Katona Gabor*
https://orcid.org/0000-0003-0783-1117

Vojvodić Slađana**
https://orcid.org/0000-0002-9772-3998

Stojiljković Dragana***
https://orcid.org/0000-0001-6275-9299

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REGULATORY ASPECTS OF COSMETIC PRODUCT TESTING AND THE ROLE OF ALTERNATIVE MODELS IN MODERN STRATEGIES

ABSTRACT: Modern regulatory frameworks in the field of cosmetic products increasingly promote the use of alternative methodologies (New Approach Methodologies, NAMs) based on *in vitro*, *ex vivo*, and *in silico* approaches, accompanied by strict limitations or complete bans on animal testing. In such a regulatory environment, interest is growing in models capable of bridging the gap between cellular systems and complex *in vivo* studies. Zebrafish (*Danio rerio*) embryos have been recognized as a potential bridging model due to their high biological relevance, testing versatility, and the more favorable ethical status of early developmental stages. This paper provides a systematized overview of regulatory frameworks in the EU, the USA, Asia, and Serbia, highlighting the degree of acceptance of alternative methods and the specific status of zebrafish in the context of the safety assessment of cosmetic products and ingredients. It analyzes the scientific advantages and limitations of the zebrafish model,

* Teaching Assistant, Pharm. Spec., PhD Candidate, University Business Academy in Novi Sad, Faculty of Pharmacy, Novi Sad, Serbia, e-mail: gabor.katona@ffns.ac.rs
** PhD, Assistant Professor, University Business Academy in Novi Sad, Faculty of Pharmacy, Novi Sad, Serbia, e-mail: sladjana.vojvodic@ffns.ac.rs

including genetic similarity to humans, embryonic transparency, and rapid development, as well as limitations that restrict its use as primary evidence in regulatory documentation. The comparative analysis indicates that zebrafish are most appropriately used as a supplementary source of data within weight-of-evidence and NAM-oriented strategies, particularly in early-stage hazard screening and the mechanistic understanding of the effects of new bioactive substances. In line with international trends and the harmonization of the national regulatory framework with EU requirements, the findings suggest that zebrafish embryos represent a valuable research and development tool, but not a substitute for validated methods that form the basis of regulatory safety assessment of cosmetic products.

Keywords: *regulatory frameworks, cosmetic product testing, new approach methodologies (NAMs), weight-of-evidence, zebrafish (Danio rerio).*

1. Introduction

Methods for assessing the safety and efficacy of cosmetic products and ingredients are most commonly classified into *in vitro* and *in vivo* approaches, with an increasingly important role of *ex vivo* and *in silico* methods in modern safety-assessment concepts (Barthe et al., 2021; Silva & Tamburic, 2022).

In the European Union (EU), testing cosmetic products and their ingredients on animals is prohibited under the Cosmetics Regulation (Regulation (EC) No 1223/2009), which clearly defines that, for any stage of product development, including product ingredients as well as evidence of safety, data obtained from animal experiments may not be used.

This regulation has stimulated the development and application of the so-called ***New Approach Methodologies*** **(NAMs)**, which enable ethically acceptable and scientifically robust safety assessments. By stimulating innovation in alternative risk-assessment methods and by combining different types of evidence, the need for classical animal testing is eliminated, while predictivity of the safety assessment is enhanced (Baltazar et al., 2020; Silva & Tamburic, 2022).

In silico methods encompass computational models, including QSAR (Quantitative Structure-Activity Relationship) and related approaches, which predict potential biological and toxicological effects of substances based on structure and existing databases, forming an important pillar of next-generation risk assessment in cosmetology (Cronin et al., 2022; Baltazar et al., 2020). ***Ex vivo*** methods use biological material, most commonly excised skin, under conditions

