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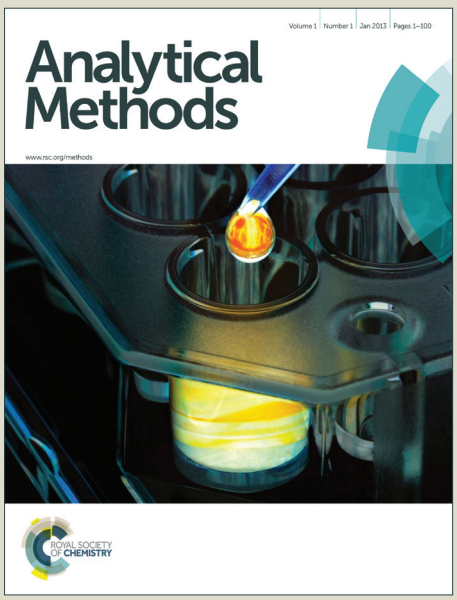

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Synthesis and characterization of molecularly imprinted silica mediated by Al^{3+} for solid phase extraction of quercetin in *Ginkgo biloba* L.

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Abstract

A bi-functionalized molecularly imprinted silica (MIS) was prepared for solid phase extraction of quercetin. To ensure specificity of molecular recognition, aluminum ions were inserted in a silica matrix to form Lewis and Brønsted acid sites and allow interaction with quercetin, as well as with 3-aminopropyltriethoxysilane as a functional monomer. A sol-gel process was used to prepare the MIS-Al with tetraethoxysilane as the crosslinker reagent. The efficiency of imprinted silica was evaluated by comparing the adsorbed amount of quercetin by MIS-Al with a non-imprinted silica (NIS-Al) and control polymers without Al. The adsorption capacity and extraction efficiency were studied with different solvents and a mixture of ethanol:water (60:40, v/v) was found to be most effective for the binding of quercetin with MIS-Al while pure ethanol was most effective for extraction. The selectivity was evaluated by HPLC using a mixture of quercetin and rutin, a molecule which was considered to have an analogous structure. Characterization of the imprinted silica and adsorption capacity tests suggested that the MIS-Al had a higher adsorption capacity and reproducibility than an MIS without Al (248.5 ± 3.5 against 159.7 ± 35.0), proving that the presence of aluminum ions improved selectivity and efficiency for quercetin extraction. Herbal medicine samples of *Ginkgo biloba* L. capsules (40 and 80 mg) were analysed by passing the extract through of an SPE-MIS-Al cartridge and the fractions collected were analysed by HPLC-PDA. The MIS-Al was shown to be selective and resulted in cleaner

chromatograms with better resolution of the quercetin peak, proving this to be an effective *clean-up* step before chromatographic analysis. By the proposed method, it was found that 40 and 80 mg capsules contained 0.25 ± 0.01 mg and 0.24 ± 0.01 mg quercetin/capsule of *Ginkgo biloba* L., corresponding to 2.6 % and 1.3 % (w/w), respectively. These results are in agreement with scientific works in literature that reports a range of 0.5 to 4.7 % mg of quercetin/capsule.

Key-words: Quercetin, molecularly imprinted polymer, silica, solid phase extraction, *Ginkgo biloba* L.

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1. Introduction

Several diseases have been linked to oxidative stress caused by a sedentary lifestyle, smoking, stress and poor diet. In the human body, natural defences such as enzymes provide most antioxidant activity; however, if levels of oxidant species are increased due to unhealthy living, exogenous antioxidants compounds can be ingested to avoid oxidative stress.[1,2]

Flavonoids are natural compounds widely founded in herbs, plants, fruits, vegetables, grains and wines, whose consumption in the human diet has been associated with potent antioxidant activity and consequent capacity to prevent damage from oxidative stress.[3] Among thousands of species of flavonoids, quercetin (Fig. 1a) is considered one of the most biologically active, with high antioxidant activity.[4] Several therapeutic properties of quercetin have been elucidated, such as antioxidant, anti-inflammatory, anticarcinogenic and antiviral effects.[5] The chemical structure of quercetin, with five hydroxyl groups attached to aromatic rings, makes it a potent antioxidant, resulting in the suppression of free radicals and the formation of stable compounds.[6]

Insert Figure 1

Quercetin is widely found in plants and herbs in its free or glycosylated form, the most common being quercetin-3-rutinoside, known as rutin (Fig. 1b).[6] Quercetin can be ingested as a tea infusion of leaves or full plants or as pills containing the dry extract of the leaves. One of the most widely consumed plants for prevention of diseases due to its antioxidant activity is *Ginkgo biloba* L.[7] Its leaves contain several active ingredients, including flavonoids, alkaloids, lipids, sterols, benzene, carotenoids, carbohydrates and terpenoids.[8] The medicinal properties of standardized dry leaf extract of *Ginkgo biloba* L. is attributed to the synergism

of two chemical classes: *ginkgo*flavonoids and terpene lactones. The commercialization of *Ginkgo biloba* L. as an antioxidant compound in Brazil is regulated by the National Agency of Sanitary Surveillance of Brazil, which preconizes that the extract must consist of 24% (w/w) ginkgo flavonoids (expressed as the sum of quercetin, kaempferol, isorhamnetin) and 6% (w/w) terpene lactones (bilobalide, ginkgolides A, B, C, D).[9] Quercetin is considered to be the major active compound in the leaves of *Ginkgo biloba* L. as it has the highest antioxidant activity of all the *ginkgo*flavonoids.

Besides its structural similarities to other *ginkgo*flavonoids, quercetin is shares basic similarities with many other polyphenols normally founded in plants. As such, to quantify the amount of quercetin in leaf samples, selective sample preparation procedures must be applied prior to determination.[2,10,11] Most studies involving determination of quercetin in leaves samples require a complex procedure of sample preparation involving solid phase extraction (SPE)[12], liquid-liquid extraction[13] or solid-phase microextraction[14] before chromatographic separation. As leaf samples are considered complex matrices, sample preparation is usually used to remove the matrix interferents or to separate the flavonoids from the matrix, before they are injected directly into the chromatographic system. However, most extraction procedures are not selective to a specific flavonoid and a group of various similar flavonoids are extracted. An efficient strategy to add selectivity to the SPE procedure is the production of a specific solid phase to extract the molecule of interest.[6,15]

An easy way to attain this objective is based on the synthesis of molecularly imprinted polymers (MIP), which have been highlighted as specific extractors due to their molecular recognition mechanism. This mechanism is based on the formation of tridimensional cavities impressed by the template molecule during the polymerization process. During synthesis, the analyte of interest, considered as a template, is bound to a functional monomer by non-covalent interactions, which remain intact during polymerization. The polymeric network is

complete after the addition of specific reagents; the polymer is then washed to completely remove the template, resulting in cavities with binding sites specific to the analyte. A number of studies have used organic synthesis methods to manufacture molecularly imprinted polymers for extraction of quercetin that can be used effectively to improve clean-up steps for analysis of complex samples, as well as for selective solid-phase extraction prior to chromatographic separation and UV detection.[16-18] There are many advantages to organic synthesis, including its simplicity, the low cost of reagents, high selectivity and robustness.[19] However, this strategy still has some restrictions, such as difficulty removing the template molecule,[20] the limitations of using an aqueous medium due to poor solubility of reagents ,[21] the difficulty to using large molecules as template,[22] the excessive use of functional monomers during synthesis resulting in nonspecific sites,[23] and low reproducibility of the imprinted polymer as an SPE.[24] So, to overcome some of the drawbacks of organic polymers, a new combination of inorganic and organic compounds has been recently published by a few groups, which exploits silica as the base of the imprint process.[25-27]

