



Determination of vitamin D3 encapsulated in liprotide β -lactoglobulin crosslink oleic acid by HPLC and SEM

Penentuan vitamin D3 dienkapsulasi liprotide dalam β -lactoglobulin berikatan silang asam oleat menggunakan HPLC dan SEM

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Article History:

Received: June 15, 2023; Revised: July 3, 2023; Accepted: July 17, 2023; Published: December 5, 2023.

Publisher:



Politeknik Kesehatan Aceh
Kementerian Kesehatan RI

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Abstract

Vitamin D is an unstable compound to light, heat, and certain chemicals, so it is easily degraded under various conditions. Therefore, instability is achieved through the encapsulation method using complex protein carrier compounds with fatty acids known as liprotide. This study aims to determine vitamin D3. Method, the determination of vitamin D3 was analyzed, including the determination of vitamin D3 standard eluents, calibration curve, and determining the retention time of vitamin D3 encapsulated in liprotide using HPLC (High-Performance Liquid Chromatography). Morphology of vitamin D3 encapsulated in liprotide using SEM (Scanning Electron Microscopy). The results showed that the standard vitamin D3 HPLC analysis using acetonitrile: methanol and acetonitrile: aquabidest obtained two peaks. Butanol: n-hexane eluent obtained one peak with a retention time of 2,170 minutes. The results of the standard calibration curve for vitamin D3 at a linearity value of $R^2 = 0,9997$ and $f(x) = 14,928x - 117,930$. The same retention time was obtained for vitamin D3 encapsulated in liprotide. Conclusion, an enlargement of the cubic structure, which occurred due to the encapsulation of vitamin D3 by the liprotide-forming micelles.

Keywords: β -lactoglobulin, HPLC, liprotide, oleic acid, SEM, vitamin D3

Abstrak

Vitamin D merupakan senyawa yang tidak stabil terhadap cahaya, panas, dan bahan kimia tertentu sehingga mudah terdegradasi dalam berbagai kondisi. Oleh karena itu, mengatasi ketidak stabilan dilakukan dengan cara metode enkapsulasi menggunakan senyawa pembawa protein kompleks dengan asam lemak yang dikenal sebagai liprotida. Penelitian bertujuan untuk menentukan vitamin D3. Metode, penentuan vitamin D3 meliputi eluen standar vitamin D3; kurva kalibrasi; dan penentuan waktu retensi vitamin D3 dienkapsulasi dalam liprotida menggunakan HPLC (*High Performance Liquid Chromatography*). Morfologi vitamin D3 yang dienkapsulasi dalam liprotida menggunakan SEM (*Scanning Electron Microscopy*). Hasil penelitian menunjukkan bahwa analisis pada HPLC standar vitamin D3 menggunakan eluen asetoneitril: metanol dan asetoneitril: aquabidest didapatkan dua puncak. Eluen butanol: n-heksana didapatkan satu puncak dengan waktu retensi 2,170 menit. Hasil kurva kalibrasi standar vitamin D3 pada nilai linieritas sebesar $R^2=0,9997$ dan $f(x) = 14,928x - 117,930$. Didapatkan waktu retensi yang sama pada vitamin D3 dienkapsulasi dalam liprotida. Kesimpulan, pembesaran struktur kubik, terjadi karena enkapsulasi vitamin D3 oleh misel pembentuk liprotida.

Kata Kunci: β -lactoglobulin, asam oleat, HPLC, liprotida, SEM, vitamin D3

Introduction

Vitamin D is a fat-soluble micronutrient that plays a role in calcium and phosphate metabolism, calcium homeostasis, vascular health, and cell differentiation and proliferation (Rafeeq et al., 2020; Rodwell et al., 2015). Vitamin D, which is also called the "sunshine vitamin," can provide benefits for the prevention of several diseases, such as multiple sclerosis, cancer, bacterial infections, and diabetes (Alloubani et al., 2019; Buyukuslu, et al., 2014). Vitamin D intake can be obtained from foods such as salmon, fortified foods such as margarine, and supplements. There are not many types of foods that contain vitamin D, and the amount of vitamin D contained in food is very small (Cribb et al., 2015).

