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Sinigrin Encapsulation in Liposomes: Influence on *In Vitro* Digestion and Antioxidant Potential

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Encapsulation of sinigrin in liposomes with the proliposomal method was performed in order to evaluate the effect of this process on *in vitro* simulated digestion and antioxidant potential of sinigrin. The recovery of sinigrin after simulated gastric and duodenal digestion of its free and liposomal forms was determined with HPLC-UV using human digestive juices. The antioxidant potential of sinigrin and sinigrin-loaded liposomes was determined with the Rancimat test as their ability to prolong oxidative stability of edible oil. The efficiency of 62% was obtained by encapsulating sinigrin in liposomes. The values of mean diameter, polydispersity index and zeta potential showed satisfactory size uniformity and physical stability of the liposomes containing sinigrin. Liposomes were shown to inhibit the digestion of sinigrin in both human gastric and intestinal juices, clearly enabling its prolonged release. Moreover, sinigrin in the liposomal form significantly prolonged the induction time of edible oil oxidation compared to its free form. The results obtained are encouraging from the point of view of a possible incorporation of the sinigrin-loaded liposomes in real functional food systems or their use as nutraceuticals.

INTRODUCTION

Glucosinolates (GSLs) are water-soluble plant secondary metabolites with an *S*-β-D-glucopyrano unit, an *O*-sulphated anomeric (*Z*)-thyohydroximate function, and a variable aglucon side chain. To date, 140 structurally different GSLs have been reported, although over 30% of them have not yet been characterized by nuclear magnetic resonance (NMR) and mass spectrometry (MS) techniques [Blažević *et al*., 2020]. GSLs are typical of Brassicaceae plants including mustard, radish, cabbage, broccoli, Brussels sprouts, cauliflower, horseradish. They are also found in other families of Brassicales, *e.g.* Capparaceae, Cleomaceae, Caricaeae [Mithen *et al*., 2010]. Due to the activity of myrosinase (β-thioglucosidase), and depending on their variable chain, GSLs break down into different products, including mostly biologically active isothiocyanates, which exhibit potent antimicrobial, antioxidant and anticancer activities [Melrose, 2019].

Sinigrin (allyl GSL or prop-2-enyl GSL, Figure 1) is one of the first known GSLs, whose name is derived from "*Sinapis nigra*" (currently known as *Brassica nigra*). It is one of the most abundant GSLs found in *Brassica* vegetables. The correct structure was proposed by Ettlinger & Lundeen [1956] while the structural issue of the geometrical isomerism at the C=N bond was shown to be *Z* (or anti-) by X-ray crystallographic analysis by Waser & Watson [1963]. It is one of the most studied GSLs thanks to its degradation product, the pharmacologically active allyl isothiocyanate, a volatile sulphur-containing compound [Blažević *et al*., 2019; Corrales *et al*., 2014]. A very important aspect of the biological activity of isothiocyanates is their antimicrobial potential against human pathogens, especially against bacteria

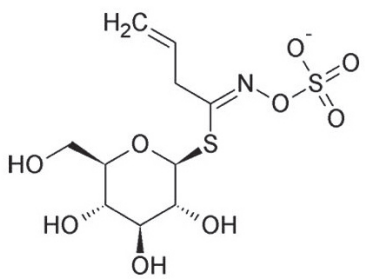

FIGURE 1. Chemical structure of sinigrin.

