**Supplementary information 2:**

A screening bio-analytical method for *Enterococcus faecalis* RNPP-type quorum sensing peptides in murine faeces.

# Methods, results and discussion Method development and method fine-tuning

In following sections, the step-by-step method development approach and method fine-tuning is provided. Per experiment, the method and results are discussed. The materials are provided in the main article. Samples were analysed in uniplicate, unless otherwise specified.

Here the numbers as provided in the Quorumpeps® database are used (see **Table 1** main text) instead of the trivial name for reasons of clarity. During method development and optimisation the concerned quorum sensing peptides were used at different concentration levels and are referred to as ‘xDev-Conc’. In **Supplementary information Table 1**, the concentration level ‘1xDev-Conc’ is provided. During the method development and optimisation a multitude of this concentration is used, thus ‘100xDec-Conc’ implies that the concentrations mentioned in **Supplementary information Table 1** must be multiplied by 100 to obtain the concentration of the concerned peptides as specified for that specific method development experiment.

**Supplementary information Table 1: RNPP *E. faecalis* quorum sensing peptides with trivial name, amino acid, peptide ID according to the Quorumpeps® database [7], and the ‘1xDev-Conc’, multiplications of this peptide concentration were used during method development and optimization.**

|  |  |  |  |
| --- | --- | --- | --- |
| **Trivial name** | **One-letter amino acid sequence** | **Peptide ID according to Quorumpeps® [7]****database** | **Peptide concentration ‘1xDev-Conc’ (nM)** |
| cAM373 | AIFILAS | Q13 | 0.1 |
| iCF10 | AITLIFI | Q14 | 0.2 |
| iPD1 | ALILTLVS | Q17 | 0.2 |
| cPD1 | FLVMFLSG | Q93 | 5.0 |
| cAD1 | LFSLVLAG | Q132 | 0.5 |
| iAD1 | LFVVTLVG | Q133 | 0.5 |
| cCF10 | LVTLVFV | Q137 | 0.2 |
| iAM373 | SIFTLVA | Q184 | 1.0 |
| cOB1 | VAVLVLGA | Q210 | 0.2 |

## Method development

The method development was divided in different sections before obtaining the final method. First the extraction solvent was determined *in silico*, followed by examining the extraction and the possibility of post-SPE sample concentration enhancement via evaporation and reconstitution. Finally, sample preparation via SPE was explored.

### Extraction solvent composition

#### Method

The initial extraction solvent (*i.e.* 10% (V/V) formic acid in water or 10% (V/V) ammonium hydroxide in water) was determined based on the isoelectric point values of the peptides. The extraction solvent was determined by the solvent that yields the largest difference in units between the pH of 10% (V/V) ammonium hydroxide (11.7; experimentally determined) and the peptide with the highest isoelectric point versus the pH of 10% (V/V) formic acid in water (1.75; experimentally determined) and the peptide with the lowest isoelectric point.

#### Results and discussion

The isoelectric point values of the considered peptides (obtained from the Quorumpeps® database) and the calculations are given in **Supplementary information Table 2**.

**Supplementary information Table 2: Iso-electric points of peptides of interest.**

|  |  |  |
| --- | --- | --- |
| **Peptide ID** | **Sequence** | **Isoelectric point** |
| Q13 | AIFILAS | 5.85 |
| Q14 | AITLIFI | 5.98 |
| Q17 | ALILTLVS | 5.92 |
| Q93 | FLVMFLSG | 5.64 |
| Q210 | VAVLVLGA | 5.94 |
| Q132 | LFSLVLAG | 5.93 |
| Q133 | LFVVTLVG | 5.99 |
| Q137 | LVTLVFV | 5.98 |
| Q184 | SIFTLVA | 5.71 |
| **Mean** | 5.88 |
| **Min** | 5.64 |
| **Max** | 5.99 |
| **Delta pH 10% formic acid in water and Min isolectric point** | 3.89 |
| **Delta pH 10% ammonium hydroxide in water and Max isolectric point** | 5.71 |

Hence, based on the data in **Supplementary information Table 2**, 10% ammonium hydroxide V/V in water was selected for the sample extraction of the consecutive method development.

### Sample extraction

#### Methods

The intended extraction of murine faeces was first verified regarding recovery. Instead of using murine faeces, 1% (m/V) bovine serum albumin in water served as the model and recoveries were calculated between a pre- and postspiked sample according to following formula:

$$Recovery \left(\%\right)=\frac{Area prespiked sample}{Area postspiked sample }x 100$$

The followed experimental procedure consisted of a blank sample (*i.e.* 1% (m/V) bovine serum albumin in water), 1% (m/V) bovine serum albumin in water spiked to a specific concentration of peptides (*i.e.* prespiked sample), and a postspiked sample (1% (m/V) bovine serum albumin in water spiked to the same concentration as the prespiked sample after the extraction procedure). To all 3 samples, ammonium hydroxide was added to obtain a solution of 10% (V/V) ammonium hydroxide in water. Consecutively, the samples were shaken on a thermoshaker (Thermomixer comfort, Eppendorf, Hamburg, Germany) at 95°C and 1400 rpm during 5 min, followed by cooling the samples for 5 min on ice. The samples were homogenized during 1 min at intensity 6. Next, the samples were shaken again at 95°C during 5 min at 1400 rpm. Finally, the samples were centrifuged (Centrifuge 5417R, Eppendorf, Hamburg, Germany) for 15 min at 20000 *g* and 20°C. Following centrifugation, the postspiked sample was spiked with peptides to the same nominal concentration as the prespiked sample before being subjected to UHPLC-MS/MS analysis.

