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Ruth McNerney[1*], Kim Mallard[1], Phyllis Ifeoma Okolo[1] and Claire Turner[2]

[1]Department of Pathogen Molecular Biology, London School of Hygiene & Tropical Medicine, London, UK.
[2] Department Life, Health and Chemical Sciences, The Open University, Milton Keynes, UK.

Corresponding author:
Dr Ruth McNerney, Department of Pathogen Molecular Biology, Faculty of Infectious and Tropical Diseases, Room 358, London School of Hygiene & Tropical Medicine, Keppel Street London, WC1E 7HT, UK
Tel: 44 (0)20 7927 2346 Fax: 44 (0)20 7637 4314
Email: Ruth.Mcnerney@lshtm.ac.uk

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Abstract

The need for improved rapid diagnostic tests for tuberculosis disease has prompted interest in the volatile organic compounds (VOCs) emitted by *Mycobacterium tuberculosis* complex bacteria. We have investigated VOCs emitted by *Mycobacterium bovis* BCG grown on Lowenstein Jensen media using selected ion flow tube mass spectrometry and thermal desorption-gas chromatography-mass spectrometry. Compounds observed included dimethyl sulphide, 3-methyl-1-butanol, 2-methyl-1-propanol, butanone, 2-methyl-1-butanol, methyl 2-methylbutanoate, 2-phenylethanol and hydrogen sulphide. Changes in levels of acetaldehyde, methanol and ammonia were also observed. The compounds identified are not unique to *M. bovis BCG* and further studies are needed to validate their diagnostic value. Investigations using an ultra-rapid gas chromatograph with a surface acoustic wave sensor (zNose) demonstrated presence of 2-phenylethanol (PEA) in the headspace of cultures of *M. bovis* BCG and *Mycobacterium smegmatis,* when grown on Lowenstein Jensen supplemented with glycerol. PEA is a reversible inhibitor of DNA synthesis. It is used during selective isolation of gram positive bacteria and may also be used to inhibit mycobacterial growth. PEA production was observed to be dependent on growth of mycobacteria. Further study is required to elucidate the metabolic pathways involved and assess whether this compound is produced during *in vivo* growth of mycobacteria.

Introduction

Mycobacteria are ubiquitous in the environment. They are slow growing bacteria that are characterised by their lipid rich, hydrophobic cell wall. In addition to non pathogenic organisms that reside in the natural environment the genus includes human and animal pathogens of considerable social and economic consequence. The most important mycobacterial pathogens belong to the *Mycobacterium tuberculosis* complex, which is a group of closely related bacteria responsible for tuberculosis disease (TB) in humans and animals. TB remains a serious threat to public health with over 9 million new cases each year and nearly two million deaths (World Health Organisation, 2010). Early diagnosis and treatment is vital to control the disease which spreads via contaminated aerosols exhaled by patients with respiratory forms of the disease. The need for low cost rapid tests has led to a renewed interest in detection of volatile organic compounds (VOC) as a means of detecting active disease (McNerney & Daley, 2011). Olfactory sensing by African pouch rats suggests that animals conditioned to detect headspace gases from *Mycobacterium tuberculosis* can identify infected sputum samples taken from patients with pulmonary tuberculosis (Weetjens*, et al.*, 2009). To improve knowledge of volatile compounds emitted by mycobacteria we examined the headspace gases above cultures of the vaccine strain *Mycobacterium bovis* Bacillus Calmette-Guérin**,** (BCG). Volatile compounds from BCG were identified by mass spectrometry and headspace from bacterial cultures was monitored in real time using a miniaturised gas chromatograph coupled to a surface acoustic wave sensor. Headspace gases from cultures were compared to those from media incubated under identical conditions but not inoculated with bacteria and with Lowenstein Jensen impregnated with *p*-nitrobenzoic acid, an inhibitor of *M. tuberculosis*. We also investigated *Mycobacterium smegmatis,* a fast growing environmental species found in soil using the rapid gas chromatographic device to compare VOC production with that of the slow growing BCG.