Silica particles are generally synthesized by the sol-gel process. This involves two steps: hydrolysis of a silane group to give silanols (Si-OH) in the presence of one catalyst (acid or basic) and the posterior condensation of silanols (-Si-O-Si-) to form the network. The process of obtaining molecular recognition is similar to that used in organic synthesis, where an analyte is used as a template. For the formation of specific cavities inside the silica, it is necessary to add reagents that bind the analyte to the silanols. Most previous studies have used 3-aminopropyltriethoxysilane (APTMS) as a basic precursor to bind the molecule template in the presence of tetraethylorthosilicate (TEOS) as a crosslinker to form a polymeric network of molecularly imprinted silica (MIS).[28] This process improves the physical, chemical and mechanical properties of the polymer in comparison to the organic

synthesis, and it is easy to control factors like the porosity, crystallinity, thermal stability and resistance to chemical action, making the synthesis procedure much simpler.[29,30]

In spite of the similar chemical structure of quercetin to the other flavonoids and polyphenols present in leaf samples, an interesting alternative way to increase the selectivity is to create more specific interaction sites for quercetin and MIS.[31] This can be achieved by the formation of complexes with metallic ions, a peculiar mechanism inherent to the quercetin molecule, which is also associated with its antioxidant activity. This interaction is due to the fact that there are three possible binding sites in the structure of quercetin molecules: the 5 and 3-hydroxicromone and 3' and 4'-o-dihydroxyl groups [32,33]. In this way, the interaction between metal and the hydroxyl groups of quercetin may be exploited to increase the selectivity in order to add another specific binding site within the matrix, allowing molecular impression and recognition mediated by ions.

The present study aims to present the synthesis of molecularly imprinted silica (MIS) mediated by Al by a sol-gel process (SGP) for extraction of quercetin. Subsequently, to assess the efficiency of extraction, selectivity and accuracy, the MIS mediated by Al was applied as an SPE cartridge for quantification of quercetin in the dry extract of *Ginkgo biloba* L. leaves.

2. Materials and methods

2.1 Instrumentation and analytical conditions

A magnetic stirrer, model Go-Stirrer MS-H-S (Go-Lab) was used for synthesis of imprinted silica. A horizontal shaker table, model Ethik (ethikechnology) was used for adsorption and selectivity tests. Spectrophotometer UV-Vis, model 8453 (Agilent) was employed in adsorption and extraction tests. A peristaltic pump, model IPC high precision multichannel dispenser (Ismatec) was used in the solid-phase extraction procedure. The HPLC-analysis was performed at ambient temperature under isocratic conditions. The

analytic column was a Brownlee C_{18} column (particle size: 5 µm, 250 x 4,6 mm, PerkinElmer) with mobile phase optimized as methanol:water with 0.5% phosphoric acid 40:60 (v/v). The solution was filtered through a 0.2 µm pore size membrane filter (Millipore) and degassed ultrasonically after mixing. Detection was performed at 375 nm and 254 nm for quercetin and rutin, respectively, in a PDA (Photodiode Array) detector. The sample volume injected was 20 µL and the flow rate was maintained at 1.3 mL min^{-1}.

2.2 Chemicals

Quercetin anhydrous (95.0%), rutin hydrate (94.0%), 3-aminopropyltriethoxysilane (APTMS) (97%) and tetraethylorthosilicate (TEOS) (99.0%) were obtained from Sigma-Aldrich. Aluminum chloride (99.0%) and acetic acid (99.0%) were obtained from Chemical Kinetics (Brazil). The reagents used as solvents were ethanol (99.9%) obtained from Scharlau (Brazil), methanol (99.0) from Tedia (Brazil), phosphoric acid (85.0%) from Vetec (Brazil) and acetone (99.5%) from Dynamics Chemical Contemporary (Brazil). The ultra-pure water was obtained from Milli-Q purification system (Millipore, USA).

2.3. MIS-Al synthesis by the sol-gel process

The molecularly imprinted silica mediated by Al for quercetin extraction was prepared according to the method proposed by Zhang et al.,[34] with some modifications, as represented in Figure 2. First, 50 mg of quercetin, as the template molecule, was dissolved in 10 mL of APTMS at room temperature until complete solubilisation. To this was added 20 mL of TEOS and 30 mL of aqueous solution of $AlCl_3$ 0.01 mol L^{-1} or distilled water. The mixture was homogenized for 10 min to complete the reaction of hydrolysis and polycondensation. The MIS-Al was dried at 105 ºC for 2 h, then macerated and sieved (< 50 µm) and subjected to an extraction process for the removal of quercetin. A glass funnel with quantitative filter

paper was used to support about 1.0 g of silica particles that was washed with 100 mL of methanol:acetic acid (20:1, v/v). To complete the removal of the quercetin, the MIS-Al was placed in a flask with 50 mL of acetone and left to stand for 6 h. Finally, the solution was filtered and the supernatant analysed by spectrophotometry in the UV-Vis region to verify the presence of quercetin. The imprinted polymers were dried at 60 °C to constant weight and stored in glass vials until use. A non-imprinted silica (NIS-Al) was synthesized simultaneously without the presence of quercetin using the same procedure described above. The synthesis of imprinted and non-imprinted silica without Al ions was carried out in parallel and these were used as control polymers (termed MIS and NIS).

Insert Figure 2

2.4. Characterization

The surface morphologies of the MIS, NIS, MIS-Al and NIS-Al were observed using a scanning electron microscope (SEM, model JEOL JSM-7001F). Each sample was fixed onto a sample port and coated with gold under inert atmosphere (Argon) for 1 h.

The surface areas, volume and pore diameter were determined through isotherm adsorption-desorption, employing a Quantachrome Nova 2200 analyzer. Preliminary drying was performed at 100°C under reduced pressure for 12 h.

The infrared spectra were analysed in a spectrophotometer (Varian, model 640-IR), with a resolution of 4 cm^{-1} by accumulation of 64 scans. MIS and NIS were macerated with KBr in a proportion of 1% (w/w) at room temperature.

In order to evaluate the ratio of Si/Al, the polymers were analysed by Spectrometer Fluorescence X-Ray Energy Dispersive (FRX-EDX); model EDX-720 (Shimadzu).

2.5. Performance of the MIS-Al

2.5.1 Adsorption experiments

To investigate the influence of the solvent during the extraction of quercetin by imprinted silica, samples of about 20.0 mg of the MIS, NIS, MIS-Al or NIS-Al were suspended in 5.0 mL of quercetin solution at a concentration of 2.5 mg L^{-1} in ethanol:water (60:40, v/v) at ambient room temperature. The vials were sealed and left stirring for 1 hour on a horizontal shaker table. At the end of this time, the solution was filtered and the supernatants were analysed by UV-Vis spectrophotometry to determine the concentration of quercetin.

