**mgr farm. Krzysztof Adam Stępień**

# Wybrane aspekty jakości niektórych suplementów diety zawierających aminokwasy

**Rozprawa na stopień doktora nauk medycznych i nauk o zdrowiu w dyscyplinie nauki farmaceutyczne**

Promotor: **dr hab. n. farm. Joanna Giebułtowicz**

**Zakład Chemii Leków, Analizy Farmaceutycznej i Biomedycznej**

WYDZIAŁ
FARMACEUTYCZNY
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# Spis treści

# Wykaz publikacji stanowiących pracę doktorską

1) **Stępień, K.A.**; Niewiarowski, J.; Harasimiuk, A. *Powszechność suplementów diety a zagrożenia związane z ich stosowaniem*; Biuletyn Wydziału Farmaceutycznego Warszawskiego Uniwersytetu Medycznego; 2019; 9; 51-59.
   DOI: https://doi.org/10.56782/pps.22
2) **Stępień, K.A**.; Giebułtowicz, J. *Application of Liquid Chromatography Coupled to Mass Spectrometry in Quality Assessment of Dietary Supplements—A Case Study of Tryptophan Supplements: Release Assay, Targeted and Untargeted Studies;* Pharmaceuticals; 2022; 15 (4); 448.
   DOI: https://doi.org/10.3390/ph15040448
   Punkty IF: 4,6.
3) **Stępień, K.A**.; Krawczyk, W.; Giebułtowicz, J. *Dietary Supplements with Proline — A Comprehensive Assessment of Their Quality*; Life; 2023; 13 (2); 263.
   DOI: https://doi.org/10.3390/life13020263
   Punkty IF: 3,2.
4) **Stępień, K.A.**; Kalicka, A.; Giebułtowicz, J. *Screening the quality of legal and illegal dietary supplements by LC-MS/MS*; Food Additives and Contaminants: Part B; 2024; 1-14.
   DOI: https://doi.org/10.1080/19393210.2024.2382221
   Punkty IF: 2,5.
5) **Stępień, K.A**.; Myslitska, D; Garbacz, G.; Paszkowska J.; Giebułtowicz, J. *Pharmaceutical availability testing – can a drug-specific test be relevant for assessing the quality of dietary supplements?;* Microchemical Journal; 2024; 112132.
   DOI: https://doi.org/10.1016/j.microc.2024.112132
   Punkty IF: 4,9.

   Sumaryczny współczynnik oddziaływania IF: **15,2**.

# Wykaz doniesień zjazdowych dotyczących pracy doktorskiej

1) Doniesienie zjazdowe nr 1

"Quality of medicines versus pharmaceutical crimes - health risks"; Międzynarodowa Konferencja Naukowo-Szkoleniowa; ISBN 978-83-946124-9-8; s. 111-115.
**Stępień, K.A.;** Wybrane aspekty jakości suplementów diety.
Warszawa; 18.11.2019 – **wystąpienie ustne**

2) Doniesienie zjazdowe nr 2

„Współczesna analityka farmaceutyczna i biomedyczna w ochronie zdrowia"; III Poznańska Konferencja Naukowo-Szkoleniowa; ISBN 978-83-7597-410-2; s. 100.
**Stępień, K.A.;** Wybrane aspekty jakości suplementów diety zawierających L-tryptofan.
Poznań, 04-05.06.2020 – **sesja plakatowa**

3) Doniesienie zjazdowe nr 3

"Synergy of interdisciplinary innovations"; Interdisciplinary Conference on Drug Science ACCORD 2022; ISBN 978-83-7637-586-1; s. 146.
**Stępień, K.A**.; Giebułtowicz, J.; Tryptophan dietary supplements - release test, targeted and untargeted screening.
Warszawa, 26-28.05.2022 – **sesja plakatowa**

4) Doniesienie zjazdowe nr 4

"High resolution mass spectrometry Fundamentals, Advances, and Applications"; International School on Mass Spectrometry (IntSMS); s. 7.
**Stępień, K.A**.; Giebułtowicz, J. Food quality assessment - a case study of tryptophan supplements: release test, targeted and non-targeted studies.
Włochy, Erice, 25-30.09.2022 – **sesja plakatowa**

5) Doniesienie zjazdowe nr 5

„Pharmaceutical education, science and practice: state, problems, prospects of development"; Conference with international participation, dedicated to the 25$^{th}$ anniversary of the pharmaceutical faculty; s. 373-374.
**Stępień, K.A**.; Giebułtowicz, J. Food quality assessment - a case study of tryptophan supplements: release test, targeted and non-targeted studies.

Bogomolets National Medical University, 19-20.12.2023 – **wystąpienie ustne**

6) Doniesienie zjazdowe nr 6

"Pharmaceutical innovations across the borders"; Interdisciplinary Conference on Drug Sciences, ACCORD 2024; ISBN 978-83-7637-632-5; s. 60.
**Stępień, K.A.**; Myslitska, D.; Garbacz, G.; Paszkowska, J.; Giebułtowicz, J. The evaluation of the quality of dietary supplements using dissolution test.
Warszawa, 23-25.05.2024 – **sesja plakatowa**

7) Doniesienie zjazdowe nr 7

"Pharmaceutical innovations across the borders"; Interdisciplinary Conference on Drug Sciences, ACCORD 2024; ISBN 978-83-7637-632-5; s. 59.
**Stępień, K.A**.; Kalicka, A.; Giebułtowicz, J. Quality Assessment of Dietary Supplements Using Liquid Chromatography Coupled with Mass Spectrometry.
Warszawa, 23-25.05.2024 – **sesja plakatowa**

# Streszczenie

Łatwość wprowadzania suplementów diety na rynek, niedostateczna kontrola ich jakości przez instytucje państwowe, takie jak Główny Inspektor Sanitarny, oraz wysoka popularność i wartość rynkowa sprawiają, że produkty te są szczególnie narażone na zaniedbania oraz zafałszowania. W najlepszym przypadku może to skutkować brakiem oczekiwanego efektu fizjologicznego/odżywczego, a w najgorszym – stanowić zagrożenie dla zdrowia, a nawet życia konsumentów. Kontrola jakości suplementów diety powinna dotyczyć tych samych podstawowych aspektów, co kontrola jakości produktów leczniczych, ponieważ w obu przypadkach preparaty przeznaczone są do użytku konsumenckiego, mogą występować w takich samych postaciach oraz mogą niejednokrotnie zawierać te same substancje czynne. Niemniej określenie jakości suplementów diety niejednokrotnie jest wyzwaniem, ponieważ mogą one zawierać wiele, czasem nieznanych składników, o odmiennych właściwościach fizykochemicznych. Niedostateczna liczba danych dotyczących jakości suplementów diety, szczególnie badania uwalniania substancji z postaci w jakiej produkt występuje, sprawia, że trudno oszacować zagrożenia wynikające z zażywania suplementów oraz prawdopodobieństwo, że podczas stosowania preparatu, wystąpi u konsumenta zamierzony efekt działania.

Celem niniejszej pracy było określenie aspektów jakości wybranych suplementów diety zawierających jako główny składnik karnitynę, prolinę, tryptofan oraz tyrozynę. Ocena jakości obejmowała a) oznaczenie jakościowe substancji obecnych w badanych suplementach diety; b) określenie zawartości głównego składnika w kapsułkach/tabletkach; c) badanie uwalniania w celu oznaczenia ilości uwolnionego głównego składnika z kapsułek/tabletek o niemodyfikowanym uwalnianiu.

Podczas analizy określono jakość 68 suplementów diety. Z wykorzystaniem techniki chromatografii cieczowej sprzężonej ze spektrometrią mas, wykryto w ich składzie związki niezadeklarowane przez producenta. Były to substancje związane z głównym składnikiem suplementu diety, między innymi produkty jego transformacji (np. metabolity tryptofanu), a także substancje, których obecność była przypadkowa, prawdopodobnie związana z nieodpowiednimi warunkami produkcji (np. melatonina). Zawartość głównego składnika suplementów diety mieściła się w przedziale od 28 % do 156 % (w stosunku do deklarowanej przez producenta). Jego uwalnianie, określone metodami farmakopealnymi, zawierało się od 1 % do 131 % dla pH 1,2 oraz od 1 % do

119 % dla pH 6,8. Zastosowanie do badania uwalniania aparatu *PhysioCell*, pozwoliło na lepsze odwzorowanie warunków występujących w przewodzie pokarmowym. Umożliwiło to potwierdzenie, że jakość formulacji suplementów diety jest w przypadku większości badanach preparatów niska, a rozpad jednostki dawkowania zależy od uwarunkowań fizjologicznych przewodu pokarmowego.

Wyniki badań przedstawione w niniejszej pracy wskazują, iż konieczne jest opracowanie standardów weryfikacji jakości suplementów diety, szczególnie w kontekście uwalniania substancji czynnej. Uwalnianie warunkuje bowiem wystąpienie efektu działania, co jest bardzo istotne ze względu na dobro konsumenta.

***Słowa kluczowe:*** *suplement diety, kontrola jakości, badanie uwalniania, aparat PhysioCell*

# Abstract

**Title: Selected quality aspects of certain dietary supplements containing amino acids**

The ease of introducing dietary supplements to the market, insufficient quality control by state institutions such as the Chief Sanitary Inspector, and their high popularity and market value make these products particularly susceptible to negligence and adulteration. In the best-case scenario, this may result in a lack of the expected physiological effect, while in the worst case, it may pose a threat to the health and even the life of consumers. Determining the quality of dietary supplements is a challenge, as they often contain numerous ingredients with varying physicochemical properties. Regardless of this, quality control of dietary supplements should cover the same aspects as the quality control of medicinal products, as both are intended for consumer use, can come in the same forms, and may often contain the same active ingredients. The insufficient data on dietary supplement quality, particularly regarding studies on the release of active substances from the product's form, is unfavorable, as it leaves consumers uncertain about achieving the intended effects when using the supplement.

The aim of this study was to determine the quality aspects of selected dietary supplements containing carnitine, proline, tryptophan, and tyrosine as their main ingredients. The quality assessment included a) the detection of substances present in the examined supplements; b) the determination of the content of the main ingredient in capsules/tablets; c) dissolution test to quantify the amount of the main ingredient released from non-modified release capsules/tablets.

During the analysis, the quality of 68 dietary supplements was assessed. Using liquid chromatography coupled with mass spectrometry, compounds not declared by the manufacturers were identified in their composition. These included substances related to the main ingredient of the dietary supplement, such as its transformation products (e.g., tryptophan metabolites), as well as substances whose presence appeared incidental, likely linked to inadequate production conditions (e.g., melatonin). The content of the main ingredient in the dietary supplements ranged from 28 % to 156 % of the amount declared by the manufacturer. Its release, determined using pharmacopoeial methods, ranged from 1 % to 131 % at pH 1.2 and from 1 % to 119 % at pH 6.8. The use of the *PhysioCell*

apparatus for release testing allowed for a more accurate simulation of gastrointestinal conditions.

The results of this study indicate the need to establish standards for verifying the quality of dietary supplements, particularly concerning substance release. Release is a critical factor for the substance to exert its intended effect, which is of utmost importance for consumer well-being.

***Keywords:*** *dietary supplement, quality control, dissolution test, PhysioCell apparatus*

# 1. Wprowadzenie

Zgodnie z prawem Unii Europejskiej oraz Stanów Zjednoczonych, suplementy diety są definiowane jako produkty będące skoncentrowanym źródłem witamin, związków mineralnych lub innych substancji o działaniu odżywczym bądź fizjologicznym (np. aminokwasów, niezbędnych nienasyconych kwasów tłuszczowych, probiotyków czy ekstraktów roślinnych), przeznaczone do uzupełnienia regularnej diety (1, 2).

Rynek suplementów diety rozwija się dynamicznie, a producenci opracowują preparaty na problemy zdrowotne, związane z funkcjonowaniem prawie wszystkich narządów. Dodatkowo, rosnąca świadomość społeczeństwa na temat znaczenia odżywiania w życiu człowieka celem utrzymania zdrowia, w połączeniu z powszechnym przekonaniem, że współczesna żywność jest uboga w witaminy są jednymi z powodów stosowania suplementów diety (3). W Polsce suplementację stosuje 30–78 % młodzieży i dorosłych (4-6) oraz około 40 % dzieci (7). Tylko jedna czwarta badanych deklaruje, że przyjmuje suplementy zalecane przez lekarza, co oznacza, iż większość z nich stosuje suplementy diety na własną rękę (8). Głównym powodem stosowania suplementów diety w Polsce wśród osób starszych jest potrzeba wzmocnienia układu odpornościowego, a wśród osób młodszych, chęć poprawy zdrowia oraz kondycji skóry i włosów (6). Dodatkowo ponad 40 % ankietowanych Polaków uważa, że przyjmowanie witamin i składników mineralnych zapobiega chorobom u osób zdrowych, a prawie 70 % twierdzi, że stosowanie przeciwutleniaczy zapobiega rozwojowi nowotworów (9).

Suplementy diety, mimo że pod względem formy dawkowania, drogi podania i wyglądu opakowania przypominają produkty lecznicze, są klasyfikowane jako środki spożywcze i podlegają odmiennym regulacjom prawnym niż leki. Niestety, konsumenci, sugerując się podobieństwem tych produktów do środków farmaceutycznych, często błędnie zakładają, że przed wprowadzeniem na rynek zostały one przebadane pod kątem skuteczności oraz bezpieczeństwa stosowania. Brak konieczności przedstawienia przez producenta Głównemu Inspektorowi Sanitarnemu wyników badań potwierdzających odpowiednią jakość suplementów diety przed ich wprowadzeniem na rynek skutkuje tym, że dane na temat jakości tych produktów są skąpe. Te, które są dostępne w piśmiennictwie oraz raportach np. Narodowego Instytutu Leków, sygnalizują niską jakość badanych preparatów. Biorąc pod uwagę właściwości suplementów diety oraz dostępne dane literaturowe, zasadniczo można wyróżnić trzy podstawowe przyczyny niskiej jakości tych

produktów. Są to obecność substancji niezadeklarowanych przez producenta (10, 11), niższa od deklarowanej zawartość głównego składnika (12, 13) oraz niskie uwalnianie substancji z formy dawkowania (14).

Pierwszą przyczyną niskiej jakości suplementów diety jest obecność substancji niezadeklarowanych przez producenta. Może być ona konsekwencją nieodpowiednich praktyk produkcyjnych lub użycia niskiej jakości surowców. Przykładem są wykryte w suplementach diety metale ciężkie (15), związki występujące w surowcach roślinnych, np. herbicydy (16), insektycydy (17), mykotoksyny (18), dioksyny (19). Niektórzy producenci, aby wzmocnić działanie suplementu diety na organizm, celowo dodają do preparatów określone związki chemiczne, których stosowanie jest zabronione w produkcji suplementów diety. Przykładami są steroidy anaboliczno-androgenne (20) oraz substancje farmakologicznie czynne np. obniżające stężenie glukozy we krwi (21), hamujące aktywność enzymu fosfodiesterazy typu 5 (22), przyspieszające redukcję masy ciała (23). Aktualnie nie są rutynowo przeprowadzane badania tożsamości substancji obecnych w suplementach. Przyczynia się to do przypadkowych lub intencjonalnych nadużyć producentów. Niestety skala zjawiska nie jest znana.

Drugą przyczyną niskiej jakości suplementów diety jest niezgodność pomiędzy deklarowaną a rzeczywistą zawartością głównego składnika. W literaturze dostępnych jest niewiele danych dotyczących tego aspektu. Badanie zawartości opisywano w przypadku suplementów diety zawierających melatoninę (24) lub luteinę (14). W obydwu badaniach stwierdzono, że zawartość głównego składnika odbiega od deklarowanej zawartości w znacznie większym zakresie (w przypadku luteiny zawartość oznaczona stanowiła od 0,12 % do 135,2 % deklarowanej zawartości), aniżeli dopuszczają wymagania farmakopealne dla zawartości substancji czynnej w produkcie leczniczym. Tak duże różnice w zawartości substancji w tabletkach/kapsułkach suplementów diety mogą być spowodowane nieodpowiednią ilością substancji użytej do produkcji lub brakiem jednolitości zawartości substancji. Jednolitość zawartości substancji jest krytycznym aspektem jakości każdego produktu. Zmienność tego parametru może być spowodowana aglomeracją cząstek, nieprawidłowościami na etapie mieszania składników lub utratą składnika (np. w wyniku adsorpcji na powierzchni sprzętu) (25). W przypadku produktów leczniczych dostępnych na rynku, które muszą spełniać wymagania zawarte w Farmakopei, brak jednolitości zawartości jest mało prawdopodobny (26). Jedyne badanie ukazujące problem w tym aspekcie, dotyczy braku jednolitości zawartości

w obrębie jednej tabletki, czyli pojawia się w przypadku dzielenia tabletek (27). Aby zapewnić konsumentom suplementów diety odpowiednią jakość produktu, producenci powinni potwierdzić, że zawartość substancji jest jednolita we wszystkich jednostkach dawkowania i zgodna z deklarowaną.

Trzecią przyczyną niskiej jakości suplementów diety jest niskie uwalnianie substancji z formy dawkowania. Badanie dostępności farmaceutycznej określa ilość substancji czynnej uwalnianej z postaci leku w jednostce czasu. Jest to obowiązkowe i najważniejsze badanie postaci leku w przypadku produktów leczniczych, ale nie jest wymagane w przypadku suplementów diety, nawet jeśli występują one w tej samej postaci co produkt leczniczy (28). Ilość uwolnionej substancji z formy dawkowania *in vivo* determinuje ilość substancji, która potencjalnie może się wchłaniać (29). Oznacza to, że niskie uwalnianie, może powodować niskie wchłanianie substancji, co może skutkować brakiem lub niedostatecznym efektem działania tej substancji na organizm. Zatem, nawet jeżeli substancja występuje w deklarowanej ilości w suplemencie diety, ale jej uwalnianie z formy dawkowania jest niskie, nie będzie możliwe osiągniecie zamierzonego efektu u konsumenta. Ze względu na to, że badanie uwalniania substancji nie jest obowiązkowe dla suplementów diety, niewiele jest danych na ten temat. Wyniki badań, które zostały opublikowane sygnalizują niskie uwalnianie składników aktywnych, takich jak węglan wapnia (30), melatonina (24), kwas foliowy (31), żelazo, cynk, mangan (32). Jednak w wymienionych badaniach przyczyny niskiego uwalniania substancji nie są znane, ze względu na brak danych dotyczących jej zawartości w jednostce dawkowania. Niskie uwalnianie może bowiem wynikać z niedostatecznej zawartości głównego składnika, niewłaściwie dobranych parametrów procesu technologicznego i/lub niewłaściwie dobranych substancji pomocniczych. Mając na uwadze powyższe, aby uzyskać wiarygodną ocenę jakości suplementu diety oraz aby konsument miał pewność, iż podczas stosowania suplementu diety wystąpi u niego zamierzony efekt działania, konieczne jest wykonanie badania uwalniania substancji z formy dawkowania.

Dokonując analizy dostępnego piśmiennictwa, można zauważyć brak danych dotyczących badania uwalniania oraz rzeczywistego składu jakościowego i ilościowego suplementów diety zawierających aminokwasy, takie jak prolina, tryptofan czy tyrozyna. W przypadku suplementów diety zawierających karnitynę, liczba dostępnych danych jest natomiast ograniczona do analizy ilościowej. Dlatego w niniejszej pracy, podjęto się uzupełnienia brakujących informacji dotyczących jakości dostępnych w Polsce

suplementów diety dokonując analizy ich składu jakościowego i ilościowego oraz wykonując badanie uwalniania głównego składnika.

Podsumowując, łatwość wprowadzania suplementów diety na rynek, niedostateczna kontrola ich jakości przez instytucje państwowe, takie jak Główny Inspektor Sanitarny, oraz wysoka popularność i wartość rynkowa sprawiają, że produkty te są szczególnie narażone na zaniedbania oraz zafałszowania. Kontrola jakości suplementów diety powinna dotyczyć tych samych podstawowych aspektów, co kontrola jakości produktów leczniczych, ponieważ w obu przypadkach preparaty przeznaczone są do użytku konsumenckiego, mogą występować w takich samych postaciach oraz mogą zawierać, w wielu przypadkach, te same substancje czynne. Niemniej określenie jakości suplementów diety niejednokrotnie jest wyzwaniem, ponieważ mogą one zawierać wiele, czasem nieznanych składników, o odmiennych właściwościach fizykochemicznych. Niedostateczna ilość danych dotyczących jakości suplementów diety jest niekorzystna, a czasem nawet niebezpieczna dla zdrowia konsumentów (33). Dlatego potrzebna jest międzynarodowa współpraca, mająca na celu pogłębianie wiedzy w tym obszarze.

## Piśmiennictwo


1. Directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of the Member States relating to food supplements. Official Journal of the European Communities: Legislation. 2002;45:51-7.
2. Dietary Supplement Health and Education Act of 1994. Editor: Public Law; 1994. p. 103-417.
3. Wierzejska RE. Dietary supplements—for whom? The current state of knowledge about the health effects of selected supplement use. International Journal of Environmental Health Research. 2021;18(17):8897.
4. Wawryk-Gawda E, Budzyńska B, Lis-Sochacka M, Chylińska-Wrzos P, Zarobkiewicz M, Jodłowska-Jędrych B. Dietary supplements–consumer assessment based on questionnaire survey. Epidemiological Review. 2018;72(1):111-20.
5. Waskiewicz A, Sygnowska E, Broda G, Chwojnowska Z. The use of vitamin supplements among adults in Warsaw: is there any nutritional benefit? Roczniki Państwowego Zakładu Higieny. 2014;65(2).
6. Suliga K, Grzelak T, Grupińska J, Pelczyńska M, Sperling M, Czyżewska K. Evaluation of using dietary supplements among polish adult people below and over 60 years of age. Journal of Medical Science. 2017;86(3):213-9.
7. Sicińska E, Pietruszka B, Januszko O, Kałuża J. Different socio-demographic and lifestyle factors can determine the dietary supplement use in children and adolescents in central-eastern Poland. Nutrients. 2019;11(3):658.
8. Ronis MJ, Pedersen KB, Watt J. Adverse effects of nutraceuticals and dietary supplements. Annual review of pharmacology and toxicology. 2018;58(1):583-601.

9. Karbownik MS, Horne R, Paul E, Kowalczyk E, Szemraj J. Determinants of knowledge about dietary supplements among polish internet users: Nationwide cross-sectional study. Journal of medical Internet research. 2021;23(4):e25228.

10. Moret S, Prevarin A, Tubaro F. Levels of creatine, organic contaminants and heavy metals in creatine dietary supplements. Food Chemistry. 2011;126(3):1232-8.

11. Costa JG, Vidovic B, Saraiva N, do Ceu Costa M, Del Favero G, Marko D, et al. Contaminants: a dark side of food supplements? Free Radical Research. 2019;53(sup1):1113-35.

12. Andrews KW, Roseland JM, Gusev PA, Palachuvattil J, Dang PT, Savarala S, et al. Analytical ingredient content and variability of adult multivitamin/mineral products: national estimates for the Dietary Supplement Ingredient Database. The American Journal of Clinical Nutrition. 2017;105(2):526-39.

13. De Ceglie C, Calvano CD, Zambonin CG. MALDI-TOF MS for quality control of high protein content sport supplements. Food Chemistry. 2015;176:396-402.

14. Anselmo CS, Mendes TC, Cabral LM, Sousa VP. Physicochemical quality profiles of commercial oral tablets and capsules containing lutein - impact of insufficient specific sanitary regulations. Annals of the Brazilian Academy of Sciences. 2018;90(3):3063-73.

15. Kowalski A, Frankowski M. Levels and potential health risks of mercury in prescription, non-prescription medicines and dietary supplements in Poland. Regulatory Toxicology and Pharmacology. 2015;73(1):396-400.

16. Nieto-García AJ, Romero-González R, Frenich AG. Multi-pesticide residue analysis in nutraceuticals from grape seed extracts by gas chromatography coupled to triple quadrupole mass spectrometry. Food Control. 2015;47:369-80.

17. Chen Y, Lopez S, Hayward DG, Park HY, Wong JW, Kim SS, et al. Determination of multiresidue pesticides in botanical dietary supplements using gas chromatography–triple-quadrupole mass spectrometry (GC-MS/MS). Journal of Agricultural and Food Chemistry. 2016;64(31):6125-32.

18. Martínez-Domínguez G, Romero-González R, Arrebola FJ, Frenich AG. Multi-class determination of pesticides and mycotoxins in isoflavones supplements obtained from soy by liquid chromatography coupled to Orbitrap high resolution mass spectrometry. Food Control. 2016;59:218-24.

19. Fernandes AR, Rose M, White S, Mortimer DN, Gem M. Dioxins and polychlorinated biphenyls (PCBs) in fish oil dietary supplements: occurrence and human exposure in the UK. Food Additives & Contaminants: Part A. 2006;23(9):939-47.

20. Martínez-Sanz JM, Sospedra I, Ortiz CM, Baladía E, Gil-Izquierdo A, Ortiz-Moncada R. Intended or unintended doping? A review of the presence of doping substances in dietary supplements used in sports. Nutrients. 2017;9(10):1093.

21. Zhou Z, Zhang J, Zhang W, Bai Y, Liu H. Rapid screening for synthetic antidiabetic drug adulteration in herbal dietary supplements using direct analysis in real time mass spectrometry. Analyst. 2011;136(12):2613-8.

22. Patel DN, Li L, Kee C-L, Ge X, Low M-Y, Koh H-L. Screening of synthetic PDE-5 inhibitors and their analogues as adulterants: analytical techniques and challenges. Journal of pharmaceutical and biomedical analysis. 2014;87:176-90.

23. Haller CA, Benowitz NL. Adverse cardiovascular and central nervous system events associated with dietary supplements containing ephedra alkaloids. New England journal of medicine. 2000;343(25):1833-8.

24. Cerezo AB, Leal Á, Álvarez-Fernández MA, Hornedo-Ortega R, Troncoso AM, García-Parrilla MC. Quality control and determination of melatonin in food supplements. Journal of Food Composition and Analysis. 2016;45:80-6.

25. Jakubowska E, Ciepluch N. Blend Segregation in Tablets Manufacturing and Its Effect on Drug Content Uniformity—A Review. Pharmaceutics. 2021;13(11):1909.

26. Farquharson A, Gladding Z, Ritchie G, Shende C, Cosgrove J, Smith W, et al. Drug Content Uniformity: Quantifying Loratadine in Tablets Using a Created Raman Excipient Spectrum. Pharmaceutics. 2021;13(3):309.

27. Ganzetti R, Logrippo S, Sestili M, Caraffa A, Cespi M, Pelliccioni G, et al. Tablet splitting in elderly patients with dementia: The case of quetiapine. Pharmaceutics. 2021;13(9):1523.

28. Al-Gousous J, Langguth P. Oral solid dosage form disintegration testing—the forgotten test. Journal of Pharmaceutical Sciences. 2015;104(9):2664-75.

29. Gray VA. Power of the dissolution test in distinguishing a change in dosage form critical quality attributes. American Association of Pharmaceutical Scientists. 2018;19(8):3328-32.

30. Brennan MJ, Duncan WE, Wartofsky L, Butler VM, Wray HL. In vitro dissolution of calcium carbonate preparations. Calcified Tissue International. 1991;49(5):308-12.

31. Younis IR, Stamatakis MK, Callery PS, Meyer-Stout PJ. Influence of pH on the dissolution of folic acid supplements. International Journal of Pharmaceutics. 2009;367(1-2):97-102.

32. Blicharska E, Szczęsna B, Kocjan R, Gumieniczek A, Komsta Ł. Analysis of dissolution profiles of iron, zinc, and manganese from complex dietary supplements by ion chromatography and chemometrics. Journal of Liquid Chromatography & Related Technologies. 2016;39(1):30-4.

33. Palaric C, Pilard S, Fontaine J-X, Cailleu D, Mesnard F, Renaud T, et al. Combined MS-NMR approach for the quality control of food supplements containing amino acids. Food Control. 2018;88:217-28.

# 2. Cel pracy

Celem niniejszej pracy było określenie aspektów jakości wybranych suplementów diety zawierających jako główny składnik karnitynę, prolinę, tryptofan oraz tyrozynę. Ocena jakości obejmowała a) oznaczenie jakościowe substancji obecnych w badanych suplementach diety; b) określenie zawartości głównego składnika w kapsułkach/tabletkach; c) badanie uwalniania w celu oznaczenia ilości uwolnionego głównego składnika z kapsułek/tabletek o niemodyfikowanym uwalnianiu. Wybór wyżej wymienionych aspektów jakości wynika z tego, że stanowią one podstawowe wyznaczniki właściwej jakości produktu. Natomiast wybór suplementów diety zawierających karnitynę, prolinę, tryptofan oraz tyrozynę wynika z tego, że nie ma dostępnych badań dotyczących jakości tych preparatów, szczególnie w zakresie badania uwalniania substancji z formy dawkowania. Dodatkowo, analiza struktury chemicznej tryptofanu, tyrozyny oraz proliny sugerowała możliwość powstania potencjalnych produktów ich transformacji, które mogłyby zostać wykryte w trakcie badań.

Cel został zrealizowany poprzez:

- przegląd piśmiennictwa dotyczącego powszechności stosowania suplementów diety oraz zagrożeń związanych z ich stosowaniem **(Publikacja 1)**;
- poszukiwanie w badanych suplementach substancji niezadeklarowanych przez producentów **(Publikacja 2, 3 i 4)**;
- oznaczenie zawartości głównego składnika w kapsułkach/tabletkach suplementów diety **(Publikacja 2, 3 i 5)**;
- oznaczenie ilości uwolnionego (do płynu akceptorowego o pH (1,2 oraz 6,8)) głównego składnika z kapsułek/tabletek o niemodyfikowanym uwalnianiu **(Publikacja 2, 3 i 5)**;
- próbę wyjaśnienia przyczyn niskiego uwalniania głównego składnika z kapsułek/tabletek o niemodyfikowanym uwalnianiu **(Publikacja 2, 3 i 5)**;
- sprawdzenie czy farmakopealne metody oceny uwalniania substancji mogą skutecznie identyfikować suplementy diety o niskiej jakości oraz określenie, czy zastosowanie aparatu *PhysioCell* pozwoli lepiej zrozumieć losy niskiej jakości produktów w przewodzie pokarmowym **(Publikacja 5)**.

# 3. Kopie opublikowanych prac wchodzących w skład rozprawy

## 3.1 Publikacja 1 (przeglądowa)

**Stępień, K. A.**; Niewiarowski, J.; Harasimiuk, A. *Powszechność suplementów diety a zagrożenia związane z ich stosowaniem*; Biuletyn Wydziału Farmaceutycznego Warszawskiego Uniwersytetu Medycznego; 2019; 9; 51-59.

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# POWSZECHNOŚĆ SUPLEMENTÓW DIETY A ZAGROŻENIA ZWIĄZANE Z ICH STOSOWANIEM

**Krzysztof Adam Stępień*, Jakub Niewiarowski, Anna Harasimiuk**

Zakład Bioanalizy i Analizy Leków, Wydział Farmaceutyczny z Oddziałem Medycyny Laboratoryjnej, Warszawski Uniwersytet Medyczny, ul. Banacha 1, 02-097 Warszawa

*autor korespondujący, tel: + 48 22 572 0949, e-mail: krzysztof.stepien@wum.edu.pl

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**STRESZCZENIE**

Suplementy diety na przestrzeni ostatniego dziesięciolecia na stałe wpisały się w świadomość konsumentów na całym świecie. Reklamę tych produktów można znaleźć w środkach masowego przekazu, takich jak, radio, telewizja, prasa, internet. Każda apteka oferuje szeroki asortyment suplementów diety, ponadto produkty te są nabywane w internecie oraz w sklepach. Według danych zawartych w raporcie statystycznym prawie 72% Polaków deklaruje przyjmowanie suplementów diety, z czego połowa przyjmuje je regularnie. Niezwykle proste jest wprowadzenie do obrotu tego typu preparatów. Jedynym wymogiem jest złożenie w siedzibie Głównego Inspektoratu Sanitarnego stosownego oświadczenia. Sprawia to, że rynek suplementów diety w Polsce rozwija się bardzo dynamicznie. Suplementy diety klasyfikowane są jako środki spożywcze. W odróżnieniu od produktów leczniczych nie są one badane pod kątem trwałości, interakcji czy potencjalnych działań niepożądanych. Badania jakości suplementów diety potwierdzają, iż niejednokrotnie preparaty te zawierają niezadeklarowane substancje lub substancje w dawce innej niż deklarowana przez producenta bądź też substancja nie uwalnia się z postaci farmaceutycznej. Stanowi to niekiedy zagrożenie dla zdrowia osób przyjmujących suplementy diety, a jednocześnie jest uzasadnieniem konieczności poddawania ich odpowiednim badaniom jakościowym.

**SŁOWA KLUCZOWE:** suplement diety, jakość, przestępczość farmaceutyczna.

**ABSTRACT**

PREVALENCE OF DIETARY SUPPLEMENTS AND THREATS ASSOCIATED WITH THEIR TAKING

Dietary supplements are widely used all over the world. Advertises these products you can find in the mass media, i.e. radio, television, press, Internet. All pharmacies offer a lot of supplements, in addition, these products are also purchased online. According to the data contained in the statistical report, almost 72% of Poles declare that they take dietary supplements. The marketing of this type of preparations is simple. Make a statement to the Chief Sanitary Inspectorate is only requirement. As a result, the market of dietary supplements is developing very dynamically in Poland. Such a significant number of dietary supplements make that checking these by sanitary inspectors is impossible. Dietary supplements are classified as foodstuffs. Unlike medicinal products, they are not tested for durability, interaction or pharmacovigilance. Studies cover the quality of dietary supplements confirmed, that these products often contain undeclared ingredients or amount of substance is incorrect than the declared one, or the substance is not released with pharmaceutical forms. This is a potential health risk for people taking dietary supplements.

**KEYWORDS:** dietary supplement, quality, pharmaceutical crime.

## 1. Wstęp

Suplementy diety stanowią grupę produktów spożywczych powszechnie stosowaną przez ludzi ze względu na ich stosunkowo niskie ceny, łatwość zakupu oraz przekonanie, iż produkty — w związku z tym, że są pochodzenia naturalnego — są bezpieczne do stosowania [1].

Zgodnie z definicją mianem suplementu diety określa się „*środek spożywczy, którego celem jest uzupełnienie normalnej diety, będący skoncentrowanym źródłem witamin lub składników mineralnych lub innych substancji wykazujących efekt odżywczy lub inny fizjologiczny, pojedynczych lub złożonych, wprowadzany do obrotu w formie umożliwiającej dawkowanie, w postaci: kapsułek, tabletek, drażetek, saszetek z proszkiem, ampułek z płynem, butelek z kroplomierzem i w innych podobnych postaciach płynów i proszków przeznaczonych do spożywania w małych, odmierzonych ilościach jednostkowych, z wyłączeniem produktów posiadających właściwości produktu leczniczego w rozumieniu przepisów prawa farmaceutycznego*”. W większości suplementy diety zawierają w swoim składzie witaminy oraz składniki mineralne. Dodatkowo posiadają substancje odżywcze lub wykazujące efekt fizjologiczny na organizm człowieka [2]. Zatem odwołując się do definicji suplementów diety, należy stwierdzić iż zażywanie ich nie ma na celu leczenia, lecz profilaktykę i wspomaganie funkcjonowania organizmu. Dostarczanie niezbędnych składników odżywczych możliwe jest bowiem dzięki codziennej, zbilansowanej diecie bez konieczności stosowania suplementów diety w postaci gotowych preparatów [3]. Suplementy diety w odróżnieniu od produktów leczniczych nie podlegają ściśle określonym wymaganiom jakościowym. Oznacza to, iż osoby kupujące tego typu preparaty nie mają pewności czy zawierają

one deklarowaną substancję czynną oraz czy określona substancja występuje w deklarowanej przez producenta ilości. Dodatkowo występuje potencjalne ryzyko obecności w preparacie substancji niedozwolonych dla suplementów diety. Nieokreślona jest także dostępność farmaceutyczna substancji czynnych. Nie można zatem jednoznacznie oszacować korzyści oraz zagrożeń związanych ze stosowaniem suplementów diety. Liczba badań dotyczących jakości tych preparatów jest niewystarczająca. Zagadnienie to jest bardzo istotne, bowiem popularność suplementów diety w Polsce i na świecie jest bardzo duża — liczba zarejestrowanych preparatów w Głównym Inspektoracie Sanitarnym wynosi ponad 81 tysięcy [4].

**2. Stosowanie suplementów diety w Polsce**

Polski rynek suplementów diety pod względem zawartości składników jest różnorodny. Zgodnie z danymi zamieszczonymi na ryc. 1 najliczniejszymi grupami suplementów diety są preparaty zawierające magnez lub bakterie probiotyczne, lub preparaty zwiększające odporność organizmu. Znaczną część rynku suplementów diety w Polsce i na świecie stanowią witaminy i składniki mineralne. Są one stosowane w celu zapobiegania niedoborom składników odżywczych w organizmie człowieka, a także obniżają ryzyko wystąpienia niektórych przewlekłych jednostek chorobowych. Istnieją wyniki badań, które potwierdzają, że w Polsce niedobory u dzieci dotyczą głównie tiaminy, niacyny, wapnia, żelaza, cynku,. Zatem pożądana i uzasadniona jest właściwa suplementacja tych składników, mająca na celu zmniejszenie ich niedoborów [5].

Mirosław Jarosz dokonał podziału suplementów diety ze względu na dwie zmienne grupujące: skład oraz przeznaczenie. Podział ze względu na skład jest następujący: suplementy zawierające witaminy i składniki mineralne; suplementy zawierające składniki roślinne i ekstrakty roślinne; suplementy zawierające niezbędne kwasy tłuszczowe; suplementy diety zawierające w składzie błonnik pokarmowy; suplementy diety zawierające w swoim składzie probiotyki i prebiotyki; suplementy zawierające aminokwasy. Z kolei podział ze względu na przeznaczenie obejmuje grupę suplementów diety wspomagających odchudzanie; wspomagających układ odpornościowy; wpływających na narządy ruchu; wpływających na opóźnienie procesów starzenia się; wpływających na układ nerwowy, koncentrację, a także wzmacniające witalność; wpływających na układ sercowo-naczyniowy; wspomagających układ pokarmowy; wspomagających prawidłowy proces widzenia; wpływających na stan skóry, włosów, paznokci; dedykowanych sportowcom; stosowanych w celu zmniejszania ryzyka osteoporozy [6].

Agencja badawcza TNS Polska, w 2014 roku przeprowadziła badania dotyczące stosowania suplementów diety w Polsce. W wyniku badania odnotowano, że 19% osób ankietowanych stosowało co najmniej jeden suplement diety w roku poprzedzającym badanie. Biorąc pod uwagę płeć badanej populacji, kobiety (24%) częściej stosowały suplementy diety niż mężczyźni (14%), natomiast uwzględniając wiek, największe spożycie suplementów diety stwierdzono u osób między 20 a 29 rokiem życia (24%). Ankietowani podali również kilka głównych powodów zażywania suplementów diety. Przeważały odpowiedzi, takie jak: uzupełnienie codziennej diety w wybrane składniki odżywcze oraz podniesienie odporności [7].

W 2014 roku agencja badawcza TNS dokonała badania wiedzy Polaków dotyczącej suplementów diety. Wyniki tego badania przedstawiają się następująco. 25% badanych osób miało problem ze zdefiniowaniem pojęcia „suplement diety". Wśród respondentów padały stwierdzenia określające suplementy diety jako „witaminy" (31%), „minerały" (8%), preparaty ułatwiające odchudzanie oraz „leki" (6%). Niespełna połowa ankietowanych osób była przekonana o właściwościach leczniczych suplementów diety, których te produkty nie posiadają [8].

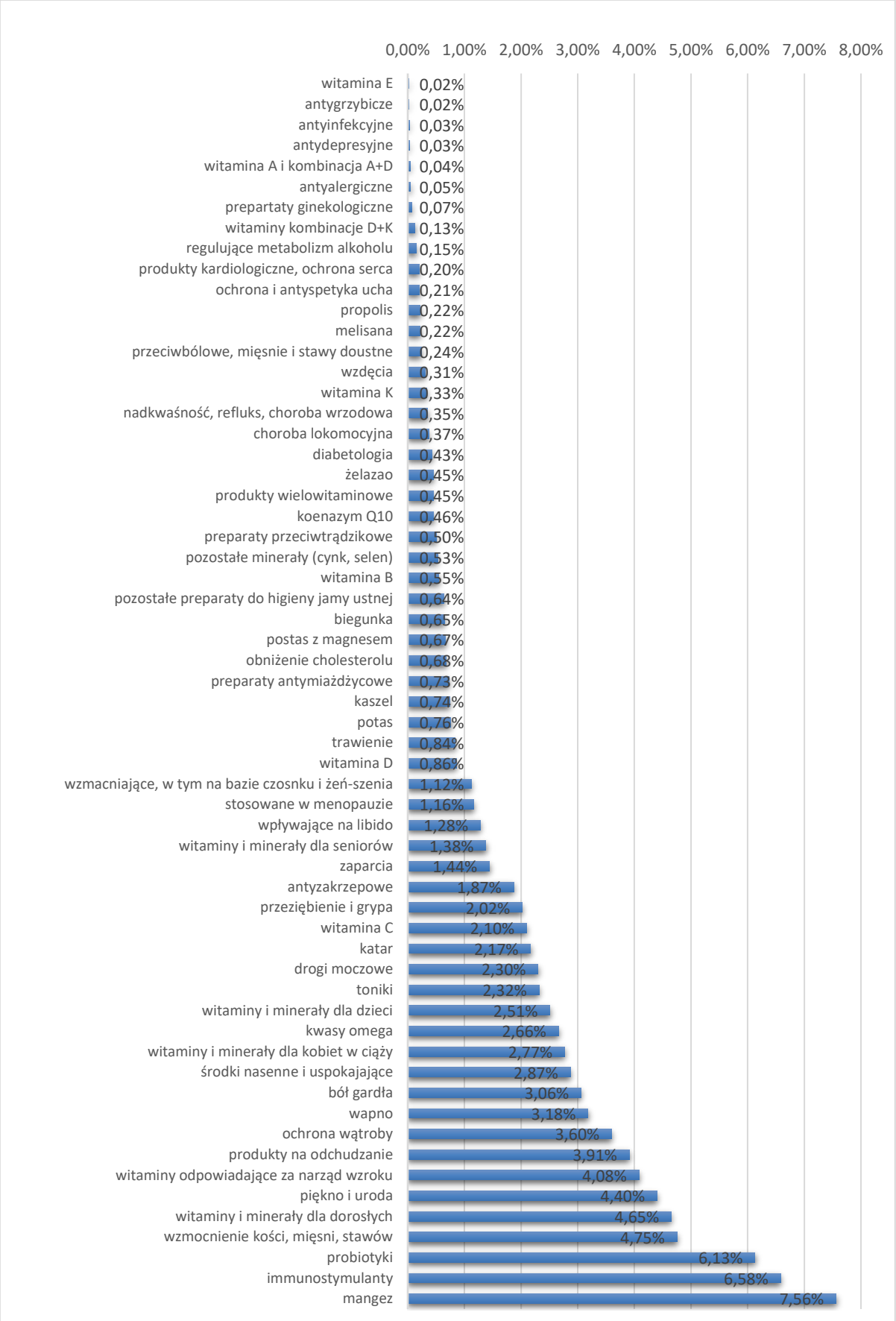
Współczesny rynek suplementów diety podlega bardzo szybkiemu rozwojowi. Ryc. 2 ukazuje tendencję wzrostową liczby rejestrowanych suplementów diety w Polsce w latach 2007–2016. Tendencja ta wynika z rosnącego zapotrzebowania konsumentów na suplementy diety. Szacuje się, iż w 2015 roku w Polsce na zakup suplementów diety konsumenci wydali kwotę około 3,5 miliarda złotych, co odpowiada 190 milionom opakowań. Zatem na statystyczną osobę przypada 6 opakowań suplementów diety o wartości około 100 złotych. Badania przewidują dalszy rozwój rynku suplementów diety do 2020 roku w tempie około 8% rocznie (ryc. 3) [9].

**3. Regulacje prawne suplementów diety**

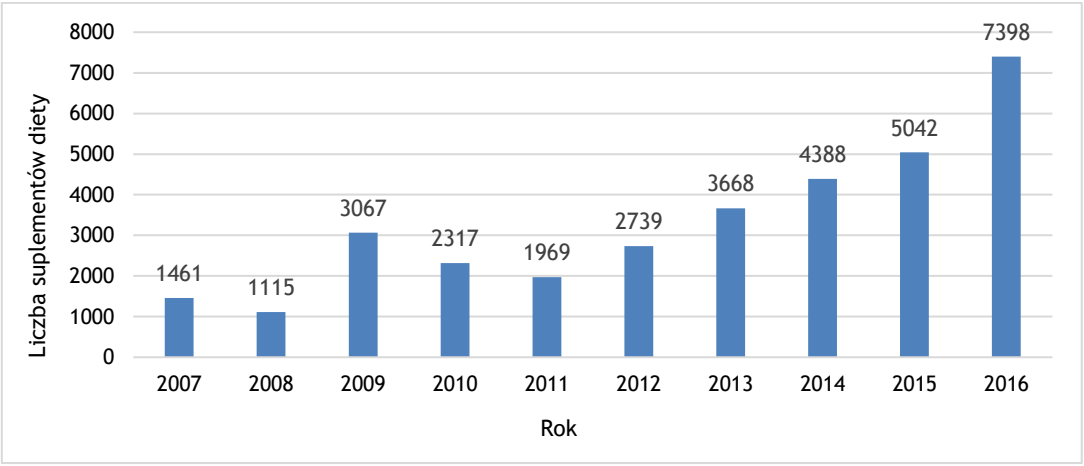
Suplementy diety są regulowane prawnie na wielu płaszczyznach. Międzynarodowy zbiór norm żywnościowych, Codex Alimentarius, klasyfikuje suplementy diety jako żywność [11]. Z kolei regulacja na poziomie europejskim obejmuje Zieloną Księgę (Generalne Zasady Prawa Żywnościowego w Unii Europejskiej), Białą Księgę Bezpieczeństwa Żywności, dyrektywy i rozporządzenia odnoszące się do higieny środków spożywczych [12,13].

W Polsce suplementy diety reguluje prawnie ustawa o bezpieczeństwie żywności i żywienia z dnia 25 sierpnia 2006 r. (Dz. U. z 2010 r. nr 136, poz. 914). Rozdział drugi tej ustawy zawiera między innymi regulacje dotyczące żywności w odniesieniu do zawartości substancji dodatkowych, enzymów spożywczych, aromatów, rozpuszczalników, zanieczyszczeń, norm napromieniania promieniowaniem jonizującym, zasad znakowania żywności, oraz oświadczenia żywieniowe i zdrowotne. Ww. ustawa określa obowiązek powiadomienia Głównego Inspektora Sanitarnego o wprowadzeniu po raz pierwszy na terytorium Rzeczypospolitej Polskiej suplementu diety oraz żywności wzbogacanej (art. 29) [2,14]. Ponadto rynek suplementów diety w Polsce regulowany jest przez rozporządzenie Ministra Zdrowia w sprawie składu oraz oznakowania suplementów diety, którego jednolity tekst zawarty jest w obwieszczeniu Ministra Zdrowia z dnia 17 września 2018 r. (Dz. U. z 2018 r., poz. 1951) [15].

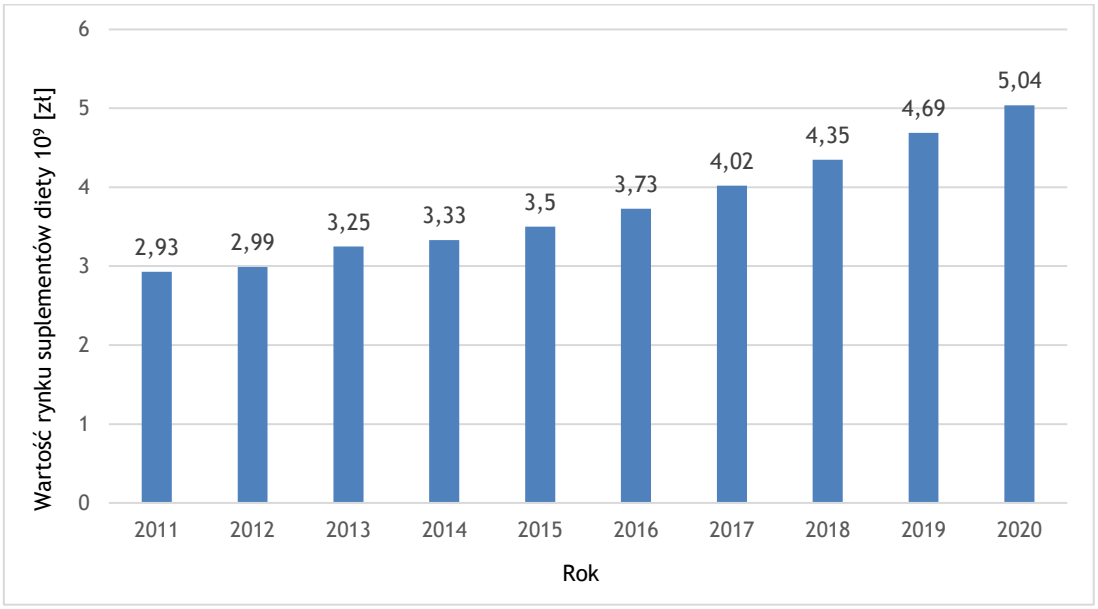
Prawo Unii Europejskiej wymaga również respektowania przez producentów suplementów diety wytycznych wspomagających przy produkcji bezpiecznej żywności. Należą do nich GMP (Dobra Praktyka Wytwarzania), GHP (Dobra Praktyka Higieniczna) oraz HACCP (Analiza Zagrożeń i Krytyczne Punkty Kontroli). Ponieważ suplementy diety są środkami spożywczymi, muszą spełniać wymagania ogólne zapisane w przepisach prawa żywnościowego dla środków spożywczych powszechnie spożywanych, a także wymagania zawarte w powyżej wymienionych przepisach. Ważna jest ich zgodność z określonymi kryteriami, takimi jak jakość sensoryczna, bezpieczeństwo oraz wartości odżywcze [16,17].




Ryc. 1. Różnorodność suplementów diety w Polsce [4].




Ryc. 2. Liczba suplementów diety zgłoszonych do rejestru GIS w latach 2007–2016 [4].




Ryc. 3. Prognozy dotyczące wartości rynku suplementów diety w Polsce w latach 2011–2020 [10].

## 4. Zagrożenia związane z nieprawidłowym stosowaniem suplementów diety

Suplementy diety stosowane są coraz częściej. Jest to związane między innymi z działaniami marketingowymi prowadzonymi przez ich producentów. Szczególnie narażeni na tego typu działalność są ludzie w podeszłym wieku. W wyniku przeprowadzonego badania na grupie osób starszych, która nie stosowała suplementów diety, wykazano, że dostarczała z pożywieniem około 315% normy witaminy A i 290% normy witaminy C. W odniesieniu do składników mineralnych największe spożycie odnotowano dla sodu (ok. 230%), a powyżej zalecanej normy znalazły się także fosfor (175%) oraz żelazo (140%). Podobny do powyższych badań przeprowadzono eksperyment z udziałem studentów Uniwersytetu Rzeszowskiego, będących odzwierciedleniem młodszej grupy wiekowej. W wyniku tego badania wykazano spożycie przekraczające normy w przypadku witaminy A (mężczyźni 190% normy, kobiety 138% normy), witaminy $B_6$ (mężczyźni 150% normy, kobiety 110% normy), witaminy $B_{12}$ (mężczyźni 184% normy, kobiety 140% normy). Średnie spożycie składników mineralnych u mężczyzn przekroczone było w przypadku sodu (170,2% normy), fosforu (198,9% normy), manganu (210,0% normy). W żywieniu kobiet odnotowano niedobory w przypadku witaminy D (49,0%), folianów (54,0%), witaminy $B_1$ (84,5%), witaminy $B_3$ (82,5%), żelaza (46%), potasu (51,4%), wapnia (55,4%) oraz magnezu (71,6%). W przypadku mężczyzn niedobory dotyczyły zawartości witaminy D (79,4%), folianów (71,6%) oraz kwasu askorbinowego (76,0%) [18]. Wyniki powyższych badań świadczą, iż dobór suplementu diety i jego stosowanie przez pacjentów często są nieuzasadnione. W niektórych przypadkach stosowanie suplementów diety może powodować przekroczenie zalecanych norm żywienia. Jest to niebezpieczne, bowiem może powodować poważne zaburzenia funkcjonowania organizmu. Spożywanie witaminy A powyżej zalecanej normy jest toksyczne dla organizmu. Może się ono objawiać m.in.: drażliwością, torsjami, zmianami skórnymi, zaburzeniami czynności śledziony i wątroby. Z kolei nadmiar żelaza może powodować choroby układu krążenia, udary, miażdżycę, choroby Alzheimera i Parkinsona [19]. Bardzo ważnym zagadnieniem są również interakcje suplementów diety z produktami leczniczymi. Producenci suplementów diety nie mają obowiązku zamieszczania na opakowaniach informacji o działaniach niepożądanych, przeciwskazaniach

czy interakcjach wytwarzanych produktów. Dodatkowo pacjenci niejednokrotnie nie przekazują informacji dotyczących przyjmowanych suplementów diety podczas konsultacji z lekarzem lub farmaceutą, co może skutkować wystąpieniem działań niepożądanych, obniżeniem lub brakiem efektu terapeutycznego lub innymi, poważnymi zagrożeniami dla zdrowia. Szczególnie niebezpieczne są interakcje występujące pomiędzy substancjami roślinnymi będącymi składnikami suplementów diety a substancjami czynnymi wchodzącymi w skład produktów leczniczych. *Ginkgo biloba*, miłorząb japoński, nie powinien być stosowany jednocześnie z lekami przeciwzakrzepowymi (warfaryną) oraz aspiryną, gdyż zwiększone jest wówczas ryzyko wystąpienia spontanicznych krwawień. *Hypericum perforatum*, dziurawiec zwyczajny, nie może być stosowany z selektywnymi inhibitorami wychwytu zwrotnego serotoniny (fluoksetyną) czy trójpierścieniowymi lekami przeciwdepresyjnymi. Połączenie takie może bowiem skutkować wystąpieniem zespołu serotoninowego. *Panax ginseng*, żeń-szeń właściwy, nasila działanie inhibitorów monoaminooksydazy (moklobemidu), substancji pobudzających ośrodkowy układ nerwowy (sybutraminy, kofeiny) oraz leków przeciwcukrzycowych (insuliny, metforminy, pochodnych sulfonylomocznika). Osłabia natomiast działanie leków stosowanych przy nadciśnieniu (amlodypiny, enalaprylu, dilitiazemu) oraz przeciwzakrzepowych (warfaryny, acenokumarolu). Magnez jest jednym z najczęściej suplementowanych pierwiastków. Obniża on biodostępność leków stosowanych w nadciśnieniu tętniczym (np. kaptoprylu), zmniejszając ich działanie. Ponadto zmniejsza absorpcję leków przeciwbakteryjnych, przeciwzakrzepowych (np. tylkopidyny), przeciwgrzybiczych (ketokonazolu), przeciwpsychotycznych, przeciwlękowych (klonazepamu), glikozydów nasercowych (digoksyny). Wapń stosowany jednocześnie z blokerami kanału wapniowego (werapamilem) może osłabiać ich działanie [20].

