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DEVELOPMENT AND VALIDATION OF ANALYTICAL METHOD FOR PALM OIL DETERMINATION IN MICROCAPSULES PRODUCED BY COMPLEX COACERVATION

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The microencapsulation of palm oil may be a mechanism for protecting and promoting the controlled release of its bioactive compounds. To optimize the microencapsulation process, it is necessary to accurately quantify the palm oil present both external and internal to the microcapsules. In this study, we developed and validated a spectrophotometric method to determine the microencapsulation efficiency of palm oil by complex coacervation. We used gelatin and gum arabic (1:1) as wall material in a 5% concentration (w/v) and palm oil in the same concentration. The coacervates were obtained at pH 4.0 ± 0.01, decanted for 24 h, frozen (−40 °C), and lyophilized for 72 h. Morphological analyzes were then performed. We standardized the extraction of the external palm oil through five successive washes with an organic solvent. We then explored the best method for rupturing the microcapsules. After successive extractions with hexane, we determined the amount of palm oil contained in the microcapsules using a spectrophotometer. The proposed method was shown to be of low cost, fast, and easy to implement. In addition, in the validation step, we confirmed the method to be safe and reliable, as it proved to be specific, accurate, precise, and robust.

Keywords: palm oil; encapsulation; spectrophotometry; gelatin; gum arabic.

INTRODUCTION

The stimulation of ileum by poorly digested foods releases gut hormones, whose actions interfere positively in the control of obesity and in its associated metabolic disorders, especially in peripheral insulin resistance.[1] Microencapsulation of palm oil by complex coacervation can be an alternative to the transport of this nutrient to ileum without being digested, which stimulates the production of intestinal hormones. This may be a prospective treatment for type 2 Diabetes Mellitus (DM), a metabolic disorder characterized by hyperglycemia and the excretion of glucose excess in the urine. Microencapsulation of palm oil can also be used as an alternative method for weight reduction.[1,2]

Microencapsulation is capable of protecting a compound and promoting its controlled release, being a process of great importance in pharmaceutical and food industries.[3] The complex coacervation is based on the associative interaction that can occur when mixing solutions of oppositely charged biopolymers.[4,5] The first step in the process of microencapsulation by complex coacervation consists in the emulsification of the core material (active compound), usually an oily phase, in an aqueous solution of the biopolymer blend (wall material). The conditions of the environment (pH, ionic strength, temperature) are then changed in order to promote coacervation, which leads to deposition of the newly formed coacervate around the core material, building a protective biopolymer capsule.[4,6-11]

For optimization of the microencapsulation process, it is necessary to quantify the core material located inside the microcapsules. Some authors proposed the determination of the core material losses in the complex coacervation process by washing the homogenizer rod and the weighing beaker with organic solvent, and then quantifying the active compound using spectrophotometry.[12] Some others authors determined the encapsulation efficiency by difference between the initial known mass of the core material in capsules and the extracting mass in the surface using organic solvent.[13,14]

Another possibility is to perform the washing of the microcapsules with organic solvent and, after its evaporation, weigh the non-microencapsulated oil. Subsequently, the same samples are submitted to a Soxhlet extraction, the solvent evaporates, and the material is weighed to quantify the microencapsulated oil. Thus, it is possible to quantify the microencapsulated and non-microencapsulated active material and estimate losses during process.[15]

In spite of the variety of methods applied to evaluate the microencapsulation efficiency through quantification of the core material allocated inside and outside the capsules, there is lack of standardization of the applied methodologies. Therefore, the objective of the present study was to develop and to validate an analytical method that is accurate for quantifying palm oil present externally and internally to the microcapsules produced by gelatin-gum arabic complex coacervation, besides being easy to use and cost-effective.

EXPERIMENTAL

Palm oil was microencapsulated by complex coacervation using bovine skin gelatin (240 *bloom*) (Gelita, Mococa, Brazil) and gum arabic (Synth, Diadema, Brazil) as wall material. The pH adjustment for encapsulation was carried out with hydrochloric acid solution (0.5 mol L^{-1}) (Impex, Diadema, Brazil).

