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Review Article

Techniques and methods to study functional characteristics of emulsion systems

Yin-Ting Hu, Yuwen Ting^{*}, Jing-Yu Hu, Shu-Chen Hsieh

Graduate Institute of Food Science and Technology, National Taiwan University, Number 1, Section 4, Roosevelt Road, Taipei, Taiwan

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ABSTRACT

With the growing popularity of the functional food market, bioactive ingredients from natural sources are discovered one after another for their ability to promote better health and prevent chronic diseases. Emulsion, widely occurring in many food systems, has become a popular vehicle to facilitate the incorporation of bioactive components into the food system. Depending on the designated functionality, an emulsion can be developed with various physical and chemical properties. To ensure the successful development of a high-quality emulsion-based system to serve their purpose in food, knowledge of the analytical methods that could efficiently evaluate their quality parameters is important for investigators who work in this field. In this work, important emulsion properties are overviewed, and techniques that are commonly used to assess them are provided. Discussions and recommendations are also included to make suggestions on advantages and disadvantages when selecting suitable techniques and methods to characterize these quality parameters of emulsion systems.

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1. Introduction

As food systems are complex matrices of various components that are not always well mixed with one another, oil–water and/or air–water interfaces are commonly found and could be stabilized using proper emulsifying or surface stabilizing agents. Thus, understanding the science and technology of emulsion systems is vital for food-related investigators because many natural and processed foods such as milk, salad

dressing, ice cream, soft drinks, and cakes are partially or exclusively made of emulsion [1]. Emulsion formed with various ingredients and processing conditions exhibit a broad spectrum of physical and chemical characteristics that allow them to provide food systems with important functionalities where stability, texture, taste, smell, appearance, and biological response can be effectively fine-tuned to meet target requirements for any specific product.

Because of its wide occurrence in food systems, emulsion has now become a popular delivery vehicle for functional

^{*} Corresponding author. Graduate Institute of Food Science and Technology, National Taiwan University, Number 1, Section 4, Roosevelt Road, Taipei, Taiwan.

E-mail address: pywting@ntu.edu.tw (Y. Ting).

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ingredients including color, flavor, preservatives, vitamins, minerals, and nutraceuticals [2]. The stability and efficacy of the abovementioned components could be effectively improved after being contained in the emulsion system, from which it could be protected and released into the biological system at optimum condition and timing. Depending on the type of ingredients used and requirement for different applications, emulsion is a highly versatile system that could be engineered to various sizes, surface structures, and electrochemical properties. Many studies have successfully demonstrated the development of emulsion-based systems, and results have already been summarized in many published reports [3–5].

The process of producing emulsion is called homogenization, which is achieved by applying sufficient energy to the oil/water interface that breaks up the bulk oil into smaller droplets. In general, the smaller the size to be achieved, the larger the energy input required [6]. As in the case for macro-emulsion, simple high-speed stirring is capable of forming droplets of a few micrometers. To further reduce the droplet size to submicron range, a larger energy input is required and could be supplied by various breakup mechanisms including rotor-stator, high-pressure, membrane, and ultrasonic systems [7]. Another important aspect of emulsion system is the selection of surfactant (emulsifier), which may determine not only the lowest achievable size, but also the stability and surface characteristics of emulsion droplets—that is, by alternating the combination of surface active materials and processing conditions, an emulsion system may contain different physical and chemical characteristics that would meet the requirement of target applications in food or many other fields [8].

For investigators to effectively design and produce emulsion systems that could meet their specific application needs, it is essential to realize the emulsion matrix and their droplet characteristics, which could strongly influence the physicochemical, functional, and sensory properties of such a system. Characteristics such as size, morphology, rheology, charge, and encapsulation capacity could be studied and evaluated using analytical instruments and standardized methodologies [9]. In this review, we aim to summarize and compare various analytical techniques and methods that are commonly used to assess the appearance, morphology, surface charge, and rheology of the emulsion system. The characteristics selected in this work are the parameters that are most commonly evaluated when determining the quality and predicting the suitability for a potential application. The purpose of this work is to provide a latest summary of general characterization for emulsion systems, and results can be used as a reference by investigators working in the field of emulsion science.

2. Emulsion stability

An emulsion is composed of two immiscible liquids, in which one of them is dispersed as droplets into the other liquid named the continuous phase [1]. In the food industry, manufacturers develop many products, such as milk, cream, butter, and margarine, which contain emulsion as part of or the entirety of its matrix. As noted earlier, all emulsions are

unstable by nature, and the two phases will eventually separate when they are allowed to stand for long enough time. The instability of emulsions may result in some undesirable effects in food including oiling off and sedimentation, which decrease the product quality and shorten shelf life. Therefore, it is important for investigators to understand the mechanisms that cause emulsion instability and to accurately evaluate the stability of such system.

Emulsion stability refers to the ability of emulsions to resist changes in its physicochemical properties over time [1]. The mechanisms that lead to emulsion instability include gravitational separation (creaming/sedimentation), flocculation, coalescence, Ostwald ripening, and phase inversion (Figure 1). The stability of emulsions is influenced by their compositional materials and processing conditions, from which different characteristics of their containing droplets would be developed. Important droplet characteristics include their concentration, size, charge, interactions, and rheological behavior [1,10]. For this reason, analytical instruments and experimental methodologies are necessary to provide information on these characteristics. In the following section, the common methods used to assess emulsion stability are summarized and compared.

2.1. Visual observation

Emulsion stability affects the products' appearance, and most of the time emulsion instability can be observed directly by the naked human eye. In this sense, visual observation is probably the simplest, cheapest, and quickest method to assess the gravitational separation of the emulsion without expensive analytical instruments [11]. Gravitational separation could be classified into two mechanisms: creaming and sedimentation [1,10]. Creaming happens when the dispersed phase that has a lower density than the continuous phase moves upward and results in a thick separated layer. Conversely, sedimentation happens if the dispersed phase has a higher density than the continuous phase, causing the droplets to move downward. The extent of creaming or sedimentation can be assessed by observing the thickness of creaming or sedimentation layer with naked eyes, and then being instrumentally measured and recorded. However, visual observation, despite its convenience, is not suitable for use to study other instability phenomena, such as flocculation, coalescence, and Ostwald ripening. More often, the creaming layer is only observed when the extent of creaming is considerable. Thus, the initial stage of emulsion stability usually requires the aid of other analytical instruments to make a precise observation.

