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Development and Validation of Analysis Methods for Retinoids, Arbutin, Nicotinamide in HPLC Cosmetics

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ABSTRACT

There are many skin lightening cosmetics on the market. Active ingredients commonly used in skin lightening cosmetics are arbutin, nicotinamide and retinoids. The purpose of this study is to determine the best conditions of HPLC that can separate several compounds and ensure the validity of the method. HPLC system that can separate several analytes, validating the method with parameters such as selectivity, linearity with a range of 25-150 ppm, LOD, LOQ, accuracy with simulation methods with 100% concentration is 100 ppm, precision with simulation methods at a concentration of 50 ppm. After the method is valid, the determination of levels is carried out on the sample. Results of this study are the best conditions that can be used to separate several analyte compounds using a KCKT system with a UV detector at a wavelength of 227 nm, a flow rate of 1ml/min and using isocratic elution with a mobile phase composition of WFI: acetonitrile: ethanol (10:65:25), the method used has been tested valid with a selectivity value of 1,590 and 4,028, with linearity values for arbutin, nicotinamide, retinoid is 0,999; 0,9980; 0,997 respectively, LOD value 7,8 ppm, LOQ 26,1 ppm, %recovery 96,863, %RSD 3,167

INTRODUCTION

Arbutin can be used as a skin lightener. The mechanism of arbutin is to work by inhibiting the formation of melanin pigment by inhibiting tyrosinase activity (Thongchai et al., 2007). The cosmetic industry uses arbutin to treat skin coloring problems such as melasma, freckles, hyperpigmentation, and others because it inhibits the formation of melanin in human skin (Rychlińska & Nowak, 2012). Arbutin works by inhibiting the enzyme tyrosinase (Mustika et al., 2020). The process by which nicotinamide works to inhibit keratinocyte factors and therefore reversibly inhibit the transfer of melanosomes from melanocytes to keratinocytes is described by Wohlrab and Kreft (2014), namely by blocking the expression of MHC-II and the formation of IL-12, TNF-a, IL-1, and nitric oxide, Nicotinemide also acts as an anti-inflammatory. According to Wohlrab and Kreft (2014), nitric oxide metabolism and prostaglandin production may be influenced by nicotinamide and its derivative N-methylnicotinamide which causes increased vascular permeability (Wohlrab & Kreft, 2014). Retinoids work by activating retinoic acid receptors (RAR), whose interaction with RAR in skin cells can stimulate the process of development of the outermost skin cells (epidermis). Formation and increase in the amount of NGAL protein (Neutrophil Gelatinase-Associated Lipocalin) where retinoic acid is able to increase the formation and increase in the amount of NGAL protein which will result in the death of sebaceous gland cells (cells that produce sebum or oil), therefore it will reduce sebum production so that it can reduce the appearance of acne (Food and Drug Supervisory Agency, 2008).

The combination of alpha arbutin and nicotinamide in cosmetic preparations is an effective choice that is widely used to brighten skin without significant side effects (Nurrosyidah et al., 2024). Retinoids are also included in skin lightening cosmetic ingredients that are currently widely used.

The use of skin lightening cosmetics must be used in accordance with the maximum levels of active ingredients used to maintain safety and minimize side effects that arise. It is very important to analyze the levels in cosmetics to find out whether the levels are in accordance with BPOM and it is necessary to validate the method to ensure that the method used can produce good results and the method used can be applied well. Methods that can be used include spectrophotometry, HPLC. Spectrophotometry has a weakness compared to HPLC, namely that it requires separation first, whereas HPLC can separate several compounds according to its working principle without having to go through a separation process first (S. Moldoveanu & David, 2022) so the time used is relatively shorter. HPLC (High Performance Liquid Chromatography) is a chromatography technique for liquid substances which is usually accompanied by high pressure. The principle of HPLC is to separate molecules based on their affinity for certain solid substances. The liquid to be separated is the liquid phase and the solid is the stationary phase. This HPLC principle is very useful for separating several compounds at one time because each compound has a selective affinity between a certain stationary phase and a certain mobile phase (S. Moldoveanu & David, 2022).

