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Versatile and reliable extraction of phytosterols employing sonochemical synthesized molecularly imprinted polymer

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ABSTRACT

Phytosterols (PSs) are bioactive compounds in the sterol family, present in numerous complex food and plant matrices in free and conjugated forms. The interest in these compounds arises for phytotherapeutic purposes, particularly for their action on cholesterol metabolism and impact on cardiovascular diseases. There is a need to develop approaches that can selectively extract target analytes and accurately identify and quantify them with high precision. This work proposed the synthesis of molecularly imprinted polymers (MIPs) for PSs with a sonochemical approach, enabling a rapid polymerization step (5 min). This proposed MIP was able to extract 8 PSs (brassicasterol, stigmastanol, campesterol, campestanol, stigmasterol, β-sitosterol, Δ^5-avenasterol, α-spinasterol) from a wide range of plant and food matrices belonging to different classes (Brassicaceae, dried fruits and Leguminosae) and was coupled to ultra-high liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS). MIP based on dispersed solid phase extraction (dSPE-MIP) and targeted analysis has proven to be particularly effective in addressing the challenges associated with the complexity of plant-derived matrices, minimising interferences. This was demonstrated by the excellent control of the matrix effect, which was within ±15 %, ensuring the robustness and reliability of the method. The identification and quantification of 8 different PSs was successfully achieved with satisfactory recovery values ranging from 65 % to 100 %. The proposed strategy offers an affordable alternative to classical methods, providing enhanced sensitivity, selectivity and overall performance.

1. Introduction

Phytosterols (PSs) are bioactive compounds found abundantly in plant-based sources such as seeds, roots, leaves and steams [1]. PSs encompass various sterols, such as β-sitosterol, campesterol and stigmasterol, and stanols like sitostanol and campestanol; these are structurally similar to cholesterol having a steroid core with a hydroxyl group in the C_3 position and a side chain in the C_{17} position but differ for the presence of a methyl or ethyl group in the C_{24}. Differences in side chain structures confer unique properties and functionalities to each of PSs. For example, β-sitosterol, which has a saturated side chain, is a potential agent for cardiovascular risk management, since it modulates cholesterol adsorption, while campesterol and stigmasterol, with unsaturated side chains, exhibit anti-inflammatory actions [2]. Moreover, they have been shown to have others pharmacological properties, including anti-obesity, antidiabetic, antimicrobial, immunomodulatory, and potentially anti-cancer effects; this identify them as promising components for the formulation of healthy foods to prevent chronic diseases and promote overall well-being [3]. Despite their promising attributes, their use in food remains a challenging task due to their low water solubility and poor stability [4]; the successful integration of PSs into functional foods requires meticulous consideration of formulation parameters and the selection of appropriate food matrices.

These complex aspects related to their structure and lipophilic characteristics have also influenced their determination. PSs are usually analysed by gas chromatography (GC) coupled with a Flame Ionization Detector (FID) [5–7], or mass spectrometry (MS) [8–10]; however, the low volatility of the compounds makes derivatization mandatory. In recent years, the combination of MS and tandem mass spectrometry (MS/MS) with liquid chromatography (LC) has emerged as a powerful

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tool for the identification and quantification of PSs in food matrices, since high sensitivity, selectivity, and ability for simultaneous identification and quantification of many analytes [11–19]. This technique offers exceptional resolution, allowing for the separation of complex mixtures of PSs and other compounds present in several matrices; however, currently published methods employ very long chromatographic runs (about 30 min) and allow simultaneous identification of a few number of PSs [12,14,20].

For the extraction phase, analytical techniques characterized by a high selectivity and specificity are considered in order to ensure maximum extraction yields, especially for complex matrices, such as plants and food. The presence in several matrices, ranging from plant tissues to aqueous environments, demands selective extraction methods that may effectively discriminate PSs from other co-existing compounds [4,21]. Conventional extraction techniques, such as solid-liquid extraction (SLE), although effective in separating components from a solid mixture, have problems such as variability in extraction efficiency, risk of solvent contamination and low selectivity. The most widely used technique for PSs extraction is solid phase extraction (SPE); in fact, it is characterized by a high selectivity and it is suitable for different uses depending on the type of stationary phase [22]. The most frequently used SPE sorbents for trace enrichment of PSs include alkyl-bonded silicas (such as C18 and C2 silica), copolymer sorbents like cross-linked polystyrene-divinylbenzene and hydrophilic-lipophilic balanced polymers [23–26]. However, the SPEs are characterized by some limitations, such as high costs and selectivity associated with the type of stationary phase chosen. Moreover, to explode the high selectivity of SPE a deep fine-tuning of the protocol is required. For example, working with a C18 cartridge, it is possible to adsorb more apolar molecules than polar, obtaining a reduction in yield; an inverse condition is obtained by working with cartridges differently functionalized, for example polymer-based, which allows to work on a wide range of polarity, increasing the matrix effect due to the adsorption of interferents with characteristics similar to the target molecules [27,28].

In the literature, molecularly imprinted polymers (MIPs) have been widely used with sample preparation techniques especially SPE and dispersion (dSPE); MIPs emerged as a revolutionary method for the selective extraction of target analytes from complex mixtures and environmental sources [29]. This inherent selectivity minimizes interference from co-existing compounds, thereby improving the purity and yield of extracted analytes [30,31].