The analytical concentration of the peptides was 10xDev-Conc (see **Supplementary information Table 1**).

#### Results and discussion

The results are provided in **Supplementary information Table 3**.

**Supplementary information Table 3: Recoveries regarding pre-SPE extraction in 10% V/V ammonium hydroxide in water. The analytical concentration of the peptides was 10xDev-Conc (see Supplementary information Table 1).**

|  |  |  |
| --- | --- | --- |
| **Peptide** | **Sequence** | **Recovery (%)** |
| Q13 | AIFILAS | 81.7 |
| Q14 | AITLIFI | 71.4 |
| Q17 | ALILTLVS | 135.1 |
| Q93 | FLVMFLSG | 131.8 |
| Q210 | VAVLVLGA | 109.2 |
| Q132 | LFSLVLAG | 88.6 |
| Q133 | LFVVTLVG | 73.8 |
| Q137 | LVTLVFV | 99.9 |
| Q184 | SIFTLVA | 72.8 |

Based on these results, the stability of the quorum sensing peptides in this extraction medium was determined sufficient and the method development was continued with 10% V/V ammonium hydroxide in water.

### Sample concentration enhancement

#### Methods

To investigate the possibility to enhance the concentration of the samples following SPE, quorum sensing peptides in 21/79 V/V acetonitrile/water were dried during 45 min at 95°C and 750 rpm in a thermoshaker. The dried samples were resuspended in 50 µl 21/79 V/V acetonitrile/water and diluted ½ in the insert with the same solvent. The postspiked sample consisted of quorum sensing peptides at the same nominal concentration. The recovery was calculated as previously described. The analytical concentration of the peptides was 30xDev-Conc (see **Supplementary information Table 1**).

#### Results and discussion

The results of the sample drying and reconstitution are given in **Supplementary information Table 4**.

**Supplementary information Table 4: Sample concentration enhancement via sample drying and consecutive reconstitution. The analytical concentration of the peptides was 30xDev-Conc (see Supplementary information Table 1).**

|  |  |  |
| --- | --- | --- |
| **Peptide** | **Sequence** | **recovery (%)** |
| Q13 | AIFILAS | 26.4 |
| Q14 | AITLIFI | 7.5 |
| Q17 | ALILTLVS | 15.8 |
| Q93 | FLVMFLSG | 25.0 |
| Q210 | VAVLVLGA | 24.0 |
| Q132 | LFSLVLAG | 25.7 |
| Q133 | LFVVTLVG | 24.0 |
| Q137 | LVTLVFV | 2.8 |
| Q184 | SIFTLVA | 25.7 |

Drying the samples under the specified conditions does not seem to be suitable to enhance the analyte concentration. It was therefore decided not to enhance the post-SPE sample concentration by drying the eluates.

### C18 SPE sample preparation: pilot experiment

#### Methods

A pilot experiment with 10% ammonium hydroxide V/V in water spiked with peptides to 100xDev-Conc was first conducted to obtain an estimation of the highest acetonitrile composition that can be applied to wash the SPE column and the minimal acetonitrile composition necessary to elute the peptides of interest. The protocol is provided in **Supplementary information Table 5**. A blank, prespiked, postspiked sample were processed.

**Supplementary information Table 5: Optimization experiment C18 SPE.**

|  |  |
| --- | --- |
| **Steps** | **C18 SPE procedure**  |
| 1 | **Condition** | 5 ml acetonitrile at 1 drop/sec |
| 2 | **Equilibration** | 5 ml water at 1 drop/sec |
| 3 | **Loading** | 1 ml sample at 1 drop/3 sec |
| 4 | **Wash 1** | 5 ml water at 1 drop/sec |
| 5 | **Elution 1** | 1 ml water at 1 drop/ 3 sec |
| 6 | **Elution 2** | 1 ml 10/90 V/V water/acetonitrile at 1 drop/ 3 sec |
| 7 | **Elution 3** | 1 ml 20/80 V/V water/acetonitrile at 1 drop/ 3 sec |
| 8 | **Elution 4** | 1 ml 30/70 V/V water/acetonitrile at 1 drop/ 3 sec |
| 9 | **Elution 5** | 1 ml 40/60 V/V water/acetonitrile at 1 drop/ 3 sec |
| 10 | **Elution 6** | 1 ml 50/50 V/V water/acetonitrile at 1 drop/ 3 sec |
| 11 | **Elution 7** | 1 ml 60/40 V/V water/acetonitrile at 1 drop/ 3 sec |
| 12 | **Elution 8** | 1 ml 70/30 V/V water/acetonitrile at 1 drop/ 3 sec |
| 13 | **Elution 9** | 1 ml 80/20 V/V water/acetonitrile at 1 drop/ 3 sec |
| 14 | **Elution 10** | 1 ml 90/10 V/V water/acetonitrile at 1 drop/ 3 sec |
| 15 | **Elution 11** | 1 ml acetonitrile at 1 drop/ 3 sec |

The elution fractions were injected as such on the UHPLC-MS/MS device and the recoveries were calculated compared to one sample containing peptides at the same nominal concentration in 10% ammonium hydroxide V/V in water, *i.e.* an analytical peptide concentration of 100xDev-Conc.