In research (Nurrosyidah et al., 2024) analysis of arbutin and nicotinamide was carried out using the mobile phase methanol and aquabides (20:80 v/v) with a flow rate of 1 ml/minute and produced results that met the method validation requirements. In research (Fajri, 2020) research has been carried out to validate the analytical method for identifying hydroquinone and retinoic acid simultaneously using ultra high performance liquid chromatography (UHPLC) in semi-solid preparations. The method used is a gradient system with a flow rate of 0.35 ml/minute and a mobile phase of 0.1% methanol-formic acid. The results met the method validation requirements. Based on the existing background, research was developed using a simpler isocratic elution system because not all HPLC systems can be carried out using a gradient system (Hidayatullah et al., 2022).

In this research, the mobile phase was also determined without using acid because the use of acid can affect the life of the column used. The analytical chromatographic conditions selected in

this study were applied to the separation of analyte samples from a combination of the active ingredients retinoid, arbutin, nicotinamide whose levels had not been tested and the method was validated with parameters of linearity, selectivity, LOD, LOQ, precision and accuracy to ensure that the method developed has been valid and can be used if there is a cream with a combination of retinoid, arbutin, nicotinamide so that it can determine analyte levels and can use a simpler isocratic elution method.

METHODS

Determination of Chromatographic Conditions

Arbutin, nicotinamide and retinoid were made into 1000 ppm by weighing 10 mg of arbutin and putting it in a 100 ml volumetric flask then dissolving it using WFI until dissolved and then mixing it with ethanol:methanol (50:50) to the limit mark (Solution A).

10 mg of nicotinamide was weighed and put into a 10 ml volumetric flask then dissolved using ethanol until dissolved then mixed with ethanol:methanol (50:50) to the limit mark (Solution B). Weighed 10 mg of retinoid and put it in a 10 ml measuring flask, then dissolved it using methanol until dissolved, then mixed it with ethanol: methanol (50:50) until the limit mark (Solution C).

Determining chromatographic conditions is done by looking for the correct mobile phase, flow rate and wavelength. Determination of the mobile phase was carried out using variations, namely WFI: acetonitrile: methanol and WFI: acetonitrile: ethanol. Determination of flow rate is carried out using variations of 0.5; 0.8; 1 ml/minute. Determination of chromatographic conditions was carried out on a mixed solution of 25 ppm concentration by taking 250 µl of each standard solution (solutions A, B and C) dyad with ethanol:methanol (50:50) in a 10ml volumetric flask. Wavelength selection was carried out by measuring the absorbance of each analyte using UV-VIS spectrophotometry with a concentration of each analyte of 100 ppm.

Method Validation

Method validation carried out includes selectivity, linearity, detection and quantification limits, accuracy and precision. The selectivity test was carried out on a 25 ppm solution and measured using the HPLC system for selected conditions and then the chromatogram resolution value was calculated. The linearity test uses a working standard solution of 25 ppm; 50ppm; 75ppm; 100ppm; 125ppm; 150 ppm was measured using the HPLC system under selected conditions and then the concentration (x) and area (y) values were plotted to obtain the regression equation and correlation coefficient. Testing of detection limits and quantification limits was carried out using calibration plots in linear regression analysis on linearity. Determination of accuracy is carried out by the method spiked placebo recovery namely by weighing 25 mg of placebo cream and adding a standard solution of 80%, 100%, 120% of the concentration used and carrying out three replications. A mixture of cream and standard solution was then prepared and at each concentration replicated three times and the area measured using HPLC so that it could be determined % recovery his. The precision test was carried out by weighing 25 mg of placebo cream and then adding 50 ppm of standard solution. This was done six times in replication. After that, the area is measured using HPLC and the RSD value is calculated.

