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Short Communication

Analysis of iodinated quorum sensing peptides by LC–UV/ESI ion trap mass spectrometry

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ABSTRACT

Five different quorum sensing peptides (QSP) were iodinated using different iodination techniques. These iodinated peptides were analyzed using a C_{18} reversed phase HPLC system, applying a linear gradient of water and acetonitrile containing 0.1% (m/v) formic acid as mobile phase. Electrospray ionization (ESI) ion trap mass spectrometry was used for the identification of the modified peptides, while semi-quantification was performed using total ion current (TIC) spectra. Non-iodinated peptides and mono- and di-iodinated peptides (NIP, MIP and DIP respectively) were well separated and eluted in that order. Depending on the used iodination method, iodination yields varied from low (2%) to high (57%).

1. Introduction

Quorum sensing peptides (QSP) are auto-inducing peptides produced by gram-positive bacteria and are used in bacterial communication [1]. These peptides are secreted as large pro-peptides by ATP-binding cassette transporters, extracellularly hydrolyzed and uptaken through permeases directly interacting with cytoplasmic receptors such as the RNPP proteins and/or sensed by membrane located receptors. Binding of the QSP to its membrane receptor causes auto-phosphorylation on a histidine residue and subsequent transfer of a phosphoryl group to a response regulator in the cytoplasm. Following phosphorylation, this response regulator will activate or deactivate transcription of target genes [2,3]. Recently, it has been demonstrated that some of these QSP are also able to influence human host cells, promoting invasion and angiogenesis of breast and colon cancer cells, thereby potentially stimulating tumor metastasis [4,5]. Radiolabeling of QSP is a convenient way to study in vitro ligand interactions as well as in vivo pharmacokinetics. Iodination of QSP with ^{125}I has already been performed to investigate the blood-brain barrier influx properties of some selected QSP; however, analytical quality information is lacking [6]. Moreover, Verbeke et al. [7] give an overview of the currently used detection methods for unmodified QSP, while their chromatographic analysis is detailed by Debunne et al. [8].

Radio-iodination (i.e. incorporation of radioactive iodine such as ^{123}I, ^{125}I or ^{131}I) is a technique commonly used for radioligand investigations, medical imaging and therapy [9,10]. Several direct and indirect iodination methods for peptides currently exist. Direct labelling is based on the iodination of tyrosine and histidine residues using Chloramine-T (CAT) [11], lactoperoxidase [12] and the Iodo-Gen® method [13]. Indirect labelling is performed by conjugation of a small radio-iodinated molecule such as the Bolton-Hunter reagent (*N*-hydroxysuccinimide ester of 3-(4-hydroxyphenyl)propionic acid) [14]. This method can be used in case of absence of tyrosine and histidine residues [15]. Iodination with ^{125}I is the method of choice if peptides are to be radiolabeled due to the high specific radioactivity and ease-of-use in counting gamma-radiation. Moreover, non-radioactive iodination is also used in the elucidation of structures of peptides and related products like foldamers [16]. While iodination is generally considered as a minimal structural modification of the peptide, it may well induce functional differences [17,18] as a consequence of the various noncovalent intermolecular interactions involving iodine atoms [19]. However, the iodination reaction can result in a mixture of peptides: unmodified as well as mono- and multiple-iodinated species, as tyrosine and histidine amino acids each can give rise to 3- and 5-diiodotyrosine, and 2- and 4-diiodohistidines, respectively [20].

