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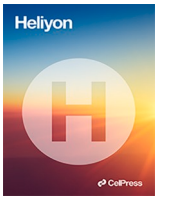

Review article

Applications of tandem mass spectrometry (MS/MS) in antimicrobial peptides field: Current state and new applications

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ABSTRACT

Antimicrobial peptides (AMPs) constitute a group of small molecular peptides that exhibit a wide range of antimicrobial activity. These peptides are abundantly present in the innate immune system of various organisms. Given the rise of multidrug-resistant bacteria, microbiological studies have identified AMPs as potential natural antibiotics. In the context of antimicrobial resistance across various human pathogens, AMPs hold considerable promise for clinical applications. However, numerous challenges exist in the detection of AMPs, particularly by immunological and molecular biological methods, especially when studying of newly discovered AMPs in proteomics. This review outlines the current status of AMPs research and the strategies employed in their development, considering resent discoveries and methodologies. Subsequently, we focus on the advanced techniques of mass spectrometry for the quantification of AMPs in diverse samples, and analyzes their application, advantages, and limitations. Additionally, we propose suggestions for the future development of tandem mass spectrometry for the detection of AMPs.

1. Introduction

Antimicrobial resistance poses a substantial threat to global health and development, with the prevalence of multidrug resistance becoming increasingly common among pathogenic bacteria. Drug-resistant pathogens are a serious concern in both hospital and non-hospital settings, presenting substantial challenges to the healthcare system, encompassing issues related to the identification, management, and control of infections caused by resistant organisms [1,2]. Therefore, the development and application of new antimicrobial drugs are imminent.

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Antimicrobial peptides (AMPs) are short, structurally diverse peptides with a broad spectrum of antibacterial, antiviral, and antifungal activity [3–5]. Genetically encoded in nature, these peptides typically comprise 10–100 amino acids and are synthesized by diverse organisms as a defense mechanism against microbial invasion [6–8]. They have been reported to be inhibitors of central dogma processes, and blocking DNA, RNA, and protein synthesis [9]. AMPs play a crucial role in the immune defense of multicellular organisms and are currently under investigation for their potential as anti-infective drugs. Their unique ability to target multiple biological targets simultaneously gives AMPs a distinct advantage over traditional antibiotics [10]. An exemplification of this phenomenon is LL-37, which exhibits effects on bacterial cell membranes, in addition to its direct microbicidal, immunomodulatory, and antibiofilm properties [11,12]. The diversity and potency of AMPs make them highly appealing for the development of antimicrobial drugs [13], Currently, many AMPs are being evaluated in clinical trials [14,15]. AMPs have pharmacodynamic properties that effectively reduce the evolution of resistance in target microorganisms. Additionally, they may exhibit synergistic effects with other antimicrobial agents and traditional antibiotics. Overall, AMPs play a critical role in maintaining the health and well-being of organisms by protecting them from infection and regulating various biological processes. Accumulating evidence indicates that AMPs can be used in the treatment of microbial infections in humans [16].

However, for the clinical application of AMPs, comprehending their inherent biological properties to minimize the potential for unintended harm and to address the current challenge of antibiotic resistance faced by traditional antibiotics is critical [17,18]. Currently, AMPs are primarily detected by using molecular biological methods, immunological methods, and mass spectrometry (MS). Immunodetection assays, including ELISA, immunohistochemistry, and western blotting [19,20], have associated limitations. These include cross-reactivity between human heterophile antibodies and the antibodies contained in reagents as well as possible autoantibodies that can lead to error in the results due to defaults or excess values [21]. Additionally, matrix differences between patient sera (hemolysis, icterus, and lipemia) and calibrators present challenges. AMPs can also be detected using molecular research platforms, including polymerase chain reaction (PCR), microarray analysis, and next-generation sequencing (NGS) [22–24]. Potential disadvantages of molecular biological detection methods, such as PCR and RT-PCR, include limited sensitivity for detecting low levels of AMPs in complex samples. This may result in false-negative results or underestimation of the true AMP levels. The utilization of molecular techniques introduces the potential for false-positive findings due to contamination. To mitigate the risk of contamination, adequate controls and rigorous quality control measures must be employed. Molecular methods, such as NGS or microarray analysis, can be costly and time-consuming. Consequently, their utility for routine diagnostics or screenings may be limited.

In recent years, MS has emerged as a robust and indispensable tool in the field of chemical and biological analysis, and has been applied in the study of AMPs. MS overcomes these interferences of the detection of immunological methods by not relying on antibodies. It detects protein directly, providing information on the expression, post-translational modifications, and degradation products of the AMPs with higher sensitivity and specificity. The detection of AMPs by tandem mass spectroscopy (MS/MS) represents a real technological revolution in clinical diagnosis and treatment. Numerous studies exist, but most research on AMPs using MS/MS has primarily focused on two aspects: (1) extracting and characterizing of biologically active peptides for the discovery of new AMPs and (2) exploring possible functional and physiological properties. Therefore, these two aspects are outlined in this review.

The detection of AMP is an important and current research focus. To our we knowledge, systematic reviews focusing on the application of MS for AMP detection are currently lacking. Therefore, we aim to comprehensively review and summarize MS techniques in AMP research and application, particularly in the discovery and characterization of novel AMPs from natural sources. Additionally, studies related to AMP discovery, quantitative analysis, characterization, activity, and therapies are emphasized. Finally, this study presents a thorouth analysis of the advantages, limitations, and future perspectives of the methods employed. The discussion serves as a valuable reference for future research endeavors.

2. Sources of AMPs

AMPs can be classified based on various criteria, such as their structure, origin, and mechanism of action. Some of the commonly recognized types of AMPs, including plant AMPs, insect AMPs, and synthetic and engineered peptides. Note that this is not an exhaustive list, and new types of AMPs are continuously being discovered and characterized all the time. According to their source, AMPs can be divided into the following.

2.1. Bacteriophage/viral AMPs

Bacteriophages, also known as phages, are viral agents that specifically target and infect bacteria. Within the realm of phage research, numerous proteins have been isolated, including endolysins, depolymerases, and holins, have been isolated and identified as potential alternative antimicrobial agents [25–27]. Phage-encoded endolysins are bacteriolytic proteins that are synthesized during the final stages of the phage lytic cycle. These proteins play a crucial role in breaking down various components of the bacterial cell wall, thereby facilitating the release of phage progeny from the host cells [28]. Two distinct categories of phage amplification systems, namely phage-encoded lysis factors and phage tail complexes, are recognized [29–31].

2.2. Bacterial

In gram-positive bacteria, reports have indicated the occurrence of ribosomal and nonribosomal synthesis of AMPs [32]. Bacteriocins, synthesized by ribosomes in bacteria, are considered as bacterial AMPs. These peptides primarily exhibit activity primarily against bacteria closely related to the producer bacteria, necessitating defense mechanisms to counteract potential harmful effects

[33]. Bacteriocins produced by gram-positive bacteria are classified into four categories: antibiotics, non-antibiotics, large bacteriocins, and bacteriocins with distinctive structures [34,35]. Numerous bacteriocins can be isolated from bacteria, with the majority originating gram-negative bacilli within the *Enterobacteriaceae bacteria* family. These AMPs have a narrow spectrum of antibacterial activity against gram-negative bacteria and are categorized as categories colicins, colicin-like, microcins, and phage tail-like bacteriocins [35].

2.3. Fungal AMPs

Fungal AMPs can be categorized into two primary groups: peptaibols and fungal defensins [36]. Peptaibols, originating mainly comes from the soil fungus Trichoderma [37], are short peptides, typically consisting of 5–21 amino acids. These peptides are characterized by a high proportion of non-proteinogenic amino acids, displaying antifungal activity [38,39]. Defensins, characterized by their short length and high cysteine content, are peptides that are ubiquitously found in microorganisms, plants, and animals [40,41]. Like other AMPs, defensins have demonstrated effectiveness as alternatives to current antifungal therapies, showing potential as novel therapeutic agents or drug leads [42,43].

