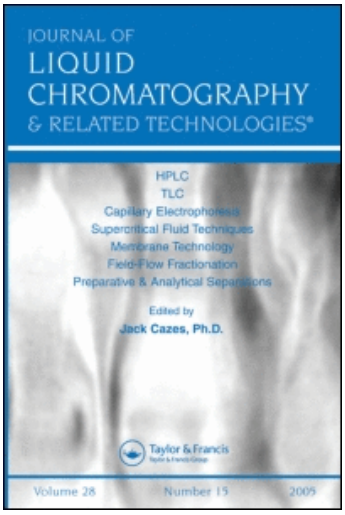

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DIRECT DETERMINATION OF ORGANIC ACIDS IN A FERROFLUID (γ-Fe_2O_3) BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A simple HPLC method has been developed for the determination of organic acids adsorbed on particles of maghemite. The advantage of this described technique is the possibility to identify and measure the ratios of differents acids bounded on the same particle. The chromatographic analysis is achieved on a sulphonated copolymer column in acidic form. Good recoveries of the acids were obtained by dosing the free acids in the supernatant and the acids adsorbed on the particles.

INTRODUCTION

Ferrofluids are colloidal suspensions of small magnetic particles (typical diameter of 7 nm) dispersed in a liquid carrier. They are involved in a large number of industrial applications.[1] One of our aims is to synthesize ferrofluids stable in a physiological medium to promote their use in the biomedical field. The ionic ferrofluids studied in the present work are sols of maghemite (γ-Fe_2O_3) particles, stabilized in aqueous medium by adsorption of α-hydroxy

organic acids at the surface of the particles. The acidic functions of the ligands which are not involved in the complexation with superficial iron ensure the stability of the ferrofluid through electrostatic charges. When different polyfunctional ligands are adsorbed on the same particle, it is possible to fix different biological active molecules on the free organic functions (SH, COOH, NH_2...). For exemple, an antibody could be fixed by the intermediary of a SH free function while on the same particle a drug could be bounded by the intermediary of free carboxylic acid function. However before their use for biomedical applications, it is in particular important to know exactly the quantity of ligand fixed on the particles.

Some indirect methods for the determination of the amount of organic ligands fixed on particles have been reported in the literature. For example, Matijevic *et al.*[2] have used a spectrophotometric method. The concentrations of oxalic and citric acids adsorbed on colloidal spherical hematite (α-Fe_2O_3) particles are deduced from the quantities of ligand present in the supernatant after centrifugation. The iron(III)-5-nitrosalicylate complex presents an absorption peak at 492 nm. When this complex is introduced in the supernatant, the citrate displaces the 5-nitrosalicylate, and so the peak at 492 nm decreases.[3] But organic acids like gluconic or glucuronic acids cannot be determined by this method because of their weak abilities to react with the complex. Furthermore, the linearity range is limited (between 10^{-5}-10^{-4} mol. L^{-1}). A radioactive exchange technique is also used to determine the amount of adsorbed labeled ^{14}C citrate on colloidal silver. The activity of the ^{14}C of this acid remaining in the solution is measured by liquid scintillation. The concentration of the fixed acid is then deduced.[4,5]

By using these methods, the concentration of organic acids is only determined in the supernatant and the amount of organic acids fixed on the particles is then deduced. In this way, validity of the measurements cannot be verified. Precise determination of the amount of ligands fixed on the particles needs a more direct method.

We have developped a simple and specific high-performance liquid chromatography (HPLC) method. This method is usually used for the determination and the separation of mixtures of organic acids in various media.[6-12] In this work, the amounts of several organic acids fixed on maghemite particles and free in the supernatant are quantified and compared with the quantity introduced, a good accuracy is obtained. This method allows also to determine simultaneously the ratios of the different organic acids linked on the particles, which is not possible by previously described methods.

MATERIALS AND METHODS

Apparatus

The chromatographic system used for HPLC analysis is equipped with a Chromatofield Model 501 pump, a Negretti injector and a IOTA refractive index monitor (Precision Instruments, Marseille). The separation is carried out on a 8% cross-linked sulphonated divinyl benzene-styren copolymer in the hydrogen form 300x7.8mm (OA 2216 Benson Polym. Inc NV). The packing particles consists of an impervious core coated with a thin shell of porous material where the exchange occurs. All analysis are carried out at room temperature.