Selectivity tests[36] were carried out using similar adsorption experiments with an analogue molecule (rutin) in a HPLC with diode array detector. For this, a proportional increase in mass sorbent and analyte concentration was necessary to obtain adequate analytical signal after 20 µl of sample injection. About 200.0 mg of MIS-Al or NIS-Al was suspended in 5.0 mL of quercetin and rutin solution at a concentration of 30.0 mg L^{-1} in ethanol:water (60:40, v/v). Assays were conducted in triplicate. The vials were sealed and left under constant stirring for 1 h on a horizontal shaker table and at the end of this period the aliquots were filtered and analysed by HPLC. Analytical curves for quercetin and rutin were prepared for the range of 5 to 40 mg L^{-1}.

2.5.2. Molecularly imprinted solid phase extraction of quercetin

Preliminary studies were carried out to evaluate the nature and volume of the solvent that should be used for extraction of quercetin in MIS-Al and the amount of polymer to be used in the production of SPE cartridge. For the assay, an SPE cartridge was prepared using syringes of 3 mL. In the base of the syringe (outlet flow) was placed a disk of sintered of polyethylene (*fritz)* recovered from commercial cartridges. Then, 200 or 400 mg of MIS-Al

was added to the syringe, along with the *fritz* on top of the cartridge accommodating the solid phase. The SPE cartridge was mounted with a support system, connecting the outlet tip of the syringe in the Tygon tube to a peristaltic pump (1.0 mL min^{-1}) to percolate to the solution through the cartridge. The cartridges were conditioned with 2 x 2.0 mL of ethanol:water (60:40 v/v) then 2.0 mL of standard solution of quercetin (10 mg L^{-1}) was percolated through the cartridge. Finally, 8 mL of ethanol was added to evaluate the efficiency of extraction. This eluate was collected and analysed in the UV-Vis spectrophotometer at 375 nm.

To extract quercetin from *Ginkgo biloba* L., two SPE cartridges were prepared containing 400 mg of MIS-Al. Initially, each cartridge was conditioned with 2 x 2.0 mL of ethanol:water (60:40). Then, 1.0 mL of the processed sample solution of *Ginkgo biloba* L. was percolated through of the SPE. Finally, 8 mL of the extractor solvent (ethanol) was used to elute the analyte. This was subsequently collected, dried with N_2 gas and redissolved in 1 mL of ethanol:water (60:40, v/v) to be analysed directly in the HPLC.

2.6.. Sample preparation

Samples of *Ginkgo biloba* L. capsules containing 40 mg and 80 mg dry extract as specified on the label were purchased from a local pharmacy. Ten capsules of *Ginkgo biloba* L. were weighed and the average weight was determined as 0.3447 g for 40 mg capsules and 0.3308 g for 80 mg capsules. On an analytical balance the mass was weighed, mixed with 10.0 mL of ethanol:water (60:40, v/v), and stirred for 10 min with the aid of a magnetic stirrer.[36] The solution was then filtered through quantitative filter paper and an aliquot of 5.0 mL of this solution was placed in a 10.0 mL volumetric flask and filled with ethanol:water (60:40, v/v). The sample and standard solutions were passed through a 0.45 µm membrane filter before MIS-Al cartridge analysis.

3. Results and discussion

3.1. Characterization

Fig. 4 (a-d) shows the surface morphologies of the control polymers (without Al) and the imprinted silica with Al after the quercetin removal step. The silica particles were observed under 100 (Fig. 3a-b) and 10.000 (Fig. 3c-d) times magnification. The synthesis procedure resulted in silica particles smaller than 50 μm that were very heterogeneous in shape and size (Fig. 3a-b), probably due to the maceration and sieving process after synthesis. Under 10.000 times magnification it was possible to visualize a slight variation in surface between MIS and MIS-Al (Fig. 3c-d). This difference could be associated with isomorphic substitution[37] of the silicon ion by aluminum in the polymeric structure, which may have altered the porogen properties of the solvent during the synthesis process.

Insert Figure 3

To investigate the surface area, volume and pore diameter of the particles in relation to the presence of Al, the particles were analysed using a gas porosimetry analyzer and adsorption/desorption isotherms. Results showed that the control polymers (without Al) and the imprinted silica with Al were similar with respect to all the parameters analysed (Table 1). This can be explained by nature of the fact that Si and Al have a similar ionic radius, so this variation should not drastically affect the polymeric structure. Overall, the surface area and pore volume of NIS and NIS-Al particles were smaller than MIS and MIS-Al particles due to the absence of the template molecule. There was no significant variation in the average pore diameter of the materials, and it was confirmed that all the molecularly imprinted silica materials could be considered microporous according to the IUPAC, with pore diameters less

than 2 nm[38]. It is probable that the lack of variation in average pore diameters obtained is due to the fact that the main parameters responsible for controlling the pore size of the polymers were the same in all the syntheses. The similarities between results of surface area and pore diameter of MIS and MIS-Al prove that the visual observation involving scanning electron micrographs may raise doubts about porosity.

Insert Table 1

The chemical structure of molecularly imprinted silica obtained by SGP was characterized by FTIR analysis (Fig. 4). Bands inherent to the silica material included stretching vibrations of the hydroxyl groups (3000-3750 cm^{-1}), a C-H stretching vibration (2931 cm^{-1}), angular vibration of water molecules (1650 cm^{-1}) and stretching of the primary amine present in the functional monomer (APTMS)(1380 cm^{-1}). Symmetric stretching of Si-O-Si and a symmetric stretch of Si-O were observed at 1050 cm^{-1} and 765 cm^{-1}, respectively. The results of FTIR spectra confirmed that all the materials had the same chemical structure with slight modifications in signal magnitude; it was not possible to distinguish the presence of aluminum in any of the materials.

Insert Figure 4

To evaluate the proportion of silicon atoms that had been replaced by aluminum during the SGP, an FRX/EDX technique was employed. Before the removal of the template, the relative percentage of Si/Al to MIS-Al was 56.59 ± 0.91 % and the relative percentage of Si/Al to NIS-Al was 51.43 ± 2.35 %. After removal of the template molecule the values were 66.94 ± 0.87 % and 69.06 ± 5.91 %, respectively. These results were very similar, with a slight increase in the Si/Al ratio for both materials after the template removal step using

methanol/acetic acid solution followed by acetone. It is likely that a small proportion of the unreacted Al was leached, along with TEOS and APTMS, increasing the ratio of Si/Al. However, a considerable amount of aluminum still remained inside the polymeric cavity to allow for specific binding with quercetin.

According to Ribeiro et al.,[39] materials that have a high Si/Al ratio have high hydrophobicity due to the large amount of silicon atoms (Si), which are more hydrophobic than aluminum atoms (Al). In this way, the insertion of Al into the MIS particles to mediate the quercetin interaction caused an increase in hydrophilic conditions. This characteristic is associated with improvement in solid resistance when the material is in direct contact with more hydrophilic substances such as water. It is important to note that replacement of Si by Al also increased the acidity of the polymers, which is related to the formation of Lewis acid sites, in addition to the presence of Brønsted acid sites.

