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Microwave pretreatment enhances the formation of cabbage sulforaphane and its bioaccessibility as shown by a novel dynamic soft rat stomach model

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ABSTRACT

Microwave (MW) pretreatment was used to increase the sulforaphane content in cabbages. *In vitro* digestion in rat stomach and shaking flask was then investigated to monitor the bioaccessible sulforaphane content in the pretreated cabbages. Microstructural changes of cabbages were observed and used to support the pretreatment and digestion results. MW treatment at 26.40 W/g for 15 s increased the sulforaphane content by 6.23 times compared with that in the fresh samples. Bioaccessible sulforaphane content after rat-stomach digestion increased 3.48–4.19 times compared with that in the pretreated samples before digestion. This increase was similar to that after shaking-flask digestion, probably because cabbage tissues are soft and can be easily digested even in a simplified batch *in vitro* digestion system. Nevertheless, sulforaphane concentration in the digested mixture during rat-stomach digestion became constant after 15 min, while that in the shaking flask reached the same concentration only after 75 min.

1. Introduction

Sulforaphane [1-isothiocyanato-4-(methylsulfinyl) butane] is well recognized to possess high anticarcinogenic activities (Guo, Yang, Wang, Guo, & Gu, 2014). Unfortunately, the compound is very heat-sensitive and easily degradable, especially in the presence of light and oxygen (Pongmalai, Devahastin, Chiewchan, & Soponronnarit, 2015). Therefore, any vegetables that can serve as the sources of sulforaphane should be prepared via the use of a suitable preparation method prior to consumption, or sulforaphane may suffer undesirable degradation. It is important to note that disruption of the plant microstructure by any pretreatment method is expected to result in the formation and release of sulforaphane from the plant cellular structure. This should in turn affect the bioaccessibility and bioavailability of the compound during subsequent consumption or digestion.

To understand the bioaccessibility of sulforaphane upon consumption, monitoring the changes in the sulforaphane content during digestion is crucial. Such an understanding can commonly be obtained via *in vitro* digestion experiments, where food is digested in synthetic digestive fluids in a shaker or a continuously stirring bioreactor (Corrêa et al., 2017; Gullon, Pintado, Fernández-López, Pérez-Álvarez, & Viuda-Martos, 2015; Kamiloglu et al., 2017; Sancho, Souza, Aliaga de Lima, & Pastore, 2017). Note, however, that most standardized *in vitro* digestion models, including SHIME, RIVM and Infogest model are static models, where food and digestive fluids are mixed and react under a controlled environment. As a result, these digestion models may not adequately simulate the real digestion system, which exhibits constantly changing biochemical reactions. Gastric mobility is also expected to result in significant structural modification and compound release from the food matrix (Thuenemann, 2015; Wu et al., 2017). For this reason, dynamic *in vitro* gastrointestinal digestion systems that offer continuous digestion reactions and closely resemble the *in vivo* digestion process have attracted increasing attention during the past decade (Krul et al., 2002). Among several dynamic gastrointestinal models, *in vitro* soft rat

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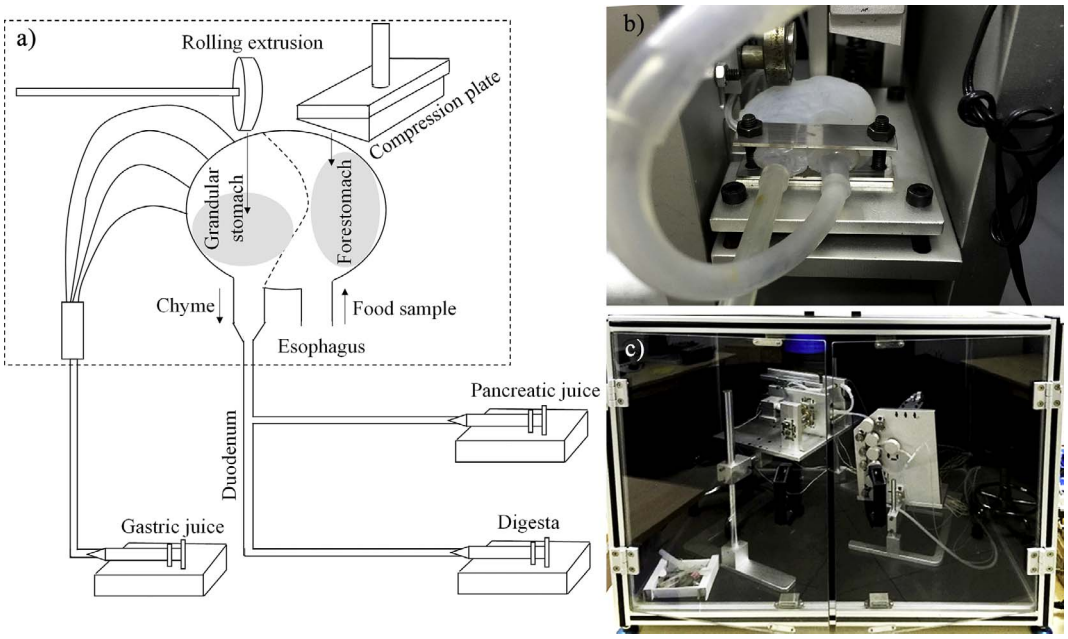

Fig. 1. Mechanized soft rat stomach model. (a) A schematic illustration of the system; (b) the soft-elastic rat stomach equipped on the apparatus; (c) the apparatus.

stomach model is an interesting option since this animal model exhibits physiological parameters very close to the *in vivo* digestion of a rat (Chen, Jayemanne, & Chen, 2013). Nevertheless, limited information is so far available both on how a suitable preparation method prior to digestion could improve the content of sulforaphane and its bioaccessibility as well as on the evolution of this bioactive compound upon digestion.

The present work aimed at studying the feasible use of microwave (MW) cooking, which is a widely used domestic food preparation method, to enhance the formation of sulforaphane. Outer leaves of white cabbage (*Brassica oleracea* L. var. *capitata*) were used as the test material. Since sulforaphane is a hydrolysis product of glucoraphanin, the evolutions of these two compounds, along with that of the activity of myrosinase, which is the enzyme responsible for the hydrolysis reaction, should first be monitored. Monitoring of such evolutions would allow the identification of a suitable MW pretreatment condition. A newly constructed mechanized soft rat stomach model was then utilized to perform dynamic *in vitro* digestion experiments of the pretreated cabbages in comparison with batch *in vitro* digestion experiments. Only gastric digestion was studied as it is the major mechanism for food disintegration and digestion and hence plays an important role on food structural modification, release and bioaccessibility of the bioactive compounds of interest. In order to understand the release and formation mechanisms of sulforaphane during dynamic *in vitro* digestion, the results were compared to that obtained with a simplified batch *in vitro* digestion system. Microstructural changes of cabbages, as observed via scanning electron microscopy (SEM) and quantified via the use of fractal dimension, were investigated and used to explain the MW pretreatment and digestion results.