Vitamin D belongs to a class of fat-soluble steroid compounds (Zenebe et al., 2014). Vitamin D as we know it is divided into 2 types, namely vitamin D₂/ ergocalciferol and vitamin D₃/ cholecalciferol (Cashman, 2015). Vitamin D₂, or ergocalciferol, is usually sourced from UV radiation. Vitamin D₃ or cholecalciferol is obtained from food and is then absorbed through the intestines using passive diffusion in the form of micelles assisted by fat and bile salts (Sharma et al., 2019). Vitamin D₃, currently consumed by the public, is vitamin D, which is unstable in light, heat, and chemicals. Certain conditions, such that it is easily degraded under various conditions. Although the availability of food sources containing vitamin D is still very limited, it is necessary to develop consumer products to meet the needs of vitamin D. Therefore, the stability of vitamin D in food products requires attention (Cribb et al., 2015).

Proteins and fatty acids are promising candidates for vitamin encapsulation (Fatemeh et al., 2017). Studies have been carried out using protein (β -lactoglobulin) and casein have been used to deliver vitamin D₃, docosahexaenoic acid, and genistein (Anjani, 2014). Research conducted by Chetta et al. (2021) and Fang et al. (2012) used α -Lactalbumin with oleic acid (OA) to form a complex compound that showed cytotoxic activity against tumor cells. Research conducted by Nadeem et al. (2019) examined the function of the protein in α -lactalbumin using a 3-dimensional structure, which has

several similarities to the β -lactoglobulin protein, especially in triple-stranded anti-parallel β -sheets. Based on these similarities, it is speculated that β -lactoglobulin and oleic acid can form complex compounds to deliver vitamin D₃.

The combination of fatty acids and proteins in complex compounds is known as a liprotide. Liprotide can kill cancer cells and bacteria, and research on liprotide has been conducted since 1995. The advantage of protein coating of liprotide is that it can increase the solubility of fatty acids (Kaspersen et al., 2014). This ability has been proven to make liprotide carriers of hydrophobic molecules in hydrophilic environments (Ismail & Csóka, 2017). Liprotides have a core-shell structure, which consists of a fatty acid core in the form of micelles and is covered by partially denatured proteins (Pedersen et al., 2020).

There is a development where apart from being a carrier of fatty acids, protein is a good target for encapsulating vitamin D. One of the fatty acids that play a role in liprotide encapsulation is oleic acid because of its abundant availability, ease of use, effectiveness, and lower cytotoxicity than cis fatty acids. Other. Oleic acid has low solubility; therefore, handling and preparation of oleic acid are very important in liprotide complexes. Oleic acid interacts with proteins in various ways. This causes variations in the oleic acid to protein ratio, which can affect the ratio (Pedersen et al., 2020). Protein function increases the solubility of fatty acids. This ability makes liprotide potent owing to the hydrophobic nature of the molecule in a hydrophilic environment. β -lactoglobulin protein with fatty acids, namely oleic acid, is a type of liprotide that can encapsulate vitamins (Frislev et al., 2016).

Pedersen et al. (2016) used protein and fatty acids to increase the stability of vitamin D₃, one of which was analyzed using HPLC (high-performance liquid chromatography) (Fang et al., 2015). However, there is no explanation in the literature regarding the determination of vitamin D₃ standards and calibration curves in HPLC. The determination of standards and calibration curves in HPLC are fundamental aspects that readers need to know to analyze the level of purity and stability

of synthesized vitamin D. Therefore, we reviewed and covered everything from sample preparation to vitamin D3 determination using HPLC. Furthermore, morphological characteristics were analyzed using Scanning Electron Microscopy (SEM). In this study, vitamin D3 was encapsulated in lipotide β -lactoglobulin-cross-linked oleic acid. The characteristics of vitamin D3 will be analyzed.

Methods

The purpose of this study was to determine vitamin D3 levels, including the determination of vitamin D3 standard eluents, calibration curve, and retention time of vitamin D3 encapsulated in lipotide using high-performance liquid chromatography (HPLC). Morphology of vitamin D3 encapsulated in lipotide using SEM (Scanning Electron Microscopy). This research was conducted at the Diponegoro University Semarang Integrated Laboratory.

Ingredients

The following materials were obtained from Sigma-Aldrich: vitamin D3/cholecalciferol (5,00936,0010), oleic acid (OA, Y0001479), and β -lactoglobulin (BLG, L0130). Support materials, such as potassium hydroxide (KOH, 1310-58-3), absolute ethanol (1,00983,2500), concentrated liquid PBS OmniPur® (6506-1LCN), ultra-centrifugal filter unit Amicon® (UFC901008), and MilliQ water.

Vitamin D3 Encapsulated in Lipotide Method

Vitamin D3 Standard Solution

A 1000 ppm Vitamin D3 solution was prepared by weighing 0,025 g of vitamin D3 and diluting it in a 25 mL measuring flask with ethanol. A vitamin D3 standard solution (1000 ppm) was prepared at 200, 400, and 800 ppm concentrations.

