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Low-cost method for quantification of salicylic acid loaded in lipid nanoparticles containing copaiba oil

Método de baixo custo para quantificação de ácido salicílico carregado em nanopartículas lipídicas contendo óleo de copaíba

Método de bajo costo para la cuantificación de ácido salicílico cargado en nanopartículas lípidas que contienen aceite de copaiba

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Abstract
The demand for analytical methods for drug quantification is an obstacle in the research and development of new products, due to the need to use simpler techniques for the identification and quantification of the actives. Although it is a very sensitive and selective technique for these determinations, high performance liquid chromatography (HPLC) requires expensive instrumentation and well-trained analysts, while spectrophotometry is fast, low-cost and hardly requires sample pre-treatment. Thus, this study aimed to develop a quantification method by UV-visible spectrophotometry for salicylic acid (SA) encapsulated in nanostructured lipid carriers (NLC) containing copaiba oil. The method was validated according to national and international criteria (ANVISA, ICH and FDA). The diluent was a mixture of absolute ethanol and phosphoric acid (99:1) and detection was performed at 310 nm. The interference of the matrix in relation to NLC-SA was minimal (0.37%), demonstrating the selectivity. Linearity was obtained in the range of 5.0 30.0 μg/mL (r = 0.99948) and the method proved to be accurate, with high recovery of SA in the presence of the other components (over 96% recovery), with precision at two levels: intra- and inter-days (DPR < 5%). Detection and quantification limits were 0.51 e 1.70 μg/mL, respectively. Finally, according to the Youden test criteria, the method was robust in the face of the proposed variations, obtaining effect values lower than the critical value (0.07). Therefore, the developed method is accessible, effective and valid for the quantification of SA in complex matrix lipid nanoparticles.
Keywords: Acne vulgaris; Analytical method; Analytical validation; Lipid nanocarriers; UV-Visible Spectrophotometry; Vegetable bioactive.

Resumo
A demanda por métodos analíticos para quantificação de fármacos é um entrave na pesquisa e desenvolvimento de novos produtos, devido à necessidade de utilização de técnicas mais simples para identificação e quantificação dos ativos. Embora seja uma técnica muito sensível e seletiva para essas determinações, a cromatografia líquida de alta eficiência (HPLC) requer instrumentação cara e analistas bem treinados, enquanto a espectrofotometria é rápida, de baixo custo e dificilmente requer pré-tratamento da amostra. Assim, este estudo teve por objetivo desenvolver um método de quantificação por espectrofotometria UV-visível para ácido salicílico (SA) encapsulado em carreadores lipídicos nanoestruturados (NLC) contendo óleo de copaíba. O método foi validado segundo critérios nacionais e internacionais (ANVISA, ICH e FDA). O diluente foi uma mistura de etanol absoluto e ácido fosfórico (99:1) e a detecção foi realizada em 310 nm. A interferência da matriz em relação ao NLC-SA foi mínima (0,37%), demonstrando a seletividade. A linearidade foi obtida na faixa de 5,0 a 30,0 μg/mL (r = 0,99948) e o método se

mostrou exato, com alta recuperação de SA na presença dos demais componentes (acima de 96% de recuperação), com precisão em dois níveis: intra e interdias (DPR < 5%). Os limites de detecção e quantificação foram 0,51 e 1,70 µg/mL, respectivamente. Por fim, de acordo com os critérios do teste de Youden, o método mostrou-se robusto diante das variações propostas, obtendo valores de efeito inferiores ao valor crítico (0,07). Portanto, o método desenvolvido é acessível, eficaz e válido para a quantificação de SA em nanopartículas lipídicas de matriz complexa.
Palavras-chave: Acne vulgaris; Método Analítico; Validação analítica; Nanocarreadores lipídicos; Espectrofotometria UV-Visível; Bioativo vegetal.

Resumen
La demanda de métodos analíticos para la cuantificación de fármacos es un obstáculo en la investigación y desarrollo de nuevos productos, debido a la necesidad de utilizar técnicas más sencillas para la identificación y cuantificación de los activos. Aunque es una técnica muy sensible y selectiva para estas determinaciones, la cromatografía líquida de alta resolución (HPLC) requiere instrumentación costosa y analistas bien capacitados, mientras que la espectrofotometría es rápida, de bajo costo y difícilmente requiere pretratamiento de la muestra. Por lo tanto, este estudio tuvo como objetivo desarrollar un método de cuantificación por espectrofotometría UV-visible para ácido salicílico (SA) encapsulado en portadores de lípidos nanoestructurados (NLC) que contienen aceite de copaiba. El método fue validado según criterios nacionales e internacionales (ANVISA, ICH y FDA). El diluyente fue una mezcla de etanol absoluto y ácido fosfórico (99:1) y la detección se realizó a 310 nm. La interferencia de la matriz con relación a NLC-SA fue mínima (0,37%), demostrando la selectividad. Se obtuvo linealidad en el rango de 5,0 a 30,0 µg/mL (r = 0,99948) y el método demostró ser exacto, con alta recuperación de AS en presencia de los demás componentes (más del 96% de recuperación), con precisión en dos niveles: intra e interdías (DPR < 5%). Los límites de detección y cuantificación fueron 0,51 y 1,70 µg/mL, respectivamente. Finalmente, según los criterios del índice de Youden, el método se mostró robusto ante las variaciones propuestas, obteniendo valores del efecto inferiores al valor crítico (0,07). Por tanto, el método desarrollado es accesible, eficaz y válido para la cuantificación de AS en nanopartículas lipídicas de matriz compleja.
Palabras clave: Acne vulgaris; Método analítico; Validación analítica; Nanocargadores lipídicos; Espectrofotometría UV-Visible; Bioactivo vegetal.

1. Introduction

Acne vulgaris is a chronic inflammatory disease that acts on the sebaceous glands of the skin, causing increased sebum secretion (Ferreira-Nunes, et al., 2019). Very common among young people, acne is characterized by comedones, papules, pustules and nodules, commonly on the face, neck and back. Patients with severe acne have a tendency to form scars that can lead to facial disfigurement (Zheng et al., 2018). Therapeutic options for the treatment mainly involve retinoids, antibiotics and hormone therapy, each acting against the disease by specific mechanisms (Charny, et al., 2017). Topical application of retinoids is limited due to their poor stability and local irritation, and oral retinoids also cause skin and mucosa dryness. Although antibiotics are effective for acne, subsequent drug resistance becomes a major challenge (Zheng et al., 2018).