2. Materials and methods

2.1. Materials and chemicals

Outer leaves of cabbages (*Brassica oleracea* L. var. *capitata*) were obtained from a local market in Suzhou; the leaves were kept at 4 °C until the time of an experiment but for no longer than 3 h. Prior to each experiment, the leaves were washed with tap water and drained on a screen to get rid of excess water. The leaves were then chopped with an electric chopper (Braun, K600, Kronberg, Germany) for 1 min and immediately introduced to a treatment process.

α-amylase from *Bacillus subtilis*, pepsin from porcine gastric mucosa and mucin from porcine stomach were obtained from Sigma-Aldrich (St. Louis, MO). Glucoraphanin potassium salt was obtained from Chromadex (Irvine, CA), while sinigrin and sulforaphane standards were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. MW treatment

Five grams of chopped cabbages were introduced into a domestic MW oven (Galanz, HC-83510FR, Guangdong, China) at an input power of either 160, 320, 480, 640 or 800 W (or specific absorbed powers of 22.46, 24.47, 24.81, 26.40 and 26.94 W/g, respectively) for a maximum of 45 s. After that the pretreated cabbages were immediately placed into a 100-mL Erlenmeyer flask containing an extraction solvent to extract the bioactive compounds and to determine the myrosinase activity. Note that 50 mL of pure methanol and 50 mL of dichloromethane were used as the extraction solvents for glucoraphanin and sulforaphane extraction, respectively. On the other hand, 50 mL of 30-mM citrate/phosphate buffer (pH 7) was prepared for myrosinase activity determination.

The cabbage temperature during MW treatment was monitored using a fiber-optic thermometer (Luxtron, m600, Santa Clara, CA); the thermometer was placed in the center of the cabbages subjecting to microwave irradiation.

2.3. Dynamic in vitro rat stomach digestion

In vitro digestion was first performed within the mechanized soft rat stomach model (Bionic Rat Model II, Nantong Dong-Concept New Material Technology Ltd., Jiangsu Province, China) following the methods of Wu et al. (2017) with some modification. Prior to the start of the digestion process, one g of the pretreated cabbages was added to the stomach through the esophagus (see Fig. 1); this was followed by adding one g of simulated rat saliva. The mixture was mixed in the stomach for 30 s before loading one g of artificial gastric juice through the esophagus. Note that the saliva solution was prepared by dissolving 0.1128 ± 0.0005 g of α-amylase in 1.01 mL of deionized water with pH 7.80 ± 0.44 adjusted by using 1-M NaOH. Artificial gastric juice was prepared by dissolving pepsin (0.27 g/L or 250 U/mL) and mucin

(1.5 g/L) in 50 mL of simulated gastric fluid, which contained KCl (37.3 g/L), KH_2PO_4 (68 g/L), $NaHCO_3$ (84 g/L), NaCl (117 g/L), $MgCl_2(H_2O)_6$ (30.5 g/L) and $(NH_4)_2CO_3$ (48 g/L) at pH 1.63 ± 0.01 (Wu, Chen, Wu, & Chen, 2014; Minekus et al., 2014).

After loading the sample, the movement of rat stomach was made by pressing it against a compression plate (at the compression amplitude of 2.6 mm) and a rolling extrusion plate, to mimic the *in vivo* movement in a rat; the frequency of these plates depended on the speed of the stepper motors, which could be adjusted between 0 and 10 rpm (Chen et al., 2013). Additional gastric juice was continuously fed from the fine tubes connecting to a syringe pump (Shenchen, SPLab01, Baoding, China) through the stomach wall at an average rate of 25 μL/min. One g of the chyme, which came out from the stomach, was collected at 15, 75 (15 + 60) and 135 (15 + 120) min; the digestion time of 15 min was noted as the initial coming out time of the chyme (cabbages + digestive fluids). The experiment was stopped at 135 min and the contents remained in the stomach were also collected.

Each sample collected at different sampling time was immediately centrifuged to separate supernatant from the remaining cabbage biomass. The supernatant was subject to a pH measurement using a pH meter (Thermo Scientific, Orion™ Star A211, Beverly, MA) and then dehydrated using a rotary evaporator (IKA, RV10, Staufen, Germany) at 50 °C for 10 min. The residue was dissolved in 2 mL of dimethyl sulfoxide; the diluted sample was kept at −80 °C in a vial until further analysis. The dry mass of the cabbage residues was determined after heating at 105 °C for 24 h in an oven (Shanghai Jing Hong, XMTD-8222, Shanghai, China). All mass measurements were carried out using a 4-digit digital balance (Sartorius, BSA224S, Goettingen, Germany).

2.4. Batch in vitro shaking flask digestion

Cabbages:saliva solution at a ratio of 1:1 was introduced into a 50-mL Erlenmeyer flask and mixed for 30 s. Four g of gastric juice was then added to the flask, which was later covered and placed in a shaking incubator (LiTekvo, THZ-98AB, Shanghai, China) at 37 °C, simulating the rat body temperature. The shaking speed was set at 100 rpm to induce slight movement of the sample mixture during the simulated digestion (Son, & Shim, 2014). The mixture was taken out from the flask at 0, 5, 15, 75 and 135 min. The liquid part was separated from the sampled mixture and added into a 13 × 100-mm test tube via the use of a 1000-μL micro pipette. The pH of the liquid was measured; the liquid was then dehydrated using the rotary evaporator at 50 °C for 10 min. The residue was dissolved in 2 mL of dimethyl sulfoxide and kept at −80 °C until further analysis. The dry mass of the cabbage residues was determined after heating at 105 °C for 24 h in the oven. All mass measurements were carried out using the 4-digit digital balance.

2.5. Extraction and quantification of glucoraphanin

Glucoraphanin content was determined following the methods of Pongmalai, Devahastin, Chiewchan, & Soponronnarit (2017) with some modification. Five grams of fresh or pretreated cabbages were placed into a 100-mL Erlenmeyer flask containing 50 mL of methanol. The mixture was then stirred for 1 min by a magnetic stirrer (Dragonlab, MS7-H550-Pro, Beijing, China) and placed into a water bath shaker at 70 °C (Zhicheng, ZWT-110X30, Shaanxi, China) for 15 min. After 15 min the mixture was cooled with tap water to room temperature (around 25 °C) and filtered through a Titan filter paper and washed with 50 mL of methanol. The methanol fraction was dehydrated using the rotary evaporator at 50 °C for 30 min. The residue was dissolved in 1 mL of methanol; the concentrated sample was kept at −80 °C in a vial until further analysis.

