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Understanding the encapsulation and release of small molecular weight model actives from alginate fluid gels

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Abstract

The inclusion of actives within a range of biopolymer-based formulated gelled systems has been receiving significant attention. Among these, encapsulation in gelled microparticles has generated substantial interest, but such systems still face challenges for scalable production. Fluid gels are a class of soft-solid particles produced via high-shear approaches that are suitable for industrial manufacture. However, their use for the entrapment of species of therapeutic/nutritional activity has been scarcely investigated. The aim of this work was to study the encapsulation/retention of different small molecular weight actives of varied hydrophilicity/hydrophobicity within alginate fluid gels (AFG). The presence of an active during AFG formation did not interfere with gel particle dimensions nor bulk rheological behaviour. Following production, encapsulation of all actives within the fluid gel particles was very low, but significantly increased for the hydrophobic ones with storage. The rate and extent of hydrophobic active loading in the AFG particles were governed by the concentration of the former and gel particle size. Release studies under sink conditions revealed that hydrophobic active content within the AFG particles is not expelled and remains fully entrapped regardless of fluid gel particle size and storage period. A hypothesis suggesting that the loading of hydrophobic actives within the AFG particles is due to changes to the solvent quality of water, is presented. Overall, the current study offers insight into the use of alginate fluid gels as encapsulation media and presents original findings that can greatly support future efforts to expand the applicability of these industrially-relevant colloidal systems.

Keywords: Alginate; Fluid gels; Encapsulation Efficiency; Release.

1   **1. Introduction**

2   Material and microstructures that can enable the controlled release of active ingredients

3   are increasingly used in both the foods and pharmaceutical industries. Microencapsulation

4   matrices, such as liposomes, microgels, emulsions and/or microemulsions, have been studied

5   for such purposes in foods (Ray *et al*., 2016). These formulations can be designed in order to

6   achieve different performances, such as to prolong the function of actives by delaying their

7   release, protect sensitive components from adverse environmental conditions (e.g. high

8   moisture content, acidity, presence of enzymes, etc.) and/or mask unwanted tastes (Wischke &

9   Schwendeman, 2008).

10   Amongst all such microstructures, hydrocolloid gel particles have received significant

11   research attention due to their biocompatibility (Burey *et al*., 2008). However, the majority of

12   current microencapsulation technologies based on hydrocolloid microparticles, face challenges

13   in terms of scalable production (Ozkan *et al*., 2019). Over the last two decades a new approach

14   for the production of biopolymer-based microparticles, commonly termed as sheared or fluid

15   gels, has been investigated (Norton *et al*., 1999; Shewan & Stokes, 2013). Fluid gels are

16   suspensions of gel particles in a non-gelled continuous medium, usually water, produced when

17   a biopolymer solution undergoes gelation whilst subjected to shear (García *et al*., 2018; Garrec

18   & Norton, 2012; Norton *et al*., 1999). Fluid gels can be produced continuously using typical

19   shear devices (e.g. pin-stirrer) and as such their scalable manufacture is less problematic

20   (Fernández Farrés *et al*., 2013). Because of their unique rheological properties/characteristics,

21   fluid gels have been mainly studied as texturing materials for applications in food products

22   (Chung *et al*., 2014; Fernández Farrés *et al*., 2014; Le Révérend *et al*., 2010; Norton *et al*.,

23   2015). Tribological studies have also shown the ability of these systems to provide lubrication,

24   suggesting that they can impart an in-mouth sensory response similar to that typically

25   associated with fat (Fernández Farrés *et al*., 2014).

26   Despite their current applications, literature on the utilisation of fluid gels for entrapment

27   and delivery purposes is extremely scarce. The current authors used alginate fluid gels to study

28   the encapsulation of tryptophan and showed that overtime such matrices can slow down the

29   release of the active (Smaniotto *et al*., 2019). In another study, ter Horst *et al*. (2019) studied

30   the entrapment of skin cells in gellan gum fluid gel systems in order to enable their delivery

31   onto burn wounds. The authors reported that cell viability was not compromised by

32   encapsulation in the gellan fluid gels nor by the subsequent spray application (ter Horst *et al*.,

33   2019). Nonetheless, the mechanism for the encapsulation of actives within fluid gel

formulations is far from understood, while key parameters that can affect both encapsulation as well as subsequent release (for example, the influence of gel particle size), have simply not been investigated.

The present work aims to understand the encapsulation and release of small molecular weight actives from alginate fluid gel (AFG) formulations, as a function of the type (hydrophilicity/hydrophobicity) and concentration of the to-be-entrapped species and AFG particle size. AFG formation was carried out within a pin-stirrer vessel both in the presence and absence of the actives. In both cases, AFG particle size and bulk rheological behaviour (shear viscosity) were measured. The encapsulation efficiency (EE) of all actives was determined within 24 h of AFG production and then monitored over a period of 2 weeks (following AFG production) during which all formulations were stored unperturbed at room temperature. The release of all actives from the AFG formulations was carried out under sink conditions for both immediately produced and stored systems. The effect of AFG particle size on the EE and release profiles for all (hydrophilic/hydrophobic) actives were studied. Overall, the current study offers new insight into the use of AFG as encapsulation matrices in an attempt to strengthen the industrial applicability of these colloidal systems.

2. Materials and methods

2.1. Materials

Sodium alginate (ALG), vanillin (VAN, ≥97%), L-tryptophan (TRP, ≥98% HPLC), and nicotinamide (NIC, ≥98%, HPLC), were purchased from Sigma-Aldrich® (UK). Calcium chloride ($CaCl_2$, anhydrous, 93%) was purchased from Alfa Aesar™ (USA). Vanillin and tryptophan were chosen as hydrophobic actives, while nicotinamide as a hydrophilic one. All material were used without further purification. Milli-Q distilled water, produced using an Elix® 5 distillation apparatus (Millipore®, USA), was used in all formulations. All concentrations are given as % w/w; percentage of the weight of any specific compound over the total weight of the system it is contained within (i.e. solution, fluid gel formulation).

2.2. Production of alginate fluid gels

2.2.1. *Blank alginate fluid gels*

The production of blank alginate fluid gels (AFG) follows the process used by Smaniotto *et al*. (2020). In brief, a 2% w/w alginate solution was prepared by dissolving the required amount of the compound in distilled water (95°C) under mild stirring for 45 min. An aqueous

1   solution of $CaCl_2$ (0.25% or 0.35% w/w) was also prepared by dissolving $CaCl_2$ at room

2   temperature under magnetic stirring. AFG were formed using a pin-stirrer device (Het Stempel,

3   Netherlands), with an available processing volume of 150 mL, operating at a pin-shaft speed

4   of 1000 rpm. The alginate solution was pumped into the pin-stirrer at 33 mL/min using a

5   peristaltic pump (Masterflex L/S Peristaltic, Germany), while the $CaCl_2$ solution was injected

6   into the pin-stirrer through a stainless steel needle (1.25 mm internal diameter) using a syringe

7   pump (Cole-Parmer Single-syringe, USA) at 4.02 mL/min.

8

9   2.2.2. *Active-containing alginate fluid gels*

10   Active-containing AFG were produced following a similar procedure. Each active

11   (0.05% or 0.10% w/w) was dissolved in distilled water at room temperature under magnetic

12   stirring, with $CaCl_2$ (0.25% or 0.35% w/w) then added to this. The alginate and active-

13   containing $CaCl_2$ solutions were pumped into the pin-stirrer as above. Preliminary tests, where

14   the actives were incorporated within the alginate (rather than the $CaCl_2$) stream for entry into

15   the pin-stirrer, produced indistinguishable active-containing AFG formulations.

16

17   **2.3. Rheological measurements**

18   The shear viscosity of all blank and active-containing AFG formulations was measured

19   using a rotational rheometer (Kinexus[™], Malvern[®], UK) equipped with a 40 mm diameter sand

20   blasted plate geometry. All analyses were carried out at 25°C using a shear rate ramp between

21   0.1 and 100 s^{-1}. All rheological measurements were carried out in triplicate and all shear

22   viscosity data are presented/plotted as mean values ± 1 standard deviation (SD). In order to

23   assess bulk viscoelasticity, the shear viscosity profiles of all AFG formulations were fitted to

24   a simple power-law model (Barnes *et al.*, 1989):

$$\eta = K_2 \, \dot{\gamma}^{\,n-1} \qquad\qquad \text{Eq. (1)}$$

25   where K is the consistency constant and n is the power-law index.

26

27   **2.4. Particle size analysis**

28   The particle size distributions (PSD) of all blank and active-containing AFG were

29   evaluated using laser diffraction (Mastersizer-2000, Malvern[®], UK) and are presented as the

30   numerical particle size percentage. Few drops of each fluid gel formulation were added to a

31   pure aqueous phase contained within the chamber of the instrument where they were mixed at

32   1300 rpm for at least 10 min (necessary for a constant value of obscuration to be obtained)

before performing the analysis. AFG particle dimensions previously determined using dynamic light scattering (Smaniotto *et al*., 2020) were in agreement to the sizes measured here. Surface weighted mean diameters ($D_{3,2}$) and span values were provided by the Mastersizer-2000 software. All PSD measurements were carried out in triplicate and all $D_{3,2}$ and span data are presented/plotted as mean values ± 1 standard deviation (SD).