2.2. Microscopy observation

As visual observation is not sufficiently capable of studying most instability mechanisms as well as droplets smaller than 100 μm [12], microscopy is used to observe the droplets that cannot be viewed by unaided eyes and to examine the factors that influence the stability of the emulsion system. For example, using microscopy, one could easily observe the distribution and dimensions of droplets, and thus obtain information on the cause of the emulsion system's instability.

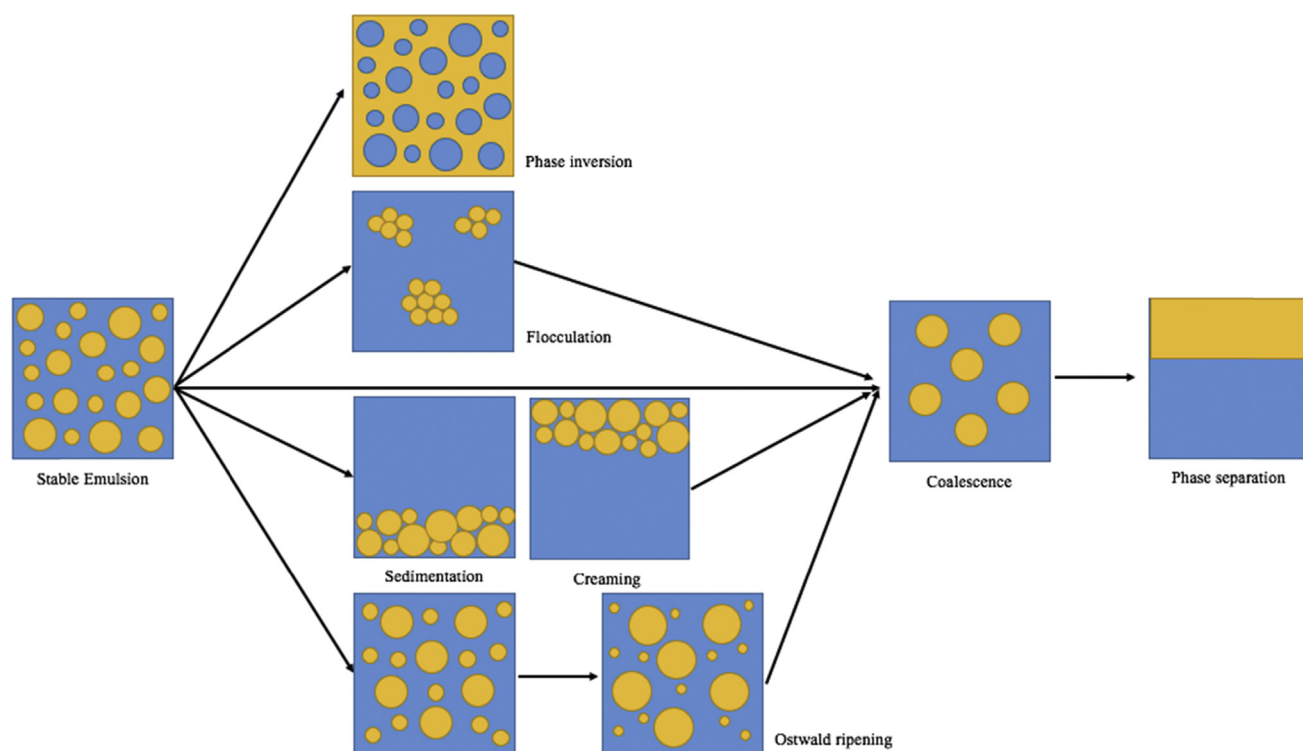


Figure 1 – Schematic representation of instability mechanisms in the emulsion system.

That is, the emulsion is nonflocculated if the droplets are homogeneously distributed in the image with a relatively small size. By contrast, a flocculated emulsion can be identified when the equally larger-sized droplets gather close to one another without merging into bigger ones. However, the emulsion may go through coalescence or Ostwald ripening if there are small and big droplets present at the same time.

To serve the need for different examinations, various types of microscopies, including optical, electron, atomic force, and confocal fluorescent microscopy, have been developed to characterize emulsion stability. Nevertheless, microscopy has several disadvantages [13]: (1) the sample for microscopy observation usually requires preparation procedures, such as dilution and slide-spreading, which may alter the structure of original emulsions; (2) the microscopy measurements are often time-consuming; (3) the results are subjective and difficult to standardize between different laboratories; and (4) multiple analyses on different regions on a single sample slide are often needed for reliable data. Consequently, it is optimal to combine information from other analytical sources such as particle size and surface charge with the microscopy observation to realize the correct instability mechanisms.

2.3. Particle size analysis

According to Stoke's law (Eq. 1) [14], the particle size of the dispersed phase is influential to the stability of an emulsion system, which is composed of two immiscible liquids with distinct density. In this sense, the emulsion with higher resistance and control to creaming should not only contain particles that are small in size, but should also be

homogeneously distributed with relatively low numbers of variations, which is the so-called particle size distribution (PSD).

$$v_{\text{Stokes}} = -2gr^2(\rho_2 - \rho_1)/9\eta_1 \quad (1)$$

In the equation, v_{Stokes} stands for the creaming velocity, r is the radius of the particle, g is the acceleration due to gravity, ρ_1 and ρ_2 are the density of two phases, and η is the shear viscosity of the target system. Following Stoke's law, the stability of emulsion would increase as the droplet size decreases. Therefore, the stability of original emulsions and the extent of gravitation separation after a certain time interval could be studied by determining the droplet size and its concentration at different regions of the sample height under specified temperature and time conditions (Figure 2). In this sense, creaming occurs when the droplet concentration increases in the higher region of the sample, whereas sedimentation occurs when particles accumulate at the lower region. Moreover, by monitoring the change in system PSD, one can also identify the incidence of undesirable droplet interaction, which causes the increase in the number of larger particles over time. However, it is still difficult to distinguish flocculation, coalescence, or Ostwald ripening by simply using particle size analysis. Thus, other analytical methods are usually carried out in combination when determining the change in sample quality during storage.