To quantify glucoraphanin in each sample, 1 mL of the extract was first introduced to Sep-Pak® Vac 6 cc cartridge (Waters, Milford, MA). The eluate was filtered through a 0.2-μm nylon filter prior to loading to an HPLC (Agilent technologies, Waldbronn, Germany). Ten microliters of the filtrate was injected into Xselect CSH C18 HPLC column (4.6 × 250 mm, Waters, Milford, MA) with 30% of methanol and 70% of 30-mM ammonium acetate buffer (pH 5) as the mobile phase; the flow rate was set at 1 mL/min. A UV detector (Agilent technologies, G4212B, Waldbronn, Germany) at a wavelength of 233 nm was used for detecting glucoraphanin. The glucoraphanin content was calculated from a standard curve of glucoraphanin potassium salt, which was prepared at the concentrations of 0–100 μg/mL.

Limit of Detection (LOD) and Limit of Quantitation (LOQ) of glucoraphanin were calculated based on the standard deviation of the intercept and slope of the calibration curve, according to the suggested methods of Şengül (2016):

$$\mathrm{LOD} = 3 \times (\sigma/\mathrm{slope}) \quad (1)$$

$$\mathrm{LOQ} = 10 \times (\sigma/\mathrm{slope}) \quad (2)$$

where σ is the standard deviation of the response or standard deviation of the intercept, and slope refers to the slope of the calibration curve.

LOD and LOQ of glucoraphanin were calculated to be 1.33 and 4.44 μg/mL, respectively. Note that the glucoraphanin concentration in our work was always higher than 35 μg/mL; such higher concentrations than LOD and LOQ indicated suitability of the employed quantification method. In addition, all calibration curves exhibited R^2 values of higher than 0.99, confirming a positive linear relationship between the concentration and peak area within the concentration range of interest.

2.6. Extraction and quantification of sulforaphane

The extraction of sulforaphane from fresh or pretreated cabbages was conducted using the methods described by Tanongkankit, Chiewchan, & Devahastin (2011) with some modification. Five grams of fresh or pretreated cabbages were extracted two times with 50 mL of dichloromethane containing 2.5 g of sodium sulfate anhydrous. The dichloromethane fraction was dehydrated using the rotary evaporator at 50 °C for 10 min. The residue was then dissolved in 2 mL of dimethyl sulfoxide; the concentrated sample was kept at −80 °C in a vial until further analysis.

Determination of the sulforaphane content was performed following the methods of Pongmalai et al. (2015) with some modification. Two milliliters of an extract was introduced to Oasis HLB, 3 cc cartridge (Waters, Milford, MA). The eluate was filtered through a 0.2-μm nylon filter. Ten microliters of the filtrate was injected into Xselect CSH C18 HPLC column (4.6 × 250 mm, Waters, Milford, MA) with 15% of acetonitrile and 85% of 1% (v/v) acetic acid as the mobile phase; the flow rate was set at 1 mL/min. A UV detector at a wavelength of 254 nm was used for detecting sulforaphane. The sulforaphane content was calculated from a standard curve of sulforaphane, which was prepared at the concentrations of 0–200 μg/mL.

2.7. Determination of myrosinase activity

Myrosinase activity was determined through a decrease in the sinigrin content via the enzymatic hydrolysis reaction following the methods of Pongmalai et al. (2017) with some modification. Fresh or pretreated cabbages were chopped by the electric chopper. Twenty-five grams of the sample was placed in a homogenizer (IKA, T18, Staufen, Germany) with 50 mL of 30-mM citrate/phosphate buffer (pH 7) and homogenized for 1 min. The mixture was filtered through a 425-μm stainless steel screen; the filtrate was then placed into a 26.35 × 102.5-mm centrifuge tube and centrifuged by a refrigerated centrifuge (Heal Force, Neofuge 18R, Shanghai, China) at 10,000 × *g* at 4 °C for 4 min. Supernatant from the tube was again filtered, and the filtrate was kept at 4 °C until further analysis.

Fifty microliters of the above-mentioned filtrate, 1.35 mL of 32.22-mM citrate/phosphate buffer (pH 6.5) with 1.07-mM EDTA and 100 μL of 37.50-mM sinigrin were prepared as the reaction mixture in a