In the synthesis of MIPs, the selection of different constituents is a key aspect of achieving the optimal performances [32]; the selection of the proper template and functional monomer represents the main parameter. Studies involving sterol-related MIPs have focused the attention on stigmasterol, brassicasterol and campesterol, using a semi-covalent imprinting strategy with β-sitosterol [33–35], stigmasterol [36,37] or stigmasterol methacrylate as a template [32,36]. In addition, other works have synthesized magnetic MIP beads using β-sitosterol as the template for the selective extraction in biological samples [8]. These MIPs have been achieved using methods such as thermal or ultrasound baths.

Thus, these approaches require a long time for synthesis (>1 h) and are limited to the PSs analysed.

Moreover, magnetic iron oxide-based MIPs can lose their specific extraction efficiency due to rapid aggregation and to formation of large clusters during application in harsh media; usually, to avoid this problem, magnetic materials are coated with stabilizing agents such as silica, metal gold (Au), oleic acid and carbon.

This work employed a high-power ultrasound to quickly produce MIP (5 min), using cholesterol as a dummy template. Compared to other methods, this speeds up chemical reactions, reduces the amount of needed reagents and initiates polymerization efficiently [38]; in addition, the use of cholesterol as dummy template significantly reduces MIP costs compared to the use of a PS standard. The dSPE-MIP procedure resulted in high selectivity for target analytes, significantly reducing the limits associated with the use of conventional SPE methods [39]. In addition, ultra-high performance liquid chromatography analysis coupled with tandem mass spectrometry (UHPLC-MS/MS) was used for the identification and quantification of 8 PSs (brassicasterol, stigmastanol, campesterol, campestanol, stigmasterol, β-sitosterol, Δ^5-avenasterol, α-spinasterol) in food and plant matrices belonging to different classes (Brassicaceae, dried fruits and Leguminosae).

2. Materials and methods

2.1. Chemicals

The selected matrices were purchased by local retailers: dried fruits (almonds, hazelnuts, walnuts and pistachio); Brassicaceae (chard, cabbage, cauliflower and broccoli) and Leguminosae (beans, chickpeas, lentils and peas).

The standards used in our research were: brassicasterol, stigmastanol, campesterol, campestanol, stigmasterol, β-sitosterol, Δ^5-avenasterol, α-spinasterol and were purchased from Vinci-Biochem Srl (Firenze, Italia). The working standard mixtures were prepared by appropriate dilution in methanol (MeOH) and stored at −20 °C.

Methacrylamide (MMA), acrylic acid (AA), methacrylic acid (MAA), 2-vinylpyridine (2-VP), ethylene glycol dimethacrylate (EGDMA), 2,2 azobis-isobutyronitrile (AIBN) and cholesterol were purchased from Sigma-Aldrich (Taufkirchen, Germany). Water (H_2O), MeOH, acetonitrile (ACN), dimethylsulfoxide (DMSO), chloroform ($CHCl_3$), ethanol (EtOH), formic acid (HCOOH), acetone (Ace), toluene (Tol), hexane (Hex), heptafluorobutyric acid (HFBA), acetic acid (CH_3COOH), all UHPLC-MS grade, were purchased from VWR (Radnor, PA, USA).

2.2. Synthesis of molecularly imprinted polymer (MIP)

The synthesis of MIP was conducted following the procedure outlined by Palmieri et al. [39,40] with slight modifications. Briefly, a solution containing 0.5 mmol l^{-1} of a dummy template (cholesterol), dissolved in 20 mL of a 50:50 (v:v) mixture of DMSO:H_2O, was prepared; then, 1.5 mmol l^{-1} of MAA was added and the mixture was gently mixed for 1 hour. Afterwards, 5.19 mmol l^{-1} of EGDMA and 0.12 mmol l^{-1} of AIBN were introduced, and the mixture was positioned at two cm from the bottom of the support, using the probe sonicator Sonifier® SFX550 (Branson, Danbury, CT, USA. Wattage output: 550 W; frequency output: 20 kHz) equipped with a Ø 13 mm disruptor horn. Sonication was carried out at 50 % amplitude, continuously for 5 min. The resulting white powder was subjected to washing with a solution of MeOH 10 % of CH_3COOH to remove the dummy template. The adsorbent powder was then separated by centrifugation and the supernatant was discarded. The obtained powder was dried at 60 °C for 2 h in an oven. The dried MIP was crushed and homogenized using a Precellys® Evolution homogenizer (Bertin Technologies SAS, Montigny-le-Bretonneux, France) at 7500 rpm for 10 cycles with 30 s stop between each one. The same procedure was performed for non-imprinted polymers (NIP) without a dummy template.

2.3. Characterization of MIP adsorption capacity

For binding experiments, 10 mg of MIP-MAA were mixed with a standard solution containing 8 PSs (100 ng g^{-1}) and stirred at room temperature (300 rpm) for different time intervals (5, 15, 30 and 60 min) and centrifuged at 12,000 g for 5 min at 10 °C; the supernatant was analysed by UHPLC-MS/MS system to assess the content of PSs not adsorbed by dispersion MIP (dSPE-MIP).

The equilibrium adsorption capacity (Q_e, mg g^{-1}) of both MIP and NIP was calculated using the equation:

$$Q_e = \frac{C_i - C_e}{m \times V}$$

where C_i and C_e are the initial and equilibrium concentrations (mg l^{-1}) of the PSs, respectively, V is the volume of the adsorption solution (L), and m is the weight of MIP or NIP (g).

