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**Co-expression of lupanine hydroxylase and pyrroloquinoline
quinone
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hydroxylase in the
Escherichia coli periplasm**

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Fig. 1

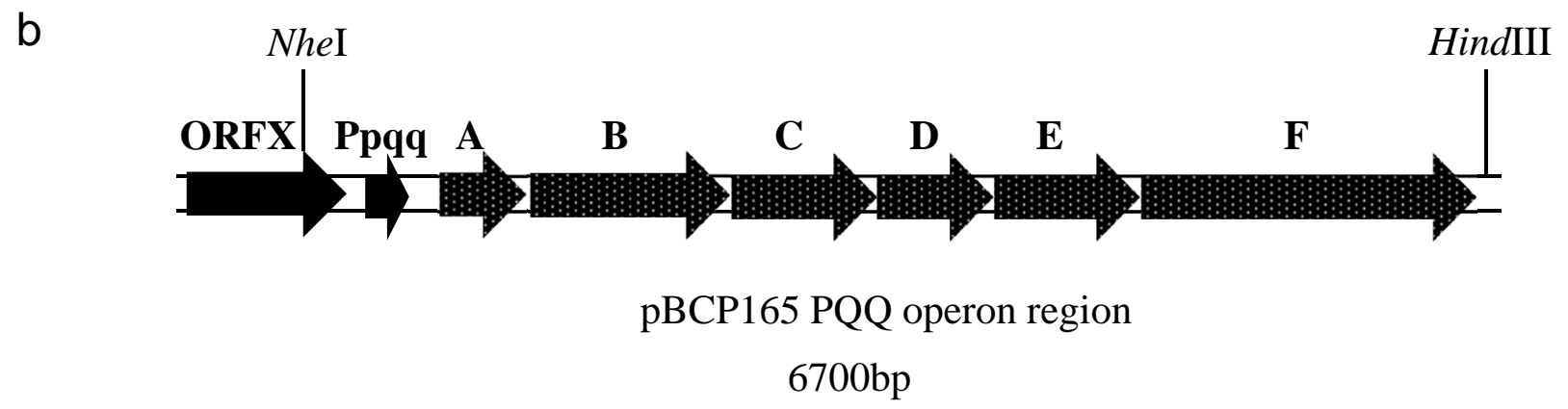
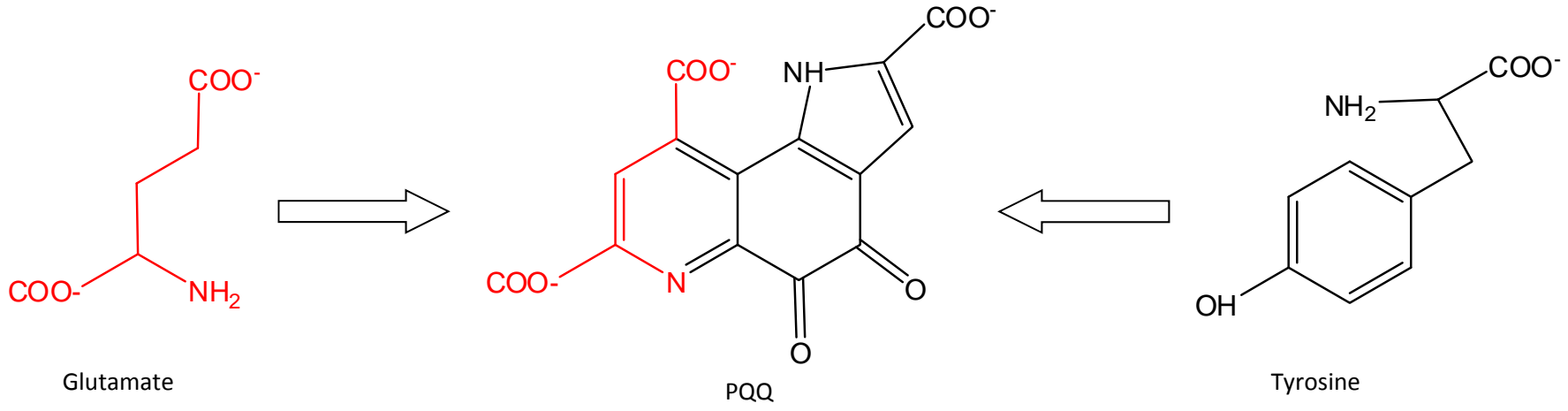
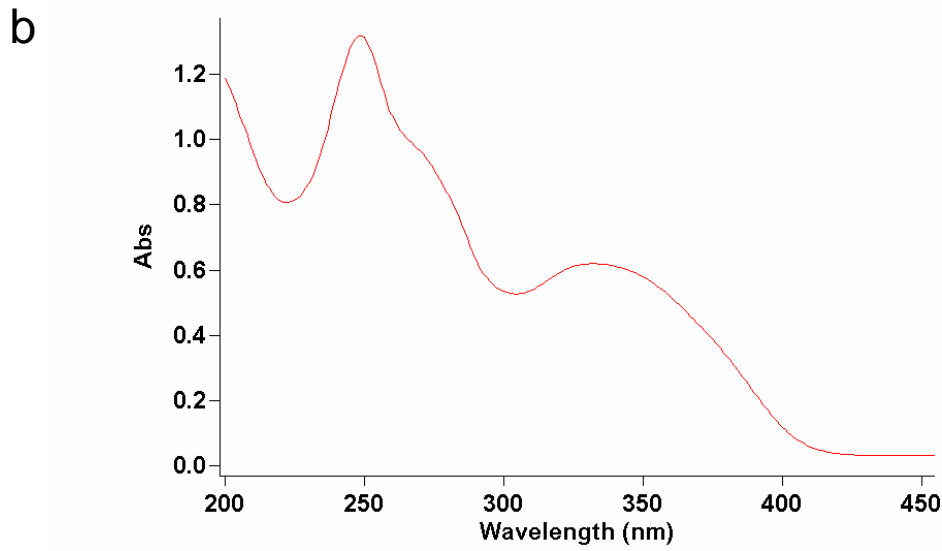
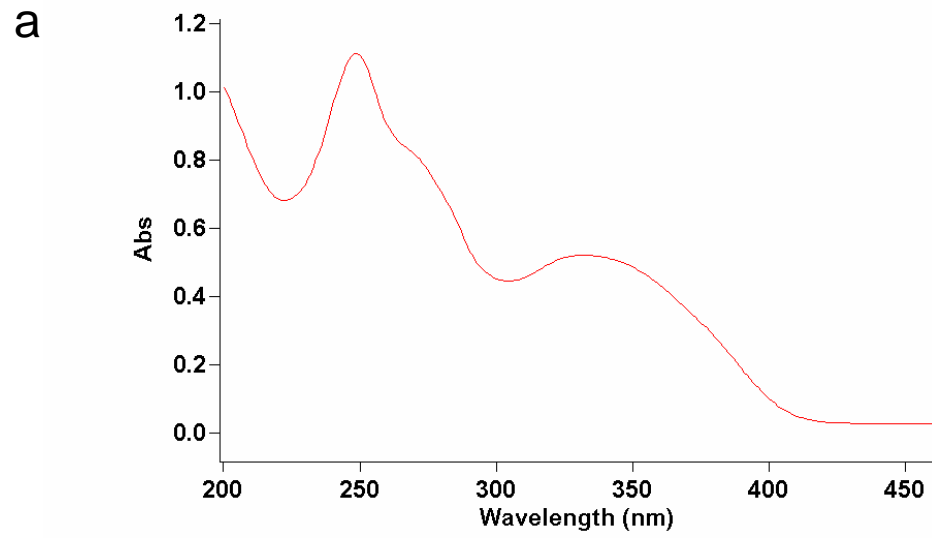


