

## S1 File:

# Thiazide and other Cl-benzenesulfonamide-bearing clinical drug affinities for human carbonic anhydrases

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2 Pharmacy Center, Institute of Biomedical Science, Faculty of Medicine, Vilnius University. Address: M. K. Čiurlionio 21/27, Vilnius, Lithuania

\*Correspondence:

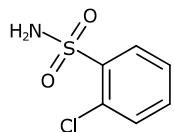
Prof. Daumantas Matulis, Department of Biothermodynamics and Drug Design, Institute of Biotechnology, Life Sciences Center, Vilnius University. Address: Saulėtekio 7, Vilnius, LT-10257, Lithuania

Email: [daumantas.matulis@bti.vu.lt](mailto:daumantas.matulis@bti.vu.lt)

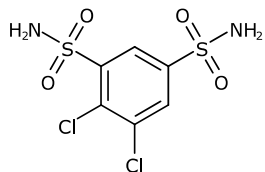
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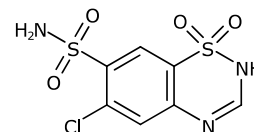
## Compounds used in this study



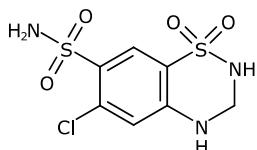
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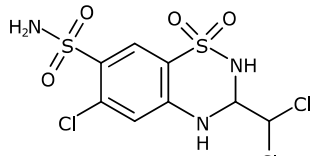
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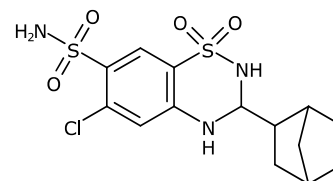
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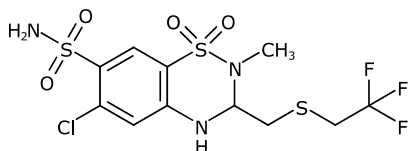
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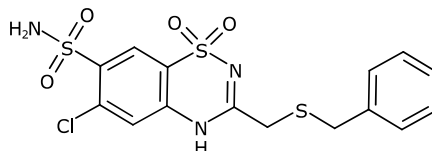
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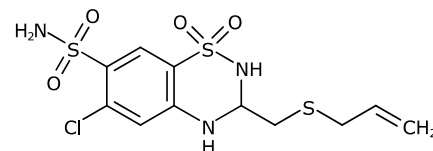
**6** cyclothiazide



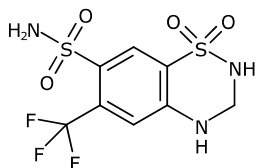
**7** polythiazide



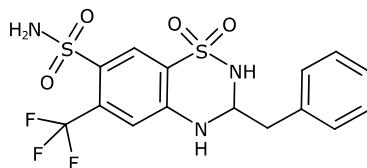
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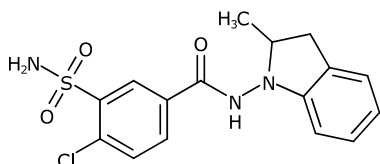
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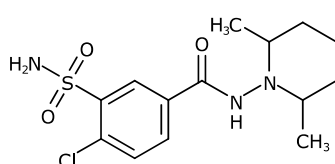
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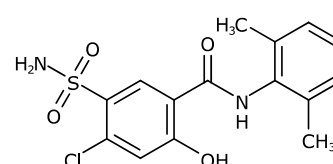
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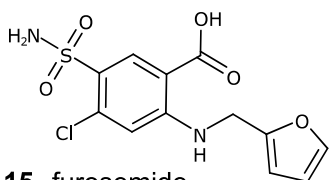
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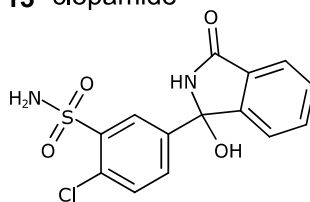
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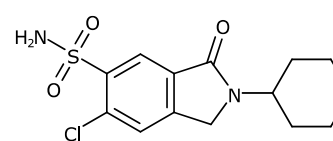
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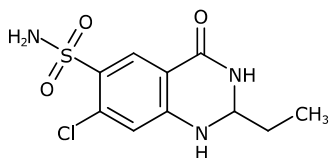
**15** furosemide



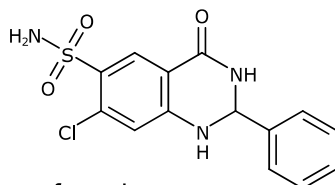
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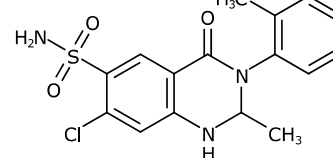
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**18** quinethazone



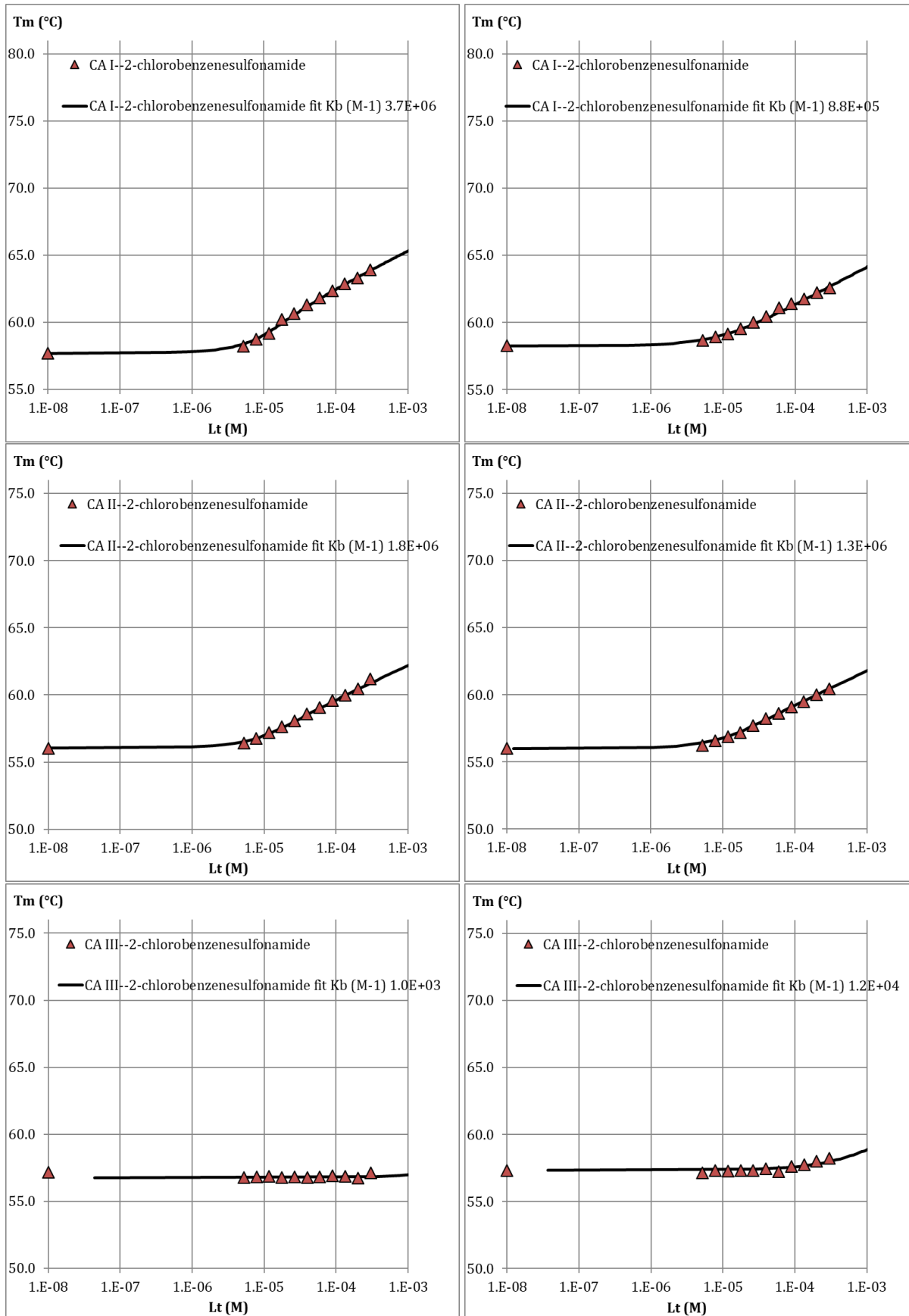
**19** fenquizone

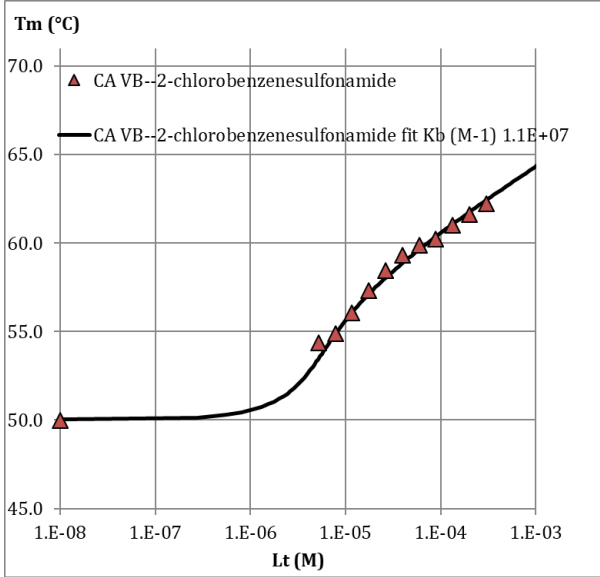
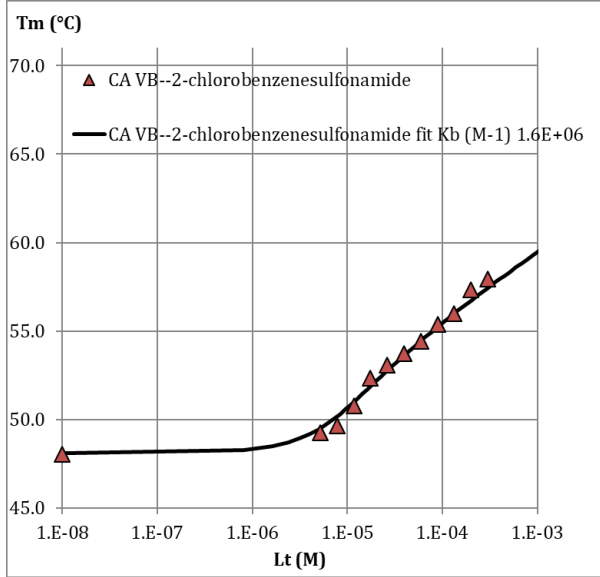
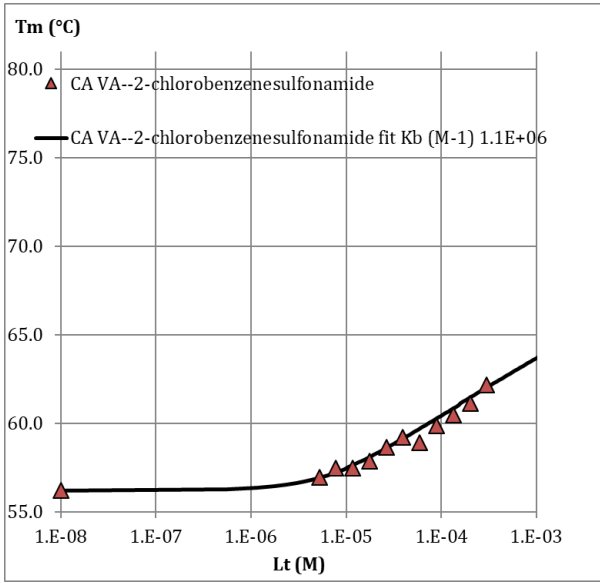
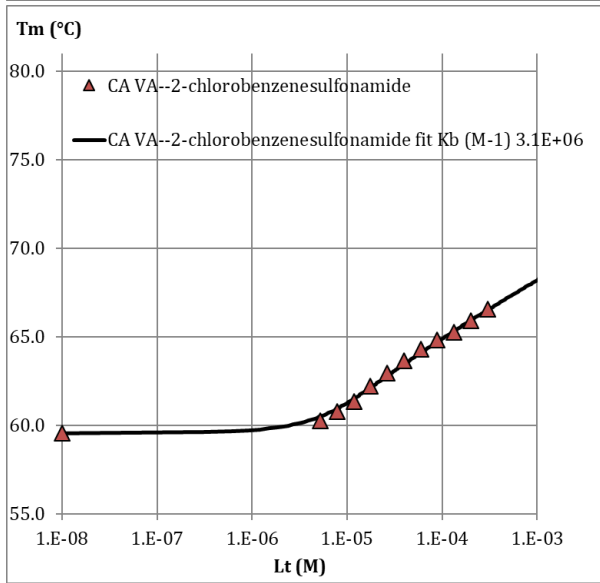
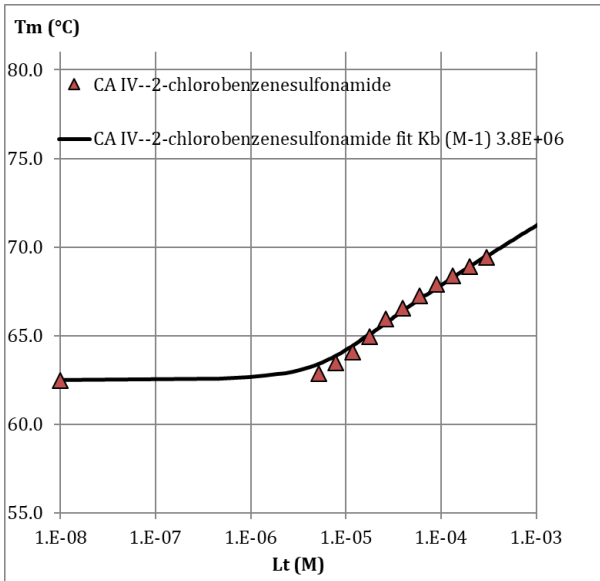
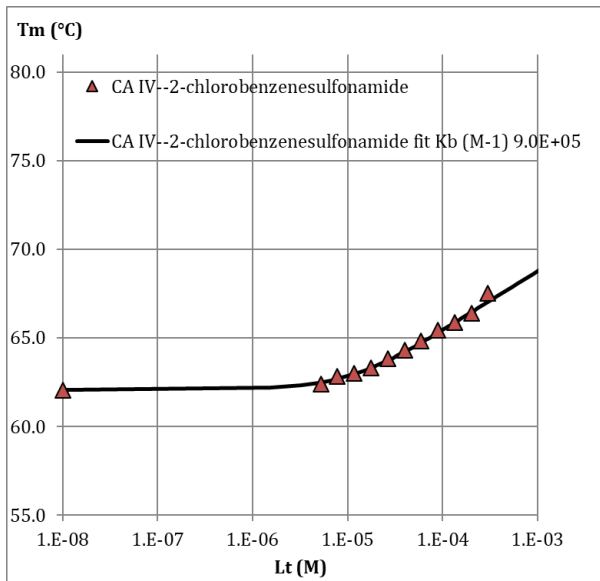


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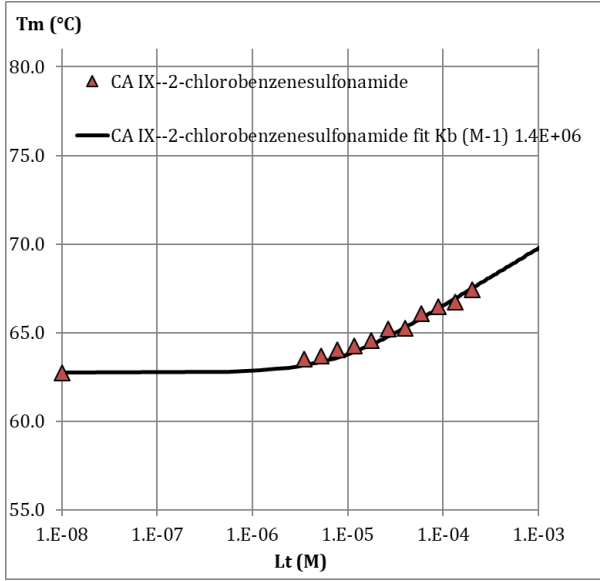
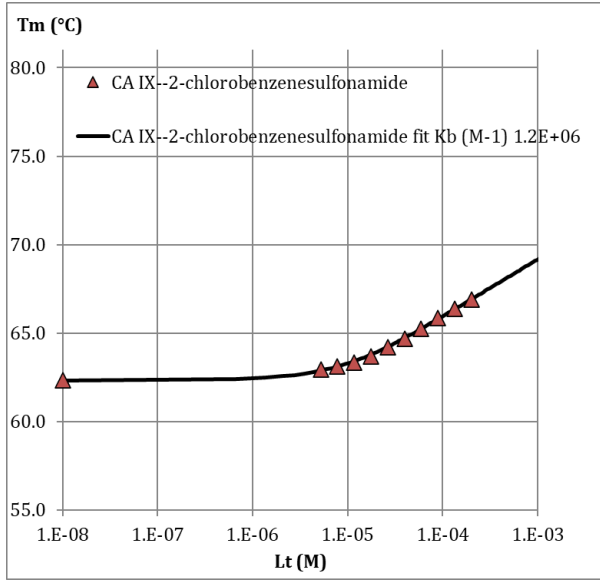
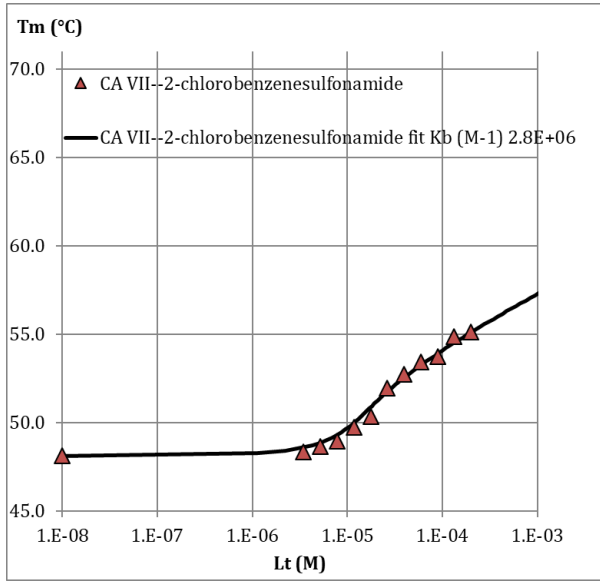
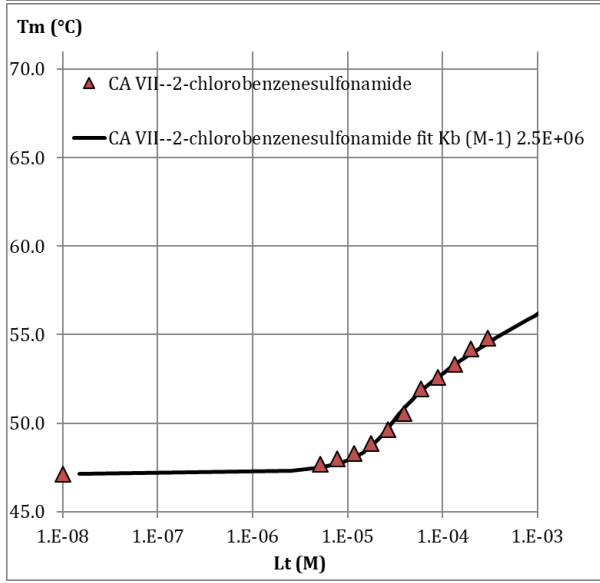
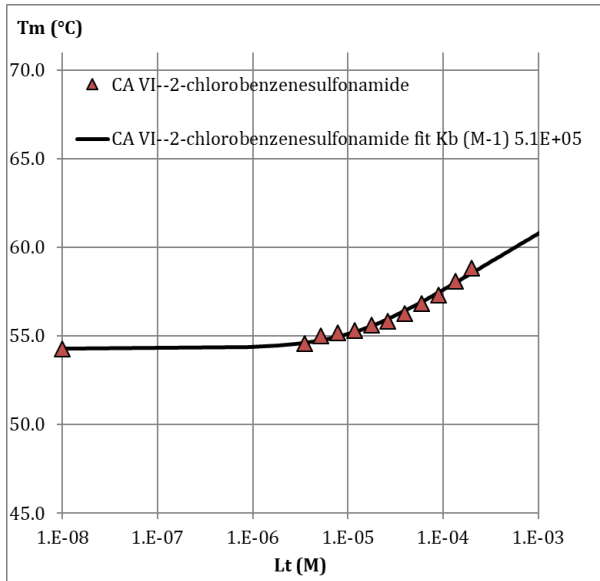
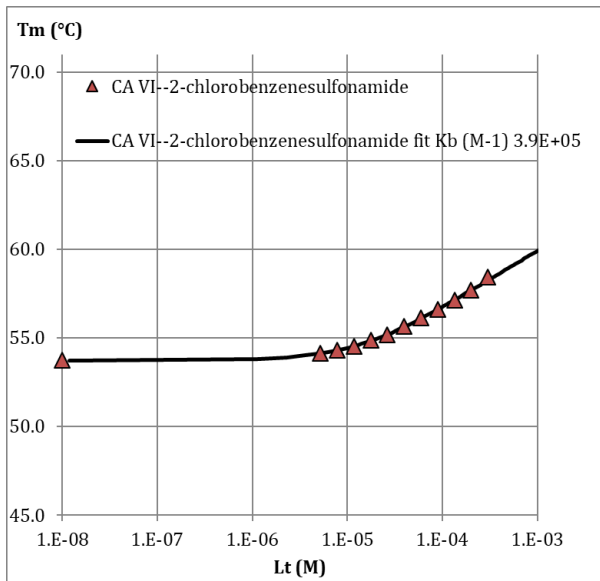
# 1. 2-chlorobenzenesulfonamide

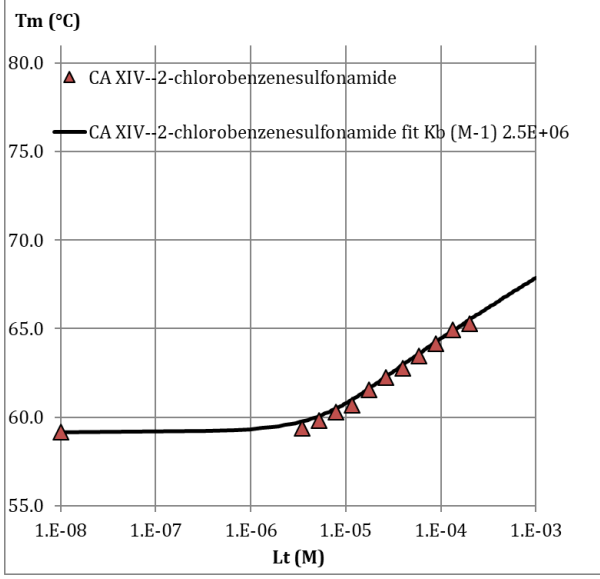
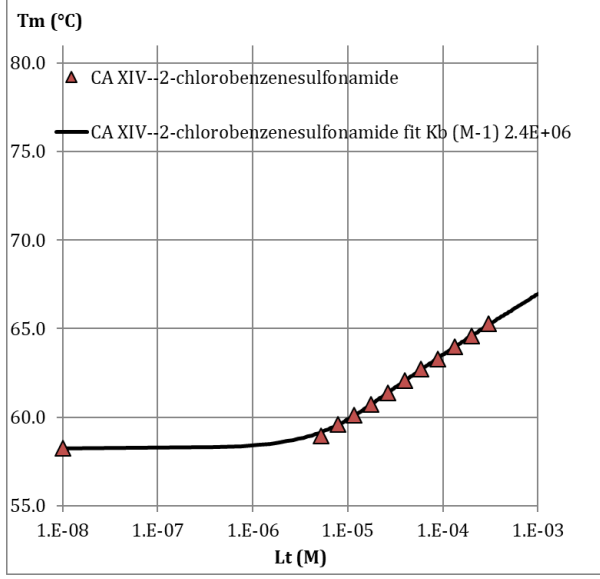
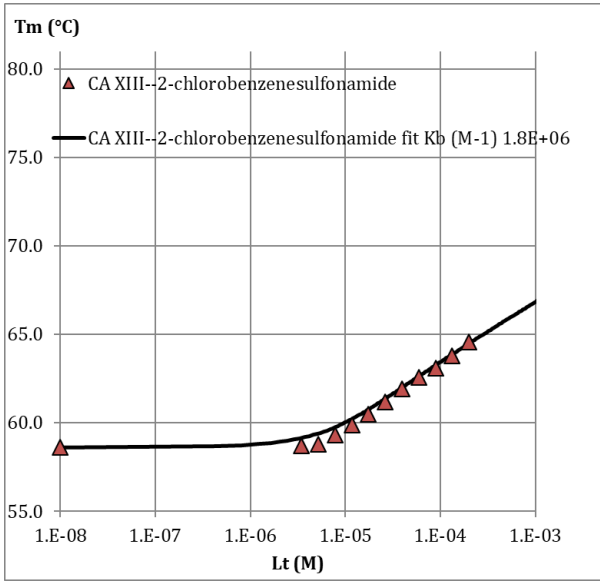
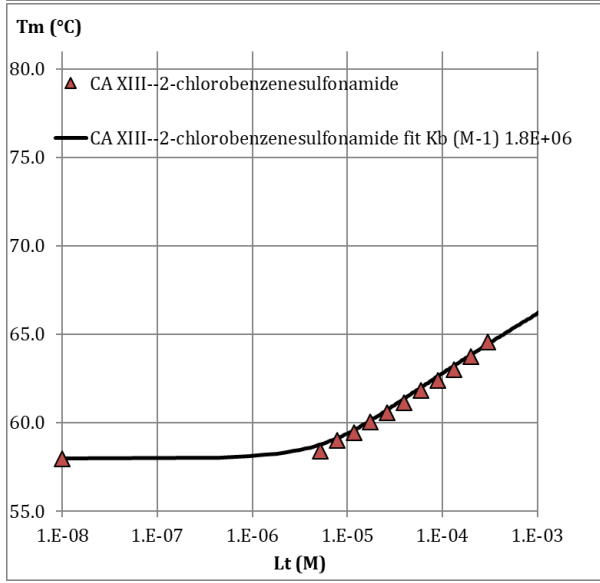
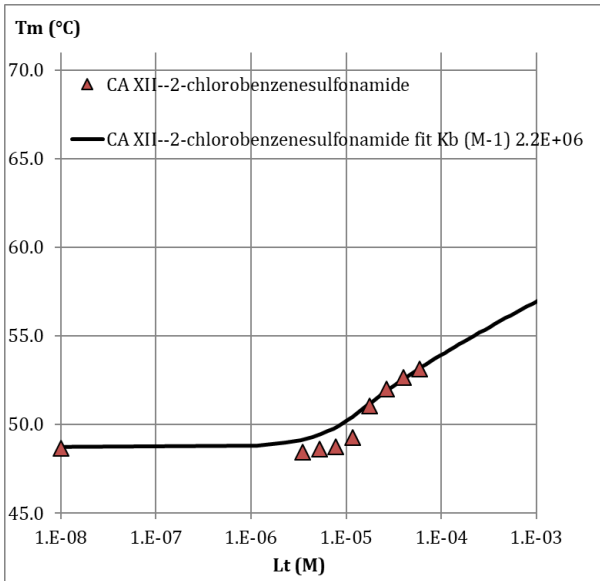
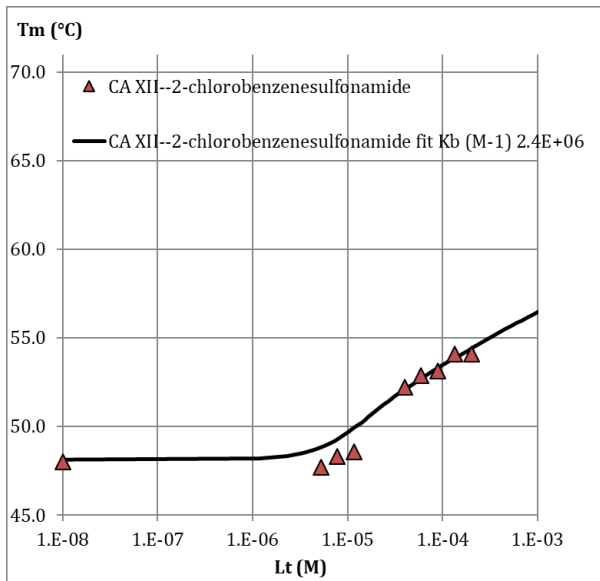
## Fluorence-based thermal shift assay





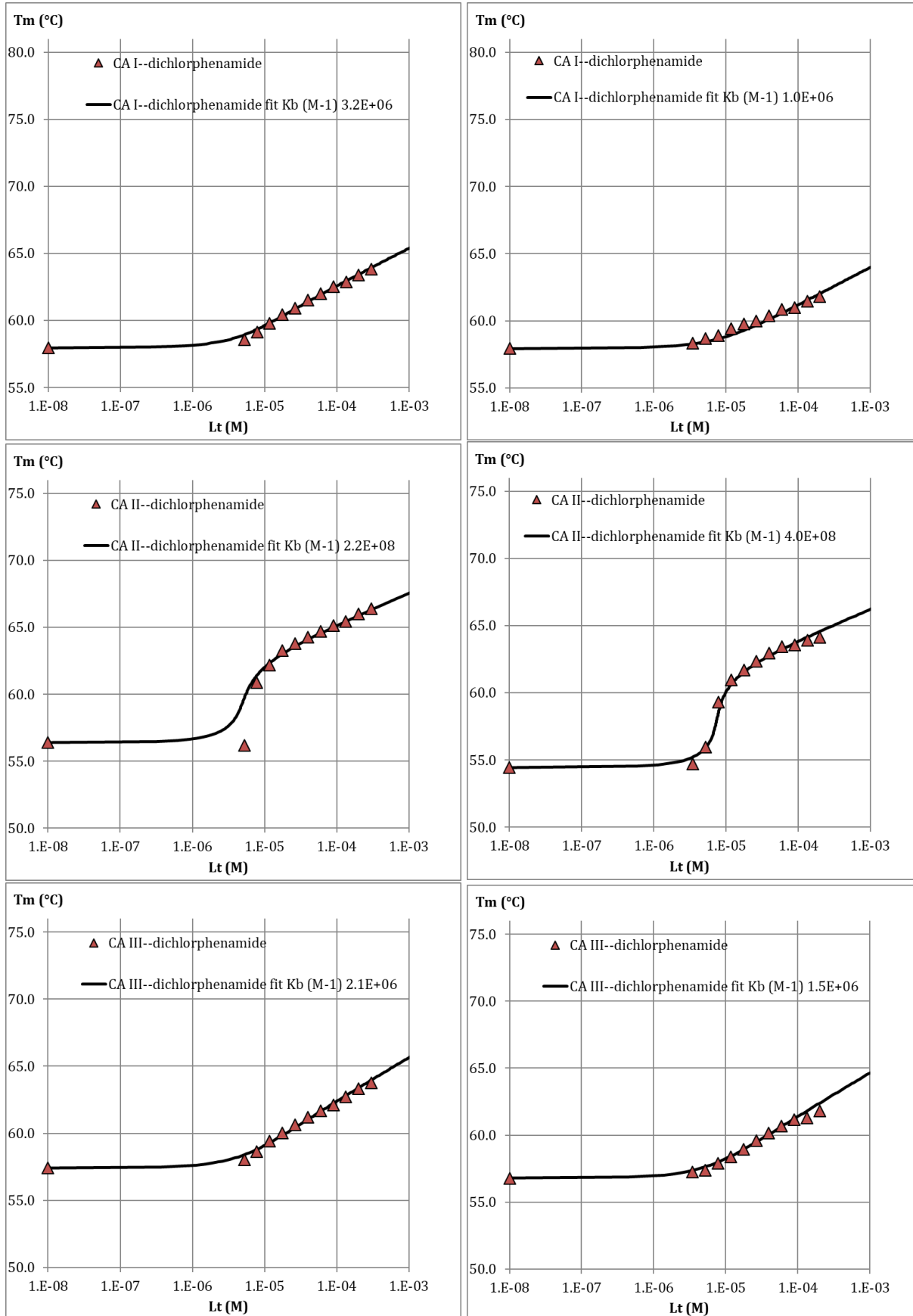


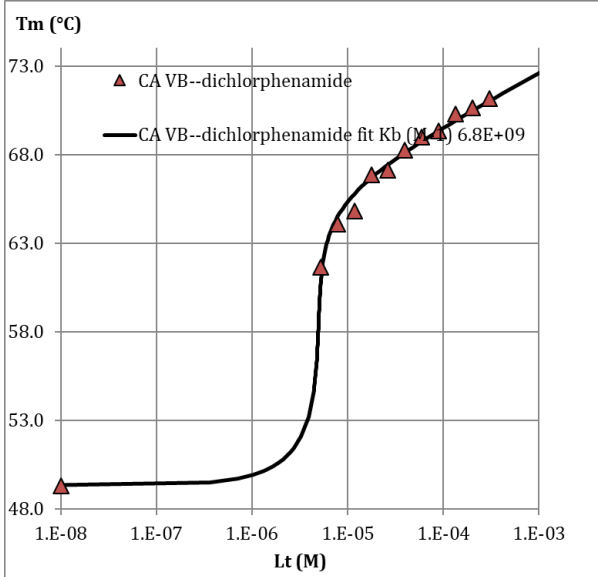
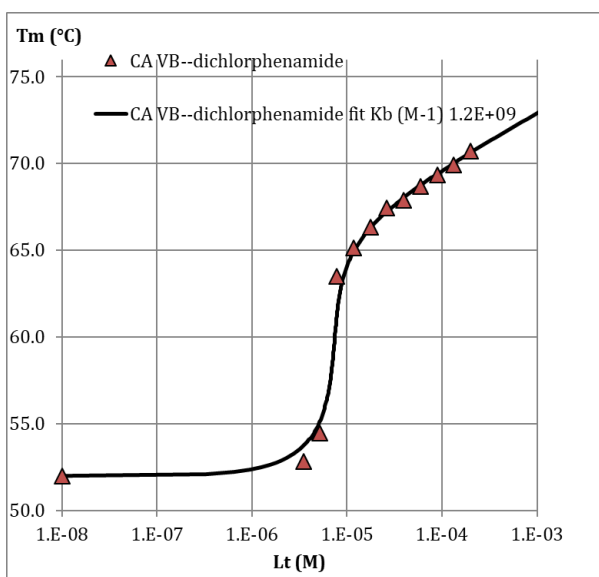
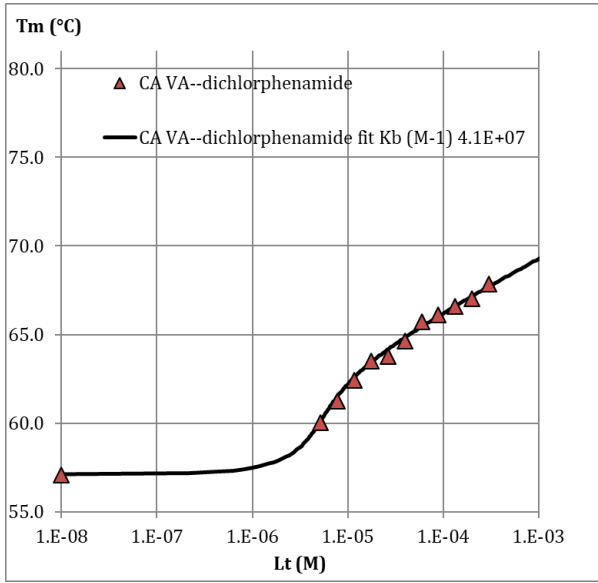
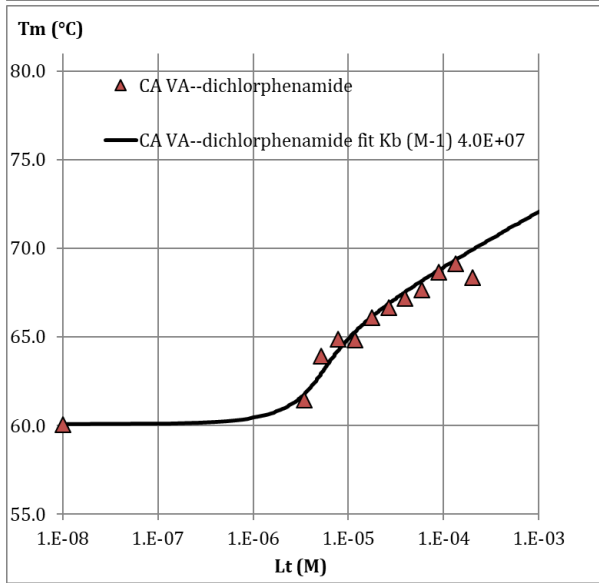
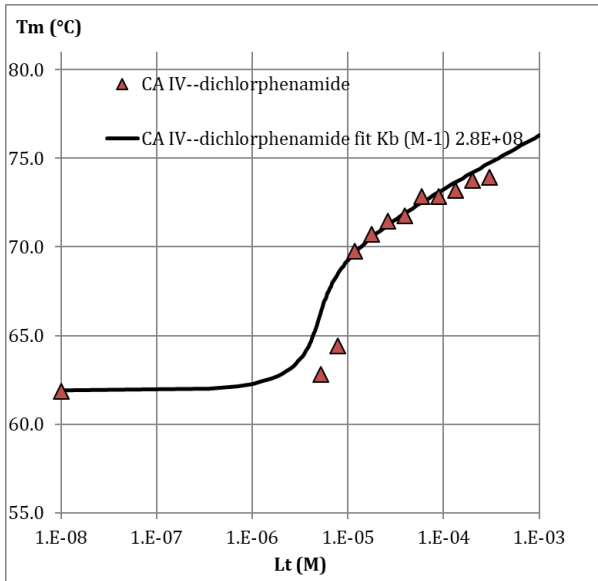
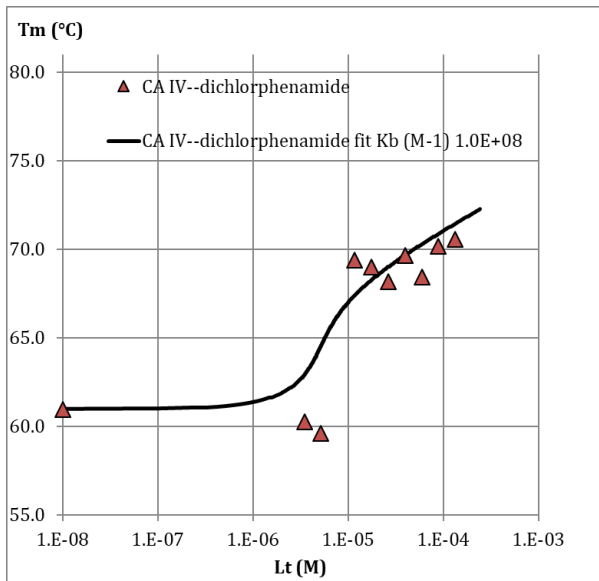


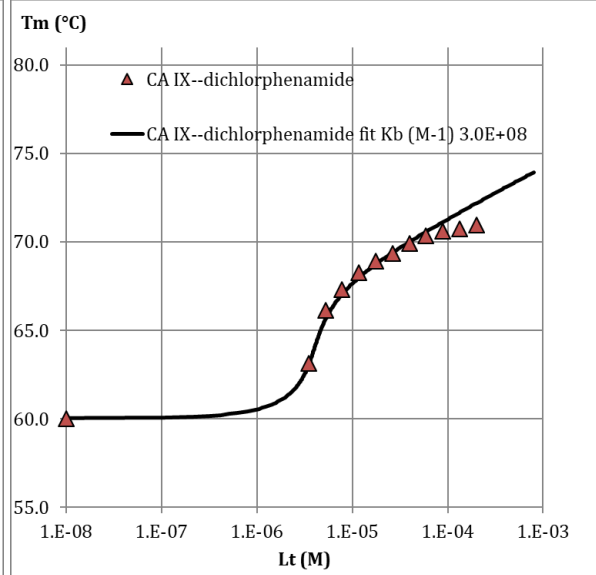
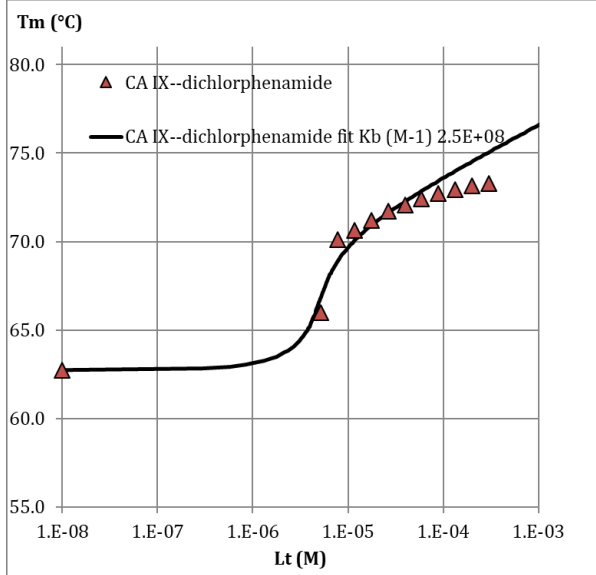
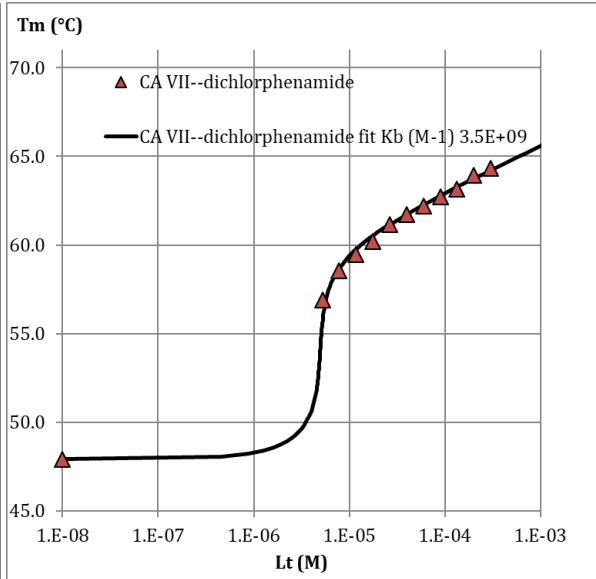
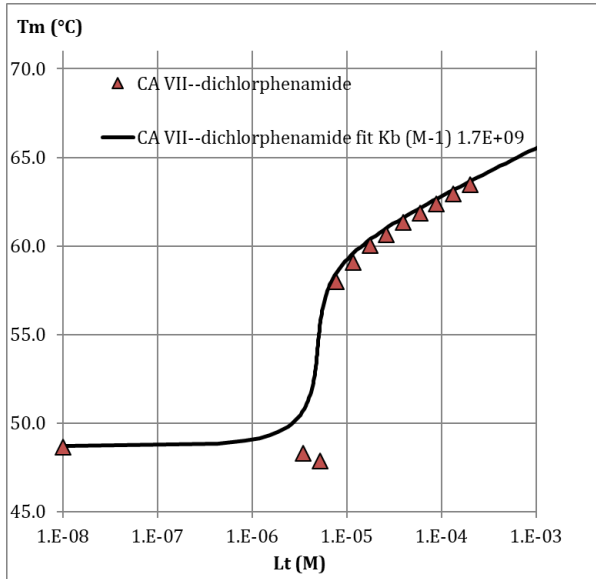
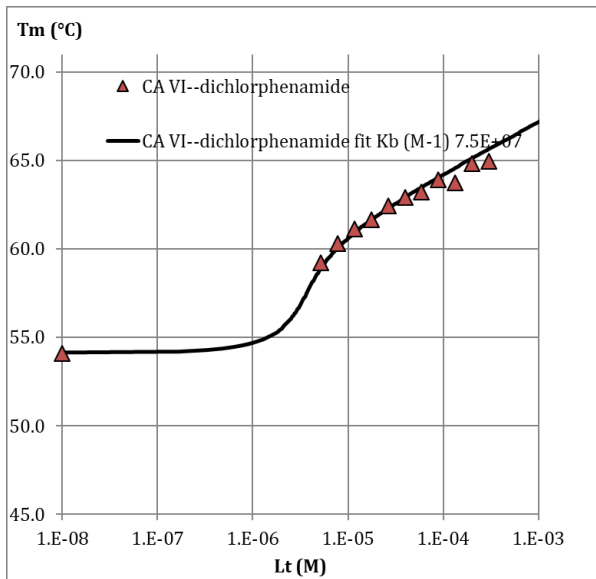
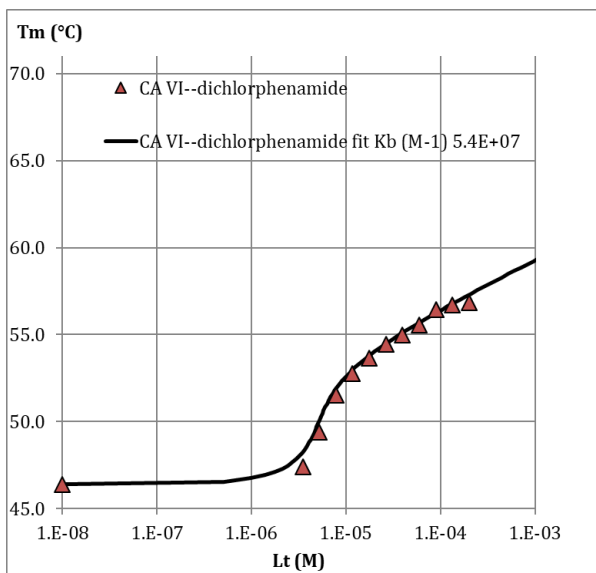


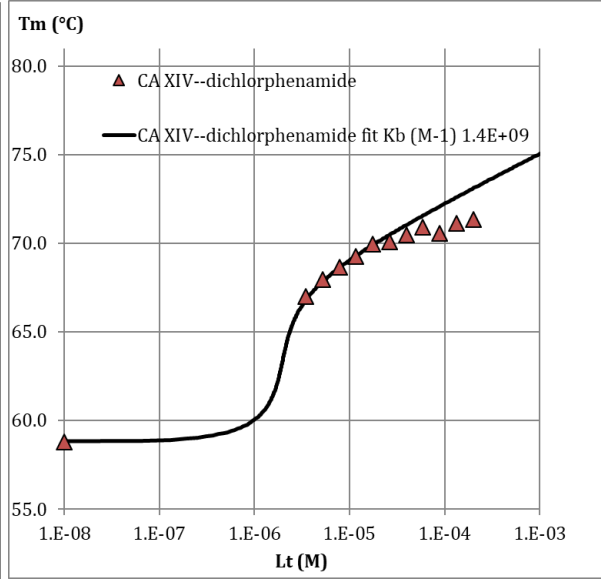
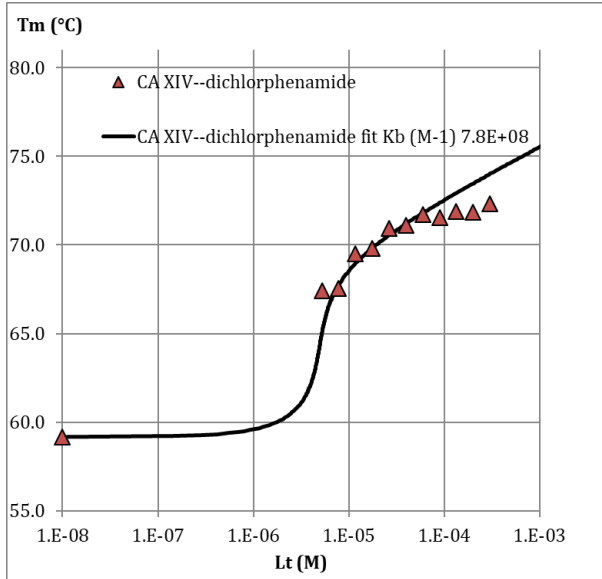
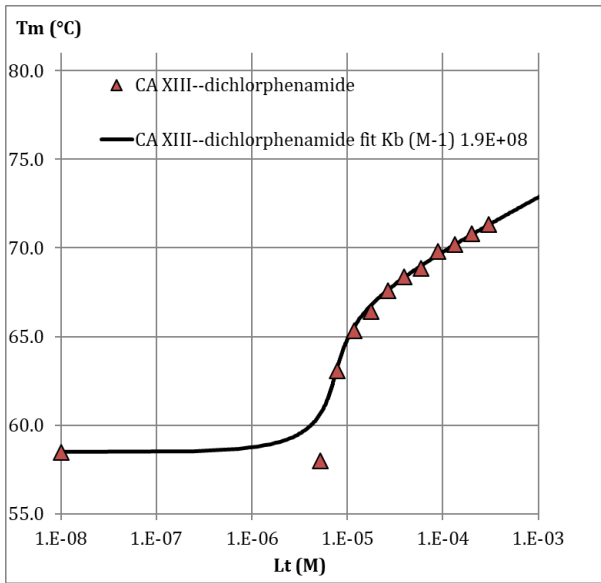
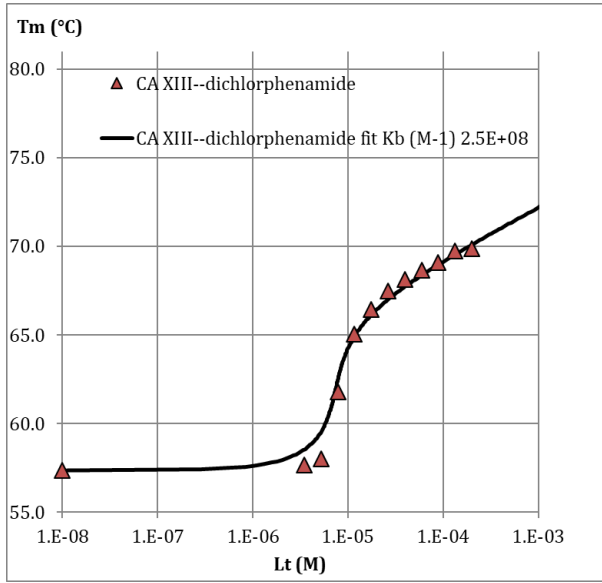
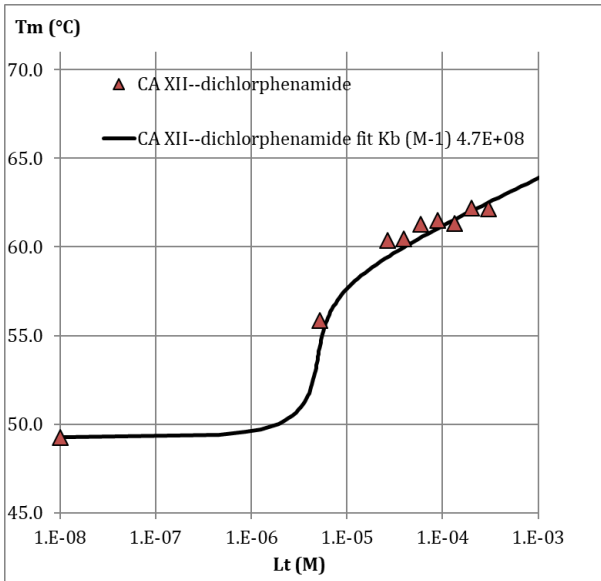
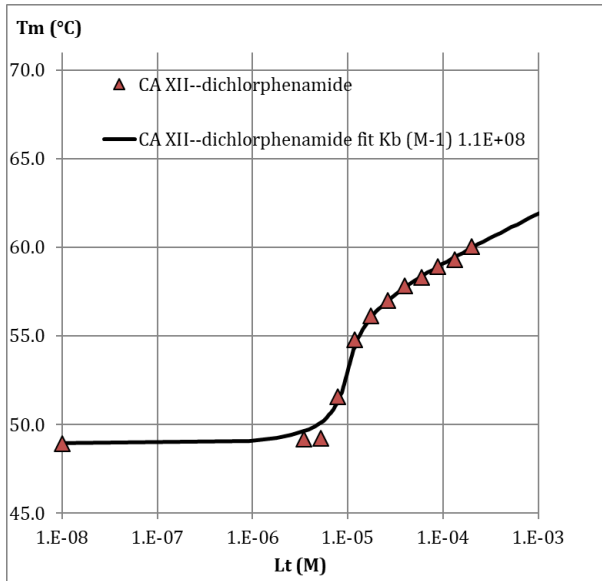
## 2. Dichlorphenamide

### Fluorence-based thermal shift assay

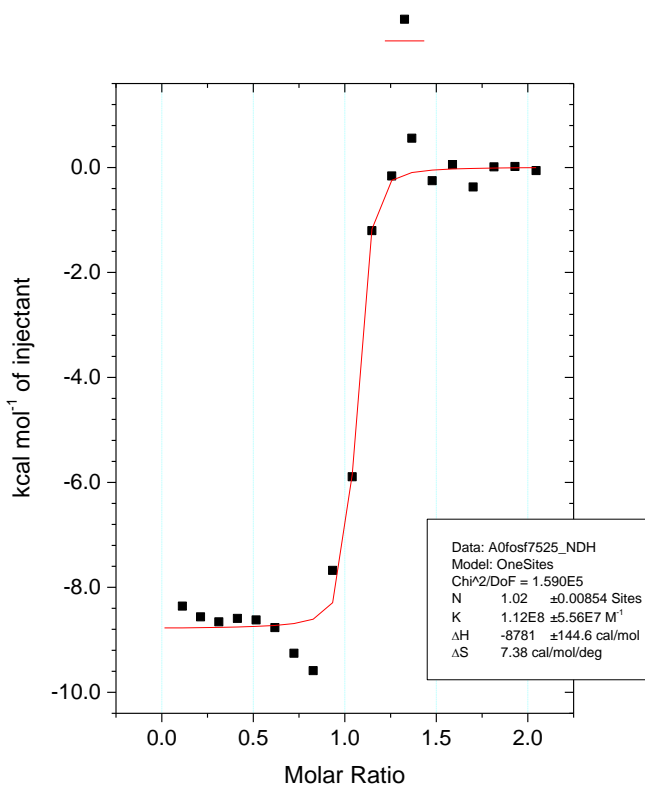
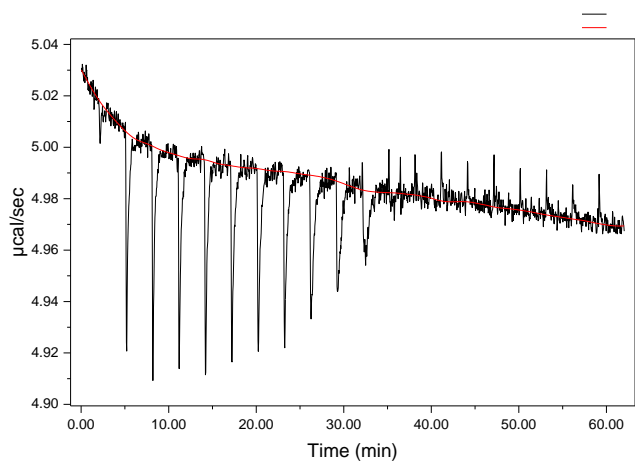




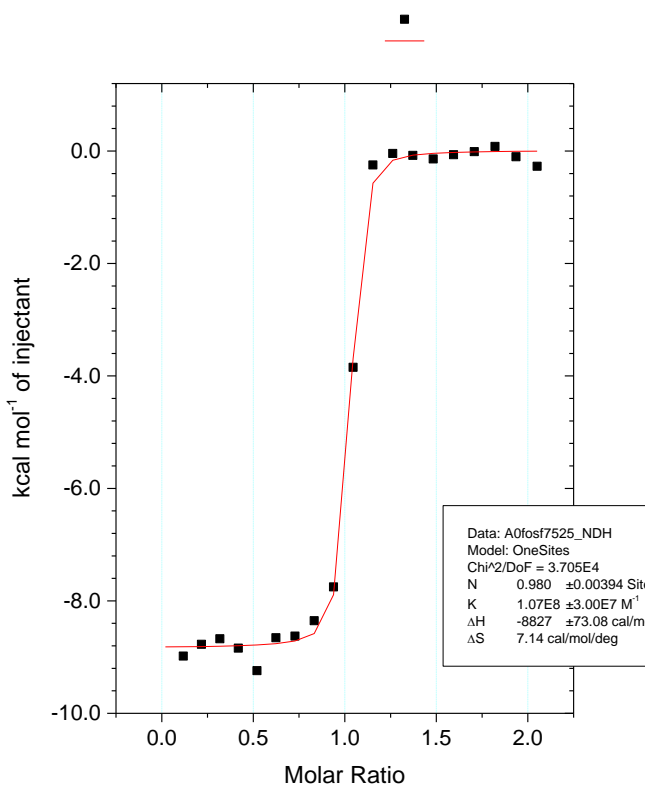
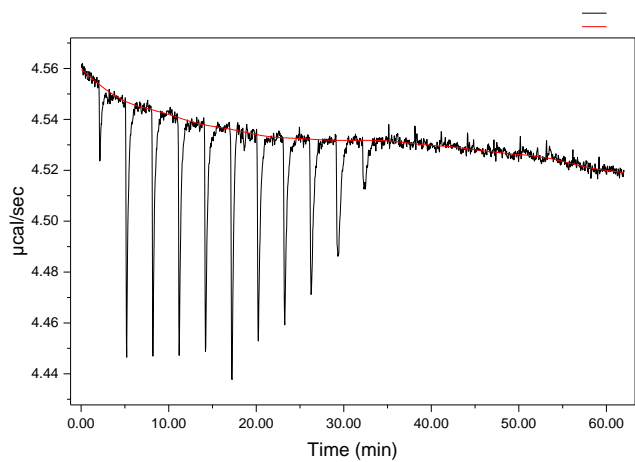




