

Development and validation of high-performance liquid chromatography method for determination of atorvastatin in polymeric nanocapsules**Desenvolvimento e validação de método de cromatografia líquida de alta eficiência para determinação de atorvastatina em nanocápsulas poliméricas**

DOI:10.34117/bjdv6n8-697

Recebimento dos originais:08/07/2020

Aceitação para publicação:31/08/2020

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ABSTRACT

This study aimed to develop and validate a HPLC based analytical methodology for determination of atorvastatin within nanocapsules containing ginger or grape seed essential oils (NCA_TG and NCA_TU, respectively). Chromatographic separation was achieved by employing a Venusil XBR C18 column as stationary phase and a ternary mixture of acetonitrile, 0.1% phosphoric acid, and methanol (60:30:10) as the mobile phase. The validated method proved to be linear for atorvastatin in the range of both particles were 10 to 50 $\mu\text{g}\cdot\text{mL}^{-1}$. Detection and quantification limits for atorvastatin were, respectively, 30 $\text{ng}\cdot\text{mL}^{-1}$ and 300 $\text{ng}\cdot\text{mL}^{-1}$ for NCA_TG, while for NCA_TU the values were 39 $\text{ng}\cdot\text{mL}^{-1}$ and 390 $\text{ng}\cdot\text{mL}^{-1}$, what assures the methodology sensitivity. The method was also precise (1.28% RSD for NCA_TG and NCA_TU), besides showing recovery rates close to 100% for both nanocapsules formulations when accuracy was accessed. Minor alterations over chromatographic (pH, mobile phase, flow and detector) setup have confirmed methodology robustness. The present methodology was able to detect and quantify atorvastatin in nanocapsules containing ginger or grape seed essential oils. Thus, proving to be an analytical alternative for the quality control of this dosage form.

Keywords: HPLC method validation, atorvastatin, nanocapsules, essential oils.

RESUMO

O objetivo deste estudo foi desenvolver e validar uma metodologia analítica baseada em HPLC para determinação de atorvastatina em nanocápsulas contendo óleos essenciais de semente de uva ou gengibre (NCA_TG e NCA_TU, respectivamente). A separação cromatográfica foi obtida empregando-se uma coluna Venusil XBR C18 como fase estacionária e uma mistura ternária de acetonitrila, 0,1% de ácido fosfórico e metanol (60:30:10) como fase móvel. O método validado mostrou-se linear para atorvastatina na faixa de partículas de 10 a 50 $\mu\text{g}\cdot\text{mL}^{-1}$. Os limites de detecção e quantificação para atorvastatina foram, respectivamente, 30 $\text{ng}\cdot\text{mL}^{-1}$ e 300 $\text{ng}\cdot\text{mL}^{-1}$ para NCA_TG, enquanto para NCA_TU os valores foram 39 $\text{ng}\cdot\text{mL}^{-1}$ e 390 $\text{ng}\cdot\text{mL}^{-1}$, o que garante a metodologia sensibilidade. O método também foi preciso (1,28% RSD para NCA_TG e NCA_TU), além de apresentar taxas de recuperação próximas de 100% para ambas as formulações de nanocápsulas quando a precisão foi determinada. Pequenas alterações na configuração cromatográfica (pH, fase móvel, fluxo e detector) confirmaram a robustez da metodologia. A presente metodologia foi capaz de detectar e quantificar atorvastatina em nanocápsulas contendo óleos essenciais de gengibre ou semente de uva. Desta forma, mostrou-se uma alternativa analítica para o controle de qualidade desta forma farmacêutica.

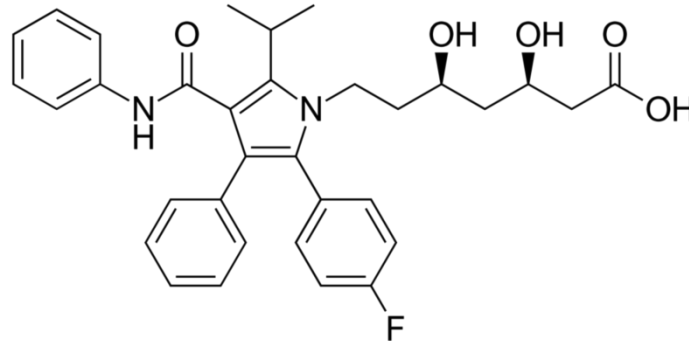
Palavras-chave: Validação de método HPLC, atorvastatina, nanocápsulas, óleos essenciais.