Materials and methods

Mycobacterium bovis BCG (BB-NCIPD*,* Sofia*,* Bulgaria*)* was maintained on Lowenstein-Jensen media (LJ) supplemented with glycerol (Media for Mycobacteria, Cardiff, UK). *Mycobacterium smegmatis* Mc^2155 (Snapper*, et al*., 1990) was maintained on Middlebrook 7H9 with 1.5% agar (BDH Becton Dickinson Diagnostic Systems, Sparks, MD) enriched with 10% oleic, albumin, dextrose and catalase supplement (Becton Dickinson Diagnostic Systems, Sparks, MD)

Mass spectrometry

Prior to analysis BCG cultures were grown on LJ medium slopes in glass universal bottles for 2 weeks at 37°C until colonies were clearly visible. Three lots of three bottles of BCG on LJ medium were placed inside sampling bags made up of 1355 mm diameter Nalophan NA tubing 25 µm thick (Kalle UK). Sample bags were 40 cm long. Three lots of three bottles of uninoculated LJ slopes were also placed inside three nalophan bags to act as control samples. After insertion inside the bags, the lids to all bottles were loosened and the bags were sealed and filled with hydrocarbon free air (BOC, Guildford, UK). The bags were then placed in an incubator at 37°C for 50 hours. After 50 hours, the headspace was analysed using selected ion flow tube mass spectrometry (SIFT-MS) and thermal desorption – gas chromatography – mass spectrometry (TD-GC-MS) as described below. *M. smegmatis* was not analysed by mass spectrometry.

SIFT-MS

SIFT-MS has been described in detail previously (Spanel & Smith, 2011). It is a real time trace gas and VOC analyser especially useful when looking at low molecular mass compounds; it is also better at obtaining quantitative data than GC-MS as the headspace is analysed directly. Analysis requires the generation of precursor ions which are produced in a microwave discharge and are selected by the first of two quadrupole mass filters before being injected into a fast flowing

helium carrier gas. These ions then react with the VOCs in the sample which is drawn into the flow tube via a heated capillary. The available precursor ion species are H_3O^+, NO^+ and O_2^+. The precursor and product ions in the carrier gas are sampled by a downstream orifice and pass into a differentially pumped second quadrupole mass spectrometer and ion counting system for analysis. A PDZ-Europa Mk 2 instrument was used in this study. Full spectra of the count rates at each m/z value were recorded for all the samples using each precursor ion. The identities and concentrations of various components were determined using an on-line database containing reaction rate coefficients (Smith & Spanel, 2005).

TD-GC-MS

The Nalophan bags were connected to a thermal desorption (TD) tube for subsequent analysis by GC-MS to pre-concentrate the headspace via an automated pump using 500 ml of BCG headspace gas. Standard stainless-steel sorbent cartridges were used, containing dual packing comprising 50% Tenax TA and 50% Carbotrap (Markes International Limited, Llantrisant, UK). Cartridges were conditioned before use by purging with helium carrier gas for 2 min at room temperature followed by 1 hour at 320 ºC.

Captured volatiles were analysed using an AutoSystem XL gas chromatograph equipped with an ATD 400 thermal desorption system and TurboMass mass spectrometer (Perkin Elmer, Wellesley, MA). CP grade helium (BOC, Guildford, UK) was used as the carrier gas throughout, after passing though a combined trap for the removal of hydrocarbons, oxygen and water vapour. Cartridges were desorbed by purging for 2 min at ambient temperature then for 5 min at 300 ºC. Volatiles purged from the cartridge were captured on a cold trap which was initially maintained at -30 ºC. Once desorption of the cartridge was complete, the trap was heated to 320 ºC using the fastest available heating rate and maintained at that temperature for 5 minutes whilst the effluent was transferred to the gas

chromatograph via a heated (180 ºC) transfer line coupled directly to the chromatographic column.