Isothermal titration calorimetry CAII –  
dichlorphenamide

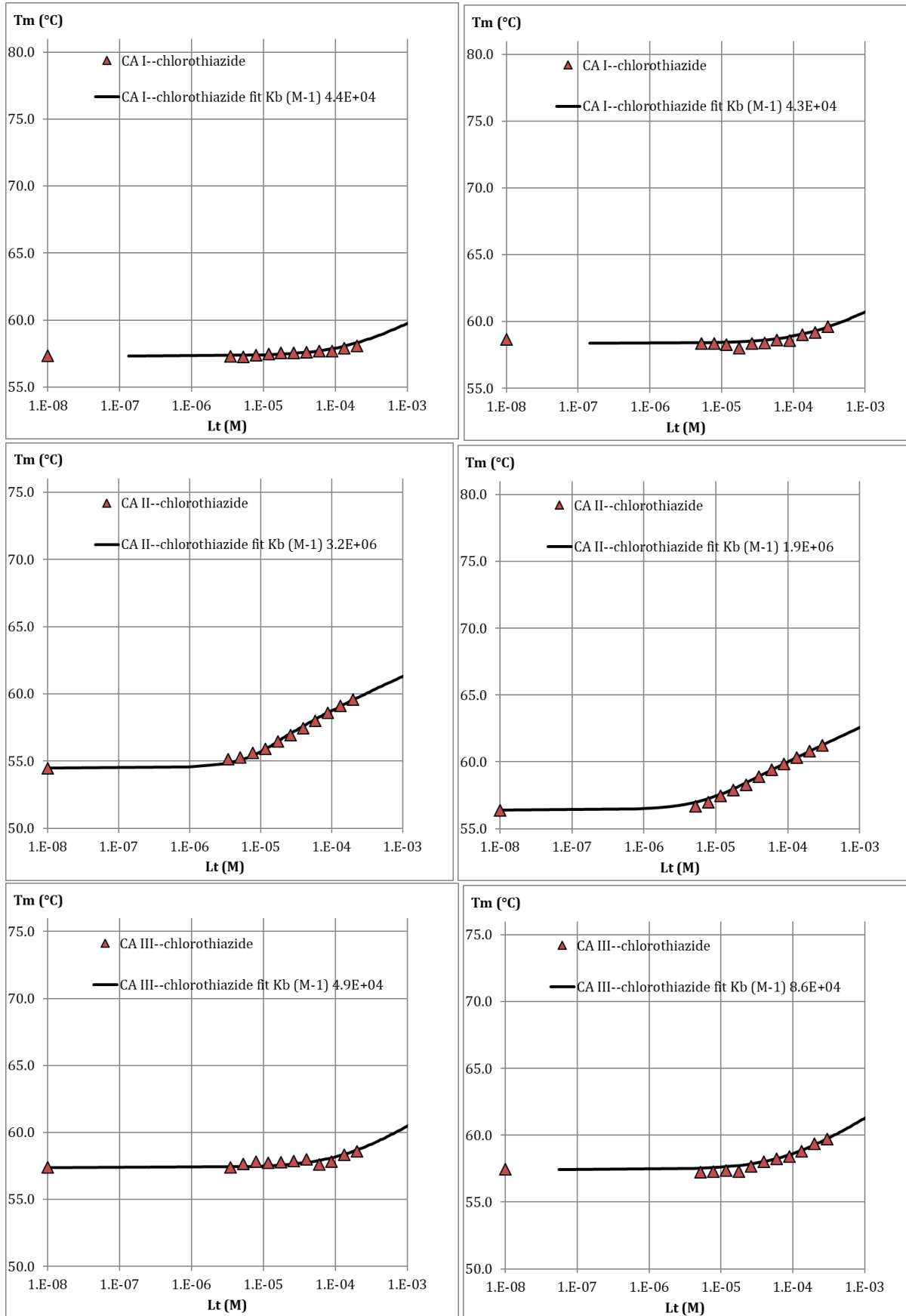


CAII – dichlorphenamide

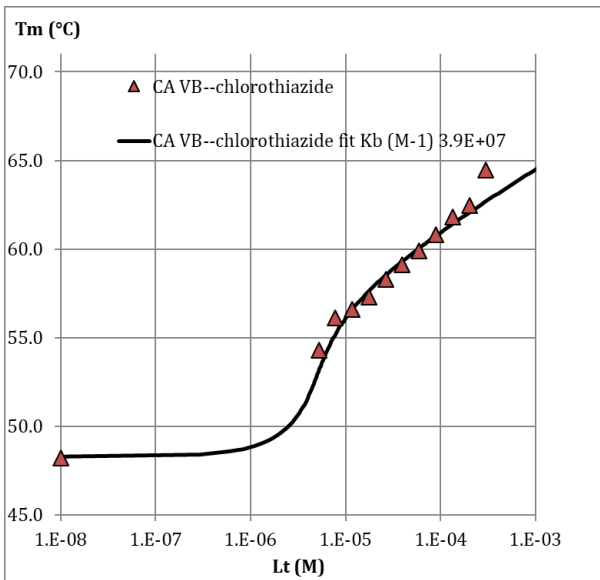
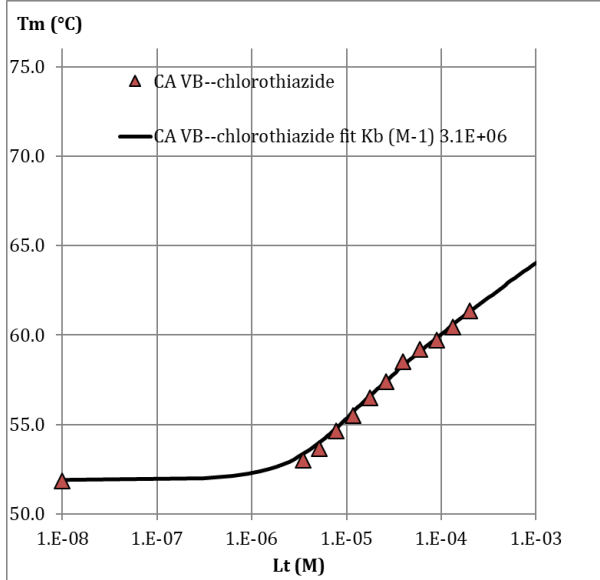
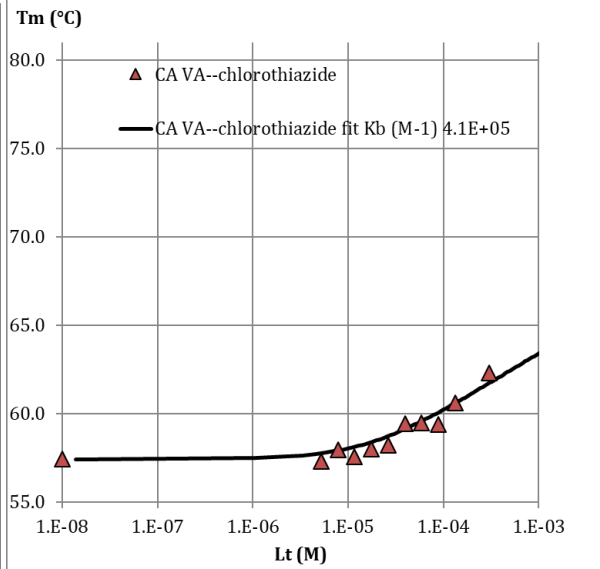
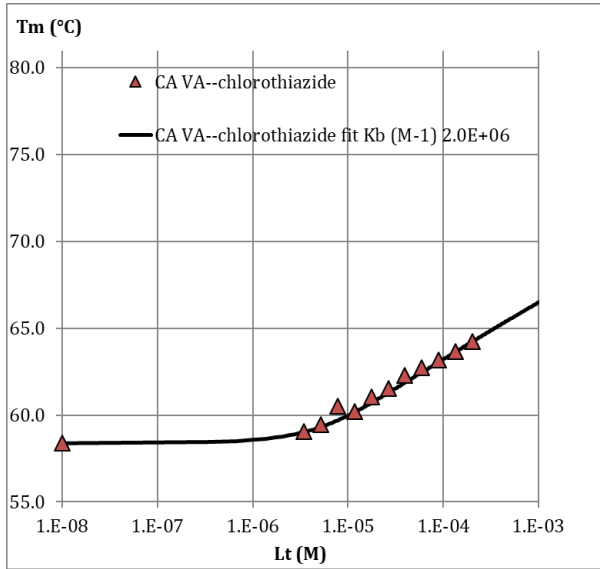
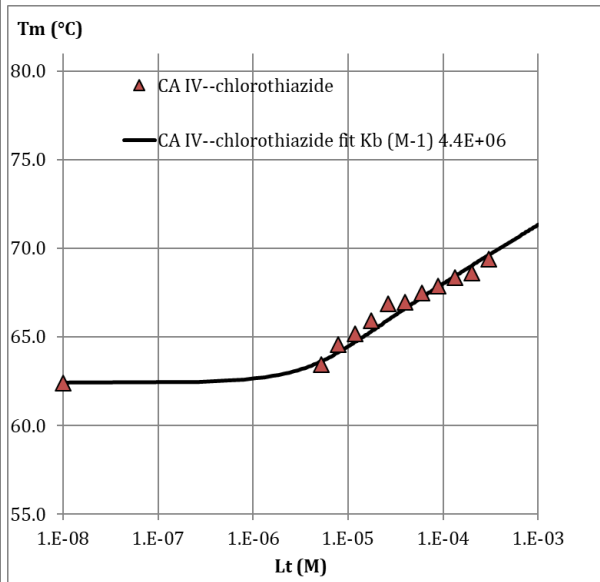
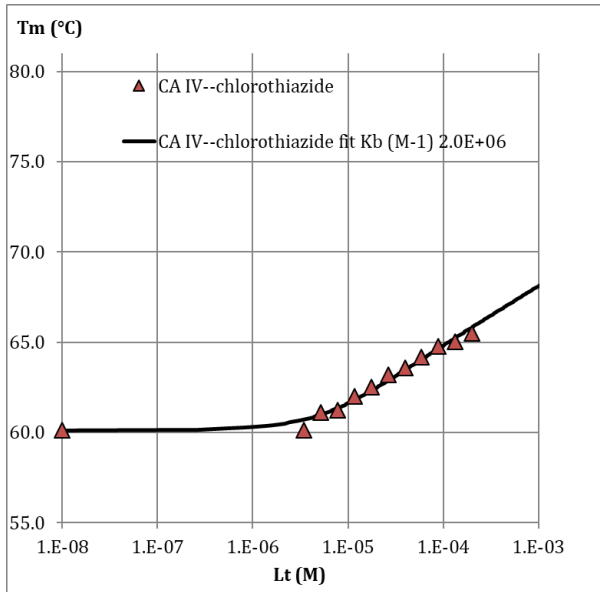


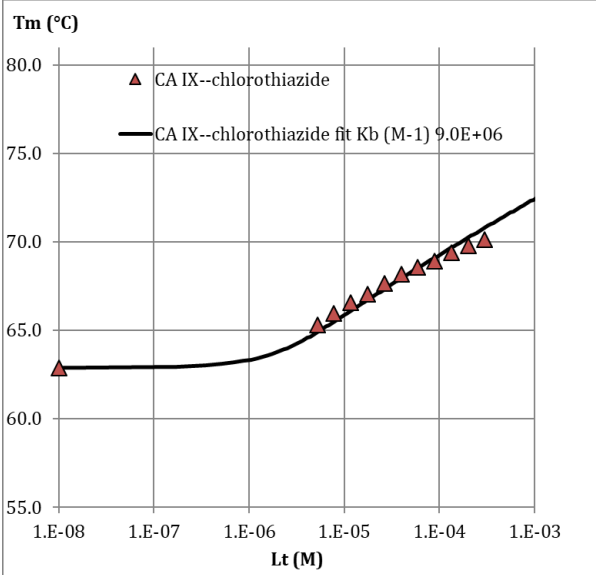
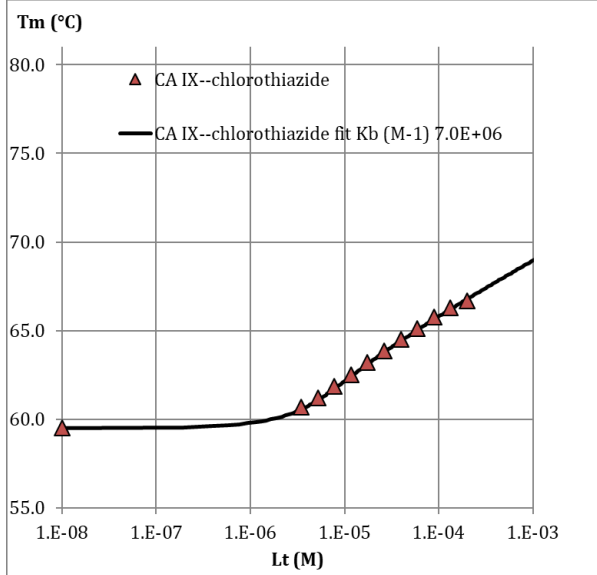
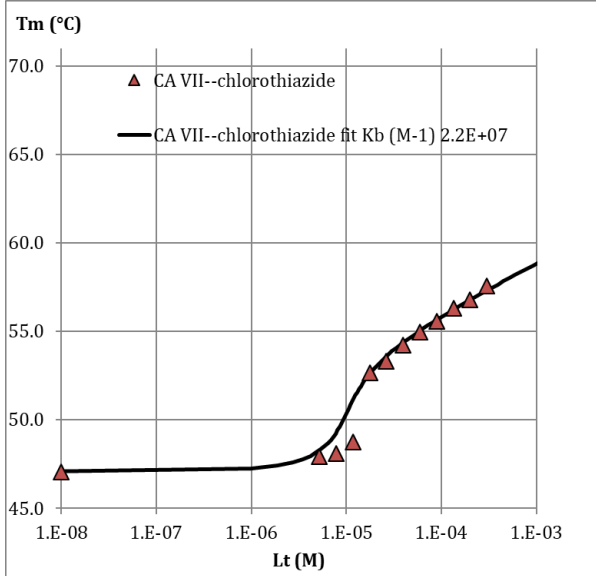
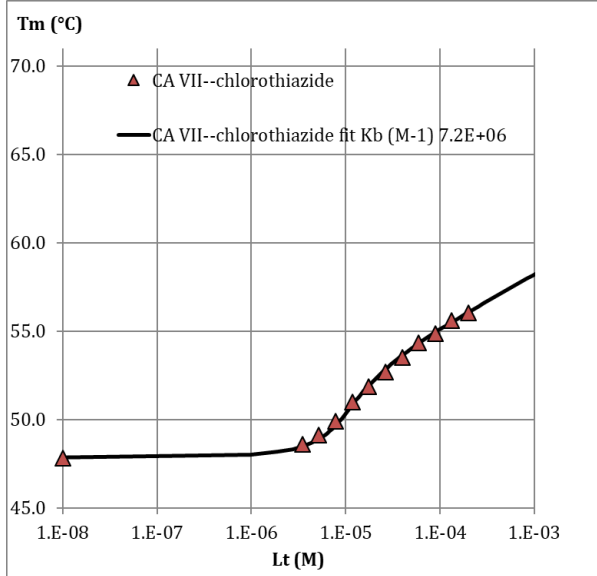
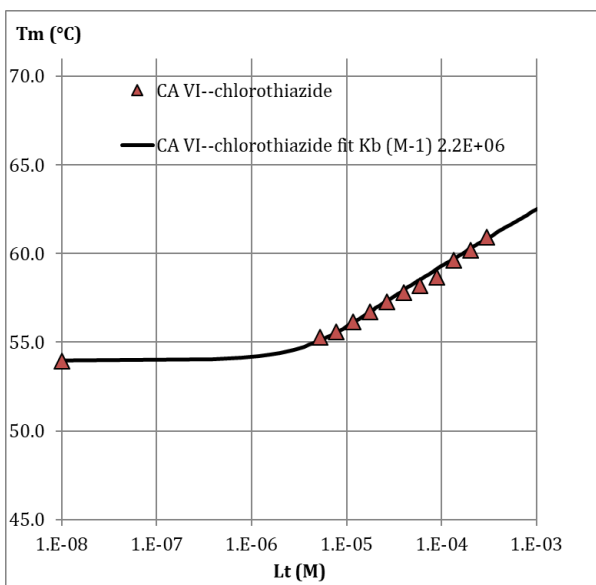
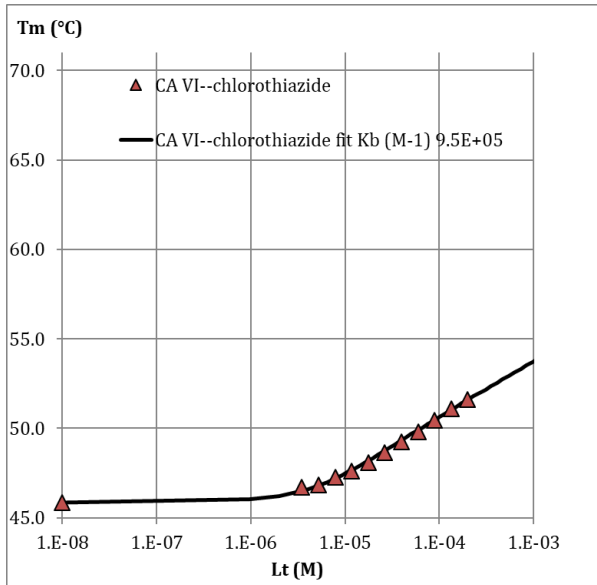
### 3. Chlorothiazide

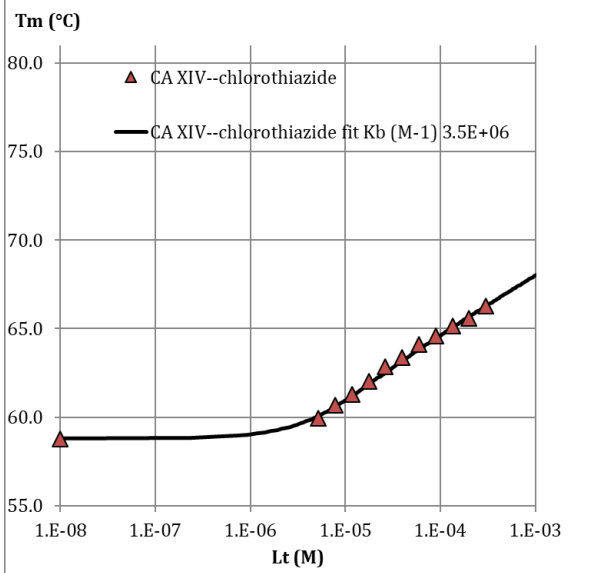
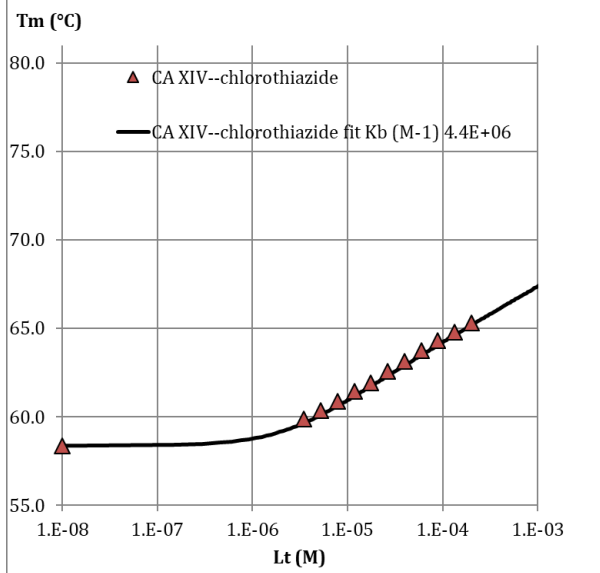
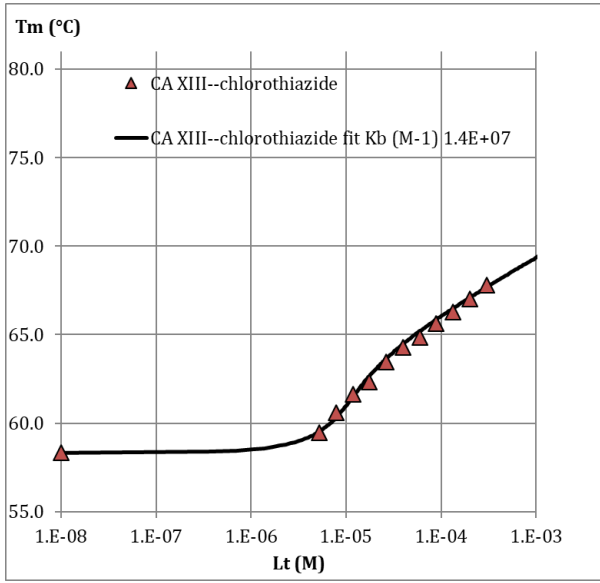
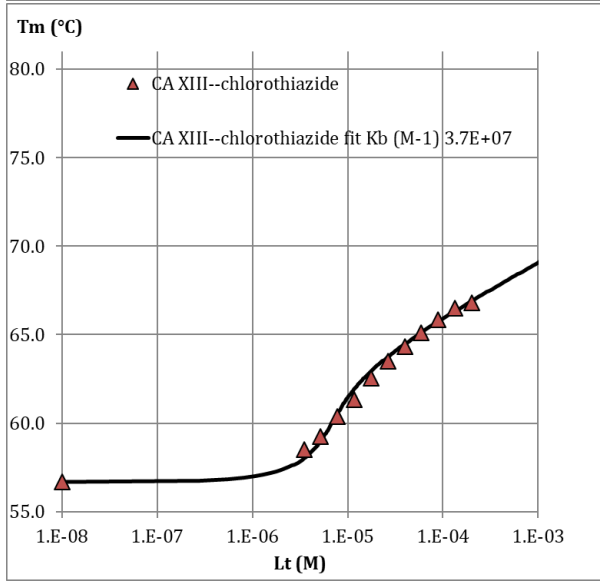
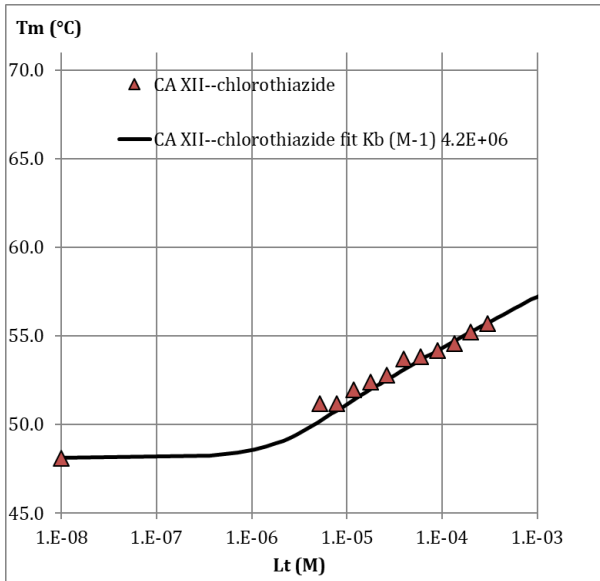
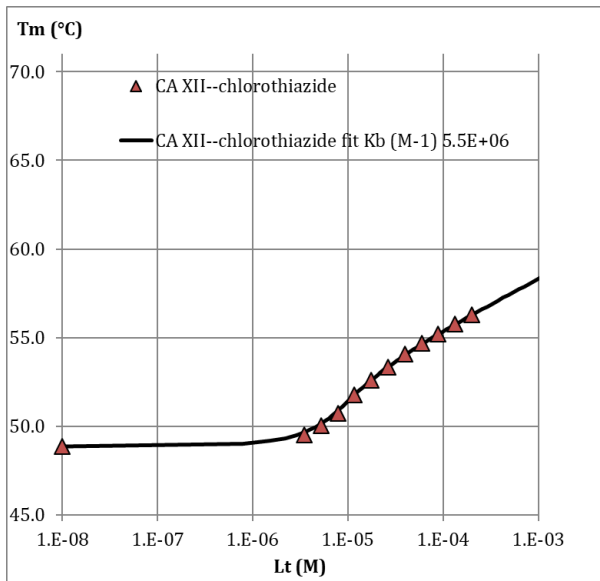
#### Fluorescence-based thermal shift assay





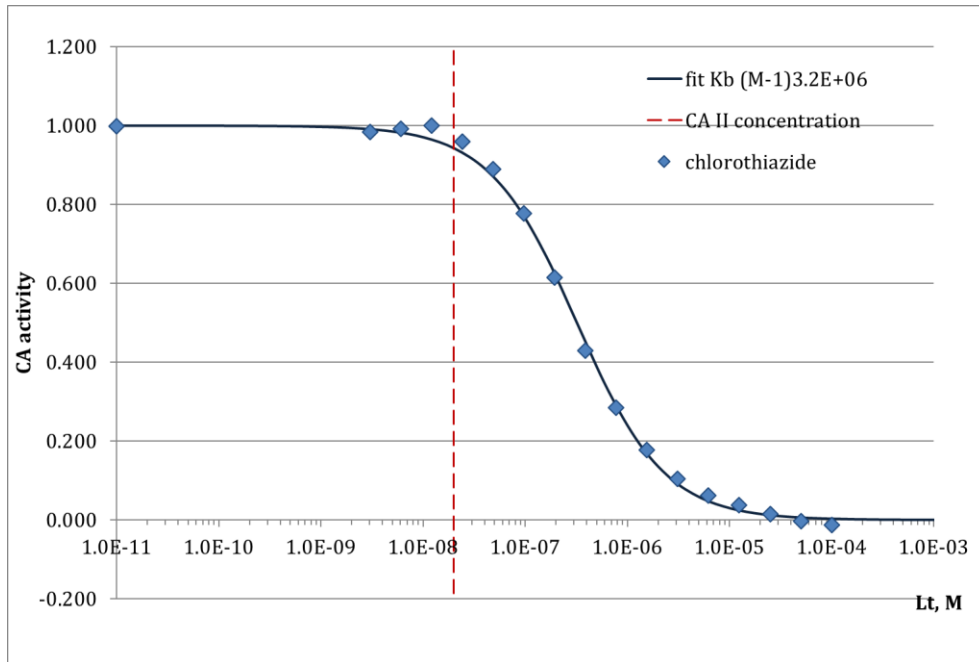




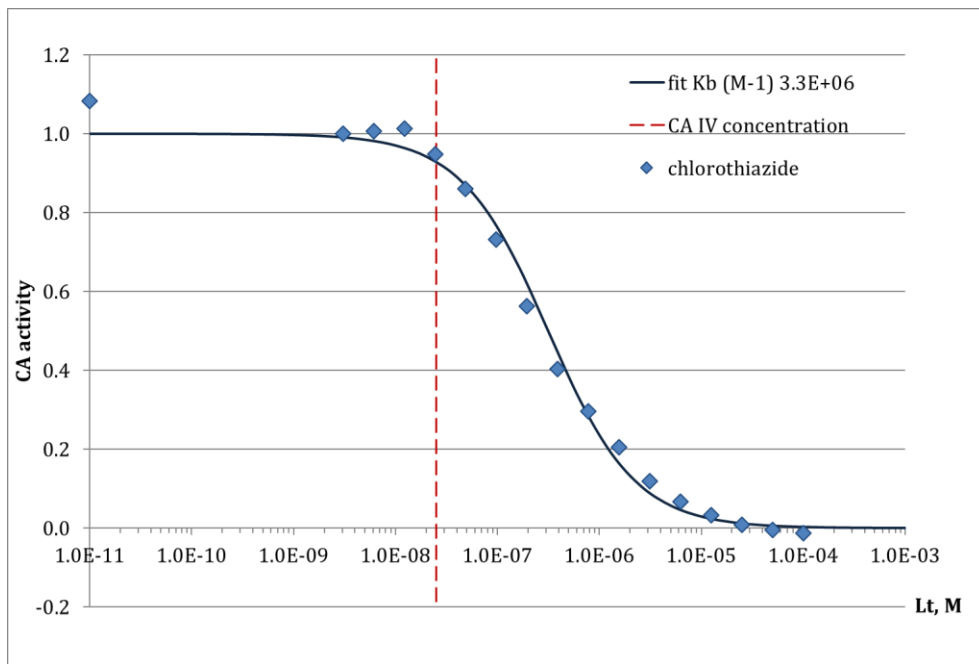


## CO<sub>2</sub> hydration activity inhibition:

### CA II - chlorothiazide

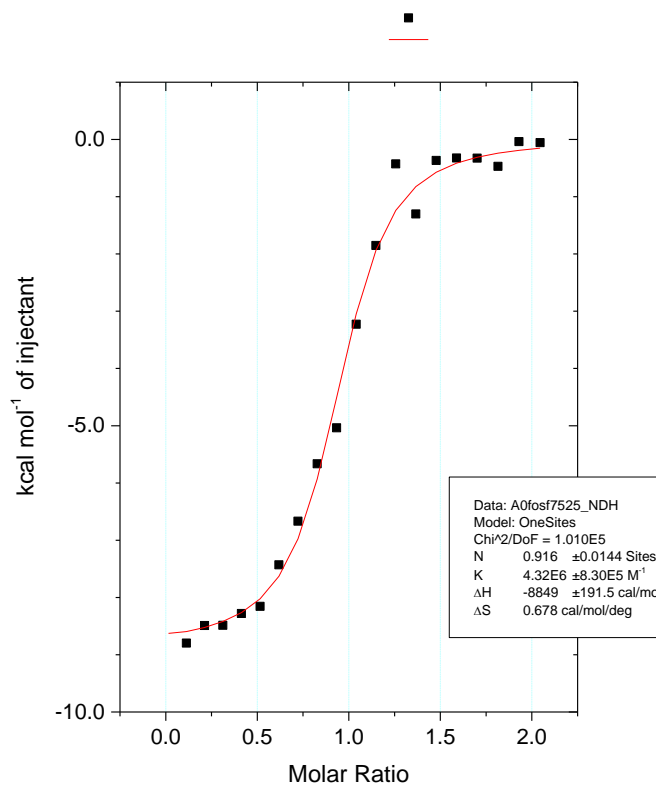
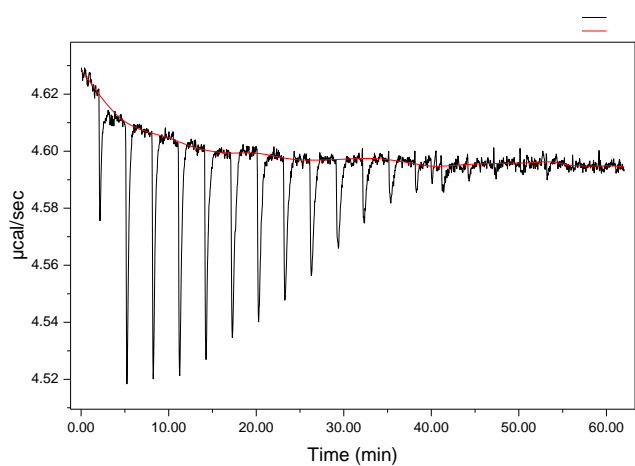


### CA IV - chlorothiazide

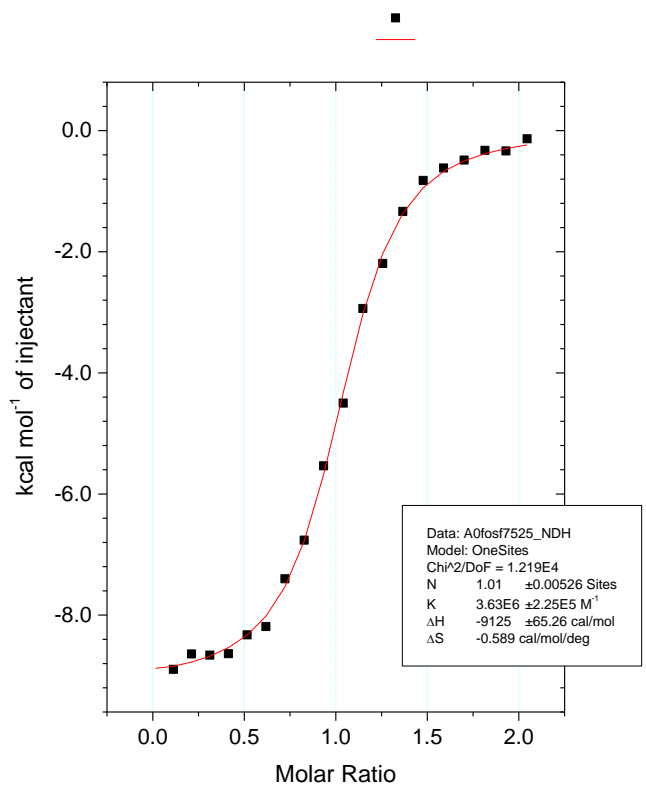
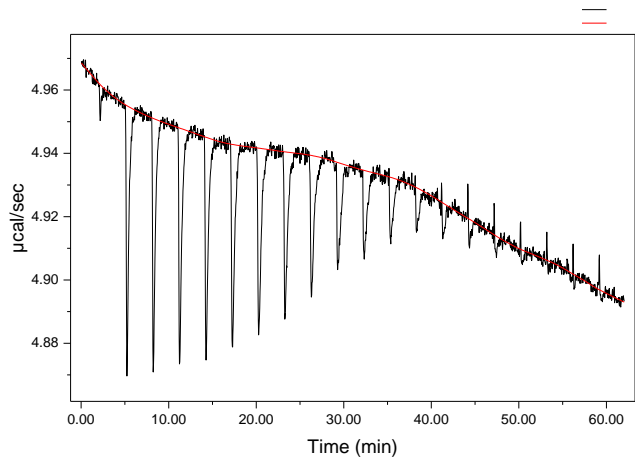


# Isothermal titration calorimetry

## CAII – chlorothiazide

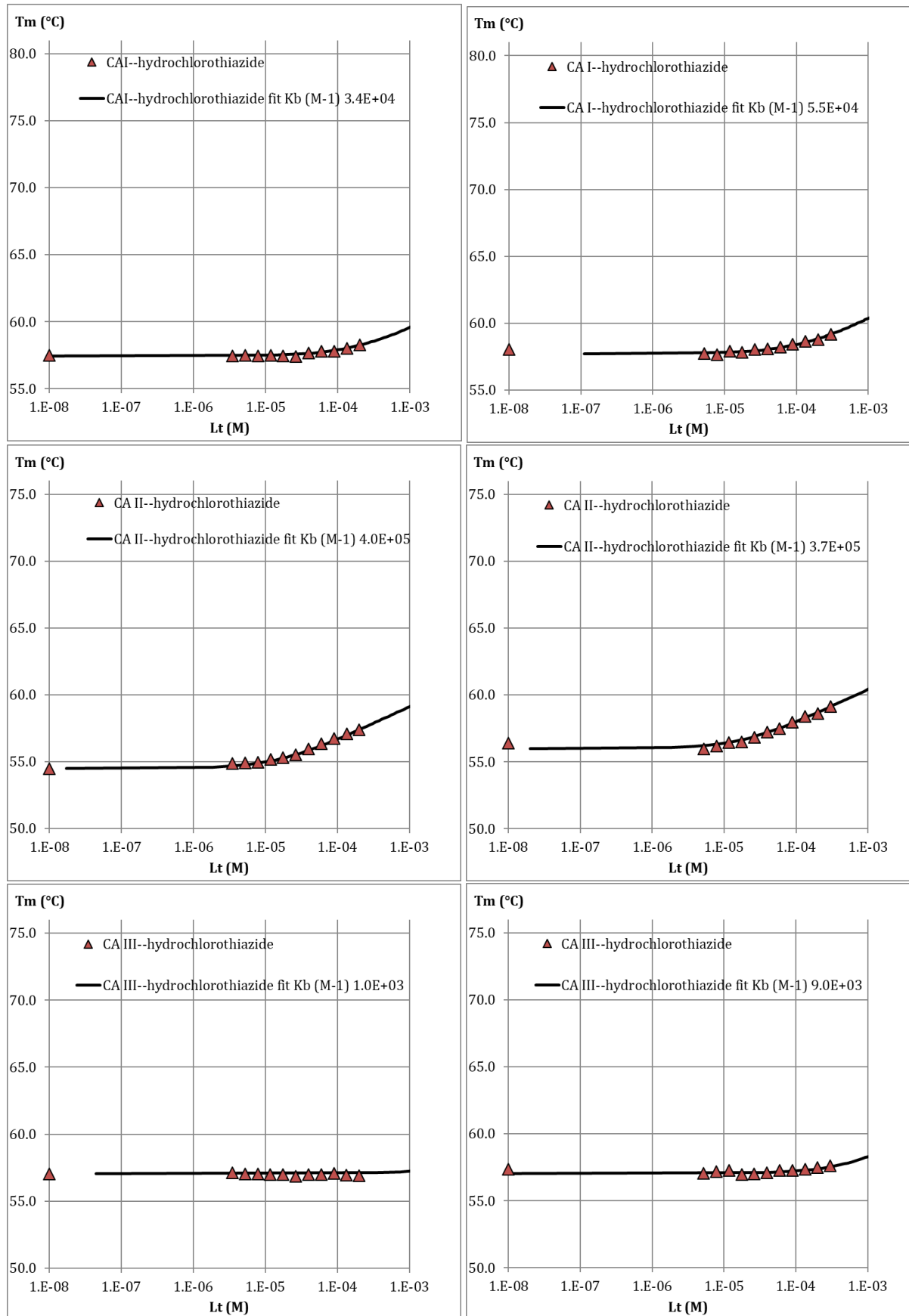


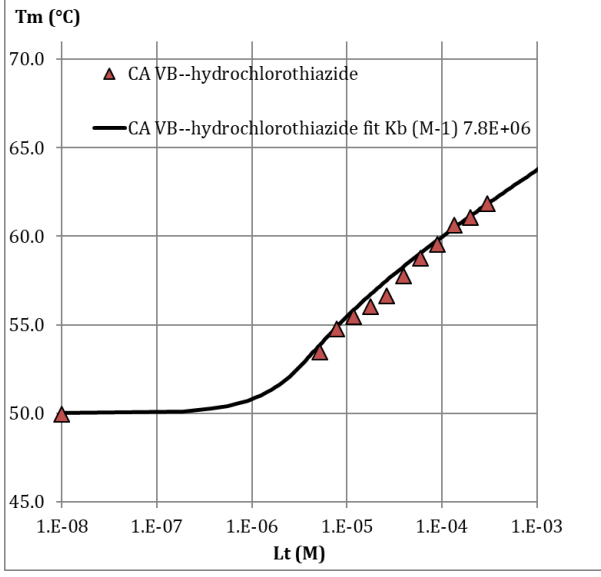
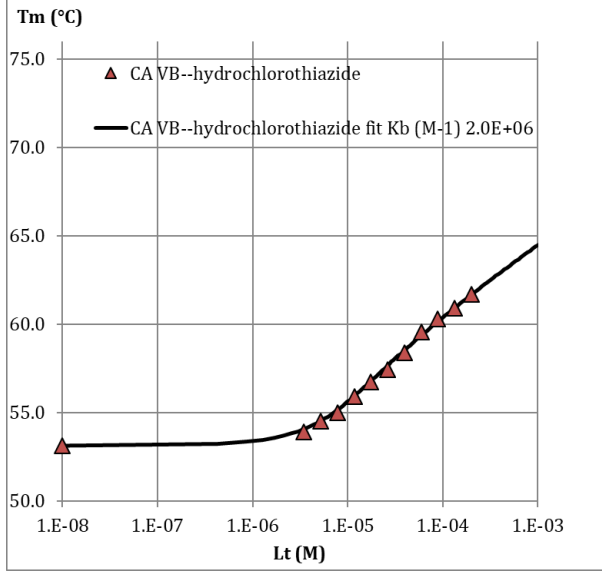
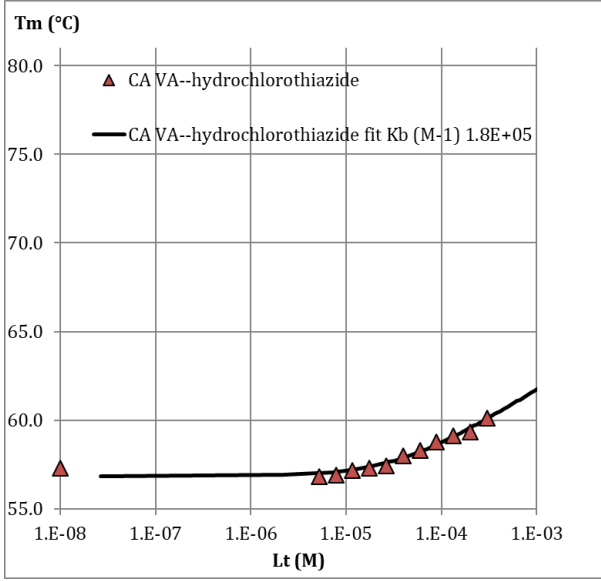
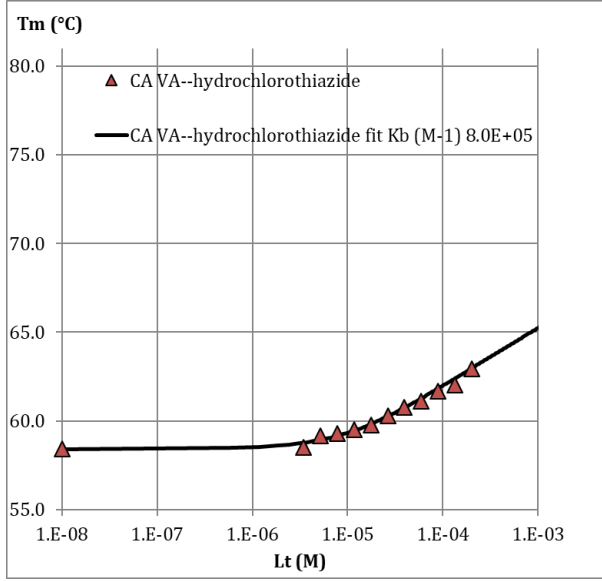
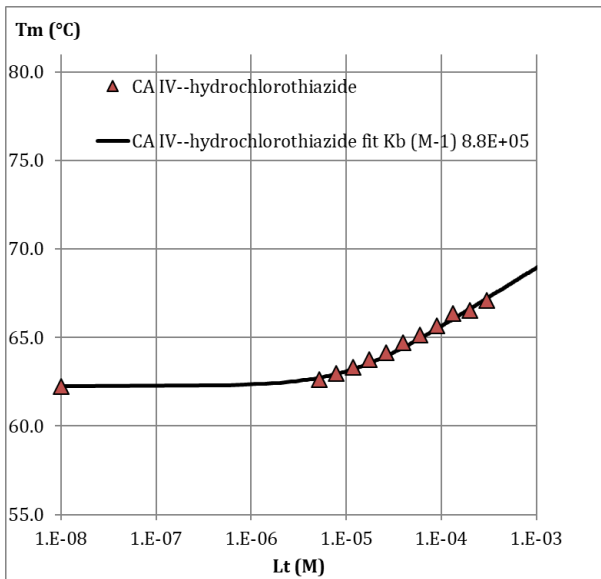
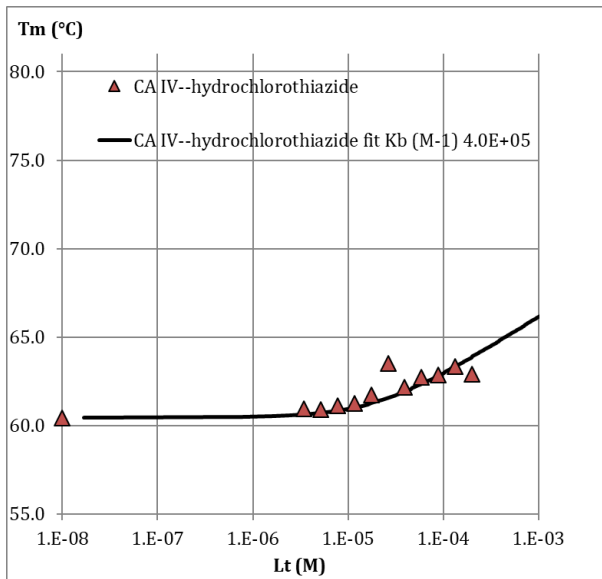
## CAII – chlorothiazide

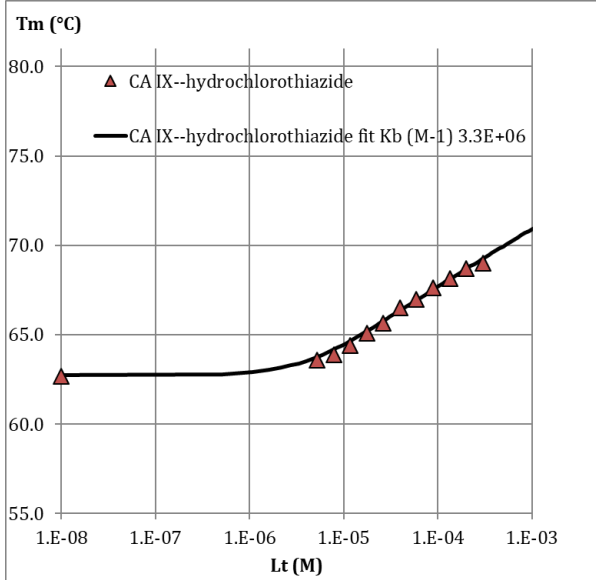
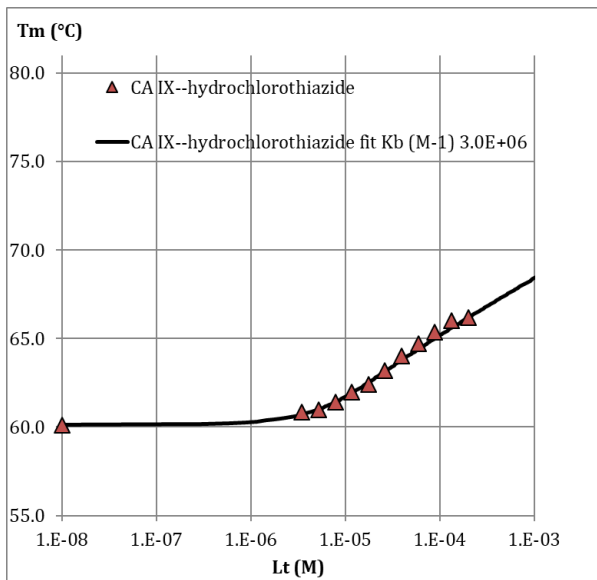
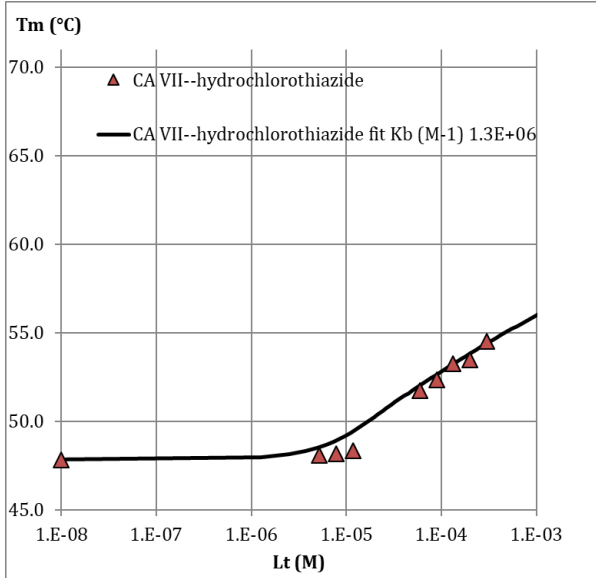
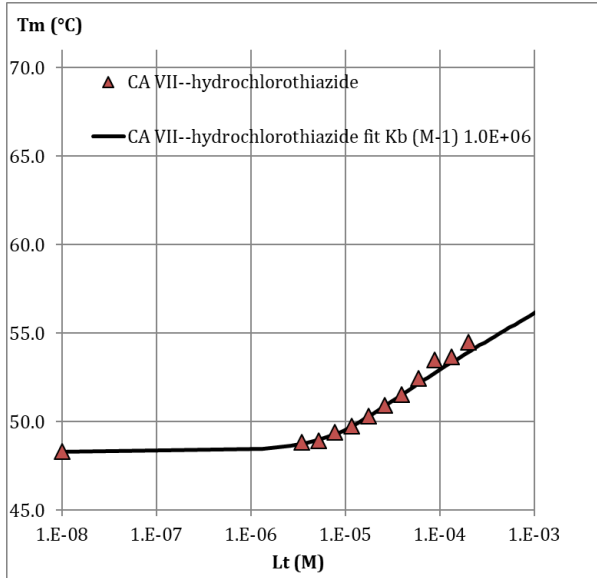
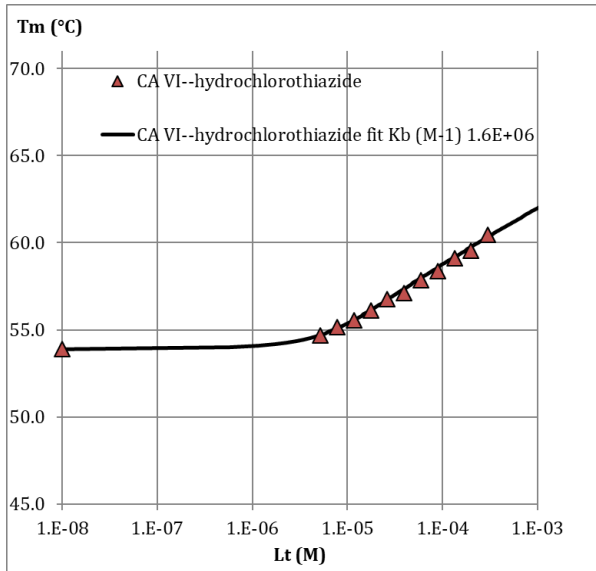
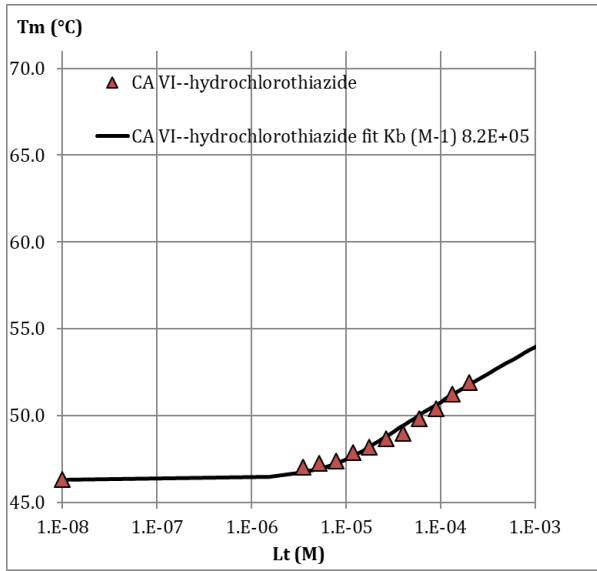


## 4. Hydrochlorothiazide

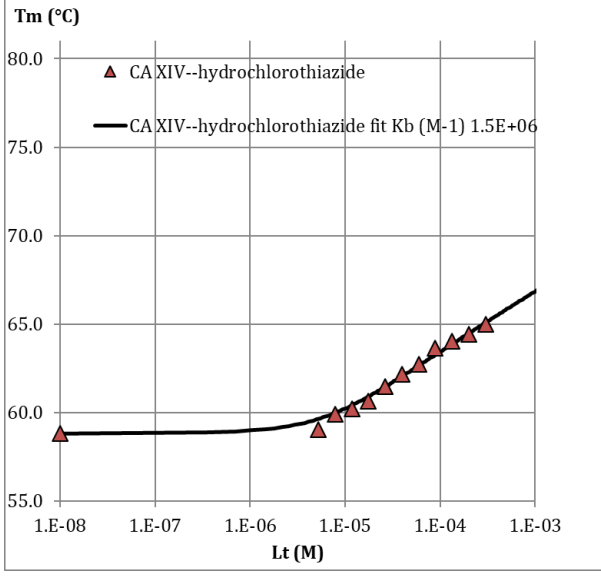
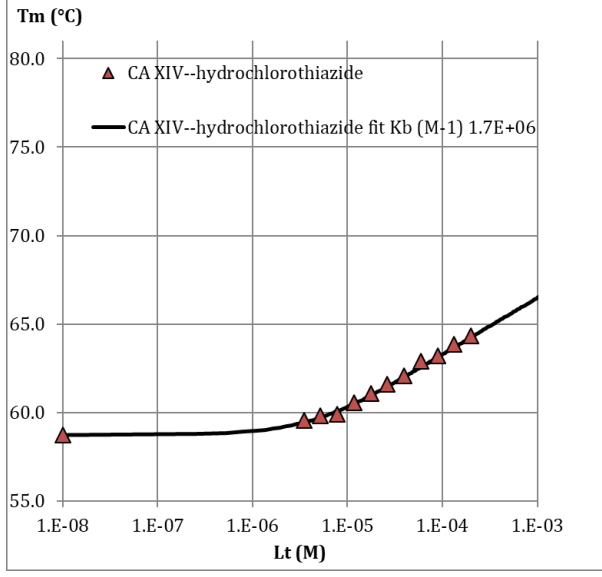
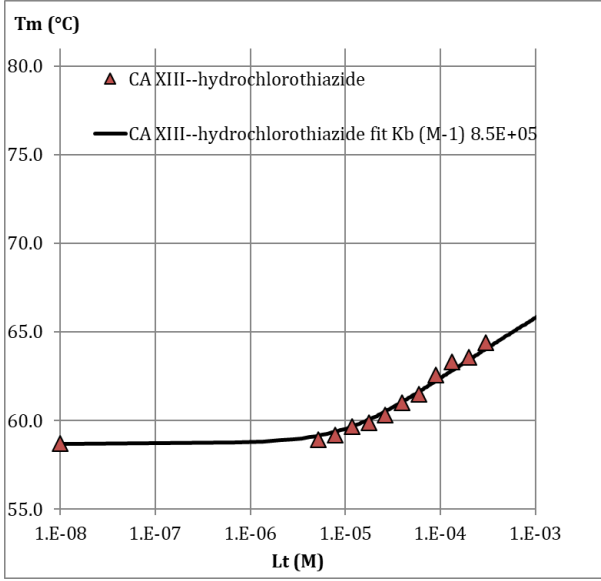
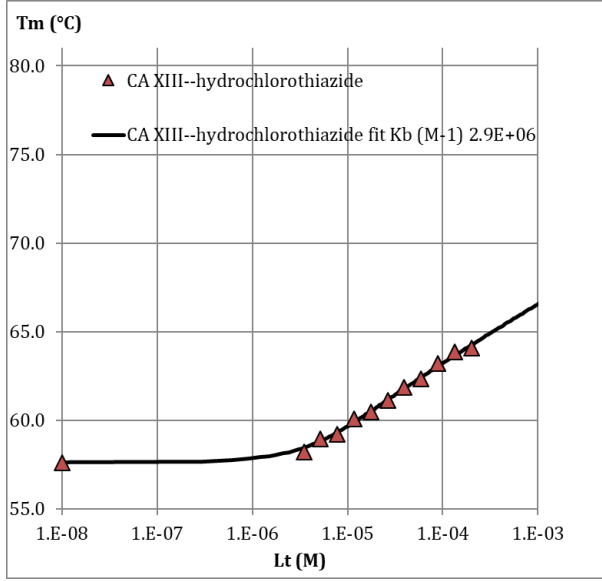
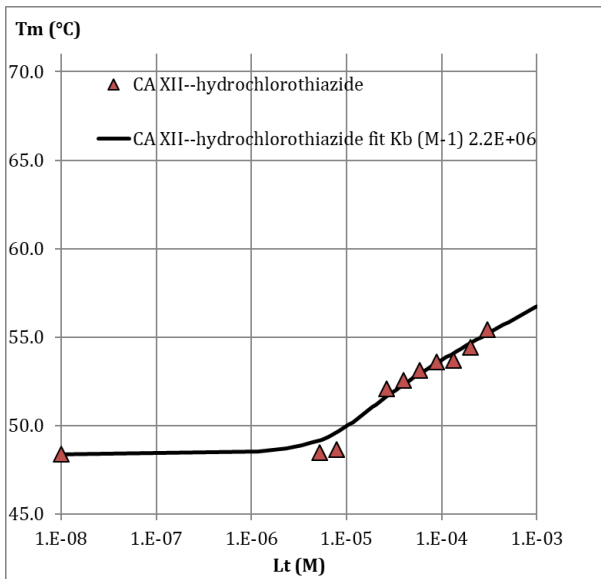
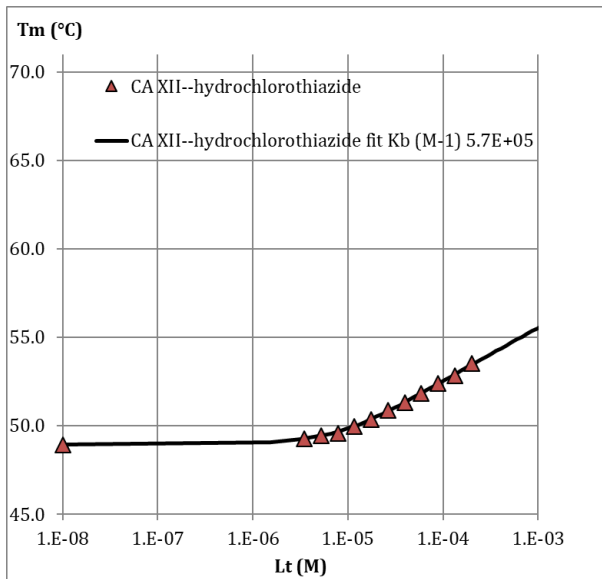
### Fluorescence-based thermal shift assay