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with multi-drug-resistance phenotypes [Romeo *et al*., 2018]. Moreover, many studies have shown that isothiocyanates exhibit anti-tumour activity by affecting multiple pathways including apoptosis, mitogen-activated protein kinase (MAPK) signaling, oxidative stress, and cell cycle progression [Jie *et al.*, 2014; Mitsioginanni *et al*., 2019; Wu *et al*., 2009]. There are many examples of the use of encapsulation techniques to enhance/protect the biological activities of GSLs and isothiocyanates: the antitumor effect against glial tumour cells by broccoli extracts [Radünz *et al*., 2020]; the antimicrobial activity of allyl isothiocyanate using different carriers such as gum Arabic, chitosan, sodium polyacrylate-coated halloysite, mesoporous silica particles [Park *et al*., 2012; Maruthupandy & Seo, 2019]; the cytotoxic activity of sinigrin-loaded phytosomes (phytolipid delivery system) against A-375 cells (human melanoma cell line) [Mazumder *et al*., 2016]; or the cytotoxic and apoptotic potential of silver nanoparticles with sinigrin [Yuan *et al*., 2018]. However, there is no study on sinigrin encapsulated in liposomes. Liposomes, *i.e.* lipid bilayers with a diameter of 50–1000 nm, are particularly attractive encapsulation systems that offer increased stability of encapsulated materials against a range of environmental, enzymatic, and chemical stresses [Emami *et al*., 2016]. What distinguishes liposomes from other encapsulation systems is their ability to encapsulate both hydrophobic (within the membrane of the particle) and hydrophilic (within the core of the liposome) substances [Gaede & Gawrisch, 2003]. The possibility of industrial production is also of great importance for their use in the food industry. However, the fact that liposomes can be produced from natural components (biocompatible, biodegradable and non-toxic) makes these systems attractive from the point of view of faster and easier implementation in cosmetic and food end products [Malheiros *et al*., 2010]. There are several reports demonstrating improved stability of liposomal formulations of commonly used nutraceuticals, such as vitamin C [Wechtersbach *et al*., 2012; Yang *et al*., 2012] during storage or processing conditions (*e.g.*, heat treatment), compared to the solutions of free nutraceuticals. Due to the delayed release of active ingredients, liposomes can improve the bioavailability of antioxidants [Takahashi *et al*., 2009] and reduce their cytotoxicity [Isailović *et al*., 2013]. Enhanced antimicrobial (*e.g.*, *Citrus limon*), antifungal (*e.g.*, *Eucalyptus camaldulensis*) and other biological activities (*e.g.*, *Artemisia arborescens* L.) of essential oils incorporated into liposomes have also been reported [Gortzi *et al*., 2007; Moghimipour *et al*., 2012]. Previously, liposomes were reported to be quite effective in enhancing the chemopreventive efficacy of phenethyl isothiocyanate [Pulliero *et al*., 2015; Sun *et al*., 2019]. This is the first report on the gastrointestinal stability of the sinigrin-loaded liposomes in the simulated two-phase digestion model (gastric and duodenal) with human digestive enzymes. There are few studies on the stability and bioaccessibility of sinigrin after a simulated gastrointestinal process and some of them evaluated the influence of human microflora in the bioconversion of sinigrin [Cheng *et al*., 2004; Girgin & Nehir, 2015]. The aim of this study was to monitor the recovery of free sinigrin and sinigrin released from liposomes after a simulated two-phase digestion process using high-performance liquid chromatography with UV detector (HPLC-UV). The effect of the liposomal form of sinigrin on the prolongation of oxidative stability of edible oil was measured with Rancimat test.

MATERIAL AND METHODS

Chemicals

Sinigrin hydrate (≥99.0% (TLC)) was obtained from Sigma Aldrich (St. Louis, MA, USA). Phospholipon 90G was supplied by Natterman Phospholipids GmbH (Köln, Germany). All other reagents and solvents were of analytical grade.

Preparation of sinigrin-loaded liposomes

Liposomes with sinigrin were prepared with the proliposome method, which allows to easily increase the scale of production to the industrial level [Liović *et al*., 2019]. We used soy lecithin Phosholipon 90G which is purified phosphatidylcholine (min 94.0% by weight), with a low content of lysophosphatidylcholine (max. 4.0% by weight) and tocopherol (max. 0.3% by weight). Phosholipon 90G was mixed with ethanol and water (1:0.8:2, *w/w/w*) at the temperature up to 60°C under continuous stirring at 800 rpm to form a homogenous mixture. The mixture was cooled down to room temperature, and sinigrin was added to the homogenous mixture under continuous stirring at 800 rpm during the next 30 min to obtain the final liposomal formulation.

