

Special Issue Reprint

Multi Methods for Detecting Natural Toxins

Edited by
Svetlana V. Malysheva

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Multi Methods for Detecting Natural Toxins

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Guest Editor

Svetlana V. Malysheva



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Guest Editor

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Contents

About the Editor	vii
Preface	ix
Svetlana V. Malysheva	
Multi Methods for Detecting Natural Toxins	
Reprinted from: <i>Toxins</i> 2026 , 18, 204, https://doi.org/10.3390/toxins18050204	1
Surangi H. Thilakarathna, Brendon Parsons and Linda Chui	
The Effect of Mitomycin C on Induction of Shiga Toxin Production in Clinical STEC Isolates	
Reprinted from: <i>Toxins</i> 2025 , 17, 267, https://doi.org/10.3390/toxins17060267	3
Svetlana V. Malysheva, Benoît Guillaume, Céline Vanhee and Julien Masquelier	
Determination of 16 Hydroxyanthracene Derivatives in Food Supplements Using LC-MS/MS: Method Development and Application	
Reprinted from: <i>Toxins</i> 2024 , 16, 505, https://doi.org/10.3390/toxins16120505	16
Lia Visintin, María García Nicolás, Sarah De Saeger and Marthe De Boevre	
Validation of a UPLC-MS/MS Method for Multi-Matrix Biomonitoring of <i>Alternaria</i> Toxins in Humans	
Reprinted from: <i>Toxins</i> 2024 , 16, 296, https://doi.org/10.3390/toxins16070296	31
Zhi Li, Bo Ma, Mengqiang Gong, Lei Guo, Lili Wang, Hua Xu and Jianwei Xie	
Sensitive Detection and Differentiation of Biologically Active Ricin and Abrin in Complex Matrices via Specific Neutralizing Antibody-Based Cytotoxicity Assay	
Reprinted from: <i>Toxins</i> 2024 , 16, 237, https://doi.org/10.3390/toxins16060237	46
Dvory Namdar, Patrick P. J. Mulder, Eyal Ben-Simchon, Yael Hacham, Loai Basheer, Ofer Cohen, et al.	
New Analytical Approach to Quinolizidine Alkaloids and Their Assumed Biosynthesis Pathways in Lupin Seeds	
Reprinted from: <i>Toxins</i> 2024 , 16, 163, https://doi.org/10.3390/toxins16030163	59
María del Carmen Pérez-Álvarez, Natalia Arroyo-Manzanares, Natalia Campillo and Pilar Viñas	
Magnetic Molecularly Imprinted Polymers for Selective Extraction of Aflatoxins from Feeds	
Reprinted from: <i>Toxins</i> 2024 , 16, 120, https://doi.org/10.3390/toxins16030120	77
Julien Masquelier, Céline Segers, Bram Jacobs, Tom Van Nieuwenhuysen, Laurence Delbrassinne and Els Van Hoeck	
Validation of a Targeted LC–MS/MS Method for Cereulide and Application in Food and Faeces	
Reprinted from: <i>Toxins</i> 2024 , 16, 13, https://doi.org/10.3390/toxins16010013	87
Marta Prygiel, Ewa Mosiej, Maciej Polak, Katarzyna Krysztopa-Grzybowska, Karol Wdowiak, Kamila Formińska and Aleksandra A. Zasada	
Challenges of Diphtheria Toxin Detection	
Reprinted from: <i>Toxins</i> 2024 , 16, 245, https://doi.org/10.3390/toxins16060245	100

About the Editor

Svetlana V. Malysheva

Svetlana V. Malysheva obtained her PhD degree in Pharmaceutical Sciences from Ghent University in Belgium. She currently works as a scientist at the Toxins unit of the Scientific Directorate Chemical and Physical Health Risks at Sciensano. Her research focuses on liquid chromatography–mass spectrometry (LC-MS) analysis of various plant toxins (pyrrolizidine alkaloids, tropane alkaloids, etc.). Dr. Malysheva has extensive expertise in method development, validation and application in food, feed and clinical matrices. Besides this, she is also a coordinator of the Exposome Working Group at Sciensano, bringing together different scientists to leverage the potential of available multidisciplinary expertise and research infrastructure to address exposome research.

Preface

This Reprint describes recently developed multi-target methods for detecting various natural toxins. It aims to provide new developments and improvements to support accurate toxin determination. The Reprint is intended for a scientific audience and is also insightful for policymakers, industry specialists or other audiences.

Svetlana V. Malysheva

Guest Editor

Editorial

Multi Methods for Detecting Natural Toxins

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Natural toxins constitute a large group of toxic compounds produced by plants, fungi, bacteria and other organisms. While these compounds are typically not poisonous to the producing organisms themselves, their transfer into food, feed, or the environment can result in significant risks to human and animal health. Their chemical diversity, occurrence at low levels, and presence in complex matrices make their reliable detection a persistent analytical challenge. Consequently, continuous advances in analytical methodologies are essential not only for accurate identification and quantification but also for exposure assessment, biomonitoring, and clinical diagnostics.

This Special Issue (SI) of the *Toxins* journal is dedicated to the theme “Multi Methods for Detecting Natural Toxins”. It provides a collection of articles that emphasise the simultaneous detection of multiple toxins, showcase innovations in sample preparation, and expand the applicability fields of toxin detection across food, feed, and human biomonitoring.

Advances in chromatography coupled to mass spectrometry have significantly enhanced the ability to simultaneously detect toxins of different origins and classes. Malysheva et al. developed and validated a sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the simultaneous quantification of hydroxyanthracene derivatives (HADs) in food supplements, addressing the urgent regulatory need for multi-analyte methods following the recent European Commission ban on specific genotoxic HADs [1]. In another study highlighting the chemical diversity of plant toxins, Namdar et al. combined the strengths of LC-MS/MS and gas chromatography-mass spectrometry techniques to establish comprehensive profiles of quinolizidine alkaloids in seeds of wild lupin species, providing essential insights into their complex biosynthesis pathways and potential for crop domestication [2].

Analysis of natural toxins involves handling complex sample matrices; therefore, the development of innovative sample preparation techniques and novel extraction methodologies to overcome the challenges posed by these matrices is highly sought after. Pérez-Álvarez et al. demonstrated the efficacy of magnetic molecularly imprinted polymers for the highly selective and efficient extraction of aflatoxins in complex pig feed matrices, eliminating the need for traditional, more labour-intensive solid-phase extraction [3].

An important highlight of this SI is the transition of analytical methods into clinical and biomonitoring applications. Visintin et al. validated a state-of-the-art ultra-high performance LC-MS/MS method for monitoring *Alternaria* toxins and their phase II conjugates across multiple human matrices, including urine, faeces, and capillary blood. Notably, the use of minimally invasive volumetric absorptive microsampling facilitates large-scale human biomonitoring studies [4]. Similarly, bridging food safety and clinical outcomes, Masquelier et al. applied a targeted LC-MS/MS method to successfully quantify the emetic *Bacillus cereus* toxin, cereulide, in both contaminated food and human faeces during a foodborne outbreak, providing a vital tool for clinical investigations [5].

Many current analytical strategies often cannot differentiate between biologically active and inactive toxins. To close this gap, Li et al. developed a high-throughput neutralising antibody-based cytotoxicity assay capable of precisely identifying and differentiating biologically active ricin and abrin in complex matrices such as milk, coffee, and plasma [6]. In clinical diagnostics, Thilakarathna et al. tackled the issue of discrepancies in results of polymerase chain reaction methods and enzymatic immunoassays for Shiga toxin-producing *Escherichia coli* by demonstrating that mitomycin C can induce Shiga toxin production in clinical isolates, thereby significantly improving diagnostic detection [7]. Highlighting ongoing diagnostic challenges, Prygiel et al. provided a comprehensive review of the evolution of diphtheria toxin detection, emphasising that while the traditional Elek test remains the gold standard, other promising assays (e.g., loop-mediated isothermal amplification), which can potentially be used for rapid point-of-care testing, have been developed [8].

Collectively, the articles of this SI constitute a cohesive scientific output that significantly advances the detection and monitoring of natural toxins. However, as some critical gaps remain, the exploration of novel areas should continue, including the development of standardised reference materials for toxins, the expansion of rapid point-of-care diagnostics, and the refinement of assays that can reliably distinguish between active and inactive toxins. By continuing to innovate in these areas, improvements and advances can be achieved in food and feed control and human biomonitoring, ultimately safeguarding human and animal health against the diverse threats posed by natural toxins.

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References

1. Malysheva, S.V.; Guillaume, B.; Vanhee, C.; Masquelier, J. Determination of 16 hydroxyanthracene derivatives in food supplements using LC-MS/MS: Method Development and application. *Toxins* **2024**, *16*, 505. [CrossRef] [PubMed]
2. Namdar, D.; Mulder, P.P.J.; Ben-Simchon, E.; Hacham, Y.; Basheer, L.; Cohen, O.; Sternberg, M.; Shelef, O. New analytical approach to quinolizidine alkaloids and their assumed biosynthesis pathways in lupin seeds. *Toxins* **2024**, *16*, 163. [CrossRef] [PubMed]
3. Pérez-Álvarez, M.d.C.; Arroyo-Manzanares, N.; Campillo, N.; Viñas, P. Magnetic molecularly imprinted polymers for selective extraction of aflatoxins from feeds. *Toxins* **2024**, *16*, 120. [CrossRef] [PubMed]
4. Visintin, L.; García Nicolás, M.; De Saeger, S.; De Boevre, M. Validation of a UPLC-MS/MS Method for multi-matrix biomonitoring of *Alternaria* toxins in humans. *Toxins* **2024**, *16*, 296. [CrossRef] [PubMed]
5. Masquelier, J.; Segers, C.; Jacobs, B.; Van Nieuwenhuysen, T.; Delbrassinne, L.; Van Hoeck, E. Validation of a targeted LC-MS/MS method for cereulide and application in food and faeces. *Toxins* **2024**, *16*, 13. [CrossRef] [PubMed]
6. Li, Z.; Ma, B.; Gong, M.; Guo, L.; Wang, L.; Xu, H.; Xie, J. Sensitive detection and differentiation of biologically active ricin and abrin in complex matrices via specific neutralizing antibody-based cytotoxicity assay. *Toxins* **2024**, *16*, 237. [CrossRef] [PubMed]
7. Thilakarathna, S.H.; Parsons, B.; Chui, L. The effect of mitomycin C on induction of Shiga toxin production in clinical STEC isolates. *Toxins* **2025**, *17*, 267. [CrossRef] [PubMed]
8. Prygiel, M.; Mosiej, E.; Polak, M.; Krysztopa-Grzybowska, K.; Wdowiak, K.; Formińska, K.; Zasada, A.A. Challenges of diphtheria toxin detection. *Toxins* **2024**, *16*, 245. [CrossRef] [PubMed]

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Article

The Effect of Mitomycin C on Induction of Shiga Toxin Production in Clinical STEC Isolates

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Abstract: Early determination of the Shiga toxin type of Shiga toxin-producing *Escherichia coli* (STEC) is crucial for guiding STEC-infected patients for proper and timely treatment and patient care. Most diagnostic microbiology laboratories rely on PCR assays to detect the presence of *stx1* and/or *stx2* and enzymatic immunoassays (EIA) to detect the presence of the Shiga toxins 1 and/or 2 in STEC-positive stool samples. Occasionally, the stool samples test positive for STEC by PCR assays but test negative for the presence of Shiga toxins. Insufficient toxin production under laboratory conditions is the main culprit of this discordance. To test whether EIA-based STEC detection could be improved, various clinical STEC strains were treated with mitomycin C, which is a commonly used inducer of Shiga toxin production. A dose-dependent increase in Shiga toxin production, in response to mitomycin C doses of up to 500 ng/mL, was observed without any bactericidal effects. Depending on the serotype, 5–50 times more Shiga toxin 2 was produced than Shiga toxin 1. Shiga toxin production was not induced by the mitomycin C treatment in certain STEC serotypes carrying the toxin subtypes *stx1a*, *stx2a*, *2b*, *2f*, or *2h*. This diversity in toxin production indicates that other factors may determine toxin expression in certain STEC strains, which warrant further exploration.

Keywords: Shiga toxin-producing *Escherichia coli* (STEC); mitomycin C; enzymatic immunoassays (EIA); STEC subtypes; Shiga toxin production; diagnostics

Key Contribution: This study evaluated mitomycin C as an inducer of Shiga toxin production from clinical STEC in broth and in stool samples.

1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) is a Gram-negative gastroenteric bacterial pathogen that can cause typical acute gastroenteritis symptoms like diarrhea, vomiting, fever, and stomach pain [1]. However, some STEC strains are highly virulent and can cause severe disease outcomes [1]. Generally, STEC strains only expressing *stx1* are associated with mild disease or asymptomatic carriage, while strains expressing both *stx1* and *stx2* are associated with greater pathogenicity, and strains expressing *stx2* alone are recognized as ‘high risk’ due to their association with severe disease outcomes such as hemolytic uremic syndrome (HUS) and bloody diarrhea [2,3]. These highly virulent *stx2* STEC strains need to be managed cautiously, as administration of antibiotics to patients infected with *stx2* STEC strains have been linked with adverse reactions and worsened severe symptoms leading

to kidney damage and even death [4]. Therefore, identifying the Shiga toxin type early in the diagnosis is crucial for STEC infection management and guiding patients for proper treatments and care.

Frontline diagnostic laboratories in Alberta use BD MAXTM (BD Life Sciences, Sparks, MD, USA), a multi-analyte nucleic acid amplification platform, to detect the presence of enteric pathogens in patient stool specimens. The BD MAXTM enteric bacterial panel includes specifically designed sets of primers and probes for *Salmonella* spp., *Shigella* spp./enteroinvasive *Escherichia coli* (EIEC), *Campylobacter* spp., and STEC [5,6]. The STEC primers and probes used in the BD MAXTM panel are designed to detect the *stx1/stx2* genes but without any differentiation between them [5]. For further differentiation between the two toxins, laboratories can perform specific PCR assays or enzyme immunoassays (EIA). The SHIGA TOXIN QUIK CHEKTM test is a qualitative EIA kit developed by TECHLAB, Inc. (Blacksburg, VA, USA) and is widely used in Canadian microbiology laboratories. Using monoclonal and polyclonal antibodies, this EIA can detect the presence of Shiga toxin 1 and Shiga toxin 2 and differentiate one from the other [7]. However, there are instances in which a stool sample tests positive for STEC by BD MAXTM, but negative for the presence of Shiga toxins using these EIA kits (personal communication). These contradictory results bring challenges to the frontline laboratories when reporting STEC infections. In such a scenario in Alberta, these stool samples are forwarded to Alberta Precision Laboratories-Provincial Laboratory for Public Health (ProvLab) for further investigations. This additional testing increases the costs and turnaround time for reporting.

A negative EIA result could be due to numerous reasons, such as a low bacterial load resulting in the reduction of toxin level or decreased expression of the *stx* gene in STEC below the detection level of the assay. If a limited *stx* expression in the stool failed to be detected by the EIA, the addition of an inducer can potentially enhance the Shiga toxin production. Application of antibiotics as enhancers to induce Shiga toxin production under laboratory settings has been previously suggested [7–9]. Antibiotic treatment is generally not recommended for patients with STEC infections, as certain antibiotics can induce Shiga toxin overproduction as a response to the triggered host SOS response [3,10]. Although this is an extremely critical condition in the clinical setting, it can be advantageous in a diagnostic setting to improve test methods such as EIA. Mitomycin C is one of the antibiotics most commonly used to induce Shiga toxin production in a laboratory setting. However, the use of mitomycin C in a clinical setting to induce Shiga toxin production in STEC isolates or STEC-positive stool samples has not been previously reported. Therefore, the objective of this study was to evaluate the effect of mitomycin C on the enhancement of Shiga toxin production by clinically relevant STEC isolates in broth and in stool samples.

2. Results

2.1. Shiga Toxin Production by Three STEC Isolates Treated with Different Concentrations of Mitomycin C

To assess the effect of mitomycin C on Shiga toxin induction, three STEC isolates with distinct Shiga toxin gene profiles were exposed to increasing concentrations of the antibiotic in trypticase soy broth (TSB), incubated overnight, and the toxin production was subsequently measured. Shiga toxin production in the three tested STEC isolates increased with the increasing mitomycin C concentrations in a dose-dependent manner (Table 1). The highest mitomycin C concentration, which was 500 ng/mL, induced the production of the highest amount of Shiga toxin in the tested STEC isolates (Supplementary Figure S1, $p < 0.05$). Real-time PCR Ct values for all tested mitomycin C concentrations stayed consistent at ~13 Ct for O5:H19. For O26:H11 and O157:H7, the Ct values ranged

from ~8–12 and decreased by ~1–3 Ct when treated with >10 ng/mL mitomycin C. These results showed that the Shiga toxin production was induced after the overnight incubation with the mitomycin C treatment. There was a difference between the production of Shiga toxin 1 and 2, as Shiga toxin 2 was increased 5–50 times more than Shiga toxin 1 after the mitomycin C treatment. Compared to the O157 *stx1/2* strain, the O26 strain was a high toxin producer, where ~10 times more Shiga toxin 2 was produced by O26:H11 treated with 500 ng/mL mitomycin C.

Table 1. Induction of Shiga toxin production by different concentrations of mitomycin C after overnight enrichment in TSB.

STEC Isolate	<i>stx</i> Subtype	Mitomycin C Conc. (ng/mL)	Avg Ct $\pm$ STDEV	Avg Toxin Conc. $\pm$ STDEV (ng/mL)	SHIGA TOXIN QUIK CHEK™
O5:H19	<i>stx1c</i>	0	13.82 $\pm$ 0.41	0.37 $\pm$ 0.03	+
		5	13.70 $\pm$ 0.18	0.84 $\pm$ 0.07	+
		10	13.81 $\pm$ 0.31	1.32 $\pm$ 0.08	+
		25	13.62 $\pm$ 0.19	2.30 $\pm$ 0.17	+
		50	13.31 $\pm$ 0.23	5.58 $\pm$ 0.92	+
		100	12.84 $\pm$ 0.11	13.81 $\pm$ 1.53	+
		500	13.17 $\pm$ 0.32	45.50 $\pm$ 4.84	+
O26:H11	<i>stx2a</i>	0	11.49 $\pm$ 0.08	21.76 $\pm$ 2.30	+
		5	11.03 $\pm$ 0.09	48.70 $\pm$ 17.07	+
		10	10.49 $\pm$ 0.11	90.88 $\pm$ 27.26	+
		25	9.67 $\pm$ 0.26	233.10 $\pm$ 92.74	+
		50	8.62 $\pm$ 0.07	698.94 $\pm$ 232.98	+
		100	7.66 $\pm$ 0.10	1947.25 $\pm$ 481.40	+
		500	9.40 $\pm$ 0.66	2582.43 $\pm$ 706.71	+
O157:H7 (<i>stx1/2</i>)	<i>stx1a</i>	0	11.64 $\pm$ 0.46	10.62 $\pm$ 0.03	+
		5	11.19 $\pm$ 0.24	10.96 $\pm$ 0.36	+
		10	10.78 $\pm$ 0.14	12.48 $\pm$ 0.36	+
		25	10.36 $\pm$ 0.07	19.89 $\pm$ 3.51	+
		50	9.94 $\pm$ 0.18	22.26 $\pm$ 1.50	+
		100	9.67 $\pm$ 0.11	29.51 $\pm$ 2.81	+
		500	9.95 $\pm$ 0.23	45.93 $\pm$ 1.38	+
	<i>stx2a</i>	0	11.59 $\pm$ 0.09	17.92 $\pm$ 1.24	+
		5	11.16 $\pm$ 0.12	41.87 $\pm$ 1.72	+
		10	10.86 $\pm$ 0.16	58.10 $\pm$ 1.97	+
		25	10.14 $\pm$ 0.12	106.68 $\pm$ 4.79	+
		50	9.52 $\pm$ 0.10	159.82 $\pm$ 1.56	+
		100	9.34 $\pm$ 0.04	204.49 $\pm$ 8.74	+
		500	9.34 $\pm$ 0.07	237.31 $\pm$ 7.60	+

Experiments and analyses were performed in triplicate on three individual days. Data reported as mean $\pm$ STDEV. '+': Positive.

2.2. Shiga Toxin Production by Clinical STEC Isolates Treated with 500 ng/mL Mitomycin C

To evaluate Shiga toxin induction in other clinical STEC isolates by the optimum mitomycin C concentration (500 ng/mL), 53 clinical STEC isolates were enriched overnight in TSB with and without the mitomycin C treatment, and their growth and toxin production were assessed. The PCR Ct values for the non-treated and the mitomycin C-treated broths were similar for ~80% of the isolates, where Δ Ct was <1 (Table 2). This was an indication that the mitomycin C treatment did not significantly affect the growth of the majority of isolates. Toxin quantification was not performed for these samples.

Table 2. Shiga toxin production by 53 STEC isolates with and without mitomycin C treatment after overnight enrichment in TSB.

Organism	Subtype	SHIGA TOXIN QUIK CHEK™				PCR (Ct)			
		NT		MC		NT		MC	
		<i>stx1</i>	<i>stx2</i>	<i>stx1</i>	<i>stx2</i>	<i>stx1</i>	<i>stx2</i>	<i>stx1</i>	<i>stx2</i>
O118:H2	<i>stx1a</i>	+		+		12.14		12.81	
O118:H16/O151	<i>stx1a</i>	+		+		11.69		11.71	
O118/O151:H16	<i>stx1a</i>	+		+		11.92		11.31	
O118/O151:H2	<i>stx1a</i>	+		+		11.11		12.07	
O121:H19	<i>stx1a</i>	+		+		11.58		11.34	
O121:H19	<i>stx1a</i>	+		+		11.94		10.84	
O121:H19	<i>stx2a</i>		+		+		12.46		11.90
O111:H8	<i>stx1a</i>	+		+		11.12		12.31	
O111:H Non-motile	<i>stx1a</i>	+		+		12.19		12.79	
O111:H Non-motile	<i>stx1a, stx2a</i>	+	+	+	+	11.44	11.92	11.34	11.21
O123/O186:H2	<i>stx1a</i>	+		+		12.09		12.03	
O123/O186:H2	<i>stx1a</i>	+		+		11.68		11.12	
O123/O186:H2	<i>stx1a, stx2a</i>	+	+	+	+	11.48	11.97	11.61	12.11
O146:H21	<i>stx1c</i>	+		+		13.64		13.69	
O146:H21	<i>stx1c, stx2b</i>	+	+	+	+	13.15	12.49	13.06	12.30
O146:H21	<i>stx1c, stx2b</i>	+	-	+	+	13.48	13.09	13.73	13.19
O26:H11	<i>stx1a</i>	+		+		11.91		12.35	
O26:H11	<i>stx2a</i>		-		-		11.90		9.65
O26:H11	<i>stx1a, stx2a</i>	+	+	+	+	11.66	11.68	11.44	11.56
O103:H2	<i>stx1a</i>	+		+		11.82		12.11	
O103:H25	<i>stx1a</i>	+		+		12.05		12.14	
O108:H21	<i>stx1c</i>	-		+		13.34		13.37	
O108:H2	<i>stx2a, stx2d</i>		+		+		12.40		9.85
O145:H Non-motile	<i>stx1a</i>	+		+		11.89		11.67	
O145:H7	<i>stx2a</i>		+		+		11.29		10.87
O156:H25	<i>stx1a</i>	+		+		11.64		11.97	
O156:H14	<i>stx2f/h/b</i>		-		-		15.21		15.71
O157:H7	<i>stx2a</i>		-		-		11.90		10.88
O157:H7	<i>stx1a, stx2c</i>	+	+	+	+	11.79	12.25	11.89	12.51
O166:H15	<i>stx2f/h/b</i>		+		+		13.83		13.70
O166:H15	<i>stx2h</i>		-		+		14.37		13.59
O177:H11	<i>stx1a</i>	+		+		11.51		11.50	
O177:H25	<i>stx2c</i>		+		+		12.65		12.14
O5:H9	<i>stx1a</i>	+		+		10.02		10.39	
O5:H9	<i>stx1a, stx2a</i>	+	+	+	+	10.95	11.46	11.35	11.51
O71:H8	<i>stx1</i>	+		+		13.00		12.31	
O71:H8	<i>stx1a, stx2c</i>	+	+	+	+	12.33	12.31	12.96	11.35
O91:H14	<i>stx2b</i>		+		+		12.91		12.56
O91:H14	<i>stx1, stx2b</i>	+	+	+	+	11.24	11.59	16.20	15.32
O98:H Non-motile	<i>stx1a</i>	+		+		11.73		12.11	
O98:H Non-motile	<i>stx1a, stx2a</i>	+	+	+	+	11.48	11.73	11.48	10.48
O85:H1	<i>stx2f/h/b</i>		+		+		13.14		13.75
O85:H2	<i>stx1a</i>	+		+		11.77		11.25	
O69:H11	<i>stx1a</i>	+		+		13.34		12.80	

Table 2. Cont.

Organism	Subtype	SHIGA TOXIN QUIK CHEK™				PCR (Ct)			
		NT		MC		NT		MC	
		<i>stx1</i>	<i>stx2</i>	<i>stx1</i>	<i>stx2</i>	<i>stx1</i>	<i>stx2</i>	<i>stx1</i>	<i>stx2</i>
O84:H Non-motile	<i>stx1a</i>	+		+		12.14		11.71	
O140:H21	<i>stx2c</i>		+ *		+ *		10.92		10.84
O17/O77/O44/O106:H45	<i>stx2d</i>		-		-		12.31		12.56
O113:H4	<i>stx2d</i>		+		+		11.44		10.91
O25:H1	<i>stx2e</i>		-		+		12.95		11.43
O2/O50:H7	<i>stx2f/h/b</i>		-		-		13.44		14.33
O75:H8	<i>stx1c, stx2b</i>	+	+	+	+	12.74	12.39	12.79	11.43
O112:H21	<i>stx1c, stx2b</i>	+	+	+	+	13.53	13.14	13.78	12.78
O128:H2	<i>stx1c, stx2b</i>	+	+	+	+	13.50	12.91	13.42	12.60
O175:H27(negative control)	-	ND	ND	ND	ND	ND	ND	ND	ND

* Very faint bands. NT: Non-treated, MC: Mitomycin C-treated. STEC serotypes with different strains are grouped. '+': Positive, '-': Negative, ND: Not detected.

Shiga toxin production was not detected by the SHIGA TOXIN QUIK CHEK™ in ~17% (9/53) of the isolates under non-treated conditions (Table 2). After an overnight enrichment with mitomycin C, 4/9 of isolates (~44%) produced sufficient amounts of toxin to be detected by EIA. The mitomycin C treatment did not enhance the toxin production of the other five isolates that showed negative EIA results. These five STEC isolates belonged to different serotypes (O26:H11 *stx2a*, O156:H14 *stx2f/h/b*, O157:H7 *stx2a*, O17/O77/O44/O106:H45 *stx2d*, and O2/O50:H7 *stx2f/h/b*), indicating lack of a direct relationship between the serotype or the toxin subtype and the limited toxin production. Two other STEC isolates (O166:H15 and O85:H1) with the *stx2f/h/b* toxin subtype were weakly detected by EIA with and without the mitomycin C treatment. It is also possible that these *stx2* toxin subtypes are not effective antigenic responders. Supplementary Figure S2 shows improved SHIGA TOXIN QUIK CHEK™ results after the mitomycin C treatment and results for high and low Shiga toxin-producing STEC isolates.

2.3. Shiga Toxin Production by STEC in Clinical Stool Samples Treated with 500 ng/mL Mitomycin C

To evaluate the impact of enrichment media and mitomycin C treatment on Shiga toxin detection from clinical stool samples, 15 STEC-positive patient-stool samples were analyzed for toxin gene profiles, growth performance, and toxin expression across two culture media: TSB and Gram-negative broth (GNB). From the tested stool samples, 7/15 were *stx1*-positive, 3/15 were *stx2*-positive, and 5/15 were *stx1/2*-positive. Based on the PCR results, GNB was the better enrichment medium for both non-treated and mitomycin C-treated stool samples (Ct values: TSB > GNB, Table 3). Only two stool samples with O157:H7 *stx1a/stx2a* (ID #2) and O26:H11 *stx1a* (ID #5) showed better growth in TSB under non-treated or mitomycin C-treated conditions (Table 3). Considering potential sampling error, TSB-GNB < -1 was not considered as a treatment effect.

The average amount of toxin produced across all STEC stool samples enriched in TSB with and without the mitomycin C treatment was statistically similar (TSB_{NT} vs. TSB_{MC}; $p > 0.05$). On the other hand, STEC stool samples enriched in GNB produced a significantly higher amount of toxin with the mitomycin C treatment compared to the non-treated samples ($p = 0.0077$). Among the two media, GNB trended toward producing more Shiga toxin compared to TSB with and without the mitomycin C treatment (mean $\pm$ SD for the amount of toxin produced (Shiga toxin 1 and 2 combined, ng/mL) by each treatment:

TSB_{NT}: 0.49 ± 0.58 , TSB_{MC}: 1.67 ± 2.77 , GNB_{NT}: 1.06 ± 1.46 , GNB_{MC}: 3.34 ± 5.16). STEC with *stx2* in clinical stool samples trended to produce more Shiga toxins compared to STEC with *stx1* (Table 4), although a statistically significant difference was not observed.

Table 3. Real-time PCR results for 15 STEC-positive stool samples after overnight enrichment in TSB and GNB with and without mitomycin C.

ID	STEC Serotypes	<i>stx</i> Subtype	PCR (Ct)											
			<i>stx1</i>						<i>stx2</i>					
			TSB		GNB		* $\Delta C_{tTSB-GNB}$		TSB		GNB		* $\Delta C_{tTSB-GNB}$	
			NT	MC	NT	MC	NT	MC	NT	MC	NT	MC	NT	MC
1	O157:H7	<i>stx2a, stx2c</i>							16.1	15.9	16.9	15.4	−0.8	0.5
2		<i>stx1a, stx2a</i>	12.6	12.6	15.2	13.4	−2.6	−0.8	13.1	11.1	15.9	12.8	−2.9	−1.8
3		<i>stx1a, stx2a</i>	16.4	15.8	16.6	15.7	−0.2	0.1	16.8	16.3	16.7	16.1	0.0	0.1
4		<i>stx1a, stx2a</i>	15.0	14.1	15.0	13.2	0	0.9	15.3	14.4	15.2	13.5	0.0	0.9
5	O26:H11	<i>stx1a</i>	18.8	18.9	23.1	23.4	−4.3	−4.5						
6		<i>stx1a</i>	20.8	20.4	18.5	18.8	2.3	1.6						
7	O103:H2	<i>stx1a</i>	15.4	14.5	14.6	15.0	0.8	−0.5						
8		<i>stx1a</i>	25.1	23.1	20.8	19.7	4.3	3.4						
9	O151/O118:H16	<i>stx1a</i>	17.3	18.0	16.8	17.2	0.5	0.8						
10		<i>stx1a</i>	26.7	26.4	23.5	23.5	3.2	2.9						
11	O91:H14	<i>stx1a</i>	15.3	14.2	13.7	14.4	1.6	−0.2						
12	O121:H19	<i>stx2a</i>							18.0	18.0	16.9	15.8	1.1	2.2
13	O9:H7	<i>stx2a</i>							13.6	13.5	12.6	12.5	1.1	0.9
14	O146:H21	<i>stx1c, stx2b</i>	16.3	16.0	14.0	14.1	2.3	1.9	15.6	15.4	13.9	13.2	1.7	2.1
15	O128:H2/O5:H9	<i>stx1a, stx1c, stx2b</i>	15.4	14.7	13.9	15.2	1.5	−0.5	15.0	14.3	13.9	14.6	1.1	−0.3

NT: non-treated, MC: mitomycin C, TSB: trypticase soy broth, GNB: Gram-negative broth. * Interpretations. Positive $\Delta C_{tTSB-GNB}$: GNB improved STEC growth, Negative $\Delta C_{tTSB-GNB}$: TSB improved STEC growth.

Table 4. SHIGA TOXIN QUIK CHEKTM and STEC-ELISA results for 15 STEC-positive stool samples in TSB and GNB with and without mitomycin C treatment after overnight enrichment.

ID	Serotype	<i>stx</i> Subtype	<i>stx1</i>								<i>stx2</i>							
			SHIGA TOXIN QUIK CHEK TM				Toxin Conc. (ng/mL Toxin)				SHIGA TOXIN QUIK CHEK TM				Toxin Conc. (ng/mL Toxin)			
			TSB		GNB		TSB		GNB		TSB		GNB		TSB		GNB	
			NT	MC	NT	MC	NT	MC	NT	MC	NT	MC	NT	MC	NT	MC	NT	MC
1	O157:H7	<i>stx2a, stx2c</i>									+	+	+	+	1.50	9.06	3.90	15.71
2		<i>stx1a, stx2a</i>	+	+	+	+	1.62	6.51	4.06	7.83	-	+	+	+	ND	137.79	11.01	582.76
3		<i>stx1a, stx2a</i>	+	+	+	+	1.36	1.11	1.72	1.94	+	+	+	+	0.81	5.05	0.83	8.41
4		<i>stx1a, stx2a</i>	+	+	+	+	0.62	1.59	0.97	4.42	+	+	+	+	0.69	14.27	1.71	12.99
5	O26:H11	<i>stx1a</i>	-	-	-	-	0.19	0.20	ND	ND								
6		<i>stx1a</i>	-	-	-	+	ND	ND	ND	0.21								
7	O103:H2	<i>stx1a</i>	-	+	-	+	ND	ND	ND	0.19								
8		<i>stx1a</i>	+	+	+	+	0.20	0.34	0.22	1.46								
9	O151/O118:H16	<i>stx1a</i>	-	+	+	+	0.23	0.30	0.39	0.44								
10		<i>stx1a</i>	-	-	-	-	ND	ND	ND	ND								
11	O91:H14	<i>stx1a</i>	+	+	-	+	0.36	0.42	0.45	0.49								
12	O121:H19	<i>stx2a</i>									+	+	+	+	0.56	3.05	2.72	12.92
13	O9:H7	<i>stx2a</i>									-	-	-	-	ND	ND	ND	ND
14	O146:H21	<i>stx1c, stx2b</i>	-	+	+	+	0.11	0.44	0.33	1.08	-	+	+	+	ND	2.86	ND	15.96
15	O128:H2/O5:H9	<i>stx1a, stx1c, stx2b</i>	-	-	-	-	0.10	0.22	0.12	ND	-	+	-	+	ND	1.98	ND	5.77

NT: non-treated, MC: mitomycin C, TSB: trypticase soy broth, GNB: Gram-negative broth, ND: not detected. ‘+’: Positive, ‘-’: Negative.

Overall, 5/15 stool samples (~33%) showed real-time PCR-positive but SHIGA TOXIN QUIK CHEKTM-negative results for both non-treated and mitomycin C-treated conditions

in both broths (Supplementary Figure S3). From those five stool samples, two had Ct values between 13–19, indicating bacterial loads of $\sim 10^6$ – 10^8 CFU/mL based on our preliminary results (unpublished data). Therefore, even with low Ct values that pointed to higher bacterial loads, some serotypes, such as O128:H2/O5:H9 *stx1/2*, O9:H7 *stx2*, O151/O118:H16 *stx1*, O103:H2 *stx1*, and O26:H11 *stx1*, produced very low amounts of toxins that were not detected by either EIA, which indicates a need for further investigation.

3. Discussion

PCR and EIA are assays commonly used in diagnostic laboratories to detect STEC in stool samples [11,12]. While PCR assays are designed to detect the STEC DNA, EIAs are designed to detect the Shiga toxins produced by STEC [11]. Therefore, in certain situations, the PCR and EIA results might not corroborate. For an example, under laboratory conditions, a PCR-positive stool sample can show a negative EIA result. This is an indication that although STEC is present in the stool sample, the expression of *stx* is at a suboptimal level. In such scenarios, Shiga toxin induction can be highly beneficial to produce a sufficient level of Shiga toxin to obtain positive EIA results. A large number of studies are available on Shiga toxin induction by various inducers, including antibiotics, to investigate clinical outcomes in patients [4,8,13–15]. However, studies performed on in vitro Shiga toxin induction for the purpose of STEC detection are limited. The current study evaluated the ability of mitomycin C treatment to improve EIA results under laboratory conditions for effective STEC detection.

This study evaluated Shiga toxin production by STEC after an overnight enrichment with mitomycin C in culture broths. Three STEC isolates that represented *stx1*, *stx2*, and *stx1/2* were selected for optimizing the mitomycin concentration to use in the experiments. We observed that overall, Shiga toxin production by *stx1* STEC isolates was low compared to *stx2* STEC isolates. The difference was more apparent with increasing mitomycin C concentrations. Our findings are in agreement with previous reports where mitomycin C induced the production of more Shiga toxin 2 than Shiga toxin 1 [16]. Shiga toxins 1 and 2 are induced by different induction methods [16]. Phage-inducing agents like mitomycin C induce the expression of *stx2* and activate Shiga toxin 2 production, while low iron conditions activate the production of Shiga toxin 1 [10,16]. Therefore, when considering enhancing Shiga toxin production in a clinical laboratory setting for STEC detection, methods that are effective in inducing both Shiga toxins need to be carefully considered.

STEC growth and toxin production can depend on the culture media used. Preliminary experiments carried out to investigate the growth of the three STEC isolates (O5:H19 *stx1*, O26:H11 *stx2*, and O157:H7 *stx1/2*) in TSB and GNB showed that the isolates in pure culture preferred TSB over GNB, which was in agreement with a previous study [17]. GNB selectively enhances the growth of Gram-negative bacteria in a heterogeneous mixture of Gram-positive and Gram-negative bacteria. Since the bacterial load inoculated into the 3 mL broth was ~ 100 CFU/mL in the current study and no other competing organisms were present in the broth, it appeared that the growth of the STEC isolates was not well supported by GNB. TSB, on the other hand, is a general medium and supported STEC isolate growth. Therefore, TSB was selected as the enrichment medium for all experiments involving pure STEC isolates (in broth media). Since different diagnostic microbiology laboratories use either GNB or TSB for overnight stool enrichment, both TSB and GNB were tested for STEC-positive stool enrichment. According to the results, STEC growth in stool samples was better supported by GNB compared to TSB, but toxin production was similar in both TSB and GNB. Random sampling error may influence the findings; however, using 10% stool suspensions in the experiments would help minimize this error. A previous

study reported better STEC growth in TSB when spiked in STEC-negative stool samples, but Shiga toxin production was not assessed in that study, preventing comparisons with our findings [17]. It is possible that stool microbiome in STEC-positive stool samples in our study contributed to the observed differences from the previous study. Contrasting with our findings, a study reported that Shiga toxin production depended on the type of broth used to culture STEC [18]. Among the eight broths tested by the authors, TSB resulted in low toxin production compared to the better-performing *E. coli* broth [18]. The results cannot be directly compared with our findings, as GNB was not tested in that study.

The *stx* expression mainly depends on prophage induction [19]. Numerous antibiotics have been tested for their prophage induction ability on STEC [15,20]. Antibiotics such as gentamicin, azithromycin, and meropenem did not induce Shiga toxin production in highly virulent *stx2a* STEC [20]. On the other hand, mitomycin C is a commonly used prophage-inducing antimicrobial [14,20]. Due to its potent effect, it has been used as a positive control to test the toxin induction ability of other antibiotics [20]. Mitomycin C can trigger the host SOS response by releasing H₂O₂ and damaging host DNA through the promotion of cross-linking events, alkylation, DNA strand breakage [14], and activation of RecA (a regulator of bacterial SOS response) [19], leading to lytic phage activation and Shiga toxin production [19]. Recovery or cell death from the DNA damage is dependent on the released H₂O₂ dose, as a low H₂O₂ dose can promote DNA repair [14]. Therefore, both the STEC growth and toxin expression can be promoted by the appropriate mitomycin C concentration. Previous studies have used mitomycin C concentrations ranging from 50 ng/mL to 50 µg/mL [9,14,20]. We observed concentration-dependent Shiga toxin production when mitomycin C was applied at 5–500 ng/mL. We tested the mitomycin C response of a limited number of STEC isolates, which is a limitation of this study. Therefore, it is challenging to generalize the optimum mitomycin C concentration or the effective range of concentrations across all STEC strains and/or *stx* subtypes.

Mitomycin C has been used in previous studies to induce Shiga toxin production in STEC subtypes. Mitomycin C increased the toxin production in different clinical STEC strains with *stx1a*, *stx2a*, *2c*, *2d* [21], and non-human STEC strains with *stx2e* subtypes [22]. From the STEC isolates tested in the current study, there was one O25:H1 with the *stx2e* subtype. In agreement with the previous findings [22], this particular STEC did not show toxin production when non-treated, but showed positive SHIGA TOXIN QUIK CHEK™ results after treatment with mitomycin C. Mitomycin C reportedly increased Shiga toxin 2 production in the *stx2a* subtype of 12 highly virulent STEC serotypes [20]. In the current study, ~20% of STEC isolates (pure culture) and ~33% of STEC strains in stool samples carried the *stx2a* subtype. However, not all of these STEC isolates with *stx2a* responded the same to the mitomycin C treatment. Shiga toxin production was not improved in O157:H7, O26:H11 (Table 2, STEC isolates in culture), or O9:H7 (Table 4, STEC in stool), after the mitomycin C treatment. Apart from *stx2a*, other serotypes with *stx2d* and *stx2f/h/b* subtypes were also not affected by the mitomycin C treatment. For example, O26:H11 with *stx2a*, O17/O77/O44/O106:H45 with *stx2d*, O2/O50:H7, and O156:H14 with *stx2f/h/b* did not produce detectable levels of toxins after the mitomycin C treatment. The selective induction of Shiga toxin production by mitomycin C in certain STEC serotypes and/or subtypes, but not in others, highlights the need for further investigation into the underlying mechanisms. Some STEC strains with a higher basal level of Shiga toxin expression are known to be less responsive to inducers such as mitomycin C [21]. This might not be directly relevant to the STEC isolates that were unaffected by the mitomycin C treatment in the current study, as they showed negative EIA results when non-treated. More STEC serotypes and subtypes

need to be tested to better understand the mitomycin C-induced Shiga toxin production by STEC.

The current study used primers and probes developed to detect *stx* gene in STEC [1,23]. Specifically, the TaqMan probes used in the real-time PCR assay differentiated the *stx1* from *stx2* [23]. Therefore, using these sets of real-time PCR primers and probes was an added advantage, as it served as a secondary assay that confirmed the EIA results. Further, when no Shiga toxins were detected by the EIA for PCR-positive samples, the secondary real-time PCR results were beneficial to confirm positive bacterial growth with either no toxin production or inability of the EIA to detect the produced toxin. The small STEC-positive stool sample size is a major limitation in our study. A larger sample size would be beneficial to minimize the effect from the observed variability in Shiga toxin production. Further, we tested only 53 STEC isolates (pure culture). Due to resource limitations, only the presence of Shiga toxin was determined for these 53 STEC isolates, without quantification by the RUO Quantitative Shiga toxin ELISA assay. This is a limitation of the study, as the amount of toxin produced was not determined for comparison with the toxin produced by different STEC serotypes and/or subtypes. To address the limitations, a more comprehensive study testing more STEC serotypes and *stx* subtypes and a larger number of stool samples needs to be performed.

4. Conclusions

This study focused on using mitomycin C, a commonly used antibiotic, as an enhancer to induce the production of Shiga toxin in clinical STEC isolates. Most of the tested STEC isolates positively responded to the mitomycin C treatment. As Shiga toxin 2 was enhanced more by the mitomycin C treatment than Shiga toxin 1, it is ideal to consider methods suitable for both *stx1* and *stx2* induction. However, some STEC isolates responded poorly to mitomycin C regardless of the *stx* type. At this point, a clear correlation between mitomycin-induced Shiga toxin production and the STEC serotype or subtype was not observed. More research is warranted to understand the effects of antibiotics on Shiga toxin induction by STEC in order to consider antibiotics as enhancers of Shiga toxin production in a diagnostic laboratory setting.

5. Materials and Methods

5.1. STEC Isolates and Growth Conditions

Clinical strains of STEC frozen in skim milk were obtained from Alberta Precision Laboratories-Provincial Laboratory for Public Health (ProvLab). Upon receipt, the isolates were plated on sheep blood agar plates (BAP, Dalynn Biologicals, Calgary, AB, Canada) and were incubated overnight at 37 °C. On the following day, a single colony from each BAP was inoculated separately into TSB (BBL™ Trypticase™ Soy Broth, Becton, Dickinson and Company, Sparks, MD, USA) and incubated at 37 °C with moderate shaking until the OD value reached ~0.5 (for ~3–4 h) (MicroScan Turbidity Meter, Siemens Healthcare Diagnostics Limited, Los Angeles, CA, USA). Our preliminary data showed a 10^{-6} dilution prepared from an enriched broth culture with a 0.5 OD was equivalent to ~1000 CFU/mL; therefore, 10^{-6} dilution was selected for the mitomycin C treatments. Using each enriched broth culture, 10-fold serial dilutions from neat to 10^{-6} were prepared in phosphate saline buffer (PBS), and 100 µL of 10^{-6} dilution was used in experiments as described in 5.5. Three clinical STEC isolates with different serotypes and *stx* types were selected to perform the experiments for determining the optimal mitomycin C concentration. They were O5:H19 (*stx1c*), O26:H11 (*stx2a*), and O157:H7 (*stx1a* and *stx2a*).

5.2. Nucleic Acid Extraction

A 100 μ L aliquot from each mitomycin C-treated and non-treated enriched broth culture was centrifuged at $17,115\times g$ for 5 min. The supernatant was removed, and the cell pellets were resuspended in 100 μ L of rapid lysis buffer (RLB; 100 mM NaCl, 10 mM Tris-HCL pH 8.3, 1 mM EDTA pH 9.0, 1% Triton X-100), then heated at 95 °C for 15 min. The suspension was centrifuged at $17,115\times g$ for 5 min, and 5 μ L of the supernatant was used as the template for the real-time PCR.

5.3. Real-Time PCR Assay Conditions

The in-house primer-probe (Integrated DNA Technologies, IDT, Skokie, IL, USA) set that targeted a conserved region of the *stx* gene [23] was used in the real-time PCR assays. Primers and probes used in the study are listed in Table 5.

Table 5. Primer and probe sequences used in this study *.

Reference Gene/Primer/Probe	Sequence 5'-3'
<i>stx1</i> -Forward	TTT GTY ACT GTS ACA GCW GAA GCY TTA CG
<i>stx1</i> -Reverse	CCC CAG TTC ARWGTR AGR TCM ACR TC
<i>stx1</i> -Probe	FAM/CTGGATGAT/ZEN/CTCAGTGGGCGTTCTTATGTAA/IABkFQ
<i>stx2</i> - Forward	TTT GTY ACT GTS ACA GCW GAA GCY TTA CG
<i>stx2</i> -Reverse	CCC CAG TTC ARWGTR AGR TCM ACR TC
<i>stx2</i> -Probe	YAkYel/TCGTCAGGC/ZEN/ACTGTCTGAAACTGCTCC/IABkFQ

* The primers and probe sequences were adapted from Perelle et al. 2004 [23]. Y is (C, T), S is (C, G), W is (A, T), R is (A, G), M is (A, C).

The real-time PCR mixture was prepared by combining 10 μ L of 2X PrimeTime[®] Gene Expression Master mix (Integrated DNA Technologies, IDT, Skokie, IL, USA), 2 μ L of nuclease-free water (Invitrogen[™], Live Technologies, Rochester, NY, USA), 3 μ L of the in-house primer-probe mixture (0.22 μ M final concentration of the probe, 0.33 μ M final concentration of each of the primers), and 5 μ L of the DNA template to contain a final reaction volume of 20 μ L. Positive DNA for the *stx* gene and a no-template control (nuclease-free water) were included in each run. Amplification conditions consisted of 95 °C for 3 min followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s on the 7500 FAST real-time PCR system (Applied Biosystems, Foster City, CA, USA). The *stx* subtypes were identified based on the predictions from Whole Genome Sequencing data using the STECFinder software version 1.1.1 (<https://github.com/LanLab/STECFinder>, accessed on 24 April 2025).

5.4. Enzymatic Immunoassays for Shiga Toxin Detection and Quantification

The presence of the Shiga toxin in the overnight-enriched TSB broth cultures was qualitatively determined using the SHIGA TOXIN QUIK CHEK[™] kits as per manufacturer's instruction. Into 650 μ L of the provided diluent, 100 μ L of the enriched broth and one drop of the provided conjugate were added. After thoroughly vortexing, 500 μ L of the mixture was added to the SHIGA TOXIN QUIK CHEK[™] cassette. After a 15 min incubation at room temperature, the reaction window was washed with the wash buffer. After adding the substrate and incubating for 10 min at room temperature, the results were visually observed and recorded.

The Shiga toxin was further quantified using the Research Use Only (RUO) Quantitative Shiga toxin ELISA kits (TECHLAB, Inc., Blacksburg, VA, USA) following the instructions provided by TECHLAB, Inc. The Quantitative Shiga toxin ELISA kits consisted of separate microplates for Shiga toxins 1 and 2, and the appropriate microplate was selected based on the STEC tested. In brief, one drop of either Shiga toxin 1 or Shiga

toxin 2 conjugate was added to each microplate assay well. The overnight-enriched broths were diluted appropriately in the diluent provided, and 100 µL of each diluted sample was added to the designated microplate well. The microplate was sealed and incubated at 37 °C for 50 min. After incubation, the wells were washed with wash buffer, and the substrate was added. After incubating for 10 min at room temperature, plates were read at 450/620 nm within 10 min after adding the stop solution. In each microplate, separate wells were designated for the Shiga toxin standards (for developing the standard curve) and the positive and negative controls.

5.5. Determination of the Optimal Mitomycin C Concentration

A single colony from each STEC strain was inoculated into TSB and incubated at 37 °C for ~3–4 h until the OD value reached ~0.5. From this neat culture, serial dilutions up to 10^{-6} were prepared in PBS. A 100 µL aliquot of the 10^{-6} PBS dilution of each STEC culture was inoculated into TSB and treated with mitomycin C solutions with concentrations ranging from 5 to 500 ng/mL in a final volume of 3 mL. Sterilized laboratory grade water was used for the non-treated control. The broth mixtures were then incubated at 37 °C overnight for enrichment. On the following day, each tube of broth mixture was subjected to real-time PCR and both EIA assays (SHIGA TOXIN QUIK CHEK™ and RUO Quantitative Shiga toxin ELISA).

5.6. Application of the Optimal Mitomycin C Concentration to Clinical STEC Isolates

Fifty-three STEC isolates were tested for their response to mitomycin C as an enhancer to induce Shiga toxin production. STEC isolates were grown and treated with mitomycin C as described in Section 5.1, at the optimal concentration as determined in Section 5.5. After an overnight enrichment at 37 °C, real-time PCR analysis was performed, and the presence of toxin was assessed by qualitative EIA assay using SHIGA TOXIN QUIK CHEK™ kits; toxin quantification was not performed.

5.7. Application of the Optimal Mitomycin C Concentration to STEC-Positive Clinical Stool Samples

Aliquots of 15 patients' stool samples submitted to ProvLab that tested positive for STEC by PCR and culture were collected and stored at 4 °C until used. A 10% stool suspension in PBS was prepared from each stool sample. Both TSB and GNB (BBL™ GN Broth, Becton, Dickinson and Company, Sparks, MD, USA) were used as the enrichment media, as frontline laboratories use both these media for stool culture. A 500 µL aliquot of each stool suspension was added to either broth with 100 µL of mitomycin C solution at the optimized concentration as determined in Section 5.5 to a final volume of 3 mL (100 µL of water was used for a non-treated control). After an overnight enrichment at 37 °C, each enriched broth was subjected to real-time PCR analysis and both EIA assays.

5.8. Statistical Analysis

Experiments to determine the optimum mitomycin C concentration were performed in triplicate on 3 separate days, and all analyses were performed in triplicate (3 biological replicates). PCR analysis and EIAs on the 53 STEC isolates and STEC-positive stool samples treated with and without mitomycin C were performed once. Each sample was analyzed in duplicate by PCR (2 technical replicates) and RUO Quantitative Shiga toxin ELISA. The antibiotic treatment effect was analyzed using one-way analysis of variance, and the treatments were compared using Bonferroni post-test. Treatment effects were considered statistically significant when $p < 0.05$. Data are presented as mean ± STDEV.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/toxins17060267/s1>, Figure S1: *SHIGA TOXIN QUIK CHEK™* images of three STEC isolates with *stx1*, *stx2*, and *stx1 and 2* incubated overnight in TSB with different concentrations of mitomycin C.; Figure S2: *SHIGA TOXIN QUIK CHEK™* images of STEC isolates enriched in TSB overnight with 500 ng/mL mitomycin C in TSB.; Figure S3: *SHIGA TOXIN QUIK CHEK™* images showing STEC-positive stool samples enriched overnight in TSB and GNB with and without 500 ng/mL mitomycin C.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

EIA	Enzymatic immune assay
GNB	Gram-negative broth
HUS	Hemolytic uremic syndrome
PCR	Polymerase chain reaction
RUO	Research use only
STEC	Shiga toxin-producing <i>Escherichia coli</i>
TSB	Trypticase soy broth

References

1. Karmali, M.A. Factors in the Emergence of Serious Human Infections Associated with Highly Pathogenic Strains of Shiga Toxin-Producing *Escherichia coli*. *Int. J. Med. Microbiol.* **2018**, *308*, 1067–1072. [CrossRef] [PubMed]
2. Tarr, P.I.; Gordon, C.A.; Chandler, W.L. Shiga-Toxin-Producing *Escherichia coli* and Haemolytic Uraemic Syndrome. *Lancet* **2005**, *365*, 1073–1086. [CrossRef] [PubMed]
3. Freedman, S.B.; van de Kar, N.C.A.J.; Tarr, P.I. Shiga Toxin-Producing *Escherichia coli* and the Hemolytic-Uremic Syndrome. *N. Engl. J. Med.* **2023**, *389*, 1402–1414. [CrossRef]
4. Tarr, P.I.; Freedman, S.B. Why Antibiotics Should Not Be Used to Treat Shiga Toxin-Producing *Escherichia coli* Infections. *Curr. Opin. Gastroenterol.* **2022**, *38*, 30–38. [CrossRef]
5. Harrington, S.M.; Buchan, B.W.; Doern, C.; Fader, R.; Ferraro, M.J.; Pillai, D.R.; Rychert, J.; Doyle, L.; Laines, A.; Karchmer, T.; et al. Multicenter Evaluation of the BD Max Enteric Bacterial Panel PCR Assay for Rapid Detection of *Salmonella* Spp., *Shigella* Spp., *Campylobacter* Spp. (*C. jejuni* and *C. coli*), and Shiga Toxin 1 and 2 Genes. *J. Clin. Microbiol.* **2015**, *53*, 1639–1647. [CrossRef]

6. BD Life Sciences Inc. *BD Max Enteric Bacterial Panel*; BD Life Sciences Inc.: Mississauga, ON, Canada, 2019. Available online: <https://www.bd.com/en-ca/products-and-solutions/products/product-page.442963> (accessed on 15 September 2024).
7. Boone, J.T.; Campbell, D.E.; Dandro, A.S.; Chen, L.; Herbein, J.F. A Rapid Immunoassay for Detection of Shiga Toxin-Producing *Escherichia coli* Directly from Human Fecal Samples and Its Performance in Detection of Toxin Subtypes. *J. Clin. Microbiol.* **2016**, *54*, 3056–3063. [CrossRef]
8. Kimmitt, P.T.; Harwood, C.R.; Barer, M.R. Toxin Gene Expression by Shiga Toxin-Producing *Escherichia coli*: The Role of Antibiotics and the Bacterial SOS Response. *Emerg. Infect. Dis.* **2000**, *6*, 458–465. [CrossRef]
9. Beutin, L.; Fach, P. Detection of Shiga Toxin-Producing *Escherichia coli* from Nonhuman Sources and Strain Typing. *Microbiol. Spectr.* **2014**, *2*. [CrossRef]
10. Pacheco, A.R.; Sperandio, V. Shiga Toxin in Enterohemorrhagic *E. coli*: Regulation and Novel Anti-Virulence Strategies. *Front. Cell Infect. Microbiol.* **2012**, *2*, 81. [CrossRef]
11. Parsons, B.D.; Zelyas, N.; Berenger, B.M.; Chui, L. Detection, Characterization, and Typing of Shiga Toxin-Producing *Escherichia coli*. *Front. Microbiol.* **2016**, *7*, 478. [CrossRef]
12. Chui, L.; Christianson, S.; Alexander, D.; Arseneau, V.; Bekal, S.; Berenger, B.; Chen, Y.; Davidson, R.; Farrell, D.; German, G.; et al. CPHLN Recommendations for the Laboratory Detection of Shiga Toxin-Producing *Escherichia coli* (O157 and Non-O157). *Can. Commun. Dis. Rep.* **2018**, *44*, 304–307. [CrossRef] [PubMed]
13. Cordonnier, C.; Le Bihan, G.; Emond-Rheault, J.G.; Garrivier, A.; Harel, J.; Jubelin, G. Vitamin B12 Uptake by the Gut Commensal Bacteria *Bacteroides Thetaiotaomicron* Limits the Production of Shiga Toxin by Enterohemorrhagic *Escherichia coli*. *Toxins* **2016**, *8*, 14. [CrossRef] [PubMed]
14. Filipiak, M.; Łoś, J.M.; Łoś, M. Efficiency of Induction of Shiga-Toxin Lambdoid Prophages in *Escherichia coli* Due to Oxidative and Antibiotic Stress Depends on the Combination of Prophage and the Bacterial Strain. *J. Appl. Genet.* **2020**, *61*, 131–140. [CrossRef]
15. McGannon, C.M.; Fuller, C.A.; Weiss, A.A. Different Classes of Antibiotics Differentially Influence Shiga Toxin Production. *Antimicrob. Agents Chemother.* **2010**, *54*, 3790–3798. [CrossRef]
16. Shimizu, T.; Ohta, Y.; Noda, M. Shiga Toxin 2 Is Specifically Released from Bacterial Cells by Two Different Mechanisms. *Infect. Immun.* **2009**, *77*, 2813–2823. [CrossRef]
17. Bording-Jorgensen, M.; Tyrrell, H.; Lloyd, C.; Chui, L. Comparison of Common Enrichment Broths Used in Diagnostic Laboratories for Shiga Toxin—Producing *Escherichia coli*. *Microorganisms* **2021**, *9*, 503. [CrossRef]
18. Rocha, L.B.; Piazza, R.M.F. Production of Shiga Toxin by Shiga Toxin-Expressing *Escherichia coli* (STEC) in Broth Media: From Divergence to Definition. *Lett. Appl. Microbiol.* **2007**, *45*, 411–417. [CrossRef]
19. Krüger, A.; Lucchesi, P.M.A. Shiga Toxins and Stx Phages: Highly Diverse Entities. *Microbiology* **2015**, *161*, 451–462. [CrossRef]
20. Ramstad, S.N.; Taxt, A.M.; Naseer, U.; Wasteson, Y.; Bjørnholt, J.V.; Brandal, L.T. Effects of Antimicrobials on Shiga Toxin Production in High-Virulent Shiga Toxin-Producing *Escherichia coli*. *Microb. Pathog.* **2021**, *152*, 104636. [CrossRef]
21. Allué-Guardia, A.; Koenig, S.S.K.; Martinez, R.A.; Rodriguez, A.L.; Bosilevac, J.M.; Feng, P.; Eppinger, M. Pathogenomes and Variations in Shiga Toxin Production among Geographically Distinct Clones of *Escherichia coli* O113:H21. *Microb. Genom.* **2022**, *8*, 000796. [CrossRef]
22. Beutin, L.; Krüger, U.; Krause, G.; Miko, A.; Martin, A.; Strauch, E. Evaluation of Major Types of Shiga Toxin 2e-Producing *Escherichia coli* Bacteria Present in Food, Pigs, and the Environment as Potential Pathogens for Humans. *Appl. Environ. Microbiol.* **2008**, *74*, 4806–4816. [CrossRef]
23. Perelle, S.; Dilasser, F.; Grout, J.; Fach, P. Detection by 5'-Nuclease PCR of Shiga-Toxin Producing *Escherichia coli* O26, O55, O91, O103, O111, O113, O145 and O157:H7, Associated with the World's Most Frequent Clinical Cases. *Mol. Cell Probes* **2004**, *18*, 185–192. [CrossRef]

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Article

Determination of 16 Hydroxyanthracene Derivatives in Food Supplements Using LC-MS/MS: Method Development and Application

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Abstract: Hydroxyanthracene derivatives (HADs) are plant substances produced by a variety of plant species, including different *Aloe*, *Rheum*, and *Rhamnus* species and *Cassia senna*. These plants are often used in food supplements to improve bowel function. However, recently, the European Commission prohibited a number of HADs due to toxicological concerns. These HADs included aloin (aloin A and aloin B), aloë-emodin, emodin, and danthron. Most of the currently available analytical methods are restricted to the analysis of only these compounds and do not include other HADs. In this view, a multi-analyte method could be useful for both regulatory analysis and dietary intake studies. To this end, such a method, employing liquid chromatography–tandem mass spectrometry and targeting 16 different HADs, was developed and validated in this study. Limits of quantification were in the range from 0.025 mg kg^{−1} to 1 mg kg^{−1}. The recovery of the method was within the acceptable range of 80% to 120%, with the exception of physcion. Repeatability varied from 0.5% to 11.6%, and the range for within-laboratory reproducibility was from 3.4% to 16.3%. The expanded measurement uncertainty was below 50% for all HADs. Subsequently, 24 commercial samples of food supplements and herbal infusions sourced in Belgium were analyzed. The results indicated that although the industry put a great effort into minimizing the amount of aloin and danthron present in food supplements, more than half of the products still exceeded the maximum tolerated levels suggested for aloë-emodin and emodin.

