactose (see supplementary information). $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactitol, $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactose, $\text{Fe}_3\text{O}_4@\text{SiN}_3$ and Fe_3O_4 , length determinations were 12.0, 11.2, 10.7 and 10.8 nm with a standard deviation of 2.6, 2.7, 2.8 and 3.1 nm respectively.

Reactions described in Fig. 2 were checked by FT-IR (Fig. 3B).

All MNPs show an intense band around 560 cm^{-1} which could be assigned to Fe–O vibration [27]. The spectrum of $\text{Fe}_3\text{O}_4@\text{SiN}_3$ shows a broad band at 980 cm^{-1} and an intense and sharp band at 2104 cm^{-1} typically assigned to Si–O vibration and the out of phase stretching vibration of azido group respectively. Those results suggest the successful modification of bare magnetite with 3-azidopropyltriethoxysilane [13, 33].

After CuAAC process the N_3 vibration band disappeared indicating that azido groups reacted with 6-O-propargyl-D-galactopyranose or 1-O-propargyl-L-galactitol. The spectra of $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactose and $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactitol also show a broad band at 1100 cm^{-1} and 1070 cm^{-1} due to the Si–O vibration, and C–O stretching from carbohydrate residue contributing to a widening of the Si–O signal. The triazole band appears at 1624 cm^{-1} . This last result also supports a successful “click” reaction [33].

The surface modification of magnetite was also evaluated by thermo gravimetric analysis (TGA) (Fig. 3C). The percentage of the organic layer surrounding the inorganic core can be

estimated by the difference between modified magnetite and bare magnetite in the residual amount after degradation. The percentage of organic layer in $\text{Fe}_3\text{O}_4@\text{SiN}_3$ represents 3.3 % while in $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactose and $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactitol represent 11.7 % and 10.4% respectively. Those results indicate the expected increase in the organic layer when galactose or galactitol is attached to magnetite. Moreover, it can be assumed that the CuAAC proceeds with a similar yield employing propargyl-galactose or propargyl-galactitol independently.

All MNPs show superparamagnetic behavior at room temperature, as it is shown in Fig. 3D. No hysteresis loop was observed when the external magnetic field was removed and a saturation magnetization was also observed at high magnetic field [34]. The saturated magnetization values were obtained after applying the Langevin function fit to the experimental data (Adj. R-Square > 0.99). The maximal saturation magnetization of Fe_3O_4 , $\text{Fe}_3\text{O}_4@\text{SiN}_3$, $\text{Fe}_3\text{O}_4@\text{Si}$ -galactose and $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactitol was 57.2, 52.9, 51.2 and 48.4 emu g^{-1} for respectively with an error of 0.4 emu g^{-1} . The percentage of nonmagnetic material on the nanoparticles was calculated from the decrease of saturation magnetization [35]. Thus, the amount of organic material calculated by VSM are 4.3, 6.0 and 8.8 w/w % for $\text{Fe}_3\text{O}_4@\text{SiN}_3$, $\text{Fe}_3\text{O}_4@\text{Si}$ -galactose and $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactitol, respectively (with an error of 0.8 w/w %). These results are in good agreement with those obtained by TGA, considering that VSM results may be influenced by the presence of non-magnetic iron oxides in the samples.

3.2 LC-MS/MS parameters

Table 1 shows the optimized parameter for each transition as well as the retention time of the chromatographic peak. High reproducibility in retention time was found for all AGs. In all compounds protonated molecular ions $[\text{M}+\text{H}]^+$ were observed.

Quantification of AMI, DSTP and TOB was conducted in a multiple reaction monitoring (MRM) by selecting each transition for the corresponding AGs.

Taking into account that the amount of each isomer of GEN in the standard is not known, quantification of GEN was carried out by using the single chromatographic peak that represents transitions of all isomers of GEN (total). However, during the optimization process percentage of recoveries was calculated from the peak area of each isomer of GEN separately, due in this case an absolute quantification is not required.

3.2. Interaction studies

3.2.1 Interaction mixture composition

Interaction mixture composition was optimized by using 1mg of sorbent ($\text{Fe}_3\text{O}_4@\text{SiN-galactose}$ or $\text{Fe}_3\text{O}_4@\text{SiN-galactitol}$), 1 μg of each AGs, 1 mL of volume and 10 min interaction time. Experimental design is depicted in Table 2S and 3S (see supplementary information).

