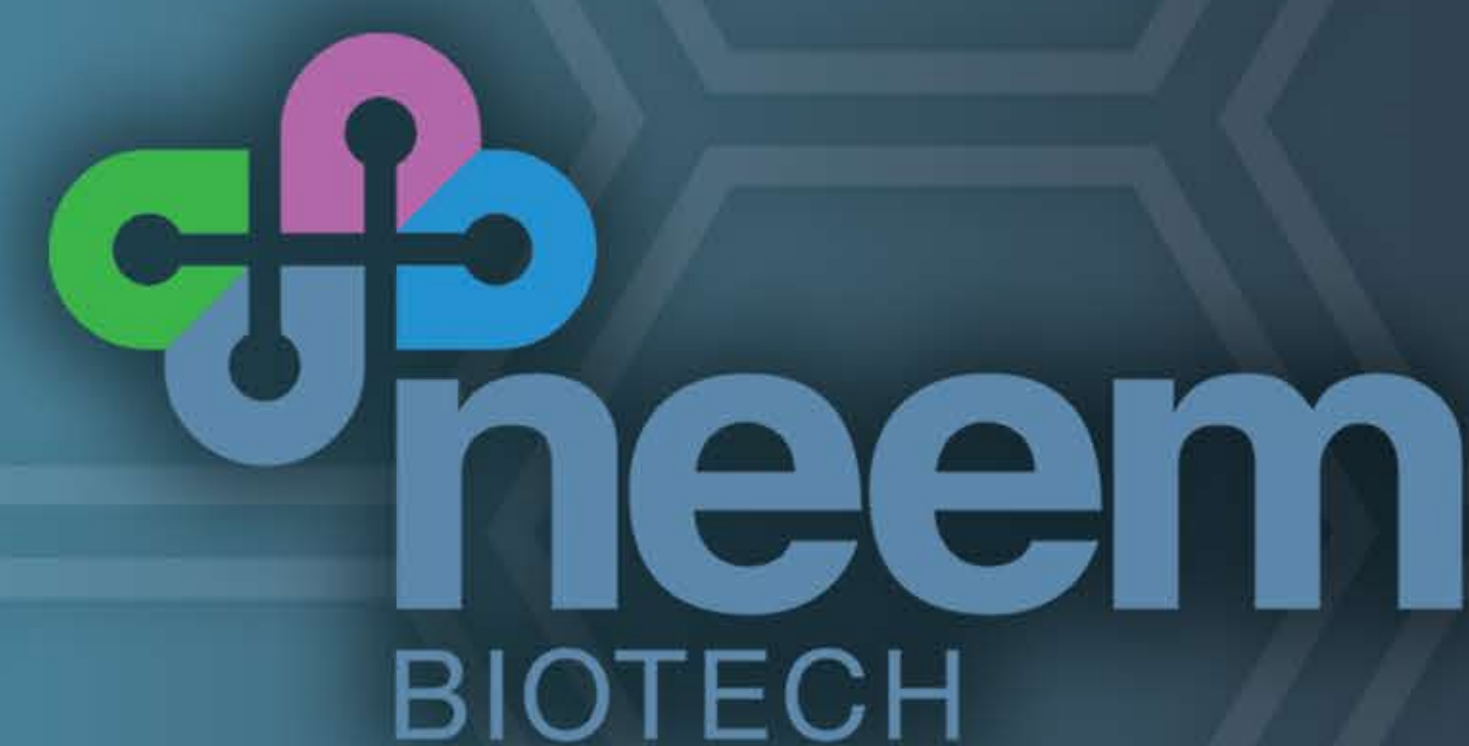


# QUANTITATIVE ANALYSIS OF AN OIL BASED NATURAL COMPOUND EXTRACTED FROM PLASMA



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## BACKGROUND

The ability to measure concentrations of a molecule underpins the building of a comprehensive knowledge of the pharmacokinetics of a pharmaceutical or nutraceutical, which is essential for the determination of its safety and efficacy. To study a compound in plasma, it must first be extracted to remove interferences including proteins and phospholipids. Following extraction, a suitable quantitative bioanalytical method, involving LC-MS/MS instrumentation, is required to determine recovery of the target compound. Compound stability in plasma is essential if the extraction and bioanalytical method are to be used for *in vivo* studies.

