Hydroxymethylfurfural determination in cereal and insect bars by high-performance liquid chromatography-mass spectrometry employing a functionalized mesostructured silica as sorbent in solid-phase extraction

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Highlights

- Development and validation of a new method to determine hydroxymethylfurfural.
- Functionalized silica-based sorbent was applied in solid-phase extraction.
- Analysis by high-performance liquid chromatography-mass spectrometry.
- High concentration of hydroxymethylfurfural was found in cereal and insect bars.

ABSTRACT

In this work a new method for the determination of 5-hydroxymethylfurfural (HMF) in cereal and insect bars has been developed and validated. The method consisted of a solid-liquid extraction (SLE) followed by a solid phase extraction (SPE), employing functionalized mesostructured silica as sorbent, and prior to high-performance liquid chromatography coupled to mass spectrometry analysis (HPLC-MS/MS). Mesostructured silica with a large pore (SBA-15-LP) functionalized with aminopropyl-groups (SBA-15-LP-NH_2), octyl- groups (SBA-15-LP-C8) and bifunctionalized with both organic ligands (SBA-15-LP-C8-NH_2) were prepared, characterized and tested for this purpose. The optimal conditions showed that the best extraction solvent was water acidified with HCl (pH 1.0) and the best material for SPE was SBA-15-LP-NH_2 (recoveries near 100%). Results were compared with other analogous commercial sorbent (Discovery® DSC-NH_2), evaluated under similar conditions, and SBA-15-LP-NH_2 sorbent showed better recoveries than the commercial one (62 ± 1%). developed method was validated and good detection and quantification limits (MDL: 11 µg $kg^{-1}$and MQL: 38 µg kg^{-1}), good precision in terms of repeatability and within-laboratory reproducibility (RSD < 8%) and good accuracy (recoveries between 99-102%) were obtained. The method was successfully applied to the determination of HMF in different samples of cereals and insect bars. In all the samples analysed, high concentrations of HMF (ranging from 336 to 962 mg kg^{-1}) have been found.

Keywords: 5-hydroxymethylfurfural; Food processing contaminants; Cereal and insect bars; Functionalized mesostructured silica; SPE; HPLC-MS/MS

1. Introduction

In the last years, food safety authorities and scientifics have paid great attention to food processing contaminants. Acrylamide, 3-monochloropropane-1,2-diol, polycyclic aromatic hydrocarbons, ethyl carbamate, heterocyclic amines and 5-hydroxymethylfurfural (HMF) are some of these food processing contaminants. Regarding HMF, this compound is formed in browning chemical reactions produced by the application of technological processes in foods, to obtain desirable sensory features or to assure their safety [1]. Two important reactions involved in the formation of HMF during thermal processing of foods, depending on the temperature, type of sugars, pH and water activity, are Maillard reaction (MR) and caramelization [2-4]. HMF is one of the most common intermediate products of the MR (considered as an early marker of MR), formed by thermal treatment of foods rich in reducing sugars in presence of proteins, peptides or amino acids [5]. Moreover, HMF is also formed by caramelization that consists of dehydration from carbohydrates (fructose, sucrose, glucose) by heating them over their melting point in absence of proteins [5, 6].

HMF is a food processing contaminant with potential harmful properties. These harmful effects are due to the transformation of HMF into other compounds such as 5-sulphoxymethylfurfural [7]. European Food Safety Authority (EFSA) has suggested that this metabolite can generates genotoxic and mutagenic effects *in vitro* [8]. Recent studies have shown nephrotoxicity and hepatotoxicity both *in vitro* and *in vivo* studies [9]. Despite this, few studies have demonstrated genotoxicity and mutagenicity *in vivo*, hence the importance of new studies to know the toxicological relevance of its exposure to ensure the health of consumers.

HMF has been evaluated as an indicator of the severity of heat treatment during food manufacturing processes, and it has been found at high concentration in some food products. Although there are not maximum values for this compound in most foods, the Codex Alimentarius and the European Union [10] have established a maximum HMF quality level in honey and in apple juice (40 and 50 mg kg^{-1}, respectively), as indicator of deterioration and heat-treatment. Consequently, a great deal of attention has been paid to the analysis of HMF in honey and juices, but also in foods like coffee, fruit puree, baby cereals, jams, dried fruits, cookies, breakfast cereals, extruded products, bakery products, vinegar, soy sauce, beverages, snacks, infant milk formulas, hazelnuts and syrups [4-7, 11-24]. In this context special attention, deserves cereals and insect bars as an example of food products with numerous ingredients used in their elaboration. In fact, besides the basic ingredients (cereal/insect flours or cereal flakes), these bars can be prepared with dried fruits, chocolate, nuts, honey, caramel, etc. HMF is already present in some of these ingredients added to cereal and insect bars, but also its content can be increased during the manufacturing process and storage. Nowadays these bars are frequently consumed as lunch, between hours, by children, adolescents or even athletes (for their high protein content in some cases). Presently, no studies have been found for HMF content in commercial cereal and insect bars. Hence the importance to develop and validate new methods of analysis for this type of samples, in order to understand their contribution to HMF daily intake.

Conventional methods for determining HMF in foods use high-performance liquid chromatography (HPLC) with UV detection (reference method of the AOAC nº 980.23) [4-7, 19-23, 25] or HPLC coupled to mass spectrometry (HPLC-MS) [13, 17]. Gas chromatography coupled to mass spectrometry (GC-MS) and capillary electrophoresis with UV detector (CE-UV) have been also applied for HMF analysis as an alternative to

HPLC [11, 16]. However, in any case, many compounds present in foods may interfere and negatively affect its quantification, so the use of an effective extraction methodology is of great importance for this task.

For sample preparation, solid-liquid extraction (SLE) [4, 6, 13-15] or liquid–liquid micro-extraction (LLME) have been used [16] employing Carrez I and II reagents as clarifiers to eliminate matrix interferences. In addition, the application of a solid-phase extraction (SPE) step for extracts purification is very useful to improve its determination. Different studies with commercial SPE cartridges have been carried out [5, 11-12, 17-19]. For example, Gokmen and Senyuva [5] used hydrophobic-lipophilic balance cartridge (Oasis® HLB) in baby foods samples before HPLC-MS analysis obtaining recoveries between 91.8 and 94.7%. Teixidó et al. [11] tested three different C18 and three polymeric commercial cartridges for the analysis of HMF in jam, honey, orange juice, breakfast cereals and biscuits, getting the best recoveries with the Isolute® ENV+ polymeric cartridges. Commercial ion exchange cartridges (Ansys SPEC SCX) were used by Xu et al. [18] for HMF determination in beverages (cola and soft drinks), showing recoveries between 99.3 and 106.2%.

Recent advances in the development of new materials have gained increasing research interest in sample preparation, due to their desirable characteristics and advantages versus traditional and commercial sorbents. For this reason, the use of new materials based on carbon, silica or magnetic nanoparticles are being widely investigated for sample preparation [26]. Thus, this type of materials have been applied for the extraction of organic contaminants, including some food processing contaminants. For example, recently Ning et al. [27] developed a new material based on dummy-surface molecularly imprinted polymer on magnetic graphene oxide particles to determine

acrylamide in potato chips, biscuit, fried instant noodles and dough sticks, obtaining recoveries between 84-97%.

Specifically, the synthesis of new silica-based materials is being a trend in the extraction of contaminants in foods, due to the advantages offered by these materials compared to commercial sorbents. Casado et al. [28] recently reviewed the application of these materials for xenobiotic analysis in different food samples. Among them, mesoporous silicas have gained increasing interest in sample preparation due to their textural properties, since they present high surface area, high pore volume and uniform and ordered pore arrangement. Their high flexibility in functionalization enables the introduction of different functional moieties on their surface, which allow an efficient and selective extraction of the target compounds. Functionalized mesostructured silicas have been successfully applied for the extraction of pesticides, veterinary drugs or steroids in foods such as fruit juices, meat and milk [28-32]. However, currently, there are not studies applying these materials in sample preparation for HMF analysis.

Therefore, the aim of this work was to optimize and validate a new HPLC-MS/MS method to determine HMF in foods, by using mesoporous silica-based materials in the sample preparation stage. Specifically, the method was applied for cereal and insect bars analysis, as they are an example of very complex food samples with numerous ingredients used in their elaboration. For this purpose, mesostructured silica with a large pore (SBA-15-LP) was synthetized, functionalized or bifunctionalized with aminopropyl- or octyl-groups (SBA-15-LP-NH_2, SBA-15-LP-C8 and SBA-15-LP-C8-NH_2) and evaluated as sorbents for SPE after a SLE. To the best of our knowledge, there are not studies that analyse HMF in cereal and insect bars.