Fig. 2



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1 **SHORT COMMUNICATION**

2 **Co-expression of lupanine hydroxylase and pyrroloquinoline quinone**
3 **leads to assembled and active recombinant lupanine hydroxylase in the**
4 ***Escherichia coli* periplasm**

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20
21 **Keywords:** pyrroloquinoline quinone, lupanine hydroxylase, *Escherichia coli*,
22 periplasmic space, quinohaemoprotein, protein export

24 ABSTRACT

25 Lupanine hydroxylase (LH) is a quinohaemoprotein responsible for the conversion of the
26 alkaloid, lupanine to 17-hydroxylupanine. Previous attempts to express the enzyme in
27 *Escherichia coli* required *in vitro* addition of the co-factor pyrroloquinoline quinone
28 (PQQ) and posed some impediments on subsequent structural studies for further
29 characterization of the enzyme. An *E. coli* clone with LH and cytochrome *c* maturation
30 operon was transformed with a third plasmid containing the PQQ operon from *Klebsiella*
31 *pneumoniae*, *luh* gene and resulted in the production of periplasmically-targeted,
32 correctly folded, PQQ and haem inserted active enzyme.

33 Interestingly, LH was less active than the *in vitro* incorporated PQQ-LH, presumably due
34 to the incorporation of PQQ precursors in the periplasm. This is a first report of an active
35 LH enzyme with *in vivo* incorporation of PQQ in *E. coli* and provides the necessary tool
36 for further enzyme structural characterization.

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46 Introduction

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3 47 Pyrroloquinoline quinone [4,5-dihydro-4,5-dioxo-1*H*-pyrrolo-[2,3-*f*] quinoline-
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5 48 2,7,9-tricarboxylic acid (PQQ) is an important non-covalently bound redox cofactor in
6
7 49 enzymes such as dehydrogenases, oxygenases and decarboxylases (Anthony 2001) along
8
9 50 with NAD(P) and flavins (Duine and Jongejan 1989a). It is an aromatic, tricyclic, *ortho*-
10
11 51 quinone and is a crucial link between compound oxidation and the respiratory chain
12
13 52 (Duine and Jongejan 1989b). Enzymes containing PQQ as a cofactor are divided into two
14
15 53 classes; Class I quinoproteins contain PQQ and Ca²⁺ as cofactors and class II
16
17 54 quinohaemoproteins, in addition to PQQ and Ca²⁺ also carry haem at their C-terminus
18
19 55 (Stoorvogel et al. 1996). In Class I quinoproteins, catalytic conversion of the substrate
20
21 56 results in PQQ accepting two electrons (PQQH₂) which are then transferred to another
22
23 57 redox protein (Davidson 2004), whereas Class II quinohaemoproteins transfer electrons
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25 58 from PQQ and then relay onto the haem moiety within the molecule before being donated
26
27 59 to an external acceptor (Oubrie et al. 2002).
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34 60 In eukaryotes, the presence of PQQ has so far been confirmed in mammalian
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36 61 tissues and milk, however, no enzymatic reaction which directly necessitates PQQ has
37
38 62 been reported yet (Steinberg et al. 2003).
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41 63 Studies in micro-organisms have revealed that a number of PQQ maturation
42
43 64 chaperones are required for its synthesis and the genes coding for these factors are all
44
45 65 organized into clusters that exhibit a high level of sequence homology (Meulenberg et al.
46
47 66 1990). Examples are *Methylobacterium extorquens* possessing seven PQQ maturation
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49 67 chaperones, *Klebsiella pneumoniae* has six and *Acinetobacter calcoaceticus* has only four
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51 68 chaperones (Puehringer et al. 2008).
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4 69 Interestingly, there is an absence of PQQ in some prokaryotes as a number of
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6 70 bacterial species are reported to express quino-enzymes lacking PQQ. An example in
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8 71 *Escherichia coli* is expression of glucose dehydrogenase minus PQQ (Matsushita et al.
9
10 72 1997) and the reason as to why *E. coli* would produce an enzyme whilst unable to
11
12 73 produce its cofactor remains a mystery. One theory is that *E. coli* is in possession of an
13
14 74 alternative pathway of PQQ production with a yet unidentified inducer (Biville et al.
15
16 75 1991). A second theory is that *E. coli* scavenges PQQ from its environment and the
17
18 76 finding that mobile *E. coli* strains move chemo-tactically towards PQQ reinforces this
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20 77 argument (DeJonge et al. 1996). Although the complete biosynthetic pathway of PQQ
21
22 78 remains elusive, tyrosine and glutamate (Fig. 1a) are the two main precursors
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24 79 (Magnusson et al. 2004).