## AIMS

- To test the feasibility of the analysis of E/Z-Ajoene in rat plasma.
- To develop a sensitive, quantitative bioanalytical method to measure recovery of E/Z-Ajoene and an internal standard from rat plasma.
- To identify a suitable internal standard.

## METHODS

A quantitative method was developed for the analysis of a natural organic molecule, Ajoene, in rat plasma and whole blood. Ajoene was purified to 99%+ levels, in a 1:1 mixture of E and Z isomers, for development of a sample preparation and bioanalysis protocol.

E/Z-Ajoene was spiked into the plasma alone and in combination with an internal standard. Extraction of E/Z-Ajoene from plasma was achieved through protein precipitation with acetonitrile.

A bioanalysis method involving reverse-phase UHPLC-DAD-MS was developed to facilitate the quantitative analysis of E/Z-Ajoene. Recovery of both E/Z-Ajoene and the internal standard was monitored, along with matrix effects.

The method was later transferred to LC-MS/MS to achieve greater sensitivity.

### UHPLC-DAD-MS method

Column: ACE Excel 2 SuperC18 (100x2.1mm, 2µm)  
Flow rate: 0.250 mL/min  
Mobile phases: Water/0.1% formic acid (A), Acetonitrile/0.1% formic acid (B)  
Gradient: 50-70%B, 5 minutes  
Column temperature: 30 °C  
Detection: UV 254nm, MSD ESI+ SIM m/z 235 & 183.

### LC-MS/MS method

Column: Agilent Zorbax SB-C18 (50x2.1mm, 3.5µm)  
Flow rate: 0.500 mL/min  
Mobile phases: Water/0.2% formic acid (A), Acetonitrile/0.2% formic acid (B)  
Gradient: 30-80%B, 3.5 minutes  
Column temperature: 40 °C  
Detection: Triple Quadrupole ESI+ MRM – E/Z-Ajoene m/z 235 (Q1) • m/z 111.0, 103.0, 67.0 (Q3). Internal standard m/z 251 (Q1) • m/z 109.0, 77.0 (Q3).

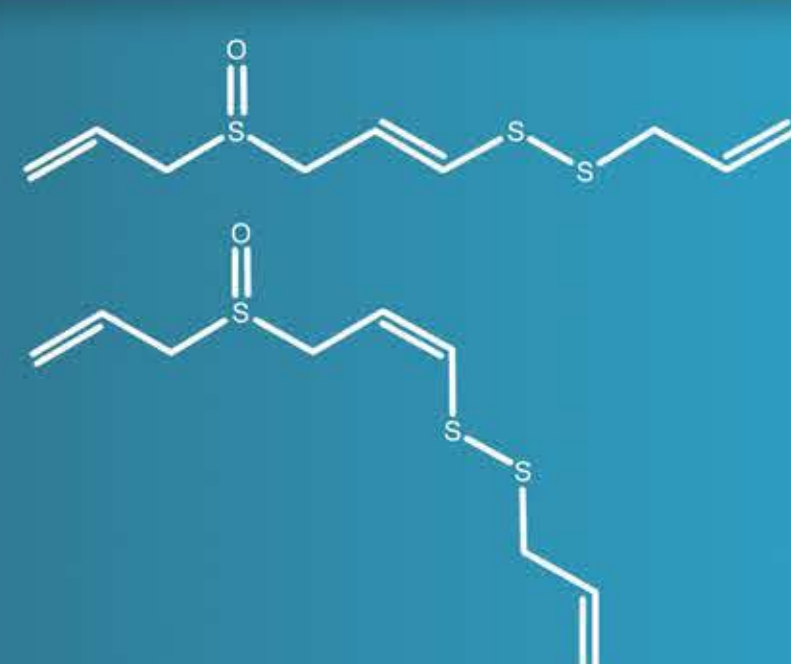


Figure 1. E-Ajoene & Z-Ajoene

## RESULTS

### 1. Preliminary testing LC-MS

E/Z-Ajoene was extracted from rat plasma following 20 minutes at room temperature. E/Z-Ajoene gave a linear response between 10-200 µg/mL and the LLOQ was 10 µg/mL (fig.3). Recovery of E/Z-Ajoene was between 28-40% and recovery of the internal standard was 44-72% (fig.4).