2. Materials and methods

2.1 Reagents and materials

High-purity (> 99%) HMF was purchased from Sigma–Aldrich (CAS 67-47-0). Stock standard solution (1000 mg L^{-1}) was prepared by diluting in methanol (MeOH) adequate amount of HMF and stored at −20 ºC in darkness. Working solutions (0.5 mg L^{-1} –100 mg L^{-1}) were prepared by appropriate dilution of the stock solutions with MeOH and were stored at 2-8 ºC. Acetonitrile (ACN), MeOH and hexane (Hex) LC–MS grade were purchased from Scharlau (Barcelona, Spain). Formic acid LC-MS grade was acquired from Fluka (Busch, Switzerland). Tetraethylorthosilicate (TEOS, 98%, 208.3 g mol^{-1}, 0.93 g mL^{-1}), poly(ethylene - glycol) - block - poly (propylene - glycol) - block-poly (ethylene - glycol) (EO20PO70EO20, Pluronic 123, 5800 g mol^{-1}, 1.019 g mL^{-1}), chloro(dimethyl)octyl-silane (C8, 97%, 206.83 g mol^{-1}), 3-aminopropyl-triethoxysilane (NH_2, ≥ 98%, 221.37 g mol^{-1}), ammonium acetate LC-MS grade, decane (≥ 95 %), potassium hexacyanoferrate and zinc sulfate analytical grade were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonium fluoride (NH_4F) was obtained from Panreac. Hydrochloric acid (HCl, 37%, 36.45 g mol^{-1}, 1.19 g mL^{-1}), acetic acid glacial (HAC, 60.05 g mol^{-1}, 1.05 g mL^{-1}), toluene, ethanol and diethyl ether were purchased from Scharlau (Barcelona, Spain). Ultra-pure water (18.2 MΩ cm quality) was obtained using a Millipore Milli-Q-System (Billerica, MA, USA). Carrez solutions used for SLE were prepared by dissolving 1.5 g of potassium hexacyanoferrate or 3 g of zinc sulfate in 10 mL of Milli-Q water (Carrez I and II, respectively). Polyethylene frits, nylon filter membranes (0.45 µm), empty syringes (3 mL) and nylon syringe filters (0.45 µm), were

acquired from Scharlau (Barcelona, Spain). Commercial Discovery® DSC-NH_2 SPE cartridges (500 mg, 3 mL) were purchased from Análisis Vínicos (Tomelloso, Spain).

2.2 Cereal and insect bars samples

Cereal and insect bars samples with different ingredients were purchased from a local market in Madrid (Spain). Samples (one bar, between 20-40 g) were ground, homogenized using liquid nitrogen and a mincer (A11 Basic analytical mill, IKA) and stored in darkness at room temperature before extraction procedure. A portion of one gram was used for the determination of HMF.

2.3 Synthesis of mesostructured silicas

SBA-15-LP was synthesized according to the method described by Tian et al. [33] with slight modifications. Firstly, 12 g of Pluronic 123® were dissolved in 420 mL of 0.1% HCl (v/v) and stirred in a silicone bath at 30 °C, until it was completely dissolved. Then, 0.14 g of NH_4F was added and stirred for 10 min at 450 rpm. After this time, 25.84 g of TEOS and 75 mL of decane were added dropwise. Immediately, the mixture was stirred for 20 h at 30 °C (300 rpm). The stirring was stopped and transferred to an autoclave at 100 °C for 48 h. The resulting product was filtered under vacuum, washed with distilled water, air dried, ground and calcined (from 20 to 540 °C at 1ºC/h, 8 h at 540 °C).

Bare SBA-15-LP silica was functionalized with aminopropyl- (SBA-15-LP-NH_2) and octyl- (SBA-15-LP-C8) groups, or bifunctionalized with both ligands (SBA-15-LP-C8-NH_2). SBA-15-LP-NH_2 and SBA-15-LP-C8 were prepared in 1:1 ratio (w/v), for this 2.2

g of SBA-15-LP was mixed with 40 mL of toluene and them 2.2 mL of aminopropyltriethoxysilane or 2,2 ml of chloro(dimethyl)octyl-silane was added. This reaction was carried out under nitrogen atmosphere at 80 °C, for 24 h and 400 rpm. The functionalized materials were filtered, washed with dried solvents (40 mL of toluene, 40 mL of ethanol and 40 mL of ethyl ether) and dried at vacuum overnight. Bi-functionalized material was prepared in the same way but, firstly SBA-15-LP-C8 was synthetized, as indicated previously, and then 1 g of this material was mixed with 40 mL of toluene and 71.6 µL of 3- aminopropyltriethoxisilane.

2.4 Characterization of mesoporous silicas

Materials were characterized by nitrogen gas adsorption-desorption isotherms, elemental analysis (EA), cross-polarization magic-angle spinning of ^{13}C (^{13}C CP-MAS-NMR), pulse decoupling angle ^{29}Si solid-state nuclear magnetic resonance spectroscopy (^{29}Si-PDA-MAS-NMR) and scanning electron microscopy (SEM). Nitrogen gas adsorption-desorption isotherms were carried out using a Micromeritics ASAP 2020 analyzer. Previous to analysis, 0.2 g of sample were dried in vacuum line overnight and afterwards, the samples were outgassed at 90 °C in vacuum during 10 h in the port of degasification of the instrument. Adsorption isotherms were measured at -196 °C over interval of relatives pressures from 10^{-4} to 0.993. EA (%H, %C, %N) was performed using a microanalyser Flash 2000 Thermo Fisher Scientific Inc. ^{13}C -CP and ^{29}Si PDA-MAS-NMR was recorded on a Bruker Advance III HD-WB spectrometer at 400 MHz as resonance frequency (^{13}C CP-MAS-NMR: 10240 transients, 12 KHz spinning speed, 3 ms contact time, 5 s press delay; ^{29}Si PDA-MAS-NMR: 15000 transients, 12 KHz spinning speed, 8 ms contact time, 5 s press delay). Finally, SEM analysis were performed

a Nova NanoSEM 230 FEI. Previously, samples were treated with sputtering method by dispersing the material in ethanol and coating the sample with a film thickness 7 nm of gold.

2.5 SLE and SPE procedures

Optimization of SLE and SPE steps was carried out by evaluating the recovery percentage of target analyte, using 3 cartridges in each experiment. Two cartridges were used for samples enriched with HMF before extraction and the other one was used for the simulated sample (sample prepared under the same conditions, but enriched with HMF by the end of the SPE stage). The recoveries obtained were calculated by comparing the peak area of HMF-enriched samples with the area of the simulated sample.

Firstly, a SLE was carried out accordingly to a previously described clean-up procedure with some modifications [13]. Solvents evaluated for SLE were MeOH, water acidified with HAC (pH 3.3) and water acidified with HCl (pH 1.0). After optimization, SLE was carried out as follows: 1 gr of sample was weighed in a 50 mL Falcon® conical tube and 10 mL of water acidified with HCl (pH 1.0) was added. The mixture was shaken with vortex for 2 min and then 0.1 mL Carrez I and Carrez II were added. This mixture was centrifuged at 6000 rpm for 10 min using a Rotofix 32A centrifuge (Hettich, Germany). The supernatant was filtered through 0.45 µm nylon filter and 1 mL of sample extract was collected and purified by SPE.

SPE was performed using a Supelco Visiprep solid phase extraction vacuum manifold 12 port model (Sigma Aldrich, St. Louis, MO, USA) connected to a vacuum pump at 7.6 psi. 3 mL polypropylene empty cartridges (length of 65 mm and i.d. 10 mm) were packed with 50 mg of SBA-15-LP-NH_2 and plugged with polyethylene frits at both ends. To prevent the material lost during sample loading, a pore size nylon filter membrane (0.45

μm) was also inserted at the bottom of the mesoporous silica bed. In order to assess the best conditions for the SPE, some preliminary experiments were run to test critical factors affecting the extraction efficiency of the procedure, including the sorbent, solvent (and volume) used for washing and elution steps and the sample volume. Under optimized conditions, the prepared SPE cartridges were conditioned with 2 mL of acidified water (pH 1.0, HCl). Then, 1 mL of sample extract was loaded (a flow rate of 0.5 mL min^{-1}) and passed through the cartridge. Prior to the elution step, a washing step with 2 mL of Hex was carried to eliminate interferences. Finally, the target analyte was eluted with 3 mL of MeOH. Eluates were evaporated to dryness under nitrogen flow and the residue was immediately reconstituted with 1.5 mL of H_2O:ACN (50:50, v/v) for subsequent analysis in the chromatographic system.