The interaction between AGs (GEN, TOB, DSTP, AMI) and carbohydrate functionalized MNPs ($\text{Fe}_3\text{O}_4@\text{SiN-galactose}$ or $\text{Fe}_3\text{O}_4@\text{SiN-galactitol}$) may be explained analogously to the phenomenon that describes hydrophilic interaction liquid chromatography (HILIC), namely, adsorption and partition. The first one refers to hydrogen-donor interaction and electrostatic mechanisms, while the former one could be explained by the presence of water molecules attracted by polar groups of the stationary phase and form an aqueous layer over the surface. Therefore, an analyte dissolved in the mobile phase partitions between the aqueous layer (semi-immobilized) and the mobile phase [36].

Acetonitrile, a polar aprotic solvent, is the most widely solvent employed in HILIC, although THF is recognized as an interesting organic solvent which is a strong hydrogen bond acceptor [37]. Thus, ACN and THF were selected to assay in the interaction mixture.

Fig. 4 describes the interaction between GEN C1, DSTP, AMI and $\text{Fe}_3\text{O}_4@\text{SiN-galactose}$ or $\text{Fe}_3\text{O}_4@\text{SiN-galactitol}$. First, it can be observed how the composition of the interaction solvent

influence the retention of aminoglycosides by the stationary phase. GEN C2 + 2A, GEN C1A and TOB showed a similar behavior as GEN C1 (see supplementary material).

Analyzing interaction between $\text{Fe}_3\text{O}_4@\text{Si-N-galactose}$ and aminoglycosides it seems that in the case of GEN C1 the interaction solvent dose not play a significant role, yielding a retention percentage of near 100 % despite the interaction mixture composition. On the other hand, interaction of DSTP is highly influenced by solvent composition. An increase in the organic solvent percentage in the interaction mixture improves DSTP retention. Moreover, ACN seems to have a more effective influence over DSTP retention compared to THF. Analyzing the case of AMI, better results are obtained when a percentage of ACN is added compared to pure water or the addition of THF. However, the influence of interaction mixture composition is less remarkable compared to DSTP.

The results also show that AGs and $\text{Fe}_3\text{O}_4@\text{SiN-galactitol}$ interaction is drastically affected by the interaction mixture composition compared to AGs and $\text{Fe}_3\text{O}_4@\text{SiN-galactose}$ interaction.

In general, when employing $\text{Fe}_3\text{O}_4@\text{SiN-galactitol}$ as sorbent, the amount of ACN required to achieve 100 % retention of AGs is higher than in the case of $\text{Fe}_3\text{O}_4@\text{SiN-galactose}$.

The interaction difference between $\text{Fe}_3\text{O}_4@\text{SiN-galactose}$ or $\text{Fe}_3\text{O}_4@\text{SiN-galactitol}$ and the different aminoglycosides is not easily explained. However, as the water layer attached to the polar hydroxyl surface of the stationary phase (galactose or galactitol) could be comparable, the experimental difference might be explained studying the adsorption mechanism between MNPs and aminoglycosides. In fact, the influence of adsorption mechanism over the binding process is depicted with the highly retained GEN and AMI and TOB when only water is used as binding solvent. In that case no partition mechanism is expected.

The better performance of ACN compared to THF could be related to the hydrogen bonds between aminoglycosides and THF which possibly decline the amount retained.

A mixture of water: ACN (50:50) was selected as the interaction solvent for the subsequent experiments.

3.2.2. Loading and elution solution

Aqueous solutions with different ionic strength and pH were assayed as eluents. The efficiency for recovery and chromatographic peak shape were evaluated (signal to noise ratio, peak width and peak high).

Fig. 5 shows the recovery of AGs from a mixture of standard solution. For this study 1 μg of each AGs, 1 mg of sorbent and 1 mL of elution solution was employed.

A high recovery of retained AMI and DSTP from $\text{Fe}_3\text{O}_4@\text{SiN-galactose}$ was achieved while GEN and TOB was poorly desorbed.

Nevertheless, all AGs could be eluted from $\text{Fe}_3\text{O}_4@\text{SiN-galactitol}$ more easily compared to the aforementioned sorbent.

