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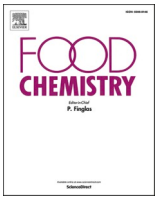

Compartmentalization vs. segregation of reactants: Accomplishment of the Maillard reaction at the water-water interface

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ABSTRACT

All-aqueous (water-in-water) emulsions are increasingly used as droplets reactors. The present communication reports that precursors of a reaction segregated by partitioning between emulsion phases can undergo reaction at the interface, *i.e.*, on droplet surface, while the interface remains liquid. Na_2SO_4-in-polyethylene glycol (PEG) emulsions were prepared, and precursors (glucose, asparagine, and tryptophan) of the Maillard reaction were partitioned either inside the droplets (co-encapsulation) or segregated between the emulsion interior and exterior phases. It was found that following the interfacial (*i.e.*, on-droplet) reaction of the segregated precursors, ~99 % of the Amadori product *N*-(1-deoxy-D-fructos-1-yl)-L-tryptophan (Fru-Trp) partitioned into the PEG phase. Also, hydrophobic advanced reaction products including β-carboline derivatives and Strecker aldehyde, alongside melanoidins, showed a clear affinity towards the PEG phase. Once the precursors were co-encapsulated within Na_2SO_4 droplets, following their generation succinimide and pyridine derivatives remained partitioned within the droplets, whereas *N*-hydroxysuccinimide, pyrrole derivatives, and melanoidins predominantly partitioned into the PEG phase.

1. Introduction

A droplet reactor is classically defined as a reaction medium confined inside a microdroplet. Droplets can be produced in different forms (sphere, slug, and plug) and formats (liquid-liquid, liquid-solid, and liquid-gas) (Wei et al., 2020). Liquid-liquid droplet reactors are by far the most investigated type of droplet reactors, and their production is essentially an emulsification process, *i.e.*, droplets are generated and kept suspended in an immiscible phase. Both large-scale emulsification and microfluidic techniques are applied for generating droplets in different configurations including single water-in-oil and oil-in-water emulsions (Zhang et al., 2023). Synthetic surfactants are usually required to decrease the interfacial tension at the aqueous-organic interface between the two phases and facilitate the disperse phase miniaturization. Alternatively, Pickering emulsions, the emulsions stabilized by solid colloidal particles instead of synthetic surfactants, have been used to run (bio)chemical reactions, such as enzymatic esterification and transesterification (Dong et al., 2019; Wang et al., 2017).

In recent years, the possibility of using all-aqueous emulsions in the context of liquid-liquid droplet reactors emerged. All-aqueous (water-in-water) emulsions are produced by size reduction of one of the phases of an aqueous two-phase system (ATPS) within the pairing immiscible phase. The ultralow interfacial tension at the water-water interface is in the range of 1–1000 μN/m (Wang et al., 2022), several orders of magnitude smaller than that at oil-water interfaces. Accordingly, size reduction (*i.e.*, droplet formation) is readily achieved by shaking/stirring at bulk scale and perturbation by pneumatic valves of aqueous-aqueous jets in microfluidic channels (Wei et al., 2020). The paired use of natural fats as the exterior phase and ATPS as the interior phase for accomplishment of a lipase-catalyzed hydrolysis at the oil-water interface has been reported (Nie et al., 2024). All-aqueous droplet reactors are relatively greener alternatives to aqueous-organic droplet reactors and can be utilized for the accomplishment of a variety of (bio) chemical reactions eliminating the use of (synthetic) surfactants and organic solvents. These reactors have been used for running coupled enzyme reactions and biocatalyzed mineralization (Aumiller Jr. et al.,

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2014; Cacace & Keating, 2013; Song et al., 2016) as well as compartmentalized cell-like bioreactors (Fuentes-Lemus et al., 2021). All-aqueous emulsions can mimic the macromolecularly crowded cytosol of cells thanks to their molecularly crowded interior phase and the exterior phase.

The Maillard reaction (MR), also known as non-enzymatic browning, is a condensation reaction that starts with the nucleophilic attack of amino acids to the electrophilic carbonyl group of carbohydrates and lipid-derived intermediates (Thorpe & Baynes, 2003). MR can take place when biological substances are exposed to high temperatures and low water activities, such as in food, and in humans due to the high concentration of sugar and amino acids present. The MR products (MRPs) significantly contribute to the flavor and color of (heat-treated and roasted) food products. MRPs have higher reducing power, taste-enhancing, and emulsifying activities (Klinchongkon et al., 2019; Shi et al., 2019). There is a great interest to generate, isolate and add the MRPs into foods to get specific techno-functional or sensory attributes. The MR also has a negative side; it may cause formation of probably carcinogenic acrylamide and mutagenic heterocyclic aromatic amines (Nowotny et al., 2018). Accordingly, it is necessary to mitigate the formation of potentially hazardous compounds, and to favor the selective production of the desired MRPs.

Along with a large amount of literature on the MR chemistry in single-phase aqueous solutions and dry or solid systems, liquid-liquid droplet reactors based on oil-water emulsions have been considered as microreactors for running the MR and generating miscellaneous products (Newton et al., 2015; Troise et al., 2016; Troise et al., 2020). Oil-water droplet reactors enable partitioning of reactants within the emulsion structure, which has an influence on the frequency of collisions between the reactants and on the reaction pathways (Fanun et al., 2001). The capacity of oil-water emulsions to modulate the spatial location of precursors allows to control the MR. However, the use of organic solvents or edible oils, also synthetic low-molecular weight surfactants that are commonly required in making oil-water emulsions is not always desired. While edible oils are generally safe for consumption, their application for the accomplishment of the Maillard reaction at elevated temperatures can lead to oxidation, producing off-flavors, harmful radicals, and altering the Maillard reaction pathways (Awada et al., 2012; Berton-Carabin et al., 2014; Villière et al., 2007; Zamora & Hidalgo, 2005).

All-aqueous emulsions have ability to partition guest molecules between emulsion phases. These emulsions have been widely used for extractive bioconversion, a process where enzymes and their substrates are co-encapsulated within the interior phase, *i.e.*, droplets, of all-aqueous emulsions, and enzymatic reaction products once generated are partitioned into the exterior phase (Madadlou et al., 2020). In addition to compartmentalized reactions, *i.e.*, reactions within the droplet phase, all-aqueous emulsions favor the occurrence of specific reactions at the interface between the two aqueous phases. This possibility is not yet fully investigated and interface solidification through the electrostatic complexation of oppositely charged entities, such as counter charged polyelectrolytes (Hann et al., 2016; Ma et al., 2016; Qu et al., 2019), biopolymers (Jiang et al., 2022), and nanoparticles (Shekhar et al., 2021), is the major case for exploitation of the aqueous-aqueous interface in different disciplines of technology. Accomplishment of an enzyme-assisted oxidation reaction at the liquid-standing aqueous-aqueous interface of a macroscopically phase-separated ATPS is worth noting (Aumiller Jr. et al., 2014).

The objective of the present study was to verify the possibility of expanding the frontiers of all-aqueous droplet reactor technology. This was achieved by investigating the development of the MRPs along with the reaction products partitioning within all-aqueous emulsions comprising polyethylene glycol (PEG) and Na_2SO_4. The MR was run either compartmentalized by co-encapsulating the Maillard reaction precursors in the emulsion interior phase, or interfacially by segregating the precursors between the two phases of the emulsions. The co-encapsulation and segregation of the reaction precursors relied on their affinity towards either of the emulsion phases.

