

**Bi-functionalized mesostructured silicas as reversed-phase/strong anion-exchange sorbents. Application to extraction of polyphenols prior to their quantitation by UHPLC with ion-trap mass spectrometry detection**

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**Microchimica Acta (2019) 186: 164**

**<https://doi.org/10.1007/s00604-019-3267-2>**

## ABSTRACT

Hybrid mesostructured silicas with wormhole-like pore structure were synthesized and bi-functionalized with n-octyl (C8) and quaternary ammonium (NR<sub>4</sub><sup>+</sup>) groups to obtain new sorbent materials for dispersive solid-phase extraction (dSPE) of polyphenols. Due to their nature of being both a reversed-phase and a strong anion-exchanger, the materials display mixed-mode retention mechanism. During the synthesis, the functionalization conditions were varied to obtain materials with different functionalization degree. The resulting materials (denoted as HMS-RPC8-SAX-1, HMS-RPC8-SAX-2 and HMS-RPC8-SAX-3) show high surface area, wormhole-like framework and controlled pore size. They were evaluated for multicomponent extraction of 22 polyphenols, including phenolic acids, flavonoids and stilbenes, from spiked juice samples. The sample extracts were analyzed by ultra-high performance liquid chromatography coupled to ion-trap tandem mass spectrometry. The adsorption capability, the amount of sorbent, the eluent and the elution volume were optimized. Best performance was achieved by using HMS-RPC8-SAX-2, which is the material with the highest fraction of NR<sub>4</sub><sup>+</sup> groups. This material has a large extraction capability and provides high recovery values of the target analytes (70-101%) as a result of its hydrophobic and anion-exchange interactions. The detection limits for the polyphenols range from 1 to 560 ng mL<sup>-1</sup> of juice.

**Keywords:** Dispersive solid-phase extraction, Mixed-mode, Flavonoids, Phenolic acids, Stilbenes, Juice, Porous sorbents, N-octyl, Quaternary ammonium, Bioactive compounds, Sample preparation

## Introduction

Current trends in analytical chemistry involve moving towards quick, cost-effective, sensitive and environmentally friendly multicomponent analytical procedures, which can provide the determination of a large number of compounds in a single run [1]. Nevertheless, meeting these requirements in food analysis is a challenging task, since food samples are highly complex matrices, which contain many components with different chemical properties. Consequently, despite the development and application of efficient powerful analytical instrumentation, food samples, generally, can not be directly analyzed because of the presence of high levels of matrix interferences that may negatively interfere in the extraction and determination of analytes. In this sense, in food analysis, sample preparation is a crucial step to minimize the negative effect of matrix interferences and to achieve good analytical results. Sample preparation includes both extraction and clean-up steps, so its main purpose is to isolate and pre-concentrate target compounds from the matrices to enhance their separation and detection [2].

Regarding sample preparation techniques, solid-phase extraction (SPE) is the most common and widely used due to its simplicity [3]. However, it requires uniform packaging of the sorbents into the SPE cartridges to avoid mass diffusion problems, which can lead to low extraction efficiency and time-consuming trials. To overcome these problems, new SPE configurations based on dispersion, such as dispersive solid-phase extraction (dSPE) or matrix solid-phase dispersion (MSPD), have emerged as alternative sample preparation techniques to SPE. In these dispersive techniques, the sorbent material is directly added into the sample solution, so interaction between the sorbent and the analytes is improved and, consequently, the fraction of sorbents and solvents used can be reduced, as well as time and labor [4].

In this context, many types of sorbents can be used in these sample preparation techniques, such as polymeric, silica-based, resin, graphene-based and carbon-based materials. Nevertheless, polymeric and silica-based materials are, generally, the most broadly used. Polymeric materials provide wide pH tolerance, but they present swelling effect in contact with organic solvents. On the other hand, silica-based sorbents have limited pH tolerance, but they are rigid, easy to modify and present good mechanical strength to shrinking and swelling, hence, they are more desirable to be used [5]. Nevertheless, one of

the challenges when developing a multicomponent extraction method is to select a suitable sorbent that allows extracting simultaneously a wide range of compounds of different nature. One way to achieve this is by using sorbents with mixed-mode interaction mechanism. Mixed-mode sorbents are dual-phase materials specifically designed to interact simultaneously with ionic and neutral species by combining both effective reversed-phase and ion exchange interaction modes [6]. The existence of a multiple interaction mechanism provides better selectivity for the enrichment of analytes, since the ion-exchange capability allows to achieve high selectivity towards basic or acid compounds by using a suitable pH combination in both extraction and elution steps. Therefore, mixed-mode sorbents can be used to improve sample preparation, since they can be helpful for both reducing matrix interferences and pre-concentrating target analytes. For instance, some authors have used commercial Oasis MAX sorbents, which are mixed-mode polymeric sorbents with anion-exchange groups (charged quaternary amine groups) and reversed-phase interactions, for SPE of polyphenols from food samples [7-10]. Polyphenols are a heterogeneous group of bioactive compounds found in fruits and vegetables which provide multiple health benefits [11]. Thus, the extraction and purification of these compounds is of high research interest due to their beneficial properties. When mixed-mode sorbents with anion-exchange capacity are used for extraction of these compounds under suitable pH conditions, the acid polyphenols, such as phenolic acids, can interact with the charged quaternary amine groups of the sorbent, while neutral and basic polyphenols, such as flavonoids, interact with the reversed-phase chemistry of the material. On the other hand, regarding silica-based materials, the development and application of new advanced sorbent materials based on functionalized mesostructured silicas has achieved a sharp increase in food sample preparation owing to their advantageous properties, such as high surface area, well-defined pore size distribution, large pore volume, modifiable surface properties and high chemical, mechanical and thermal stability, allowing their use in a wide pH range [12]. For these reasons, several functionalized mesostructured silicas have been explored as sorbents for the analysis of food samples [13-25]. Mesostructured silicas with mixed-mode retention mechanism can also be prepared by their bifunctionalization with different ligands. For instance, Li et al. [26] prepared a reversed-phase/anion-exchange mixed-mode dendrimer-sorbent by functionalization of a KIT-6 (Korea Advanced Institute of Science and Technology No. 6) mesostructured silica type, which was applied as SPE sorbent for the

extraction of 3 acid drugs from environmental and biological samples. As well, in a previous work of our research group, we developed novel reversed-phase/cation-exchange mixed-mode sorbents using a SBA-15 (Santa Barbara Amorphous No. 15) mesostructured silica type for the multi-residue SPE of 26 veterinary drugs from meat samples [14]. However, up to date, mixed-mode mesostructured silicas have not been evaluated as sorbents for extraction of polyphenols. Moreover, there are other types of mesostructured silicas which have not yet been bi-functionalized with this purpose, such as the HMS (Hexagonal Mesoporous Silica) type, which presents some advantages over other silicas, since its synthesis is more cost-effective and environmentally friendly, as well as it provides mesostructures with thicker pore walls that improve its stability [27]. In one work of our research group, HMS showed under same conditions better extraction efficiency than other mesostructured silicas, such as SBA-15, MCM-41 (Mobil Composition of Matter No. 41), ethane-PMO (Periodic Mesoporous Organosilica) and MSU-2 (Michigan State University No. 2), for extraction of veterinary drugs from meat samples [15]. This fact was attributed to its small particle size and spherical morphology, which provide better access to the wormhole-like framework confined mesopores in adsorption processes, so promotes better retention of analytes.

For these reasons, the aim of this work was to prepare novel mixed-mode sorbents using a wormhole-pore HMS type mesostructured silica and applied them as dSPE sorbents for the multicomponent extraction of polyphenols from food samples. For this purpose, HMS mesostructured silica was bi-functionalized with n-octyl (C8) chains and quaternary ammonium ( $\text{NR}_4^+$ ) groups. The resulting hybrid silicas were evaluated and compared as dSPE sorbents with reversed-phase/strong anion-exchange mixed-mode retention mechanism for the multicomponent extraction of 22 polyphenols belonging to different chemical families (phenolic acids, flavonoids and stilbenes) from juice samples, combined with their analysis by ultra-high performance liquid chromatography coupled to ion-trap tandem mass spectrometry (UHPLC-IT-MS/MS). To best of our knowledge, this is the first time that bi-functionalized mesostructured silicas with wormhole-pore structure have been prepared and applied as reversed-phase/strong anion-exchange mixed-mode sorbents in the preparation of food samples. Moreover, mixed-mode cartridges with both reversed-phase and ion-exchange retention modes, have not been reported so far for the extraction of phenolic compounds in food samples by dSPE.