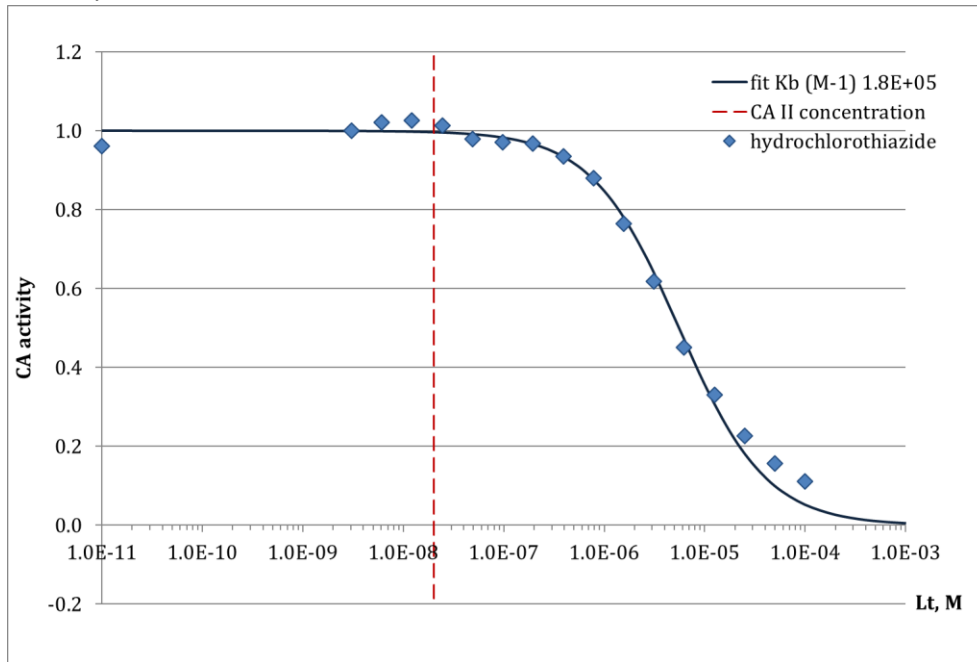




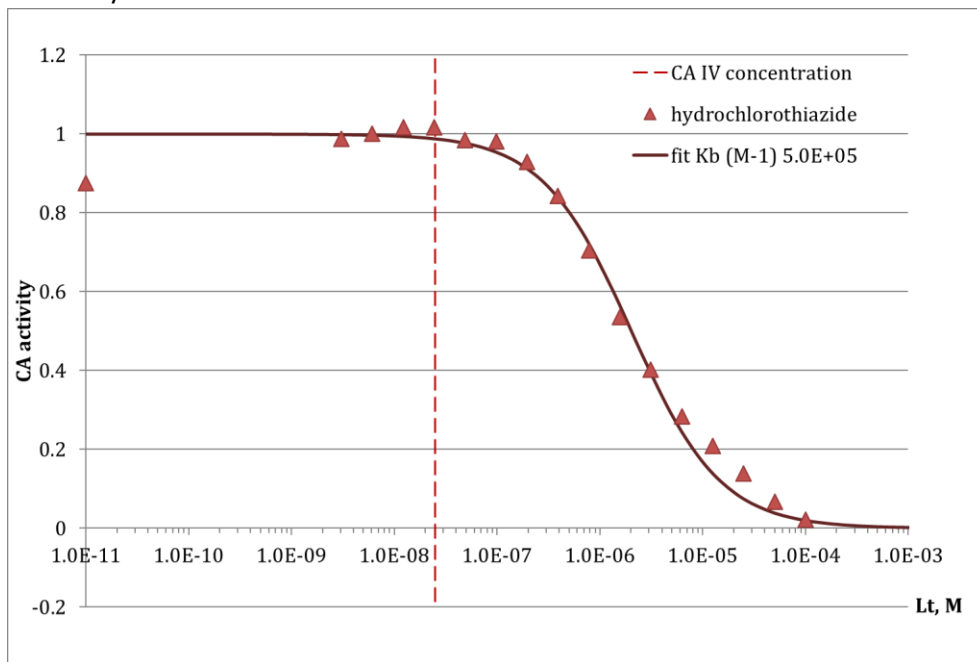


## CO<sub>2</sub> hydration activity inhibition:

### CA II- hydrochlorothiazide

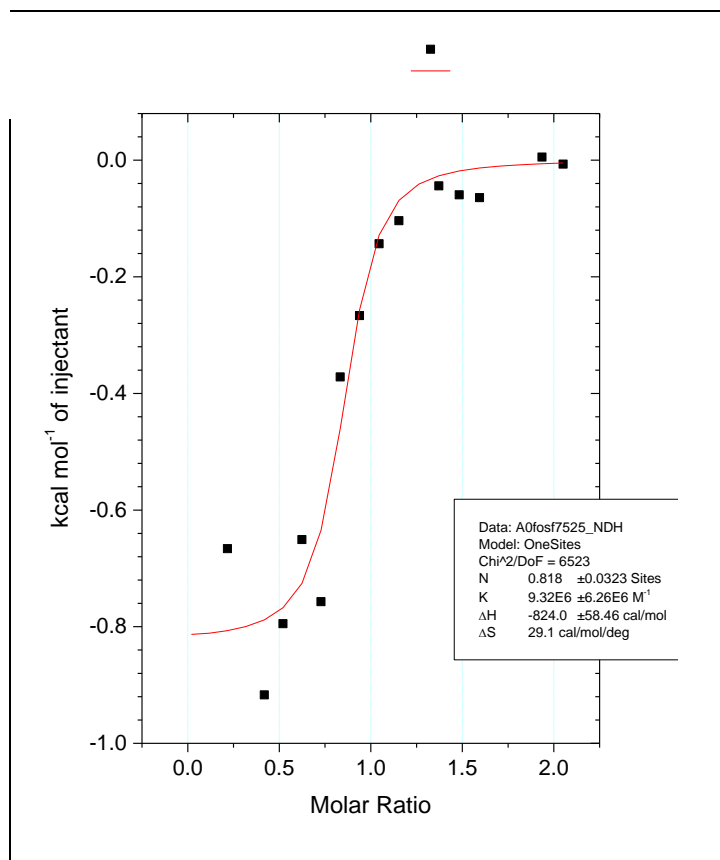
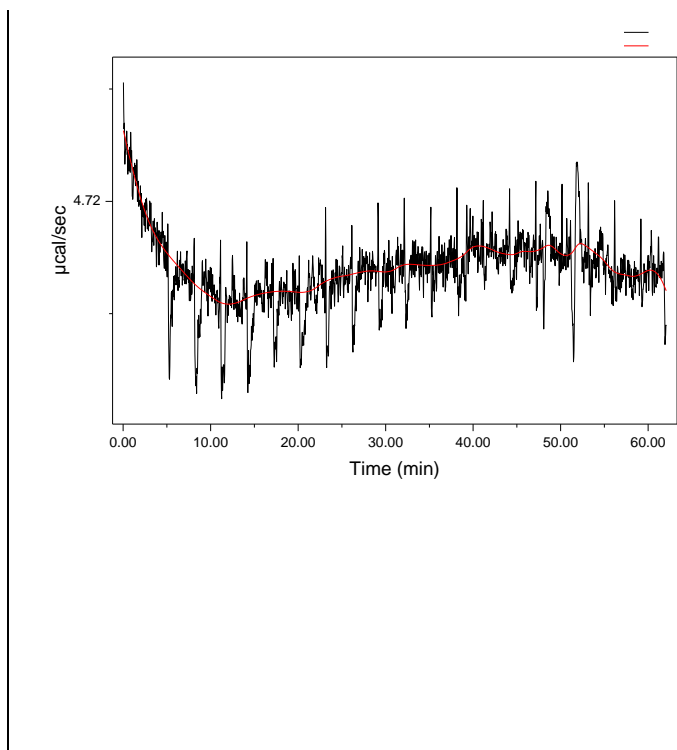


### CA IV - hydrochlorothiazide



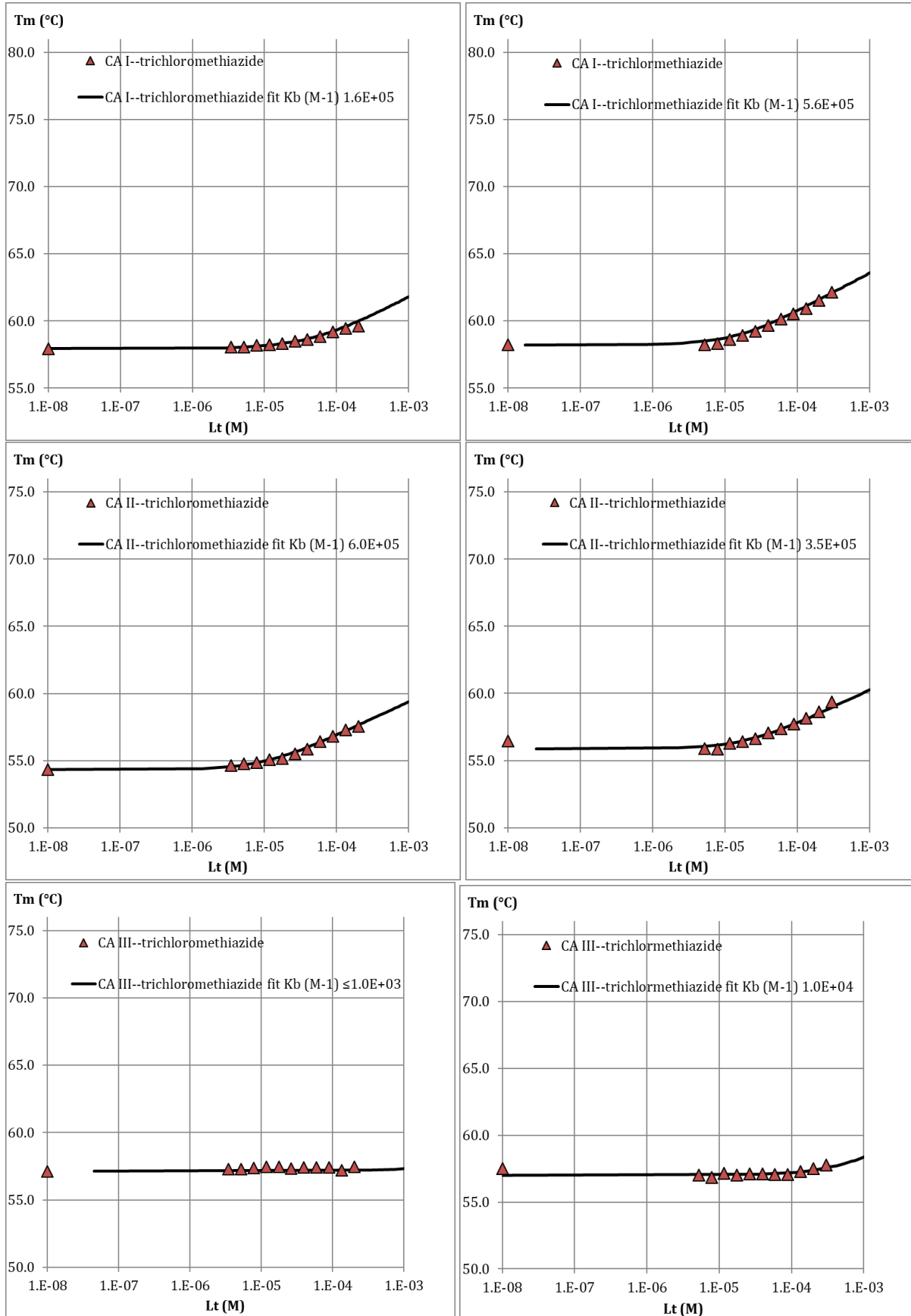
# Isothermal titration calorimetry

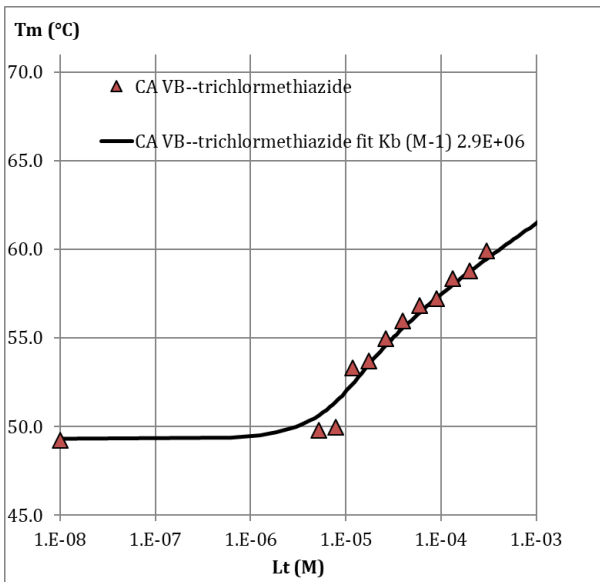
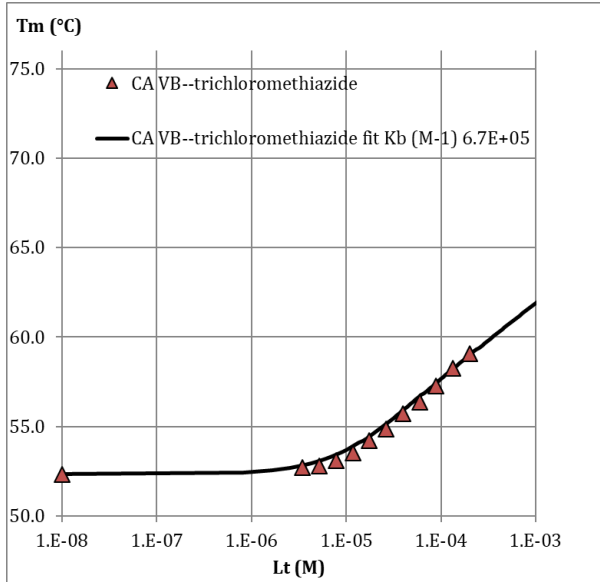
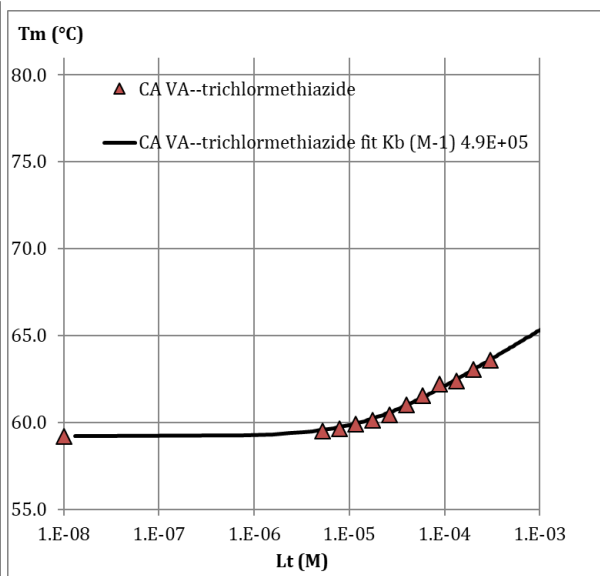
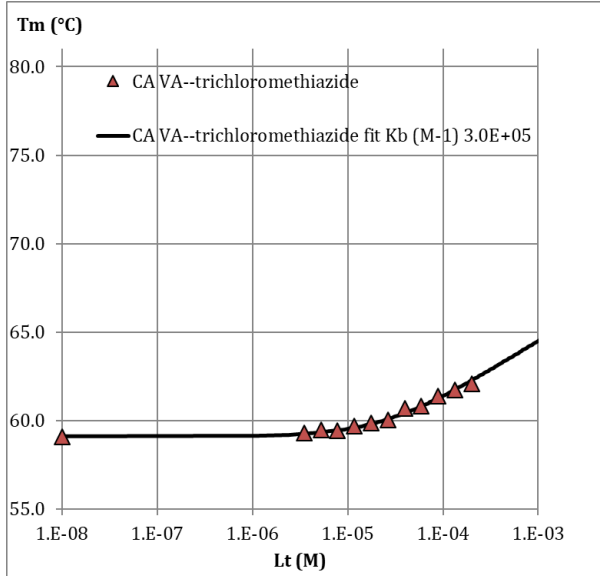
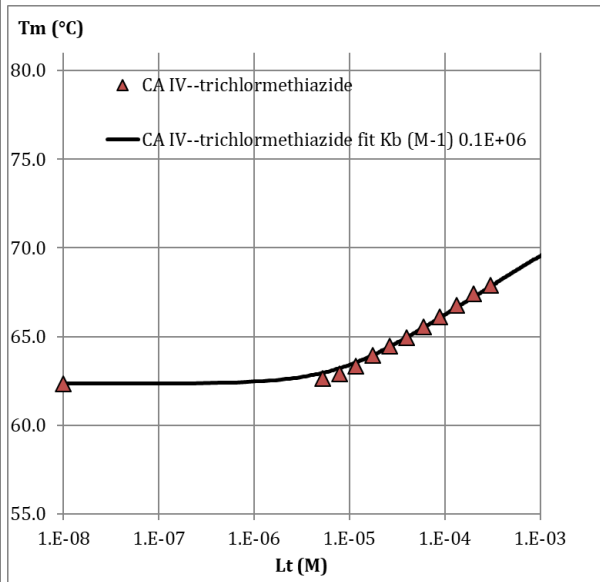
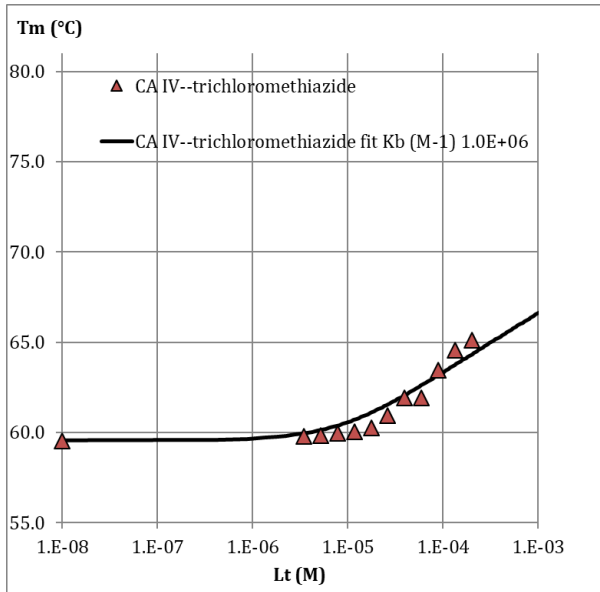
## CA II – hydrochlorothiazide

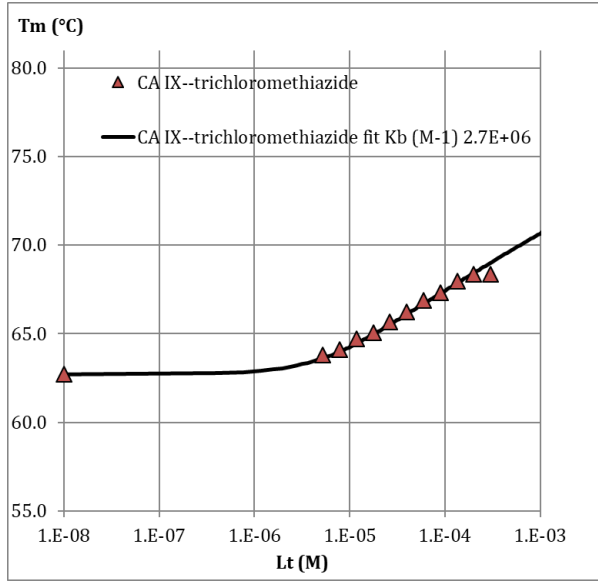
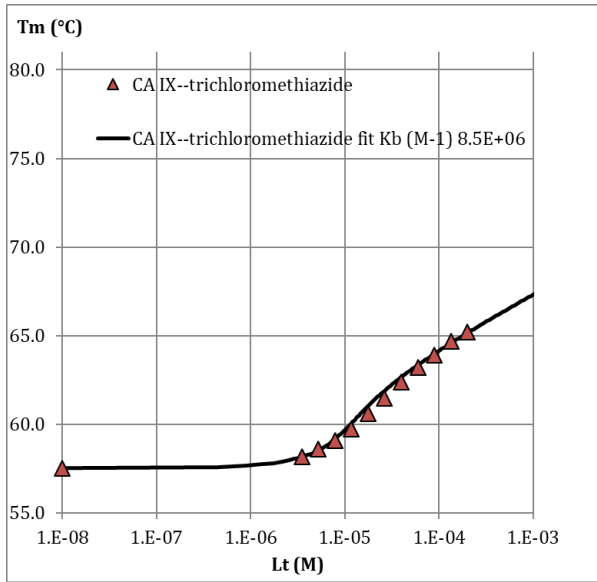
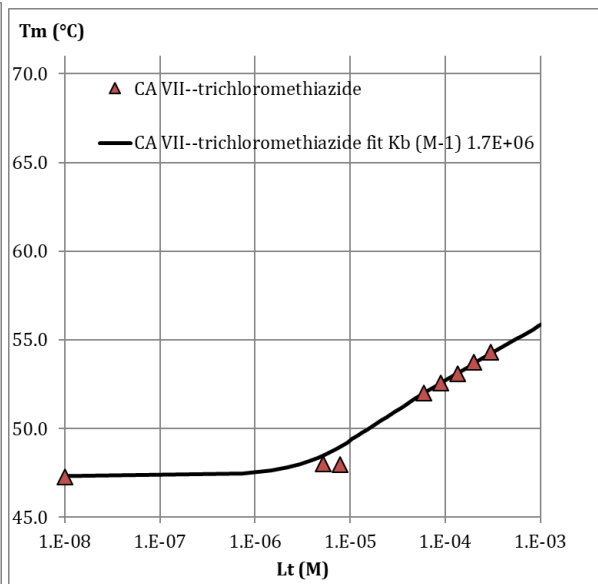
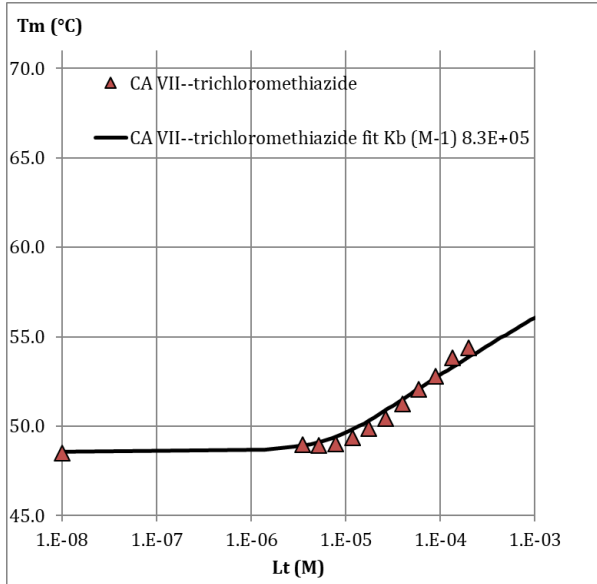
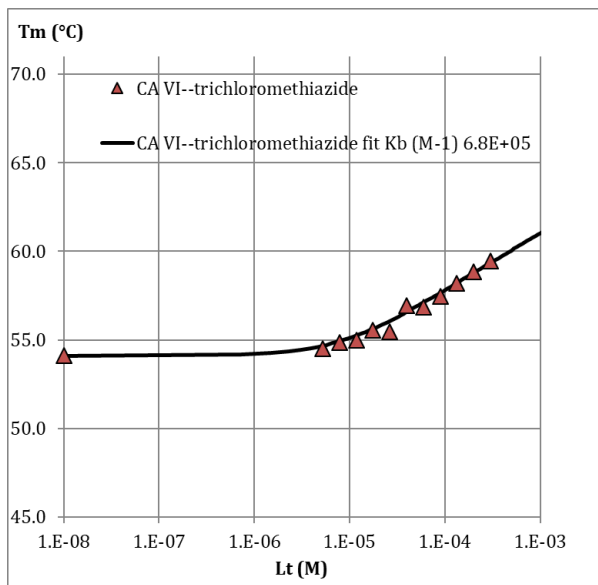
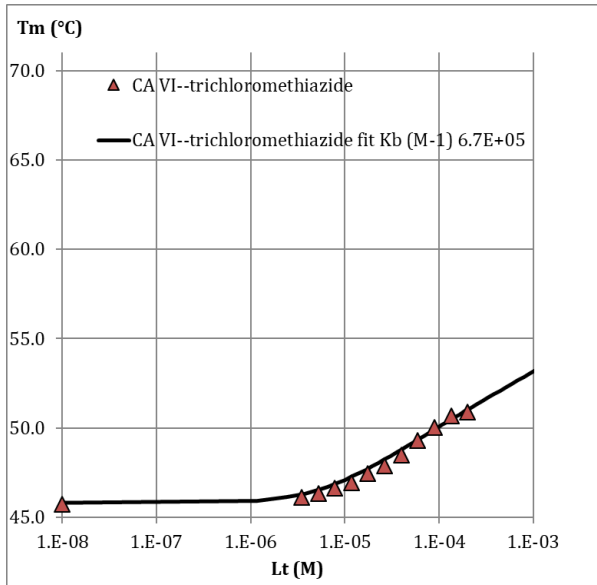


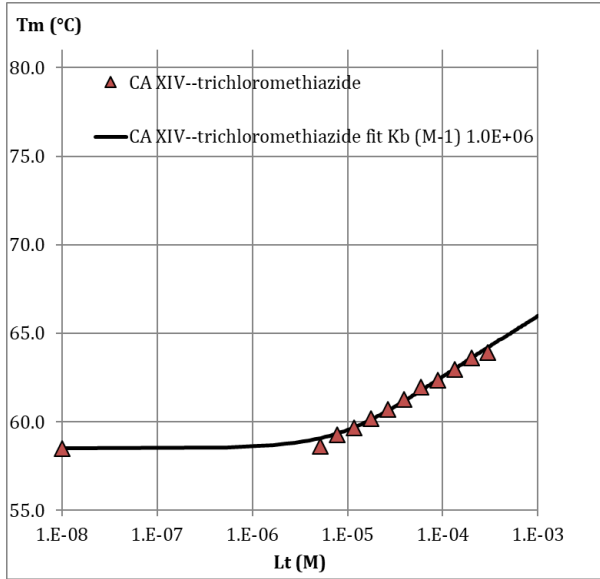
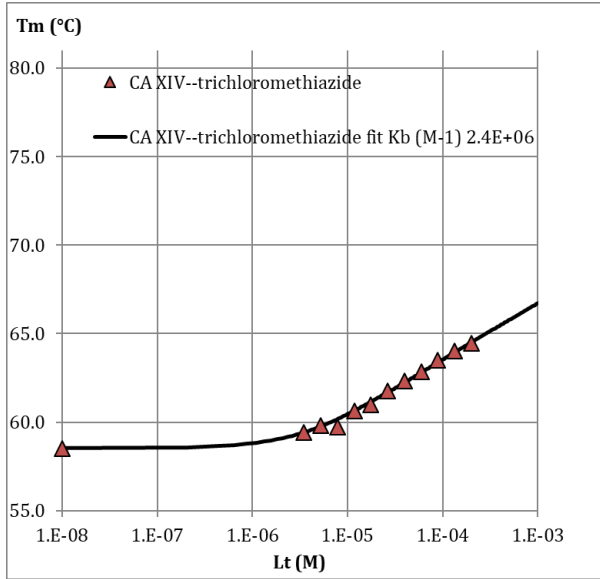
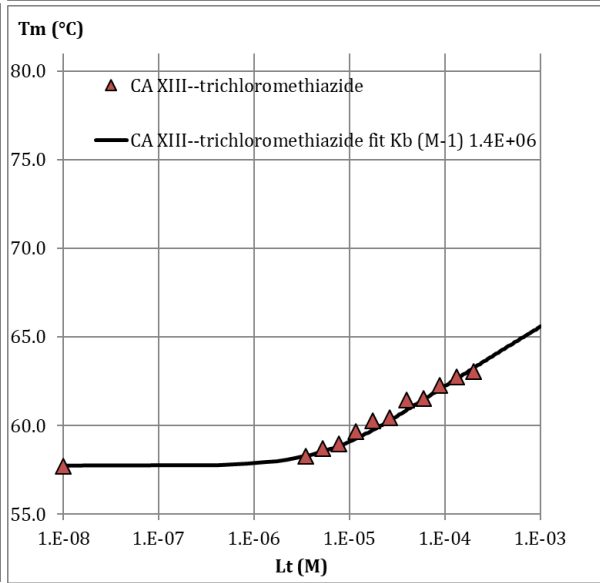
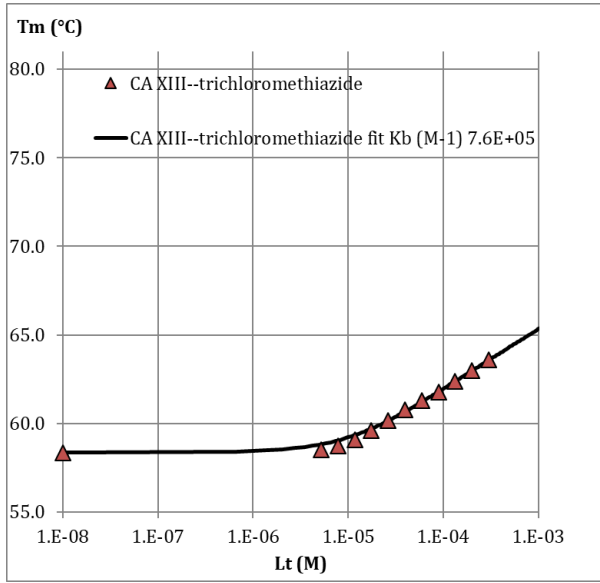
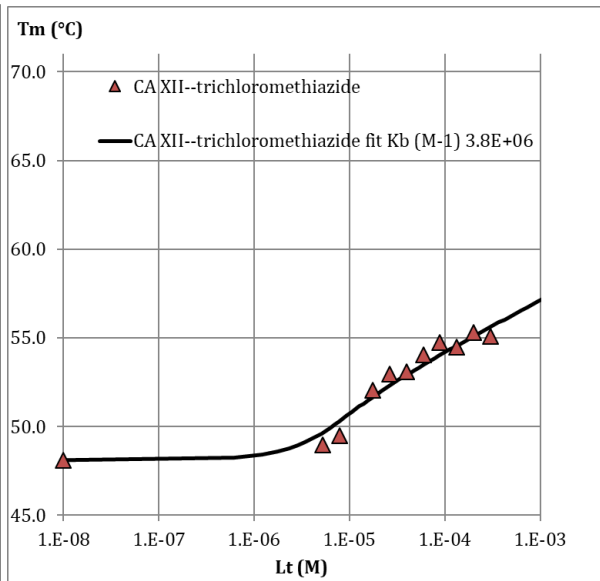
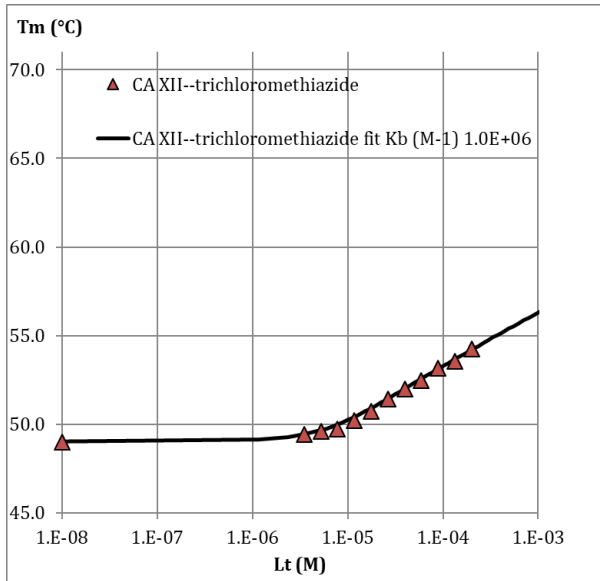
# 5. Trichloromethiazide

## Fluorence-based thermal shift assay



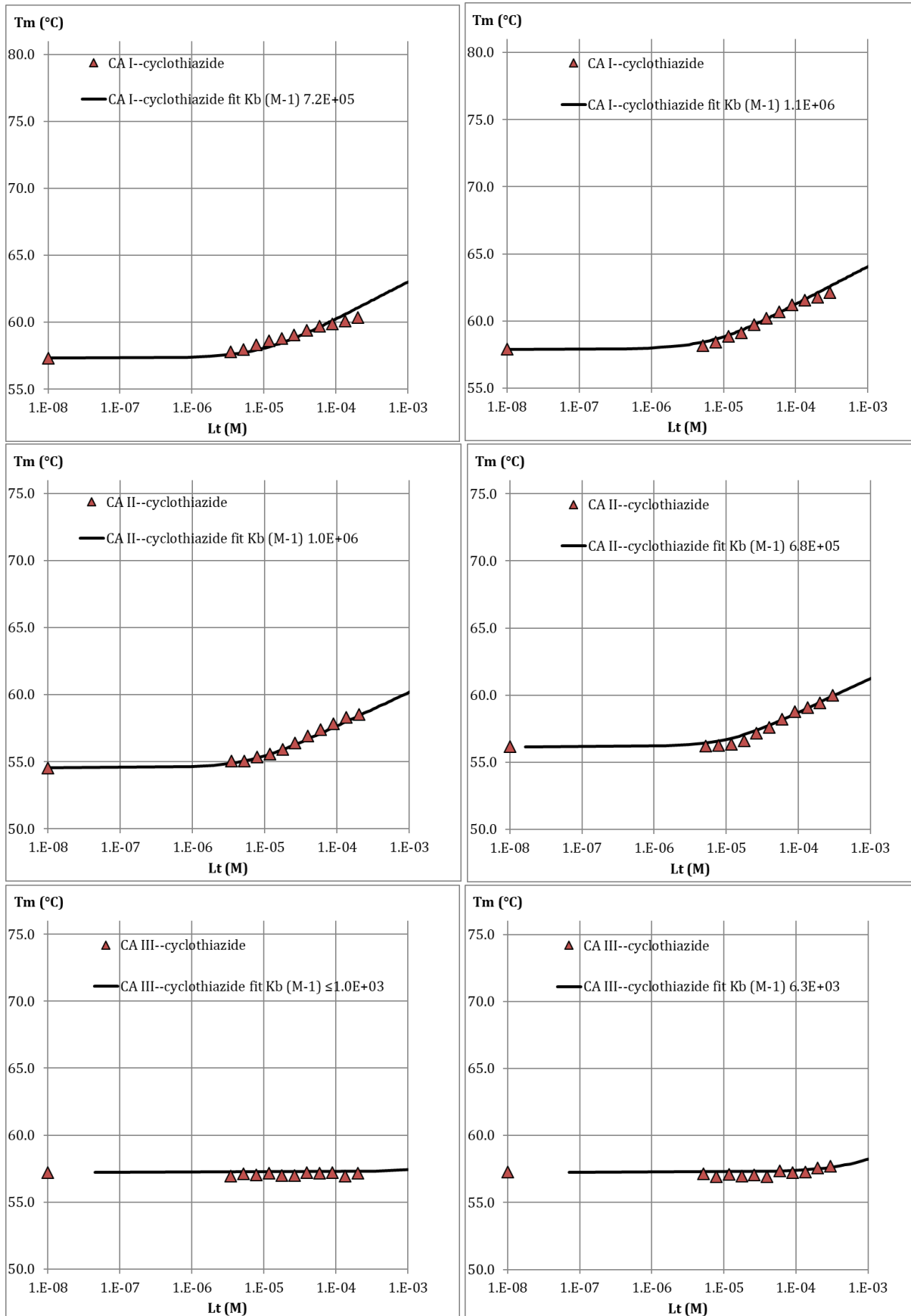




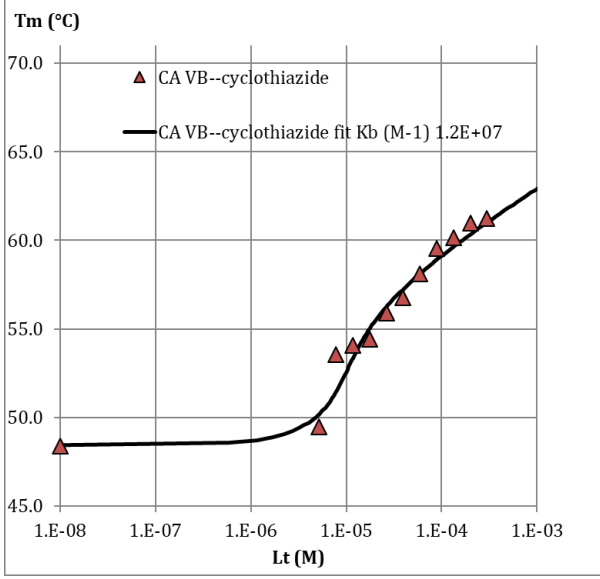
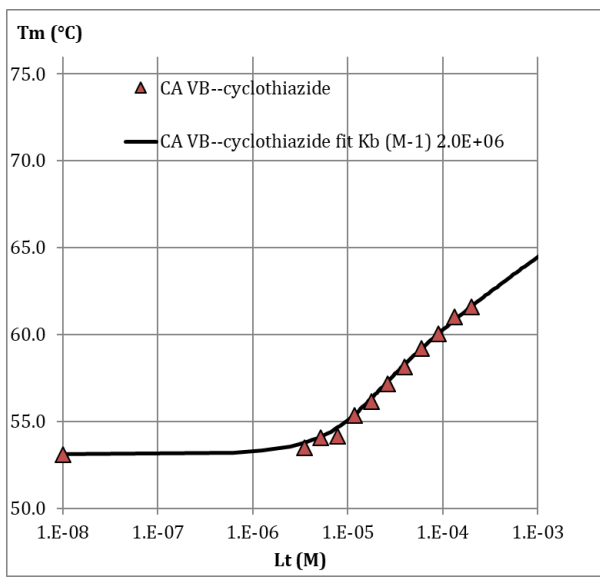
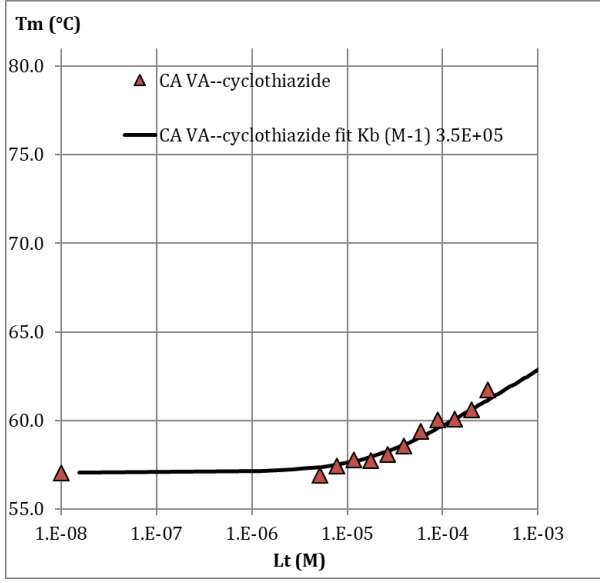
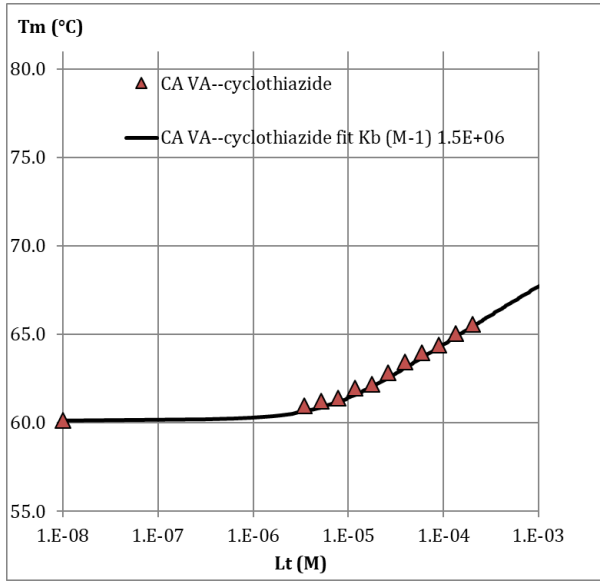
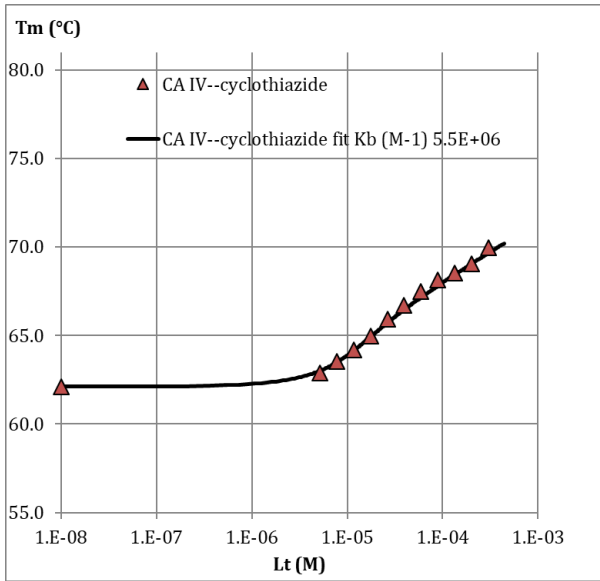
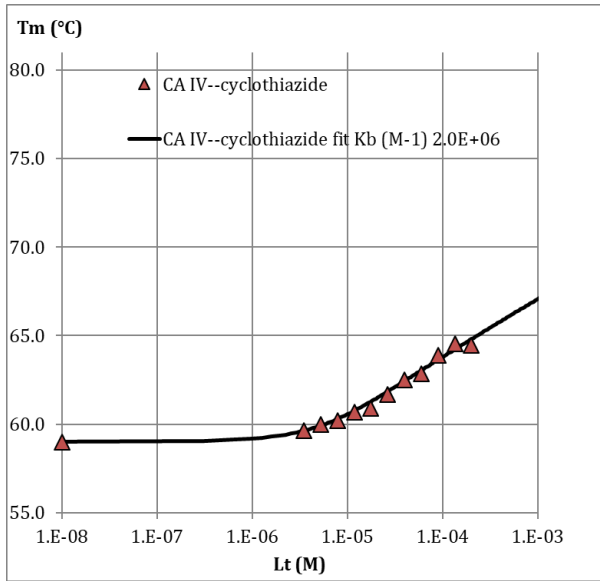


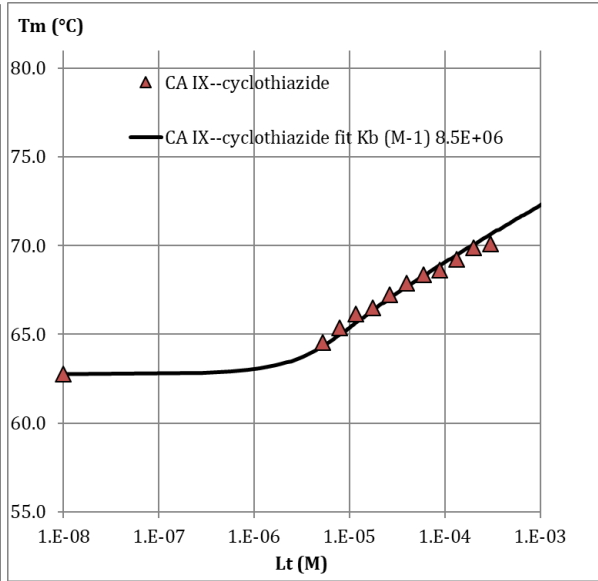
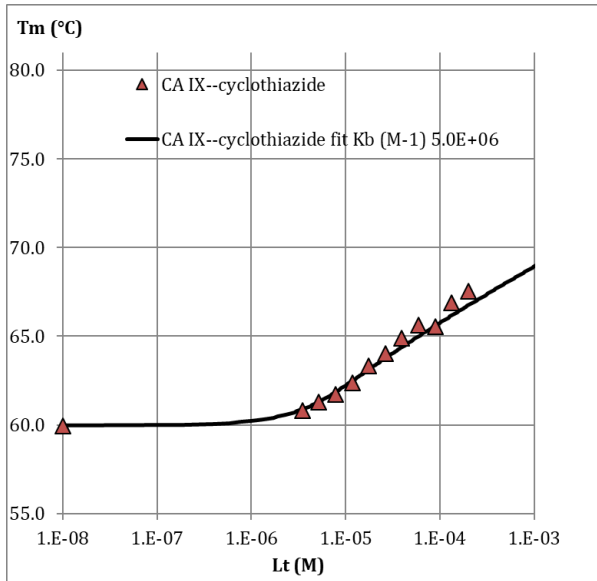
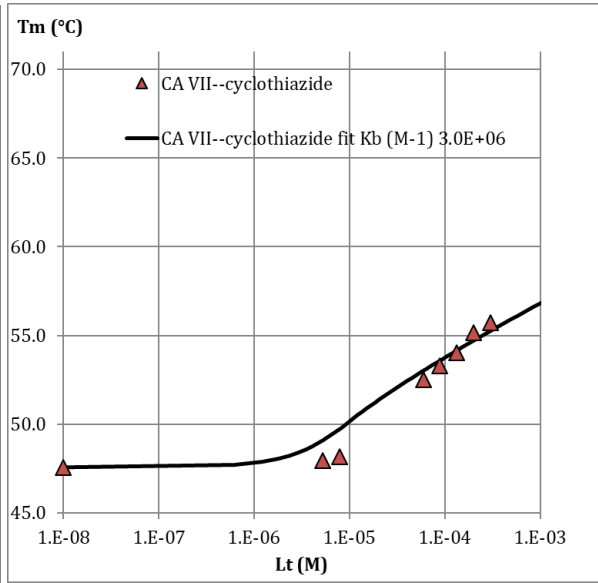
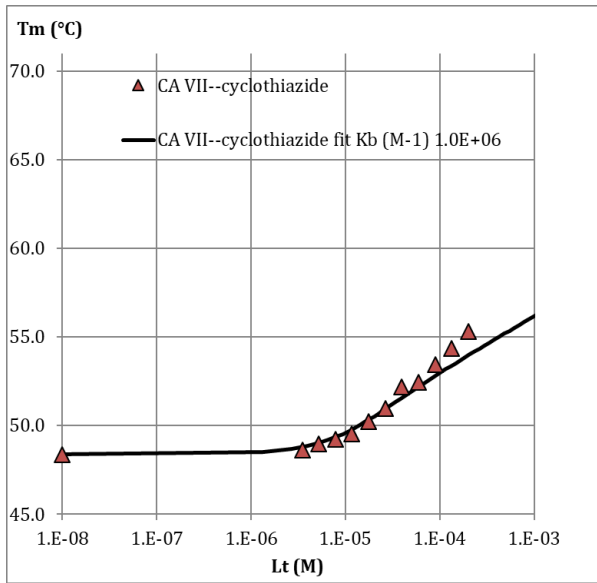
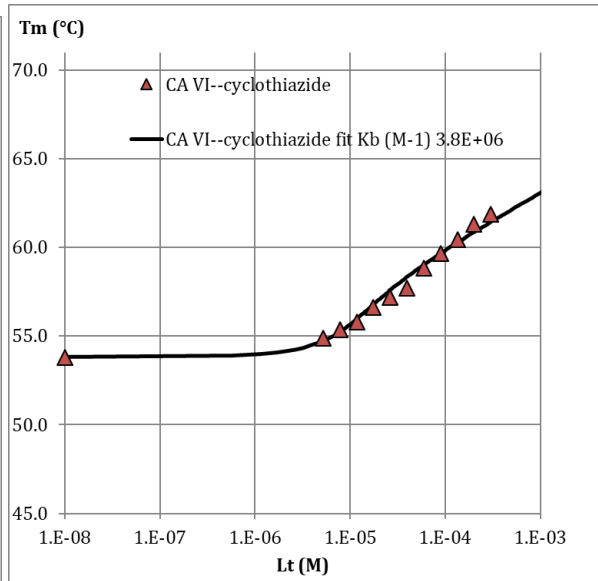
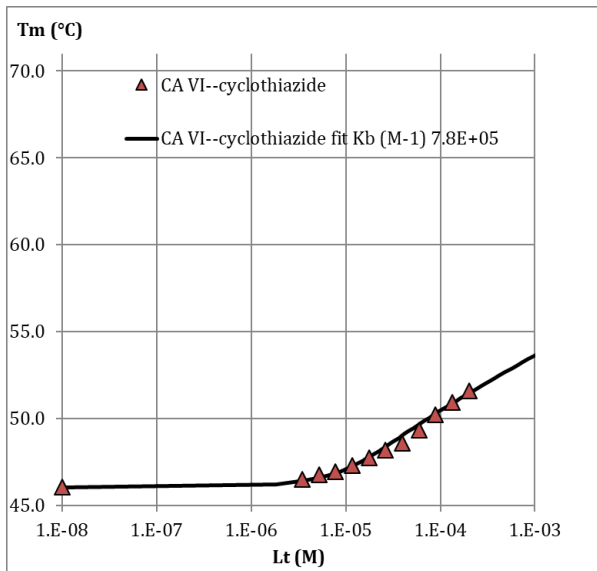
## 6. Cyclothiazide

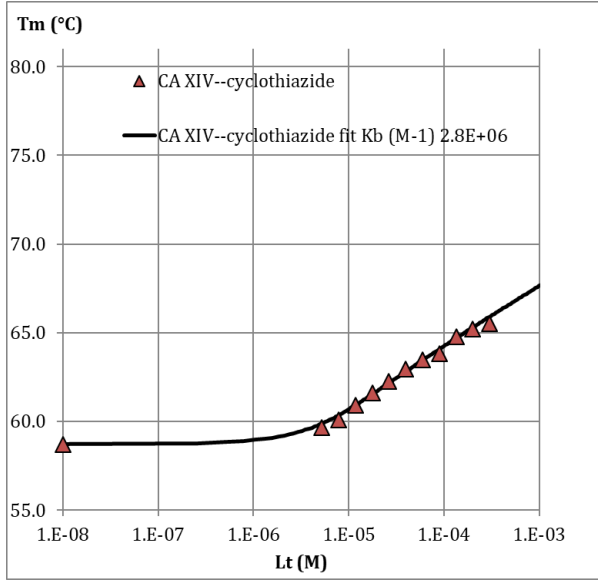
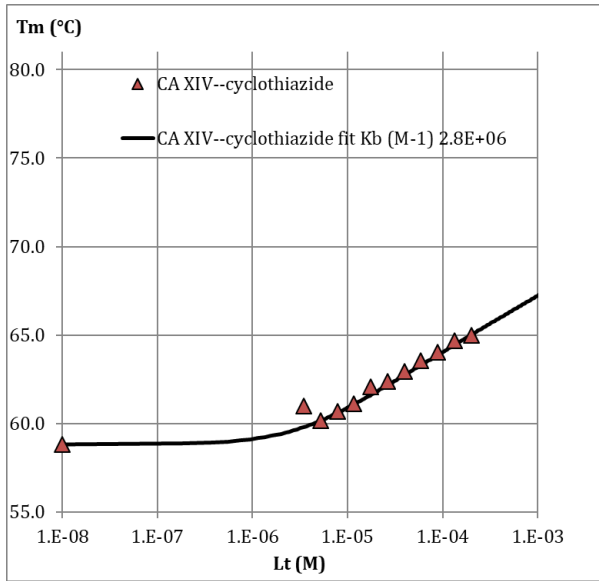
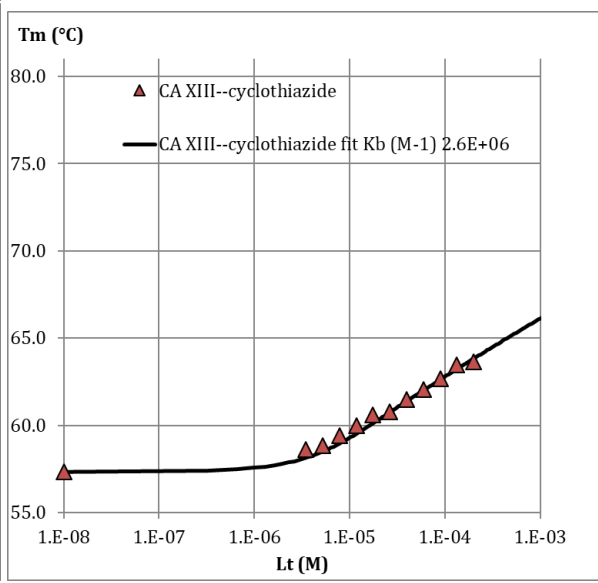
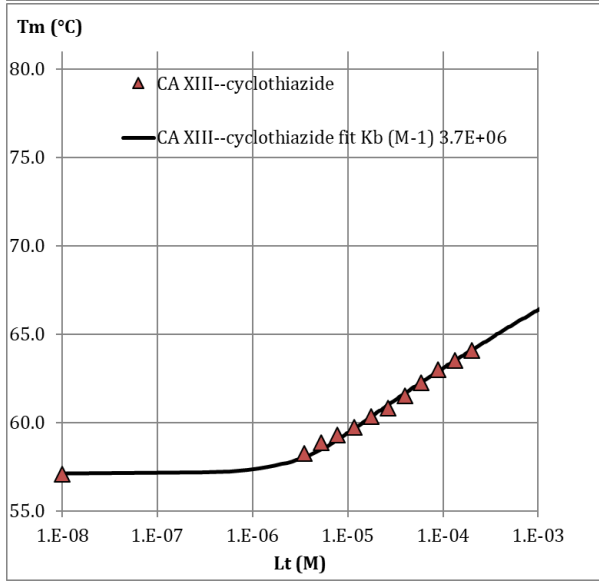
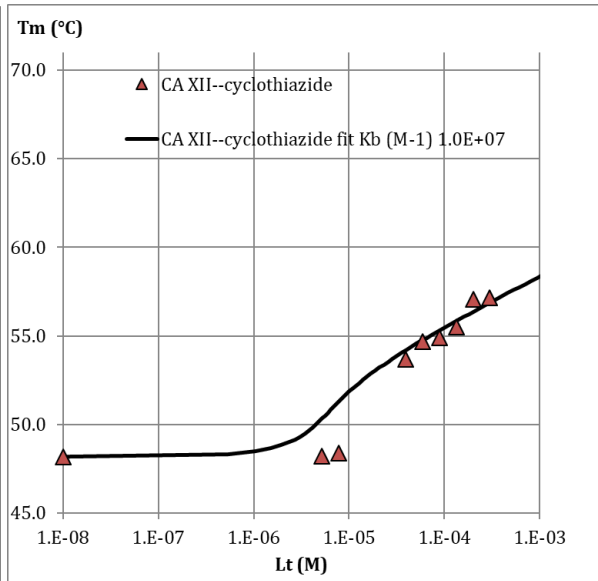
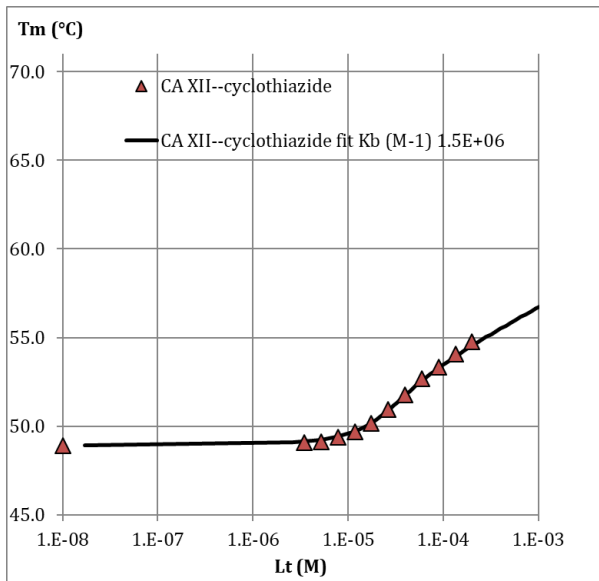
### Fluorescence-based thermal shift assay