2. Materials and methods

2.1. Materials

D-Glucose, L-tryptophan, L-asparagine, polyethylene glycol (PEG) 8 kDa, sodium sulfate (Na_2SO_4), phosphate buffer powder, and fluorescein isothiocyanate-dextran conjugate (FITC-labelled dextran) 500 kDa were purchased from Sigma-Aldrich (Amsterdam, The Netherlands). *N*-(1-deoxy-D-fructos-1-yl)-L-tryptophan (Fru-Trp) and *N*-(1-deoxy-D-fructos-1-yl)-L-asparagine (Fru-Asn) were purchased from Toronto Research Chemicals (Toronto, Canada). All chemicals used in this study were analytical grade, except for liquid chromatographic solvents that were of mass spectrometry grade.

2.2. Preparation of all-aqueous droplet reactors and the reactants partitioning

Phosphate buffer stock solution (0.1 M, pH 7.4) was prepared by reconstituting 8.60 g phosphate buffer powder within 500 mL Milli-Q® water. Then, aqueous stock solutions of Na_2SO_4 (15 wt%) and PEG (30, 40, and 50, wt%) were prepared by dissolving each of the compounds within the phosphate buffer solution. All-aqueous droplet reactors (emulsions) were prepared by mixing the Na_2SO_4 and PEG stock solutions at a volume ratio of 1:4 at 1000 RPM, for 1 h. Next, the Maillard reactants *i.e.*, glucose, tryptophan, and asparagine powders were added separately into the emulsion reactors, followed by stirring the samples overnight. The emulsion samples were subsequently allowed to phase separate (no stirring) at room temperature (25 °C) for 4 h.

After phase separation, glucose was quantified using a glucose oxidase/peroxidase assay kit, and the amino acids were quantified by the *o*-phthaldialdehyde method (Nielsen et al., 2001) at both phases.

The partition coefficient (K) of the reactants was calculated by:

$$K = C_{top}/C_{bottom} \quad (1)$$

where C_{top} and C_{bottom} represent the concentrations of the reactant at the top (PEG) and bottom phases (Na_2SO_4), respectively.

The partition extent (E_{PEG}) at the PEG phase was calculated as:

$$E_{PEG} = C_{top} \times V_{top} / \left(C_{top} \times V_{top} + C_{bottom} \times V_{bottom}\right) \quad (2)$$

where C denotes reactant concentration, V represents phase volume, and the subscripts correspond to the top and bottom phases.

2.3. Microscopic imaging of the emulsion droplets, droplet size analysis, and determination of aqueous two-phase diagrams

The Na_2SO_4 stock solution (15 wt%) was supplemented (0.25 mg/mL) with FITC-labelled dextran and mixed with the PEG stock solution (50 wt%) at a volume ratio of 1:4. The mixture was stirred at 1000 rpm for 1 h. Subsequently, the emulsion was imaged on glass slides by using a fluorescence microscope (Leica DMi8, Leica Microsystems, Wetzlar, Germany) working at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. The imaging was carried out to determine the type of the all-aqueous emulsions, *i.e.*, Na_2SO_4-in-PEG or PEG-in-Na_2SO_4. The concentration of FITC-labelled dextran at the top and bottom phases was measured by a luminescence spectrophotometer (LS50B, Perkin Elmer, Waltham, MA) and using a standard curve.

The droplet size distribution was determined by the ImageJ (NIH, Bethesda, MD) software. Two replicates of emulsion reactors were freshly prepared, and the droplet size of 100 randomly selected droplets was measured per replicate.

The binodal curve of PEG-Na_2SO_4 mixtures was plotted at 95 °C using the cloud point method (Hatti-Kaul, 2008). An 18 wt% aqueous stock

solution of Na_2SO_4 and a 40 wt% aqueous stock solution of PEG 8000 were prepared to determine the phase diagrams.

2.4. Running the MR in emulsion reactors and single-phase solutions

Na_2SO_4-in-PEG emulsions were supplemented with the Maillard reactants (glucose and either asparagine or tryptophan) at a final concentration of 40 mM. The emulsions were stirred overnight at 25 °C for complete dissolution of the reactants. In addition to the emulsion reactor samples (two-phase systems), single-phase solutions including phosphate buffer solution, and mixtures of phosphate buffer solution and either of Na_2SO_4 or PEG stock solutions (at volume ratios comparable to the emulsions) were prepared and supplemented with the reactants.

The reactant-supplemented emulsions (and single-phase solutions) were poured (5 mL) into gas chromatography vials (20 mL), and the vials were sealed. Then the samples were heated at 95 °C for 0–5 h while being stirred at 1000 RPM. Next, the samples were cooled within an ice bath and stored at 25 °C for 4 h to let complete phase separation. The phase-separated top and bottom phases were diafiltrated at 14,000 ×g 5 times (30 min each time, molecular weight cut-off 3000 Da) for maximizing the recovery of the reaction products smaller than 3 kDa and removing PEG. The permeate was collected and freeze-dried (Alpha 2–4 LD plus, Martin Christ, Osterode am Hartz, Germany) until analysis. For comparison purposes, the emulsion and PEG solutions supplemented with the Maillard reactants were stored at 25 °C for 0 h to 6 h while being stirred and then subjected to diafiltration and analysis.

2.5. Quantification of sugars, amino acids, and Amadori products

The concentration of glucose and fructose at the top and bottom phases of the emulsions in subsequent to their phase separation were determined using ultra-performance liquid chromatography combined with evaporative light scattering detection (UPLC-ELSD) (Schouten et al., 2022). Samples were diluted 10 times with the solution water/acetonitrile (50/50, *v*/v), filtered, and then injected into a UPLC system (Waters Co., Milford, MA) equipped with a Waters ACQUITY UPLC BEH Amide column (1.7 μm, 100 mm × 2.10 mm). The injection volume was 1.3 μL and the column temperature was kept at 35 °C. The mobile phase consisted of acetonitrile/water (80/20, v/v) with 0.2 % TEA (solvent A) and acetonitrile/water (30/70, v/v) with 0.2 % TEA (solvent B); sugars were separated through the following gradient (t in [min]/[%A]): (0.00/100), (6.00/40), (6.01/100), and (18.00/100), with a flow rate of 0.25 mL/min. External standard procedure was used for quantitation. The pressure of ELSD conditions was 40 psi with a drift tube temperature of 40 °C and a data rate of 10 pps.

Tryptophan, asparagine, and their Amadori products were quantified by a Nexera U-HPLC system coupled with a LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan), equipped with an electrospray ionization interface. Two different chromatographic separations were used for amino acids and Amadori products, while tandem mass spectrometry conditions were applied as previously reported by Zhang et al. (2021) with some modifications (see below). In both cases, the samples were diluted (5000 times and 100 times for amino acids and Amadori products analysis, respectively) with water/acetonitrile (50/50, *v*/v), filtered using cellulose acetate filters (0.20 μm, Phenomenex, Torrance, CA) and then injected by a SIL-30 AC autosampler (Shimadzu Corporation, Kyoto, Japan) at 4 °C.

For tryptophan and asparagine analysis, 5 μL of samples were injected into a SeQuant® ZIC HILIC 3.5 μm, 4.6 × 150 mm attached to a SeQuant® ZIC HILIC PEEK coated guard column 20 × 2.1 mm (Merck KGaS, 64,271, Darmstadt, Germany). The flow rate was set at 0.7 mL/min, and the column temperature was 40 °C. The chromatographic separation of tryptophan and asparagine was achieved by 0.1 % formic acid (solvent A) and acetonitrile with 0.1 % formic acid (solvent B) through the following gradient (t in [min]/[%B]): (0.0/90), (4.0/70), (10.0/20), (13.0/20), (15.0/90), and (18.0/90).