Buffer solutions at different pH were prepared using potassium hydrogen phthalate ($C_8H_5KO_4$) 0.1 mol L^{-1} (Impex, Diadema, Brazil), potassium phosphate (KH_2PO_4) 0.1 mol L^{-1} (Êxodo Científica, Hortolândia, Brazil), sodium hydroxide (NaOH) 0.1 mol L^{-1} (Proquímios, Rio de Janeiro, Brazil), ammonia (NH_3) 1 mol L^{-1} (Impex, Diadema, Brazil), ammonium chloride (NH_4Cl) 1 mol L^{-1} (Proquímios, Rio de Janeiro, Brazil), and hydrochloric acid (HCl) 0.1 mol L^{-1}, all analytical grade reagents. Hexane (Dinâmica, Diadema, Brazil) was used as solvent for the extraction of palm oil.

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The materials were weighed on an analytical balance (Marconi / JK 200, Brazil).

Two commercial palm oils were used for specificity tests: Hemmer® (Blumenau, Santa Catarina, Brazil), and Marabá®, palm oil export quality, containing 25% of soybean oil (Mauá, São Paulo, Brazil).

Production of palm oil microcapsules

The methodology used to obtain the microcapsules by complex coacervation is schematized by the flowchart in Figure 1. Gelatin and gum arabic were used in the same proportion, and the quantity of total polymer was fixed in 5.0% (w/v) on a dry basis. The ratio between the quantity of filling (palm oil) and wall materials was 1:1. The gelatin solution was heated to 50 ± 3 °C, the palm oil was incorporated into the system and homogenized in a disperser (IKA, T25D Ultra-Turrax, Germany) at 15,000 rpm, for 5.0 min, to obtain an emulsion. Then, gum arabic solution (50 ± 3 °C) and water (twice the volume of the system) were added in a magnetic stirrer. Temperature was controlled (50 ± 3 °C) throughout the process. HCl (0.5 mol L^{-1}) was used to adjust pH (4.0 ± 0.01), using a digital pH meter (Tecnal, TEC-3MP, Brazil) and the system was immersed in an ice bath, keeping constant agitation to slow cooling, until 10 ± 2 °C. The system was covered with aluminum foil to protect it from light and was kept in a refrigerator (5 °C) for 24 h to complete precipitation of the microcapsules produced. Subsequently, excess of water was eliminated; the decanted particles were arranged in aluminum trays and frozen in ultra-freezer (Liobrás, FV 500, Brazil) at -40 °C, for 24 h. Drying was carried out by lyophilization. The samples remained in the lyophilizer (Liobrás, L101, Brazil), for about three consecutive days. Then, the samples were ground in a mortar, wrapped for protection against the light, and stored in a desiccator for the tests.

Morphology of palm oil microcapsules

The morphology of the palm oil microcapsules was analyzed by light microscope (Bioval, L-2000, Brazil) coupled with a camera (Sony, DSC-W150, Brazil), 8.1 mega pixels. The microcapsule samples were spread over glass slides and were coated with cover slips.