#### Results and discussion

An estimation of the highest acetonitrile composition that can be applied to wash the SPE column and the minimal acetonitrile composition necessary to elute the peptides of interest was investigated. The recoveries are provided in **Supplementary information Table 6**.

**Supplementary information Table 6: Recoveries optimization experiment C18 SPE. The selected wash (10/90 V/V acetonitrile/water) and elution step (50/50 V/V water/acetonitrile) are indicated in purple. The analytical concentration of the peptides was 100xDev-Conc (see Table 1).**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **C18 SPE procedure** | **Recoveries (%)1** | **Mean** | **Median** | **Standard deviation** |
| **Q13** | **Q14** | **Q132** | **Q137** | **Q17** | **Q133** | **Q210** | **Q184** | **Q93** |
| 5 ml acetonitrile at 1 drop/sec | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 5 ml water at 1 drop/sec | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 1 ml sample at 1 drop/3 sec | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 5 ml water at 1 drop/sec | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 1 ml water at 1 drop/ 3 sec | 0.38 | 0.26 | 0.67 | 0.38 | 0.70 | ND | ND | ND | 0.17 | 0.26 | 0.26 | 0.27 |
| 1 ml 10/90 V/V water/acetonitrile at 1 drop/ 3 sec | 0.20 | ND | 0.22 | 0.12 | ND | 0.08 | 0.41 | 0.46 | 0.11 | 0.12 | 0.12 | 0.16 |
| 1 ml 20/80 V/V water/acetonitrile at 1 drop/ 3 sec | 15.30 | 2.60 | 3.29 | 3.51 | 3.13 | 2.27 | 25.64 | 21.54 | 0.54 | 3.29 | 3.29 | 9.54 |
| 1 ml 30/70 V/V water/acetonitrile at 1 drop/ 3 sec | 48.40 | 40.06 | 37.19 | 42.76 | 34.22 | 46.09 | 56.45 | 51.67 | 30.29 | 42.76 | 42.76 | 8.49 |
| 1 ml 40/60 V/V water/acetonitrile at 1 drop/ 3 sec | 57.97 | 45.41 | 49.17 | 50.68 | 52.98 | 60.22 | 70.91 | 65.79 | 56.46 | 56.46 | 56.46 | 8.16 |
| 1 ml 50/50 V/V water/acetonitrile at 1 drop/ 3 sec | 67.74 | 58.10 | 68.32 | 68.32 | 68.45 | 72.76 | 88.67 | 78.19 | 68.48 | 68.45 | 68.45 | 8.45 |
| 1 ml 60/40 V/V water/acetonitrile at 1 drop/ 3 sec | 75.40 | 63.90 | 64.07 | 68.94 | 66.88 | 75.23 | 85.70 | 84.45 | 64.01 | 68.94 | 68.94 | 8.61 |
| 1 ml 70/30 V/V water/acetonitrile at 1 drop/ 3 sec | 73.77 | 52.26 | 56.42 | 64.50 | 61.34 | 69.16 | 70.91 | 72.66 | 75.76 | 69.16 | 69.16 | 8.20 |
| 1 ml 80/20 V/V water/acetonitrile at 1 drop/ 3 sec | 72.64 | 43.81 | 75.98 | 65.49 | 70.93 | 75.36 | 85.25 | 78.11 | 72.57 | 72.64 | 72.64 | 11.57 |
| 1 ml 90/10 V/V water/acetonitrile at 1 drop/ 3 sec | 76.83 | 52.26 | 74.27 | 75.11 | 69.42 | 80.03 | 86.61 | 75.24 | 73.06 | 75.11 | 75.11 | 9.35 |
| 1 ml acetonitrile at 1 drop/ 3 sec | 33.42 | 32.96 | 35.37 | 36.52 | 32.66 | 36.95 | 40.53 | 36.60 | 31.73 | 35.37 | 35.37 | 2.78 |

1: ND=not determinable; signal-to-noise below 10.

10/90 V/V acetonitrile/water was selected as wash step 2 and 50/50 V/V water acetonitrile as the elution step.

### C18 SPE sample preparation: elution and wash solvent confirmation

#### Methods

The selection of wash step 2 and the elution condition was confirmed by repeating the experiment with the aforementioned conditions (see **Supplementary information Table 7**). The peptides were spiked to a concentration of 100xDev-Conc (see **Supplementary information Table 1**).