For the quantification of Fru-Trp and Fru-Asn, the sample (5 μL) was injected into a core-shell Kinetex HILIC column (2.6 μm, 2.1 mm × 100 mm, Phenomenex) thermostated at 30 °C, with a flow rate of 0.4 mL/min. The mobile phases consisted of 0.1 % formic acid (solvent A), 0.1 % formic acid in acetonitrile (solvent B), and 50 mmol/L ammonium formate (solvent C). The gradient was as follows (t in [min]/[%B]): (0.0/80), (3.5/40), (6.5/40), (8.0/80), and (11.0/80). Solvent C was kept at 10 % to maintain ionic strength.

Positive ionization multiple reaction monitoring (MRM) mode was used for the tandem MS analysis of both chromatographic separations. The spray voltage used for amino acids and Amadori products analysis was 4.0 kV and 3.0 kV, respectively. Amino acids (dwell time, 4 ms) and Amadori products (dwell time, 25 ms) were analyzed using the collision energies (CE), the mass transitions and in bold the transition used for the quantitation (quantifier ions) in parentheses: tryptophan (*m/z* 205 ➔ **188**, 146, CE: 12 and 18), asparagine (*m/z* 133 ➔ 87, **74** CE: 12 and 15), Fru-Trp (*m/z* 367 ➔ 229, **349,** CE: 15 and 12), Fru-Asn (*m/z* 295 ➔ 211, **259,** CE: 16 and 12). Along with qualifier ions, the other transitions were used for the structural confirmation based on the optimized fragmentation patterns. Profile data were acquired and analyzed through LabSolutions (Shimadzu Corporation, Kyoto, Japan).

2.6. Liquid chromatography-high resolution mass spectrometry (LC-MS/MS) for the detection of advanced products

The formation of advanced markers of asparagine and tryptophan degradation was screened in targeted mode in emulsion systems after phase separation. Both upper and lower phases were diluted in 50 % aqueous acetonitrile then centrifuged (18,000 x*g*, 4 °C, 10 min). LC-MS/MS data were acquired using an Exploris 120 quadrupole Orbitrap high-resolution mass spectrometer interfaced to a Vanquish Core liquid chromatographic system (Thermo Fisher Scientific, Bremen, Germany). A list of putative chemical structures arising from the reaction between asparagine and glucose, tryptophan and glucose along with their degradation products was built in Compound Discoverer and TraceFinder environment (Thermo Fisher Scientific). Target compounds were separated at 35 °C through a zwitterionic sulfobetaine column (Atlantis Premier BEH, *Z*-HILIC, 100 × 2.1, 1.7 μm, Waters) with the following gradient of solvent B (minutes/%B): (0/5), (1.5/5), (10/50), (13/50). Mobile phases consisted of 0.1 % formic acid in acetonitrile (solvent A) and 0.1 % formic acid in water (solvent B) and the flow rate was 0.2 mL/min. For positive ion mode, H-ESI interface parameters were as follows: static spray voltage 3.3 kV, ion transfer tube and vaporizer temperature were both at 280 °C; sheath gas flow and auxiliary gas flow were 30 and 15 arbitrary units, respectively. Glycation, oxidation and fragmentation end-products were identified in product ion scan mode screening the precursor ions according to an in-house mass list generated in Trace Finder (v. 5.1, Thermo Fisher Scientific, Waltham, MA). For product ion scan, normalized collision energy was set to 35 %, Orbitrap resolution at 60,000 (FWHM at *m/z* 200) and the quadrupole resolution was set at 1.

2.7. Color development

The glass vials containing emulsion and single-phase samples were photographed after phase separation.

2.8. Statistical analysis

The results were analyzed using SPSS Statistics 28.0 (IBM software, New York, NY). Analysis of Variance (ANOVA) testing at a significance level of $P < 0.05$ was applied to test for significant differences between the samples.

3. Results and discussion

3.1. Amino acids partitioning in all-aqueous emulsions

The amino acids asparagine and tryptophan were dissolved in parallel within the emulsions prepared by mixing Na_2SO_4 (15 wt%) and PEG 8000 (30, 40, and 50, wt%) solutions at a volume ratio of 1:4, followed by determining the amino acids partitioning between the emulsion phases. A set of preliminary experiments showed that the higher the PEG concentration, the lower was the partition coefficient (K) of asparagine (see Fig. S1). When a PEG solution of 50 wt% was used in emulsion preparation, asparagine concentration in the Na_2SO_4 phase was approximately 12.5 folds higher than that in the PEG phase. On the contrary, the partition coefficient (K) of tryptophan increased with increasing PEG concentration; when a PEG solution of 50 wt% was used, the partition coefficient of tryptophan was about 6, and approximately 97 % of the added tryptophan into the emulsion partitioned within the PEG phase. Under this condition, tryptophan concentration in the PEG phase was 43.2 ± 0.2 mM, which was comparable to the concentration (40 mM) added into the whole emulsion. The difference in the preferential partitioning of the amino acids between the emulsion phases was attributed to the difference in their hydrophobicity. LogP (logarithm of octanol-water partition coefficient) values of tryptophan and asparagine are −1.06 and − 3.82, respectively (Chmelík et al., 1991), (Hansch et al., 1995). Based on these data, a PEG solution of 50 wt% and a Na_2SO_4 solution 15 wt% were used for the following experiments.

Partition coefficient (K) of glucose was 0.21 ± 0.03, indicating that glucose concentration in the Na_2SO_4 phase was roughly 5 times higher than that in the PEG phase (Table S1). In summary, in the conditions used, tryptophan very efficiently partitioned into the exterior PEG phase, whereas glucose and asparagine significantly partitioned into the interior Na_2SO_4 (droplet) phase.

It is worth noting that the partitioning values are at 25 °C, and the molecules partitioning at 95 °C (temperature used to run the MR experiments) could be different. Hydrophobic interactions between the reactants and PEG mainly governed the partitioning of the reactants, and the hydrophobicity of PEG increases with increasing temperature (Dormidontova, 2002). We expected that the partition coefficient of tryptophan would increase, whereas those of asparagine and glucose would decrease at higher temperatures compared to the measured values (Fig. S1 and Table S1). Running the MR at 95 °C could enhance the compartmentalization of glucose and asparagine within the droplets