Analytical characterization of iodinated peptides has been scarcely reported. Vergote et al. compared the iodination of obestatin using the lactoperoxidase, Iodo-Gen® and chloramine-T methods [21]. A

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comparison of different iodination procedures using a variety of peptides has also been made by this group [20]. Loot et al. reported the HPLC analysis of iodinated angiotensin-(1-7) using the Chloramine-T method [18]. The analysis of iodinated salmon calcitonin using reversed phase HPLC has been reported by Lee et al. [22]. De Blois et al. [23] give a clear overview of iodinated somatostatin analogues. However, the analytical characterization of iodinated QSP has not yet been reported. In this study, five QSP were iodinated using different techniques and their analytical characterization was investigated. These five peptides are situated in three different clusters (clusters 2A, 2B and 3A) of the quorum sensing peptide chemical space [24] and are involved in mediating cell death (Q19) [25], plasmid transfer (Q132, Q184) [26,27], expressing virulence factors (Q164) [28] and inhibition of Rap phosphatases (Q206) [29]. Q19 and Q164 bind a membrane associated receptor while Q132, Q184 and Q206 are internalized and bind to a cytoplasmic receptor.

2. Materials and methods

2.1. Reagents and peptides

Bolton-Hunter reagent (N-Succinimidyl 3-(4-hydroxyphenyl)propionate) and disodium hydrogen phosphate dihydrate were purchased from VWR (Oud-Heverlee, Belgium). Pre-coated Iodo-Gen® tubes were purchased from Thermo Scientific (Erembodegem, Belgium). ULC/MS grade acetonitrile and LC–MS grade formic acid were purchased from Biosolve (Valkenswaard, The Netherlands). Chloramine-T (N-Chloro-p-toluenesulfonamide sodium salt hydrate) and DMSO were purchased from Sigma-Aldrich (Diegem, Belgium). All other reagents were purchased from Merck (Darmstadt, Germany). Water (18.2 MΩ/cm) was purified in the lab by a Sartorius arium® pro purification system (Goettingen, Germany). All peptides were purchased from GL Biochem (Shanghai, China) (Table 1). The peptide numbering used in the Quorumpeps® database is retained in this study [30].

2.2. Peptide iodination using Bolton-Hunter

To 10 μL of sodium iodide (4.5 mg/mL), 15 μL of Bolton-Hunter reagent (0.5 mg/mL), 15 μL of sodium hydroxide (0.1 M) and 15 μL of chloramine-T (4 mg/mL) were added. After 60 s, 15 μL of sodiummetabisulphite (8 mg/mL) was added to stop the reaction. Finally, 50 μL of the peptide solution (1 mM in 25 mM sodium phosphate buffer pH 7.4) was added to the mixture and incubated overnight at room temperature.

2.3. Peptide iodination using Iodo-Gen®

50 μL of sodium iodide (1.1 mM) and 15 μL of 0.1 M sodium hydroxide were added to a rinsed pre-coated Iodo-Gen® tube. This mixture was incubated for 6 min at room temperature. The reaction mixture was added to 50 μL of peptide solution (1 mM in 25 mM sodium phosphate buffer pH 7.4) and further incubated for 6 min at room temperature.

Table 1
Quorum sensing peptides under investigation.

Quorumpeps ID[a]	Sequence	Molecular weight M_r (Da)[b]
Q19	NNWNN	660.66
Q132	LFSLVLAG	819.01
Q164	SDLPFEH	843.89
Q184	SIFTLVA	749.90
Q206	SYPGWSW	881.94

[a] Identification number in Quorumpeps database [30].
[b] Molecular weight calculated using the abridged atomic weights [31].

2.4. Peptide iodination using Chloramine-T (CAT)

This method was based on the method described by Wynendaele et al. [6]. Briefly, to 50 μL of 1 mM peptide solution, 20 μL of 4.5 mg/mL NaI in 100 mM sodium phosphate buffer, 30 μL of 4 mg/mL chloramine-T in 100 mM phosphate buffer and 17 μL of a 0.1 M sodium hydroxide solution were added. After 40 s incubation, the reaction was stopped by adding 30 μL of sodiummetabisulphite solution (8 mg/mL).