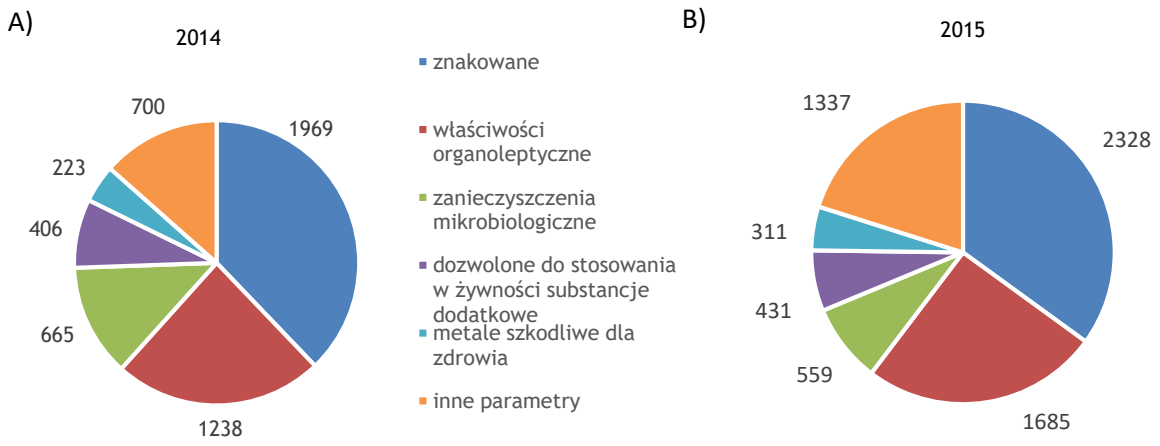
## 5. Zagrożenia związane z brakiem kontroli jakości suplementów diety

Suplementy diety nie podlegają obowiązkowej kontroli jakości, takiej jakiej podlegają produkty lecznicze. Nie jest wymagane sprawdzanie trwałości tych preparatów oraz badanie ich interakcji z produktami leczniczymi. Główny Inspektorat Farmaceutyczny nie sprawuje kontroli nad suplementami diety, co oznacza, że nie są one także monitorowane pod względem wywoływania potencjalnych działań niepożądanych [21]. Zgodnie z obowiązującym prawem każdy przedsiębiorca może wprowadzić suplement diety na rynek. Warunkiem jest złożenie stosownego oświadczenia w siedzibie Głównego Inspektoratu Sanitarnego. W przypadku otrzymania zgłoszenia o potencjalnej wadzie jakościowej suplementu diety, Główny Inspektorat Sanitarny wszczyna kontrolę danego preparatu. Postępowanie weryfikacyjne może trwać kilka lat, a podczas jego przebiegu, producent nie ma obowiązku wycofania suplementu z obrotu [22].

Najwyższa Izba Kontroli wykazała, że wobec połowy zgłoszeń rejestracyjnych suplementów diety z lat 2014-2016, tj. około 6 tysięcy preparatów, nie rozpoczęto żadnego procesu weryfikacji, co oznacza, że produkty te nie były w jakikolwiek sposób sprawdzone pod względem bezpieczeństwa stosowania przez konsumentów. Wyniki badań jakościowych wybranych suplementów diety dostarczają informacji, iż obecne w sprzedaży suplementy mogą nie spełniać podstawowych wymagań jakościowych. Przykładem są suplementy diety wspomagające odchudzanie, w których wykryto niedozwolone składniki, takie jak roślinę *Acacia rigidula* zawierającą dimetylotryptaminę. Jest to substancja psychoaktywna wymieniona w ustawie o przeciwdziałaniu narkomanii. W wyniku tego badania wycofano z obrotu 316 opakowań suplementu, podczas gdy jego sprzedaż przez jednego importera przekroczyła 10 tysięcy opakowań. Zakres badań oraz liczbę badanych próbek suplementów diety przez GIS przedstawia ryc. 4 [22].

Czystość mikrobiologiczna suplementów diety była przedmiotem badań na Poznańskim Uniwersytecie Medycznym. W wyniku tych badań stwierdzono iż, 6,5% z 1165 przebadanych próbek suplementów diety produkowanych przez zakłady farmaceutyczne na terenie województwa wielkopolskiego nie spełniała obowiązujących wymogów. Główną nieprawidłowością było przekroczenie zawartości bakterii tlenowych, rzadziej grzybów. W sześciu przypadkach stwierdzono obecność bakterii *Escherichia coli*, wywołującej zakażenia układu pokarmowego oraz moczowego [23].

Wyniki innych badań weryfikujących jakość suplementów diety również odkrywają nieprawidłowości dotyczące ich zawartości. Suplementy diety Chińskiej Medycyny Naturalnej zawierające substancje pochodzenia roślinnego, zanieczyszczone były prometazyną, klometiazolem lub diklofenakiem [24]. Analizy jakości suplementów diety potwierdzają także, iż nie tylko zawarte w nich substancje czynne




Ryc. 4. Zakres badań oraz liczba badanych próbek suplementów diety w ramach urzędowej kontroli żywności. A) w 2014 roku, B) w 2015 roku [22].

mogą być niezgodne z deklarowanymi. Odnotowano przypadki preparatów, w których główną substancją pomocniczą był gips, a barwnikiem tusz używany w drukarkach komputerowych [25].

Badanie sześciu suplementów diety z grupy probiotyków przeprowadzone przez Najwyższą Izbę Kontroli, dowiodło, iż tylko w dwóch preparatach zawartość bakterii probiotycznych była zgodna z ilością deklarowaną przez producenta [22]. Doniesienia z innych krajów wskazują na podobną sytuację w przypadku suplementów diety zawierających nienasycone kwasy tłuszczowe [26] czy substancje roślinne [27]. Również preparaty zawierające melatoninę w 71% przebadanych próbek nie spełniały kryterium zawartości substancji czynnej. Przebadane suplementy diety zawierające niacynę, witaminę $B_6$, kwas askorbinowy, tokoferol w większości przypadków spełniły kryterium deklarowanej zawartości [28]. Prawie wszystkie z analizowanych preparatów zawierających kwasy eikozapentaenowy i dokozaheksaenowy, zakupionych w Nowej Zelandii, spełniały stosowne wymagania [29].

Ograniczona jest ilość informacji na temat dostępności farmaceutycznej suplementów diety. Część z nich dowodzi jednak, że suplementy diety zawierające luteinę [30], żelazo [31] czy witaminę A [32] charakteryzują się niskim uwalnianiem substancji czynnej z postaci leku.

**6. Zagrożenia związane z przestępczością farmaceutyczną**

Przestępczość farmaceutyczna rozwija się dynamicznie zarówno w Polsce, jak i na świecie. Fałszowanie leków i suplementów diety jest działaniem wysoce opłacalnym [33]. Niewielka jest również wykrywalność procederu. W 2016 r. postawiono zarzuty w 404 postępowaniach w związku z przestępstwami związanymi z podrabianiem leków [34].

Suplementy diety będące przedmiotem fałszowania można podzielić na siedem grup:

- stosowane w dysfunkcji erekcji, zawierające sildenafil, tadalafil, wardenafil i ich pochodne,
- środki na szybki przyrost masy mięśniowej, zawierające hormony anaboliczne,
- odchudzające, zawierające sibutraminę i inne pochodne amfetaminy,
- nielegalne i sfałszowane surowce ziołowe i produkty Tradycyjnej Medycyny Chińskiej,
- nielegalne i sfałszowane suplementy i kosmetyki oferowane w sex-shopach, zawierające kantarydynę, johimbinę, benzokainę czy lidokainę,
- „cudownie" działające suplementy leczące każdą chorobę,
- „dopalacze", zawierające N-benzylopiperazynę [35].

Tabela 1. Sfałszowane suplementy diety (wpływające na odchudzanie lub na dysfunkcję erekcji) wykryte w krajach UE w latach 2008–2009 [35].

| Nazwa produktu | Deklarowany status produktu | Deklarowany skład produktu | Stwierdzona substancja czynna |
|---|---|---|---|
| **Super Slim** | suplement diety | zioła | sibutramina |
| **Miaozi** | suplement diety | zioła | sibutramina |
| **Meizitanc** | suplement diety | zioła | sibutramina |
| **Meitang** | suplement diety | zioła | sibutramina |
| **Lida Daidahuajiao Nang** | suplement diety | zioła | sibutramina lub monodezmetylosibutramina |
| **Paiyouji** | suplement diety | zioła | sibutramina, fenoloftaleina |
| **ErMax Power Plus** | suplement diety | zioła | tadalafil |
| **VPXL No1** | suplement diety | zioła, witaminy, soja | tadalafil, sildenafil |
| **Astra-SX** | suplement diety | aminokwasy, witaminy, minerały | hydroksyacetyldenafil |
| **Herbat Viogra** | produkt ziołowy | zioła | tadalafil |
| **VPXL** | produkt ziołowy | zioła, witaminy, soja | sildenafil |
| **Kosttillskott** | produkt ziołowy | zioła | tadalafil |
| **Herbat Viogour** | produkt ziołowy | zioła | tadalafil |
| **China Vigour** | produkt ziołowy | zioła | Nor-acetyldenafil |
| **Natura Vigour** | produkt ziołowy | zioła | wardenafil |
| **Libidfit** | suplement diety | zioła | acetyldenafil |
| **Satibo** | suplement diety | zioła | N-metylopiperazynosildenafil |
| **Viamax** | suplement diety | zioła | N-piperydynowardenafil |

Tabela 2. Suplementy diety dedykowane sportowcom, w których stwierdzono obecność steroidów anabolicznych (38).

| Nazwa produktu | Firma | Wykryte steroidy anaboliczno-androgenne |
|---|---|---|
| **BCAA** | Ulimate Nutrition | dehydroepiandrosteron |
| **L-Carnitine 1000** | Ulimate Nutrition | dehydroepiandrosteron, androstendion |
| **Fat Bioc Ulitimate** | Ulimate Nutrition | dehydroepiandrosteron, androstandiendion, androstendion, norandrostendion |
| **Pure OKG** | Vitalife | androstendion |
| **Ultra Ripped** | Vitalife | androstendion |
| **Tribugain** | Vitalife | 4-norandrostendion |
| **Super L-Carnitine** | Vitalife | 4-norandrostendion |
| **All-in-one** | Nutrisearch | dehydroepiandrosteron |
| **Fattack Maximuscle** | Maximuscle | dehydroepiandrosteron, 4-norandrostendion |
| **Speed Creatin Kautab** | All Stars | dehydroepiandrosteron, 4-orandrostendion |
| **Tri plex Zell Maxim** | All Stars | dehydroepiandrosteron, 4-norandrostendion |
| **Zell Tech Optimizer** | All Stars | dehydroepiandrosteron |

Tabela 3. Suplementy diety zawierające inhibitory 5-fosfodiesterazy wykryte w Stanach Zjednoczonych [36,39,45].

| Suplement diety | Niezadeklarowana substancja |
|---|---|
| Apexx | sildenafil |
| Diamond 3500 | sildenafil, tadalafil |
| Eros Power Zone 1900 | karbodenafil, dapoksetyna |
| OrgaZen 3000 | tadalafil |
| Extreme Diamond 3000 | desmethyl carbodenafil |
| Rhino Big Horn 3000 | carbodenafil, sildenafil |
| King of Romance | sildenafil |
| Triple MiracleZen Plus 1,5mg | sildenafil, tadalafil, dapoksetyna |
| Triple Power Zen Gold | sildenafil, tadalafil |
| X Again Platinum | sildenafil, tadalafil, dapoksetyna |
| Xtr Zone 2400 | sildenafil, tadalafil |
| Black Ant | sildenafil |
| Real Skill | sildenafil |
| Stree Overlord | sildenafil |
| Weekend Prince | sildenafil |
| African Black Ant | sildenafil |
| Liu Bian Li | sildenafil |
| Super Cheetach | sildenafil |
| Love raider | tadalafil |
| VitaliKOR | dapoksetyna, |
| Sex Plus | aminotadalafil |
| Full Throttle on Demand | sildenafil, thiosildenafil |
| RezzRX | propoksyfenylosildenafil |

Zgodnie z polskim prawem legalne suplementy diety muszą spełniać wymagania jakościowe i ilościowe zadeklarowane przez producenta na opakowaniu. Bardzo często dochodzi jednak do naruszenia prawa w tym względzie. Znacznie gorsza sytuacja jest w przypadku sfałszowanych suplementów diety. Takie produkty mogą nie zawierać substancji aktywnej zadeklarowanej na opakowaniu, mogą zawierać jej mniejszą lub większą ilość, lub zawierać inną substancję aktywną, której zastosowanie w suplemencie diety jest niedozwolone. W preparatach na odchudzanie stwierdzono obecność zakazanych przez polskie prawo sibutraminy, monodezmetylosibutraminy czy didezmetylosibutraminy [35], których stosowanie obarczone jest wystąpieniem poważnych działań niepożądanych. Innymi wykrytymi substancjami były benzodiazepiny, diuretyki, antydepresanty [36] oraz fenoloftaleina, która została wycofana z amerykańskiego rynku już w 1999 r. [37]. Dostępne są także wyniki badań, które potwierdzają, że w preparatach dla sportowców, deklarowanych jako witaminy i aminokwasy, stwierdzono zawartość steroidów anabolicznych, m.in. androstendionu, dehydroepiandrosteronu, metandienonu, stanozololu, oxandrolonu, mających znaczny wpływ na gospodarkę hormonalną organizmu. Potwierdzono również obecność zanieczyszczeń betametylofenyloetyloaminą, substancją o podobnym działaniu do zabronionej w stosowaniu amfetaminy [38]. Innym przykładem preparatów, w których stwierdzono niezgodność składu deklarowanego ze składem rzeczywistym są ziołowe suplementy diety mające wpływać na sprawność seksualną. Zamiast deklarowanej substancji roślinnej wykryto w nich inhibitory 5-fosfodiesterazy, takie jak sildenafil czy tadalafil oraz inhibitory wychwytu zwrotnego serotoniny, jak dapoksetyna [39]. Inhibitory 5-fosfodiesterazy wykryte w suplementach diety w Stanach Zjednoczonych przedstawia tabela 3. W preparatach o sugerowanym działaniu łagodzącym ból stawów oraz nerwoból stwierdzono obecność kortykosteroidu deksametazonu oraz fenylobutazonu zaliczanego do grupy niesteroidowych leków przeciwzapalnych o bardzo silnym działaniu [38]. Przykładem nieprawidłowości związanych ze składem jakościowym suplementów diety są także preparaty ułatwiające zasypianie, które zamiast deklarowanej przez producenta melatoniny zawierały serotoninę [40]. W ziołowych suplementach diety wspomagających proces odchudzania stwierdzono obecność substancji niedozwolonych, tj. takich jak johimbina, winkamina, hupercyna A. Sfałszowane suplementy wspomagające odchudzanie wykryte w latach 2008–2009 przedstawia tabela 1 [41-43]. W odżywkach dla sportowców potwierdzono obecność pochodnych steroidów anabolicznych, co przedstawia tabela 2 [38,44].

## 7. Podsumowanie

Nadużywanie suplementów diety przez konsumentów stanowi obecnie poważny problem. Wzrostowi spożycia tych preparatów przez społeczeństwo sprzyja m.in. ich nieograniczona dostępność na rynku. Ilość suplementów diety dostępna w aptekach i punktach aptecznych ciągle wzrasta. Pacjenci najczęściej sięgają po suplementy diety zawierające witaminy oraz elektrolity.

Niewielka ilość suplementów diety dostępnych na rynku jest badana pod kątem bezpieczeństwa ich stosowania, m.in. obecności substancji niezadeklarowanych na opakowaniu. Brak stosownych badań jakościowych suplementów diety, tj. obecności i ilości deklarowanych substancji, a także parametrów procesu technologicznego oraz użytych substancji pomocniczych, stanowi poważne zagrożenie dla zdrowia i życia osób, które je stosują. Problem niskiej jakości większości suplementów diety związany jest bezpośrednio z brakiem obligatoryjnej kontroli tych preparatów przez ich wytwórców. Aktualne zasady prawne nie wymagają od producentów suplementów diety wykonywania rzetelnych kontroli wytwarzanych preparatów.

Biorąc pod uwagę ilość zażywanych preparatów, których jakość nie jest w odpowiedni sposób określona, nie można dokonać jednoznacznej oceny ich wpływu na organizm człowieka. Nie jest możliwe także określenie korzyści zdrowotnych oraz potencjalnego ryzyka wystąpienia działań niepożądanych związanych z ich stosowaniem. Konieczne jest zatem podejmowanie działań mających na celu dokonanie precyzyjnej oceny jakości suplementów diety.

## 8. Wykaz skrótów

| | |
|---|---|
| GIS | Główny Inspektorat Sanitarny |
| GMP | Dobra Praktyka Wytwarzania (ang. *Good Manufacturing Practice*) |
| GHP | Dobra Praktyka Higieniczna (ang. *Good Hygienic Practice*) |
| HACCP | Analiza Zagrożeń i Krytyczne Punkty Kontroli (ang. *Hazard Analysis and Critical Control Points*) |
| FDA | Agencja Żywności i Leków (ang. *Food and Drug Administration*) |

## 9. Bibliografia


1. Cerezo AB, Leal Á, Álvarez-Fernández MA, Hornedo-Ortega R, Troncoso AM, García-Parrilla MC. Quality control and determination of melatonin in food supplements. Journal of Food Composition and Analysis. 2016, 45, 80-86.
2. Ustawa z dnia 25 sierpnia 2006r. o bezpieczeństwie żywności i żywienia.
3. Krajowa Rada Suplementów i Odżywek [na:] https://www.krsio.org.pl.
4. Najwyższa Izba Kontroli. Suplementy diety w świetle kontroli NIK. 12.04.2017 [25.11.2018]. https://www.nik.gov.pl/plik/id,13978, vp,16418.pdf.
5. Bojarowicz H, Dźwigulska P. Suplementy diety. Część II. Wybrane składniki suplementów diety oraz ich przeznaczenie. Hygeia Public Health. 2012, 47(4), 433-441.
6. Jarosz M. Suplementy diety a zdrowie – porady lekarzy i dietetyków. Wydawnictwo PZWL. Warszawa, 2008, 16-24.
7. Raport TNS Polska. Styczeń 2014. www.lekiczysuplementy.pl
8. Badanie TNS http://www.rynekzdrowia.pl.
9. Raport NIK. nik.gov.pl/RynekZdrowia.
10. Raport PMR. Rynek suplementów diety w Polsce 2015. Prognozy rozwoju na lata 2015–2020.
11. Codex Alimentarius. http://www.fao.org.
12. Stankiewicz D. Reforma prawa żywnościowego w UE — Biała Księga dotycząca bezpieczeństwa żywności. 2001, 1-3.
13. Dragan A. Zielone i Białe księgi Komisji Europejskiej. Kancelaria Senatu — Dział Analiz i Opracowań Tematycznych. 2010, 5-8.
14. https://gis.gov.pl.
15. Obwieszczenie Ministra Zdrowia z dnia 17 września 2018 r. w sprawie ogłoszenia jednolitego tekstu rozporządzenia Ministra Zdrowia w sprawie składu oraz oznakowania suplementów diety. Dz. U. 2018 poz. 1951.
16. Brzozowska A, Roszkowski W, Pietruszka B, Kałuża J. Witaminy i składniki mineralne jako suplementy diety. Żywność. Nauka. Technologia. Jakość. 2005, 4 (45), 5-16.
17. Obwieszczenie Marszałka Sejmu Rzeczypospolitej Polskiej z dnia 7 lutego 2005 r. w sprawie ogłoszenia jednolitego tekstu ustawy o warunkach zdrowotnych żywności i żywienia. Dz.U. 2005, nr 31, poz. 265.
18. Gil M, Głodek E, Rudy M. Ocena spożycia witamin i składników mineralnych w całodziennych racjach pokarmowych studentów uniwersytetu rzeszowskiego. Roczniki Państwowego Zakładu Higieny. 2012, 63(4), 441-446.

19. Orkusz A, Gorla B. Ocena sposobu żywienia mieszkańców domu pomocy społecznej z terenu województwa dolnośląskiego. Nauki Inżynierskie i Technologie. 2016, 3(22), 51-62.
20. Bojarowicz H, Dźwigulska P. Suplementy diety. Część III. Interakcje suplementów diety z lekami. Hygeia Public Health. 2012, 47(4), 442-447.
21. Bojarowicz H, Dźwigulska P. Suplementy diety. Część I. Suplementy diety a leki — porównanie wymagań prawnych. Hygeia Public Health. 2012, 47(4), 427-432.
22. Najwyższa Izba Kontroli. Informacja o wynikach kontroli. Dopuszczenie do obrotu suplementów diety. 03.02.2017 [01.12.2018]. LLO.430.002.2016 Nr ewid. 195/2016/P/16/078/LLO https://www.nik.gov.pl/plik/id,13031,vp,15443.pdf.
23. Ratajczak M, Kubicka MM, Kamińska D, Długaszewska J. Microbiological Quality of Foods Supplements. Acta Polonia Pharmacie. 2015, 72(2), 383-387.
24. Brown AC. An overview of herb and dietary supplement efficacy, safety and government regulations in the United States with suggested improvements. Part 1 of 5 series. Food and Chemical Toxicology. 2017, 107(Pt A), 449-471.
25. Fijałek Z, Sarna K, Błazewicz A, Marin J. Counterfeit phosphodiesterase type 5 inhibitors-growing safety risks for public health. Roczniki Państwowego Zakładu Higieny. 2010, 61(3), 227-235.
26. Kutzner L, Ostermann AI, Konrad T, Riegel D, Hellhake S, Schuchardt JP. Lipid Class Specific Quantitative Analysis of n-3 Polyunsaturated Fatty Acids in Food Supplements. Journal of Agricultural and Food Chemistry. 2017, 65(1), 139-147.
27. Li J, Yuan C, Pan L, Benatrehina PA, Chai H, Keller WJ. Bioassay-Guided Isolation of Antioxidant and Cytoprotective Constituents from a Maqui Berry (Aristotelia chilensis) Dietary Supplement Ingredient As Markers for Qualitative and Quantitative Analysis. Journal of Agricultural and Food Chemistry. 2017, 65(39), 8634-8642.
28. Andrews KW, Roseland JM, Gusev PA, Palachuvattil J, Dang PT, Savarala S. Analytical ingredient content and variability of adult multivitamin/mineral products: national estimates for the Dietary Supplement Ingredient Database. The American Journal of Clinical Nutrition. 2017, 105(2), 526-539.
29. Bannenberg G, Mallon C, Edwards H, Yeadon D, Yan K, Johnson H. Omega-3 Long-Chain Polyunsaturated Fatty Acid Content and Oxidation State of Fish Oil Supplements in New Zealand. Scientific Reports - Nature. 2017, 7(1), 1488.
30. Anselmo CS, Mendes TC, Cabral LM, Sousa VP. Physicochemical quality profiles of commercial oral tablets and capsules containing lutein - impact of insufficient specific sanitary regulations. Annals of the Brazilian Academy of Sciences. 2018, 90(3), 3063-3073.
31. Patrício JP, Santos C, Cerdeira R. In vitro dissolution profile of two commercially available iron preparations. Drugs in R & D. 2012, 12(1), 35-40.
32. Davydova N, Stippler E, Jin P, Giancaspro G. Development and validation of a dissolution test method for vitamin A in dietary supplement tablets. Journal of Pharmaceutical and Biomedical Analysis. 2010, 53(3), 295-301.
33. Rynekaptek.pl [15.02.2018]. http://www.rynekaptek.pl/marketing-i-zarzadzanie/sfalszowane-leki-zabijaja-najszybciej,19867_4.html
34. Krajowa Organizacja Weryfikacja Autentyczności Leków (KOWAL). [20.12.2018]. http://www.nmvo.pl/pl/o-nas
35. Fijałek Z, Sarna K. Wybrane aspekty jakości produktów leczniczych i suplementów diety –produkty substandardowe, nielegalne i sfałszowane. Rynek farmaceutyczny. 2009, 65(7), 467-475.
36. Couzin-Frankel J. The supplement sleuth. Science. 2015, 349(6250), 780-783.
37. Tucker J, Fischer T, Upjohn L, Mazzera D, Kumar M. Unapproved Pharmaceutical Ingredients Included in Dietary Supplements Associated With US Food and Drug Administration Warnings. JAMA Netw Open. 2018, 1(6), 1-11.
38. Kwiatkowska D. Suplementy diety, odżywki i dopalacze a doping. Zakład Badań Antydopingowych Instytutu Sportu w Warszawie. Jurata2010, 10-46.
39. Chiang J, Yafi FA, Dorsey PJ, Hellstrom WJ. The dangers of sexual enhancement supplements and counterfeit drugs to "treat" erectile dysfunction. Translational Andrology and Urology. 2017, 6(1), 12-19.
40. Erland LA, Saxena PK. Melatonin Natural Health Products and Supplements: Presence of Serotonin and Significant Variability of Melatonin Content. Journal of Clinical Sleep Medicine. 2017, 13(2), 275-281.
41. Ustawa o zmianie ustawy – Prawo Farmaceutyczne oraz niektórych innych ustaw z 19 grudnia 2014r. Dz.U. 2015 poz. 28.
42. Venhuis BJ, Zwaagstra ME, Keizers PH, de Kaste D. Dose-to-dose variations with single packages of counterfeit medicines and adulterated dietary supplements as a potential source of false negatives and inaccurate health risk assessments. Journal of Pharmaceutical and Biomedical Analysis. 2014, 89, 158-165.
43. Zovko Končić M. Getting More Than You Paid For: Unauthorized "Natural" Substances in Herbal Food Supplements on EU Market. Planta Medica. 2018, 84(6-07), 394-406.
44. Cho SH, Park HJ, Lee JH, Do JA, Heo S, Jo JH. Determination of anabolic-androgenic steroid adulterants in counterfeit drugs by UHPLC-MS/MS. Journal of Pharmaceutical and Biomedical Analysis. 2015, 111, 138-146.
45. ElAmrawy F, ElAgouri G, Elnoweam O, Aboelazayem S, Farouk E, Nounou MI. Adulterated and counterfeit male enhancement nutraceuticals and dietary supplements pose a real threat to the management of erectile dysfunction: a global perspective. Journal of Dietary Supplements. 2016, 13(6), 660-693.

## 3.2 Publikacja 2 (oryginalna)

**Stępień, K.A**.; Giebułtowicz, J. *Application of Liquid Chromatography Coupled to Mass Spectrometry in Quality Assessment of Dietary Supplements—A Case Study of Tryptophan Supplements: Release Assay, Targeted and Untargeted Studies;* Pharmaceuticals; 2022; 15 (4); 448.

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# Application of Liquid Chromatography Coupled to Mass Spectrometry in Quality Assessment of Dietary Supplements—A Case Study of Tryptophan Supplements: Release Assay, Targeted and Untargeted Studies

**Krzysztof Adam Stępień and Joanna Giebułtowicz ***

Department of Bioanalysis and Drugs Analysis, Faculty of Pharmacy, Medical University of Warsaw, 1 Banacha, 02-097 Warsaw, Poland; krzysztof.stepien@wum.edu.pl

* Correspondence: joanna.giebultowicz@wum.edu.pl; Tel.: +48-22-572-0949

**Abstract:** Dietary supplements are widely consumed in the EU and the USA. Based on their similarity to pharmaceuticals, consumers mistakenly believe that dietary supplements have also been approved for safety and efficacy. However, in the absence of mandatory testing, data on supplement quality is scarce. Thus, we applied liquid chromatography coupled with tandem mass spectrometry to analyse the quality of dietary supplements containing tryptophan (Trp). We examined 22 supplements in tablets or capsules, produced in the USA, Great Britain, Germany, France, Czech Republic, and Poland. Trp release, crucial for bioavailability and efficiency, was assessed. Additionally, we performed a qualitative analysis of the main ingredient and screened for contaminants. Among the contaminants, we detected Trp's metabolites, condensation products of Trp and carbonyl compounds, Trp degradation products, degradation products of kynurenine, and other contaminants such as glucosamine and melatonin. The main ingredient content was in the range of 55–100% in capsules and 69–87% in tablets. Surprisingly, almost no Trp release was noted from some supplements. Our study confirms the need to advance research on supplements. We believe that the high-quality analysis of supplements based on reliable analytical techniques will be an important contribution to the discussion on the regulatory framework of these products.

**Keywords:** dietary supplement; food supplement analysis; LC-MS/MS; release test; quality control; food composition



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## 1. Introduction

The consumption of dietary supplements is increasing globally, due to low prices, broad prescription-free distribution, and a common belief in efficacy and safety [1]. In the EU, they are classified as food and contain ingredients with nutritional or physiological effects [2]. Similarly, in the USA, dietary supplements are also classified as food and contain amino acids, herbal substances, vitamins, minerals, and enzymes [3]. Although they are foods, they are sold in typically pharmaceutical dosage forms such as tablets, capsules, sachets, and others designed to be taken in small and defined unit quantities. Based on their similarity to pharmaceuticals, consumers mistakenly believe that dietary supplements have also been approved for safety and efficacy before marketing. However, in the absence of mandatory quality testing, data on supplement quality is scarce. When the data do appear, they indicate some issues such as the presence of contaminants [4,5], the content of the main ingredient lower than the declared one [6,7], or low release from the formulation [8]. The most commonly described contaminants are heavy metals [9], anabolic steroids in preparations for athletes [10], and dioxins in dietary supplements containing fish oil [11]. The amount of the main ingredient in the dietary supplements was only examined for melatonin supplements [1] and supplements containing eicosapentaenoic

acid (EPA) and docosahexaenoic acid (DHA) [12]. Release of the main constituent from the formulation was conducted for formulations with calcium carbonate [13], melatonin [14], folic acid [15,16], iron [17], triiodothyronine [18], trans-resveratrol [19] and lutein [8]. The paucity of data on supplement quality is disadvantageous [20]. Therefore, the need for international collaboration to advance the knowledge on supplements has recently been emphasized. Not only the efficacy but also the quality of dietary supplements should be evaluated, which is essential to improve the regulatory framework [21].

Depression, the most common mental illness, is one of the most common disorders for which supplementation is also used. This disorder affects over 300 million people around the world, of different ages, and in all communities [22]. The prevalence of the illness increases with age and is more common in women and people with higher education [23]. Depression is treated pharmacologically, often with moderate efficacy [24]. This is why some people also use supplementation. There are numerous mood-enhancing supplements on the market, mainly containing the neutral amino acid tryptophan (Trp) [25]. It is one of the 20 L-amino acids incorporated into proteins during mRNA translation [26] and a precursor of serotonin (5-hydroxytryptamine), niacin (niacinamide), and melatonin. Trp enters the kynurenine pathway and is a precursor of the coenzyme NAD(P)+ [27]. It is an exogenous amino acid whose [28] deficiency leads to the insufficient synthesis of the neurotransmitter serotonin, which worsens mood. Lower levels of Trp in peripheral blood have been confirmed in patients suffering from depression. Trp supplementation significantly improved the symptoms of the disease [29]. Trp has a positive effect on mood, cognitive functions [30], sleep [31], and a decrease or maintenance of a healthy weight [32]. Response to supplementation is individual and may be influenced by genetic factors [33]. The potential efficacy of Trp in depression patients led us to select supplements with this ingredient to evaluate their quality.

Western diet usually contains about 0.5 g of Trp per day. However, only 2–3% of this amount enters the brain for conversion, via 5-hydroxytryptophan, to serotonin. It is due to extensive metabolism and competition with other long-chain neutral amino acids, e.g., histidine, isoleucine, leucine, methionine, phenylalanine [34]. Trp is an ingredient of dietary supplements [35]. Sometimes, during Trp supplementation, dose-independent side effects occur, i.e., tremors, dry mouth, mild nausea, dizziness. Contaminants, present in commercially available Trp for nutritional use (feed-grade Trp in raw materials of different manufacturers), were investigated [36]. The contaminants detected and identified in commercially available Trp sources were the known metabolites of this amino acid, oxidation products of Trp, condensation products of Trp with carbonyl compounds [37]. So far, there are little data on the quality of Trp preparations. Additionally, no data have been published on the content and release of Trp from supplements, and most studies on supplements rely on simple analytical techniques.

This study aimed to apply liquid chromatography coupled with mass spectrometry as a highly reliable analytical technique to evaluate the quality of dietary supplements containing Trp in tablets or capsules ($n$ = 22) produced in the USA, UK, Germany, France, Czech Republic, and Poland. This evaluation was performed by (i) assessment of Trp release, a key parameter for bioavailability and efficacy, (ii) qualitative analysis of the main ingredient, and (iii) screening for contaminants. We believe that a high-quality analysis of supplements will be an important contribution to the discussion of the regulatory framework for these products and that the new analytical approach will have broad applicability in the assessment of supplement quality.

## 2. Results and Discussion

### *2.1. Tentative Contaminants Present in Trp Supplements*

In addition to Trp, twenty-two compounds were detected in the analysed supplements in the range of 0.02% to 43.89% of the main ingredient area (Table 1, Figure A1). Their molecular formula, retention time, experimental and theoretical mass, fragmentation, and tentative names are presented in Table 1. None of these compounds was listed on

the package as a component of the supplement. Detected compounds were classified into five groups: (A) Trp's metabolites, (B) condensation products of Trp and carbonyl compounds, (C) Trp degradation products, (D) degradation products of kynurenine and (E) other contaminants.

The first group (group A) includes products of the main metabolic pathways of Trp: anthranilic acid (**I4**), indole-3-acetaldehyde (**I7**), indole acetic acid (**I9**), 5-hydroxyTrp (**I16**), formylkynurenine (**I19**) (Figure 1).

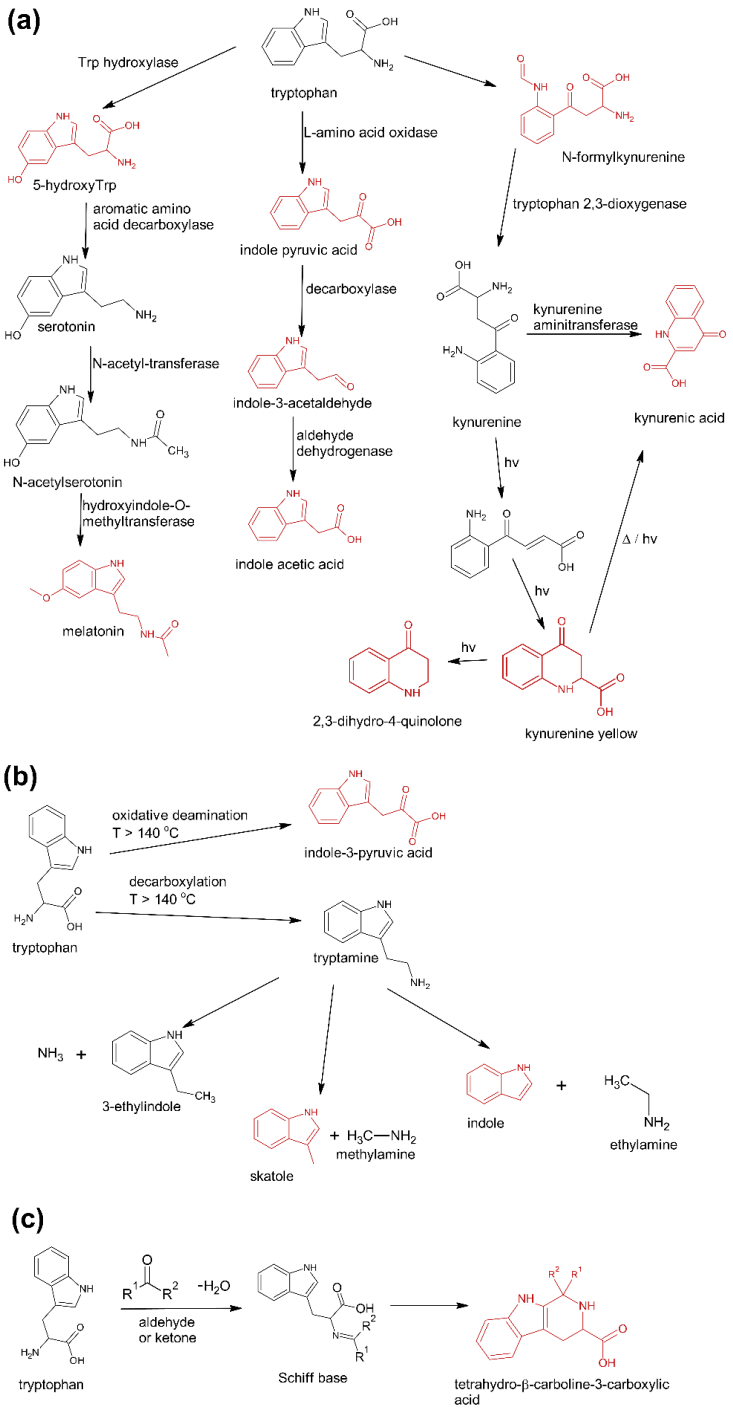


**Figure 1.** Pathways reasoning for the presence of specific contaminants in Trp supplements. (**a**) Major metabolic pathways downstream of Trp (**b**), Trp degradation products formed after exclusive exposure to heat (**c**), and reaction products of Trp with aldehydes and ketones. Compounds marked in red were detected in this study [37].

**Table 1.** Tentative identification of contaminants found in Trp supplements and their MS parameters.

| Code | Formula | Neutral Mass Calculated from the Formula [Da] | Neutral Mass Calculated from the Measured *m*/*z* [Da] | ΔMass [ppm] | RT [min] | Identification Confidence Level | Fragments [*m*/*z*] | Dietary Supplements Containing Contaminant (% of the Analysed) | Tentative Name | % of the Main Ingredient Area |
|---|---|---|---|---|---|---|---|---|---|---|
| **I1** | C8H7N | 117.05785 | 117.05792 | 0.6 | 5.0 | 3 | 91.05414 | All, (100%) | Indole | 23.19 |
| **I2** | C9H9N | 131.07350 | 131.07350 | 0.0 | 5.0 | 2 | 117.06720; 130.06493; 131.07260 | All (100%) | Skatole | 6.68 |
| **I3** | C8H7NO | 133.05276 | 133.05243 | 2.5 | 3.1 | 2 | 79.05412; 106.06493 | C1; C2; C8; C9; T1; T3; T4; T6; T7; T8; T9; T10 (55%) | Oxindole | 2.41 |
| **I4** | C7H7NO2 | 137.04768 | 137.04766 | 0.2 | 3.1 | 2 | 92.04936; 94.06511; 110.06001 | C8; C9; T1; T3; T5; T6; T7; T8; T9; T10 (45%) | Anthranilic acid | 0.42 |
| **I5** | C9H7NO | 145.05276 | 145.05284 | 0.6 | 5.0 | 2 | 91.05412; 117.05762; 118.06503 | All (100%) | 3-formy-lindole | 43.89 |
| **I6** | C9H9NO | 147.06841 | 147.06827 | 1.0 | 3.9 | 3 | 120.04422; 130.03930; 130.06487 | T6 (4.5%) | 2,3-dihydro-4-quinolone | 0.02 |
| **I7** | C10H9NO | 159.06841 | 159.06845 | 0.3 | 5.0 | 2 | 130.06490; 132.080610; 142.06616 | All (100%) | Indole-3-acetaldehyde | 2.50 |
| **I8** | C10H10N2O | 174.07931 | 174.07924 | 0.4 | 3.9 | 3 | 132.04401; 147.09129; 157.07565 | C1, C2, C3, C4, C5, C6, C7, C8, C10, C11, C12, T1, T2, T3, T4, T5, T6, T7, T8, T9, T10 (95%) | 1-Phenyl-3-methyl-5-pyrazolone | 0.20 |
| **I9** | C10H9NO2 | 175.06333 | 175.06326 | 0.4 | 3.9 | 2 | 130.06479; 146.05980; 158.05960 | C7, C11, T1, T2, T3, T4, T6, T7, T8, T8 (45%) | Indole acetic acid | 0.15 |
| **I10** | C6H13NO5 | 179.07937 | 179.07938 | 0.1 | 3.1 | 1 | 127.03854; 144.06540; 145.04945 | C8, T3 (9%) | Glucosamine | 0.07 |

**Table 1.** *Cont.*

| Code | Formula | Neutral Mass Calculated from the Formula [Da] | Neutral Mass Calculated from the Measured *m*/*z* [Da] | ΔMass [ppm] | RT [min] | Identification Confidence Level | Fragments [*m*/*z*] | Dietary Supplements Containing Contaminant (% of the Analysed) | Tentative Name | % of the Main Ingredient Area |
|---|---|---|---|---|---|---|---|---|---|---|
| **I11** | C10H7NO3 | 189.04259 | 189.04239 | 1.1 | 8.5 | 2 | 162.05463; 172.03886; 173.04672 | T4, T6 (9%) | Kynurenic acid | 2.33 |
| **I12** | C10H9NO3 | 191.05824 | 191.05811 | 0.7 | 4.0 | 3 | 150.05463; 164.07000; 174.05472 | C4, C5, C7, C8, C9, C10, C11, C12, T1, T2, T3, T4, T6, T7, T8, T9, T10 (77%) | Kynurenine yellow | 0.03 |
| **I13** | C11H10N202 | 202.07423 | 202.07414 | 0.4 | 3.9 | 3 | 130.06480; 157.07570; 185.07106 | C1, C2, C3, C5, C7, C8, C9, C10, C12, T1, T2, T3, T4, T5, T6, T7, T10 (77%) | Unsaturated Trp | 0.38 |
| **I14** | C11H9NO3 | 203.05824 | 203.05849 | 1.2 | 3.9 | 3 | 160.07555; 176.07080; 186.05499 | C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, C12, T2, T3, T5, T6, T7, T8, T9, T10 (91%) | Indole pyruvic acid | 0.03 |
| **I15** | C12H12N2O2 | 216.08988 | 216.08986 | 0.1 | 8.0 | 3 | 171.09120; 173.10748; 188.07051 | C2, C4, C6, C7, C8, C9, C11, C12, T1, T2, T3, T4, T5, T6, T7, T8, T9, T10 (82%) | Tetrahydro-β-carboline-3-carboxylic acid | 0.23 |
| **I16** | C11H12N2O3 | 220.08479 | 220.08467 | 0.5 | 3.9 | 2 | 130.06488; 158.05981; 175.08636 | All (100%) | 5-hydro-xyTrp | 0.18 |
| **I17** | C13H14N2O2 | 230.10553 | 230.10533 | 0.9 | 8.2 | 3 | 168.08034; 188.07034; 214.08580 | C2, C8, C9, C11, T1, T2, T3, T4, T5, T6, T7, T8, T9 (59%) | 1-methyl-tetrahydro-β-carboline-3-carboxylic acid | 0.02 |
| **I18** | C13H16N2O2 | 232.12118 | 232.12099 | 0.8 | 9.1 | 1 | 174.09070; 204.10060; 216.10116 | C9 (4.5%) | Melatonin | 1.62 |

**Table 1.** *Cont.*

| Code | Formula | Neutral Mass Calculated from the Formula [Da] | Neutral Mass Calculated from the Measured *m*/*z* [Da] | ΔMass [ppm] | RT [min] | Identification Confidence Level | Fragments [*m*/*z*] | Dietary Supplements Containing Contaminant (% of the Analysed) | Tentative Name | % of the Main Ingredient Area |
|---|---|---|---|---|---|---|---|---|---|---|
| **I19** | C11H12N2O4 | 236.07971 | 236.07932 | 1.6 | 3.9 | 3 | 146.05975; 173.06980; 203.08099 | All (100%) | n-formylky-nurenine | 0.49 |
| **I20** | C20H19N3O2 | 333.14773 | 333.14739 | 1.0 | 8.8 | 3 | 188.07037; 205.09702; 217.09743 | C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, T1, T2, T3, T4, T5, T6, T7, T8, T9, T10 (95%) | 2-(3-Methyle-neindole)Trp | 0.02 |
| **I21** | C21H19N3O2 | 345.14773 | 345.14745 | 0.8 | 9.0 | 3 | 283.12204; 285.13794; 329.12610 | C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, T1, T2, T3, T4, T5, T6, T7, T8, T9, T10 (95%) | 1-(3-methylene-indole)-tetrahydro-β-carboline -3-carboxylic acid | 0.10 |
| **I22** | C22H23N3O4 | 393.16886 | 393.16878 | 0.2 | 8.5 | 3 | 251.31799; 277.11810; 358.15448 | C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, T1, T2, T3, T4, T5, T6, T7, T8, T9, T10 (95%) | 1-(2-Trp)-1-(3-indole) propane diol | 0.03 |

These compounds are formed during the fermentation of Trp in biotechnological production through the activity of Trp-degrading enzymes. The second group (group B) of contaminants corresponds to the condensation of Trp with carbonyl compounds: tetrahydro-β-carboline-3-carboxylic acid (**I15**) (condensation with formaldehyde), 1-methyl-tetrahydro-β-carboline-3-carboxylic acid (**I17**) (condensation with acetaldehyde), 1-(3-methyleneindole)-tetrahydro-β-carboline-3-carboxylic acid (**I21**) (condensation with indole-3-acetaldehyde (**I7**)). Reactions of tryptophan with aldehydes/ketones to form tetrahydro-beta-carbolines (tetHβCs), known as the Pictet–Spengler reaction, is one of the most common reactions of tryptophan with organic compounds. The transformation is usually acid-catalysed and takes place at low pH and high temperatures [38]. Highly reactive aldehydes are generated during the fermentation processes, thus tetHβCs may be detected in any biotechnologically derived Trp [39]. In the summary, the first and second groups of contaminants are associated with the process of Trp production using fermentation. The third group (group C) contains indole (**I1**), skatole (**I2**), oxindole (**I3**), 3-formylindole (**I5**), unsaturated Trp (**I13**), indole pyruvic acid (**I14**), 2-(3-Methyleneindole)Trp (**I20**), 1-(2-Trp)-1-(3-indole)propanediol (**I22**), and originate from Trp degradation [37]. Indole (**I1**), skatole (**I2**), and indole pyruvic acid (**I14**) are Trp degradation products formed after exclusive exposure to heat. At temperatures above 140 °C, decarboxylation and oxidative deamination of Trp occurs, forming tryptamine and indole pyruvic acid (**I14**). Tryptamine can degrade further, to form a possible product: indole (**I1**) or skatole (**I2**) [39]. Additionally, contaminants from this group may be the precursors in the Trp production process as chemical synthesis (indole (**I1**), 3-formylindole (**I5**)), enzymatic synthesis (indole (**I1**)), biotechnological synthesis (indole (**I1**), anthranilic acid (**I4**)). The next group of contaminants (group D) is degradation products of kynurenine following irradiation and heat: 2,3-dihydro-4-quinolone (**I6**), kynurenic acid (**I11**), kynurenine yellow (**I12**). Thermal and UV radiation cause a cascade of reactions of kynurenine, and it transforms to yield kynurenine yellow (**I12**) and 4-quinolone. Kynurenine yellow (**I12**) can react further, undergoing either oxidative decarboxylation to also afford 4-quinolone or oxidation to kynurenic acid (**I11**) [40]. Compounds from the last group (group E) are probably accidental contaminants related to production conditions, packaging method or quality, transport conditions. The contaminants include 1-phenyl-3-methyl-5-pyrazolone (**I8**), glucosamine (**I10**), melatonin (**I18**). The properties of some of them can be found in the literature. Glucosamine (**I10**) is used in the treatment of osteoarthritis [41]. Melatonin (**I18**) is centrally produced by the pineal gland and directly released in the blood, acting as a hormone. In mammals, yeast, and bacteria, melatonin **(I18)** is synthesized from tryptophan. Melatonin (**I18**) has a lot of functions: circadian and seasonal timing of organism; sleep and wakefulness cycle; endocrine functions, such as energy metabolism, glycaemic control, blood lipid profile and reproduction [42]. Glucosamine (**I10**) and melatonin (**I18**) are ingredients of many dietary supplements. Manufacturers of C8 and T3 (where we detected glucosamine (**I10**)) produce also dietary supplements containing glucosamine (**I10**). Thus, its presence (**I10**) in C8 and T3 may be the result of the insufficient purification (e.g., washing) before the manufacturing process. The same conclusion can be made in case of melatonin contaminant (**I18**).

To better visualize the results the heat map was prepared (Figure 2). We can observe the following:

a) Dietary supplements in capsules contained mainly contaminants from group C (Trp degradation products), which may indicate that Trp was obtained by chemical synthesis;
b) Dietary supplements in tablets contained mainly contaminants belonging to groups A (Trp's metabolites) and B (condensation products of Trp and carbonyls), which may indicate that Trp was obtained by biotechnology;
c) Trp from C1 and C3 dietary supplements might be produced by the same manufacturer. The supplements contained the same contaminants (difference in **I3**—Trp degradation product, which may be related to different storage conditions);

d) Trp from C6 and C11 dietary supplements were produced by the same manufacturer, supplements contained the same contaminants (difference **I17**—condensation product of Trp and carbonyls);
e) Trp from C5 and C10 dietary supplements were produced by the same manufacturer, supplements contained similar contaminants, the differentiating contaminants were classified as Trp degradation products and can be generated during supplement storage.

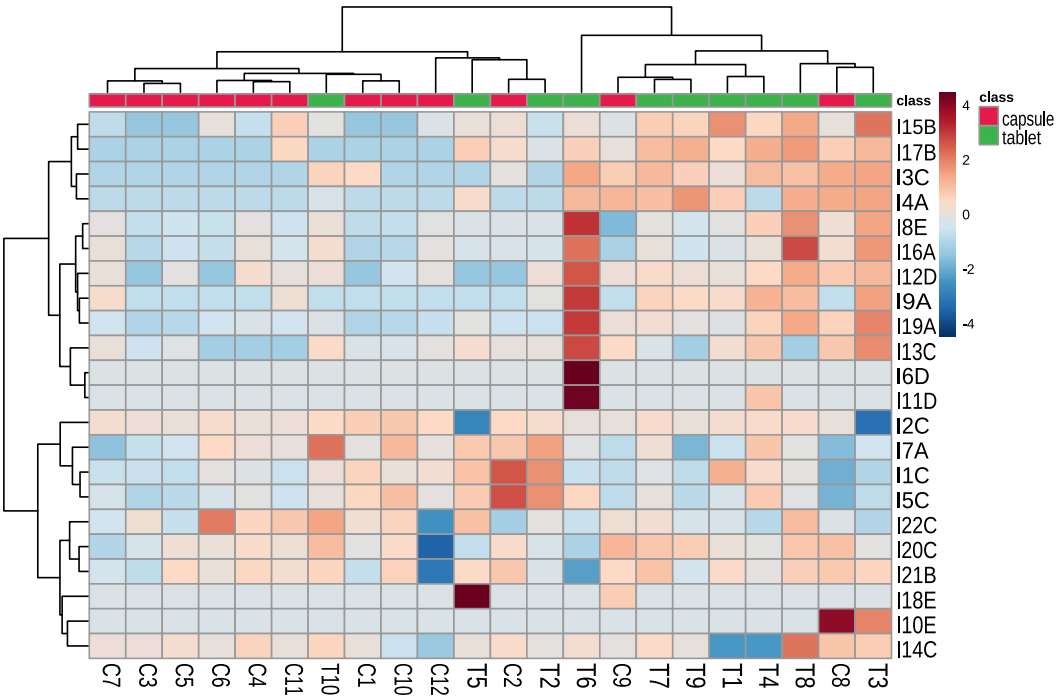


**Figure 2.** Clustering result of the tested supplements and detected contaminants (using Euclidean distance and clustering algorithm using Ward's method). The level of contaminant is presented as a heatmap (red colour indicates higher concentration and blue colour indicates lower concentration than the average) T—tablet, C—capsule, I—contaminant (with the name of a group of contaminants, i.e., A—Trp's metabolites, B—condensation products of Trp and carbonyls, C—Trp degradation products, D—degradation products of kynurenine, E—other contaminants).

To summarize, twenty-two compounds were detected in the analysed supplements in the range of 0.02% to 43.89% of the main ingredient area. Among the contaminants, there were Trp's metabolites, condensation products of Trp and carbonyl compounds, Trp degradation products, degradation products of kynurenine, and other contaminants. Some of Trp's contaminants have been already described in Trp raw material of different manufacturers [39], and melatonin supplements [1]. Melatonin can be synthesized from tryptophan by yeast and bacteria, so the occurrence of the contaminants was expected. The biological effect of Trp-related contaminants is unknown. Some Trp degradation products can impact cellular metabolism. **I12** a degradation product of Trp was shown to induce apoptosis in a human natural killer cell line. **I15** and **I17** act as antioxidants and free radical scavengers. However, the dose of **I12**, **I15**, **I17** needed to have a specific effect on cellular metabolism is unknown. The contaminants were present rather in small amounts, so they may not cause significant side effects [37].

Contamination can occur accidentally, due to poor manufacturing practices or contaminants originating from the supplement ingredients, or intentionally being added by manufacturers. The first group covers heavy metals [9] or substances found in raw materials, e.g., herbicides [43], insecticides [44], mycotoxins [45], and dioxins [11]. All detected contaminants in our study were from this group. Most of them were generated during manufacturing, under storage or transport of supplements/Trp, but some were found in the

preparation by accident. Heavy metal analysis was not performed because it requires other analytical techniques such as ICP (inductively coupled plasma) or ASA (atomic absorption analysis). Moreover, these contaminants are mainly detected in herbal-based dietary supplements. Similarly, targeted screening for pesticides and mycotoxins (which were detected in herbal formulations), dioxins (detected in fish oil formulations), cyanobacterial neurotoxins (detected in shark cartilage) and microcystins (detected in algae) because they were not warranted, was not conducted in the study. For instance, pesticides were previously detected in supplements with Ginkgo [44,46] and Ginseng [47,48], whereas mycotoxins were in supplements with Ginkgo and grapes (Table A1). Many of these compounds require targeted screening as well as isolation and enrichment from the complex matrix to obtain a reliable signal [49]. The isolation methods include solid-phase extraction [50], dispersive solid-phase extraction [51], liquid-phase microextraction [52], microwave-assisted extraction [53], microwave-assisted saponification combined with simultaneous unsaponifiable extraction [54]. In our study, a simple extraction was performed without enrichment.

The second group of contaminants includes substances that are prohibited in dietary supplements and are intentionally used by the manufacturer to enhance the observed effect (anabolic steroids [10], hypoglycemic drugs [55], drugs used in potency disorders [56], weight loss products [57]). For Trp supplements, we screened for antidepressants because Trp is often used for depression [28], but no such substances were detected.

The liquid chromatography with mass spectrometry used in this experiment in the untargeted analysis is one of the most frequently used for that purpose. The increased bioavailability of high-resolution instruments improved the detection and identification of compounds in food including dietary supplements. However, confidence in these identifications varies between studies and substances, since it is not always possible or even meaningful to synthesize each substance or confirm them via complementary methods [58]. Thus, we applied the confidence identification level for our data (Table 1). To minimize the risk of false-positive identification it is recommended to search dedicated "small size" MS databases including compounds with a realistic probability to be observed [59]. In our case, the database consisted of degradation products of Trp was used. To decrease further the risk of false-positive identification, all detected compounds were fragmented to achieve a confidence level of at least 3. However, for unexpected compounds such as melatonin or glucosamine, we confirmed the structure with the reference standards. The differences in retention times of these compounds in the samples and reference standard were 0.01 min for glucosamine and 0.03 min for melatonin. The isotopic and fragmentation patterns were similar. The fragmentations are shown in Figure 3.

