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Foreword

We would like to express our gratitude to the Almighty God for His grace and guidance that have enabled the publication of this journal, "Eruditio: Indonesia Journal of Food and Drug Safety." This journal is part of the Indonesian Food and Drug Authority (BPOM) responsibility as the institution that oversees the development of the Pharmaceutical and Food Supervisory (PFM) functional position, providing a platform for BPOM employees to develop their profession in the scope of drug and food surveillance.

Research results and findings in the field of drug and food surveillance in Indonesia are crucial in making decisions and policies to address challenges and issues in the drug and food surveillance sector. Therefore, Eruditio: Indonesia Journal of Food and Drug Safety, Volume 4, No. 2, June 2024 Edition, presents seven articles to address these challenges.

These seven articles include: (1) Strengthening the Surveillance of 1,4-Dioxane Contaminants in Cosmetics through Harmonization of Analysis Methods and Networking of Cosmetics Laboratories in Indonesia, by Susan Gracia Arpan, Sri Purwaningsih, Hasti Kusuma, Erita Lusianti, and Yustina; (2) Performance Characteristics of the Quantitative Method for Staphylococcus aureus in Food Products corresponds to ISO 16140-3:2021 by Fannisa Putri, Sri Surati, Aditya Anugerah Marusaha Sitorus, Kemala S. Nagur, Eni Cahyaningsih, Yulin Wilasti, and Maria Arieni Eka Devina Sihotang; (3) Optimization and Validation of Analytical Method for Detection of Shigella sp. in Oral Preparations of Quasi-Drugs in the Form of Lozenges by Desty Herawati, Eko Aprianto, Eni Cahyaningsih, and Nur Miftahurrohmah; (4) Development and Validation of a Method for Detecting and Quantifying Mitragynine in Kratom Samples Using HPLC-PDA by Neni Isnaeni, Asep Saefumillah, and Antonius Herry Cahyana; (5) Pengujian Analysis of the Effectiveness of Food Safety of School Snacks Program in Changing Knowledge, Attitude, and Behavior of School Children in Southeast Sulawesi by Dewi Amni Idrus, and Dian Reni Agustina; (6) Public Engagement on the Instagram Social Media Account Format of the Indonesian Food and Drug Supervisory Authority Regional Office in Ambon in 2023 by Mohammad Viva Agusta and Nugroho Budi Santoso; (7) Analysis of ZnPtO in Anti-dandruff Shampoo by High-Performance Liquid Chromatography - Photo Diode Array by Zahara, Farida Kurniawati, and Erita Lusianti.

We would like to thank all the authors, reviewers, and all parties who have contributed to the publication of Eruditio: Indonesia Journal of Food and Drug Safety, Volume 4, No. 2, June 2024 Edition. We welcome all readers to read this journal, and constructive suggestions and criticisms are highly appreciated for the improvement of this journal in the next editions. Hopefully, the articles presented in this edition of Eruditio: Indonesia Journal of Food and Drug Safety can provide new knowledge and perspectives to contribute to drug and food surveillance.

Editorial Team

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Strengthening the Surveillance of 1,4-Dioxane Contaminants in Cosmetics through Harmonization of Analysis Methods and Networking of Cosmetics Laboratories in Indonesia

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ABSTRACT / ABSTRAK

Laboratories play a strategic role in protecting public health by ensuring the quality and safety of drugs, food, and cosmetics. The Centre of National Quality Laboratory of Drugs and Food (CNQLDF), as the central laboratory of the Indonesian Food and Drug Authority (Indonesian FDA), continuously develops reliable, selective, sensitive, and accurate analytical methods (AM) for cosmetic testing in line with advancements in cosmetic formulation technology. However, both the Indonesian FDA and external cosmetic laboratories need more human resources and infrastructure, hindering the development of analytical method and the testing of cosmetic products. Strengthening these laboratories through stakeholder networks is essential. Indonesia actively participates in the ASEAN Cosmetic Testing Laboratories Committee (ACTLC) and the Indonesian Cosmetic Laboratory Network (ICLN). One of the main tasks of CNQLDF is to develop a new ASEAN Cosmetic Method (ACM) for 1,4-Dioxane contaminants in cosmetics. Currently, CNQLDF, along with the Indonesia National Standard Body (INSB), Ministries and External Laboratories, in which together became members of ICLN, harmonized the analytical method of 1,4-Dioxane at the national level, leading to the issuance of the Indonesian National Standard (INS). Both the INS and ACM have main purpose to standardize analytical methods across laboratories, ensure the safety and quality of cosmetic products and enhance national product competitiveness. This study examines method to strengthen pre-market cosmetic surveillance through harmonizing the analytical method of 1,4-Dioxane at the national and ASEAN regional levels, using a qualitative approach based on the Indonesian FDA's internal data and stakeholder information on 1,4-Dioxane testing capabilities.

Laboratorium mempunyai peranan yang sangat strategis dalam melindungi kesehatan masyarakat dengan menjamin mutu dan keamanan obat dan makanan, termasuk kosmetikyang beredar di masyarakat. Pusat Pengembangan Pengujian Obat dan Makanan Nasional (PPPOMN) sebagai laboratorium pusat Badan Pengawas Obat dan Makanan (BPOM) terus mengembangkan metode analisis (MA) pengujian kosmetika yang handal, selektif, sensitif dan akurat seiring dengan perkembangan teknologi formulasi kosmetik. Namun, hingga saat ini laboratorium kosmetik, baik di Badan POM maupun laboratorium eksternal masih dihadapkan pada keterbatasan sumber daya manusia maupun sarana dan prasarana dalam melakukan pengembangan MA dan pengujian sampel produk kosmetik yang beredar. Untuk itu diperlukan perkuatan laboratorium pengujian kosmetikantara lain melalui jejaring dengan melibatkan stakeholder terkait. Saat ini jejaring laboratorium pengujian kosmetik yang sudah diikuti oleh Indonesia secara aktif adalah ASEAN Cosmetic Testing Laboratories Committee (ACTLC) dan Jejaring Laboratorium Kosmetik Indonesia (JLKI). Salah satu peran

Indonesia di ACTLC dalam hal ini didelegasikan kepada PPPOMN adalah menjadi negara yang bertanggung jawab dalam pengembangan MA cemaran 1,4-Dioksan dalam kosmetik menjadi ASEAN Cosmetic Method (ACM) yang baru. Saat ini PPPOMN dan BSN serta Kementerian dan Laboratorium Eksternal yang tergabung dalam JLKI sedang berproses melakukan harmonisasi MA 1,4-Dioksan pada tingkat nasional berupa penerbitan Standar Nasional Indonesia (SNI). Dengan adanya SNI dan ACM ini seluruh laboratorium memiliki metode analisis standar untuk menjamin keamanan dan mutu produk kosmetik yang beredar serta meningkatkan daya saing produk bangsa. Penelitian ini dilakukan untuk mempelajari metode penguatan pengawasan pre market kosmetik oleh industri dan laboratorium eksternal melalui harmonisasi MA 1,4 Dioksan di tingkat nasional maupun regional ASEAN.. Penelitian ini disusun dengan menggunakan pendekatan metode kualitatif berdasarkan data internal BPOM maupun data stakeholder yang terkait regulasi dan kemampuan pengujian 1,4-Dioksan tingkat nasional maupun regional.

Keywords: 1,4-Dioxane, Cosmetic, Pre-market Surveillance, Laboratory Network, Indonesian FDA
Kata Kunci: *1,4-Dioksan, Kosmetik, Pengawasan Pre-market, Jejaring Laboratorium, BPOM*

1. Introduction

As the backbone of drug and food control, laboratories have a strategic role in ensuring the quality and safety of drug and food products circulating in the community, including cosmetic products. Cosmetics are one of the commodities inherent in our daily lives, from the moment we wake up until we go back to sleep. This suggests that cosmetics have become a fundamental necessity for individuals of all ages, encompassing both women and men. With the advancement of cosmetics industry technology, ease of transportation, and access to information today, more diverse types of cosmetics are circulating in the community, with innovations in various formulations. To protect the public from cosmetics with health risks, the Indonesian FDA conducts comprehensive supervision starting from pre-market evaluation in the form of product notification before marketed in the national market to post-market control or when the product is circulated in the community. The extensive process of cosmetic product supervision generally starts with preparing standards, registration or notification, inspection or examination of facilities and products, laboratory testing, and law enforcement (Indonesian FDA Regulation No. 21/2020).

The Indonesian FDA Regulation No. 21/2022 on the Procedure for Submitting Cosmetics Notification states that every cosmetic product distributed in the territory of Indonesia must have a distribution permit in the form of a notification from the Head of the Indonesian FDA. Until October 2023, around 471,121 cosmetic products were notified at the Indonesian FDA, positioning it at the first rank in Indonesia's registered food and drug products. This is a challenge for cosmetics testing laboratories to build capacity and test capabilities to ensure the quality and safety of cosmetic products in circulation BPOM, 2022).

One of the efforts to answer these challenges, the Centre of National Quality Laboratory of Drugs and Food (CNQLDF) continues to develop analytical methods for cosmetics testing, both simple and sophisticated techniques, resulting in the test being carried out quickly, accurately, effectively and efficiently. However, to date, cosmetic laboratories, both at the Indonesian FDA and external laboratories, have the same obstacles

regarding limited resources in developing analytical methods and testing cosmetic product samples, as well as both human resources and testing infrastructure. Therefore, it is necessary to strengthen the cosmetic testing laboratory, among others, through networking by involving relevant stakeholders to synergize in improving public health protection from health-threatening cosmetic products. This aligns with the penta helix model of drug and food supervision as the key to the more effective drug and food supervision by involving five elements including business actors, the community like non-governmental organizations, government, academics, and the media (BPOM Regulation No. 9 of 2020).

Indonesia has actively participated in the regional cosmetics testing laboratory network, the ASEAN Cosmetic Testing Laboratories Committee (ACTLC). ACTLC is one of the committees of the ASEAN Cosmetic Committee (ACC) working group. ACTLC's main task including to conduct studies or develop analytical method related to ingredients that are or will be created for use and prohibited ingredients in cosmetics by the ASEAN Cosmetic Directives (ACD).

In the ACTLC network, Indonesia represented by CNQLDF, was responsible for developing the analytical method of Determination of 1,4-Dioxane in cosmetics, which is currently already published as a new ASEAN Cosmetic Method (ACM). The 2019 Indonesian FDA regulation on contaminants in cosmetics categorizes 1,4-Dioxane as a chemical contaminant, a hazardous substance from chemical elements or compounds that can harm and endanger human health. 1,4-Dioxane is a contaminant produced as a by-product in manufacturing certain cosmetic ingredients. It is produced when ethoxylated chemicals are used in cosmetic products. During the ethoxylation or alkoxylation process, unwanted side reactions may accidentally produce 1,4-Dioxane. 1,4-Dioxane is commonly found in products with PEG, polyethylene, polyethylene glycol, polyoxyethylene, -ethylene (e.g., laureth sulfate), -et- or -oxynol- listed on the product label ((Zhou, 2019 and U.S. Food and Drug Administration, 2022). These contaminants may be present in cosmetic products such as shampoo, body wash, baby lotion, hair lotion, and bubble baths. Furthermore, in a survey conducted by the U.S. FDA in 2018, 2 out of 82 commercial products had 1,4-dioxane concentrations above ten ppm. While in a study conducted by Castor et al. (2021) stated that 53% of detergents, 59% of shampoos, 62% of body cleaners, and 69% of dish soaps contained 1,4-Dioxane above one ppm.

The risks to health from 1,4-Dioxane contamination include irritation of the eyes, nose, and throat, and can trigger kidney and liver damages (SCCS, 2015). The International Agency for Research on Cancer (IARC) and the Environmental Protection Agency (EPA) designated 1,4-Dioxane as a compound that may cause carcinogens in humans (Group 2B) in 1999 based on the results of animal carcinogenicity tests conducted through oral administration to rats, mice, and guinea pigs. According to the Scientific Committee on Consumer Safety (SCCS) in 2015, cosmetics with 1,4-Dioxane contaminant levels less than 10 ppm (g/g) are considered safe.

This study was conducted to strengthen pre-market surveillance through a network of cosmetics laboratories, mainly industrial laboratories and external laboratories, through harmonization of AM 1,4-Dioxane used for cosmetic testing. Through this laboratory network, all cosmetic laboratories are expected to have the same analysis standard, thus it prevent disputes regarding test results. The more AMs being harmonized, the more it will help stakeholders and the public ensure the safety and quality of cosmetic products in circulation. This harmonization includes the stage of method transfer to all cosmetic testing

network laboratory members, one of which is by increasing the competence of external laboratory testing staff related to the method. The strengthening of external laboratories and industry can encourage the industry to be fully responsible for ensuring its products' safety, quality, and efficacy. Thus, post-market surveillance carried out by Indonesian FDA can focus more on prohibited and restricted ingredients critical to endangering public health.

2. Methodology

This research was prepared using a qualitative method approach. The data used in this study are internal data of the Indonesian FDA and related stakeholders data in the form of primary data and secondary data, including the following data:

a. Comparison of international regulations on dioxane control from ASEAN, European Union, and America
b. The capability of laboratories in Indonesia and ASEAN to conduct 1,4-Dioxane testing
c. Development of regulations in Indonesia regarding 1,4-Dioxane from year to year
d. CNQLDF cosmetic laboratory collaboration test result data conducted in 2022

3. Result and Discussion

3.1 Description and Safety Evaluation of 1,4-Dioxane

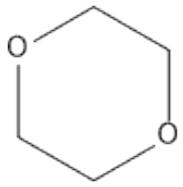

Figure 1. Structure of 1,4-Dioxane

1,4-Dioxane ($C_4H_8O_2$) is a clear liquid that dissolves easily in water. 1,4-Dioxane is commonly used as a solvent in some manufacturing processes and as a laboratory reagent. 1,4-Dioxane is a potential contaminant in some dietary supplements, water supplies contaminated with 1,4-Dioxane, cosmetics, detergents, and shampoos (Wilbur et al., 2012).

Regarding the toxicity of 1,4-Dioxane, it is classified in the European Union as a category two carcinogen (suspected of causing cancer) by IARC as a group 2B carcinogen (this agent is probably carcinogenic in humans) based on sufficient evidence of carcinogenicity in animals and insufficient evidence of carcinogenicity in humans and by the U.S. EPA in group B2 (Probable human carcinogen). Meanwhile, the U.S. NIOSH considers 1,4-Dioxane a potential carcinogen in the workplace. Based on the results of the toxicity evaluation of ingredients, (the Scientific Committee on Consumer Safety (SCCS) thinks that the level of 1,4-dioxane in cosmetic products representing LCR not more than 10^{-5} is considered safe for consumers. Thus, 1,4-Dioxane levels in cosmetic products of not more than 10 ppm are considered secure (SCCS, 2015).

Table 1. Toxicological Evaluation of 1,4-Dioxane Exposure

No	Type of Exposure	Toxicology Evaluation
1	Short-term exposure	Exposure to low levels and short periods of 1,4- dioxane may cause eye and nc irritation. Exposure to massive doses can cause kidney and liver damage and ev death.
2	Long-term exposure	Rodents: inhalation of vapors, ingestion of contaminated water, or skin contact w 1,4-dioxane, which mainly affects the nasal cavity, liver, and kidneys.
3	Possibility of Cancer	Long-term 1,4-Dioxane carcinogenicity studies in rats orally and by inhalati showed a positive correlation in the form of liver and kidney tumors or cancers. Liver tumors are believed to be related to cytotoxicity, which can be explained reactive metabolites such as HEAA and its related metabolite, hydroxyethoxyacetaldehyde.
4	Reproductive health/ baby	Miscarriages and stillbirths: There is a trend towards increased rates of spontanec abortion and stillbirth associated with occupational exposure to the combination 1,4-Dioxane with other substances. Breast milk transfer: A nursing mother exposed to high amounts of 1,4-Dioxane m pass it on to the infant through breast milk.

According to SCCS (2015), daily exposure values considered safe are presented in Table 2.

Table 2. Summary of Safety Assessment for 1,4-Dioxane

No.	Summary of Safety Assessment for 1,4-Dioxane	Daily Exposure Level Considered Safe	Type of Exposure
1	Canada – CMP assessment	85 µg/day	Aggregate exposure, 100% inhalation, 3.4% dermal absorption based on LOAEL/NOAEL
2	Europe	217 µg/day	Aggregate exposure, three scenarios, 100% inhalation, 50% dermal absorption based on NOAEL
3	Australia	420 µg/day	Aggregate exposure from up to 10 products based on NOAEL
4	Japan	4.3 µg/day	Estimation of general population exposure using Monte Carlo simulation based on MOE of NOAELs
5	California	30 µg/day	Based on LCR 10^{-5}
6	SCCS	55 µg/day	Based on LCR 10^{-5}

3.2 Mechanism of Formation of 1,4-Dioxane Contaminants in Cosmetics

1,4 Dioxane contamination in cosmetics comes from the manufacturing by-products of cosmetic raw materials made through ethoxylation processes, such as ethylene glycol, polyethylene glycol, and ethoxylated surfactants (such as Sodium Laureth Sulphate, polysorbates or fatty alcohol ethoxylates) ((Hayes et al., 2022). Ethoxylated surfactants are widely found in personal care and household products, such as detergents, body cleansers, shampoos, dishwashing detergents, and toothpaste. The mechanism of 1,4-Dioxane contamination can be seen in Figure 2.

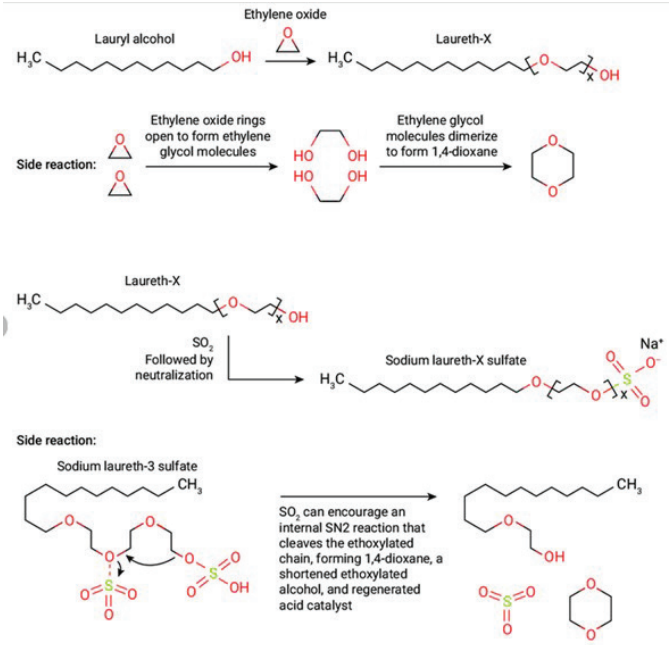

Figure 2. Ethoxylation Process Formation of By-Product 1,4-Dioxane (Ron Honnold et al., 2021)

3.3. International Regulation on 1,4-Dioxane

Several countries have established regulations related to 1,4-dioxane. Although 1,4-Dioxane has been banned from use as an ingredient in cosmetic products, 1,4-Dioxane is still allowed to be present as a contaminant at low levels, as summarized in Table 3 as follows:

Table 3. Regulation of 1,4-Dioxane in Various Countries

No.	Organization	Country	Exposure	Matrices	Results
1	Association of Southeast Asian Nations (ASEAN)	Brunei, Kamboja, Indonesia, Laos, Malaysia, Myanmar, Filipina, Singapore, Thailand and Vietnam	-	Cosmetic	NMT of 10 ppm
2	Food and Drug Administration (FDA)	United States of America	-	Cosmetic	Not yet set
3	Environmental Protection Agency (EPA)	United States of America	-	Drinking water	35 µg/L
4	The National Institute for Occupational Safety and Health (NIOSH)	United States of America	Workplace	-	One ppm; (3.6 mg/m3) upper boundary (30 minutes)
5	Occupational Safety and Health Administration (OSHA)	United States of America	Workplace	-	100 ppm, (360 mg/m3) 8-hour time-weighted average; Skin
5	Taiwan FDA	Chinese Taipei	-	Cosmetic	NMT of 100 ppm
6	European Medicine Agency (EMA)	Australia	-	Pharmacy	380 ppm

No.	Organization	Country	Exposure	Matrices	Results
7	Dutch Expert Committee on Occupational Safety	Netherlands	Workplace	-	20 mg/m3 (6 ppm)
8	Health Canada	Canada	-	-	Banned
9	Federal Department of Health	Australia			
10	Scientific Committee for Consumer Safety (SCCS)	European Union and England		Cosmetic	NMT of 10 ppm
11	Department of Environmental Conservation	The state of New York, United States of America		Cosmetic	NMT of 10 ppm
				Household cleaning and personal care products	Two ppm

*NMT = No more than

The U.S Environmental Protection Agency (EPA) has classified 1,4-dioxane as "possibly carcinogenic to humans" based on finding sufficient evidence of carcinogenicity in animals intentionally exposed to 1,4-Dioxane but insufficient evidence of carcinogenicity in humans. ((U.S. Food and Drug Administration (FDA), 2022). The U.S FDA has not set limits on the amount of dioxane allowed in cosmetics. Although it has not conducted an independent risk assessment, the FDA has periodically monitored dioxane levels in cosmetic products sold in the United States since the late 1970s. Following recommendations to implement changes in manufacturing processes to reduce these contaminants, the FDA has reported significantly reduced levels of dioxane in cosmetics over the years (Ramos, n.d.).

On the other hand, the state of New York, through New York Senate Bill No. S4389B, for the first time, set limits on dioxane in cosmetics, household cleaning products, and personal care due to concerns about the general public's exposure to dioxane through drinking water. This law limits dioxane levels to 10 ppm in cosmetics starting December 31st, 2022 (Ramos, n.d.). moreover, the European Commission, when preparing policies and proposals related to consumer safety, health, and the environment, relies on independent Scientific Committees to provide scientific advice. One is the European Commission Scientific Committee on Consumer Safety (SCCS). The SCCS thinks that levels of 1,4-Dioxane in cosmetic products of ≤10 ppm are considered safe for consumers (SCCS, 2015). Meanwhile, the Association of Southeast Asian Nations (ASEAN) has updated the Cosmetic Contaminant Limit Guidelines to Version 3.0. At the 30th ACSB Meeting held in Nay Pyi Taw, Myanmar, June 18th-19th, 2019, it was decided that from June 19th, 2020, cosmetic products in ASEAN countries should not contain more than 25 ppm of 1,4-Dioxane as a contaminant, and from June 19th, 2023, the limit is reduced to no more than ten ppm (ASEAN, 2019).

3.4 1,4-Dioxane Testing Capability of Indonesian FDA Laboratories in Indonesia

Since 2022, the Indonesian FDA has started implementing a laboratory regionalization pilot project to strengthen Indonesia's food and drug control system. The

Indonesian FDA Magazine Edition XII/2022 stated that laboratory regionalization is defined as a grouping of laboratories based on region and testing specialization to increase effectiveness and efficiency while prioritizing the validity and speed of testing to accelerate supervisory follow-up. There is a change in policy direction in the concept of laboratory regionalization in 2023, making it mandatory for all POM Centers/Branches to test all chemical testing parameters using gas chromatography-tandem mass spectrometry instruments. Throughout 2022, the supervision of the Agency in testing for 1,4-Dioxane contamination has been running quite well (Figure 3), where the Indonesian FDA provincial office in Palembang held the most significant number of testing samples for the 1,4-Dioxane parameter, while the smallest number of testing samples was held by the Indonesian FDA provincial office in Medan (Annual Report of Indonesian FDA Provincial Offices in Palembang and Medan in 2022).

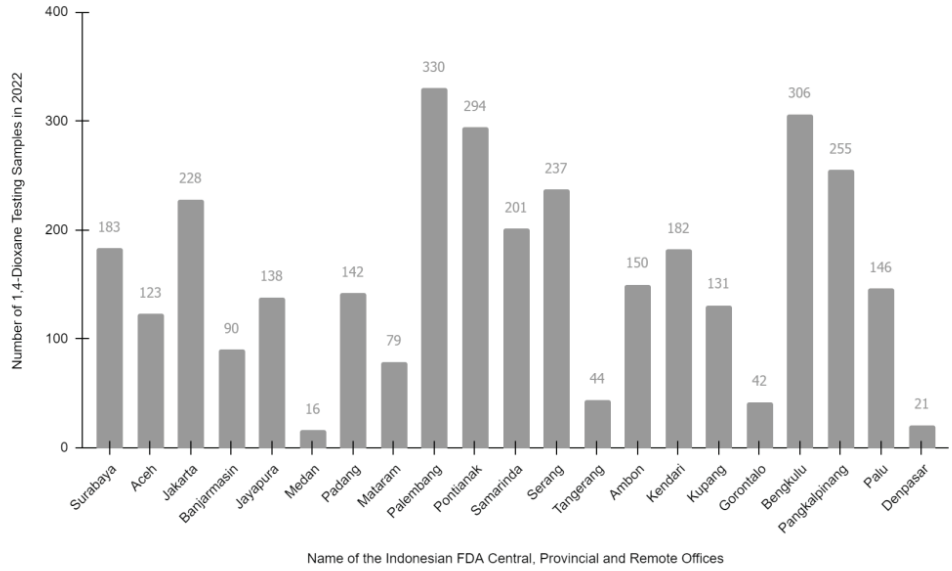

Figure 3. Testing Chart of 1,4-Dioxane Samples at The Indonesian FDA Central Office and Provincial Offices Across Indonesia

The planning and implementation of sampling and testing of traditional medicines, quasi medicines, health supplements, and cosmetics is stipulated by the deputy for supervision of traditional medicines, health supplements, and cosmetics, which is a reference for work units in carrying out sampling and testing of traditional medicines, quasi medicines, health supplements, and cosmetics. This guideline regulates product types and sample categories that must be tested for dioxane content, as shown in Table 4.

Table 4. Product Types and Categories of Cosmetics with 1,4-Dioxane Test Parameters

No.	Product Type	Category
1	Cream, emulsion, liquid, viscous liquid, gel, oil for skin (face, hands, feet, etc.)	Other baby preparations
2	Bath soap, antiseptic body wash, etc.	Baby bath soap, solid
		Hand soap, solid
		Bath soap, solid
3	Bath preparations (bath salts, foam, oils, gels, etc.)	Liquid body wash
		Hand wash soap (liquid)
		Bath foam
		Bath oil
		Bath salt
		Bath powder
		Other bathing preparations
		Baby bath soap, liquid
4	Hair Preparations	Shampoo
		Dry Shampoo
		Dandruff Shampoo
		Hair and body wash
		Baby hair and body wash
		Hair conditioner
		Hair Creambath
		Hair Mask
		Baby shampoo
		Other baby hair preparations
5	Oral and dental care preparations	Dentrifice
		Mouthwashes
		Mouth freshener

In this guideline, the cosmetics that should be tested for 1,4-Dioxane content contain ingredients made through the ethoxylation process. It should also be noted that cosmetics containing polyethylene glycol (PEG), polyethylene, polyoxyethylene, or oxynol may contain 1,4-Dioxane (Wilbur et al., 2012).