A current alternative for the treatment of mild and moderate acne is salicylic acid (SA). SA is a beta-hydroxy acid commonly used in chemical peeling, as it acts as a keratolytic, anti-inflammatory and reduces sebum production, with a comedolytic effect. Thus, this agent has been used in the complementary treatment of acne (Kantikosum, et al., 2019). However, SA is poorly soluble in water, and tends to precipitate in aqueous and alcoholic solutions with low pH, which limits its use in the form of aqueous solutions or hydrogels. The recrystallization of SA in the formulation decreases its bioavailability and may increase the skin irritation (Lu et al., 2019; Zheng et al., 2018).

Topical formulations based on nanocarriers aiming the target to the pilosebaceous units can considerably reduce the adverse effects of the encapsulated drugs, such as SA. Lipid nanoparticles are nanocarriers capable of incorporating these bioactives of interest, directing them to the desired target and releasing them in a controlled ratio. Among the lipid nanoparticles, the nanostructured lipid carriers (NLC) are nanostructures composed of a solid lipid matrix containing a mixture of solid and liquid lipids stabilized by surfactants, capable of promoting the controlled release of encapsulated molecules and protection against degradation, while increasing the long-term stability (Danaei et al., 2018; Garcês, et al., 2018; Ghate, et al., 2016; Krambeck, et al., 2020; Mu & Holm, 2018; Patel & Prabhu, 2020).

In recent years, NLC have been widely used for encapsulation of vegetable oils. Since the oils are mostly lipophilic, they have a good miscibility with the solid lipids in the lipid matrix, resulting in a high amount of oil encapsulated within the nanosystem (Falcão et al., 2015, p. 568). Thus, these carriers have the potential to protect the labile and sensitive components of the oil from chemical, photochemical or oxidative degradation, since, due to immobilization by solid lipids, nanocarriers serve as a barrier between the encapsulated oil and the environment Another intended feature of the use of NLC as a carrier of vegetable oils is to provide a controlled release pattern, seeking to improve their bioavailability, reduce toxicity and thus increase patient compliance (Lammari, et al., 2021; Vieira et al., 2020).

Copaiba oleoresin (CO) is a vegetable oil widely used in folk medicine, with indications for its wound healing, anti-inflammatory, antinociceptive and antimicrobial activity, which can be encapsulated in NLC in order to protect its components. The main components of this oleoresin from Copaifera reticulata Ducke are the sesquiterpenes α-copean (3-4%), β-caryophyllene (38-45%), β-elemene (1-2%), cadinene (2-3%), β -bisabolene (2- 4%), α-humulene (5-7%), and diterpenic acids (14-16%). Thus, as the NLC formulation has oils in its constitution, an interesting strategy is the use of bioactive vegetable oils as matrix constituents, especially those with low toxicity, such as CO. These formulations can also be enriched with other encapsulated actives, such as SA, in order to obtain a combined effect (Arruda et al., 2019; Svetlichny et al., 2015; Teixeira et al., 2017).

One of the main challenges in the research of new drug delivery systems is the development of analytical methods that allow not only the dosage of drugs, but also their unequivocal quantification in the presence of excipients and other additives in the formulations (Ferreira-Nunes et al., 2019). High performance liquid chromatography (HPLC) is a physical-chemical separation technique that, coupled with ultraviolet-visible (UV-Vis) detection, has become one of the most used methods for quantitative analysis of various bioactives, due to its high sensitivity, selectivity and versatility. However, HPLC requires preliminary sample preparation, such as solvent extraction and separation of interfering metal ions, which makes this analysis more time consuming, in addition to demanding a high cost associated with the use of an expensive equipment, along with specific analyst training requirements (Albuquerque et al., 2020; Ferreira et al., 2020).

These limitations can be overcome by employing simpler techniques, such as UV-Vis absorption spectrophotometry. Spectrophotometric determinations are simple and accessible, accurate, reproducible and inexpensive, as they do not require the use of sophisticated analytical instruments, in addition to providing fast, reliable and easy-to-interpret results (Ferreira et al., 2020; Nalini & Kumar, 2020). However, since this technique may lack selectivity, this parameter should be controlled, especially when the analyte is in the presence of a complex matrix, such as the case of vegetable oils. Few studies have proposed the use of spectrophotometry for direct quantification of SA, including the direct determination of SA associated in tablets with acetylsalicylic acid, salicylamide, caffeine, and phenacetin (Clayton & Thiers, 1966); SA determination in aspirin tablets by second derivative analysis (Kitamura & Majima, 1982; Kokot & Burda, 1998); in pharmaceutical formulations by using copper (II) acetate as a color developer (Saha & Baksi, 1985); in a resorcinol and lactic acid solution (Jessner solution), and in a hydrogel matrix by first and second derivative analyses (Ramos, et al., 2005); in aspirin and dipyridamole association tablets by second derivative analysis (El-Ragehy, et al., 2016). However, to the moment, reported studies with the spectrophotometric quantification of SA in lipid nanoparticles have not been found. Thus, the main goal of this study was to develop and validate a spectrophotometric method for the determination of SA incorporated in NLC containing CO, according to the parameters established by national and international legislation.

2. Methodology

2.1 Materials

Salicylic acid was supplied by Xiamen Fine Chemical Import & Export Co. Ltd (Xiamen, China). Copaiba oleoresin (Copaifera reticulata Ducke) was obtained from Laszlo (Belo Horizonte, Brazil). The other components of the lipid nanoparticles were the surfactants Span 80 (sorbitan monooleate) and Tween 80 (polysorbate 80), kindly provided by Croda Inc (Edison, USA); and Capmul GDB EP/NF (mixture of diglycerides, mainly glyceryl dibehenate, together with mono- and triglycerides), kindly provided by IMCD Brasil (São Paulo, Brazil).

Tetrahydrofuran (THF) P.A./ACS, used to solubilize the nanoparticle formulations, was supplied by Neon Comercial (Suzano, Brazil). The solvent utilized in the validation studies were absolute ethanol, from Ciclo Farma (São Paulo, Brazil) and Itajá (Goianésia, Brazil); and phosphoric acid (85% purity), from ISOFAR (Rio de Janeiro, Brazil) and Dinâmica Química Contemporânea (Indaiatuba, Brazil).