Conceivably, they are a promising sorbent for sample preparation due to their improved selectivity and capacity.

## Materials and Methods

### Chemicals, reagents and standard solutions

Tetraethylorthosilicate 98% (TEOS,  $M = 208.33 \text{ g mol}^{-1}$ ,  $d = 0.934 \text{ g mL}^{-1}$ ), dodecylamine 98% (DDA,  $M = 185.36 \text{ g mol}^{-1}$ ), chloro(dimethyl)octylsilane 97% ( $M = 206.83 \text{ g mol}^{-1}$ ,  $d = 0.873 \text{ g mL}^{-1}$ ), 3-chloropropyltriethoxysilane 95% (3CPTS,  $M = 240.8 \text{ g mol}^{-1}$ ,  $d = 1.00 \text{ g mL}^{-1}$ ), N,N-dimethylformamide 99% ( $M = 73.09 \text{ g mol}^{-1}$ ,  $d = 0.944 \text{ g mol}^{-1}$ ) toluene and diethyl ether were purchased from Sigma-Aldrich (St. Louis, MO, USA, [www.sigmaaldrich.com](http://www.sigmaaldrich.com)). N,N-dimethyl-ethylamine 99% ( $\text{CH}_3\text{CH}_2\text{N}(\text{CH}_3)_2$ ,  $M = 73.14 \text{ g mol}^{-1}$ ,  $d = 0.673 \text{ g mol}^{-1}$ ) was from Merck (Darmstadt, Germany, [www.merckgroup.com](http://www.merckgroup.com)). Methanol (MeOH) LC-MS grade, hydrochloric acid 35% (HCl), ethanol and ammonia solution 32% were obtained from Scharlab (Barcelona, Spain, [www.scharlab.com](http://www.scharlab.com)). Formic acid and ammonium acetate LC-MS grade were from Fluka (Busch, Switzerland, [www.lab-honeywell.com/products/brands/fluka](http://www.lab-honeywell.com/products/brands/fluka)). Water (resistivity  $18.2 \text{ M}\Omega \text{ cm}$ ) was obtained from a Millipore Milli-Q-System (Billerica, MA, USA, [www.merckmillipore.com](http://www.merckmillipore.com)). Individual standard solutions of the target analytes were all high purity grade ( $\geq 90\%$ ) and were purchased from Acros Organics (Geel, Belgium, [www.acros.com/](http://www.acros.com/)) and Sigma-Aldrich (St. Louis, MO, USA, [www.sigmaaldrich.com](http://www.sigmaaldrich.com)).

### Synthesis of mesostructured silicas

Mesostructured silica HMS type with wormhole framework structure was prepared according to Pérez-Quintanilla et al. [28] with slight modifications. Briefly, 30 g of DDA were dissolved in 388.8 mL of Milli-Q water and 227.4 mL of ethanol. The solution was stirred until homogenization and, subsequently, 124.8 g of TEOS were added drop by drop. The mixture was stirred for 18 h, yielding a thick white suspension which was filtered and, afterwards, dried at  $80 \text{ }^\circ\text{C}$  for 1 h. The resultant solid was heated at reflux in ethanol with a Soxhlet for 8 h to remove the remaining amine. Finally, it was calcinated at  $550 \text{ }^\circ\text{C}$  for 18 h to remove the residual surfactant.

HMS mesostructured silica was bi-functionalized with hydrophobic (C8) and quaternary ammonium ( $\text{NR}_4^+$ ) groups, obtaining materials with different properties and different functionalization degree ( $L_0$ ), which were denoted HMS-RPC8-SAX-1, HMS-RPC8-SAX-2 and HMS-RPC8-SAX-3. HMS-RPC8-SAX-1 was prepared using 8 g of the HMS silica previously obtained and adding 60 mL of toluene with a mixture of 1.56 mL (6.4 mmol) of chloro(dimethyl)octylsilane and 1.62 mL (6.4 mmol) of 3CPTS with vigorous stirring under nitrogen atmosphere. The reaction was allowed to continue for 24 h. The material was recovered by filtration and was washed successively with two fractions of 50 mL of toluene, ethanol and diethyl ether. Then, it was dried at 80 °C overnight. Afterwards, the solid was placed in a flask and 60 mL of N,N-dimethyl-formamide and an excess of 5 mL (45.5 mmol) of N,N-dimethyl-ethylamine were added under nitrogen atmosphere. The reaction was performed at 80 °C for 24 h. The resulting material was filtered and washed successively with two fractions of 50 mL of ethanol and diethyl ether. Finally, it was dried at 80 °C overnight. In order to obtain materials with different  $L_0$ , HMS-RPC8-SAX-2 and HMS-RPC8-SAX-3 were prepared in the same way, but using, 1.56 mL (6.4 mmol) of chloro(dimethyl)octylsilane and 3.24 mL (12.8 mmol) of 3CPTS for HMS-RPC8-SAX-2 and 3.12 mL (12.8 mmol) of chloro(dimethyl)octylsilane and 1.62 mL (6.4 mmol) of 3CPTS for HMS-RPC8-SAX-3. Fig. 1a depicts the bi-functionalization reaction procedure.

### **Characterization of mesostructured silicas**

All the mesostructured silicas were characterized. Scanning electron micrographs (SEM) and morphological analysis were carried out on a XL30 ESEM Philips with an energy-dispersive spectrometry system (EDS). The samples were treated with a sputtering method with the following parameters: sputter time 100 s, sputter current 30 mA, and film thickness 20 nm using sputter coater BAL-TEC SCD 005. Conventional transmission electron microscopy (TEM) was carried out on a TECNAI 20 Philips microscope operating at 200 kV, with a resolution of 0.27 nm and  $\pm 70^\circ$  of sample inclination, using a BeO sample holder. Nitrogen gas adsorption-desorption isotherms were performed using a Micromeritics ASAP 2020 analyzer.  $^{13}\text{C}$  and  $^{29}\text{Si}$  magic-angle spinning solid-state nuclear magnetic resonance (MAS-NMR) spectra were recorded on a Bruker Avance III/HD spectrum of 9.4 Tesla with a  $^1\text{H}$  proton resonance frequency of 400 MHz using a probe of 4 mm at room temperature.

The  $^{13}\text{C}$  MAS-NMR spectra was recorded under the following conditions, sequence: cross-polarization (CP), 100 MHz resonance frequency, 4096 transients, contact time 3 s, pulse delay 3 s, spinning speed 8 KHz. For the  $^{29}\text{Si}$  MAS-NMR spectra, sequence: high-power decoupling pulse (HPDEC), 79 MHz resonance frequency, 1000 transients, pulse delay 60 s, spinning speed 10 KHz. A Mettler Toledo Star System TGA/DSC 1 thermogravimetric analyser was used to study the thermal stability of the materials, using a 70  $\mu\text{L}$  platinum crucible and synthetic air atmosphere. The temperature increased from 25  $^{\circ}\text{C}$  to 800  $^{\circ}\text{C}$  at a speed of 10  $^{\circ}\text{C}$  per minute. Elemental analysis (%H, %C and %N) was performed using a microanalyser model Flash 2000 Thermo Fisher Scientific Inc. The elemental analysis data was used to estimate the  $L_0$  of the different materials synthesized.  $L_0$  indicates the amount of ligand attached to the silica surface (mmol of ligand attached per g of material). The amount of  $\text{NR}_4^+$  groups was estimated using the nitrogen percentage, according to the following formula:

$$\text{Functionalization degree of } \text{NH}_4^+ \text{ groups } (L_0 \text{ NH}_4^+) = \frac{\%N \times 10}{14}$$

Then, by stoichiometry the amount of carbon related to the  $\text{NR}_4^+$  ligand was calculated (assuming an average of 10 carbon atoms in each amine group attached to the silica surface) and it was subtracted from the total carbon percentage measured. This way, the difference obtained corresponds to the carbon that comes from the C8 ligand. Thus, dividing the result between 10 (since there are 10 carbon atoms in every C8 group), it is possible to estimate the amount of C8 groups joined to the silica surface:

$$\text{Functionalization degree of C8 groups } (L_0 \text{ C8}) = \frac{\left[ \left( \frac{\%C \times 10}{12} \right) - 10 \times \left( \frac{\%N \times 10}{14} \right) \right]}{10}$$

### **Preparation of juice samples**

A commercial juice made of fruits and vegetables treated by high pressure processing (HPP) technique was purchased from a local supermarket in Madrid (Spain) and stored at 4  $^{\circ}\text{C}$  until analysis. There are no standard methods or certified reference materials available for the analysis of polyphenols which can be used to compare the extraction methods developed in this work. For this reason, the optimization processes were carried out by spiking the sample with the standard solutions at a known



concentration level ( $0.02 - 2 \mu\text{g mL}^{-1}$ ) according to the system and method sensitivity. The spiked concentration and the limit of detection of each analyte are listed in Table S1. In this sense, in each set of experiments 3 samples were spiked with the working standard solution containing the target analytes, allowing a 30-min period for equilibration prior to the sample extraction procedure, while other sample (denoted simulated sample) was extracted in the same way than the other, but it was spiked with the target analytes at the end of the sample treatment process. This way, recovery values were calculated by comparing the areas of the spiked samples with the areas obtained for the simulated sample. All experiments were performed in triplicate.