3.2. Performance of MIS-Al

3.2.1. Adsorption experiments

As evident in Table 2, the MIP-Al exhibited the highest adsorption capacity compared to the MIS and its respective non-imprinted silica (NIS) when suspended in a solution of quercetin in ethanol:water (60:40 v/v). This result shows that the MIS-Al had more binding sites for quercetin and this was probably due to the presence of Al inside the cavities, which promoted specific molecular recognition mediated by metallic ions. Moreover, the solvent mixture based on ethanol:water had a better adsorption capacity than methanol due to the increase in hydrophilicity of the new material, as described above.

Insert Table 2

The imprinting factor (IF) of the MIS-Al in ethanol:water (60:40) may also indicate a bifuncionality in the molecular recognition process, since the MIS also demonstrated a value of IF that resulted from interaction with APTMS. This means that the molecular recognition of quercetin by MIS-Al is possible at two kinds of binding site inside the cavities: one of them being the interaction between the functional groups and hydroxyls (Lewis bases) of quercetin with the Al (complex formation) and another related to the hydrogen bonds between the nitrogen atoms in the APTMS. Furthermore, it was observed that the standard deviation of the capacity adsorption values of the MIS-Al in ethanol:water (60:40 v/v) was significantly lower compared to that of NIS-Al in the same medium, while for the methanol solvent, the results obtained lower accuracy for all the materials. These observations are in agreement with the higher hydrophilicity of the material in the presence of Al, which allows more reproducible and effective interactions when quercetin is in an aqueous medium. It is important to note that quercetin is slightly soluble in water and using an ethanol aqueous solution improves its solubility. In light of the fact that MIS-Al performed better results than the other materials tested, subsequent experiments were performed only with MIS-Al.

Among all the properties of an imprinted polymer, selectivity is by far the most important. To assess the selectivity of MIS-Al, adsorption capacity studies were carried out with binary mixture solutions of quercetin and rutin in the presence of MIS-Al and NIS-Al (Table 3). Rutin (Fig. 1b) is a compound which coexists with quercetin in *Ginkgo biloba* L. leaves and has very similar chemical structure, the only difference being a glycosyl group in C3. It was found that MIS-Al adsorbed more of the quercetin than its structural analogue (rutin). Both molecules have hydroxyl groups which are capable of interacting with the amino groups of APTMS and forming hydrogen bonds, as well as being capable of interacting with Al ions located within the structure of MIS-Al. However, because rutin has a bulky group in

the equatorial position, this occupies a large area inside the cavity and impedes specific interactions, resulting in poor extraction. This was not observed for NIS-Al, which showed approximately the same affinity for quercetin as for rutin (84.9 ± 59.1 and 94.9 ± 8.3 mg g^{-1}, respectively). The imprinting factor confirmed that the adsorption capacity of the MIS-Al by quercetin was almost four times greater than for rutin, indicating that the recognition cavities were selective for quercetin in accordance with the template used in the synthesis process.

Insert Table 3

3.2.2. Molecularly imprinted solid phase extraction of quercetin

The SPE procedure was realized by employing 3 mL syringes with cartridges and fritz from renewable commercial cartridges, as described in section 2.5.2. To evaluate the mass of MIS-Al to be used in the cartridge, adsorption and recovery tests were evaluated using 200 and 400 mg of material. A standard solution of quercetin (10.0 mg L^{-1}) was percolated in ethanol:water (60:40 v/v) and after loading, a elution solution of pure ethanol was used, which in preliminary studies showed high desorption of the quercetin molecule. Cartridges containing 400 mg of MIS-Al obtained much more interactions than 200 mg cartridges, adsorbing 29.4 ± 2.0 % in comparison to 3.6 ± 0.2 % of the quercetin. This result was calculated by the difference in the concentration added and monitoring the eluate after percolation by UV-VIS spectrophotometry. The difference between the two cartridges could also be associated to different sampling and elution times provided by amount of sorbent mass and consequently the interaction time. Although the MIS-Al did not have a high extraction efficiency, percentages values are rarely stated in literature reporting the development of MIS technologies, probably due to differences in synthesis procedures, materials and templates.

Thus, with an extraction efficiency of about 29% and good specificity and precision, this material is highly recommendable for application with real samples.

After the elution step with ethanol solution (8.0 mL), the recovery of quercetin adsorbed was of 99.6 % for the 400 mg cartridge and 49.5 % for the 200 mg cartridge. Recovery was calculated in relation to the amount of quercetin adsorbed into the MIS-Al, as described above, revealing that the extraction procedure was very efficient for 400 mg cartridges. To verify the efficacy of the elution step, studies were carried out with four different volumes of ethanol in 400 mg cartridges under the same conditions for sample percolation described above. The accuracy of the SPE was also evaluated using three cartridges for each volume studied. The results confirmed good reproducibility (r.s.d. < 2.5%) in relation to recovery of quercetin adsorbed when 8.0 mL of ethanol was used (101.4 ± 0.3 %). In view of these observations, the extraction of quercetin in *Ginkgo biloba* L. was conducted with pure ethanol as an eluent solvent in an SPE cartridge containing 400 mg of MIS-Al.

3.2.3. Application

Cartridges containing 400 mg of MIS-Al were used to extract quercetin from *Ginkgo biloba* L. capsule samples as described in the materials and methods section. As noted previously, the extraction efficiency of MIS-Al was about 29%; it was then necessary to apply the same conditions for construction of the analytical curve during sample analysis. In this way, two analytical curves were processed with quercetin standard solutions varying from 5 to 40 mg/L (Fig. 5) in HPLC, as described in section 2.5.2. For comparison and verification of the losses during the SPE procedure, curve A was estimated without percolation and curve B was estimated after percolation of the standard solution through the cartridge containing MIS-Al (Fig. 5). Both curves showed good linear coefficients with adjustments of 0.9998 and

0.9992, for curves A and B, respectively. This result indicates a decrease in the analytical signal and lower sensitivity of Curve B, which was to be expected. The detection limit (LOD) and quantification (LOQ) were estimated as 1.19 mg L^{-1} and 1.64 mg L^{-1} for curve A, 1.72 mg L^{-1} and 2.67 mg L^{-1} for curve B. The LOD and LOQ estimation were based on the sensitivity of the method and average instrumental noise measured before and after the chromatographic peak, taking into account the lowest concentration in the standard solutions[40].

Insert Figure 5

Figures 6a and 6b show the chromatograms of the *Ginkgo biloba* L. capsules corresponding to 40 mg and 80 mg of flavonoids, respectively. It can be observed that the chromatograms for samples that were not passed through MIS-Al resulted in many peaks of unknown species and the two main components rutin (1) and quercetin (2), with probable co-elution with other species. After the solid phase extraction procedure with the MIS-Al cartridge, chromatograms showed peaks of lower intensity but with less interference of the sample matrix. Furthermore, better resolution for the quercetin peak was observed. These results prove that the proposed extraction method was efficient to separate quercetin from the overlapping interfering species in both samples, being an effective *clean-up* step before chromatographic analysis.