2.4. Plant AMPs

Cysteine-rich AMPs, widely distributed in plants, which form part of the plant defense systems [44]. Seeds and fruits are

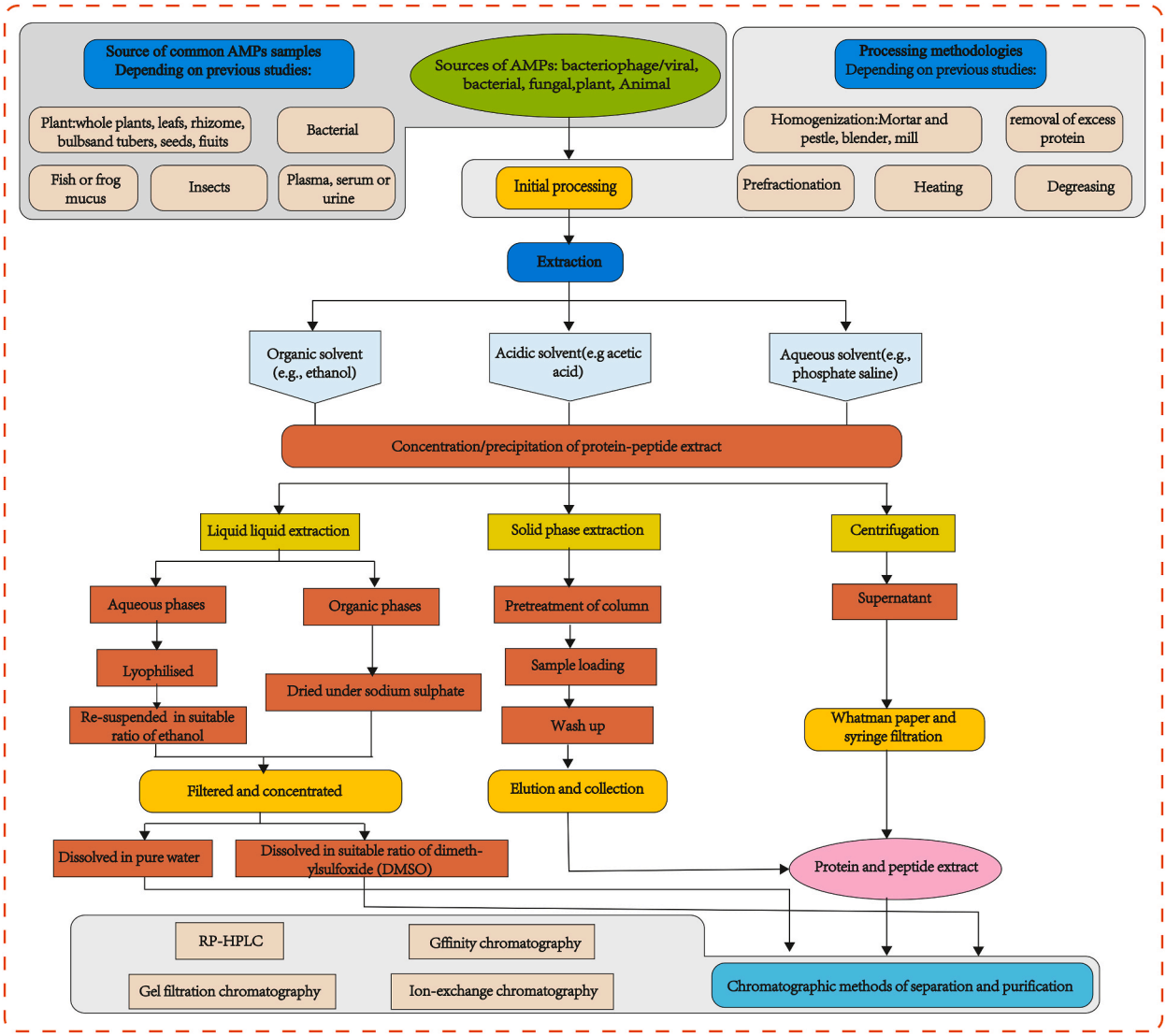

Fig. 1. Typical procedures for isolation and purification of antimicrobial peptides [47,62–65]

particularly valuable sources of diverse AMPs in plants. Cysteine-rich AMPs exhibit persistent broad-spectrum antimicrobial activity, enabling plants to effectively defend against a wide array of pathogens [44,45]. Currently, plant AMPs are primarily classified according to their structure into α-hairpin proteins, defensins, heme-like peptides, desmin-type peptides, lipid transfer proteins, thionin, snake proteins and unclassified cysteine-rich AMP [46,47].

2.5. Animal AMPs

Various animals, including amphibians, fish, reptiles, birds, mammals, produce AMPs. Invertebrates, lacking adaptive immunity, rely on AMPs as a a vital component of their immune defense. In the case of the invertebrates under investigation, these peptides play a significant role in the immune response [48,49]. These invertebrate AMPs include defensins and cecropin found in insects, defensins found in mollusks and nematodes, macrodefensins found in horseshoe crabs, and invertebrate β-defensins found in crustaceans [50–52]. Vertebrate antimicrobial peptides (AMPs) exhibit a range of sizes, spanning from 15 to 200 residues, and fulfill crucial roles in the immediate defense response against pathogens [53]. These AMPs are found in fish, amphibians, reptiles, birds, and mammals. Fish, in particular, serve as a valuable source of AMPs, expressing various major classes such as cathelicidins, defensins, histone-derived peptides, hepcidins, and a fish-specific class of the cecropin family known as piscidins [54]. Reptiles and birds predominantly express cathelicidin and defensin family members as their AMPs [55,56].

The cathelicidin and defensin families constitute the primary groups of AMPs found in mammals. While additional mammalian AMPs, including platelet antimicrobial proteins, hepcidin, and dermocidin, exist outside cathelicidin or defensin families [57], LL-37 is the sole member of the cathelicidin family of AMPs in humans and it currently represents the most well-studied cathelicidin [58,59].

3. Application of MS in AMPs study

3.1. AMP isolation

Several pretreatment methods, such as liquid−liquid extraction, protein precipitation, and SPE, are employed for the analysis of biologically small molecular substances using MS/MS detection. Our previous study demonstrated that the magnetic bead method not only automates the sample pretreatment but also greatly reduces the cost compared with that of the SPE method [60]. However, the methods used for protein and peptide extraction are relatively complex. Obtaining peptides, derived from sources such as skin and limb secretions and bacteria, is a relatively complex process. Thus, the extraction of peptides suitable for research, along with considerations of quantity and purity of peptides, constitutes the current focus and challenge in AMPs research. The extraction and purification of AMPs present challenges and yield low quantities, thereby limiting their application in industry and scientific research [61]. Typically, AMPs possessing protease inhibitor activity are isolated through a series of purification steps, including salt extraction, ultrafiltration, and C18 reverse-phase chromatography.

Nevertheless, the majority of existing methods are designed to target specific structural types of AMPs, restricting their ability to

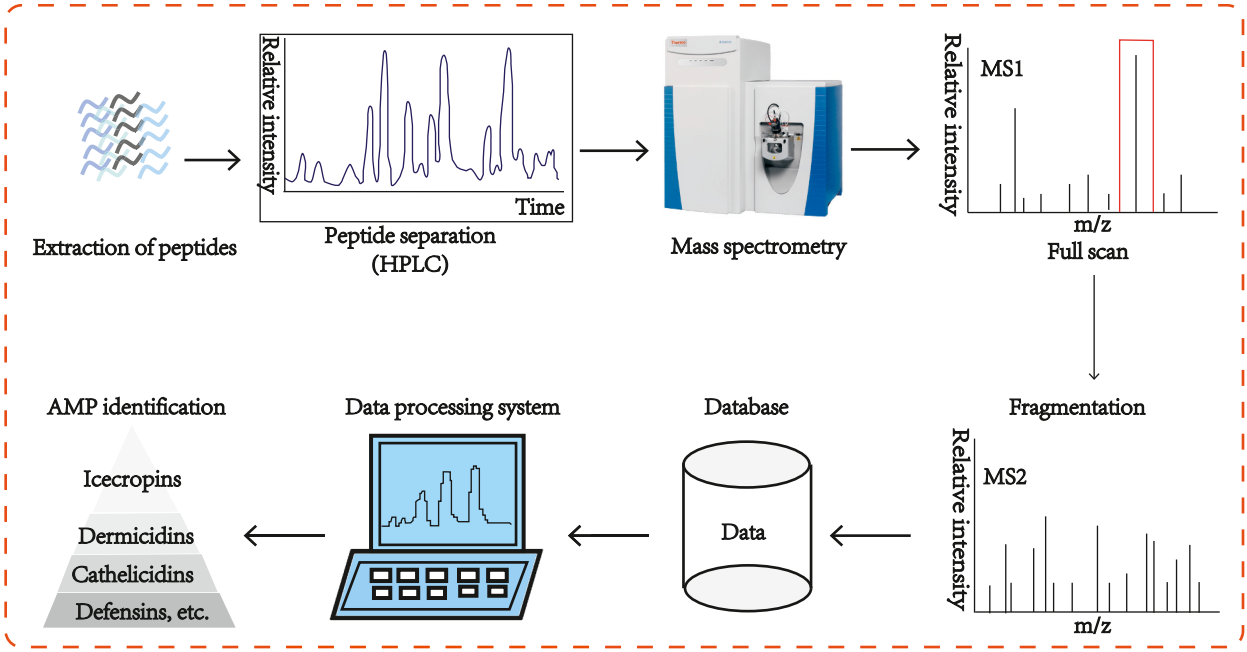

Fig. 2. Workflow diagram for the detection of antimicrobial peptides (AMPs) through mass spectrometry (MS).