### **Sample extraction procedure**

Sample treatment involved first a solvent extraction procedure followed by dSPE. First, 10 mL of juice were centrifuged at 6000 rpm for 10 min to separate the aqueous phase which was transferred to a polypropylene tube. Afterwards, the solid residue (0.5 g) was extracted in two extraction cycles with 5 mL of MeOH (pH 2), vortexed for 1 min and centrifuged at 6000 rpm for 10 min. The resultant organic supernatants were collected in the same tube containing the aqueous phase, obtaining a final sample extract of 20 mL which was subsequently subjected to dSPE. In order to achieve the mixed-mode interaction between the sorbent and the analytes, the sample extract was basified to pH 9 with ammonia solution prior to extraction.

For evaluation of bi-functionalized HMS mesostructured silicas as sorbents, different extraction parameters were tested, such as type of sorbent, pH, extraction technique, sorbent amount, elution solvent and elution volume. The optimal extraction conditions were achieved using HMS-RPC8-SAX-2 as dSPE sorbent as follows: 50 mg of HMS-RPC8-SAX-2 were first conditioned with 1 mL of MeOH:water (50:50, v/v pH 9) and stirred for 10 min at 300 rpm to enable dispersion of the material. Subsequently, 5 mL of sample extract (pH 9) were added. The suspension was stirred for 20 min at 300 rpm. Then, the material concentrated with the analytes was recovered by filtration using a  $0.45 \mu\text{m}$  nylon filter. Elution of analytes was performed by passing  $3 \times 2$  mL of MeOH:water (95:5, v/v pH 1) through the nylon filter. The eluate was then evaporated to dryness and re-dissolved with 500  $\mu\text{L}$  of MeOH for subsequent analysis in the chromatographic system.

## **Chromatographic UHPLC-IT-MS/MS analysis**

Separation was achieved using a UHPLC system (Dionex UltiMate 3000, Thermo Scientific, MA, USA) coupled to an ion-trap tandem mass spectrometer detector (Bruker) and an ACE Excel 2 C18-PFP column (100 mm x 2.1 mm, 2  $\mu\text{m}$  particle size, ACE, UK) at 30  $^{\circ}\text{C}$ . The injection volume was 10  $\mu\text{L}$  and the flow rate 0.25  $\text{mL min}^{-1}$ . A gradient elution was carried out by combining phase A (MeOH) and phase B (Milli-Q water), containing both phases 0.1% formic acid and 2 mM ammonium acetate, in the following way: initial composition of phase A 20% increased linearly to 100% in first 9 min, then returned to initial conditions in 2 min and was equilibrated for 1 min prior to next injection, yielding a total analysis time of 12 min. Mass spectrometry (MS) acquisition was performed using electrospray ionization interface (ESI) operating in negative ion mode. Capillary voltage was held at -4500 V and the end plate offset at -500 V. The nebulizer was set at 20 psi, the dry gas at 10  $\text{L min}^{-1}$  and the dry temperature at 200  $^{\circ}\text{C}$ . ESI source parameters were optimized by direct infusion of each analyte in pure standard solutions (5  $\mu\text{g mL}^{-1}$ ) at a flow rate of 4  $\mu\text{g min}^{-1}$ . Different parameters were manually optimized to obtain the maximum total ion current (TIC) signal in negative operation mode within the mass range of 70-700  $m/z$ . Fig. S1 shows the extracted ion chromatograms and mass spectra of each analyte in the spiked juice sample extract. Table S1 lists pKa values, retention time, mass spectrum parameters and product ions of the target analytes under the chromatographic conditions described (see Electronic Supplementary Material).

## **Results and discussion**

### **Characterization of mesostructured silicas**

HMS mesostructured silica was characterized before and after bi-functionalization. SEM micrographs of HMS reveal its typical spherical morphology, with mean diameters between 0.5-1  $\mu\text{m}$  (Fig. S2a). TEM micrographs of HMS show irregularly mesopores throughout the material with relatively uniform pore sizes, what it is known as wormhole-like pore arrangement (Fig. S2b). SEM and TEM micrographs of bi-functionalized HMS silicas confirm the maintenance of the morphology, particle size and pore distribution of the materials after functionalization of HMS. Therefore, these

results suggest that the modification process of HMS does not affect the structural properties of the materials. In the same way, the nitrogen adsorption-desorption isotherms of all the silicas synthesized are of type IV, according to the I.U.P.A.C. classification, with a narrow hysteresis loop representative of mesoporous materials. As well, it is observed that the capillary condensation/evaporation step of all the materials is not very sharp, thus, the pore size is not too uniform with more than one maximum, providing bimodal pore size distribution (Fig. S3). Regarding the textural properties (Table 1), all materials show high Brunauer, Emmett and Teller specific surface area ( $S_{\text{BET}}$ ), with pore volumes and average Barret, Joyner and Halenda (BJH) pore diameters typical of surfactant-assembled mesostructures [29]. However, surface modification of HMS significantly decreases the  $S_{\text{BET}}$  and pore volume, and to a lesser extent, the pore diameter of the materials (Table 1). These results suggest that the organic moieties (C8 and  $\text{NR}_4^+$  groups) are both grafted on the external surface and inside the mesopores of the mesostructured silicas. To verify the correct attachment of the organic moieties, elemental analysis was performed in all the mesostructured silicas which were prepared. In this sense, the  $L_0$  ( $\text{mmol g}^{-1}$ ) of C8 and  $\text{NR}_4^+$  groups were estimated from the percentages of carbon and nitrogen measured with elemental analysis (Table 1), as it has been previously explained in the Materials and Methods section. Results reveal differences in the  $L_0$  of the different materials (Table 1). The content of hydrophobic groups in the bi-functionalized silicas HMS-RPC8-SAX-1, HMS-RPC8-SAX-2 and HMS-RPC8-SAX-3 is estimated in 0.53, 0.46 and 0.74  $\text{mmol g}^{-1}$  of C8, respectively (Table 1). On the other hand, the amount of  $\text{NR}_4^+$  groups of HMS-RPC8-SAX-1, HMS-RPC8-SAX-2 and HMS-RPC8-SAX-3 is estimated in 0.33, 0.37 and 0.17  $\text{mmol g}^{-1}$ , respectively (Table 1). As it can be seen, adding the double of C8 in HMS-RPC8-SAX-3 significantly increases the amount of C8 in comparison with the other silicas. On the other hand, when the amount of 3CPTS added during bi-functionalization is higher than the amount of C8, the  $\text{NR}_4^+$  loading slightly increases, while the C8 loading slightly decreases.

## **Evaluation of bi-functionalized HMS**

### ***Type of sorbent and pH effect***

Bi-functionalized HMS mesostructured silicas were prepared with the aim of developing mixed-mode sorbents that allow extracting simultaneously polyphenols of different nature belonging to

different chemical families (phenolic acids, flavonoids and stilbenes). Phenolic acids have pKa values ranging between 3-5, while flavonoids and stilbenes are basic compounds with pKa values in the range of 8-10, except rutin, myricetin, quercetin 3- $\beta$ -D-glucoside and quercetin, which are neutral compounds (Table S1). In this sense, HMS mesostructured silica was bi-functionalized with C8 and NR<sub>4</sub><sup>+</sup> groups in order to obtain novel sorbents with different *L*<sub>0</sub> and a reversed-phase/strong anion-exchange mixed-mode retention mechanism.

