Preparation and Characterization of Water-in-Oil-in-Water Emulsions Containing a High Concentration of L-Ascorbic Acid

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This study sought to encapsulate a high concentration of L-ascorbic acid, up to 30% (w/v), in the inner aqueous phase of water-in-oil-water (W/O/W) emulsions using soybean oil as the oil phase. Two-step homogenization was conducted to prepare W/O/W emulsions stabilized by a hydrophobic emulsifier and 30% (w/v) of W/O droplets stabilized by a hydrophilic emulsifier. First-step homogenization prepared W/O emulsions with an average aqueous droplet diameter of 2.0 to 3.0 μm. Second-step homogenization prepared W/O/W emulsions with an average W/O droplet diameter of 14 to 18 μm and coefficients of variation (CVs) of 18% to 25%. The results indicated that stable W/O/W emulsions containing a high concentration of L-ascorbic acid were obtained by adding gelatin and magnesium sulfate in the inner aqueous phase and glucose in both aqueous phases. L-Ascorbic acid retention in the W/O/W emulsions was 40% on day 30 and followed first-order kinetics.

Key words: W/O/W emulsions; L-ascorbic acid; high concentration; stability; microstructure

Double emulsions are categorized as complex dispersion systems and are regarded as emulsions of emulsions.1 Double emulsions are further classified into water-in-oil-in-water (W/O/W) and oil-in-water-in-oil (O/W/O) emulsions. Most industrial applications utilize W/O/W emulsions, whereas the applicability of O/W/O emulsions is still limited, mainly due to solubility issues. W/O/W emulsions have attracted a great deal of interest for various applications, including foods,2 pharmaceuticals,3 and cosmetics.4 For instance, they have been utilized as controlled-release drug-delivery systems (DDS),5 as templates for preparing microcapsules with enhanced stability and controlled release of functional food components,6 and in the formulation of reduced-calorie food emulsions.7

Food-grade W/O/W emulsions are usually prepared by two-step emulsification using conventional mechanical emulsification devices (e.g., rotor-stator homogenizers and high-pressure homogenizers). They are mostly polydisperse, and structurally heterogeneous. W/O/W emulsions with high monodispersity can be prepared by membrane emulsification8,9 or microchannel emulsification10,11 as the second-step emulsification. In W/O/W emulsions, oil droplets containing inner aqueous droplets as dispersed phase are further dispersed in an outer aqueous phase. Compared to single emulsions, W/O/W emulsions are capable of encapsulating hydrophilic components with higher entrapment yields and stability against degradation.12 These advantages make them useful as dispersion systems for encapsulating food-grade hydrophilic components.

Stabilizing double emulsions is a major challenge for the food, cosmetic, and pharmaceutical industries. In general, several major factors (e.g., Ostwald ripening, coalescence, flocculation, and creaming) are responsible for destabilizing emulsions. Moreover, the stability of W/O/W emulsions is critically influenced by the osmotic pressure balance between the inner and the outer aqueous phase,12 the volume ratio balance between the oil and the outer aqueous phase, and the selection of hydrophilic and hydrophobic emulsifiers.13,14

L-Ascorbic acid (vitamin C), an important water-soluble vitamin, is the most widely used vitamin supplement in the world.15 It plays an important role in the biosynthesis of collagen, carnitine, and neurotransmitters.16 Plants and most animals synthesize L-ascorbic acid for their own requirements, but primates and humans are unable to synthesize L-ascorbic acid due to lack of the enzyme gulonolactone oxidase.17 L-Ascorbic acid is a labile molecule, so it can be lost from foods during cooking, processing, and preservation.15 Cooking loss of L-ascorbic acid depends upon many factors (e.g., heating at high temperature, surface area exposed to water, oxygen, pH, and the presence of transition metals).18 Synthetic L-ascorbic acid is available in a wide variety of supplements in the forms of tablets, capsules, chewable tablets, crystalline powder, effervescent tablets, and liquids.