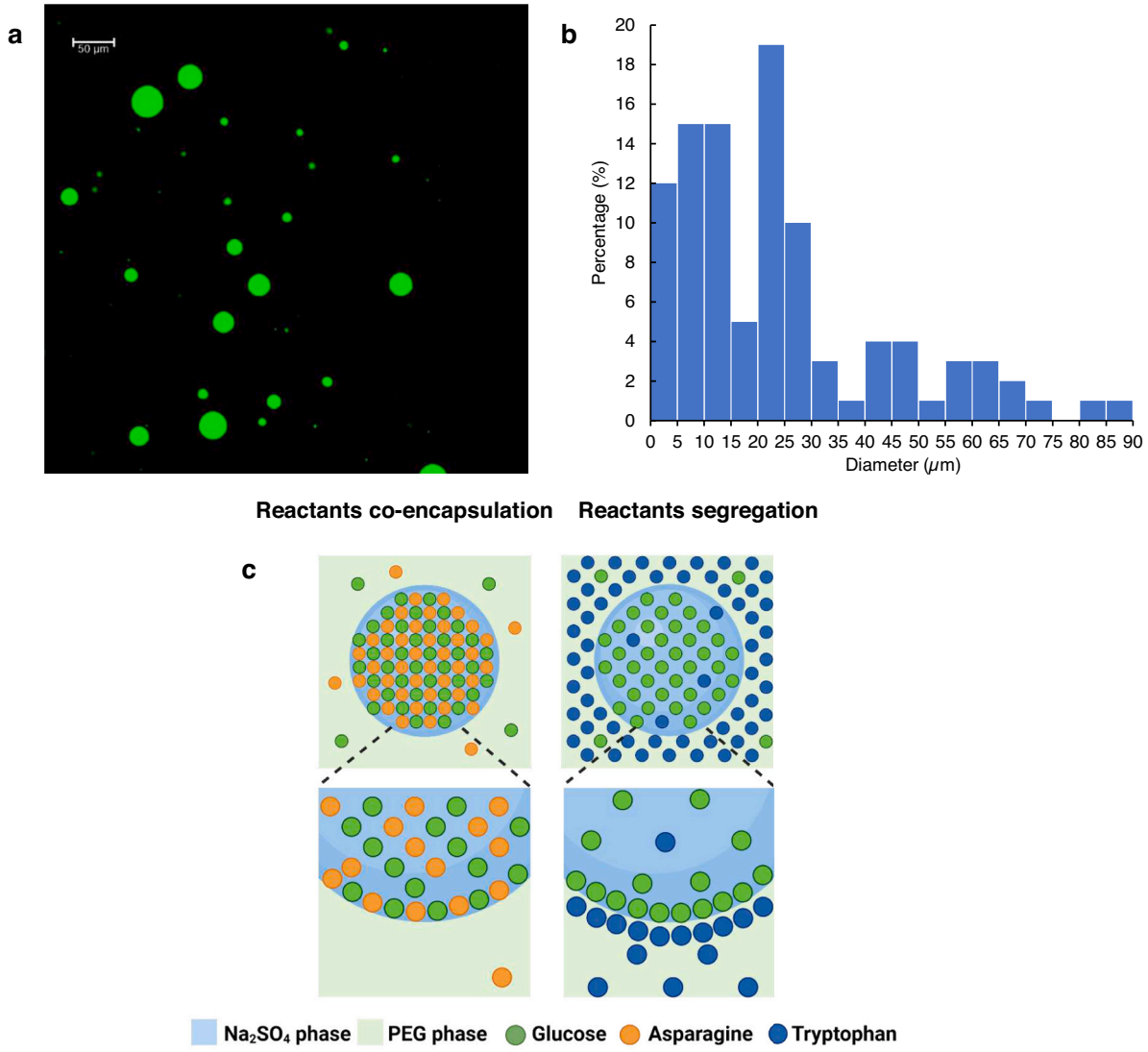

Fig. 1. A representative epi-fluorescent microscopy image (a); droplet size distributions of Na_2SO_4-in-PEG emulsions, with average droplet diameter of 23.30 ± 18.88 μm (b); and a schematic illustration of co-encapsulation and segregation of reactants in Na_2SO_4-in-PEG emulsions for accomplishing the Maillard reaction (c). The partition coefficient (K) of FITC-labelled dextran indicated that it preferentially partitioned within the Na_2SO_4 phase of the emulsion.

and preferential partitioning of tryptophan into the exterior PEG phase, further favoring the reactants segregation. Higher temperatures are also favorable for ATPS formation of polymer-electrolyte mixtures because polymer-water interactions decrease by increasing temperature (Chen et al., 2023). The binodal curve of PEG-Na_2SO_4 mixtures at 95 °C (Fig. S2) indicates that the transition from single-phase to two-phase systems took place at lower concentrations of the comprising components compared to those reported by González-Amado et al. (2016) at 20 °C and 35 °C. The change in the phase boundary transition as a consequence of temperature is also consistent with the observation by

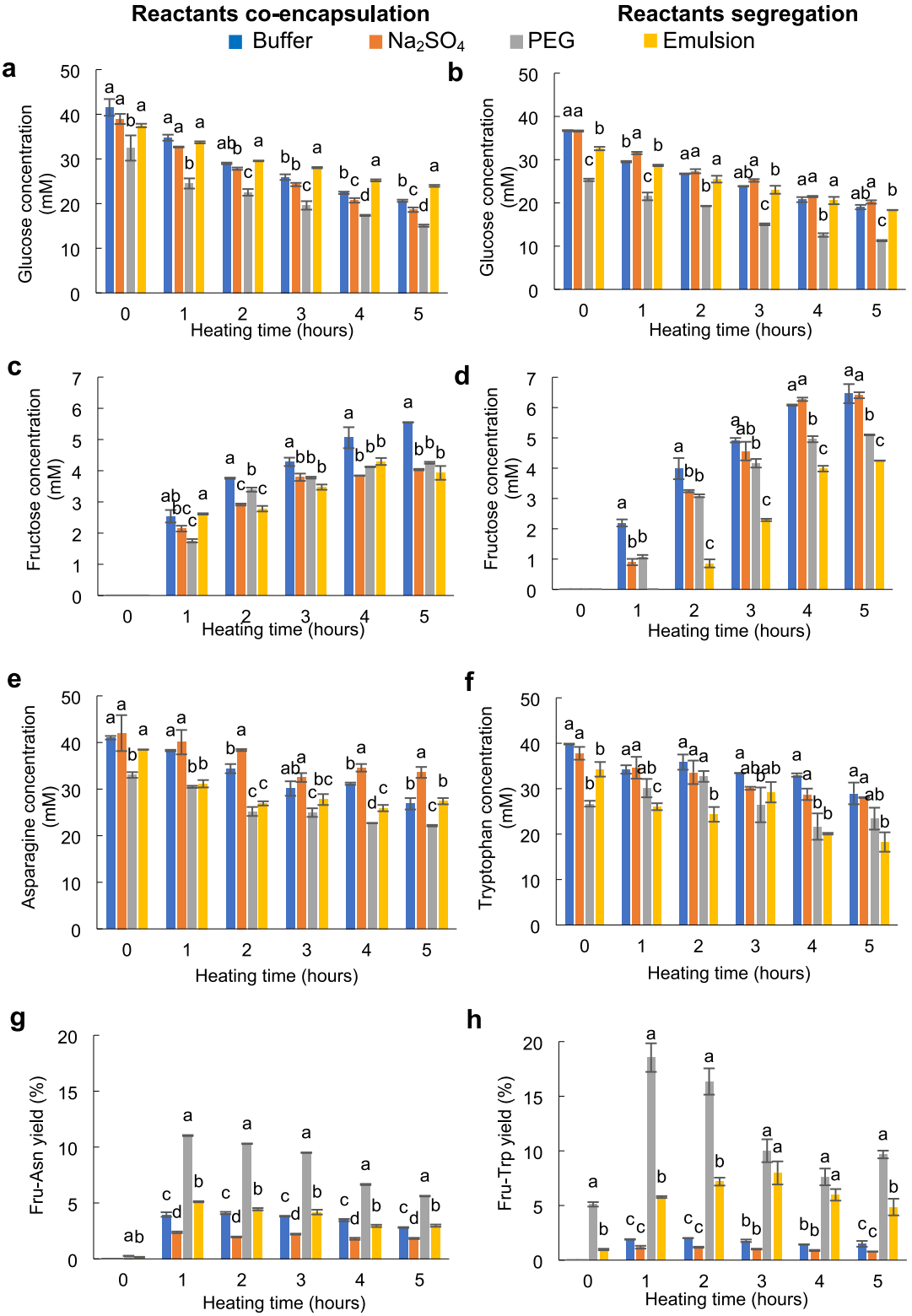

Fig. 2. The concentration of the Maillard reaction precursors (glucose, and either asparagine or tryptophan), glucose isomerization product, *i.e.*, fructose, and yield of Amadori products in all-aqueous emulsions and single-phase solutions after 0–5 h of the Maillard reaction. The concentration of precursors was 40 mM. Different letters indicate the difference in reaction media ($P < 0.05$).

González-Amado et al. (2016) that increasing the temperature from 20 °C to 35 °C extended the biphasic region of PEG8000-Na_2SO_4 systems.

