ORIGINAL ARTICLE

Quality evaluation of synthetic quorum sensing peptides used in R&D

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Abstract Peptides are becoming an important class of molecules in the pharmaceutical field. Closely related peptide-impurities in peptides are inherent to the synthesis approach and have demonstrated to potentially mask biomedical experimental results. Quorum sensing peptides are attracting high interest in R&D and therefore a representative set of quorum sensing peptides, with a requested purity of at least 95.0%, was evaluated for their purity and nature of related impurities. In-house quality control (QC) revealed a large discrepancy between the purity levels as stated on the supplier's certificate of analysis and our QC results. By using our QC analysis flowchart, we demonstrated that only 44.0% of the peptides met the required purity. The main compound of one sample was even found to have a different structure compared to the desired peptide. We also found that the majority of the related impurities were lacking amino acid(s) in the desired peptide sequence. Relying on the certificates of analysis as provided by the supplier might have serious consequences for peptide research, and peptide-researchers should implement and maintain a thorough in-house QC.

1. Introduction

Peptides are becoming an important class of molecules in the biomedical and pharmaceutical fields owing to their high affinity, strong selectivity for their targets and low toxicity [1]. Despite potential limitations such as overall low oral bio-availability, low metabolic resistance, potential immunogenicity, poor membrane permeability and financial aspects, several peptide drugs have entered the market [2,3]. The promising future of peptide therapeutics is further highlighted by the number of peptides in clinical and preclinical phases [4]. In 2012, approximately 200 peptides entered the clinical phase, while another 400 were at advanced preclinical stages [4–6].

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Quorum sensing peptides are a group of peptides currently attracting high interest. Quorum sensing, the process of cell-to-cell communication between bacteria, has been the subject of a great number of scientific research papers [7–9]. Three main groups of quorum sensing molecules can be distinguished: *N*-acylhomoserine lactone derivatives (AHL or auto-inducer-1), quorum sensing peptides and boron-furan derivatives (auto-inducer-2). The quorum sensing peptides, mainly found in Gram-positive bacteria, show a large structural diversity: short, linear fragments as well as cyclic (thiolacton) derivatives, with or without post-translational isoprenyl modifications, are observed [10]. These peptides bind to (i) bacterial membrane associated receptors, or (ii) cytoplasmatic receptors, after which the transcription of the target genes is activated. Interfering with this bacterial quorum sensing pathway might open interesting application perspectives.

Chemical peptide synthesis for medicinal purposes has become economically viable [11]. The possibility to produce small, medium (5–20 residues) to large (20–50 residues) peptides has evolved dramatically, hereby frequently outperforming the biotechnological approaches as they are known to date [6]. In 1965, Merrifield cleared the way for Solid-Phase Peptide Synthesis (SPPS), thus introducing and facilitating a totally new concept in peptide synthesis [12]: a peptidic chain, fixed to a solid support, is created by the consecutive addition of the appropriate amino acids. The reaction can be automated and possible solubilization-issues are avoided due to the fixation of the peptide to the solid matrix [13]. During SPPS, different side-reactions can occur, resulting in several types of peptide impurities [14]: *e.g.* (i) diketopiperazine structures [15,16], (ii) aspartimide residues [17–19], (iii) cysteine racemization [16], (iv) diastereoisomeric products [20,21], (v) dimers [22], (vi) acid precursors and protected sequences [20], (vii) oxidation and reduction of amino acids [19,23], (viii) amino acid deletions [15,23–26], (ix) amino acid insertions [23,24], (x) products of side chain reactivity and (xi) amino acid modifications during cleavage [16,19,23,24,26–30]. Thus, the crude peptides obtained from SPPS mostly will contain many by-products. These impurities, if present even after purification, have to be quantified and characterized to meet pharmaceutical regulatory requirements, but they need to be under control as well during the unregulated biomedical research and discovery phases [31,32]. Due to budgetary constraints, peptides used in research are often purchased at undefined or low purity levels, *e.g.* 70.0% [31]. Generally, the peptide purity used for biomedical research and discovery purposes ranges from as low as 50.0% to more than 95.0% [22,26]. However, closely related impurities of the target peptide may possess stronger binding affinities compared to the native peptide [26], thus potentially causing erroneous conclusions. Investigations by de Beukelaar et al. [33] indicated different immune responses of a protein-spanning peptide pool of 70.0% pure peptides due to the impurities. False-positive results in a HIV-vaccine trial were also ascribed to impurities in the peptide-mixtures [34]. Zhang et al. [35] were unable to reproduce their initial obestatin results in vitro, possibly due to impurities present in the initially examined peptide. Additionally, Verbeken et al. [31] observed different biofunctional responses in a set of tissue-organ bath experiments caused by impurities of the examined peptides. Impurities are thus able to potentially mask biomedical experimental outcomes and may cause false negative or positive results.

Quorum sensing peptides are currently being actively investigated, *i.e.* for their possible role in the crosstalk between the microbiome and its host [36,37]. Therefore, a thorough in-house QC analysis of a selected set of quorum sensing peptides will be conducted, followed by identification of the observed impurities. These data not only demonstrate the need for routine QC in peptide research, but also can help in building a global overview of expected related impurities in peptides.

2. Materials and methods

2.1. Chemicals and reagents

A set of 98 representative peptides was selected from the Quorumpeps database [38]. Linear peptide synthesis was conducted by an international supplier, while another supplier synthesized the cyclic peptides, both by means of Fmoc-SPPS. A minimal purity of 95.0% was requested at order. The sequences of the 98 peptides are provided in Table 1. Acetonitrile of HPLC–MS and UPLC-MS grades were purchased from Fisher Scientific (Aalst, Belgium). Formic acid (LC–MS grade) and DMSO (p.a. $\geq$ 99.9%) were obtained from Sigma-Aldrich (Diegem, Belgium). Trifluoroacetic acid (TFA), $NaH_2PO_4 \cdot H_2O$ and Na_2HPO_4 were

Table 1 Peptides information.

Quorumpeps ID	Sequence	Molecular weight	Solvent	Column[a]	Purity (%)[b]
2	FNTIPSY	839.95	Water	C_{18}-FA	83.31
5	Ac-CGSLF, thiolacton linkage between C1 and F5	549.72	Water+50% DMSO	C_{18}-FA	88.30
7	FNTWPSY	913.00	Water	C_{18}-FA	98.46
10	ADLPFEF	837.93	Water	C_{18}-FA	99.20
11	AGTKPQGKPASNLVECVFSLFKKCN	2667.14	Water	C_{18}-FA	72.93
13	AIFILAS	733.91	Water+58% DMSO	C_{18}-FA	97.82
14	AITLIFI	790.01	Water+60% DMSO	C_{18}-FA	72.48
15	AKDEH	598.61	Water	C_{18}-TFA	69.26
16	AKTVQ	545.64	Water	C_{18}-TFA	96.20
17	ALILTLVS	829.05	Water+60% DMSO	C_{18}-FA	94.43
18	ARNQT	588.62	Water		92.33

Table 1 (*continued*)

