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Dissecting the contributions of composition and mesostructure on the release of thymol from silica-based mesopowders

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ABSTRACT

In pharmaceutics, mesoporous silica (MS)- based powders are used as high capacity sorbents for active agents, sometimes with controlled release properties. In literature, MS powder-adsorbed substances display faster or slower release compared to unformulated compounds. In order to understand this controversial phenomenon, we carried out a comprehensive study by investigating the behavior of thymol (TH, a model compound with acaricide properties against the *Varroa destructor* bee parasite) in different conditions, as a pure powder and when adsorbed onto four silica-based mesopowders from commercial (Sil-Sol 6035®, Neusilin US2®, Silica Gel 60737®) and synthetic (MCM-41) origin, and characterized by different chemical composition and nano/mesostructure. All MS-powders displayed high TH loading capacity (between 0.35 and 0.72 g/g SiO_2), related to their mesopore volume. Stabilization of physisorbed TH in amorphous form was achieved by all except Neusilin US2®. Adsorption onto MCM-41 and SiliSol® or Silica Gel 60737® led to tenfold and twofold reduction of sublimation rate at 33 °C (the average beehive temperature during treatment), respectively. Adsorption onto the three retentive MS-powders also reduced the energy of activation (Ea) of TH sublimation, providing explanation on the controversial effects on the release of bioactive agents adsorbed onto MS powders described by the literature. In field applications, the effect exerted by the retentive powders should permit to cover the entire *Varroa* reproduction cycle by a single administration. Reduction of the sublimation Ea brings the additional advantage of reducing sensitivity of the sublimation process to the environment temperature fluctuations.

1. Introduction

The term *mesoporous silica* (MS) refers to forms of silica having large surface area and pores with size in the range of 2–50 nm. In pharmaceutics, colloidal MS powders find several uses among which as sorbents for liquids and as drug carriers [1]. Commercially available colloidal MS (e.g. Aerosil®, Sylysia®, Florite®, Sil-Sol®) are synthesized either from $SiCl_4$ in a hydrogen and oxygen atmosphere at high temperature (pyrogenic, fumed) or by sulfuric acid mediated precipitation of sodium silicate solutions. Alternatively, *ordered* mesoporous materials, largely investigated for their tunable properties [2–5], can be obtained by synthetic routes in which silica polymerization is induced in the presence of templating micelle-forming surfactants. In these solids, the mesostructure is highly ordered and is characterized by the presence of channels, cages, or pores with amorphous silica walls (MCM41, SBA15 or SBA16) [6].

In general, and independent of the synthetic route, the large surface area MS permits absorption of large amounts of compounds, while the porous structure, depending on pore size, can favor stabilization of the amorphous state of the adsorbed molecule and modify its release profile [7–9]. However, the influence of MS powder adsorption on release remains a subject of controversy. Indeed, it was found that upon adsorption onto MS, the release rate of active agents either accelerates [10–12] or slows down [4,13–15], depending on the chemical-physical nature of the adsorbed molecule and the nanostructure of the silica (size and volume of the pores and tortuosity of the channels) [6].

In this work we aimed at further exploring how MS powders affect the adsorption and release of physisorbed molecules. To this end, we

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conducted a comprehensive investigation on the sorption and release modifying properties of four MS powders exhibiting distinct chemical composition and nano/mesostructure. As model active compound we used thymol (TH), a phenolic monoterpenoid found in thyme extracts with bactericidal and antiseptic properties [16,17] whose use could benefit from controlled release strategies. TH also exhibits efficacy against *Varroa destructor* (Varroidae), an ectoparasite that infests honeybees [18] which is considered a major threat to the bees worldwide. Thymol-based products are commercially available to beekeepers in gel form (Apiguard®) or adsorbed onto a cellulose (Thymovar®) carrier or combined with other essential oils adsorbed onto a vermiculite carrier (ApiLifeVar®). The excipients used on these products serve as "simple" carriers and do not exert any control on TH release, so that during hot summers its sublimation is often too fast and multiple treatments are necessary to cover the whole parasite reproduction cycle; on the contrary, when the temperatures drop down (as in late summer nights or in the fall) TH sublimation slows down to the point that its efficacy is diminished [19].

Several formulation strategies have been proposed to regulate TH release with the aim of stabilizing it, reducing its volatilization and improve efficacy. For instance, encapsulation employing chitosan nanoparticles showed enhanced *in vitro* efficacy against fungi [20], and the use of β-cyclodextrins or poly lactide-co-glycolide nanoparticles was capable to attenuate TH release. [21–23]. Few studies [1,24] have also shown that adsorption of essential oils and TH onto mesoporous powders can slow down their release in the environment [25–28] and/or improve their biocidal efficacy [27,29–31]. However, to our knowledge, all investigations in this context have been carried out using *ordered* mesoporous materials (MCM-41 or SBA15), which are more costly than pyrogenic or precipitated porous silicas [6] and have not yet received approval from regulatory agencies [5].

With the twofold objective of a) gaining further understanding on how MS powder features affect the behavior of adsorbed molecules and b) identify potential TH/MS blends suitable for field application, we compared four MS powders from commercial and synthetic origin, namely one synthetic ordered MCM-41 [11], two MS of commercial origin (Pharma grade Sil-Sol 6035®, and a Silica Gel 60737® used in chromatography) and a pharma-grade colloidal aluminum-magnesium silicate (Pharma-grade Neusilin US2®) [32] widely employed as sorbent. Powders were compared in terms of loading capability, the ability to stabilize TH in the amorphous phase, and the ability to modify its rate of volatilization. The energy of activation (Ea) of TH sublimation when adsorbed onto the different powders was also calculated and compared to that of the unformulated compound. Results were also discussed in relation to the potential field application of the retentive blends. To this end, MS powder erodibilities in aqueous environment were also tested to rule out potential risks of bioaccumulation in case of exposure in the hives.

2. Materials and methods

2.1. Materials

Thymol (purity 99 %) was obtained from ACEF (Italy). Tetraethoxysilane (TEOS, (purity 98 %)), Hexadecyl-trimethyl-ammonium bromide (CTAB, (purity 98 %)), Silica Gel 60737® (Merck-Sigma Aldrich code n. 60737) and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). SilSol™ 6035 (lot n.1000292277, produced by Grace Corp - USA) was a gift from Evotec (Italy) and Neusilin US2® (lot. n.307061) was a gift from FujiChemical (Japan). Milli-Q grade water was used throughout the experiments. Absolute ethanol (EtOH (purity 100 %)) was purchased from Carlo Erba (Italy).