Determination of the encapsulation efficiency of sinigrin by liposomes

The proportion of sinigrin entrapped within liposomes was determined according to the following procedure. Centrifugation of 10 times diluted suspension of liposome at 21,952×*g* for 1 h at 10°C (Eppendorf centrifuge 5804R, Hamburg, Germany) was used to separate supernatant (containing non-encapsulated sinigrin) from the precipitate (liposomes with encapsulated sinigrin). The resulting precipitate was dissolved in 10 mL of methanol, filtered through 0.2 μm pore size filter and transferred to a clean tube for further analysis on HPLC-UV. Based on HPLC-UV analysis, the percentage of encapsulated sinigrin was calculated, as the percentage of sinigrin entrapped in liposomes relative to the total amount of added sinigrin.

In vitro release kinetics of sinigrin from liposomes

In vitro monitoring of the release kinetics of sinigrin from the liposome formulation was performed in a Franz diffusion cell (PermeGear, Inc., Hellertown, PA, USA), containing donor and receptor cell separated with an acetate-cellulose membrane, based on the procedure given in Isailović *et al*. [2013]. In brief, after Franz cell thermostating, we placed 1 mL of liposome dispersions containing sinigrin as a donor phase in diffusion cell chamber. Magnetic stirring speed and temperature were set at 600 rpm and 37°C, respectively. During the next 6 h, an aliquot of the sample (0.5 mL for each time point) was taken from the receptor section at different time intervals (5, 10, 15, 30, 45, 60, 90, 120, 180, 200, 240 and 360 min) for HPLC-UV analysis and replaced by the same volume of distilled water. The results were expressed as C/C_e against time (where C is sinigrin concentration at time t and C_e is sinigrin

equilibrium concentration). The release profile of sinigrin from liposomes was compared with the profile of a sinigrin aqueous solution that was used as a control (with identical sinigrin concentration as the one used for liposomes preparation).

Size and zeta potential measurements

The average particle size (hydrodynamic weighted mean diameter, z-average) and polydispersity index (PDI) of 1000 times diluted liposome suspension was measured on a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) at 25±0.1°C, using the dynamic light scattering (DLS), so-called photon correlation spectroscopy (PCS). The same instrument was used for the zeta-potential measurement as described in Liović *et al*. [2019]. The physical stability test was performed during 14 days with the liposomal preparation stored at 4°C.

Light microscopy analysis

Empty liposomes and liposomes loaded with sinigrin were viewed under a Motic light microscope (BA 210, Xiamen, China) equipped with a Moticam digital camera (1 SP, 1,3 MP) and a Motic Images Plus 2.0 software.

***In vitro* digestion method**

Prior to the *in vitro* digestion procedure, the collection of human digestive juices from the stomach and duodenum of healthy donors was carried out using an endoscope. The collected juices were stored in a sterile tube and then centrifuged in a mySpin 12 microcentrifuge (Thermo Scientific, Waltham, MA, USA) at room temperature, for 10 min at 7,700×*g* to remove mucus and cell debris. To reduce inter-individual variations, batches of pooled gastric and duodenal juices were prepared and then stored at –20°C until use. The enzymatic activity of collected digestive juices was determined using the spectrophotometric method described by Almaas *et al*. [2006]. According to this method, one unit of enzyme activity (U) is defined as the amount of enzyme that causes the absorbance change of 1 between the blank and the sample, after 20 min at 37°C. The *in vitro* digestion procedure was performed according to Furlund *et al*. [2013]. The incubation temperature was 37°C. The simulated digestion process was carried out in a horizontal shaking bath (1,200 rpm). The adjustment of appropriate pH (2.5 for gastric phase and 7.5 for duodenal phase) was done using 1 M HCl and 2 M NaOH. Incubation intervals were 60 min for the gastric phase, and 120 min for the duodenal phase. Due to the liquid nature of the sample, the incubation interval of 60 min for gastric phase was long enough. According to the previously mentioned spectrophotometric method, the enzymatic activity of 1 U was equivalent to 20 μL of human gastric juice and 25 μL of human duodenal juice. The quantities of digestive juices used in this experiment were as follows: 200 mL for the gastric phase, and 800 mL for the duodenal phase. The *in vitro* digestion process was stopped on ice for the period of 5 min. After the digestion, the samples were centrifuged at room temperature, for 10 min at 13,000×*g*. The undigested sample (control) and digested samples were kept at –20°C. All digestive processes were run in duplicate. The recovery of sinigrin was calculated as follows [Girgin & Nehir, 2015]:

$$\text{Recovery of sinigrin (\%)} = (S/C) \times 100$$

where: S – sinigrin content after *in vitro* digestion; C – sinigrin content before *in vitro* digestion.