Method Application

Sample testing was carried out by weighing 100 mg of sample cream, then dissolving it using ethanol:methanol (50:50) in a 10 ml flask and sonicating until homogeneous.

RESULTS AND DISCUSSION

1. Determination of Chromatographic Conditions

Determining chromatographic conditions is carried out to find chromatographic conditions that can separate the analytes in the matrix. The analytes in this study were arbutin, nicotinamide, retinoid.

Determining chromatographic conditions includes determining the mobile phase, flow rate and wavelength. The mobile phase is the main parameter in HPLC analysis conditions. Determination of the mobile phase in HPLC is based on the polarity of the sample to be separated. The separation system in this research refers to research (Thongchai et al., 2007) and (Wang et al., 2015) is to use a reverse phase where the mobile phase is polar and the stationary phase is non-polar.

The stationary phase used in this research is C18 which is non-polar. The mobile phase (WFI:acetonitrile:ethanol) with a ratio of (10:65:25)

and a flow rate of 1ml/minute is very good for use in this study because it has a short retention time (1.05 for arbutin, 1.45 for nicotinamide and 2 .6 for retinoids) and has good resolution values (1.590 and 4.028) which means it can separate each analyte well (AOAC International, 2019) as in Figure 1. The WFI composition is the smallest among the other mobile phases due to elution in HPLC phase reverses faster when the mobile phase contains more organic solvent and less water (S. C. Moldoveanu & David, 2016). In previous research (Fajri, 2020) who conducted research on the analysis of hydroquinone and retinoic acid using UHPLC with gradient elution resulting in a retention time of 1.93 minutes for hydroquinone and 11.99 for retinoids. The development of the elution method in this research is The isocratic elution method is a method with a constant mobile phase so it is more concise because there is no need to set the time for the flow of the mobile phase like the gradient elution method. Peak The first to appear was arbutin with a retention time of 1.05 minutes, then nicotinamide with a retention time of 1.45 minutes and the last was retinoid with a retention time of 2.6 minutes. The retention time in this study was shorter, that is, everything eluted in less than 5 minutes with isocratic elution and reverse phase HPLC systems. HPLC with a reverse phase system will elute the most polar substances to come out first. The log P value is the distribution ratio of compounds in polar or non-polar solvents (Yusuf et al., 2013). The log P value of nicotinamide was -0.4; log P arbutin was 2.05; log P retinoid was 2.8. The smaller the log P value, the higher the hydrophilicity of the compound or the more polar it will be. The order of polarity of the analytes in this study was nicotinamide, arbutin and retinoid. Arbutin elutes earlier compared to nicotinamide because arbutin is dissolved in WFI, WFI has a higher polarity level compared to ethanol so that arbutin can elute from the column first.

Wavelength selection is carried out using UV-VIS spectrophotometry. The wavelength chosen in this HPLC system is 227 nm because this wavelength provides maximum absorption so that it can read arbutin, nicotinamide and retinoids. The wavelength used in this research has shifted to be shorter than the wavelength usually used for arbutin analysis, namely 280 nm with water as a solvent (Scientific Committee on Consumer Safety, 2011), nicotinamide analysis, namely 254 nm with solvents and retinoid analysis, namely 365 nm with methanol solvent (U.S Pharmacopedia The United States Pharmacopedia, 2018). This shift in wavelength to 227 nm is called hypsochromic, which is a shift in absorbance to a shorter wavelength area due to substitution or solvent effects because this research used a more polar mixed solvent, namely ethanol:methanol (50:50). This shift in absorption peaks is caused by the $\eta \rightarrow \pi^*$ transition which is caused by the presence of unsaturated groups which provide π orbitals in the analytes arbutin, nicotinamide and retinoid which meet a greater solvent polarity so that the energy required is smaller. The hypsochromic shift or shift in wavelength to a shorter wavelength is caused by increased solvation of electron pairs, resulting in a decrease in energy (Gandjar & Rohman, 2012). The molar absorptivity for the $\eta \rightarrow \pi^*$ excitation peaks is generally low, ranging from 10 to 100 Lcm^{-1} mol^{-1}.