1 INTRODUCTION

Atorvastatin (3R,5R)-7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-propan-2-ylpyrrol-1-yl]-3,5-dihydroxyheptanoic acid) (ATV) (Fig.1) is a very utilized drug in the treatments of dyslipidemia and others cholesterol disorders (Ferreira and Marques da Silva 2016). ATV was launch in the 90's was a huge commercial success due it new and promising action mechanism for the time. The ATV act by the inhibition of the HMG-CoA reductase enzyme (3-hidroxi-3-

metilglutaril-CoA reductase) in convert the subtract HMG-CoA in mevalonic acid, lowering the hepatic cholesterol production (Arrigoni, Del Re *et al.* 2017).

Fig. 1 Atorvastatin chemical structure



The ATV demonstrates a high efficacy and its acceptance by the general public is tolerable (Gomez-Gerique, *et al.* 2003). However it may cause some side effects as: myalgia, gastrointestinal disorders and in long terms diabetes risk (Joy and Hegele 2009). Beyond that, the plasmatic distribution of ATV shows some physicochemicals problems such as: low solubility in water and low bioavailability. This fact may lead to less pharmacological effectiveness (J B, *et al* 2017).

Dyslipidemia and atherosclerosis are chronic pathologies and in many cases are identified too late or when some clinical symptom appears and affect the patient life (Siqueira *et al.*, 2020; Zidiotti *et al.*, 2020). The use of ATV, when indicated, will be to be use in long periods of the patient life (Peng *et al* 2018). Thus, the development of carrier systems in order to improving the therapeutic effect of drugs administered, such as ATV, is an interesting alternative. Among these systems, it is possible highlights the polymeric nanoparticles (NPs). NPs are vesicular or matricial colloids based on polymers. These vesicular carriers constituted of an oil core surrounded by a polymeric wall are named nanocapsules (Colomé *et al.*, 2017; Bernardi *et al.*, 2011)

The optimization of ATV in NPs, more specifically in nanocapsules (NC), have for objective formulate new therapeutics systems in the combat of diseases related to cholesterol. For this purpose, establish a new analitic method capable of quantified the ATV in NC is indispensable. Some analytical methods already exist in the literature for quantification of statins (Patel, Macwana *et al.* 2012; J.B. *et al* 2017), however, none of then about extraction and quantification of ATV in polymeric NCs containing polycaprolactone (PCL) and who involves quantification in HPLC.

2 MATERIALS AND METHODS

2.1 REAGENTS

Atorvastatin (98.97% purity) was purchased from Galena (Brazil). Poly(ϵ -caprolactona) (PCL) (Mw = 80,000), Sorbitan Monostearate and Polysorbate 80 were purchased from Sigma-Aldrich (Brazil). Acetone was obtained from Nuclear (Brazil). HPLC-grade acetonitrile (ACN) and methanol (MeOH) was acquired from Tedia (Brazil). Purified water was prepared using a Mili-Q Plus (Millipore, US). Essential oils from grape seed (*Vitis vinifera L.*) and ginger (*Rosmarinus officinalis*) were purchased from Quinari (Brazil).

2.2 EQUIPMENT AND CHROMATOGRAPHIC CONDITIONS

High performance liquid chromatograph Prominence[®] (Shimadzu), equipped with quaternary pump, automated injector, photodiode array detector (PDA) and LC software v. 1.22 SP1. For the separation of the compounds we used a C18 reverse phase column (Venusil[®] XBP, 4.6 \times 150 mm, 5 μ m) and mobile phase composed of acetonitrile, 0.1% phosphoric acid (pH 3) and methanol (60:30:10 v / v / v). The flow rate was 1 mL.min⁻¹. Injection volume of 20 μ L and ATV detection at 245nm.