HPLC-UV analysis

The identification and quantification of the encapsulated sinigrin, as well as the monitoring of its release from the obtained formulations, was performed using HPLC-UV according to the modified procedure by Tsao *et al*. [2000]. The analysis was performed on the Ultimate 3000 UHPLC system (Thermo Fisher Scientific, Schwerte, Germany). By optimizing the elution conditions (flow rate 1 mL/min, isocratic conditions) and selecting the mobile phase (acetonitrile/0.025 M ammonium acetate, 1:99, *v/v*), the detection and quantification of sinigrin in the tested formulations at 228 nm (λ_{max}) was successfully performed using the obtained standard curve in methanol and water (80:20). Prior to HPLC-UV analysis, all samples were filtered through 0.2 μm syringe filters and prepared as described above.

Antioxidant potential evaluation

The antioxidant potential of sinigrin, and sinigrin-loaded liposomes was determined as their ability to inhibit the olive oil oxidation. The oxidation of olive oil (pure and with antioxidants) was carried out using the Rancimat apparatus (Methrom 743, Herisau, Switzerland) [ISO 6886:1996] at 120°C (DT=1.4°C) and the constant airflow of 20 L/h. The quantity of olive oil used for this experiment was 2.5 g. Concentrations of samples (sinigrin, liposomes with sinigrin, and pure liposomes) in the olive oil were 0.2 and 0.1% (*w/w*). The conductivity was measured as a function of time, and the results were expressed as induction time. All measurements were performed in triplicate, and the results were expressed as mean value ± standard deviation.

RESULTS AND DISCUSSION

Nowadays, encapsulation technologies have proved to be competitive tools in the development of new nutraceuticals or the properties and functions of commonly used ingredients in the food industry, as they are able to protect active ingredients, improve their stability and prolong their release in the gastrointestinal tract. Encapsulation in liposomes has a number of advantages that are important for the food industry. These include the ability to carry a variety of bioactive compounds, and the health benefits of natural liposomal ingredients such as phospholipids and sphingolipids for humans [Emami *et al*., 2016]. This is the first study on the sinigrin encapsulation in liposomes, the effect on recovery after *in vitro* digestion process, and its antioxidant potential by prolonging the oxidative stability of olive oil.

Characterization of sinigrin-loaded liposomes

Encapsulation efficiency of 62±3% has been achieved *via* encapsulation of sinigrin in liposomes by the proliposome method using a commercially available mixture of Phospolipon 90G. In addition to the reports showing that proliposome method provides better encapsulation efficacy in comparison