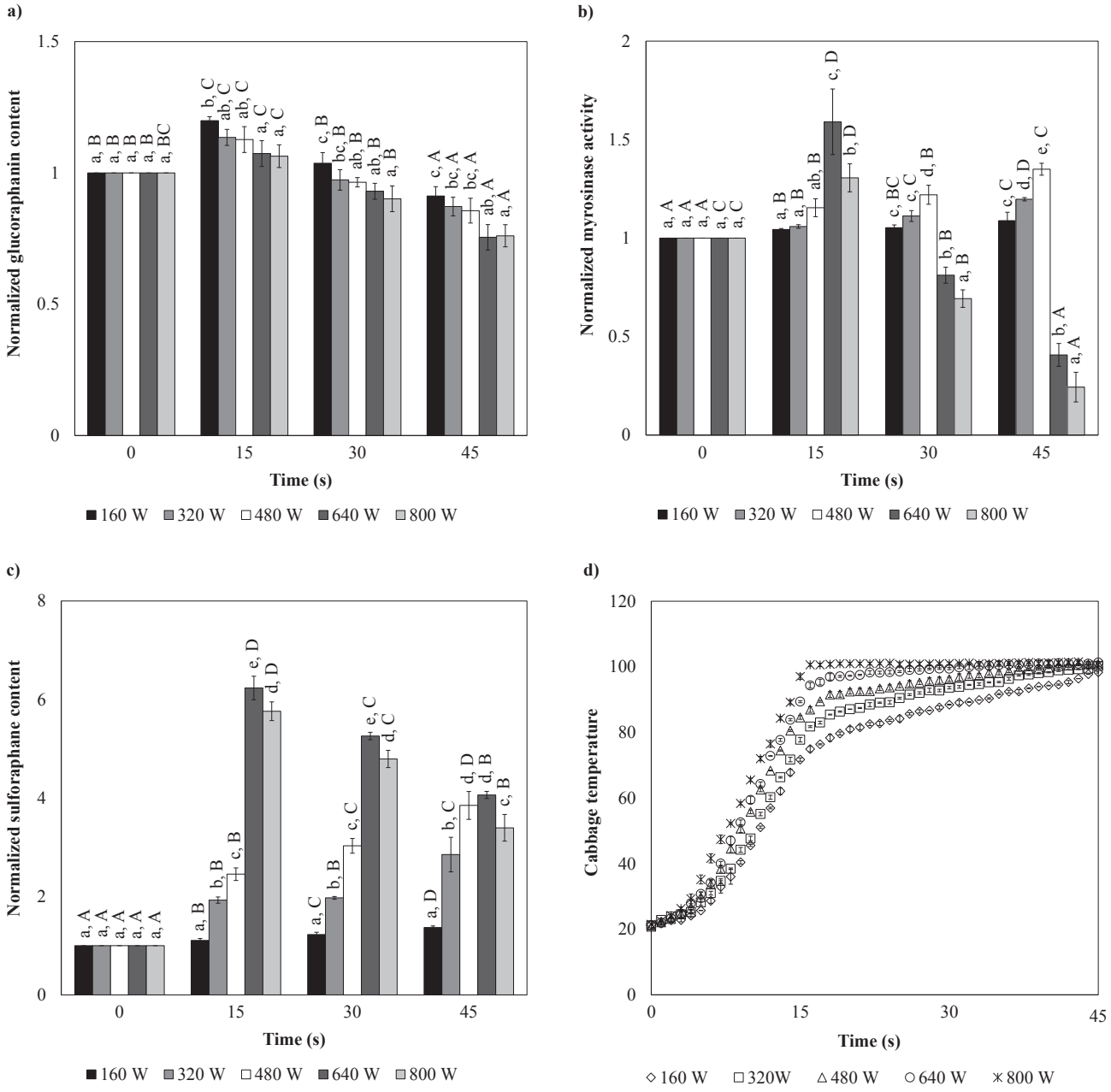

Fig. 2. Evolutions of normalized bioactive compounds contents, myrosinase activity and temperature of cabbages pretreated by MW at different powers. (a) Glucoraphanin content; (b) myrosinase activity; (c) sulforaphane content and (d) cabbage temperature. Same lowercase letters at the same treatment time and same uppercase letters at the same MW power indicate that values are not significantly different ($p \geq 0.05$).

0.8 × 13.5-cm test tube at 25 °C. The reference mixture contained 100 µL of deionized water instead of the sinigrin solution. Then, the tube was vortex mixed for 30 s. The sample mixture was placed into a cuvette and had their absorbance measured at 227 nm at every 5-s interval for a period of 5 min after the mixing via the use of a spectrophotometer (Molecular Devices, SpectraMax® M5, Sunnyvale, CA). Myrosinase activity was determined from the linear slope of a plot between the absorbance of sinigrin and the reaction time, which represents the disappearance of sinigrin from the reaction mixture. A unit (U) of activity is defined as the disappearance of 1 µmol sinigrin per min.

2.8. Microstructure evaluation

Cabbages were placed on a 15 × 100 mm petri dish and covered with aluminum foil prior to being placed in a freezer at −80 °C for 4 h. After that, the sample was taken out to dehydrate in a freeze dryer (Xiangyi, LGJ-10D, Hunan, China) for 48 h. The sample was then coated with platinum in an ion sputter coater (Hitachi, MC1000, Tokyo, Japan). The sample microstructure was observed using a scanning electron microscope (Hitachi, SU1510, Tokyo, Japan) operated at 5 kV at a magnification of 350×.

2.9. Determination of fractal dimension

Fractal dimension (FD) of each microstructural image was calculated using a box counting method via the use of ImageJ software (version 1.47v, National Institutes of Health, Bethesda, MD). An image was equally divided into four sub-images and FD of each sub-image was determined; the final FD value of each image is then reported in terms

of the average value and standard deviation. This was performed to assign each image a numerical value, so that a statistical analysis could be performed to determine whether FDs of different images and hence the microstructures of different samples were statistically different (Niamnuy, Devahastin, & Soponronnarit, 2014).

2.10. Statistical analysis

The experimental data were subject to the analysis of variance (ANOVA) and are presented as mean values with standard deviations. Differences between the mean values were established using Duncan's new multiple range tests; the differences were considered at a confidence level of 95%. All statistical analyses were performed using SPSS® software (version 17, SPSS Inc., Chicago, IL). All experiments were independently performed at least in triplicate.

3. Results and discussion

3.1. Optimization of MW treatment condition

Normalized values of bioactive compounds contents and myrosinase activity are reported in this study to avoid unnecessary variation in the experimental data caused by the different batches of cabbages. Normalized glucoraphanin and sulforaphane contents are defined as the contents at any instant to those of the fresh cabbage outer leaves as shown in Eq. (3). Normalized myrosinase activity is defined as the activity at any instant to that in the fresh sample (Eq. (4)).

$$\text{Normalized bioactive compounds content} = \frac{\text{Instantaneous compound content (mg/g(d. b.))}}{\text{Compound content in fresh sample (mg/g(d. b.))}} \quad (3)$$

$$\text{Normalized myrosinase activity} = \frac{\text{Instantaneous myrosinase activity (μmol/min·g(d. b.))}}{\text{Myrosinase activity in fresh sample (μmol/min·g(d. b.))}} \quad (4)$$

The evolutions of the normalized glucoraphanin content, sulforaphane content and myrosinase activity in pretreated cabbages as well as that of the cabbage temperature during MW treatment are shown in Fig. 2. Normalized glucoraphanin content increased in almost all cases when the cabbages were subject to MW irradiation for 15 s. This is due to the rapid intracellular heating, which resulted in cell rupture and release of the compound (Pongmalai et al., 2015). The increase in the normalized glucoraphanin content during this period (15 s) indicated that the release rate of the compound from the cabbage matrix was higher than the rate of the enzymatic reaction that converted glucoraphanin into sulforaphane. However, higher MW powers seemed to result in the lower contents of glucoraphanin (Fig. 2a). The contents of glucoraphanin after MW treatments at 640 and 800 W for 15 s were similar to the initial value, probably because the released glucoraphanin from the cabbage matrix was converted to sulforaphane due to the increased activity of myrosinase (Fig. 2b). While it is true that a higher MW power could induce more damage to the cabbage structure, resulting in a higher release of glucoraphanin from the cells, higher rate of the hydrolysis reaction might also occur since myrosinase could as well be released at a larger extent from the more damaged cells.

The normalized myrosinase activity at 15 s of MW treatment at 160–480 W slightly increased when compared with the initial value (Fig. 2b). Although the temperature of cabbages during MW treatment (Fig. 2d) increased beyond the inactivation temperature of myrosinase (around 50 °C), exposure time and microwave power are reported to also affect the enzyme inactivation (Owusu-Ansah, & Marianchuk, 1991). As such, myrosinase might not be completely inactivated. In addition, inhomogeneous heating during the MW treatment might result in the non-uniform distribution of heat in the cabbages. Therefore, it is possible that active myrosinase could still exist in the pretreated cabbages, even after being treated at a higher temperature. These active enzyme could in turn induce the hydrolysis reaction of glucoraphanin. Because of the released glucoraphanin and remaining active myrosinase, an increase in the normalized sulforaphane content was observed after 15 s of MW treatment at 160–480 W (Fig. 2c). Although the rapid increase in the sample temperature could lead to degradation of sulforaphane, the period during which the cabbage temperature increased beyond the degradation temperature (of around 60 °C) was rather short. In addition, epithiospecifier protein (ESP), which is the myrosinase cofactor that results in the formation of undesirable sulforaphane nitrile instead of sulforaphane, might also be inactivated; note that the activation temperature of ESP is around 20–40 °C (Matusheski, Juvik, & Jeffery, 2004).