3.5 Development of 1,4-Dioxane Regulations in Indonesia

Regulations in Indonesia regarding 1,4-Dioxane yearly have developed based on the latest developments. Initially, 1,4-Dioxane was a prohibited ingredient in cosmetics according to the Regulation of the Head of the Food and Drug Administration of the Republic of Indonesia No. HK.00.05.42.1018 concerning Cosmetic Ingredients in 2008 Appendix I List of Prohibited Cosmetic Ingredients No. 476. This regulation is further amended into the Head of Food and Drug Administration Regulation No. 8/2015, 1,4-Dioxane is listed in Appendix V List of Prohibited Ingredients in Cosmetics No. 433. In the Food and Drug Administration Regulation No. 23 Year 2019 on Technical Requirements for Cosmetic Ingredients, 1,4-Dioxane is listed in Appendix V of the List of Unauthorized Ingredients in Cosmetics No. 433. In addition to the above regulations, 1,4-Dioxane is regulated in the Food and Drug Administration Regulation No. 12 Year 2019 on Contaminants in Cosmetics. 1,4-Dioxane is included as a contaminant, which is something that enters cosmetics unintentionally and unavoidably originating from processing, storage, and carried from raw materials. 1,4-Dioxane is a chemical contaminant from cosmetics containing ingredients made through ethoxylation processes, such as Sodium Laureth Sulphate or Polyethylene Glycol. In this regulation, the limit of 1,4-Dioxane is no more than 25 mg/kg or 25 mg/L (25 ppm). This regulation on cosmetic ingredients may undergo changes based on discussions at the ASEAN Cosmetic Scientific Body (ACSB) session, where it was agreed that the 1,4-Dioxane contamination limit would change to no more than 10 mg/kg or ten mg/L (10 ppm) and came into effect on June 19, 2023.

Table 5. Dioxane Regulatory Data and Analysis Methods CNQLDF

No.	Regulation	1,4-Dioxane Requirements	CNQLDF Analytical Methods	Results
1	Indonesian FDA Regulation No. Hk.00.05.42.1018/2018 about cosmetic ingredients	1,4-Dioxane as a Prohibited Cosmetic Ingredient Number 476	No. 12/KO/10	-
2	Indonesian FDA Regulation No.18/2015 about technical requirements for cosmetic ingredients	1,4-Dioxane as a Prohibited Ingredient in Cosmetics No. 433	No. 44/KO/MA-PPPOMN/18	LOD = 0,12 mg/kg
3	Indonesian FDA Regulation No. 23/2019 about technical requirements for cosmetic ingredients	1,4-Dioxane as an Unauthorized Ingredient in Cosmetics No. 433	-	-
4	Indonesian FDA Regulation No. 12/2019 about contaminants in cosmetics	1,4-Dioxane contamination limit not more than 25 mg/kg or 25 mg/L (25 bpj)	No. 22/KO/MA-PPPOMN/20	LOQ = 0,29 mg/kg
6	Discussion at the ASEAN Cosmetic Scientific Body meeting	Maximum 1,4-Dioxane requirement of 10 ppm as of June 19, 2023	No. 01 /KO/MA-PPPOMN/22	LOQ = 0,27 mg/kg

CNQLDF, in overseeing Regulations related to cosmetic ingredients, also made adjustments in the development of analytical method for testing 1,4-Dioxane. In 2010,

analytical method No. 12/KO/10 on the Identification of 1,4-Dioxane in cosmetic products by Gas Chromatography-Mass Spectroscopy was developed. Then it followed the updated one on No. 44/KO/MA-PPPOMN/18 with a shifted purpose, from identification to determination of levels in anticipation of changes in the regulation of 1,4-Dioxane as a contaminant that has a maximum limit, then refined by adding internal standard (IS) Tetrahydrofuran written in AM number 22/KO/MA-PPPOMN/20. Finally, this analytical method has been refined again by replacing the IS using the isotope of 1,4-Dioxane, 1,4-Dioxane-d8, with AM No. 01 /KO/MA-PPPOMN/22. This IS replacement regarded to feedback from several ASEAN members to create the ASEAN Cosmetic Method (ACM). The comparison between the applicable regulations and the developed AM as illustrated in Table 5.

3.6 Regional Harmonization of 1,4-Dioxane Methods

To strengthen the testing capabilities of 1,4-Dioxane, stakeholders need an analytical method that can be used for both pre and post-market surveillance. Indonesia, in this case, CNQLDF, was appointed as the leader in the development of 1,4-Dioxane AM for ASEAN (ASEAN Cosmetic Method, ACM). The process of establishing this ACM has gone through a long process, according to the ASEAN Guideline on Establishing the ASEAN Cosmetic Method (ACM). The establishment of ACM is carried out through several stages of activities, starting from identifying ASEAN member states (AMS) with related MAs, then comparing these AMs, and selecting the best AM, including the validation results. Indonesian AM have the best sensitivity, thus it was chosen due to Gas Chromatography Tandem Mass Spectrometry-Head Space (GCMS-HSS) has been selected as the preferred instrument to become ACM candidates. The next step was to refine the MA, in this case, making internal changes to the standards used, according to input from other AMS in the ACTLC meeting. AM revalidation was then carried out using the internal standard isotope of 1,4-Dioxane 1,4-Dioxane-d8. After the AM revalidation stage was carried out, the collaboration test of the AM was continued. Furthermore, a manuscript report was made along with a draft ACM. Input was sought from other AMS on the report manuscript and draft ACM, which were then finalized by adopting the AM into ACM.

The collaborative study conducted from August to September 2022. It was attended by 10 participants, consisting of three ASEAN member countries, namely the Philippines, Thailand, and Viet Nam, and seven laboratories in Indonesia, namely Indonesian FDA provincial offices in Padang, DKI Jakarta, Pontianak, Denpasar, Mataram, Pangkal Pinang and CNQLDF. The test objects were taken from products on the market and met the homogeneity and stability test requirements. The collaboration test results are sr=0.14393; sR=1.74388; RSDR=14.5010; PRSD=11.00388; HORRAT=1.318, or it can be concluded that the reproducibility of the method meets the requirements ($0.5 < \text{HORRAT} \leq 1.5$). The AM validation results for determining 1,4-Dioxane levels in cosmetic products using GCMS-HSS gave the following values (Table 6).

Table 6. 1,4-Dioxane Validation Parameter Value

No.	Validation Parameters	Acceptance Condition	GCMS Study
1	Selectivity	Resolution ≤ 1.5	Rt 1,4- Dioxane 8.816, Rt 1,4- Dioxane -d8 8.704 m/z 1,4-Dioksan 88, 58, 43 m/z 1,4- Dioxane -d8 96, 64, 46
2	Linearity	Vx0 ≤ 5.0	r = 1.000; Vx0 = 1.4%
3	Precision	RSD 12.5% (1.21 µg/g) ≤ 7,3	0,3%
		RSD 100% (10.28 µg/g) ≤ 7,3	1,1%
		RSD 125% (13.09 µg/g) ≤ 7,3	0,2%
4	Accuracy	% Recovery 12.5% (1.21 µg/g) = 80 – 110	94,6 – 95,7
		% Recovery 100% (10.28 µg/g) = 80-110	99,4 – 101,0
		% Recovery 125% (13.09 µg/g) = 80-110	101,7 – 102,1
5	LOD	-	0,08 µg/g
6	LOQ	-	0,27 µg/g

3.7 Harmonization of 1,4-Dioxane Method at the National Level

Through the notification system, the industry is fully responsible for the quality, safety, and benefits of the products produced. One of the efforts to strengthen pre-market supervision is to build cross-sectoral cooperation to strengthen industrial laboratories and external laboratories that carry out cosmetic testing.

To date, 15 external laboratories have their cosmetic testing performance accredited ISO 17025: 2017. Of these 15 laboratories, only two laboratories could carry out 1,4-Dioxane testing. Therefore, it is necessary to strengthen pre-market supervision by conducting cross-sector collaboration within the ICLN to equalize the ability of cosmetic testing among laboratories. Some of the collaboration steps taken were as follows:

a. Training on validation of analytical methods to equalize perception in carrying out AM validation
b. Technical training on cosmetic testing to improve personnel competency in conducting cosmetic testing or implementing new AM.
c. Implementation of proficiency test to assess laboratory performance
d. Organization of collaboration tests to assess the robustness of analytical methods
e. Development of the ICLN website as a forum for all members to exchange information about cosmetic testing
f. Harmonization of AM, both at the national, ASEAN regional, and international levels

Harmonization of analytical methods at the national, regional, and international levels is an effort to harmonize existing analytical methods so that all laboratories have the same reference standard for analytical methods when conducting sample testing. The objectives of standardization according to RI Law No. 20/2014 were as follows:

a. Improve quality assurance, production efficiency, national competitiveness, fair and transparent business competition in trade, business certainty, and the ability of business actors, as well as the ability of technological innovation.
b. Increasing protection to consumers, business actors, labor, and other communities, as well as the state, both from the aspects of safety, security, health, and preservation of environmental functions
c. Increase certainty, smoothness, and efficiency of trade transactions in goods and services domestically and abroad

Harmonization of cosmetic analysis methods at the national level is carried out through the issuance of Indonesian National Standards (INS). INS on Cosmetics can prevent disputes over test results and increase the competitiveness of the nation's products. The formulation of analytical methods into INS involves various stakeholders who are members of the Cosmetics Technical Committee, consisting of representatives from NQCLDF, Directorate of Standardization and Quality Control - Ministry of Trade, Center for Standardization and Services for the Chemical, Pharmaceutical and Packaging Industries - Ministry of Industry, Academic Experts from the University of Indonesia, PT Saraswati Indo Genetech, PT SGS Indonesia, PT Angler BioChemlab, Indonesian Cosmetics Association (Perkosmi), Indonesian Consumers Foundation (ICF).

To increase the capacity of 1,4-Dioxane testing in Indonesia, the Indonesian FDA needs to harmonize the analytical testing methods for 1,4-Dioxane contamination at the national level by issuing the Indonesian National Standards (INS). This ACM method is intended to be widely utilized by stakeholders and external laboratories for pre-market supervision of cosmetic products to ensure product quality and safety.

4. Conclusion

The analytical method for determining 1,4-Dioxane levels in cosmetics by GCMS-HSS developed by CNQLDF has been collaborated and harmonized at the regional level into ACM 011 and in the process of harmonization at the national level into INS. With the harmonization of this method, all cosmetics laboratories in Indonesia and ASEAN, including those incorporated in JLKI, have standardized analytical methods for testing 1,4 Dioxane to strengthen pre-market supervision of cosmetic products in ensuring product quality and safety.

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Performance Characteristics of the Quantitative Method for *Staphylococcus aureus* in Food Products corresponds to ISO 16140-3: 2021

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ABSTRACT / ABSTRAK

Good food sanitation is one of the main pillars for achieving food security goals. High levels of *Staphylococcus aureus* in food can indicate poor hygiene and handling practices during food production, processing, or storage. Before testing, the laboratory needs to verify the quantitative method for *Staphylococcus aureus* to produce valid data to ensure food safety and quality. The Centre of National Quality Laboratory of Drugs and Food, Indonesian Food and Drug Authority (FDA) has never verified the *Staphylococcus aureus* quantification method based on the latest ISO 16140-3:2021. Following the guidelines established in ISO 16140-3:2021, method verification is accomplished by calculating the Interlaboratory Reproducibility Standard Deviation (S_{IR}) for implementation validation and the eBias value for verifying the method's suitability for assessing specific food items. This research was conducted to confirm the ISO 6888-1:2021 as the designated reference method for quantifying *Staphylococcus aureus* in food products. The cheese was utilized as the test food item to verify the implementation of the method. At the same time, several products, including condensed milk, margarine, baby porridge, cassava chips, and ready-to-eat sausage, were examined as challenging food types. Every chosen food item was artificially contaminated with *Staphylococcus aureus* WDCM 00034. The S_{IR} value obtained was $0.04 \leq 2\times 0.11$ (the lowest mean of SR value from ISO 6888-1:2021), which indicated that the Centre of National Quality Laboratory of Drugs and Food Indonesian FDA was able to implement the method very well. In addition, the eBias value for all types of food tested was below 0.5log10, which showed that the quantitative method for coagulase-positive *Staphylococci* (*Staphylococcus aureus* and other types) could be applied in the Centre of National Quality Laboratory of Drugs and Food laboratory for the extensive scope of food.

Sanitasi pangan yang baik merupakan salah satu pilar utama untuk mencapai tujuan food security. Tingginya jumlah Staphylococcus aureus dalam makanan dapat mengindikasikan buruknya sanitasi selama proses produksi atau penyimpanan pangan. Sebelum melakukan pengujian, sangat penting bagi laboratorium untuk melakukan verifikasi terhadap metode kuantitatif Staphylococcus aureus guna menghasilkan data yang valid dalam rangka memastikan keamanan dan kualitas pangan. Laboratorium Pusat Pengembangan Pengujian Obat dan Makanan Nasional (PPPOMN) Badan Pengawas Obat dan Makanan (BPOM) belum pernah melakukan verifikasi metode kuantifikasi Staphylococcus aureus berdasarkan ISO terbaru 16140-3:2021. Sesuai dengan pedoman yang ditetapkan dalam ISO 16140-3:2021, verifikasi metode dilakukan melalui penghitungan, yakni Standar Deviasi Reprodusibilitas Intralaboratorium

(SIR) untuk verifikasi implementasi dan nilai eBias untuk memverifikasi kesesuaian metode dalam menilai berbagai jenis pangan. Penelitian ini dilakukan dengan tujuan mengkonfirmasi ISO 6888-1:2021 sebagai metode acuan yang ditetapkan dalam menghitung jumlah Staphylococcus aureus pada pangan olahan. Keju digunakan sebagai sampel uji untuk verifikasi implementasi di laboratorium, sedangkan sejumlah produk termasuk susu kental manis, margarin, bubur bayi, keripik singkong, dan sosis siap makan digunakan sebagai jenis pangan yang menantang dalam rangka memverifikasi jenis pangan. Setiap jenis produk pangan yang digunakan dalam verifikasi, dicemari Staphylococcus aureus WDCM 00034. Nilai SIR yang diperoleh adalah 0.04 ≤ 2× 0.11 (rata-rata nilai SR terendah dari ISO 6888-1:2021) yang menandakan laboratorium PPPOMN Badan POM mampu mengimplementasikan metode dengan baik. Selain itu, nilai eBias untuk semua jenis pangan yang diujikan berada di bawah 0.5log10, yang menunjukkan bahwa metode kuantitatif untuk Staphylococci positif koagulase (Staphylococcus aureus dan jenis lainnya) dapat digunakan di Laboratorium PPPOMN dalam ruang lingkup pangan yang lebih luas.

Keywords: ISO 16140-3: 2021, *Staphylococcus aureus*, microbiology, eBias, food item, S_{IR}, verification
Kata Kunci: *ISO 16140-3: 2021, Staphylococcus aureus,* mikrobiologi, eBias, jenis pangan, S_{IR}, verifikasi

1. Introductions

Staphylococcus aureus is a potential foodborne pathogen. It can produce toxins that cause food poisoning when ingested. Therefore, the presence of this bacterium in food products can pose a significant food safety risk (Fetsch & Johler, 2018). Consuming food contaminated with *Staphylococcus aureus* can lead to foodborne illnesses, including symptoms such as nausea, vomiting, diarrhea, and abdominal cramps (CDC, 2023). The severity of these symptoms can vary depending on the level of contamination and the individual's susceptibility, potentially leading to hospitalization in severe cases (Hennekinne et al., 2012). The impact of these illnesses can be severe, depending on the amount of toxin produced and the individual's immune response, making it crucial to prevent *Staphylococcus aureus* contamination in the food supply chain (Kadariya et al., 2014). High levels of *Staphylococcus aureus* in food can indicate poor hygiene and handling practices during food production, processing, or storage (Gutiérrez et al., 2012). Outbreaks of *Staphylococcus aureus* food poisoning have been reported worldwide, underlining the need for rigorous monitoring and control measures (Balaban & Rasooly, 2000).

Many regulatory agencies and governments have established maximum allowable limits for *Staphylococcus aureus* in certain food products. In Indonesia, the maximum permissible limits for *Staphylococcus aureus* in food products are regulated in Indonesian FDA Regulation No. 13, 2019. Enumeration testing helps determine the level of *Staphylococcus aureus* contamination and assess the potential consumer risk. Monitoring the enumeration of this bacterium is an essential quality control measure to ensure that food products meet specific quality and safety standards (International Organization for Standardization (ISO), 2021a). Strict surveillance of pathogenic microorganisms in food products is necessary to prevent food-borne diseases from consuming contaminated food (Hoorfar, 2011). It is essential to ensure compliance with these legal requirements. To maintain food safety and quality, verifying the quantitative method of *Staphylococcus aureus* is very important. According to Wang et al. (2022), global food safety requires continuous monitoring of pathogenic microorganisms to prevent foodborne illnesses and ensure consumer health.

Method verification is a critical step in ensuring that all methods used, especially in laboratory testing and research, meet the standards required for data reliability and accuracy (Abdel & El-Masry, 2021). It ensures that a particular testing or analytical method can produce accurate and precise results. This means the method's measurements are close to the actual value, and repeated measurements under the same conditions yield consistent results (Sushila Dagadu Chavan & Deepa Mahendra Desai, 2022). Method verification is a critical component of quality control in laboratories. It helps monitor and maintain the quality and reliability of testing processes, ensuring that the methods are suitable for their intended purpose (Taverniers et al., 2004). In situations where different laboratories may use the same method, method verification helps ensure that the results are comparable and that the method is applied consistently across various settings. Moreover, method verification across multiple laboratories helps ensure consistency in results, which is particularly important in the global food trade where standardized practices must be followed (Elliott et al., 2020).

ISO 6888-1:2021 provides guidelines for enumerating coagulase-positive *Staphylococci* (*Staphylococcus aureus* and other species) using microbiological culture methods. The principle is to take a food or feed product sample, inoculate it into a suitable growth medium, and count the bacterial colonies that grow after a certain incubation period. Referring to the guidelines set out in ISO 16140-3:2021 is a critical approach to ensure the accuracy and reliability of the quantitative method for *Staphylococcus aureus* in food products. ISO 16140-3:2021 is a specific part of the ISO 16140 series that guides validating alternative (proprietary) methods for microbiological testing. The standard provides a structured process for verifying proprietary testing methods against a reference method. This verification ensures that alternative methods are as reliable as established reference methods (International Organization for Standardization (ISO), 2021b). According to Zhang et al. (2020), the standardization of microbiological methods like ISO 16140-3:2021 is essential to ensuring the safety of food products globally.

There are two parameters used in the context of quantitative microbial testing: the Interlaboratory Reproducibility Standard Deviation (S_{IR}) for implementation verification and the eBias value for food item verification (International Organization for Standardization (ISO), 2019). By evaluating these performance characteristics, laboratories can verify and validate their quantitative methods for *Staphylococcus aureus* in food products, ensuring that the methods are accurate, reliable, and suitable for their intended purposes, as recommended by ISO 16140-3:2019.

This study will present the general procedure of verifying the enumeration method of coagulase-positive *Staphylococci* and assign the performance characteristics referred to ISO 16140-3: 2021. Moreover, this study will also outline the critical criteria supporting the method verification's fruitfulness.

2. Methodology

2.1 Research Material

The reference strain *Staphylococcus aureus* WDCM 00034 was purchased from Microbiologics, USA. Cultural media purchased from Merck, Germany, prepared by ISO 6888-1:1999+A2:2018. The sample used for implementation verification (S_{IR} determination) is the type of food selected from the scope of validation in ISO 6888-1:1999+A2:2018 Annex A (Cheese). Food item verification (eBias determination) uses

samples from several food items selected based on various characteristics of matrix types stated in Indonesian FDA regulation No. 13/2019 (Table 1).

Table 1. Food Items Intended for Verification Studies Aimed at Determining eBias (Indonesian FDA, 2019)

Food Item	Food Type	Food Category
Condensed Milk	Condensed Milk and Its Analogues	Dairy products and their analogs
Margarine	Fat Emulsions, Especially Water-in-Oil Emulsion Type	Fats, Oils, and Oil Emulsions
Baby Porridge	Food for babies and growing children	Processed food for particular nutritional needs
Cassava Chips	Snacks made from potatoes, tubers, cereals, flour, or starch (from tubers and nuts)	Ready-to-eat snacks
Ready-to-eat Sausage	Processed meat products, poultry meat, and ground game meat	Meat and meat products, including poultry and game

2.2 Preparation of Reference Strain as Artificial Contamination and Initial Suspension

The results of cultivating the reference strain on an overnight Tryptic Soy Agar (TSA) plate were prepared to make a 0.8% NaCl suspension with a turbidity of 1 Mc Farland. Serial dilution made The suspension into low, medium, and high-level concentrations. A sterile filter bag was filled with 25 grams of the sample. The sample was mixed with 225 mL of Peptone Salt Solution (PSS). A stomacher homogenized the sample at 230 rpm for 30 seconds to achieve 1:10 dilution.

2.3 Verification Method

2.3.1 Implementation Verification for S_{IR} Determination

Ten test portions were prepared in duplicate and deliberately contaminated in the initial suspension with different levels of bacterial inoculum, including low, medium, and high concentrations. The quantification of *Staphylococcus aureus* followed the guidelines outlined in ISO 6888-1:2021. Two technicians conducted The verification process simultaneously using two different batches of media and equipment, including incubators, vortex mixers, and pipettes.

2.3.2 Food Item Verification for eBias Determination

The food products underwent artificial contamination with inoculums at three distinct levels in the initial suspension, with each level tested in duplicate. Enumeration of the artificially contaminated food items and the inoculum suspension used for contamination was performed per ISO 6888-1:2021. The analysis was conducted simultaneously under replicable conditions by a single technician.

Following the guidelines outlined in ISO 16140-3:2021, an eBias evaluation was conducted. The eBias value was assessed by calculating the absolute difference between the logarithmic counts of *Staphylococcus aureus* in contaminated food items and the bacterial inoculum suspension.

2.4 Data Analysis

2.4.1 Implementation Verification for S_{IR} Determination

The number of *Staphylococcus aureus* was calculated using the formula by ISO 16140-3:2021 to analyze the intralaboratory reproducibility standard deviation (S_{IR}) values.

$$S_{IR} = \sqrt{\frac{1}{2n}\sum_{i=1}^{n}(y_{iA} - y_{iB})^2}$$

2.4.2 Food Item Verification for eBias Determination

In compliance with ISO 16140-3:2021, an eBias analysis was carried out. The absolute difference between the log numbers of *Staphylococcus aureus* from contaminated food items and bacterial inoculum suspension was used to analyze the estimated Bias (eBias) value.

3. Results and Discussion

Verification of the enumeration of coagulase-positive *Staphylococci* (*Staphylococcus aureus* and other species) has been carried out by ISO 16140-3: 2021 by determining the Interlaboratory Reproducibility Standard Deviation (S_{IR}) parameters for implementation verification and eBias for food item verification. The validation study on ISO 6888-1:1999+A2:2018 used three types of food: cheese, meat, and egg powder, with the lowest average S_R value of egg powder (0.11) (Table 2). Implementation verification in this study was carried out using cheese samples with a S_{IR} value of 0.04 (Table 3 and Table 4).

For implementation verification, the S_{IR} of the verified method shall be equal to or less than twice the lowest mean value of the reproducibility standard deviation (S_R) of the (food) items used in the validation study stated in ISO 6888-1:2021. This study noted that *the* S_{IR} value for cheese was less than twice the average S_R value for egg powder, which, this indicates that the laboratory was able to implement the method very effectively.

Table 2. Summary of S_R values from the validation study for ISO 6888-1

(Food) item	S_R values from the validation study			
	Low Inoculation Level	Medium Inoculation Level	High Inoculation Level	The mean value of three Inoculation levels
Cheese	0.19	0.16	0.24	0.20
Meat	0.17	0.17	0.14	0.16
Egg Powder	0.11	0.10	0.11	**0.11**

Table 3. Result of S_{IR} Compared to S_R Value from ISO 6888-1:2021

Laboratory Sample Number	Result A	Result B	Log_{10} Result A yiA = log_{10}(xiA)	Log_{10} Result B yiB = log_{10}(xiB)	Absolute Difference |yiA – yiB|	Squared Difference |yiA – yiB|^2
1	550	570	2.74	2.76	-0.0155	0.0002
2	520	590	2.72	2.77	-0.0548	0.0030
3	5900	5700	3.77	3.76	0.0150	0.0002
4	6,200	5,500	3.79	3.74	0.0520	0.0027
5	6,300	6,100	3.80	3.79	0.0140	0.0002
6	11,000	11,000	4.04	4.04	0.0000	0.0000
7	10,000	10,000	4.00	4.00	0.0000	0.0000
8	13,000	9,900	4.11	4.00	0.1183	0.0140
9	43,000	55,000	4.63	4.74	-0.1069	0.0114
10	46,000	56,000	4.66	4.75	-0.0854	0.0073
					SUM	0.0391
					Sum/(2xn)	0.0020
					S_{IR}	**0.0442**

Table 4. The result of S_{IR} compared to the S_R value from ISO 6888-1:2021

Food item	S_{IR} Value	The mean value of SR ISO 6888-1:2021
Cheese	0.04	0.11
Acceptance Criteria		$S_{IR} \leq 2 \times 0.11$
		0.04 < 0.22 (comply with the requirement)

The verified method is for counting *Staphylococcus aureus* and other *Staphylococci* that can coagulase plasma. The coagulase test is one of the methods used to differentiate highly pathogenic *Staphylococcus aureus* from other less pathogenic *Staphylococcal* species (Chamberlain, 2009). This study used *Staphylococcus aureus* WDCM 00034 to contaminate the samples artificially due to the difficulties of finding cheese naturally contaminated with *Staphylococcus aureus*.