For isothermal binding experiments, 10 mg of MIP-MAA were suspended in 1 mL of PSs solution with concentration levels ranging from 0.5 to 1000 ng mL^{-1}. After agitation for 30 min, the suspension was centrifuged and the residual PSs concentration was determined using UHPLC-MS/MS.

Moreover, to investigate the effect of polymer quantity, different amounts of polymer (1, 3, 5 and 10 mg) were incubated with 50 ng mL^{-1} of PSs solution for 30 min.

2.4. Morphological characterization

For the physical characterization, MIP and NIP were mounted on aluminium pegs and sputter-coated using an Emitech K950 metallizer. The metallizer was set to 3 V, 80 A, with 3 pulses of 3 s each, producing a graphite coating approximately 25 nm thick.

Subsequent scanning electron microscopy (SEM) analysis was performed using a FEI Quanta 400 scanning electron microscope from FEI Company. Secondary electron images were captured at magnifications ranging from 80X to 1600X, with the SEM operating at 20 kV.

2.5. PSs extraction

The procedure was applied on dried fruits (almonds, hazelnuts, walnuts and pistachio), Brassicaceae (chard, cabbage, cauliflower and broccoli) and Leguminosae (beans, chickpeas, lentils and peas). Initially, each sample was previously lyophilized, to remove the water component, and blended to ensure greater homogenization; except for the dried fruits, which were directly homogenized with a blender.

1 g of each sample was then weighed and extracted with 5 mL of $CHCl_3$:H_2O (2:1 v:v) by ultrasonic water bath (UAE) for 30 min at 25 °C and centrifuged for 10 min at 10,000 g; the supernatant was taken and the pellet was extracted again under the same conditions. The supernatants were pooled and the extract was dried by SpeedVac Vacuum Concentrator system (Thermo Fischer, Waltham, Massachusetts, USA) and treated with dSPE-MIP procedure.

2.6. MIP-based dispersive solid-phase extraction (dSPE-MIP)

The dried samples were resuspended in 1 mL of H_2O:ACN solution (90:10 v:v); 10 mg of dried MIP-MAA were weighed and added to sample solution and incubated under orbital shaking for 30 min (300 rpm) and centrifuged at 10,000 g for 10 min at 4 °C. The supernatant was discarded and 1 mL of a MeOH:H_2O:ACN solution (45:5:50 v:v) was added for the desorption phase of the target analytes under orbital agitation (300 rpm) for 15 min. The samples were centrifuged and the eluted analytes were analysed using the UHPLC-MS/MS system.

2.7. UHPLC-MS/MS analysis

An Acquity UPLC H—Class System from Waters Corporation (Milford, Connecticut, United States) coupled with a 4500 QTrap mass spectrometer (Sciex, Toronto, ON, Canada) equipped with an atmospheric pressure chemical ionization (APCI) source was used. A Kinetex 2.6 μm Polar C18 (100 mm × 2.1 mm) column (Phenomenex, Torrance, CA, USA) was used for the separation of the analytes. The mobile phases were: MeOH:H_2O (90:10 v:v) (A) and ACN (B). The gradient was set as follows: start with 50 % B; held for 2.0 min; linear increase to 95 % B in 1.5 min; isocratic step at 95 % B for 1 min; back to the initial conditions (50 % B) in 0.5 min. The running time was 7.0 min; the flow rate was set at 0.700 mL min^{-1} and the injection volume was set at 5 μL. For all the selected analytes, instrumental parameters, such as declustering potential (DP), focusing potential (FP), entrance potential (EP), collision energy (CE) and cell exit potential (CXP) were tuned by infusion of each single standard methanolic solution (10 ng mL^{-1}) at a flow rate of 10 μL min^{-1} as shown in Table S1. For each analyte, two precursor ion/product ion transitions were chosen. All PSs were detected in positive ionization with a capillary voltage of 5500 V, nebulizer gas (air) at 40 psi, turbo gas (nitrogen) at 40 psi and source temperature at 500 °C. The acquisition of target analytes was performed in multiple reaction monitoring (MRM) acquisition mode. Data collection and processing were performed by Analyst 1.7.2 software while quantification by Multiquant 3.0.3 (Sciex) software.

2.8. Method validation

The validation of the analytical method for the determination of PSs was carried out on 12 food matrices (almonds, hazelnuts, walnuts, pistachio, chard, cabbage, cauliflower, broccoli, beans, chickpeas, lentils and peas), according to the guidelines established by the U.S. Food and Drug Administration (FDA) [41]. Extensive tests were conducted to evaluate linearity, carry-over, limit of detection (LOD), limit of quantification (LOQ), recovery, matrix effect, accuracy and precision. The samples used for the validation set were obtained by spiking the 12 food matrices with standards at different concentrations.

2.8.1. Calibration standards, quality controls, linearity and carry-over

In order to create calibration curves, calibration standards (CSs) were made in triplicate ($n = 3$) using MeOH: H_2O:ACN solution (45:5:50 v:v) at ten levels (0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 100 and 250 ng mL^{-1}). In addition, quality control (QC_0) samples were prepared in triplicate, as blank to ascertain the endogenous PS concentrations. The reported analytical procedure was then performed for all quality controls (QCs) ($n = 5$, for each concentration) by spiking analytical standards at target concentrations of 25, 150 and 250 μg Kg^{-1}. For each analyte, linearity was assessed from the instrumental limit of quantification (iLOQ) to 250 ng mL^{-1} using the previously mentioned calibration curves that were created on each validation day. The quantifier transition analyte response was calibrated using linear least square regression and standardized to recovery and matrix effect impact. After highly concentrated QC and CS, carryover was evaluated by injecting a blank MeOH:H_2O:ACN solution.