L-Ascorbic and dehydroascorbic acid are the major dietary forms of vitamin C. All forms of ascorbic acid are soluble in water, except for ascorbyl palmitate, which

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is used in commercial antioxidant preparations due to its greater lipid solubility.\textsuperscript{19} L-Ascorbic acid and its fatty acid esters are used as food additives, antioxidants, browning inhibitors, reducing agents, flavor stabilizers, dough modifiers, and color stabilizers.\textsuperscript{20,21}

Encapsulating L-ascorbic acid in emulsion systems is highly recommended due to its hydrophilic nature and rapid degradation in bulk aqueous systems. Both single- and double-emulsion systems are used to encapsulate L-ascorbic acid. Several research groups have encapsulated L-ascorbic acid at low concentrations (up to 5\%) in aqueous droplets using the W/O/W and O/W/O techniques.\textsuperscript{22–26} Nevertheless, in order to formulate food-based products such as high energy drinks, high concentrations of active components are required.

The primary objective of this study was to prepare stable W/O/W emulsions containing a high concentration of L-ascorbic acid in the inner aqueous phase. We investigated the effects of type of sugar in the outer aqueous phase and the addition of gelatin in the inner aqueous phase on the preparation and stability of W/O/W emulsions and the retention kinetics of L-ascorbic acid in W/O/W emulsions. The W/O/W emulsions prepared were also characterized by microscopic analysis, and the results were employed to evaluate physical stability.

Materials and Methods

Materials. L-Ascorbic acid (>99.6\% purity), refined soybean oil, magnesium sulfate (MgSO\textsubscript{4}), gelatin, glucose, fructose, sucrose, and methanol were purchased from Wako Pure Chemical Industries (Osaka, Japan). Xanthan gum was purchased from Tokyo Chemical Industry (Tokyo, Japan). These reagents were of analytical grade.

Methanol were purchased from Wako Pure Chemical Industries (Osaka, Japan). The coefficient of variation (CV) was used as an indicator of the droplet size distribution of the emulsions prepared. CV was used as an indicator of the droplet size distribution of the emulsions prepared. CV was used as an indicator of the droplet size distribution of the emulsions prepared.
W/O/W Emulsions Containing l-Ascorbic Acid

Table 1. Fluid Properties of Three-Phase Systems Containing High Concentrations of l-Ascorbic Acid, Glucose, and Gelatin Used for Preparing W/O/W Emulsions

<table>
<thead>
<tr>
<th>l-Ascorbic acid concentration(%\ (w/v))</th>
<th>Viscosity (mPa s)</th>
<th>Interfacial tension (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inner aq. phase(b)</td>
<td>Oil phase(c)</td>
</tr>
<tr>
<td>10</td>
<td>2.0 ± 0.01</td>
<td>66.1 ± 0.10</td>
</tr>
<tr>
<td>20</td>
<td>2.8 ± 0.02</td>
<td>66.1 ± 0.10</td>
</tr>
<tr>
<td>30</td>
<td>3.3 ± 0.01</td>
<td>66.1 ± 0.10</td>
</tr>
</tbody>
</table>

\(a\) Initial concentration of l-ascorbic acid in the inner aqueous phase.
\(b\) Containing 10–30% (w/v) l-ascorbic acid with 1% (w/w) MgSO\(_4\) and gelatin 1% (w/v).
\(c\) Containing TGCR 5% (w/w) in soybean oil.
\(d\) Containing MgSO\(_4\) 1% (w/w), glucose 10–30% (w/v), and DGM 1% (w/w).

Measurement of fluid properties. The viscosity and density of each phase and the interfacial tension between two contacting phases were measured at 25 °C. Viscosity was measured with a vibroviscometer (SV-10, A&D, Tokyo, Japan). Density was measured using a density meter (DA-130 N, Kyoto Electronics Manufacturing, Kyoto, Japan). Interfacial tension was measured with a Fully Automatic Interfacial Tensiometer (PD-W, Kyowa Interface Sciences, Saitama, Japan) by the pendant drop method. Table 1 lists the major fluid properties of the three-phase systems used to prepare the W/O/W emulsions.