Formic acid 190 mM was selected as elution solvent due to the recoveries achieved and for the chromatographic peak (Fig. 9S, see supplementary information). It can be concluded that an increase in the ionic strength has a deleterious effect in the peak shape. Furthermore, neutral or basic solutions such as ammonium acetate or ammonium formate did not have adequate recoveries and present a wide and distorted peak.

The elution volume was also studied to evaluate the AGs concentration factor. The addition of 50 μL of elution solution allows the recovery of AGs with comparable results at 1 mL. However, in order to yield reproducible results and to ensure enough volume in the LC vial to repeat the analysis, 150 μL of elution solution was employed.

The loading volume was optimized from 1 to 8 mL. When $\text{Fe}_3\text{O}_4@\text{SiN-galactitol}$ as sorbent is used, the recovery performance of DSTP decrease with increasing the loading volume employed (Fig. 6), while this effect is much less remarkable when $\text{Fe}_3\text{O}_4@\text{SiN-galactose}$ is employed.

Fig. 6 also depicts the recovery of AGs after three different incubation times. A better recovery of DSTP from MSPE with Fe₃O₄@SiN-galactitol in 4 mL at 120 min was obtained in comparison to 10 min. However, from a compromise between percentage of recovery and time of analysis, a 10 min of interaction time was selected.

3.3. Binding isotherm study

Adsorption isotherm data were obtained after incubation of each AGs separately with Fe₃O₄@SiN-galactose or Fe₃O₄@SiN-galactitol for twenty four hours. It has to be noted, that total gentamicin (GEN C1, GEN C1A, GEN C2+2A) was quantified and plotted in the adsorption isotherm although it is not a single compound.

All isotherm curves depicted a lack of saturation. Adsorption capacities (Q_{eq}) are defined as the amount of sorbate (AMI, TOB, DSTP or GEN) per mass unit of sorbent (Fe₃O₄@SiN-galactitol or Fe₃O₄@SiN-galactose) and determined as follows: $Q_{eq} = (C_0 - C_{eq})V/m$, where C_0 and C_{eq} are the initial concentration and the equilibrium concentration of AGs in the interaction solution respectively ($\mu\text{g mL}^{-1}$), V is volume of the interaction solution (mL) and m is the sorbent mass (mg) [38].

Experimental data fitted better for the Freundlich isotherm model compared to other models tested. Fit estimators (Reduced Chi-Sqr and Residual Sum of Squares) were lower compared to those obtained from Langmuir or Sips models (see supplementary information).

The Freundlich model predicts a heterogeneous sorbent surface, without taking into consideration saturation of binding sites. According to this model, in first term high affinity binding sites are occupied followed by the low affinity one. Equation 1 depicts the Freundlich isotherm model.

$$Q_{eq} = K C_{eq}^n \quad (1)$$

The factor K is directly related to the relative adsorption capacity of the sorbent and the parameter n is the measure of the intensity of the sorption [39].

Table 2 summarizes the Freundlich isotherm parameters and the correlation coefficient for all AGs tested. Fig. 10S and 11S depict the adsorption experimental data and the fitted isotherm (see supplementary material).

A similar relative adsorption capacity was found for all AGs when Fe₃O₄@SiN-galactitol was employed as sorbent. On the other hand, heterogeneous values were found when Fe₃O₄@SiN-galactose was employed as sorbent. The value of n remained < 0.7 for the entire systems tested.

3.4. Method validation

Three sets of calibration standards were used to study the presence of matrix effect and method recovery in three different days. The first set (A) consisted of standards of each AGs in water. The second set (B) was obtained by spiking the AGs (in the same concentration employed for set A) in aliquots obtained after honey samples extraction as described in section 2.4. On the other hand, in set (C) AGs were spiked in honey samples before extraction and subjected to the entire procedure.

