

Confocal Laser Staining Microscopy for Microstructure Observation on Starch-Based Food Systems

Gusti Setiavani^{1,2}, Sugiyono³, Adil Basuki Ahza⁴, Nugraha Edi Suyatma⁵

^{1,3,4,5}Department of Food Science and technology, IPB University (Bogor Agricultural University), Dramaga, Bogor, East Java, PO BOX 220 Bogor 16002, Indonesia

²Agricultural Development Polytechnic of Medan, Jalan Binjai KM. 10, Medan, North Sumatera, PO BOX 2000, Indonesia



Abstract - The research on starch-based food products using CLSM remains limited. Therefore, this paper provides the basic principles of CLSM coloring methods which is the development of a conventional confocal microscope, and its application in the starch-based food field. The principle of CLSM reflects on certain light to produce 3D images, which uses fluorescent probes with the right wavelength to clearly visualize objects. Some fluorescent probes often used to color starch are APTS and FITC. Aside from its function in observing the structure of starch, CLSM tends to provide changes in starch granules due to the processing, storage and presence of other components such as protein, fat, and hydrocolloids. Furthermore, in a complex system consisting of various constituent materials, it requires modifications in its coloring technique to assure that the structure of the system is clear and intact. Therefore, CLSM is an excellent microscopy approach to observe the microstructure of starch-based food.

Keywords - Starch, Confocal Microscope, Fluorescents, APTS, FITC.

I. INTRODUCTION

Starch is a polysaccharide that plays a very important role in the food system. These past few decades, research conducted on it has broadly expanded to include microscopy. Analysis on food is an archetypal multidisciplinary subject that reflects the need for a holistic scientific approach, which combines biology, chemistry and physics, and scientific disciplines, such as microscopy [1]. The microscopy approach helps to correlate food structures at the meso scale with physicochemical properties, textures and sensory attributes. Several approaches are used to observe the microstructure of starch or emulsions such as optical microscopy (OM), SEM, TEM, atomic force microscopy (AFM), and confocal laser staining microscopy (CLSM) [2].

Confocal Laser Scanning Microscope (CLSM) is one of the widely used optical equipment due to its ability to provide 3D images better than other devices. It is a form of optical

microscope with many similarities to the epifluorescence. Internationally, the CLSM application has been highly developed, and research based on it, is not only limited to the structure of living cells, but to other various fields. In Indonesia, research on CLSM is currently biological which tends to observe plant, animal or biomedical cell microstructure living in tissue. Furthermore, its applications in the food sector, especially in the starch-based products remain limited due to lack of socialization and knowledge.

II. BASIC PRINCIPLES OF CONFOCAL MICROSCOPES AND THEIR DEVELOPMENT

The original source of confocal microscopy was in 1884, when Paul Nipkow designed a rotating disk with holes in the spiral which made it possible to scan images and send them via telegraph cable. In 1980, Petran adopted the Nipkow disk to develop a confocal microscope [3]. However, the principle of confocal imaging was first patented by American cognitive

scientist Marvin Lee Minsky in 1975. This principle uses an epi-illuminated design to remove the unfocused light by scanning its source which crosses the specimen with a pinhole to remove it from the detector. A point source of light is produced by a pinhole placed in front of the zirconium arc source which focuses on the objective lens in the specimen. The light is also focused on the second objective lens with a pinhole, similar to the first [4]. This principle forms the basis for the configuration of the modern confocal system used for fluorescent images. In the Minsky microscope, the image formed is not real but the output of the photodetector translated in the observed region. Further, the image was built on a military surplus oscilloscope screen without facilities for hard copy, which makes its use in biological observations with reference to moving specimens limited.

Several technologies for improving the Minsky confocal microscope have been developed since 1955. These include the use of bright and stable laser light sources, efficient

mirrors and more precise filters, improved scanning and electronic methods for data collection, quantum photodetector for low noise, improved specimen preparation methods, increased ability of computers to process images quickly, elegant software solutions for analyzing images, high-resolution digital screens and color printers, as well as bioinformatics methods for managing images [4]. The weakness associated with Minsky's confocal microscopy system is corrected by scanning the beam across stationary specimens (beam scanning). This method is more practical and produces configurations for imaging biological specimens. Furthermore, the beam scanning methods is divided into single and multiple types. The single beam scanning is achieved using a mirror which is controlled by a galvanometer computer to direct the excitation light to the sample. This method is commonly used in CLSM.