2.6 HPLC-MS/MS analysis

For the determination of HMF by HPLC, a Varian 1200/1200L LC-MS/MS was used (Varian Ibérica, España) containing two solvent deliver module ProStar 210/215, a ProStar 410 autosampler (equipped with a 100 μL loop), a thermostatic column compartment, and a 1200L TQ triple cuadrupole mass spectrometer with an electrospray ionization (ESI) ion source (data acquisition system MS Workstation version 6.3). Chromatographic separation was carried out using a C18 Kromaphase 100 column (150 mm x 2.0 mm, 3.5 μm particle size) at 30ºC. The injection volume was 10 μL (partial injection) and the flow rate 0.3 mL min^{-1}. A gradient elution was performed by combining solvent A (MeOH) and solvent B (H_2O), containing both phases 0.1% formic acid and 2 mM ammonium acetate. The gradient elution started at 10% A and 90 % B, then eluent A increased linearly to 90 % in 10 min and returned to the initial conditions in 5 min. The

column was then equilibrated with 10% A for 2 min prior to the next run. MS data acquisition was performed using ESI in positive ion mode using the following parameters: drying gas N_2 (350 ºC and 22 psi), nebulizer gas pressure (60 psi), ion spray voltage (-5000 V for capillary and -600 V for shield). Collision gas was set at 1.90 mTorr and electron multiplier was set at 1480 V. The ionization source parameters were optimized by direct infusion of standard solution of HMF in MeOH (10 µg mL^{-1}) at a flow rate of 20 µL min^{-1}. Multiple reaction monitoring (MRM) mode was used for HMF detection (mass peak width Q1 2.5; mass peak width Q3 2.5; scan width in MRM 0.70). The precursor ion was 127 m/z (40 V cone voltage) and the product ions were 108.8 m/z, 80.6 m/z and 53 m/z with a collision energy of 10 V, 14 V and 22 V, respectively and a dwell time of 0.5 s. The quantification was carried out with the product ion 108.8 m/z.

2.7 Instrumental and method validation

Standard working solutions were analyzed in the HPLC-MS/MS to evaluate the instrumental linear range, repeatability, reproducibility, detection and quantification limits (LOD a LOQ). Repeatability and reproducibility were evaluated at three levels of concentration, low (0.5 mg L^{-1}), medium (2.6 mg L^{-1}) and high (10 mg L^{-1}). Repeatability was carried out in one day, analyzing by HPLC-MS/MS six times each standard working solution (n = 6) in the three levels of concentration. Reproducibility was evaluated in three different days, analyzing each day three times each standard working solution (n = 9) in the three levels of concentration. LOD and LOQ was determinate as three and ten times S/N, respectively, corresponding to the lowest concentration standard working solution analyzed (0.1 mg L^{-1}).

Method validation (SLE, SPE and HPLC-MS/MS) was performed for the assessment of the linear range, precision, accuracy, method detection and quantification limits (MDL and MQL) and robustness. Firstly, the linear range of the method was evaluated, and for this purpose, matrix-matched calibration curves were prepared. These calibration curves were obtained by plotting peak areas versus HMF concentration in spiked sample extracts obtained under the optimized extraction conditions (concentrations ranging from 0.77 - 100 μg g^{-1}). The precision and accuracy were evaluated by spiking the samples at three levels of concentration, low (7.7 μg g^{-1}), medium (40 μg g^{-1}) and high (153 μg g^{-1}). The medium level corresponds to the HMF amount legislated in honey [10]. Precision was evaluated in terms of repeatability and reproducibility, applying the optimized SLE and SPE process indicated in section 2.5. Repeatability was carried out in one day using six different samples (n = 6) and reproducibility was evaluated on three different days, with three different samples (n = 9). The accuracy was expressed as recovery percentage (%) and was obtained by spiking six different samples (n = 6) at three concentration levels (low, medium and high) and subjecting them to the proposed method. The method detection limit (MDL) and the method quantification limit (MQL) were estimated as the concentration that yields S/N of 3 or 10 for the chromatographic response measured in the HPLC-MS/MS, using the lowest concentration of the matrix-matched calibration curve (0.77 μg g^{-1}).

3. Results and discussion

3.1 Characterization of mesoporous silicas

SBA-15-LP was characterized before and after functionalization. Nitrogen gas adsorption-desorption isotherms showed type IV isotherms (Fig. 1a-d), according to the

IUPAC classification, with a type H1 hysteresis loop typical of mesoporous materials. The formation of this hysteresis loop represents the capillary condensation of nitrogen within the uniform mesoporous structure. Results obtained indicated a BET specific surface area (S_{BET}) of 510 $m^2 g^{-1}$ and a pore volume of 1.6 $cm^3 g^{-1}$ for the bare silica, and between 382-429 $m^2 g^{-1}$ and 1.4-1.6 $cm^3 g^{-1}$ for the functionalized materials (Table 1). Anymore, the results showed pore diameters centered at 38 Å and 153 Å for SBA-15-LP material and between 36-37 Å and 126-152 Å for the functionalized materials (Fig. 1a-d). After functionalization, materials possessed lower S_{BET}, pore volume and pore diameter, as consequence of the functional groups attached in their surface or inside the mesopores. On the other hand, the pore diameter distribution of the materials (insets in Fig. 1a-d) was narrower for SBA-15-LP-C8 (similar to SBA-15-LP) than for SBA-15-LP-NH_2. This fact can be attributed to the presence of the C8 groups mainly on the silica surface and the NH_2 groups on the interior of the mesopores.

EA of the materials revealed different functionalization degree (L_0) in the mesostructured silicas. Thus, regarding the %C and %N in the mono-functionalized silicas, it was estimated the presence of 0.25 mmol g^{-1} of C8 groups and 0.93 mmol g^{-1} of NH_2 groups attached on the SBA-15-LP-C8 and SBA-15-LP-NH_2, respectively. These results revealed that the functionalization with NH_2 groups allows a higher L_0 of the mesostructured silicas, in comparison with the functionalization with C8 groups, probably because of its shorter chain. On the other hand, regarding de %C and %N in the bi-functionalized mesostructured silica (SBA-15-LP-C8-NH_2), it was estimated the presence of 0.24 mmol g^{-1} of C8 and 0.36 mmol g^{-1} of NH_2.

Fig. 2 show the solid state ^{29}Si MAS-NMR spectrum for SBA-15-LP (Fig. 2a) and SBA-15-LP-NH_2 (Fig. 2b) that confirm the union between the ligand and silanol groups on the surface of silica. SBA-15-PE showed three peaks at -110, -101 and -95 (*sh*) ppm

assigned to Q^4 ($(SiO)_4Si$), Q^3 ($(SiO)_3SiOH$) and Q^2 ($(SiO)_2Si(OH)_2$) silanol sites. Q^4 is the most abundant peak which is characteristic of materials with a great degree of condensation. The spectrum of SBA-15-LP-NH_2 (Fig. 2b) showed a decrease in the intensity of Q^3 and Q^2, which verified the functionalization of the material through the silanol groups. Moreover, two new peaks at -62 and -52 (*sh*) ppm were assigned to T^2 ($(SiO)_2$ *Si*OH-R) and T^3 ($(SiO)_3$ *Si*-R) sites corresponding to the union of the organic ligand with the silica. The ^{13}C-CP-MAS-NMR spectrum for SBA-15-LP-NH_2 (Fig.2c) showed five signals due to C atoms denoted as C1 - C5 at 14, 57, 7, 24, 42 ppm, respectively. These peaks correspond to the expected immobilization of the 3-aminopropyl-triethoxysilane on the silica surface. Finally, SEM images (Fig.2d) were used to determine the morphology of the material and the particle size. The SBA-15-LP-NH_2 material presented spherical or quasi-spherical particles with sizes between 50 nm and 250 nm grouped in cluster forms (of around 880 x 940 nm). Fig. 1S (supplementary material) shows the histogram of particle size distribution in these clusters.

3.2 HMF standard solution stability

A stability study was carried out to check the possible degradation of HMF. To carry out this study, HMF standard solutions stored under different conditions were analyzed by HPLC-MS/MS. Each day, for five days, two standard solutions of 2.6 mg L^{-1} and 10 mg L^{-1} of HMF in MeOH were analyzed. The HMF standard solutions were stored in freezing (-18 ºC), in refrigeration (5 ºC) and at room temperature (20-25 ºC). Peak area obtained showed a RSD < 10%, for both levels in the different conditions tested, demonstrating that this compound is stable under the storage conditions and time evaluated.