Table 5. The eBias Results from Food Item Verification

Food Item	eBias Value		
	Low	Medium	High
Condensed Milk	0.09	0.02	0.01
Margarine	0.00	0.05	0.07
Baby Porridge	0.05	0.19	0.01
Cassava Chips	0.05	0.17	0.12
Ready-to-eat Sausage	0.18	0.13	0.07

The successful implementation verification using cheese, as demonstrated by the low SIR value, underscores the robustness of the ISO 6888-1:2021 method in detecting *Staphylococcus aureus*. The importance of low SIR values cannot be overstated as they reflect the method's reproducibility, which is crucial for ensuring consistent results across different laboratories (Latimer, 2023). This consistency is particularly vital in the global food

industry, where standardized methods are necessary to maintain food safety and ensure compliance with international regulations (Elliott et al., 2020).

Food item verification compared artificially contaminated samples and inoculum control. In this study, five types of food were used to cover the extensive scope of food products. These five types of food have represented challenging food, for example, samples with a high-fat content (margarine), high sugar level (condensed milk), and high microbiota content (baby porridge, cassava chips, and ready-to-eat sausage).

Food matrices can vary significantly in composition, including fat content, sugar levels, microbiota, pH, and more (Aryani et al., 2016). By selecting food items representing a broad spectrum of these characteristics, the verification process becomes more representative of real-world scenarios. Different food matrices can pose unique challenges to testing methods. For example, high-fat content in some foods can interfere with microbial detection, and high-sugar foods can impact the growth of microorganisms (Hamad, 2012). By including a variety of matrices, the verification process can evaluate the method's ability to handle these challenges and provide accurate results in a range of conditions. By testing the method across different matrices, it can be confirmed that the method is specific to the target microorganism and not unduly influenced by the matrix components (Latimer, 2023). The ability of the method to produce consistent and accurate results, regardless of the food matrix, demonstrates its robustness and versatility, making it a valuable tool for food safety laboratories worldwide. This study's findings also emphasize the importance of using well-characterized reference materials and adhering to strict biosafety protocols, especially when working with pathogenic microorganisms like *Staphylococcus aureus* (Kadariya et al., 2014).

According to ISO 16140-3:2021, the absolute difference between the artificially contaminated food and the inoculum suspension as positive control is equal to or less than 0.5 log10. The results in Table 5 showed that the eBias value between five food items was diverse and complied with the requirement. From those results, it was noted that each level of contamination between the absolute difference between the contaminated samples and positive control was less than 0.5 log10. In conclusion, the user laboratory successfully verified the enumeration of coagulase-positive Staphylococci.

Considering several factors that may contribute to the verification process, this study used well-characterized and purified reference materials to prevent non-conformities. In this case, using commercial references should be considered. Moreover, proper biosafety practices should be applied since coagulase-positive *Staphylococci* are known as pathogenic bacteria. Another factor identified during this study was how to appropriately treat challenging food as the representatives for food item verification based on ISO 16140-3: 2021. Those challenging food samples should be prepared and homogenized according to ISO 6887 (all parts): 2017. In addition, fully understanding the essential statistical element applied in ISO 16140-3: 2021 is also essential in this study because the data analysis sometimes needs conversion, especially for the test portion.

The enumeration of *Staphylococcus aureus* in this verification process is done meticulously using the standardized formula outlined in ISO 16140-3:2021. ISO 16140-3:2021's stringent guidelines emphasize the analysis of interlaboratory reproducibility standard deviation (S_{IR}) and eBias values, which are crucial for assessing consistency across different laboratories. Careful S_{IR} calculations and analysis guarantee accuracy and

reproducibility, enhancing the method's reliability and integrity. This meticulous adherence to international standards instills confidence in the verification process's ability to accurately detect and count *Staphylococcus aureus*, a critical step in ensuring food safety and quality.

Nonconformities may occur during verification, and personnel must be competent to identify the problem. This proactive approach not only rectifies immediate discrepancies but also acts as a preventative measure to ensure that similar problems do not recur. Moreover, when verifying quantitative methods, it is essential to assess the measurement uncertainty of the S_{IR} value concurrently to reduce the factors that can increase uncertainty. This approach makes measurement uncertainty more evident and enhances the reliability of laboratory performance, thereby increasing consumer trust.

4. Conclusion

This work discussed how to meet the requirements of performance characteristics of the enumeration method for coagulase-positive *Staphylococci* (*Staphylococcus aureus* and other species) by ISO 16140-3: 2021. Both implementation verification and food item verification showed satisfactory results, meaning the user laboratory could implement the ISO 6888-1: 2021 well. In line with implementation verification, food item verification provided satisfactory results, reflecting an accurate and reliable method to be applied in the user laboratory. Since Centre of National Quality Laboratory of Drugs and Food Indonesian FDA has been equipped with calibrated instruments, competent personnel, and pure references, this study's SIR and eBias value complied with the verification criteria. In conclusion, this meticulous verification process, as outlined in ISO 16140-3, ensures that laboratories are well-prepared to maintain the highest standards of accuracy and reliability when enumerating coagulase-positive *Staphylococci*, ultimately contributing to enhanced food safety and quality control.

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Optimization and Validation of Analytical Method for Detection of *Shigella* sp. in Oral Preparations of Quasi-Drugs in the Form of Lozenges

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ABSTRACT / ABSTRAK

Lozenges, a quasi-drug used to relieve sore throats, must meet the safety and quality requirements according to Indonesian FDA Regulation No. 7 of 2023 concerning the criteria and procedures for quasi-drug registration, which stipulates that oral preparations of quasi-drugs must be free from *Shigella* spp—microbial contamination. Contamination by *Shigella* bacteria can cause diseases such as shigellosis, characterized by symptoms such as diarrhoea, vomiting, and fever. This study aims to validate the *Shigella* spp—detection method in quasi-drug lozenges using the WHO (Quality Control Methods for Herbal Materials) reference method. Validation was carried out to ensure that this method can be used for routine analysis in the Indonesian FDA laboratory. The guidelines used to validate the microbiological method are the Singapore Accreditation Council (SAC) Guidance Notes C&B AND ENV 002 of 2019 and refer to pharmacopoeias. The study results showed that the WHO (Quality Control Methods for Herbal Materials) method in detecting *Shigella* sp. has a sensitivity and specificity of 100%, with false positive and false negative rates of 0% each. The detection limit obtained was 3 cfu/g, which meets the requirements (below 10 cfu/g). The method suitability test (optimization) showed that the initial solvent used for sample homogenization in *Shigella* detection was sufficient using Tryptone Soya Broth (1:10) without additional neutralization or modification procedures at the sample homogenization stage. Based on these results, the *Shigella* spp. The WHO reference (Quality Control Methods for Herbal Materials) detection method is accurate. It can be applied for routine microbial contamination testing analysis in the Food and Drug Supervisory Agency laboratories.

Tablet hisap obat kuasi yang digunakan untuk meredakan sakit tenggorokan harus memenuhi persyaratan keamanan dan mutu sesuai Peraturan Badan POM No. 7 Tahun 2023 tentang kriteria dan tata laksana registrasi obat kuasi, yang menetapkan bahwa sediaan oral obat kuasi harus bebas dari cemaran mikroba Shigella spp. Kontaminasi oleh bakteri Shigella dapat menyebabkan penyakit seperti shigellosis yang ditandai dengan gejala seperti diare, muntah, dan demam. Penelitian ini bertujuan untuk memvalidasi metode deteksi Shigella spp. pada tablet hisap obat kuasi menggunakan metode acuan WHO (Quality Control Methods for Herbal Materials). Validasi dilakukan untuk memastikan metode ini dapat digunakan untuk analisis rutin di laboratorium BPOM. Pedoman yang digunakan untuk memvalidasi metode mikrobiologi adalah Singapore Accreditation Council (SAC) Guidance Notes C&B AND ENV 002 Tahun 2019 serta farmakope. Hasil penelitian menunjukkan bahwa metode WHO (Quality

Control Methods for Herbal Materials) dalam mendeteksi Shigella sp. memiliki sensitivitas dan spesifisitas sebesar 100%, dengan tingkat positif palsu dan negatif palsu masing-masing 0%. Limit deteksi yang diperoleh adalah 3 cfu/g, yang memenuhi persyaratan (di bawah 10 cfu/g). Dari uji kesesuaian metode (optimasi) yang dilakukan menunjukkan bahwa pelarut awal yang digunakan untuk homogenisasi sampel pada deteksi Shigella cukup menggunakan Tryptone Soya Broth (1:10) tanpa perlu prosedur penetralan atau modifikasi tambahan pada tahapan homogenisasi sampel. Berdasarkan hasil ini, metode deteksi Shigella spp. menggunakan metode acuan WHO (Quality Control Methods for Herbal Materials) dinyatakan akurat dan dapat diterapkan untuk analisis rutin pengujian cemaran mikroba di laboratorium-laboratorium Badan Pengawas Obat dan Makanan.

Keywords: *Method validation, Shigella, quasi-drug, lozenges, microbiological examination of non-sterile product, SAC-Singlas*
Kata Kunci: Validasi metode, *Shigella,* obat kuasi, tablet hisap, pengujian mikrobiologi produk nonsteril, SAC-Singlas

1. Introduction

According to Indonesian FDA regulation No.7 of 2023, quasi-drugs are preparations containing active ingredients with pharmacological effects that are non-systemic or local to treat minor complaints. Quasi-drug preparations consist of 2 types, namely topical and oral. Various kinds of quasi-medicinal products are circulating in Indonesia, so monitoring these products' physical, chemical and biological safety aspects is necessary. Quasi-drugs circulating in Indonesia must meet the safety and quality requirements of finished quasi-drug products listed in the latest Indonesian FDA regulation No.7 of 2023 concerning criteria and procedures for registration of quasi-drugs. One of the requirements in the regulation states that oral quasi-medicinal products must not contain *Shigella* contamination.

Based on the website (https://cekbpom.pom.go.id/obat_kuasi, accessed on October 23, 2023), the Indonesian FDA has issued 12 quasi-medicinal products in the form of lozenges. Adults and children widely use quasi-medicinal lozenges to treat minor throat complaints (Rathod et al., 2018). Quasi-medicinal lozenges commonly circulated in the market are generally complex candy types, also known as lozenges. Hard candy-type lozenges have hygroscopic properties, so if not packaged properly, they will increase the water content in the lozenges, which can potentially cause the growth of microorganisms (Choursiya & Andheriya, 2018).

Microbial contamination in quasi-medicinal products can potentially reduce or even activate therapeutic activity and harm one's health (Aini et al., 2021). *Shigella* bacteria are capable of producing toxins and causing diseases such as dysentery, diarrhoea, and even death. *Shigella* is a gram-negative bacterium in the *Enterobacteriaceae* family that causes Shigellosis (Muzembo et al., 2023). Shigellosis is a disease caused by *Shigella* species bacteria that infect the intestinal tract and rectum (Kotloff et al., 2018). In a study in East Africa, *Salmonella* and *Shigella* were the main pathogenic bacteria most commonly reported as contaminants in traditional medicines (Walusansa et al., 2021). *Shigella* was a bacterial contaminant in traditional medicine samples studied in Nairobi, Kenya (Korir et al., 2017). *Shigella*e are highly infectious; the infective dose is about 103 organisms compared to 105-108 for *Salmonella* and Vibrio (Brooks et al., 2012).

Quasi-medicinal products before circulating to the market and those that have circulated in the market must meet safety, efficacy and quality requirements. One safety requirement

that oral quasi-drugs must meet is that they must not contain *Shigella* microbial contamination; this can be proven through *Shigella* detection testing in the laboratory. Therefore, the laboratory needs a validated analytical method to detect microbial contamination. Method validation is an essential factor in obtaining analytical results that are valid, reliable, and can be scientifically accounted for by the intended use (Faridah et al., 2018).

In 2023, the Food and Drug Administration did not have a test method for detecting *Shigella* quasi-drugs in lozenges. Indonesian FDA only has a validated *Shigella* detection analysis method (MA PPOMN No.52/MI/15) for testing traditional medicine samples because there were no regulations requiring microbial contamination limits for finished quasi-medicine products. According to ISO 17025:2017, the laboratory must validate the reference method used if it is used outside the intended scope, outside its designation, or modified. In this study, the WHO issued the reference method for *Shigella* detection analysis on quality control methods for herbal materials (World Health Organization, 2011). The method issued by WHO is intended for herbal/traditional medicine samples, so if the laboratory wants to apply the technique to quasi-medicine samples, validation testing of the method is needed before use.

This study focuses on validating *Shigella* sp detection methods in quasi-medicinal products in the form of lozenges using conventional culture-based methods based on the methods recommended by WHO. The selection of this method considers various factors such as cost, equipment, reagents/media, human resources, as well as accommodation and environmental conditions in the Indonesian FDA Provincial Offices throughout Indonesia. In addition, based on the United States Pharmacopeia Convention (USP) 2022 in appendix <1223>, if there are differences in test results between alternative and conventional microbiological methods, the traditional/culture method is still used as a gold standard reference.

However, recent developments have shown that several alternative molecular based (DNA) methods offer higher speed and accuracy in detecting *Shigella*. For example, a study by Yang et al. (2020) compared the performance of conventional PCR, real-time PCR, and droplet digital PCR (ddPCR) in detecting *Shigella* in food, where ddPCR showed the highest sensitivity with a detection limit as small as 0.1 cfu/mL. Another rapid method is real-time Loop-Mediated Isothermal Amplification (LAMP), which, according to a study by Liew et al. (2014), can detect *Shigella* within 90 minutes. Still, it has a relatively large detection limit of 5.9 x 10^5 cfu/mL.

In this study, the culture-based WHO method will be validated to prove that the process meets the requirements of the predetermined validation parameters. This study aims to produce a valid analytical method to detect *Shigella* contamination in quasi-oral drug samples in the form of lozenges. A valid analytical process is expected to protect the public from products that pose health risks, and this method can later be used routinely in testing laboratories.

2. Methodology

2.1. Time and Place of Research

This research was conducted at the Microbiology and Molecular Biology Laboratory, National Food and Drug Testing Development Center, Indonesian FDA. The study was conducted from February to March 2023.

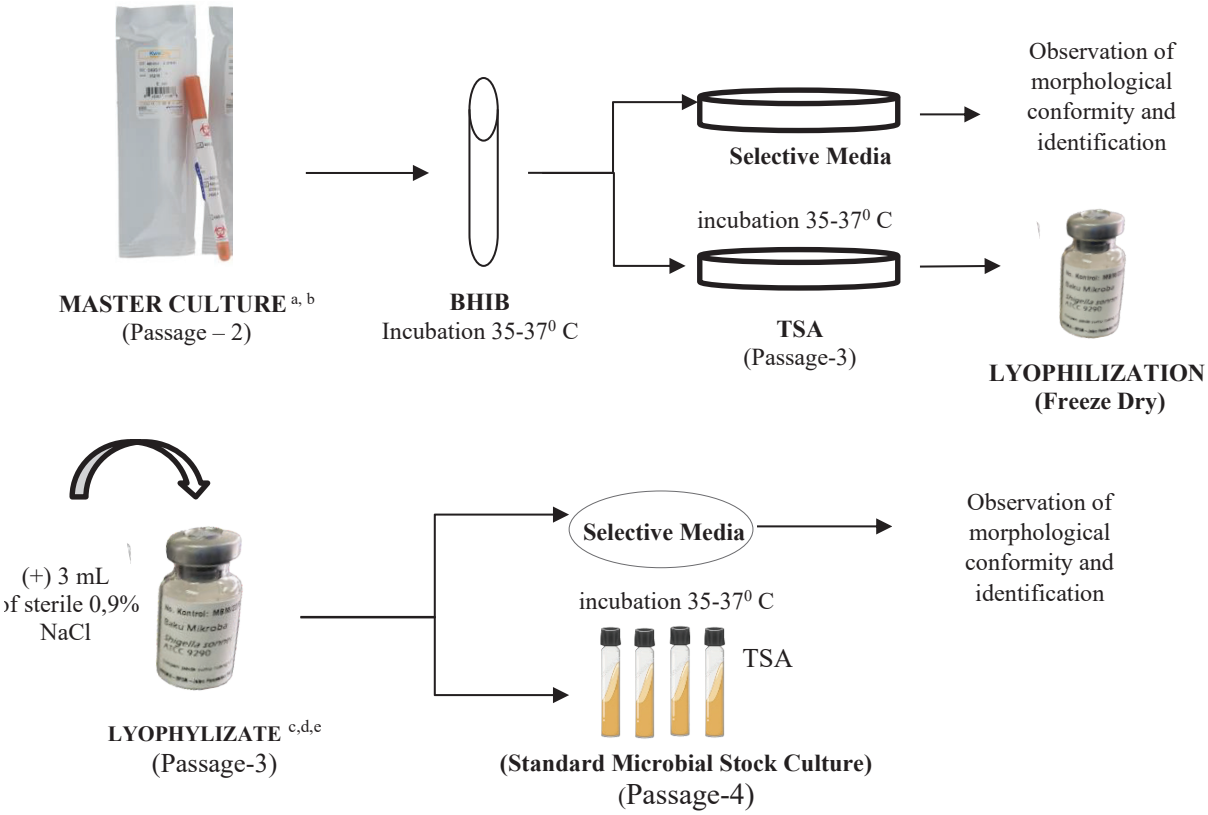
2.2. Materials and Research Instruments

The materials used in this study include Tryptone Soy Broth (TSB), *Enterobacteriaceae* Enrichment Broth Mossel (EEB-Mossel), Mac Conkey Agar (MCA), Xylose Lysine Desoxycholate Agar (XLD), Kligler Iron Agar (KIA), Tryptone Soy Agar (TSA), Buffered Sodium Chloride-Pepton Solution pH 7.0, physiological NaCl solution 0, 9%, Mc Farland standard, GN VITEK® card, quasi oral lozenges that have Indonesian FDA marketing license with the same batch number, raw microbes *Shigella sonnei* ATCC 9290, *Shigella dysentriae* ATCC 13313, *Shigella flexneri* ATCC 9199, *Escherichia coli* ATCC 8739 and *Salmonella typhimurium* ATCC 14028.

The tools used in this study include a set of glassware, oven, autoclave sterilization and deconstruction, incubator, microscope, pH meter, top loading scale, stomacher, Ose needle, Ose incinerator, Biosafety Cabinet (BSC), vortex mixer, micropipette and pipette tips—biochemical identification of bacteria using VITEK® 2 System instrument.

2.3. Preparation of Raw Microbial Cultures

Standard commercially available microbes such as ATCC (American Type Culture Collection) or other collection cultures are used. Standard microbes used for validation should not exceed 5 passages (United States Pharmacopeia Convention, 2022). The standard microbes *Shigella sonnei*, *Salmonella typhimurium* and *Escherichia coli* used in this study used the National Food and Drug Testing Development Center standard microbial lyophilization derived from the master Microbiologics® ATCC pasase-2 culture.



[a] *Shigella dysentriae* ATCC 13313, [b] *Shigella flexneri ATCC 9199*, [c] *Shigella sonnei* ATCC 9290, [d] *Escherichia coli* ATCC 8739, [e] *Salmonella* TyphimuriumATCC 1402

Figure 1. Schematic of Raw Microbial Culture Preparation

The preparation of standard microbial cultures that will be used for validation is illustrated in Figure 1. Indonesian FDA standard microbial lyophilization (pasase-3) was dissolved by adding 0.9% physiological NaCl and then waited for 10-15 minutes. It was then streaked onto several TSA and selective media, such as MCA and XLD, to observe its morphology. Colonies that grow on TSA media (please-4) can be used as raw microbial cultures for spiking.

2.3. Sample Preparation

The samples used were complex candy-type lozenges with Indonesian FDA marketing authorization on the market. The lozenges were aseptically crushed using a sterile mortar and pestle until smooth, then weighed 10 g into a sterile stomacher bag.

2.4. *Shigella* Detection Analysis Using WHO Reference Method

Shigella detection was performed using the reference method from WHO (2011) Quality Control Methods for Herbal Materials listed in Chapter 18: Determination of Microorganisms. The test began with sample preparation, followed by adding 90 mL of TSB as an enrichment medium, then homogenized using a stomacher and incubated at 30-37°C for 2-5 hours (pre-enrichment stage). From the pre-enrichment suspension, 10 mL was pipetted into 90 mL of EEB-Mossel, then incubated at 35-37°C for 18-24 hours. One ose of the enrichment results (EEB-Mossel) was inoculated on the surface of XLD and MCA media and then incubated at 35-37°C for 18-24 hours.

Observations were made of the specific colonies that grew. Cultures were considered *Shigella* positive if there were colonies with the following characteristics:

- On XLD: small round, red, 1-2 mm in diameter.
- On MCA: convex round, colourless, 2-3 mm diameter.

If no colonies grow on MCA and XLD, then *Shigella* is negative. Specific colonies were propagated by inoculating 3 colonies onto TSA/NA slant, incubated at 35-37°C for 24 hours for a confirmation test. The growing colonies were confirmed through Gram staining, KIA/TSIA biochemical media, and automatic bacterial identification using VITEK 2 Compact with GN reagent cards.

The following *Shigella* detection analysis procedure in schematic form is presented in Figure 2.

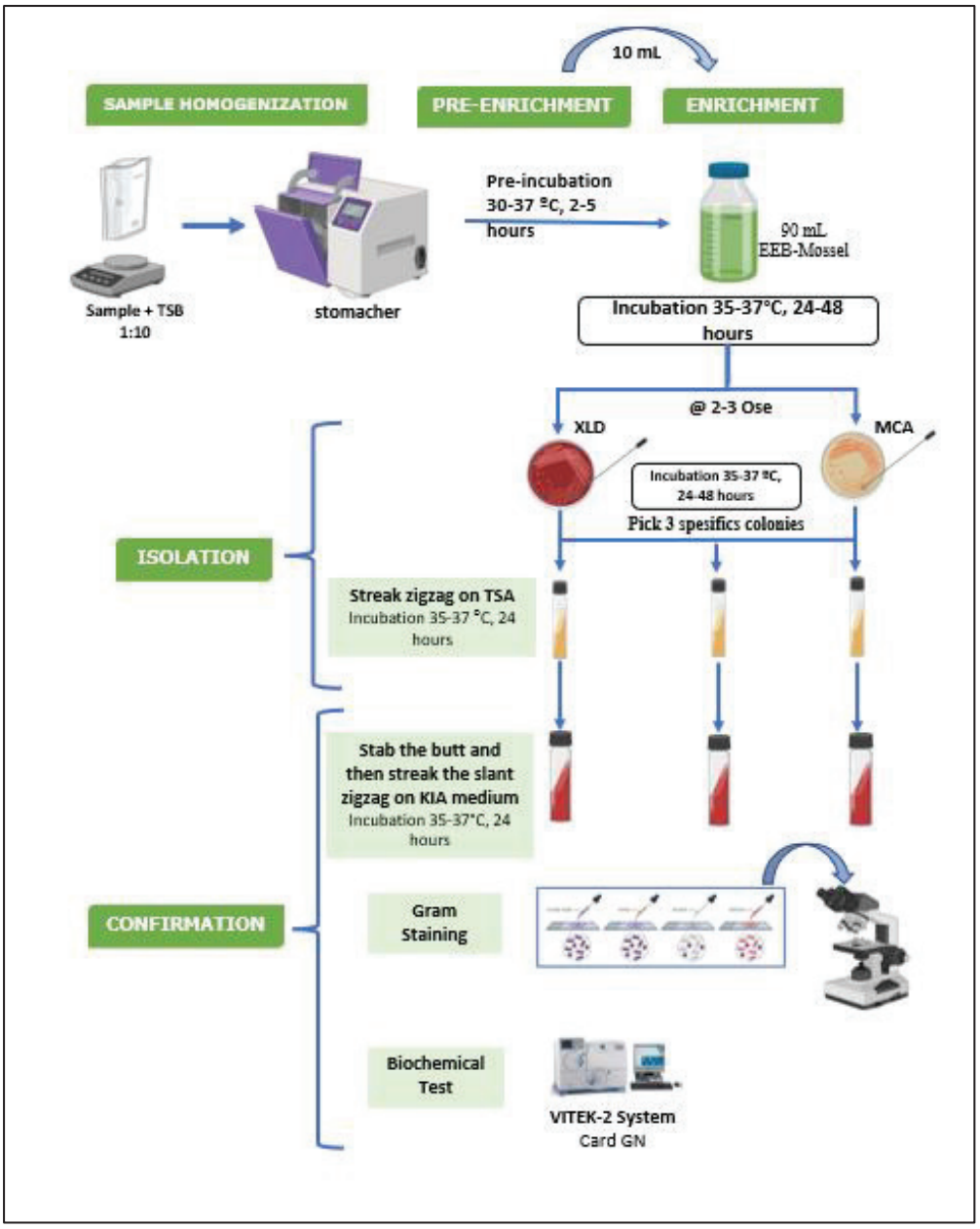


Figure 2. *Shigella* Detection Analysis Test Scheme (World Health Organization, 2011)

2.5. Preparation of contaminant inoculum

Preparation of bacterial suspensions for contamination is based on the calculation of the number of target bacteria (*Shigella sonnei*, *Shigella dysentriae*, *Shigella flexneri*) using the Total Plate Numbers method with 1 McFarland standard or UV-Vis Spectrophotometer (% Transmittance) λ 580 nm. One Ose of standardized microbial culture was inoculated onto TSA media and incubated at 37°C for 24 hours. The growing colonies were suspended in 0.9% physiological NaCl solution until the turbidity reached 1 McFarland. The number of colonies was counted using the Total Plate Count technique. This standardized microbial suspension was inoculated into the sample preparation with a volume according to the required concentration level.

2.6. Method Conformance Test (Optimization)

The suitability test will be conducted for each new product or sample using microbiological methods. This test aims to ensure that the product or sample does not interfere with detecting the analysis results. The test is performed by analyzing negative samples (samples without contamination), positive samples (samples contaminated with *Shigella* sp. standard microbes), and positive controls (solvents without samples contaminated with *Shigella* sp. standard microbes). Positive samples and positive controls are contaminated with a standardized microbial inoculum of no more than 100 cfu and then tested according to the reference analytical method with the shortest incubation period. The positive samples' and controls' test results must be positively detected according to the indicative reaction described in the reference method. The suitability test is declared successful if the positive sample and positive control can grow *Shigella* sp. microbes while the negative sample shows no growth of *Shigella* sp. microbes.

2.7. Measurement or Data Collection

Validation of the *Shigella* test method refers to the microbiology method validation guide (Singapore Accredited Council, 2019). The laboratory must meet acceptance parameters, including 100% sensitivity and specificity and 0% false positive and negative rates. In addition, the method's detection limit (LOD) must be below 100 cfu.