2. Method Validation

Validation is an act of proving that the method that has been used is selective, sensitive, linear, accurate and thorough. Method validation is necessary so that the method used can be trusted and accounted for. Methods that have been declared valid and valid will be acceptable for certain analytical purposes. This method was validated using parameters that correspond to category I (determining the levels of main components in medicinal raw materials or compounds) as in Indonesian Pharmacopoeia, namely selectivity, linearity, range, accuracy, precision (Ministry of Health of the Republic of Indonesia, 2020), but in this study coupled with detection limit and quantification limit tests. Table 1 is the result of the method validation carried out in this research.

Table 1. Results of Method Validation Parameters

Parameter	Results
Selectivity (resolution value)	1,590 and 4,028
Linearity (R)	0,999; 0,998; 0,997
Detection limit (ppm)	7,8 ppm
Quantification limit (ppm)	26,1 ppm
Accuracy (%*Recovery*)	96,863%
Precision (%RSD)	3,167%

A. Selectivity

The selectivity test is a test to measure the ability of the method used to carefully and thoroughly separate the analytes in the matrix. The selectivity test aims to separate mixed solutions containing the analytes arbutin, nicotinamide and retinoid. Selectivity refers to the resolution value. The resolution value is a value that shows whether two chromatogram peaks that are located next to each other can be separated or not. The required resolution value is >1.5 (AOAC, 2019). The selectivity test in this study used a concentration of 25 ppm and the resolution values obtained were 1.590 and 4.028. Figure 1 is an image of the chromatogram produced in the selectivity test.

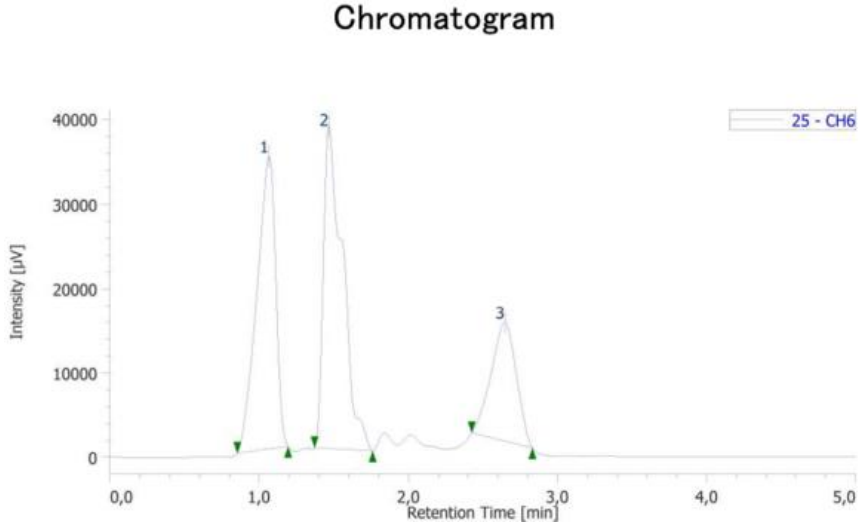

Figure 1. Chromatogram of Mixed Standard Solution with Peaks of Arbutin (1), Nicotinamide (2), Retinoid (3) 25 PPM for Selectivity Test

Linearity

Linearity aims to determine the relationship between concentration and area. The linearity test was carried out by creating a standard curve for arbutin, nicotinamide and retinoid which were tested using selected HPLC conditions. Linearity was carried out at concentrations of 25 ppm, 50 ppm, 75 ppm, 100 ppm, 125 ppm, 150 ppm which were injected into the HPLC system. The plot of concentration (x) and area (y) produces a linear regression for arbutin, nicotinamide, retinoid with the respective values being y=14018x-59423 (R^2 = 0.999), y=1308x-27177 (R^2 = 0.998), y =9491x-7552 (R^2 = 0.997). Figure 2, Figure 3, Figure 4 are images of the standard curves for arbutin, nicotinamide and retinoids.

