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The synthesis of certain fatty acid ester derivatives of trehalose and an investigation of their emulsifying properties and bioactivities

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ABSTRACT

Bio-based surfactants exerting both functional and nutritional properties are needed in the food industry. This study reports the one-pot synthesis, using a Novozym 435-catalyzed *trans*-esterification protocol, of a new family of such compounds, namely a homologous series of 6-*O*-acylated trehalose and 6,6′-di-*O*-acylated trehalose derivatives incorporating long-chain fatty acid residues (12–22 carbons). These mono- and di-esters exhibited HLB values within the 8.3 to 14.7 range and their performance as surface-active compounds, penetration enhancers and anti-cancer agents have been evaluated. They were found to be excellent emulsifiers, maintaining oil-in-water emulsions with 566–742 nm mean oil-particle sizes and with the stability of these only gradually deteriorating over a 15-day storage period and during *in vitro* digestion. Mono-esters **2**–**4** embodying shorter side-chains (12–16 carbons) proved to be excellent foaming agents while congeners **4**–**6** incorporating longer side-chains (16–20 carbons) served as penetration enhancers capable of reversibly opening tight junctions in Caco-2 cell mono-layers. Mono-esters **5**–**7**, as well as di-esters **11**–**13**, exerted weak cytotoxic effects on seven human cancer cell lines. This work should serve as a useful guide for the development of the multi-functional properties of such trehalose esters in the food and related industries.

1. Introduction

Trehalose, a non-reducing disaccharide, is readily obtained from a range natural sources including mushrooms, bacteria, insects and shrimp. Since it is 55% less sweet than sucrose (Schiraldi et al., 2002), trehalose has long been employed in the food industry as, *inter alia*, an alternative sweetener, a flavor enhancer and a preservative. (Kopjar et al., 2008; Richards et al., 2002). It is favored by diabetics because of its low glycemic index. Given this, and its reduced carcinogenicity, trehalose is attracting attention as a key component of "sugar reduction" health regimes (Maki et al., 2009). Other health-promotion effects of trehalose have been noted including its anti-aging properties (Berry et al., 2020) and its anti-cancer effects (Chaitanya et al., 2021). Additionally, neuroprotective capacities (Béranger et al., 2008; Khalifeh et al., 2021; Liu et al., 2005) and reductions in susceptibility to seizures (Sinha et al., 2021) have been attributed to trehalose. It also plays an important role in the cosmetics industry by serving as a stabilizer, skin moisturizer and odor-masking agent (Ohtake & Wang, 2011).

The eight free hydroxyl groups present in trehalose result in it having poor lipid solubility and so restricting its direct exploitation in the above-mentioned settings but the esterification of this sugar using fatty acids would provide derivatives with higher lipophilicities. Such derivatives would be very similar in structure to their sucrose ester counterparts that are deployed extensively as emulsifiers. Accordingly, they are also expected to serve as novel non-ionic surfactants (Smeds et al., 2007).

Although the preparation of trehalose fatty acid esters can be realized by both chemical (Hill, 2007) and biochemical means (Chen et al., 2005; Hill, 2007), the seemingly inevitable contamination (by catalysts and/or organic solvents) of those products obtained by the former

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method complicates the evaluation of their physicochemical and biological properties. As such, biochemical techniques, especially enzymatic *trans*-esterification ones, are preferred for obtaining pure materials, at least at the laboratory scale (Li et al., 2019; Xie et al., 2021; Zhu et al., 2020; Zhu et al., 2022). Indeed, the lipase Novozyme 435 has been used to catalyze the synthesis of trehalose esters under solvent-free (Ogawa et al., 2019) or other conditions (Ji et al., 2020) while Marathe et al. (2020) have described the synthesis of such esters from caprylic, lauric and palmitic acids using lipase Fermase CALB™ 10,000. However, these previous studies have been concerned primarily with the optimization of the synthetic process rather than the full evaluation of the surface-active and other properties of the derived trehalose esters.

Given the foregoing, we now report a one-pot, Novozyme 435-catalyzed synthesis of certain mono- and di-esters of trehalose incorporating different fatty acid-derived residues and the subsequent, systematic assessment of these compounds as surfactants (*viz*. as foaming agents and emulsifiers). The evaluation of these same esters in a gastrointestinal behavior model (GITBM) was also undertaken, as was an in-depth evaluation of their potential to serve as penetration enhancers and as anti-cancer agents. The comprehensive structure-property profile so-developed lays the foundation for the rational deployment of trehalose esters in the manufacture of foodstuffs, medicines and associated products.

2. Materials and methods

2.1. Materials

Trehalose (99.5%) was purchased from Macklin Co. Ltd. (Shanghai, China) while vinyl acetate (98.0%), vinyl laurate (>99.0%), vinyl myristate (>99.0%), vinyl palmitate (>96.0%), vinyl stearate (>95.0%), oleic acid (>85.0%) and erucic acid (>85.0%) were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). The commercial sucrose esters P-1570 and S-970 were obtained from the Mitsubishi Chemical Foods Corporation (Tokyo, Japan). Dihydrolevoglucosenone (Cyrene™) and *t*-BuOH were purchased from CIRCA Group Pty Ltd (Victoria, Australia) and the Energy Chemical Co., Ltd. (Shanghai, China), respectively. Novozyme 435 was obtained from Novo Nordisk (Bagværd, Denmark) whereas Lipase (Type II, L3126 from porcine pancreatic pancreatin, pepsin (77,160, from porcine gastric mucosa) and mucin (Type II, M2378 from porcine stomach) were all supplied by Sigma-Aldrich (Shanghai, China). Virgin olive oil (Olivia™, *ex*. Tunisia) was purchased from a local supermarket. All solutions and emulsions were prepared using double-distilled water produced using a Milli-Q water purification system (Millipore, Billerica, MA). Other reagents and solvents employed in this study were all analytical reagent (AR) grade and were used as received.

2.2. Enzymatical synthesis of trehalose esters 2–13

Syntheses of the target mono- and di-esters of trehalose were carried out using enzymatic *trans*-esterification methods as summarized in Fig. 1. Briefly, a magnetically stirred and anhydrous solution of trehalose (2.9 mmol) in 1:1 v/v *t*-BuOH:Cyrene™ was treated with Novozym 435 (1.0 g) then one of the seven, above-mentioned fatty acid vinyl esters (11.6 mmol). After being stirred at 60 °C for 48 h the reaction mixtures were then cooled, filtered and concentrated under reduced pressure. The residue thus obtained was subjected to flash column chromatography (silica, 9:1 *v/v* CH_2Cl_2/MeOH elution) to afford, after concentration of appropriate fractions, the relevant trehalose ester **2**–**13** all of which were obtained as white, amorphous solids. ^1H and ^{13}C NMR spectroscopic analyses of these materials established that they were >95% purity (see Supplementary Materials for details).

2.3. Characterization of surface-active properties

In order to investigate the potential of trehalose esters **2**–**13** as surfactants, their abilities to stabilize a given interface were established using the following range of measurements and techniques.

2.3.1. Hydrophilic-lipophilic balance (HLB)

The HLB values of compounds **2**–**13** were determined using minor modifications of the method reported by Li et al. (2019). Specifically, a sample of each ester (100 mg) was dissolved in 10 mL of a 100:5 v/v solution of DMF/benzene at 25 ± 1 °C and the resulting solution treated, dropwise, with distilled water until irreversible turbidity was observed. For calibration proposes, the HLB values of a series of controls were also measured and determined to be as follows: Span 80 (4.3), Span 40 (6.7), Span 20 (8.6), Tween 80 (15.0), Tween 40 (15.6), and Tween 20 (16.7). The theoretical HLB values were calculated using Griffin's method (Griffin, 1949).