2.2. Synthesis of the MCM-41 mesopowder

MCM-41 mesoporous nanopowder was prepared through base-catalysed hydrolysis of TEOS (the alkoxysilane precursor) in the presence of CTAB (Table 1) as templating agent, according to Ref. [11]. Briefly, in a round bottom glass flask, CTAB was dissolved in a H_2O:EtOH:32 % ammonia mixture (100:100:25 vol ratio) at a final concentration of 21 mg/mL (58 mM). TEOS was then added to this solution and the mixture was allowed to stir for 2 h at room temperature. The resulting suspension was centrifuged (4420g, rt, 10 min), the supernatant removed and the solid powder was washed first with water and then with absolute EtOH, dried in vacuo (at room temperature, RT) and heated at 70 °C for 3 h. Template removal was then performed by calcination at 600 °C for 12 h and verified by infrared (IR) analysis by the absence of the characteristic CTAB absorption bands (in the spectral range of 2900–2800 cm^{-1}) (Jasco FT/IR 4200 with ATR PRO ONE diamond crystal attachment, absorption spectra recorded in the 400-4000 cm^{-1} range with an accuracy of 4 cm^{-1}).

The formation of an ordered mesoporous structure was verified by XRD diffraction. X-ray diffraction patterns (XRD) were collected on a diffractometer Panalytical X'Pert 3 with a copper Kα radiation (0.15418 nm), in the 1.5–10° range with a step size of 0.02°.

2.3. Surface area and pore size evaluation

Specific surface area measurements and mesopores analyses were performed in an Autosorb 1Q (Quantachrome Instruments) through N_2 physisorption at 77K. Samples were degassed at 90 °C for 1 h (heating rate 5 °C/min), followed by a second step at 300 °C for 6 h (heating rate 2 °C/min). Nitrogen absorption isotherm data were analysis by BET (Brunauer-Emmett-Teller) method to retrieve the specific surface area. The pore size distribution and total pore volume were evaluated by the NLDFT (non-linear density functional theory) and Gurvitch methods, respectively.

2.4. Silica dissolution test

MS powder dissolution was investigated at 37 °C by immersing them (5 mg in silica) into 40 mL of 10 mM tris hydroxymethylaminomethane (TRIS), 150 mM NaCl, pH 7.4 buffer, or 0.1M HCl (pH 2.0) in a shaking water bath. At scheduled times, small fractions of the solution (800 μl) were removed for silicic acid quantification and replaced by fresh buffer. Silicic acid concentration was quantified using the molybdenum-blue colorimetric test. Data were mathematically elaborated and were plotted as (Mt/Mtot) % vs time, where Mt is the amount of silica released at time t, and Mtot is the total silica contained in the sample. These analyses were conducted using a buffer/powder volume ratio that allows free dissolution of the silica without reaching saturation (sink conditions for silica) [11,33,34].

2.5. Loading MS powders with thymol

2.5.1. Immersion method

This procedure [35] was adopted along the preliminary tests to quantify the powder's loading capability for TH. In details, 200 mg of mesopowder (MS-powder) were immersed overnight under magnetic stirring (room temperature RT, 200 round/min - RPM) in 5 mL of abs ethanol solutions at different concentrations of thymol (160, 320 and 640 mg/mL, corresponding to TH/MS-powder w/w ratios = 4,8, and 16). After overnight incubation, the suspensions were centrifuged (4420 g, RT, 10 min) and the supernatant removed. To remove the not-physisorbed TH, powders were rapidly rinsed with 1 mL of abs.

Table 1
Reagents and amounts used for the preparation of 1 g of MCM-41 MS powder.

CTAB solution	113 mL
TEOS	3.47 mL
TEOS:H_2O:EtOH:NH_3:CTAB	1:178.6:55.2:6.7:0.42 (molar ratios)

EtOH and immediately centrifuged (4420g, RT, 10 min). After removal of the supernatant, powders were dried in vacuo.

2.5.2. *Incipient wetness impregnation method*

This procedure, which was used to prepare larger amounts of TH/MS-powder blends for the release and XRD tests, was carried out once the powder loading capability had been quantified. In this procedure the MS-powders were blended with their maximum loadable TH dissolved in the minimum volume of ethanol necessary to wet the powder. The latter was determined (Table S1) according to Ref. [36]. Immediately after impregnation, the solvent was removed by vacuum evaporation, and mixtures were kept in a closed container at 4 °C until further investigations. In case of Neusilin US2® an additional blend was prepared at TH/MS powder ratio of 0.4 (w/w).

2.5.3. *TH quantification in TH/MS powder blends*

To quantify the amount of thymol loaded in the TH/MS-powder blends, 10 mg of TH/MS sample were immersed in 1 mL of abs ethanol, gently shaken for 10 min and centrifuged (16000 g, rt, 10 min). The supernatant was analyzed for TH content by UV spectroscopy at 276 nm, according to a calibration curve. The procedure was repeated until powder exhaustion. This analysis was performed onto 3–5 samples/each TH/MS-powder blend to assess homogeneity. Data were elaborated according to the following equation:

Thymol loading (TH/MS w/w%) = mg TH in 10 mg TH/MS-powder blend/(10 – mg TH in 10 mg TH/MS-powder blend) x 100 Eq.1

2.6. *XRD analysis*

X-ray diffraction patterns were registered at different time points after blend preparation (0, 2, 4, 6 and 11 weeks). Analyses were performed on 100 mg of total powder on a Bruker D8 Advance Plus diffractometer, with a copper Kα radiation ($\lambda = 1.5406$ Å, 40 kV, and 40 mA). Patterns were collected in the 5–60° 2Θ range (0.02°/step, 1s/step).

2.7. *Transmission electron microscopy*

To investigate the porosity and dimension of the mesoporous powders, silica samples were suspended at 1 mg/mL in water and analyzed with a Tecnai G2 transmission electron microscope (FEI; Thermo Fisher Scientific, Waltham, MA, USA) operating at 100 kV. Images were captured with a Veleta digital camera (Olympus Soft Imaging System; Münster, Germany).