There are many instrumental particle sizing techniques (Figure 3) including light scattering (static or dynamic light scattering), electrical pulse counting, and ultrasonic spectrometry, which are commonly used [1]. These instruments

have several advantages: (1) they are easy to use; (2) they are fully automated; and (3) they provide substantial information about the droplet size within a short period. Even so, the factors used to convert the experimental data to PSD have to be chosen carefully because different instruments are operated under distinct physical principles.

The light scattering method measures both the droplet concentration and size by detecting the percentage and angle of backscattered light when a monochromatic beam of near-infrared light is directed through the sample [15]. As a larger percentage of backscattered light indicates higher concentration, the particle size is usually angular dependent on where its scattering pattern (number of scattering angles vs. corresponding intensity) can give information on its size distribution. Based on the differences in the operating principle, two types of light scattering instruments are commercially available for measuring particle size: static and dynamic light scattering instrument.

The static light scattering method operates based on Mie's light scattering theory [16] using precisely inputted particle characteristics, such as refractive index, shape, and dimension, to determine the particle size within the range of 100 nm to 1000 μm [9]. Meanwhile, the dynamic light scattering technique measures the intensity fluctuation of scattered light at a specified time assuming smaller particles move faster than larger ones and create a larger rate of intensity flocculation. Dynamic light scattering instruments usually operate using particular scattering angle (fixed or variable) and then mathematically convert the flocculation intensity into PSD, which is used to detect particles of 3 nm to 5 μm in size [9].

For both types of light scattering instruments, the information on size distribution is more accurate if the particle concentration is low, which could prevent the occurrence of multiple scattering effects. The multiple scattering phenomenon is a common problem that causes overestimation of particle size, and thus, the sample is usually diluted before being subjected to light scattering measurement [17].

Electrical pulse counting techniques measure the emulsion droplet size and distribution by detecting the magnitude of electrical conductive variation when passing the emulsion through a small aperture in the electrode [18]. Because oil has a much lower electrical conductivity than water, the electrical current decreases each time when an emulsion droplet passes through. The size of emulsion droplet could be calculated

based on the fact that larger particles move slower and create larger electrical pulses. The setting of the electrical pulse counting techniques include two electrodes contained in a glass tube with a tiny hole, from which the emulsion sample is sucked and passed between the two electrodes. This method is suitable for measuring particles with a diameter range between 0.4 μm and 1200 μm [9].

However, the measurement is limited by the hole size on the glass tube and may require constant changes in the comparable apparatus to accommodate the entire size range. Moreover, the samples used for this analytical method usually should be diluted because the measurement is strongly affected if it is too concentrated and does not allow smooth passage of single particles into the glass tube. Thus, this method, in particular, is suitable for particle size analysis but not for studying the flocculation of the system because the dilution will break up the gathered particles and lead to misinterpretation of the sample stability.

Ultrasonic spectrometry, unlike the light scattering method and electrical pulse counting techniques, can be used to measure the sample with higher particle concentration up to 50% [19]. Ultrasonic spectrometry measures the level of attenuation in the frequency of ultrasonic velocity and relates it to the PSD and concentration in the sample—that is, the ultrasonic spectrum varies when the ultrasound wave is transmitted through the continuous and dispersed phases, where velocity decrease and attenuation increase at contact with the disperse particles [20]. Ultrasonic spectroscopy is able to determine the particle diameters within the range of 10 nm to 1000 μm at relatively high droplet concentrations [9]. Furthermore, the fact that the sample need not be diluted makes ultrasonic spectroscopy more advantageous than other particle sizing instruments where nondestructive and noninvasive characterization of the concentrated and optically opaque sample is possible.

2.4. Charge analysis

Another important factor that affects the stability and physicochemical behavior of emulsion is the electrical charge on its droplet surface. The magnitude and sign of the charge on the dispersed emulsion particle stem from the ionization characteristic of the absorbing emulsifier molecule on its surface [1]. Moreover, the charge characteristic is, most of the time, sensitive to the pH and presence of other

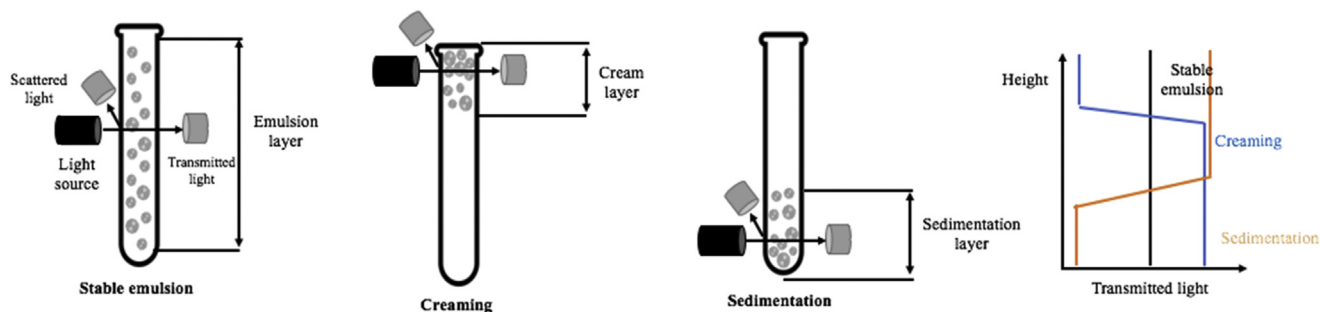


Figure 2 – Schematic representation of studying emulsion stability to creaming and sedimentation using light scattering method.