When using mixed-mode sorbents is important to control pH of the whole procedure because, depending of the stage, pH may enable retention or elution of analytes. In this sense, analytes with the appropriate charge can interact with the ion-exchange phase of the material, retaining them during the extraction process. Then, pH of the elution solvent can be adjusted to reduce the charge on the analytes and release them from the ion-exchange groups of the silica. Since, analytes may be also retained by the reversed-phase of the sorbent, the organic percentage of the elution solvent should be adjusted to achieve selective elution. This way, in the case of the reversed-phase/strong anion-exchange mixed-mode HMS silicas, under basic extraction conditions, acidic compounds may experience a reversed-phase interaction with the C8 groups and an anion-exchange interaction with the NR<sub>4</sub><sup>+</sup> groups of the material, while basic and neutral compounds only interact with the hydrophobic C8 chains of the silica (Fig. 1b). According to this, bi-functionalized HMS mesostructured silicas were first evaluated and compared as dSPE sorbents using 50 mg of them and performing extraction of analytes from juice samples under basic conditions (pH 9), in order to achieve the anion-exchange interaction between the acidic compounds and the NR<sub>4</sub><sup>+</sup> groups of the material. Afterwards, analytes retained in the material were eluted with 6 mL of a combination of MeOH:water (95:5, v/v) acidified at pH 2 in order to break the strong anion-exchange interaction, since pKa of phenolic acids range between 3-5 (Table S1). However, as Fig. 3a shows, under these conditions recoveries for phenolic acids were low with the three bi-functionalized HMS mesostructured silicas (16-67%), except for chlorogenic acid and caffeic acid, which ranged between 86-100%, and vanillic acid (75-80%) in the case of HMS-RPC8-SAX-1 and HMS-RPC8-SAX-2. These results suggest, that pH 2 used for elution is not enough to break the strong anion-exchange interaction between the acidic analytes and the NR<sub>4</sub><sup>+</sup> groups of the silica. Additionally, low recovery values were also observed for other non-acidic analytes, such as epigallocatechin,

epigallocatechin gallate, piceid, *trans*-resveratrol, myricetin and naringenin (8-62%). Therefore, in view of these results, a more acidic pH was tested for elution, using 6 mL of MeOH:water (95:5, v/v) acidified at pH 1. Under these conditions (Fig. 3b) recovery values, in general, significantly improve for all the analytes, particularly in the case of phenolic acids. Therefore, from these results it can be deduced the existence of the mixed-mode retention mechanism in the HMS bi-functionalized silicas.

Comparing the three materials, best results are achieved with HMS-RPC8-SAX-2 mesostructured silica (Fig. 3), which is the material with the highest amount of  $\text{NR}_4^+$  groups (Table 1). Thus, the higher presence of  $\text{NR}_4^+$  groups enhances retention of phenolic acids, particularly of gallic acid, caftaric acid, protocatechuic acid, 4-hydroxybenzoic acid, syringic acid and *p*-coumaric acid (Fig. 3b). In contrast, the other silicas provide lower recovery values and, although, both present similar retention for phenolic acids, HMS-RPC8-SAX-3 shows low recoveries for some non-acidic compounds, such as epigallocatechin, epigallocatechin gallate, piceid, *trans*-resveratrol, myricetin and naringenin. Therefore, HMS-RPC8-SAX-2 mesostructured silica was selected as the most effective mixed-mode sorbent to perform the multicomponent extraction of the 22 target analytes using MeOH:water (95:5, v/v) acidified at pH 1 as elution solvent. However, for gallic acid, caftaric acid, protocatechuic acid, 4-hydroxybenzoic acid and syringic acid recoveries are a little bit lower than 80% (ranging between 70-78%). This is due to different reasons, for instance weak interaction with the sorbent, lack of sorbent, incomplete elution, or inadequate elution solvent. Therefore, different extraction parameters were tested using HMS-RPC8-SAX-2 as sorbent, since, at it has been reported (Fig. 3b), it was the most effective material to perform the extraction.

### ***Evaluation of the extraction technique***

Since there are no previous studies which use this type of materials in dSPE, the extraction procedure using HMS-RPC8-SAX-2 as sorbent was performed on SPE in order to verify if the mixed-mode interaction between the sorbent and the analytes can take place in a more suitable way in the SPE cartridge configuration than by dispersion. For this purpose, the SPE procedure was as follows: SPE cartridges were packed with 100 mg of HMS-RPC8-SAX-2 and plugged with polyethylene frits at both ends. A nylon filter membrane of 0.45  $\mu\text{m}$  was also inserted at the bottom of the mesostructured silica

bed to avoid material lost during sample loading. Cartridges were conditioned by passing 2 x 2 mL of MeOH:water (50:50, v/v) at pH 9. Subsequently, the sample extract (20 mL) was passed through the cartridges at a flow rate of 0.5 mL min<sup>-1</sup>. Once the entire extract was loaded, cartridges were dried for 20 min. Elution was performed by passing 3 x 2 mL of MeOH:water (95:5, v/v) acidified at pH 1. Finally, the eluate was evaporated to dryness and re-dissolved with 500 µL of MeOH for its analysis in the UHPLC-IT-MS/MS system.

As Fig. 4a shows, results verify that SPE is not better than dSPE in terms of recoveries. Moreover, better recovery values are achieved with dSPE for gallic acid, protocatechuic acid, epigallocatechin, 4-hydroxybenzoic acid, vanillic acid, *p*-coumaric acid, *trans*-resveratrol and myricetin. The differences observed among both techniques are probably due to fact that in dSPE, the sorbent is directly added and dispersed into the sample extract, instead of being packed in a cartridge (Fig. 4b). Therefore, contact and interaction between the analytes and the moieties of the silica is more effective than in SPE. Additionally, the dSPE procedure presents other advantages over SPE. For instance, dSPE was performed in 20 min, while the SPE procedure needed approximately 60 min. On the other hand, dSPE technique uses fewer amounts of sample (5 mL of sample extract), solvents (16 mL) and sorbents (50 mg) than SPE, which requires 20 mL of sample extract, 20 mL of solvents and 100 mg of sorbent. Therefore, it can be concluded that dSPE procedure is quicker and more cost-effective than SPE.

### ***Method optimization***

Since the interaction contact was not the problem for the lower recoveries obtained for some of the acid compounds, a study to evaluate the sorbent amount, the elution volume and the elution solvent was carried out. Respective data and Figures are provided in the Electronic Supplementary Material (SM. 1, Fig. S4 and S3). Nevertheless, despite optimizing different parameters, the best experimental conditions were found to be 50 mg of HMS-RPC8-SAX-2 mesostructured silica and 6 mL of MeOH:water (95:5, v/v) acidified at pH 1 as elution solvent, providing good recovery values ranging between 70 -101%. Therefore, these conditions were selected as the best ones to achieve the multicomponent extraction of polyphenols.

## **Comparison of bi-functionalized HMS-RPC8-SAX-2 mesostructured silica with other sorbents**

In a previous work of our research group, a reversed-phase sorbent was prepared by the mono-functionalization of HMS with n-octadecyl (C18) groups, which was denoted HMS-C18, and it was evaluated as dSPE sorbent for the multicomponent extraction of 20 polyphenols from juice and smoothie samples [30]. By comparing the results obtained with HMS-C18 and HMS-RPC8-SAX-2 under the optimized extraction conditions described for each of them, an improvement is evidenced with HMS-RPC8-SAX-2 silica in the retention of some phenolic acids, such as gallic acid, caftaric acid, ferulic acid and 4-hydroxybenzoic acid, because of the strong anion-exchange interactions provided by the material. As well, the retention of other non-acidic compounds, such as epigallocatechin, epigallocatechin gallate, naringin, quercetin 3- $\beta$ -D-glucoside, trans-resveratrol, myricetin and naringenin, significantly improves when using HMS-RPC8-SAX-2 as sorbent, probably due to the higher amount of hydrophobic groups in HMS-RPC8-SAX-2 silica (0.79 mmol g<sup>-1</sup> of C8) than in HMS-C18 (0.23 mmol g<sup>-1</sup> of C18). On the other hand, when comparing HMS-RPC8-SAX-2 with other commercial cartridges reported for extraction of polyphenols in foods (Table 2), it can be observed that there are no works which perform extraction of these compounds by dSPE, instead, they use SPE. Moreover, many of these works are not validated or optimized, so most do not provide recovery values for the target analytes. In general, octadecyl (C18) bonded silica reversed phase and Oasis® MAX cartridges have been the most common choice for extracting polyphenols from food samples. Comparing the results obtained in this study with the works which provide recovery values [8, 31, 33, 36], it is observed that recovery values of HMS-RPC8-SAX-2 are better or within the range of the results reported by other authors. For instance, in the works of Pérez-Magariño et al. [31] and Mattonai et al. [36], despite analyzing a higher number of compounds, it is observed that their range of recoveries is worse than the one obtained with HMS-RPC8-SAX-2, achieving very low values under 70% for some analytes. On the other hand, in the work of Capriotti et al. [33], despite having similar recovery values, they only analyzed 7 polyphenols using 500 mg of sorbent, which is a significantly higher amount in comparison to the 50 mg used in this work for the analysis of 22 polyphenols. In the case of Vallverdú-Queralt and co-workers [8], they used mixed-mode Oasis MAX cartridges, which have the same basis