To maintain the column efficiency and so to obtain reproducible results, metallic ions (Fe^{2+}, Fe^{3+}, Na^{+}...) are removed from the samples. They are retained on cations exchange columns (poly-prep) (Biorad AG 50W-X8 resin).[13] These columns are regenerated with three bed volumes of HCl 3 $mol.L^{-1}$ and washed with distilled water until pH is neutral.

The absence of iron in the samples is verified by atomic absorption using a Perkin Elmer 373 spectrophotometer. The calibration curve is done for four concentrations of $(Fe(SO_4)_2(NH_4)_2,6H_2O)$ which are $2\cdot10^{-6}$, $5\cdot10^{-6}$,10^{-5} and$2\cdot10^{-5}$ $mol.L^{-1}$.

Reagents

HPLC mobile phase

The mobile phase is a sulfuric acid solution prepared from reagent grade sulfuric acid and distilled water. The range of concentration is 0.006-0.02 $mol.L^{-1}$. The mobile phase is used at a flow rate of 0.3 mL / min or 0.5 mL / min (table 1).

Organic acids solutions

Four organic ligands are studied: tartaric acid (pKa: 2.88 ;3.94), citric acid (pKa: 2.79 ;4.30 ;5.65), glucuronic acid (pKa: 3.20 ;12.50), and gluconic acid (pKa: 3.56)[14] (Prolabo RP Normapur). Sodium gluconate is used to prepare the acidic solution because it is easier to handle than the viscous acid. The sodium ions are then exchanged with H^{+} on a cation exchange column to

protect the HPLC column. The original acid solutions are titrated with NaOH 0.1 mol.L^{-1}. They are prepared to obtain a final concentration of about 3.10^{-2} mol.L^{-1}.

Ferrofluid

The ionic aqueous ferrofluids used in this present work are cationic sols of γ-Fe_2O_3 macro-ions. They are synthesized according to a method described elsewhere,[15] by alkalizing an aqueous mixture of iron (II) chloride and iron (III) chloride. The precipitate, consisting in anionic magnetite particles (Fe_3O_4), is isolated by centrifugation and acidified by a solution of nitric acid. The particles are then oxidized in maghemite by ferric nitrate, centrifuged and dispersed in water. The ionic ferrofluid so obtained is composed of magnetic particles positively charged with H^+ at the surface. The molar ratio of superficial protonated sites to total iron is $2.44.10^{-2}$.[16] The iron concentration determined by chemical analysis[17] is 1.03 mol.L^{-1}. The polydisperse system is constituted of roughly spherical particles which the mean diameter, obtained by X-ray diffraction, is 8.3 nm.

Sample Preparation

A known volume of organic acids is added to 4 mL of this ferrofluid, the final volume is adjusted to 100 mL with distilled water. The molar ratio of organic acids added to total iron is noted R in this text. To determine the quantity of ligands adsorbed on the surface of the particles, it is necessary to isolate the particles from the supernatant 10 mL of the sample are centrifuged at 4000 r.p.m during 20 minutes. The supernatant and the solid are then separated.

The solid degraded by 1 ml of HCl 6 mol.L^{-1}, is adjusted to 10 mL with distilled water. 4 mL of this solution are passed two times through the ions exchange columns to eliminate the ferric ions, and the column is then washed with distilled water until a final volume of 10 mL.

Two drops of HCl 6 mol.L^{-1} are added to 3 mL of supernatant to displace the possible complexes of Fe(II) or Fe(III) with organic acids. Then the supernatant is also passed through the ions exchange column, and adjusted to 10 mL.

The absence of iron in these latter samples is confirmed by an atomic adsorption method (iron concentration is lower than 10^{-6} mol.L^{-1}).

