

Intercalation of Phenylalanine, Isocoumarin and Ochratoxin A (OTA) into LDH's

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Abstract

Phenylalanine, isocoumarin and Ochratoxin A (OTA) have been intercalated within the interlayer space of layered double hydroxides. Synthesis of these nanocompounds was achieved via co-precipitation. Their physicochemical properties were studied by element chemical analysis, powder X-ray diffraction, infrared spectroscopy and thermal analyses. The presence of OTA in the interlayer is demonstrated by the study of LC-FD Analysis. On the other hand, these studies allow to check how some of the toxin is on the surface of the nanocomposite.

Keywords

Mycotoxins, Ochratoxin A, Layered Double Hydroxide, Intercalation

1. Introduction

Ocratoxin A (OTA) is a mycotoxin produced by mycomicetes fungi of the genus *Aspergillus* and *Penicillium* which is widely distributed as a natural contaminant of cereals, beans and other foods and which has shown a great diversity of toxic effects in experimental studies [1]. The main contributors to OTA intake in humans are cereals and cereal products since they are the major group of food commodities where the toxin is of the greatest impact. This mycotoxin is resistant to technological processes and may persist in foodstuffs even after mould destruction [2]. Due to its physicochemical properties, OTA is easily absorbed in the gastrointestinal tract, with a bioavailability greater than 50% in all of the mammalian species tested. It shows a high affinity for the plasmatic proteins, which results in a long persistence in the organism. The main metabolites are eliminated via the renal and hepatobiliar routes, as well as through lacteal secretion. The chronic ingestion of OTA leads to the appearance of a renal toxic effect in all the monogastric mammalian species tested. Diverse studies have evidenced toxic effects of OTA on the immune system and on the nervous system. The International Agency for Research on Cancer (IARC) has classified this mycotoxin as a potential group 2B carcinogen to humans [3]. Ochratoxin A

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is not produced commercially [4]; however, in 2010, it was available from 16 suppliers worldwide, including eigth U.S. suppliers [5].

The most frequently used analytical method to determine OTA is liquid chromatography (LC) coupled to fluorescence detection (FD) [6] [7].

The molecular structure consists of an isocumarin nucleus bonded to a L-phenylalanine unit by an amide bond (Figure 1).

The IUPAC name is L-phenylalanine-N-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1*H*-2-benzopyran-7-yl)carbonyl]-(*R*)-isocoumarin and its chemical abstract specification (CAS) is 303-47-9 [8]. OTA forms colorless crystals of empirical formula C₂₀H₁₈O₆NCl, which has a molecular mass of 403.822 Da [9].

Hydrotalcite-like compounds, also known as layered double hydroxides (LDHs), are a family of the so-called anionic clays. The structure of LDHs is similar to that of brucite where each Mg^{2+} ion is octahedrally surrounded by six OH ions and the different octahedras share edges to form infinite sheets. In hydrotalcite-like materials a partial substitution of divalent by trivalent cations has taken place. The chemical composition can be represented by the general formula $[M^{II}_{-x}M^{III}_{x}(OH)_{2}](A^{n-})_{x/n}$ mH₂O, where M(II) and M(III) are divalent and trivalent cations, respectively, in the octahedral positions within the hydroxide layers, x is the $M^{III}/(M^{II} + M^{III})$ molar ratio which value ranges between 0.2 to 0.4 [10], and A^{n-} is an exchangeable interlayer anion [11]. The nature of M(II) and M(III) can be changed within broad ranges. These compounds exhibit a high anisotropy in their chemical bonds, which results in strong interactions within a given brucite-like layer and weak hydrogen bondings between different layers. This allows the intercalation and exchange of a wide range of organic [12]-[16] or inorganic [17] [18] anionic guests, with further influence on the properties and reactivity of the LDH material [10]. Hybrid organic-inorganic LDHs are of high interest because of their chemical, physical and mechanical properties. Applications of other types of hydrotalcite-like compounds remain in water decontamination, catalysis, photochemistry, agricultural, environmental and medicinal chemistry [19]-[22].