Quorumpeps ID	Sequence	Molecular weight	Solvent	Column[a]	Purity (%)[b]
				Organic Acid	
19	NNWNN	660.64	Water	C_{18}-TFA	92.66
22	CVGIW, thiolacton linkage between C1 and W5	558.78	Water+50% DMSO	C_{18}-FA	60.39
24	CTFTLPGGGGVCTLTSECIC	1962.3	Water+1% DMSO	C_{18}-FA	12.21
25	CVFSLFKKCN	1188.47	Water	C_{18}-FA	47.92
28	DIRHRINNSIWRDIFLKRK	2480.91	Water	C_{18}-FA	93.68
30	DLRGVPNPWGWIFGR	1770.03	Water	C_{18}-FA	91.62
31	DLRNIFLKIKFKKK	1791.26	Water	C_{18}-FA	76.74
32	DMCNGYF, thiolacton linkage between C3 and F7	831.04	Water+50% DMSO	C_{18}-FA	58.05
34	DRVGA	516.55	Water	C_{18}-TFA	98.30
40	DSVCASYF, thiolacton linkage between C4 and F8	873.06	Water+50% DMSO	C_{18}-FA	31.61
42	DWRFLNSIRDLIFPKRK	2204.61	Water	C_{18}-FA	97.86
44	EKMIG	576.71	Water	C_{18}-FA	99.41
45	EMRISRIILDFLFLRKK	2178.71	Water	C_{18}-FA	81.91
46	EMRKSNNNFFHFLRRI	2109.44	Water	C_{18}-FA	96.43
47	EMRLPKILRDFIFPRKK	2187.72	Water	C_{18}-FA	98.46
49	EQLSFTSIGILQLLTIGTRSCWFFYCRY	3346.92	Water+17% DMSO	C_{18}-FA	52.58
50	ERGMT	592.67	Water	C_{18}-TFA	98.76
51	ERNNT	632.63	Water	Organic Acid	98.24
52	ERPVG	556.62	Water	C_{18}-TFA	99.75
53	ESRLPKILLDFLFLRKK	2116.62	Water	C_{18}-FA	65.11
54	ESRLPKIRFDFIFPRKK	2177.62	Water	C_{18}-FA	99.30
55	ESRVSRIILDFLFQRKK	2135.54	Water	C_{18}-FA	96.90
56	VNYGNGVSCSKTKCSVNWGQAFQERYTAGINSFVSGVASGAGSIGRRP	4969.46	Water	C_{18}-FA	33.57
58	DSRIRMGFDFSKLFGK	1904.22	Water	C_{18}-FA	95.72
62	ESRISDILLDFLFQRKK	2108.47	Water	C_{18}-FA	99.27
71	QNCPNIFGQWM, lacton linkage between S3 and M11	1319.70	Water+50% DMSO	C_{18}-FA	77.64
75	SINSQIGKATSNLVECVFSLFKKCN	2731.2	Water	C_{18}-FA	30.03
76	SNLVECVFSLFKKCN	1731.06	Water	C_{18}-FA	80.63
81	FNTIPKY	881.04	Water	C_{18}-FA	99.29
82	NTIPKY	733.87	Water	C_{18}-FA	69.22
84	FFNTCPSY	978.09	Water	C_{18}-FA	96.54
85	FNTCPSY	830.91	Water	C_{18}-FA	92.98
92	FHWWQTSPAHFS	1530.66	Water	C_{18}-FA	99.54
93	FLVMFLSG	913.14	Water+33% DMSO	C_{18}-FA	91.31
97	QNSPNIFGQWM, lacton linkage between S3 and M11	1303.63	Water+50% DMSO	C_{18}-FA	43.95
99	GKAEF	550.61	Water	C_{18}-FA	99.33
100	GKATSSISKCVFSFFKKC	1968.36	Water+33% DMSO	C_{18}-FA	28.65
101	GLWEDILYSLNIIKHNNTKGLHHPIQL	3167.67	Water	C_{18}-FA	96.92
102	GLWEDLLYNINRYAHYIT	2254.53	Water+33% DMSO	C_{18}-FA	97.98
103	GNWNN	603.59	Water	C_{18}-FA	97.70
105	GSQKGVYASQRSFVPSWFRKIFRN	2846.25	Water	C_{18}-FA	99.31
107	GVNACSSLF, thiolacton linkage between C5 and F9	879.13	Water+50% DMSO	C_{18}-FA	39.15
111	GWWEDFLYRFNIIEQKNTKGFYQPIQL	3434.91	Water+50% DMSO	C_{18}-FA	89.72
121	ILSGAPCIPW	1056.29	Water	C_{18}-FA	77.04
123	IRFVT	634.78	Water	C_{18}-FA	99.53
125	KSSAYSLQMGATAIKQVKKLFKKWGW	2985.59	Water	C_{18}-FA	66.39
132	LFSLVLAG	819.01	Water+33% DMSO	C_{18}-FA	96.92

Table 1 (*continued*)

Quorumpeps ID	Sequence	Molecular weight	Solvent	Column[a]	Purity (%)[b]
133	LFVVTLVG	847.06	Water+60% DMSO	C_{18}-FA	97.74
134	LPFEF	651.76	Water+50% DMSO	C_{18}-FA	17.17
135	LPFEH	641.72	Water	C_{18}-FA	94.23
137	LVTLVFV	790.01	Water+50% DMSO	C_{18}-FA	84.02
138	MAGNSSNFIHKIKQIFTHR	2229.59	Water+17% DMSO	C_{18}-FA	81.90
140	MKAEH	614.72	Water	C_{18}-FA	66.91
143	MPFEF	669.79	Water	C_{18}-FA	86.24
146	NEVPFEF	880.95	Water	C_{18}-FA	99.90
147	NGWNN	603.59	Water	C_{18}-FA	94.53
148	YSTCDFIM, thiolacton linkage between C4 and M8	961.25	Water+50% DMSO	C_{18}-FA	44.40
151	NNGNN	531.48	Water	C_{18}-TFA	96.79
152	NNNWNNN	888.85	Water	C_{18}-FA	98.42
153	NNWGN	603.59	Water	C_{18}-FA	99.10
154	NNWNG	603.59	Water	C_{18}-FA	99.33
155	NWN	432.44	Water	C_{18}-FA	98.40
156	FNTIP	589.69	Water	C_{18}-FA	99.27
157	FNTWP	662.75	Water	C_{18}-FA	99.23
160	QKGMY	625.74	Water	C_{18}-FA	99.04
162	QRGMI	603.74	Water	C_{18}-FA	94.36
164	SDLPFEH	843.89	Water	C_{18}-FA	91.37
165	SDMPFEF	871.96	Water	C_{18}-FA	99.44
166	SGSLSTFFLLFNRSFTQALGK	2321.66	Water	C_{18}-FA	92.99
174	SGSLSTFFRLFNRSFTQALG	2236.52	Water	C_{18}-FA	97.41
176	SGSLSTFFRLFNRSFTQALGK	2364.69	Water	C_{18}-FA	98.96
177	SGSLSTFFRLFNRSFTQALGKIR	2634.04	Water	C_{18}-FA	55.68
180	SGSLSTFFRLFNRSQTQALGK	2345.65	Water	C_{18}-FA	99.06
184	SIFTLVA	749.90	Water+33% DMSO	C_{18}-FA	93.17
186	SKDYN	625.64	Water	C_{18}-TFA	92.88
188	SLSTFFRLFNFSFTQALG	2083.37	Water+33% DMSO	C_{18}-FA	85.61
191	SRKAT	561.64	Water	C_{18}-TFA	78.31
192	SRNAT	547.57	Water	Organic Acid	81.74
193	SRNVT	575.62	Water	C_{18}-TFA	81.49
206	SYPGWSW	881.94	Water	C_{18}-FA	99.45
207	TAGPAIRASVKQCQKTLKATRLFTVSCKGKNGCK	3595.31	Water	C_{18}-FA	32.42
208	TNRNYGKPNKDIGTCIWSGFRHC	2668.01	Water	C_{18}-FA	89.19
210	VAVLVLGA	740.94	Water+33% DMSO	C_{18}-FA	92.98
212	VPFEF	637.73	Water	C_{18}-FA	97.65
214	WPFAHWPWQYPR	1670.89	Water	C_{18}-FA	58.94
215	FNTWPKY	954.10	Water	C_{18}-FA	95.52
218	YNPCSNYL, thiolacton linkage between C4 and L8	955.18	Water+50% DMSO	C_{18}-FA	16.87

[a] C_{18}-FA: acquity UPLC BEH 300 C_{18} column (100 mm × 2.1 mm, 1.7 µm). C_{18}-TFA: Vydac Everest C_{18} column (250 mm × 4.6 mm, 5 µm). Organic acid: Grace Alltech Prevail Organic Acid column (250 mm × 4.6 mm, 5 µm).
[b] Purity assigned based on UV chromatogram with internal normalization to the most abundant peak.