Stability of W/O/W emulsions. The W/O/W emulsions prepared were observed in order to evaluate consistency, color, homogeneity, and eventually phase separation during 35 d of storage under refrigeration at 4 °C. After centrifugation, samples were inspected for eventual phase separation. Testing was replicated at time intervals previously determined.

Retention kinetics of l-ascorbic acid in W/O/W emulsions. l-Ascorbic acid encapsulated in the W/O/W emulsions was determined by the spectrophotometric method reported by Zeng et al.,\(^{28}\) which was originally used to measure l-ascorbic acid solubilized in aqueous solution. All spectral measurements of methanolic extracts of W/O/W emulsions were carried out with a UV/VIS/NIR spectrophotometer (V-570, Jasco, Hachioji, Japan). Next, 1 mL of W/O/W emulsion was extracted with 10 mL of methanol, followed by ultrasonication for 20 min. The methanolic extract was then centrifuged (KN-70, Kubota) at 2000 rpm for 15 min. A 1-mL aliquot of the supernatant was diluted to 20 mL with methanol, and the diluted solution was then injected into a quartz cell with a 10-mm pass length. The absorbance of l-ascorbic acid in this solution was measured at 247 nm using an appropriate blank. A representative standard curve of absorbance versus concentration gave linear least-squares regression with a coefficient of determination \(r^2\) of 0.9996. All experiments were repeated in triplicate and mean values were calculated. The retention ratio, defined as \(R = (C_{\text{AA}}/C_{\text{AA,0}}) \times 100\), was calculated as the ratio of the l-ascorbic acid concentration measured at a given storage time \(C_{\text{AA}}\) to the initial l-ascorbic acid concentration \(C_{\text{AA,0}}\).

Statistical analysis. Analysis of variance (ANOVA) tests were used to analyze the characterization data at a confidence level of 95% (\(p < 0.05\)). A least-significant difference (LSD) test was used to compare the effect of adding gelatin on the average oil droplet diameter of the W/O/W emulsions and the effect of storage time on the droplet diameter of the W/O and W/O/W emulsions. LSD was calculated by the method described by Steel et al.,\(^{29}\)

Results and Discussion

Effects of the compositions of aqueous phases on the preparation of W/O/W emulsions

Osmotic pressure plays a very important role in the stability of W/O/W emulsions, and Laplace pressure works against the stability of W/O emulsions.\(^{30}\) The addition of a small quantity of electrolyte to the disperse phase has a stabilizing effect on W/O emulsions, by counteracting the Laplace pressure effect. Kanouni et al.,\(^{12}\) found that stabilization of W/O/W emulsions required a balance between the osmotic pressures of the outer and the inner aqueous phase. Considering these results, the outcomes of adding various sugars in the outer aqueous phase and adding gelatin in the inner aqueous phase were investigated.