Keywords: hydroxyanthracene derivatives; aloë-emodin; emodin; sennosides; LC-MS/MS; food supplements; validation; occurrence

Key Contribution: Dedicated and validated analytical methods for the determination of multiple HADs in food are scarce but are in high demand due to a recent update in the regulation. For the first time, this study provides a sensitive and quantitative method for the simultaneous analysis of 16 HADs in food supplements or related substances.

1. Introduction

Hydroxyanthracene derivatives (HADs) are a class of compounds naturally present in numerous plant species belonging to different botanical families and genera, among which are *Aloe*, *Rheum*, *Senna*, *Cassia*, *Frangula*, and *Rhamnus* spp. HADs are considered important biologically active components exerting a range of biological activities, including their recognized effect on the gastrointestinal system. Herbal products containing HADs are mostly appreciated for their laxative effects. According to a recent study, 40% of the worldwide population suffers from chronic gastrointestinal problems, among which 12% from chronic constipation [1].

In 2013, the European Food Safety Authority (EFSA) concluded that HADs in food could improve bowel function, but at the same time, warned that long-term use of these products and consumption at high doses may lead to potential electrolyte imbalance, impaired bowel function and dependence on laxatives [2]. In recent years, more concerns have been raised regarding the safety of HAD-containing products. Based on available scientific data, in 2018, EFSA concluded that certain HADs are genotoxic and may pose an increased risk for colorectal cancer [3]. Eventually, in 2021, aloe-emodin (ALE), emodin (EMO), the structurally related substance danthron (DAN), and aloe preparations were banned by the European Commission (EC), while preparations from certain *Rheum* sp., *Cassia senna* L. and *Rhamnus* sp. containing HADs were put under scrutiny [4]. In 2024, the EFSA Panel on Nutrition, Novel Foods and Food Allergens concluded that preparations with rhubarb, frangula, cascara, and senna pose safety concerns for genotoxicity [5].

Consequently, the industry introduced filtration/purification processes aiming to remove HADs, although these methodologies are not completely efficient. Nevertheless, in order to guarantee the safety of foodstuffs on the market, analytical methods for HADs were implemented. Several literature reports exist describing methods for quantification of ALE, EMO, aloin A (ALA), aloin B (ALB), and some other HADs in botanical preparations [6]. Not all these methods have been validated, or only partial validation was performed, or they focused solely on the HADs mentioned above. A great part of the methods made use of the high-performance liquid chromatography (HPLC) technique with ultraviolet (UV) or diode-array detectors (DAD) [7–12]. Kline et al. developed and validated a quantitative HPLC-UV method for the analysis of ALA, ALB, and ALE in *Aloe vera* raw materials and finished products [7]. Using the same technique, Yilmaz and Kolak quantified RHE, EMO, CHR, and PHY in *Rhamnus petiolaris* fruit extracts [8], while Al-Hamoud et al. analyzed CHR in *Senna occidentalis* extract [9]. The latter study made use of ultrasound-assisted extraction, which was optimized using response surface methodology. ALE in *Aloe vera* extracts and commercial formulations was determined by Mandrioli et al. using HPLC with tandem UV absorption and fluorescence detection (FD) [10]. The authors emphasized that coupling UV and FD allowed comparison of the data obtained with both detection methods, elevating the level of confidence in the results. A rapid method utilizing UHPLC coupled to a photodiode array detector (PDA) was developed for the quantification of five anthraquinones (ALE, EMO, PHY, RHE, and CHR) in *Polygonum multiflorum* [13]. Ultrasonic extraction followed by dispersive solid-phase extraction (dSPE) were used for the preparation of the samples. This method aided in demonstrating large differences in the HAD content in *Polygonum multiflorum* samples from different regions. Liquid (LC) or gas chromatography (GC) combined with mass spectrometry (MS) was also used for the analysis of HADs in botanical preparations [14–17]. Loschi et al. developed an LC-DAD-MS method for the analysis of five HADs, namely ALA, ALB, ALE, EMO, and DAN, in food supplements and plant materials [11]. A GC-MS method was developed by Dai et al. for the determination of four anthraquinones, ALE, EMO, CHR and PHY, in rhubarb, the traditional Chinese medicinal herb [14]. Osthole was used as an internal standard, and the MS detection was accomplished in selected ion monitoring mode to increase the sensitivity. Recently, a UHLC-MS/MS quantification of HADs in *Aloe vera* (L.) Burm. f. gel commercial beverages was presented by Di Minno et al. [18]. The method targeted only ALA, ALB, EMO, and ALE and was characterized by a fast separation of the analytes, which was achieved within 5 min. Another analytical method was validated for the simultaneous quantification of ALE, EMO, PHY, RHE, and CHR among two other physiologically relevant active components and eight adulterants [16]. As an analytical technique, HPLC-MS/MS was optimized, and the target matrices included slimming foods and herbal products. The heteronuclear multiple quantum correlation spectroscopy technique was used for the determination of the total HAD content in plant materials [19], while a two-dimensional quantitative nuclear magnetic resonance was utilized to determine total sennosides and separately individual content of SENA, SENA1, and SENB in senna leaflets, pods and tablets [20]. The method performance was satisfactory, except for precision for SENA1

and SENB, which was mainly attributed to the low resolution of the heteronuclear single quantum correlation spectroscopy signals for these HADs. Overall, the main drawbacks of the published methods are either a lack of specificity and/or sensitivity or a limited number of HADs included. Having diverse HADs in the method is pivotal to having a more complete picture of the amount and type of HADs present in the samples. Indeed, a previous study performed by Fierens and Corthout [21] on food supplements retrieved from the Belgian market showed large variability in total HAD content but lacked data on individual HADs. Consequently, the estimation of the exposure to HADs from food supplements based on the recommended daily doses of these products was only possible for a limited number of derivatives due to this lack of data for the other HADs [3].

The objective of this work was to develop and validate an analytical method based on ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) for the detection and quantification of individual HADs in a single run. The advantages of this technique include high sensitivity, specificity, and the possibility to analyze many analytes simultaneously. The target compounds included ALA, ALB, ALE, chrysophanol (CHR), DAN, EMO, frangulin A (FRA), frangulin B (FRB), glucofrangulin A (GFA), glucofrangulin B (GFB), physcion (PHY), rhein (RHE), sennoside A (SENA), sennoside A1 (SENA1), sennoside B (SENB), and sennoside C (SENC).

2. Results and Discussion

2.1. Optimization of LC-MS/MS Conditions

For the optimization of the MS signal of HADs, mobile phases with methanol (MeOH) and acetonitrile (ACN) as organic modifiers and with formic acid (HCOOH), acetic acid (CH₃COOH), ammonium formate (HCOONH₄), and ammonium bicarbonate (NH₄HCO₃) as additives were tested (Supplementary Figure S1). Mobile phases with no additives were also investigated. The spectra were recorded in positive (ESI(+)) and negative (ESI(−)) electrospray ionization modes using flow injection analysis. It was apparent that, overall, MeOH with or without additives generated a higher MS signal compared to ACN. The mobile phases without an additive generated poor signal for DAN, CHR, and ALE. Among the tested additives, HCOOH at 0.1% provided a better signal for the majority of HADs. A few exceptions were FRB and GFA showing a better signal in the mobile phase with 10 mM NH₄HCO₃ (MeOH as an organic modifier) and ALE demonstrating a higher signal in the mobile phase with 0.1% HCOOH and ACN as an organic modifier. As a compromise, the mobile phase with MeOH and 0.1% HCOOH was selected for further optimization.

Regarding the selection of ionization mode, aloins, frangulins, ALE, CHR, DAN, and PHY were better ionized in ESI(+), generating abundant [M+H]⁺ precursor ions (Table 1). A sufficiently intense signal in ESI(+) was also obtained for GFB, SENA, and SENA1 through the formation of [M+Na]⁺ and [M+NH₄]⁺ (the latter was only for sennosides) adducts. Knowing that sodium adducts are generally less robust, ESI(−) was preferred for the analysis of these three HADs, as they also formed abundant deprotonated molecules. ESI(−) was also chosen for the detection of EMO, GFA, RHE, SENB, and SENC. Notably, the most abundant signal for both glucofrangulins was obtained using formic acid adducts (Table 1). SENB and SENC were well ionized in both ESI(+) and ESI(−) modes, but somewhat better sensitivity was achieved in ESI(−). In the final method, for higher specificity and sensitivity, the data were acquired in multiple reaction monitoring (MRM) mode. Table 1 summarizes the optimized precursor and product ions for the MS/MS detection of HADs.

LC columns, previously used in multi-target analysis of some HADs and other natural toxins [13,22–26], namely ACQUITY™ UPLC™ BEH C18 (1.7 µm, 2.1 × 100 mm), ACQUITY UPLC HSS T3 (1.8 µm, 2.1 × 100 mm), and ACQUITY UPLC BEH Phenyl (1.7 µm, 2.1 × 100 mm), were tested for the separation of HADs using the optimal mobile phase, and similar satisfactory results were obtained. Finally, the first-listed column was chosen for the method, and the gradient program was described in Section 4.4. Figure 1 displays the separation profile of HADs under the optimized LC conditions, while information on the retention time is summarized in Table 1. Importantly, the peaks of HADs with the

same target precursor and product ions, namely, sennosides SENA, SENA1, and SENB, and aloins ALA and ALB were baseline separated.

Table 1. Retention times and optimized multiple reaction monitoring (MRM) parameters for the UHPLC-ESI-MS/MS analysis of HADs.

HAD	Retention Time (min)	Ionization Mode	Precursor Ion (<i>m/z</i>)	Cone Voltage (V)	Product Ion (<i>m/z</i>)	Collision Energy (eV)
Aloin A (ALA)	5.53	ESI(+)	419.2 [M+H] ⁺	15	239.0 ¹ 211.0	10 25
Aloin B (ALB)	5.30	ESI(+)	419.2 [M+H] ⁺	20	239.0 ¹ 211.0	10 25
Aloe-emodin (ALE)	7.11	ESI(+)	271.0 [M+H] ⁺	15	225.0 ¹ 197.0	25 30
Chrysophanol (CHR)	9.11	ESI(+)	255.0 [M+H] ⁺	50	152.0 ¹ 181.0	35 25
Danthron (DAN)	8.29	ESI(+)	241.0 [M+H] ⁺	30	213.1 ¹ 167.1	25 25
Emodin (EMO)	8.67	ESI(−)	269.2 [M−H] [−]	30	225.1 ¹ 241.1	30 30
Frangulin A (FRA)	8.44	ESI(+)	417.0 [M+H] ⁺	15	271.0 ¹ 197.0	15 40
Frangulin B (FRB)	8.12	ESI(+)	403.0 [M+H] ⁺	20	271.0 ¹ 197.0	20 45
Glucofrangulin A (GFA)	6.05	ESI(−)	623.3 [M+HCOOH−H] [−]	30	269.0 ¹ 431.0	60 30
Glucofrangulin B (GFB)	5.81	ESI(−)	609.1 [M+HCOOH−H] [−]	30	431.0 ¹ 269.0	20 30
Physcion (PHY)	9.60	ESI(+)	285.1 [M+H] ⁺	30	168.0 ¹ 139.2	35 55
Rhein (RHE)	7.71	ESI(−)	283.0 [M−H] [−]	30	183.1 ¹ 211.1	30 30
Sennoside A (SENA)	4.76	ESI(−)	861.3 [M−H] [−]	30	224.2 ¹ 386.3	50 50
Sennoside A1 (SENA1)	4.57	ESI(−)	861.3 [M−H] [−]	30	224.2 ¹ 386.3	50 50
Sennoside B (SENB)	4.24	ESI(−)	861.3 [M−H] [−]	30	224.2 ¹ 386.3	50 50
Sennoside C (SENC)	4.54	ESI(−)	847.0 [M−H] [−]	30	224.2 ¹ 386.3	50 50
Aloe-emodin- <i>d</i> ₄ (ALE-D4)	7.09	ESI(+)	275.1 [M+H] ⁺	15	229.1 ¹ 245.1	20 20
Danthron- <i>d</i> ₄ (DAN-D4)	8.27	ESI(+)	245.1 [M+H] ⁺	20	143.0 ¹ 161.1	35 30
Emodin- <i>d</i> ₄ (EMO-D4)	8.65	ESI(−)	273.1 [M−H] [−]	30	229.0 ¹ 245.1	30 30
Physcion- <i>d</i> ₃ (PHY-D3)	9.58	ESI(+)	288.1 [M+H] ⁺	20	168.0 ¹ 242.0	40 30

¹ The most abundant product ion.

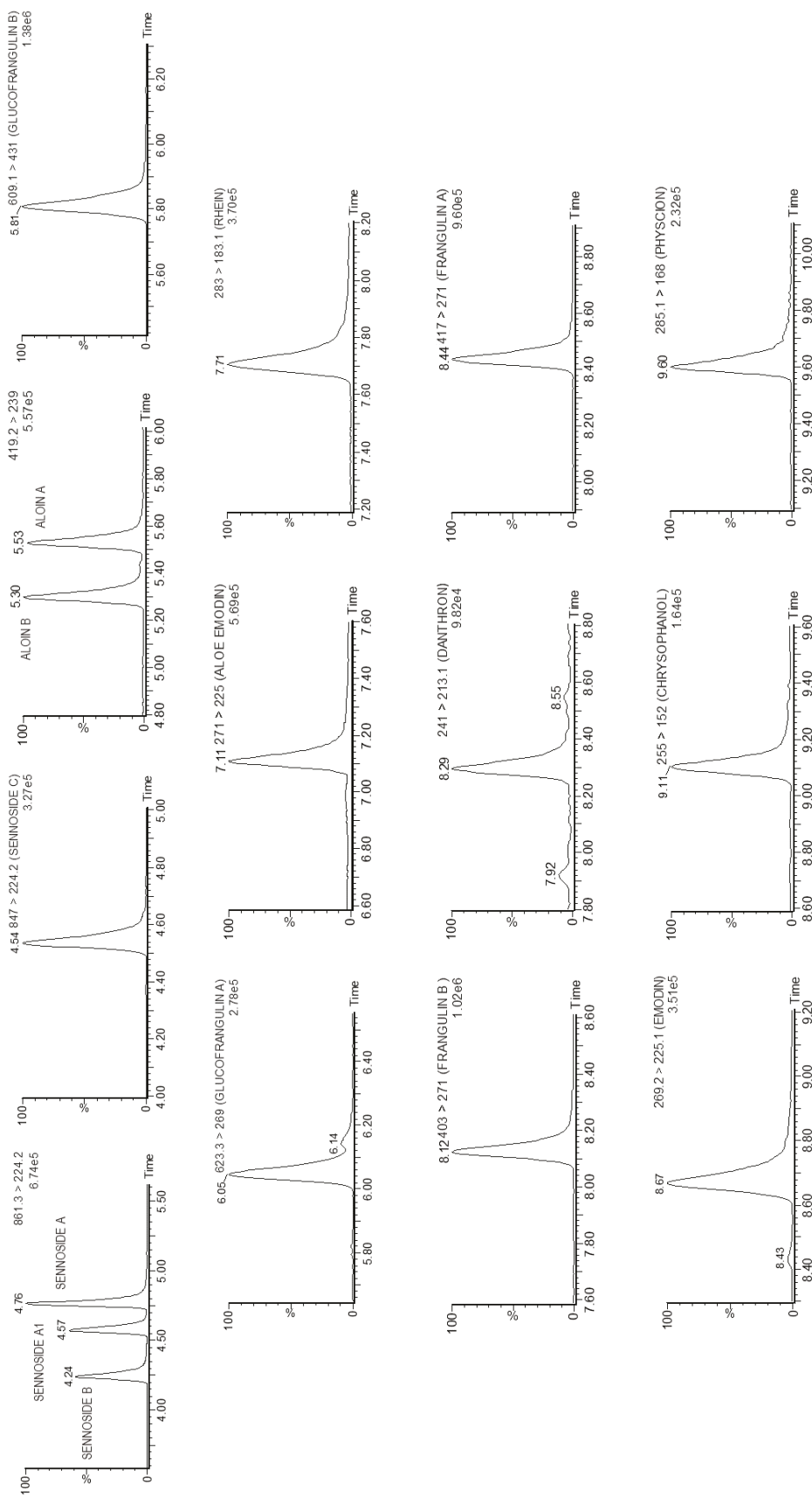


Figure 1. LC-MS/MS multiple reaction monitoring (MRM) chromatograms of a blank food supplement fortified with 16 HADs at the limit of quantification (LOQ) level. The LOQ levels for each HAD are specified in Table 2. For each analyte, the most abundant MRM transition is displayed. The vertical axes represent relative peak intensity (normalized to 100%), while the horizontal axes display retention time (in min). The chromatographic conditions used were as described in Section 4.4.

Table 2. Limit of quantification (LOQ), matrix effect, and linearity data for the analysis of HADs in food supplements.

HAD	LOQ (mg kg ^{−1})	LOQ (mg L ^{−1})	Matrix Effect (%)	Linear Range (mg kg ^{−1})	Coefficient of Determination R ²	Regression Model	Internal Standard Used
Aloin A (ALA)	0.05	0.002	40	0.05–0.5	0.9923	Linear	ALE-D4
Aloin B (ALB)	0.1	0.004	32	0.1–1.0	0.9925	Linear	ALE-D4
Aloe-emodin (ALE)	0.2	0.008	60	0.2–2.0	0.9922	Linear	ALE-D4
Chrysophanol (CHR)	0.1	0.004	82	0.1–1.0	0.9920	Linear	ALE-D4
Danthron (DAN)	0.2	0.008	114	0.2–2.0	0.9931	Linear	DAN-D4
Emodin (EMO)	0.025	0.001	85	0.025–0.25	0.9942	Linear	EMO-D4
Frangulin A (FRA)	0.1	0.004	70	0.1–1.0	0.9920	Linear	ALE-D4
Frangulin B (FRB)	0.1	0.004	72	0.1–1.0	0.9921	Linear	ALE-D4
Glucofrangulin A (GFA)	0.1	0.004	33	0.1–1.0	0.9969	Linear	None
Glucofrangulin B (GFB)	1.0	0.042	42	1–10	0.9962	Linear	None
Physcion (PHY)	0.1	0.004	58	0.1–1.0	0.9951	Linear	None
Rhein (RHE)	0.1	0.004	87	0.1–1.0	0.9958	Linear	EMO-D4
Sennoside A (SENA)	1.0	0.042	47	1–10	0.9939	Linear	None
Sennoside A1 (SENA1)	0.75	0.031	51	0.75–7.5	0.9930	Linear	None
Sennoside B (SENB)	0.75	0.031	47	0.75–7.5	0.9920	Linear	None
Sennoside C (SENC)	0.5	0.021	42	0.5–5.0	0.9905	Linear	None

2.2. Optimization of Sample Preparation

The sample preparation procedure was optimized using fine powder of a multi-component plant-based food supplement intended to support intestinal function.

Different organic solvents and/or their combinations were tested for the extraction of HADs from the plant-based material and included MeOH, ACN, ethanol (EtOH), and acetone (ACE) (Supplementary Figure S2). EtOH or ACE with 30% H₂O, commonly used in the extraction of anthraquinones [13,27,28], demonstrated satisfactory results with regard to extraction efficiency. In the case of EtOH, the addition of H₂O greatly improved the recovery of aloins, ALE, glucofrangulins, and sennosides. Nonetheless, the estimated limits of quantification (LOQs) for SENA and PHY using EtOH/H₂O (70/30, *v/v*) for the extraction were considered rather high. ACN alone or in combination with H₂O in different proportions showed lower extraction efficiency for ALB and sennosides.

The use of MeOH as an extraction solvent resulted in a somewhat lower yield for SENA, SENA1, and SENB. The addition of 1% CH₃COOH only slightly improved the

recovery of these sennosides, while the use of MeOH in combination with 1% HCOOH provided good results for all HADs (extraction efficiency > 70%). With this latter extraction mixture, good estimated LOQ values were obtained; however, the downside of this protocol was the distorted LC peak shape for sennosides due to the higher elution strength of the sample solvent compared to the mobile phase. The peaks of the other HADs were not affected.

Another protocol, with the addition of H₂O to the extraction solvent MeOH, was also tested. Surely, the higher percentage of H₂O led to a better peak shape for sennosides; however, at 50% H₂O in the solvent mixture, the recovery of PHY, DAN, CHR, and EMO reduced down to about 20–45%. The optimal combination in terms of extraction efficiency and sensitivity was found to be 20% H₂O in MeOH.

Different sample-to-solvent ratios (between 1:7.5 (*w/v*) to 1:15 (*w/v*)) were investigated, and the final selection was the ratio of 1:15 (*w/v*), providing good sensitivity.

Furthermore, different clean-up methods with or without subsequent extract evaporation were tested in order to improve the method sensitivity even further and/or allow for the solvent switch before injection into the LC system for better peak shape. None of the target HADs were detected after the application of solid-phase extraction (SPE) on the graphitized carbon cartridges (Supelclean ENVI-Carb). Low to no recovery of the analytes was observed in the case of dSPE based on C18 and/or primary secondary amine (PSA) sorbents. SPE performed on the Oasis HLB cartridges demonstrated low efficiency for EMO, DAN, CHR, and PHY using MeOH for the elution. Acidification of the elution solvent with 1% HCOOH resulted in improved efficiency for the problematic HADs but still reached only about 30% for PHY and required an increased elution volume (3 × 3 mL). Similar results were obtained for the Discovery DSC-18 cartridges. In the end, considering the unsatisfactory efficiency for some HADs and the lack of clear benefit compared to the protocol with only extraction, the purification of sample extracts was dismissed.

The final sample preparation procedure for food supplements consisted of a single-step solid-liquid extraction with MeOH/H₂O (80/20, *v/v*), subsequent extract dilution with H₂O to reach its volume concentration of 50% in the extract, and filtration (see Section 4.3). The dilution was necessary to resolve the issue of distorted LC peak shape for sennosides.

2.3. Method Validation

The method validation data for the analysis of HADs in food supplements are summarized in Tables 2 and 3.

The selectivity of the method was assessed by analyzing different blank plant-based matrices. No interfering peaks in the region of the analyte elution, which necessitated further improvement of the LC separation, MS detection, or sample preparation procedure, were present (Supplementary Figure S3). The matrix effect ranged from 32% to 114%. It was revealed that all HADs, with the exception of DAN, had high to moderate signal suppression. The signal of ALB and GFA was affected by the matrix the most (Table 2). The utilization of the isotopically labeled internal standards (IS), namely aloe-emodin-*d*₄ (ALE-D4), danthron-*d*₄ (DAN-D4), emodin-*d*₄ (EMO-D4), and physcion-*d*₃ (PHY-D3), in the control of matrix effects was evaluated. Such analogs were not commercially available for each of the target HADs. While for a number of HADs the matrix effects could be improved, for sennosides and glucofrangulins, none of the IS was suitable. Initially, for PHY, its analog PHY-D3 was used, but due to inconsistent day-to-day method validation results using this IS, it was eventually excluded. This could be attributed to the position of deuterium in the structure and its stability. Unlike ALE-D4, DAN-D4, and EMO-D4, PHY-D3 produced no MS fragments with the labeled site on the portion of the molecule that is being quantitated. The final choice of the IS is given in Table 2.

Matrix-matched calibration was used for quantification, and the starting calibration point for all HADs corresponded to the respective LOQ level. Mandel's test showed that the linear regression model was the best fit, and the coefficients of determination were all above 0.99. The linear range is specified in Table 2. The LOQs varied from 0.025 mg kg⁻¹ to

1 mg kg⁻¹, with EMO being the most sensitive compound and GFB and SENA demonstrating the lowest sensitivity (Table 2). Compared to other studies, the current method demonstrated much lower LOQs for sennosides, aloins, frangulins, ALE, EMO, CHR, PHY, DAN, and RHE [11–13,26,29–34]. While Loschi et al. achieved concentrations for aloins, ALE, EMO, and DAN only as low as between 42 ng mL⁻¹ and 94 ng mL⁻¹ [11], the current study presented LOQs in the range from 1.04 ng mL⁻¹ to 8.33 ng mL⁻¹. In a previous report, frangulins were quantified at the lowest concentration of 61.4 ng mL⁻¹ [26], which was much higher than the LOQ of 4.17 ng mL⁻¹ obtained in the current study. However, Fung et al. obtained a more sensitive detection for SENA, SENB, and ALE at 4.73 ng mL⁻¹, 4.32 ng mL⁻¹, and 1.51 ng mL⁻¹, respectively, in comparison to 41.67 ng mL⁻¹, 31.25 ng mL⁻¹, and 8.33 ng mL⁻¹, respectively, achieved in the current study [22]. Concurrently, Fung et al. obtained much lower sensitivity for PHY (10.29 ng mL⁻¹), CHR (8.44 ng mL⁻¹), and EMO (2.08 ng mL⁻¹). LOQs for these HADs in the current method were as low as 4.17 ng mL⁻¹, 4.17 ng mL⁻¹, and 1.04 ng mL⁻¹, respectively. Compared to the data of Wu et al. [32], the method described in the current study achieved approximately three to five times lower LOQs for SENA, RHE, and ALE. LOQ values for GFA and GFB were not reported in previous studies. Overall, the LOQs obtained in the present study were considerably lower than the threshold of 1 ppm proposed by the EC for reliable quantification of ALE, EMO, and the sum of ALA and ALB [35].

The method was validated at three concentration levels, including the LOQ level, and the recoveries ranged from 80.1% (CHR) to 119.3% (GFB) (Table 3) and thus were within the acceptance limit of 80%–120% [36]. PHY was the only exception, with a mean recovery of 50.7%; for this reason, a recovery correction was implemented for the quantification of this HAD. Previously published studies reported recovery of HADs in the range from 71.5% to 118.4% [13,14,16,18,29,30]. Although the recovery value for PHY in the current research was below the desired limit, it was amply repeatable (mean: 2.3%) and reproducible (mean: 12.4%), eventually allowing for reliable recovery correction in the quantification of samples. Further optimization of extraction and/or clean-up procedures with the purpose of improving the recovery of PHY proved to be unrewarding due to the fact that the method covered analytes with different physico-chemical properties. The final sample preparation protocol was a compromise for having all target HADs analyzed using an easy-to-apply protocol and with good sensitivity.

The range for repeatability was from 0.5% (SENA) to 11.6% (DAN), while within-laboratory reproducibility varied from 3.6% (SENA) to 16.3% (DAN) (Table 3). Commission Implementing Regulation (EU) 2021/808 provides acceptance criteria for within-laboratory reproducibility that correspond to the validated concentration level [36]. This parameter was much lower than the specified acceptance limit for all HADs and for all concentration levels (Table 3). The expanded measurement uncertainty (MU) ranged from 12.2% (GFA) to 49.2% (DAN) (Table 3). It has to be noted that the uncertainty of bias was not included in the calculation of expanded MU for PHY due to the application of recovery correction in quantification. Overall, the method was found to be fit for purpose. It is important to mention that this validated method covers a range of HADs beyond two to five usual HADs, which were targeted by other methods, thus providing a more comprehensive assessment of HAD content of plant-based samples. The developed method was subsequently applied to the analysis of HADs in commercial products.

2.4. Method Application

The validated LC-MS/MS method was utilized for the analysis of 24 commercial samples of food supplements and dry herbal infusions purchased on the Belgian market. Most of the acquired samples contained senna extracts and rhubarb and were intended to support bowel function. Supplementary Table S1 provides detailed information on the samples' ingredients, while Figure 2 and Supplementary Table S2 summarize the measured concentrations of HADs in these products.

Table 3. Recovery, repeatability (relative standard deviation (RSD_r)), reproducibility (RSD_{RW}), and expanded measurement uncertainty (MU) for the analysis of HADs in food supplements.

HAD	Concentration Level (mg kg ⁻¹)	Recovery (%)	RSD_r (%)	RSD_{RW} (%)	Acceptance Criteria for RSD_{RW} (%) [36]	Expanded MU (%)
Aloin A (ALA)	0.05	101.1	7.1	13.4	25	37.2
	0.15	108.5	4.9	6.3	22	24.8
	0.25	99.5	2.6	7.3	22	20.1
Aloin B (ALB)	0.1	104.5	6.5	15.1	25	43.4
	0.3	112.1	5.0	5.0	22	28.2
	0.5	102.9	2.3	4.5	22	13.8
Aloe-emodin (ALE)	0.2	98.6	7.2	14.3	22	39.2
	0.6	103.7	4.5	6.0	22	18.3
	1.0	96.3	2.2	5.5	22	16.7
Chrysophanol (CHR)	0.1	80.1	7.5	11.1	25	48.5
	0.3	88.9	7.3	7.8	22	30.0
	0.5	81.5	1.2	5.1	22	39.1
Danthron (DAN)	0.2	87.5	11.6	16.3	22	49.2
	0.6	96.9	5.9	9.0	22	25.1
	1.0	92.7	4.0	7.8	22	25.3
Emodin (EMO)	0.025	83.8	8.3	14.1	25	48.4
	0.075	94.0	8.0	15.3	25	42.5
	0.125	87.5	3.4	13.5	22	42.9
Frangulin A (FRA)	0.1	84.0	9.1	14.3	25	48.6
	0.3	103.7	4.9	5.4	22	16.9
	0.5	95.3	2.5	4.4	22	15.0
Frangulin B (FRB)	0.1	81.1	8.8	11.2	25	47.1
	0.3	99.8	4.9	6.8	22	18.7
	0.5	92.0	2.7	5.5	22	21.6
Glucofrangulin A (GFA)	0.1	105.9	1.6	13.3	25	39.3
	0.3	106.3	1.5	5.4	22	19.7
	0.5	101.3	1.3	4.3	22	12.2
Glucofrangulin B (GFB)	1.0	108.7	1.9	14.3	22	44.5
	3.0	119.3	1.1	5.4	16	41.9
	5.0	116.3	0.8	4.4	16	35.0
Physcion (PHY) ¹	0.1	49.2	3.0	8.3	25	17.5
	0.3	50.9	1.6	13.4	22	28.2
	0.5	52.0	2.4	15.4	22	32.5
Rhein (RHE)	0.1	103.9	6.1	6.4	25	19.7
	0.3	107.4	4.2	8.3	22	27.8
	0.5	100.7	2.3	7.0	22	19.4
Sennoside A (SENA)	1.0	87.9	0.6	13.4	22	42.4
	3.0	96.0	1.2	5.9	16	17.8
	5.0	93.4	0.9	3.6	16	16.1
Sennoside A1 (SENA1)	0.75	95.9	1.5	6.0	22	18.1
	2.25	93.4	0.6	7.4	16	23.7
	3.75	91.0	0.5	7.2	16	26.3
Sennoside B (SENB)	0.75	93.5	2.1	6.1	22	20.8
	2.25	93.9	1.2	4.7	16	17.5
	3.75	92.5	0.7	5.0	16	20.0
Sennoside C (SENC)	0.5	100.5	1.7	13.8	22	38.0
	1.5	107.2	1.0	9.0	16	29.3
	2.5	103.1	0.8	8.4	16	24.3

¹ The recovery was calculated using the uncorrected measured concentration in order to demonstrate that for the quantification recovery correction was necessary. The uncertainty of bias was not included in the calculation of the expanded MU.

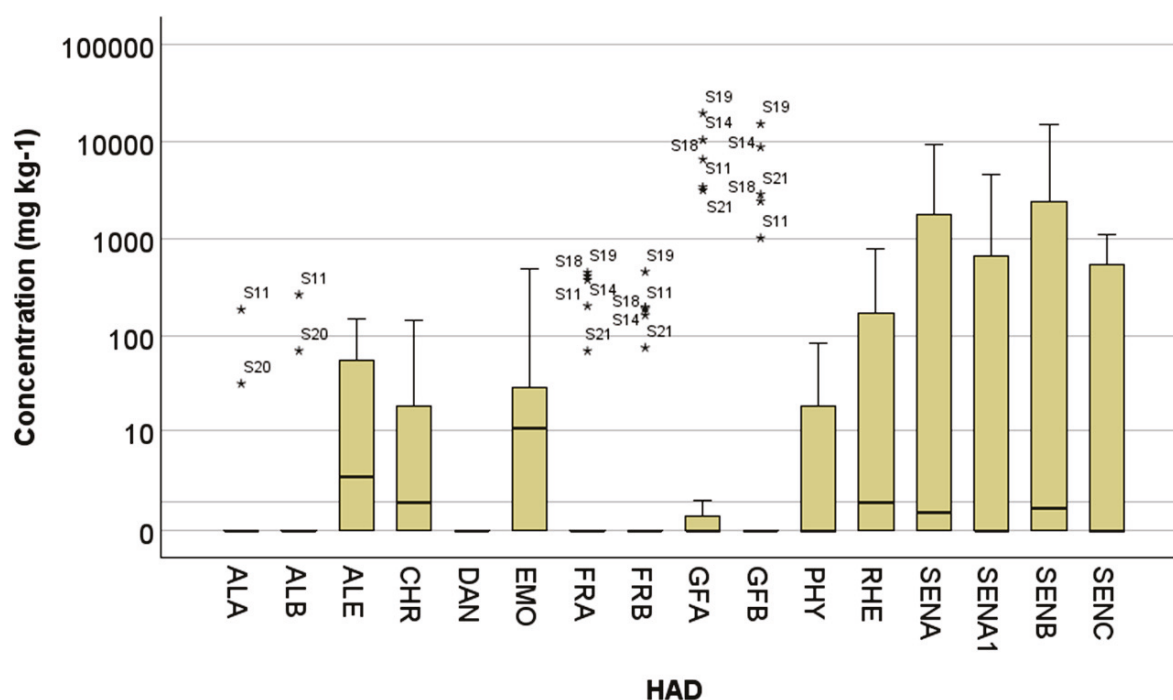


Figure 2. Distribution of HAD content in commercial food supplements and herbal infusions. The bottom and top of the box correspond to the lower quartile (25th percentile) and the upper quartile (75th percentile), respectively. The line inside the box represents the median (50th percentile). The ends of the whiskers correspond to the lowest and highest observations that are still within 1.5-fold of the interquartile range (corresponding to the length of the box). Observations beyond the ends of the whiskers are marked with an asterisk and labeled with a sample code. The spacings between the different parts of the box give an indication of the degree of spread and skewness of the data. Given the large distribution in HAD concentrations, a logarithmic scale is applied to the y-axis for better visualization.

The target HADs in the samples were identified in accordance with the requirements of Commission Implementing Regulation (EU) 2021/808 [36]. This included correspondence of the retention time and ion ratio of the analyte in the sample to that of the matrix-matched standards with a tolerance of 0.1 min and a relative deviation of 40%, respectively. In case an internal standard is used, a maximum deviation for the relative retention time of the analyte in the sample is set to 1%. For the analysis and data processing, the most abundant product ion was used for quantification, while the second ion was used for confirmation of the detected HADs (see Table 1). For samples with concentrations exceeding the working range of the method, a re-analysis was performed by diluting the sample extract with a blank matrix extract used for the preparation of the matrix-matched calibration curve. The dilution factor was selected using the estimated concentration from the initial analysis with the intention of obtaining a response within the working range.

In 7 out of 24 samples, no HADs were detected above the LOQ level. Among the different analytes, only DAN was not found in the samples, which can be explained by the synthetic origin of this HAD. The highest number of HADs present in the same sample was equal to 13 in a food supplement containing buckthorn, rhubarb, and senna and in a herbal infusion with senna. Eleven HADs simultaneously occurred in food supplements containing *Frangula alnus*, *Aloe vera*, and senna. Ten HADs were found in a food supplement containing, among others, senna leaves and rhubarb. The level of 1 ppm was exceeded in 14 out of 24 analyzed samples (58%) for EMO, 12 samples (50%) for ALE and 2 samples (8%) for the sum of ALA and ALB.

The most frequently detected HAD was EMO (67% of samples), followed by CHR (58%) and RHE (54%). ALE, SENA, and SENB were measured in 50% of the samples, while

the least detected HADs were ALA (8%) and ALB (8%). The individual concentrations of HADs in positive samples ranged from 0.13 mg kg⁻¹ to 19.81 g kg⁻¹. The highest levels were found for GFA (19.81 g kg⁻¹) and GFB (15.39 g kg⁻¹) in a food supplement based on dirt tree bark, SENB (15.39 g kg⁻¹) in a food supplement containing, among others, senna pods and rhubarb root extract, and GFA (10.55 g kg⁻¹) in a herbal infusion of *Rhamnus frangula*. As regards the sample with senna pods and rhubarb extract, on the package, it is stated that the product contains 18 mg sennosides per tablet of 345 mg, which corresponds to 52.2 g kg⁻¹ of total sennosides. The current analysis demonstrated that this product contained 30.2 g kg⁻¹ of sennosides, the sum of SENA, SENA1, SENB, and SENC, implying that other isomers not covered by the current method might be present [20]. Notably, this sample also showed the highest total concentration of HADs in this work (sum of HADs: 41.05 g kg⁻¹). Other highly contaminated samples were a food supplement with dirt tree bark (sum of HADs: 36.69 g kg⁻¹) and *Rhamnus frangula* herbal infusion (20.09 g kg⁻¹).

It is important to mention that, with the exception of a few samples with low HAD concentrations, if one sennoside was detected in a sample, the other targeted sennosides were also present. This observation was also valid for ALA with ALB, and FRA with FRB, GFA and GFB.

The results of this small-scale market study undoubtedly demonstrate the importance of putting in place analytical methods for HADs in food supplements and other plant-based matrices. In vitro genotoxicity testing suggested that HADs, particularly ALE, interact with mammalian DNA under certain conditions. Based on different toxicological studies, EFSA concluded that HADs should be considered genotoxic and carcinogenic unless there are specific data to the contrary, such as for RHE [3]. Although recent in vivo studies on dried *Aloe ferox* juice and ALE indicated against DNA damage [37,38], the conclusion of EFSA on the safety of HADs remained unchanged [5]. At present, no daily intake of HADs that does not give rise to health concerns can be established. Given that about half of the commercial products in the current study exceeded the tolerated limit for ALE and EMO, this occurrence data certainly require more attention, as they highlight a health concern for the consumers [35]. Additionally, it should not be overlooked that two products contained 13 out of 16 target HADs simultaneously, highlighting the necessity of performing combined dietary exposure assessment to guarantee a high level of public health protection.

3. Conclusions

In this study, a UHPLC-MS/MS method for the simultaneous quantification of 16 HADs in food supplements was developed and validated. The validation study showed satisfactory performance parameters and good sensitivity of the method. Analysis of commercial food supplements and herbal infusions revealed the presence of sennosides, aloins, frangulins, glucofrangulins, and other target HADs, except for DAN. About 60% of the samples significantly exceeded the threshold for the content of a number of HADs proposed by the EC, thus stressing the importance of this method and the collected occurrence data from a controlling and regulatory point of view. The presented method is of critical importance for the scientific community and industry since the recent prohibition of certain HADs in preparations. The method can also be used as a tool in collecting more occurrence data for a wider range of HADs in plant-based products and facilitating a more accurate dietary exposure assessment.

4. Materials and Methods

4.1. Reagents and Standards

MeOH OptimaTM LC/MS from Fisher Scientific (Merelbeke, Belgium) was used. HCOONH₄ ULC/MS, HCOOH ULC/MS, CH₃COOH ULC/MS, ACN ULC/MS, ACE, and 2-propanol LC-MS were supplied by Biosolve BV (Valkenswaard, The Netherlands). Dimethyl sulfoxide (DMSO) and NH₄HCO₃ LC-MS LiChropurTM were obtained from Merck (Zwijndrecht, The Netherlands), while EtOH was purchased from VWR International

(Leuven, Belgium). Waters (Antwerp, Belgium) was the supplier of Oasis[®] HLB Vac cartridges (3 cc, 60 mg). H₂O was purified using a Milli-Q purification system (Millipore Corp., Bedford, MA, USA). Phenex-RC syringe filters (15 mm, 0.2 µm) were acquired from Phenomenex (Utrecht, The Netherlands). Single-use 3 mL syringes HENKE-JECT[®], and PP 50 mL centrifuge tubes were supplied by VWR International. Supelclean[™] ENVI-Carb[™] (3 mL, 250 mg) SPE tubes and Discovery[®] DSC-18 (6 mL, 500 mg) tubes were acquired from Merck. Bondesil C18 (40 µm) and Bondesil PSA (40 µm) bulk sorbents were obtained from Agilent (Diegem, Belgium).

Reference standards of the native HADs were purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany), while the labeled analogs were supplied by LGC Standards (Molsheim, France). ALE, CHR, RHE, SENA, SENA1, and SENC were dissolved in DMSO at 1 mg mL⁻¹. ALA, ALB, GFA, and EMO-D4 were dissolved in MeOH at 1 mg mL⁻¹. EtOH was used to dissolve DAN and DAN-D4 at 0.5 mg mL⁻¹. FRA, FRB, and PHY-D3 were dissolved at 0.5 mg mL⁻¹ in DMSO/2-propanol (50/50, *v/v*), MeOH/DMSO (90/10, *v/v*), and DMSO, respectively. The stock solutions of PHY and SENB were prepared at 0.4 mg mL⁻¹ in DMSO and MeOH, respectively. EMO was dissolved in MeOH at 0.25 mg mL⁻¹, while ALE-D4 was prepared in MeOH at 0.1 mg mL⁻¹. GFB was dissolved in MeOH at 0.04 mg mL⁻¹. The intermediate and working solutions were prepared in MeOH. All solutions of HADs and IS were stored at −20 °C.

4.2. Samples

The majority of products for the analysis of HADs were purchased in local health food shops and supermarkets, while others were obtained from online pharmacies. A detailed overview of the samples is given in Supplementary Table S1. Prior to extraction, samples were finely ground and/or homogenized.

4.3. Sample Preparation

Two grams of a food supplement were weighed in a 50 mL PP extraction tube. Thirty mL of MeOH/H₂O (80/20, *v/v*) was added, and the sample was extracted for 45 min on an overhead shaker (Heidolph Reax 2, Fisher Scientific). After centrifugation for 15 min at 3181 × *g*, 900 µL of the supernatant was combined with 540 µL H₂O. Prior to injection into the LC-MS/MS system, the diluted extract was filtered through 0.2 µm Phenex-RC filter using a syringe and spiked with a mixture of IS at levels corresponding to 1.79 mg kg⁻¹ for ALE-D4, 1.19 mg kg⁻¹ for DAN-D4 and 0.07 mg kg⁻¹ for EMO-D4.

4.4. LC-MS/MS Conditions

Analysis of HADs involved separation on an ACQUITY UPLC H-class system (Waters, Milford, MA, USA) equipped with an ACQUITY UPLC BEH C18 column (1.7 µm, 2.1 × 100 mm; Waters, Antwerp, Belgium) and an ACQUITY UPLC BEH C18 guard column (1.7 µm, 2.1 × 5 mm; Waters, Antwerp, Belgium). The column temperature was kept at 60 °C. The mobile phase used was (A) H₂O containing 0.1% HCOOH and (B) MeOH containing 0.1% HCOOH at a flow rate of 0.4 mL min⁻¹. The gradient program started with 5% B, linearly increasing to 85% B in 9 min. This mobile phase ratio was kept for 1.5 min and changed to 5% B within 0.5 min. Finally, the column was equilibrated with 5% B for 3 min. The total run time was 15 min, and the injection volume was 10 µL.

After separation, the analytes were detected by a Xevo TQ-S triple quadrupole mass spectrometer (Waters, Milford, MA, USA) operated in ESI(+) and ESI(−) modes. The MS parameters for both ionization modes were as follows: capillary voltage: 1.00 kV, source temperature: 150 °C, desolvation temperature: 450 °C, cone gas flow: 150 L h⁻¹, desolvation gas flow: 1000 L h⁻¹, collision gas flow: 0.15 mL min⁻¹, source offset: 30 V. The data were acquired in MRM mode, and the optimized MRM parameters are given in Table 1.

4.5. Validation

Method validation was performed on a multi-component fiber-based food supplement for supporting intestinal function, and the following parameters were determined: matrix effects, linearity, sensitivity, recovery, repeatability, within-laboratory reproducibility, and measurement uncertainty.

For the evaluation of matrix effects, matrix-matched calibration curves and curves dissolved in the solvent, i.e., MeOH/H₂O (50:50, *v/v*), were prepared. The slopes of the curves were compared by means of a *t*-test, and the matrix effect was calculated as a relative percentage of the ratio of the curve slopes. For the linearity, matrix-matched calibration curves of at least six fortification levels were prepared and analyzed in triplicate, and Mandel's test was used to assess the suitability of linear and quadratic regression models.

Sensitivity in terms of LOQ was defined as the lowest concentration level for which the method performance criteria were satisfied. The determination of recovery, repeatability, within-laboratory reproducibility, and measurement uncertainty was performed based on analyte-free matrix samples fortified in triplicate at three concentration levels (see Table 3). This analysis set-up was repeated on two other days by the same operator. Recovery was calculated as the relative percentage of the difference between the measured concentration and the fortified concentration. Repeatability and within-laboratory reproducibility are expressed as RSD under repeatability and reproducibility conditions, respectively. The expanded MU was calculated as a square root of the sum of squares of the uncertainties of bias and reproducibility and was multiplied by a coverage factor of 2 for 95% confidence.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/toxins16120505/s1>, Table S1: Overview of commercial samples purchased for the analysis of HADs, Table S2: Concentrations of HADs in commercial food supplements and herbal infusions, Figure S1: Influence of the different mobile phase modifiers and additives on the MS signal intensity of HADs. Figure S2: Efficiency (%) of the different solvents tested for the extraction of HADs from food supplements, Figure S3: LC-MS/MS multiple reaction monitoring (MRM) chromatograms of neat solvent MeOH/H₂O (50/50, *v/v*); (lower chromatogram), blank extract of food supplement (middle chromatogram) and blank extract of food supplement spiked with individual HADs at levels corresponding to 0.075 mg kg⁻¹ EMO, 0.15 mg kg⁻¹ ALA, 0.3 mg kg⁻¹ ALB, CHR, GFA, FRA, FRB, PHY and RHE, 0.6 mg kg⁻¹ ALE and DAN, 1.5 mg kg⁻¹ SENC, 2.25 mg kg⁻¹ SENA1 and SENB, and 3 mg kg⁻¹ GFA and SENA in a sample (upper chromatogram).

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References

1. Sperber, A.D.; Bangdiwala, S.I.; Drossman, D.A.; Ghoshal, U.C.; Simren, M.; Tack, J.; Whitehead, W.E.; Dumitrascu, D.L.; Fang, X.; Fukudo, S.; et al. Worldwide prevalence and burden of functional gastrointestinal disorders, results of Rome Foundation global study. *Gastroenterology* **2021**, *160*, 99–114. [CrossRef] [PubMed]

2. EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies). Scientific Opinion on the substantiation of a health claim related to hydroxyanthracene derivatives and improvement of bowel function pursuant to Article 13(5) of Regulation (EC) No 1924/2006. *EFSA J.* **2013**, *11*, 3412. [CrossRef]
3. EFSA ANS Panel (EFSA Panel on Food Additives and Nutrient Sources added to Food); Younes, M.; Aggett, P.; Aguilar, F.; Crebelli, R.; Filipic, M.; Frutos, M.J.; Galtier, P.; Gott, D.; Gundert-Remy, U.; et al. Scientific Opinion on the safety of hydroxyanthracene derivatives for use in food. *EFSA J.* **2018**, *16*, 5090. [CrossRef]
4. European Commission, Directorate-General for Health and Food Safety. Commission Regulation (EU) 2021/468 of 18 March 2021 amending Annex III to Regulation (EC) No 1925/2006 of the European Parliament and of the Council as regards botanical species containing hydroxyanthracene derivatives. *OJ* **2021**, *96*, 6–8.
5. EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA); Turck, D.; Bohn, T.; Castenmiller, J.; De Henauw, S.; Hirsch-Ernst, K.I.; Knutsen, H.K.; Mangelsdorf, I.; McArdle, H.J.; Naska, A.; et al. Scientific Opinion on additional scientific data related to the safety of preparations of *Rheum palmatum* L., *Rheum officinale* Baill. and their hybrids, *Rhamnus purshiana* DC., *Rhamnus frangula* L. and *Cassia senna* L., submitted pursuant to Article 8(4) of Regulation (EC) No 1925/2006. *EFSA J.* **2024**, *22*, e8766. [CrossRef] [PubMed]
6. EURL-MP-Report_002, Inventory Analytical Methods Hydroxyanthracene Derivatives. 2020. Available online: https://www.wur.nl/en/show/1-eurl-mp-report_002-inventory-analytical-methods-hydroxyanthracene-derivatives.htm (accessed on 9 September 2024).
7. Kline, D.; Ritruhai, V.; Babajanian, S.; Gao, Q.; Ingle, P.; Chang, P.; Swanson, G. Quantitative analysis of aloins and aloin-emodin in *Aloe vera* raw materials and finished products using high-performance liquid chromatography: Single-laboratory validation, First Action 2016.09. *J. AOAC Int.* **2017**, *100*, 661–670. [CrossRef]
8. Yılmaz, P.K.; Kolak, U. Development and validation of a SPE–HPLC method for quantification of rhein, emodin, chrysophanol and physcion in *Rhamnus petiolaris* Boiss. & Balansa. *J. Chromatogr. Sci.* **2023**, *62*, 872–877. [CrossRef]
9. Al-Hamoud, G.A.; Alam, P.; Fantoukh, O.I.; Hawwal, M.F.; Akhtar, A. HPLC–UV analysis of chrysophanol in *Senna occidentalis* extract obtained by using the RSM-optimized ultrasonic extraction process. *Processes* **2023**, *11*, 1410. [CrossRef]
10. Mandrioli, R.; Mercolini, L.; Ferranti, A.; Fanali, S.; Raggi, M.A. Determination of aloe emodin in *Aloe vera* extracts and commercial formulations by HPLC with tandem UV absorption and fluorescence detection. *Food Chem.* **2011**, *126*, 387–393. [CrossRef]
11. Loschi, F.; Faggian, M.; Sut, S.; Ferrarese, I.; Maccari, E.; Peron, G.; Dall’Acqua, S. Development of an LC–DAD–MS-based method for the analysis of hydroxyanthracene derivatives in food supplements and plant materials. *Molecules* **2022**, *27*, 1932. [CrossRef]
12. Xue, Y.; Liang, J. Screening of bioactive compounds in *Rhizoma polygoni cuspidati* with hepatocyte membranes by HPLC and LC–MS. *J. Sep. Sci.* **2014**, *37*, 250–256. [CrossRef] [PubMed]
13. Xu, Y.; Yu, X.; Gui, J.; Wan, Y.; Chen, J.; Tan, T.; Liu, F.; Guo, L. Ultrasonic solvent extraction followed by dispersive solid phase extraction (d-SPE) cleanup for the simultaneous determination of five anthraquinones in *Polygonum multiflorum* by UHPLC–PDA. *Foods* **2022**, *11*, 386. [CrossRef] [PubMed]
14. Dai, H.; Chen, Z.; Shang, B.; Chen, Q. Identification and quantification of four anthraquinones in rhubarb and its preparations by Gas Chromatography–Mass Spectrometry. *J. Chromatogr. Sci.* **2018**, *56*, 195–201. [CrossRef] [PubMed]
15. Nesměrák, K.; Kudláček, K.; Čambal, P.; Štícha, M.; Kozlík, P.; Červený, V. Authentication of senna extract from the eighteenth century and study of its composition by HPLC–MS. *Monatsh Chem.* **2020**, *151*, 1241–1248. [CrossRef]
16. Shi, Y.; Zhong, Y.; Sun, A.; Gao, B.; Sun, C.; Xiong, J. Validation of a rapid and simple high-performance liquid chromatography electrospray ionization-mass spectrometry method for simultaneous analysis of 15 key chemicals in slimming foods and herbal products. *J. Chromatogr. Sci.* **2018**, *56*, 912–919. [CrossRef]
17. Tao, Y.; Zhou, X.; Li, W.; Cai, B. Simultaneous quantitation of five bioactive ingredients in raw and processed *Fallopia multiflora* by employing UHPLC–Q–TOF–MS. *J. Chromatogr. Sci.* **2019**, *57*, 618–624. [CrossRef]
18. Di Minno, A.; Morone, M.V.; Ullah, H.; Sommella, E.; Buccato, D.G.; De Lellis, L.F.; Campiglia, P.; De Filippis, A.; Galdiero, M.; Daglia, M. Liquid chromatography-tandem mass spectrometry (LC–MS/MS)-based quantification of hydroxyanthracene derivatives in *Aloe vera* (L.) Burm. f. gel commercial beverages and preliminary safety evaluation through in vitro genotoxicity studies. *Food Safe Health* **2024**, *2*, 489–496. [CrossRef]
19. Girreser, U.; Ugolini, T.; Çiçek, S.S. Quality control of *Aloe vera* (*Aloe barbadensis*) and *Aloe ferox* using band-selective quantitative heteronuclear single quantum correlation spectroscopy (bs-qHSQC). *Talanta* **2019**, *205*, 120109. [CrossRef]
20. Çiçek, S.S.; Cardenas, C.M.; Girreser, U. Determination of total sennosides and sennosides A, B, and A1 in senna leaflets, pods, and tablets by two-dimensional qNMR. *Molecules* **2022**, *27*, 7349. [CrossRef]
21. Fierens, C.; Corthout, J. Anthranoid-containing medicines and food supplements on the Belgian market: A comparative study. *J. Pharm. Belg.* **2014**, *96*, 40–49.
22. Fung, H.Y.; Lang, Y.; Ho, J.-M.; Wong, T.-L.; Ma, D.-L.; Leung, C.-H.; Han, Q.-B. Comprehensive quantitative analysis of 32 chemical ingredients of a Chinese patented drug Sanhuang tablet. *Molecules* **2017**, *22*, 111. [CrossRef] [PubMed]
23. Namdar, D.; Mulder, P.P.J.; Ben-Simchon, E.; Hacham, Y.; Basheer, L.; Cohen, O.; Sternberg, M.; Shelef, O. New analytical approach to quinolizidine alkaloids and their assumed biosynthesis pathways in lupin seeds. *Toxins* **2024**, *16*, 163. [CrossRef] [PubMed]
24. Rodríguez-Cañás, I.; González-Jartín, J.M.; Alfonso, A.; Alvarino, R.; Vieytes, M.R.; Botana, L.M. Application of a multi-toxin detect method to analyze mycotoxins occurrence in plant-based beverages. *Food Chem.* **2024**, *434*, 137427. [CrossRef] [PubMed]

25. Rosenthal, I.; Wolfram, E.; Meier, B. New UHPLC—Method to determine Aloin A and B in *Aloe capensis*. *Planta Med.* **2013**, *79*, PK34. [CrossRef]
26. Rosenthal, I.; Wolfram, E.; Peter, S.; Meier, B. Validated method for the analysis of frangulins A and B and glucofrangulins A and B using HPLC and UHPLC. *J. Nat. Prod.* **2014**, *77*, 489–496. [CrossRef] [PubMed]
27. Hemwimol, S.; Pavasant, P.; Shotipruk, A. Ultrasound-assisted extraction of anthraquinones from roots of *Morinda citrifolia*. *Ultrason. Sonochem.* **2006**, *13*, 543–548. [CrossRef]
28. Glavnik, V.; Vovk, I. Extraction of anthraquinones from Japanese knotweed rhizomes and their analyses by high performance thin-layer chromatography and mass spectrometry. *Plants* **2020**, *9*, 1753. [CrossRef]
29. Aichner, D.; Ganzer, M. Analysis of anthraquinones in rhubarb (*Rheum. palmatum* and *Rheum. officinale*) by supercritical fluid chromatography. *Talanta* **2015**, *144*, 1239–1244. [CrossRef]
30. Brown, P.N.; Yu, R.; Kuan, C.H.; Finley, J.; Mudge, E.M.; Dentali, S. Determination of aloin A and aloin B in *Aloe vera* raw materials and finished products by high-performance liquid chromatography: Single-laboratory validation. *J. AOAC Int.* **2014**, *97*, 1323–1328. [CrossRef]
31. Tang, W.; Wan, M.; Zhu, Z.; Chen, G.; Huang, X. Simultaneous determination of eight major bioactive compounds in Dachengqi Tang (DT) by high-performance liquid chromatography. *Chin. Med.* **2008**, *3*, 5. [CrossRef]
32. Wu, T.-Y.; Chang, F.-R.; Liou, J.-R.; Lo, I.-W.; Chung, T.-C.; Lee, L.-Y.; Chi, C.-C.; Du, Y.-C.; Wong, M.-H.; Juo, S.-H.H.; et al. Rapid HPLC quantification approach for detection of active constituents in modern combinatorial formula, San-Huang-Xie-Xin-Tang (SHXXT). *Front. Pharmacol.* **2016**, *7*, 374. [CrossRef] [PubMed]
33. Xu, Y.; Wan, Y.; Liu, F.; Chen, J.; Tan, T.; Guo, L. Simultaneous determination of seven anthraquinones in *Cassiae semen* by natural deep eutectic solvent extraction. *Phytochem. Anal.* **2022**, *33*, 1246–1256. [CrossRef] [PubMed]
34. Yan, J.; Wang, Y.; Wu, H.; Sun, Z.; Tan, S.; Wang, W.; Gong, L.; Xia, X.; Li, S. Development of a method for simultaneous determination of two stilbenes and four anthraquinones from *Polygonum cuspidatum* by RP-HPLC. *J. AOAC Int.* **2019**, *102*, 69–74. [CrossRef] [PubMed]
35. European Commission. Standing Committee on Plants, Animals, Food and Feed Section General Food Law, 5 October 2020, Summary Report: Sante.ddg2.g.5(2020)7913268. Available online: https://food.ec.europa.eu/document/download/081fe590-a0b8-432b-84ba-3c7e6efcb3f9_en?filename=reg-com_gfl_20201005_sum.pdf (accessed on 9 September 2024).
36. European Commission, Directorate-General for Health and Food Safety. Commission Implementing Regulation (EU) 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. *OJ* **2021**, *180*, 84–109.
37. Galli, C.L.; Cinelli, S.; Ciliutti, P.; Melzi, G.; Marinovich, M. Lack of in vivo genotoxic effect of dried whole *Aloe ferox* juice. *Toxicol. Rep.* **2021**, *8*, 1471–1474. [CrossRef]
38. Galli, C.L.; Cinelli, S.; Ciliutti, P.; Melzi, G.; Marinovich, M. Aloe-emodin, a hydroxyanthracene derivative, is not genotoxic in an in vivo comet test. *Regul. Toxicol. Pharmacol.* **2021**, *124*, 104967. [CrossRef]

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Article

Validation of a UPLC-MS/MS Method for Multi-Matrix Biomonitoring of *Alternaria* Toxins in Humans

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Abstract: Mycotoxins, natural toxins produced by fungi, contaminate nearly 80% of global food crops. *Alternaria* mycotoxins, including alternariol (AOH), alternariol monomethylether (AME), and tenuazonic acid (TeA), present a health concern due to their prevalence in various plants and fruits. Exposure to these toxins exceeds the threshold of toxicological concern in some European populations, especially infants and toddlers. Despite this, regulatory standards for *Alternaria* toxins remain absent. The lack of toxicokinetic parameters, reference levels, and sensitive detection methods complicates risk assessment and highlights the necessity for advanced biomonitoring (HBM) techniques. This study addresses these challenges by developing and validating ultra-high performance liquid chromatography method coupled with tandem mass spectrometry to quantify AOH, AME, TeA, and their conjugates in multiple biological matrices. The validated method demonstrates robust linearity, precision, recovery (94–111%), and sensitivity across urine (LOD < 0.053 ng/mL), capillary blood (LOD < 0.029 ng/mL), and feces (LOD < 0.424 ng/g), with significantly lower LOD for TeA compared to existing methodologies. The application of minimally invasive microsampling techniques for the blood collection enhances the potential for large-scale HBM studies. These advancements represent a step toward comprehensive HBM and exposure risk assessments for *Alternaria* toxins, facilitating the generation of data for regulatory authorities.

Keywords: *Alternaria* toxins; multi-matrix; UPLC-MS/MS; human biomonitoring; VAMS

Key Contribution: A UPLC-MS/MS method was validated for the quantification of *Alternaria* toxins and their phase II conjugates in urine, blood, and fecal material with the aim of closing the gap between existing quantification methods and accurate human biomonitoring for risk assessment.

