Food Chemistry 360 (2021) 129979

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical validation of an ultraviolet–visible procedure for determining vitamin D_3 in vitamin D_3-loaded microparticles and toxigenetic studies for incorporation into food

Tamires Barlati Vieira da Silva [a], Anielle de Oliveira [a], Thaysa Fernandes Moya Moreira [a], Kelly Cristina da Silva [b], Rodolfo Campos Zanin [b], Evandro Bona [a], Odinei Hess Gonçalves [a,c], Marianne Ayumi Shirai [b], Ana Paula Peron [d], Fernanda Vitória Leimann [a,c,*]

[a] Program of Food Technology (PPGTA), Federal University of Technology – Paraná (UTFPR-CM), via Rosalina Maria Dos Santos, 1233 CEP 87301-899, Campo Mourão, Paraná, Brazil
[b] Program of Food Technology (PPGTAL), Federal University of Technology-- Paraná (UTFPR-LD), Avenida dos Pioneiros 3131, CEP 86036-370, Londrina Paraná, Brazil
[c] Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus Santa Apolónia, 5300-253 Bragança, Portugal
[d] Department of Biodiversity and Nature Conservation (DABIC), Federal University of Technology – Paraná (UTFPR-CM), via Rosalina Maria Dos Santos 123 CEP 87301-899 POBox 271, Campo Mourão, Paraná, Brazil

ARTICLE INFO

Keywords:
Vitamin
Carnauba wax
Spectroscopy
Hot homogenization
Encapsulation
Cytotoxicity
Genotoxicity

ABSTRACT

Vitamin D is a water-insoluble compound presented in two main forms (D_2 and D_3), susceptible to environmental conditions. Microencapsulation is an alternative to supplements and preserve vitamin D properties in foods. Entrapment efficiency (EE) is the main property to evaluate the encapsulation effectiveness and therefore it is of interest the study of analytical methods for the identification and quantification of this compound within the particle. This paper describes a low cost UV–Vis methodology validation to the identification and quantification of vitamin D_3 in microparticles produced by hot homogenization. The method was validated following the International Conference on Harmonization (ICH) guidelines. To guarantee safe application in foodstuff, microparticles toxigenicity was evaluated with *Allium cepa* L. *in vivo* model, showing no cytotoxic nor genotoxic potential. High entrapment efficiency was obtained, the results also demonstrated that the concentration of vitamin D_3 in microparticles can be safely accessed by the validated method.

1. Introduction

Vitamin D is a fat-soluble bioactive with steroid hormone function that plays significant role in human health controlling the intestinal absorption of calcium and phosphorus. On the other hand, vitamin D deficiency leads to bone softness as well as infectious diseases, autoimmune diseases, hypertension, cardiovascular disease, and diabetes while its excessive intake increases the risk of hypercalcemia and kidney problems (Park et al., 2017; Verkaik-Kloosterman et al., 2017). There are two major physiologically forms of vitamin D that exhibit similar physiological effect: vitamin D_2 (ergocalciferol) synthesized by ultraviolet radiation from plants ergosterol, and vitamin D_3 (cholecalciferol) synthesized in human skin and found in oily fish, egg yolk and cod liver oil (Lee et al., 2008).

Salmon, sardines, shiitake mushrooms, and egg yolk are foods that naturally contain substantial amounts of vitamin D. Furthermore, because of the well-recognized association between skin cancer and exposure to sunlight, and insufficient ultraviolet energy during winter days, especially in northern latitudes, the world population is facing declining levels of vitamin D in the blood, leading to problems associated to vitamin D deficiency (Jannasari et al., 2019; Lee et al., 2008; Paucar et al., 2016). As a result, the food industry has developed vitamin D fortified foods and supplements to overcome vitamin D deficiency. However, this vitamin is susceptible to environmental conditions such as high temperature, oxidation, and acidic media, moreover, its hydrophobicity reduces the vitamin D bioavailability and also does not allow

* Corresponding author at: Program of Food Technology (PPGTA), Federal University of Technology – Paraná (UTFPR-CM), via Rosalina Maria Dos Santos, 1233 CEP 87301-899, Campo Mourão, Paraná, Brazil.
E-mail address: fernandaleimann@utfpr.edu.br (F.V. Leimann).

https://doi.org/10.1016/j.foodchem.2021.129979
Received 20 August 2020; Received in revised form 19 March 2021; Accepted 26 April 2021
Available online 3 May 2021

its incorporation into aqueous food or beverages (Guttoff et al., 2015; Hasanvand et al., 2015).

Encapsulation represents a good alternative to increase the stability and preserve the properties of vitamin D during processing and storage. In this process the bioactive (core) is protected by a coating shell to increase their protection. Besides, encapsulation might further provide target delivery and controlled release of vitamin, enhancing bioactivity and reducing side effects of potential hypervitaminosis by controlling administration doses (Gonnet et al., 2010; Huang et al., 2010). Microparticles containing vitamin D_3 have the potential to be used in fortified food as an alternative to consumers to supplement their dietary needs.

The entrapment efficiency is one of the most important parameters in the encapsulation field. It represents the amount of the core that was successfully entrapped into the particle and effectively protected. Among many methods available for entrapment efficiency measurements, dissolution assays are one of the most widely used in which particles are disintegrated to release the bioactive compound followed by its quantification using an appropriate analytical method. Chromatographic methods are usually employed to determine the concentration of vitamin D_3, offering sensitivity and specificity (Abbasi et al., 2014; Maurya & Aggarwal, 2019; Paucar et al., 2016). However, its disadvantages compared to spectroscopic methods include high operating costs, the need for large amounts of samples and solvents, and the generation of hazardous wastes such as organic solvents that are expensive to dispose of and can have significant environmental impacts (Deconinck et al., 2011).