that retain some physiological relevance, which is particularly important for studies of dermal penetration and local effects (Barthe et al., 2021***). In vitro*** methods are conducted on isolated cells, tissues or reconstructed 3D models of human skin, providing high control of experimental conditions and good standardization, although with limitations regarding systemic complexity of an organism (Barthe et al., 2021; Silva & Tamburic, 2022). ***In vivo*** approaches in the field of cosmetics refer, in practice, to controlled studies in human volunteers, with strict ethical requirements and caution in data interpretation, especially regarding historically used irritation and sensitization tests (Scientific Committee on Consumer Safety [SCCS], 2015; Barthe et al., 2021; Robinson, McFadden & Basketter, 2001). Due to ethical and regulatory considerations, modern strategies for evaluating cosmetic ingredients increasingly rely on combining *in vitro*, *ex vivo*, and *in silico* evidence within an integrated **"weight-of-evidence"** approach (Silva & Tamburic, 2022; Baltazar et al., 2020).

In the context of developing new bioactive compounds relevant to cosmetic products, particularly in the early screening phase of effects and safety, **zebrafish models** may represent a useful intermediate level of biological complexity between cell-based systems and classical vertebrate models (Zon & Peterson, 2005; MacRae & Peterson, 2015; Bauer, Mally & Liedtke, 2021). The Zebra fish (*Danio rerio*) is a freshwater species originating from South Asia, naturally distributed across parts of India, Bangladesh, Nepal, Myanmar and Pakistan (Lawrence, 2007; Whiteley et al., 2011). The introduction of zebrafish as a laboratory model intensified during the 1980s, with the work of Streisinger and colleagues representing a foundational milestone in modern zebrafish genetics (Streisinger et al., 1981). Zebrafish has become a widely accepted vertebrate model due to its small size, low maintenance cost, external embryonic development, optical transparency, and suitability for high-throughput testing (Penberthy, Shafizadeh & Lin, 2002; MacRae & Peterson, 2015). Genomic comparisons show that approximately 70% of human genes have at least one clear ortholog in zebrafish, further supporting its translational potential (Howe et al., 2013). Embryogenesis and early organogenesis proceed rapidly, while high fecundity enables the production of large numbers of embryos in a short period, making the model well suited for toxicological and pharmacological screening (Russo et al., 2022). Transparent embryos facilitate direct observation of developmental changes and phenotypic effects of tested substances, and exposure to hydrophilic compounds is often easily achieved by simple addition of the substance to the water (Bauer et al., 2021; MacRae & Peterson, 2015). Thus, the zebrafish embryo model can be viewed as an intermediate step between *in vitro* systems and more complex *in vivo* approaches in the

research and development of active substances, including those with potential dermatological and pigmentation effects (Russo et al., 2022; Qu et al., 2023).

Modern safety assessment and evidence-based substantiation of cosmetic product efficacy increasingly rely on combining *in vitro*, *ex vivo*, and *in silico* approaches, with cautious, ethically strictly controlled use of *in vivo* testing in human volunteers. This shift results from regulatory bans and the strong conceptual orientation toward the 3R principles (Replacement, Reduction, Refinement), alongside the development of integrated risk-assessment strategies that include "weight-of-evidence" and IATA (Integrated Approaches to Testing and Assessment) approaches. Within this framework, zebrafish embryos gain importance as a potential bridging model between reduced cell systems and complex organism-level responses, providing relatively rapid and high-throughput insight into early toxicity signals and certain functional effects of bioactive substances (Strähle et al., 2012; MacRae & Peterson, 2015; Bauer et al., 2021).

2. Aim of the study

Despite considerable progress in developing alternative methodologies and regulatory shifts limiting the use of animal models in cosmetic testing, there remains a pronounced need for reliable, ethically acceptable, and scientifically relevant approaches that can bridge the gap between *in vitro* and *in vivo* systems. Particularly in the early phases of research and development of new bioactive compounds, existing methods are often insufficient to fully characterize the hazard profile, while the regulatory framework simultaneously requires a high degree of scientific rigor. In this context, the motivation for the present study arises from the need to critically examine the regulatory, methodological and practical landscape in which zebrafish models may be applied as scientifically justified components of "weight-of-evidence" strategies in safety and biological-activity assessment of cosmetic ingredients, especially considering that zebrafish aligns with key requirements of modern approaches aimed at 3R compliance and enhancing the predictive value of alternative models.