1. Introduction

Mycotoxins, naturally occurring toxic compounds produced by certain fungi, present a challenge to global food safety and public health. Among these, toxins produced by *Aspergillus*, *Fusarium*, *Alternaria*, and *Penicillium* are particularly concerning due to their prevalence in a wide array of food crops. Notably, Eskola et al. (2020) [1] highlighted that nearly 80% of global food crops are tainted with some level of mycotoxin contamination. This situation is compounded by climate change, which is expected to increase the spread of mycotoxins in Europe, thus escalating the health risks associated with their exposure [2]. Adding to the concern, the most recent annual report from the European Rapid Alert System for Food and Feed (RASFF) underscores mycotoxins as a significant hazard category, highlighting the issue as a major cause of economic loss and emphasizing the necessity for vigilant monitoring and regulatory action to safeguard public health [3].

Alternaria alternata, a species of particular concern, secretes mycotoxins such as alternariol (AOH), alternariol monomethylether (AME), and tenuazonic acid (TeA), predominantly affecting numerous crops, fruits, and vegetables. The European Food Safety Authority (EFSA) reports indicate that dietary exposure to TeA significantly exceeds that of other *Alternaria* toxins within the European population. Alarming, exposure levels for infants and toddlers surpass the established threshold of toxicological concern (TTC), indicating a potential health threat. For the adult population, both high dietary exposure scenarios (upper bound and 95th percentile) for AME and AOH are above the TTC, underscoring the public health risk posed by these contaminants [4,5]. In the same study, EFSA suggested the need for continued efforts to generate more analytical data on *Alternaria* toxins using sensitive methods to reduce uncertainty in exposure assessments.

The toxicity of these mycotoxins extends to acute and chronic adverse effects on microbes, plants, humans, and animals [6,7]. TeA is known for its acute toxicity, evidenced by adverse outcomes in rodents ($LD_{50} = 81\text{--}186\text{ mg/kg bw}$) and chicken embryos ($LD_{50} = 0.55\text{ mg/egg}$), as well as in various animal-feeding trials causing vomiting and hemorrhages in lungs and the gastrointestinal system. Furthermore, *in vitro* studies have demonstrated the cytotoxic effects of TeA, particularly when combined with AME, in human intestinal epithelial cells (Caco-2) and hepatocytes (HepG2) [8]. AOH and AME were demonstrated to be cytotoxic and genotoxic [9]. Moreover, several studies confirmed the ability of AOH and AME to cause DNA damages already at low concentrations [10–13]. Despite the evident health risks, *Alternaria* toxins remain unregulated in food in any country, though the European Commission has set indicative alert levels for these mycotoxins in specific food commodities, prompting investigations into the presence of *Alternaria* toxins and the impact of food processing on their levels [4,5,14]. Investigation on the metabolism and excretion profiles of *Alternaria* toxins has been limited [15–17], with TeA being the only toxin with known toxicokinetic (TK) parameters in pigs and broiler chickens [18] and partially known in humans [19]. The assessment of dietary co-exposure to *Alternaria* toxins is currently based on direct mycotoxin quantification in food. A comprehensive risk assessment at individual level is challenged by the multiple metabolic pathways involved and hampered by the lack of toxicokinetic parameters and health-based evidence values. This challenge is further exacerbated by the diverse chemical groups and physicochemical properties of *Alternaria* toxins, complicating the validation of a unified analytical method, especially considering the peculiar acidity and tautomerism [20] of TeA, as displayed in Figure 1.

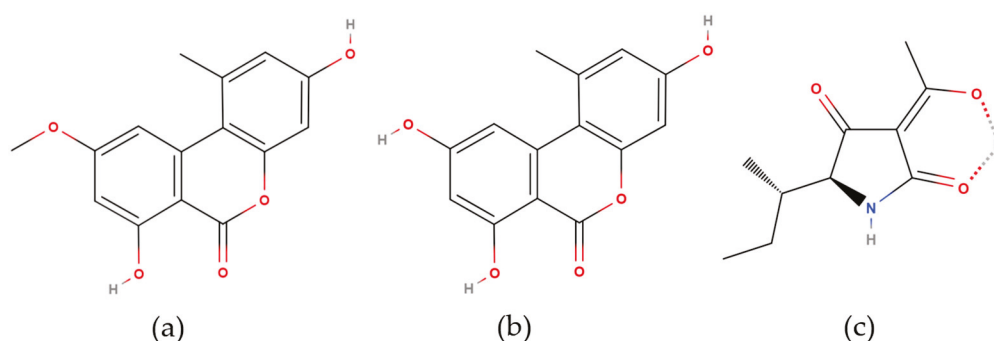


Figure 1. Chemical structures of (a) alternariol monomethyl ether (AME), (b) alternariol (AOH), and (c) the predominant form of tenuazonic acid in organic solvents and water (TeA) according to Mikula et al. (2013) [20]. Structures created with MolView.org.

Understanding the potential health consequences in humans necessitates the capability of conducting accurate mycotoxin exposure assessments at the individual level and determining population toxicokinetics. In response to this growing need for accurate mycotoxin exposure assessment, human biomonitoring (HBM) has been evolving, with a shift toward methodologies that are less invasive and more patient-friendly. This transition is pivotal

for enhancing our capacity to monitor exposure to *Alternaria* mycotoxins in a manner that is both efficient and acceptable to the public. Capillary blood microsampling, particularly using volumetric absorptive microsampling (VAMS), allows home and remote sampling by providing a minimally invasive alternative to the traditional blood withdrawal [21,22]. Microsampling significantly enhances the feasibility of large-scale human biomonitoring (HBM) studies [23] and facilitates more comprehensive pharmacokinetic trials [24] by increasing patient participation and enabling more frequent sample collection. Moreover, owing to improvements in the analytical performances in detection and quantification of analytes, the analysis of capillary blood on VAMS, whole blood, serum, and plasma provides comparable results [24,25]. These factors make whole blood microsampling a valid alternative compared to traditional hematic matrices aligning with current trends in analytical methodology that prioritize patient comfort and study feasibility while maintaining analytical rigor. The development of quantitative methods for analyzing multi-mycotoxins in capillary blood, collected via volumetric absorption microsampling (VAMS) techniques such as Mitra[®] tips, represented a significant advance in this field [25]. As it is known, mycotoxins present a wide range of polarities and diverse physicochemical properties that require compromising the simultaneous quantification of different classes of mycotoxins and/or the sensitivity of the methods. In fact, the quantification method proposed by Vidal et al. (2021) [25] for 24 mycotoxins was not validated for TeA and the sensitivity for AOH and AME is not sufficient for HBM ($LOQ_{AOH} = 2.74 \text{ ng/mL}$; $LOQ_{AME} = 3.72 \text{ ng/mL}$), especially if considered in relation with the TTC value of the two toxins (2.5 ng/kg bw).

Metabolization of AOH, AME, and TeA were proved to involve phase I and II reactions [15,17,26], indicating the necessity for quantifying not only the parent mycotoxins but also their metabolites for accurate exposure assessment. Aichinger et al. (2020) [27] highlighted the importance of screening for glucuronide metabolites of AOH and AME in urine for effective HBM. This approach implies being able to quantify accurately mycotoxins and their metabolites. Unfortunately, this remains a challenge given the lack of commercially available reference standards for these metabolites. An approach to quantify phase II metabolites is introducing an enzymatic hydrolysis step to convert any conjugate into the parent molecules, as tentatively performed by Asam et al. (2013) [19]. Nonetheless, previous methods have not validated the co-quantification of *Alternaria* toxins, considering the conversion of phase II conjugates [28,29].

HBM is not often performed in human fecal material, although studies in rats demonstrated the scarce bioavailability of AOH and AME that are preferentially excreted through the gastrointestinal tract [30]. Krausová et al. (2021) [31] made advances in detecting multi-mycotoxins in toddler stools, but the method was validated only for AOH and AME, not TeA. In Appendix A, a resume of validation parameters of the mentioned analytical methods for the quantification of one or more *Alternaria* toxin in biological matrices is provided.

In response to these challenges, this study aims to advance HBM of *Alternaria* mycotoxins, offering validated extraction procedures and a state-of-the-art ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) method. This technique allows for the simultaneous and quantitative biomonitoring of AOH, AME, and TeA, along with their phase II metabolites in urine, capillary blood collected via Mitra[®] tips (VAMS), and feces. The quantification of phase II conjugates in biological samples is achieved by a double quantification—preparing samples with and without enzymatic hydrolysis employing β -glucuronidase by *Helix pomatia*. This advancement represents a pivotal step toward refining exposure assessments, informing regulatory standards, and ultimately enhancing public health.

2. Results and Discussion

2.1. Method Optimization

The blood extraction from Mitra[®] tips (VAMS) was optimized based on the method described by Vidal et al. (2021) [25]. Various extraction solvents were tested, including

CH₃CN, CH₃OH, CH₃CN/H₂O (60/40 *v/v*), and CH₃OH/H₂O (60/40 *v/v*) added with 0, 0.1, and 1% CH₃COOH. Pure CH₃OH demonstrated to be the best extraction solvent, as the presence of water resulted in turbid extracts with high chromatographic noise, and the presence of acid negatively affected the recovery of TeA. A novel enzymatic hydrolysis step, based on the method published by Amante et al. (2021) [32], was introduced after the extraction to convert phase II conjugates into the parent molecule. The original sample preparation foresaw the evaporation of the extract and reconstitution of the residue in 50 µL of injection solvent. Following the same procedure, the samples would have an overall four-fold dilution. For this reason, in the optimized procedure, the residues are reconstituted in 25 µL (two-fold dilution) of injection solvent and transferred into glass total recovery vials (Waters, Manchester, UK). Additionally, the centrifugal filtration was replaced with a centrifugation (10 min at 10,000× *g*) to avoid loss of sample since the CH₃OH extracts resulted cleaner than with the watery extraction solvents.

Urine sample preparation was based on the salting-out assisted liquid–liquid extraction (SALLE) described by Vidal et al. (2018) [33], which was extended to include *Alternaria* toxins without further optimization, as the performances resulted fit for purpose.

The extraction of feces samples was developed using a design of experiments (DoE) approach. The solvents and extractions techniques considered were retrieved from the methods published by Krausová et al. (2021), Lauwers et al. (2019), Miró-Abella et al. (2019), and Puntsher et al. (2019a; 2019b) [16,30,31,34,35]. The DoE, performed using D-Optimal from the Chemometric Agile Tool (CAT) in the R environment [36,37], provided 26 independent experiments with different levels of CH₃CN, H₂O, CH₃OH, CH₃COOH, use of solid phase extraction (SPE) cartridges (Strata C-18, Phenomenex, Woerden, The Netherlands; Oasis, Waters, Manchester, UK), and use of SALLE. Figure 2 displays the coefficients of the DoE models computed for each analyte using the chromatographic peak area, demonstrating that solid liquid extraction (SLE) with CH₃CN was the best approach, and the addition of CH₃COOH positively influenced the extraction of AME and AOH. Accordingly, the selected extraction procedure involved SLE with CH₃CN containing 1% CH₃COOH (*v/v*).

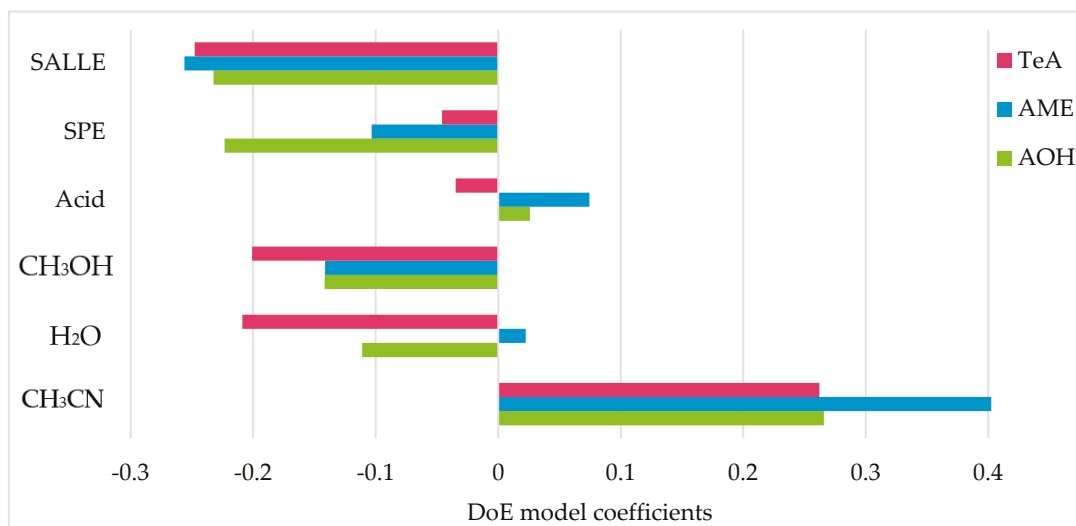


Figure 2. Normalized coefficients for the factors considered during the design of experiments process performed to optimize the extraction of TeA, AME, and AOH from human feces.

Finally, a novel liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis method was developed using an Acquity UPLC system coupled to a Waters Xevo[®] TQ-XS tandem quadrupole mass spectrometer (Waters, Manchester, UK) equipped with an electrospray interface. MS/MS analysis was carried out using multiple reaction monitoring (MRM) with negative electrospray ionization (ESI[−]). The MRM parameters for ionization

and fragmentation were optimized by tuning the compounds of interest. For the three *Alternaria* mycotoxins, the formation of the $[M-H]^-$ quasi-molecular ion led to higher precursor ion intensities. The two most intense product ions were selected for the MRM transitions in the MS method after applying different collision energies.

The mobile phase (mp) composition, flow, gradient, column type, injection volume, and strong and weak wash solvent composition and volumes were evaluated based on peak shape, area, and signal-to-noise ratios. Optimal separation and sensitivity was obtained using 1% CH_3COOH (*v/v*) in H_2O (mp A) and pure CH_3CN (mp B) and alternatively with $\text{H}_2\text{O}/\text{CH}_3\text{OH}/\text{CH}_3\text{COOH}$ (94/5/1, *v/v/v*, mp A) and $\text{CH}_3\text{OH}/\text{H}_2\text{O}/\text{CH}_3\text{COOH}$ (97/2/1, *v/v/v*, mp B) adjusted with 5 mM of $\text{CH}_3\text{COONH}_4$. The first set of mps was chosen since it aligns more closely with green chemistry principles [38]. The column choice was governed mostly by the peak shape of TeA that tended to tail. Overall, the Acquity UPLC[®] HSS T3 (1.8 μm $\times$ 2.1 $\times$ 100 mm) column paired with a pre-column Acquity UPLC[®] HSS T3 VanGuard[™] (1.8 μm) (Waters, Manchester, UK) provided the best chromatographic separation and narrowest peaks. Moreover, accordingly with the lower volume of blood samples available for the analysis, the loop overfilling factor of the Acquity UPLC system was set to 1.5.

2.2. Method Validation

The validation of analytical methods for the quantification of *Alternaria* toxins—AOH, AME, TeA—and their phase II conjugates in human urine, blood, and feces was conducted with a focus on ensuring sensitivity, recovery, and applicability across the biological matrices considered. The calibration model was fit by applying a quadratic $1/x$ weighed fitting. The performance of the methods were demonstrated through a range of metrics including the coefficient of determination (R^2), limit of detection (LOD, ng/mL), calibration range - expressed as the lower limit of quantification (LLOQ, ng/mL)–upper limit of quantification (ULOQ, ng/mL) interval - signal suppression enhancement (SSE, %), apparent recovery (R_A , %), and extraction efficiency (R_E , %), recovery (%), repeatability (RSD_r , %), and intermediate precision (RSD_R , %). Table 1 summarizes these performance indicators, underscoring the robustness of the method across all tested matrices. The validation results demonstrate robust linearity and goodness-of-fit with R^2 above the acceptance threshold (>0.990). The LODs obtained in urine and capillary blood were notably <0.1 and <0.05 ng/mL, confirming the feasibility of the calibration range chosen (0.1–10 ng/mL for urine and 0.05–10 ng/mL for blood) and affirming the sensitivity of the method. For fecal samples, higher LOD values (0.204–0.424 ng/mL) were obtained reflecting the complexity of the matrix and associated chromatographic noise, yet remain coherent within the calibration range. The LLOQ for the three analytes was set as the concentration of the lowest calibration point and verified for recovery, repeatability, and intermediate precision. Accordingly, the obtained recovery (%), repeatability (RSD_r , %), and intermediate precision (RSD_R , %) were within the acceptable ranges for the three matrices. No interference peaks and carry-over were detected at the retention time $\pm 2.5\%$ of the analytes with S/N values ≥ 3 . The introduction of the enzymatic hydrolysis, achieved by the use of β -glucuronidase by *Helix pomatia* on the urine and blood extract, did not influence negatively the performances of the method. The methods were successfully validated and obtained results comparable with the procedures without hydrolysis. Remarkably, the LOD values obtained with hydrolysis were slightly lower than the ones without hydrolysis, confirming the suitability of the approach.

Table 1. Results of the method validation for the quantification of alternariol (AOH), alternariol monomethyl ether (AME) and tenuazonic acid (TeA) in urine, capillary blood collected via VAMS Mitra® tips, and feces. The enzymatic hydrolysis was performed by the use of β -glucuronidase by *Helix pomatia*. The performance of the method is reported as the determination coefficient (R^2), limit of detection (LOD, ng/mL), calibration range—expressed as the lower limit of quantification (LLOQ, ng/mL)—upper limit of quantification (ULOQ, ng/mL), interval—signal suppression enhancement (SSE, %), apparent recovery (R_A , %), and extraction efficiency (R_E , %), recovery (%), repeatability (RSD_r , %), and intermediate precision (RSD_R , %). The results were obtained by testing the same calibration curve three times for 3 days ($n = 9$).

Analyte	Matrix	Enzymatic Hydrolysis	R ²	LOD	Calibration Range	SSE	R _A		R _E		Recovery		RSD _r		RSD _R			
							without IS		with IS		LLOQ		ULOQ		LLOQ		ULOQ	
							ng/mL *	ng/mL *	%	%	%	%	%	%	%	%	%	%
					LLOQ-ULOQ													
AOH	Urine	No	0.9960	0.053	0.1–10	22.1	15.5	94.71	70.3	107.2	100.9	14.0	2.0	0.7	0.6			
AME	Urine	No	0.9989	0.050	0.1–10	73.0	18.8	96.3	25.7	105.5	100.3	10.0	5.0	7.4	3.1			
TeA	Urine	No	0.9971	0.016	0.1–10	104.1	95.4	95.8	95.7	107.6	98.3	4.0	4.1	3.2	4.7			
AOH	Urine	Yes	0.9983	0.042	0.1–10	101.3	64.4	93.55	63.6	102.2	103.1	19.0	3.5	3.0	1.9			
AME	Urine	Yes	0.9994	0.043	0.1–10	24.5	24.8	108.9	101.4	103.6	100.9	16.5	4.3	15.4	4.5			
TeA	Urine	Yes	0.9983	0.048	0.1–10	80.3	80.01	97.12	99.6	96.22	101.1	11.5	2.1	17.1	1.9			
AOH	Blood	No	0.9988	0.027	0.05–10	93.6	89.6	92.6	95.7	106.7	100.1	10.0	2.0	7.7	0.8			
AME	Blood	No	0.9967	0.007	0.05–10	62.9	63.3	93.3	100.4	111.1	99.6	11.1	2.3	2.4	1.5			
TeA	Blood	No	0.9984	0.029	0.05–10	93.6	89.6	105.6	95.7	108.9	99.6	8.9	1.5	8.8	1.5			
AOH	Blood	Yes	0.9963	0.01	0.05–10	70.2	43.3	97.8	61.6	96.3	101.1	6.3	0.4	4.5	4.4			
AME	Blood	Yes	0.9957	0.003	0.05–10	113.8	88.8	102.6	78.0	103	102.4	17.8	0.6	12.3	2.1			
TeA	Blood	Yes	0.9912	0.026	0.05–10	105.3	64.9	99.6	61.6	98.0	101.1	19.6	3.1	9.5	3.1			
AOH	Feces	No	0.9934	0.412	1.25–20	91.7	77.2	98.4	78.9	103.9	100.3	1.2	0.1	4.2	3.5			
AME	Feces	No	0.9919	0.424	1.25–20	82.0	74.9	99.2	89.7	94.5	94.4	1.3	5.0	8.3	5.3			
TeA	Feces	No	0.9946	0.204	0.63–20	69.3	62.3	100.1	75.2	101.1	102.1	5.9	3.7	5.9	3.7			

* for feces, the concentration is expressed as ng/g of dry sample.

2.3. Comparison with Previously Published Methods

The validation of our methods presents significant improvements over previous studies, particularly in the detection limits for *Alternaria* toxins in human biological samples. Comparing the LOD values obtained for AME and AOH in urine with the existing literature [28,29], the results align well, broadcasting similar sensitivities. Notably, the present study marks a significant improvement in detecting TeA, with LODs (0.016 ng/mL in urine; 0.048 ng/mL in urine after hydrolysis) an order of magnitude lower than previously published methods [19,28,30]. This enhancement in sensitivity indicates a substantial advancement in our ability to detect and quantify TeA at low concentration and is crucial for accurately assessing low levels of exposures that are greatly influencing left-censored data. Similar conclusions can be drawn for feces samples; the present method shows comparable or improved performance relative to those validated by Krausová et al. (2021) [31] in infant feces and Puntischer et al. (2019) [30] in rat feces, indicating its applicability in complex matrices and providing a new tool to investigate HBM in this type of matrix. The method optimization for capillary blood, collected via Mitra® tips, extends the sample preparation technique of Vidal et al. (2021) [25] to include TeA and enhances the quantification performance for AOH and AME. Overall, the sensitivity of the methods presented opens the possibility to detect and quantify *Alternaria* toxins in samples of subjects exposed at or below the TTC. Moreover, the introduction of the hydrolysis step allows for the quantification of both parent *Alternaria* toxins and their phase II conjugates in urine and capillary blood. In fact, the enzyme chosen provides glucuronidase and sulfatase activity that can be exploited for the comprehensive assessment of total toxin exposure, including glucuronidated and sulfonated forms of *Alternaria* toxins that are extensively described in the literature [15,17,26,27]. The successful validation of a quantification method for phase II conjugates in various biological matrices is a significant advancement in HBM of *Alternaria* toxin. By accounting for both parent compounds and their conjugated forms, the developed method provides an accurate tool to assess exposure and to possibly investigate their toxicokinetics, which is essential for conducting reliable risk assessments and establishing regulatory standards.

2.4. Matrix Effects

Extraction efficiency was acceptable for all mycotoxins extracted from the three matrices, except for AME in urine prepared without enzymatic hydrolysis (25.7%). The investigation of the signal suppression enhancement showed diminishment of the signals of AME in urine prepared with enzymatic hydrolysis and AOH in urine prepared without enzymatic hydrolysis, 24.5% and 22.1%, respectively. To compensate for the matrix effects, isotope-labeled AME ($^{13}\text{C}_{15}$ -AME) was added as internal standard (IS) to the samples before the extraction. The apparent recovery, achieved by considering the responses, i.e., the ratio of the areas of the analyte and the internal standard corrected by the concentration of the internal standard, was ensured within 92.6–108.9% for the three toxins for all the extraction procedures. This demonstrates that any losses due to incomplete extraction or SSE were proportional systematic errors [39]. Therefore, the addition of $^{13}\text{C}_{15}$ -AME as an internal standard accounted for the losses, thus preserving the overall recovery.

2.5. Stability

Stability tests for urine and capillary blood collected via VAMS Mitra® tips are reported in Table 2. The three toxins did not show any sign of degradation in urine, with recovery ranging 87.0–116.0% considering all the conditions tested. Vidal et al. (2021) [25] reported no loss of AME and AOH at 10 ng/mL after storing of the capillary blood for 7–21 days at 4 °C or at room temperature (recovery ranging between 83.6 and 118.0%). The stability test performed revealed no significant loss of AME, confirming the same results. On the contrary, the recovery obtained for AOH after 5 days of storage at 20 °C was 64.1% and substantially differing from the one reported in the literature [25], namely 118%. TeA is generally stable in both capillary blood and urine; however, the recovery after

storage at 20 °C for 5 days for the capillary blood at 10 ng/mL was reduced to 76.5%. Overall, the stability of the three toxins in capillary blood on Mitra[®] tips and urine is good, since no extensive decline in the recovery was proven. Avoiding the storage of capillary blood samples collected via VAMS Mitra[®] tips at room temperature or higher, however, is advisable to circumvent any loss of TeA and AOH.

Table 2. Results of the stability test of alternariol (AOH), alternariol monomethylether (AME), and tenuazonic acid (TeA) in urine and capillary blood collected via VAMS Mitra[®] tips.

Analyte	Stability in Urine (%)				Stability in Capillary Blood (%)			
	0.1 ng/mL	10 ng/mL	0.1 ng/mL	10 ng/mL	0.05 ng/mL	5 ng/mL	0.05 ng/mL	5 ng/mL
	−20 °C 21 Days	−20 °C 21 Days	4 °C 5 Days	4 °C 5 Days	4 °C 21 Days	4 °C 21 Days	20 °C 5 Days	20 °C 5 Days
AOH	93.0	99.5	87.0	89.8	104.5	94.9	99.2	64.1
AME	115.0	95.8	107.0	95.9	108.9	111.3	104.0	93.9
TeA	101.6	101.0	116.0	96.2	101.2	99.9	89.6	76.5

2.6. Application to Real Samples

The validated methods were applied to real samples obtained from a small-scale exposure study on three volunteers that consumed a watery bolus containing one of the three *Alternaria* toxins investigated at the TTC.

TeA was detectable between 15 min and 4 h from the exposure in blood collected via VAMS Mitra[®] tips ($n = 5$) and between 1 and 8 h in urine ($n = 6$). The maximum concentrations of TeA were 0.40 and 29.37 ng/mL, in blood and urine, respectively. Notably, the hydrolysis step led to an increase in the concentrations of TeA, leading to a maximum concentration of 1.04 ng/mL in blood and 31.00 ng/mL in urine. Due to the lower TTC of AOH and AME, these toxins were quantified at lower concentrations compared to TeA. AOH was detectable after 23 h in six blood samples of which four could be quantified; the maximum concentration found was 0.18 ng/mL before hydrolysis. AOH was detected in three samples in urine between 23 and 39 h from the exposure, but was quantifiable only in one with a concentration of 0.12 ng/mL before hydrolysis. Similar to TeA, an increment in the concentrations followed the application of the hydrolysis; the maximum concentration for total AOH was 0.27 ng/mL in blood and 0.16 ng/mL in urine. Additionally, AOH could be quantified in one more urine sample after hydrolysis (0.11 ng/mL). AME was detected in several blood samples collected ($n = 7$), and quantified in four of them with a maximum concentration of 0.16 ng/mL before hydrolysis and 0.21 ng/mL after hydrolysis. No urine sample contained AME above the LLOQ, only after hydrolysis two samples were quantified at 0.11 and 0.12 ng/mL. Regarding the analysis of stool samples, AME was detected for the first time after 35 h from the exposure at a concentration of 7.86 ng/g of dry matrix. On the contrary, TeA and AOH were not detected. While this was an expected outcome for TeA, since a study in rats demonstrated that only up to 2% of the dose administered is excreted via the gastro-intestinal tract, AOH was supposed to be detected since its excretion in the same study was of 89% of the dose administered in this matrix [30]. However, the only sample provided by the volunteer was collected just 13 h after the ingestion of AOH, which was probably an insufficient time frame between the exposure and the collection to record the excretion.

The concentrations of AOH and AME after exposure at a dose equal to the TTC reached levels at the limit of the validated method. However, the results demonstrated the applicability of the method for direct risk assessment and large-scale HBM. On the other hand, the concentrations obtained for TeA meet perfectly the validation range of the methods, thus opening the possibility for a broader toxicokinetic study. Moreover, the data highlight the crucial role of the hydrolysis step in accurately assessing the content of *Alternaria* toxins in urine and blood, as it allows for the quantification of both free and

conjugated forms of the toxins. This represents an important novelty and aligns with the opinion of the scientific community in the context of the HBM of *Alternaria* toxins [27].

3. Conclusions

In conclusion, the developed and validated UPLC-MS/MS method represents a significant advancement in the biomonitoring of *Alternaria* toxins in human biological matrices. This multi-matrix approach, which includes urine, capillary blood, and feces, provides a comprehensive tool for assessing exposure to AOH, AME, TeA, and their phase II conjugates. The incorporation and validation of an enzymatic hydrolysis step using β -glucuronidase is a novel aspect that allows a more accurate quantification of toxin levels by converting phase II conjugates back to their parent compounds, ensuring a more realistic representation of exposure and avoiding underestimation [27].

The validated methods showed improved sensitivity for AOH and AME, and included for the first time the quantification of TeA in capillary blood and human feces, addressing critical gaps in current research. The methods were successfully applied to real samples collected during a small-scale toxicokinetic study, confirming their applicability for risk assessment. Moreover, the results showed the potential for determination of the toxicokinetic properties of TeA through carefully designed human toxicokinetic trials, further enhancing our understanding of its behavior in the human body. The lower LODs achieved are crucial for generating reliable exposure data to inform regulatory standards and public health authorities. By providing left-censored data at an acceptable level and operating on multiple matrices, the method enables the possibility to perform accurate HBM and comprehensive risk assessment associated with *Alternaria* toxin exposure. Additionally, the application of minimally invasive microsampling techniques, such as the use of Mitra[®] tips for capillary blood collection, aligns with the trend toward less invasive HBM methods and facilitates the implementation of large-scale studies.

In summary, the development of a multi-matrix UPLC-MS/MS method, coupled with the application of minimally invasive microsampling techniques, represents a significant step forward in the HBM of *Alternaria* toxins. It provides the precise quantification of these natural toxins in different matrices, promoting the possibility of conducting comprehensive risk assessment and large-scale HBM studies, ultimately contributing to the protection of public health.

4. Materials and Methods

4.1. Chemicals and Reagents

One milligram of the individual mycotoxin (AOH, AME, and TeA, Figure 1) solid standards was obtained from Fermentek (Jerusalem, Israel), while 25.1 μ g of isotope-labeled AME (¹³C₁₅-AME) as internal standard (IS) was purchased from Merck (Darmstadt, Germany). All the mycotoxins and isotope-labeled mycotoxins solid standards were dissolved in methanol (CH₃OH) to reach the final concentration of 1 mg/mL and 25.1 μ g/mL, respectively, and were stored at -20 °C. From the individual stock solutions, a standard mixture of TeA, AME, and AOH, 10 ng/mL each, and a ¹³C₁₅-AME solution of 50 ng/mL were prepared in CH₃OH. Water (H₂O) was obtained from an Aurim[®] Pro water system from Sartorius (Brussels, Belgium). LC-MS grade (99.95%) methanol (CH₃OH) and acetonitrile (CH₃CN) were purchased from BioSolve (Valkenswaard, The Netherlands). Glacial acetic acid (CH₃COOH, 100%), formic acid (HCOOH, 98–100%), ammonium acetate (CH₃COONH₄, 99%), and sodium acetate (CH₃COONa, 99%) were supplied by Merck (Darmstadt, Germany). Sodium chloride (NaCl, 99%) and magnesium sulfate (MgSO₄, 99%) were obtained from VWR Chemicals (Leuven, Belgium). β -glucuronidase by *Helix pomatia* type HP-2 aqueous solution ($\geq 100,000$ units/mL) was purchased from Merck (Darmstadt, Germany). VAMS Mitra[®] tips were obtained from Trajan Scientific and Medical (Torrance, CA, USA).

4.2. Sample Collection

EDTA-anticoagulated blood samples for method development and validation purposes were supplied by Rode Kruis Vlaanderen (Ghent, Belgium). The blood samples were aliquoted in 0.5 mL cryogenic tubes and stored at -80°C . Once the blood was defrosted and homogenized, the samples were prepared by dipping the tip into the whole blood. After completely filling the tips, the devices were dried in the accompanying clamshells for one night at room temperature and stored at -80°C until the day of the sample preparation. Individual urine samples were donated by 20 healthy subjects in plastic containers and pooled together. The samples were stored at -20°C until the day of the sample preparation. Individual feces samples were donated by 8 healthy subjects in plastic containers equipped with screw caps with spoon and pooled together. The samples were freeze-dried for 24–36 h at 0.300 mbar (Büchi, Uster, Switzerland) and stored at -80°C until the day of the sample preparation. Real samples were obtained via a small-scale human exposure study performed as described by Vidal et al. (2018) and Visintin et al. (2023) [17,33]. Briefly, three volunteers ingested a watery bolus containing either TeA or AOH, or AME at the TTC, after a washout period of 2 days. The volunteers were then asked to collect individual blood (via VAMS Mitra[®] tips), urine, and fecal samples for 48 h. The blood was collected following a schedule: at 15, 30 min, 1, 2, 4, 6, 8, 12, 24, 36, and 48 h after the bolus consumption. Throughout the study, the volunteers followed a nutritional regime developed specifically for minimizing the dietary intake of *Alternaria* toxins. Samples were aliquoted and stored at -80°C until the day of the sample preparation. The study was conducted according to the guidelines in the Declaration of Helsinki and was approved by the Ethical Committee of the Ghent University Hospital (B670201630414). Informed consent was obtained from all the volunteers before participation.

4.3. Sample Extraction

4.3.1. Capillary Blood Samples

The blood extraction from Mitra[®] tips (VAMS) was performed as described by Vidal et al. (2021) [25] after optimization of the extraction solvent. After separation of the polymeric tips from the plastic handlers, the tips were transferred into Eppendorf Safe-Lock Tubes 1.5 mL (Eppendorf Belgium N.V.-S.A., Aarschot, Belgium). The calibration curves were built by spiking 9 tips at different concentration levels with the mycotoxin standard mix and IS solutions. The final concentrations were 0.00 (blank), 0.05, 0.10, 0.20, 0.50, 1.00, 2.00, 5.00, and 10.00 ng/mL for AOH, AME, and TeA, and 2.50 ng/mL for the IS. The tips were left air-drying in a cool and dark place to ensure the complete absorption of the mycotoxins on the polymeric material. The extraction was carried out by adding 550 μL of CH_3OH , ultrasonicated for 20 min at room temperature (Branson Ultrasonic Corporation, Danbury, CT, USA), and shaking the samples for 30 min at room temperature using an overhead shaker (Agilitec, Paris, France). Different extraction solvents (mix of H_2O and CH_3CN or CH_3OH with a different percentage of CH_3COOH) were considered. Based on matrix effect and recovery, CH_3OH was eligible as the best extraction solvent for the three mycotoxins. The tips were removed, and the supernatant was split in two aliquots and evaporated under a gentle stream of N_2 at room temperature using a Turbovap LV Evaporator (Biotage, Charlotte, NC, USA). The extract of the first aliquot was reconstituted in 25 μL of injection solvent ($\text{CH}_3\text{CN}/\text{H}_2\text{O}/\text{CH}_3\text{COOH}$, 25/74/1, $v/v/v$), vortexed, and centrifuged for 10 min at $10,000\times g$. Finally, samples were transferred into glass total recovery vials (Waters, Manchester, UK). The extract of the second aliquot was subjected to hydrolysis using β -glucuronidase by *Helix pomatia* to hydrolyze phase II conjugates into the original mycotoxin. The extracts were reconstituted in 250 μL H_2O and 25 μL acetate buffer 0.05 M pH 5; 5 μL of β -glucuronidase solution was added to the solution, and the tubes were vortexed for 1 min and incubated at 55°C while shaking at 1400 rpm using a Thermo-Shaker TS-100 (Biosan, Riga, Latvia). The reaction was stopped after 1 h by protein precipitation obtained by adding 250 μL of ice-cold CH_3CN . The suspension was vortexed for 30 s and centrifuged for 5 min at $10,000\times g$. The supernatant was transferred in a

tube and evaporated under a gentle stream of N₂ at room temperature. The residue was reconstituted in 25 µL of injection solvent (CH₃CN/H₂O/CH₃COOH, 25/74/1, *v/v/v*), vortexed, and centrifuged for 10 min at 10,000× *g*. Finally, samples were transferred into glass total recovery vials (Waters, Manchester, UK).

4.3.2. Urine

The urine sample preparation was based on the salting-out assisted liquid–liquid extraction (SALLE) as described by Vidal et al. (2018) [33]. Briefly, 2 mL of the thawed urine sample was transferred to a 50 mL extraction tube. The calibration curves were built by spiking 9 samples at different concentration levels with the mycotoxin standard mix and IS solutions. The final concentrations were 0.00 (blank), 0.10, 0.20, 0.50, 1.00, 2.00, 5.00, and 10.00 ng/mL for AOH, AME, and TeA, and 1.00 ng/mL for the IS. To the samples, 18 mL of CH₃CN/H₂O/HCOOH (52/45/3, *v/v*), 4 g of anhydrous magnesium sulfate, and 1 g of sodium chloride were added. The samples underwent 1 min of vortex shaking, 30 min of overhead shaking at room temperature (Agilitec, Paris, France), and centrifugation at 4000× *g* for 10 min (Sigma 3-16PK, Osterode am Harz, Germany). The organic fraction was transferred to a glass tube and evaporated under a gentle stream of N₂ at 40 °C. The residue was re-dissolved in 250 µL of injection solvent (CH₃CN/H₂O/CH₃COOH, 25/74/1, *v/v/v*), vortexed, and centrifuged for 10 min at 10,000× *g* (Sigma 3-16PK, Osterode am Harz, Germany). Finally, samples were transferred into UPLC vials.

The enzymatic hydrolysis of the urine samples was performed analogously to the hydrolysis performed on the blood samples. Briefly, 125 µL of the thawed urine sample was transferred into Eppendorf Safe-Lock Tubes 1.5 mL. The calibration curves were built by spiking 8 samples at different concentration levels with the mycotoxin standard mix and IS solutions. The final concentrations were 0.00 (blank), 0.10, 0.20, 0.50, 1.00, 2.00, 5.00, and 10.00 ng/mL for AOH, AME, and TeA, and 1.00 ng/mL for the IS. The samples were combined with 12.5 µL acetate buffer (0.05 M, pH 5) and 2.5 µL of β-glucuronidase, and vortexed for 1 min. After incubation for 1 h at 55 °C while shaking at 1400 rpm using a Thermo-Shaker TS-100 (Biosan, Riga, Latvia), the reaction was stopped by protein precipitation obtained by adding 125 µL of ice-cold CH₃CN. The suspension was vortexed for 30 s and centrifuged for 5 min at 10,000× *g* (Sigma 3-16PK, Osterode am Harz, Germany). The supernatant was transferred in a tube and evaporated under a gentle stream of N₂ at room temperature. The residue was reconstituted in 50 µL of injection solvent (CH₃CN/H₂O/CH₃COOH, 25/74/1, *v/v/v*), vortexed, and centrifuged for 10 min at 10,000× *g* (Sigma 3-16PK, Osterode am Harz, Germany). Finally, samples were transferred into UPLC vials.

4.3.3. Feces

Accordingly to the optimized extraction of *Alternaria* toxins from feces obtained applying the DoE approach, one gram of freeze-dry feces samples were transferred in 50 mL extraction tubes. Eight blank samples were used as calibrants and spiked at different concentration levels with the mycotoxin standard mix and IS solutions. The final concentrations were 0.00 (blank), 0.63, 1.25, 2.50, 5.00, 7.50, 10.00, and 20.00 for TeA, and 5.00 ng/g for the IS. After 30 min of equilibration in a cool and dark place, the samples were extracted with 20 mL of CH₃CN/CH₃COOH (99/1, *v/v*). The tubes were vortexed for 1 min, ultrasonicated for 15 min at room temperature (Branson Ultrasonic Corp., Danbury, CT, USA), and overhead shaking at room temperature (Agilitec, Paris, France). After centrifugation for 15 min at 4000× *g*, the supernatant was transferred to a new tube and evaporated under a gentle N₂ flow. The residues were re-suspended in 250 µL of injection solvent (CH₃CN/H₂O/CH₃COOH, 25/74/1, *v/v/v*) and vigorously vortexed. Finally, the samples were filtered with Durapore® PVDF 0.22µm centrifugal filters (Merk Millipore, Cork, Ireland) and transferred into UPLC vials.

4.4. UPLC-MS/MS Analysis and Method Validation

The optimized UPLC-MS/MS analysis was carried out using a Acquity UPLC system coupled to a Waters Xevo® TQ-XS tandem quadrupole mass spectrometer (Waters, Manchester, UK) equipped with an electrospray (ESI) interface. The detection was performed using multiple reaction monitoring (MRM) with ESI[−]. Chromatographic separation was achieved using an Acquity UPLC® HSS T3 (1.8 µm × 2.1 × 100 mm) column paired with a pre-column Acquity UPLC® HSS T3 VanGuard™ (1.8 µm) (Waters, Manchester, UK). Column and autosampler temperature were set at 35 and 10 °C, respectively. Gradient elution was established with a mobile phase consisting of 1% CH₃COOH (*v/v*) in H₂O (mobile phase A) and pure CH₃CN (mobile phase B) at a flow rate of 0.4 mL/min. A run started with an increase in B from 25% to 85% during 3 min with a concave profile, followed by an instant increase to 100% B that was maintained until 4.5 min, after which % B was linearly decreased until 25% at 6 min, finally the column was allowed to re-equilibrate for 1.5 min, resulting in a total run time of 7 min. The optimized MRM parameters and the retention times are reported in Table 3 for each compound. The capillary voltage was set at 0.80 kV; desolvation gas temperature and flow were 600 °C and 1000 L/h. The cone curtain and nebulizer gas flow were 150 L/h and 7.0 Bar, respectively. The nebulizer and curtain gas used was N₂, while the collision gas was Ar. Masslynx®. Targetlynx® software 4.2 (Waters Corp., Milford, CT, USA) was used for data acquisition and processing.

Table 3. Multiple reaction monitoring (MRM) parameters used for the quantification of alternariol (AOH), alternariol monomethylether (AME), and tenuazonic acid (TeA). Retention time (Rt), cone voltage, mass-to-charge ratio of quasi-molecular ion ([M-H][−] *m/z*), mass-to-charge ratio of the product ions *m/z*, and collision energy (CE).

Analyte	Rt (min)	Cone (V)	[M-H] [−] <i>m/z</i>	CE (eV)	Product Ion <i>m/z</i>
AOH	2.80	73	257.0	23	213.0
				25	215.0
TeA	2.68	50	196.1	23	112.1
				18	139.0
AME	3.44	60	271.1	31	255.0
				22	256.0
¹³ C ₁₅ -AME	3.44	60	286.1	31	269.0
				22	270.0

The methods were validated to meet the criteria of European Commission (EC) decision no. 2002/657, Commission Implementing Regulation (EU) no. 2023/2782, and ISO 5725-1:2023 as guidance [40–42]. The goodness-of-fit of the linear regression (coefficient of determination, R²), limit of detection (LOD), calibration range, recovery (%), intra-day precision (repeatability, RSD_r, CV %), and inter-day precision (intermediate precision, RSD_R, CV %) were calculated based on the results of 3 days of analysis in which 3 calibration curves were run each day for each matrix, for a total of 9 samples for each concentration level considered. The linearity and the goodness-of-fit of the linear regression were assessed by calculating the coefficient of determination (R²) [43] between the spiked concentrations of the analytes and the corresponding instrument response, which was calculated as the ratio of the analyte peak area to the IS peak area, multiplied by the concentration of the IS. Matrix effects, namely signal suppression enhancement (SSE), apparent recovery (R_A), and extraction efficiency (R_E), were assessed following the approach described by Sulyok et al. (2006) [39]. Specificity was determined by evaluating the presence of interfering chromatographic peaks in representative blank samples. The carry-over was evaluated by injecting injection solvent after the highest calibration point into the LC-MS/MS system.

The stability was investigated at two concentration levels in duplicates to simulate a high- and low-exposure scenario in urine and capillary blood collected via VAMS Mitra® tips. The urine was spiked and stored at 4 °C for 5 days and −20 °C for 21 days, while the VAMS devices were stored at 20 °C for 5 days and 4 °C for 21 days. The hypothesis of influence of hematocrit (Hct) was previously tested and disproved, meaning that the recovery from VAMS Mitra® tips is not hematocrit [25].

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Appendix A

Table A1. Resume of validation parameters from published analytical methods for the quantification of one or more *Alternaria* toxin in biological matrices. The parameters reported are limit of detection (LOD, ng/mL or ng/g), limit of quantification (LOQ, ng/mL or ng/g), calibration range (ng/mL or ng/g), extraction efficiency (R_E , %), intermediate precision (RSD_R , %), repeatability (RSD_r , %), signal suppression enhancement (SSE, %), signal suppression enhancement with internal standard compensation (SSE_{IS} , %), and apparent recovery (R_A , %).

Reference	Matrix	Analyte	LOD	LOQ	Linear Range	R_E	RSD_R	RSD_r	SSE	SSE_{IS}	R_A
			ng/mL	ng/mL	ng/mL	%	%	%	%	%	%
De Ruyck et al. (2020) [28]	Human urine	AME	0.037	0.063							
		AOH	0.015	0.061							
		TeA	0.180	0.250							
Asam et al. (2013) [19]	Human urine	TA-DNPH	0.2	0.6	1–100		6.7				
Krausová et al. (2021) [31]	Infant feces *	AOH	0.8	1.6	10–30	68–78	28–8	40–9	96		
		AME	0.03	0.06	0.7–2.2	40–49	27–16	23–7	83		
Vidal et al. (2021) [25]	Human blood (VAMS)	AOH	1.37	2.74	10–250				13.2	105	136–92.8
		AME	1.86	3.72	10–100				10.4	83.1	124–118
Martins et al. (2019) [29]	Human urine	AOH	0.4	0.9	0.1–50		7.8	5.9			89.7
		AME	0.5	1	0.1–50		9.6	8.2			87
Puntscher et al. (2019) [30]	Rat urine	AOH	0.05	0.1	0.1–100	43	8	8	62		
		AME	0.005	0.01	0.02–20	31	9	8	66		
		TeA	0.1	1	1.2–1200	117	17	7	86		
	Rat feces **	AOH	0.5	1	1–1000	65	19	11	42		
		AME	0.3	0.6	0.2–200	56	18	7	19		
		TeA	30	60	12–12,000	89	23	13	109		

* LOD, LOQ, and linear range are expressed as ng/g. ** LOD, LOQ, and linear range are expressed as ng/g of dry feces after conversion from ng/mL of dry blank feces suspended in water (1:10), which accounted for a ten-fold higher spiking concentration.

References

1. Eskola, M.; Kos, G.; Elliott, C.T.; Hajšlová, J.; Mayar, S.; Krska, R. Worldwide Contamination of Food-Crops with Mycotoxins: Validity of the Widely Cited 'FAO Estimate' of 25%. *Crit. Rev. Food Sci. Nutr.* **2020**, *60*, 2773–2789. [CrossRef] [PubMed]
2. Moretti, A.; Pascale, M.; Logrieco, A.F. Mycotoxin Risks under a Climate Change Scenario in Europe. *Trends Food Sci. Technol.* **2019**, *84*, 38–40. [CrossRef]
3. European Commission. *2021 Annual Report. Alert and Cooperation Network*; Publications Office of the European Union: Luxembourg, 2022; p. 28. [CrossRef]
4. Arcella, D.; Eskola, M.; Gómez Ruiz, J.A. Dietary Exposure Assessment to Alternaria Toxins in the European Population. *EFSA J.* **2016**, *14*, e04654. [CrossRef]
5. EFSA Panel on Contaminants in the Food Chain. Scientific Opinion on the Risks for Animal and Public Health Related to the Presence of *Alternaria* toxins in Feed and Food. *EFSA J.* **2011**, *9*, 2407. [CrossRef]
6. Kumari, A.; Turkey, N.N. Tenuazonic acid: A potent mycotoxin. In *Recent Trends in Human and Animal Mycology*; Springer: Berlin/Heidelberg, Germany, 2019; pp. 203–211. ISBN 9789811394355.
7. Commission Regulation (EC) No 1881/2006, European Commission. EFSA Contam Panel. *Off. J. Eur. Union* **2006**, 5–23.
8. den Hollander, D.; Holvoet, C.; Demeyere, K.; De Zutter, N.; Audenaert, K.; Meyer, E.; Croubels, S. Cytotoxic Effects of Alternariol, Alternariol Monomethyl-Ether, and Tenuazonic Acid and Their Relevant Combined Mixtures on Human Enterocytes and Hepatocytes. *Front. Microbiol.* **2022**, *13*, 849243. [CrossRef]
9. Bensassi, F.; Gallerne, C.; Sharaf El Dein, O.; Hajlaoui, M.R.; Bacha, H.; Lemaire, C. Cell Death Induced by the Alternaria Mycotoxin Alternariol. *Toxicol. Vitro.* **2012**, *26*, 915–923. [CrossRef] [PubMed]
10. Fehr, M.; Pahlke, G.; Fritz, J.; Christensen, M.O.; Boege, F.; Altemöller, M.; Podlech, J.; Marko, D. Alternariol Acts as a Topoisomerase Poison, Preferentially Affecting the II α Isoform. *Mol. Nutr. Food Res.* **2009**, *53*, 441–451. [CrossRef]
11. Pfeiffer, E.; Eschbach, S.; Metzler, M. Alternaria Toxins: DNA Strand-Breaking Activity in Mammalian Cells in Vitro. *Mycotoxin Res.* **2007**, *23*, 152–158. [CrossRef]
12. Schwarz, C.; Kreutzer, M.; Marko, D. Minor Contribution of Alternariol, Alternariol Monomethyl Ether and Tenuazonic Acid to the Genotoxic Properties of Extracts from *Alternaria alternata* Infested Rice. *Toxicol. Lett.* **2012**, *214*, 46–52. [CrossRef]
13. Solhaug, A.; Wisbech, C.; Christoffersen, T.E.; Hult, L.O.; Lea, T.; Eriksen, G.S.; Holme, J.A. The Mycotoxin Alternariol Induces DNA Damage and Modify Macrophage Phenotype and Inflammatory Responses. *Toxicol. Lett.* **2015**, *239*, 9–21. [CrossRef] [PubMed]
14. European Commission. *Commission Recommendation (EU) 2022/553*; European Commission: Luxembourg, 2022; pp. 90–92.
15. Burkhardt, B.; Pfeiffer, E.; Metzler, M. Absorption and Metabolism of the Mycotoxins Alternariol and Alternariol-9-Methyl Ether in Caco-2 Cells in Vitro. *Mycotoxin Res.* **2009**, *25*, 149–157. [CrossRef] [PubMed]
16. Puntschner, H.; Hankele, S.; Tillmann, K.; Attakpah, E.; Braun, D.; Kütt, M.-L.; Del Favero, G.; Aichinger, G.; Pahlke, G.; Höger, H.; et al. First Insights into Alternaria Multi-Toxin in Vivo Metabolism. *Toxicol. Lett.* **2019**, *301*, 168–178. [CrossRef] [PubMed]
17. Visintin, L.; García Nicolás, M.; Vangeenderhuysen, P.; Goessens, T.; Alladio, E.; Pomian, B.; Vanhaecke, L.; De Saeger, S.; De Boevre, M. Unraveling Biomarkers of Exposure for Tenuazonic Acid through Urinary Metabolomics. *Food Chem. Toxicol.* **2023**, *182*, 114183. [CrossRef] [PubMed]
18. Fraeyman, S.; Devreese, M.; Broekaert, N.; De Mil, T.; Antonissen, G.; De Baere, S.; De Backer, P.; Rychlik, M.; Croubels, S. Quantitative Determination of Tenuazonic Acid in Pig and Broiler Chicken Plasma by LC-MS/MS and Its Comparative Toxicokinetics. *J. Agric. Food Chem.* **2015**, *63*, 8560–8567. [CrossRef] [PubMed]
19. Asam, S.; Habler, K.; Rychlik, M. Determination of Tenuazonic Acid in Human Urine by Means of a Stable Isotope Dilution Assay. *Anal. Bioanal. Chem.* **2013**, *405*, 4149–4158. [CrossRef] [PubMed]
20. Mikula, H.; Horkel, E.; Hans, P.; Hametner, C.; Fröhlich, J. Structure and Tautomerism of Tenuazonic Acid—A Synergetic Computational and Spectroscopic Approach. *J. Hazard. Mater.* **2013**, *250–251*, 308–317. [CrossRef] [PubMed]
21. Stove, C.P.; Ingels, A.-S.M.E.; De Kesel, P.M.M.; Lambert, W.E. Dried Blood Spots in Toxicology: From the Cradle to the Grave? *Crit. Rev. Toxicol.* **2012**, *42*, 230–243. [CrossRef] [PubMed]
22. De Kesel, P.M.M.; Lambert, W.E.; Stove, C.P. Does Volumetric Absorptive Microsampling Eliminate the Hematocrit Bias for Caffeine and Paraxanthine in Dried Blood Samples? A Comparative Study. *Anal. Chim. Acta* **2015**, *881*, 65–73. [CrossRef]
23. Bastos-Moreira, Y.; Ouédraogo, L.; De Boevre, M.; Argaw, A.; de Kok, B.; Hanley-Cook, G.T.; Deng, L.; Ouédraogo, M.; Compaoré, A.; Tesfamariam, K.; et al. A Multi-Omics and Human Biomonitoring Approach to Assessing the Effectiveness of Fortified Balanced Energy–Protein Supplementation on Maternal and Newborn Health in Burkina Faso: A Study Protocol. *Nutrients* **2023**, *15*, 4056. [CrossRef]
24. Delahaye, L.; De Baerdemaeker, L.; Stove, C.P. Determination of Paracetamol and Its Metabolites via LC-MS/MS in Dried Blood Volumetric Absorptive Microsamples: A Tool for Pharmacokinetic Studies. *J. Pharm. Biomed. Anal.* **2021**, *206*, 114361. [CrossRef] [PubMed]
25. Vidal, A.; Belova, L.; Stove, C.; De Boevre, M.; De Saeger, S.; Vidal, A.; Belova, L.; Stove, C.; De Boevre, M.; De Saeger, S. Volumetric Absorptive Microsampling as an Alternative Tool for Biomonitoring of Multi-Mycotoxin Exposure in Resource-Limited Areas. *Toxins* **2021**, *13*, 345. [CrossRef] [PubMed]
26. Pfeiffer, E.; Schebb, N.H.; Podlech, J.; Metzler, M. Novel Oxidative in Vitro Metabolites of the Mycotoxins Alternariol and Alternariol Methyl Ether. *Mol. Nutr. Food Res.* **2007**, *51*, 307–316. [CrossRef] [PubMed]

27. Aichinger, G.; Del Favero, G.; Warth, B.; Marko, D. Alternaria Toxins—Still Emerging? *Compr. Rev. Food Sci. Food Saf.* **2021**, *20*, 4390–4406. [CrossRef] [PubMed]
28. De Ruyck, K.; Huybrechts, I.; Yang, S.; Arcella, D.; Claeys, L.; Abbeddou, S.; De Keyzer, W.; De Vries, J.; Ocke, M.; Ruprich, J.; et al. Mycotoxin Exposure Assessments in a Multi-Center European Validation Study by 24-Hour Dietary Recall and Biological Fluid Sampling. *Environ. Int.* **2020**, *137*, 105539. [CrossRef] [PubMed]
29. Martins, C.; Vidal, A.; De Boevre, M.; De Saeger, S.; Nunes, C.; Torres, D.; Goios, A.; Lopes, C.; Assunção, R.; Alvito, P. Exposure Assessment of Portuguese Population to Multiple Mycotoxins: The Human Biomonitoring Approach. *Int. J. Hyg. Environ. Health* **2019**, *222*, 913–925. [CrossRef] [PubMed]
30. Puntischer, H.; Aichinger, G.; Grabher, S.; Attakpah, E.; Krüger, F.; Tillmann, K.; Motschnig, T.; Hohenbichler, J.; Braun, D.; Plasenzotti, R.; et al. Bioavailability, Metabolism, and Excretion of a Complex Alternaria Culture Extract versus Alternotoxin II: A Comparative Study in Rats. *Arch. Toxicol.* **2019**, *93*, 3153–3167. [CrossRef] [PubMed]
31. Krausová, M.; Ayeni, K.I.; Wisgrill, L.; Ezekiel, C.N.; Braun, D.; Warth, B. Trace Analysis of Emerging and Regulated Mycotoxins in Infant Stool by LC-MS/MS. *Anal. Bioanal. Chem.* **2022**, *414*, 7503–7516. [CrossRef]
32. Amante, E.; Alladio, E.; Rizzo, R.; Di Corcia, D.; Negri, P.; Visintin, L.; Guglielmotto, M.; Tamagno, E.; Vincenti, M.; Salomone, A. Untargeted Metabolomics in Forensic Toxicology: A New Approach for the Detection of Fentanyl Intake in Urine Samples. *Molecules* **2021**, *26*, 4990. [CrossRef]
33. Vidal, A.; Claeys, L.; Mengelers, M.; Vanhoorne, V.; Vervaet, C.; Huybrechts, B.; De Saeger, S.; De Boevre, M. Humans Significantly Metabolize and Excrete the Mycotoxin Deoxynivalenol and Its Modified Form Deoxynivalenol-3-Glucoside within 24 Hours. *Sci. Rep.* **2018**, *8*, 5255. [CrossRef]
34. Lauwers, M.; De Baere, S.; Letor, B.; Rychlik, M.; Croubels, S.; Devreese, M. Multi LC-MS/MS and LC-HRMS Methods for Determination of 24 Mycotoxins Including Major Phase I and II Biomarker Metabolites in Biological Matrices from Pigs and Broiler Chickens. *Toxins* **2019**, *11*, 171. [CrossRef] [PubMed]
35. Miró-Abella, E.; Herrero, P.; Canela, N.; Arola, L.; Ras, R.; Borrull, F.; Fontanals, N. Optimised Extraction Methods for the Determination of Trichothecenes in Rat Faeces Followed by Liquid Chromatography-Tandem Mass Spectrometry. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2019**, *1105*, 47–53. [CrossRef] [PubMed]
36. Leardi, R.; Melzi, C.; Polotti, G. CAT (Chemometric Agile Tool). Available online: <http://gruppochemiometria.it/index.php/software> (accessed on 14 January 2024).
37. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria. Available online: <https://www.r-project.org/> (accessed on 27 September 2022).
38. Anastas, P.T.; Warner, J.C. *Green Chemistry*; Oxford University Press: Oxford, UK, 1998; ISBN 9780198506980.
39. Sulyok, M.; Berthiller, F.; Krska, R.; Schuhmacher, R. Development and Validation of a Liquid Chromatography/Tandem Mass Spectrometric Method for the Determination of 39 Mycotoxins in Wheat and Maize. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 2649–2659. [CrossRef] [PubMed]
40. 2002/657/EC: Commission Decision of 12 August 2002 Implementing Council Directive 96/23/EC Concerning the Performance of Analytical Methods and the Interpretation of Results (Text with EEA Relevance) (Notified under Document Number C(2002) 3044). Available online: <https://www.legislation.gov.uk/eudn/2002/657> (accessed on 11 November 2021).
41. European Commission. Commission Implementing Regulation (EU) 2023/2782 of 14 December 2023 on the Methods of Sampling and Analysis for the Control of the Levels of Mycotoxins in food and Repealing Regulation (EC) No 401/2006. *Off. J. Eur. Union* **2023**, *2782*, 1–44.
42. ISO 5725-1:2023; International Organization for Standardization Accuracy (Trueness and Precision) of Measurement Methods and Results. JSA Group Webdesk: Tokyo, Japan, 2023.
43. Di Bucchianico, A. Coefficient of Determination (R²). In *Encyclopedia of Statistics in Quality and Reliability*; Wiley: Hoboken, NJ, USA, 2007.

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Article

Sensitive Detection and Differentiation of Biologically Active Ricin and Abrin in Complex Matrices via Specific Neutralizing Antibody-Based Cytotoxicity Assay

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Abstract: Ricin and abrin are highly potent plant-derived toxins, categorized as type II ribosome-inactivating proteins. High toxicity, accessibility, and the lack of effective countermeasures make them potential agents in bioterrorism and biowarfare, posing significant threats to public safety. Despite the existence of many effective analytical strategies for detecting these two lethal toxins, current methods are often hindered by limitations such as insufficient sensitivity, complex sample preparation, and most importantly, the inability to distinguish between biologically active and inactive toxin. In this study, a cytotoxicity assay was developed to detect active ricin and abrin based on their potent cell-killing capability. Among nine human cell lines derived from various organs, HeLa cells exhibited exceptional sensitivity, with limits of detection reaching 0.3 ng/mL and 0.03 ng/mL for ricin and abrin, respectively. Subsequently, toxin-specific neutralizing monoclonal antibodies MIL50 and 10D8 were used to facilitate the precise identification and differentiation of ricin and abrin. The method provides straightforward and sensitive detection in complex matrices including milk, plasma, coffee, orange juice, and tea via a simple serial-dilution procedure without any complex purification and enrichment steps. Furthermore, this assay was successfully applied in the unambiguous identification of active ricin and abrin in samples from OPCW biotoxin exercises.

Keywords: ricin; abrin; activity; neutralizing antibody; cytotoxicity assay; complex matrices

Key Contribution: The present study has successfully developed a neutralizing antibody-based cytotoxicity assay for the sensitive and efficient detection and differentiation of active ricin and abrin in complex matrices via a simple serial-dilution step, addressing a significant limitation of other methods that may not distinguish between active and inactive toxins.