A Zebron ZB624 chromatographic column (dimensions 30m×0.4mm×0.25mm ID) was used (Phenomenex, Torrance, CA). The gas chromatograph oven was maintained at 50 ºC for 4 min following injection and was then raised at 10 ºC.min^{-1} to 220 ºC for 9 min. Separated products were transferred by heated line to the mass spectrometer and ionised by electron bombardment. The spectrometer was set to carry out a full scan from mass/charge ratios (m/z) 33 to 350 using a scan time of 0.3s with a 0.1s scan delay. The resulting mass spectra were combined to form a total ion chromatogram (TIC) by the GCMS integral software (TuboMass ver 4.1) and resolved compounds were identified using AMDIS software and the NIST mass spectral database. The data obtained by MS were analysed to determine the compounds which were present in more than one of the cultures and absent in the medium controls.

zNose

The zNose™ combines miniaturised gas chromatograph separation technology with a temperature controlled surface acoustic wave (SAW) detector to provide rapid monitoring of volatile compounds (Staples, 2000). Two instruments were used, a Model 7100 bench top vapour analysis system fitted with a capillary DB-624 column (Electronic Sensor Technology, Newbury Park, CA) and a Model 4200 system fitted with a DB5 column (TechMondial, London, UK). The two columns vary in their polarity, the DB-624 (6% cyanopropylphenyl, 94% dimethyl polysiloxane) being more highly polar than DB5 (5% diphenyl, 95% dimethyl polysiloxane). Liquid samples to be tested were placed in glass bottles sealed with screw caps with integral PTFE/silicone septa (Supelco, Gillingham, UK). LJ cultures to be tested were grown in universal tubes with septum caps. Headspace samples were withdrawn from the sealed bottles via a side hole Luer needle inserted through the septum. Ten second samples were taken at a flow rate of 0.5 ml sec^{-1}. All samples were taken at ambient

temperature. The DB-624 column was ramped at temperatures from 40 ºC to 140ºC at 10 ºCs^{-1} in a helium flow of 3.00 cm^3. The DB-5 column was ramped at from 40 ºC to 160ºC at 10 10 ºCs^{-1} with the same carrier gas flow. The SAW sensor operated at a temperature of 60 ºC and data were collected every 0.02 s. On encountering compounds exiting the column the SAW detector registers a depression in the frequency of the acoustic wave at its surface relative to a reference sensor. Derivativisation is performed automatically by the Microsense software (EST, Newbury Park, CA) and retention time and peak sizes are plotted. After each data sampling period the sensor was baked for 30s at 150 ºC to remove any residual deposit and an air blank was run to ensure cleaning of the system and a stable baseline. Each sampling run was completed in under two minutes. A reference standard alkane mixture supplied by the manufacturers was run at beginning of each day to ensure continuity of performance. Pure chemicals were also run to confirm that the retention times of experimental peaks identified were identical to that of the target compounds identified by TD-GC-MS.

Results

MS analysis

By carefully examining those compounds detected by TD-GC-MS in at least two of the *M. bovis* BCG cultures, but which were absent in the LJ medium controls, seven potential markers of *M. bovis* BCG were identified and are given in Table 1. In addition, SIFT-MS analysis of the culture headspace indicated that hydrogen sulphide, H_2S, was produced by *M. bovis* BCG but was absent in the medium controls. The headspace of the BCG cultures also contained significantly more acetaldehyde and methanol than was present in the headspace of the controls. Ammonia (NH_3) was significantly depleted in the culture headspace compared with the medium, indicating utilization by the mycobacteria.