### *2.2. Determination of Trp in Dietary Supplements*

Following the Polish Pharmacopoeia VI, the content of an active substance in tablets or capsules should not exceed the following: (1) ±10% for units with the declared active substance content below 100 mg or (2) ±5% for units with the declared content of the active substance of 100 mg and above. These requirements apply to pharmaceuticals. Due to the lack of specific guidelines for dietary supplements and the fact that dietary supplements appear in the same form as drugs, the same criteria for Trp content were adopted in this study. Therefore, none of the formulations contained the amount of Trp declared by the manufacturer (Table 2), i.e., it was not within 90–110% in each tablet or capsule. The lowest (55% of the declared content) Trp content was in supplement C6, followed by C8 (60%) and T5 (69%). The amount of Trp was within the range of 70–79% in nine supplements and 80–90% in the other nine supplements. The amount of Trp ranged from 70–79% in nine supplements and 80–90% in the other nine supplements. The low Trp content may be due to the lower amount of active ingredients used in production. The highest average Trp content (i.e., 100.45% of the claimed content) was observed in supplement C2. However, the amount of Trp in each capsule varied significantly and ranged from 174 to 251 mg/unit (CV = 19%), indicating improper mixing of the capsule mass. The concentration of the main ingredient in C6 and T2 supplements also had a high coefficient of variation: C6

(CV = 32%), T2 (CV = 35%), but the average amount of Trp was 55% and 87% of the claimed content, respectively. In these cases, both the wrong amount of active ingredient used and improper mixing of the tablet or capsule mass during the manufacturing process may be the reason for inadequate quality.

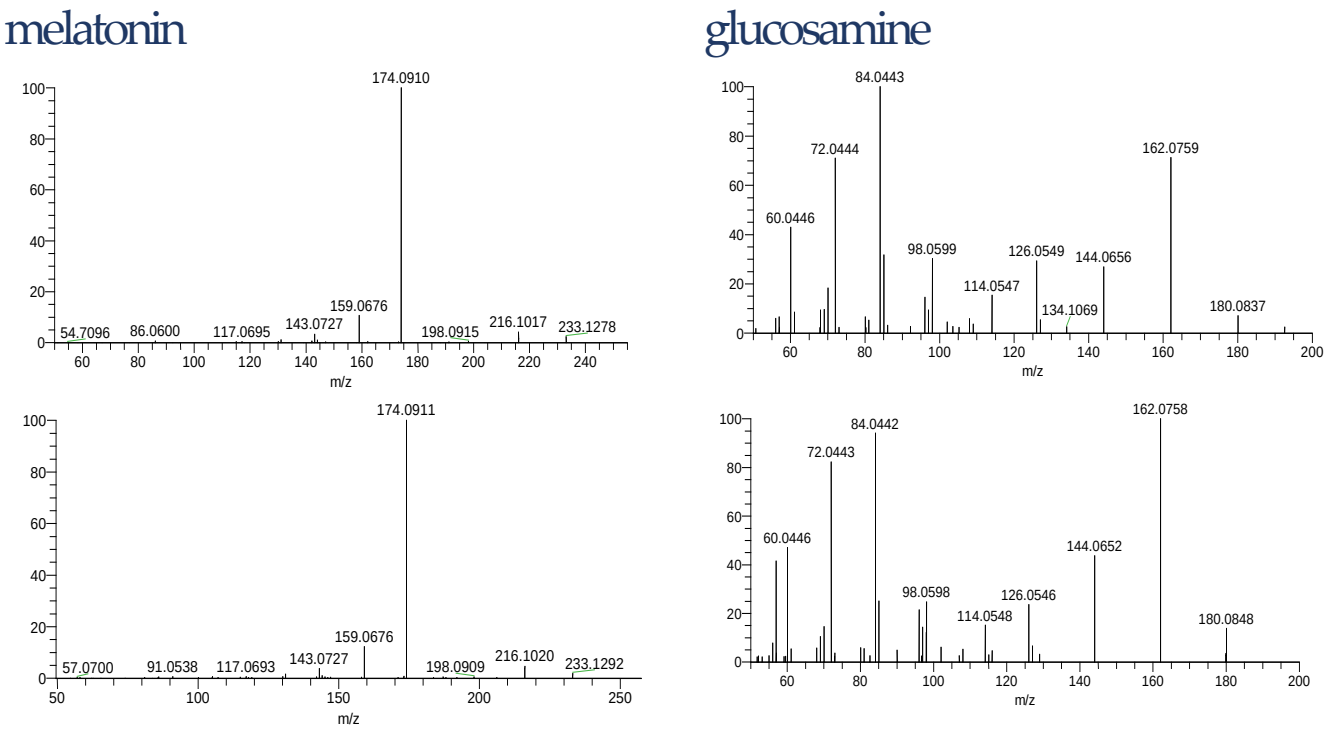


**Figure 3.** Glucosamine and melatonin fragmentation pattern in samples (**top**) and reference standards (**down**).

**Table 2.** Content of Trp in the dietary supplements (maximum error value above 40 was bolded).

| Code | Dosage Form | Source | Declared Trp Content [mg/unit] | Determined Trp Content [mg/unit] [a] | Maximum Error [%] |
|---|---|---|---|---|---|
| **C1** | capsule | United Kingdom | 250 | 205 (CV = 5.0%) | −21 |
| **C2** [b] | capsule | France | 220 | 221 (CV = 19%) | −21 |
| **C3** | capsule | United States | 500 | 398 (CV = 4.1%) | −24 |
| **C4** | capsule | Poland | 500 | 368 (CV = 4.1%) | −29 |
| **C5** | capsule | Poland | 500 | 415 (CV = 13%) | −27 |
| **C6** | capsule | No label | 500 | 277 (CV = 32%) | **−74** |
| **C7** | capsule | Czech Republic | 160 | 143 (CV = 16%) | −29 |
| **C8** | capsule | Germany | 50 | 29.8 (CV = 6.6%) | **−47** |
| **C9** | capsule | Poland | 100 | 77.3 (CV = 8.4%) | −32 |
| **C10** | capsule | United Kingdom | 500 | 432 (CV = 5.4%) | −19 |
| **C11** | capsule | United States | 500 | 350 (CV = 14%) | **−44** |
| **C12** | capsule | United States | 500 | 443 (CV = 8.7%) | −20 |
| **T1** | tablet | Poland | 100 | 72.6 (CV = 10%) | −32 |
| **T2** | tablet | United States | 1000 | 870 (CV = 35%) | **−48** |
| **T3** | tablet | Poland | 40 | 32.6 (CV = 14%) | −27 |

**Table 2.** *Cont.*

| Code | Dosage Form | Source | Declared Trp Content [mg/unit] | Determined Trp Content [mg/unit] [a] | Maximum Error [%] |
|---|---|---|---|---|---|
| **T4** | tablet | Poland | 100 | 71.4 (CV = 7.0%) | −33 |
| **T5** | tablet | Poland | 167 | 115.5 (CV = 7.1%) | −36 |
| **T6** | tablet | Poland | 50 | 37.0 (CV = 9.0%) | −33 |
| **T7** | tablet | Poland | 50 | 41.4 (CV = 8.1%) | −24 |
| **T8** | tablet | Poland | 50 | 41.1 (CV = 16%) | −29 |
| **T9** | tablet | Poland | 50 | 37.1 (CV = 3.6%) | −29 |
| **T10** | tablet | No label | 200 | 155 (CV = 15%) | −35 |

CV—coefficient of variation; [a]—mean (standard deviation $n = 3$); [b]—three capsules were analysed, results (251 mg, 239 mg; 174 mg).

Inconsistency between the declared and determined content of the main ingredient has been previously reported for melatonin supplements [1] and lutein [8]. None of the lutein supplements ($n = 10$) and 41% of the melatonin supplements ($n = 17$) met our criteria. However, it is not clear whether the melatonin or lutein content was evenly distributed among the units. Therefore, no conclusions could be drawn regarding quality.

Content uniformity is an important critical quality attribute. High variability in active ingredient content can be caused by the following: improper particle distribution (e.g., agglomeration); poor macro- and microblending at the powder mixing stage; loss of a component (e.g., due to adsorption to the equipment surface); thief sampling and analytical errors; segregation of well-mixed blends during powder transfer, handling or further operations [60]. The controlling of all this process is required. In the case of pharmaceuticals available on the market, content uniformity is not likely to occur [61,62]. The only study showing the problem with this attribute concern tablets splitting [63]. However, a large number of articles on content uniformity and the ways of continuous monitoring tablet content uniformity [64] suggest that it is a difficult task to achieve.

For supplements with Trp, we observed only a slightly higher level of the active ingredient for capsules (79%) than for tablets (77%). Similar results were noted for melatonin (capsules—91%, tablets—87%) [1] and lutein (capsules—122%, tablets—42%). The supplements with lutein from Brazil (e.g., 0.12% or 135%) had lower quality than those from the USA (112%, 113%) [8].

### *2.3. Dissolution Test for Trp Tablets and Capsules*

The Food and Drug Administration provides guidelines for drug testing. According to the dissolution test requirements, the active ingredient should release from the immediate-release oral solid drug at least 80% of its claimed content after 30 min of the release test [65].

Dietary supplements do not have dedicated guidelines for dissolution testing. Therefore, the same criteria for the dissolution test were used in this study. Trp release higher than 80% was determined for supplements C6, C7, C10 at pH 1.2 and supplement T9, T10 at pH 6.8. (Table 3, Figure 4). Supplement C11 had the lowest release (1.22%) at gastric pH (pH 1.2), while at pH 6.8 the release reached 60.2%. Supplements C2 and C3 were characterized by a release of no more than 5% Trp at both pHs (1.2 and 6.8). Thus, only up to 5% of the claimed dose of Trp could be absorbed from these supplements across biological membranes to produce a physiological effect (Figure 4a). Trp release between 10 and 20% regardless of dissolution medium was determined for C8 and between 20% and 30% for T3 and T7. In summary, Trp release for 10 of the 22 supplements was determined between 1.22 and 59.9% at both pHs (Figure 4).

**Table 3.** Comparison of the amount of Trp determined and released from tablets and capsules in two pH (gastric, pH = 1.2 and intestinal, pH = 6.8) with the expanded uncertainty.

| Code | The Average Percentage of Trp Amount Released from a Dosage Form (Standard Deviation $n$ = 6) | | Expanded Uncertainty Parameters | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | pH 1.2 | | | pH 6.8 | | |
| | pH 1.2 | pH 6.8 | $\lvert x_1-x_2\rvert$ | $U(x_1-x_2)$ | Equal [a] | $\lvert x_1-x_2\rvert$ | $U(x_1-x_2)$ | Equal [a] |
| **C1** | 60 (11) | 66.0 (8.1) | 55.38 | 25.29 | No | 39.99 | 136.07 | Yes |
| **C2** | 2.65 (0.55) | 2.3 (2.2) | 215.43 | 48.12 | No | 216.31 | 8.53 | No |
| **C3** | 3.08 (0.76) | 4.8 (2.6) | 382.17 | 19.07 | No | 373.66 | 57.22 | No |
| **C4** | 53 (13) | 41.2 (3.8) | 100.87 | 52.04 | No | 162.59 | 117.62 | No |
| **C5** | 36.2 (7.5) | 17.9 (3.7) | 234.17 | 68.74 | No | 325.37 | 113.25 | No |
| **C6** | 84.3 (8.3) | 76.4 (2.2) | 144.78 | 187.05 | Yes | 105.26 | 233.65 | Yes |
| **C7** | 81 (18) | 40.6 (3.3) | 13.18 | 37.53 | Yes | 77.73 | 9.87 | No |
| **C8** | 17.9 (1.8) | 15.58 (0.70) | 20.81 | 3.89 | No | 21.98 | 0.29 | No |
| **C9** | 75.5 (3.1) | 46.6 (6.6) | 1.75 | 10.06 | Yes | 30.65 | 15.13 | No |
| **C10** | 90.4 (9.4) | 68.9 (7.3) | 19.93 | 49.18 | Yes | 87.62 | 435.58 | Yes |
| **C11** | 1.22 (0.31) | 60.2 (8.1) | 344.27 | 80.11 | No | 49.64 | 541.99 | Yes |
| **C12** | 47.7 (5.8) | 18.0 (1.5) | 204.17 | 55.25 | No | 352.75 | 17.95 | No |
| **T1** | 71.9 (9.1) | 78.0 (5.1) | 0.7 | 11.42 | Yes | 5.41 | 9.64 | Yes |
| **T2** | 77.9 (5.4) | 76.0 (5.7) | 91.2 | 355.46 | Yes | 109.37 | 1071.86 | Yes |
| **T3** | 27 (16) | 21.3 (6.9) | 21.9 | 7.22 | No | 24.12 | 3.37 | No |
| **T4** | 54.1 (12.2) | 79.3 (8.8) | 17.3 | 11.55 | No | 7.93 | 26.61 | Yes |
| **T5** | 22.8 (2.9) | 39.3 (6.2) | 77.3 | 10.29 | No | 49.75 | 36.69 | No |
| **T6** | 32.9 (4.9) | 59.9 (6.1) | 20.5 | 4.31 | No | 7.01 | 3.94 | No |
| **T7** | 12.1 (1.0) | 26.1 (2.4) | 35.3 | 3.87 | No | 28.37 | 1.06 | No |
| **T8** | 47.7 (3.6) | 68.3 (5.0) | 17.3 | 7.84 | No | 6.80 | 2.85 | No |
| **T9** | 67.4 (7.1) | 80.0 (5.9) | 3.4 | 3.26 | No | 2.76 | 3.74 | Yes |
| **T10** | 71.0 (7.0) | 81.4 (2.9) | 13.3 | 28.43 | Yes | 7.49 | 11.42 | Yes |

[a] amount of Trp in the formulation and amount of Trp released are equal (yes) or not (no) within the uncertainty.

Trp was completely released from C6, C10, T1, T2, T11 supplements at both pHs (Table 3, Figure 4). The other five supplements released Trp at only one pH: gastric (supplement C7, C9) or intestinal (supplement C1, C11, T4, T9). Thus, in these supplements, the amount of Trp released was limited only by the content of the main ingredient (Table 2). No negative effect of technological parameters and excipients was observed. One of these supplements, i.e., C11 was probably designed by the manufacturer as an enteral form, which was not even mentioned in the packaging. For this supplement, a release of less than 10% was observed at pH 1.2 (as recommended by the guidelines) and a complete release was observed at pH 6.8. However, due to the lower Trp content, the complete release did not reach 80% of the claimed content as recommended.

The low release of Trp from C2 (pH = 1.2, release 2.65%; pH = 6.8, release 2.3%) and C3 (pH = 1.2, release 3.08%; pH = 6.8, release 4.8%) was mainly due to improperly selected process parameters and/or improperly selected excipients. This is because the content of Trp in the dosage form was much higher than the amount of Trp released (Figure 4a). In the remaining formulations (i.e., C4, C6, C8, C9, C11, T1, T4, T5, T6, T9), the low Trp release was due to both low compound content in the formulation and inappropriate preparation technology (poorly selected technological parameters or excipients) (Figure 4). Referring to

in vivo conditions, units characterized by low release will enter the gastric juice but will not release the substance. Thus, no physiological effect will be observed.

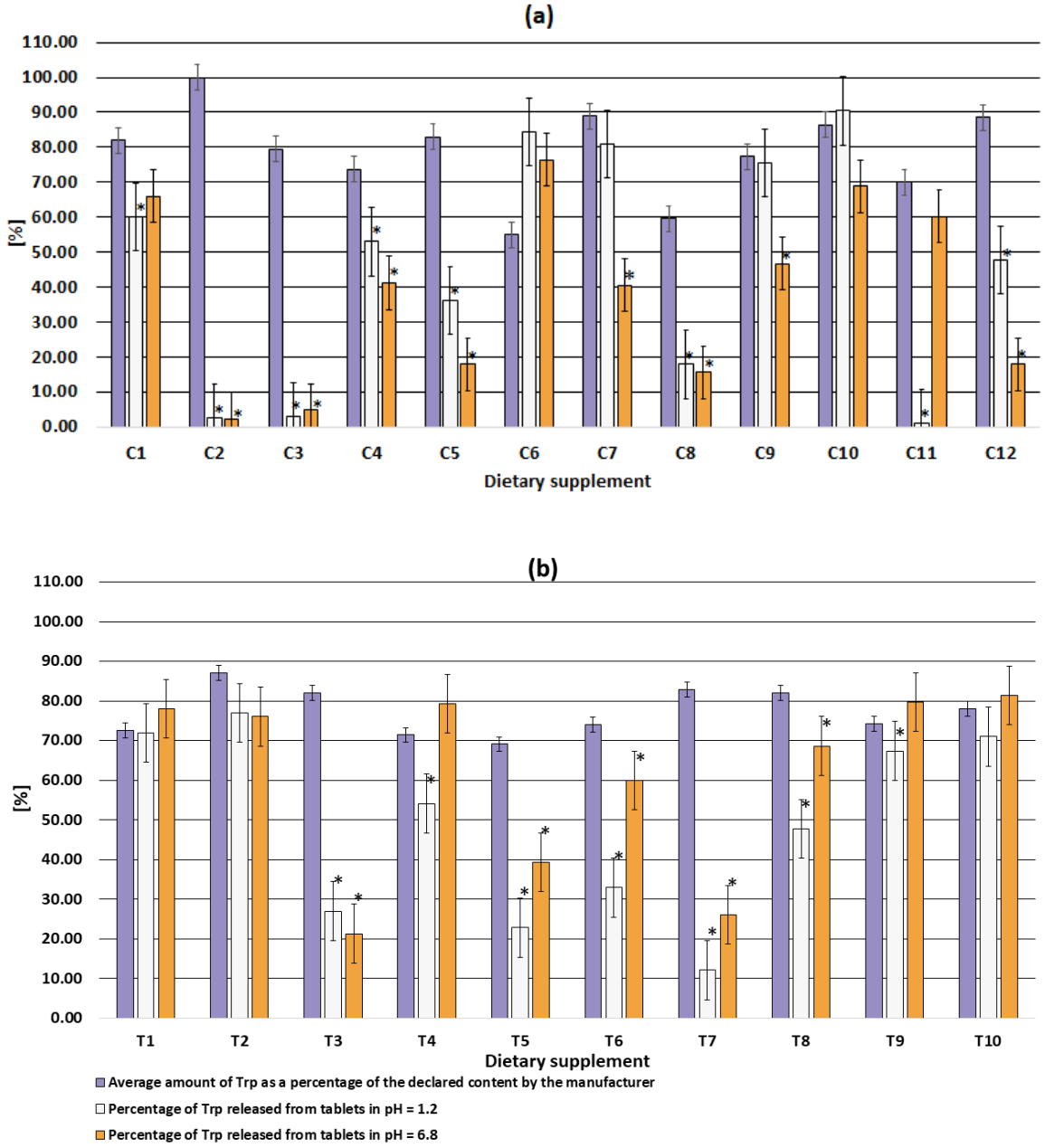


**Figure 4.** Comparison of the amount of Trp released at pH 1.2 (simulated gastric conditions), pH 6.8 (simulated intestinal conditions) with the amount determined in dietary supplements in capsules (**a**) and tablets (**b**); * significant differences (results not equal within the uncertainty) of the amount released with the amount detected.

In summary, none of the analysed dietary supplements contained 80% or more Trp (for each tablet/capsule), which means that these formulations do not meet the release requirements for medicinal products. Comparative data on Trp release from other dietary supplements are not available. Applying our criteria to dietary supplements containing lutein [8], it also the case that none of these dietary supplements would meet these requirements. However, a comparison between the two studies is not easy because the release of the lutein supplements was performed using unconventional parameters. The dissolution test fluid for tablets was 2% P80 (*w*/*v*) and for capsules 2% P80 (*w*/*v*) with 25%

ethanol. In the case of Trp supplements, the release test fluid was 0.1 mol/L hydrochloric acid (simulated gastric conditions) and 0.05 mol/L phosphate buffer (pH 6.8, simulated intestinal conditions) regardless of the form of the dietary supplement. Low release of active ingredients such as calcium carbonate [13], melatonin [14], folic acid [15,16], iron, zinc, manganese [17] and Grape seed extract [19], have also been observed in other dietary supplements in solid form (Table A2). However, in these cases, the reasons for the low release are not known due to the lack of data on the content of the main ingredient in these supplements. Low release may be due to insufficient content of the main ingredient, improperly selected process parameters, and/or improperly selected excipients. Only for food supplements containing triiodothyronine ($n$ = 3) or prehormone thyroxine ($n$ = 1) was the main component release above 93% [18].

In our study, Trp release was higher from tablets (12.1–81.4%) than from capsules (1.22–90.4%). Similar results were previously obtained for lutein [8] and folic acid [16]. For lutein supplements, release from capsules (made in the USA), despite containing adequate amounts of lutein, showed alarming results due to poor dissolution properties (less than 20% after 180 min of testing). These results may contribute to the lack of bioavailability of lutein. Unlike the capsules, the lutein tablets (made in Brazil) released more than 80% of the lutein within 180 min.

Analysis of the content, identity, and release of active ingredients from products is important to assess their quality [66]. The dissolution test determines the amount of active substance released and is mandatory for solid drug forms, but not for the same forms of dietary supplements [67]. The in vivo absorption of the active ingredient from solid formulations can be predicted to some extent using this assay [68]. A low release rate means low absorption and no intended effect. Thus, even the substance is in labelled amounts in the supplement but is not released, the consumer will not be able to achieve the effect.

Ease of marketing the supplement and low level of control combined with high popularity and high market value make dietary supplements a group of products particularly vulnerable to negligence or intentional manipulation, which poses a threat to consumers' interests and sometimes even their health [21,69,70]. Determining the quality of dietary supplements is challenging and can be more difficult than for pharmaceutical products because such products often contain multiple vitamins, minerals [71], many of which are derived from plants [72] or other biological sources [73]. However, quality control of supplements should meet the same standards as pharmaceutical products because in both cases they are intended for consumer use [74]. The results of our studies developed with the use of a gold standard in analytics—mass spectrometry coupled with liquid chromatography, provide important data on the quality of the analysed dietary supplements. We hope that our results will encourage further research and increase public awareness about the purposefulness and safety of taking dietary supplements. An informed consumer will choose tested supplements, which will encourage manufacturers to test. In the case of the Food and Drug Administration, Good Manufacturing Practice in Manufacturing, Packing, Labelling, or Holding Operations for Dietary Supplements were already established. Applying GMPs to dietary supplements would be a further step to ensure products are consistently produced and controlled to the quality standards appropriate to their intended use [74].

A limitation of our study is the inability to detect compounds present at very low concentrations. These compounds require appropriate sample preparation. In addition, the targeted analysis should be chosen over non-targeted screening in their case.

## 3. Materials and Methods

### *3.1. Samples*

The study was conducted on twenty-two Trp supplements, which is 10% of all dietary supplements with Trp in tablets or capsules registered in Poland, and all available on the market. There were two types of dosage forms: capsules (C1–C12) and tablets (T1–T10). All supplements were manufactured in the EU (Poland, UK, France, Germany, Czech Republic)

and the USA. Six supplements were purchased in a Polish online e-commercial platform, the rest in pharmacies or online pharmacies in Poland.

### *3.2. Reagents*

L-Trp (≥99%) (standard) and doxepin hydrochloride (internal standard) (≥98%) were purchased from Merck (Darmstadt, Germany). Hydrochloric acid (35–38%) solution pure p.a., sodium hydroxide (≥98.8%) pure p.a., potassium phosphate monobasic (≥99.5%) pure p.a. were purchased from Chempur (Piekary Śląskie, Poland). HPLC-grade methanol, acetonitrile, and formic acid were purchased from Merck (Darmstadt, Germany).

### *3.3. Sample Preparation*

Three tablets or capsules were randomly selected from each supplement. The total weight of three tablets, or of the contents of three capsules, were determined. For tablets, a grinding step was applied. In the next step, the tablet's mass or capsule content equivalent to 10 mg Trp was weighed and 1.00 mL of acetonitrile/methanol/water (1:1:1; *v*/*v*/*v*) mixture was added. The mixture was sonicated for 15 min and centrifuged for 5 min. The supernatant was then diluted with mobile phase to a concentration of 500 ng/mL or 100 ng/mL for qualitative and quantitative analysis, respectively. For quantitative analysis, an internal standard (doxepin) was added in the last step to a final concentration of 500 ng/mL.

### *3.4. Qualitative Analysis*

Instrumental analysis was performed using a UHPLC Dionex Ultimate 3000 with a Q-Exactive hybrid quadrupole-orbitrap mass spectrometer system equipped with heat electrospray ionization (HESI), an online vacuum degasser, a quaternary pump, an autosampler, and a thermostatted column compartment. The HESI was operated in positive mode. Full MS scans were acquired over the $m/z$ 100–1400 range with a resolution of 70,000 ($m/z$ 200). Fragmentation was performed in different runs with a normalized collision energy of 20, 35, 50 eV. The ion selection threshold was $8 \times 10^3$ counts, and the maximum allowed ion accumulation times were set to auto both for full MS scans and for the tandem mass spectrum. Standard mass spectrometric conditions for all experiments were: spray voltage, 3.5 kV; sheath gas pressure: 60 arb; aux gas pressure: 20 arb; sweep gas pressure: 0 arb, heated capillary temperature: 320 °C; loop count: 3; isolation window: $m/z$ 1.0; and dynamic exclusion: 6.0 s. For all full scan measurements, lock-mass ions from ambient air ($m/z$ 445.1200 and 291.2842) were used as internal calibrants.

Chromatographic separation was achieved with an Accucore C-18 column (100 mm × 4.6 mm, 2.6 μm) supplied by Thermo Fisher Scientific (Waltham, MA, USA) equipped with a security guard. The column was maintained at 40 °C at a flow rate of 0.3 mL/min. The mobile phases consisted of HPLC grade water with 0.1% formic acid as eluent A and acetonitrile with 0.1% formic acid as eluent B. The gradient (% B) was as follows: 0 min 10%; 1 min 10%; 10 min 95%; 15 min 95%. The volume of injection was 10 μL.

The results obtained were analysed using Compound Discoverer 3.0 software supplied by Thermo Fisher Scientific (Waltham, MA, USA).

The structures of the metabolites were proposed based on:

1. The $m/z$ of the compound. The difference between experimental and theoretical molecular weight should be no higher than 5 ppm;
2. The isotopic pattern. The relative intensity tolerance to be used for the isotope search was set at 30%;
3. Fragmentation of the compound. The fragmentation spectrum was compared with experimental data found in the mass spectra library or the literature (confidence level 2), in silico fragmentation (confidence level 3) or reference standard (confidence level 1).

### *3.5. Quantitative Analysis*

The instrumental analysis was performed using an Agilent 1260 Infinity (Agilent Technologies, Santa Clara, CA, USA), equipped with a degasser, autosampler, and binary pump coupled to a QTRAP 4000 hybrid triple quadrupole/linear ion trap mass spectrometer (AB Sciex, Framingham, MA, USA). The Turbo Ion Spray source was operated in positive mode. The curtain gas, ion source gas 1, ion source gas 2, and collision gas (all high-purity nitrogen) were set at 0.24 MPa, 0.41 MPa, 0.28 MPa, and "medium" instrument units, respectively. The ion spray voltage and source temperature were 4500 V and 600 °C, respectively. The target compounds were analysed in multiple reaction monitoring (MRM) mode. The compounds parameters, viz. declustering potential (DP), collision energy (CE), entrance potential (EP), and collision exit potential (CXP), were 76, 27, 12 V and 71, 25, 14 V for Trp and doxepin, respectively.

Chromatographic separation was achieved with a Kinetex C18 column (100 mm × 4.6 mm, 2.6 µm, Phenomenex, Milford, MA, USA). The injection volume was 10 µL. The flow rate was 0.75 mL/min. The mobile phases consisted of HPLC grade water with 0.2% formic acid as eluent A and acetonitrile with 0.2% formic acid as eluent B. The gradient (% B) was as follows: 0 min 5%; 1 min 5%; 2 min 95%; 3 min 95%.

The analysis of the Trp content in dietary supplements was preceded by method validation. The parameters tested were selectivity, precision, accuracy, linearity, and limit of quantification. The range of the calibration curve was selected as 0.01–10 µg/mL. Accuracy and precision were determined in triplicate at four concentration levels (0.01, 0.05, 5.0 and 10.0 µg/mL).

Calculations were made using the Analyst 1.6.3 software (AB Sciex, Framingham, MA, USA).

### *3.6. Dissolution Test for Tablets or Capsules*

Trp release study was performed using a USP II Varian VK 7025 or USP I Varian VK 7025 dissolution tester (Erweka GmbH, Heusenstamm, Germany) for tablets and capsules, respectively. Six tablets or capsules were randomly selected and individually placed in the dissolution vessels. Each vessel contained 900 mL of dissolution medium. The stirring speed of 50 rpm or 100 rpm was used for tablets and capsules, respectively. The temperature was set at 37 ± 0.5 °C. Aliquots (1.5 mL) of the medium were manually collected using 5 mL syringes after 30 min of the test and filtered through a Millex-HA 0.45 µm filter. Each aliquot withdrawn was replaced with 1.5 mL of fresh medium. The experiment was performed both in hydrochloride acid pH 1.2 (simulated gastric conditions) and phosphate buffer pH 6.8 (simulated intestinal conditions). The Trp content was measured as described in Section 3.5 (Quantitative analysis).

### *3.7. Expanded Uncertainty*

To assess whether the amount of Trp in the dosage unit and amount of the compound released is equal within the uncertainty range, extended uncertainty was determined using Equation (1).

$$U(x_1 - x_2) = 2\sqrt{[u(x_1)]^2 + [u(x_2)]^2} \quad (1)$$

The measurement results were equal if:

$$|x_1 - x_2| < U(x_1 - x_2)$$

$x_1$—mean [mg] Trp content determined in dosage unit using quantitative analysis ($n$ = 3).

$x_2$—mean [mg] amount of Trp released from six dosage units.

$u(x_2), u(x_1)$—standard uncertainties of the measured values: $x_1$ and $x_2$ determined according to the formula:

$$u(x_1) = \frac{S}{\sqrt{n}}$$

*S*—standard deviation of the average amount of Trp in dosage unit [mg] or standard deviation of the released amount of Trp [mg].

*n*—the number of tablets or capsules analysed.

## 4. Conclusions

A new analytical approach based on liquid chromatography coupled to mass spectrometry provided the opportunity to obtain reliable results on the quality of dietary supplements. The quality of supplements is lower than that of pharmaceuticals with lower than claimed amounts of the main ingredient and a lack of uniform distribution between units. Sometimes, the release of the main ingredient is low, resulting in a lower probability of absorption and physiological effect. Contaminants were detected in all dietary supplements analysed, based on untargeted analysis. These substances, in the amounts determined, may not affect health or show significant unknown effects. The study confirms issues with the quality of dietary supplements and provides an important contribution to the discussion on the regulation of dietary supplements. We believe that the new analytical approach will have broad applicability in the assessment of supplement quality.

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## Appendix A

**Table A1.** A review of research on contaminants in dietary supplements.

| Type of Dietary Supplement | Number of Supplements | Contaminants | Country of Sale | Year | Method Applied | Ref. |
|---|---|---|---|---|---|---|
| **Plant based (e.g., Ginkgo biloba, Ginseng, flower pollen), algae** | 24 | Cd, Pb, Hg | Mexico | 2007 | ASA | [75] |
| **Mainly plant-based (herbs or botanicals as major components)** | 95 | As, Cd, Pb, Hg | USA | 2003 | ICP-MS | [76] |
| **Mainly plant-based (e.g., ginger, gingko biloba, ephedra), minerals** | 40 | Hg | USA | 2005 | ASA | [77] |
| **Plant-based and algae (e.g., gingko biloba)** | 16 | As | Denmark | 2013 | ICP-MS, LC-ICP-MS | [78] |
| **Iron supplements** | 15 | As | Brazil/Spain | 2017 | LC-ICP-MS | [79] |
| **Multimineral supplements** | 168 | Pb | Poland | 2018 | MIP-OES | [80] |

**Table A1.** *Cont.*

| Type of Dietary Supplement | Number of Supplements | Contaminants | Country of Sale | Year | Method Applied | Ref. |
|---|---|---|---|---|---|---|
| **Herbal (improve hair, skin, and nails; regulate glucose levels)** | 24 | Hg | Poland | 2018 | ASA | [81] |
| **Prenatal and children supplements** | 10 | As | USA | 2014 | IC-ICP-MS | [82] |
| **Prenatal vitamin supplements** | 51 | As, Cd, Pb, Hg | Canada | 2018 | ICP-MS | [83] |
| **Health clays products** | 27 | As, Cd, Pb, Hg | Netherlands | 2013 | ICP-MS | [84] |
| **Calcium supplements** | 45 | Pb | USA | 2007 | ICP-MS | [85] |
| **Shark cartilage powder** | 16 | Cyanobacterial toxin (N-methylamino-L-alanine) and its isomers (2,4-diaminobutyric acid and N(2-aminoethy) glycine)), Hg | USA | 2014 | LC-FLD, LC–MS, CVAFS | [86] |
| **Ginkgo** | 9 | 250 toxic substances including pesticides (e.g., hymexazol, tebufenozide) and mycotoxins (e.g., aflatoxin B1, aflatoxin B2, T-2 toxin), Insecticides, Fungicides, Herbicides | Spain, Poland, USA | 2015 | LC-HRMS | [46] |
| **Grape** | 24 | Mycotoxin (Ochratoxin A) | Italy | 2015 | LC–FLD | [87] |
| **Different plants (used for liver problems, menopause, for general health improvement)** | 69 | 57 mycotoxins (e.g., zearalenone, enniatins) | Czech Republic, USA | 2015 | LC-MS | [88] |
| **Brewer's yeast** | 51 | Mycotoxin (Ochratoxin A) | Germany | 2002 | LC-FLD | [89] |
| **Blue green algae** | 17 | Microcystins | Italy | 2012 | LC–MS, ELISA | [90] |
| **Blue green algae and Chlorella** | 18 | Microcystins | Germany | 2012 | PPIA, ELISA, LC–MS | [91] |
| **Ginseng** | 23 | Insecticide, Fungicides | USA | 2016 | GC-MS | [44] |
| **Soya** | 14 | Herbicides | Spain | 2016 | LC-MS | [92] |
| **Fish, seal and vegetable** | 30 | Insecticides | Canada | 2009 | GC-MS | [93] |
| **Omega-3** | 9 | Polychlorinated dibenzo-p-dioxins | Spain | 2017 | GC-MS | [94] |
| **Plant-based (weight loss)** | 11 | Sibutramine and its analogues, phenolphthalein | China | 2008 | LC-MS | [95] |
| **Plant-based (weight loss)** | 24 | Sibutramine and its analogues, rimonabant, phenolphthalein | Netherland | 24 | LC-DAD-MS | [96] |
| **Plant-based (naturally enhance sexual performance)** | 74 | PDE-5 inhibitors and their analogues | USA | 2013 | LC-DAD-MS | [97] |

**Table A1.** *Cont.*

| Type of Dietary Supplement | Number of Supplements | Contaminants | Country of Sale | Year | Method Applied | Ref. |
|---|---|---|---|---|---|---|
| **Plant-based (enhance sexual potency)** | 23 | PDE-5 inhibitors and their analogues | Netherland | 2013 | LC-DAD-MS | [98] |
| **Tryptophan** | 22 | Untargeted screening, Trp products generated during production, storage, transport | Poland | 2022 | LC-HRMS | Current study |

ASA—atomic absorption spectrometry, CVAFS—cold vapor atomic fluorescence spectrometry, DAD—diode array detection, ELISA—enzyme linked immuno-system, GC—gas chromatography, IC—ion chromatography, ICP-MS—inductively coupled plasma—mass spectrometry, LC-liquid chromatography, LC-HRMS—liquid chromatography–high resolution mass spectrometry, LC-FLD—liquid chromatography fluorescence detector, MIP-OES—microwave-induced plasma optical emission spectrometry, MS—mass spectrometry, PPIA—phosphatase inhibition assay.

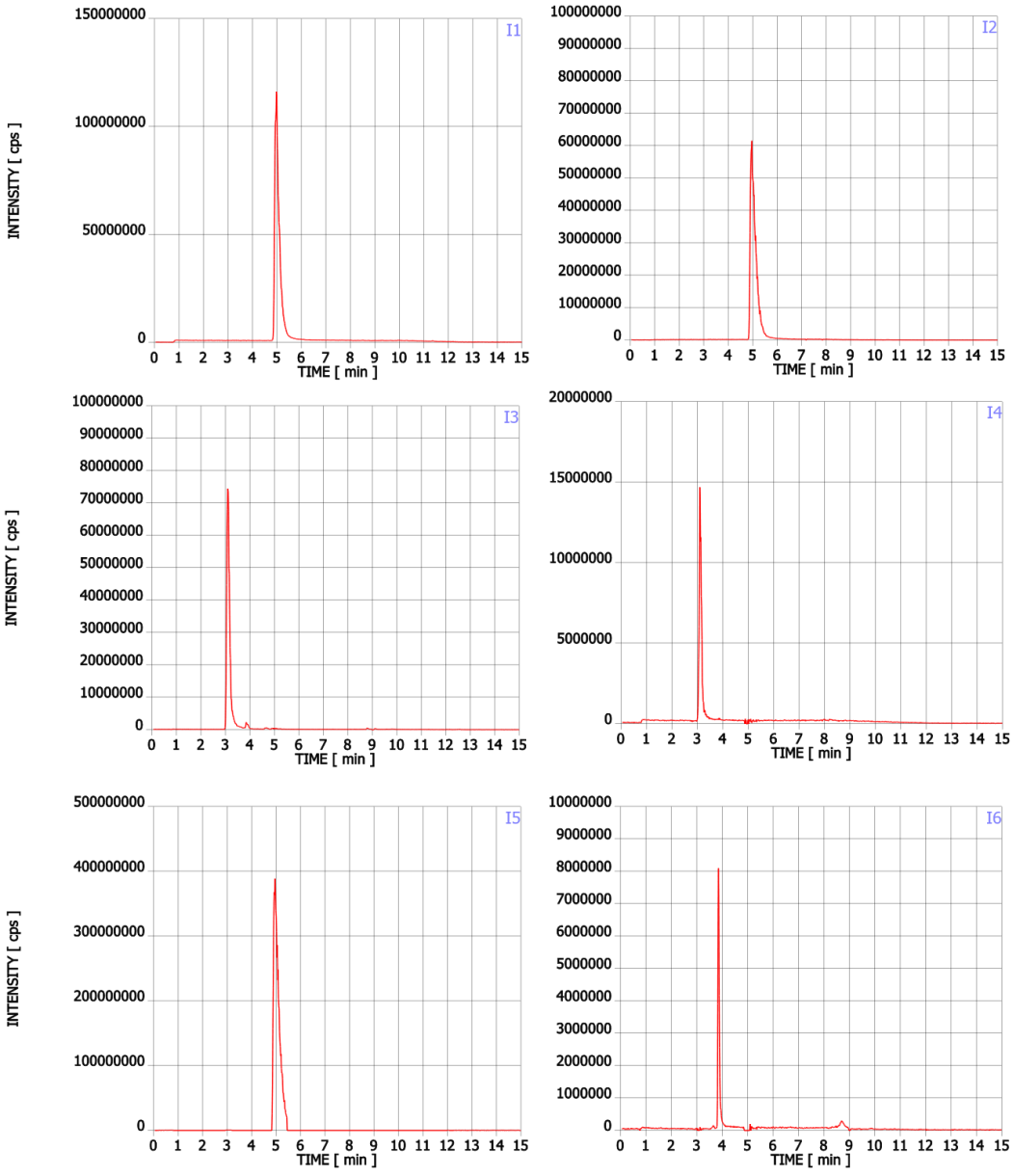


**Figure A1.** *Cont.*

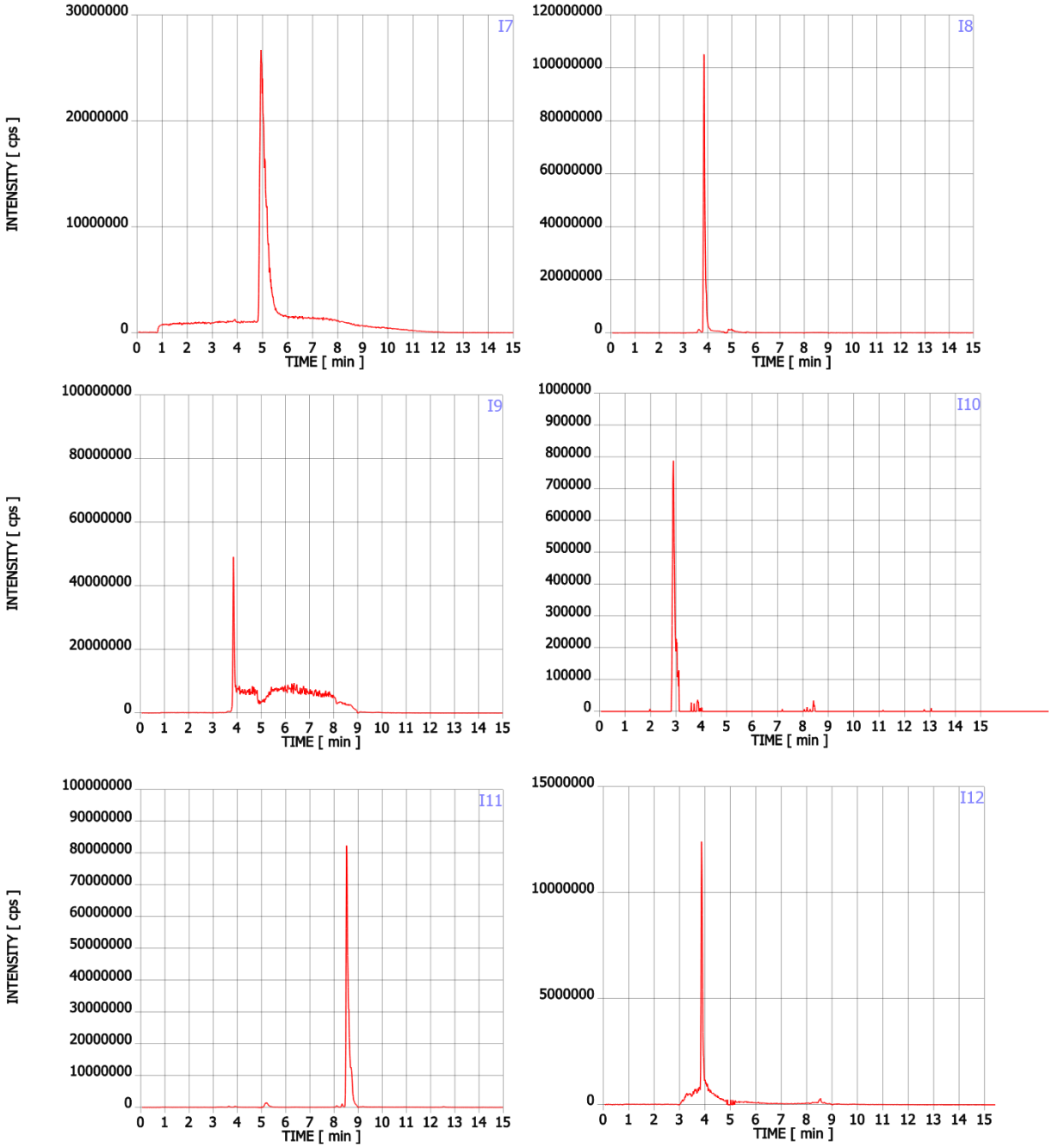


**Figure A1.** *Cont.*

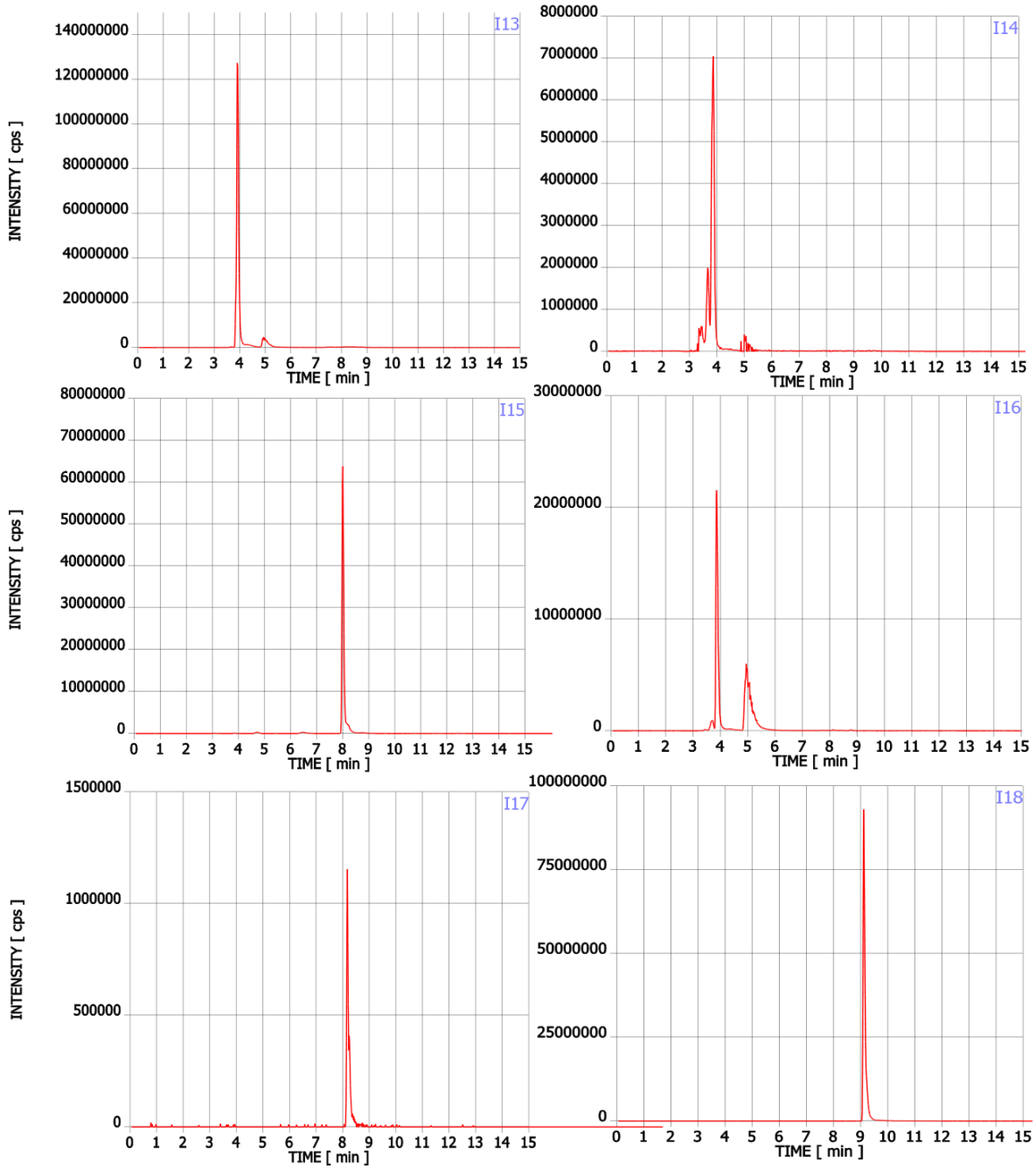


**Figure A1.** *Cont.*

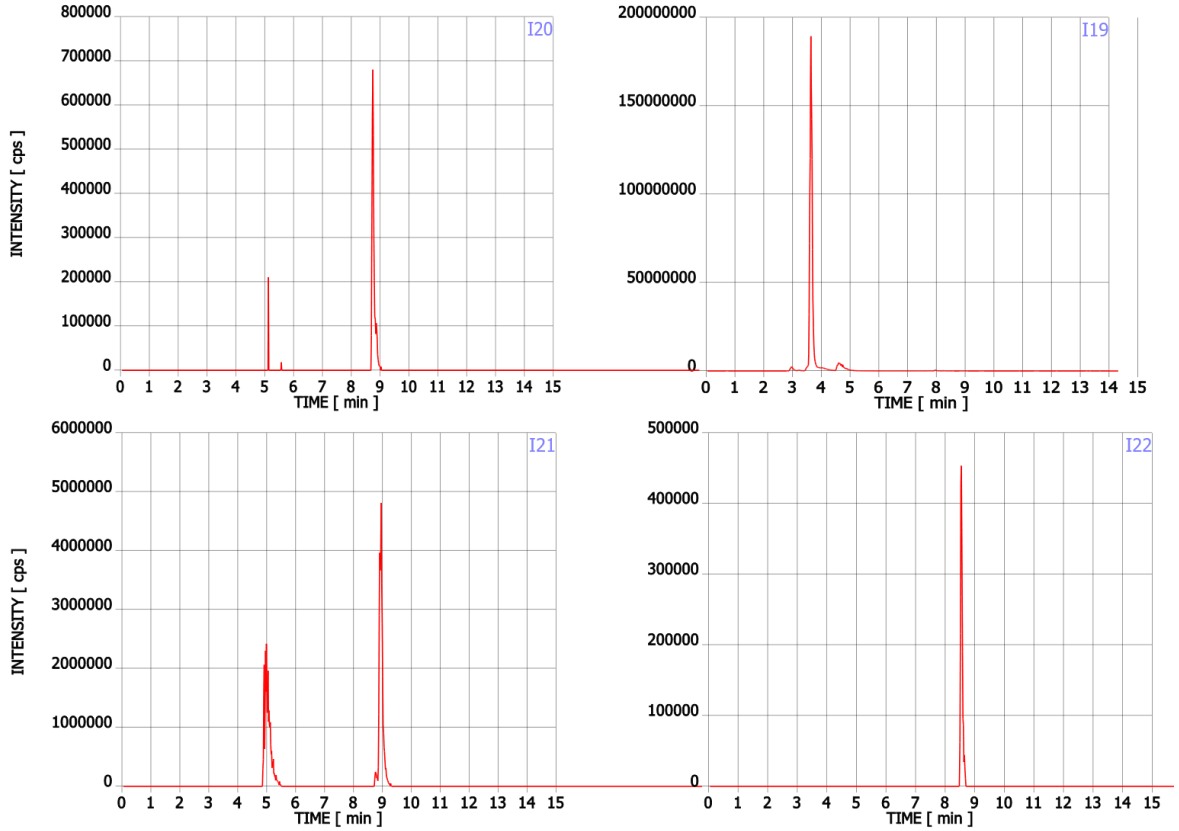


**Figure A1.** Chromatograms of Trp contaminants (I1-I22) detected in Trp dietary supplements.

**Table A2.** Review of studies on the release assay of the active substance from dietary supplements.

| Main of Ingredient Dietary Supplement | Year | Country of Sale | Dosage Form | Number of Supplements | Dissolution Test | The Average Percentage of Trp Amount Released from a Dosage Form (Dissolution Medium) | Reference |
|---|---|---|---|---|---|---|---|
| **Calcium Carbonate** | 1990 | USA | tablet | 27 | Yes | 5/27—below 75% (HCl pH 1.0) 4/27—between 33–75% (HCl pH 1.0) 18/27—less than 33% (HCl pH 1.0) | [13] |
| **Melatonin** | 1999 | USA | Immediate-release | 9 | Yes | 4/9 above 75% (HCl pH 1.0) | [14] |
| | | | Controlled-release | 2 | | 1/2 above 90% (HCl pH 1.0) | |
| **Folic Acid** | 2001 | United Kingdom | capsule tablet | 11 | Yes | 6/11—below 70% (0.1 M sodium hydroxide) 4/11—above 70% (0.1 M sodium hydroxide) | [15] |
| **Folic Acid** | 2009 | USA | tablet | 14 | | 45.0% (NaCl, pH 1.5) 104.5% (phosphate buffer, pH 7.5) | [16] |
| | | | capsule | 1 | | 15.2% (NaCl, pH 1.5) 47.4% (phosphate buffer, pH 7.5) | |

**Table A2.** *Cont.*

| Main of Ingredient Dietary Supplement | Year | Country of Sale | Dosage Form | Number of Supplements | Dissolution Test | The Average Percentage of Trp Amount Released from a Dosage Form (Dissolution Medium) | Reference |
|---|---|---|---|---|---|---|---|
| **Iron, zinc, manganase** | 2016 | Poland | tablet | 4 | Yes | Iron—1/4 above 80% (HCl, pH 1.2) Zinc—1/4 above 80% (HCl, pH 1.2) Manganase—4/4–60% or less (HCl, pH 1.2) | [17] |
| **Lutein** | 2018 | Brazil USA | tablet | 4 | Yes | 41.7% (2% polysorbate 80) | [8] |
| | | | capsule | 6 | | 122.5% (2% polysorbate 80 with 25% ethanol) | |
| **Triiodothyronine** | 2019 | United Kingdom | tablet | 3 | Yes | Above 93.5% (fasted-state simulated gastric fluid) | [18] |
| **Prehormone thyroxine** | 2019 | United Kingdom | tablet | 1 | Yes | Above 97.4% (fasted-state simulated gastric fluid) | [18] |
| **Grape seed extract** | 2021 | USA | capsule | 1 | Yes | 73.09, 67.9, 71.06, 59.75% of gallic acid, catechin, procyanidin B2, and epicatechin, respectively (acetate buffer pH 4.6), 96.49, 89.09, 87.65, 78.84% of gallic acid, catechin, procyanidin B2, and epicatechinin, respectively (HCl pH 1.2) | [19] |
| **Trans-resveratrol** | 2021 | China | capsule | 1 | Yes | Above 75% (acetate buffer pH 4.6) Above 75% (HCl pH 1.2) | [19] |
| **Tryptophan** | 2022 | Poland | tablet | 10 | Yes | 48.5% (HCl, pH 1.2) 61.0% (phosphate buffer pH 6.8) | Current study |
| | | | capsule | 12 | | 46.1% (HCl, pH 1.2) 38.2% (phosphate buffer pH 6.8) | |

## References


1. Cerezo, A.B.; Leal, Á.; Álvarez-Fernández, M.A.; Hornedo-Ortega, R.; Troncoso, A.M.; García-Parrilla, M.C. Quality control and determination of melatonin in food supplements. *J. Food Compos. Anal.* **2016**, *45*, 80–86. [CrossRef]
2. EC. Directive 2002/46/EC of the European parliament and of the council of 10 June 2002 on the approximation of the laws of the member states relating to food supplements. *Off. J. Eur. Communities Legis.* **2002**, *45*, 51–57.
3. FDA. *Dietary Supplement Health and Education Act of 1994*; FDA: St. Louis, MO, USA, 1995; Volume 1.
4. Moret, S.; Prevarin, A.; Tubaro, F. Levels of creatine, organic contaminants and heavy metals in creatine dietary supplements. *Food Chem.* **2011**, *126*, 1232–1238. [CrossRef]
5. Tumir, H.; Bošnir, J.; Vedrina-Dragojević, I.; Dragun, Z.; Tomić, S.; Puntarić, D.; Jurak, G. Monitoring of metal and metalloid content in dietary supplements on the Croatian market. *Food Control* **2010**, *21*, 885–889. [CrossRef]
6. Andrews, K.W.; Roseland, J.M.; Gusev, P.A.; Palachuvattil, J.; Dang, P.T.; Savarala, S.; Han, F.; Pehrsson, P.R.; Douglass, L.W.; Dwyer, J.T.; et al. Analytical ingredient content and variability of adult multivitamin/mineral products: National estimates for the dietary supplement ingredient database. *Am. J. Clin. Nutr.* **2017**, *105*, 526–539. [CrossRef]
7. De Ceglie, C.; Calvano, C.D.; Zambonin, C.G. MALDI-TOF MS for quality control of high protein content sport supplements. *Food Chem.* **2015**, *176*, 396–402. [CrossRef] [PubMed]
8. Anselmo, C.S.; Mendes, T.C.; Cabral, L.M.; Sousa, V.P. Physicochemical quality profiles of commercial oral tablets and capsules containing lutein—Impact of insufficient specific sanitary regulations. *An. Acad. Bras. Cienc.* **2018**, *90*, 3063–3073. [CrossRef] [PubMed]