Formulation Liprotides

OA (38 mg/ml) was then dissolved in ethanol. Then, 6 mg/ml β -lactoglobulin was mixed with 1.5 mg/ml OA in KOH (pH 10,5) and incubated for 30 minutes at 45°C. Liprotides (β -lactoglobulin: oleic acid) are cooled after their pH is regulated to 7,4 using PBS solution. Next

stage sample liprotide (β -lactoglobulin: oleic acid) was vortexed (Fang et al., 2015).

Vitamin D3 Encapsulated in Liprotides

Vitamin D3 is dissolved in ethanol, and the resulting liprotide is mixed with different amounts of vitamin D3. The sample (Vitamin D3: liprotide) was vortexed and centrifuged using an ultra-centrifugal filter unit for 8 minutes at 30°C, and 4000 rpm. Hereafter referred to as the end product, Vitamin D3 encapsulation (Fang et al., 2015).

HPLC (High-Performance Liquid Chromatography) Analysis

Optimization

The master solution (1000 ppm) was prepared by weighing the dissolved vitamin D3 in ethanol. This condition was used to determine the standard Vitamin D3 retention time as described in several previous studies.

Analysis was performed using HPLC Shimadzu corp LC20AD® (Serial Number: L20105130725, Kyoto Japan) with an HPLC column C-18 (5 μ m). The HPLC conditions used are listed in Table 1.

Determination of Curve Calibration

The determination of curve calibration on the solution parent vitamin D3 with ethanol made with the diluted solution was 1.000 ppm more first. Prepare standard Vit D3 solutions with various concentrations (200, 400, and 800 ppm).

Furthermore, measurements were performed using HPLC analysis to optimize the standard vitamin D3 eluent results with a maximum UV wavelength detector of 265 nm.

Analysis of Vitamin D3 Encapsulated in Liprotides

End product Vitamin D3 encapsulation with lipotide was analyzed to optimize the standard vitamin D3 eluent results with a maximum UV wavelength detector of 265 nm.

SEM (Scanning Electron Microscopy) Analysis

Encapsulated vitamin D3 morphology with β -lactoglobulin was analyzed using SEM (Jeol JSM-6510LA, Japan®) with 5,000 \times magnification of pure Vitamin D3 samples, lipids, and products end vitamin D3 encapsulation with lipotide.

Result and Discussion

Determination of Vitamin D3 in Liprotide

Before creating a calibration curve using a standard solution of Vitamin D3, it was necessary to determine the optimum conditions under which a single peak chromatogram could be obtained (Table 1). This study used seven conditions to determine the retention time of the standard Vitamin D3. In HPLC 1-4 conditions, a

mixture of eluents (acetonitrile: methanol) was used, referring to several previous studies, but with some variations in composition (Fatemeh et al., 2017). The determination of Vitamin D3 using a mobile phase as a mixture of butanol was based on previous studies, but with variations in eluent composition (Chetta et al., 2021; Fang et al., 2012). Specifications of the HPLC conditions used are listed in Table 2.

Table 1. Optimization standard vitamin D3

Condition	Temperature (°C)	Flow Rate (mL/ min)	Analysis Time (minutes)	Injection Volume (µL)	Mobile Phases
1	35	1	15	50	Acetonitrile: Methanol (40:60)
2	35	1	15	50	Acetonitrile: Methanol (70:30)
3	35	1	15	50	Acetonitrile: Methanol (90:10)
4	28	1	15	50	Acetonitrile: Methanol (60:40)
5	40	1	20	20	Acetonitrile: Aquabidest (99:1)
6	30	1.5	20	20	Butanol: n -hexane (6:94)
7	30	1	10	5	Butanol: n -hexane (6:94)

Detector (UV): 265 nm

Table 2. Determination of standard vitamin D3 using various eluent composition

Condition	Solvent	Temperature (°C)	Flow Rate (mL/min)	Analysis Time (minutes)	Injection Volume (µL)	Mobile Phases	Retention Time (minutes)
1	Ethanol	35	1	15	50	Acetonitrile: Methanol (40:60)	8,910 and 9,495
2	Ethanol	35	1	15	50	Acetonitrile: Methanol (70:30)	10,504 and 11,145
3	Ethanol	35	1	15	50	Acetonitrile: Methanol (90:10)	11,766 and 12,476
4	Ethanol	28	1	15	50	Acetonitrile: Methanol (60:40)	11,766 and 12,476
5	Ethanol	40	1	20	20	Acetonitrile: Aquabidest (99:1)	12,928 and 13,876
6	Ethanol	30	1.5	20	20	Butanol: n-hexane (6:94)	1,476 and 1,707
7	Ethanol	30	1	10	5	Butanol: n-hexane (6:94)	2,170