2.5. LC–MS instrumentation and conditions

The HPLC–UV/MS apparatus consisted of a Spectra System SN4000 interface, a Spectra System SCM1000 degasser, a Spectra System P1000XR pump, a Spectra System AS3000 auto sampler and a Finnigan LCQ Classic ion trap mass spectrometer in positive ion mode (Thermo, San José, CA, USA) equipped with a Waters 2487 dual wavelength UV detector (Waters, Milford, MA, USA) and XCalibur 2.0 software (Thermo, San José, CA, USA) for data acquisition. The dwell volume of this setup was 1.7 mL.

Different HPLC methods were optimized for the different peptides to obtain the shortest run time as possible. The first method used an XBridge™ BEH300 C_{18} (250 mm × 4.6 mm, 5 μm) stationary phase (Waters, Milford, MA, USA). Column temperature was maintained at 45 °C. The mobile phase consisted of (A) 95/5% (v/v) H_2O/acetonitrile supplemented with 0.1% (m/v) formic acid and (B) 5/95% (v/v) H_2O/acetonitrile supplemented with 0.1% (m/v) formic acid. Elution was performed as described in Table 2 using a flow rate of 1 mL/min.

The second and third methods used a Vydac Everest C_{18} column (250 mm × 4.6 mm i.d., 5 μm) (Grace, Columbia, MD, USA). Column temperature was maintained at 30 °C. The mobile phases consisted of H_2O and acetonitrile supplemented with 0.1% (m/v) formic acid. Elution was performed as described in Table 2 using a flow rate of 1 mL/min. Detection was performed in all these methods using UV at 210 nm and ESI-MS set in positive mode (*m/z*: 100–2000). The ESI-MS used a spray voltage of 4.5 kV and a capillary temperature of 250 °C. N_2 was used as sheath and auxiliary gas with flow rates of respectively 80 and 20 mL/min. Peptide solutions resulting from the iodination were directly injected on the LC–MS apparatus without prior purification.

3. Results and discussion

3.1. MS spectral data

The experimental MS spectra obtained with the peptides and the iodinated derivatives corresponded to their respective molecular structures (Table 3). The addition of a Bolton-Hunter molecule caused a *m/z* shift in $[M + H]^+$ of + 148 Da, while addition of a iodine-labeled Bolton-Hunter molecule caused a *m/z* shift of +274 Da. Using the Iodo-Gen® method and CAT method, a shift of + 126 Da was observed from non-iodinated peptide (NIP) to mono-iodinated peptide (MIP) and a further shift of + 126 Da to di-iodinated peptide (DIP). The chemistry of

Table 2
Gradient program of HPLC methods.

HPLC method	Run time (min)	Gradient program
1	45	5% (5 min) → 58% ACN (30 min)
2	45	2% (5 min) → 50% ACN (30 min)
3	30	15% (5 min) → 45% ACN (20 min)

Table 3
Experimental MS spectral data obtained on iodinated QSP vs expected theoretical data.

Peptide	Experimental vs theoretical *m/z* value ($[M + H]^+$)					
	NIP	NIP-BH[a]	BH(I)[b]	BH(2I)[c]	MIP	DIP
Q19	661.26 vs 661.67	809.12 vs 809.82	934.99 vs 935.71	*NA*	*NA*	*NA*
Q132	819.31 vs 820.02	989.52 vs 990.15	1115.39 vs 1116.04	*NA*	*NA*	*NA*
Q164	844.27 vs 844.90	992.39 vs 993.05	1118.21 vs 1118.94	*NA*	*NA*	*NA*
	844.29 vs 844.90	*NA*	*NA*	*NA*	970.10 vs 970.79	1095.83 vs 1096.68
Q184	750.24 vs 750.91	920.43 vs 921.04	1046.34 vs 1046.93	1172.22 vs 1172.82	*NA*	*NA*
Q206	882.27 vs 882.95	*NA*	*NA*	*NA*	1008.17 vs 1008.84	1134.01 vs 1134.73

NA = Not applicable.
[a] Conjugation of Bolton-Hunter reagent.
[b] Conjugation of iodine labeled Bolton-Hunter reagent.
[c] Conjugation of two iodine atoms coupled to a Bolton-Hunter reagent.