2.7.1. Determination of Limit of Detection

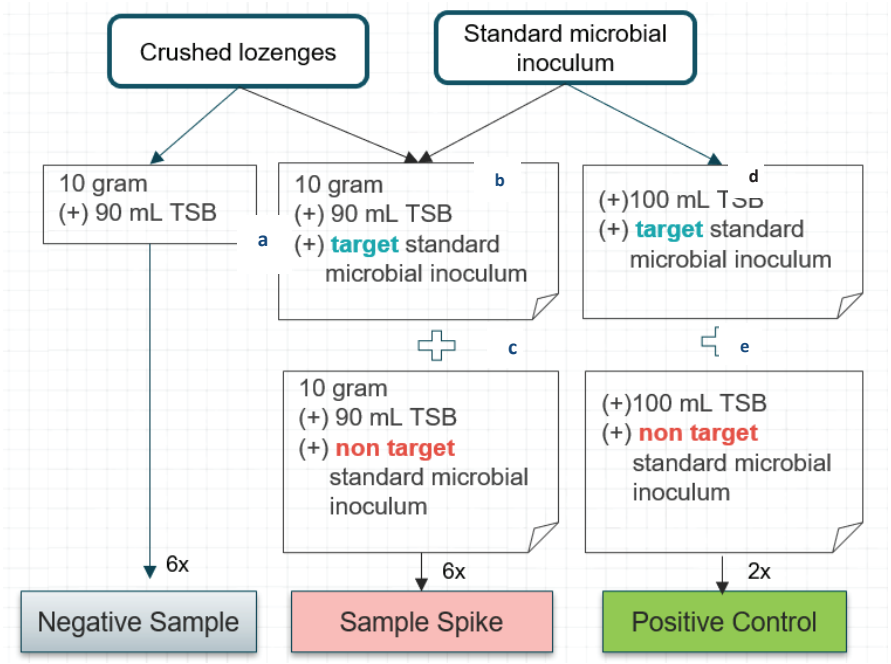
The detection limit was determined by analyzing samples spike at low concentrations of ±1, 3, 9 cfu/g. Each concentration level was tested for 6x replicates using the reference method. The limit of detection was determined by observing the response that gave all positive results from 6x replicates at the slightest concentration. The Singapore Accredited Council validation guidelines, 2019, only mention that it must be detectable below 100 cfu, which means that if the sample weighing is 10 g, the lowest concentration will be below 10 cfu/g. The most minor concentration is 1 cfu/g, while 3 and 9 are multiples of 3.

2.7.2. Determination of Sensitivity, Specificity, False Positive & Negative Rate

Determination of sensitivity, specificity, false positive and false negative rates was carried out according to validation guidelines (Singapore Accredited Council, 2019) by analyzing negative samples, samples spike and positive controls. As shown in Figure 3, the test scheme for sensitivity, specificity, and false positive and false negative rates was carried out by contaminating samples spike and positive controls using microbial inoculum according to the concentration obtained from determining the limit of detection. Samples spike and positive controls were contaminated with three and two non-target microbial strains. Tests were performed using the reference analytical method, with six replicates for samples and two for controls. Negative samples containing only crushed lozenges and diluted with TSB without microbial inoculum were tested six times.

- Negative sample = sample without the addition of any contaminants
- Sample positive spike = sample contaminated with target standard microbes (*Shigella sonnei*, *Shigella dysentriae*, *Shigella flexneri*) and non-target standard microbes (*Escherichia coli*, *Salmonella* Typhimurium)
- Positive control = Solvent media contaminated with target standard microbes (*Shigella sonnei*, *Shigella dysentriae*, *Shigella flexneri*) and non-target standard microbes (*Escherichia coli*, *Salmonella* Typhimurium)

- Negative control = media blank



Description: The difference in treating negative samples, approving samples, and positive controls to determine sensitivity, specificity, and false positive and negative favourable rates lies in the sample homogenization or initial dilution stage.

a. Negative sample = 10 g sample + 90 mL TSB (6x replicates)
b. Target Sample Spike = 10 g sample + 90 mL TSB + target microbial inoculum (*Shigella sonnei*, *Shigella dysentriae*, *Shigella flexneri*) (6x replicates)
c. Non-Target Sample Spike = 10 g sample + 90 mL TSB + non-target microbial inoculum (*Escherichia coli* and *Salmonella* Typhimurium) (6x replicates)
d. Target Positive Control = 100 mL TSB + target microbial inoculum (*Shigella sonnei*, *Shigella dysentriae*, *Shigella flexneri*) (2x replicates)
e. Non-target Positive Control = 100 mL TSB + non-target microbial inoculum (*Escherichia coli* and *Salmonella* Typhimurium) (2x replicates)

Figure 3. Test Scheme of Sensitivity, Specificity, False Positive and Negative Rate

2.8 Data Analysis

Data analysis was performed using descriptive statistical analysis. A descriptive-analytical method is a method that serves to describe or give an overview of the object under study through data or samples that have been collected as is without analyzing and making conclusions that apply to the public (Sugiyono, 2013). Validation categories and calculation formulas refer to Tables 1 and 2 (Singapore Accredited Council, 2019).

Table 1. Validation Categories (Singapore Accredited Council, 2019)

Number of test results	Presumptive (+)	Presumptive (-)	
Confirmed (+)	a	b	a+b
Confirmed (-)	c	d	c+d
	a+c	b+d	n

Description: n = total number of tests
Category a = number of confirmed positive presumptive positives (true positives)
Category b = number of presumptive negatives confirmed positive (false negatives)
Category c = number of presumptive positive confirmed negatives (false positives)
Category d = number of confirmed negative presumptive negatives (true negatives)

After calculating the number of categories a, b, c, and d, input the category using the calculation formula according to Table 2. to determine whether the method validation we have done meets the acceptance requirements.

Table 2. Calculation Formula

Parameter	Calculation Formula
Sensitivity	a/(a+b) x 100%
Specificity	d/(c+d) x 100%
False positive rate	c/(a+c) x 100%
False negative rate	d/(c+d) x 100%

3. Results and Discussion

3.1. Method suitability test (optimization)

The method suitability test is carried out to ensure that no product/sample effect will obscure the analysis results (United States Pharmacopeia Convention, 2022). The results of the method suitability test for *Shigella* detection analysis using TSB solvent media (1:10) with a concentration of 50 cfu/g are presented in Table 3.

Table 1. Method Conformance Test Results

Test Microbe	Inoculum Level	Positive Control	Positive Sample	Negative Sample
Shigella sonnei	50 cfu/g	+	+	-
Shigella flexneri	50 cfu/g	+	+	-
Shigella dysentriae	50 cfu/g	+	+	-

Description:
(+) = there is growth
(-) = no growth

The results of the method suitability test in Table 3. show that all *Shigella* sp. test microbes, namely *Shigella sonnei*, *Shigella flexneri*, and *Shigella dysenteriae* with an inoculum level of 50 cfu/g (below 100 cfu/g), showed growth (+) in the positive control and positive samples. This indicates that the method used can detect the presence of these microbes under the specified conditions.

In the negative sample, there was no microbial growth (-), meaning there was no contamination or unwanted microbial growth under supposedly sterile conditions. This indicates that the test method works well and is reliable in detecting or identifying the tested microbes as intended.

Thus, the analysis of *Shigella* detection in lozenge samples analyzed using the WHO method with the initial solvent TSB (1:10) met the method's suitability requirements without needing prior neutralization/modification procedures for the sample. Microbiological method suitability requirements, as listed in the USP pharmacopoeia guidelines, usually cover several essential aspects, including the ability of the method to detect the growth of target microorganisms under appropriate conditions without interference from external factors such as the physicochemical properties of the sample. In this context, the starting

solvent used was TSB at a ratio of 1:10, a commonly used enrichment medium in microbiology to support the growth of various microorganisms, including *Shigella*.

This method is said to meet the suitability requirements of the technique because:

- Appropriate Growth (Recovery): One of the main criteria of microbiological method validation is the growth of the target microorganism in positive controls and positive samples. In this case, the growth of *Shigella* in both the control and sample indicates that there are no inhibiting factors from the sample that affect the detection results.
- No Neutralization or Modification Required: Usually, in some cases, samples with specific physicochemical properties may inhibit the growth of microorganisms, so neutralization or modification of the procedure is required. However, in this case, the lozenge sample does not require any additional adjustments, which means that the standard method used is optimal for detecting *Shigella*.
- Proven Method Suitability: Validation was performed by ensuring that the method could detect *Shigella* in positive controls (without the influence of the sample) and positive samples (containing both the sample and the target microorganism). The method was qualified without additional modification when consistent growth was observed in both conditions.

Therefore, this validation showed that the WHO method effectively detected *Shigella* in lozenge samples and met the requirements per international standards.

3.2. Limit of Detection (LOD) Determination

A test with a limit of detection was conducted to determine the sensitivity of the test method. The detection limit was determined by analyzing positive samples from various low inoculum concentrations of 1, 3, and 9 cfu per gram. The LOD value was determined based on the lowest concentration of test microbial inoculum in the sample, which could still be detected in all replicates.

Table 2. Detection Limit Determination Test Results

Contaminant Concentration	Number of positive results / Number of tests	Positive Response
Sample + *S.sonnei* 9 cfu/g	6 / 6	100 %
Sample + *S.sonnei* 3 cfu/g	6 / 6	100 %
Sample + *S.sonnei* 1 cfu/g	5 / 6	83,3 %

Based on Table 4, samples contaminated with *Shigella sonnei* at concentrations of 9 and 3 cfu/g showed positive results in all 6 test samples (100%). However, at a concentration of 1 cfu/g, only 5 out of 6 test samples (83.3%) gave positive results, while 1 other sample did not detect the presence of *Shigella* sp.

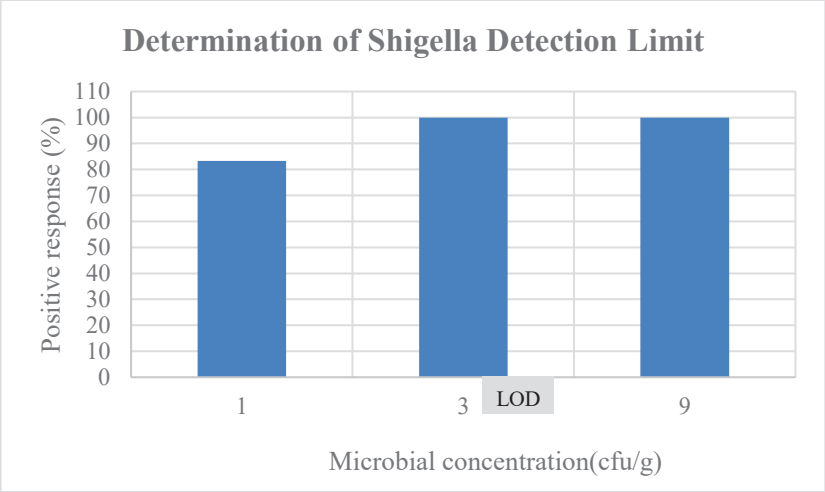


Figure 1. Determination of Limit of Detection

Based on the detection limit concentration determination test (Figure 4), the LOD value of the *Shigella* spp. The detection method was obtained at 3 cfu/g sample. This concentration is the lowest, giving 100% of the test microbes detected. According to the Indonesian Pharmacopeia (2020), microbial detection methods must be able to detect microbes with numbers below 100 colonies per gram of sample. Still, the actual detection limit has never been determined quantitatively because many variables can affect microbial recovery in preparations. The results of the limit of detection determination can be used as a reference for the LOD value for contaminant inoculum concentration in the validation and verification of an analytical method.

3.3.Method Validation Results

Data from the sensitivity, specificity, false positive rate and false negative rate tests on the validation of the *Shigella* detection analysis method are presented in Table 5 and the sum of the results in Table 6.

Table 3. Method Validation Testing Results

Analysis	Repeat	Presumptive	Confirmation	Category
Negative Sample	1	Negative	Negative	d
	2	Negative	Negative	d
	3	Negative	Negative	d
	4	Negative	Negative	d
	5	Negative	Negative	d
	6	Negative	Negative	d
Positive Sample *S.sonnei* (target microbe)	1	Positive	Positive	a
	2	Positive	Positive	a
	3	Positive	Positive	a
	4	Positive	Positive	a
	5	Positive	Positive	a
	6	Positive	Positive	a
Positive Control *S.sonnei* (target microbe)	1	Positive	Positive	a
	2	Positive	Positive	

Analysis	Repeat	Presumptive	Confirmation	Category
				a
Positive Sample *S.flexneri* (target microbe)	1	Positive	Positive	a
	2	Positive	Positive	a
	3	Positive	Positive	a
	4	Positive	Positive	a
	5	Positive	Positive	a
	6	Positive	Positive	a
Positive Control *S. flexneri* (target microbe)	1	Positive	Positive	a
	2	Positive	Positive	a
Positive Sample *S.dysentriae* (target microbe)	1	Positive	Positive	a
	2	Positive	Positive	a
	3	Positive	Positive	a
	4	Positive	Positive	a
	5	Positive	Positive	a
	6	Positive	Positive	a
Positive Control *S.dysentriae* (target microbe)	1	Positive	Positive	a
	2	Positive	Positive	a
Positive Sample *E.coli* (non-target microbes)	1	Negative	Negative	d
	2	Negative	Negative	d
	3	Negative	Negative	d
	4	Negative	Negative	d
	5	Negative	Negative	d
	6	Negative	Negative	d
Positive Control *E.coli* (non-target microbe)	1	Negative	Negative	d
	2	Negative	Negative	d
Positive Sample *S.*Typhimurium (non-target microbe)	1	Negative	Negative	d
	2	Negative	Negative	d
	3	Negative	Negative	d
	4	Negative	Negative	d
	5	Negative	Negative	d
	6	Negative	Negative	d
Positive Control *S.*Typhimurium (non-target microbe)	1	Negative	Negative	d
	2	Negative	Negative	d

Based on Table 5, the presumptive determination of *Shigella* sp. is as follows:

- In negative samples, the presumptive result is negative.
- The presumptive result was positive in positive samples and positive controls contaminated with target microbes (*Shigella sonnei*, *Shigella flexneri* and *Shigella dysentriae*).
- The presumptive results were negative in positive samples and positive controls contaminated with non-target microbes (*E. coli* and *Salmonella* Typhimurium).

Negative samples did not show colony growth, so the confirmation result was negative. In contrast, a complete confirmation test (MCH scratch stain, Gram stain, and automatic identification with VITEK 2) was performed to approve samples and positive controls and

determine whether the colonies growing on MCA and XLD selective media were positive or negative.

After obtaining the presumptive-confirmation test results from each treatment, the results were grouped as follows: Negative presumptive results that were confirmed negative were categorized as "d". Positive presumptive results that were confirmed positive were classified as "a". No test results fell into categories "b" or "c".

Table 4. Number of Validation Test Result Categories

Number of test results	Presumptive (+)	Presumptive (-)	
Confirmed (+)	a = 24	b = 0	24
Confirmed (-)	c = 0	d = 22	22
	24	22	n = 46

After grouping and categorizing the test results, as shown in Table 6. there are 24 for Category A and 22 for Category D, with 46 tests.

Tabel 5. Calculation of Sensitivity, Specificity, False Positive and Negative Rates

Parameter Validation	Calculation	Conclusion
Sensitivity	$\frac{a}{a+b} = \frac{24}{24+0} = 100\ \%$	Qualified
Qualified	$\frac{d}{c+d} = \frac{22}{0+22} = 100\ \%$	Qualified
False Positive Rate	$\frac{c}{a+c} = \frac{0}{24+0} = 0\ \%$	Qualified
False Negative Rate	$\frac{b}{b+d} = \frac{0}{0+22} = 0\ \%$	Qualified

In the calculation based on Table 7. the sensitivity and specificity values obtained the results of 100%. These results indicate that the method used meets the requirements for detecting *Shigella* sp. microbes, specifically in the presence of other components in the sample matrix. According to the Singapore Accredited Council (2019), the difference between sensitivity and specificity is that sensitivity is the ability of the test method to give positive results on samples that have been contaminated with target microbes (*Shigella sonnei*, *Shigella flexneri* and *Shigella dysentriae*) while specificity is the ability of the test method to give negative results on samples that do not contain target microbes or samples contaminated with non-target microbes (*Escherichia coli* and *Salmonella typhimurium*).

The test results show that the false positive and false negative rate is 0%. All samples that should be positive gave positive results, and all samples that should be negative gave negative results. Thus, the *Shigella* spp. The detection method tested using the WHO method is reliable. The false positive rate test was performed to determine the possibility of a negative sample giving a positive result due to the influence of reagents, media used, or contamination due to non-aseptic procedures. Conversely, a false negative test is performed to determine the possibility of a positive sample giving a negative result due to the influence of the sample matrix or reagent (Ismail, 2009).

This method qualifies validation parameters, including sensitivity, specificity, and false positive and negative rates (World Health Organization, 2011). Therefore, the WHO method

is very applicable in testing laboratories throughout the POM Agency. With a sensitivity result of 100% and a detection limit of 3 cfu/g, this method allows for routine analysis.

The WHO analytical method (Quality control methods for herbal materials) for the detection of *Shigella* in quasi-medicinal lozenges that have been validated in this study can be proposed as an analytical method text (MA-PPPOMN) and formalized in the MA-PPPOMN discussion session. This method can then be officially used in all Indonesian FDA laboratories in Indonesia to monitor *Shigella* microbial contamination in quasi-medicines

4. Conclusion

This study aims to validate the method used for *Shigella* detection analysis in quasi-medicinal lozenges using the WHO reference method (Quality control methods for herbal materials). The results showed that the technique had a sensitivity and specificity of 100% and a false positive and false negative rate of 0%. The limit of detection obtained was 3 cfu/g, which meets the acceptability requirements of microbiological analysis methods (below 100 cfu/g). With these validation results, the process is declared accurate and can be applied for routine *Shigella* spp—testing analysis in the Food and Drug Administration laboratories.

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Development and Validation of a Method for Detecting and Quantifying Mitragynine in Kratom Samples Using HPLC-PDA

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ABSTRACT / ABSTRAK

Kratom (Mitragyna speciosa Korth) has been identified as a New Psychoactive Substance (NPS) by the United Nations Office on Drugs and Crime (UNODC) and included in the list of prohibited ingredients in food supplements and traditional medicine by Indonesian FDA. Therefore, a rapid, easy, and reliable analytical method is necessary to detect and quantify this plant and its products. This study developed a method for the detection and quantification of kratom products based on a unique compound, mitragynine, as a biomarker. A previous survey of determining mitragynine in Kratom using GC-MS, LC-MS/MS, UPLC, and HPLC-PDA. Previously, the HPLC-PDA method used a C8 column. Yet, for efficiency, it is also necessary to develop a test method using a C18 column. Analysis was performed using an HPLC - PDA system with Waters Atlantis T3-C18 (250 x 4.6 mm, 5 µm) column. The mobile phase comprises acetonitrile and formic acid 0.05%, pH 5.0 (75:25 v/v), delivered at a 1.0 mL/min flow rate. Detection was carried out at a wavelength of 225 nm. The analytical method was validated with test parameters of selectivity, system suitability, accuracy, precision, linearity, detection limit, and quantification limit. The validation study demonstrated an excellent linear concentration range of 1.96 - 6.01 µg/mL with a correlation coefficient of 0.9996; the detection limit is 0.14 µg/mL, while the limit of quantification is 0.45 µg/mL—accuracy method of 98.88 - 101.44% and a bias of 0.27%. The percent relative standard deviation for six independent assay determinations was 0.67%, and the intermediate precision was 1.56% on two days. The mitragynine amounts in these materials ranged from 6.01 to 6.31 mg/g of dried leaf material. Based on the research results, it can be concluded that the method developed provides quick, simple, reliable, accurate, and valid, and has an advantage over existing methods in terms of simplicity of sample preparation, short analysis time, and cost-effectiveness compared to GCMS and LCMS/MS and can be applied for future analysis in Kratom samples.

Kratom (Mitragyna speciosa Korth) telah ditetapkan sebagai senyawa psikoaktif baru (NPS) oleh United Nations Office on Drugs and Crime (UNODC) dan digolongkan ke dalam daftar bahan terlarang dalam suplemen makanan dan obat tradisional oleh BPOM. Oleh karena itu, diperlukan metode analisis yang cepat, mudah, dan andal untuk mendeteksi dan menetapkan kandungan tanaman tersebut dan produk turunannya. Pada penelitian ini dikembangkan metode untuk mendeteksi dan menetapkan kadar produk kratom berdasarkan senyawa unik, mitragynine sebagai biomarker. Penelitian sebelumnya yang telah dikembangkan menggunakan GC-MS, LC-MS/MS, UPLC dan HPLC-PDA. Metode HPLC-PDA yang tersedia menggunakan kolom C8, namun untuk efisiensi, perlu juga dikembangkan metode pengujian dengan menggunakan kolom C18. Analisis dilakukan menggunakan sistem KCKT - PDA yang dilengkapi dengan kolom Waters Atlantis T3-C18 (250 x 4,6 mm, 5µm). Fase gerak terdiri dari asetonitril dan asam

format 0,05%, pH 5,0 (75:25 v/v) dengan laju alir 1,0 mL/menit. Deteksi dilakukan pada panjang gelombang 225 nm. Validasi metode ditunjukkan dengan parameter uji: selektifitas, akurasi, presisi, linieritas, batas deteksi dan batas kuantifikasi. Metode identifikasi dan penetapan kadar mitragynine secara KCKT-PDA menunjukkan hasil uji linier pada rentang konsentrasi 1,96 - 6,01 µg/mL dengan koefisien korelasi 0,9996. Batas deteksi metode yaitu 0,14 µg/mL, sedangkan batas kuantifikasi 0,45 µg/mL. Uji akurasi metode adalah 98,88 - 101,44% dan bias 0,27%. Presisi metode dan intermediet secara berturut-turut yaitu adalah 0,67% dan 1,56%. Kandungan mitragynine berkisar antara 6,01 hingga 6,31 mg/g bahan daun kering. Berdasarkan hasil penelitian, dapat disimpulkan bahwa metode yang dikembangkan cepat, sederhana, andal, akurat, dan valid serta memiliki keunggulan dibandingkan metode yang ada dalam hal kesederhanaan persiapan sampel, waktu analisis yang singkat, dan efektivitas biaya dibandingkan dengan GCMS dan LCMS/MS dan dapat diaplikasikan untuk analisis sampel Kratom.

Keywords: Kratom, Mitragynine, Mitragyna speciosa, Detection, Quantification, HPLC-PDA
Kata kunci: Kratom, Mitragynine, Mitragyna speciosa, deteksi, kuantifikasi, KCKT-PDA

1. Introduction

Kratom (*Mitragyna speciosa* Korth) is a plant of the Rubiaceae family from Southeast Asia (Muang Thai, Indonesia, Malaysia, Myanmar, Philippines) and Papua New Guinea. It is a Putussibau, Kapuas Hulu, and West Kalimantan herb characteristic. The leaves are most widely used in Kratom, and it is consumed by chewing, smoking, and boiling like tea. (Sanagi et al., 2013).

Figure 1. Kratom plant (a). Kratom leaves, (b) and (c). Kratom tree

Kratom has been identified as a New Psychoactive Substance (NPS) by the United Nations Office on Drugs and Crime (UNODC) in the World Drug Report since 2013 (UNODC, 2013). Accordingly, the Indonesian Food and Drug Authority (FDA) issued an Announcement Letter Number. HK. 04.4.42.421.09.16.1740 concerning the Prohibition of Mitragyna speciosa (Kratom) in Traditional Medicines and Health Supplements. This is according to the Decree of the Head of the Indonesian FDA Number HK.00.05.23.3644 Appendix 3 concerning Basic Provisions for Supervision of Food Supplements and Regulation of the Head of Indonesian FDA Number HK.00.05.41.1384 Attachment 4, "Criteria and Procedure for Registration of Traditional Medicines, Standardized Herbal Medicines, and Phytopharmaca." It was stated that Mitragyna speciosa (Kratom) is included in the list of prohibited ingredients in food supplements and traditional medicine (BPOM, 2005a, 2005b, 2016). However, the National Narcotics Board granted a transition period

until 2024 to classify Kratom into the Class I Narcotic List published by the Ministry of Health (BNN, 2020).

Kratom can be obtained easily in online shops with low prices; therefore, Kratom and its product are abused as alternatives to other narcotics like heroin or marijuana. (Elsa, 2016). The pharmacological effects were reported as pain relievers. (Carpenter et al., 2016; Reanmongkol et al., 2007; Shamima et al., 2012), sedative (Novindriani, 2014), stimulant, antidepressant (Moklas et al., 2008), anti-inflammatory (Tohar et al., 2019), antidiarrheal (Suhaimi, S.; Kartikasari, 2020), antioxidant, and antibacterial (Parthasarathy et al., 2009). Kratom contains alkaloid indole, mitragynine, and 7-hydroxy mitragynine with antinociceptive pharmacological properties. (Shamima et al., 2012). However, some adverse effects associated with the exposure have been reported, such as psychosis, seizures, intrahepatic cholestasis, other medical problems, and fatalities. (Fluyau & Revadigar, 2017). Therefore, a rapid, easy, and reliable analytical method is necessary to detect and quantify this plant and its products.

Mitragynine (Fig. 2), a major alkaloid kratom, binds to the μ-opioid and δ-opioid receptors, while 7-hydroxy mitragynine has a stronger affinity to μ-opioid receptor than other receptors (Matsumoto, 2006). Mitragynine showed 16 times more affinity for μ-opioid and δ-opioid receptors and 200 times less affinity than morphine (Yue et al., 2018), resulting in analgesic opioid activity for both mitragynine and 7-hydroxy mitragynine (Swogger & Walsh, 2018). Mitragynine has stimulant effects at low doses and sedative effects at high doses (Swogger & Walsh, 2018), and it is not found in other plants (Kikura-Hanajiri et al., 2009). This compound is exclusively found in M. speciosa but not in any other genus of Mitragyna (Lesiak et al., 2014; Sanagi et al., 2013). Therefore, it may be used as a marker compound for identifying Kratom.