The highest activity of myrosinase was noted when MW at 640 W was applied for 15 s (Fig. 2b). The highest activity resulted in the maximum content of sulforaphane (around 6.23 times of the initial value, Fig. 2c). The highest content of sulforaphane in our pretreated cabbages was 1.1-fold higher than the value reported by Pérez, Barrientos, Román, & Mahn (2014) who investigated the effect of blanching on the content of sulforaphane in broccoli florets. Although a higher MW power (800 W) might have resulted in a more release of bioactive compounds and myrosinase from the cabbage cells, some inactivation of myrosinase might have occurred since the period during which the cabbage temperature reached and stayed beyond the myrosinase inactivation temperature was longer (Fig. 2d).

After 15 s of MW treatment, the normalized glucoraphanin content in the pretreated cabbages decreased in all cases, possibly due to the enzymatic hydrolysis reaction. Higher MW power led to a lower content of glucoraphanin (Fig. 2a), indicating that the released glucoraphanin might have been converted to sulforaphane. Note that an increase in the cabbage temperature over a short period of only 45 s was probably not long enough to cause degradation of glucoraphanin, since this compound is quite stable to heat (Pongmalai et al., 2017). MW treatment at 160 W for 30–45 s did not result in any significant differences in the normalized myrosinase activity, probably due to the use of low MW power over a shorter period of time. On the other hand, the treatments at 320–480 W for 30 and 45 s led to a continuous increase in the enzyme activity toward the end of the treatment (Fig. 2b). When the MW power increased to 640 and 800 W, decrease in the myrosinase activity was observed after treatment for 30 and 45 s (Fig. 2b), which indicated possible enzyme inactivation. Due to the decrease in the myrosinase activity, lower sulforaphane formation was observed when compared with the value obtained after 15-s treatment (Fig. 2c). Lower sulforaphane content might also be due to thermal degradation of the compound upon extended pretreatment, especially at a higher MW power.

From the above-mentioned arguments, MW treatment at 640 W for 15 s was noted to be able to enhance the formation of sulforaphane in cabbages by up to 6.23 times of the initial value in the fresh sample. The sample pretreated at this condition was therefore used for *in vitro* digestion tests as will be reported in the next sections.

3.2. In vitro digestion in soft rat stomach

3.2.1. Bioaccessibile sulforaphane content

Fig. 3a shows the normalized bioaccessible sulforaphane content, which is defined as the content after digestion at any time to that before digestion, and its accumulated amount before, during and after digestion. The content in the pretreated cabbages right after MW treatment (before digestion) was 1. After digestion for 15 min, the mixture (cabbages + digestive fluids) contained only a small amount of sulforaphane. Sulforaphane was nevertheless continuously released from the cabbage matrix until the end of digestion (135 min); the accumulated bioaccessible content of sulforaphane, which was the total amount of sulforaphane in all three collected samples (at 15, 75 and 135 min), was around 2.15. The digestion process also led to a gradually decreasing pH of the mixture (as also shown in Fig. 3a and will be discussed in the

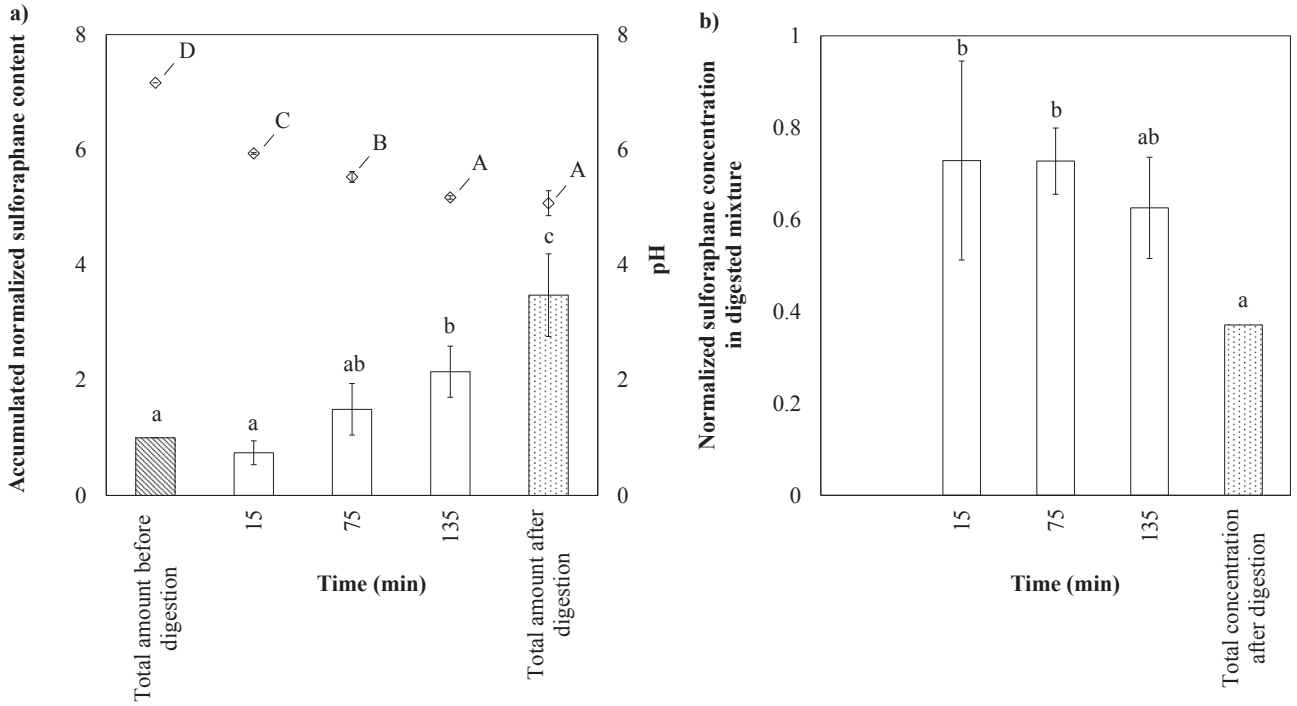

Fig. 3. (a) Normalized sulforaphane content and its accumulation, pH of the mixture (◇) as well as (b) normalized sulforaphane concentration in digested mixture before, during and after digestion in the in vitro rat stomach model. Same lowercase letters at the same treatment time and same uppercase letters at the same pH indicate that values are not significantly different ($p \geq 0.05$).

next subsection) and induced the softening of cabbage tissues. In addition, enzymatic reactions, catalyzed by a continuous feeding of fresh enzymes such as pepsin into the gastric juice, resulted in the breaking down of proteins in the cabbage tissues (Boarder, Newby, & Navti, 2010). These were expected to help facilitate the release of sulforaphane from the cabbage structure, resulting in an increase in its bioaccessible content.