Insert Figure 6

Table 4 shows the concentration of quercetin obtained from the two samples of *Ginkgo biloba* L. extract when injected directly into the HPLC, analysed by curve A (Fig.5)

and after the passage though MIS-AL by curve B (Fig. 5). It was found that the values obtained by the two curves are different in a 95% confidence level, according to the Student's t-test. This was expected once that interfering species of the sample matrix were co-eluted with quercetin in direct analysis by HPLC. An official and selective standard procedure to determine quercetin in leaves samples was not applied considering that the most works uses HPLC as comparative results. Table 4 shows that the values obtained from both analytical curves for quercetin are very precise, which can be considered as a good result due to the complexity of the sample and heterogeneity of the MIS-Al particles.

Insert Table 4

Considering the specifications of the analysed capsules (*Ginkgo biloba* L. 40 mg and 80 mg), the total flavonoid content should be represented as 9.6 mg and 19.2 mg, respectively; however, the concentration of quercetin is not specified for either of the samples. Based on the proposed method, it was found that 0.25 ± 0.01 mg and 0.24 ± 0.01 mg quercetin/capsule corresponds to 2.6 % and 1.3 % of the 40 mg and 80 mg capsules, respectively. These values are consistent with those presented in the literature,[41] which found that *Ginkgo biloba* L. contained between 0.5 and 4.7 % quercetin. High variation between samples of the same extract is very common, since a wide range of concentrations can be found in natural samples.

In terms of the performance of the MIS-Al as SPE, the same efficiency in extraction was obtained after five times of reutilization of the cartridge with the same SPE procedure. Studies for increasing the reutilization times are in development. One of the concerns about the MIS-Al was the presence of aluminum ions after extensive utilization and washing. Studies involving analysis of MIS-Al by XRF/ EDX and TG after reuse have shown that loss

of Al ions during adsorption/desorption was only 4.75 x 10^{-5} mmol (1.12%) after five complete procedures, which means that selective adsorption of quercetin should still be possible after several uses of the SPE.

4. Conclusions

A new approach to molecular recognition based on metallic ion mediation is proposed. The presence of Al inside the recognition cavity increased selectivity to quercetin in comparison to its molecular analogue (rutin), due to the formation of specific bonds (chelation). Although MIS-Al was found to have an extraction efficiency of about 29%, the solid phase was efficiently applied to real samples. Characterization studies by FTIR exhibited similarity between all the spectra after the washing step, resulting in a matrix with the same structure without quercetin. Furthermore, the SEM photos showed relevant differences between the imprinted silica with and without aluminum ions. Analysis by FRX/EDX confirmed that the Al remained present in the silica matrix after extraction and reutilization. Lewis and BrØnsted acid sites were generated inside the silica imprinted cavity by Al addition, increasing the selectivity to quercetin. The synthesis of MIP-Al based in the SGP was successfully developed for the selective extraction of quercetin in *Ginkgo biloba* L. samples. The analytical results showed excellent resolution for the quercetin peak without overlap with interfering species. Another great advantage offered by MIS-Al was the possibility of reusing the cartridge until five times without reducing the efficiency of adsorption/desorption of quercetin.

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References

1. G. Cao, E.Sofic, R.L.Prior, *Free Radic Biol. Med*., 1997, **22**, 749-760.

2. E.B. Behling, M.C. Sendão, H.D.C. Francescato; L.M.G. Antunes.; M.L.P. Bianchi, *Alim. Nutr*., 2004, **15**, 285-292.

3. Y. F. Wang, X.H. Wang, Y.T. Zhu, *Nat.Prod.Res.Dev*., 2003, **15**, 171-173.

4. B. Ekberg, K. Mosbach, *Trends Biotechnol*., 1989, **7**, 92-96.

5. N. Russo, M. Toscano, N. Uccella, *J Agric. Food Chem.,* 2000, **48**, 3232-3237.

6. M. Biesaga, K. Pyrznska, *Crit. Ver. Anal. Chem*., 2009, ***39***, 95-107.

7. Y. Ji, Q. Xu, Y. Hu, Y. Heyden, *J. Chromatogr. A* 2005, **1066**, 97-104.

8. B.J. Diamond, S.C. Shiflett, N. Feiwel, R.J. Matheis, O. Noskin, J.A. Richards, N.E. Schoenberger, *Amh Phys Mad Rehabil*., 2000, **81**, 668-678.

9. Brazil. Ministry of Health. Resolution RE nº 89, de 20 de janeiro de 2004. "Product List of Simplified Registration".

10. S.G. Dmitrienko, V.A. Kudrinskaya, V.V. Apyari, *J. Anal. Chem*., 2012, **67**, 299-311.

11.F. Deng, S.W. Zito, *J. Chormatogr. A.*, 2003, **986**, 121-127.

12.C. F. Poole, *Trends Anal. Chem*., 2003, **22**, 362-373.

13. L. Michodjehoun-Mestres, J-M. Souquet, H. Fulcrand, C.Bouchut, M. Reynes, J. Brillouet, Food Chem., 2009, **112**, 851-857.

14.A. Kumar, A.K. Malik, D.K. Tewary, *Anal. Chim. Acta.*, 2009, **631**, 177-181.

15.E. Rijke, P. Out, W. M. A. Niessen, F. Ariese, C. Gooijer, U.A.T. Brinkman, *J. Chromatogr.* A., 2006, **1112,** 31-63.

16.V. Pakade, S. Lindahl, L. Chimuka, C. Turner, *J. Chromatogr A.*, 2012, **1230**, 15-23.

17.X. Song, J. Li, J. Wang, L. Chen, *Talanta*., 2009, **80**, 694-702.

18.A. Molinelli, R. Weiss, B. Mizaikoff, *J. Agric. Food Chem*., 2002, **50**, 1804-1808.

19. J.O. Mahony, K. Nolan, M.R. Smyth, Mizaikoff, B., A*nal. Chim. Acta*., 2005, **534**, 31-40.

20. R.A. Lorenzo, A.M. Carro, C. Alvarez-Lorenzo, A. Concheiro, *Int. J. Mol. Sci.*, 2011, **12**, 4327-4347.

21. S.A. Piletsky, H.S. Andersson, I.A. Nicholls, *J. Mol. Recognit*., 1998, **11**, 94-97.

22. L. Levi, S. Srebnik, *J. Phys. Chem. B.*, 2010, **114**, 107-114.

23. M. Yan, Molecularly imprinted materials: Science and Technology Marcel Dekker, New York, 2005.

24. F. X. Qiao, H. W. Sun, H. Y. Yan, K.H. Row, *Chromatogr.*, 2006, **64**, 625-634.

25. M.E. Diáz-Garcia, R.B. Laino, *Microchim. Acta*., 2005, **149**, 19-36.

26. P. Yang, W.D. Hou, H.D. Qiu; X. Liu, S.X. Jiang, *Chin. Chem. Lett.*, 2012, **23**, 615.

H. Tsai,. M. Syu, *Chem. Eng. J.*, 2011, **168,** 1369-1376.

27. K. Farrington, F. Regan, *Talanta.*, 2009, **78**, 653-659.

28. K.J.Shea, D.A.Loy, *Chem. Mater.*, 2001, **13**, 3306-3319.

29.T. Hsuan-Ang, S. Mei-Jywan, *Chem. Eng. J.*, 2011, **168**, 1369-1376.

30. L.T. Arenas, T.A.S. Aguirre, A. Langaro, Y. Gushikem, E.V Benvenutti, T.M.H. Costam, *Polymer.*, 2003, **44**, 5521-5525.