Validation of an analytical procedure is very important for the determination of vitamin D_3 encapsulation efficiency in order to guarantee reliability, traceability, or comparability of results (Silva et al., 2017). Thus, UV–Vis spectroscopic methods are frequently preferred in most encapsulation systems due to its fast and reliable results (Kaushik, Dowling, Barrow, & Adhikari, 2015; Silva-Buzanello et al., 2015; Silva et al., 2017).

Another important factor to be evaluated for the safe and effective application of micro/nanoparticles release systems is their cytotoxic and genotoxic potential. The *Allium cepa* L. root meristems model is internationally accepted as a toxicogenetic test of substances and it can be applied to investigate safety aspects of food additives. Biomarkers such as the mitotic index (cell division index) may evaluate the different levels of cytotoxicity, while the frequency of chromosome and mitotic spindle changes are indicative of genotoxicity (Herrero et al., 2012; Silva et al., 2020). Results obtained employing the bioassay with *A. cepa* have a good correlation with the results observed in genetic tests carried out in other bioassays, such as those with mammals and in cell culture (Herrero et al., 2012; Sales et al., 2017). The objective of this work was to develop and validate an analytical method based on UV–Vis spectroscopy to determine the vitamin D_3 concentration in carnauba wax microparticles obtained by hot homogenization technique, and *Allium cepa* L. root meristems model was used to access cytotoxicity and genotoxicity.

2. Material and methods

2.1. Materials

Vitamin D_3 (90% purity, kindly gifted by Pincredit Bio-tech Co.), carnauba wax (Sigma-Aldrich, USA) was used as the encapsulating agent and sodium caseinate (Sigma-Aldrich, USA) was used as stabilizing agent in the microparticles preparation. Ethanol (99.5%, P.A., Neon, Brazil), was used as received for the encapsulation efficiency determination.

2.2. Preparation of the vitamin D_3-loaded carnauba wax microparticles

Microparticles were obtained using the procedure described by Venturini et al. (2018). The aqueous phase was prepared by dissolving 55 mg of sodium caseinate in 50 g of deionized water at 95 °C. Separately, carnauba wax (3.350 g) was placed into a borosilicate vessel connected to a thermostatic bath at 95 °C. Then, vitamin D_3 (1.650 g) was incorporated under magnetic stirring for 1 min to produce the molten mixture. Next, the aqueous phase was added to the molten mixture and homogenized at 16,000 rpm for 5 min using an Ultra-Turrax disperser (IKA, T25, USA). The obtained mixture was cooled in an ice bath for particle formation. Finally, the dispersed particles were freeze-dried (Liotop, L101, Brazil), and then stored at − 18 °C protected from light. One formulation was produced without adding vitamin D_3 to obtain blank microparticles.

2.3. Validation procedure

In order to demonstrate that no interference occurred due to the other microparticles constituents, specificity tests (ICH Harmonised Tripartite Guideline, 2005) was carried out through the obtainment of the UV–Vis spectra of vitamin D_3 and the carnauba wax microparticles without vitamin D_3 (blank microparticles sample). For blank microparticles analysis, 50 mg of microparticles were washed with 5 mL of ethanol, vacuum filtered, and analyzed by UV–Vis spectrometry. Pure vitamin D_3 was also dissolved in ethanol (40 mg L^{-1} solution) and analyzed by UV–Vis spectrometry (Ocean Optics, model USB-650-UV–VIS Red Tide).

2.3.1. Linearity

Linearity was investigated by using Vitamin D_3 solutions in ethanol in five different concentration levels ranging from 1 mg sL^{-1} to 40 mg L^{-1}. Experiments were carried out in triplicate using UV–Vis at 266 nm wavelength. The linear regression was evaluated using the correlation coefficient (R^2) of the calibration curve, and adjusted correlation coefficient (R^2_{adj}), as well as the lack of fit (ICH Harmonised Tripartite Guideline, 2005; Silva et al., 2017; Silva-Buzanello et al., 2015).

2.3.2. Quantification limit

Quantification limit (QL) (Araujo, 2009; ICH Harmonised Tripartite Guideline, 2005) was determined according to Eq. (1), where "b" is the angular coefficient from the calibration curve and "s" is the standard deviation calculated from seven blank samples (no analyte added).

$$QL = s \times \frac{10}{b} \quad (1)$$

2.3.3. Detection limit

The detection limit (DL) is determined by the analysis of samples with known analyte levels as stated in Eq. (2), where "s" is the standard deviation from samples without analyte, and "b" is the angular coefficient of calibration curve equation (ICH Harmonised Tripartite Guideline, 2005).

$$DL = s \times \frac{3.3}{b} \quad (2)$$

2.3.4. Accuracy

Accuracy (González, Herrador, & Asuero, 2010; ICH Harmonised Tripartite Guideline, 2005) was evaluated by the recovery method analyzing three different concentrations of vitamin D_3 solutions in ethanol (2, 8, and 40 mg L^{-1}). Samples were analyzed by UV–Vis spectrometry in triplicate on two different days and the respective concentrations were recalculated from the calibration curve.

2.3.5. Precision

Intermediate precision (different days and different analysts) and reproducibility (inter-laboratory studies) (ICH Harmonised Tripartite Guideline, 2005) were determined with six scans of three vitamin D_3 solutions at 1, 4, and 40 mg L^{-1} and expressed as relative standard deviation percentage (RSD%). UV–Vis spectra were acquired using an

Ocean Optics equipment (model USB-650-UV–VIS Red Tide) in Laboratory of Food Analysis. Reproducibility analyses were performed by the same analyst in the Laboratory of Spectroscopy using a second equipment of the same model mentioned above.

2.3.6. Validation of equipment and statistical analyses

For the method validation, an ultraviolet–visible spectrophotometer was used (Ocean, USB-650-UV–VIS Red Tide) and scanning was performed from 200 to 850 nm with a 1 nm resolution. Analysis of variance (ANOVA), adjusted determination coefficient, and standard error of estimate were used to evaluate the calibration curve ($p = 0.01$). The effects of concentration, day, and their interaction were tested by factorial ANOVA ($p = 0.05$). One-way ANOVA was implemented to determine the influence of laboratory and analyst ($p = 0.05$). All analyses were performed by using the software Statistica 7.0 (Statsoft, USA).

