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Review

Quantitative bioanalysis of peptides by liquid chromatography coupled to (tandem) mass spectrometry

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ABSTRACT

With the growing interest for peptides and proteins in different kinds of fields, e.g. pharmacy, clinical diagnostics or food industry, the quantification of these compounds is becoming more and more important. Quantitative analysis of these analytes in biological matrices, however, remains a challenging task, due to the complexity of both the matrix and the analytical characteristics of these large bio-molecules. Liquid chromatography coupled to (tandem) mass spectrometry (LC–MS or LC–MS/MS) is the preferred analytical technique for peptide analysis as it allows very selective and sensitive measurements. This article summarizes the numerous published LC–MS applications for the quantification of peptides in biological matrices and discusses all different issues herewith concerned. This includes chromatographic aspects as the selection and effects of mobile and stationary phase, flow rate and temperature, as well as mass spectrometric characteristics such as ionization and detection modes, collision-induced dissociation of peptides and factors influencing the mass spectrometric response. For both techniques the main properties of all described methods have been listed, creating a comprehensive overview with the peptide analytes divided into different classes. Likewise, all other issues concerned with quantitative bioanalysis have been evaluated in detail, including extensive consideration of several different applied sample pre-treatment techniques and reflection of subjects as the choice for an internal standard and assay validation. Furthermore, several issues which are of particular interest for the quantitative bioanalysis of peptide compounds like peptide adsorption and degradation have been regarded.
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1. Introduction

Peptides and proteins are becoming increasingly important in different types of biomedical research. For example, in the pharmaceutical industry peptides are emerging as a novel class of therapeutic agents, especially in cancer therapy [1]. These peptide therapeutics are often synthetic analogs of endogenous peptides, such as hormones or neurotransmitters [2–5] or are derived from natural bio-active peptides, mainly of marine origin or with antimicrobial activity [6–10]. Besides the usefulness of peptides as templates for novel pharmaceutical compounds, endogenous peptides can also provide information about physiological conditions or important functional processes in the body and are therefore extensively explored for applications as diagnostic tools for different types of diseases [11,12].

With this growing interest in peptides, techniques for the quantification of these compounds in their biological environment are required. For peptide therapeutics, quantitative bioanalytical techniques have been developed for therapeutic drug monitoring or to obtain pharmacokinetic information about the compounds during both clinical and pre-clinical drug development. Furthermore, bioanalytical assays have been described for the quantification of endogenous peptides with diagnostic potential and of peptide digests of proteins to measure the absolute amount of the intact protein. The measurement of these smaller peptides is favored as quantification of large proteins by mass spectrometry is complicated and quantification with immunoassays entails major drawbacks. In addition, quantitative bioassays have been reported for monitoring peptide degradation [13] or quantifying peptides in food [14,15].

Although mainly employed for the quantification of large proteins, several immunoassays have conventionally also been employed for the analysis of peptide drugs [16–21]. Immunological methods can be highly sensitive and provide rapid analysis with high sample throughput, but a major disadvantage is the risk of cross-reactivity, as the used antibodies cannot discriminate between structurally related peptides. Accurate quantification is therefore limited and data should be interpreted cautiously.

Other bioanalytical procedures, specifically used in the bioanalysis of peptide therapeutics are liquid chromatography (LC) combined with ultraviolet (UV) [22–30], fluorescence [31–39] or electrochemical [40–42] detection. However, these detection methods have limited sensitivity and specificity. Separation of the analyte from interfering matrix components prior to detection makes sample pre-treatment and long and complicated LC-gradient runs necessary. Fluorescence and electrochemical detection are also dependent on fluorescent or electro-active properties of the compound and do otherwise require derivatization.

Nowadays, mass spectrometry is the method of choice for sensitive and selective detection of peptides. This technique can discriminate co-eluting peptides with different masses and by performing tandem mass spectrometry even compound specific masses of fragments can be detected and used for quantification.

Several reviews on the quantitative (bio)analysis of peptides appeared during the last years [43–45]. With the rapidly increased number of publications of LC–MS and LC–MS/MS bioassays for peptides, this review provides an expanded overview of the applications for the quantification of peptides in biological matrices. Different types of peptides, like (potential) therapeutics or biomarkers, have been categorized according to their origin. The summarized LC–MS procedures additionally include peptides that have been analyzed after *ex vivo* digestion as a measure of the total amount of intact polypeptide or protein. Furthermore, the effect of peptide degradation on the quantification has been discussed, as well as other general aspects on the bioanalysis of peptides such as a detailed summary of sample pre-treatment techniques. A general scheme of a bioanalytical assay with the selected items for this review is shown in Fig. 1.

2. Sample pre-treatment

2.1. Adsorption problems

Peptides are known to adsorb on surfaces, including reaction vials, pipette tips and parts of the LC–MS system. To minimize the loss of peptides, resulting in poor responses and non-linear correlations, experiments should be performed cautiously with the potential adsorption behavior in mind. Factors effecting peptides adsorption are either container material or peptide and solvent properties, such as peptide conformation and concentration or solvent pH, temperature and ionic strength.

The interaction of peptides with solvents and surfaces depends largely on the specific side chains of the amino acids of the peptide. Positively charged peptides will easily exhibit electrostatic interaction with for example glass surfaces, carrying a negative potential. Neutral compounds, on the other hand, will more easily exhibit hydrophobic interactions with hydrophobic materials, e.g. polypropylene. It was demonstrated that from a set of proteolytic peptides, the effect of adsorption was most pronounced for hydrophobic peptides using polypropylene vials, as a result of

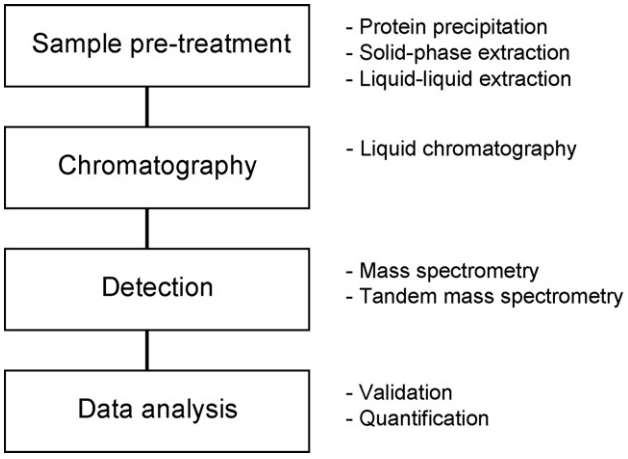


Fig. 1. General steps in a bioanalytical assay with several examples that are subjected to discussion in this review.

Table 1
SPE procedures for extraction of peptides, including (potential) therapeutics, biomarkers and other peptides, from biological matrices

Analyte	MW	Matrix	SPE sorbent	Elution solvent (ratio, % (v/v) or molarity)	Recovery (%)	Remarks[a]	Reference
Opioid peptides							
DADLE	569	Plasma	C_{18}	A–W (7:3)	79 ± 11[c]	+ Flu	[68]
DADLE	569	Plasma	PVP-DVB	A, T (0.1%)	76 ± 4[c]		[68]
DALDA	611	Plasma	C_{18}	A–W (3:7)	71–76[c]	Indirect MS	[69]
Dansyl-PQR-NH_2	631	Brain tissue	C_{18}	A–W, AA (1:1, 3%)	×[c]		[70]
[Dmt[a]] DALDA	639	Plasma	PVP-DVB	M	80		[71,72]
DPDPE	648	CSF	C_{18}	M	97–102		[73]
DSLET	686	Plasma	C_{18}	A–W, T (8:2, 0.1%)	85		[74]
LE-R	712	CSF	C_{18}	M, T (0.1%)	73 ± 2	+ RIA	[75]
ME-RF	877	CSF	C_{18}	M, T (0.1%)	61 ± 2	+ RIA	[75]
ME-RGL	900	Plasma	C_{18}	A–W–T (8:2, 0.1%)	65		[74]
Hormone analogs and antagonists							
Taltirelin	405	Plasma	PVP-DVB	M	91–95		[76]
Tabimorelin	529	Serum	C_8	×	×		[77]
Bradykinin 1-5	572	Plasma	MA-DVB	M, HCl (0.001 M)	×		[78]
IRI-514	671	Plasma	C_{18}	A–W, T (3:7, 0.1%)	>70[c]		[79]
GHRP-6	873	Serum	C_{18}	M, T (0.5%)	87 ± 2	+ ED	[40]
Substance P_{1-7}	899	CSF	C_{18}	M, T (0.1%)	72 ± 2	+ RIA	[75]
Antagonist G	951	Plasma	C_2	M–W, NA (9:1, 1 M)	71–100	+ ED/UV	[23,41]
Melanotan-II	1024	Urine	C_8	A–W, P (3:7, 0.1 M)	×	+ UV	[28]
Melanotan-II	1024	Plasma	PVP-DVB	A–W, T (9:1, 0.1%)	>90	96w	[80]
Leuprorelin	1209	Serum/urine	C_{18}	E–W, AA (86:14, 4%)	88–98	+ RIA	[20]
Substance P	1347	CSF	C_{18}	M, T (0.1%)	71 ± 4	+ RIA	[75]
Cetrorelix	1430	Plasma/urine	C_8	M–W, T (9:1, 0.1%)	60–70		[55]
Antagonist D	1517	Plasma	C_8	M–W, T, NA (8:2, 0.1%; 1 M)	44-74	+ ED	[41]
Melanotan-I	1647	Plasma	C_{18}	A–W, P (3:7, 0.1M)	±50	+ UV	[26]
Hepcidin-25	2789	Plasma/serum	PVP-DVB	A–W, T (8:2, 0.05%)	59[c]	96w	[81]
B201	2814	Plasma	C_{18}	A–W, T (8:2, 0.1%)	78		[82]
C-peptide	3020	Plasma	C_8	A–W, P (35:65, 0.025 M)	78–89		[83]
Thymosin-α1	3108	Serum	C_{18}	E–W, AA (86:14, 4%)	×		[63]
C-peptide-31	3020	Serum	C_{18}	A–W, T (1:1, 0.1%)	±80	Indirect MS	[58]
C-peptide-33	3304						
Insulin	5807						
Pro-insulin	9388						
Insulin	5808	Blood	C_{18}	A–W, AA (4:6, 0.05 M)	95		[53]
Antimicrobial peptides[b]							
Caspofungin	1093	Plasma/urine	OH	M, T, NH (0.1%; 0.25 M)	76–84	+ MS/Flu	[35,84]
Caspofungin	1093	Plasma	C_8	M, T, NH (0.05%; 0.25 M)	76[e]; 84[f]	96w + Flu	[85]
LY303366	1140	Plasma	PH	A–W, P (6:4, 0.05 M)	78–90	+ UV	[30]
Cyclosporin A	1203	Blood	C_{18}	IPA-H (1:1)	72.5[c]		[86]
Cyclosporin A	1203	Blood	×	A	×[c]	96w; OAs	[87]
Cyclosporin A	1203	Blood	C_{18}	M–W (9:1)	±90[c]		[88]
Cyclosporin A	1203	Saliva	C_{18}	IPA-H (1:1)	85 ± 3[c]		[89]
Cyclosporin A	1203	Blood	C_{18}	IPA-H (1:1)	81 ± 5[c]		[90]
Cyclosporin A	1203	Plasma	PVP-DVB	M	84 ± 10		[91]
Actinomycin-D	1255	Plasma	PVP-DVB	M	±80	OAs	[92,93]
Vancomycin	1449	Plasma	C_{18}	A–W, P (1:1, 0.05 M)	92–98	+ UV	[24]
Vancomycin	1449	Serum	C_{18}	1) A–W, P (7:3, 0.05 M) 2) A–W (1:1)	×	+ UV	[22]
Vancomycin	1449	Serum	SCX	M, NH	85–96		[94]
Enfuvirtide	4492	Plasma	PVP-DVB	A–W, T (6:4, 0.1%)	79–99	+ Metabolite	[95,96]
Tifuvirtide	5037	Plasma	PVP-DVB	A–W, T (6:4, 0.1%)	74–110		[95]
Marine derived anticancer peptides							
TZT-1027	702	Plasma	C_{18}	A-W, T (6:4, 0.05%)	63–88[d,e]		[97]
Aplidine	1110	Plasma/blood	C_{18}	ACE	55[5]; 60[g]	+ Flu	[37,98]
Thiocoraline	1156	Plasma	C_{18}	A	67–80[c]		[65]
Kahalalide F	1478	Plasma	C_{18}	M, HCl (0.1M)	91 ± 0.5		[99]
Other							
Bivalirudin	2180	Plasma/urine	C_{18}	M	85–89	+ Flu	[32]

The peptides have been classified according to their origin. A: acetonitrile; AA: acetic acid; ACE: acetone; B201: bradykinin antagonist; CSF: cerebrospinal fluid; DADLE: D-Ala^2,D-Leu^5 enkephalin; DALDA: Tyr-D-Arg-Phe-Lys-NH_2; dansyl-PQR-NH_2: neuropeptide FF antagonist; DPDPE: D-penicillamine^{2,5} enkephalin; DSLET: (D-Ser^2) Leu-enkephalin-Thr^6; E: ethanol; ED: electrochemical detection; Flu: fluorescence detection; GHRP-6: His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2; H: heptane; HCl: hydrochloric acid; IPA: isopropyl alcohol; IRI-514: Ac-Arg-Pro-Asp-Pro-Phe-NH_2; LE-R: leucine enkephaline-Arg^6; LY303366: lipopeptide antifungal echinocandin B analog; M: methanol; MA-DVB: methacrylate-divinylbenzene copolymer; ME-RF: methionine enkephalin-Arg^6-Phe^7; ME-RGL: methionine enkephalin-Arg-Gly-Leu; NA: ammonium acetate; NH: ammonium hydroxide; OAs: other analytes simultaneously quantified; OH: Diol-SPE sorbent; P: phosphate; PH: phenyl-SPE sorbent; PVP-DVB: polyvinylpyrrolidone- divinylbenzene copolymer; RIA: radioimmunoassay; SCX: strong cation exchange sorbent; T: trifluoroacetic acid; TZT-1027: dolastatin-10 derivative; UV: ultraviolet detection; W: water; ×: not reported; 96w: In 96 wells format.

[a] MS(/MS) detection is employed, unless otherwise stated.

[b] Antimicrobial peptides (AMP's) include peptides with antibiotic, antifungal, antiviral and immunosuppressive activity.

[c] In combination with PP.

[d] In combination with LLE.

[e] In plasma.

[f] In urine.

[g] In blood.

mainly hydrophobic interactions with the vial material [46]. However, it remains difficult to predict the effect of tube material on a specific peptide. Adsorption of the decapeptide cetrorelix was found to be less to plastic than to glass vials [47], whereas for other peptides loss was described to be minimized by using glassware [48] or not to be influenced by tube materials [49,50].