31.R.S. Hiratsuka, C.V Santilli, S.H. Pulcinelli, *Quím. Nova.*, 1995, **18**, 171-180..

32. A. Katz, M. E. Davis, *Macromol.*, 1999, **32,** 4113-4121.

33. G. Wulff, S. Schauhoff., *J. Org. Chem.*, 1991, **56**, 395-400.

34.Y. Zhang, Y. Zhang, *Braz. J. Chem. Eng*., 2008, **25**, 201-206.

35. T. Li, P. Chen, P. Nien, C. Lin, R. Vittal, T. Ling, K. Ho, *Anal. Chim. Acta* 2012, **711**, 83-90.

36. Y. Jin, Q. Xu, Y., Hu, Y. V. Heyden, *J. Chomatogr. A*, 2005, **1066**, 97-104.

37. M. Müller, G. Harvey, R. Prins, *Microporous Mesoporous Mater.*, 2000, **34**, 135-147.

38. IUPAC: commission on colloid and surface chemistry incluiding catalysis. *Pure Appl. Chem.*, 1994, **66**, 1739-1758.

39. F.R. Ribeiro, F. Alvarez, C. Henriques, F. Lemos, J.M. Lopes, M.F Ribeiro, *J. Mol. Catal. A: Chem.* 1995, **96**, 245-270.

40. M. Ribani, C.B.G. Bortolli, C.H. Collins, I.C.S.F. Jardim, L.F.C. Melo, *Quím. Nova*, 2004, **27**, 771-780.

41. F. Deng, S.W. Zito, *J. Chomatogr. A*, 2003, **986**, 121-127.

Figure Captions

Figure 1. Chemical structures of quercetin (a) and rutin (b).

Figure 2. Mechanism proposal of MIS-Al synthesis. Steps of hydrolysis and polycondensation reaction between APTMS/Quercetin/TEOS in solution aqueous of Al, followed by removal of quercetin and adsorption/extraction of quercetin.

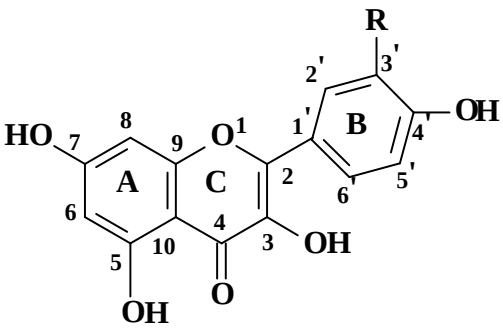
Figure 3. Scanning electron micrographs of MIS (a) and MIS-Al (b) at magnification of 100 times, MIS (c) and MIS-Al (d) at magnification of 10.000 times.

Figure 4. FTIR spectra of the imprinted and non-imprinted silica.

Figure.5. Analytical curve of quercetin before (curve A) and after (curve B) pass through MIS-Al SPE cartridge.

Figure 6. Chromatograms of *Ginkgo biloba* L. of 40 mg (a) and 80 mg (b) before (—) and after (—) pass through the SPE cartridge with MIS-Al. Identification of peaks (1) rutin and (2) quercetin.