2.3.2. Interfacial properties

To assess the ability (or otherwise) of trehalose esters **2**–**13** to stabilize the interface of two immiscible liquid phases, various relevant parameters were measured using an UpHSV1220 automatic interfacial tensiometer (Dataphysics, OCA, Filderstadt, Germany) according to the method of Zhu et al. (2022). These parameters included the critical micelle concentrations (CMC), the critical surface tensions corresponding to the CMC (γ_{CMC}), the oil–water interfacial tension ($\gamma_{O/W}$) and the contact angle (θ).

2.3.3. Surface tension reduction efficiency (pC_{20})

The potential of compounds **2**–**13** to act as surfactants could be quantitated by measuring the reduction efficiency of the surface tension

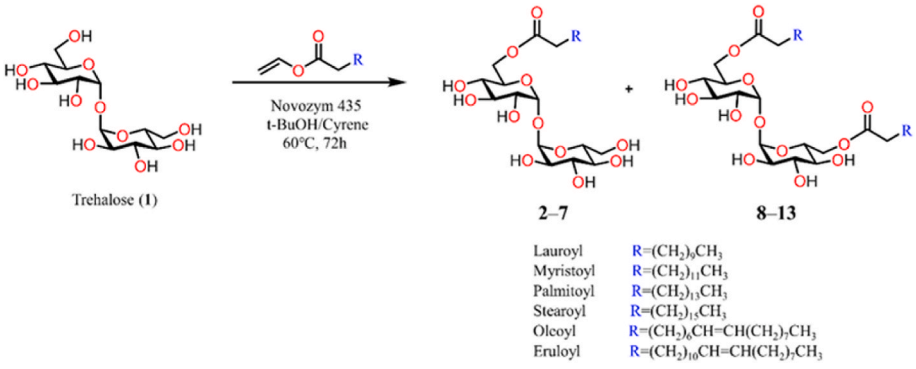

Fig. 1. Enzymatic syntheses of trehalose esters **2**–**13.** Mono-esters: 6-*O*-Lauroyltrehalose mono-ester (**2**); 6-*O*-Myristoyltrehalose mono-ester (**3**); 6-*O*-Palmitoyltrehalose mono-ester (**4**); 6-*O*-Stearoyltrehalose mono-ester (**5**); 6-*O*-Oleoyltrehalose mono-ester (**6**); 6-*O*-Erucicacyltrehalose mono-ester (**7**); and Di-esters: 6,6′-*O*-Lauroyltrehalose di-ester (**8**); 6,6′-*O*-Myristoyltrehalose di-ester (**9**); 6,6′-*O*-Palmitoyltrehalose di-ester (**10**); 6,6′-*O*-Stearoyltrehalose di-ester (**11**); 6,6′-*O*-Oleoyltrehalose di-ester (**12**); 6,6′-*O*-Erucicacyltrehalose di-ester (**13**).

at a given interface using equation (1) (Zhu et al., 2022)

$$pC_{20} = -\log_{10} C_{20} \quad (1)$$

where C_{20} is the concentration of trehalose esters that reduces the surface tension by 20 mN/m, relative to a system lacking any surfactant.

2.4. Evaluation of foaming properties

As an important metric for judging surface-active effects, the so-called foamabilities and foaming stabilities of the trehalose esters **2**–**13** were measured at 25 ± 1 °C using a minor modification of the method reported by Li et al. (2019). In short, aqueous solutions of esters **2**–**13** and of commercial controls S-970 and P-1570 (20 mL of each as either 0.2% or 0.5% w/w solutions) were each placed in 100 mL conical flasks and the original heights (H_0) measured. Thereafter, each solution was stirred, using a blender, at 25,000 rpm for 3 min and the resulting foam height (H_2) and the total height (H_1) were immediately measured. The reduced foam height (H_3) of each sample was measured again after each had been allowed to stand for 10, 20 and 30 min. The foamability and foaming stability values were then calculated using equations (2) and (3):

$$\text{Foamability}\ (\%) = \frac{H_1 - H_0}{H_0} \times 100\% \quad (2)$$

$$\text{Foaming stability}\ (\%) = \frac{H_3}{H_2} \times 100\% \quad (3)$$

2.5. Evaluation of emulsifying capacities

Six mono- and di-trehalose ester cocktails (TL-75 to TE-75) were prepared by mixing, in a 3:1 ratio, samples of each of these embodying the same fatty acid residue (Table 1). This ratio follows from the most popular commercial Ryoto™ sucrose ester S-1670 containing 75% of a mono-ester, 20% of the corresponding di-ester and 5% of higher-order esters. Aqueous solutions (90.0 mL of a 0.5% w/w mixture) of each of these cocktails and of the pure trehalose esters **2**–**13**, as well as of the commercial sucrose ester controls P-1570 and S-970, were placed in 200 mL beaker. Dimethyl sulfoxide (2.0 mL) was then added (as a solubilizer) and this was followed by the addition of olive oil (10 mL). Following established protocols (Li et al. (2019), the resulting mixtures were firstly homogenized and then passed through a high-pressure (1200 bar) homogenizer for three cycles to produce the final emulsions. These could be stored in the dark for 15 days.

To characterize the oil droplets in the prepared emulsions, each sample was diluted 100-fold before being evaluated using a laser diffraction particle size analyzer (SALD-2300, Shimadzu, Japan) equipped with an automatic stirring device. Three measurements were carried out on each sample over three days. The mono- and di-ester cocktails TL-75 to TE-75 derived emulsions were examined using a fluorescence confocal scanning microscope (LSM880, Carl Zeiss, Germany) (Xie et al., 2021).

Table 1
Mixing 6-*O*-acyltrehalose and 6,6′-di-*O*-acyltrehalose for the preparation of trehalose ester cocktails.

Sample	Fatty acid-derived residue	Component (wt.%)	
		6-*O*-acyltrehalose	6,6′-di-*O*-acyltrehalose
TL-75	Laurate	75	25
TM-75	Myristate	75	25
TP-75	Palmitate	75	25
TS-75	Laurate	75	25
TO-75	Oleic acid	75	25
TE-75	Erucic acid	75	25

2.6. Assessment of in vitro digestibility

During the digestion of emulsions, the secretions from the gastrointestinal tract (GIT) may alter the interfacial compositions and further influence the breakdown and absorption of oil (Wei et al., 2020). A gastrointestinal behavior model (GITBM) was developed according to Xie et al. (2021) to mimic the *in vitro* digestion, in the human gut, of emulsions stabilized by the mono- and di-ester cocktails TL-75 to TE-75. The digestibility of each emulsion was assessed based on the fraction of released free fatty acids (FFA) in the small intestinal phase of the GITBM. Fluorescence confocal scanning microscopy was also employed so as to directly observe the morphology and dispersive state of the emulsions at different phases of the GITBM (Ma et al., 2018).

2.7. Assessment of the impact of trehalose esters on the permeability of Caco-2 cell monolayers

Sugar esters, particularly those derived from sucrose and lactose, have recently come to be regarded as potential mucosal permeation enhancers that facilitate the transportation of bioactive components across such membranes (Lucarini et al., 2016; Maher et al., 2019; Perinelli et al., 2018). Based on the method of Perinelli et al. (2018), the capacities of the trehalose esters in enhancing mucosal permeability were assessed *in vitro* by measuring *trans*-epithelial electrical resistance (TEER). In short, Caco-2 cell monolayers (TEER >800 Ω cm^2) were transferred to Hanks' balanced salt solution (HBSS) and equilibrated for 30 min at 37 °C in an atmosphere containing 5% CO_2. After measuring the baseline TEER values using an EVOM volt-ohmmeter (*ex*. World Precision Instruments, UK) equipped with a pair of chopstick electrodes, a Caco-2 monolayer was treated with 25 μM of aqueous solutions of each of the pure trehalose esters **2**–**13** for 3 h. Measurements of TEER were carried out every 0.5 h. After 3 h the Caco-2 monolayers were drained, washed with PBS buffer and resuspended in HBSS media. Following a 24 h incubation period, final TEER measurements were undertaken so as to establish the reversibility of the electrical resistance. Pure trehalose was employed as the control while the background TEER values caused by filters (100–110 Ω cm^2) were subtracted from all measurements, these being performed in triplicate.