Matrix effects and recovery were evaluated by comparing the slopes of the calibration curves obtained from set B and A, and C and B respectively and expressed as the average of three different days. The difference between both slopes was statistically evaluated by applying a Student's *t*-test [40, 41]. Process efficiency (4), was also calculated for each sorbent.

$$\text{Matrix effects (\%)} = \frac{B}{A} \times 100 \quad (2)$$

$$\text{Recovery (\%)} = \frac{C}{B} \times 100 \quad (3)$$

$$\text{Process efficiency (\%)} = \frac{C}{A} \times 100 \quad (4)$$

A slight suppression effect was found for TOB while a small enhance response was found for AMI and DSTP for both MSPE system employed ($\text{Fe}_3\text{O}_4\text{@SiN-galactitol}$ or $\text{Fe}_3\text{O}_4\text{@SiN-galactitol}$). It is worth to note that matrix effect over GEN was highly remarkable for $\text{Fe}_3\text{O}_4\text{@SiN-galactose}$ MSPE; on the contrary for $\text{Fe}_3\text{O}_4\text{@SiN-galactitol}$ GEN enhanced matrix effect was lower.

During the optimization process for MSPE using $\text{Fe}_3\text{O}_4\text{@SiN-galactose}$, recovery values for DSTP and AMI was close to 100 % while for TOB and GEN recovery was near 40%. However, low recovery from honey samples for all AGs was found when $\text{Fe}_3\text{O}_4\text{@SiN-galactose}$ was used in MSPE procedure.

In the case of MSPE- $\text{Fe}_3\text{O}_4\text{@SiN-galactitol}$, higher recoveries ranging from 69.9 to 88.3 % were found for AMI, TOB and GEN though recovery for DSTP was remarkably lower than the former ones, even lower than for MSPE- $\text{Fe}_3\text{O}_4\text{@SiN-galactose}$. Those results are almost comparable to those recoveries from 4 mL of spike water (Fig. 6).

Hence, $\text{Fe}_3\text{O}_4\text{@SiN-galactitol}$ as stationary phase seems not to be severely affected by natural honey components when MSPE is performed. On the other hand, MSPE performance is drastically affected when it is performed from honey samples by using $\text{Fe}_3\text{O}_4\text{@SiN-galactose}$.

Taking into account the observed matrix effect and the recovery values, a matrix-matched standard calibration curves (MMSCC) was proposed to quantify samples and correct for both effects.

For MMSCC, calibration standards are prepared by spiking blank samples before extraction with AGs at different concentration and then submitted to the entire procedure. MMSCC has been employed for AGs analysis in food samples [26] as it is also suggested for quantifying

drugs in food by the “proposed draft guidelines on performance characteristics for multiple-residues methods” by FAO/WHO Food Standards Programme of the Codex Alimentarius Commission.

To test linearity, the experimental F value corresponding to the ratio of residual variance to squared pure error did not exceed the tabulated critical F at a significance level of 0.05, which support the adequacy of the linear model in all cases. This statistic test is preferred than the correlation coefficient of a calibration graph for assessing its linearity [42]. In addition, linear regression analysis of all calibration curves showed correlation coefficient higher than 0.99.

Validation data is resumed in Table 4.

The limit of detection (LOD) was calculated with blank samples fortified at low concentration and submitted to the entire extraction procedure by using a signal to noise ratio of 3:1. Limit of quantification (LOQ) was calculated with a signal to noise ratio of 10:1.

For trueness and precision honey blank samples were spiked at four levels over three different days with three replicates. Those levels were selected from the work of Diez *et al.* [26] taking into account that maximum residue levels (MRLs) are not established for AMI, TOB, DSTP and GEN in honey samples.

Trueness was expressed for each level as an average of the inter day analysis, as the ratio between the predicted value of AGs and its nominal value.

Satisfactory values for trueness were found for AMI, TOB, DSTP and GEN when Fe₃O₄@SiN-galactitol was employed in MSPE for all levels (84% - 109%). However, for DSTP the low level proposed by Diez *et al.* [26] (15 µg Kg⁻¹) was below LOQ (19 µg Kg⁻¹), hence it was not possible to determine trueness. Moreover, as it was presented in Table 3, DSTP recovery from honey samples was remarkably low which supports the lack of trust in the determination of DSTP at such low concentration.

On the other hand, the values for trueness ranged from 82% to 107% for AMI and DSTP when Fe₃O₄@SiN-galactose was employed. However, the values for trueness when GEN and TOB are

analyzed were not satisfactory. Those results may also be explained in terms of low recovery for GEN and TOB when Fe₃O₄@SiN-galactose is used (Table 3).