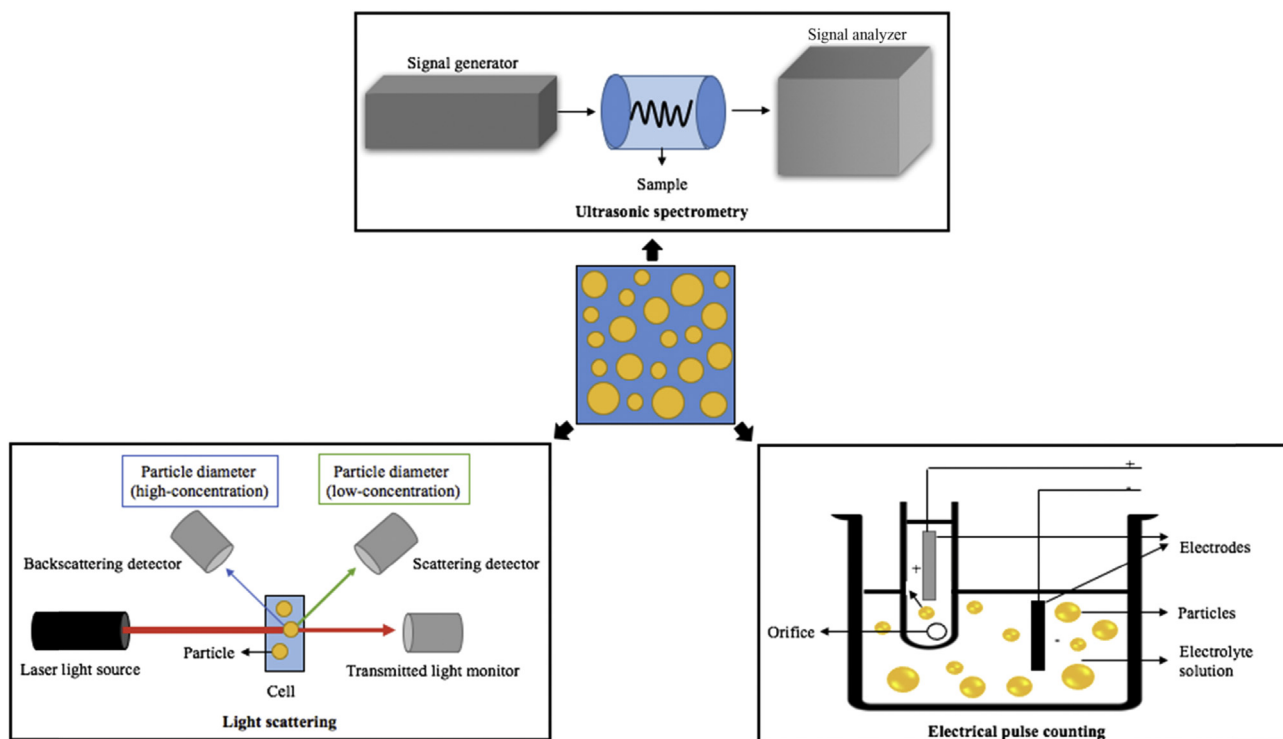


Figure 3 – Schematic representation of evaluating particle size distribution and stability of emulsion system using light scattering, ultrasonic spectrometry, and electrical probe counting.

charged species in the surrounding aqueous phase. Thus, the surface charge of the droplet often determines the nature of the interaction between the droplets and other charged species close by. Electrostatic repulsive forces occur between the droplets with the same sign of surface charge, which will deter particle flocculation and inhibit the propagation of most of the emulsion instability pathway [1,10]. By contrast, electrostatic attractive forces appear when particles with opposite charges come in contact and, depending on the magnitude of the attraction force, the phenomenon of droplet instability may result from different destabilizing mechanisms. That is, droplets tend to gather close to one another but do not physically merge into a larger particle when the magnitude of the attractive force is weak. However, coalescence occurs when the attractive forces are strong, leading to the appearance of a bigger droplet at the expense of smaller ones. Consequently, the particle charge could strongly affect the emulsion stability and, thus, it becomes especially important for investigators to study the electrical properties of droplets contained in an emulsion system. To serve this purpose, two commercially available analytical instruments are used to measure the electrical charge and its distribution on the surface of emulsion droplet: microelectrophoretic techniques and electroacoustic spectroscopy.

Microelectrophoretic techniques measure the electrochemical characteristics of a droplet surface based on the velocity and direction of that charged droplet in an emulsion move when an electrical field is applied [21]. The sign of the electrical charge is determined by monitoring the direction of the droplets' movement because charged droplets are

attracted to oppositely charged electrodes. The velocity, at which the charged droplets move toward the opposite charged electrode, is dependent on the magnitude of the electrical charge on the droplet surface. Moreover, light scattering measurement is usually combined in this analytical method to examine the concentration and distribution of the charged particle in a given sample volume [22]. Again, a sample analyzed using light scattering techniques should be diluted to a lower particle concentration to avoid multiple scattering effects. However, one should be especially careful when selecting the dilution medium because the electrical characteristic is sensitive to changes in the surrounding aqueous environment.

Electroacoustic spectroscopy operates in a similar manner to most ultrasonic techniques, which is usually nondestructive to the sample being analyzed. Unlike the microelectrophoretic method, which requires sufficient dilution of a sample to make a meaningful measurement, the electroacoustic technique can be used to study concentrated samples that contain up to 50% of particles in a given volume. This technique couples ultrasonic and electric field and is designed in two operating modes: Electro-Sonic Amplitude (ESA) and Colloid Vibration Potential (CVP) [23]. For instruments using the ESA mode, radio frequency signal is applied to an emulsion causing particle oscillation, generating a responsive acoustic signal, which is later recorded. In the CVP mode, emulsion is hit by a longitudinal acoustic pulse, inducing the vibration of charged particles and thus generate an electric signal to be recorded [24]. The recorded signal from both ESA and CVP mode could be converted to zeta potential using a suitable mathematical model.