3.2. Characterization of the droplet phase in the emulsions

The adopted experimental conditions were expected to result in an ATPS where Na_2SO_4 and PEG make the interior (droplet) and exterior phases of the emulsions, respectively. This was confirmed by epifluorescence microscopy imaging using FITC-labelled dextran as probe.

(Fig. 1a). The partition coefficient (K) of FITC-labelled dextran was $\sim 2.97 \times 10^{-3}$, therefore, it partitioned into the Na_2SO_4 phase of the emulsions. The droplet size data reported in Fig. 1b show a remarkable heterogeneity because in our study the emulsions were not stabilized, and the droplets underwent coalescence during sample preparation for microscopic imaging. It is worth reminding that over the course of the MR the emulsions were kept stirred, thus the droplets did not coalesce, enabling us to run the reaction either as compartmentalized (co-encapsulation) or segregated (touching at the interface) (Fig. 1c).

3.3. The role of macromolecular crowding

Emulsion reactors, as well as all the control systems (single-phase solutions of buffer, mixed buffer and Na_2SO_4 solution, and mixed buffer and PEG solution) were prepared. Sample preparation procedure involved a hydration step for the reactants before the heat treatment. For this purpose, samples were prepared and stored (while being stirred) for 19 h at 25 °C (Fig. S3a). The stored Na_2SO_4 and buffer solutions had comparable concentrations of glucose and amino acids to the amounts added during sample preparation (Fig. 2a, b, e, f). The solutions did not contain fructose, and the Amadori products Fru-Asn and Fru-Trp. On the contrary, in the stored PEG solution, glucose concentration was ~19 % (when supplemented together with asparagine) or ~36 % (when supplemented together with tryptophan) less than the amount initially added (Fig. 2a, b). Likewise, in the stored PEG solution the amino acid concentrations were lower than the amounts added, and both the Amadori products were formed (Fig. 2e-h). Once the.

sample preparation procedure was modified aiming to shorten the whole procedure, the Amadori products were still measurable in the PEG solution after 2 h of storage for glucose-asparagine combination, also right after the addition of the reactants (time zero) for glucose and tryptophan combination (Fig. S3b, c). All together, these results indicate that PEG facilitated the condensation reaction between amino acids and glucose (and the succeeding Amadori rearrangement) and heating was not required to initiate the MR. While the formation of the Amadori products in food products can be initiated at room temperature, it is conventionally associated with prolonged storage (Mastrocola & Munari, 2000).

Macromolecular crowding due to high concentration of polymers could non-specifically and non-linearly speed up some (bio)chemical reactions (Perusko et al., 2015), this is explained by the excluded volume theory (Zimmerman & Minton, 1993). This observation opens new possibilities for the synthesis of Amadori products and the control of the whole MR. Our results on the condensation reaction of free amino acids with glucose in the PEG solution are in line with those previously reported by Perusko and coworkers. In their study using PEG (6 kDa) as a crowding agent, the authors showed a significant enhancement of whey proteins arabinosylation, highlighting the positive impact of macromolecular crowding on the protein modification (Perusko et al., 2015). Macromolecular crowding is a common phenomenon in biological systems including food and cells.

3.4. The role of reactants partitioning

Fig. 1c schematically shows the two different ways of partitioning the reactants within the all-aqueous emulsion reactors. Co-encapsulation involved compartmentalizing the reactants asparagine and glucose inside the Na_2SO droplets, while segregation involved partitioning the reactants tryptophan and glucose between the two aqueous phases. How the reactants were partitioned (*i.e.*, co-encapsulation or segregation) influenced their concentration at the end of the storage step and before the heat treatment. When co-encapsulated, the concentrations of the reactants were comparable with the amounts initially added; even though the Amadori product Fru-Asn was detected, its concentration was negligible (Fig. 2g). On the other hand, when segregated, the concentrations of the reactants were ~ 17 % less than the amounts added into the emulsions. The insignificant effect of the reactants co-encapsulation and the stimulating effect of the reactants segregation on the MR during storage was unexpected. It could be argued that the reactants co-encapsulation within droplets did not trigger the MR during storage because Na_2SO_4 does not cause microenvironmental crowding. The segregation nevertheless caused concentration of the reactants at the aqueous-aqueous interface, rather than the reactants isolation in two discrete compartments. In all-aqueous emulsions, polymers are depleted from the interface by 5 % to 30 % when compared to the bulk phases. Our data suggests that the depletion which leads to elevated water content at the interface, favors solvation and concentration of solutes at the interface (Dickinson, 2019), advancing the MR. In summary, tryptophan-glucose condensation, Schiff base formation and the consequent Amadori rearrangement during emulsions storage (while being stirred) was due to concentration of tryptophan (and possibly glucose) at the interface favoring the collision of the reactants.

3.5. Progress of the MR over time

The MR development at 95 °C was monitored for up to 5 h. Concentration of glucose and amino acids in the single-phase solutions decreased over time (except a slight increase in tryptophan at 1 h and 2 h in the PEG solution) (Fig. 2a, b, e, f). Also, fructose was formed in the samples, most likely due to glucose isomerization (van Boekel & Brands, 2005). The Amadori products Fru-Asn and Fru-Trp were found in all single-phase solutions and their concentrations in buffer and Na_2SO_4 solutions were always lower than those in the PEG solution. The Amadori products maximum yield was higher when the MR was run in the PEG solution compared with the emulsion reactors. Also, the maximum yield of the Amadori products were obtained at an earlier time (1 h *vs* 3 h) in the PEG solution than the emulsion reactor by segregated reactants (Fig. 2g, h). As already explained, PEG could facilitate the MR by macromolecularly crowding the solution and increasing the reactants effective concentration. In the emulsion reactors, the reaction was either run within Na_2SO_4 droplets or at the water-water interface which were deficient in PEG. Compared to reactants segregation, the effect of Na_2SO_4 on hindering the accumulation of Amadori products was more pronounced. Na_2SO_4 could increase the ratio of zwitterion in amino acids and thus retard the amino-carbonyl reaction (Yamaguchi et al., 2009).

3.6. Partitioning of the Amadori products

All-aqueous emulsion reactors enable fractionation of reaction products between the two phases of emulsions after reaction termination and emulsion phase separation. The partitioning of Amadori products was investigated by determining the partition coefficient (K) and partition extent into the top phase (E_{PEG} %) following the MR for up to 5 h (Fig. S4). At 0 h, the concentration of Fru-Asn was roughly 17 times higher in the Na_2SO_4 phase than the PEG phase, while the concentration of Fru-Trp in the PEG phase was approximately 10 folds higher than that in the Na_2SO_4 phase, with 98.5 % of Fru-Trp partitioning into the PEG phase. Although K of both Fru-Asn and Fru-Trp experienced an increase after heat treatment for up to 5 h, their primary location in emulsions remained unchanged. Therefore, the all-aqueous emulsion reactors facilitated the Amadori products

concentration by preferentially partitioning Fru-Asn and Fru-Trp into Na_2SO_4 phase and PEG phase, respectively. Similarly, asparagine efficiently partitioned into the Na_2SO_4 phase and tryptophan strongly partitioned into the PEG phase, suggesting that the partitioning of the Amadori products paralleled that of the respective amino acid precursors (Fig. S1).