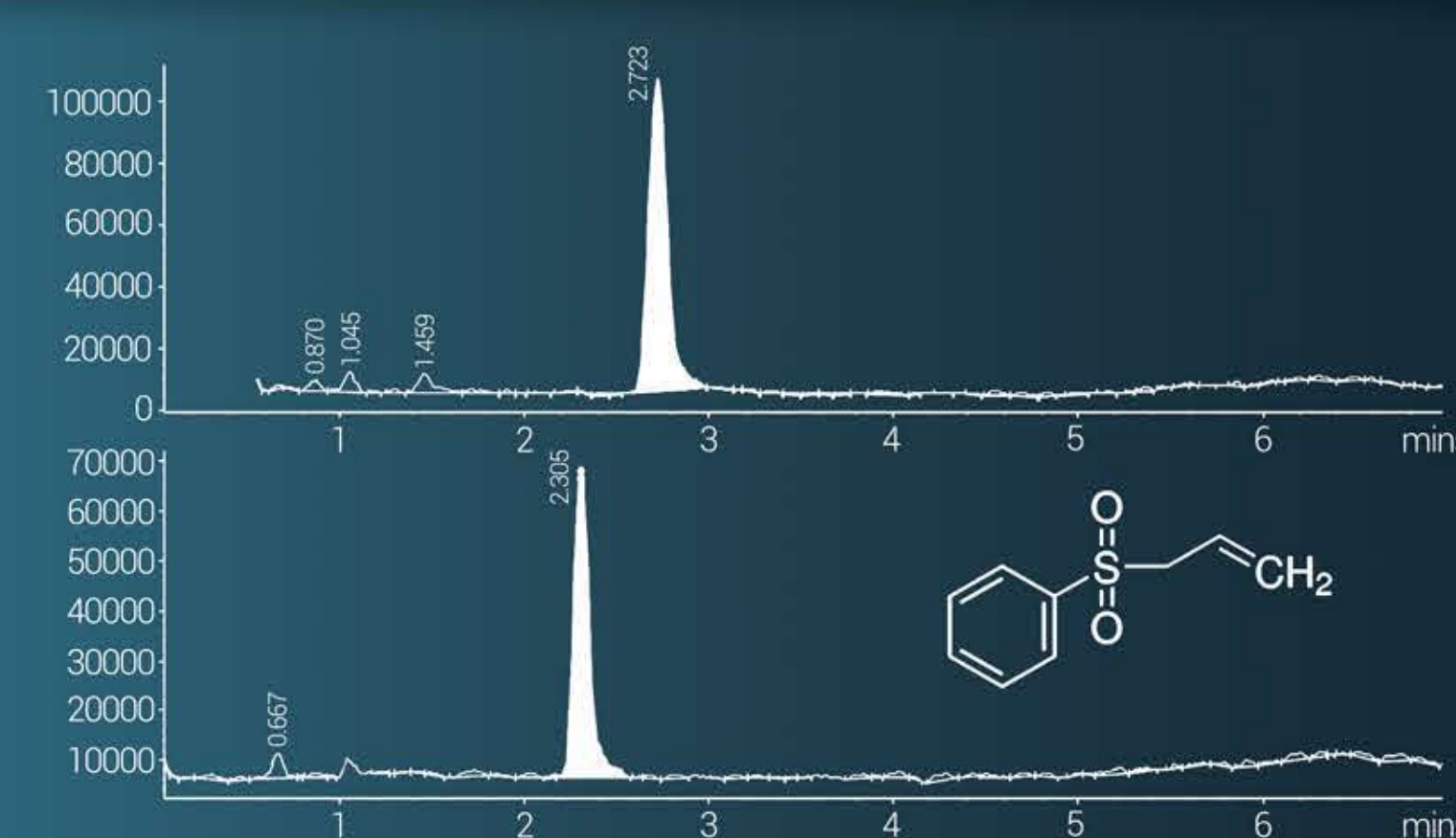


Figure 2. Total ion chromatogram in positive ESI mode for E/Z-Ajoene (upper) and internal standard Allyl Phenyl Sulfone (lower).