2.4. Determination of the entrapment efficiency in the microparticles

The vitamin D_3 loaded microparticles (50 mg) were flushed with 5 mL of ethanol using Whatman n° 1 filter paper. The collected solution was submitted to UV–Vis spectrometry. After that, the previously constructed calibration curve was used to determine the real concentration of Vitamin D_3 non entrapped in the microparticles. Finally, the encapsulation efficiency (EE (%)) was determined according to Eq. (3).

$$EE(\%) = \left[\frac{(VitD_3\ total\ amount - VitD_3 non\ entrapped\ amount)}{VitD_3 total amount}\right] \times 100 \quad (3)$$

2.5. Microparticle characterization

Differential scanning calorimetry (DSC, Perkin Elmer 4000) analyses were carried out to investigate the thermal behavior of samples. The samples were accommodated in sealed aluminum pans with nitrogen flow (50 mL min^{-1}) and heated from 20 to 360 °C at a rate of 10 °C min^{-1}.

Morphological features of the microparticles were visualized under a Scanning Electron Microscope (Philips, FEI Quanta 200 model, Japan) with electron source of tungsten and detectors of secondary and backscattered electrons at 20 kV. Microparticles samples (vitamin D_3 loaded microparticles and blank microparticles) were coated in gold using a sputter coater (BAL-TEC, SCD-050, Balzers, Liechtenstein).

The microparticles (vitamin D_3 loaded microparticles and blank microparticles) and vitamin D_3 crystalline characteristics were investigated by XRD. The analysis was performed using a diffractometer (Bruker, D2 Phaser), with copper Kα radiation generated at 1.1 kVA. The scanning of samples was performed from $2\theta = 10°$ to $2\theta = 60°$, with ramping at 5.9°/min and slit of 0.2 mm.

2.6. Cytotoxic and genotoxic analysis in Allium cepa L.

For cytotoxicity and genotoxicity assessment of vitamin D_3 and the microparticles containing the vitamin D_3, five onion bulbs (variety *beta cristal*, from an organic garden) were placed in vials with distilled water, constantly aerated, to obtain roots of 2.0 cm in length. Before putting the roots in contact with their respective treatments, some roots were collected and fixed to serve as control of the bulb itself, which was identified as time of analysis 0 h or control of the bulb itself (Co − 0 h). Then, the other roots were put in their respective treatments for 24 and 48 h, procedures called exposure times 24 and 48 h, where roots were collected every 24 h.

Positive control was prepared with methyl methanesulfonate (MMS), a known cytotoxic and genotoxic substance to the *A. cepa* test system, at 4×10^{-4} mol L^{-1}. All roots collected during the experiment were fixed in 3: 1 Carnoy solution (ethanol:acetic acid) for up to 24 h. For estimates of the mitotic index, cells in interphase, prophase, metaphase, anaphase, and telophase were counted to determine the cytotoxic potential. The mitotic index or cell division index was calculated by Eq. (4).

$$MI(\%) = \left[\frac{(total\ number\ of\ dividing\ cells)}{total\ number\ of\ cells}\right] \times 100 \quad (4)$$

The genotoxic potential was assessed by frequency of cell alterations such as micronuclei, colchicine metaphases, anaphase and telophase bridges, gene amplifications, cells with adhesions, nuclear buds, and multipolar anaphases. *A. cepa* results were analyzed by ANOVA and the mean values were compared by the Scott-Knott test with the significance level (p) of 0.05.

3. Results and discussion

3.1. Analytical validation

Firstly, the specificity test was performed to verify the absence of interferences from the microparticles constituents on the analysis of vitamin D_3. In Fig. 1(A) the UV–Vis spectra of pure vitamin D_3 diluted in ethanol (40 mg L^{-1}) and the ethanol collected after blank and vitamin D_3 loaded microparticles "washing" procedure are presented. It is possible to observe an intense peak with a maximum located at 266 nm, which is in accordance with reported by Olds, McKinley, Moore, & Kimlin (2008) as the optimal wavelength for vitamin D_3 detection. It must be highlighted that the blank microparticles spectra did not show any potential interference on 266 nm, in other words, the method showed 100% selectivity. It also can be highlighted by the vitamin D3 loaded microparticles scanning, where it is possible to detect an increase in the absorbance at 266 nm, due to the presence of unencapsulated vitamin D_3.

According to the calibration curves (triplicate) for vitamin D_3, a linear relationship was found between the absorbance and the concentration of vitamin D_3 in the range of 1–40 mg L^{-1}. After the regression analysis, linearity was estimated by the coefficients of determination, $R^2 = 0.9957$ and $R^2_{adj} = 0.9954$, indicating excellent linearity. The representative linear equation was $y = 40.632x + 0.0116$, linear and angular coefficients of calibration curve found were 0.0116 ± 0.0129 and 40.632 ± 2.759 L mg^{-1}, respectively. Residuals presented normal distribution and outliers were not detected. ANOVA showed that the calculated F value was 331.6 times higher than the critical F value at a significance level of 1%, the linear regression was significant in the concentration ranges studied ($p = 9.039 \times 10^{-17}$) and the mathematical model for vitamin D_3 did not present lack of fit ($p = 0.874$) proving to be appropriate to perform quantifications. The standard error of estimate was 0.0419 mg L^{-1}, which means that linearity can be assumed.