Table 1

Retention Times and Operating Conditions, $k' = (t_r - t_0) / t_0$, Capacity Factor $\alpha = (t_{ra} - t_0)/(t_{rb} - t_0)$, Selectivity Factor

Sample Type	pH (Eluent)	Flow (mL/min)	t_r	k'	α
Tartaric Acid	2.24	0.5	11'9"	0.50	
Citric Acid	2.24	0.5	10'19"	0.27	
D-Glucuronic Acid	2.24	0.5	9'40"	0.28	
Gluconic Acid	2.24	0.5	10'50"	0.44	
Citric + Tartaric	2.24	0.5	t_r citric = 10'11" t_r tartaric = 11'09"	0.27 0.49	1.35
Citric + Gluconic	2.57	0.5	t_r citric = 9'05" t_r gluconic = 10'02"	0.25 0.38	1.52
Citric + Glucuronic	2.24	0.5	t_r citric = 10'31" t_r D-glucuronic = 9'40"	0.39 0.28	1.41

The calibration curves are established from four standard solutions for each acid at the concentrations of 3.10^{-4}, 6.10^{-4}, 9.10^{-4}, $1.2.10^{-3}$ mol.L^{-1}. The corresponding heights of the peaks obtained on the chromatograms are plotted versus the concentration of acid. The calibration curves are checked every day with freshly prepared organic acids solutions. For the different organic acids, the calibration curves indicate a linear response over a range of concentration of 5.10^{-5}-$1.2.10^{-3}$ mol.L^{-1}. The curves indicate also that as low as 5.10^{-5} mol.L^{-1} of ligand can be quantified reliably by the HPLC method.

Quantification of Organic Acids in Samples

20 μL of the sample are injected and the amount of organic acid is then obtained directly from the calibration curve. The operating conditions are

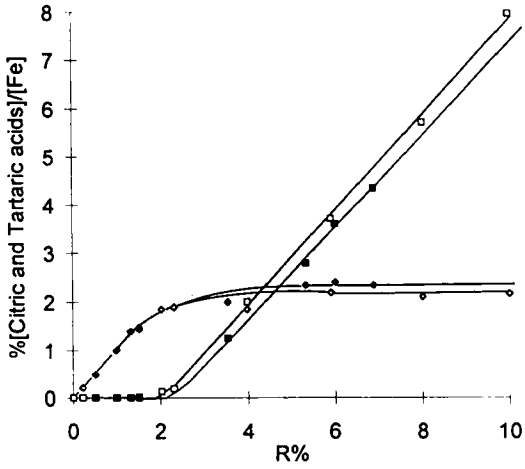

Figure 1. Quantities of adsorbed tartaric acid (◆), citric acid (◇) and free tartaric acid (■), citric acid (□) in the supernatant, versus the quantities of organic acid introduced.

summarized in the Table 1. The organic acids are resolved as a single peak with no interference from other compounds used in this work, which confirm the specificity of the method for organic acids. The identity of the organic acid peaks is assigned by its relative retention time and by spiking with standards.

Recovery

The knowledge of the quantity of organic acids added to the ferrofluid and the determination of adsorbed and free organic acids, allow to calculate the ratios of recovery of ligands from samples. Results are given in the Tables 2-3.

RESULTS AND DISCUSSIONS

The adsorption of organic acids at the oxide surface is based on exchange where the ionizable sites of the particles are replaced by the organic anions which complex the surface iron atoms.[5,18-20] The ligands adsorption on the particles depends on the nature of both oxide surface and ligands. For these acids several complexation models have been proposed in the literature.[21-28]

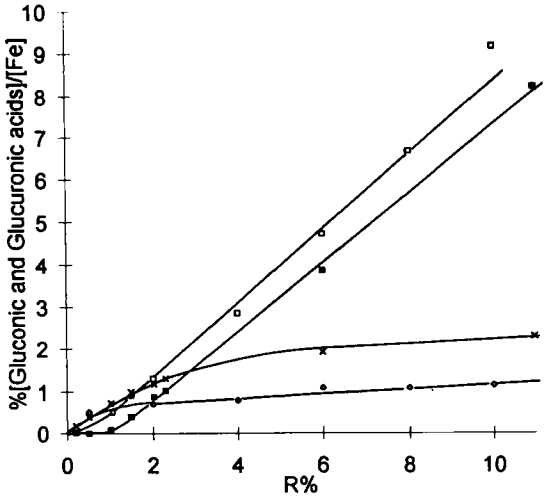

Figure 2. Quantities of adsorbed gluconic acid (✕), glucuronic acid (◇) and free gluconic acid (■), glucuronic acid (□) in the supernatant, versus the quantities of organic acid introduced.