**Supplementary information Table 7: Confirmation optimization experiment C18 SPE.**

|  |  |
| --- | --- |
| **Steps** | **C18 SPE procedure**  |
| 1 | **Condition** | 5 ml acetonitrile at 1 drop/sec |
| 2 | **Equilibration** | 5 ml water at 1 drop/sec |
| 3 | **Loading** | 1 ml sample at 1 drop/3 sec |
| 4 | **Wash 1** | 5 ml water at 1 drop/sec |
| 5 | **Wash 2** | 5 ml 10/90 V/V water/acetonitrile at 1 drop/sec |
| 6 | **Elution**  | 1 ml 50/50 V/V water/acetonitrile at 1 drop/ 3 sec |

#### Results and discussion

The wash and elution conditions were confirmed for the majority of the peptides (see **Supplementary information Table 8**).

**Supplementary information Table 8: Confirmation recoveries optimization experiment C18 SPE of selected wash and elution solvent composition. Analytical peptide concentration postspiked sample at 100xDev-Conc and prespiked sample to 100xDev-Conc.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **C18 SPE procedure** | **Recoveries (%)1** | **Mean** | **Median** | **Standard deviation** |
| **Q13** | **Q14** | **Q132** | **Q137** | **Q17** | **Q133** | **Q210** | **Q184** | **Q93** |
| 5 ml acetonitrile at 1 drop/sec | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 5 ml water at 1 drop/sec | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 1 ml sample at 1 drop/3 sec | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 5 ml water at 1 drop/sec | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 5 ml 10/90 V/V water/acetonitrile at 1 drop/sec | 15.97 | ND | 3.27 | 2.04 | 0.82 | 0.86 | 66.05 | 46.89 | ND | 15.10 | 2.04 | 24.44 |
| 1 ml 50/50 V/V water/acetonitrile at 1 drop/ 3 sec | 88.30 | 105.47 | 77.01 | 182.93 | 79.33 | 98.81 | 52.32 | 55.89 | 104.16 | 93.80 | 88.30 | 38.56 |

1: ND=not determinable; signal-to-noise below 10.

### C18 SPE sample preparation: wash solvent adjustment

#### Methods

The selected wash step and elution condition proved suitable to continue SPE method development for the majority of peptides. However, Q210 and Q184 were confronted with a considerable loss during the wash step. Therefore the experiment from **Supplementary information Table 7** was repeated with wash step 2 consisting of 95/5 V/V water/acetonitrile (see **Supplementary information Table 9**). The peptides were spiked to a concentration of 100xDev-Conc (see **Supplementary information Table 1**).

**Supplementary information Table 9: Adjustment wash step 2 confirmation optimization experiment C18 SPE.**

|  |  |
| --- | --- |
| **Steps** | **C18 SPE procedure**  |
| 1 | **Condition** | 5 ml acetonitrile at 1 drop/sec |
| 2 | **Equilibration** | 5 ml water at 1 drop/sec |
| 3 | **Loading** | 1 ml sample at 1 drop/3 sec |
| 4 | **Wash 1** | 5 ml water at 1 drop/sec |
| 5 | **Wash 2** | 5ml 5/95 V/V water/acetonitrile at 1 drop/sec |
| 6 | **Elution**  | 1 ml 50/50 V/V water/acetonitrile at 1 drop/ 3 sec |

#### Results and discussion

Only run 2 (Q210 and Q184 are analyzed during run 2) was injected onto the UHPLC-MS/MS system since the peptides in run 1 already have proven to remain retained on the SPE column when 10/90 V/V acetonitrile/water is applied as wash step. The results are provided in **Supplementary information Table 10**.

**Supplementary information Table 10: Recoveries with adjusted wash step 2 with prespiked and postspiked sample at 100xDev-Conc.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **C18 SPE procedure** | **Recoveries (%)1** | **Mean** | **Median** | **Standard deviation** |
| **Q133** | **Q210** | **Q184** | **Q93** |
| 5 ml acetonitrile at 1 drop/sec | ND | ND | ND | ND | ND | ND | ND |
| 5 ml water at 1 drop/sec | ND | ND | ND | ND | ND | ND | ND |
| 1 ml sample at 1 drop/3 sec | ND | ND | ND | ND | ND | ND | ND |
| 5 ml water at 1 drop/sec | ND | ND | ND | ND | ND | ND | ND |
| 5 ml 5/95 V/V water/acetonitrile at 1 drop/sec | 4.91 | 2.54 | 0.93 | 5.44 | 3.45 | 3.72 | 2.10 |
| 1 ml 50/50 V/V water/acetonitrile at 1 drop/ 3 sec | 85.98 | 91.47 | 85.97 | 81.75 | 86.29 | 85.98 | 3.99 |

1: ND=not determinable; signal-to-noise below 10.

### SPE with faeces extract

#### Methods

Following the protocol provided in **Supplementary information Table 9**, which have been executed with water samples (thus lacking faeces matrix), the experiment was repeated with undiluted murine faeces extract, *i.e.* 25 mg murine faeces + 1000 µl 10% V/V ammonium hydroxide in water, shaked 15 min 1400 rpm at 21°C, 1 min homogenisation at intensity 6, shaked for 15 min 1400 rpm at 21°C, and centrifuged for 15 min 20 000 *g* at 21°C) and spiked to a peptide concentration of 100xDev-Conc.