2.2 Prepare of the Lipid Nanoparticles

The NLC formulations were prepared by the hot emulsification method followed by ultrasonication. First, the oily phase (OP), containing 500 mg Capmul GDB (solid lipid), 100 mg Tween 80 + 100 mg Span 80 (surfactant mixture) and 500 mg CO (bioactive oil), was heated to 85 °C. Next, water preheated to the same temperature q.s. 20 mL batch was poured into the OP, stirred for 2 minutes and immediately subjected to high-intensity sonication (40% amplitude) for 10 minutes in an ultrasound probe (Q55 sonicator; Qsonica, Church HillRoad, Newton, USA). After this time, the formulations were cooled to room temperature for the formation of the lipid nanoparticles. NLC-SA were prepared by including SA in the OP and blank NLC (i.e., NLC without SA) were prepared by removing SA from the OP composition.

2.3 Interference of the Diluent in the SA Quantification

To determine the interference of the diluent in the absorbance of SA, three solvents were assessed: distilled water, absolute ethanol and a mixture of absolute ethanol and phosphoric acid (ET:AF) in a ratio of 99:1. SA solutions were prepared in each solvent at the nominal concentration of 20.0 µg/mL and the absorbance were determined in a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, USA) at wavelength ranging between 200 – 400 nm, using a quartz cuvette with optical path of 1 cm.

2.4 Validation of the Analytical Method

The spectrophotometric method for the quantification of SA in the lipid nanoparticles was developed and validated according to national and international criteria: RDC 166/2017 from Agência Nacional de Vigilância Sanitária (ANVISA) (2017), the Brazilian Health Regulatory Agency; Guideline Q2 (R1) from the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) (2005); and the Analytical Procedures and Methods Validation for Drugs and Biologics, from the U.S. Food and Drug Administration (FDA) (2015). Thus, the parameters selectivity, linearity, detection limit, quantification limit, precision (intra- and inter-days), accuracy and robustness were determined.

2.4.1 Selectivity

To determine the selectivity of the method, the absorption spectrum between 200 – 400 nm of three preparations at the same concentration was obtained: an SA solution and the lipid nanoparticles containing or not SA diluted in the same way. The

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SA solution was prepared directly in the diluent at 20 µg/mL. The NLC-SA was first solubilized in THF and then diluted to 20 µg/mL with the diluent. Blank NLC was diluted in the same way as NLC-SA. A THF solution at the same concentration used in the solubilization of the nanoparticles was prepared in the diluent, as a negative control. All spectra were overlaid and the percentage of interference at a wavelength of 310 nm was also determined.

2.4.2 Linearity

For the determination of linearity, a stock solution of SA was prepared at the concentration of 1 mg/mL. From this solution, 6 independent dilutions were obtained in triplicate, ranging from 5.0 to 30.0 µg/mL, whose absorbance was determined at 310 nm. Linear regression was performed using the least squares method and tests for linearity departure and regression significance were also performed ($\alpha = 0.05$). The analytical curve was plotted, its equation was determined, and the slope significance was also determined ($\alpha = 0.05$). The data were evaluated ($\alpha = 0.05$) for the presence of outliers (Jackknife residuals), normality (Ryan Joiner test), homoscedasticity (modified Levene's test) and residual independence (Durbin-Watson test).

2.4.3 Detection and Quantification Limits

The detection limit (DL) represents the lowest detectable concentration of SA but not necessarily quantitated, while the quantification limit (QL) represents the lowest amount of SA that can be quantitatively determined with suitable reliability. The LOD and LOQ values were estimated based on the analytical curves, according to the following equations 1 and 2:

$$LOQ = \frac{10\,\sigma}{S} \tag{1}$$

$$LOD = \frac{3{,}3\,\sigma}{S} \tag{2}$$

where σ is the residual standard deviation of the regression line and S is the slope of the calibration curve.

2.4.4 Accuracy and Precision

The accuracy of the method was assessed from the recovery of SA in the presence of the other components of the lipid matrix. Exact amounts of SA were then added to the blank NLC to obtain different concentration levels: 5.0, 20.0 and 30.0 µg/mL. The test was performed in triplicate and the percent recovery was determined through equation 3:

$$Recovery\ (\%) = \frac{Measured\ SA\ amount}{Added\ SA\ amount} \times 100 \tag{3}$$

Precision was assessed at two levels: intra- and inter-days. Intra-assay repeatability was determined from the analysis of six SA solutions prepared on the same day and under identical experimental conditions, at the concentration of 20.0 µg/mL. Inter-days precision was determined from the analysis of twelve SA solutions at 20.0 µg/mL, each group of six solutions prepared on different days and under identical experimental conditions. All results were recorded and the relative standard deviation (DPR) was calculated, which must be less than 5% (Agência Nacional de Vigilância Sanitária [ANVISA], 2003).

2.4.5 Robustness

The robustness of the SA quantification method was determined using the method proposed by Youden and Steiner (Youden & Steiner, 1975). Seven analytical parameters were selected and small variations were induced in the nominal values

of the method (Table 1): the spectrophotometer used in the measurements (A/a), wavelength (B/b), Et supplier (C/c), PhA supplier (D/d), solvent ratio (Et:PhA) (E/e), ambient temperature (F/f), and reading time (G/g).

Then, the parameters were combined in eight spectrophotometric tests (s to z), according to the factorial combination matrix (Table 1), and were performed in random order, in order to determine the influence of each parameter on the final result. A standard solution at the nominal concentration of 20.0 μg/mL was prepared and its absorbance was determined in triplicate under the conditions expressed in each combination assay. The standard curve equation was used to determine the measured concentration.

Table 1 – Parameters varied in the robustness test with the nominal and alternative conditions. The robustness combination assays (*s* to *z*) were performed according to the factorial combination matrix.

Analytical parameter	Nominal condition	Alternative condition	Factorial combination							
			1	2	3	4	5	6	7	8
Spectrophotometer	Agilent Cary 60 (A)	Tecnal UV 5100 (a)	A	A	A	A	a	a	a	a
Wavelength	310 nm (B)	311 nm (b)	B	B	b	b	B	B	b	b
Et supplier	Itajá (C)	Ciclo Farma (c)	C	c	C	c	C	c	C	c
PhA supplier	Isofar (D)	Dinâmica (d)	D	D	d	d	d	d	D	D
Solvent ratio (Et:PhA)	99:1 (E)	98:2 (e)	E	e	E	e	e	E	e	E
Ambient temperature	22 ºC (F)	25 ºC (f)	F	f	f	F	F	f	f	F
Reading time	1 s (G)	10 s (g)	G	g	g	G	g	G	G	g
	Combination assays		*s*	*t*	*u*	*v*	*w*	*x*	*y*	*z*

Source: Authors.