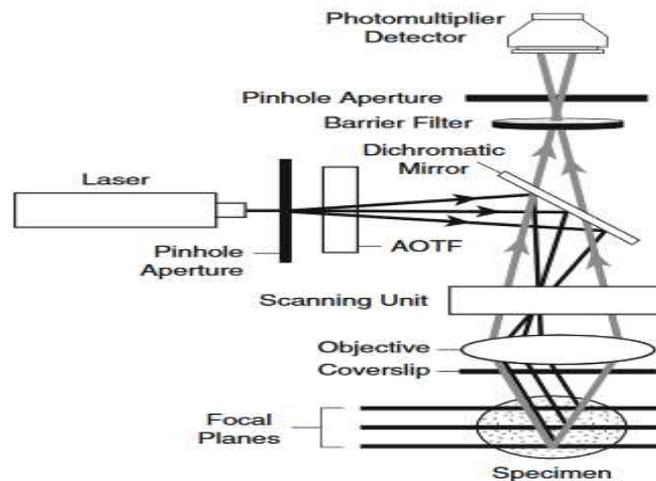


Figure 1. The main components of a modern CLSM laser scan that reflects light.

Light from one or more lasers passes through the eye of the needle, attenuates through AOTF, bounces off the chromatic mirror, and enters the scanning unit. Furthermore, the beam enters the focal plane behind the objective lens, which focuses light at a point in the specimen. The reflected light from the excitation of the flouochrome passes through the objective lens and the scanning unit again. Due to the long wavelength, it passes through a chromatic mirror, cleaned by a barrier filter and finally focused on the second pinhole. Every light passing through the pinhole faces a low noise photomultiplier detector, and the signal is transmitted to the computer imaging system from the confocal microscope [4].

CLSM is an advanced development of conventional confocal microscopy, with many similarities to the epifluorescence. It uses lasers as light sources, sensitive photomultiplier detector tubes (PMT), and computers to control scanning and facilitate collection and presentation [4]. CLSM uses the basic principle of a confocal microscope while the laser beam is focused on the specimen using an objective microscope which excites fluorescence. In a pinhole detector, only certain fluorescent light are detected through a dichroic mirror which produces an image [1]. According to Reference[3], CLSM has a mode of epi-fluorescence and epi-reflexion. When working in fluorescence mode, the laser beam is filtered to select different monochromatic wavelengths from one or more lasers (360 nm, 458 nm, 488

nm, 543 nm, 568 nm, 633 nm, 647 nm). Furthermore, a multi-dichroic mirror which reflects the excitation and transmission wavelength is used to produce a specific fluorescence signal covering up to four detectors. Its epireflection mode does not require filter wavelengths. Conversely, semi-transparent mirrors reflect 50% of the laser beam emitted from the objective lens to reach the specimen which is further collected by the objective lens, into the detector. In modern confocal microscopes, images are made from photomultiplier tube output or captured using a CCD camera. It is directly processed in a computer imaging system, displayed on a high-resolution video monitor, and recorded on modern hard copy devices, with results in 2D or 3D [4]. The signal captured through the CCD is amplified and converted from the intensity of photon electrons with varying sizes of 512×512 or 1024×1025 pixels to provide 2D images. The motors connected to the sample, acquire successive x-y focus planes through the z-plane, and produces 3D datasets that are recombined and rendered into 3D projections [1]. The laser source is focused by an objective lens to illuminate a single point, which is observed in the specimen. The scanning device deflects the beam in X / Y, X / Z, or Y / Z dimensions for the scan to focus on the point in the specimen to create an image of the focal plane X / Y, X / Z, or Y / Z [5]. Computer controlled image production allows image analysis and processing while also being used to calculate 3-D surface or volume reconstructions given to specimens [3].

According to Reference [6], CLSM makes the provision of excellent optical cross-sections possible (i.e., deep imaging of structures). It also enables the interference-free of three-dimensional observation of non-defective samples, which cannot be observed with classical Light Microscopy. In addition, computer control and data acquisition creates excellent possibilities for image analysis and processing. According to [1], [4], the advantages of CSLM compared to conventional light microscopes include: (a) Pictures taken in digital form where images of 3D samples with optical parts and digital reconstruction, (b) minimal sample preparation and disturbance, (c) better resolution than conventional optical microscopes, (d) sensitive detection of multiple flux probes, (e) dynamic processes are easy to learn under controlled environmental conditions using appropriate sample stages and rapid acquisition rates, (e) no physical separation is required. Conversely, its weaknesses include: (a) food components usually require labeling with the appropriate fluorescence, which may involve solvents and some processing steps capable of affecting the sample, (b) the surface need to be folded to ensure proper illumination and

signal emission across the field of view and, (c) lateral diffraction resolution is limited to ~ 200 nm.