Temperature might also be expected to influence peptide adsorption, but its effect has never been described. Only the observation of no difference in adsorption to glass between 4 and 37 °C for the peptide calcitonin has been reported [51].

The adsorption problem will be more severe at low peptide concentrations, especially in aqueous solutions. These (aqueous) solutions with very low peptide concentrations should therefore be avoided, if possible, or treated carefully. The composition of the peptide solution can be optimized to reduce peptide adsorption to container surfaces by addition of displacement agents that compete with the analyte for surface binding sites, e.g. structural analogs [52] or protein-rich solutions, like plasma or serum albumin [35,49,50,53–58]. Addition of organic solvents [34,46–48,59], acids [34,47] or salts [23,41,60] has also been mentioned to reduce

Table 2
PP procedures for extraction of peptides, including (potential) therapeutics, biomarkers and other peptides, from biological matrices

Analyte	MW	Sample matrix	Precipitation solvent (volume relative to sample)	Recovery (%)	Remarks[a]	Reference
Opioid peptides						
DAMGO	513	Plasma	A (2)	×	No LC	[101]
DADLE	569	Plasma	A (1.8)	76 ± 4[c]		[68]
DALDA	611	Plasma	A (1.3–5)	71–76[c]	Indirect MS	[69]
Dansyl-PQR-NH_2	631	Bh/plasma	A (4)	×[c]		[70]
[Dmt[a]]DALDA	639	Plasma	A (2)	×		[71,72]
DE-γ-endorphin	1304	Plasma	TCA (0.3)	50[5]	+ Flu	[38]
LVV-hemorphin-7	1310	Plasma	M, HCl * (6.7)	×	Indirect MS	[102]
Hormone analogs and antagonists						
ACE-inhibiting peptides	276-625	Plasma	10% T (0.02)	×	+ Heating	[50]
IRI-514	671	Plasma	A (2)	>70[c]		[79]
Melanotan-II	1024	Plasma	W, $HClO_4$ (0.3)	×	+ UV	[27]
Melanotan-II	1024	Bh/plasma	A (4)	>90[f]; ∼60[i]		[103]
RC-3095	1106	Plasma	A* (2)	73–90		[104]
[M1]-PTH(1–14) NH_2	1721	Plasma	4% TCA (1)	77		[105]
Endothelin peptide-1	2494	HUVEC	A (1.6)	75–90[e]		[56]
Endothelin peptide-2	2549	Supernatant				
Endothelin peptide-3	2645					
Leu[13]-motilin	2681	Plasma	A* (×)	95		[60]
Motilin	2699	Plasma	A (×)	×		[54]
Hepcidin-25	2789	Serum	4% TCA (1)	34		[105]
Hepcidin-25	2789	Plasma/serum	A (1)	59[c]	96w	[81]
Desacyl-ghrelin	3245	Plasma/serum/cs	A-W, SSA (0.5)	101 ± 1[e]		[106]
Ghrelin	3371			96 ± 12[e]		
Salcatonin	3430	Plasma	A (1)	×		[107]
Salcatonin	3430	Serum	A (2)	×		[108]
Glucagon	3483	Plasma	A (2)	68 ± 4		[49]
Urodilatin [95–126]	3506	Urine	E* (2)	90–104	+ RIA	[16]
Antimicrobial peptides[b]						
Actinomycin-D	1255	Plasma	A (6)	76–96	OAs	[109]
NR58-3.14.3	1358	Serum	A* (4)	×		[110]
Vancomycin	1449	Plasma	M (1)	91 ± 8	+ ED	[42]
Vancomycin	1449	Plasma	M-W, T (3)	×		[111]
Human HEM-γ	1935	Plasma	A* (2)	84 ± 3		[112]
Enfuvirtide	4492	Plasma	A, T, NNGP (9)	50–75	+ Flu	[33]
Enfuvirtide	4492	Plasma	A (9)	116–124	+ Metabolite	[113]
Enfuvirtide	4492	Plasma	A, T (9)	102–107	+ Flu	[31]
Marine derived anticancer peptides						
Aplidine	1110	Plasma/urine	A, FA (4)	69[d,f], 91[d,g]		[114]
Aplidine	1110	Plasma/blood/urine	A, FA (4)	54–70[d,f,h] 46-60[4,7]		[115]
Thiocoraline	1156	Plasma	A (2)	101	+ Flu	[36]
Thiocoraline	1156	Plasma	A (0.75)	67–80[c]		[65]

The peptides have been classified according to their origin. A: acetonitrile; Bh: brain homogenate; cs: cell supernatant; DADLE: D-Ala^2,D-Leu^5 enkephalin; DALDA: Tyr-D-Arg-Phe-Lys-NH_2; DAMGO: Tyr-D-Ala-Gly-*N*-methyl-Phe-Gly-ol; Dansyl-PQR-NH_2: Neuropeptide-FF antagonist; DE-γ-endorphin: Desenkephalin-γ-endorphin; E: ethanol; ED: electrochemical detection; Flu: fluorescence detection; FA: formic acid; HCl: hydrochloric acid; $HClO_4$: perchloric acid; HUVEC: human umbilical vein endothelial cells; IRI-514: Ac-Arg-Pro-Asp-Pro-Phe-NH_2; M: methanol; [M1]-PTH(1–14)NH_2: parathyroid hormone analog; NNGP: *n*-nonyl-β-D-glucopyranoside; NR58-3.14.3: panchemokine inhibtor; OAs: other analytes simultaneously quantified; RC-3095: bombesin/gastrin releasing peptide antagonist; RIA: radioimmunoassay; SSA: sulfosalicylic acid; T: trifluoroacetic acid; TCA: trichloroacetic acid; UV: ultraviolet detection; W: water; ×: not reported; *: icecold; 96w: in 96-wells format.

[a] MS(/MS) detection is employed, unless otherwise stated.
[b] Antimicrobial peptides (AMP's) include peptides with antibiotic, antifungal, antiviral and immunosuppressive activity.
[c] In combination with SPE.
[d] In combination with LLE.
[e] In combination with on-line pre-treatment.
[f] In plasma.
[g] In urine.
[h] In whole blood.
[i] In brain homogenate.

adsorptive losses, mainly by improving the peptide's solubility. The use of surfactants to reduce adsorptive losses has been frequently reported [25,33,47,61], but these compounds are not compatible with MS measurements and have not been used in quantitative LC–MS assays [46].

Furthermore, adsorption of the peptide in the LC-system can cause analytical problems and is frequently observed by showing a carry-over effect. To reduce this carry-over effect, extensive rinsing of the injection system has been reported several times [62–67], as well as column-switching that enables extensive washing of the autosampler and the extraction column during the analysis [62].

The adsorption phenomenon of peptides is obviously a serious issue that can drastically affect the peptide's analysis. However, it seems hard to predict the effect of adsorption of a specific peptide on the analytical procedure. Therefore, careful evaluation of the adsorption characteristics and potential complications of a specific peptide is recommended for any peptide.

2.2. Sample pre-treatment techniques

Although sensitive and specific mass spectrometric detection limits the demands for sample pre-treatment, it still remains a challenging part of LC–MS method development, since matrix components can cause severe ion suppression of the analyte or contamination of the system. Commonly used techniques for the extraction of peptides from biological matrices are solid-phase extraction (SPE), protein precipitation (PP) and liquid–liquid extraction (LLE).

SPE is a chromatographic procedure based on the interaction of the analyte with the sorbent of the SPE cartridges. Solid-phase sorbents with non-polar, polar, ion-exchange and mixed properties are available and the possibility to select optimal SPE sorbent and solvents makes SPE a very selective, sensitive and versatile technique. The correct choice of SPE material depends mainly on peptide polarity and charge. Table 1 summarizes several SPE methods for the extraction of peptides from biological matrices. C_{18}-modified silica is the most commonly applied SPE sorbent, for both small and large peptides. Nevertheless, polymeric columns have been more frequently applied for the more recent SPE procedures. The elution solvents contain a high percentage of organic solvent, usually acetonitrile or methanol.

PP is a simple sample pre-treatment technique, based on the addition of a precipitating solvent, acid or salt. Several different PP methods for peptide analytes are shown in Table 2. A widely applied PP procedure consists of the addition of acetonitrile in twice (or more) the volume of the biological sample. If very strong acids are applied, precipitation solvent volumes smaller than the volume of the biological samples can be used. In the precipitation step of cyclosporin A, $ZnSO_4$ is frequently added to improve recovery, although it is a main cause of ionization suppression [100]. Table 3 shows several specified PP methods for this immunosuppressive peptide.

After addition of the precipitation solvent, samples are centrifuged and the supernatant is further processed. It is an easy and inexpensive procedure and it might be sufficient when selective MS detection is operational. However, the risk for inclusion of the analyte by PP is of specific concern for the analysis of peptides. Furthermore, remaining matrix components might lead to contamination of the LC–MS system and can also cause ion suppression. PP is therefore often performed in combination with another sample pre-treatment procedure (Tables 2 and 3).

LLE is a sample pre-treatment method based on distribution differences of the analyte between aqueous and organic solvents. Since the matrix is usually aqueous, LLE is especially suited for apolar compounds. However, most peptides exhibit ionic activity and the usefulness of LLE is therefore limited (Table 4) to peptides that are uncharged at a specific pH, like peptides with a cyclic structure or peptides with modified C- and N-termini (Fig. 2). Nevertheless, an LLE method for the quantification of the decapeptide cetrorelix in human plasma with fluorescence detection has been reported [34]. This peptide is acetylated at the N-terminus and amidated at the C-terminus, but still contains charged groups and LLE recovery was low ($50.1 \pm 4.9\%$). A higher recovery (60–70%) for the same peptide has been described by Niwa et al. [55], using SPE before LC–MS analysis. On the other hand, the recovery of substance P, substance P_{1-7}, Met-enkephalin-Arg^6-Phe^7 and Leu-enkephalin-Arg^6 from cerebrospinal fluid (CSF), highly increased by the use of LLE in stead of SPE [75]. Moreover, the recovery of these four peptides could be further increased by the addition of HCl to the CSF sample prior to LLE.

In addition to SPE, PP or LLE, alternative sample pre-treatment techniques have been reported to recover peptides from biologi-

Table 3
Different protein precipitation methods for cyclosporin A, eventually preceding other cleanup procedures, before LC–MS analysis

Matrix	Sample amount (μl)	Precipitation reagent	Molarity (M) $ZnSO_4$ (volume ratio organic solvent:$ZnSO_4$)	Volume ratio sample:reagent	Further extraction	Recovery (%)	Other analytes	Reference
Blood	100	A–W (7:3)	–	1:2	SPE	72.5	No	[86]
Blood	1000	M–$ZnSO_4$	0.4 (4:1)	1:1	On-line SPE	90–96	Yes	[116–118]
Blood	350	A–$ZnSO_4$	0.1 (7:3)	1:3	SPE	×	Yes	[87]
Blood	100	M–$ZnSO_4$	0.2 (7:3)	1:2	On-line SPE	91 ± 10	Yes	[119]
Blood	300	M–$ZnSO_4$	0.4 (4:1)	1:1	SPE	±90	No	[88]
Blood	×	M	–	1:9	–	89–95	No	[120]
Serum	100			1:4				
Blood	250	A	–	25:35	On-line SPE	×	Yes	[121]
Blood	300	M–$ZnSO_4$	0.2 (4:1)	1:2	On-line SPE	±96	Yes	[122]
Blood	100	A	–	1:2	–	102–119	No	[123]
Blood	100	M–$ZnSO_4$	0.3 (7:3)	1:2	–	71	Yes	[124]
Blood	10	A–$ZnSO_4$	0.4 (5:2)	1:14	–	102–119	Yes	[125]
Blood	100	M–$ZnSO_4$	0.2 (4:1)	1:2	On-line SPE	98–103	Yes	[126]
Blood	50	M–$ZnSO_4$	0.5 (4:1)	1:2	TFC	97	Yes	[127]
Saliva	500	A–W	–	1:2	SPE	85 ± 3	No	[89]
Blood	50	A–$ZnSO_4$	0.1 (7:3)	1:3	SPE	81 ± 5	No	[90]
Blood	500	A–$ZnSO_4$	Xx (1:1)	1:3	SA-SPE	100 ± 6	Yes	[128]
Blood	100	M–$ZnSO_4$	0.00005 (4:1)	1:6	–	77–101	Yes	[129]
Blood	50	M–$ZnSO_4$	0.05 (1:1)	1:20	–	84–119	Yes	[100]

A: acetonitrile; M: methanol; SA-SPE: semi-automated SPE; TFC: turbulent-flow chromatography; W: water; ×: not reported.

Table 4
Different LLE procedures for the extraction of endogenous peptides and peptide therapeutics from biological matrices

Analyte	MW	Sample matrix	Organic solvent	Recovery (%)	Remarks[a]	Reference
Opioid peptides						
LE-R	712	CSF	(1) EA-HCl	94 ± 18	+ RIA	[75]
ME-RF	877		(2) EA-PE	97 ± 17		
			(3) ACE-PE			
			(4) EA			
Hormones and hormone analogs						
CP-80, 794	620	Serum	BCl	×		[52]
Substance P_{1-7}	899	CSF	(1) EA, HCl	102 ± 13	+ RIA	[75]
Substance P	1347		(2) EA-PE	112 ± 17		
			(3) ACE-PE			
			(4) EA			
Cetrorelix	1430	Plasma	EA-B	50 ± 5	+ Flu	[34]
Antimicrobial peptides[b]						
Apicidin	624	Serum	BME	87–97		[130]
Cyclosporin A	1203	Blood	(1) EE	88		[131]
			(2) W-A-M-H			
Cyclosporin A	1203	Blood	MTBE	×	96w; OAs	[132]
Marine derived (anticancer) peptides						
Dolastatin-10	785	Plasma	BCl	85–90		[133]
TZT-1027	702	Plasma	EA	74–99[c]		[97]
FR901228	540	Plasma	EA	×		[134]
FR901228	540	Plasma	EA	60–70		[135]
Aplidine	1110	Plasma/urine	Cf	±69[d,e]; ±91[d,f]		[114]
Aplidine	1110	Blood/plasma/urine	Cf	54-70[d,e]; 46–55[d,g]		[115]
Aplidine	1110	Plasma	BME	88–117		[64]

The peptides have been classified according to their origin. A: acetonitrile; ACE: acetone; Am: ammonia; B: 1-butanol; BCl: *n*-butyl chloride; BME: butyl-*t*-methylether; Cf: chloroform; CP-80, 794: renin inhibitor; CSF: cerebrospinal fluid; E: ethanol; EA: ethyl acetate; EE: ethyl ether; Flu: fluorescence detection; FR901228: antitumor depsipeptide; H: *n*-heptane; HCl: hydrochloric acid; LE-R: leucine enkephalin-Arg[6]; M: methanol; ME-RF: methionine enkephalin-Arg[6]-Phe[7]; MTBE: methyl-*tert*-butyl ether; OAs: other analytes simultaneously quantified; PE: petroleum ether; RIA: radioimmunoassay; TZT-1027: dolastatin-10 derivative; W: water; ×.: not reported; 96w: in 96-wells format.