purchased from Merck (Overijse, Belgium). Water was purified using an Arium 611 purification system (Sartorius, Gottingen, Germany) yielding ≥ 18.2 MΩ cm quality water. Eppendorf Protein LoBind 2.0 and 1.5 mL tubes were purchased from Eppendorf (Nijmegen, the Netherlands). UPLC/HPLC vials and inserts were purchased from Waters (Milford, MA, USA). The Vydac Everest C_{18} (250 mm × 4.6 mm, 5 µm) column and Alltech Prevail Organic Acid column (250 mm × 4.6 mm, 5 µm), both protected with guard columns, were obtained from Grace (Deerfield, IL, USA). The Acquity UPLC BEH300 C_{18} column (100 mm × 2.1 mm, 1.7 µm) with guard column was purchased from Waters (Milford, MA, USA).
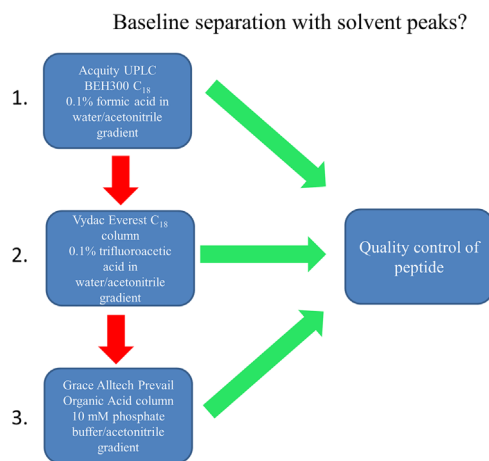
Baseline separation with solvent peaks?

Fig. 1 (U)HPLC column flow chart for quorum sensing peptides analysis.
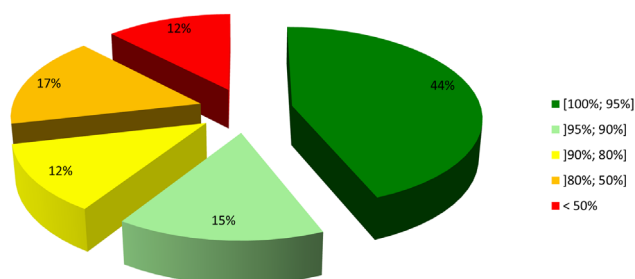
Fig. 2 Purity levels of quorum sensing peptides determined by in-house QC.

Fig. 3 The in-house QC control shows a distinct difference in peptide purity. (A) Chromatogram of supplier; Kromasil C_{18}-5 (250 mm × 4.6 mm, 5 μm), quantification at 220 nm; (B) chromatogram of in-house QC; Vydac Everest C_{18} column (250 mm × 4.6 mm, 5 μm), quantification at 210 nm.

2.2. Sample preparation

0.5–1 mg of each lyophilized, linear peptide was dissolved in water or a mixture of water and DMSO, depending on the calculated log P values and the solubility in water, to obtain a concentration of approximately 1.0 mg/mL (Table 1). The quantity of DMSO used was determined experimentally, by adding DMSO stepwise to the peptide-water suspension until no more precipitate was observed. The dissolved peptides were stored at −35 °C. The cyclic peptides (*i.e.* ID5, 22, 32, 40, 71, 97, 107, 148 and 218) were dissolved in DMSO-water (50/50, V/V) to obtain a concentration of approximately 0.5 mg/mL.

2.3. HPLC and UPLC

2.3.1. QC purity profiling

Three LC systems were consecutively applied in the analysis. To avoid carry-over peaks, 50% acetonitrile in water was always used as needle wash.

Each peptide was first analyzed with the Acquity UPLC BEH300 C_{18} column. Sample compartment was kept at 5 °C (± 2 °C) and column was maintained at 30 °C (±2 °C). Injection volume was set at 2 μL for the linear and 5 μL for the cyclic peptides. Mobile phases consisted of 0.1% (m/V) formic acid in water (A) and 0.1% (m/V) formic acid in acetonitrile (B). A general linear gradient was applied running from 95% A to 20% A during 22 min, followed by returning
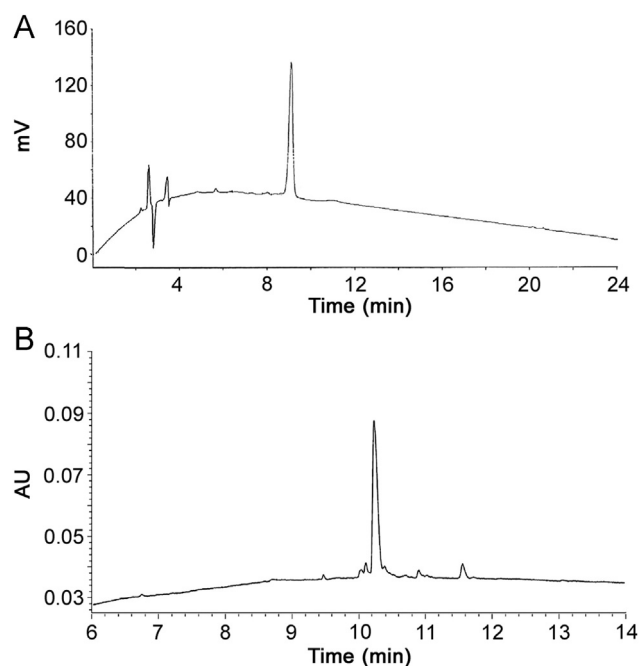
to initial condition and re-equilibration with a flow rate set at 0.5 mL/min. Analysis was conducted using a Waters Acquity UPLC Class BioQuaternary Solvent Manager, a Waters Acquity Bio-sample Manager, combined with a Flow Through Needle and a Waters Acquity UPLC PDA (500 nL to 10 mm path length analytical flow cell) detector (Waters, Milford, MA, USA). Empower™ 2 software was employed for data acquisition and analysis. Peptides and related impurities were quantified by area-normalization at 210 nm, using a sampling rate of 20 points/s and a detector time constant of 0.1 s. A reporting threshold (*i.e.* 0.1% of main peak AUC in the UV chromatogram) and identification threshold (*i.e.* 0.5% of main peak AUC in the UV chromatogram) as stated in the European Pharmacopoeia were applied [39]. Solvent and system peaks, observed in the blank (*i.e.* the solvent used to dissolve a certain peptide, Table 1), were excluded.