2.5. Rheology

According to Stoke's law [14], the resistance of the emulsion system to instabilities caused by particle flocculation is higher when the viscosity increases. Thus, the flow properties of emulsions, which are also known as rheological behaviors, are necessary physical attributes that significantly affect the quality of these products. For better product control, many analytical instruments including shear and compression devices are developed to monitor and characterize the rheological behavior of an emulsion system [25]. Besides playing an important role in product stability, rheological characteristics are also important factors when assessing mixing efficiency, power consumption, pump ratings, etc., and thus provide solutions to some technical problems encountered during manufacturing.

Factors that could affect the rheological properties of emulsions include viscosity and chemical composition (pH, electrolyte concentration, etc.) of the continuous phase, volume fraction of the dispersed phase, droplet characteristics (size distribution, deformability, internal viscosity, concentration, and particle–particle interaction), and elasticity of the emulsifier film, which is dependent on the concentration and type of emulsifier used [26]. The flow properties of emulsions could be described and specified by many moduli. For example, linear viscoelastic properties are commonly characterized by frequency-dependent storage and loss moduli as well as time-dependent creep moduli. Shear viscosity, a nonlinear steady-state property, is considered a Weissenberg effect. In addition, effects of different parameters on the rheology of emulsions can be investigated and predicted based on the Krieger–Dougherty microstructure/viscosity equation.

A rheometer, or viscometer, is an important instrument for measuring the rheological properties of emulsions. Depending on the concepts and applications, rheometers applying either shearing or compressing deformation force could be engineered to study liquids, solids, or other viscoelastic samples giving information on viscosity, yield stress, elastic modulus, and fracture behavior [27]. Shear devices, such as shear viscometers and dynamic shear rheometers, are more suitable for samples that are fluid and viscoelastic fluidlike emulsions, whereas compression devices are better for testing viscoelastic, plastic, or solidlike emulsions [28].

Because rheological behavior highly depends on product temperature, the shear device giving information on the relationship between stress and strain usually includes a temperature-controlled measurement cell [28], in which sample is placed and then subjected to the controlled shear stress being applied at a predetermined rate. According to the application needs, the measurement cells could be further divided into different types: concentric cylinder, parallel plate, cone, and vane. For current applications, shear devices are most often used to study the elastic modulus, yield stress, fracture stress, and fracture strain of solid samples; the apparent viscosity of a fluid at constant stress; and the complex dynamic shear modulus of a solid, fluid, or viscoelastic sample at sinusoidal stress.

Compression devices are commonly used to analyze solid or semisolid foods such as food gels, butter, margarine, and

ice cream [29]. Devices including capillary rheometers, rotational rheometer, falling-ball rheometers, oscillatory rheometers, torque rheometers, and interfacial rheometers, are the commercially available types of rheometers that utilize compression force for sample analysis [30]. Compression devices include a fixed plate where samples could be placed and a vertically movable probe, which could be a flat plate, spike, blade, or a set of teeth. According to the dimensions of samples and the force applied by instruments, compression devices analyze the stress and strain that samples receive and then convert them to numerical information, such as elastic modulus, fracture stress, fracture strain, and yield stress. However, it should be noted that error may occur during data mining because the friction between the fixed plates and the analyzed sample could also generate shear and compression forces [31]. Thus, control of measuring condition and calibration procedure should be undertaken carefully.

3. Emulsion appearance and morphology

Emulsification gives food products the characteristic appearance and sensory properties that significantly influence the consumer's perception of such products. As human sensation is complex, food processors usually require numerical standards for better quality control. In addition, quality standards become even more important when manufacturers develop new product formulation or adapt innovative processing procedures. The first impression of the consumer toward any food product is usually formed when the buyer is viewing its outward appearance. Thus, having a standardized presentation is critical for food producers to secure their customers' repeated purchase of food products. As visual qualities can be easily observed by the naked eye—product color, light transmittance, flow consistency, presence and absence of phase separation, and sedimentation—it is especially important for the product to include emulsion as part of its matrix. Thus, in this section, the analytical methods used to study the appearance as well as macro- and micromorphology of the emulsion system are overviewed.

3.1. Appearance

The appearance of food emulsion depends on its interactions, such as scatter, absorb, reflect, and transmit, with the light waves in the visible region of the electromagnetic spectrum [32]. When a light beam irradiates onto the emulsion surface, most of the light is transmitted into the emulsion system with a part of it being scattered by emulsion particles and a part of it being absorbed by chromophores in either the continuous phase or the dispersed phase [33]. The amount of light being scattered mainly determines the lightness of emulsion, which can be perceived as turbidity, cloudiness, and opacity. By contrast, the fraction and wavelength of light being absorbed by the system decide the color of the emulsion, such as blueness, greenness, redness, or yellowness [34]. According to the aforementioned aspects, the combination of lightness, turbidity, and color together make up the major part of emulsion product appearance. Thus, methods used to

evaluate the turbidity, lightness, and color of emulsion are presented.

3.2. Absorbance and turbidity spectra, and spectral reflectance

The ability to absorb and scatter light are the two most critical factors that determine emulsion turbidity, which directly influences the appearance of emulsion. Therefore, researchers usually study the absorbance and turbidity spectra of emulsion systems and relate the data to its perceivable appearance [35]. The UV–visible spectrophotometer is most often used when detecting the absorbance and turbidity level of the emulsion. To conduct analysis, a sample is placed in a quartz cuvette of 1-cm width, where the electromagnetic wave at the visible region (380–780 nm) would perform a thorough scanning of the emulsion sample. Once the scanning is complete, the absorbance and turbidity spectra of measured values are corrected using the value for distilled water, which is defined as the most transmittable liquid [36]. As for spectral reflectance, the sample is measured using the UV–visible spectrophotometer with an integrating sphere attachment, and the value is corrected relatively to a standard white plate made of barium sulfate (BaSO_4) [36]. This method is popular and standardized among researchers in this field. Thus, the data obtained from such analysis are comparable across different research facilities.