III. FLUORESCENCE LABEL AND STAINING METHODS

The use of fluorescence labels or probes is very necessary to visualize objects in CLSM because not all food ingredients are autofluorescent. It is a relatively small modifying agent used to modify proteins, nucleic acids, and other molecules [7]. CLSM requires the right fluorescence probe with the right wavelength to visualize the microstructure properly [1], [3]. According to [3] many fluorochromes are sensitive to laser lighting therefore, they tend to whiten the image. Fluorochromes molecules are rich in chromophores, absorb photons and emit longer wavelengths. According to [1], fluorescent light emissions occur at peak wavelengths that are longer than excitation laser light, this phenomenon is known as the Stokes shift. These emissions from fluorescent molecules on excitation with photons form a light source through the initial dye molecule which was in a ground state, S_0 . Photons excite for the dye molecule to absorb energy (EV) and reach an excited state level (S_1') for approximately 1 to 10 nanoseconds. This was followed by dissipation of energy molecules (EM), to an intermediate state S_1 . The dye molecule returns to the S_0 energy level by removing EC with its dissipation occurring as fluorescence energy, in the form of photons [1]. Therefore, the excitation laser beam used in CLSM should be adjusted or close to the wavelength of the dye molecule. The choice of laser excitation rays to be used depends on the specific wavelength of the fluorescent dye molecule without overlapping [2].

There are several types of fluorosens/probe/dye used to label starch such as Aminofluorophore, 8-amino-1,3,6-pyrenetrisulfonic acid (APTS) [8]–[13], Fluorescein isothiocyanate isomer I (FITC) [14]–[19], fluorescamine [9] and Nile blue [20], [21], with APTS and FITC widely utilized. According to ref [22] APTS is one of the specific dyes that reacts with the reduction of amylose and amylopectin in starch granules. It has a more intense ability to color hilum area which contains greater amount of amylose compared to other regions [23]. While FITC is more intensively colored compared to starch in water [17]. It has the ability to dissolve in DMF and used as stock solution before adding the aqueous mixture. This isothiocyanate group is generally quite stable in aqueous solution for a short time, but its activity decreases when stored. Storage of FITC need to be dried, and protected from light at -20°C [7]. According to [21] water-soluble basic oxazine Nileblue dye is a fluorescence staining agent which is very suitable for coloring native starch granules. Each of these probes has different wavelengths (table 1).

Table 1. Wavelength of excitation and emission of fluorescents for staining

Probe Fluoresens	Excitation Wavelength	Emission Wavelength	Reference
APTS	488 nm	425 to 475 nm	[16]
		500 to 530 nm	[10], [11]
		500-600 nm	[13]
FITC	488 nm	500-525 nm	[14], [17]–[19]
Flourescamine	405 nm	406-493 nm	[9]
<i>Nile Red Blue</i>	638 nm	650 nm	[20], [21]

There are three main ways used to label food components with flouochromes, namely; 1. Generic or non-specific labeling through electrostatic bonds (commonly used to label proteins/polysaccharides and hydrophobic modifications for fat), 2. Specific labeling for proteins (immuno labeling), and polysaccharides (bonding lectins), 3. Covalent labeling for protein and polysaccharides. Among the three techniques, specific methods of covalent labeling are often used in several studies of starch [1]. The covalent staining method was used by [10], [24] on a ternary system involving more than two components of a colored material. APTS was further utilized to color starch the RITC for carrageenan, and Alexa for protein, which makes it possible to observe the role of starch in building product structures [10]. References [24] also used CLSM to observe the structure of polysaccharide and starch gel composites using the non-covalent Rhodamin B staining method, and FA for pectin and FITC for carrageenan. Besides the covalent and non-covalent methods, double staining using situ, bulk water, drop, and the rapid freezing techniques were utilized. This method used to label starch and phosphorus molecules inside granules, with APTS, used to color the starch, while the phosphorus was stained with Pro-Q Diamond dye. This double staining method of starch provides very good contrast and clearly shows the structural features in detail along with its molecular distribution [22]. Reference [25] also use the double staining method with FITC for starch and nile red for fat. [26] developed the bulk water, drop, and rapid freezing techniques to research the interaction between components in bread colored with rhodamine. Lucas showed that the technique of rapid freezing and drop significantly influences rheology and microstructure, therefore, it is not recommended. However, the bulk water technique provides realistic and invasive results. Ref [27] used an in situ method to dye polysaccharides which did not require a slicing, freezing and rehydration process for the preparation of the specimen. The colors used are monoclonalrate IgA, JIM7, ConA-Alexa, Flour 488 conjugate, WGA-Alex Flour 488