3.3 Optimization of SLE and SPE conditions

Cereal and insect bars have very complex matrices due to the high number of ingredients that make them up, which provide lipids, proteins and carbohydrates, making difficult their analysis. In this sense, the development of the extraction and clean up methodology is important, so in this work different sorbent materials were applied to purify the sample extracts by SPE prior to analysis by HPLC.

Firstly, a SLE was performed and different polar extraction solvents were tested, taking advantage of the high polar nature of the analyte. Solvents evaluated (with 0.1 mL of Carrez I and II) for SLE were: MeOH, water acidified with HAC (pH 3.3) and water acidified with HCl (pH 1.0). SPE conditions were: 50 mg of sorbent (SBA-15-LP, SBA-15-LP-NH2, SBA-15-LP-C8 and SBA-15-LP-C8-NH2), 2 mL of the extraction solvent (conditioning), 1 mL of the extract after the SLE process (charge), 2 mL of Hex or 2 mL of water (washing) and 3 x 3 mL MeOH (elution). The conditioning step was always selected according to the solvent used during the SLE, considering that the pH of the cartridge must be the same as that of the extraction medium, so that the functional groups in the silicas surface were activated and interacted with HMF during the loading stage.

Table 2 shows the results obtained in these experiments. As it can be seen, low recovery percentages were obtained with MeOH as SLE solvent (22 - 43%), which can be attributed to the high amount of matrix interferences extracted with this solvent. On the other hand, with acidified water (pH 3.3 with 0.1% HAC) results showed an improvement, in all cases, but especially with the most polar SPE sorbents (85 ± 2% for SBA-15-LP and 72 ± 2% for SBA-15-LP-NH_2). In addition, water acidified to pH 1.0 (with HCl) was also evaluated as SLE solvent. In these conditions, the best recoveries

were achieved, and for SBA-15-LP-NH_2 material recovery percentages near 100% were found (sorbent washing with Hex). In the case of SBA-15-LP sorbent, satisfactory mean recovery percentages were also achieved, under similar conditions, but with large dispersion in the results (88 ± 29%). This fact can be attributed to the physical characteristics of the SBA-15-LP, so the high static electricity and the agglomeration of the material caused non-homogeneous packaging of the cartridge, generating preferential channels that affect good results. Finally, water was also evaluated as wash solution according to other works [5, 11]. It is well known that in SPE, the washing step is very important. In this stage impurities are rinsed through with wash solutions that are strong enough to remove them, but weak enough to leave the target analyte behind the cartridge. Hex was firstly selected as washing solvent, in order to eliminate fats from the cartridge, and as it can be seen in Table 2 recoveries between 64 and 98% were obtained in these conditions. However, lower recovery percentages (0 to 23%) were observed when water was used for this purpose, this fact can be due to the breakdown of the analyte-sorbent interaction due to high polarity of the HMF.

Additionally, non-acidified water and ACN were also tested as SLE solvents (only with SBA-15-LP-NH_2 as SPE sorbent). Water extracts showed turbidity after centrifugation process, showing low recovery percentage (26 ± 9%). The turbidity of the extract can saturate the SPE cartridges, and this can explain that the analyte recoveries were reduced. Furthermore, the ACN solvent was tested showing a clean supernatant after SLE, but recovery percentages were near to zero. Finally, the amount of Carrez I and II in the SLE stage was increased from 0.1 mL to 0.5 mL (using acidified water pH 1.0 as solvent extraction and SBA-15-LP-NH_2 as SPE sorbent) and the recovery percentage decreased from 98 to 62 %. This fact can be due to an adsorption of the analyte in the precipitate surface formed by these reagents.

Secondly, once SLE stage was optimized (water acidified to pH 1.0 with HCl containing 0.1mL of Carrez I and II) and SBA-15-LP-NH_2 was selected as the best sorbent (and Hex as washing solvent), other SPE conditions were re-optimized. Thus, during loading, 5 and 1 mL extract volumes were tested, obtaining similar recovery percentages. For this reason, 1 mL of extract was selected for the loading stage. Regarding elution, 1 x 3 mL of MeOH was evaluated and compared with 3 x 3mL MeOH, showing that recoveries near 100% were achieved in both cases. For this reason, 1 x 3 mL of MeOH was selected for the elution stage.

The different composition of the materials, in terms of the type of ligand and functionalization degree, means that they are not going to have a similar adsorption performance as can be seen in Table 2. Firstly, in the case of the SBA-15-LP-NH_2 material, we can find several types of interactions. The main interactions are ion-dipole, between the positively charged amino groups of the surface and the polar molecules of HMF. At the same time, because we are working at pH lower than the PZC of the silica (point of zero charge, between 2 - 4) the surface charge of the adsorbent (SBA-15-LP-NH_2) would be positive, so the polar molecules of HMF can also be adsorbed by the silica surface, again through ion-dipole interactions. Therefore, the high amount of amino groups (0.93 mmol/g) in this material and the ion-dipole interactions with the charged surface explains the biggest recovery observed (98 ± 2). Secondly, in the case of the SBA-15-LP-C8 the main interactions that we can encounter are the hydrophobic ones, between the alkyl organic chain of the C8 groups and the HMF, being this latter interaction weaker than the former one (ion-dipole). In addition, the ion-dipole forces between the material surface and the HMF could be also found. In this material, the sum of all the possible interactions is weaker than in the case of SBA-15-LP-NH_2 being, therefore, the recovery percentage of HMF lower (72 ± 15). Finally, in the case of SBA-15-LP-C8-NH_2, it would

be expected to be found the highest recoveries of HMF, because all the above stated interactions are presented. Nevertheless, the recoveries obtained are the lowest (64 ± 1). This fact can be explained as C8 groups are bulkier than the amino groups, provoking steric hindrance, that does not allow that all the amino groups can interact with the HMF. Therefore, the ion-dipole interactions are much weaker in this material than in SBA-15-LP-NH_2. Even the surface is less accessible than in SBA-15-LP-C8, because of the higher functionalization degree (0.36 mmol/g of C8 in SBA-15-LP-C8-NH_2 instead of the 0.25 of C8 in SBA-15-LP-C8). Therefore, in this material, the main interactions are only the hydrophobic ones, and because of the lower C8 functionalization degree, the lower HMF recovery percentage of all the materials evaluated in this work.

Finally, a last study was carried out comparing the material that offered the best results (SBA-15-LP-NH_2) with a commercial one (Discovery® DSC-NH_2) functionalized with the same groups (aminopropyl phase polymerically bonded on amorphous silica). Fig.3 compares the commercial material with the best material proposed in this article, showing in all cases better recoveries for the SBA-15-LP-NH_2 silica. In the best conditions of SLE (water acidified to pH 1 with HCl and 0.1 mL Carrez l and II), the SBA-15-LP-NH_2 has a recovery near to 100%, while the commercial sorbent only has 59 ± 1%. These differences can be attributed to the greater degree of functionalization for SBA-15-LP-NH_2 (0.93 mmol ligand g^{-1}) than for the commercial sorbent (0.83 mmol ligand g^{-1}). Moreover, the good textural properties of the mesostructured silicas (Table 1), such as high surface area, pore volume and pore diameter with respect to the amorphous silica (commercial material), provide better recovery percentages with this material.

3.4 Instrumental and method validation

Firstly, standard working solutions were analyzed in the HPLC-MS/MS to evaluate the instrumental linear range, repeatability, reproducibility, detection and quantification limits (LOD a LOQ). A linear range between 0.5 and 100 mg L^{-1} ($y = 1.68 \cdot 10^8 x + 3.99 \cdot 10^9$, $R^2 \geq 0.993$) was found (supplementary material, Table S1). Repeatability and reproducibility were evaluated at three levels of concentration. Good precision was achieved with RSD (%) values between 1.7 and 2.2% for repeatability and between 2.3 and 4.6% for reproducibility. For LOD and LOQ, low values were obtained (18 μg L^{-1} and 59 μg L^{-1}, respectively).

Method validation was performed for the assessment of the linear range, precision, accuracy, method detection and quantification limits (MDL and MQL) and robustness (Table SM1). Firstly, matrix-matched calibration curves were obtained and good linear regression ($y = 1.23 \cdot 10^8 x + 2.30 \cdot 10^8$) was found ($R^2 \geq 0.995$). Low RSD (%) values were observed (between 6 - 8%) for method repeatability and reproducibility, which demonstrated its good precision (Table SM1). Recovery percentages that ranged between 99 to 102%, demonstrated good accuracy of the method for the three evaluated levels. Low values were obtained for MDL (11 μg kg^{-1}) and MQL (38 μg kg^{-1}).