Root mean square error (RMSE) was employed to evaluate MSPE performance between Fe₃O₄@SiN-galactitol and Fe₃O₄@SiN-galactose, as a measure of concordance between predicted and nominal values of AGs in the test samples, $RMSE = \sqrt{\frac{\sum_{n=1}^N (C_{nom} - C_{pred})^2}{N}}$,

where, C_{nom} and C_{pred} are the nominal and predicted concentration of the sample n, and N is the number of test samples. Table 4 shows all the RMSE values. Accordingly, MSPE with Fe₃O₄@SiN-galactitol showed lower RMSE values for AMI TOB and GEN than MSPE conducted with Fe₃O₄@SiN-galactose. On the other hand, MSPE carried out with Fe₃O₄@SiN-galactose lead to lower values of RMSE for DSTP than MSPE with Fe₃O₄@SiN-galactitol.

Inter-day precision ranged from 4% to 28% for both MSPE methodologies but precision for MSPE with Fe₃O₄@SiN-galactitol was in general better than MSPE with Fe₃O₄@SiN-galactose.

In summary, MSPE carried out with Fe₃O₄@SiN-galactitol seems to be suitable for the analysis of AMI, TOB, DSTP and GEN at concentration higher than 15, 20, 20 and 60 µg Kg⁻¹ respectively. In contrast MSPE with Fe₃O₄@SiN-galactose was not suitable for TOB and GEN analysis although quantification procedure of AMI and DSTP at concentration higher than 15 µg Kg⁻¹ was acceptable.

The lower recovery of TOB, AMI and GEN in honey after MSPE using Fe₃O₄@SiN-galactose compared to Fe₃O₄@SiN-galactitol (Table 3), might partially explain the difference between Fe₃O₄@SiN-galactose and Fe₃O₄@SiN-galactitol in terms of precision and trueness. Moreover, the aforementioned lower recovery of TOB and GEN with Fe₃O₄@SiN-galactose might be explained due to a not complete elution step (Fig. 5). On the other hand, the reported trueness and precision of MSPE with Fe₃O₄@SiN-galactose for DSTP might be better-quality compared to Fe₃O₄@SiN-galactitol also due to the higher recovery of DSTP with Fe₃O₄@SiN-galactose compared to Fe₃O₄@SiN-galactitol.

Table 5 resume recent methodologies concerning AGs determination, emphasizing those that employ any kind of SPE and honey as matrix. The proposed method showed comparative LOQ, trueness and matrix effect similar to the previously methodologies reported. In terms of total analysis time, the proposed method requires only 10 min of MSPE (and a previous centrifugation step of 20 min) plus 5 min of chromatographic analysis. Total analysis time in previous reported methods involving SPE ranges from 10 min to one day plus the chromatographic analysis (Table 5).

Figure 7 depicts the chromatograms of each AGs analysis after MSPE. The use of a triple quadrupole provide a good selectivity, however some natural components of honey can be observed in the same transition of GEN after MSPE with $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactose but not after MSPE with $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactitol. Nevertheless, those compounds and GEN are resolved by LC.

4. Conclusion

$\text{Fe}_3\text{O}_4@\text{SiN}$ -galactitol and $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactose showed a manifest interaction with AMI, TOB, DSTP and GEN which is useful for magnetic solid phase extraction in honey samples. The conducted adsorption experiments revealed the dependence of the interaction solvent over the adsorption of AGs in the MSPE procedure. Additionally, the desorption efficiency of AGs was also different depending on the selected sorbent, $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactitol or $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactose.

Regarding the analysis of AGs in honey the MSPE performed with $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactitol displayed a better-quality performance over $\text{Fe}_3\text{O}_4@\text{SiN}$ -galactose in terms of precision and trueness.

Compared to other SPE methods previously reported for AGs analysis, the present one employed a minimum amount of sorbent (1 mg) and sample (0.2 g). The final optimized

method was validated for the analysis of four aminoglycosides in honey with acceptable and reliable results.

It is expected that these carbohydrate functionalized magnetic nanoparticles might be of interest as stationary phase for other analytical applications concerning hydrophilic interactions beyond the one described here.

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Table 1. Fragment ions with their optimized parameters, and retention time.