2.3 NANOCAPSULES PREPARATION

Atorvastatin nanocapsules (NCA_t) containing essential oils from grape seed (*Vitis vinifera L.*) (NCA_tU) or ginger (*Rosmarinus officinalis*) (NCA_tG) were prepared by the preformed polymer precipitation method, according to Venturini *et al.* (2011) and Bender *et al.* (2012). The technique consists in the preparation of two phases, one of them is organic containing acetone, the polymer PCL, sorbitan monostearate (SPAN 60), grape seed oil (*Vitis vinifera L.*) or Ginger oil (*Zingiber officinale*) and the drug atorvastatin. The aqueous phase is formed by distilled water and polysorbate 80 (Tween 80). After the total solubilization of both phases of the formulation, the organic phase was poured with magnetic agitation under the aqueous phase using a small funnel. Utilizing a rotary evaporator (Büchi, Switzerland), the organic solvent and a part of the aqueous phase were concentrated.

2.4 SAMPLE AND STANDARD SOLUTIONS

In the drug content evaluation, stock solution of ATV (1000 μ g.mL⁻¹) was prepared by dissolving accurately weighed 10 mg of the drug in methanol using 10 mL volumetric flask. Standard solution was prepared by dilution of the diluted stock solution with mobile phase to obtain solutions in a final concentration of 10 μ g.mL⁻¹. To prepare the sample solutions, 0.10 mL of NCA_tG

or NCA_tU (1 mg.mL⁻¹ of ATV in both) were transferred into a 10 mL volumetric flask and completed with acetonitrile, obtaining the final concentration of 10 µg.mL⁻¹. This flask was kept in an ultrasonic bath for 60 min, causing partial dissolution of the polymer and release of the active contained into the nanostructure. The solutions were filtered through a 0.45 µm membrane filter and analyzed by HPLC, determining the total concentration of ATV.

2.5 VALIDATION OF ANALYTICAL METHOD

All validation was performed following the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH, 2005). The parameters evaluated in the development of this work were: Linearity, selectivity, limit of quantification (LQ), limit of detection (LD), precision, accuracy and robustness.

2.6 LINEARITY

The linearity study was evaluated by linear regression using the R² of the calibration curve (n = 3) which was constructed using the ATV 1000 µg.mL⁻¹ stock solution and NCA_tG or NCA_tU samples at the dilutions of 10, 15, 20, 25 and 50 µg.mL⁻¹.

2.7 SELECTIVITY

The selectivity of the method has the purpose to evaluated if the constituents of the nanocapsules interfered in the quantification of ATV. The experiment was performed by injecting a midpoint of the ATV calibration curve (15µg.ml⁻¹) with the sample solutions (NCA_tG and NCA_tU) and of empty nanocapsule suspension (without drug), called (NCU_v e NCG_e). At the end, the chromatograms obtained in the analysis were compared, observing the drug retention time and the analytical signal intensity.

2.8 QUANTITATION LIMIT (LQ) AND DETECTION (LD)

The limits were calculated by the signal-to-noise ratio comparing the two encapsulated ATV measurements (NCA_tG and NCA_tU) and the free drug. LD was considered a 3:1 signal-to-noise ratio, while LQ was considered a 10:1 signal-to-noise ratio. All analyzes were performed at least five times (n = 5). Relative standard deviation (RSD) was calculated across all ATV determinations.

2.9 PRECISION

The precision was evaluated by repeatability and intermediate precision. This way six samples from same concentration (15 µg.mL⁻¹ NCA_tG and NCA_tU), were analyzed in a single day (day 1/n=6). After, the analyses were respited through three dais whit a different analyst (day 2/n=

3 and day 3/n= 3). The results were expressed as percentage of the drugs content (%) and by the relative standard deviation (RSD).

2.10 ACCURACY

The accuracy was evaluated utilizing the drug recovering method. This way, three samples of NCGe and NCUv were utilized as blank NC (without ATV), and after that, concentrations of 10, 20 and 30 $\mu\text{g}\cdot\text{mL}^{-1}$ of the standard solution of ATV were add to the formulations. Each of these concentrations were prepared in triplicate (n=3) and the recuperation of the drug was evaluated.

2.11 ROBUSTNESS

Methodology robustness was evaluated by means of minor and deliberated alterations of chromatographical conditions. Thus, we modified the analysis parameters of NCA_tG and NCA_tU formulations such as pH (2.8 and 3.1), mobile phase proportions (32.5: 7.5: 60 and 27.5: 12.5: 60), detector (λ :243 and 247 nm) and flow rate. injection (0.8 and 1.1 $\text{mL}\cdot\text{min}^{-1}$). Robustness confirmation was evaluated in terms of the RSD value obtained from determination at each condition when compared to the original setup.