2.8. *Thymol release*

Considering that the only volatile component in the TH/MS blends was TH, its release upon air exposure was quantified by following the time evolution of the samples weight (up to a maximum of 33 days). In details, 10 mg of thymol as a pure powder or in TH/MS blends (n = 7–10) were placed in 2 mL polypropylene microtubes which were left open while incubated in a thermostatic plate placed under a chemical hood to simulate the flow of air present in the hive. Samples were weighted daily up to a maximum time of 33 days. This procedure was validated by comparing, at selected time points, the TH content of the samples calculated by weight loss to that measured by solvent extraction (2.3.3). The assay was carried out at three temperatures, 33 °C (the average temperature in the beehive), 45 °C (5 °C below TH melting temperature) and 60 °C (10 °C above TH melting temperature).

2.8.1. *Data elaboration*

Weight loss data were elaborated according to Eq.2

Q = ((Mtot-Mt)/Mtot)) %/time Eq.2

Where:

- Q is the TH daily release;
- M_t is the amount of thymol released at time t;
- M_{tot} is the total amount of thymol in the sample;
- time is expressed in days.

Data were further mathematically elaborated and plotted according to the Arrhenius equation (ln(Q) versus 1/T (°K)). The energy of activation (Ea, in kJ) of the release process was derived from the slope of the Arrhenius plot according to Eq.(3).

Ea = -(ln(Q) x T) x R Eq. 3

Where:

- T is temperature in Kelvin;
- R = Universal Gas Constant = $8.314\text{x}10^{-3}$ kJ $K^{-1}mol^{-1}$.

3. Results

3.1. *Properties of the mesopowders*

The properties of the silica powders used in this investigation are summarized in Table 2, Fig. 1 and S1.

MCM-41 is a synthetic silica belonging to the ordered mesoporous materials. In particular, the material here used, previously described in the literature [11], is made of round-shaped nanoparticles of about 330 ± 100 nm (Fig. 1), and is characterized by a specific surface area of about 1218 m^2/g, mesopore volume of 0.62 cm^3/g and mesopore diameter in the range of 2–8 nm (NLDFT), with a peak value around 3 nm, as determined by physisorbed analysis (Fig. S2). The XRD pattern (Fig. S3) shows a diffraction peak at 2.6°, which suggests the mesopores are organized in a hexagonal lattice. This mesostructure was confirmed by TEM analysis (Fig. 1A) which showed nanoparticles with average diameter of 371+/142 nm, channels and ordered mesopores with diameter (measured by imageJ software) of 3.17 ± 1.72 nm.

Sil-Sol6035® (Sil-Sol ®) is a USP approved mesoporous silica powder commercialized as pharma excipient to improve the properties of poorly soluble active ingredients. Optical microscopy images indicate an average particle size of 42.7 ± 13.8 μm, while BET analysis indicates a surface area of 600 m^2/g, a pore volume of 0.74 cm^3/g and pore size between 2 and 12 nm (Fig. S2). The nanoscale TEM morphological analysis (Fig. 1B) shows clusters of nanosized porous particles of about 20 nm, with no mesopore order.

Neusilin US2® (Neusilin®) (Fig. 1C and S1) is a colloidal magnesium aluminum silicate widely used in the pharma industry as adsorbent and stabilizing agent. Optical microscopy (Fig. S1) reveals clusters of microparticles with average size of 70.3 ± 32.7 μm. Physisorption analysis (Fig. S2) indicates a specific surface area of 401 m^2/g, a mesopore volume and pore diameter (DFT) of about 1.36 cm^3/g and 10 nm (peak

Table 2
Properties of the MS-powders here investigated.

MS-Powder	Composition	Specific surface area (m^2/g)	Mesopore volume (cm^3/g)	Pore range Ø (nm) (peak value)
MCM-41	Silica (SiO_2)	1218	0.62	2-8 (3)
Neusilin US2®	MgO-Al_2O_3-SiO_2 (SiO_2 30 %)	401	1.36	6-80 (10)
Silica Gel 60737®	Silica (SiO_2)	389	0.72	5-20 (6.5)
Sil-Sol 6035®	Silica (SiO_2)	600	0.74	2-12 (6.5)

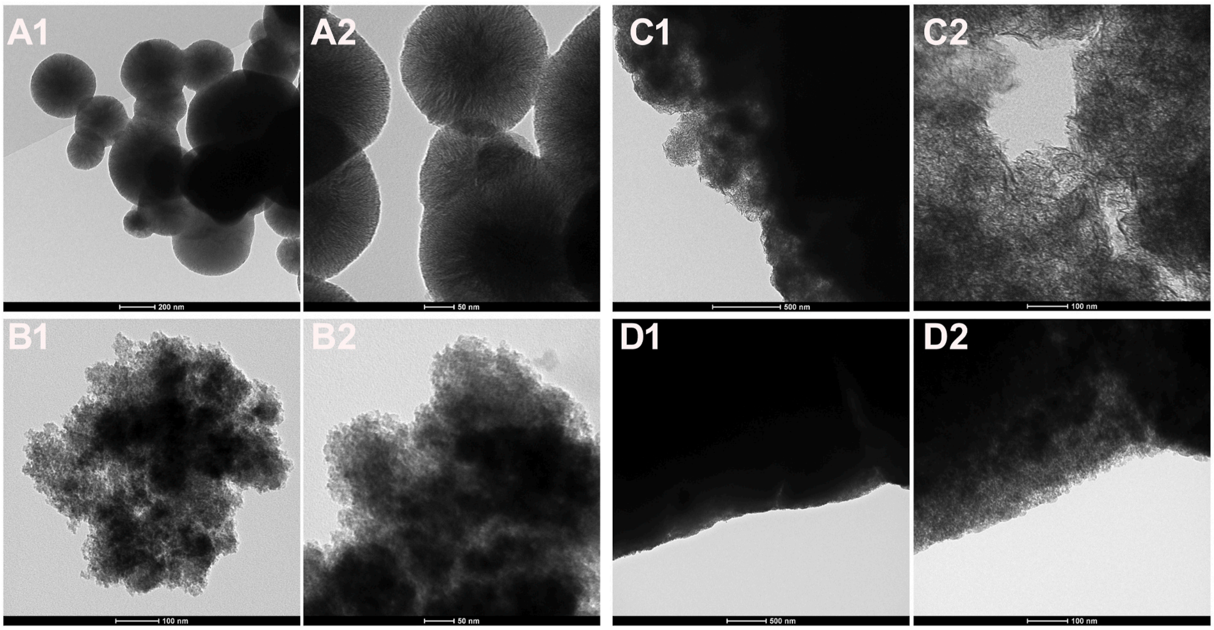

Fig. 1. TEM images of the powders used in this investigation A) MCM-41; B) Sil-Sol6035®; C) NeusilinUS2®; D) Silica Gel ® at different magnifications. bars: A1: 200 nm; B1, C2: 100 nm, A2, B2, D2) 50 nm, C1, D1) 500 nm.

value), respectively, in line with earlier reports [32].