3.7. Partitioning of the MR advanced products and melanoidins

The possibility of selective fractionation of the MR advanced oxidation, glycation, and degradation products between the two phases of all-aqueous emulsion reactors was investigated by using HILIC high resolution tandem mass spectrometry in targeted product ion scan mode. Fig. 3 shows the peak area values of succinimide, *N*-hydroxysuccinimide, two pyrrole derivatives and two pyridine derivatives during the development of the MR for 0–5 h between the co-encapsulated reaction precursors (*i.e.*, glucose and asparagine). For succinimide and both pyridine derivatives, namely 3-(2-aminopyridin-4-yl)propanamide and 3-(2-(3-aminopropyl)pyridin-4-yl)propanamide, a preferential affinity to the Na_2SO_4 phase was observed (Fig. 3a, e, f). This behavior was comparable to that of the reactants glucose and asparagine. Conversely, *N*-hydroxysuccinimide (*i.e.*, hydroxylated form of succinimide) and two pyrrole derivatives (*i.e.*, 1*H*-pyrrole-2-carbaldehyde and 1*H*-pyrrole-2-carboxamide), strongly partitioned within the PEG phase (Fig. 3b, c, d). These results suggested that following the reactants co-encapsulation, a part of advanced products could migrate into the PEG phase, thus concentrating reaction products in different phases according to their chemical nature.

Fig. 4 displays the peak area values of the oxidation products (*i.e.*, β-carboline derivatives and 4-indolecarbaldehyde) after running the MR for 0–5 h between the segregated reaction precursors (*i.e.*, glucose and tryptophan). Similar to tryptophan, five distinct β-carboline derivatives, namely OH-β-carboline (β-carbinol), hydroxyethyl (HET)-β-carboline, hydroxymethyl (HME)-β-carboline, β-carboline-2,3-butandione, and

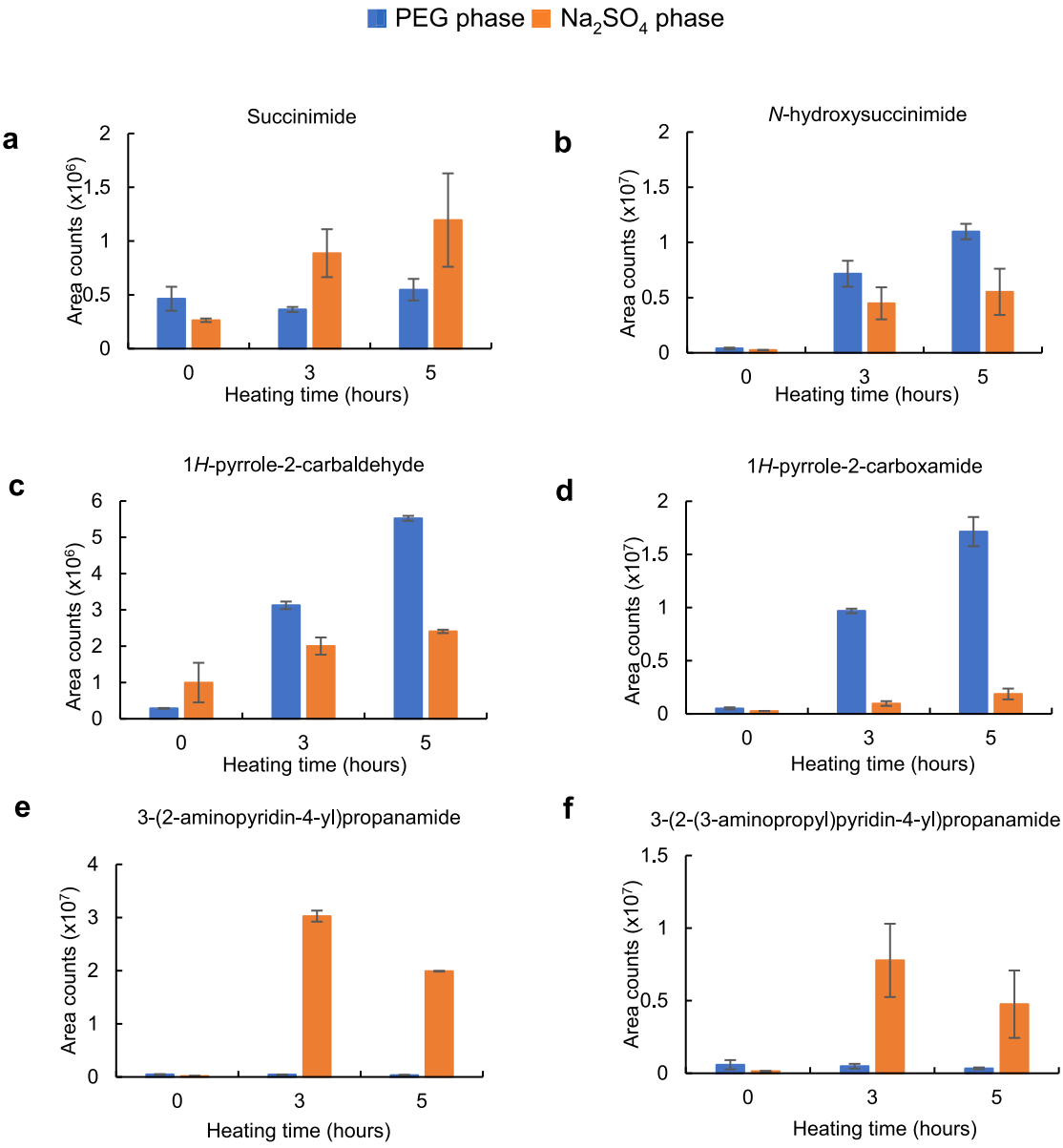

Fig. 3. Area counts of advanced product signals detected in the PEG and Na_2SO_4 phases of all-aqueous emulsions after running the Maillard reaction for 0–5 h between the co-encapsulated reaction precursors (*i.e.*, glucose and asparagine) and the subsequent phase separation of the emulsions. Succinimide, *N*-hydroxysuccinimide, pyridine derivatives, and pyrrole derivatives were selected according to the reaction pathways of asparagine and reducing sugars (Yaylayan et al., 2003).