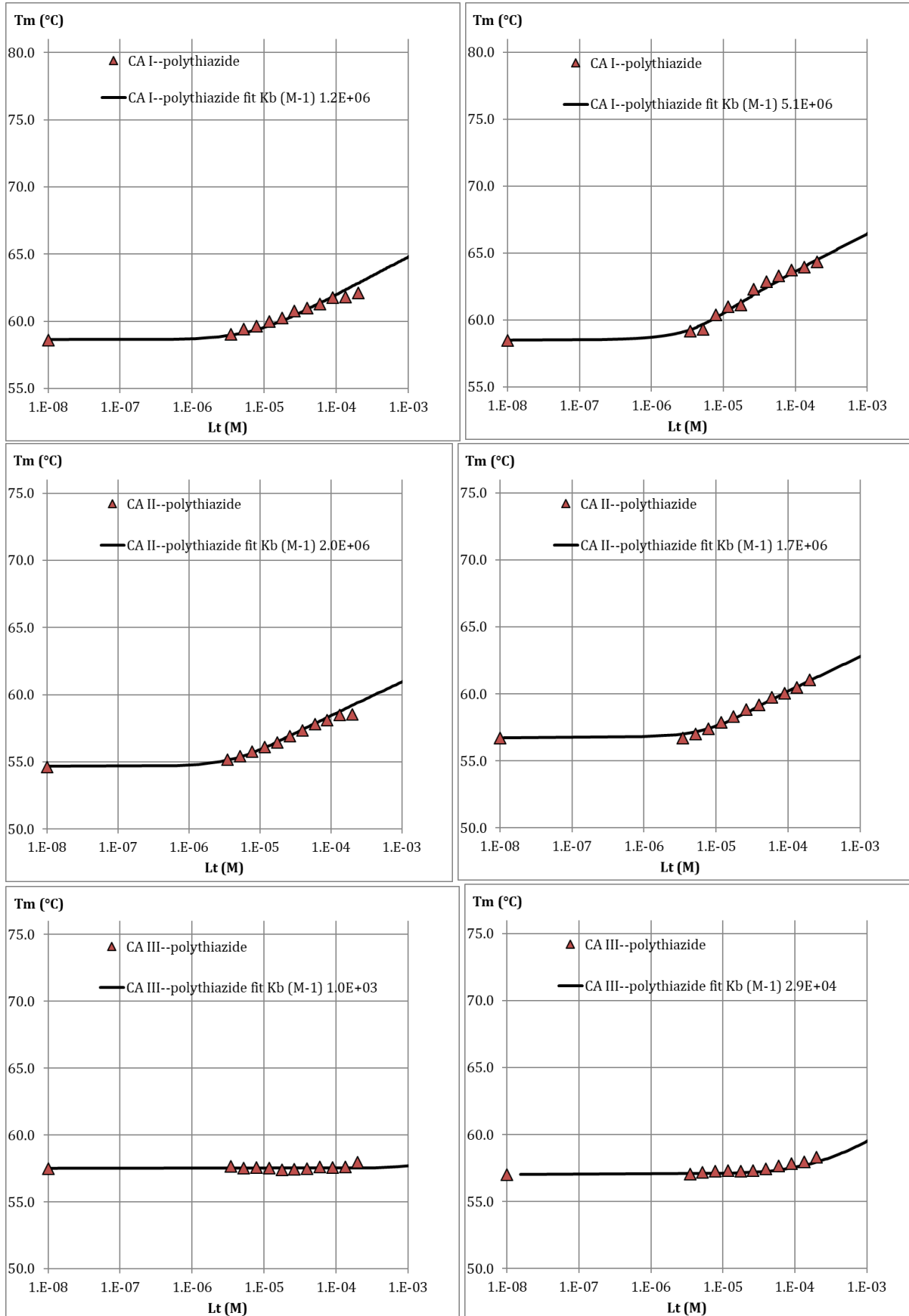


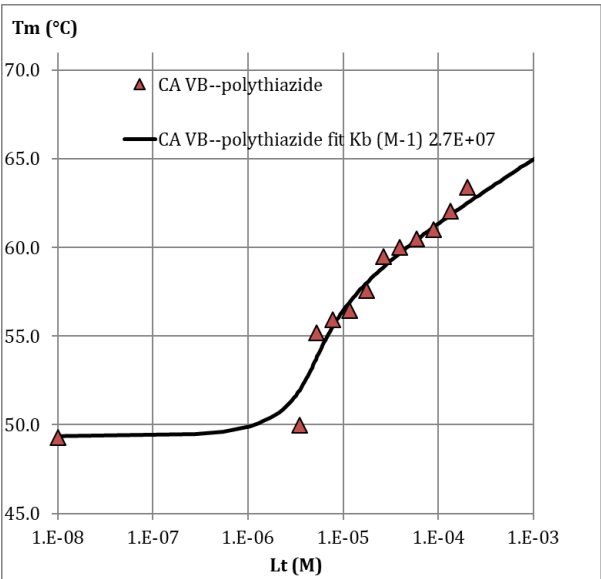
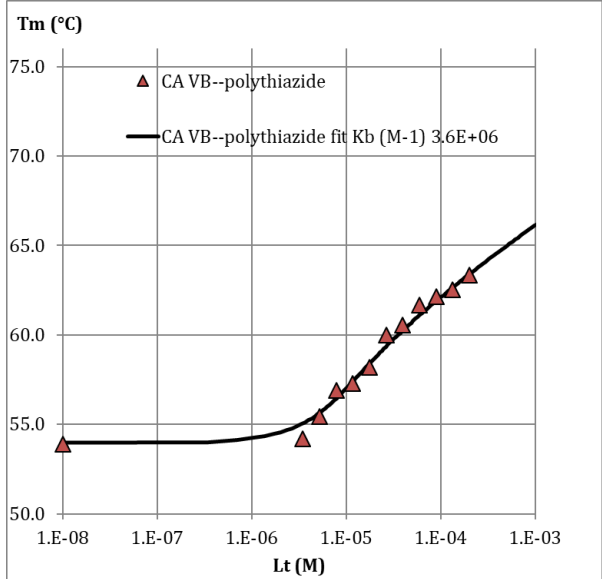
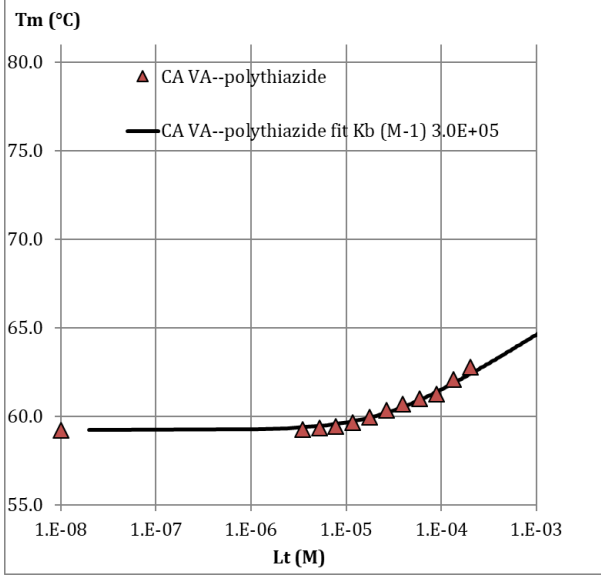
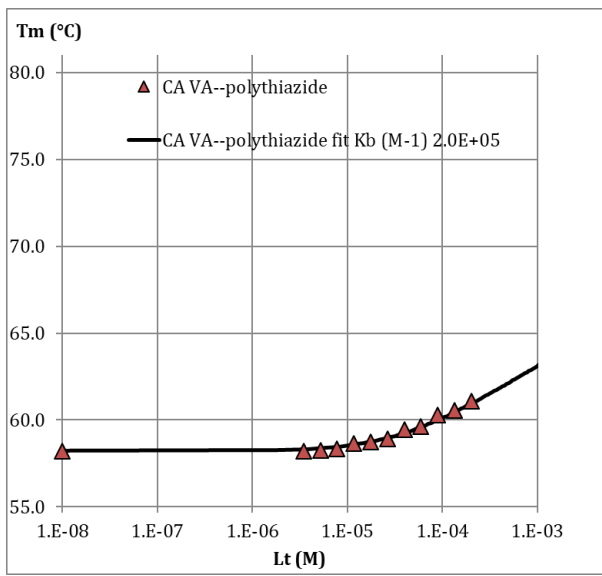
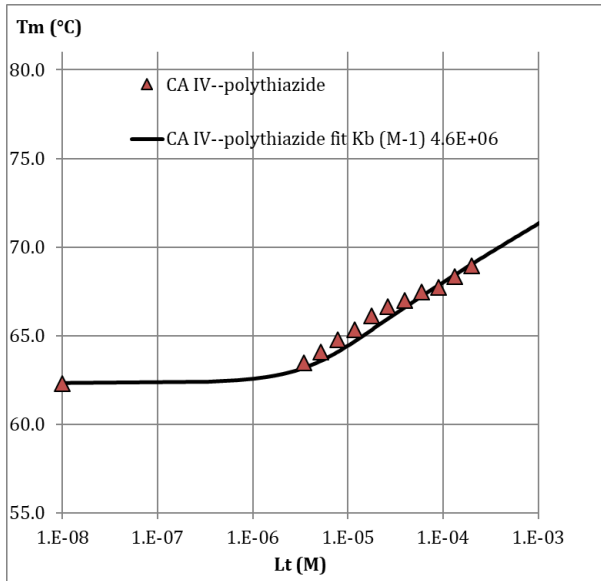
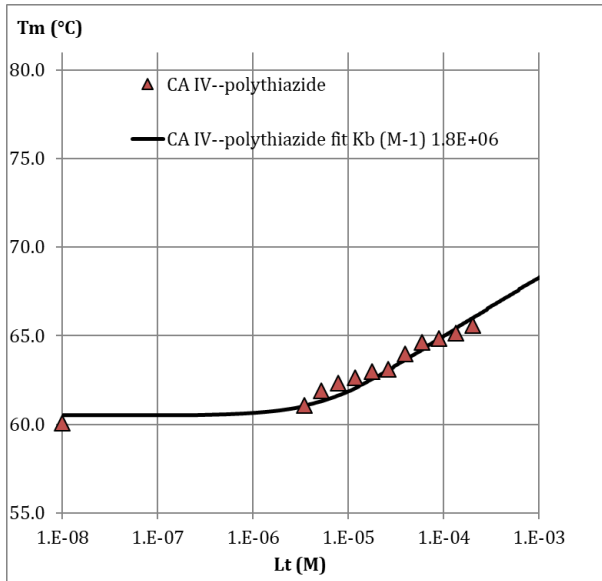


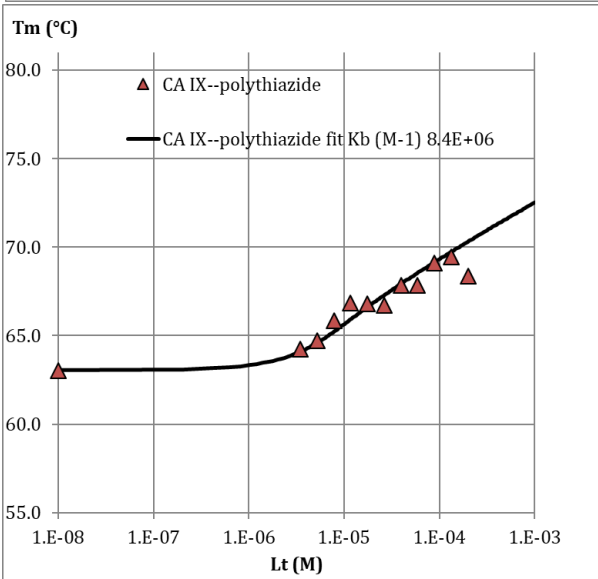
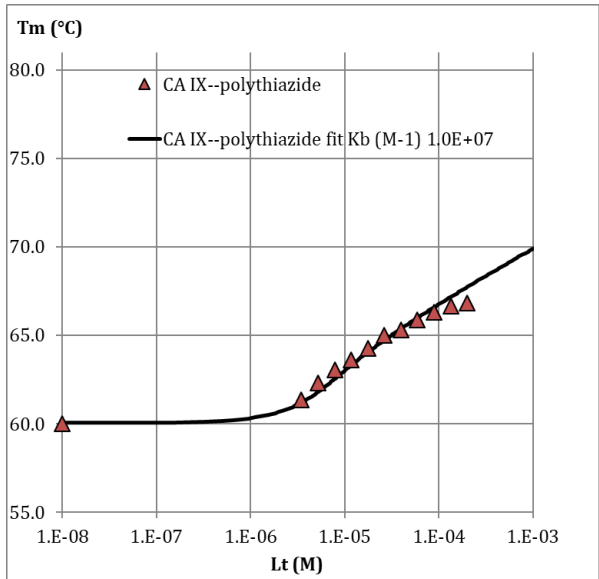
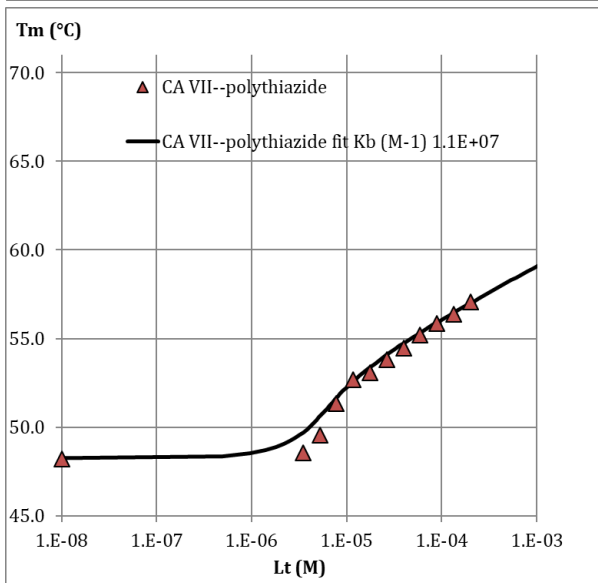
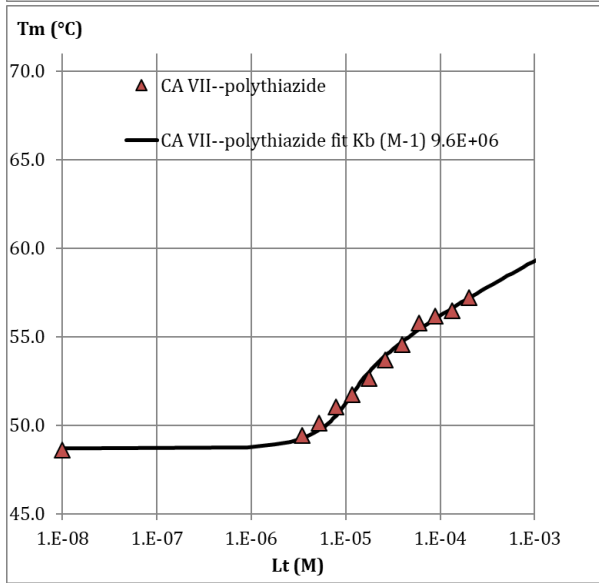
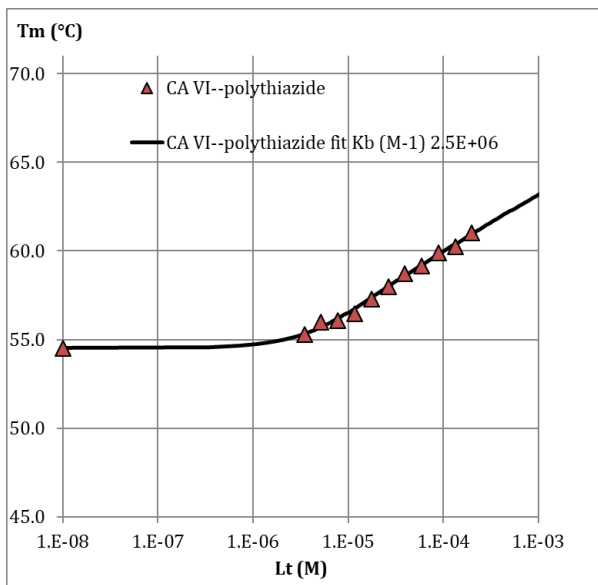
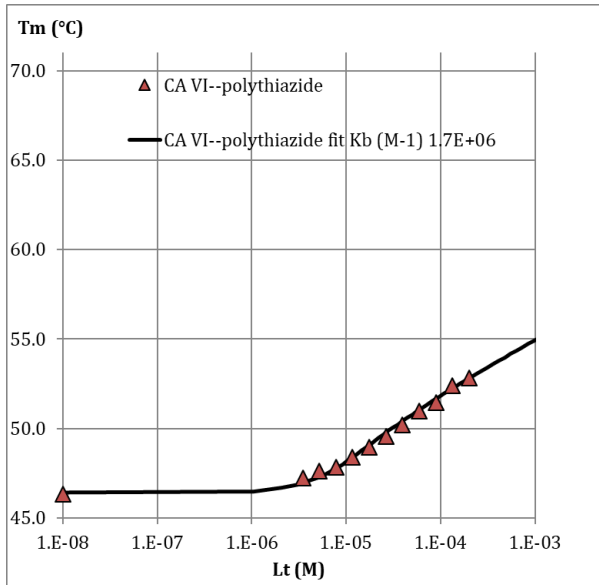


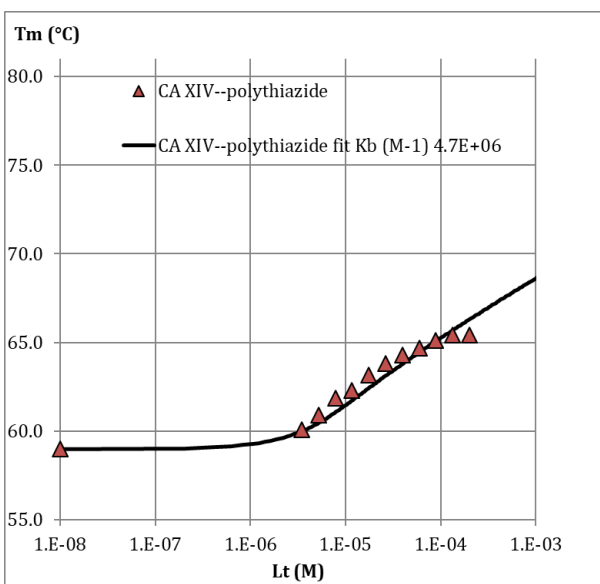
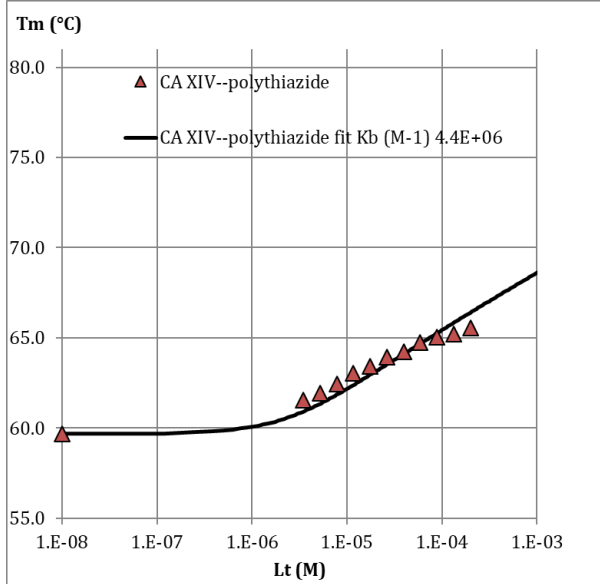
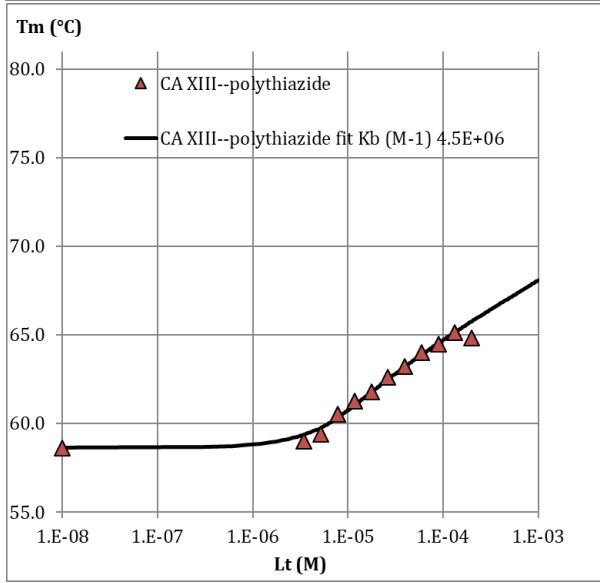
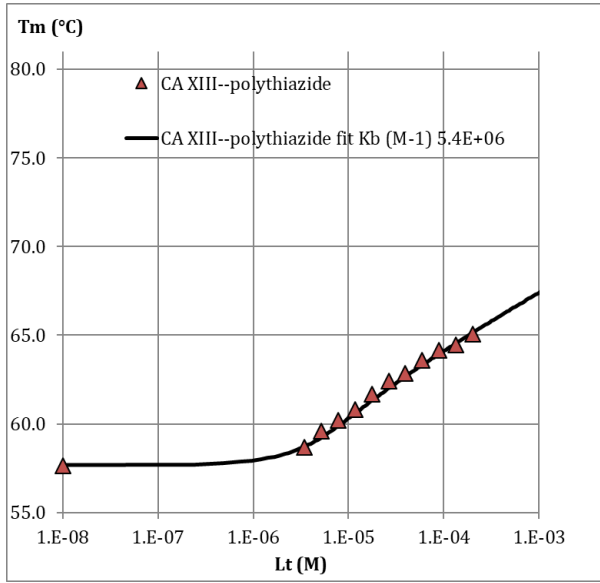
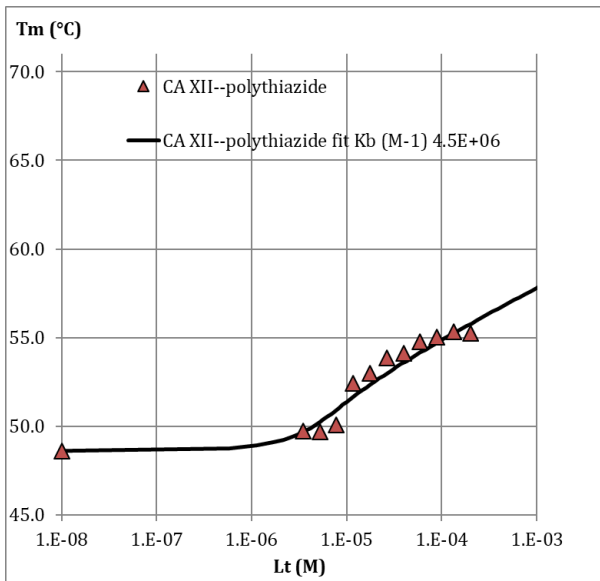
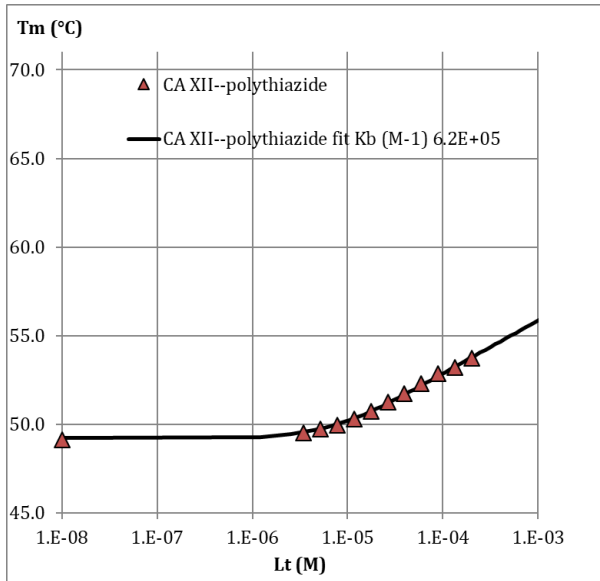
# 7. Polythiazide

## Fluorecence-based thermal shift assay



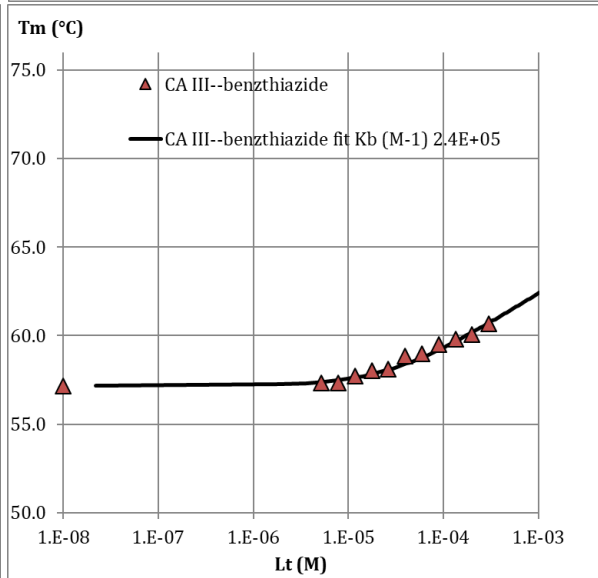
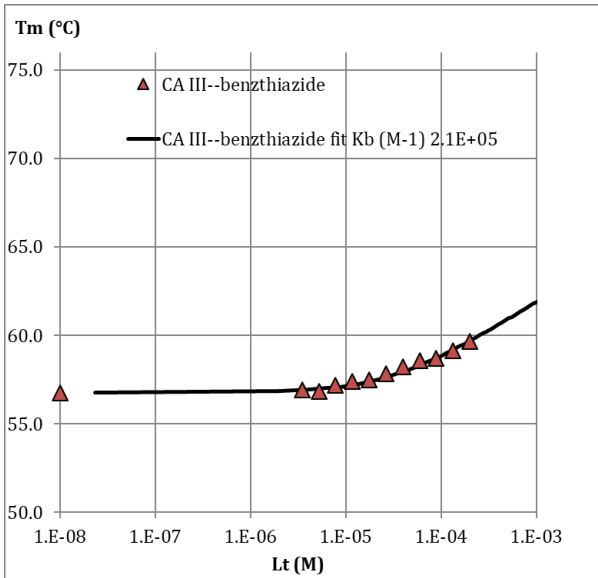
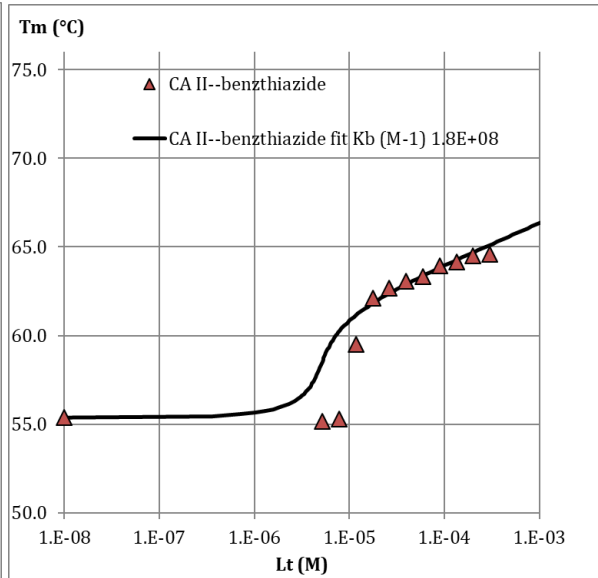
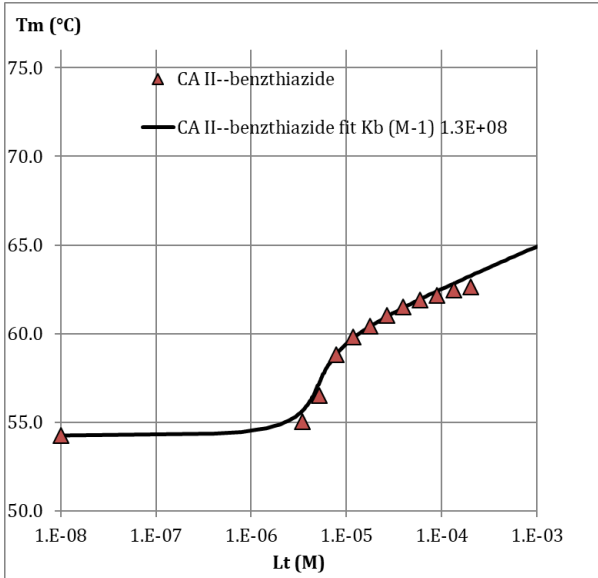
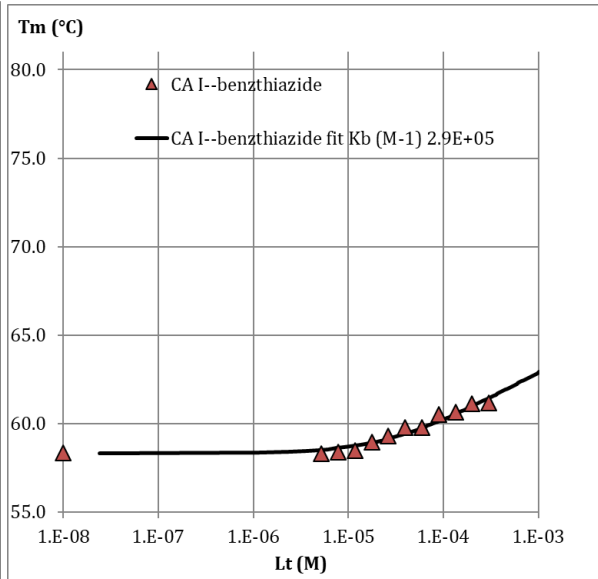
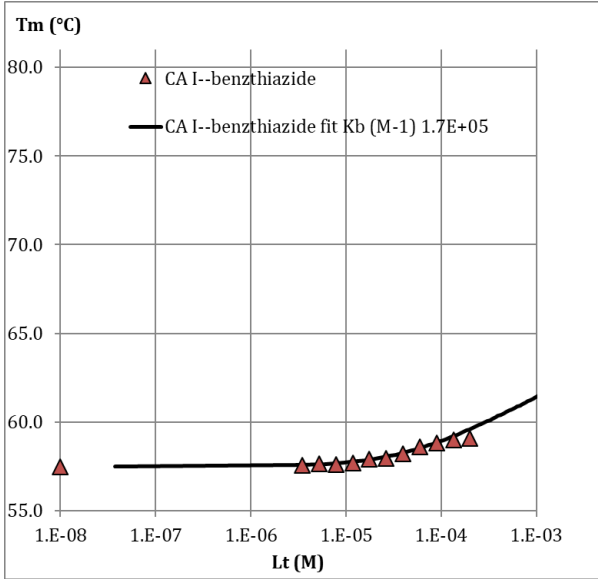




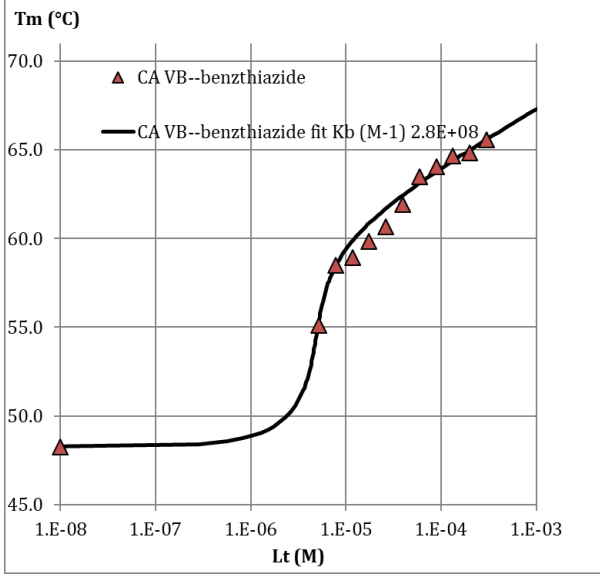
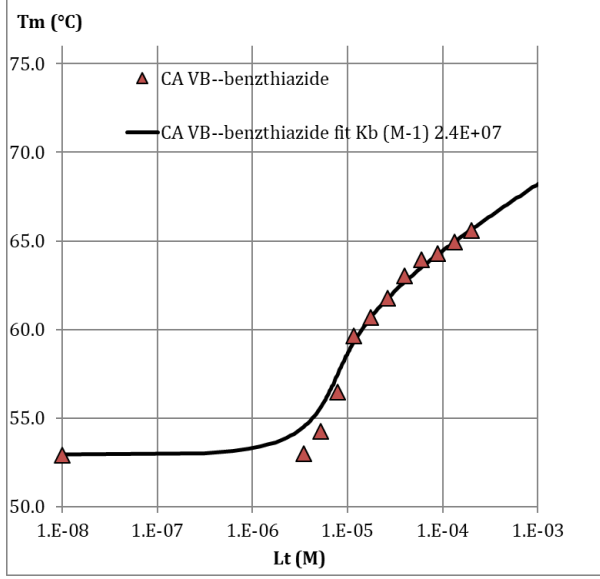
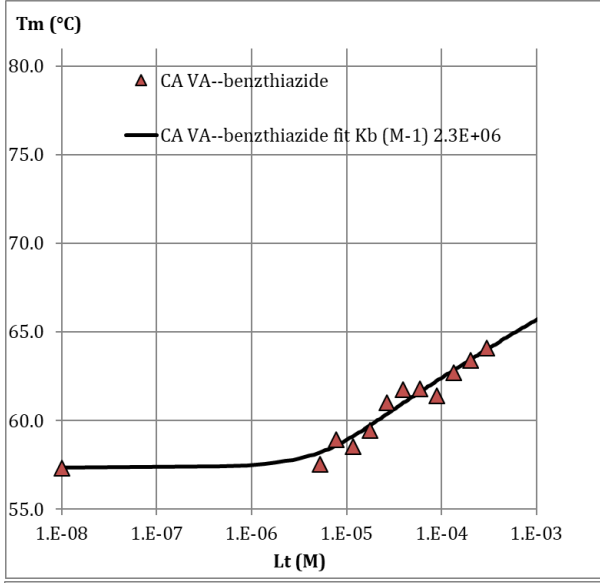
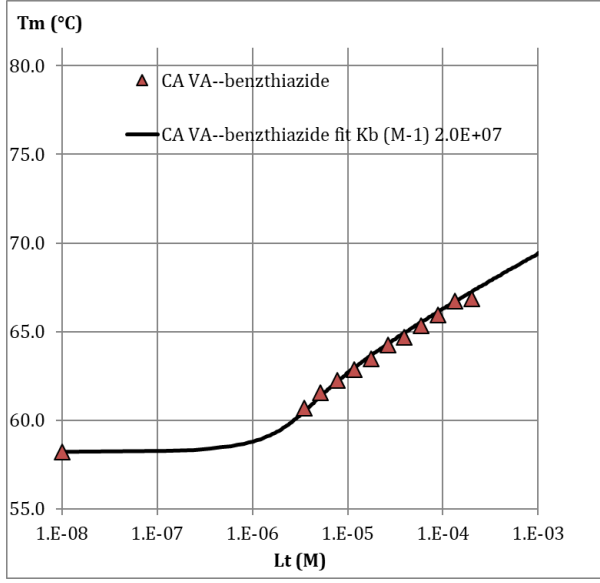
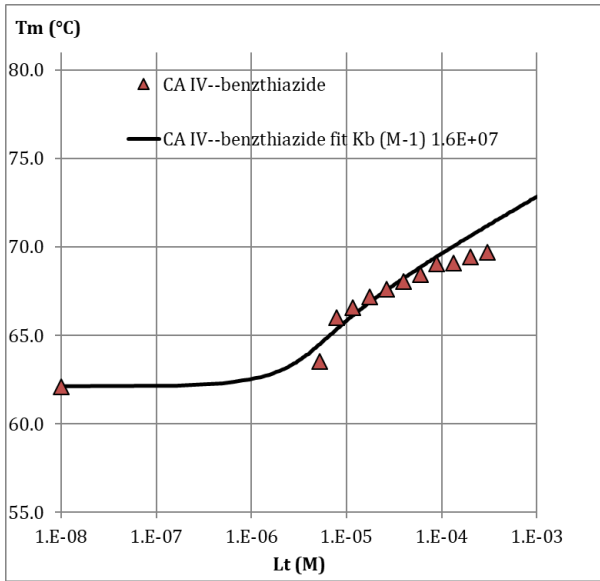
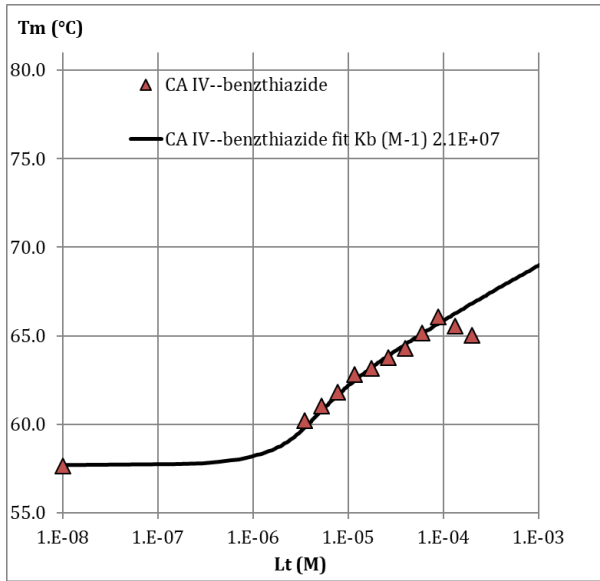


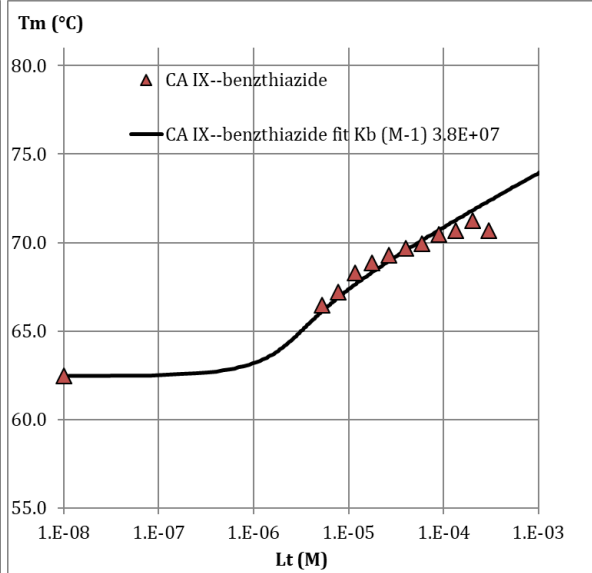
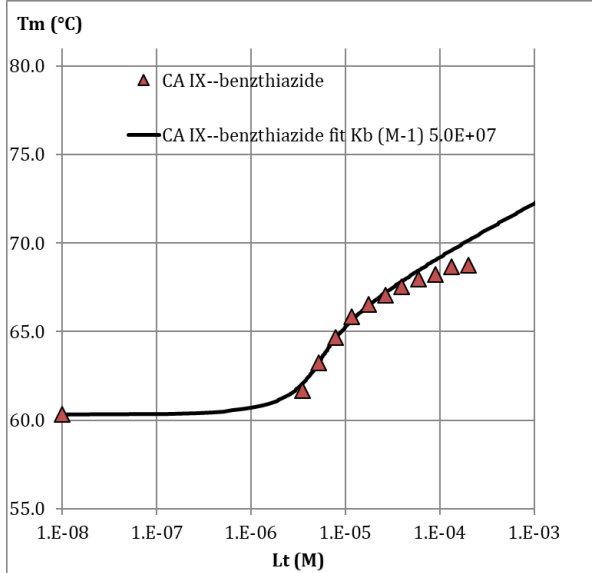
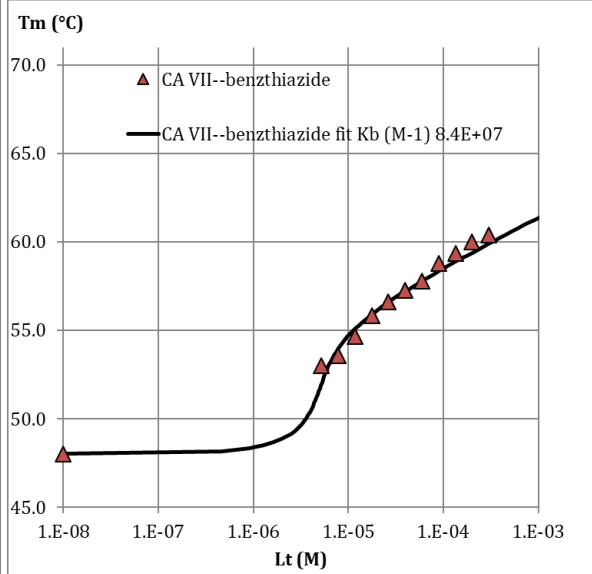
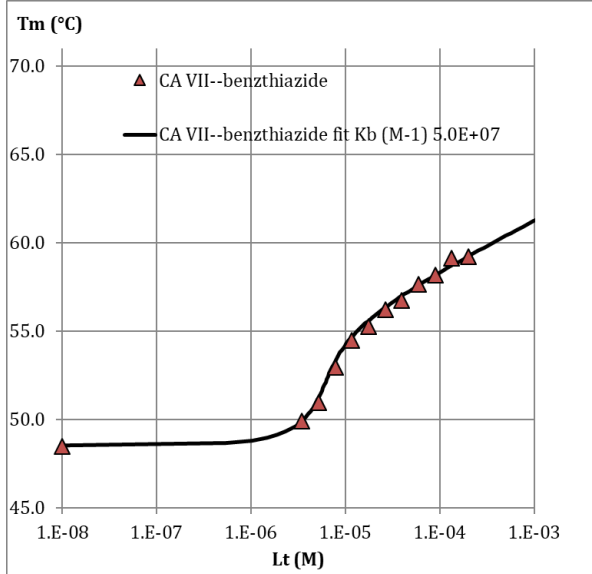
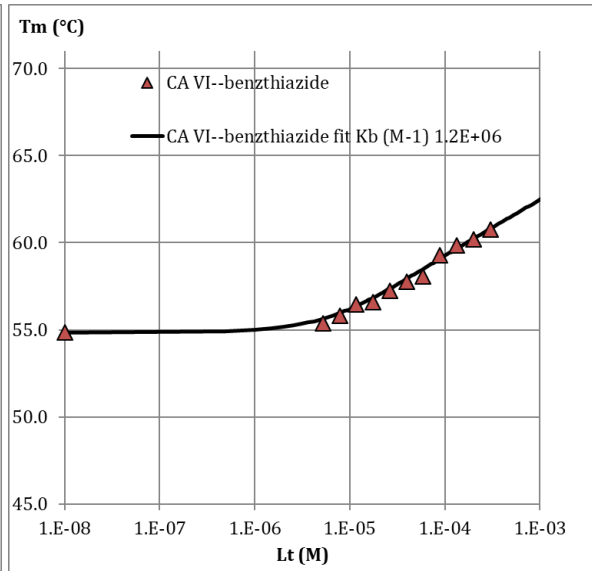
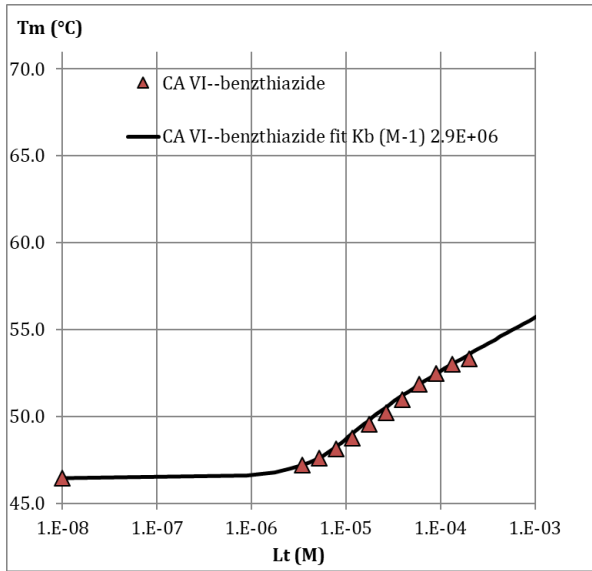
# 8. Benzthiazide

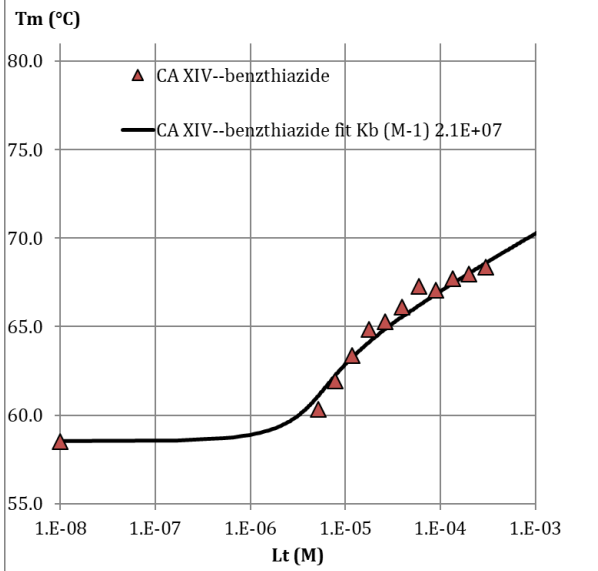
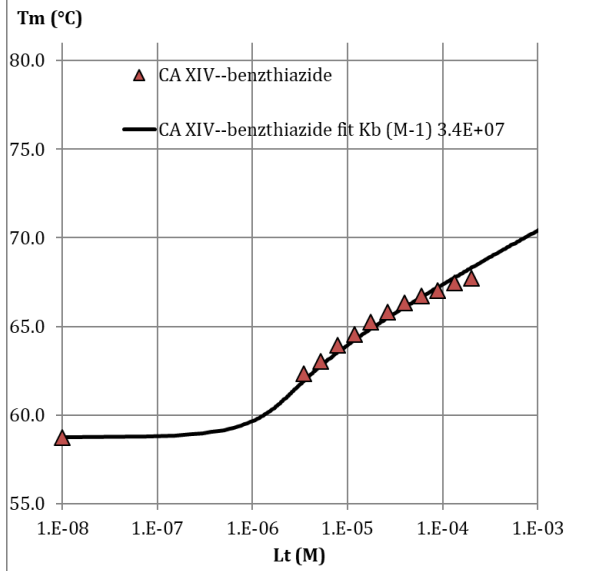
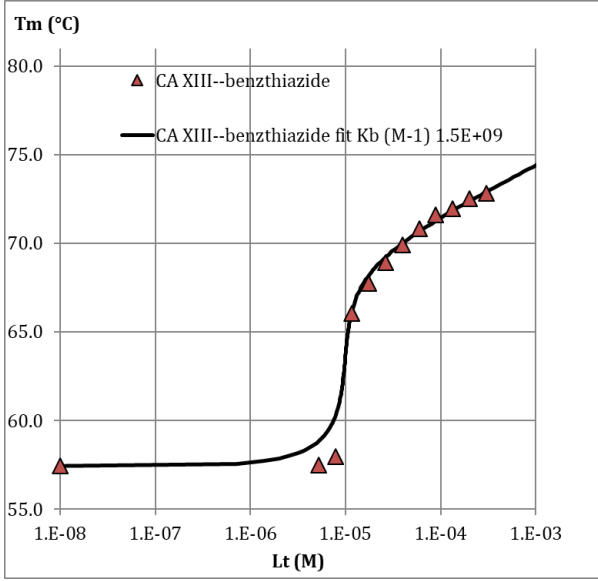
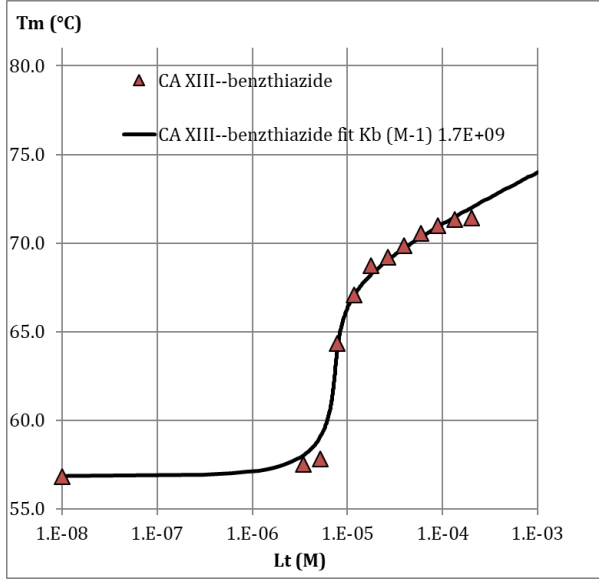
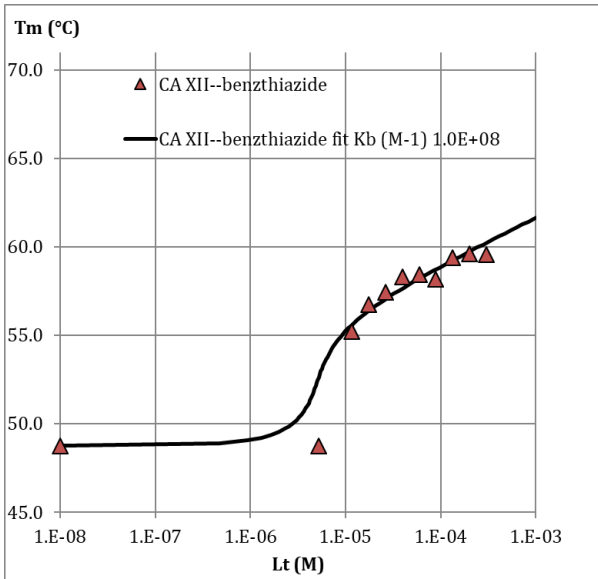
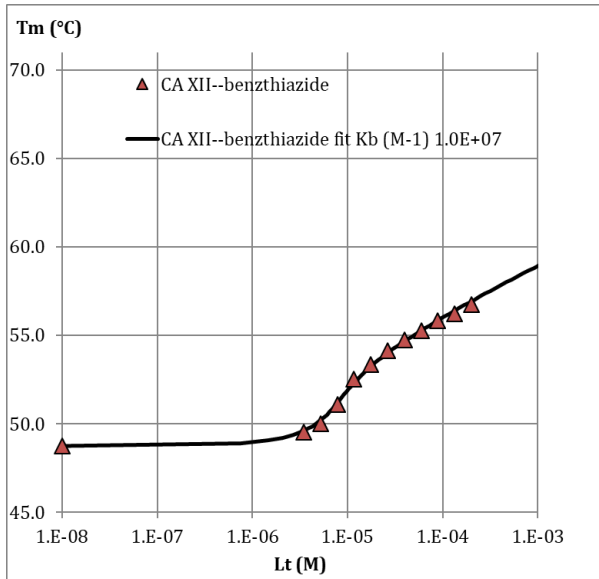
## Fluorecence-based thermal shift assay





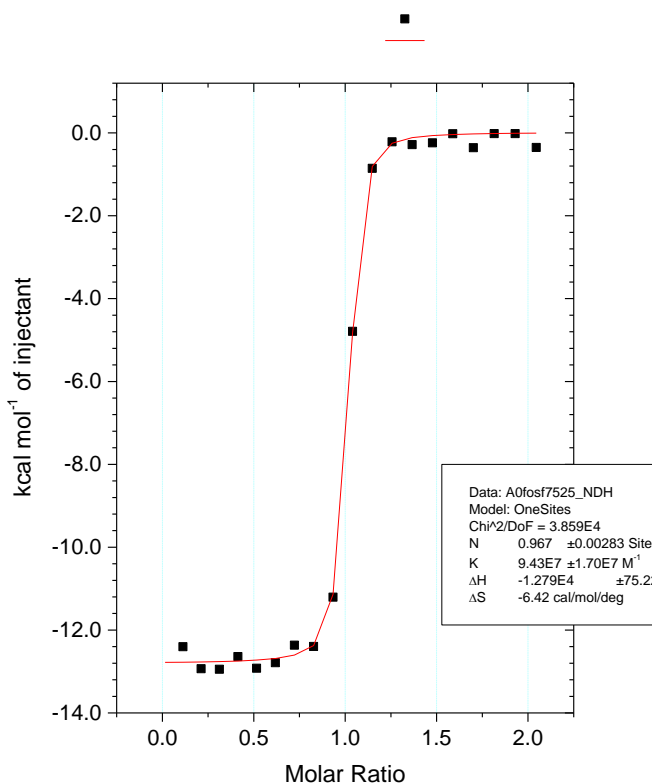
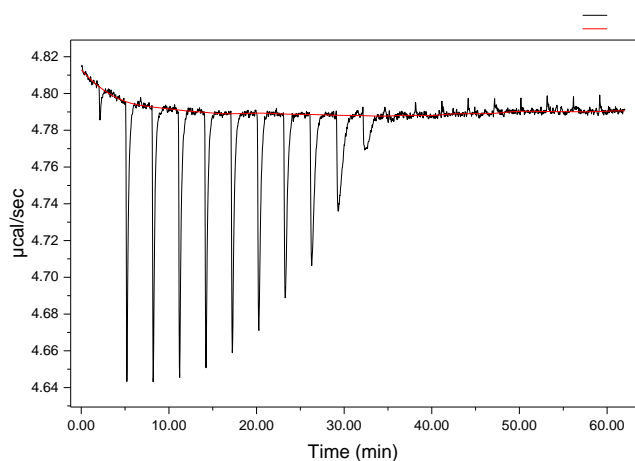




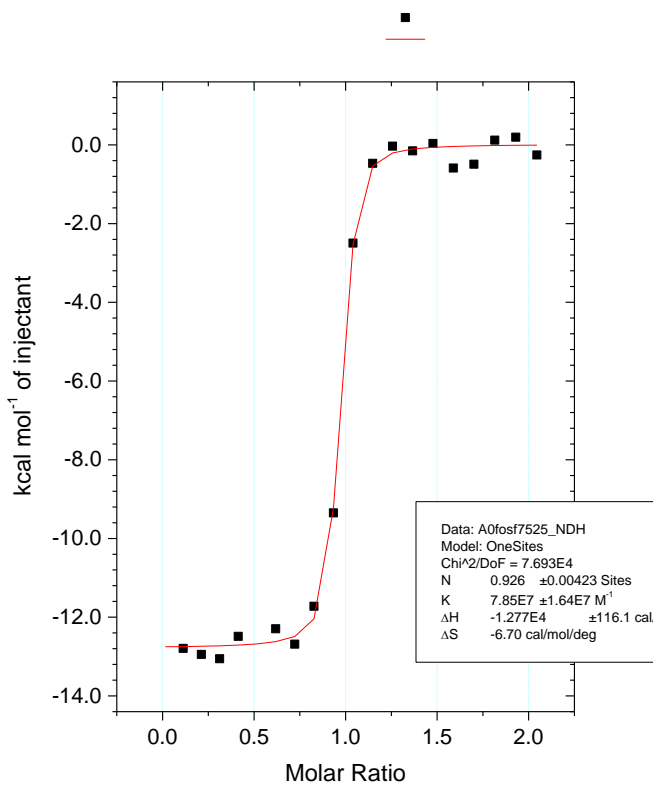
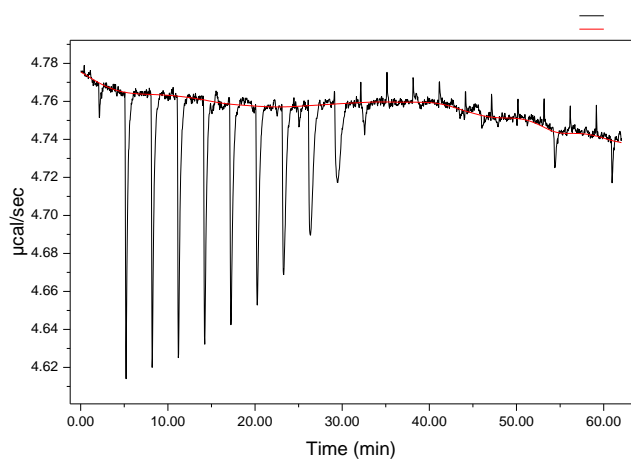


# Isothermal titration calorimetry

## CA II – benzthiazide

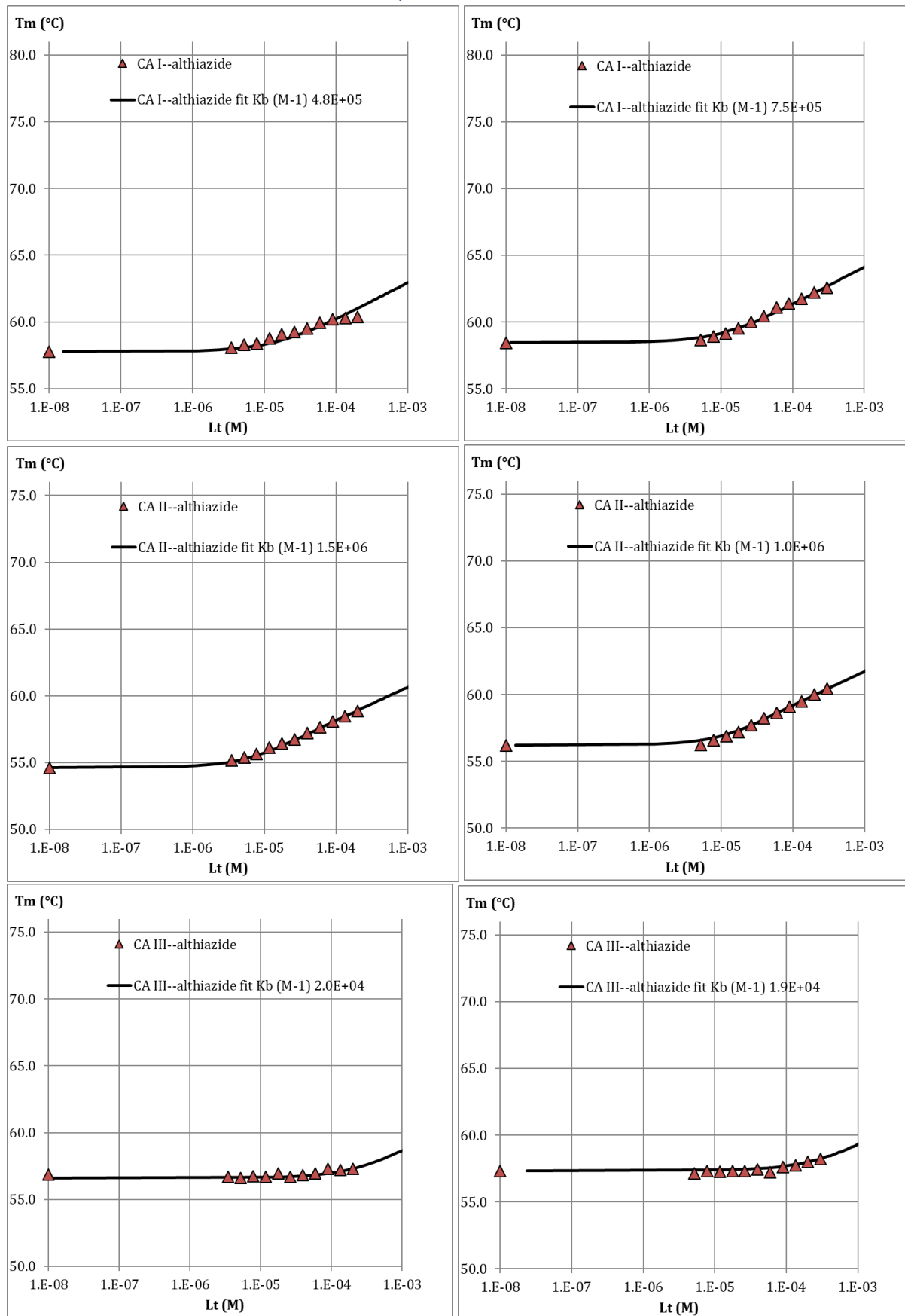


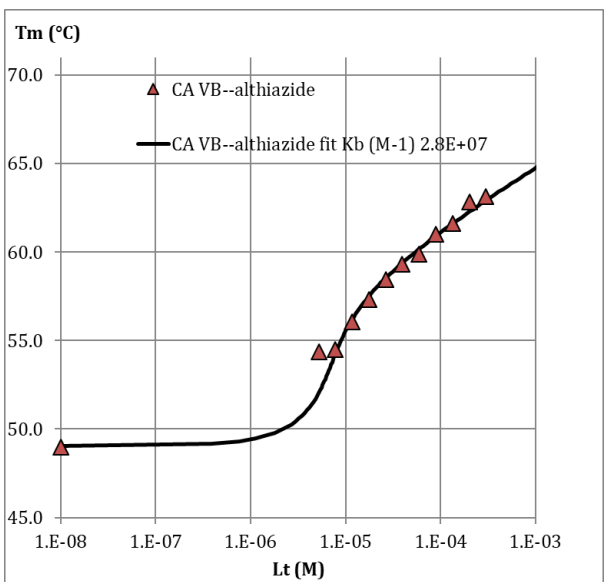
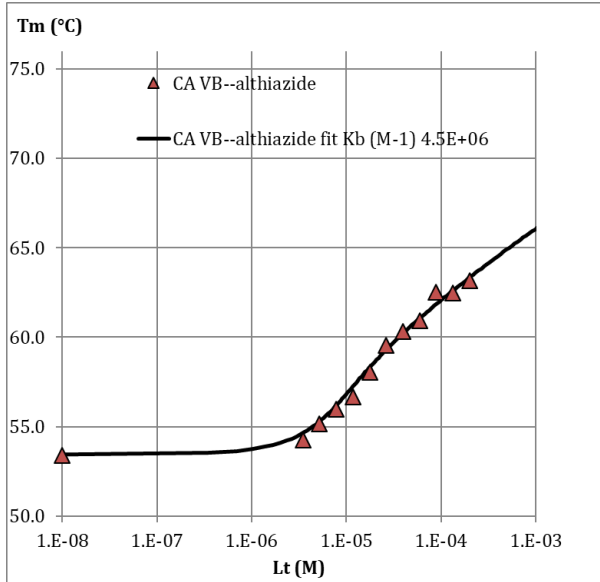
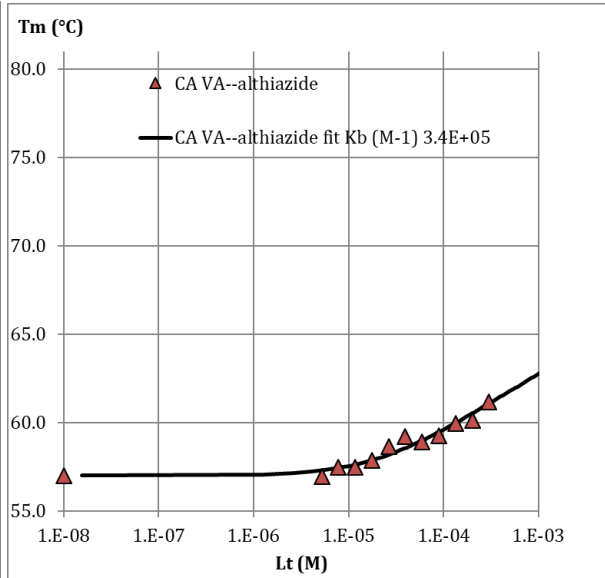
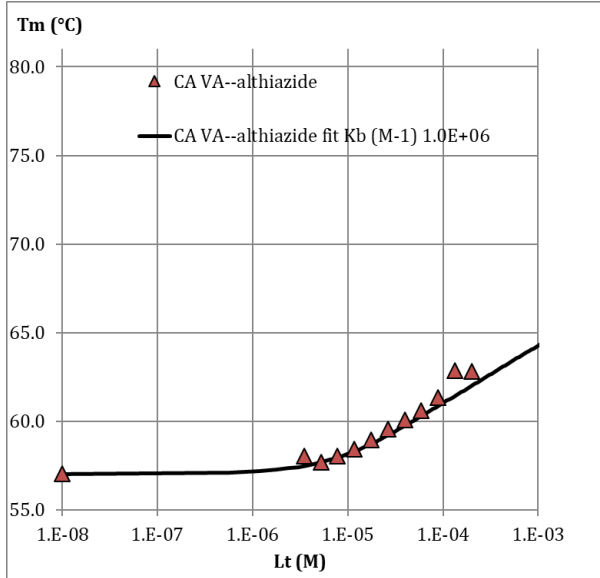
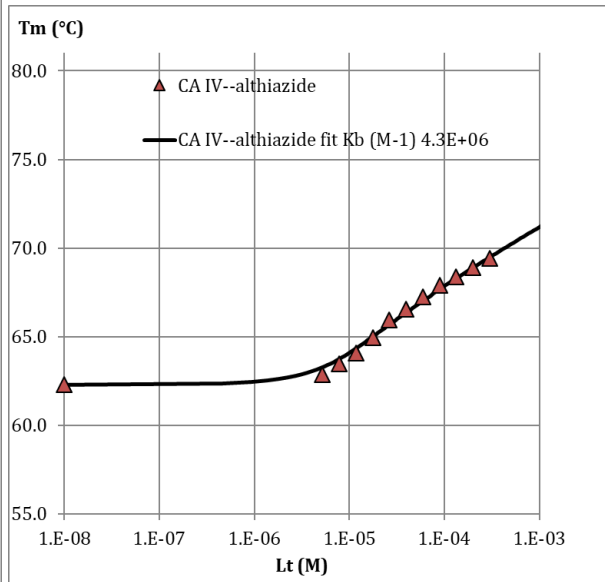
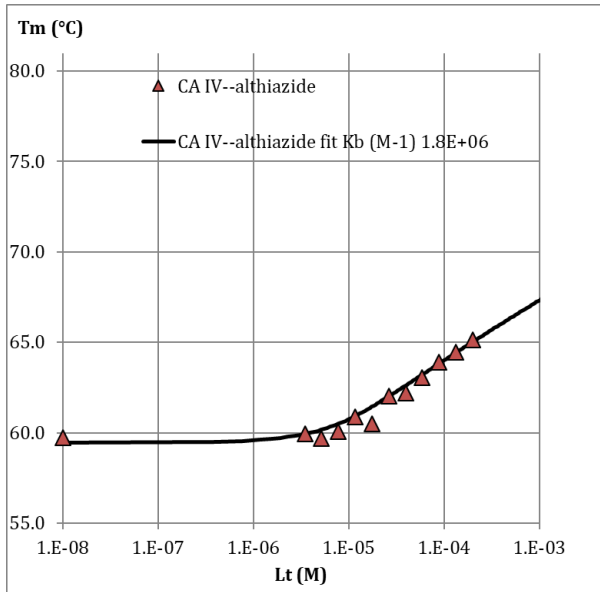
## CA II – benzthiazide