Detector (UV): 265 nm

The results of HPLC (Figure 1) show that the different eluent compositions as the mobile phase give different chromatogram results (Sahu et al., 2018). The stationary phase influences the separation that occurs in the HPLC process the column used in the C-18 column but also the composition of the eluent as the mobile phase (Yin et al., 2019). Under HPLC conditions 1, 2, 3, and 4, where variations in the components of acetonitrile and methanol were carried out, a chromatogram with a single peak was not produced. However, there were two peaks with different retention times. This is due to the lack of polarity range of the two eluent

mixtures used, both acetonitrile and methanol. Are polar solvent groups. Variations in the composition of acetonitrile and methanol as eluents did not provide significant changes to the resulting chromatogram, where the resulting chromatogram retained the retention time of two different peaks. In the 5th High-Performance Liquid Chromatography (HPLC) condition, almost all acetonitrile solvent was used with a ratio of 99:1 to aquabidest but still produced a chromatogram with two peaks. This indicates that using acetonitrile as the eluent did not provide optimal separation results. Vitamin D3 is a group of vitamins that are soluble in fat (Almarria et al., 2017).

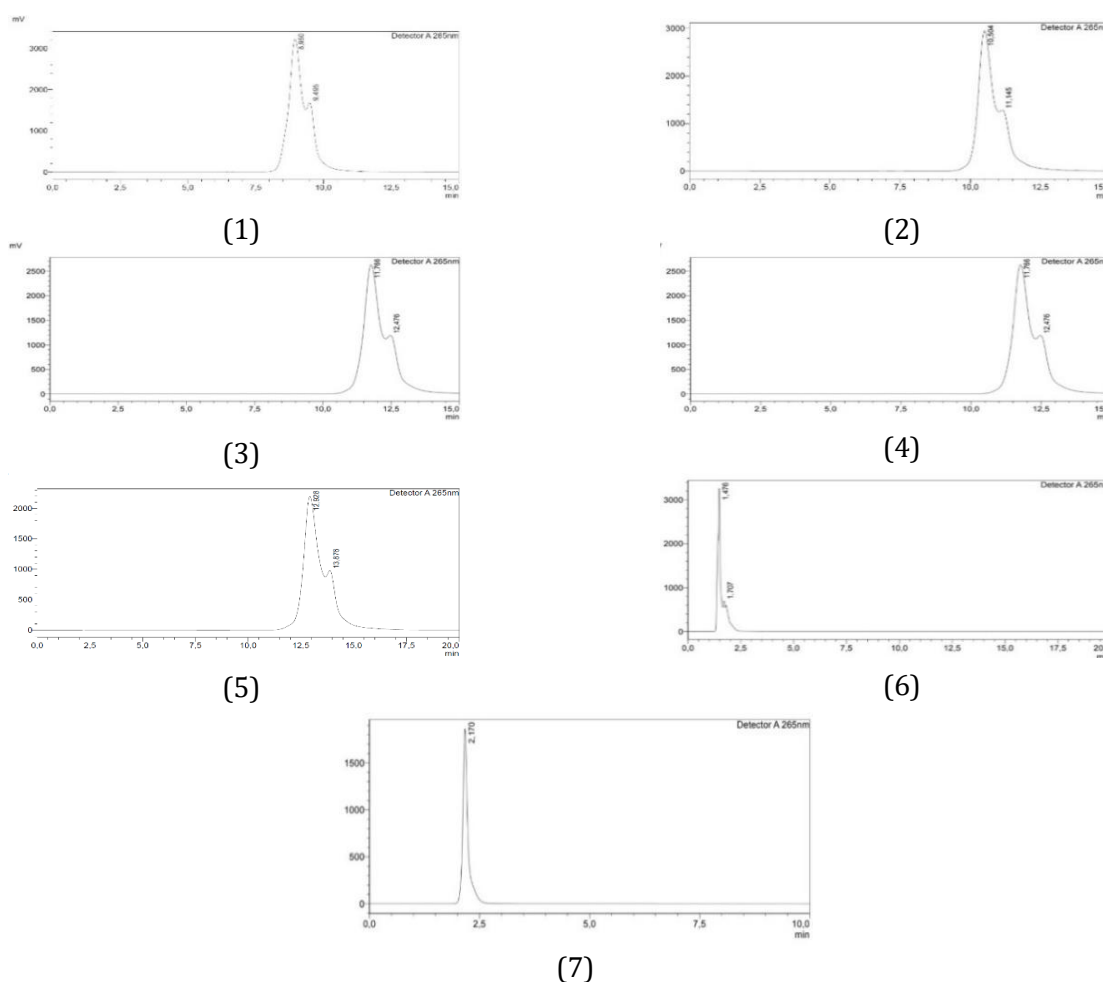


Figure 1. Chromatogram results from the optimization of standard vitamin D3 HPLC

Acetonitrile, which is used as the mobile phase, cannot provide maximum separation results because of the nature of acetonitrile itself, which is classified as an aprotic polar solvent with a fairly high dielectric constant and does not dissolve well in acetonitrile (Lanin et

al., 2020). The difference in polarity between vitamin D3 and acetonitrile as the mobile phase (mobile phase) causes suboptimal separation (Malik, 2020). Acetonitrile has good solubility in organic solvents, such as ethanol, butanol, DMSO, and vitamin D3. Especially for vitamin

D3, using organic solvents such as acetonitrile is good because of the long-chain group in the structure of vitamin D3 (Almarria et al., 2017). In HPLC conditions 6 and 7, where a mixture of butanol and n-hexane (6:94) solvents was used, better separation results were obtained.