3.3. Color

Color aspect is probably the most often described attribute of a given food system. However, it is rather difficult for humans to perceive quantify and precisely describe the color variation of a given sample. Hence, the quantification of color in food is usually carried out using a specially designed instrument, which provides numerical information by evaluating the “tristimulus coordinates” of the specimen [34]. In the case of emulsion, the tristimulus coordinates are often measured by an instrumental colorimeter that uses the L , a , b color space system to represent the color information. The L aspect represents the lightness, whereas a and b represent the color coordinates. Normally, the L value ranges from 0 to 100, and as the L value gets closer to 100, it means that the color of emulsion is lightest, and vice versa. Furthermore, the a axis extends from red ($+a$) to green ($-a$), whereas the b axis ranges from blue ($-b$) to yellow ($+b$) [34]. Color is affected by certain factors such as droplet size, concentration, refractive index of the dispersed phase and the aqueous phase, and the type of dye used and its concentration.

3.4. Morphology

Morphology, the microstructure of emulsion, could not be observed by unaided eyes once it is smaller than $100\ \mu\text{m}$ [12,13]. Therefore, various types of microscopes are used to observe and obtain structural information on the surface and interior of the emulsion. Even though simple emulsion is a mixture of two immiscible liquids, such systems are commonly altered to contain various features that serve

specific functions including stabilizing, encapsulating, protecting, and controlling release kinetics, and enhancing the absorption of food components. In this sense, various types of emulsion systems—such as nanosized emulsion, solid lipid emulsion, self-emulsifying emulsion, layer-by-layer emulsion, swell-resistant emulsion, gel emulsion, and pH-responsive emulsion—are developed for various applications [3]. The study of emulsion structure using microscopic techniques is especially used to obtain direct and reliable information. Moreover, microscopic observation can also provide useful information on emulsion particle size distribution and the progression of instability. In the following section, various types of microscopic techniques are summarized and compared in terms of their uses for different samples and conditions.

3.4.1. Optical microscopy

Optical microscopy is the most widely used type of microscopy for characterizing emulsion microstructures because it is relatively inexpensive, easy to operate, and available in most research facilities. Most of the time, the optical microscope is linked to a digital camera for capturing images of the emulsion microstructure, which is later stored and further analyzed by a computer. The optical microscope could be the first screening tool to obtain information on the shapes and size of emulsion droplets. However, optical microscopes have many limitations when used in emulsion science. For example, most optical images of the emulsion microstructure require flat sample preparation, and most of the time they only show the spherical drops, and therefore much useful information is missing. With the level of resolution, it is difficult to distinguish the compositions of emulsion system—whether it is made of proteins, polysaccharides, or other amphiphilic small molecules. Detailed information on the physical status of the dispersed phase and its three-dimensional microstructures such as fat crystals, air bubbles, and ice crystals are also limited when only the optical microscope is used for analysis. In addition, the contrast between different components in emulsions is often poor because they have similar refractive indexes, which makes it difficult to distinguish one from the other.

To overcome these problems, the optical microscope is usually combined with additional sample preparation techniques. Many stains and dyes that absorb light in the visible region of the electromagnetic spectrum are usually added to specific components or dissolved in either the oil phase or the aqueous phase of emulsion before samples are subjected to optical microscope observation [37]. The inclusion of a coloring procedure prior to observation allows the components of interest to stand out from the rest of the emulsion system being highlighted and efficiently studied. However, special attention should be given in the selection of the coloring agent because some staining agents may interact with the compositional material and alter the emulsion microstructure. Alternatively, selecting an optical microscope with specialized feature changes observation conditions, which could also improve contrast when suitable chemical stains are unavailable. Phase contrast or differential interference contrast microscopy can improve contrast by using special lenses that

convert even small differences in the refractive index into meaningful differences in light intensity [37]. Polarization light microscopy is another useful tool when examining the morphology and structure of fat crystals present in an emulsion sample [13].

3.4.2. Confocal laser scanning microscopy

The confocal laser scanning microscope was developed to overcome the limitations of the traditional wide-field fluorescence microscope. In the confocal microscope, point illumination and the spatial pinhole at the confocal plane of the lens could eliminate out-of-focus light and result in better optical resolution and contrast compared with the wide-field fluorescence microscope [38]. A confocal microscope can also construct good three-dimensional images and surface profiles of samples using its special three-dimensional scanning feature. However, confocal microscopic observation may be undesirable for samples that are sensitive to light or are constantly changing because it requires longer exposure time for enough signal intensity.

As some samples naturally fluoresce, a nonfluorescing sample requires the incorporation of a fluorescence agent to be seen in the confocal microscope (Figure 4). Various types of fluorescent dyes can be used for distinguishing the distribution of different components in the emulsion when subjected to fluorescent or confocal fluorescent microscopy. For instance, oil-soluble Nile red that can be excited at 488 nm and then emitted above 515 nm [39] is commonly used to stain the oil phase, whereas ANS is fluorescent stain specific for proteins. According to Yang et al [40], using Rhodamine B (red) and Nile blue (green) as fluorescent dyes to respectively stain the protein and oil phases gives a clear indication of the special arrangement of the emulsion in their system, in which the oil phase is green, the protein phase is red, and their overlapping region is yellow.

3.4.3. Electron microscopy

Electron microscopy (EM) is one of the most powerful tools used to investigate the morphology and microstructure of emulsions. There are two types of electron microscope: transmission electron microscope (TEM) and scanning electron microscope (SEM). TEM obtains high-resolution images by directing a beam of electrons onto the specimen, in which the electron is transmitted and interacts with the molecule contained [41]. With TEM, the investigator can examine the fine details of the microstructure, crystalline state, and projection of the specimen. For a more efficient transmittance of electrons, the thickness of the sample used for TEM should be ultrathin, usually < 200 nm [42]. Unlike TEM, which relies on the transmission of electrons through the specimen, SEM is used to scan the specimen with a focused beam of electrons that interact with atoms in the sample and then produce images of the sample, giving information on its surface topography and composition [43]. Because electrons do not have to be physically transmitted through the sample, the thickness of specimen is usually not strictly specified and the acceleration voltage of electrons could also be much lower than that in TEM. Although EM can provide the three-dimensional surface structure and detailed microstructure (< 5 nm) of the specimen, sample preparation is time consuming and may alter the structure if the proper pre-experiment procedure is not followed.