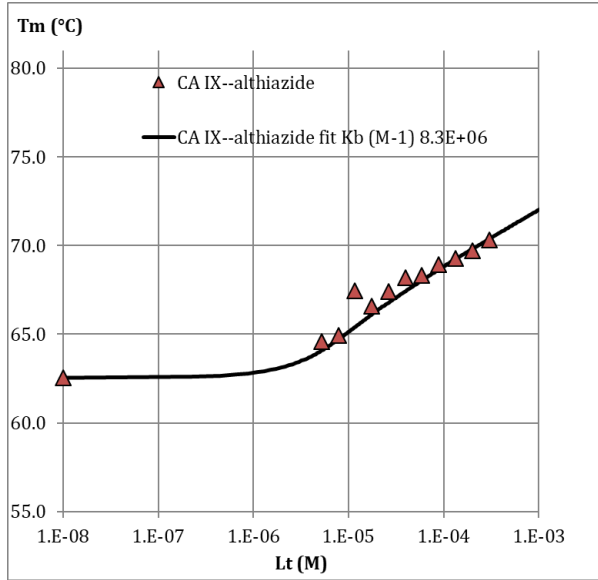
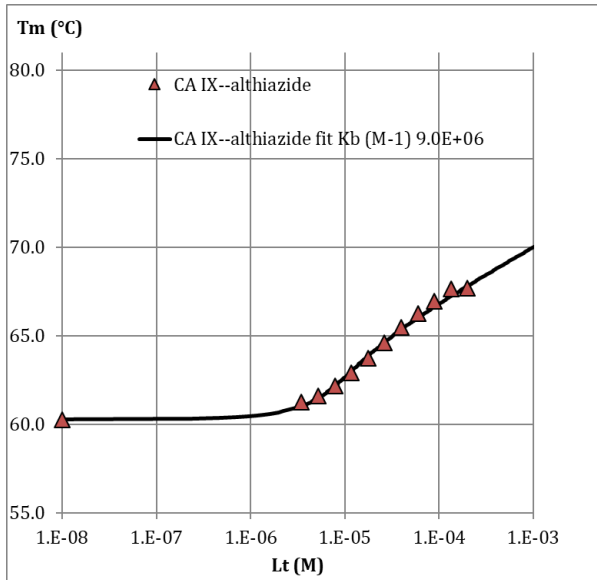
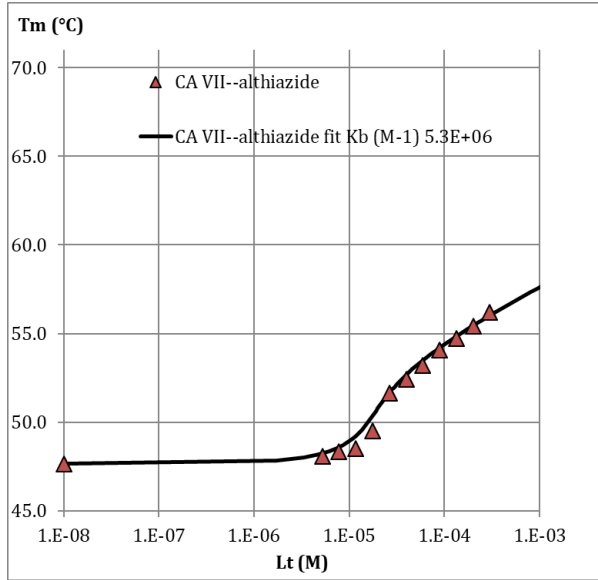
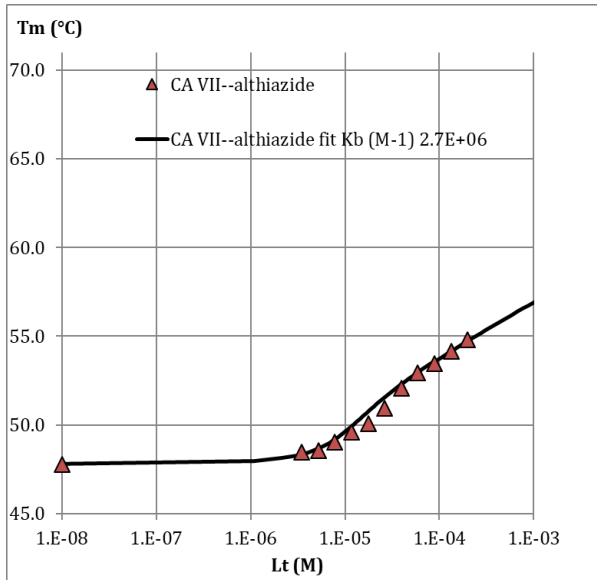
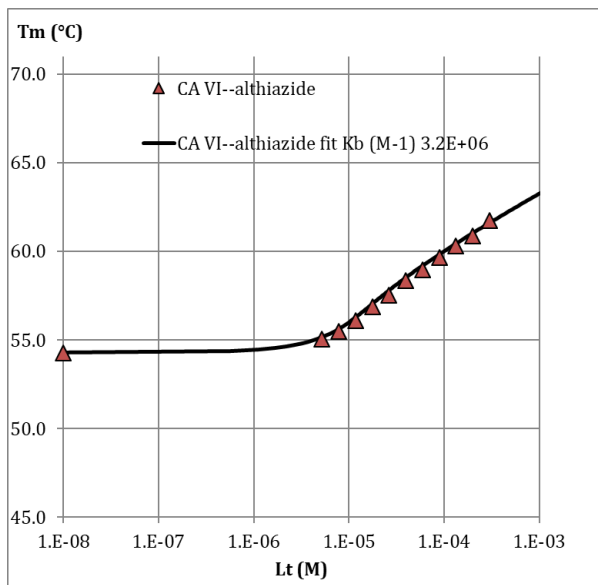
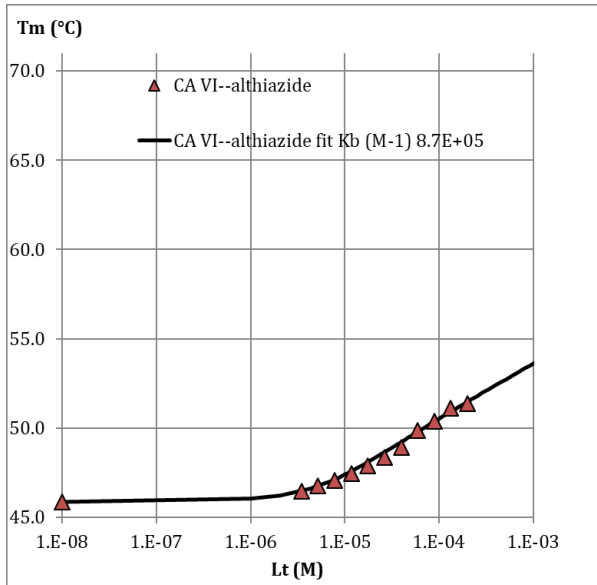


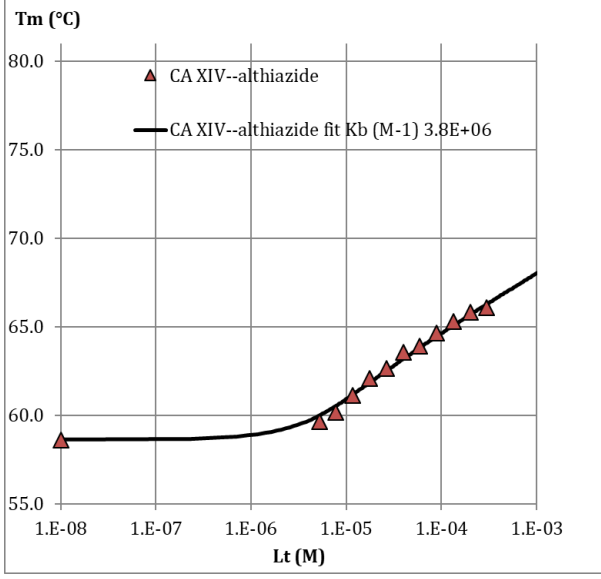
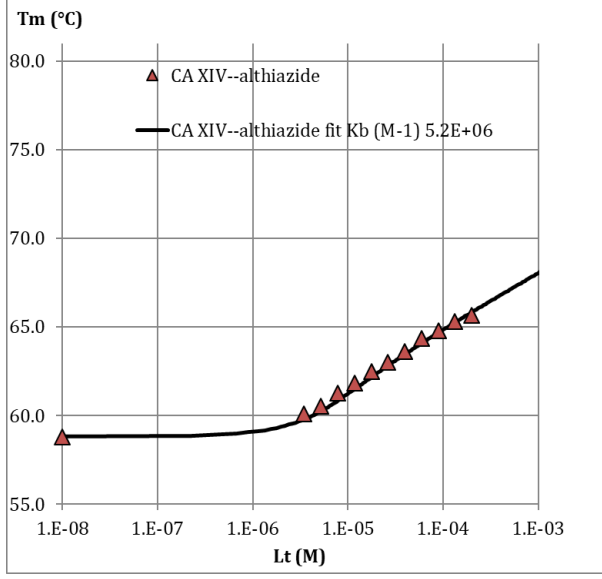
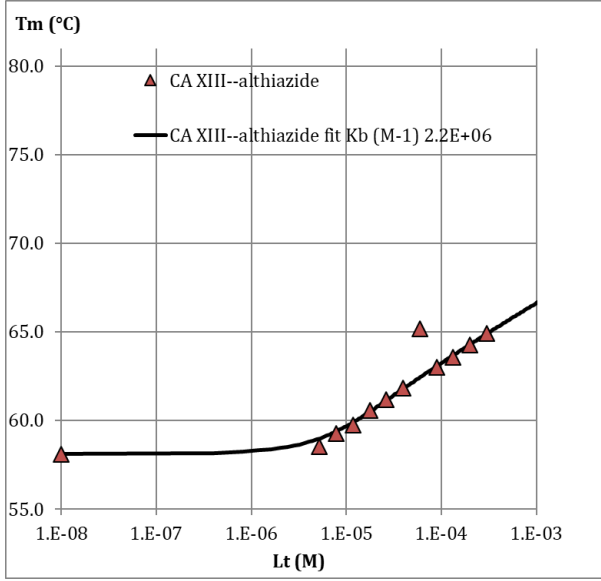
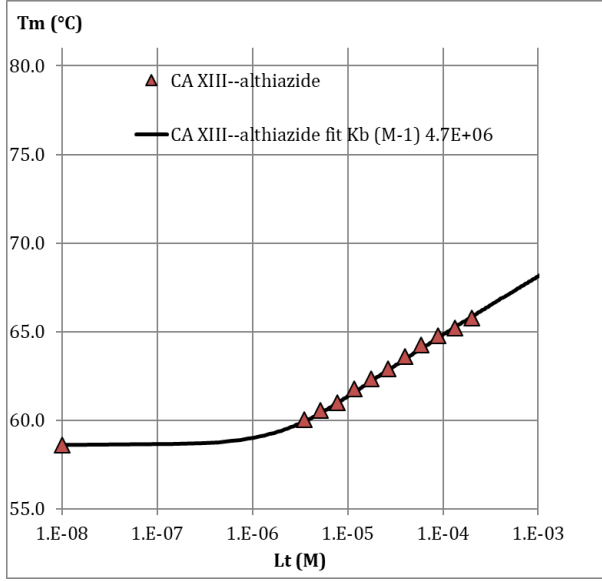
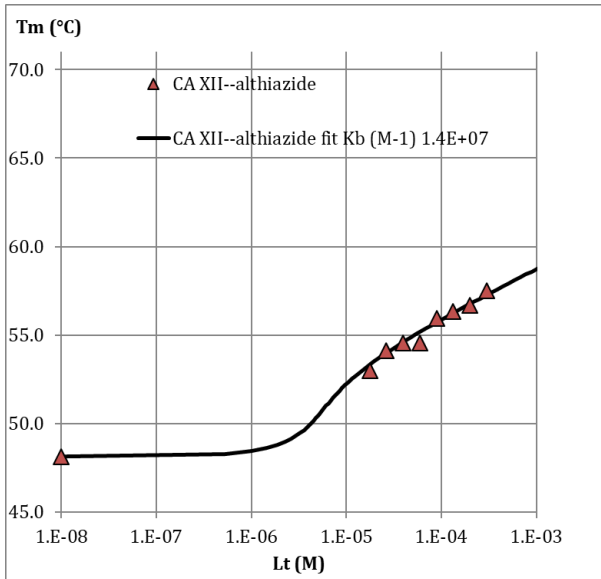
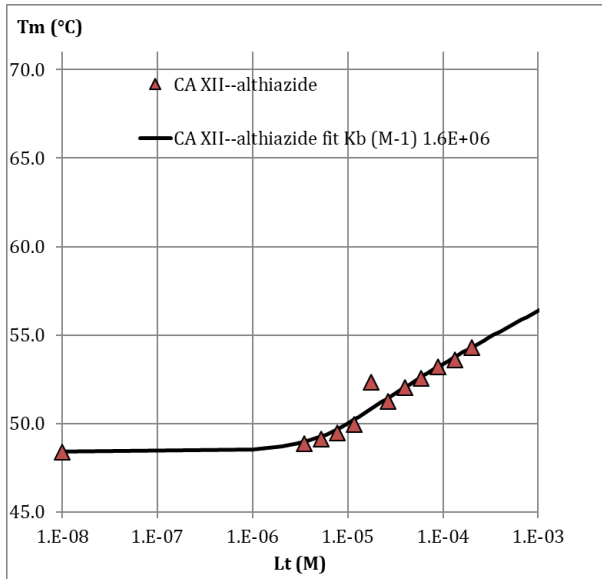
# 9. Athiazide

## Fluorence-based thermal shift assay





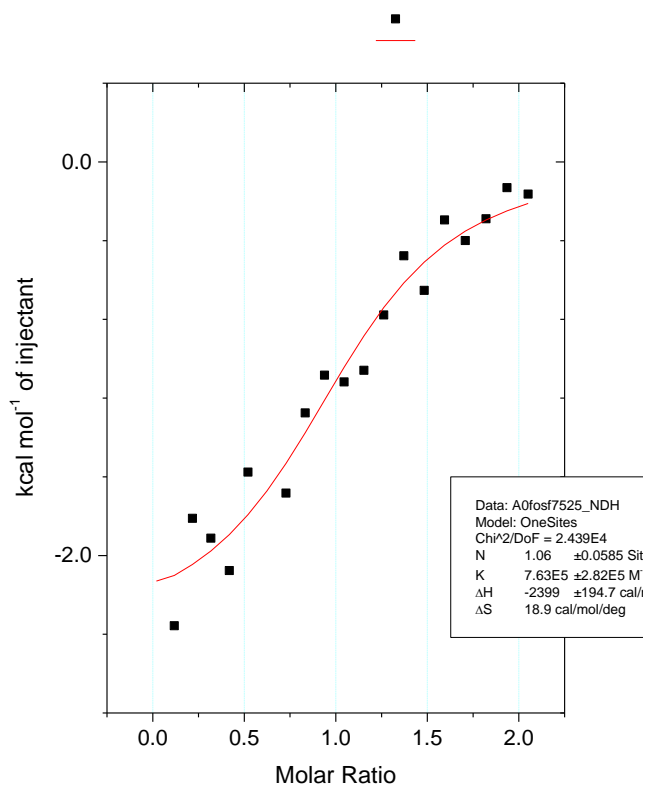
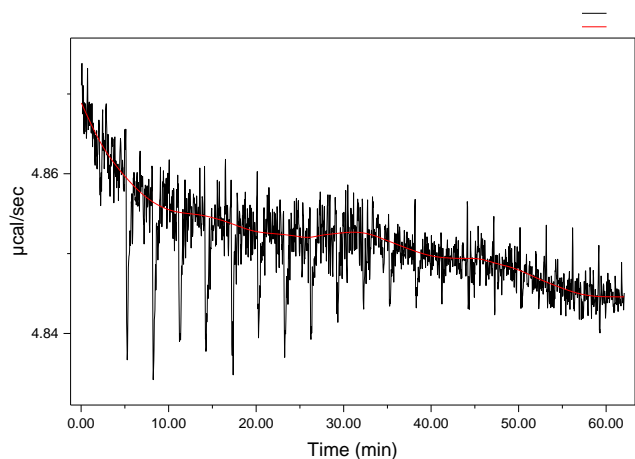




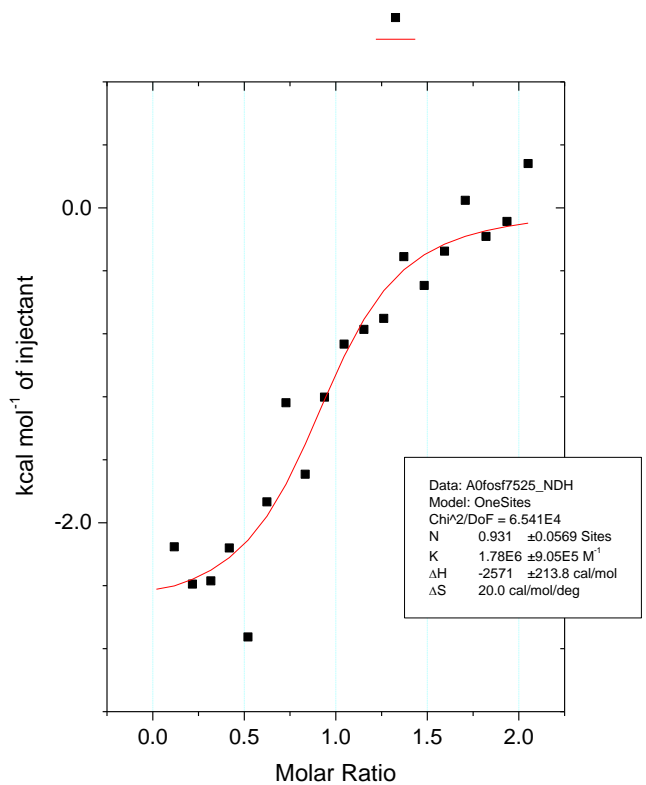
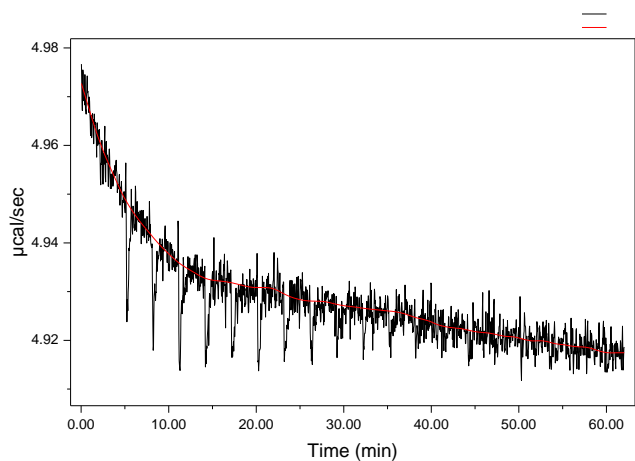


# Isothermal titration calorimetry

CA II- althiazide

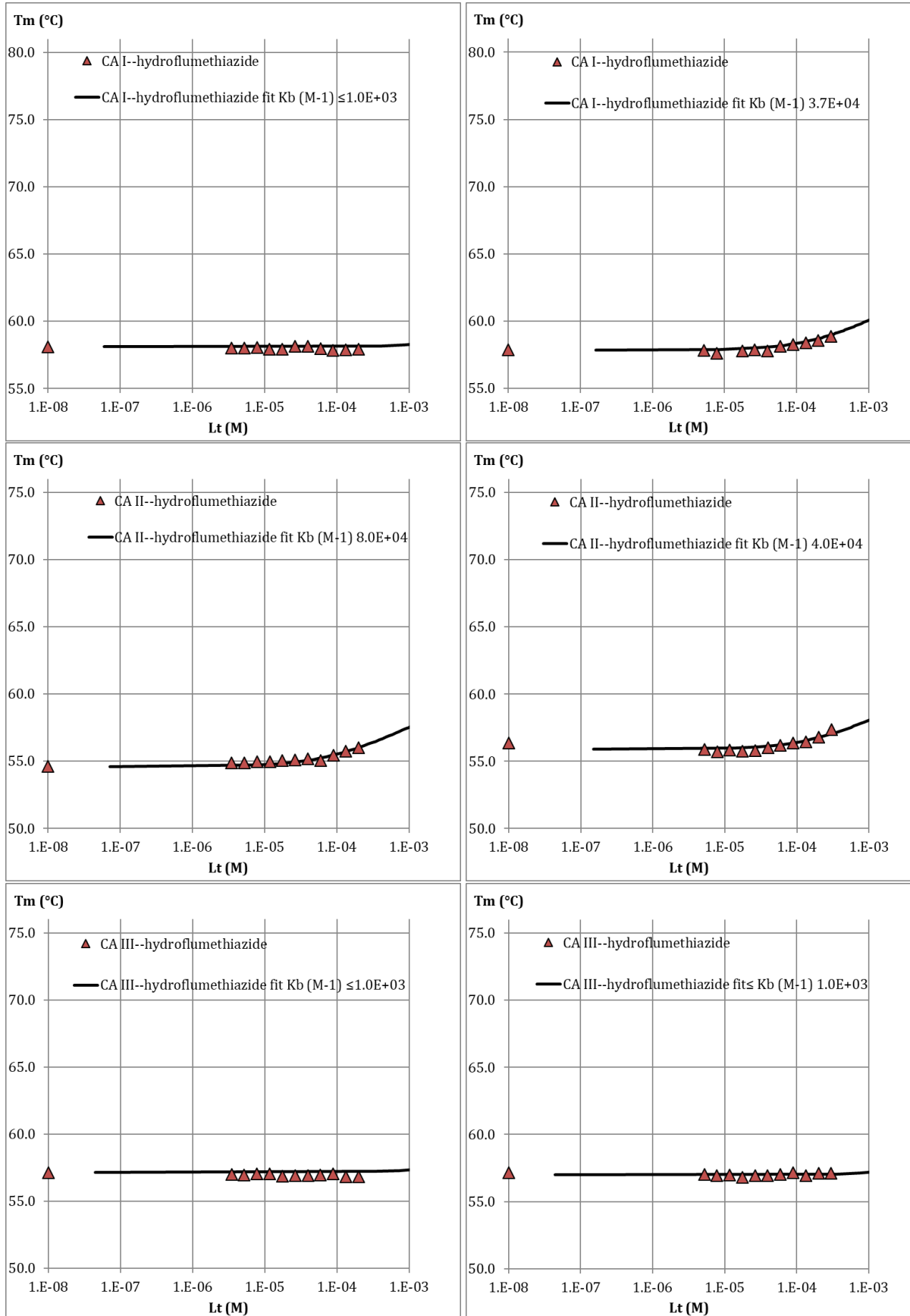


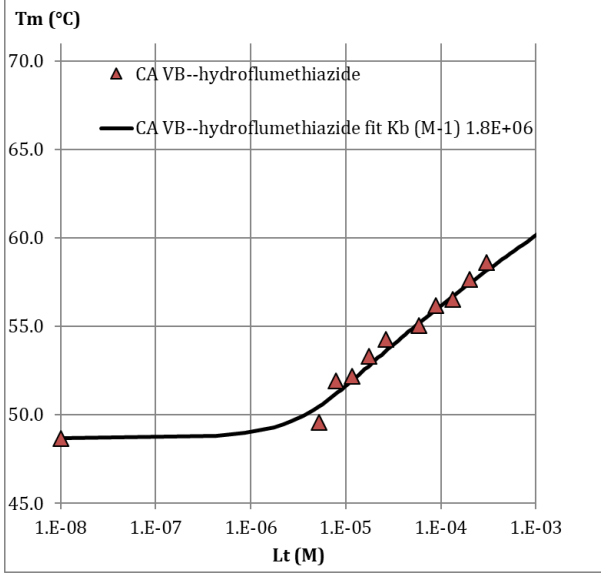
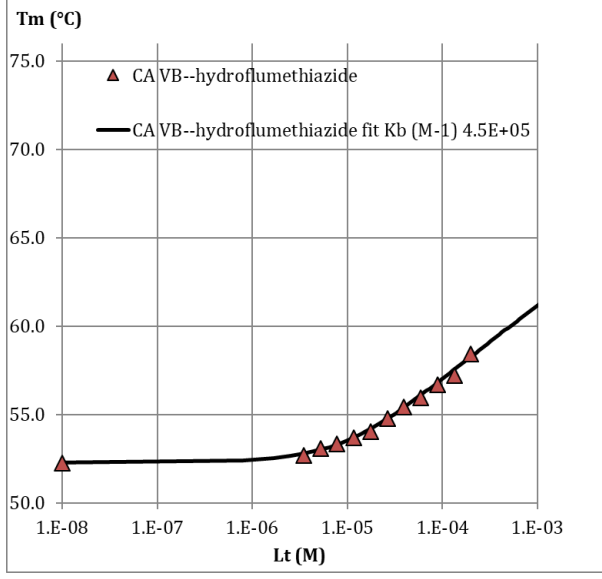
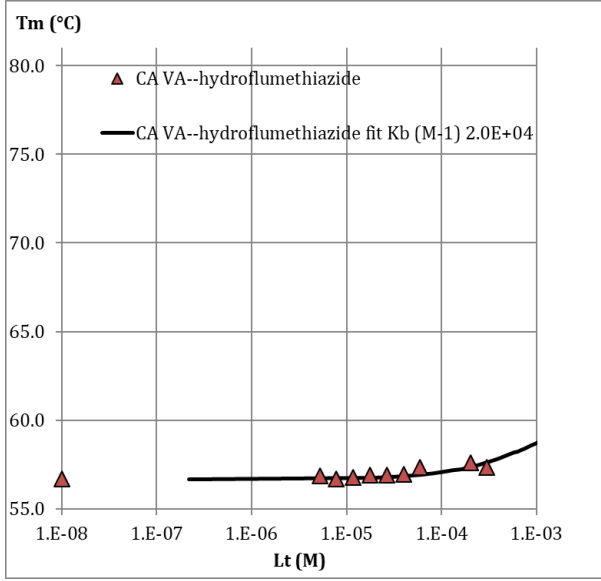
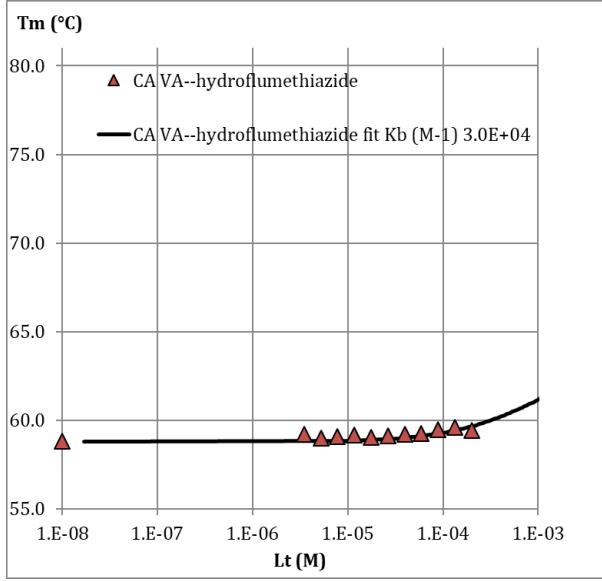
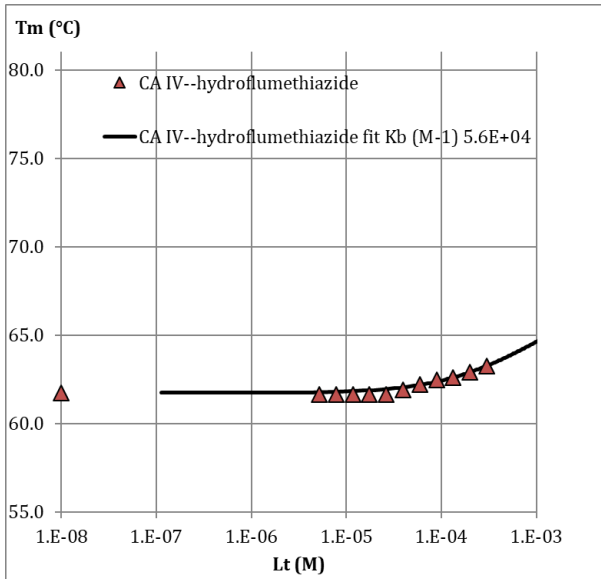
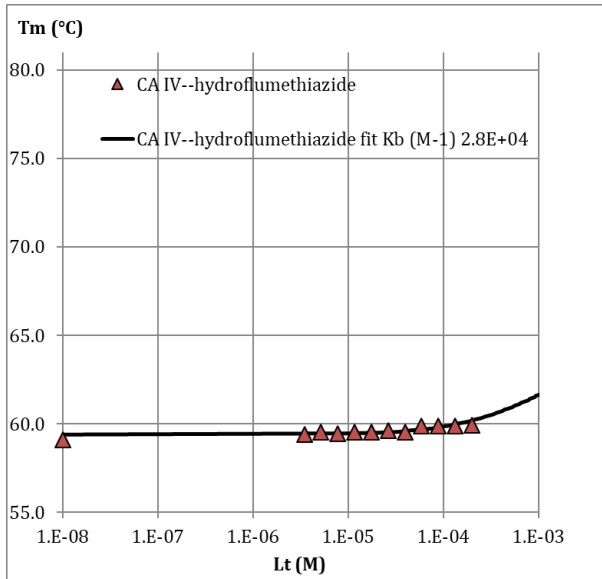
CA II- althiazide

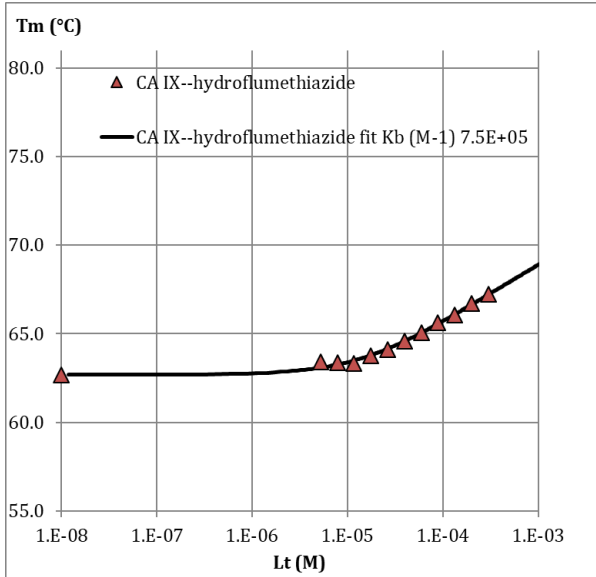
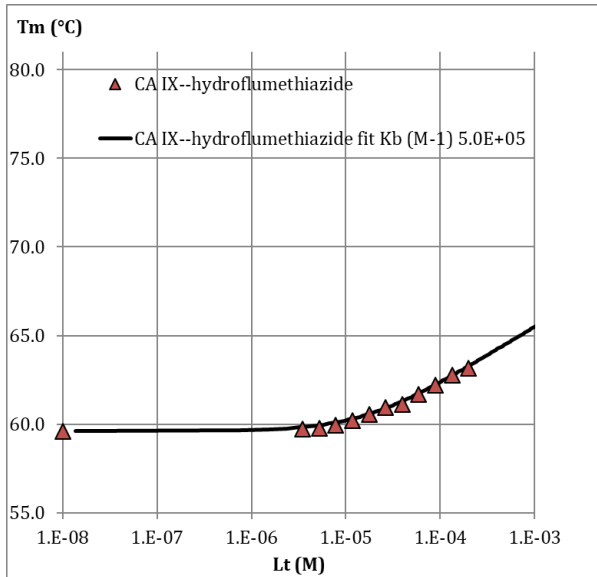
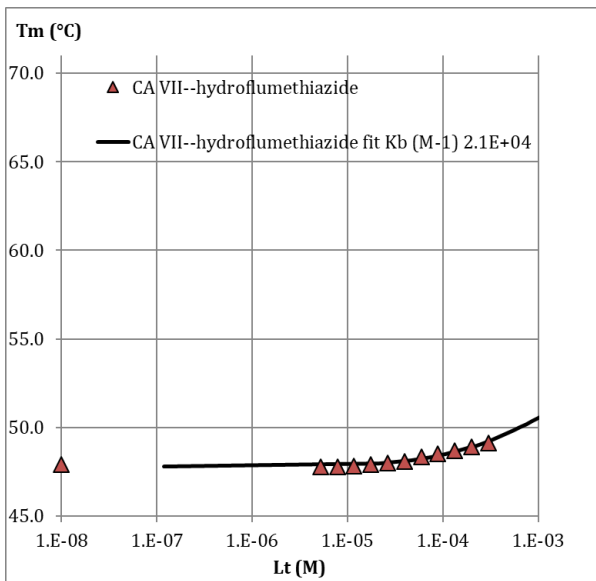
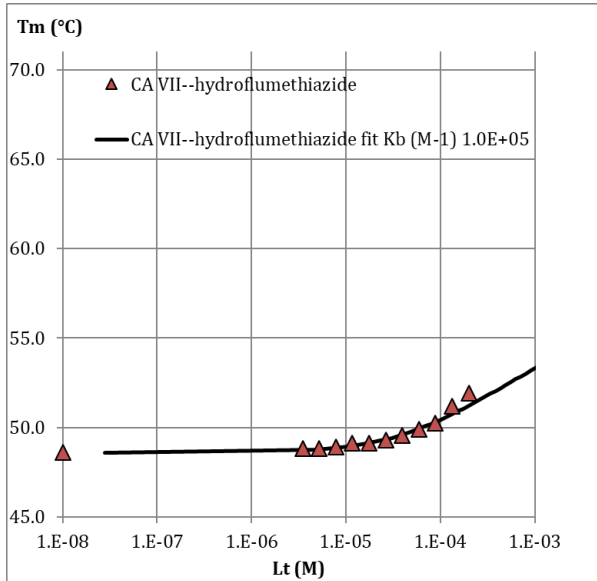
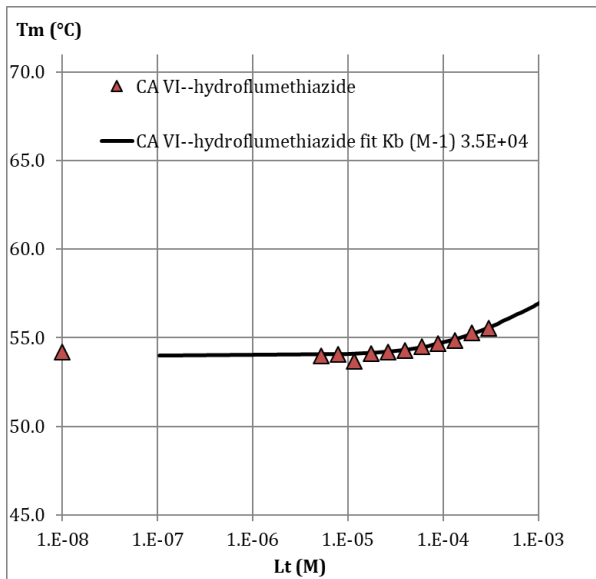
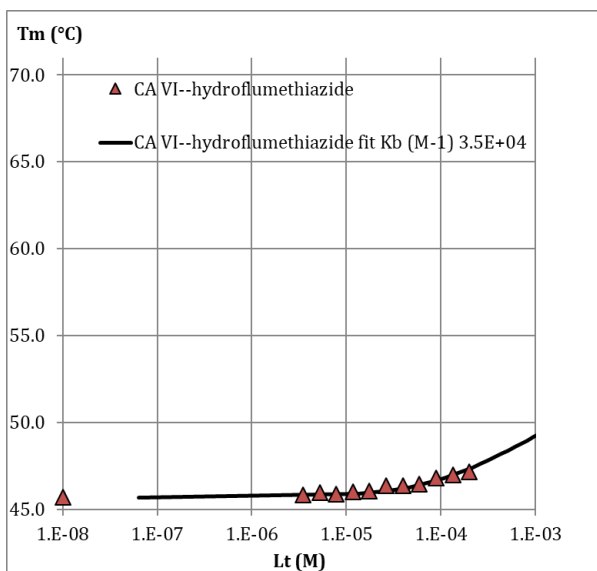


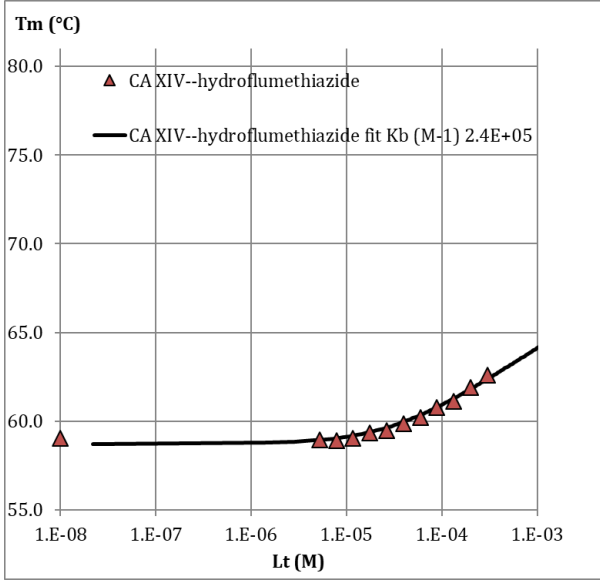
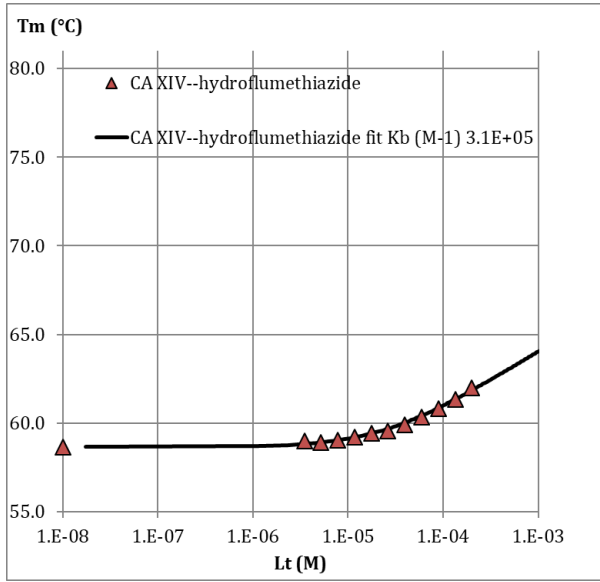
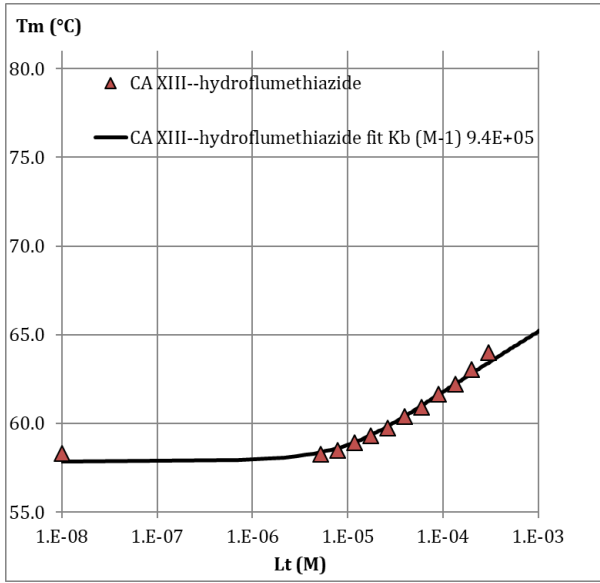
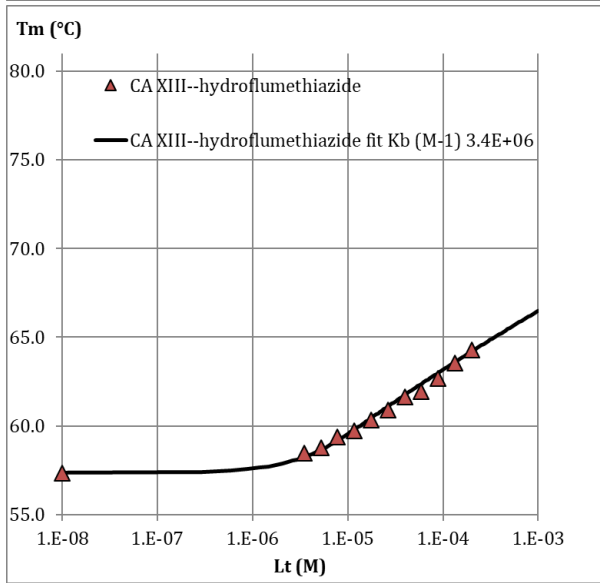
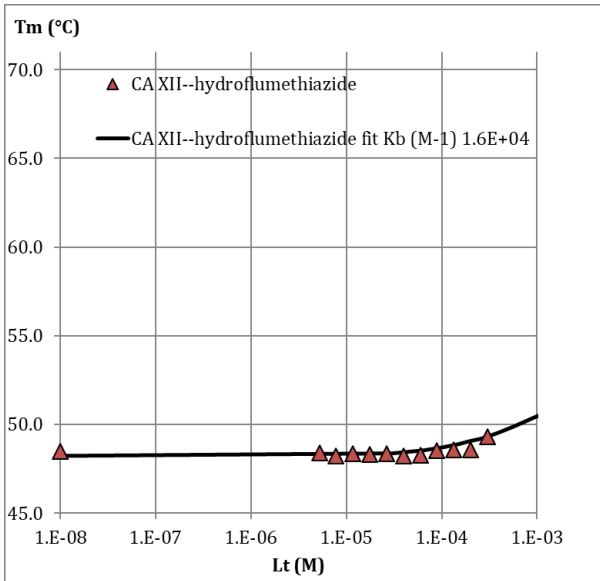
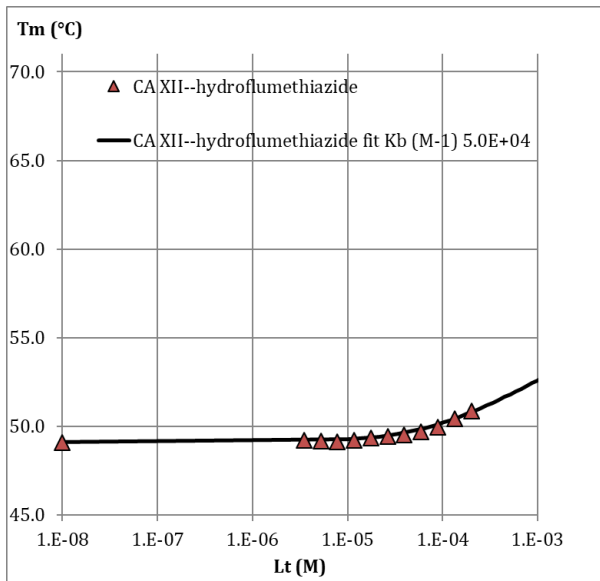
# 10. Hydroflumethiazide

## Fluorence-based thermal shift assay



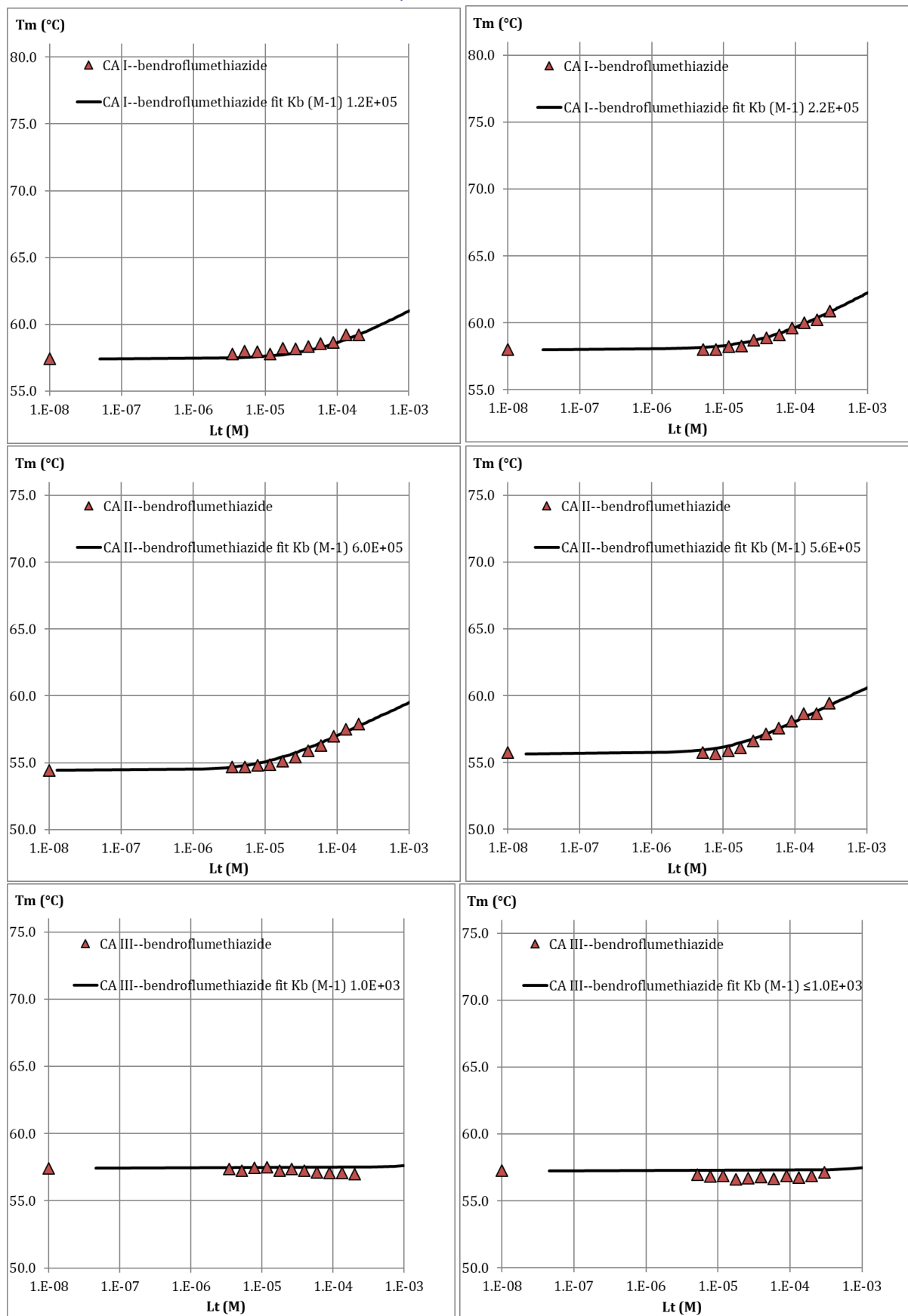


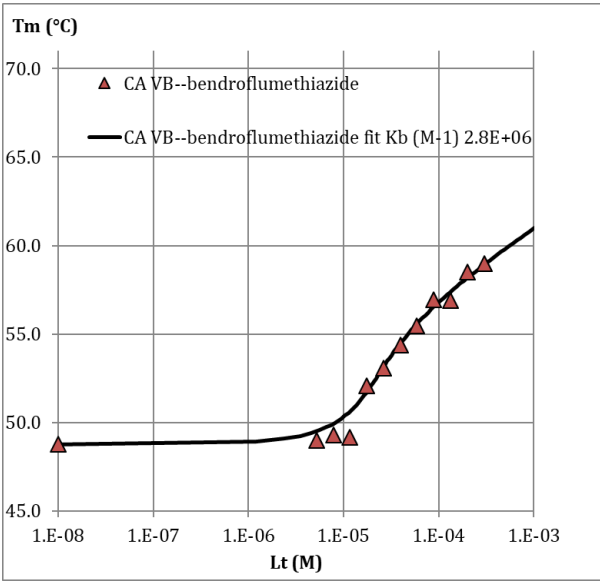
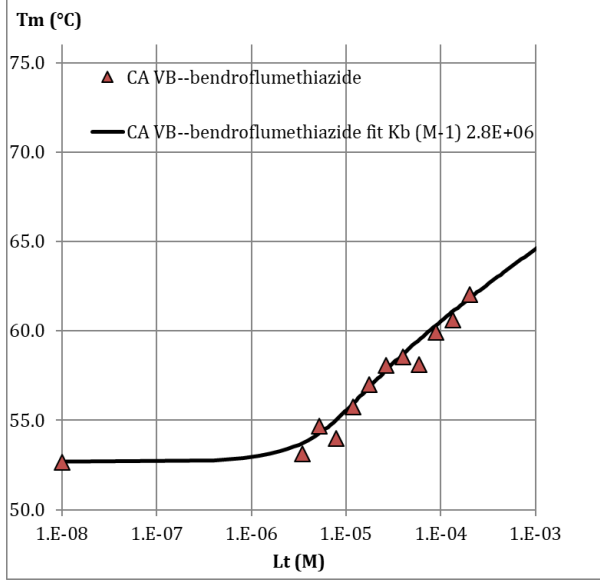
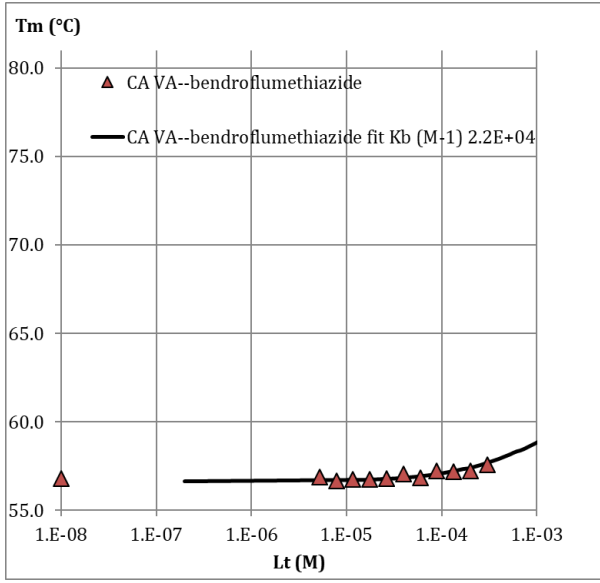
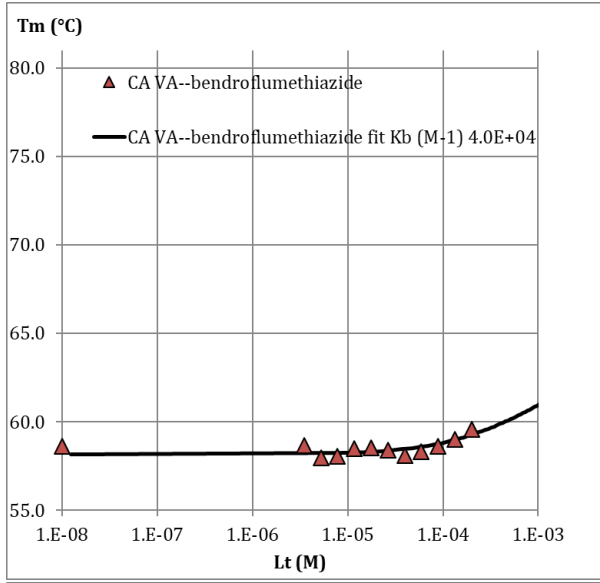
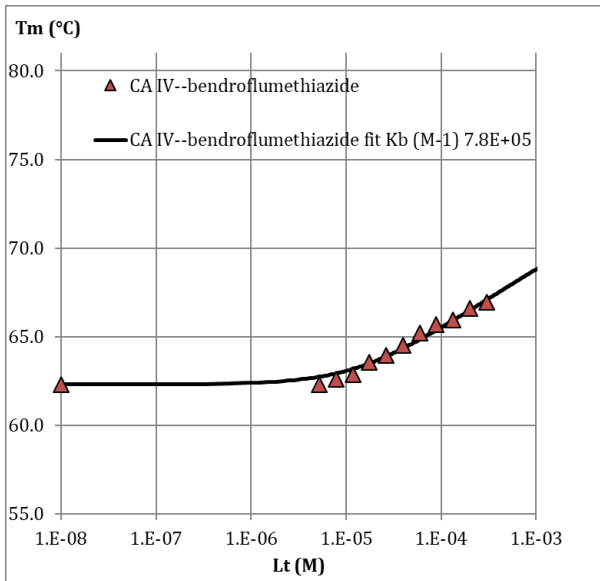
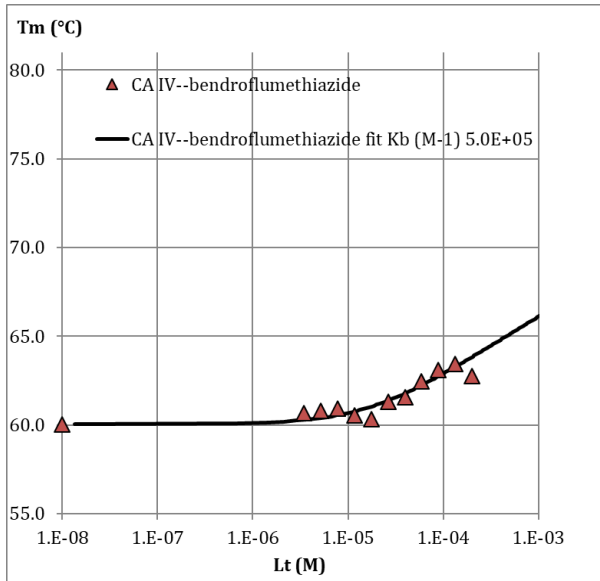