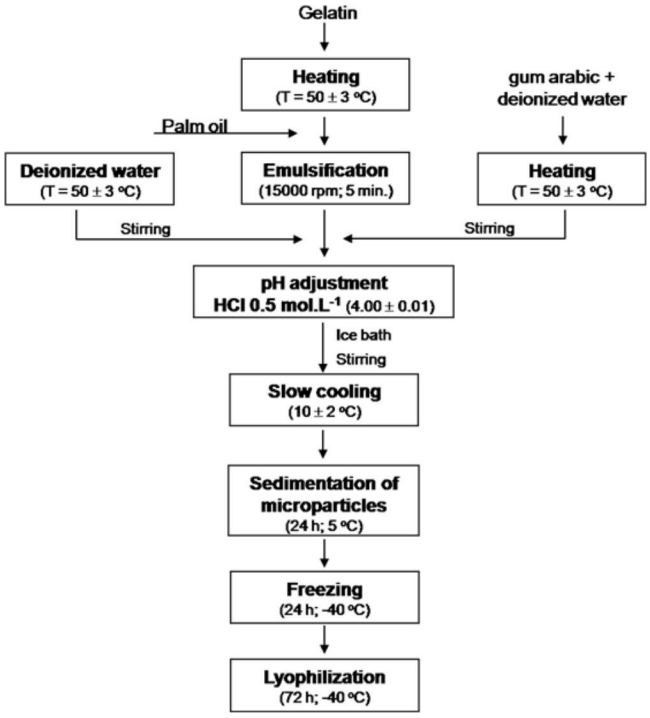

Figure 1. *Flowchart of the production of palm oil microcapsules by complex coacervation*

Ultrastructural analysis was carried out with a scanning electron microscope (SEM). The lyophilized samples were placed in a sample holder under carbon tape and metalized with a thin layer of gold and palladium (Polaron, SC7620 Sputter, UK). The SEM analysis was conducted in a Scanning Electron Microscope (Magellan XHR, 400L, US), in the Laboratory of Structural Characterization (LCE) of the Department of Materials Engineering (DEMa) from the Federal University of São Carlos (UFSCar).

Particle size

Particle size was determined by a Laser Diffraction Particle Size Distribution Analyzer LA-950 (HORIBA, Japan) using ethanol (PA) as the dispersing medium.

The results were expressed in terms of average particle diameter ($D_{(4,3)}$) which is obtained by equation (1). The calculation of $D_{(4,3)}$ considers the average diameter of each particle (D_i) which is obtained by the square root of the product between the largest and smallest diameter (geometric mean) of n particles analyzed.

$$D_{(4,3)} = \frac{\sum_{i=1}^{i=n} D_i^4 v_i}{\sum_{i=1}^{i=n} D_i^3 v_i} \quad (1)$$

where: $D_i = \sqrt{D_s d_i}$; v_i = percentage of particles with the same D_i to a total of n particles; D_s = projection of the largest dimension of a particle; d_i = projection of the smallest dimension of a particle.

Choice of solvent and selection of optimum wavelength

Ethanol and hexane were tested as potential solvents for palm oil through qualitative assays. A sample of palm oil was put into a test tube with 10 mL of solvent and stirred for 2.0 min. for complete dissolution.

For selection of the optimum wavelength, a solution was prepared with palm oil and hexane (1200 µg mL^{-1}) and the absorption spectrum was determined from 400 to 690 nm in UV-visible spectrophotometer (Hach, DR/2010, USA). The same absorption spectrum was determined for a sample of 0.2000 g of microcapsules without palm oil, produced according to the flowchart of Figure 1.

Solubility of microcapsules

Palm oil microcapsules (0.2000 g) were stirred in acetic acid, in heated water at 60 °C, or in sodium hydroxide solution (pH = 12), for 1.0 min. and, then, 10 mL of hexane was used for palm oil extraction. Assays were performed in triplicate and analyzed qualitatively.[16]

Choice of the best pH for palm oil releasing

A 0.1000 g sample of the microcapsules was placed in a separatory funnel (125 mL) with 10 mL of buffer of specific pH and stirred for 1.0 min. Then, 5 mL of hexane was added and, again, stirred for 1.0 min. The system was set aside until total segregation. Aqueous and organic phases (hexane + palm oil) were separated and the absorbance was read. Buffer pH was between 0.95 and 12.05.

Standardizing time and number of external washes of the microcapsules

A method of successive extractions was proposed to quantify palm oil present externally to the microcapsules (not microencapsulated).

To determine the time required for washing, 12 tubes containing 0.2000 ± 0.0023 g of sample and 5 mL of hexane were prepared. The tubes were divided into four groups. The first group was stirred for 30 s, the second for 1.0 min, the third for 1.5 min, and the last for 2.0 min, continuously.

After stirring, the organic phase was transferred to a 25 mL volumetric flask and the volume was completed with hexane. Absorbance measurements were performed to quantify the palm oil extracted from the outside of the microcapsules using UV-visible spectrophotometer (Hach, DR/2010, USA) at a wavelength of 446 nm.