Effects of adding sugars in the outer aqueous phase

A well-balanced composition of aqueous phases is needed in order to prepare stable W/O/W emulsions. Figure 2 indicates the variation in the average W/O...
droplet diameters ($d_{av,w/o/w}$) of freshly prepared W/O/W emulsions in the presence and the absence of a sugar (fructose, sucrose, or glucose) containing L-ascorbic acid (10% w/v) in the inner aqueous droplets. The W/O emulsions prepared by first-step homogenization had an average aqueous droplet diameter ($d_{av,w/o}$) of 2.5 μm and a CV$$_{w/o}$ of 20%. The W/O droplets of the resulting W/O/W emulsions had a $d_{av,w/o/w}$ of 12 to 20 μm and a CV$$_{w/o/w}$ of 20 to 25%. Adding sugar caused a 20 to 30% reduction in the $d_{av,w/o/w}$ value. These CV values for the W/O/W emulsions containing fructose or sucrose were somewhat greater than the values for those containing glucose and no sugar. Freshly prepared W/O/W emulsions containing no sugar or glucose did not undergo creaming for 2 d but those containing sucrose and fructose underwent rapid creaming soon after preparation (Fig. 3a). This creaming may have occurred due to considerable flocculation of the W/O droplets, which causes much faster creaming of aggregated droplets (flocs). The micrographs in Fig. 3b depict aggregation of W/O droplets in the outer aqueous phase containing fructose or sucrose. Stokes’ law gives a good description of the terminal creaming rate of isolated emulsion droplets and flocs, provided that there is little movement of the liquid within the droplets.31) This condition is fulfilled when the viscosity of the droplets (the dispersed phase) is significantly greater than that of the continuous phase, or when the droplets are surrounded by an emulsifier membrane that resists deformation.31) The terminal creaming rate of an individual droplet is defined as $v_{dr} = -gd_{dr}^2Δρ/18η_c$, and that of a floc is defined as $v_{floc} = -gd_{floc}^2Δρ/18η_c$, where $g$ is gravitational acceleration, $d_{dr}$ is droplet diameter, $d_{floc}$ is floc diameter, $Δρ$ is the density difference between the droplet or floc and the continuous phase, and $η_c$ is the viscosity of the continuous phase. The formation of flocs caused by droplet aggregation greatly increases the creaming rate of emulsions.

Adding glucose leads to increased molecular movement, interfacial film formation, and the setting up of physical barriers with components due to the non-Newtonian behavior of emulsions.32,33) Creaming and oiling-off are the results of a variety of physicochemical mechanisms that occur within W/O/W emulsions, including gravitational separation, flocculation, and coalescence. Schmidts et al.34) reported that it was difficult to obtain physically stable W/O/W emulsions using hydrophilic sucrose esters in the continuous aqueous phase. Added fructose or sucrose in the continuous aqueous phase can interact with the hydro-

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**Fig. 3.** Photographs and Optical Micrographs of the W/O/W Emulsions Prepared in the Presence and Absence of a Sugar in the Continuous Aqueous Phase.

a. Photographs. b. Symbols: A, no sugar, B with fructose, C with sucrose, and D with glucose.
phobic emulsifier at the oil-water phase and weaken the repulsive interaction between the adjacent interfacial films that prevents rapid creaming.

**Effects of adding gelatin to the inner aqueous phase**

The effects of adding gelatin to the inner aqueous phase on the droplet size and droplet size distribution of the W/O/W emulsions prepared is depicted in Fig. 2. Adding gelatin did not significantly affect (p > 0.05) the $d_{av,w/o}$ of the W/O emulsions (Fig. 2a). The $d_{av,w/o}$ of the W/O/W emulsion in the presence of gelatin was greater than that of the W/O/W emulsion in the absence of gelatin, whereas the CV of the W/O/W emulsions was unaffected by added gelatin. The W/O/W emulsion containing gelatin had smooth mobility with a good organoleptic profile in terms of color, flowability, and stability in comparison to that without gelatin. The W/O/W emulsion prepared in the presence of gelatin had better stability against creaming than that in the absence of gelatin (data not shown).

Adding gelatin to the aqueous phase of the W/O emulsions resulted in a narrow size distribution with improved stability and a good organoleptic profile. Evison et al. have reported that adding 1% (w/w) gelatin in the inner aqueous phase of W/O/W emulsions led to a substantial increase in the yield of an encapsulated compound and better stability against the coalescence of both inner aqueous and oil droplets. Biopolymers such as gelatin that are incorporated in W/O/W emulsions also act as chelating agents that can reduce the release rate of encapsulated compounds.