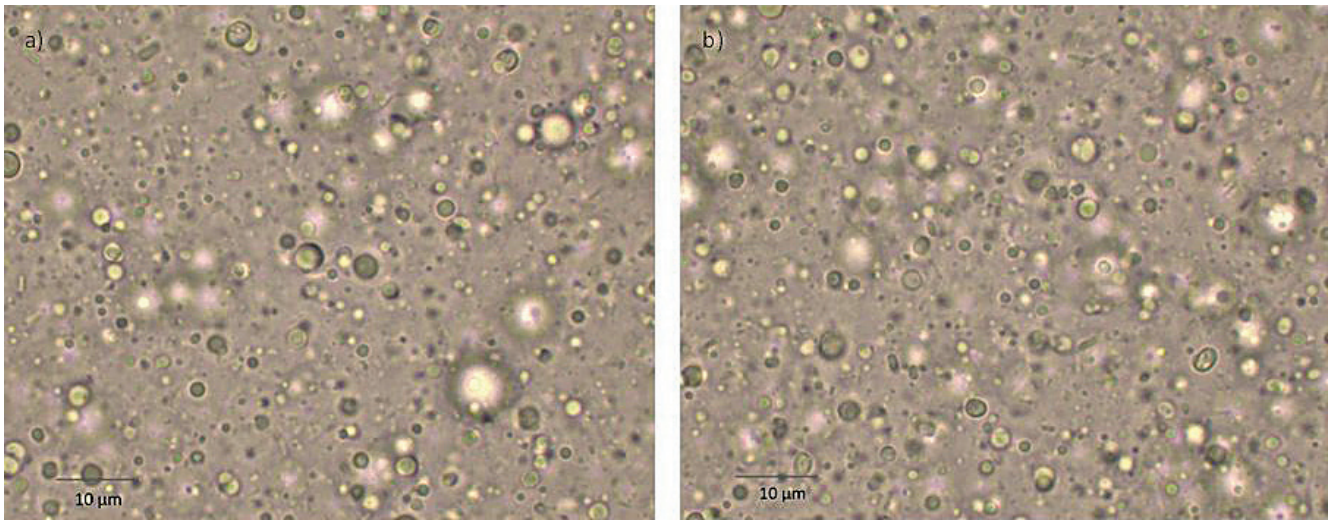

FIGURE 2. Micrographs of a) empty liposomes and b) liposomes with ensapuslated sinigrin obtained by a light microscope (1,000×magnification).

to some other methods for liposomes preparation, *e.g.* ethanol injection method [Chen *et al*., 2012] or thin-film method [Isailović *et al*., 2013], it was chosen in this study as a method that is easily applicable on the industrial scale [Đorđević *et al*., 2015]. It has been reported that in the case of polyphenols, the efficiency of encapsulation in liposomes varied between 10 and 70% depending on the molecular weight of the encapsulated compound (and consequently the ability to leak out from liposomes) and its molecular structure and the affinity to phospholipidic membranes [Đorđević *et al*., 2015]. For the lipophilic molecules, such as resveratrol, encapsulation efficiency up to 97% was achieved using the proliposome method [Isailović *et al*., 2013]. Similar results to those demonstrated in this study for encapsulation of sinigrin, as a water-soluble compound, were obtained in the case of (+)-catechin encapsulation in liposomes (approximately 70%). Furthermore, the entrapment efficiency of phenolic compounds of algal extract in soy lecithin liposomes was shown to be 50.2% by Savaghebi *et al*. [2020], while an entrapment efficiency of 47.5% was obtained for encapsulation of garlic extract in liposomes [Pinilla *et al*., 2017]. The slight variation may be associated with a difference in the encapsulation method or lecithin concentration applied [Rashinidejad *et al*., 2014; Savaghebi *et al*., 2020]. Although obtained encapsulation efficacy in this study is in accordance with the results of other reports and could be accepted as satisfying, further studies should investigate the effects of the addition of different components in liposomes preparation, since they have been recognized as important in encapsulation efficiency improvement. For instance, the addition of chitosan coatings on liposomes was found to be beneficial in encapsulation efficiency or slow-release improvement [Akgün *et al*., 2020; Li *et al*., 2015].

Figure 2 (a-b) shows the micrographs obtained by a light microscope of the produced empty liposomes (control) and liposomes loaded with sinigrin. We have noticed the similarity in control and sinigrin-containing liposomes at the first glance, both revealing spherical structures, which have been typically observed for phosphatidylcholine liposomes [Pinilla *et al*., 2017]. Since the used microscopic technique could not provide quantitative data or detailed insight into the morphology, like other more sophisticated techniques – atomic force microscopy or transmission electron microscopy, further characterization of the liposomes prepared in this study relied on dynamic light scattering technique.

Determination of mean diameter, PDI and zeta potential has revealed satisfying size uniformity of liposomes containing sinigrin. Compared with the control, the encapsulation of sinigrin did not lead to a statistically significant change in surface charge, as well as the mean diameter (Table 1). The obtained results can be explained by the incorporation

TABLE 1. Zeta potential, polydispersity index (PDI) and mean diameter of liposomes with and without encapsuled sinigrin during 2-week storage at 4°C.