Silica-Gel® (Merck Sigma-Aldrich# 60737) (Fig. 1D and S1) is a high purity silica powder used as a separating medium in flash chromatography, with particle size of 63.0 ± 15.3 µm (Fig. S1), a surface area of 389 m^2/g, a pore size distribution between 5 and 20 nm (peak value of 6.5 nm) and a mesopore volume of 0.72 cm^3/g (Fig. S2).

3.2. Silica dissolution

These materials are to be used in beehives, so it is vital to verify that they can be degraded into silicic acid when in contact with biological fluids to avoid risks of bioaccumulation. Dissolution tests were therefore carried out at both acidic pH (HCl 0.1M, pH 2.0) - mimicking the stomach environment - and neutral pH - simulating all other body compartments. At acidic pH, no dissolution was observed (data not shown) for all MS powders, in line with the documented poor solubility of silicic acid in acidic conditions. At pH 7.4 (Fig. 2), all materials showed a similar pattern of dissolution in which a slower initial lag-phase was followed by a second one linear with the square root of time. This pattern is compatible with a diffusion related mechanism and the slower early lag-phase may be due to a higher degree of silanol condensation at the material's surface. The various powders differed both for the duration of the early lag phase and the slope of the diffusion related process. In general, the slower the dissolution, the longer was the lag-phase. We estimated the time necessary for total silica exhaustion (SiO_2-t_{ex}) from the slopes registered in the second phase. The slowest dissolving material was Neusilin US2® with estimated SiO_2-t_{ex} > 100 days. All other materials, which are composed of pure silica, dissolved much faster with SiO_2-t_{ex} between 1 and 4 days, the fastest one being MCM-41.

3.3. Thymol loading

Loading was performed using solvent-driven impregnation methods in which the MS powders are imbibed with solutions of TH and then the solvent is removed by evaporation in vacuo. Impregnation methods rely on the drug molecules diffusing into the MS pores together with the solvent and remaining trapped after solvent removal. Since loading depends on the mutual interactions between the active agent, the MS powder and the solvent [6,37], the nature of the solvent affects the loading capability. Here we used ethanol, which is both safe and an excellent solubilizer for TH so that high concentrated solutions can be

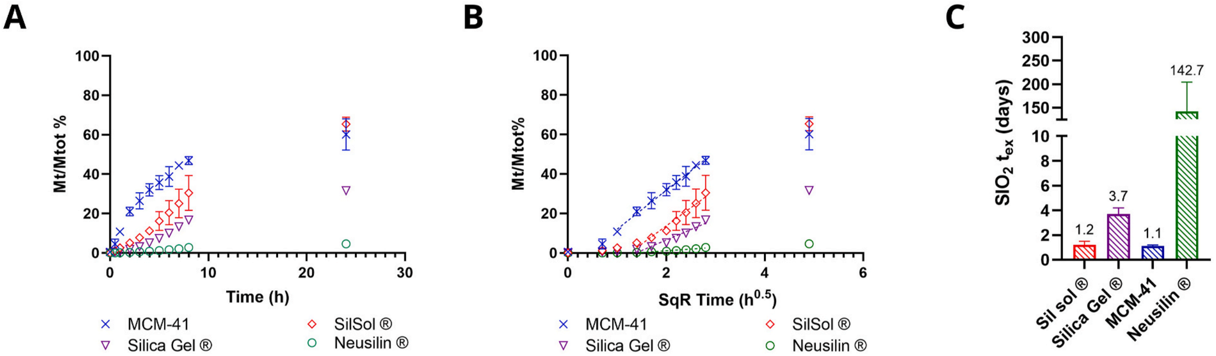

Fig. 2. Silica dissolution in buffer. A) SiO_2 dissolution as a function of time; B) release as a function of the square root of time; C) The dissolution rate was calculated from the linear part of the curves of panel B and used to compute the theoretical time (in days) necessary to reach total silica dissolution (time for silica exhaustion - t-ex). The dissolution test was performed at 33 °C using a buffer/silica powder volume ratio that allows free dissolution of the silica without reaching saturation [41].

generated for efficient loading.

3.3.1. *Quantification of loading capability*

In general, absorption follows the Langmuir equilibrium, in that - at defined solvent/MS powder/active agent - the amount of compound adsorbed increases with its concentration in the adsorption medium until surface saturation is reached. In order to assess the loading capability for TH at MS-powders' saturation we prepared a series of TH/MS-powder blends using the *solvent immersion method* [6], namely by equilibrating the MS-powders with increasing concentrations of TH in ethanol. To remove non physisorbed TH molecules which would recrystallize outside the MS pores, a rapid rinsing step [6,37]was introduced between the absorption and drying steps. XRD analysis indeed confirmed that this step was necessary to remove non physisorbed molecules, which otherwise recrystallize already the day after preparation. (See also supplementary Information S.I.).

For all materials, an increase in TH load was registered up TH concentration in ethanol = 320 mg/mL (TH/MS powder = 8 w/w), and no significant change was registered when the concentration in the absorption medium was raised from 320 to 640 mg/mL (Fig. 3). The MS-powder loading capabilities were calculated by averaging the TH content of samples obtained at 320 and 640 mg/mL. Depending on the MS material, the loading capability (Table 3) was between 0.35 and 0.72 TH/MS-powder (w/w) and was in line with the materials pore volume (Neusilin® > MCM-41 > SilSol® = SilicaGel®). By combining the mesopore information (Table 2) with TH molecular footprint of 0.29 nm^2 [38], we can postulate that in the case of MCM-41, Sil-Sol ® and Silica Gel®, TH adsorption occurs in monolayer, while the larger pores of Neusilin® can accommodate it in multilayers (Table 3).