Selected AFG systems were also visualised using an optical microscope (DM 2500 LED, Leica®, CH) to, amongst others, confirm the particle size data obtained by laser diffraction. For the purpose of this, AFG samples were firstly diluted with distilled water using a water-to-sample ratio of 10:1 and these were subsequently mixed using a vortex mixer (VM20, Rigal Bennet®, UK) for 20 seconds. Differential Interference Contrast (DIC) settings were set up to increase the contrast, allowing for clearer visualisation of the particles and their dimensions. Images were captured using a CCD camera (DFC450C, Leica®, CH) coupled to the microscope (Figure 1). Image analysis to evaluate the dimensions of the microparticles was carried out using the Leica Application Suite (LAS 4.8.0, Leica®, CH).
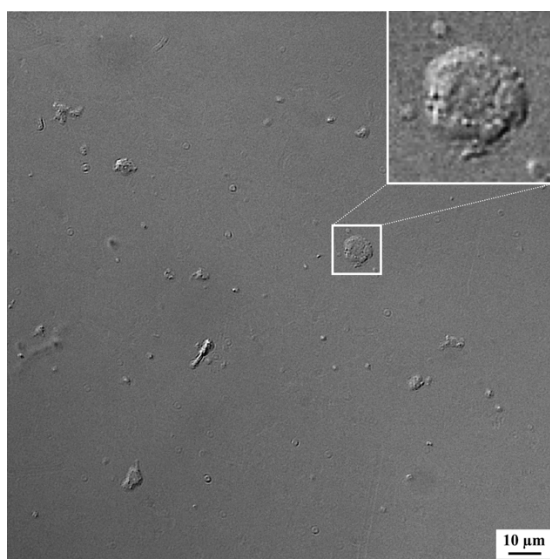
Figure 1. Optical micrograph of AFG particles produced in a pin-stirrer device, using a 2% w/w and 0.35% w/w alginate and $CaCl_2$ aqueous solutions, respectively.

2.5. Ultraviolet–visible (UV-Vis) spectroscopy

Ultraviolet–visible (UV-Vis) spectroscopy was used to determine the concentration of non-entrapped actives using the procedure employed by Smaniotto *et al*. (2019). In brief, the absorbance of all actives was measured at room temperature using an Orion AquaMate 8000 UV-Vis spectrophotometer (Thermo-Scientific®, UK), at 300 nm for vanillin, 278 nm for tryptophan and 214 nm for nicotinamide. Absorbance was converted to concentration using the

Beer-Lambert equation and correlation parameters obtained from active-specific calibration curves. All UV-Vis measurements were carried out in triplicate.

2.6. Encapsulation efficiency

The encapsulation efficiency (EE) of all actives in the AFG particles was determined following the procedure employed by Smaniotto *et al.* (2019). In brief, ~15g of fluid gel sample were subjected to ultracentrifugation for 45 minutes, at 21°C and 21,000 rpm, using a Sigma 3K-30 refrigerated centrifuge (Sigma®, Denmark), equipped with a 12150 rotor. A known amount of the formed supernatant (~1.5 g) was placed in a 100 mL volumetric flask and filled to full capacity with distilled water. The mass of the active in the supernatant was finally determined via UV-Vis analysis. EE was then calculated as:

$$EE\% = \frac{W_i - W_t}{W_i} \times 100 \qquad \text{Eq. (2)}$$

where W_i is the amount of active initially added to the fluid gel formulation and W_t is the amount of active in the supernatant (non-encapsulated active) of the same fluid gel formulation at different time intervals t. The ($W_i - W_t$) difference therefore corresponds to the amount of active that is encapsulated within the AFG particles (Piacentini, 2016).

2.7. Release studies

Approximately 2.5 g of an active-containing AFG formulation were enclosed in a dialysis sack (cellulose dialysis tubing membrane, width 43 mm, MW cut-off of 14000 Da; Sigma–Aldrich, UK) which was then placed in 500 mL of distilled water (acceptor phase) at room temperature (~ 21°C), under mild mixing provided by the use of a magnetic stirrer at 150 rpm. At regular intervals, aliquots of 2 mL were withdrawn from the acceptor phase and measured using UV-Vis spectroscopy to determine the concentration/amount of released active; following their UV-Vis measurement, each withdrawn aliquot was added back into the acceptor phase. All measurements were carried out in triplicate and all release data are presented/plotted as mean values ± 1 standard deviation (SD). Release data were fitted to the Ritger-Peppas model (Ritger & Peppas, 1987):

$$\frac{M_t}{M_\infty} = k_r\, t^{\,n_r} \qquad \text{Eq. (3)}$$

where M_t and M_∞ are the cumulative amount of active released at time t and at infinite time (complete release), respectively, k_r is a kinetic constant, and n_r is the diffusional exponent characteristic of the release mechanism.

2.8. Statistical analysis

All data are presented as mean values ± one standard deviation (SD). Statistical significance was determined by performing Student's t-test. Results were considered statistically significant at p-values ≤ 0.05.

3. Results and discussion

3.1. Effect of active type and concentration

Alginate fluid gels (AFG) were produced in the presence of actives and the effect of active type (hydrophilic/hydrophobic) and concentration on AFG particle size (PSD), rheological behaviour, encapsulation efficiency (EE) and release behaviour (under sink conditions) were studied. Samples were produced by fixing the alginate and $CaCl_2$ concentrations at 2% w/w and 0.35% w/w, respectively. A previous study by the current authors showed that, at these concentrations, AFG particles (no active present) in the size range of 0.5-5 μm can be obtained (Smaniotto *et al*., 2020). The model actives used were selected based on their water-solubility (hydrophilicity/hydrophobicity); vanillin and tryptophan were chosen to represent low water-solubility actives (11 mg/mL and 13 mg/mL, respectively) and nicotinamide to correspond to a model active of high water-solubility (500 mg/mL). Vanillin is a natural flavour, tryptophan is an essential amino acid and nicotinamide is a form of vitamin B3 (Gibbs *et al*., 1999; Jelena *et al*., 2010). The concentrations of all actives tested here were matched and kept below the aqueous saturation limit of the least water soluble one (i.e. vanillin). As such, active concentrations of 0.05% to 0.10% w/w for VAN (VFG_0.05% and VFG_0.10%), TRP (TFG_0.05% and TFG_0.10%), and NIC (NFG_0.05% and NFG_0.10%) were chosen.

3.1.1. *Fluid gel particle size and rheological behaviour*

All AFG produced in the presence of different actives were characterised in terms of particle size (PSD) and bulk rheological behaviour (shear viscosity). Results were compared to PSD and shear viscosity curves of blank AFG systems (formed in the absence of an active). Size measurements, reported in Figure 2, revealed that all AFG formulations have practically the same PSD, independently of the type and/or concentration of the active used. Blank and active-containing AFG particles exhibited similar $D_{3,2}$ (1-3 μm) and span (1-2) values.

7
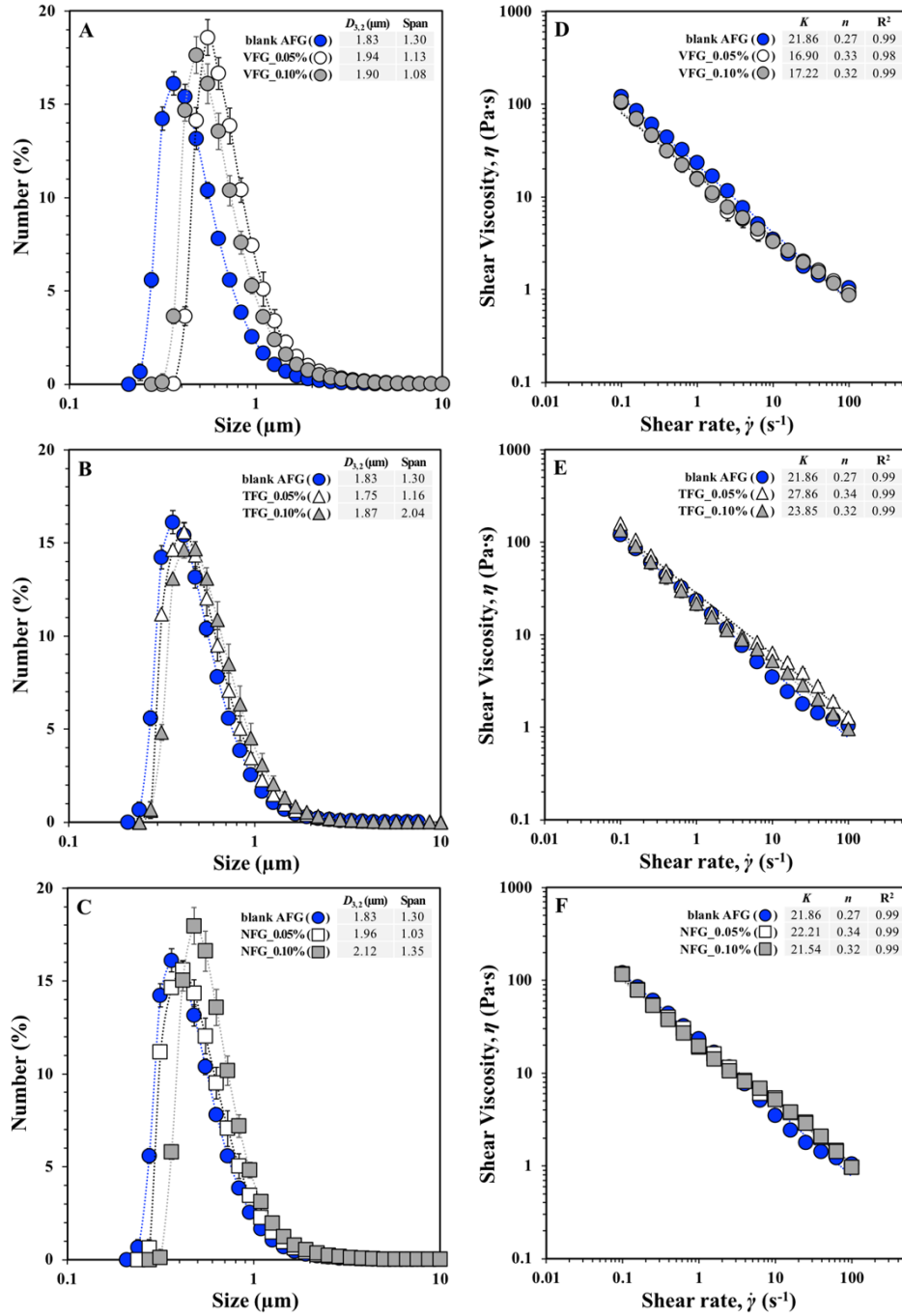
Figure 2. Particle size distributions (**A**, **B** and **C**) and shear viscosity curves (**D**, **E** and **F**) for AFG formed in the absence (blank AFG) and the presence of different actives (vanillin, VFG; tryptophan, TFG; nicotinamide, NFG) as a function of active concentration (0.05% and 0.10% w/w). All data are given as mean values ± 1 SD (error bars).