Total sulforaphane content, which included sulforaphane in the digestive fluids and residual sulforaphane in the rat stomach after digestion was also investigated. The accumulated total amount of bioaccessible sulforaphane in the collected samples at 15, 75, 135 min and the residual sulforaphane in the rat stomach was 3.48 times higher than the value prior to the digestion (as shown in Fig. 3a). It should be noted that the accumulated amount of sulforaphane was calculated based on the amounts collected at four sampling points. During an experiment, around 1 mL of the reactant was allowed to naturally flow out of the stomach to mimic the *in vivo* digestion process. Experimental analysis showed that the normalized sulforaphane concentration in the efflux was 0.71 mg/mg/g on average, corresponding to a normalized sulforaphane content of around 0.71 mg/mg. As such, the total normalized released amount of sulforaphane should be 3.48 with the additional 0.71. Such an increase in the accumulated total amount of bioaccessible sulforaphane after digestion will be later explained via the changes in the cabbage microstructure upon digestion.

3.2.2. pH of mixture during digestion

The change in the pH of the mixture during digestion was investigated and is shown in Fig. 3a. The initial pH of the mixture of pretreated cabbages and saliva solution was around 7.16. Upon digestion, the pH of the mixture gradually decreased due to the continuous feeding of the gastric juice with a lower pH (around 1.63). The pH of the reactant reached 5.07 by the end of the *in vitro* digestion experiment. The decrease in the pH of the mixture resulted in the changes of the cabbage microstructure, facilitating the release of glucoraphanin and myrosinase from the cabbage matrix, and inducing the conversion of glucoraphanin into sulforaphane. Note that the range of a suitable pH for the hydrolysis reaction is around 5–7 (Redovniković, Glivetić, Delonga, & Vorkapić-Furač, 2008).

3.2.3. Normalized sulforaphane concentration in digested mixture

The values of the normalized sulforaphane concentration, which is defined as the normalized bioaccessible sulforaphane content in each sample over the total mass of the sample, during and after digestion are presented in Fig. 3b. Sulforaphane was steadily released from the cabbages, starting as early as 15 min after the digestion started. The release was quite stable until 135 min, which might reflect the actual release of sulforaphane during the *in vivo* digestion. On the other hand, the sulforaphane content in the digested sample decreased to a relatively low level when compared with the values at 15 and 75 min. This indicated that the release of sulforaphane content almost reached the maximum for the tested condition. Nevertheless, as mentioned earlier, the accumulated amount of sulforaphane in the four samples was higher than that extractable from the sample prior to the digestion by the standard dichloromethane method by 3.48 times.

3.3. In vitro digestion in shaking flask model

3.3.1. Bioaccessibile sulforaphane content

To unveil the mechanisms underlying the significant increase in the sulforaphane content during dynamic *in vitro* digestion, batch *in vitro* digestion in a shaking flask was conducted, as this simplified digestion system might help aid understanding of the changes in the cabbage structure as well as the formation and release of sulforaphane.

Fig. 4a presents the evolution of the normalized bioaccessible sulforaphane content before, during and after digestion in the shaking flask. The normalized content continuously increased until reaching its maximum value of around 3.71 when the digestion time was 75 min and pH of the mixture was around 5.67 throughout the digestion experiments. Although there was neither continuous feeding of gastric juice as in the case of the dynamic digestion process nor the feeding out of the digested mixture, the lower pH of the mixture as a result of the larger amount of gastric juice in the digestive system, along with the

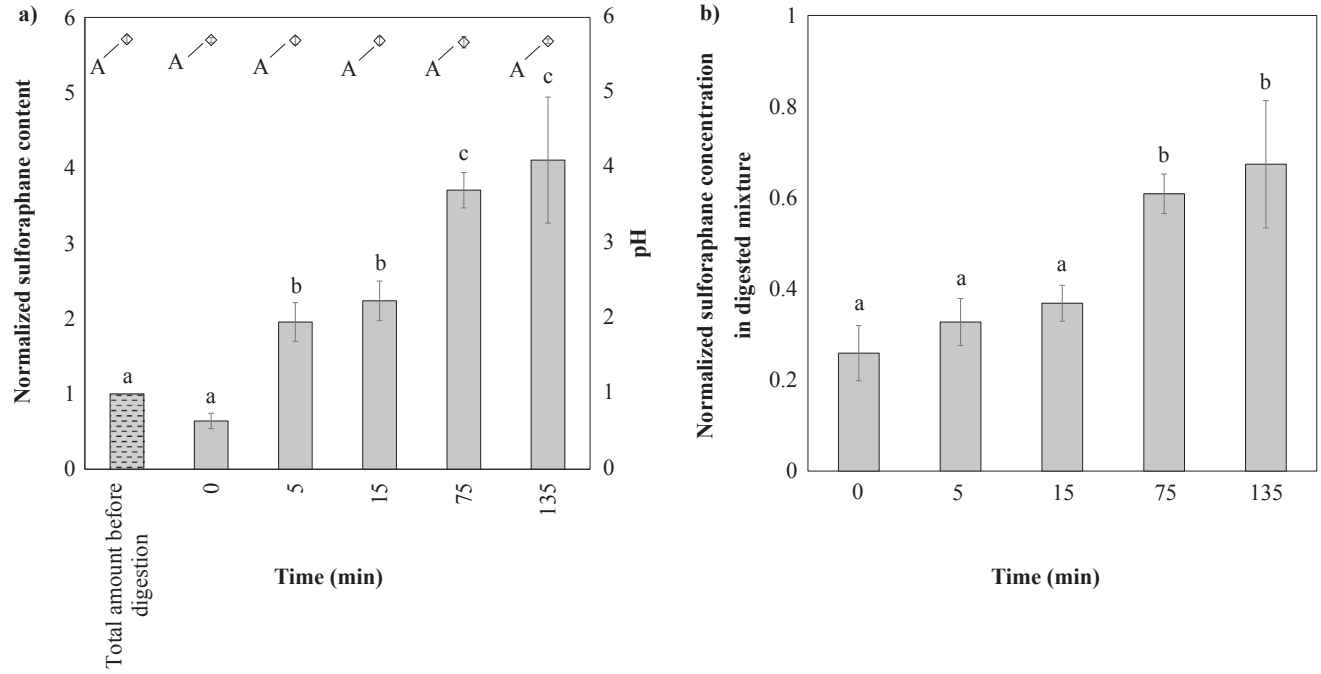

Fig. 4. (a) Normalized sulforaphane content, pH of the mixture (◇) as well as (b) normalized sulforaphane concentration in digested mixture before, during and after digestion in the shaking flask system. Same lowercase letters at the same digestion time and same uppercase letters at the same pH value indicate that values are not significantly different ($p \geq 0.05$).