The influence of each parameter on the variation of the final result was determined by comparing the average of the four values corresponding to the nominal conditions (uppercase letters) with the average of the four values corresponding to the altered conditions (lowercase letters). For instance, to evaluate the effect of changing the spectrophotometer in the analysis (A/a), the results of the combinations s, t, u, and v (with nominal conditions for "A") were compared to the results of the combinations w, x, y and z (with alternative conditions for "a"), as expressed in the following equation 4:

$$E(A/a) = \left(\frac{s+t+u+v}{4}\right) - \left(\frac{w+x+y+z}{4}\right) \quad (4)$$

The effect was considered relevant when its absolute value was higher than the critical value defined by the expression $s\sqrt{2}$, where s is the standard deviation obtained from the eight combination assays (Enéas et al., 2020; Ferreira-Nunes et al., 2019). Thus, by performing the Youden's test, it is possible to identify the parameters with the greatest influence on the final results and so have a more rigorous control in avoiding variations in critical parameters during the routine analysis (César & Pianetti, 2009; Ferreira-Nunes et al., 2019).

3. Results and Discussion

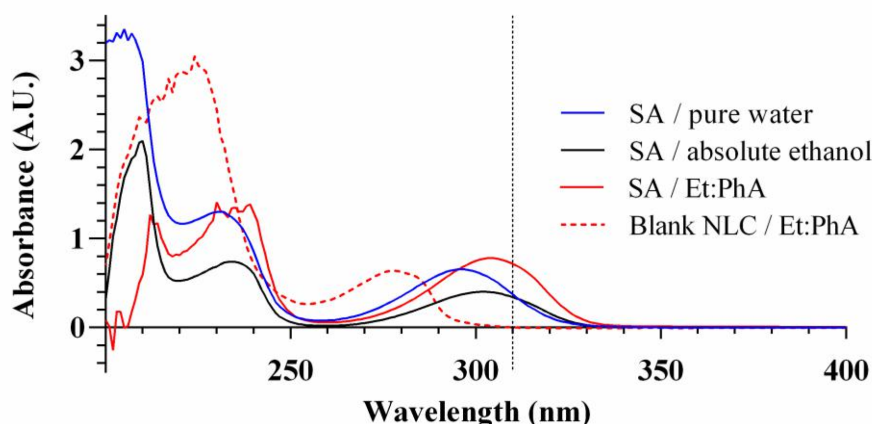
3.1 Selectivity

The quantification of pure active substances in a complex matrix composed of multiple constituents, as is the case of lipid nanoparticles, is a major concern during the development of this type of formulations, due to the need for a reliable analysis method to quantify a given analyte in the presence of matrix components. When a bioactive oil, such as copaiba oil, is included in this matrix, this concern becomes even greater, since bioactive vegetable oils by themselves are already sufficiently

complex matrices (Ferreira-Nunes et al., 2019; Leandro et al., 2012). Thus, in this study the use of a low-cost spectrophotometric analytical method is proposed for the quantification of SA in the face of a complex lipid matrix of NLC containing copaiba oil.

Selectivity was then the first parameter to be evaluated, that is, the ability to quantify the analyte against other components, such as impurities, degradation products and matrix components (ICH, 2005). The absorption spectra of SA were evaluated together with the blank NLC (equivalent to the lipid matrix without SA) between 200 and 400 nm, performed in three different diluents. The SA absorption spectrum presented a profile with two regions in all solvents evaluated (Figure 1): some absorption peaks in the range of 200-250 nm and one peak with absorption maximum defined around 300 nm. The maximum absorption of the peak was at 301 nm in distilled water, at 302 nm in absolute ethanol, and at 304 nm in the Et:PhA (99:1) mixture. Compared with the spectrum of absolute ethanol, a bathochromic shift is observed with the addition of phosphoric acid in the mixture Et:PhA (99:1), probably associated with the greater number of protonated SA species. At this peak, the highest specific absorption coefficient for SA was observed in the Et:PhA mixture (39.15 L g^{-1} cm^{-1}), followed by distilled water (30.65 L g^{-1} cm^{-1}) and absolute ethanol (20.20 L g^{-1} cm^{-1}).

Figure 1 – SA absorption spectra in the range of 200-400 nm, performed in three different diluents and blank NLC absorption spectrum in the least interfering solvent at 310 nm (Et:PhA mixture).



Source: Authors.

The lowest percentage of lipid matrix interference was found at 310 nm (highlighted in Figure 1) and using the Et:PhA mixture as diluent (0.37%), but was drastically higher in absolute ethanol (14.8%) and in distilled water (165.1%). The absorption spectrum of blank NLC in Et:PhA mixture showed an absorption peak with a maximum at 280 nm, which would represent a possibility of interference. However, minimal interference was observed at the wavelength of 310 nm, which was the reason that this diluent and this wavelength were defined to proceed with the method validation. Due to this low interference, there was no need to apply second derivatives or chemometric methods to evaluate these data, thus obtaining a simple and direct spectrophotometric methodology.

Previous studies obtained a similar profile in the SA absorption spectra, with peaks at 200-250 nm and near 300 310 nm (Clayton & Thiers, 1966; El-Ragehy et al., 2016). However, most of these methods required some additional actions, such as complexation with copper II (Saha & Baksi, 1985), the use of first and second derivatives (Kitamura & Majima, 1982; Kokot & Burda, 1998; Ramos et al., 2005), or using chemometrics tools (El-Ragehy et al., 2016). Even thus, the proposed detection wavelength was similar: 316 nm in 1% chloroacetic acid-ethanol solution (Kitamura & Majima, 1983), 328 nm in

acetonitrile-formic acid solution (99:1) (Kokot & Burda, 1998), 320 nm for absolute ethanol and 317 nm for hydrogel matrix in 0.1 N sulfuric acid (Ramos et al., 2005).

3.2 Linearity

An appropriate analytical method should exhibit the best linear correlation between the analytical response and the concentration of the analyte in the sample. Thus, linear regression using the method of least squares was applied to fit the standard curve (Figure 2A) and showed an excellent correlation ($r > 0.99$) in the range of 5 – 30 μg/mL (Table 2), as recommended by guidelines such as the ICH (ICH, 2005). Since the regression coefficient is closer to 1.0, the dispersion of the set of experimental points is considered smaller, with low uncertainty of the estimated regression coefficients (Abrão et al., 2020; ANVISA, 2017; ICH, 2005).