2.8.2. Limit of detection and limit of quantification

LODs and LOQs were determined by normalizing the instrumental limits of detection and quantification (iLODs and iLOQs) with dilution factors, recoveries and matrix effects, because some target analytes could be present as endogenous molecules [42]. The iLODs and iLOQs were estimated using a signal-to-noise (S/N) ratio of 3 and 10, respectively.

2.8.3. Accuracy and precision

QCs were prepared for each concentration and analysed in duplicate on three different days; accuracy was calculated as Bias%, considering the endogenous concentration. For this purpose, the following formula was used:

$$Bias\% = \frac{\overline{x}_i - \left(\mu + \overline{x}_{QC_0}\right)}{\left(\mu + \overline{x}_{QC_0}\right)} \times 100$$

Where $\overline{x}_i$ is the mean of measured concentrations, μ corresponds to the theoretical concentration, and $\overline{x}_{QC_0}$ corresponds to the endogenous concentration for that sample set. Moreover, precision was calculated as the coefficient of variation (CV), by the following formula:

$$CV = \frac{\sigma}{\overline{x}i} \times 100$$

Where σ is the standard deviation and $\overline{x}_i$ is the mean of measured concentrations. For intra-day precision, the values obtained for each day and each QC class were considered. The values of all five days were

considered for each QC group for the determination of intraday and inter-day precision.

2.8.4. Recovery and matrix effects

Recovery (Rec%) and matrix effects (ME%) were evaluated for each QC concentration by spiking matrices before the extraction step and processed with the same procedure; different reference mixtures were made for each QC. The method used to calculate the recovery was based on the ratio between the area of the analytes in the samples spiked before the MIP extraction ($\overline{A}_i$) and the area of the samples spiked after the MIP procedure ($\overline{A}_f$); ME% was calculated as the ratio of the area of the sample spiked after the MIP procedure ($\overline{A}_f$), corrected by the area of the QC_0 ($\overline{A}_e$), and to the reference mixture area ($\overline{A}_r$):

$$Rec\% = \frac{\overline{A}_i}{\overline{A}_f} \times 100$$

$$ME\% = \frac{\overline{A}_f - \overline{A}_e}{\overline{A}_r} \times 100$$

3. Results and discussion

3.1. UHPLC–MS/MS method development

Several experiments were performed for the development of the LC-MS/MS method according to similar approaches with sterol compounds [43,44]. An isocratic approach was employed, using MeOH as phase A and ACN as phase B, with a Kinetex XB C18 column (2.6 µm 100 × 2.1 mm) from Phenomenex (Macclesfield, UK). However, despite using a 30 min chromatographic run, satisfactory separation of the target analytes was not achieved. Therefore, other mobile phases were tested, involving different combinations of aqueous and organic phases and evaluating different types of acids to obtain the most suitable ionization conditions for the target analytes. Moreover, a gradient approach using different phases such as H_2O, H_2O 0.1 % HCOOH, H_2O 0.1 % HFBA as phase A and ACN, ACN with 0.1 % HCOOH, and ACN with 0.1 % HFBA as phase B was evaluated. The same conditions were tested on columns with different stationary phases, specifically with a mixed ligand column ACE Excel 2 C18-PFP (10 cm x 2.1 mm) packed with 2 µm particles from Advanced Chromatography Technologies (Aberdeen, United Kingdom) and a Kinetex Polar C18 column (2.6 µm 100 × 2.1 mm) from Phenomenex.

The results obtained were still unsatisfactory, particularly due to significant tailing phenomena for most analytes and noticeable carry-over. Good results were achieved with a gradient using MeOH as phase A and ACN as phase B. Building upon these conditions, various amounts of isopropanol and H_2O were added to promote analyte distribution in the mobile phase and eliminate carryover. Finally, the best result was obtained using a gradient with MeOH with 10 % H_2O (A) and ACN (B); the Polar C18 column gave good retention and peak shape, thus providing separation of the analytes of interest in 7 min of chromatographic run time (Fig. S1).

3.2. MIPs performance evaluation

To obtain an effective fast MIP synthesis, an evaluation of the functional monomers was performed. To this aim, AA, MAA, MMA and MAA-VP were tested in DMSO using EGDMA as cross-linker and AIBN as initiator. During the MIPs extraction, some important influencing factors were studied to achieve good extraction efficiency, including the shaking rate, adsorption solvent, extraction time, desorption solvent and desorption time.

For the evaluation of adsorption solvents, it was chosen to work with different pH, to increase the adsorption of PSs; for this purpose, H_2O: ACN pH 3 (90:10 v:v), H_2O:ACN (90:10 v:v) and H_2O:ACN (10:90 v:v) were tested; H_2O:ACN (90:10 v:v) resulted in the best solvent for the adsorption capacity of MIP-MAA and MIP-VP-MAA. However, MAA had more specific adsorption capacity for target analytes than the monomer mixture VP-MAA, as shown in Fig. 1. As desorption solvent, ACN:H_2O (50:50 v:v), MeOH:H_2O:ACN (45:5:50 v:v) and EtOH:CH_3COOH (90:10 v:v) were tested; MeOH:H_2O:ACN (45:5:50 v:v) for 15 min was chosen for the best results in terms of time-consuming and recovery.