In this work we report results of studies of the separate intercalation of both components of OTA (namely, phenylalanine and isocumarin), as well as the intercalation of OTA in layered double hydroxides; the samples have been characterized and LC-FD has been used for detection of OTA. To the authors' knowledge, this is the first report in the literature about the interaction between OTA and LDHs.

2. Experimental

2.1. Materials and Methods

Zinc nitrate 6-hydrate (purity 98%) and aluminum nitrate 9-hydrate (purity 98%) were supplied by Panreac (Spain). D-L phenylalanine (purity 99%) and isocoumarin (purity 98%) were from Fluka (China and France). Ochratoxin A from *Aspergillus ochraceus* was supplied by Sigma Aldrich (Israel). Methanol (purity 99.8%) was from Scharlau (Spain). Sodium hydroxide pellets were from Panreac (Spain). All reactants were used as received, without further purification.

2.2. Preparation of Hydrotalcite-Intercalated by Coprecipitation

The samples were prepared by the coprecipitation method [16] [23] using zinc nitrate $Zn(NO_3)_2$ ·6H₂O (0.05 mol)





and aluminum nitrate $Al(NO_3)_3 \cdot 9H_2O$ (0.025 mol) as precursors, dissolved in 150 mL of decarbonated water. Other solutions (100 mL) were prepared using decarbonated water for phenylalanine (0.05 mol) and isocuomarin (0.05 mol). 0.00248 mole of $Zn(NO_3)_2 \cdot 6H_2O$ and 0.00124 mole of $Al(NO_3)_3 \cdot 9H_2O$ were dissolved in 50 mL of decarbonated water and the solution mixed with another formed by 50 mL of methanol containing OTA (0.0124 mmol) because this is insoluble in water, but is moderately soluble in organic solvents such as chloroform, ethanol, methanol, and xylene [24] [25]. Three samples with a nominal Zn:Al molar ratio of 2:1 varying the intercalated organic anions, phenylalanine, isocuomarin or OTA, were synthesized (**Table 1**). The solution of cations was slowly added to a basic solution of the organic anions whose pH was previously adjusted to 8 by addition of NaOH 1M, maintaining the pH by a feedback loop pH-meter. Once the addition was completed the suspension was vigorously stirred under nitrogen atmosphere at room temperature for 24 h. The suspension was then centrifuged to 3000 rpm for 5 min and the solid was washed several times with decarbonated water. Finally the solid was dried under vacuum at room temperature.

The sample containing OTA was prepared from the very small amount provided by the supplier (5 mg) because of its very high toxicity. For phenylalanine and isocumarin the anion/Al³⁺ molar ratio was 2:1, *i.e.*, an excess was used, as the stoichiometric amount corresponds to a molar ratio of 1:1. Table 1 includes the names given to the samples prepared, the amounts of metal cations and organic component reacted and other data related to characterization of the samples.