[a] MS(/MS) detection is employed, unless otherwise stated.

[b] Antimicrobial peptides (AMP's) include peptides with antibiotic, antifungal, antiviral and immunosuppressive activity.

[c] In combination with SPE.

[d] In combination with PP.

[e] In plasma.

[f] In blood.

[g] In urine.

cal matrices, including immunoaffinity purification [66,136–141] and ultrafiltration [57]. Furthermore, the above-mentioned most commonly applied techniques have become more frequently automated or placed in-line with the LC–MS system as these manual techniques are relative laborious and time-consuming. On-line SPE allows direct injection of the biological sample as it couples an extraction column with the analytical column, usually by column-switching. This technique has frequently been described for quantitative assays of the immunosuppressant cyclosporin A [116–119,122,126,142], as well as for several other peptides [56,62,67,106,143–146]. Turbulent flow chromatography (TFC) is an on-line SPE technique using extraction columns with large particle sizes, allowing high flow rates. The turbulent flow in the extraction column causes smaller molecules to bind to the absorbent while large molecules are not retained and are removed from the sample matrix. An application of TFC in peptide bioanalysis is the extraction of cyclosporin A from blood [127]. Eventually, the automated procedures can be preceded by off-line protein precipitation [56,116–119,121,122,126,127]. Besides on-line automation to speed up the time limiting sample pre-treatment, off-line extraction methods have been (semi-)automated for several applications in 96-well format [80,81,87,125,132,147].

The various matrices in which the analytes exist can affect the choice of the applied type of sample pre-treatment. For example, the quantification of desmopressin has been reported in human skin samples [148] and this uncommon matrix required alternative sample pre-treatment by extraction with a water–ethanol–methanol mixture. Furthermore, quantification of peptides from CSF has sometimes been performed without any purification step [149–151], as CSF is a substantial cleaner matrix. Nevertheless, also an LC–MS assay without any purification has been reported for urine samples [152].

2.3. Enzymatic digestion

All above-mentioned sample pre-treatment techniques are required to reduce potential interferences of matrix compounds with the analyte of interest. Another technique that is sometimes applied before the LC–MS measurement of peptides is *enzymatic digestion*, performed to yield peptide compounds rather then cleaning-up the matrix. This sample handling step involves cleavage of a large peptide or protein into smaller peptide fragments by a specific proteolytic enzyme under specific conditions. The amount of the proteolytic peptide fragment can be quantified by LC–MS and serve as a representation of the total amount of the intact peptide or protein. This procedure specifically offers opportunities for the quantification of proteins as quantification by mass spectrometry will be more sensitive and less complicated for the smaller peptides compared to the large protein.

Several applications of enzymatic digestion for the quantification of large peptides or proteins in biological matrices have been reported [96,141,153–161], mostly with the intention to show the abilities of this technique for absolute protein quantification rather then to provide a sensitive, validated bioanalytical assay. Therefore, and because of the complicated sample handling steps involved with enzymatic digestion, further sample clean-up of the

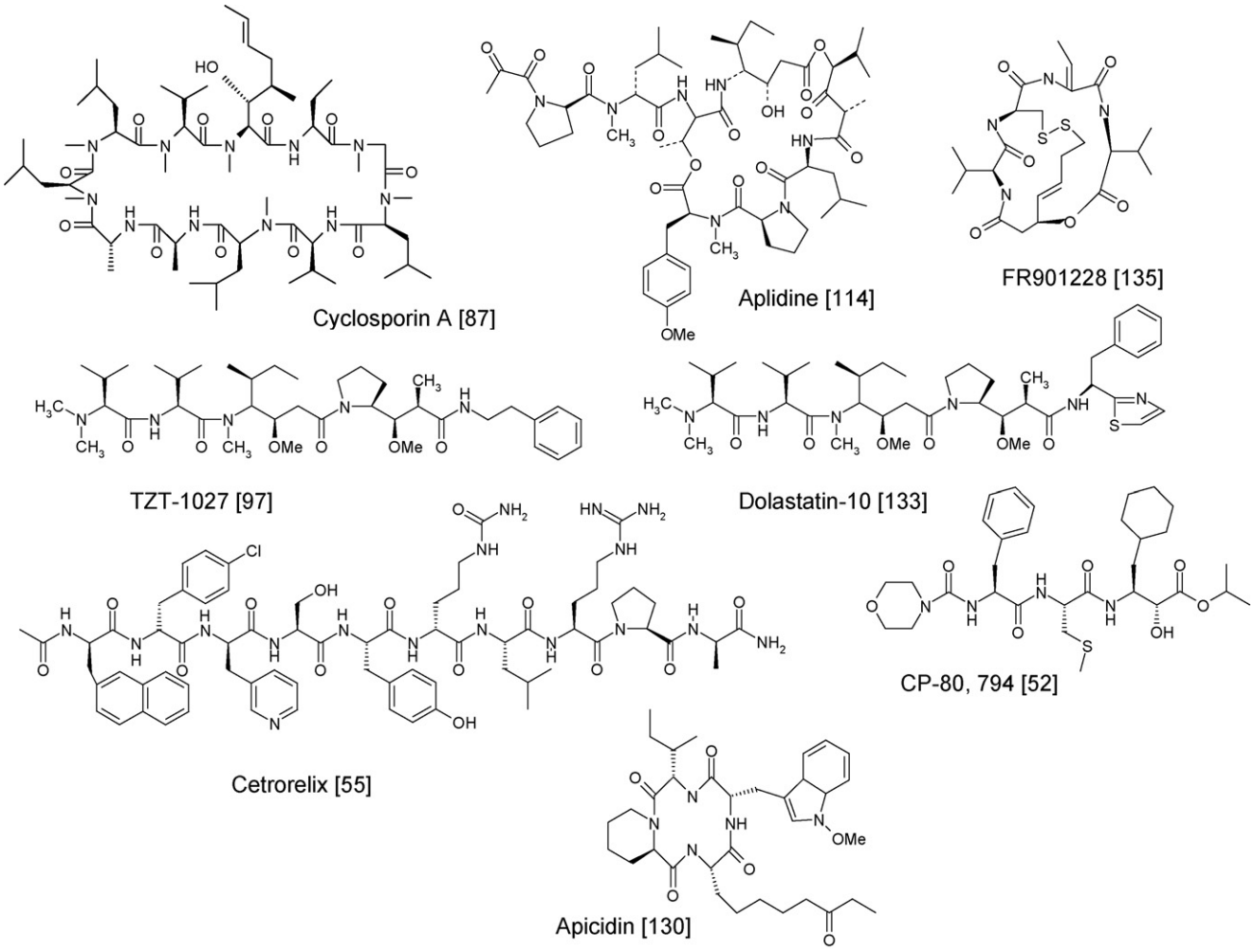


Fig. 2. Examples of chemical structures of peptides for which LLE procedures have been reported.

biological matrices has not always been performed. Nevertheless, depletion of the most abundant serum proteins using immunoaffinity prior to the digestion procedure has been reported a few times [141,158,161]. Enzymatic digestion is mostly preceded by denaturation, reduction and alkylation of the protein with trypsin as the preferred proteolytic enzyme for the digestion step.

Cleavage of the intact peptide or protein by enzymatic digestion results in the formation of multiple smaller peptide fragments, of which only one or a few are selected for the LC–MS quantification. Proper selection of this proteolytic peptide fragment is very important as this peptide serves as a representation of the total amount of intact peptide or protein. The digestion should therefore ideally be completed to yield reproducible fragments.

Except for the on-line digestion of bovine serum albumin [155], a stable isotopically labeled copy of the selected peptide fragment (SIL-F) is used as internal standard. The ratio of the unlabeled and labeled proteolytic fragment is then used for further quantification. However, it should be realized that this SIL-F will not show similar characteristics as the analyte until after the digestion procedure.

3. Liquid chromatography

3.1. Stationary phases

Reversed phase LC (RP-LC), using hydrophobic stationary phases and aqueous mobile phases, is normally used for the chromatography of peptides. Normal phase LC (NP-LC) uses polar stationary phases with non-aqueous mobile phases, but is normally not used for quantification and separation of peptides. An alternative can be the use of polar stationary phases in combination with aqueous mobile phases, providing better solubility for peptides. This technique is referred to as hydrophilic-interaction liquid chromatography (HILIC). Several peptide separations using HILIC have been reviewed by Yoshida [162], but no quantitative HILIC assays have been described so far.

For quantitative LC–MS methods for peptides, silica-based C_{18} stationary phases are mostly used, as listed in Table 5. This type of RP-LC stationary phases consists of a silica support to which the hydrophobic octadecyl group is attached. This group can be either monomerically or polymerically bonded by chlorosilanes, but will normally not occupy all silanols on the silica support. To avoid interactions of basic peptides with these underivatized silanol groups, resulting in peak tailing, end-capped materials are normally used.

Another type of RP-LC stationary phases consists of polymeric support. An advantage of polymeric stationary phases, such as polystyrene-divinylbenzene (PS-DVB), is that they allow a larger pH range than silica-based phases. Nevertheless, the use of polymer based stationary phases for peptide quantification has not been reported so far.

The use of a polymer-coated mixed-function (PCMF) column for the quantitative analysis of the 27 amino acid peptide

Table 5
LC conditions of bioanalytical assays for peptides, including (potential) therapeutics, biomarkers and proteolytically derived peptides, coupled with (tandem) mass spectrometric detection

Analyte	MW	SP	Column length × i.d. (mm)	Mobile phase A	Mobile phase B	Flow rate μl/min	Col T (°C)	IS	LLOQ (ng/ml)	Detection	Remarks	Reference
Opioid peptides												
Leu-enkephalin	555	C_{18}	150 × 0.3	W, FA	A–W, FA	6.5	38	Other	0.6	MS^2	OSP	[146]
Met-enkephaliln	573											
DADLE	569	C_{18}	50 × 2.1	W, FA	A, FA	200	25	SA	0.5	MS^2	OAs	[68]
Gluten Exorphin B5	595	C_{12}	150 × 2.0	W, AA	A	250	×	SA	0.8	MS		[150]
Gluten exorphin A5	599	C_{18}	150 × 4.6	W, AA	A–M	400	×	Other	1.5	MS		[151]
Dansyl-PQR-NH_2	631	C_{18}	50 × 0.3	W, AA	A	10	×	SA	×	MS^2	NV	[70]
Dansyl-PQR-NH_2	631	C_{18}	50 × 3.0	W–A, AA	–	250	×	SA	×	MS^2	NV	[70]
[Dmt][a]DALDA	639	C_{18}	100 × 0.075	A–W, FA	–	0.5	×	SIL	×	MS^2	NV	[71]
[Dmt][a]DALDA	639	C_{18}	8 × 0.075	A–W, FA	–	0.5	×	SIL	0.64	MS^2		[72]
DPDPE	648	C_{18}	30 × 2.2	M, NF	–	400	×	SA	1	MS		[73]
DSLET	686	C_{18}	50 × 1.0	W, NA	W–A	50	×	SIL	5	MS^2	NV	[74]
ME-RGL	900								1			
Hormones and hormone analogs												
ACE inhibiting peptides	276–652	C_{18}	150 × 2.1	W, T	A, T	200	60	SIL	0.01/0.05	MS^2	PCA	[50]
Taltirelin	405	C_{18}	150 × 2.0	M–W, FA	M–W, FA	200	40	SA	0.017	MS^2		[76]
Bradykinin [1–5]	572	C_{18}	50 × 2.1	W–A, AA	A, AA	78	×	SIL	×	MS^2		[78]
CP-80, 794	620	C_{18}	150 × 3.9	M–W	–	1000	×	SA	0.05	MS		[52]
IRI-514	671	×	250 × 2.0	W–M–A, NF	–	250[b]	×	SIL	2	MS		[79]
Angiotensin IV	775	C_{18}	150 × 0.075	W–A, FA	A–W, FA	30	×	SIL	0.16	MS^2	OSP	[145]
Melanotan-II	1024	C_8	50 × 2.1	W–A, NF, T	W–A, NF, T	200	×	Other	5	MS^2		[80]
Melanotan-II	1024	C_4	50 × 4.6	W–A, NA, FA	A–W, NA, FA	600	×	Other	0.5[c]	MS^2		[103]
Bradykinin	1060	C_{18}	150 × 0.5	W–A, FA	A–W, FA	250	×	SA	0.2	MS	OSP	[67]
Desmopressin	1068	C_{18}	125 × 2.0	A–W, FA, NA	–	200	×	Other	40	MS		[148]
RC-3095	1106	C_8	100 × 4.6	W, T	A, T	1000	RT	None	20	MS^2		[104]
Arg-bradykinin	1216	C_{18}	150 × 0.5	W–A, FA	A–W, FA	250	×	SA	0.2	MS	OSP	[67]
Goserelin	1269	C_{18}	150 × 1	A–W, ac	A–W, ac	×	×	None	300	MS^2	NV	[166]
NR58-3.14.3	1358	C_{18}	150 × 3.0	W, FA	A	500	35	SA	200	MS		[110]
Cetrorelix	1430	C_{18}	150 × 2.1	W–A, T	–	200	×	SA	1[c]; 2.1[d]	MS		[55]
[M1]-PTH(1–14) NH_2	1721	C_{18}	250 × 2.0	W, F	A, F	200	×	SA	0.2	MS^2		[105]
Endothelin peptide-1	2494	C_{18}	100 × 0.075	W–A, AA, T	A–M–W, AA,T	0.55	×	SA	0.125	MS^2	OSP	[56]
Endothelin peptide-2	2549											
Endothelin peptide-3	2645											
Leu^{13}-motilin	2681	C_{18}	150 × 2.0	W–A, NF	–	200	×	SA	0.5	MS		[60]
Motilin	2699	C_{18}	50 × 2.0	W, T, AA	A, T, AA	200	60	None	10	MS		[54]
Hepcidin-25	2789	C_{18}	250 × 2.0	W, F	A, F	200–300	×	SIL	5	MS^2		[105]
Hepcidin-25	2789	C_{18}	35 × 2	W–HI, AA	M–A–W–HI, AA	200	30	Other	1	MS^2		[81]
B201	2814	C_8	50 × 2.0	W, T	A, T	200	×	Other	1[g]	MS^2		[82]
C-peptide	3017	C_{18}	150 × 2.1	W–A–P, FA, T	W–A–P, FA, T	170	×	SIL	×	MS^2		[57]
C-peptide	3020	C_{18}	50 × 2	W–M–IPA, FA	A	150–400	40	SIL	0.16	MS	2D	[83,152]
Thymosin-α1	3108	C_{18}	50 × 2	W, NA, FA	A, NA, FA	300	×	Other	0.5	MS^2		[63]
GLP-1 [7–36]	3297	C_{18}	150 × 1	M–W, FA	W–M, FA	20–40	RT	SA	11,000[i]	MS	OAs;MET	[139]
Desacyl-ghrelin	3245	C_{18}	100 × 4.6	M–W, NA, AA	W–M, NA, FA	800	×	SA	0.35	MS^2	OSP	[106]
Ghrelin	3371	ML							0.07			
Human calcitonin	3417	C_{18}	150 × 0.32	W, T	A–W, T	3	×	SA	10	MS	NV	[137]
Salcatonin	3430	C_{18}	150 × 2.1	A, T	W, T	200	RT	None	10	MS		[108]
Salcatonin	3430	C_{18}	250 × 0.3	W, T	A, T	5	×	SIL	10[d]; 25[c]	MS		[107]
Glucagon	3483	C_{18}	50 × 2.0	W, AA, T	A, AA, T	200	60	None	25	MS		[49]
GIP [1–42]	4983	C_{18}	100 × 1	M–W, FA	W–M, FA	20–40	RT	SA	5000[i]	MS	MET	[138]
GIP [1–42]	4983	C_{18}	150 × 1	M–W, FA	W–M, FA	20–40	RT	SA	5000[i]	MS	OAs;MET	[139]
Insulin	5808	C_{18}	150 × 2.1	W, T	A	300	×	SA	1	MS		[53]