Comparing the initial results obtained, it is evident that the adsorption and desorption solvents used are different from those reported in the literature; comparable adsorption and desorption capacities are achieved yet. In Table S2 are reported the works present in the literature that use MIPs or magnetic MIPs for selective extraction of PSs. Indeed, Schwarz et al. [24] synthesized stigmasterol-imprinted polymers using covalent imprinting strategies and Hashim et al. [36] synthesized stigmasterol-imprinted polymers using both covalent and non-covalent imprinting strategies, focusing on recognition and selectivity towards stigmasterol. They employed H_2O:ACN (10:90 v:v) and EtOH:CH_3COOH (90:10 v:v) as adsorption and desorption solvents in SPE obtaining 99 % of recovery of stigmasterol. Similarly, Zang et al. [8] prepared β-sitosterol magnetic molecularly imprinted polymer beads for the selective extraction of stigmasterol and β-sitosterol, using toluene and ACN as adsorption and desorption solvents in dSPE obtaining 70–80 % of recovery.

3.3. Kinetic adsorption isotherm capacity

According to the literature [19,32,35], the binding adsorption capacities of MIP and NIP for the 8 studied compounds were systematically investigated as a function of the initial PSs amount in the 0.1–1000 ng mL^{-1} range (Fig. 2). Under increasing PSs concentrations, the PS uptake of the MIP was significantly higher compared with NIP. An outstanding reproducibility was obtained for all the analytes for the whole concentration ranges studied (RSD $\leq$ 2 %, $n = 3$). The imprinting factors (*IF*) of this MIP, defined as the ratio between recoveries on MIP and NIP were also calculated. The data, reported in Fig. 2, indicated that MIP-MAA gave *IF* values higher than 4–5 for the target compounds, demonstrating the superior performance of the MIP compared to NIP. This likely occurred because the MIP has multi-electrostatic interaction sites and spatial structures that recognize the target molecules, while the NIP give rise only to non-specific adsorption. Comparing the literature with our study, it was evident that few works calculated the *IF* for all the analysed PSs (see Table S2). Hashim et al. [36] calculated the *IF* of stigmasterol to be 5.15, while Schwarz et al. [32], evaluating the ability of MIP to selectively capture PSs as stigmasterol, campesterol and brassicasterol, obtained *IFs* of 4, 10.5, and 7.1, respectively. Our MIP underlined a loss of *IF* for specific PSs, but we wish to underline that, in this work, the *IF* was determined on the basis of the binding capacity in a

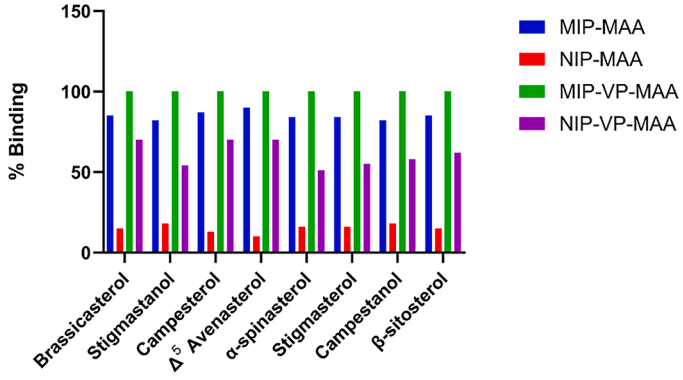

Fig. 1. Histogram of% Binding capacity with H_2O:ACN (90:10) at a concentration of 50 ng mL^{-1} of phytosterols. The percentage indicates for% of phytosterols binding to the MIP.