3.3.2. *Large scale preparation by the incipient wetness impregnation process*

The *solvent immersion* method is useful to assess the MS loading capability but it is time consuming, necessitates relatively large amounts of solvent and generates waste of solute. Once the MS-powder capabilities for TH were identified, larger scale MS-powder/TH blends were prepared using the *incipient wetness impregnation process*, a faster technique in which the powder is blended with the maximum loadable compound (Table 3) dissolved in the minimum volume of solvent necessary to wet the powder (Table S1). After impregnation, no washing step is necessary and the solvent is removed by vacuum evaporation. This method [37] is more convenient than the previous one as the whole solution is adsorbed by the MS powder by capillary forces, avoiding filtration steps, minimizing waste and reducing the time necessary for solvent evaporation. To evaluate whether this process lead to TH deposition at the internal MS pores similar to the immersion method, we carried out XRD analysis the day after preparation (Fig. 4) confirming the absence of crystalline TH indicating that all compound was physisorbed at the MS mesosurface and no molecules remained outside the pores. In addition, blends prepared by the two methods were also compared in terms of release of TH, showing essentially the same behavior (Fig. S6). These findings concurrently confirm the ability of all powders to amorphize the adsorbed TH, the interchangeability between the two methods of impregnation and thus the suitability of the incipient wetness impregnation technique for the preparation of TH/MS blends in a timely manner.

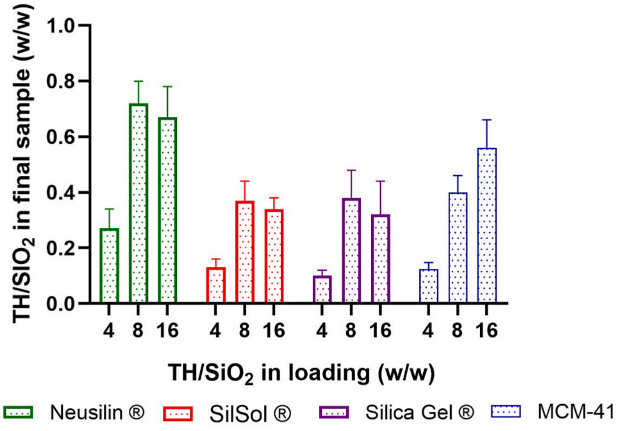

Fig. 3. MS powder loadings with in equilibrium adsorption experiments. MS powders (N = 3) were incubated overnight (40 mg/mL in MS powder) with TH solutions in absolute ethanol at 160, 320 and 640 mg/mL (TH/MS powder in adsorption = 4, 8 and 16 w/w). After supernatant removal, samples were rapidly rinsed with fresh ethanol (1mL/200 mg of dry MS powder), the wash supernatant removed by centrifugation and samples were dried removed by vacuum evaporation. The TH content was then evaluated by extraction.

Table 3

MS-powder loading capabilities for TH. Values were calculated form the loading values measured on samples obtained by equilibrium impregnation with TH ethanolic solutions at 320 and 640 mg/mL (samples/group = 6).

MS powder	Neusilin®	MCM-41	Sil-Sol ®	Silica Gel®
Loading capability (TH/ MS-powder, w/w)	0.72 ± 0.09	0.48 ± 0.08	0.35 ± 0.05	0.36 ± 0.11
Theoretical surface area in monolayer - nm^2 (*)	838 ± 104	559 ± 93	407 ± 58	419 ± 128
Modality of adsorption	Multilayer	Monolayer	Monolayer	Monolayer

(*) assuming a TH foot print of 0,29 nm^2 (PDB ID 1E06, [39], see also Fig. S5.).

XRD analyses were then repeated periodically (up to 11 weeks after preparation). Blended thymol remained amorphous throughout the entire investigation in mixtures with MCM-41, Silica Gel 6®, and Sil-Sol®. Conversely, in the case of the TH/Neusilin® blend, reversion to the crystalline form occurred within 6 weeks of storage. This is likely due to the relatively large pore of Neusilin® and the fact that prevention from re-crystallization generally necessitates confinement in a space that is approximately less than 20 times the diameter of the sorbed compound [6]. To support this hypothesis, we estimated the critical diameter of nuclei *d* needed for thymol crystal formation by the Gibbs Thompson analysis, according to Knapik et al., [40] (S.I.). In the conditions of storage (4 °C; 277.2 K) this dimension is indeed smaller than the pore dimension of Neusilin®, larger than the mesopore size of MCM-41 and close to the average pore diameter of Silica Gel® and Sil-Sol® (Fig. S8).

3.4. *Thymol release*

The results of the release assays measured from samples loaded with TH at their maximum loading capability (Table 3) are summarized in Fig. 4. For all samples and at all temperatures investigated (33 °C, 45 °C and 60 °C), TH release occurred with zero-order kinetics, in agreement with the fact that sublimation is a zero-order process, in which the rate of mass loss is constant as long as the process occurs at constant temperature and pressure, with unchanging mass transport conditions and constant surface area exposure [41,42].

The sublimation rates (Q, expressed in % TH release/day, Table S2) were calculated from the slopes of the curves from which the time required to reach total depletion (exhaustion) of thymol (TH-tex) was derived (Fig. 5D, and Table S3).

The results reveal significant differences/similarities among the various preparations. In the experimental conditions adopted, the daily sublimation (Q) of unformulated thymol was 2.8 %, 9.4 % and 29.7 % at 33 °C, 45 °C and 60 °C, respectively. When adsorbed onto pure silica-based MS powders (loading between 0.35 and 0.48 TH/MS powder w/w), the release rate was always slower than that of unformulated TH, with differences among the different powders. The most retentive MS powder was MCM-41 (with Q = 0.3 %, 0.62 % and 1.4 % daily release at 33 °C, 45 °C and 60 °C), while Sil-Sol® and Silica Gel® showed very similar retentive behavior (Q = 1.5–1.7 % at 33 °C, 3.6–3.9 % at 45 °C