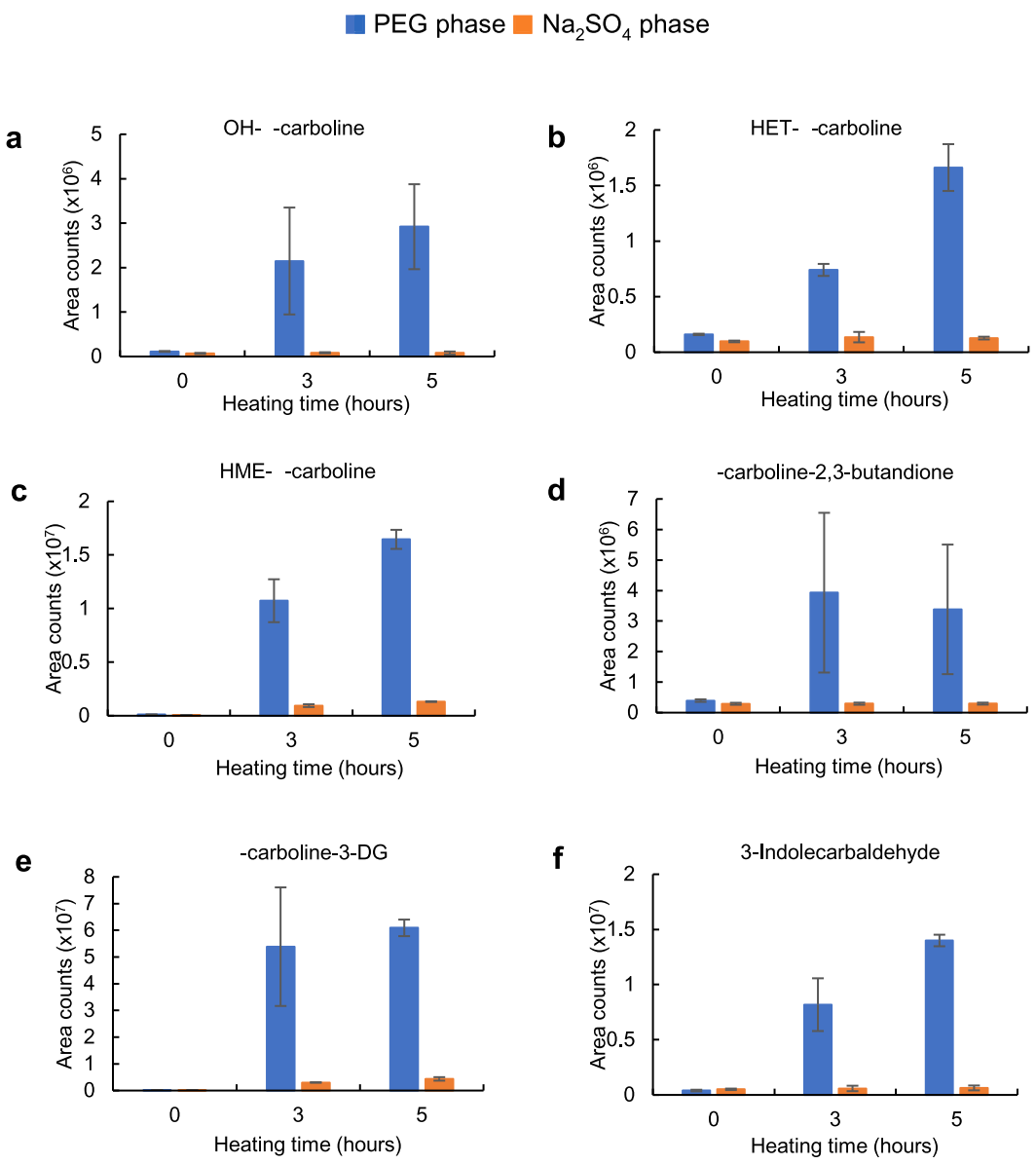

Fig. 4. Area counts of advanced product signals detected in the PEG and Na_2SO_4 phases of all-aqueous emulsions after running the Maillard reaction for 0–5 h between the segregated reaction precursors (*i.e.*, glucose and tryptophan) and subsequent phase separation of the emulsions. β-carboline derivatives and 4-indolecarbaldehyde were used as markers of two interrelated reaction pathways including dicarbonyls-mediated oxidation of indole rings and Strecker degradation (Herraiz et al., 2022).

β-carboline-3-deoxyglucosone (β-carboline-3-DG), as well as 3-indolecarbaldehyde strongly partitioned into the PEG phase (Fig. 4a-f). Hence, it can be concluded that when the reactants could interfacially react, the advanced reaction products showed a clear affinity towards the PEG phase. Notably, this eliminates the inhibitory influence of Na_2SO_4, as compared to the reactants co-encapsulation.

The capability to selectively fractionate melanoidins, the brown polymeric final products of the MR, between the two phases of all-aqueous emulsion reactors is demonstrated in Fig. 5. After 5 h of heating and the subsequent phase separation of emulsion reactors, the top phase (*i.e.*, PEG phase) exhibited a dark brown color. On the contrary, the bottom phase (*i.e.*, Na_2SO_4 phase) was either lightly colored (when the reactants were co-encapsulated) or completely transparent (when the reactants were segregated). The color difference between the top and bottom phases indicated that irrespective of the reactants location and mode of partitioning (*i.e.*, co-encapsulation or segregated and interfacially touching), the brown pigments, *i.e.*, melanoidins consistently concentrated into the PEG phase.

3.8. Concluding remarks on the progress of MR in the emulsion reactors

Fig. 6 schematically shows the main location of the reactants, Amadori products, advanced products, and the presumed formation mechanism of the Amadori products and melanoidins in the Na_2SO_4-in-PEG emulsions. The reaction precursors co-encapsulation within the Na_2SO_4 phase enabled running the MR as compartmentalized. The partitioning preference of glycosylamine (the Schiff base) is similar to its precursors, namely glucose and asparagine. Therefore, glycosylamine significantly partitioned into the Na_2SO_4 phase and subsequently underwent the Amadori rearrangement. The thermal degradation of Amadori product Fru-Asn resulted in succinimide, followed by generation of pyrrole (partitioned into the PEG phase) and pyridine derivatives (partitioned into the Na_2SO_4 phase). As the reaction progressed, pyrrole, pyridine derivatives, and other heterocyclic structures could undergo

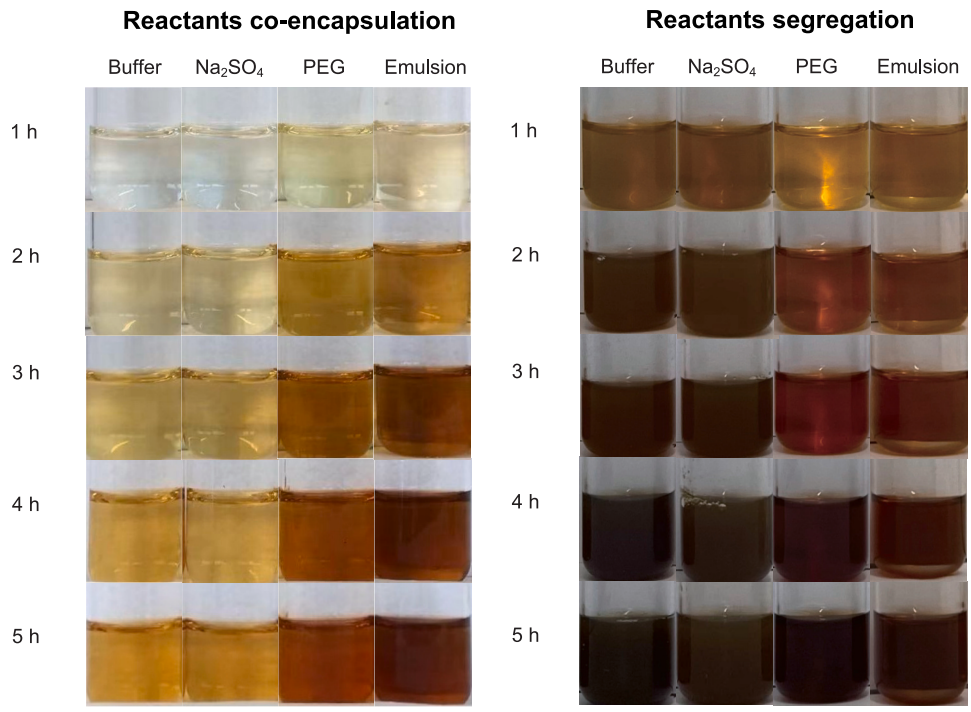

Fig. 5. Browning development in the all-aqueous emulsions and single-phase solutions. Images were captured after storing samples at 25 °C for 4 h to allow phase separation of the all-aqueous emulsions.