The results obtained for the limits of detection (LD) and quantification (LQ) were equal to 0.039 mg L^{-1} and 0.8132 mg L^{-1}, respectively. Zhang et al. (2018) validated a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to quantify vitamin A, vitamin D_3, and α-tocopherol, and they obtained detection limit of 0.4 ng mL^{-1} and quantification limit of 1.2 ng mL^{-1} for vitamin D_3. Kaushik, Sachdeva, Arora, & Wadhwa (2014) reported a detection limit of 0.001 ng and a quantification limit of 0.01 ng of vitamin D_2 in fortified milk by HPLC. Even though the chromatographic method detects lower concentration comparing to spectroscopy methods, the detection and quantification limits determined in this work are adequate to quantify vitamin D_3 in microparticles since this compound is usually not added in lower concentrations in encapsulation processes. In addition, the spectroscopic methods are interesting because they require less time and are also specific for data processing and can be used in conjunction with encapsulation techniques.

In Table 1 data used to calculate recovery rates and accuracy are presented. The quantification limit determined was 0.8132×10^{-3} mg mL^{-1}. Recovery rate values varied from 104.61% to 84.37% and RSD% from 1.08% and 2.39%. In analytical validation the acceptable recovery

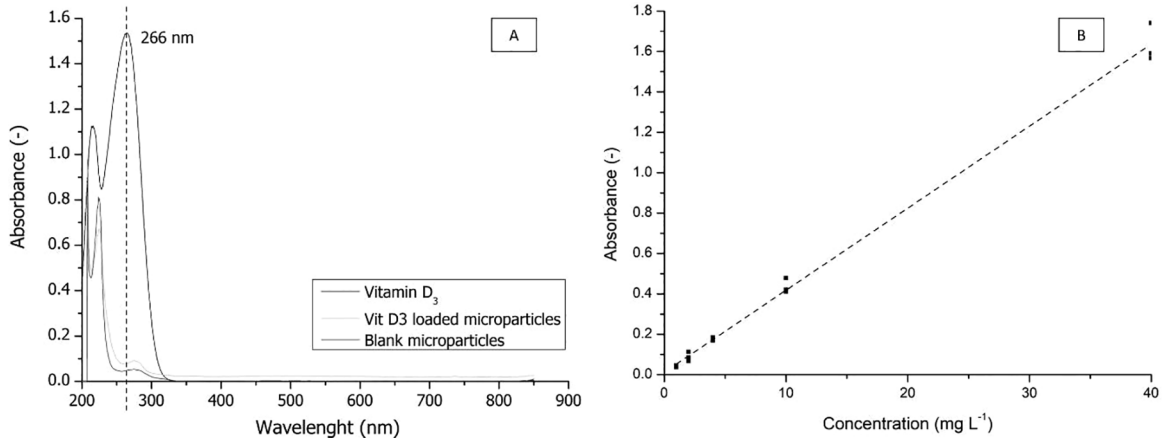

Fig. 1. (A) UV–Vis spectra of pure vitamin D_3 (40 mg mL^{-1}), carnauba wax nanoparticles without vitamin D_3 (blank nanoparticles) and vitamin D_3 loaded microparticles; (B) Calibration curves determined for vitamin D_3 (triplicate).

Table 1
Accuracy study for the analytical validation of the vitamin D_3 determination method.

Concentration of added vitamin D_3 (mg L^{-1})	Sample (day)	Concentration of vitamin D_3 found (mg L^{-1})	Average of vitamin D_3 concentrations found (mg L^{-1})	Recovery rate mean (%) (confidence interval 95%)
40	1	37.5	37.8	94.51 ± 1.08
	1	37.5	RSD% = 1.15%	RSD% =
	1	37.3		1.15%
	2	37.8		
	2	38.2		
	2	38.5		
8	1	8.1	8.4	104.61 ±
	1	8.5	RSD% = 2.21%	2.33
	1	8.6		RSD% =
	2	8.6		2.23%
	2	8.5		
	2	8.4		
2	1	1.7	1.7	84.37 ± 2.39
	1	1.6	RSD% = 2.80%	RSD% =
	1	1.7		2.83%
	2	1.7		
	2	1.7		
	2	1.7		

rate is within the range of 70% to 120% (Silva-Buzanello et al., 2015), and this result is considered consistent with the results of other studies on the determination of vitamin D (Kaushik et al., 2014; Kazmi et al., 2007). The RSD% values determined in the solution with 2 mg L^{-1} of vitamin D_3 indicate that this method is less sensitive to low vitamin D_3 concentration. On the other hand, this UV–Vis spectroscopy method for vitamin D_3 quantification showed increased sensibility for the highest and medium vitamin D_3 concentration levels.

In Table 2 are shown the results for repeatability (intra-day), intermediate precision (inter-day) and reproducibility (intra-laboratory). Repeatability assays were carried out by the same analyst on the same day (Analyst I, 1st day) and RSD% values remained between 1.14% and 4.25%. The maximum value of relative standard deviation obtained in intermediate precision assays (between analysts) was 3.72% and in reproducibility assays (between laboratories) was 3.33%.

RSD% of intermediate (4 mg L^{-1}) and low (1 mg L^{-1}) vitamin D concentrations were similar and greater than the highest concentration of vitamin D_3 (40 mg L^{-1}). Nevertheless, these results are satisfactory for vitamin D quantification in encapsulation systems, where the concentration of encapsulated active substances usually ranges from 1% to 10% (Silva et al., 2017). Other authors reported values of RSD% between 1.07 and 1.31% for vitamin D_3 quantification by HPLC-UV (Almarri et al., 2017), and between 0.822 and 0.896% RSD% for vitamin D_3 by supercritical fluid chromatography (Andri et al., 2017).

3.2. Application of the validated method and microparticles characterization

3.2.1. Entrapment efficiency

The entrapment efficiency of vitamin D_3 loaded in carnauba wax microparticles was determined and a considerable value was obtained, 83.9 ± 1.2%. This parameter is one of the most important to evaluate the effectiveness of the encapsulation procedure because it shows the amount of core that is within the particle; otherwise the core is not

Table 2
Precision levels (intermediate precision and reproducibility) for the analytical validation of the vitamin D_3 determination method.