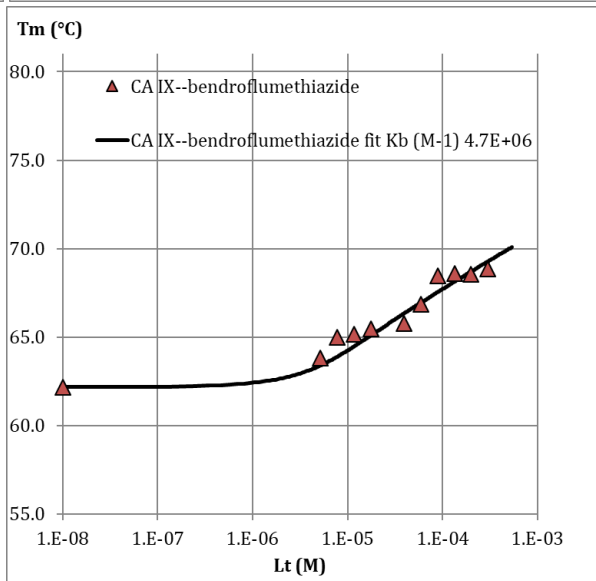
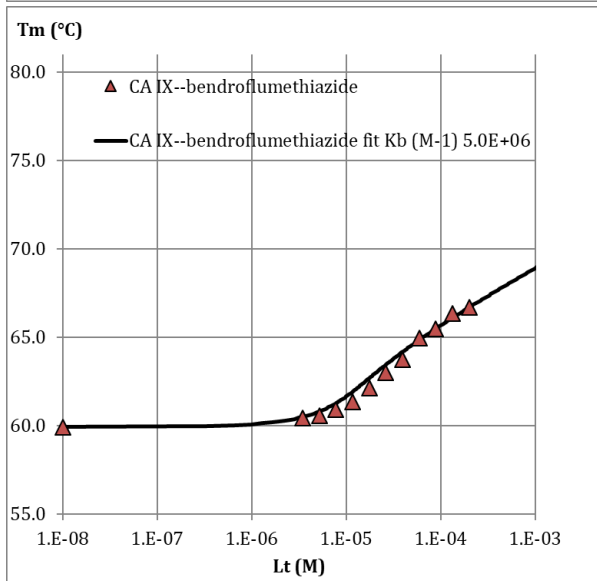
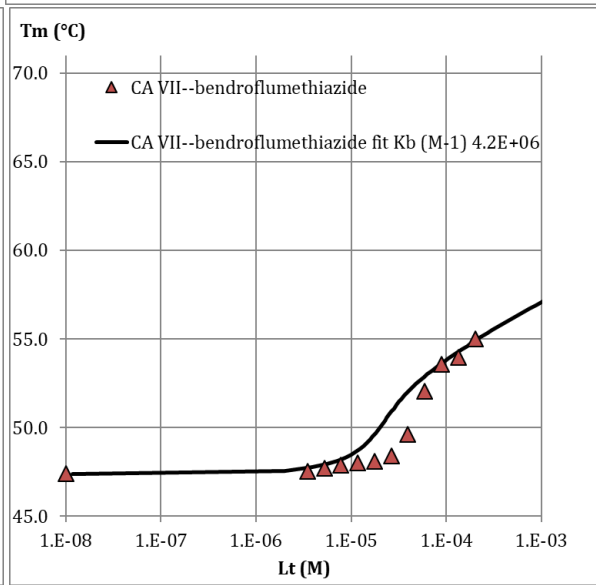
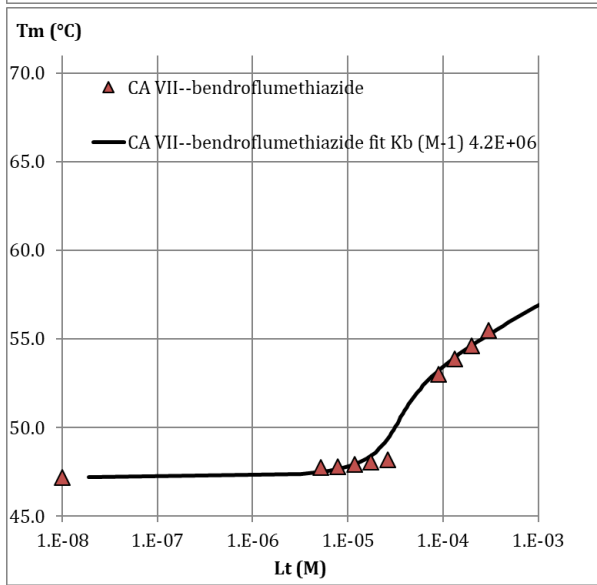
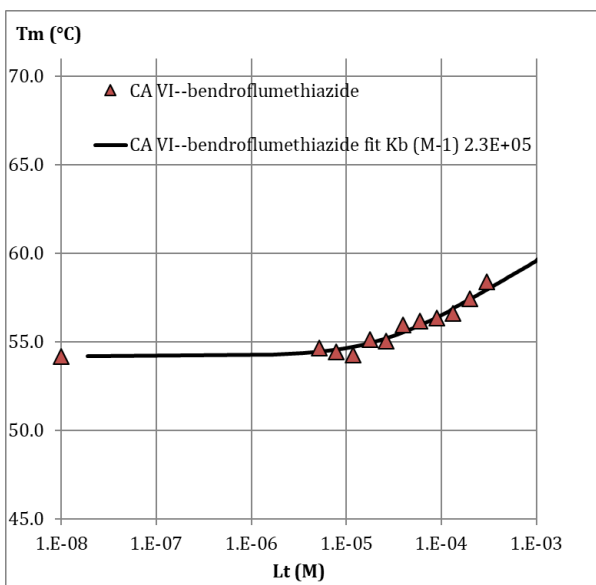
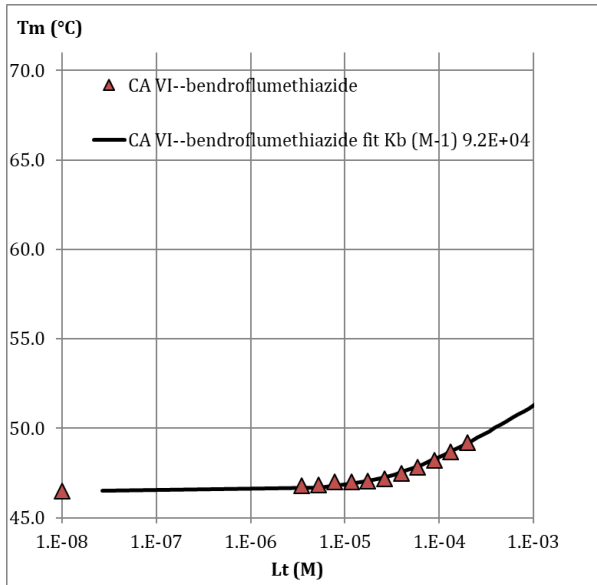


# 11. Bendroflumethiazide

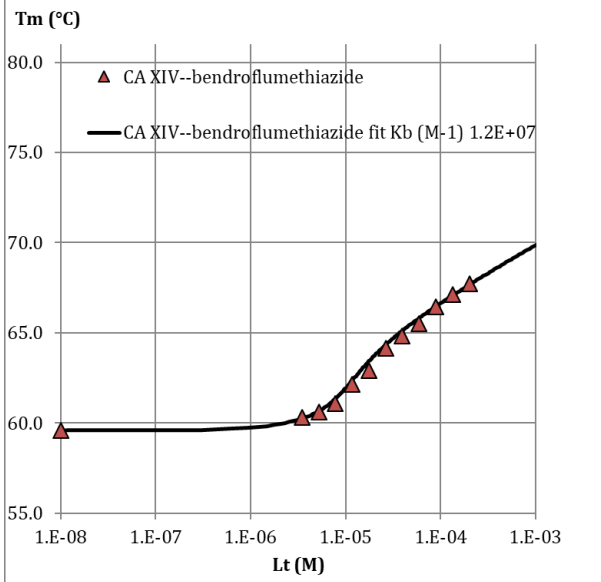
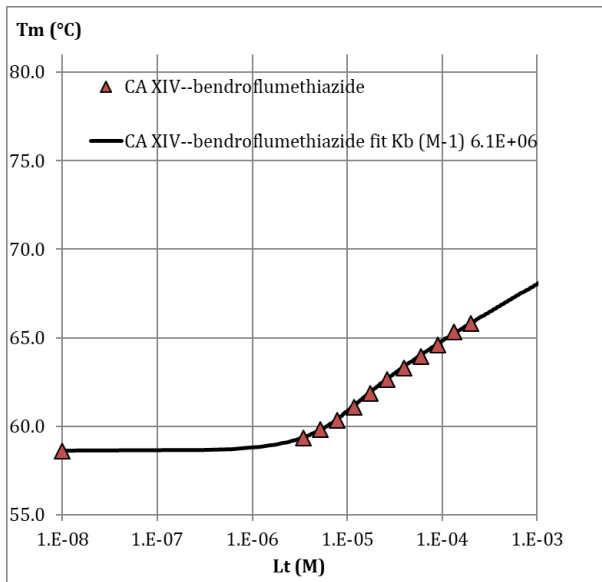
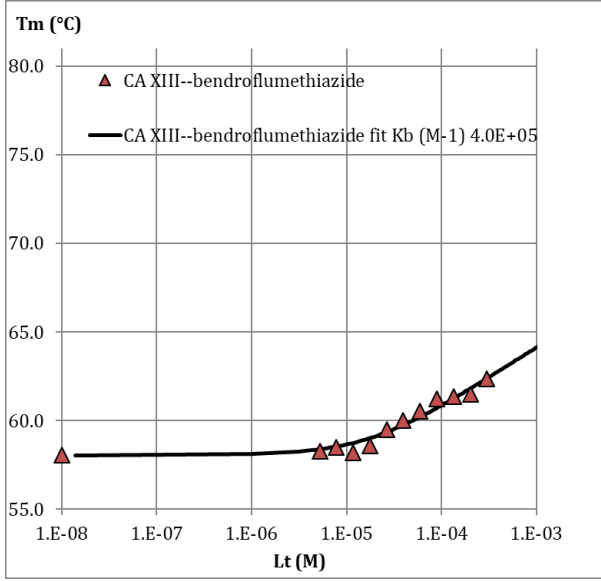
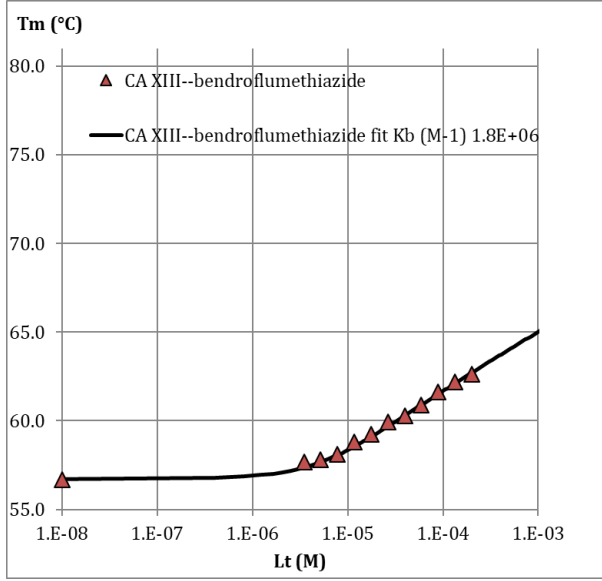
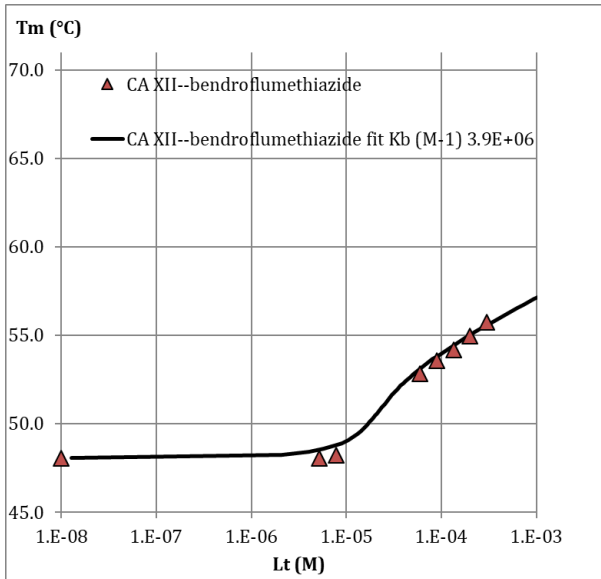
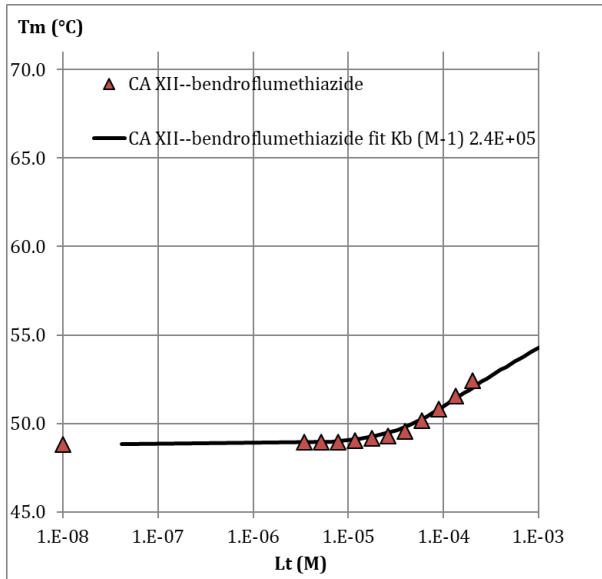
## Fluorecence-based thermal shift assay





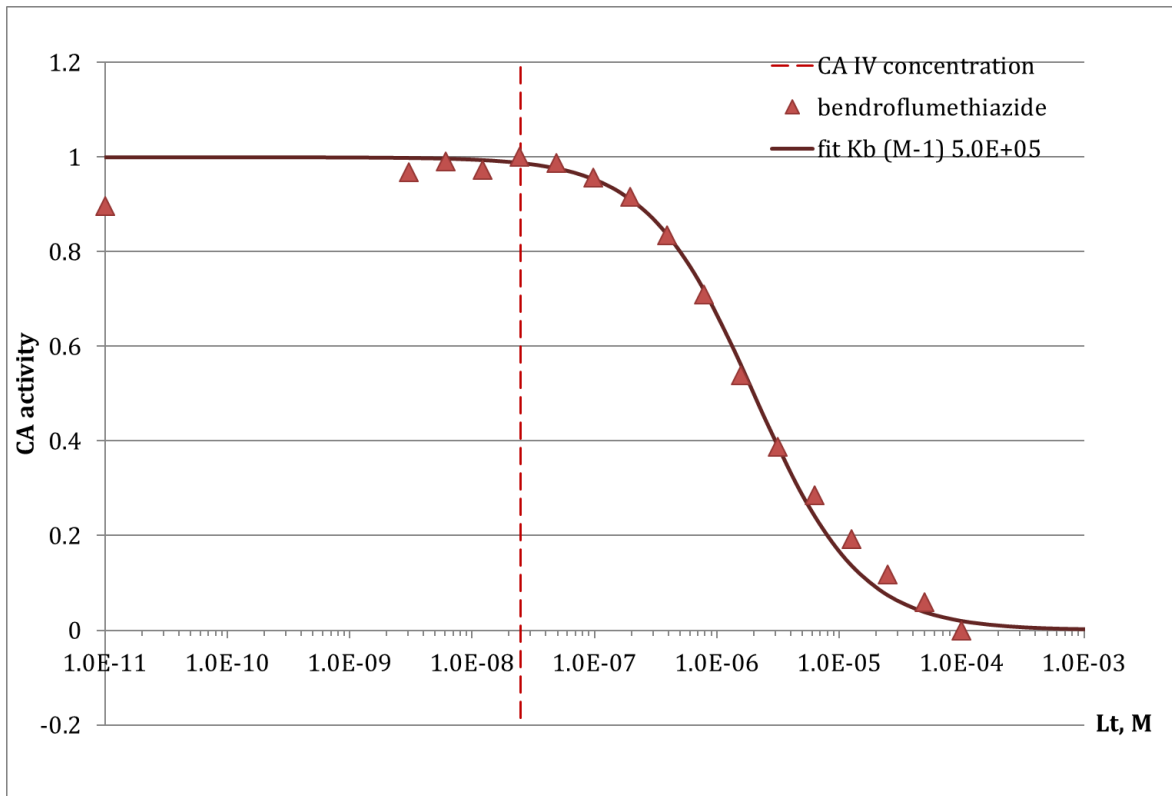






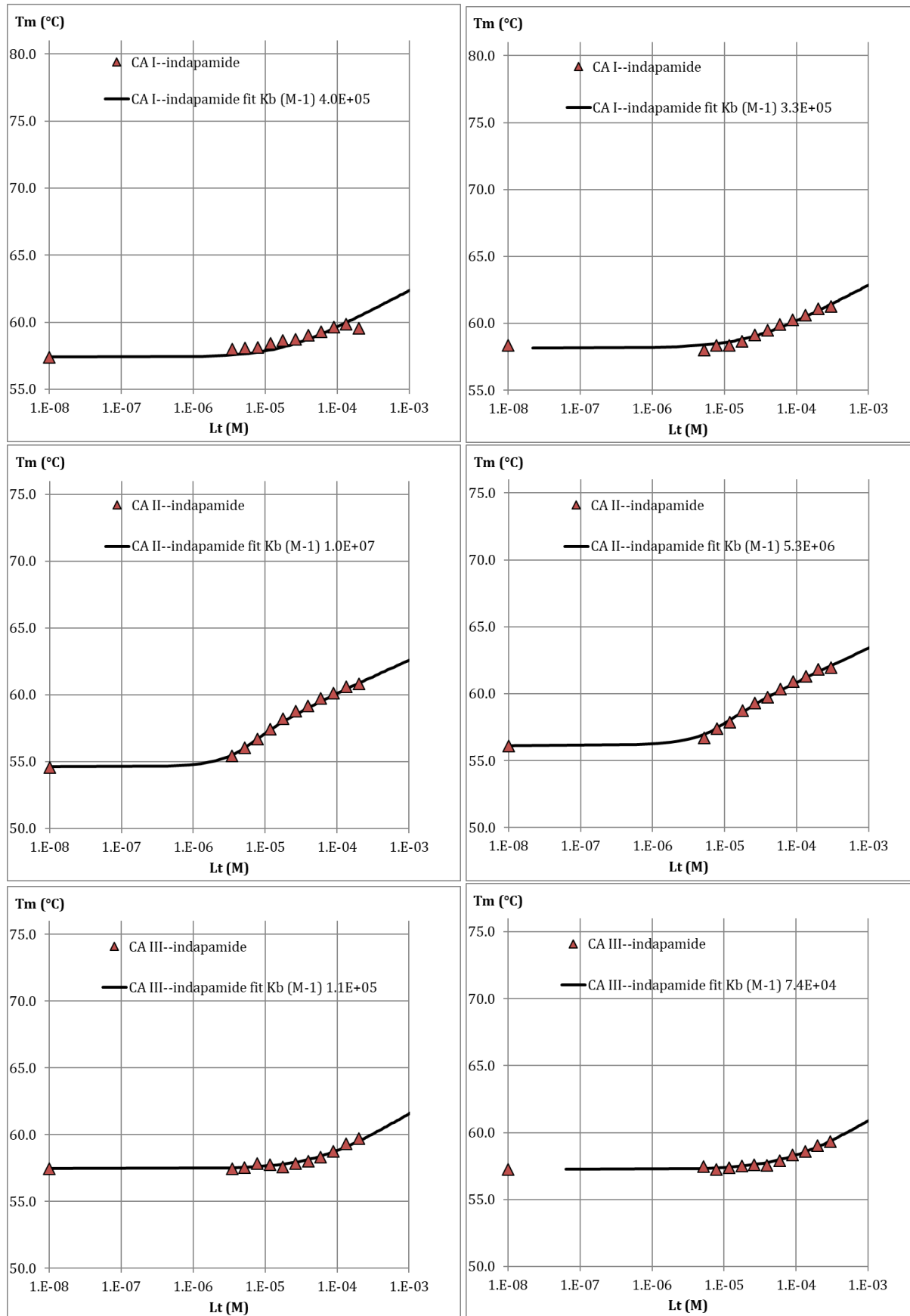
# CO<sub>2</sub> hydration activity inhibition

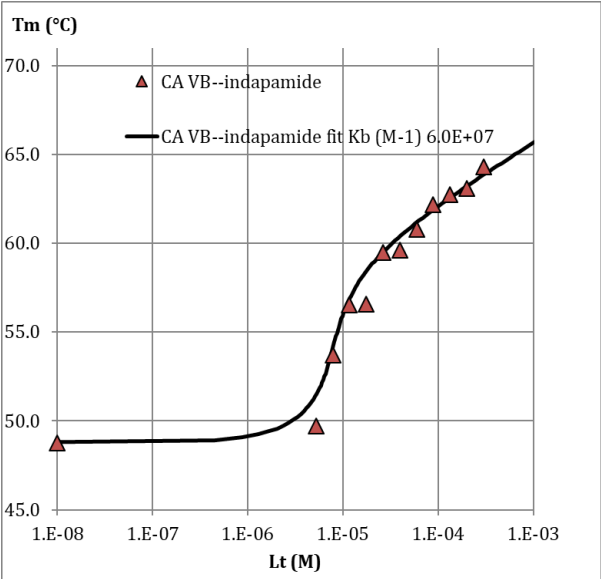
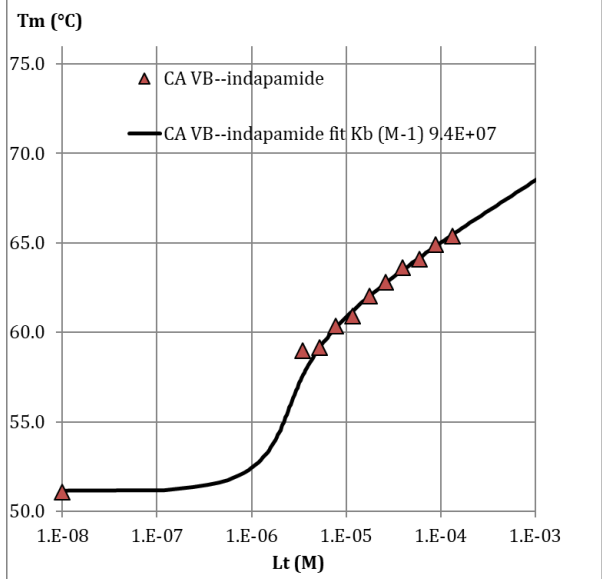
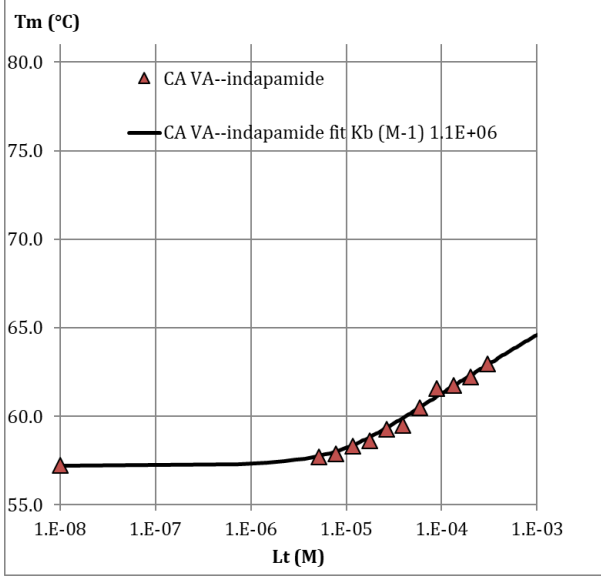
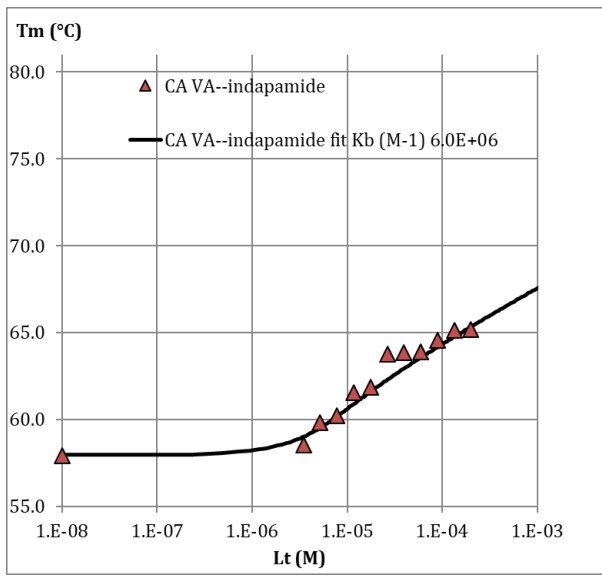
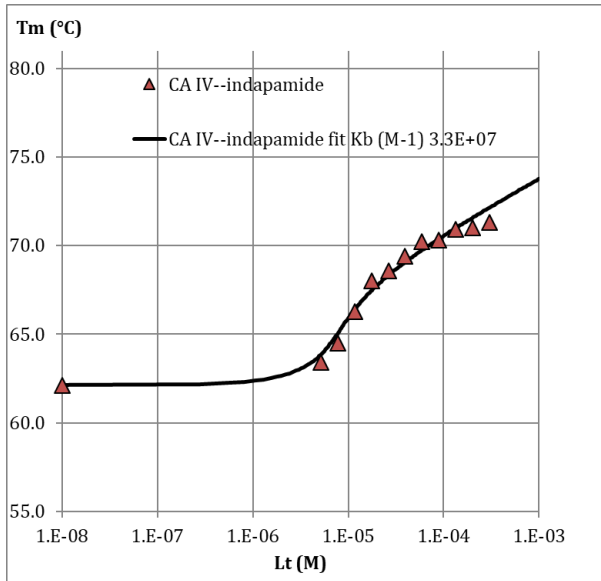
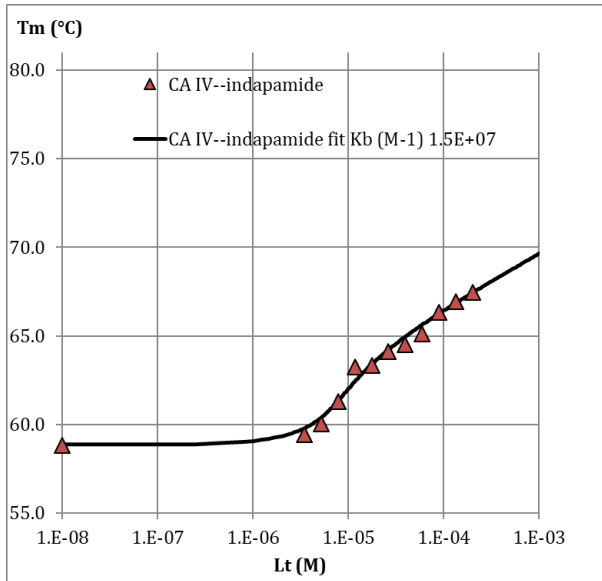
## CA IV - bendroflumethiazide

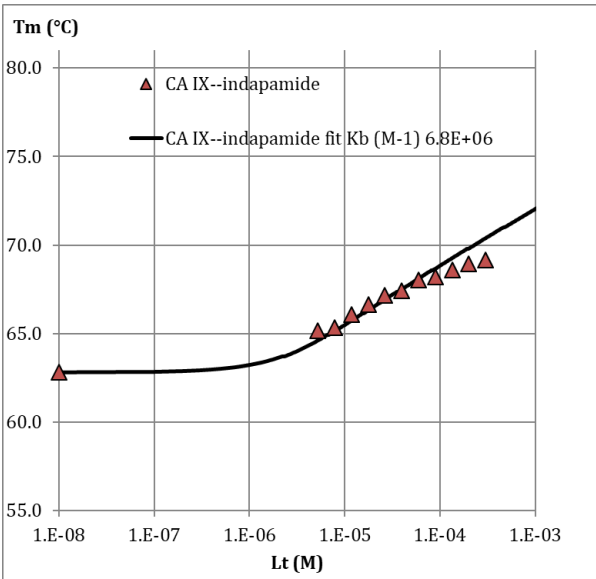
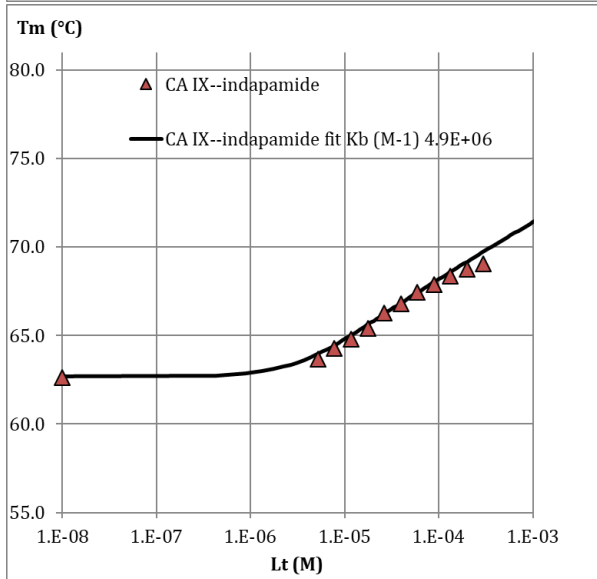
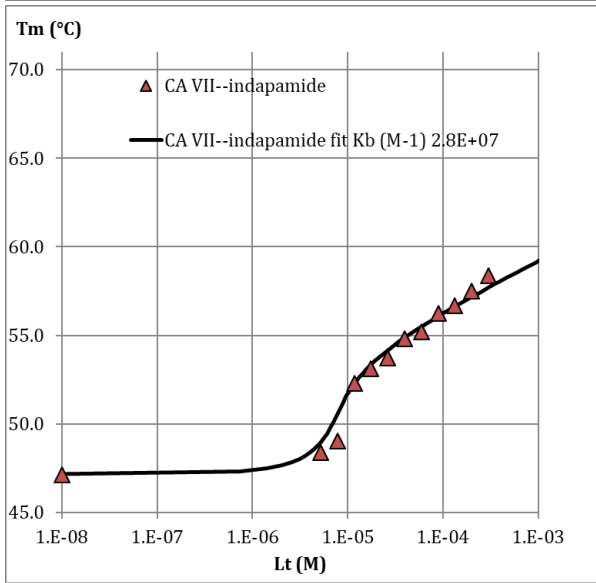
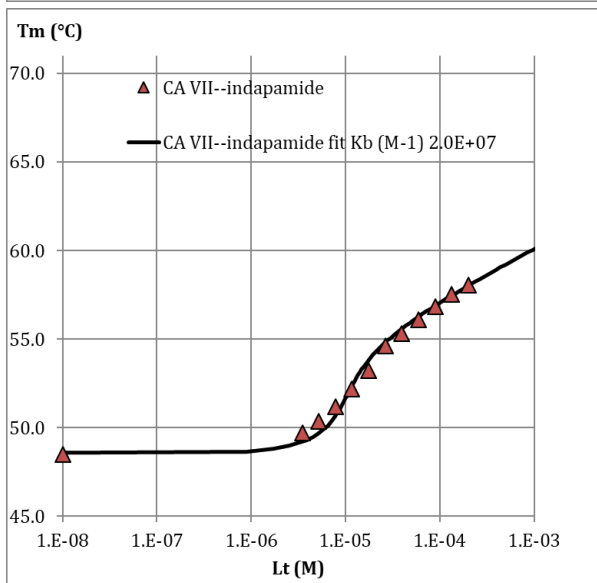
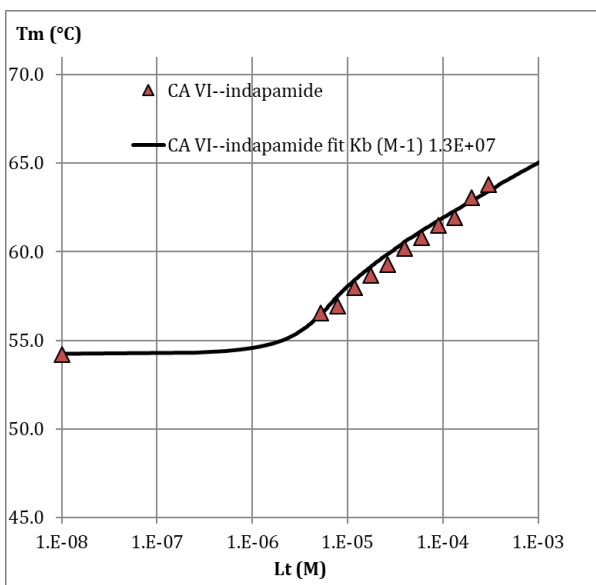
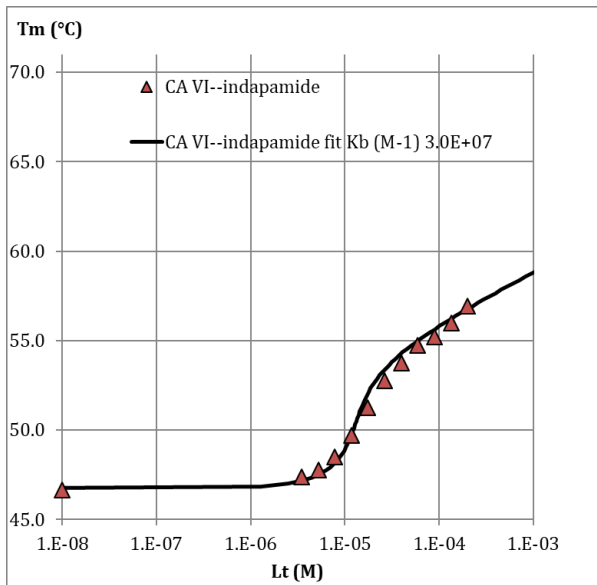


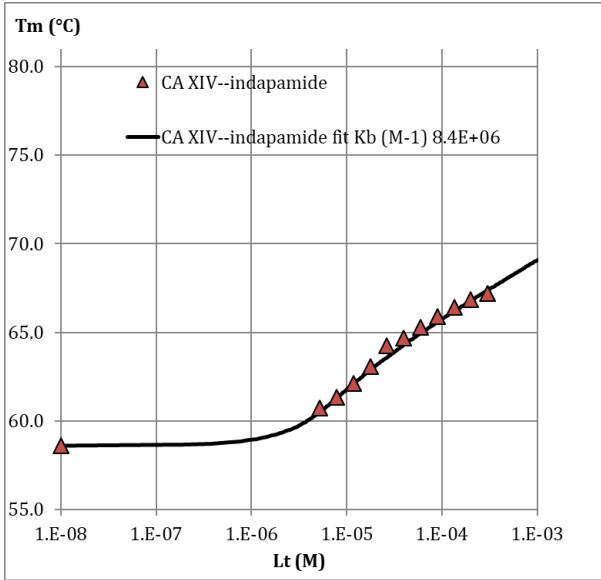
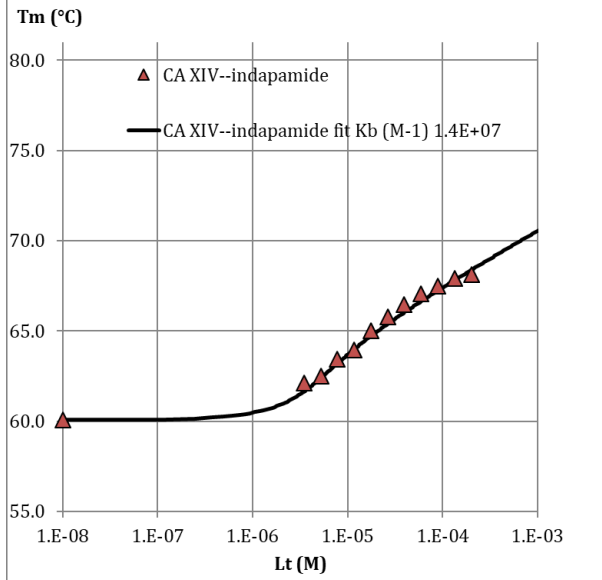
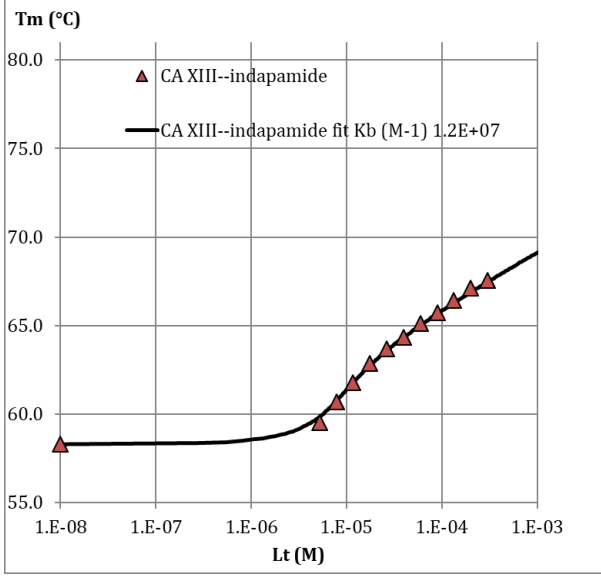
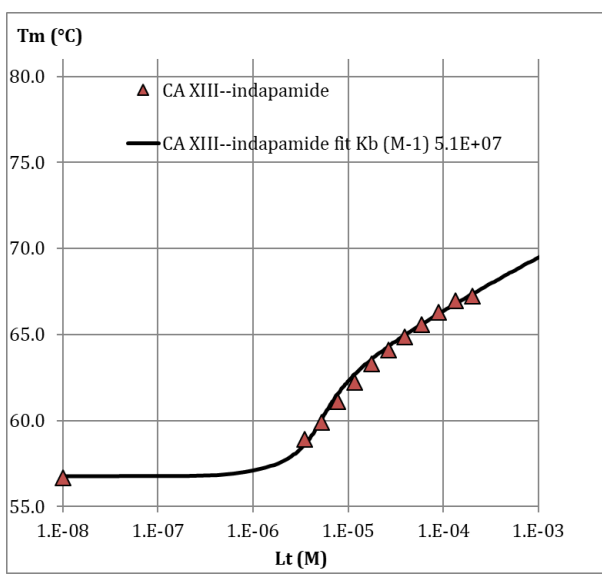
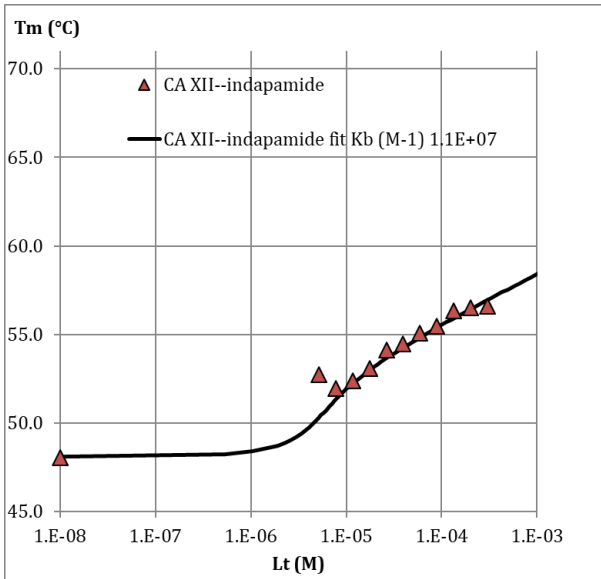
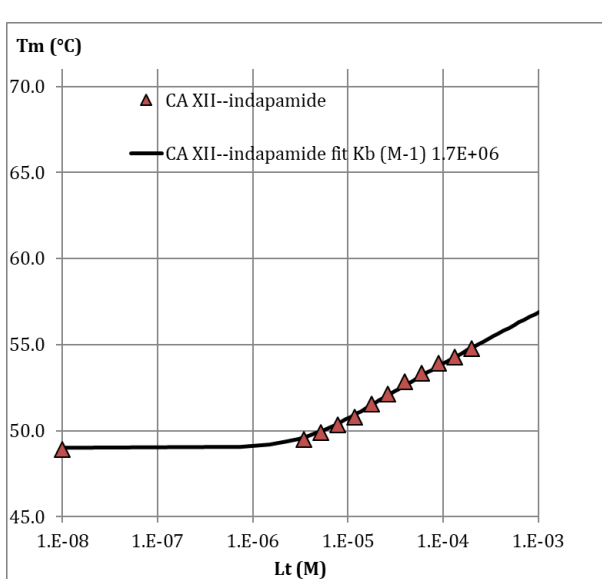
## 12. Indapamide

### Fluorescence-based thermal shift assay



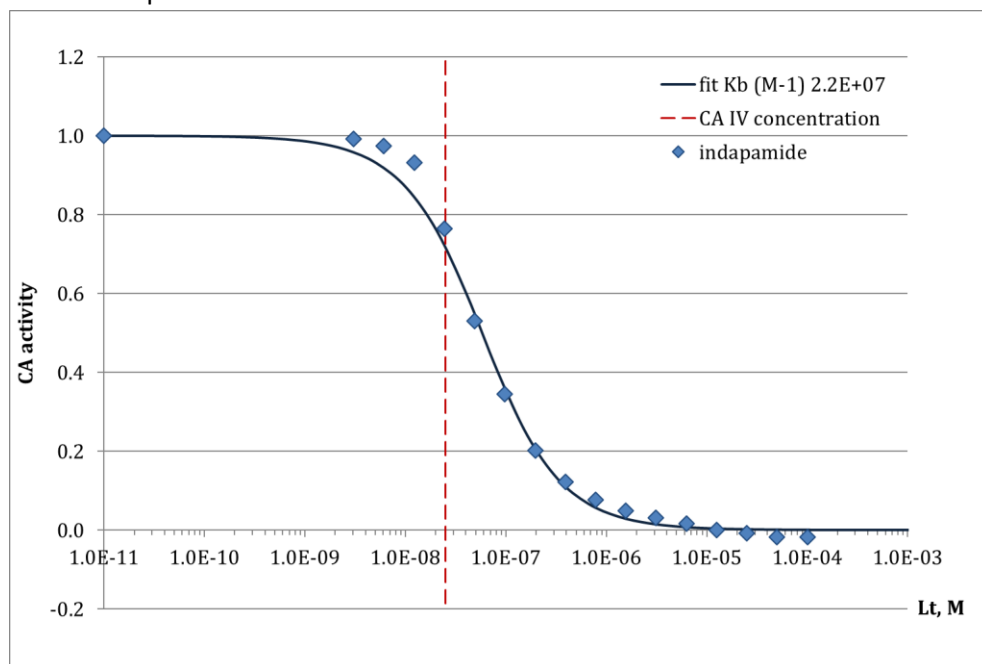




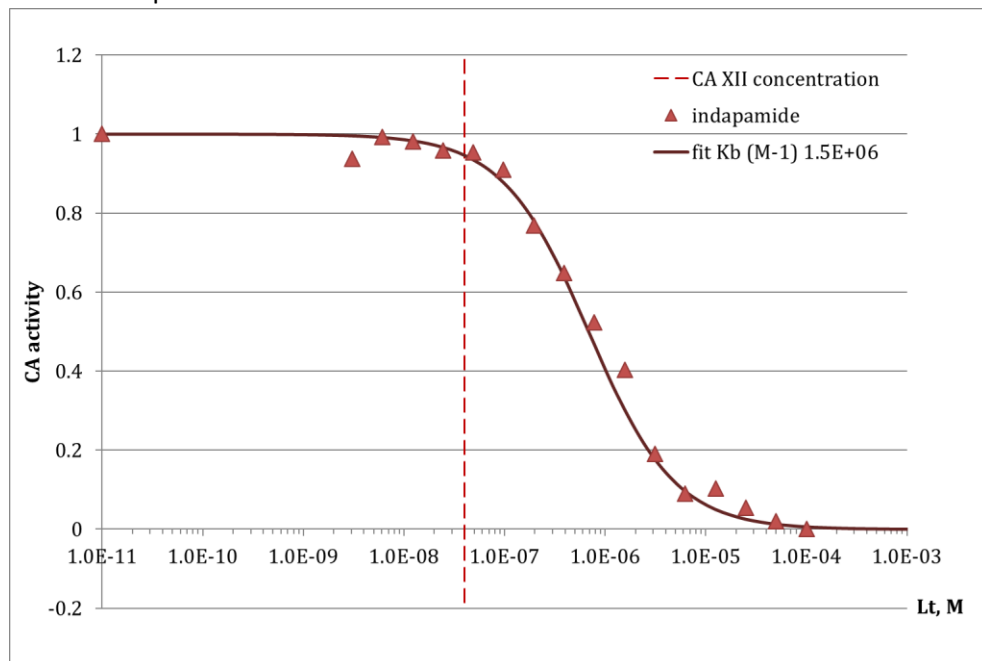


## CO<sub>2</sub> hydration activity inhibition

### CA IV - indapamide

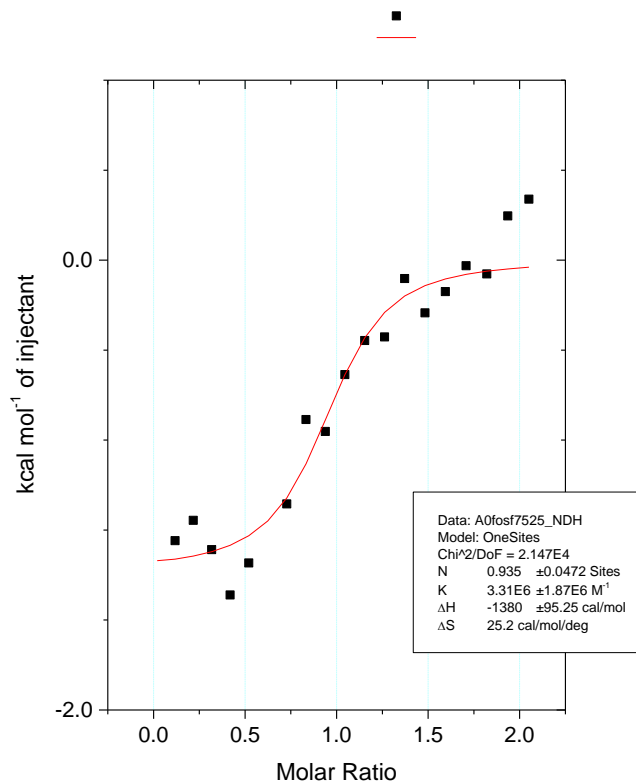
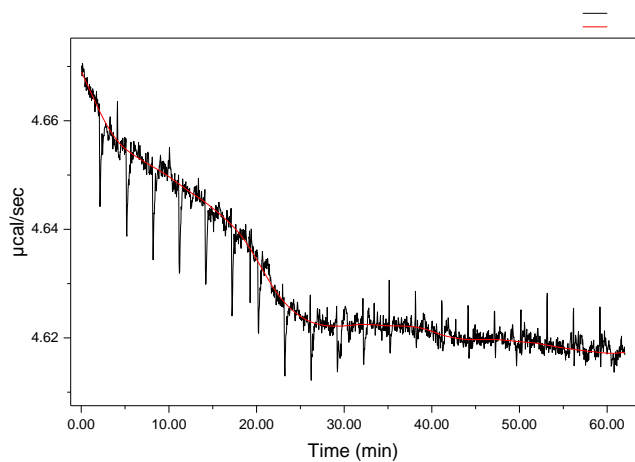


### CA XII - indapamide

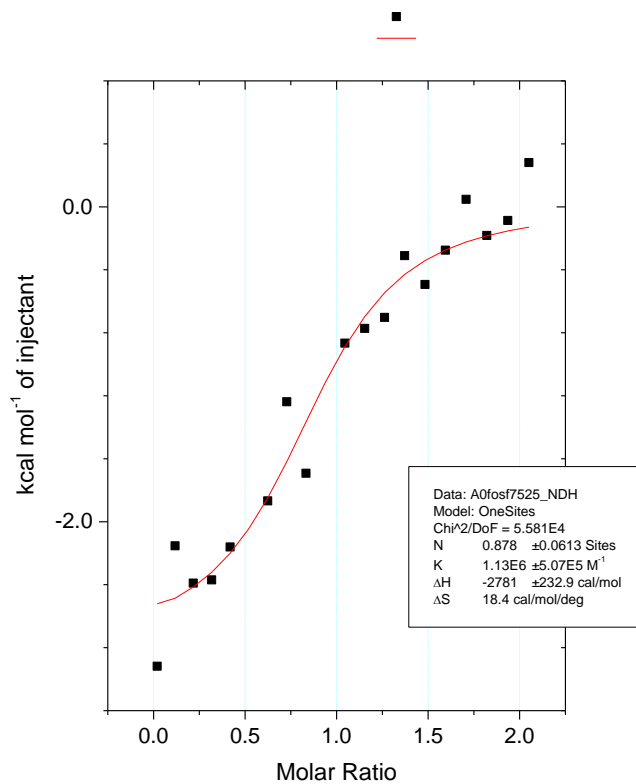
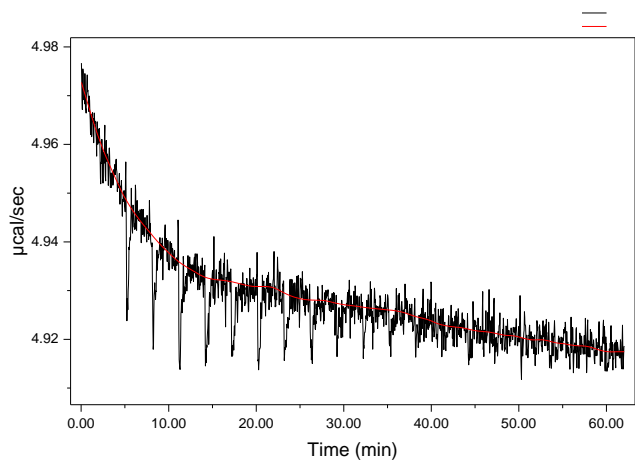


# Isothermal titration calorimetry

## CA II – indapamide



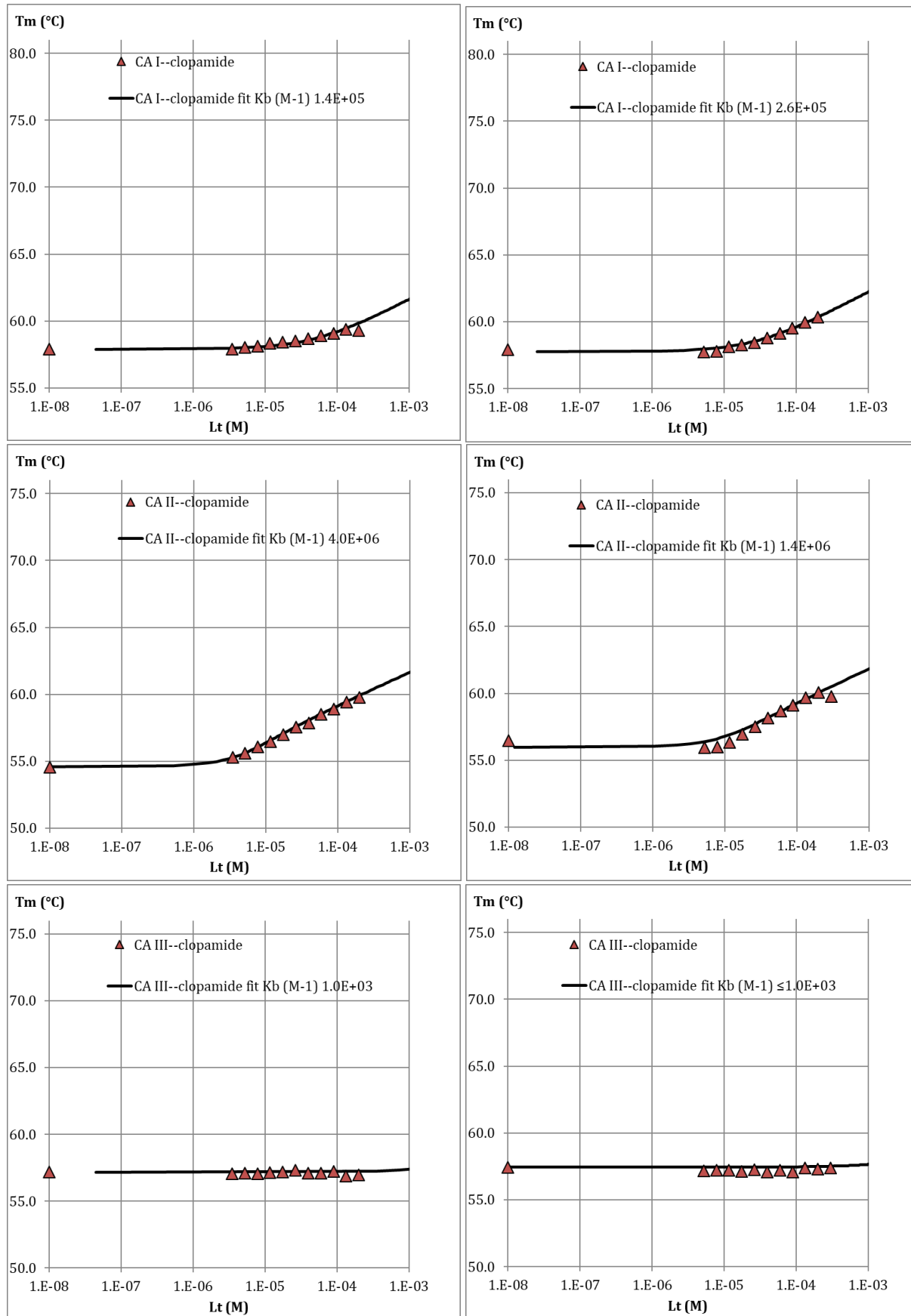
## CA II – indapamide

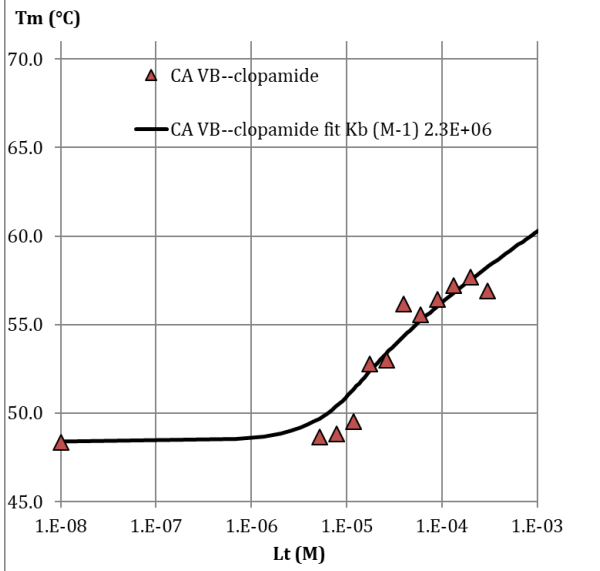
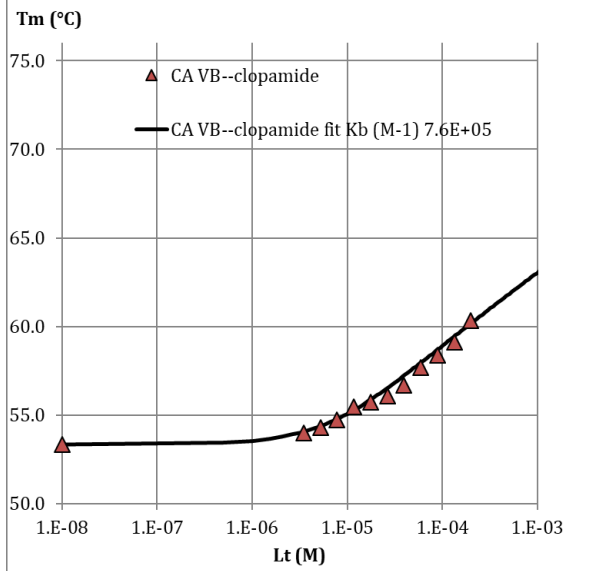
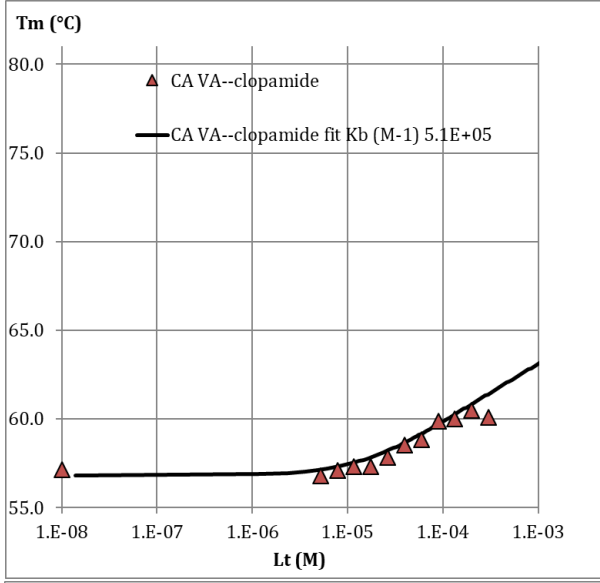
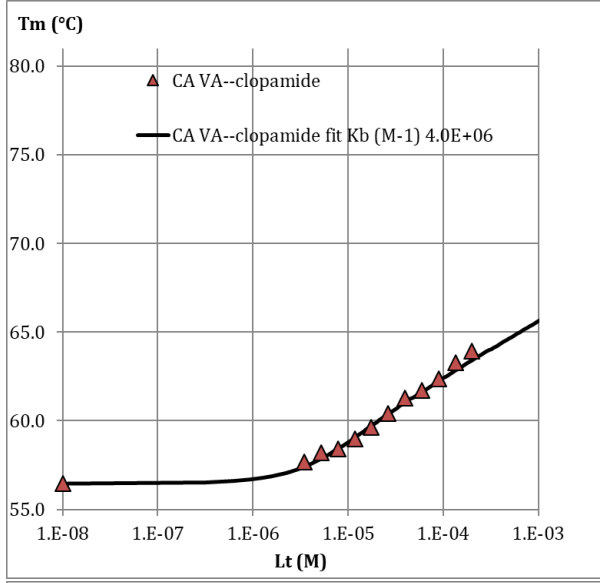
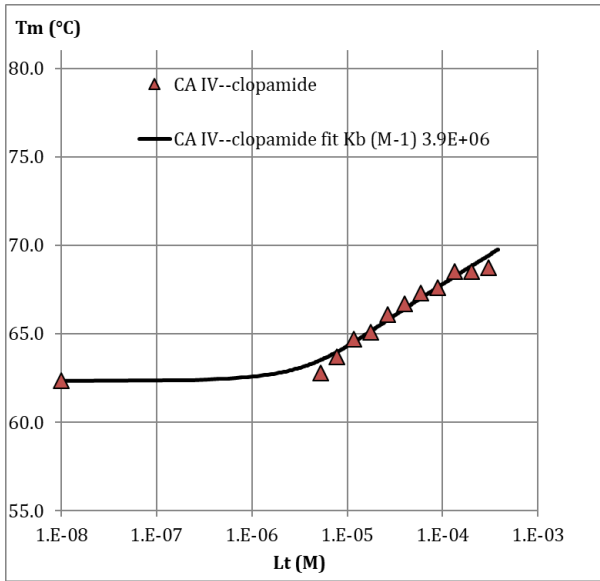
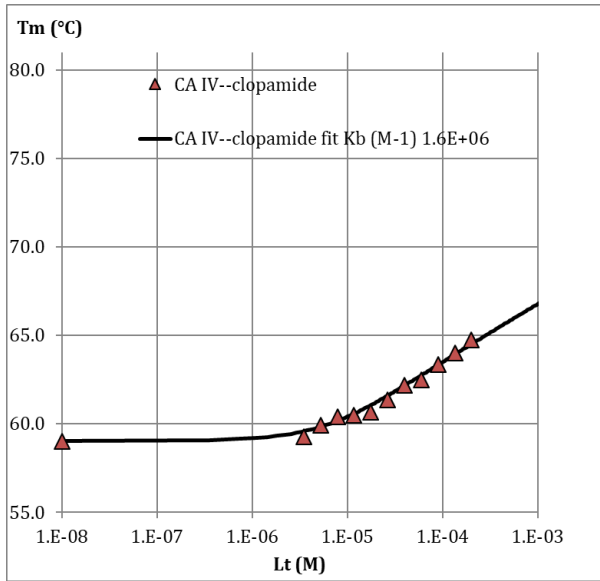