IGF-1	7649	C_{18}	50 × 1	W, AA, T	A–W, AA, T	100	40	SA	50	MS^2	OAs	[140]
Antimicrobial peptides[a]												
Antimicrobial peptides[a]												
Apicidin	624	C_{18}	150 × 2.0	A, AA	–	250	40	Other	0.5	MS^2		[130]
Caspofungin	1093	C_8	150 × 4.6	W–A, T, NH	–	1200[b]	×	SA	10[e];2.5[f]	MS^2		[84]
Caspofungin	1093	CN	30 × 4	W, FA	A	400	40	None	200	MS^2	OSP	[62]
Cyclosporin A	1203	C_{18}	250 × 2	W–M, NA	M–A–W, NA	120[b]	50	SA	5	MS^2		[131]
Cyclosporin A	1203	C_8	100 × 2.1	M–W, NA	–	300[b]	70	SA	5	MS^2		[86]
Cyclosporin A	1203	C_{18}	250 × 2	M–W	–	200	35	None	1.5	MS	OAs; OSP	[117]
Cyclosporin A	1203	C_{18}	250 × 2	M–W	–	350	35	None	0.5	MS	OAs; OSP	[118]
Cyclosporin A	1203	C_{18}	250 × 2	M–W	–	200	35	None	2	MS	OAs; OSP	[116]
Cyclosporin A	1203	C_{18}	125 × 4	A–W, NA	–	1000	55	SIL	6.95	MS	OAs	[87]
Cyclosporin A	1203	C_8	50 × 4.6	W, FA, SF	M	400	65	SA	7.5	MS	OAs; OSP	[119]
Cyclosporin A	1203	C_{18}	150 × 2	W–M	–	100[b]	35	SIL	1	MS		[88]
Cyclosporin A	1203	C_8	75 × 4.6	M–W	–	500	×	None	×	MS		[120]
Cyclosporin A	1203	C_{18}	50 × 2	A–W, NA	–	200	40	SIL	5.23	MS^2	OAs	[132]
Cyclosporin A	1203	C_{18}	250 × 2	M–W	–	250	33	Other	5	MS	OAs; OSP	[122]
Cyclosporin A	1203	CN	33 × 3.0	W–A, NA, FA	–	500	30	Other	1	MS^2		[123]
Cyclosporin A	1203	C_{18}	150 × 3.0	M–W, NA	–	800	65	SA	5	MS^2	OAs	[124]
Cyclosporin A	1203	C_{18}	4.0 × 3.0	W–M, FA, NA	M	600	55	Other	10	MS^2	OAs; OSP	[126]
Cyclosporin A	1203	PH	50 × 2.1	M–W, AA, NA	–	600	40	SA	4.5	MS^2	OAs; OSP	[127]
Cyclosporin A	1203	C_{18}	150 × 3.0	M–W, NA	–	500	65	SA	1	MS^2		[89]
Cyclosporin A	1203	C_{18}	50 × 2.1	M, NA	–	400[b]	70	SIL	10	MS^2		[90]
Cyclosporin A	1203	C_8	150 × 3.9	A–W, AA	–	400–700	60	SA	5	MS		[91]
Cyclosporin A	1203	C_{18}	30 × 3.2	A–W	–	500	75	SA	25	MS	OAs	[128]
Cyclosporin A	1203	C_8	475 × 4.6	W, AA	M	1000	80	SA	1	MS	MET	[129]
Cyclosporin A	1203	π	20 × 3	M	W, NF	800	65	SA	20	MS^2	OAs	[100]
Cyclosporin A	1203	C_{18}	50 × 2.1	M–W, FA, NF	M–W, FA, NF	400	50	Other	5	MS	OSP	[142]
Actinomycin D	1255	C_8	50 × 2	W, AA, NH	M	200	×	SA	1	MS		[109]
Actinomycin D	1255	C_8	50 × 2	W, AA, NH	M	×	×	SA	0.5	MS^2	OAs	[92]
Actinomycin D	1255	C_{18}	100 × 2	W, NA, AA	M	250	RT	SA	0.05	MS^2	OAs	[93]
Vancomycin	1448	C_{18}	100 × 2.1	W–A, AA	–	200	40	None	10	MS^2		[111]
Vancomycin	1448	C_{18}	40 × 2	W, FA	A, FA	250	65	SA	5	MS^2	MP; OSP	[143]
Vancomycin	1448	C_8	50 × 3	A–W, FA	–	200	40	Other	5	MS		[94]
Human HEM-γ	1935	C_{18}	150 × 1	W, T	W–A, T	30	RT	None	290	MS^2	MP	[112]
Enfuvirtide	4492	C_{18}	50 × 2	W–A, T	A–M, AA, T	400–1000	×	SIL	10	MS^2	MET	[113]
Enfuvirtide	4492	C_{18}	50 × 2.1	W, FA	A, FA	200	0	SIL	20	MS^2	MET	[95]
Sifuvirtide	4727	×	×	W, FA	A, FA	200	×	SA	4.9	MS^2	OSP	[144]
Tifuvirtide	5037	C_{18}	50 × 2.1	W, FA	A, FA	200	0	SIL	20	MS^2		[95]
Marine derived (anticancer) peptides												
FR901228	540	C_8	50 × 4.6	M–A–W, NA	–	550[b]	×	Other	0.1[g]	MS^2		[134]
FR901228	540	C_8	50 × 4.6	A–W, T, NA	–	1000[b]	×	Other	1	MS^2		[135]
TZT-1027	702	C_{18}	250 × 4.6	W–A, T	–	1000	RT	SIL	0.25[g]	MS		[97]
Dolastatin-10	786	C_{18}	50 × 1	W	A	75	RT	SA	0.005	MS		[133]
Aplidine	1110	C_{18}	100 × 3.0	W, FA	A, FA	500	RT	SA	5[c] 1.25[d]	MS^2		[114]
Aplidine	1110	C_{18}	100 × 3.0	A, FA	W, FA	500	RT	SA	1	MS^2		[115]
Aplidine	1110	C_{14}	50 × 2	A–W, FA, NA	–	200	60	SA	0.5	MS^2		[64]
Thiocoraline	1156	C_{18}	50 × 2	W–M, FA	–	300	4	SA	0.1	MS^2		[65]
Kahalalide F	1478	C_{18}	150 × 2.1	A–W, NH	–	200[b]	30	SA	1	MS^2		[99]
Protein fragments after *in vivo* proteolysis												
Amyloid β [1–40]	4330	C_8	50 × 0.5	W–A, AA, T	A–W–Pr, AA, T	30	×	None	×	MS		[136]
Amyloid β [1–42]	4514											
Amyloid β [1–40]	4330	C_{18}	150 × 2.1	W, NH	A–W, NH	200–300	25	SIL	0.4	MS^2		[66]
Amyloid β [1–42]	4514								0.2			
Protein fragments after *ex vivo* proteolysis												
Enfuvirtide [30–33]	475	C_{18}	50 × 2.1	W, FA	M, FA	200	30	SIL-F	100[h]	MS^2		[96]

Table 5 (*Continued*)

Analyte	MW	SP	Column length × i.d. (mm)	Mobile phase A	Mobile phase B	Flow rate µl/min	Col T (°C)	IS	LLOQ (ng/ml)	Detection	Remarks	Reference
NTproBNP [18–23]	756	C_{18}	35 × 1	W, FA	M–A, FA	180	55	SIL-F	0.1[h]	MS^2	OSP	[141]
Rhodopsin [140–148]	902	C_{18}	50 × 1.0	W, AA	A, AA	150	×	SIL-F	×	MS^2	NV	[153]
IGFBP-3 [226–233]	945	C_{18}	150 × 2.1	W, AA	A	400[b]	40	SIL-F	×	MS^2	NV	[157]
BSA [598–607]	1002	C_{18}	150 × 2.0	W–A, FA	A–W, FA	200	×	None	280[h,i]	MS	NV; OSP	[155]
PSA [1–9]	1020	C_{18}	50 × 2.1	W, AA	M, AA	250	×	SIL-F	×	MS^2	NV	[154]
Human CRP [14–23]	1128	C_{18}	100 × 0.075	W, FA	A, FA	0.2	×	SIL-F	×	MS^2	NV; 2D	[158]
GST-A1 [42-52]	1218	C_{18}	50 × 2	W, AA	A, AA	400	×	SIL-F	×	MS^2	NV	[160]
GST-M1 [18–30]	1591											
Apo A1 [42–64]	1400	C_{18}	100 × 2.1	W–A, FA	A–W, FA	0.3	×	SIL-F	×	MS^2	NV	[156]
IGF-1 [22–36]	1667	C_{18}	150 × 2.1	W, AA	A	400[b]	40	SIL-F	×	MS^2	NV	[157]
Vitellogenin [34–49]	1700	C_{18}	50 × 2	W, AA	A, AA	400[b]	×	SIL-F	×	MS^2	NV	[159]
HmAb [102–122]	2202	C_8	150 × 2.1	W, FA	A, FA	200	×	SIL-F	2000[h]	MS^2		[161]
Others												
AM336	2756	MF C_8	4.6 × 50	W, TBAH, T	A, TBAH, T	300–1000	RT	None	200	MS^2		[149]

The peptides have been classified, according to their origin. A: acetonitrile; AA: acetic acid; ac: acidified; ACE: angiotensin-I-converting enzyme; AM336: potent *N*-type calcium channel blocker; B201: bradykinin antagonist; BSA: bovine serum albumin; CN: cyanopropyl; Col T: column temperature; CP-80, 794: renin inhibitor; CRP: C-reactive protein; DADLE: D-Ala^2,D-Leu^5 enkephalin; Dansyl-PQR-NH_2: neuropeptide FF antagonist; [DMT^1]-DALDA: Dmt-D-Arg-Phe-Lys-NH_2; DPDPE: D-penicillamine^{2,5} enkephalin; DSLET: (D-Ser^2) Leu-enkephalin-Thr^6; FA:formic acid; FR901228: antitumor depsipeptide; GIP: glucose-dependent insulinotropic polypeptide; GLP-1: glucagon-like peptide 1; GST: glutathione S-transferase; HI: hexafluoroisopropanol; HmAb: human monoclonal antibody; i.d.: internal diameter; IGF-1: insulin-like growth factor-1; IGFBP-3: insulin-like growth factor binding protein 3; IRI-514: Ac-Arg-Pro-Asp-Pro-Phe-NH_2; LLOQ: lower limit of quantification; M: methanol; ME-RGL: methionine enkephalin-Arg-Gly-Leu; MET: metabolite(s) simultaneously quantified; MF: mixed function; ML: monolithic column; MP: multiplexing, use of two column system; [M1]-PTH(1–14)NH_2: parathyroid hormone analog; NA: ammonium acetate; NF: ammonium formate; NH: ammonium hydroxide; NR58-3.14.3: panchemokine inhibitor; NTproBNP: N-terminal pro-brain natriuretic peptide; NV: no or limited validation results presented; OAs: other (non-peptide) analytes simultaneously quantified; OSP: includes on-line sample pre-treatment; PCA: post-column additive; PH: phenyl; Pr: *n*-propanol; PSA: prostate-specific antigen; RC-3095: bombesin/gastrin releasing peptide antagonist; RT: room temperature; SA: structural analog internal standard; SF: sodium formate; SIL: stable isotopically labeled internal standard; SP: stationary phase; T: trifluoroacetic acid; TBAH: tetrabutylammonium hydroxide; TZT-1027: dolastatin-10 derivative; W: water; ×: not reported; 2D: preceded by a first dimension purification.

[a] Antimicrobial peptides (AMP's) include peptides with antibiotic, antifungal, antiviral and immunosuppressive activity.

[b] Flow is split before MS detection.

[c] In plasma.

[d] In urine.

[e] Using ionspray.

[f] Using turbo ionspray.

[g] In *human* plasma.

[h] Amount of the intact peptide/protein.

[i] In nmol/l.

AM336 has been described by Bu et al. [149]. In this stationary phase, hydrophilic and hydrophobic groups are bonded simultaneously to the coated silica. The outer surface prevents large bio-molecules from accessing the inner layer by size exclusion and hydrophilic-interaction, while smaller molecules are separated on the hydrophobic sorbent in the inner layer. AM336, with a molecular weight of 2756 Da, was small enough to enter the inner layer and direct injection of the peptide in CSF was herewith possible.

Particle sizes of the used stationary phases are generally between 3 and 5 μm. Smaller packing particles can increase column efficiency and speed of analysis, but will also cause higher pressures. The pore sizes of the column beds vary between 80 and 300 Å. The LC particles with large pore sizes (∼300 Å) have been especially developed for the separation of large molecules, as these compounds are too large to enter smaller pores. For peptide separations, the smaller pore sizes have therefore only been used for the smaller peptides (<2000 Da), whereas the larger pores have been particularly applied for the larger peptides.