zNose analysis

Headspace from LJ cultures of BCG and *M. smegmatis* was tested and the resultant chromatograms compared to LJ slopes that were not inoculated. Of the

seven VOC previously identified by TD-GC-MS one was observed to be present exclusively in the headspace of cultures. The remaining six VOC did not have retention times that coincided with peaks that varied according to growth, either they were not present in sufficient quantity or retention times coincided those of other interfering compounds in culture headspace. The observed peak ran concurrently with reference samples of phenylethyl alcohol (PEA) (Sigma-Aldrich, Gillingham, UK) on both columns. A retention time of 7.06 sec was recorded when using the zNose 7100 (Figure 1) and 3.50 sec with the zNose 4200 (Figure 3). PEA production was dependent on growth of the bacteria and when testing with the ZNose was observed when sufficient bacteria were present to be seen by eye. For *M. bovis* BCG PEA was observed in culture headspace a minimum of five days following inoculation with a 10µl loop of culture. The time taken for PEA to appear in the headspace of *M. smegmatis* culture was between one and two days. Peaks increased in size as the culture within the bottle increased and decreased when cultures reached confluence. For *M. smegmatis* the peaks were observed for a period of less than 1 week, whereas for BCG peaks were observed for up to 5 weeks (Figure 2). Growth of bacteria and production of PEA was encouraged if caps on the culture bottles were loosened during the incubation to allow exchange of gases (data not shown). The PEA peak was not observed when LJ was inoculated with heat killed bacteria. Following inoculation of LJ slopes containing compounds inhibitory to the growth of mycobacteria belonging to the *M. tuberculosis* complex, such as 0.5mg/ml p-nitro benzoic acid, PEA production was absent for BCG but present for *M. smegmatis* (Figure 3). PEA was not observed when LJ slopes were inoculated with *E. coli* DH5 or when mycobacteria were grown on Middlebrook 7H9 agar slopes (data not shown).

Discussion

MS analysis of headspace gases from cultures of BCG grown on LJ slopes demonstrated the production of volatile organic compounds including: dimethyl sulphide, 3-methyl-1-butanol (isoamyl alcohol), 2-methyl-1-propanol (isobutanol), butanone (methyl ethyl ketone), 2-methyl-1-butanol (active amyl alcohol), methyl

2-methylbutanoate, 2-phenylethanol (phenylethyl alcohol), and hydrogen sulphide. Changes in levels of acetaldehyde, methanol and ammonia were also observed. These compounds are not unique to mycobacteria and will be of limited value as individual markers for detecting *M. tuberculosis* complex bacteria. Their value may increase if used in combination as components of a mycobacterial VOC profile or 'fingerprint'. Technical difficulties also arise from the variety and size of the compounds to be investigated, which range from organic compounds to simple gases. Whereas the zNose may be used for real time detection of VOC production from bacterial cultures (Casalinuovo*, et al.*, 2006, Dawson*, et al.*, 2011), concurrent measurement of gases such as ammonia will require sophisticated analytical instrumentation not readily available to microbiology laboratories. SIFT-MS and GC-MS are large expensive instruments well suited to these types of analysis. However, although sensitive and with the ability to resolve several hundreds of compounds they are not readily suited to field deployment. The z-nose, in comparison is small, rapid and much less expensive. However, it is less able to differentiate compounds and sensitivity is lower.

It has been suggested that VOC may be used to detect tuberculosis disease. Detecting mycobacterial VOC in the headspace of clinical materials or in breath will be challenging as VOC markers produced by mycobacteria in vitro may not be detected in vivo. In addition, the relatively low concentration of such markers produced in vivo may make their detection in the presence of host VOCs difficult (Syhre*, et al.*, 2009). A more robust approach is likely to be achieved by obtaining a whole spectra of samples for TB diagnosis, and subjecting these to multivariate analysis and extensive validation to derive diagnostic algorithms. The dependency of PEA production on growth of the bacteria suggests that it could be used to assist LJ based tests for susceptibility to anti-tuberculosis drugs. However, when directly testing headspace for PEA with the zNose large numbers of bacteria were needed and for rapid drug resistance testing a VOC pre-concentration step or a more sensitive detection method would be required.