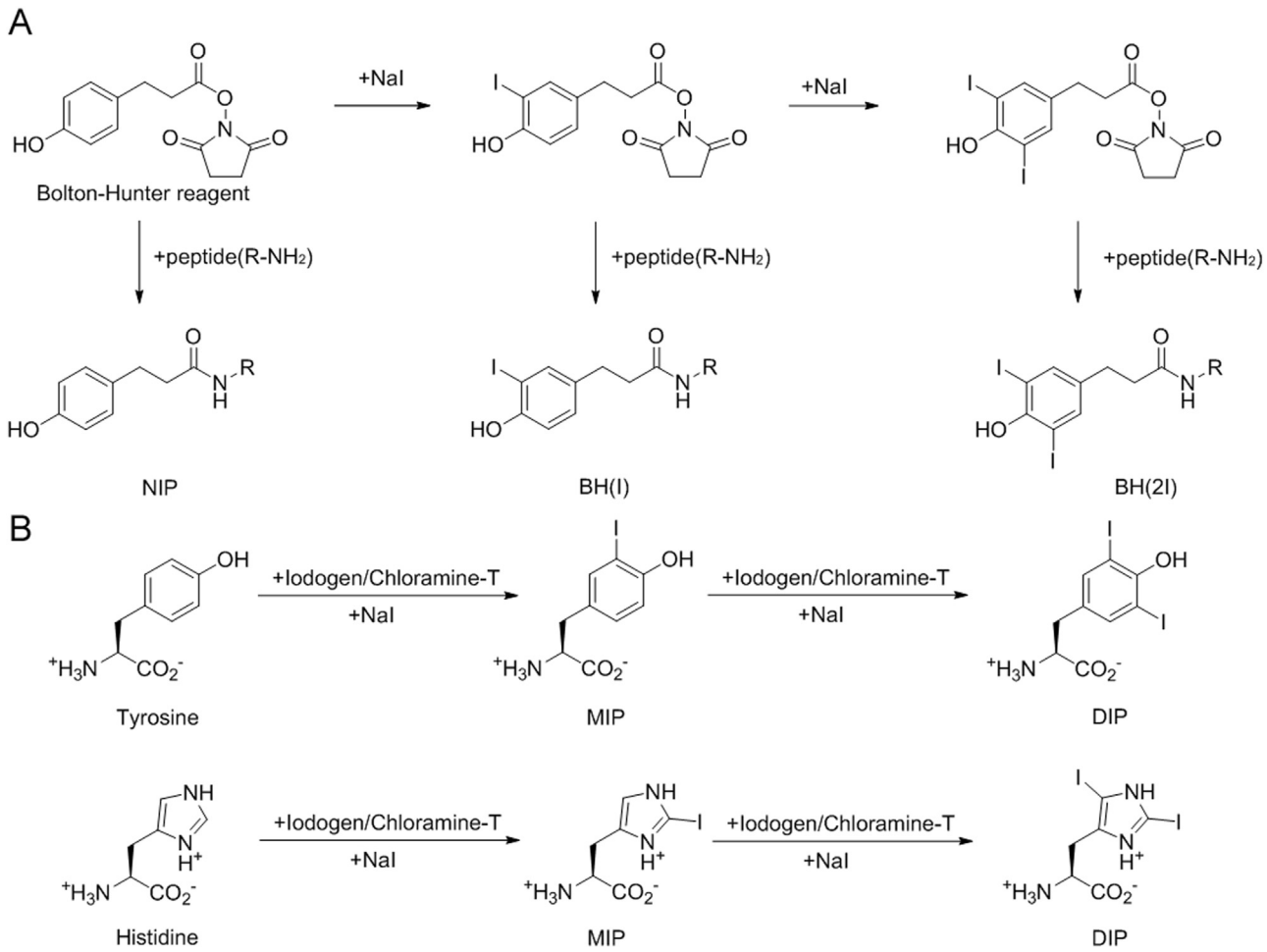

Fig. 1. (A) Iodination using the Bolton-Hunter method. The Bolton-Hunter reagent was iodinated using NaI, the iodinated Bolton-Hunter molecule reacted with the amino-terminus of the peptides. (B) Iodination using the Iodo-Gen®- or Chloramine-T method. Peptides were iodinated on the Tyrosine (Q206) and or Histidine (Q164) residues.