2.3. Sample Characterization

Element chemical analysis was carried out in Servicio General de Análisis Químico Aplicado (Universidad de Salamanca, Spain) by atomic absorption in a Mark 2 ELL-240 apparatus after previous dissolution of the samples in nitric acid. Carbon, nitrogen and hydrogen contents were determined through a LECO CHNS-932 equipment in Centro de Microanálisis Elemental (Universidad Complutense de Madrid, Spain). Powder X-ray diffraction (PXRD) patterns were recorded on a Siemens D-500 apparatus equipped with a Daco-MP microprocessor and Diffrac-AT software, using graphite-filtered CuK α radiation ($\lambda = 1.54$ Å). The instrument was set at a current of 30 mA, operating voltage of 40 kV (power 1200 W), and a scanning speed of 2°/min (step size 0.05° and step time 1.5 s) in the 2° - 70° range (2θ scale). Identification of the crystalline phases was made by comparison with the JCPDS files [26] and literature data. The Fourier-Transform infrared spectra (FT-IR) of the samples were recorded on a Perkin-Elmer 1600 spectrometer, in the 4000 to 400 cm^{-1} range, with a nominal resolution of 4 cm⁻¹ and averaging 100 scans to improve the signal-to-noise ratio. The samples were pressed in KBr (Merck) pellets (sample/KBr mass ratio ca. 1:300). Thermal Analysis (DTA) and Thermogravimetric Analysis (TG) of the samples were carried out in air in Perkin-Elmer DTA 1700 and TGS-2 apparatuses, respectively, coupled to a Perkin-Elmer 3600 Data Station, at a heating rate of 10°C/min. LC-FD analysis of the mycotoxin was carried out in a Varian chromatographic system (Walnut Creek, CA), which consisted of a 9012Q pump, a 9100 auto injector, and a 9075 fluorescence detector. Separation of ochratoxin A was performed on a 150×3.9 mm i.d., 4 µm (Waters Nova-Pack, Milford, MA) reverse phase C18 column. All the chromatographic information was reprocessed on a Star Workstation (ver. 4.5) supplied by Varian. Chromatographic conditions were as follows: mobile phase flow rate 1 mL/min from 0 to 12.3 min, 0.7 mL/min for 5 min, and then 1 mL/min until stop time; injection volume, 50 µL; and solvents, A water/acetonitrile/methanol (83:8.5:8.5), and B water/acetonitrile/acetic acid (49.5:49.5:1). The elution program was as follows: 100% A isocratically for 12 min, 100% of B in A for 0.3 min, and 100% B isocratically for 8.7 min. Detection was carried out using a wavelength program with excitation and emission wavelengths of 332 and 478 nm, respectively, for ochratoxin A.

 Table 1. Nomenclature of the samples, amounts reacted and positions of the PXRD maxima corresponding to diffraction by planes (003).

Sample	mmol Zn ²⁺	mmol Al ³⁺	mmol of phenylalanine	mmol of ac. isocuomarin	mmol of OTA	PXRD d ₀₀₃ (Å)
ZnAlph	50	25	50			17
ZnAlc	50	25		50		18
ZnAlOTA	2.48	1.24			1.24×10^{-2}	16

3. Results and Discussions

3.1. Element Chemical Analyses

Element chemical analyses data for metals, carbon, nitrogen and hydrogen are included in **Table 2**. Despite the Zn/Al ratio in the starting solutions was 2 in all cases, it decreased in the solids to values amounting 87%, 79% and 75% for samples ZnAlc, ZnAlph and ZnAlOTA, respectively, indicating an enrichment of the trivalent cation in the brucite layers. The lack of full agreement between these ratios is rather common in the literature and has been ascribed to a preferential precipitation of one or another cation as a hydroxide during the stirring process [18]. As concluded from other characterisation techniques applied (ee below), samples ZnAlph and ZnOTA contain a contamination by nitrate from the original reagents.

3.2. Powder X-Ray Diffraction

The PXRD patterns for samples are shown in **Figure 2**. All patterns indicate the formation of a well-crystallized hydrotalcite-like phase, although with impurities in some cases, with harmonics due to the basal planes (00*l*), which confirms a layered structure. Crystallinity is high in ZnAlph and ZnAlc, as concluded from the marked sharpness of the maxima. The patterns for samples ZnAlph and ZnAlc show characteristic (003) reflections corresponding to spacings of 17 and 18 Å (**Table 1**), respectively, well above the value expected for a nitrate- containing sample with a low Zn/Al molar ratio, 8.6 Å [27]. These values are consistent with the formation of swelled structures as a result of intercalation of the rather large organic anions [28]-[30].