A number of other volatile compounds have recently been reported as potential markers for *M. tuberculosis* complex bacteria including 1-methylnaphthalene, 3-heptanone; methylcyclododecane; 2,2,4,4,6-pentamethyl heptane (isododecane); benzene, 1-methyl-4-(1-methylethyl)-; cyclohexane, 1,4-dimethyl-; 3,5-dimethylamphetamine; butanal, 3-methyl- (isopentanal); 2-hexene; trans-anti-1-methyldecahydronaphthalene (Phillips*, et al.*, 2007) and methyl phenylacetate, methyl p-anisate, methyl nicotinate and o-phenylanisole which are metabolites of nicotinic acid (Syhre & Chambers, 2008). It is important to note that VOC described in the published studies differ, suggesting that growth conditions and media may have a major effect on the levels of these small metabolites. The impact of such compounds on their environment and possible role during infection remains to be investigated.

Further examination of VOC in the headspace of mycobacterial cultures using the zNose found that 2-phenylethanol (PEA) was produced during growth of mycobacteria. This observation is surprising as the compound is used as an inhibitor of mycobacterial growth (Fraud*, et al.*, 2003). PEA is bacteriostatic, causing reversible inhibition of the synthesis of bacterial deoxyribonucleic acid (Berrah & Konerzka, 1962, Woodley*, et al.*, 1981). It is recommended for the selective isolation of gram-positive bacteria as it inhibits gram-negative bacteria, including *Salmonella, Shigella, Aerobacter, Klebsiella, Escherichia, Pseudomonas,* and *Proteus* (Lilley & Brewer, 1953). However, it has been reported that some gram-negative non-sporulating anaerobes are relatively resistant (Dowell*, et al.*, 1964). PEA can be produced by yeasts and some bacteria (Etschmann*, et al.*, 2002) and has been observed in gram-negative members of the *Achromobacter* genus, but not from *Moraxella* and *Acinetobacter* (Chen & Levin, 1974). PEA production has previously been reported in *Mycobacterium lepraemurium* when grown on Ogawa yolk medium (Mori & Aishima, 1992). Further investigation is required to ascertain whether PEA is produced in sufficient quantity to inhibit bacterial growth, either of the mycobacteria themselves or of other bacteria, in which case PEA production

could offer a competitive advantage. That PEA was not observed from mycobacteria growing on Middlebrook medium suggests that its production is dependent on the nutrient sources available and the metabolic pathway adopted by the mycobacteria (Barclay & Wheeler, 1989, Warner & Mizrahi, 2008). Further study is required to elucidate the metabolic pathways involved in and whether PEA is produced during *in vivo* growth of pathogenic mycobacteria.

In summary, we have identified a number of VOC produced when is BCG cultured in vitro and that PEA is produced during mycobacterial growth on an egg-based medium. Further study is required to determine the utility of VOC for the detection of mycobacteria and assess their potential role as diagnostic biomarkers.

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Table 1. Potential marker compounds determined by GC-MS. Compounds were observed in the headspace of cultures of *M. bovis* BCG, but not in uninoculated Lowenstein Jensen bottles.

Compound	**CAS No**	**Number of samples where present**	**Relative abundance***
Dimethyl sulphide	75-18-3	3/3	++++
3-Methyl-1-butanol	123-51-3	3/3	++++
2-Methyl-1-propanol	78-83-1	3/3	+++
2-methyl-1-Butanol	137-32-6	3/3	++
2-Phenylethanol	60-12-8	3/3	++
Butan-2-one	78-93-3	2/3	+
Methyl 2-methylbutanoate	868-57-5	2/3	+

***Relative amounts observed, where + refers to a headspace concentration of less than 0.3 parts per million (ppm); ++ refers to 0.3 to 0.5 ppm; +++ refers to 0.5-1 ppm and ++++ refers to more than 1 ppm.