If no retention of the peptides was observed using the Acquity UPLC BEH300 C_{18} column, peptides were analyzed with the Vydac Everest C_{18} column on a Waters Alliance 2695 HPLC apparatus equipped with a Waters 2695 Separations Module, combined with a Flow Through Needle, and a Waters 2996 Photodiode Array Detector with Empower™ 2 software for data acquisition. Mobile phases consisted of 0.1% (m/V) TFA in water (A) and 0.1% (m/V) TFA in acetonitrile (B). A general linear gradient was applied running from 95% A to 20% A during 30 min, followed by returning to initial condition and re-equilibration with a flow rate set at 1.0 mL/min. For peptide ID191, a slightly modified linear gradient was applied, *i.e.* running from 98% A to 80% A during 10 min, followed by returning to initial conditions and re-equilibration with a flow rate set at 1.0 mL/min. Sample compartment was kept at 5 °C (±2 °C) and column was maintained at 30 °C (±2 °C); 20 μL of the samples dissolved in suitable solvent composition were injected. UV detection was performed at 215 nm using a sample rate of 1.0 point/s combined
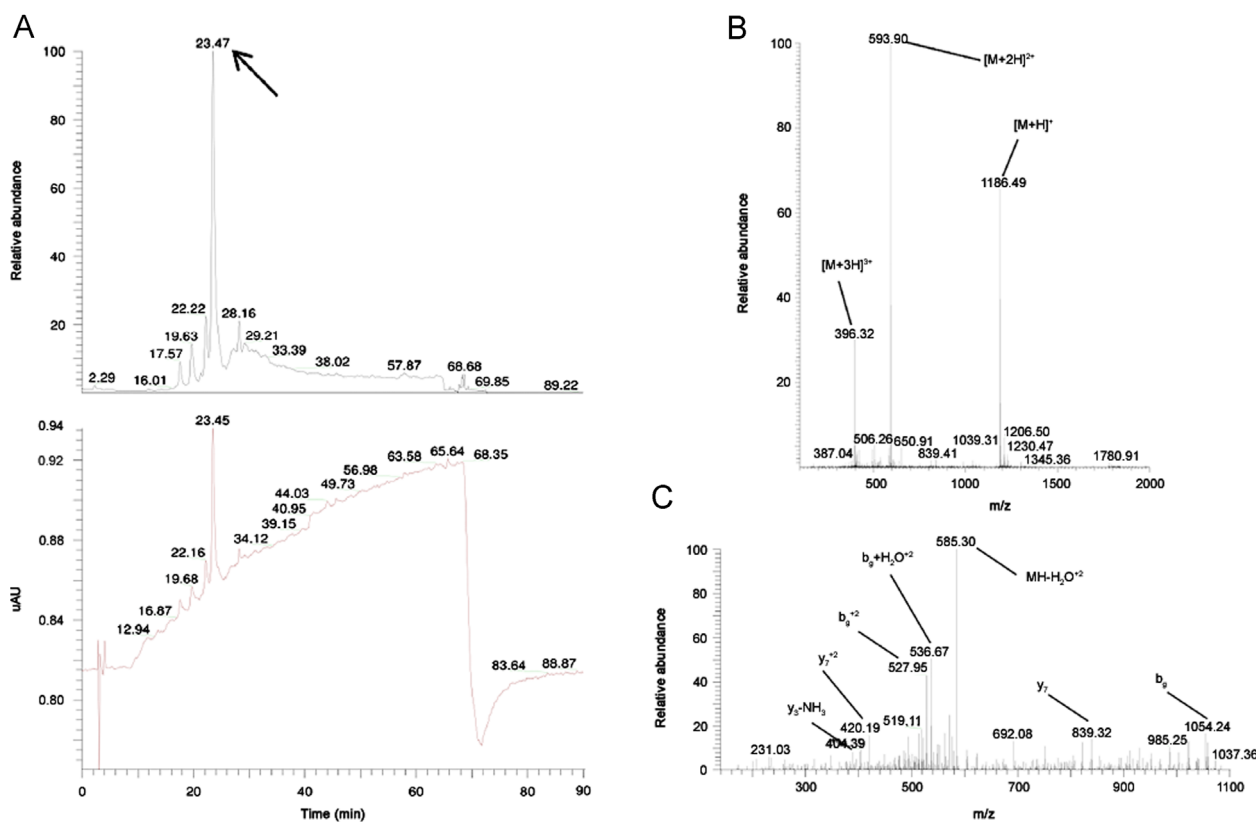
Fig. 4 TIC and UV chromatogram (A), MS^1 (B) and MS^2 (C) spectra of the related impurity C$_1$VFSLFKKC$_9$N (disulfide bond between C$_1$ and C$_9$) of peptide ID25 at a retention time of 23.47 min with appointed isotopic mass patterns and b- and y-fragmentation are allocated.
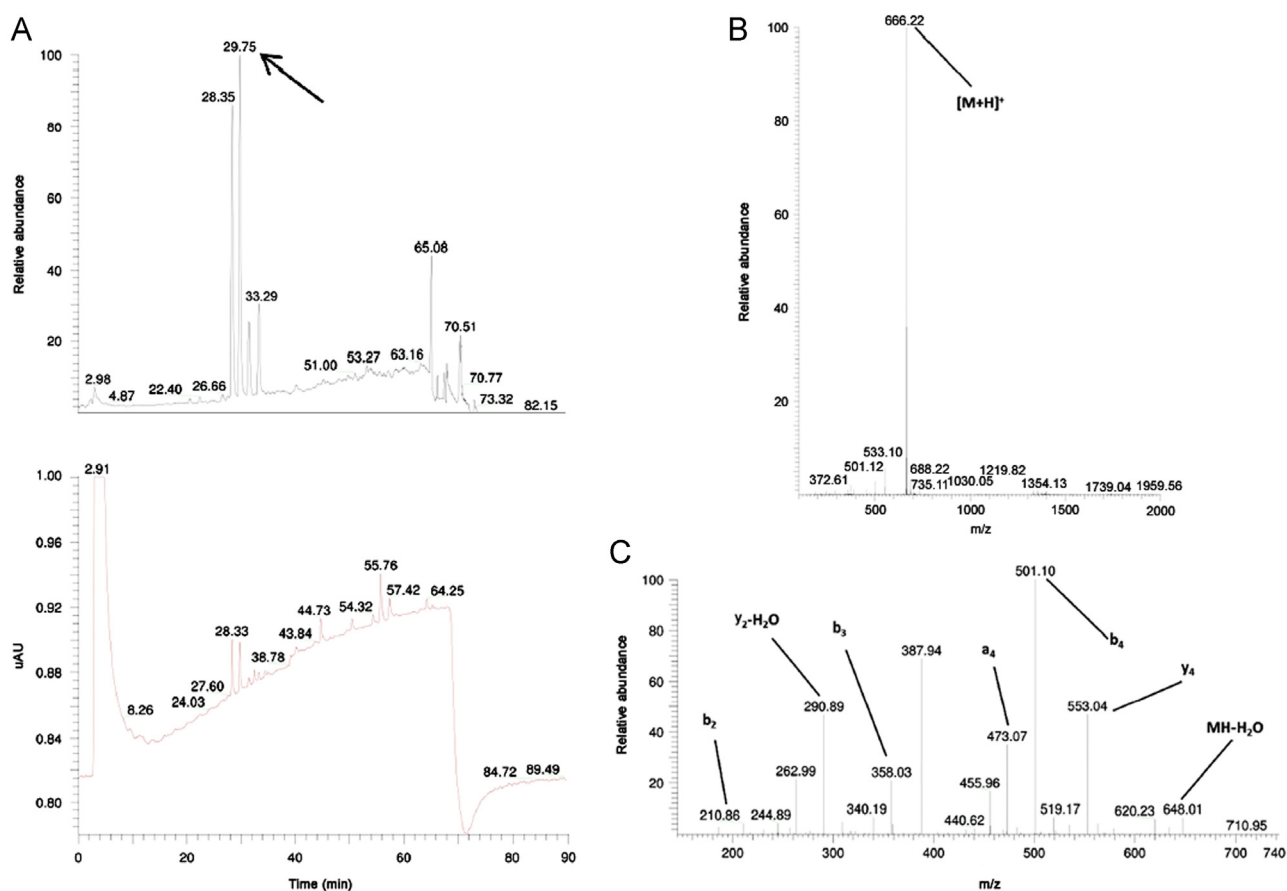

Fig. 5 TIC and UV chromatogram (A), MS^1 (B) and MS^2 (C) spectra of the related impurity LPFE(methyl)F of peptide ID134 at a retention time of 29.75 min with appointed isotopic mass patterns and b- and y-fragment allocation.

with a detector time constant of 0.2 s. The flow rate was set to 1.0 mL/min. The peptide purity was quantified through normalization by calculating the area (UV) of the most abundant peak as a percentage of total peak areas.

Finally, peptides ID18, 51 and 192 were analyzed using a Grace Alltech Prevail Organic Acid column (250 mm × 4.6 mm, 5 μm), using the same Waters Alliance 2695 HPLC equipment as used with the Vydac column. Mobile phases consisted of 10 mM phosphate buffer (pH 7) (A) and acetonitrile (B). A general linear gradient was applied running from 95% A to 60% A during 20 min, followed by returning to initial condition and re-equilibration with a flow rate set at 1.0 mL/min. Sample compartment was kept at 5 °C (±2 °C) and column maintained at 30 °C (±2 °C). 20 μL of the samples dissolved in suitable solvent composition were injected. UV detection was performed at 215 nm, using a sample rate of 1.0 point/s combined with a detector time constant of 0.2 s. The peptide purity was quantified through normalization by calculating the area (UV) of the most abundant peak as a percentage of total peak areas.