2.8. Determination of cytotoxicity

The cytotoxicities of trehalose esters **2**–**13** were evaluated using an MTT assay that assesses the capacity of the test compounds to cause mitochondrial damage and so compromising cell viability. Specifically, then, Dulbecco's modified Eagle's medium (DMEM) was further modified according to Vater et al. (2020) by treating this with 1% penicillin-streptomycin and 10% fetal bovine serum. Seven cell lines (comprising MCF-7 breast cancer cells, A549 alveolar adenocarcinoma cells, HepG2 liver cancer cells, Caco-2 colorectal adenocarcinoma cells, N2A mouse brain neuroma cells, SW480 colon cancer cells or HEK293 human embryonic kidney cells) were then cultivated in the modified DMEM for 24 h at 37 °C under an atmosphere containing 5% CO_2 then distributed (@ 3000 cells/well) in seven 96-well plates containing DMEM individually supplemented with different concentrations (0, 25, 50, 100, 300, 800 and 1200 μM) of trehalose esters **2**–**13** or doxorubicin (positive control) while DMEM without supplements was used as the blank. After 48 h of incubation, 20 μL of MTT stock solution (5 mg/mL) was added to each well and the resulting mixture was left to stand for another 4 h. Thereafter, the supernatant liquid was removed from each well and replaced by dimethyl sulfoxide (DMSO, 150 μL). The 96-well plates were shaken for 20 min and the absorbance in each well then measured at 570 nm using an Epoch™ 2 Microplate Spectrophotometer (*ex*. Biotek Instruments, Winooski, VT, USA). The IC_{50} values thereby obtained represent the dose of the test compound causing a 50% loss of cell viability (relative to the blank).

2.9. Statistic analysis

Experiments were performed in triplicate while results are presented as a mean ± standard deviation (SD). One-way analysis of variance (ANOVA) as well as Duncan's new multiple range test (MRT) were performed using SPSS software (SPSS, Inc., Chicago, IL) to identify any significant deviations. Differences were considered significant when $p < 0.05$.

3. Results and discussion

3.1. Surface-active properties

The surface-active properties of the trehalose esters **2**–**13** were assessed using the six parameters shown in Table 2. HLB values, which are important classification standards for surfactants and used to quantitatively define the hydrophobicities and hydrophilicities of surfactants, are usually determined experimentally or predicted based on structure (Li et al., 2019). As can be discerned from Table 2, the experimentally measured HLB values of mono-esters **2**–**7** varied from 14.7 to 11.5, these being significantly higher than those of the corresponding di-esters **8**–**13** which ranged from 10.6 to 8.3. The calculated HLB values also varied in a similar manner. These outcomes indicate, as is to be expected, that the HLB values of the trehalose esters are determined by both the length of the associated side-chain and the degree of esterification but not, to any significant extent, by the varying degrees of unsaturation within the side-chains (compare esters **5** and **6**). Given the trehalose esters prepared for the purposes of this study showed HLB values within the 8–18 range, they would be expected to serve as effective oil-in-water surfactants (Chun & Martin, 1961).

CMC values are defined as the minimum surfactant concentration required to form micelles in given solvent (Polat & Linhardt, 2001) and can be regarded as another useful indicator of surface-active properties. The CMCs of esters **2**–**13** correlated positively with the corresponding HLB values (Table 2) and, moreover, trehalose di-esters generally exhibited lower CMC values than those of their corresponding mono-counterparts, a trend attributable to the additional side-chain enhancing intermolecular hydrophobic interactions and resulting in greater aggregation and micelle formation (Soultani et al., 2003). The trend in γ_{CMC} values was, however, different. The values within both the mono- and di-ester series decreased at first then increased again as the carbon content of the alkyl side-chain reached 22. For example, the γ_{CMC} values across the series of mono-esters **2**–**7** fell from 37.2 to 34.3 mN/m then rose to 35.1 mN/m. This U-shaped profile may result from those esters incorporating ultra-long alkyl side-chains tending to fold back on themselves, as a result of van der Waals interactions, and so reducing their effective hydrophobicities while simultaneously increasing surface tension at the CMC (Ferrer et al., 2002; Zhu et al., 2022). As for the corresponding $\gamma_{o/w}$ values, the trehalose mono-esters **2**–**7** also showed a similar U-shaped profile while the values for di-esters **8**–**13** continuously increased from 11.1 to 13.7 mN/m as the associated alkyl side-chains became longer. This trend may derive from those systems incorporating higher degrees of esterification being hindered in their interactions with the oil droplets and a consequent inability in fully perform as surfactants at the oil-water interface.

Table 2
The HLB values, CMC, γ_{CMC}, $\gamma_{O/W}$, pC20, and contact angle (θ) of pure trehalose esters **2**–**13** [a].

Ester	HLB Measured[a]	HLB Calcd.[b]	CMC μM	γ_{CMC} (mN/m)	$\gamma_{o/w}$ (mN/m)	pC20	θ (°)
2	14.7 ± 0.1	13.05	570	37.2 ± 0.5	7.3 ± 0.2	5.0 ± 0.4	31.1 ± 1.0
3	13.7 ± 0.3	12.39	410	35.6 ± 1.3	6.9 ± 0.3	5.2 ± 0.2	28.5 ± 0.4
4	13.0 ± 0.2	11.79	310	35.1 ± 2.7	4.1 ± 0.1	5.3 ± 0.2	27.3 ± 1.5
5	12.4 ± 0.2	11.25	270	34.3 ± 1.4	5.5 ± 0.2	5.7 ± 0.1	18.1 ± 0.7
6	12.3 ± 0.4	11.28	270	34.2 ± 1.1	5.6 ± 0.3	5.4 ± 0.2	19.4 ± 0.4
7	11.5 ± 0.1	10.33	83	35.1 ± 0.7	6.2 ± 0.6	6.0 ± 0.1	25.5 ± 0.8
8	10.6 ± 0.2	9.68	91	42.5 ± 0.4	11.1 ± 0.4	4.7 ± 0.3	14.1 ± 0.8
9	9.9 ± 0.3	8.97	72	41.6 ± 0.9	12.3 ± 0.5	4.9 ± 0.2	13.3 ± 0.9
10	9.1 ± 0.3	8.36	41	40.8 ± 1.3	12.4 ± 0.7	4.8 ± 0.3	11.7 ± 1.3
11	8.4 ± 0.4	7.82	38	39.7 ± 0.8	12.9 ± 0.2	5.0 ± 0.4	11.1 ± 0.8
12	8.6 ± 0.1	7.86	37	39.8 ± 0.7	13.1 ± 0.3	5.2 ± 0.1	10.3 ± 1.4
13	8.3 ± 0.6	6.96	15	41.1 ± 1.1	13.7 ± 0.4	5.4 ± 0.1	10.7 ± 1.3

[a] Value = mean ± SD, n = 3. HLB values determined using the water number method.
[b] HLB values calculated by Griffin's method.

pC_{20} values were negatively correlated with the HLB and CMC measurements and thus indicating the superior performance of mono- over di-esters in reducing the interfacial surface tension. Using this metric, ester **7** (pC_{20} = 6.0) was considered to be most effective surfactant with the associated hydrophobic effect arising from the presence of the erucic acid residue (Ferrer et al., 2002; Tawfik, 2015).

