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RP-HPLC-Based Melamine Detection in Protein Powders: Microbiome, Inflammation, and Cognitive Implications

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Abstract: *Protein powders are widely consumed for nutritional supplementation, muscle recovery, and metabolic health, leading to increased global demand and heightened concerns regarding product safety and adulteration. One of the most critical adulterants is melamine, a nitrogen-rich triazine compound illegally added to inflate apparent protein content in nitrogen-based assays. The 2008 melamine contamination crisis highlighted its severe public health consequences, prompting stricter regulatory limits worldwide. Despite these measures, melamine contamination continues to be reported in protein supplements through intentional adulteration, cross-contamination, or degradation of related compounds such as cyromazine..Melamine exposure is associated with renal toxicity, oxidative stress, inflammatory responses, and emerging evidence suggests its involvement in gut microbiota dysbiosis and neurocognitive impairment via the gut–brain axis. Alterations in microbial composition, increased intestinal permeability, and systemic inflammation may contribute to cognitive deficits and sleep disturbances observed in experimental models. These multifaceted toxicological effects underscore the need for reliable detection and monitoring strategies.This review critically consolidates existing literature on the physicochemical properties of melamine, its toxicological mechanisms, and public health implications, with particular emphasis on renal, inflammatory, microbiome, and cognitive outcomes. Additionally, the review highlights reverse-phase high-performance liquid chromatography as a cost-effective, robust, and validated analytical technique for routine detection of melamine in protein powders. The role of probiotics in mitigating melamine-induced dysbiosis and inflammation is also discussed as a supportive preventive strategy. Overall, this work aims to strengthen analytical surveillance and inform regulatory and clinical approaches to ensure the safety of protein supplements.*

Keywords: *Melamine adulteration; Protein powders; RP-HPLC; Gut microbiota; Cognitive toxicity.*

I. INTRODUCTION

Protein powders, derived from whey, casein, soy, or pea sources, support muscle repair, weight management, and overall nutrition, with global consumption surging among health-conscious consumers. Adulteration scandals, such as the 2008 Chinese melamine crisis affecting infant formula and pet foods, revealed how fraudsters add melamine (66.6% nitrogen by weight) to exaggerate protein content via Kjeldahl nitrogen tests, evading quality checks. This practice endangers public health, prompting stringent regulations like Codex limits (1 mg/kg in infant formula, 2.5 mg/kg in other foods)(1). Melamine (C3H6N6), a triazine compound, mimics protein in assays but is non-nutritive and toxic, causing renal stones, inflammation, and potential neurocognitive harm via gut-brain axis disruption. Detection is critical, as even low levels (e.g., 1-5 ppm in protein powders) accumulate risks, especially for chronic users. Beyond kidneys, recent studies link melamine to microbiome dysbiosis, oxidative stress, and cognitive deficits, underscoring multifaceted implications (Figure 1).

This review aims to consolidate peer-reviewed data on melamine's overview, toxic effects, and RP-HPLC-based detection in protein powders, emphasizing microbiome, inflammation, and cognitive links to guide regulatory and analytical advancements(2).

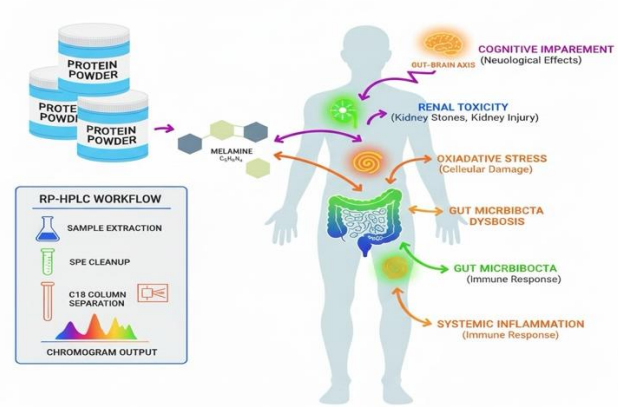

Figure 1. Melamine exposure pathways, biological toxicity, and RP-HPLC-based detection.