Disruption standardization procedure and number of extractions of microcapsules

To determine the number of extractions necessary for rupturing the capsules, 0.2000 g of sample, 5 mL of buffer (pH = 9.5 ± 0.2), and 5 mL of hexane were added to five tubes and stirred for 1.0 min. The organic phase was removed and subjected to absorbance reading at 446 nm. Then, 5 mL of hexane was added again and the process was repeated until the sixth extraction.

Validation of proposed analytical methodology

The validation parameters of the proposed analytical procedure were performed according to recommendations of the current legislation of Resolution RE 899, of May 25, 2003 and of the document DOQ-CGCRE-008, according to Category I - quantitative tests for determination of active ingredient in pharmaceutical products or raw materials.[17,18] Thus, specificity, linearity and range, precision (repeatability), intermediate precision (interday precision), accuracy, and robustness were evaluated.

Specificity

Samples of 0.2030 ± 0.0010 g of microcapsules produced without palm oil were subjected to the proposed analytical methodology. The organic phases obtained from the washing of the microcapsules and after the disruption had their absorption spectrum determined in the range between 400 and 690 nm, using a spectrophotometer (Hach, DR/2010, USA). Aliquots of 0.0300 g of two commercial palm oil samples were dissolved in 25 mL of hexane and the absorption spectrum was determined in the same conditions. All assays were performed in triplicate.

Linearity and range

The linearity was evaluated from the preparation of three calibration curves in the interval 200-3200 μg mL^{-1}, for two different days. A stock solution was prepared by weighing approximately 0.5000 g of the palm oil used for microencapsulation in a 10 mL beaker and transferred to a 25 mL volumetric flask using hexane as the solvent. The resulting stock solution had a concentration of 20,000 μg mL^{-1} and, from this, six dilutions were made with hexane in a volumetric flask of 10 mL, resulting in concentrations of 200, 400, 600, 800, 1600, and 3200 μg mL^{-1}. Each volumetric flask was stirred until complete mixing of the samples. Absorbance of each dilution was read at a wavelength of 446 nm in a random fashion, in order to avoid cross contamination. The values obtained were used to establish the calibration curve, and the coefficient of determination (R^2) was used to assume linearity of points. The limits of detection (LD) and quantification (LQ) were determined from equations 2 and 3, using the data from the two analytical curves.[17]

$$LD = \frac{3s}{\alpha} \quad (2)$$

$$LQ = \frac{10s}{\alpha} \quad (3)$$

where *s* is the standard deviation of the intercept with the y axis in the two calibration curves and α is the slope of the line.

Precision

To determine the intra-run precision (repeatability), six solutions were prepared at an intermediate concentration of the linearity curve (1600 μg mL^{-1}), by the same analyst, and the absorbance obtained in the spectrophotometer was used throughout all this study (Hach, DR/2010, USA). To determine the intermediate or inter-run precision (reproducibility), twelve replicates were prepared at a concentration of 1600 μg mL^{-1} by another analyst, and absorbance measurements were done in two different spectrophotometers (Instrutherm, UV-1000A, Brazil and Biospectro, SP22, Brazil). For both repeatability and intermediate precision, the coefficient of variation (CV), expressed by equation 4, was considered as the criteria for acceptance.

$$CV = \left(\frac{s}{\bar{X}}\right) 100\% \quad (4)$$

where *s* is the standard deviation and $\bar{X}$ the average of n measurements.

Accuracy

To determine the accuracy of the method, a known aliquot of palm oil was mixed with a known mass of empty microcapsules. External washing of this mixture was performed, as proposed, for determination of the external palm oil (non-microencapsulated oil). Assays were performed in triplicate (n = 3) and, using the calibration curve, the recovery percentage of the original palm oil was calculated.

Robustness and Stability

To determine robustness of the method, three spectrophotometers of different suppliers were used: Hach (DR/2010, USA), Instrutherm (UV-1000A, Brazil), and Biospectro (SP22, Brazil). The stability of solutions was evaluated at room temperature (25 °C) for 12, 24, and 36 h and at different storage conditions. Solutions at a concentration of 1600 μg mL^{-1} were prepared and stored in volumetric flasks, protected and non-protected from light.