9. Kowalski, A.; Frankowski, M. Levels and potential health risks of mercury in prescription, non-prescription medicines and dietary supplements in Poland. *Regul Toxicol. Pharm.* **2015**, *73*, 396–400. [CrossRef] [PubMed]
10. Martinez-Sanz, J.M.; Sospedra, I.; Ortiz, C.M.; Baladia, E.; Gil-Izquierdo, A.; Ortiz-Moncada, R. Intended or unintended doping? A review of the presence of doping substances in dietary supplements used in sports. *Nutrients* **2017**, *9*, 1093. [CrossRef] [PubMed]
11. Fernandes, A.R.; Rose, M.; White, S.; Mortimer, D.; Gem, M. Dioxins and polychlorinated biphenyls (PCBs) in fish oil dietary supplements: Occurrence and human exposure in the UK. *Food Addit. Contam.* **2006**, *23*, 939–947. [CrossRef]
12. Bengtson Nash, S.M.; Schlabach, M.; Nichols, P.D. A nutritional-toxicological assessment of Antarctic krill oil versus fish oil dietary supplements. *Nutrients* **2014**, *6*, 3382–3402. [CrossRef] [PubMed]
13. Brennan, M.J.; Duncan, W.E.; Wartofsky, L.; Butler, V.M.; Wray, H.L. In vitro dissolution of calcium carbonate preparations. *Calcif. Tissue Int.* **1991**, *49*, 308–312. [CrossRef] [PubMed]
14. Hahm, H.; Kujawa, J.; Augsburger, L. Comparison of melatonin products against USP's nutritional supplements standards and other criteria. *J. Am. Pharm. Assoc.* **1999**, *39*, 27–31. [CrossRef]
15. Sculthorpe, N.; Davies, B.; Ashton, T.; Allison, S.; McGuire, D.; Malhi, J. Commercially available folic acid supplements and their compliance with the British pharmacopoeia test for dissolution. *J. Public Health* **2001**, *23*, 195–197. [CrossRef] [PubMed]
16. Younis, I.R.; Stamatakis, M.K.; Callery, P.S.; Meyer-Stout, P.J. Influence of pH on the dissolution of folic acid supplements. *Int. J. Pharm.* **2009**, *367*, 97–102. [CrossRef]
17. Blicharska, E.; Szczęsna, B.; Kocjan, R.; Gumieniczek, A.; Komsta, Ł. Analysis of dissolution profiles of iron, zinc, and manganese from complex dietary supplements by ion chromatography and chemometrics. *J. Liq. Chromatogr. Relat. Technol.* **2016**, *39*, 30–34. [CrossRef]
18. Bowerbank, S.L.; Carlin, M.G.; Dean, J.R. Dissolution testing of single-and dual-component thyroid hormone supplements. *Separations* **2019**, *6*, 18. [CrossRef]
19. Lyu, W.; Omar, T.; Patel, H.; Rodriguez, D.; Ferruzzi, M.G.; Pasinetti, G.M.; Murrough, J.W.; Muzzio, F.J.; Simon, J.E.; Wu, Q. Dissolution study on grape polyphenol hard gelatin capsule dietary supplements. *Front. Nutr.* **2021**, *8*, 780260. [CrossRef] [PubMed]
20. Palaric, C.; Pilard, S.; Fontaine, J.-X.; Cailleu, D.; Mesnard, F.; Renaud, T.; Petit, A.; Molinié, R. Combined MS-NMR approach for the quality control of food supplements containing amino acids. *Food Control* **2018**, *88*, 217–228. [CrossRef]
21. Dwyer, J.T.; Coates, P.M.; Smith, M.J. Dietary supplements: Regulatory challenges and research resources. *Nutrients* **2018**, *10*, 41. [CrossRef] [PubMed]
22. Marozoff, S.; Veugelers, P.J.; Dabravolskaj, J.; Eurich, D.T.; Ye, M.; Maximova, K. Diet quality and health service utilization for depression: A prospective investigation of adults in alberta's tomorrow project. *Nutrients* **2020**, *12*, 2437. [CrossRef] [PubMed]
23. Park, S.J.; Lee, D.K.; Kim, B.; Na, K.S.; Lee, C.H.; Son, Y.D.; Lee, H.J. The association between omega-3 fatty acid intake and human brain connectivity in middle-aged depressed women. *Nutrients* **2020**, *12*, 2191. [CrossRef] [PubMed]
24. Galts, C.P.C.; Bettio, L.E.B.; Jewett, D.C.; Yang, C.C.; Brocardo, P.S.; Rodrigues, A.L.S.; Thacker, J.S.; Gil-Mohapel, J. Depression in neurodegenerative diseases: Common mechanisms and current treatment options. *Neurosci. Biobehav. Rev.* **2019**, *102*, 56–84. [CrossRef]
25. Lieberman, H.R.; Agarwal, S.; Fulgoni, V.L., 3rd. Tryptophan intake in the US adult population is not related to liver or kidney function but is associated with depression and sleep outcomes. *J. Nutr.* **2016**, *146*, 2609S–2615S. [CrossRef] [PubMed]
26. Palego, L.; Betti, L.; Rossi, A.; Giannaccini, G. Tryptophan biochemistry: Structural, nutritional, metabolic, and medical aspects in humans. *J. Amino Acids* **2016**, *2016*, 8952520. [CrossRef] [PubMed]
27. Fukuwatari, T. Possibility of amino acid treatment to prevent the psychiatric disorders via modulation of the production of tryptophan metabolite kynurenic acid. *Nutrients* **2020**, *12*, 1403. [CrossRef] [PubMed]
28. Soh, N.L.; Walter, G. Tryptophan and depression: Can diet alone be the answer? *Acta Neuropsychiatr.* **2014**, *23*, 3–11. [CrossRef]
29. Samad, N.; Yasmin, F.; Manzoor, N. Biomarkers in drug free subjects with depression: Correlation with tryptophan. *Psychiatry Investig.* **2019**, *16*, 948–953. [CrossRef] [PubMed]
30. Jenkins, T.A.; Nguyen, J.C.; Polglaze, K.E.; Bertrand, P.P. Influence of tryptophan and serotonin on mood and cognition with a possible role of the gut-brain axis. *Nutrients* **2016**, *8*, 56. [CrossRef]
31. Binks, H.; Vincent, G.E.; Gupta, C.; Irwin, C.; Khalesi, S. Effects of Diet on Sleep: A Narrative Review. *Nutrients* **2020**, *12*, 936. [CrossRef]
32. Strasser, B.; Fuchs, D. Diet versus Exercise in Weight Loss and Maintenance: Focus on Tryptophan. *Int. J. Tryptophan Res.* **2016**, *9*, 9–16. [CrossRef] [PubMed]
33. Gibson, E.L. Tryptophan supplementation and serotonin function: Genetic variations in behavioural effects. *Proc. Nutr. Soc.* **2018**, *77*, 174–188. [CrossRef] [PubMed]
34. Thomas, S.R. l-Tryptophan as an antidepressive agent in the management of treatment-resistant unipolar depression in borderline personality disorder: Three case reports. *Adv. Integr. Med.* **2015**, *2*, 68–71. [CrossRef]
35. European Parliament; Council of the European Union. Regulation (EU) No 609/2013 of the European parliament and of the council of 12 June 2013 on food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control and repealing council directive 92/52/EEC, commission directives 96/8/EC, 1999/21/EC, 2006/125/EC and 2006/141/EC, directive 2009/39/EC of the European parliament and of the council and commission regulations (EC) No 41/2009 and (EC) No 953/2009. *Off. J. Eur. Union* **2013**, *181*, 35–56.

36. Simat, T.J.; Kleeberg, K.K.; Müller, B.; Sierts, A. Contamination of commercially available L-tryptophan by related substances. *Eur. Food Res. Technol.* **2003**, *216*, 241–252. [CrossRef]
37. Bellmaine, S.; Schnellbaecher, A.; Zimmer, A. Reactivity and degradation products of tryptophan in solution and proteins. *Free. Radic. Biol. Med.* **2020**, *160*, 696–718. [CrossRef] [PubMed]
38. Sheng, X.; Himo, F. Enzymatic pictet-spengler reaction: Computational study of the mechanism and enantioselectivity of norcoclaurine synthase. *J. Am. Chem. Soc.* **2019**, *141*, 11230–11238. [CrossRef]
39. Simat, T.; Van Wickern, B.; Eulitz, K.; Steinhart, H. Contaminants in biotechnologically manufactured L-tryptophan. *J. Chromatogr. B Biomed. Sci. Appl.* **1996**, *685*, 41–51. [CrossRef]
40. Zelentsova, E.A.; Sherin, P.S.; Snytnikova, O.A.; Kaptein, R.; Vauthey, E.; Tsentalovich, Y.P. Photochemistry of aqueous solutions of kynurenic acid and kynurenine yellow. *Photochem. Photobiol. Sci.* **2013**, *12*, 546–558. [CrossRef] [PubMed]
41. Gugliandolo, E.; Peritore, A.F.; Impellizzeri, D.; Cordaro, M.; Siracusa, R.; Fusco, R.; D'Amico, R.; Di Paola, R.; Schievano, C.; Cuzzocrea, S. Dietary supplementation with palmitoyl-glucosamine co-micronized with curcumin relieves osteoarthritis pain and benefits joint mobility. *Animals* **2020**, *10*, 1827. [CrossRef] [PubMed]
42. Amaral, F.G.d.; Cipolla-Neto, J. A brief review about melatonin, a pineal hormone. *Arch. Endocrinol. Metab.* **2018**, *62*, 472–479. [CrossRef]
43. Nieto-García, A.J.; Romero-González, R.; Frenich, A.G. Multi-pesticide residue analysis in nutraceuticals from grape seed extracts by gas chromatography coupled to triple quadrupole mass spectrometry. *Food Control.* **2015**, *47*, 369–380. [CrossRef]
44. Chen, Y.; Lopez, S.; Hayward, D.G.; Park, H.Y.; Wong, J.W.; Kim, S.S.; Wan, J.; Reddy, R.M.; Quinn, D.J.; Steiniger, D. Determination of multiresidue pesticides in botanical dietary supplements using gas chromatography–Triple-quadrupole mass spectrometry (GC-MS/MS). *J. Agric. Food Chem.* **2016**, *64*, 6125–6132. [CrossRef] [PubMed]
45. Martínez-Domínguez, G.; Romero-González, R.; Arrebola, F.J.; Frenich, A.G. Multi-class determination of pesticides and mycotoxins in isoflavones supplements obtained from soy by liquid chromatography coupled to orbitrap high resolution mass spectrometry. *Food Control* **2016**, *59*, 218–224. [CrossRef]
46. Martinez-Dominguez, G.; Romero-Gonzalez, R.; Frenich, A.G. Determination of toxic substances, pesticides and mycotoxins, in ginkgo biloba nutraceutical products by liquid chromatography orbitrap-mass spectrometry. *Microchem. J.* **2015**, *118*, 124–130. [CrossRef]
47. Yue, K.X.; Li, H.L.; Pi, Z.F.; Song, F.R.; Wang, Y.P.; Liu, Z.Y. Multi-residue screening of pesticides in panax ginseng CA meyer by ultra-high-performance liquid chromatography with quadrupole time-of-flight mass spectrometry. *J. Sep. Science.* **2022**, 1–9. [CrossRef]
48. Hou, X.; Liu, L.; Wei, L.; Feng, D.; Lv, M.; Wang, X.; Yu, X.; Lu, Z.; Hou, Z. A Novel Analysis Method for Simultaneous Determination of 31 Pesticides by High-Performance Liquid Chromatography-Tandem Mass Spectrometry in Ginseng. *J. Anal. Methods Chem.* **2022**, *2022*, 4208243. [CrossRef]
49. Nasiri, M.; Ahmadzadeh, H.; Amiri, A. Sample preparation and extraction methods for pesticides in aquatic environments: A review. *TrAC Trends Anal. Chem.* **2020**, *123*, 115772. [CrossRef]
50. Montone, C.M.; Moneta, B.G.; Aita, S.E.; Aulenta, F.; Cavaliere, C.; Cerrato, A.; Fazi, S.; Laganà, A.; Paolini, V.; Petracchini, F. Untargeted analysis of contaminants in river water samples: Comparison between two different sorbents for solid-phase extraction followed by liquid chromatography-high-resolution mass spectrometry determination. *Microchem. J.* **2022**, *172*, 106979. [CrossRef]
51. Nemati, M.; Tuzen, M.; Farazajdeh, M.A.; Kaya, S.; Mogaddam, M.R.A. Development of dispersive solid-liquid extraction method based on organic polymers followed by deep eutectic solvents elution; application in extraction of some pesticides from milk samples prior to their determination by HPLC-MS/MS. *Anal. Chim. Acta* **2022**, *1199*, 339570. [CrossRef]
52. Zohrabi, P.; Shamsipur, M.; Hashemi, M.; Hashemi, B. Liquid-phase microextraction of organophosphorus pesticides using supramolecular solvent as a carrier for ferrofluid. *Talanta* **2016**, *160*, 340–346. [CrossRef] [PubMed]
53. Alvi, T.; Asif, Z.; Khan, M.K.I. Clean label extraction of bioactive compounds from food waste through microwave-assisted extraction technique-A review. *Food Biosci.* **2022**, *46*, 101580. [CrossRef]
54. Moret, S.; Scolaro, M.; Barp, L.; Purcaro, G.; Conte, L.S. Microwave assisted saponification (MAS) followed by on-line liquid chromatography (LC)—Gas chromatography (GC) for high-throughput and high-sensitivity determination of mineral oil in different cereal-based foodstuffs. *Food Chem.* **2016**, *196*, 50–57. [CrossRef] [PubMed]
55. Zhou, Z.; Zhang, J.; Zhang, W.; Bai, Y.; Liu, H. Rapid screening for synthetic antidiabetic drug adulteration in herbal dietary supplements using direct analysis in real time mass spectrometry. *Analyst* **2011**, *136*, 2613–2618. [CrossRef]
56. Patel, D.N.; Li, L.; Kee, C.-L.; Ge, X.; Low, M.-Y.; Koh, H.-L. Screening of synthetic PDE-5 inhibitors and their analogues as adulterants: Analytical techniques and challenges. *J. Pharm. Biomed. Anal.* **2014**, *87*, 176–190. [CrossRef] [PubMed]
57. Haller, C.A.; Benowitz, N.L. Adverse cardiovascular and central nervous system events associated with dietary supplements containing ephedra alkaloids. *N. Engl. J. Med.* **2000**, *343*, 1833–1838. [CrossRef] [PubMed]
58. Schymanski, E.L.; Jeon, J.; Gulde, R.; Fenner, K.; Ruff, M.; Singer, H.P.; Hollender, J. Identifying small molecules via high resolution mass spectrometry: Communicating confidence. *Environ. Sci. Technol.* **2014**, *48*, 2097–2098. [CrossRef] [PubMed]
59. Rochat, B. Proposed confidence scale and ID score in the identification of known-unknown compounds using high resolution MS data. *J. Am. Soc. Mass Spectrom.* **2017**, *28*, 709–723. [CrossRef] [PubMed]

60. Jakubowska, E.; Ciepluch, N. Blend segregation in tablets manufacturing and its effect on drug content uniformity—A review. *Pharmaceutics* **2021**, *13*, 1909. [CrossRef]
61. Farquharson, A.; Gladding, Z.; Ritchie, G.; Shende, C.; Cosgrove, J.; Smith, W.; Brouillette, C.; Farquharson, S. Drug content uniformity: Quantifying loratadine in tablets using a created raman excipient spectrum. *Pharmaceutics* **2021**, *13*, 309. [CrossRef]
62. Khashaba, P.Y.; Abdelgaleel, M.; Derayea, S.M.; Nagi, D.M. Development of a simple validated spectrofluorometric method for the assay of midodrine in tablets dosage form; Application to content uniformity testing. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **2022**, *273*, 121046. [CrossRef] [PubMed]
63. Ganzetti, R.; Logrippo, S.; Sestili, M.; Caraffa, A.; Cespi, M.; Pelliccioni, G.; Blasi, P.; Bonacucina, G. Tablet splitting in elderly patients with dementia: The case of quetiapine. *Pharmaceutics* **2021**, *13*, 1523. [CrossRef] [PubMed]
64. Peeters, M.; Peeters, E.; De Leersnyder, F.; Van Hauwermeiren, D.; Cogoni, G.; Liu, Y.; De Beer, T. Determination and understanding of dynamic lead lag between in-line nir tablet press feed frame and off-line nir tablet measurements. In Proceedings of the 2019 AIChE Annual Meeting, Orlando, FL, USA, 12 November 2019.
65. FDA. *Dissolution Testing and Acceptance Criteria for Immediate-Release Solid Oral Dosage form Drug Products Containing High Solubility Drug Substances Guidance For Industry*; FDA: Rockville, MD, USA, 2018.
66. Yu, L.X.; Amidon, G.; Khan, M.A.; Hoag, S.W.; Polli, J.; Raju, G.; Woodcock, J. Understanding pharmaceutical quality by design. *AAPS J.* **2014**, *16*, 771–783. [CrossRef]
67. Al-Gousous, J.; Langguth, P. Oral solid dosage form disintegration testing—The forgotten test. *J. Pharm. Sci.* **2015**, *104*, 2664–2675. [CrossRef] [PubMed]
68. Gray, V.A. Power of the dissolution test in distinguishing a change in dosage form critical quality attributes. *AAPS PharmSciTech* **2018**, *19*, 3328–3332. [CrossRef] [PubMed]
69. Binns, C.W.; Lee, M.K.; Lee, A.H. Problems and prospects: Public health regulation of dietary supplements. *Annu. Rev. Public Health* **2018**, *39*, 403–420. [CrossRef] [PubMed]
70. Jędrejko, K.; Lazur, J.; Muszyńska, B. Risk associated with the use of selected ingredients in food supplements. *Chem. Biodivers.* **2021**, *18*, e2000686. [CrossRef] [PubMed]
71. Moses, G. The safety of commonly used vitamins and minerals. *Aust. Prescr.* **2021**, *44*, 119. [CrossRef]
72. Fibigr, J.; Šatínský, D.; Solich, P. Current trends in the analysis and quality control of food supplements based on plant extracts. *Anal. Chim. Acta* **2018**, *1036*, 1–15. [CrossRef] [PubMed]
73. Rautiainen, S.; Manson, J.E.; Lichtenstein, A.H.; Sesso, H.D. Dietary supplements and disease prevention—A global overview. *Nat. Rev. Endocrinol.* **2016**, *12*, 407–420. [CrossRef] [PubMed]
74. Costa, J.G.; Vidovic, B.; Saraiva, N.; do Ceu Costa, M.; Del Favero, G.; Marko, D.; Oliveira, N.G.; Fernandes, A.S. Contaminants: A dark side of food supplements? *Free Radic. Res.* **2019**, *53*, 1113–1135. [CrossRef] [PubMed]
75. Garcia-Rico, L.; Leyva-Perez, J.; Jara-Marini, M.E. Content and daily intake of copper, zinc, lead, cadmium, and mercury from dietary supplements in Mexico. *Food Chem. Toxicol.* **2007**, *45*, 1599–1605. [CrossRef] [PubMed]
76. Dolan, S.P.; Nortrup, D.A.; Bolger, P.M.; Capar, S.G. Analysis of dietary supplements for arsenic, cadmium, mercury, and lead using inductively coupled plasma mass spectrometry. *J. Agric. Food Chem.* **2003**, *51*, 1307–1312. [CrossRef] [PubMed]
77. Levine, K.E.; Levine, M.A.; Weber, F.X.; Hu, Y.; Perlmutter, J.; Grohse, P.M. Determination of mercury in an assortment of dietary supplements using an inexpensive combustion atomic absorption spectrometry technique. *J. Autom. Methods Manag. Chem.* **2005**, *2005*, 211–216. [CrossRef] [PubMed]
78. Hedegaard, R.V.; Rokkjær, I.; Sloth, J.J. Total and inorganic arsenic in dietary supplements based on herbs, other botanicals and algae—A possible contributor to inorganic arsenic exposure. *Anal. Bioanal. Chem.* **2013**, *405*, 4429–4435. [CrossRef] [PubMed]
79. Araujo-Barbosa, U.; Peña-Vazquez, E.; Barciela-Alonso, M.C.; Ferreira, S.L.C.; Dos Santos, A.M.P.; Bermejo-Barrera, P. Simultaneous determination and speciation analysis of arsenic and chromium in iron supplements used for iron-deficiency anemia treatment by HPLC-ICP-MS. *Talanta* **2017**, *170*, 523–529. [CrossRef]
80. Poniedziałek, B.; Niedzielski, P.; Kozak, L.; Rzymski, P.; Wachelka, M.; Rzymska, I.; Karczewski, J.; Rzymski, P. Monitoring of essential and toxic elements in multi-ingredient food supplements produced in European union. *J. Consum. Prot. Food Saf.* **2018**, *13*, 41–48. [CrossRef]
81. Brodziak-Dopierała, B.; Fischer, A.; Szczelina, W.; Stojko, J. The content of mercury in herbal dietary supplements. *Biol. Trace Elem. Res.* **2018**, *185*, 236–243. [CrossRef] [PubMed]
82. Wolle, M.M.; Rahman, G.M.; Pamuku, M. Speciation analysis of arsenic in prenatal and children's dietary supplements using microwave-enhanced extraction and ion chromatography—Inductively coupled plasma mass spectrometry. *Anal. Chim. Acta* **2014**, *818*, 23–31. [CrossRef]
83. Schwalfenberg, G.; Rodushkin, I.; Genuis, S.J. Heavy metal contamination of prenatal vitamins. *Toxicol. Rep.* **2018**, *5*, 390–395. [CrossRef] [PubMed]
84. Reeuwijk, N.M.; KLerx, W.N.; Kooijman, M.; Hoogenboom, L.; Rietjens, I.M.; Martena, M.J. Levels of lead, arsenic, mercury and cadmium in clays for oral use on the Dutch market and estimation of associated risks. *Food Addit. Contam. Part A* **2013**, *30*, 1535–1545. [CrossRef] [PubMed]
85. Kauffman, J.F.; Westenberger, B.J.; Robertson, J.D.; Guthrie, J.; Jacobs, A.; Cummins, S.K. Lead in pharmaceutical products and dietary supplements. *Regul. Toxicol. Pharmacol.* **2007**, *48*, 128–134. [CrossRef] [PubMed]

86. Mondo, K.; Glover, W.B.; Murch, S.J.; Liu, G.; Cai, Y.; Davis, D.A.; Mash, D.C. Environmental neurotoxins β-N-methylamino-l-alanine (BMAA) and mercury in shark cartilage dietary supplements. *Food Chem. Toxicol.* **2014**, *70*, 26–32. [CrossRef] [PubMed]
87. Solfrizzo, M.; Piemontese, L.; Gambacorta, L.; Zivoli, R.; Longobardi, F. Food coloring agents and plant food supplements derived from Vitis vinifera: A new source of human exposure to ochratoxin A. *J. Agric. Food Chem.* **2015**, *63*, 3609–3614. [CrossRef] [PubMed]
88. Veprikova, Z.; Zachariasova, M.; Dzuman, Z.; Zachariasova, A.; Fenclova, M.; Slavikova, P.; Vaclavikova, M.; Mastovska, K.; Hengst, D.; Hajslova, J. Mycotoxins in plant-based dietary supplements: Hidden health risk for consumers. *J. Agric. Food Chem.* **2015**, *63*, 6633–6643. [CrossRef] [PubMed]
89. Gareis, M. Ochratoxin a in brewer's yeast used as nutrient supplement. *Mycotoxin Res.* **2002**, *18*, 128–131. [CrossRef] [PubMed]
90. Vichi, S.; Lavorini, P.; Funari, E.; Scardala, S.; Testai, E. Contamination by microcystis and microcystins of blue–green algae food supplements (BGAS) on the italian market and possible risk for the exposed population. *Food Chem. Toxicol.* **2012**, *50*, 4493–4499. [CrossRef] [PubMed]
91. Heussner, A.H.; Mazija, L.; Fastner, J.; Dietrich, D.R. Toxin content and cytotoxicity of algal dietary supplements. *Toxicol. Appl. Pharmacol.* **2012**, *265*, 263–271. [CrossRef]
92. Domingos Alves, R.; Romero-González, R.; López-Ruiz, R.; Jiménez-Medina, M.; Garrido Frenich, A. Fast determination of four polar contaminants in soy nutraceutical products by liquid chromatography coupled to tandem mass spectrometry. *Anal. Bioanal. Chem.* **2016**, *408*, 8089–8098. [CrossRef] [PubMed]
93. Rawn, D.F.; Breakell, K.; Verigin, V.; Nicolidakis, H.; Sit, D.; Feeley, M. Persistent organic pollutants in fish oil supplements on the Canadian market: Polychlorinated biphenyls and organochlorine insecticides. *J. Food Sci.* **2009**, *74*, T14–T19. [CrossRef] [PubMed]
94. Garcia-Bermejo, A.; Herrero, L.; Gonzalez, M.-J.; Gomara, B. Occurrence and estimated dietary intake of PCBs and PCDD/Fs in functional foods enriched with omega-3 from Spain. *J. Agric. Food Chem.* **2017**, *65*, 3396–3405. [CrossRef] [PubMed]
95. Wang, J.; Chen, B.; Yao, S. Analysis of six synthetic adulterants in herbal weight-reducing dietary supplements by LC electrospray ionization-MS. *Food Addit. Contam.* **2008**, *25*, 822–830. [CrossRef] [PubMed]
96. Reeuwijk, N.M.; Venhuis, B.J.; de Kaste, D.; Hoogenboom, R.L.; Rietjens, I.M.; Martena, M.J. Active pharmaceutical ingredients detected in herbal food supplements for weight loss sampled on the Dutch market. *Food Addit. Contam. Part A* **2014**, *31*, 1783–1793. [CrossRef] [PubMed]
97. Campbell, N.; Clark, J.P.; Stecher, V.J.; Thomas, J.W.; Callanan, A.C.; Donnelly, B.F.; Goldstein, I.; Kaminetsky, J.C. Adulteration of purported herbal and natural sexual performance enhancement dietary supplements with synthetic phosphodiesterase type 5 inhibitors. *J. Sex. Med.* **2013**, *10*, 1842–1849. [CrossRef] [PubMed]
98. Reeuwijk, N.M.; Venhuis, B.J.; de Kaste, D.; Hoogenboom, L.; Rietjens, I.M.; Martena, M.J. Sildenafil and analogous phosphodiesterase type 5 (PDE-5) inhibitors in herbal food supplements sampled on the Dutch market. *Food Addit. Contam. Part A* **2013**, *30*, 2027–2034. [CrossRef] [PubMed]

## 3.3 Publikacja 3 (oryginalna)

**Stępień, K.A**.; Krawczyk, W.; Giebułtowicz, J. *Dietary Supplements with Proline — A Comprehensive Assessment of Their Quality*; Life; 2023; 13 (2); 263.

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# Dietary Supplements with Proline—A Comprehensive Assessment of Their Quality

**Krzysztof Adam Stępień, Weronika Krawczyk and Joanna Giebułtowicz ***

Department of Drugs Chemistry, Faculty of Pharmacy, Medical University of Warsaw, 1 Banacha, 02-097 Warsaw, Poland

* Correspondence: joanna.giebultowicz@wum.edu.pl; Tel.: +48-22-572-0949

**Abstract:** Dietary supplements are food products commonly used worldwide to obtain nutritional and physiological effects. They can contain a wide variety of active substances and can be administered for health and disease. Their use can be beneficial if justified, and their quality is adequate. Unfortunately, data on the quality of supplements is scarce. As part of this work, we assess the quality of seven dietary supplements containing proline. The preparations were produced in the EU and the USA. The quality assessment consisted of the detection of potential impurities, the determination of the content of the main ingredient, and the release of proline. The technique used to analyse impurities and proline (Pro) content was liquid chromatography coupled with tandem mass spectrometry. We detected five contaminants. The main ingredient content was in the range of 73–121% in capsules and 103–156% in tablets. Five of the seven analysed dietary supplements released below 80% Pro (for each tablet/capsule at pH 1.2). One of the supplements may be inactive because a very low release of Pro was reported. The results, we hope, will increase consumer awareness of the quality of these preparations and result in a change in the regulations governing the marketing of these preparations, at least by making release testing mandatory.

**Keywords:** dietary supplement; proline; food supplement analysis; dissolution test; quality control



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## 1. Introduction

Dietary supplements are growing in popularity worldwide due to the increasing need for health and body care [1]. Using these products is equated with a safe and easy way to live a healthy lifestyle [2]. Dietary supplements are intended to correct nutritional deficiencies and maintain an adequate intake of certain nutrients to produce a nutritional or physiological effect. They may contain vitamins and minerals, plant ingredients and extracts, omega-3 fatty acids [3], probiotics and prebiotics [4], and amino acids [5]. Consumers use dietary supplements prophylactically or in the course of a specific disease. Depending on the intended use, dietary supplements can be divided into those that support weight loss [6], the immune system [7], nervous system [8], cardiovascular system [9], digestive system [10], skin, hair, nails [11], and reducing the risk of osteoporosis [12].

Dietary supplements are often sold in tablet and capsule form, resembling medicinal products [13]. However, in the EU, they are classified as food [14], so their quality is not subject to the exact requirements of drugs [15]. Recently, some reports appeared on the inadequate quality of dietary supplements. The low quality of these preparations may be a consequence of impurities [16]. Contaminants such as heavy metals [17], mycotoxins [18], insecticides [19], herbicides [20], degradation products of the main ingredient [21], and active substances such as sibutramine [22] or sildenafil [23] have often been detected in such products. Another aspect of low-quality supplements is the incorrect content of active ingredients, as shown for tryptophan [21] or melatonin [24]. In addition, if supplements are in the form of tablets or capsules, consumers expect that the substance contained in them can be released from this form at an appropriate level, thus guaranteeing the possibility

of obtaining the right effect [25]. Unfortunately, since the manufacturer is not required to conduct a release study, the release of the active substance is sometimes shallow. Then there is no possibility that the substance contained in the supplement will have any effect on the consumer. Low release of the substance contained in the supplement has been described in the case of supplements with tryptophan [21], lutein [26], melatonin [27], and folic acid [28]. However, the amount of data on the quality of supplements is still scarce. For instance, the release of the active substance was analysed for only a few types of supplements. In many cases, a single supplement was analysed [29,30]. The analysis of more than five supplements of one kind was carried out only for calcium carbonate [31], melatonin [27], folic acid [32], lutein [26] and tryptophan [21]. There are even fewer studies in which a comprehensive assessment was made: content, release, and impurities; we found such data only for tryptophan [21].

Since the quality of dietary supplements seems to be a crucial aspect in the context of consumers' widespread use, dietary supplements containing proline were analysed in this study. Pro is one of the 20 essential L-$\alpha$-amino acids that build proteins [33], playing a crucial role in building the collagen chain. It participates in the construction of collagen [34]. Collagen's polypeptide chains are made of repeating Gly-X-Y triples, where any amino acid can occupy the X and Y positions. However, Pro and its derivative, 4-hydroxyproline, are by far the most common, accounting for about 20% of collagen [35]. Pro is also present in the central nervous system [36], and participates in redox balance, affecting the survival or death of cells and cancer development, constituting an element of their so-called metabolic re-programming [37]. Diseases caused by congenital deficiency of enzymes involved in Pro metabolism are relatively rare [38]. However, they clearly illustrate the multidirectional function of Pro and the complexity of its metabolic pathways, which still hide interdependencies that are not fully explained.

The role of Pro in collagen synthesis is why manufacturers of dietary supplements recommend it for use by physically active people who take care of their hair, skin, and nails.

So far, data on the quality of dietary supplements containing Pro are lacking. This study aimed to fill this gap by assessing the quality of dietary supplements containing Pro in tablet or capsule form and manufactured in the EU and the US. All supplements with Pro that can be purchased in Poland were included in the study ($n$ = 7). This assessment was covered by performing qualitative analysis of Pro and unknown screening to detect contaminants. Additionally, we assessed the release of Pro from tablets or capsules. In all tests, the most reliable analytical method was applied: liquid chromatography coupled with mass spectrometry. We believe that the results of our study will be an essential argument in any discussion of dietary supplements.

## 2. Materials and Methods

### *2.1. Samples*

We included in our study seven dietary supplements with Pro in capsules (Pro1-Pro6) or tablets (Pro7). Supplements were manufactured in Poland (L-Prolina; Biocaps COLLAGEN, Prolina), Germany (L-Prolina), and the US (Włosy, Skóra, Paznokcie; L-Proline). Supplements were purchased in Polish pharmacies, online pharmacies, or on an online e-commerce platform.

### *2.2. Reagents*

L-Pro (≥98%) (standard) was purchased from LGC (Luckenwalde, Germany). Sodium hydroxide (≥98.8%), hydrochloric acid (35–38%), and potassium phosphate monobasic (≥99.5%) we bought from Chempur (Piekary Śląskie, Poland). HPLC-grade acetonitrile, formic acid, and methanol were from Merck (Darmstadt, Germany).

### *2.3. Sample Preparation*

The sample was prepared based on Stepien and Giebultowicz [21]. Briefly, for each supplement, we randomly selected three tablets or capsules, determined the total weight of

the tablets' or capsules' content. The tablets were ground into a fine powder in a mortar. Next, we weighed the capsule content or tablet's mass equivalent to 10 mg of Pro, and added 1.00 mL mixture containing acetonitrile, methanol, and water in a ratio of 1:1:1; $v/v/v$. Then, the mixture was sonicated (15 min) and centrifuged (5 min). The supernatant was diluted with the mobile phase to a concentration of 500 ng/mL or 100 ng/mL for qualitative and quantitative analysis.

### *2.4. Qualitative Analysis*

Instrumental analysis was performed using a UHPLC Dionex Ultimate 3000 with a Q-Exactive spectrometer as described previously [21]. Briefly, the HESI source was operated in positive mode over $m/z$ 100–1400 with a resolution of 70,000 ($m/z$ 200). The collision energies were set to 20, 35, and 50 eV.

An Accucore C-18 column (100 mm × 4.6 mm, 2.6 µm, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a security guard was applied for chromatographic separation. The mobile phases consisted of eluent A (0.1% formic acid) and eluent B (acetonitrile with 0.1% formic acid). The gradient (% B) was as follows: 0 min 10%; 1 min 10%; 10 min 95%; 15 min 95%, 16 min 10%; 17 min 10%. The injection volume was 10 µL, the column temperature was 40 °C, and the mobile phase flow rate was 0.3 mL/min.

The data were analyzed using Compound Discoverer 3.3 software (Thermo Fisher Scientific, Waltham, MA, USA).

### *2.5. Quantitative Analysis*

Agilent 1260 Infinity (Agilent Technologies, Santa Clara, CA, USA), coupled to a QTRAP 4000 hybrid triple quadrupole/linear ion trap mass spectrometer (AB Sciex, Framingham, MA, USA), was used for quantitative analysis. The turbo ion-spray source was operated in positive mode. The curtain gas, ion source gas 1, ion source gas 2, and collision gas (all high-purity nitrogen) were set at 0.24 MPa, 0.41 MPa, 0.28 MPa, and "medium" instrument units, respectively. The ion spray voltage and source temperature were 5500 V and 600 °C, respectively. The target compounds were analysed in multiple reaction monitoring modes using the 116.0/67.9 transition. We optimized the declustering potential (DP = 56 V), collision energy (CE = 41 V), entrance potential (EP = 10 V), and collision exit potential (CXP = 10 V). The samples were injected at a volume of 10 µL.

A Kinetex C18 column (100 mm × 3.0 mm, 2.6 µm, Phenomenex, Milford, USA) was used for separation. The mobile phases consisted of eluent A (0.1% formic acid) and eluent B (methanol with 0.1% formic acid). The gradient (%B) was: 0 min 10%; 1 min 10%; 2 min 70%; 3 min 70%; 4 min 10%; 5 min 10%. The mobile phase flow rate was 0.5 mL/min.

The concentrations of Pro in all analysed samples were within the range of the calibration curve, i.e., from 0.01 to 1 µg/mL.

Analyst 1.6.3 software (AB Sciex, Framingham, MA, USA) was used for calculations. Following the Polish Pharmacopoeia VI, the content of an active substance in tablets or capsules should not exceed ±10% (if less than 100 mg of the active substance is declared in one unit) or ±5% otherwise. These requirements apply to medicines. For dietary supplements no exist specific guidelines to assess their quality and take point of the fact that dietary supplements are manufactured in the same form as medicines, the same criteria for the content of Pro were adopted in this study.

### *2.6. Dissolution Test for Tablets or Capsules*

The dissolution test was based on Stepien and Giebultowicz [21]. For tablets and capsules, we used USP II Varian VK 7025 and USP I Varian VK 7025 dissolution testers (Erweka GmbH, Heusenstamm, Germany), respectively. We randomly selected six tablets or capsules for each supplement and individually placed them in the dissolution vessels containing 900 mL of dissolution medium. The stirring speed was 50 rpm for tablets and 100 rpm for capsules; the temperature was 37 ± 0.5 °C. Aliquots (1.5 mL) of the medium were manually collected using 5 mL syringes after 30 min of the test and filtered through

a Millex-HA 0.45 µm filter. Each aliquot withdrawn was replaced with 1.5 mL of fresh medium. The experiment was performed in 0.1 mol/L hydrochloride acid (simulated gastric conditions) and 0.05 mol/L phosphate buffer (pH 6.8, simulated intestinal conditions).

### *2.7. Expanded Uncertainty*

To verify whether the release of Pro is equal to the amount of Pro detected in the dosage unit, we used Equation (1).

$$U(x_1 - x_2) = 2\sqrt{[u(x_1)]^2 + [u(x_2)]^2} \quad (1)$$

The measurement results were equal if:

$$|x_1 - x_2| < U(x_1 - x_2)$$

$x_1$—mean [mg] Pro content determined in tablet/capsule ($n$ = 3);
$x_2$ —mean [mg] amount of Pro released;
$u(x_2), u(x_1)$—standard uncertainties of the measured values: $x_1$ and $x_2$ determined according to the formula:

$$u(x_1) = \frac{S}{\sqrt{n}}$$

$S$—standard deviation of the amount of Pro in dosage unit [mg] or standard deviation of the released amount of Pro [mg];
$n$—the number of tablets or capsules used for analysed.

## 3. Results and Discussion

### *3.1. Tentative Contaminants Present in Pro Supplements*

In addition to Pro and lysine (an ingredient listed in Pro 7) five contaminants were detected in the analysed supplements. The level of their content was in the range of 0.94% to 7.61% of the Pro area (Table 1). Surprisingly, we have not noticed any transformation products of Prothat can be generated, e.g., during storage.

Among the possible contaminants are substances with various properties. Potentially present in each sample is 4-ethylguaiacol (A1). A1 is a compound naturally occurring in high concentrations in coffee and can be used in industry as a flavour and fragrance [39]. Namely, it is used in vanilla flavour. Its intentional addition to a dietary supplement cannot be excluded. However, it is not included in the composition list as recommended [40]. None of the tested preparations was declared to contain any flavouring agent. Cross-contamination between different production lines is also a likely explanation.

**Table 1.** Tentative* identification of contaminants detected in Pro supplements and their MS parameters.

| Code | Formula | Neutral Mass Calculated from the Formula [Da] | Δ Mass [Da] | Δ Mass [ppm] | RDBE [a] | H/C [b] | SFit [c] [%] | PC [d] [%] | X/Pro [e] [%] | Fragments [*m*/*z*] | Dietary Supplements Containing Contaminant (% of the Analysed) | Tentative Name |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A1 | C9 H12 O2 | 152.08373 | 0.00002 | 0.16 | 4 | 1.3 | 81.54 | 99.48 | 3.51–7.61 | 83.04901; 93.06975; 107.08539; 111.04391; 125.05946; 135.07991 | All (100%) | 4-ethylguaiacol |
| A2 | C6 H7 N O3 S | 173.01466 | 0.00005 | 0.27 | 4 | 1.2 | 70.72 | 98.46 | 2.18 | 93.05725 | Pro7 (14%) | 4-methylpyridine-3-sulfonic acid |
| A3 | C11 H13 N3 O3 S | 267.06776 | −0.00027 | −1.01 | 7 | 1.2 | 63.45 | 100.00 | 2.53 | 92.04922; 108.04426; 113.07071; 156.01105 | Pro7 (14%) | Sulfisoxazole |
| A4 | C18 H37 N O | 283.28751 | −0.00002 | −0.07 | 1 | 2.1 | 91.29 | 99.90 | 5.65 | 88.07558; 102.09121; 116.10651 | Pro3 (14%) | Stearamide |
| A5 | C22 H43 N O | 337.33446 | 0.00003 | 0.08 | 2 | 2.0 | 65.66 | 99.82 | 0.94–1.25 | 83.08537; 97.10140; 109.10137; 149.13242; 303.30444; 321.31512 | Pro3, Pro5 (28%) | Erucamide |

[a] Rings and double bonds equivalent, [b] hydrogen versus carbon atoms ratio, [c] spectral similarity score between measured and theoretical isotope pattern in %, [d] matched intensity percentage of the theoretical pattern, [e] % of the main ingredient area, *—identification was performed based on *m*/*z*, isotopic pattern and fragmentation spectra (coverage by in silico fragmentation). The results were not validated using the analytical standard.

Stearamide (A4) and erucamide (A5) were found in two supplements from the United States. A4 and A5 are fatty acid amides with a wide range of applications, e.g., as anti-adhesive and coating components. In addition, the FDA classifies them as indirect food additives, i.e., substances that are assumed to come into contact with foodstuffs during their packaging, storage, or processing but are not intended to be used as a direct food ingredient [41]. Their presence in dietary supplements may be the result of the manufacturing process or migration from packaging. During the quality analysis, we also detected compounds with similar $m/z$ and fragmentation as 4-methylpyridine-3-sulfonic acid (A2). A2 is used by industry and is an irritant to mucous membranes and the upper respiratory tract [42]. In the same supplement, a compound with a similar structure to sulfisoxazole was noted (A3). Sulfisoxazole is a pharmacologically active substance belonging to the group of sulfonamides. Compounds of this class are part of the antibacterial drugs currently used to a small extent, mainly topically, because of the resistance of microorganisms and severe side effects, such as allergic reactions and bone marrow damage [43]. Due to its current limited use, cross-contamination during production seems unlikely. Thus, both A2 and A3 can be somehow related to the addition of methylsulfonylmethane (which is labelled an ingredient in Pro 7) to the supplement and be its contaminants and/or products of the reaction between different ingredients.

Here, we suggested only the probable structure of contaminants based on the $m/z$, isotopic pattern, and fragmentation. However, identification requires further analysis using comparative standards. Additional to these results, in the literature, there are some reports on the identified impurities, which are considered a significant problem not only in Poland but also in other countries of the world. As an example, tryptophan's dietary supplements contained some contaminants like tryptophan's metabolites, condensation products of tryptophan and carbonyl compounds, tryptophan degradation products, degradation products of kynurenine, and other contaminants, e.g., melatonin or glucosamine [21].

One of the contaminants sought and detected by researchers in dietary supplements are mycotoxins [16,18], microbial contaminants [44], pesticides [45], and metals [46,47]. However, they can be found in herbal products, so it makes no sense to determine these contaminants in Pro products. Non-labelled substances may appear in dietary supplements as unintentional impurities and deliberate adulterations with pharmacologically active ingredients. Particularly exposed to their occurrence are preparations from which consumers usually expect immediate and noticeable effects: presented as supporting sexual performance, slimming, or building muscle mass (also physical performance, intended for athletes) [16,48]. It is also not the case with Pro products, which are designed as skin and hair conditioning agents.

Pro contaminants are relatively low (up to 7.6%). Thus, no adverse effects are expected. However, since the quantitative analysis was not performed, no conclusion on the safety of Pro supplements can be drawn.

### *3.2. Determination of Pro in Dietary Supplements*

According to manufacturers, analysed supplements contained Pro in the range of 25–520 mg (Table 2). None of the dietary supplements analysed had Pro content within the accepted range for all three dosage forms studied. In the case of Pro3 (CV = 5.9%), Pro6 (CV = 7.2%), and Pro7 (CV = 16.4%) supplements, 1 out of 3 analysed units contained the amount of Pro that met the adopted criteria. The lowest Pro content (75% declared by the manufacturer) was determined for the Pro2 preparation. An insufficient amount of Pro and low variation (CV = 2%) suggest a lower amount of Pro added during product manufacturing rather than an inadequate distribution of the active substance. This may be considered a premeditated act to the consumer's disadvantage.

**Table 2.** Results of quantitative analysis of Pro in dietary supplements (maximum error value above 20 is bolded).

| Code | Dosage Form | Source | Declared Pro Content [mg/unit] | Determined Pro Content [mg/unit] [a] | Maximum Difference [%] |
|---|---|---|---|---|---|
| Pro1 | Capsule | Poland | 520 | 546 (CV = 9.9%) | 15 |
| Pro2 | Capsule | Poland | 500 | 375 (CV = 2.0%) | −27 |
| Pro3 | Capsule | United States | 500 | 522 (CV = 5.9%) | 9 |
| Pro4 | Capsule | Germany | 400 | 375 (CV = 9.8%) | −16 |
| Pro5 | Capsule | United States | 500 | 587 (CV = 2.6%) | 21 |
| Pro6 | Capsule | Poland | 270 | 263 (CV = 7.2%) | −11 |
| Pro7 | Tablet | United States | 25 | 33 (CV = 16.4%) | 56 |

CV—coefficient of variation; [a]—mean (standard deviation $n = 3$).

In two cases, higher content of Pro than declared was noted. The highest content of Pro (132% of the declared content by the manufacturer) was observed for the Pro7 supplement. The reason may be an incorrect (too high) amount of Pro used during manufacture. The coefficient of variation equal to 16.4% for this supplement also indicates improper mixing of the tablet mass.

To sum up, during the quantitative analysis, we encountered two essential factors: different than declared Pro content and an inhomogeneous mixing of the tablet or capsule mass at the manufacturing stage. The difference between the stated and determined content of the main component has been previously described for tryptophan [21], melatonin [24] and lutein [26] supplements. Twenty out of 22 tryptophan supplements, 10 out of 10 lutein supplements and 7 out of 17 melatonin supplements did not meet the criteria applied in the current work. For dietary supplements with tryptophan, the content of the main ingredient ranged from 55–100% in capsules and 69–87% in tablets. Moreover, low content uniformity was observed [21]. In the case of melatonin or lutein supplements, it is unclear whether the homogeneity of the distribution of the melatonin or lutein among the dosage units was checked. Therefore, it was impossible to determine the quality of these preparations comprehensively.

### *3.3. Dissolution Test for Pro Tablets and Capsules*

The Food and Drug Administration (FDA) presents guidelines for testing medicines. Following the requirements concerning the dissolution test for the unmodified release of solid oral medicine, the active substance should be released at 80% or more of the declared content within 30 min of conducting the dissolution test. There is a lack of specific guidelines for conducting release tests in the case of dietary supplements. For this reason, the same criteria for the dissolution test were used for this study. The manufacturers of the analysed dietary supplements did not provide information on the packaging that the preparation has a modified release. Therefore, Pro should be released in the accepted range (above 80%) at pH 1.2 in all analysed dietary supplements. pH 1.2 corresponds to the gastric conditions and in this place of the gastrointestinal tract, Pro should be released from the formulation. Additionally, a dissolution test at pH 6.8 corresponding to the small intestine conditions was performed to verify the potential place of Pro release in the analysed supplements.

Among the supplements analysed, we found two that met the criteria at pH 1.2; for Pro3 and Pro5, all units tested ($n$ = 6) had Pro release above 80%. For Pro2, Pro6 and Pro7, only the average release was above 80%, with some units releasing less. The release of Pro lower than 50% was noted for two products. However, one of them (i.e., Pro 1) released a high amount of Pro in intestinal pH, indicating that most of the Pro in the product will be available to the consumer. The quality of Pro4 is most questionable. For this formulation, a release of only 11% of the active ingredient in an acidic environment, and 10% in an alkaline environment was reported (Table 3). Given the high Pro content of the supplement (94%), we conclude that the low level of Pro release may have been due to improperly selected manufacturing process parameters and inadequate excipients (Figure 1).

**Table 3.** Comparison of the results of the amount Pro released from the dosage form and the Pro amount determined in the units using the expanded uncertainty.

| Code | The Average Percentage of Pro Amount Released from a Dosage Form (Standard Deviation $n$ = 6) | | Expanded Uncertainty Parameters | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | pH 1.2 | | | pH 6.8 | | |
| | pH 1.2 | pH 6.8 | $\lvert x_1 - x_2 \rvert$ | $U(x_1 - x_2)$ | Equal [a] | $\lvert x_1 - x_2 \rvert$ | $U(x_1 - x_2)$ | Equal [a] |
| Pro1 | 47 (42) | 85.0 (7.4) | 300.1 | 187.6 | No | 104.4 | 70.1 | No |
| Pro2 | 82 (13) | 79 (16) | 33.2 | 52.3 | Yes | 19.1 | 61.9 | Yes |
| Pro3 | 90.0 (5.2) | 74.9 (4.8) | 71.8 | 41.5 | No | 147.4 | 40.7 | No |
| Pro4 | 11 (12) | 10 (10) | 328.9 | 57.6 | No | 334.6 | 52.9 | No |
| Pro5 | 131 (30) | 118.6 (5.9) | 65.4 | 122.5 | Yes | 5.8 | 29.5 | Yes |
| Pro6 | 91 (12) | 2.0 (1.1) | 17.8 | 32.9 | Yes | 257.9 | 21.9 | No |
| Pro7 | 105 (19) | 77 (12) | 6.7 | 7.3 | Yes | 13.9 | 6.7 | No |

[a] Amount of Pro in the formulation and amount of Pro released are equal (yes) or not (no) within the uncertainty.

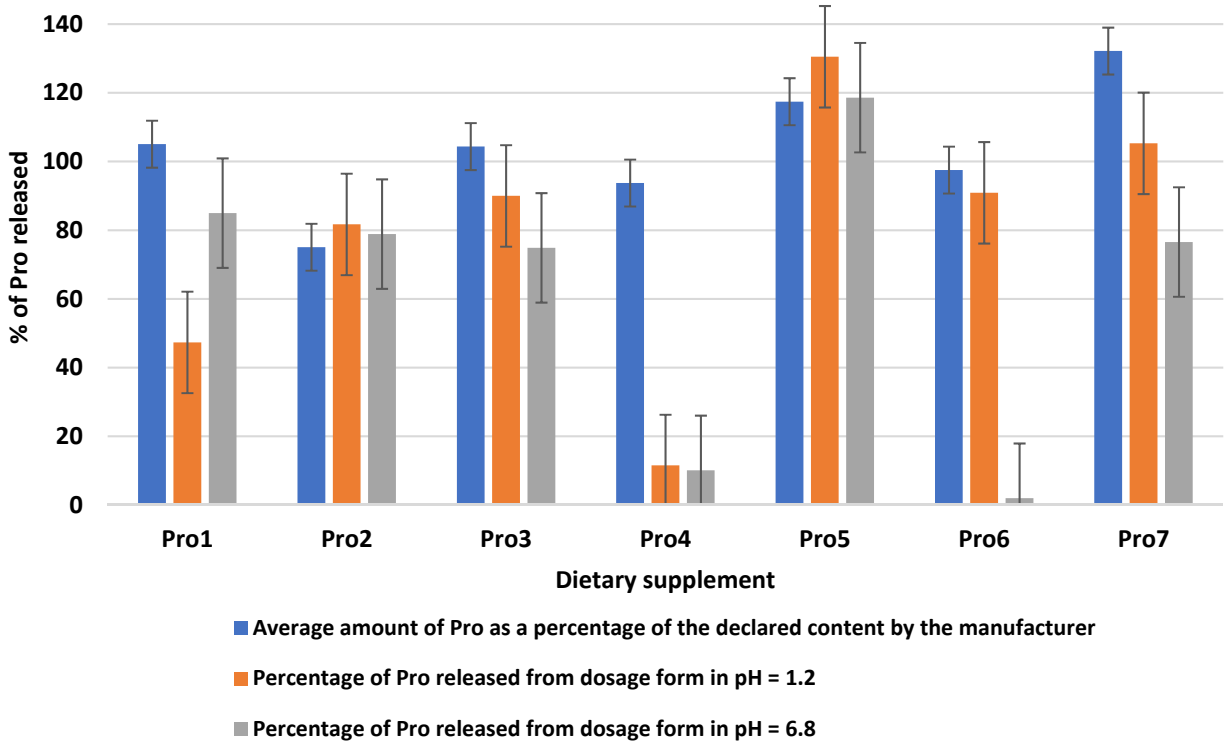


**Figure 1.** Comparison of the dissolution test results in pH 1.2 (simulated gastric conditions), pH 6.8 (simulated intestinal conditions) with the Pro content analysis for dietary supplements in the dosage form. Comparison of the amount of Pro released and the Pro amount determined in the units, using the extended uncertainty can be found in Table 3.

In summary, 2 out of 7 of the Pro supplements released a minimum of 80% Pro (in the case of each analysed capsule or tablet). This means that these preparations met the requirements for releasing the active substance in medicinal products. It is impossible to compare the Pro release data we have obtained with other dietary supplements because supplements containing this amino acid have not been studied. The quality of these supplements is much better than that of tryptophan [21] or lutein [26], as none of these dietary supplements meet these requirements. Low release of active ingredients such as iron, zinc, manganese [49], grape seed extract [30], melatonin [27], folic acid [28,32], and calcium carbonate [31] have also been reported. The reason is the low content of active substances or incorrect technological parameters.

In our study, Pro release was higher from tablets (105%) than from capsules (11–131%). In the literature, we found similar results for tryptophan [21], lutein [26] and folic acid [28]. This indicates that manufacturers have more problems with maintaining quality in the case of capsule formulation.

Qualitative analysis, quantitative analysis and the release of active ingredients from products are essential to ensuring good quality [50]. Dissolution testing measures the amount of active ingredient that passes from the drug into the fluid. It is the basic test for determining the quality of a medicinal product. However, this test is not required for dietary supplements. [51]. This assay can somewhat predict in vivo absorption [25]. A low release rate means low absorption. So even if the active ingredient is present in the dietary supplement in the amount declared by the manufacturer, but the release of this ingredient is low, no physiological effect will be observed.

The lack of a legal framework can be considered the main reason for the low quality of dietary supplements. Since formally they are nutrients (both in Poland and other EU countries [14] and in the United States [52]), they do not have to meet the exact requirements as medicinal products—despite the same form, availability in pharmacies and intensive advertising that may create additional confusion for consumers.