3 RESULTS AND DISCUSSION

Aiming the reliability in the quantification of encapsulated ATV in polymeric nanocapsules was realized the validation of the analytic method. Other studies that determined the actual ATV content by liquid chromatography were based on the use of the isolated asset. (Silva *et al.* 2019; Courlet *et al.* 2019). In our study, the process of validation of the analytic method considered the possible interferences of the compounds of the nanocapsules, such as adjuvants of the polymeric wall and the essential oils (grape seed and ginger) in the nucleus. For this purpose, we test some mobile phase proportions aiming to obtain the best chromatographic results (Reolon *et al.*, 2018) (Table 1).

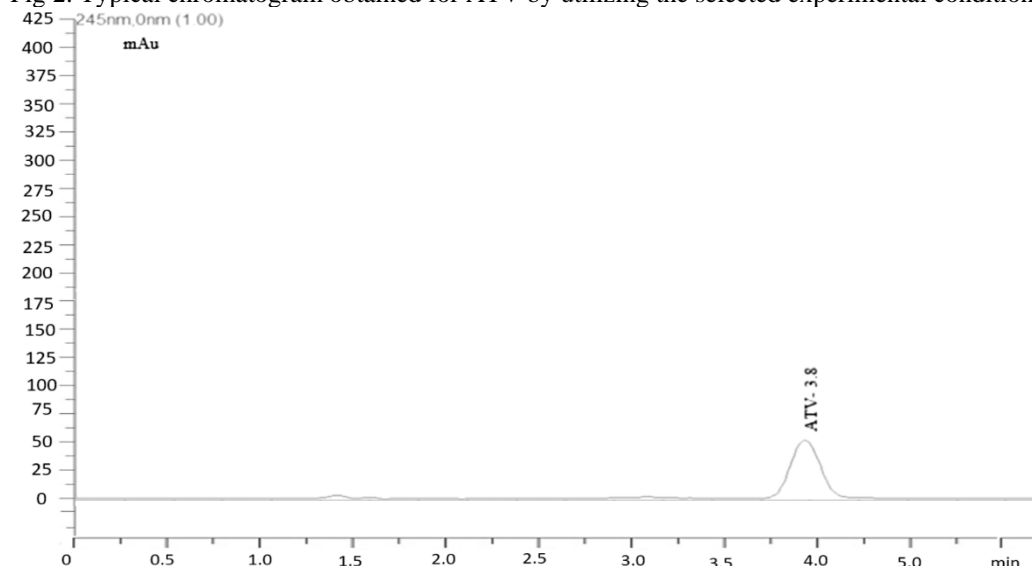
Table 1. Experimental proportions of mobile phase for better quantification of ATV.

Analyses	Flow (mL/min)	RT (min)	MF (%)	TEA (mL)
1°	1.0	2.90	13;11;73	0
2°	0.8	6.25	15;35;50	0
3°	1.0	2.70	40;20;40	1.0
4°	1.2	3.70	20;30;50	1.5
5°	1.0	3.60	10;30;60	2.0

RT= Retention time; MF= Mobile Fase; TEA= Trietilamine

Using the PDA detector, it was possible to analyze the purity peaks of the ATV, obtaining a purity of 0.9998. This result indicate an adequate chromatographic separation between the ATV and some interferents. The Figure 2 shows a common chromatogram from the ATV analyses.

Fig 2. Typical chromatogram obtained for ATV by utilizing the selected experimental conditions.



3.1 LINEARITY

The linearity of the ATV determination in nanocapsules was demonstrated according the type of nanocarrier. In this case, calibration curves were plotted to NCA_tG and NCA_tU using two analytic curves. The method showed to be linear in the range of 10 to 50 $\mu\text{g}\cdot\text{mL}^{-1}$ for ATV, with the linearity coefficient (R^2) 0.9987 and 0.9944 for NCA_tG and NCA_tU, respectively. This confirms that the response of the equipment is indeed proportional to the drug concentrations in the samples. (ICH, 2005). The validity of the assays was verified by ANOVA analysis, which demonstrated significant linear regression and no significant linearity deviation (Table 2).