1. Introduction

Ricin and abrin, two of the most lethal plant toxins, are mainly derived from the seed of *Ricinus communis* and *Abrus precatorius*, respectively. Both of them belong to the family of type II ribosome-inactivating proteins (RIPs) with high structural similarity and sequence homology. Ricin is the only biotoxin covered by the Chemical Weapons Convention (CWC) as well as the Biological and Toxin Weapons Convention (BTWC). In addition, both ricin and abrin are categorized as category B biothreat agents by the USA Center for Disease Control and Prevention (CDC) [1–3]. Their high toxicity, easy availability, and lack of effective antidotes make them potential bioweapons and bioterrorism agents, underscoring the critical need for convenient and sensitive detection methods.

A variety of analytical techniques have been developed for detecting ricin and abrin, based on their intrinsic physicochemical and immunological properties [4–6]. Mass spectrometric approaches can provide sequence and structural information achieved through the accurate identification of unique peptides after enzymatic digestion, which are necessary for the unambiguous confirmation of the toxins [7,8]. Nonetheless, these methods are usually time-consuming, costly, not sufficiently sensitive, susceptible to matrix interference,

and require sophisticated extraction and enrichment procedures and professional personnel. Immunological methods, such as enzyme-linked immunosorbent assay (ELISA) and lateral flow assay (LFA), are favored for their simplicity, high sensitivity, and efficiency [9–11]. However, a major disadvantage of these methods is the inability to distinguish between biologically active and inactive toxin. The discrimination of functionally active or inactive toxin is extremely important to determine the risk level and appropriate management of a suspected toxin exposure incident, because the inactivated toxin no longer poses a threat [12]. Both ricin and abrin are composed of a catalytically active A chain and a lectin B chain, which are linked by a single disulfide bond. It is known that the A chain with *N*-glycosidase activity (EC 3.2.2.22) is required for ribosome inactivation through depurinating the 28S rRNA of the 60S ribosomal subunit and thereby inhibiting protein synthesis. The galactose-specific B chain is necessary for binding, internalization, and trafficking of the toxin into cells [13]. Generally, the depurination activity of the A chain can be detected via adenine-release assay or rabbit reticulocyte lysate translation assay [14,15]. The enzyme-linked lectin assay determines the glycan binding activity of the B chain [16]. However, activity detection of the separated chain only provides limited information on the toxicity of the intact toxin, because neither the A chain nor the B chain are significantly toxic in isolation. Only intact and active ricin and abrin have sufficiently strong potency to induce cell death in various cell lines. Consequently, cell-based cytotoxicity assays emerge as a promising strategy, offering a clear advantage in displaying the toxin's full biological activity. Currently, cytotoxicity can be detected using different endpoint measurements, such as colorimetry, fluorescence, or luminescence, or a real-time assay based on an impedance sensor technique to monitor cell viability and proliferation without labeling [17–20]. However, the sensitivity of a cytotoxicity assay is directly influenced by the choice of cell line and its susceptibility to the toxin [21,22]. In addition, most assays lack specific functional neutralizing antibodies to exclude cytotoxicity caused by irrelevant matrix components or to differentiate the cytotoxicity from other RIP toxins, particularly in the case of abrin detection [23–25].

In this study, we selected nine human cell lines, each originating from distinct organs, to assess the sensitivity of the cytotoxicity assay. Among all these cell lines, HeLa cells were found to be the most sensitive to both ricin and abrin, with the lowest IC₅₀ values (1.18 and 0.14 ng/mL) and limits of detection (LODs) (0.3 and 0.03 ng/mL). High sensitivity, good robustness, rapid proliferative capacity, and ease of cultivation make HeLa cells an optimal choice for toxin detection. Using their specific functional blocking antibodies, it is feasible to differentiate ricin and abrin and exclude cytotoxicity caused by other toxic compounds. Moreover, this assay is capable of detecting both toxins in a variety of complex matrices such as plasma, milk, coffee, orange juice, and tea through a simple serial-dilution procedure without any complex purification and enrichment steps. Furthermore, the developed method was successfully applied for the sensitive and effective identification and differentiation of active ricin and abrin from unknown samples of biotoxin exercises conducted by the Organization for the Prohibition of Chemical Weapons (OPCW).

2. Results and Discussion

2.1. HeLa Cells Exhibited the Highest Sensitivity to Both Ricin and Abrin Exposures

Based on the main target organs injured through ricin and abrin intoxication, nine human cell lines were exposed to the two toxins at concentrations ranging from 0.01 to 300 ng/mL for the evaluation of cytotoxicity and selection of the cell line with highest sensitivity. The selected cell lines were HeLa, HepG2, A549, HEK-293, HCT-8, BGC-823, HUVEC, AC16, and SH-SY5Y, derived from the cervix, liver, lung, kidney, intestine, stomach, endothelium, heart, and nervous system, respectively. Our findings indicated significant variability in the cytotoxicity of ricin and abrin across different cell lines. HeLa cells were found to be the most sensitive to both ricin and abrin, achieving the lowest IC₅₀ values (the concentrations required for a 50% reduction in cell survival) of 1.18 and 0.14 ng/mL, with corresponding LODs at 0.3 and 0.03 ng/mL (Figure 1, Table 1). A549,

HCT-8, HUVEC, AC16, and SH-SY5Y cells displayed resistance to cell death, with no significant increase in cell mortality observed upon elevating the toxin concentration to 300 ng/mL.

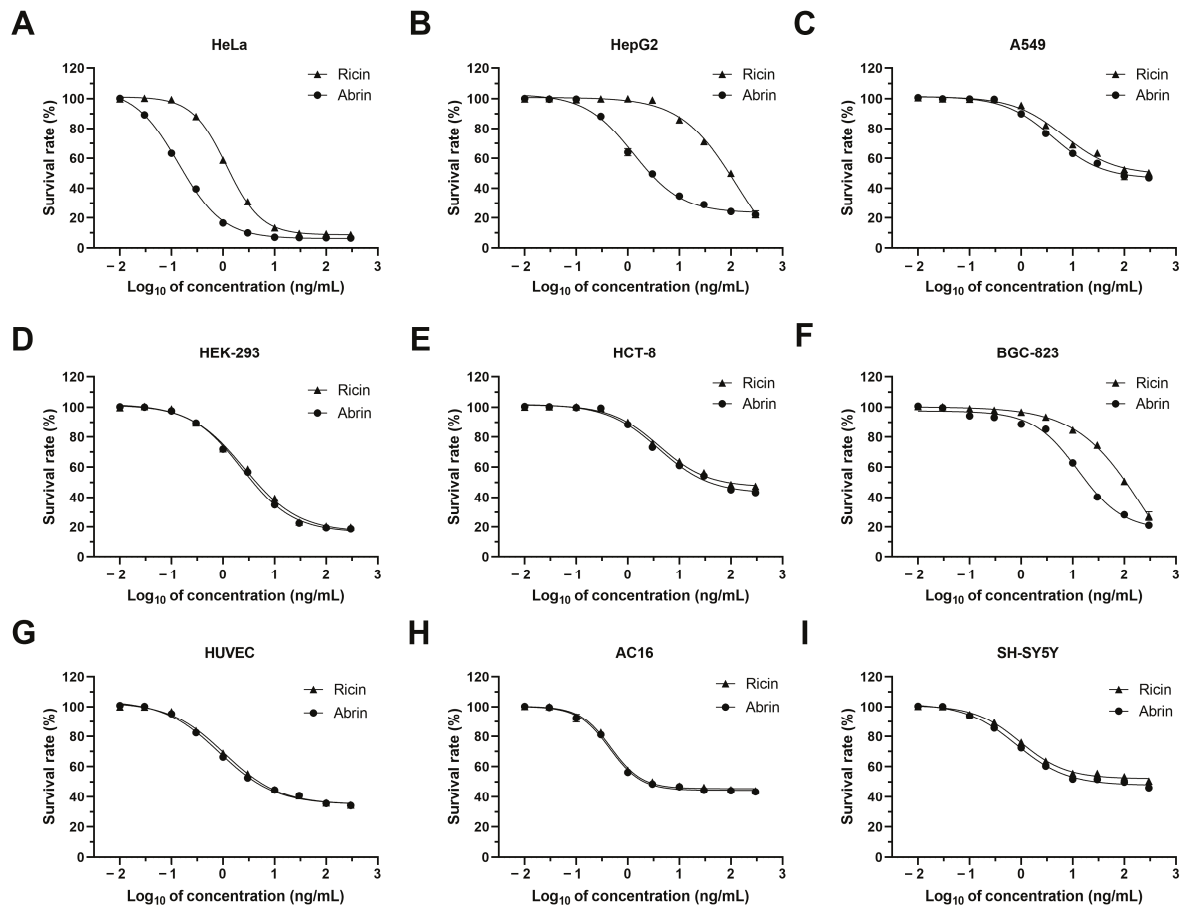


Figure 1. Survival curves of nine human-derived cell lines upon exposure to ricin and abrin. HeLa (A), HepG2 (B), A549 (C), HEK-293 (D), HCT-8 (E), BGC-823 (F), HUVEC (G), AC16 (H), and SH-SY5Y (I) cells were seeded into 96-well cell culture plates at a density of 1.0×10^4 cells per well and were then exposed to a range of ricin and abrin concentrations, from 0.01 to 300 ng/mL, for 24 h. Cell viability was assessed and survival curves were generated using the CCK-8 assay.

Table 1. The IC₅₀ values and LODs for ricin and abrin in nine human-derived cell lines.

Cell Type	Ricin IC ₅₀ (ng/mL)	Ricin LOD (ng/mL)	Abrin IC ₅₀ (ng/mL)	Abrin LOD (ng/mL)
HeLa	1.18	0.30	0.14	0.03
HepG2	130.20	10.00	1.30	0.30
A549	>300.00	3.00	67.61	1.00
HEK293	2.57	0.30	2.34	0.30
HCT-8	74.99	1.00	36.52	1.00
BGC-823	250.40	10.00	12.80	1.00
HUVEC	5.01	0.30	4.22	0.30
AC16	3.16	0.30	2.70	0.30
SH-SY5Y	>300.00	0.30	42.17	0.30

Upon exposure, the majority of cell lines exhibited the classic morphological hallmarks of apoptosis, characterized by cell shrinkage, membrane blebbing, and the formation of apoptotic bodies (Figure 2). Previous studies have reported that ricin and abrin can induce apoptosis through caspase activation, suggesting that the observed morphological changes

in various cell lines in the present study were likely to have been due to apoptosis [26,27]. We propose that the variable cytotoxicity observed across different cell lines may stem from disparities in the quantity of glycan-binding sites on the cell membrane or variations in the toxins' cell binding affinity. Such differences could affect the internalization and intracellular amounts of toxins, leading to varied cytotoxic outcomes [21,28–30]. Additionally, the different expression levels of key regulatory proteins in apoptotic signaling pathways among various cell lines may be another possible reason [22]. Notably, a minority of the cell lines, including HeLa, HepG2, and BGC-823, showed markedly different IC_{50} values for ricin and abrin. The mechanisms underlying this phenomenon require further investigation. Elucidating these mechanisms may be essential for the identification of more specific toxic effects and the development of cell lines with even greater sensitivity. Given its exceptional sensitivity, good robustness, rapid proliferative capacity, and ease of cultivation, the HeLa cell line was selected as the optimal model for toxin detection in subsequent experiments.

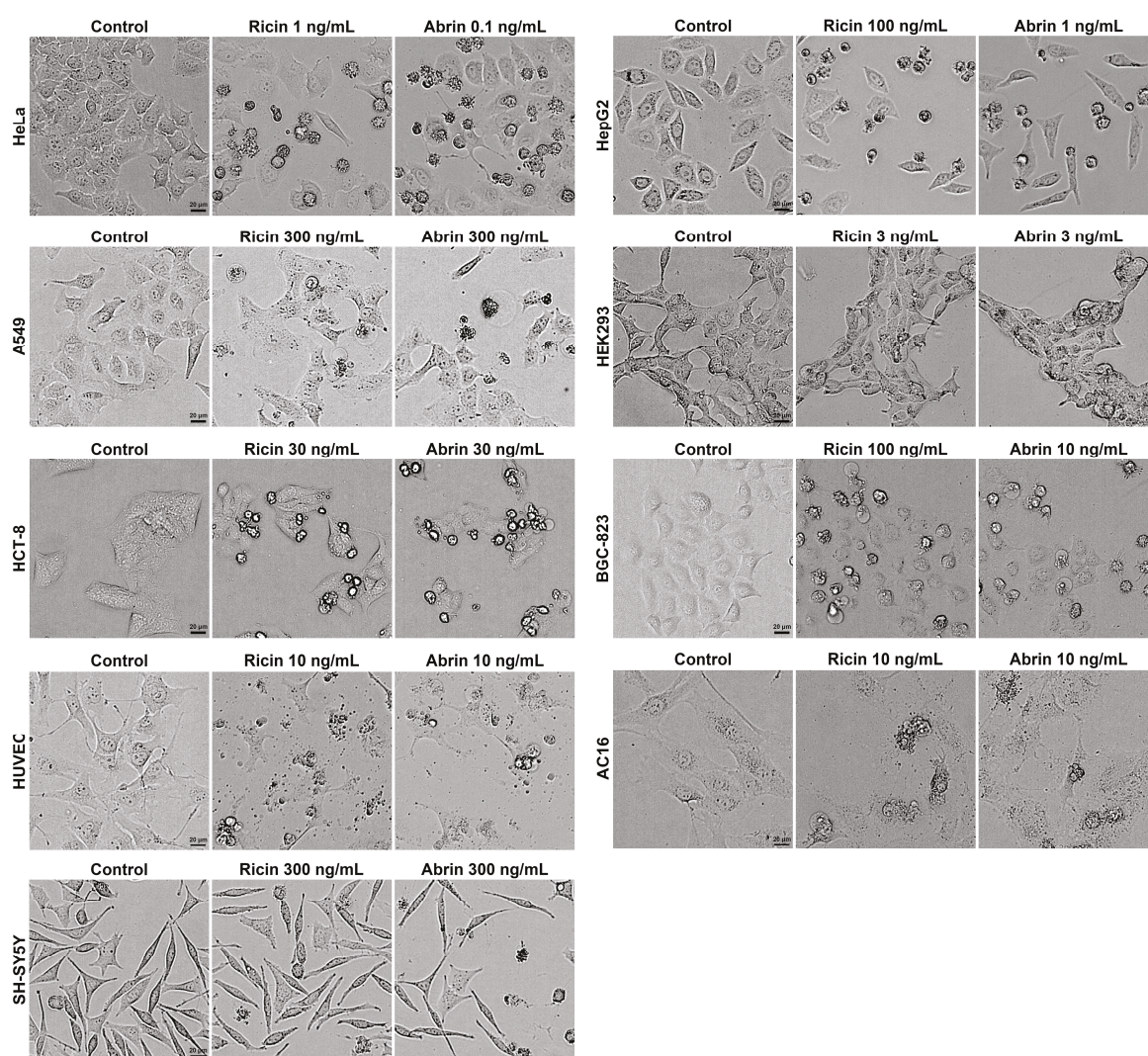


Figure 2. Different cell lines were seeded into 96-well cell culture plates at a density of 1.0×10^4 cells per well and exposed to ricin and abrin at indicated concentrations for 24 h. Cellular morphology was visualized and recorded using a high-content cell analysis system in bright-field mode. Scale bars: 20 μ m.

2.2. Optimization of Neutralizing Antibodies Concentrations for Ricin and Abrin-Specific Detection

A distinct advantage of the cytotoxicity assay is its ability to measure the full biological activity of intact toxin. Furthermore, the assay is also high-throughput and highly sensitive. However, the assay's selectivity and specificity may be compromised by similar cytotoxic effects of other toxic agents. To address this problem, pre-incubation of the sample with toxin-specific blocking antibodies was essential to exclude cytotoxicity induced by irrelevant compounds and make a distinction between ricin and abrin. HeLa cells were subjected to different concentrations of ricin and abrin preincubated with or without monoclonal antibodies (mAbs) MIL50 or 10D8 at final concentrations of 5, 10, and 20 $\mu\text{g}/\text{mL}$ for 1 h at 37 °C. Results demonstrated that MIL50 or 10D8 treatment significantly inhibited cytotoxicity induced by ricin or abrin. Specifically, MIL50 at 5 or 10 $\mu\text{g}/\text{mL}$ completely abolished cytotoxicity induced by ricin at concentrations up to 3 or 10 ng/mL, respectively. However, at concentrations exceeding 30 ng/mL, increasing MIL50 concentration to 20 $\mu\text{g}/\text{mL}$ provided only marginal additional protection against cytotoxicity (Figure 3A). Similarly, 10D8 at 5 or 10 $\mu\text{g}/\text{mL}$ fully prevented cytotoxicity caused by abrin at concentrations below 1 or 3 ng/mL, respectively, with limited improvement observed at 20 $\mu\text{g}/\text{mL}$ for abrin at concentrations exceeding 10 ng/mL (Figure 3B). Based on these results, a neutralizing antibody concentration of 10 $\mu\text{g}/\text{mL}$ was determined to be optimal and used in subsequent experiments.

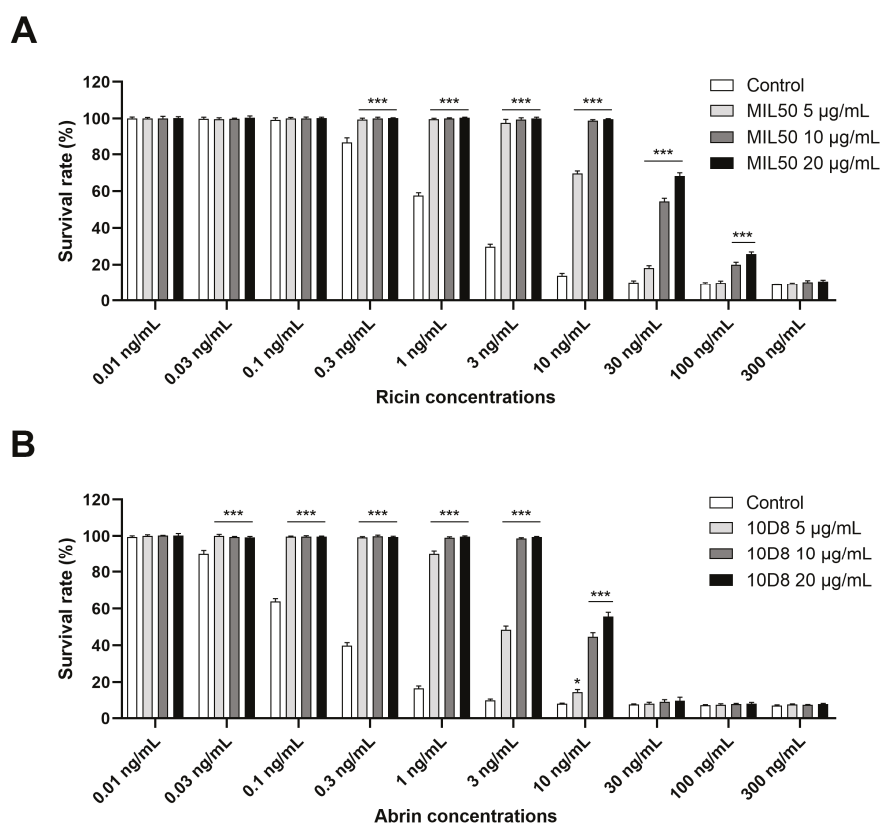


Figure 3. Optimization of neutralizing antibody concentrations for ricin and abrin-specific detection. Ricin (A) and abrin (B) at concentrations ranging from 0.01 to 300 ng/mL were freshly prepared in serum-free medium and preincubated with mAbs MIL50 or 10D8, respectively, at final concentrations of 5, 10, and 20 $\mu\text{g}/\text{mL}$ for 1 h at 37 °C. Toxins with or without antibody pretreatment were added to HeLa cells cultured in 96-well plates at a density of 1.0×10^4 cells per well and incubated for 24 h at 37 °C. The cell survival rate was assessed using CCK-8 assay, with data presented as the mean $\pm$ SD ($n = 3$). Statistical significance was evaluated using one-way ANOVA (* $p < 0.05$, *** $p < 0.001$ vs. control).

2.3. Assessment of Neutralizing Antibodies' Cross-Reactivity

The specificity of neutralizing antibodies is crucial for the accurate identification and differentiation of toxins. To determine the specificity and cross-reactivity of mAbs, ricin and abrin at indicated concentrations were pre-treated with both MIL50 and 10D8 at a final concentration of 10 $\mu\text{g}/\text{mL}$. Results showed that pretreatment with MIL50 effectively neutralized cytotoxicity induced by different concentrations of ricin. In contrast, 10D8 did not exhibit any protective effect against ricin's cytotoxicity. Conversely, 10D8 pretreatment was able to eliminate cytotoxicity caused by abrin exposure, whereas MIL50 had no such effect (Figure 4). These results underscore the high specificity of the two mAbs. Consequently, the assay described herein was able to reliably distinguish between ricin and abrin using their respective blocking mAbs.

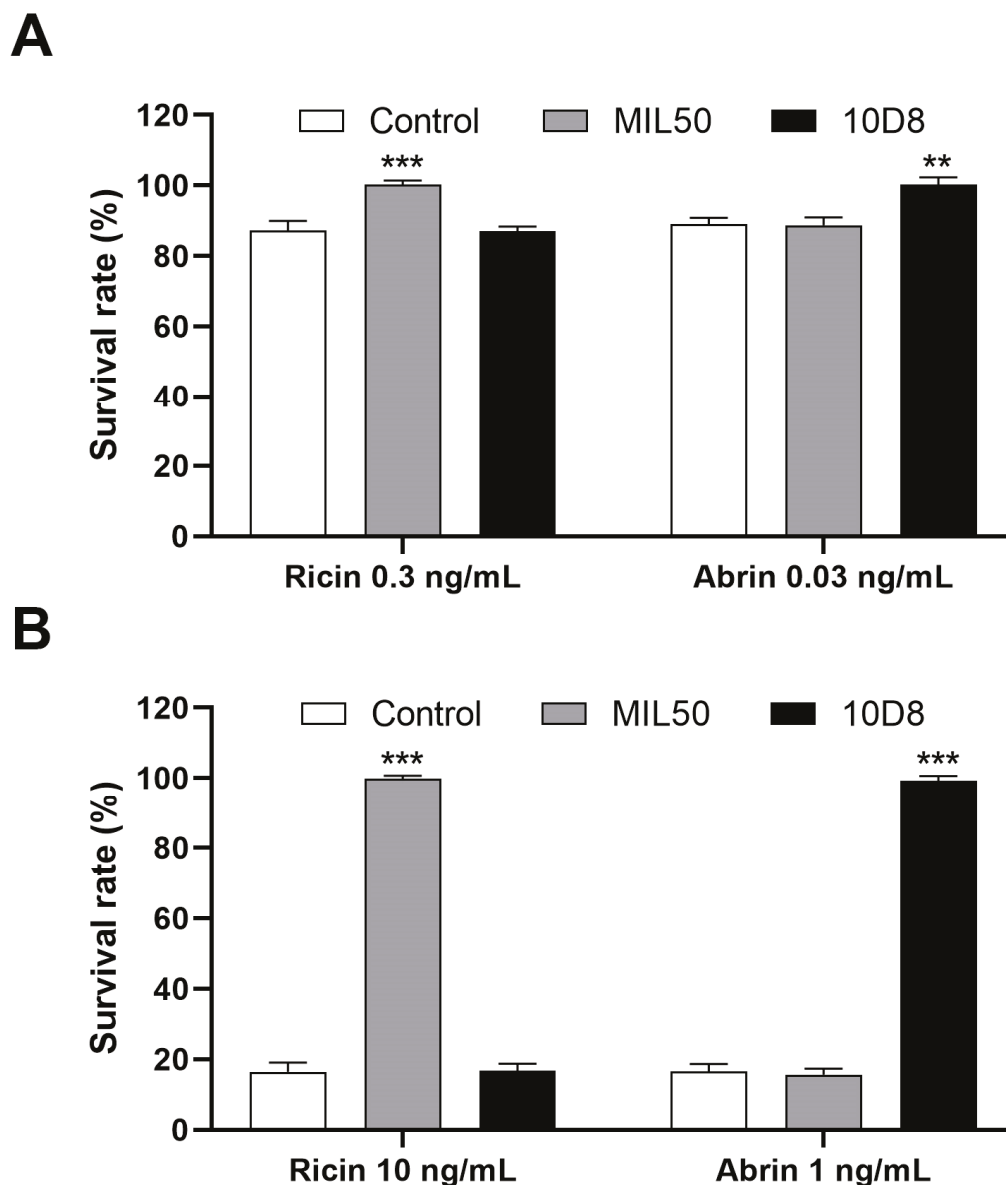


Figure 4. Assessment of neutralizing antibodies' cross-reactivity. Low (A) and high (B) concentrations of ricin and abrin were treated with both MIL50 and 10D8 at a final concentration of 10 $\mu\text{g}/\text{mL}$ for 1 h at 37 $^{\circ}\text{C}$, then added to HeLa cells and incubated for 24 h at 37 $^{\circ}\text{C}$. The survival rate of cells was calculated via CCK-8 assay. Data are presented as the mean $\pm$ SD ($n = 3$). Statistical significance was evaluated using one-way ANOVA (** $p < 0.01$, *** $p < 0.001$ vs. control).

2.4. Impact of Complex Matrices on Cell Growth and Toxin Detection

Firstly, the impact of various complex matrices on cell growth were assessed through serially diluting plasma, milk, orange juice, green tea, and coffee with serum-free RPMI-1640 medium. Notably, milk at a 1:10 dilution slightly promoted cell growth, whereas other matrices exhibited no significant impact (Figure 5). An appropriate dilution ratio is crucial to minimize matrix effects on cell growth, facilitating the accurate and reliable detection of toxins. Based on these findings, it is feasible to detect toxin samples in complex matrices by adjusting the dilution to a range of 1:10 to 1:1000 for subsequent analysis.

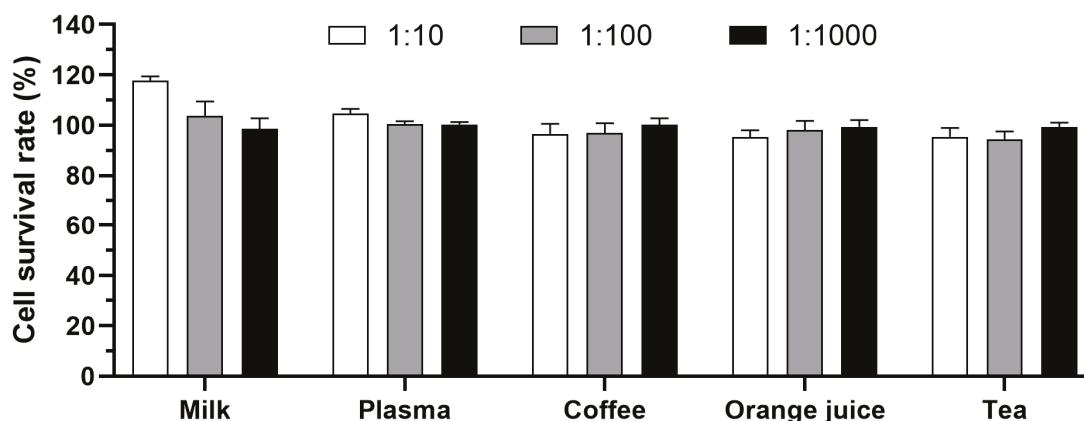


Figure 5. Impact of complex matrices on cell growth. Milk, plasma, coffee, orange juice, and tea were serially diluted to 1:10, 1:100, and 1:1000 with serum-free RPMI-1640 medium. The diluted samples were centrifuged, filtered, added to HeLa cells, and incubated for 24 h at 37 °C. The survival rate of cells was calculated via CCK-8 assay.

Next, the impact of various complex matrices on toxin detection was assessed through spiking ricin and abrin into the diluted matrices, with or without pre-treatment with mAbs MIL50 or 10D8. Notably, the findings revealed that a 1:10 diluted milk matrix significantly reduced the cytotoxicity of ricin or abrin by approximately 30- or 10-fold, respectively (Figure 6A,B). However, no significant effects were observed with other matrices. The observed reduction in cytotoxicity may be attributed to the interaction between the galactose present in milk and the toxin, which could have potentially interfered with and inhibited the toxin's ability to bind to cells [31–34]. This result is important as it highlights the need for appropriate dilution for detecting toxins in milk samples. If the milk is diluted 1:10, ricin and abrin can be detected down to 10 ng/mL and 0.3 ng/mL, corresponding to concentrations of 100 ng/mL and 3 ng/mL in undiluted milk. Meanwhile, a 1:100 dilution did not interfere with the assay, allowing detection down to 0.3 ng/mL and 0.03 ng/mL, corresponding to 30 ng/mL and 3 ng/mL in the original milk sample (Figure 6A,B). Consequently, for the detection of toxins in milk samples, a 100-fold dilution is recommended to mitigate matrix interference and enhance assay sensitivity. For other complex matrices like plasma, tea, orange juice, and coffee, a 10-fold dilution is sufficient for sensitive toxin detection (Figure 6C–J). In summary, the developed assay provided LODs of 30 ng/mL for ricin and 3 ng/mL for abrin in milk, and 3 ng/mL and 0.3 ng/mL in other matrices including plasma, tea, orange juice, and coffee. The established detection limits for ricin and abrin in complex matrices provide valuable data for future reference and comparison.

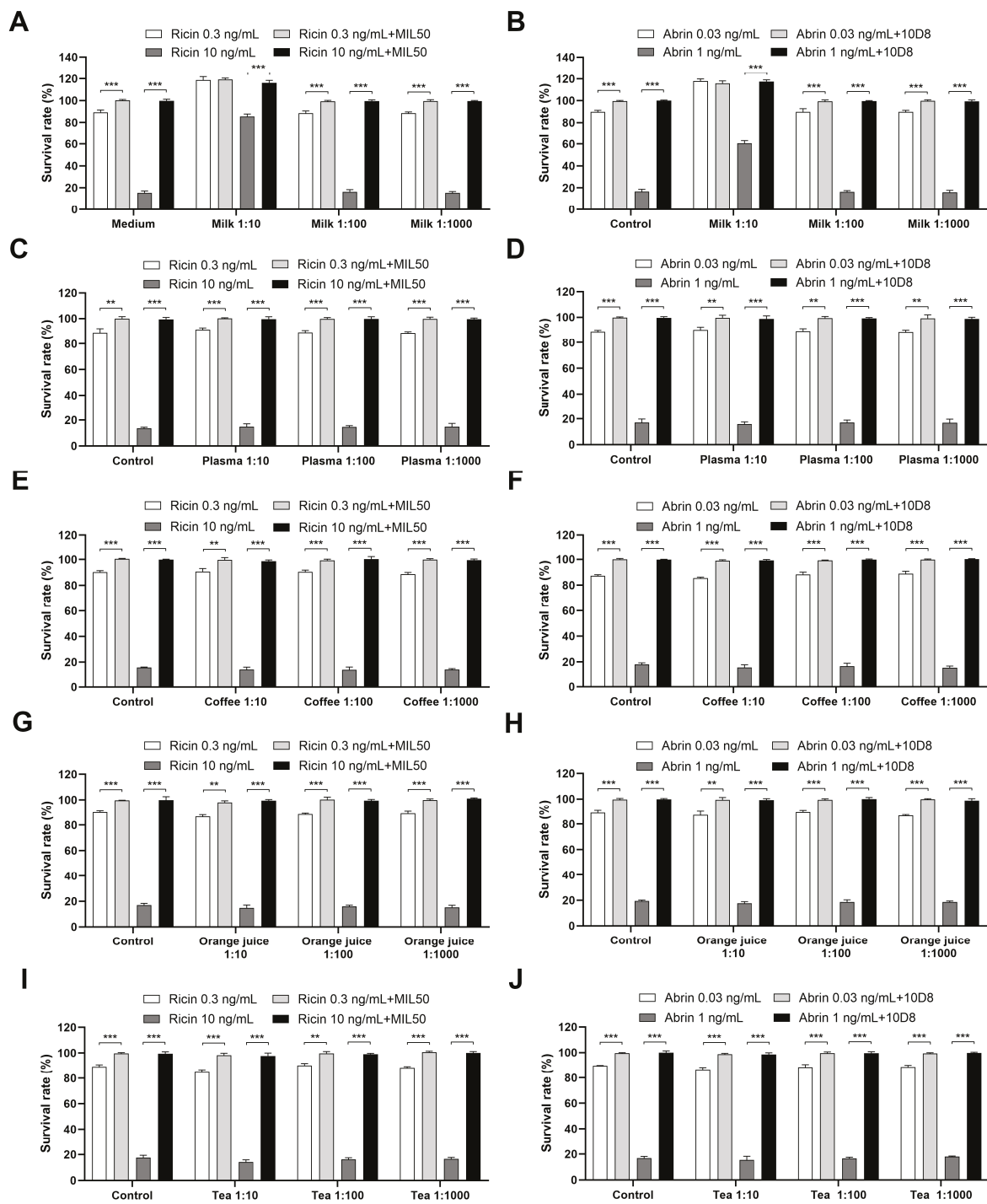


Figure 6. Impact of complex matrices on toxin detection. Ricin and abrin at indicated concentrations were spiked into the diluted milk (A,B), plasma (C,D), coffee (E,F), orange juice (G,H), and tea (I,J). The samples were preincubated with or without mAbs MIL50 or 10D8, respectively, at a final concentration of 10 µg/mL for 1 h at 37 °C, added to HeLa cells, and incubated for 24 h at 37 °C. The cell survival rates were calculated via CCK-8 assay. Data are presented as the mean ± SD ($n = 3$). Statistical significance was determined using Student's t test (** $p < 0.01$, *** $p < 0.001$).

2.5. Detection of Ricin and Abrin from the Samples of OPCW Biotoxin Exercises

The developed method was successfully used for the unambiguous identification of ricin and abrin in unknown samples from OPCW biotoxin exercises (Table 2). Specifically, during the third biotoxin exercise, the method effectively detected ricin and

abrin spiked into protein powder samples, utilizing a 1:1000 dilution ratio after dissolving for optimal sensitivity. For sample BT18.2, abrus agglutinin rather than purified abrin was spiked into the protein powder. It is important to note that abrus agglutinin is a 40% ammonium sulfate precipitate from the preparation of abrin. Consequently, the sample was expected to contain abrin. However, the accurate concentration of abrin within this sample was presumed to be significantly lower than that of the abrus agglutinin present. Based on our results, treatment with either MIL50 or 10D8 antibodies individually resulted in the inhibition of cytotoxicity. Moreover, simultaneous application of both MIL50 and 10D8 antibodies led to a synergistic effect and further increased cell survival. Given these neutralization profiles, it is evident that sample BT18.2 contained both ricin and abrin toxins (Figure 7A). In the sample from the fourth biotoxin exercise, ricin was spiked into spray buffer, which consisted of PBS with Tween-20 (PBST) and various organic solvents (including polypropyleneglycol monobutylether, PEG200, glycerol, ethanol, and isopropanol), at a concentration of 30 µg/mL. Notably, the spray buffer itself exhibited significant cytotoxicity at dilution ratios below 1:5000, potentially interfering with the detection of ricin. However, the challenge was overcome and ricin was still sensitively detected at a 1:20,000 dilution ratio, where the cytotoxicity caused by the spray buffer was no longer apparent (Figure 7B). In the fifth biotoxin exercise, all ricin samples spiked into human plasma at concentrations varying from 1 to 16 µg/mL were successfully detected using a 1:1000 dilution ratio (Figure 7C). These results further demonstrate the method's robustness and reliability in detecting real samples in the presence of complex matrices. Future work should focus on expanding the assay's applicability to other toxins and further improving its robustness against the challenges posed by various complex matrices.

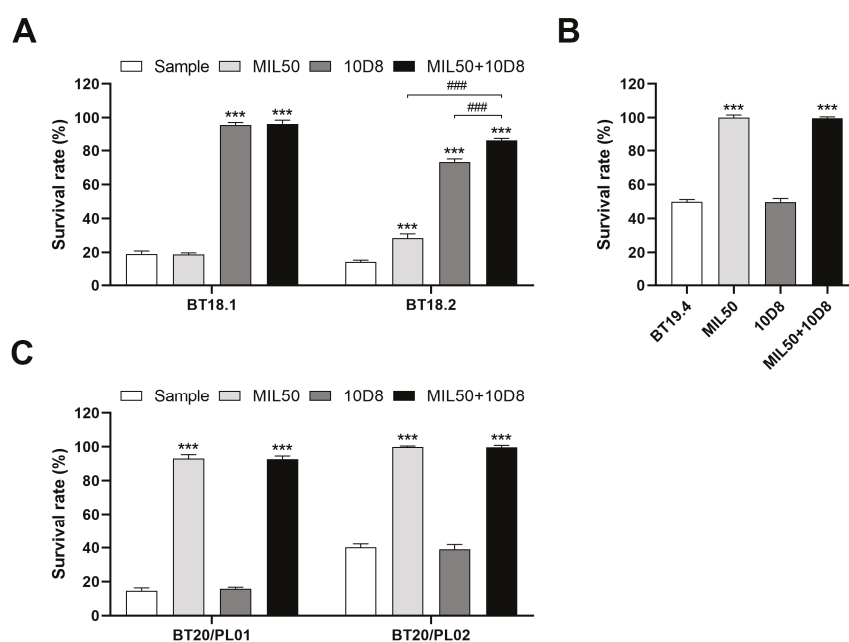


Figure 7. Detection of ricin and abrin from the samples of OPCW biotoxin exercises. (A) A 20 mg aliquot of each sample was dissolved in 1 mL PBS buffer, vortexed, and centrifuged. The resulting supernatant was subsequently diluted to a final ratio of 1:1000 with serum-free RPMI-1640 medium. (B) Spray buffer sample BT19.4 was diluted to a final ratio of 1:20,000 with serum-free RPMI-1640 medium. (C) Human plasma samples BT20/PL01 and BT20/PL02 were prepared via dilution to a final ratio of 1:1000 in serum-free RPMI-1640 medium. The diluted samples were preincubated with mAbs MIL50 or 10D8, either individually or in combination, at a final concentration of 10 µg/mL for 1 h at 37 °C and then added to cells. The cell survival rate was calculated via CCK-8 assay. Data are shown as the mean $\pm$ SD ($n = 3$). Statistical significance was determined using one-way ANOVA (***) $p < 0.001$ vs. sample, ### $p < 0.001$.

Table 2. The detection results of samples from OPCW biotoxin analysis exercises.

No.	Sample Name	Spiking Chemicals (Concentration)	Matrices	Dilution Ratio	Detection Results
3rd	BT18.1	Abrin (150 µg/g)	Protein powder	1:1000	Abrin
	BT18.2	Ricin (15 µg/g) Abrus agglutinin (300 µg/g) *	Protein powder	1:1000	Ricin Abrin
4th	BT19.4	Ricin (30 µg/mL)	Spray buffer	1:20,000	Ricin
5th	BT20/PL01	Ricin (16 µg/mL)	Human plasma	1:1000	Ricin
	BT20/PL02	Ricin (1 µg/mL)	Human plasma	1:1000	Ricin

* Abrus agglutinin is the 40% ammonium sulfate precipitate from the same preparation and contains abrin.

3. Conclusions

The present study successfully developed a sensitive, convenient, and high-throughput cytotoxicity assay for the efficient detection and differentiation of active ricin and abrin in complex matrices. The assay employs a straightforward serial-dilution procedure, circumventing the need for laborious purification or enrichment processes. The current method could be extended to a range of other non-cytotoxic or cytotoxic matrices with appropriate dilution ratios, providing a valuable tool for the detection and identification of ricin and abrin in diverse and challenging environments.

4. Materials and Methods

4.1. Materials

Ricin and abrin were prepared in house and purified to an estimated purity higher than 95% as described previously [35–37]. All toxins were handled by well-trained personnel wearing appropriate protective equipment in a clean fume hood. Toxin-containing solutions and consumable materials were decontaminated with sodium hydroxide or inactivated through autoclaving. Ricin-specific neutralizing monoclonal antibody (mAb) MIL50 was provided by Prof. Jiannan Feng's team at the Academy of Military Medical Sciences (China). Abrin-specific neutralizing mAb 10D8 was prepared in our previous research [38].

4.2. Cell Culture

HepG2, HeLa, HCT-8, and BGC-823 cells were maintained in Roswell Park Memorial Institute 1640 medium (RPMI-1640, Gibco, Grand Island, NY, USA). HEK-293 and SH-SY5Y cells were propagated in minimum essential medium (MEM, Sigma-Aldrich, St. Louis, MO, USA). A549 cells were cultured in F-12K Nutrient Mixture (Gibco, Grand Island, NY, USA). HUVEC and AC16 cells were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA). Each medium was supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin (Gibco, Grand Island, NY, USA). The cells were incubated at 37 °C in an atmosphere containing 5% CO₂ in a humidified incubator.

4.3. Cytotoxicity Evaluation

Cells were cultured in 96-well plates at a density of 1.0×10^4 cells per well. Ricin and abrin, which were freshly prepared in sterile PBS, were serially diluted with serum-free medium to concentrations ranging from 0.01 to 300 ng/mL and introduced to each well after the removal of serum-containing growth media. Following a 24 h incubation at 37 °C, cell morphology was visualized and recorded using a high-content cell analysis system (Opera Phenix, PerkinElmer, Waltham, MA, USA) in bright-field mode. CCK-8 reagent (Beyotime, Shanghai, China) was added into each well and incubated for an additional hour at 37 °C. Absorbance at 450 nm was measured using a microplate reader (Infinite M1000 pro, TECAN, Männedorf, Switzerland). The cell survival rate was calculated using the following formula: survival rate % = $(A_{450_{\text{sample}}} - A_{450_{\text{background}}}) / A_{450_{\text{control}}}$

– $A_{450_{\text{background}}} \times 100\%$. The survival curve was derived from the “log(inhibitor) vs. response-viable slope (four parameters)” nonlinear regression model, represented by the equation: $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogIC}_{50} - X) * \text{HillSlope})})$. IC_{50} value (the concentration required for a 50% reduction in cell survival) was determined through interpolation from the equation using the “interpolate unknowns from standard curve” function in GraphPad Prism with a Y value set to 50%. The limits of detection (LODs) were determined based on the lowest concentrations of toxin used in the experiments that produced a significant cytotoxic effect.

4.4. Antibody Treatment

Ricin and abrin at concentrations ranging from 0.01 to 300 ng/mL were freshly prepared in serum-free medium and preincubated with or without mAbs MIL50 or 10D8, respectively, at final concentrations of 5, 10, and 20 $\mu\text{g/mL}$ for 1 h at 37 °C. Alternatively, for the evaluation and determination of cross-reactivity of mAbs, ricin and abrin were treated with both MIL50 and 10D8 at a final concentration of 10 $\mu\text{g/mL}$ for 1 h at 37 °C. Toxins with or without antibody pretreatment were added to HeLa cells cultured in 96-well plates at a density of 1.0×10^4 cells per well and incubated for 24 h at 37 °C. CCK-8 reagent was added into each well and incubated for an additional hour at 37 °C. Absorbance at 450 nm was measured and the cell survival rate was calculated.

4.5. Analysis of Complex Matrix Samples

The matrices used in this study were whole milk (Yili, Hohhot, China), NFC (not from concentrate) orange juice (Nongfu Spring, Hangzhou, China), Oriental Leaf green tea (Nongfu Spring, Hangzhou, China), and TANBING no sugar black coffee beverage (Nongfu Spring, Hangzhou, China). To determine the impact of complex matrices on cell growth, plasma, milk, orange juice, green tea, and coffee were serially diluted to 1:10, 1:100, and 1:1000 with serum-free RPMI-1640 medium. The diluted samples were centrifuged, filtered through a 0.22 μm filter, and then added to cells grown in 96-well cell culture plates at a density of 1.0×10^4 cells per well. For the analysis of toxin-spiked complex matrix samples, ricin and abrin were spiked into the diluted matrices at the indicated concentrations. The samples were preincubated with or without mAbs MIL50 or 10D8, respectively, at a final concentration of 10 $\mu\text{g/mL}$ for 1 h at 37 °C and then added to cells. After 24 h incubation at 37 °C, CCK-8 reagent was introduced into each well and incubated for an additional hour at 37 °C. Absorbance at 450 nm was measured and the cell survival rate was calculated.

4.6. Analysis of OPCW Biotoxin Exercise Samples

For protein powder samples BT18.1 and BT18.2, obtained from the third OPCW biotoxin exercise, a 20 mg aliquot of each sample was dissolved in 1 mL PBS buffer, vortexed, and centrifuged. The resulting supernatant was subsequently diluted to a final ratio of 1:1000 with serum-free RPMI-1640 medium. The spray buffer sample BT19.4, derived from the fourth OPCW biotoxin exercise, was diluted to a final ratio of 1:20,000 with serum-free RPMI-1640 medium. Human plasma samples BT20/PL01 and BT20/PL02, from the fifth OPCW biotoxin exercise, were prepared through dilution to a final ratio of 1:1000 in serum-free RPMI-1640 medium. The diluted samples were preincubated with mAbs MIL50 or 10D8, either individually or in combination, at a final concentration of 10 $\mu\text{g/mL}$ for 1 h at 37 °C and then added to cells. After 24 h incubation at 37 °C, CCK-8 reagent was added into each well and incubated at 37 °C for 1 h. Absorbance at 450 nm was measured and the survival rate of cells was calculated.

4.7. Statistical Analysis

Data are presented as the mean $\pm$ standard deviation (SD) from three independent experimental replicates. Statistical analyses were conducted using Student's *t* test for two-group comparisons or one-way analysis of variance (ANOVA) followed by Tukey's multiple

comparisons test with GraphPad Prism 8 software (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

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References

1. Felder, E.; Mossbrugger, I.; Lange, M.; Wolfel, R. Simultaneous detection of ricin and abrin DNA by real-time PCR (qPCR). *Toxins* **2012**, *4*, 633–642. [CrossRef] [PubMed]
2. Stirpe, F.; Battelli, M.G. Ribosome-inactivating proteins: Progress and problems. *Cell Mol. Life Sci.* **2006**, *63*, 1850–1866. [CrossRef] [PubMed]
3. Janik, E.; Ceremuga, M.; Saluk-Bijak, J.; Bijak, M. Biological Toxins as the Potential Tools for Bioterrorism. *Int. J. Mol. Sci.* **2019**, *20*, 1181. [CrossRef] [PubMed]
4. Bozza, W.P.; Tolleson, W.H.; Rivera Rosado, L.A.; Zhang, B. Ricin detection: Tracking active toxin. *Biotechnol. Adv.* **2015**, *33*, 117–123. [CrossRef] [PubMed]
5. Dorner, B.G.; Zeleny, R.; Harju, K.; Hennekinne, J.-A.; Vanninen, P.; Schimmel, H.; Rummel, A. Biological toxins of potential bioterrorism risk: Current status of detection and identification technology. *TrAC Trends Anal. Chem.* **2016**, *85*, 89–102. [CrossRef]
6. Robb, C.S. The analysis of abrin in food and beverages. *TrAC Trends Anal. Chem.* **2015**, *67*, 100–106. [CrossRef]
7. Kalb, S.R.; Schieltz, D.M.; Becher, F.; Astot, C.; Fredriksson, S.A.; Barr, J.R. Recommended Mass Spectrometry-Based Strategies to Identify Ricin-Containing Samples. *Toxins* **2015**, *7*, 4881–4894. [CrossRef] [PubMed]
8. Liang, L.H.; Yang, Y.; Geng, S.; Cheng, X.; Yu, H.L.; Liu, C.C.; Liu, S.L. Rapid Differential Detection of Abrin Isoforms by an Acetonitrile- and Ultrasound-Assisted On-Bead Trypsin Digestion Coupled with LC-MS/MS Analysis. *Toxins* **2021**, *13*, 358. [CrossRef]
9. Simon, S.; Worbs, S.; Avondet, M.A.; Tracz, D.M.; Dano, J.; Schmidt, L.; Volland, H.; Dorner, B.G.; Corbett, C.R. Recommended Immunological Assays to Screen for Ricin-Containing Samples. *Toxins* **2015**, *7*, 4967–4986. [CrossRef]
10. He, X.; Patfield, S.; Cheng, L.W.; Stanker, L.H.; Rasooly, R.; McKeon, T.A.; Zhang, Y.; Brandon, D.L. Detection of Abrin Holotoxin Using Novel Monoclonal Antibodies. *Toxins* **2017**, *9*, 386. [CrossRef]
11. Worbs, S.; Kampa, B.; Skiba, M.; Hansbauer, E.M.; Stern, D.; Volland, H.; Becher, F.; Simon, S.; Dorner, M.B.; Dorner, B.G. Differentiation, Quantification and Identification of Abrin and Abrus precatorius Agglutinin. *Toxins* **2021**, *13*, 284. [CrossRef] [PubMed]
12. Becher, F.; Duriez, E.; Volland, H.; Tabet, J.C.; Ezan, E. Detection of functional ricin by immunoaffinity and liquid chromatography-tandem mass spectrometry. *Anal. Chem.* **2007**, *79*, 659–665. [CrossRef] [PubMed]
13. Wu, Y.; Taisne, C.; Mahtal, N.; Forrester, A.; Lussignol, M.; Cintrat, J.C.; Esclatine, A.; Gillet, D.; Barbier, J. Autophagic Degradation Is Involved in Cell Protection against Ricin Toxin. *Toxins* **2023**, *15*, 304. [CrossRef] [PubMed]
14. Bevilacqua, V.L.; Nilles, J.M.; Rice, J.S.; Connell, T.R.; Schenning, A.M.; Reilly, L.M.; Durst, H.D. Ricin activity assay by direct analysis in real time mass spectrometry detection of adenine release. *Anal. Chem.* **2010**, *82*, 798–800. [CrossRef] [PubMed]
15. Hale, M.L. Microtiter-based assay for evaluating the biological activity of ribosome-inactivating proteins. *Pharmacol. Toxicol.* **2001**, *88*, 255–260. [CrossRef] [PubMed]
16. Dawson, R.M.; Paddle, B.M.; Alderton, M.R. Characterization of the Asialofetuin microtitre plate-binding assay for evaluating inhibitors of ricin lectin activity. *J. Appl. Toxicol.* **1999**, *19*, 307–312. [CrossRef]
17. Brzezinski, J.L.; Craft, D.L. Evaluation of an in vitro bioassay for the detection of purified ricin and castor bean in beverages and liquid food matrices. *J. Food Prot.* **2007**, *70*, 2377–2382. [CrossRef] [PubMed]
18. Wahome, P.G.; Bai, Y.; Neal, L.M.; Robertus, J.D.; Mantis, N.J. Identification of small-molecule inhibitors of ricin and shiga toxin using a cell-based high-throughput screen. *Toxicon* **2010**, *56*, 313–323. [CrossRef] [PubMed]
19. Stechmann, B.; Bai, S.K.; Gobbo, E.; Lopez, R.; Merer, G.; Pinchard, S.; Panigai, L.; Tenza, D.; Raposo, G.; Beaumelle, B.; et al. Inhibition of retrograde transport protects mice from lethal ricin challenge. *Cell* **2010**, *141*, 231–242. [CrossRef]

20. Pauly, D.; Worbs, S.; Kirchner, S.; Shatohina, O.; Dorner, M.B.; Dorner, B.G. Real-time cytotoxicity assay for rapid and sensitive detection of ricin from complex matrices. *PLoS ONE* **2012**, *7*, e35360. [CrossRef]
21. Oda, T.; Komatsu, N.; Muramatsu, T. Cell lysis induced by ricin D and ricin E in various cell lines. *Biosci. Biotechnol. Biochem.* **1997**, *61*, 291–297. [CrossRef] [PubMed]
22. Saxena, N.; Phatak, P.; Chauhan, V. Differential toxicity of abrin in human cell lines of different organ origin. *Toxicol. In Vitro* **2022**, *78*, 105250. [CrossRef] [PubMed]
23. Rasooly, R.; He, X. Sensitive bioassay for detection of biologically active ricin in food. *J. Food Prot.* **2012**, *75*, 951–954. [CrossRef] [PubMed]
24. Pasetto, M.; Barison, E.; Castagna, M.; Della Cristina, P.; Anselmi, C.; Colombatti, M. Reductive activation of type 2 ribosome-inactivating proteins is promoted by transmembrane thioredoxin-related protein. *J. Biol. Chem.* **2012**, *287*, 7367–7373. [CrossRef]
25. Tolleson, W.H.; Jackson, L.S.; Triplett, O.A.; Aluri, B.; Cappozzo, J.; Banaszewski, K.; Chang, C.W.; Nguyen, K.T. Chemical inactivation of protein toxins on food contact surfaces. *J. Agric. Food Chem.* **2012**, *60*, 6627–6640. [CrossRef] [PubMed]
26. Sowa-Rogozinska, N.; Sominka, H.; Nowakowska-Golacka, J.; Sandvig, K.; Slominska-Wojewodzka, M. Intracellular Transport and Cytotoxicity of the Protein Toxin Ricin. *Toxins* **2019**, *11*, 350. [CrossRef]
27. Makdasi, E.; Laskar, O.; Milrot, E.; Schuster, O.; Shmaya, S.; Yitzhaki, S. Whole-Cell Multiparameter Assay for Ricin and Abrin Activity-Based Digital Holographic Microscopy. *Toxins* **2019**, *11*, 174. [CrossRef] [PubMed]
28. Sandvig, K.; Olsnes, S.; Pihl, A. Kinetics of binding of the toxic lectins abrin and ricin to surface receptors of human cells. *J. Biol. Chem.* **1976**, *251*, 3977–3984. [CrossRef] [PubMed]
29. Leonard, J.E.; Grothaus, C.D.; Taetle, R. Ricin binding and protein synthesis inhibition in human hematopoietic cell lines. *Blood* **1988**, *72*, 1357–1363. [CrossRef]
30. Chan, L.N.; Li, J.S.; Liu, S.Y. Differential effects of abrin on normal and tumor cells. *J. Cell Physiol.* **1985**, *123*, 132–138. [CrossRef]
31. Nagatsuka, T.; Uzawa, H.; Ohsawa, I.; Seto, Y.; Nishida, Y. Use of lactose against the deadly biological toxin ricin. *ACS Appl. Mater. Interfaces* **2010**, *2*, 1081–1085. [CrossRef] [PubMed]
32. Rasooly, R.; He, X.; Friedman, M. Milk inhibits the biological activity of ricin. *J. Biol. Chem.* **2012**, *287*, 27924–27929. [CrossRef]
33. Lumor, S.E.; Deen, B.D.; Ronningen, I.; Smith, K.; Fredrickson, N.R.; Diez-Gonzalez, F.; Labuza, T.P. Assessment of the inhibition of ricin toxicity by lactose in milk. *J. Food Prot.* **2013**, *76*, 2037–2039. [CrossRef]
34. Brandon, D.L.; Adams, L.M. Milk matrix effects on antibody binding analyzed by enzyme-linked immunosorbent assay and biolayer interferometry. *J. Agric. Food Chem.* **2015**, *63*, 3593–3598. [CrossRef] [PubMed]
35. Hegde, R.; Maiti, T.K.; Podder, S.K. Purification and characterization of three toxins and two agglutinins from *Abrus precatorius* seed by using lactamyl-Sepharose affinity chromatography. *Anal. Biochem.* **1991**, *194*, 101–109. [CrossRef] [PubMed]
36. Tang, J.; Xie, J.; Shao, N.; Yan, Y. The DNA aptamers that specifically recognize ricin toxin are selected by two in vitro selection methods. *Electrophoresis* **2006**, *27*, 1303–1311. [CrossRef]
37. Luo, L.; Yang, J.; Li, Z.; Xu, H.; Guo, L.; Wang, L.; Wang, Y.; Luo, L.; Wang, J.; Zhang, P.; et al. Label-free differentiation and quantification of ricin, abrin from their agglutinin biotoxins by surface plasmon resonance. *Talanta* **2022**, *238*, 122860. [CrossRef]
38. Li, Z.; Xu, H.; Ma, B.; Luo, L.; Guo, L.; Zhang, P.; Zhao, Y.; Wang, L.; Xie, J. Neutralizing Monoclonal Antibody, mAb 10D8, Is an Effective Detoxinant against Abrin-a Both In Vitro and In Vivo. *Toxins* **2022**, *14*, 164. [CrossRef]

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Article

New Analytical Approach to Quinolizidine Alkaloids and Their Assumed Biosynthesis Pathways in Lupin Seeds

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Abstract: Alkaloids play an essential role in protecting plants against herbivores. Humans can also benefit from the pharmacological effects of these compounds. Plants produce an immense variety of structurally different alkaloids, including quinolizidine alkaloids, a group of bi-, tri-, and tetracyclic compounds produced by *Lupinus* species. Various lupin species produce different alkaloid profiles. To study the composition of quinolizidine alkaloids in lupin seeds, we collected 31 populations of two wild species native to Israel, *L. pilosus* and *L. palaestinus*, and analyzed their quinolizidine alkaloid contents. Our goal was to study the alkaloid profiles of these two wild species to better understand the challenges and prospective uses of wild lupins. We compared their profiles with those of other commercial and wild lupin species. To this end, a straightforward method for extracting alkaloids from seeds and determining the quinolizidine alkaloid profile by LC–MS/MS was developed and validated in-house. For the quantification of quinolizidine alkaloids, 15 analytical reference standards were used. We used GC–MS to verify and cross-reference the identity of certain alkaloids for which no analytical standards were available. The results enabled further exploration of quinolizidine alkaloid biosynthesis. We reviewed and re-analyzed the suggested quinolizidine alkaloid biosynthesis pathway, including the relationship between the amino acid precursor L-lysine and the different quinolizidine alkaloids occurring in seeds of lupin species. Revealing alkaloid compositions and highlighting some aspects of their formation pathway are important steps in evaluating the use of wild lupins as a novel legume crop.

Keywords: *Lupinus pilosus*; *Lupinus palaestinus*; quinolizidine alkaloids; LC–MS/MS; GC–MS; toxicity; biosynthesis

Key Contribution: A comprehensive analysis of quinolizidine alkaloids in *L. pilosus* and *L. palaestinus* was achieved using LC–MS/MS and GC–MS. Using this, the QA assumed biosynthesis pathways were reviewed.

1. Introduction

Over millions of years, the long evolutionary tradeoff between plants and the animals that feed on them has led to the production of numerous phyto-compounds that prevent

plants from being over-eaten [1]. A prominent and evolutionarily stable strategy in plants is the production of toxic, repellent, or anti-nutritive metabolites [2]. These substances protect plants against herbivores, limit plant feeding, and decrease plant biomass loss [3]. Many such compounds are toxic to herbivores and vertebrates, including humans. They are also harmful to other microorganisms, such as viruses, bacteria, and fungi, and thus, could be natural biocides to help combat pathogens and treat diseases, including cancers [3].

Quinolizidine alkaloids (QAs) are a group of these toxic compounds. QAs are characterized by their quinolizidine ring structure [4] and are produced as secondary metabolites by members of the genus *Lupinus* [5,6]. QAs offer plants efficient protection from insect pests, and their accumulation in significant quantities complicates lupin consumption [7]. The EU has not regulated a QA human/mammal consumption threshold. However, the following QAs will likely be of interest for future legislation: albine, anagryne, angustifoline, multiflorine, sparteine, lupanine, isolupanine, and 13-hydroxylupanine. Except for anagryne, all of these are QAs typically found in white and blue lupins (*L. albus* and *L. angustifolius*, respectively), the two main species that are consumed in the EU. To date, only Australia and New Zealand have regulated the allowable QA threshold level in lupins, limiting it to 200 mg per kg of lupin product [8].

Despite their potential uses for medicine and plant protection, information on the toxicity of QAs occurring in *Lupinus* species is limited [4,9], and so far, no comprehensive human health risk has been established [4,10–13]. Of the various QAs found in edible lupins, sparteine is generally regarded as the most poisonous, although its association with the intake of industrially produced lupin foods has not been reported [4,14].

Four lupin species have been domesticated and are commercially grown as legume crops: *L. angustifolius* (blue or narrow-leaved lupin), *L. luteus* (yellow lupin), *L. albus* (white lupin), and *L. mutabilis* (pearl or Andean lupin), of which, *L. angustifolius* is the most produced [7]. The chemical structures of many QAs are well characterized [15], and lupin cell cultures and enzyme assays have been employed to identify some biosynthetic enzymes and pathway intermediates [7]. However, a complete understanding of the QA biosynthetic pathway remains unresolved. QA biosynthesis is light regulated, being stimulated during the day, and occurs in the stroma of leaf chloroplasts [16]. Once produced, QAs are phloem transported and stored in vacuoles of plant organs [17]. Stems and leaves accumulate alkaloids, but the alkaloids disappear during leaf senescence. Lupin seeds, on the other hand, accumulate QAs as they mature.