Table 2 – Linearity and regression parameters.

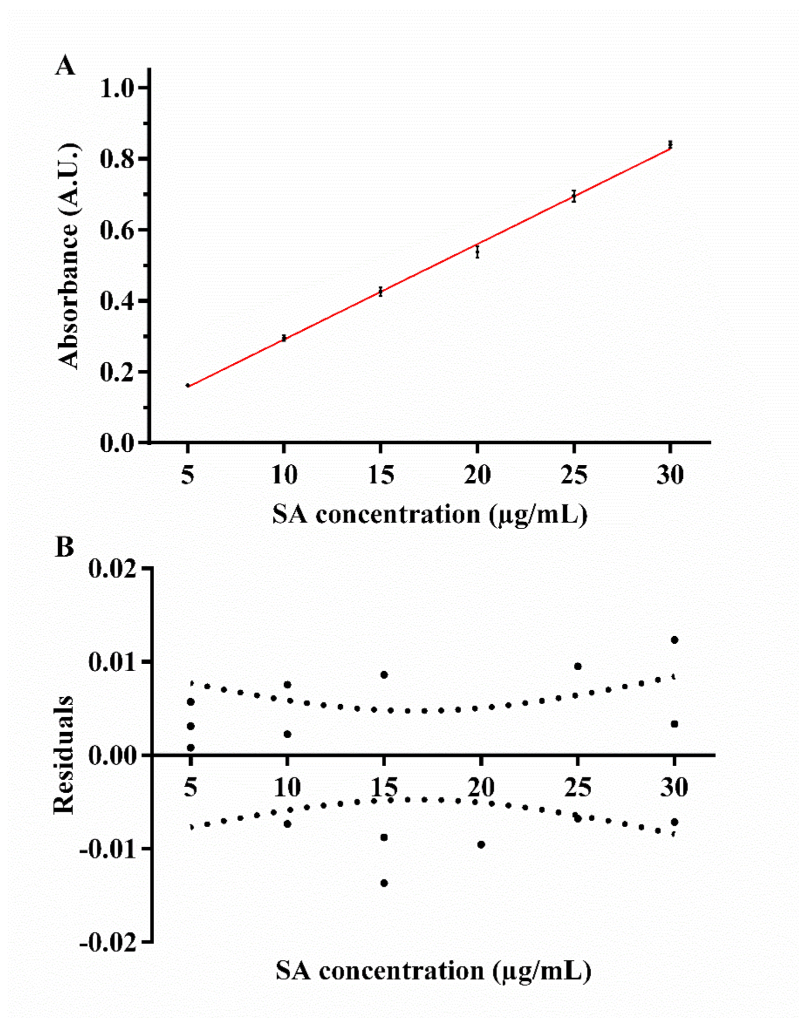
Regression parameters	Results
Slope ± s.d.	0.0271 ± 0.00024
Curve intersection ± s.d.	0.0233 ± 0.0046
Correlation coefficient ®	0.99948
Concentration range	5 to 30 μg/mL
Number of points	6
Statistical analysis	**Result (p-value)**
Significance of regression	Significant (8.67×10^{-21})
Linearity departure	Not significant (0.63)
Slope significantly non-zero	Significant (< 0.0001)
Ryan Joiner test (normality)	Normal distribution (> 0.05)
Modified Levene's test (homoscedasticity)	Homoscedasticity (0.27)
Durbin-Watson test (residual independence)	Significant (< 0.05)

Note: *s.d. = standard deviation. Source: Authors.

The linear regression equation was $y = 0.02711x + 0.2346$, where y is the absorbance and x is the nominal concentration of SA (μg/mL). The regression was statistically significant ($p < 0.05$) and no lack of fit to the linear model was observed ($p > 0.05$), and the slope was considered significantly non-zero ($p < 0.05$).

In the Jackknife standardized residuals test, three values were found to exceed the critical t-value and were therefore excluded, which is less than the limit established by the test (22% of the data). In addition, the residues were randomly distributed (Figure 2B) and independent ($p < 0.05$), the responses of the spectrophotometric method showed normal distribution ($p > 0.05$) and homoscedasticity ($p > 0.05$) (Almeida, et al., 2017; Souza et al., 2018; ICH, 2005; Souza & Junqueira, 2005).

Figure 2 – Linearity results. Analytical curve (A) and residuals plot (B) of the developed method.



Source: Authors.

Spectrophotometric methods previously developed for SA analysis in face of other substances also showed high correlation coefficient (r > 0.99) and detection in similar linear ranges. Ramos et al. (2005) observed linearity in the range of 12-24 µg/mL for SA quantification in the presence of resorcinol and lactic acid in ethanol, and 8-32 µg/mL for the analysis in a hydrogel matrix, using 0.1 N sulfuric acid solution as diluent. For SA analysis in the presence of acetylsalicylic acid, as in tablets, the linear range was 1-10 µg/mL in 1% chloroacetic acid-ethanol solution (Kitamura & Majima, 1982) and 0.5-40 µg/mL in acetonitrile–formic acid solution (99:1) (Kokot & Burda, 1998). A low and narrow range (1-3 µg/mL) was proposed for the analysis of SA in the presence of the components of acetylsalicylic acid and dipyridamole tablets (El-Ragehy et al., 2016).

3.3 Detection and Quantification Limits

DL and QL were estimated from the relationship between the standard deviation of the regression line and the slope of the standard curve. The DL and QL values obtained in this study were respectively 0.51 and 1.70 µg/mL, indicating that the proposed spectrophotometric method was sensitive for small amounts of SA (ICH, 2005). Previous studies showed DL and QL values in the same concentration range: 0.51 and 1.71 µg/mL (DL and QL) in hydrogel (Ramos et al., 2005), 0.19 and 0.65 µg/mL (DL and QL) using second derivative analysis in Jessner solution, and 0.12 µg/mL (DL) also using second derivative to quantify SA in acetylsalicylic acid tablets (Kitamura & Majima, 1982).