Table 1
General characteristics of AMPs detected by mass spectrometry in the most recent application.

Type of Matrix	Pretreatment methods	Mobile phase	Column	Source	Mass Analyzer	Mass Spectrometry Technique	Country	Year	References
Insects hemolymph	SDS-PAGE, LP-SEC, Acetone Protein precipitation, trypsin digestion	–	a glass column (150 mm*20 mm)	ESI+	Aligent TOF/Q-TOF	FSCAN	India	2022	[124]
Insect hemolymph	Organic solvent extraction, followed by size exclusion RPLC and SDS-PAGE	50% acetonitrile containing 0.05% TFA	ZORBAX-XDB-C18 analytical (4.6 × 250 mm) column (Agilent)	MALDI	MALDI-TOF	–	India	2021	[125]
Food	Protein precipitation, Protease digestion, Ultrafiltration, HPLC, gel filtration chromatography	A, 100% acetonitrile; B, was water with 0.1% (m/v) trifluoroacetic acid (TFA)	A C18 semi-prep column (10 × 250 mm, YMC, Japan)	ESI+	QExactive (Thermo Fisher Scientific, USA)	FSCAN	China	2019	[126]
Human plasma or urine	SPE (Oasis WCX cartridge)	A, 0.2% FA in water; B, 0.2% FA in acetonitrile	Acquity UPLCR HSS T3 (2.1 mm × 100 mm, 1.8 μm)	ESI	AB 5500 triple quad MS	MRM	China	2020	[127]
Mouse plasma	Carboxylic acid magnetic bead affinity capture	A, 0.1% DFA (Difluoroacetic acid) in water;B, 0.1% DFA in acetonitrile	Waters 2.1 × 100 mm, 3.5 μm XSelect Peptide CSH C18 column	–	TSQ Quantiva triple Quad MS (Thermo Scientific)	SRM	USA	2020	[128]
DBS	Liquid liquid extraction, filtration	A, 0.2% FA in acetonitrile; B, 0.2% FA in water	XB-C18 (100 mm × 2.1 mm I.D., 2.6 μm) column Phonomenx Kinetex	ESI+	4500 Triple Quad MS	MRM	China	2022	[129]
Lactobacillus	Precipitation at 80% saturation of ammonium sulfate, separated with ultrafiltration, RPC	A, 1% MeOH, 0.1% FA in pure water; B, 99.9% AcN, 0.1% FA in pure water	XP C18 trap column (3 μm, 120A 350 μm 0.5 mm) and a 3C18-CL-120 separation column (3 μm, 120A, 75 μm 150 mm)	Nano spray ESI+	Triple TOF 5600+ MS	MRM	Russia	2020	[111]
Mouse serum and epithelial lining fluid	Protein precipitation	A, 0.1% formic acid in water; mobile phase B, 100% acetonitrile	Acquity UPLC HSS C18 column (100 mm × 2.1 mm internal diameter, 1.7 mm)	ESI+	API 5500-Qtrap Triple Quad MS	MRM	USA	2013	[130]
Cation-adjusted Mueller-Hinton broth (CAMHB), human and rat plasma	Protein precipitation (vortexing, sonicating, and re-vortexing, followed by centrifugation)	A, water with 0.5% FA and 0.01% TFA; B, 50/50 ACN/methanol with 0.5% FA and 0.01% TFA	Waters XTerra MS™ column (C18 3.5 μm, 2.1 mm × 100 mm); Waters XSelect CSH™ column (C18 3.5 μm, 2.1 mm × 100 mm)	ESI+	API 3000 Triple Quad MS	MRM	USA	2017	[131]
Goat milk	Protein precipitation	A, 0.1% (v/v) formic acid; B, 0.1% formic acid and 2% water in Optima acetonitrile	C-18, 3 μm nano-trap column (Bruker magic C18AQ, 0.1 × 150 mm, 3 μm partials size, and 200 A° pore size)	Captive spray +	Impact II QTOF	FSCAN	India	2022	[123]
Fish mucus	Acid solvent extraction, RP-HPLC	A, 0.1% formic acid B, acetonitrile	C4 column (Vydac, 300A, 5 μm, 4.6 × 250 mm)	ESI+	LC-MS/MS-QTOF	FSCAN	Tunisia	2023	[132]
Sea bass mucus	SPE	A, formic 5 acid in water (pH 2.55; B, and acetonitril	analytical C4 column (Vydac, 300A, 5 μm- 4.6 × 250 mm)	ESI+	Agilent MSD 1946B Simple Quad MS	FSCAN	Tunisia	2013	[120]

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Table 1 (*continued*)

Type of Matrix	Pretreatment methods	Mobile phase	Column	Source	Mass Analyzer	Mass Spectrometry Technique	Country	Year	References
Bacterial isolates	Trypsin digestion and Centrifugal filtration	A, 0.1% FA, 5% ACN, and 95% H2O; B, 0.1% FA, 95% ACN, and 5% H2O	C18, Agilent catalog no. G4240-62010 (160 nl, 150 mm)	ESI+	LC-MS/MS Orbitrap Fusion/ spectrometer Thermo Fisher	MRM	USA	2019	[122]
polymyxin B products	Centrifugal protein precipitation	A, water with 0.5% FA and 0.01% TFA; B, 50/50 ACN/ methanol with 0.5% FA and 0.01% TFA	C18, 3.5 μm, 2.1 mm × 100 mm	ESI+	API 3000 Triple Quad MS	MRM	USA	2018	[133]
Human plasma and epithelial lining fluid	Protein centrifugation precipitation	A, 0.1% (v/v) FA in water; B, 0.1% (v/v) FA in acetonitrile	C18 column (50 × 2.1 mm, 3 μm)	ESI+	4500 Triple Quad MS	MRM	China	2023	[134]
Ligilactobacillus salivarius cell-free supernatants	Protein centrifugation precipitation and RP-FPLC	A, 0,1% AF in H_2O; B, 0.1% AF in ACN	C18 Picofrit column (Easy Spray Column, PepMap RSLC C18n)	ESI	Q-Exactive HF high-resolution MS	FSCAN	Spain	2023	[135]
The strain Bacillus amyloliquefaciens Ba168	Protein centrifugation precipitation, trypsin digested, desalted on C18 containers , centrifuged, and concentrated	A, 0.1% formic acid; B, 84% acetonitrile and 0.1% formic acid	C18 reversed-phase analytical column (10 cm length, 75 μm inner diameter, 3 μm resin)	ESI+	Q Exactive MS	–	China	2022	[136]
Moss Physcomitrella	Homogenized, gel filtration, Protein precipitation and SPE on reverse-phase DSC18 cartridges	A, 0.1% (v/v) formic acid; B, 80% acetonitrile, 0.1% for mic acid	C18 trap column (3 μm, 120 Å, 350 m × 0.5 mm) and C18 column with a diameter of 75 μm (3 μm, Acclaim)	NanoSpray III ESI+	QTRAP 4500 triple quadrupole MS	MRM	Russia	2019	[137]
Saccharomyces cerevisiae autolysate	Homogenized, protein precipitation, filtered, SDS-PAGE, FPLC Gel Filtration	A, water; B, acetonitrile	C18-AQ 3 μm column (15 cm length, 75 μm inner diameter)	ESI+	Quadrupole-Orbitrap Q-Exactive Plus MS	FSCAN	Brazil	2022	[138]
Polymyxin B products	Centrifugation precipitation, SPE	acetonitrile:water:formic acid (30:70:0.1%; v/v/v)	C18 (1.8 μm, 2.1 mm i.d. x 50 mm)	ESI+	Alliance e2695 Quattro triple-quadrupole MS	MRM	Kuwait	2020	[117]
Rubing cheese	Homogenization, Centrifugation precipitation, gastric trypsin digestion, Ultrafiltration	A, 0.1% formic acid in water; B, 0.1% formic acid acetonitrile solution	RP C18 (50 μm × 15 cm)	–	Q Exactive™ Plus-Orbitrap mass spectrometer	FSCAN	China	2022	[118]
Egg white filtrate	Homogenization, ultrafiltration, nanofiltration and centrifugation	A, 2% v/v acetonitrile, 0.08% v/v formic acid and 0.01% v/v TFA in deionized wate; B, 95% v/v acetonitrile, 0.08% v/v formic acid, and 0.01% v/v TFA in deionized water	concentrated on a C18 column (5 μm 100 Å pore, 300 μm i.d., 5 mm length; peptide separation on a C18 column (3 μm, 100 Å pore, 75 μm i.d., 150 mm length)	Nano spray ESI+	Q Exactive MS	–	U.K	2021	[115]
Seahorse peptide extracts	Homogenization, sonication pulses, centrifugation, filtration, reduction and alkylation reaction	5%–38% ACN in 0.1% formic acid	ReproSil-Pur C18-AQ 3 μm resin (Dr. Maisch GmbH HPLC)	–	Fusion Lumos Orbitrap MS	FSCAN	Brazil	2023	[139]
Lactiplantibacillus plantarum B21	concentration, n-butanol extraction and cation exchange chromatography	A, 0.1% (v/v) formic acid; B, 3% (v/v) ACN	C_{18} analytical column (length 15 cm, 5 μm)	ESI+	LTQ™ Orbitrap Elite ETD	FSCAN	Australia	2020	[116]