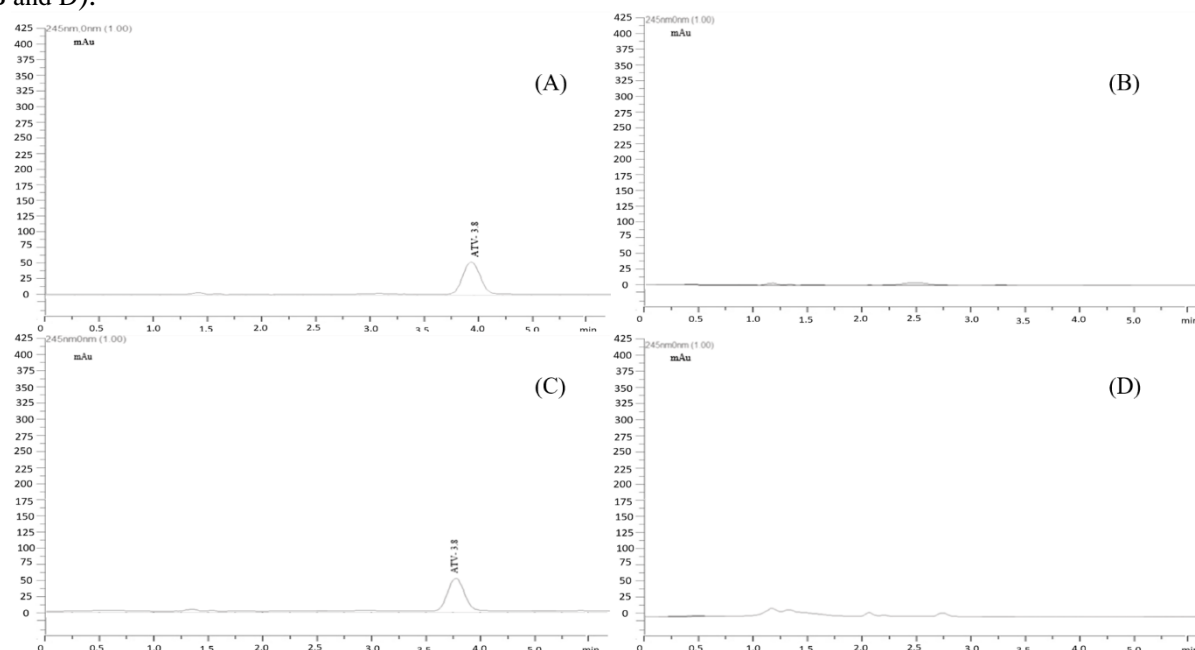
Table 2: Results concerning methodology linearity by the ANOVA analysis for ATV in NCA_tG and NCA_tU.

	NCA _t U	NCA _t G
Correlation coefficients	0.9944	0.9987
Line equations	$Y = 3717 \cdot X + 21617$	$Y = 7370 \cdot X - 13122$
Linearity range	10-50 $\mu\text{g}\cdot\text{mL}^{-1}$	10-50 $\mu\text{g}\cdot\text{mL}^{-1}$
Linear regression	$F_{\text{calculated}} = 565.1 > F_{\text{critical}} = 1.3$	$F_{\text{calculated}} = 2918 > F_{\text{critical}} = 1.3$

3.2 SELECTIVITY

The selectivity is the capacity of evaluate unambiguously the analyte in the presence of compounds whose may be present, and may interfere in the quantification, increasing the tendency of the analyses error (Ermer and Nethercote , 2015). According the chromatograms obtained for all formulations containing ATV (NCA_tG and NCA_tU) when compared to the without the drug (NCG_e and NCU_v), it was possible to observe that the different constituents of the nanoformulations do not interfered in the ATV quantification (Fig.3). In both cases there was no overlap of peaks between these formulations with and without ATV, demonstrating the selectivity of the method

Fig 3. Chromatograms of formulations with ATV: NCA_tU and NCA_tG (A and C) and without ATV: NCU_v and NCG_e (B and D).



3.3 LIMIT OF DETECTION (LD) AND QUANTIFICATION (LQ)

To determine the methodology sensitivity, LD and LQ were assessed. LD values were 30 ng.mL⁻¹ and 39 ng.mL⁻¹ for ATV in NCA_tG and NCA_tU, respectively. While LQ values were 300 ng.mL⁻¹ and 390 ng.mL⁻¹ for ATV in NCA_tG and NCA_tU, respectively. These demonstrating that the method is sensible enough to determine ATV in polimeric nanoparticles.