The pattern for sample ZnAlph indicates the presence of a phase responsible for maxima at 17 and 8.6 Å, which should correspond to diffraction by planes (003) and (006), respectively, of the phenylalanine-intercalated LDH. However, as it can be concluded from the data in Table 2, the molar C/N ration for this sample is ca. 6, a value much lower than that corresponding to phenylalanine, 9, suggesting the presence of an additional phase containing nitrogen. As the Zn and Al precursors were used as nitrate salts, the presence of an additional, minor

Table 2. Element Composition of the samples synthesized.										
Sample	Al^{a}	Zn ^a	\mathbf{C}^{a}	H^{a}	N^{a}	Zn^{2+}/Al^{3+b}				
ZnAlph	6.06	25.08	22.89	4.17	4.59	1.58				
ZnAlc	6.20	26.24	21.38	3.59	0.22	1.75				
ZnAlOTA	7.67	27.63	2.82	2.90	5.37	1.50				

^aMass percentage; ^bMolar ratio.



Figure 2. X-ray diffraction patterns.

phase containing intercalated nitrate cannot be discarded. This phase would give rise to a maximum, due to diffraction by the (003) planes, close to 8.6 Å and would be obscured by the intense maximum in the same position due to diffraction by planes (006) of the phenylalanine-containing phase. A single maximum is recorded at high 2θ (60.5°) values, due to diffraction by planes (110) [11] although two maxima are recorded usually in this region, due to diffraction by planes (110) and (113); a single maximum is usually recorded for LDHs containing intercalated organic anions.

These results are similar to those previously reported by Seftel *et al.* [31] for Mg, Al LDHs (molar ratio 3) intercalated with phenylalanine. Fei-Peng *et al.* [32] reported a (003) spacing of 16.8 Å for CuZnAl LDHs containing intercalated phenylalanine prepared by reconstruction [33] [34]. Aisawa *et al.* [35] have reported a slightly larger swelling (18.6 Å) for ZnAl hydotalcite with a 2/1 molar ratio prepared by coprecipitation at pH = 8. When the pH was raised to 9 for this same Zn/Al molar ratio the recorded value for d(003) was 18.1 Å [36] [37]. When Zn-Al hydrotalcites were prepared by the calcination-rehydration reaction method the result obtained depends on the pH values of work; d(003) of 15.8 Å (pH = 7), 15.8 Å (pH = 9.5) and 8.0 Å (pH = 10.5) were obtained [38]. However, when the layer cations were Mg²⁺ and Al³⁺ and the synthesis was performed at pH = 10 the main diffraction maxima were observed at 8.6 and 4.3 Å [35] [38].

Assuming a value of 4.8 Å for the width of the brucite-like layer [23], the height of the interlayer space in our sample results 12.2 Å, larger than the length of the phenylalanine molecule. By using Chemlab (Chem. Office Ultra 9.0, 2005), the calculated dimensions for the phenylalanine molecule are $8.8 \times 5.8 \times 3.1$ Å. The difference between the height of the gallery and the maximum dimension of the intercalated entity is ca. 3.4 Å. Consequently, instead of a single layer of intercalated phenylalanine molecules, formation of a tilted bilayer of molecules with side-by-side $\pi - \pi$ interactiond between the phenyl groups, has been previosuly proposed [35] [37].

Direct intercalation of isocoumarin by co-precipitation afforded organo-LDHs with an average interlayer separation of 18 Å. The PXRD pattern also showed broad maxima which can be assigned to diffraction by planes (006) and (009) at 8.6 and 5.7 Å, respectively. A single, well-crystallized, hydrotalcite-like structure was identified in this case. Contamination by nitrate, if any, should be extremely low, as concluded from element chemical analysis data (**Table 2**). Taking again 9.1 Å for the length of the anion and a value of 4.8 Å for the width of the brucite-like layer, the interlayer distance calculated was 13.2 Å. Probably, the organic molecules were intercalated with their carboxylate group towards the hydroxyl layers. Therefore, it is proposed that molecules probably formed an overlaped bilayer in the LDHs gallery.

Finally, **Figure 2** also includes the PXRD diagram of the OTA-intercalated sample. As mentioned above, the amount of OTA in the reaction medium was very low, and so intercalation of the molecules was very low too. Actually, the main maxima in the PXRD diagram of this sample correspond to those of a nitrate-LDH phase, and only a minor, very weak diffraction maximum is recorded at 16 Å, which might be tentatively assigned to a OTA-LDH phase.