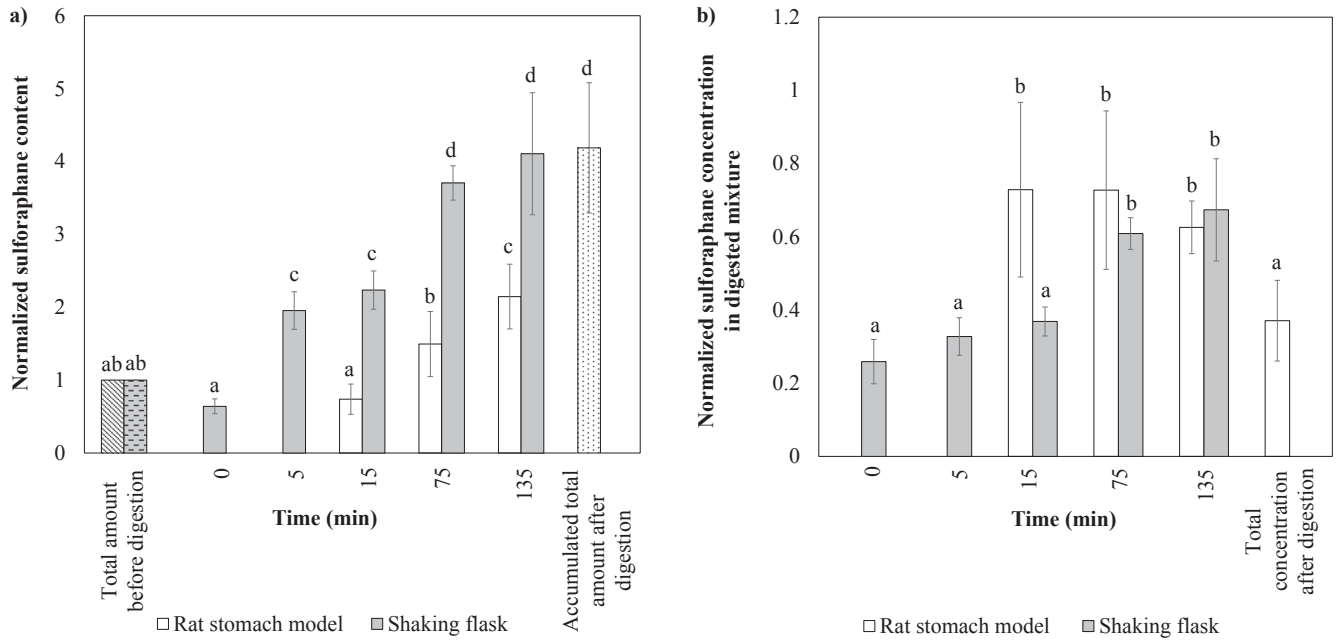

Fig. 5. Comparison of (a) normalized sulforaphane contents and (b) normalized sulforaphane concentration in digested mixture between rat stomach model and shaking flask. Same letters at the same digestion time indicate that values are not significantly different ($p \geq 0.05$).

larger amount of enzymes in the gastric juice as well as the mechanical process rendered by the shaker affected the disruption of cabbage cellular structure (as demonstrated later in Fig. 6d). These results corresponded with the data on the formation and release of sulforaphane. Our results exhibited a similar trend to those of Trancoso-Reyes et al. (2016) who observed the effect of MW pretreatment on physicochemical and structural properties as well as bioaccessibility of β-carotene in dried sweet potato flour. MW treated samples exhibited higher bioaccessibility of β-carotene when compared with the control flour. This was attributed to the more damaged cellular structure of the MW-treated flour, which allowed easier access of the digestive enzymes and hence the enhanced bioaccessibility during digestion.

The existence of myrosinase activity in the MW-pretreated cabbages might also lead to the conversion of glucoraphanin into sulforaphane during the digestion. Similar observation was made by Sarvan, Kramer, Bouwmeester, Dekker, & Verkerk (2017) who investigated the bioaccessibility of sulforaphane in steamed broccoli during *in vitro* digestion. Increased sulforaphane formation in steamed broccoli was observed during gastric digestion. The initial short period to adjust the pH of the sample to gastric condition might be sufficient for the hydrolysis of glucoraphanin by the remaining myrosinase. However, myrosinase was later inactivated at the lower pH; this inactivation was not reversible.

After 75 min the normalized bioaccessible sulforaphane content showed a slight increase until the end of the digestion (4.11 at