With hydroxy carboxylic acids, both carboxylate and deprotonated hydroxyl groups may participate in the surface complexes. In our experiments, the medium is acidic, the superficial charges of the oxide is then assured by protons and the adsorption of ligands as anionic form is enhanced following the reaction: $MOH_2^+ + L^- \rightarrow ML + H_2O$. The number of ionizable sites (MOH_2^+) on the surface oxide (refered to the total iron) has been determined previously ($[MOH_2^+]/[Fe] = 2.44\%$).[16] In the following section, we will first discuss the results obtained when only one kind of hydroxy acid is adsorbed, and then when two acids are used competitively.

Only One Kind of Ligand Adsorbed on the Particles

The concentrations of organic acids adsorbed on the particles and free are refered to the concentration of total iron, and they are in good accuracy with the introduced quantity as shown in Table 2.

For R lower than 2%, the whole introduced acid is fixed on the particles for tartaric and citric acids (Figure 1). In the case of gluconic and glucuronic

Table 2

Sample Type and Recovery of Organic Acids Introduced

Sample type	R (%)	Recovery (%)	Sample type	R (%)	Recovery (%)
Tartaric acid	0.49	102	Gluconic acid	0.21	100
	0.98	102		0.51	80
	1.31	106		1.02	82
	1.51	99		1.51	93
	3.53	92		2.02	101
	5.32	96		2.30	100
	6.00	97		6.00	96
	6.87	91		10.95	96
	8.96	91		15.47	97
				20.36	96
Citric acid	0.21	100	Glucuronic acid	0.20	95
	0.51	100		1.02	90
	1.01	101		1.51	92
	1.50	98		2.01	100
	2.02	98		4.00	91
	2.30	91		6.00	97
	3.99	96		8.00	97
	5.90	100		10.00	103
	8.00	97		12.51	103
	10.00	101		15.00	101
				17.50	103
				20.10	97
				25.01	106

acids (Figure 2), the introduced acids are partly fixed on the particles, and it remains free ligands in the supernatant. Then the adsorption of anions reachs a maximum. The value of this maximum expressed in adsorbed quantity of ligand refered to iron is about 2.3%. This value agrees with the number of ionizable sites at the oxide surface. The quantity of ligands added to reach this maximum depends on the nature of the ligands. This quantity increases following the order tartaric acid (R=5%) < citric acid (R=6%) < gluconic acid (R=10%) < glucuronic acid (R=20%), indicating that the stability of the surface complexes formed decreases from tartaric to glucuronic acid. This trend is in agreement with the stability constants of the corresponding complexes in

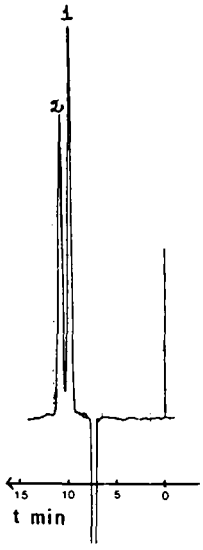

FIGURE 3A

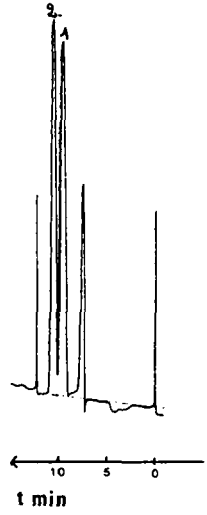

FIGURE 3B

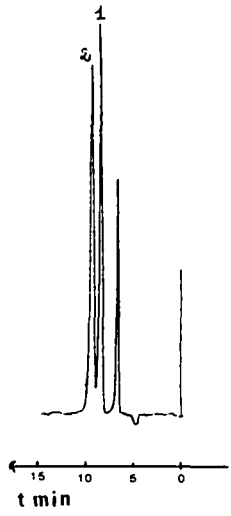

FIGURE 3C

Figure 3. Chromatograms of mixtures of organic acids: A: citric acid (1)- tartaric acid (2) B: glucuronic acid (1)- citric acid (2), C: citric acid (1)- gluconic acid (2).

solution.[27] The weak ability for glucuronic acid to complex superficial iron may be due to its structure. Indeed, among the four ligands studied it is the only one which have no hydroxyl group in a of the carboxylate function. The stability of surface complexe is then lower than with the others ligands which may be coordinated to the surface involving both hydroxyl and carboxylate groups.