these iodination reactions is displayed in Fig. 1. A typical MS spectrum for the MIP of Q164 using the Iodo-Gen® method, together with its corresponding MS^2 spectrum is shown in Fig. 2. Peaks were observed at *m/z* values 970.10 Da and 485.73 Da, attributed to the $[M + H]^+$ and $[M + 2H]^{2+}$ ions, respectively. MS spectra of the other peptides are given in the Supplementary material.

3.2. Chromatography

Due to the addition of Bolton-Hunter reagent and iodine molecules, lipophilicity of the peptides increased and an increase in retention time of these iodinated peptides was expected. An overview of the retention time of iodinated peptides is given in Table 4. All peptides could be analyzed using standard HPLC

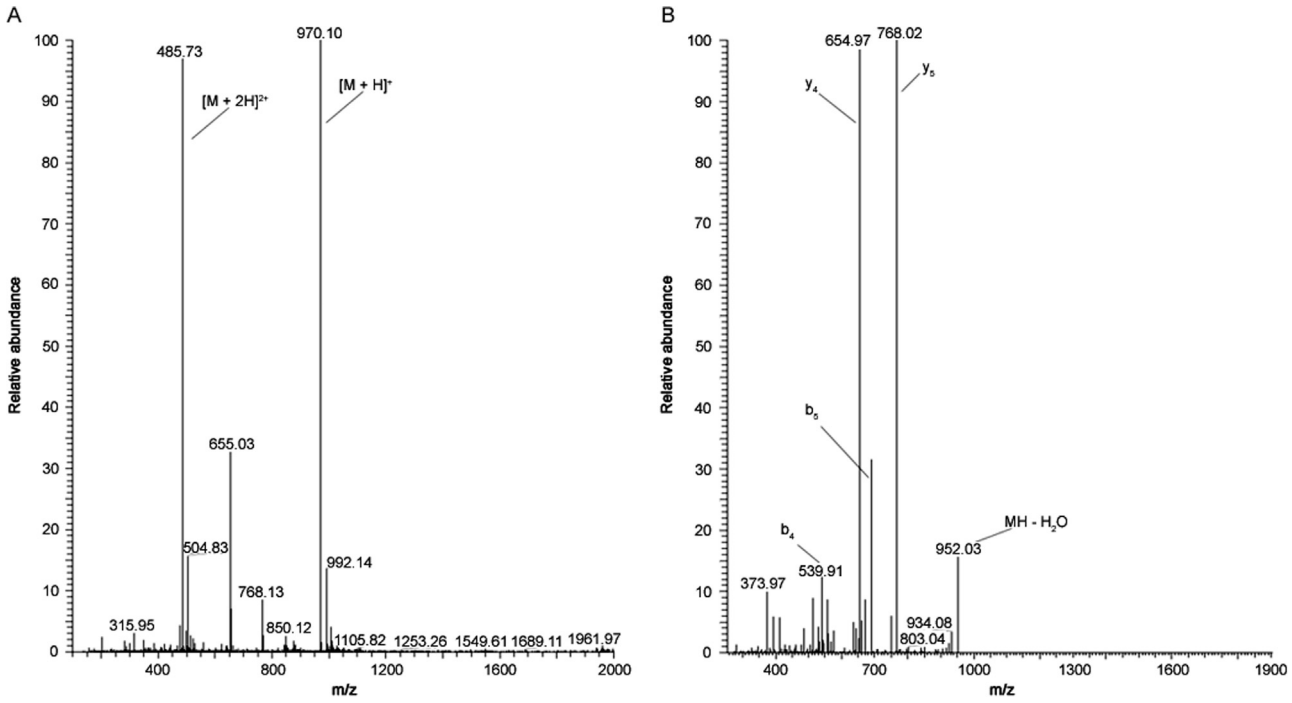

Fig. 2. (A) LC-MS spectrum of mono-iodinated Q164 using the Iodo-Gen® method and (B) MS^2 spectrum of mono-iodinated Q164 with $[M + H]^+$ as parent-ion.

Table 4
LC data on iodinated QSP.

Peptide	HPLC method	Iodination method	Retention time (min)					
			NIP	NIP-BH	BH(I)	BH(2I)	MIP	DIP
Q19	2	Bolton-Hunter	11.05	17.74	20.73	*NA*	*NA*	*NA*
Q132	1	Bolton-Hunter	24.94	32.60	34.82	*NA*	*NA*	*NA*
Q164	1	Bolton-Hunter	17.87	21.88	24.45	*NA*	*NA*	*NA*
	1	Iodo-Gen®	17.93	*NA*	*NA*	*NA*	18.91	22.37
Q184	1	Bolton-Hunter	24.27	29.52	31.96	34.28	*NA*	*NA*
Q206	3	CAT	15.26	*NA*	*NA*	*NA*	16.84	17.78

NA = Not applicable.

methods with acetonitrile, water and 0.1% (m/v) formic acid as mobile phase constituent. A relationship between the ratio of the percentage acetonitrile at the retention time of MIP vs NIP and DIP vs MIP and the molecular mass of the iodinated peptide divided by the number of charges in acidic medium has been observed by Vergote et al. [20]. An example of the UV chromatogram together with a total ion current (TIC) chromatogram of iodinated Q164 using the Iodo-Gen® method is given in Fig. 3. Chromatograms of the other peptides are given in the Supplementary material.

3.3. Iodination yields