RESULTS AND DISCUSSION

Figures 2A and 2B show the morphological characteristics of palm oil microcapsules obtained by complex coacervation in light and scanning electron microscopy, respectively.

The images reveal that the microcapsules are spherical, of varied sizes, present solid wall and have average diameter ($D_{(4,3)}$) 284.74 ± 207.26 μm.

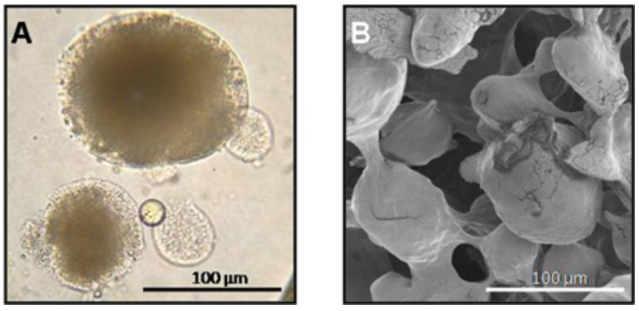

Figure 2. *Micrographs of the microcapsules of palm oil produced by complex coacervation* ***A)*** *Light microscopy.* ***B)*** *Scanning Electron Microscopy*

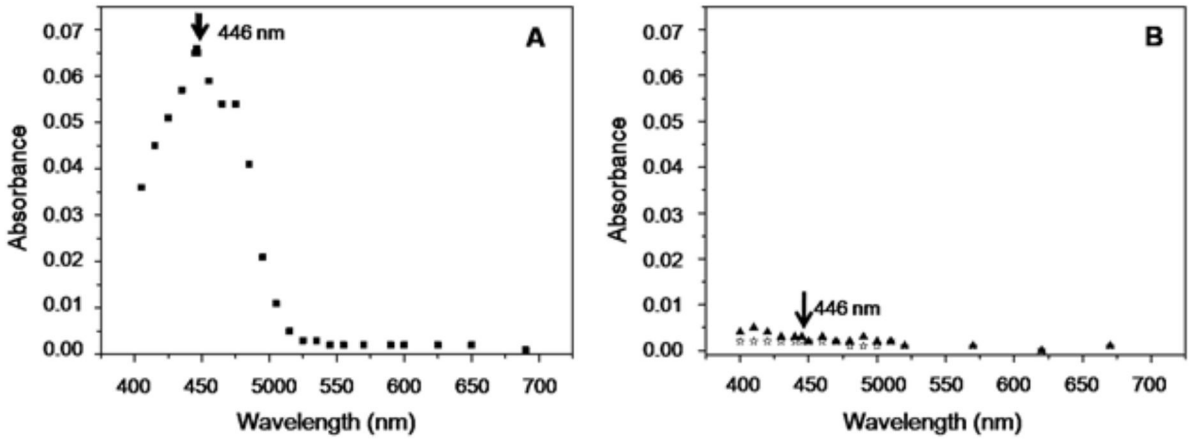

Figure 3. A) *Absorption spectrum (400 to 690 nm) to determine the best wavelength for palm oil detection in solution (1200 μg mL^{-1}).* ***B)*** *Absorption spectrum of the microcapsules without the addition of palm oil. (▲ = without disruption; ☆ = after disruption)*

Development of the analytical method for determination of palm oil in microcapsules produced by complex coacervation

Choice of solvent and selection of the optimum wavelength

The palm oil samples were analyzed visually for the occurrence of phase separation, after testing their solubility in ethanol and hexane. A single phase was obtained only in samples where the palm oil was dissolved in hexane, thus being chosen as the most suitable solvent. The optimum wavelength for palm oil detection in solution was determined as being 446 nm (Figure 3A).

Microcapsules without the addition of palm oil were prepared and a sample of 0.2000 g was subjected to extraction of the surface oil with hexane (without disruption of the microcapsules). This procedure was then followed by disruption of the capsules and extraction of the oil located inside them. The wall material did not interfere with the absorbance values over the entire range of wavelengths investigated, indicating that the wavelength of 446 nm can be used for quantification of palm oil (Figure 3B).