Some European researchers suggest that ensuring the quality of dietary supplements should be based on rules similar to or the same as those for medicinal products, e.g., by introducing the obligation to comply with GMP rules in production, as is the case in the United States [16,53]. This solution combines the guidelines for food with those for medicinal products, including those concerning sanitary conditions, the quality of the operations carried out, the prevention of adulteration, and ensuring the final product's purity, composition, and quality. Where possible, compliance with the requirements established in official compendia, such as USP [53–55], would be expected. It is also considered necessary to raise the awareness of the public, especially healthcare professionals, about the potential benefits and risks of taking dietary supplements [53].

Although there are undeniable dietary supplements on the market that take the consumer into account, the main problem is finding them among preparations of dubious quality, which can also be sold. It is necessary to amend the current legislation, as the ease of marketing and low standard of inspection of dietary supplements make them vulnerable to negligence during production. This poses a threat to the interests and health of consumers. The dissolution test here estimates the amount of proline released from dietary supplements in the gastrointestinal tract. However, the in vitro analysis did not include enzymes and surfactants that can be found in body fluids. Therefore, the in vivo release may be different. Moreover, not all released doses may be absorbed into the circulation and generate a physiological effect. Therefore, the study's next step should be to determine the bioavailability of proline from supplements. In addition, an important aspect to investigate is the effect of detected impurities using a cytotoxicity assay on cell lines or animals.

## 4. Conclusions

Most of the Pro supplements are of acceptable quality. The number and level of impurities were low. The amount of Pro in one case was 25% lower than declared. In other

cases, it was similar or even higher. A more significant problem is the proper preparation of the dosage unit. For example, one supplement may be inactive because a deficient release of Pro was reported. Therefore, a legal framework should be established to promote the appropriate quality of supplements for consumers.

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## References

1. Thakkar, S.; Anklam, E.; Xu, A.; Ulberth, F.; Li, J.; Li, B.; Hugas, M.; Sarma, N.; Crerar, S.; Swift, S. Regulatory landscape of dietary supplements and herbal medicines from a global perspective. *Regul. Toxicol. Pharmacol.* **2020**, *114*, 104647. [CrossRef] [PubMed]
2. Binns, C.W.; Lee, M.K.; Lee, A.H. Problems and prospects: Public health regulation of dietary supplements. *Annu. Rev. Public Health* **2018**, *39*, 403–420. [CrossRef] [PubMed]
3. Wierzejska, R.E. Dietary supplements—For whom? The current state of knowledge about the health effects of selected supplement use. *Int. J. Environ. Health Res.* **2021**, *18*, 8897. [CrossRef]
4. Marasco, G.; Cirota, G.G.; Rossini, B.; Lungaro, L.; Di Biase, A.R.; Colecchia, A.; Volta, U.; De Giorgio, R.; Festi, D.; Caio, G. Probiotics, prebiotics and other dietary supplements for gut microbiota modulation in celiac disease patients. *Nutrients* **2020**, *12*, 2674. [CrossRef] [PubMed]
5. Master, P.B.Z.; Macedo, R.C.O. Effects of dietary supplementation in sport and exercise: A review of evidence on milk proteins and amino acids. *Crit. Rev. Food Sci. Nutr.* **2021**, *61*, 1225–1239. [CrossRef]
6. Watanabe, M.; Risi, R.; Masi, D.; Caputi, A.; Balena, A.; Rossini, G.; Tuccinardi, D.; Mariani, S.; Basciani, S.; Manfrini, S. Current evidence to propose different food supplements for weight loss: A comprehensive review. *Nutrients* **2020**, *12*, 2873. [CrossRef] [PubMed]
7. Del Cornò, M.; Gessani, S.; Conti, L. Shaping the innate immune response by dietary glucans: Any role in the control of cancer? *Cancers* **2020**, *12*, 155. [CrossRef] [PubMed]
8. Sivamaruthi, B.S.; Suganthy, N.; Kesika, P.; Chaiyasut, C. The role of microbiome, dietary supplements, and probiotics in autism spectrum disorder. *Int. J. Environ. Res. Public Health* **2020**, *17*, 2647. [CrossRef]
9. Lau, S.O.; Georgousopoulou, E.N.; Kellett, J.; Thomas, J.; McKune, A.; Mellor, D.; Roach, P.D.; Naumovski, N. The effect of dietary supplementation of green tea catechins on cardiovascular disease risk markers in humans: A systematic review of clinical trials. *Beverages* **2016**, *2*, 16. [CrossRef]
10. Wobith, M.; Weimann, A. Oral Nutritional Supplements and Enteral Nutrition in Patients with Gastrointestinal Surgery. *Nutrients* **2021**, *13*, 2655. [CrossRef]
11. Sadowska, A.; Świderski, F. Sources, bioavailability, and safety of silicon derived from foods and other sources added for nutritional purposes in food supplements and functional foods. *Appl. Sci.* **2020**, *10*, 6255. [CrossRef]
12. Rondanelli, M.; Faliva, M.A.; Barrile, G.C.; Cavioni, A.; Mansueto, F.; Mazzola, G.; Oberto, L.; Patelli, Z.; Pirola, M.; Tartara, A. Nutrition, physical activity, and dietary supplementation to prevent bone mineral density loss: A food pyramid. *Nutrients* **2021**, *14*, 74. [CrossRef] [PubMed]
13. Rajakumari, R.; Oluwafemi, O.S.; Thomas, S.; Kalarikkal, N. Dietary supplements containing vitamins and minerals: Formulation, optimization and evaluation. *Powder Technol.* **2018**, *336*, 481–492. [CrossRef]
14. European Community. Directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of the Member States relating to food supplements. *Off. J. Eur. Communities Legis.* **2002**, *45*, 51–57.
15. Dwyer, J.T.; Coates, P.M.; Smith, M.J. Dietary Supplements: Regulatory Challenges and Research Resources. *Nutrients* **2018**, *10*, 41. [CrossRef]
16. Costa, J.G.; Vidovic, B.; Saraiva, N.; do Ceu Costa, M.; Del Favero, G.; Marko, D.; Oliveira, N.G.; Fernandes, A.S. Contaminants: A dark side of food supplements? *Free Radic. Res.* **2019**, *53*, 1113–1135. [CrossRef]
17. Schwalfenberg, G.; Rodushkin, I.; Genuis, S.J. Heavy metal contamination of prenatal vitamins. *Toxicol. Rep.* **2018**, *5*, 390–395. [CrossRef] [PubMed]

18. Veprikova, Z.; Zachariasova, M.; Dzuman, Z.; Zachariasova, A.; Fenclova, M.; Slavikova, P.; Vaclavikova, M.; Mastovska, K.; Hengst, D.; Hajslova, J. Mycotoxins in plant-based dietary supplements: Hidden health risk for consumers. *J. Agric. Food Chem.* **2015**, *63*, 6633–6643. [CrossRef]
19. Chen, Y.; Lopez, S.; Hayward, D.G.; Park, H.Y.; Wong, J.W.; Kim, S.S.; Wan, J.; Reddy, R.M.; Quinn, D.J.; Steiniger, D. Determination of multiresidue pesticides in botanical dietary supplements using gas chromatography–triple-quadrupole mass spectrometry (GC-MS/MS). *J. Agric. Food Chem.* **2016**, *64*, 6125–6132. [CrossRef]
20. Domingos Alves, R.; Romero-González, R.; López-Ruiz, R.; Jiménez-Medina, M.; Garrido Frenich, A. Fast determination of four polar contaminants in soy nutraceutical products by liquid chromatography coupled to tandem mass spectrometry. *Anal. Bioanal. Chem.* **2016**, *408*, 8089–8098. [CrossRef]
21. Stępień, K.A.; Giebułtowicz, J. Application of Liquid Chromatography Coupled to Mass Spectrometry in Quality Assessment of Dietary Supplements—A Case Study of Tryptophan Supplements: Release Assay, Targeted and Untargeted Studies. *Pharmaceuticals* **2022**, *15*, 448. [CrossRef]
22. Reeuwijk, N.M.; Venhuis, B.J.; de Kaste, D.; Hoogenboom, R.L.; Rietjens, I.M.; Martena, M.J. Active pharmaceutical ingredients detected in herbal food supplements for weight loss sampled on the Dutch market. *Food Addit. Contam.-Chem. Anal. Control Expo. Risk Assess.* **2014**, *31*, 1783–1793. [CrossRef] [PubMed]
23. Reeuwijk, N.M.; Venhuis, B.J.; de Kaste, D.; Hoogenboom, L.; Rietjens, I.M.; Martena, M.J. Sildenafil and analogous phosphodiesterase type 5 (PDE-5) inhibitors in herbal food supplements sampled on the Dutch market. *Food Addit. Contam.-Chem. Anal. Control Expo. Risk Assess.* **2013**, *30*, 2027–2034. [CrossRef] [PubMed]
24. Cerezo, A.B.; Leal, Á.; Álvarez-Fernández, M.A.; Hornedo-Ortega, R.; Troncoso, A.M.; García-Parrilla, M.C. Quality control and determination of melatonin in food supplements. *J. Food Compos. Anal.* **2016**, *45*, 80–86. [CrossRef]
25. Gray, V.A. Power of the dissolution test in distinguishing a change in dosage form critical quality attributes. *AAPS J.* **2018**, *19*, 3328–3332. [CrossRef]
26. Anselmo, C.S.; Mendes, T.C.; Cabral, L.M.; Sousa, V.P. Physicochemical quality profiles of commercial oral tablets and capsules containing lutein—Impact of insufficient specific sanitary regulations. *An. Acad. Bras. Cienc.* **2018**, *90*, 3063–3073. [CrossRef] [PubMed]
27. Hahm, H.; Kujawa, J.; Augsburger, L. Comparison of melatonin products against USP's nutritional supplements standards and other criteria. *J. Am. Pharm. Assoc.* **1999**, *39*, 27–31. [CrossRef] [PubMed]
28. Younis, I.R.; Stamatakis, M.K.; Callery, P.S.; Meyer-Stout, P.J. Influence of pH on the dissolution of folic acid supplements. *Int. J. Pharm.* **2009**, *367*, 97–102. [CrossRef] [PubMed]
29. Bowerbank, S.L.; Carlin, M.G.; Dean, J.R. Dissolution Testing of Single-and Dual-Component Thyroid Hormone Supplements. *Separations* **2019**, *6*, 18. [CrossRef]
30. Lyu, W.; Omar, T.; Patel, H.; Rodriguez, D.; Ferruzzi, M.G.; Pasinetti, G.M.; Murrough, J.W.; Muzzio, F.J.; Simon, J.E.; Wu, Q. Dissolution Study on Grape Polyphenol Hard Gelatin Capsule Dietary Supplements. *Front. Nutr.* **2021**, *8*, 780260. [CrossRef]
31. Brennan, M.J.; Duncan, W.E.; Wartofsky, L.; Butler, V.M.; Wray, H.L. In vitro dissolution of calcium carbonate preparations. *Calcif. Tissue Int.* **1991**, *49*, 308–312. [CrossRef] [PubMed]
32. Sculthorpe, N.; Davies, B.; Ashton, T.; Allison, S.; McGuire, D.; Malhi, J. Commercially available folic acid supplements and their compliance with the British Pharmacopoeia test for dissolution. *J. Public Health* **2001**, *23*, 195–197. [CrossRef] [PubMed]
33. Szabados, L.; Savouré, A. Proline: A multifunctional amino acid. *Trends Plant Sci.* **2010**, *15*, 89–97. [CrossRef]
34. Li, P.; Wu, G. Roles of dietary glycine, proline, and hydroxyproline in collagen synthesis and animal growth. *Amino Acids* **2018**, *50*, 29–38. [CrossRef] [PubMed]
35. Krane, S.M. The importance of proline residues in the structure, stability and susceptibility to proteolytic degradation of collagens. *Amino Acids* **2008**, *35*, 703–710. [CrossRef]
36. Schulz, D.; Morschel, J.; Schuster, S.; Eulenburg, V.; Gomeza, J. Inactivation of the mouse L-proline transporter PROT alters glutamatergic synapse biochemistry and perturbs behaviors required to respond to environmental changes. *Front. Mol. Neurosci.* **2018**, *11*, 279. [CrossRef]
37. Liu, W.; Le, A.; Hancock, C.; Lane, A.N.; Dang, C.V.; Fan, T.W.-M.; Phang, J.M. Reprogramming of proline and glutamine metabolism contributes to the proliferative and metabolic responses regulated by oncogenic transcription factor c-MYC. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 8983–8988. [CrossRef]
38. Chen, S.; Yang, X.; Yu, M.; Wang, Z.; Liu, B.; Liu, M.; Liu, L.; Ren, M.; Qi, H.; Zou, J. SIRT3 regulates cancer cell proliferation through deacetylation of PYCR1 in proline metabolism. *Neoplasia* **2019**, *21*, 665–675. [CrossRef]
39. Yu, J.-M.; Chu, M.; Park, H.; Park, J.; Lee, K.-G. Analysis of volatile compounds in coffee prepared by various brewing and roasting methods. *Foods* **2021**, *10*, 1347. [CrossRef]
40. EUR-Lex. Regulation (Eu) No 1169/2011 of the European Parliament and of the Council of 25 October 2011. 2011. Available online: https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2011:304:0018:0063:EN:PDF (accessed on 12 December 2022).
41. Food Ingredient and Packaging Terms. Available online: https://www.fda.gov/food/food-ingredients-packaging/food-ingredient-packaging-terms (accessed on 12 December 2022).
42. chemBlink. Available online: http://www.matrixscientific.com/media/msds/14/42/30/MxMSDS_144230.pdf (accessed on 12 December 2022).
43. Connor, E.E. Sulfonamide antibiotics. *Prim. Care Update Ob. Gyns* **1998**, *5*, 32–35. [CrossRef]

44. Ratajczak, M.; Kubicka, M.M.; Kamińska, D.; Długaszewska, J. Microbiological quality of food supplements. *Acta Pol. Pharm.* **2015**, *72*, 383–387.
45. Páleníková, A.; Martínez-Domínguez, G.; Arrebola, F.J.; Romero-González, R.; Hrouzková, S.; Garrido Frenich, A. Occurrence of pesticide residues and transformation products in different types of dietary supplements. *Food Addit. Contam.-Chem. Anal. Control Expo. Risk Assess.* **2015**, *32*, 849–856. [CrossRef] [PubMed]
46. Filipiak-Szok, A.; Kurzawa, M.; Szłyk, E. Determination of toxic metals by ICP-MS in Asiatic and European medicinal plants and dietary supplements. *J. Trace Elem. Med. Biol.* **2015**, *30*, 54–58. [CrossRef]
47. Brodziak-Dopierała, B.; Fischer, A.; Szczelina, W.; Stojko, J. The content of mercury in herbal dietary supplements. *Biol. Trace Elem. Res.* **2018**, *185*, 236–243. [CrossRef] [PubMed]
48. Rocha, T.; Amaral, J.S.; Oliveira, M.B.P. Adulteration of dietary supplements by the illegal addition of synthetic drugs: A review. *Compr. Rev. Food Sci. Food Saf.* **2016**, *15*, 43–62. [CrossRef] [PubMed]
49. Blicharska, E.; Szczęsna, B.; Kocjan, R.; Gumieniczek, A.; Komsta, Ł. Analysis of dissolution profiles of iron, zinc, and manganese from complex dietary supplements by ion chromatography and chemometrics. *J. Liq. Chromatogr. Relat.* **2016**, *39*, 30–34. [CrossRef]
50. Yu, L.X.; Amidon, G.; Khan, M.A.; Hoag, S.W.; Polli, J.; Raju, G.; Woodcock, J. Understanding pharmaceutical quality by design. *AAPS J.* **2014**, *16*, 771–783. [CrossRef] [PubMed]
51. Al-Gousous, J.; Langguth, P. Oral solid dosage form disintegration testing—The forgotten test. *J. Pharm. Sci.* **2015**, *104*, 2664–2675. [CrossRef]
52. Kapoor, A.; Sharfstein, J.M. Breaking the gridlock: Regulation of dietary supplements in the United States. *Drug Test. Anal.* **2016**, *8*, 424–430. [CrossRef]
53. Abdel-Tawab, M. Do we need plant food supplements? A critical examination of quality, safety, efficacy, and necessity for a new regulatory framework. *Planta Med.* **2018**, *84*, 372–393. [CrossRef]
54. Sarma, N.; Giancaspro, G.; Venema, J. Dietary supplements quality analysis tools from the United States Pharmacopeia. *Drug Test. Anal.* **2016**, *8*, 418–423. [CrossRef] [PubMed]
55. Wallace, T.C. Twenty years of the dietary supplement health and education act—How should dietary supplements be regulated? *J. Nutr.* **2015**, *145*, 1683–1686. [CrossRef] [PubMed]

## 3.4 Publikacja 4 (oryginalna)

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# Screening the quality of legal and illegal dietary supplements by LC-MS/MS

Krzysztof Adam Stępień, Agnieszka Kalicka & Joanna Giebułtowicz

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# Screening the quality of legal and illegal dietary supplements by LC-MS/MS

Krzysztof Adam Stępień, Agnieszka Kalicka, and Joanna Giebułtowicz

Faculty of Pharmacy, Department of Drug Chemistry, Pharmaceutical and Biomedical Analysis, Medical University of Warsaw, Warsaw, Poland

**ABSTRACT**

Dietary supplements are widely consumed. However, the lack of mandatory testing results in limited data on their quality, particularly in Eastern Europe. In this study, 21 legally registered and 9 illegal supplements, seized from an underground facility by the Polish Police, were examined. Contaminants were screened by utilising high-performance liquid chromatography coupled with untargeted mass spectrometry. The analysis identified 32 contaminants in the 30 dietary supplements examined. Untargeted analysis revealed a concerning issue: the intentional adulteration of both legal and illegal supplements with pharmacologically active substances that are prohibited in this category of products. This study indicated that many dietary supplements are of low quality due to deliberate adulteration or inadequate manufacturing conditions. The presence of unregistered or unapproved substances in these supplements poses serious health risks. Strong legal regulations are essential to address this issue effectively.

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## Introduction

The utilisation of novel analytical techniques opens a myriad of possibilities, with particular emphasis on methods capable of detecting compounds at low concentrations. These methods can be used to analyse the quality of medicinal products and dietary supplements, both those produced legally and illegally. So far, various analytical methods have been employed to detect adulterants in dietary supplements. These include thin-layer chromatography (Muschietti et al. 2020), liquid chromatography (LC) with UV (Hemdan and Tawakol 2018), with diode array (Rebiere et al. 2012), or with corona discharge detection (Poplawska et al. 2013). Nowadays, a widely popular technique for analysing compounds illicitly used to adulterate dietary supplements, is mass spectrometry coupled with LC (Al Lawati et al. 2017; Jairoun et al. 2021; Stępień and Giebułtowicz 2022), or coupled with gas chromatography (Dastjerdi et al. 2018; Lin et al. 2018). Other techniques include Raman spectroscopy (Mateescu et al. 2017; Muschietti et al. 2020), infrared spectroscopy (Deconinck et al. 2018), X-ray powder diffractometry (Stypułkowska et al. 2011), nuclear magnetic resonance (Hachem et al. 2016), and capillary electrophoresis (Moreira et al. 2014).

There are two main reasons for the low quality of dietary supplements related to the presence of undeclared substances on the label. Firstly, there is the presence of contaminants which may occur inadvertently due to substandard manufacturing practices or the presence of contaminants originating from supplement ingredients. Secondly, intentional addition of undeclared ingredients by manufacturers is another possibility. The first category encompasses heavy metals (Kowalski and Frankowski 2015) or substances present in raw materials, such as herbicides (Nieto-García et al. 2015), insecticides (Chen et al. 2016), mycotoxins (Martínez-Domínguez et al. 2016) and dioxins (Fernandes et al. 2006). For example, pesticides were previously identified in supplements containing Ginkgo (Martinez-Dominguez et al. 2015) and Ginseng (Yue et al. 2022), while mycotoxins were present in supplements with Ginkgo and grapes. The second category of contaminants consists of substances that are prohibited in dietary supplements and are intentionally added by the manufacturer to enhance the observed effects (Stępień et al. 2019). This is a way of adulterating dietary supplements, similarly to adulteration of medicinal products (Jabłońska and Stępień 2019). This includes anabolic steroids (Martínez-Sanz et al. 2017), hypoglycaemic drugs (Zhou et al. 2011), drugs used in potency disorders (Patel et al. 2014) and weight loss products (Haller and Benowitz 2000). Given uncertainties regarding potential adulteration/contamination, highly accurate techniques like high-resolution mass spectrometry are essential for analysing such products, identifying compounds even at low concentrations.

**CONTACT** Joanna Giebułtowicz joanna.giebultowicz@wum.edu.pl
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The presence of undeclared substances in dietary supplements indicates poor quality and potential risks to consumers health. The lack of global harmonisation in defining and classifying dietary supplements leads to varied regulatory approaches across different jurisdictions. Despite these inconsistencies, ensuring consumer safety remains a shared objective among all countries (Thakkar et al. 2020). The regulations governing dietary supplements in the European Union (EU) are implemented nationally under Directive 2002/46/EC, commonly known as the Food Supplements Directive (FSD). According to this directive, dietary supplements are considered foodstuffs intended to complement the normal diet by providing concentrated sources of nutrients or other substances with nutritional or physiological effects. These supplements are available in various forms including capsules, tablets, powders, and liquids. The FSD specifies permitted vitamins and minerals, as well as criteria for their forms and dosages (European Commission 2002).

Under Directive 2002/46/EC, within the EU various Member States have developed different systems for monitoring the introduction of dietary supplements into the market, varying in terms of regulatory freedom. Some countries lack any notification procedures altogether. However, in the vast majority notifying the relevant authorities of the intention to market dietary supplements is mandatory, although not all jurisdictions require verification of these notifications. Unlike Poland, certain countries such as Greece, Belgium, or Cyprus require additional approvals or administrative fees as part of this process (Wróbel et al. 2022). It is particularly important to include on the packaging of dietary supplements the content of vitamins, minerals and other substances with nutritional or physiological effects. This information should be presented numerically or graphically per the manufacturer's declared daily dose. Additionally, it should be expressed as a percentage of the reference intake values, which are listed in point 1 of Part A of Annex XIII to Regulation (EU) No 1169/2011 (European Commission 2011). The manufacturer of a dietary supplement is obligated to ensure that the product meets legal requirements and is consistent with the information presented on the packaging (Brzezińska and Grembecka 2021). There is no legal requirement to conduct studies confirming durability, effectiveness, interactions with other products, or adverse effects prior to market introduction (Bojarowicz and Dźwigulska 2012). Consumer safety relies solely on the responsible use of dietary supplements according to the label's recommendations.

In Poland, the Chief Sanitary Inspectorate (GIS) supervises dietary supplements, which includes verifying notifications and if doubts arise conduct clarification proceedings. Due to the large number of notifications and inadequate staffing levels relative to market size, verifications are often significantly delayed and actual inspections can take a considerable amount of time. As a result, consumers unknowingly purchased unverified dietary supplements for years. According to a report by the Supreme Audit Office (NIK), only 11% of products from 63,000 notifications submitted to GIS between 2017 and May 2021 were analysed (Supreme Audit Office 2023).

In the United States, the Food and Drug Administration (FDA) serves as the main regulatory authority for dietary supplements. However, unlike pharmaceutical products, which must demonstrate safety and efficacy approved by the FDA before being released to the market, dietary supplements are presumed to be safe until proven otherwise by the FDA. Only then a potentially harmful product can be removed from the market (2023). The Dietary Supplement Health and Education Act mandates adherence to good manufacturing practice principles for ensuring the quality of dietary supplements (Pawar and Grundel 2017; Mathews 2018). However, manufacturers are left to establish their own standards, leading to non-uniformity among similar supplements from different producers. Conscientious dietary supplement manufacturers can benefit from pharmacopeial guidelines. The United States Pharmacopeia (USP) sets official standards for both medicinal products and dietary supplements, offering quality benchmarks for these substances. With over 500 monographs developed for supplement ingredients and their final forms, along with guides, the USP aims to inform manufacturers and safeguard consumers. Importantly, compliance with pharmacopeial requirements for dietary supplements is optional, not mandatory (Sarma et al. 2016).

In many countries, there is an ongoing discussion about the poor quality of dietary supplements. There are literature reports confirming the use of LC-MS/MS to detect substances prohibited in dietary supplements or undeclared by manufacturers. Data come from the USA (Li et al. 2021), Korea (Lee et al. 2020), Japan (Tachi et al. 2022), Canada (Dos Santos et al. 2016), China (Sheng et al. 2023), Vietnam (Hoa et al. 2020), Brazil (dos Santos et al. 2018) and from European countries such as Belgium (Van Hassel et al. 2022), UK (Al-Khadhra 2020), Spain (Pallarés et al. 2022), Italy (Pascali et al. 2018), France (Fabresse et al. 2021), Portugal (Paíga et al. 2017), the Netherlands (Biesterbos et al. 2019), Germany (Parr et al. 2008), Turkey (Dincel et al. 2020) and Croatia (Mornar et al. 2020).

In Eastern Europe there is limited data on the use of this technique to analyse dietary supplements. Recently, we published some data regarding the quality of dietary supplements containing tryptophan (Stępień and Giebułtowicz 2022) or proline (Stępień et al. 2023). In Poland, besides these studies, other ones on the quality of dietary supplements have also been conducted for several years, but despite being reported by state institutions, these results lack publication in scientific journals, so analytical methods and supplement selection criteria cannot be compared. Effective methods should be sought for ensuring supplement quality. Moreover, continuous market analysis, particularly in Eastern European countries lacking supplement quality data, is essential.

Thus, this study aimed to apply liquid chromatography coupled with mass spectrometry as a highly reliable analytical technique to screen for contaminants and detect adulteration of legal and illegal dietary supplements containing a variety of substances. Twenty-one registered supplements, sourced from various European countries, available in Poland, along with nine illegal supplements recovered by the Polish Police from a closed illicit facility, originating from different global locations, were analysed. A comprehensive examination of these samples will significantly contribute to ongoing discussions about the regulatory framework for these products. Additionally, it is anticipated that the advanced analytical approach employed in this study will show its value due to its broad applicability to conduct a thorough evaluation of supplement quality.

## Materials and methods

### Samples

The study encompassed 30 dietary supplements containing a variety of substances, including vitamins, amino acids, minerals, herbs, glucosamine, collagen, chondroitin, hyaluronic acid, citicoline, caffeine, yohimbine, 1,3-dimethylamine, MK 2866, 13-ethyl-3-methoxy-gona-2,5(10)-diene-17-one, halodrol, or carbopol. These substances were enclosed in either capsule or tablet forms and were manufactured in the Czech Republic, France, Germany, Poland, Sweden, the United Kingdom, and the United States. Twenty-one of these dietary supplements were registered in Poland (L1 – L21), readily available on the market and procured from pharmacies, online pharmacies, a drugstore in Poland, or a Polish online e-commerce platform. In contrast, nine dietary supplements (IL1 – IL9) were deemed illegal, as they lacked registration in Poland, but are still available from online sources. These products were manufactured in various countries, including China, Cyprus, India, Singapore, the UK, and the USA. Basic information on the analysed samples is presented in Table A1 of the Supplementary material. Figure 1 shows a photograph of selected legal dietary supplements and all illegal dietary supplements analysed. Illegal products were confiscated from an illegal drug facility that was closed by the Polish Police and submitted to the Counterfeit Medicines Task Force of the Medical University of Warsaw as part of cooperation for analysis and scientific research purposes. Nevertheless, these illicit products remain accessible for purchase online as they are offered from abroad.

### Sample preparation

Three tablets or capsules were randomly selected from each supplement and their respective total weight or content was determined. Tablets were turned into powder by means of a mortar and subsequently an amount equivalent to 10 µg to 10 mg, depending on the product, of the selected ingredient was weighed and 1.00 mL of an acetonitrile/methanol/water mixture (1:1:1; v/v/v) was added. The resulting mixture was sonicated for 15 min and then subjected to centrifugation for 5 min. The supernatant was subsequently diluted with the mobile phase.

### Qualitative analysis

Instrumental analysis was performed using a Dionex Ultimate 3000 UHPLC connected to a Q-Exactive hybrid quadrupole-orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) system equipped with heat electrospray ionisation (HESI) in positive mode, an online vacuum degasser, a quaternary pump, an autosampler and a thermostatted column compartment. Full MS scans were acquired over the m/z 100–1400 range with a resolution of 70,000 (m/z 200). Fragmentation was performed in different runs with a normalised collision energy of 20, 35, and 50 eV. The ion selection threshold was 8.00E + 03 counts and the maximum allowed ion accumulation times were set to auto, both for full MS scans and the tandem mass spectrum. Standard mass spectrometric conditions for all experiments were: spray voltage, 3.5 kV; sheath gas pressure: 60 arb; aux gas pressure: 20 arb; sweep gas pressure: 0 arb, heated capillary temperature: 320°C; Loop count: 3; Isolation window: m/z 1.0; and Dynamic exclusion: 6.0 s. For all full scan measurements lock-mass ions from ambient air (m/z 445.1200 and 291.2842) were used as internal calibrants. Chromatographic separation was achieved with an Accucore C-18 column (100 mm ×


**Figure 1.** a) Selected legal dietary supplements, b) all analysed illegal dietary supplements.

4.6 mm, 2.6 µm) supplied by Thermo Fisher Scientific (Waltham, MA, USA) equipped with a security guard. The column was maintained at 40°C at a flow rate of 0.3 mL/min. The mobile phases consisted of HPLC-grade water with 0.1% formic acid as eluent A and acetonitrile with 0.1% formic acid as eluent B. The gradient (% B) was as follows: 0 min 10%; 1 min 10%; 10 min 95%; 15 min 95%. The injection volume was 10 µL. The obtained results were processed with Compound Discoverer 3.3 software supplied by Thermo Fisher Scientific (Waltham, MA, USA). HPLC-grade methanol, acetonitrile, and formic acid were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

The structures of the contaminants were proposed based on:

(1) The *m/z* of the compound. The difference between experimental and theoretical molecular weight should be not higher than 5 ppm.
(2) Isotopic pattern. The relative intensity tolerance to be used for the isotope search was set at 30%.
(3) Fragmentation of the compound (Figure A1 of the Supplementary material). The fragmentation spectrum was compared with reference standards (confidence level 1), either experimental data available in mass spectra libraries or scientific literature (confidence level 2) or in silico fragmentation (confidence level 3).

Trueness, precision, recovery and selectivity of the method was validated in accordance with Regulation (EU) 2021/808 (European Commission 2021).

## Results and discussion

The analytical results unveiled the presence of 32 substances that were not declared by the manufacturer on the product packaging. Table 1 provides the essential data for these substances, including their molecular formulas, retention times, experimental and theoretical masses, fragmentation data and tentative names. For compounds for which reference standards were available in the laboratory, a quantitative analysis was conducted to estimate the impact of the determined dose on the human body. Substances such as proline, leucine, arginine, caffeine, tryptophan and rutin were determined in small amounts (Table 2), which will not have a negative effect on the body, but they should be declared on the packaging by the manufacturer. On the contrary, substances such as salbutamol, sildenafil or tadalafil can have an effect on the body even at low levels. The scheme for dietary supplement analysis with

**Table 1.** Tentative identification of contaminants in legal and illegal supplements and their MS parameters.

| Code | Formula | Neutral mass calculated from formula [Da] | Neutral mass calculated from measured [Da] | Δ Mass [ppm] | RT [min] | Identification confidence level * | Fragments [*m/z*] | Found in dietary supplements | Tentative name |
|---|---|---|---|---|---|---|---|---|---|
| C1 | C5H13NO | 103.09971 | 103.09948 | −2.2 | 2.8 | 2 | 58.0653; 60.0809 | L5, L8, L12 | Choline |
| C2 | C6H11NO | 113.08406 | 113.08409 | 0.3 | 7.7 | 2 | 68.0494; 79.0542; 96.0805;97.0646 | L2 | Caprolactam |
| C3 | C5H9NO2 | 115.06333 | 115.06325 | −0.7 | 3.0 | 1 | 70.0651; 88.0756; 93.0367 | L16 | Proline |
| C4 | C5H11NO2 | 117.07898 | 117.07888 | −0.9 | 2.9 | 2 | 58.0654; 59.0732 | L5, L12 | Betaine |
| C5 | C6H6N2O | 122.04801 | 122.04799 | −0.2 | 3.0 | 2 | 78.0335; 80.0494; 96.0442 | IL4 | Nicotinamide |
| C6 | C6H11NO2 | 129.07898 | 129.07895 | −0.2 | 3.0 | 2 | 56.0498; 67.0545; 84.0807 | L12 | Pipecolic acid |
| C7 | C4H9N3O2 | 131.06948 | 131.06952 | 0.3 | 2.9 | 2 | 87.0554; 90.0549; 114.0662; 115.0502 | L8 | Creatine |
| C8 | C6H13NO2 | 131.09463 | 131.09465 | 0.2 | 4.4 | 1 | 69.0699; 72.9370; 86.0962; 90.9475; 108.9582; 113.9635 | L12 | Leucine |
| C9 | C11H16O | 164.12012 | 164.12005 | −0.4 | 12.4 | 3 | 55.0546; 67.0543; 79.05412; 81.06979; 93.06992; 95.04895; 105.06955; 109.06465; 119.08520; 121.10093; 123.08043; 137.09593; 147.11667 | L2 | Jasmone |
| C10 | C11H8N2 | 168.06875 | 168.06890 | 0.9 | 12.2 | 2 | 115.0543; 142.0654; 168.0680 | L18, L19 | Norharman |
| C11 | C8H11NO3 | 169.07389 | 169.07384 | −0.3 | 3.0 | 1 | 55.9347; 70.9578; 88.9681; 93.0571; 96.9607; 111.9842; 113.9636; 128.9504; 141.9814; 146.9611 | L14, L15 | Norepinephrine |
| C12 | C12H11N | 169.08915 | 169.08917 | 0.1 | 13.8 | 2 | 65.0390; 92.0493; 93.0572; 153.0692 | IL6 | Diphenylamine |
| C13 | C11H11NO | 173.08406 | 173.08385 | −1.2 | 11.1 | 3 | 91.05431; 103.05415; 117.05742; 130.06497; 131.07274; 144.08020 159.06760 | L12 | Pyroquilon |
| C14 | C6H14N4O2 | 174.11168 | 174.11142 | −1.5 | 2.8 | 1 | 60.0558; 70.0651; 72.0808; 112.0869; 116.0705; 130.0975; 158.0921 | L6, L12 | Arginine |
| C15 | C7H8N4O2 | 180.06473 | 180.06468 | −0.3 | 4.1 | 2 | 67.0290; 69.0442; 108.0554; 110.0712; 135.0665; 137.0819; 138.0660; 163.0611 | L8 | Theobromine |
| C16 | C7H8N4O2 | 180.06473 | 180.06465 | −0.4 | 5.1 | 2 | 69.0448; 96.0575; 124.0504; 142.0610 | L8 | Paraxanthine |
| C17 | C8H10N4O2 | 194.08038 | 194.08043 | 0.3 | 8.4 | 1 | 83.0600; 110.0713; 138.0659 | L15, L17 | Caffeine |
| C18 | C11H12N2O2 | 204.08988 | 204.08984 | −0.2 | 6.1 | 1 | 57.0700; 118.0648; 132.0803; 144.0804; 146.0598; 149.0229; 159.0913; 170.0597; 188.0701 | L12 | Tryptophan |
| C19 | C10H8O5 | 208.03717 | 208.03719 | 0.1 | 8.3 | 2 | 107.0491; 149.0233; 163.0389; 194.0208 | L5 | Fraxetin |
| C20 | C12H18O3 | 210.12559 | 210.12553 | −0.3 | 13.2 | 2 | 79.0541; 81.0697; 83.0853; 91.0539; 95.0853; 97.0647; 105.0696; 109.0645; 119.0852;121.1009; 131.0854; 133.1010; 137.0594; 139.0746; 147.1165; 151.1115; 165.1271; 175.1116; 193.1219; | L2 | Jasmonic acid |
| C21 | C13H21NO3 | 239.15214 | 239.15199 | −0.6 | 3.5 | 1 | 57.0702; 103.0540; 121.0646; 130.0649; 148.0754; 166.0860; 222.1483 | IL3 | Salbutamol |
| C22 | C15H14O4 | 258.08921 | 258.08895 | −1.0 | 9.4 | 2 | 55.0181; 71.0125; 95.0488; 107.0490; 121.0646; 123.0440; 135.0438; 149.0596; 153.0697 157.0648; 181.0643; 199.0750; 213.0907; 226.0617; 227.0695; | IL1 | Isorhapontigenin |
| C23 | C15H12O5 | 272.06847 | 272.06804 | −1.6 | 8.3 | 2 | 67.0178; 91.0539; 107.0487; 119.0489; 123.0436; 147.0437; 153.0178; | IL4 | Naringenin |
| C24 | C16H22O4 | 278.15181 | 278.15169 | −0.4 | 17.2 | 2 | 57.0703; 95.0492; 109.0649; 121.0282; 149.0234; | IL1 | Diisobutyl phthalate |
| C25 | C21H23NO | 305.17796 | 305.17711 | −2.8 | 11.3 | 2 | 115.0539; 117.0696; 129.0696; 145.0647; 157.0645; 165.0691; 183.0801; 215.0844; 233.0957; 261.1267; | L21 | Dapoxetine |
| C26 | C20H27ClO2 | 334.16996 | 334.17015 | 0.6 | 13.4 | 2 | 81.0699; 93.0700; 105.0696; 107.0858; 109.1012; 121.1010; 149.1324; 155.0256; 161.1323; 169.0415; 181.0420; 207.0572; 317.1664 | IL9 | Chlorodehydromethyltestosterone |
| C27 | C16H18O9 | 354.09508 | 354.09416 | −2.6 | 8.8 | 2 | 89.0382; 117.0326; 135.0432; 145.0275; 163.0379 | L12 | Chlorogenic acid |
| C28 | C22H19N3O4 | 389.13756 | 389.13765 | 0.2 | 12.2 | 1 | 99.0556; 130.0654; 135.0442; 169.0762; 192.0812; 205.0892; 233.0832; 240.1132; 250.0859; 262.0863; 263.0944; 268.1080; 274.0853; 302.0810 | L18, L19 | Tadalafil |
| C29 | C23H30N6O3 | 438.23794 | 438.23768 | −0.6 | 9.9 | 2 | 99.0913; 165.0181; 311.1133; 339.1447; | L21 | Desmethyl carbodenafil |
| C30 | C23H30N6OS2 | 470.19225 | 470.19338 | 2.4 | 11.5 | 2 | 343.0687; 371.1005; 414.1426; 440.1575 | L21 | Dithio-desmethyl-carbodenafil |
| C31 | C22H30N6O4S | 474.20492 | 474.20491 | 0.0 | 10.3 | 1 | 58.0654; 70.0650; 99.0916; 100.099; 255.1246; 283.1188; 311.1505 | L18, L20 | Sildenafil |
| C32 | C27H30O16 | 610.15338 | 610.15343 | 0.1 | 10.0 | 1 | 71.0491; 85.0283; 97.0282; 129.0542; 153.0180; 229.0483; 257.0438; 303.0492; 465.1006 | L12 | Rutin |

*1- the fragmentation spectrum was compared with reference standards; 2 - the fragmentation spectrum was compared with experimental data available in mass spectra libraries or literature; 3 - the fragmentation spectrum was received in silico fragmentation.

**Table 2.** Results of quantitative analysis and validation parameters for substances for which standards were available in the laboratory.

| Substance | Single dose available in capsules/tablets [mg] | Quantity [mg] | ILD[1] [pg] | $R^2$ | Precision [%] | Repeatability [%] |
|---|---|---|---|---|---|---|
| Arginine | 500–1000 | 2.6 (L6)<br>51 (L12) | 13 | 0.9999 | 0.48–1.8 | 99–102 |
| Caffeine | 200 | 33 (L15)<br>2 (L17) | 17 | 0.9999 | 0.31–3.2 | 98–102 |
| Leucine | 500 | 3.1 | 71 | 0.9991 | 1.1–2.2 | 97–103 |
| Norepinephrine | not applicable | 39 (L14)<br>4 (L15) | 16 | 0.9997 | 0.33–4.3 | 98–102 |
| Proline | 25–520 | 3.7 | 35 | 0.9996 | 0.024–4.8 | 99–105 |
| Rutin | 25–100 | 2.7 | 22 | 0.9999 | 0.064–4.7 | 98–101 |
| Salbutamol | 2–4 (32)[2] | 3.1 | 11 | 0.9991 | 0.21–0.92 | 96–105 |
| Sildenafil | 25–100 (100)[2] | 110 (L20)<br>66 (L18) | 5.4 | 0.9999 | 0.39–2.4 | 99–105 |
| Tadalafil | 2.5–20 (20)[2] | 47 (L18)<br>21 (L19) | 2.1 | 0.9996 | 0.69–4.4 | 98–104 |
| Tryptophan | 40–1000 | 0.4 | 70 | 0.9996 | 0.12–4.4 | 101–105 |

[1]Instrument Detection Limit, [2]maximum daily dose is provided in brackets.

the results is presented in Figure 2. The obtained results could be sorted out in three different categories, which are described in the three following paragraphs:

### *Legal substances in legal supplements*

Undeclared legal substances detected in legal supplements were divided into three categories: 1) amino acids, 2) stimulants, and 3) others.

The first category, undeclared amino acids, consisted of the findings of proline (m/z 116.0706, 3.7 mg/unit), leucine (m/z 132.1019, 3.1 mg/unit), arginine (m/z 175.1185, 2.6–51 mg/unit), and tryptophan (m/z 205.0972, 0.40 mg/unit). While dietary supplements may contain amino acids, their presence must be clearly indicated on the product packaging (Dz 2017 poz. 979). The presence of these amino acids in the supplements could result rather from contamination during the manufacturing process than intentional addition to enhance physiological effects (Binks et al. 2020). As an example, tryptophan was detected in melatonin supplements, at doses at least 100 times lower than those specified as an ingredient on the packaging. Cerezo et al. (2016) also detected tryptophan in 6 out of 16 melatonin supplements. However, due to the lack of a quantitative study, it remains unclear whether the detected amount could induce a physiological effect. Tryptophan as such is considered to be safe. However, excessive consumption or supplementary tryptophan can lead to various adverse effects, including nausea and upset stomach, drowsiness, serotonin syndrome, allergic reactions, medication interactions, and weight gain (Fernstrom 2012). Arginine in dietary supplements mainly occurs in doses of 500–1000 mg per unit. It can increase the secretion of gastric hydrochloric acid, exacerbating conditions such as heartburn, ulcers, digestive disorders, and intensifying cold symptoms (Grimble 2007). In the present study, it was found in at least 10-times lower concentrations. Leucine mainly

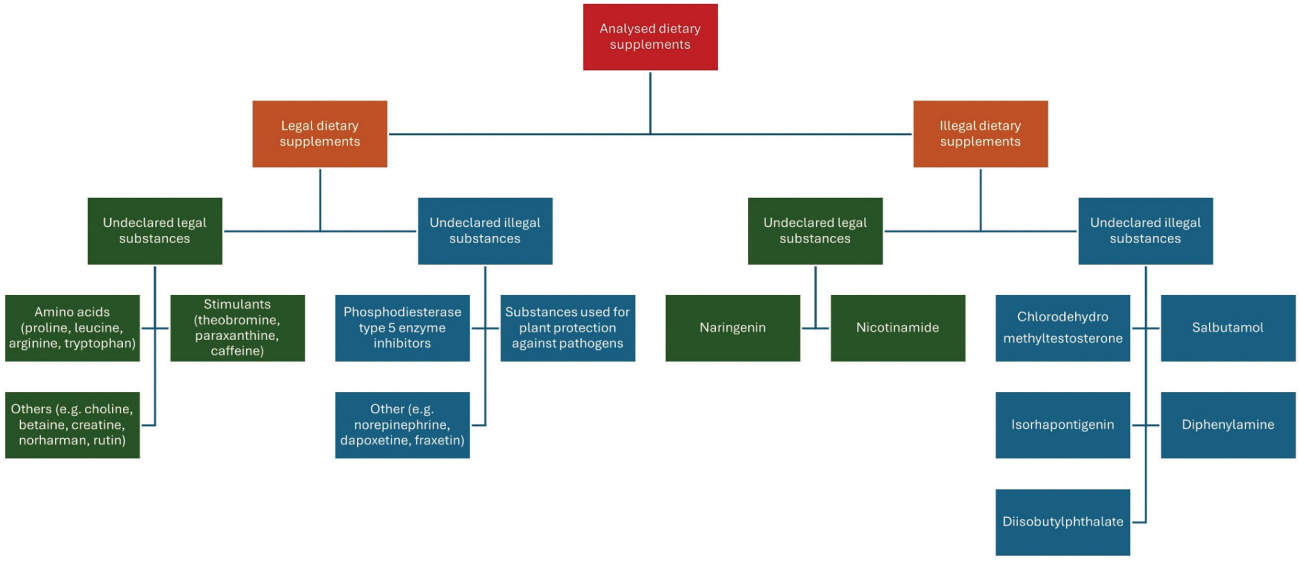


**Figure 2.** Scheme for dietary supplement analysis with obtained results.

occurs in dietary supplements in doses of 500 mg per unit. In the present study, at least 160-times lower concentrations were found. When consumed in excess, it may lead to coordination disorders, chronic fatigue, anxiety, or mood swings. Very high doses can even be toxic and elevate ammonia levels in the blood. Proline in dietary supplements occurs usually in doses ranging from 25 to 520 mg per unit. In the present study, it was found in at least 6-times lower concentrations. Excessive proline consumption weakens bones, cartilage, joints, and skin, while also reducing the elasticity of blood vessel walls (Stępień et al. 2023).

The second category of undeclared substances are stimulants, which encompass theobromine (m/z = 187.0715), paraxanthine (m/z = 181.0725) and caffeine (m/z = 195.0877, 2–33 mg/unit). These substances belong to the methylxanthine family and can be components of dietary supplements. The maximum amount of caffeine in dietary supplements is specified: caffeine can be used in an amount of up to 400 mg per day, divided in portions, provided that the product does not contain other ingredients with a synergistic effect. A serving of caffeine consumed at one time cannot exceed 200 mg (Uchwała nr 2019). When consumed in moderate amounts, it alleviates fatigue, enhances mood and concentration, boosts physical endurance, and heightens attention by improving cognitive functions such as working memory (Nawrot et al. 2003). Paraxanthine and theobromine share similar central nervous system stimulant properties with caffeine. Caffeine, theobromine and paraxanthine are often present in numerous dietary supplements designed to enhance energy, concentration, and physical performance or support weight reduction. Excessive consumption of caffeine, theobromine, and paraxanthine can lead to various side effects, the most common include anxiety, hypertension, certain drug interactions, and withdrawal symptoms (Daly 2007).

The third category of undeclared substances consists of compounds with diverse properties. One such compound is choline (*m/z* 104.3936), of which numerous studies have suggested that it is involved in memory processes, so its deficiency has been linked to dementia and Alzheimer's disease (Aguree et al. 2023). Another substance within this category is betaine (*m/z* 118.0667), recognised as a generally safe component in dietary and supplements. Potential observed side effects may include reduction in homocysteine, along with a minute lowering of diastolic blood pressure and uplifting of low-density lipoprotein and total cholesterol in serum (Kaur et al. 2019). Creatine (m/z 132.1999) is another substance commonly found in dietary supplements. The most prevalent side effect is temporary water retention during the initial phases of supplementation. Furthermore, the presence of norharman was identified in two plant-based dietary supplements. The exact role of norharman in the human body remains complex and not fully understood. Nevertheless, research suggests that norharman may have a potential role in reducing the incidence of Parkinson's disease (Piechowska et al. 2019). Rutin (m/z 609.6430, 2.7 mg), commonly used in dietary supplements as well, was also found, far below the daily dose for adults of 25–100 mg. Its potential adverse effects encompass gastrointestinal disturbances and allergic reactions.

All these undeclared substances mentioned above may be legally included in dietary supplements, provided they are accurately listed on the product label, as they exhibit nutritional or other physiological effects.

### *Illegal substances in legal supplements*

In the present study, also substances with therapeutic effects were identified. These do not naturally occur in food and exert effects beyond nutritional or physiological functions. Therefore, they are illegal in dietary supplements. These particular substances are strictly prohibited in dietary supplements. The substances found in the analysed dietary supplements were divided into three classes: 1) phosphodiesterase type 5 enzyme inhibitors, 2) substances used for plant protection against pathogens and 3) other substances.

Compounds belonging to the first class encompass sildenafil (m/z 475.2125), tadalafil (m/z 390.1459), desmethylcarbodenafil (m/z 439.2451) and dithiodesmethylcarbodenafil (m/z 471.1991). Sildenafil and tadalafil are pharmaceutical ingredients used to address erectile dysfunction and are characterised by potent pharmacological effects. The unlabelled presence of these compounds carries a significant risk of potential drug interactions, which in turn can lead to adverse effects such as hypotension or myocardial dysfunction. In sample L20 an amount of 110 mg of sildenafil was found in 1 tablet, whereas the maximum single and daily dose is 100 mg. In sample L19 an amount of 21 mg of tadalafil was found, whereas the maximum single and daily dose is 20 mg. Both in case L19 and L20 the single and daily dose was exceeded, while the manufacturer even did not declare their presence. In sample L18 amounts of 47 mg of tadalafil and 66 mg of sildenafil were found. The combination of these two substances in a single product is atypical, as there is currently no legal formulation for such a composition. The potential adverse effects of combining these active ingredients are also unknown. The single and daily dose of tadalafil in this dietary supplement was exceeded more than

twice. The use of these substances in such high doses and in one preparation may cause serious side effects. It should be emphasised that these substances are contraindicated for individuals with severe renal impairment, cardiovascular disease, congestive heart failure (NYHA class II, III, or IV) and uncontrolled arrhythmia (Kloner et al. 2018). Desmethylcarbodenafil and dithiodesmethylcarbodenafil are structural analogues of sildenafil, but they are not registered as active substances that can be used in medicinal products. The presence of PDE 5 inhibitors and their analogues in dietary supplements claimed to be of plant origin and marketed as natural enhancers of sexual potency has been documented in the scientific literature (Kee et al. 2018). Consumption of PDE 5 inhibitors analogues such as desmethylcarbodenafil may cause executive hypotension to death (Bakota et al. 2017). Dithiodesmethylcarbodenafil was found by Yun et al. (2018) in a dietary supplement, which claimed to contain a natural sexual enhancer. It can be expected that side effects like cardiovascular disorders associated with its use will be similar to those caused by other PDE5-inhibitors analogues, but these have not been described in the literature. While applying LC-MS, Campbell et al. (2013) found tadalafil and/or sildenafil in 40 out of 74 investigated dietary supplements of plant origin purchased in the USA, of which 18 contained above 110% of the highest approved drug product strength. The other 34 samples contained PDE5-inhibitor analogs, whereas all 74 did not claim synthetic substances on the labelling. This also shows that despite its detection a decade ago, the regulatory landscape appears to be largely unchanged, as these supplements continue to be legally distributed worldwide.

The second class of compounds includes pipecolic acid (m/z 130.0862), pyroquilon (m/z 174.0911) and jasmonic acid (m/z 211.1328). These are present in plant-based supplements. Pipecolic acid is an important regulator of immunity in plants and humans alike and occurs naturally in the organisms of many plants (Koc and Dinler 2022). Pyroquilon, also known as quinochlorine or *N*-[(4-chlorophenyl) methyl]pyrazolidine-3,5-dione, is a synthetic chemical compound that does not occur naturally. It is a commercial blasticide that binds in the naphthol pocket of the fungal trihydroxynaphthalene reductase active site (Liu et al. 2022). Jasmonic acid is an organic chemical compound which is representative of the plant growth and development regulators group (Ghorbel et al. 2021). Such substances should not be present in dietary supplements. They are probably a remnant of the plant breeding stage. In the literature, there are also data confirming the presence of other substances used at the plant breeding stage in plant-based supplements, such as herbicides (Chen et al. 2016), fungicides, and insecticides (Costa et al. 2019).

The third class of substances consists of compounds with diverse properties. The first substance described in this group is norepinephrine (m/z 170.0811; 4–39 mg/unit), classified as a very potent substance. It is a sympathomimetic amine formed under physiological conditions in postganglionic adrenergic neurons and chromaffin cells of the adrenal medulla. However, it is impossible to assess its effect on the body after oral administration, because it is rapidly inactivated in the digestive tract. In medicine, it is administered parenterally only at the hospital (French et al. 2021). Another substance with a pronounced effect on the body is dapoxetine (m/z 306.1844). It is typically found in medications available only by prescription and is employed in the treatment of premature ejaculation in males aged 18 to 64. It functions by delaying the release of serotonin, a neurotransmitter responsible for ejaculation. It should be noted that the off-label use of antidepressant SSRIs (selective serotonin reuptake inhibitors) for premature ejaculation is common practice, whereas antidepressants, including SSRIs, may increase the risk of suicidal thoughts and behaviours in children, adolescents, and young adults (McMahon 2012). Furthermore, dapoxetine was identified in 14 out of 353 sexual enhancement supplements adulterated in the USA, constituting approximately 4.0% of the analysed samples (Tucker et al. 2018). Other substances within this category exert a milder influence on the organism. Fraxetin (m/z 209.0444) is a simple coumarin and serves as a phytochemical found in medicinal plants like *Fraxinus rhynchophylla* and *Cortex Fraxini*. Within plants, it functions as a regulator of iron homoeostasis. Fraxetin is associated with various health-promoting effects, including anticancer, neuroprotective, and antibacterial properties (Sarfraz et al. 2017). Chlorogenic acid (m/z 355.1014), known for its diverse potential health benefits, including antioxidant activity, antibacterial, hepatoprotective, cardioprotective, anti-inflammatory, antipyretic, neuroprotective, anti-obesity, antiviral, anti-microbial, anti-hypertension, free radicals scavenger, and a central nervous system stimulator. Side effects associated with chlorogenic acid are typically rare and mild (Naveed et al. 2018). Jasmone (m/z 165.1273) finds application in the manufacturing of perfumes and cosmetics (PubChem 2023). On the other hand, caprolactam (m/z 114.0913) is utilised in the production of high-temperature-resistant synthetic packaging materials, including those used for medical product packaging. Animal studies have indicated potential toxicity from

caprolactam, particularly with prolonged and high-level exposure (Kumar et al. 2019).

### Legal & illegal substances in illicit supplements

In illicit dietary supplements, seven undeclared substances were found. Of these, only naringenin (m/z 273.0753) and nicotinamide (m/z 123.0553) can be included in dietary supplements, as these are legal substances. Naringenin exhibits anti-inflammatory properties, strengthens blood vessels, and reduces cholesterol levels. It is generally regarded as safe and does not have any known side effects when consumed in typical dietary quantities (Salehi et al. 2019). Nicotinamide is considered a safe dietary component. However, it can elicit adverse effects in specific cases. Potential adverse effects of nicotinamide include liver toxicity, teratogenicity, oncogenicity, bullos lesions of the skin, toxic amblyopia, and hypotensive reactions. The other five detected substances are not allowed for use in dietary supplements, so these are illegal. These are chlorodehydromethyltestosterone, salbutamol, isorhapontigenin, diphenylamine, and diisobutylphthalate.