Measuring the contact angles (θ) of aqueous solutions of trehalose esters **2**–**13** allowed for the quantification of their wetting abilities. The θ values so-obtained negatively correlated with ester hydrophobicity as defined by the length of the associated alkyl side-chains and degrees of esterification. For instance, the θ values of mono-esters were all above 18° while those of di-esters **8**–**13** fell from 14.1° to 10.7°. So, solutions of trehalose esters possessing higher HLB values tended to spread more easily, a trend consistent with the recent report of Zhu et al. (2022).

3.2. Foaming properties

As a suspension of fine air bubbles in an aqueous phase, foams are supported by surface-active agents that reduce liquid surface tensions or/and prevent coalescence of bubbles (Grenni et al., 2018). Foams contribute to the characteristic (and desirable) structures in a multitude of food products including whipped cream and mousses (Cao et al., 2018). Surfactants with excellent foaming properties also have wide applications in the detergents and cosmetics fields. To fully evaluate the potential of trehalose esters **2**–**13** as foaming agents, their foamabilities (Fig. 2A) and foaming stabilities (Fig. 2B) were measured with the two commercial sucrose ester surfactants P-1570 and S-970 being used as positive controls.

As can be discerned from Fig. 2A, trehalose ester **2** (embodying a 12-carbon side-chain) displayed the best foamability, reaching 45.0% and 61.7% at 0.2% and 0.5% w/w concentrations, respectively while esters **3** (14-carbon side-chain) and **4** (16-carbon side-chain) showed comparable or even significantly better foamability than the commercial products P-1570 and S970. Normally, the foamability of solutions containing sugar esters (such as those derived from raffinose, lactose and sucrose) increases significantly at higher concentrations (Li et al., 2019; Liang et al., 2018; Zhu et al., 2022) but this was not so for the trehalose di-esters and especially in the case of compound **13** incorporating two 22-carbon side-chains. It is possible that the "excessive" molecular size and hydrophobicity of this material slows down its adsorption and transport rates and so limiting its performance at the air-water interface (van Kempen et al., 2014a).

With regard to the foaming stabilities (FSs) revealed in Fig. 2B, the shorter side-chain-containing systems **2** and **3** were also the best in terms of maintaining more than 60% of the original foam height even after 30

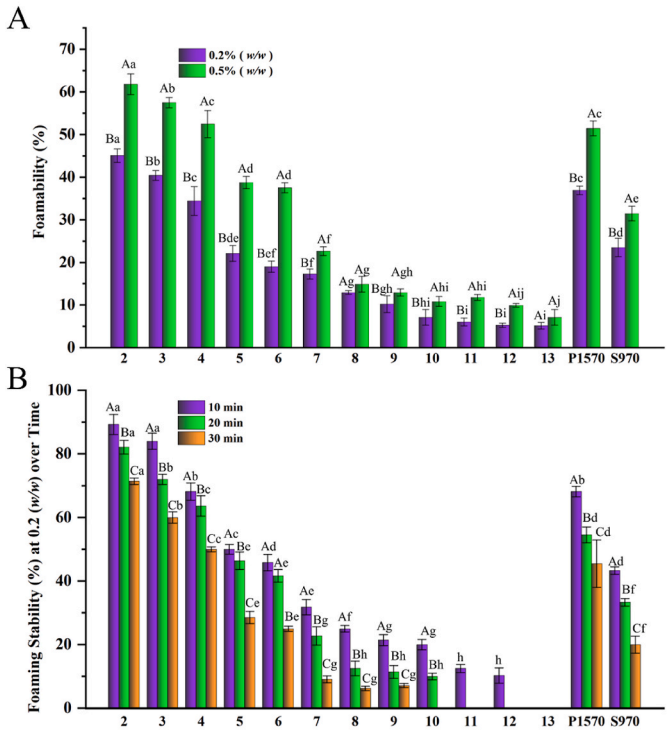

Fig. 2. Foamability of pure trehalose esters **2**–**13** and controls P-1570 and S-970 at 0.2% and 0.5% (w/w) concentrations (A). Foam stability of the same compounds at 0.2% (w/w) concentration after standing for 10, 20 and 30 min (B). All data are shown as mean ± SD (n = 3). *Capital letters indicate comparisons of the same surfactant at different concentrations (A) or different storage periods (B). Lower-case letters indicate comparisons of different surfactants at the same concentration.

min. The FSs of esters **5** and **6** (each incorporating 18 carbon side-chains) were comparable to the control S-970 and so indicating that the effect of different degrees of unsaturation is minimal. In contrast to the foregoing, di-esters **8**–**13** showed very limited capacities to stabilize foams and so suggesting that low water solubilities and high hydrophobicities adversely impact foaming properties (Zhang et al., 2015). Kanokkarn et al. (2017) have reported that surfactants which effectively reduce interfacial tension normally display excellent foaming properties. Fully consistent with the observations reported here, others have also found that only esters incorporating alkyl side-chains of intermediate length (*viz.* those containing 10–16 carbons) are capable of forming dense and uniform foams (Li et al., 2019; Liang et al., 2018; van Kempen et al., 2014b).

Given the foregoing it would appear that certain trehalose monoesters, especially congeners **2** (with a 12 carbon side-chain) and **3** (with a 14 carbon side-chain), have considerable potential as foaming agents that could be exploited in the food industry.

3.3. Emulsifying properties

Emulsions are comprised of two or more immiscible phases and can be stabilized by amphiphilic molecules (*viz.* emulsifiers) that generally act in at least two ways. On the one hand, emulsifiers effectively reduce the interfacial tension between the immiscible phases and on the other they can simultaneously increase steric hindrance and/or electrostatic repulsion and so preventing the coalescence of, for example, oil droplets in an oil-in-water emulsion (Matos et al., 2016). The periods over which emulsions remain stable largely determine their potential utility.

As can be discerned from Table 3, trehalose di-esters **8**–**13** showed the weakest emulsifying capacities given that the derived emulsions stratified within 6 days. In contrast, monoesters **3**–**6** and the mono- and di-ester cocktails TL-75 to TO-75 proved to be very effective emulsifiers as evidenced by the absence of any stratification of the derived emulsions during extended storage and so establishing that these materials were superior to the positive controls P1570 and S-970. The robustness of the derived emulsions was also evident from the monitoring of the mean oil diameters which only increased at a very slow rate over time (Nilsson & Bergenståhl, 2007). Of particular note, the mono- and di-ester cocktails TL-75 to TO-75 formed emulsions with significantly smaller particle sizes (ranging from 566 to 589 nm) than the others and these profiles were effectively maintained through to the end of the 15-day storage (at which point a 591–661 nm range was observed).

Overall, then, the ranked performance of the trehalose esters as emulsifiers is TL-75 to TE-75 > mono-esters **2**–**7** > di-esters **8**–**13**. Clearly, then, adding 25% di-esters to the pure mono-esters significantly improves the emulsifying properties. This result is consistent with those shown in Table 2 in that the hydrophobicity (in terms of the degree of esterification) of the surfactant negatively correlates with CMC values. So, in emulsions, the CMCs of those derived from a trehalose mono-ester

Table 3
Mean diameters (nm) of oil droplets associated with emulsions derived from pure trehalose esters **2**–**13**, ester cocktails TL-75 to TE-75 and the commercial controls S-970 and P-1570 over a 15-day storage period [a].