Due to the lack of tyrosine and histidine residues, the peptides Q19, Q132 and Q184 were iodinated using the Bolton-Hunter method [14]. Q164 and Q206 contain a histidine or tyrosine residue and were iodinated using a direct method. The iodination yields are given in Table 5. Yields are calculated by comparing the peak area of the iodinated peptide to the total peak area of the peptides in the TIC spectrum. Iodination of Q19 using Bolton-Hunter resulted in an iodination yield of around 2.4%. For Q132, a low iodination yield of around 6% was obtained. For Q164, two iodination methods could be used. Using the Iodo-Gen® method, an iodination yield of ± 20% could be obtained compared to only ± 2% using the Bolton-Hunter method. For Q184 a total iodination yield of around 5% was obtained. The Chloramine-T method for Q206 resulted in a high iodination yield of ± 57%. These iodination yields using Bolton-Hunter are rather low as literature shows that yields up to 40% can be achieved using Bolton-Hunter [32].

In most pharmacokinetic studies using iodine labelling, no isolation of mono-iodinated peptides is performed. A mixture of non-iodinated, mono-iodinated and multiple-iodinated peptides is used [6,33,34]. When saturable transport mechanisms are used, the presence of non-labeled peptide will underestimate the transport properties of the peptide as less radio-labeled peptide is transported due to competition of the NIP transport [35]. The observed effect can also be the result of multiple-iodinated peptides. These peptides underwent the biggest changes as iodination with multiple ^{125}I atoms increases the peptide size and hydrophobicity and can also affect the secondary structure of the peptide, thereby also changing the physico-chemical as well as the pharmacokinetic and pharmacodynamic properties [36,37]. Furthermore, radiolabeling of peptides can influence the biological properties such as receptor affinity, biodistribution, internalization and cell dissociation [15]. For most biomedical and pharmaceutical applications, single iodinated peptides are desirable as they are expected to functionally behave closest to the unmodified peptide compared to the multiple-iodinated species. Therefore, it is advantageous to isolate the mono-iodinated peptide for biomedical studies of these QSP.