In order to evaluate robustness of the method, three different batches of the SBA-15-LP-NH_2 material were synthesized (on different days), characterized and evaluated as sorbents under the optimized conditions (see section 3.3). As it can be seen in Table 3, the materials showed very similar functionalization degree, pore volume, pore diameter and surface area. After the SLE, SPE and HPLC-MS/MS protocol, recoveries obtained were 101 ± 3 (batch 1), 97 ± 4 (batch 2) and 96 ± 2 (bach 3). These results confirmed the good robustness of the method.

The analytical performance of the proposed method was compared with other methods previously published (Table 4). As to date, there are no scientific articles in the literature that describe the use of mesostructured silica as SPE sorbent for HMF determination. In addition, there are not studies that analyse HMF in cereal and insect bars. Therefore, we have compared the developed method with other proposed methods [5, 11, 12, 17, 19] used for the determination of HMF in different food samples (e.g. cereal-based baby foods, coffee, juice, breakfast cereals, toasts, honey, chocolate, biscuits). In these methods, commercial SPE sorbents (e.g. Oasis® HLB, Bon Elut® C18, Strata® X, Isolute® ENV+, etc.) were used after SLE, and analysis was carried out by HPLC, GC or MECK. Compared with other methods reported in the literature, the proposed method displayed similar [11] or lower [5, 12, 17, 19] MDLs than other protocols. Additionally, with the proposed method best recoveries were obtained, despite the analysed bar samples have high complexity based on their composition (high amounts of starch, sugars, proteins and fats).

3.5 Application of the method

Different samples of cereal and insect bars were analysed to demonstrate de applicability of the proposed method (the list of ingredients for each bar is showed in supplementary material, Fig. 4, Table 5). Results obtained for the cereal and insect bars analysed are summarised in Table 5, besides the HMF content of other foods previously reported in the literature [6, 11, 12, 19, 20, 23]. Additionally, and for comparative purpose, a sample of breakfast cereals (rice and wheat-based cereals) was analysed with the proposed method. As it can be seen in Table 5, HMF was found at concentrations levels between 336 to 962 mg kg^{-1} in the cereal and insect bars. For breakfast cereals, the

HMF concentration was 88 mg kg^{-1} that is in agreement with those reported in the literature for this kind of samples [6, 11].

The high levels of HMF in cereal and insect bar samples can be due to different chemical reactions (MR and caramelization) that occurs during their processing, but also as a result of its presence in high amounts in some of the bars ingredients (see Table S2). In fact, these bars are prepared with extruded cereals, and different studies have confirmed that the extrusion of cereals can generates high amounts of HMF in the final product [13]. Moreover, other ingredients such as dried fruits, syrups and chocolate can contribute to the high levels of HMF in cereal and insect bar samples. As it can be seen in Table 5, Murkovic and Pichler [19] found up to 2200 mg kg^{-1} of HMF in dried fruits. Corn or cane syrups, widely used in these food products as sweeteners and because their addition provide stability to the bar, have HMF levels between 407 and 2121 mg kg^{-1} according to Andrade et al. [23]. Finally, chocolate is another ingredient that can contribute to the high concentration of HMF in the bars. In fact, the cereal bar with chocolate and orange analysed in this work has the highest concentration of HMF (up to 962 mg kg^{-1}) In these sense, Teixidó et al. [12] found up to 165 mg kg^{-1} in milk chocolate (Table 5).

4. Conclusions

Functionalized mesostructured silicas have been evaluated for the first time as sorbents in sample preparation for the analysis of HMF in foods. An efficient SLE and SPE extraction procedures were achieved using SBA-15-LP functionalized with aminopropyl- groups as sorbent in the SPE step. The proposed SLE, SPE and HPLC-MS/MS method showed good linearity, precision, accuracy, robustness, detection and quantitation limits. Its applicability was demonstrated for the determination of HMF in

cereal and insect bars. Moreover, as HMF has been evaluated for the first time in this type of foods, the high HMF values observed (between 336 to 962 mg kg^{-1}) suggest that more studies are need to develop a reliable database for the HMF content in different commercial cereal and insect bars.

Declaration of Competing Interest

The authors have declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version.

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Figure captions:

Fig. 1. Nitrogen adsorption-desorption isotherms and pore size distributions (inset) of: a) SBA-15-LP, b) SBA-15-LP-C8-NH_2, c) SBA-15-LP-C8 and d) SBA-15-LP-NH_2.

Fig. 2. Characterization of functionalized mesostructured silica: a) ^{29}Si PDA-MAS-NMR spectra of SBA-15-LP, b) ^{29}Si PDA-MAS-NMR spectra of SBA-15-LP-NH_2, c) ^{13}C CP-MAS-NMR spectra of SBA-15-LP-NH_2 and d) SEM of SBA-15-LP-NH_2.

Fig. 3. Recovery of HMF in cereal bars under different SLE conditions using SBA-15-LP-NH_2 or a commercial sorbent as SPE sorbent.

Fig. 4. Extracted chromatogram obtained, by the proposed method, for the product ion 108.8 m/z: a) Cereal bar with chocolate and orange, b) cereal bar with hazelnut and c) insect bar with apple, cinnamon and caramel.

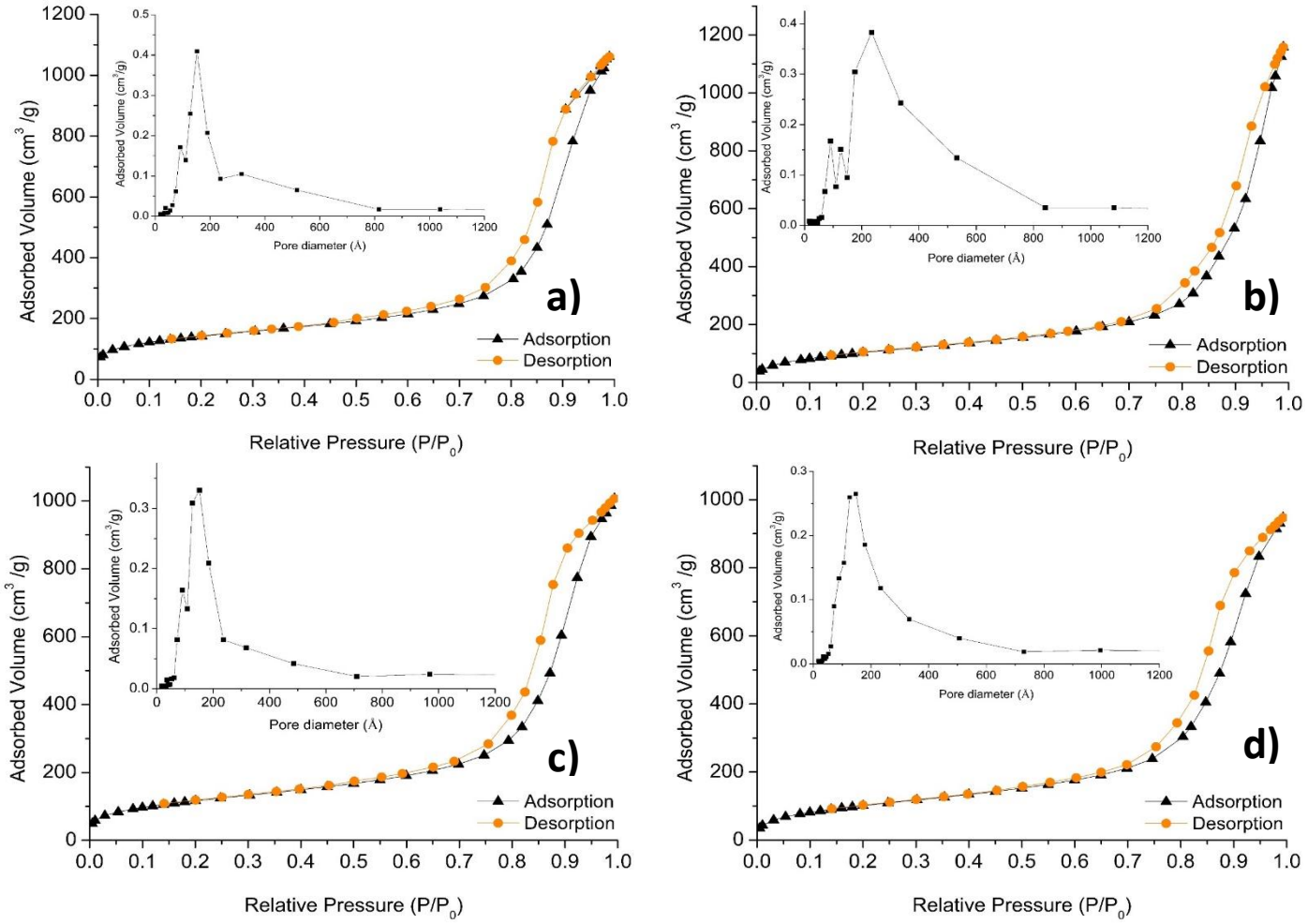
a)
b)
c)
d)
Adsorbed Volume (cm³/g)
Relative Pressure (P/P0)
Adsorption
Desorption
Pore diameter (Å)