We wish to explore the ability to use wild lupin species as a means to enhance diversity and resilience in local crops [18] or as a supplementary protein crop [19]. However, lupin bitterness and health risks are mainly attributed to their alkaloid content and QA profile ([4] and references therein). To cultivate wild lupins into a food crop, a thorough evaluation, and ultimately, control over the alkaloid concentrations and profile, must be achieved. To control legumes' bitterness, people have often used debittering techniques such as fermentation, soaking, or salt-rich cooking, or they have focused on breeding sweeter seeds [7]. Generally, breeding for sweeter lupins usually aims to relocate QA production and accumulation to lupin vegetative tissues to preserve insect resistance, while reducing its production in seeds to keep the QA levels within industry demands [20,21]. Another strategy is to screen for wild lupin populations naturally low in QA levels and to grow them in suitable agricultural conditions. A third approach is to manipulate the QA biosynthesis pathway by blocking the biosynthesis pathway of non-desired alkaloids or of their precursors. In all of these scenarios, obtaining knowledge regarding the QA profile, particularly its biosynthesis, is crucial for transforming wild lupins into an industrial crop and food ingredient.

The QAs present in the abovementioned species may be divided into four major structural groups: lupanine (including angustifoline), lupinine, multiflorine, and sparteine [4]. A fifth, structurally independent group has an alpha-pyridone structure, including thermopsine, cytisine, and anagryne, and is considered highly toxic. The structural diversity of QAs is largely species dependent, and each lupin species has a unique alkaloid profile [15,17,22]. This quinolizidine-based chemo-diversity is considerable. For example, 46 alkaloids were

identified in 22 cultivars derived from three different Italian species grown in Sicily, including some alkaloids not shared with other edible cultivars [22]. Quantitative analyses demonstrated large differences in the total QA content of lupin species, ranging from 0.003 to 32.8 mg/g in the fresh leaves of eight species [17]. Many questions, such as edibility (i.e., bitterness and toxicity) and pharmaceutical potential, are strongly related to what governs the vast diversity of both QA content and relative concentration, namely the alkaloid biosynthesis mechanism. In a recent DNA molecular study, Bermúdez-Torres and colleagues [23] showed that QA diversity development is affected by geographical and evolutionary origin [23]. Nevertheless, the molecular mechanisms underlying the biosynthesis of lysine-derived alkaloids, including QAs, are not yet fully understood [16]. Advances in the understanding of lysine-derived alkaloids led researchers to suggest a theoretical biosynthesis pathway [7,13,16]. According to this hypothesis, lysine decarboxylase catalysis is the first step in the biosynthetic pathway of QAs [16]. This model also refines the molecular role of lysine decarboxylase activity in plants, highlighting lysine decarboxylase as the enzyme catalyzing the first initiated step of QA biosynthesis and depicting the likely relationships between the different types of QAs and derivatives [16].

Most studies on lupin-derived QAs focus on the four cultivated species. In this study, we evaluated the QA composition of two wild species native to Israel—*L. pilosus* and *L. palaestinus*. *L. pilosus*, common in Mediterranean shrublands, which have large seeds and a high tolerance to various soil conditions, while *L. palaestinus* prospers on sandy calcareous soils. Only limited information on the QA content of *L. pilosus* and *L. palaestinus* is available in the literature [22,24]. Among other *Lupinus* species, Świącicki et al. [25] studied the QA content of *L. pilosus* and *L. palaestinus* accessions from seed bank collections. Here, we studied the QA profiles of these two species in 31 wild populations. We then compared the QA compositions of the wild populations with samples of the four cultivated lupin species. For this, we developed a new LC–MS/MS method for determining QAs in lupin seeds. The method was validated in-house using 15 QAs available from commercial sources. In addition, we used GC–MS to identify some QAs for which no analytical standard was available. We analyzed the inner statistical correlations between the various QAs identified in the different species and their connection with the precursor amino acid lysine to gain new insights into QA phyto-production.

2. Results

2.1. Analytical Method

A novel LC–MS/MS method was developed and validated in-house using 15 QA standards (see Supplementary Table S1 for information on vendors). A straightforward one-step sample extraction procedure using acidified methanol/water was applied, leading to recoveries between 80 and 105% for all QAs except 13-trans-cinnamoyloxylupine, which displayed yields between 45 and 55% (Supplementary Table S2). In-house validation showed relative standard deviations ($n = 6$) between 2 and 14% for all compounds at the three spiking levels tested (1–5–25 mg/kg). However, QA levels in lupin seeds can be very high, exceeding 10,000 mg/kg for individual QAs in some bitter varieties, far above the level that can be realistically spiked in a sample. To check recovery at such high concentrations, several samples were extracted twice with the extraction solvent. The presence of only small amounts of QAs in the second extract (<10%) confirmed the high extraction efficiencies obtained with the spiked samples. Calibration lines showed good to excellent linearity (>0.995) over the full calibration range of 0–200 µg/L for all QAs (Supplementary Table S3). Due to the huge range of QA concentrations in the lupin seeds, sample extracts were prepared with overall standard dilution factors (DFs) of 2000, 10,000, and 50,000. This approach ensured that all QAs could be quantified against the same set of calibration lines by selecting the best-fitting diluted extract for each QA.

The available standards covered the majority of QAs present in the lupin species. Nevertheless, some relevant QA commercial standards were not available, including 11,12-seco-12,13-didehydromultiflorine and 13-hydroxymultiflorine, and several esters

of 13-hydroxymultiflorine and 13-hydroxylupanine. These QAs were semi-quantified by taking a structurally related QA with high structural similarity as a reference. Identification of the QAs without reference compounds is described in the ‘Materials and Methods’ section below.

2.2. Quinolizidine Alkaloid Profiles

Total QA compositions by LC–MS/MS are detailed in the Supplementary Materials (Table S4). The QA amounts reported here are averages for each of the 31 tested wild *L. pilosus* and *L. palaestinus* populations. The total QA composition of four cultivated lupin species—*L. angustifolius*, *L. luteus*, *L. mutabilis*, and two cultivars of *L. albus*, as well as two control groups—chickpea and soybeans—are also reported. This research uses these species as a reference group for the wild lupins (*L. pilosus* and *L. palaestinus*), as they are more thoroughly studied. Twenty-one quinolizidine alkaloids are identified and (semi)-quantified in the samples. In Supplementary Figure S2, representative LC–MS/MS chromatograms are provided for the six lupin species in this study.

Kruskal–Wallis tests on the total QA amounts reveal the uniqueness of each species tested and the apparent differences between the analyzed varieties or species (Figure 1). All populations relating to the same species show a close statistical correlation in the total amount and the profile of alkaloids produced. *L. pilosus* and *L. palaestinus* show a lower total QA content than the seeds of the other tested species, except for the *L. albus* sweet variety (Figure 1).

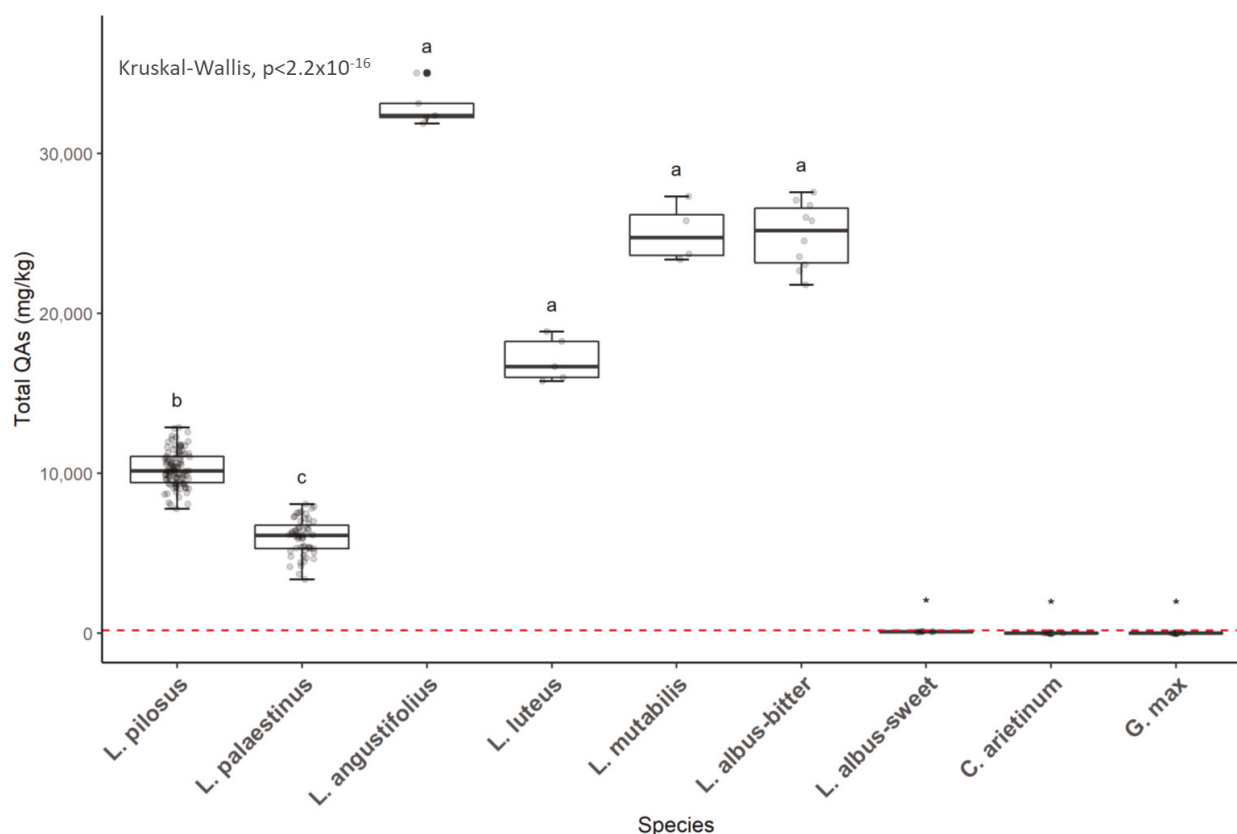


Figure 1. Total quinolizidine alkaloid (QA) levels were detected in wild local species *L. palaestinus* and *L. pilosus* (in the grey box) compared with other lupin or legume species. The Australian regulatory threshold for human consumption (200 mg QAs/kg seed) is marked with a dashed red line. Kruskal–Wallis significance ($p > 0.05$) is marked with small letters. Groups marked with an asterisk were used as control groups and thus were not included in the statistical analysis.

The heat map of all the QAs detected (Figure 2A) shows a very high inner correlation among species. Among *L. pilosus* and *L. palaestinus*, all populations relating to the same

species showed a close inner correlation in QA compositions, although grown in different ecological systems (Figure 2). The two lupin species show a much closer statistical correlation to one another than to the other species, which produces a different array of QAs (Figure 2B). Regarding individual compounds, the dominant QAs in *L. pilosus* and *L. palaestinus* are multiflorine and epilupinine, the latter being less abundant in *L. palaestinus*. Other important marker QAs for both species are 11,12-seco-12,13-didehydromultiflorine and 13 α -hydroxymultiflorine. Together, these four QAs comprise 95–97% of the QA composition in the two species. In the other species, the contribution of these four QAs to the total content is between 0.2% (*L. mutabilis*) and 10–11% (*L. albus*).

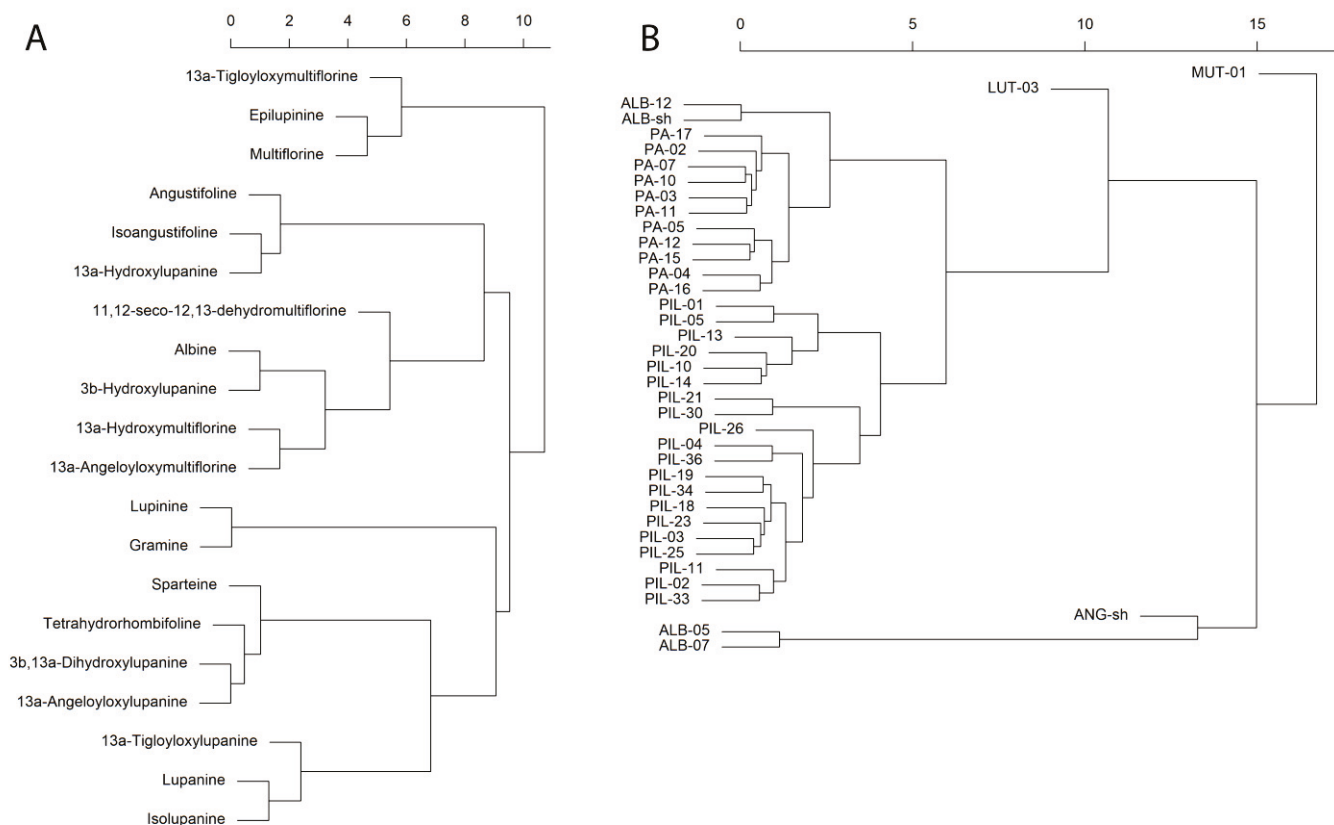


Figure 2. Hierarchical clustering of the studied lupin species and their QAs. The hierarchical clustering (A) shows the statistical distances between the various QAs identified. The grouping of the different lupin populations (B) is based on their complete QA profiles, as correlated in (A). Analysis was carried out using the dendrogram function of the *heatmap* package after scaling using the scale base function.

In *L. pilosus* and *L. palaestinus*, sparteine is present at moderate levels (Figure 3). This is also the situation for other lupin species besides *L. mutabilis*, in which sparteine is one of the marker QAs (Figure 3). Other marker QAs in *L. mutabilis* are lupanine, 13 α -hydroxylupanine, and 3 β ,13 α -dihydroxylupanine. Together, these four QAs are responsible for approx. 88% of the QA content in *L. mutabilis*.

Lupanine and 13 α -hydroxylupanine are major compounds in *L. albus* and *L. angustifolius* (Figure 3). In *L. albus*, they are complemented with albine and 13 α -hydroxymultiflorine (accounting for 80–87% in total), and in *L. angustifolius* with angustifoline (representing 96% in total). Finally, *L. luteus* displays a QA profile different from all the other lupin species, the marker QAs being lupinine, gramine, and, present in smaller amounts, epilupinine, which together make up 99% of the QA content. The results show that the seeds of the six species studied have distinctively different QA profiles with different QAs dominating. *L. pilosus* and *L. palaestinus* are closely related as they contain the same QAs. Nevertheless,

they can be differentiated based on the ratio of epilupinine to multiflorine in the seeds, which for *L. pilosus* and *L. palaestinus* contain $44 \pm 12\%$ and $12 \pm 8.5\%$, respectively.

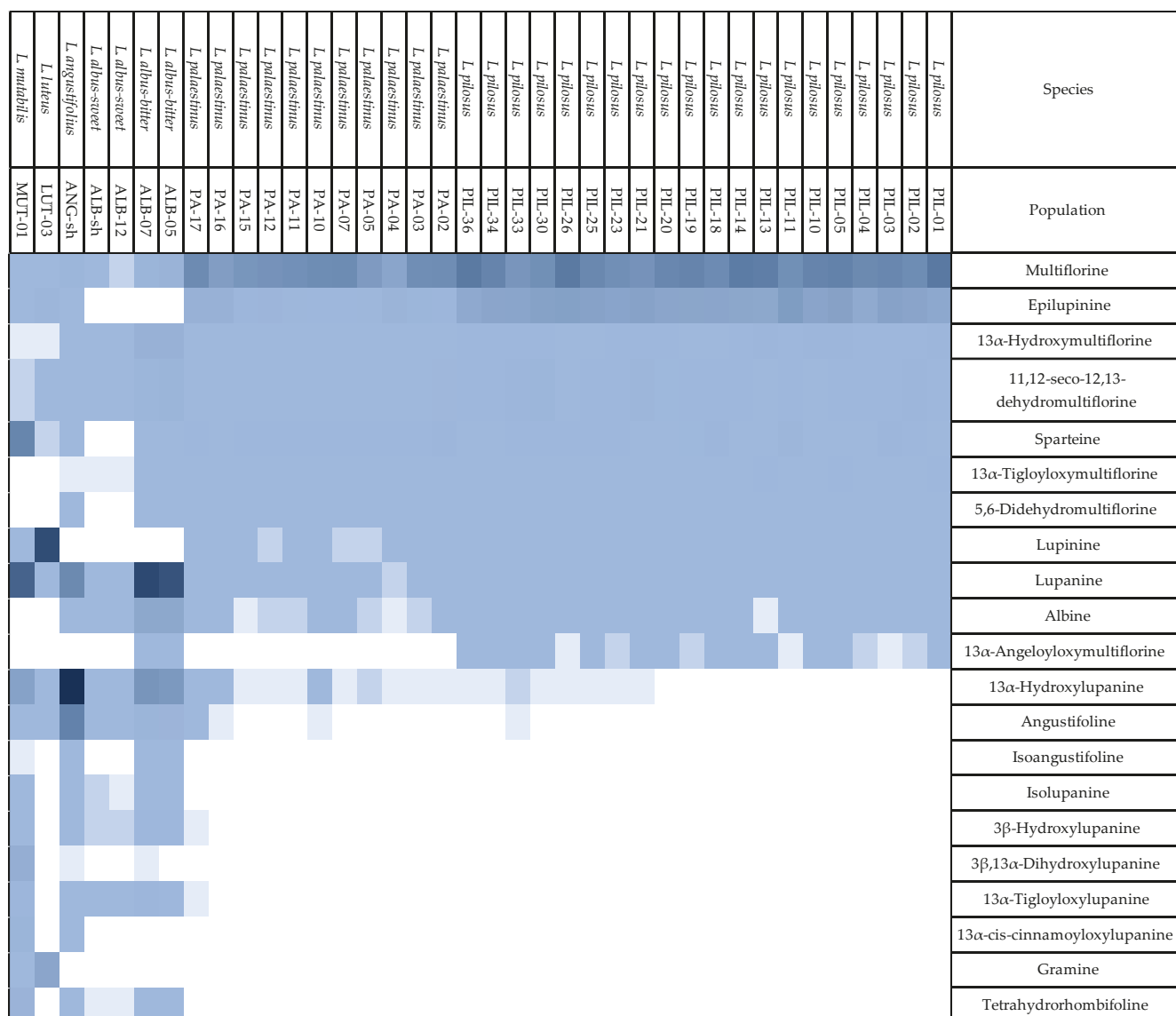


Figure 3. Heat map of the relative ranked quantity of the individual QAs in the analyzed lupin populations and control groups. Results are shown in a grayscale relative gradient, from the highest concentration (in blue) to the lowest/non-detected concentrations (in white). For complete details see Supplementary Material Table S4.

Another heat map reveals the significance of the statistical correlation shown in Figure 3, pointing out the correlation between the different species among themselves and compared with other analyzed lupin species.

2.3. Correlation with L-Lysine

Correlation plots between l-lysine amounts (*w/w%* of DW) and the amounts of four main alkaloid groups present in *L. pilosus* and *L. palaestinus*—(epi)lupinine, lupanine, multiflorine, and sparteine, were constructed to reveal the connection between l-lysine levels and alkaloid production (Figure 4). (Epi)lupinine and multiflorine correlate negatively with l-lysine levels, suggesting that when lupinine and multiflorine are produced, l-lysine is consumed and its concentration drops. No significant correlation was demonstrated between l-lysine and lupanine or l-lysine and sparteine. As l-lysine is the precursor of all

QA derivatives, including lupanine and sparteine, the lack of correlation may be attributed to their very low levels, suggesting that the two are under-produced in these lupin species.

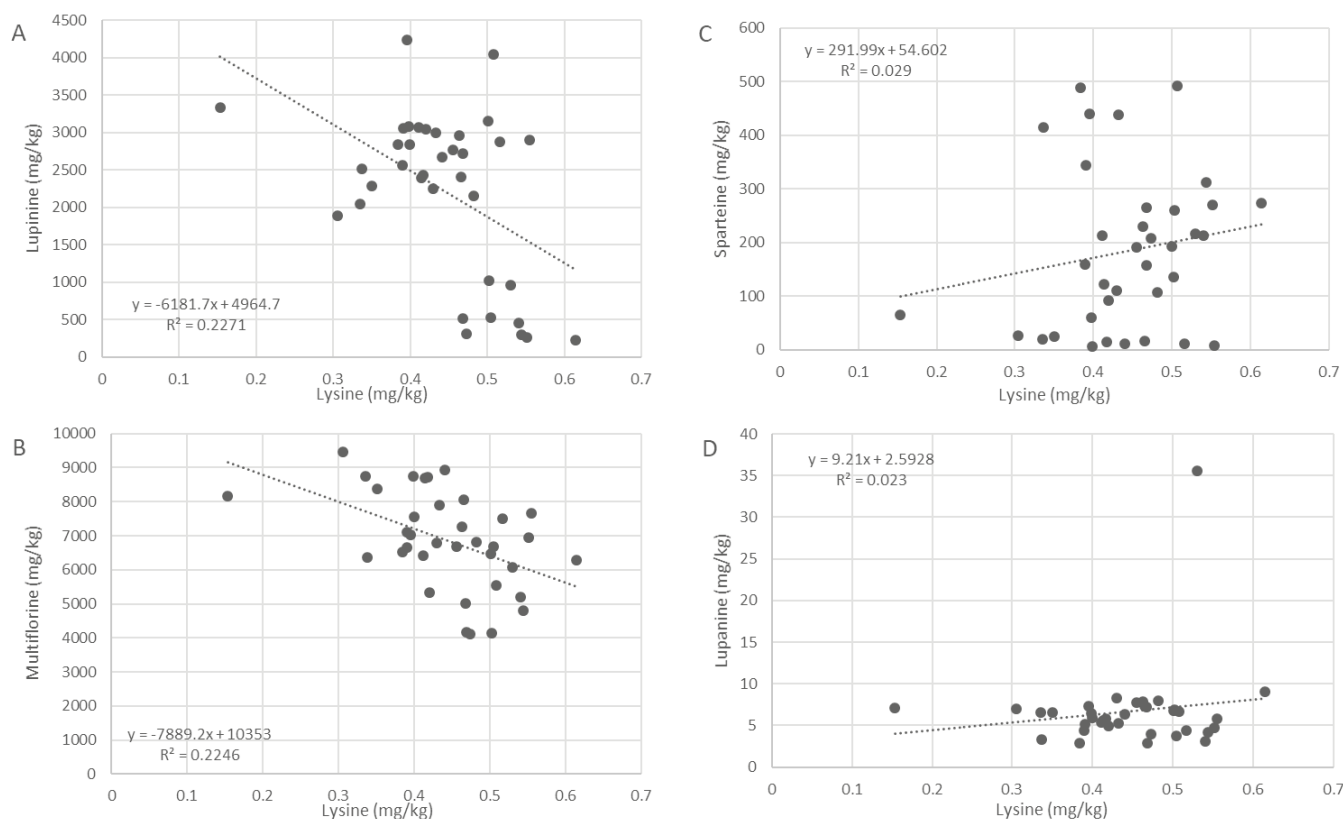


Figure 4. Correlation plots between l-lysine amounts ($w/w\%$ of DW) and the amounts of the four main alkaloid groups—(A) (epi)lupinine, (B) multiflorine, (C) sparteine, and (D) lupanine, in *L. pilosus* and *L. palaestinus*.

3. Discussion

The new LC–MS/MS method developed and presented here proved effective for the detection, identification—and, most importantly—accurate quantification of QAs. Whenever a QA reference standard was unavailable, GC–MS complemented the identification. Thus, we were able to identify 21 QAs in six different species. Ten of these 21 QAs could be quantified employing a reference standard, while the others were semi-quantified based on structurally related QAs. When previously analyzed by GC–MS as a stand-alone method, relative quantification was achievable for these species [25–28]. GC–MS is often the gold standard for QA analysis in lupin seeds. This is partly because GC-based methods have been used for over 40 years, and extensive databases that tabulate the various QAs in *Lupinus* and related genera have been compiled. Only recently have the first LC–MS analytical methods started to appear [11,12,29]. The limitation of many studies, particularly GC–MS-based methods, is that primarily semi-quantitative results are reported due to the lack of suitable reference standards. Lupanine is often used as a reference standard for quantitation. Still, it should be acknowledged that the detector response is strongly dependent on the specific chemical structure of a compound as well as the measurement conditions. This variability in detector response is also an essential factor in LC–MS/MS analysis, as can be seen for the QAs incorporated in the method (Supplementary Table S3). For the QA calibration lines, the slope differences amounted to a factor of 12. Nevertheless, a large part of the QAs could be quantified using this method, resulting in a more detailed assessment of the QA content in lupin seeds.

The QA profiles obtained for the six lupin species generally agreed with data reported in the literature [15,25–28,30]. However, some differences were observed. Swieci-

cki et al. [25] reported that their accessions of *L. luteus* contained primarily lupinine and sparteine with smaller amounts of gramine. Similar results were reported by Wink et al. [15]. Magelhaes et al. [31], on the other hand, reported the absence of lupinine in several *L. luteus* cultivars. In our sample, sparteine was only present at trace levels. This suggests that a wider variability within *L. luteus* may exist regarding the production of marker QAs.

Interestingly, it was found that *L. pilosus* and *L. palaestinus* produce epilupinine rather than lupinine. The literature so far provides contradicting results. For instance, Wink et al. [15] and Świącicki et al. [25] reported lupinine as the predominant epimer. However, Ainouche et al. [32] reported epilupinine as the epimer present in both species. Our study confirms the original findings of Thomas et al. [33], who specifically reported epilupinine and not lupinine as present in *L. pilosus*. Epilupinine and lupinine have close retention times in most GC–MS methods, which may have led to misinterpretation of the data by some authors.

The average total QA content determined in the studied *L. pilosus* and *L. palaestinus* populations was $10,250 \pm 1000$ and 6040 ± 1090 mg/kg, respectively. Swiecicki et al. [25] reported a range from 872 to 4717 mg/kg for *L. pilosus* (six accessions) and a range from 774 to 2308 mg/kg for *L. palaestinus* (six accessions). The statistical differences between the accessions were significant ($p < 0.01$), which was attributed by Swiecicki et al. [25] to different gene bank origins of the samples. The levels reported by Swiecicki et al. [25] are substantially lower than those determined in our current study, and differences between accessions were also not statistically significant. Swiecicki et al. [25] used lupanine as the single standard for quantification of the different QAs detected in the seeds, and it cannot be excluded that the QA content was underestimated due to differences in detection response between the QAs present in *L. pilosus* and *L. palaestinus*, and lupanine.

Some publications regarding alkaloid biosynthesis in plants, including genera closely related to *Lupinus*, suggested some QA biosynthesis pathways in lupins [7,16]. These suggested QA biosynthesis pathways in lupins were extrapolated from known pathways of different alkaloids and their derivative compounds produced by other (non-lupin) species. Our results present a new comprehensive dataset derived from QAs in lupin species, which directly supports our refined suggested pathway (Figure 5).

Statistical data analysis allows us to revisit the QA biosynthesis mechanisms in lupin seeds. As mentioned above, the QA compositions and QA correlations within species observed here confirm the QA formation biosynthetic pathway (Figure 5). By integrating all the accumulated data, we suggest that the previously suggested biosynthesis pathways of QAs in lupins are a targeted path. The statistical analysis of individual QAs at the species level allows us to explore further nuances in lupin QA biosynthesis. Plots of the QA compositions produced by the different species show that any given species produces a set of QAs and refrains from producing other QAs (Figure 3, Table S4). For example, *L. pilosus*, as observed in the various populations sampled, produces relatively high amounts of multiflorine but almost no lupanine or related derivatives, including angustifoline. On the other hand, *L. albus* seeds contain relatively high amounts of lupanine but hardly any (epi)lupinine (Figure 3, Table S4).

This preferential production of certain assemblages of QAs is consistent in all species studied. We show that (1) only certain QAs will be produced for any variety of lupin, and others will not, and (2) there is a link between the main QAs that will be produced by a certain lupin species (Figure 4). From this, we can extrapolate that the synthases governing QA production are not randomly activated but are rather controlled by a ‘switch on/switch off’ system. We suggest that lupins produce specific dual QA combinations, i.e., they switch on only two out of the four main QA pathways at any given time. These combinations may be either (epi)lupinine coupled with multiflorine (as in *L. pilosus* and *L. palaestinus*), multiflorine and lupanine (as in *L. albus* and *L. angustifolius*), sparteine and lupanine (as in *L. mutabilis*), or lupinine and sparteine (as in *L. luteus*). Thus, even though the common precursor L-lysine can produce all QAs detected in lupins, it seems that when lupanine and its derivatives are produced, only a determined dual combination will result for each

variety. Similar observations, leading to similar conclusions, were independently drawn by Swiecicki et al. [25], although their analysis was based on a smaller number of lupin populations and fewer QAs.

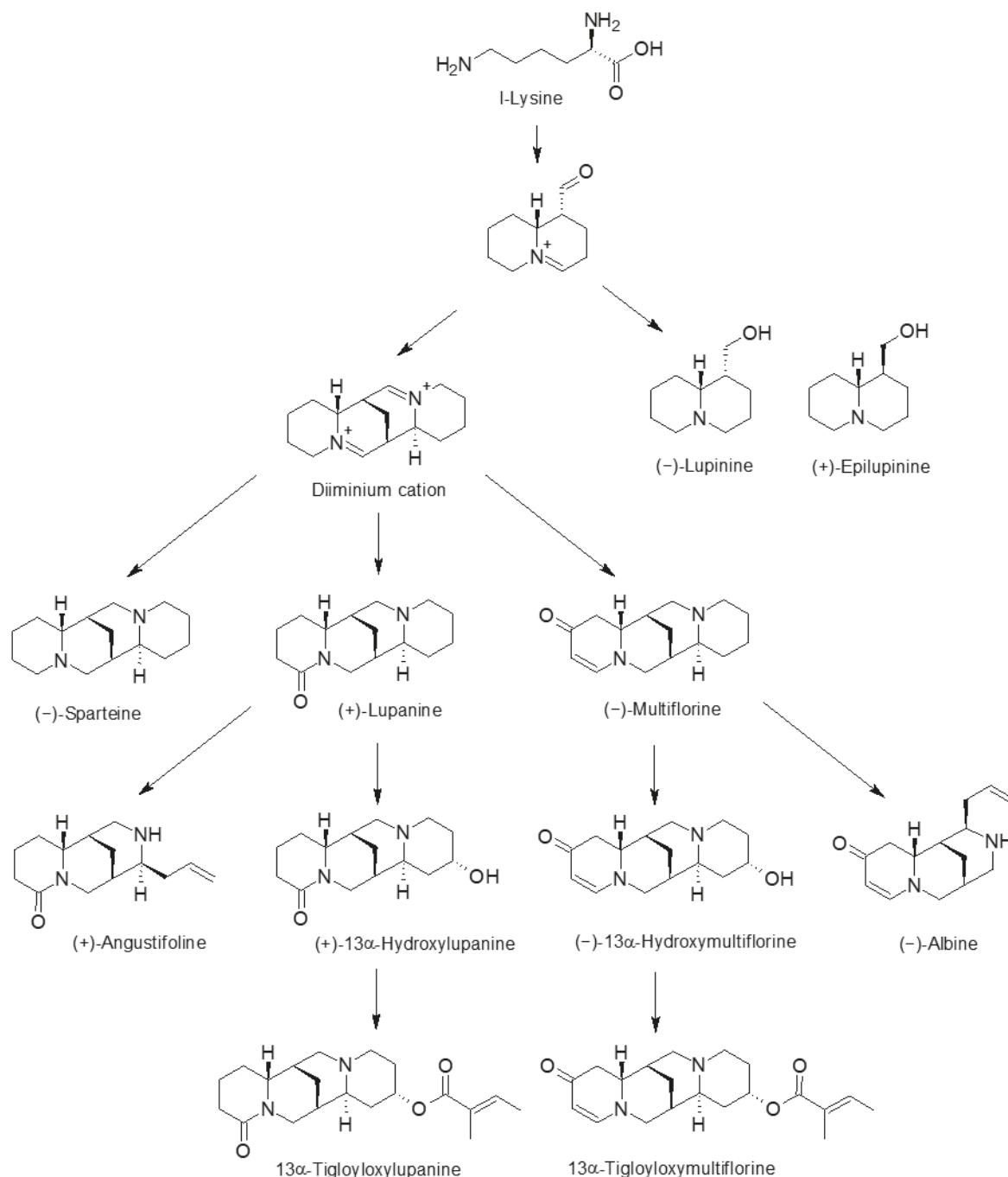


Figure 5. Proposed biosynthesis pathway of quinolizidine alkaloids in lupin seeds based on the observations described in the text.

The previously suggested pathway for QA production in lupins identified the amino acid l-lysine as the precursor of most QAs studied. Here, we propose a new way to examine the QA biosynthesis pathway. Our results support the concept of l-lysine as the amino acid precursor for QA biosynthesis in lupin seeds, as demonstrated in Figure 5. For epilupinine and multiflorine, which are the dominant alkaloids in both *L. pilosus* and *L. palaestinus*, the lower the l-lysine amount detected in the seeds, the higher the total alkaloids accumulated (Figure 4A,B). This inverse correlation reinforces the suggestion that

l-lysine is the precursor for alkaloids produced by lupins. Notably, sparteine and lupanine, produced in negligible or low amounts in the two wild species, showed no correlation with l-lysine concentrations (Figure 4C,D). This accords with the recognition of l-lysine as the precursor for QA biosynthesis and further supports a preferential or alternate production when one production flow is dominantly executed over the other, as described in Figure 4. In *L. pilosus* and *L. palaestinus*, for example, it appears that l-lysine is preferably transformed into multiflorine and epilupanine, while the production of lupanine is very low. Lupanine derivatives were not detected in these species when screened for using GC–MS. In contrast, 13 α -tigloyloxymultiflorine and 13 α -tigloyloxylupanine, two prominent multiflorine and lupanine ester derivatives, were detected in the lupin seed extracts (Figure 5).

4. Conclusions

This paper is an essential step in describing the QA profiles of local wild lupin varieties to be used as the basis to transform them into agricultural crops, either for human consumption or for stock animal feed. We can gain some insights from the cultivation process of *Lupinus angustifolius* in the 1930s. For many centuries, until the appearance of its low-alkaloid mutants, *L. angustifolius* was used mainly as a green manure because the high QA content precluded its use as a feed ingredient. This is a bi-facial phenomenon as low-alkaloid (widely named ‘sweet’) genotypes are mandatory for edible crops but make the plants more susceptible to pathogens and diseases. Some researchers [34] suggested creating ‘bitter/sweet’ dual varieties, where some varieties of the same species will be high in QA content (bitter) and can be used for green manure, while low-QA varieties will be used for food and feed. Creating varieties with high levels of QAs in the vegetative organs while retaining low QA concentrations in edible seeds [34] may lead to a dual-purpose plant that preserves its adaptive fungicidal, antibacterial, and insecticidal capabilities.

Another important motivation for this effort is the desired biological activity that QAs have demonstrated in multiple studies [35]. Many QAs show beneficial and desired qualities such as antioxidant, antimicrobial, anti-carcinogenic, and anti-inflammatory activities [23,36–47]. However, several individual alkaloids, especially sparteine, anagryne, and cysteine, have undesired or even toxic activity, thus their concentrations in edible lupins must be strongly regulated [4,47]. However, cysteine and anagryne concentrations in the wild lupin species studied here were below the detection limit of the LC–MS/MS (0.2 mg/kg). Thus, we conclude that the wild populations of native lupins in Israel might have a future as promising legume crops.

5. Materials and Methods

5.1. Materials

Seven lupin species were included in this study. Mature pods of two wild populations of (A) *L. pilosus* and (B) *L. palaestinus* were sourced in Israel during late spring (see Section 5.2.1 below) (Table 1). Twenty populations of *L. pilosus* and eleven populations of *L. palaestinus* were sampled. Additionally, seven different species or varieties of lupins were included as a reference: four different sources of *L. albus* (two bitter and two sweet), *L. angustifolius*, *L. luteus*, and *L. mutabilis*. Two non-lupin plants—chickpea (*Cicer arietinum*) and soybean (*Glycine max*)—were included as controls, as they do not contain QAs. The low levels of QAs in these control samples are most likely due to minor contamination during sample preparation/analysis. Five repetitions (containing different seeds each time) per each of the 40 samples were collected to compose an entire set of 200 seed samples. The sampling unit was a fixed number of seeds adjusted to each species and cultivation.

Table 1. List of the samples included in this study.

Species	Regional Distribution	Population Code	Collection Site/Notes
<i>Lupinus pilosus</i>	Golan Heights	PIL-1	Mapalim Junction
	Hula Valley	PIL-2	Nahal Hamdal
	Judean Hills	PIL-3	Tel Socho South
	Golan Heights	PIL-4	Ofir Viewpoint
	Golan Heights	PIL-5	South-west Hispin
	Golan Heights	PIL-10	Hazeka Road
	Judean Hills	PIL-11	Matta
	Golan Heights	PIL-13	Fares Road (vineyard)
	Golan Heights	PIL-14	Tel Fazra
	Judean Hills	PIL-18	Khirbet Kanim
	Judean Hills	PIL-19	Zechariya (Tel e-Sharia)
	Golan Heights	PIL-20	Avital
	Carmel Mountain	PIL-21	Makura
	Samarea Mountains	PIL-23	Awartha
	Samarea Mountains	PL-25	Shechem Mountains
	Samarea Mountains	PIL-26	Kedumim
	Carmel Mountain	PIL-30	Kerem Maharal South
	Judean Hills	PIL-33	Sarisa
	Lower Galilee	PIL-34	Ahuzat Barak
	Lower Galilee	PIL-36	Nau'ra
<i>Lupinus palaestinus</i>	Sharon—Coastal Plain	PA-2	Pardes Hana
	Sharon—Coastal Plain	PA-3	Ilanot Forest West
	Coastal Plain (East)	PA-4	Yashresh
	Sharon—Coastal Plain	PA-5	Bnei-Tzion North
	Sharon—Coastal Plain	PA-7	Ilanot Forest East
	Sharon—Coastal Plain	PA-10	Netanya
	Sharon—Coastal Plain	PA-11	Hirbet Samara
	Sharon—Coastal Plain	PA-12	Hod Ha-Sharon
	Sharon—Coastal Plain	PA-15	Tel Mond—Kurkar
	Coastal Plain (South)	PA-16	Ashqelon
	Coastal Plain (East)	PA-17	Sitriya
<i>Lupinus albus</i> (bitter)	Egypt, Central Nile	ALB-05 *	
	Egypt, Central Nile	ALB-07 *	
<i>Lupinus albus</i> (sweet)	Commercial Product 1	ALB-12	
	Commercial Product 2	ALB-sh	
<i>Lupinus angustifolius</i>	Sharon—Coastal Plain	ANG-sh *	Haogen
<i>Lupinus luteus</i>		LUT-03	
<i>Lupinus mutabilis</i>	The Netherlands	MUT-01 **	<i>Lupinus mutabilis</i> , var. <i>cruckshankii</i> . Vreeken's Zaden Seed Company, Dordrecht, The Netherlands, www.vreeken.nl (accessed on 12 March 2024)
<i>Glycine max</i> (soybean)		MAX-01	Organic soybeans certified by Pro-Cert 2020 Crop year, Thompson Limited Canada, Net—25 kg, HOSSQ21-24, Packed 03/2021, Sell by 03/2022
<i>Cicer arietinum</i> (chickpea)		ARI-01	Organic Chickpeas by Tvuot, www.tvuot.co.il (accessed on 12 March 2024), 500 g package Best before 01/06/2022

* Non-edible variety (high QA levels). ** Non-edible species (used as an ornamental garden species).

5.2. Methods

5.2.1. Seed Collection Method

Wild populations of *L. pilosus* and *L. palaestinus* were surveyed in natural areas in Israel, representing a range of environmental conditions (including various climate and soil conditions) typical of Eastern Mediterranean ecosystems. Populations were identified and marked in early spring (February–early March) at the beginning of the flowering period. In late spring (April–May), matured lupin pods were collected before seed dispersal occurred. Around 100 individuals were sampled at each site. Matured pods standing on plants were collected into paper bags and brought to Tel Aviv University laboratories. Once in the lab, pods from each population were placed in separate containers and left to dry at room temperature until all pods opened naturally. Dried lupin pods were removed, and seeds from each population were kept in dry and cool conditions.

5.2.2. Analytical Standards

Fifteen quinolizidine alkaloids were available as analytical standards for this study: albine, anagyrine, angustifoline, 13 α -*trans*-cinnamoyloxylupanine, cytisine, epilupanine, gramine, 13 α -hydroxylupanine, isolupanine, lupanine, lupinine, methylcytisine, multiflorine, sparteine, and thermopsine. See Supplementary Table S1 for further details on vendors and purity. Individual QA stock solutions of 200 μ g/mL were prepared in methanol. Mixed QA solutions in methanol (5 μ g/mL, 500 ng/mL) were prepared from the individual stocks. LC–MS-grade methanol was obtained from Actu-All (Oss, The Netherlands). Analytical-grade formic acid and ammonium carbonate were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) for HPLC analyses. Ethyl acetate and ammonia (NH₄OH, 25%) were HPLC grade. Deionized water (Milli-Q, Merck Millipore, Darmstadt, Germany) with a minimum resistance of 18.2 M Ω was used.

5.2.3. Sample Pretreatment

Samples were ground to a particle size of <1 mm using a FOSS grinder (FOSS CM-290 Cemotec, Labtec, Auckland, New Zealand). Five replicates of 2 g were weighed into 50 mL polypropylene tubes with screw caps, and 40 mL of methanol/water (1:1 *v/v*) containing 1% formic acid was added. The mixture was shaken vigorously for 30 min on a rotary tumbler and then centrifuged for 15 min at 3500 rpm. A total of 0.5 mL of the supernatant was transferred and diluted with water up to 50 mL, and 500 μ L of the diluted sample extract was transferred to a 500 μ L vial. Extracts containing QAs exceeding the range of the calibration line (see below) were diluted 5- and 25-fold.

5.2.4. Liquid Chromatography–Tandem Mass Spectrometry (LC–MS/MS)

Analysis was performed with an LC–MS/MS system consisting of a Waters Acquity UPLC coupled to a Xevo TQ-S tandem mass spectrometer (Waters, Milford, MA, USA). The alkaloids were separated on an Acquity UPLC BEH C18, 1.7 μ m, 100 $\times$ 2.1 mm column (Waters, Milford, MA, USA). The solvents used were (A) ammonium carbonate buffer (10 mmol/L, pH 9.0 $\pm$ 0.1) and (B) methanol. At a flow rate of 0.4 mL/min, the linear gradient conditions were 0.0–1.0 min, 0% B; 8.0 min, 40% B; 12.0 min, 80% B; 12.2 min, 0% B; and 12.2–14.2 min, 0% B. The injection volume was 2 μ L, and the column oven temperature was 50 $^{\circ}$ C. A solvent discard was included at 0–1.5 min and 13–14.2 min.

The instrument was run in the positive electrospray ionization (ESI) mode. The capillary voltage used was 3.0 kV with 30 V cone voltage. The source temperature was set at 150 $^{\circ}$ C, desolvation temperature at 600 $^{\circ}$ C, cone gas flow at 150 L/h, and desolvation gas flow at 800 L/h. Argon was used as the CID gas at a pressure of 10^{−3} mbar (0.17 mL/min). Two to three multiple reaction monitoring (MRM) transitions were measured per analyte. See Table 2 for detailed information on MRM transitions, MS settings, and retention times. Masslynx 4.2 and TargetLynx 4.1 software (Waters, Milford, MA, USA) were used for data acquisition and processing, respectively.

Table 2. MS/MS MRM transitions.

No.	Compound	Precursor Ion (<i>m/z</i>)	Product Ion 1 (<i>m/z</i>)	Col. Energy 1 (eV)	Product Ion 2 (<i>m/z</i>)	Col. Energy 2 (eV)	Product Ion 3 (<i>m/z</i>)	Col. Energy 3 (eV)	Indicative RT (min)	MRM	Compound Quantified
Standard QAs											
1	Gramine	130.0	77.0	20	103.0	25			7.35	1	1
2	Epilupanine	170.2	96.0	30	152.0	20	124.0	25	5.50	1	2
3	Lupanine	170.2	124.0	25	152.0	20	96.0	30	6.00	1	3
4	Cytisine *	191.2	133.0	30	148.0	20			5.70	1	4
5	Methylcytisine *	205.2	58.2	20	108.0	20			7.20	1	5
6	Albine	233.2	112.0	20	138.0	20	150.0	30	7.15	2	6
7	Angustifoline	235.2	112.0	20	193.0	30	114.0	30	8.15	2	7
8	Sparteine	235.2	98.0	30	233.0	30	70.0	30	7.65	2	8
9	Anagyrene *	245.2	70.0	45	98.0	35			9.40	2	9
10	Thermopsine *	245.2	70.0	45	98.0	35			9.50	2	10
11	Multiflorine	247.2	70.0	40	112.0	25	134.0	20	7.65	2	11
12	Lupanine	249.2	114.0	30	136.0	30	98.0	30	8.10	3	12
13	Isolupanine	249.2	84.0	30	98.0	30	136.0	30	10.50	3	13
14	13 α -Hydroxylupanine	265.2	114.0	30	152.0	30	84.0	40	6.95	3	14
15	13 α -trans-Cinnamoyloxylupanine *	395.2	112.0	30	247.0	30	98.0	40	11.55	1	15
Non-standard QAs											
16	Isoangustifoline	235.2	112.0	20	193.0	30	114.0	30	8.00	2	7
17	5,6-Didehydromultiflorine	245.2	70.0	45	98.0	35			7.60	2	9
18	11,12-seco-12,13-Didehydromultiflorine	247.2	98.0	30	112.0	25	70	40	9.95	2	11
19	Tetrahydrothombifoline	249.2	114.0	30	136.0	30	166.0	30	11.40	3	13
20	13 α -Hydroxymultiflorine	263.2	112.0	30	245.0	20	70.0	40	5.65	3	14
21	3 β -Hydroxylupanine	265.2	114.0	30	152.0	30	112.0	35	6.80	3	14
22	3 β ,13 α -Dihydroxylupanine	281.2	130.0	25	152.0	25	101.0	35	5.95	3	14
23	13 α -Tigloyloxymultiflorine	345.2	112.0	30	245.0	20	70.0	40	10.10	1	15
24	13 α -Angeloyloxymultiflorine	345.2	112.0	30	245.0	20	70.0	40	10.20	1	15
25	13 α -Tigloyloxylupanine	347.2	112.0	30	247.0	30	98.0	40	10.70	1	15
26	13 α -Angeloyloxylupanine	347.2	112.0	30	247.0	30	98.0	40	10.85	1	15
27	13 α -cis-Cinnamoyloxylupanine	395.2	112.0	30	247.0	30	98.0	40	11.15	1	15

* Standard is included in the method but not detected in any of the samples.

Lupin QAs for which an analytical standard was available were quantified by external matrix calibration using a diluted blank soybean extract. A nine-point calibration line (0–200 ng/mL) was constructed for each analyte and injected before and after each set of samples. The linearity of the calibration lines was >0.999 . The recovery was monitored by spiking a blank soybean sample with a mixture of the QA standards (5 µg/mL) corresponding to a level of 10 mg/kg. The amount retrieved after extraction was used to correct the results for recovery. The LOQ of the method was defined as the lowest concentration validated (1 mg/kg). The LOD was not determined in detail but was visually estimated to be between 0.1 and 0.5 mg/kg, depending on the QA. Due to the wide range of concentrations found for individual QAs in the seeds (1 → 10,000 mg/kg), the sample extracts were measured undiluted, as well as 5- and 25-fold dilutions. This corresponds to concentration ranges of 0–400 mg/kg, 0–2000 mg/kg, and 0–10,000 mg/kg, respectively.

QAs for which no analytical standard was available were semi-quantified by comparison with a standard of similar structure, as indicated in Table 2.

Each set of 50 extracts included 4 lupin extracts with known content as quality control (QC) samples. These QC samples were used to check the reproducibility of the measurements. Reproducibility for individual compounds varied between 2 and 16%, and for the total PA content, between 4 and 8%, showing good LC–MS/MS system stability and reproducibility between the measurements.

5.2.5. Validation of the LC–MS/MS Method

The method was validated in-house by spiking finely ground samples (0.5 g) at three levels (1, 5, 25 mg/kg, six replicates each) of a mixture of QAs. Because no blank lupin seeds were available, soybeans were used as the surrogate blank material. Soybeans were chosen as they have a composition comparable to lupin seeds but do not contain QAs. The results for recovery and repeatability of the method are presented in Supplementary Table S2. The linearity of the combined calibration lines (run before and after the samples) is shown in Supplementary Table S3.

5.2.6. Gas Chromatography Coupled with Mass Spectroscopy (GC–MS)

Sample preparation, GC–MS analysis, and QA identification followed established protocols [23,48–50], specifically the procedure entitled A2 by the authors in [50]. In summary, the main steps are described below.

Lupin samples (15 g from each lupin species included in the project, see Table 1) were freeze-dried for 48 h (Freeze dryer LMC-2, Martin Christ, Osterode, Germany) and then ground to a fine powder using a FOSS grinder (FOSS CM-290 Cemotec, FOSS Ltd., Nils Foss Allé 1, DK-3400 Hilleroed, Denmark). The QA extraction method was developed in-house following the procedure described by Kushnareva et al. [50]. A total of 2 g of ground lupin seeds were weighed into a test tube, and 9 mL of a solvent mixture consisting of ethyl acetate and ammonia solution (NH₄OH, 25%), in an 8:1 volumetric ratio, was added to the ground lupin samples and left for 18 h in a refrigerator (4 °C). The extracts were filtered twice, once through a 0.45 µm membrane filter paper, followed by a 0.22 µm membrane filter, and directly injected into the GC–MS equipment.

GC–MS analyses were carried out using an Agilent 7890B gas chromatograph coupled to a 5977A mass spectrometer (electron multiplier potential 2 kV, filament current 0.35 mA, electron energy 70 eV), and the spectra were recorded over the range of m/z 40 to 500. An Agilent 7683 autosampler was used for the sample introduction. A 1 µL aliquot of each sample was injected into the GC–MS equipment using a 1:10 split-ratio injection mode. Helium was the carrier gas at a constant flow of 1.1 mL/s. An isothermal hold at 50 °C was maintained for 2 min, followed by a heating gradient of 6 °C/min to 300 °C, and the final temperature was held for 4 min. A 3-minute solvent delay was applied. A 30 m × 0.25 mm ID, 5% cross-linked phenylmethyl siloxane capillary column (HP-5MS) with a 0.25 µm coating thickness was used for separation, and the injection port temperature was 220 °C. The MS interface temperature was 280 °C.

Peaks were assigned with a spectral library (NIST 17.0) and compared with MS data obtained from injecting 12 analytical standards. For identification and relative quantification, 10 µL of the QA standard mixtures (5 µg/mL, corresponding to a level of 10 mg/kg) were injected into the GC–MS equipment, and their retention time and elution order were set. Identification of QAs present in LC–MS/MS chromatograms, for which no correlating standards were available for confirmation, was made based on cross-referencing with 2 NIST libraries (NIST2008, NIST2014) and verified for molecular ion mass and typical six dominant breakdown ions using the library search engine. In this way, the identity could be confirmed of 12 QAs in the LC–MS/MS measurements.

Ten lupin seeds for each of the lupin varieties included in the project (see Table 1) were freeze-dried for 24 h (Freeze dryer LMC-2, Martin Christ, Osterode, Germany), then milled to a fine powder using a FOSS grinder (FOSS CM-290 Cemotec, FOSS Ltd., Nils Foss Allé 1, DK-3400 Hilleroed, Denmark). A total of 50 mg of dried powder was added to 300 mL of distilled water to allow the extraction of the proteins. Protein hydrolysis was carried out using ETHOS EASY Advanced Microwave system with an HPR-3000/1 rotor [50]. Lysine content was determined by using 2,4-dinitrofluorobenzene (DNFB) for derivatization. The amino-acid-derived samples were separated on a reversed-phase C18 column (Luna® Omega 3 µm, 100 Å; 150 × 4.6 mm) using an Ultimate 3000 UHPLC (Thermo Fisher Scientific, 168 Third Avenue, Waltham, MA, USA, 02451) following the method by [51].

The boxplot comparison analysis and hierarchical clustering were performed using R software (version 4.0.2, Team 2020). To compare the total QA variable, the assumptions of normality and homogeneity of residual variance were first assessed by visually plotting sample quantiles against theoretical quantiles and residuals against fitted values, followed by performing Shapiro–Wilk and Levene’s tests. The variable for total QAs did not meet the above assumptions, even after applying square root and log transformations and, therefore, was treated as nonparametric. Due to the nonparametric nature of the data, a Kruskal–Wallis test followed by a Dunn post-hoc test was used to assess differences between species using the *kruskal.test* base function and *dunn.test* function of the *FSA* package [52]. Due to the absence of QAs in the *L. albus* sweet samples, these were removed from the analysis presented in Figure 3 to avoid statistical bias and, therefore, marked with an asterisk sign (*) instead of letters as with the other tested species.

The hierarchical clustering and production of heat maps for the different lupin species and QAs were carried out using the *as.dendrogram* function of the *pheatmap* package [53] after being scaled using the *scale* base function.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/toxins16030163/s1>. Table S1a. Analytical QA standards; Table S1b. QAs tentatively identified in the samples; Table S2. Validation results: recovery and repeatability ($n = 6$) for soybeans spiked at three concentration levels; Table S3. Linearity of calibration lines in blank soybean extract, 0–200 ng/mL, eight-point calibration, combined lines before and after analysis of the samples; Table S4. Quinolizidine alkaloids were detected in the lupin species and control plants when analyzed using both LC–MS/MS and GC–MS. Amounts are given in mg/kg of seed dry weight; Supplementary Figure S1A. Quinolizidine alkaloid structures are included in the analytical method; Supplementary Figure S1B. Additional quinolizidine alkaloids are included in the method; Supplementary Figure S2A–G: Representative LC–MS/MS MRM chromatograms of the six main lupin species analyzed. S2A. A mixture of QA reference standards (25 ng/mL in blank soy extract). S2B. *Lupinus pilosus* (PIL-18). S2C. *Lupinus palaestinus* (PA-02). S2D. *Lupinus albus* (ALB-05) S2E. *Lupinus angustifolius* (ANG-sh) S2F. *Lupinus luteus* (LUT-03). S2G. *Lupinus mutabilis* (MUT-01).