conjugate. JIM 7 is used to visualize pectin, while polyclonal antibodies are stable under stress conditions and able to color carrageenan properly. WGA tends to localize chitosan, while ConA shows affinity for some hydrocolloids and bound to gelatin.

IV. CONFOCAL LASER STAINING MICROSCOPY APPLICATION IN OBSERVATION OF STARCH BASED FOOD MICROSTRUCTURE

Confocal Laser Scanning Microscopy has been used in food research since the 80th century [28]. Its use in food product research was first published to determine the distribution of water in fat and mayonnaise, as well as microstructure of cheese and bread dough[6]. The results created a new era of food product research. Since then, the use of CLSM in food research has tremendously increased and is used as a primary tool t, which integrates the results of chemical and physical analysis of food products [1](Auty 2013). Its application in food research is quite extensive, at the structure and in observing the effect of processing treatment. Confocal Laser Staining Microscopy is widely used to observe changes in food systems that are affected by processing conditions, storage time, absorption and digestion [2].

4.1 Starch structure and morphology

Confocal Laser Staining Microscopy is a very useful technique for analyzing carbohydrate structure[3]. Starches consist of granules which have varying shapes and sizes such as round, oval, truncated, polyginal, dependent on the source [29]. It is composed of amylose and amylopectin [29]–[31]. The structure of the starch granules is represented by semicrystalline which shows a hierarchical structure layered between the crystalline growth ring and almorph [29], [30]. Amylose is mostly distributed in hilum and circumference of heterogeneous starch granules, and abundant in aggregate as well as elongated granules compared to individuals [32].

CLSM clearly identifies the growth rings and hilum of barley starch granules. Hilum is an area that contains a lot of amylose compared to other parts [23]. The higher the starch amylose content, the higher the fluorescence. CLSM observes the cross section and internal structure of starch granules without disturbing its microstructure [11]. The growth ring, internal channel, and central part of the amorphous are seen with fluorescence intensity in CLSM [9], [22].

When the starch granules are heated, its semicrystalline and 3D architecture are gradually disrupted, resulting in a transition phase from the irregular structure called gelatinization, which are observed by microstructure [33]. Reference [12] observed the internal structure of starch and the phase transition of corn with various amylose and amylopectin ratios during the gelatinization process which is illustrated by the brightness level of starch granules that decreases with increase in temperature. Starch growth rings are clearly observed after it is added to HCl. The semicrystalline growth ring of waxy maize has a slightly higher thickness than normal starch, while those with high amylose, it is not visible. Starch amylose is known to be in the middle of the granule as indicated by a greater level of fluorescence intensity [12], [18]. Furthermore, as the heating temperature increases, > 130 °C the intense fluorescence becomes gradually weaker and the darker area spreads outwards. This indicates damage to the amylose component which gradually disappears [18]. A decrease in ionic strength leads to fewer starch granules in the CLSM, which shows that the gelatinization process gradually occurs from the middle to the outside [34].

Further use of CLSM to observe the deposition of molecular starch has also been carried out. Deposition of potato is a normal and transgenic technique which was modified by using Environmental Scanning Electron Microscopy (ESEM) and CLSM. Unlike the ESEM which presents data on the surface topography of starch, CLSM presents the internal structure of the starch granules more extensively with the right coloring by [11]. In addition, CLSM is used to detect the presence of a minor component such as phosphorus, and minerals. Surface proteins are distributed together in the edge layers of starch granules, forming thin layers or films with strong fluorescence intensity [9].