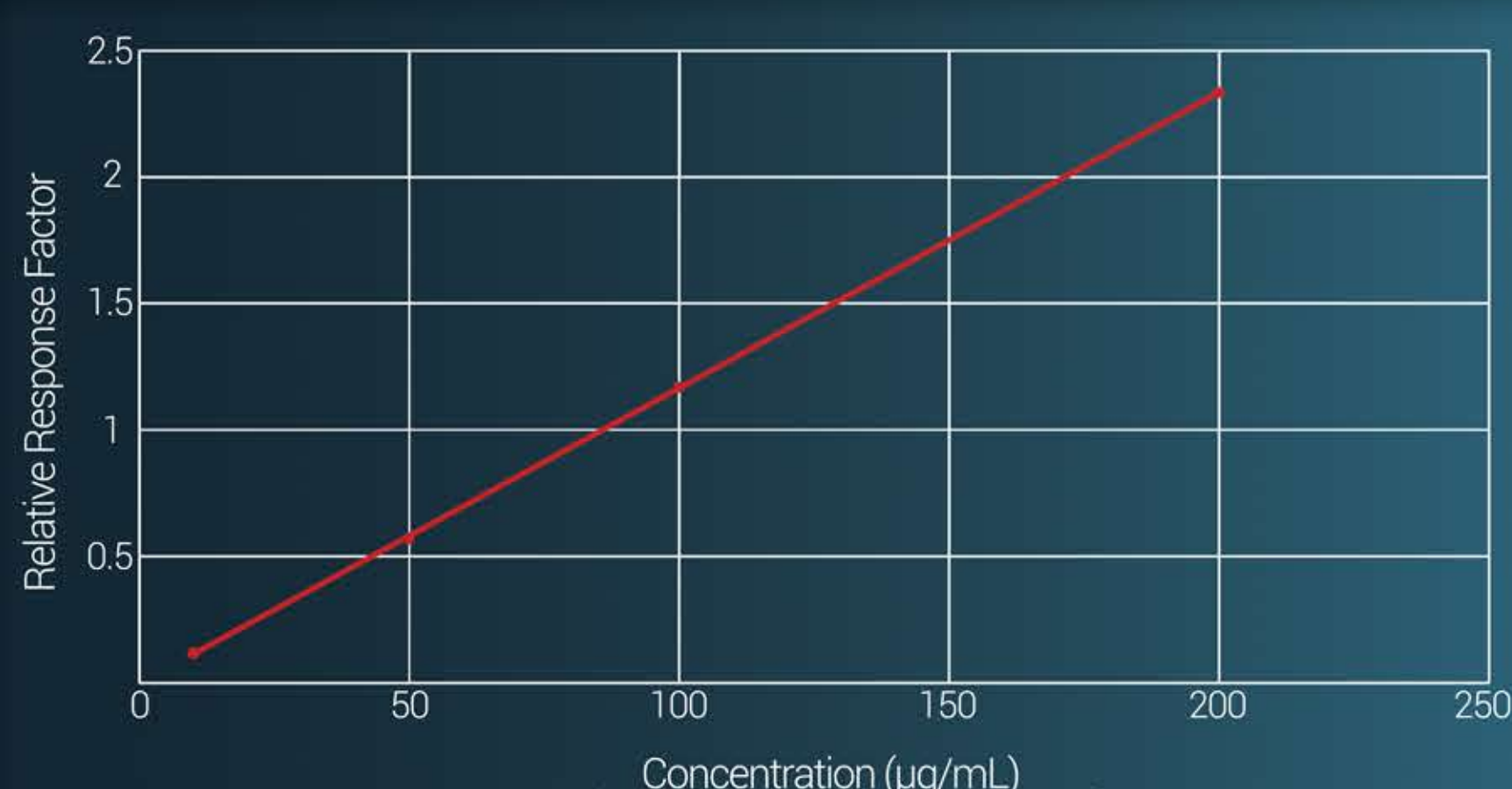


Figure 3. Calibration graph for E/Z-Ajoene (10-200 µg/mL) in rat plasma plotting relative response factor against standard concentration by reverse phase LC-MS.

Figure 4. Recovery and matrix effects calculated for E/Z-Ajoene, Allyl phenyl sulfone (IS) and the two compounds combined when extracted from rat plasma.

Sample	Recovery in plasma	Matrix effects
E/Z - Ajoene	39.5 %	27.8 % Suppression
Allyl phenyl sulfone (IS) only	44.1 %	12.6 % Enhancement
E/Z-Ajoene+ Internal standard	28.4 % E/Z-Ajoene 72.4% IS	2.1 % Enhancement 3.5% Suppression

### 2. LC-MS/MS

To quantitatively measure the lower concentrations expected in *in-vivo* samples, a more sensitive method was required. The preliminary LC-MS method was transferred to LC-MS/MS.

An alternative internal standard was introduced, S-phenyl benzenethiosulfonate, due to its greater retention on C18 and recovery from rat plasma.