Only recently a quantitative LC–MS/MS assay for the quantification of ghrelins, gastrointestinal peptides, has been described using a monolithic column [106], whereas all other quantitative LC-assays for peptides so far had used columns with spherical support. Monolithic supports can offer advantages for high throughput analysis by reducing analysis time without loss in performance. These columns consist of one piece of a totally porous support material that has only internal porosity consisting of mesopores (±13 nm) and macropores (±2 μm). The unique properties of this material allow columns to be operated at very high flow rates and very low back-pressures. Furthermore, as all the eluent is forced to flow through the pores of the separation medium, mass transport is enhanced, improving chromatographic efficiency [163]. Improved chromatographic resolution of peptides using an octadecylated monolithic PS-DVB has been demonstrated by analyzing tryptic protein digests and standard mixtures of peptides and proteins [164,165].

3.2. Column dimensions

Narrow-bore (±2 mm ID) columns are most frequently used, with column lengths varying from 50 to 150 mm (Table 5). Miniaturization of the column inner diameter tends to increase ionization efficiency of electrospray for MS detection, by providing a low volumetric flow rate and sample pre-concentration. Although column efficiency is not improved and analysis time not reduced, it can facilitate the coupling of LC to MS, saves solvents and is advantageous for the analysis of small amounts of biological samples [167]. However, only a few applications for the quantification of peptides using capillary columns (±75 μm ID) have been described [56,71,72,145]. A drawback of capillary LC is the need for complex instrumentation.

Human and salmon calcitonins have been quantified using columns with an internal diameter of ±0.3 mm [107,137]. These microbore columns allow flow rates of 3–5 μl/min, but total analysis of one sample takes longer than 10 min.

The performance of ESI-MS with packed capillary LC has been compared with normal bore LC for the analysis of a tryptic digest of myoglobin by Banks [168], showing that the LC-ESI-MS signal with a 0.25 mm ID column was 163 times higher than that obtained using a 4.6 mm ID column. Nevertheless, for the neuropeptide FF antagonist Dansyl-Pro-Gln-Arg-NH_2 improvement in the detection limit was only small when capillary-bore was compared to narrow-bore LC and the latter has therefore been preferred for its higher ruggedness [70].

3.3. Mobile phases

The choice of the correct mobile phase and mobile phase additives in RP-LC of peptides is very important to achieve good chromatographic separation as well as mass spectrometric performance. When LC is coupled to MS with electrospray ionization (ESI) the mobile phase has to be compatible with the ESI source and, furthermore, the additives have to enhance or at least not to suppress ionization of the analyte. A suitable mobile phase for ESI-MS contains an organic modifier (methanol or acetonitrile) and should not contain non-volatile buffers or mobile phase additives. The addition of ion-pairing reagents to the mobile phase improves the chromatographic separation of peptides, by forming ion pairs with charged groups and thereby increasing the hydrophobicity of the peptides and effecting interaction with the stationary phase [169]. However, the ion-pairing agent most commonly used for peptide separations, trifluoroacetic acid (TFA), is also known to suppress the ion formation in the ESI source [170].

A study of Giorgianni et al. [171] with peptide mixtures of four angiotensin peptide standards and two tryptic digests from horse myoglobin and bovine catalase, showed higher sensitivity and shorter gradient times with methanol compared to acetonitrile in nano-LC-ESI-MS/MS analysis. On the contrary, most LC–MS applications for peptides use a water–acetonitrile mobile phase, mostly acidified with TFA, formic acid or acetic acid.

3.4. Other LC conditions

Besides the choice for a stationary and mobile phase that best meets the specific analytical requirements of the analyte of interest concerning good chromatographic and mass spectrometric performance, several other LC conditions can be optimized. Depending on the application of the assay, the best compromise in speed, simplicity, sensitivity and accuracy can be accomplished by optimizing column temperature, column dimension, gradient system and flow rate.

Although it is well known that temperature is a critical parameter on the retention mechanism in RP-LC, only little is described about the influences of column temperature on the LC–MS performance of peptides. Several LC–MS assays for peptide drugs use elevated column temperatures (Table 5), but the chosen temperature is barely rationalized. A possible explanation might be the acceptance that elevated temperatures improve resolution and decrease retention time, as a result of a lower mobile phase viscosity and increased mass transfer [172]. Column temperatures for the LC analysis of cyclosporin A vary to a maximum of 80 °C. Temperatures above 65 °C have been reported to reduce peak broadening of cyclosporin due to incomplete separation of conformers [119]. Also for the anticancer drug aplidine quantitative LC applications have described the temperature-dependent equilibrium of two conformers. This cyclic depsipeptide (MW 1110 Da) has a *cis*- and *trans*-isomer at the pyruvoyl proline residue, which are separated as two chromatographic peaks at ambient temperature, but can be merged into one as the column temperature reaches 60 °C [37,64,114,115].

Chen et al. [173] studied the effect of temperature on the chromatographic properties of four series of peptides with different peptide conformations and demonstrated reduced retention with increasing temperature for all peptides. However, the extent of this effect differed, due to conformation differences of the peptides.

On the contrary, increased retention by elevating temperatures has also been observed. This “reverse effect” can appear when the eluent pH is close to the pK_a of the analytes and changes in column temperature can have a drastic effect on the ratio of charged and uncharged amino acid residues in the molecule [174]. van den

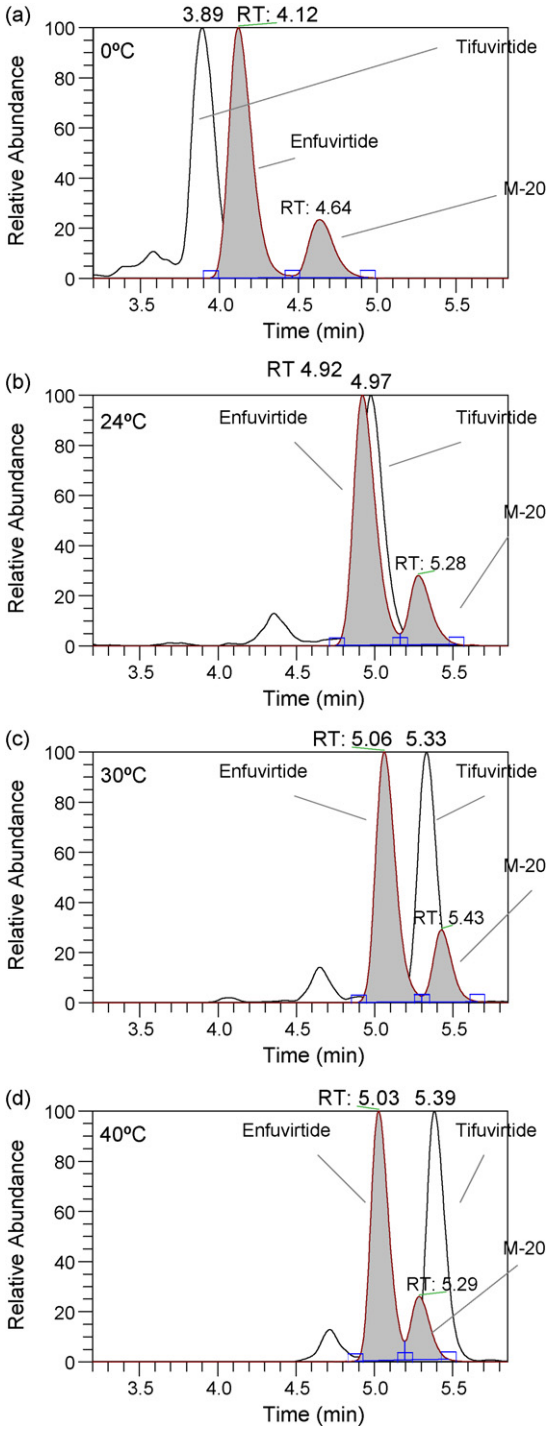


Fig. 3. Increased retention by elevated column temperatures for the HIV1-fusion inhibitors enfuvirtide, tifuvirtide and a metabolite of enfuvirtide, M-20 [95]. (*With permission*).

Broek et al. [95] have reported the decrease in retention time for the HIV-fusion inhibitors enfuvirtide and tifuvirtide by lowering column temperature to 0 °C. Additionally, improvement of the chromatographic resolution between enfuvirtide and its metabolite was observed by decreasing column temperature (Fig. 3).

Other parameters influencing retention and thus analysis time are flow and gradient rate. Coupling of LC to MS makes chromatographic resolution less critical, because the MS detector can discriminate co-eluting compounds with different masses. Short columns and fast gradients are therefore commonly applied. To gain time it is often advantageous to use higher flow rates than the recommended flow rates based on the column's internal diameter. Increasing the flow rate by a factor two not only reduces run time as much as using a column with half the length, but also means less reduction of plate number than when reducing column length [175]. When using higher flow rates, back-pressure and compatibility with the MS interface should be concerned as MS interfaces may not allow higher flow rates. Post-column splitting of the LC-eluent can then be applied.

An alternative approach to gain time without increasing the flow rate is the use of a multiplexing LC-system, enabling analysis on one column while the other column is washed and equilibrated. In peptide analysis, two-column LC-systems have been applied for the quantification of the glycopeptide vancomycin (MW 1448 Da) [143] and the antimicrobial human polypeptide HEM-γ 130–136 (MW 1935 Da) [112].

Polypeptide chromatography is mostly performed by gradient elution, which is especially suited for the analysis of complex mixtures, providing good resolution with short run times. However, isocratic chromatography has also been performed, mainly for smaller peptides (<2000 Da). An exception is KW-5139, a variant of motilin with a MW of 2681 Da, which is eluted isocratically [60].

4. Mass spectrometry

4.1. Ionization

As MS detection is only possible when the peptides exist as ions in the gaseous state, coupling of LC to MS necessitates the volatilization of these large bio-molecules from the LC-eluent. A large number of different LC–MS interfaces have been developed to achieve this goal, of which nowadays atmospheric pressure ionization (API) is generally accepted as the optimum interface for on-line LC–MS coupling. Two main API sources are the electrospray ionization (ESI) and the atmospheric pressure chemical ionization (APCI) source. In ESI, charged droplets are formed by spraying the sample solution through a high voltage (2–5 kV) capillary in the presence of a strong electric field. The charged droplets move towards the mass spectrometer inlet, generating analyte ions during evaporation and droplet fission. In APCI a heated pneumatic nebulizer probe is used for nebulization and a high voltage-needle is used to produce a corona discharge for ionization of the evaporated solvent, reacting with the analyte.

Quantitative LC–MS analysis of peptides is mostly performed by ESI, although some methods have been described using APCI [52,87,97,121,129]. The main reason for using APCI was either to overcome complications like matrix effects and ionization suppression [121], or better sensitivity for a simultaneously quantified compound [87]. Adduct formation in ESI has also been a reason to select APCI as the ionization source [129]. ESI and APCI performances have been compared for some other peptides as well, resulting in selection of ESI as it was easier in use [134] or showed significantly lower limits of detection [70].

Another API technique is atmospheric pressure photoionization (APPI). With APPI the analyte is ionized by photons emitted from a vacuum-ultraviolet lamp. APPI has been compared with APCI and ESI for the determination of cyclosporin A in plasma and resulted in equivalent analytical performances [176].

Besides the very mild ionization by electrospray, which easily ionizes polar, non-volatile, high molecular mass and thermo labile compounds it enables the formation of multiply charged ions of the type $[M+nH]^{n+}$. As the MS system separates ions according to their mass-over-charge ratio (m/z), detection of compounds with

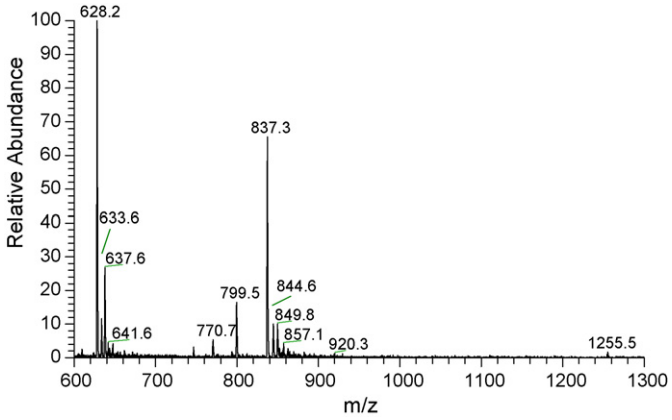


Fig. 4. Example of a typical ESI-MS spectrum, showing the multiply charged ions $[M+2H]^{2+}$ (m/z 1255.5), $[M+3H]^{3+}$ (m/z 837.3) and $[M+4H]^{4+}$ (m/z 628.2) of a peptide fragment [256–278] of human apolipoprotein A-IV (MW 2509 Da). Furthermore, the sodium- and potassium ion adducts $[M+3H+Na]^{4+}$ (m/z 633.6), $[M+3H+K]^{4+}$ (m/z 637.6), $[M+2H+Na]^{3+}$ (m/z 844.6) and $[M+2H+K]^{3+}$ (m/z 849.8) can be seen.

high molecular masses in lower m/z ranges is thereby possible. This makes ESI a very suitable technique for ionization of large biomolecules like peptides. Series of ions can be observed, such as the molecular ion with different charge states and different types of adduct ions of which sodium-, potassium- and ammonium-adducts are regularly formed (Fig. 4). For quantitative assays the most abundant ion is normally chosen for MS measurements. This is mostly the molecular ion but can also be an adduct ion. Cyclosporin A, for example, is reported to be measured as $[M+Na]^+$ or $[M+NH_4]^+$ and the decapeptide cetrorelix as the trifluoroacetic acid adduct (Table 6). Alternatively, Zhou et al. [120] could accurately quantify the amount of cyclosporin A by measuring the sum of H^+-, Na^+- and K^+-adducts.

API can be performed in either the positive or negative ionization mode, measuring either positively or negatively charged ions. Proteins or peptides can contain multiple basic amino acid residues that are readily protonated and are therefore often analyzed in the positive ion mode. Nevertheless, a few LC-API-MS applications for peptides in the negative ion mode have been described [52,55,57,66].

Another ion source, commonly used for mass spectrometric analysis of peptides in proteomic studies, is matrix-assisted laser desorption ionization (MALDI). In MALDI, samples are co-crystallized onto a sample plate with a small organic matrix compound that can absorb at the wavelength of the laser. However, the loading and crystallization of the sample in the target prohibits on-line application of MALDI with LC separation.