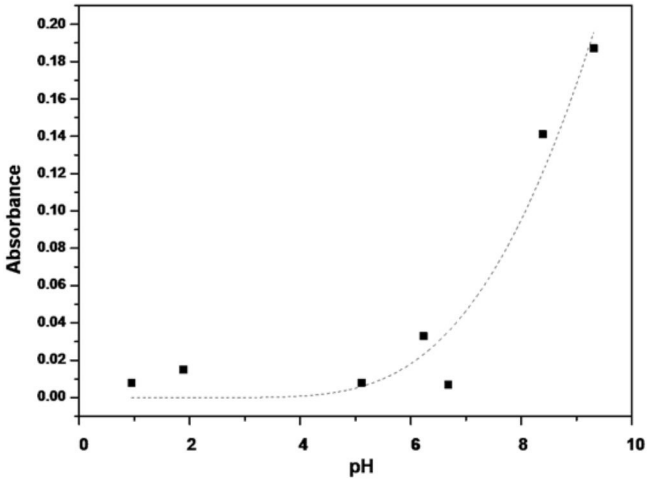

Figure 4. *Influence of pH on solubility of the palm oil microcapsules*

Solubility of microcapsules

After qualitative analysis of solubility tests, it was observed that, among the solvents studied, only the solution of NaOH (pH = 12.05 ± 0.10) resulted in a yellowish solution due to the release of the microencapsulated palm oil. After these tests, the best pH for the rupture of the palm oil microcapsules was studied.

Choice of the best pH for palm oil releasing

Figure 4 shows a clear trend to higher solubility of the palm oil microcapsules at higher pH values. In pH of 12.05 ± 0.10, the resulting solution showed high viscosity, which prevented the separation of the solvent and made it difficult to measure absorbance. The pH of 9.5 ± 0.2 was, therefore, adopted to subsequent measurements.

The trend line shown in Figure 4 is the result of the adjustment of an exponential function (Equation 5) that best fit the experimental data ($R^2 > 0.95$).

$$y = 20.85e^{\frac{-45.8}{x+0.5}} \quad (5)$$

Standardizing time and number of external washes of the microcapsules

After washing the microcapsules uninterruptedly from 30 s to 2.0 min, no significant difference was observed among absorbance values using ANOVA and t-test for significance ($p < 0.05$). Thus, the results suggest that, for a period of up to 2.0 min, the microcapsules may be washed extracting the same amount of palm oil as in a shorter time interval (Table 1), indicating that the solvent is capable of solubilizing the non-microencapsulated oil without interacting with the filling of the microcapsules.

As microcapsules with significant amount of non-microencapsulated palm oil may be present, it is possible that only one wash results in saturation of the solvent without removing external palm oil entirely. Therefore, four washes of 30 s each were standardized and, in each extraction, the organic phase is removed to a 25 mL volumetric flask and a new aliquot of solvent (5 mL) is added. Thus, the total time of contact between solvent and sample does not exceed 2.0 min. After the extractions, hexane was used to complete the volume of the flasks (25 mL).

Disruption standardization and number of extractions of microcapsules

The absorbance data obtained after each extraction of microencapsulated palm oil is shown in Figure 5. After each new extraction, there is a gradual decrease in absorbance values, tending to zero after the sixth extraction. Qualitative analysis of this data allows us to observe that, after the second extraction, similar amounts of palm oil were extracted from inside the microcapsules for all the samples. On the other hand, in the first two extractions, it was observed that, in some samples, higher amounts of palm oil were extracted in the first wash than in the second one, whereas the other samples showed opposite behavior. This fact can be associated with the interaction between the buffer and the sample, which can result in a more viscous

Table 1. Absorbance values as affected by the time of external washing of the microcapsules

Time (min)	Absorbance 1	Absorbance 2	Absorbance 3	Average ± standard deviation
0.5	0.033	0.034	0.034	0.034[a]±0.001
1.0	0.035	0.035	0.032	0.034[a]±0.002
1.5	0.034	0.037	0.036	0.036[a]±0.002
2.0	0.036	0.035	0.034	0.035[a]±0.001