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Table 1 (*continued*)

Type of Matrix	Pretreatment methods	Mobile phase	Column	Source	Mass Analyzer	Mass Spectrometry Technique	Country	Year	References
Crude cheese whey	Protease digestion. filtration and centrifugation	A, 10 mM HCl; B, 10 mM HCl containing 80 mL 100 mL-1 acetonitrile	Inertsil ODS-3 (4.6 mm × 250 mm)	ESI+	LCQ or LCMS 8040	–	Japan	2019	[140]
Rhipicephalus microplus saliva	SDS-PAGE, Protease digestion and ultrafiltration centrifugation	A, 0.1% formic acid; B, 0.1% formic acid, 84% acetonitrile	C18 column (75 μm × 100 mm, 3 μm)	Nanocaptive spray ESI+	Q Exactive MS	FSCAN	China	2019	[141]
Paenibacillus peoriae IBSD35 culture broth	70% ammonium sulfate enriched, distilled water and desalted, centrifugation, and column chromatography, RP-HPLC, digests	A, 5% acetonitrile, 0.1% formic acid; B, 95% acetonitrile, 0.1% formic acid	15 cm PicoFrit column (360 μm outer diameter, 75 μm inner diameter, 10 μm tip) filled with 1.9 μm of C18-resin	Nanocaptive spray ESI+	Q Exactive MS	FSCAN	India	2021	[142]
B. velezensis 9D-6, liquid LB culture metabolites	liquid/liquid extraction, C18 RP-HPLC	A, 0.1% formic acid; B, acetonitrile	C18 RHLC on a 1260 Infinity Series	–	Q-Exactive Quadrupole-Orbitrap MS	FSCAN	Canada	2019	[143]
Chicken Muscle and Egg	Homogenization, centrifugation, filtration	A, 1% formic acid in water; B, 1% formic acid in acetonitrile	C18 (100 × 2.1 mm, 1.9 μm)	ESI+	TSQ Quantis triple quadrupole MS	SRM	India	2021	[144]
Egg yolk hydrolysate	liquid/liquid extraction, Protease digestion	A, 0.05% (v/v) trifluoroacetic acid B, 0.05% trifluoroacetic acid in acetonitrile	C18 column, C18AQ-15 mm, 30 × 250 mm	CaptiveSpray+	micro-TOF-Q II MS	SRM	Thailand	2023	[145]
Metabolites in the culture supernatants of Entomopathogenic bacterial symbiont	Centrifugation and filtration	A, 0.1% trifluoroacetic acid in water; B, acetonitrile	C_{18} analytical column,5 μm, 4.6 mm by 150 mm	ESI+	Agilent 6120 quadrupole MS	–	France	2022	[146]
Peptides R9, penetratin	Homogenization, SPE	A, H2O/0.1% FA; B, ACN/ 0.1% FA	HSS T3 (2.1 × 50 mm, 1.8 μm) column	ESI+	AB/Sciex 5500 or 6500 triple quadrupole system	MRM	USA	2019	[119]
Buthus occitanus crude venom	centrifugation precipitation, filtration, reduction and carboxamido methylated	A, [0.1% (v/v) formic acid; B, acetonitrile (ACN) and 0.1% (v/v) FA	C4 PepMap 300 particles with 5 μm size and 300 A pores)	Spray+	Orbitrap Fusion MS	FSCAN	Morocco	2021	[147]
Skin secretion of the fungoid frog	RP-HPLC	A, water with 30% acetonitrile and 0.1% TFA	C-18 analytical column (Vydac 238 TP, 150 × 4.6 mm)	Nanocaptive spray ESI	SYNAPT G2 High Definition MS	–	India	2018	[148]
Fungus culture medium	Protein extraction, precipitation, chromatographic column separation	A, 0.1% formic acid in water; B, 0.1% formic acid in acetonitrile	C-18 analytical column (75 μm internal diameter and 70 cm in length)	Nanocaptive spray ESI	Q-Exactive Plus MS	FSCAN	USA	2021	[112]
Chinese yogurt	Ammonium sulfate precipitation or Organic solvent extraction, RP-HPLC	A, (2% acetonitrile, and 0.1% Formic acid; B, 80% acetonitrile, and 0.1% Formic acid	C18 reverse-phase column (Column, 10 cm long, 75 μm inner diameter, 3 μm resin)	ESI	Q Exactive MS	–	China	2023	[149]
Tree frog skin secretion	desalted and concentrated	A, 0.1% FA; B1, 80% ACN in 0.1% FA	C-18 column (15 cm × 50 μm Acclaim)	–	Q Exactive MS	FSCAN	Brazil	2021	[150]
Pichia pastoris	precipitated with ammonium sulfate, desalted by gel	–	Ziptips C18 (Millipore)	ESI+	triple quadrupole/ion	MRM	Spain	2019	[151]

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Table 1 (*continued*)