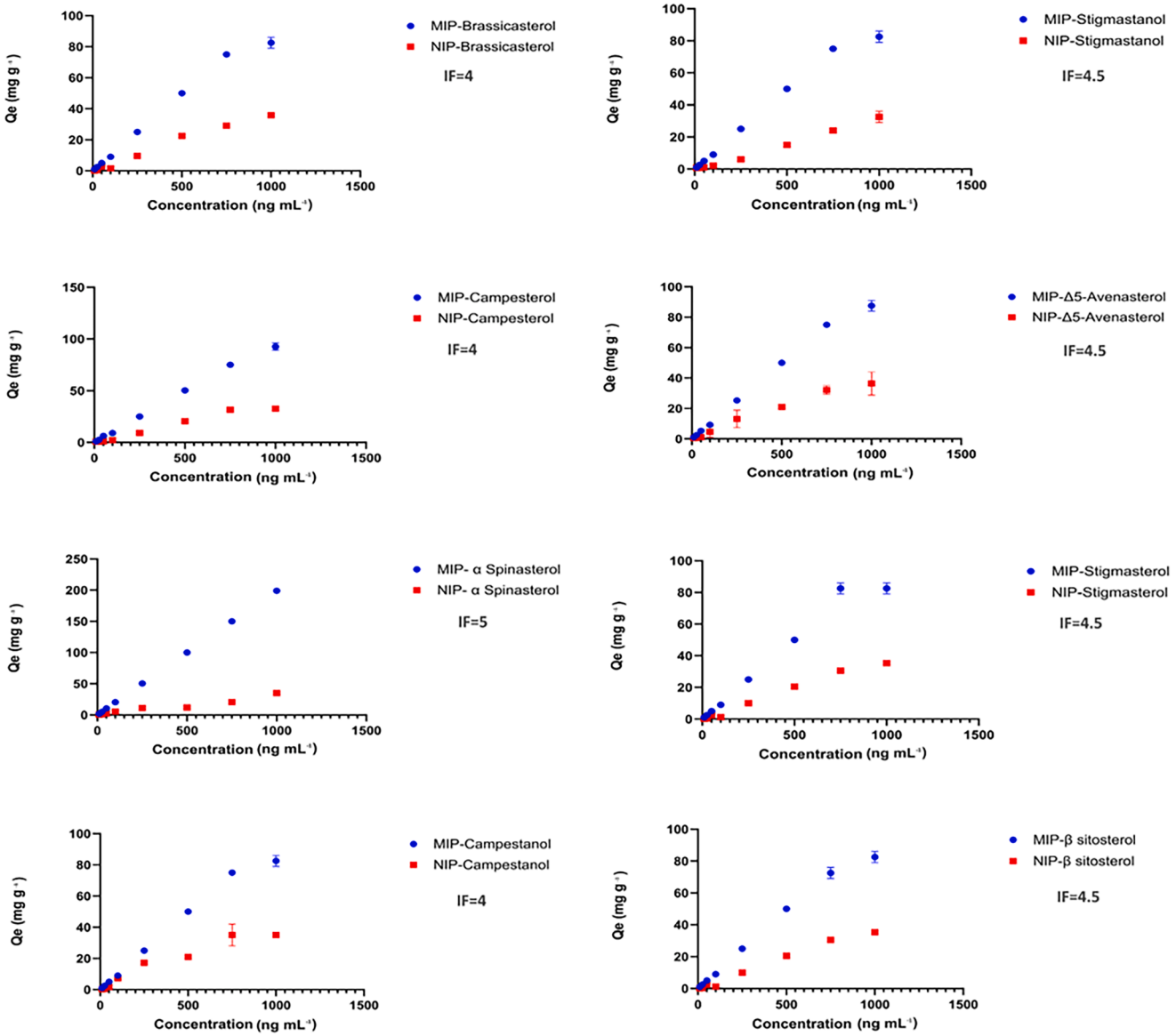

Fig. 2. Adsorption capacity expressed as Q_e (ng mL^{-1}) of the MIP (red) and NIP (blue) was obtained by analysing different phytosterols amounts (from 0.1 to 1000 ng mL^{-1}).

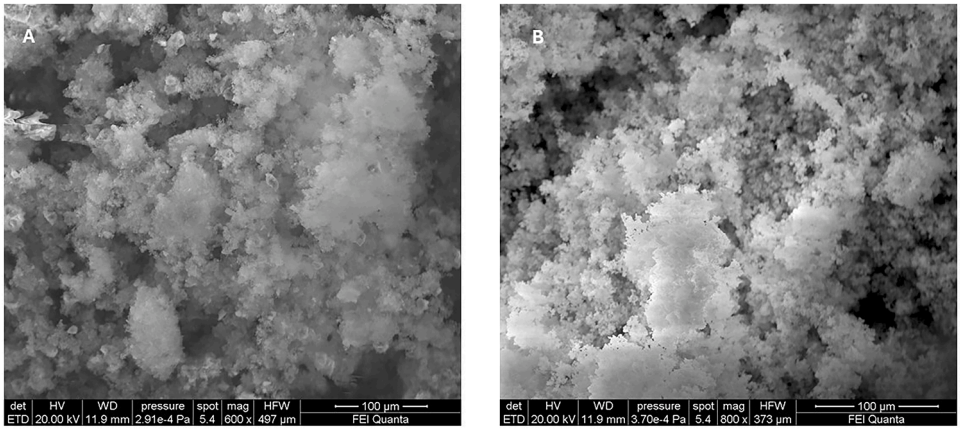

Fig. 3. Scanning electron micrographs of the of MIP (A) and NIP (B) particles prepared by sonochemical polymerization. Magnifications: (a) 200X.

mixture of 8 different PSs and the results were satisfactory (*IF*>1 for all PSs) to obtain a class selective MIP.

3.4. Physical characteristics

The proposed MIP was prepared by fast sonochemical synthesis according to the procedure of previous works [40,45]. Cholesterol was used as a dummy template because of the similar structure in the steroid nucleus to PSs, that may allow the class selectivity of the method. Homogeneous surface morphology is essential for achieving good extraction capacity and reproducibility when using MIPs in analytical procedures. SEM was employed for the analysis of the particle size and morphology of polymers under different magnifications of 200-fold and 10,000-fold, respectively. Fig. 3 displays scanning electron micrographs illustrating the morphology of both imprinted MIP (A) and non-imprinted counterpart (B). MIP and the corresponding non-imprinted control were synthesized using identical methods, except that the template was omitted in NIP. Both polymers were ground to a particles size of 100–200 μm. The images reveal noticeable differences in polymer morphology. For both polymers, agglomerates of microparticles of different sizes were obtained. The non-imprinted polymer exhibited a uniform and smooth shape, while the imprinted polymer displayed an irregular, rough morphology resembling microparticles with small cavities.

3.5. Extraction step

Solvents with decreasing polarity were selected for the extraction step, to allow the extraction of PSs in the 12 matrices analysed (Brassicaceae, dried fruits and Leguminosae), to ensure a high extraction yield and test the selectivity of MIP. $CHCl_3$, Tol, Ace and Hex were tested following the procedure reported in Section 2.5. The supernatants were pooled, and subjected to the dSPE-MIP procedure, as reported in Section 2.6 and injected into the LC-MS/MS platform. As shown in Fig. S2, the best extraction yield expressed in% was obtained with $CHCl_3$:H_2O (2:1 v:v).