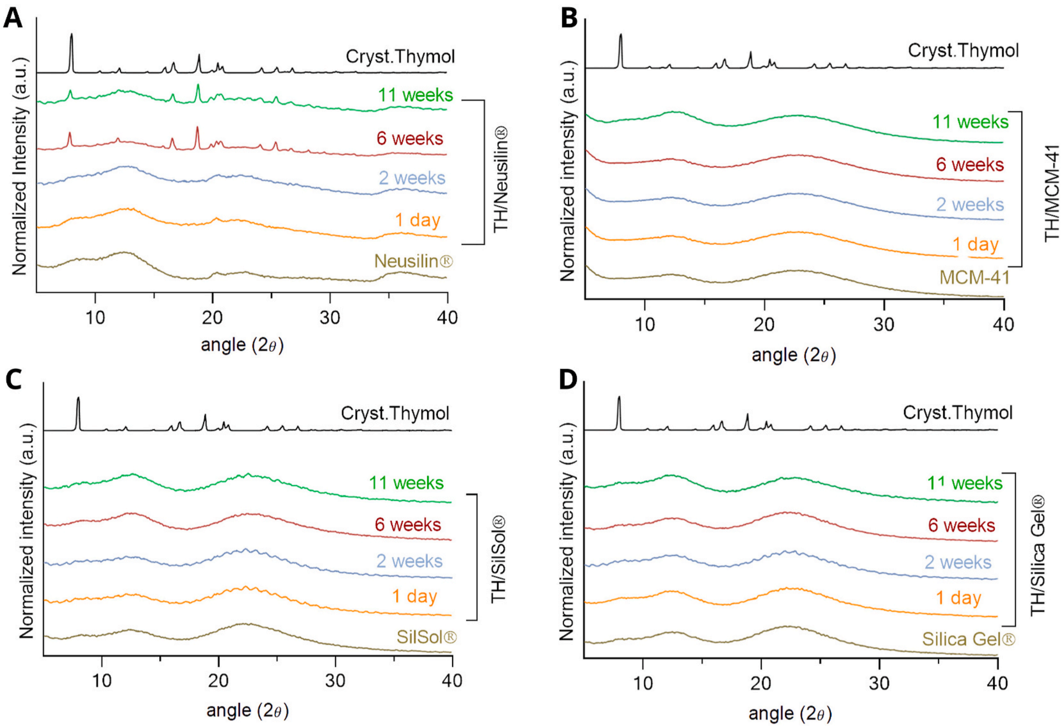

Fig. 4. XRD patterns of TH/MS-powder blends. Patterns were registered the day after preparation and upon storage at 4 °C for up to 11 weeks. The pattern of TH-free MS powders and of free crystalline TH are also reported for reference.

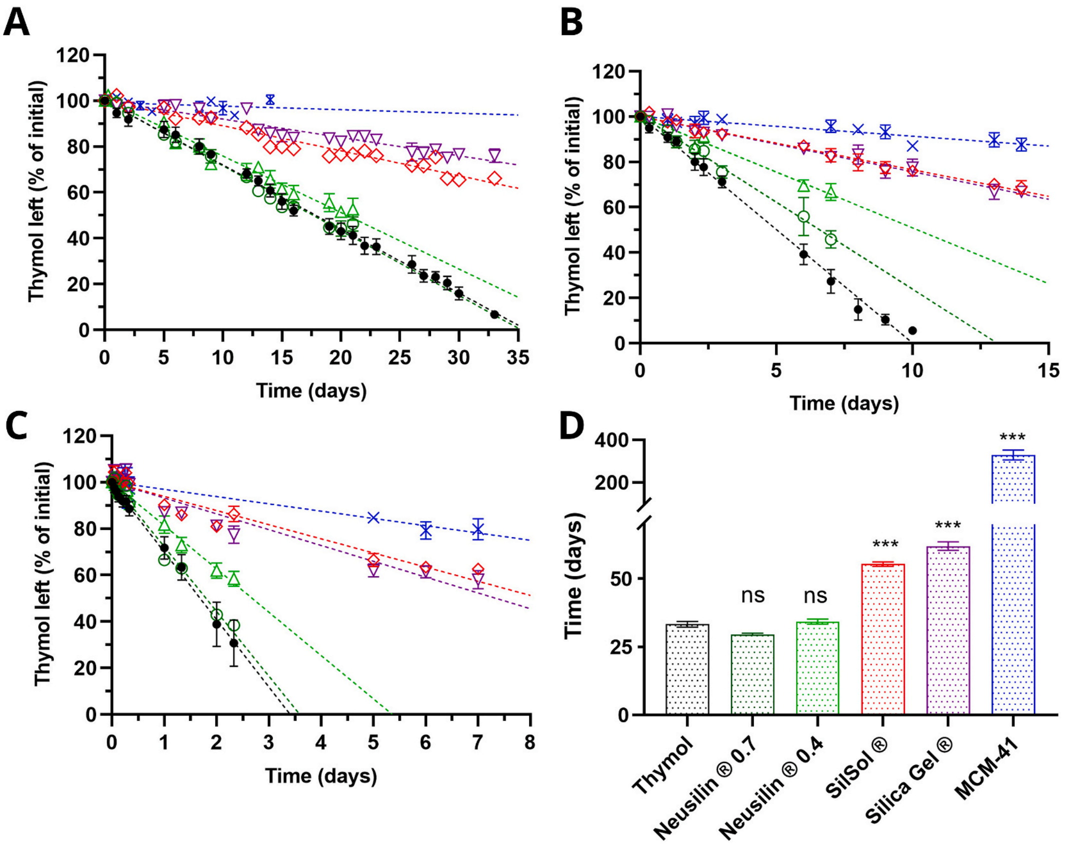

Fig. 5. Thymol release/sublimation. Kinetics of thymol release in open air as a free crystal powder (black full circles) and upon adsorption onto the MS-Powders. Neusilin green (open circles, dark: 0.7 loading, triangles, light: loading 0.4); red diamonds Silsol®; purple inverted triangles SilicaGel®; blue cross MCM-41. Release assays were carried out at three temperatures: A) 33 °C, B) 45 °C, C) 60 °C; D) Calculated time for complete thymol exhaustion at 33 °C. Significance: One way ANOVA test *versus* unformulated TH: ***p < 0,005; ns: non-significant. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and 7.9–8.2 % at 60 °C). In case of the Al_2O_3-MgO-SiO_2 Neusilin® MS-powder, which showed higher loading capacity than the others, we tested two TH blends, one having TH/MS-powder = 0.72 w/w (the maximum capability identified along the loading experiments) and a second with TH/MS-powder = 0.4 w/w (in the same range of the silica-based MS powder blends, which should lead deposition mostly in monolayer). In both cases, TH sublimation rate was close to that of the unformulated compound (see also Table S2 and S3).