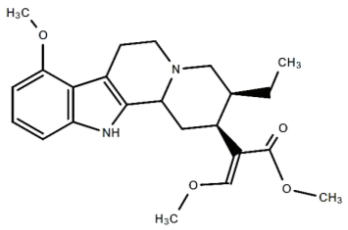


Figure 2. Structure molecule of mitragynine

Some methods for analyzing mitragynine in kratom plant material have been reported. (Elsa, 2016; Flores-Bocanegra et al., 2020; Kikura-Hanajiri et al., 2009; Lelono et al., 2021; Lesiak et al., 2014; Mudge & Brown, 2017; Parthasarathy et al., 2013). Lelono et al. Identified mitragynine in kratom leaves by TLC, GCMS, and LCMS/MS. Mudge, E. M., and Brown determined mitragynine in Mitragyna speciosa raw materials and finished products by liquid chromatography and UV detection. (Mudge & Brown, 2017). Furthermore, Casey et al. conducted a quantitative and qualitative analysis of mitragynine in Kratom (Mitragyna speciosa) by GC-MS, LC-MS/MS, and UPLC-PDA. Mitragynine in kratom products was quantitated by UPLC and LCMS/MS using a mobile gradient phase of 0.1% aqueous formic acid and acetonitrile. Parthasarathy et al. developed and validated an analytical method using an HPLC-PDA system with Inertsil C8 (150 x 4.6 mm, 5 μm) as the column and a mix of acetonitrile and formic acid, 50:50 (v/v), as the mobile phase. Analysis with the HPLC method is more straightforward, sensitive, and inexpensive than GCMS, LC-

MS/MS, and UPLC. Previous studies by HPLC have been limited to using C8 columns, so it is necessary to research different types of columns to provide alternative columns. Considering this, this study aims to develop and validate a method for identifying and quantifying the amount of mitragynine in Kratom leaves using HPLC - PDA, which is faster, more inexpensive, and more sensitive but remains accurate, specific, and selective. Different types of columns were also used to add alternative column usage. This method was validated on the test parameters of selectivity, system suitability, accuracy, precision, linearity, detection limit, quantification limit, robustness, and stability study according to ICH guidelines.

2. Methodology

2.1. Plant Material

Dried leaves of M. speciosa Korth. (Rubiaceae) were collected from Putussibau, Kapuas Hulu, West Kalimantan Province, Indonesia, in December 2020.

2.2. Chemicals

Mitragynine standard Supelco and 7-Hydroxymitragynine standard Supelco were purchased from Cerriliant Corporation (USA), reagents for analysis: methanol, chloroform, n-hexane, ethyl acetate, ammonia 25%, methanol gradient grade for LC, acetonitrile gradient grade LC, formic acid 98-100%, sodium hydroxide solution 1 N (Merck, German), water for HPLC was prepared using Milli-Q water purifier system (Merck, German), TLC, HPTLC silica gel 60 F_{254}, Silica Gel 60 (0.063 – 0.200 mm) for column chromatography (Merck, German), membrane filter PTFE 0.45 µm Agilent (USA).

2.3. Chromatographic conditions

The method was developed on a Shimadzu LC-20AD Prominence HPLC system coupled to a photodiode array detector (Shimadzu, Japan). Chromatographic separation was achieved at 40°C on a Waters Atlantis T3-C18 column (250 x 4.6 mm, 5 µm) (Waters, MA, USA). The mobile phase was acetonitrile and 0.05% formic acid (adjusted to pH 5 with NaOH 1 N), 75:25 (v/v), running in an isocratic mode at a 1.0 mL/min flow rate. The volume injection of the sample was 20 µL, and the total analytical run time was 8 min with mitragynine eluting at 6.2 min. Detection was carried out at 200 - 400 nm, while the UV signal at 225 nm was extracted for quantification. Mitragynine identification compares the retention time of the HPLC and the UV spectrum of the analyte with that of the mitragynine standard. Peak purity was obtained by PDA chromatogram.

2.4. Validation of the HPLC method

This method was validated according to ICH guidelines on the test parameters of selectivity, system suitability, accuracy, precision, linearity, detection limit, quantification limit, and robustness.

A blank solution was methanol HPLC grade. Subsequently, 100 µg/mL of mitragynine-certified standard reference solution in methanol and 100 µg/mL of 7-hydroxy mitragynine-certified standard reference solution in methanol. The specificity solution was a mixture of 7-hydroxy mitragynine and mitragynine standard in methanol (4.0 µg/mL). Working standard solutions of 2.0, 3.0, 4.0, 5.0, and 6.0 µg/mL mitragynine standard were prepared. A calibration curve was created on each day of analysis. Six replicates of mitragynine isolate were analyzed on two consecutive days to evaluate the within-day and between-day precision. Accuracy was assessed as trueness (% bias) between the content of mitragynine

standard quantified with calibration curve and at the certificate. Recovery was performed by spiking the standard to sample with a ratio (30:70) at three-level concentrations. Accuracy and recovery were calculated using Eq. (1-3) below.

$$\%\ accuracy\ (\%\ bias) = \frac{K_b - K_d}{Kb}\ x\ 100\% \quad (1)$$

$$K_d = \frac{a}{b}\ x\ 100\% \quad (2)$$

where :
$a = calculated\ concentration\ based\ on\ calibration\ curve$
$b\ = theoretical\ concentration$
$K_b = content\ at\ certificate$
$K_d = calculated\ content$

$$\%\ recovery = \frac{total\ concentration\ of\ mitragynine - \ concentration\ of\ spike\ sample}{concentration\ of\ standard}\ x\ 100\% \quad (3)$$

The robustness method was used by quantifying mitragynine at varying flow rates (0.8 mL/min, 1.0 mL/min, and 1.2 mL/min) and wavelengths (223 nm, 225 nm, and 227 nm). The data were analyzed using a single ANOVA factor.

2.5. Preparation and analysis of M. speciosa samples

The analytical method was developed and applied with authentic samples from *M. speciosa*. The dried leaves of *Mitragyna speciosa* Korth (400 g) were powdered and extracted sequentially using n-hexane, chloroform, and methanol corresponding to the Mustafa et al. method. In the current study, the ratio of solid to solvent is 1:10. For each solvent, extraction assisted ultrasonication for 10 minutes and extraction for 2 hours at room temperature with an orbital shaker. The methanol extract was concentrated with a rotary evaporator to yield the residue (67.94 g). In addition, the methanol extract (2 g) was subjected to column chromatography using silica gel 60 (0.063 - 0.200 mm) eluting with n-hexane - ethyl acetate - ammonia 25% (30:15:1) to yield fraction A-G. Fraction G was separated by HPTLC silica gel 60 F 254 (n-hexane - ethyl acetate - ammonia 25% (30:15:1)) to obtain isolate (8.10 mg).

The samples of kratom were prepared by sequential extraction according to the preceding method. First, the methanol extract (50 mg) was dissolved in 25.0 mL methanol with sonication assistance for 2 minutes. Next, 0.5 mL of the solution was diluted in 10.0 mL of methanol and filtered through a PTFE syringe filter (Agilent, USA). Finally, samples were injected into an HPLC system. Mitragynine content was quantified by HPLC-PDA based on the calibration curve of mitragynine standard and isolate. Data results were compared statistically with Anova's single factor.

2.6. Stability of mitragynine

The short-term stability of mitragynine at a concentration of 4 µg/mL in methanol was evaluated at room temperature (24 - 26°C) during 0 - 72 hours.

3. Result and Discussion

3.1. Optimisation of the chromatographic conditions

Some methods for analyzing mitragynine in kratom plant material using TLC, GCMS, LCMS/MS, HPLC, and UPLC have been reported. (Elsa, 2016; Flores-Bocanegra et al., 2020; Kikura-Hanajiri et al., 2009; Lelono et al., 2021; Lesiak et al., 2014; Mudge & Brown, 2017; Parthasarathy et al., 2013). Analysis with the HPLC method is a simple, fast, easy-to-use, selective, accurate, and cost-effective method for testing Mitragynine. (Janchawee et al., 2007; Mudge & Brown, 2017; Parthasarathy et al., 2013)Parthasarathy et al. developed and validated an analytical method using an HPLC-PDA system with Inertsil C8 (150 x 4.6 mm, 5 µm) as the column. Therefore, this study chose HPLC-PDA.

Previous study (Janchawee et al., 2007) The characteristics of mitragynine were reported in various chromatographic conditions, particularly in the mobile phase selection. With >80% methanol in the mobile phase, mitragynine elutes quickly but interferes with other peaks in the sample. With methanol composition < 80%, mitragynine was well separated from the polar interferences, but broader peaks were observed. The use of a short column (150 mm) resulted in a run time of <10 min but poor resolution; as such, the analysis was carried out using a long column (250 mm) with a total run time of 30 min.

In this study, various HPLC column and mobile phase systems were assessed. Among the chromatographic columns evaluated, i.e., Sunfire C18, 4.6 mm x 250 mm, 5 µm (Waters, MA, USA), Symmetry C8, 4.6 mm x 150 mm, 5 µm (Waters, MA, USA) and Atlantis T3 C18, 4.6 mm x 250 mm, 5 µm (Waters, MA, USA). With Sunfire C18, mitragynine was eluted rapidly and not well separated from the solvent peak. Furthermore, symmetry C8 can separate mitragynine from other peaks despite poor resolution and broader peaks. Atlantis T3 C18 provided the best chromatography separation and shape of mitragynine. Several mobile phase compositions of acetonitrile with 0.05% formic acid (adjusted to pH 5 with NaOH) were also evaluated, i.e., ACN - 0.05% formic acid 50:50 (v/v), 65:35 (v/v), 70:30 (v/v), and 75:25 (v/v). The results indicated that the optimal mobile phase was ACN - 0.05% formic acid, 75:25 (v/v). It gave the best resolution and peak shape, while the total run time was 8 minutes. So, the column used in the study, T3 C18, differs from previous studies by Janchawee et al., 2007; Mudge & Brown, 2017; Parthasarathy et al., 2013. This aims to provide another alternative column that also has good performance.

3.2. Method validation

The method was selective and specific, in which the chromatographic peak of mitragynine was free of interferences originating from the sample matrix and blank. This was confirmed through the PDA chromatogram's peak purity curve, which evaluates the consistency of UV spectra at the start and end peaks. The purity curve shows that impurity was not detected with a peak purity index of 1.000000 for 7-hydroxy mitragynine (Fig. 3) and mitragynine (Fig. 4). Therefore, there are no interferences at retention time for both analytes. Furthermore, their peak profile was similar for 7-hydroxy mitragynine and mitragynine.

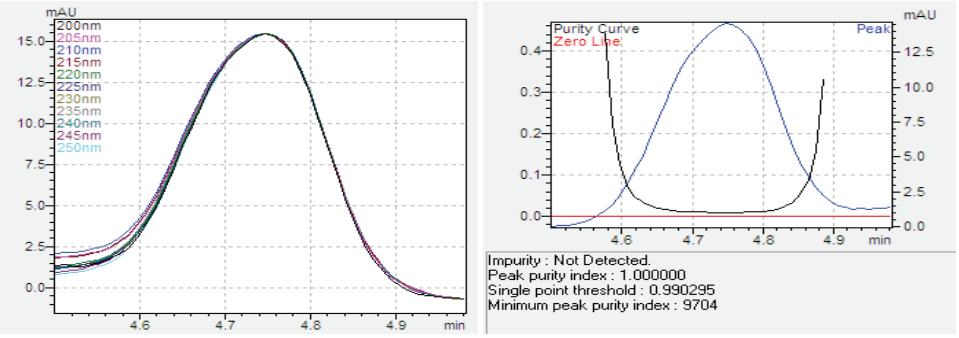


Figure 3. Purity curve and peak profile 7-hydroxy mitragynine at a variable wavelength

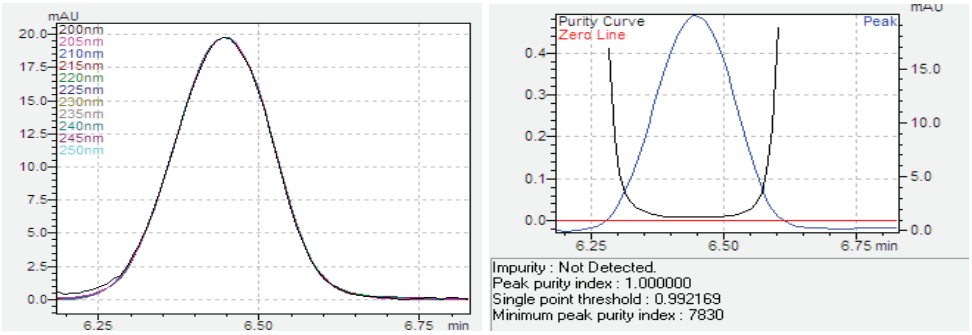
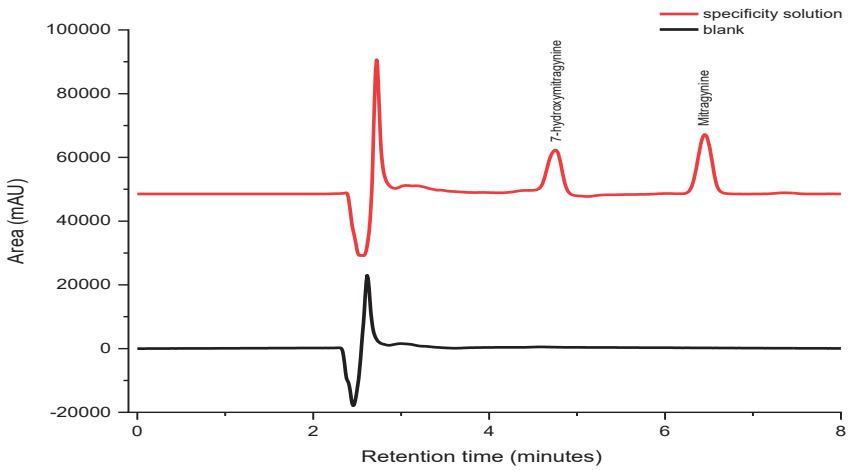


Figure 4. Purity curve and peak profile mitragynine at a variable wavelength

In Fig. 5, blank, 7-hydroxy mitragynine, and mitragynine peaks were well separated with a retention time of 2.5 minutes, 4.74 minutes, and 6.44 minutes, respectively. This method produced the best resolution between 7-hydroxy mitragynine and mitragynine at 5.58. In previous studies, hydroxy mitragynine was not used to see whether the separation conditions were good. This means that the method demonstrated its best ability to separate 7-hydroxy mitragynine, mitragynine, and other alkaloids in kratom samples. Moreover, the short analytical run time in this study is an advantage for routine detection and quantification of mitragynine in kratom products.



Peak	Retention time (minute)	Area	Tailing factor	Theoretical plate	Resolution
7-hydroxy mitragynine	4.744	151727	0.928	3903	--
Mitragynine	6.439	206295	1.011	7213	5.58

Figure 5. Blank, 7-hydroxy mitragynine, and mitragynine chromatogram

Identification was carried out by comparing the UV spectrum of the analyte with that of the mitragynine standard within 210 - 400 nm (Fig. 6). A representative chromatogram of the standard and samples is given in Fig. 11. The calibration curve of mitragynine was found to be linear over the concentration range of 1.959 - 6.010 µg/mL with a mean equation of y = 40,933.2072x + 3,747.2000 with R^2 of 0.9992 and Vx_0 value of 1.4%. The calculated lower limit of detection and quantitation were 0.14 µg/mL and 0.45 µg/mL, respectively. The sensitivity was higher than the previously reported methods. (Fowble & Musah, 2019; Parthasarathy et al., 2013) To analyze mitragynine in Kratom and its products. The range and limit of quantification were satisfactory for quantifying the level of mitragynine in the isolate and kratom extract. The precision and suitability system of the method was satisfied with the result in Table 1.

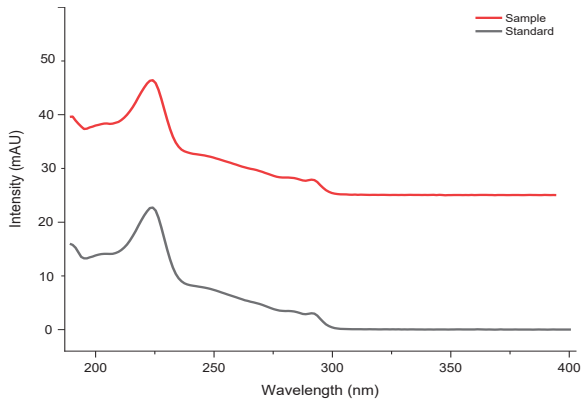


Figure 6. UV spectrum of standard and analyte in kratom sample which have a retention time that corresponds to mitragynine peak

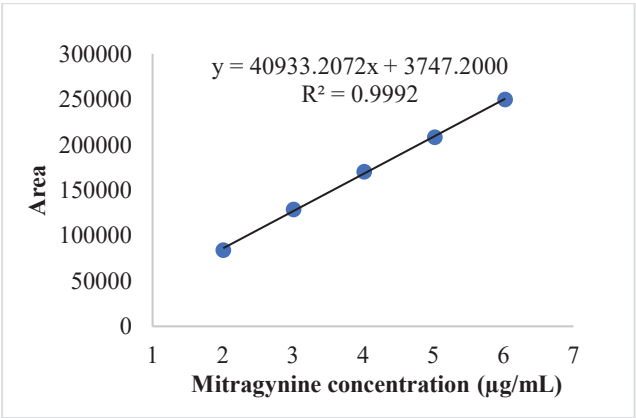


Figure 7. Linearity curve of standard mitragynine and sample (30:70)

Resolution between peaks due to 7-hydroxy mitragynine and mitragynine of 5.58 was well apart. The RSD of retention time and area at the system suitability solution was less than 2%. Repeatability for six determinations and intermediate precision were 0.69% and 1.57% at two consecutive days, respectively. The injection was replicated to ensure that the measured area or retention time was correct since the analysis results were valid. (USP, 2020). Suppose repeated injections produce variable area and retention time and do not meet the requirements; the resulting peak area of the chromatogram may be inappropriate, and the analysis will result in invalid data. The tailing factor and theoretical plate of the method also met the criteria.

Table 1. Precision and suitability system

Parameter	Criteria (USP, 2020)	Result	
		7-Hydroxymitragynine	Mitragynine
Resolution between 7-Hydroxymitragynine and Mitragynine	≥ 1.5	-	5.58
% RSD of retention time	≤ 2%	0.11	0.14
% RSD area	≤ 2%	0.31%	0.09%
Tailing factor	≤ 2	0.93	1.01
Theoretical plates	≥ 2000	3903	7213
Repeatability (n = 6)	≤ 2%	-	0.69% and 1.27%
Intermediate precision (2 days consecutive)	≤ 2%	-	1.54 %

This study's accuracy was measured by recovering the mitragynine standard in the spiked sample (30:70) and calculating the percent bias of the mitragynine standard using a calibration curve and certificate. The recovered mitragynine standard in the spiked sample ranged from 99.54% to 100.54%, and the percent bias (accuracy) was 0.27%. The percentage recovery and bias requirements were 98-102% and did not exceed 2%, respectively. (Ahuja, 2005; AOAC, 2002). It indicates that the methodology was accurate and valid for quantifying mitragynine in the kratom sample.

Table 2. Accuracy and recovery of mitragynine

Concentration (µg/mL)	% Recovery
3	99.64
4	100.54
5	99.79
Mean	99.49
% RSD	0.48
Bias (%)	0.27

In this study, robustness was in dire straits of analytical technique capability to stay unaffected. However, deliberate variations in technique parameters show its responsibility throughout normal usage. (ICH, 2022). Parameters were used, such as flow rate variation (0.8 mL/min, 1.0 mL/min, and 1.2 mL/min) and wavelength (223 nm, 225 nm, and 227 nm). The data were statistically analyzed using the Anova single factor. Robustness results are shown in Fig. 8 and Table 3 since Fcal < F crit was provided. This signifies that the change in flow rate and wavelength on mitragynine quantification using HPLC-PDA did not

significantly affect the assay of mitragynine. The analytical method for the quantification of mitragynine was robust.

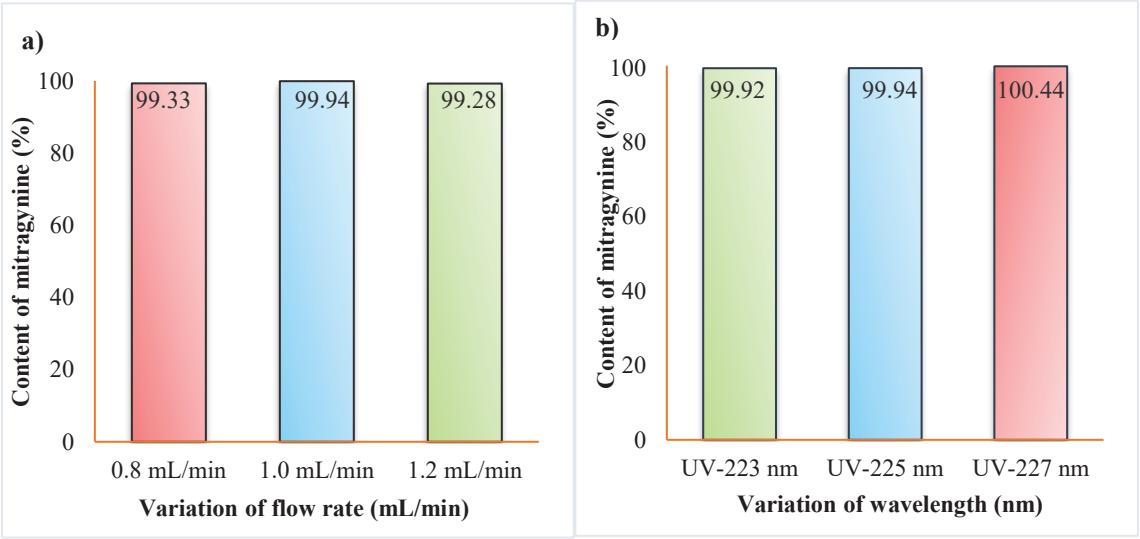


Figure 8. Robustness mitragynine analytical method with a). variable of flow rate; b). variable of wavelength

Table 3. *Anova single factor* robustness for quantification of mitragynine

Parameter	Fcalculated	Frit	P-value
Flow rate (±0.2 mL/min)	0.436149	5.143253	0.6655
Wavelength (±2 nm)	0.595003	5.143253	0.5811

3.3. Stability study of mitragynine

The result of the short-term stability study showed that mitragynine was stable in methanol at room temperature (24 - 26ºC) over 54 hours (Fig. 9). Statistical analysis with a single Anova factor demonstrated Fcal < Fcrit (2.260 < 2.604) for the 1 – 54-hour stability study. However, after 54 hours, the concentration of mitragynine decreased, resulting in stability tests of over 60 hours, Fcal > Fcrit (2.672 > 2.507).

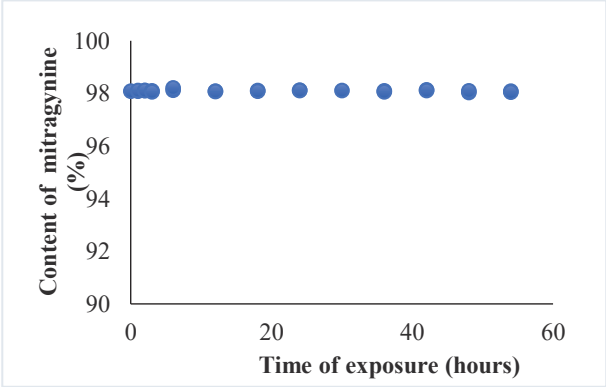


Figure 9. Short-term stability study of mitragynine in methanol solution

A long-term stability study of mitragynine in methanol solution at four ºC was reported by Parthasarathy et al., where mitragynine was stable over one month (Parthasarathy et al., 2013).

3.4. Sample analysis

This method was used to determine and quantify mitragynine in the kratom samples obtained from Putussibau, Kapuas Hulu, West Kalimantan Province, Indonesia, in 2020. Assay of mitragynine in Kratom using mitragynine standard and isolate. Fig. 10 shows a similar slope for both calibration curves. This means the calibration curve between the standard and the isolate was comparable. Analytical statistics using Anova single factor was obtained for Fcal < Fcrit (1.302 < 7.709) and a P-value of 0.318 > 0.05. Therefore, the mitragynine content in kratom material using standards and isolates was not significantly different.

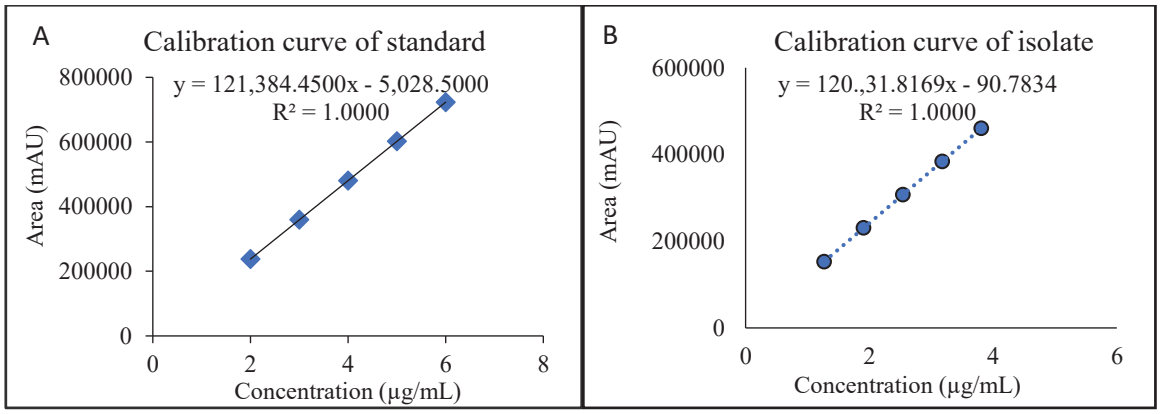


Figure 10. Calibration curve of standard (A) and isolate mitragynine (B)

The result (Table 4) showed that mitragynine content of 6.01 - 6.31 mg/g corresponded to the Prozialeck et al. (2020) study, where the content in kratom products and their derivatives varies in the range of 3.9 - 62.1 mg/g (Prozialeck et al., 2020) And commercial kratom product extracted by ultrasonication was 0.8 - 62.6 mg/g (Kikura-Hanajiri et al., 2009). The mitragynine content obtained in this study was lower than in previous studies because the samples used were in dried leaf form.