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29 80 LH is a class II, type I quinohaemoprotein which contains equimolar amounts of
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31 81 PQQ, Ca²⁺ and haem as co-factors (Stampolidis et al. 2009). It is a 72 kDa monomeric
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33 82 enzyme responsible for the initial conversion of the alkaloid lupanine to 17-
34
35 83 hydroxylupanine (Toczko et al. 1963). Heterologous expression of the *luh* gene in *E. coli*
36
37 84 also necessitated co-expression of the cytochrome *c* maturation machinery (Thony-Meyer
38
39 85 2003), responsible for haem insertion into the apoform of the enzyme (Stampolidis et al
40
41 86 2009). Based on these factors, we investigated the co-expression and *in vivo*
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43 87 incorporation of a third plasmid coding for PQQ into the above *E.coli* clone co-
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45 88 expressing LH and cytochrome *c* maturation machinery.
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52 53 90 **Materials and methods**

54 55 91 **Bacterial strains, growth conditions and plasmids**

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3 92 Starter cultures of *E. coli* TB1 [F^- *ara* $\Delta(lac-proAB)$ ($\phi 80dlac$ $\Delta(lacZ)M15$) *rpsL(Str^r)* *thi*
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5 93 *hsdR*] (New England BioLabs) clones harbouring plasmids pEC86 (cytochrome *c*
6
7 94 maturation operon), pEV-LH32 (untagged LH) and pINK-LH-His₄ (tetra-His tagged LH)
8
9 95 were cultured in Luria Bertani broth (1% (w/v) Tryptone, 0.5% (w/v) yeast extract and
10
11 96 1% (w/v) NaCl) containing 75 μ g mL⁻¹ ampicillin and/or 50 μ g mL⁻¹ chloramphenicol
12
13 97 were grown to saturation for 16h at 30⁰C and applied as 2% (v/v) inoculum for batch
14
15 98 cultivation in the MOPS medium (Karim et al. 1993) with the appropriate antibiotics and
16
17 99 orbital agitation at 125 rev min⁻¹ for 18h at 21⁰C. The clone pK-187-PQQ, pEC86/
18
19 100 pK187-PQQ and pEC86/ pINK-LH-His₄/ pK187-PQQ for PQQ synthesis, was induced in
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21 101 MOPS in the presence of 1% (w/v) Na-gluconate, 50 μ M IPTG and kanamycin at 50 μ g
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23 102 mL⁻¹ and grown as above.
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30 103 Plasmid pBCP165 was provided by J. C. Arents (Velterop et al. 1995) and
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32 104 plasmids pEV-LH32, pINK-LH-His₄ and pEC86 were from Dr M. A. Kaderbhai
33
34 105 laboratory.
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39 107 **Periplasmic extraction from clones**

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41 108 Periplasmic extract was prepared by the osmotic shock method as described previously
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43 109 (Kaderbhai et al. 2012) and was recovered by centrifugation at 10,000g for 5min and
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45 110 stored frozen at -80⁰C.
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50 112 **His-tagged LH purification using Qiagen Ni-NTA**

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52 113 His-tagged LH was routinely purified from periplasmic extracts of clone pEC86/pINK-
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54 114 LH-His₄ via passage through Qiagen Ni-NTA column and eluted with 100mM imidazole
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3 115 (pH 8.0) and 100mM EDTA. Untagged LH was purified from the pEC86/pEV-LH32
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6 116 clone on DE-52-DEAE-cellulose columns as described previously (Stampolidis et al.
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8 117 2009).
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119 **PQQ purification using DEAE Sepharose Fast Flow**

120 A 10mL volume of periplasmic extract from 2L culture of pKK-187-PQQ (5ml) was
121 mixed with DEAE Sepharose slurry (10ml) and was gently shaken overnight at 4⁰C. The
122 suspension was packed in a column, washed twice with 0.5M KPO₄ (pH 7.4) and eluted
123 in 2mL total volume of 2M NaCl in 25mM Tris-HCl (pH 8). The salt was removed from
124 the eluent by PQQ binding to a 2mL bed volume of C18 reverse phase column followed
125 by two washes with 5mM HCl (pH 2.0) and final elution with 20% (v/v) methanol. PQQ
126 was detected by diode-array detection and ESI-MS as described in Comont et al. (2012).
127