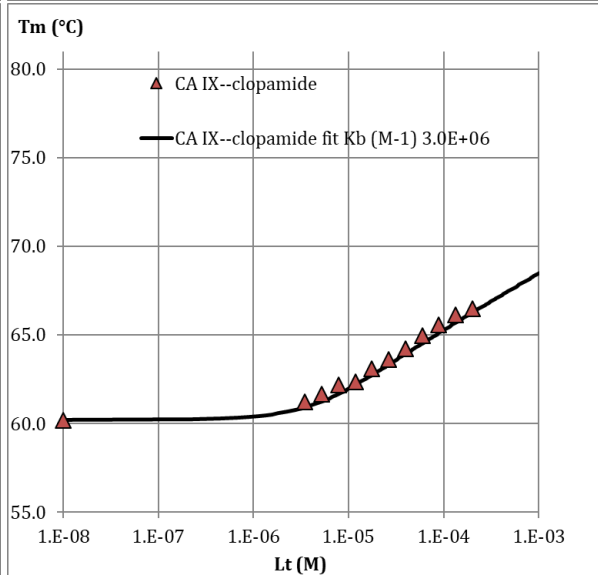
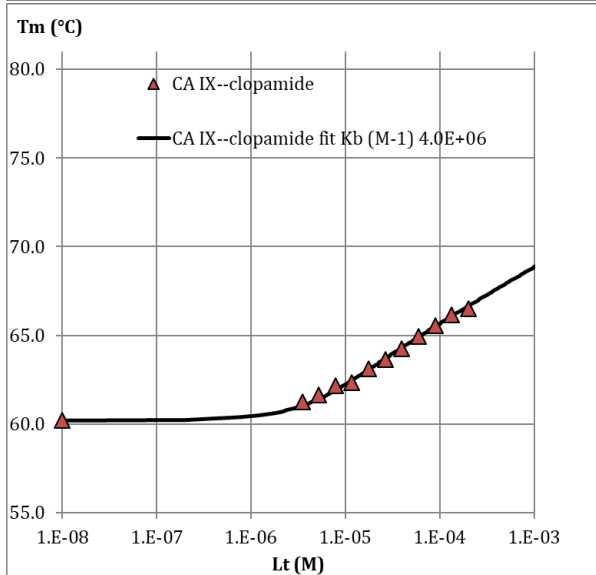
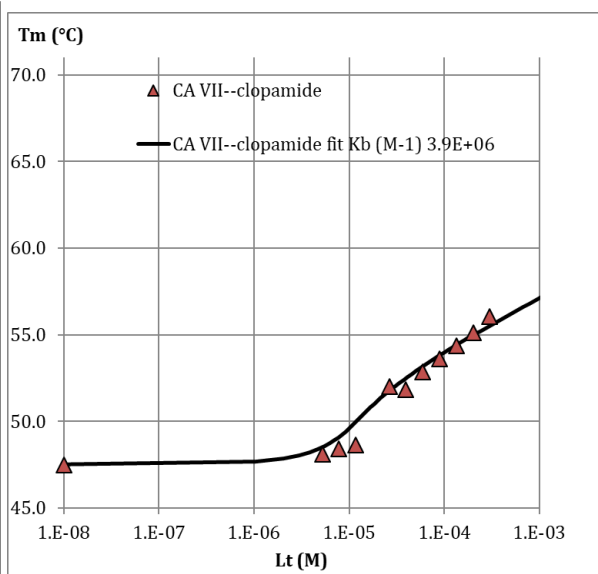
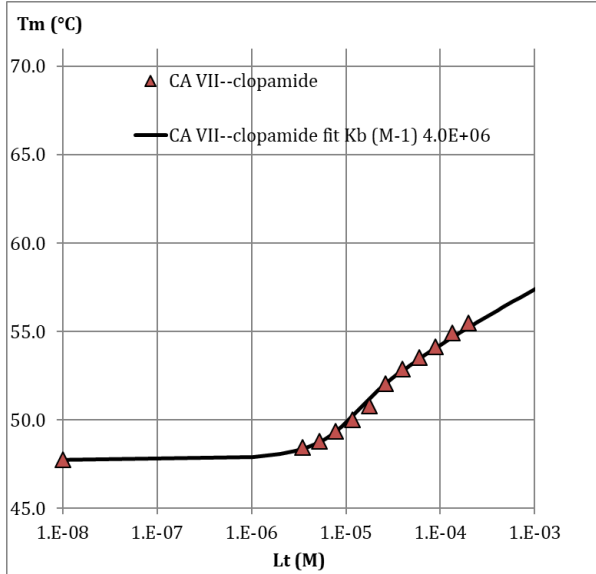
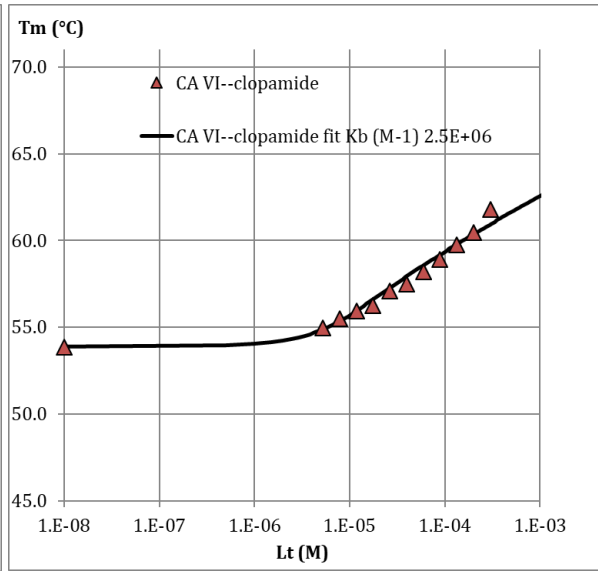
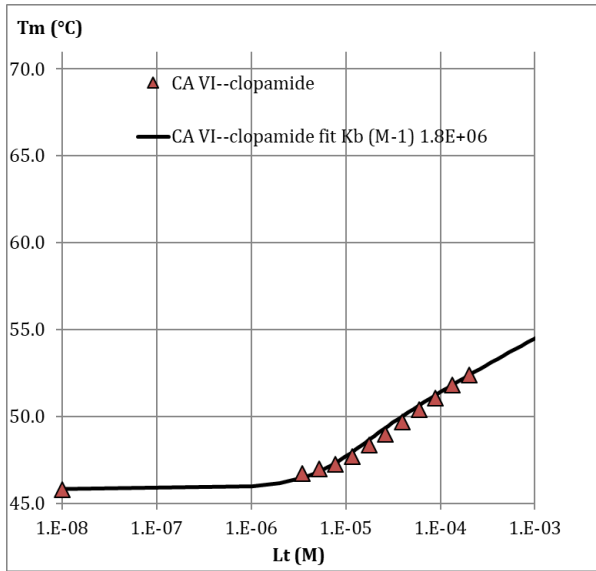


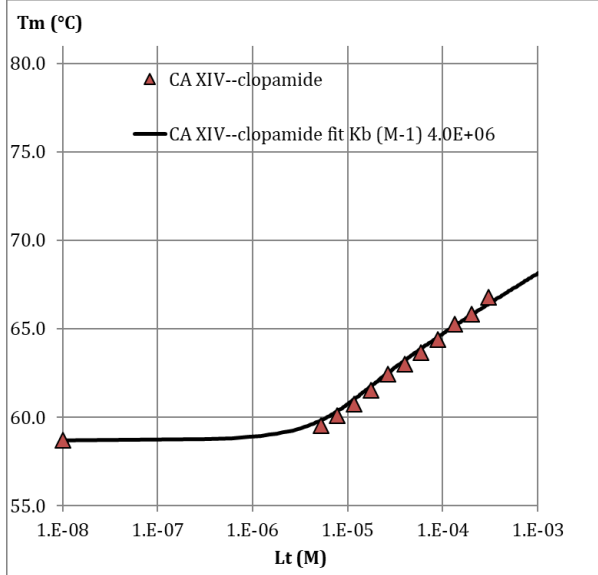
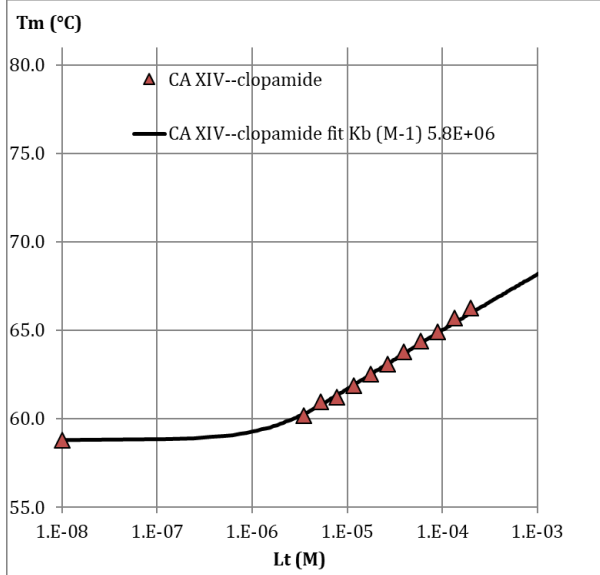
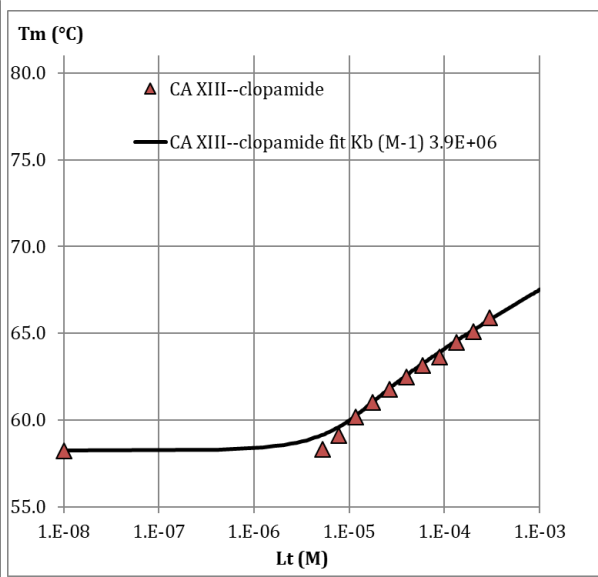
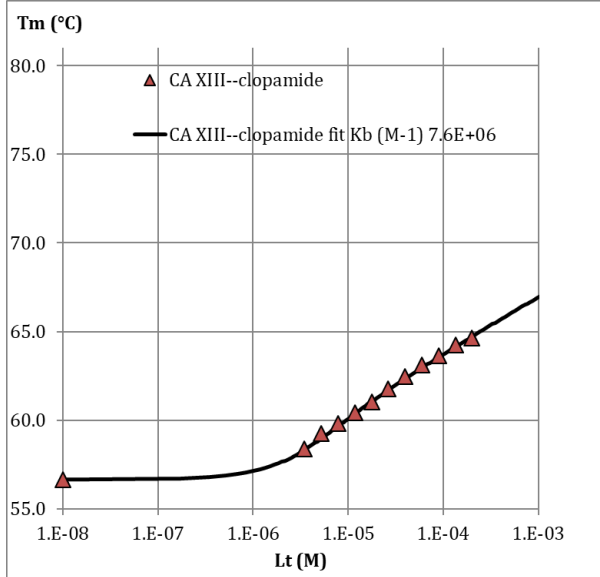
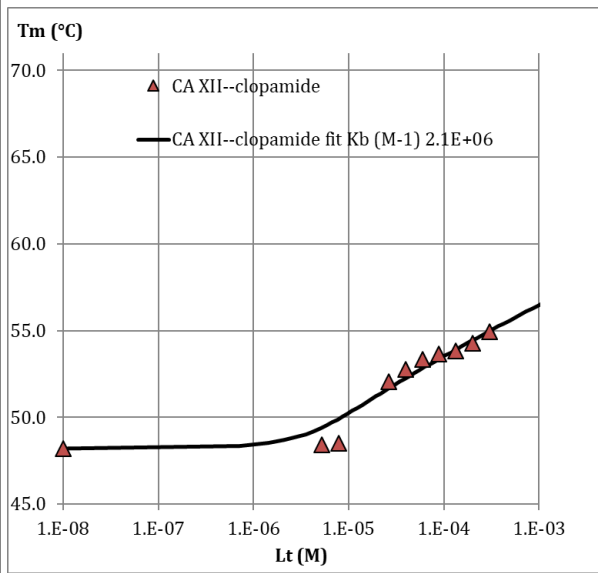
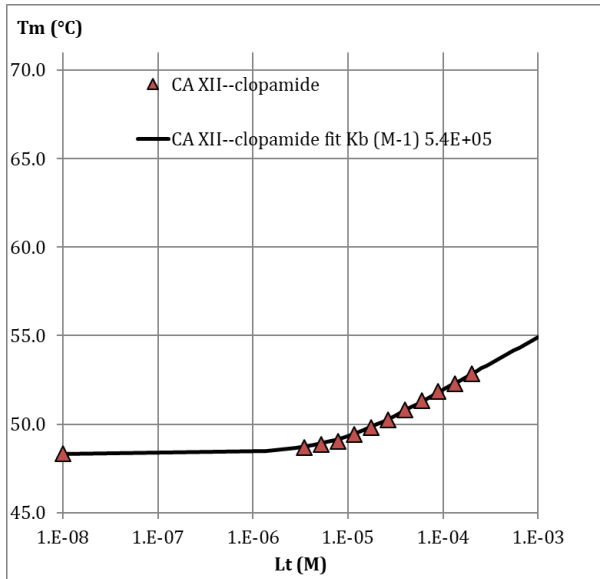
# 13. Clopamide

## Fluorence-based thermal shift assay



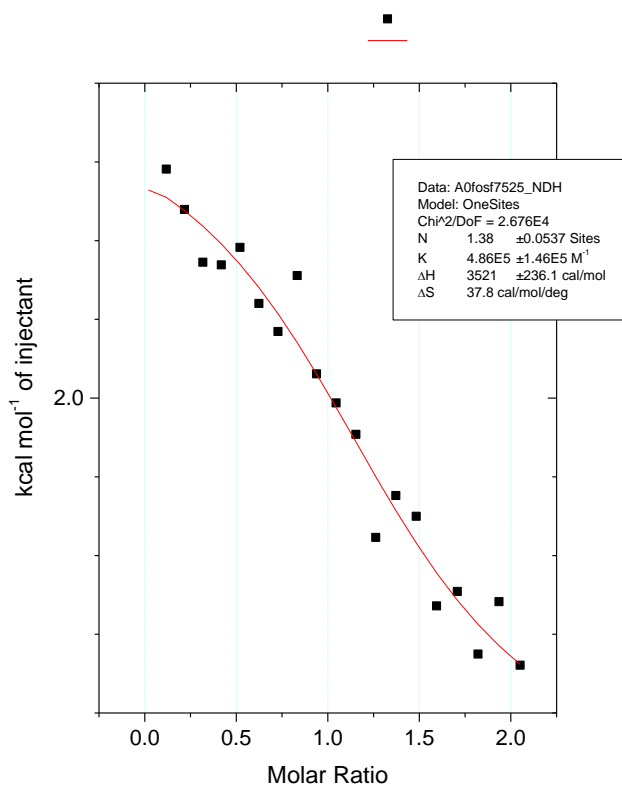
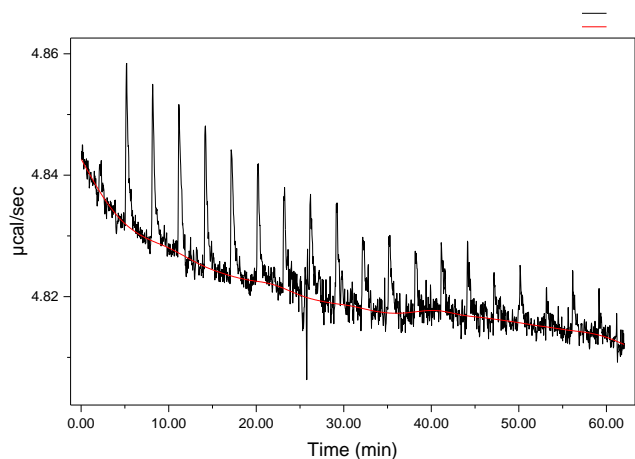




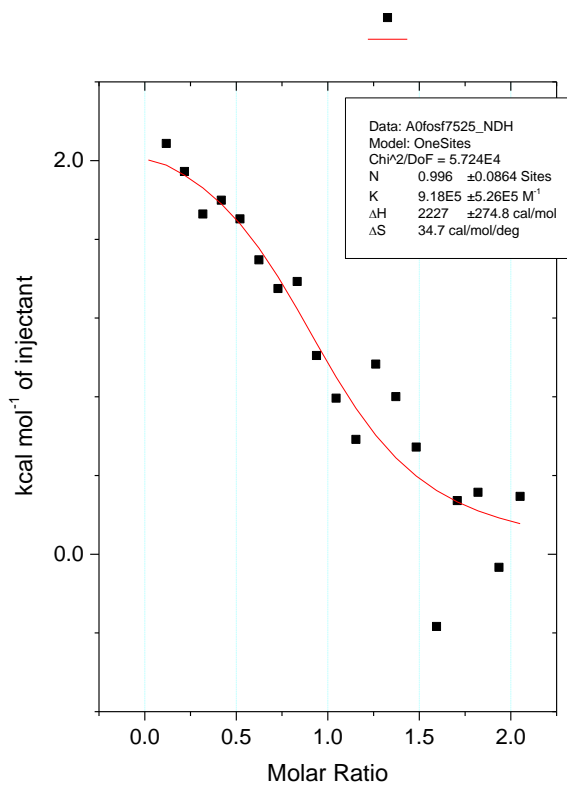
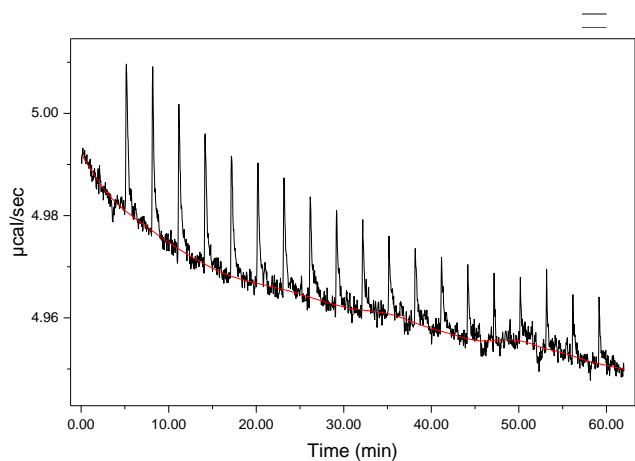


# Isothermal titration calorimetry

## CA II – clopamide

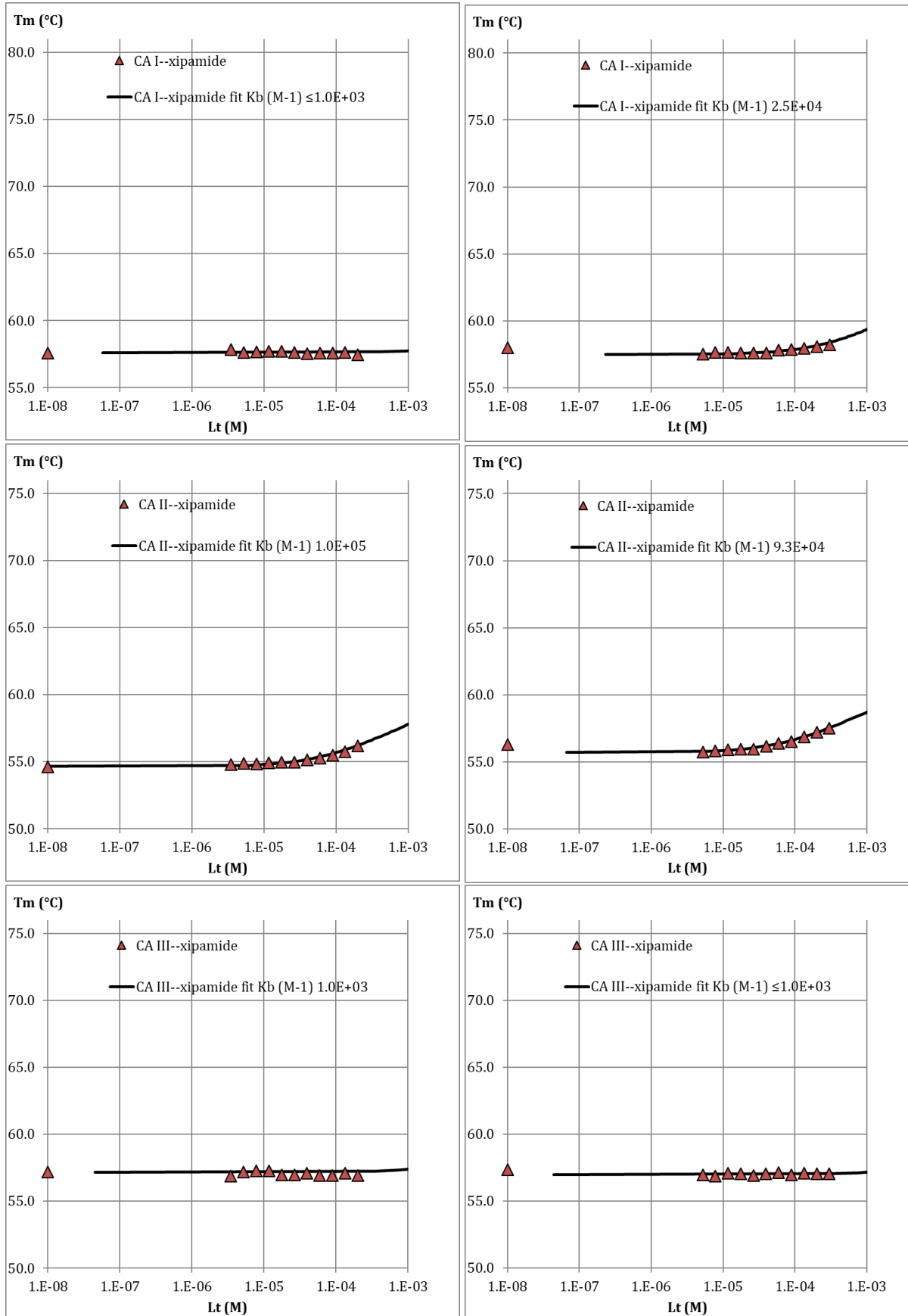


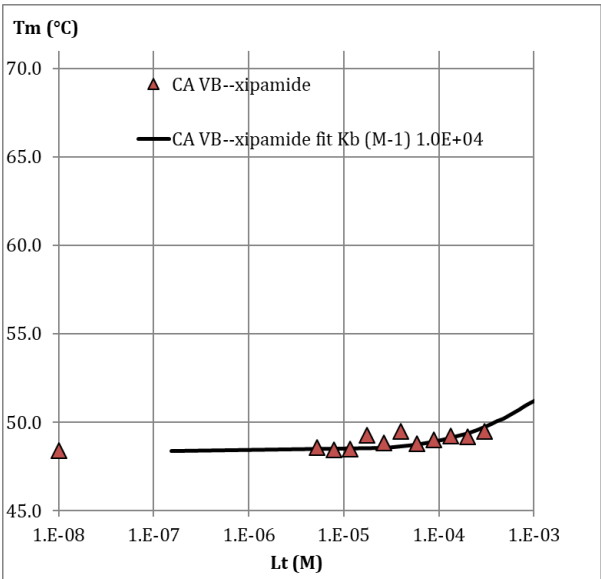
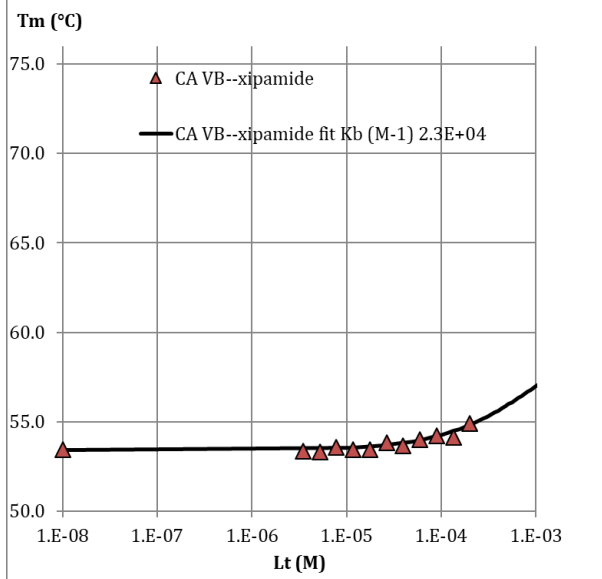
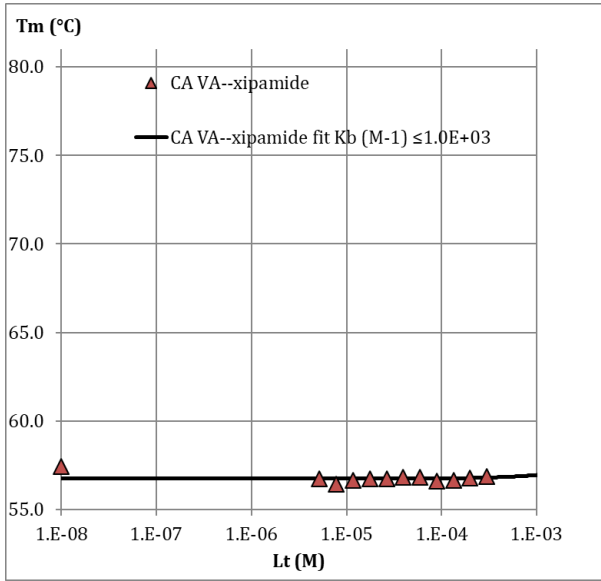
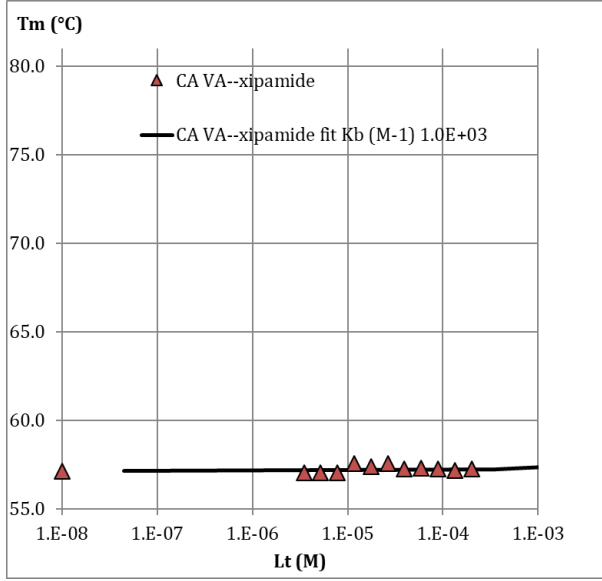
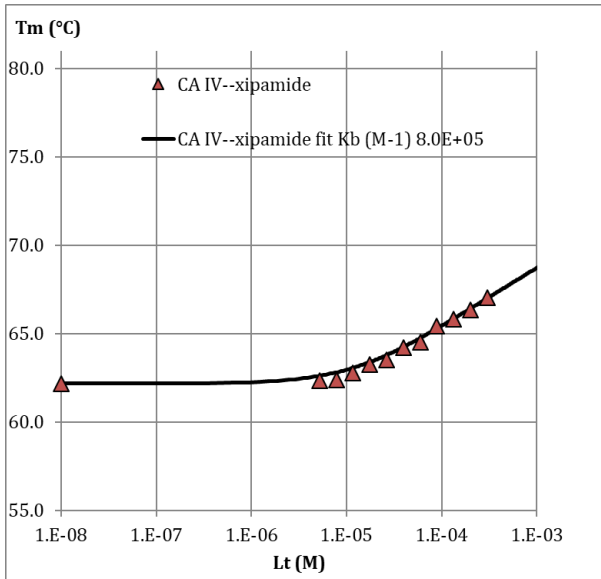
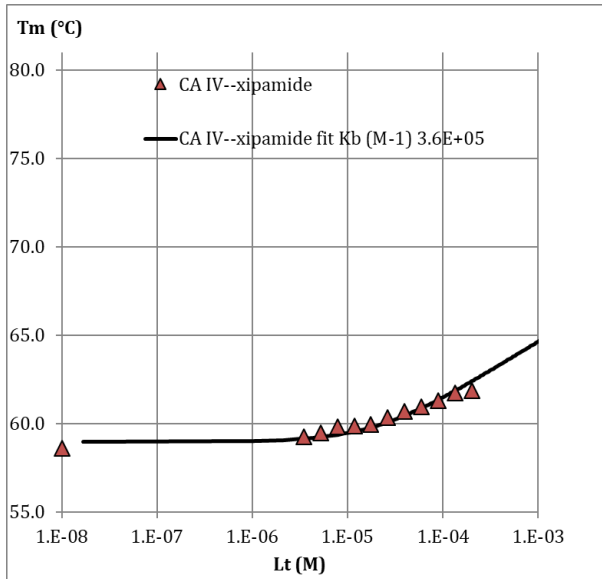
## CA II – clopamide

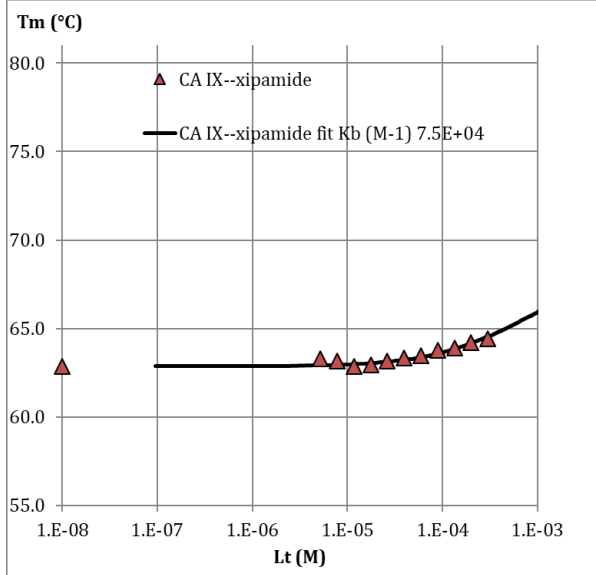
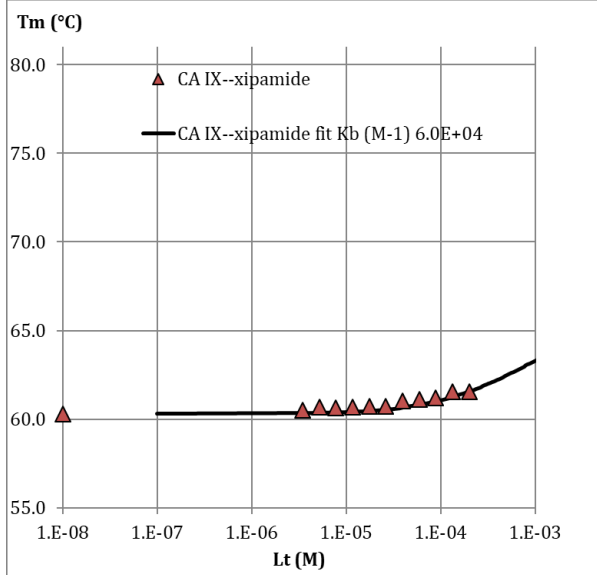
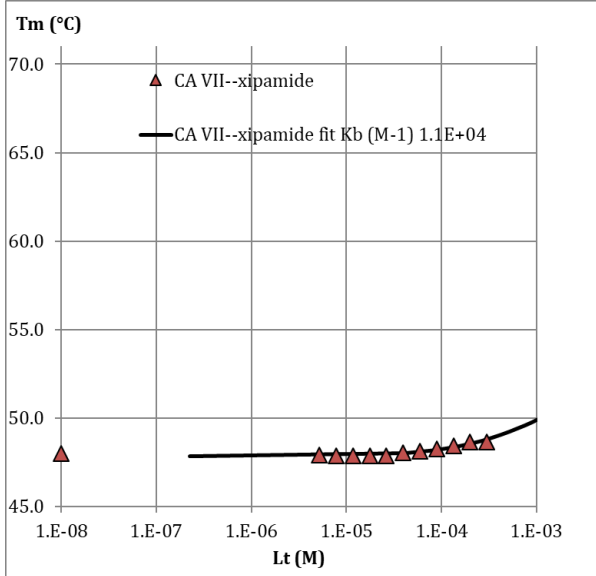
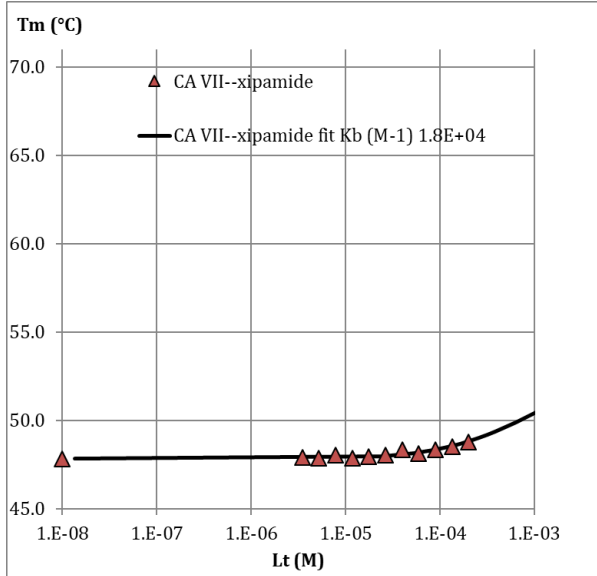
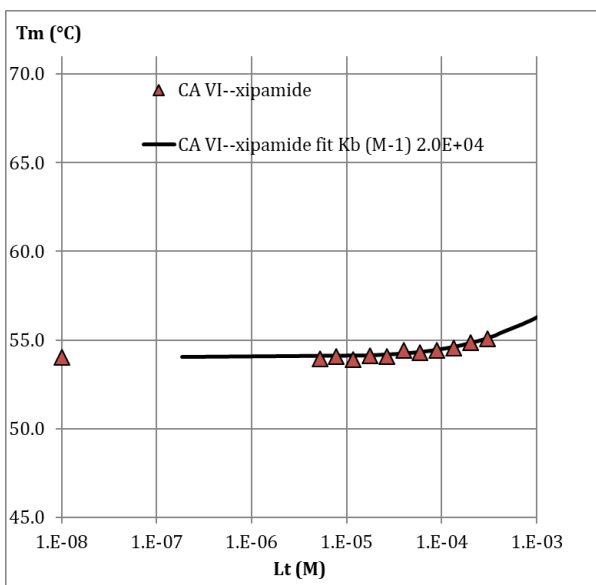
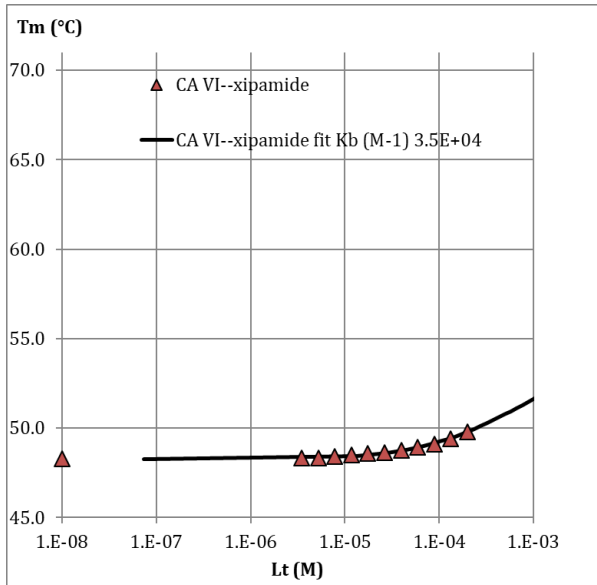


# 14. Xipamide

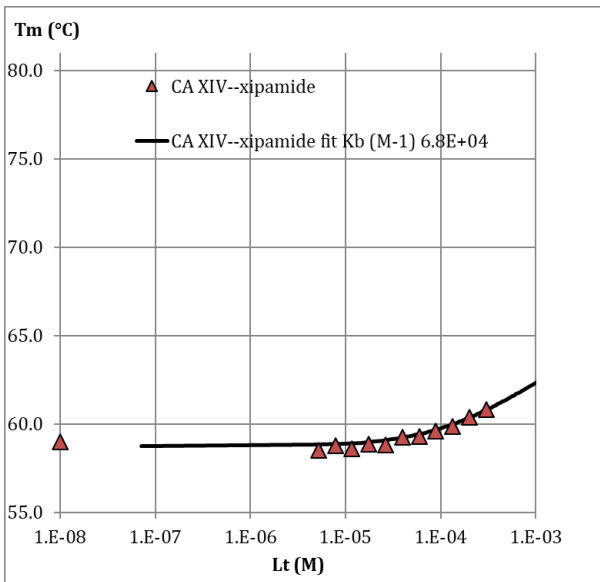
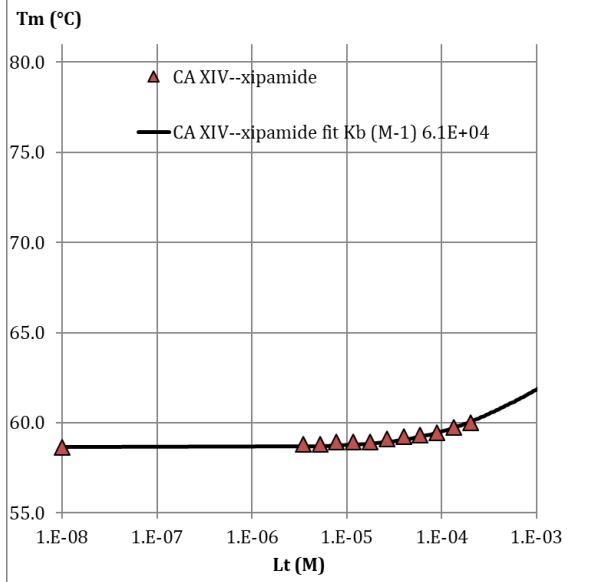
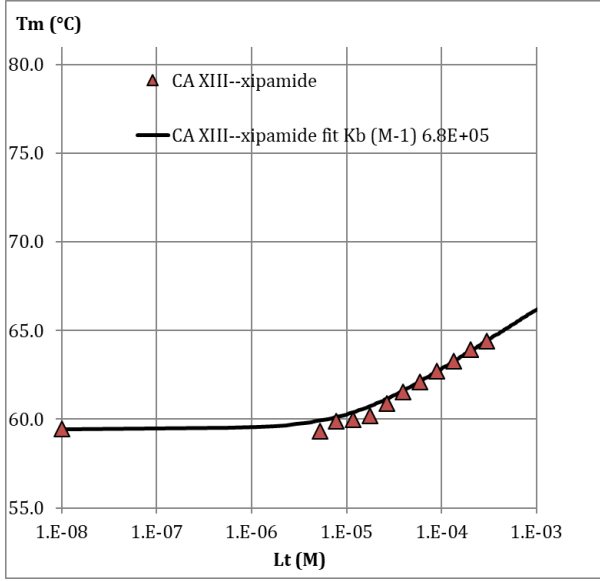
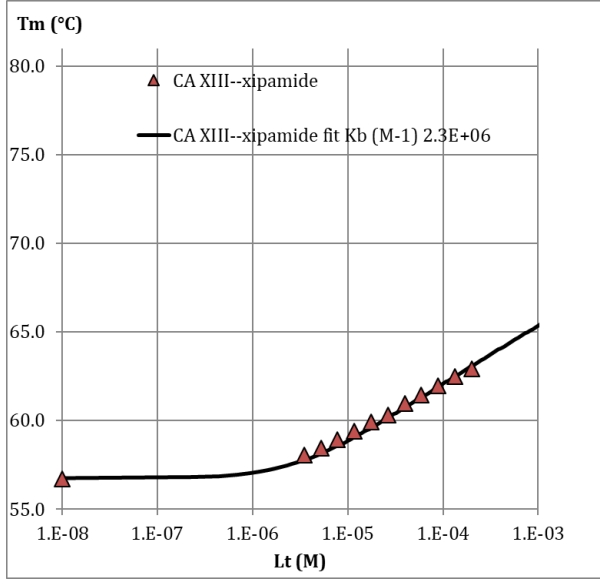
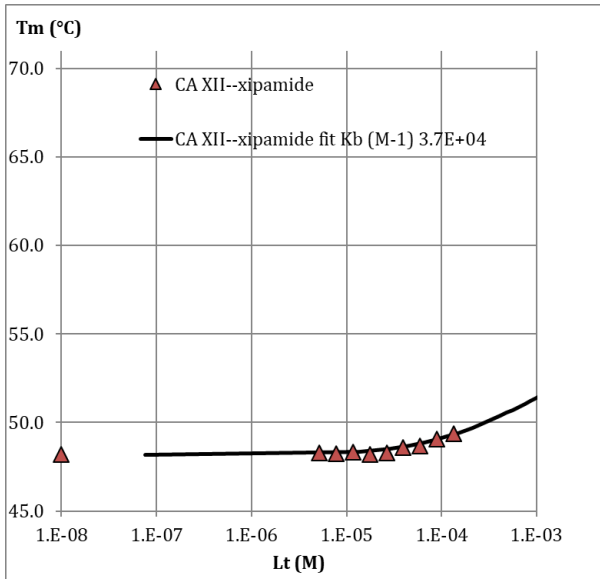
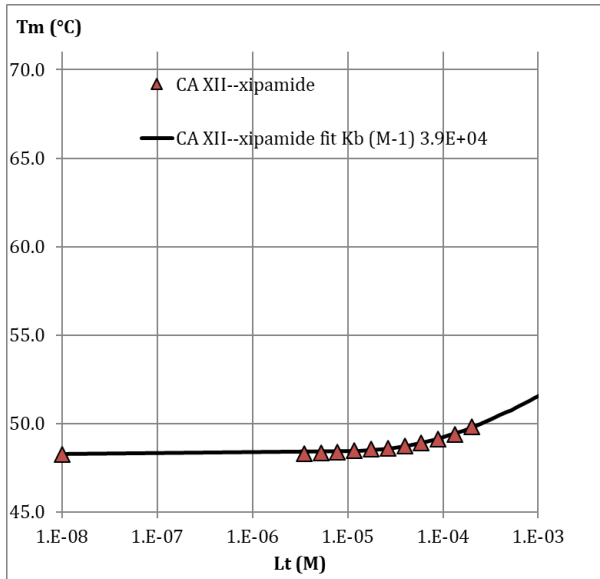
## Fluorence-based thermal shift assay





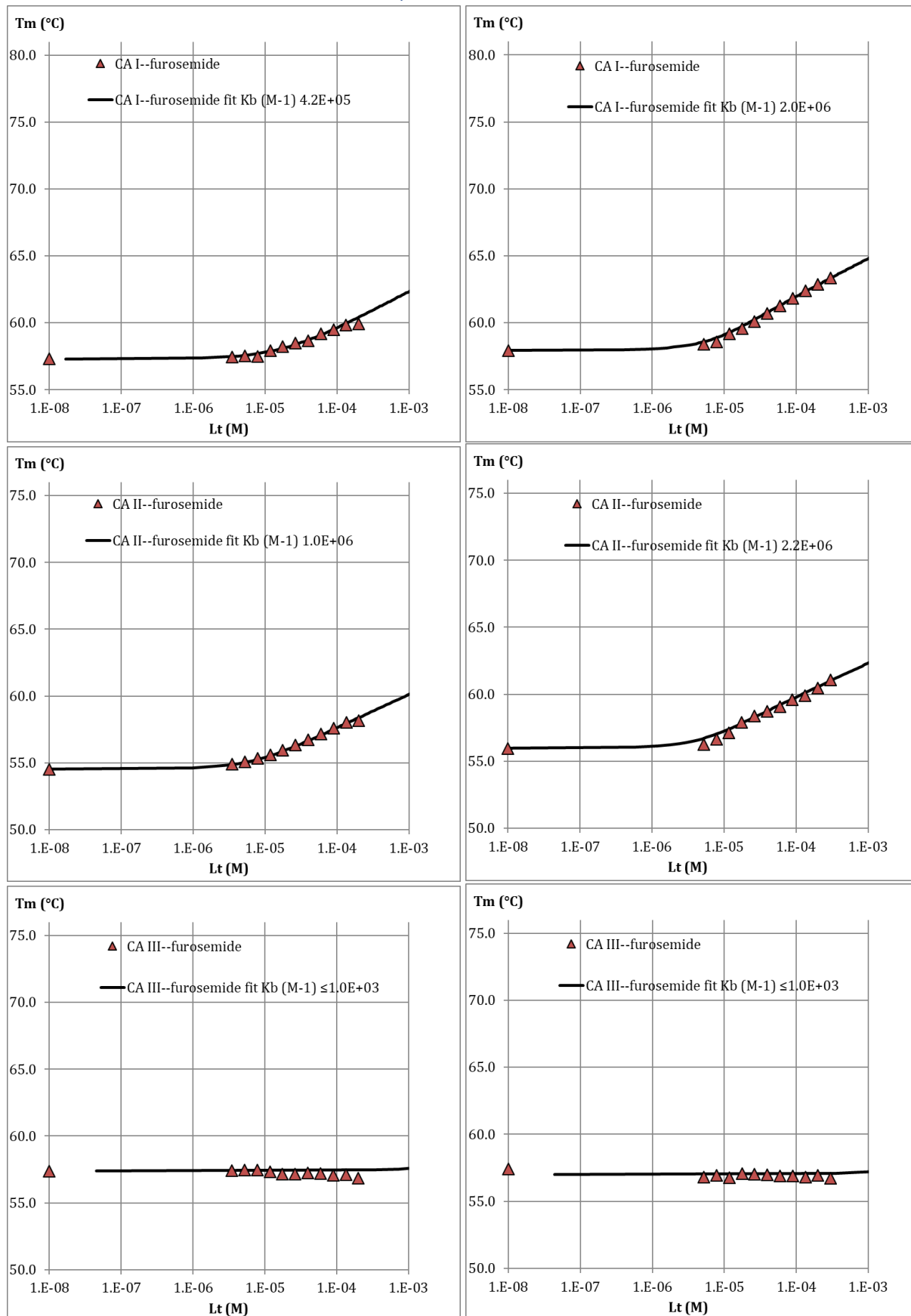


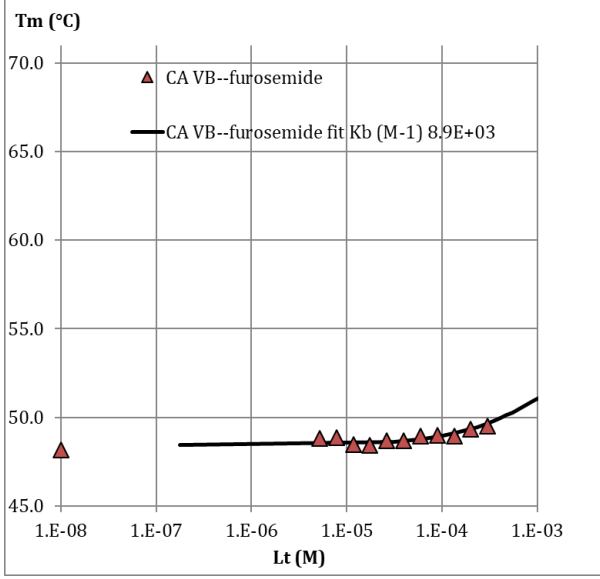
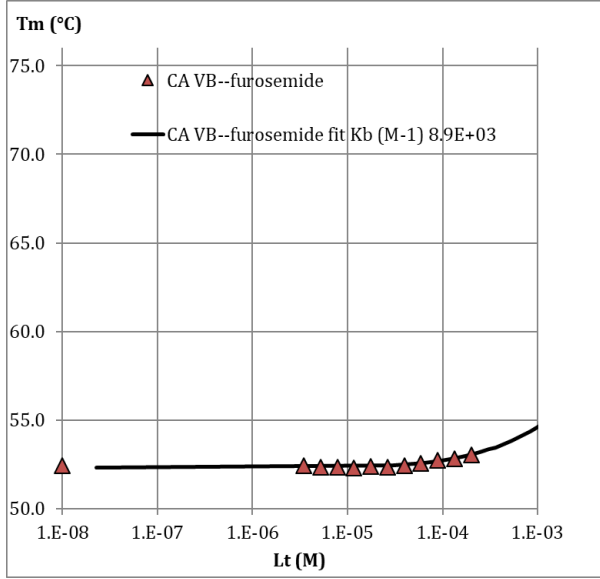
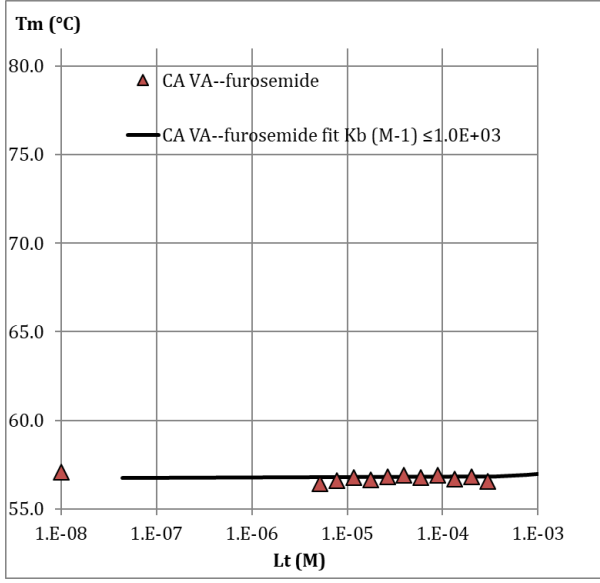
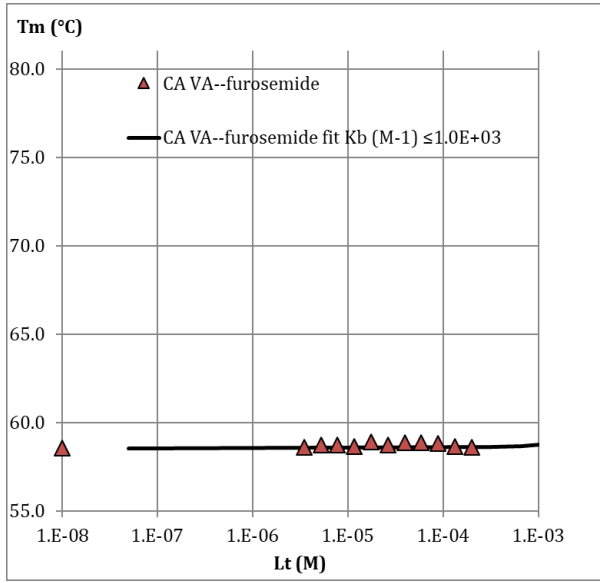
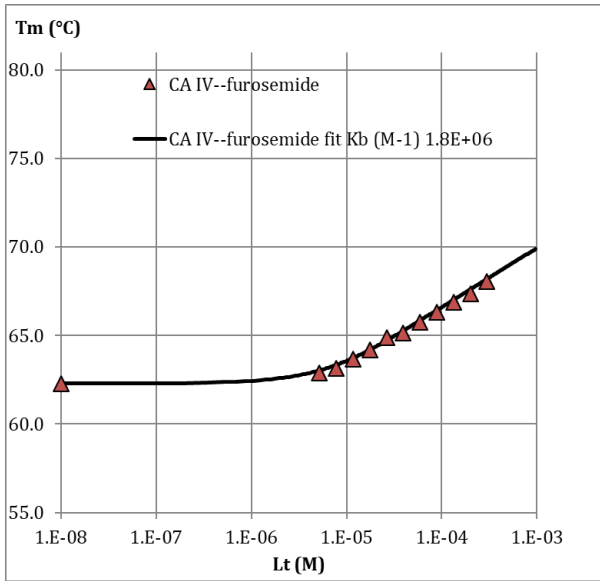
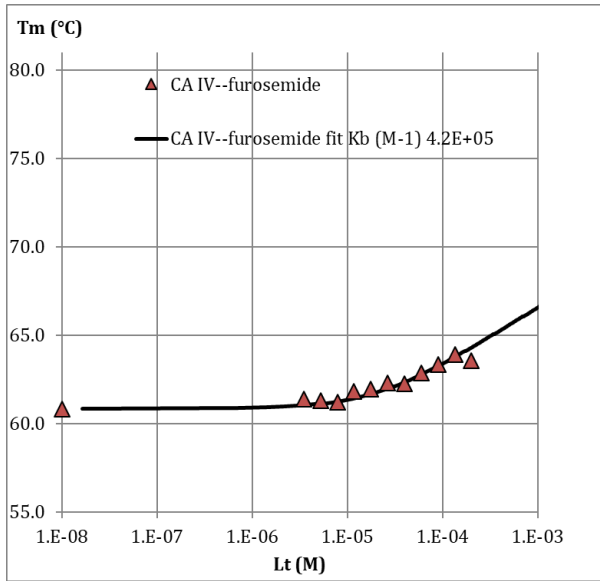


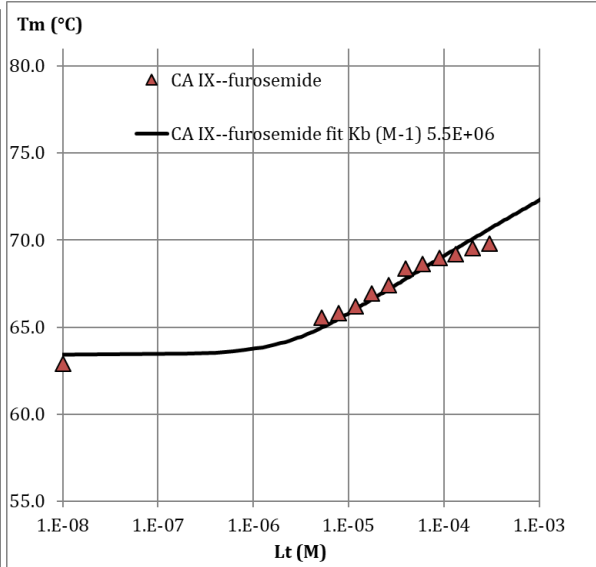
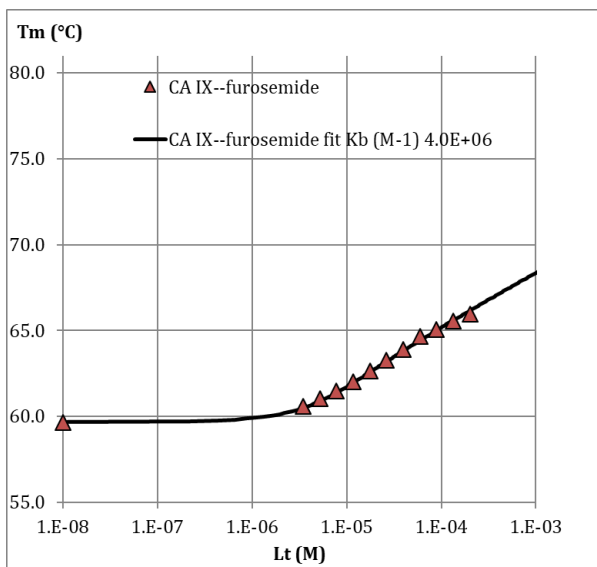
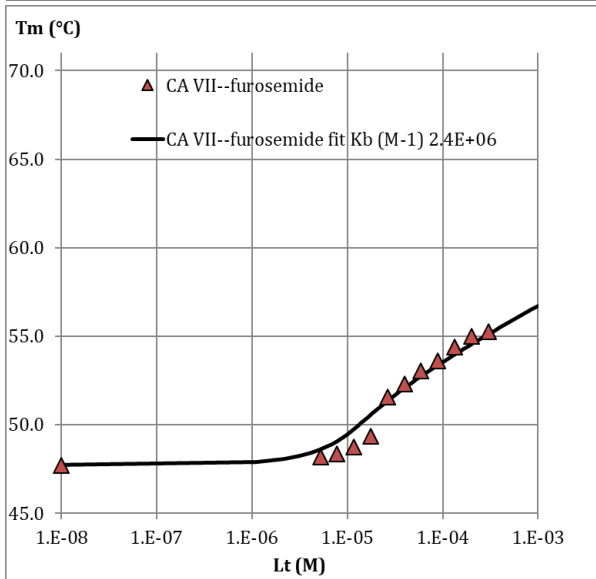
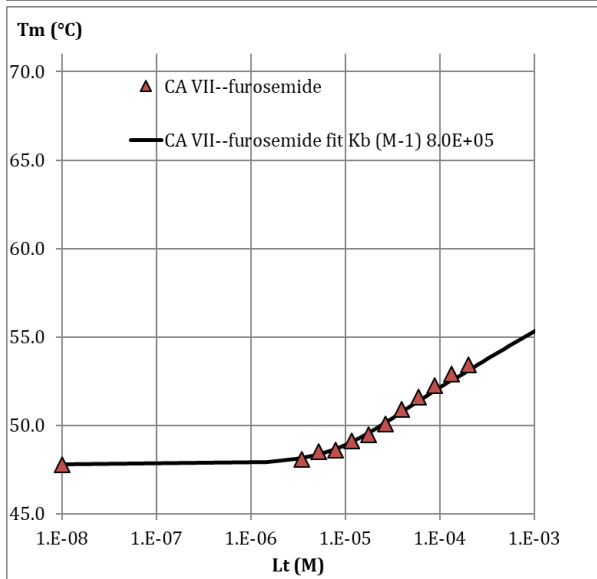
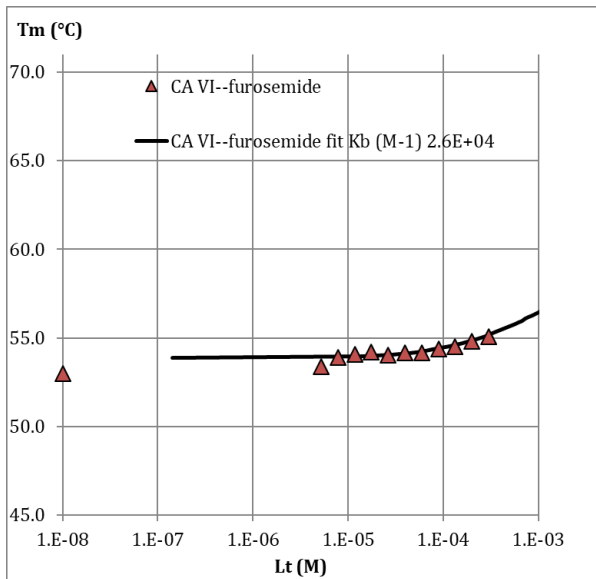
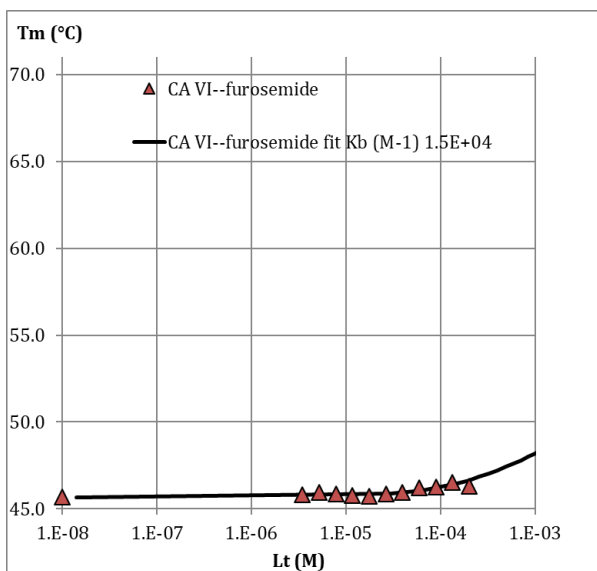