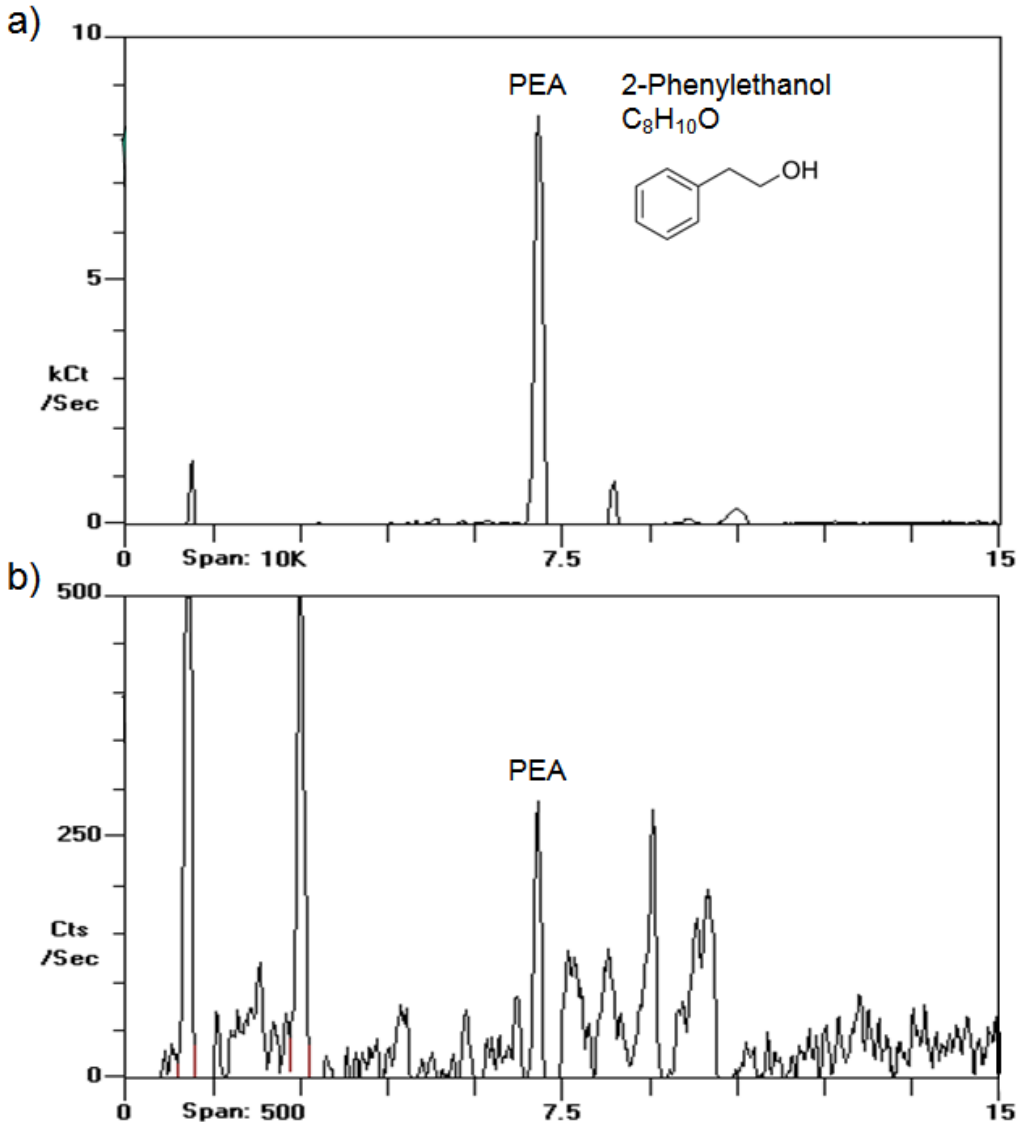
Figure legends

Figure 1. Detection of volatile 2-phenylethanol (PEA) from *M. bovis* BCG culture by ultra rapid gas chromatography. Headspace gases were tested using the zNose 7100 instrument with a DB624 column: a) PEA reference standard. One drop, diluted in 10 ml methanol; b) BCG culture growing on Lowenstein Jensen media supplemented with glycerol. A PEA peak was observed with a retention time of 7.06 sec.