3.4 Accuracy, Intra-day and Inter-day Precision

The accuracy of an analytical method indicates its ability to provide experimental responses as close as possible to the real and expected values. The average recovery, the relation between the experimental concentration obtained as response of the method and the expected concentration, is one of the ways to demonstrate the method accuracy. The average recovery from SA solutions at 5.0, 20.0 and 30.0 µg/mL was found to be near 100% (Table 3), and within the range of 80-120%, the limits usually considered in the literature and established by regulatory agencies (Food and Drug Administration [FDA], 1994; ICH, 2005), which underscores the accuracy of the method. Previous studies involving different matrices and concentration ranges considered valid the methods that showed recovery in the range of 99.7-100.1% for the concentration of 1-3 µg/mL (El-Ragehy et al., 2016), 99.3-103.0% for ethanolic solution in the concentration of 2-5 µg/mL and 98.0-100.8% for hydrogel at 4-10 µg/mL (Ramos et al., 2005).

Precision indicates the ability of the analytical method to maintain a constant response of a given analyte solution within the linear range regardless of when quantification is performed and/or the analyst responsible for the experiment. The results obtained with intra-day and inter-day precision were expressed as the relative standard deviation of the six determinations obtained on the same day (repeatability) and of the twelve determinations obtained on two different days (intermediate precision). All these RSD values were below 5% (Table 3), as established by regulatory agencies (ANVISA, 2003).

Table 3 – Accuracy, intra-day and inter-day precision for SA quantification.

Accuracy		
Added SA concentration (µg/mL)	**Measured SA concentration (µg/mL)**	**Recovery (%)**
5.20	5.00 ± 0.15 [3.04%]	96.1 ± 2.92 [3.04%]
20.40	21.12 ± 0.61 [2.90%]	103.5 ± 3.00 [2.90%]
30.60	30.20 ± 0.17 [0.57%]	98.7 ± 0.57 [0.57%]
Precision		
Inter-days (n=6)		**Intra-day [n=12]**
20.25 ± 0.63 [3.13%]	19.82 ± 0.47 [2.39%]	20.04 ± 0.30 [1.51%]

Note: *Results expressed as average values ± standard deviation, with each calculated relative standard deviation (%RSD) inside brackets. Source: Authors.

3.5 Robustness

Robustness indicates the ability of the analytical method to withstand small and deliberate variations in analytical conditions. The methodology proposed by Youden and Steiner (1975) was used to verify the effect of each variation in the SA absorbance (Table 4), through an experimental design of factorial combination. The most relevant differences indicated that the corresponding factors have a greater influence than the others on the method response. Differences greater than the critical value ($s\sqrt{2}$, where s is the standard deviation of the eight determinations) in absolute values were considered significant in the effect analyses (ANVISA, 2017; Enéas et al., 2020; Youden & Steiner, 1975).

Table 4 – Effects of the analytical parameters on the absorbance (U.A.) for the SA quantification method.

Parameter	Nominal condition	Alternative condition	Effect on Absorbance
Spectrophotometer	Agilent Cary 60 (A)	Tecnal UV 5100 (a)	-0.01
Wavelength	310 nm (B)	311 nm (b)	0.01
Et supplier	Itajá (C)	Ciclo Farma (c)	0.02
PhA supplier	Isofar (D)	Dinâmica (d)	-0.08
Solvent ratio (Et:PhA)	99:1 (E)	98:2 (e)	0.00
Ambient temperature	22 ºC (F)	25 ºC (f)	-0.01
Reading time	1 s (G)	10 s (g)	-0.04

Source: Authors.

The critical value obtained was 0.07, so that none of the effects had absolute value greater than the critical value (Table 4). Thus, the influence of the variations introduced in the method were negligible within the analyzed conditions and the spectrophotometric method was considered robust in relation to the variations in the spectrophotometer used in the measurements, wavelength, Et supplier, PhA supplier, solvent ratio (Et:PhA), ambient temperature, and reading time.

4. Conclusion

Even after several years, UV-Vis spectrophotometry is still a useful technique in the identification and quantification of drugs and other bioactives, due to its simplicity and agility. The spectrophotometric method proposed in this study was developed and validated according to criteria established by national and international regulatory agencies and was able to quantify salicylic acid present in nanostructured lipid carriers containing copaiba oil, a bioactive vegetable oil. This analytical method was selective for the quantification of salicylic acid in the presence of the complex lipid matrix of the nanocarrier and copaiba oil, with linearity demonstrated in the range of 5 - 30 μg/mL, and sensitivity for its detection and quantification in low concentrations. Precision, accuracy and robustness requirements were also reached under the conditions tested.

The proposed method is simple to perform, fast, low cost and capable of obtaining reliable results in routine quality control analyses. In addition to the analysis of total content and encapsulation efficiency of SA in the lipid nanoparticles, this analytical method can still be applied to other tests, such as the determination of the controlled release of SA and stability analysis. Furthermore, this technique of identification and quantification of SA has the potential for application in other matrices of pharmaceutical formulations, such as cosmeceutical oils, nanoemulsions and liposomes.

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References

Abrão, L. C. de C., Silveira, A. T., de Faria, H. D., Machado, S. C., Mendes, T. V., Plácido, R. V., Marciano, L. P. de A., & Martins, I. (2020). Toxicological analyses: analytical method validation for prevention or diagnosis. Toxicology Mechanisms and Methods, 31(1), 18–32. https://doi.org/10.1080/15376516.2020.1839612. 10.1080/15376516.2020.1839612

Albuquerque, V. J. A., Reinaldo, A. T. G., Silva, D. A., Silva, S. G. F., Azevedo Filho, C. A., Lima, E. N., & Sousa, C. E. M. (2020). Development and validation of a method for quantification of sildenafil citrate in UV-HPLC. Brazilian Journal of Health Review, 3(1), 286–299. https://doi.org/10.34119/bjhrv3n1-021. 10.34119/bjhrv3n1-021

Almeida, O. P., Marcial, S. P. S., Gouveia, F. P. P., & Carneiro, G. (2017). Validation of a chromatographic analytical method for quantification of benznidazole incorporated in nanostructured lipid formulations. Journal of the Brazilian Chemical Society, 28(2), 236–241. https://doi.org/10.5935/0103-5053.20160168. 10.34119/bjhrv3n1-021

Agência Nacional de Vigilância Sanitária. (2003). Resolução RE no 899, de 29 de maio de 2003. https://bvsms.saude.gov.br/bvs/saudelegis/anvisa/2003/res0899_29_05_2003.html.