of interaction than HMS-RPC8-SAX-2. As it can be observed, recoveries are similar among both works, but it is noteworthy to point out that in our work we analyzed a higher number of compounds, despite using a little bit more of sorbent. Additionally, comparing the results obtained for the common analytes of both works, better recoveries for quercetin and naringenin are achieved using HMS-RPC8-SAX-2. Therefore, it can be concluded that HMS-RPC8-SAX-2 can be used as an alternative sorbent to commercial materials.

## Conclusions

In this work, different HMS type mesostructured silicas were synthesized and bi-functionalized with C8 and NR<sub>4</sub><sup>+</sup> groups in order to obtain novel sorbent materials with reversed-phase/strong anion-exchange mixed-mode retention mechanism and different  $L_0$ . The resulting hybrid materials were successfully applied and evaluated as dSPE sorbents for the multicomponent extraction of 22 polyphenols from juice samples and its analysis by UHPLC-IT-MS/MS. Best results were achieved with the HMS-RPC8-SAX-2, which showed big extraction potential providing high recovery values of all the target analytes (70-101%) as a result of the hydrophobic and anion-exchange interactions provided by the material. Additionally, the dSPE method has showed some advantages over SPE since it has proved to be quicker and cost-effective. Therefore, this method can be used to improve food sample preparation and perform multicomponent extraction of bioactive compounds in food products. As well, the method application can be extended to other compounds such as drugs or pollutants, as long as they have affinity for non-polar groups or are negatively charged.

## Acknowledgments

Authors thank financial support from the Comunidad of Madrid and European funding from FEDER program (Project S2013/ABI-3028, AVANSECAL).

## Compliance with ethical standards

**Conflict of interest** Authors declare no conflict of interest.



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## Figure Captions

**Fig. 1** (a) Bi-functionalization reaction procedure of HMS and (b) mixed-mode interaction mechanism proposal between bi-functionalized HMS mesostructured silicas and the target analytes.

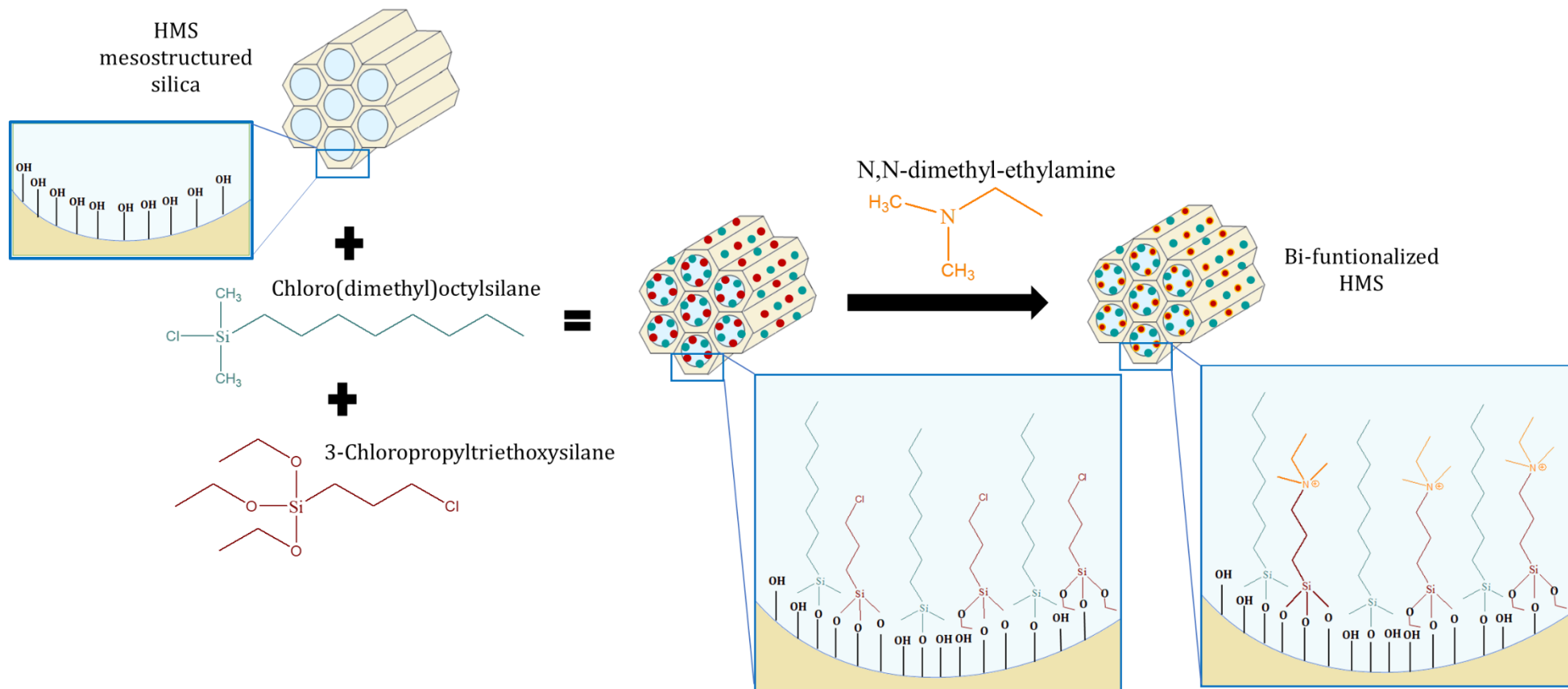
**Fig. 2** (a)  $^{29}\text{Si}$  and (b)  $^{13}\text{C}$  MAS-NMR spectra of HMS-RPC8-SAX-2.

**Fig. 3** Comparison of recovery percentages obtained from the analysis of spiked juice samples ( $0.02 - 2 \mu\text{g mL}^{-1}$ ) extracted by dSPE using 50 mg of HMS-RPC8-SAX-1, HMS-RPC8-SAX-2 and HMS-RPC8-SAX-3 and performing elution with 6 mL of MeOH:Water (95:5, v/v) at (a) pH 2 and (b) pH 1. Spiked concentrations of each analyte are listed in Table S1. Error bars represent the standard deviation of sample replicates ( $n = 3$ ).

**Fig. 4** (a) Comparison of recovery percentages obtained by dSPE and SPE using 50 and 100 mg of HMS-RPC8-SAX-2, respectively, as sorbent in spiked juice samples ( $0.02 - 2 \mu\text{g mL}^{-1}$ ). Spiked concentrations of each analyte are listed in Table S1. Error bars represent the standard deviation of sample replicates ( $n = 3$ ). (b) Graphic representation of dSPE and SPE techniques.