Sample	day 0	day 3	day 6	day 9	day 12	day 15
2	671 ± 1.1	684 ± 2.4	689 ± 1.4	698 ± 1.8	717 ± 3.1	SE
3	641 ± 0.9	648.5 ± 1.4	655 ± 3.7	664 ± 4.2	677 ± 2.8	699 ± 2.6
4	627 ± 2.3	631 ± 3.8	638 ± 4.7	644 ± 2.3	647 ± 1.1	667 ± 4.1
5	631 ± 1.9	653 ± 1.7	658 ± 4.1	663 ± 0.9	680 ± 2.9	692 ± 5.0
6	635 ± 1.7	649 ± 2.2	657 ± 1.2	662 ± 4.5	686 ± 2.0	711 ± 6.2
7	691 ± 3.1	703 ± 2.2	720 ± 0.5	745 ± 1.1	SE	SE
8	713 ± 3.1	SE	SE	SE	SE	SE
9	691 ± 5.5	727 ± 4.1	SE	SE	SE	SE
10	686 ± 4.2	722 ± 3.9	SE	SE	SE	SE
11	697 ± 3.9	717 ± 2.1	SE	SE	SE	SE
12	721 ± 4.5	739 ± 1.1	SE	SE	SE	SE
13	742 ± 1.0	SE	SE	SE	SE	SE
TL-75	587 ± 2.7	621 ± 1.0	629 ± 2.3	630 ± 1.0	639 ± 2.2	649 ± 1.8
TM-75	589 ± 0.6	612 ± 1.5	627 ± 1.1	631 ± 3.1	633 ± 4.2	657 ± 1.4
TP-75	566 ± 2.1	570 ± 4.6	574 ± 3.3	577 ± 2.4	582 ± 5.5	591 ± 3.8
TS-75	579 ± 3.3	599 ± 1.1	601 ± 0.8	613 ± 2.1	622 ± 0.9	646 ± 3.1
TO-75	581 ± 1.5	593 ± 5.1	621 ± 4.1	642 ± 1.1	651 ± 3.8	661 ± 1.1
TE-75	605 ± 1.4	623 ± 1.0	637 ± 1.7	653 ± 2.0	686 ± 6.0	SE
P-1570	587 ± 2.5	611 ± 3.3	633 ± 2.1	647 ± 4.4	674 ± 2.8	SE
S-970	637 ± 1.9	667 ± 3.1	733 ± 0.6	SE	SE	SE

[a] SE: stratified emulsion. Value = mean ± SD, n = 3.

decreases on admixing it with the corresponding di-ester, indicating that the latter aggregates more readily at lower concentrations. Soultani et al. (2003) have reported that the CMC values of emulsions formed from fructose mono-esters are larger than those formed from its admixture with di-esters. This phenomenon can be attributed to mono-ester/di-ester interactions operating *via* a bridging micelle mechanism (Husband et al., 1998).

Pure trehalose esters **2**–**13** and the mono- and di-ester cocktails TL-75 to TE-75 displayed similar U-shape profiles in stabilizing emulsions. The mean oil-droplet sizes in these emulsions continuously decreased until the trehalose esters incorporating side-chains with more than 16 carbons (*viz*. Ester **4** and TS-75) were used as emulsifiers. Similar U-shape profiles were found in esters derived from maltotriose and sucrose (Xie et al., 2021; Zhu et al., 2020; Zhu et al., 2022). Fortuitous combinations of relative molecular sizes and hydrophobicities resulting in effective adsorption and transport at the oil-water interface may explain these profiles (van Kempen et al., 2014a).

3.4. *In vitro digestion*

Emulsion-based delivery systems are important in the pharmaceutical industry because they are capable of encapsulating and transporting bioactive but hydrophobic compounds such as fat-soluble vitamins and drugs (McClements & Li, 2010; Porter et al., 2008). This mode of delivery can enhance bioavailability in the human gastrointestinal tract. Accordingly, a GITBM was performed in this study to investigate the "fate" of trehalose ester-derived emulsions during digestion in *vitro*. Outcomes are shown in Figs. 3 and 4.

3.4.1. *The stability of emulsions during digestion*

The mean sizes of oil droplets in emulsions derived from trehalose esters **2**–**13** and the mono- and di-ester cocktails TL-75 to TE-75, as well as two controls P-1570 and S-970, were measured at different stages of the GITBM. Overall, and as shown in Fig. 3, the droplet sizes continuously increased during digestion and thus signalling a reduction in stability of the trehalose ester-derived emulsions. So, the oil-droplets sizes in emulsions derived from the trehalose mono-esters increased, on average, from 0.65 μm (before digestion) to 17.52 μm (at the small intestine phase). Indeed, most of the esters tested in this study performed comparably during the first three digestive phases. Significantly, the stability of emulsions derived from the mono- and di-ester cocktails decreased dramatically at the small intestine stage (Fig. 3D) with the oil-particle sizes ranging from 18.15 to 24.88 μm while for their pure mono- and di-ester counterparts the values ranged from 15.33 to 20.63 μm and from 13.76 to 16.35 μm, respectively. Clearly, then, those emulsions derived from the mono- and di-ester cocktails are more "digestible", in the small intestine phase, than the others.

To further investigate the fate of emulsions stabilized by TL-75 to TE-75, their microstructures were examined using confocal microscopy, the results of which are shown in Fig. 4 and wherein the oil particles are represented in red while the green lines indicate the distribution of oil droplets in the emulsion. After simulated oral exposure, there were no significant changes in the oil droplet sizes (Fig. 3) and distributions (Fig. 4) and nor any notable changes in morphology and so suggesting the emulsion is stable under the near neutral conditions involved (Ozturk et al., 2015).

By contrast, in the simulated stomach phase, the same emulsions assumed a bimodal distribution of oil particle sizes and this change was accompanied by slight flocculation and/or coalescence (Fig. 4) and so indicating stability had been compromised. This change could be attributed to, (i), the protease (*viz*. pepsin) present in the simulated gastric juice cleaving the trehalose esters and thus resulting in insufficient emulsifier being available to form an effective interface around the oil particles (Chung et al., 2019) and, (ii), changes in pH and ionic strength adversely affecting electrostatic repulsions between the constituent oil droplets (Mun et al., 2017). As a consequence, and consistent with outcomes reported by Xie et al. (2021), coalescence and "oiling-off" inevitably occur with an accompanying increase in the mean sizes of the oil droplets (Fig. 3).

After their exposure to the simulated small intestinal digestive phase, the oil-particle sizes in all the emulsions under study increased significantly (Fig. 3). This was especially so for those derived from the mono- and di-ester cocktails, the average oil-droplet particle size being 21.3 μm which compares to a value of 16.4 μm for those emulsions stabilized by the control S-970. As can be discerned from Fig. 4, all of the emulsions displayed multi-modal oil-particle distributions as well as the obvious signs of flocculation and/or coalescence (of the oil particles). Such dramatic reductions in the stability of the emulsions can be ascribed to the presence, in the intestinal fluid, of highly surface-active compounds such as bile salts and phospholipids that probably displace, to some extent at least, the trehalose esters at the oil-water interface. As a consequence, the trehalose esters could attach to more than one oil droplet and so promoting bridging flocculation (Mun et al., 2007). These amphiphilic molecules could also be digested/hydrolysed to release more fatty acids and so alter the composition of the oil phase (Porter et al., 2008; Wei et al., 2020). Interestingly, even though the emulsions formed using the mono- and di-ester cocktails were more "digestible" than those derived from their pure mono- and di-ester counterparts, they still performed more effectively, at the end-stage of the GITBM, than the ones prepared using the control P-1570.

3.4.2. *The release of free fatty acids*

Free fatty acid (FFA) release was used to monitor digestion of the trehalose ester-derived emulsions in the simulated small intestine (Fig. 5). So, at a lipase concentration of 24 mg/mL, FFA release within all emulsions occurred rapidly over the first 20 min then slowed thereafter, an observation consistent with results reported by Xie et al. (2021) and Li et al. (2011). This opening "burst" of FFA release likely results from the presence of bile salts and lipases in the small intestine excretion that effectively increase the solubility of ester molecules and replace them with lipases. Thereafter, the lipases attach to the surface of the oil particles and so furthering hydrolysis (McClements & Li, 2010). Hu et al. (2022) have noted the important role of bile salts in digesting *β*-sitosterol-based sugar esters.