128 **Identification of PQQ**

129 Presence of PQQ in the isolates was detected spectrophotometrically at 257nm
130 absorbance.
131

132 **Measurement of *in vivo* and *in vitro* LH enzyme activity**

133 LH was routinely activated on addition of 4mM CaCl₂ and incubation at room
134 temperature for 1h. Activation of LH apoform *in vitro* necessitated addition of 200μM
135 PQQ. LH was assayed at 25⁰C using horse heart cytochrome *c* as the electron acceptor by
136 increase in absorbance at 550nm due to its reduction as described by Stampolidis et al.
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3 137 (2009). Assays were performed in duplicates, the reaction rate being linear during this
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5 138 time.
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10 140 **Results**

13 141 **Construction and expression of PQQ in *E. coli***

14 142 Plasmid pBCP165 comprising a 6.7 Kbp PQQ operon under the transcriptional control of
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18 143 a *K. pneumoniae pqq* promoter was cleaved with *NheI* (blunted) and *HindIII* restriction
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20 144 endonucleases (Fig. 1b). The resulting fragment allowed closer proximity of the operon
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22
23 145 to the exogenous *lac* promoter present in the host plasmid. Colony PCR with primers,
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25 146 For-PQQ-Screen: 5'-GCCATCCTGCGGCAGC-3' and Rev-PQQ-Screen: 5'-
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27 147 CCCCCGGCCATTAATCCC-3' using part of the PQQ operon (For-PQQ-Screen) and
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30 148 part of the plasmid vector (Rev-PQQ-Screen) along with gene mapping with *EcoRI* and
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32 149 *HindIII* confirmed the presence of the entire PQQ operon in a positive clone designated
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34 150 as pK187-PQQ.
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37 151 A red eluted band was obtained from the periplasmic extract of pKK187-PQQ
38
39 152 after passage through a DEAE Sepharose Fast Flow column and gave a spectral scan with
40
41 153 a major peak at 257nm, characteristic of PQQ. Comparison of this spectrum with a
42
43 154 control spectrum of PQQ from *Methylophilus methylotrophus* showed no significant
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45
46 155 difference indicating that the PQQ produced in pKK187-PQQ was of good quality and
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48 156 purity (Fig. 2b).
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51 157 The PQQ from pKK187-PQQ showed a major peak that eluted at 17 minutes
52
53 158 following separation by C₁₈ reverse phase chromatography and had a molecular mass of
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56 159 331.1 *m/z* [M+H]⁺ using ESI-MS (supplementary data 1).
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56 161 **Co-expression of PQQ and LH in *E.coli***
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8 162 A fully functional LH with *in vivo* PQQ incorporation was obtained with the construction
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10 163 of a three plasmid system. Isolated plasmid pK187-PQQ (Kam^r), LH encoding pINK-LH-
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12 164 His₄ (Amp^r) and cytochrome *c* maturation machinery encoding pEC86 (Cm^r) were co-
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14
15 165 transformed into *E. coli* TB1 cells to generate clone pEC86/ pINK-LH-His₄/ pK187-
16
17 166 PQQ. The TB1 strain, a JM83 derivative, carries the *hsdR* mutation which facilitates
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20 167 transformation efficiency and is reported to provide plasmid stability in conjunction to
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22 168 protein expression and purification (Yanisch-Perron et al. 1985; Belo et al.
23
24 169 1996). Successful transformants were expressed in the MOPS phosphate-limited medium
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26
27 170 containing 1% (w/v) Na-gluconate and 50μM IPTG in the presence of the three
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29 171 antibiotics (see Materials and Methods) for 18h at 21⁰C. Periplasmic extracts from
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31
32 172 osmotically shocked cells were prepared as described in the Materials and Methods
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34 173 section.

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39 175 **Comparative activity of *in vivo* PQQ incorporated LH apoenzyme and *in vitro***
40
41 176 **activated LH apoenzyme**
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43 177 Comparison of tetra-His-tagged LH activity with the pEC86/pEV-LH32 untagged LH
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45 178 showed no significant difference in enzymatic activity (Table 1). Comparison of the
46
47 179 purified LH activity from clone pEC86/ pINK-LH-His₄/ pK187-PQQ with LH from clone
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49 180 pEC86/ pINK-LH-His₄ showed *in vivo* activated form exhibited 10% of the activity of *in*
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51 181 *vitro* activated form. Periplasmic extracts from clones pEC86, pK187-PQQ and pEC86/
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3 182 pK187-PQQ were also assayed for LH activity as controls and gave no significant
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5 183 enzyme activity.
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8 184 Interestingly, although *in vivo* form of LH exhibits only 10% activity of its *in vitro*
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10 185 counterpart, comparison of the PQQ levels of production between the two respective host
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12 186 clones appeared to be similar.
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18 188 **Discussion**