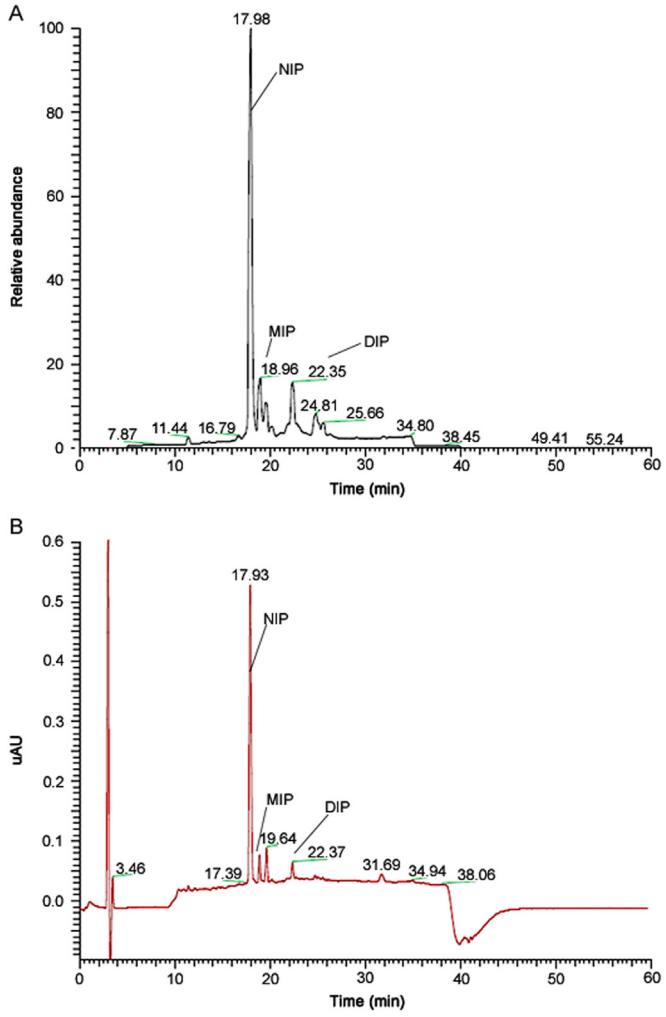

Fig. 3. (A) TIC chromatogram of Q164 using the Iodo-Gen® method and (B) UV chromatogram of Q164. Peaks at 17.93, 18.91 and 22.37 min were identified as NIP, MIP and DIP respectively.

Table 5
Iodination yields of quorum sensing peptides.

Peptide	Amount (nmol)	Iodination method	Area % (TIC)					
			NIP	NIP-BH	BH(I)	BH(2I)	MIP	DIP
Q19	5	Bolton-Hunter	75.9	21.7	2.4	*NA*	*NA*	*NA*
Q132	5	Bolton-Hunter	86.9	7.3	5.8	*NA*	*NA*	*NA*
Q164	5	Bolton-Hunter	82.6	15.6	1.8	*NA*	*NA*	*NA*
		Iodo-Gen®	80.7	*NA*	*NA*	*NA*	9.6	9.6
Q184	5	Bolton-Hunter	95.0	0.2	2.2	2.6	*NA*	*NA*
Q206	5	CAT	42.6	*NA*	*NA*	*NA*	34.7	22.7

NA = Not applicable.

4. Conclusions

Iodinated quorum sensing peptides were separated by HPLC using a C_{18} column with water-acetonitrile mobile phase gradients and 0.1% (m/v) formic acid. Iodinated peptides were identified using mass spectrometry and quantitatively estimated using normalization of the peak areas from the total ion current chromatograms. Iodination yields were variable, ranging from 2% to 57% depending on the used iodination method.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpha.2017.09.001.

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