4.2. Factors influencing ionization

Different factors might influence peptide's ionization efficiency and thus the sensitivity and accuracy and precision of the assay. The intensity of the most abundant ion in the mass spectrum mostly depends on the ionizability of the peptide and can be affected by formation of different ion types, i.e. many adducts or many different charge states. Sometimes little alterations in, for example, capillary voltage [49], orifice potential [86] or mobile phase composition can favor the formation of a specific ion type. For example, the addition of ammonium acetate has been reported to eliminate the sodium adduct of the cyclic tetrapeptide apicidin [130], whereas addition of ammonium or sodium is described to enhance formation of the ammonium or sodium adduct of cyclosporin A, respectively [119,121]. Changes in pH of the eluent can also favor the formation of a specific ion, as with lower pH values higher charged species are usually more pronounced [60,63].

As smaller peptides contain less amino acid residues and are less "sensitive" to multiple charging, the ionization efficiency might be expected to be higher for smaller peptides. However, MS detection of five different chymotryptic fragments of the polypeptide enfuvirtide (4492 Da) after enzymatic cleavage did not result in better ionization properties of the smaller peptide fragments compared to the intact peptide [96]. Despite the measurement of smaller and lower charged ions, the ionization properties appeared merely to depend on the specific characteristics of the peptide fragments. On the other hand, the formation of several different peptides from one peptide by this approach increases the chance of formation of a very easily ionized compound with better sensitivity.

While peptide's size could not directly be related to ESI response, the surface activity of the analyte can. Analytes with high affinity for the surface of ESI droplets are generally accepted to have higher ESI response [179]. Cech et al. compared the ESI response of six tripeptides with different C-terminal residues and observed increased ESI response for peptides with more non-polar side chains [180]. According to the equilibrium partitioning model, more hydrophobic peptides are more easily located at the surface of the ESI droplet, whereas more polar peptides remain in the interior of the droplet. The hydrophobic peptides are therefore able to carry a greater fraction of the excess charge, resulting in higher ESI response.

The composition of the mobile phase is also an important aspect on the analyte's ESI response. Solvent pH affects the protonation or deprotonation of the analyte. Acidic solvents are therefore ideally used for the analysis in the positive ion mode of compounds with basic functional groups. However, protonated ions can also be observed when ESI-MS analysis is performed with basic solvents and deprotonated ions can be observed in the ESI-MS analysis of acidic solutions. A possible explanation can be gas-phase-ion–molecule reactions or collision-induced dissociation of base- or acid-adduct ions in basic or acidic solutions, respectively [181]. An example of this "wrong-way-round ionization' is the quantification of the marine derived anticancer drug kahalalide F, a cyclic tridecapeptide with a molecular mass of 1478 Da, in the positive ion mode in alkaline mobile phase [99]. In addition, the decapeptide cetrorelix (MW 1430 Da) is quantified using negative ionization and an acidified solution. In this case, the peptide formed a negative $[M+TFA-H]^-$ ion with the anions present in the acidified solution [55].

Furthermore, other mobile phase components might influence ESI response. Salts and non-volatile organic compounds can lead to decreased signal intensities and signal-to-noise ratios as well as contamination of the ion source. Also TFA, the ion-pairing agent most widely used for the separation of peptides, unfortunately leads to signal suppression and low sensitivity when ESI-MS detection is employed [170]. Post-column addition of propionic acid and 2-propanol (70:30 v/v) for peptide quantification has been reported by van Platerink et al. to overcome the suppressive effect of TFA [50]. Different mobile phase additives and their effect on LC-ESI-MS sensitivity of peptides have been reviewed by García [169], but a solid alternative for TFA could not be proposed. ESI response can also be affected by the percentage of aqueous or organic modifier in the mobile phase. For the quantification of the pentapeptide drug IRI-514, the post-column addition of organic modifier has been described, as sensitivity was reduced by the highly aqueous mobile phase [79]. Also co-eluting matrix components can be a reason of suppression of the ionization of the analyte in the ionization source, showing the importance of good sample preparation and chromatographic separation prior to MS detection.

Table 6
MS conditions of bioanalytical assays for peptides, including (potential) therapeutics, biomarkers and proteolytically derived peptides, after LC separation

Analyte	MW	Matrix	Sample pre-treatment	Ioni-zation	Mass analyzer	Scan mode	Selected (parent) ion(s)	Selected product ion(s)[a]	Remarks	Reference
Opioid peptides										
Leu-enkephalin	555	CSF	OSP	ESI	Q3	MRM	$[M+H]^+$	*m/z* 278; *m/z* 397		[146]
Met-enkephalin	573									
DADLE	569	Plasma	PP + SPE	ESI+	Q3	MRM	$[M+H]^+$	$a_1{}^*$		[68]
Gluten Exorphin B5	595	CSF	None	ESI+	Q1	SIM	$[M+H]^+$	–		[150]
Gluten Exorphin A5	599	CSF	None	ESI+	Q1	SIM	$[M+H]^+$	–		[151]
Dansyl-PQR	631	Brain tissue	PP + SPE	ESI+	Q-IT	FS-P	$[M+H]^+$	$c_1+b_2+b_3$		[70]
		Plasma	PP							
[Dmt][a]DALDA	639	Plasma	PP + SPE	ESI+	Q-TOF	MRM	$[M+2H]^{2+}$	z_3		[71]
[Dmt][a]DALDA	639	Plasma	PP + SPE	nESI+	IT	MRM	$[M+2H]^{2+}$	$[M+2H\text{-}NH_3]^{2+}$		[72]
DPDPE	648	CSF	SPE	ESI+	Q1	SIM	$[M+H]^+$	–		[73]
DSLET	686	Plasma	SPE	ESI+	Q3	MRM	$[M+H]^+$	a_5		[74]
ME-RGL	900	Plasma	SPE	ESI+	Q3	MRM	$[M+2H]^{2+}$	a_1		[74]
Hormones and hormone analogs										
ACE inhibiting peptides	276–652	Plasma	AH	ESI+	Q3	MRM	$[M+H]^+$	Various[b]		[50]
Taltirelin	405	Plasma	SPE	ESI+	Q3	MRM	$[M+H]^+$	*m/z* 264	TISP	[76]
Bradykinin [1–5]	572	Plasma	SPE	ESI+	Q3	MRM	$[M+2H]^{2+}$	b_4		[78]
CP-80, 794	620	Serum	LLE	APCI–	Q3	SIM	$[M-H]^-$	–		[52]
IRI-514	671	Plasma	PP + SPE	ESI+	Q1	SIM	$[M+H]^+$	–		[79]
Angiotensin IV	775	Brain dialysate	OSP	nESI+	Q3	MRM	$[M+H]^+$	*m/z* 513		[145]
Melanotan-II	1024	Plasma	SPE	ESI+	Q3	MRM	$[M+2H]^{2+}$	*m/z* 436	TISP	[80]
Melanotan-II	1024	Brain tissue	PP + F	ESI+	Q3-LIT	MRM	$]M+2H]^{2+}$	*m/z* 128	TISP	[103]
		Plasma	PP					*m/z* 110		
Bradykinin	1060	Muscle tissue	D + OSP	ESI+	TOF	SIM	$[M+2H]^{2+}$	–		[67]
Desmopressin	1068	Skin samples	Ext	ESI+	IT	SIM	$[M+H]^+$	–		[148]
RC-3095	1106	Plasma	PP	ESI+	Q3	MRM	$[M+2H]^{2+}$	*m/z* 144		[104]
Arg-bradykinin	1216	Muscle tissue	D + OSP	ESI+	TOF	SIM	$[M+H]^{3+}$	–		[67]
Goserelin	1269	Plasma	PP	ESI+	Q-TOF	MRM	$[M+2H]^{2+}$	*m/z* 608		[166]
NR58-3.14.3	1358	Serum	PP	ESI+	IT	SIM	$[M+H]^+$; $[M+2H]^{2+}$; $[M+3H]^{3+}$	–		[110]
Cetrorelix	1430	Plasma/urine	SPE	ESI–	Q1	SIM	$[M+CF_3COOH\text{-}H]^-$	–		[55]
[M1]-PTH(1–14)NH_2	1721	Plasma	PP	ESI+	Q3	MRM	$[M+H]^{3+}$	*m/z* 58	TISP	[105]
Endothelin peptide-1	2294	HUVEC supernatant	PP + OSP	μESI+	IT	MRM	$[M+H]^{3+}$	$b_{20}{}^{2+}$		[56]
Endothelin peptide-2	2549									
Endothelin peptide-3	2645									
Leu^{13}-motilin	2681	Plasma	PP	ESI+	Q3	SIM	$[M+3H]^{3+}$	–		[60]
Motilin	2699	Plasma	PP	ESI+	Q3	SIM	$[M+4H]^{4+}$	–		[54]
Hepcidin-25	2789	Serum	PP	ESI+	Q3	MRM	$[M+5H]^{5+}$	*m/z* 120 i(Phe)	TISP	[105]
Hepcidin-25	2789	Serum/plasma	PP + SPE	ESI+	Q3	×	×	×	ISP	[81]
B201	2814	Plasma	SPE	ESI+	IT	MRM	$[M+3H]^{3+}$	*m/z* 816		[82]
C-peptide	3017	Urine	UF	ESI–	Q3	MRM	$[M\text{-}2H]^{2-}$	*m/z* 1320		[57]
C-peptide	3020	Plasma	SPE + OSP	ESI+	Q3	SIM	$[M+3H]^{3+}$	–	TISP	[83]
C-peptide	3020	Urine	Dilution	ESI+	Q3	MRM	$[M+3H]^{3+}$	*m/z* 147	TISP	[152]
Thymosin-α1	3108	Serum	SPE	ESI+	Q3	MRM	$[M+3H]^{3+}$	*m/z* 316	TISP	[63]
GLP-1 [7–36]	3297	Plasma	IAP	ESI+	Q1	SIM	$[M+3H]^{3+}$; $[M+4H]^{4+}$	–	ISP	[139]
Desacyl-ghrelin	3245	Plasma/serum/cs	PP + OSP	ESI+	Q3	MRM	$[M+4H]^{4+}$	$b_{20}{}^{2+}$; $y_{23}{}^{3+}$; $y_{24}{}^{3+}$	TISP	[106]
Ghrelin	3371							$b_{20}{}^{2+}$; $y_{23}{}^{3+}$; $y_{24}{}^{3+}$; $y_{25}{}^{3+}$		
Human calcitonin	3417	Plasma	IAP	ESI+	Q3	SIM	$[M+3H]^{3+}$	–		[137]
Salcatonin	3430	Plasma	PP	ESI+	IT	SIM	$[M+3H]^{3+}$	–		[108]
Salcatonin	3430	Serum	PP	ESI+	TOF	SIM	$[M+3H]^{3+}$	–		[107]
Glucagon	3483	Plasma	PP	ESI+	Q3	SIM	$[M+4H]^{4+}$	–		[49]
GIP [1–42]	4983	Plasma	IAP	ESI+	Q1	SIM	$[M+5H]^{5+}$	–	ISP	[138,139]
Insulin	5808	Blood	SPE	ESI+	Q-IT	SIM	$[M+3H]^{3+}$; $[M+4H]^{4+}$	–		[53]
IGF-1	7649	Plasma	IAP	ESI+	Q3-LIT	MRM	$[M+6H]^{6+}$	*m/z* 70 i(Pro)	QDA	[140]