Shear viscosity profiles for all AFG formulations are also presented in Figure 1 together with their fit to Eq. (1) and obtained model parameters. The shear viscosity curves for all systems were comparable with similar K and n parameters; the latter also indicating that all formulations exhibit a similar degree of shear thinning behaviour (Fox *et al*., 2011). Overall, AFG particle size and rheological performance does not appear to be affected by the presence or

1 concentration of either a hydrophilic or a hydrophobic active; at least within the range of
2 concentrations tested here. PSD and shear viscosity profiles, for all fluid gel systems, were
3 monitored over a period of 2 weeks following manufacture, and no significant deviation to
4 either of these parameters was observed (data not shown here).
5
6 3.1.2. *Encapsulation efficiency*
7   The data on the encapsulation efficiency (EE) for all active-containing AFG formulations
8 as a function of active type (hydrophilic/hydrophobic) and concentration are reported in Table
9 1. EEs were determined within 24 h of AFG production and then monitored over a period of 2
10 weeks, during which all formulations were stored unperturbed at room temperature; t_s is used
11 to denote the period under storage.
12
13 **Table 1**. Encapsulation efficiency (EE) in AFG formulations produced in the presence of different
14 actives (vanillin, VFG; tryptophan, TFG; nicotinamide, NFG), as a function of active type and
15 concentration, and storage time (t_s). Differences between EE values (for the same active) marked with
16 the same superscripted letter are not statistically significant ($p > 0.05$).

Active-containing AFG formulation				Storage period, t_s		
Active type	Active conc. (% w/w)	Sample		1 Day	1 Week	2 Weeks
Vanillin	0.05	VFG_0.05%		22.5% (±3.2)[a]	48.3% (±3.1)[b]	69.2% (±3.4)[c]
	0.10	VFG_0.10%		28.9% (±3.2)[a]	25.2% (±2.9)[a]	43.9% (±3.2)[b]
Tryptophan	0.05	TFG_0.05%	EE (±SD)	12.3% (±3.5)[a]	21.4% (±3.2)[b]	33.1% (±3.7)[c]
	0.10	TFG_0.10%		19.8% (±3.9)[a,b]	22.1% (±3.2)[b]	16.2% (±5.7)[a,b]
Nicotinamide	0.05	NFG_0.05%		0.0% (±0.0)[a]	0.0% (±0.0)[a]	0.0% (±0.0)[a]
	0.10	NFG_0.10%		4.1% (±1.3)[b]	5.6% (±1.1)[b]	3.6% (±1.7)[b]

18   The data shows that within the first 24 h following the production of all active-containing
19 AFG, EE was very low. Unlike typical gel particle formation approaches (e.g. emulsion
20 templating, electrostatic extrusion, spray drying) where systems are produced via the gelation
21 of a liquid precursor of comparable (to the resulting particle) dimensions (Lengyel *et al*., 2019),
22 fluid gel particles are formed via a nucleation and growth mechanism (Norton *et al*., 1999;
23 Fernández Farrés *et al*., 2014). As such, the opportunity to already have an active contained
24 within a domain (e.g. droplet) that is then gelled, is not pertinent to fluid gels, where particle
25 formation and active entrapment would take place in tandem. With this in mind and given the
26 small molecular dimensions of all actives tested here, it is not surprising that initial EE is
27 significantly compromised.

1   However, when these systems are stored over a period of 2 weeks, the fraction of active

2   species entrapped within the fluid gel particles (still expressed as EE) increases as a function

3   of t_s, in the case of hydrophobic actives (vanillin and tryptophan). In contrast, the EE of the

4   hydrophilic active (nicotinamide) remains practically unchanged over the same period;

5   maintaining the same extremely low EE levels initially measured. Therefore, although not

6   initially encapsulated upon AFG production, both hydrophobic actives (but not the hydrophilic

7   one) migrate from the surrounding aqueous phase to the formed gel particles over the time that
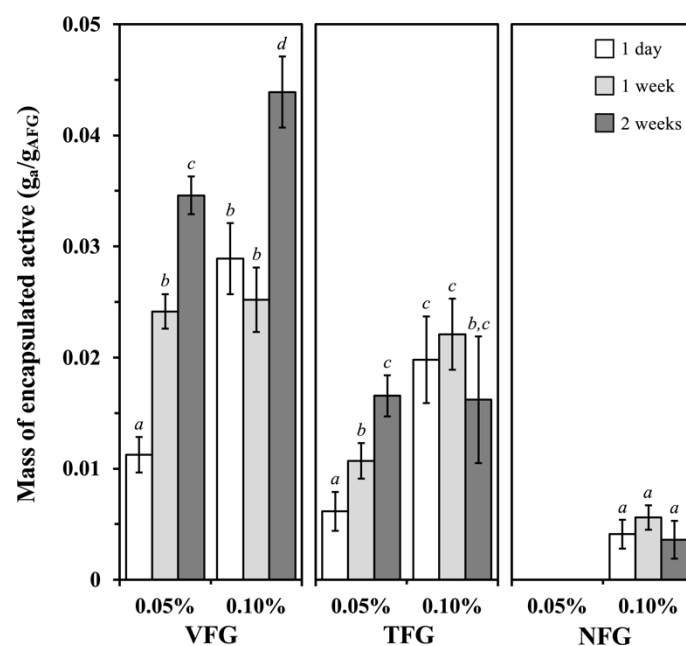
8   the formulations are stored.

9

10

Figure 3. Mass of actives encapsulated in AFG produced in the presence of different actives (vanillin,
VFG; tryptophan, TFG; and nicotinamide, NFG), as a function of active type and concentration, and
storage period t_s. Data are reported as mass of encapsulated active (g_a) over mass of active-containing
AFG formulation (g_{AFG}). All data are given as mean values $\pm$ 1 SD (error bars). Differences between
values (for AFG formulations containing the same type of active) marked with the same letter are not
statistically significant ($p > 0.05$).

17

18   Furthermore, the EE data reveals that this transferal takes place more efficiently (i.e.

19   resulting in a higher EE) when a lower active concentration (0.05% w/w) is used (Table 1).

20   Nonetheless, by only considering the mass of active that is entrapped into the gel particles (as

21   a function of t_s), formulations prepared at a higher active content (0.10% w/w) yield AFG

22   particles with a higher total mass of encapsulated species (Figure 3). Considering that AFG

23   particle dimensions for these formulations were shown not to be affected by the presence of

24   the active nor its concentration (Figure 2), it can be assumed that all systems would possess a

1 comparable number of particles per unit of volume. Therefore, the data presented in Figure 3

2 suggests that increasing the concentration of the active present during AFG formation results

3 in particles that, over the same time period, can be loaded with a higher mass of active.

4

5 3.1.3. *Release performance*

6   Release tests for all active-containing AFG formulations as a function of active type,

7 active concentration and storage time (t_s), were carried out under sink conditions and the results

8 are presented in Figure 4. Release testing was carried out on 'as-produced' AFG formulations,

9 without separation of the formed fluid gel particles from their continuous water phase nor

10 dilution. Results were not adjusted to take into account the fraction of encapsulated active

11 within the fluid gel particles, as determined by EE analysis. As such, release data reported here

12 correspond to the cumulative release of each active (total amount of active detected in the

13 acceptor phase) and are expressed as a percentage of the active content initially used during the

14 production of all AFG formulations. Additionally, release experiments for simple aqueous

15 solutions of the actives (vanillin, tryptophan and nicotinamide aqueous solutions, denoted as

16 VAS, TAS and NAS, respectively) were carried out at a 0.10% w/w active concentration. All

17 release data were fitted to Eq. (3) and the values for the obtained model parameter n_r are

18 reported in Table 2.