In contrast, HPLC images for conditions 6 and 7 showed that a chromatogram with a single peak was produced. Optimization was performed using the 7th HPLC condition, where the flow rate was 1 mL/min. A chromatogram with a sharp and single peak indicated that the separation of vitamin D3 under HPLC conditions was good. The resulting retention time was also relatively shorter than that under the previous conditions. Under the 7th HPLC condition, the retention time was approximately 2,17 minutes. Compared with HPLC conditions before using a mixture of butanol and n-hexane, the retention time was approximately 12 min. A shorter retention time would make preparing grades using HPLC more efficient and effective.

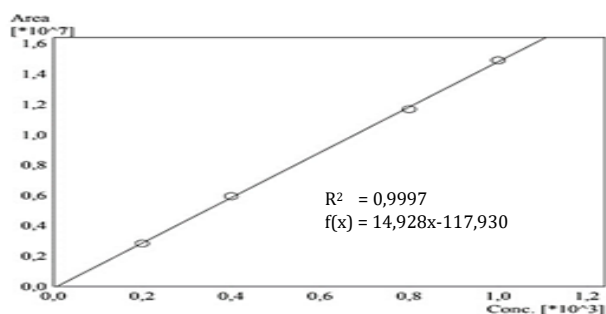


Figure 2. Results of the vitamin D3 solution calibration curve using the most optimal HPLC conditions

Optimal HPLC conditions were used to create a calibration curve for the vitamin D3 solution. As shown in Figure 2, the results of the calibration curve of the Liprotide Encapsulated Vitamin D3 standard solution (200, 400, 800, and 1000 ppm) using optimal HPLC conditions (7th condition) obtained a value of $R^2 = 0,9997$, which indicates that the calibration curve is quite good and valid for use in determining vitamin D3 levels in samples (Temova & Roškar, 2016).

The optimal HPLC conditions were obtained from the vitamin D3 solution (Table 2). The retention time of vitamin D3 encapsulated by liprotide was 2,107 min, which was not much different from the retention time of HPLC vitamin D3 in the seventh condition. The

difference is only visible from the width of the chromatogram, which allows the encapsulated vitamin D3 to take longer to separate in the HPLC column, resulting in a wider chromatogram. The HPLC chromatogram in Figure 3 shows that the presence of liprotide as a vitamin D3 binder does not interfere with measurements using HPLC, as can be seen from the absence of chromatograms other than the chromatogram at $t = 2,107$ min, which is the retention time of vitamin D3.