Table 4. Mitragynine content in kratom samples

Kratom sample	Mitragynine standard		Isolate	
	Mitragynine concentration (µg/mL)	Amount of mitragynine (mg/g)	Mitragynine concentration (µg/mL)	Amount of mitragynine (mg/g)
1	3.59	6.12	3.57	6.01
2	4.15	6.19	4.13	6.10
3	4.56	6.31	4.54	6.22

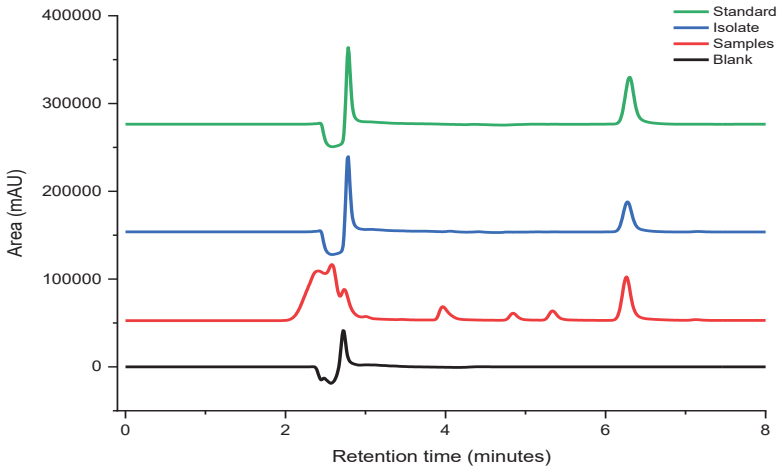


Figure 11. HPLC chromatogram, obtained on an Atlantis T3 C18 column (250 x 4,6 mm; 5µm) using a mobile phase of ACN – formic acid 0.05% pH 5.0 (75:25 v/v)

4. Conclusion

A simple, quick, and reliable HPLC-PDA method has been developed and validated to identify and quantify mitragynine in Mitragyna speciosa and its products. This method has an advantage over existing methods in terms of simplicity of sample preparation, short analysis time, and cost-effectiveness compared to GCMS and LCMS/MS. Therefore, it is suitable for routine screening of M. speciosa products in the market, especially Kratom materials. The amount of mitragynine in the Kratom samples obtained ranged from 6.01 to 6.31 mg/g.

Acknowledgments

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Public Engagement on the Instagram Social Media Account Format of Indonesian Food and Drug Authority Regional Office in Ambon in 2023

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ABSTRACT / ABSTRAK

Public awareness regarding the importance of safe drugs and food is crucial. However, products still have the potential to be unsafe, so the community needs to be equipped with knowledge through Information, Education, and Communication (IEC). Due to advances in information technology and the increasing number of internet users, IEC can be conducted through electronic and social media to reach a wider audience. Instagram is one of the platforms used by the Indonesian Food and Drug Authority (Indonesian FDA) Regional Office in Ambon for IEC. It has the potential to serve as a medium for communicating, disseminating information, and educating the public about safe drugs and food. However, an analysis of Instagram user engagement with the content created is needed. This study aims to determine Instagram user engagement with content formats on the Indonesian FDA Regional Office in Ambon. Instagram account using quantitative research methods with a descriptive approach. The research-dependent variable is the content format (video, photo, infographic), and the independent variable is engagement (like, comment, save, share). Engagement analysis was conducted on 509 posts during 2023, consisting of 51 (10.02%) video content, 174 (34.19%) photos, and 284 (55.80%) infographics. The results of the analysis show that photo content formats have higher engagement than videos and infographics, with an average number of likes (25.03), saves (0.29), and shares (0.69). Meanwhile, video content formats have a higher average number of comments (0.14) compared to photos and infographics. This study concludes that photo and video content have higher engagement, so it can be chosen as priority content to increase follower engagement on the Indonesian FDA Regional Office in Ambon Instagram account.

Kesadaran masyarakat tentang pentingnya obat dan makanan yang aman sangatlah vital. Namun produk yang beredar masih memiliki potensi untuk tidak aman, sehingga masyarakat perlu dibekali pengetahuan tentang produk yang aman melalui Komunikasi, Informasi, dan Edukasi (KIE). Sering kemajuan teknologi informasi dan peningkatan jumlah pengguna internet, KIE dapat dilakukan melalui media elektronik dan media sosial untuk menjangkau audiens yang lebih luas. Salah satu media KIE yang digunakan oleh Balai Pengawas Obat dan Makanan (Balai POM) di Ambon adalah melalui Instagram. Instagram berpotensi menjadi media untuk berkomunikasi, menyebarkan informasi, dan mengedukasi masyarakat tentang obat dan makanan aman. Namun diperlukan analisis terhadap keterlibatan pengguna Instagram (engagement) terhadap format konten yang dibuat. Penelitian ini bertujuan untuk mengetahui engagement pengguna Instagram terhadap format konten di akun Instagram Balai POM di

Ambon menggunakan metode penelitian kuantitatif dengan pendekatan deskriptif. Variabel terikat penelitian adalah format konten (video, foto, infografis) dan variabel bebas adalah engagement (like, comment, save, share). Analisis engagement dilakukan terhadap 509 postingan selama tahun 2023, terdiri dari 51 (10,02%) format konten video, 174 (34,19%) foto, dan 284 (55,80%) infografis. Hasil analisis menunjukkan format konten foto memiliki engagement lebih tinggi dibandingan video dan infografis dengan rata-rata jumlah like (25,03), save (0,29), dan share (0,69). Sedangkan format konten video memiliki rata-rata jumlah comment lebih tinggi dibandingkan foto dan infografis sebanyak 0,14. Kesimpulan penelitian ini bahwa konten foto dan video memiliki engagement lebih tinggi sehingga bisa dipilih sebagai konten prioritas untuk meningkatkan engagement pengikut di akun Instagram Balai POM di Ambon.

Keywords: Engagement, Instagram, Content Format, Indonesian FDA Regional Office in Ambon, IEC
Kata Kunci: *Keterlibatan, Instagram, format konten, Balai POM di Ambon, KIE*

1. Introduction

Public awareness of safe drugs and food must be realized because the products in distribution lines are still potentially unsafe, so the public is required to be smart in choosing and using drugs and food that are safe, quality, beneficial. As consumers, the public is expected to select and use drugs and food that meet established standards, necessitating education through Information, Education, and Communication (IEC). (Food and Drug Authority, 2020)

Advances in information technology and the increase in internet users allow IEC can be conducted through electronic and social media, reaching broader audiences In 2024, the number of internet users in Indonesian reached 221,563,479 out of a total population of 278,696,200 Indonesians in 2023, with an internet penetration rate of 79.5% (APPJI, 2024). Although the internet penetration rate in Maluku-Papua of 69.91% (Katadata, 2024), which is below the national average, it is still substantial and is expected to grow.

One of the IEC media used by Indonesian FDA Regional Office in Ambon is Instagram, which expands public exposure to information about safe food and drug information due to the limitations of offline IEC related to resources (budget, time, and human resources). Additionally, Indonesian FDA Regional Office in Ambon used Instagram to promote its profile and to highlight special activities unrelated to drugs and food in collaboration with various sectors. Instagram is the second-largest social media platform used in Indonesia, with the country ranking fourth globally for Instagram users at 89.15 million (Emeilia et al., 2024).

Instagram can potentially be used as a medium for communication, disseminating information, and public education about safe drugs and food to the public. However, it is necessary to analyze user engagement on Instagram in terms of the content formats created. To provide targeted content, it is essential to study which content formats (videos, photos, and infographics) have the highest levels of effectiveness to determine priority formats.

Research by Wijayanti (2022) on user engagement with library Instagram accounts during COVID-19 measured engagement based on likes and comments from each post. This study found that photo content received more likes and comments than video content. In another study by Hellberg (2015) on visual brand communication on Instagram, defining engagement as user interaction through likes, comments, shares and follows. The results

indicated that visual content that personally engages users can evoke emotions, inspire, or have a strong appeal, resulting in more positive response from Instagram users. Another study by Bonilla-Quanja et al. (2023) on consumer engagement on Instagram with popular fashion brands based on likes and comments states that content in which products are displayed (photos) has higher engagement than video content formats.

The Indonesian Food and Drug Authority (Badan POM) promotes its programs through Instagram due to its potential to reach a wider community. However, this potential must be studied in terms of the effectiveness of Instagram in attracting user interest in the posted content by measuring engagement. To date, in Indonesia, there has been no research that specifically examining engagement on the Instagram account of the Badan POM RI or Indonesian FDA Regional Office in Ambon (id@bpom.ambon) to asses interest in the posted content format. The research aims to determine what content formats—videos, photos, or infographics—are more populars on the Indonesian FDA Regional Office in Ambon Instagram account, based on likes, comments, saves, and shares.

This research is preliminary and seeks to understand Instagram user engagement with the content format of the Indonesian FDA Regional Office in Ambon Instagram account. The results will help identify content formats that can be prioritized to enhance both quantity and quality, ensuring that the objectives of IEC (Educational Information Communication) through Instagram effectively conveyed and easily understood by the public.

2. Methodology

This research was conducted on the Instagram social media account of Indonesian FDA Regional Office in Ambon with the identifier @bpom.ambon with 4,480 followers (2023). The study employs a quantitative research design with a descriptive approach. The variables in this study are content format (dependent variable) and engagement (independent variable). The posted content is categorized according to the content format: videos, photos, and infographics. The video format includes IEC content, documentation of activities, and other content produced in video form. The photo format consists of content containing documentation of activities. The infographic format presents IEC content by combining image illustrations with descriptive narratives.

Engagement analysis was conducted by calculating the number of likes, comments, shares, and saves on c a total of 509 content posts during 2023, comprising 51 videos posts, 174 photos, and 284 infographics. Data collection was performed using Instagram insight, following the monitoring and evaluation procedures established by Badan POM RI. The data were processed using descriptive analysis, and the results were analyzed using SPSS Statistics 29.0.2.0 (IBM) software.

3. Results and Discussion

The results of the data collection from the insights of the Indonesian FDA Regional Office in Ambon Instagram account are presented in Table 1.

Engagement analysis was conducted on the Instagram account of Indonesian FDA Regional Office in Ambon, examining 509 posts from January to December 2023. This comprised 51 (10.02%) video posts, 174 (34.19%) photo posts, and 284 (55.80%) infographic posts (see Table 1). The lower number of video posts can be attributed to the significant challenges involved in their production compared to photo and infographic content. Additionally, the primary responsibilities of the Indonesian FDA Regional Office

in Ambon social media manager limited the time available for creating video content. The volume of photo posts reflects the numerous activities related to Indonesian FDA Regional Office in Ambon that require public awareness, while the higher number of infographic posts is due to their flexibility, depending on the information conveyed and the creativity of the Instagram account manager. Each month, the CIE theme established by the central administration facilitates the creation of infographic content for the Indonesian FDA Regional Office in Ambon social media manager. Overall, the 509 posts garnered an average of 16.60 likes, 0.10 comments, 0.24 saves, and 0.99 shares.

Table 1. Recapitulation of Indonesian FDA Regional Office in Ambon Instagram content data in 2023

Content Format	Total	*Like*	*Comment*	*Save*	*Share*
Video	51	1.091	7	6	23
Photo	174	4.356	19	51	120
Infographics	284	3.004	24	65	164
Total	509	8.451	50	122	307

Table 2. Average Engagement of Indonesian FDA Regional Office in Ambon Instagram Account Year 2023

Content Format	*Nilai*	*Like*	*Comment*	*Save*	*Share*
Video (n=51)	*Mean*	21,39±28,942	0,14±0,491	0,12±0,325	0,45±1,222
Photo (n=174)	*Mean*	25,03±19,442	0,11±0,510	0,29±0,559	0,69±2,064
Infographics (n=284)	*Mean*	10,58±10,363	0,08±0,429	0,23±0,601	0,58±1,489

Table 2 shows that photo content formats have higher average likes (25.03±19.942), saves (0.29±0.559), and shares (0.69±2.064) compared to videos and infographics, although the lower percentage of photo posts (174) relative to infographics (284). Additionally, video content formats have a higher average number of comment (0.14±0.491) compared to both photos and infographics. The elevated levels of likes, saves, and shares for photo content can be attributed to the brevity of time required to view photos, which quickly capture users' attention, thereby enhancing interactions through likes, saves, and shares. Conversely, the longer duration of video content, along with the need for narration and audio elements, may limit the engagement of Instagram users. This observation aligns with previous research by Bonilla-Quanja et al. (2023) on consumer engagement with well-known fashion brands on Instagram, which found that content featuring products (photos) generated higher engagement in terms of likes compared to video formats.

The high standard deviation for video (28.942), photo (19.442), and infographic (10.363) content formats suggests considerable variability in the number of likes, indicating an inconsistency in user engagement. On average, video posts receive 21.9 likes, with a maximum of 165 and a minimum of just 1. In comparison, photo posts average 25.03 likes, peaking at 194 and also dropping to 1. Infographic posts average 10.58 likes, with a highest of 56 and a lowest of 1. The standard deviation for comments and saves is relatively low, reflecting a narrow range between the highest (5) and lowest (0) values. However, the standard deviations for shares are more pronounced, with video content at 1.222, photos at

2.064, and infographics at 1.489. Notably, the highest share count was 16 for photo content, while the lowest was 0, indicating a diverse level of content sharing.

Content such as the Handover of Retirement Charter and Gold Pin post, which received 194 likes, and the Intensification of Takjil Ramadhan 1444 H post, garnering 56 likes, demonstrates that engaging topics resonate with followers and meet their informational needs, often featuring well-recognized figures. Conversely, the Happy Kartini Day post received only 1 like, likely due to its common nature and lack of novelty.

The post with the highest shares was the inauguration of the new Head of the Indonesian FDA Regional Office in Ambon, as followers deemed the information valuable for others. In contrast, the WHO Assessment of the Maturity Level of Balai POM saw low shares because it did not align with follower interests and failed to provide perceived added value.

As illustrated in Figure 1, the diagram shows the average monthly trend of engagement across different conten formats on the BPOM in Ambon Instagram account.

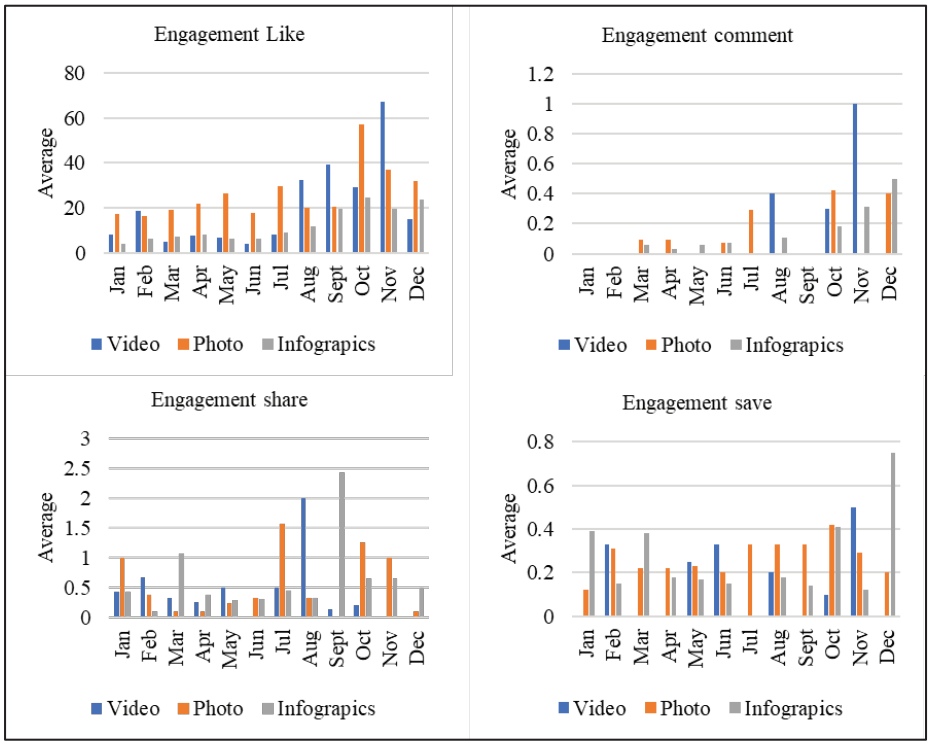

Figure 1. Diagram of average engagement

Figure 1 illustrates an upward trend in engagement for likes, indicating a growing appreciation for the content starting in July and reaching its peak in November 2023. Notably, the video content format recorded its highest average likes in November, averaging 67.50, with a standout post related to BPOM's Net Zero Carbon Program activities garnering 76 likes. This particular initiative involved a collaborative mangrove planting event with the Regional Leadership Communication Forum of Maluku Province, local government organizations, professional groups, universities, schools, community organizations, and all Indonesian FDA Regional Office in Ambon employees. Additionally, another video focused on the internalization of bureaucratic reform activities aimed at achieving a Corruption Free Zone (WBK) for Indonesian FDA Regional Office in Ambon, themed "Reformasi Birokrasi

(RB) On Stage," also engaged all Balai POM employees. The high levels of engagement can be attributed to the personal connection users felt with the content, as their faces were featured prominently, fostering interaction and encouraging likes.

The presence of the Instagram account of the Head of Indonesian FDA Regional Office in Ambon (@pugya.tam) and the Agent of Change (AOC) account (@nyongnona.manise) as collaborators significantly contributed to the increase in engagement likes. Other events that garnered high likes due to collaboration include the release of participants from the Sulawesi-Maluku Regional Pertikawan Contingent of the Scout Movement Provincial Council of Maluku in 2023, which received 59 likes; a Healthy Talkshow in collaboration with Ambon Express Radio, with 58 likes; Guidance on Management of Service Facilitation in West Seram District, achieving 53 likes; Pesta Kenari Pro UMKM which is an innovation of BPOM in Ambon in assisting the acceleration of product certification and registration for micro, small and medium businesses, with 48 likes; and Guidance on Food Poisoning, which received 49 likes. This observation aligns with research conducted by Hellbert (2015), which found that collaboration with well-known figures who have large followings can help reach a broader audience, potentially increasing the number of likes, comments, and shares. Additionally, Aulia et al. (2017) noted that the involvement of public figures as Instagram content collaborators can enhance public engagement in conversations. Santoso (2017) also indicated that content featuring activities or events significantly influences engagement, as followers or non-followers can directly interact with the account owner.

In January, the content exhibited the lowest average likes for photo posts, at 17.25, likely due to less engaging content that did not resonate with audience interests. The trend of increasing comments was observed in July, peaking in November before declining in December 2023. Notably, the video content format recorded the highest average comments in November related to the BPOM Net Zero Carbon Bureaucratic Reform Program (RB) On Stage, averaging 1.00 comments compared to other content formats. The average comment is tiny compared to the average likes of the exact content at 67.50. The overall average for comments across 509 posts was a modest 0.09. Comments represent a deeper form of interaction with followers or potential followers; thus, creating trending content that captivates the community or involves collaborators with substantial followings is crucial to enhancing user engagement.

Figure 1 indicates an increasing trend in the average content save value, peaking in December at 0.75 from eight infographic posts. The most significant contributor to saves was the post titled “List of Traditional Medicines Containing Medicinal Chemicals (BKO),” which was saved three times due to its valuable information deemed essential for future reference. Furthermore, the content was attractively designed, featuring appealing colors, layouts, and product photos. Research by Arifah (2023) also supports the notion that engagement is influenced by visual elements, including color, typography, illustrations, and layout.

Lastly, Figure 1 illustrates that the trend for content shares fluctuated throughout 2023, with the highest average shares occurring in September for infographic content, totaling 35 shares from 14 posts. This content, which focused on motivation and education regarding intelligent product selection through Cek KLIK (Check the Packaging, Label, Distribution Permit and Expiration date), was perceived as both attractive and essential information worthy of sharing.

To optimize the management of the Indonesian FDA Regional Office in Ambon Instagram account, it is essential to provide training for the team to enhance their competencies in conceptualizing material and designing content aligned with the daily posting agenda. Furthermore, additional research is needed to compare engagement data based on content topics and the demographic profiles of followers, including age, gender, and location. This analysis aims to identify specific content that aligns with the preferences and needs of the audience, ultimately fostering increased engagement and loyalty.

4. Conclusion

Photo content formats exhibit higher average engagement, with likes averaging 25.03, saves at 0.29, and shares at 0.69, compared to videos and infographics, despite the lower percentage of photo posts relative to infographics. In contrast, video content formats yield a higher average of comments at 0.14 than both photos and infographics. To enhance engagement on the Indonesian FDA Regional Office in Ambon Instagram account, priority should be given to photo and video content. Involving collaborators in content creation can further boost engagement. Additionally, incorporating users' personal experiences by featuring their faces in the content can encourage greater interaction through likes, saves, comments, and shares. The engagement data obtained in this study demonstrates that content format and various other factors significantly influence user interaction, including likes, comments, saves, and shares. Key elements include the presence of notable figures, events that involve large groups, and collaborations with influential partners.

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Analysis of the Effectiveness of Food Safety of School Snacks Program in Changing Knowledge, Attitude, and Behavior of School Children in Southeast Sulawesi

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ABSTRACT / ABSTRAK

Snacks are trendy foods with school-age children to fulfil their energy and nutrition needs in the school environment and must be ensured to be safe from biological, chemical, and physical contamination. However, food safety of school snacks program (PJAS) in Indonesia is still a reasonably concerning problem. One of the efforts made by the government to protect school-age children from unsafe PJAS is by increasing the knowledge, behavior, and attitudes of school children in choosing, buying, and consuming food through the Safe PJAS Program. This study aimed to examine the effect of the Safe PJAS program on the knowledge, attitudes, and behavior of school children in Southeast Sulawesi and the effectiveness of the Safe PJAS program in terms of the Knowledge, Attitudes, and Behavior of school children. The research methodology used was the quantitative analysis with Wilcoxon Test and Oneway Anova Test and literature review. The research results showed it was found that the Safe PJAS program carried out in Southeast Sulawesi in 2023 in Wakatobi and Central Buton Regencies had an impact and was influential in increasing the knowledge, attitudes, and behavior of school children in choosing and consuming snack foods. The author's recommendation for the Safe PJAS Program is that the Indonesian FDA can improve coordination and communication with the Ministry of Education and Culture to include food safety material in the independent learning curriculum and increase advocacy to obtain support from the Regional Government in allocating a budget for improving canteen facilities and infrastructure and to replicate the Safe PJAS program.

Pangan jajanan merupakan pangan yang sangat digemari oleh anak usia sekolah dalam pemenuhan energi dan gizinya saat anak berada dalam lingkungan sekolah. Pangan jajanan yang dikonsumsi harus dipastikan aman dari cemaran biologi, kimia dan fisik. Namun, Keamanan pangan jajanan anak sekolah (PJAS) di Indonesia masih menjadi masalah yang cukup memprihatinkan. Salah satu upaya yang dilakukan pemerintah untuk melindungi anak usia sekolah dari PJAS yang tidak aman dengan meningkatkan pengetahuan, perilaku dan sikap anak sekolah dalam memilih, membeli dan mengkonsumsi pangan melalui Program PJAS Aman. Tujuan penelitian ini untuk mengkaji pengaruh program PJAS Aman terhadap pengetahuan, sikap dan perilaku anak sekolah di Sulawesi Tenggara serta efektivitas program PJAS Aman ditinjau dari Pengetahuan, Sikap, dan Perilaku anak sekolah. Metodologi penelitian yang digunakan yaitu metode analisa kuantitatif dengan Uji Wilcoxon dan Uji Oneway Anova dan kajian literatur. Hasil penelitian menunjukkan bahwa program PJAS Aman yang dilakukan di Sulawesi Tenggara pada tahun 2023 di Kabupaten Wakatobi dan Buton Tengah berpengaruh

dan efektif terhadap peningkatan pengetahuan, sikap dan perilaku anak sekolah dalam memilih dan mengkonsumsi pangan jajanan. Rekomendasi penulis terhadap Program PJAS Aman adalah agar Badan POM dapat meningkatkan koordinasi dan komunikasi dengan Kementerian Pendidikan dan Kebudayaan guna memasukkan materi keamanan pangan dalam kurikulum pembelajaran merdeka dan meningkatkan advokasi untuk mendapatkan dukungan Pemerintah Daerah dalam mengalokasikan anggaran perbaikan sarana dan prasarana kantin serta untuk mereplikasi program PJAS Aman.

Keywords: Safe PJAS Program, Southeast Sulawesi, Student
***Kata kunci**: Program PJAS Aman, Sulawesi Tenggara, Anak Sekolah*

1. Introduction

Food safety of school snacks (PJAS) in Indonesia is still a concern. Sampling data from the Safe PJAS Program of the Indonesian FDA Regional Office in Kendari in 2023 showed that 15.67% did not meet the microbiological requirements of 185 PJAS samples tested (Balai POM in Kendari, 2023). Microbial contaminated food can come from bacteria, fungi, protozoa, and viruses that can cause foodborne diseases. Foodborne diseases symptoms include fever, headache, nausea, vomiting, abdominal pain, and diarrhoea (WHO, 2022). Foodborne diseases can lead to an Extraordinary Event of Food Poisoning (KLB KP). According to KP outbreak data in 2022 (Figure 1), the causative agent of most KP outbreaks was microbial contamination, with 55 incidents, consisting of 50 suspected microbiology (69.44%) and five confirmed microbiology (6.95%) due to *Salmonella* and *Staphylococcus aureus* contamination. Based on the distribution of KP outbreaks, schools ranked second at 38.89%, after residential homes at 40.28% (Badan POM RI, 2022).

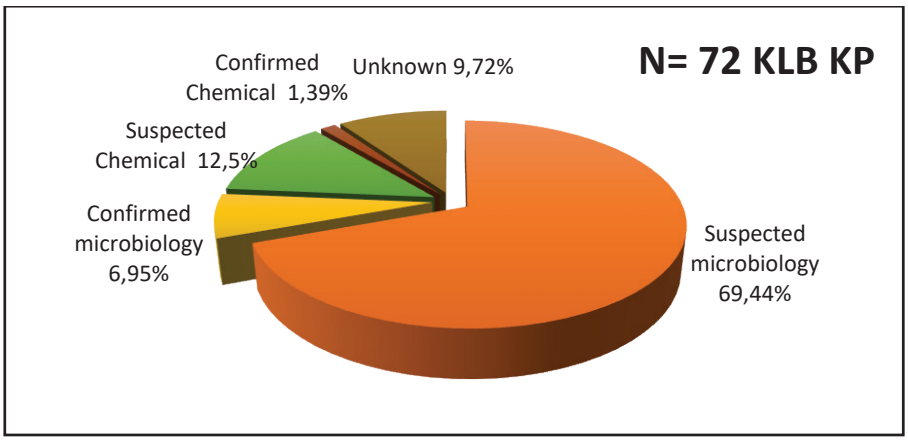

Figure 1. Distribution data of the causes of KP outbreaks in 2022 (Badan POM RI, 2022)

Problems in food safety include microbial contamination due to poor sanitation and hygiene in the production and preparation process of PJAS and chemical contamination problems due to the misuse of hazardous chemicals in food (Syah et al., 2015). Microbial contamination can occur due to unhygienic behavior and attitudes of food vendors, such as not washing hands or not using mouth/head/hand covers. Meanwhile, contamination due to unfulfilled sanitation can cause cross-contamination of processed food from unclean facilities/equipment. These problems can be prevented by increasing the knowledge of food vendors in canteen, teachers, and school children about choosing and consuming PJAS.