4.2 Starch - protein system

Starch and protein often interact in systems that affect their physicochemical properties, such as whey protein isolates which changes the structure of tissue in the corn starch matrix. Reference [35] observed the interaction of corn

starch with the addition of whey protein using CLSM. The structure which was initially compact and homogeneous became more complex and fragmented. Also the number of whey protein isolates added, increases the cross-linking between starch and protein [35]. The effect of milk protein on the waxy microstructure of corn starch using CLSM, with emulsion staining and gradual labeling had been studied. A mixture of corn starch and milk protein is labeled using FITC. The results showed an interaction of α -caseinate and β -caseinate with corn waxy granules seen in CLSM images. Further interactions were observed using fluorescence labeling for proteins (using 3-(4-carboxybenzoyl) quinoline-2-carboxaldehyde (CBQCA)). The CLSM image shows that with the addition of α -caseinate, the protein is no longer limited to the continuous phase or the surface of the starch granules but it is penetrated which is extended to the middle in rare cases [36].

Specific image analysis methods are combined with CLSM to analyze structural changes in the starch-protein system. References [37] tried to link the mechanical properties with the microstructure of the Pea composite. This research combines the starch-protein microstructural observations with matlab program to analyze the total area and perimeter of the particles with the image matrix. This analysis method allows structural changes such as protein aggregate fragmentation or starch degradation that are applicable in establishing the composite morphology.

4.3 Starch-fat system

Lipids with certain chain lengths tend to interact with amylose to form amylose-lipid complexes. The impact of this affects not only the nature of the starch paste but also the gel texture [38]. Confocal laser staining microscopy also used to study the stability of free and encapsulated ascorbil palmitate in corn starch before and after spray drying under accelerated storage conditions. Ascorbil palmitate was stained using Nile red and the reverse was the case for maize starch. Scans were carried out at an excitation wavelength of 448 nm and emissions of 550 nm. Fluorescence has the ability to interact with ascorbil palmitate before enzymatic hydrolysis and unable after the procedure. This research captures the possibility of ascorbil palmitate trapped in starch and it is the structure of amylose which indicates the possibility of amylose-lipid complex [39].

The process of frying high-carbohydrate (starch) foods cause heat transfer from oil to ingredients, it also leads to absorption by the material and expulsion of water. This results in structural transformation of the material during frying. Distributing oil to these ingredients increases the fat content.

The location of the oil is observed directly by using CLSM with minimal intrusion methods. This shows that oil on the skin of materials such as the egg-box causes dehydration without penetration [40]. Reference [8] used CLSM to determine the distribution of oil in the starch-oil-water system. Starch was previously used to fry with soybean oil at 180 ° C for 20 minutes. The starch was stained with Nile red probe, and treated with 10, 20, 40, 80% humidity. Samples treated with 10 and 20% humidity had irregular shapes and rough surfaces while those with 40 and 80% exhibit melt and aggregate shapes. This research proves the effect of water on the distribution of oil in starch which cannot be observed with a Scanning electron microscope (SEM) and Polarized light microscope (PLM).

4.4 Starch-hydrocolloid system

The use of hydrocolloid in food is under developed and has become a topic of interest to researchers. These studies are aimed at uncovering interactions or effects of hydrocolloid such as xanthan, guar gum, cytosan, sodium alginate, etc. on starch gelatinization and retrogradation. Some researchers used CLSM to confirm and complete the results in terms of molecular structure. However, a recent research was conducted on three types of hydrocolloid namely galactomannan, XN, and arabic gum which were observed for their effect on retrogradation of lotus starch using a rheometer, NMR, atomic force microscope (AFM) and CLSM. The sample concentration used was 6% lotus starch, and 0.4% hydrocolloid. In addition, observation with CLSM utilized FITC as a dye shot with a 480 nm argon laser at 500-525 nm emission. The results showed the formation of more compact structures in lotus starch and arabic gum samples which confirmed and explained the increase in the value of G' and the rate of retrogradation of lotus starch [41], [14]. Similar research was also carried out by [17], on the effect of hydrocolloid arabic, guar, and xanthan gums on the gelatinization of native corn starch with various analytical methods including CLSM. The previous starch was heated to 90 ° C in RVA and stained using FITC. The results showed guar and xanthan gums significantly reduced swelling of starch during heating. Previously, ref [19] analyzed the effect of corn fiber gum (CFG) pasting properties and thermal characteristics of starch RVA (pasting properties), DSC (thermal), starch swelling, and CLSM (microstructure). The addition of 1.0% CFG looks more compact. Also, this finding is in line with the results of starch swelling analysis and swelling power.