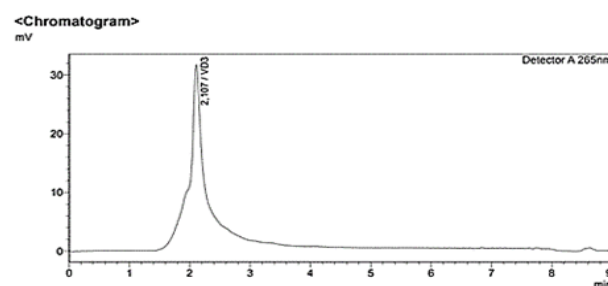


Figure 3. Vitamin D3 encapsulated chromatogram with liprotide formulation

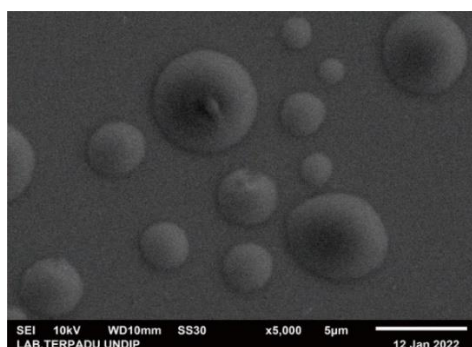
Morphology Encapsulation by SEM (Scanning Electron Microscopy)

From the SEM results of Vitamin D3 encapsulation using Liprotide, the morphology of the vitamin D3 encapsulation results can be seen. In Figure 4 (A), it can be seen that the SEM results for Vitamin D3 in ethanol are in a round granular form. From the morphology of the SEM, the granular form appears to have a fairly large size and a non-uniform size, indicating that Vitamin D3 can be dissolved in ethanol but not very well.

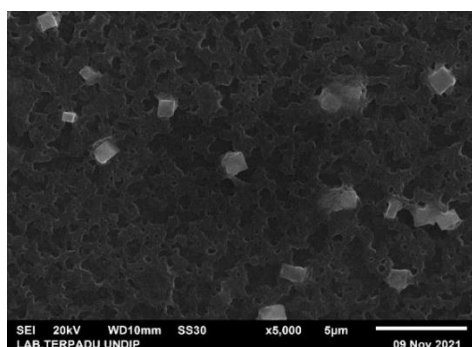
Therefore, large granules indicate that there are molecules in vitamin D3 that have not completely dissolved homogeneously in the solution. In ethanol. In Figure 4 (B), the SEM results of liprotide show the morphology in the form of sheets with several small cubes (cubical morphology). The morphology in the form of sheets is characteristic of β -lactoglobulin, a protein with a primary, secondary, tertiary, and quaternary structure (Salah et al., 2020). The tertiary structure of proteins allows for a structure that fills the 3-dimensional space formed from secondary structures that fold together. The quaternary structure of a protein consists of a combination of one or more polypeptide chains that operate as a unit. Figure

4(C) shows a morphological image of liprotide encapsulating vitamin D3.

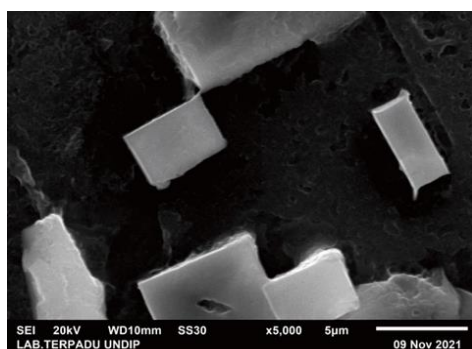
The SEM results show that a cubic morphology is formed with a size much larger than the cubic size in the liprotide SEM results shown in Figure 1. This shows that the liprotide encapsulation process against vitamin D3 was successful. The enlarged cubical size is possible because of the encapsulation of vitamin D3 by liprotide to form micelles (Li et al., 2022).



(A)



(B)



(C)

Figure 4. Morphology with 5000x magnification (A) Vitamin D3 Morphology, (B) Morphology Liprotide (β -lactoglobulin: Oleic Acid), (C) Morphology Vitamin D3 encapsulation with Liprotide

Conclusion

In order to determine vitamin D3 using the HPLC method, optimal HPLC was obtained; in condition 7, a mixture of butanol and n-hexane (6:94) solvents resulted in a chromatogram with a single peak and a retention time of approximately 2,170 min. The results of the Vitamin D3 standard solution calibration curve using optimal HPLC conditions obtained. The morphology determined by SEM showed that vitamin D3 is attached to liprotide, and the enlarged cubical size is possible due to the encapsulation of vitamin D3 by liprotide to form micelles.

Further research is suggested for morphological analysis in SEM accompanied by analysis in the form of EDX-mapping to provide colored morphological results. Hopefully, this product can be further developed for in vivo and in vitro testing.

Acknowledgments

The authors thank the Indonesian Ministry of Education, Culture, Research, and Technology (Grant Number: No.187-13/UN7.6.1/PP/2021).

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