Figure 2. Production of volatile 2-phenylethanol from cultures of mycobacteria over time. Levels of 2-phenylethanol in the headspace of Lowenstein Jensen cultures of a) *M. smegmatis* and b) *M. bovis* BCG as measured by sequential sampling. Triplicate culture bottles (A, B and C) were inoculated with a 10µl loop of bacteria. Bottles were sampled daily (*M. smegmatis*) or weekly (BCG) using the zNose 4200 instrument.

Figure 3. Differential inhibition of 2-phenylethanol production by mycobacteria with *p*-nitro benzoic acid. VOC profiles from the zNose 4200 instrument of a) 2-phenylethanol reference standard displaying a retention time of 3.5 sec. b) *M. bovis* BCG 5 days post subculture on LJ; c) *M. smegmatis* 18 hr post subculture on LJ containing 0.5mg ml^{-1} *p*-nitrobenzoic acid; d) *M. bovis* BCG 5 days post subculture on LJ with 0.5mg ml^{-1} *p*-nitrobenzoic acid. PEA peaks were observed in samples b and c, but not from sample d where BCG was sub-cultured onto LJ media containing inhibitory levels of *p*-nitrobenzoic acid.

Figure 1


a)
10
5
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kCt
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PEA
2-Phenylethanol
$C_8H_{10}O$
OH
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Span: 10K
7.5
15
b)
500
250
0
Cts
/Sec
PEA
0
Span: 500
7.5
15

Figure 2

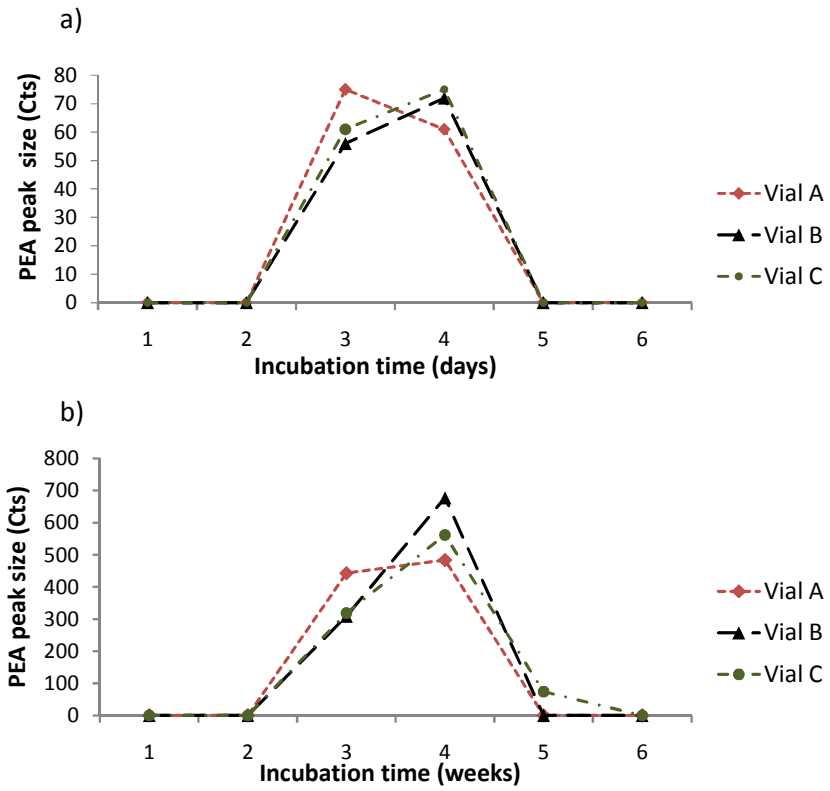
a)
PEA peak size (Cts)
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
Incubation time (days)
Vial A
Vial B
Vial C
b)
PEA peak size (Cts)
800
700
600
500
400
300
200
100
0
1
2
3
4
5
6
Incubation time (weeks)
Vial A
Vial B
Vial C

Figure 3