19
20 189 As a result of the inability of *E. coli* to endogenously produce PQQ, initial efforts to
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22 190 express quinoproteins, heterologously, in a simple and inexpensive way were impeded. In
23
24 191 an effort to tackle this issue, cloning of the entire operon for PQQ from *Klebsiella*
25
26 192 *pneumonia* to *E. coli* was carried out (Meulenberg et al. 1990). Expression of the 6.7 kbp
27
28 193 operon consisting of genes *pqq A, B, C, D, E* and *F* resulted in *in vivo* incorporation of
29
30 194 PQQ into glucose dehydrogenase and subsequent optimization by Velterop and co-
31
32 195 workers (Velterop et al. 1995) with incorporation of plasmid pBCP165 in *E. coli* strain
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34 196 *JA221* resulted in greater yields in the region of 180nM.
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39 197 Periplasmic targeting of LH in an earlier study (Stampolidis et al. 2009) resulted
40
41 198 in homogeneous production of the apoform of the enzyme and was readily transformed
42
43 199 into holoform upon subsequent *in vitro* addition of PQQ. However, this approach posed
44
45 200 some impediments to further work in characterization of the enzyme. Preliminary
46
47 201 attempts to examine the effect of replacing Ca²⁺ in the active site of the enzyme with
48
49 202 other ions proved inconclusive due to micro quantities of other ions in PQQ preparation.
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51 203 Moreover, X-ray diffraction data from crystals of *in vitro* correctly folded LH apoform
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53 204 failed to provide meaningful diffraction patterns.
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3 205 In this study, co-expression of the engineered LH and cytochrome *c* with PQQ
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6 206 resulted in the production of active LH with successful *in vivo* incorporation of PQQ. The
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8 207 10% activity of the *in vivo* incorporated PQQ-LH might be due to the incorporation of
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10 208 PQQ precursors into the active site of the enzyme. This occurrence of PQQ precursors is
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12 209 not uncommon and has been observed in alcohol dehydrogenase from *Pseudomonas*
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14 210 *testosteroni* with similar effects on the enzyme activity and no change in the quaternary
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16 211 structure of the protein (Jongejan et al. 1989). Another probable cause for this
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18 212 phenomenon could be extracellular targeting of PQQ which limits cofactor availability in
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20 213 the periplasm for apoform incorporation.
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24 214 Future attempts to produce LH crystals suitable for X-ray diffraction will
25
26 215 facilitate studies on the structure of quinohaemoproteins and increase our understanding
27
28 216 on the catalytic breakdown of the alkaloid, lupanine. The outcome of our approach is a
29
30 217 first report of *in vivo* recombinant LH apoenzyme synthesized in *E. coli* and provides the
31
32 218 necessary tools for further LH enzymology studies.
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49 225 **Conflict of interest:**

50
51 226 The authors declare no conflict of interest.
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56 228 **References**

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298 **Figure Legends**

299 **Fig. 1: Generation of PQQ in *E. coli*.**

300 **a) Precursors of PQQ biosynthesis.** Glutamate and tyrosine have been identified as the
301 two precursors of PQQ. PQQ maturation requires at least four different chaperones

302 **b) The PQQ operon in pBCP165.** ORFX is schematic diagram of an open-reading frame
303 coding for an unidentified protein (not essential for PQQ expression), Ppqq; PQQ
304 promoter and A-F; PQQ maturation genes.

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306 **Fig. 2: PQQ spectra.**

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3 307 Spectral comparison of PQQ purified preparation from **a)** *Methylophilus methylotrophus*
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5 308 (control) and **b)** *Escherichia coli* pK187-PQQ.
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For Peer Review

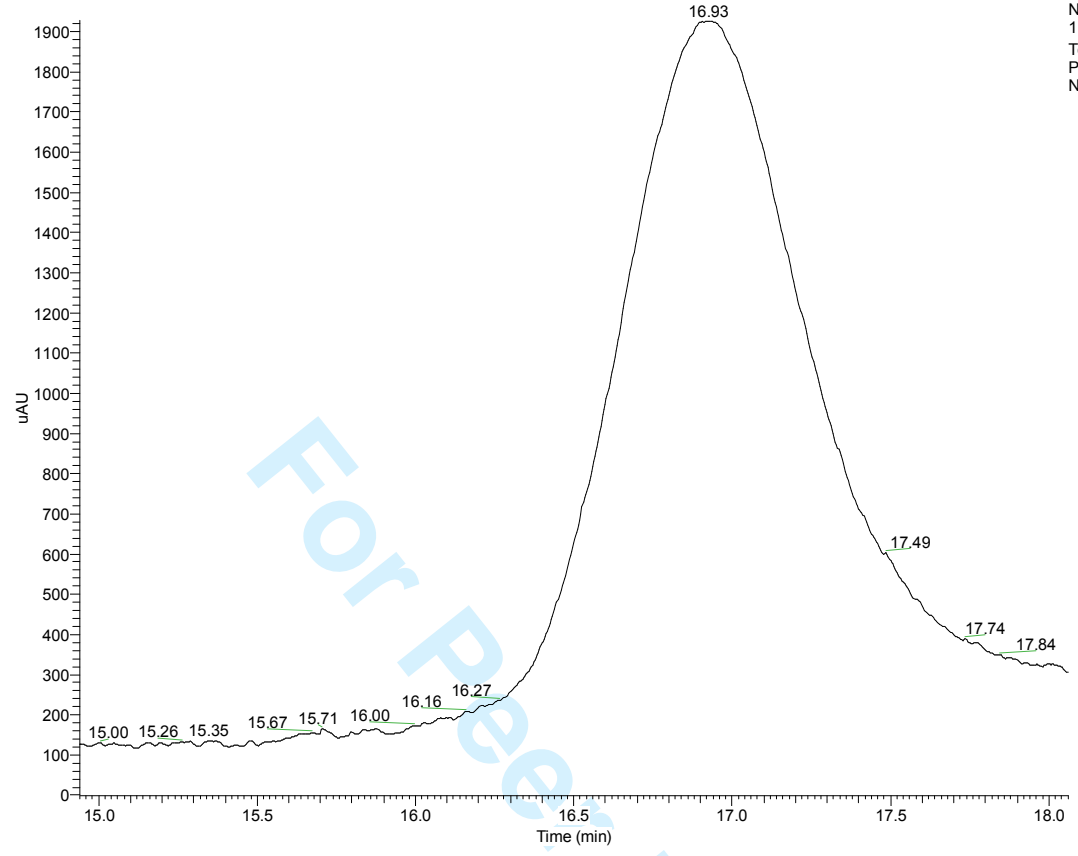
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5 311 **Table 1: Lupanine hydroxylase (LH) enzyme activity in *Escherichia coli* clones in the presence and/or absence of PQQ and**
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7 312 ***Cytc* operons.**
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Clone name and description	LH expression	PQQ production	LH activity (units/mg periplasmic protein)
pEC86/pEV-LH32-untagged LH and <i>Cytc</i> operon	+	-	66
pEC86/pINK-LH-His ₄ tagged- <i>Cytc</i> operon	+	-	54
pEC86/pINK-LH-His ₄ tagged /pK187-PQQ and <i>Cytc</i> operons	+	+	6.8
pEC86- <i>Cytc</i> operon	-	-	0.15
pK187-PQQ operon	-	+	0
pEC86/pK187-PQQ and <i>Cytc</i> operons	-	+	0.30