In practical terms, adsorption onto Neusilin® has no impact on TH sublimation, adsorption onto Sil-sol® and Silica Gel® approximately doubled the time necessary for full TH release at 33 °C (the average temperature in a hive), and adsorption onto MCM-41 increased it of about 10-fold. Notably, the relative rates were different when comparing the phenomenon at 45 °C and 60 °C. Therefore, the kinetic data were mathematically elaborated to generate Arrhenius plots (Fig. 6), from which the energies of activation (**Ea**) (Fig. 7) of the process were calculated. The Arrhenius curves of Neusilin®-adsorbed TH obtained at both TH loadings were almost superimposable to that of unformulated TH and no significant difference was registered in the sublimation process Ea (about 70 kJ/mol). On the contrary, the sublimation Ea from the other TH/MS powder blends was significantly lower and similar (about 50 kJ/mol) among them, despite their differences in their TH release rates.

It is important to note that, even if at the temperatures tested the MS-powder blended TH displayed sublimation rates slower than the unformulated compound, this situation is expected to invert at temperatures above the point of intersection between their Arrhenius plots (Interchange temperature- ***Ti***) (Table 4). The temperatures at which this intersection occurs vary between the different blends. Interestingly, in case of the Sil-Sol® blend, this interchange occurs at a ‘convenient’ temperature (18.0 ± 1.0 °C), which is likely to occur often in the field during the recommended period for thymol treatment (summer/early fall).

4. Discussion

The mesoporous powders and the active agent (TH) tested along this work were selected with the twofold aim of basic and applied research purposes. On one hand, by selecting ordered and non-ordered materials, with pores having different size and volume, we aimed at evaluating the role of the material mesostructure on the active agent physisorption and sublimation and produce data having a general scientific valence. By carrying out the extensive investigation on TH release behavior at different temperatures we also aimed at finding some justification to the contrasting behaviors reported in the literature in terms of the release rate of adsorbed compounds (accelerated or slowed-down *versus* non-formulated molecules).

On the other hand, since TH is an active substance adopted in the honey bee management whose use is limited by its fast sublimation rate, a goal was also to provide some potential solutions to improve its use in the field. Therefore, by testing also commercial powders (one also USP approved) our goal was also to generate information which, in principle, could be translated to the field. To this end a first important information was that regarding the erodibility in sink conditions, an indicator of the fate of the materials if ingested/inhaled by the bees or if ingested by humans due to residues in honey. In this respect, the dissolution behavior of all MS powders indicate that they are safe. Even if no dissolution was observed upon immersion at acidic pH (data not shown), silica hydrolysis at neutral pH was quantitive within 1–2 days for the pure silica-based and 12 days for the Mg-Al silicate Neusilin®. Notably, from a biosafety perspective the behavior at neutral pH is the most relevant one. In fact, even if upon ingestion the initial conditions encountered by the MS powders would be acidic, the gastrointestinal (GI) environment would turn neutral to slightly basic after passing the stomach. In case adsorption of particles from the GI tract would occur (a phenomenon which may occur for smaller particles - as in the case of the MCM-41 material), penetration would likely occur via the transcellular pathway and bring the particles to the neutral environment of the epithelial cell basolateral pole [43]. In case some MS particles may reach the bloodstream or would be inhaled, the pH encountered would be, again, neutral.

From the TH adsorption and release perspective, most of the results are in line with the literature on mesoporous materials. For example, TH loadability nicely correlates to the materials pore volume, the highest one achieved by Neusilin® with both loading capability and mesopore volume twice as big as the other MS powders. Despite this, Neusilin® was unable to stabilize the TH amorphous state, its pore size is too large to prevent TH recrystallization after physisorption and amorph stabilization was indeed achieved by all other powders which are all characterized by pores ≤10 nm.

Release of TH in the air followed zero-order kinetics both as a free molecule and when adsorbed onto all MS-powders. Again, in the case of the large pore Neusilin® sublimation occurred with similar rates as to the unformulated compound. When stabilization of the amorphous phase occurred, the sublimation rate was slowed down. Significant differences were registered among the different powders which correlate well with material mesostructures (pore size, surface area and the presence of ordered channels).

One important novel finding is that in all formulations having retentive properties, TH adsorption was accompanied with a reduction in its sublimation Ea. All pure silica based mesopowders led to the same change in Ea occurred, independently of their retention capability. The effect on Ea is therefore independent of the material mesostructure. Likely, this is due to the ability of the monolayered thymol to create

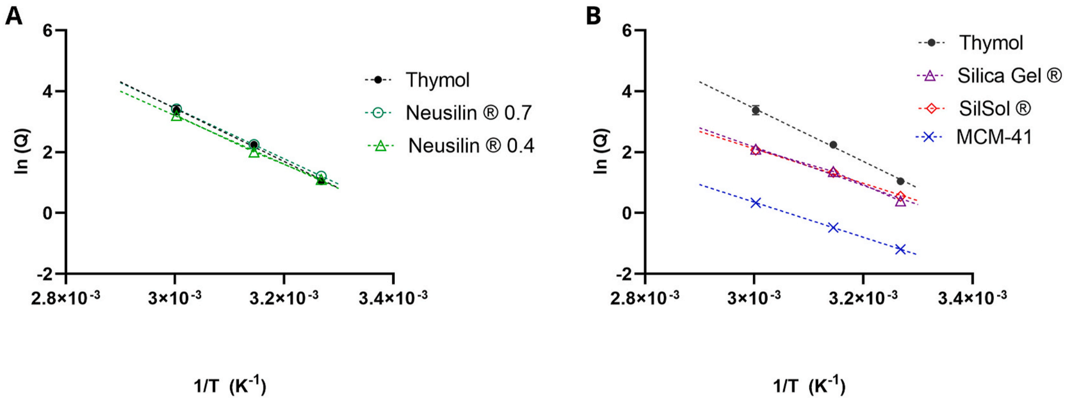

Fig. 6. Arrhenius Plots of the thymol sublimation process when in crystal form or adsorbed onto MS powders. A) comparison between thymol in the free form and when adsorbed onto Neusilin® at TH/MS-powder ratios of 0.4 and 0.72 w/w); B) comparison between thymol in the free form and when adsorbed onto SilSol® (TH/MS-powder ratio of 0.35 w/w), Silica Gel® (TH/MS-powder = 0.36 w/w); D and CM-41 (TH/MS-powder = 0.4 w/w).