In our study, this gelation and chelating action of gelatin were assumed to be responsible for increasing the stability of the W/O/W emulsions against sedimentation and creaming. The additional gelatin can also act as a buffer to balance the effect of a high concentration of t-ascorbic acid in W/O/W emulsions.

**Effects of t-ascorbic acid concentration on the preparation of W/O/W emulsions**

Figure 4 presents the average diameters ($d_{av,w/o}$ and $d_{av,w/o/w}$) and CV of the oil droplets and inner aqueous droplets in prepared W/O/W emulsions containing various concentrations of t-ascorbic acid. The concentration of t-ascorbic acid in the inner aqueous phase was varied from 10 to 30% (w/v), and gelatin was added to all the inner aqueous phase solutions at a concentration of 1% (w/v). The concentration of glucose in the outer aqueous phase was also varied from 10 to 30% (w/v), depending on the preceding t-ascorbic acid concentration. The W/O emulsions, with a $\phi_w$ of 30%, used in second-step homogenization, had a $d_{av,w/o}$ of 2.3 to 2.8 $\mu$m and a CV of 10 to 16%. The $d_{av,w/o}$ value increased slightly with increasing t-ascorbic acid concentrations, probably due to the increased viscosity of the inner aqueous phase (Table 1). The interfacial tension between the inner aqueous phase and the oil phase was independent of the t-ascorbic acid concentration (Table 1). W/O/W emulsions were successfully prepared regardless of the t-ascorbic acid concentration applied. The $d_{av,w/o/w}$ of the resulting W/O/W emulsions increased gradually from 12 to 18 $\mu$m as the concentration of t-ascorbic acid increased. The increase in $d_{av,w/o/w}$ of the W/O/W emulsions corresponds to an increase in the viscosity of the outer aqueous phase (Table 1), as the interfacial tension between the oil phase and the continuous aqueous phase was unaffected by the t-ascorbic acid concentration. The results shown in Fig. 4 indicate the successful preparation of W/O/W emulsions containing a high concentration of t-ascorbic acid (up to 30% w/v) in the inner aqueous phase, considerably higher than those previously reported.

**Stability of W/O/W emulsions containing high concentrations of t-ascorbic acid**

**Physical stability of the W/O/W emulsions**

The W/O/W emulsion samples were stored at 4°C for 35 d. Immediately after preparation, those containing concentrations of t-ascorbic acid were whitish in color and showed good flowability. In addition, the W/O/W emulsions did not exhibit any change in physical appearance or homogeneity during the storage period, confirming increased stability. A centrifugation test indicated no phase separation in the W/O/W emulsions containing gelatin in the inner aqueous phase even after 30 d of storage. In contrast, those without gelatin resulted in phase separation after 36 h of storage as indicated by the centrifugation test. This suggests a beneficial effect of gelatin on the stabilization of W/O/W emulsions containing 30% (w/v) t-ascorbic acid.
Retention kinetics of L-ascorbic acid in the W/O/W emulsions

Figure 6 shows the rate of L-ascorbic acid loss in the W/O/W emulsions during storage under refrigeration at 4 °C. The freshly prepared W/O/W emulsions had an initial retention of L-ascorbic acid (10–30% w/v) of higher than 90%. The L-ascorbic acid levels in the W/O/W emulsions decreased gradually with time and exhibited about 40% retention at day 28. The L-ascorbic acid in a bulk Milli-Q water stored at 4 °C had retention of <5% at day 28, as rapid ionization in aqueous solutions results in a rapid loss of L-ascorbic acid. Lee et al. encapsulated 5% (w/w) L-ascorbic acid in W/O/W emulsions using electrolytes, and concluded that W/O/W emulsions were revealed as efficient tool for improving L-ascorbic acid stability. The prepared W/O/W emulsions had L-ascorbic acid concentrations (R) of 3.88 g/100 mL, 8.02 g/100 mL, and 12.06 g/100 mL, at 10, 20, and 30% (w/v), after 30 d of storage time. They were subjected to centrifugation at 15000 rpm for 20 min to determine the amounts of phase separation. There was hardly any phase separation in them during 30 d of storage, and the degradation of L-ascorbic acid took place with a rate constant of 0.03 (0.75%) (Table 2). The formation of an oil-emulsifier interface across the inner aqueous phase containing L-ascorbic acid may protect the linkage of L-ascorbic acid to the outer aqueous phase. Similarly, L-ascorbic acid also appear to act as a pro-antioxidant protecting against L-ascorbic acid leakage and enhancing the stability W/O/W emulsions.