Type of Matrix	Pretreatment methods	Mobile phase	Column	Source	Mass Analyzer	Mass Spectrometry Technique	Country	Year	References
	filtration, cation exchange, RP-FPLC				trap MS 5500 QTRAP				
skin mucus of greater amberjack	Protein precipitation, 2-D Clean-Up Kit, SDS-PAGE	A, 0.1% formic acid; B, 80% acetonitrile, 0.1% formic acid	300 μm × 5 mm Acclaim Pepmap precolumn (Thermo Scientific)	Nanocaptive spray ESI+	Orbitrap Fusion MS	–	Spain	2021	[152]
Human Gut Bacterium Blautia obeum A2-162	Immunoprecipitation, bound peptides eluted	A, water/0.1% formic acid; B, acetonitrile	C18 LC Column (C18, 2 μm, 500 × 0.75 mm)	Nanocaptive spray ESI+	Orbitrap Fusion MS	–	UK	2018	[153]
Leptodactylus latrans Amphibian Skin Secretions	Lyophilized and resuspended	A, 0.1% formic acid in water; B, 0.1% formic acid in acetonitrile (ACN)	C18 column (75 μm × 10 cm, 1.7 μm BEH column)	Nanocaptive spray ESI+	LTQ OrbitrapVelos MS	FSCAN	Argentina	2018	[154]
Animal tissues	Centrifugation, sonication, HLB SPE, HPLC-UV	A, ACN; B, 50% ACN in water; C, 50% ACN in water containing 0.02% FA	column (50 mm × 2.1 mm i.d., 2.6 μm)	ESI+	Triple Quad 5500 triple-quadrupole MS	MRM	China	2022	[155]
Crude bacteriocins from both cell culture supernatant	Ammonium sulfate precipitation	–	C18 analytical column (180 mm length × 76 μm i.d., 3 μm)	–	Q Exactive MS	(DDA mode)	China	2018	[156]
Peptide Production from Donkey Milk by Selected Strains	centrifugation, filtration, SPE	A, 0.1% (v/v) formic acid in water; B, 0.1% (v/v) formic acid in acetonitrile	C18 column (0.5 × 100 mm, 2.7 μm)	DuoSpray Ion Source	5600+ TripleTOF MS	(DDA)	Italy	2021	[157]
Moringa oleifera seeds	freeze-dried, Centrifugation, filtration	A, 2 % acetonitrile (containing 0.1 % formic acid) in water; B, 80 % acetonitrile (containing 0.1 % formic acid) in water	C18 column (75 μm × 250 mm	ESI+	Q-Exactive MS	–	China	2023	[158]
plasma samples	HLB SPE	A, 0.1% formic acid in distilled water (v/v); B, 0.1% formic acid in acetonitrile (v/v)	XBridgeTM HILIC 3.5 μm 3 × 150 mm column	ESI+	LCMS-8040 triple quadrupole MS	MRM	Thailand	2020	[159]
Ascidian tunic	Homogenized, centrifuged and digested with enzymes	A, 0.1% FA; B, 80% ACN	EASY-Spray column (ES800, PepMap RSLC, C18, 3 μm)	ESI+	Q-Exactive Hybrid Quadrupole-Orbitrap MS	FSCAN	Portugal	2020	[160]
Fungal rice cultures	Sonication, centrifugation and filtration	A, 5 mM ammonium formate and 0.1% (v/v) formic acid water; B, 5 mM ammonium formate and 0.1% (v/v) formic acid in methanol	BEH C18 column (100 × 2.1 mm i.d., 1.7 μm particle size)	ESI	TQD triple quadrupole MS	–	Poland	2020	[161]
Fungal rice cultures	Centrifugation and filtration	A, 5 mM ammonium formate; B, (5 mM of ammonium formate in MeOH/water, 95:5, v/v)	C18 column (75 × 2.1 mm; 2.6 μm particles)	–	Q-Exactive Fourier-transform high-resolution	FSCAN	Poland	2020	[161]
Chicken Plasma	Lyophilized, centrifugation, gel filtration and reverse-phase chromatography	A, 0.1% formic acid in water; B, 0.1% formic acid in 80% acetonitrile	C18 column (2 μm, 100 Å, nanoViper, 75 μm i.d. × 15 cm)	Nanocaptive spray ESI+	Hybrid quadrupole Q-Tof MS	–	Thailand	2022	[162]
Plasma	Ultrafiltration, centrifugation	–	–	ESI	Agilent Technologies 6490 Triple Quad	–	Belgium	2019	[163]

(*continued on next page*)

Table 1 (*continued*)

Type of Matrix	Pretreatment methods	Mobile phase	Column	Source	Mass Analyzer	Mass Spectrometry Technique	Country	Year	References
Canine Saliva	Centrifugation, digested	A, formic acid (0.1% v/v); B, acetonitrile with formic acid (0.1% v/v)	150 mm Acclaim PepMap100 C18 column	–	LTQ-Orbitrap electron-transfer dissociation (ETD)	FSCAN	UK	2022	[164]
Serum samples	Protein precipitation	A, water with 0.1 % (v/v) formic acid; B, acetonitrile	column (ShimPack GIST-HP 150 mm × 3.0 mm)	ESI+	AB Sciex Qtrap 3200	MRM	Russia	2021	[165]
Octopus Skin Mucus	Protein centrifugation precipitation, SDS-PAGE, trypsin digested	A, 0.1% FA; B, 98% acetonitrile (98% ACN) with 0.1% FA	column (EASY-Spray column, 50 cm × 75 μm ID, PepMap C18, 2 μm particles)	ESI+	LTQ-Orbitrap-Elite MS	–	Spain	2023	[166]
Speckled anemone	Homogenization, centrifugation, sonication and filtration	A, 0.1% FA; B, 80% acetonitrile, 0.1% FA	Thermo RSLC pepmap100, 75 μm id, 100 Å pore size, 50 cm reversed-phase nano column	–	Q-Exactive™ Orbitrap MS	FSCAN	Australia	2020	[167]
Octopus ink sac	Homogenization, centrifugation, SDS-PAGE, trypsin digestion, chromatographic separation	A, 0.1% FA; B, 98% acetonitrile (98% ACN) with 0.1% FA	column (EASY-Spray column, 50 cm × 75 μm ID, PepMap C18, 2 μm particles)	ESI+	LTQ-Orbitrap-Elite MS	–	Spain	2023	[168]
Burkholderia cepacia Complex	Centrifugation, filtration and digested	A, 0.1% FA, 2% ACN, 98% H2O; B, 0.1% FA, 100% ACN	HALO 2 Peptide ES-C18 2.1 × 100 mm 2 μm	ESI	Agilent ChipCube 6495 Triple Quadrupole MS	MRM	USA	2020	[169]
Samples from in vitro and in vivo	HLB-SPE	A, acetonitrile containing 0.1% formic acid; B, water with 0.1% formic acid	2.5 μm, 2.1 mm × 50 mm XBridge™ Ethylene Bridged Hybrid (BEH) column	ESI+	AgilentVarian 320 triple quadruple MS	MRM	Belgium	2020	[170]
Mice and human plasma	WCX- SPE	A, water containing 0.05 % formic acid; B, ACN containing 0.05 % formic acid	CSH C18 column, (75 × 3.0 mm, 2.5 μ)	ESI+	API 5500 Q trap MS	MRM	India	2019	[171]
Animal feed	HLB-SPE	A, 0.1% formic acid in water; B, 0.1% formic acid in acetonitrile	BEH C18 column (2.1 × 50 mm, 1.7 μm)	ESI	TSQ triple quadrupole MS and Q-ToF MS	MRM	France	2021	[172]
Trichoderma	cold maceration and liquid extraction	A, 0.1% solution of acetic acid; B, methanol	C18 column (Phenomenex, 250 × 4.6 mm, 5 μm)	ESI+	microtof II, Bruker Daltonics	–	Brazil	2023	[173]
Strains culture medium	Organic extraction, HPLC	A, 0.1% FA in ACN solution; B, 0.1% FA in water solution	Phenomenex Kinetex Biphenyl column (50 mm × 2.1 mm i.d., 2.6 μm)	ESI+	Triple Quad 5500 MS	MRM	China	2022	[155]

Note: "-" indicates not described.

Abbreviations: SELDI-TOF, Surface-enhanced laser desorption/ionization time-of-flight; IDM, Interaction discovery mapping; RP-HPLC, Reverse-phase high-pressure liquid chromatography; DBS, Dried blood spots; SPE, Solid phase extraction; MALDI, Matrix-assisted laser desorption/ionization; FSCAN, Full scan; MRM, Multiple reaction monitoring; SRM, Selective reaction monitoring; DDA, Data-dependent acquisition; LTQ, Linear trap quadrupole; LP-SEC: Low-pressure size exclusion chromatography; SDS-PAGE: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IEC: Ion exchange chromatography; FPLC: Fast-performance liquid chromatography.

capture the full molecular diversity of AMPs within a given sample matrix. Barashkova, A S et al. [62] studied a general extraction method for the isolation of plant AMPs. The isolation of AMPs involves three main stages: 1) homogenization of material, 2) extraction, and 3) saturation purification of the extract. The extracts are typically fractionated using a series of liquid chromatography (LC) methods. The commonly used sample pretreatment and AMP extraction methods are presented in Fig. 1.

3.1.1. Sample preparation

AMPs can be derived from various parts of the plants such as roots, tubers, flowers, fruits, seeds, or the whole plant. Additionally, AMPs can be obtained from soil actinomycetes, molds, insects, and marine vertebrates. We extensively reviewed the preparation of AMPs samples from various sources. Several methods have been used to isolate AMPs, no uniform protocol capable of isolating all structural families of AMPs exists.