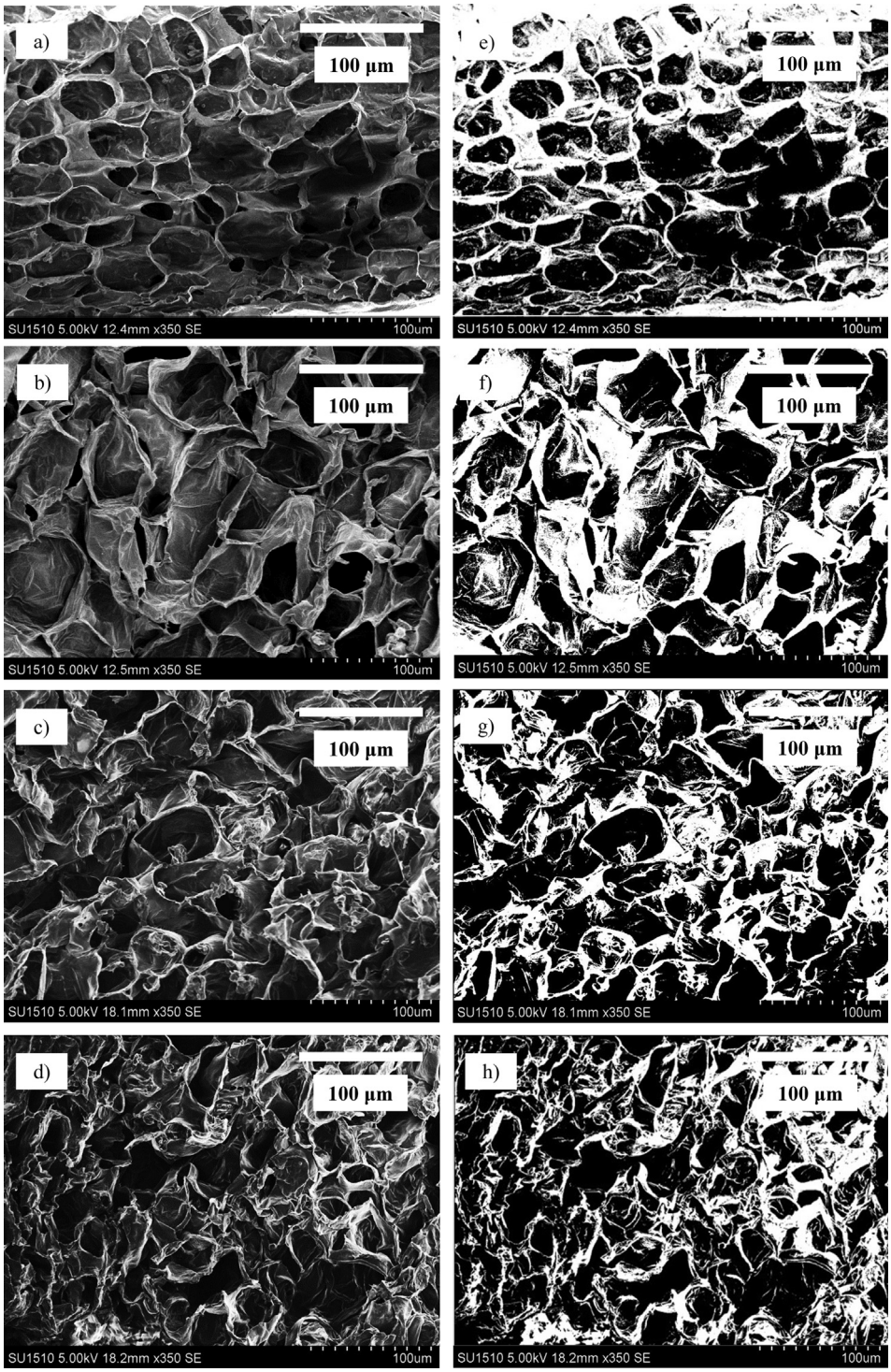

Fig. 6. Cross-section SEM images of cabbage microstructures before/after in vitro digestion (a–d) and their corresponding binary images (e–h). (a) Fresh cabbages; (b) cabbages undergone MW treatment (before digestion); (c) cabbages after digestion in the in vitro rat stomach model; (d) cabbages after digestion in shaking flask at 75 min. FDs of images (e) to (h) are 1.86 ± 0.01a, 1.87 ± 0.00b, 1.89 ± 0.01c and 1.90 ± 0.00c, respectively. Same letters indicate that values are not significantly different ($p \geq 0.05$).

135 min), but the value was not statistically different from that at 75 min. This is probably because the sulforaphane that was released into the digestive solution might become saturated; no driving force for further mass transfer then existed.

3.3.2. Normalized sulforaphane concentration in digested mixture

Fig. 4b presents the normalized sulforaphane concentrations during and after digestion via the use of the shaking flask. Sulforaphane was gradually released from the cabbage structure into the digested mixture during 15 min of the digestion. The normalized sulforaphane concentration reached its maximum value at 75 min; the concentration then remained almost unchanged until the end of the digestion.

3.4. Comparison between rat-stomach and shaking-flask digestion

Fig. 5a presents the normalized bioaccessible sulforaphane content and its accumulation before, during and after digestion in the soft rat stomach in comparison with those obtained from the shaking flask. The

maximum normalized bioaccessible sulforaphane content after rat-stomach digestion (of around 3.48–4.19) was not significantly different from that after shaking-flask digestion (around 3.71–4.11). The similar values might be due to the soft texture of cabbages, which are composed of small contents of polysaccharides and proteins that can be easily digested even during the simplified shaking-flask process.

The maximum normalized sulforaphane concentration after rat-stomach digestion was also not significantly different from that after shaking-flask digestion as shown in Fig. 5b. Nevertheless, it is important to note that during rat-stomach digestion sulforaphane was steadily released into the digested mixture and reached the maximum concentration only after 15 min of the digestion, which may better represent the *in vivo* digestion. The released sulforaphane during shaking-flask digestion was, on the other hand, gradually accumulated in the reactant mixture. As digestion progressed further, the released sulforaphane during shaking-flask digestion reached the similar maximum concentration at 75 min. Although the total sulforaphane yield was similar, the distinct release profiles demonstrated the difference between a continuous release process and a batch reaction process. The release profile may be an important factor to consider during certain digestion processes, for example, the release of glucose in functional foods for diabetes, or the controlled release of active drug ingredients through oral administration.

3.5. Microstructural changes of cabbages

SEM images of fresh and MW treated cabbages as well as those of the digested cabbages (both via digestion in the rat stomach and batch shaking flask) are shown in Fig. 6. Pretreated cabbages before digestion exhibited slight cell rupture with slightly more open-porous structures (Fig. 6b) compared with the fresh sample (Fig. 6a). This was expected as MW is known to cause cellular disruption due to rapid evaporation of cellular water (Pongmalai et al., 2017). After digestion in the rat stomach, more collapse of the cellular structure and more open pores with irregular shape were observed (Fig. 6c). Enzymes in the digestive solution and the dynamic digestion process might have led to these changes as mentioned in Section 3.1. In the case of digestion carried out in the shaking flask, the cell structure suffered notable damages after 75 min (Fig. 6d) when compared with the sample before digestion.

FD of each microstructural image was calculated to quantitatively evaluate the microstructural changes of cabbages after digestion in comparison with that of the pretreated sample before digestion. The corresponding binary images used for the FD calculation are illustrated in Fig. 6e–h. FD of the fresh and pretreated cabbages before digestion was around 1.86 ± 0.01 and 1.87 ± 0.00, respectively. FD values of the samples after digestion via rat stomach model and shaking flask significantly increased to 1.89 ± 0.01 and 1.90 ± 0.00, respectively. FD of the images of cabbage microstructure after shaking flask digestion was not significantly different from that after rat stomach digestion. The increase in the FD corresponded to the disruption of cabbage microstructure by MW irradiation and the result of the normalized sulforaphane content as reported in previous sections.

4. Conclusions

The evolutions of the normalized glucoraphanin content, sulforaphane content and myrosinase activity in pretreated cabbages as well as that of the cabbage temperature during MW treatment to increase the sulforaphane content in cabbages were investigated. *In vitro* digestion via dynamic soft rat stomach model and batch shaking flask was then performed to monitor the bioaccessible sulforaphane content during and after digestion.

MW treatment at 640 W for 15 s helped enhance the formation of sulforaphane in cabbages by up to 6.23 times of the initial value in the fresh sample due to the released glucoraphanin and myrosinase with the highest activity from the cabbage structure. *In vitro* soft rat stomach model resulted in the increase in the accumulated total amount of bioaccessible sulforaphane up to 3.48–4.19 times in comparison with the value prior to digestion. This content was nevertheless not significantly different from that obtained in the shaking flask of around 3.71–4.11, probably because of the soft and easily digested texture of cabbages. Nevertheless, sulforaphane concentration in the digested mixture during rat-stomach digestion became constant after 15 min, while that in the shaking flask reached the same concentration level only after 75 min. Cabbages undergone digestion in the rat stomach model and shaking flask suffered similar damage; the results therefore well supported the obtained digestion data.

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