2.3.2. Identification of main LC peak and related impurities

Mass spectroscopic characterization was performed with a Thermo HPLC system consisting of a Waters 2487 dual λ absorbance UV/VIS-detector (UV at 215 nm), a Finnigan LCQ ion trap mass spectrometer with electrospray ionization (ESI) and Xcalibur™ software version 2.0 for data acquisition. The LC–MS equipment is yearly qualified using a tune solution and qualification solution according to the EDQM guideline [40]. The tune solution consists of 100 μL 1.0 mg/mL caffeine (Sigma-Aldrich, Diegem, Belgium) in methanol, 15 μL 5 nmol/μL MRFA (Met-Arg-Phe-Ala) (Research Plus Inc., Atlantic City, NJ, USA) in 50/50 methanol/water (V/V), 2.5 mL 0.1% (V/V) Ultramark 1621 (Alfa Aesar, Karlsruhe, Germany) in acetonitrile, 50 μL glacial acetic acid (Riedel de Haen, Diegem, Belgium) and 2.34 mL of 50/50 methanol/water (V/V). The EDQM qualification solution consists of a 10.0 μg/mL reserpine (Flandria, Ghent, Belgium) solution in acidified methanol (i.e. 1% acetic acid in methanol (V/V)). The reserpine solution is infused in the mass spectrometer with a flow rate of 3 μL/min with a collision energy of 35.00 eV. The m/z value of 609.4 is selected as parent ion and the m/z range for the daughter ions is set to m/z 165.0-800.0. The obtained m/z values are compared with the acceptance criteria as set by the EDQM.

For the analysis of the quorum sensing peptides, column temperature was set at 30 °C (±2 °C) and samples were kept at room temperature. 20 μL of aqueous peptide solution was injected into the HPLC system and a flow rate of 1.0 mL/min was applied. All 15 selected quorum sensing peptides were analyzed using the Vydac Everest C_{18} column (250 mm × 4.6 mm, 5 μm). Mobile phases consisted of 0.1% (m/V) formic acid in water (A) and 0.1% (m/V) formic acid in acetonitrile (B) with a flow rate of 1.00 mL/min. An isocratic period consisting of 95% A during 5 min was maintained, followed by a linear gradient running from 95% A to 40% A in 60 min and returning to the initial conditions and re-equilibration afterwards. The ion transfer capillary was operated at 250 °C, nitrogen was used as sheath gas (80 arbitrary units corresponding to 1.2 L/min) and helium
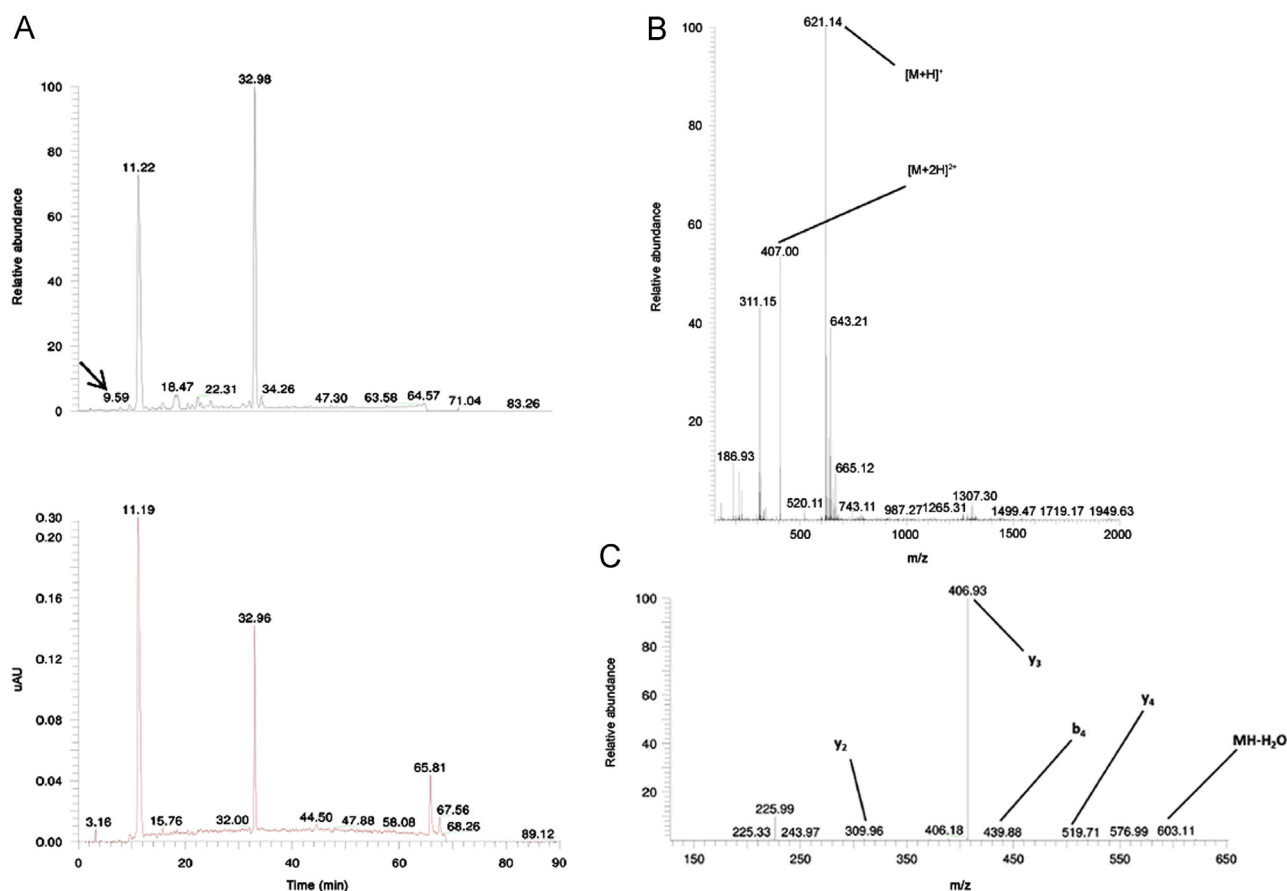

Fig. 6 TIC and UV chromatogram (A), MS^1 (B) and MS^2 (C) spectra of the related impurity TIPKY of peptide ID82 at a retention time of 9.59 min with appointed isotopic mass patterns and b- and y-fragment allocation.

as auxiliary gas (20 arbitrary units corresponding to 0.2 L/min). The spray voltage was 4.50 kV and the capillary voltage was 46 V. MS^1 (100–2000 m/z) and MS^2 (data dependent, *i.e.* highest abundant MS^1 m/z value for MS^2) data were obtained. All m/z values possessing an abundance higher than 25% in the according MS^1 spectrum were subject to identification via their respective MS^2 fragmentation pattern. Theoretical MS^2 fragmentation patterns of the peptides were calculated *in silico* with ProteinProspector [41] (University of California, San Francisco, CA, USA). The proposed sequences of the related impurities were confirmed using the ProteinProspector generated MS^2 spectra. The nomenclature originally proposed by Roepstorff and Fohlman [42] was used to annotate MS^2 fragments.