[a] values with same letter did not show significant difference using ANOVA and t-test ($p < 0.05$).

suspension, hindering the contact of the solvent with the entire sample. After data analysis and visual observation of the samples, two new steps were proposed to this extraction method. The first modification was related to the sequence of actions in the first extraction; rather than adding 5 mL of buffer and 5 mL of hexane and stirring for 1.0 min., the sequence adopted started with addition of buffer, followed by stirring for 1.0 min., subsequent addition of hexane, and finally stirring for 1.0 min. Thus, there is greater contact between the buffer and the sample. The second modification was to insert a new step after the second extraction, which consisted of adding 3 mL of buffer, followed by stirring for 1.0 min., addition of hexane, and further stirring for 1.0 min. Thus, the objective was to facilitate the stirring process (decreasing viscosity) and increase the contact between the sample and the buffer, in order to maximize the microcapsules rupture and, hence, the extraction of microencapsulated palm oil. After the sixth extraction, only a small quantity of solute was observed in the organic phase.

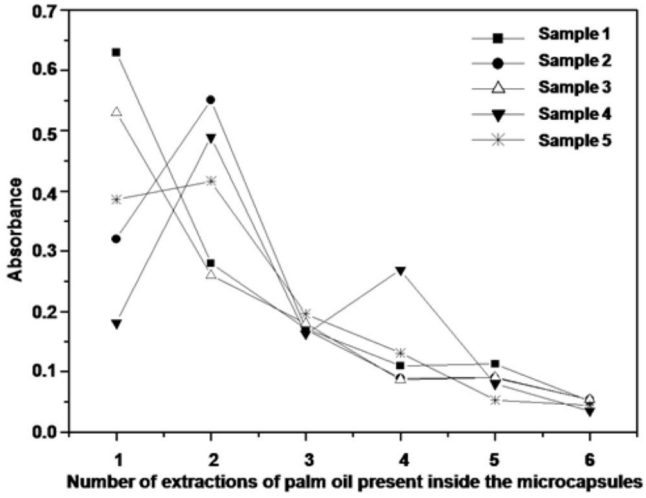

Figure 5. *Absorbance values as affected by the number of extractions of palm oil present inside the microcapsules produced by complex coacervation*

Validation of the analytical method

Specificity

The specificity of the spectrophotometric method proposed in the wavelength of 446 nm was confirmed, as the wall material of the microcapsules did not interfere in absorbance (Figures 3A and 3B). Moreover, Figure 6 shows the absorption spectra of palm oil of trademarks Marabá® and Hemmer®, in which it is possible to observe the highest value of absorbance at a wavelength of 446 nm for both samples. The absorbance values of trademark Marabá® were lower, since this oil presents 25% soybean oil in its composition.

Linearity and interval

The calibration curves showed correlation coefficient (r) higher than 0.9998, indicating a linear relationship between absorption and

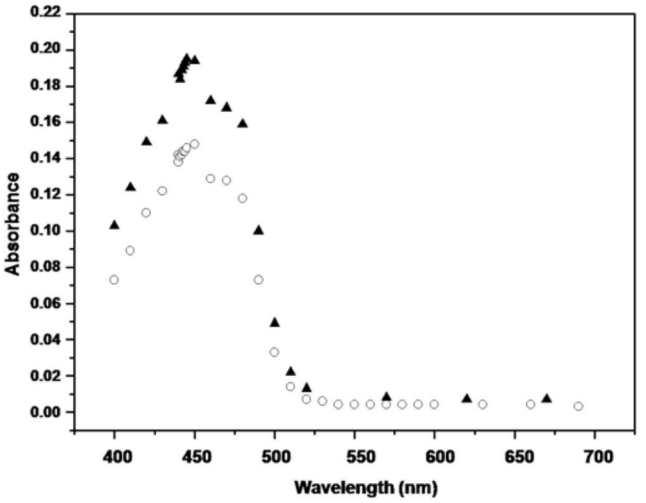

Figure 6. *Absorption spectra (400 to 690 nm) of solutions of palm oil of two different brands. (○ = Marabá®; ▲ = Hemmer®)*

palm oil concentration (Figure 7). The values found for the lower limit of detection (LD) and lower limit of quantification (LQ) were 36.21 and 120.71 μg mL^{-1}, respectively. These two values were lower than the quantification value of the method (200 μg mL^{-1}). This value was determined by the authors and corresponds to the minimum concentration that can be present in these microcapsules, thus proving that it is suitable for the proposed objectives.