Fig. 1

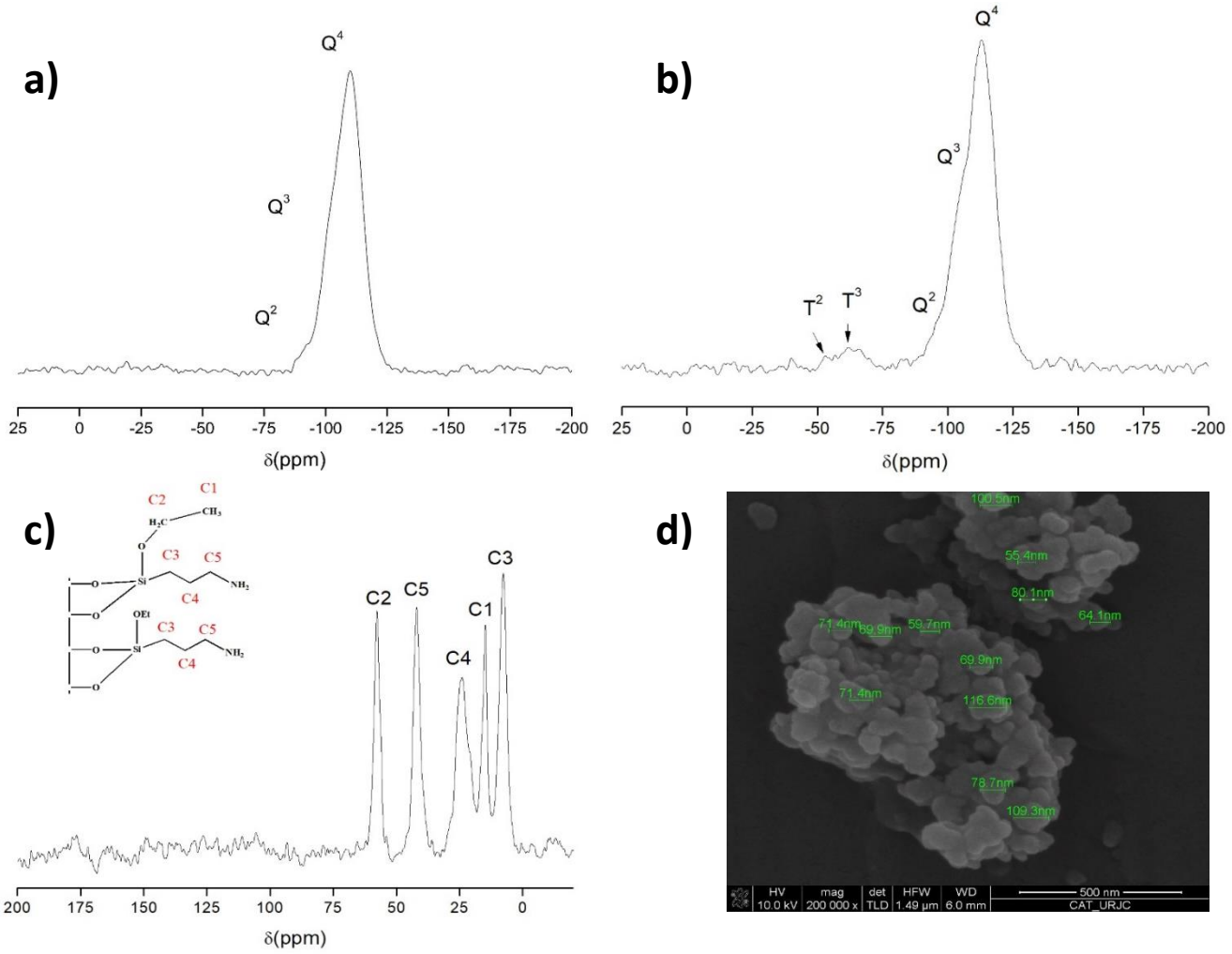
a)
Q4
Q3
Q2
25 0 -25 -50 -75 -100 -125 -150 -175 -200
δ(ppm)
b)
Q4
Q3
T2 T3 Q2
25 0 -25 -50 -75 -100 -125 -150 -175 -200
δ(ppm)
c)
C1 C2 C3 C4 C5
C2 C5 C1 C3 C4
200 175 150 125 100 75 50 25 0
δ(ppm)
d)
100.5nm
55.4nm
80.1nm
64.1nm
71.4nm
69.9nm
59.7nm
69.9nm
71.4nm
116.6nm
78.7nm
109.3nm
HV 10.0 kV
mag 200 000 x
det TLD
HFW 1.49 µm
WD 6.0 mm
500 nm
CAT_URJC

Fig. 2

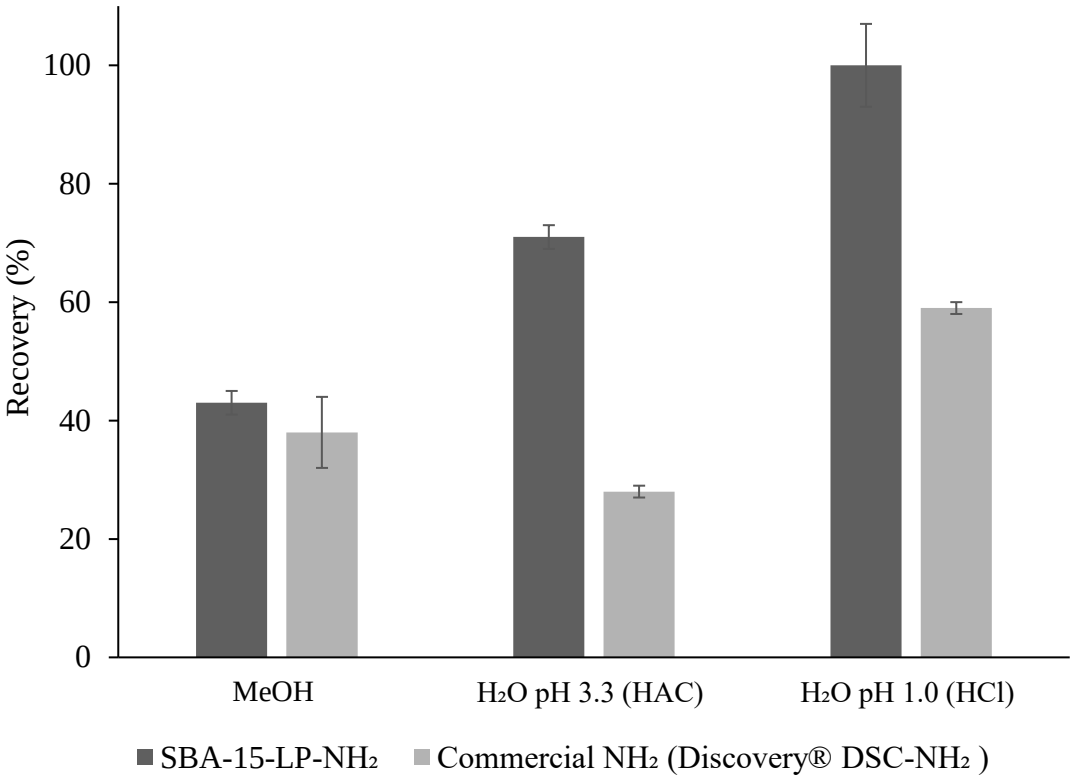
100
80
60
40
20
0
Recovery (%)
MeOH
H2O pH 3.3 (HAC)
H2O pH 1.0 (HCl)
SBA-15-LP-NH2
Commercial NH2 (Discovery® DSC-NH2)

Fig 3.