4.5 Application of confocal Laser Scanning Microscopy in complex systems

Confocal Laser Scanning Microscopy is also widely used to observe the interaction of food components in a more complex mixture system. Reference [10] analyzed the interaction between starch-milk-carrageenan using three different dyes for each food component, namely APTS for starch, carrageenan, and protein. Furthermore, Reference [42] observed a microstructure of a cross-environmental mixture of corn starch, whey protein isolates and k-carrageenan. The staining technique utilized covalent and non-covalent methods, when starch is stained with FITC, carrageenan and protein are stained with RITC and ANS respectively. CLSM is also an appropriate tool for visualizing composite structures consisting of starch and polysaccharides and for monitoring their evolution during preparation [24]. According to [21] CLSM combined with thermal treatment through special stages makes it possible to analyze the structure of more complex systems such as bread. CLSM have been used to determine the structure of food and interactions between components of bread [26]. It is also applied to analyze starch-fat-protein systems in extruded products by analyzing the process of starch-fat-protein reorganization in wheat extrusion products using CLSM. The results show that temperatures <130 °C are required to protect and encapsulate lipids in the liquid oat matrix [43].

The use of CLSM is to observe starch in more complex systems that tend to be more complicated due to the duration needed to color each component. Therefore, the double staining method is often used. The characterization of microstructure of several starch-based product formulations which are fried to determine fat absorption, with FITC used to stain starch, and oil with Nile red. In this research, 0.01% (w / w) FITC solution was poured into the bread mixture, printed and fried into oil with Nile red coloring of 0.05 g / l, and double fluorescent labeling combined with CSLM observation. The deeper of microstructure products formulated based on gluten and potato flakes are observed and analyzed. This technique also allows non-invasive characterization of the food matrix and oil deposition through image analysis, to understand the relationship between the inner microstructure and oil absorption capacity [25]. Reference [44] used CLSM to determine the damage to the fat layer in pastry products. The staining method used is non-invasive double staining, with fat used to stain Nile Red and Alexa to dye protein, leaving starch. Both dyes were dissolved into dimethylformamide and applied to the sample (Danish paste). Besides the colored layers of fat and protein, CLSM images also found a lot of empty space thought to be

starch and pastry dough. The same method was carried out to determine the microstructural properties of cake dough as an impact of formulations after it is prepared and baked. Two probes namely red Nile dye to dye fat, and Alexa to dye protein are mixed into one solution in dimethylformamide. In this research it was found that the roasting process affects the distribution of each dough component. After swelling, the starch is surrounded by a network of proteins, while the oil phase separates and forms droplets of various sizes to form heterogeneous structures [45].

V. CONCLUSION

Confocal Laser Scanning Microscopy is a development of conventional confocal microscopy. In principle, it is an epifluorescence, where the rays emitted with certain wavelengths, produce 3D images. To function properly, the CLSM samples are colored. Some of the popular dyes used to analyze starch-based microstructure are APTS, and FITC with different wavelengths of excitation and emission. CLSM application is used to determine the morphological structure of starch, as well as to observe changes that occur due to the presence of other components. In the starch-fat system, CLSM makes it possible to determine complex oil distribution processes. The effect of protein on starch tissue structure and the hydrocolloid on starch gelatinization is explained using CLSM. Furthermore, in more complex systems CLSM has been applied to observe the microstructure of bread dough, pastry, and fat distribution in fried starch.

ACKNOWLEDGMENT

We would like to thank Agricultural Extension and Professionals Development Agency, The Ministry of Agriculture Indonesia for the funding.

REFERENCES

[1] Auty, M. A. E. (2013). *Confocal microscopy: Principles and applications to food microstructures*.
 [2] Drapala, K. P., Mulvihill, D. M., & J. A. O'Mahony. (2018). A review of the analytical approaches used for studying the structure, interactions and stability of emulsions in nutritional beverage systems, *Food Struct.* 16:27–42.
 [3] Dürrenberger M. B. (2001). Visualization of food structure by confocal laser scanning microscopy (CLSM). *Leb. und-Technologie*, 17(1):11–17.
 [4] Paddock S. W. & Eliceiri K. W. (2014) *Chapter 2 Laser Scanning Confocal Microscopy : History , Applications , and Related Optical Sectioning Techniques*, 1075.
 [5] Aguilera, J. (2000). Microstructure and food product engineering. *Food Technol.* 54(11): 56–64.