3. Results and discussion

3.1.   Quorum sensing peptide analysis flow chart

To determine the purity of the quorum sensing peptides, we developed a UPLC/HPLC-analysis flow chart (Fig. 1). Each peptide was analyzed first with the Acquity UPLC BEH300 C_{18} column, using the method described above. A C_{18} column with a 0.1% (m/V) formic acid in water/acetonitrile gradient is widely used for peptide analysis and was therefore selected as the initial method of analysis. If no retention of the peptides was observed, the peptides were analyzed with the Vydac Everest C_{18} column with TFA as ion-pairing reagent, which is known to have

a narrowing effect on peak width and an increased retention. Finally, if no retention was observed with the aforementioned columns, the Prevail Organic Acid column (mobile phase of pH 7) with a 10 mM phosphate buffered water/acetonitrile gradient was used for peptide quality control. Previous experience indicated that peptides which had no retention using standard C_{18} peptide columns might have appropriate retention using a polar-embedded column for highly hydrophilic compounds, like the Prevail Organic Acid column.

3.2.   QC purity assay

Despite a requested purity of at least 95.0%, only 43 out of 98 peptides met these purity specifications (Fig. 2). However, all the 98 peptides were accompanied by a certificate of analysis stating at least 95.0% purity. Our QC analysis upon arrival showed purity levels sometimes far below the demanded purity level (Table 1). In Fig. 3, the chromatogram of ID76, as provided by the supplier on the certificate of analysis versus our QC analysis, is given. The mobile phases of the supplier consisted of 0.1% (m/V) TFA in acetonitrile (A) and 0.1% (m/V) TFA in water (B). A general linear gradient was applied running from 22% A to 47% A during 22 min with a Kromasil C_{18}-5 (250 mm × 4.6 mm, 5 μm) column with quantification at 220 nm. The peptide was analyzed in our laboratory with the Acquity UPLC BEH300 C_{18} column, using the operational details as described above.
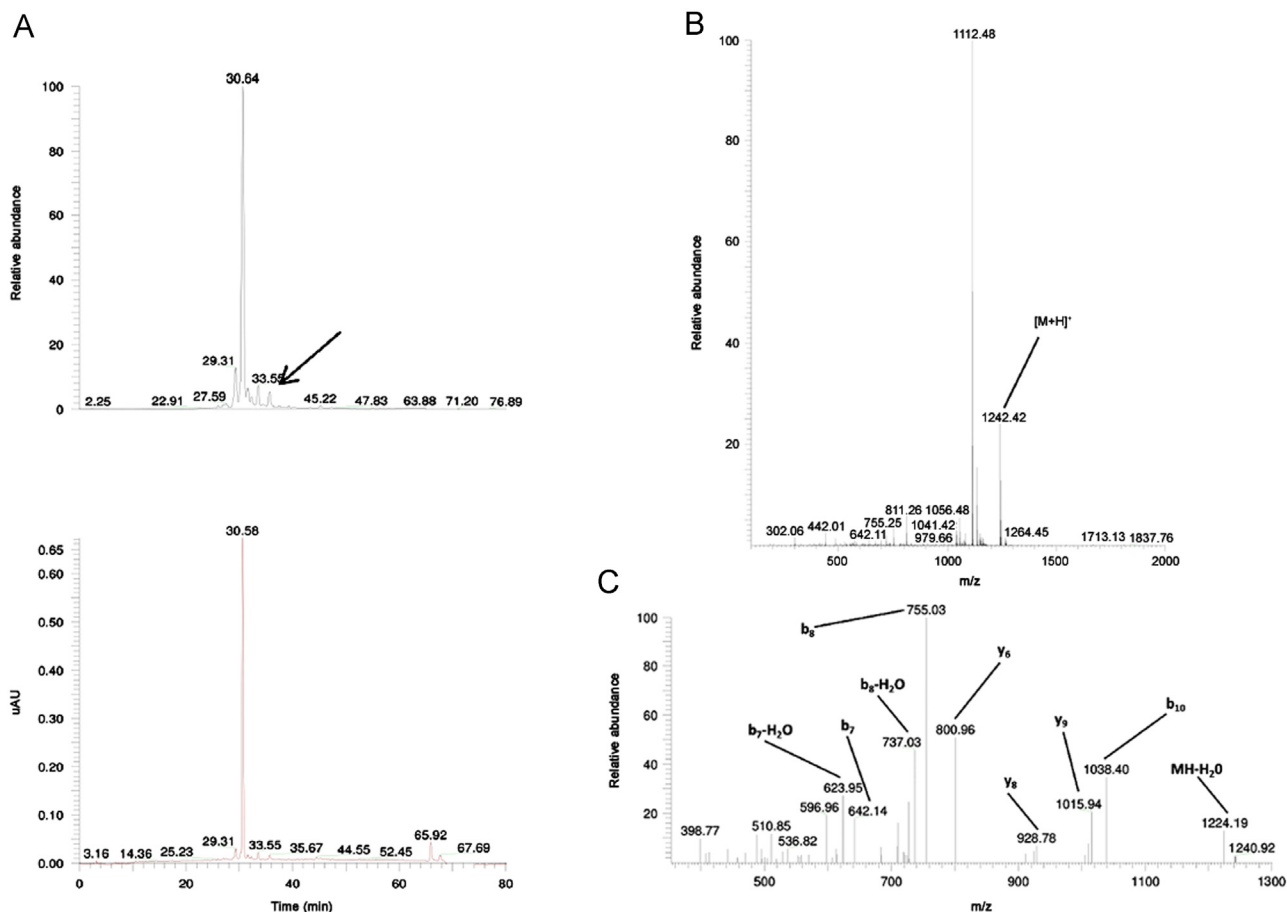

Fig. 7   TIC and UV chromatogram (A), MS^1 (B) and MS^2 (C) spectra of the related impurity ILSGAPCIPWW of peptide ID121 at a retention time of 35.70 min with appointed isotopic mass patterns and b- and y-fragment allocation.

The method applied by the supplier clearly lacked selectivity to determine the purity level of the peptide. We demonstrated the necessity of a reliable QC method, *i.e.* the need of a suitable chromatographic system to analyze peptide purity. The supplier claimed ≥ 95.0% purity using its system, where we determined a purity level of 80.6%.

Especially the cyclic peptides ($n=9$) were characterized by low purity levels: none of the peptides had a purity exceeding 90.0%. Of all cyclic peptides, 1 peptide had a purity between 80.0% and 90.0%, whereas the other 8 had purity levels below 80.0%. Nevertheless, each of these peptides was accompanied by a certificate of analysis stating a purity of at least 95.0%. After communication with the suppliers, they revealed that they were unable to produce these cyclic peptides at the requested purity level.

3.3. Identification of related impurities

A set of 15 peptides with varying purities was selected to determine the identity of the impurities (Table 1, depicted in red). The main peak was characterized as the desired peptide for 14 out of 15 peptides. The main peak from peptide ID25 was not the requested amino acid sequence. Instead of CVFSLFKKCN, a peptide with a mass difference of -2.1 Da compared with the requested peptide sequence was noted. Therefore, the formation of a disulfide bond (and thus cyclisation) between C_1 and C_9 is suggested. Because sulfur-containing amino acids are prone to oxidation [43], a spontaneous cyclisation is suspected for this peptide (Fig. 4).

The MS^1 spectrum of a related peptide impurity of peptide ID134 (retention time of 29.75 min) showed an impurity with a monoisotopic mass of 666.22 Da (Fig. 5). The difference with the monoisotopic mass of the requested peptide sequence (LPFEF) amounted to 14.04 Da, a mass difference that could be associated with the addition of a methyl group. The MS^2 fragmentation pattern of the related peptide impurity (Fig. 5C) showed a difference of $+14$ Da with the requested peptide sequence fragmentation pattern at b_4, y_2-H_2O, y_4, MH-H_2O, while the b_3 fragment of requested peptide and related impurity were the same, thus suggesting the addition of a methyl group to Glu4.