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32 314 Signs represent presence (+) or absence (-) of LH expression and PQQ production.
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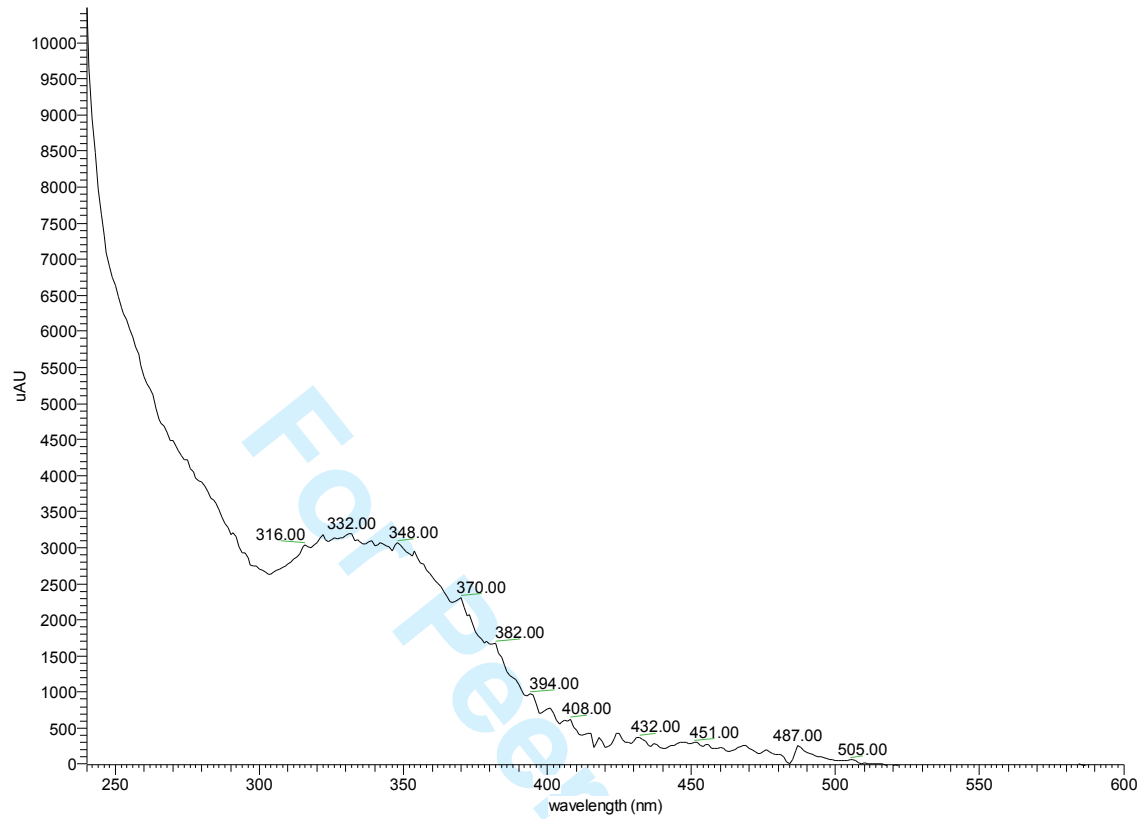
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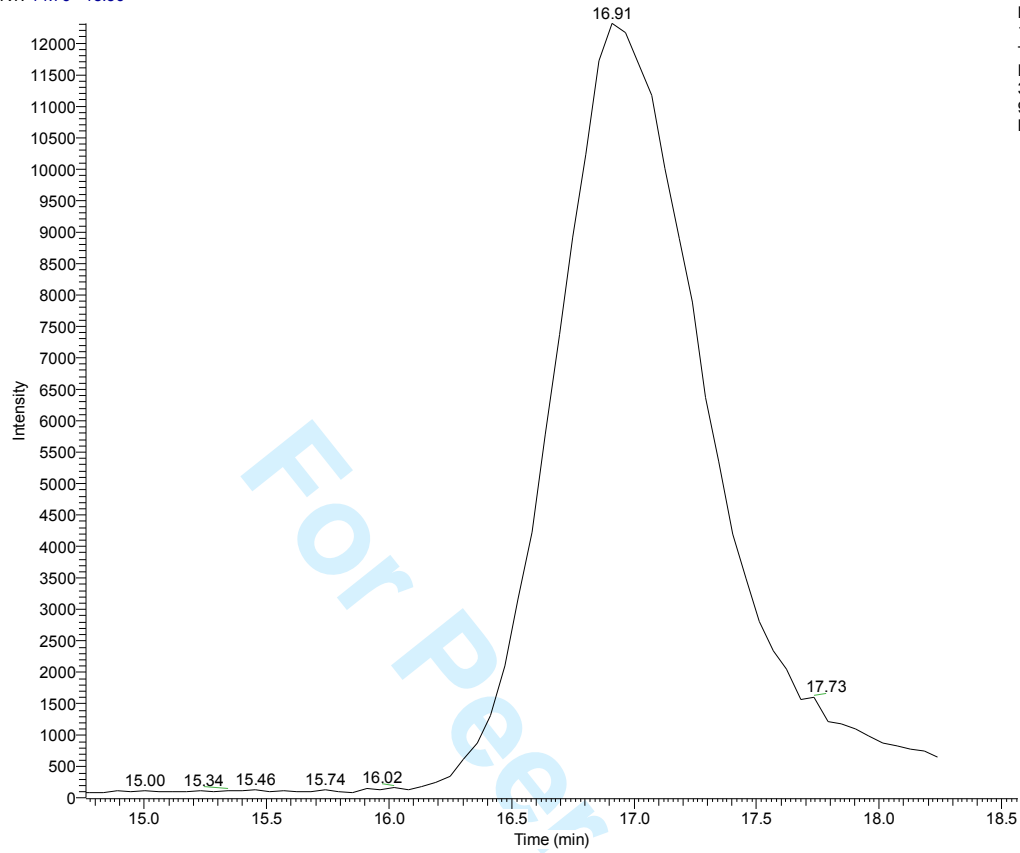
NL:
1.93E3
Total Scan
PDA
Naheed1