The aim of this study is to systematize and compare regulatory frameworks in the EU, USA, Asia and Serbia concerning the acceptability of alternative methods and the regulatory status of zebrafish in cosmetic-product testing; to analyze the scientific advantages and limitations of the zebrafish embryonic model in the context of cosmetic safety and efficacy assessment; and to identify realistic possibilities for integrating zebrafish into NAM-oriented strategies. Additionally, the study aims to provide an evidence-based

position on how these data can be used as supplementary, but not primary, evidence in regulatory documentation, particularly in light of harmonization with EU requirements and modern "weight-of-evidence" approaches.

3. Regulatory frameworks and the status of zebrafish (Danio rerio) in cosmetic-product testing

In the European Union, the regulatory context for such an approach is defined by Regulation (EC) 1223/2009 (Regulation (EC) No 1223/2009) and the associated history of the complete ban on animal testing of cosmetic products and ingredients, with the marketing ban fully enforceable since 2013 (European Commission, 2013). Consequently, validated *in vitro* methods, particularly reconstructed human skin systems and defined approaches for key toxicological endpoints, have become central pillars of safety justification, with the Scientific Committee on Consumer Safety (SCCS) guidelines serving as a practical regulatory compass (SCCS, 2015). Concurrently, the European animal welfare framework legally protects fish developmental stages up to the free-feeding stage, which practically allows early embryonic zebrafish testing to be treated more favorably than classical vertebrate models when used in clearly defined research objectives (Strähle et al., 2012). Methodologically, the OECD TG 236 *Fish Embryo Acute Toxicity test* represents a standardized international framework for fish embryonic acute toxicity testing, primarily in chemical and ecotoxicological domains, but its rationale can also be applied as supplementary evidence in early screening of cosmetic raw materials, with cautious regulatory positioning (Organisation for Economic Co-operation and Development [OECD], 2019; European Union Reference Laboratory for alternatives to animal testing [EURL ECVAM], 2014).

In **the United States (USA)**, the federal framework does not require animal testing of cosmetic products as a condition for market placement, whereas the Modernization of Cosmetics Regulation Act (MoCRA) strengthens manufacturers' responsibility to maintain adequate safety substantiation (U.S. Food and Drug Administration [FDA], 2022). At the same time, regulatory practice is becoming more complex due to state-level bans on the sale of animal-tested cosmetics, with California being one of the most prominent examples (Steptoe, 2018). In terms of laboratory animal welfare, it is important to note that *the Animal Welfare Act* does not cover fish, while *the Public Health Service* policy institutionally mandates oversight for vertebrate species used in research funded by relevant sources. Practically, this means that the ethical status of zebrafish and their developmental stages may vary

depending on the institution and funding regime (Office of Laboratory Animal Welfare [OLAW], 2021). In this environment, zebrafish embryos hold real value as an early screening tool in research and internal safety strategies, although without the role of primary regulatory evidence in a cosmetic dossier.

The **Asian** regulatory landscape is heterogeneous, but the dominant trend in major markets is toward reducing or eliminating mandatory animal tests for cosmetic products. China, through *the Cosmetics Supervision and Administration Regulation* (CSAR) and accompanying technical documents, has modernized its safety assessment system and allowed exemptions from mandatory animal tests for certain categories of general cosmetics under defined conditions (Cosmetics Supervision and Administration Regulation [CSAR], 2021; EU SME Centre, 2021). South Korea implemented a ban on animal testing for cosmetic products and ingredients, effective from 2018 (Cruelty Free International, 2018). Japan does not have a general statutory requirement for animal testing of cosmetic products and supports validation and regulatory acceptance of alternative methods through the Japanese Center for the Validation of Alternative Methods – JaCVAM (Japanese Center for the Validation of Alternative Methods [JaCVAM], 2020). India banned cosmetic animal testing in 2014 (Government of India, 2014). In this context, zebrafish embryos gain particular development value as a relatively rapid organismal model for early hazard screening and mechanistic investigation of new bioactive substances, with industry motivation to provide evidence compatible with multiple international markets.