Sample	Zeta potential (mV)			PDI			Mean diameter (nm)		
	0 days	7 days	14 days	0 days	7 days	14 days	0 days	7 days	14 days
Liposomes without sinigrin (control)	-34.6±0.5	-33.8±0.4	-32.0±0.8	0.454±0.01	0.450±0.01	0.461±0.03	1070±267	1260±432	1365±402
Liposomes with encapsulated sinigrin	-24.0±0.5	-28.4±0.8	-30.1±0.1	0.497±0.07	0.566±0.04	0.509±0.03	1196±267	1227±553	1263±308

All measurements were made in triplicate and results are reported as mean ± standard deviation.

of the bioactive compound within the aqueous core of liposomes, and only slightly between the bilayer membranes of liposomes or their surface. The PDI determines the particle size distribution and system homogeneity. A system whose PDI ranges from 0 to 0.5 is considered homogeneous [Balanč *et al*., 2016]. Zeta potential is a physicochemical parameter that represents the charge on the particle surface. It correlates with the stability of colloidal suspensions: thus, a high absolute value of the zeta potential indicates a more stable system [Mateos *et al*., 2019]. Our results regarding zeta potential of sinigrin-loaded liposomes are comparable with the results from other studies on the encapsulation of different bioactive ingredients in lecithin liposomes [Akgün *et al*., 2020; Pinilla *et al*., 2017; Savaghebi *et al*., 2020]. The obtained negative values of the zeta potential (Table 1), revealed satisfying electrostatic stabilization of the preparation, preventing aggregation of liposomes [Lin *et al*., 2018]. This was confirmed by the results of stability testing, since the mean diameter of liposomes with sinigrin did not change for more than 6% during 2-week storage at 4°C (Table 1). The observed slight changes in zeta potential could be a result of rearrangement of phospholipids, which are responsible for negative zeta potential values, due to the presence of phosphate groups (PO_4^{3-}) in phospholipids.

The physical stability of sinigrin liposomes and prolonged release of sinigrin over 5 h, as shown by the sinigrin release kinetic curves (Figure 3), indicated that the obtained liposomes made of natural phospholipids present potentially adequate carriers for this compound. In the control solution of sinigrin, distribution of sinigrin happened rapidly, reaching maximum content in acceptor cell after 180 min. As expected, sinigrin release from liposomes was slower, achieving the maximum content in the acceptor compartment after more than 300 min. In the release profile of sinigrin from liposomes in an aqueous medium there was the initial burst, which was probably associated with the small amount of surface-bound sinigrin. The initial burst (which lasted 15 min) was followed by a slow lag phase (which lasted another 15 min) and then a second burst release phase. It is generally believed that the molecules entrapped within liposomes are released primarily by three mechanisms [Pothakamury & Barbosa-C'anovas, 1995]. These are 1) diffusion of molecules through the intact liposomal membrane into the surrounding environment, 2) erosion of liposomal membrane caused by phospholipid degradation, and 3) swelling of pores in the liposomal membrane allowing the leakage of entrapped molecules [Liu *et al*., 2020]. The existence of lag-phase in the release pattern of sinigrin-loaded liposomes in this study may indicate that, apart from diffusion, some changes of the liposomal membranes, like erosion or swelling, were involved, too.

In vitro digestion and antioxidant potential of sinigrin loaded liposomes

The digestion of food *in vivo* is a complex process and includes mouth, stomach, and small intestine, as compartments where the digestion can be done. Liquid food stays in the mouth for a very short time because it does not need to undergo chewing. Thus, the digestion of sinigrin solution and liposomes with sinigrin as samples that are in the liquid

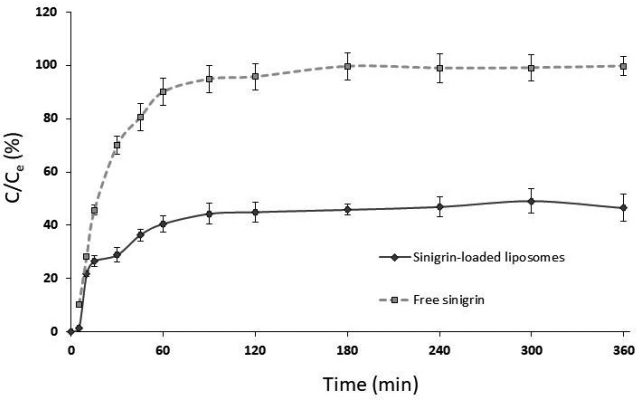

FIGURE 3. Kinetic curve of release of sinigrin from liposomes and prolife of free sinigrin in aqueous solution against time (control). C: sinigrin concentration at time t; C_e: sinigrin equilibrium concentration. Measurements were made in triplicate and results are shown as mean and standard division.