AGs	Transitions (m/z)	Collision energy (eV)	Tube lends (V)	Retention time (min)	RSD (ret time)
AMI	586 $\rightarrow$ 163	29	82	2.20	0.4
	586 $\rightarrow$ 425	17	82		
DSTP	584 $\rightarrow$ 246	40	119	2.34	0.3
	584 $\rightarrow$ 263	33	119		
TOB	468 $\rightarrow$ 163	24	84	2.14	0.7
	468 $\rightarrow$ 324	15	84		
GEN C1	478 $\rightarrow$ 160	25	81	2.13	0.03
	478 $\rightarrow$ 322	15	81		
GEN C1A	450 $\rightarrow$ 160	25	76	2.13	0.8
	450 $\rightarrow$ 322	15	76		
GEN	464 $\rightarrow$ 160	25	80	2.13	0.8
C2+2A	464 $\rightarrow$ 322	15	80		
GEN (total)	(478,450,464) $\rightarrow$ 322	15	81, 76, 80	2.13	0.03

Table 2. Freundlich isotherm model parameters.

Sorbent	AGs	K ($\mu\text{g}^{1-n} \text{mL}^n \text{mg}^{-1}$)	n	Reduced Chi-Sqr	Residual Sum of Squares	Adj. R-Square
$\text{Fe}_3\text{O}_4@\text{SiN-galactitol}$	AMI	8.39	0.46	20.5	61.5	0.98
	TOB	8.24	0.43	33.5	167.6	0.98
	DSTP	8.71	0.27	5.7	23.0	0.98
	GEN	10.65	0.68	17.0	68.3	0.99
$\text{Fe}_3\text{O}_4@\text{SiN-galactose}$	AMI	10.75	0.45	50.3	201.3	0.99
	TOB	23.00	0.17	3.8	11.4	0.98
	DSTP	7.52	0.47	51.8	311.2	0.96
	GEN	14.54	0.61	35.5	177.7	0.99

Table 3. Matrix effect, recovery and process efficiency for MSPE of AGs.

Sorbent	AGs	Matrix effect (RSD)	Recovery (RSD)	Process efficiency
Fe ₃ O ₄ @SiN-galactitol	AMI	108 (2) *	88,3 (21)	95.4
	TOB	92 (5) *	69,9 (0,3)	64.3
	DSTP	106 (3)	12 (10)	12.7
	GEN	138(4) *	80,3 (22)	110.8
Fe ₃ O ₄ @SiN-galactose	AMI	108 (2)	45 (8)	48.7
	TOB	95 (2)	3,9 (0,9)	3.7
	DSTP	121 (9) *	26,8 (1,0)	32.6
	GEN	225 (17) *	8,1 (15)	18.2

* Significant (p < 0.05)

Table 4. Validation data.

Sorbent	AGs	LOD ($\mu\text{g Kg}^{-1}$)	LOQ ($\mu\text{g Kg}^{-1}$)	Low level ($\mu\text{g Kg}^{-1}$)	Trueness % (RSD)	Level 1 ($\mu\text{g Kg}^{-1}$)	Trueness % (RSD)	Level 2 ($\mu\text{g Kg}^{-1}$)	Trueness % (RSD)	Level 3 ($\mu\text{g Kg}^{-1}$)	Trueness % (RSD)	RMSE
$\text{Fe}_3\text{O}_4@\text{SiN-galactitol}$	AMI	1	2	15	89 (13)	20	98 (6)	40	102 (4)	60	100 (9)	2.7
	TOB	1	3	20	92 (20)	40	87 (9)	80	95 (17)	120	103 (9)	8.7
	DSTP	6	19	15	*	20	93 (28)	40	95 (16)	60	84 (21)	8.7
	GEN	4	10	60	84 (10)	100	90 (7)	200	109 (5)	300	101(12)	14
$\text{Fe}_3\text{O}_4@\text{SiN-galactose}$	AMI	1	3	15	82 (21)	20	107 (5)	40	96 (20)	60	87 (5)	5.7
	TOB	11	37	20	*	40	48 (9)	80	86 (9)	120	65 (5)	28
	DSTP	5	15	15	88 (16)	20	97 (5)	40	101 (4)	60	98 (13)	4
	GEN	28	94	60	*	100	44 (27)	200	111 (5)	300	58 (28)	68

* Not applicable the spike level is below LOQ

Table 5. Previously reported methodologies for AGs analysis.