The slowing of FFA release after 20 min could be attributed to the gradual digestion of triglycerides. McClements and Li (2010) have reported that lipid hydrolysis is hampered when the oil-droplet surface becomes covered with free fatty acids. Moreover, such hydrolysis would alter the composition and, thereby, the stability of the oil-water interface (Figs. 3 and 4) within emulsions encountering the small intestine digestion stage of GITBM (Wei et al., 2020). In the event of oil-water separation, the lipases could no longer easily attach to the oil particles and so resulting in significantly reduced FFA release rates.

The FFA release profiles from emulsions prepared using trehalose di-esters **8**–**13** were all very similar (Fig. 5 B). In contrast, the analogous profiles for those emulsions prepared from ester cocktails TL-75, TM-75, TP-75, TS-75, and TE-75 (Fig. 5 C) were highly varied, with the overall extent of FFA release being 73.1%, 75.7%, 82.6%, 66.1%, 64.4%, and 58.8%, respectively (this compares with values of 68.0% and 55.9% for those systems prepared from the controls P-1570 and S-970, respectively). These values are significantly higher than those recorded for the emulsions formed using the corresponding mono- and di-esters. This trend suggests bile salts have better capacities to displace trehalose mono-esters from the oil-water interfaces in the presence of di-esters. Precisely why this might be so remains to be fully understood.

3.5. *Penetration-enhancing potency*

Although previous studies have established *in vitro* bioactivities of a plethora of natural substances, their deployment as drugs can be thwarted by a multitude of factors including, for example, low bioavailability and/or rapid metabolism (Brglez Mojzer et al., 2016). On

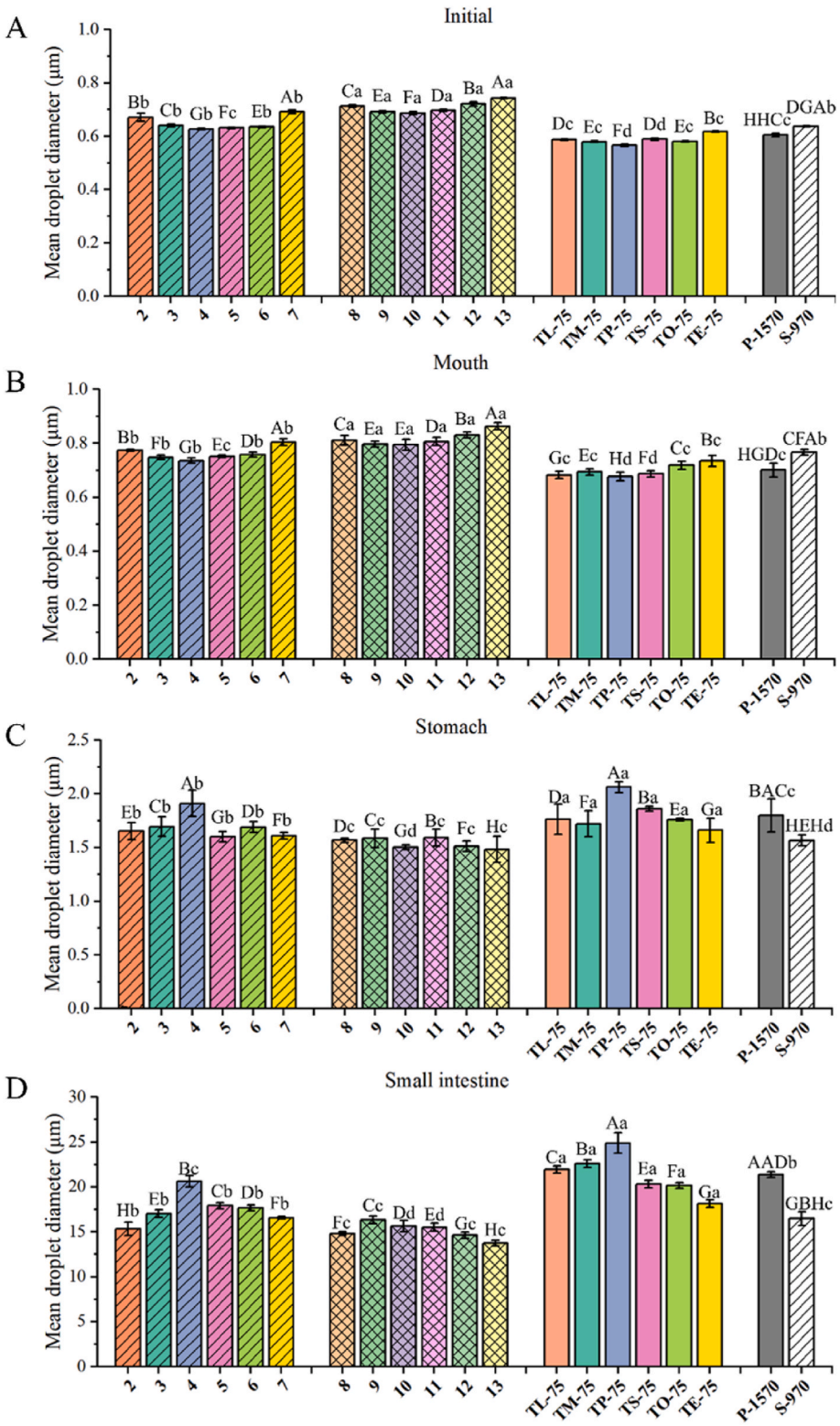

Fig. 3. Mean sizes of oil particles in emulsions stabilized by pure trehalose mono- and di-esters **2**–**13**, cocktails TL-75 to TE-75 and the controls P-1570 and S-970, at initial (A) and three different stages (B, C, and D) of a gastrointestinal behavior model (GITBM). All data are shown as mean ± SD (n = 3). *Capital letters indicate a comparison of emulsions stabilized by the surfactants with the same degrees of esterification but different fatty acid-derived residues at the same digestion stages; Lower-case letters indicate a comparison of emulsions stabilized by same fatty acid-derived residues but different degrees of esterification at the same digestion stages.