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References

1. Dyer, L.A.; Philbin, C.S.; Ochsenrider, K.M.; Richards, L.A.; Massad, T.J.; Smilanich, A.M.; Forister, M.L.; Parchman, T.L.; Galland, L.M.; Hurtado, P.J.; et al. Modern approaches to study plant-insect interactions in chemical ecology. *Nat. Rev. Chem.* **2018**, *2*, 50–64. [CrossRef]
2. Mithöfer, A.; Boland, W. Plant defense against herbivores: Chemical aspects. *Ann. Rev. Plant Biol.* **2012**, *63*, 431. [CrossRef]
3. Adamski, Z.; Bufo, S.A.; Milella, L.; Scrano, L. Identification and Functional Characterization of Plant Toxins. *Toxins* **2021**, *13*, 228. [CrossRef] [PubMed]
4. EFSA Panel on Contaminants in the Food Chain (CONTAM); Schrenk, D.; Bodin, L.; Chipman, J.K.; del Mazo, J.; Grasl-Kraupp, B.; Hogstrand, C.; Hoogenboom, L.(R.); Leblanc, J.; Nebbia, C.S.; et al. Scientific opinion on the risks for animal and human health related to the presence of quinolizidine alkaloids in feed and food, in particular in lupins and lupin-derived products. *EFSA J.* **2019**, *17*, e05860. [PubMed]
5. Michael, J.P. *The Alkaloids*; Knölker, H.-J., Ed.; Academic Press: London, UK, 2016; Volume 75, pp. 1–498.
6. Wink, M.; Witte, L. Cell-free synthesis of the alkaloids ammodendrine and smipine. *Z. Für Naturforschung C* **1987**, *42*, 197–204. [CrossRef]
7. Boschin, G.; Resta, D. Alkaloids Derived from Lysine: Quinolizidine (a Focus on Lupin Alkaloids). In *Natural Products*; Ramawat, K.G., Mérillon, J.-M., Eds.; Springer: Berlin/Heidelberg, Germany, 2013.
8. Frick, K.M.; Kamphuis, L.G.; Siddique, K.H.M.; Singh, K.B.; Foley, R.C. Quinolizidine alkaloid biosynthesis in lupins and prospects for grain quality improvement. *Front. Plant Sci.* **2017**, *8*, 87. [CrossRef] [PubMed]
9. Resta, D.; Boschin, G.; D’Agostina, A.; Arnoldi, A. Evaluation of total quinolizidine alkaloids content in lupin flours, lupin-based ingredients, and foods. *Mol. Nutr. Food Res.* **2008**, *52*, 490–495. [CrossRef]
10. Boschin, G.; Tesio, E.; Arnoldi, A. A field case of pig poisoning by accidental feed contamination by alkaloid-rich lupin seeds. *J. Appl. Anim. Res.* **2022**, *50*, 725–731. [CrossRef]
11. Yovo, K.; Huguet, F.; Pothier, J.; Durand, M.; Breteau, M.; Narcisse, G. Comparative Pharmacological Study of Sparteine and its Ketonic Derivative Lupanine from Seeds of *Lupinus albus*. *Planta Medica* **1984**, *50*, 420–424. [CrossRef]
12. Otterbach, S.L.; Yang, T.; Kato, L.; Janfelt, C.; Geu-Flores, F. Quinolizidine alkaloids are transported to seeds of bitter narrow-leaved lupin. *J. Exp. Bot.* **2019**, *70*, 5799–5808. [CrossRef]
13. Engel, A.M.; Engel, A.M.; Klevenhusen, F.; Klevenhusen, F.; Moenning, J.-L.; Moenning, J.-L.; Numata, J.; Numata, J.; Fischer-Tenhagen, C.; Fischer-Tenhagen, C.; et al. Investigations on the transfer of quinolizidine alkaloids from *Lupinus angustifolius* into the milk of dairy cows. *J. Agric. Food Chem.* **2022**, *70*, 11749–11758. [CrossRef]
14. Mancinotti, D.; Frick, K.M.; Geu-Flores, F. Biosynthesis of quinolizidine alkaloids in lupins: Mechanistic considerations and prospects for pathway elucidation. *Nat. Prod. Rep.* **2022**, *39*, 1423. [CrossRef]
15. Bundesinstitut für Risikobewertung. *Risk Assessment of the Occurrence of Alkaloids in Lupin Seeds*; BfR Opinion No 003/2017; 2017; Volume 1, pp. 1–35. Available online: <https://mobil.bfr.bund.de/cm/349/risk-assessment-of-the-occurrence-of-alkaloids-in-lupin-seeds.pdf> (accessed on 12 February 2024).
16. Wink, M.; Meißner, C.; Witte, L. Patterns of quinolizidine alkaloids in 56 species of the genus *Lupinus*. *Phytochemistry* **1995**, *38*, 139–153. [CrossRef]
17. Bunsupa, S.; Katayama, K.; Ikeura, E.; Oikawa, A.; Toyooka, K.; Saito, K.; Yamazaki, M. Lysine decarboxylase catalyzes the first step of quinolizidine alkaloid biosynthesis and coevolved with alkaloid production in leguminosae. *Front. Plant Sci.* **2012**, *3*, 239. [CrossRef]
18. Cely-Veloza, W.; Quiroga, D.; Coy-Barrera, E. Quinolizidine-based variations and antifungal activity of eight *Lupinus* species grown under greenhouse conditions. *Molecules* **2022**, *27*, 305. [CrossRef] [PubMed]
19. Shelef, O.; Weisberg, P.J.; Provenza, F.D. The value of native plants and local production in an era of global agriculture. *Front. Plant Sci.* **2017**, *8*, 2069. [CrossRef] [PubMed]

20. Hernández-López, I.; Ortiz-Solà, J.; Alamprese, C.; Barros, L.; Shelef, O.; Basheer, L.; Rivera, A.; Abadias, M.; Aguiló-Aguayo, I. Valorization of local legumes and nuts as key components of the mediterranean diet. *Foods* **2022**, *11*, 3858. [CrossRef] [PubMed]
21. Martiniello, P.; Teixeira da Silva, J.A. Physiological and bio-agronomical aspects involved in growth and yield components of cultivated forage species in Mediterranean environments: A review. *Eur. J. Plant Sci. Biotech.* **2011**, *5*, 64–98.
22. Guilengue, N.A. Genetic and Morphological Diversity of Germplasm Collection from *Lupinus mutabilis* Sweet Grown under Mediterranean Climate. Ph.D. Thesis, Universidade de Lisboa, Lisbon, Portugal, 2021.
23. Bermúdez-Torres, K.; Ferval, M.; Hernández-Sánchez, A.M.; Tei, A.; Gers, C.; Wink, M.; Legal, L. Molecular and chemical markers to illustrate the complex diversity of the genus *Lupinus* (Fabaceae). *Diversity* **2021**, *13*, 263. [CrossRef]
24. Romeo, F.V.; Fabroni, S.; Ballistreri, G.; Muccilli, S.; Spina, A.; Rapisarda, P. Characterization and antimicrobial activity of alkaloid extracts from seeds of different genotypes of *Lupinus* spp. *Sustainability* **2018**, *10*, 788. [CrossRef]
25. Świącicki, W.; Czepiel, K.; Wilczura, P.; Barzyk, P.; Kaczmarek, Z.; Kroc, M. Chromatographic Fingerprinting of the Old World Lupins Seed Alkaloids: A Supplemental Tool in Species Discrimination. *Plants* **2019**, *8*, 548. [CrossRef]
26. Heisteringer, A.; Pistrick, K. 'Altreier Kaffee': *Lupinus pilosus* L. cultivated as coffee substitute in northern Italy (Alto Adige/Südtirol). *Genet. Resour. Crop Evol.* **2007**, *54*, 1623–1630. [CrossRef]
27. Carvajal-Larenas, F.E.; Linnemann, A.R.; Nout, M.J.R.; Koziol, M.; van Boekel, M.A.J.S. *Lupinus mutabilis*: Composition, uses, toxicology, and debittering. *Crit. Rev. Food Sci. Nutr.* **2016**, *56*, 1454–1487. [CrossRef]
28. Kamel, K.A.; Świącicki, W.; Kaczmarek, Z.; Barzyk, P. Quantitative and qualitative content of alkaloids in seeds of a narrow-leaved lupin (*Lupinus angustifolius* L.) collection. *Genet. Resour. Crop Evol.* **2016**, *63*, 711–719. [CrossRef]
29. Kroc, M.; Rybiński, W.; Wilczura, P.; Kamel, K.; Kaczmarek, Z.; Barzyk, P.; Świącicki, W. Quantitative and qualitative analysis of alkaloids composition in the seeds of a white lupin (*Lupinus albus* L.) collection. *Genet. Resour. Crop Evol.* **2017**, *64*, 1853–1860. [CrossRef]
30. Eugelio, F.; Palmieri, S.; Fanti, F.; Messuri, L.; Pepe, A.; Compagnone, D.; Sergi, M. Development of an HPLC-MS/MS Method for the Determination of Alkaloids in Lupins. *Molecules* **2023**, *28*, 1531. [CrossRef]
31. El-Shazly, A.; Ateya, A.M.M.; Wink, M. Quinolizidine Alkaloid Profiles of *Lupinus varius orientalis*, *L. albus albus*, *L. hartwegii*, and *L. densiflorus*. *Z. Für Naturforschung C* **2001**, *56*, 21–30. [CrossRef]
32. Magalhães, S.C.; Fernandes, F.; Cabrita, A.R.; Fonseca, A.J.; Valentão, P.; Andrade, P.B. Alkaloids in the valorization of European *Lupinus* spp. seeds crop. *Ind. Crop. Prod.* **2017**, *95*, 286–295. [CrossRef]
33. Ainouche, A.; Greinwald, R.; Witte, L.; Huon, A. Seed alkaloid composition of *Lupinus tassilicus* Maire (Fabaceae: Genisteae) and comparison with its related rough seeded lupin species. *Biochem. Syst. Ecol.* **1996**, *24*, 405–414. [CrossRef]
34. Thomas, A.F.; Vipond, H.J.; Marion, L. The papilionaceous alkaloids: Xxi. The alkaloids of *Lupinus pilosus* walt. And the structure of tetralupine. *Can. J. Chem.* **1955**, *33*, 1290–1294. [CrossRef]
35. Zhang, J.; Liu, Y.Q.; Fang, J. The biological activities of quinolizidine alkaloids. In *The Alkaloids: Chemistry and Biology*; Academic Press: Cambridge, MA, USA, 2023; Volume 89, pp. 1–37.
36. Vishnyakova, M.A.; Kushnareva, A.V.; Shelenga, T.V.; Egorova, G.P. Alkaloids of narrow-leaved lupine as a factor determining alternative ways of the crop's utilization and breeding. *Vavilov J. Genet. Breed.* **2020**, *24*, 625–635. [CrossRef]
37. Gavilan, J.; Mennickent, D.; Ramirez-Molina, O.; Triviño, S.; Perez, C.; Silva-Grecchi, T.; Godoy, P.A.; Becerra, J.; Aguayo, L.G.; Moraga-Cid, G.; et al. 17 Oxo Sparteine and Lupanine, Obtained from *Cytisus scoparius*, Exert a Neuroprotection against Soluble Oligomers of Amyloid- β Toxicity by Nicotinic Acetylcholine Receptors. *J. Alzh. Dis.* **2019**, *67*, 343. [CrossRef]
38. Lu, A.; Wang, T.; Hui, H.; Wei, X.; Chui, W.; Zhou, C.; Li, H.; Wang, Z.; Guo, J.; Ma, D.; et al. Natural products for drug discovery: Discovery of gramines as novel agents against a plant virus. *J. Agric. Food Chem.* **2019**, *67*, 2148. [CrossRef] [PubMed]
39. Mironets, R.V.; Garazd, Y.L.; Garazd, M.M. Synthesis of Conjugates of the Alkaloids Cytisine and Lupinine. *Chem. Nat. Compd.* **2019**, *55*, 1115. [CrossRef]
40. Tsutsumi, T.; Karanjit, S.; Nakayama, A.; Namba, K. A concise asymmetric total synthesis of (+)-Epilupinine. *Org. Lett.* **2019**, *21*, 2620. [CrossRef]
41. Xue, S.; Xu, H.; Sun, Z.; Shen, H.; Chen, S.; Ouyang, J.; Zhou, Q.; Hu, X.; Cui, H. Depletion of TRDMT1 affects 5-methylcytosine modification of mRNA and inhibits HEK293 cell proliferation and migration. *Biochemic. Biophysic. Res. Commun.* **2019**, *520*, 60. [CrossRef]
42. Lehner, Z.; Stadlbauer, K.; Brunmair, B.; Adorjan, I.; Genov, M.; Kautzky-Willer, A.; Scherer, T.; Scheinin, M.; Bauer, L.; Fürnsinn, C. Evidence that the multiflorine-derived substituted quinazolidine 55P0251 augments insulin secretion and lowers blood glucose via antagonism at α 2-adrenoceptors in mice. *Diabetes Obes. Metab.* **2020**, *22*, 290. [CrossRef] [PubMed]
43. Lucio FN, M.; da Silva, J.E.; Marinho, E.M.; Mendes FR, D.S.; Marinho, M.M.; Marinho, E.S. Methylcytisine alcaloid potentially active against dengue virus: A molecular docking study and electronic structural characterization. *Int. J. Res. Gran.* **2020**, *8*, 221. [CrossRef]
44. Mazumder, K.; Nabila, A.; Aktar, A.; Farahnaky, A. Bioactive variability and in vitro and in vivo antioxidant activity of unprocessed and processed flour of nine cultivars of Australian lupin species: A comprehensive substantiation. *Antioxidants* **2020**, *9*, 282. [CrossRef]
45. Nurmaganbetov, Z.S.; Savelyev, V.A.; Gatilov, Y.V.; Nurkenov, O.A.; Seidakhmetova, R.B.; Shulgau, Z.T.; Mukusheva, G.K.; Fazylov, S.D.; Shults, E.E. Synthesis and analgesic activity of 1-[(1, 2, 3-triazol-1-yl) methyl] quinolizines based on the alkaloid lupinine. *Chem. Heterocycl. Compd.* **2021**, *57*, 977. [CrossRef]

46. Lu, X.; Lu, F.; Yu, J.; Xue, X.; Jiang, H.; Jiang, L.; Yang, Y. Gramine promotes functional recovery after spinal cord injury via ameliorating microglia activation. *J. Cell. Mol. Med.* **2021**, *25*, 7980. [CrossRef]
47. Petruczynik, A.; Wróblewski, K.; Misiurek, J.; Plech, T.; Szalast, K.; Wojtanowski, K.; Mroczek, T.; Szymczak, G.; Waksmundzka-Hajnos, M.; Tutka, P. Determination of Cytisine and N-Methylcytisine from Selected Plant Extracts by High-Performance Liquid Chromatography and Comparison of Their Cytotoxic Activity. *Toxins* **2020**, *12*, 557. [CrossRef] [PubMed]
48. Aly, S.H.; Elissawy, A.M.; Eldahshan, O.A.; Elshanawany, M.A.; Singab, A.N.B. Variability of the Chemical Composition of the Essential Oils of Flowers and the Alkaloid Contents of Leaves of *Sophora secundiflora* and *Sophora tomentosa*. *J. Essent. Oil Bear. Plants* **2020**, *23*, 442–452. [CrossRef]
49. Kushnareva, A.V.; Shelenga, V.; Perchuk, I.N.; Egorova, G.P.; Malyshev, L.L.; Kerv, Y.A.; Shavarda, A.L.; Vishnyakova, M.A. Selection of an optimal method for screening the collection of narrow-leaved lupine held by the Vavilov Institute for the qualitative and quantitative composition of seed alkaloids. *Vavilov J. Genet. Breed.* **2020**, *24*, 829. [CrossRef] [PubMed]
50. Reichelt, M.; Hummert, C.; Luckas, B. Hydrolysis of microcystins and nodularin by microwave radiation. *Chromatographia* **1999**, *49*, 671–677. [CrossRef]
51. Li, N.; Liu, Y.; Zhao, Y.; Zheng, X.; Lu, J.; Liang, Y. Simultaneous HPLC determination of amino acids in tea infusion coupled to pre-column derivatization with 2, 4-dinitrofluorobenzene. *Food Anal. Met.* **2016**, *9*, 1307–1314. [CrossRef]
52. Ogle, D.H.; Doll, J.C.; Wheeler, P.; Dinno, A. FSA: Fisheries Stock Analysis. R Package. 2021. Available online: <https://CRAN.R-project.org/package=FSA> (accessed on 12 February 2024).
53. Kolde, R. *Pheatmap: Pretty Heatmaps*. R Package Version 1.0. 12. 2019. 2019. Available online: <https://rdrr.io/cran/pheatmap/> (accessed on 12 February 2024).

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Article

Magnetic Molecularly Imprinted Polymers for Selective Extraction of Aflatoxins from Feeds

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Abstract: Magnetic molecularly imprinted polymers (MMIPs) have fused molecular imprinting technology with magnetic separation technology, emerging as an innovative material capable of recognizing specific molecules and efficiently separating target substances. Their application to the extraction and purification of mycotoxins has great potential, due to the toxicity and economic impact of these contaminants. In this work, MMIP has been proposed as a sample treatment for the determination of main four aflatoxins (B_1 , B_2 , G_1 and G_2) in pig feed. The MMIP was formed through the integration of magnetic material (Fe_3O_4) with commercial molecularly imprinted polymers, avoiding the synthesis step and, therefore, simplifying the process. The analyses were carried out by high-performance liquid chromatography with fluorescence detection and the method was validated and limits of quantification (LOQs) between 0.09 and 0.47 ng/g were obtained, below the allowed or recommended levels by the European Union. Repeatability and intermediate precision showed relative standard deviations lower than 10% in all cases and trueness ranged from 92 to 111%. Finally, the proposed method was applied to 31 real pig feed samples, detecting aflatoxins with concentrations between 0.2 and 3.2 ng/g.

Keywords: aflatoxins; feeds; magnetic molecularly imprinted polymers; magnetic separation technology; HPLC-FLD

Key Contribution: Magnetic molecularly imprinted polymers (MMIPs) has been proposed as a sample treatment for the determination of main four aflatoxins in pig feed.

1. Introduction

Magnetic molecularly imprinted polymers (MMIPs) are formed through the integration of inorganic nanoparticles with molecularly imprinted polymers (MIPs). These MMIPs are developed in a core-shell configuration, where nanoparticles constitute the core, while selective polymeric layers serve as the shell enveloping the core surface. Essentially, MMIPs embody a synthesis of the optimal features from both inorganic nanoparticles and MIPs. The specificity of cavities imprinted on the MIP combines with the superparamagnetic attributes of nano-magnetite, which facilitates the extraction process of various analytes in complex matrices [1].

Recently, MMIPs have attracted wide attention since possess several promising characteristics in comparison with other media used for separation and adsorption [2]. These sorbents offer extensive surface areas, excellent biocompatibility, easy handling and straightforward customization. The inherent magnetic responsiveness of MMIPs establishes them as a potent and effective approach for streamlining the pre-concentration, separation and manipulation of analytes, particularly in large-scale operations. Because they can be effortlessly isolated from the sample matrix using an external magnetic field, the need for high-speed centrifugation and additional filtration is obviated when employing MMIPs

as affinity adsorbents [3]. Some key applications include environmental contaminant extraction from soils and water, analysis of food contaminants, sample preparation in clinical diagnostics, water quality monitoring, detection of drugs in biological samples and forensic analysis of illicit substances. MMIPs enhance the efficiency and selectivity of analytical processes in various fields, showcasing their versatility in addressing complex sample matrices and the selective extraction of important contaminants such as aflatoxins (AFs).

AFs are toxic secondary metabolites produced by some species of *Aspergillus fungi*, especially *A. flavus* and *A. parasiticus*. They can be produced before, but especially after, harvest and can contaminate a variety of crops, including cereals, nuts, spices as well as feed [4]. Within AFs, about 30 compounds have been identified, the main ones being AFs B₁, B₂, G₁ and G₂. These mycotoxins are thermostable compounds that can persist during the milling, washing and processing of foodstuffs so that their content is not reduced in the final products.

AFs are recognized as genotoxic carcinogens by the Scientific Committee on Food [5]. Their descending order of toxicity is as follows: AFB₁ > AFG₁ > AFB₂ > AFG₂. Notably, AFB₁ has been established as carcinogenic in all experimental animals and has held the classification as a human carcinogen by the World Health Organization (WHO) since 1988. As a consequence, the International Agency for Research on Cancer (IARC) has categorized AFB₁ as a Group 1 substance, indicating substantial evidence of its carcinogenicity in humans [6].

Contamination by AFs can affect animal and human health and cause various adverse effects. In the case of animal feed, only AFB₁ is regulated by European legislation, the maximum content fixed is 20 ng/g for pig, poultry and ruminant feed, 10 ng/g for calves and lambs and, for milk-producing animals and young animals, the limit is set at 5 ng/g [7]. In addition, codes of good hygienic practice are also established in the production and storage chain in order to achieve a reduction in the exposure of the population.

Compound feeds usually contain a variety of ingredients, such as grains, feed by-products, minerals and additives. The diversity of components can complicate the identification and quantification of AFs during the analysis, due to the high number of interferents implying a significant matrix effect. In addition, the composition of feed can vary considerably depending on the brand, batch and geographic region. This variability makes it difficult to establish standardized analysis methods and interpret the results. Therefore, very selective extraction methods are required where MMIPs could have great potential.

A recent review summarizes studies that applied advanced MMIPs for the analysis of mycotoxins in food [2]. This novel sample treatment has been applied for the extraction of AFs [8,9] and other mycotoxins, such as ochratoxins [10], sterigmatocystin [11], patulin [12,13] and zearalenone [14–18], mainly in cereals (wheat, corn, rice, millet and coix lacryma-jobi), but also in wine, soy sauce, vinegar or juice.

The most frequently used magnetic material is Fe₃O₄ [12,14,16,17,19,20], although applications have also been described magnetically modified hydroxyapatite (Fe₃O₄-HAP) [18] or the use of Fe₃O₄ as the magnetic core with the addition of chitosan (CS) and SiO₂, to improve the biocompatibility, stability and dispersibility of the MIP adsorbent and graphene oxide (GO), since is an excellent supporting material for synthesizing MIP (Fe₃O₄@SiO₂@CS-GO) [15]. Magnetic halloysite nanotubes [13], magnetic nanofibrous and porous carbon microsphere [10], or a magnetic core of Co₃O₄ with nanoporous carbon [11], have also been proposed. Regarding the template used for the generation of MIPs, 5,7-dimethoxycoumarin or ethyl 3-coumarincarboxylate have been used for the aflatoxin-specific MMIPs.

In this work, the potential of MMIPs for the extraction and preconcentration of AFs in feed samples continues to be explored. To the best of our knowledge, this is the first time that these sorbents have been applied for the determination of mycotoxins in these complex matrices.

2. Results and Discussion

2.1. Optimization of Sample Treatment

Although the use of MMIPs has multiple advantages over other sample treatment methods, the synthesis of MIPs can complicate the procedure, and sometimes the use of expensive templates is required to achieve effective recognition of the analyte molecule. Therefore, in this study, the use of commercial MIPs has been chosen, although it has the disadvantage that its composition and the template used for its preparation are not known. The MIPs utilized are commercially available as solid phase extraction (SPE) cartridges. The proposal involves magnetizing these MIPs and utilizing them in a procedure based on magnetic dispersive micro-solid phase extraction, which offers several practical advantages over traditional SPE, including faster extraction times, reduced sample volumes and improved efficiency in handling complex sample matrices. The preparation procedure for the MMIPs is described in Section 4.3.

To verify the effectiveness of MMIPs to extract and preconcentrate AFs, they were applied and compared with the direct use of the magnetic material Fe_3O_4 without MIPs and the use of the commercial MIPs using the same procedure but, in this last case, introducing a centrifugation process for the isolation of the MIPs. For this purpose, a pig feed sample free of AFs was used, which was spiked at a concentration of 100 ng/g. The results are shown in Figure 1. As can be seen, in addition to their multiple advantages, MMIPs considerably improve the efficiency of extraction. MMIPs exhibit the selectivity of MIPs and as they are prepared by the polymerization of functional monomers in the presence of a molecular template and when the template is removed, MIPs have specific binding sites that confer molecular recognition to the polymer, having therefore a high affinity to aflatoxins than the magnetic material.

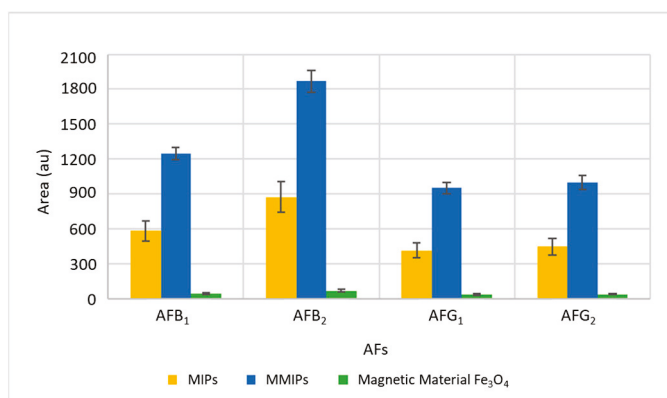


Figure 1. Comparison of the extraction efficiency of MMIPs, magnetic material without MIPs coating and MIPs. The bars show the area obtained for each AF in each experiment and error bars indicate the maximum and minimum of the replicas.

The binding of MIPs to magnetic particles facilitates the isolation and recovery of the analytes of interest with respect to the rest of the analytes present in the matrix using a magnetic field, which can be a useful tool for the analysis of complex samples. Magnetic property reduces extraction steps and therefore prevents sorbent losses improving recovery compared to using only MIPs.

In order to optimize the procedure based on magnetic dispersive micro-solid phase extraction, both adsorption and desorption efficiency were evaluated, and the amount of mass of MMIPs, sample amount, the addition of salt to promote extraction, adsorption and desorption times and nature and volume of desorption solvent were investigated. The optimal values were selected based on the highest extraction recovery, i.e., the largest area obtained for the four AFs.

The optimization of the parameters associated with the adsorption (amount of MMIPs, adsorption time and salt addition) was carried out using a multivariate study because

these parameters are closely related. A central composite design (2^3 + star, face centered), with three spaced central points, involving 17 runs, was used as an approach to generate the response surface, using the area as an analytical response. The different factors were studied in the following ranges: amount of MMIPs (10–50 mg); adsorption time (1–20 min); and percentage of NaCl (0–10% *m/v*). The correlation coefficients R^2 were in the range of 70.7 to 93.5%, which proves the suitability of the experiment. According to the Pareto diagrams, all variables were significant for all or some of the mycotoxins: the adsorption time significantly influenced the extraction of four AFs, while salt content and mass MMIPs were significant in the extraction of AFB₁ and AFB₂, and AFB₁ and AFG₁, respectively.

The desirability function (Figure 2) was used for multiple response optimization resulting in the following optimum values: 10 mg of MMIPs, an adsorption time of 10 min and 0% of NaCl.

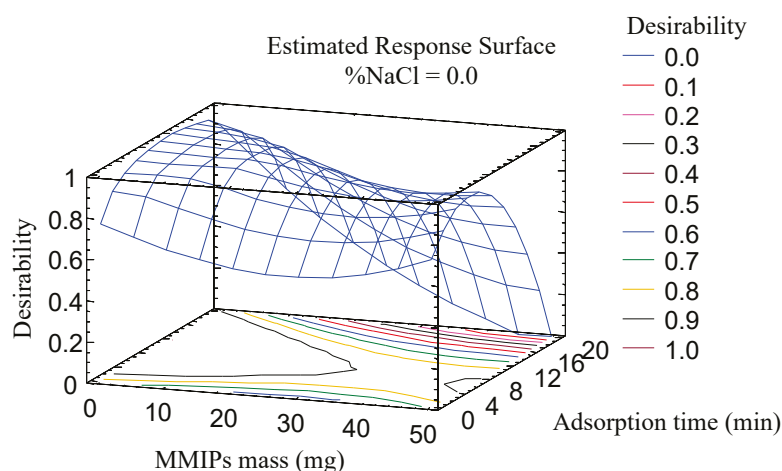


Figure 2. Estimated response surface for the study of significant variables using MMIPs by multiple response optimization. This optimization was based on a central composite design (2^3 + star, face centered), with three spaced central points, involving 17 runs, which was used as an approach to generate the response surface, using the area as an analytical response.

To optimize the desorption stage, the nature of the desorption solvent was initially investigated, and the following solvents were studied: acetonitrile (ACN), methanol (MeOH), ethyl acetate, ethanol (EtOH), acetone and methyl isobutyl ketone. All solvents gave rise to similar desorption efficiencies, and ethyl acetate was therefore selected as optimal since it allowed a subsequent rapid drying step, allowing reconstitution in a smaller volume, thereby improving the preconcentration factor of the sample treatment process.

Subsequently, the volume of ethyl acetate was studied in the range between 1 and 4 mL. The results are shown in Figure 3, where it can be seen that the intensity of the signals increased with the desorption volume up to 3 mL; from this volume, the signal was practically maintained, so larger volumes will not improve the extraction efficiency and 3 mL was chosen as optimal. The desorption time was also studied between 2 and 15 min, selecting 10 min as optimal because no significant differences were found using longer times.

Drying the desorption solvent and reconstituting it in a smaller volume would improve the preconcentration factor, although it would also increase the matrix effect. To evaluate this process, reconstitution volumes between 250 and 500 μ L using a mixture of MeOH/water (50:50, *v/v*) were studied. A volume of 250 μ L was selected for further experiments, the minimum that allows good filtering and handling of the extract. Finally, and again looking for better preconcentration factors, the sample quantity was investigated between 0.5 and 2 g. It was found that increasing the mass, the areas of the AFs increased; however, above 1.5 g, interferences began to be observed in the chromatogram that worsened the selectivity of the method, so this amount was selected as optimum. A typical

chromatogram corresponding to a spiked pig feed sample submitted to the proposed method is shown in Figure 4.

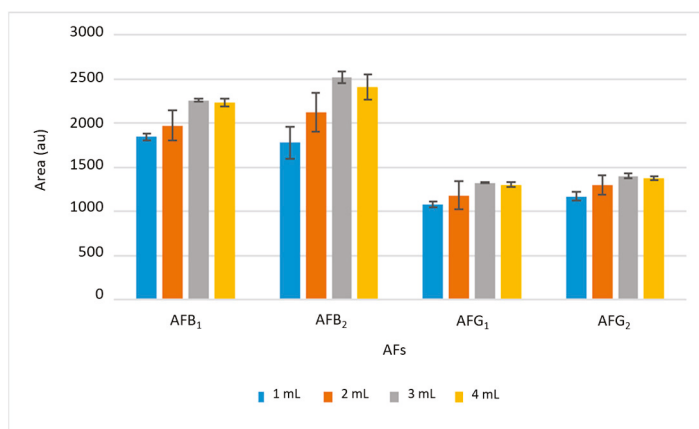


Figure 3. Optimization of desorption solvent volume. The bars show the area obtained for each AF in each experiment and error bars indicate the maximum and minimum of the replicas.

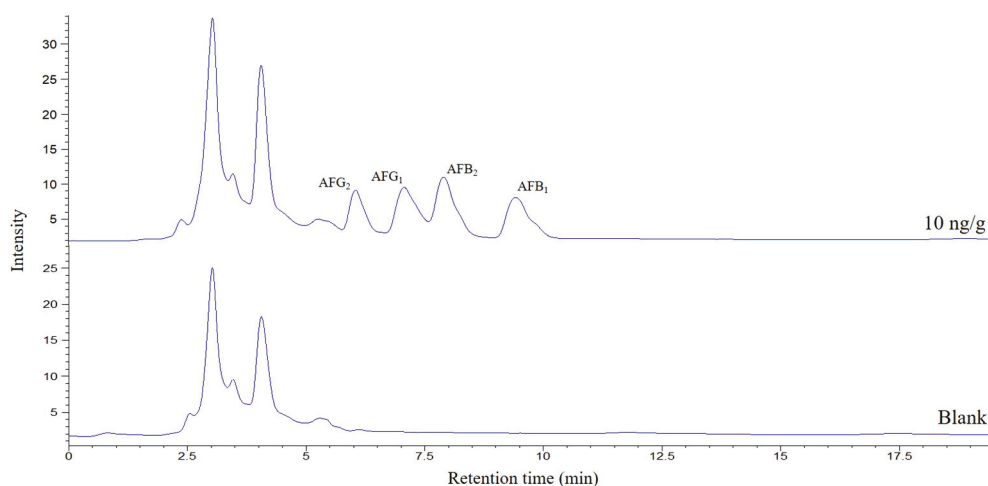


Figure 4. Chromatograms obtained from MMIPs isolation and HPLC-FLD analysis under the finally selected conditions of a pig feed sample spiked at 10 ng/g and a blank sample.

2.2. Validation of the Analytical Method

To validate the method, linear dynamic ranges, the limit of detection (LOD) and quantification (LOQ), precision and trueness were evaluated. For this, blank samples were used (previously analyzed to establish the absence of detectable AFs) that were spiked with the different analytes. Table 1 shows the results of this validation.

To assess the linearity of the method, blank samples were spiked with the different analytes. In total, six different concentration levels between 0.5 and 100 ng/g were included, and the analytical procedure was performed in duplicate at each concentration. Satisfactory determination coefficients ($R^2 > 0.99$) were obtained, confirming that all analytical responses were linear over the studied ranges. The LODs and LOQs were determined as the minimum concentration of AFs in the spiked blank samples with a signal-to-noise ratio of 3 and 10, respectively. In this case, LOD varied between 0.03 and 0.14 ng/g, and the LOQ was between 0.09 and 0.47. The proposed methodology allows obtaining LOQs lower or similar to those described for the determination of AFs in feed using HPLC-FLD [19–21].

The precision of the method was studied in terms of repeatability on the same day and between days. For this purpose, spiked feed samples were analyzed at three different concentration levels (0.5, 10 and 50 ng/g). Relative standard deviations (RSD) were lower than 10%.

Table 1. Method characterization data for AFs determination in feeds.

AFs	Equation (x, ng/g)		R ²	LOD (ng/g)	LOQ (ng/g)	
AFB ₁	y = 19.28x − 0.2953		0.9999	0.0711	0.237	
AFB ₂	y = 23.45x + 2.291		0.9993	0.0594	0.198	
AFG ₁	y = 13.16x + 22.96		0.9949	0.0273	0.091	
AFG ₂	y = 12.34x − 1.317		0.9993	0.1425	0.475	
Intra-day precision, RSD (%) (n = 9)			Inter-day precision, RSD (%) (n = 9)			
	0.5 ng/g	10 ng/g	50 ng/g	0.5 ng/g	10 ng/g	50 ng/g
AFB ₁	3.5	4.1	3.1	9.6	4.4	4.5
AFB ₂	2.4	4.4	5.2	8.3	4.9	5.4
AFG ₁	7.2	9.3	3.3	8.7	9.7	4.6
AFG ₂	6.9	3.7	3.8	7.3	9.0	4.6
Trueness (RSD, %)						
	0.5 ng/g		10 ng/g		50 ng/g	
AFB ₁	102.7 (4.0)		100.1 (1.3)		110.1 (2.4)	
AFB ₂	110.9 (4.7)		105.5 (1.7)		103.8 (3.2)	
AFG ₁	107.0 (3.9)		107.0 (5.7)		110.4 (2.8)	
AFG ₂	91.7 (5.0)		96.7 (4.8)		99.8 (2.7)	

Finally, the trueness of the method was studied using recovery studies and the analysis of a reference material. For the recovery study, fortification of blank samples was carried out at three concentration levels (0.5, 10 and 50 ng/g). The observed signal was plotted against the actual concentration. The measured concentration was determined using the obtained calibration curves, and the recovery was calculated as $100 \times \text{measured concentration} / \text{actual (added) concentration}$. Recoveries were between 91.7 and 110.9% in all the cases, fulfilling the current legislation [22].

As previously indicated, the method was also validated using certified reference material for AFs in feed, which was cereal-based and supplied in a finely milled form. The proposed method was applied for the analysis of this reference material and the results are shown in Table 2. In addition, to ensure the suitability of the method, the certified material was also analyzed using the same sample treatment and ultra-high-performance liquid chromatography with high-resolution mass spectrometry (UHPLC-HRMS) analysis to ensure the identification of the compounds. A total of three experimental replicates were analyzed. The results were compared using the one-way repeated measures analysis of variance test. No significant differences were found with a *p*-value greater than 0.05 (*p* = 0.1978), which demonstrates the validity of the developed method.

Table 2. Analysis of the reference material by UHPLC-HRMS and HPLC-FLD (concentration expressed in ng/g).

AFs	Certified Concentration *	Concentration Obtained by HPLC-FLD	Concentration Obtained by UHPLC-HRMS
AFB ₁	12.9 ± 1.8	13.8 ± 0.6	14.6 ± 0.7
AFB ₂	0.68 ± 0.1	0.58 ± 0.08	0.73 ± 0.09
AFG ₁	5.2 ± 0.8	5.6 ± 0.3	5.8 ± 0.3
AFG ₂	----	ND	ND

* The certified reference material included only aflatoxins B1, B2, G1 and G2. ND: not detected.

2.3. Analysis of Feed Samples

The validated method was applied for the analysis of 31 real pig feed samples, which were analyzed in duplicate and the obtained mean value and standard deviation are shown in Table 3.

Table 3. Analysis of real samples with the proposed methodology (concentration expressed in ng/g).

Sample	AFB ₁	AFB ₂	AFG ₁	AFG ₂
M1	0.43 ± 0.05	<LOQ	ND	<LOQ
M2	0.24 ± 0.08	0.20 ± 0.04	ND	<LOQ
M3	<LOQ	ND	ND	<LOQ
M4	0.53 ± 0.04	<LOQ	ND	ND
M5	0.278 ± 0.001	<LOQ	ND	<LOQ
M6	0.25 ± 0.01	<LOQ	ND	ND
M7	0.75 ± 0.02	0.358 ± 0.005	ND	<LOQ
M8	3.2 ± 0.1	0.85 ± 0.06	ND	<LOQ
M9	0.58 ± 0.08	0.3 ± 0.1	ND	ND
M10	<LOQ	1.14 ± 0.08	ND	ND
M11	ND	0.69 ± 0.02	ND	<LOQ
M12	0.58 ± 0.02	0.69 ± 0.06	ND	<LOQ
M13	0.727 ± 0.009	0.9 ± 0.1	ND	ND
M14	0.68 ± 0.03	0.221 ± 0.006	ND	<LOQ
M15	0.62 ± 0.09	<LOQ	ND	<LOQ
M16	0.59 ± 0.09	<LOQ	ND	<LOQ
M17	0.46 ± 0.01	0.301 ± 0.002	ND	<LOQ
M18	<LOQ	1.2 ± 0.1	ND	0.52 ± 0.03
M19	0.313 ± 0.002	<LOQ	ND	ND
M20	<LOQ	0.82 ± 0.07	ND	<LOQ
M21	0.28 ± 0.05	<LOQ	ND	<LOQ
M22	1.8 ± 0.1	1.30 ± 0.02	ND	<LOQ
M23	0.7 ± 0.1	0.74 ± 0.04	ND	<LOQ
M24	<LOQ	0.63 ± 0.01	ND	0.78 ± 0.02
M25	<LOQ	0.86 ± 0.09	ND	<LOQ
M26	<LOQ	0.270 ± 0.003	ND	<LOQ
M27	<LOQ	0.6 ± 0.1	ND	<LOQ
M28	<LOQ	<LOQ	ND	<LOQ
M29	0.24 ± 0.04	<LOQ	ND	<LOQ
M30	0.29 ± 0.02	<LOQ	ND	<LOQ
M31	0.41 ± 0.03	<LOQ	ND	<LOQ

ND: not detected; <LOQ: value between the LOD and the LOQ.

AFB₁ and AFB₂ were present in 96.8% of the samples and AFG₂ in 80.6%. In contrast, none of the samples showed concentrations above the LOD for AFG₁. AFB₁ is the one with the highest concentrations ranging between 0.24 and 3.2 ng/g, followed by AFB₂ ranging between 0.2 and 1.3 ng/g and, finally, AFG₂ with values below 0.78 ng/g. Compared to the legislated levels, it is found that all samples comply with the requirements for maximum AFB₁ content [8]. In addition, in all samples, more than one AF was detected. Specifically, 26% of the analyzed samples present cooccurrence of 2 AFs, and 74% of samples presented 3 of the studied AFs.

3. Conclusions

Magnetic separation technology based on the use of MMIPs has shown to have great potential to determine AFs in feed, a matrix that today represents a great challenge due to the variety of its ingredients and therefore its high matrix effect. The proposed MMIPs have allowed the extraction and purification of AFs in an excellent manner, allowing LOQs below the current limits established in the legislation. The union of the MIPs to the magnetic material provides a simplification of the analysis method, avoiding the use of SPE, reducing volumes of solvent and fulfilling the principles of Green Analytical Chemistry. In addition, the use of already commercial MIPs also helps to simplify sample treatment because the synthesis process is also avoided, which allows for reducing uncertainty and obtaining a very exact (truthful and precise) method. The application of the method for the analysis of 31 real pig feed samples has shown that in all samples more than one AF was detected,

AFB₁ was the one with the highest concentrations ranging between 0.24 and 3.2 ng/g, but in all cases, the limits established by legislation were not exceeded.

4. Materials and Methods

4.1. Chemicals and Reagents

MeOH, ACN, ethyl acetate, acetone, EtOH and methyl isobutyl ketone were obtained from Sigma Aldrich (St. Louis, MO, USA). Commercial Affinisep MIPs (selective SPE cartridges format 6 mL-200 mg for AFs, Ochratoxin A, HT-2, T-2, Fumonisin, Zearalenone and Deoxynivalenol), Mohr's salt, iron trichloride hexahydrate and ammonium hydroxide were also supplied by Sigma-Aldrich and used to prepare the MMIPs. A certified reference material (ERM-BE376 compound feedingstuff) was obtained from Sigma-Aldrich and was used for method validation.

The water used for the analyses was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

Individual standards of AFs (AFB₁, AFB₂, AFG₁ and AFG₂) at 1 mg (98% purity) were obtained from Sigma-Aldrich. Solutions of each AF at 1000 µg/mL in ACN were prepared and stored at −20 °C. Intermediate dilutions of the AFs mixture were prepared weekly at 10 µg/mL in ACN.

4.2. Instrumentation and Software

The instrument used was an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany), equipped with a quaternary pump (G1311A) and coupled to two detectors, one diode array (G4212B) and one fluorescence detector (G1321A). The HPLC and detectors are connected to a UVE photochemical reactor (LCTech, Obertaufkirchen, Germany). The chromatographic column used was an ACE Excel 3 C18 (15 cm × 4.6 mm, 3 µm), made of high-purity silica by Advanced Chromatography Technologies (Aberdeen, Scotland).

For UHPLC-HRMS analysis, an Agilent 1290 Infinity II Series HPLC (Agilent Technologies, Santa Clara, CA, USA) with a high-speed binary pump coupled to an Agilent 6550 QTOF mass spectrometer and an Agilent dual jet stream electrospray ionization source (AJS-Dual ESI) was used. Agilent Technologies MassHunter software (Version B.07.00, Santa Clara, CA, USA) was used to obtain the data.

For sample treatment, an IKA-KS-130 Basic Orbital Shaker (Staufen, Germany) and a Horizon Technology Xcelvap air-drying system (Salem, MA, USA) were used. The permanent magnets used were blocks made of Nd-Fe-B with a strength of 33 kg, a weight of 86 g and dimensions of 50 × 15 × 15 mm obtained from Supermagnete (Gottmadingen, Germany). For sample filtration, 0.22 µm × 25 mm nylon syringe filters obtained from Agela Technologies (New York, NY, USA) were used.

The statistical tools used for data processing were Microsoft Office Excel version 16.82 (Microsoft, Redmond, WA, USA), SigmaPlot 13.1 (Systat, Software Inc., San Jose, CA, USA) and Statgraphics (Statistical Graphics, Rockville, MD, USA).

4.3. Preparation of MMIP

To obtain the MMIPs, 0.85 g of Mohr's salt and 0.42 g of iron trichloride hexahydrate were weighed and dissolved in 250 mL of Milli-Q water. Then, 0.5 g of commercial MIPs were added and heated at 50 °C for 20 min in an ultrasonic bath. After this time, 25 mL of 8 M ammonium hydroxide was added dropwise, keeping the temperature at 50 °C. The mixture was kept in the bath without ultrasounds at 50 °C for another 30 min. Finally, the MMIPs were collected with the help of a magnet and washed twice with Milli-Q water and twice with EtOH. They were left to dry at 70 °C and, once dry, were stored in glass vials at room temperature until further use.

4.4. Samples

A total of 31 pig feed samples were analyzed, all from the region of Murcia (Spain). In addition, one pig feed sample free of AFs was used for the optimization and validation of the method. All samples were stored at room temperature until analysis.

4.5. Sample Treatment

The treatment consisted of weighing 1.5 g of feed sample into a 15 mL centrifuge tube, adding 5 mL of Milli-Q water and then shaking for 20 s. Then, 10 mg of the MMIPs were added. The mixture was orbitally shaken for 10 min, the MMIPs were removed with the help of a magnet and the supernatant was decanted. For desorption of the analytes, 3 mL of ethyl acetate was added and orbitally shaken again for 10 min. After this time, with the help of an external magnet, the MMIPs were also removed and the supernatant solution was collected, placed in a test tube and dried completely with the help of an air stream at 35 °C. After drying, the sample was reconstituted by adding 250 µL of a MeOH: water mixture (50:50, *v/v*). Finally, the extract was filtered through a 0.22 µm nylon filter into a 2 mL vial with a 250 µL insert and analyzed by HPLC-FLD.

4.6. HPLC-FLD Analysis

The separation was carried out on an ACE Excel 3 C18 column (15 cm × 4.6 mm, 3 µm). The mobile phase consisted of a mixture of Milli-Q water, MeOH and ACN in a 65:20:15 (*v/v/v*) ratio, respectively, working in isocratic mode. The flow rate of the mobile phase was set at 1 mL/min and the injection volume of 20 µL. In the fluorescence detection system, 360 nm was selected as the excitation wavelength and 440 nm as the emission wavelength.

4.7. UHPLC-HRMS Analysis

For validation, a UHPLC was used, using a column ZORBAX RRHD Eclipse Plus C18 column (10 cm × 2.1 mm, 1.8 µm particle size) and a 0.3 µm inline filter, both from Agilent Technologies. The mobile phase used was composed of solvent A (water/MeOH (95:5, *v/v*)) and solvent B (MeOH/water (95:5, *v/v*)). Both solvents contained 0.3% formic acid and 5 mM ammonium formate. The gradient profile was as follows: 0–0.5 min 40% B, 0.5–5.5 min 40–70% B, 5.5–6 min 70–40% B and 6–8 min 40% B at a flow rate of 0.4 mL/min. The sample volume injected was 20 µL. Positive mode operation was used in the mass spectrometer. The drying gas flow rate was 16 L/min at a temperature of 130 °C and a nebulizer gas pressure of 30 psi. The enveloping gas flow rate was 11 L/min and the temperature was 300 °C. For data acquisition, MS scans were configured in the range of 100–1000 *m/z*. Three collision energies (0, 10 and 40 V) were used in each cycle.

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References

- da Fonseca Alves, R.; da Silva, L.N.C.; Matos Neto, G.; Ierick, I.F.; Ferreira, T.L.; Sotomayor, M.D.P.T. Magnetic MIPs: Synthesis and applications. *Methods Mol. Biol.* **2021**, 2359, 85–96. [CrossRef] [PubMed]
- Jia, B.; Feng, F.; Wang, X.; Song, Y.; Zhang, F. Recent advances in magnetic molecularly imprinted polymers and their application in the food safety analysis. *J. Future Foods* **2024**, 4, 1–20. [CrossRef]
- Xu, L.; Pan, J.; Dai, J.; Li, X.; Hang, H.; Cao, Z.; Yan, Y. Preparation of thermal-responsive magnetic molecularly imprinted polymers for selective removal of antibiotics from aqueous solution. *J. Hazard. Mater.* **2012**, 233, 48–56. [CrossRef] [PubMed]
- Mahato, D.K.; Lee, K.E.; Kamle, M.; Devi, S.; Dewangan, K.N.; Kumar, P.; Kang, S.G. Aflatoxins in food and feed: An overview on prevalence, detection and control strategies. *Front. Microbiol.* **2019**, 10, 30–45. [CrossRef]
- Scientific Committee on Food. Opinion on aflatoxins, ochratoxin A and patulin. *Rep. Sci. Comm. Food* **1996**, 35, 45–50.
- IARC. International Agency for Research on Cancer. Available online: <http://www.iarc.fr> (accessed on 16 January 2024).
- The European Parliament and the Council of the European Union. Directive 2002/32/EC on undesirable substances in animal feed. *Off. J. Eur. Union* **2002**, 140, 1–10.
- Wang, S.; Shao, R.; Li, W.; Li, X.; Sun, J.; Jiao, S.; Dai, S.; Dou, M.; Xu, R.; Li, Q.; et al. Three-dimensional ordered macroporous magnetic inverse photonic crystal microsphere-based molecularly imprinted polymer for selective capture of aflatoxin B1. *ACS Appl. Mater. Interfaces* **2022**, 14, 18845–18853. [CrossRef]
- Wu, C.; He, J.; Li, Y.; Chen, N.; Huang, Z.; You, L.; He, L.; Zhang, S. Solid-phase extraction of aflatoxins using a nanosorbent consisting of a magnetized nanoporous carbon core coated with a molecularly imprinted polymer. *Microchim. Acta* **2018**, 185, 515. [CrossRef]
- Hu, M.; Huang, P.; Suo, L.; Wu, F. Polydopamine-based molecularly imprinting polymers on magnetic nanoparticles for recognition and enrichment of ochratoxins prior to their determination by HPLC. *Microchim. Acta* **2018**, 185, 300. [CrossRef] [PubMed]
- Wang, R.; Wu, P.; Cui, Y.; Fizir, M.; Shi, J.; He, H. Selective recognition and enrichment of sterigmatocystin in wheat by thermoresponsive imprinted polymer based on magnetic halloysite nanotubes. *J. Chromatogr. A* **2020**, 1619, 460952. [CrossRef] [PubMed]
- Zhao, M.; Shao, H.; Ma, J.; Li, H.; He, Y.; Wang, M.; Jin, F.; Wang, J.; Abd El-Aty, A.M.; Hacımuftuoğlu, A.; et al. Preparation of core-shell magnetic molecularly imprinted polymers for extraction of patulin from juice samples. *J. Chromatogr. A* **2020**, 1615, 460751. [CrossRef]
- Sun, J.; Guo, W.; Ji, J.; Li, Z.; Yuan, X.; Pi, F.; Zhang, Y.; Sun, X. Removal of patulin in apple juice based on novel magnetic molecularly imprinted adsorbent Fe₃O₄@SiO₂@CS-GO@MIP. *LWT* **2020**, 118, 108854. [CrossRef]
- Huang, Z.; He, J.; Li, H.; Zhang, M.; Wang, H.; Zhang, Y.; Li, Y.; You, L.; Zhang, S. Synthesis and application of magnetic-surfaced pseudo molecularly imprinted polymers for zearalenone pretreatment in cereal samples. *Food Chem.* **2020**, 308, 125696. [CrossRef]
- Fu, H.; Xu, W.; Wang, H.; Liao, S.; Chen, G. Preparation of magnetic molecularly imprinted polymers for the identification of zearalenone in grains. *Anal. Bioanal. Chem.* **2020**, 412, 4725–4737. [CrossRef] [PubMed]
- Wang, M.; He, J.; Zhang, Y.; Tian, Y.; Xu, P.; Zhang, X.; Li, Y.; Chen, J.; He, L. Application of magnetic hydroxyapatite surface-imprinted polymers in pretreatment for detection of zearalenone in cereal samples. *J. Chromatogr. B* **2022**, 1201, 123297. [CrossRef] [PubMed]
- Hu, M.; Ge, W.; Liu, X.; Zhu, Y. Preconcentration and determination of zearalenone in corn oil by a one-step prepared polydopamine-based magnetic molecularly imprinted polymer (MIP) with high-performance liquid chromatography with fluorescence (HPLC-FLD) detection. *Anal. Lett.* **2022**, 55, 343–354. [CrossRef]
- Zhang, Y.; Liu, D.; Peng, J.; Cui, Y.; Shi, Y.; He, H. Magnetic hyperbranched molecularly imprinted polymers for selective enrichment and determination of zearalenone in wheat proceeded by HPLC-DAD analysis. *Talanta* **2020**, 209, 120555. [CrossRef] [PubMed]
- Muñoz-Solano, B.; González-Peñas, E. Mycotoxin determination in animal feed: An LC-FLD method for simultaneous quantification of aflatoxins, ochratoxins and zearalenone in this matrix. *Toxins* **2020**, 12, 374. [CrossRef] [PubMed]
- Arroyo-Manzanares, N.; Huertas-Pérez, J.F.; García-Campaña, A.M.; Gámiz-Gracia, L. Aflatoxins in animal feeds: A straightforward and cost-effective analytical method. *Food Control* **2015**, 54, 74–78. [CrossRef]
- Kim, H.J.; Lee, M.J.; Kim, H.J.; Cho, S.K.; Park, H.J.; Jeong, M.H. Analytical method development and monitoring of Aflatoxin B1, B2, G1, G2 and Ochratoxin A in animal feed using HPLC with Fluorescence detector and photochemical reaction device. *Cogent Food Agric.* **2017**, 3, 1419788. [CrossRef]
- European Commission. Commission Regulation (EC) No. 401/2006 of laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. *Off. J. Eur. Union* **2006**, 70, 12–34.

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Article

Validation of a Targeted LC–MS/MS Method for Cereulide and Application in Food and Faeces

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Abstract: Cereulide is an emetic toxin produced by some strains of *Bacillus cereus*. This bacterial toxin, a cyclic 1.2 kDa dodecadepsipeptide, is stable to heat and acids and causes nausea and vomiting when ingested via contaminated food. This work aimed to develop and validate a targeted analytical method applying liquid chromatography-tandem mass spectrometry (LC–MS/MS) to quantify this toxin in food and human faeces. Samples were extracted with acetonitrile in the presence of ¹³C₆-cereulide, a labelled internal standard, and purified by centrifugation and filtration. The limits of quantification were 0.5 and 0.3 µg kg^{−1} for food and faeces, respectively. The linearity of the method was very good, with calculated R² values above 0.995. The mean recovery of the method was within the acceptable range of 70.0%–120.0%, the repeatability was not higher than 7.3%, and the highest intra-laboratory reproducibility was 8.9%. The estimated range for the expanded measurement uncertainty was between 5.1% and 18.0%. The LC–MS/MS method was used to analyse one food sample (rice) from a Belgian foodborne outbreak and five faecal samples from patients with clinical symptoms after consumption of the contaminated rice. The levels of cereulide were 12.22 µg g^{−1} for food and between 6.32 and 773.37 ng g^{−1} for faecal samples.

Keywords: cereulide; LC-MS/MS; validation; food; faeces; bacterial toxin

Key Contribution: This work describes the validation of a reliable analytical LC–MS/MS method for the quantification of the emetic toxin from *Bacillus cereus*; cereulide in food and human faeces and its application during a Belgian foodborne outbreak. Most published cereulide quantification methods have yet to be validated for human samples. This method could help investigate faecal samples from potentially contaminated patients with generic gastro-intestinal clinical symptoms.

1. Introduction

Bacillus (*B.*) *cereus sensu lato* (*s.l.*) is a Gram-positive, spore-forming bacteria that poses a significant safety risk in the food industry, with the associated potential production of toxins [1]. According to the European Food Safety Authority (EFSA) annual report, more than 10% of food poisoning outbreaks caused by bacterial toxins are attributed to *B. cereus*, which is more than for *Clostridium* or *Staphylococcus* toxins [2]. With 87 registered outbreaks in the EU in 2021, *B. cereus* is well known for causing foodborne outbreaks due to ingesting foods contaminated by bacteria, toxins, or both. Moreover, the incidence of food poisoning caused by *B. cereus* is certainly underestimated since symptoms are not specific and self-limiting, and reporting procedures may vary between countries [3]. *B. cereus* spores are typically found in soil and can, therefore, contaminate almost all raw materials and unprocessed food. They are responsible for two types of gastrointestinal diseases: emetic or diarrheal syndromes [4]. The diarrheal form of food poisoning is mainly characterised by diarrhoea and abdominal cramps, similar to food poisoning by *Clostridium perfringens* [5]. The enterotoxins known to be involved in this syndrome are

hemolysin BL (Hbl), nonhemolytic enterotoxin (Nhe), and cytotoxin K (CytK) [6]. Diarrheic symptoms occur after approximately 10–16 h, and the enterotoxins are produced by viable bacteria inside the human intestine [7]. The emetic form of food poisoning is mainly characterised by nausea, vomiting, and sometimes abdominal cramps combined with diarrhea. Sometimes, fatal emetic cases have been reported. The emetic symptoms, similar to those of *Staphylococcus aureus* intoxication, may appear from 1 to 5 h following ingestion of contaminated food and can continue for approximately 24 h [8]. Due to its ubiquitous nature, *B. cereus* can be found in a wide variety of foods, and the emetic syndrome is due to the ingestion of a preformed toxin, cereulide, mainly produced by *B. cereus* in rice, pasta, potato-based meals, or dairy products [9,10]. Even if *B. cereus*-associated gastrointestinal and emetic diseases are often associated with mild or limited symptoms and are self-limiting with a short duration, severe and fatal outbreaks were also reported in some cases [11–14]. Moreover, the preformed emetic toxin, cereulide, can also be detected directly in food in such outbreaks. Indeed, low levels of cereulide in food (from 5 to 10 ng g⁻¹) have already been reported as illness-inducing doses, while higher levels (µg range) were also reported with various symptoms [12,15–18].

Cereulide was first described by Agata et al. in 1994 [19]. More recently, other isoforms of cereulide have also been described as showing variabilities in cytotoxicity: isocereulide A shows around 8–10-fold higher cytotoxicity compared to cereulide, while isocereulide B is almost non-toxic [20]. Cereulide is a ring-structured dodecadepsipeptide with a molecular mass of 1.2 kDa, containing three repetitions of four amino acids: D-Oxy-Leu—D-Ala—L-Oxy-Val—L-Val [21]. This small peptide is synthesised by non-ribosomal cereulide synthetase machinery encoded in the *ces* gene cluster [22] and is chemically very stable and highly resistant to heat (>120 °C during 90 min), acid, and the proteolytic activity of pepsin and trypsin [23,24]. Only highly alkaline pHs can reach inactivation. Consequently, once present in the food chain, cereulide is very difficult to eradicate. Moreover, *B. cereus* spores can survive after thermal treatments, and germinated vegetative cells can grow and produce toxins under favourable conditions [25]. Based on the chemical structure, cereulide is comparable to valinomycin, and both are potassium ion-selective ionophores [26]. Subsequently, it can cause a potassium-dependent drop in the transmembrane potential of mitochondria with the expulsion of H⁺ and stimulation of mitochondrial respiration [27]. However, the precise mechanism of the emetic effect of cereulide remains to be decrypted [28].

The classical microbiological methods for detection of *B. cereus* show bacterial contamination but do not offer any information on the presence or potential concentration of the emetic toxin cereulide that may be produced. Therefore, it is very important to quantify cereulide in food samples, particularly in cases of emetic outbreaks. Typical toxin detection methods depend on enzyme-linked immunosorbent assays (ELISA), but the development of an antibody against cereulide is still challenging. A semi-quantitative system to detect cereulide via a computer-assisted sperm analysis system has already been reported [29], as well as other non-specific bioassays based on Hep-2 cells [30,31], but chemical methods, like LC–MS/MS or Matrix-Assisted Laser Desorption Ionisation–Time of Flight (MALDI–TOF) MS, are now gold standards for its quantification [32–38]. Chemical methods were used to establish an ISO standard method based on stable isotope MS-based dilution [39], using commercially available synthetic cereulide as an internal standard instead of related compounds such as valinomycin [40,41], allowing precise absolute quantification. Using the original standard effectively decreases matrix effects and interferences in the electrospray ionisation step that could appear when using surrogate standards [42]. ISO 18465:2017 can be applied to analyse the toxin in products intended for human consumption [43].

Numerous analytical approaches for the analysis of cereulide in food are currently well-described and available. However, a similar method covering a wide range of food matrices as well as human clinical samples is an added value to assess potential human consumption and related effects. More particularly, the analytical methods describing cereulide quantification in human samples are very limited in the literature. Even if cereulide was

detected in 2010 in human clinical samples (stool, urine, gastric fluid, and blood serum) of outbreak patients by Hep-2 Assay [44], none of the existing methods combine the quantification of cereulide in leftover food and faeces from patients. This study aimed to develop and perform the validation of straightforward and sensitive analytical LC–MS/MS methods in both food and human samples in support of an additional collection of quantitative and occurrence data associated with the intake of food contaminated with cereulide. The method should be able to analyse a broad range of matrices, including lasagna, hotdog sausage, baby food, and rice. Furthermore, it should also be applicable to human samples (faeces), which is a real novelty as there is a real lack of studies on the analysis of cereulide in human samples. This validated method in faeces in combination with food analysis could certainly be useful for further characterisation of *B. cereus* foodborne outbreaks.

2. Results and Discussion

2.1. LC–MS/MS Use and Optimisation of LC–MS/MS Parameters

Using Liquid Chromatography–Mass Spectrometry (LC–MS) for cereulide detection offers several distinct advantages, making it the preferred analysis method. The rapidity of the method is a huge advantage, as this method could be performed within a couple of hours. High sensitivity allows for detecting cereulide at very low concentrations, which is crucial when dealing with potent toxins like cereulide that can be harmful even in trace amounts. The selectivity of mass spectrometry provides high selectivity by measuring the mass-to-charge ratio of ions. This enables accurate identification and differentiation of cereulide from other compounds in complex food or human matrices. LC–MS can also provide structural information about cereulide molecules. Through fragmentation patterns observed during mass spectrometry analysis, the chemical structure of cereulide can be confirmed, ensuring accurate identification. LC–MS also allows for accurate quantification of cereulide levels in samples. Its linear response over a wide concentration range ensures reliable quantification even at low levels. Food and human samples often contain complex matrices with various interfering compounds. The chromatographic separation capabilities of liquid chromatography resolve interferences and enhance the accuracy of cereulide quantification. Furthermore, this technique can be applied to various samples, including solid, liquid, and semi-solid samples. This versatility allows for the analysis of various matrices, from food products to human samples. Regulatory agencies have recognised and accepted LC–MS methods for cereulide detection, making them suitable for compliance with food safety and quality standards. Modern LC–MS systems also offer rapid analysis times, making it possible to process many samples in a relatively short period of time. The benefits of LC–MS in terms of rapidity, sensitivity, selectivity, and versatility make it a powerful tool for accurate cereulide detection in various matrices.

For the optimisation of LC–MS/MS parameters, an acidic aqueous mobile phase (10 mM ammonium formate (HCOONH_4) with 0.1% formic acid (HCOOH)) in combination with acetonitrile (ACN) with 0.1% HCOOH as an organic phase was used. Due to the addition of HCOONH_4 , cereulide and its internal standard were always detected as $[\text{M} + \text{NH}_4]^+$ adduct.

Considering the MS part of the method, data acquisition were performed in multiple reaction monitoring (MRM) mode to improve selectivity and sensitivity. MRM parameters were adjusted by flow injection analysis in the ESI-positive mode of individual standards (cereulide and $^{13}\text{C}_6$ -cereulide), followed by collision-induced dissociation experiments to identify the most intense product ions. It should be emphasised that the injection of extracts (food and faeces) into the column exhibited parameters comparable with those described in ISO 18465:2017 [43]. Table 1 provides the optimised MRM transitions, cone voltages, and collision energies for each transition and analyte.

Table 1. Retention time and optimised MRM parameters for the detection of cereulide and $^{13}\text{C}_6$ -cereulide in food and faeces.

Standard	Precursor ion	Cone Voltage	Product Ions (<i>m/z</i>) * [Collision Energy in eV]
Cereulide	1170.5	60	314.2 [65]
		60	499.3 [65]
$^{13}\text{C}_6$ -cereulide	1176.5	60	172.1 [90]
		60	315.4 [75]

* The daughter ions highlighted in bold were used as quantifiers, while the second ions were considered qualifiers.

To separate the compounds of interest using LC, the suitability of the UHPLC column with Acquity BEH C18 stationary phase, in combination with the acidic mobile phases consisting of ACN with 0.1% HCOOH and the aqueous mobile phase with 10 mM HCOONH₄ and 0.1% HCOOH, was demonstrated, and good peak shapes were obtained in food and faecal samples using a gradient elution (see Section 4) (Figure 1).

2.2. Optimisation of Sample Preparation

A simple ACN extraction procedure described in ISO 18465:2017 was tried for the preparation of food samples [43]. The method demonstrated favourable sensitivity and recovery across various food matrices, such as lasagna, hotdog sausage, baby food, and rice. Because of the complexity of the faecal matrix, the application of a clean-up step in the faecal sample preparation procedure was tested to reduce matrix interference and increase sensitivity. To clean up the extract, solid-phase extraction (SPE) was chosen, and two SPE types, the Strata-X cartridge with a polymeric cation exchange sorbent and the Bond Elut C18 Solid cartridge with a broad retention spectrum among bonded silica sorbents, were tested. Furthermore, several extraction solvents were also tried in combination with these two SPE models. ACN, methanol, and ethanol were used to evaluate the method's recovery. Unfortunately, these SPE clean-up steps were not found suitable for the purification of faecal samples regarding recovery after purification. In addition, because freeze-drying of faecal samples has emerged as a valuable tool for metabolome research [45–47], a freeze-drying step was also tested for this matrix. Even if results were good for higher fortified concentrations, the recoveries obtained for low fortified concentrations (e.g., 0.3 ng g^{−1}) were unsuitable (above 120%). Therefore, a simple ACN extraction gave the best recovery results without the SPE purification step on the fresh matrix. Consequently, the application of the ISO 18465:2017 protocol [43] to faecal samples required an adjustment of the volumes used (see Section 4).