There is a relationship between the knowledge and behavior of school children in choosing healthy snacks (Febrianto, 2016). Education can instill the concept of behavior change to prevent consuming unhealthy snacks (Surya Syarifuddin, 2022).

The government has made efforts to improve the safety of PJAS through various programs, one of which is the Safe PJAS Program. This program started in 2011, and in 2017, the program was integrated into the Healthy Living Community Movement (GERMAS SAPA), initiated by President of the Republic of Indonesia. Prioritizing promotive and preventive efforts that involve all components of the nation to promotes a healthy paradigm. Safe PJAS aims to improve the knowledge, attitudes, and behavior of school children, PJAS traders, and teachers related to the safety of snacks so that they can protect themselves and their families from unsafe and unhealthy food to realize superior and competitive human resources towards Golden Indonesia 2045 Vision (BPOM, 2023). Evaluation of the objectives achievement of the Safe PJAS program needs to be done to assess the effectiveness of program implementation and increase knowledge, attitudes, and behavior, which is one of the critical indicators in the success of the Safe PJAS program. Based on this, related research is needed in schools intervened by the Balai POM in Kendari in 2023.

Wakatobi and Central Buton Regency are the focus locations of the Safe PJAS Program intervention in 2023 implemented by Indonesian FDA Regional Office in Kendari. Health problems occur in these two regencies, such as a high prevalence of diarrhoea. Based on data from the Central Bureau of Statistics in 2023, diarrhoea ranked 4th most cases of diseases that occurred in 2021 in Wakatobi Regency with 653 instances and 9th in Central Buton Regency (Anjani et al., 2023; Maulana et al., 2024).

This study examines the effect of the Safe PJAS Program on the knowledge, attitude, and behavior of school children in Southeast Sulawesi. In addition, the effectiveness of the Safe PJAS program will also be reviewed based on the knowledge, attitude, and behavior of school children.

2. Methodology

The study used a quantitative analysis method based on the survey assessments conducted before and after the intervention (pre- and post-interventional study). The survey was conducted directly by food safety cadres in school by distributing questionnaires containing questions for assessing Knowledge, Attitude, and Behavior with ten questions each. The results obtained are quantitative data, which are then statistically analysed using descriptive analysis methods, comparison tests, and variance analysis to obtain descriptions of the patterns of knowledge, attitudes, and behavior of school children. Furthermore, a literature review was conducted from reports related to school snacks in national and international journals.

2.1. Research Design

Descriptive with a longitudinal survey type to see changes before and after implementing the Safe PJAS Program in Schools.

2.2. Population and Sample

The population in this study were elementary, junior high, and high school students in Wakatobi and Central Buton Regency who received the Safe PJAS Program from Balai POM in Kendari. The samples were taken by 120 children divided into 60 children in

Wakatobi Regency and 60 in Central Buton Regency. Samples were taken from 6 schools in each regency, each involving ten children.

2.3. Time and Place

This study was conducted in May-July 2023 in the focus location of the intervention of Safe PJAS Program in 2023, namely Wakatobi and Central Buton Regency, with details of schools according to Table 1.

Table 1. Intervention Schools of PJAS Program of Balai POM in Kendari in 2023

No.	Intervention School	
	Wakatobi Regency	Central Buton Regency
1.	SDN 1 Mandati	SDN 1 Lakudo
2.	SDN 1 Pongo	SDN 7 Lakudo
3.	SMPN 1 Wangi-wangi	SDN 9 Lakudo
4.	SMPN 3 Wangi-wangi Selatan	SDN 17 Lakudo
5.	MTSN 1 Wakatobi	SMPN 3 Buton Tengah
6.	MAN 1 Wakatobi	MAN 1 Buton Tengah

2.4.Data Collection Technique

Data collection was collected based on secondary data from the results of implementing the PJAS program in 2023 by giving questionnaires directly to school children. The questionnaire given to respondents consisted of 30 questions divided into three assessment components: Knowledge, Attitude, and Behavior. The questionnaire was given before and after the PJAS program intervention by the Balai POM in Kendari in Wakatobi and Central Buton Regency, which was implemented in 2023. The pre-intervention survey was conducted before school children's food safety socialization activities. The post-intervention survey was conducted after school children received Information, Education, and Communication (IEC) from food safety cadres in school.

2.5. Data Analysis Technique

Data analysis was conducted using the SPSS 26 application, paired-samples T-Test, to compare differences in knowledge, attitude, and behavior of school children before and after the intervention of the Safe PJAS program. The data must be normally distributed as a requirement for paired-sample T-test analysis, so a normality test was conducted using the Kolmogorov-Smirnov method. If the data is not normally distributed, proceed with a non-parametric test using the Wilcoxon Test method. Normality test was conducted on knowledge, attitude, and behavior score data.

Furthermore, the data were analyzed using the Oneway Anova method to determine the increase in knowledge, attitude, and behavior before and after the PJAS program intervention. A further test was conducted using the Games Howell method because the data was not homogeneous. This test aims to determine the school that experienced the best improvement.

3. Results and Discussion

3.1 Respondent Characteristics

The respondents in this study amounted to 120 schoolchildren from elementary/junior high/high school in Wakatobi Regency (50%) and Central Buton Regency (50%). The distribution of schoolchildren based on education level is elementary school (50%), junior

high school (33.33%), and high school (16.67%). Meanwhile, the distribution of schoolchildren by gender was male (48.33%) and female (51.67%).

3.2 Knowledge of school children about the safety of PJAS

Knowledge of school children before and after the intervention of food safety program in Wakatobi Regency (Chart 1) and Central Buton Regency (Chart 2), with the average value of knowledge in 5 schools in Wakatobi Regency increased, except for one school that decreased its score, namely MAN 1 Wakatobi. Likewise, the average knowledge score in Central Buton Regency increased in 5 schools. One school's score did not change before or after the Safe PJAS Program intervention, namely MAN 1 Central Buton. From both regencies, at the secondary school level there was no increase in knowledge (the same or decreased). This can be caused by several possibilities, such as school children already having good food safety knowledge before the intervention, school children being less interested in the socialization material provided, and the presentation method being less attractive. Hence, participants are less interested in the information provided.

The PJAS program's effect on school children's knowledge score was further analyzed using the Wilcoxon test. Based on the results of the analysis of the Wilcoxon test, the significance of 0.000 is smaller than 0.05 (Sig < α). Statistically, the Safe PJAS Program has an influence on school children's knowledge of PJAS food safety in Southeast Sulawesi in 2023. Sixty-one school children experienced increased knowledge, with an average increase of 36.72.

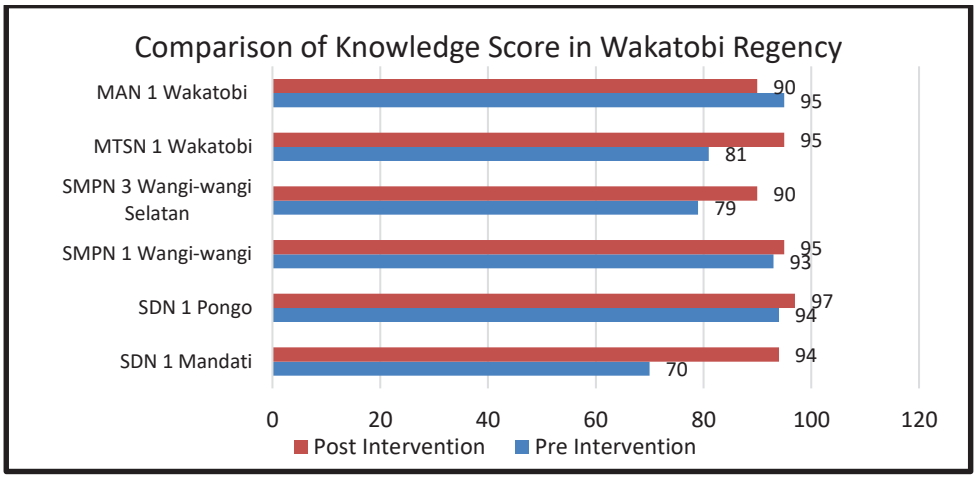

Chart 1. Comparison of Mean Knowledge Score Before and After Safe PJAS Program Intervention in Wakatobi Regency

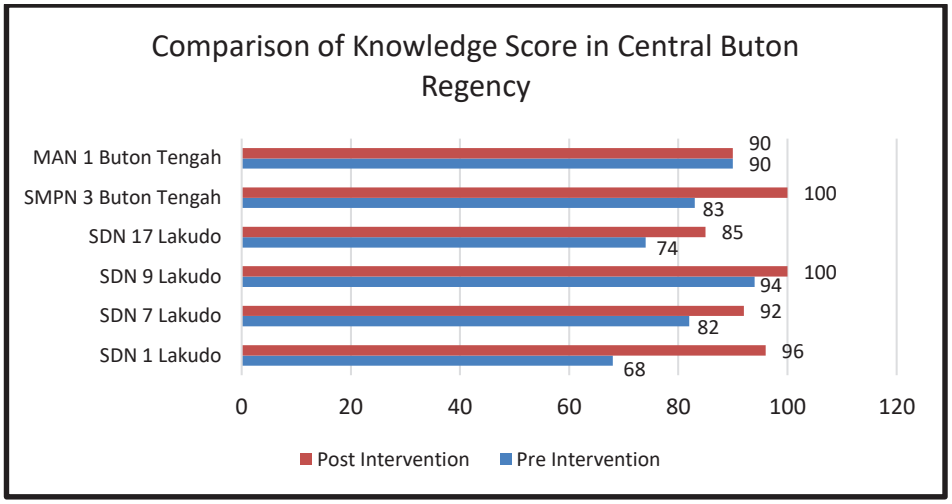

Chart 2. Comparison of Average Knowledge Score Before and After Safe PJAS Program Intervention in Central Buton Regency

The Oneway Anova test was conducted to determine the differences between schools. H0 in the Oneway Anova test is the increase in the score of knowledge of school children in all schools is the same, after the intervention of Safe PJAS Program, while H1: The increase in in the score of school children's knowledge is different in each school after the Safe PJAS Program intervention. The test results (Table 2) obtained Sig 0.000, Significance <0.05 indicates that H0 is rejected and Ha is accepted. From this test, it can be said that statistically, the increase in knowledge scores of school children was different in each school after the Safe PJAS Program intervention.

Table 2. Oneway Anova Test of Knowledge Score of School Children

Difference between pre- and post-knowledge	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10409.167	11	946.288	4.139	.000
Within Groups	24690.000	108	228.611		
Total	35099.167	119			

Based on the Howel Games Test with α 5%, the results show that the score improvement at SDN 1 Mandati, SMPN 3 Wangi-Wangi, and MTsN 1 Wakatobi is better than other schools in Wakatobi Regency. Meanwhile, in Central Buton Regency, significant improvements occurred at SDN 1 Lakudo, SDN 7 Lakudo, SDN 17 Lakudo, and SMPN 3 Central Buton.

Providing information on food safety to school children showed positive results in improving the food safety knowledge of school children (Anggitasari et. al., 2014). Food safety knowledge is an essential aspect of a person's understanding of food safety. One of the best methods to increase a person's knowledge is through the provision of Information, Education, and Communication (IEC) in the form of lectures, leaflets, and videos. The higher a person's knowledge, the better their behavior and attitude when choosing, buying, and consuming snacks.

In the Safe PJAS Program, intervened schools will be provided with food safety information in the form of posters, leaflets, food safety videos, banners, and IEC on the introduction of food safety hazards, recognizing and choosing safe food (5 Keys to Food Safety) and Tips for Safe Food Consumption by Paying Attention to Nutritional Value Information and Checking Packaging, Label, Distribution Permit, and Expiration.

3.2. Attitude of school children on the safety of PJAS

Questionnaire-based scores of school children's attitudes before and after food safety program intervention in Wakatobi Regency (Chart 3) and Central Buton Regency (Chart 4). The average value of Attitude in Wakatobi Regency has increased in 5 schools, and one school has not changed, namely SMPN 3 Wangi-Wangi Selatan. Meanwhile, the increase in the mean score of attitude in Central Buton Regency occurred in all schools intervened by the Safe PJAS Program.

The PJAS program's effect on school children's attitude score was analyzed using the Wilcoxon test method, preceded by the Kolmogorov-Smirnov test. The normality test results obtained a significance of 0.000, Asymp Sig (2-tailed) < α; the data is not normally distributed. Based on the results of the analysis of the Wilcoxon test, the significance of 0.000 is smaller than 0.05 (Sig < α), so statistically, there is an influence of the Safe PJAS Program on the attitude of school children about PJAS food safety in Southeast Sulawesi in 2023. Sixty-four school children experienced an increase in attitude, with an average increase of 41.10.

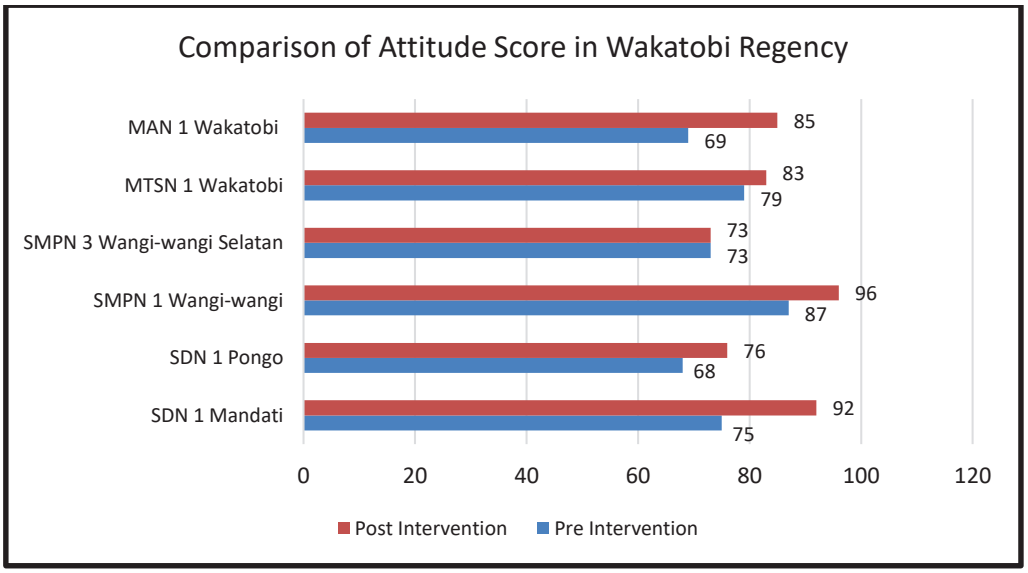

Chart 3. Comparison of the Mean Score of Attitude Before and After Safe PJAS Program Intervention in Wakatobi Regency

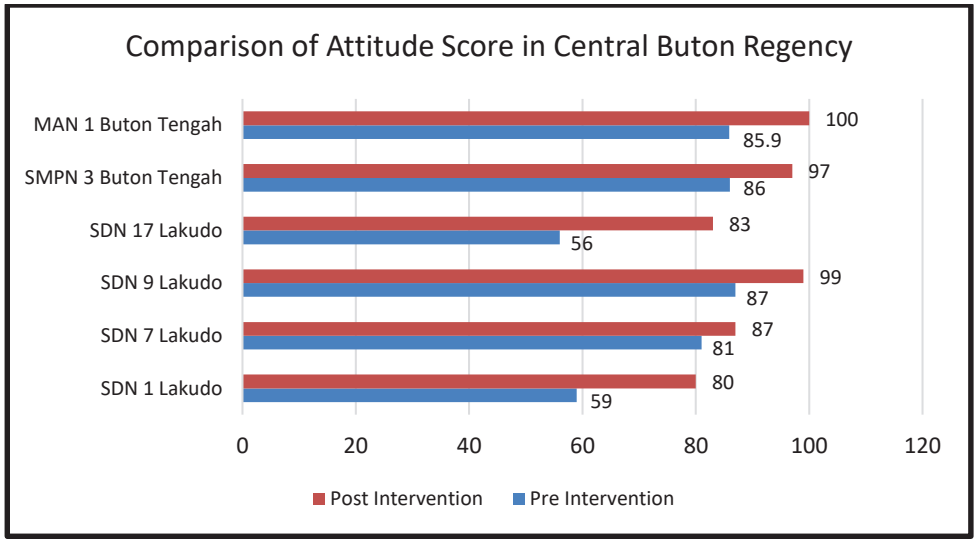

Chart 4. Comparison of Mean Score of Attitude Before and After Safe PJAS Program Intervention in Central Buton Regency

The Oneway ANOVA test was conducted to determine the difference in attitude score in each school. H0: The increase in the attitude score of school children in all schools is the same after the Safe PJAS Program intervention, while Ha: The increase in the attitude score of school children is different in each school after the intervention of the Safe PJAS Program. The test results (Table 3) obtained Sig 0.030, Significance <0.05 indicates that H0 is rejected and Ha is accepted. Statistically, the increase in the score of attitude of school children is different in each school after the intervention of the Safe PJAS Program.

Table 3. Oneway Anova Test of Attitude Score of School Children

Difference between Pre and Post Attitude	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6213.092	11	564.827	2.057	.030
Within Groups	29648.900	108	274.527		
Total	35861.992	119			

Based on descriptive analysis, the best improvement in attitude scores of school children occurred at SDN 17 Lakudo, with a difference in pre-and post-test scores of 27 points. This was followed by the Games Howell test, with α 5% significant difference compared to SMPN 3 Wangi Selatan. However, there is insufficient evidence that SDN 17 Lakudo is better than other schools.

Children's decisions to buy snacks are usually influenced by price, desire to try and the color of the food; the more striking the color, the more interested children are in buying. The Safe PJAS program in intervention schools is expected to change children's attitudes toward food purchasing decisions. In addition, the support of teachers, parents, and food vendors in canteen is critical so that safe snacks are easily obtained.

3.3. Behavior of School Children on the Safety of PJAS

Chart 5 and 6 show that the average behavior score increased in all schools in the Wakatobi and Central Buton Regency. The most significant increase occurred at SDN 1 Lakudo and SDN 17 Lakudo.

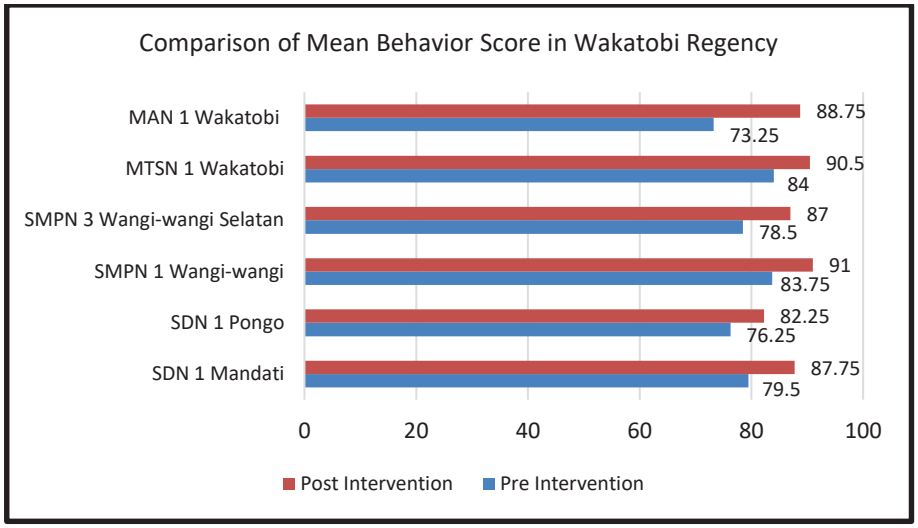

Chart 5. Comparison of Mean Behavior Score Before and After Safe PJAS Program Intervention in Wakatobi Regency

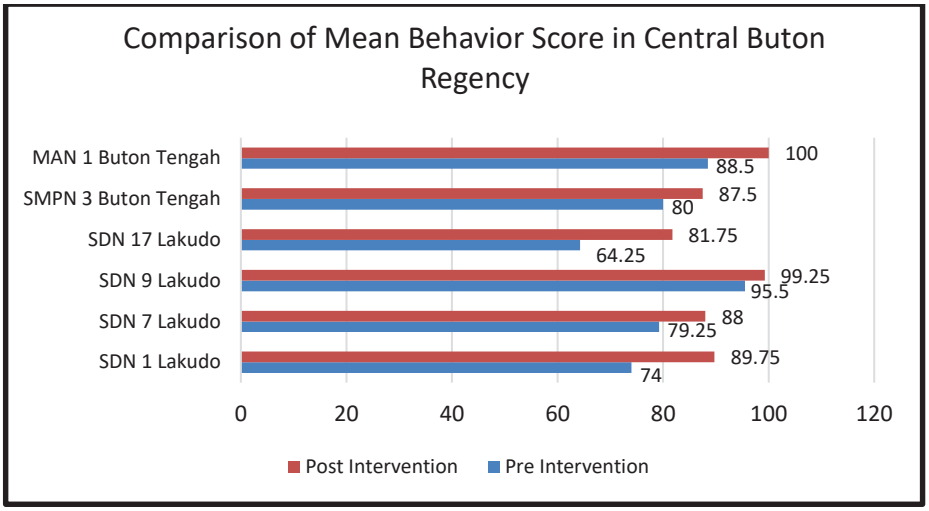

Chart 6. Comparison of Mean Behavior Score Before and After Safe PJAS Program Intervention in Central Buton Regency

The Wilcoxon Test method analyzed the PJAS program's effect on school children's behavior value. In the Wilcoxon Test of Attitude Value, H0 is that there is no influence of the Safe PJAS Program on the behavior of school children about PJAS food safety in Southeast Sulawesi in 2023. Based on the analysis results, the significance of 0.000 is smaller than 0.05 (Sig < α); statistically, there is an influence of the Safe PJAS Program on the behavior of school children regarding PJAS food safety in Southeast Sulawesi in 2023. Ninety-eight school children experienced an increase in behavior, with an average increase of 55.14.

The Oneway ANOVA test was conducted to determine the difference in behavioral values in each school. H0: The increase in the value of school children's behavior in all schools is the same after the Safe PJAS Program intervention. Ha: The increase in the value of school children's behavior is not the same in each school after the intervention of the Safe PJAS

Program. The test results (Table 4) obtained Sig 0.012, Significance <0.05 indicates that H0 is rejected and Ha is accepted. Statistically, the increase in the value of school children's behavior is not the same in each school after the intervention of the Safe PJAS Program.

Table 4. Oneway Anova Test of Behavior Score of School Children

Difference between Pre and Post Behavior	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2089.323	11	189.938	2.366	.012
Within Groups	8670.625	108	80.284		
Total	10759.948	119			

Further tests to determine scholls that experienced a better increase in behavioral values than other schools. Howel's Games test with α 5%, the results of the analysis obtained that the increase in behavior scores that are better than other schools for Wakatobi Regency occurred in SDN 1 Mandati, SMPN 1 Wangi-Wangi, MTS 1 Wakatobi, MAN 1 Wakatobi. Meanwhile, significant increase occurred in the Central Buton Regency at SDN 1 Lakudo, SDN 17 Lakudo, SMP 3 Central Buton, and MAN 1 Central Buton compared to other schools. Some schools, such as SDN 1 Pongo, SMPN 1 Wangi-Wangi, SDN 7 Lakudo, and SDN 9 Lakudo, did not experience significant increase in behavior after the intervention. This is due to the suboptimal role of food safety cadres in implementing the food safety program action plan. The success of health programs is influenced by trained human resources (cadres) and good supervision (Pratiwi, 2021). The safe PJAS Program can run because of the contribution of PJAS cadres, namely teachers and little doctors, as role models who provide examples to other school children (Imara Ihsan et al., 2024).

Interventions in the Safe PJAS Program provided at elementary, junior, and senior high school education levels in Wakatobi and Central Buton Regencies use the same stages of activities, namely by conducting direct socialization of food safety to students, showing food safety videos, installing banners and food safety posters. However, education through the snakes and ladders game is only done at the primary school level. Intervention through providing education with media such as flipcharts, posters, and audio-kinetic (gymnastics) with the theme of good snack foods and drinks can improve snacking behavior to be better in school children (Briawan, 2016).

Knowledge is a factor that supports school children in terms of healthy snack selection behavior (Febriyanto, 2016). The increase in school children's behavior in food safety practices in intervention schools can be caused by an increase in school children's knowledge about food safety, so it is expected that the Safe PJAS program can continue to be carried out in intervention schools independently.

3.4. Effectiveness of Safe PJAS Program at Indonesian FDA Regional Office in Kendari

Based on the research results, the Safe PJAS Program implemented in Wakatobi and Central Buton Regency effectively improves knowledge, attitudes, and behavior in choosing and consuming snacks (S value < a). The PJAS Program intervention begins with advocating for the Local Government to deliver the program, followed by food safety socialization activities, food safety technical guidance for food safety cadres in school, providing food safety education packages, monitoring the empowerment of food safety cadres in school, and certifying schools with safe PJAS (BPOM, 2023). This program also prepares

commitment documents from school principals and forms school food safety teams. In addition, food safety education packages such as posters, educational books, leaflets, banners, etc., are provided. This is in line with research conducted by Rahayu (2015), which showed that poster installation and the formation of food safety teams in schools had a significant effect on changes in the attitudes of elementary school children in western Indonesia, while in eastern Indonesia, another critical factor was food safety counseling. However, 25% of schools had no improvement in knowledge or attitude, namely MAN 1 Wakatobi, MAN 1 Buton Tengah, and SMPN 3 Wangi-Wangi Selatan. School children's health empowerment programs are less effective in changing children's behavior and attitudes toward consuming PJAS because children's decisions to choose food are influenced by teachers, parents, friends, and children's character (Anna Triwijayati et al., 2016). Therefore, food safety materials must be included in the school learning curriculum to carry out education continuously.

The success of the Safe PJAS program implemented by the Balai POM in Kendari was due to several factors, such as the role of food safety cadres in carrying out food safety program action plan through direct of face-to-face socialization, food safety videos, installation of banners and posters in the school environment and supervision of snacks in school canteens. According to Imara Ihsan et.al.. (2024), in a case study of an elementary school (SD 1 Banjar) found that the PJAS program was running because of the contribution of PJAS Cadres (teachers and little doctors). In addition, innovative educational media also influence the effectiveness of the Safe PJAS program, such as integration with P5 and ladders games. Such as the education conducted by Andriani et al. (2023) at the Pilot Elementary School, Meulaboh, West Aceh, with educational and persuasive media using snakes and ladders games, showed an increase in school children's knowledge about healthy snacks.