Vitamin D3 concentration (mg·L^{-1})	Analyst I			Analyst II	Inter-analyst reproducibility RSD% (%)
	1st day RSD% (%)	2nd day RSD% (%)	Inter-day (n = 6) RSD% (%)	1st day RSD% (%)	
40	1.14	1.68	1.41	1.68	1.41
4	2.83	4.62	3.72	4.62	3.72
1	4.25	3.68	2.96	3.16	3.70
Vitamin D3 concentration (mg. L^{-1})	Laboratory I		Laboratory II	Inter-Laboratory Reproducibility RSD% (%)	
	1st day RSD% (%)		1st day RSD% (%)		
40	0.65		4.56	2.61	
4	4.84		1.81	3.33	
1	4.25		1.86	3.05	

protected and consequently subject to evaporation and oxidation (Ré, 1998). This entrapment efficiency value shows that the carnauba wax presented good encapsulation properties that protect the core due to wax ordered crystal packing that results in considerable long term stability (Jenning & Gohla, 2000). In addition, the method employed for the microencapsulation and also the proportion encapsulant:core used was suitable in protecting vitamin D_3 since entrapment efficiency is highly affected by the properties of the shell and its concentration, and the method of encapsulation. Even though there are reports in which higher entrapment efficiencies were found (Rao et al., 2014; Relkin et al., 2014), the optimization of this parameter was not the goal of this work.

3.2.2. Microparticles characterization

The Differential Scanning Calorimetry thermograms of the microparticles and Vitamin D_3 are presented in Fig. 2(A).

The melting point of the encapsulant (carnauba wax) was found at 83 and 84 °C, which is in accordance with the literature (Lacerda et al., 2011; Rocha et al., 2020). The same is valid for Vitamin D_3 which melting point was 88 °C (Hasanvand et al., 2018). The peak corresponding to Vitamin D_3 was not found in the microparticles suggesting that it was efficiently entrapped in the carnauba wax matrix in an amorphous state.

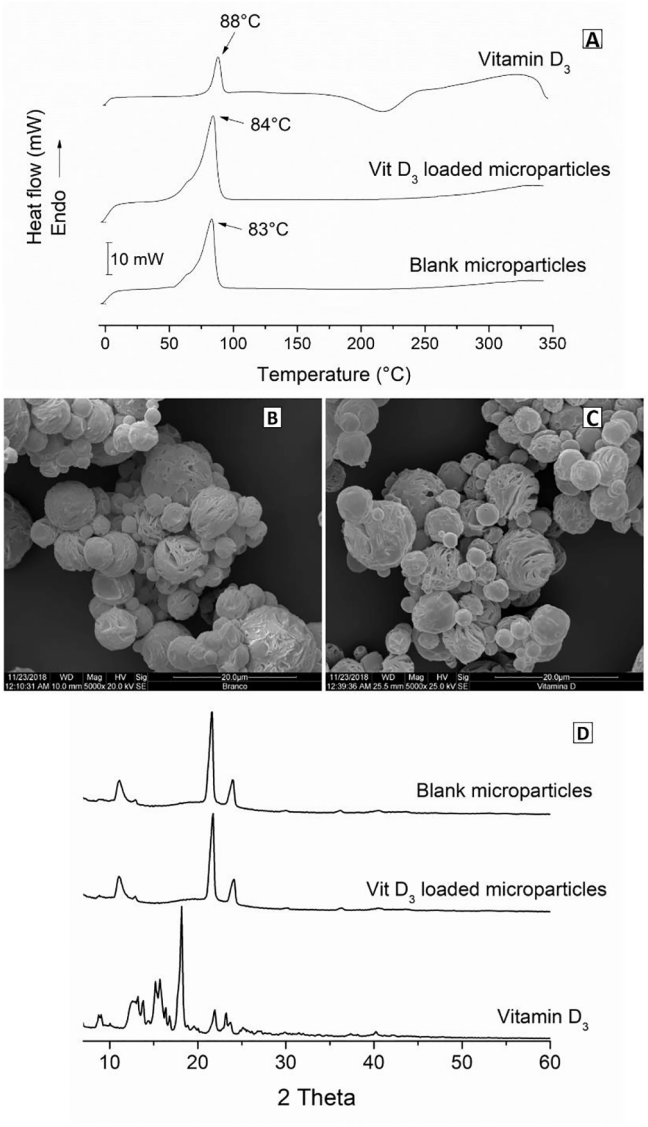

Fig. 2. Microparticles characterization: (A) DSC thermograms of Vitamin D_3, Vitamin D_3 loaded microparticles and blank microparticles (no vitamin added); (B) SEM images of blank carnauba wax microparticles (without vitamin D_3) (Magnification = 5000×); (C) SEM images of Vitamin D_3 loaded carnauba wax microparticles. (Magnification = 5000×); (D) Diffractograms of pure Vitamin D_3, Blank carnauba wax microparticles (without vitamin D_3), and Vitamin D_3 loaded carnauba wax microparticles.

The SEM images showing the microstructure of the carnauba wax microparticles loaded with vitamin D_3 are presented in Fig. 2(B) and (C).

Microparticles presented spherical shape, shriveled, and apparent fine crevices on them. Such characteristics affect the particle flowability because their irregular structure and aggregation prevent their free flow due to mechanical interlocking (Walton & Mumford, 1999). The particles presented similar microstructure independently of vitamin D_3 presence which means that the presence of vitamin D_3 did not change the carnauba wax crystallization behavior during the cooling step as demonstrated by XRD analysis (Fig. 2(D)). Moreover, the microparticles are aggregated due to the adhesion characteristic of carnauba wax (Hu et al., 2015; Jenning & Gohla, 2000).