During the homogenization stage, some of the samples withstand mechanical damage. The disintegration method is selected based on the physical properties of the sample material. For instance, plant seeds and the dried material are ground in a coffee grinder, whereas frozen portions are crushed in a mortar and pestle in liquid nitrogen. Animal mucus secretions are ground in a blender with an extraction buffer [66]. The extraction process may include additional steps, such as heating [67], removal of excess protein, pre-fractionation [68], or removal of tannins [69], or degreasing [70], if necessary for large protein peptides or small molecules with higher content. Protein and peptides can be extracted using simple solvent extraction techniques [71]. The conventional methods for peptide extraction are detailed further, starting with simple solvent extraction, including aqueous, organic, and acidic extraction. Subsequently, multiple rounds of centrifugation and subsequent retrieval were performed using methodologies such as ultrafiltration, precipitation, or chromatography.

3.1.2. Selection of solvent

Currently, there are three distinct categories of extractants are employed for the extraction of AMP. The first category comprises water and water-based solutions, including salts and buffers. The second category encompasses organic-based solutions, specifically water solutions of ethanol, and the third category is acidic extractions (Fig. 1).

3.1.2.1. Aqueous extractions. Buffer was the first extractant used to isolate AMPs. The solvents most frequently employed in these investigations included saline, water, ammonium bicarbonate, and Tris-buffered saline. A maximum diversity of proteins and peptides are isolated during the extraction process. However, using of buffer as an extraction agent results in the extraction of various peptides, such as carbohydrates and secondary metabolites dissolved in water, reducing the total yield of the target compound.

Although aqueous extraction is the most commonly used extraction method, but the extract exhibits the lowest antimicrobial capacity. This is evident in studies comparing other extraction methods. In some studies, the aqueous extract did not show any antimicrobial activity [72–74]. Al-Rashed et al. (2018) [72] demonstrated that the aqueous extract did not display antimicrobial activity against the tested bacteria. Based on the well diffusion method, the acidic crude extract exhibited different antimicrobial activities on agar plates. Phosphate buffer is the most common extractant for isolating AMP [75–77]. Ben Brahim, R et al. (2022) [78] revealed that antimicrobial activity was not observed in extracts obtained using sulfuric acid, dichloromethane, and phosphate buffer extraction. However, some strains were growth inhibited by acetate and sodium acetate extracts. Other studies have reported a growth inhibitory effect on gram-negative and gram-positive bacteria with aqueous extracts [79–84]. Gikas, E et al. (2023) [85] demonstrated the significant antimicrobial activity of urinary aqueous extracts against *Escherichia coli* and their relatively safety against mammalian cells. In a study by Ogbole, OO et al. (2021) [86], peptide fractions isolated using aqueous extracts from Euphorbia hirta leaf showed high antimicrobial activity against Coxsackievirus A13, Coxsackievirus A20, and Enterovirus C99.

Therefore, when detecting the biological activity of AMPs, special attention should be paid to the selection of extraction methods based on the characteristics of AMPs.

3.1.2.2. Organic extractions. Among organic extractions, ethanol is the most common solvent, followed by dichloromethane. Organically extracted AMPs have shown high antimicrobial activity [87,88]. This effectiveness can be attributed to two main factors: (1) the presence of hydrophobic groups in antimicrobial molecules, contributing to their affinity for membranes and ability to disrupt them [89]; (2) the use of organic solvents, which reduce interactions between hydrophobic groups in these extracts. Therefore, these extracts contain a high proportion of hydrophobic molecules, thereby hindering the aggregation of such hydrophobic molecules and facilitating their separation [90].

Studies have shown that both gram-positive and gram-negative bacteria were inhibited [91–93]. However, in a study by Křížkovská, B et al. (2023) [94], the majority of ethanol extracts from European herbs showed low or no toxicity against all strains. Subsequently, clinical multidrug-resistant (MDR) strains were tested to determine if ethanol extract could reverse drug-resistance phenotypes. The synergistic effects of breakpoint concentrations of antibiotics and nontoxins were observed. Neither the antibiotic nor the extract was active against the MDR strain when cultured alone. However, a combination of antibiotics and extracts reduced the viability of the MDR strain.

3.1.2.3. Acidic extractions. The most common solvent used for acid extraction was acetic acid, followed by trifluoroacetic acid. AMPs are also known as cationic host defense peptides [95,96]. Therefore, the first step in the extraction process was the extraction of proteins and peptides with basic properties during the aqueous extraction of the acid, thus simplifying further separation. The first thionins were isolated using sulfuric acid solutions in the 1970s [97]. Subsequently, the first AMP isolation and purification scheme

was proposed. This scheme was modified and applied to the isolation of synthetic anti-LPS peptide Pep19–2.5 [98]. After the addition of 270 μL hydrochloric acid (10 M), the sample was hydrolyzed for 24 h at 120 °C. Subsequently, the solvent was evaporated in a vacuum evaporator for 4 h at 40 °C, and the pellet was resuspended in water to enrich to the applied concentration.

Overall, acidic extracts exhibit better antimicrobial activity than other extraction methods [72,78,99,100]. Ben Brahim, R. (2022) [78] tested the antimicrobial activity of *Anthyllis sericea* and *Astragalus armatus* obtained from different extractions against six bacterial strains. Acetate and sodium acetate extracts were active against the studied strains. However, the extraction products based on sulfuric acid, phosphate buffer, and dichloromethane did not show any activity against the different tested strains. Al-Rashed et al. (2018) [72] found that aqueous extracts of fish epidermal mucus did not show any antibacterial activity against the studied bacteria. However, acidic mucus extracts showed strong and variable inhibitory effects on the growth of the tested bacteria. Mucus samples inhibited the growth of *Staphylococcus aureus, Escherichia coli, Salmonella, Bacillus subtilis, and Aeromonas hydrophila*, but the sensitivity was not high.

3.1.3. Concentration and purification

After extraction, the next step involves saturation, purification, and concentration, which are crucial in the purification stage of downstream processing [101]. The typical purification methods include chemical extraction, salt precipitation, membrane separation and chromatography [65].

When adopting salt buffer, water, or acid solution as the extraction agent, most protein peptide saturation is achieved by ammonium sulfate solution [102–104]. When seeds with a high total protein content were used, this was done in one or two stages [103,105]. Dialysis is required to remove excess salt, which can be held against water, buffer solutions used during extraction, or solutions needed for further analysis [106]. Dialysis, as a protein separation and purification method, possesses limited purification capabilities. Therefore, a variety of separation methods should be used to effectively separate and purify proteins while preserving their biological properties. In recent years, desalination (low-pressure chromatography) has emerged as an alternative to dialysis. This technique involves the use of hydrophobic adsorbents, such as phenyl-agarose, reverse phase C8, and C18 [107–109]. RP-HPLC has become a primary purification method for peptides and other small compounds due to its good selectivity and high separation performance. Depending on the separation efficiency of each method, a combination of methods enables the purification of peptides with different purities or molecular weight ranges.

The combination of the aforementioned purification methods often enhances peptide purity. Currently, commonly used purification methods include a combination of gel filtration, ultrafiltration, ion exchange chromatography, and RP-HPLC [110–113] (Fig. 1). In addition to the well-established peptide chemical extraction methods, there are emerging protein and peptide extraction methods such as microwave-assisted extraction, pulsed electric field, and ultrasound-assisted tooth extraction [114]. However, these methods are rarely used in currently studies. Therefore, summarizing all the accumulated experience seems meaningful. The existence of isolation protocols that allow extraction of a wide variety of peptides and comparison of results provides more possibilities for AMP studies.

3.2. AMPs detected through MS

MS technology can identify and analyze AMPs with high sensitivity, high selectivity and high resolution, offering crucial technical assistance for their research and application. The detection of AMPs typically encompasses sample preparation, liquid chromatography separation, tandem mass spectrometry analysis, and data interpretation, as illustrated in Fig. 2.