19   The release curves for all fluid gel formulations follow a similar trend; an initially fast

20 release that eventually reaches a plateau, in all cases, after ~3 h. However, the cumulative

21 release value for this plateau varies with the type and concentration of the active as well as with

22 t_s. While the percentage of cumulative release for nicotinamide (hydrophilic) reaches 100%

23 (the full content of the active initially contained in the AFG formulation is eventually detected

24 in the release medium), the release plateau for vanillin or tryptophan (both hydrophobic) is

25 lower and is further reduced as storage time increases. It is worth noting that the release of both

26 hydrophilic and hydrophobic actives from their simple aqueous solutions also progresses to

27 their full transferal (100%) in the release medium. Furthermore, analysis of the release data

28 (Table 2) suggests that, in all cases, active transport to the acceptor phase takes place

29 predominantly by diffusion; for all systems the exponent n_r is close to 0.5 (Korsmeyer *et al.*,
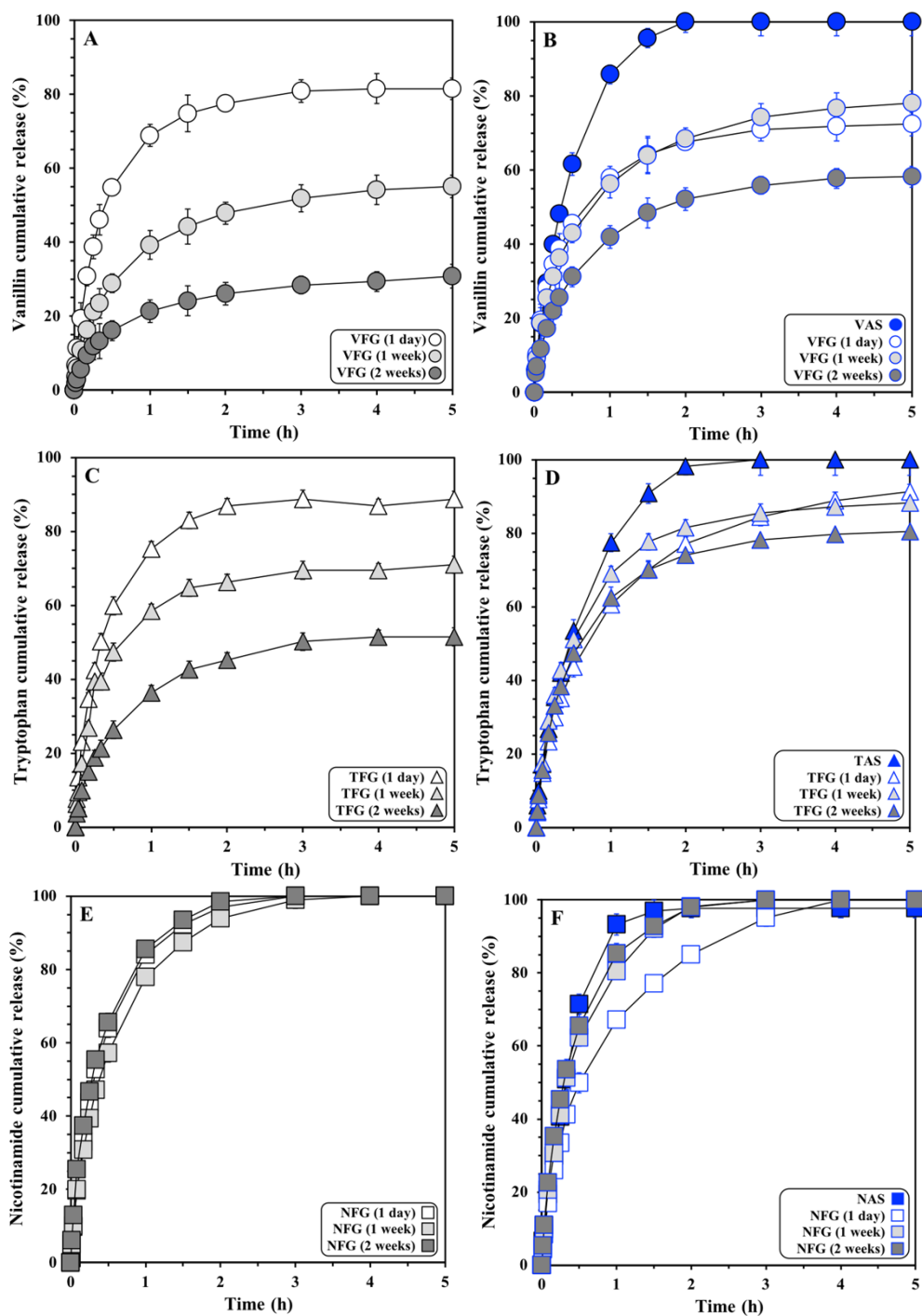
30 1983).

31

Figure 4. Cumulative release profiles under sink conditions for AFG formulations produced in the presence of different actives; vanillin (VFG; A & B), tryptophan (TFG; C & D) and nicotinamide (NFG; E & F). Release data for all AFG formulations are presented as a function of storage time (t_s) and active concentration; 0.05% w/w (A, C & E) and 0.10% w/w (B, D & F). Release profiles of aqueous solutions of vanillin (VAS), tryptophan (TAS) and nicotinamide (NAS), at a 0.10% w/w active concentration are also shown. All data are given as mean values ± 1 SD (error bars).

12

Table 2. Model parameter n_r (mean value ± 1 SD) as a function of storage time (t_s), obtained from the best-fit to Eq. (3) of the cumulative release data from active-containing AFG formulations shown in Figure 4. Goodness-of-fit (R^2) values are also provided.

Active-containing AFG formulation

Active type	Active conc. (% w/w)	Sample	t_s	n_r	R^2
Vanillin	0.05	VFG_0.05%	1 day	0.58 (± 0.06)	0.99
			1 week	0.56 (± 0.06)	0.99
			2 weeks	0.58 (± 0.09)	0.99
	0.10	VFG_0.10%	1 day	0.45 (± 0.08)	0.98
			1 week	0.47 (± 0.06)	0.99
			2 weeks	0.50 (± 0.04)	0.99
Tryptophan	0.05	TFG_0.05%	1 day	0.55 (± 0.06)	0.99
			1 week	0.55 (± 0.14)	0.97
			2 weeks	0.56 (± 0.06)	0.99
	0.10	TFG_0.10%	1 day	0.62 (± 0.06)	0.99
			1 week	0.60 (± 0.09)	0.99
			2 weeks	0.61 (± 0.07)	0.99
Nicotinamide	0.05	NFG_0.05%	1 day	0.68 (± 0.14)	0.99
			1 week	0.66 (± 0.07)	0.99
			2 weeks	0.62 (± 0.10)	0.99
	0.10	NFG_0.10%	1 day	0.63 (± 0.07)	0.99
			1 week	0.66 (± 0.08)	0.99
			2 weeks	0.67 (± 0.09)	0.99