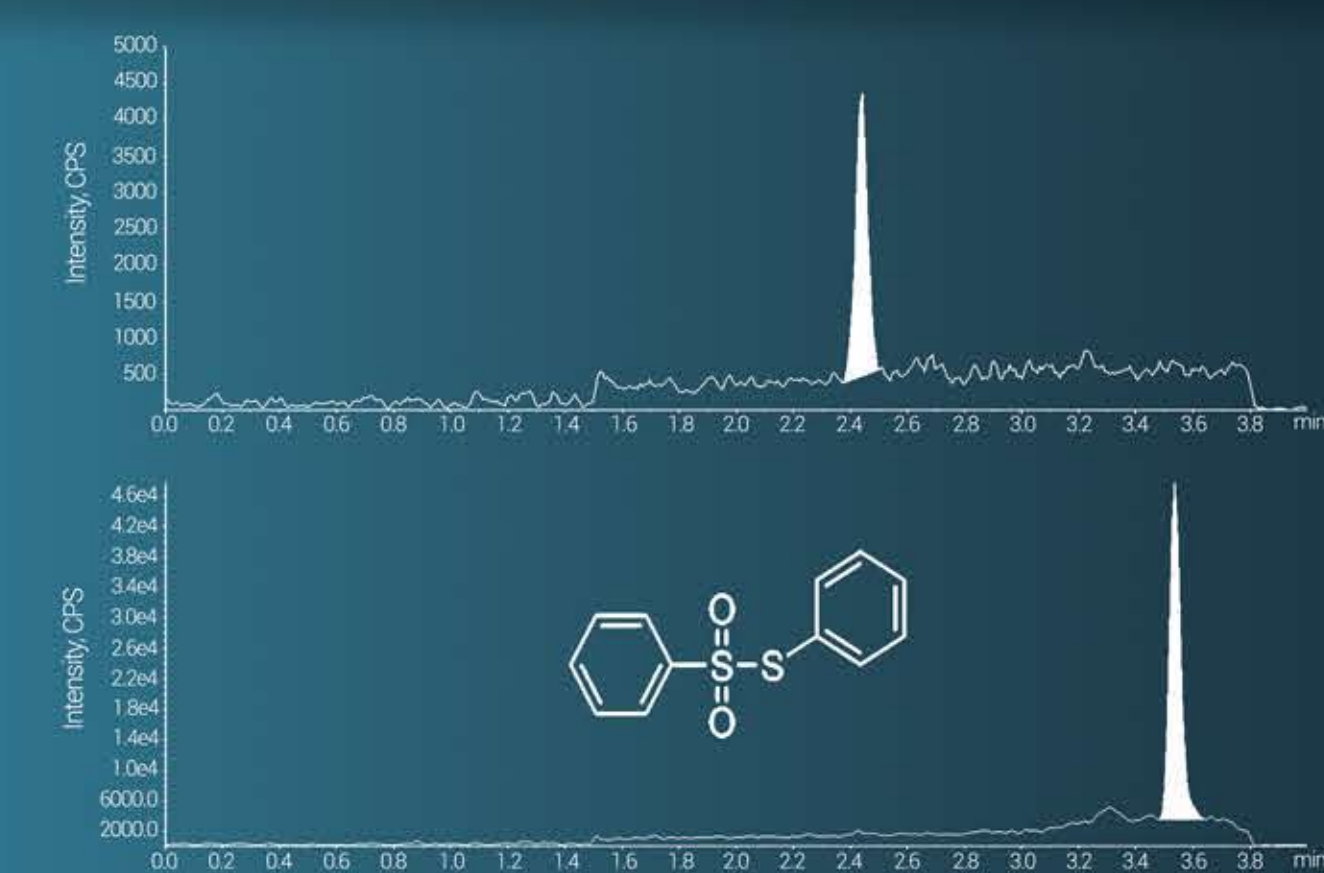


Figure 5. Total ion chromatogram in positive ESI mode for E/Z-Ajoene (upper) and internal standard S-phenyl benzenethiosulfonate (lower).

### 3. Stability

Stability was tested for E/Z-Ajoene at 10 µg/mL in rat plasma over 1 hour and 3 hours (fig.6). E/Z-Ajoene was extracted by protein precipitation and found to have degraded by 99%.

Various reagents were tested to stabilise E/Z-Ajoene in plasma over 1 hour and 3 hours (fig.6). E/Z-Ajoene was stabilised in 1% phosphoric acid on ice. Following stabilisation, E/Z-Ajoene recovery was 90-100% with an LLOQ of 10 ng/mL for rat plasma.

Linearity was tested over concentration range 10-5000 ng/mL (fig.7). The response for E/Z-Ajoene was linear from 10-2000 ng/mL. Internal standard response was consistent and repeatable over multiple injections.

Figure 6. Reagents tested for stabilisation of E/Z-Ajoene in rat plasma and their purpose

Reagent	Purpose
50mM Ascorbic Acid	Inhibit oxidation of Ajoene.
0.5% Ammonium Hydroxide	Enzyme inhibition / preventing biological degradation.
6M Guanidine	Denatures enzymes preventing non-specific binding or adherence of Ajoene to the solid pellet.
1% Phosphoric Acid	Enzyme inhibition / preventing biological degradation.
10mM Dithiothreitol	Inhibit oxidation of Ajoene.