Chlorodehydromethyltestosterone (m/z = 335.1772), also known as oral turinabol, is a synthetic anabolic steroid that was long since used as a doping agent in sports, particularly in athletics. It is considered a controversial and prohibited substance in international sports due to its potential for enhancing physical performance and muscle development. It was identified in the IL9 formulation at a concentration of less than 1 mg per capsule. As stipulated on the product packaging, consumers are instructed to ingest three capsules daily. Consequently, the potential adverse effects of this substance on the human body, when following this advice, cannot be excluded. The adverse effects of chlorodehydromethyltestosterone can encompass various side effects typical of anabolic steroids. It may increase the risk of cardiovascular diseases such as hypertension, coronary artery disease, and heart attacks. Other side effects cover liver problems, urinary system problems, aggression, depression, and irritability. Due to its illegal and hazardous nature, chlorodehydromethyltestosterone is banned in many countries and sports organisations (Singh 2017; Shelley et al. 2019). Its presence raises concerns that the illicit manufacturer may have produced other items with higher concentrations of this substance. In the examined preparation, this compound was the sole contamination detected from the production stage. It is noteworthy that, in accordance with the information provided on the packaging, 13-ethyl-3-methoxy-gona-2,5(10)-diene-17-one should also be present in IL9, but analyses did not confirm it.

Salbutamol (m/z = 240.1592, 3.1 mg) stimulates the sympathetic system, causing the relaxation of bronchial muscles and is primarily used in the treatment of bronchial asthma. Salbutamol medications are available only with a physician's prescription. The suggested oral dosage is 4 mg taken 3 or 4 times daily. The physician may incrementally raise this to a maximum of 8 mg, administered three or four times per day. Using salbutamol may lead to side effects such as urticaria, hypotension, collapse, hypokalaemia, and tremor (Marques and Vale 2022). Salbutamol was detected in a dietary supplement marketed as a fat burner at therapeutic level. It may influence the process of thermogenesis, leading to an increase in body temperature. While this process can enhance calorie burning and aid in weight loss, it is not a safe method to lose weight. Therefore, it is emphasised that using salbutamol for weight loss or slimming is dangerous and contradicts medical principles (Fragkaki et al. 2013).

Isorhapontigenin (m/z 259.0962) was identified in a plant-based dietary supplement designed for fat removal, toxin elimination, and slimming aid purposes. It is an analog of resveratrol, which can be used in dietary supplements. Various biological activities of resveratrol and its derivatives have been previously described in the context of both cancer and inflammation (Pecyna et al. 2020).

Diphenylamine (m/z 170.0965), which is an aromatic amine containing two phenyl substituents, was used as a fungicide to treat superficial burns on apples and pears, but is no longer approved for this purpose in the European Union (Kokulnathan et al. 2022). This substance was detected in a plant-based dietary supplement, and it was probably used during plant breeding.

Diisobutylphthalate (m/z = 279.1590) is used as a plasticiser because of its flexibility and durability. It is commonly found in various industrial and personal care products like polishes, nail polishes, and cosmetics. Its impact on the human body is currently under investigation, as this compound can be released from the products and it may be present in the environment (Yost et al. 2019).

### Summary

To summarise, 32 contaminants were detected in 30 analysed dietary supplements. 25 contaminants were detected in 13 legal products and 7 contaminants in 5 illegal preparations. Among the contaminants detected were substances with a strong pharmacological effect, which are dispensed only based on a physician's

prescription. In the case of legal dietary supplements, these were: norepinephrine, dapoxetine, and phosphodiesterase type 5 enzyme inhibitors. Illegal supplements contained salbutamol and a testosterone derivative. Other contaminants in legal dietary supplements included amino acids, caffeine, theobromine, paraxanthine, substances used to protect plants against pathogens, rutin, and substances that are components of the packaging (caprolactam) or used in cosmetics (jasmone). In illegal dietary supplements substances such as naringenin, isorhapontigenin, diphenylamine, and diisobutylphthalate were detected. In 9 out of 21 legal dietary supplements, undeclared and prohibited substances were found and in 4 out of 9 illegal preparations, undeclared and prohibited substances for the production of dietary supplements were found. These findings highlight the importance of assessing the quality of dietary supplements, as the majority of tested preparations contained additional substances, some of which may have a potent impact on the body. The data presented in this paper are not isolated. A similar study was conducted in Italy, where high-resolution LC-MS was applied to analyse 110 food products that were collected from the online market or during official inspections. Out of these samples, 5 (4.5%) contained undeclared substances like progesterone, dehydroepiandrosterone, paracetamol or sildenafil. These findings suggest a need to strengthen the monitoring of dietary supplements to detect adulteration, as these pose a potential threat to consumers health (Giannetti et al. 2023). Undoubtedly, the scale of this phenomenon would be smaller if there were detailed requirements for the assessment of the quality of dietary supplements and if they were legally sanctioned. Only then the consumer would be sure about good quality of the consumed products.

## Conclusions

Liquid chromatography coupled with untargeted mass spectrometry provided valuable information about dietary supplements on the EU market. Compounds permitted for use in supplements were found at concentrations 6–160 times lower than those used for physiological effects, but missed obligatory declaration on the label. Untargeted analysis also revealed the presence of intentionally adulterated supplements, whether legal or illegal, with pharmacologically active substances that are not legally allowed in these supplements. Compounds like sildenafil, tadalafil, dapoxetine, salbutamol, desmethyl carbodenafil, dithiodesmethyl-carbodenafil, and chlorodehydromethyltestosterone, in quantifiable levels may have implications for human health or exhibit significant yet unknown effects. Especially PDE5 inhibitors analogues, since these are not approved for medical use and therefore have not been assessed for safe consumption. This study raises concern regarding the quality of dietary supplements, thereby making a substantial contribution to the ongoing discourse on supplement regulation. The newly established analytical approach holds considerable potential for widespread applicability in the assessment of supplement quality and thus will help to develop relevant legislation.

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## ORCID

Joanna Giebułtowicz http://orcid.org/0000-0002-2567-4453

## References

Aguree S, Zolnoori M, Atwood TP, Owora A. 2023. Association between choline supplementation and Alzheimer's disease risk: a systematic review protocol. Front Aging Neurosci. 15. doi: 10.3389/fnagi.2023.1242853.

Al Lawati HA, Al Busaidi I, Kadavilpparampu AM, Suliman FO. 2017. Determination of common adulterants in herbal medicine and food samples using core-shell column coupled to tandem mass spectrometry. J Chromatogr Sci. 55(3):232–242. doi: 10.1093/chromsci/bmw175.

Al-Khadhra RS. 2020. The determination of common anabolic steroid and stimulants in nutritional supplements by HPLC-DAD and LC-MS. J Chromatogr Sci. 58(4):355–361. doi: 10.1093/chromsci/bmz121.

Bakota EL, Kelly AT, Walterscheid JP, Phatak DR. 2017. A case report of fatal desmethyl carbodenafil toxicity. J Anal Toxicol. 41(3):250–255. doi: 10.1093/jat/bkw128.

Biesterbos JW, Sijm DT, van Dam R, Mol HG. 2019. A health risk for consumers: the presence of adulterated food supplements in the Netherlands. Food Addit Contam Part A. 36(9):1273–1288. doi: 10.1080/19440049.2019.1633020.

Binks H, Ev G, Gupta C, Irwin C, Khalesi S. 2020. Effects of diet on sleep: a narrative review. Nutrients. 12(4):936. doi: 10.3390/nu12040936.

Bojarowicz H, Dźwigulska P. 2012. Suplementy diety. Część I. Suplementy diety a leki– porównanie wymagań prawnych. Hygeia Public Health. 47(4):427–432.

Brzezińska J, Grembecka M. 2021. Suplementy diety – specyficzna żywność. Postępy Higieny i Medycyny

Doświadczalnej. 75(1):655–673. doi: 10.2478/ahem-2021-0011.
Campbell N, Clark JP, Stecher VJ, Thomas JW, Callanan AC, Donnelly BF, Goldstein I, Kaminetsky JC. 2013. Adulteration of purported herbal and natural sexual performance enhancement dietary supplements with synthetic phosphodiesterase type 5 inhibitors. J Sex Med. 10 (7):1842–1849. doi: 10.1111/jsm.12172.
Cerezo AB, Á L, Álvarez-Fernández MA, Hornedo-Ortega R, Troncoso AM, García-Parrilla MC. 2016. Quality control and determination of melatonin in food supplements. J Food Compos Anal. 45:80–86. doi: 10.1016/j.jfca.2015.09.013.
Chen Y, Lopez S, Hayward DG, Park HY, Wong JW, Kim SS, Wan J, Reddy RM, Quinn DJ, Steiniger D. 2016. Determination of multiresidue pesticides in botanical dietary supplements using gas chromatography–triple-quadrupole mass spectrometry (GC-MS/MS). J Agric Food Chem. 64(31):6125–6132. doi: 10.1021/acs.jafc.6b00746.
Costa JG, Vidovic B, Saraiva N, Do Ceu Costa M, Del Favero G, Marko D, Oliveira NG, Fernandes AS. 2019. Contaminants: a dark side of food supplements? Free Radic Res. 53(sup1):1113–1135. doi: 10.1080/10715762.2019.1636045.
Daly J. 2007. Caffeine analogs: biomedical impact. Cell Mol Life Sci. 64(16):2153–2169. doi: 10.1007/s00018-007-7051-9.
Dastjerdi AG, Akhgari M, Kamali A, Mousavi Z. 2018. Principal component analysis of synthetic adulterants in herbal supplements advertised as weight loss drugs. Complemen Ther Clin Pract. 31:236–241. doi: 10.1016/j.ctcp.2018.03.007.
Deconinck E, Aouadi C, Bothy J, Courselle P. 2018. Detection and identification of multiple adulterants in plant food supplements using attenuated total reflectance—infrared spectroscopy. J Pharm Biomed Anal. 152:111–119. doi: 10.1016/j.jpba.2018.01.047.
Dincel D, Olgan H, Canbaloğlu Z, Yalcin S, Erkuçuk A, Tırıs G, Gören AC. 2020. Determination of dihydrocapsaicin adulteration in dietary supplements using LC-MS/MS. J Chem Metrol. 14(1):77–82. doi: 10.25135/jcm.36.20.01.1532.
dos Santos MK, Gleco E, Davidson JT, Jackson GP, Limberger RP, Arroyo LE. 2018. DART-MS/MS screening for the determination of 1, 3-dimethylamylamine and undeclared stimulants in seized dietary supplements from Brazil. Forensic Chem. 8:134–145. doi: 10.1016/j.forc.2018.03.005.
Dos Santos VB, Daniel D, Singh M, Do Lago CL. 2016. Amphetamine and derivatives in natural weight loss pills and dietary supplements by capillary electrophoresis-tandem mass spectrometry. J Chromatogr B. 1038:19–25. doi: 10.1016/j.jchromb.2016.10.019.
Dz U. 2017. Dziennik Ustaw Rzeczypospolitej Polskiej. Ministerstwo Zdrowia. 979:1–9.
European Commission. 2002. Directive 2002/46/EC of the European parliament and of the council of 10 June 2002 on the approximation of the laws of the member states relating to food supplements. Off J Eur Communities: Legislation. 45:51–57.
European Commission. 2011. Regulation (EU) No 1169/2011 of the European parliament and of the council of 25 October 2011 on the provision of food information to consumers. Off J Eur Communities. 304:18–63.
European Commission Implementing Regulation (EU). 2021. 2021/808 of 22 March 2021 on the performance of analytical methods for residues of pharmacologically active substances used in food-producing animals and on the interpretation of results as well as on the methods to be used for sampling and repealing decisions 2002/657/EC and 98/179/EC. Off J Eur Union. 180:84–109.
Fabresse N, Gheddar L, Kintz P, Knapp A, Larabi IA, Alvarez J-C. 2021. Analysis of pharmaceutical products and dietary supplements seized from the black market among bodybuilders. Forensic Sci Int. 322:110771. doi: 10.1016/j.forsciint.2021.110771.
Fernandes AR, Rose M, White S, Mortimer DN, Gem M. 2006. Dioxins and polychlorinated biphenyls (PCBs) in fish oil dietary supplements: occurrence and human exposure in the UK. Food Addit Contam. 23(9):939–947. doi: 10.1080/02652030600660827.
Fernstrom JD. 2012. Effects and side effects associated with the non-nutritional use of tryptophan by humans. J Nutr. 142(12):2236S–2244S. doi: 10.3945/jn.111.157065.
Food and Drug Administration. 2023. Questions and answers on dietary supplements. [accessed 2023 Nov 29]. https://www.fda.gov/food/information-consumers-using-dietary-supplements/questions-and-answers-dietary-supplements.
Fragkaki A, Georgakopoulos C, Sterk S, Nielen M. 2013. Sports doping: emerging designer and therapeutic β2-agonists. Clin (Rome) Acta. 425:242–258. doi: 10.1016/j.cca.2013.07.031.
French WB, Rothstein WB, Scott MJ. 2021. Time to use peripheral norepinephrine in the operating room. Anesth & Analg. 133(1):284–288. doi: 10.1213/ANE.0000000000005558.
Ghorbel M, Brini F, Sharma A, Landi M. 2021. Role of jasmonic acid in plants: the molecular point of view. Plant Cell Rep. 40(8):1471–1494. doi: 10.1007/s00299-021-02687-4.
Giannetti L, Gallo V, Necci F, Marini F, Giorgi A, Sonego E, D'Onofrio F, Neri B. 2023. LC-HRMS analysis of 13 classes of pharmaceutical substances in food supplements. Food Addit Contam Part B. 16(3):253–265. doi: 10.1080/19393210.2023.2214883.
Grimble GK. 2007. Adverse gastrointestinal effects of arginine and related amino acids. J Nutr. 137(6):1693S–1701S. doi: 10.1093/jn/137.6.1693S.
Hachem R, Assemat G, Martins N, Balayssac S, Gilard V, Martino R, Malet-Martino M. 2016. Proton NMR for detection, identification and quantification of adulterants in 160 herbal food supplements marketed for weight loss. J Pharm Biomed Anal. 124:34–47. doi: 10.1016/j.jpba.2016.02.022.
Haller CA, Benowitz NL. 2000. Adverse cardiovascular and central nervous system events associated with dietary supplements containing ephedra alkaloids. N Engl J Med. 343 (25):1833–1838. doi: 10.1056/NEJM200012213432502.
Hemdan A, Tawakol SM. 2018. HPLC–UV chromatographic methods for detection and quantification of undeclared withdrawn synthetic medications in counterfeit herbal medicines with confirmation by HPLC–PDA and mass spectrometry. Chromatographia. 81(5):777–783. doi: 10.1007/s10337-018-3502-8.
Hoa DTM, Mai PTN, Anh HQ, Khanh CC, Ngoc N, Hoa M, Son T, Anh N, Hao L. 2020. Simultaneous determination of

sibutramine and its derivatives in weight loss dietary supplements by LC-MS/MS. Vietnamese J Food Control. 3 (2):104–114. doi: 10.47866/2615-9252/vjfc.106.

Jabłońska AE, Stępień KA. 2019. Fałszowanie produktów leczniczych oraz zagrożenia wynikające z ich stosowania. Prospects Pharm Sci. 17(11):66–71. doi: 10.56782/pps.19.

Jairoun AA, Al-Hemyari SS, Shahwan M, Zyoud Se H. 2021. Adulteration of weight loss supplements by the illegal addition of synthetic pharmaceuticals. Molecules. 26(22):6903. doi: 10.3390/molecules26226903.

Kaur S, Sharma N, Vyas M, Mahajan R, Satija S, Mehta M, Khurana N. 2019. A review on pharmacological activities of betaine. Plant Arch. 19:1021–1034.

Kee C-L, Ge X, Gilard V, Malet-Martino M, Low M-Y. 2018. A review of synthetic phosphodiesterase type 5 inhibitors (PDE-5i) found as adulterants in dietary supplements. J Pharm Biomed Anal. 147:250–277. doi: 10.1016/j.jpba.2017.07.031.

Kloner RA, Goldstein I, Kirby MG, Parker JD, Sadovsky R. 2018. Cardiovascular safety of phosphodiesterase type 5 inhibitors after nearly 2 decades on the market. Sex Med Rev. 6(4):583–594. doi: 10.1016/j.sxmr.2018.03.008.

Koc FN, Dinler BS. 2022. Pipecolic acid in plants: biosynthesis, signalling, and role under stress. Botan Lith. 28(1):4–14. doi: 10.35513/Botlit.2022.1.2.

Kokulnathan T, Vishnuraj R, Chen S-M, Pullithadathil B, Ahmed F, Hasan P, Bilgrami AL, Kumar S. 2022. Tailored construction of one-dimensional TiO2/Au nanofibers: validation of an analytical assay for detection of diphenylamine in food samples. Food Chem. 380:132052. doi: 10.1016/j.foodchem.2022.132052.

Kowalski A, Frankowski M. 2015. Levels and potential health risks of mercury in prescription, non-prescription medicines and dietary supplements in Poland. Regul Toxicol Pharmacol. 73(1):396–400. doi: 10.1016/j.yrtph.2015.08.001 .

Kumar R, Shah S, Paramita Das P, Bhagavanbhai GGK, Al Fatesh A, Chowdhury B. 2019. An overview of caprolactam synthesis. Catal Rev. 61(4):516–594. doi: 10.1080/01614940.2019.1650876.

Lee JH, Min AY, Han JH, Yang YJ, Kim H, Shin D. 2020. Development and validation of LC-MS/MS method with QuEChERS clean-up for detecting cannabinoids in foods and dietary supplements. Food Addit Contam Part A. 37 (9):1413–1424. doi: 10.1080/19440049.2020.1769200.

Li C, Xu D, Moezzi B. 2021. Identification of erectile dysfunction drugs in dietary supplements by liquid chromatography ion trap mass spectrometry. J Diet Suppl. 18 (3):261–277. doi: 10.1080/19390211.2020.1758274.

Lin Y-P, Lee Y-L, Hung C-Y, Chang C-F, Chen Y, Mudiam MKR. 2018. Detection of adulterated drugs in traditional Chinese medicine and dietary supplements using hydrogen as a carrier gas. PLoS One. 13(10): e0205371. doi: 10.1371/journal.pone.0205371.

Liu R, Meng X, Mo C, Wei X, Ma A. 2022. Melanin of fungi: from classification to application. World J Microbiol Biotechnol. 38(12):228. doi: 10.1007/s11274-022-03415-0.

Marques L, Vale N. 2022. Salbutamol in the management of asthma: a review. Int J Mol Sci. 23(22):14207. doi: 10.3390/ijms232214207.

Martínez-Domínguez G, Romero-González R, Arrebola FJ, Frenich AG. 2016. Multi-class determination of pesticides and mycotoxins in isoflavones supplements obtained from soy by liquid chromatography coupled to orbitrap high resolution mass spectrometry. Food Control. 59:218–224. doi: 10.1016/j.foodcont.2015.05.033.

Martinez-Dominguez G, Romero-Gonzalez R, Frenich AG. 2015. Determination of toxic substances, pesticides and mycotoxins, in ginkgo biloba nutraceutical products by liquid chromatography orbitrap-mass spectrometry. Microchem J. 118:124–130. doi: 10.1016/j.microc.2014.09.002.

Martínez-Sanz JM, Sospedra I, Ortiz CM, Baladía E, Gil-Izquierdo A, Ortiz-Moncada R. 2017. Intended or unintended doping? A review of the presence of doping substances in dietary supplements used in sports. Nutrients. 9 (10):1093. doi: 10.3390/nu9101093.

Mateescu C, Popescu AM, Radu GL, Onisei T, Raducanu AE. 2017. Spectroscopic and spectrometric methods used for the screening of certain herbal food supplements suspected of adulteration. Adv Pharm Bull. 7(2):251. doi: 10.15171/apb.2017.030.

Mathews NM. 2018. Prohibited contaminants in dietary supplements. Sports Health. 10(1):19–30. doi: 10.1177/1941738117727736.

McMahon CG. 2012. Dapoxetine: a new option in the medical management of premature ejaculation. Ther Adv Urol. 4 (5):233–251. doi: 10.1177/1756287212453866.

Moreira APL, Martini M, de Carvalho LM. 2014. Capillary electrophoretic methods for the screening and determination of pharmacologic adulterants in herbal-based pharmaceutical formulations. Electrophoresis. 35(21–22):3212–3230. doi: 10.1002/elps.201400059.

Mornar A, Buhač T, Klarić DA, Klarić I, Sertić M, Nigović B. 2020. Multi-targeted screening of phytoestrogens in food, raw material, and dietary supplements by liquid chromatography with tandem mass spectrometry. Food Anal Methods. 13(2):482–495. doi: 10.1007/s12161-019-01653-x.

Muschietti L, Redko F, Ulloa J. 2020. Adulterants in selected dietary supplements and their detection methods. Drug Test Anal. 12(7):861–886. doi: 10.1002/dta.2806.

Naveed M, Hejazi V, Abbas M, Kamboh AA, Khan GJ, Shumzaid M, Ahmad F, Babazadeh D, FangFang X, Modarresi-Ghazani F. 2018. Chlorogenic acid (CGA): a pharmacological review and call for further research. Biomed & Pharmacother. 97:67–74. doi: 10.1016/j.biopha.2017.10.064.

Nawrot P, Jordan S, Eastwood J, Rotstein J, Hugenholtz A, Feeley M. 2003. Effects of caffeine on human health. Food Addit Contam. 20(1):1–30. doi: 10.1080/0265203021000007840.

Nieto-García AJ, Romero-González R, Frenich AG. 2015. Multi-pesticide residue analysis in nutraceuticals from grape seed extracts by gas chromatography coupled to triple quadrupole mass spectrometry. Food Control. 47:369–380. doi: 10.1016/j.foodcont.2014.07.041.

Paíga P, Rodrigues MJ, Correia M, Amaral JS, Oliveira MBP, Delerue-Matos C. 2017. Analysis of pharmaceutical adulterants in plant food supplements by UHPLC-MS/MS. Eur J Pharm Sci. 99:219–227. doi: 10.1016/j.ejps.2016.12.024.

Pallarés N, Berrada H, Font G, Ferrer E. 2022. Mycotoxins occurrence in medicinal herbs dietary supplements and exposure assessment. J Food Sci Technol 59(7):2830–2841. doi: 10.1007/s13197-021-05306-y.

Parr MK, Koehler K, Geyer H, Guddat S, Schänzer W. 2008. Clenbuterol marketed as dietary supplement. Biomed Chromatogr. 22(3):298–300. doi: 10.1002/bmc.928.

Pascali JP, Fais P, Vaiano F, Bertol E. 2018. Application of HRAM screening and LC–MS/MS confirmation of active pharmaceutical ingredient in "natural" herbal supplements. Forensic Sci Int. 286:e28–e31. doi: 10.1016/j.forsciint.2018.03.014.

Patel DN, Li L, Kee C-L, Ge X, Low M-Y, Koh H-L. 2014. Screening of synthetic PDE-5 inhibitors and their analogues as adulterants: analytical techniques and challenges. J Pharm Biomed Anal. 87:176–190. doi: 10.1016/j.jpba.2013.04.037.

Pawar RS, Grundel E. 2017. Overview of regulation of dietary supplements in the USA and issues of adulteration with phenethylamines (PEAs). Drug Test Anal. 9(3):500–517. doi: 10.1002/dta.1980.

Pecyna P, Wargula J, Murias M, Kucinska M. 2020. More than resveratrol: new insights into stilbene-based compounds. Biomolecules. 10(8):1111. doi: 10.3390/biom10081111.

Piechowska P, Zawirska-Wojtasiak R, Mildner-Szkudlarz S. 2019. Bioactive β-carbolines in food: a review. Nutrients. 11(4):814. doi: 10.3390/nu11040814.

Poplawska M, Blazewicz A, Bukowinska K, Fijalek Z. 2013. Application of high-performance liquid chromatography with charged aerosol detection for universal quantitation of undeclared phosphodiesterase-5 inhibitors in herbal dietary supplements. J Pharm Biomed Anal. 84:232–243. doi: 10.1016/j.jpba.2013.06.018.

PubChem National Center for Biotechnology Information, 2023. Bethesda (MD) 20894 (USA): U.S. National Library of Medicine; [accessed 2023 Nov 9]. https://pubchem.ncbi.nlm.nih.gov/compound/Jasmone#section=Uses.

Rebiere H, Guinot P, Civade C, Bonnet P-A, Nicolas A. 2012. Detection of hazardous weight-loss substances in adulterated slimming formulations using ultra-high-pressure liquid chromatography with diode-array detection. Food Addit Contam Part A. 29(2):161–171. doi: 10.1080/19440049.2011.638676.

Salehi B, Fokou PVT, Sharifi-Rad M, Zucca P, Pezzani R, Martins N, Sharifi-Rad J. 2019. The therapeutic potential of naringenin: a review of clinical trials. Pharmaceuticals. 12(1):11. doi: 10.3390/ph12010011.

Sarfraz I, Rasul A, Jabeen F, Younis T, Zahoor MK, Arshad M, Ali M. 2017. Fraxinus: a plant with versatile pharmacological and biological activities. Evid-Based Complement Altern Med. 2017:1–12. doi: 10.1155/2017/4269868.

Sarma N, Giancaspro G, Venema J. 2016. Dietary supplements quality analysis tools from the United States pharmacopeia. Drug Test Anal. 8(3–4):418–423. doi: 10.1002/dta.1940.

Shelley J, Moir HJ, Petroczi A. 2019. The use and misuse of testosterone in sport: the challenges and opportunities in doping control. Nutr Enhanced Sports Perform. 48:571–580.

Sheng Y, Xue Y, Wang J, Liu S, Jiang Y. 2023. Fast screening and identification of illegal adulteration in dietary supplements and herbal medicines using molecular networking with deep-learning-based similarity algorithms. Anal Bioanal Chem. 415(16):3285–3293. doi: 10.1007/s00216-023-04708-5.

Singh S. 2017. Doping in sports: an overview of ancient and modern history of doping. Int J Phys Educ, Sports Health Stud. 4(1):289–292.

Stępień KA, Giebułtowicz J. 2022. Application of liquid chromatography coupled to mass spectrometry in quality assessment of dietary supplements—A case study of tryptophan supplements: release assay, targeted and untargeted studies. Pharmaceuticals. 15(4):448. doi: 10.3390/ph15040448.

Stępień KA, Krawczyk W, Giebułtowicz J. 2023. Dietary supplements with proline—A comprehensive assessment of their quality. Life. 13(2):263. doi: 10.3390/life13020263.

Stępień KA, Niewiarowski J, Harasimiuk A. 2019. Powszechność suplementów diety a zagrożenia związane z ich stosowaniem. Prospects Pharm Sci. 17(9):51–59. doi: 10.56782/pps.22.

Stypułkowska K, Błażewicz A, Maurin J, Sarna K, Fijałek Z. 2011. X-ray powder diffractometry and liquid chromatography studies of sibutramine and its analogues content in herbal dietary supplements. J Pharm Biomed Anal. 56(5):969–975. doi: 10.1016/j.jpba.2011.08.028.

Supreme Audit Office. 2023. Najwyższa Izba Kontroli 2021, Warszawa. Wprowadzanie do obrotu suplementÓw diety (placing on the market dietary supplements. [accessed 2023 Nov 29]. https://www.nik.gov.pl/plik/id,25355,vp,28114.pdf.

Tachi M, Kobayashi S, Tomita K, Tanahashi T, Imanishi SY. 2022. Hierarchical clustering of liquid chromatography-tandem mass spectrometry data for screening of phosphodiesterase type 5 inhibitors and their analogues in adulterated dietary supplements. J Chromatogr A. 1678:463366. doi: 10.1016/j.chroma.2022.463366.

Thakkar S, Anklam E, Xu A, Ulberth F, Li J, Li B, Hugas M, Sarma N, Crerar S, Swift S. 2020. Regulatory landscape of dietary supplements and herbal medicines from a global perspective. Regul Toxicol Pharmacol. 114:104647. doi: 10.1016/j.yrtph.2020.104647.

Tucker J, Fischer T, Upjohn L, Mazzera D, Kumar M. 2018. Unapproved pharmaceutical ingredients included in dietary supplements associated with US food and drug administration warnings. JAMA Netw Open. 1(6):e183337. doi: 10.1001/jamanetworkopen.2018.3337.

Uchwała nr. 2019. Zespół Do Spraw Suplementów Diety. 16/2019.

Van Hassel WHR, Ahn A-C, Huybrechts B, Masquelier J, Wilmotte A, Andjelkovic M. 2022. LC-MS/MS validation and quantification of cyanotoxins in algal food supplements from the Belgium market and their molecular origins. Toxins. 14(8):513. doi: 10.3390/toxins14080513.

Wróbel K, Milewska AJ, Marczak M, Kozłowski R. 2022. Assessment of the impact of scientific reports published by EFSA and GIS on functional foods newly placed on the market in Poland. Int J Environ Res Public Health. 19(7):4057. doi: 10.3390/ijerph19074057.

Yost EE, Euling SY, Weaver JA, Beverly BE, Keshava N, Mudipalli A, Arzuaga X, Blessinger T, Dishaw L, Hotchkiss A. 2019. Hazards of diisobutyl phthalate (DIBP) exposure: a systematic review of animal toxicology studies. Environ Int. 125:579–594. doi: 10.1016/j.envint.2018.09.038.

Yue KX, Li HL, Pi ZF, Song FR, Wang YP, Liu ZY. 2022. Multi-residue screening of pesticides in panax ginseng CA meyer by ultra-high-performance liquid chromatography with quadrupole time-of-flight mass spectrometry. J Sep Sci. 1–9. doi: 10.2139/ssrn.3921725.

Yun J, Shin KJ, Choi J, Kwon K, Jo C-H. 2018. Identification and structural elucidation of a new sildenafil analogue, dithiopropylcarbodenafil, from a premixed powder intended as a dietary supplement. J Chromatogr B. 1072:273–281. doi: 10.1016/j.jchromb.2017.11.029.

Zhou Z, Zhang J, Zhang W, Bai Y, Liu H. 2011. Rapid screening for synthetic antidiabetic drug adulteration in herbal dietary supplements using direct analysis in real time mass spectrometry. Analyst. 136(12):2613–2618. doi: 10.1039/c0an01047b.

# Screening the quality of dietary supplements using liquid chromatography coupled with mass spectrometry

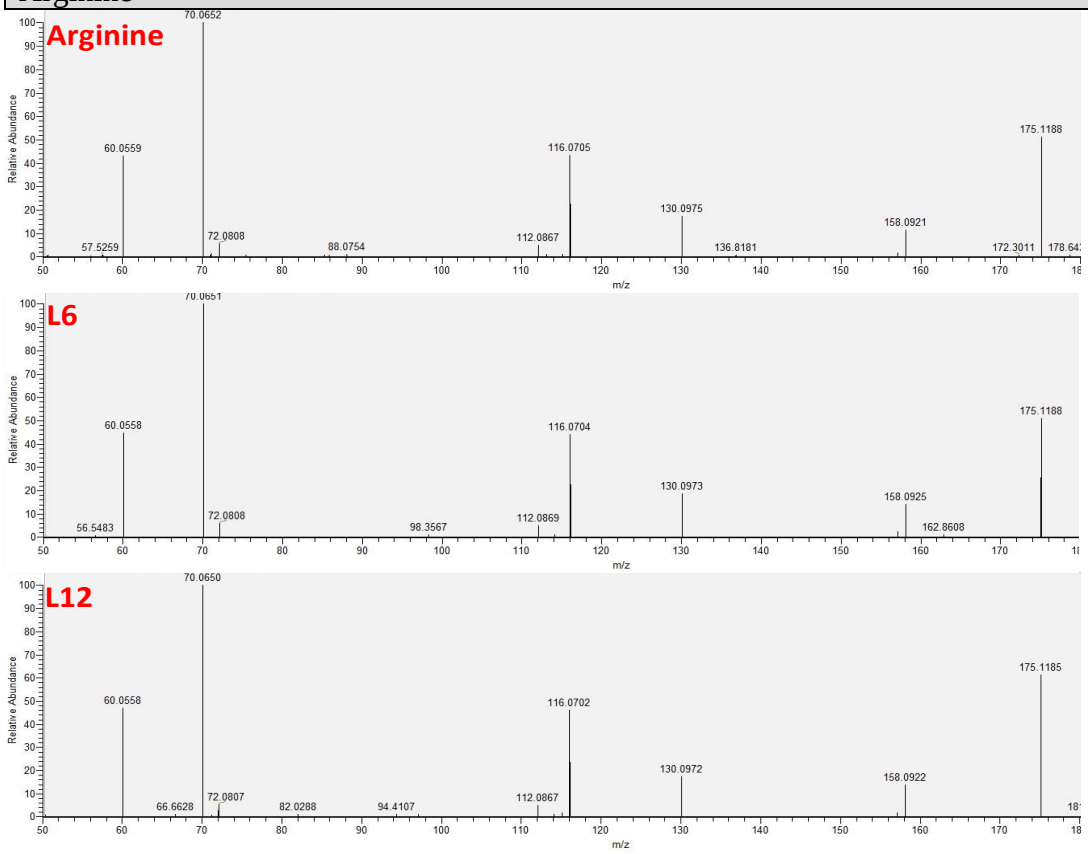
Krzysztof Adam Stępień[a], Agnieszka Kalicka[a], Joanna Giebułtowicz[a]*

[a] *Department of Drug Chemistry, Pharmaceutical and Biomedical Analysis, Faculty of Pharmacy, Medical University of Warsaw, 02-097 Warsaw, Poland.*

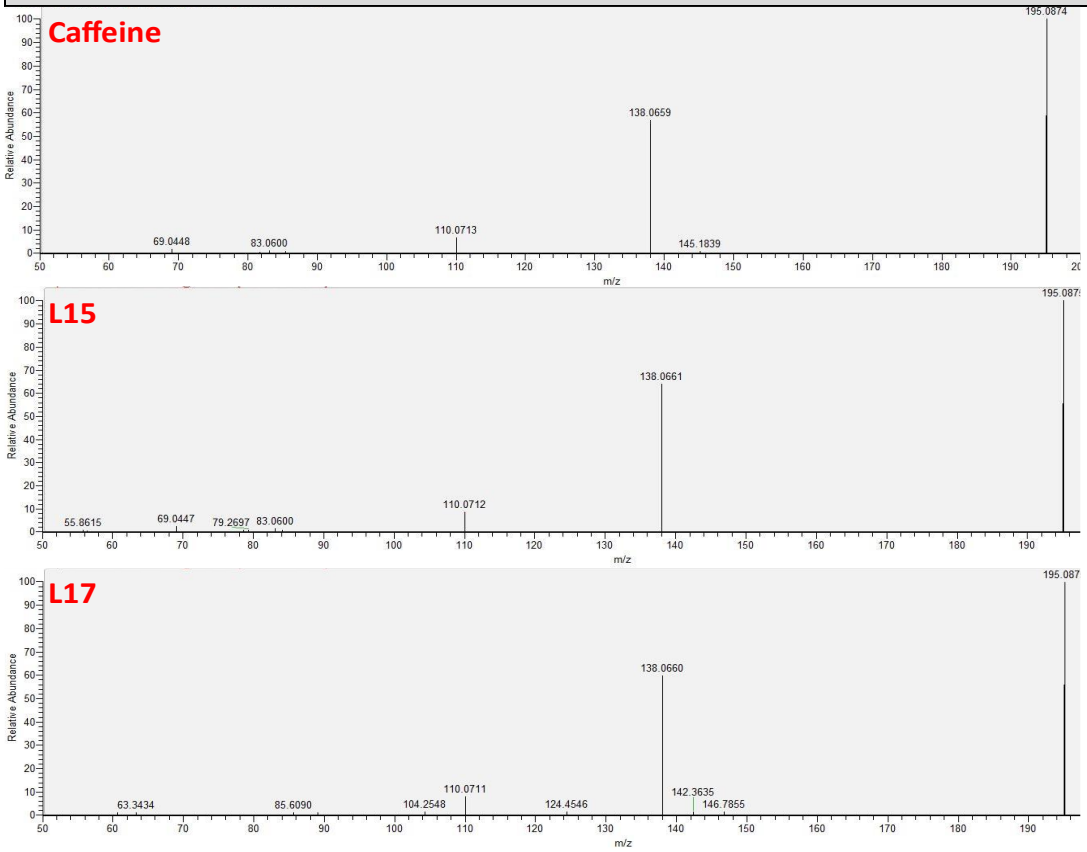
*corresponding author: joanna.giebultowicz@wum.edu.pl

Fragmentation patterns (confidence level 1)

Arginine



## Caffeine

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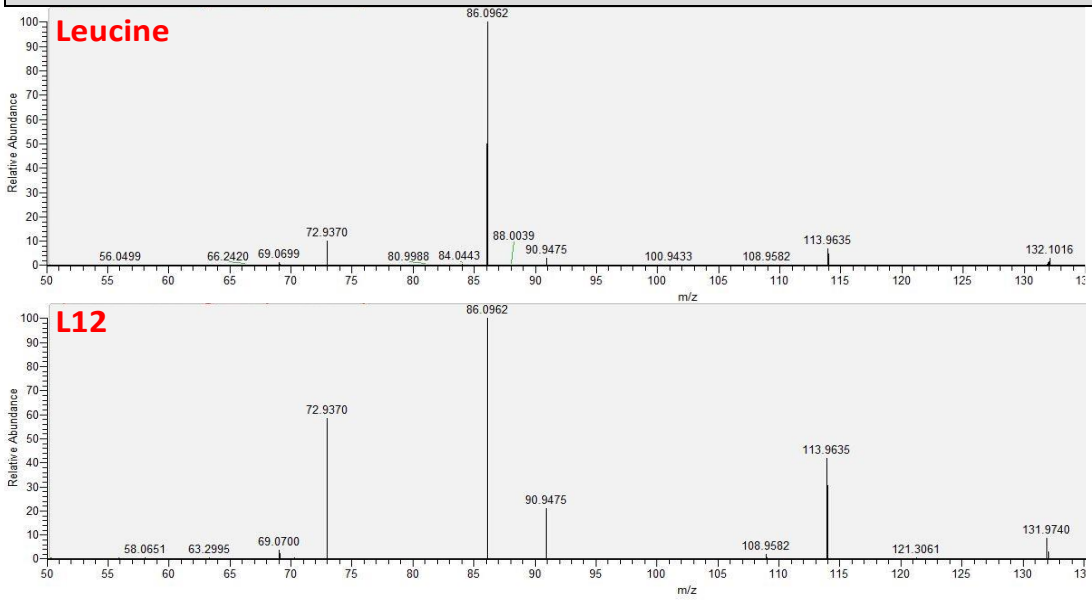

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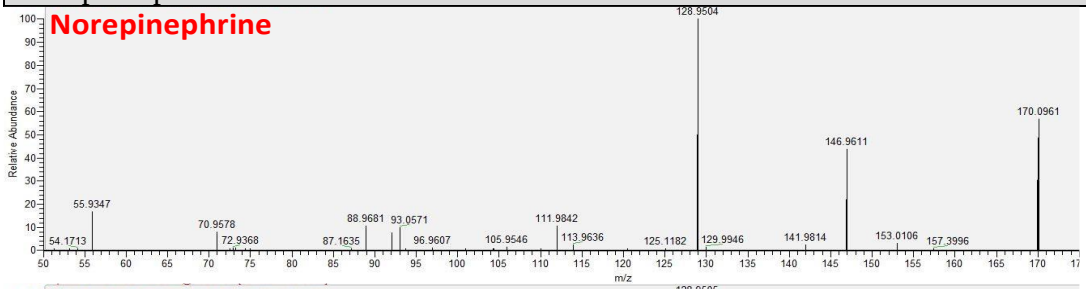
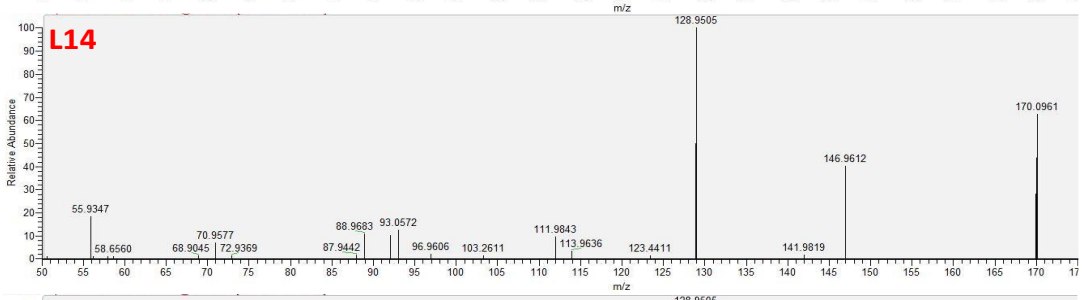
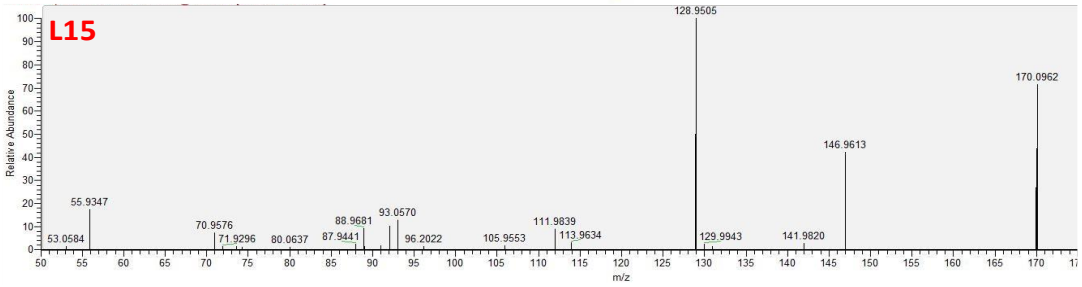
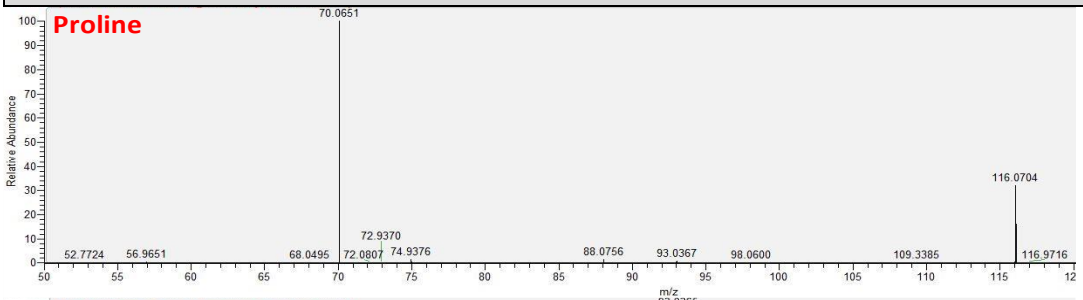
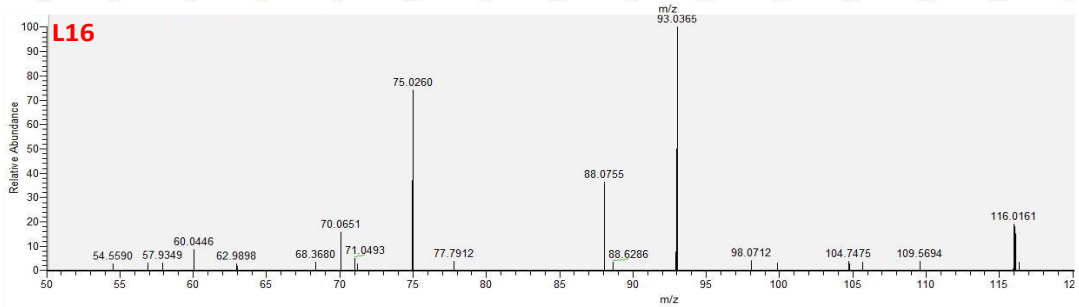


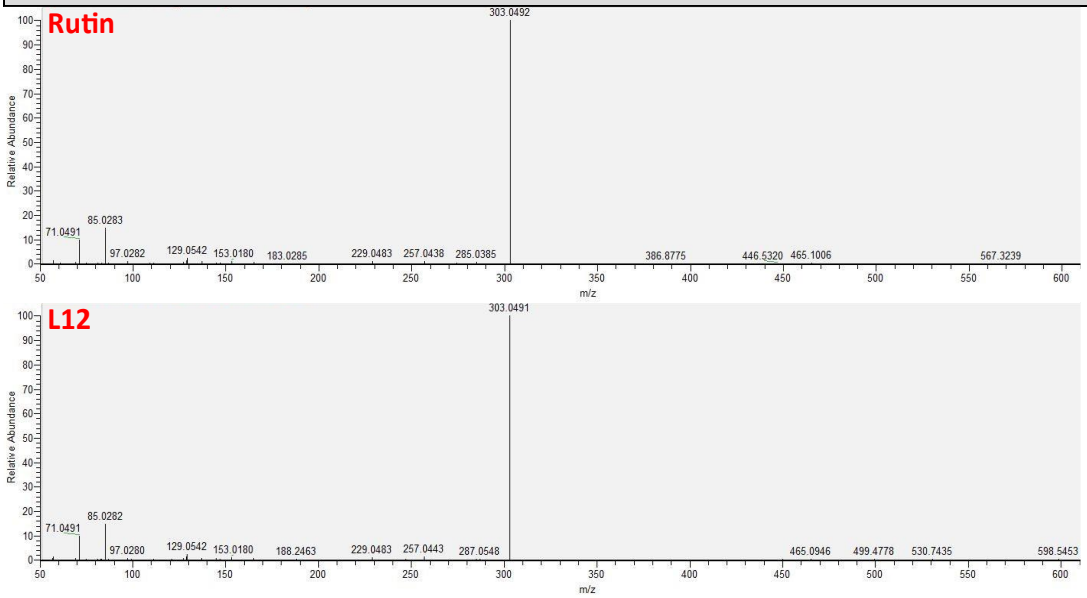




## Proline



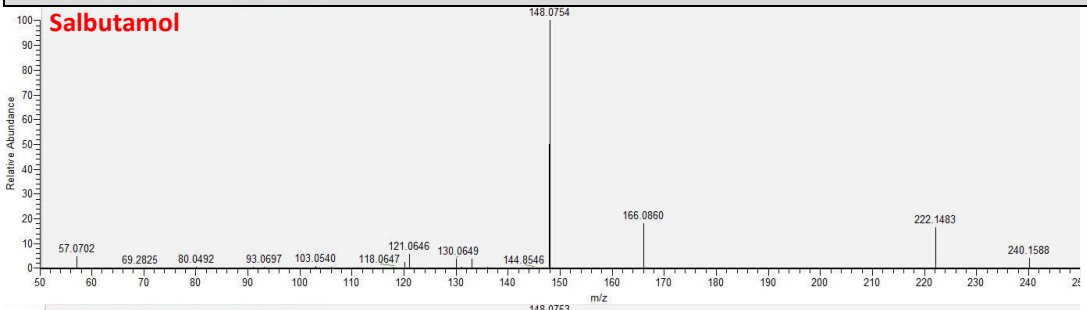
## Rutin

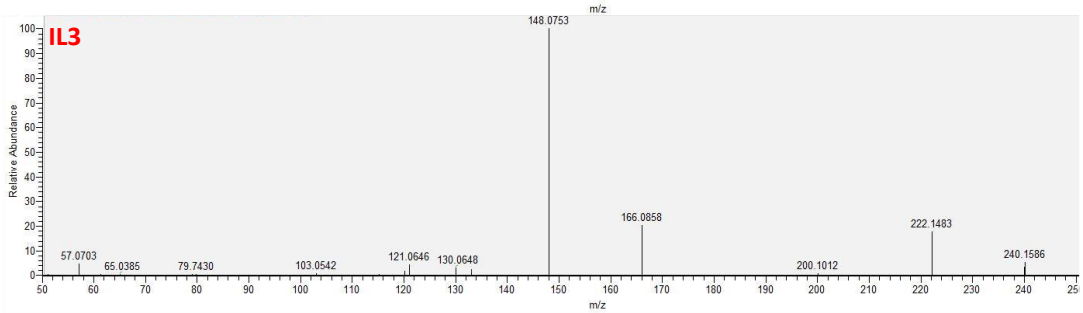




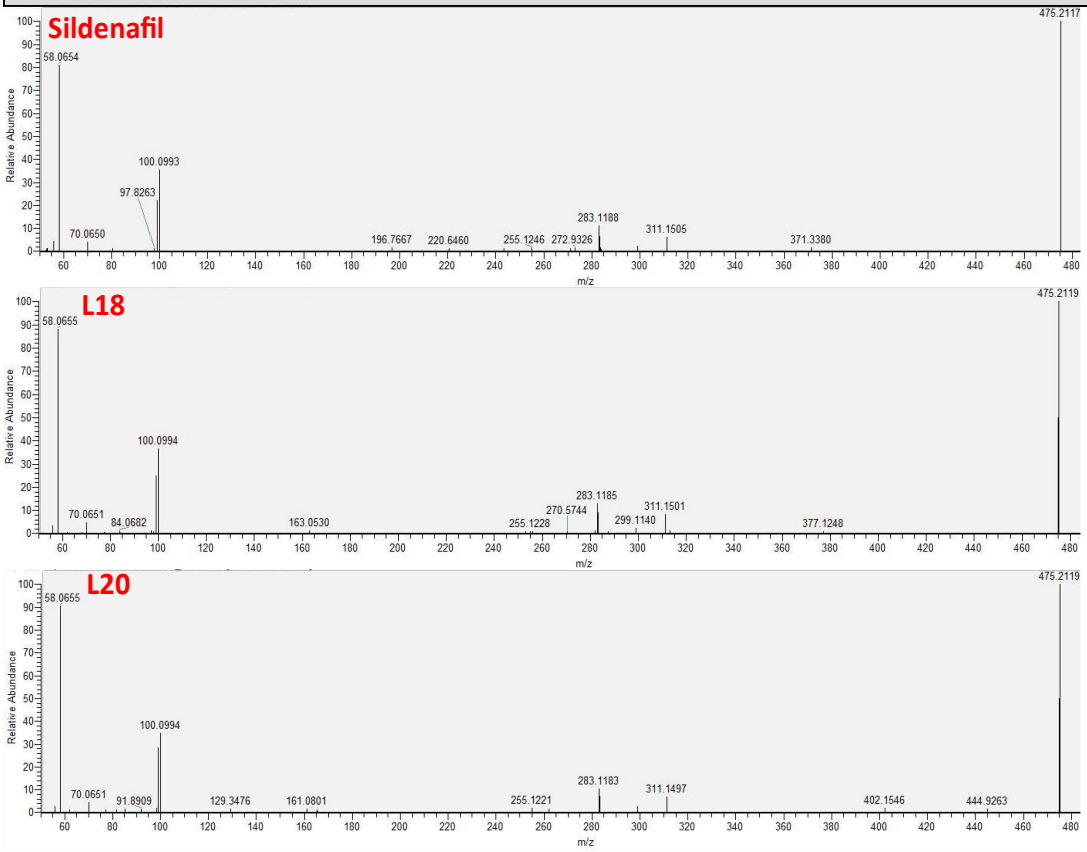


## Salbutamol

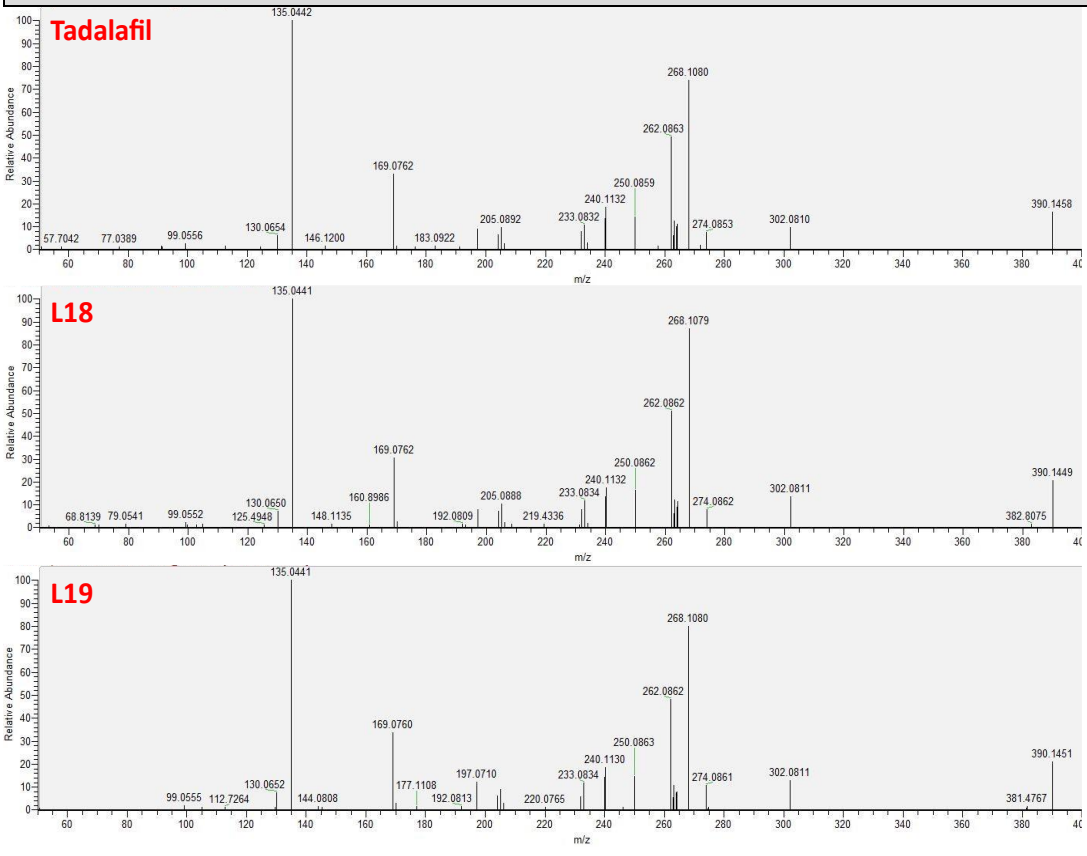


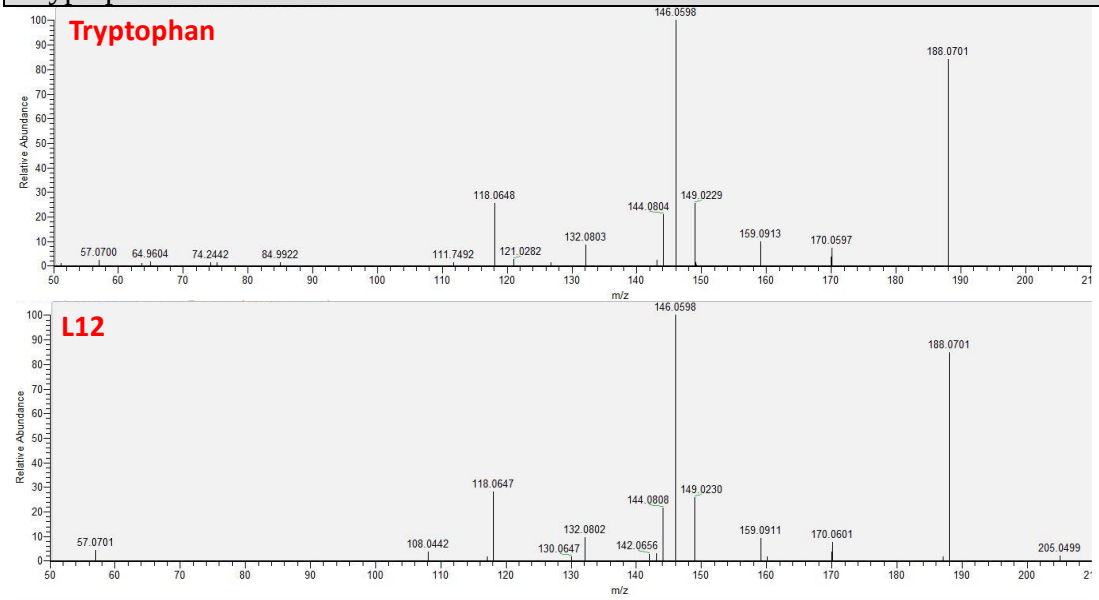
## Sildenafil




## Tadalafil

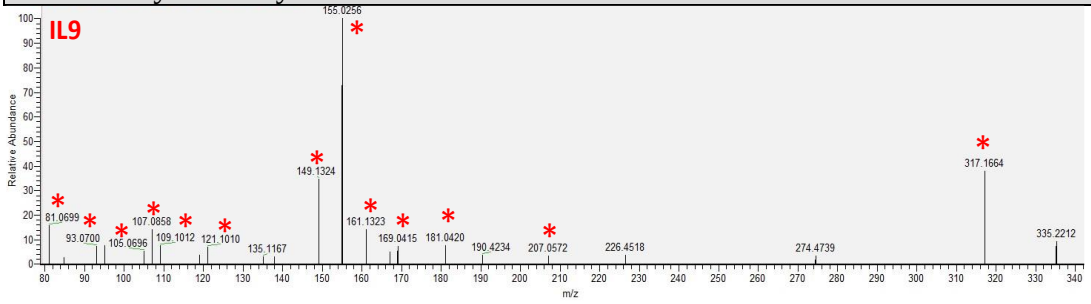


## Tryptophan

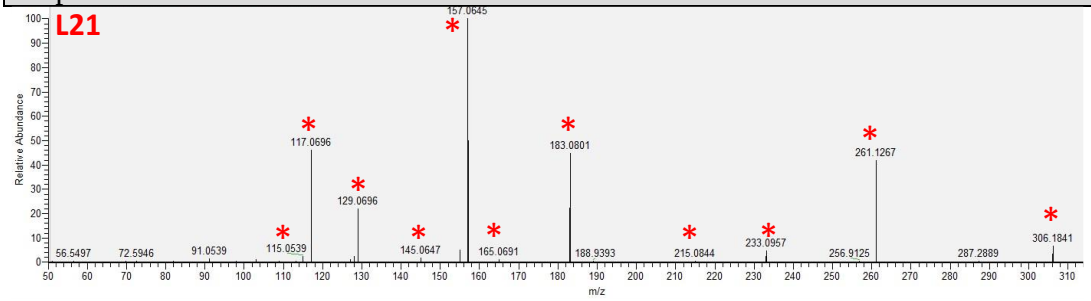



# Fragmentation patterns (confidence level 2)

## Chlorodehydromethyltestosterone




## Dapoxetine



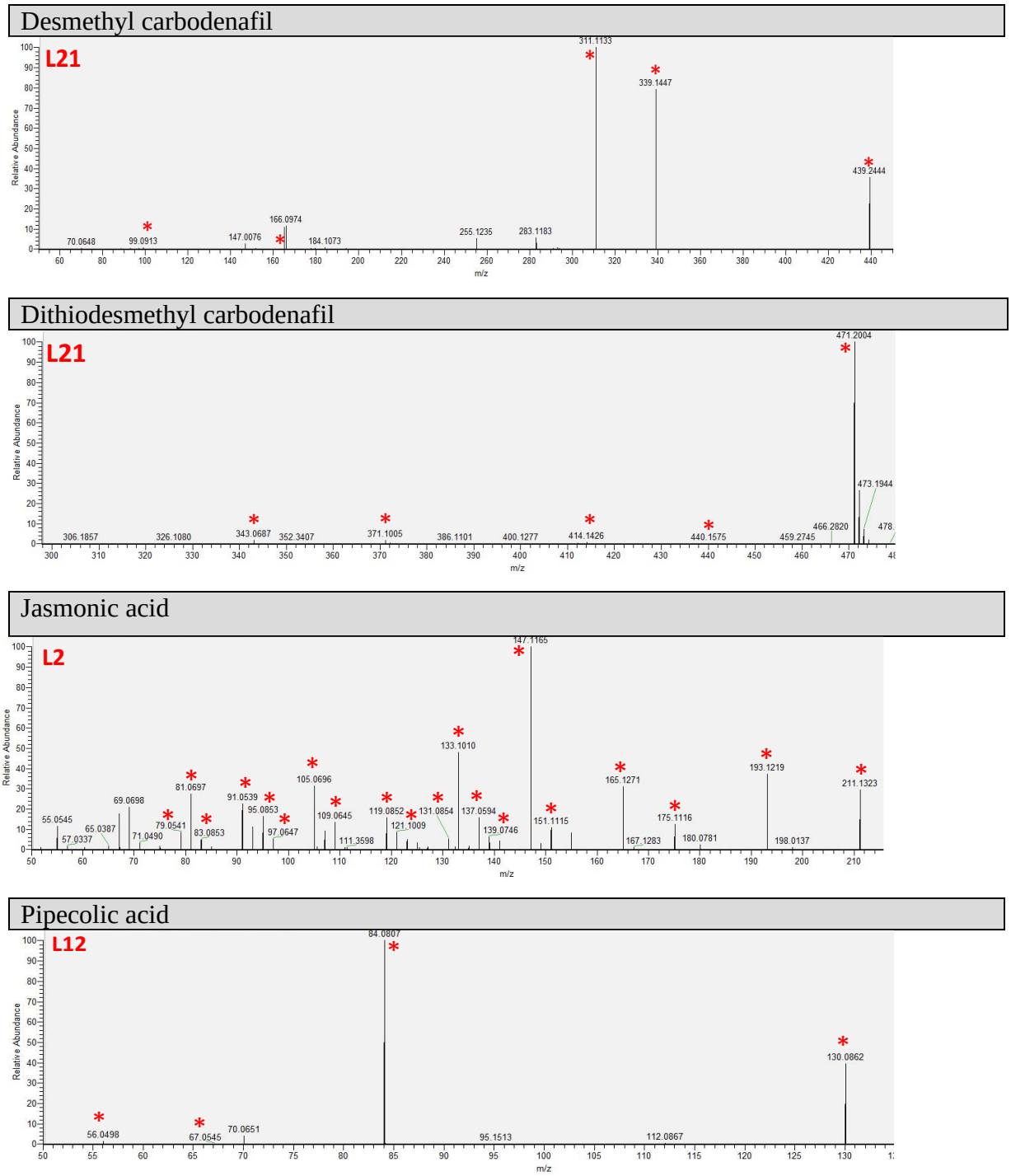



Figure A1. Contaminants fragmentation pattern in references standards and samples. Fragments labeled with a red asterisk on the spectrum were compared with either experimental data available in mass spectra libraries or the literature (if we did not have a reference standard for substances potentially harmful to the organism).