A related impurity of peptide ID82 (retention time 9.34 min) possessed an *m/z* value of 621.14 (Fig. 6). Compared with the desired peptide, *i.e.* NTIPKY, there was a mass difference of 114.02 Da, a mass difference which could be bridged by the deletion of Asn1. The suggested sequence TIPKY was confirmed by the MS^2 spectrum in Fig. 6C. On the other hand, we also demonstrated a related impurity characterized by the incorporation of an additional amino acid at the C-terminus of peptide ID121 (retention time 35.70 min) (Fig. 7). The MS^2 spectrum (Fig. 7C) confirmed the addition of an extra Trp residue at the C-terminus of peptide ID121, thus resulting in the related impurity ILSGAP-CIPWW. Another example of a related peptide impurity was the combination of both deleted and additionally inserted amino acids in the desired peptide sequence. In Fig. 8, such an example is
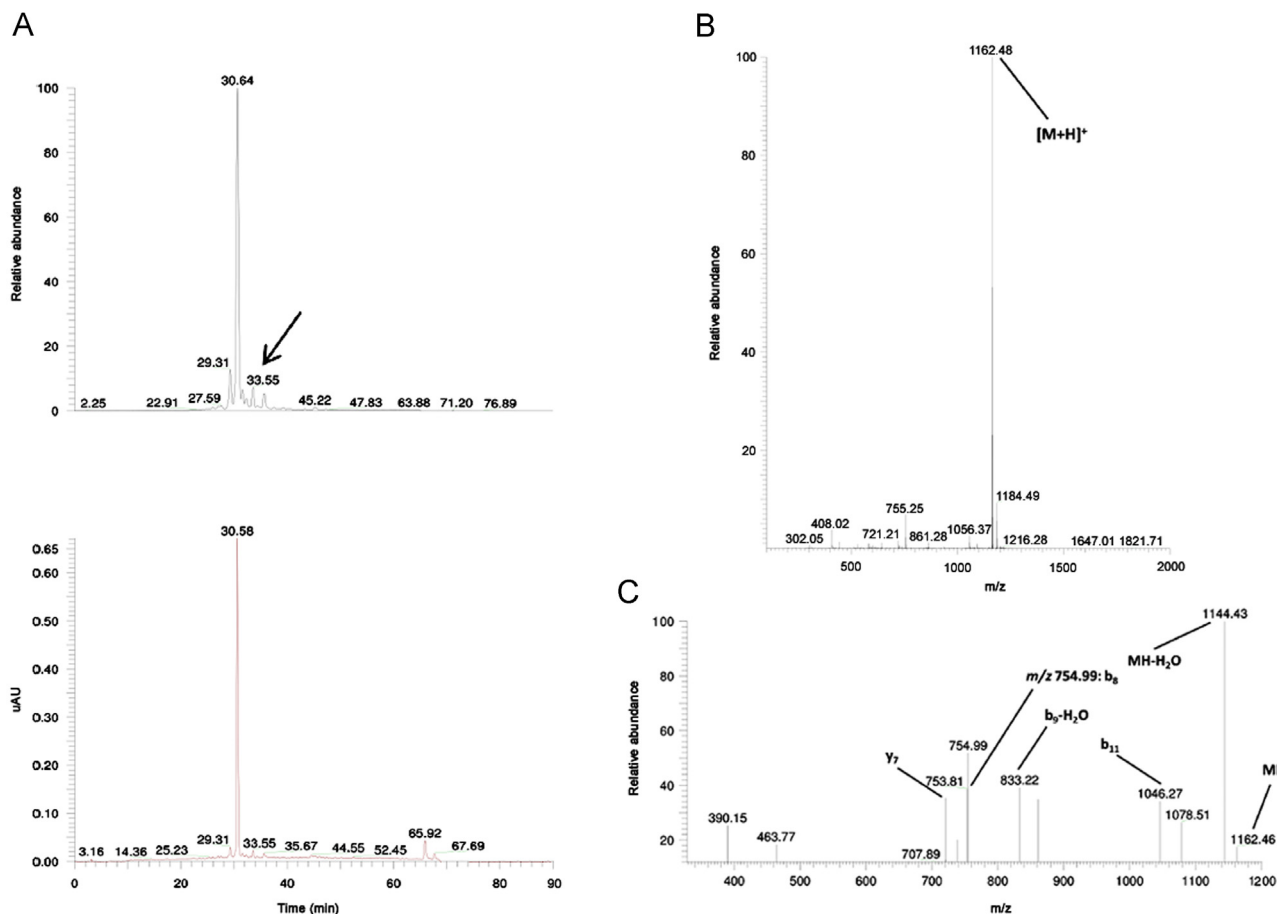

Fig. 8 TIC and UV chromatogram (A), MS^1 (B) and MS^2 (C) spectra of the related impurity ILSGAPCIPPPP of peptide ID121 at a retention time of 33.55 min with appointed isotopic mass patterns and b- and y-fragment allocation.

Table 2   Overview of the observed peptides (blue) and impurities (purple).

Peptide ID	Purity (%)[1]	M_r (exp.) (Da)	Impurity sequence[3]	Retention time (min)	Alteration type
19 NNWNN	92.66	660.12	NNWNN	4.59	-
		661.10	Deamidated	5.73	Not yet identified
44 EKMIG	99.41	576.19	EKMIG	3.83	-
76 SNLVECVFSLFKKC N	80.63	1778.20	**X**FSLFKKCN	35.18	Not yet identified
		1761.98	Not yet identified	38.31	Not yet identified
		1728.69	SNLVECVFSLFKKCN	40.59	-
		1641.10	*NLVEC$_5$VFSLFKKC$_{13}$N, disulfide bond between C$_5$ and C$_{13}$	45.59	Deletion + cyclisation
134 LPFEF	17.17	518.10	LPE(methyl)F	22.40	Deletion + functional group
		651.18	LPFEF	28.35	-
		665.22	LPFE(methyl)F	29.75	+ functional group
		709.28	LPFEF**X**	31.49	Not yet identified
		679.21	Not yet identified	33.29	Not yet identified
162 QRGMI	94.36	-	ND[4]	4.15	-
		546.07	QRMI	4.94	Deletion
		603.27	QRGMI		
		474.98	RGMI		Deletion
		-	ND[4]	5.80	-
		586.17	QR(ammonia-loss)GMI	14.82	Ammonia loss
		2143.56	QRGMRR**X**GMRGMRGMGMI	17.74	Not yet identified
153 NNWGN	99.10	546.05	NNWN	4.56	Deletion
		603.06	NNWGN	5.05	-
		604.05	NNWGGG	6.90	Deletion + insertion
25 CVFSLFKKCN	47.92[2]	985.42	FSLFKKCN	17.57	Deletion
		1084.45	VFSLFKKCN	19.53	Deletion
		1187.46	CVFSLFKKCN	21.22	-
		1086.42	C$_1$FSLFKKC$_8$N, disulfide bond between C$_1$ and C$_8$		Deletion + cyclisation
		1185.50	C$_1$VFSLFKKC$_9$N, disulfide bond between C$_1$ and C$_9$	23.47	Cyclisation
		1027.39	FKLFKKCN or acetyl-FSLFKKCN	25.26	Deletion + insertion or deletion + acetylation
		1219.36	C$_1$VFSFFKKC$_9$N, disulfide bond between C$_1$ and C$_9$	25.74	Deletion + insertion + cyclisation
		1235.41	CFFSLFKKCN	28.16	Deletion + insertion
		1057.35	C$_1$VFSLFC$_8$N, disulfide bond between C$_1$ and C$_8$		Deletion + cyclisation
		1126.56	KVFLFKKCN	29.21	Deletion + insertion
		1213.38	KVFSLFKKCN	31.52	Deletion + insertion
164 SDLPFEH	91.37	696.16	SDLPEH	4.12	Deletion
		746.10	SDLFEH	14.72	Deletion
		570.06	acetyl-PFEH	16.31	Acid precursor + deletion
		714.27	SDLPFH		Deletion
		728.95	SLPFEH		Deletion
		785.22	SD**X**LPL	16.87	Not yet identified
		470.00	SDLH		Deletions
		529.05	PFEH	17.36	Deletions
		843.24	SDLPFEH		-
		641.17	LPFEH		Deletions
		756.15	DLPFEH	18.33	Deletion
		939.30	SDLPPFEH or PSDLPFEH	26.76	Insertion
		1308.75	**X**LPFEH or LPFEH**X**	31.06	Not yet identified
53 ESRLPKILLDFLFLR KK	65.11	2115.99	ESRLPKILLDFLFLRKK	29.87	-
		1620.81	SKILLDFLFLRKK	31.11	Deletion
		1959.46	ESRLPKILLDFLFLKK	35.89	Deletion
		1430.67	**X**DFLFLRKK	38.98	Not yet identified
		2131.14	Not yet identified	39.73	Not yet identified
		1627.78	**X**LDFLFLRKK	41.53	Not yet identified
		1740.71	**X**LLDFLFLRKK	43.52	Not yet identified
138 MAGNSSNFIHKIKQI FTHR	81.90	-	ND[4]	4.11	-
		2027.64	GNSSNFIHKIKQIFTHR	21.09	Deletion
		2229.01	MAGNSSNFIHKIKQIFTHR		-
		-	ND[4]	23.01	-
		2091.64	MAGNSSNFIHKIKQIFTR	23.90	Deletion
		1834.12	MAGNSSNFIHKIKQIF		Deletion
		1307.38	HKIKQIFTHR		Deletion
		2159.99	MAGNS**X**IHKIKQIFTHR	24.53	Not yet identified
		2123.85	RNSSNFIHKIKQIFTHR	25.34	Deletion + insertion
		1995.71	MANNFIHKIKQIFTHR		Deletion