3.2.1. MS detection

The peptide extracts were First separated and purified through chromatography. Methanol and acetonitrile are the most commonly used organic phases for HPLC [115–119]. Depending on the types of AMPs detected, an appropriate amount of formic acid, at a low proportion, can be added to either the aqueous phase or organic phase. The flow rate of liquid phase can then be adjusted appropriately for better substance retention. The most commonly used ion source is ESI [120–122]. After separation through LC, the peptide samples underwent ionization and filtration through the ion transport channel and were subsequently detected by the mass detector. The mass-to-charge ratio of the ionized peptides was detected using the mass spectrometer to obtain the primary mass spectrum. It was further detected through secondary MS, which is similar to the primary MS. The secondary MS material is selected using the quadrupole mass analyzer and transported to the collision cell for secondary fragmentation, producing a large number of secondary fragments. The secondary MS detects the fragmented peptide ions to obtain the secondary mass spectrum, whereas the primary MS detects the complete peptide ions.

Previous studies indicate that over 3100 natural AMPs have been described from groups representing life on Earth [4]. However, the research on the application of AMPs through MS primarily focuses on the discovery of new AMPs and the elucidation of their structures, often using high-resolution MS. Additionally, LC−MS/MS has been used to quantitatively detect AMPs. Iram, D et al. (2022) [123] reported the characterization of AMPs from *Lactobacillus rhamnosus* (C25) fermented goat milk, using high-resolution MS. Nanoflow LC−MS/MS analysis was performed on a Captive spray-Maxis-HD impact II QTOF (Bruker, Germany). Peptides were separated using a C-18, 3 μm nano-trap column (Bruker Magic C18AQ, 0.1 × 150 mm, 3 μm partials size, and 200 A° pore size). A gradient method was used, starting with 2% B at a flow rate of 5 μL/min for 2 min. The gradient increased was increased to 20% B at 46 min, followed by 45% B at 61 min. The gradient was then increased to 95% B in 2 min, which was maintained for 5 min, followed by 2% B at 12 min, resulting in a total run time of 84 min. Analytes were detected using LC−MS in positive ion mode.

Currently, the MS technology used in AMPs research primarily high-resolution MS, which is mainly used to discover new AMPs and study the amino acid composition of newly discovered AMPs. The quantitative detection of AMPs is mainly performed using LC−MS/

MS. In this review, studies on the determination of AMPs through LC−MS over the past five years are summarized in Table 1.

3.2.2. Data analysis

Three commonly used methods exist for the analysis of MS data. The first is the database search [151,156,168], which involves comparing theoretical enzyme digestion with theoretical fragments to identify peptides that match the MS spectrum. The identification information obtained through this method is derived from the sequences generated by known coding regions in the database. Cochet, MF et al. (2021) [115] used the database to match the mass spectra for data analysis, identifying peptides from the MS/MS spectra using the X!Tandem pipeline software [Plateforme d'Analyze Proteomique de Paris Sud-Ouest (PAPPSO) at INRAE, Jouy-en-Josas, Fragnce; http://pappso.inra.fr]. The search was conducted against a database consisting of reviewed proteins of *Gallus gallus*, which included 2262 proteins downloaded from the common Repository of Adventitious Protein (http://thegpm.org/crap). The database search parameters were defined as follows: non-specific enzyme cleavage was employed, allowing a mass error of 0.05 Da was allowed for fragment ions and 10 ppm for parent ions. Putative modifications considered in the search included methionine oxidation and serine phosphorylation. Furthermore, a minimum score corresponding to an e-value below 0.05 was deemed necessary for the accurate identification of peptides.

The second method is de novo sequencing [174,154], which is a type of secondary spectrum that doed no rely on the database for directly deducing suitable peptides matching. It addresses the challenge of parsing peptides that are not present in the database, such as peptides produced by variable bases in non-coding regions. The de novo sequencing steps for AMPs analysis included mass spectrum data acquisition, peak identification, mass-to-charge ratio extraction, fragment spectrum extraction, fragment spectrum analysis, deduction of amino acid sequence, generation of candidate sequence, evaluation of candidate sequence, and verification of the sequence. De novo sequencing is a complex task that dependent on instrumental resolution, data quality, and analyst experience. Professional mass spectrophotologists usually possess extensive experience in this area to ensure that the inferred sequence is accurate and reliable. It can be combined with other experimental techniques and database information to improve the reliability of amino acid sequence prediction. Grady, EN et al. (2019) [143] reported the utilization of MS/MS spectra for de novo sequencing of peptide-containing compounds to validate potential compounds identified using Antibase.

The third method is Spectrum library search [139,142], which involves is the rapid detection and identification of known peptides in the sample, followed by obtaining by pre-defined peptide screens through spectrum search. Feng, LL et al. (2019) [141] reported that LC–MS/MS data obtained in its raw form underwent a search using the Mascot engine v.2.2 (Matrix Science, London, UK) against established protein libraries that have been previously published. Proteins were obtained from the Uniprot database (Uniprot_Ixodidae_80119_20180302.fasta; 80119 protein sequences were downloaded on March 02, 2018). The Mascot search parameters utilized were as follows: trypsin was used as the enzyme, with a maximum of two allowed missed cleavages; peptide mass tolerance was set at 20 ppm; and fragment mass tolerance was set at 0.1 Da. Fixed modifications included carbamidomethyl (C), iTRAQ8plex (N-term), and iTRAQ8plex (K). Peptides were considered identified when the false discovery rate (FDR) was below 0.

With the rapid development of instrumental technology and the combined application of multi-omics techniques, the scale and complexity of data are increasing. Faced with of the challenge of processing and analyzing high-throughput protein and peptide MS data, many comprehensive data analysis software platforms are available to assist us in the efficient and time-saving analysis and processing of such data. PEAKS Studio software (Waterloo, ON, Canada) is commonly used for data acquisition and analysis [145,175–178]. Additionally, software such as Proteome Discoverer [179,160], Mascot software [180,157], and ProteinLynx Global SERVER [148,181,182] are widely used in protein polypeptide analysis.

3.3. Application of MS in the functional research of AMPs

The role of mass spectrometers in the field of AMP research is primarily focused on the following aspects.

3.3.1. Discovery and characterization of new AMPs from natural sources

Over the past few decades, a variety of AMPs have been found, with peptides exhibiting similar properties were extracted from diverse organisms, including mammals, insects, birds, fish, and plants. Due to their low concentration in bodily fluids, a comprehensive purification approach is necessary for obtaining pure peptides. The limited quantity of peptides obtained necessitates the use of highly sensitive analytical techniques like MS for sequencing purposes, making MS one of the main methods for their structure identification [183,184]. Liu, J et al. (2017) [183] outlines the basic steps of an accurate mass determination method in the positive ion mode of offline low-energy CID ESI Qq-TOF MS/MS. This method is commonly used for de novo sequencing of peptides to elucidate the structure of AMPs. Rodriguez, A et al. (2023) [185] performed peptide sequencing analysis with NanoLC-ESI−MS/MS for peptides from the marine shellfish. Punginelli, D et al. (2023) [186] demonstrated that nine new peptides were further identified through high-resolution MS analysis and database searches. Two synthetic peptides, one from a green leaf and one from a rhizome of Oceania, were identified and showed anti-biofilm activities against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*.

Research in this area uses MS-based screening approaches to identify new AMPs from natural sources, such as plants, animals, and microbes. This represents the most common approach currently employed in studies applying MS to detect AMPs.

3.3.2. Quantitative analysis of AMPs

Research in this area is dedicated to developing methods for quantifying AMPs accurately in complex samples, including biological fluids and tissues. The goal is to identify potential biomarkers for infectious or inflammatory diseases by measuring AMP levels under different conditions. Wang, Y et al. (2023) [187] have effectively developed a method using UPLC−MS/MS for the precise

measurement of colistin levels in both plasma and kidney tissue samples. This method has been successfully used to analyze the pharmacokinetics of colistin sulfate in rats, and the relationship between the renal accumulation of colistin and the administration time has been explored.