Fig. 1.

a)



b)

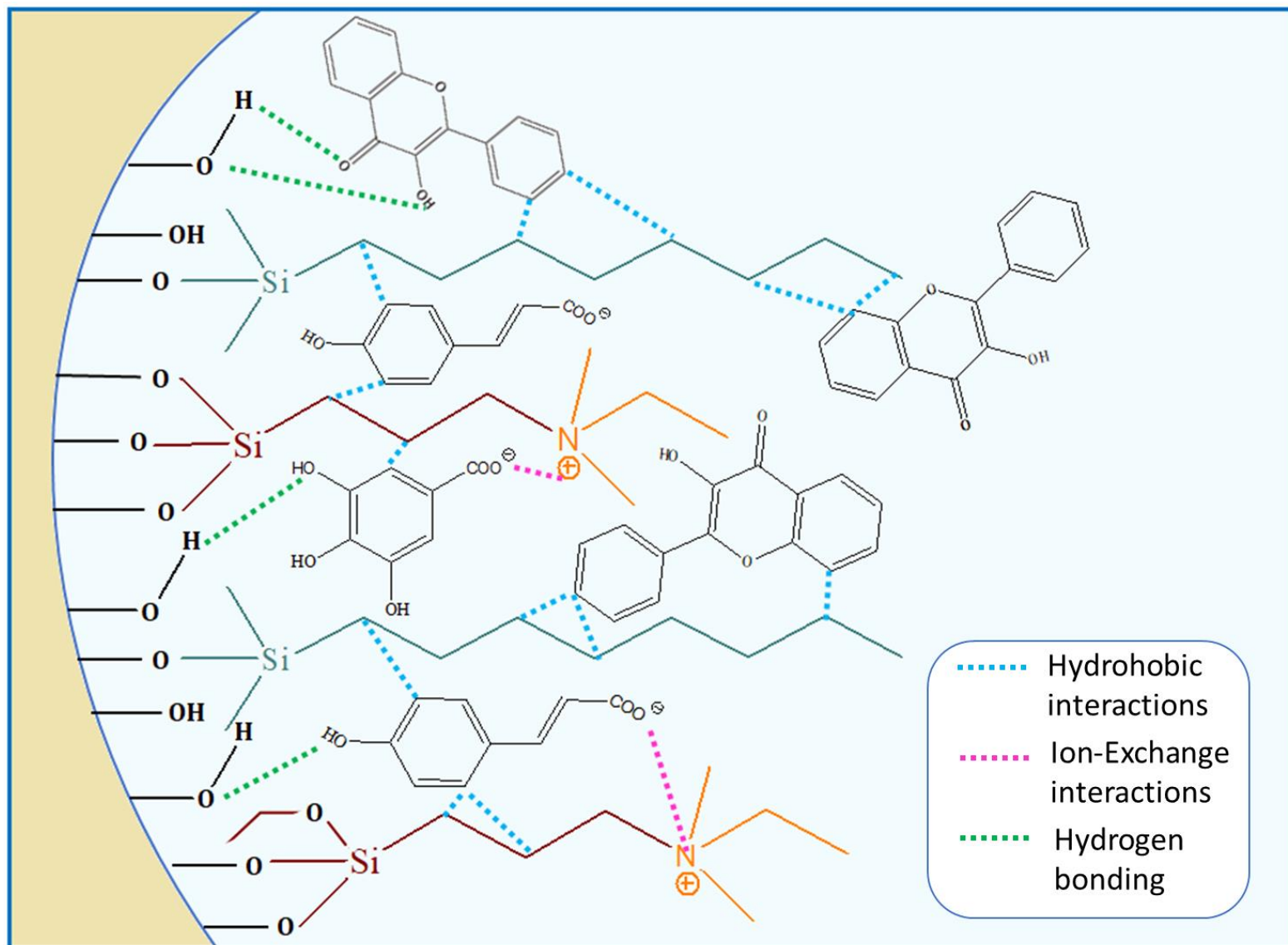
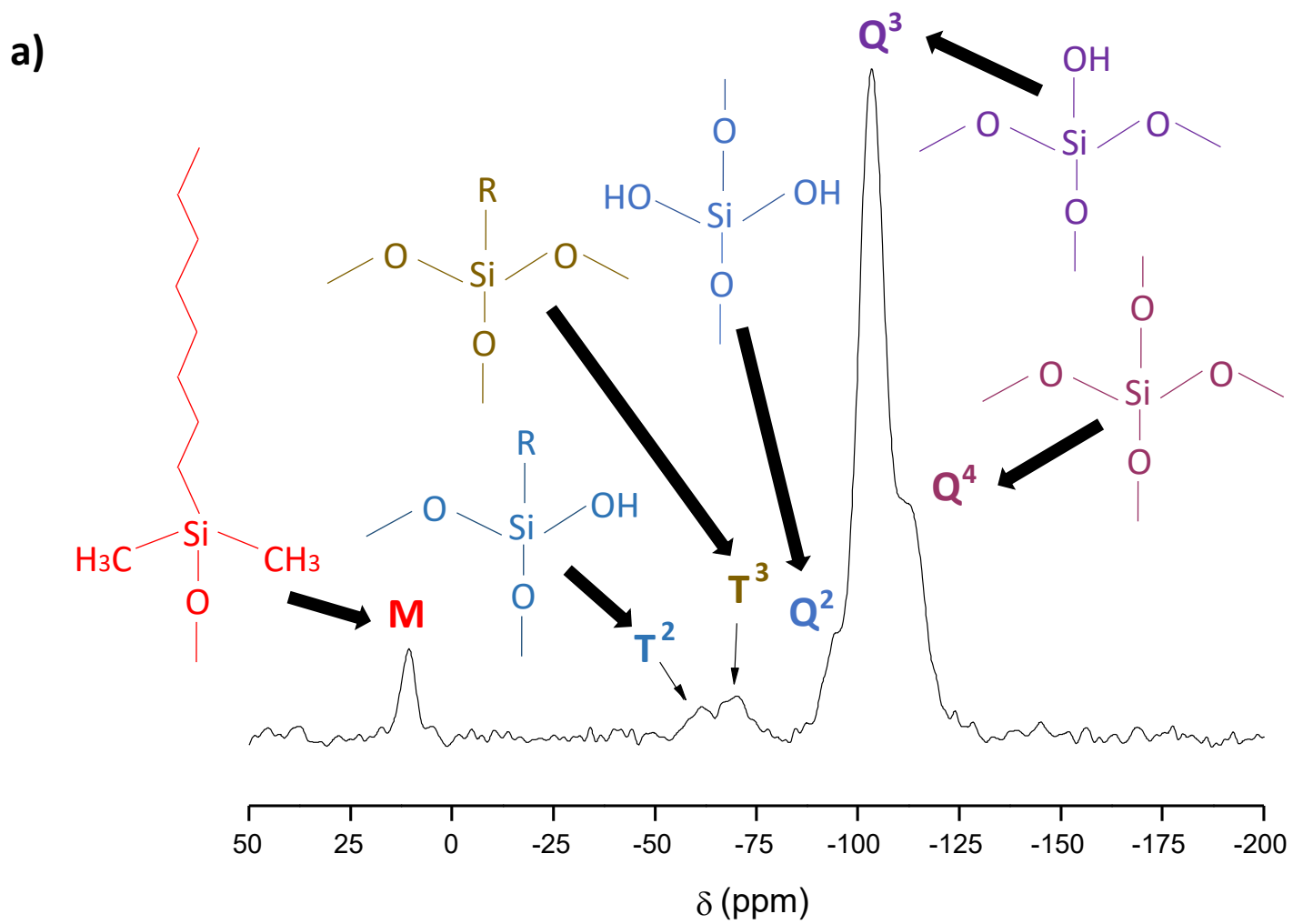
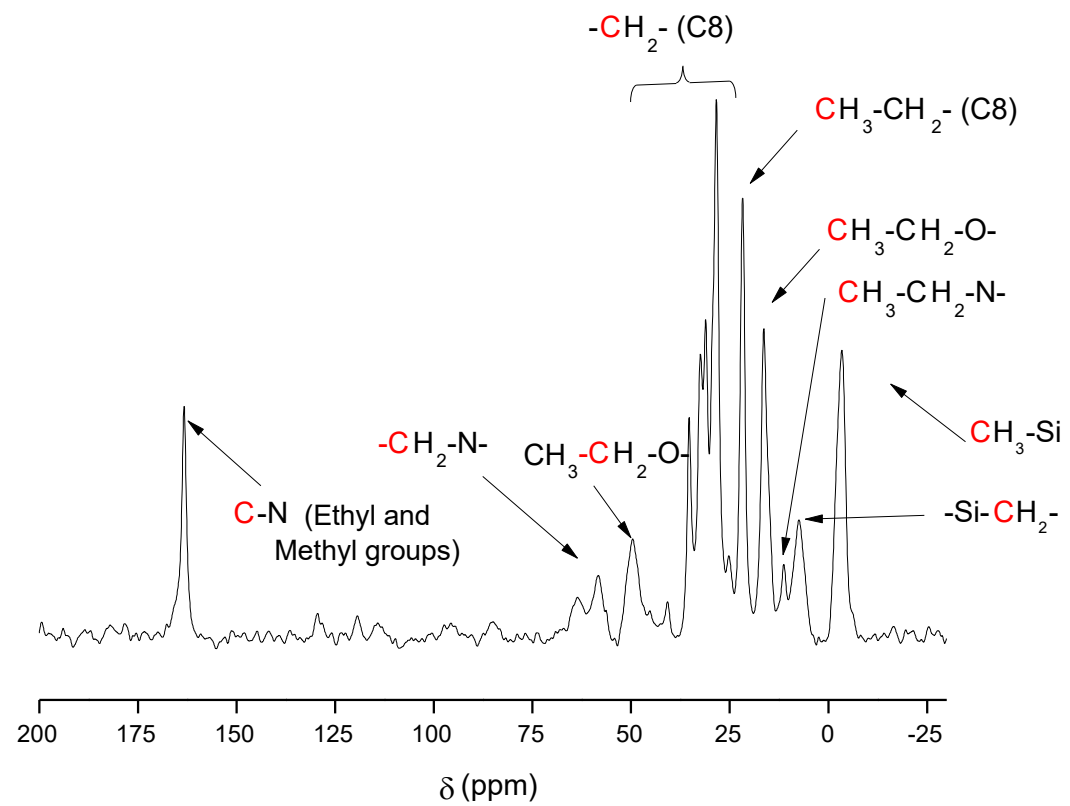