Even so, there are several obstacles faced by officers in implementing the Safe PJAS program, such as the lack of motivation of food safety cadres in school to implement food safety action plans due to the busy teaching schedule with government programs in schools. Not only that, the availability of facilities and infrastructure in school canteens, hygiene and sanitation issues in school canteens, and the lack of support from local governments to replicate PJAS Program activities are some of the constraints in budget limitations. There are several technical problems in PJAS supervision, namely some not knowing the role in PJAS supervision; lack of coordination in the implementation of PJAS supervision programs; limited number of human resources, budgets, and infrastructure; and the presence of food sellers outside the school environment (Febrianis, 2023).

In this study, the authors provide recommendations for the implementation of the Safe PJAS program to be more effective and sustainable, among others: (1) Badan POM can improve coordination and communication with the Ministry of Education and Culture so that the materials contained in the Safe PJAS Program can be included in the independent learning curriculum to be socialized and taught to all schools in Indonesia; (2) Increase advocacy to get local government support in allocating a budgets for improving canteen facilities and infrastructure and to replicate the Safe PJAS program.

4. Conclusion

There was an increase in knowledge, attitudes, and behavior of school children regarding PJAS safety after receiving the Safe PJAS intervention program at their school. The

increased occurred in all regencies in Southeast Sulawesi that were intervened, both Wakatobi and Central Buton. However, out of 12 schools, there were 3 schools that did not see an increase in knowledge or attitudes after being intervened with the Safe PJAS Program.

The Safe PJAS program at Indonesian FDA Regional Office in Kendari in 2023 was declared effective as assessed by the increase in Knowledge, Attitide amd Behavior of the school children who were intervened. This increase is due to the active role of food safety cadres in school in developing and implementing food safety program action plans through direct face-to-face socialization, food safety videos, installation of banners and posters in the school environment, and monitoring snack foods in school canteen. This effort can be improved through innovation by integrating programs and using educational and persuasive media. This Safe PJAS Program can be replicated by the City/Regency Government so that the impact felt by school children will be broader.

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Analysis of ZnPtO in Anti-dandruff Shampoo by High-Performance Liquid Chromatography - *Photo Diode Array*

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ABSTRACT

ZnPtO is widely used as an active substance in anti-dandruff shampoo. Instead of describing its benefits, many articles have reported that ZnPtO can harm the environment and human health. Through the Food and Drugs Administration, Indonesia has been regulated to have a maximum limit of 2.0% for rinse-off hair products. The research aims to develop an accurate and reliable method to determine product ZnPtO level. ZnPtO was analyzed using High-Performance Liquid Chromatography (HPLC) with Photo Diode Array (PDA) at a wavelength of 257.9 nm. This research used a C18 column with dimensions of 250 x 4.6 nm and 5 µm in particle size. The mobile phase consisted of acetonitrile and a mixture of potassium dihydrogen phosphate solution and disodium EDTA at pH 4.0 (30:70). The column temperature was maintained at 40°C at a 1.0 ml/min flow rate. The results showed that ZnPtO was detected at a retention time of 7 minutes. The method's correlation coefficient and residual deviation were 0.999% and 0.65%, respectively. Method precision at 20, 100, and 160 µg/ml was 0.6694, 0.4511, and 0.4728%, respectively. Method accuracy at those levels was 98.3 to 100.9%. All validation parameters have fulfilled the qualification. ZnPtO levels contained in anti-dandruff shampoos were 0.0081%, 0.0040%, and 0.016%, respectively. The developed method has proven selective, accurate, and reliable. It can be used to control the quality and safety of anti-dandruff shampoo due to pre-market and post-market surveillance.

ZnPtO merupakan senyawa aktif dalam produk sampo anti ketombe. Meskipun memiliki banyak kegunaan, namun beberapa artikel ilmiah menyebutkan adanya dampak penggunaan senyawa ini baik terhadap lingkungan maupun terhadap hewan uji. Sebagai zat aktif dalam produk anti ketombe, Indonesia melalui Badan Pengawas Obat dan Makanan (BPOM) telah mengatur batas aman kandungan ZnPtO maksimal sebesar 2,0% dalam produk sediaan rambut bilas. Penelitian ini dilakukan untuk mengembangkan metode analisis yang akurat dan handal untuk menguji kandungan senyawa ZnPtO di dalam produk sampo anti ketombe. ZnPtO dapat dianalisis menggunakan instrumen Kromatografi Cair Kinerja Tinggi (KCKT) menggunakan detektor Photo Diode Array (PDA) pada panjang gelombang 275,9 nm dengan kolom C18 (250 x 4,6 mm dengan ukuran partikel 5 µm) menggunakan fase gerak asetonitril dan campuran larutan larutan kalium dihidrogen fosfat – dinatrium EDTA pH 4,0 (30 :70). Suhu kolom dijaga pada 40°C dan laju alir 1,0 ml/menit. Hasil analisis menunjukkan baku ZnPtO terdeteksi pada waktu retensi sekitar 7 menit. Nilai koefisien korelasi (r) dan deviasi residual (Vx0) pada penetapan linieritas metode berturut-turut adalah 0,999% dan 0,65%. Presisi metode pada konsentrasi 20; 100; dan 160 µg/ml berturut – turut adalah 0,6694%; 0,4511% dan 0,4728%. Akurasi metode pada tiga

konsentrasi tersebut berada pada rentang 98,3 – 100,9%. Seluruh parameter validasi telah memenuhi syarat. Hasil uji kadar ZnPtO dalam sampel sampo anti ketombe yaitu 0,0081%, 0,0040% dan 0,016%. Metode analisis yang dikembangkan terbukti selektif, akurat dan andal, sehingga dapat digunakan sebagai metode uji dalam rangka kontrol kualitas dan keamanan sebelum dan selama produk beredar.

Keywords: HPLC-PDA, method validation, Zinc Pyrithione
Kata Kunci: *KCKT – PDA, validasi metode, Zink Pirition*

1. Introduction

In everyday life, humans are inseparable from using cosmetics both for caring purposes, changing or improving appearance, and covering body odor (Sharma et al., 2018). According to the Food and Drug Authority Regulation No. 17 of 2022 concerning Amendments to the Food and Drug Authority Regulation No. 23 of 2019 concerning Technical Requirements for Cosmetic Ingredients, cosmetics are materials or products intended for use on the external parts of the human body such as the epidermis, hair, nails, lips, and external genital organs, or teeth and oral mucous membranes, especially for cleaning, perfuming, changing appearance, and/or improving body odor or protecting or taking care the body in good condition. (BPOM RI, 2022). One type of cosmetic that is widely used in daily life is shampoo.

Shampoo is the most common hair product used by the public. Shampoo cleans the oil/sebum, sweat, the dirt that sticks to the scalp, and other cosmetic products used for hair styling (George & Potlapati, 2021). In addition to cleaning the hair's dirt, shampoo also used to remove dandruff. Dandruff is excessive flaking of the scalp accompanied by the presence of fatty impurities, itching, and hair loss. Dandruff is caused by infection process with microorganisms, including *Malassezia furfur*, *Malassezia globosa,* and *Malassezia restricta*. (Das & Khubdikar, 2019) (Pertiwi et al., 2020).

The compound that is generally added in anti-dandruff shampoos is *zinc pyrithione* (ZnPtO) (Leong et al., 2020). This compound is an active ingredient with low irritation and sensitization potential in anti-dandruff. ZnPtO is an anti-fungal ingredient widely used in shampoos to treat seborrheic dermatitis and dandruff symptoms (Mangion et al., 2021). Although the risk of sensitization is low, cases of allergic dermatitis are still found due to skin contact with ZnPtO compounds (Mangion et al., 2021). Apart from being used in anti-dandruff shampoo formulations, ZnPtO can also be used in topical formulations to treat localized psoriasis.

ZnPtO ($ZnC_{10}H_8N_2O_2S_2$) is a coordination complex compound with a 2-valence zinc cation (Zn^{2+}) as central atom with two bound ligands, namely pyrithion anions (Kim et al., 2018). The molecular weight of this compound is 317.7 g/mol and its logP is 0.88. These properties make the ZnPtO molecule very *permeable* when applied to the skin. However, the permease properties of ZnPtO on the skin are limited because this compound has a low solubility in water. This makes ZnPtO ideal for use in shampoo formulations (Mangion et al., 2021). As an active ingredient for anti-dandruff shampoos, ZnPtO are often combined with other compounds, such as climbazole, to provide anti-dandruff efficacy with more significant benefits (Turner et al., 2013).

Even though ZnPtO has good effectiveness as an anti-fungal in anti-dandruff shampoo, contact or ingestion of this compound to a certain extent level can have negative impact ; where according to the *Scientific Committee on Consumer Safety,* above 2000 mg/kg of

ZnPtO level can cause acute dermal toxicity (SCCS, 2020). In laboratory studies, ZnPtO compounds are reported to trigger various responses, such as DNA damage in skin cells (Park et al., 2020). ZnPtO, as an active ingredient in anti-dandruff shampoo, has the potential to adhere on the scalp, which can at least cause mild irritation even if shampoo is used by rinsing. Determination of ZnPtO levels in the scalp showed that shampoos containing a combination of ZnPtO and climbazole gave more ZnPtO deposits than shampoos using only a single ZnPtO (Chen et al., 2015).

In the Indonesian Food and Drug Authority (BPOM) Regulation No. 17 of 2022, the requirements for ZnPtO are listed in Appendix 1 of the List of Ingredients Allowed for Use in Cosmetics with Restrictions and Requirements for Use. The content of ZnPtO in the hair rinse off product as anti-dandruff should not be more than 2.0% (BPOM RI, 2022). To determine the quality and safety of anti-dandruff shampoo, it is necessary to establish an analytical method to detect the product's ZnPtO level. Currently, the analytical method available as.

BPOM's *in-house method* for testing the ZnPtO content in anti-dandruff shampoo is a manual titration method with a long processing time, a complicated preparation process, and requires a large amount of reagents. This method is considered ineffective and inefficient for monitoring many products circulated in the market. In addition, the manual titration method requires high accuracy from each laboratory staff to determine the endpoint of the titration, allowing for potential errors in concluding the test results. To minimize this potency, it is necessary to develop a new analytical method to determine the ZnPtO content in anti-dandruff shampoo quickly, precisely, and accurately.

Previous research reported that ZnPtO content in anti-dandruff shampoo can be determined using complex and potentiometric titration methods. The complexometric test procedure uses several reagents, including hydrochloric acid and hydrogen peroxide. Ammonia and aqueous solution are added for pH adjustment to reach a pH of 10. EDTA solution (0.01 M) is used as a titrant with eriochrome black T as the indicator. The endpoint is reached if a color changes from purple to blue. In potentiometric testing, the titrant solution used is iodine solution (0.05 M), where the endpoint observation is carried out using a platinum electrode (Egurrola et al., 2021).. The use of complexometric and potentiometric titration methods is less sensitive and allows for errors in determining the endpoint of the titration.

Several other studies have used high-performance liquid chromatography (HPLC) techniques to analyze ZnPtO content. The analysis technique generally focuses on preparing to obtain ZnPtO compounds with good solubility before being analyzed on KCKT. The method developed uses a normal phase HPLC system using Porasil column and was reported to provide good precision and accuracy values. In that study, the preparation was carried out through a liquid liquid extraction technique using 10 mM copper sulfate solution and methylene chloride solution (1:1, v/v), and 5 ml of isopropanol in 2 liters of methylene chloride as mobile phase. (Fenn & Alexander, 1988).

Another study mentioned that used of RP-18 column with a mobile phase of acetonitrile water containing phosphate-buffered and *ethylenediaminetetraacetic acid* (EDTA) can determine the level of ZnPtO. Sample preparation was carried out using a methanol-water solvent mixture containing acetic acid and EDTA to increase the solubility of ZnPtO. The detection limit value obtained of 2 ng calculated based on the signal per noise ratio (Gagliardi et al., 1998).

In another study, the application of reversed-phase HPLC preliminary with the extraction process using dichloromethane and methanol was reported. The HPLC analysis used oxalic acid/EDTA in water (pH 4) and acetonitrile as mobile phases (Mildau, 2018).

The procedure to form ZnPtO complex compounds as copper complex compounds was also reported. The procedure carried out by adding copper sulfate solution and extraction using chloroform, and then analyzed uisng Reversed-phase HPLC. (Nakajima et al., 1990).

In the LC-MS/MS analysis of ZnPtO, sample preparation was reported carried out by dissolving the sample in a chloroform-methanol mixture (2:1, v/v). (Kim et al., 2018).

In other LC-MS/MS analysis of ZnPtO, samples are prepared by first washing them with water to remove surfactants and water-soluble impurities, then ultrasonically extracting them with acetonitrile-methanol (Gu et al., 2014). (Gu et al., 2014).

A reversed-phase HPLC technique with simple preparation technique has also been developed, which uses methanol as a solvent and provides a method LOD value of 4.10 µg/ml (Kachchhi et al., 2020).

Another analytical methods for testing ZnPtO content in shampoo that have been reported generally have complexity regarding sample preparation procedures, where the solvent and mobile phase combine several reagents. This study aims to find a more straightforward and faster preparation and analysis procedure of ZnPtO in anti-dandruff shampoo through chelate formation using EDTA solution so that it can be used as quality control and safety of anti-dandruff shampoo products before the products are circulated in the market and to facilitate BPOM as a regulator in the testing process in the context of supervision when the product circulating on the market to ensure the safety and quality of the product.

2. Methodology

2.1.Reagents and materials

The zinc pyrithione standard was obtained from the Center for National Quality Control Laboratory of Drugs and Food (PPPOMN BPOM). All reagents were used directly without further purification. Potassium dihydrogen phosphate (KH_2PO_4) analysis *grade* and *ethylenediaminetetraacetic acid disodium salt dihydrate* ($Na_2EDTA.2H_2O$) *reagent grade* from Sigma Aldrich, dimethyl sulfoxide (DMSO) analysis grade; methanol HPLC grade; orthophosphoric acid analysis grade from Merck and deionized water (18.2 MΩ cm at 25° C),– PTFE membrane filters of 0.45 µm were used to separate the target analyte from the interfering matrix.

2.2.Equipment

High-Performance Liquid Chromatography system was a Waters Alliance e2695 with an automatic injection system, pump, automatic sampler, thermal compartment, and a *Photo Diode Array* detector (2998 PDA). *Empower* software was used for system control, data processing, and collection. An octadecylsilane (C18) column with dimensions of 250 mm x 4.6 mm and a particle size of 5 µm was used for the separation process. The column temperature was maintained at 40° C during the analysis; the flow rate was 1.0 ml/min with an injection volume of 20 µl.

2.3.Solution Preparation

Preparation of Solvent and Mobile Phase. The solvents used were dimethyl sulfoxide (DMSO) as first solvent and HPLC grade methanol as second solvent. The mobile phase consisted of HPLC grade methanol (**A**) and a mixture of potassium dihydrogen phosphate

10 mM - sodium EDTA 25 mM pH 4.0 solution (**B**) with the ratio of A and B being 30 : 70. The pH of solution **B** was adjusted to 4.0 by the addition of orthophosphoric acid solution.

Preparation of Standard Solution. Zinc pyrithione standard stock solution was prepared at a concentration of 2000 µg/ml using first solvent in a volumetric flask. Series standard solutions were prepared by pipetting 1.0 ml, 2.0 ml, 4.0 ml, 5.0 ml, 7.0 ml, and 8.0 ml of the standard stock solution into separate 20 ml-flasks and diluted to volume with the first solvent. Furthermore, each solution was diluted with second solvent to obtain serial standard solutions with concentrations of 20 µg/ml, 40 µg/ml, 80 µg/ml, 100 µg/ml, 140 µg/ml and 160 µg/ml, respectively.

Sample Preparation. Samples of cosmetic hair rinse off product (shampoo) were obtained from the samples circulated on the market. A 0.5 gram of sample was dissolved and diluted with first solvent in a 20-ml volumetric flask. Then, 1.0 ml of the sample solution was pipetted and diluted with second solvent in a 5-ml flask.

2.4.Method Validation

The method's selectivity was carried out by injecting the ZnPtO standard solution and methylisothiazolinone standard simultaneously, and then the resolution values of the two standard peaks were observed. Linearity, precision, and accuracy were analyzed using sample solution spiked with a standard solution so that the final concentration of solution equal to the concentration of serial standard solution. A serial standard calibration curve was created by comparing the concentration of ZnPtO in the solution (x-axis) with the area of ZnPtO obtained from the analysis (y-axis). Precision was established by analyzing samples spiked with known concentration of ZnPtO standard, and the percent repeatability was calculated. Accuracy was determined by calculating the percent recovery of the ZnPtO standard added to the sample. A standard solution of methylisothiazolinone with a concentration of 100 µg/ml was used for method selectivity analysis. The limit of quantitation was established through dilution of the sample spiked with ZnPtO standard solution so that the precision and accuracy of ZnPtO response met the requirements. (AOAC Internacional, 2023).

3. Results and Discussion

3.1.Validation of Analysis Methods

Methylisothiazolinone standard solution was used as a selectivity standard solution to develop this analysis method. Methylisothiazolinone was used as a selectivity standard because this compound has a similar wavelength as the wavelength of ZnPtO when analyzed with a *Photo Diode Array* (PDA) detector (Pham Ngoc Thuy et al., 2021). This compound is also commonly used as a preservative in cosmetic anti-dandruff shampoo products containing ZnPtO compounds (Tomás et al., 2020). Figure 1 shows the results of the selectivity analysis of the analysis method.

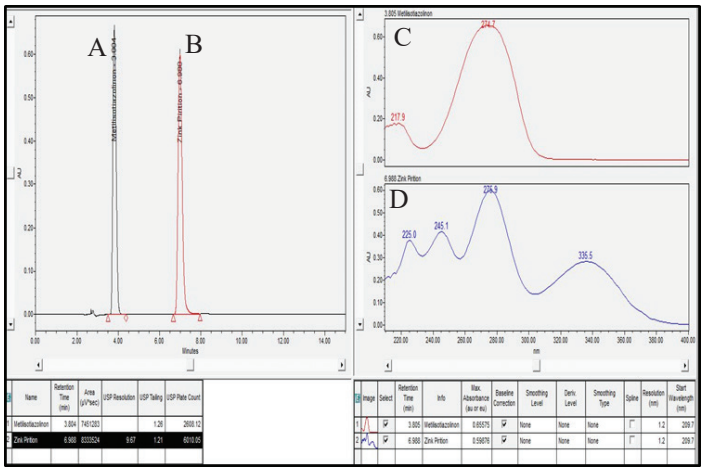

Figure 1. Chromatograms of (A) ZnPtO; (B) Methylisothiazolinone and PDA spectra of (C) Methylisothiazolinone ; (D) ZnPtO

Linearity, precision, and accuracy were analyzed by adding a certain amount of ZnPtO standard solution with concentrations of 20 µg/ml, 40 µg/ml, 80 µg/ml, 100 µg/ml, 140 µg/ml, and 160 µg/ml to the sample. The concentration range selected in the validation method was adjusted to the levels of ZnPtO compounds commonly contained in circulating samples after orientation process of several samples and and also adjusted to the current regulations, where the level of ZnPtO in shampoo is not more than 2.0%. The concentration range above reflects the levels of ZnPtO contained in the shampoo of 0.4%, 0.8%, 1.6%, 2%, 2.8%, and 3.2%. With the selection of these concentration ranges, the analytical method is expected can be used for testing the ZnPtO content in anti-dandruff shampoo at low and high concentration conditions. Table 1 shows the solution codes used to determine the method's linearity, precision, and accuracy.

Table 1. Solutions for Determination of Linearity, Precision, and Accuracy

Validation Parameters	Solution Concentration (µg/ml)*					
	20	***40***	***80***	***100***	***140***	***160***
Linearity	P	Q	R	S	T	U
Precision	P	-	-	S	-	U
Accuracy	P	-	-	S	-	U

*Linearity was achieved by making two solutions for each concentration level (duplo), while precision and accuracy were achieved triple. Solutions were made 1x for all validation parameter determinations.

From the linearity analysis, the correlation coefficient (r) value obtained is 0.9999, and the residual deviation value (V_{x0}) is 0.65%. Based on the literature, the requirements for the value of the correlation coefficient and residual deviation (V_{x0}) are more than 0.999 and less than 5%, respectively (Indrayanto, 2018). While other literature states that the requirements for the correlation coefficient (r) and residual deviation (V_{x0}) are more than and equal to (≥) 0.995 and less than (<) 5% (Yuwono & Indrayanto, 2005). So that the linearity parameters of this validation method has meet the requirements ~~set~~. The calibration curve of ZnPtO Standard and series of ZnPtO concentration in spiked sample for linearity analysis can be seen in Figure 2.

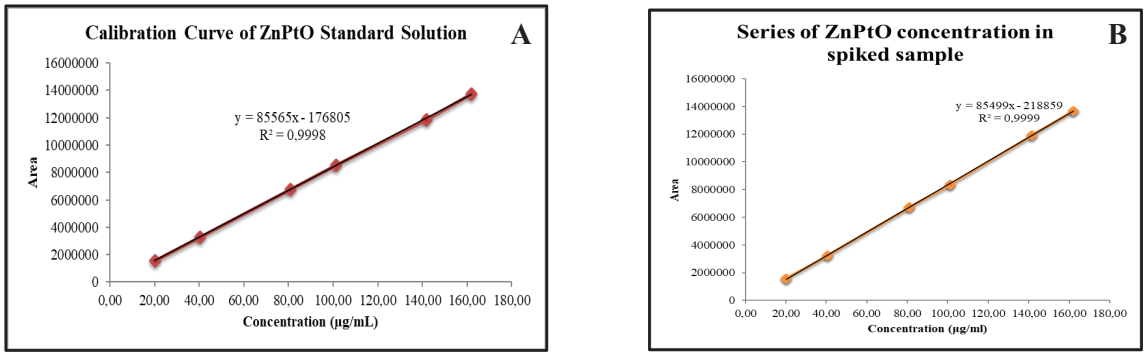

Figure 2. Calibration curve of ZnPtO standard (A); and series of ZnPtO concentration in spiked sample (B)

The method repeatability is expressed as a percentage of *Relative Standard Deviation* (%RSD). The %RSD value is a value that indicates the precision/repeatability of several data in a series test. The greater the %RSD value indicates that the precision/repeatability method is poor. This means that the method cannot be used for testing because one of the validation parameters does not meet the requirements stated in the *Official Methods of Analysis of AOAC International* (AOAC International, 2023). Measurement of the %RSD value is one of the validation parameters that must be measured to prove that the analytical method developed is robust with a good repeatability. The %RSD value in determining the method's precision was obtained as 0.6694%, 0.4511%, and 0.4728% at three different concentration levels. Meanwhile, the recoveries (%recoveries) in determining the method's accuracy were obtained in the range of 99.4 - 100.9%, 98.3 - 98.9%, and 99.3 - 100.0% for the three injected concentration levels. The limit of quantitation (LoQ) value obtained in this study was 0.23 µg/ml.

Table 2. Results of Linearity, Precision, and Accuracy Determination

Validation Parameters	Requirements	Results	Concentration (µg/ml)
Linearity	r < 0,995 V_{x0} <5%	r: 0,999 V_{x0}: 0.65%	20 - 160
Precision	≤ 7,3 ≤5,3 ≤5,3	0,6694% 0,4511% 0,47288%	20 100 160
Accuracy	80 - 110 % 90 - 107 % 90 - 107 %	99,4 - 100,9 % 98,3 - 98,9 % 99,3 - 100,0%	20 100 160

3.2.Analysis Result of ZnPtO Content in Samples

This developed analysis method for determining of ZnPtO content using a high-performance liquid chromatography (HPLC) instrument was carried out through chelate formation with Na_2EDTA compound. ZnPtO contained in the sample reacts with the mobile phase of the EDTA solution to form chelates according to the following reaction:

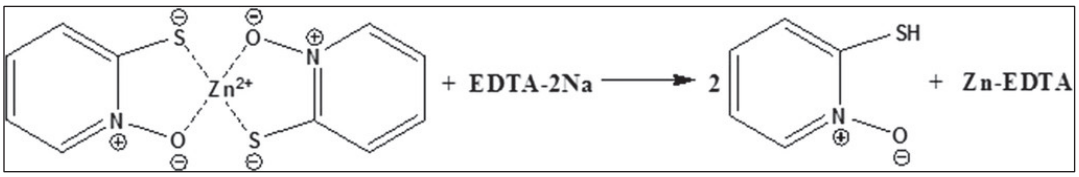

Figure 3. ZnPtO reaction with EDTA

EDTA solution will chelate the Zinc cation on ZnPtO so that it decomposes into pyrothione anion (Kim et al., 2018). With the formation of the Zn-EDTA chelate, ZnPtO compounds can be analyzed quickly using a HPLC instrument.

The sample preparation process in the developed analytical method is relatively simple because it is only carried out by dissolving the sample within a short analysis time.

The validation results of the method have proven its validity so that it can be used to analyze the active substance content of ZnPtO in 3 (three) anti-dandruff shampoo samples on the market.

The analysis results on several shampoo samples obtained randomly from the market are shown in Table 3.

Table 3. Analysis result of zinc pyrithione content in anti-dandruff shampoo

Sample Name	ZnPtO area	*Average Area of ZnPtO*	*ZnPtO concentration (µg/ml)*	*ZnPtO concentration (%)*
Sample X	6732381			
	7009619	6791789	81,44	0,0081
	6633366			
Sample Y	3227070			
	3240873	3227596	39,78	0,0040
	3214845			
Sample Z	13681325			
	13553106	13607980	161,10	0,016
	13589509			

Based on the test results of 3 (three) samples, the ZnPtO content in each sample was 0.0081%, 0.0040%, and 0.016%, respectively. These results indicate that all samples tested are still below the specified requirement of 2.0% (BPOM RI, 2022).

The developed analytical method can be used to analyze anti-dandruff shampoo samples circulating on the market. However, with the diverse matrix composition of anti-dandruff shampoos circulated on the market, it is necessary to apply the method to several other types of anti-dandruff samples. Thus, more representative test data regarding analytical methods for monitoring shampoo products on the market will be obtained.

4. Conclusion

The analytical method for determining the level of ZnPtO in anti-dandruff shampoo developed using the High-Performance Liquid Chromatography (HPLC) instrument has met all the validation parameters requirements so that the method can be used to analyze the ZnPtO content in anti-dandruff shampoo, both for quality control and product safety before and during the product circulating on the market due to guarantee public protection against

shampoo products that do not meet the requirements. Preparation of ZnPtO standard and samples using the developed method is more straightforward because it only goes through a dissolution process using a simple solvent with analytical conditions that are also relatively easy but can be applied to various anti-dandruff shampoo sample matrices.

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