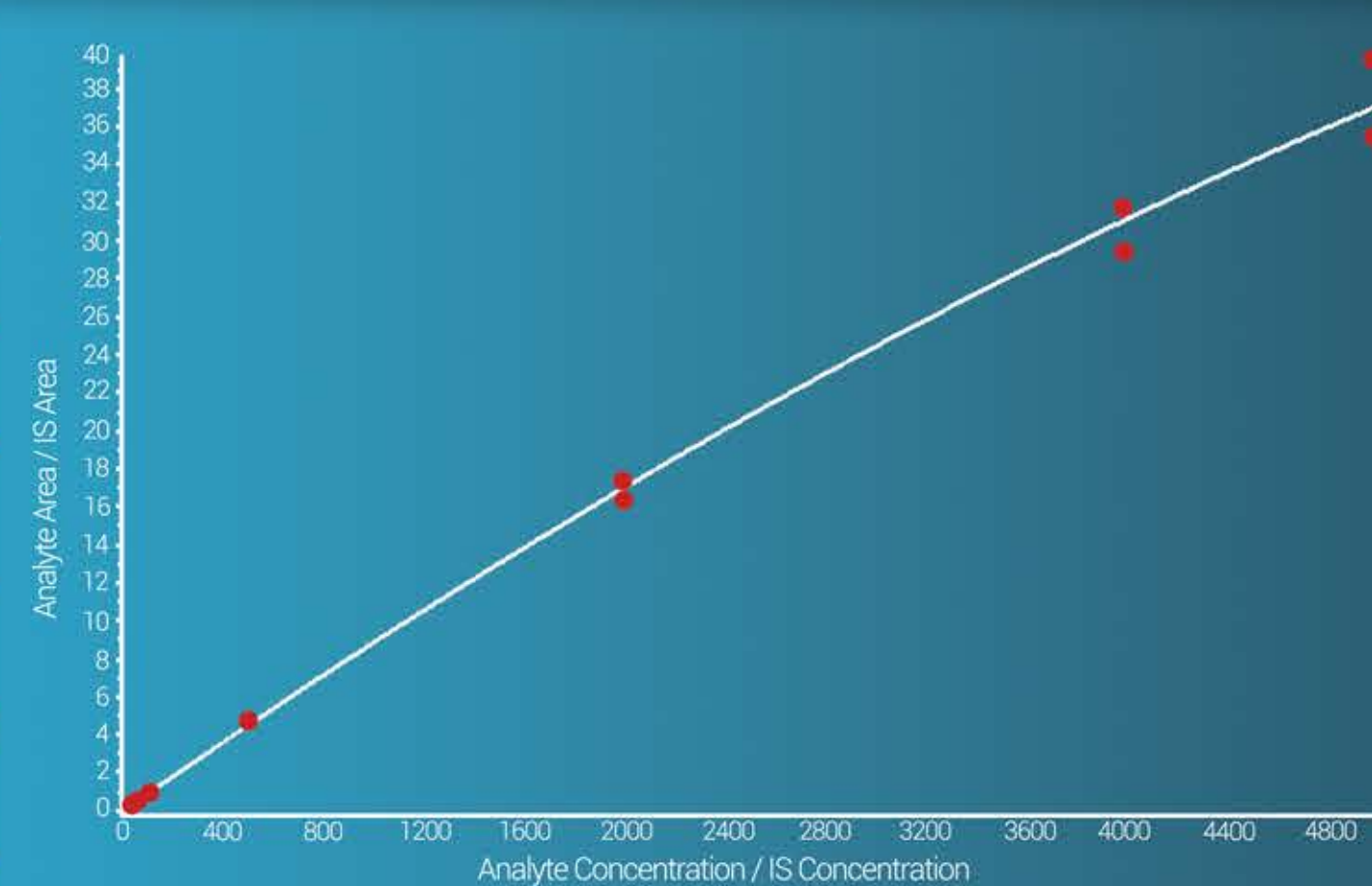


Figure 7. Calibration graph of E/Z-Ajoene (10-5000 ng/mL) in rat plasma by LC-MS/MS.

### 4. Whole blood

The stability of E/Z-Ajoene in whole blood was tested for 15 minutes on ice with and without stabiliser. Without the presence of a stabiliser, E/Z-Ajoene had degraded by 99%. The addition of 0.2% and 1% phosphoric acid caused gel formation.

E/Z-Ajoene was stabilised in whole blood by diluting the whole blood at a 1:2 ratio in water acidified with 1.5% phosphoric acid. The blood was added as soon as possible to the acidified aqueous solution and kept on ice.

A calibration range of 10-500 ng/mL of E/Z-Ajoene in whole blood was tested. The linear range was 10-250 ng/mL with a correlation coefficient of  $\geq 0.999$ .

## CONCLUSIONS

- Feasibility of quantitative analysis of E/Z-Ajoene in plasma was determined.
- A sensitive LC-MS/MS method was developed and used to monitor recovery of E/Z-Ajoene from plasma and whole blood.
- A suitable internal standard was found and showed repeatability in response through multiple analyses.
- A plasma assay has been developed but is not suitable to support *in-vivo* studies due to the instability of E/Z-Ajoene.
- A blood assay was developed that is suitable to support *in-vivo* studies.