state would start in the gastric compartment and then be continued in the intestinal section. However, in this study, it was only possible to perform digestion discontinuously, starting from gastric or intestinal conditions simulation, and the percentage of unbroken molecules of sinigrin was monitored. We are aware of the limitation of such kind of analysis, so the establishment of reliable *in vitro-in vivo* correlation could not be expected. However, we believe that even preliminary data obtained from the two-phase digestion could contribute to further optimization in the development of sinigrin-loaded liposomes and their incorporation in real food matrices. The results of the *in vitro* recovery of free sinigrin and sinigrin released from liposomes after simulated gastric and duodenal digestion phases are given in Table 2 and Table 3, respectively. The recovery of free sinigrin was high after simulated gastric (87.39%) and duodenal digestion (83.31%) (Table 2). The recovery of free sinigrin in simulated acidic medium without human digestive enzymes was lower (71.23%), while in simulated slightly basic medium free sinigrin was almost completely stable (98.15%) (Table 2). High gastrointestinal stability of sinigrin was also reported by other authors who investigated its stability after a simulated digestion process with commercial digestive enzymes [Kuljarachanan *et al*., 2020]. Hwang *et al*. [2019] reported high stability of sinigrin in kale after gastric digestion with commercial digestive enzymes, but in contrast, the authors reported a low stability rate of sinigrin in kale after simulated duodenal digestive phase. This discrepancy can be explained by the effect of plant matrix on the duodenal stability of sinigrin and/or the differences between *in vitro* studies based on human and commercial digestive enzymes. Discrepancies between *in vitro* digestion studies with human and commercial digestive enzymes have already been reported [Aarak *et al*., 2013; Zorić *et al*., 2016]. Table 3 shows results of the recovery of sinigrin released from liposomes. Sinigrin loaded in liposomes was protected from the influence of pH and digestive enzymes. Namely, starting from 38% of available sinigrin in the case of liposomes, after the digestion process, the percentage of free sinigrin increased to 52.50% in the gastric medium and 52.71% in the duodenal medium. A similar portion of available sinigrin after digestion

TABLE 2. Concentration and recovery of sinigrin after its two-phase *in vitro* digestion.

Digestion phase	Concentration (μM)	Recovery (%)
Befor digestion (control)	61.03±1.55	
After gastric digestion	53.34±2.26	87.39
After duodenal digestion	50.85±0.23	83.31
pH 2.5 (without digestive enzymes)	43.48±0.41	71.23
pH 7.5 (without digestive enzymes)	59.91±0.15	98.15

The quantification of sinegrin was performed by HPLC-UV. Results are expressed as mean ± standard deviation (n=3).

TABLE 3. Concentration and recovery of sinigrin after a two-phase *in vitro* digestion of sinigrin-loaded liposomes.

Digestion phase	Concentration (μM)	Recovery (%)
Befor digestion (control)	1.08±0.12	38.00
After gastric digestion	1.49±0.32	52.50
After duodenal digestivom	1.50±0.47	52.71
pH 2.5 (without digestive enzymes)	1.43±0.41	50.16
pH 7.5 (without digestive enzymes)	1.24±0.22	43.74

The quantification of sinigrin was performed by HPLC-UV. Results are expressed as mean ± standard deviation (n=3).