In order to better understand the effect of active type and concentration on the obtained release profiles, EE data were also considered. For each AFG formulation, non-encapsulated active fraction, as determined from EE data, was compared to the plateau value (cumulative release percentages after 5 h) from the corresponding release profile, and results are presented in Figure 5. The comparison revealed that these two quantities, regardless of the type and concentration of the active and/or the period of storage, were practically the same. It is, therefore, apparent that the amount of active detected during the release experiments was, in all cases, the fraction of the species that was not entrapped in the first place. This further implies that once an active is entrapped within the alginate fluid gel particles, then its release simply does not take place, even under sink conditions. As the mass of active within the AFG particles increases with t_s, the capacity of these microstructures to retain the entrapped species is maintained. This is obviously the case only for the systems exhibiting some level of entrapment and as such this behaviour was not observed for the nicotinamide-containing AFG formulations (NFG). For the latter systems release is indeed slightly delayed, in comparison to that observed simply for an aqueous solution of the active, most likely as a result of the increased viscosity of the AFG formulations (compared to water). Nonetheless, release does result in complete expulsion of the active (100%); realised approximately after 90 minutes for NAS and 120 minutes for NFG.
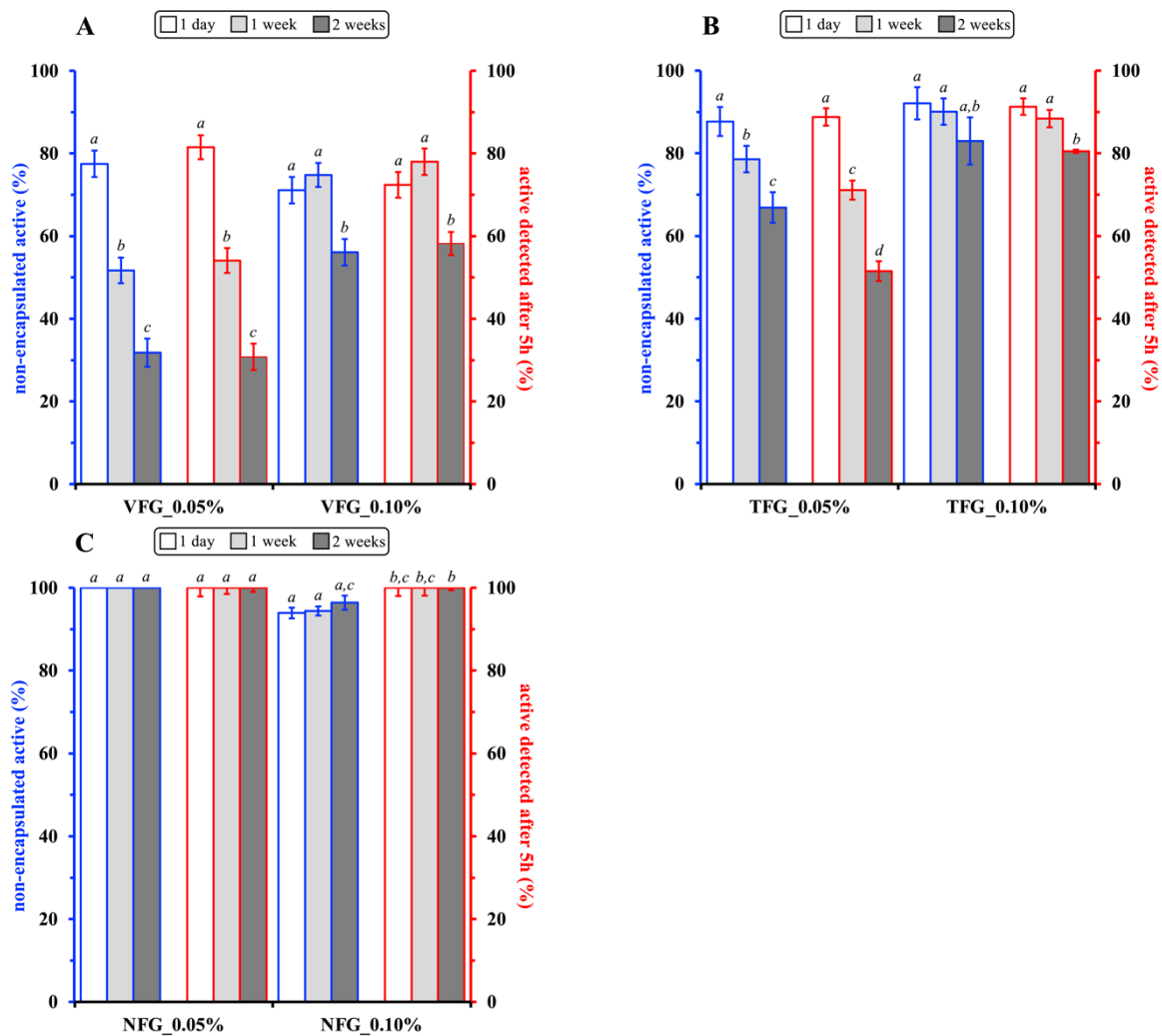
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Figure 5. Non-encapsulated active (%) in the AFG formulations (blue) and active detected in the acceptor phase after 5 h of release testing (red), as a function of active type (**A**: vanillin, VFG; **B**: tryptophan, TFG; **C**: nicotinamide, NFG), active concentration (0.05 and 0.10% w/w) and storage time t_s (1 day, 1 week, 2 weeks). All data are given as mean values ± 1 SD (error bars). Differences between values (for AFG formulations containing the same type and concentration of active) marked with the same letter are not statistically significant ($p > 0.05$).

3.2. Effect of fluid gel particle dimensions

The observation that alginate fluid gels particles appear to preferentially entrap small molecular weight hydrophobic actives (but not hydrophilic ones) over a storage period of 2 weeks, is further explored in this section with reference to the role of AFG particle dimensions; AFG particle sizes tested in the previous section were all of equivalent dimensions (~2 μm; see Figure 2). In this part, AFG formulations of two different gel particle sizes were produced in the presence of a hydrophobic active. As the aforementioned influence was observed regardless of the specific hydrophobic active and its concentration, a single active (vanillin; which displayed the highest sensitivity to this effect, in comparison to tryptophan) at a single concentration (0.10% w/w; the highest of those previously tested) were only considered. AFG

14

1 formulations were produced in the presence (and absence) of vanillin and the effect of the

2 active on AFG particle size (PSD), rheological behaviour, EE and release behaviour (under

3 sink conditions) were studied.

4

5 3.2.1. *Fluid gel particle size and rheological behaviour*

6 AFG formulations were produced with $CaCl_2$/alginate ratios of 0.125 and 0.175 (alginate

7 concentration fixed at 2% w/w and $CaCl_2$ concentration at 0.25 and 0.35% w/w; samples

8 VFG_A and VFG_B, respectively), as these fractions have been previously reported to give

9 AFG particles of distinctively different dimensions (Smaniotto *et al*., 2020). The two

10 $CaCl_2$/alginate fractions gave AFG particles with $D_{3,2}$ values of ~200 nm (ratio of 0.125) and

11 ~2 μm (ratio of 0.175), regardless of whether vanillin was present (VFG) or not (AFG) during

12 their production; the particle size distributions for all fluid gel formulations are presented in

13 Figure 6. AFG particle dimensions for the lower $CaCl_2$/alginate ratio (0.125) were in agreement

14 to the size of alginate nano-assemblies reported by Badita *et al*. (2016) to form under $CaCl_2$

15 and alginate contents comparable to those used here. The latter study concludes that at such

16 relatively low calcium concentrations, gelation is limited and alginate aggregates of spherical

17 shape and rough surfaces are formed with a radius of gyration of ~300 nm.

18 The shear viscosity data, together with their fit to the simple power-law model of Eq. (1),

19 for AFG formulations of both gel particle sizes, produced in the presence of vanillin or not

20 (blank), are also shown in Figure 6. The viscosity profiles for all formulations are very similar

21 and no influences, relating to the different gel particle sizes or the presence of the active, can

22 be observed. Moreover, the values for the power law fitting parameter *n* for all fluid gel

23 formulations suggest a similar shear thinning behaviour. Viscosity data presented here are in

24 agreement to the flow curves reported by Fernández Farrés & Norton (2014), although alginate

25 fluid gels in the latter study were formed by slowly releasing calcium from $CaCO_3$ using

26 glucono-δ-lactone (GDL). It is worth noting that both the PSD and shear viscosities profiles,

27 for all AFG formulations, were monitored over a period of 2 weeks, following manufacture,

28 and no significant deviation to either of these characteristics was observed.

29 As the concentration of vanillin used in these systems is very low, it is not surprising that

30 the flow behaviour of the active-containing AFG formulations is equivalent to that of their

31 'blank' counterparts. Although, the observation that the same flow characteristics are also not

32 affected by gel particle dimensions might appear to be an unexpected one, the reason for this

1   relates to the microstructural attributes of these systems. The flow behaviour in the case of fluid

2   gels is largely dominated by the high fraction of dispersed particles typically obtained in these

3   formulations (reported to be close to the maximum packing fraction) and particle-specific

4   mechanical properties (deformability or modulus) (Garrec & Norton, 2012; Fernández Farrés

5   *et al*., 2014). The latter dominate FG flow behaviour at high particle fractions (higher frequency

6   of particle-particle contacts/interactions) and are proportional to the biopolymer concentration

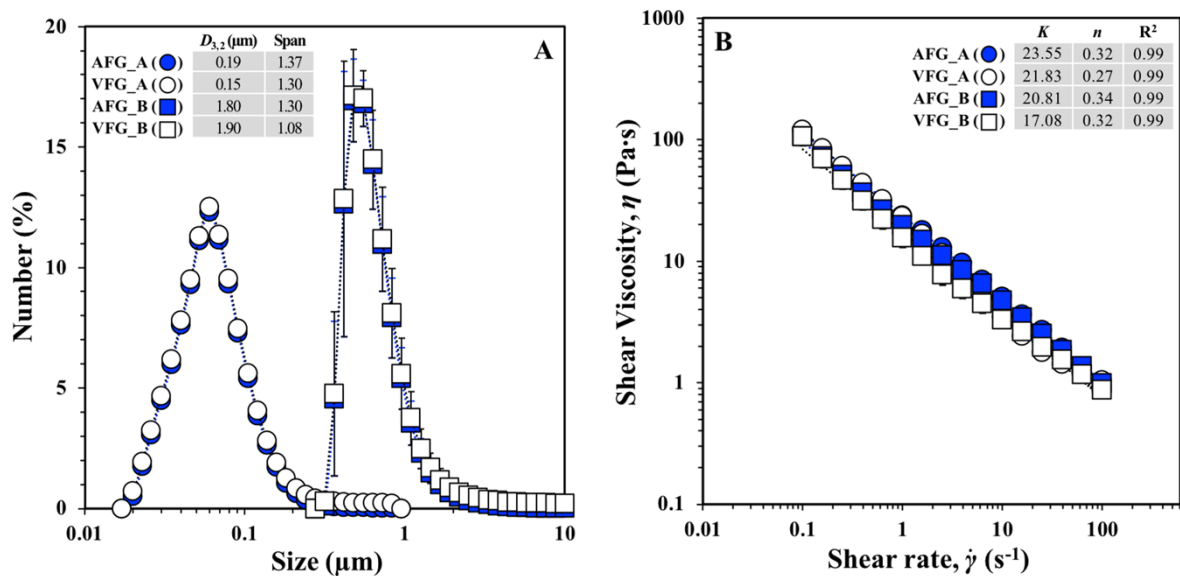7   (Garrec & Norton, 2012; Fernández Farrés *et al*., 2014).

8

9
10 **Figure 6.** Particle size distributions (**A**) and shear viscosity curves (**B**) for alginate fluid gels, formed in
11 the absence (AFG; *blank*) and the presence of vanillin (VFG), as a function of the used $CaCl_2$/alginate
12 ratio; 0.125 (AFG_A and VFG_A) and 0.175 (AFG_B and VFG_B). All data are given as mean values
13 $\pm$ 1 SD (error bars).