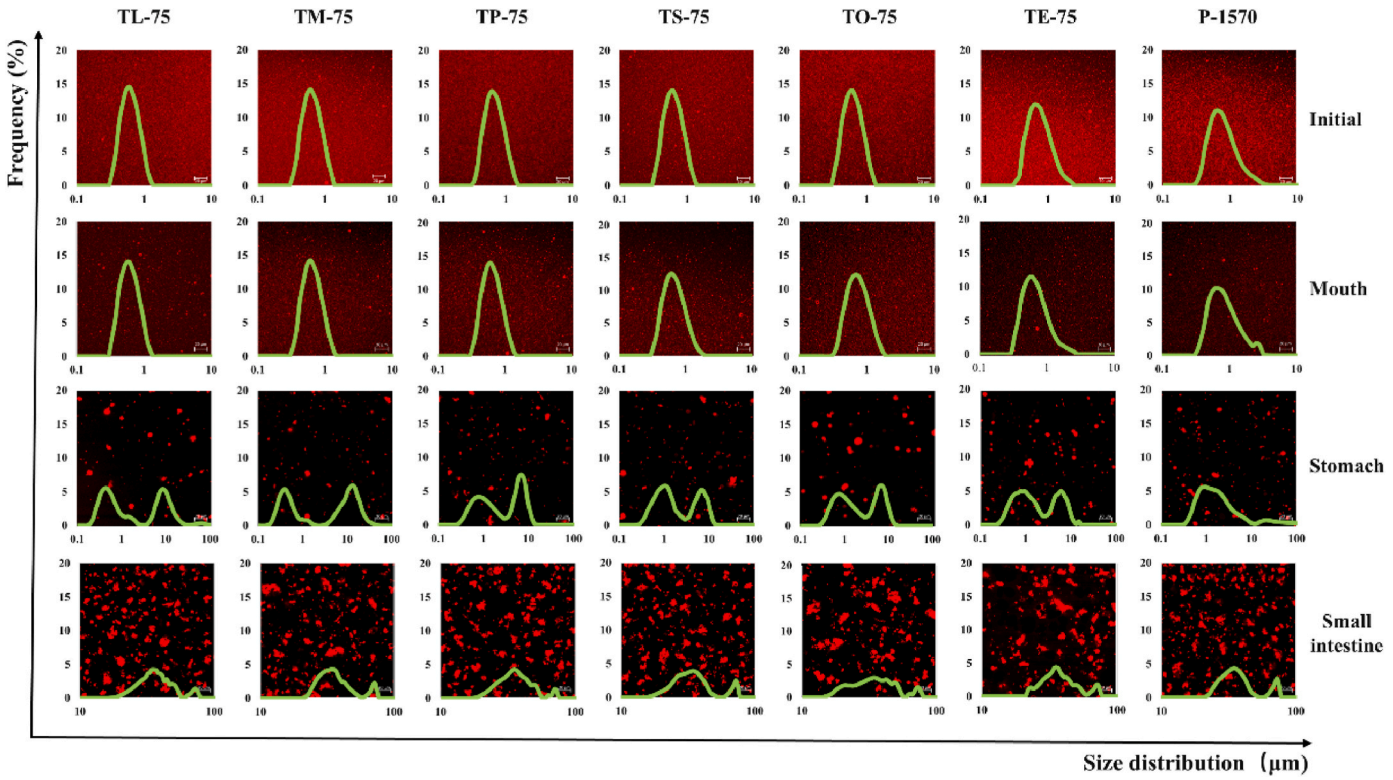

Fig. 4. Microstructures and droplet size distributions of emulsifier-stabilized emulsions prepared by mixing trehalose esters and positive controls P-1570 at different stages of a gastrointestinal behavior model (GITBM).

this basis, the buccal mucosa is regarded as one of the ideal absorption sites since those substances crossing the buccal epithelium will be delivered by the bloodstream into the internal jugular vein and so avoiding first-pass metabolism in the liver and small intestine (Nicolazzo et al., 2005). While the buccal mucosa acts, like skin, as a barrier to the permeation of xenobiotics, the application of certain chemical penetration enhancers (CPEs), including non-ionic surfactants, can circumvent such blocking effects. Indeed, various sugar ester-based surfactants have already been comprehensively evaluated as CPEs including ones derived from sucrose (Ayala-Bravo et al., 2003) and lactose (Lucarini et al., 2016; Perinelli et al., 2018). On this basis, trehalose esters **2**–**13** would seem to have considerable potential as CPEs that could be exploited in the food and related industries.

The permeability-enhancing potencies of trehalose esters **2**–**13** were assessed by determining their TEER values (Fig. 6). In the relevant assay, as detailed above, trehalose **1** was used as the control and observed to exert a modest change of 4.9% during the first 30 min. This was also the case for esters **8** (7.6%) and **9** (9.7%) and which are thus characterized as having weak penetration capacities. However, the TEER values of esters **4**, **5**, **6** as well as the corresponding di-esters **10**, **11**, and **12** had values ranging, after 30 min, from 27.2% to 38.8% and so demonstrating that these compounds can effectively break intercellular tight junctions. This was especially so for ester **6** that sustained a 35.1% reduction over a further 120 min. Notably, the TEER values of all the tested compounds fully recovered after 180 h and so indicating that the effects of the compounds on the membrane permeability of the Caco-2 cell monolayer are reversible and cause no damage. Others have reported (Perinelli et al. (2018) that fatty acid-derived lactose esters are also able to interact with tight junctions and safely enhance membrane permeability. Ujhelyi et al. (2012) concluded that by employing Labrasol and Polysorbate 20 in the same manner that these surfactants could reduce the integrity of cell mono-layers and so promote the redistribution of linker proteins with the end result being effective penetration through the paracellular pathway. The ensuing enhanced membrane fluidity of lipid layers could be contributing to the observed effects (Li et al., 2016).

3.6. Cytotoxicity

Sugar esters are considered ideal surfactants and have massive market value because of their biodegradable and non-toxic properties (Teng et al., 2021). Furthermore, a range of sugar esters exhibit excellent anti-cancer properties including those derived from maltotriose (Zhu et al., 2020), lactose (Liang et al., 2018) and glucose (El-Baz et al., 2021). In order to evaluate, *in vitro*, the cytotoxicity of the trehalose esters under study here, seven cell lines were employed for this purpose and the outcomes of doing so are presented in Table 4. Although trehalose mono-esters **3**–**7** and di-esters **10**–**13** exhibited some degree of cytotoxicity against these cell lines, none of them proved as effective as the positive control doxorubicin. Nevertheless, mono-esters **6** and **7** as well as the corresponding di-esters **12** and **13** exerted some noteworthy effects, especially the first of these (possessing an 18 carbon side-chain) with IC_{50} values against all cell lines falling with the range 32.2–58.1 μM. Since Zhu et al. (2020) have established that the structurally related 6″-*O*-stearoylmaltotriose (also possessing an 18 carbon side-chain) exerted anti-cancer effects by arresting the G1 phase of the tumor cell cycle, congener **6** is presumed to act in the same way.

Consistent with the findings of Liang et al. (2018) and Zhu et al. (2022), the cytotoxicities of those compounds embodying side-chains greater than 18 carbons in length were not significant. Importantly, the di-esters **10**–**13** showed higher IC_{50} values than their corresponding CMC values (Table 1) and so suggesting that these are safe surfactants. That said, it should also be noted that sugar esters are, in general, regarded as completely safe surfactants since they are easily metabolized into the constituent carbohydrates and fatty acids prior to being absorbed and entering the bloodstream (Verboni et al., 2021).

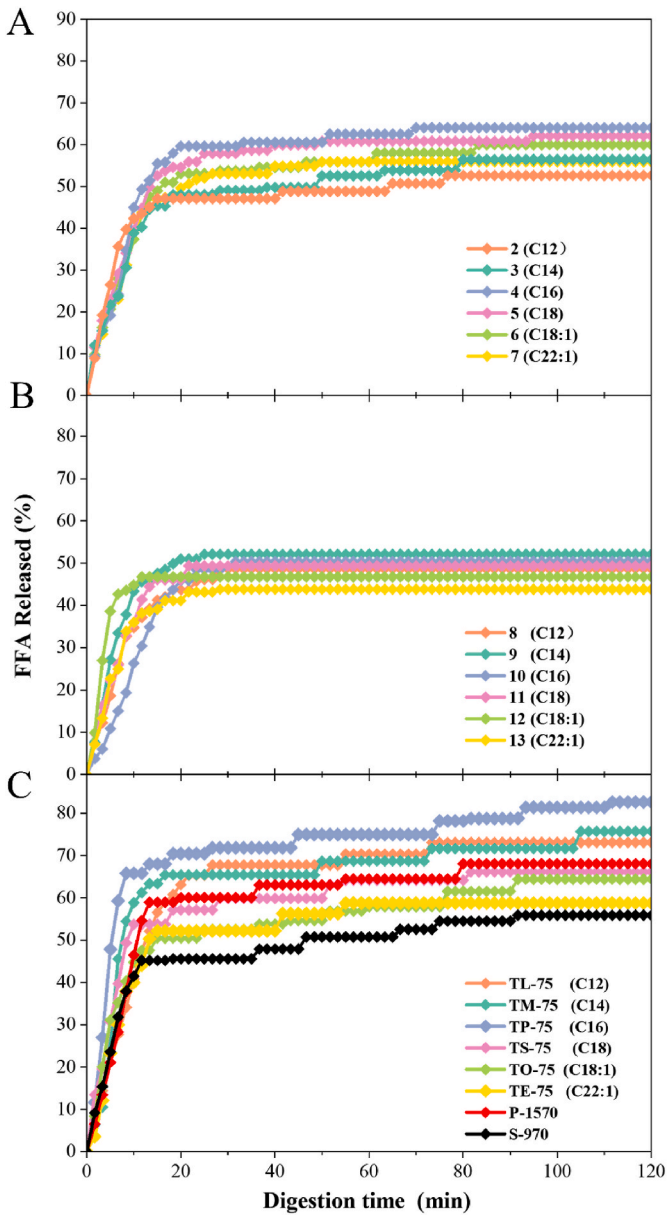

Fig. 5. Percentage of free fatty acids (FFA) released from the emulsions prepared from esters **2**–**13** and cocktails TL-75 to TE-75 at the simulated small intestine stage of a gastrointestinal behavior model (GITBM).