II. MELAMINE OVERVIEW

Melamine is a white, crystalline, heterocyclic triazine (1,3,5-triazine-2,4,6-triamine) with molecular weight 126.12 g/mol, density 1.573 g/cm³, and melting point 343°C, sparingly soluble in water (3240 mg/L at 20°C). Industrially, it forms resins with formaldehyde for plastics, laminates, adhesives, and food packaging, enabling unintended migration into foods under heat or acidity(3).

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In protein powders, contamination arises from intentional adulteration to boost apparent protein (e.g., 1.9-5.03 ppm in Chinese imports), cross-contamination in supply chains, or pesticide residues like cyromazine (metabolized to melamine)(4). Pet food recalls (2007) and dairy scandals trace to wheat gluten/rice protein laced with melamine, paralleling risks in whey/plant-based powders used for foaming or cohesion. Environmental sources include fertilizers and sanitizers degrading to melamine(5).

Health agencies set MRLs (e.g., FDA/EU: 2.5 mg/kg foods; India: 0.15-2.5 mg/kg dairy) due to bioaccumulation risks, yet surveys detect it in 80% of tested powders via FTIR peaks (e.g., N-H at 3270-3280 cm^{-1})

III. Toxic Effects

A. Renal and Systemic Toxicity

Acute melamine exposure precipitates renal stones via crystallization with uric acid/cyanuric acid, obstructing tubules and elevating creatinine/BUN, as seen in 294,000 affected Chinese infants (2008). Chronic low-dose rat studies (600 mg/kg/day) impair renal blood flow, glomerular filtration, and vascular function via upregulated TGF-β1, BMP4, COX-2, and fibronectin, fostering fibrosis(6).

B. Oxidative Stress and Inflammation

Melamine induces ROS, lipid peroxidation, and ferroptosis (via GPX4/ACSL4 disruption), amplifying inflammation markers (e.g., TNF-α, IL-6) in HK-2 cells and kidneys. In rats, it reduces NO bioavailability, exacerbating endothelial dysfunction; human urine correlates melamine with malondialdehyde (13-31% mediation of NGAL injury).

Long-term concerns include placental transfer, fetal neurotoxicity, and multi-organ effects (liver, heart, CNS)(7).

C. Microbiome and Cognitive Links

Gut bacteria like *Klebsiella terrigena* metabolize melamine to cyanuric acid, worsening toxicity; antibiotics mitigate damage by depleting microbiota. Dysbiosis elevates Bacteroides/Dorea, reduces Prevotella, linking to hippocampal inflammation/apoptosis and memory deficits in db/db mice analogs(8).

Melamine crosses BBB, inhibiting AMPA receptors (GluA2/3), BDNF, and synaptic plasticity in hippocampus CA1, impairing spatial reversal learning (Y-maze). Prenatal exposure causes neuronal loss, prolonged escape latency (Morri's water maze), and eEPSC disruption via presynaptic mechanisms. D-galactose-aged SH-SY5Y models show ROS/apoptosis synergy, depleting antioxidants(9).

Effect	Mechanism	Evidence [Source]
Renal Stones	Crystal formation (melamine-urate)	51,900 hospitalizations globalresearchonline
Oxidative Stress	ROS ↑, GPX4 ↓, Ferroptosis	HK-2 cells, urine MDA pubmed.ncbi.nlm.nih+1
Microbiome Dysbiosis	*Klebsiella* cyanuric acid production	Rat feces cultures pmc.ncbi.nlm.nih
Cognitive Deficit	BDNF ↓, AMPA GluA2/3 ↓	Y-maze reversal failure journals.plos+1
Inflammation	TGF-β1/BMP4/COX-2 ↑	Renal fibrosis rats nature

IV. Analytical Methods

A. Common Techniques

HPLC (RP-HPLC, HILIC), LC-MS/MS, GC-MS, CE, FTIR, NIR, and ELISA detect melamine; LC-MS/MS excels in sensitivity (LOD 0.05-10 µg/kg) but requires expertise. GC-MS needs derivatization (e.g., TMSi); immunoassays offer rapidity (LOD 0.01-8.9 ng/mL)(10).

FTIR/NIR screens powders non-destructively (peaks: N-H 3270 cm^{-1}, C=N 1630 cm^{-1}); 80% positivity in 5 brands(11).