3.6. Method validation

The proposed approach allowed to combine the selectivity of a dSPE-MIP procedure with the sensitivity of LC-MS/MS analysis, allowing low LOD and LOQ values to be achieved (Table S3). To determine these values, calibration curves were constructed for each standard in the range between the lower LOQ and 250 ng mL^{-1}; a coefficient of determination $R^2 \geq 0.99$ was obtained for all analytes (Table S3). In addition, method accuracy, expressed as Bias%, was estimated at 3 levels (25, 150 and 250 μg kg^{-1}); the values obtained, within +2 - +5 %, demonstrate the good performance of the method across the entire calibration range (Table S4). To assess precision, three replicates of the extraction experiment were performed at 3 levels (25, 150 and 250 μg kg^{-1}); for each PSs on the same day (intra-day) and different days (inter-day) +5 % was obtained, as showed in Table S5.

In the literature, few articles use LC-MS/MS technique for the identification of PSs in plant and food matrices. Some studies have focused on some food matrices, while other works have primarily investigated matrices of a biological nature [46,47]. Regarding food, Zang et al. [17] have developed a simultaneous determination of eight bioactive compounds containing 2 PSs (stigmasterol and β-sitosterol) in edible oil using ultrasound-assisted saponification and liquid-liquid extraction; recoveries ranged from 87 % to 89 %. Nabeshima et al. [48] developed a method for the identification of 5 PSs in *Aloe vera* plants, achieving recoveries ranging from 95 % to 105 %; Nzekoue et al. [49] reported the identification of 3 PSs (β-sitosterol, campestanol and campesterol) in black beans, with recoveries of 87 % to 91 %. These values are comparable to those obtained from the analysis of dried fruits, Brassicaceae and Leguminosae (70–110 %) matrices (see Table S6); in this study a good recovery was also achieved for the other PSs analysed (70–100 %) (Table S6), proving the ability to quantify simultaneously a greater number of PSs in different food samples.

Despite the complexity of food matrices, the proposed method exhibits low matrix effects (< ±15 %) for all samples (Table S6). This is usually a big challenge for complex matrices such as Leguminosae, characterized by a high protein content, dried fruits, rich in fatty acids, or Brassicaceae, rich in fibers; the ability to reduce matrix effects can be attributed to the high effectiveness of dSPE-MIP in selectively binding analytes and in removing most interfering compounds. This ensures a strong and effective sample extraction and clean-up.

3.7. Real samples

Food samples were analysed in targeted analysis using MRM acquisition mode. The results are shown in Table 1. Quantitative analysis performed on Brassicaceae samples identified campestanol as the major PS (about 60–70 % w/w) in all the samples analysed. Whereas β-sitosterol and stigmasterol accounted for about 10–15 % (w/w) in all the selected samples. Minor amounts of brassicasterol and campesterol, 5 % (w/w) and 3 % (w/w) respectively [50–52], were found. The PSs most commonly present in walnut samples was brassicasterol, which constituted 72 % (w/w) of the identified PS content. In addition, the analysis also allowed the quantification of analytes such as stigmasterol, β-sitosterol and stigmastanol constituting 8–10 % (w/w) [53,54] of the PSs content and campesterol, Δ^5-avenastenol and α-spinasterol present in smaller amounts (about 1–2 % w/w) [55]. Analysis of hazelnuts also identified brassicasterol as the most prevalent PS, 84 % (w/w) of the quantified PSs. Also in pistachio and almonds, the most prevalent analyte is brassicasterol which accounts for 53 % (w/w) and 43 % (w/w), respectively [56]. The analysis of Leguminosae resulted in high brassicasterol content in all samples analysed (55–65 % w/w) [52,57]. In addition, Leguminosae are characterized by a content of stigmastanol, stigmasterol and β-sitosterol around 11–15 % (w/w) of the total content of PSs. Δ5-avenastenol and α-spinasterol are present at lower amounts (1–3 % w/w) in beans, chickpeas and lentils; they were not detected (<LOQ) in peas [43,48].

3.8. MIP reusability

In this work, the reusability of MIP was directly evaluated for the different matrices analysed using the dSPE-MIP procedure reported above, in a total of 6 adsorption–regeneration cycles. In order to regenerate the MIPs several washing cycles using desorption solvent (MeOH:H_2O:ACN 45:5:50 v:v) were performed.

Each sample was split after extraction in different aliquots ($n = 7$), which were used for the test. The first extraction concentration, reported in Table 1, was taken as the reference. As reported in Fig. 4, the error remained in the range of acceptability up to the third cycle, with respect to the FDA guidelines in term of relative error; from the fourth use onward, a decrease in performance was observed. However, this result is significant because, to our knowledge, the parameter of reusability has not been evaluated in the literature for this class of compounds.

4. Conclusion

The focus of this work was to develop a highly efficient method for the selective extraction and quantification of PSs in 12 different food and plant matrices, belonging to classes with distinctive characteristics such as nuts (almonds, hazelnuts, walnuts and pistachio), Brassicaceae (beets, cabbage, cauliflower and broccoli) and Leguminosae (beans, chickpeas, lentils and peas), by exploiting the potential of coupling selective extraction by dSPE-MIP with UHPLC-MS/MS analysis. Despite the long time required for the extraction and clean-up steps (min), the proposed MIP synthesis featured a rapid polymerization step (5 min) for the synthesis of dSPE-MIP cavities selective for PSs and the development of a

Table 1
PSs identified and quantified with the targeted method in each sample. Data are reported in mg g $^{-1}$.