The protocol with undiluted faeces extract yielded no recoveries. Therefore the experiment was repeated with murine faeces extract as previously specified but diluted 1/10 with 10% V/V ammonium hydroxide in water prior to spiking the peptides to 100xDev-Conc and simultaneously exploring different SPE procedures (see **Supplementary information Table 11**).

**Supplementary information Table 11: Optimization experiment C18 SPE with murine faeces extract.**

|  |  |  |
| --- | --- | --- |
| **SPE** | **Steps** | **C18 SPE procedure**  |
| **Sample 1** | **Sample 2** | **Sample 3** |
| 1 | **Condition** | 5 ml acetonitrile at 1 drop/sec | 5 ml acetonitrile at 1 drop/sec | 5 ml acetonitrile at 1 drop/sec |
| 2 | **Equilibration** | 5 ml water at 1 drop/sec | 5 ml water at 1 drop/sec | 5 ml water at 1 drop/sec |
| 3 | **Loading** | 1 ml sample at 1 drop/3 sec | 1 ml sample at 1 drop/3 sec | 1 ml sample at 1 drop/3 sec |
| 4 | **Wash 1** | 5 ml water at 1 drop/sec | 5 ml water at 1 drop/sec | 5 ml water at 1 drop/sec |
| 5 | **Wash 2** | 5 ml 5/95 V/V water/acetonitrile at 1 drop/sec | 5 ml 20/80 V/V water/acetonitrile at 1 drop/sec | 51 ml 5/95 V/V water/acetonitrile at 1 drop/sec |
| 6 | **Elution**  | 2 ml 30/70 V/V water/acetonitrile at 1 drop/ 3 sec | 2 ml 50/50 V/V water/acetonitrile at 1 drop/ 3 sec | 2 ml 50/50 V/V water/acetonitrile at 1 drop/ 3 sec |

The recovery was compared to one sample containing peptides in 21/79 V/V acetonitrile/water and peptides spiked to a concentration of 100xDev-Conc.

#### Results and discussion

When undiluted faeces was extracted and consecutively spiked (to assess SPE recoveries), none of the peptides demonstrated recoveries > 1%. Hence, it was assumed that undiluted faeces extract caused sample matrix breakthrough.

Therefore the experiment was repeated with 1/10 diluted faeces extract (to minimize sample matrix breakthrough), the wash step 2 and elution step were examined at different acetonitrile/water V/V conditions (to minimize matrix elution). The results are provided in **Supplementary information Table 12**.

**Supplementary information Table 12: Recoveries of faeces extract processed with SPE under different wash step 2 and elution conditions outlined in Table 12. Prespiked sample had peptides present at 100x-Dev-Conc. Recovery was calculated to a postspiked sample with peptides at 100xDev-Conc.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Experiment** | **Recoveries (%)1** | **Mean** | **Standard deviation** | **Median** |
| **Q13** | **Q14** | **Q132** | **Q137** | **Q17** | **Q133** | **Q210** | **Q184** | **Q93** |
| Sample 1 | 58.31 | 23.02 | 36.94 | 35.68 | 57.21 | 42.72 | 42.70 | 77.60 | ND | 41.58 | 20.96 | 42.70 |
| Sample 2 | 6.55 | 6.95 | 3.90 | 7.97 | 7.27 | 5.22 | 2.00 | 5.30 | 0.45 | 5.07 | 2.39 | 5.30 |
| Sample 3 | 49.84 | 21.84 | 10.34 | 30.13 | 27.72 | 18.37 | 12.55 | 53.75 | 0.61 | 25.01 | 16.68 | 21.84 |
| Sample 1 wash | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| Sample 2 wash | 57.52 | 20.56 | 13.59 | 37.92 | 32.66 | 24.31 | 19.25 | 64.24 | ND | 30.00 | 19.54 | 24.31 |
| Sample 3 wash | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |

1: ND=not determinable; signal-to-noise below 10.

**S**ample 1 (see **Supplementary information Table 11** and **12**) yielded the highest overall mean and median recovery, hence this SPE approach was retained for further optimization.

## Method fine-tuning

### Fine-tuning C18 SPE procedure: elution fraction analysis and confirmation

#### Methods

Previously (see **Supplementary information Table 11**), the samples were eluted with 2 ml of elution solvent. To assess whether 2 ml is necessary to elute the peptides, the elution step was assessed by collecting the elution step in fractions of 250 µl (referred to as fraction 1, 2, …). The sample preparation procedure is given in **Supplementary information Table 13** and peptides were again spiked in the prespiked sample to 100xDev-Conc.