Some diffraction maxima due to $NaNO_3$ formed along the synthesis step were also recorded; this means that it was not removed during washing because less washing cycles were applied in this case to avoid removal of mycotoxin.

According to Lee *et al.* [39], the dimensions of the OTA molecule are 16.0×5.0 Å, while the height of the interlayer space, as calculated from the spacing of the reflection due to planes (003) and the width of the brucite-like layers, is 11.8 Å, suggesting again a tilted orientation of the bilayer, **Figure 3**. When a zeolite Linde type L is used as sorbent OTA is located within the channels because their dimension the channels are formed by columns of zeolite units diameter of the units is 1.26 nm and the diameter of the connecting windows 0.71 nm, so large enough to accommodate the OTA molecular, with diameter of 0.5 nm and a length of 1.60 nm.

3.3. FT-IR Spectra

The FT-IR spectra of the solids prepared are included in **Figure 4**. For all three samples the spectra show typical bands of the layered components, composed of absorption bands due to normal vibration modes of layer hydroxyl groups, water molecules, M-O and M-O-M' stretching vibrations, which have already been reported in the literature [40] [41].

The FT-IR spectrum of sample ZnAlph shows absorption bands which demonstrate incorporation of phenylalanine within the LDH structure. The amine N-H stretching mode is recorded at 2930 cm⁻¹. The C-H stretching



Figure 3. Probable orientation of the OTA between the ZnAl-hydroxide layers.



Figure 4. FTIR spectra of the samples.

mode is recorded at 3042 cm⁻¹ [35]. The intense bands recorded at 1580 cm⁻¹ and 1400 cm⁻¹ are assigned to the antisymmetric and symmetric stretching modes, respectively, of the carboxylate group, and the weak absorptions due to mono substituted benzene are recorded in the 770 - 680 cm⁻¹ range [38]. The presence of nitrate species can be concluded from the peak superimposed to the band of the symmetric stretching mode of the carboxylate group at 1385 cm⁻¹.

No bands due to the presence of nitrate species were recorded in the spectrum of sample ZnAlc, further confirming the purity of this sample, suggested by the element chemical analysis and the PXRD results. The most significant bands are those recorded at 1637 and 1600 cm⁻¹, due to the lactone, although they are shifted with respect to the positions of free lactones with six-member rings [42]. The bands between 1176 and 1000 cm⁻¹ can be ascribed to the pirane C-O moiety. The stress introduced by the ring in the lactone increases the energy of the carbonyl group stretching mode.

In the spectrum of sample ZnAlOTA the band due to the C-Cl stretching mode is expected at 730 - 720 cm⁻¹, but it is buried in the stronger bands due to the M-O stretching modes. Two weak bands at 3000 and 2840 cm⁻¹ are due to N-H and C-H stretching modes, respectively. A more intense band at 1640 cm⁻¹ is due to the presence of the secondary amide and overlaping of the band due to the carbonyl group. A new weak band at 1760 cm⁻¹, not recorded in the spectra of the previously discussed samples, is due to the C=O stretching vibration from the carbonyl and the carboxylic acid groups. A band at 3380 cm⁻¹ is ascribed to the hydroxyl groups, and its position

coincides within the broad band for the OH groups of the hydrotalcite structure.

The presence of nitrate is confirmed by the sharp band at 1367 cm⁻¹, due to mode v_3 , and another one at 1066 cm⁻¹, due to mode v_1 , which activity is due to a decrease in the symmetry (originally D_{3h}) of the intercalated nitrate anion.

3.4. Thermal Analyses

The analysis was performed from room temperature up to 900°C. The curves are displayed in **Figure 5** and **Figure 6**. At a first sight, all curves are similar to each other, showing three mass losses, although differently defined. The first step corresponding to the removal of adsorbed and interlayer water extends up to 220°C, and the second step, due to both the dehydroxylation of the LDH basal layer and the decomposition of anion organic is recorded in the 220°C - 500°C range. The third step corresponds to the combustion of the intercalated organic ions. Total mass losses were 62%, 54%, and 42%, respectively, for samples ZnAlph, ZnAlc, and ZnAlOTA. As the organic anions were burnt off during the analysis, the residues at the maximum temperature reached were a



Figure 5. Thermogravimetric analysis curves.