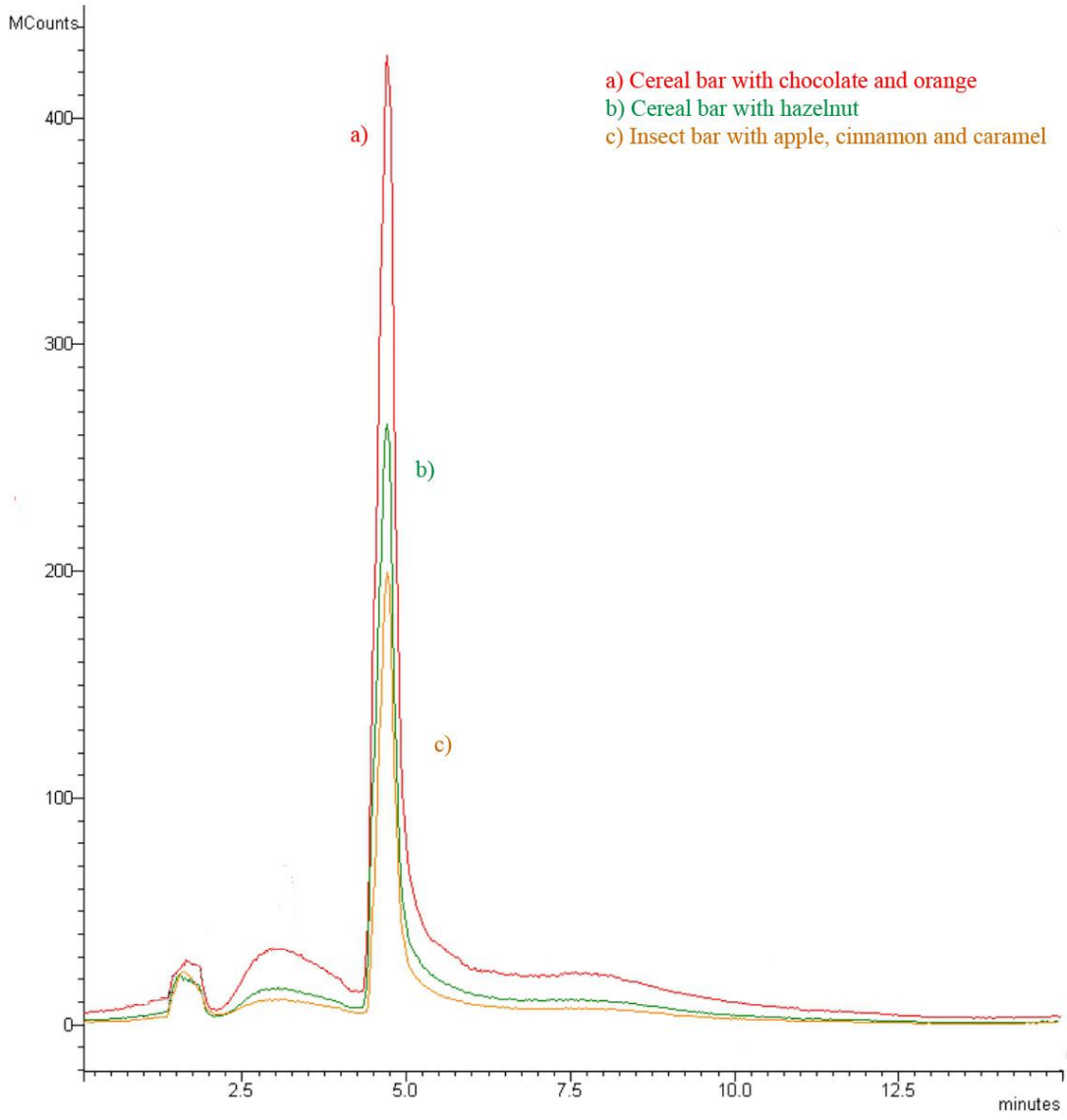
MCounts
400
300
200
100
0
a)
b)
c)
a) Cereal bar with chocolate and orange
b) Cereal bar with hazelnut
c) Insect bar with apple, cinnamon and caramel
2.5
5.0
7.5
10.0
12.5
minutes

Fig. 4.

Table 1

Textural properties of different materials.

Material	S_{BET} (m^2 g^{-1})[a]	Pore volume (cm^3 g^{-1})[b]	Pore size (Å)[c]	L_0 (mmol ligand g^{-1})[d]
SBA-15-LP	510	1.6	38 153	-
SBA-15-LP-NH_2	382	1.4	36 148	0.93
SBA-15-LP-C8	429	1.5	37 152	0.25
SBA-15-LP-C8-NH_2	389	1.6	36 126	0.24 (NH_2) 0.36 (C8)
Discovery® DSC-NH_2	293	0.7	72	0.83

[a] S_{BET}: Specific surface area calculated by Brunauer-Emmett-Teller (BET) method.

[b] Pore volume: Total pore volume was measured at relative P/P_0=0.97.

[c] Pore size: Pore diameter estimated by Barret-Joyner-Halenda (BJH) model applied in the desorption branch.

[d] L_0: Functionalization degree estimated by elemental analysis.

Table 2

Recovery of HMF obtained in the analysis of cereal bars under different LLE-SPE conditions using bare and functionalized mesostructured silicas as sorbents.

	Recovery (% ± SD)			
Sorbent	MeOH [a]	H_2O [a] (pH 3.3, HAC)	H_2O [a] (pH 1.0, HCl)	
	Hex[b]	Hex[b]	Hex[b]	Water[b]
SBA-15-LP	26 ± 3	85 ± 2	88 ± 29	23 ± 1
SBA-15-LP-NH_2	43 ± 2	71 ± 2	98 ± 2	4 ± 1
SBA-15-LP-C8	22 ± 1	54 ± 1	72 ± 15	19 ± 1
SBA-15-LP-C8-NH_2	34 ± 1	56 ± 1	64 ± 1	0

[a] Extraction solvent with 0.1 mL of Carrez I and II (1 gr of sample).

[b] Washing solvent in the SPE.

Table 3

Robustness of the LLE-SPE-HPLC-MS/MS and textural properties of different batches of SBA-15-LP-NH_2.

Material	S_{BET} (m^2 g^{-1})[a]	Pore Volume (cm^3 g^{-1})[b]	Pore Size (Å)[c]	L_0 (mmol ligand g^{-1})[d]	Recovery (% ± SD)[e]
SBA-15-LP-NH_2-B1	382	1.4	36 148	0.93	101 ± 3
SBA-15-LP-NH_2-B2	345	1.2	36 126	0.74	97 ± 4
SBA-15-LP-NH_2-B3	330	1.2	36 109	0.73	96 ± 2

[a] S_{BET}: Specific surface area calculated by Brunauer-Emmett-Teller (BET) method.

[b] Pore volume: Total pore volume were measured at relative P/P_0=0.97.

[c] Pore size: Pore diameter estimated by Barret-Joyner-Halenda (BJH) model applied in the desorption branch.

[d] L_0: Functionalization degree.

[e] Recovery percentage under optimized conditions

Table 4

Comparison of proposed method with others that use commercial cartridges for the determination of HMF.

Sample	Extraction procedure	Sorbent (amount)	Analytical technique	Recovery (%)	MDL[a]	[Ref.]
Cereal-based baby foods	SLE-SPE	Oasis® HLB (30 mg)	HPLC-MS	91.8 - 94.7 (cereal- based baby foods)	Not shown	[5]
Dried fruits, fruit bread and coffee	SLE- SPE	Bon Elut® C18 (200 mg)	HPLC-UV	89 (coffee)	35 µg kg^{-1} (coffee)	[19]
Orange juice, multifloral honey, breakfast cereals, plum jam, biscuits and oranges	SLE-SPE	Oasis® HLB (200 mg) Strata® X (200 mg) Bon Elut® C18 (100 mg) ENV+ (200 mg) ENVI-18 (200 mg) Discovery® DSC-18 (100 mg)	GC-MS	100 (standard solution, ENV+)	12 µg kg^{-1} (orange juice)	[11]
Orange juices, honey, breakfast cereals, plum jams, biscuits and oranges	SLE-SPE	Isolute® ENV+ (200 mg)	HPLC-MS/MS	Not shown	276 µg kg^{-1} (breakfast cereals) 235 µg kg^{-1} (biscuits)	[17]

					141 µg kg^{-1} (orange juice) 157 µg kg^{-1} (honey) 216 µg kg^{-1} (jam)	
Breakfast cereals, toasts, honey, orange juice, apple juice, jam, coffee, chocolate, biscuits	SLE-SPE	Isolute® ENV+ (200 mg)	MECK-DAD[b]	Not shown	0.7 mg kg^{-1} (orange juice)	[12]
Cereal and insect bars and breakfast cereals	SLE-SPE	SBA-15-LP (50 mg) SBA-15-LP-NH_2 (50 mg) SBA-15-LP-C8 (50 mg) SBA-15-LP-C8-NH_2 (50 mg)	HPLC-MS/MS	99 - 102 (cereal bar with hazelnut, SBA-15-LP-NH_2)	11 µg kg^{-1} (cereal bar with hazelnut)	This work

[a] Method detection limit (MDL)

[b] Micellar electrokinetic chromatography

Table 5

HMF content in different samples.