Fig. 2



b)



**Fig. 3**

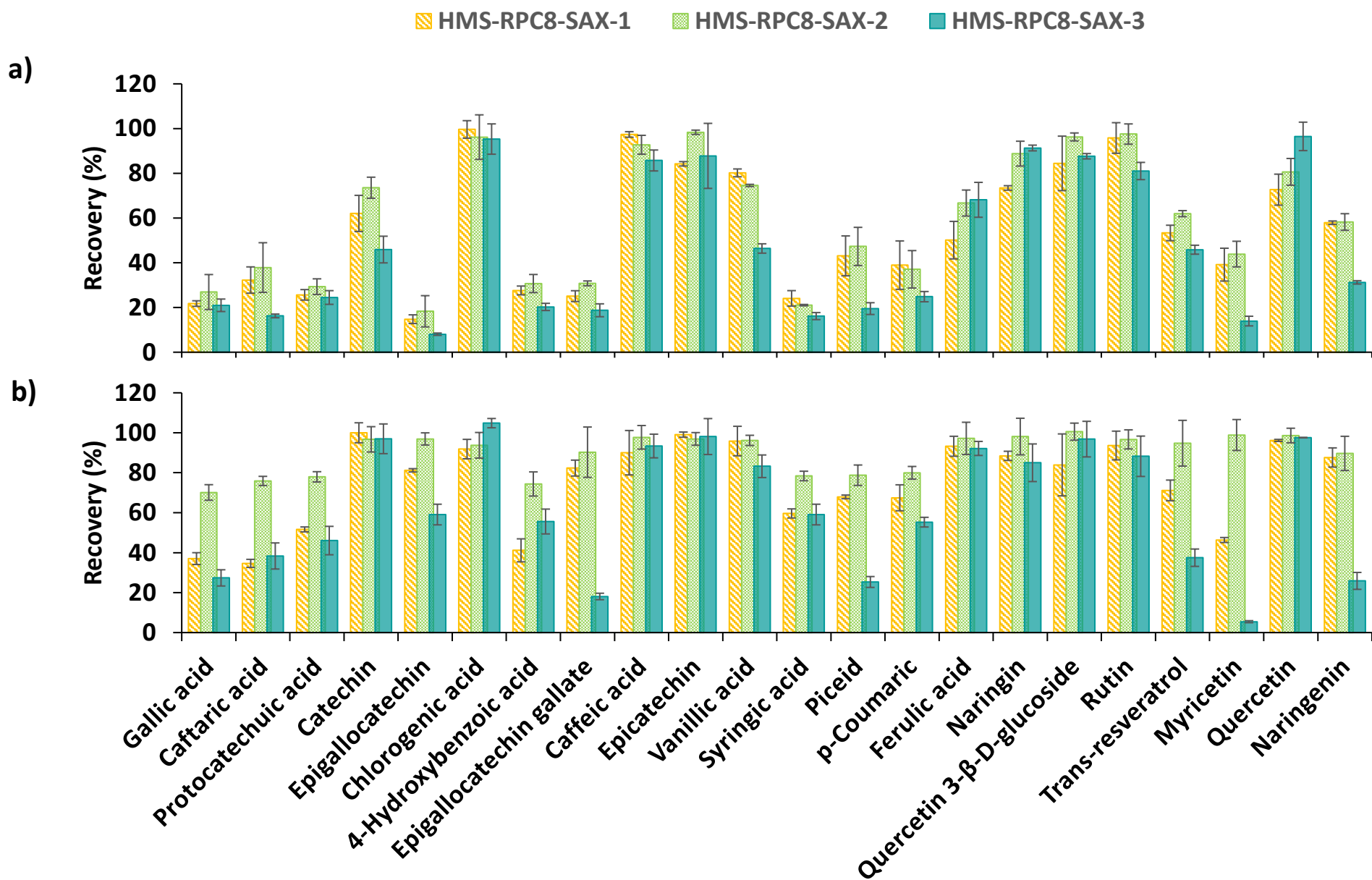
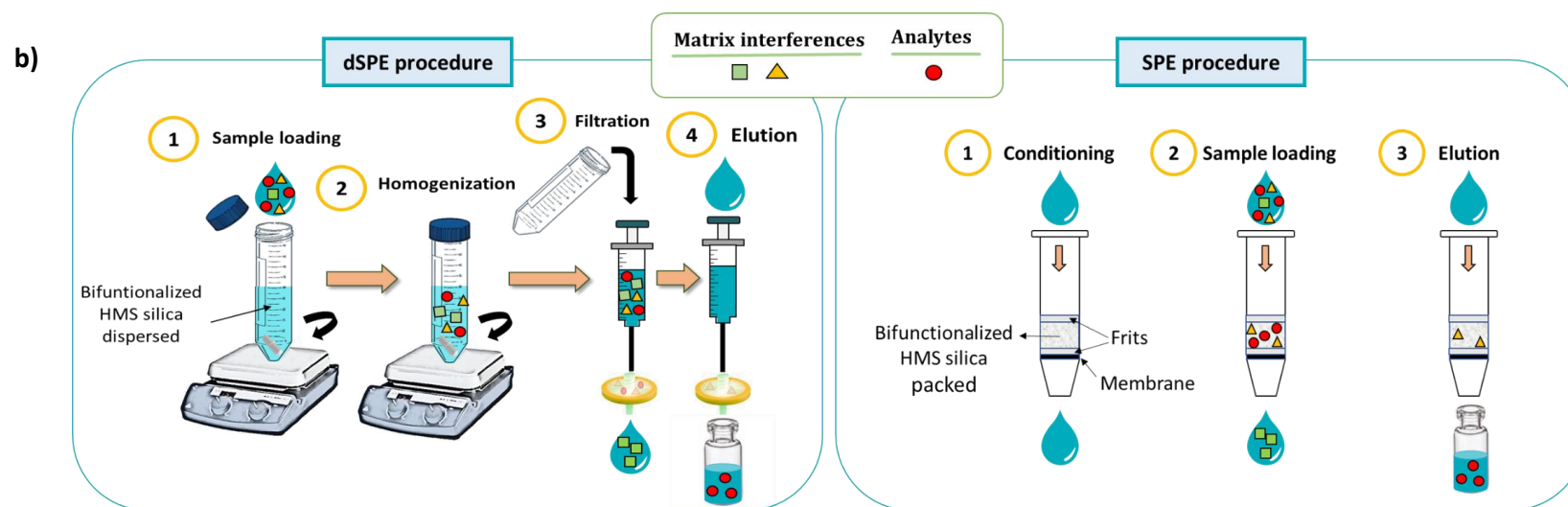
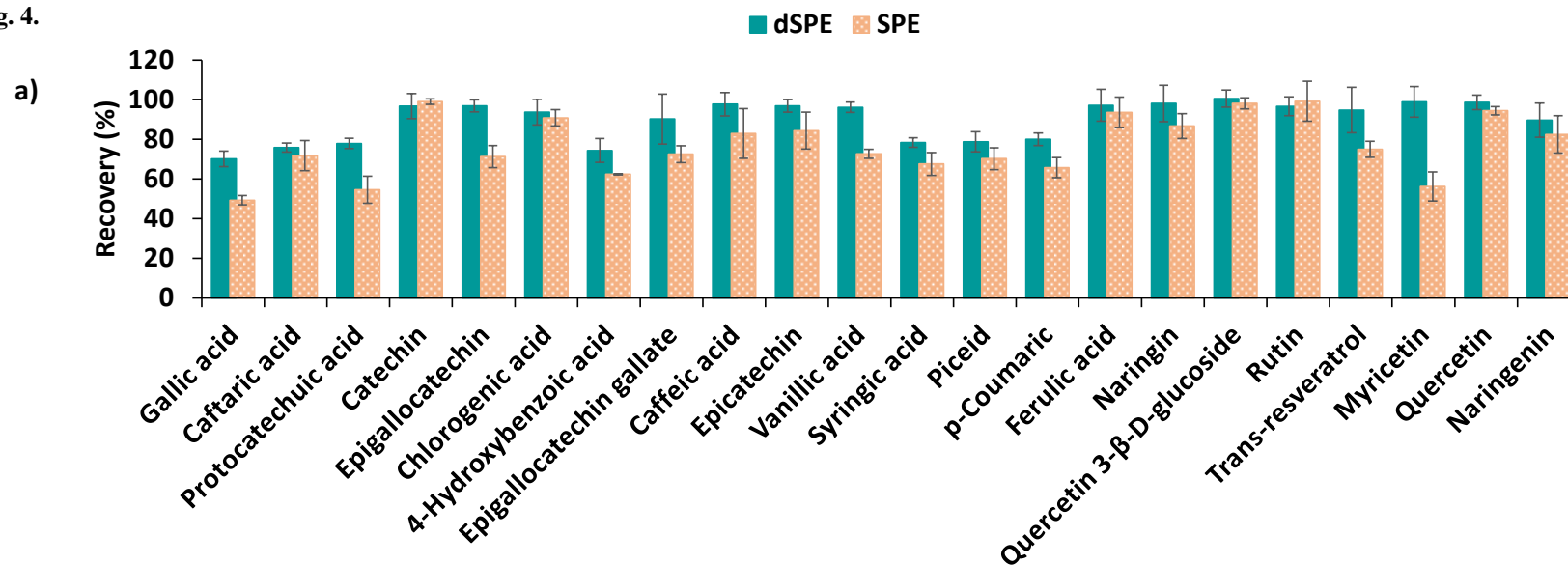