a



b

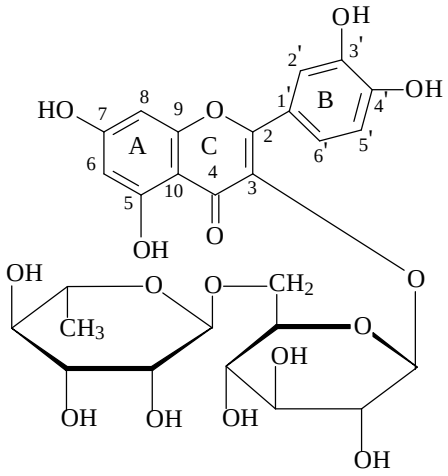


Figure 1

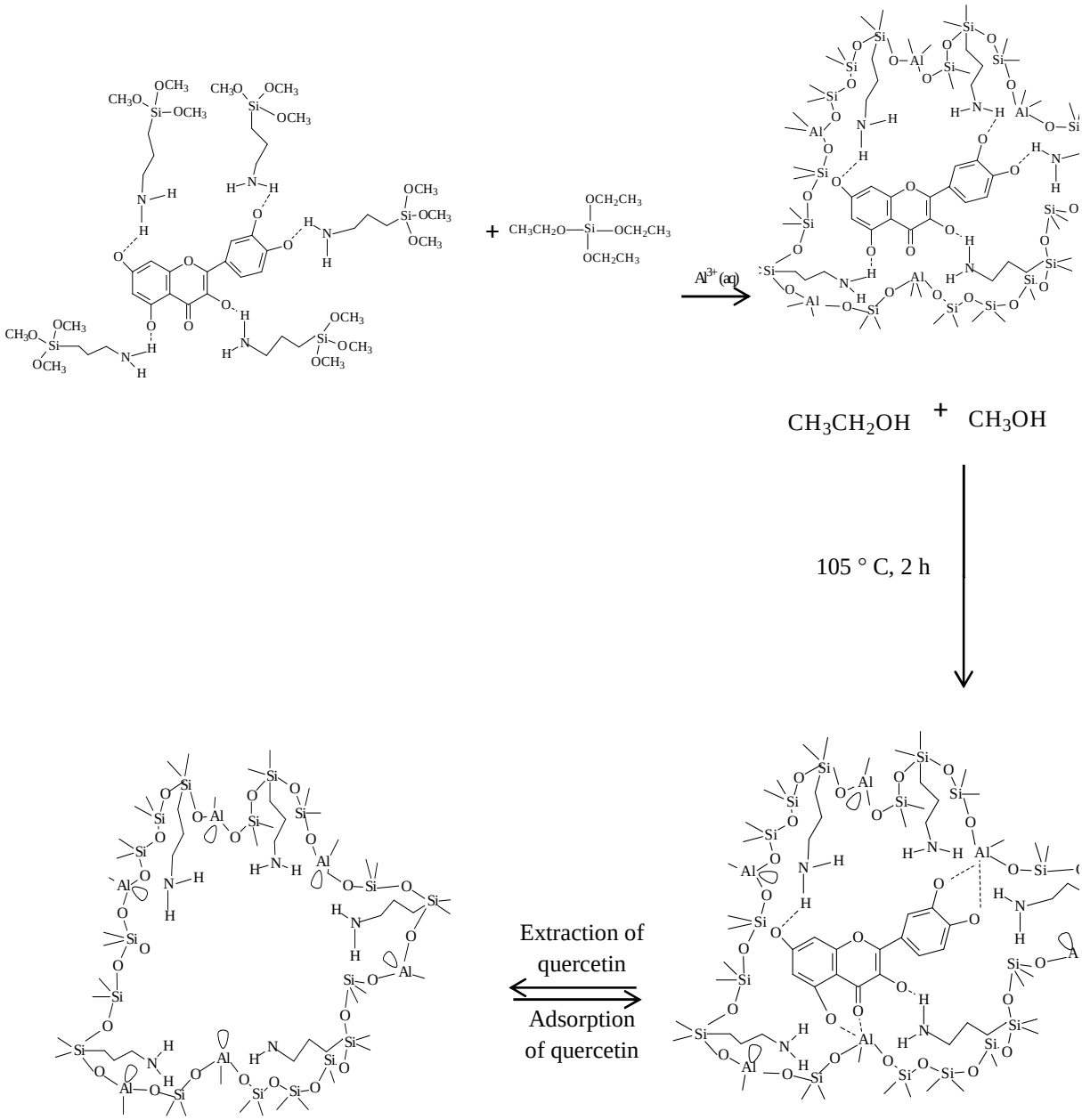


Figure 2

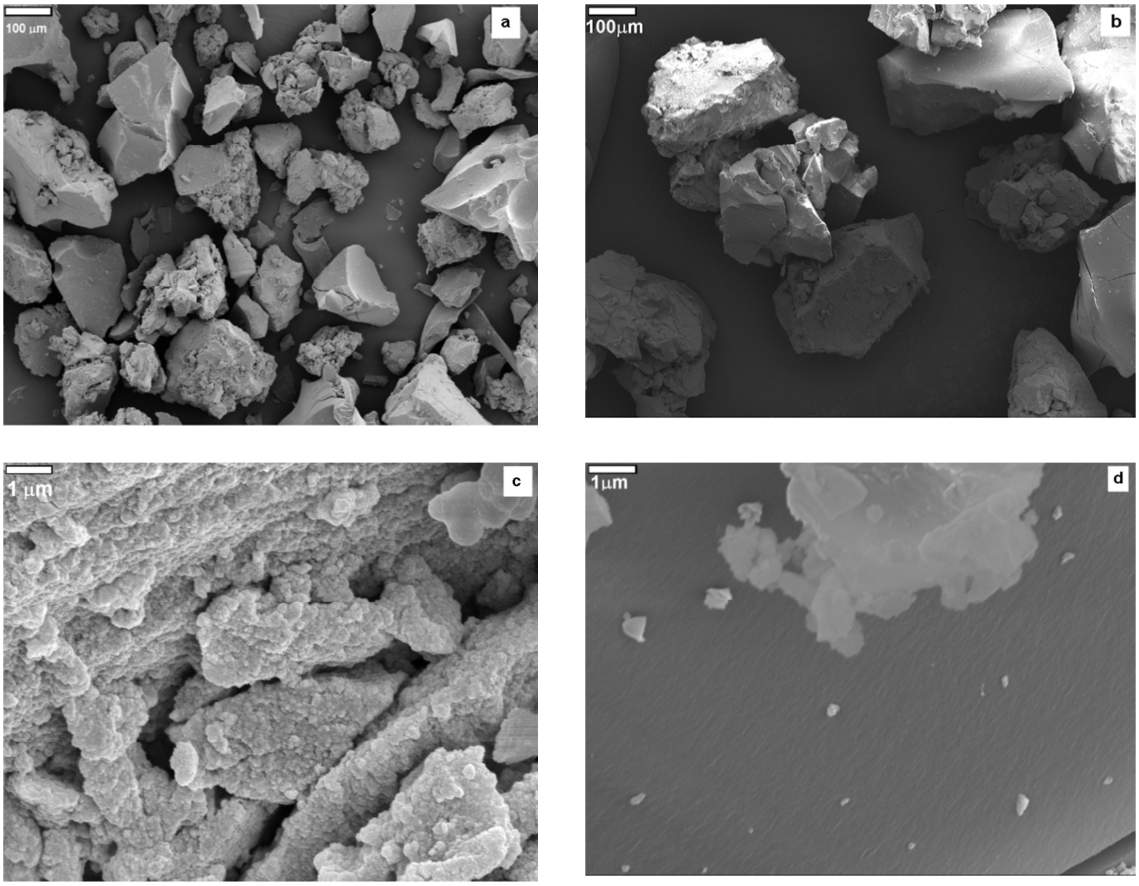


Figure 3

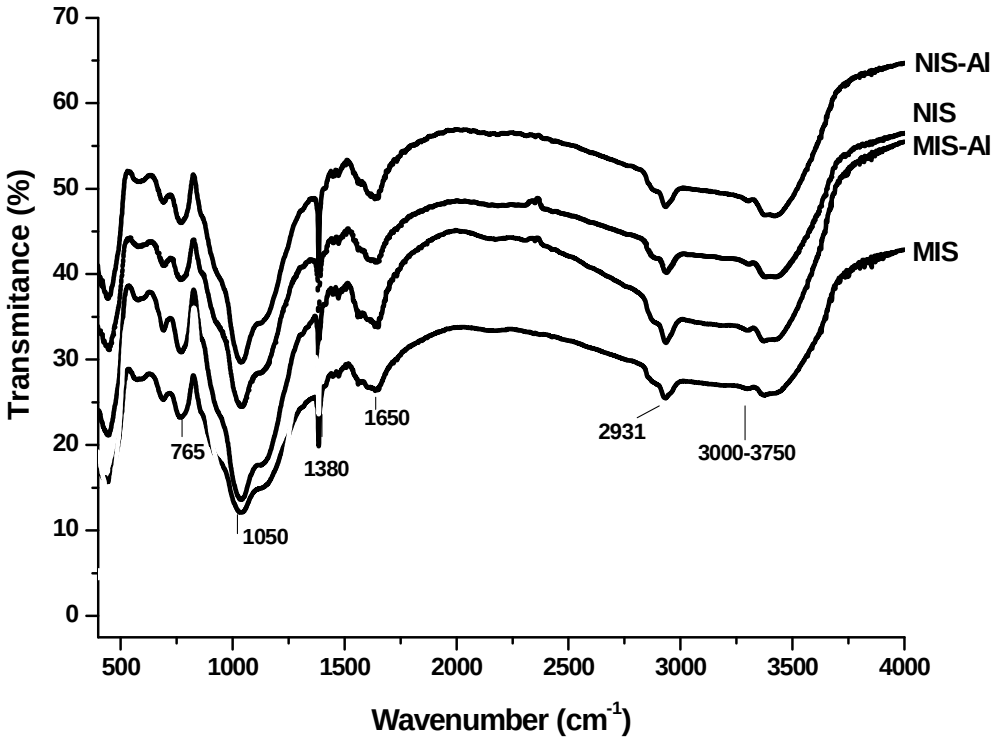


Figure 4.