14

15   The influence of particle deformability on the flow behaviour of fluid gels or high phase

16 volume suspensions of microgel particles, has also been reported in whey protein fluid gels

17 (Moakes *et al*., 2015) and (high phase volume suspensions of) a range of agar microgel particles

18 (Adams *et al*., 2004). Although, none of the fluid gel formulations studied here were diluted

19 (i.e. they should all possess a high gel particle phase fraction) and all were formed using the

20 same alginate concentration (2% w/w), the calcium content of the VFG_A (or AFG_A) fluid

21 gel nano-particles and the VFG_B (or AFG_B) fluid gel micro-particles are different. Albeit at

22 slightly higher concentrations to those studied here, Fernández Farrés & Norton (2014)

23 reported that increasing the calcium content leads to an enhanced AFG particle stiffness.

24 However, Moakes *et al*. (2015) suggest that deformability is also a function of size, with larger

16

particles being easier to deform compared to the more rigid smaller ones. It is not clear whether either of these contrasting effects apply to the low calcium concentrations and/or the small gel particle dimensions investigated here. It might be that both these arguments do apply and eventually cancel each other out; i.e. the alginate micro-particles, produced at the higher calcium concentration, are indeed stiffer than the alginate nano-particles, but at the same time the former are more deformable than the latter due to their larger dimensions. Whatever the case, it is evident that the flow behaviour of these systems is greatly affected by the large particle phase volumes typically encountered in fluid gel formulations, an effect that appears to dwarf any other influences.

3.2.2. Encapsulation efficiency

The data on the EE for all vanillin-containing AFG formulations as a function of fluid gel particle size are reported in Table 3; note that formulations corresponding to samples VFG_B (Table 3) and VFG_0.10% (see Table 1) are the same. Regardless of particle size, both VFG_A and VFG_B fluid gel formulations exhibit an EE enhancement with increasing storage time t_s. This trend of increasing EE under storage is similar to what was earlier shown for AFG formulations containing either 0.05% or 0.10% w/w of vanillin or tryptophan (Table 1). Nonetheless, the rate and magnitude of the EE increase (of the same active) observed in this case, appear to be significantly influenced by the dimensions of the AFG particles.

More specifically, the EE increase for the VFG_A formulation appears to progress more rapidly and reaches a higher value than that for VFG_B. As vanillin is much smaller (radius of gyration of ~ 0.47 nm; Yaws & Leh, 2009) than the reported average pore size of the alginate gel network (~ 5 nm; Lee & Mooney, 2012), it can be assumed that the observed migration of this active, from the bulk aqueous phase of the fluid gel formulations to the interior of the fluid gel particles, should take place predominantly by diffusion. In this case, Fick's second law would be sufficient to describe this process. As such diffusion of vanillin would be expected to depend on three parameters: the active's concentration difference between the exterior and the interior of the particle, the diffusion coefficient or diffusivity of the particle structure and the diffusion length or path (Li & Mooney, 2016). As the vanillin concentration used and the initial EEs for the VFG_A and VFG_B formulations are more or less the same (***Table 3***), a similar concentration differential (between bulk aqueous phase and AFG particles) driving diffusive migration is expected. However, although particles in both systems would consist of similar 'building blocks' (i.e. assemblies of alginate polymer chains), the presence of a higher

17

Ca^{2+} concentration in the VFG_B system would suggest the presence of an alginate network with a higher degree of cross-linking; and thus lower diffusivity, as suggested by literature (Lee & Mooney, 2012). What is more, the diffusion path for vanillin to be transported into the AFG_A fluid gel particles is significantly shorter than that in the case of AFG_B, because of the difference in their dimensions. In conclusion, the observed variance in the amount of vanillin that is accumulated in the VFG_A over the VFG_B AFG particles during the studied storage period, can be explained primarily due to the much smaller sizes of the former (VFG_A) and also because of the reduced diffusivity of the polymer network in the latter (VFG_B).

Table 3. Encapsulation efficiency (EE; mean value ± 1 SD) of vanillin-containing AFG formulations as a function of gel particle size ($D_{3,2}$) and storage time (t_s). Differences between EE values (for the same active) marked with the same superscripted letter are not statistically significant ($p > 0.05$).

Vanillin-containing AFG formulation				Storage time, t_s		
CaCl$_2$/ALG ratio	$D_{3,2}$ (µm)	Sample		1 Day	1 Week	2 Weeks
0.125	0.15	VFG_A	EE (±SD)	24.2% (±2.5)[a]	49.5% (±3.3)[b]	73.3% (±2.9)[c]
0.175	1.90	VFG_B		28.9% (±3.2)[a]	25.2% (±2.9)[a]	43.9% (±3.2)[b]

3.2.3. *Release behaviour*

Release testing for vanillin-containing AFG formulations as a function of FG particle size and storage time (t_s), were carried out under sink conditions and the results are presented in Figure 7. Release data were fitted to Eq. (3) and the obtained model parameter n_r is reported in Table 4. The shape of the release curves is similar to that exhibited by all other active-containing AFG formulations discussed previously (see Figure 4); an initial fast discharge that progresses over time to reach a plateau in terms of cumulative release. The value for this plateau, earlier shown to vary with the type and concentration of the active, is in addition revealed here to be affected by AFG particle size, while the strong dependency on t_s is retained. Although a final cumulative release of approximately 75% (w/w) of the initial vanillin content (same for both AFG formulations) is detected 1 day following their formation, this is ultimately suppressed (upon increasing the storage period to 2 weeks) to 30% and 58% (w/w) for the AFG nano-particles (VFG_A) and micro-particles (VFG_B), respectively (Figure 7). However, regardless of AFG particle dimensions or storage period, analysis of the data in Figure 7 suggests that vanillin release takes place predominantly by diffusion ($t_r \approx 0.5$; Table 4).

18

1   What is more, for both the VFG_A and VFG_B AFG formulations, the non-encapsulated

2 vanillin fraction, as determined from the EE analysis (Table 3), and the maximum vanillin

3 cumulative release, as detected in the aqueous acceptor phase after 5 h, are practically identical

4 (Figure 8). Therefore, as discussed earlier, the amount of vanillin that is released from the AFG

5 formulations, despite changes to the fluid gel particle size, corresponds to the fraction of active

6 that was not entrapped. As such, the previously established capacity of the fluid gel particles

7 to fully retain (under sink conditions) their enclosed hydrophobic species regardless of the

8 active concentration used, is shown here to further persist irrespective of the dimensions of the
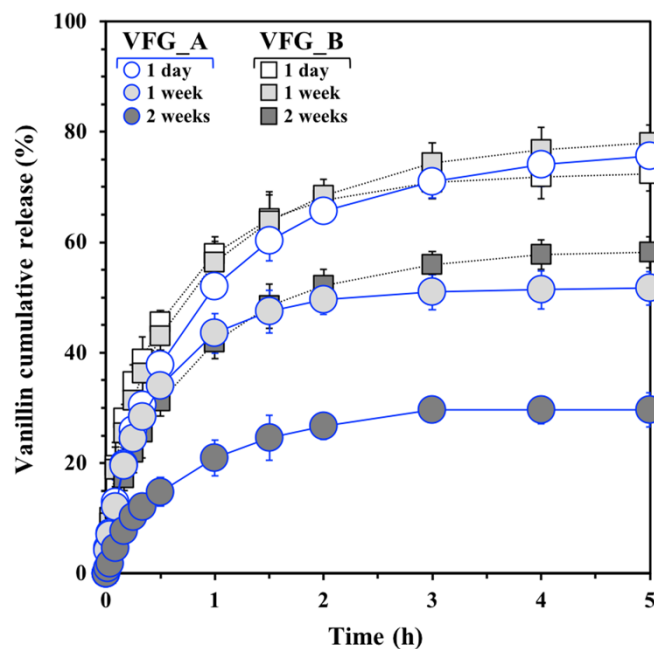
9 AFG particles themselves.

10

11
12 **Figure 7.** Vanillin cumulative release from vanillin-containing AFG (VFG) formulations of different
13 gel particle sizes, produced at $CaCl_2$/alginate ratios of 0.125 (VFG_A; AFG nano-particles) and 0.175
14 (VFG_B; AFG micro-particles), as a function of storage time (t_s). All data are given as mean values
15 $\pm$ 1 SD (error bars).

16

17 **Table 4.** Model parameter n_r (mean value $\pm$ 1 SD) obtained from the best-fit to Eq. (3) of the cumulative
18 release data from the vanillin-containing AFG (VFG) formulations (Figure 7), as a function of gel
19 particle size ($D_{3,2}$) and storage time (t_s). Goodness-of-fit (R^2) values are also provided.