of sinigrin-loaded liposomes in the gastric and duodenal medium was somewise unexpected result. Namely, it is generally accepted that liposomal structural integrity remains practically unchanged under gastric conditions, while the lipid digestion and consequent liposomes destruction occur primarily in the duodenum [Liu *et al*., 2020]. Since gastric juice in this study was obtained from human volunteers, it may be speculated that the samples were rich in gastric lipases. On the other hand, it is known that apart from the disruption of liposomal structure by pancreatic enzymes, bile salts contribute crucially to the digestion of lipids in duodenal medium, since the activity of phospholipase A_2 and lipase requires the presence of bile salts [Liu *et al*., 2020]. The low level of bile salts in the intestinal juices collected from healthy human donors could be a possible explanation for the obtained results on liposomes digestion. Furthermore, since the bile salts are very potent surface-active compounds, their content in the human juices used may not be sufficient for the complete lipid digestion and liposomes degradation, but adequate for rearrangement of the liposomes to smaller ones still encapsulating sinigrin. Although it is considered that without digestive enzymes, the change of pH through gastrointestinal system does not influence the liposomes' structure [Liu *et al*., 2019], the results on the available amount of sinigrin after incubation in a medium with pH 2.5 and 7.5 indicate that not just diffusion, but also swelling of liposomes in acidic/basic media and their transformation to the gelled state could contribute to sinigrin release. Overall, the obtained results proved the protective effect of liposomes on sinigrin digestion under various conditions, since a high portion of sinigrin remains encapsulated and thus is available for further release and transformation to highly bioactive isothiocyanates. This indicates real potential for liposomes application as a prolonged delivery system of sinigrin in food matrices. For that sake, besides the use of free sinigrin, in this study we have tested its potential after encapsulation in liposomes for the preservation of oxidative stability of edible oil.

Results presented in Table 4 show the effect of free sinigrin (at the concentration of 0.2%) on the prolongation of the oxidative stability of olive oil (the percentage of the prolongation was 23.5%). This effect was higher with sinigrin loaded in liposomes (the percentage of the prolongation of the oxidative stability was 38.68%, at the concentration of 0.2%). Weil *et al*. [2004] reported that sinigrin inhibited lipid peroxidation by 71% *in vitro*. The effectiveness of liposome systems in prolonging the induction period was reported by Gortzi *et al*. [2008]. The low oxidative stability of pure liposomes (without bioactive compound) (Table 4) can be explained by the lipophilic components present in lipid bilayers, which stabilized the liposome membranes but also underwent to oxidation firstly due to the presence of double bonds prone to oxidative degradation [Huang *et al*., 2019]. It is already known that encapsulated antioxidants (for instance, essential oils and polyphenols) protect lipid bilayers from oxidation to some extent [Balanč *et al*., 2016; Detoni *et al*., 2012]. In our case, encapsulated sinigrin in liposomes partially stabilized the liposomal membrane, protecting itself from degradation, so it could prevent lipid peroxidation of olive oil.

TABLE 4. Induction time of oxidation of olive oil with the addition of sinigrin, singrin loaded in liposomes and pure liposomes in Rancimat test. The quantity of olive oil was 2.5 g. Concentrations of samples (sinigrin, sinigrin-liposomes complex, and pure liposomes in olive oil) were 0.2 and 0.1% (*w/w*).

Sample	Induction time (h)	
	0.2%	0.1%
Sinigrin	8.10±0.11	7.60±0.28
Sinigrin-liposomes	10.11±0.55	9.24±0.40
Liposomes	3.22±0.48	4.15± 0.31

The induction time of pure olive oil oxidation (control) was 6.20±0.31 h. All measurements were made in triplicate and results are reported as mean ± standard divisoin.

The application of the sinigrin-loaded liposomes as a protective agent against lipid peroxidation might be of great interest in lipid-based food systems.

CONCLUSION

The present study demonstrated the high stability of sinigrin after simulated digestion with human gastric and duodenal medium, and the influence of a slightly basic medium on the stability of sinigrin. The use of liposomes enabled the protection of sinigrin under the conditions of the simulated digestive process (stomach and duodenum) and prolonged its release in the gastric and duodenal medium. Moreover,

the liposomal form enhanced the effect of sinigrin on prolonging the induction time of edible oil compared to the free form of sinigrin. The innovative potential of the sinigrin-loaded liposomes in real functional food systems or their use as dietary supplements deserves further investigation.

RESEARCH FUNDING

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

COMPLICANCE WITH ETHICAL STANDARDS

This article contains a study with human digestive juices. The approval for the collection of digestive juices was obtained from the Ethics Committee of the University Hospital Centre Split.

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