Two Kinds of Ligands Adsorbed on the Particles

The following mixtures have been prepared [ferrofluid + citric acid + ligand L] to study the competition for the oxide surface between citric acid and L, L being tartaric, gluconic or glucuronic acid. The molar ratio of ligand added to the particles refered to total iron of the oxide is noted R_1 for citric acid and R_2 for the ligand L. Increasing quantities of citric acid (R_1 varying from 0 to 6%) are added to the ferrofluid and L is then introduced (R_2 = 6%). The quantities of ligands on the particles and free in the supernatant are then

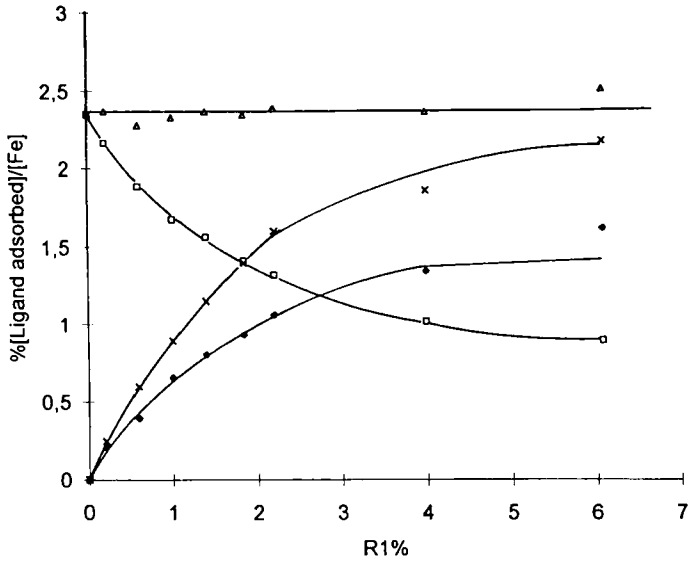

Figure 4. (✕) citric acid adsorbed when only citric acid is added. Quantities adsorbed of: (●) citric acid, (□) tartaric acid, (△) citric and tartaric versus R_1 when citric acid (R_1=0 to 6%) and tartaric acid (R_2=6%) are added to the ferrofluid.

determined by HPLC in the same way as indicated previously for only one ligand. This HPLC method allows a good separation between two ligands, so the selectivity factor is about 1.4 (Table 1), and good recoveries (Table 3). Typical chromatograms are shown in Figure 3. As shown on the Figures 4-6, the first ligand introduced is adsorbed on the surface and when the second ligand is added, there is a competition for surface sites between the two acids, the second ligand introduced displacing a part of the previously adsorbed ligand. The same observation is reported by Waite and Morel[5] for the competition of citrate and phosphate on lepidocrocite (γ-FeOOH) particles.

Citric acid (1)-tartaric acid (2)

In this experiment (Figure 4), the adsorbed quantity of citric acid increases progressively but is smaller than previously when only citric acid was added to the ferrofluid. The quantity of tartaric acid adsorbed decreases, but the total quantity of ligands on the particles is about the same as ionizable

Table 3

Sample Type and Recovery of Organic Acids Introduced, Ferrofluid + Citric Acid ($0 < R_1 < 6\%$) + A: Tartaric Acid ($R_2 = 6.01\%$) B: Gluconic Acid ($R_2 = 5.88\%$), C: Gluconic Acid ($R_2 = 6.57\%$) Rcv_i Represent the Recovery of the Ligand i

A: Tartaric Acid

$R_1\%$	0.20	0.60	1.00	1.39	1.83	2.10	3.99	6.07
Rcv_1	105	105	107	109	97	97	97	104
Rcv_2	102	106	106	105	95	110	99	106

B: Gluconic Acid

$R_1\%$	0.21	0.63	1.04	1.46	1.91	2.30	4.18	5.99
Rcv_1	124	100	98	86	87	90	85	93
Rcv_2	101	102	106	107	108	103	90	97

C: Glucuronic Acid

$R_1\%$	0.20	0.50	0.82	1.10	1.40	2.51	4.00	6.00
Rcv_1	115	82	106	111	96	97	93	102
Rcv_2	92	91	90	94	93	96	93	102

surface sites. This result indicates that citric acid can displace tartaric acid on the particles.