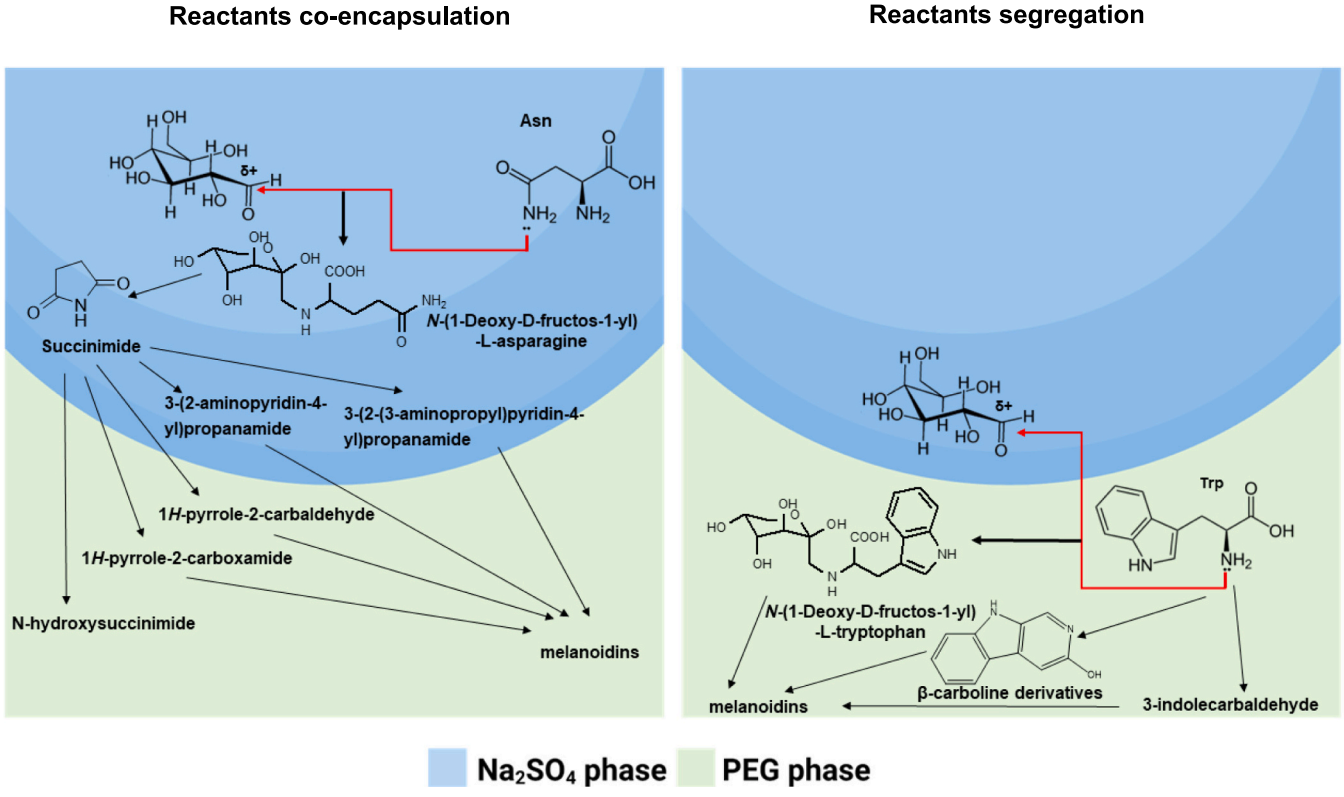

Fig. 6. The main location of the Maillard reaction precursors (glucose, and either asparagine or tryptophan), Amadori products, advanced products, and the presumed formation mechanism of Amadori products, advanced products, and melanoidins in all-aqueous emulsion reactors.

polymerization and copolymerization reactions to form melanoidins which are more hydrophobic compounds, thus they mostly migrated into the PEG phase.

The reaction precursors segregation led to the reaction of tryptophan and glucose at the interface, forming the corresponding glycosylamine. This compound moved into the PEG phase owing to the aromatic side group from tryptophan. Therefore, once formed at the interface, it migrated into the PEG phase and underwent the Amadori rearrangement

to yield Fru-Trp. Simultaneously, the reaction between methylglyoxal and tryptophan led to the formation of hydroxyethyl (HET)-β-carboline, while in the case of hydroxymethyl (HME)-β-carboline, tryptophan reacted with glyoxal. It is hypothesized that other α-dicarbonyls, such as 2,3-butanedione and 3-deoxyglucosone, may follow a similar reaction pathway, resulting in the formation of β-carboline-2,3-butanedione and β-carboline-3-DG. Considering the location of glucose and its isomerization product fructose, we hypothesized that sugar autoxidation occurred in the Na_2SO_4 phase with the consequent formation of α-dicarbonyls. Indole rings of tryptophan can scavenge α-dicarbonyls both at the interface and directly in the PEG phase, in the case that these highly reactive compounds arise from oxidative fragmentation of Fru-Trp. In both situations, the reaction of α-dicarbonyls and indole rings lead to the formation of β-carboline (Ghassem Zadeh & Yaylayan, 2019).

Tryptophan is also converted into 3-indolecarbaldehyde, a Strecker aldehyde usually associated with leathery and tar odor attributes. Volatile and semi-volatile aldehyde can be efficiently partitioned in the PEG phase, thus suggesting the potential use as flavor generation system. As the reaction progressed, β-carboline derivatives, 3-indolecarbaldehyde, and other heterocyclic structures may undergo polymerization and copolymerization reactions, ultimately forming melanoidins that predominantly remained in the PEG phase. These findings open interesting possibilities in the downstream process in many processes used in food industry to produce flavor and aroma compounds through the MR.

It is noteworthy that the presence of high concentrations of the polymer PEG and Na^+ ions (which can be replaced by K^+ions in other studies) can mimic the biological milieu in cells; hence, some evidence of the MR in all-aqueous emulsions can be useful to investigate *in vitro* the fate of glycation compounds in cells and to the possibility that some compounds as indole rings serve as scavengers for reactive α-dicarbonyls.

4. Conclusions

The present study explored the use of all-aqueous emulsions as microreactors for accomplishment of the MR. Our method ensured consistent concentrations of buffer, PEG, and Na_2SO_4 across the reaction medium. While PEG mainly partition within PEG phase in ATPS, our quantification showed that the PEG concentration in the PEG phase was only 6 % higher than in PEG solution, which we believe is not sufficiently high to cause unreasonable comparisons. If using the top/bottom phases after phase separation, the top phase includes little salt and the bottom phase contains little PEG, which makes the solution binary, even though single-phase. The introduction of PEG in the emulsion systems resulted in a noticeable acceleration of the formation of Amadori products. Conversely, Na_2SO_4 exhibited either the opposite effect (in the case of Fru-Asn) or no effect (in the case of Fru-Trp). It was demonstrated that the manipulation of reactant location enables the modulation of the Amadori compound formation and the reaction pathways leading to the formation of pyrroles, pyridines, Strecker aldehyde and β-carbolines. Additionally, the use of all-aqueous emulsions allowed for the fractionation of MR products including Amadori products and end-products after phase separation. Table S2 reports the concentration of PEG and Na_2SO_4, as well as the highest yield and the corresponding concentrations of Fru-Asn and Fru-Trp in these reaction systems. PEG can be effectively removed through methods such as ultrafiltration (diafiltration) and dialysis, while sodium sulfate can be removed by ion-exchange resins, making all-aqueous emulsions feasible for large-scale use. Our findings indicate that all-aqueous emulsions could serve as effective tools for controlling the location of both reactants and products, as well as MR pathways, in comparison to single-phase solutions. These results hold significant implications for the industrial utilization of all-aqueous emulsions for the controlled synthesis and extraction of MR products. Moving forward, we will continue to optimize the reaction systems to further increase yield and ensure even greater applicability.

The all-aqueous emulsions used in this study mimic macromolecular crowding and compartmentalization of intracellular environment and therefore could have important implications for bioreactions compartmentalization by molecular partitioning. Additionally, the positive influence of macromolecular crowding on the condensation reaction between carbonyl and amino groups is noteworthy, suggesting the feasibility of developing an all-aqueous-emulsion-based microreactor for precisely controlled synthesizing Amadori products under mild conditions.

CRediT authorship contribution statement

Kangni Chen: Writing – original draft, Methodology, Investigation, Formal analysis. **Antonio Dario Troise:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **Sabrina De Pascale:** Writing – review & editing, Methodology, Formal analysis. **Andrea Scaloni:** Writing – review & editing, Funding acquisition. **Vincenzo Fogliano:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Ashkan Madadlou:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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.foodchem.2024.142089.

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