The samples of vitamin D_3, blank microparticles (carnauba wax), and microparticles containing vitamin D_3 were evaluated by XRD and the diffractogram is shown in Fig. 2(D). Vitamin D_3 showed two intense peaks at 15.1 and 15.6, and another at 18.1. The intense peaks of pure vitamin D_3 were not observed in the blank microparticles and vitamin D_3 microparticles, suggesting that it was successfully encapsulated by carnauba wax.

The presence of vitamin D_3 did not affect the crystallization pattern of carnauba wax once comparing the diffractograms of both blank microparticles where only carnauba wax is present and microparticles containing vitamin D_3, typical carnauba wax crystalline structure with two narrow peaks (2θ between 20° and 25°) remained unchanged with no region characteristic of amorphous materials and crystal disorder. It is interesting to note that in contrast to other authors (Westesen et al., 1997) although the carnauba wax kept its crystal arrangement it did not result in the expulsion of vitamin D_3 which may be observed by the high entrapment efficiency. Also, the crystalline structure is particularly important concerning the increased stability of the particles that improve the protection of the core against oxidation reactions and evaporation (Bhandari & Howes, 1999; Roos, 2010).

3.2.3. Cytotoxic and genotoxic analysis in Allium cepa L.

Table 3 present the results obtained for the *in vivo* tests of

Table 3
Mitotic indices (%) and Indices of cellular changes observed in root meristems of *Allium cepa* L. exposed to the Vitamin D_3 loaded microparticles and Vitamin D_3 for 24 and 48 h exposure times.

Positive control			**MI (%)± SD**	
MMS (4×10^{-4} mol L^{-1})			6.51 ± 0.34	
			MI (%)± SD	
	Treatment	**Co (0 h)**	**24 h**	**48 h**
	Vitamin D_3 loaded microparticles	16.80[a] ±0.13	15.53[a] ± 0.39	23.71[a] ± 0.13
	Vitamin D_3	16.43[a] ± 0.72	26.80[a] ± 1.78	15.79[a] ± 1.30
Positive control			**CAI (%)**	
MMS (4×10^{-4} mol L^{-1})			8.95	
			TE ± CAI (%)	
	Treatment	**Co (0 h)**	**24 h**	**48 h**
	Vitamin D_3 loaded microparticles	0.01 [a]	0.18[a] ± 0.71	0.04 [a] ± 0.71
	Vitamin D_3	0.01 [a]	0.09[a] ± 0.36	0.09 [a] ± 0.20

cytotoxicity and genotoxicity of the microparticles and Vitamin D_3, respectively.

Based on the results obtained in Table 3, under the established analysis conditions, vitamin D_3 and encapsulated Vitamin D_3 did not alter the cell division index of the meristematic cells of *Allium cepa* L., in the two exposure times evaluated. These results are important since they suggest the protection of Vitamin D_3 to the mechanisms of checking/regulating the cell cycle and, therefore, the protection of the functioning of the root meristems. It is worth noting that pure vitamin D_3 and encapsulated vitamin D_3 did not cause chromosomal changes and disturbances to the mitotic spindle of meristem cells (Table 3), being non-genotoxic.

4. Conclusions

A UV–Vis spectroscopic procedure for vitamin D_3 determination in microparticles was successfully developed and validated. The method presented detection and quantification limits satisfactory for the vitamin D_3 quantification on the microparticles. UV–Vis spectroscopy method was shown to be a promising technique for microparticle evaluation with fast and reliable results. The specificity test showed that carnauba wax employed as encapsulant material did not represent a potential interference on UV–Vis spectra of pure vitamin D_3. Micrographs showed that the presence of vitamin D_3 did not cause significant difference in particle morphology and maintained their crystalline form without losses of vitamin D_3, supported by the high entrapment efficiency detected. Besides, the *in vivo* tests suggested that the microparticles presented no cytotoxicity and were non-genotoxic, which is important for future safe application in the cosmetic and food industries.

CRediT authorship contribution statement

Tamires Barlati Vieira da Silva: Validation, Investigation, Writing - original draft. **Anielle de Oliveira:** Validation, Investigation, Writing - original draft. **Thaysa Fernandes Moya Moreira:** Validation, Investigation, Writing - original draft. **Kelly Cristina da Silva:** Investigation, Writing - original draft. **Rodolfo Campos Zanin:** Investigation, Software, Writing - original draft. **Evandro Bona:** Software, Formal analysis, Writing - original draft. **Odinei Hess Gonçalves:** Formal analysis, Writing - original draft, Supervision. **Marianne Ayumi Shirai:** Conceptualization, Writing - review & editing, Supervision. **Ana Paula Peron:** Conceptualization, Writing - review & editing, Supervision. **Fernanda Vitória Leimann:** Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

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

Acknowledgements

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001. The authors thank the Multiuser Laboratory of Federal University of Technology – Paraná – Campus Londrina, and the "Central Analítica Multiusuário da UTFPR Campo Mourão" (CAMulti-CM) for the analyses. Also, to CNPq for the financial support (Project n° 420055/2018-5) and scholarship.

References

Abbasi, A., Emam-Djomeh, Z., Mousavi, M. A. E., & Davoodi, D. (2014). Stability of vitamin D3 encapsulated in nanoparticles of whey protein isolate. *Food Chemistry, 143*, 379–383. https://doi.org/10.1016/j.foodchem.2013.08.018.

Almarri, F., Haq, N., Alanazi, F. K., Mohsin, K., Alsarra, I. A., Aleanizy, F. S., & Shakeel, F. (2017). An environmentally benign HPLC-UV method for thermodynamic solubility measurement of vitamin D3 in various (Transcutol + water) mixtures. *Journal of Molecular Liquids, 242*, 798–806. https://doi.org/10.1016/j.molliq.2017.07.011.

Andri, B., Lebrun, P., Dispas, A., Klinkenberg, R., Streel, B., Ziemons, E., Marini, R. D., & Hubert, P.h. (2017). Optimization and validation of a fast supercritical fluid chromatography method for the quantitative determination of vitamin D3 and its related impurities. *Journal of Chromatography A, 1491*, 171–181. https://doi.org/10.1016/j.chroma.2017.01.090.