Table 2. (*continued*)

Peptide ID	Purity (%)[1]	Mr (exp.) (Da)	Impurity sequence[3]	Retention time (min)	Alteration type
121 ILSGAPCIPW	77.04	-	Not yet identified	24.73	Not yet identified
		-	Not yet identified	25.85	Not yet identified
		614.10	ISIPW or LSIPW or PCIPW	26.06	Deletion
		830.13	SGAPCIPW	27.25	Deletion
		-	GAPCIP**X**PCIPW		Not yet identified
		754.22	ILSGAPCI(dehydrated)	27.59	Deletion + loss of water
		1027.41	**X**PW		Not yet identified
		942.27	ILSGAPCPW	29.31	Deletion
		952.41	ILSGAPIPW		Deletion
		969.88	ILSGAPCIPT		Insertion
		1041.37	Deamination product	30.64	Not yet identified
		1055.46	ILSGAPCIPW		-
		927.34	ILSPCIPW	31.71	Deletion
		998.40	ILSAPCIPW		Deletion
		968.35	ILGAPCIPW	32.56	Deletion
		1161.48	ILSGAPCIPPPP	33.55	Deletion + insertion
		-	Not yet identified	35.70	Not yet identified
		1241.42	ILSGAPCIPWW	35.70 / 36.25	Insertion
125 KSSAYSLQMGATAI KQVKKLFKKWGW	66.39	2912.62	y	29.63	Not yet identified
		2984.46	KSSAYSLQMGATAIKQVKKLFKKWGW	29.73	-
		3089.70	KSSAYYSLQMATAIKQVKKLFKKWGW	32.36	Deletion + insertion
31 DLRNIFLKIKFKKK	76.74	1675.62	LRNIFLKIKFKKK	19.42	Deletion
		1790.03	DLRNIFLKIKFKKK	20.19	-
		1633.78	DLNIFLKIKFKKK	21.96	Deletion
82 NTIPKY	69.22	644.18	**X**KY	4.12	Not yet identified
		620.22	TIPKY	9.59	Deletion
		406.07	PKY	11.22	Deletion
		734.24	NTIPKY		
		897.30	NTIPKYY	15.78	Insertion
		569.09	YPKY or PKYY		Deletion + insertion
		605.87	NTIPY(hydroxylated)	16.14	Deletion + hydroxylation
		662.18	acetyl-TIPKY	18.12 / 18.47	Protection group + deletion
		1275.50	NTIPKTTIPKY or TIPKYTIPKNT or TIPKTNTIPKY	18.52	Insertion
		517.05	K(ammonia-loss)PKY	24.73	Deletion + insertion + modification
		976.34	NNK NKN KNN -TIPKY Or NNQ NQN QNN -TIPKY	32.98	Insertion
75 SINSQIGKATSNLVE CVFSLFKKCN	30.03	2421.00	Not yet identified	28.40	Not yet identified
		2743.23	Not yet identified	33.63	Not yet identified
		2256.32	SINSQIGKATSNLVECVFSLF	35.20	Deletion
		2729.40	SINSQIGKATSNLVECVFSLFKKCN	40.99	-
		2786.42	SINSQIGGKATSNLVECVFSLFKKCN	46.27	Insertion

[1]Purity assigned based on UV chromatogram with internal normalization to the most abundant peak.

[2]The peptide $C_1VFSLFKKC_9N$ (disulfide bond between C_1 and C_9) was the most abundant peak and was not the desired sequence.

[3]Structural deviations are defined as follows: (a) Chemical modifications of an amino acid are depicted in between brackets following the modified amino acid; (b) **X** signals a "semi-identified" impurity with already part of the peptide sequence allocated in the MS[2] fragmentation pattern.

[4]Solely observed in UV spectrum, no signal in TIC.

given: ILSGAPCIPPPP is a related impurity of peptide ID121 and lacks the C-terminal Trp residue, but is characterized by 3 additional Pro residues at the C-terminus.

In total, 84 impurities (Table 2) were observed and 73% could be assigned a structure related to the requested peptide by means of MS[2]. Analysis of these identified impurities revealed a high abundance of impurities with at least one deletion (*i.e.* in 74% of all identified impurities is involved) (Fig. 9). Inappropriate deletion of blocking groups during synthesis results in such sequences, thereby lacking at least 1 amino acid [20,44]. A minority of the observed peptide impurities were characterized by the incorporation of additional amino acids. During synthesis, an excess of amino acid equivalents used to assure a maximum coupling efficiency might result in the incorporation of additional amino acids [20,45]. Impurities can also be a combination of alteration types, *e.g.* one or more desired amino acid(s) might be missing at the N-terminus whilst at the C-terminus additional amino acid(s) are incorporated.
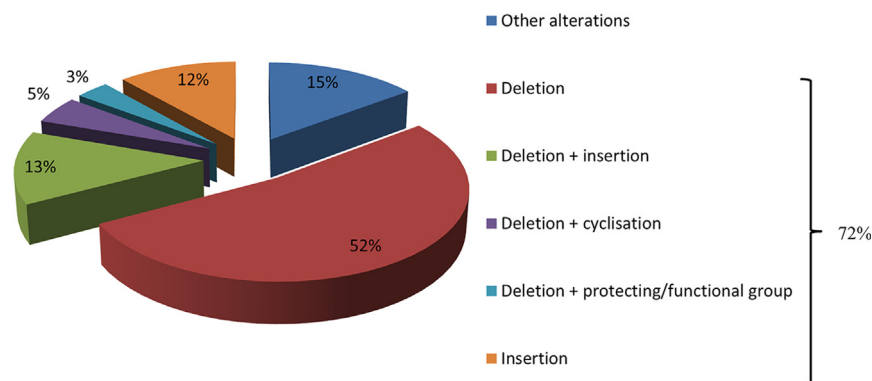
Fig. 9 Types of related peptide impurities found in the 61 experimentally determined sequences.

4. Conclusions

In conclusion, "purified" synthetic peptides still contain a considerable amount and wide variety of related impurities, with peptides missing amino acid(s) being the most prominent. As a consequence, a thorough in-house quality characterization of peptides, already used in basic biomedical research, is mandatory. Remarkably, there is a large discrepancy between the certificates of analysis provided by the suppliers and the in-house quality control, which can at least partly be explained by the methods used. Finally, researchers should also be aware that the main peak observed in the chromatogram might be an impurity instead of the requested peptide; hence MS is requested in this QC.

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