3.4 PRECISION

Repeatability and intermediate precision results are expressed as percentage of the drug content, and also as RSD (Table 3). Repeatability assessment was conducted by analyzing six different samples (NCA_tG and NCA_tU) at the same day, and the same experimental conditions.

RSD values were 1.28 % for NCA_tG and NCA_tU. Intermediate precision was evaluated by a total amount of twelve analyzes for three consecutive days, where RSD values were 1.64% for NCA_tG and 1.97% for NCA_tU. Recovered content were about 90.23 % and 92.32 % for NCA_tG and NCA_tU, respectively, in all assays. All RSD values were lower than 2%, what is within the established limits, asserting the methodology precision (ICH, 2005).

Table 3: Content values for NCA_tG and NCA_tU, expressed as % and RSD, representing repeatability and intermediate precision.

	NCA _t G	NCA _t U
	Repeatability	
Content (%)	98	93
RSD 1	1.28	1.28
Content (%)	98	91
RSD 2	0.78	0.32
Content (%)	105	101
RSD 3	1.11	0.58
	Intermediate precision	
Content (%)	90.2	92.3
RSD Intermediate	1.64	1.97

RSD: relative standard deviation

3.5 ACCURACY

Accuracy has assessed recovery percentage after adding known amounts of ATV at three different concentrations on NCG_e or NCU_v (NC without active) (Table 4). Values of recovery percentages were satisfactory, since it remained between 91.48 and 100.93 % for NCA_tG, as well as 96.45 and 105.36 for NCA_tU. The RSDs values was less than 2% for both, asserting the methodology accuracy, according to the established standards (ICH, 2005).

Table 4: Percentage (%) content values and RSD for atorvastatin quantification in polymeric nanocapsules, at the three evaluated concentrations, representing the method accuracy.

	NCG _e	NCU _v
	ATV (%)*	
Low concentration	91.48 ± 1.23	102.41 ± 1.31
Middle concentration	100.93 ± 1.25	105.36 ± 1.16
High concentration	98.58 ± 0.95	96.45 ± 0.07

*Values expressed as mean ± RSD; ATV: atorvastatin; RSD: relative standard deviation; NCG_e and NCU_v: nanocapsules suspension without ATV.

3.6 ROBUSTNESS

The achievement of small and deliberate changes in the conditions of analysis allowed to determine the methodological robustness. We observed that, for the selected chromatographic conditions, there was no impediment affecting the determination of ATV (Table 5). The peak areas

showed no significant changes and, the content stood close to 100%. In addition, RSD values were lower than 2% for all the varied conditions tested (pH, mobile phase ratio, flow and detector). In this case, the data obtained suggest that the methodology is robust according to the variations evaluated for quantify ATV from nanocapsules.

Table 5: ATV content and their respective RSD values obtained by the chromatographic condition variation in the nanocapsules systems.

Condition	Content (%)	
	NCA _t G	NCA _t U
No changes	98.01	93.02
pH 2.8	94.98	95.44
pH 3.1	94.75	94.44
Mobile phase ratio 60:32.5:7.5	94.60	94.03
Mobile phase ratio 60:27.5:12.5	95.13	95.85
Flow 0.8 mL.min ⁻¹	94.47	95.21
Flow 1.1 mL.min ⁻¹	95.25	94.67
Detector (λ) 243 nm	94.30	95.59
Detector (λ) 247 nm	95.43	94.30
Mean	94.94	94.86
RSD	0.68	0.55

4 CONCLUSIONS

Considering the ICH determined standards, the analytical method for the detection and quantification of the ATV was linear, precise, accurate, robust and specific in the concentration range from 10 to 50 $\mu\text{g.mL}^{-1}$. The obtained limits of quantification and detection indicated that the method is effective to quantify the ATV for a minimum concentration of 300 ng.mL^{-1} and 390 ng.mL^{-1} in NCA_tG and NCA_tU, respectively. Thus, the validated method was suitable for quantification of ATV in polymeric nanocapsules based on ginger or grape seed essential oils.

ACKNOWLEDGEMENTS

The authors thank CAPES (Brazil) for providing a master student fellowship. Financial support from the PRONEX FAPERGS/CNPq 12/2014.

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