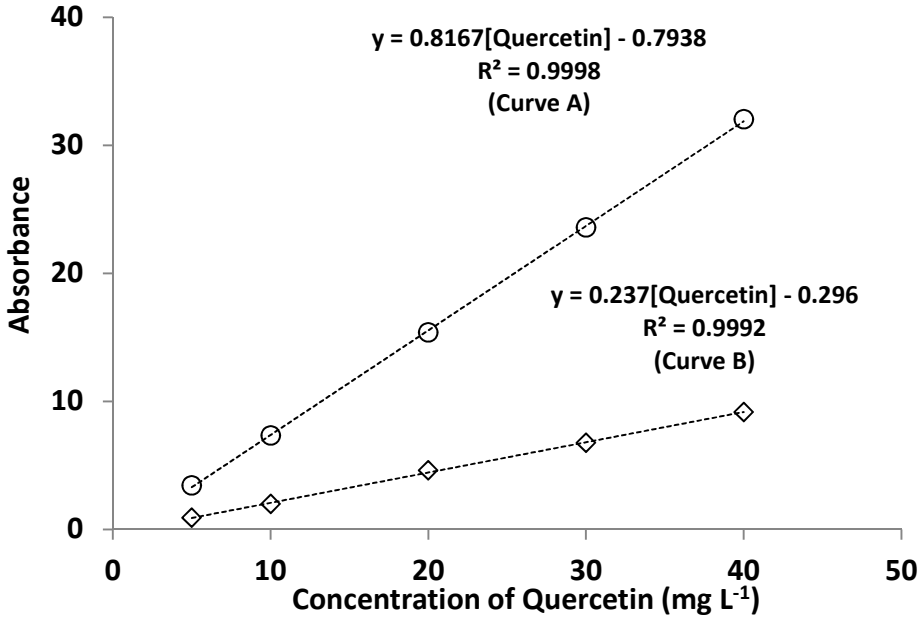
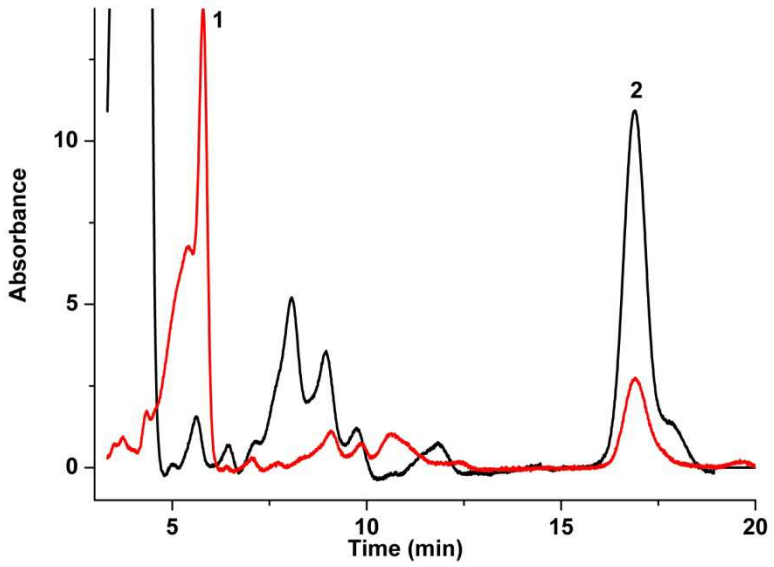


Figure 5

(a)



(b)

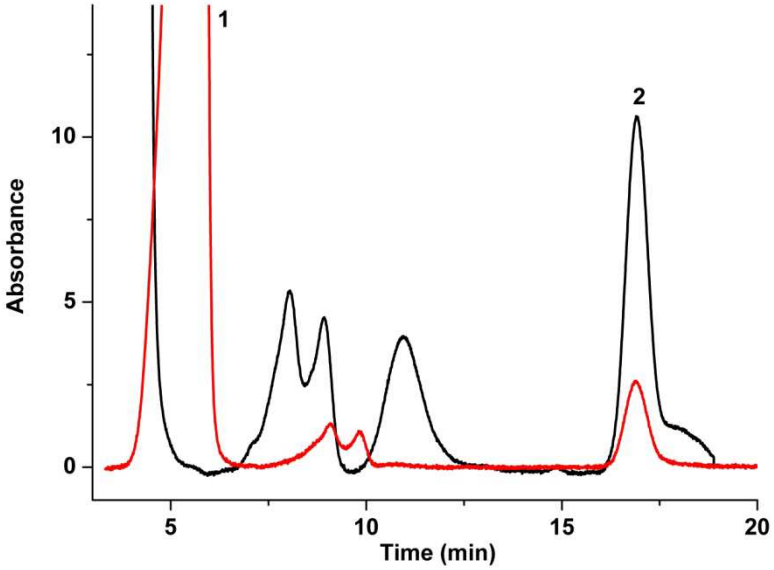


Figure 6

Table 1. Results of porosimetry analysis of the imprinted and non-imprinted silica.

Synthesis	Surface area (m^2/g)	Volume of pore (cm^3/g)	Pore diameter (nm)
MIS	49.266	0.044	1.675
NIS	42.142	0.037	1.532
MIS-Al	41.343	0.035	1.679
NIS-Al	32.816	0.028	1.677

Table 2. Results of adsorption capacity of imprinted and non-imprinted silica in ethanol and methanol:water (60:40, v/v) to quercetin extraction.

Synthesis	C_o (mg L^{-1})	Cs (mg L^{-1})		Q (µg g^{-1})		IF	
		Ethanol:water	Methanol	Ethanol:water	Methanol	Ethanol:water	Methanol
MIS	2.5	1.86 ± 0.14	2.06 ± 0.13	159.7 ± 35.0	109.41 ± 33.44	1.27	0.74
NIS		2.00 ± 0.03	1.91 ± 0.19	125.7 ± 7.4	148.70 ± 48.15		
MIS-Al	2.5	1.52 ± 0.01	1.93 ± 0.10	248.5 ± 3.5	142.10 ± 24.68	2.06	1.08
NIS-Al		2.02 ± 0.16	1.97 ± 0.19	120.2 ± 38.9	132.10 ± 48.63		

$Q = (C_o - C_s) \times 1000V/m$, C_0 – represents the concentration of the compound in the initial solution and C_s is the average of concentrations in the final solutions; V is the volume of the solution (5.0 mL); m is the weight of the adsorbent (20.0 mg); Q is the adsorption capacity of the imprinted silica. Co and Cs are concentrations before and after adsorbed by imprinted silica, respectively. $IF = Q_{MIP}/Q_{NIP}$, IF means the imprinting factor of MIS or MIS-Al.

Table 3. Evaluation of the selectivity of imprinted and non-imprinted silica by HPLC.

	C_o (mg/L)		C_s (mg/L)		Q_{MIS-Al}	Q_{NIS-Al}	IF
	MIS-Al	NIS-Al	MIS-Al	NIS-Al	(μg g^{-1})	(μg g^{-1})	
Quercetin	30		23.8 ± 1.7	26.6 ± 2.9	155.5 ± 25.0	84.9 ± 59.1	1.8
Rutin			28.1 ± 1.0	26.2 ± 0.3	47.2 ± 16.2	94.9 ± 8.3	0.5

$Q = (C_o - C_s) \times 1000V/m$, C_0 – represents the concentration of the compound in the initial solution and C_s is the average of concentrations in the final solutions; V is the volume of the solution (5.0 mL); m is the weight of the adsorbent (200.0 mg); Q is the adsorption capacity of the imprinted silica by quercetin and rutin. Co and Cs are concentrations before and after adsorbed by MIS-Al or NIS-Al, respectively. $IF = Q_{MIP}/Q_{NIP}$, IF means the imprinting factor of MIS-Al.

Table 4. Results of *Ginkgo biloba* L. analysis estimated by direct injection in HPLC (Curve A) and after passage through MIS-Al (Curve B) expressed as quercetin concentration (mg L^{-1}).

***Ginkgo biloba* L.**	**Direct injection by HPLC**	**After MIS-Al**
40 mg	15.0 ± 0.2	12.3 ± 0.4
80 mg	14.4 ± 0.2	12.1 ± 0.4