Matrix	AGs	LOQ $\mu\text{g Kg}^{-1}$	Trueness (%)	ME (%)	Amount of sample/ extraction time	Extraction procedure/ amount of sorbent	Analysis	Ref.
Honey	GEN C1a	69.1	92.7-96.9	99	10 g/30 min	MISPE / 50 mg	CE-MS ²	[18]
	DSTP	15.7	93.7-99.8	96				
Honey	GEN	10	92.5-100.2	110	5g / >26 min	Polymeric RP cartridge / 200 mg	LC-QTOF	[19]
	DSTP	10	85.7- 98	85				
Honey	DSTP	10	76.4-94.5	120-90	5g	WCE or PCE + evaporation	LC-MS/MS	[20]
	GEN C1a	25	92.1-103.1					
	TOB	25	79.7-101.4					
Honey	DSTP	0.66	73.8-85.0	-	1g /40 min	SPME + evaporation	LC-MS/MS	[21]
	AMI	0.69	88.7-102					
	TOB	0.97	96.6-110					
Milk	GEN C1a	49	76-106	92-99	2g	MISPE/ 50 mg	UHPL-MS/MS	[22]
	DSTP	7.7	70-96	90-93				
	AMI	31.3	70-92	94-101				
	TOB	45.5	33-65	85-96				
Honey	GEN	8	91-100	-	2g	MISPE / 200mg	LC-MS/MS	[23]
	DSTP	6	92-100					
Muscle	TOB	4	82-95	95	10 g / one day	On-line in tube solid phase micro extraction	LC-ELSD	[24]
Honey	DSTP	9.4	95-110	-	2 g	PVA-silica cartridge	LC-MS/MS	[25]
Honey	AMI	4	99	32-113	2 g / 30 min	WCE / 500 mg	LC-MS/MS	[26]
	GEN C1a	39	92					
	DSTP	2	84					
	TOB	13	82					
Milk	DSTP	5 $\mu\text{g L}^{-1}$	95-102	88-97	2 mL / 25 min	RAM-DSPE / 16 mg	LC-MS/MS	[27]
Honey	DSTP	3.04	82-95.5	-	2 g / 10 min	PVA-MNPs MG@mSiO ₂ - APB Composites / 40 mg	LC-MS/MS	[28]

MISPE: Solid phase extraction with molecularly imprinted polymers

WCE: Weak cation exchange

PCE: Polymeric cation exchange

SPME: Solid phase micro extraction

RAM-DSPE: restricted access matrix dispersive solid phase extraction

PVA: Poly vinyl alcohol

MG@mSiO₂-APB: magnetic graphene/mesoporous silica composites with boronic acid functionalized pore-walls

Dihydrostreptomycin

Amikacin

Tobramycin

Gentamicin C1

Gentamicin C1a

Gentamicin C2a

Fig. 1. Structures of the selected aminoglycosides.

Fig. 2. Schematic synthesis of Fe₃O₄@SiN-galactose and Fe₃O₄@SiN-galactitol. The distribution between toluene and water of Fe₃O₄@SiN₃ changes after CuAAC to yield Fe₃O₄@SiN-galactose and Fe₃O₄@SiN-galactitol.

Fig. 3. (A) TEM images of $\text{Fe}_3\text{O}_4@SiN\text{-galactitol}$. (B) FT-IR spectra, (C) TGA analysis and (D) magnetization curves of all MNPs.

Fig. 4. AGs retained percentage in sorbent. First row: Fe₃O₄@SiN-galactose, second row: Fe₃O₄@SiN-galactitol. 1 $\mu\text{g mL}^{-1}$ of each AGs, total volume 1 mL. 1 mg of MNPs was employed.

Fig. 5. AGs recover percentage by using different elution solution. First row: Fe₃O₄@SiN-galactose. Second row: Fe₃O₄@SiN-galactitol. Initial loading of 1 $\mu\text{g mL}^{-1}$ of each AGs(1 mL). Elution solution volume: 1 mL.

Fig. 6. Loading volume effect over recovery. In all cases 1 μg of each AGs was used.

Fig. 7. LC-MS/MS analysis of real sample spiked at level 1 after MSPE. Left column: MSPE with $\text{Fe}_3\text{O}_4@\text{SiN-galactose}$. Right column: MSPE with $\text{Fe}_3\text{O}_4@\text{SiN-galactitol}$.