Table A1. Basic information on the analyzed legal and illegal dietary supplements.

| Code | Type of dietary supplement | Dosage Form | Source / Country of producing | Place of purchase / Source in the Polish market | Recommended for consumer |
|---|---|---|---|---|---|
| **L1** | Vitamins, minerals | Tablet | Poland | Pharmacy | A comprehensive set of vitamins and minerals for mature people over 50 |
| **L2** | Vitamins, minerals | Tablet | France | Online pharmacy | Intended for use by physically active people and those suffering from chronic fatigue and exhaustion |
| **L3** | Vitamin C | Capsule | Poland | Online pharmacy | Helps in maintaining the proper functioning of the immune system |
| **L4** | Vitamin C | Tablet | Poland | Online pharmacy | Recommended that adults supplement the normal diet with vitamin C |
| **L5** | Plant-based (Nasturtium herb extract, Horseradish root extract, Elderberry flower extract, Verbena herb extract, Gentian root extract, Mullein flower extract) | Tablet | Czech republic | Pharmacy | Supports the respiratory system and immunity |
| **L6** | Glucosamine sulfate, hydrolyzed collagen, chondroitin sulfate, hyaluronic acid | Capsule | Poland | Pharmacy | Supports the mobility and flexibility of joints |
| **L7** | Citicoline | Tablet | Poland | Pharmacy | Supports the nervous system. |
| **L8** | Vitamin, mineral, Korean ginseng extract | Capsule | Poland | Online pharmacy | Intended for adults who exercise recreationally and competitively, practicing strength and endurance sports, e.g. combat sports. |

| | | | | | |
|---|---|---|---|---|---|
| **L9** | Vitamins | Tablet | Germany | Drugstore | Preparation containing vitamins |
| **L10** | Vitamin $B_{12}$ | Tablet | Sweden | Pharmacy | Preparation supplements the diet with vitamin $B_{12}$ |
| **L11** | Glucosamine sulfate, type II collagen | Capsule | Poland | Pharmacy | Supports the articular cartilage |
| **L12** | Plant-based (Hop cone extract, Lemon balm herb extract, Cultivated crocus stigma extract), vitamin | Tablet | Poland | Pharmacy | Supports good sleep |
| **L13** | L-histidine | Capsule | United States | Online pharmacy | ----- |
| **L14** | Amino acids, vitamins | Capsule | Poland | Online pharmacy | Helps to remove fatigue caused by prolonged physical effort, supports concentration and speed of thinking |
| **L15** | Amino acid, vitamins, plant-based (Ginseng extract, Guarana extract) | Capsule | France | Online Pharmacy | Contributes to the reduction of fatigue |
| **L16** | Amino acid, plant-based (Green tea extract, Bitter orange extract, Guarana extract, Black pepper extract) | Capsule | Poland | Online Pharmacy | Recommended for adults, especially for physically active people and sportsmen |
| **L17** | L-carnitine | Tablet | Poland | Pharmacy | For adults seeking to reduce body fat, slimming |
| **L18** | Herbal (Ginseng root extract), minerals | Tablet | United Kingdom | Online e-commercial platform | Erectile dysfunction |
| **L19** | Herbal (Ginseng root extract), minerals | Tablet | United Kingdom | Online e-commercial platform | Erectile dysfunction |
| **L20** | Herbal (Ginseng root extract), minerals | Tablet | United Kingdom | Online e-commercial platform | Erectile dysfunction |

| | | | | | |
|---|---|---|---|---|---|
| **L21** | plant-based (Rhodiola root, Oat stalks, Fenugreek seeds, Ginseng root, Sarsaparilla root), minerals | Capsule | United States | Online e-commercial platform | Erectile dysfunction |
| **IL1** | Plant-based | Capsule | China | Illegal factory | Slimming aid. Reduces the activity of the fat enzyme in the alimentary duct. It prevents the absorption of food fat, Increases the effectiveness of fat metabolism and the BMR. The product removes fat and toxin. |
| **IL2** | Caffeine, plant-based | Capsule | United States | Illegal factory | Fat loss, aids in appetite suppression |
| **IL3** | Yohimbine, caffeine | Capsule | United Kingdom | Illegal factory | Fat burner |
| **IL4** | Plant-based, 1,3-dimethylamine (1,3-DMAA), yohimbine, caffeine | Capsule | Cyprus | Illegal factory | Weight loss and energy booster |
| **IL5** | Plant-based, caffeine anhydrous | Capsule | United States | Illegal factory | Fat burner, metabolic incinerator |
| **IL6** | Plant-based, caffeine | Capsule | United States | Illegal factory | Energy, weight loss |
| **IL7** | Yohimbine, caffeine | Capsule | United Kingdom | Illegal factory | Fat burner |
| **IL8** | MK 2866 | Capsule | India | Illegal factory | No label |
| **IL9** | 13-ethyl-3-methoxy-gona-2,5(10)-diene-17-one (Max LMG), Halodrol, Carbopol, plant-based | Capsule | Singapore | Illegal factory | Rapid weight loss increases exercise, capacity and prevents the accumulation of adipose tissue |

## 3.5 Publikacja 5 (oryginalna)

**Stępień, K.A**.; Myslitska, D.; Garbacz, G.; Paszkowska, J.; Giebułtowicz, J. *Pharmaceutical availability testing – can a drug-specific test be relevant for assessing the quality of dietary supplements?;* Microchemical Journal; 2024; 112132.

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# Pharmaceutical availability testing – can a drug-specific test be relevant for assessing the quality of dietary supplements?

Krzysztof Adam Stępień [a], Daria Myslitska [b], Grzegorz Garbacz [b,c], Jadwiga Paszkowska [b], Joanna Giebułtowicz [a,*]

[a] Department of Drug Chemistry, Pharmaceutical and Biomedical Analysis, Faculty of Pharmacy, Medical University of Warsaw, 02-097 Warsaw, Poland
[b] Physiolution Polska, 74 Piłsudskiego St., 50-020 Wroclaw, Poland
[c] Physiolution GmbH, 49a Walther-Rathenau-Straße, 17489 Greifswald, Germany

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Release profile

ABSTRACT

We investigated the dissolution performance of selected dietary supplements using standard pharmacopoeial methods and biopredictive tools (PhysioCell). We tested commercially available tablets and capsules containing proline, tryptophan, tyrosine, carnitine, and vitamin C that were obtained from the EU, Switzerland, and the USA.

The results indicate striking differences among the investigated products that are considered exchangeable. The release levels of some formulations were low in pharmacopoeial dissolution tests. The implementation of PhysioCell enabled a more comprehensive understanding of the formulation's behavior within the gastrointestinal (GI) tract. The simulation of GI motility using the PhysioCell apparatus led to enhanced release levels, especially for capsules (from 11 to 106 percentage points). It indicates that the dissolution of the tested formulations can be influenced by the GI motility which may contribute to high variability of the drug delivery performance *in vivo*.

It becomes obvious that the implementation of well-defined and consistent dissolution methods for dietary supplements could improve their reliability and biopharmaceutical quality.

## 1. Introduction

Dissolution represents a crucial parameter of oral drugs, focusing on assessing the release of active substances from dosage forms such as tablets and capsules [1]. These studies are essential for ensuring precise drug dosages, minimizing treatment inefficiency, and preventing potential toxicities [2]. Moreover, these investigations optimize dosage form design by considering the physicochemical properties of the substance for effective release and absorption of active substances, thereby enhancing therapeutic outcomes [3]. The effective release of active substances is pivotal for achieving desired therapeutic outcomes [4].

Solid dosage forms like tablets and capsules require efficient disintegration for optimal release. Without this process, delayed absorption occurs, impacting therapeutic outcomes. Formulation intricacies, such as excipient selection and compression forces, are critical to avoid lackluster drug release [5]. Coated or encapsulated forms may encounter hindrances if the shell is overly thick, hindering interaction with gastrointestinal fluids. Controlled-release formulations might not disintegrate or release as intended, undermining their time-dependent efficacy [6].

Dissolution testing, a formal examination utilized by pharmacopoeias, evaluates the release of drugs from solid and semisolid dosage forms. This fundamental technique involves introducing a dosage form into a dissolution apparatus with a physiologically relevant medium to precisely measure the drug release over time [7]. Pharmacopoeial methods are highly standardized, cost-effective, and easily executable means. Nonetheless, their limitation lies in their limited ability to accurately mimic the conditions within the human body. Recently, much effort has been put into developing dissolution devices that can more accurately replicate the conditions prevailing within gastrointestinal tract compared to pharmacopeial methods [8,9]. One example of such devices is PhysioCell that can simulate physiological factors such as pH changes, variable fluid flow, temperature gradient and mechanical agitation [10]. The latter is applied as pressure waves exerted on a solid dosage form, which often affect the degree of tablet/capsule disintegration. The PhysioCell was successfully implemented as a tool for

* Corresponding author.
*E-mail address:* joanna.giebultowicz@wum.edu.pl (J. Giebułtowicz).

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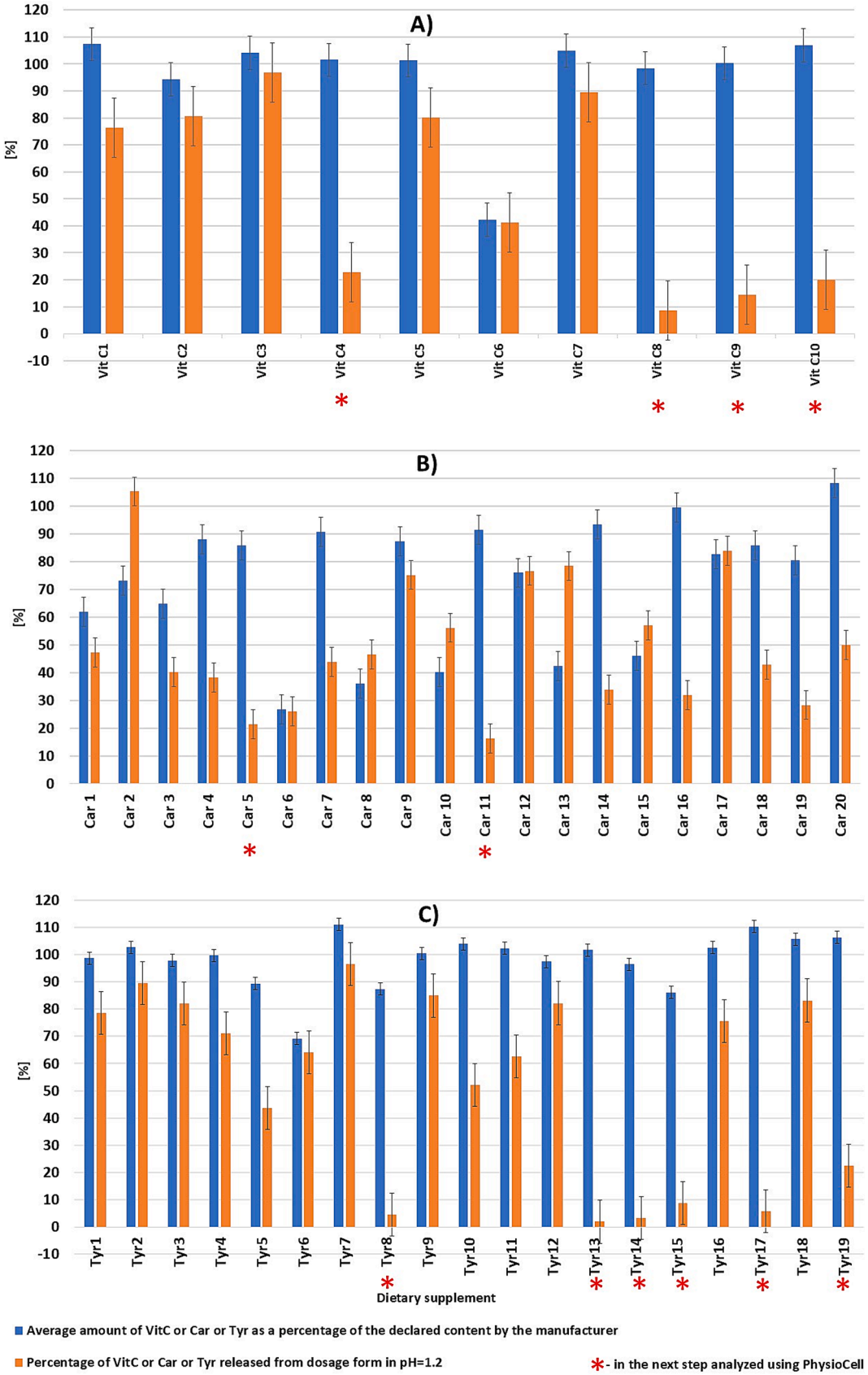


**Fig. 1.** Comparison of the results of dissolution test (pharmacopeial conditions) in pH 1.2 (simulated gastric conditions) with the content analysis for dietary supplements containing A) vitamin C, B) carnitine and C) tyrosine. The error bars represent the standard error of the mean (SEM).

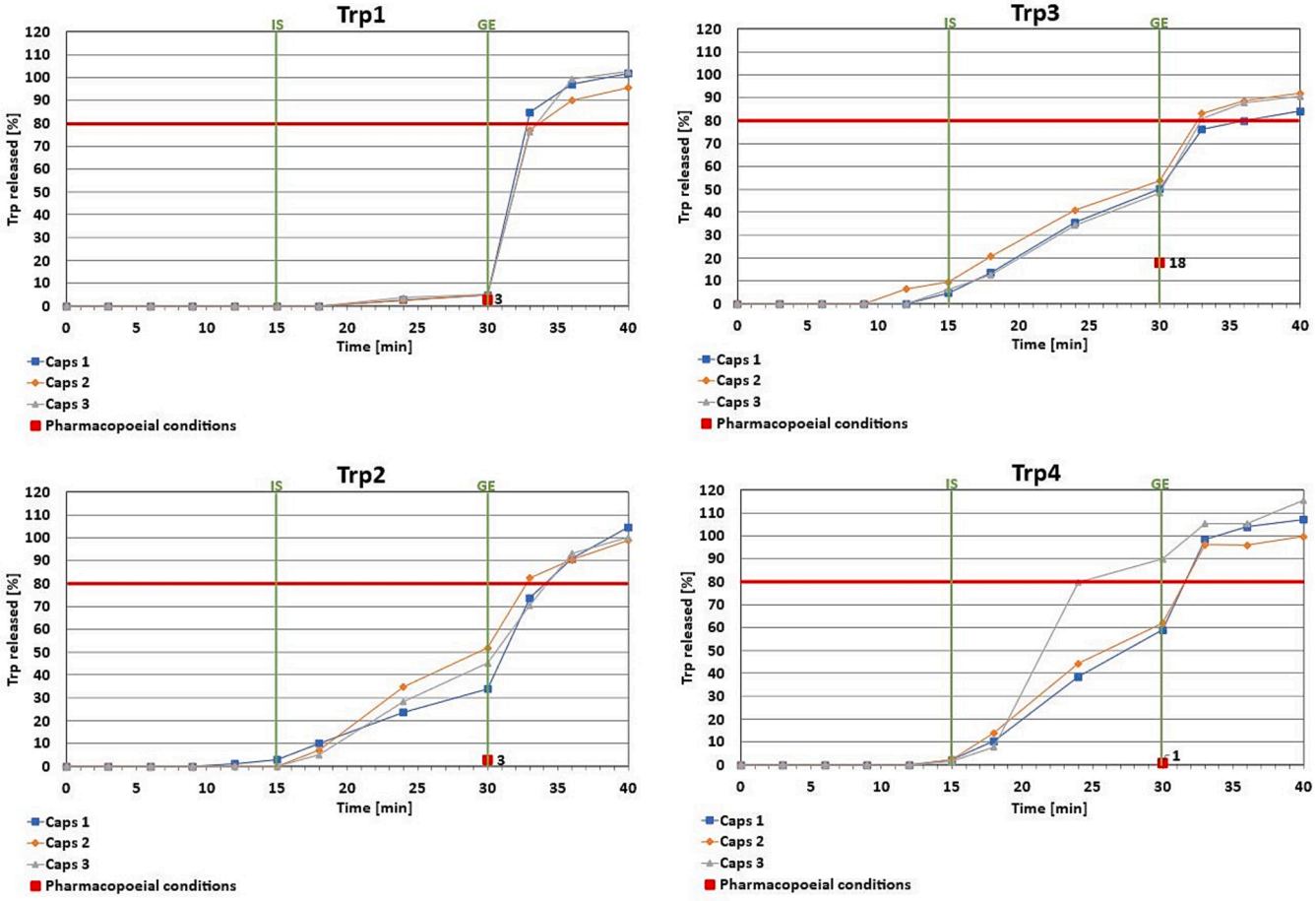


**Fig. 2.** Release profiles of tested dietary supplements under biorelevant conditions, with average percent release under pharmacopeial conditions indicated; (IS - intragastric stress – 100 mbar, GE - gastric emptying – 300 mbar).

biopredictive dissolution testing of immediate release products [9].

Though classified as food, dietary supplements often come in the form of tablets and capsules. Due to their resemblance to pharmaceuticals, consumers often mistakenly assume that dietary supplements undergo rigorous safety and efficacy evaluations prior to being marketed. However, due to the absence of mandatory quality testing, there is a scarcity of data available regarding the quality of these supplements [11]. When data does become available, it frequently reveals various issues, including the presence of contaminants, discrepancies between the declared content and the actual content of the primary ingredient, or suboptimal release rates from the supplement formulations [12].

Notably, inadequate release of active ingredients, such as tryptophan (Trp) [13], proline (Pro) [14], calcium carbonate [15], melatonin [16], folic acid [17], iron, zinc, manganese [18], and Grape seed extract [19], has also been observed in solid-form dietary supplements. Potential factors contributing to low release may include insufficient primary ingredient content, improperly selected process parameters, and/or poorly chosen excipients. The most common issue with supplements, which are relatively inexpensive, is the inadequate release of the active ingredient. In this case, there is typically no evidence of falsification in terms of content or the addition of undisclosed active substances with pharmacological activity. However, data on this topic is still limited.

In the present work, we assessed the content and specifically the release amount of the active component, of supplements (manufactured in the EU, Switzerland and USA) containing tyrosine (Tyr, $n = 19$), carnitine (Car, $n = 20$), and vitamin C (VitC, $n = 10$) using standard pharmacopoeial methods. We re-analyzed the samples with the lowest dissolution level (less than 25 %) using PhysioCell as the reference method, considering the impact of gastrointestinal motility on solid dosage form dissolution [10]. In addition to our current study, we incorporated six supplements containing Trp and one with Pro, whose release level was found to be lower than 25 % in our previous research [13,14]. Our primary objective was to investigate whether pharmacopoeial methods for testing dosage forms can effectively identify low-quality dietary supplements based on the release of active substances, and to determine if apparatuses such as the PhysioCell can provide new insights into supplement quality. Notably, the dissolution test has not been previously performed on dietary supplements using the PhysioCell apparatus. Additionally, there is a lack of literature reporting the application of pharmacopoeial methods in conjunction with the PhysioCell apparatus for the quality assessment of dietary supplements.

## 2. Materials and methods

### 2.1. Samples

The study included 56 dietary supplements containing different active compounds: Trp (Trp1-Trp6), Tyr (Tyr1-Tyr19), Car (Car1-Car20), VitC (VitC1-VitC10) and Pro (Pro1). The supplements came in two dosage forms: capsules ($n = 40$) and tablets ($n = 16$). These products are registered in Poland and widely available on the market. They were purchased from Polish pharmacies, online pharmacies or an online e-commerce platform. The origin of the supplements includes the EU (Belgium, Czech Republic, France, Germany, Poland), Switzerland, UK

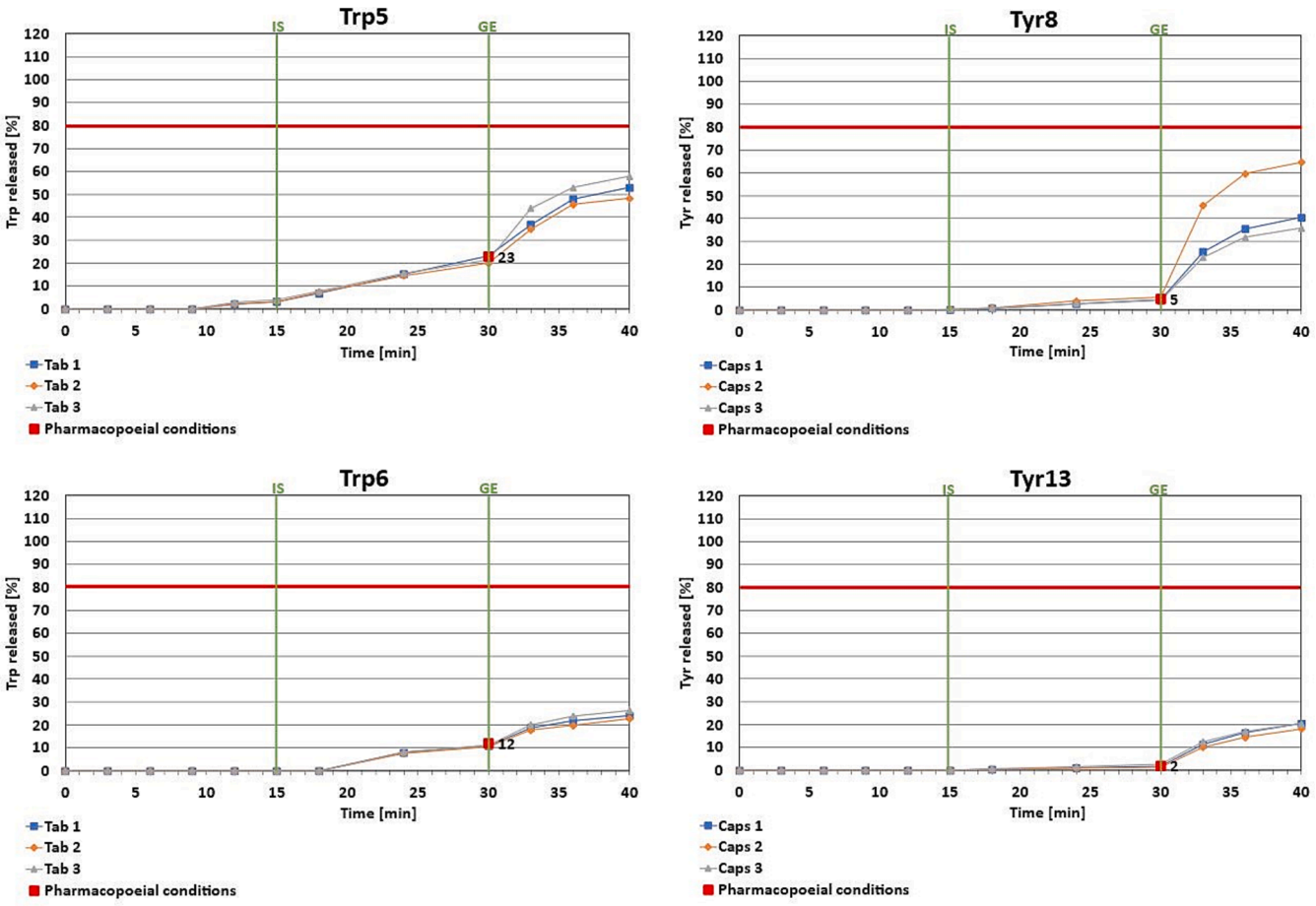


**Fig. 2.** (*continued*).

and the USA. Dietary supplements containing Trp and Pro, which exhibited a release rate lower than 25 % after 30 min (using a dissolution medium of 0.1 mol/L hydrochloric acid), were selected based on our previous examinations [13,14]. They were produced in: France (Trp1, dose 220 mg, release 2.65 %), Germany (Trp3, dose 50 mg, release 17.9 % and Pro1, dose 400 mg, release 11.0 %), Poland (Trp5, dose 167 mg, release 22.8 % and Trp6, dose 50 mg, release 12.1 %) and USA (Trp2, dose 500 mg, release 3.08 % and Trp4, dose 500 mg, release 1.22 %).

### 2.2. Reagents

L-Trp (≥99 %) (standard), L-Tyr (≥99 %) (standard) and VitC (≥99 %) (standard) were purchased from Merck (Darmstadt, Germany). L-Car (98 %) (standard) and L-Pro (≥98 %) (standard) were purchased from LGC (Luckenwalde, Germany). Hydrochloric acid (35–38 %) solution pure p.a., sodium hydroxide (≥98.8 %) pure p.a., potassium phosphate monobasic (≥99.5 %) pure p.a. and ammonium acetate (≥97 %) pure p. a were purchased from Chempur (Piekary Śląskie, Poland). HPLC-grade methanol, acetonitrile, acetic acid and formic acid were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Millipore water purification system (Milli-Q water).

### 2.3. Sample preparation

Three tablets or capsules were randomly selected from each supplement. The total weight of three tablets or three content of capsules were determined. For tablets, a grinding step was applied. In the next step, the tablet's mass or capsule content equivalent to 10 mg Trp, Tyr, Car, Pro or VitC was weighed, and 1 mL of acetonitrile/methanol/water (1:1:1; *v/v/v*) mixture for Trp and Pro, 1 mL of water for Car, 1 mL of hydrochloric acid (1 mol/L) for Tyr, or 1 mL of hydrochloric acid (0.1 mol/L) for VitC was added. The mixture was sonicated for 15 min and centrifuged (10 000g, 5 min). The supernatant was then diluted with the mobile phase to a final concentration of 1 mg / mL (for VitC) or 1 μg / mL (for other analytes).

### 2.4. Determination of the Car, Pro, Trp, Tyr and VitC content

The instrumental analysis for Car, Pro, Trp, Tyr were performed using an Agilent 1260 Infinity (Agilent Technologies, Santa Clara, CA, USA), equipped with a degasser, autosampler, and binary pump coupled to a QTRAP 4000 hybrid triple quadrupole/linear ion trap mass spectrometer (AB Sciex, Framingham, MA, USA). The turbo ion-spray source was operated in positive mode. The operating parameters of the apparatus are presented in Table A1. The target compounds were analyzed in multiple reaction monitoring (MRM) mode. The method parameters are presented in Table A2. VitC was determined using a high-performance liquid chromatography (HPLC) with UV–Vis detection (Varian, Middelburg, Netherlands). The chromatographic separation parameters for individual analytes are presented in Table A3. The re-analysis of Tyr, Trp and VitC after the biorelevant dissolution tests was performed using a HPLC with UV–Vis detection (Shimadzu, Kyoto, Japan). The chromatographic separation parameters for individual compounds are presented in Table A4. Calculations were made using the Analyst 1.6.3 software (AB Sciex, Framingham, MA, USA). In the case of Vit C calculations were performed using MS Excel.

The method was validated for selectivity, linearity, precision and accuracy. The limit of quantitation (LOQ) was determined as the lowest concentration of an analyte with a signal-to-noise ratio of 10:1. Calibration curved ($n = 3$) were constructed by plotting the analyte peak

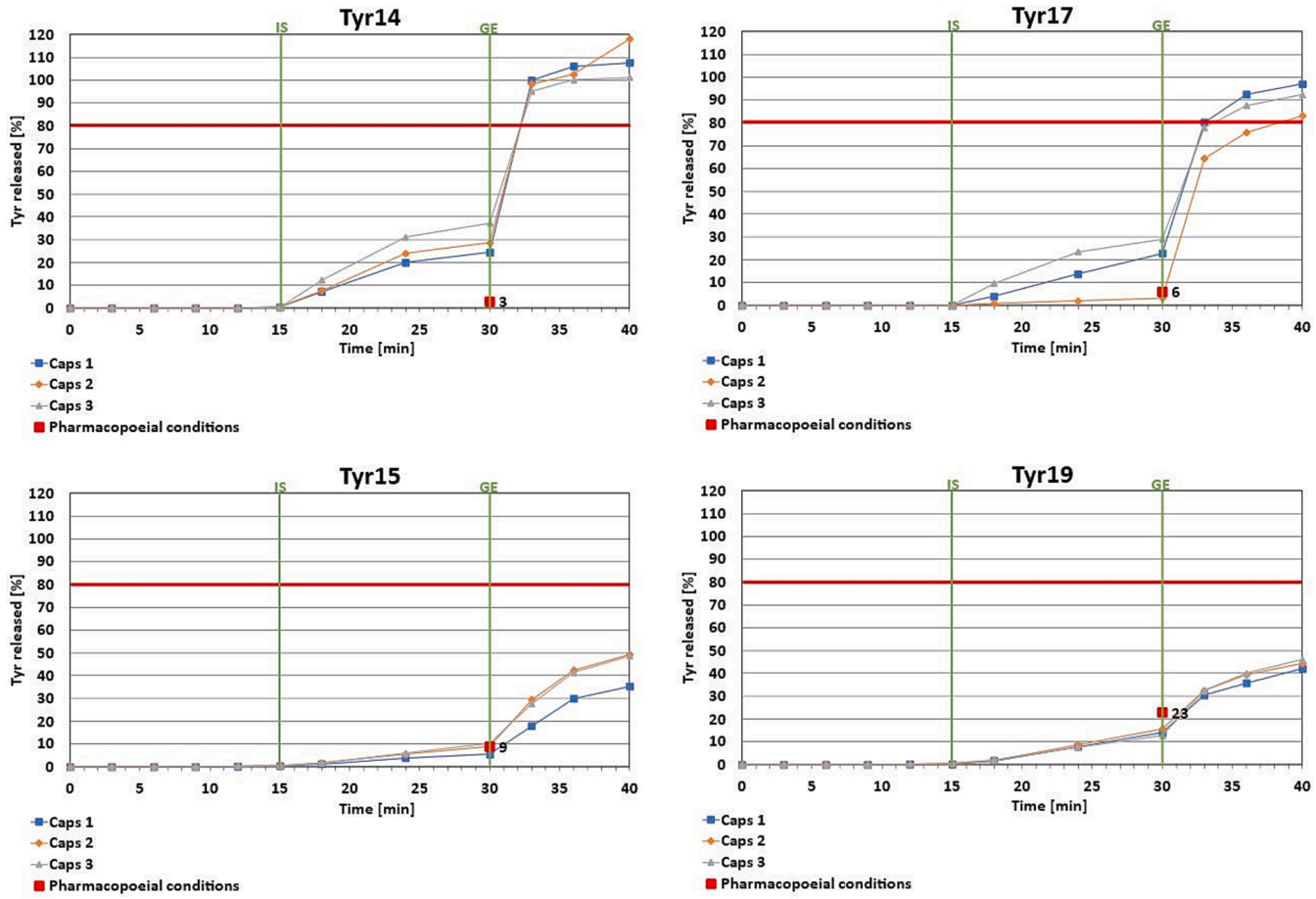


**Fig. 2.** (*continued*).

area against the analyte nominal concentration. Precision and accuracy were assessed by analyzing nine replicates of quality control samples at three concentration levels (at the lower end, at the midpoint, and at the upper end of the calibration curve) over three consecutive days. Accuracy was determined by comparing calculated values to nominal concentrations, while precision was expressed as the coefficient of variation for the calculated concentrations. Selected validation results are presented in Table A5.

### 2.5. Dissolution test for tablets or capsules according to the Pharmacopoeia

Trp, Tyr, Car, Pro or VitC release studies were performed using a USP II Varian VK 7025 or USP I Varian VK 7025 dissolution tester (Erweka GmbH, Heusenstamm, Germany) for tablets and capsules, respectively. Six tablets or capsules were randomly selected and individually placed in the dissolution vessels. Each vessel contained 900 mL of dissolution medium. The stirring speed of 50 rpm or 100 rpm was used for tablets and capsules, respectively. The temperature was set at 37 ± 0.5 °C. Aliquots (1.5 mL) of the medium were manually collected using 5 mL syringes after 30 min of the test and filtered through a Millex-HA 0.45 μm filter. Each aliquot withdrawn was replaced with 1.5 mL of fresh medium. The experiment was performed both in 0.1 mol/L hydrochloric acid (simulated gastric conditions) and 0.05 mol/L phosphate buffer (pH 6.8, simulated intestinal conditions). The amount of dissolved active compound was determined as described in Section 2.4.

### 2.6. Dissolution test simulating biorelevant conditions

The biorelevant dissolution tests were performed in the PhysioCell apparatus in a closed-loop configuration consisting of a StressCell connected to a Collection Vessel. The dietary supplement dissolution was tested in triplicate in total 900 mL of HCl pH 1.2 in temperature and flow rate gradients. At the beginning of the test the Stress Cell was filled with the medium at the rate of 50 mL/min, which gradually decreased to 8 mL/min within 15 min. The temperature of the dissolution medium increased from room temperature to 37 ± 0.5 °C within the first 15 min of the experiment. Pressure waves were applied at 15 min (100 mbar) and 30 min (3 x 300 mbar) to simulate the intragastric stress (IS) and gastric emptying (GE) events, respectively. The samples were manually collected from Collection Vessel at the 3, 6, 9, 12, 15, 18, 24, 30, 36 and 40 min using 5 ml syringe with a cannula and a prefilter (Cannula Filters, 1 μm, UHMW PE, Dissolution accessories). Then, without delay, the samples were filtered through a syringe filter (Minisart® RC 0.22 μm, Sartorius, Goettingen, Germany) into a vial. The concentration of the main ingredient was measured without dilution using the HPLC method as described in Section 2.4.

### 2.7. Expanded uncertainty

To assess whether the amount of analysed ingredient (Tyr, Car, VitC) in the dosage unit and amount of the compound released is equal within the uncertainty range, extended uncertainty was determined using Eq. (1).

$$U(x_1 - x_2) = 2\sqrt{[u(x_1)]^2 + [u(x_2)]^2} \quad (1)$$

The measurement results were equal if:

$$|x_1 - x_2| < U(x_1 - x_2)$$

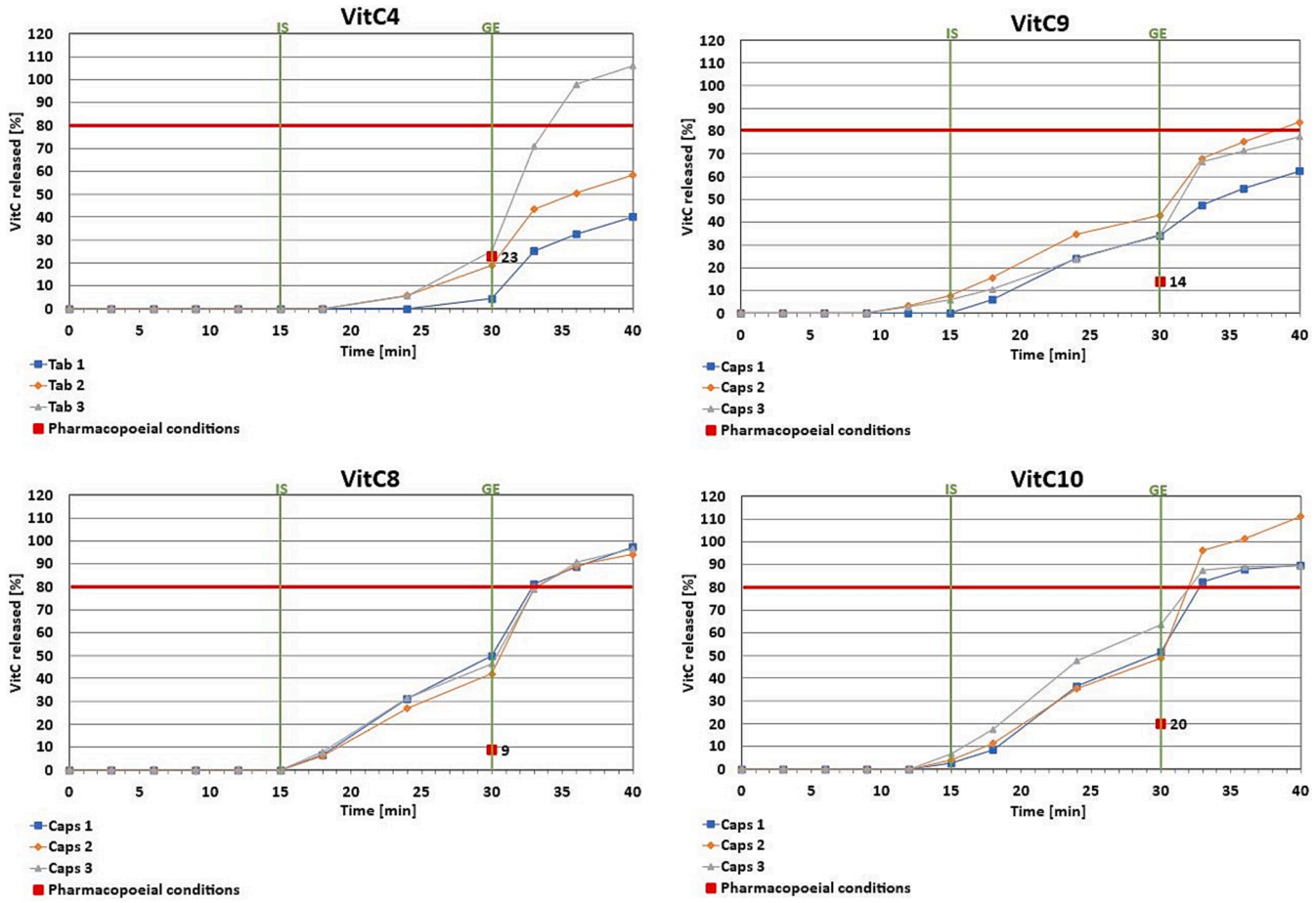


**Fig. 2.** (*continued*).

$x_1$– mean [mg] analysed ingredient (Tyr, Car, VitC) content determined in dosage unit using quantitative analysis ($n = 3$)

$x_2$– mean [mg] amount of analysed ingredient (Tyr, Car, VitC) released from six dosage units.

$u(x_2), u(x_1)$- standard uncertainties of the measured values: $x_1$ and $x_2$ determined according to the formula:

$$u(x_1) = \frac{S}{\sqrt{n}}$$

$S$- standard deviation of the average amount of analysed ingredient (Tyr, Car, VitC) in dosage unit [mg] or standard deviation of the released amount of analysed ingredient (Tyr, Car, VitC) [mg]

$n$ – the number of tablets or capsules analysed.

## 3. Results

### 3.1. Determination of the Car, Pro, Trp, Tyr and VitC content

Based on the guidelines provided in the Polish Pharmacopoeia VI for pharmaceuticals, the acceptable content range of the active substance in tablets or capsules is outlined as follows:

For units with a declared active substance content below 100 mg: The acceptable range is ± 10 %. This indicates that the actual content of the active substance in each tablet or capsule should not deviate more than 10 % from the amount stated on the label.

For units with a declared active substance content of 100 mg and above: The acceptable range is ± 5 %. In this case, the actual content of the active substance in each tablet or capsule should not deviate more than 5 % from the amount stated on the label.

These specific criteria are designated for pharmaceuticals. However, in this study, we applied the same criteria to dietary supplements due to the absence of specific guidelines for this category, considering their similar form to drugs (tablets or capsules).

Our analysis covered a range of dietary supplements, all of which, as indicated by the manufacturer's declaration, contained above 100 mg of Tyr, Car, and VitC. For supplements containing Tyr, we noted that the lowest average Tyr content was 69.1 % (Tyr6, CV = 1.7 %), while the highest reached 111.0 % (Tyr7, CV = 5.6 %) of the declared amount. Out of the 19 tested dietary supplements, 17 failed to meet the criteria outlined in this study for the main ingredient's content (i.e., falling outside the range of 95–105 % for all examined tablets/capsules). In the case of Car, none of the tested dietary supplements met the criteria outlined in this study for the main ingredient's content. The lowest average Car content was 27.7 % (Car6, CV = 44 %), while the highest reached 108.4 % (Car20, CV = 13 %) of the declared amount. With respect to dietary supplements containing VitC, 6 out of 10 tested dietary supplements did not meet the criteria established in this study for the main ingredient's content. The lowest average VitC content was 42.3 % (VitC6, CV = 7.4 %), and the highest was 107.3 % (VitC1, CV = 4.3 %) of the declared amount. In the case of 5 out of 20 and 1 out of 10 analysed supplements, the Car or VitC content respectively was below 50 %. Fig. 1 presents the mean ($n = 3$) measured content of Car, Tyr, and VitC in the tested tablets and capsules, expressed as a percentage of the declared content by the manufacturer, along with the average release percentage ($n = 6$) of these substances. The high deviation from the mean is attributed to significant variations in the active ingredient

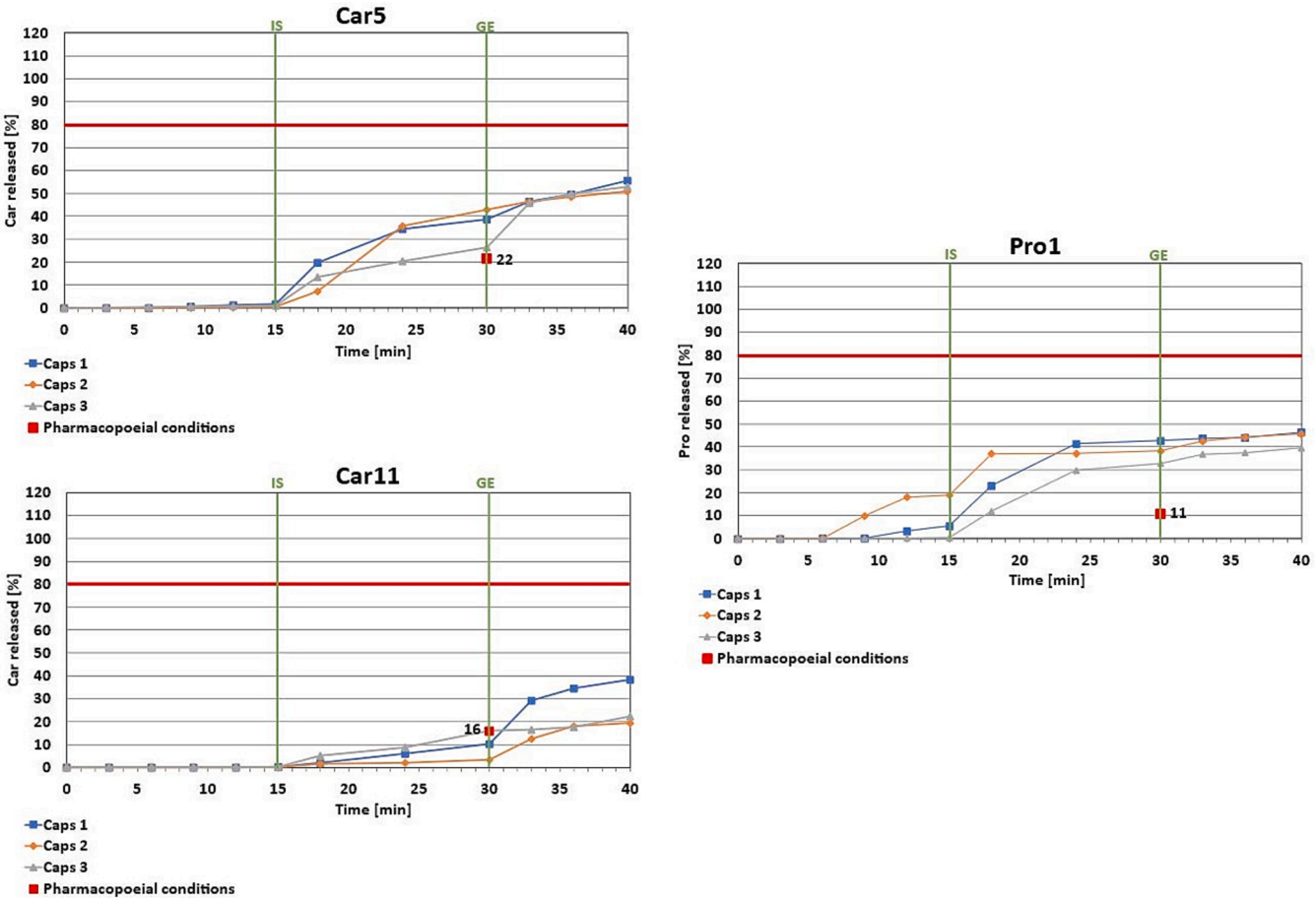


**Fig. 2.** (*continued*).

content among tablets of the same dietary supplement. This variability can be caused by several factors, including improper particle distribution (e.g., agglomeration), inadequate macro- and microblending during the powder mixing phase or loss of components (e.g., due to adsorption onto equipment surfaces).

Results of quantitative analysis of Car, Tyr and VitC in dietary supplements are also presented in Table A6. Dietary supplements with Trp or Pro also did not met the criteria set as previously shown [13,14].

The outcomes of this study provide valuable insights into the compliance of dietary supplements with the stipulated content criteria, aligning with pharmaceutical standards, and contribute to our understanding of the quality and consistency of these products in the market.

### 3.2. Dissolution test

The release test was performed in two steps: A) using pharmacopoeial guidelines and B) simulating biorelevant conditions. The results of the dissolution test using pharmacopoeial methods for Car, Tyr and VitC are presented in Table A7. The average release of Car, Tyr and VitC ranged from 16.4-105.3 %, 1.94–96.6 % and 8.6–96.8 %, respectively. It should be noted that some supplements were characterized by low substance release, i.e. below 25 %. They were six dietary supplements in the form of capsules containing Tyr, six dietary supplements containing Trp (four in capsule form, two in tablet form), two capsule-form supplements containing Car, one capsule-form supplement containing Pro, and four supplements containing VitC (three in capsule form, one in tablet form). In summary, considering the various forms in which they were presented, a total of 16 capsule-form supplements and 3 tablet-form supplements were evaluated. If the main ingredient release of dietary supplements fell below 25 % in pharmacopoeial test, a dissolution test simulating biorelevant conditions was performed. Given the physiological characteristics of the stomach, the application of mechanical stress on the drug formulation during the release test proceeded as follows. No additional pressure waves were applied during 0–15 min, 1 x 100 mbar was applied in the 15th minute (simulation of IS), and 3 x 300 mbar were applied in the 30th minute of the test (simulation of GE). The acquired results are highly intriguing and are outlined below, presented in the form of release profiles illustrated in Fig. 2.

Generally, the omission of the pressure factor led to release outcomes that were either lower or equal to those achieved under pharmacopoeial conditions, irrespective of whether the supplement was in the form of capsules or tablets. The application of IS in the context of dietary supplements in tablet form yielded release percentage values comparable to those obtained through analysis under pharmacopoeial conditions (the difference was no more than 7 percentage points). Results indicated that for tablet-form supplements, releases were low in both pharmacopoeial dissolution tests (Trp5 – 23 %; Trp6 – 12 %; VitC4 – 23 %) and biorelevant conditions (Trp5 – 53 %; Trp6 – 24 %; VitC4 – 68 %). In the case of dietary supplements in capsules i.e. Trp1, Car11, Tyr8, Tyr13,Tyr 15 and Tyr19, the application of IS, similar release (the difference was no more than 9 percentage points) were obtained, while for supplements like Tyr2, Tyr3, Tyr4, Car5, Tyr14, Tyr17, VitC8, VitC9, VitC10, and Pro1, higher values (from 12 to 69 percentage points) were recorded compared to those achieved using pharmacopoeial conditions. The simulation of GE resulted in increased (from 6 to 95 percentage points) release of substances for all examined dietary supplements. In eight out of sixteen tests of capsule-form supplements, a release of over 80 % was observed, with five formulations exceeding 95 %. Conversely, in the remaining eight out of sixteen tested supplements, the release was below 80 % under the applied conditions, with average values observed for

Car5 (53 %), Car11 (27 %), Tyr8 (50 %), Tyr13 (20 %), Tyr15 (44 %), Tyr19 (44 %), VitC9 (74 %), and Pro1 (44 %). Section S.3.2. of the manuscript provides comprehensive details on the dietary supplements tested, categorized by their dosage forms.

## 4. Discussion

The application of pharmacopoeial conditions for release testing enables the assessment of the quality of dietary supplements, as concluded from the analysis of the results. We were able to detect dietary supplements characterized by low (below 80 %) or variable and non-reproducible release of the active compound, indicating their suboptimal quality. Low substance release represents a technological challenge and can result from various factors, including inadequate dosage form disintegration stemming from improper tablet/capsule manufacturing, suboptimal excipient or production parameter selection. Pharmacopoeial methods serve as valuable tools for rapid quality assurance assessment. However, they are designed with specific assumptions, such as their simplicity, which limit their ability to account for certain factors occurring during the passage of a formulation through the gastrointestinal tract. For instance, the pressure exerted on the tablet/capsule in the stomach, a critical factor affecting substance release, is not taken into consideration within these methods. As a result, certain dietary supplements may demonstrate low release based on pharmacopeial methods, whereas *in vivo*, the substance is released to a greater extent. This thesis was confirmed in our research. Applying conditions similar to physiological ones, such as pressure (using the PhysioCell apparatus), sometimes led to the disintegration or accelerated disintegration of the dosage form compared to the pharmacopeial method.