Citric acid (1)-glucuronic (or gluconic) acid (2)

The gluconic acid comportement in the mixture (Figure 6) is the same as the glucuronic one (Figure 5). For $R_1 < 1.8\%$, all the amount of citric acid added is adsorbed on the particles and when the glucuronic (or the gluconic) acid is added, no deplacement of the citric acid is observed. For $R_1 > 2\%$, the quantity of adsorbed citric acid is lower than for citric acid introduced alone, gluconic (or glucuronic) acid displacing a little part of citric acid. At the beginning of the curves the total organic acids on the particles is smaller than in the precedent mixtures. This agrees with the weak ability of glucuronic (or gluconic) acid to complex the surface iron when it is not introduced in a large excess (10% and 20% for gluconic and glucuronic acids respectively). Therefore, when the quantity of citric acid introduced is higher than 3% the

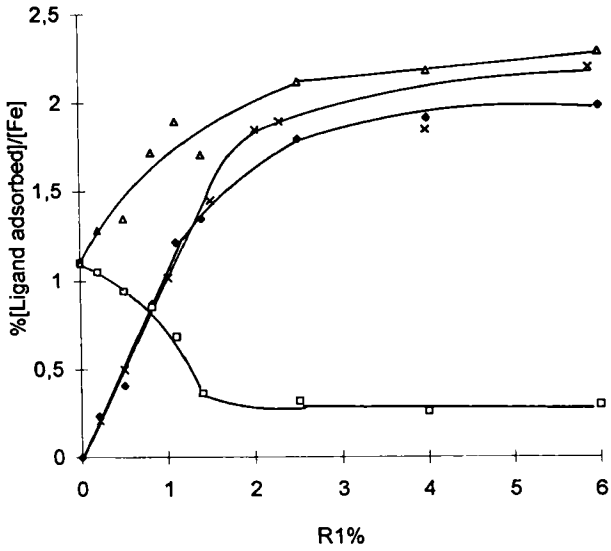

Figure 5. (✕) citric acid adsorbed when only citric acid is added, Quantities adsorbed of (◆) citric acid, (□) glucuronic acid, (△) citric and glucuronic when citric acid (R_1=0 to 6%) and glucuronic acid (R_2=6%) added to the ferrofluid.

total quantity of adsorbed ligands reaches a maximum (2.1% and 2.25% for mixture with gluconic and glucuronic acids respectively) which is a little smaller than for mixture with tartaric acid.

By this method we have shown that it was possible to fix simultaneously two ligands on the same particles. Citric acid is always fixed to the surface, but it can be partly displaced by the others ligands following the order tartaric > gluconic > glucuronic. This trend agrees with the order of stability of surface complexes which has been seen previously.

CONCLUSION

In summary, then, the developed method possesses all the features of a successful analytical method. The recoveries of organic acids from various samples are high, as shown in Tables 2 and 3. By means of this method, the quantity of adsorbed organic acids on the particles of maghemite can be

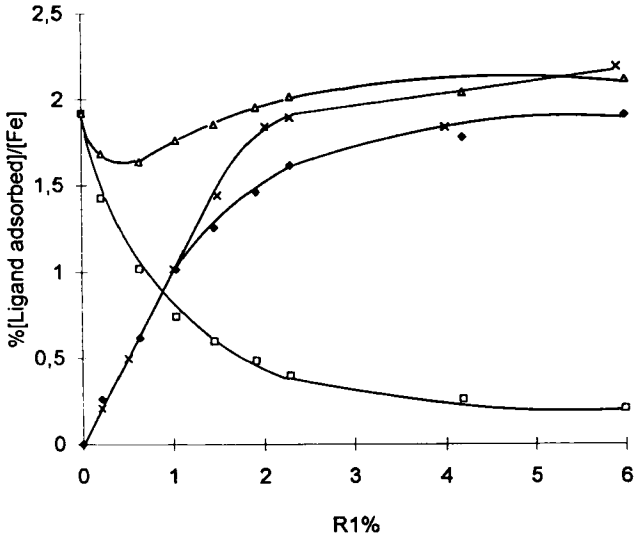

Figure 6. (✕) citric acid adsorbed when only citric acid is added. Quantities adsorbed of: (●) citric acid, (□) gluconic acid, (△) citric and gluconic when citric acid (R_1=0 to 6%) and gluconic acid (R_2=6%) added to the ferrofluid.

directly determined. The ability ot separate and identify the diffrerent organic acids is an advantage for the analysis of our systems in which two diffrerent ligands are adsorbed on a single particle. The ratio of adsorption for each ligand is a function of its ability to complex the surface.

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