For Peer Review

Naheed1 #4979-5183 RT: 16.59-17.27 AV: 205 NL: 1.05E4 microAU

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RT: 14.76 - 18.56

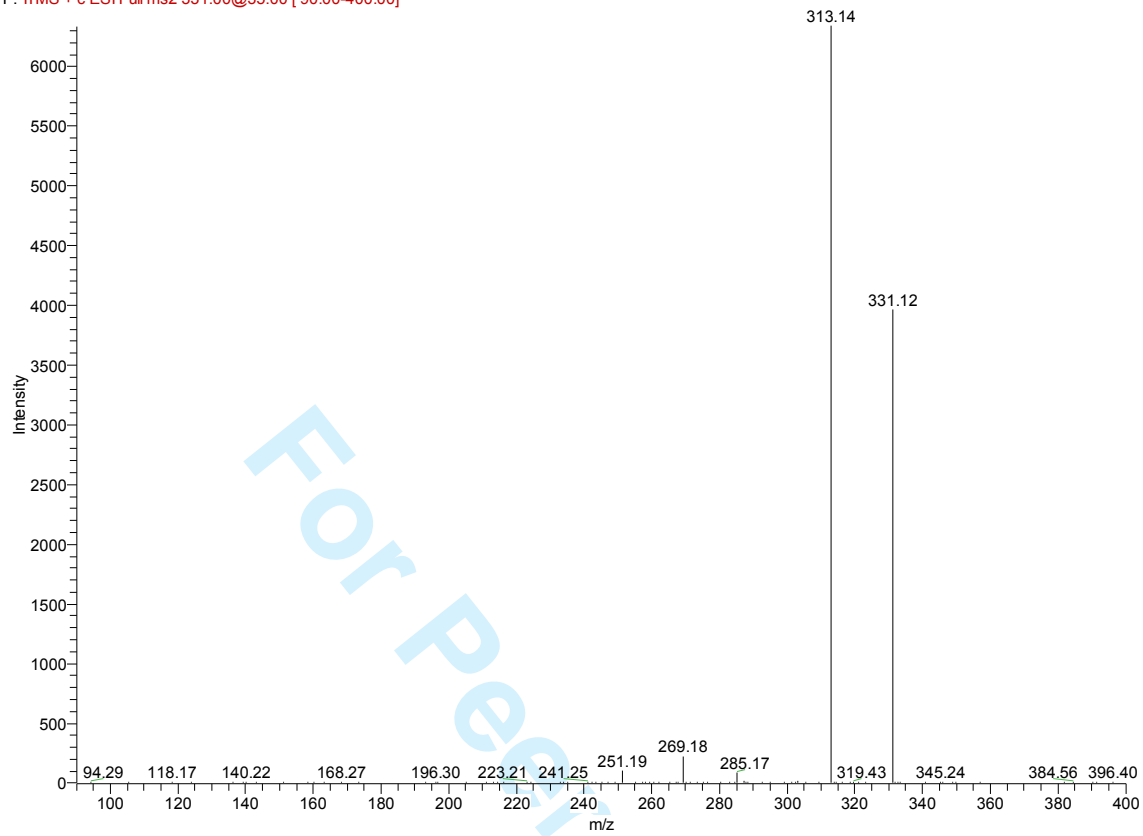


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1.23E4
TIC F: ITMS + c
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331.00@35.00 [
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MS Naheed1