PhysioCell device is used in dissolution test simulating biorelevant conditions to mimic physical conditions during oral administration through the gastrointestinal tract. The primary factor in this device is pressure, simulating the forces exerted by the gastric or intestinal wall's motility, shear forces during propagation, and loss of contact with water when the dosage form is in an intestinal air pocket [10]. Employing conditions that mimic the body's environment during release testing is crucial due to the stomach's unique physiology. The stomach constitutes a highly variable system, and its internal parameters are subject to numerous stimuli, primarily related to the properties of the ingested food and the body's reactions. Mechanical tensions in the fasting stomach are generated by cyclic contractions, forming part of the migrating motor complex (MMC). Each cycle consists of four phases. In the first phase of the cycle, the stomach exhibits the lowest activity, and its smooth muscles remain relaxed, lasting for 45 to 60 min. In the second phase of the MMC, lasting for 30 min, stomach contractions begin, inducing pressure up to 150 mbar [20], with an increasing frequency; peristalsis spreads from the stomach and propagates along the small intestine. The subsequent phase is characterized by the highest intensity. Strong stomach contractions occur during this period, aimed at removing food residues and lingering fluid. It is during this phase that tablets, ingested on an empty stomach and remaining undissolved, are typically expelled. Contractions in this phase can generate pressures of around 460–500 mbar, representing the highest pressures in the gastrointestinal tract and often leading to tablet disintegration. The final phase of the MMC involves the gradual fading of contractions and transition to the first phase of the next cycle [21–23]. This suggests that the intragastric stresses experienced on a tablet or capsule, and consequently on its disintegration, can vary depending on the anatomical and physiological conditions of the stomach, as well as food consumption. As a result, the release of the active substance may also vary accordingly, be unique and difficult to estimate, what indicate low quality of the formulation.Therefore, tablets or capsules should be designed so that their disintegration is not dependent only on occurrence of intragastric stresses.

Release is a critical process influencing substance absorption and the potential effect *in vivo*. Therefore, this parameter should be meticulously determined during the quality analysis of dietary supplements. While previous research has focused on analyzing the composition of dietary supplements, detecting heavy metal [24], mycotoxins, pesticides [25], and unauthorized substances [26], as well as quantifying major components like lutein [27] and melatonin [28], the evaluation of substance release from dietary supplements has been less explored [29]. Nevertheless, previous studies using pharmacopoeial methods often confirmed observed here the low quality of some formulations [30,31].

To sum up, our study using PhysioCell is pioneering and provides a definitive assessment of supplement quality in terms of substance release. Specifically, if the substance release after applying PhysioCell exceeds 80 %, whereas it was low (below 80 %) using pharmacopoeial methods, then for these supplements, we can anticipate significant pharmacokinetic variability and infer that substance release is influenced by many individual factors. However, if the substance release after using PhysioCell and pharmacopoeial methods was low (below 80 %), it unequivocally indicates that the probability of the active substance release from the supplement is low. Given that both high-quality, well-formulated supplements and those with unsatisfactory composition and release exist on the market, it is imperative to establish procedures aimed at eliminating low-quality supplements and/or promoting high-quality products. Establishing guidelines for mandatory testing of manufactured supplements is essential, but it is crucial to employ reliable methods for a credible and unequivocal assessment of supplement quality. Here, we demonstrated that basic pharmacopeial apparatuses can serve this purpose. This result was validated using PhysioCell, which has been previously shown to accurately mimic *in vivo* conditions [32].

## 5. Conclusions

This is the first time the PhysioCell apparatus has been used in the analysis of dietary supplements. This approach allows for a better understanding of how a tablet or capsule, identified as low-quality through pharmacopoeial tests, will actually behave in the gastrointestinal tract. It also provides insight into how the conditions in the gastrointestinal tract will affect the release of the active substance.

Here, we conclude that basic pharmacopoeial methods can be used to evaluate the quality of dietary supplements. However, whether assessments are conducted using pharmacopoeial standards or the PhysioCell apparatus, the observed low active substance content or inadequate release in dietary supplements highlights the substandard quality of certain products. Consequently, there is a pressing need to implement stringent quality control measures for these formulations, addressing not only content parameters but also release parameters.

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## CRediT authorship contribution statement

**Krzysztof Adam Stępień:** Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Daria Myslitska:** Writing – review & editing, Investigation, Formal analysis. **Grzegorz Garbacz:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Jadwiga Paszkowska:** Writing – review & editing, Supervision, Methodology. **Joanna Giebułtowicz:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

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## Appendix

### *S.3.1. Determination of the Car, Pro, Trp, Tyr and VitC content*

**Table A1**
Mass spectrometric analysis parameters. The turbo ion-spray source was operated in positive mode.

| Parameter | Car | Trp, Tyr | Pro |
|---|---|---|---|
| Model | | QTRAP 4000 | |
| Source temperature [℃] | 600 | 600 | 600 |
| Ion spray voltage [V] | 5500 | 4500 | 5500 |
| Curtain gas [psi] | 35 | 35 | 35 |
| Collision gas | medium | medium | medium |
| Ion source gas 1 [psi] | 30 | 60 | 30 |
| Ion source gas 2 [psi] | 50 | 40 | 50 |

**Table A2**
Method parameters (MRM).

| Parameter | Car | Trp | Tyr | Pro |
|---|---|---|---|---|
| Q1 Mass [Da] | 162 | 205 | 182 | 116 |
| Q3 Mass[Da] | 56 | 146 | 165 | 68 |
| DP [V] | 76 | 76 | 56 | 56 |
| CE [eV] | 85 | 27 | 15 | 41 |
| EP [V] | 10 | 10 | 10 | 10 |
| CXP [V] | 8 | 12 | 12 | 10 |

Declustering potential (DP), collision energy (CE), entrance potential (EP), and collision exit potential (CXP).

**Table A3**
LC-QTRAP 4000 and HPLC Varian liquid chromatograph parameters.

| Parameter | Car | Trp, Tyr | Pro | VitC |
|---|---|---|---|---|
| Pomp | | Agilent 1260 Binary Pump | | Varian ProStar 230 |
| Mobile phases | A: 0.02 mol/L ammonium acetate (aqueous)<br>B: acetonitrile: formic acid (998:2, *v/v*),<br>A : B 15:85 *(v/v)* | A: water: formic acid (998:2, *v/v*),<br>B: acetonitrile: formic acid (998:2, *v/v)* | A: water: formic acid (999:1, *v/v*),<br>B: methanol: formic acid (991:1, *v/v)* | methanol: water: acetic acid (23:67:10, *v/v/v*) |
| Column | SeQuant Zic HILIC 100 mm x 2.1mm, 5 μm Merck | Kinetex 2.6μm C 18 100 Å 100 x 4.6 mm, Phenomenex | Kinetex 2.6μm C 18 100 Å 100 x 3.0 mm, Phenomenex | Luna 5 μm C8 (250 mm x 4.6 mm) |
| Temperature [℃] | 40 | 40 | 40 | 20 |
| Autosampler temperature [℃] | 4 | 4 | 4 | 20 |
| Injection volume [μl] | 5 | 10 | 10 | 25 |
| Flow rate [μl/min] | 500 | 750 | 500 | 500 |

The gradients (%B) were as follows, Trp and Tyr: 0 min 5 %; 1 min 5 %; 3 min 95 %; 5 min 95 %; 5.1 min 5 %; 7 min 5 %, whereas for Pro: 0 min 10 %; 1 min 10 %; 2 min 70 %; 3 min 70 %; 4 min 10 %; 5 min 10 %. Car and Vit C were analysed in isocratic mode for 5 and 7 min, respectively.

**Table A4**
HPLC Shimadzu liquid chromatograph parameters.

| Parameter | Trp, Tyr | VitC |
|---|---|---|
| **Specifications** | LC-2030C 3D Plus | LC-2050C 3D |
| **Mobile phases** | A: water: formic acid (998:2, *v/v*),B: acetonitrile: formic acid (998:2, *v/v)* | A: water: trifluoroacetic acid (999:1, *v/v*),B: methanol: formic acid (999:1, *v/v)* |
| **Column** | Phenomenex Kinetex 5 um EVO C18 100A, 250 × 4.6 mm, 5 μm | Dr. Maisch GmbH ReproSil-Pur 120 C18-AQ, 100 × 3 mm, 2.4 μm |
| **Temperature [℃]** | 40 | 25 |
| **Autosampler temperature [℃]** | 4 | 20 |
| **Injection volume [μl]** | 10 (Trp) and 5 (Tyr) | 1 |
| **Flow rate [μl/min]** | 1200 | 300 |

The gradients (%B) for Trp and Tyr were as follows: 0.01 min 5 %; 1 min 5 %; 6 min 95 %; 8 min 95 %; 10 min 5 %; 13.5 min 5 %. Vit C was analysed in an isocratic mode (5 min, 2 % B). Trp and Tyr detection was performed at the wavelength of 276 nm, whereas Vit C was measured at 275 nm. The analytical methods were found to be linear in the Trp, Tyr and VitC concentration range as follows: Trp 0.005 – 0.5 mg/mL, Tyr 0.002 – 0.5 mg/mL and VitC 0.025 – 1 mg/mL.

**Table A5**
Selected validation parameters of the analytical methods using an Agilent 1260 Infinity or HPLC Varian .

| Substance | Limit of Quantification [μg/ml] | Range [μg/ml] | Correlation coefficient of determination ($R^2$) | Precision [%] | Accuracy [%] |
|---|---|---|---|---|---|
| Car | 0.06 | 0.10 – 5.0 | 0.9995 | 3.1 – 4.8 | 96 – 99 |
| Pro | 0.03 | 0.25 – 5.0 | 0.9999 | 2.8 – 3.6 | 97 – 105 |
| Trp | 0.03 | 0.25 – 5.0 | 0.9988 | 0.27 – 4.9 | 99 – 105 |
| Tyr | 0.03 | 0.25 – 5.0 | 0.9992 | 2.1 – 4.8 | 95 – 99 |
| VitC | 120 | 200 – 1250 | 0.9995 | 0.63 – 3.6 | 97 – 103 |

**Table A6**
Results of quantitative analysis of Car, Tyr and VitC in dietary supplements, maximum error value above 20 was bolded.

| Code | Dosage form | Source | Declared main ingredient content [mg/unit] | Determined main ingredient content [mg/unit][a] | Maximum error [%] |
|---|---|---|---|---|---|
| Car1 | capsule | Poland | 1000 | 620 (CV = 18 %) | **−50** |
| Car2 | tablet | Belgium | 612 | 448 (CV = 8 %) | **–32** |
| Car3 | tablet | European Union | 612 | 396 (CV = 28 %) | **−60** |
| Car4 | tablet | European Union | 667 | 588 (CV = 9 %) | −19 |
| Car5 | capsule | Poland | 600 | 515 (CV = 5 %) | −19 |
| Car6 | capsule | Poland | 940 | 251 (CV = 44 %) | **−89** |
| Car7 | capsule | European Union | 500 | 454 (CV = 15 %) | **−25** |
| Car8 | tablet | United Kingdom | 1024 | 370 (CV = 7 %) | **−66** |
| Car9 | capsule | United states | 500 | 437 (CV = 14 %) | **−24** |
| Car10 | capsule | Belgium | 333 | 260 (CV = 18 %) | **−35** |
| Car11 | capsule | United Kingdom | 680 | 622 (CV = 11 %) | **–23** |
| Car12 | capsule | Germany | 333 | 253 (CV = 19 %) | **−36** |
| Car13 | capsule | European Union | 780 | 330 (CV = 5 %) | **−60** |
| Car14 | tablet | European Union | 682 | 637 (CV = 16 %) | **–22** |
| Car15 | capsule | Switzerland | 612 | 282 (CV = 10 %) | **−60** |
| Car16 | tablet | United states | 500 | 497 (CV = 9 %) | −12 |
| Car17 | capsule | United states | 500 | 413 (CV = 22 %) | **−34** |
| Car18 | capsule | Poland | 1000 | 859 (CV = 10 %) | **−26** |
| Car19 | capsule | Poland | 667 | 538 (CV = 12 %) | **–33** |
| Car20 | tablet | Poland | 300 | 325 (CV = 13 %) | **25** |
| Tyr1 | Capsule | Germany | 500 | 493 (CV = 13 %) | −15 |
| Tyr2 | Capsule | Poland | 500 | 514 (CV = 9.0 %) | 11 |
| Tyr3 | Capsule | United States | 100 | 97.8 (CV = 8.2 %) | −8 |
| Tyr4 | Tablet | European Union | 500 | 498 (CV = 5.3 %) | −6 |
| Tyr5 | Capsule | United States | 500 | 447 (CV = 7.4 %) | −18 |
| Tyr6 | Capsule | Poland | 570 | 394 (CV = 1.7 %) | **–32** |
| Tyr7 | Capsule | United States | 500 | 555 (CV = 5.6 %) | 15 |
| Tyr8 | Capsule | Poland | 600 | 523 (CV = 18 %) | **−28** |
| Tyr9 | capsule | United States | 500 | 502 (CV = 6.0 %) | −6 |
| Tyr10 | capsule | United States | 500 | 520 (CV = 4.7 %) | 10 |
| Tyr11 | capsule | European Union | 375 | 383 (CV = 3.6 %) | 5 |
| Tyr12 | tablet | Poland | 500 | 487 (CV = 7.3 %) | −10 |
| Tyr13 | capsule | United States | 500 | 508 (CV = 4.4 %) | 6 |
| Tyr14 | capsule | Germany | 400 | 386 (CV = 9.3 %) | −14 |
| Tyr15 | capsule | Poland | 840 | 723 (CV = 5.2 %) | −18 |
| Tyr16 | capsule | European Union | 500 | 513 (CV = 7.9 %) | 12 |
| Tyr17 | capsule | Poland | 100 | 110 (CV = 4.2 %) | 15 |
| Tyr18 | capsule | France | 250 | 264 (CV = 6.3 %) | 13 |
| Tyr19 | capsule | Poland | 500 | 532 (CV = 3.4 %) | 9 |
| VitC1 | tablet | Poland | 200 | 215 (CV = 4.3 %) | 13 |
| VitC2 | tablet | Poland | 200 | 189 (CV = 2.2 %) | −7 |
| VitC3 | tablet | Poland | 200 | 208 (CV = 3.1 %) | 7 |
| VitC4 | tablet | Poland | 200 | 203 (CV = 4.2 %) | 5 |
| VitC5 | tablet | Poland | 200 | 203 (CV = 1.9 %) | 3 |
| VitC6 | capsule | Poland | 1000 | 423 (CV = 7.4 %) | **−61** |
| VitC7 | capsule | Poland | 1000 | 1050 (CV = 3.4 %) | 7 |
| VitC8 | capsule | Poland | 1000 | 984 (CV = 2.0 %) | −4 |
| VitC9 | capsule | Poland | 1000 | 1003 (CV = 1.2 %) | 2 |
| VitC10 | capsule | Poland | 1000 | 1070 (CV = 0.7 %) | 8 |

CV − coefficient of variation; a − mean (standard deviation, n = 3).

*S.3.2. Dissolution test*

**Table A7**
Comparison of the results of amount Car, Tyr and VitC release from the dosage form and the Car, Tyr and VitC amount determined in the units using the expanded uncertainty.

| Code | The average percentage of main ingredient amount released from a dosage form (standard deviation n = 6) | | Expanded uncertainty parameters | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | pH 1.2 | | | pH 6.8 | | |
| | pH 1.2 | pH 6.8 | $\|x_1 - x_2\|$ | $U(x_1 - x_2)$ | Equal[a] | $\|x_1 - x_2\|$ | $U(x_1 - x_2)$ | Equal[a] |
| Car1 | 47.3 (6.7) | 10.6 (3.3) | 147 | 142 | No | 514 | 134 | No |
| Car2 | 105.3 (7.5) | 81.2 (5.7) | 196 | 56 | No | 49 | 50 | Yes |
| Car3 | 40.3 (4.7) | 37.5 (2.4) | 150 | 130 | No | 167 | 128 | No |
| Car4 | 38.4 (4.4) | 30 (11) | 332 | 65 | No | 385 | 83 | No |
| Car5 | 21.6 (5.6) | 15.0 (2.0) | 386 | 40 | No | 425 | 31 | No |
| Car6 | 26.1 (8.3) | 28.3 (9.7) | 6 | 142 | Yes | 14 | 148 | Yes |
| Car7 | 43.8 (5.7) | 25.3 (4.3) | 235 | 79 | No | 327 | 78 | No |
| Car8 | 52 (14) | 44 (24) | 108 | 106 | No | 36 | 175 | Yes |
| Car9 | 123.0 (7.9) | 105 (21) | 60 | 71 | Yes | 116 | 86 | No |
| Car10 | 56.2 (2.1) | 60.8 (2.9) | 73 | 54 | No | 58 | 54 | No |
| Car11 | 16.4 (1.3) | 8.5 (1.2) | 510 | 79 | No | 563 | 79 | No |
| Car12 | 77 (19) | 60 (12) | 2 | 76 | Yes | 55 | 65 | Yes |
| Car13 | 78.4 (4.6) | 69.0 (5.8) | 281 | 34 | No | 208 | 41 | No |
| Car14 | 33.8 (2.2) | 23.8 (1.1) | 407 | 122 | No | 414 | 122 | No |
| Car15 | 57.1 (1.9) | 57.1 (5.3) | 67 | 34 | No | 67 | 42 | No |
| Car16 | 31.9 (2.1) | 39.3 (1.3) | 338 | 52 | No | 301 | 51 | No |
| Car17 | 84.0 (3.9) | 85 (11) | 6 | 104 | Yes | 10 | 112 | Yes |
| Car18 | 43.0 (1.5) | 30.5 (2.8) | 429 | 99 | No | 554 | 101 | No |
| Car19 | 28.4 (5.5) | 15.7 (2.8) | 348 | 80 | No | 433 | 75 | No |
| Car20 | 50.1 (1.3) | 68.9 (2.6) | 175 | 50 | No | 119 | 50 | No |
| Tyr1 | 78.6 (5.4) | 57.6 (4.1) | 100.3 | 37.9 | No | 205.4 | 35.1 | No |
| Tyr2 | 89.6 (6.6) | 61.4 (3.5) | 65.7 | 59.8 | No | 206.3 | 55.2 | No |
| Tyr3 | 82.0 (2.1) | 78.5 (2.7) | 15.7 | 9.4 | No | 19.3 | 9.5 | No |
| Tyr4 | 71.1 (3.7) | 49.1 (2.1) | 142.6 | 33.9 | No | 252.7 | 31.5 | No |
| Tyr5 | 37 (22) | 56.79 (0.54) | 228.3 | 97.4 | No | 162.8 | 38.1 | No |
| Tyr6 | 64.1 (4.8) | 48.3 (1.7) | 28.8 | 23.6 | No | 119.0 | 10.7 | No |
| Tyr7 | 96.6 (5.2) | 56.7 (7.8) | 72.3 | 41.5 | No | 271.3 | 47.8 | No |
| Tyr8 | 4.5 (2.3) | 1.47 (0.50) | 496.8 | 107.1 | No | 514.9 | 106.5 | No |
| Tyr9 | 85 (14) | 14 (11) | 77.3 | 64.3 | No | 433.4 | 53.9 | No |
| Tyr10 | 53 (16) | 35 (17) | 258.5 | 70.2 | No | 346.4 | 72.7 | No |
| Tyr11 | 63 (10) | 34.9 (8.0) | 148.7 | 33.2 | No | 252.6 | 29.0 | No |
| Tyr12 | 82.2 (3.4) | 38.8 (4.0) | 76.5 | 43.2 | No | 293.4 | 44.0 | No |
| Tyr13 | 1.94 (0.74) | 29.5 (8.9) | 498.3 | 25.8 | No | 360.6 | 44.4 | No |
| Tyr14 | 3.3 (4.3) | 17 (20) | 372.7 | 43.5 | No | 321.4 | 74.6 | No |
| Tyr15 | 8.9 (3.0) | 1.58 (0.26) | 648.7 | 47.6 | No | 710.0 | 43.1 | No |
| Tyr16 | 76 (14) | 27.6 (9.5) | 134.8 | 71.4 | No | 375.1 | 60.8 | No |
| Tyr17 | 5.89 (0.82) | 0.58 (0.12) | 104.4 | 5.4 | No | 109.7 | 5.4 | No |
| Tyr18 | 83.2 (6.4) | 30 (36) | 56.3 | 23.2 | No | 188.8 | 75.7 | No |
| Tyr19 | 22.6 (7.6) | 3.88 (0.84) | 418.6 | 37.2 | No | 512.1 | 20.9 | No |
| VitC1 | 76 (12) | 35.7 (7.0) | 62 | 22 | No | 143 | 12 | No |
| VitC2 | 80.7 (5.6) | 22.3 (5.5) | 27 | 10 | No | 144 | 6 | No |
| VitC3 | 96.8 (8.2) | 24.1 (4.7) | 15.3 | 14.9 | Yes | 160 | 8 | No |
| VitC4 | 22.7 (2.5) | 15.52 (0.48) | 158 | 11 | No | 172 | 10 | No |
| VitC5 | 80 (16) | 17.34 (0.88) | 42 | 25 | No | 168 | 5 | No |
| VitC6 | 41.2 (2.9) | 2.959 (0.013) | 11 | 43 | Yes | 393 | 36 | No |
| VitC7 | 89.6 (8.6) | 78.9 (5.4) | 153 | 81 | No | 260 | 59 | No |
| VitC8 | 8.6 (6.0) | 6.6 (1.2) | 898 | 54 | No | 918 | 25 | No |
| VitC9 | 14.39(0.95) | 11.1 (1.7) | 859 | 15 | No | 892 | 19 | No |
| VitC10 | 20.1 (4.5) | 17.6 (2.8) | 869 | 38 | No | 893 | 24 | No |

[a] amount of Car or Tyr or VitC in the formulation and amount of Car or Tyr or VitC released are equal (yes) or not (no) within the uncertainty

For the dietary supplements in tablet form ($n = 3$), the percentage release of Trp (Trp5, Trp6) or VitC (VitC4) was below 10 % at the 15-minute mark of the test, both under pharmacopeial conditions and biorelevant dissolution test. At 15th minutes of testing under biorelevant dissolution test, intragastric stress (IS) (100 mbar) was applied. However, this modification did not result in significant release, as it remained at an average of 10 % to 20 %, similar to the release under pharmacopeial conditions. At 30th minutes, gastric emptying (GE) (3 x 300 mbar) was simulated. These conditions led to an increase in release compared to the previous stage (15–30 min) and averaged 24 % (Trp6), 53 % (Trp5) and 68 % (VitC4) at the end of the study. However the 80 % release criterion was not met by these supplements. During the test, it was observed that the examined tablets (Trp5, Trp6, VitC4) underwent unpredictable changes. Some tablets did not disintegrate, some only partially disintegrated, and others disintegrated but remained in the form of large particles.

Dissolution test results for dietary supplements in the capsule form ($n = 16$) are more differing. For the dietary supplements in capsule form containing Trp, the application of IS led to increased substance release compared to pharmacopeial conditions (except Trp1). However, for none of the analyzed preparations did this result in exceeding 80 % of the active substance content. For Trp, the average percentage release was 44 % (Trp2), 51 % (Trp3), and 70 % (Trp4). The use of these conditions resulted in the opening of the capsules. At 30 min of the test, GE was applied. These conditions led to increased release in release compared to the previous stage (15–30 min), and for all analyzed preparations containing Trp, the percentage release exceeded 80 %. For the dietary supplements containing VitC, the application of IS conditions also led to increased substance release compared to pharmacopeial conditions for all analysed supplements. However, for none of the analyzed preparations did this result in exceeding 80 % of the active

substance content. For VitC, the average percentage release was 46 % (VitC8), 37 % (VitC9), and 55 % (VitC10). The use of these conditions resulted in the opening of the capsules. The application of GE for VitC8 and VitC10 resulted in achieving release above 80 %. In the case of VitC9, the release of the substance was 78 %. In the case of dietary supplements containing Tyr, the application of IS conditions resulted in the observation of capsule opening. The release of Tyr between 15 and 30 min during testing was comparable or slightly higher than under pharmacopeial conditions. Upon the application GE conditions, a release exceeding 80 % was attained for Tyr14 and Tyr17. For the remaining formulations, the release was significantly higher than under pharmacopeial conditions, although it did not surpass 80 %. The average release of Car under pharmacopoeial conditions was 22 % and 16 % for Car5 and Car11, respectively. Following the implementation of IS for Car5, an average release of 36 % was observed, while under GE conditions, it rose to 53 %. For Car11, the average release after applying IS conditions was 10 %, increasing to 27 % under GE conditions. Regarding Pro1, the average release under pharmacopeial conditions was 11 %. Upon application of IS conditions, the average release reached 38 %, and with GE conditions, it was 44 %.In summary, the implementation of biorelevant conditions PhysioCell for capsule-form supplements led to an enhanced release of substances (from 11 to 106 percentage points) compared to pharmacopoeial dissolution tests.

## Data availability

Data will be made available on request.

## References


[1] C. Andreas, J. Rosenberger, J. Butler, P. Augustijns, M. McAllister, B. Abrahamsson, J. Dressman, Introduction to the OrBiTo decision tree to select the most appropriate in vitro methodology for release testing of solid oral dosage forms during development, Eur. J. Pharm. Biopharm. 130 (2018) 207–213.

[2] V.A. Gray, Power of the dissolution test in distinguishing a change in dosage form critical quality attributes, AAPS Pharm. Sci. Tech. 19 (8) (2018) 3328–3332.

[3] M. Shrivas, D. Khunt, M. Shrivas, M. Choudhari, R. Rathod, M. Misra, Advances in in vivo predictive dissolution testing of solid oral formulations: how closer to in vivo performance? J. Pharm. Innov. 15 (3) (2020) 296–317.

[4] K. Sugano, A. Okazaki, S. Sugimoto, S. Tavornvipas, A. Omura, T. Mano, Solubility and dissolution profile assessment in drug discovery, Drug Metab. Pharmacokinet. 22 (4) (2007) 225–254.

[5] F. Abay, T. Ugurlu, Orally disintegrating tablets: a short review, J. Pharm. Drug Devel. 3 (3) (2015) 303.

[6] A. Seidlitz, W. Weitschies, In-vitro dissolution methods for controlled release parenterals and their applicability to drug-eluting stent testing, J. Pharm. Pharmacol. 64 (7) (2012) 969–985.

[7] P.M. Salve, S.V. Sonawane, M.B. Patil, R.K. Surawase, Dissolution and dissolution test apparatus a review, Indianjournals.com 11 (3) (2021) 229–236, https://doi.org/10.52711/2231-5659.2021.00037.

[8] M. Koziolek, G. Garbacz, M. Neumann, W. Weitschies, Simulating the postprandial stomach: biorelevant test methods for the estimation of intragastric drug dissolution, Mol. Pharm. 10 (6) (2013) 2211–2221.

[9] M. Romański, M. Staniszewska, J. Paszkowska, J. Dobosz, S. Romanova, J. Pieczuro, M. Kątny, D. Roznerska, J. Szczepański, M. Schraube, Application of a novel PhysioCell apparatus for biopredictive dissolution tests of oral immediate release formulations–A case study workflow for in vitro-in vivo predictions, Int. J. Pharm. 641 (2023) 123061.

[10] M. Staniszewska, M. Romański, J. Dobosz, B. Kołodziej, U. Lipski, G. Garbacz, D. Danielak, PhysioCell—a novel, bio-relevant dissolution apparatus: hydrodynamic conditions and factors influencing the dissolution dynamics, AAPS Pharm. Sci. Tech. 24 (2) (2023) 65.

[11] J.T. Dwyer, P.M. Coates, M.J. Smith, Dietary supplements: regulatory challenges and research resources, Nutrients 10 (1) (2018), https://doi.org/10.3390/nu10010041.

[12] K.A. Stępień, J. Niewiarowski, A. Harasimiuk, Powszechność suplementów diety a zagrożenia związane z ich stosowaniem, Prospects in Pharm. Sci. 17 (9) (2019) 51–59.

[13] K.A. Stępień, J. Giebułtowicz, Application of Liquid Chromatography Coupled to Mass Spectrometry in Quality Assessment of Dietary Supplements—A Case Study of Tryptophan Supplements: Release Assay, Targeted and Untargeted Studies. Pharmaceuticals 15 (4) (2022) 448.

[14] K.A. Stępień, W. Krawczyk, J. Giebułtowicz, Dietary supplements with proline—a comprehensive assessment of their quality, Life 13 (2) (2023) 263.

[15] M.J. Brennan, W.E. Duncan, L. Wartofsky, V.M. Butler, H.L. Wray, In vitro dissolution of calcium carbonate preparations, Calcif. Tissue Int. 49 (5) (1991) 308–312.

[16] H. Hahm, J. Kujawa, L. Augsburger, Comparison of melatonin products against USP's nutritional supplements standards and other criteria, J. Am. Pharm. Assoc. 39 (1) (1999) 27–31.

[17] N. Sculthorpe, B. Davies, T. Ashton, S. Allison, D. McGuire, J. Malhi, Commercially available folic acid supplements and their compliance with the British Pharmacopoeia test for dissolution, J. Public Health 23 (3) (2001) 195–197.

[18] E. Blicharska, B. Szczęsna, R. Kocjan, A. Gumieniczek, Ł. Komsta, Analysis of dissolution profiles of iron, zinc, and manganese from complex dietary supplements by ion chromatography and chemometrics, J. Liq. Chromatogr. Relat. 39 (1) (2016) 30–34.

[19] W. Lyu, T. Omar, H. Patel, D. Rodriguez, M.G. Ferruzzi, G.M. Pasinetti, J. W. Murrough, F.J. Muzzio, J.E. Simon, Q. Wu, Dissolution study on grape polyphenol hard gelatin capsule dietary supplements, Front. Nutr. (2021) 8.

[20] M. Koziolek, G. Garbacz, M. Neumann, W. Weitschies, Simulating the postprandial stomach: physiological considerations for dissolution and release testing, Mol. Pharm. 10 (5) (2013) 1610–1622.

[21] G. Garbacz, S. Klein, Dissolution testing of oral modified-release dosage forms, J. Pharm. Pharmacol. 64 (7) (2012) 944–968.

[22] M. Koziolek, F. Schneider, M. Grimm, C. Modeβ, A. Seekamp, T. Roustom, W. Siegmund, W. Weitschies, Intragastric pH and pressure profiles after intake of the high-caloric, high-fat meal as used for food effect studies, J. Control. Release 220 (2015) 71–78.

[23] F. Schneider, M. Grimm, M. Koziolek, C. Modeß, A. Dokter, T. Roustom, W. Siegmund, W. Weitschies, Resolving the physiological conditions in bioavailability and bioequivalence studies: Comparison of fasted and fed state, Eur. J. Pharm. Biopharm. 108 (2016) 214–219.

[24] A. Kowalski, M. Frankowski, Levels and potential health risks of mercury in prescription, non-prescription medicines and dietary supplements in Poland, Regul. Toxicol. Pharm. 73 (1) (2015) 396–400, https://doi.org/10.1016/j.yrtph.2015.08.001.

[25] G. Martínez-Domínguez, R. Romero-González, F.J. Arrebola, A.G. Frenich, Multi-class determination of pesticides and mycotoxins in isoflavones supplements obtained from soy by liquid chromatography coupled to Orbitrap high resolution mass spectrometry, Food Control 59 (2016) 218–224.

[26] J.M. Martínez-Sanz, I. Sospedra, C.M. Ortiz, E. Baladía, A. Gil-Izquierdo, R. Ortiz-Moncada, Intended or unintended doping? a review of the presence of doping substances in dietary supplements used in sports, Nutrients 9 (10) (2017) 1093.

[27] C.S. Anselmo, T.C. Mendes, L.M. Cabral, V.P. Sousa, Physicochemical quality profiles of commercial oral tablets and capsules containing lutein - impact of insufficient specific sanitary regulations, An. Acad. Bras. Cienc. 90 (3) (2018) 3063–3073, https://doi.org/10.1590/0001-3765201820170972.

[28] A.B. Cerezo, Á. Leal, M.A. Álvarez-Fernández, R. Hornedo-Ortega, A.M. Troncoso, M.C. García-Parrilla, Quality control and determination of melatonin in food supplements, J. Food Compos. Anal. 45 (2016) 80–86, https://doi.org/10.1016/j.jfca.2015.09.013.

[29] J.P. Patrício, C. Santos, R. Cerdeira, In vitro dissolution profile of two commercially available iron preparations, Drugs RD 12 (1) (2012) 35–40, https://doi.org/10.2165/11631560-000000000-00000.

[30] J. Đuriš, B. Čalija, B. Vidović, V. Dobričić, J. Milić, S. Ibrić, Comparative analysis of mechanical and dissolution properties of single-and multicomponent folic acid supplements, J. Food Compos. Anal. 60 (2017) 17–24.

[31] M. Mikulić, M.A. Krstonošić, B. Gaćeša, T. Vojnović, S. Jovanović, J. Cvejić, Quality assessment and dissolution properties of dietary supplements with isoflavones, J. Food & Nutrition Research 62 (2) (2023).

[32] G. Garbacz, R.-S. Wedemeyer, S. Nagel, T. Giessmann, H. Mönnikes, C.G. Wilson, W. Siegmund, W. Weitschies, Irregular absorption profiles observed from diclofenac extended release tablets can be predicted using a dissolution test apparatus that mimics in vivo physical stresses, Eur. J. Pharm. Biopharm. 70 (2) (2008) 421–428.

# 4. Podsumowanie i wnioski

1. Podczas analizy suplementów diety z wykorzystaniem techniki chromatografii cieczowej sprzężonej ze spektrometrią mas, wykryto w ich składzie substancje niezadeklarowane przez producenta. Były to substancje związane z głównym składnikiem suplementu diety, między innymi produkty jego transformacji np. produkty przemian metabolicznych tryptofanu, produkty kondensacji tryptofanu ze związkami karbonylowymi oraz produkty degradacji kinureniny. Wykryto także związki, których obecność była przypadkowa i prawdopodobnie związana z nieodpowiednimi warunkami produkcji, takie jak noradrenalina (suplement zawierający tyrozynę), melatonina, glukozamina (suplementy zawierające tryptofan), 4-etylogwajakol, sulfizoksazol, steramid, erukamid (suplementy zawierające prolinę), kofeina (suplementy zawierające tyrozynę lub karnitynę).
2. W wyniku analizy zawartości głównego składnika suplementów diety, uzyskano następujące zakresy średniej procentowej zawartości (średnia zawartość oznaczona w stosunku do zawartości deklarowanej przez producenta) oraz zakresy współczynników zmienności (CV) pomiędzy różnymi jednostkami dawkowania tego samego preparatu:
   a) dla tryptofanu: średnia: 55 % - 100 %; CV: 3,7 % - 58 %;
   b) dla proliny: średnia: 75 % – 132 %; CV: 2,1 % - 17 %;
   c) dla tyrozyny: średnia: 69 % – 111 %; CV: 1,4 % - 15 %;
   d) dla karnityny: średnia: 27 % - 108 %: CV: 4,9 % - 44 %.

   Podsumowując, spośród 68 badanych suplementów diety, w przypadku 16 preparatów średnia zawartość głównego składnika mieściła się w przedziale 95 – 105 % (przy deklarowanej zawartości 100 mg lub więcej w jednostce) lub 90 – 110 % (przy deklarowanej zawartości poniżej 100 mg w jednostce). Jednak tylko w przypadku 2 preparatów wszystkie analizowane tabletki lub kapsułki zawierały ilość głównego składnika mieszczącą się w powyższych zakresach. Dodatkowo w przypadku suplementów z tryptofanem i karnityną wykazano istotną zmienność zawartości pomiędzy jednostkami dawkowania niektórych preparatów.
3. Badanie z wykorzystaniem aparatu łopatkowego lub koszyczkowego, umożliwiło ocenę uwalniania głównego składnika z formy dawkowania suplementu diety do płynu akceptorowego o pH 1,2 oraz o pH 6,8. Otrzymano następujące zakresy

średniego procentowego uwalniania oraz zakresy współczynników zmienności (CV) pomiędzy różnymi jednostkami dawkowania tego samego preparatu:

a) tryptofan;

- średnie uwalnianie: 1 % – 90 %; CV: 0,36 % – 60 % (pH 1,2);
- średnie uwalnianie: 2 % – 81,4 %; CV: 2,9 % – 96 % (pH 6,8);

b) prolina;

- średnie uwalnianie: 11 % – 131 % ; CV: 5,8 % – 110 % (pH 1,2);
- średnie uwalnianie: 2 % – 118,6 %; CV: 5,0 % – 100 % (pH 6,8);

c) tyrozyna;

- średnie uwalnianie: 2 % – 96,6 %; CV: 2,6 % – 140 % (pH 1,2);
- średnie uwalnianie: 1 % – 78,5 %; CV: 0,96 % – 130 % (pH 6,8);

d) karnityna;

- średnie uwalnianie: 16,4 % – 123,0 %; CV: 2,6 % – 32 % (pH 1,2);
- średnie uwalnianie: 9 % – 105 %; CV: 3,4 % – 55 % (pH 6,8).

Podsumowując, spośród 68 badanych preparatów, średni procent uwolnienia głównego składnika do płynu akceptorowego niższy niż 80 % odnotowano aż dla 51 (pH = 1,2) oraz 61 (pH = 6,8) suplementów diety. Natomiast jedynie w przypadku 6 suplementów (pH 1,2) oraz 1 suplementu (pH 6,8) procent uwolnienia głównego składnika przekraczał 80 % we wszystkich analizowanych tabletkach lub kapsułkach.

4. Wyznaczenie parametru niepewności rozszerzonej umożliwiło określenie czy zawartość głównego składnika suplementu diety oraz jego uwolniona ilość są równe w granicach niepewności. W przypadku 53 na 68 badanych preparatów ww. zawartości nie były równe w granicach niepewności. Oznacza to, iż w przypadku tych suplementów, niższa ilość uwolnionej substancji, wynikała z negatywnego wpływu zastosowanych parametrów procesu technologicznego wytwarzania kapsułek/tabletek oraz/lub użytych substancji pomocniczych. W przypadku pozostałych preparatów (15 suplementów diety) ilość uwolnionej substancji z tabletek/kapsułek była warunkowana zawartością głównego składnika.
5. Farmakopealne metody badania uwalniania substancji są dobrym narzędziem do oceny jakości postaci, w których występują suplementy diety.
6. Zastosowanie aparatu *PhysioCell* do badania uwalniania głównego składnika, pozwoliło na lepsze odwzorowanie warunków panujących w przewodzie pokarmowym. Wyniki potwierdziły, że jakość formulacji suplementów diety

w większości analizowanych preparatów była niska, a proces ich rozpadu zależał od warunków panujących w przewodzie pokarmowym, charakteryzujących się dużą zmiennością zarówno wewnątrz-, jak i międzyosobniczą.

7. Niska jakość suplementów diety wynikała z obecności substancji niezadeklarowanych przez producenta, różnej (z reguły niższej) od deklarowanej zawartości głównego składnika oraz niskiego poziomu uwalniania substancji.
8. Konieczne jest opracowanie standardów dotyczących weryfikacji jakości suplementów diety, szczególnie w kontekście uwalniania substancji, ponieważ uwalnianie warunkuje wystąpienie efektu działania substancji na organizm konsumenta.

# 5. Oświadczenia wszystkich współautorów publikacji

## 5.1 Publikacja 1 (przeglądowa)

**Stępień, K. A.**; Niewiarowski, J.; Harasimiuk, A. *Powszechność suplementów diety a zagrożenia związane z ich stosowaniem*; Biuletyn Wydziału Farmaceutycznego Warszawskiego Uniwersytetu Medycznego; 2019; 9; 51-59.

Warszawa, 17.12.2024 r.
(miejscowość, data)

Krzysztof Adam Stępień
(imię i nazwisko)

**OŚWIADCZENIE**

Jako współautor pracy pt. „**Powszechność suplementów diety a zagrożenia związane z ich stosowaniem**" (Biuletyn Wydziału Farmaceutycznego Warszawskiego Uniwersytetu Medycznego; 2019; 9; 51-59) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie w formie publikacji stanowi: **opracowanie koncepcji, przegląd piśmiennictwa, wyciągnięcie wniosków, redagowanie i korekta manuskryptu, prowadzenie korespondencji z redakcją.**

Mój udział procentowy w przygotowaniu publikacji określam jako 80%.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Krzysztofa Stępnia.**

Krzysztof Stępień
(podpis oświadczającego)

Warszawa, 16.12.2024
(miejscowość, data)

Anna Harasimiuk
(imię i nazwisko)

**OŚWIADCZENIE**

Jako współautor pracy pt. „**Powszechność suplementów diety a zagrożenia związane z ich stosowaniem**" (Biuletyn Wydziału Farmaceutycznego Warszawskiego Uniwersytetu Medycznego; 2019; 9; 51-59) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie w formie publikacji stanowi: **dokonanie przeglądu części piśmiennictwa.**

Mój udział procentowy w przygotowaniu publikacji określam jako 10%.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Krzysztofa Stępnia.**

Anna Harasimiuk

(podpis oświadczającego)

Warszawa, 13.12.2024 r.
(miejscowość, data)

Jakub Niewiarowski
(imię i nazwisko)

**OŚWIADCZENIE**

Jako współautor pracy pt. **„Powszechność suplementów diety a zagrożenia związane z ich stosowaniem”** (Biuletyn Wydziału Farmaceutycznego Warszawskiego Uniwersytetu Medycznego; 2019; 9; 51-59) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie w formie publikacji stanowi: **dokonanie przeglądu części piśmiennictwa.**

Mój udział procentowy w przygotowaniu publikacji określam jako **10%.**

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Krzysztofa Stępnia.**

Jakub Niewiarowski
(podpis oświadczającego)

## 5.2 Publikacja 2 (oryginalna)

**Stępień, K.A**.; Giebułtowicz, J. *Application of Liquid Chromatography Coupled to Mass Spectrometry in Quality Assessment of Dietary Supplements—A Case Study of Tryptophan Supplements: Release Assay, Targeted and Untargeted Studies;* Pharmaceuticals; 2022; 15 (4); 448.

Warszawa, 17.12.2024
(miejscowość, data)

Krzysztof Adam Stępień
(imię i nazwisko)

**OŚWIADCZENIE**

Jako współautor pracy pt. „**Application of Liquid Chromatography Coupled to Mass Spectrometry in Quality Assessment of Dietary Supplements—A Case Study of Tryptophan Supplements: Release Assay, Targeted and Untargeted Studies**" (Pharmaceuticals; 2022; 15 (4); 448) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie w formie publikacji stanowi: **udział w opracowaniu metodyki badania, wykonanie badań, analiza wyników, interpretacja wyników i wyciągnięcie wniosków, redagowanie i korekta manuskryptu.**

Mój udział procentowy w przygotowaniu publikacji określam jako 80%.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Krzysztofa Stępnia.**

Krzysztof Stępień
(podpis oświadczającego)

Warszawa, 17.12.2024
(miejscowość, data)

Joanna Giebułtowicz
(imię i nazwisko)

**OŚWIADCZENIE**

Jako współautor pracy pt. „**Application of Liquid Chromatography Coupled to Mass Spectrometry in Quality Assessment of Dietary Supplements—A Case Study of Tryptophan Supplements: Release Assay, Targeted and Untargeted Studies**" (Pharmaceuticals; 2022; 15 (4); 448) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie w formie publikacji stanowi: **opracowanie koncepcji badań, opracowanie metodyki badania, nadzór nad przebiegiem badania, analiza wyników, redagowanie i korekta manuskryptu.**

Mój udział procentowy w przygotowaniu publikacji określam jako 20%.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Krzysztofa Stępnia.**

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(podpis oświadczającego)

## 5.3 Publikacja 3 (oryginalna)

**Stępień, K.A**.; Krawczyk, W.; Giebułtowicz, J. *Dietary Supplements with Proline — A Comprehensive Assessment of Their Quality*; Life; 2023; 13 (2); 263.

Warszawa, 17.12.2024 r.
(miejscowość, data)

Krzysztof Adam Stępień
(imię i nazwisko)

**OŚWIADCZENIE**

Jako współautor pracy pt. „**Dietary Supplements with Proline—A Comprehensive Assessment of Their Quality**" (Life; 2023; 13 (2); 263) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie w formie publikacji stanowi: udział w **opracowaniu koncepcji badań, metodyki badania, wykonanie badań, analiza wyników, interpretacja wyników i wyciągnięcie wniosków, przygotowanie pierwszej wersji manuskryptu.**

Mój udział procentowy w przygotowaniu publikacji określam jako 80%.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Krzysztofa Stępnia.**

Krzysztof Stępień
(podpis oświadczającego)

Pruszków, 17.12.2024
(miejscowość, data)

Weronika Krawczyk
(imię i nazwisko)

**OŚWIADCZENIE**

Jako współautor pracy pt. **„Dietary Supplements with Proline—A Comprehensive Assessment of Their Quality”** (Life; 2023; 13 (2); 263) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie w formie publikacji stanowi: **wykonanie części badań.**

Mój udział procentowy w przygotowaniu publikacji określam jako 5%.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Krzysztofa Stępnia.**

Weronika Krawczyk
(podpis oświadczającego)

Warszawa, 17.12.2024 r.
(miejscowość, data)

Joanna Giebułtowicz
(imię i nazwisko)

# OŚWIADCZENIE

Jako współautor pracy pt. „**Dietary Supplements with Proline—A Comprehensive Assessment of Their Quality**" (Life; 2023; 13 (2); 263) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie w formie publikacji stanowi: **opracowanie koncepcji badań, opracowanie metodyki badania, nadzór nad przebiegiem badania, analiza wyników, redagowanie i korekta manuskryptu.**

Mój udział procentowy w przygotowaniu publikacji określam jako 15%.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Krzysztofa Stępnia.**

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(podpis oświadczającego)

## 5.4 Publikacja 4 (oryginalna)

**Stępień, K.A.**; Kalicka, A.; Giebułtowicz, J. *Screening the quality of legal and illegal dietary supplements by LC-MS/MS*; Food Additives and Contaminants: Part B; 2024; 1-14.

Warszawa, 17.12.2024 r.

(miejscowość, data)

Krzysztof Adam Stępień

(imię i nazwisko)

**OŚWIADCZENIE**

Jako współautor pracy pt. „**Screening the quality of legal and illegal dietary supplements by LC-MS/MS**" (Food Additives and Contaminants: Part B; 2024; 1-14) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie w formie publikacji stanowi: udział w **opracowaniu koncepcji badań, wykonanie części badań, analiza wyników, interpretacja wyników i wyciągnięcie wniosków, redagowanie i korekta manuskryptu.**

Mój udział procentowy w przygotowaniu publikacji określam jako 65%.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Krzysztofa Stępnia.**

Krzysztof Stępień

(podpis oświadczającego)

Warszawa, 17.12.2024 r.
(miejscowość, data)

Agnieszka Kalicka
(imię i nazwisko)

**OŚWIADCZENIE**

Jako współautor pracy pt. „**Screening the quality of legal and illegal dietary supplements by LC-MS/MS**" (Food Additives and Contaminants: Part B; 2024; 1-14) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie w formie publikacji stanowi: **udział w opracowani metodyki badania, wykonanie części badań, analiza wyników, udział w redagowaniu pierwszej wersji manuskryptu.**

Mój udział procentowy w przygotowaniu publikacji określam jako 25%.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Krzysztofa Stępnia.**

Agnieszka Kalicka
(podpis oświadczającego)

Warszawa, 17. 12. 2024 r.
(miejscowość, data)

Joanna Giebułtowicz
(imię i nazwisko)

# OŚWIADCZENIE

Jako współautor pracy pt. „**Screening the quality of legal and illegal dietary supplements by LC-MS/MS**" (Food Additives and Contaminants: Part B; 2024; 1-14) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie w formie publikacji stanowi: **opracowanie koncepcji badań, opracowanie metodyki badania, nadzór nad przebiegiem badania, redagowanie i korekta manuskryptu.**

Mój udział procentowy w przygotowaniu publikacji określam jako 10%.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Krzysztofa Stępnia.**

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Farmaceutycznej i Biomedycznej
dr hab. n. farm. Joanna Giebułtowicz

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(podpis oświadczającego)

## 5.5 Publikacja 5 (oryginalna)

**Stępień, K.A**.; Myslitska, D.; Garbacz, G.; Paszkowska, J.; Giebułtowicz, J. *Pharmaceutical availability testing – can a drug-specific test be relevant for assessing the quality of dietary supplements?;* Microchemical Journal; 2024; 112132.

Warszawa, 27.12.2024 r.
(miejscowość, data)

Krzysztof Adam Stępień
(imię i nazwisko)

**OŚWIADCZENIE**

Jako współautor pracy pt. „**Pharmaceutical availability testing – can a drug-specific test be relevant for assessing the quality of dietary supplements?**" (Microchemical Journal; 2024; 112132) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie w formie publikacji stanowi: **udział w opracowaniu metodyki badania, wykonanie części badań, analiza wyników, interpretacja wyników i wyciągnięcie wniosków, redagowanie pierwszej wersji manuskryptu.**

Mój udział procentowy w przygotowaniu publikacji określam jako 55%.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Krzysztofa Stępnia.**

Krzysztof Stępień
(podpis oświadczającego)

Wrocław, 13.12.2024
(miejscowość, data)

Daria Myslitska
(imię i nazwisko)

**OŚWIADCZENIE**

Jako współautor pracy pt. „**Pharmaceutical availability testing – can a drug-specific test be relevant for assessing the quality of dietary supplements?**" (Microchemical Journal; 2024; 112132) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie w formie publikacji stanowi: **wykonanie części badań, redagowanie i korekta manuskryptu.**

Mój udział procentowy w przygotowaniu publikacji określam jako 15%.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Krzysztofa Stępnia.**

Daria Myslitska
(podpis oświadczającego)

Wrocław, 13.12.2024
(miejscowość, data)

Jadwiga Paszkowska
(imię i nazwisko)

**OŚWIADCZENIE**

Jako współautor pracy pt. „**Pharmaceutical availability testing – can a drug-specific test be relevant for assessing the quality of dietary supplements?**" (Microchemical Journal; 2024; 112132) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie w formie publikacji stanowi: **udział w opracowaniu metodyki badania, nadzór nad przebiegiem badania, redagowanie i korekta manuskryptu.**

Mój udział procentowy w przygotowaniu publikacji określam jako 10%.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Krzysztofa Stępnia.**

.....

(podpis oświadczającego)

Wrocław, 13.12.2024
(miejscowość, data)

Grzegorz Garbacz
(imię i nazwisko)

# OŚWIADCZENIE

Jako współautor pracy pt. „**Pharmaceutical availability testing – can a drug-specific test be relevant for assessing the quality of dietary supplements?**" (Microchemical Journal; 2024; 112132) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie w formie publikacji stanowi: **udział w opracowaniu koncepcji badań, nadzór nad przebiegiem badania, redagowanie i korekta manuskryptu.**

Mój udział procentowy w przygotowaniu publikacji określam jako 5%.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Krzysztofa Stępnia.**

.....

(podpis oświadczającego)

Warszawa, 17.12.2024 r.
(miejscowość, data)

Joanna Giebułtowicz
(imię i nazwisko)

# OŚWIADCZENIE

Jako współautor pracy pt. „**Pharmaceutical availability testing – can a drug-specific test be relevant for assessing the quality of dietary supplements?**" (Microchemical Journal; 2024; 112132) oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie w formie publikacji stanowi: **opracowanie koncepcji badań, nadzór nad przebiegiem badania, redagowanie i korekta manuskryptu.**

Mój udział procentowy w przygotowaniu publikacji określam jako 15%.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej **mgr Krzysztofa Stępnia.**

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Farmaceutycznej i Biomedycznej
dr hab. n. farm. Joanna Giebułtowicz

(podpis oświadczającego)

# Wykaz innych publikacji naukowych

a) Rycerz, K.; **Stępień, K.A**.; Czapiewska, M.; Arafat, B.T.; Habashy, R.; Isreb, A.; Peak, M.; Alhnan, M.A. *Embedded 3D printing of novel bespoke soft dosage form concept for pediatrics*; Pharmaceutics; 2019; 11 (12); 1-15.
DOI: https://doi.org/10.3390/pharmaceutics11120630
Punkty IF: 4,4

b) Jabłońska, A.E.; **Stępień, K.A.** *Fałszowanie produktów leczniczych oraz zagrożenia wynikające z ich stosowania*; Biuletyn Wydziału Farmaceutycznego Warszawskiego Uniwersytetu Medycznego; 2019; 11; 66-71.
DOI: https://doi.org/10.56782/pps.19

# Wykaz innych doniesień zjazdowych

a) Doniesienie zjazdowe nr A1

„Przestępczość farmaceutyczna w okresie pandemii COVID-19”; Światowy tydzień walki ze sfałszowanymi lekami; ISBN 978-83-959554-1-9; s. 55-59.
Kalicka, A.; **Stępień, K.A.** Zastosowanie metody LC-MS/MS do identyfikacji substancji czynnych w sfałszowanych i nielegalnych produktach leczniczych.
Warszawa, 07-13.12.2020 – **wystąpienie ustne**

b) Doniesienie zjazdowe nr A2

“Synergy of interdisciplinary innovations”; Interdisciplinary Conference on Drug Science ACCORD 2022; ISBN 978-83-7637-586-1; s. 159.
Kalicka, A.; **Stępień, K.A.**; Giebułtowicz, J.; Fijałek, Z. Use of liquid chromatography associated with mass spectrometry (LC-MS/MS) to determine the composition of illegal pharmaceutical products containing anabolic-androgenic steroids.
Warszawa, 26-28.05.2022 – **sesja plakatowa**

c) Doniesienie zjazdowe nr A3

“Synergy of interdisciplinary innovations”; Interdisciplinary Conference on Drug Science ACCORD 2022; ISBN 978-83-7637-586-1; s. 97.
Franczak-Rogowska, M.K.; **Stępień, K.A.**; Pieńko, T.; Grudzień, M.E.; Grudzień, M.D.; Mazurek, A.A.; Mazurek, A.P. Possibility of using hetero-derivatives of fullerene $C_{60}$ as carriers of drugs containing benzene, naphthalene or anthracene ring - endohedral complexes $@C_{54}Y_6$ and $@C_{55}Y_5$.
Warszawa, 26-28.05.2022 – **sesja plakatowa**

d) Doniesienie zjazdowe nr A4

„Współczesna analityka farmaceutyczna i biomedyczna w ochronie zdrowia”; IV Poznańska Konferencja Naukowo-Szkoleniowa; ISBN 978-83-959554-9-5; s. 26.
Kalicka, A.; **Stępień, K.A.** The use of high-performance liquid chromatography coupled with mass spectrometry (LC-MS/MS) to identify pharmacologically active substances in falsified medicinal products and dietary supplements.
Poznań, 23-24.10.2023 – **wystąpienie ustne**