Serbia is regulatory-wise closest to the **European model**. The current framework for cosmetic products and general-use items is largely harmonized with the EU approach, with transitional periods concluded and full implementation of stricter requirements for safety documentation and the Product Information File (PIF) from April 2023 (General-Use Products Act, 2019; Rulebook on Cosmetic Products, 2019; CMS, 2025). The Animal Welfare Act (2009) regulates general principles of animal experimentation but does not provide detailed thresholds for fish embryonic development identical to European interpretations, leaving practical discretion to ethical committees according to the 3R principle and specific study design.

Comparatively, Serbia shares the same strategic direction as the EU and much of the contemporary Asian regulatory landscape, while differing from the USA primarily in the explicitness of prohibitions, and less in scientific logic of safety assessment. In this context, it makes sense to consider zebrafish, particularly zebrafish embryos, as a research and development tool in domestic academic and industrial settings. Their greatest value lies in early filtering

of potentially hazardous raw materials, supplementary hazard profiling, and strengthening "weight-of-evidence" packages for innovative active substances, including biologically active complexes relevant to dermatological and pigmentation indications (MacRae & Peterson, 2015; Bauer et al., 2021). Nevertheless, from a regulatory perspective in Serbia, as in the EU, it is most rational to position zebrafish embryo data as supplementary rather than primary evidence of safety for cosmetic raw materials and finished products, relying on validated *in vitro* methods and clearly structured toxicological assessment within the PIF documentation (European Commission, 2013; SCCS, 2015; Rulebook on Cosmetic Products, 2019; General-Use Products Act, 2019).

The comparative regulatory analysis clearly indicates that regulatory approaches to cosmetic-product testing in the EU, USA, Asia and Serbia are increasingly converging toward NAM-oriented paradigms, with the EU remaining the most detailed and restrictive framework regarding the ban on animal testing and the explicit reliance on validated *in vitro* approaches within PIF and SCCS guidance, while the USA retains federal flexibility alongside growing state-level restrictions, and key Asian markets are rapidly approaching a "cruelty-free" model (European Commission, 2013; SCCS, 2015; FDA, 2022; Steptoe, 2018; CSAR, 2021; Cruelty Free International, 2018).

Comparative assessment of these frameworks allows for precise understanding of the acceptability of alternative methods and the position zebrafish embryos may occupy in different regulatory contexts, confirming that their formal status varies, but trends increasingly favor models that complement the existing NAM system.

A novel practical insight relevant to the region is that zebrafish embryos, thanks to international standardization through OECD TG 236 and the favorable ethical status of early developmental stages in European interpretations, can be rationally positioned as bridging research evidence in early selection and mechanistic validation of innovative cosmetic raw materials, but not as a primary regulatory pillar of safety (OECD, 2019; EURL ECVAM, 2014; Strähle et al., 2012; MacRae & Peterson, 2015).

In Serbia, which has been essentially aligned with EU requirements for safety documentation since April 2023, this creates a logical space for using zebrafish embryos as a supplementary element of the "weight-of-evidence" approach in academic and industrial development, while highlighting the need for clearer national professional guidance to standardize ethical committee expectations and the role of these data in a cosmetic dossier (General-Use Products Act, 2019; Rulebook on Cosmetic Products, 2019; CMS, 2025; Animal Welfare Act, 2009).

4. Conclusion

Analysis of the scientific literature confirms that the zebrafish embryonic model has clear advantages, such as low maintenance costs, optical transparency, and justified translational potential, but also identifies limitations that prevent it from being recognized as primary regulatory evidence. Within this framework, the realistic possibilities for integrating zebrafish into NAM strategies relate primarily to its positioning as a supplementary data source within a "weight-of-evidence" approach, confirming the most practical role of zebrafish in early research and developmental screening, where it contributes to better-informed decision-making and reduction of uncertainty in subsequent assessment phases. With proper methodological standardization and ethical compliance, zebrafish can rationally contribute to research and development processes, but it remains clear that its use cannot replace mandatory validated methods forming the basis of regulatory safety assessment.