For both food and faecal samples, a stable isotope labelled internal standard of cereulide, $^{13}\text{C}_6$ -cereulide, was used to compensate for possible losses during sample preparation and matrix effects, hence optimising the quantification. Using a $^{13}\text{C}_6$ -cereulide internal standard gives better ion suppression correction, accuracy, and precision than using similar compounds [48].

2.3. Method Validation

2.3.1. Selectivity and Specificity

For cereulide and $^{13}\text{C}_6$ -cereulide quantitative and qualitative transitions, no interferences were detected in the analysis of blank extracts from blank lasagna, hotdog sausage, baby food, and rice. However, for faecal samples, while the analysis of blank extract was clean for cereulide transitions, an interfering compound was present (Figure 1F) at the ion transition 1176.5 > 172.1 ($^{13}\text{C}_6$ -cereulide quantitative transition). Nevertheless, both peaks were well resolved, and there was no identification of any interfering compound with a signal-to-noise ratio greater than three at the retention time of the target compound. Furthermore, all samples fortified with cereulide satisfied the retention time and ion ratio criteria (Commission Decision 2021/808/EC).

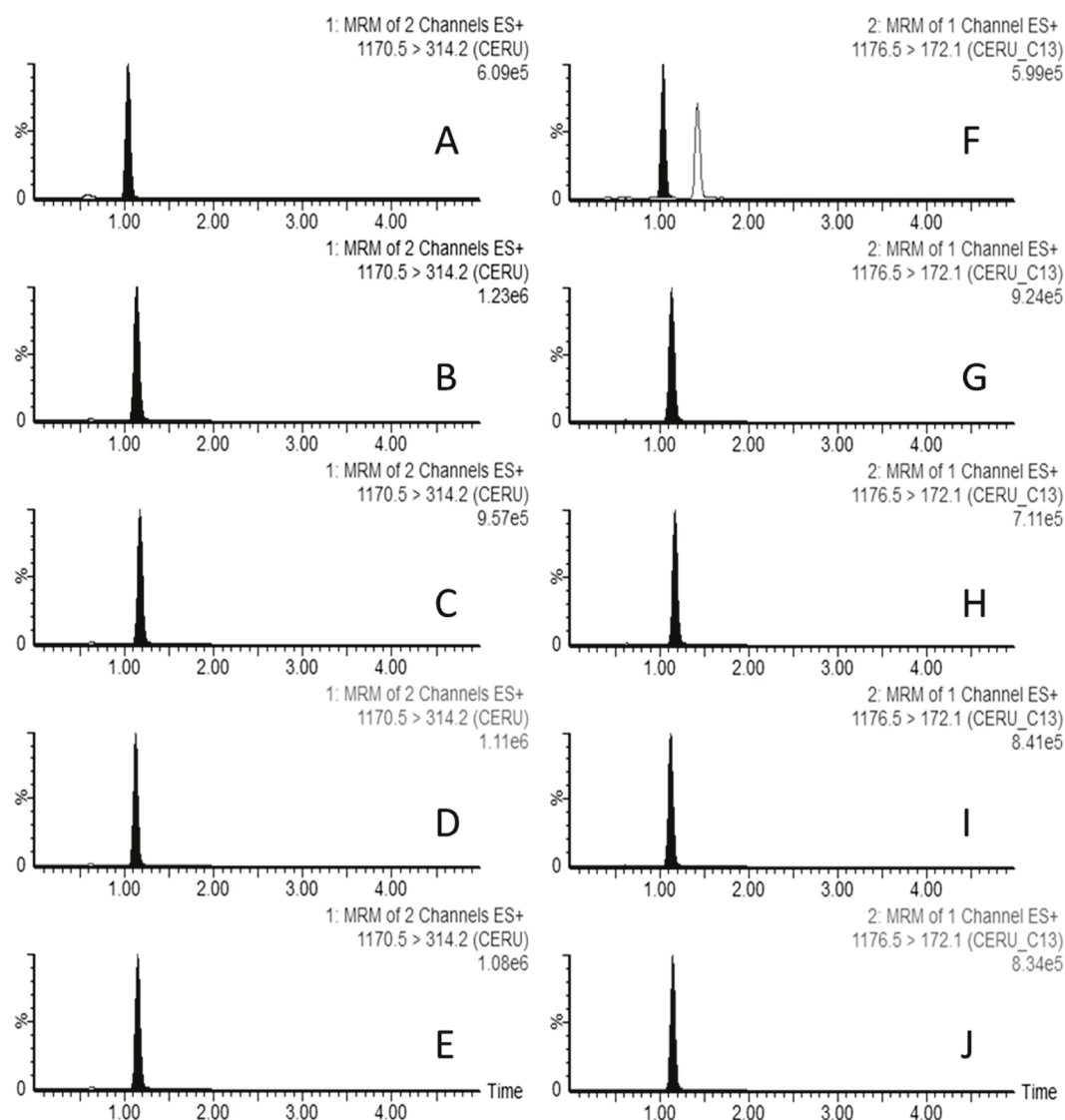


Figure 1. LC–MS/MS multiple reaction monitoring (MRM) chromatograms of a single injection of a cereulide fortified in faeces (A), at a concentration of 3 ng g^{-1} , rice (B), baby food (C), hotdog sausage (D), and lasagna (E), at a concentration of 5 ng g^{-1} and $^{13}\text{C}_6$ -cereulide in faeces (F), rice (G), baby food (H), hotdog sausage (I), and lasagna (J), at a concentration of 1.7 ng g^{-1} . The most prevalent transition is presented for each matrix. The vertical axes represent relative peak intensity (normalised to 100%), while the horizontal axes indicate retention time (in min). The chromatographic conditions used are detailed in Section 4.

2.3.2. Matrix Effect and Linearity

For the analysis of food samples, and based on the protocol described in ISO 18465:2017, calibration curves were made in ACN. For faecal samples, after an evaluation of the slopes of calibration curves prepared in solvent and in blank matrix extract, the statistical *t*-test (at 95% confidence) illustrated that there is no significant difference between the slopes in the solvent and in the matrix and, therefore, no presence of matrix effects (Table 2). Consequently, the calibration curves were established extemporaneously in the solvent (ACN) for food and faecal samples.

Table 2. Evaluation of matrix effects and linearity for the quantification of cereulide in faeces.

Matrix			
Matrix effect			
	<i>t</i> -value	<i>t</i> _{crit} (95%)	Matrix effect
Faeces	1.37	2.03	No
Linearity			
	<i>t</i> -value	<i>F</i> _{crit}	Linear model
Solvent	6.73	7.82	Yes

The linearity of the calibration curves of cereulide in solvent was evaluated with Mandle's fitting test. The linear regression model was selected as the test value was lower than the *F*_{crit} value (Table 2). Moreover, coefficients of determination above 0.995 were always obtained during the analyses. The linear regression was assessed using a calibration range between 0.01 ng mL^{−1} and 8.3 ng mL^{−1}, equivalent to 0.1 µg kg^{−1} and 83.3 µg kg^{−1} in matrix (food and faeces).

2.3.3. Limits of Detection and Limits of Quantification

The LOD and LOQ were determined as the lowest concentration in the calibration curve or the lowest level meeting the validation criteria, respectively, which, in our opinion, ensures the method quality. The LOD was 0.1 µg kg^{−1} for both food and faecal samples, corresponding to the lowest concentration in the calibration curve. The LOQ was set at 0.3 and 0.5 µg kg^{−1} for faeces and food, respectively, as these were the lowest validated concentrations. The average signal-to-noise values for the LOD were always above three, while the values for the LOQ were consistently higher than ten. The limits of detection and/or quantification of the validated method were equivalent or better compared to several previously described methods in food [35,49–51].

2.3.4. Apparent Recovery, Repeatability, Reproducibility, and Measurement Uncertainty

The apparent recovery values for every fortified sample consistently exceeded 70%. This was independent of the concentration level and matrix tested. The mean values (Table 3) fell within the acceptable range of 70% to 120%, and the lowest observed apparent recovery was 92.1% for cereulide at the intermediate level in food (i.e., 5.0 µg kg^{−1}). At the lowest concentration levels (0.5 and 0.3 µg kg^{−1} for food and faeces, respectively), apparent recoveries were 109.2% and 100.9% for food and faecal samples, respectively. The highest apparent recovery was 111.0% in faeces at an intermediate concentration level (2.9 µg kg^{−1}).

Table 3. Validation data for cereulide in food (lasagna, hotdog sausage, baby food, and rice) and faeces matrices (linear range, apparent recovery, repeatability, intra-laboratory reproducibility, and measurement uncertainty).

Analyte	Linear Range (µg kg ^{−1})	Concentration Level (µg kg ^{−1})	Apparent Recovery (%)	Repeatability (%)	Reproducibility (%)	Measurement Uncertainty (%)
Food	0.1–83.3	0.5	109.2	5.2	5.2	10.4
		5.0	92.1	1.8	5.4	10.7
		15.0	96.4	3.0	3.7	7.3
		80.0	98.3	2.8	4.2	8.5
Faeces	0.1–83.3	0.3	100.9	4.9	5.3	10.6
		2.9	111.0	7.3	8.9	18.0
		29.2	105.4	1.5	2.5	5.1

Furthermore, Horwitz ratios determined the maximum acceptable repeatability and reproducibility in each matrix. The repeatability and reproducibility calculated with the results from the validation were below these maximum values. The method's repeatability was lower than 7.3%, and the highest intra-laboratory reproducibility was 8.9% in faeces at an intermediate concentration level (Table 3). The highest measurement uncertainty (MU) was 18% in faeces.

In general, the criteria for apparent recovery, repeatability, intra-laboratory reproducibility, and measurement uncertainty were satisfied at every concentration level of cereulide and for all the tested matrices during the validation process (including lasagna, hotdog sausage, baby food, rice, and faeces).

In brief, an LC–MS/MS method for quantification of *Bacillus cereus* emetic toxin, cereulide, was successfully validated in food (lasagna, hotdog sausage, baby food, and rice) and faeces.

2.3.5. Method Application to Food and Human Samples

In 2022, in Belgium, a dish of cooked rice was served among other dishes during a party. Several people became sick after eating this rice. The Belgian National Reference Laboratory for Foodborne Outbreaks also received faeces from five patients with clinical signs after ingesting the contaminated rice. The faecal samples were collected between one and five days after the onset of symptoms. The developed and validated LC–MS/MS method was applied to analyse one food sample (rice) and five faecal samples from patients with clinical symptoms after the consumption of contaminated rice. The rice sample was highly contaminated, with a quantification of cereulide at $12.22 \mu\text{g g}^{-1}$. This level of contamination (range of $\mu\text{g g}^{-1}$ for cereulide in food) is usual in foodborne outbreaks. Several other cases were reported in a similar high-level range [12,18,50,52].

The samples were analysed following the validated method, and results ranged between 6.32 and 773.37 ng g^{-1} in faeces, confirming the cause of the symptoms due to the ingestion of contaminated meals and the subsequent excretion of the toxin (Figure 2).

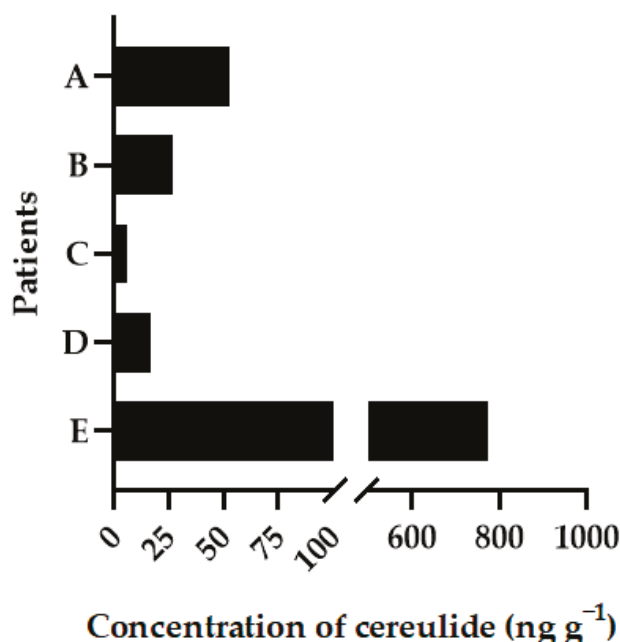


Figure 2. Quantification of cereulide in faeces samples of five patients (A–E) with symptoms after ingestion of contaminated rice.

These results suggest that the concentrations of cereulide in the human faecal sample after ingestion of contaminated meal are still relatively high (higher than 700 ng g^{-1}) and consistently detectable with this validated method when patients show typical clinical

signs such as vomiting or diarrhea. Therefore, reliable monitoring of faeces from patients suspected to be intoxicated with cereulide-contaminated food could be helpful in the assessment of intoxication.

The quantity of publications on LC–MS/MS detection methods focusing on food or foodborne outbreak samples is increasing in the literature. Even if there is no official regulation for cereulide levels in food, reaching low levels of LOQ and satisfactory performance is essential to detecting even limited amounts. Nevertheless, there is a lack of methods for the detection of cereulide in human samples (urine, plasma, faeces, etc.). Such detection methods could be helpful to characterise the origin of contamination, given that symptoms are often similar after bacterial toxins have been contaminated.

3. Conclusions

In conclusion, this study focused on the development and validation of a sensitive analytical LC–MS/MS method for the quantification of *Bacillus cereus* emetic toxin, cereulide, in various food matrices (lasagna, hotdog sausage, baby food, and rice) and human faecal samples. The method demonstrated several advantages, including high sensitivity, selectivity, and versatility, making it a powerful tool for cereulide detection in complex matrices. The validation process confirmed the method's robustness, meeting the criteria for apparent recovery, repeatability, reproducibility, and measurement uncertainty across different concentration levels and matrices. The method's sensitivity was demonstrated by its low limits of detection and quantification, making it suitable for detecting cereulide even at trace levels in food and faeces. The method was successfully applied to analyse a highly contaminated rice sample from a foodborne outbreak and human faecal samples from patients with clinical symptoms after consuming contaminated rice. The results indicated significant cereulide contamination in food and human samples, confirming its role in food poisoning outbreaks and its excretion in detectable amounts in faeces.

Overall, this validated LC–MS/MS method provides a valuable tool for monitoring and assessing cereulide contamination in food and human samples, enhancing our understanding of *Bacillus cereus*-related foodborne illnesses and their impact on public health.

4. Materials and Methods

4.1. Standards, Reagents, and Consumables

Analytical standards of cereulide and an isotopically labelled internal standard, $^{13}\text{C}_6$ -cereulide, were purchased from Chiralix B.V. (Nijmegen, The Netherlands). Individual stock solutions of standards were bought as acetonitrile solutions with a concentration of $50\text{ }\mu\text{g mL}^{-1}$ and $20\text{ }\mu\text{g mL}^{-1}$ for cereulide and $^{13}\text{C}_6$ -cereulide, respectively. Intermediate solutions were prepared by diluting the stock solutions in ACN. Intermediate and working solutions were made at 250 and 10 ng mL^{-1} for cereulide and 1000, 100, and 10 ng mL^{-1} for $^{13}\text{C}_6$ -cereulide by diluting the individual stock solutions in ACN.

All standard solutions were always stored at $-20\text{ }^{\circ}\text{C}$.

Acetonitrile ULC-MS grade (ACN), formic acid 99% LC-MS grade (HCOOH), and ammonium formate ULC-MS grade (HCOONH_4) were purchased from Biosolve (Valkenswaard, The Netherlands). Water (H_2O) was purified using a Milli-Q system (Millipore, Overijse, Belgium). The water exhibited a resistivity of $18.2\text{ }\Omega$ and a total organic carbon content ranging from 2 to 3 ppm. Two mL syringes for filtration and 50 mL centrifugal tubes were acquired from VWR (Leuven, Belgium), and the $0.2\text{ }\mu\text{m}$ polytetrafluoroethylene (PTFE) syringe filters were purchased from Phenomenex (Utrecht, The Netherlands). Two mL amber glass injection vials were purchased from Waters (Waters, Milford, MA, USA).

4.2. Instrumentation and LC–MS/MS Conditions

The liquid chromatography instrument employed for this study was an ACQUITY UPLC H-Class system (Waters, Milford, MA, USA) fitted with a quaternary pump, sampler manager, and column oven. The LC separation of cereulide was obtained on a reversed-phase ACQUITY UPLC BEH C18 column ($50 \times 2.1\text{ mm}$, $1.7\text{ }\mu\text{m}$) (Waters) with a pre-filter

(0.22 μm) (Waters, Milford, MA, USA). The temperature of the column was adjusted to 40 °C. The aqueous mobile phase was made up of H_2O with the inclusion of 10 mM HCOONH_4 and 0.1% of HCOOH , while the organic mobile phase was ACN with the addition of 0.1% of HCOOH . For the food sample, the elution was conducted with a flow rate of 1 mL min^{-1} employing the subsequent gradient program: 0–1.2 min: 92% B; 1.2–1.8 min: 92–100% B; 1.8–2 min: 100–92% B. The total run time was 2 min, and the injection volume was 10 μL . For the faecal samples, the elution was performed at a flow rate of 1 mL min^{-1} using the following gradient program: 0–1.2 min: 92% B; 1.2–2 min: 92–100% B; 2–4.8 min: 100% B; 4.8–5 min: 92% B. The total run time was 5 min, and the injection volume was 10 μL .

Following the separation by liquid chromatography, cereulide and $^{13}\text{C}_6$ -cereulide were detected with an XEVO TQ-S triple quadrupole mass spectrometer (Waters, Milford, MA, USA) fitted with an electrospray ionisation (ESI) source operated in positive mode. The optimised configurations for the MS instrument were as follows: source temperature 150 °C, desolvation temperature 550 °C, desolvation gas flow 1200 L h^{-1} , cone gas flow 150 L h^{-1} , collision gas flow 0.17 mL min^{-1} with argon as collision gas. The value of 60 V was set for the source offset and 3 kV for the capillary voltage. The acquisition of MS data were performed in Multiple Reaction Monitoring (MRM) mode. The selected MRM parameters for cereulide quantification are listed in Table 1. The transition displaying the highest signal intensity (quantifier) was employed for quantifying the analyte, while the second most intense ion transition was utilised for identification (qualifier).

4.3. Preparation of Calibration Standards and Quality Control Standards

Quality control (QC) measures were established to guarantee the daily method performance and ensure the reliability of analytical results, including the analysis of fortified samples and replicate injections of one of the calibration points—system suitability checks (SSC). For the apparent recovery of the sample, during each analytical run, a blank sample fortified with cereulide standard before extraction is analysed following the same protocol as the unknown samples. The accepted range of the apparent recovery of the QC sample was 70–120%. This sample is used to verify whether the sample preparation step has worked properly. The criteria for the identification of cereulide in samples for analysis were according to Regulation (EU) 2021/808 (Commission Decision 2021/808) and ISO 18465:2017 [43,53]. For SSC, at the beginning of each analytical run, a control with a concentration of 0.83 ng mL^{-1} was injected at least three times to verify that the LC–MS was working correctly, i.e., signal intensity variation for consecutive injections and retention time variation between injections.

4.4. Samples

The food sample, a cooked rice dish, was taken by the Belgian Federal Agency for the Safety of the Food Chain following similar complaints from several consumers. This sample was ground and homogenised before the extraction step. The faecal samples were obtained from patients admitted to the hospital following clinical symptoms (including diarrhoea and vomiting) after the consumption of the contaminated rice. Volunteers provided blank samples of human faeces.

4.5. Sample Preparation

For the preparation of food samples, the process is based on the ISO 18465:2017 method [43]. Briefly, a solid-liquid extraction (SLE) was applied. 3 ± 0.05 g of sample were weighted in a 50 mL centrifugal tube, fortified with an internal standard solution, and extracted with 30 mL of ACN after 30 min of equilibration. The process is adapted from ISO 18465:2017 for the preparation of faecal samples preparation [43]. An SLE was also applied, but 1 ± 0.02 g of faeces was weighted in a 50 mL centrifugal tube, fortified with an internal standard solution, and extracted with 10 mL of ACN after 30 min of equilibration.

For both sample types, after shaking for one hour and centrifugation for 10 min, the extracts were filtered through a PTFE filter (0.2 μm) fixed on a syringe into an amber glass vial. The extracts were then analysed by LC–MS/MS.

4.6. Method Validation

The method validation was assessed in four food matrices (lasagna, hotdog sausage, baby food, and rice) and human faeces.

The following parameters were determined during method validation: selectivity and specificity, linearity, sensitivity (limits of detection (LOD) and quantification (LOQ)), repeatability, intra-laboratory reproducibility, and measurement uncertainty. The method's selectivity and specificity were assessed by analysing and comparing blank matrix samples (food and faecal samples) and fortified matrix samples. These parameters were evaluated, showing that on the one hand, no compound interferes with or can disrupt the identification and quantification of the cereulide in the blank matrix or injection solvent, and on the other hand, when cereulide is fortified in the sample or injection solvent, the presence of a chromatographic peak is confirmed at the retention time expected for this compound without interference. To assess linearity, standard solutions of cereulide were formulated in the injection solvent (ACN). The linearity was evaluated with Mandel's test to compare the linear and quadratic regression models. The linear regression model was preferred if the test value was lower than the critical value, F_{crit} . If the test value was higher than F_{crit} , the quadratic model was chosen. At least six calibration points in triplicate (on three different days) were injected to assess the linearity. To determine apparent recovery, repeatability, and intra-laboratory reproducibility, the fortified matrix samples were prepared at 0.5, 5, 15, and 80 $\mu\text{g kg}^{-1}$ for food matrices. For faecal samples, the validation was performed at 0.3, 2.9, and 29.2 $\mu\text{g kg}^{-1}$.

For lasagna, hotdog sausage, baby food, and rice, a one-day validation for each matrix was performed with the samples fortified at each concentration level in triplicate. For faeces, the samples were prepared at each concentration level in triplicate, and the experiment was repeated over three different days. The apparent recovery was calculated by taking the ratio between the measured and the fortified concentration and expressing it in percentages (%). Repeatability and intra-laboratory reproducibility were expressed as the relative standard deviation (RSD). The measurement uncertainty was determined by calculating intra-laboratory reproducibility and applying a coverage factor of 2 for 95% confidence. The LOD was determined as the lowest concentration of cereulide with a signal-to-noise ratio (S/N) of at least three, and the lowest point of the calibration curve was selected. At the same time, the LOQ was defined as the lowest concentration of cereulide, which can be quantified by the method and which met the validation criteria.

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References

1. Tirloni, E.; Stella, S.; Celandroni, F.; Mazzantini, D.; Bernardi, C.; Ghelardi, E. *Bacillus Cereus* in Dairy Products and Production Plants. *Foods* **2022**, *11*, 2572. [CrossRef] [PubMed]
2. European Food Safety Authority; European Centre for Disease Prevention and Control. The European Union One Health 2021 Zoonoses Report. *EFSA J.* **2022**, *20*, e07666. [CrossRef]
3. Cayemitte, P.E.; Raymond, P.; Aider, M. *Bacillus Cereus* as an Underestimated Foodborne Pathogen and New Perspectives on Its Prevalence and Methods of Control: Critical and Practical Review. *ACS Food Sci. Technol.* **2022**, *2*, 1196–1212. [CrossRef]
4. Stenfors Arnesen, L.P.; Fagerlund, A.; Granum, P.E. From Soil to Gut: *Bacillus Cereus* and Its Food Poisoning Toxins. *FEMS Microbiol. Rev.* **2008**, *32*, 579–606. [CrossRef] [PubMed]
5. *Bacillus Cereus* Infections and Intoxications. In *Red Book Atlas of Pediatric Infectious Diseases*; Tan, T.Q., Ed.; American Academy of Pediatrics: Itasca, IL, USA, 2022; ISBN 978-1-61002-630-7.
6. Fagerlund, A.; Lindbäck, T.; Granum, P.E. *Bacillus Cereus* Cytotoxins Hbl, Nhe and CytK Are Secreted via the Sec Translocation Pathway. *BMC Microbiol.* **2010**, *10*, 304. [CrossRef] [PubMed]
7. Jessberger, N.; Dietrich, R.; Granum, P.E.; Märklbauer, E. The *Bacillus Cereus* Food Infection as Multifactorial Process. *Toxins* **2020**, *12*, 701. [CrossRef] [PubMed]
8. Meng, J.-N.; Liu, Y.-J.; Shen, X.; Wang, J.; Xu, Z.-K.; Ding, Y.; Beier, R.C.; Luo, L.; Lei, H.-T.; Xu, Z.-L. Detection of Emetic *Bacillus Cereus* and the Emetic Toxin Cereulide in Food Matrices: Progress and Perspectives. *Trends Food Sci. Technol.* **2022**, *123*, 322–333. [CrossRef]
9. Ellouze, M.; Buss Da Silva, N.; Rouzeau-Szynalski, K.; Coisne, L.; Cantergiani, F.; Baranyi, J. Modeling *Bacillus Cereus* Growth and Cereulide Formation in Cereal-, Dairy-, Meat-, Vegetable-Based Food and Culture Medium. *Front. Microbiol.* **2021**, *12*, 639546.
10. Rajkovic, A.; Jovanovic, J.; Monteiro, S.; Decler, M.; Andjelkovic, M.; Foubert, A.; Beloglazova, N.; Tsilla, V.; Sas, B.; Maddar, A.; et al. Detection of Toxins Involved in Foodborne Diseases Caused by Gram-positive Bacteria. *Compr. Rev. Food Sci. Food Saf.* **2020**, *19*, 1605–1657. [CrossRef]
11. Dierick, K.; Van Coillie, E.; Swiecicka, I.; Meyfroidt, G.; Devlieger, H.; Meulemans, A.; Hoedemaekers, G.; Fourie, L.; Heyndrickx, M.; Mahillon, J. Fatal Family Outbreak of *Bacillus Cereus*-Associated Food Poisoning. *J. Clin. Microbiol.* **2005**, *43*, 4277–4279. [CrossRef]
12. Naranjo, M.; Denayer, S.; Botteldoorn, N.; Delbrassinne, L.; Veys, J.; Waegenaere, J.; Sirtaine, N.; Driesen, R.B.; Sipido, K.R.; Mahillon, J.; et al. Sudden Death of a Young Adult Associated with *Bacillus Cereus* Food Poisoning. *J. Clin. Microbiol.* **2011**, *49*, 4379–4381. [CrossRef] [PubMed]
13. Tschiedel, E.; Rath, P.-M.; Steinmann, J.; Becker, H.; Dietrich, R.; Paul, A.; Felderhoff-Müser, U.; Dohna-Schwake, C. Lifesaving Liver Transplantation for Multi-Organ Failure Caused by *Bacillus Cereus* Food Poisoning. *Pediatr. Transplant.* **2015**, *19*, E11–E14. [CrossRef] [PubMed]
14. Thery, M.; Cousin, V.L.; Tissieres, P.; Enault, M.; Morin, L. Multi-Organ Failure Caused by Lasagnas: A Case Report of *Bacillus Cereus* Food Poisoning. *Front. Pediatr.* **2022**, *10*, 978250. [CrossRef] [PubMed]
15. Agata, N.; Ohta, M.; Yokoyama, K. Production of *Bacillus Cereus* Emetic Toxin (Cereulide) in Various Foods. *Int. J. Food Microbiol.* **2002**, *73*, 23–27. [CrossRef] [PubMed]
16. Delbrassinne, L.; Andjelkovic, M.; Rajkovic, A.; Dubois, P.; Nguessan, E.; Mahillon, J.; Van Loco, J. Determination of *Bacillus Cereus* Emetic Toxin in Food Products by Means of LC–MS². *Food Anal. Methods* **2012**, *5*, 969–979. [CrossRef]
17. Zuberovic Muratovic, A.; Tröger, R.; Granelli, K.; Hellenäs, K.-E. Quantitative Analysis of Cereulide Toxin from *Bacillus Cereus* in Rice and Pasta Using Synthetic Cereulide Standard and 13C6-Cereulide Standard—A Short Validation Study. *Toxins* **2014**, *6*, 3326–3335. [CrossRef] [PubMed]
18. Delbrassinne, L.; Botteldoorn, N.; Andjelkovic, M.; Dierick, K.; Denayer, S. An Emetic *Bacillus Cereus* Outbreak in a Kindergarten: Detection and Quantification of Critical Levels of Cereulide Toxin. *Foodborne Pathog. Dis.* **2015**, *12*, 84–87. [CrossRef] [PubMed]
19. Agata, N.; Mori, M.; Ohta, M.; Suwan, S.; Ohtani, I.; Isobe, M. A Novel Dodecadepsipeptide, Cereulide, Isolated from *Bacillus Cereus* Causes Vacuole Formation in HEp-2 Cells. *FEMS Microbiol. Lett.* **1994**, *121*, 31–34. [CrossRef]
20. Marxen, S.; Stark, T.D.; Frenzel, E.; Rüttschle, A.; Lücking, G.; Pürstinger, G.; Pohl, E.E.; Scherer, S.; Ehling-Schulz, M.; Hofmann, T. Chemodiversity of Cereulide, the Emetic Toxin of *Bacillus Cereus*. *Anal. Bioanal. Chem.* **2015**, *407*, 2439–2453. [CrossRef]
21. Suwan, S.; Isobe, M.; Ohtani, I.; Agata, N.; Mori, M.; Ohta, M. Structure of Cereulide, a Cyclic Dodecadepsipeptide Toxin from *Bacillus Cereus* and Studies on NMR Characteristics of Its Alkali Metal Complexes Including a Conformational Structure of the K⁺ Complex. *J. Chem. Soc. Perkin Trans.* **1995**, *7*, 765–775. [CrossRef]
22. Lücking, G.; Frenzel, E.; Rüttschle, A.; Marxen, S.; Stark, T.D.; Hofmann, T.; Scherer, S.; Ehling-Schulz, M. Ces Locus Embedded Proteins Control the Non-Ribosomal Synthesis of the Cereulide Toxin in Emetic *Bacillus Cereus* on Multiple Levels. *Front. Microbiol.* **2015**, *6*, 1101. [CrossRef] [PubMed]
23. Schoeni, J.L.; Wong, A.C.L. *Bacillus Cereus* Food Poisoning and Its Toxins. *J. Food Prot.* **2005**, *68*, 636–648. [CrossRef] [PubMed]
24. Rajkovic, A.; Uyttendaele, M.; Vermeulen, A.; Andjelkovic, M.; Fitz-James, I.; in 't Veld, P.; Denon, Q.; Vérhe, R.; Debevere, J. Heat Resistance of *Bacillus Cereus* Emetic Toxin, Cereulide. *Let. Appl. Microbiol.* **2008**, *46*, 536–541. [CrossRef] [PubMed]
25. Logan, N.A. *Bacillus* and Relatives in Foodborne Illness. *J. Appl. Microbiol.* **2012**, *112*, 417–429. [CrossRef] [PubMed]
26. Makarassen, A.; Yoza, K.; Isobe, M. Higher Structure of Cereulide, an Emetic Toxin from *Bacillus cereus*, and Special Comparison with Valinomycin, an Antibiotic from *Streptomyces fulvissimus*. *Chem.—Asian J.* **2009**, *4*, 688–698. [CrossRef] [PubMed]

27. Mikkola, R.; Saris, N.-E.L.; Grigoriev, P.A.; Andersson, M.A.; Salkinoja-Salonen, M.S. Ionophoretic Properties and Mitochondrial Effects of Cereulide. *Eur. J. Biochem.* **1999**, *263*, 112–117. [CrossRef] [PubMed]
28. Zhong, W.; Shahbaz, O.; Teskey, G.; Beever, A.; Kachour, N.; Venketaraman, V.; Darmani, N.A. Mechanisms of Nausea and Vomiting: Current Knowledge and Recent Advances in Intracellular Emetic Signaling Systems. *Int. J. Mol. Sci.* **2021**, *22*, 5797. [CrossRef]
29. Rajkovic, A.; Uyttendaele, M.; Deley, W.; Van Soom, A.; Rijsselaere, T.; Debevere, J. Dynamics of Boar Semen Motility Inhibition as a Semi-Quantitative Measurement of *Bacillus Cereus* Emetic Toxin (Cereulide). *J. Microbiol. Methods* **2006**, *65*, 525–534. [CrossRef]
30. Finlay, W.J.J.; Logan, N.A.; Sutherland, A.D. Semiautomated Metabolic Staining Assay for *Bacillus Cereus* Emetic Toxin. *Appl. Environ. Microbiol.* **1999**, *65*, 1811–1812. [CrossRef]
31. Hughes, S.; Bartholomew, B.; Hardy, J.C.; Kramer, J.M. Potential Application of a HEp-2 Cell Assay in the Investigation of *Bacillus Cereus* Emetic-Syndrome Food Poisoning. *FEMS Microbiol. Lett.* **1988**, *52*, 7–11. [CrossRef]
32. in 't Veld, P.H.; van der Laak, L.F.J.; van Zon, M.; Biesta-Peters, E.G. Elaboration and Validation of the Method for the Quantification of the Emetic Toxin of *Bacillus Cereus* as Described in En-ISO 18465—Microbiology of the Food Chain—Quantitative Determination of Emetic Toxin (Cereulide) Using Lc-MS/Ms. *Int. J. Food Microbiol.* **2019**, *288*, 91–96. [CrossRef] [PubMed]
33. Koike, H.; Kanda, M.; Hayashi, H.; Matsushima, Y.; Ohba, Y.; Nakagawa, Y.; Nagano, C.; Sekimura, K.; Hirai, A.; Shindo, T.; et al. Identification and Quantification of Cereulide in Cow's Milk Using Liquid Chromatography-Tandem Mass Spectrometry. *Food Addit. Contam. Part A* **2018**, *35*, 2424–2433. [CrossRef] [PubMed]
34. Decler, M.; Rajkovic, A.; Sas, B.; Madder, A.; De Saeger, S. Development and Validation of Ultra-High-Performance Liquid Chromatography–Tandem Mass Spectrometry Methods for the Simultaneous Determination of Beauvericin, Enniatins (A, A1, B, B1) and Cereulide in Maize, Wheat, Pasta and Rice. *J. Chromatogr. A* **2016**, *1472*, 35–43. [CrossRef] [PubMed]
35. Rønning, H.T.; Asp, T.N.; Granum, P.E. Determination and Quantification of the Emetic Toxin Cereulide from *Bacillus Cereus* in Pasta, Rice and Cream with Liquid Chromatography–Tandem Mass Spectrometry. *Food Addit. Contam. Part A* **2015**, *32*, 911–921. [CrossRef] [PubMed]
36. Doellinger, J.; Schneider, A.; Stark, T.D.; Ehling-Schulz, M.; Lasch, P. Evaluation of MALDI-ToF Mass Spectrometry for Rapid Detection of Cereulide From *Bacillus Cereus* Cultures. *Front. Microbiol.* **2020**, *11*, 511674. [CrossRef] [PubMed]
37. Walser, V.; Kranzler, M.; Dawid, C.; Ehling-Schulz, M.; Stark, T.D.; Hofmann, T.F. Distribution of the Emetic Toxin Cereulide in Cow Milk. *Toxins* **2021**, *13*, 528. [CrossRef] [PubMed]
38. Ulrich, S.; Gottschalk, C.; Dietrich, R.; Märtlbauer, E.; Gareis, M. Identification of Cereulide Producing *Bacillus Cereus* by MALDI-TOF MS. *Food Microbiol.* **2019**, *82*, 75–81. [CrossRef] [PubMed]
39. Ramarao, N.; Tran, S.-L.; Marin, M.; Vidic, J. Advanced Methods for Detection of *Bacillus Cereus* and Its Pathogenic Factors. *Sensors* **2020**, *20*, 2667. [CrossRef]
40. Huang, Y.; Flint, S.H.; Loo, T.S.; Palmer, J.S. Emetic Toxin Production of *Bacillus Cereus* in a Biofilm. *LWT* **2022**, *154*, 112840. [CrossRef]
41. Cui, Y.; Liu, Y.; Liu, X.; Xia, X.; Ding, S.; Zhu, K. Evaluation of the Toxicity and Toxicokinetics of Cereulide from an Emetic *Bacillus Cereus* Strain of Milk Origin. *Toxins* **2016**, *8*, 156. [CrossRef]
42. Bauer, T.; Stark, T.; Hofmann, T.; Ehling-Schulz, M. Development of a Stable Isotope Dilution Analysis for the Quantification of the *Bacillus Cereus* Toxin Cereulide in Foods. *J. Agric. Food Chem.* **2010**, *58*, 1420–1428. [CrossRef] [PubMed]
43. ISO 18465; Microbiology of the Food Chain—Quantitative Determination of Emetic Toxin (Cereulide) Using LC-MS/MS 2017. International Organization for Standardization: Geneva, Switzerland, 2017.
44. Shiota, M.; Saitou, K.; Mizumoto, H.; Matsusaka, M.; Agata, N.; Nakayama, M.; Kage, M.; Tatsumi, S.; Okamoto, A.; Yamaguchi, S.; et al. Rapid Detoxification of Cereulide in *Bacillus Cereus* Food Poisoning. *Pediatrics* **2010**, *125*, e951–e955. [CrossRef] [PubMed]
45. Moosmang, S.; Pitscheider, M.; Sturm, S.; Seger, C.; Tilg, H.; Halabalaki, M.; Stuppner, H. Metabolomic Analysis—Addressing NMR and LC-MS Related Problems in Human Feces Sample Preparation. *Clin. Chim. Acta* **2019**, *489*, 169–176. [CrossRef] [PubMed]
46. Staley, C.; Hamilton, M.J.; Vaughn, B.P.; Graiziger, C.T.; Newman, K.M.; Kabage, A.J.; Sadowsky, M.J.; Khoruts, A. Successful Resolution of Recurrent *Clostridium Difficile* Infection Using Freeze-Dried, Encapsulated Fecal Microbiota; Pragmatic Cohort Study. *Am. J. Gastroenterol.* **2017**, *112*, 940–947. [CrossRef] [PubMed]
47. Molnar, A.; Lakat, T.; Hosszu, A.; Szebeni, B.; Balogh, A.; Orfi, L.; Szabo, A.J.; Fekete, A.; Hodrea, J. Lyophilization and Homogenization of Biological Samples Improves Reproducibility and Reduces Standard Deviation in Molecular Biology Techniques. *Amino Acids* **2021**, *53*, 917–928. [CrossRef] [PubMed]
48. Berg, T.; Strand, D.H. ¹³C Labelled Internal Standards—A Solution to Minimize Ion Suppression Effects in Liquid Chromatography–Tandem Mass Spectrometry Analyses of Drugs in Biological Samples? *J. Chromatogr. A* **2011**, *1218*, 9366–9374. [CrossRef] [PubMed]
49. Biesta-Peters, E.G.; Reij, M.W.; Blaauw, R.H.; in 't Veld, P.H.; Rajkovic, A.; Ehling-Schulz, M.; Abee, T. Quantification of the Emetic Toxin Cereulide in Food Products by Liquid Chromatography-Mass Spectrometry Using Synthetic Cereulide as a Standard. *Appl. Environ. Microbiol.* **2010**, *76*, 7466–7472. [CrossRef] [PubMed]
50. Yamaguchi, M.; Kawai, T.; Kitagawa, M.; Kumeda, Y. A New Method for Rapid and Quantitative Detection of the *Bacillus Cereus* Emetic Toxin Cereulide in Food Products by Liquid Chromatography-Tandem Mass Spectrometry Analysis. *Food Microbiol.* **2013**, *34*, 29–37. [CrossRef]

51. Delbrassinne, L.; Andjelkovic, M.; Rajkovic, A.; Botteldoorn, N.; Mahillon, J.; Van Loco, J. Follow-up of the *Bacillus Cereus* Emetic Toxin Production in Penne Pasta under Household Conditions Using Liquid Chromatography Coupled with Mass Spectrometry. *Food Microbiol.* **2011**, *28*, 1105–1109. [CrossRef]
52. Yang, S.; Wang, Y.; Liu, Y.; Jia, K.; Zhang, Z.; Dong, Q. Cereulide and Emetic *Bacillus Cereus*: Characterizations, Impacts and Public Precautions. *Foods* **2023**, *12*, 833. [CrossRef]
53. Publications Office of the European Union. *Commission Implementing Regulation (EU) 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*; Publications Office of the European Union: Luxembourg, 2009; pp. 4535–4539.

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Challenges of Diphtheria Toxin Detection

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Abstract: Diphtheria toxin (DT) is the main virulence factor of *Corynebacterium diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis*. Moreover, new *Corynebacterium* species with the potential to produce diphtheria toxin have also been described. Therefore, the detection of the toxin is the most important test in the microbiological diagnosis of diphtheria and other corynebacteria infections. Since the first demonstration in 1888 that DT is a major virulence factor of *C. diphtheriae*, responsible for the systemic manifestation of the disease, various methods for DT detection have been developed, but the diagnostic usefulness of most of them has not been confirmed on a sufficiently large group of samples. Despite substantial progress in the science and diagnostics of infectious diseases, the Elek test is still the basic recommended diagnostic test for DT detection. The challenge here is the poor availability of an antitoxin and declining experience even in reference laboratories due to the low prevalence of diphtheria in developed countries. However, recent and very promising assays have been developed with the potential for use as rapid point-of-care testing (POCT), such as ICS and LFIA for toxin detection, LAMP for *tox* gene detection, and biosensors for both.

Keywords: *Corynebacterium*; diphtheria toxin; methods of detection; in vivo methods; tissue culture cytotoxicity assays; Elek test; immunoassays; PCR; LAMP; biosensors

Key Contribution: The detection of the DT is the most important test in the diagnosis of diphtheria. This paper review present various methods for DT detection described in the scientific literature.

1. Introduction

Diphtheria is a serious infectious disease caused by *Corynebacterium diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis*, although *C. diphtheriae* is indicated as the most common factor [1]. These Gram-positive bacteria most often colonise the mucous membranes of the throat and nose, sometimes also the larynx and trachea, less frequently the stomach, lungs, conjunctiva or mucous membranes of the genitals [2]. The bacteria may also infect skin and cutaneous lesions. Diphtheria toxin (DT) is the main virulence factor of *C. diphtheriae*, although toxigenic *C. ulcerans* and *C. pseudotuberculosis* strains have also been described. The toxin is extremely potent, with a minimal human lethal dose of less than 100 ng/kg of body weight [3]. The DT produced by the pathogens enters the bloodstream, spreads throughout the body and injures distant organs—mainly the heart, liver and central nervous system. The majority of deaths from diphtheria which result from severe complications of toxin absorption include acute systemic toxicity, myocarditis, cardiac failure, neurologic complications, primarily peripheral neuritis, and paralysis [4]. Recently, new *Corynebacterium* species with the potential to produce DT have been described, such as *C. silvaticum*, *C. rouxii* and *C. ramonii* [5,6]. DT is an approximately 58 kDa protein encoded by the *tox* gene of bacteriophage origin. *Tox* expression is regulated in response to changes in the Fe²⁺ level by the DtxR protein—a product of the DT repressor gene (*dtxR*) that presents on the

bacterial chromosome [7,8]. There are three domains present in the structure of mature DT: catalytic, transmembrane, and receptor-binding. The N-terminal catalytic domain with ADP-ribosyltransferase activity is named fragment A, and the transmembrane and receptor-binding domains determine fragment B, which are linked to each other by a disulfide bond [9]. The lethal effect of the toxin begins with the interaction of the receptor-binding domain with a receptor on the eukaryotic cell surface, which induces endocytosis. Inside the endosome, the disulfide bond is reduced, and the low pH causes a change in the conformation of the DT, which increases its affinity for membrane lipid molecules. The transmembrane domain is then incorporated into the endosomal membrane, thus allowing for cytosolic exposure of the catalytic domain and initiation of the specific toxicity reaction. Translation of host proteins is inhibited by inactivation of elongation factor 2 (EF-2) due to its ADP-ribosylation, resulting in cell death [9,10].

Since 1888, when Pierre Emile Roux and Alexandre Yersin isolated the DT and demonstrated that systemic disease manifestations were related to the toxin presence, much research on DT detection has been conducted [11]. The detection of the toxin is the most important test in the microbiological diagnosis of diphtheria [12]. Below, we present various methods for DT detection described in the scientific literature (Figure 1).

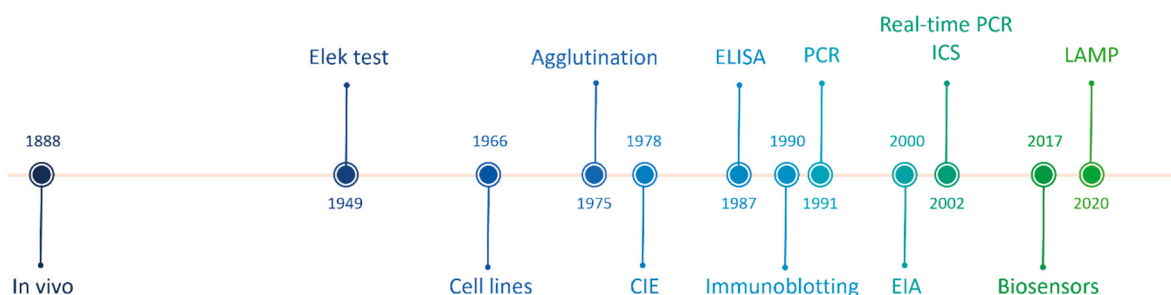


Figure 1. Diphtheria toxin detection in history.

2. Animal Models

The in vivo detection of DT is the traditional “gold standard”; however, currently, the test is used very rarely due to the availability of alternative methods. The detection of DT using in vivo methods is only possible if it retains the properties of a potent and lethal exotoxin. The first reports of the use of animals in diphtheria research date back to the second half of the nineteenth century, when Löffler reported the growth of diphtheria culture and postulated that the damage to the nasopharyngeal resulted from a soluble toxin. By 1888, Roux and Yersin soon confirmed the pathogenicity of *C. diphtheriae* for guinea pigs [13]. Animals injected with free filtrates of broth cultures of *C. diphtheriae* developed all manifestations of human diphtheria. This demonstrated that diphtheria exotoxin was the major virulence factor of *C. diphtheriae* [14]. The first studies using animals focused on understanding the mechanism of action of DT [13]. It was found that the sensitivity and resistance of different animal species to DT vary greatly. Man, dogs, rabbits, monkeys, and guinea pigs are susceptible species, while rats and mice are relatively resistant. Rats and mice can withstand $100 \times$ the dose of the toxin that is a minimally lethal dose for guinea pigs. Interestingly, humans have been shown to be five times more sensitive to the deadly effects of toxin than guinea pigs [15]. DT is known to be lethal to susceptible animals and unvaccinated humans at very low doses of 100 ng/kg or even less [16]. The action of the toxin is known to inhibit protein synthesis, but its detailed biochemical involvement in the pathogenesis of the disease has not been fully known until recently [13]. It is also known that the toxin does not affect all tissues equally and some organs are more sensitive to DT than others. Cardiac failure and myocarditis are recognised complications of severe diphtheria in humans. Inhibition of protein synthesis in the guinea pig myocardium is similar to the clinical manifestations of the disease in man. Therefore, the guinea pig was selected as a model organism for DT toxicity testing [17]. Since then, the subcutaneous virulence test and the dermatonecrosis assay have been applied in guinea pigs to examine

the toxigenicity of diphtheria isolates [18,19]. The presence of the toxin is recorded by the appearance of specific necrotic skin lesions (after 48 h) or the death of the animal (after 2 to 5 days). A rabbit skin test for investigation of *C. diphtheriae* virulence has also been proposed but is more often used for the assessment of diphtheria antitoxin [20].

In vivo tests are specific and sensitive but difficult to perform, of long duration, and with an inconvenient dependence on laboratory animal facilities. Before the introduction of in vitro cytotoxicity assays, animal assays were the only biological activity available from the DT detection method and are still considered the “gold standard” for toxigenicity tests [21]. It should be remembered that in vitro methods reflect only in vitro data, and it cannot be ruled out that isolates that are not toxic (e.g., non-toxic *tox* gene-bearing (NTTB) isolates) or weakly toxigenic in vitro are capable of producing DT during in vivo experiments [21]. The WHO Manual for the Laboratory Diagnosis of Diphtheria from 1994 mentioned the in vivo subcutaneous test for virulence in guinea pigs as the most reliable method for detection of DT production, but it did not recommend it for inexperienced laboratory staff. The current WHO laboratory manual for the diagnosis of diphtheria and other related infections, published in 2021 [22], does not mention in vivo toxin testing due to the availability of an alternative test (reducing animal suffering) such as the Elek test.

3. In Vitro Tissue Culture Cytotoxicity Assays

In vitro cytotoxicity assays are a suitable alternative to the in vivo virulence test. The first studies on DT using tissue cultures investigated the biochemical mode of action of the toxin [23]. These findings provided important data to justify the clinical symptoms that occur in humans. The information that DT inhibits protein synthesis was confirmed for the first time in vitro in the HeLa cervical cancer cell line by Strauss and Hendee [23]. Subsequent studies using guinea pig heart muscle tissue confirmed that biochemical heart injury was correlated with cardiac failure and tissue pathology observed in many cases of fatal diphtheria infections in humans [23]. Numerous studies have shown that cells from animals sensitive to DT are more sensitive to the toxin than cells from resistant animals; for example, guinea pig tracheal cells are 500 times more susceptible to the action of DT than murine fibroblastic cell line L929 [24,25]. Interestingly, resistance to the toxin has also been demonstrated in guinea pig embryos [24]. The inhibitory effect of DT on protein synthesis was also observed in tissue from skeletal muscle, liver, and brain. The results obtained with human cells and rabbit reticulocytes suggest that the reaction of inhibition of protein synthesis by DT is probably common to all sensitive mammalian cells [24]. Several tissue culture methods were developed to detect DT produced by live *C. diphtheriae*. Many research teams have tested a number of different cell lines for cytotoxicity of DT testing, among which the Vero line (African green monkey kidney cells) was probably the most popular [19,26,27]. Other cell lines used for the test primarily included rabbit kidney tissue culture cells, Chinese hamster ovary (CHO) cell line and HeLa cells [28–30]. The cytotoxic action of DT on cultured mammalian cells is based on the inhibition of protein synthesis, which leads to cell death [31]. In general, in most tissue culture methods for DT detection, *C. diphtheriae* culture filtrates or whole broth cultures are added to cell monolayers and incubated between 24 h to 5 days. A negative control (a toxin neutralised by an antitoxin) is used to exclude non-specific cytotoxic effects. Using dilutions of bacterial culture supernatants, cytotoxicity titres can be determined, which will enable quantitative determination of DT production by a given inoculum of the appropriate *C. diphtheriae* strain. Cytotoxic effects are usually directly visible or calorimetrically measurable. Moreover, Andre et al., in their study on monkey kidney tissue culture cells, confirmed that material with *C. diphtheriae* directly from throat swabs could be examined for cytotoxic activity [32].

The studies conducted by many research teams have confirmed that the results of optimised tissue culture cytotoxicity assays for DT detection demonstrate compatibility with the results of in vivo methods. The in vitro assays were confirmed to be specific, accurate and reliable in the detection and the quantification of biologically active DT produced by *C. diphtheriae* isolates. Moreover, the in vitro cytotoxicity assays are an order

of magnitude more sensitive than the intradermal tests *in vivo*. The limits of DT detection *in vitro* are in the range between 10 and 100 pg/mL and are dependent on the method applied [19,27,29,33]. The *in vitro* tissue culture cytotoxicity assay is currently mainly used for measuring serum antitoxin levels. The use of the Vero cell line is recommended [22].

4. Immunological Assays

Immunoassay methods are based on the antigen–antibody reaction and are usually performed with mouse monoclonal antibodies (mAb) and, more rarely, equine polyclonal antibodies (pAb) directed against subunit A or B of DT. The limit of detection (LOD) for those methods is 0.1–4.0 ng/mL [34].

4.1. Elek and Modified Elek Tests

The immunodiffusion (or immunoprecipitation) assay commonly called Elek test was developed by Orjan Ouchterlony and Stephen D. Elek independently in 1948 [35,36]. The test is based on a precipitation phenomenon that occurs between the toxin and the antitoxin, creating visible precipitation lines on semi-solid media. The sensitivity of the test is comparable with that of the *in vivo* test on guinea pigs. In the test, toxin-positive, toxin-negative, and tested strains are spread on the agar surface in a straight line across the plate and at an angle to the paper strip soaked in the antitoxin and placed on the medium with bacteria inoculum. Following the reaction, precipitation lines are formed between the antitoxin strip and the line of bacterial growth, indicating toxin production [36,37]. The test results are visible after 24 to 48 h. The recommended basal medium is the improved agar base supplemented with serum. The medium might be supplemented with rabbit, calf, adult bovine serum and newborn bovine serum, with the best standard recommended by the WHO being newborn bovine serum [12,22]. The reliability of the test is strongly related to the quality of the medium and antitoxin used, and therefore each batch of medium, serum and antitoxin should be checked before application for the diagnostic test [12,22,37].

The first modification of the Elek test was proposed by Feldman et al. [38]. They used discs soaked with an antitoxin around which the culture of the strains was inoculated. This test was used in countries of the USSR during the diphtheria epidemic in the 1990s. [39]. Other modifications of the Elek test was proposed in the 90s by Engler et al. [40]. In their study, they paid attention to the volume of the medium, concentrations of the antitoxins, inoculum density and the distance of inoculation from antitoxin disks. By changing all these parameters, they were able to reduce the incubation time. A thinner medium with less density and a closer distance between the antitoxin disc and inoculum reduced the time to obtain the results of the test to within 16 to 24 h. Moreover, the authors suggested that extending the incubation time may result in the occurrence of non-specific lines of precipitation. Further modifications proposed in the scientific literature concerned the medium used for the test, for example, the application of Columbia blood agar base instead of Elek base medium [41] and the application of a modified medium for simultaneous Cystinase and Elek tests [42].

The test originally proposed by Elek replaced the *in vivo* virulence test in guinea pigs, the use of which, due to high costs, the risk of accidental self-inoculation and extended waiting times, has been reduced in most laboratories [21]. Currently, the Elek test (and the modified Elek test) is recommended as the “gold standard” in diphtheria diagnostics. Both conventional and modified Elek tests are recommended in the current version of the WHO laboratory manual for the diagnosis of diphtheria and other related infections [22]. The manual presents a step-by-step procedure for performing the test, including important quality recommendations. However, it must be underscored that the tests require experienced staff to avoid misinterpretation of the results. The inconvenience of using the Elek method in routine diagnostics may be the problem of limited access to supplies of diphtheria antitoxin. Antitoxin is available from only a few sources worldwide, mostly from India [22]. It is therefore extremely important to look for alternative, more easily achieved methods.

4.2. Counterimmunoelectrophoresis

Counterimmunoelectrophoresis (CIE) was originally described by Bussard and Huer in 1959 and used for the detection of hepatitis virus antigens (HBsAg) [43]. Almost 20 years later, the method was proposed for the detection of DT in *C. diphtheriae* cultures [44]; this method refers to the formation of a precipitation line in agar. The process is a modification of immunoelectrophoresis and takes place under the influence of an electric field. In CIE, the antigen and antibody move in opposite directions, forming precipitates at the point of their connection. In the obtained results, the presence of a precipitation line indicates the corresponding antigen to the antibodies. In the CIE test, the DT production can be detected within 16 to 24 h. The test was developed using five toxigenic *C. diphtheriae* strains, one non-toxigenic *C. diphtheriae* strain, one *C. hemolyticum* and three unspiciated diphtheroid isolates, and the influence of the culture medium and culture conditions on the test results was indicated.

4.3. Agglutination Methods

The application of various agglutination-based tests has been proposed for DT detection, including latex agglutination, coagglutination, slide agglutination and haemagglutination. Holmes and Perlow developed a reverse passive haemagglutination (RPHA) test for the detection of DT in 1975 [45]. The RPHA test was conducted in microtiter plates with V-bottom wells. The purified antitoxic antibodies were coupled to sheep erythrocytes and agglutination occurred after overnight incubation at 4 °C when DT was present in *C. diphtheriae* culture supernatant. The detection limit of DT by the RPHA test was about 200 pg/mL. However, the study was conducted only on the reference strain *C. diphtheriae* PW8 and on the mutants of the strain C7 of *C. diphtheriae* and compared to an immunodiffusion method. Jalgaonkar and Saoji applied a coagglutination test, in which Cowan I strain of *Staphylococcus aureus* was used as a carrier particle to coat antibodies [46]. An antibody to the DT was coupled to protein A of *Staphylococcus aureus*. Protein A combines with the Fc portion of the antibody, enabling the antigenic Fab regions to be freely exposed on the surface of the *S. aureus* cells. The sensitised *S. aureus* cells were mixed with the bacterial culture supernatants on a slide and agglutinate within 10 min if the DT was present. The test results were consistent with the conventional guinea pig pathogenicity test, but it was verified only on eight *C. diphtheriae* clinical isolates, including five toxinogenic isolates. A reversed passive latex agglutination (RPLA) test for the detection of toxigenic *C. diphtheriae* isolates was developed in 1997. In this assay, the affinity-purified antibodies were attached to latex particles and reacted with the soluble DT [47]. For the RPLA assay, bacterial strains were grown in Elek broth medium with 10% calf foetal serum at 34 °C with shaking. It was demonstrated that 4 h of cultivation was not enough for DT production in slow toxin producer isolates, while 6 h of cultivation was enough to obtain a clearly positive result. In a U-bottom microtiter plate, the bacterial culture supernatants were serially diluted twofold. Subsequently, an equal volume of sensitised latex was added to each well. The microtiter plate was covered, shaken thoroughly and incubated at room temperature. Agglutination was assessed visually after 6 h. If DT was present in the tested supernatants, agglutination occurred as a result of the formation of a molecular lattice and a dispersed layer was formed at the bottom of the well. In the absence of DT, the latex particles sedimented to the bottom of the well and formed a tight button. The DT titre was determined as the reciprocal of the highest dilution in which agglutination was recorded. The RPLA assay enables the rapid detection of the DT in 12 h, of which 6 h are required for bacteria cultivation for toxin production, and an additional 6 h are needed to observe latex agglutination. The limit of detection of DT by the RPLA assay was about 5 ng/mL. The assay results were in agreement with the results of PCR and the Vero cell cytotoxicity assays. But the assay was evaluated only with seven *C. diphtheriae* isolates—five positive and two negative for toxin production.

4.4. Enzyme-Linked Immunosorbent Assay

The enzyme-linked immunosorbent assay (ELISA) for DT determination was described in [48]. ELISA can be a qualitative or quantitative method, with quantitative immunoassay methods based on mAbs being easier to standardise [34]. The results of sandwich ELISA cover the results of other methods for DT detection, such as the Elek test or genetic identification of the *tox* gene, and are characterised by high sensitivity (approximately 99%) and specificity (100%). But occasional false positive results were also documented. The detection limit of DT is of less than 1 ng/mL [34]. The use of ELISA to determine the level of the toxin production by strains may be helpful in assessing the pathogenic potential of isolates [49]. This is very important because diphtheria epidemic isolates cause more severe forms of infection in unvaccinated children when they secrete greater amounts of the toxin [50]. There are many difficulties in the detection of a weakly toxigenic strain of *C. diphtheriae*. Some authors have suggested that ELISA may be useful as a reference in vitro method for detecting isolates with questionable toxin production. The research carried out by Simonova et al. showed that *Corynebacterium* strains with low levels of DT were not detected by the Elek test and gave positive results in ELISA [34]. Although the ELISA method is suitable for detecting toxigenic diphtheria isolates in routine practice, this is a labour-intensive and time-consuming assay. Another disadvantage is that there are no commercially available kits and all ELISA tests are based on the validation of the internal method.

4.5. Enzyme Immunoassay

The enzyme immunoassay (EIA) is a modification of the ELISA test for rapid diagnostics [18]. EIA is also characterised by a very high diagnostic sensitivity (LOD is 0.1 ng/mL). In a comparative study of 220 toxigenic *Corynebacterium* isolates, the results obtained by the EIA method correlate 100% with the results obtained by the Elek test [51]. Engler et al. also reported 100% consistency of the results of both methods [51]. This shows that it is a highly accurate and specific method for the detection of toxigenicity. EIA provided a result within 3 h of colony selection compared to 24 h for the modified Elek test and 48 h for the conventional Elek test. Furthermore, the interpretation of the EIA results was easier than for that of the Elek test. Isolates that showed weak precipitation lines in the Elek test gave a good visible colour change in the EIA [51]. To increase the detection of isolates with the biological activity of DT, the standardisation of the density of the inoculum and the incubation time in broth are very important, particularly among isolates producing low levels of the toxin [51].

4.6. Immunoblotting

Whereas the ELISA and EIA methods are good for testing culture supernatants, the immunoblotting method is better suited for studies of whole lysates of pathogenic corynebacteria, and this is only a qualitative method. Nitrocellulose membranes are used as solid phases that bind to protein more strongly than solid plastic phases (ELISA, EIA). The LOD (analytical sensitivity) of this method is about 5–10 ng/mL [52]. Immunoblot detection can be characterised by a low percentage of false positive results for nonspecific binding. To improve the specificity of the assay, proteins were/are denatured and separated by SDS-PAGE electrophoresis before detection. The results can be obtained after 11 h, but for weakly toxigenic isolates, the test must be extended to approximately 24 h [52]. The limitation of immunoblotting assays is the inability to distinguish between biologically active and inactive toxins. Despite many advantages (high specificity and sensitivity), due to time and labour consumption, immunoblotting is not recommended for routine diagnostic use [19,52,53].