[6] Heertje, I. Van Der Vlist, P., Hendrickx, H. A. C. M, and Brakenhoff G. J. (1987). Confocal scanning laser microscopy in food research : some observations. 6(2).
 [7] Hermanson, G. (2013). Fluorescent probes, in *Bioconjugate Techniques*, 395–463.
 [8] Chen, L. Tian, Y. Sun, B. Cai, C. Ma, R. & Jin Z. (2017). Measurement and characterization of external oil in the fried waxy maize starch granules using ATR-FTIR and XRD. *Food Chem.*
 [9] Naguleswaran, S. Li, J. Vasanthan, T. & Bressler, D. and corn starches as revealed by Confocal Laser Scanning Microscopy. 88(1): 87–94.
 [10] Matignon A. Moulin G, Barey P, Desprairies M, Mauduit S, Sieffermann JM. (2014) Starch/carrageenan/milk proteins interactions studied using multiple staining and Confocal Laser Scanning Microscopy. *Carbohydr. Polym.*, 99:345–355.
 [11] Blennow, A., Hansen, M., Schulz, A. Jørgensen, K., Donald, A. M., & Sanderson, J. (2003). The molecular deposition of transgenically modified starch in the starch granule as imaged by functional microscopy. *J. Struct. Biol.*, 143(3):229–241.
 [12] Chen, P., Yu, L., Simon, G. P., Liu, X., Dean, K., & Chen, L. (2011). Internal structures and phase-transitions of starch granules during gelatinization, *Carbohydr. Polym.*, 83(4):1975–1983.
 [13] Chung Y., & Lai, H. (2006). Molecular and granular characteristics of corn starch modified by HCl-methanol at different temperatures, 63:527–534.
 [14] Zheng, M., Su, H., You, Q., Zeng, S., & Zheng, B. (2019). An insight into the retrogradation behaviors and molecular structures of lotus seed starch-hydrocolloid blends, *Food Chem.* 295: 548–555.
 [15] Chen, B., Zhang, B., Li, M., Xie, Y., & Chen, H. (2018). Effects of glutenin and gliadin modified by protein-glutaminase on pasting, rheological properties and microstructure of potato starch, *Food Chem.*
 [16] Thaiudom S. & Pracham, S. (2016). The influence of rice protein content and mixed stabilizers on textural and rheological properties of jasmine rice pudding, *Food Hydrocoll.*
 [17] Zhang, Y., Gu, Z., Zhu, L., & Hong, Y. (2018). Comparative study on the interaction between native corn starch and different hydrocolloids during gelatinization, *Biol. Macromol.*
 [18] Chen, X., Du, X., Chen, P., Guo, L., Xu, Y & Zhou, J. (2017). Morphologies and gelatinization behaviours of high-amylose maize starches during heat treatment,” *Carbohydr. Polym.*, 157:637–642.
 [19] Qiu, S. Yadav, M. P., Chen, H., Liu, Y., Tatsumi, E., &