Fig. 4.



**Table 1.** Textural properties and functionalization degrees of the synthesized materials.

<b>Mesostructured silica</b>	<b>S<sub>BET</sub><sup>a</sup> (m<sup>2</sup> g<sup>-1</sup>)</b>	<b>Pore volume (cm<sup>3</sup> g<sup>-1</sup>)</b>	<b>BJH<sup>b</sup> pore diameter (Å)</b>	<b>Carbon (%)</b>	<b>Nitrogen (%)</b>	<b>L<sub>0</sub> C8<sup>c</sup> (mmol g<sup>-1</sup>)</b>	<b>L<sub>0</sub> NR<sub>4</sub><sup>+</sup><sup>d</sup> (mmol g<sup>-1</sup>)</b>
HMS	910	0.89	25.9	-	-	-	-
HMS-RPC8-SAX-1	796	0.60	21.5	10.46	0.47	0.53	0.33
HMS-RPC8-SAX-2	746	0.59	23.1	9.93	0.52	0.46	0.37
HMS-RPC8-SAX-3	785	0.49	21.9	10.92	0.23	0.74	0.17

<sup>a</sup> S<sub>BET</sub>: Specific surface area calculated by Brunauer-Emmett-Teller (BET) method.

<sup>b</sup> BJH: Pore size distribution calculated by Barret-Joyner-Halenda (BJH) method in the desorption branch.

<sup>c</sup> L<sub>0</sub> C8: Functionalization degree of C8 groups.

<sup>d</sup> L<sub>0</sub> NR<sub>4</sub><sup>+</sup>: Functionalization degree of NR<sub>4</sub><sup>+</sup> groups  $-(\text{CH}_2)_3\text{N}^+(\text{CH}_3)_2(\text{CH}_2\text{CH}_3)$

**Table 2.** Comparison of the proposed method with other reported methods employing commercial sorbents.

Sample	Analytes	Extraction procedure	Sorbent <sup>a</sup> (Amount) <sup>b</sup>	Optimal extraction conditions	Recovery <sup>b</sup> (%)	Analytical Technique	Limit of detection <sup>b</sup>	Ref.
Tomatoes and tomato-based product	12 polyphenols	SPE	Oasis MAX (30 mg)	Load: 1 mL sample extract reconstituted + 1 mL acidified water Elution: 1.8 ml MeOH with 2% formic acid	n. p.	HPLC-TQ-MS/MS	n. p.	[7]
Tomatoes	10 polyphenols	SPE	Oasis MAX (30 mg)	Load: 1 mL sample extract reconstituted + 1 mL acidified water Elution: 1.8 ml MeOH with 2% formic acid	78 - 98	HPLC-TQ-MS/MS	0.13 – 0.66 µg/g fresh weight	[8]
Beer	47 polyphenols	SPE	Oasis MAX (30 mg)	Load: 1 mL of dealcoholized acidified beer Elution: 1.8 ml MeOH with 2% formic acid	n. p.	HPLC- LTQ-Orbitrap-MS and HPLC-PDA	n. p.	[9]
Bistort Rhizoma	31 polyphenols	SPE	Oasis MAX (30 mg)	Load: 1 mL sample reconstituted and basified Elution: 1 ml 5% ammonia, 1 ml MeOH and 1 ml MeOH containing 5% formic acid	n. p.	HPLC-QTOF-MS	n. p.	[10]
Red wines	33 polyphenols	SPE	HLB (500 mg)	Load: 1 mL wine sample Elution: 5 ml diethyl ether, 5 ml ethyl acetate and 5 ml MeOH	26 - 103	HPLC-DAD	0.01 – 0.88 mg/L	[31]
<i>Chaenoemeles</i> fruits	24 polyphenols	SPE	Supelclean ENVI-18 (n. p.)	Load: 20 mL sample extract reconstituted Elution: MeOH	n.p.	HPLC-DAD and HPLC-IT-MS/MS	n. p.	[32]
Virgin olive oil	7 polyphenols	SPE	Diol (500 mg)	Load: 6 g sample + 6 mL hexane Elution: 10 ml MeOH:H <sub>2</sub> O (80:20, v/v)	75 – 104	HPLC–DAD and HPLC–QTOF	n. p.	[33]
Banana	19 polyphenols	SPE	Sep-Pak Plus C18 (360 mg)	Load: 3 mL sample reconstituted Elution: ethanol (0-100%)	n. p.	HPLC-LTQ-Orbitrap-MS and HPLC-DAD	0.007 – 0.16 µg/mL	[34]
Mulberry	41 polyphenols	SPE	Sep-Pak C18 (n. p.)	Load: 50 mL sample reconstituted Elution: ethyl acetate, and MeOH	n. p.	UHPLC–DAD and UHPL-LTQ-Orbitrap-MS/MS	n. p.	[35]
Honey	24 polyphenols	SPE	Amberlite XAD-2 (n. p.)	Load: 1 g sample +5 mL acidified water Elution: 3 ml MeOH	5 – 99	HPLD-DAD and HPLC-QTOF-MS/MS	1 – 4000 ng/g	[36]
Fruit and vegetables-based HPP juice	22 polyphenols	dSPE	HMS-RPC8-SAX-2 (50 mg)	Load: 5 mL sample extract Elution: 6 mL MeOH:water (95:5, v/v) at pH 1	70 - 101	UHPLC-IT-MS/MS	1 – 560 ng/mL	This work

<sup>a</sup> HLB: hydrophilic-lipophilic balance polymeric material sorbent; Oasis MAX: mixed-mode reversed-phase/strong anion-exchange polymeric sorbent; Supelclean ENVI-18: silica gel base sorbent with C18 (n-octadecyl) bonding; Diol: polar sorbent with short alkyl chains and diol groups; Sep-Pack: silica-based sorbent with strong hydrophobicity end-capped; Amberlite XAD-2: hydrophobic crosslinked polystyrene copolymer resin (polymeric sorbent); HMS-RPC8-SAX-2: mesostructured silica HMS bi-functionalized with C8 (n-octyl) chains and quaternary ammonium (NR<sub>4</sub><sup>+</sup>) groups;

<sup>b</sup> n. p.: not provided.