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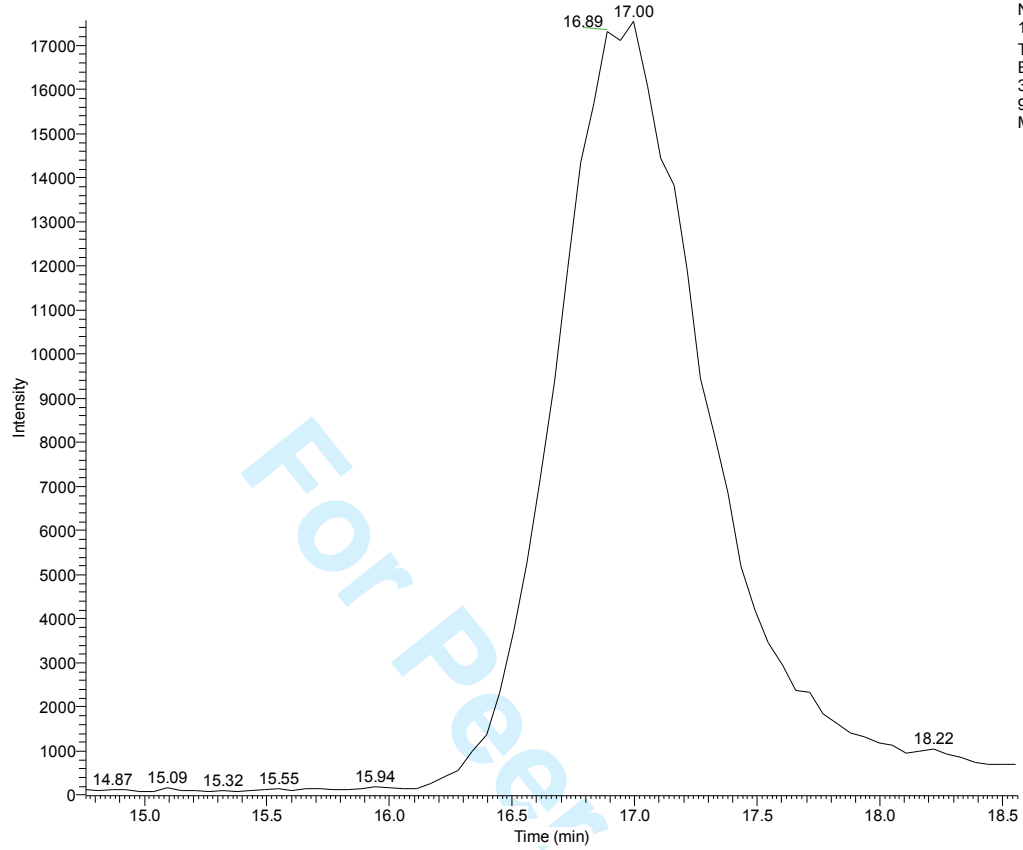
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Naheed1 #722-747 RT: 16.75-17.18 AV: 9 NL: 6.33E3
F: ITMS + c ESI Full ms2 331.00@35.00 [90.00-400.00]



For Peer Review

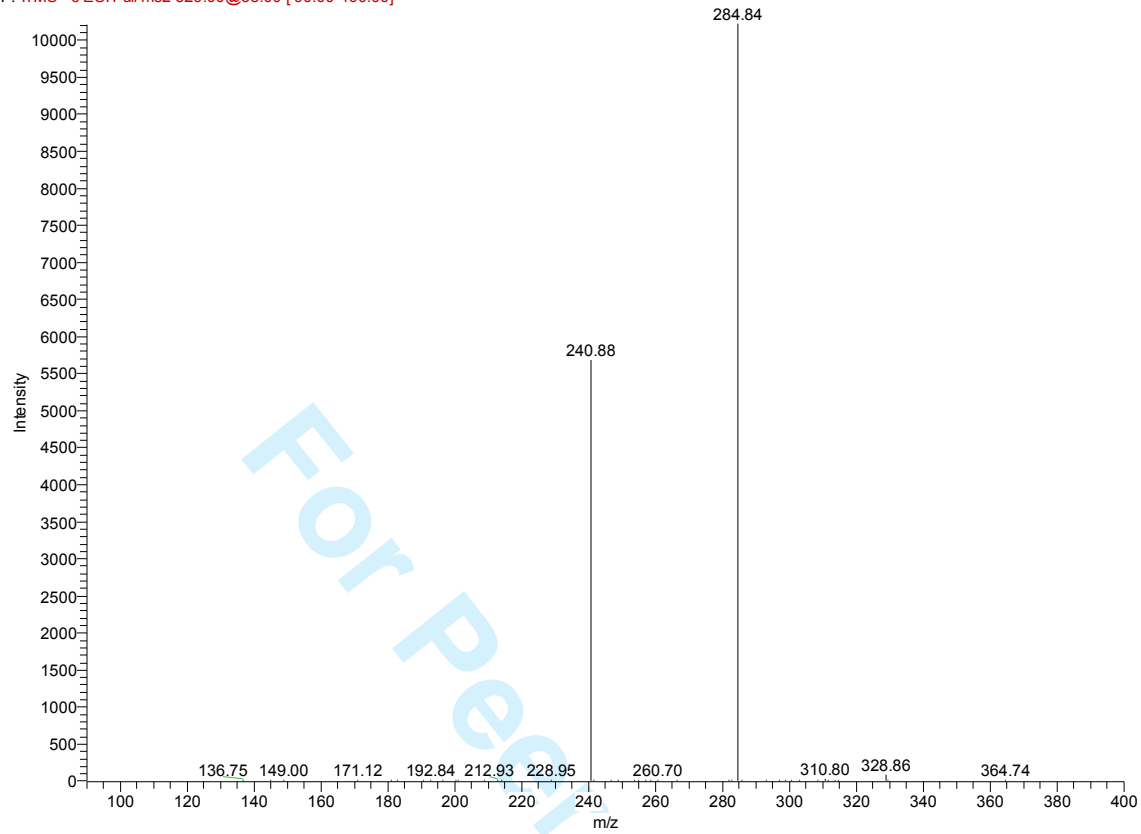
RT: 14.76 - 18.56



NL:
1.76E4
TIC F: ITMS - c
ESI Full ms2
329.00@35.00 [
90.00-400.00]
MS Naheed1

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3 Naheed1 #727-746 RT: 16.83-17.16 AV: 7 NL: 1.02E4
4 F: ITMS - c ESI Full ms2 329.00@35.00 [90.00-400.00]



15% SDS-PAGE OF pEC86, pKK-187, pEC86/pEVLH32 (1), pEC86/pINK-LH-His₄ (2)

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