Despite the advancement in microscopic instrumentation, the optical microscope is still by far the observatory instrument used most in many laboratories because it is relatively inexpensive and easy to operate. Therefore, it is frequently used to obtain information on the shapes and size of droplets in the emulsion in the initial formulation stage. However, because optical microscopes do not provide enough information for identifying the emulsion compositions, fluorescence microscopes, such as a confocal microscope, are usually

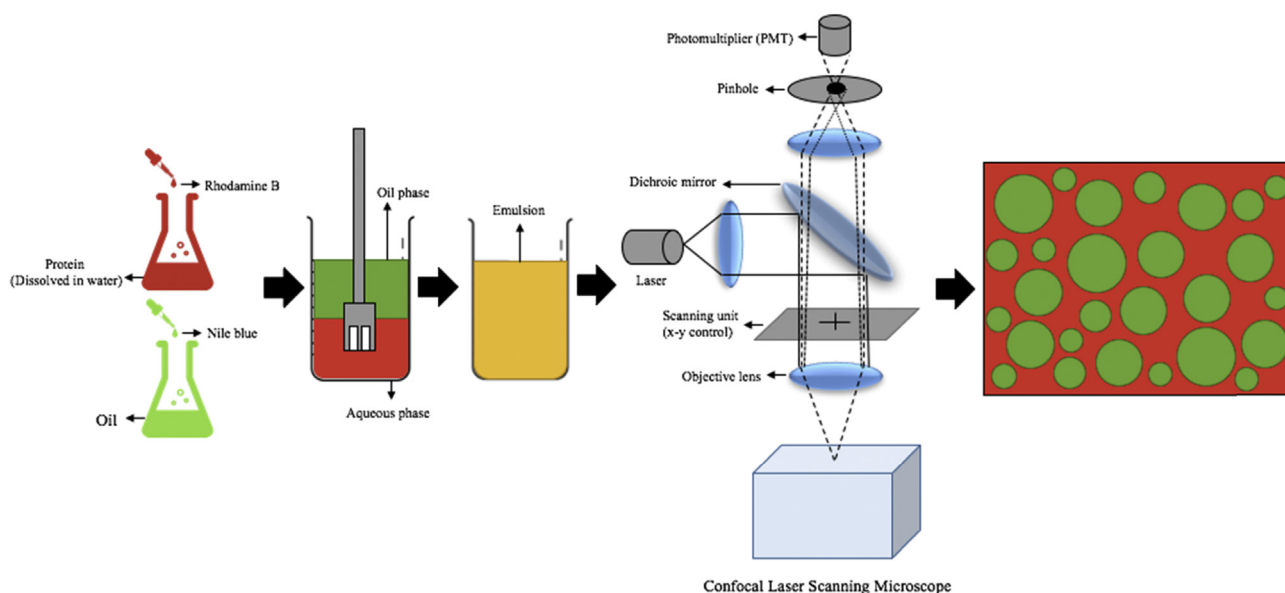


Figure 4 – Schematic representation of characterizing the emulsion microstructure using confocal microscopy with the addition of fluorescence dye.

used in combination to provide more useful information on the distribution and structure of the compositional materials. To further examine the topological structure of specimens, TEM and SEM are also used to supplement the study.

To study emulsion microstructure, all types of microscopic observations share common shortcomings: (1) sample preparation alter the original structure; (2) microscopy analysis are often time consuming; (3) the results are subjective; and (4) large sampling size is required to obtain reliable data. Consequently, it is necessary for investigators to select suitable instruments for their specific samples and to follow proper sample preparation procedure when performing the analysis.

3.5. Emulsion encapsulation

In recent decades, scientists have expended huge efforts to discover health-promoting bioactive ingredients from natural sources. These bioactive compounds are nowadays known as nutraceuticals, which include compounds in a wide spectrum of chemical structures such as protein, peptides, polysaccharides, fiber, fatty acids, and phenolics [44,45]. The efficacy of nutraceuticals to promote better health conditions and to prevent the development of chronic diseases have been extensively researched and published in numerous papers. In order to obtain health benefits from nutraceuticals, repeated ingestion is necessary and, thus, the most feasible way for the general population to consume such compounds is through the oral route as it is noninvasive and convenient for a liberal dosing schedule [3].

However, bioactive compounds isolated from natural sources often cannot be added directly into food systems for the following reasons: (1) incompatibility with the food matrix; (2) rapid degradation during food processing; and (3) vulnerability to digestion activity in the biological system [3]. Thus, these bioactive ingredients often need to be encapsulated for better protection and easier incorporation into the food matrix. For instance, bioactive proteins could degrade rapidly in the gastrointestinal (GI) tract as a result of their instability in the acidic environment and be digested by proteolytic enzymes. Moreover, hydrophobic compounds, such as flavonoids, have a poor aqueous solubility that causes challenges in their incorporation into food as well as being absorbed in the digestion tract.

Luckily, many research studies also reported that the absorption of hydrophobic compounds significantly increases their bioavailability in the biological system when consumed with lipids. With lipids contained in their matrix, emulsions that are commonly seen in many foods have become a favorable vehicle for encapsulation, in which nutraceuticals can be protected, stabilized, and integrated into real food systems [46]. Besides nutraceuticals, flavor compounds that are either highly volatile or easily oxidized are another compound category that can be incorporated into the emulsion system for higher flavor preservation in food products [47]. Encapsulated flavor compounds, such as citral, have shown better stability and longer retention of their characteristic aroma [48].