**Supplementary information Table 13: Elution fraction analysis C18 SPE with murine faeces extract.**

|  |  |
| --- | --- |
| **Faeces extraction** | 25 mg murine faeces + 1000 µl 10% V/V ammonium hydroxide in water15 min 1400 rpm at 21°C1 min homogenization at intensity 615 min 1400 rpm at 21°C15 min 20.000 g at 21°CSupernatant diluted 1/10 with 10% V/V ammonium hydroxide in water and spiked with quorum sensing peptides to 100xDev-Conc |
| **SPE** | **Steps** | **C18 SPE procedure** |
| 1 | **Condition** | 5 ml acetonitrile at 1 drop/sec |
| 2 | **Equilibration** | 5 ml water at 1 drop/sec |
| 3 | **Loading** | 1 ml sample at 1 drop/3 sec |
| 4 | **Wash**  | 5 ml 5/95 V/V water/acetonitrile at 1 drop/sec |
| 5 | **Elution** | 2 ml 30/70 V/V water/acetonitrile at 1 drop/ 3 sec; collected in fractions of 250 µl |

The recovery was compared to one sample containing peptides at the same nominal concentration in 21/79 V/V acetonitrile/water, *i.e.* 100xDev-Conc.

#### Results and discussion

The recoveries in **Supplementary information Table 14** show that fraction 5 yielded the highest mean and median recoveries with recoveries often exceeding 100%, hence indicating peptide concentration enhancement due to the loading volume of 1 ml versus elution in 250 µl.

**Supplementary information Table 14: Recoveries of fraction analysis. Highest recovery per peptide is indicated in purple (peptide concentration postspiked sample and prespiked sample 100xDev-Conc).**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Fraction** | **Recovery (%)1** | **Mean** | **Median** | **STDEV** |
| **Q13** | **Q14** | **Q132** | **Q137** | **Q17** | **Q133** | **Q210** | **Q184** | **Q93** |
| Fraction 1 | ND | ND | ND | ND | ND | ND | ND | 3.4 | ND | 0.4 | ND | 0.4 |
| Fraction 2 | ND | ND | ND | ND | ND | ND | ND | 4.7 | ND | 0.6 | ND | 0.6 |
| Fraction 3 | 10.9 | 4.6 | ND | 2.6 | 3.1 | 1.7 | 7.6 | 45.5 | ND | 9.5 | 3.8 | 9.5 |
| Fraction 4 | 145.3 | 155.9 | ND | 69.0 | 82.2 | 19.4 | 85.3 | 239.3 | ND | 101.0 | 83.7 | 101.0 |
| Fraction 5 | 211.3 | 467.5 | 51.0 | 191.6 | 248.2 | 56.8 | 72.9 | 220.0 | ND | 189.9 | 201.5 | 189.9 |
| Fraction 6 | 77.2 | 459.1 | 52.6 | 137.3 | 168.3 | 48.7 | 13.5 | 44.1 | ND | 125.1 | 64.9 | 125.1 |
| Fraction 7 | 15.2 | 182.4 | 20.0 | 45.0 | 51.0 | 19.3 | 2.6 | 8.4 | ND | 43.0 | 19.7 | 43.0 |
| Fraction 8 | 3.4 | 52.4 | 5.4 | 11.1 | 13.9 | 5.0 | ND | 2.2 | ND | 11.7 | 5.2 | 11.7 |

1: ND=not determinable; signal-to-noise below 10.

### Resolving low total recovery

#### Methods

Prior, murine faeces extract was spiked with quorum sensing peptides. However, when murine faeces was spiked with quorum sensing peptides prior to extraction, the total peptide recovery went down. To circumvent this, an experiment was conducted to investigate the effect of an adapted extraction solvent, *i.e.* 10/10/80 V/V/V acetonitrile/ammonium hydroxide/water. Additionally, the different fractions of 1/10 diluted sample were assessed and the recovery and LOQ were calculated to obtain the most suitable extraction solvent and most suitable elution fraction. Murine faeces was spiked with a quantity of quorum sensing peptides that would yield a nominal concentration of 100xDev-Conc after extraction. Faeces was spiked by adding the µl-range stock solution to the faeces and allowing during 15 min the adsorption in the faeces prior to extraction.

#### Results and discussion

Initial experiments on peptide recovery were carried out by spiking faeces extract, this approach was considered justified since the recovery of the peptides needed to be assessed solely regarding the SPE procedure. If faeces would have been spiked and consecutively extracted and SPE processed, then the overall recovery of the sample preparation method would have been assessed. To assess the faeces extraction efficiency, a suitable SPE method must first have been established to purify the samples prior to UHPLC-MS/MS analysis.

When spiked faeces was extracted with consecutively SPE processing, a notable drop in the peptide recoveries compared to spiked faeces extract was observed. This could indicate absorbance of the peptides to components in the faeces matrix (solid components) that are removed when the faeces samples are centrifuged.