- L. Yin, (2015). Effects of corn fiber gum (CFG) on the pasting and thermal behaviors of maize starch,” *Carbohydr. Polym.*, 115:246–252.
- [20] Schirmer, M., Höchstötter, A., Jekle, M., Arendt, E., & Becker, T. (2013). Physicochemical and morphological characterization of different starches with variable amylose/amylopectin ratio, *Food Hydrocoll.*, 32(1):52–63.
- [21] Schirmer, M., Jekle, M., & Becker, T. (2011). Quantification in starch microstructure as a function of baking time,” *Procedia Food Sci.*, 11.
- [22] Naguleswaran, S., Vasanthan, T., Hoover, R. & Bressler, D. (2013). The susceptibility of large and small granules of waxy , normal and high-amylose genotypes of barley and corn starches toward amylolysis at sub-gelatinization temperatures, *FRIN*, 51(2): 771–782.
- [23] Li W., Xiao, X., Zhang, W., Zheng, J., Luo, Q., Ouyang, S., & Zhang, G. (2014). Compositional , morphological , structural and physicochemical properties of starches from seven naked barley cultivars grown in China, *Food Res. Int.*, 58:7–14.
- [24] Savary, G., Handschin, S., Conde-Petit, B., Cayot, N., & Doublier, J.L. (2008). Structure of polysaccharide-starch composite gels by rheology and confocal laser scanning microscopy: Effect of the composition and of the preparation procedure, *Food Hydrocoll.*, 22(4):520–530.
- [25] Moreno M. C. & Bouchon, P. (2013). Microstructural characterization of deep-fat fried formulated products using confocal scanning laser microscopy and a non-invasive double staining procedure, 118:238–246.
- [26] Lucas, I., Stauner, B., Jekle, M., & Becker, T. (2018). Staining methods for dough systems – Impact on microstructure and functionality, *LWT - Food Sci. Technol.*, 88:139–145.
- [27] Arltoft, D., Madsen, F., & Ipsen, R. (2007). Screening of probes for specific localisation of polysaccharides, *Food Hydrocoll.* 21(7): 1062–1071.
- [28] Rincon, J., Cardona, H. C., & L. M. (2013). *Applications of Confocal Laser Scanning Microscopy (CLSM) in Foods. Confocal Laser Microscopy - Principles and Applications in Medicine, Biology, and the Food Sciences.*
- [29] Amagliani, L., . O’Regan, J. Kelly, A. L., & O’Mahony, J. A. (2016). Chemistry, structure, functionality and applications of rice starch,” *J. Cereal Sci.*, 70:291–300.
- [30] Copeland, L., Blazek, J., Salman, H. & Tang, M. C. (2009). Form and functionality of starch, *Food Hydrocoll.*, 23(6):1527–1534.
- [31] Tako, M., Tamaki, Y., Teruya, T., & Takeda, Y. (2014). The principles of starch gelatinization and retrogradation, *Food Nutr. Sci.*, 05(03): 280–291.
- [32] Cai, J., Man, J., Huang, J., Liu, Q., Wei, W. & Wei, C. (2015). Relationship between structure and functional properties of normal rice starches with different amylose contents, *Carbohydr. Polym.*, 125:35–44.
- [33] Noisuwan, A., Hemar, Y., Wilkinson, B., & Bronlund, J. E. (2011). Adsorption of milk proteins onto rice starch granules,” *Carbohydr. Polym.*, 84(1):247–254.
- [34] Suda, I., Oki, T., Masuda, M., & Kobayashi, M. (2003). Physiological functionality of purple-fleshed sweet potatoes containing anthocyanins and their utilization in foods, 37:167–173.
- [35] Yang, C., Zhong, F., Goff, H. D., & Li, Y. (2018). Study on starch-protein interactions and their effects on physicochemical and digestible properties of the blends., *Food Chem.*
- [36] K. AP, C. V, F. SM, M. ER, O. JA, and F. MA, “Influence of milk proteins on the pasting behaviour and microstructural characteristics of waxy maize starch,” *J. Food Hyd.*, vol. 30, no. 2, pp. 661–671, 2013.
- [37] Jebalia I. Maigret J.E., Reguerre, A.L., Novales, B., Guessasma, S., Lourdin, D., Della Valle, G., & Kristiawan M. (2019). Morphology and mechanical behaviour of pea-based starch-protein composites obtained by extrusion, *J. Carb. Pol.*
- [38] Ohashi, K., Goshima, G., Kusada, H., & Tsuge, H. (1980). Effect of embraced lipid on the gelatinization of rice starch, *Starch - Stärke*, 32 (2): 54–58.
- [39] Bamidele O. P., & Emmambux, M. N. (2019). Storage stability of encapsulated ascorbyl palmitate in normal and high amylose maize starches during pasting and spray dryin, *Carbohydr. Polym.* 216:217–223.
- [40] Pedreschi, F., Aguilera, J., & Arbildua, J. (1999). Confocal Laser Staining Microscopy (CLSM) study of oil location in fried potato slices, *Microsc. Anal.* 37: 21–22.
- [41] Li, Z., Liu, W., Gu, Z., Li, C., Hong, Y., & Cheng, L. (2015). The effect of starch concentration on the gelatinization and liquefaction of corn starch, *Food Hydrocoll.*, 48:189–196.
- [42] Espinosa-dzib, A. Ramírez-gilly, M., & Tecante, A. (2012). Viscoelastic behavior and microstructure of aqueous mixtures of cross-linked waxy maize starch , whey protein isolate and k -carrageenan, *Food Hydrocoll.*, 28(2):248–257.
- [43] Moisis, T., Forsell, P., Partanen, R., Damerau, A., & Hill, S. (2015). Reorganisation of starch, proteins and lipids in extrusion of oats, *J Cereal Sci*, 64: 48–55.
- [44] Bousquieres, J., Deligny, C., Challoy, S., & Lucas, T. (2014). Using confocal laser scanning microscopy to

examine the breakdown of fat layers in laminated dough,” *FRIN*, 62:359–365.

- [45] Hesso, N., Garnier, C., Loisel, C., Chevallier, S., Bouchet, B., & Le-Bail, A. (2015). Formulation effect study on batter and cake microstructure: Correlation with rheology and texture, *Food Struct.* 5:31–41.