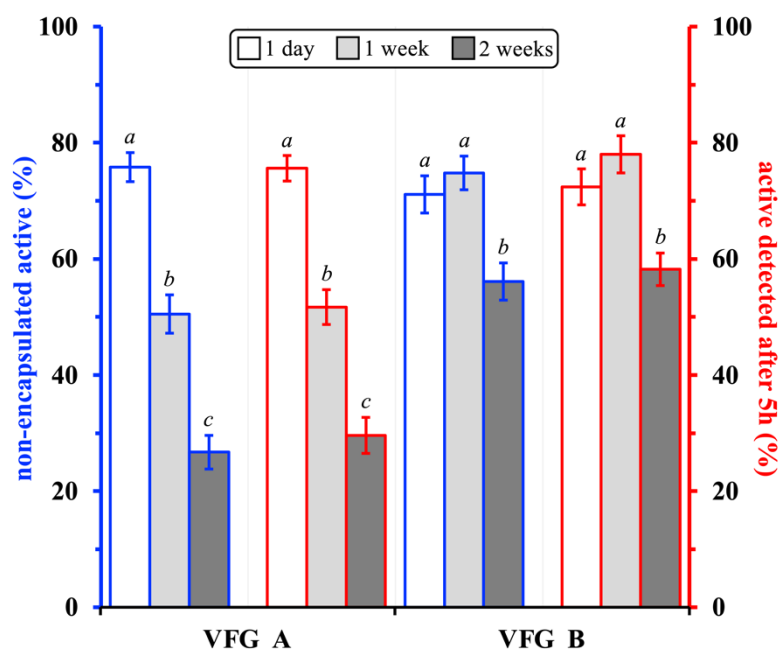| Vanillin-containing AFG formulation					
$CaCl_2$/ALG ratio	**$D_{3,2}$ (μm)**	**Sample**	**t_s**	**n_r**	**R^2**
0.125	0.15	VFG_A	1 day	0.54 ($\pm$ 0.05)	0.99
			1 week	0.49 ($\pm$ 0.08)	0.98
			2 weeks	0.51 ($\pm$ 0.04)	0.99
0.175	1.90	VFG_B	1 day	0.45 ($\pm$ 0.08)	0.98
			1 week	0.47 ($\pm$ 0.06)	0.99
			2 weeks	0.50 ($\pm$ 0.04)	0.99

Figure 8. Non-encapsulated active (%) in the vanillin-containing AFG formulations (blue) and active detected in the acceptor phase after 5 h of release testing (red), as a function of AFG particle size and storage time t_s (1 day, 1 week, 2 weeks). FG formulations produced at CaCl$_2$/alginate ratios of 0.125 (VFG_A; fluid gel nano-particles) and 0.175 (VFG_B; fluid gel micro-particles). All data are given as mean values ± 1 SD (error bars). Differences between values (for the same vanillin-containing AFG formulations; VFG_A or VFG_B) marked with the same letter are not statistically significant ($p > 0.05$).

3.3. Proposed mechanism for the encapsulation and release behaviour of small molecular weight hydrophobic actives in alginate fluid gels

It is proposed here that the migration of small molecular weight hydrophobic actives (vanillin, tryptophan) to the interior of the AFG particles occurs via diffusion and storage of these systems post production and over a period of 2 weeks, leads to significant increases in active loading/encapsulation. Moreover, release (under sink conditions) from the AFG formulations is only limited to the fraction of the active that is not entrapped within the gel particles. This overall behaviour in terms of the encapsulation and release of small molecular weight hydrophobic actives in AFG is schematically presented in Figure 9.

Based on Fick's second law, it is suggested that the diffusional loading of the hydrophobic actives within the fluid gel particles will depend on the active's concentration difference between the exterior and the interior of the particle, the diffusion coefficient of the particle structure and diffusion length (Li & Mooney, 2016). These influences were discussed earlier to explain the variation in the rate and magnitude of loading exhibited by AFG particles of different dimensions; AFG nano- (VFG_A) and micro-particles (VFG_B) (Table 3). Nonetheless, the rate of loading observed here is much lower than that reported for other

hydrophobic actives within alginate gel particles. Tanaka *et al.* (1984) studied the diffusion of several species of different molecular weights, both into and from calcium-alginate gel beads, and reported that an equilibrium concentration of tryptophan in the particles was obtained after only 30 min of placing blank beads into an aqueous buffer solution of the active. Tu *et al.* (2005) prepared alginate microparticles by a spray-coagulation method and studied the loading and release of 4-phenylazoaniline (PAA). Active loading was carried out by suspending the blank alginate particles (110 - 212 μm), in an ethanol solution of PAA for a 24 h period. Although, PAA loading efficiency was not reported it is suggested that the duration of the used loading stage was sufficient to induce the highest possible level of active inclusion into the particles. Elsewhere, Hariyadi *et al.* (2010) studied the diffusional loading and release of ibuprofen within alginate gel microparticles produced via an impinging aerosols method. In this case, an aqueous suspension of alginate gel microparticles was added to an ibuprofen water solution and stored at room temperature for 24 h; ibuprofen loading was within the range of 23-29% w/w.

In the present study, active loading takes place within 'intact' AFG formulations; i.e. AFG particles were not separated from their continuous aqueous medium and the formulation was not diluted. Therefore, although the diffusion coefficient of the particle structure can be assumed to be the rate-limiting step for active loading in other studies (and expected to be comparable to that observed for the AFG particles here), what appears to primarily govern the incorporation of the actives within the formulations studied here is the rate of diffusion through the continuous phase; i.e. the rate by which actives can diffuse to the surface of the AFG particles for entrapment to take place. This is not necessarily expected to directly relate to the much higher bulk viscosity of the AFG formulations (compared to that of water). In addition to this what should also be taken into account is the significant impact to the diffusion path of the active from the presence of a very high fraction of dispersed particles; even though the continuous medium surrounding these particles can be assumed to possess a 'local' viscosity that approaches that of water. In many ways, this phenomenon is equivalent to what has been termed a "cage effect" in suspensions of hard-sphere-like colloidal particles at high fractions ($\varphi > 0.52$). (Kasper *et al.*, 1998).

The data in the current work suggest that, once the hydrophobic actives diffuse to the AFG particles, they are eventually incorporated within the particle structure. Paradoxically, this was not observed for hydrophilic actives. This is despite the fact that both alginate quiescent gels and alginate gel particles are overwhelmingly regarded in literature as ideal

1 structures for the enclosure of hydrophilic species (e.g. Josef *et al*., 2010). Release experiments

2 carried out for vanillin-containing AFG were replicated for simple aqueous solutions of the

3 active (0.10% w/w) in the presence of alginate (2% w/w), but without the addition of calcium;

4 i.e. no alginate gel formation (Figure 10). It is evident that, regardless of the storage time

5 imposed, alginate alone only slightly delays the release rate of vanillin (compared to that from

6 a simple vanillin aqueous solution) and in all cases the active is fully discharged into the

7 acceptor phase after 2-3 h. Therefore, the retention of vanillin observed earlier (see Figure 4

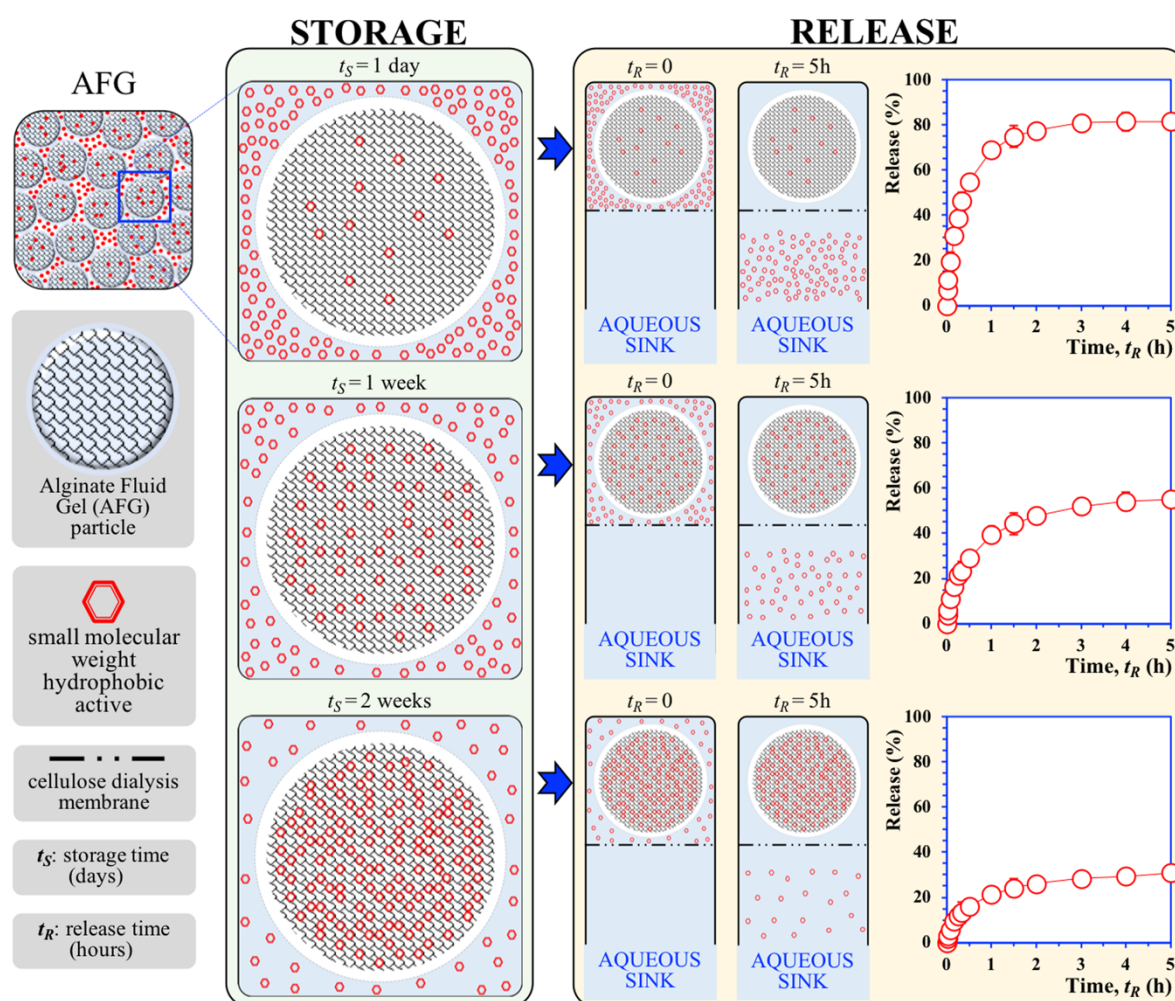8 and Figure 7) appears to be directly related to the existence of the alginate gel network.