Antimicrobial peptides[c]										
Apicidin	624	Serum	LLE	ESI+	Q3	MRM	$[M+H]^+$	m/z 84	TISP	[130]
Caspofungin	1093	Plasma/urine	SPE	ESI+	Q3	MRM	$[M+H]^+$	m/z 1034	ISP; TISP	[84]
Caspofungin	1093	Serum	OSP	ESI+	IT	MRM	$[M+2H]^{2+}$	$[M-H_2O]^{2+}$		[62]
Cyclosporin A	1203	Blood	LLE	ESI+	Q3	MRM	$[M+NH_4]^+$	$[M+H]^+$	ISP	[131]
Cyclosporin A	1203	Blood	PP + SPE	ESI+	Q3	MRM	$[M+H]^+$	m/z 425	ISP	[86]
Cyclosporin A	1203	Blood	PP + OSP	ESI+	Q1	SIM	$[M+Na]^+$	–		[116–119,122]
Cyclosporin A	1203	Blood	PP + SPE	APCI+	Q3	SIM	$[M+H]^+$	–		[87]
Cyclosporin A	1203	Blood	PP + SPE	ESI+	MnS	FS	$[M+Na]^+$	–		[88]
Cyclosporin A	1203	Blood/serum	PP	ESI+	Q1	SIM	$[M+H]^+$; $[M+K]^+$; $[M+Na]^+$	–		[120]
Cyclosporin A	1203	Blood	PP + OSP	APCI+	Q3	MRM	$[M+NH_4]^+$	$[M+H]^+$	No LC	[121]
Cyclosporin A	1203	Blood	LLE	ESI+	Q3	MRM	$[M+NH_4]^+$	$[M+H-(CH_3OH+H_2O)]^+$		[132]
Cyclosporin A	1203	Blood	PP	ESI+	Q3	MRM	$[M+NH_4]^+$	$[M+H]^+$		[100,123,125]
Cyclosporin A	1203	Blood	PP	ESI+	Q3	MRM	$[M+H]^+$	m/z 425	TISP	[124]
Cyclosporin A	1203	Blood	PP + OSP	ESI+	Q3	MRM	$[M+NH_4]^+$	$[M+H]^+$	TISP	[126,127]
Cyclosporin A	1203	Saliva	PP + SPE	ESI+	Q3	MRM	$[M+NH_4]^+$	$[M+H]^+$	TISP	[89]
Cyclosporin A	1203	Blood	PP + SPE	ESI+	Q3	MRM	$[M+NH_4]^+$	$[M+H]^+$		[90]
Cyclosporin A	1203	Plasma	SPE	ESI+	Q1	SIM	$[M+2H]^{2+}$	–		[91]
Cyclosporin A	1203	Blood	PP + SPE	ESI+	Q1	SIM	$[M+Na]^+$	–		[128]
Cyclosporin A	1203	Blood	PP	APCI+	Q3	SIM	$[M+H]^+$	–		[129]
Cyclosporin A	1203	PBMCs	Ext + OSP	ESI+	Q1	SIM	$[M+Na]^+$	–		[142]
Actinomycin D	1255	Plasma	PP	ESI+	Q3	SIM	$[M+H]^+$	–	TISP	[109]
Actinomycin D	1255	Plasma	SPE	ESI+	Q3	MRM	$[M+H]^+$	m/z 857/m/z 858	(TISP)	[92,93]
Vancomycin	1448	Plasma	PP	ESI+	Q3	MRM	$[M+2H]^{2+}$	m/z 144	TISP	[111]
Vancomycin	1448	Serum/urine	OSP	ESI+	Q3	MRM	$[M+2H]^{2+}$	m/z 144	TISP	[143]
Vancomycin	1448	Serum	SPE	ESI+	LIT-FT	FS	$[M+2H]^{2+}$	–		[94]
Human HEM-γ	1935	Plasma	PP	ESI+	IT	MRM	$[M+2H]^{2+}$	y_5; ${b_{15}}^{2+}$; ${b_{16}}^{2+}$; $(b_{16}+H_2O)$; y_8; b_8; $[M-H_2O+2H]^{2+}$; y_9; y_{12}; b_{12}		[112]
Enfuvirtide	4492	Plasma	PP	ESI+	Q3	MRM	$[M+4H]^{4+}$	${b_{33}}^{3+}$		[113]
Enfuvirtide	4492	Plasma	SPE	ESI+	Q3	MRM	$[M+4H]^{4+}$	${b_{33}}^{3+}$		[95]
Sifuvirtide	4727	Plasma	OSP	ESI+	IT	MRM	$[M+5H]^{4+}$	${b_{33}}^{5+}$		[144]
Tifuvirtide	5037	Plasma	SPE	ESI+	Q3	MRM	$[M+5H]^{5+}$	${b_{38}}^{4+}$		[95]
Marine derived (anti cancer) peptides										
FR901228	540	Plasma	LLE	ESI+	Q3	MRM	$[M+H]^+$	m/z 272		[134,135]
TZT-1027	702	Plasma	SPE + LLE	APCI+	Q1	SIM	$[M+H]^+$	–		[97]
Dolastatin-10	786	Plasma	LLE	ESI+	×	SIM	$[M+H]^+$	–		[133]
Aplidine	1110	Plasma/urine/blood	PP + LLE	ESI+	Q3	MRM	$[M+H]^+$	m/z 295	TISP	[114,115]
Aplidine	1110	Plasma	LLE	ESI+	Q3	MRM	$[M+H]^+$	m/z 295	TISP	[64]
Thiocoraline	1156	Plasma	PP + SPE	ESI+	Q3	MRM	$[M+H]^+$	m/z 215	TISP	[65]
Kahalalide F	1478	Plasma	SPE	ESI+	Q3	MRM	$[M+2H]^{2+}$	b_1		[99]
Protein fragments after *in vivo* proteolysis										
Amyloid β [1–40]	4330	Cell lysates	IAP	ESI+	MnS	SIM	$[M+4H]^{4+}$	–		[136]
Amyloid β [1–42]	4514									
Amyloid β [1–40]	4330	CSF	IAP	ESI–	IT	MRM	$[M-4H]^{4-}$	$[M-3H_2O-4H]^{4-}$		[66]
Amyloid β [1–42]	4514									
Protein fragments after *ex vivo* proteolysis										
Enfuvirtide [30–33]	475	Plasma	SPE[d]	ESI+	Q3	MRM	$[M+H]^+$	y_1; z_1		[96]
NTproBNP [18-23]	756	Serum	IAP[d]	ESI+	Q3	MRM	$[M+2H]^{2+}$	y_4		[141]
Rhodopsin [140–148]	902	ROS membrane	None[d]	ESI+	Q3	MRM	$[M+H]^+$	b_7		[153]
IGFBP-3 [226–233]	945	Serum	None[d]	ESI+	Q3	MRM	$[M+2H]^{2+}$	y_4; y_6		[157]
BSA [598–607]	1002	Plasma/urine	OSP[d]	ESI+	IT	FS	$[M+H]^+$	–		[155]
PSA [1–9]	1020	Serum	None[d]	ESI+	Q3	MRM	$[M+2H]^{2+}$	y_7		[154]
Human CRP [14–23]	1128	Serum	IAP[d]	nESI+	Q3	MRM	$[M+H]^+$	y_3; y_4; y_5; y_6; y_8		[158]
GST-A1 [42–52]	1218	Liver cytosol	None[d]	ESI+	Q3	MRM	$[M+2H]^{2+}$	y_9	TISP	[160]
GST-M1 [18–30]	1591						$[M+2H]^{2+}$	y_{10}		
Apo A1 [42–64]	1400	Serum	PP[d]	nESI+	Q3-LIT	MRM	$[M+2H]^{2+}$	y_4	ISP	[156]
IGF-1 [22–36]	1667	Serum	None[d]	ESI+	Q3	MRM	$[M+3H]^{3+}$	${y_{12}}^{2+}$; ${y_{13}}^{2+}$		[157]

Table 6 (*Continued*)

Analyte	MW	Matrix	Sample pre-treatment	Ioni-zation	Mass analyzer	Scan mode	Selected (parent) ion(s)	Selected product ion(s)[a]	Remarks	Reference
Vitellogenin [34–49]	1700	Plasma	None[d]	ESI+	Q3	MRM	$[M+2H]^{2+}$	y_{10}	TISP	[159]
HmAb [102-122]	2202	Serum	IAP[d]	ESI+	Q3-LIT	MRM	$[M+3H]^{3+}$	y_8		[161]
Others										
AM336	2756	CSF	None	ESI+	Q3	SIM	$[M+3H]^{3+}$	–		[149]

The peptides have been classified according to their origin. ACE: angiotensin-I-converting enzyme; AM336: potent *N*-type calcium channel blocker; APCI: atmospheric pressure chemical ionization; AH: acidification and heating; BSA: bovine serum albumin; B201: bradykinin antagonist; cs: cell supernatant; CSF: cerebrospinal fluid; CP-80, 794: renin inhibitor; CRP: C-reactive protein; D: dialysis; DADLE: D-Ala^2,D-Leu^5 enkephalin; dansyl-PQR-NH_2: neuropeptide FF antagonist; [Dmt]^{1}DALDA: Dmt-D-Arg-Phe-Lys-NH_2; DPDPE: D-penicillamine^{2,5} enkephalin; DSLET: (D-Ser^2) Leu-enkephalin-Thr^6; EDg: enzymatic digestion.; ESI: electrospray ionization; Ext: extraction; F: filtration; FR901228: antitumor depsipeptide; FS: full scan; FS-P: full scan product ion; GIP: glucose-dependent insulinotropic polypeptide; GLP-1: glucagon-like peptide 1; GST: glutathione S-transferase; HmAb: human monoclonal antibody; HN: heated nebulizer; HUVEC: human umbilical vein endothelial cells; IAP: immunoaffinity purification; IGF-1: insulin-like growth factor-1; IGFBP-3: insulin-like growth factor binding protein 3; IRI-514: Ac-Arg-Pro-Asp-Pro-Phe-NH_2; ISP: ionspray; IT: ion trap; LIT-FT: hybrid linear ion trap fourier transform mass spectrometer; ME-RGL: methionine enkephalin-Arg-Gly-Leu; MnS: magnetic sector; MRM: multiple reaction monitoring; [M1]-PTH(1–14)NH_2: parathyroid hormone analog; NR58-3.14.3: panchemokine inhibitor; NTproBNP: N-terminal pro-brain natriuretic peptide; OSP: on-line sample pre-treatment; PBMCs: peripheral blood mononuclear cells; PSA: prostate-specific antigen; QDA: qualitative detection of analogues; Q1: single quadrupole; Q3: triple quadrupole; Q3-LIT: hybrid triple quadrupole/linear ion trap; RC-3095: bombesin/gastrin releasing peptide antagonist; ROS: rod outer segments; SIM: selected ion monitoring; TISP: turbo ionspray; TOF: time-of-flight; TZT-1027: dolastatin-10 derivative; UF: ultrafiltration; ×: not reported.

[a] Notation according to Biemann [177,178]. Ions are denoted with * for the loss of ammonia. When the fragment ion is not specified or could not be notated properly, the corresponding *m/z* value is listed.

[b] Two product ions are used for each peptide.

[c] Antimicrobial peptides (AMP's) include peptides with antibiotic, antifungal, antiviral and immunosuppressive activity.

[d] Sample pre-treatment includes enzymatic digestion procedure, eventually preceded by denaturation, reduction and/or alkylation.

A way to improve ESI response can be derivatization, either by making the analyte more easily charged or by increasing its surface activity. Mirzaei and Regnier [182] describe the enhanced ionization efficiency of many peptides, by introducing a label that contains both a charged quaternary amino group as well as a hydrophobic octyl group.

To fulfill the increasing expectations of the ESI response, novel advances in ESI techniques have been introduced. Electrospray sources that allow low flow rates, e.g. micro-ESI and nanospray, have been developed to improve ESI response. Miniaturization of the ESI interface should result in a larger fraction of ions entering the mass detector and thus in increased sensitivity [183]. Also different types of ESI sources are available, all claiming more efficient ion transfer into the mass spectrometer. Examples applied in the bioanalysis of peptides are the z-spray, an orthogonal spray from Micromass [49,54,67,71,125,146], pneumatically assisted electrospray (ionspray; ISP) and pneumatically and thermally assisted electrospray (turboionspray; TISP).

4.3. Mass analysis

The three most common mass analyzers are quadrupole, time-of-flight (TOF) and ion trap. Mass analyzers separate ions by their mass-over-charge ratio (*m/z*). In quadrupole mass spectrometers specific radio frequency (RF) and direct current (DC) voltages are be applied on four parallel rods, allowing only ions with a specific *m/z* values to move between the rods and to reach the detector. TOF analyzers accelerate the ions by a short voltage gradient and measure the flight time of the ions which is proportional to the square root of the *m/z*. Ion-trap mass spectrometers trap the ions in an electric field, where specific ions can be activated and ejected by manipulation of this electric field.

Tandem mass spectrometers are made of two successive mass analyzers with a collision cell in between that can select specific ions, induce their fragmentation in the collision cell, and measure the *m/z* of the fragment ions. The most common tandem mass spectrometer is the triple quadrupole, where the second quadrupole is the collision cell and the first and third quadrupole are the actual mass analyzers. Quadrupoles are very suitable for quantitative measurements, because of their relative high ion transmission efficiency, large dynamic range and high sensitivity when used in the static mode, i.e. no full scan mode. Ion traps can also perform multiple MS (MS^n) as the trapped ions can be fragmented inside the trap. Although ion traps are small and relatively cheap with high sensitivity, due to the ion concentrating capability, and able to provide full-scan MS/MS spectra they are not often used for quantitative assays. A disadvantage of an ion trap can be the limited number of ions that can be trapped, reducing linearity [72]. Controversially, Shipkova et al. [184] have proposed the ion trap to be advantageous compared to triple quadrupole instruments, especially for the quantification of large peptides. Due to differences in fragmentation processes, the large peptides generated fewer fragments with higher relative abundance on the ion trap, resulting in lower limits of detection. Several applications of ion traps for the quantification of peptides have been described, performing either MS or MS/MS (Table 6). Also combinations of quadrupoles and ion traps, such as the hybrid triple quadrupole/linear ion trap mass spectrometer in the most recently described applications [103,140,156,161], have been reported. This instrument combines the benefits of both mass analyzers, i.e. the triple quadrupole's highly selective tandem MS scans and the ion trap's high sensitivity product scans, within the same platform.

TOF analyzers are ideal for measurement of high molecular masses and offer high resolution and thereby high selectivity and accuracy. However, TOF analyzers generally provide lower

sensitivity and only few peptide applications of TOF analyzers or combinations with TOF analyzers have been described [67,71,107,166]. Wan et al. describe the use of an ion-trap mass spectrometer for the quantification of [Dmt^1]DALDA, which makes the assay more sensitive compared to an earlier Q-TOF assay [71,72].

A less commonly used type of mass analyzer is Fourier transform mass spectrometry. The application of a hybrid linear ion trap-Fourier transform mass spectrometer has been reported for the quantification of vancomycin [94].

An important parameter in mass analysis is the mass resolution of the mass spectrometer. The resolution defines the ability of the mass spectrometer to distinguish different masses and is commonly described as the width of a mass peak, half way up the height of the peak (FWHM). Base line separation of two peaks with 1 amu mass difference is approximately achieved at 0.7 FWHM or unit resolution. Measuring multiply charged ions, a lower FWHM value will be required when distinction between the different isotope peaks is desired. However, the applied resolution for the quantitative assay is not often mentioned. Unit resolution on triple quadrupoles has been reported for singly [52,64,68,115,130] as well as doubly [80] or multiply charged peptides [105]. For quantification of higher charged peptides on triple quadrupoles, higher FWHM values (and thus lower resolution) have been mentioned [54,95,149] in order to combine different isotopes in one signal. Sometimes higher resolution is essential. For example, the quantification of the LHRH analog goserelin, a decapeptide with a molecular mass of 1269 Da, was complicated by a contamination at almost similar m/z value as the analyte. As these masses could not be separated on a triple quadrupole, a Q-TOF analyzer was used [166].

The measurement of ions in MS and MS/MS can occur in different scan modes. In the MS full scan (FS) mode a complete mass spectrum is generated, showing the molecular ion in different charge states and the different adduct ions. Collection of all mass spectral information can be useful when specific information is required, for example to monitor possible interfering ions. For quantitative assays both sensitivity and specificity of the method are usually increased when only one or few specific m/z ions are selected in the so-called selected ion monitoring (SIM) mode. To overcome the problem of irreproducible quantification in the SIM mode due to variable intensities of the differently charged molecular ions, a summation of all charged molecular ions can be used [110,120].

Also in MS/MS a full scan of the fragments of the selected ion can be recorded, but for very selective and sensitive MS/MS quantification the selection of one or few fragment ions is favored. This is called multiple reaction monitoring (MRM) and eliminates the interference of co-eluting contaminants more efficiently than SIM, especially in the case of peptides with high molecular masses. However, fragmentation of these larger peptides during collision-induced dissociation usually requires higher collision energies, thereby more commonly producing multiple fragments, providing lower sensitivity [185]. Therefore, sometimes SIM is preferred despite the ability to perform MRM [49,129]. For the same reason, Rogatsky et al. [185] showed higher sensitivity using two-dimensional HPLC with SIM detection than using single column HPLC with MRM detection.

For the quantification of cyclosporin A, both SIM and MRM analyses have been developed. Several MRM analyses measure the $[M+NH_4]^+$ ion as the parent ion with the molecular ion as product ion after collision-induced dissociation (CID) [90,100,121,123,125–127,131,132], as no stable abundant fragment of cyclosporin A could be measured. However, MRM analysis with the $[M+H]^+$ ion as parent ion has also been reported, measuring an undefined product ion with m/z 425.4 [86,124].

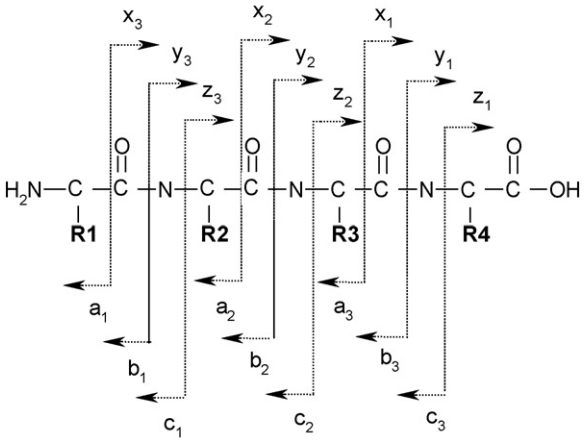


Fig. 5. Nomenclature of common ion types after fragmentation of peptides by (tandem) mass spectrometry. Cleavage at the amide bonds generates b- and y-ions, containing the N- or C-terminus, respectively, plus one or more additional amino acid residues. Ion pairs formed after cleavages at other positions in the backbone can be a- and x-ions or c-and z-ions.