Samples	Brassicasterol		Stigmastanol		Campesterol		Δ^5-Avenastenol		α-Spinasterol		Stigmasterol		β-Sitosterol		Campestanol	
	mg g $^{-1}$	±DevSt	mg g $^{-1}$	±DevSt	mg g $^{-1}$	±DevSt	mg g $^{-1}$	±DevSt	mg g $^{-1}$	±DevSt	mg g $^{-1}$	±DevSt	mg g $^{-1}$	±DevSt	mg g $^{-1}$	±DevSt
Leguminosae																
Beans	12.38	1.06	1.38	0.07	0.16	0.01	0.75	0.06	0.83	0.04	2.25	0.00	2.24	0.03	<LOQ	n.d.
Chickpeas	2.16	0.03	0.43	0.01	0.08	0.01	0.39	0.01	0.46	0.00	1.38	0.02	1.39	0.04	<LOQ	n.d.
Lentils	1.89	0.15	0.46	0.02	0.08	0.00	0.07	0.01	0.05	0.01	0.49	0.00	0.50	0.03	<LOQ	n.d.
Peas	0.64	0.00	0.17	0.01	0.02	0.00	<LOQ	–	<LOQ	–	0.14	0.00	0.16	0.00	0.16	n.d.
Dried fruits																
Walnuts	1.72	0.05	0.18	0.01	0.00	0.00	0.04	0.00	<LOQ	–	0.23	0.02	0.20	0.01	<LOQ	n.d.
Hazelnuts	1.48	0.02	0.08	0.00	<LOQ	–	0.02	0.00	<LOQ	–	0.10	0.01	0.10	0.01	<LOQ	n.d.
Pistachio	1.82	0.17	0.44	0.01	0.01	0.00	0.11	0.01	<LOQ	–	0.53	0.03	0.51	0.01	<LOQ	n.d.
Almonds	1.73	0.04	0.53	0.02	0.01	0.00	0.26	0.00	<LOQ	–	0.76	0.00	0.71	0.02	<LOQ	n.d.
Brassicaceae																
Broccoli	1.56	0.01	<LOQ	–	0.90	0.00	0.03	0.00	<LOQ	–	4.33	0.02	2.90	0.01	18.89	0.01
Cauliflower	1.54	0.02	<LOQ	–	0.91	0.02	0.04	0.00	<LOQ	–	4.30	0.02	2.91	0.02	18.91	0.03
Chard	0.93	0.02	<LOQ	–	0.42	0.02	0.02	0.00	<LOQ	–	2.52	0.01	2.66	0.00	10.64	0.03
Cabbage	0.95	0.01	<LOQ	–	0.40	0.01	0.02	0.00	<LOQ	–	2.50	0.01	2.63	0.01	10.60	0.06

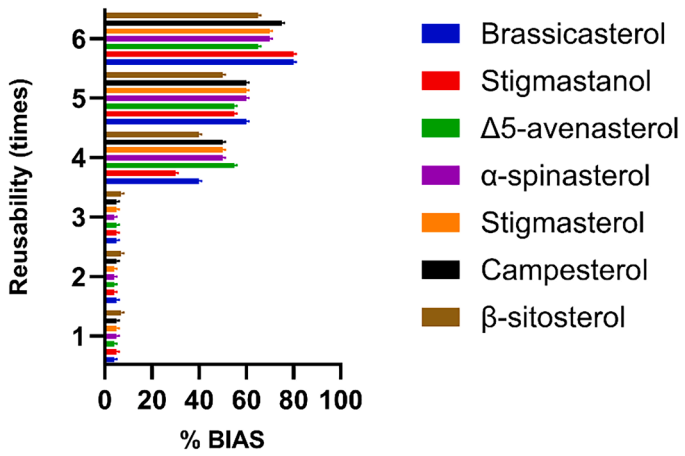

Fig. 4. Reusability was evaluated 6 times by dSPE-MIP procedure. Data are expressed as Bias% of each analyte analysed in 12 matrices.

targeted UHPLC-MS/MS analysis protocol, enabling the identification and quantification of 8 PSs. Moreover, the synthesis allowed the production of a high amount of polymers (>2 g), sufficient for several analyses (10 mg of MIP for each analysis) with satisfactory value of *IF* for each target PS (≥4). Additionally, it is important to note that the MIPs can be reused up to 3 times. In addition, the validation data demonstrate the method selectivity, with impressive extraction performance (65–100 %) and minimal matrix effect (<±15 %), effectively eliminating the potential interferents present in complex food and plant matrices. This approach represents a significant advancement in PS analysis, offering a selective, and reliable technique with broad applicability in both scientific research and industrial applications, facilitating accurate assessment of the phytochemical composition in diverse plant and food products.

CRediT authorship contribution statement

Eleonora Oliva: Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Sara Palmieri:** Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Francesco Della Valle:** Software, Investigation. **Fabiola Eugelio:** Writing – review & editing, Software, Formal analysis. **Federico Fanti:** Writing – review & editing, Visualization, Conceptualization. **Alessandro Ciccola:** Writing – review & editing, Validation, Formal analysis. **Manuel Sergi:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Michele Del Carlo:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Dario Compagnone:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability statement

Data are available from the corresponding author on reasonable request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcoa.2024.100174.

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