9

11 **Figure 9**. Schematic representation of the loading/encapsulation and release behaviour of small
12 molecular weight hydrophobic actives in AFG particles.

13

14   Despite the entropic penalty relating to disproportionately placing vanillin in the water

15 phase associated with the AFG particles (in comparison to the aqueous continuous phase), this

16 migration does take place and should therefore provide an enthalpic advantage. It has been

1 previously shown that the addition of small amounts of alcohol (ethanol) decreases the aqueous

2 solubility of vanillin due to the reinforcement of water structuring, whereas higher alcohol

3 concentrations increase vanillin solubility as a result of water structure disruption (Kappatos *et*

4 *al*.,1996). Probst et al (1985) showed that Ca^{2+}, due to its size and strength of the ion-water

5 interaction, is also able to disrupt the structure of the water quite effectively. It is therefore

6 proposed that the presence of calcium could facilitate an enhancement to the capacity of water

7 to solubilise vanillin. As calcium is predominantly associated with the alginate gel matrix, any

8 such improvement in the solvent quality of water would be localised; primarily confined to

9 water situated within the gel porous structure. Such a difference in vanillin solubility would

10 indeed offer a significant enthalpic incentive for the migration of the active into the alginate
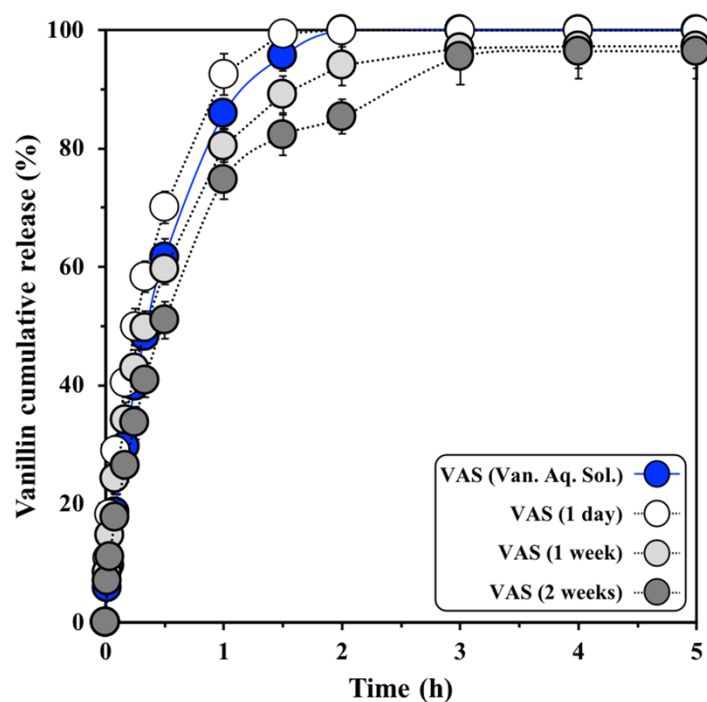
11 gel matrix.

12

Figure 10. Vanillin cumulative release from a vanillin (0.10% w/w) aqueous solution (VAS) and vanillin (0.10% w/w) aqueous solutions also containing alginate (VAN-ALG), the latter as a function of storage time (t_s). All data are given as mean values ± 1 SD (error bars).

18 The hypothesis outlined above would also be relevant to explain why active entrapment

19 persists even when the AFG formulations are placed within an aqueous sink; the release of the

20 encapsulated fraction of the hydrophobic species is hindered. In addition to solvent-specific

21 enthalpic contributions to the retainment of vanillin (or tryptophan) within the AFG particles,

22 what should also be considered is the influence(s) of interactions between the hydrophobic

23 actives and alginate and/or calcium in the gel matrix. Vanillin has been reported to interact

23

with amino acids and proteins via Schiff base formation (covalent binding of the aldehydic function of vanillin to the amino groups of proteins), hydrogen bonding, hydrophobic and electrostatic interactions (Weerawatanakorn *et al*., 2015). It has been shown that vanillin can interact with a range of proteins, including β-lactoglobulin, sodium caseinate, bovine serum albumin, ovalbumin, whey, milk, soy and pea protein (Chobpattana *et al*., 2002; Houde *et al*., 2018; Wang *et al*., 2011; Weerawatanakorn *et al*., 2015). Mikheeva *et al*. (1998) reported that vanillin can interact with β-lactoglobulin, bovine serum albumin, and ovalbumin through electrostatic interactions. Chobpattana *et al*. (2002) showed that hydrophobic interactions are the main forces between vanillin and bovine serum albumin, while the interactions between vanillin and casein or sodium caseinate are primarily driven by hydrogen bonding. Finally, interactions between calcium and the quadrupole moment of the aromatic ring in vanillin or tryptophan (cation-π interactions) could also become important (Mahadevi & Sastry, 2013). Although, the data in Figure 10 could be interpreted to suggest that the influence of any such interactions between vanillin and the alginate chains on the release kinetics of the active is minimal to practically non-existent, this might not be the case within an AFG particle setting. Considering events taking place within any single pore of the AFG particle network, the plurality and proximity of functional sites/groups would enhance the probability of such interactions taking place, and thus could impact on the retention of the actives in question.

4. Conclusions

Although the encapsulation of active components in gelled microparticles is generating great interest, the scalable production of such systems still remains challenging. Fluid gels are a class of soft-solid gel particles that can be produced via high-shear approaches that fully align with industrial manufacture. As such, there is a (currently unmet in open literature) need to study fluid gel formulations for their capacity to entrap actives.

In this work, the encapsulation of vanillin and tryptophan, as hydrophobic small molecular weight model actives, and nicotinamide, as a hydrophilic one, in cross-linked alginate fluid gel (AFG) particles has been investigated. It was demonstrated that the presence of an active does not affect the dimensions of the AFG particles (maintaining a mean diameter, $\bar{D}_{3,2}$, of 1.91±0.12 µm) nor their bulk rheological properties (a shear thinning behaviour with an average power law index , n, of 0.32±0.02). The encapsulation efficiency of all small molecular weight actives in the AFG particles is initially (after 1 day of storage) extremely low (<30%), since both active entrapment and particle formation take place in tandem. However,

hydrophobic actives were shown to migrate into the AFG particles post formation and over a storage period of 2 weeks; with EE values of up to 70% (in the case of vanillin) at the end of the storage period. It is proposed that the calcium ions, located within the alginate gel network, disrupt the structure of water present therein, thus enhancing the aqueous solubility of the hydrophobic species. By essentially confining this enhancement to the water's solvent quality within the AFG particles, an enthalpic advantage that encourages the migration of the hydrophobic species, from the continuous aqueous medium to the water located within the alginate gel matrix, is created. The rate of the migration of the hydrophobic actives seen here is much lower than that typically observed during the diffusional loading of hydrophobic actives into alginate gel microparticles. The reason for this delayed loading is the much greater bulk viscosity of the medium over which diffusion takes place as well as the significantly increased diffusion path for a small molecular weight active within an aqueous phase containing a high fraction of closely packed gel particles. The rate and magnitude of the incorporation of the hydrophobic actives within the alginate fluid gel particles is however accelerated by increasing the concentration of the species initially added in the formulation or by decreasing the size of the gel particles; EE values for vanillin entrapment in AFG nano- (VFG_A) and micro-particle (VFG_B) systems were 73.3% and 43.9%, respectively. Thus, both the initially imposed concentration difference of the active between the suspending aqueous medium and the interior of the AFG particles as well as and diffusion path within the particles' gel network, also seem to impact upon the hydrophobic species' loading rate and loading extent. Release under sink conditions revealed that encapsulated hydrophobic content is essentially confined within the fluid gel particles and only the non-encapsulated fraction of the species is detected in the acceptor phase. It is argued that in addition to the enhanced solvent quality of water in the interior of the particles, the occurrence of a number of interactions that have been previously reported to occur between these or similar hydrophobic compounds and hydrocolloids and/or calcium, exaggerated by the proximity and plurality of these species within each pore of the gel network, could also impact on the retention of the actives. Future efforts would obviously need to focus on studying the release of such hydrophobic species (from AFG formulations) in simulated gastric and intestinal media.

The present findings show clear evidence that AFG can be preferentially loaded with small molecular weight hydrophobic species and the hypotheses reported here for the first time, offer insight that is valuable to future efforts into achieving control over these phenomena. Regulating the loading/encapsulation and release of actives from AFG will significantly

intensify the applicability of these formulations, a class of colloidal systems with demonstrated potential in terms of industrial manufacture.

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