However, several challenges persist. First, the variability of sample preparation and the cumbersome nature of sample pretreatment before MS quantitative detection are notable. To achieve a specific detection concentration and purity of the target substance, pretreatment conditions should be explored before applying each quantification method [188,189]. Second, the detection range of MS is limit despite its high sensitivity and specificity [171,190,191]. After establishing the methodology, the performance of the linear detection range was verified. He, J et al. (2013) [130] established an MS method for the quantification of polymyxin B in mouse serum, demonstrating a linear concentration range of 0.0065–3.2 mg/L. Third, the complexity of the sample matrix is a critical consideration. A diverse range of biological samples, such as blood, urine, cerebrospinal fluid, skin mucus, and tissue, are used for the extraction of AMPs. Numerous substances present in these biological samples can exert matrix effects on the target substances. These matrix effects pose challenges to the direct analysis of MS, particularly when conducting prompt quantitative investigations [192]. In a study conducted by Song et al. (2019) [193], various cyclic polypeptide antibiotics exhibited significant matrix effects in different feeds, resulting in signal inhibition up to 50%. Matrix effect studies constitute one of the crucial factors in the development of various MS methodologies [194,129].

3.3.3. Localization of AMPs in tissues and cells

Mass spectrometry imaging (MSI) is an emerging technology with substantial potential in biomedicine, including advancements in biomarker discovery, metabolomics studies, drug applications, and clinical diagnostics [195,196]. Considerable achievements have been realized in using MSI to visualize the distribution of AMPs in tissues and cells.

Lu, J et al. (2023) [197] performed MSI [MALDI-7090 tandem time-of-flight (TOF)-MS] analysis of rat spinal cord 1 h after polymyxin B was administered via intraventricular injection at a clinically relevant dose of 0.5 mg/kg. MSI of brain slices revealed a distinct distribution pattern of polymyxin B, with most of it clustered in the lateral ventricle on the injection side and subsequently into the third and fourth ventricles. No polymyxin B was detected in the left ventricle and brain tissue. This mechanistic understanding, combined with pharmacokinetic/pharmacodynamic drug delivery strategies, will provide valuable insights for the development of secure and efficient intraventricular/intrathecal polymyxin regimens for treating central nervous system (CNS) infections caused by MDR gram-negative bacteria. Nilsson, A et al. (2015) [198] used these techniques to measure the distribution of polymyxin drugs and their metabolites. MSI provides a powerful alternative for tissue homogenization analysis and labeling or antibody imaging, enabling simultaneous detection of the spatial distribution of drugs and drug metabolites. Gonzalez, DJ et al. (2012) [199] identified novel phenol-soluble regulatory protein derivatives in methicillin-resistant *Staphylococcus aureus* using MSI. This work suggests that MSI can be used as a useful tool to extend our understanding of an important family of small peptide virulence factors.

3.3.4. Development of AMP-based therapies

This line of research focuses on using MS to optimize the pharmacokinetics and efficacy of AMP-based therapies. The rapid detection of AMPs in body fluids through LC−MS/MS can be used for the monitoring of therapeutic drugs. The application of AMPs in therapeutic monitoring primarily involves studying antibacterial activity against different pathogens in vitro.

Wang, X et al. (2023) [158] successfully isolated a novel AMP, named MCNDCGA peptide (also referred to as MOp3), from the seeds of *Moringa oleifera*. This peptide exhibited a significant inhibitory effect against *Staphylococcus aureus*, with a minimum inhibitory concentration (MIC) of 2 mg/mL. Parra, ALC et al. (2022) [200] investigated AMPs from ornamental tobacco floral nectar, selecting six peptides for additional characterization, synthesis, and assessment of their antimicrobial efficacy against plant pathogenic fungi and bacteria. The results demonstrated that all six peptides exhibited certain antibacterial activity. Cuesta, SA et al. (2021) [201] reported two novel crizole septin-16 and septin-17 peptides. These peptides, initially discovered through molecular cloning and MS/MS, were synthesized using solid-phase peptide synthesis. Their MICs and hemolytic activity were subsequently assessed through experimental testing. The results showed that crizocseptin exhibited antibacterial activity against *Escherichia coli, Staphylococcus aureus*, and *Candida albicans*, with low hemolytic effects. Polymyxin is predominantly used in the treatment of MDR infections in clinical practice, with numerous studies focusing on the detection of polymyxin through LC−MS/MS [111,127,131,134].

The objective is to explore the structure and activity of the new AMPs and to identify potential therapeutic applications.

Overall, research on AMPs through MS involves a range of techniques, including LC, MS/MS, MALDI, imaging, and other advanced MS-based approaches. The specific type of research will depend on the specific questions being addressed and the available resources. In this review, we provide a comprehensive summary of the studies conducted on the detection of AMPs using MS, encompassing different types and species (Table 1).

4. Limitations and future perspectives

4.1. Challenges in the application of MS for the detection of AMP

Currently, the application of LC−MS/MS in AMPs research primarily centers on the discovery of new AMPs, quantification, and antibacterial activity detection. The mass of peptides required to assess AMP mechanisms, structures, and functions may vary from a few micrograms to many milligrams. Therefore, a key challenge in AMP research lies in optimizing extraction or production methods to effectively fulfill the requirements for characterization and functional exploration. simultaneously, the selection of extraction methods and solvents during sample pretreatment and extraction greatly influences the amount and activity of AMPs, posing another

research challenge. Researchers must tailor pre-treatment and extraction conditions according to the properties of the respective AMP and subsequently choose the appropriate method.

The application of MS technology to investigate the localization of AMPs in tissues or cells represents a new research hotspot. MSI technology facilitates the localization and semi-quantitatively analysis of AMP expression and its interaction with other biomolecules. While the development of MSI technology brings new prospects, it also introduces challenges for researchers. On the one hand, a higher level of professional and technical expertise is required among scientific research staff. On the other hand, research on MS equipment and auxiliary tools demands elevated standards, entailing substantial investments in equipment and space.

4.2. Prospects of MS applied to AMP detection

Recent studies have demonstrated the utilization of "applied proteomics" approaches to investigate the diverse biological activities of AMPs. Digital mining using proteomics technology combined with bioinformatics analysis has revealed the medicinal value of various AMPs. To enhance the practical application of AMPs, a comprehensively analysis of their societal cost-effectiveness is essential. Studies have identified several AMPs with cytolytic activities. The discovery and development of AMPs possessing biological functions and devoid of toxic side effects in the human body for clinical application necessitate extensive clinical experiments for validation. Future research endeavors can focus on innovating novel AMPs, considering genetic engineering techniques focus on synthesis of high-value AMPs for biomedical applications. Furthermore, given recent advancements in precision genome editing technology, the synthesize of AMPs with no cytotoxicity becomes feasible, offering a potential avenue for treating infectious diseases and addressing the global drug resistance problem. With the in-depth study of AMPs, the effect of AMPs on the human immune system has emerged as a key research focus. In the future, the integration of MS/MS technology with other immunodetection technologies, such as immunoassay, can enhance the dimension and depth of AMPs research.

5. Conclusion

MS holds immense potential in the study of protein peptides due to its high sensitivity and specificity, particularly with the recent advancements in high-resolution MS technology. It not only facilitates peptide isolation but also serves the purpose of species identification. The capability to analyze multiple samples simultaneously contributes time and cost savings, overcoming many limitations associated with alternative technologies.

The review aimed to comprehend the methodological studies on the detection of AMP using MS/MS technology, encompassing the entire operation cycle from sample separation to MS identification. Despite sincere efforts to summarize comprehensive information and laboratory processes experience, this review is not exhaustive. Given the complexity and diversity of AMP sources, which present additional challenges to the technical application of MS/MS, a truly comprehensive review is nearly impossible. However, the content is anticipated to be sufficient to provide insights for the future stud on AMPs.

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Ethics declarations

Review and approval by an ethics committee was not needed because this review article was prepared using already available references and does not involve new human and animal experiments.

Data availability statement

Data sharing is not applicable to this article as no new data were created or analyzed in this review article.

CRediT authorship contribution statement

Panpan Fang: Writing – original draft, Project administration, Funding acquisition, Data curation, Conceptualization. **Songlin Yu:** Writing – review & editing, Software, Project administration, Methodology, Formal analysis. **Xiaoli Ma:** Writing – original draft, Visualization, Validation, Resources, Investigation, Data curation. **Lian Hou:** Supervision, Resources, Methodology. **Tiewei Li:** Validation, Resources, Investigation, Data curation. **Kaijie Gao:** Investigation, Funding acquisition, Data curation. **Yingyuan Wang:** Resources, Project administration, Methodology. **Qianqian Sun:** Visualization, Validation, Software. **Lujun Shang:** Resources, Investigation. **Qianqian Liu:** Visualization, Methodology. **Manjie Nie:** Supervision, Investigation. **Junmei Yang:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

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