Araujo, P. (2009). Key aspects of analytical method validation and linearity evaluation. *Journal of Chromatography B, 877*(23), 2224–2234. https://doi.org/10.1016/j.jchromb.2008.09.030.

Bhandari, B. R., & Howes, T. (1999). Implication of glass transition for the drying and stability of dried foods. *Journal of Food Engineering, 40*(1-2), 71–79. https://doi.org/10.1016/S0260-8774(99)00039-4.

Deconinck, E., Crevits, S., Baten, P., Courselle, P., & De Beer, J. (2011). A validated ultra high pressure liquid chromatographic method for qualification and quantification of folic acid in pharmaceutical preparations. *Journal of Pharmaceutical and Biomedical Analysis, 54*(5), 995–1000. https://doi.org/10.1016/j.jpba.2010.11.036.

Gonnet, M., Lethuaut, L., & Boury, F. (2010). New trends in encapsulation of liposoluble vitamins. *Journal of Controlled Release, 146*(3), 276–290. https://doi.org/10.1016/j.jconrel.2010.01.037.

González, A. G., Herrador, M.Á., & Asuero, A. G. (2010). Intra-laboratory assessment of method accuracy (trueness and precision) by using validation standards. *Talanta, 82*(5), 1995–1998. https://doi.org/10.1016/j.talanta.2010.07.071.

Guttoff, M., Saberi, A. H., & McClements, D. J. (2015). Formation of vitamin D nanoemulsion-based delivery systems by spontaneous emulsification: Factors affecting particle size and stability. *Food Chemistry, 171*, 117–122. https://doi.org/10.1016/j.foodchem.2014.08.087.

Hasanvand, E., Fathi, M., & Bassiri, A. (2018). Production and characterization of vitamin D3 loaded starch nanoparticles: Effect of amylose to amylopectin ratio and sonication parameters. *Journal of Food Science and Technology, 55*(4), 1314–1324. https://doi.org/10.1007/s13197-018-3042-0.

Hasanvand, E., Fathi, M., Bassiri, A., Javanmard, M., & Abbaszadeh, R. (2015). Novel starch based nanocarrier for vitamin D fortification of milk: Production and characterization. *Food and Bioproducts Processing, 96*, 264–277. https://doi.org/10.1016/j.fbp.2015.09.007.

Herrero, O., Pérez Martín, J. M., Fernández Freire, P., Carvajal López, L., Peropadre, A., & Hazen, M. J. (2012). Toxicological evaluation of three contaminants of emerging concern by use of the Allium cepa test. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 743*(1-2), 20–24. https://doi.org/10.1016/j.mrgentox.2011.12.028.

Hu, L., Gao, N., Li, J., Sun, Y., & Yang, X. (2015). Development and evaluation of novel microcapsules containing poppy-seed oil using complex coacervation. *Journal of Food Engineering, 145*, 272–277. https://doi.org/10.1016/j.jfoodeng.2015.03.027.

Huang, Q., Yu, H., & Ru, Q. (2010). Bioavailability and delivery of nutraceuticals using nanotechnology. *Journal of Food Science, 75*(1), R50–R57. https://doi.org/10.1111/j.1750-3841.2009.01457.x.

ICH Harmonised Tripartite Guideline. (2005). Validation of analytical procedures: text and methodology q2(r1). International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use, 1–17. 10.1590/s1984-82502011000100012.

Jannasari, N., Fathi, M., Moshtaghian, S. J., & Abbaspourrad, A. (2019). Microencapsulation of vitamin D using gelatin and cress seed mucilage: Production, characterization and in vivo study. *International Journal of Biological Macromolecules, 129*, 972–979. https://doi.org/10.1016/j.ijbiomac.2019.02.096.

Jenning, V., & Gohla, S. (2000). Comparison of wax and glyceride solid lipid nanoparticles (SLN®). *International Journal of Pharmaceutics, 196*(2), 219–222. https://doi.org/10.1016/S0378-5173(99)00426-3.

Kaushik, P., Dowling, K., Barrow, C. J., & Adhikari, B. (2015). Microencapsulation of omega-3 fatty acids : A review of microencapsulation and characterization methods Microencapsulation of omega-3 fatty acids : A review of microencapsulation and characterization methods. *Journal of Functional Foods, 19*, 868–881. https://doi.org/10.1016/j.jff.2014.06.029.

Kaushik, R., Sachdeva, B., Arora, S., & Wadhwa, B. K. (2014). Development of an analytical protocol for the estimation of vitamin D2 in fortified toned milk. *Food Chemistry, 151*, 225–230. https://doi.org/10.1016/j.foodchem.2013.11.085.

Kazmi, S. A., Vieth, R., & Rousseau, D. (2007). Vitamin D3 fortification and quantification in processed dairy products. *International Dairy Journal, 17*(7), 753–759. https://doi.org/10.1016/j.idairyj.2006.09.009.

Lacerda, S. P., Cerize, N. N. P., & Ré, M. I. (2011). Preparation and characterization of carnauba wax nanostructured lipid carriers containing benzophenone-3. *International Journal of Cosmetic Science, 33*(4), 312–321. https://doi.org/10.1111/j.1468-2494.2010.00626.x.

Lee, J. H., O'Keefe, J. H., Bell, D., Hensrud, D. D., & Holick, M. F. (2008). Vitamin D Deficiency. *Journal of the American College of Cardiology, 52*(24), 1949–1956. https://doi.org/10.1016/j.jacc.2008.08.050.

Maurya, V. K., & Aggarwal, M. (2019). A phase inversion based nanoemulsion fabrication process to encapsulate vitamin D3 for food applications. *The Journal of Steroid Biochemistry and Molecular Biology, 190*, 88–98. https://doi.org/10.1016/j.jsbmb.2019.03.021.