4. Conclusion

A homologous series of 6-*O*-acyltrehalose and 6,6′-di-*O*-acyltrehalose derivatives embodying fatty acid side-chains were synthesized for the first time using a simple and highly selective enzymatic transesterification protocol that proceeds in good to high yield (59–78%). The trehalose derivatives **2**–**13** so-formed have been shown to effectively reduce oil-water interfacial tensions, this being especially so for mono-esters **5**–**7** that possess moderate HLB values. Their foaming and emulsifying properties as well as their robustness on being subjected to the three simulated digestive phases of a GITBM were also evaluated. Monoesters **2**–**4** showed comparable foamability and foaming stability properties to the best commercial control P1570. Trehalose mono-esters **3**–**6** and the mono- and di-trehalose ester cocktails TL-75 to TO-75 showed excellent emulsifying potencies under *in vitro* digestion conditions, although the emulsions derived from the latter group were more easily digested in the simulated small intestine phase. Mono-esters **4**–**6**

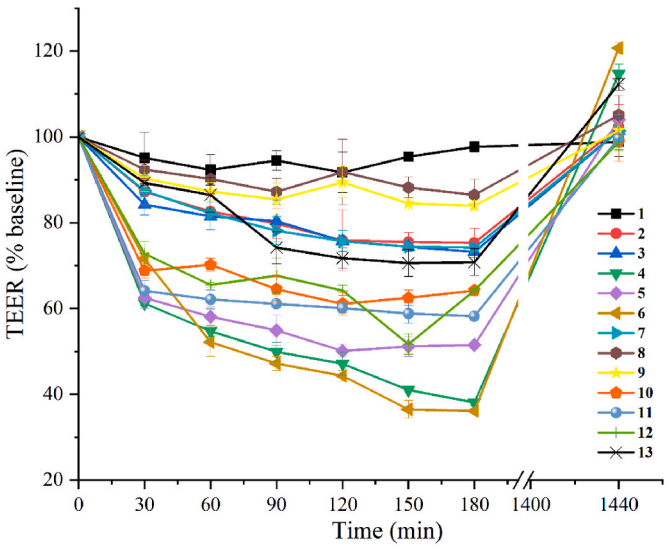

Fig. 6. Effects of trehalose and its ester derivatives on the electrical resistance of Caco-2 cell monolayers as determined using a TEER assay. All data are shown as mean ± SD (n = 3).

Table 4
The half maximal inhibitory concentrations (IC_{50}, in μM) of trehalose esters **2**–**13** and the positive control doxorubicin against seven cell lines. [a]

Sample	Cell Lines						
	MCF-7	A549	HepG2	Caco-2	N2A	SW480	HEK-293
2	>256	>256	>256	>256	>256	>256	>256
3	151.8 ± 17.5	168.2 ± 22.8	>256	125.6 ± 9.7	137.1 ± 13.4	>256	>256
4	83.2 ± 4.2	78.1 ± 5.2	91.1 ± 6.2	78.4 ± 5.9	74.8 ± 3.9	94.2 ± 4.2	55.4 ± 7.8
5	64.2 ± 7.9	58.2 ± 3.9	51.9 ± 8.2	42.9 ± 7.6	44.9 ± 5.8	58.7 ± 6.3	49.2 ± 3.3
6	58.1 ± 9.4	56.1 ± 3.7	42.3 ± 4.2	32.2 ± 3.7	47.7 ± 6.7	42.6 ± 7.7	52.3 ± 4.8
7	67.1 ± 5.7	61.3 ± 5.9	63.4 ± 3.5	46.2 ± 1.5	52.3 ± 4.9	55.8 ± 7.6	57.3 ± 6.1
8	>256	>256	>256	>256	>256	>256	>256
9	>256	>256	>256	>256	>256	>256	>256
10	102.5 ± 8.3	104.2 ± 14.9	154.7 ± 24.4	114.8 ± 14.2	106.5 ± 11.8	92.4 ± 2.7	73.6 ± 6.5
11	87.5 ± 5.4	76.5 ± 7.5	87.1 ± 8.0	76.9 ± 3.9	61.5 ± 9.8	67.2 ± 8.1	58.6 ± 3.5
12	65.6 ± 5.3	64.6 ± 8.9	62.5 ± 3.0	55.3 ± 8.3	55.6 ± 3.4	52.7 ± 6.3	50.6 ± 8.2
13	61.5 ± 7.2	54.1 ± 5.0	47.7 ± 2.1	57.4 ± 8.2	41.9 ± 1.3	45.2 ± 6.3	46.1 ± 5.9
DOX[b]	5.3 ± 0.7	2.2 ± 0.3	12.4 ± 1.6	<1.0	8.6 ± 0.5	15.2 ± 1.4	11.2 ± 0.7

[a] Value = mean ± SD, n = 3.
[b] DOX = doxorubicin.

were considered the best chemical penetration enhancers and acted without causing cellular damage. Certain of these (*viz.* compounds **5**–**7**) together with di-esters **11**–**13** showed moderate cytotoxic effects on seven human cancer cell lines. In the aggregate, these results will serve as a useful guide for applying the title trehalose esters, which incorporate a range of different fatty acid residues and vary in their degrees of esterification, to the diverse needs of the food processing and related industries.

CRediT authorship contribution statement

Jia-Qing Chen: Writing – original draft, (in Chinese), Investigation,

Formal analysis, Visualization. **Yao-Wen Hai:** Writing – review & editing, Conceptualization. **Chun Qing:** Investigation, Formal analysis. **Min-Yi Liang:** Conceptualization, Methodology. **Martin G. Banwell:** Writing – review & editing, Conceptualization, Supervision, Project administration, Funding acquisition. **Ping Lan:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The article entitled "**The Synthesis of Certain Fatty Acid Ester Derivatives of Trehalose and an Investigation of their Emulsifying Properties and Bioactivities**" is the result of original work and describes the enzymatic preparation, rigorous characterization of the surfactant properties and biological potencies of a homologous series of 6-*O*-acyltrehalose and 6,6′-di-*O*-acyltrehalose bearing long (fatty acid) side-chains. The properties of such esters were exhaustively characterized so as to reveal their structure-property profiles as surfactants. Furthermore, their surface-active performances in terms of foaming and emulsifying properties, including being subjected to the three simulated digestive phases of a GITBM, were evaluated in detail. Moreover, biological evaluations revealed that these same esters inhibited the proliferation of a range of cell lines and that some of them proved to be excellent penetration enhancers. As such, this study establishes, for the first time, that trehalose esters bearing long side-chains have potential multifunctional applications within the food industries, as they can simultaneously serve as good emulsifiers. Moreover, their demonstrated inhibition of tumor cell growth lays the foundations for new directions in anti-tumor research. In short, these results will serve as a useful guide for deploying certain trehalose esters in the food and related industries based on different needs. As such, we feel that the work described is clearly within the scope of the journal and appeal to its readers.The authors are Jia-Qing Chen, Yao-Wen Hai, Chun Qing, Min-Yi Liang, Martin G. Banwell (corresponding author) and Ping Lan (corresponding author) declare they have no conflicts of interest. All authors have agreed to the submission of this paper in its current forms.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2023.115369.

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