The added value of these findings is that the zebrafish model is not proposed as a substitute for validated *in vitro* methods, but as a functional "intermediate step" that can enhance the predictivity of early development and complement integrated risk assessment strategies (IATA), particularly in areas where scientific gaps remain.

In this sense, the study supports the broader concept of contemporary regulatory trends that a "weight-of-evidence" approach requires combining multiple complementary sources of data, including information zebrafish can provide. This contributes to more rational and scientifically justified integration of this model, in compliance with the 3R principles and international standards.

Overall, the findings of this study confirm the set objectives and indicate that zebrafish is a valuable alternative model when used as a strategic complement within integrated NAM approaches, strengthening the scientific basis for safety assessment and promoting further harmonization with contemporary global regulatory trends.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization, G.K., S.V., and D.S.; methodology, G.K.; formal analysis G.K. and S.V.; writing – original draft preparation, G.K.; writing – review and editing, S.V. and D.S. All authors have read and agreed to the published version of the manuscript.

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Katona Gabor
Univerzitet Privredna akademija u Novom Sadu, Farmaceutski fakultet, Novi Sad, Srbija

Vojvodić Slađana
Univerzitet Privredna akademija u Novom Sadu, Farmaceutski fakultet, Novi Sad, Srbija

Stojiljković Dragana
Univerzitet Privredna akademija u Novom Sadu, Farmaceutski fakultet, Novi Sad, Srbija

REGULATORNI ASPEKTI TESTIRANJA KOZMETIČKIH PROIZVODA I ULOGA ALTERNATIVNIH MODELA U SAVREMENIM STRATEGIJAMA

APSTRAKT: Savremeni regulatorni okviri u oblasti kozmetičkih proizvoda sve snažnije podstiču primenu alternativnih metodologija (New Approach Methodologies, NAMs) zasnovanih na *in vitro*, *ex vivo* i *in silico* pristupima, uz stroga ograničenja ili potpunu zabranu testiranja na životinjama. U takvom okruženju raste interesovanje za modele koji mogu popuniti praznine između ćelijskih sistema i kompleksnih *in vivo* ispitivanja. Embrioni zebrice (*Danio rerio*) prepoznati su kao potencijalni mostovni model zahvaljujući visokoj biološkoj relevantnosti, mogućnostima

testiranja i povoljnijem etičkom statusu ranih razvojnih stadijuma. Ovaj rad daje sistematizovan pregled regulatornih okvira u EU, SAD, Aziji i Srbiji, naglašavajući stepen prihvatljivosti alternativnih metoda i specifičan status zebrice u kontekstu procene bezbednosti kozmetičkih proizvoda i sastojaka. Analiziraju se naučne prednosti i ograničenja zebrice, uključujući genetsku srodnost sa ljudima, transparentnost embriona i rapidan razvoj, ali i nedostatke koji ograničavaju njenu upotrebu kao primarni dokaz u regulatornoj dokumentaciji. Uporedna analiza pokazuje da se zebrica najracionalnije može koristiti kao dopunski izvor podataka u okviru *weight-of-evidence* i NAM-orijentisanih strategija, posebno u ranoj fazi hazard skrininga i mehanističkog razumevanja dejstva novih bioaktivnih supstanci. U skladu sa međunarodnim trendovima i harmonizacijom domaćeg okvira sa EU zahtevima, rezultati ukazuju da embrioni zebrice predstavljaju vredan razvojno-istraživački alat, ali ne i zamenu za validirane metode koje čine osnovu regulatorne procene bezbednosti kozmetičkih proizvoda.

***Ključne reči*:** *regulatorni okviri, testiranje kozmetičkih proizvoda, alternativne metodologije (NAMs), weight-of-evidence, zebrica (Danio rerio).*

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