Olds, W. J., McKinley, A. R., Moore, M. R., & Kimlin, M. G. (2008). In vitro model of vitamin D3 (Cholecalciferol) synthesis by UV radiation: Dose–response relationships. *Journal of Photochemistry and Photobiology B: Biology, 93*(2), 88–93. https://doi.org/10.1016/j.jphotobiol.2008.07.004.

Park, S. J., Garcia, C. V., Shin, G. H., & Kim, J. T. (2017). Development of nanostructured lipid carriers for the encapsulation and controlled release of vitamin D3. *Food Chemistry, 225*, 213–219. https://doi.org/10.1016/j.foodchem.2017.01.015.

Paucar, O. C., Tulini, F. L., Thomazini, M., Balieiro, J. C. C., Pallone, E. M. J. A., & Favaro-Trindade, C. S. (2016). Production by spray chilling and characterization of solid lipid microparticles loaded with vitamin D 3. *Food and Bioproducts Processing, 100*, 344–350. https://doi.org/10.1016/j.fbp.2016.08.006.

Rao, M. P., Manjunath, K., Bhagawati, S. T., & Thippeswamy, B. S. (2014). Bixin loaded solid lipid nanoparticles for enhanced hepatoprotection – Preparation, characterisation and in vivo evaluation. *International Journal of Pharmaceutics, 473* (1-2), 485–492. https://doi.org/10.1016/j.ijpharm.2014.07.027.

I Ré, M. (1998). MICROENCAPSULATION BY SPRAY DRYING. *Drying Technology, 16*(6), 1195–1236.

Relkin, P., Shukat, R., & Moulin, G. (2014). Encapsulation of labile compounds in heat- and high-pressure treated protein and lipid nanoparticles. *Food Research International, 63*, 9–15. https://doi.org/10.1016/j.foodres.2014.03.038.

Rocha, B. A.d., Francisco, C. R. L., Almeida, M.d., Ames, F. Q., Bona, E., Leimann, F. V., Gonçalves, O. H., & Bersani-Amado, C. A. (2020). Antiinflammatory activity of carnauba wax microparticles containing curcumin. *Journal of Drug Delivery Science and Technology, 59*, 101918. https://doi.org/10.1016/j.jddst.2020.101918.

Roos, Y. H. (2010). Glass transition temperature and its relevance in food processing. *Annual Review of Food Science and Technology, 1*(1), 469–496.

Sales, I. M. S., Barbosa, J. S., dos Santos, F. K. S., da Silva, F. C. C., Ferreira, P. M. P., de Castro e Sousa, J. M., & Peron, A. P. (2017). Assessment of grape, plum and orange synthetic food flavourings using in vivo acute toxicity tests. Food Technology and Biotechnology, 55(1), 131–137. 10.17113/ftb.55.01.17.4770.

Silva-Buzanello, R. A.d., Ferro, A. C., Bona, E., Cardozo-Filho, L., Araújo, P. H. H.d., Leimann, F. V., & Gonçalves, O. H. (2015). Validation of an Ultraviolet–visible (UV–Vis) technique for the quantitative determination of curcumin in poly(l-lactic acid) nanoparticles. *Food Chemistry, 172*, 99–104. https://doi.org/10.1016/j.foodchem.2014.09.016.

Silva, J. T. D. P., Silva, A. C. D., Geiss, J. M. T., de Araújo, P. H. H., Becker, D., Bracht, L., Leimann, F. V., Bona, E., Guerra, G. P., & Gonçalves, O. H. (2017). Analytical validation of an ultraviolet–visible procedure for determining lutein concentration and application to lutein-loaded nanoparticles. *Food Chemistry, 230*, 336–342. https://doi.org/10.1016/j.foodchem.2017.03.059.

Silva, T. D. S., Silva, A. P. S. E., de Almeida Santos, A., Ribeiro, K. G., Souza, D. C. D., Bueno, P. A. A., Marques, M. M. M., Almeida, P. M. D., & Peron, A. P. (2020). Cytotoxicity, Genotoxicity, and Toxicity of Plant Biostimulants Produced in Brazil: Subsidies for Determining Environmental Risk to Non-Target Species. *Water Air Soil Pollut, 231*(5). https://doi.org/10.1007/s11270-020-04614-x.

Venturini, L. H., Moreira, T. F. M., da Silva, T. B. V., de Almeida, M. M. C., Francisco, C. R. L., de Oliveira, A., … Leimann, F. V. (2018). Partial Substitution of Margarine by Microencapsulated Chia Seeds Oil in the Formulation of Cookies. *Food and Bioprocess Technology, 2014*. https://doi.org/10.1007/s11947-018-2188-0.

Verkaik-Kloosterman, J., Seves, S. M., & Ocké, M. C. (2017). Vitamin D concentrations in fortified foods and dietary supplements intended for infants: Implications for vitamin D intake. *Food Chemistry, 221*, 629–635. https://doi.org/10.1016/j.foodchem.2016.11.128.

Walton, D. E., & Mumford, C. J. (1999). Spray dried products—Characterization of particle morphology. *Chemical Engineering Research and Design, 77*(1), 21–38.

Westesen, K., Bunjes, H., & Koch, M. H. J. (1997). Physicochemical characterization of lipid nanoparticles and evaluation of their drug loading capacity and sustained release potential. *Journal of Controlled Release, 48*(2-3), 223–236. https://doi.org/10.1016/S0168-3659(97)00046-1.

Zhang, H., Quan, L.i., Pei, P., Lin, Y.e., Feng, C., Guan, H., Wang, F., Zhang, T., Wu, J., & Huo, J. (2018). Simultaneous determination of Vitamin A, 25-hydroxyl vitamin D 3 α-tocopherol in small biological fluids by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B, 1079*, 1–8. https://doi.org/10.1016/j.jchromb.2017.12.017.