To evaluate the efficiency of an emulsion-based system when encapsulating bioactive compounds, parameters including encapsulation efficiency, storage stability, and

release profile are of specific interest among investigators. To determine these parameters, it is necessary to quantify the amount of target compounds remaining in the emulsion system right after processing, at a specific time of storage, and during digestion. When determining their encapsulation efficiency, the amount of bioactive components contained in the unit barrier of such a system is quantified relative to the original input concentration. Later, the release profiles—meaning the amount of components released from the barrier at a particular time or environment—are also another critical factor when describing the behavior of encapsulation in the biological environment. As an example, Choi et al [49] encapsulated fish oil by β -cyclodextrin using an emulsion–diffusion method where they defined these essential parameters including actual loading, encapsulation efficiency, and loading content as follows:

$$\text{Actual loading (g)} = \text{Total content (g)} - \text{free content (g)} \quad (2)$$

$$\text{Encapsulation efficiency (\%)} = [\text{Actual loading (g)}/\text{Total content (g)}] \times 100 \quad (3)$$

$$\text{Loading content (\%)} = [\text{Actual loading (g)}/\text{Total mass of system (g)}] \times 100 \quad (4)$$

In reference to these equations, higher loading of the target components in the barrier (emulsion droplet) is positively related to higher encapsulation efficiency. Moreover, emulsion systems can be specially formulated with functionality. One of the interesting mechanisms is the target release properties, where the system will disintegrate at a particular time or site, and thus release the molecules in a designated time and location [47]. Therefore, to evaluate the successfulness of an emulsion system in meeting its target functionality in the food system, it is very critical for investigators to quantify the concentration of bioactive compounds in the system and in the surrounding environment. To evaluate the compounds contained in the system, several common methods used across different laboratories are summarized in following sections.

3.5.1. UV–visible spectrophotometry

UV–visible spectrophotometry is usually used for qualitative analysis of analytes with chromophores, such as nitromethane, and auxochrome, such as benzene. The wavelength UV light ranges from 200 nm to 400 nm, whereas the wavelength of visible light is 400–800 nm. These analytes could absorb energy from electromagnetic waves of 200–800 nm, and excite their peripheral electrons to change from ground state to excited state. To return to the ground state, the excited electrons would simultaneously release the absorbed energy [50]. These analytes with different chemical structures would absorb energy from a particular light wavelength, and, thus, could be differentiated according to their characteristic absorption spectrum [51]. However, caution must be taken when analytes absorb light wavelength at a similar region, as the identification of these compounds may not be accurate.

UV–visible spectrophotometry can also be used for qualitative analysis based on Beer–Lambert's law:

$$A = \varepsilon \times l \times c, \quad (5)$$

where ε is the molar absorbance coefficient of analytes (L/mol cm), l is the path length (cm), and c is the concentration of analytes (molarity). Besides Beer–Lambert's law, the carefully constructed standard curve and standard addition method is also valid for the qualitative analysis of target compounds. When determining the encapsulation efficiency of emulsion, encapsulated compounds that contain chromophore and auxochrome can be extracted by solvents and then analyzed using UV–visible spectrophotometry. Common equipment available for detecting the absorbance of encapsulated analytes at a particular light wavelength include the spectrophotometer, ELISA (enzyme-linked immunosorbent assay) reader, photodiode array detector, and nanodrop. When using the spectrophotometry method, particular caution should be taken when choosing the extraction solvent because the absorption spectrum may deviate when a different solvent is used.

3.5.2. Chromatography

Chromatography is especially useful when separation of pure compounds from a mixture is required [52]. In this method, compounds are separated from mixtures based on the partitioning of a sample between a mobile phase and a stationary phase. With the advancement in technology, chromatography has been developed to handle quantitative and qualitative analysis of samples based on the fact that different compounds are partitioned differently and thus the length being retained on stationary phase is variable. There are many methods to classify chromatography. With the difference in the mobile phase properties, chromatography could be subdivided into liquid chromatography (LC), gas chromatography (GC), and supercritical fluid chromatography. With different stationary phases, thin layer chromatography, paper chromatography, and column chromatography are available for different analytical purposes [53]. Lastly, chromatography can also be engineered utilizing partition, absorption, ion exchange, size exclusion, or affinity as their force of separation.

Among all chromatography instruments available, high-performance LC (HPLC) and GC are more commonly used for the quantitative and qualitative analyses of encapsulated compounds. In GC, the stationary phase is a microscopic layer of liquid or polymer on an inert solid support, whereas the mobile phase is gas. Only compounds that are volatile and nonpolar and have a low boiling point are suitable for GC analysis because they are to be eventually vaporized into gas for detection. In HPLC, the stationary phase is solid and the mobile phase is liquid. HPLC is an effective and convenient technique to analyze encapsulated compounds if they can be separated by their difference in polarities [54]. However, the fact that the chromatography method separates analytes by their retention time in the stationary phase may cause overestimation of the compound concentration if there are compounds with similar retention strength present at the same time. Thus, proper sample preparation and selection of stationary/mobile phase

are important for the accuracy of this method. For better quality control, researchers usually combine mass spectrometry and chromatography to validate the result.

4. Conclusion

As wide variety of food products contain emulsion as part of their complex matrix, it is reasonable for investigators to select emulsion as a convenient vehicle to incorporate functional ingredients that can provide food with better physico-chemical, sensory, nutrient, and health-promoting qualities. This review article highlighted the common characteristics that would affect the quality and effectiveness of the designated emulsion system to meet its requirement to serve as a medium adding value to existing food systems. The overview of techniques and methods that are used to analyze and study such characteristics are provided and compared. Owing to the complexity of the food system, the evaluation of certain critical features usually requires more than one analytical means to provide unbiased information of such delicate systems. With the references provided in the work, one can obtain a good understanding of the different analytical methods and make decisions to select the most efficient means of obtaining useful information that can help to develop an effective emulsion system for various uses.

Conflicts of interest

The authors declare no conflict of interest.

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