4.7. Immunochromatographic Strip Tests

The reagents previously used in the enzyme immunoassay (EIA) [51] were applied to develop an immunochromatographic strip test (ICS). The ICS test uses equine polyclonal

antitoxin coated onto nitrocellulose membrane as the capture antibody, and colloidal-gold-labelled monoclonal antibodies specific for fragment A of the DT molecule as the detection antibody. The assay enables rapid detection of DT from pure *C. diphtheriae* clinical isolates as well as from clinical specimens (throat swabs) [54]. Using the ICS test, a definitive toxicity result is available within 3.5 h for pure isolates. *C. diphtheriae* colonies from Columbia blood agar should be suspended in serum-supplemented Elek broth (SSEB) and incubated for 3 h at 37 °C. In the case of toxicity testing directly from throat swabs, 16 h of incubation in the SSEB medium is required. Subsequently, an ICS test strip should be added to each broth tube, and the result is available within 10 min. In addition, 100% consistency has been demonstrated between ICS and Elek test results for 915 pure clinical *Corynebacterium* isolates (851 *C. diphtheriae* and 64 *C. ulcerans*), and 99% agreement for results obtained for throat swabs directly inoculated into broth cultures (848 of 850 specimens), which results in a sensitivity and specificity for the ICS test of 98% and 99%, respectively. The limit of detection of the ICS test is 0.5 ng of DT per ml. In conclusion, the ICS test is reliable, highly sensitive and specific. The test is simple to perform and enables the rapid detection of DT. Moreover, this method does not require any specialised equipment and the results of ICS test are easy to interpret. Unfortunately, the production of the ICS strips ceased [55].

Another study performed by Melnikov et al. [56] presented lateral flow immunoassay (LFIA) as a method for detection of DT, in which a pair of monoclonal antibodies specific to subunit B of DT, previously used to develop the ELISA assay by Simonova et al. [34], was applied. In the case of the ICS test, the monoclonal antibody against subunit A of DT was used as the detecting antibody [54]. LFIA test strips were manufactured under the H2020-MSCA-IF-2018 (843405-DIFTERIA) research programme by Senova (Weimar, Germany). A pair of mouse monoclonal antibodies to the DT B subunit—IgG1 anti-DT 675-3 (capture antibody on the solid support) and IgG1 anti-DT 676-3 labelled with colloidal gold (detection antibody) and polyclonal goat anti-mouse IgG (control antibody)—were applied in LFIA. The test strips were placed into lateral flow cassettes. LFIA for DT detection was validated with the use of 322 corynebacterial cultures as well as 360 simulated diphtheria specimens, which were obtained by adding test strains of corynebacteria to human throat samples. In addition, 24 h corynebacterial cultures on Columbia blood agar were inoculated on Elek broth, and after 6 h of incubation at 37 °C, 100 µL of the liquid culture was added to the LFIA cassette, with the result being available within 15 min. The simulated diphtheria specimens were plated on selective Hoyle's tellurite agar and cultivated for 18–24 h at 37 °C. Next, a mixture of 5–10 grey/black colonies, characteristic of corynebacteria, were inoculated on Elek broth and then cultivated for 6 h at 37 °C. It was shown that LFIA detected DT in corynebacterial cultures with high accuracy. The LFIA results obtained for 321 of the 322 corynebacterial strains were consistent with the results of the Elek test. It was demonstrated that the diagnostic sensitivity of the LFIA for DT detection on bacterial cultures was 99.35%, and the specificity was 100% [56]. Compared to the Elek test, the LFIA test is much simpler and faster to perform. Implementation of the LFIA into routine usage may speed up the detection of toxigenic corynebacterial isolates.

5. Nucleic Acid Amplification Tests

Nucleic acid amplification tests (NAATs) are rapid molecular techniques that enable the detection of small amounts of genetic material in tested samples. NAATs for toxigenicity determination are based on the detection of the *tox* gene encoding DT [22]. Since the presence of the *tox* gene does not necessarily determine the production of DT—as non-toxigenic strains bearing the *tox* gene (NTTB) of *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* have been reported [19,57–61]—all positive NAATs results in patients suspected of diphtheria must be phenotypically confirmed, for example, by the Elek test [22]. However, *tox*-positive NAATs results can be considered for further preventive treatment of patients without waiting for the Elek test results [22,62]. In contrast, *tox*-negative results are considered definitive and do not require confirmation by other methods [22,62]. The use of NAATs allows for the quick exclusion of toxigenicity in tested isolates and

prevents unnecessary further phenotypic measures for toxin presence. Moreover, unlike phenotypic tests, NAATs can detect NTTB isolates, which pose a potential epidemiological threat as they could theoretically reactivate *tox* expression after phage conversion or DNA recombination [22].

5.1. Polymerase Chain Reaction

The first NAATs used for *tox* detection were based on conventional polymerase chain reaction (PCR), in which fluorescently stained PCR products are resolved on an agarose gel, and were described in the 1990s [63–66]. Among the conventional PCR systems, those developed by Pallen et al. [64], Hauser et al. [65], and Nakao and Popovic [66] have likely been the most widely used [57]. The PCR system described by Pallen et al. [64] focuses on the amplification of fragment A of the *tox* gene (*toxA*), encoding the catalytic subunit of the toxin. In contrast, the system by Nakao and Popovic [66], an extension of the previous system with an additional primer set, also amplifies fragment B of the gene (*tox*B), which encodes the translocation and receptor-binding domains [63,66]. The PCR by Hauser et al. [65] targets both the A and B fragments in a single amplicon (*tox*AB) [65]. Most assays using these protocols have been performed on pure bacterial cultures, but direct *tox* detection from clinical specimens has also been reported [57,66,67]. The protocols can be used on preparations of simple boiled cells or pure extracted DNA [22]. The LOD calculated for the Nakao and Popovic [66] test ranges from 50 to 1500 CFU/PCR mixture [66,67]. The *toxA* amplifying primer set was found to be more sensitive than the *tox*B primer set [66,68]. Data from numerous studies have shown 100% correlation between the results of these three conventional PCR systems and phenotypic methods, such as in vivo ADP-ribosylation activity and the Elek test [19,57,64–67,69–72]. However, it should be noted that NTTB strains identified by PCR but not by phenotypic methods have been described [19,58,59]. The three most commonly used conventional PCR systems, although originally designed for *C. diphtheriae* toxicity testing, have frequently been used for the effective amplification and detection of the *C. ulcerans tox* gene. This occurred despite approximately 5% divergence between the *C. ulcerans* and *C. diphtheriae tox* gene nucleotide sequences [57,73]. Specific detection of the *C. ulcerans tox* gene alone—without the *C. diphtheriae tox* gene—was possible using primers developed by Sing et al. [57,73,74].

Faster and more sensitive detection of toxigenicity compared to conventional PCR was achieved using real-time PCR, a method that monitors the amplification of target DNA during the process. The first real-time PCR assay for toxin detection was developed at the Centers for Disease Control and Prevention (CDC) and published in 2002 [22,67]. This method involved the amplification of *toxA* and *tox*B fragments and their real-time detection using hydrolysis probes (TaqMan). The analytical sensitivity of the assay was estimated at 2 CFU (17 fg of genomic DNA), which was 750-fold higher than that of the conventional PCR method by Nakao and Popovic [66,67]. Higher sensitivity of the test compared to conventional PCR was also demonstrated in clinical specimens [57,67]. In the authors' evaluation, the test achieved 100% sensitivity and specificity, although further studies showed that it was unreliable in assessing the toxicity of some *C. ulcerans* isolates, resulting in atypical or false-negative results [75,76]. These observations led Schuëgger et al. [74] to design primers as well as TaqMan hydrolysis probes targeting regions of the *tox* gene that are conserved in both *C. diphtheriae* and *C. ulcerans* [57,74]. This assay demonstrated 100% sensitivity and specificity with a detection limit of 100 fg of DNA per reaction, making it 25 times more sensitive than the conventional PCR designed by Hauser et al. [65,74]. This method has also been successfully used by others to identify the *tox* gene of both *C. diphtheriae* and *C. ulcerans* [57,77]. Real-time PCR for the *tox* gene was also designed in another popular real-time detection format using LightCycler hybridisation probes [57,71]. Evaluation showed that the method had 100% sensitivity and specificity with a detection limit of 100 fg per reaction. Moreover, LightCycler melting curve analysis allowed for the differentiation of *C. diphtheriae* and *C. ulcerans* species [71]. However, based on the LightCycler melting curve analysis using fluorescence resonance energy transfer (FRET)

probes, a new group of feline *C. diphtheriae* strains—whose *tox* gene sequence is more similar to *C. ulcerans* *tox* than to the *tox* of human *C. diphtheriae* strains—may be misclassified as *C. ulcerans* [57,71]. Importantly, due to recent changes in the taxonomy of *C. diphtheriae*, these atypical, feline-derived strains have been proposed to be reclassified into a novel species, *Corynebacterium rouxii* [78]. To overcome the problems related to *tox* gene heterogeneity, Mancini et al. [79] developed a diagnostic algorithm combining two different real-time PCR assays based on the *tox* and *rpoB* gene amplifications with LightCycler melting curve analysis. The analysis of melting curve profiles of the *rpoB* amplicons (the gene encoding the β subunit of RNA polymerase) enabled species differentiation between *C. diphtheriae* and *C. ulcerans*, while the analysis of the *tox* gene effectively distinguished the *tox* from *C. diphtheriae* and *C. ulcerans*/*C. pseudotuberculosis* species [79]. However, as the authors noted, potential misclassification cannot be ruled out, as shown by the unexpected results of melting temperature obtained for single strains of *C. ulcerans* and *C. pseudotuberculosis*.

In addition to the above, multiplex PCR (mPCR) assays, based on conventional or real-time systems, have been designed to combine *tox* gene detection with identification of corynebacterial species [79–84]. The species-specific conventional mPCR proposed by Pimenta et al. [80] combined the detection of *tox*A and *tox*B with *C. diphtheriae* identification using primers targeting *dtxR*, the product of which acts as a global regulator of metabolism in both toxigenic and non-toxigenic strains, including the regulation of *tox* expression [80]. Another conventional mPCR based on the amplification of the *tox* and *dtxR* genes was described by Sunarno et al. [84], which, in addition to *tox* detection and differentiation of three potentially toxigenic corynebacterial species (*C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis*), was also used to predict some NTTB type strains (deletions at position 25 or 55 of the *tox* gene) [84]. The conventional pentaplex PCR assay proposed by Torrez et al. [81], in addition to *tox*-specific primers (a region between fragments A and B), contains primers for detection of *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis*. These primers correspond to the following genes: *rpoB* for *Corynebacterium* spp. identification, 16S rRNA sequence specific for *C. ulcerans* and *C. pseudotuberculosis*—*pld* a *C. pseudotuberculosis*-specific sequence (gene encoding PLD exotoxin), and the *dtxR* gene of *C. diphtheriae*. De Zoysa et al. [83] developed and validated a quadruplex real-time PCR targeting fragment A of *tox*-carrying corynebacteria, a *rpoB* fragment specific for *C. diphtheriae*, and a second *rpoB* fragment specific for *C. ulcerans*/*C. pseudotuberculosis* [22,83]. To test PCR inhibition, an internal control in the form of green fluorescent protein (*gfp*) gene amplification after addition of control DNA to the samples was included in the assay. The assay demonstrated 100% diagnostic sensitivity, high specificity (98–100%), and 100% concordant results with those of phenotypic tests (excluding NTTB strains) and the conventional PCR of Pallen et al. [64]. The method correctly identified *C. ulcerans* strains that had previously yielded false-negative results in the Mothershed et al. test [67]. The limit of detection was lower than 1 copy per reaction for all three targets [22,83]. The results were available 3–4 h after receiving the isolate, significantly reducing the time to report a result compared to conventional PCR [83]. This test has been used in Public Health England since 2014 and is currently recommended by the WHO for identification of potentially toxigenic corynebacteria. Since the use of *gfp* as an internal control did not provide information on the efficiency of bacterial DNA extraction, this method was recently updated by replacing the *gfp* control with amplification of a 16S rRNA universal fragment [82]. This modified pentaplex real-time PCR has been subsequently validated, and its usefulness was confirmed in studies on both bacterial strains and clinical samples—on both pure DNA preparations and boiling preparations [82]. Other assays using real-time multiplex PCR have also been described for *tox* gene identification and discrimination of potentially toxigenic corynebacteria using a set of primers and probes corresponding to the *tox* and *rpoB* genes [61,85] or to the *tox* and *dtxR* genes [86].

5.2. Loop-Mediated Isothermal Amplification

Another molecular technique that has been recently used to detect *C. diphtheriae* and to differentiate between toxigenic and non-toxigenic strains is loop-mediated isothermal amplification (LAMP) [87]. In general, the LAMP method is a highly specific amplification reaction using strand-displacing DNA polymerase, 4–6 primers recognising 6–8 distinct regions of target DNA, which generate concatemeric products under constant temperature conditions [88]. Zasada et al. designed two LAMP assays for the detection of *tox* and *dtxR* genes [87]. The developed assays were highly specific and sensitive (100% specificity and 100% sensitivity compared to conventional PCR and Elek test), with a low detection limit estimated at 2.84 pg of DNA per reaction when the amplification was carried out for 60 min. The sensitivity of the assays decreased about 10-fold for every 10 min reduction in the reaction time and 2.84 ng of the template was the detection limit when the reaction incubation time was 30 min. The proposed tests, in addition to being simple and quick to perform, were cost-effective—they do not require a very precise heating device, as the tests were effective at temperatures from 62 to 70 °C, and their results were successfully visualised by a colour change in the reaction mixture. The authors proposed the application of various colorimetric indicators, such as hydroxynaphthol blue (HNB), QuantiFluor and calcein, as well as immunochromatographic strips when the labelled primers were used for the reaction. Colorimetric indicators added to the reaction mixture before incubation enabled a faster detection of the positive reaction in the real-time mode [87]. The proposed LAMP assay has the potential for Point-of-Care Testing (POCT) diagnosis of diphtheria and other *C. diphtheriae* infections, although this would require prior testing of their performance directly on clinical specimens.

6. Biosensors

Biosensors are a new technology that has been developed for diagnostic purposes in recent years. Biosensors are specific analytical probes consisting of a biological part that functions as a sensor and a transducer that converts biological changes into a signal that is proportional to the concentration of the detected agent [89]. There are two main groups of biosensors: (i) optical biosensors, that often use surface plasmon resonance (SPR), colorimetry and fluorescence; and (ii) electrochemical biosensors that are usually based on impedance, amperometry and voltammetry [90]. According to the literature, there are only a few biosensors developed for detecting DT or the DT gene. The function of biosensors for DT detection is similar to the technology used in ELISA tests, whereas the biosensors for *tox* gene detection are based on the phenomenon of specific hybridisation of DNA strands. Moreno-Bondi et al. [91] designed a biosensor consisting of a set of specific IgG antibodies that were immobilised on borosilicate microscope slides by the interaction of lysine residues of the antigens with the N-hydroxysuccinimidyl ester terminus. This biosensor allows the analysis of patients' serum to detect the DT, tetanus toxin, staphylococcal enterotoxin B and hepatitis B [91]. Detection was carried out using Cy5-labelled goat anti-human IgG secondary antibodies and measuring the fluorescence level. The tests showed detection limits in the range of 0.2–3 µg of specific IgG per mL, with an absolute optical detection limit of approximately 100 fg [91]. However, the authors of the cited article indicated that a significant problem of the designed biosensor is the possibility of absorbing non-specific antigens that may be present in the serum in excess of the detected antigens. Furthermore, the high protein content as well as the high viscosity of undiluted serum may also interfere with the test. Further research is necessary to improve the diagnostic potential. Another type is the optical biosensor proposed by Zeinoddini et al. [92]. A localised surface plasmon resonance (LSPR) nanobioprobe was used to design it, in which monoclonal anti-DT IgG antibody and gold nanoparticles were conjugated. In order, fluorescence spectroscopy was used to detect territorial structural alterations in the antibody in the presence of DT [92]. The authors of the article indicate that the sensitivity of this method is about 10 ng/mL, and the time necessary to perform the test is about 1–2 h [92]. Therefore, the LSPR biosensor has high potential to be used for rapid DT detec-

tion compared to traditional methods. However, it seems that electrochemical biosensors would be the most optimal due to their simplicity of use, low measurement costs and desirable miniature size. Ziółkowski et al. [93] present an in-depth analysis, which resulted in an electrochemical biosensor with anti-DT IgG antibodies, for the immobilisation of which an intermediate cysteamine layer and a crosslinker—glutaraldehyde—were used. The antibody-based receptor layer was tethered to gold nanospheres, and BSA acted as a blocking agent to reduce non-specific adsorption [93]. The signal change against DT was calculated on the basis of reduction peak currents and charge transfer resistance, derived from square-wave voltammetry and impedance spectroscopy. The linear dependence of redox current change against DT was recorded within 10^{-4} to 10^{-1} Lf/mL, where the Lf is defined as the antigen-content-forming 1:1 ratio against 1 unit of antitoxin [93]. Even better specificity and detection level were obtained using an electrochemical biosensor in which the gold electrode surface was modified by co-adsorption of HS-L-Biotin and diethyldithiocarbamate [94]. This special layer was characterised by reduced nonspecific protein adsorption and high conductivity. Anti-DT IgG antibodies were immobilised by a biotin-streptavidin reaction. The mechanism of detection based on the differential analysis of square wave voltammetry (SWV) allowed the authors to obtain a lower detection limit at the level of 5×10^{-6} µg/mL [94]. A very interesting concept was proposed by Ameku et al. [95]. An electrochemical immunosensor was developed in which a dipeptide consisting of two epitopes of DT was conjugated to the surface of a pencil-lead electrode integrated into a portable electrode holder. This system was able to capture anti-DT IgG antibodies for measurement by an indirect immunoassay using secondary antibodies conjugated with alkaline phosphatase, which, in turn, enzymatically generates hydroquinone, a redox molecule measurable by SWV. Under the optimised working conditions, a limit of detection of 5×10^{-6} IU/mL was achieved [95]. For detection of the *tox* gene, Marchlewicz et al. [96] proposed an electrochemical genosensor (DNA biosensor) with the potential to be used in a portable device. The detection is based on the combination of asymmetric PCR (aPCR) and a specific stem-loop structure DNA probe immobilised on the electrode surface. The probe hybridises to the aPCR product, resulting in the changes in the electrochemical signal. The detection limit is 20.8 nM of the target (*tox* gene fragment) in 5 min or 0.5 nM in 30 min.

7. Variations in the Nucleotide and Amino Acid Sequences of DT

Changes in the nucleotide sequences of the *tox* gene and in the amino acid sequences of the toxin may strongly influence the sensitivity of NAAT and immunological-based diagnostics, respectively. As has been mentioned in the beginning of this paper, the DT gene (*tox*) occurs in some corynephages (mainly β) and by lysogenic conversion, and it may be integrated with the chromosome of corynebacteria, primarily *Corynebacterium diphtheriae* but also *C. pseudotuberculosis*, *C. ulcerans* and others [6,97]. Comparative genomic studies of 12 *C. diphtheriae* clinical strains in relation to references *C. diphtheriae* NCTC 13129 showed great diversity of the sequenced *tox*-positive corynephage integrated with the bacterial genomes but not along its entire length. The right side of the prophage genomes is characterised by the greatest nucleotide sequence similarity. This region harbours the *tox* gene and is specified by a decreased GC content [98]; hence, there is practically no variability observed within the DT gene [99]. Even though *tox* is extrinsic, it is regulated by a chromosomally encoded protein DtxR (DT repressor), which is a transcriptional regulator that uses Fe^{2+} as a corepressor [100].

Research on identifying the sequence of the DT and DtxR structural genes began in the early 1980s [101,102] and 1990s [7], respectively. This paved the way to analysing the evolution of these genes among isolates originating from different places and at different times. PCR-single-strand conformation polymorphism analysis (PCR-SSCP) of 72 *C. diphtheriae* clinical strains isolated in Russia and Ukraine showed heterogeneity of the *tox* and *dtxR* genes [103]. Further analysis by sequencing revealed that in the case of the *tox* gene, there were three different point mutations: one in the A subunit and three in the B subunit,

but all four were silent. Significantly more heterogeneity was detected in the *dtxR* gene, namely 35 point mutations were detected, 9 of which resulted in amino acid substitutions in the carboxyl-terminal half of DtxR and 26 of which were silent [66].

Much more recent research performed in Indonesia confirms these observations [99]. Among 89 throat swabs from patients with a clinical diagnosis of diphtheria, 10 toxin-producing *C. diphtheriae* isolates were detected, for which the *tox*B sequence was analysed. Nine isolates showed an SNP mutation (G30A), but the mutation did not change the amino acid sequence of the toxin. In consequence, the authors point to other factors that could have reduced the efficacy of the diphtheria vaccine used in Indonesia, for example, mutations in the toxin promoter/operator region (*tox* PO) [99]. The results of the research by Kolodkina et al. showed that the increase in the level of DT production is determined by the mutation located in the 9-bp palindrome, which overlaps the −10 sequence of the promoter and the operator region [104].

Despite the emphasis on the fact that DT produced by *C. diphtheriae* is highly conserved at the amino acid level [66,99,105], it is worth noting that there are few reports showing heterogeneity of *tox* sequences between and within *Corynebacterium* species [73,106]. Comparative analysis of *tox* sequences of the *C. diphtheriae* and three *C. ulcerans* strains resulted in remarkable differences at the nucleotide but also at the amino acid level, mainly in the B fragment of DT. Inter- and intra-species variability has been proven and it has also been shown that DT amino acid sequences from a *C. ulcerans* isolate associated with classical diphtheria are less well conserved than those from *C. diphtheriae* [106]. It is extremely important to take this fact into account when diagnosing diphtheria and carefully design primers and probes for detecting the *C. ulcerans* *tox* gene using NAATs [74].

8. Conclusions

In the history of diphtheria diagnostics, three turning points might be identified: (i) the demonstration that DT is a major virulence factor of *C. diphtheriae* and development of the toxigenicity test using an animal model in 1888; (ii) the development of agar immunodiffusion assay for the detection of DT production in 1948, commonly called the Elek test; and (iii) the development of PCR in 1991, which enables the detection of the *tox* gene. Many other various methods for the detection of DT and identification of toxigenic isolates have been proposed but the sensitivity, repeatability and reproducibility of most of them have not been confirmed on a sufficiently large group of bacterial isolates or clinical samples (Table 1).

Regulations in force in the European Union (EU) require implementation of the following three principles concerning the use of animals for laboratory tests: replacement, reduction and refinement (the 3Rs) [107]. Therefore, the toxigenicity test using an animal model is not used any more in the EU for diagnostic purposes. Despite enormous progress in science and diagnostics of infectious diseases, after almost 80 years, the Elek test is still the basic recommended diagnostic test, enabling the detection of the main pathogenicity factor of *C. diphtheriae*, which is a highly potent toxin. Although the diphtheria vaccine has been known for many decades and has been broadly used in many countries, the disease has not been eradicated. In the 21st century, diphtheria occurs not only as single cases but also as epidemics in some regions of the world. Moreover, new *Corynebacterium* species with the potential to produce DT were described [5,6]. Therefore, a rapid, easy to interpret and modern method for the detection of DT-producing isolates is needed, especially considering that the availability of diphtheria antitoxin—which is a main cure and a necessary reagent for current diagnostics methods—is extremely limited. The very promising tools are ICS and LFIA for toxin detection, and isothermal amplification methods, such as LAMP, for the detection of *tox* gene. Also, biosensors represent a promising technology for the rapid DT detection in clinical samples, but they still face challenges that must be addressed to maximise the sensitivity and selectivity of the test and to eliminate cross-reactivity.

Table 1. Detection methods for diphtheria toxin.

Technique	Method	LOD [ng/mL]	Time to Obtain the Result f	Experience of Laboratory Staff	Comments
In vivo ^a	Animal model	ND	48–120 h	very high	traditional “gold standard”; animal facility required; not recommended due to the 3R rule
Cell lines ^a	In vitro tissue culture cytotoxicity	0.01–0.1	24–120 h	very high	specialized laboratory facilities required; mainly used for measuring serum antitoxin levels
	Elek test	ND ^d	24–48 h 16–24 h ^g	very high	basic recommended diagnostic test; prone to misinterpretation; technically demanding
	Counterimmunoelectrophoresis (CIE)	ND	16–24 h	average	evaluated on a small number of isolates
	Agglutination methods	0.2–5	12 h	average	evaluated on a small number of isolates
Immunological ^a	Enzyme-linked immunosorbent assay (ELISA)	<1	22 h	high	no commercially available kits; recommended by WHO for detection and quantification of anti-DT antibodies
	Enzyme immunoassay (EIA)	0.1	3 h	average	easy to interpret
	Immunoblotting	5–10	11–24 h	average	not recommended for routine diagnostic use
	Immunochromatographic strip test (ICS)	0.5	3.5–6.5 h	low	easy to apply and interpret
	Polymerase Chain Reaction (PCR)	2.5 ^e	4–5 h ^h	average	allows to detect NTTB strains; must be confirmed by the Elek test; specialized laboratory facilities required
Molecular ^b	Real-time PCR	<0.017 ^e	3–4 h ^h	high	not require a very precise heating device; potential Point-of-Care Testing; allows to detect NTTB strains; must be confirmed by the Elek test
	Loop-mediated isothermal amplification (LAMP)	2.84 ^e	1 h ^h	average	potential Point-of-Care Testing, allows to detect NTTB strains (genosensors); genosensors’ results must be confirmed by the Elek test
Optical/electrochemical ^c	Biosensors	0.005–10	1–2 h	low	potential Point-of-Care Testing, allows to detect NTTB strains (genosensors); genosensors’ results must be confirmed by the Elek test

LOD—limit of detection; ND—not determined; ^a—DT detection; ^b—tox gene detection; ^c—depends of type, DT or tox gene detection; ^d—modified Elek test may detect toxin even in low-toxicogenic strains; ^e—pg of genomic DNA/reaction; ^f—time counted from the moment of obtaining a pure culture; ^g—modifications; ^h—without DNA isolation.

According to the definition for the purposes of epidemiological surveillance, a confirmed diagnosis of diphtheria requires meeting both clinical and laboratory criteria. The laboratory criterion includes the isolation of toxin-producing corynebacteria from clinical material. Local public health laboratories deal with initial diagnostics consisting of isolation and molecular identification. Due to the fact that PCR only allows for the detection of the gene encoding DT but does not provide information on the production of DT, it is necessary to perform the Elek test in all *tox*-positive isolates to confirm whether the strain is toxigenic. Diagnostic laboratories are recommended to submit *tox*-positive isolates to the National Reference Laboratory to confirm the production of DT with the Elek test. Currently, the role of Reference Laboratories is crucial for diphtheria diagnostics because these are the only laboratories with access to diphtheria antitoxin necessary for the Elek test and where staff have the necessary experience in the conduction and result interpretation of the Elek test. However, the necessity to transport the sample to the Reference Laboratory requires additional time, which results in a delay in obtaining information about the toxigenicity status of the isolate, introducing an appropriate therapy to the patient, and taking actions to prevent the spread of the infection. The challenges in the development of new test for DT detection include, among others, the following: (i) the test should be independent of the availability of diphtheria antitoxin; (ii) it should be easy-to-perform and the results should be easy-to-interpret so that local laboratories would be able to carry out the test even without any experience in diphtheria diagnostics; (iii) it should be rapid; (iv) it might be implemented as a point-of-care test, especially in cases when an outbreak is suspected; and (v) it should be easy to store and maintain activity for long time because diphtheria cases occur very rarely in some countries.

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References

1. Riegel, P.; Ruimy, R.; Briel, D.; Prevost, G.; Jehl, F.; Christen, R.; Monteil, H. Taxonomy of *Corynebacterium diphtheriae* and related taxa, with recognition of *Corynebacterium ulcerans* sp. nov. nom. rev. *FEMS Microbiol. Lett.* **1995**, *126*, 271–276. [CrossRef]
2. Hadfield, T.L.; McEvoy, P.; Polotsky, Y.; Tzinslerling, V.A.; Yakovlev, A.A. The Pathology of Diphtheria. *J. Infect. Dis.* **2000**, *181*, S116–S120. [CrossRef]
3. Chenal, A.; Nizard, P.; Gillet, D. Structure and Function of Diphtheria Toxin: From Pathology to Engineering. *J. Toxicol. Toxin Rev.* **2002**, *21*, 321–359. [CrossRef]
4. Ryan Kenneth, J.; Ray George, C. *Sherris Medical Microbiology: An Introduction to Infectious Diseases*, 4th ed.; McGraw-Hill: New York, NY, USA, 2004.
5. Crestani, C.; Arcari, G.; Landier, A.; Passet, V.; Garnier, D.; Brémont, S.; Armatys, N.; Carmi-Leroy, A.; Toubiana, J.; Badell, E.; et al. *Corynebacterium ramonii* sp. nov., a Novel Toxigenic Member of the *Corynebacterium diphtheriae* Species Complex. *Res. Microbiol.* **2023**, *174*, 104113. [CrossRef] [PubMed]
6. Prygiel, M.; Polak, M.; Mosiej, E.; Wdowiak, K.; Formińska, K.; Zasada, A. New *Corynebacterium* Species with the Potential to Produce Diphtheria Toxin. *Pathogens* **2022**, *11*, 1264. [CrossRef] [PubMed]
7. Boyd, J.; Oza, M.N.; Murphy, J.R. Molecular Cloning and DNA Sequence Analysis of a Diphtheria Tox Iron-Dependent Regulatory Element (*dtxR*) from *Corynebacterium diphtheriae*. *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 5968–5972. [CrossRef]
8. Tao, X.; Schiering, N.; Zeng, H.; Ringe, D.; Murphy, J.R. Iron, DtxR, and the Regulation of Diphtheria Toxin Expression. *Mol. Microbiol.* **1994**, *14*, 191–197. [CrossRef] [PubMed]
9. Gill, D.M.; Pappenheimer, A.M. Structure-Activity Relationships in Diphtheria Toxin. *J. Biol. Chem.* **1971**, *246*, 1492–1495. [CrossRef]
10. Yamaizumi, M.; Mekada, E.; Uchida, T.; Okada, Y. One Molecule of Diphtheria Toxin Fragment a Introduced into a Cell Can Kill the Cell. *Cell* **1978**, *15*, 245–250. [CrossRef]
11. Aboul-Enein, B.H.; Puddy, W.C.; Bowser, J.E. The 1925 Diphtheria Antitoxin Run to Nome-Alaska: A Public Health Illustration of Human-Animal Collaboration. *J. Med. Humanit.* **2019**, *40*, 287–296. [CrossRef]

12. Burkovski, A. Diphtheria and Its Etiological Agents. In *Corynebacterium Diphtheriae and Related Toxigenic Species*; Burkovski, A., Ed.; Springer: Dordrecht, The Netherlands, 2014; pp. 1–9. ISBN 978-94-007-7623-4.
13. Plotkin Stanley, A.; Orenstein Walter, A.; Offit Paul, A. *Plotkin's Vaccines*, 7th ed.; Elsevier: Philadelphia, PA, USA, 2017.
14. Murphy John, R. *Medical Microbiology*, 4th ed.; Elsevier: Galveston, TX, USA, 1996; Volume 32.
15. Barksdale, L.; Garmise, L.; Horibata, K. Virulence, Toxigenicity, and Lysogeny in *Corynebacterium diphtheriae*. *Ann. N. Y. Acad. Sci.* **1960**, *88*, 1093–1108. [CrossRef] [PubMed]
16. Pappenheimer, A.M. Diphtheria Toxin. *Annu. Rev. Biochem.* **1977**, *46*, 69–94. [CrossRef] [PubMed]
17. Bonventre, P.F.; Saelinger, C.B.; Imhoff, J.G. Studies on the Effect of Diphtheria Toxin on Protein Synthesis in Mice. *J. Med. Microbiol.* **1973**, *6*, 169–176. [CrossRef] [PubMed]
18. Brooks, R. *Guidelines for the Laboratory Diagnosis of Diphtheria*; World Health Organization: Geneva, Switzerland, 1981.
19. Efstratiou, A.; Engler, K.H.; Dawes, C.S.; Sesardic, D. Comparison of Phenotypic and Genotypic Methods for Detection of Diphtheria Toxin among Isolates of Pathogenic Corynebacteria. *J. Clin. Microbiol.* **1998**, *36*, 3173–3177. [CrossRef] [PubMed]
20. Fraser, D.T.; Wigham, H.E. The Use of Rabbits for Intracutaneous Virulence Tests: Of B.Diphtheriae or Titration of Diphtheria Antitoxin: Preliminary Note. *JAMA* **1924**, *82*, 1114–1115. [CrossRef]
21. Efstratiou, A.; Engler, K.H.; Mazurova, I.K.; Glushkevich, T.; Vuopio-Varkila, J.; Popovic, T. Current Approaches to the Laboratory Diagnosis of Diphtheria. *J. Infect. Dis.* **2000**, *181*, S138–S145. [CrossRef]
22. *WHO Laboratory Manual for the Diagnosis of Diphtheria and Other Related Infections*; World Health Organization: Geneva, Switzerland, 2021.
23. Bonventre, P.F.; Imhoff, J.G. Studies on the Mode of Action of Diptheria Toxin. *J. Exp. Med.* **1966**, *124*, 1107–1122. [CrossRef] [PubMed]
24. Bowman, C.G.; Bonventre, P.F. Studies on the Mode of Action of Diptheria Toxin. *J. Exp. Med.* **1970**, *131*, 659–674. [CrossRef] [PubMed]
25. Gabliks, J.; Falconer, M. Interaction of Diphtheria Toxin with Cell Cultures from Susceptible and Resistant Animals. *J. Exp. Med.* **1966**, *123*, 723–732. [CrossRef]
26. Miyamura, K.; Nishio, S.; Ito, A.; Murata, R.; Kono, R. Micro Cell Culture Method for Determination of Diphtheria Toxin and Antitoxin Titres Using VERO Cells. *J. Biol. Stand.* **1974**, *2*, 189–201. [CrossRef]
27. Hoy, C.S.; Sesardic, D. In Vitro Assays for Detection of Diphtheria Toxin. *Toxicology In Vitro* **1994**, *8*, 693–695. [CrossRef] [PubMed]
28. Schubert, J.H.; Bickham, S.T.; Wiggins, G.L. Tissue Culture Method for Toxigenicity Testing of *Corynebacterium diphtheriae*. *Appl. Microbiol.* **1968**, *16*, 1748–1752. [CrossRef] [PubMed]
29. Murphy, J.R.; Bacha, P.; Teng, M. Determination of *Corynebacterium diphtheriae* Toxigenicity by a Colorimetric Tissue Culture Assay. *J. Clin. Microbiol.* **1978**, *7*, 91–96. [CrossRef] [PubMed]
30. Laird, W.; Groman, N. Rapid, Direct Tissue Culture Test for Toxigenicity of *Corynebacterium diphtheriae*. *Appl. Microbiol.* **1973**, *25*, 709–712. [CrossRef] [PubMed]
31. Moehring, T.J.; Moehring, J.M.; Kuchler, R.J.; Solotorovsky, M. The Repsonse of Cultured Mammalian Cells to Diptheria Toxin. *J. Exp. Med.* **1967**, *126*, 407–422. [CrossRef] [PubMed]
32. Andre, J.; Audebaud, G.; Chambon, L. [Diagnosis of diphtheria on tissue culture]. *Ann. Inst. Pasteur (Paris)* **1960**, *99*, 179–187.
33. Lennox, E.S.; Kaplan, A.S. Action of Diphtheria Toxin on Cells Cultivated in Vitro. *Exp. Biol. Med.* **1957**, *95*, 700–702. [CrossRef]
34. Simonova, M.A.; Melnikov, V.G.; Lakhtina, O.E.; Komaleva, R.L.; Berger, A.; Sing, A.; Zavriev, S.K. Determination of Diphtheria Toxin in Bacterial Cultures by Enzyme Immunoassay. *Diagnostics* **2022**, *12*, 2204. [CrossRef] [PubMed]
35. Ouchterlony, O. In Vitro Method for Testing the Toxin Producing Capacity of Diptheria Bacteria. *Acta Pathol. Microbiol. Scand.* **1948**, *25*, 186–191. [CrossRef]
36. Elek, S.D. Recognition of Toxicogenic Bacterial Strains in Vitro. *BMJ* **1948**, *1*, 493–496. [CrossRef]
37. Elek, S.D. The Plate Virulence Test for Diphtheria. *J. Clin. Pathol.* **1949**, *2*, 250–258. [CrossRef] [PubMed]
38. Fel'dman, I.M.; Makhaneva, L.G.; Glushkevich, T.G. [Determination of the toxigenicity of *Corynebacterium diphtheriae* by using paper indicator disks]. *Zhurnal Mikrobiologii Epidemiologii Immunobiologii* **1987**, *4*, 32–34.
39. Melnikov, V.G.; Berger, A.; Sing, A. Detection of Diphtheria Toxin Production by Toxigenic Corynebacteria Using an Optimized Elek Test. *Infection* **2022**, *50*, 1591–1595. [CrossRef] [PubMed]
40. Engler, K.H.; Glushkevich, T.; Mazurova, I.K.; George, R.C.; Efstratiou, A. A Modified Elek Test for Detection of Toxigenic Corynebacteria in the Diagnostic Laboratory. *J. Clin. Microbiol.* **1997**, *35*, 495–498. [CrossRef]
41. Fitriana, F.; Nugroho, F.S.; Herawati, M.H. Columbia Blood Agar Base as an Alternative Medium for the Elek Test. *Jundishapur J. Microbiol.* **2022**, *15*, e121537. [CrossRef]
42. Fitriana, F.; Sunarno, S.; Syarif, A.K.; Karyana, M.; Rosana, Y.; Moehario, L.H. A New Modified Medium for Simultaneous Cystinase and Elek Tests of Bacteria Causing Diphtheria. *Bali Med. J.* **2019**, *8*, 334–340. [CrossRef]
43. Tilton, R.C. Counterimmunoelectrophoresis in Biology and Medicine. *CRC Crit. Rev. Clin. Lab. Sci.* **1978**, *9*, 347–365. [CrossRef]
44. Thompson, N.L.; Ellner, P.D. Rapid Determination of *Corynebacterium diphtheriae* Toxigenicity by Counterimmunoelectrophoresis. *J. Clin. Microbiol.* **1978**, *7*, 493–494. [CrossRef] [PubMed]
45. Holmes, R.K.; Perlow, R.B. Quantitative Assay of Diphtherial Toxin and of Immunologically Cross-Reacting Proteins by Reversed Passive Hemagglutination. *Infect. Immun.* **1975**, *12*, 1392–1400. [CrossRef]

46. Jalgaonkar, S.V.; Saoji, A.M. Coagglutination for Rapid Testing of Toxin Producing *Corynebacterium diphtheriae*. *Indian J. Med. Res.* **1993**, *97*, 35–36.
47. Toma, C.; Sisavath, L.; Iwanaga, M. Reversed Passive Latex Agglutination Assay for Detection of Toxigenic *Corynebacterium diphtheriae*. *J. Clin. Microbiol.* **1997**, *35*, 3147–3149. [CrossRef] [PubMed]
48. Nielsen, P.B.; Koch, C.; Friis, H.; Heron, I.; Prag, J.; Schmidt, J. Double-Antibody Sandwich Enzyme-Linked Immunosorbent Assay for Rapid Detection of Toxin-Producing *Corynebacterium diphtheriae*. *J. Clin. Microbiol.* **1987**, *25*, 1280–1284. [CrossRef] [PubMed]
49. Titov, L.; Kolodkina, V.; Dronina, A.; Grimont, F.; Grimont, P.A.D.; Lejay-Collin, M.; De Zoysa, A.; Andronescu, C.; Diaconescu, A.; Marin, B.; et al. Genotypic and Phenotypic Characteristics of *Corynebacterium diphtheriae* Strains Isolated from Patients in Belarus during an Epidemic Period. *J. Clin. Microbiol.* **2003**, *41*, 1285–1288. [CrossRef] [PubMed]
50. Kombarova, S.I.; Mazurova, I.K.; Mel'nikov, V.G.; Kostiuikova, N.N.; Volkovoi, K.I.; Borisova, O.I.; Platonova, T.V.; Efstratiou, A. [Genetic structure of *Corynebacterium diphtheriae* strains isolated in Russia during epidemics of various intensity]. *Zhurnal Mikrobiologii Epidemiologii Immunobiologii* **2001**, *3*, 3–8.
51. Engler, K.H.; Efstratiou, A. Rapid Enzyme Immunoassay for Determination of Toxigenicity among Clinical Isolates of *Corynebacterium diphtheriae*. *J. Clin. Microbiol.* **2000**, *38*, 1385–1389. [CrossRef] [PubMed]
52. Pietrzak, J.; Muehlestein, S.; Gasser, M. Sandwich-Dot Immunobinding Assay (Sandwich-DIA), a New Immunological Method for the Detection of Diphtheria Toxin. *Zentralblatt Bakteriologie* **1990**, *274*, 61–69. [CrossRef] [PubMed]
53. Hallas, G.; Harrison, T.G.; Samuel, D.; Colman, G. Detection of Diphtheria Toxin in Culture Supernates of *Corynebacterium diphtheriae* and *C. ulcerans* by Immunoassay with Monoclonal Antibody. *J. Med. Microbiol.* **1990**, *32*, 247–253. [CrossRef] [PubMed]
54. Engler, K.H.; Efstratiou, A.; Norn, D.; Kozlov, R.S.; Selga, I.; Glushkevich, T.G.; Tam, M.; Melnikov, V.G.; Mazurova, I.K.; Kim, V.E.; et al. Immunochromatographic Strip Test for Rapid Detection of Diphtheria Toxin: Description and Multicenter Evaluation in Areas of Low and High Prevalence of Diphtheria. *J. Clin. Microbiol.* **2002**, *40*, 80–83. [CrossRef] [PubMed]
55. Neal, S.E.; Efstratiou, A. International External Quality Assurance for Laboratory Diagnosis of Diphtheria. *J. Clin. Microbiol.* **2009**, *47*, 4037–4042. [CrossRef]
56. Melnikov, V.G.; Berger, A.; Dangel, A.; Sing, A. Lateral Flow Immunoassay-Based Laboratory Algorithm for Rapid Diagnosis of Diphtheria. *Open Res. Eur.* **2023**, *3*, 62. [CrossRef]
57. Berger, A.; Hogardt, M.; Konrad, R.; Sing, A. Detection Methods for Laboratory Diagnosis of Diphtheria. In *Corynebacterium diphtheriae and Related Toxigenic Species*; Burkovski, A., Ed.; Springer: Dordrecht, The Netherlands, 2014; pp. 171–205; ISBN 978-94-007-7623-4.
58. Bonmarin, I.; Guiso, N.; Le Flèche-Matéos, A.; Patey, O.; Grimont Patrick, A.D.; Levy-Bruhl, D. Diphtheria: A Zoonotic Disease in France? *Vaccine* **2009**, *27*, 4196–4200. [CrossRef] [PubMed]
59. Wagner, K.S.; White, J.M.; Neal, S.; Crowcroft, N.S.; Kuprevičienė, N.; Paberza, R.; Lucenko, I.; Jöks, U.; Akbaş, E.; Alexandrou-Athanassoulis, H.; et al. Screening for *Corynebacterium diphtheriae* and *Corynebacterium ulcerans* in Patients with Upper Respiratory Tract Infections 2007–2008: A Multicentre European Study. *Clin. Microbiol. Infect.* **2011**, *17*, 519–525. [CrossRef]
60. Zakikhany, K.; Neal, S.; Efstratiou, A. Emergence and Molecular Characterisation of Non-Toxigenic Tox Gene-Bearing *Corynebacterium diphtheriae* Biovar Mitis in the United Kingdom, 2003–2012. *Eurosurveillance* **2014**, *19*, 22. [CrossRef] [PubMed]
61. Williams, M.M.; Waller, J.L.; Aneke, J.S.; Weigand, M.R.; Diaz, M.H.; Bowden, K.E.; Simon, A.K.; Peng, Y.; Xiaoli, L.; Cassidy, P.K.; et al. Detection and Characterization of Diphtheria Toxin Gene-Bearing *Corynebacterium* Species through a New Real-Time PCR Assay. *J. Clin. Microbiol.* **2020**, *58*, e00639–20. [CrossRef]
62. Sharma, N.C.; Efstratiou, A.; Mokrousov, I.; Mutreja, A.; das B, Ramamurthy T. Diphtheria. *Nat. Rev. Dis. Primers* **2019**, *5*, 81. [CrossRef] [PubMed]
63. Pallen, M.J. Rapid Screening for Toxigenic *Corynebacterium diphtheriae* by the Polymerase Chain Reaction. *J. Clin. Pathol.* **1991**, *44*, 1025–1026. [CrossRef]
64. Pallen, M.J.; Hay, A.J.; Puckey, L.H.; Efstratiou, A. Polymerase Chain Reaction for Screening Clinical Isolates of *Corynebacterium* for the Production of Diphtheria Toxin. *J. Clin. Pathol.* **1994**, *47*, 353–356. [CrossRef]
65. Hauser, D.; Popoff, M.R.; Kiredjian, M.; Boquet, P.; Bimet, F. Polymerase Chain Reaction Assay for Diagnosis of Potentially Toxinogenic *Corynebacterium diphtheriae* Strains: Correlation with ADP-Ribosylation Activity Assay. *J. Clin. Microbiol.* **1993**, *31*, 2720–2723. [CrossRef] [PubMed]
66. Nakao, H.; Mazurova, I.K.; Glushkevich, T.; Popovic, T. Analysis of Heterogeneity of *Corynebacterium diphtheriae* Toxin Gene, Tox, and Its Regulatory Element, dtxR, by Direct Sequencing. *Res. Microbiol.* **1997**, *148*, 45–54. [CrossRef]
67. Mothershed, E.A.; Cassidy, P.K.; Pierson, K.; Mayer, L.W.; Popovic, T. Development of a Real-Time Fluorescence PCR Assay for Rapid Detection of the Diphtheria Toxin Gene. *J. Clin. Microbiol.* **2002**, *40*, 4713–4719. [CrossRef]
68. Kobaidze, K.; Popovic, T.; Nakao, H.; Quick, L. Direct Polymerase Chain Reaction for Detection of Toxigenic *Corynebacterium diphtheriae* Strains from the Republic of Georgia after Prolonged Storage. *J. Infect. Dis.* **2000**, *181*, S152–S155. [CrossRef] [PubMed]
69. Aravena-Román, M.; Bowman, R.; O'Neill, G. Polymerase Chain Reaction for the Detection of Toxigenic *Corynebacterium diphtheriae*. *Pathology* **1995**, *27*, 71–73. [CrossRef] [PubMed]
70. Mikhailovich, V.M.; Melnikov, V.G.; Mazurova, I.K.; Wachsmuth, I.K.; Wenger, J.D.; Wharton, M.; Nakao, H.; Popovic, T. Application of PCR for Detection of Toxigenic *Corynebacterium diphtheriae* Strains Isolated during the Russian Diphtheria Epidemic, 1990 through 1994. *J. Clin. Microbiol.* **1995**, *33*, 3061–3063. [CrossRef] [PubMed]

71. Sing, A.; Berger, A.; Schneider-Brachert, W.; Holzmann, T.; Reischl, U. Rapid Detection and Molecular Differentiation of Toxigenic *Corynebacterium diphtheriae* and *Corynebacterium ulcerans* Strains by LightCycler PCR. *J. Clin. Microbiol.* **2011**, *49*, 2485–2489. [CrossRef]
72. Sulakvelidze, A.; Kekelidze, M.; Gomelaury, T.; Deng, Y.; Khetsuriani, N.; Kobaidze, K.; De Zoysa, A.; Efstratiou, A.; Morris, J.G.; Imnadze, P. Diphtheria in the Republic of Georgia: Use of Molecular Typing Techniques for Characterization of *Corynebacterium diphtheriae* Strains. *J. Clin. Microbiol.* **1999**, *37*, 3265–3270. [CrossRef]
73. Sing, A.; Hogardt, M.; Bierschenk, S.; Heesemann, J. Detection of Differences in the Nucleotide and Amino Acid Sequences of Diphtheria Toxin from *Corynebacterium diphtheriae* and *Corynebacterium ulcerans* Causing Extrapharyngeal Infections. *J. Clin. Microbiol.* **2003**, *41*, 4848–4851. [CrossRef]
74. Schuëgger, R.; Lindermayer, M.; Kugler, R.; Heesemann, J.; Busch, U.; Sing, A. Detection of Toxigenic *Corynebacterium diphtheriae* and *Corynebacterium ulcerans* Strains by a Novel Real-Time PCR. *J. Clin. Microbiol.* **2008**, *46*, 2822–2823. [CrossRef]
75. Tiwari, T.S.P.; Golaz, A.; Yu, D.T.; Ehresmann, K.R.; Jones, T.F.; Hill, H.E.; Cassidy, P.K.; Pawloski, L.C.; Moran, J.S.; Popovic, T.; et al. Investigations of 2 Cases of Diphtheria-Like Illness Due to Toxigenic *Corynebacterium ulcerans*. *Clin. Infect. Dis.* **2008**, *46*, 395–401. [CrossRef] [PubMed]
76. Cassidy, P.K.; Pawloski, L.C.; Tiwari, T.; Sanden, G.N.; Wilkins, P.P. Analysis of Toxigenic *Corynebacterium ulcerans* Strains Revealing Potential for False-Negative Real-Time PCR Results. *J. Clin. Microbiol.* **2008**, *46*, 331–333. [CrossRef]
77. Hall, A.J.; Cassidy, P.K.; Bernard, K.A.; Bolt, F.; Steigerwalt, A.G.; Bixler, D.; Pawloski, L.C.; Whitney, A.M.; Iwaki, M.; Baldwin, A.; et al. Novel *Corynebacterium diphtheriae* in Domestic Cats. *Emerg. Infect. Dis.* **2010**, *16*, 688–691. [CrossRef]
78. Badell, E.; Hennart, M.; Rodrigues, C.; Passet, V.; Dazas, M.; Panunzi, L.; Bouchez, V.; Carmi-Leroy, A.; Toubiana, J.; Brisse, S. *Corynebacterium rouxii* sp. nov., a Novel Member of the Diphtheriae Species Complex. *Res. Microbiol.* **2020**, *171*, 122–127. [CrossRef] [PubMed]
79. Mancini, F.; Monaco, M.; Pataracchia, M.; Von Hunolstein, C.; Pantosti, A.; Ciervo, A. Identification and Molecular Discrimination of Toxigenic and Nontoxigenic Diphtheria *Corynebacterium* Strains by Combined Real-Time Polymerase Chain Reaction Assays. *Diagn. Microbiol. Infect. Dis.* **2012**, *73*, 111–120. [CrossRef]
80. Pimenta, F.P.; Hirata, R.; Rosa, A.C.P.; Milagres, L.G.; Mattos-Guaraldi, A.L. A Multiplex PCR Assay for Simultaneous Detection of *Corynebacterium diphtheriae* and Differentiation between Non-Toxigenic and Toxigenic Isolates. *J. Med. Microbiol.* **2008**, *57*, 1438–1439. [CrossRef] [PubMed]
81. Torres, L.D.F.C.; Ribeiro, D.; Hirata, R., Jr.; Pacheco, L.G.C.; Souza, M.C.; Santos, L.S.D.; Santos, C.S.D.; Salah, M.; Costa, M.M.D.; Ribeiro, M.G.; et al. Multiplex Polymerase Chain Reaction to Identify and Determine the Toxigenicity of *Corynebacterium* spp. with Zoonotic Potential and an Overview of Human and Animal Infections. *Memórias Inst. Oswaldo Cruz* **2013**, *108*, 272–279. [CrossRef] [PubMed]
82. Badell, E.; Guillot, S.; Tulliez, M.; Pascal, M.; Panunzi, L.G.; Rose, S.; Litt, D.; Fry, N.K.; Brisse, S. Improved Quadruplex Real-Time PCR Assay for the Diagnosis of Diphtheria. *J. Med. Microbiol.* **2019**, *68*, 1455–1465. [CrossRef] [PubMed]
83. De Zoysa, A.; Efstratiou, A.; Mann, G.; Harrison, T.G.; Fry, N.K. Development, Validation and Implementation of a Quadruplex Real-Time PCR Assay for Identification of Potentially Toxigenic *Corynebacteria*. *J. Med. Microbiol.* **2016**, *65*, 1521–1527. [CrossRef] [PubMed]
84. Sunarno; Khariri, Muna, F.; Sariadji, K.; Rukminiati, Y.; Febriyana, D.; Febrianti, T.; Saraswati, R.D.; Susanti, I.; Puspadari, N.; et al. New Approach for the Identification of Potentially Toxigenic *Corynebacterium* sp. Using a Multiplex PCR Assay. *J. Microbiol. Methods* **2021**, *184*, 106198. [CrossRef] [PubMed]
85. Rosana, Y.; Inta Prilandari, L.; Ajisman, R.; Sarry Hartono, T.; Yasmon, A. Detection of Toxin-Producing *Corynebacterium diphtheriae* from Throat Swabs of Diphtheria Patients Using Duplex Real-Time PCR. *Iran. J. Microbiol.* **2020**, *12*, 508–515. [CrossRef] [PubMed]
86. Sunarno, S.; Hartoyo, Y.; Amalia, N.; Sofiah, S.N.; Rizki, A.; Puspadari, N.; Febriyana, D.; Febrianti, T.; Saraswati, R.D.; Muna, F.; et al. Development and Application of Dtxr and Tox Genes Targeting Real-Time PCR to Identify *Corynebacterium diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* Simultaneously. *Jundishapur J. Microbiol.* **2022**, *15*, e121256. [CrossRef]
87. Zasada, A.A.; Wiatrzyk, A.; Czajka, U.; Brodzik, K.; Formińska, K.; Mosiej, E.; Prygiel, M.; Krysztopa-Grzybowska, K.; Wdowiak, K. Application of Loop-Mediated Isothermal Amplification Combined with Colorimetric and Lateral Flow Dipstick Visualization as the Potential Point-of-Care Testing for *Corynebacterium diphtheriae*. *BMC Infect. Dis.* **2020**, *20*, 308. [CrossRef]
88. Notomi, T. Loop-Mediated Isothermal Amplification of DNA. *Nucleic Acids Res.* **2000**, *28*, 63e. [CrossRef] [PubMed]
89. Dincer, C.; Bruch, R.; Costa-Rama, E.; Fernández-Abedul, M.T.; Merkoçi, A.; Manz, A.; Urban, G.A.; Güder, F. Disposable Sensors in Diagnostics, Food, and Environmental Monitoring. *Adv. Mater.* **2019**, *31*, 1806739. [CrossRef] [PubMed]
90. Sivakumar, R.; Lee, N.Y. Recent Advances in Airborne Pathogen Detection Using Optical and Electrochemical Biosensors. *Anal. Chim. Acta* **2022**, *1234*, 340297. [CrossRef] [PubMed]
91. Moreno-Bondi, M.C.; Taitt, C.R.; Shriver-Lake, L.C.; Ligler, F.S. Multiplexed Measurement of Serum Antibodies Using an Array Biosensor. *Biosens. Bioelectron.* **2006**, *21*, 1880–1886. [CrossRef] [PubMed]
92. Zeinoddini, M.; Azizi, A.; Bayat, S.; Tavasoli, Z. Localized Surface Plasmon Resonance (LSPR) Detection of Diphtheria Toxoid Using Gold Nanoparticle-Monoclonal Antibody Conjugates. *Plasmonics* **2018**, *13*, 583–590. [CrossRef]
93. Ziółkowski, R.; Jarczewska, M.; Drozd, M.; Zasada, A.A.; Malinowska, E. Studies on the Development of Electrochemical Immunosensor for Detection of Diphtheria Toxoid. *J. Electrochem. Soc.* **2019**, *166*, B472–B481. [CrossRef]

94. Ziółkowski, R.; Kaczmarek, A.; Kośnik, I.; Malinowska, E. Reduced Nonspecific Protein Adsorption by Application of Diethyldithiocarbamate in Receptor Layer of Diphtheria Toxoid Electrochemical Immunosensor. *Bioelectrochemistry* **2020**, *132*, 107415. [CrossRef] [PubMed]
95. Ameku, W.A.; Ataide, V.N.; Costa, E.T.; Gomes, L.R.; Napoleão-Pêgo, P.; William Provance, D.; Paixão, T.R.L.C.; Salles, M.O.; De-Simone, S.G. A Pencil-Lead Immunosensor for the Rapid Electrochemical Measurement of Anti-Diphtheria Toxin Antibodies. *Biosensors* **2021**, *11*, 489. [CrossRef] [PubMed]
96. Marchlewicz, K.; Ostrowska, I.; Oszwaldowski, S.; Zasada, A.; Ziółkowski, R.; Malinowska, E. Molecular Diagnostic of Toxigenic *Corynebacterium diphtheriae* Strain by DNA Sensor Potentially Suitable for Electrochemical Point-of-Care Diagnostic. *Talanta* **2021**, *227*, 122161. [CrossRef]
97. Sangal, V.; Hoskisson, P.A. Corynephages: Infections of the Infectors. In *Corynebacterium diphtheriae and Related Toxigenic Species*; Burkovski, A., Ed.; Springer: Dordrecht, The Netherlands, 2014; pp. 67–81. ISBN 978-94-007-7623-4.
98. Trost, E.; Blom, J.; De Castro Soares, S.; Huang, I.-H.; Al-Dilaimi, A.; Schröder, J.; Jaenicke, S.; Dorella, F.A.; Rocha, F.S.; Miyoshi, A.; et al. Pangenomic Study of *Corynebacterium diphtheriae* That Provides Insights into the Genomic Diversity of Pathogenic Isolates from Cases of Classical Diphtheria, Endocarditis, and Pneumonia. *J. Bacteriol.* **2012**, *194*, 3199–3215. [CrossRef]
99. Rosana, Y.; Gabriella Lusiana, D.I.; Yasmon, A. Genetic Characterization of Diphtheria Tox B to Evaluate Vaccine Efficacy in Indonesia. *Iran. J. Microbiol.* **2022**, *14*, 606–610. [CrossRef] [PubMed]
100. Mokrousov, I. *Corynebacterium diphtheriae*: Genome Diversity, Population Structure and Genotyping Perspectives. *Infect. Genet. Evol.* **2009**, *9*, 1–15. [CrossRef] [PubMed]
101. Greenfield, L.; Bjorn, M.J.; Horn, G.; Fong, D.; Buck, G.A.; Collier, R.J.; Kaplan, D.A. Nucleotide Sequence of the Structural Gene for Diphtheria Toxin Carried by Corynebacteriophage Beta. *Proc. Natl. Acad. Sci. USA* **1983**, *80*, 6853–6857. [CrossRef] [PubMed]
102. Ratti, G.; Rappuoli, R.; Giannini, G. The Complete Nucleotide Sequence of the Gene Coding for Diphtheria Toxin in the Corynephage Omega (Tox⁺) Genome. *Nucleic Acids Res.* **1983**, *11*, 6589–6595. [CrossRef] [PubMed]
103. Nakao, H.; Pruckler, J.M.; Mazurova, I.K.; Narvskaja, O.V.; Glushkevich, T.; Marijevski, V.F.; Kravetz, A.N.; Fields, B.S.; Wachsmuth, I.K.; Popovic, T. Heterogeneity of Diphtheria Toxin Gene, Tox, and Its Regulatory Element, dtxR, in *Corynebacterium diphtheriae* Strains Causing Epidemic Diphtheria in Russia and Ukraine. *J. Clin. Microbiol.* **1996**, *34*, 1711–1716. [CrossRef] [PubMed]
104. Kolodkina, V.L.; Titov, L.P.; Sharapa, T.N.; Drozhzhina, O.N. Point Mutations Sites in Tox Promoter/Operator and Diphtheria Toxin Repressor (DtxR) Gene Associated with the Level of Toxin Production by *Corynebacterium diphtheriae* Strains Isolated in Belarus. *Mol. Genet. Microbiol. Virol.* **2007**, *22*, 24–33. [CrossRef]
105. Grosse-Kock, S.; Kolodkina, V.; Schwalbe, E.C.; Blom, J.; Burkovski, A.; Hoskisson, P.A.; Brisse, S.; Smith, D.; Sutcliffe, I.C.; Titov, L.; et al. Genomic Analysis of Endemic Clones of Toxigenic and Non-Toxigenic *Corynebacterium diphtheriae* in Belarus during and after the Major Epidemic in 1990s. *BMC Genom.* **2017**, *18*, 873. [CrossRef]
106. Sing, A.; Bierschenk, S.; Heesemann, J. Classical Diphtheria Caused by *Corynebacterium ulcerans* in Germany: Amino Acid Sequence Differences between Diphtheria Toxins from *Corynebacterium diphtheriae* and *C. ulcerans*. *Clin. Infect. Dis.* **2005**, *40*, 325–326. [CrossRef]
107. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the Protection of Animals Used for Scientific Purposes L 276/33. Available online: <https://eur-lex.europa.eu/legal-content/en/NIM/?uri=CELEX:32010L0063> (accessed on 1 February 2024).

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