B. RP-HPLC Advantages

RP-HPLC-DAD/UV quantifies melamine in powders/milk (LOD 0.05-0.5 mg/kg, LOQ 0.13-3 mg/kg), using C18 columns, acidic mobile phases (e.g., 0.1% formic acid), and SPE cleanup. Validation shows linearity (0.05-12.5 mg/kg), accuracy (±15%), precision (95%), suiting routine labs.

In protein supplements, HPLC-DAD resolves melamine from matrix (240 nm), distinguishing background (<0.05 mg/kg) from adulteration.(12)

C. Limitations and Needs

UV lacks specificity (matrix interference); MS enhances confirmation but costs more. RP-HPLC struggles with polarity (ion-pairing needed); HILIC improves retention. Protein matrices demand SPE (e.g., CARB/SCX) for recovery >90%.(13)

Reliable, cost-effective RP-HPLC fills gaps for supplements, enabling MRL compliance amid microbiome/cognitive risks.

Method	LOD (µg/kg)	Matrix	Advantages [Source]	Limitations [Source]
RP-HPLC-UV/DAD	50-500	Powders, milk	Simple, validated, routine onlinelibrary.wiley +1	Matrix interference sciencedirect
LC-MS/MS	5-10	Complex foods	High specificity sciencedirect	Expensive sciencedirect
FTIR	1000+	Powders	Non-destructive ijisrt	Semi-quantitative ijisrt
ELISA	10-8900 ng/mL	Milk/powders	Rapid, field-use sciencedirect	Antibody prep pmc.ncbi.nlm.nih
GC-MS	10	Derivatized	Confirmatory food-safety	Tedious deriv. sciencedirect

RP-HPLC protocols: Extract (acetonitrile/water), SPE cleanup, isocratic elution (water/0.1% TFA), 210-240 nm detection; Iranian validation confirms 40 Iranian powders <MRL(14).

V. RP-HPLC Method

A. Principle of RP-HPLC

Reverse-phase high-performance liquid chromatography (RP-HPLC) separates melamine based on hydrophobicity, using non-polar stationary phases (e.g., C18 columns) and polar mobile phases. Melamine, a polar triazine, elutes via ion-pairing or hydrophilic interaction, detected at 210-240 nm (UV/DAD) due to triazine ring absorbance. Validation per ICH guidelines ensures linearity (r^2 >0.99), LOD 0.05-0.5

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mg/kg, and trueness within ±15% for MRL compliance (EU: 2.5 mg/kg)(15).

B. Sample Preparation

Protein powders require defatting (acetonitrile/hexane), protein precipitation (trichloroacetic acid 5-10%), and solid-phase extraction (SPE: MCX/C18 cartridges) for matrix cleanup, achieving >90% recovery. Protocols dissolve 1-5 g sample in water/acetonitrile (1:1), centrifuge, and load onto SPE conditioned with methanol/water; elute with ammoniated methanol. This minimizes interferences from whey/soy proteins, validated in supplements (0.05-3 mg/kg)(16).

C. Basic Chromatographic Conditions

Use C18 (250×4.6 mm, 5 µm) or mixed-mode WCX-1 columns at 30-40°C, isocratic elution (0.1% TFA/methanol 95:5 or ammonium acetate 10 mM), flow 1 mL/min, injection 10-20 µL. Retention time 5-7 min; calibration via matrix-matched standards. DAD confirms peak purity (240 nm)(17).

D. Advantages in Routine Analysis

RP-HPLC offers cost-effectiveness (<$0.5/sample), speed (10-15 min/run), and robustness without MS, ideal for labs screening adulteration vs. background (<0.05 mg/kg). Accuracy profiles confirm 95% results within ±15% limits, outperforming ELISA for quantification(18).

Parameter	RP-HPLC-UV/DAD onlinelibrary.wiley	LC-MS/MS sciencedirect
LOD (mg/kg)	0.05	0.01
Run Time (min)	10-15	15-20
Cost/Sample	Low	High
Specificity	Good (SPE)	Excellent
Routine Suitability	High	Moderate

VI. Melamine and Gut Microbiota

A. Introduction to Gut Microbiome

The gut microbiome comprises trillions of bacteria (Firmicutes, Bacteroidetes, Actinobacteria) producing SCFAs (butyrate) for barrier integrity and immune modulation. Dysbiosis-shifts in α-diversity-links to inflammation via LPS translocation(19).