Therefore, an experiment was conducted to investigate the effect of an adapted extraction solvent, *i.e.* 10/10/80 V/V/V acetonitrile/ammonium hydroxide/water. Additionally, the different fractions of a 1/10 diluted sample (*i.e.* 25 mg murine faeces + 1000 µl 10% V/V ammonium hydroxide in water, shaked 15 min 1400 rpm at 21°C, 1 min homogenisation at intensity 6, shaked 15 min 1400 rpm at 21°C, centrifugation for 15 min 20.000 g at 21°C, and 1/10 V/V diluted in 10/10/80 V/V/V acetonitrile/ammonium hydroxide/water) were assessed and the recovery and limit of quantification (LOQ) were calculated to obtain the most suitable extraction solvent and elution fraction. The reassessment of the elution fractions is justified since extracting the sample in extraction solvent containing 10% V/V acetonitrile might alter the interactions of the peptides with the stationary phases and hence resulting in earlier/later elution. The LOQ was estimated based on the signal-to-noise (S/N) ratio and peptide concentration. By extrapolating the S/N to 10, the LOQ was estimated starting from a nominal concentration after extraction of 100xDev-Conc. The results are provided in **Supplementary information Table 15**.

**Supplementary information Table 15: Recoveries of experiment to resolve apparent adsorption of peptides to faecal material. Nominal peptide concentration after extraction of 100xDev-Conc.**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Extraction solvent****Dilution factor faeces****Elution faction** |  | **Peptide1** | **Mean** | **Median** | **Standard deviation** |
| **Q13** | **Q14** | **Q132** | **Q137** | **Q17** | **Q133** | **Q210** | **Q184** | **Q93** |
| 10/10/80 V/V/V acetonitrile/ammonium hydroxide/ water1/10 diluted4th | Recovery (%) | 185.3 | 109.9 | 54.3 | 70.5 | 116.9 | 114.1 | 150.7 | 190.2 | 10.7 | 111.4 | 114.1 | 59.5 |
| LOQ (nM) | 1.2 | 6.2 | 42.4 | 7.5 | 8.6 | 16.8 | 2.5 | 6.6 | 1174.3 | 140.7 | 7.5 | 387.8 |
| 10/10/80 V/V/V acetonitrile/ammonium hydroxide/ water1/10 diluted5th | Recovery (%) | 138.1 | 100.3 | 99.7 | 78.7 | 129.1 | 103.1 | 93.0 | 84.3 | 26.3 | 94.7 | 99.7 | 32.1 |
| LOQ (nM) | 1.2 | 3.7 | 20.4 | 5.5 | 2.5 | 8.0 | 6.0 | 17.7 | 261.0 | 36.2 | 6.0 | 84.6 |
| 10/10/80 V/V/V acetonitrile/ammonium hydroxide/ water1/10 diluted6th | Recovery (%) | 13.2 | 27.2 | 30.9 | 17.3 | 32.2 | 39.7 | 9.1 | 13.1 | 32.2 | 23.9 | 27.2 | 10.9 |
| LOQ (nM) | 9.0 | 10.7 | 100.1 | 9.0 | 8.1 | 5.8 | 28.7 | 83.9 | 287.0 | 60.3 | 10.7 | 92.2 |
| 10/10/80 V/V/V acetonitrile/ammonium hydroxide/ water1/10 diluted4-6th (combined) | Recovery (%) | 88.8 | 57.6 | 72.1 | 48.4 | 112.7 | 90.7 | 89.6 | 104.1 | 23.0 | 76.3 | 88.8 | 28.8 |
| LOQ (nM) | 1.3 | 6.4 | 68.9 | 6.2 | 2.9 | 23.1 | 6.3 | 16.9 | 424.1 | 61.8 | 6.4 | 137.5 |
| 10/90 V/V ammonium hydroxide/ water1/10 diluted4th | Recovery (%) | 72.0 | 0.5 | 0.4 | 1.1 | 10.0 | 32.8 | 13.0 | 166.7 | ND | 33.0 | 10.0 | 55.4 |
| LOQ (nM) | 6.8 | 886.0 | 7978.3 | 1041.4 | 66.8 | 71.3 | 18.3 | 19.7 | ND | 1261.1 | 69.0 | 2746.7 |
| 10/90 V/V ammonium hydroxide/ water1/10 diluted5th | Recovery (%) | 3.6 | 0.0 | 0.2 | 0.2 | 2.6 | 6.3 | 3.0 | 4.5 | ND | 2.2 | 2.6 | 2.3 |
| LOQ (nM) | 6.2 | 1045.6 | 9819.7 | 543.9 | 24.1 | 32.4 | 32.9 | 0.0 | ND | 1643.6 | 32.9 | 3626.5 |
| 10/90 V/V ammonium hydroxide/ water1/10 diluted6th | Recovery (%) | 3.6 | 0.0 | 0.2 | 0.2 | 2.6 | 6.3 | 3.0 | 4.5 | ND | 2.2 | 2.6 | 2.3 |
| LOQ (nM) | 30.9 | 0.0 | 14008.0 | 1254.0 | 179.9 | 57.4 | 388.2 | 10675.3 | ND | 3799.1 | 388.2 | 5928.9 |
| 10/90 V/V ammonium hydroxide/ water1/10 diluted4-6th (combined) | Recovery (%) | 37.2 | 0.2 | 0.3 | 0.8 | 9.4 | 26.7 | 11.4 | ND | ND | 9.5 | 0.8 | 13.7 |
| LOQ (nM) | 5.2 | 2602.5 | 14226.6 | 700.5 | 60.3 | 98.4 | 25.5 | ND | ND | 2531.3 | 98.4 | 5241.2 |

1: ND=not determinable; signal-to-noise below 10.