The retention kinetics of L-ascorbic acid in the W/O/W emulsions stored at 4 °C was also evaluated as \( \ln \frac{C_{AV,W/O/W}}{C_{AV,W/O/W}} = -kt \), where \( k \) is the rate constant and \( t \) is the storage time. The rate constant of first-order kinetics was calculated by applying the regression function of Microsoft Excel 2010, obtaining best-fit results. The lines fitted by the retention kinetics equation are presented in Fig. 7. The calculated \( r^2 \) values for all the W/O/W emulsions were >0.95, indicating that the fitting application of the first-order kinetics is plausible. The observed half-life for W/O/W emulsions containing high concentrations of L-ascorbic acid at 4 °C was about 24 d (Table 2). Our results indicate that the L-ascorbic acid retention kinetics depended on the composition of the external aqueous phase used in the W/O/W emulsion preparation. Diffusion of L-ascorbic acid across the interface can play an important role in producing high concentrations of L-ascorbic acid at 4 °C. The samples containing L-ascorbic acid were stored at 4 °C. The samples containing L-ascorbic acid in the inner aqueous phase. The L-ascorbic acid in a bulk Milli-Q water stored at 4 °C had retention of <5% at day 28, as rapid ionization in aqueous solutions results in a rapid loss of L-ascorbic acid. Lee et al. encapsulated 5% (w/w) L-ascorbic acid in W/O/W emulsions using electrolytes, and concluded that W/O/W emulsions were revealed as efficient tool for improving L-ascorbic acid stability. The prepared W/O/W emulsions had L-ascorbic acid concentrations (R) of 3.88 g/100 mL, 8.02 g/100 mL, and 12.06 g/100 mL, at 10, 20, and 30% (w/v), after 30 d of storage time. They were subjected to centrifugation at 15000 rpm for 20 min to determine the amounts of phase separation. There was hardly any phase separation in them during 30 d of storage, and the degradation of L-ascorbic acid took place with a rate constant of 0.03 (0.75%) (Table 2). The formation of an oil-emulsifier interface across the inner aqueous phase containing L-ascorbic acid.
Retention kinetics of L-ascorbic acid in the W/O/W emulsions. Fig. 7. Rate constants, coefficients of determination, and half-life of the fitted lines are presented in Table 2. C_{AA,0} initial concentration of L-ascorbic acid in the inner aqueous phase. C_{AA,0} of 10% is indicated by ●, 20% by ■ and, 30% by ▲.

In conclusion, food-grade W/O/W emulsions containing a high concentration of L-ascorbic acid were successfully prepared using suitably selected compositions, including gelatin in the inner aqueous phase and glucose in both aqueous phases. In particular, the formulations containing gelatin contributed to better flowability and higher L-ascorbic acid retention. The prepared W/O/W emulsions containing high concentrations of L-ascorbic acid were physically stable with slight changes in droplet size and size distribution during 35 d of storage at 4°C. The W/O/W emulsions also demonstrated relatively high retention of L-ascorbic acid after 30 d of storage, and their retention kinetics followed a first-order kinetics equation. Our results provide a better understanding of W/O/W emulsions containing high concentration of vital vitamins such as L-ascorbic acid, as W/O/W emulsions have promising applications in the food industry, due to better protection of active ingredients and functionality than simple W/O emulsions.

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