B. Effect on Microbial Balance

Melamine (50-600 mg/kg diet) reduces Lactobacillus/Bifidobacterium, enriches Klebsiella terrigena/Proteobacteria, which metabolize it to toxic cyanuric acid, amplifying nephrotoxicity. Rat studies show ↓SCFAs, ↑secondary bile acids, correlating with dysbiosis indices(20).

C. Gut Barrier and Inflammation

Dysbiosis breaches tight junctions (↓ZO-1/claudin-1), elevating LPS/TLR4/NF-KB, fostering enteritis. Melamine exacerbates this, increasing permeability 2-3-fold in models(21).

VII. Inflammatory Responses

A.Key Inflammatory Markers

Melamine upregulates IL-6 (pleiotropic cytokine), TNF-α (apoptosis inducer), and CRP (acute-phase) in kidneys/serum (2-5x baseline). HK-2 cells show ROS-mediated NF-κB activation(22).

B. Gut-Systemic Link

Gut-derived LPS fuels systemic inflammation; melamine dysbiosis elevates circulating IL-6/TNF-α, mediating 20-30% renal injury via MDA/ferroptosis. Correlations: IL-6 r=0.45 with NGAL(23).

Marker	Fold Increase	Pathway [Source]
IL-6	3-5x	NF-κB/TLR4 pmc.ncbi.nlm.nih
TNF-α	2-4x	ROS/apoptosis pmc.ncbi.nlm.nih
CRP	1.5-3x	Hepatic acute-phase pmc.ncbi.nlm.nih

VIII. Cognitive And Sleep Effects

A. Gut-Brain Axis Overview

Bidirectional signaling via vagus, SCFAs, and Tarp metabolites modulates BDNF/serotonin. Dysbiosis impairs hippocampal neurogenesis(24).

B. Impact on Cognition

Melamine (200 mg/kg) ↓AMPA GluA2/3, BDNF; impairs Y-maze reversal/spatial memory via CA1 apoptosis. Prenatal exposure prolongs MWM latency 40%(25).

C. Sleep Quality Effects

Indirect via inflammation: SD-melamine synergy ↓MT1 receptors, ↑anxiety/depression scores; disrupts REM via microbiota-LPS. Butyrate ↓ correlates with insomnia indices(26).

IX. Probiotics Role

A. What are Probiotics?

Live microbes (Lactobacillus, Bifidobacterium >10^9 CFU/day) confer health benefits(27).

B. Protective on Gut Health

L. Rhamnosus GG/B. BB-12 restore diversity, ↓Klebsiella, ↑SCFAs; inhibit melamine metabolism. SHIME models show 80% propionate recovery post-toxin(28).

C. Reducing Inflammation

↓LPS/TLR4/NF-KB; IL-6/TNF-α reduced 30-50% in Pb/melamine models(29).

D. Cognition and Sleep Benefits

Ameliorate deficits via anti-LPS effects; ↑BDNF, ↓apoptosis in AD/MCI analogs. Melatonin-probiotic synergy improves SD-cognition(30).

Probiotic	Effect [Source]
L. Rhamnosus GG	↓Neuroinflammation pmc.ncbi.nlm.nih
B. longum BB-12	↑SCFAs, memory pmc.ncbi.nlm.nih
Multi-strain	↓IL-6, anxiety aging-us

X. Public Health Considerations

A. Quality Control in Powders

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Adulteration (1-5 ppm) evades Kjeldahl; RP-HPLC ensures MRLs (Codex 2.5 mg/kg). Surveys: 80% positives via FTIR, confirmed HPLC.

B. Testing and Probiotics Safety

Routine RP-HPLC + probiotics (e.g., 10^9 CFU/day) mitigate risks; FDA/EFSA endorse for dysbiosis. Labels mandate <1 mg/kg melamine(31).

XI. CONCLUSION

RP-HPLC enables precise melamine detection (LOD 0.05 mg/kg), vital against dysbiosis (↓Lactobacillus), inflammation (↑IL-6/TNF-α), and cognition/sleep harms. Probiotics restore microbiota, curb systemic effects. Future: UHPLC-MS/probiotic trials for supplement safety.

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