Food	Type	HMF (mg kg^{-1})	[Ref]
Cereal bar	Cereal bar with chocolate and orange	962 ± 5	This work
Cereal bar	Cereal bar with hazelnut	336 ± 7	
Insect bar	Insect bar with apple, cinnamon and caramel	379 ± 1	
Breakfast cereal	Rice and wheat flakes	88 ± 8	
Corn salty bar	Puffcorn bars	2.2 ± 0.3	[20]
Bar shaped Japanese snack	Salty bar	0.7 ± 1	
Bar shaped Japanese snack	Caramelized bar	91 ± 2	
Dried fruits	Dried plums	1600 - 2200	[19]
Dried fruits	Dried date	1000	
Dried fruits	Dried apple	80	
Bread	Bread with dried fruits	450	
Chocolate	With milk A	87 ± 10	[12]
Chocolate	Dark A	42 ± 9	
Chocolate	With milk B	165 ± 34	
Chocolate	Dark B	76 ± 13	
Chocolate	White	99 ± 14	
Syrup	Corn syrup	407 - 2121	[23]
Syrup	Cane syrup	109 - 893	
Breakfast cereal	Honey rings	47 ± 2	[11]
Breakfast cereal	Corn flakes	25 ± 1	
Breakfast cereal	Rice	49	[6]
Breakfast cereal	Wheat and corn	12	
Breakfast cereal	Wheat	29 - 48	
Breakfast cereal	Corn	28 - 61	

Supplementary material

Hydroxymethylfurfural determination in cereal and insect bars by HPLC-MS/MS employing a functionalized mesostructured silica as sorbent in solid-phase extraction

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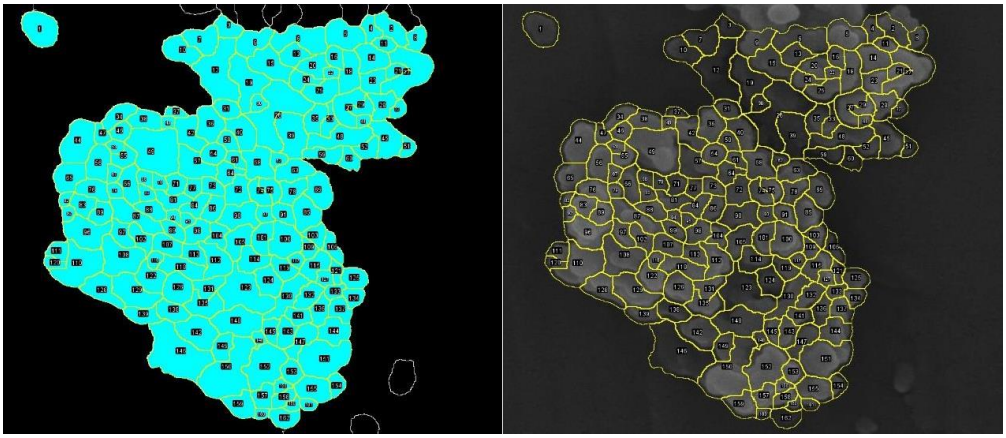
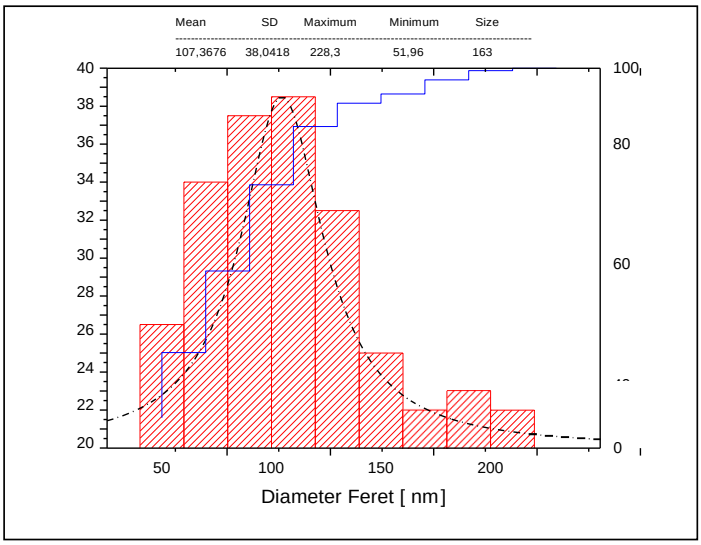
a)





b)

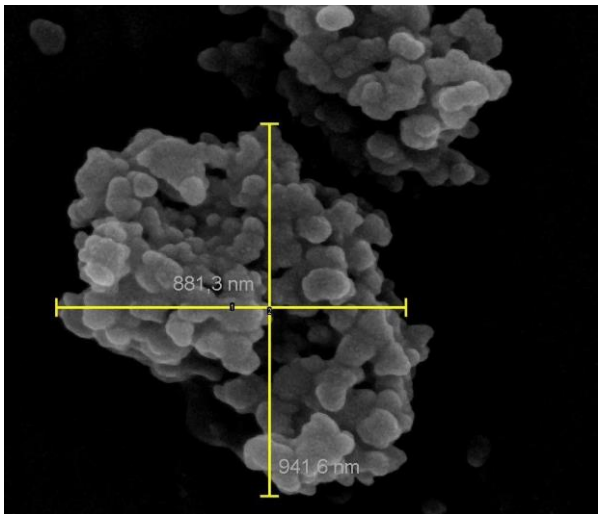


Figure S1. a) Histogram of particle size distribution and b) dimension of the clusters.

Table S1

Analytical characteristics of the developed method.

Parameters		Instrumental parameters [a]	Method parameters [b]
Linear range		0.5-100 mg L^{-1}	0.77 - 100 µg g^{-1}
Linearity		$y = 1.68 \cdot 10^8 x + 3.99 \cdot 10^9$	$y = 1.23 \cdot 10^8 x + 2.30 \cdot 10^8$
R^2		0.993	0.995
Recovery (% ± SD)	Low	-	100 ± 7
	Medium	-	99 ± 4
	High	-	102 ± 4
Repeatability (% RSD)	Low	2	6
	Medium	2	7
	High	2	8
Within-laboratory reproducibility (% RSD)	Low	2	7
	Medium	5	6
	High	4	8
LOD/ LOQ [c]		18 µg L^{-1} / 59 µg L^{-1}	-
MDL/MQL [d]		-	0.011 µg g^{-1} / 0.038 µg g^{-1}

[a] HMF working standard solution (prepared in MeOH). Speaking levels: 0.5 mg L^{-1} (low), 2.6 mg L^{-1} (medium), 10 mg L^{-1} (high).
[b] Matrix-matched standard solution (prepared in cereal bar matrix). Speaking levels: 7.7 µg g^{-1} (low), 40 µg g^{-1} (medium), 153 µg g^{-1}(high).
[c] Limit of detection (LOD) and limit of quantification (LOQ).
[d] Method detection limit (MDL) and method quantification limit (MQL).

Table S2. Ingredients of samples

Samples	Ingredients
Cereal bar with chocolate and orange	Wheat flour (18%), glucose and fructose syrup, rolled oatmeal flakes (9%), chocolate chips (8%), corn (7%), sugar, rice (5%), rice flour (3%), sunflower oil, juice orange (1%), lean cocoa powder, fructose syrup, concentrated apple puree, moisturizer, wheat starch, dextrose, salt, wheat malt flour, barley malt flour, emulsifier: sunflower lecithin, malt extract barley, acidulant (E330), natural orange aroma, orange pulp, cocoa butter, gelling agent: pectins, aroma
Cereal bar with hazelnut	Integral oatmeal flakes (32%), glucose and fructose syrup, corn flakes (8%), roasted hazelnuts (8%), extruded wheat and rice (6%), rice flakes (6%), sunflower oil (1%), hazelnut paste, maltodextrin, emulsifier: sunflower lecithin, natural aroma.
Insect bar with apple, cinnamon and caramel	Cricket flour, apple, cinnamon and caramel.
Breakfast cereals	Rice, wheat, sugar, barley, malt flour, aroma and salt.