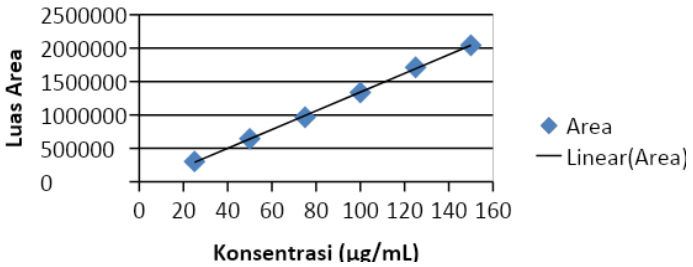

Figure 2. Arbutin Standard Curve

Figure 2, namely the standard curve for arbutin, produces a correlation value (R) of 0.999, which means >0.997, thus indicating a linear relationship between concentration and response (area).

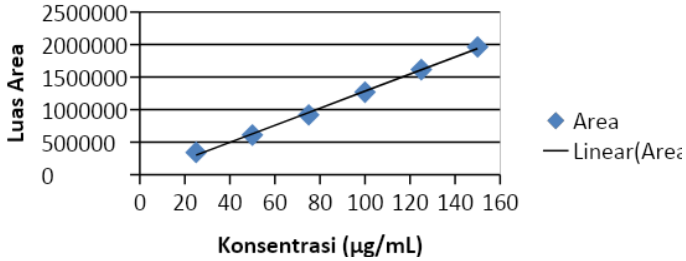

Figure 3. Nicotinamide Standard Curve

Figure 3, namely the nicotinamide standard curve, produces a correlation value (R) of 0.998, which means >0.997, thus indicating a linear relationship between concentration and response (area).

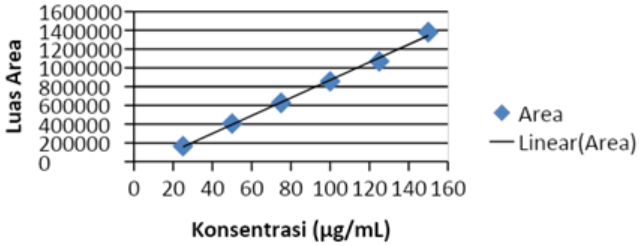

Figure 4. Retinoid Standard Curve

Figure 4, namely the standard curve for arbutin, produces a correlation value (R) of 0.997, which shows that there is a linear relationship between concentration and response (area).

A. Detection Limits and Quantification Limits

Detection limits and quantification limits are carried out to determine the concentration limits of analytes that can be detected and to determine the concentration limits of analytes that can be quantified by the tool. The detection limit is calculated by 3.SD/S and the quantification limit is calculated by 10.SD/S where SD is the standard deviation in the linear regression plot and S is the slope value in the linear regression equation. The detection limit and quantification limit were 3.411 ppm and 11.71 ppm for arbutin; 6,654 ppm and 22.18 ppm for nicotinamide and 7,834 and 26.11 for retinoids.

B. Accuracy

Accuracy is a method used to determine the closeness of the concentration value of test results in experiments that have been carried out to the actual concentration or actual concentration. Accuracy in this study used a simulation method with the addition of 80%, 100%, 120% standard solutions to the placebo cream. Accuracy was carried out using three

replications at each concentration. Table 2 is the result of accuracy with actual concentrations of 80, 100, 120 ppm. Based on the results of testing the accuracy of arbutin, nicotinamide, retinoid was found to be average %recovery namely 96.863 as in table 3, which means it meets the accuracy requirements because the accuracy requirements are %recovery for concentrations ranging from 0.01% it is in the range of 85% to 110% (AOAC, 2019). Table 2 is a table of requirements regarding %Recovery and %RSD. %Recovery calculated by formula (1)

$$\%\ Recovery = \frac{real\ rate}{theoretical\ rate} \times 100\%$$

Table 2. Acceptable Range Requirements in % Recovery and % RSD Accepted

Concentration	Recoupment (%)	RSD (%)
100%	98-101	1
10%	95-102	1,5
1%	92-105	2
0,1%	90-108	3
0,01%	85-110	4
10 µg/g (ppm)	80-115	6
1 µg/g (ppm)	75-120	8
10 µg/kg (ppb)	70-125	15