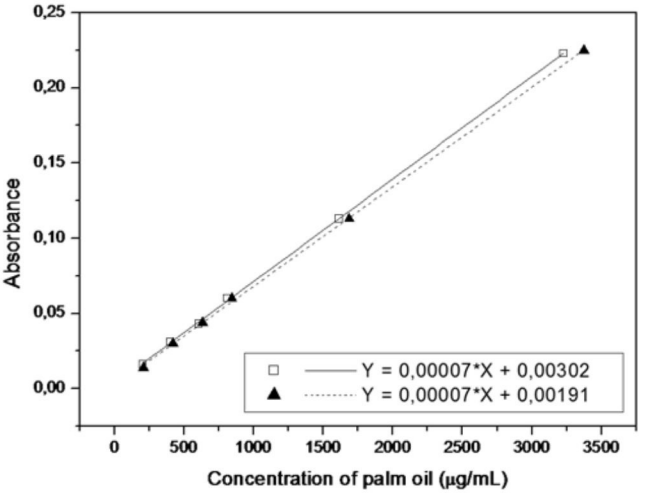

Figure 7. *Calibration curve for palm oil solutions*

Precision

The analytical parameters of precision (repeatability) and intermediate precision (reproducibility) were evaluated using the coefficient of variation (CV) and both were considerably lower than 5% (Table 2). These results are satisfactory according to RE 899 of May 29, 2003.[17] It can be stated that the method is precise intra-run and inter-run.

Accuracy

The result obtained for accuracy is shown in Table 2 and lies within the acceptance criteria, namely between 98 and 102%.[7] A coefficient of variation (CV) of 0.98% was observed, being lower than 5.0%.[19] According to the rules of the ICH (International Conference on Harmonization), the accuracy is confirmed as long as precision, linearity, and specificity are established. Thus, the proposed spectrophotometric method for quantifying the amount of palm oil present outside and inside the microcapsules is accurate, since the accuracy, the linearity, and specificities showed to be adequate.[20]

Robustness and Stability

The robustness of the spectrophotometric method was demonstrated through the conditions employed in the intermediate precision test, using different analysts and equipment. The modifications did not change the results, which remained within the acceptance criteria, indicating that the method is accurate for both types of precision evaluated (Table 2).

The solutions stored in volumetric flask and volumetric flask protected from light for up to 36 h at room temperature (25 ± 2 °C) showed stable absorbance values, allowing more convenience in the execution of the method.

Table 2. Results of validation of the spectrophotometric method for quantification of palm oil microencapsulated by complex coacervation

Validation parameters	Spectrophotometric method
Linearity	r=0.99989
	r=0.99984
LD (μg mL^{-1})	36.21
LQ (μg mL^{-1})	120.71
Repeatability (n=6)*	CV = 1.50%
Intermediate precision (n=12)	CV = 3.86%
Accuracy (n=3)	101.87 ± 0.99%

*The repeatability of the method can be verified by two ways: minimum of 6 (six) determinations at 100 % concentration of the test or minimum of 9 (nine) determinations contemplating the linear range of the method, i.e. 3 (three) concentrations low, medium and high with 3 (three) responses each.[17]

CONCLUSION

It was possible to quantify the palm oil present externally through successive extractions with hexane and absorbance reading at 446 nm. It was also possible to rupture the microcapsules using buffer at pH 9.5 ± 0.2 and to quantify the core material through six extractions with the same solvent. The proposed analytical method meets the guidelines of the current legislation, being economical, quick, easy to perform, and suitable for the purposes.

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