Agência Nacional de Vigilância Sanitária. (2017). Resolução da Diretoria Colegiada (RDC) no 166, de 24 de julho de 2017. http://portal.anvisa.gov.br/documents/10181/2721567/RDC_166_2017_COMP.pdf/d5fb92b3-6c6b-4130-8670-4e3263763401

Arruda, C., Aldana Mejía, J. A., Ribeiro, V. P., Gambeta Borges, C. H., Gomes Martins, C. H., Sola Veneziani, R. C., Ambrósio, S. R., & Bastos, J. K. (2019). Occurrence, chemical composition, biological activities and analytical methods on Copaifera genus—A review. Biomedicine and Pharmacotherapy, 109(July 2018), 1–20. https://doi.org/10.1016/j.biopha.2018.10.030. 10.1016/j.biopha.2018.10.030

Castro e Souza, M. A., Reis, N. F. A., de Souza Batista, L., da Costa César, I., Fernandes, C., & Pianetti, G. A. (2018). An Easy and Rapid Spectrophotometric Method for Determination of Chloroquine Diphosphate in Tablets. Current Pharmaceutical Analysis, 16(1), 5–11. https://doi.org/10.2174/1573412914666180730123426. 10.2174/1573412914666180730123426

César, I. D. C., & Pianetti, G. A. (2009). Robustness evaluation of the chromatographic method for the quantitation of lumefantrine using Youden's test. Brazilian Journal of Pharmaceutical Sciences, 45(2), 235–240. https://doi.org/10.1590/S1984-82502009000200007. 10.1590/S1984-82502009000200007

Charny, J. W., Choi, J. K., & James, W. D. (2017). Spironolactone for the treatment of acne in women, a retrospective study of 110 patients. International Journal of Women's Dermatology, 3(2), 111–115. https://doi.org/10.1016/j.ijwd.2016.12.002. 10.1016/j.ijwd.2016.12.002

Clayton, A. W., & Thiers, R. E. (1966). Direct spectrophotometric determination of salicylic acid, acetylsalicylic acid, salicylamide, caffeine, and phenacetin in tablets or powders. Journal of Pharmaceutical Sciences, 55(4), 404–407. https://doi.org/10.1002/jps.2600550411. 10.1002/jps.2600550411

Danaei, M., Dehghankhold, M., Ataei, S., Hasanzadeh Davarani, F., Javanmard, R., Dokhani, A., Khorasani, S., & Mozafari, M. R. (2018). Impact of Particle Size and Polydispersity Index on the Clinical Applications of Lipidic Nanocarrier Systems. Pharmaceutics, 10(2), 1–17. https://doi.org/10.3390/pharmaceutics10020057. 10.3390/pharmaceutics10020057

El-Ragehy, N. A., Yehia, A. M., Hassan, N. Y., Tantawy, M. A., & Abdelkawy, M. (2016). Chemometrics tools in detection and quantitation of the main impurities present in aspirin/dipyridamole extended-release capsules. Journal of AOAC International, 99(4), 948–956. https://doi.org/10.5740/jaoacint.16-0082. 10.5740/jaoacint.16-0082

Enéas, P. C. R., Costa, A. L. O., Souza, D. E. R., Alvesa, J. C., Magalhães, M., Fialho, S. L., César, I. C., & Pianetti, G. A. (2020). Simultaneous quantitation of efavirenz, lamivudine and tenofovir disoproxil fumarate in fixed dose combination tablet by high performance liquid chromatography. Brazilian Journal of Health and Pharmacy, 2(4), 38–50. https://doi.org/10.29327/226760.2.4-5. 10.29327/226760.2.4-5

Falcão, D. Q., Mourão, S. C., Araujo, J. L. de, Pereira, P. A. K., Cardoso, A. C. A., Almeida, K. B. de, Zibetti, F. M., & Lima, B. G. (2015). Challenges In Development Of Essential Oil Nanodelivery Systems And Future Prospects. In J. Naik (Org.), Nano Based Drug Delivery (p. 557–578). IAPC Publishing. https://doi.org/10.1016/B978-0-12-811037-9.00023-0. 10.1016/B978-0-12-811037-9.00023-0

Food and Drug Administration. (1994). Reviewer Guidance - Validation of chromatographic methods. CDER. Center for Drug Evaluation and Research, 22(3), 1–30. http://www.scopus.com/inward/record.url?eid=2-s2.0-0031924265&partnerID=tZOtx3y1

Food and Drug Administration. (2015). Analytical Procedures and Methods Validation for Drugs and Biologics Guidance for Industry Analytical Procedures and Methods Validation for Drugs and Biologics Guidance for Industry (Número July).

Ferreira-Nunes, R., Ferreira, L. A., Gratieri, T., Cunha-Filho, M., & Gelfuso, G. M. (2019). Stability-indicating analytical method of quantifying spironolactone and canrenone in dermatological formulations and iontophoretic skin permeation experiments. Biomedical Chromatography, 33(11), 1–10. https://doi.org/10.1002/bmc.4656. 10.1002/bmc.4656

Ferreira, C. P., Techera Antunes, F. T., Rebelo, I. N., da Silva, C. A., Vilanova, F. N., Corrêa, D. S., & de Souza, A. H. (2020). Application of the UV–vis spectrophotometry method for the determination of glutamate in the cerebrospinal fluid of rats. Journal of Pharmaceutical and Biomedical Analysis, 186. https://doi.org/10.1016/j.jpba.2020.113290. 10.1016/j.jpba.2020.113290

Garcês, A., Amaral, M. H., Sousa Lobo, J. M., & Silva, A. C. (2018). Formulations based on solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) for cutaneous use: A review. European Journal of Pharmaceutical Sciences, 112(September 2017), 159–167. https://doi.org/10.1016/j.ejps.2017.11.023. 10.1016/j.ejps.2017.11.023

Ghate, V. M., Lewis, S. A., Prabhu, P., Dubey, A., & Patel, N. (2016). Nanostructured lipid carriers for the topical delivery of tretinoin. European Journal of Pharmaceutics and Biopharmaceutics, 108, 253–261. https://doi.org/10.1016/j.ejpb.2016.07.026. 10.1016/j.ejpb.2016.07.026

International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. (2005). GESTÃO DO RISCO À QUALIDADE. Fase 4, 1–19. https://www.gov.br/anvisa/pt-br/acessoainformacao/institucional/relacoes-internacionais/convergencia-regulatoria/arquivos/1558json-file-1