Figure 6. Differential thermal analysis curves.

mixture of Zn and Al oxides.

Differential thermal analysis of sample ZIAlph shows two strong exothermal effects at 208°C and 466°C. The very large intensity of these peaks does not allow a clear identification of the weaker endothermic effect around 100°C due to removal of adsorbed water molecules. The DTA trace of phenylalanine only shows three thermal effects, one of them endothermic due to the decomposition, and two exothermic effects at 385°C and 528°C due to combustion of the organic molecule. When the amino acid was intercalated within the LDH layers combustion also gave rise to two effects, but at temperatures lower than those for pure phenylalanine.

This combustion is carried out in two steps, where the first peak implies the removal of the lateral chain and the second one is associated to the loss of organic mass adhered to the layer in the interlayer space. However, the curve for sample ZnAlc shows a single, strong, exothermic effect at 424°C, due to combustion of the organic anion.

Finally, the curve for sample ZnAlOTA shows a weak endothermic effect which can be tentatively ascribed to removal of the nitrate anions existing in a large concentration in this sample [27]. This is immediately followed by an exothermic effect at 220°C, and another exothermic effect is recorded at 530°C. These are, however, weaker than for the other two samples, probably because of the much lower concentration of organic anion in this sample. The process of combustion of the organic interlayer anions is carried out in two steps as observed for the phenylalanine sample. The first endothermic peak was at 220°C and the second peak was the combustion of the organic residue at 530°C.

3.5. LC-FD Analysis

As mentioned above, OTA is a mycotoxin which handling required a strict control, because of its high toxicity. In this study the control of the toxin is extremely important and caution should be taken to handle it very carefully.

In this way very small amounts of OTA result detectable [43]-[45]. OTA retention time was 17 min [44], although this depends on the specific experimental and instrumental conditions used, resulting in values of 11.30 min [46] [47], 12.26 min [45] or 13.25 min at low pH values a [48].

The chromatograms recorded are shown in Figure 7. The chromatogram was first obtained for a sample after 24 h ageing after addition of the Zn and Al cations solution to the OTA solution (Figure 7(a)); the amount of OTA corresponds to the total amount added as the sample has not been centrifuged nor washed.



Figure 7. HPLC chromatograms corresponding to ZnAlOTA. (a) Sample after 24 h ageing; (b) Supernatant liquid.

The amount of OTA is so high that it exceeds the saturation level of the instrument; the peak is recorded at 17.29 min. An additional signal at 15.98 min is difficult to be assigned, although it might correspond to OTA too, as this is the single detectable species. Probably one signal corresponds to OTA adsorbed on the external surface of the crystallites and the other to OTA from the interlayer. The sample was centrifuged at 3000 rpm after 24 h. However, the supernatant liquid was actually a suspension probably containing LDH microparticles. The chromatogram of the supernatant liquid is shown in **Figure 7(b**); it shows two peaks at 17.29 and 15.98 min; this last peak is much weaker than that in **Figure 7(a)**, suggesting that OTA has become actually intercalated between the LDH layers. From the integrated areas of these peaks, we may conclude that the amounts of intercalated and externally adsorbed OTA are rather close to each other. One advantage of this technique is that it permit to discriminate between the anions existing inside or outside the layers.

4. Conclusion

Well crystallized hydrotalcites with two organic anions which reaction leads to formation of OTA have been prepared and characterized following several physicochemical techniques. A sample containing intercalated OTA has been also prepared, and it is shown that the mycotoxin is partially intercalated and partially adsorbed on the external surface of the crystallites. The results suggest that LDHs may be suitable materials to remove OTA existing as contaminants in several foods, as LDHs are not toxic, are easily prepared and are cheap.

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