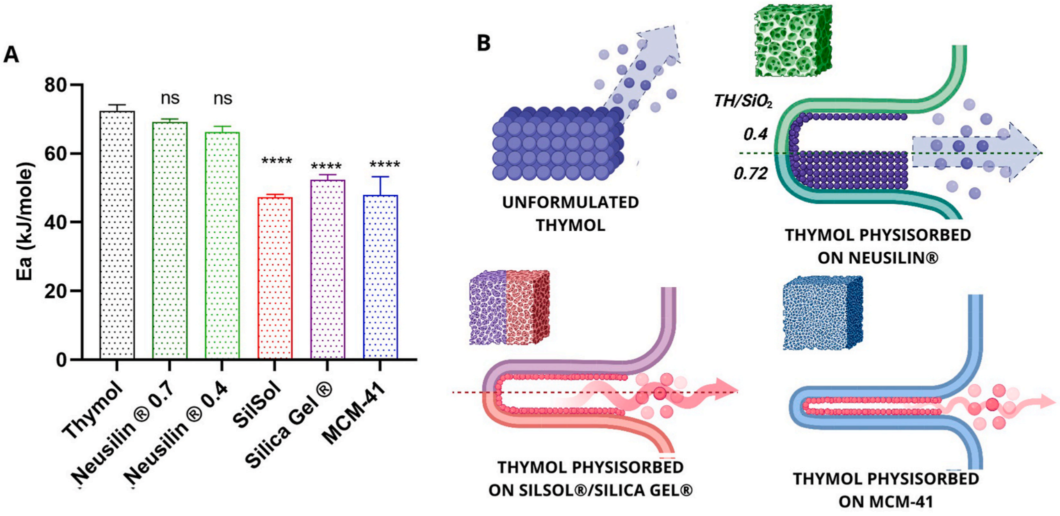

Fig. 7. Impact of MS adsorption on the Energy of activation of TH sublimation A) Eas of TH sublimation as pure compound and upon adsorption onto the different MS powders. Significance: One-way ANOVA test *versus* unformulated TH: ***$p < 0{,}005$; ns: non-significant; **B)** cartoon depicting the process of TH release in air when non-formulated and when physisorbed. Spheres represent physisorbed TH, with (purple) or without (blue) establishing the weak chemical interactions with the MS-powder surface that affect sublimation Ea. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 4

Sublimation Ea of TH differently formulated and Interchange temperatures, namely the temperatures below which MS-powder physisorbed TH sublimation is expected to occur faster than the non-formulated compound.

Properties	TH	TH/Neu 0.72	TH/Neu 0.4	TH/Sil-Sol® 0.35	TH/Silica gel® 0.36	TH/MCM-41 0.48
Ea (kJ)	72.44 ± 1.7	69.26 ± 0.77	66.3 ± 1.58	47.22 ± 0.88	53.39 ± 1.47	47.95 ± 5.28
Interchange Temp (°C) (***Ti***)	–	n.c.	n.c.	18.0 ± 1.0	10.6 ± 0.5	−25.7 ± 11.0

n.c. not calculated.

weak bonds with the surface silanols, this phenomenon occurring at length scales below those of the mesostructure.

Use of the Arrhenius plot allows to predict the sublimation behavior at temperatures beyond those tested experimentally. Notably, when a change in Ea occurs, sublimation of powder-adsorbed TH results either slower of faster than that of unformulated TH depending on the temperature, whether above or below ***Ti***. In other words, when adsorption onto a MS powder leads to a change in Ea, assuming that the porosity of the MS powder will always generate some resistance to the release process, release of the adsorbed compound will be slower than that of the non-formulated compound at $T > Ti$, but the opposite will occur at $T < Ti$. Considering that ***Ti*** values vary with both mesostructure and the nature of the compound adsorbed, this finding can explain why MS adsorption in literature – where normally experiments are carried out at one single temperature - has been accompanied to both faster or slower release rates of the adsorbed elements.

From a translational perspective, it has to be noted that, even if the synthetic ordered mesoporous MCM-41 was the best performer in terms of TH retention, this material is currently too costly and is not yet approved as a pharmaceutical excipient, so that application to the field would require a long regulatory pathway. Both Silica Gel® and Sil-Sol ® are significantly less costly than MCM-41 and are available in large scale. The two showed similar behavior on TH and the effects they exert, even if less pronounced than those of MCM-41, are surely sufficient to justify future field testing. Sil-Sol® is also approved as pharma excipient, so that, in principle this translation is already feasible also from a regulatory perspective.

5. Conclusions

This work led to new insights on the mechanisms underlying the effects of MS-powder adsorption of compounds on their release properties. Concurrently, we demonstrated that some TH/MS powder blends have superior properties than unformulated TH in environmental conditions that mimic those of its field application, opening the way towards future field experimentations. Slower release rates at the typical temperatures of the hive during the treatment season will permit to prolong the intervals between applications and this may improve beekeepers willingness to adopt this soft acaricide. In addition, lower Ea values imply that the sublimation rate will be less affected by temperature changes, as for example the daily temperature excursions or the temperature drops that can occur during late summer season. In principle, this should guarantee a more constant TH concentration in the hive and, therefore, more consistency in treatment efficacy.

CRediT authorship contribution statement

Simone Bernardotto: Writing – review & editing, Investigation, Data curation. **Elena Colusso:** Investigation, Writing – review & editing. **Ylenia Calogero:** Investigation. **Ilona Barker:** Investigation. **Erica Franceschinis:** Writing – review & editing, Funding acquisition. **Nicola Realdon:** Resources. **Elisa Schiavon:** Investigation. **Martina Stocco:** Investigation. **Tommaso Barbon:** Investigation. **Marzio Rancan:** Resources, Investigation. **Alessandro Martucci:** Resources, Methodology, Writing – review & editing. **Margherita Morpurgo:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization, Funding acquisition, Resources.

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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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jddst.2025.107066.

Data availability

Data will be made available on request.

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