4.4. Collision-induced dissociation (CID) of peptides

During fragmentation of the peptides in (tandem) MS, different types of fragment ions are produced that can be described by a specific nomenclature (Fig. 5) [177,178,186].

As the cleavage of the peptide backbone commonly occurs at the peptide amide bond, N-terminal b-ions as well as C-terminal y-ions are most typically observed in tandem MS spectra of peptides. Similar ion types are formed for positive and negative ions. High energy CID generates more fragments, such as ions resulting from cleavage of the side-chain bond in addition to backbone cleavage(s) that are named as d-, v- and w-ions [177]. In addition, ions which have lost an ammonia (−17 Da) or water (−18 Da) molecule can be seen in the CID spectra, especially in low energy CID. Furthermore, internal fragments can be observed as a result of double backbone cleavages. Immonium ions are internal fragments that can be observed at low m/z values, generated by cleavage at both the C- and N-terminus of an amino acid residue. Muroa et al. have reported more sensitive detection using an immonium ion as product ion compared to other types of product ions and have related the occurrence of the immonium ions to the sample clean-up precipitation procedure with TCA [105]. Also Chang et al. [113] have reported higher intensity of an immonium product ion, but this ion was not selected for SRM as it showed interference with endogenous substances in the matrix.

The fragmentation of peptides is affected by the number, the nature and the position of the amino acid residues in the peptide and has been reviewed in detail by Papayannopoulos [187]. For example, the presence of proline causes preferential fragmentation adjacent to proline, often resulting in relative abundant y-ions [187]. For cyclic peptides all amino acid residues present in the peptide can occur at the C- and N-terminus after ring opening, thereby producing different types of b- and y-ions.

5. Data analysis and quantification

5.1. Validation

Validation of a bioanalytical LC–MS assay is required to ensure accurate and precise data. Procedures for bioanalytical liquid chromatographic method validation have been reviewed by Rosing et al. [188]. The guideline for bioanalytical method validation, published by the Food and Drug Administration (FDA) counts as a standard

for the examination of validation parameters, such as linearity, accuracy, precision, selectivity and specificity, stability and recovery [189]. The guideline includes LC–MS and LC–MS/MS assays, for which it specifically describes the validation of matrix effects. This guideline can also be applied for the bioanalysis of peptides.

The sensitivity of an assay can be expressed as the lower limit of quantification (LLOQ), which is defined as the lowest amount of analyte that can be quantified with a precision and accuracy equal to or better than 20%. Most bioanalytical assays for peptides show an LLOQ between 0.5 and 10 ng/ml, with some lower LLOQs mainly for smaller peptides (Table 5). Lower LLOQs have sometimes been achieved by using columns with smaller diameters or by decreasing background interferences. For example, for both C-peptide and actinomycin-D improved sensitivity has been reported by reducing matrix effects, either by using two-dimensional chromatography [83,152] or by changing the internal standard and eluent composition [92]. Improved sensitivity of LC–MS assays has also been reported for several other compounds. The LLOQ for the quantification of the depsipeptide FR901228 could be decreased from 1 to 0.1 ng/ml plasma by an improved extraction procedure and modified internal standard [134,135]. The sensitivity of a quantitative assay for the α-melanocyte-stimulating hormone analog melanotan-II could be improved by the incorporation of a more sensitive hybrid triple quadrupole linear ion trap MS instrument, decreasing the LLOQ from 5 to 0.5 ng/ml plasma [80,103]. For the anti-fungal peptide caspofungin however, the advanced measurement by the implementation of a fully automated on-line extraction system resulted in a higher LLOQ than an earlier developed method [62,84].

Some assays have been described with lower sensitivity and LLOQs above 10 ng/ml, particularly when quantification in lower concentration ranges is not required. However, also instrumentation seems to play an important role in the sensitivity of the assay. This is illustrated for the few assays with the highest LLOQ (>200 ng/ml) of which three have been based on measurement with an ion trap mass spectrometer [62,110,112].

5.2. Internal standards

Internal standards (IS) are commonly used in quantitative bioanalysis, and may correct for variabilities during all different steps of the bioanalytical process, e.g. recovery, dilution, adsorption, evaporation, degradation, derivatization or instrumental performances. For quantitative bioanalytical LC–MS methods, the main reason for the use of an IS is correction for MS detection, as MS response may vary largely due to several parameters that are difficult to control. For example, the ionization of the analyte may be influenced drastically by the presence of matrix components causing ion suppression of the analyte, especially when electrospray ionization (ESI) is employed. Other parameters influencing ionization efficiency are the temperature or gas flow of the ion source. To correct for all these variabilities the ideal IS should have the same characteristics as the analyte, showing similar extraction recovery, but also similar LC retention for identical ESI conditions and similar MS ionization. Therefore, stable isotopically labeled (SIL) analogs of the peptide are commonly used as IS for bioanalytical peptide LC–MS assays. These compounds differ from the analyte only by mass, thereby allowing simultaneous MS detection.

However, the synthesis of SIL compounds is very expensive and this makes the use of structural analogs a useful and cheaper alternative. Structural peptide analogs can differ from the analyte by an exchange or removal/addition of amino acids or a small modification in one or more side chains. For the quantification of a panchemokine inhibitor, the use of a diastereomer as IS has been described [110]. The use of a diastereomer however necessitates chromatographic separation from the analyte.

Comparisons of SIL-ISs with alternative ISs have turned out in favor of the SIL-IS [145,190]. For the analysis of kahalalide F, a cyclic depsipeptide with a molecular mass of 1478 Da, the use of a SIL-IS resulted in significantly lower variance compared to the use of a butyric acid analog [99]. Improved linearity, accuracy and precision with a SIL-IS was observed for the quantification of angiotensin IV in dialysates by nano LC–MS/MS, as well as the ability to correct for peptide degradation in the microdialysates [145].

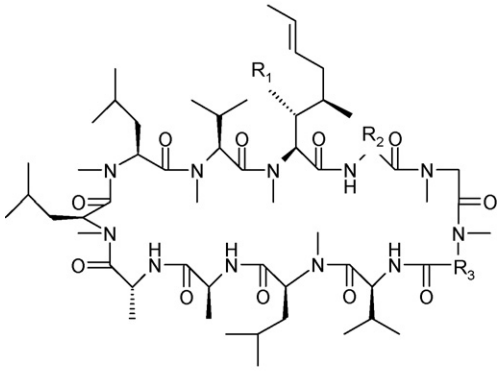
Compounds that might initially seem completely different from the analyte have also been described to be used as an IS, some being peptides [63,81,82,103,151], but some even being non-peptidic, e.g. morphine as IS for the quantification of the nonapeptide vasopressin analog desmopressin (MW 1068 Da) from human skin samples [148]. For the quantification of depsipeptide FR901228, an antitumor agent, t-boc-α-d-glutamic acid benzyl ester has initially been used as IS [135]. Li and coworkers [134] have described the improvement of this method, using N-t-boc-Met-Leu-Phe as IS. This IS still differs structurally from the analyte, although it is a peptide, but it improved sensitivity since the initial IS was unstable in certain biological matrices. For the quantification of apicidin, the structurally different trazodone was favored as IS instead of the structural analog HC-toxin, which showed too low extraction recovery [130].

Although the use of ISs for quantitative LC–MS bioanalysis of peptides is well accepted, some assays are reported that do not use an IS (Table 5). Quantification of the polypeptides glucagon (MW 3483 Da) and motilin (MW 2699 Da) without the use of an IS has been described by Delinsky et al. [49,54], as a SIL analog was considered too expensive and no other polypeptide or structural analog was found to exhibit the same extraction properties. As for the other assays, the use of an IS is mostly preferred, but merely simply not possible as no suitable analog can be found. The accurateness of the quantification of salmon calcitonin (MW 3430 Da) without IS [108] could simply be improved by the use of a SIL analog [107].

The high number of different bioanalytical assays for the quantification of cyclosporin A offers an insight in the variety and performances of used ISs. The use of a SIL-IS is preferred, because it best mimics the absolute recovery, fragmentation and, most importantly, the ionization efficiency of cyclosporin A. Alternatively, structural analogs, like cyclosporin B, C, D or G have been used (Fig. 6). Structurally different ISs have been used, because of the restricted availability of the structural analogs or because one IS is preferred for the simultaneous quantification of several analytes [121–123,125,142]. However, the use of an IS for cyclosporin A quantification has also been reported to be unnecessary [116–118,120], particularly when no off-line SPE step is performed. At the end, validation of the bio-analytical assay has to show whether the proposed internal standard sufficiently corrects for the different variabilities.

5.3. Peptide degradation

The quantification of peptides in biological samples can be complicated by various degradation processes, not only in the biological matrix, but during all steps of the analytical procedure. The chemical instability of peptides and proteins is defined by modifications in the amino acid residues, such as oxidation, reduction, hydrolysis or de-amidation. The physical instability of peptides and proteins involves changes in the secondary, tertiary and quaternary structures. Factors influencing both chemical and physical instability of peptides and proteins, and amino acid residues most sensitive to the specific degradation pathways have been described and specified by Reubsaet et al. [191,192].



	R1	R2	R3
Cyclosporin A	OH		
Cyclosporin B	OH		
Cyclosporin C	OH	HO	
Cyclosporin D	OH		
Cyclosporin G	OH		
PSC833	O		

Fig. 6. Chemical structures of cyclosporin A (CsA) and analogs used as internal standards.

In biological matrices, the presence of different metabolic enzymes, e.g. esterases or proteases, will easily cause both *in vivo* and *ex vivo* degradation of peptides and proteins. *In vivo* degradation of the analyte might necessitate simultaneous quantification of one or more metabolites along with the parent compound, especially when these metabolites exhibit (un-) desired activity. For example, the de-amidated metabolite of the polypeptide HIV-fusion inhibitor enfuvirtide, M-20, only differing from the parent compound by a de-amidated C-terminus and a 1 amu mass difference, has approximately 20% of *in vitro* activity of the parent compound and has been successfully quantified together with the parent drug [95,113].

Degradation of peptides can also occur *ex vivo*. For several peptides instability in the biological matrix at room temperature has been reported [53,65,80,82,141,144,150,151]. Biological samples with peptides may be stored frozen for periods up to 1 year without significant losses. To prevent *ex vivo* degradation of peptides in the biological matrix, addition of protease or other inhibitors has been reported [138,144,150,151], as well as complete preparation of the samples in on ice [65].

Limitations for quantitative assays caused by both *in vivo* and *ex vivo* alterations of peptides have been illustrated for the endogenous vasoactive nonapeptide bradykinin. As bradykinin is rapidly metabolized and also artifactual produced during blood sampling, its stable metabolite BK1-5 has been selected for quantification as a marker for bradykinin production [78]. The gastrointestinal peptide ghrelin has also shown limited stability and has therefore been simultaneously quantified with its deacylated analog [106].

Once extracted from the biological environment, peptides and proteins can still suffer from different types of degradation processes. A complete validation of the LC–MS (/MS) method should therefore include different in-process stability experiments, such as stability of the peptide in the dried extracts or reconstitution solvent. Limited storage in the autosampler has been reported due to different degradation or solubility characteristics of the analyte and its IS [95,99,115].

6. Future perspectives

The bioanalysis of peptides is continuously developing with novel or improved techniques being introduced. For peptide separation LC columns become more advanced or miniaturized, such as the monoliths discussed before. Other advances are the production of LC columns with sub-2 μm particles and LC-systems that can handle the higher pressures associated with these columns. This technique offers advantages, such as improved speed, sensitivity and resolution. For peptide analyses, this can be a suitable technique to improve the separation of complex mixtures, as demonstrated for peptides from an enzymatic digest on a 27 cm long capillary column packed with 1.0 μm particles [193]. The quantification of a tryptic digest of apolipoprotein A1 on a column with 1.7 μm particles showed comparable sensitivity as a capillary column (75 μm i.d.), but a 20-fold reduction in run time [156].

Also *mobile* phase alterations can improve traditional LC. In supercritical fluid chromatography (SFC) a fluid, maintained at its critical state is used as mobile phase. This mobile phase has a low viscosity and high diffusivity and thereby offers advantages such as speed and use of longer columns. As the most commonly used fluid CO_2 is relatively non-polar, SFC has been applied for mainly non-polar compounds. Nevertheless, Zheng et al. [194] showed the applicability of SFC with MS detection for peptides by the elution of polypeptides up to 40-mers.

Current mass spectrometric detection has been improved by several novel techniques for the different stages in the mass spectrometric process. Several advances in ionization sources have already been mentioned. A technique that pre-separates ions between the ion source and the mass spectrometer is high-field asymmetric waveform ion mobility spectrometry (FAIMS). With FAIMS ions are separated by their differences in ion mobility in the alternately applied strong and weak electric fields [195]. This technique can thereby reduce chemical background and consequently improve signal-to-noise ratios, as has also been demonstrated for peptide ions [196,197].

Mass analyzers have been improved to provide higher resolution and to increase sensitivity and signal-to-noise ratios. Also novel techniques to improve the ion transmission, collision or detection in the mass spectrometer have been developed and are under further development.

7. Conclusions

LC–MS is a suitable technique for the quantification of peptides from biological samples and is still under further development. Due to the complexity of the biological matrices, the sample

pre-treatment is a crucial, but also time-consuming step in the bioanalytical process. The development of (semi-)automated sample pre-treatment techniques will be a way to reduce the total analysis time. Other features of peptides, complicating their quantification, are their ability to adsorb to different sorbents during the analytical steps, their sensitivity to degradation and the difficulty to find a suitable IS.

LC is necessary to separate the peptide from interfering components, although selective MS detection diminishes the chromatographic requirements. LC columns have been developed in ways to improve analysis speed and chromatographic performance. The LC effluent usually enters the MS by ESI. However, during ionization a part of the sensitivity of the MS detection can be lost as the signal is deviated over multiple charges, isotopes or adduct ions. To improve sensitivity, the ionization has to be sufficiently optimized. Tandem MS can improve the selectivity by selecting a specific product of the peptide after dissociation.

The different parts of the MS remain susceptible to developments and improvements, likely to make LC–MS and/or LC–MS/MS the most versatile technique for peptide quantification in biological matrices.

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