Kantikosum, K., Chongpison, Y., Chottawornsak, N., & Asawanonda, P. (2019). The efficacy of glycolic acid, salicylic acid, gluconolactone, and licochalcone a combined with 0.1% adapalene vs adapalene monotherapy in mild-to-moderate acne vulgaris: A double-blinded within-person comparative study. Clinical, Cosmetic and Investigational Dermatology, 12, 151–161. https://doi.org/10.2147/CCID.S193730.10.2147/CCID.S193730

Kitamura, K., & Majima, R. (1982). Determination of Salicylic Acid in Aspirin Powder by Second Derivative Ultraviolet Spectrometry. Analytical Chemistry, 55(1), 54–56. https://doi.org/10.1021/ac00252a017. 10.1021/ac00252a017

Kokot, Z., & Burda, K. (1998). Simultaneous determination of salicylic acid and acetylsalicylic acid in aspirin delayed-release tablet formulations by second-derivative UV spectrophotometry. Journal of Pharmaceutical and Biomedical Analysis, 18(4–5), 871–875. https://doi.org/10.1016/S0731-7085(98)00225-8. 10.1016/S0731-7085(98)00225-8

Krambeck, K., Santos, D., Otero-Espinar, F., Sousa Lobo, J. M., & Amaral, M. H. (2020). Lipid nanocarriers containing Passiflora edulis seeds oil intended for skin application. Colloids and Surfaces B: Biointerfaces, 193(May), 111057. https://doi.org/10.1016/j.colsurfb.2020.111057. 10.1016/j.colsurfb.2020.111057

Lammari, N., Louaer, O., Meniai, A. H., Fessi, H., & Elaissari, A. (2021). Plant oils: From chemical composition to encapsulated form use. International Journal of Pharmaceutics, 601, 1–34. https://doi.org/10.1016/j.ijpharm.2021.120538. 10.1016/j.ijpharm.2021.120538

Leandro, L. M., De Sousa Vargas, F., Barbosa, P. C. S., Neves, J. K. O., Da Silva, J. A., & Da Veiga-Junior, V. F. (2012). Chemistry and biological activities of terpenoids from copaiba (Copaifera spp.) oleoresins. Molecules, 17(4), 3866–3889. https://doi.org/10.3390/molecules17043866. 10.3390/molecules17043866

Lu, J., Cong, T., Wen, X., Li, X., Du, D., He, G., & Jiang, X. (2019). Salicylic acid treats acne vulgaris by suppressing AMPK/SREBP1 pathway in sebocytes. Experimental Dermatology, 28(7), 786–794. https://doi.org/10.1111/exd.13934. 10.1111/exd.13934

Mu, H., & Holm, R. (2018). Solid lipid nanocarriers in drug delivery: characterization and design. Expert Opinion on Drug Delivery, 15(8), 771–785. https://doi.org/10.1080/17425247.2018.1504018. 10.1080/17425247.2018.1504018

Nalini, C. N., & Kumar, V. (2020). A Review of Different Analytical Techniques for Fexofenadine Hydrochloride and Montelukast Sodium in Different Matrices. Critical Reviews in Analytical Chemistry, 51(3), 232–245. https://doi.org/10.1080/10408347.2019.1709410. 10.1080/10408347.2019.1709410

Patel, R., & Prabhu, P. (2020). Nanocarriers as versatile delivery systems for effective management of acne. International Journal of Pharmaceutics, 579, 119140. https://doi.org/10.1016/j.ijpharm.2020.119140. 10.1016/j.ijpharm.2020.119140

Ramos, T. R., Santoro, M. I. R. M., Kedor-Hackmann, E. R. M., & Singh, A. K. (2005). Validação de um método analítico para a determinação de substâncias ativas em formulaçõ es farmacêuticas empregadas em "peelings" químicos. Revista Brasileira de Ciencias Farmaceuticas/Brazilian Journal of Pharmaceutical Sciences, 41(2), 229–235. https://doi.org/10.1590/s1516-93322005000200011. 10.1590/s1516-93322005000200011

Saha, U., & Baksi, K. (1985). Spectrophotometric determination of salicylic acid in pharmaceutical formulations using copper(II) acetate as a colour developer. The Analyst, 110(6), 739–741. https://doi.org/10.1039/an9851000739. 10.1039/an9851000739

Souza, S. V. C., & Junqueira, R. G. (2005). A procedure to assess linearity by ordinary least squares method. Analytica Chimica Acta, 552(1–2), 25–35. https://doi.org/10.1016/j.aca.2005.07.043. 10.1016/j.aca.2005.07.043

Svetlichny, G., Kulkamp-Guerreiro, I. C., Cunha, S. L., Silva, F. E. K., Bueno, K., Pohlmann, A. R., Fuentefria, A. M., & Guterres, S. S. (2015). Solid lipid nanoparticles containing copaiba oil and allantoin: Development and role of nanoencapsulation on the antifungal activity. Pharmazie, 70(3), 155–164. https://doi.org/10.1691/ph.2015.4116. 10.1691/ph.2015.4116

Teixeira, F. B., Silva, R. D. B., Lameira, O. A., Webber, L. P., Souza, R., Couto, D. A., Martins, M. D., & Lima, R. R. (2017). Copaiba oil-resin (Copaifera reticulata Ducke) modulates the inflammation in a model of injury to rats ' tongues. 1–8. https://doi.org/10.1186/s12906-017-1820-2. 10.1186/s12906-017-1820-2

Vieira, R., Severino, P., Nalone, L. A., Souto, S. B., Silva, A. M., Lucarini, M., Durazzo, A., Santini, A., & Souto, E. B. (2020). Sucupira Oil-Loaded Nanostructured Lipid Carriers (NLC): Lipid Screening, Factorial Design, Release Profile, and Cytotoxicity. Molecules, 25(3), 1–22. https://doi.org/10.3390/molecules25030685. 10.3390/molecules25030685

Youden, W. J., & Steiner, E. H. (1975). Statistical manual of AOAC. In Association of Official Analytical Chemistry.

Zheng, Y., Yin, S., Xia, Y., Chen, J., Ye, C., Zeng, Q., & Lai, W. (2018). Efficacy and safety of 2% supramolecular salicylic acid compared with 5% benzoyl peroxide/0.1% adapalene in the acne treatment: a randomized, split-face, open-label, single-center study. Cutaneous and Ocular Toxicology, 38(1), 48–54. https://doi.org/10.1080/15569527.2018.1518329. 10.1080/15569527.2018.1518329