Based on these results, extraction of faeces in 10/10/80 V/V/V acetonitrile/ammonium hydroxide/water was chosen and the fractions 4 and 5 were determined to be pooled prior to analysis.

### Dilution factor faeces and LOQ/LOD estimation

#### Methods

Following, the optimal faeces dilution was reassessed by extracting murine faeces as previously described. Since the extraction solvent was adjusted, the added acetonitrile might extract more/less/other matrix compounds and therefore the optimal dilution of the faeces extract was reassessed. The followed approach is provided in **Supplementary information Table 16**.

**Supplementary information Table 16: Protocol for determination of murine faeces dilution factor.**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Blank 1/10 diluted | Blank 1/2 diluted | Prespiked 1/10 diluted | Prespiked 1/2 diluted | Postspiked 1/10 diluted | Postspiked 1/2 diluted |
| 25 mg faeces spiked with 4 µl water5 min 21°C 300 rpm1000 µl 10/10/80 V/V/V acetonitrile/ammonium hydroxide/water15 min 1400 rpm 21°C30 sec homogenisation intensity 615 min 1400 rpm 21°C15 min 20.000 *g* at 21°C | 25 mg faeces spiked with 4 µl peptide stock 5000xDev-Conc5 min 21°C 300 rpm1000 µl 10/10/80 V/V/V acetonitrile/ammonium hydroxide/water15 min 1400 rpm 21°C30 sec homogenisation intensity 615 min 1400 rpm 21°C15 min 20.000 *g* at 21°C | 25 mg faeces spiked with 4 µl water5 min 21°C 300 rpm1000 µl 10/10/80 V/V/V acetonitrile/ammonium hydroxide/water15 min 1400 rpm 21°C30 sec homogenisation intensity 615 min 1400 rpm 21°C15 min 20.000 *g* at 21°C |
| Supernatant diluted 1/10 with 10/90 V/V ammonium hydroxide/water | Supernatant diluted 1/2 with 10/90 V/V ammonium hydroxide/water | Supernatant diluted 1/10 with 10/90 V/V ammonium hydroxide/water | Supernatant diluted 1/2 with 10/90 V/V ammonium hydroxide/water | Supernatant diluted 1/10 with 10/90 V/V ammonium hydroxide/water | Supernatant diluted 1/2 with 10/90 V/V ammonium hydroxide/water |
| **Steps** | **C18 SPE procedure** |
| 1 | **Condition** | 5 ml acetonitrile at 1 drop/sec |
| 2 | **Equilibration** | 5 ml water at 1 drop/sec |
| 3 | **Loading** | 1 ml sample at 1 drop/3 sec |
| 4 | **Wash 1** | 5 ml 5/95 V/V water/acetonitrile at 1 drop/sec |
| 5 | **Wash 2** | 0.75 ml 30/70 V/V water/acetonitrile at 1 drop/ 3 sec; |
| 6 | **Elution** | 0.5 ml 30/70 V/V water/acetonitrile at 1 drop/ 3 sec |
| 90 µl eluate + 10 µl water | 90 µl eluate + 10 µl water | 90 µl eluate + 10 µl peptide stock to obtain concentration of prespiked samples |

#### Results and discussion

During this experiment, the dilution factor (1/10 or 1/2) of the murine faeces after extraction in 10/10/80 V/V/V acetonitrile/ammonium hydroxide/water with 10/90 water/ammonium hydroxide was determined (see **Supplementary information Table 17**).

**Supplementary information Table 17: Determination of murine faeces dilution factor.**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Dilution factor** |  | **Peptide1** | **Mean** | **Median** | **Standard deviation** |
| **Q13** | **Q14** | **Q132** | **Q137** | **Q17** | **Q133** | **Q210** | **Q184** | **Q93** |
| 1/10(*i.e.* 2xDev-Conc analytical concentration) | Recovery (%) | 198.7 | 60.7 | 48.1 | 46.2 | 70.9 | 130.2 | 165.5 | 118.4 | 17.7 | 104.8 | 94.7 | 61.1 |
| LOQ (nM) | 1.7 | 3.7 | 47.1 | 16.7 | 13.3 | 15.7 | 6.8 | 17.3 | 1366.1 | 15.3 | 14.5 | 450.5 |
| ½(*i.e.* 10xDev-Conc analytical concentration) | Recovery (%) | 161.5 | 65.8 | 64.6 | 44.8 | 85.8 | 114.1 | 231.7 | 68.1 | ND | 104.5 | 76.9 | 68.5 |
| LOQ (nM) | 0.8 | 3.6 | 96.0 | 33.4 | 7.4 | 7.1 | 4.9 | 18.0 | ND | 21.4 | 7.2 | 31.9 |

1: ND=not determinable; signal-to-noise below 10.

Given the lowest median LOQ of the 1/2 dilution of murine faeces, this dilution factor was chosen. Based on this final experiment, the method as described in the main article was determined to be established.