Table 3. Results % Recovery Accuracy with Simulation Method

Theoretical concentration	Actual concentration			%Recovery		
	Arbutin	Nkt	Retinoid	Arbutin	Nkt	Retinoid
80	85,434	70,848	68,713	106,792	88,560	85,89
80	78,321	84,498	72,682	97,901	105,622	90,852
80	88,137	74,56	70,337	110,171	93,199	87,921
100	101,696	86,567	99,176	101,696	86,567	99,176
100	99,497	98,893	98,672	99,497	98,893	98,672
100	111,938	89,283	103,664	111,938	89,283	103,664
120	112,373	107,139	109,398	93,645	89,283	91,165
120	120,970	117,185	110	100,809	97,654	91,667
120	121,605	116,816	115,331	101,337	97,347	96,109
Σ				102,643	94,045	93,902

C. Precision

Precision is used to measure the degree of agreement between the results of each test. In this research, the method used is the simulation method. The precision test was carried out using six replications at a time according to ICH 2 guidelines (ICH, 2022). The requirements for acceptance of the precision test are in the %RSD in table 2. The precision test was carried out at a concentration of 50 ppm added with 25 mg of placebo cream using the selected HPLC system. Based on the precision test results, the average %RSD result was 3.167 as in table 4, which means that the results met the requirements because the requirements for precision levels at concentrations ranging from 0.01% were less than 4%. (AOAC, 2019). %RSD for precision test is calculated by formula (2)

$$RSD = \frac{SD}{Rate-rate} \times 100\%$$

Table 4. Precise % RSD Results with a Concentration of 50 PPM

Replication	Actual concentration		
	Arbutin	Nkt	Retinoid
1	51,353	57,719	49,879
2	50,53	55,031	53,153
3	52,404	55,045	54,117
4	51,792	53,561	49,258
5	49,249	54,016	52,022
6	48,923	53,708	49,523
%RSD	2,76	2,82	4,01
Σ %RSD		3,167	

D. Application of the Method

The aim of developing this HPLC method is so that it can be applied to determine arbutin, nicotinamide and retinoids in cosmetic cream samples. The cosmetic cream samples used were creams containing arbutin, nicotinamide and retinoid which were prepared and tested using the selected HPLC system. In sample A there is nicotinamide and retinoid while in sample B there is arbutin, nicotinamide and retinoid with percentages as in table 5. The arbutin contained in sample B is around 0.13% which means <2% so it is still within the safe limit for using cream sample B (Scientific Committee on Consumer Safety, 2011). Arbutin works by inhibiting tyrosinase activity, resulting in inhibition of melanin formation. Nicotinamide levels in samples A and B ranged from 13% in accordance with the standard use of nicotinamide which can be effective in the skin ranging from 2-20% by blocking the transfer of melanosomes from melanocytes to keratinocytes (J et al., 2002) while the retinoid level in sample A is around 0.2% and sample B is around 0.6%, which means >0.05%, which means it has the potential to be teratogenic so this sample cannot be used in pregnant women (Drug Control Agency and Food, 2008).

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CONCLUSION

The HPLC system in this research can be used to analyze combination substances, namely arbutin, nicotinamide and retinoid in cosmetic cream samples. The method in this research meets the method validation requirements. This HPLC system method is simple, fast, precise, accurate and thorough so it is appropriate when applied to test cosmetic samples with combination active substances.

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