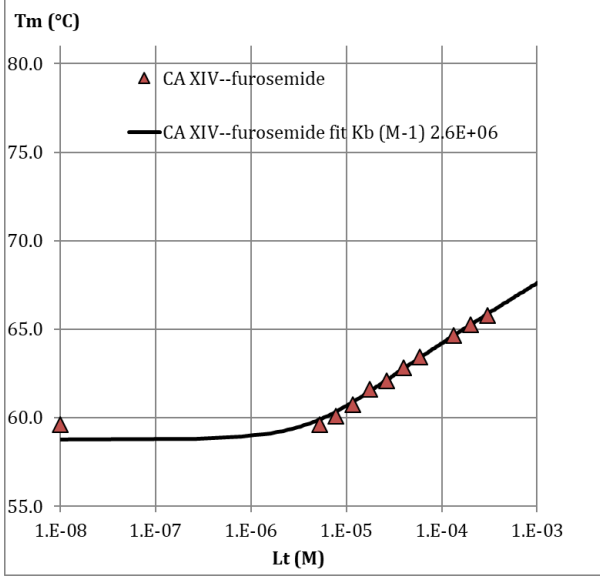
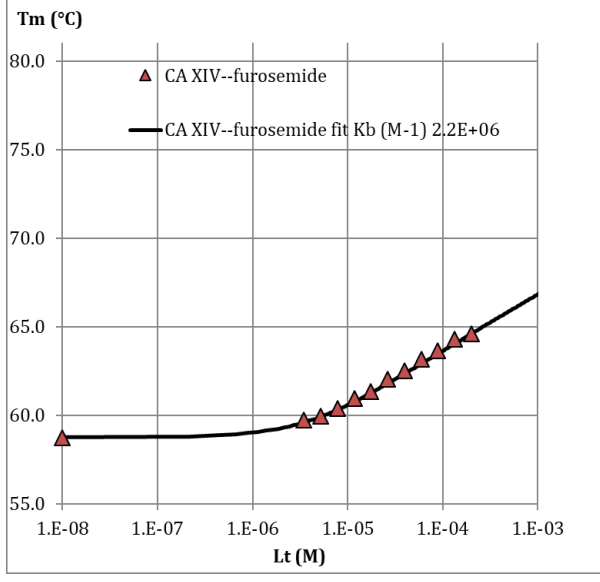
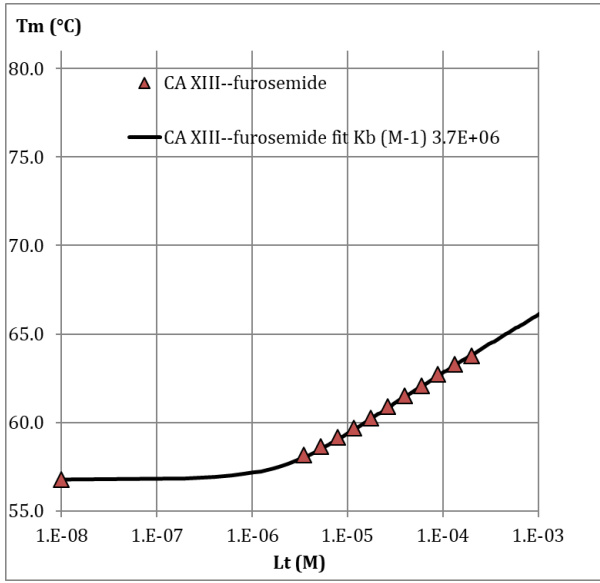
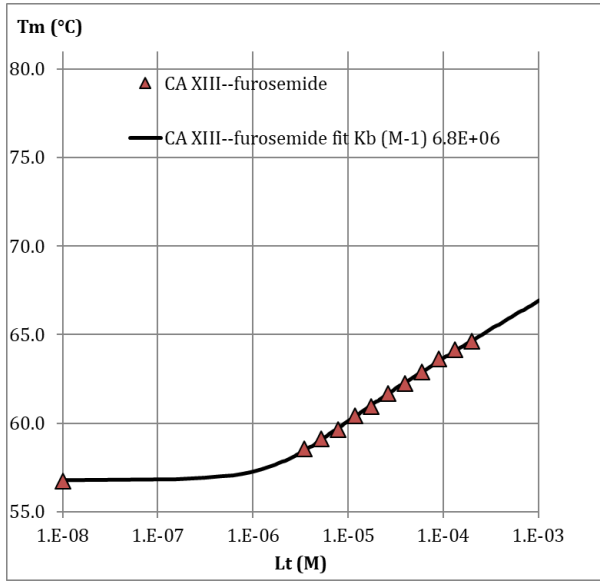
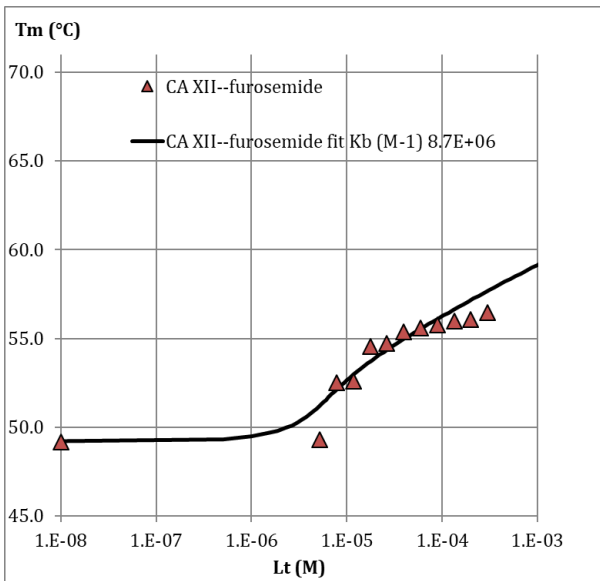
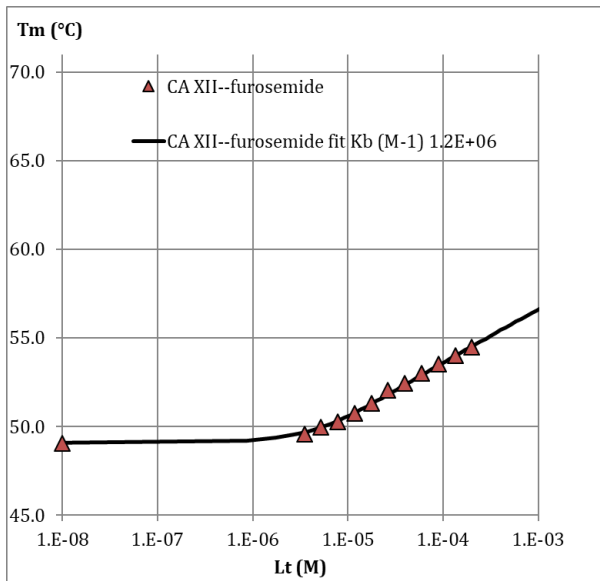
# 15. Furosemide

## Fluorecence-based thermal shift assay



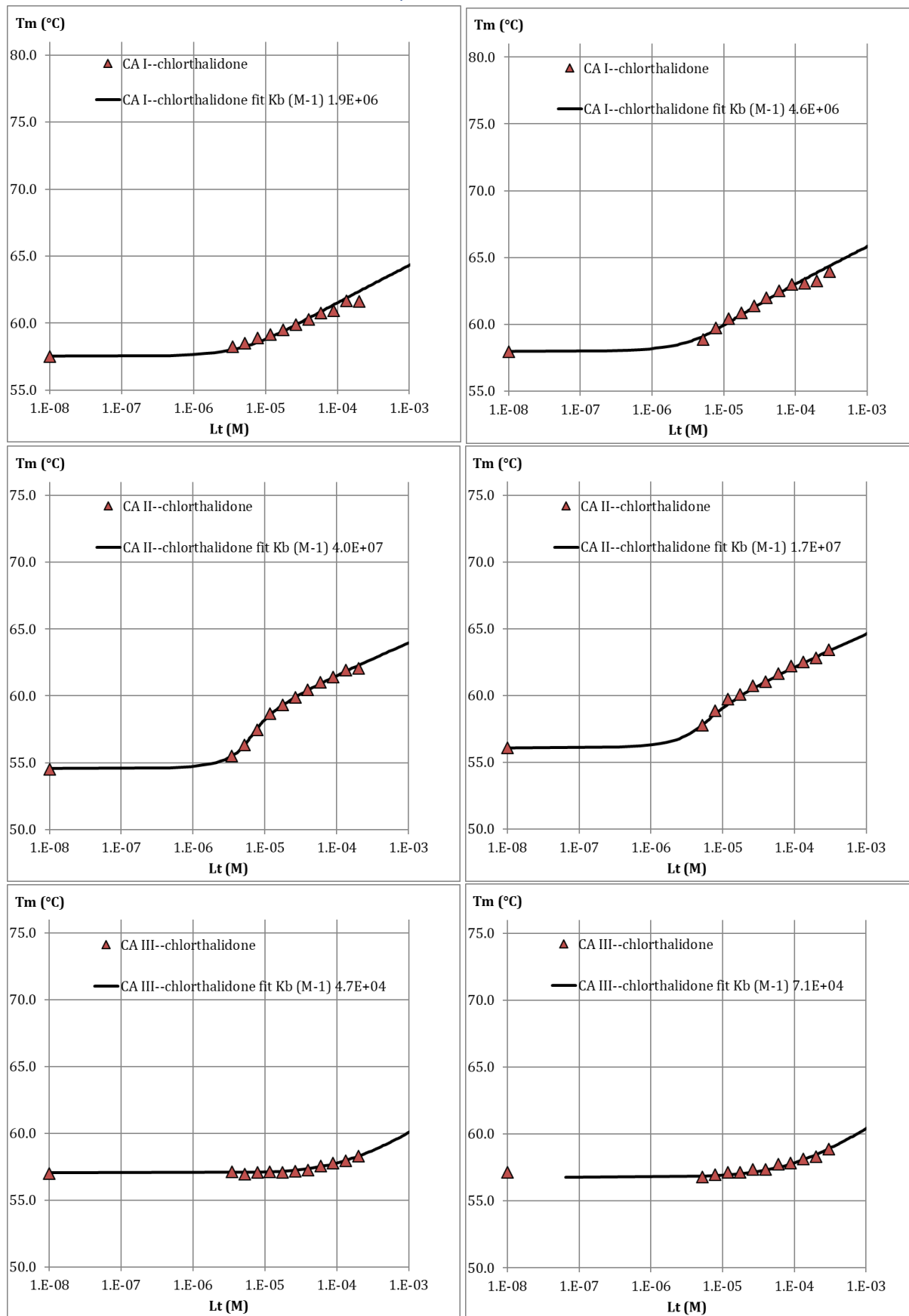


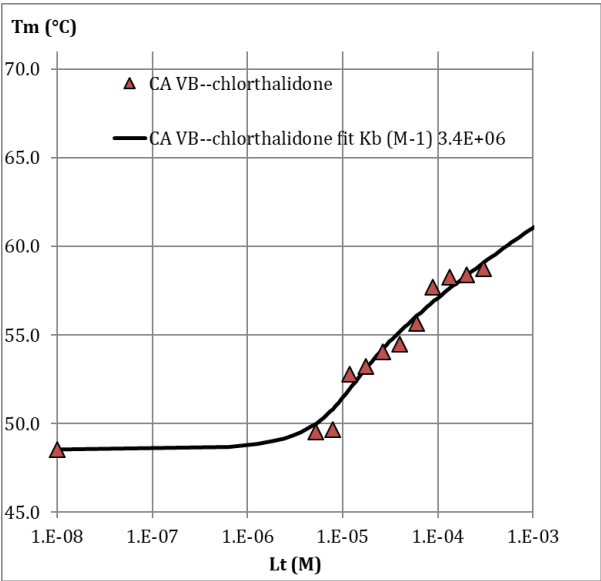
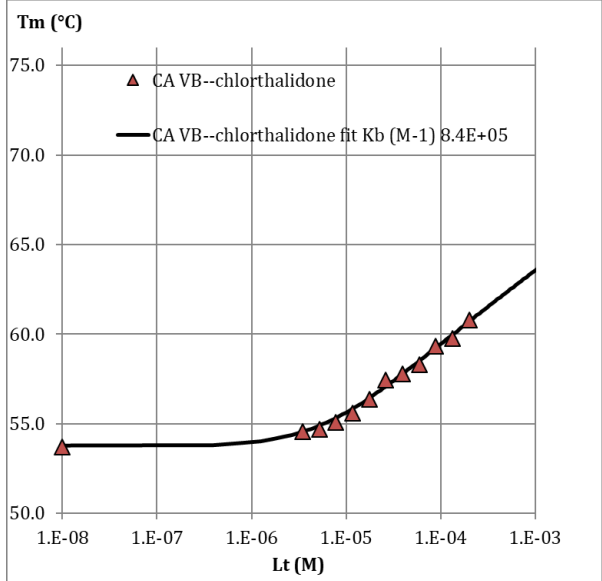
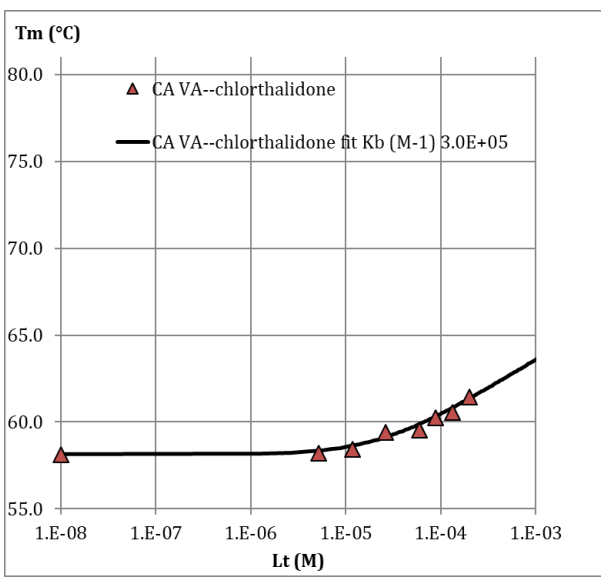
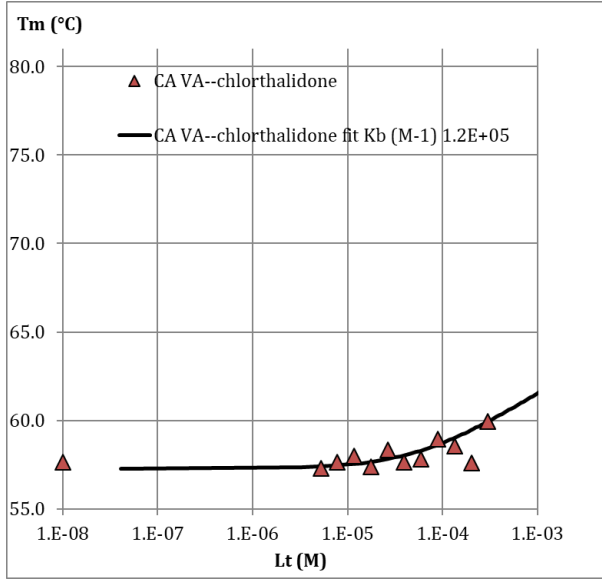
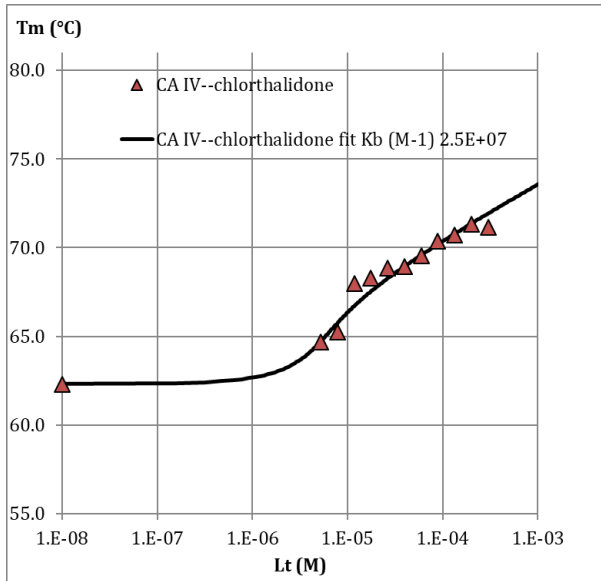
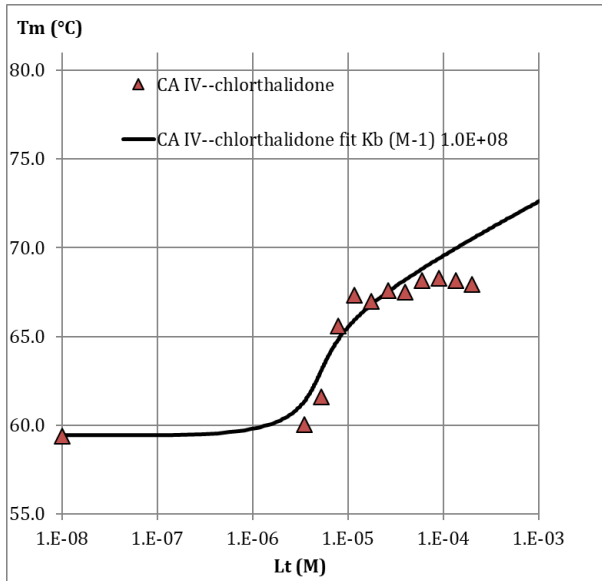


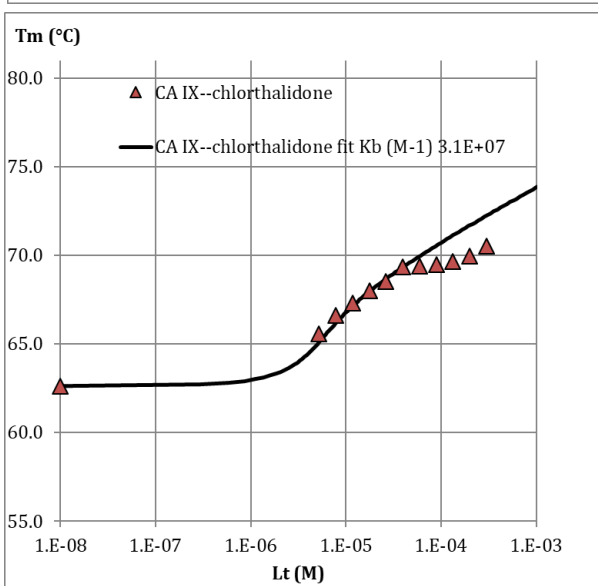
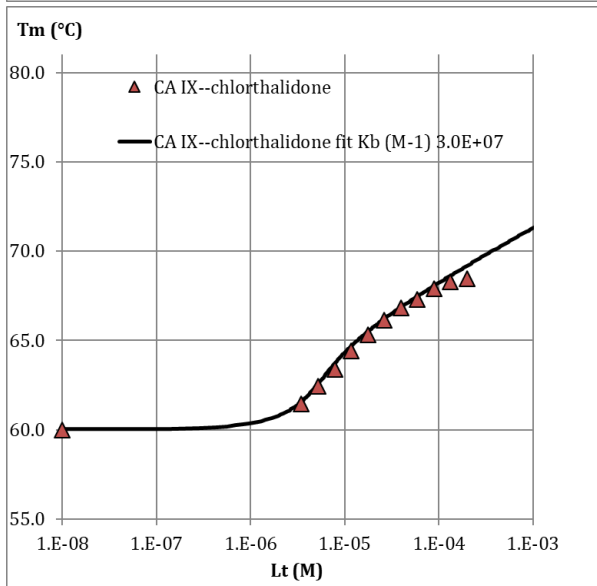
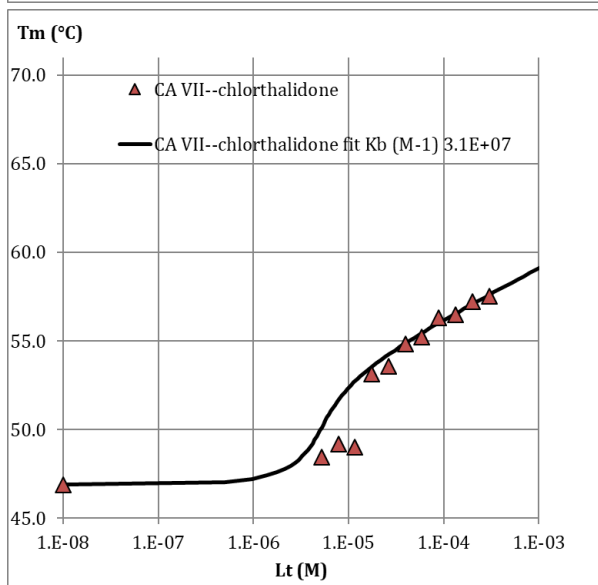
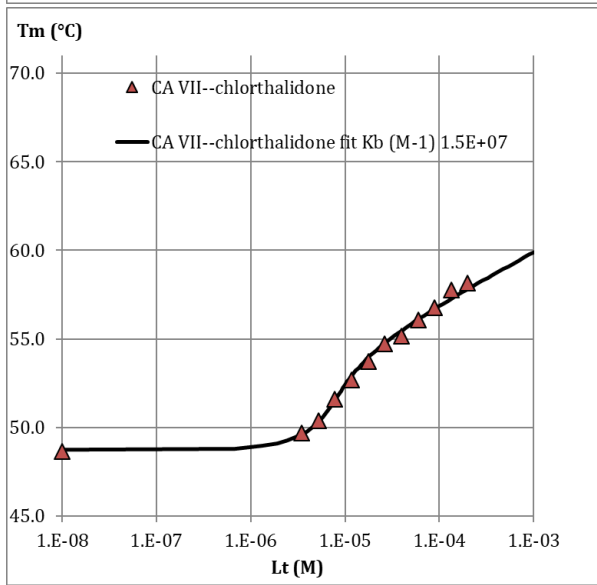
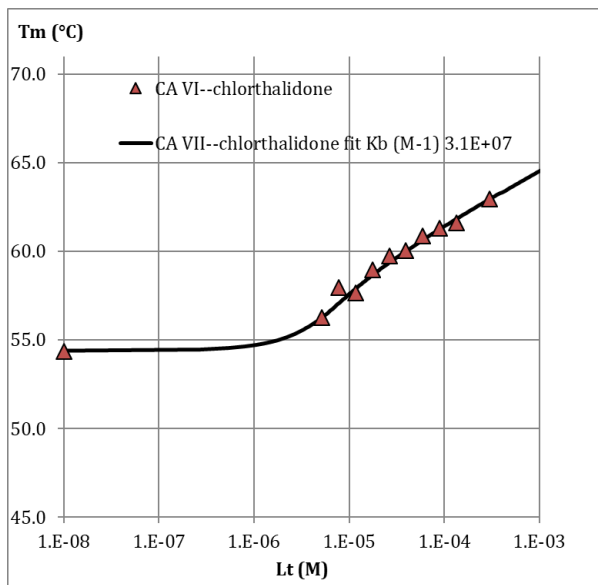
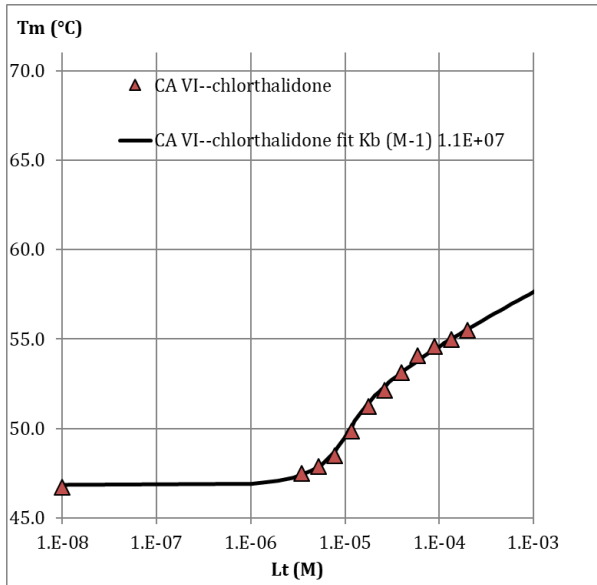


# 16. Chlorthalidone

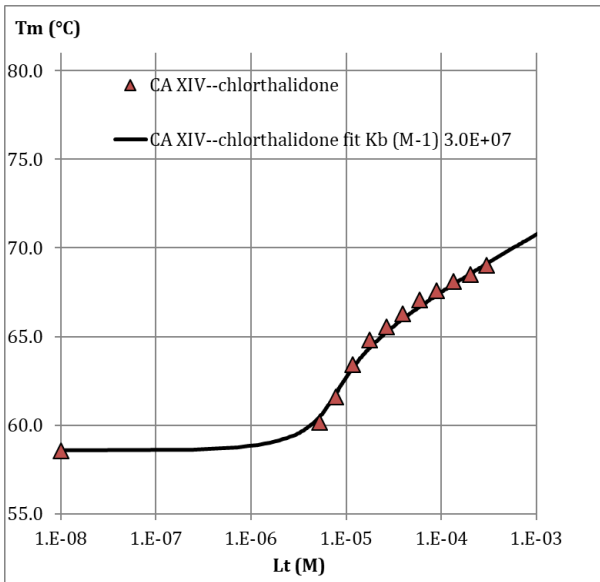
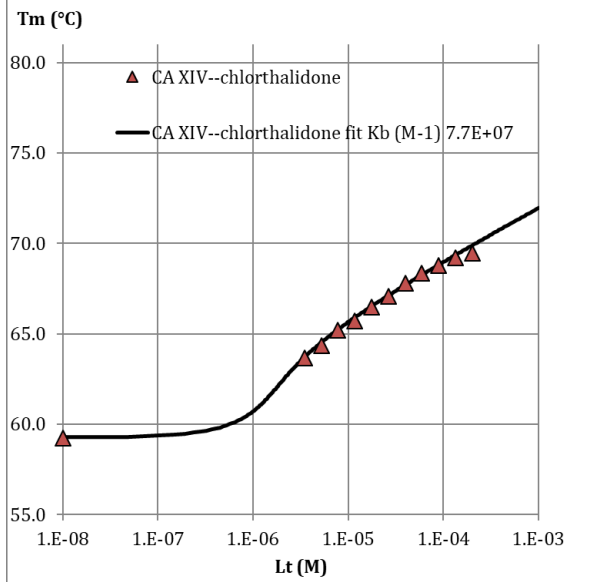
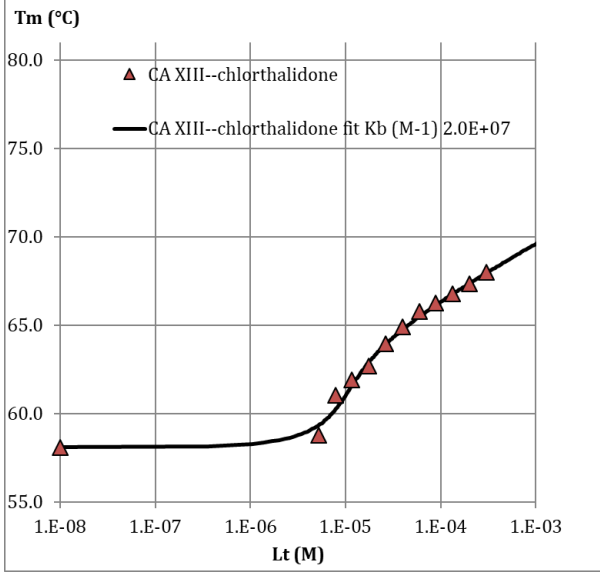
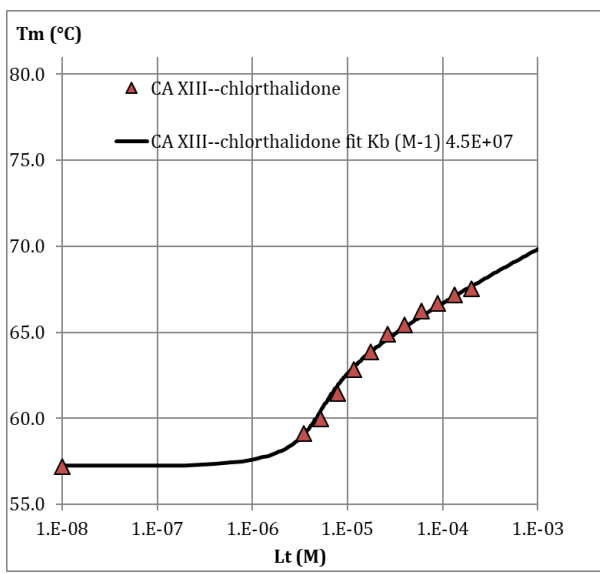
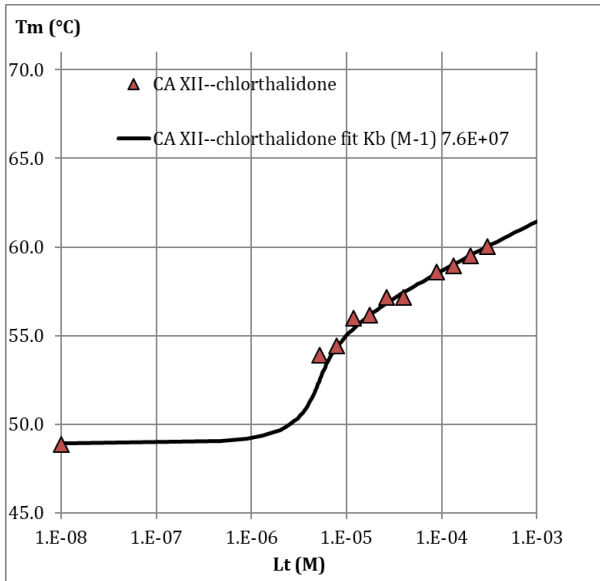
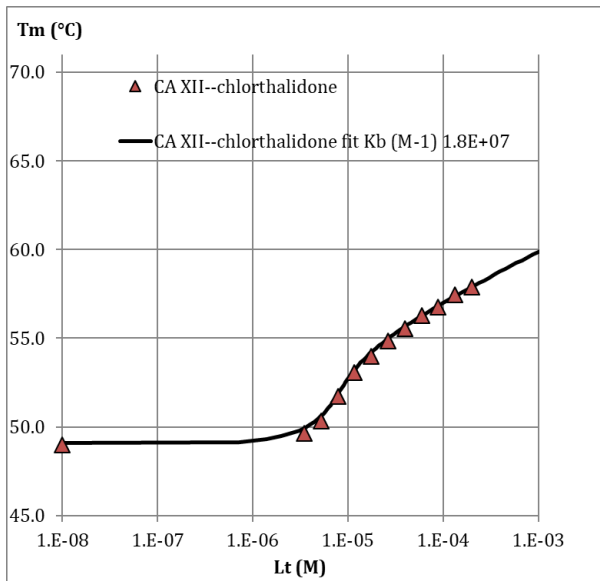
## Fluorecence-based thermal shift assay





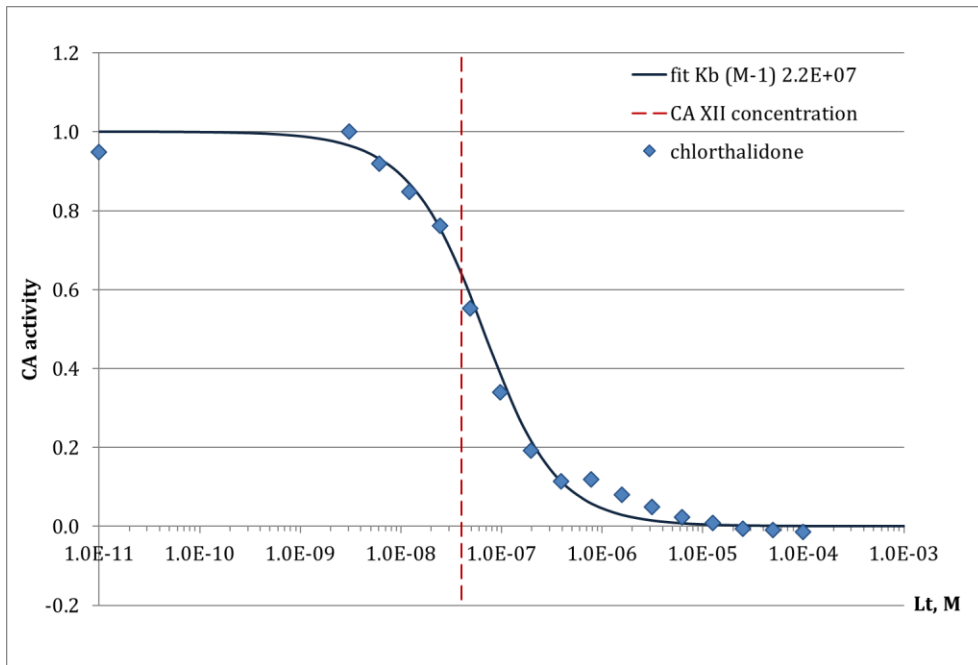






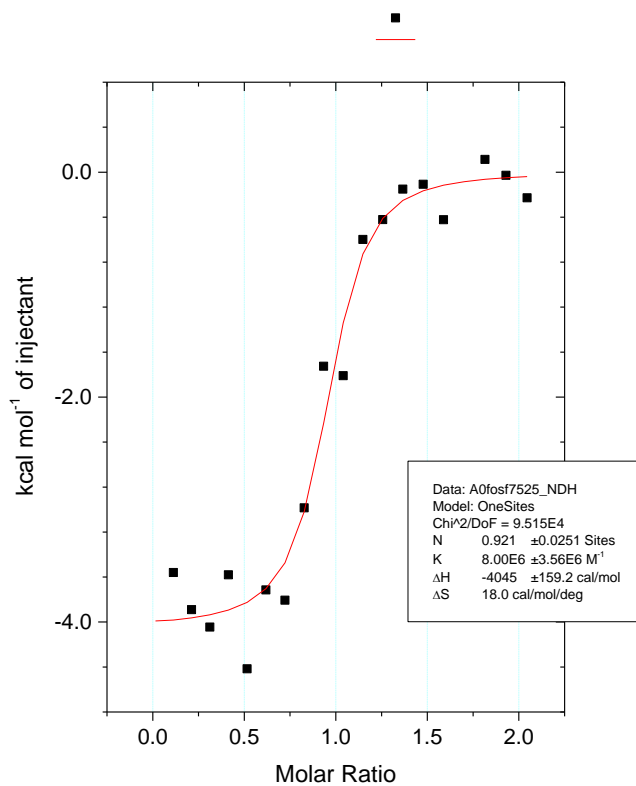
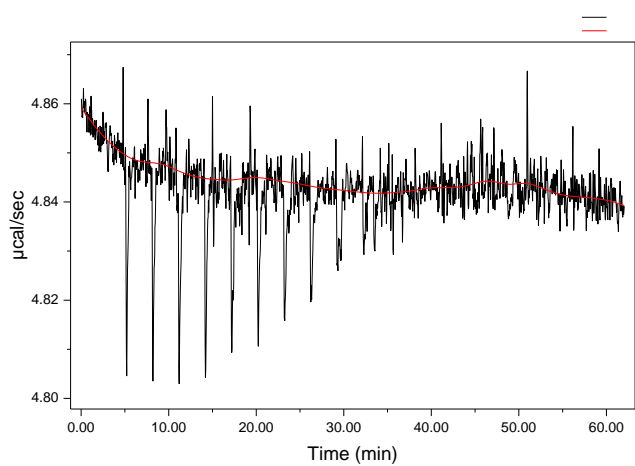
# CO<sub>2</sub> hydration activity inhibition

## CA XII - chlorthalidone

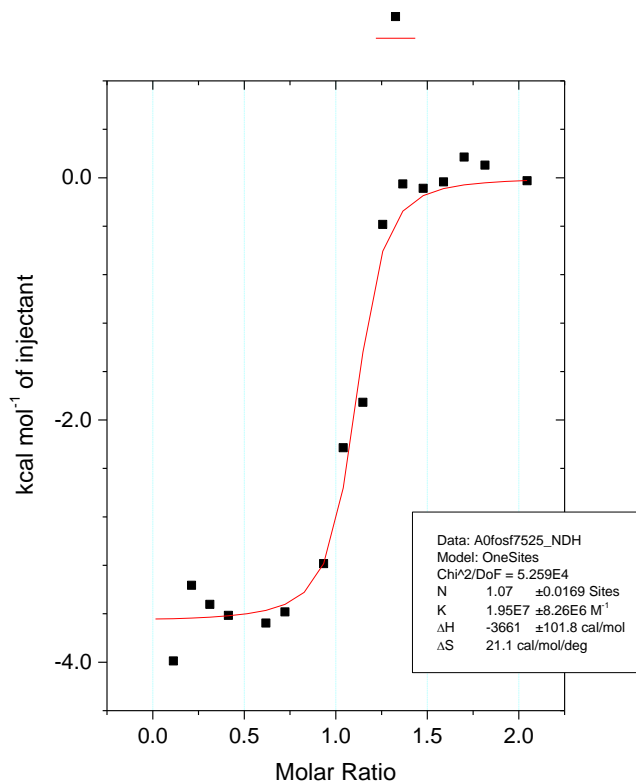
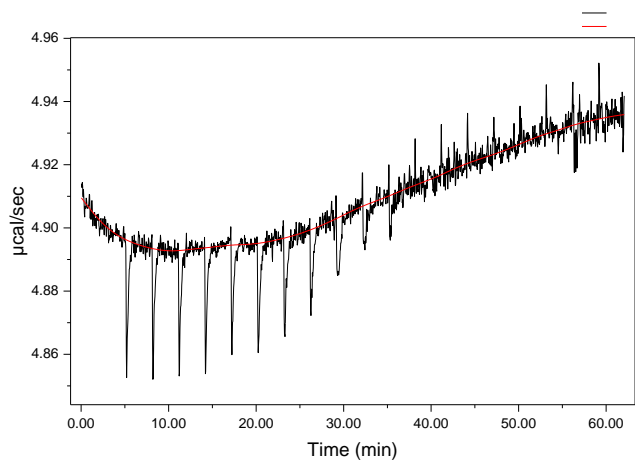


# Isothermal titration calorimetry

## CA II – chlorthalidone

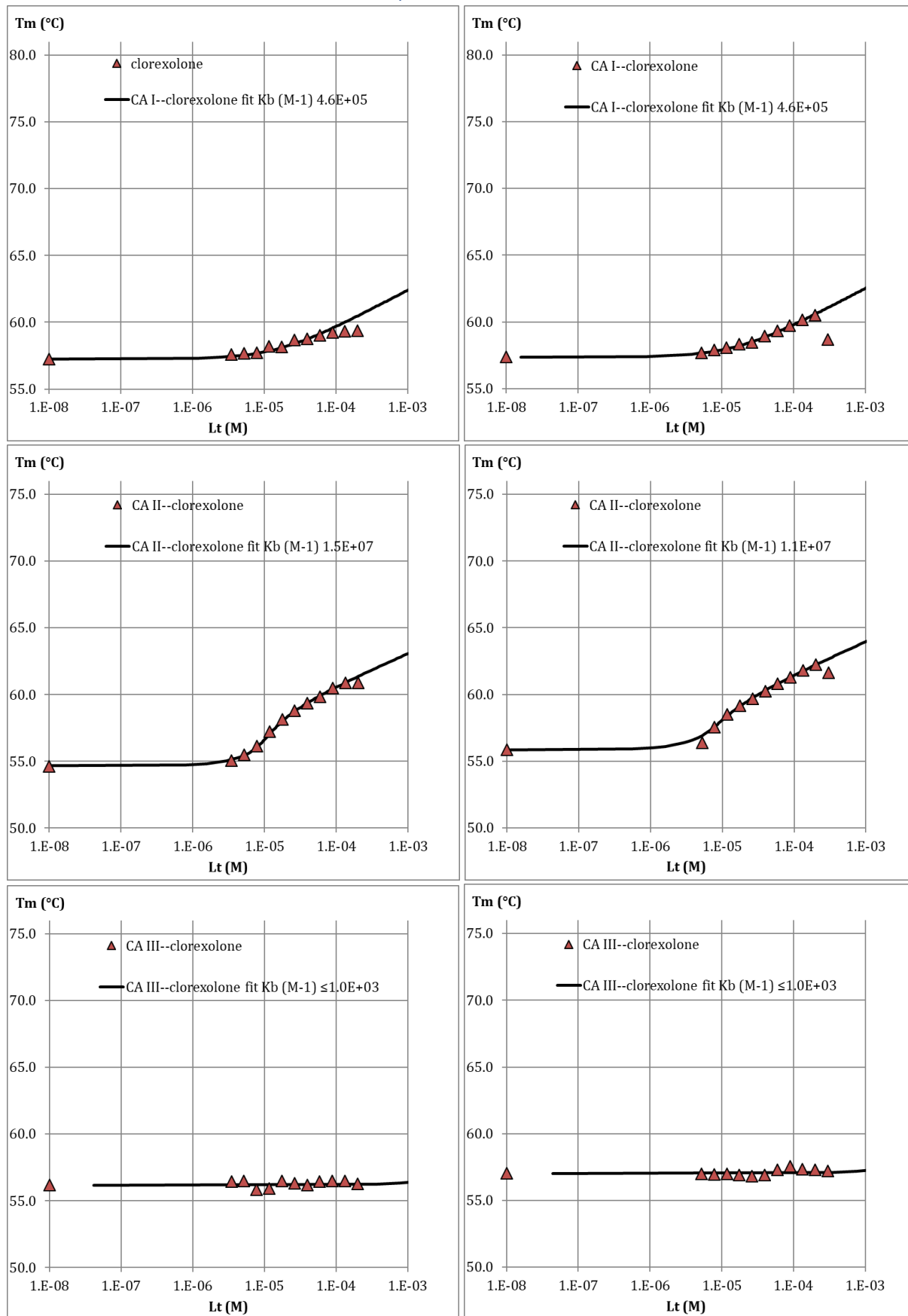


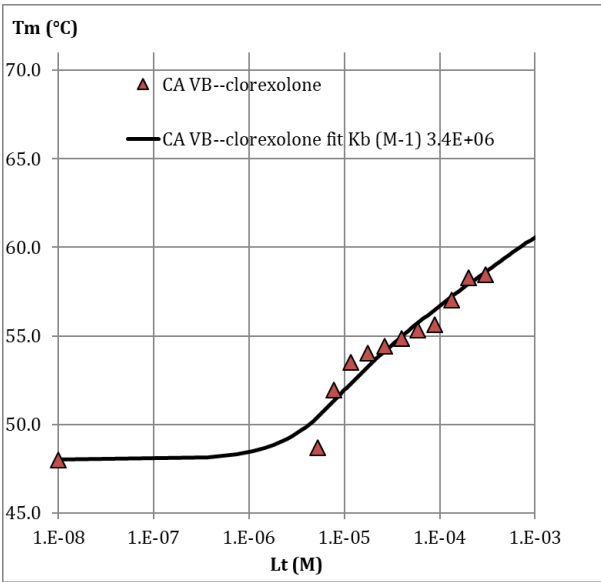
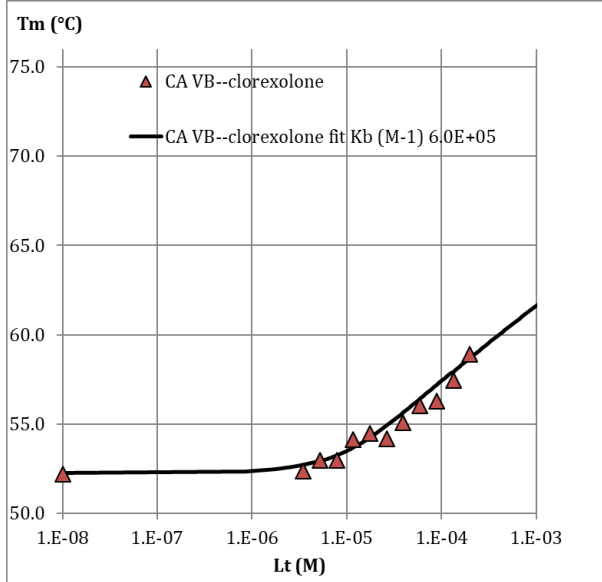
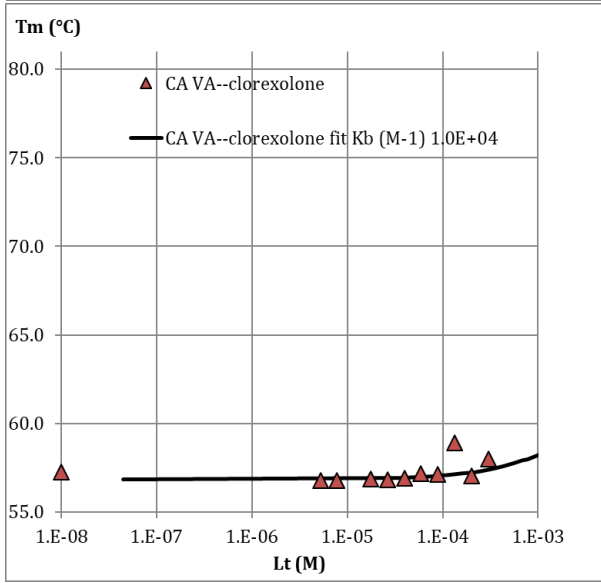
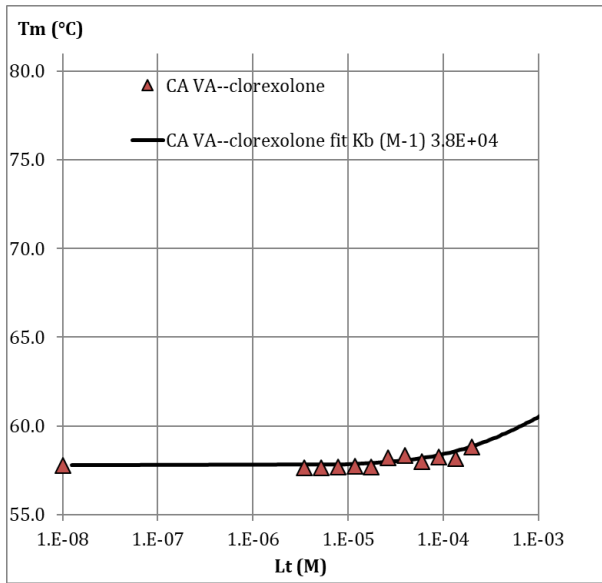
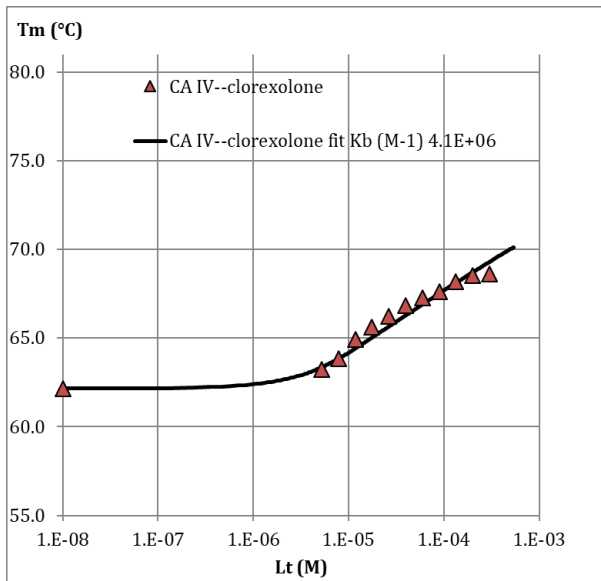
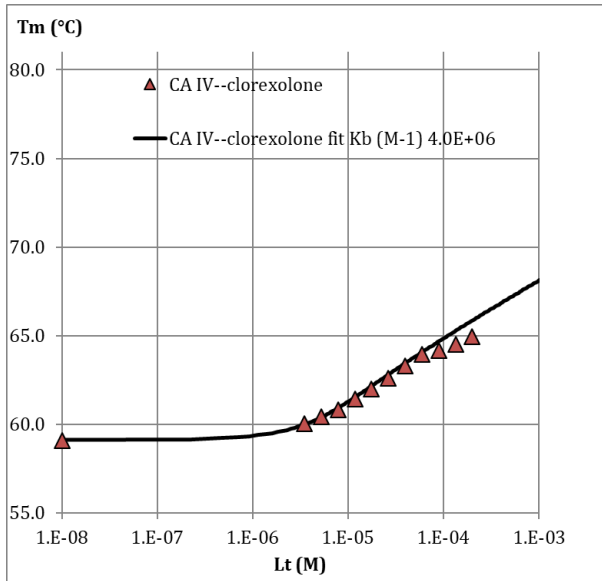
## CA II – chlorthalidone

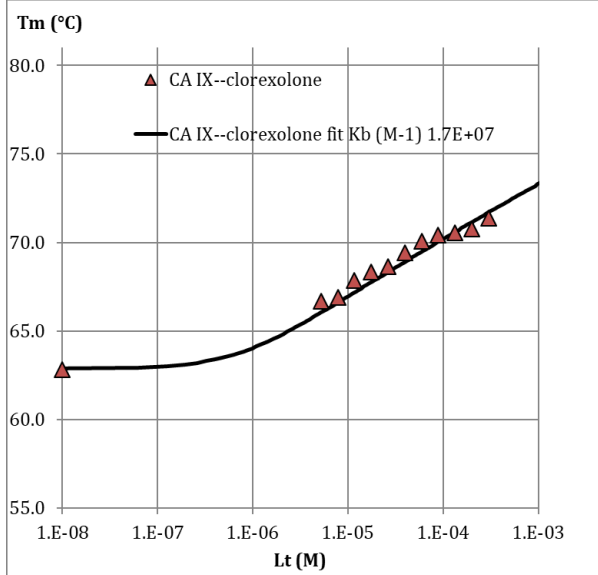
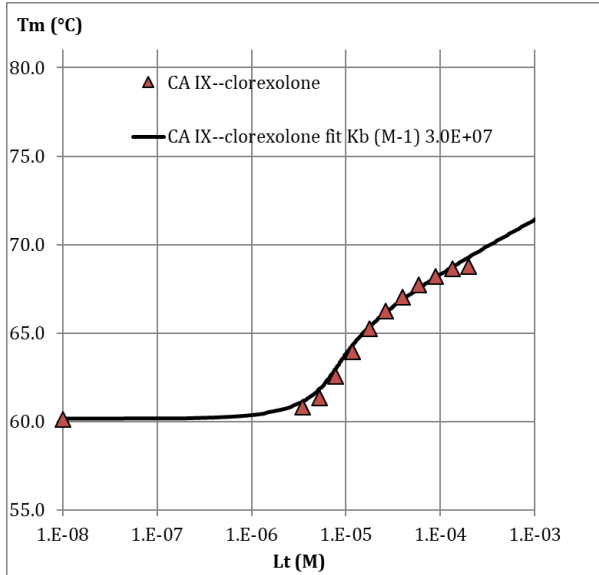
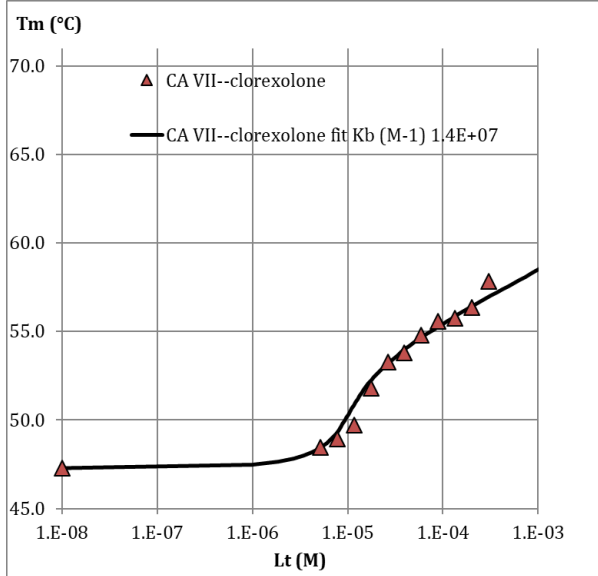
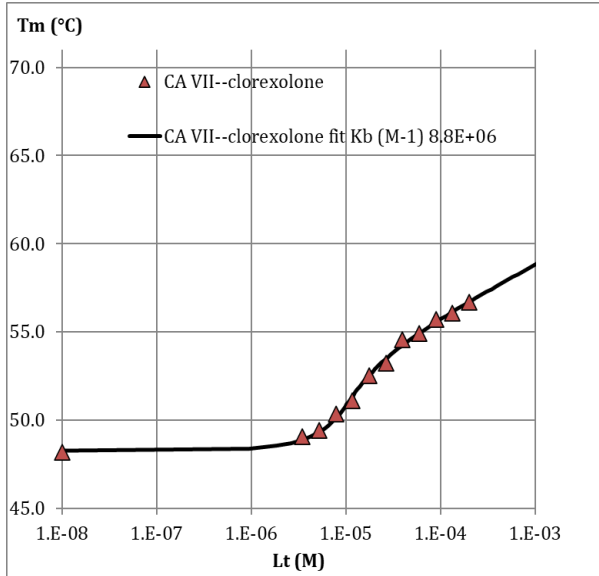
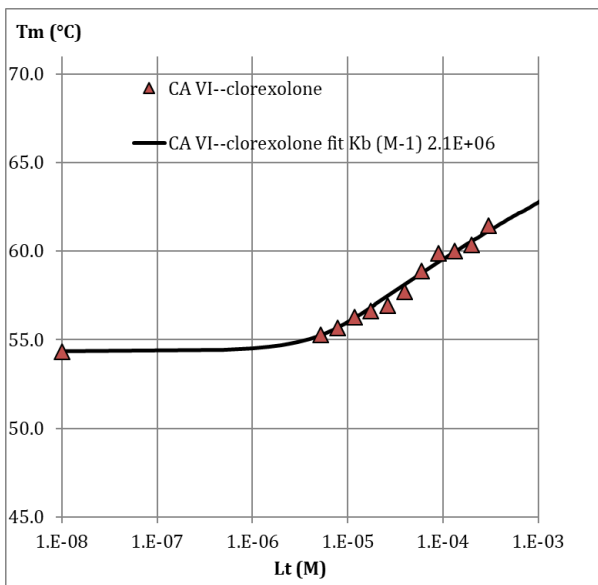
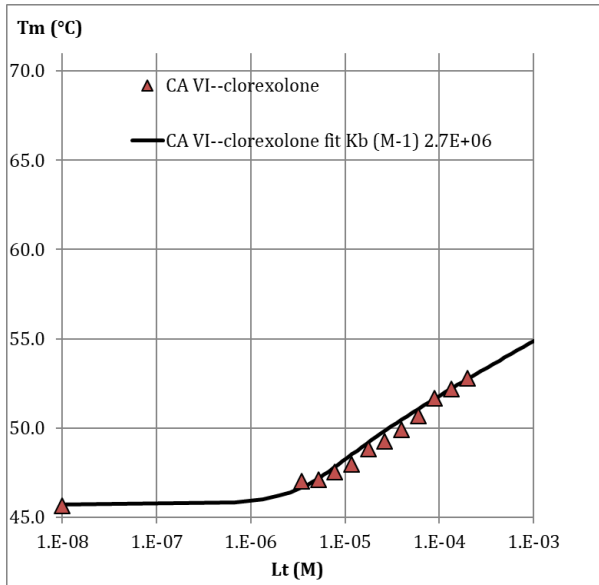


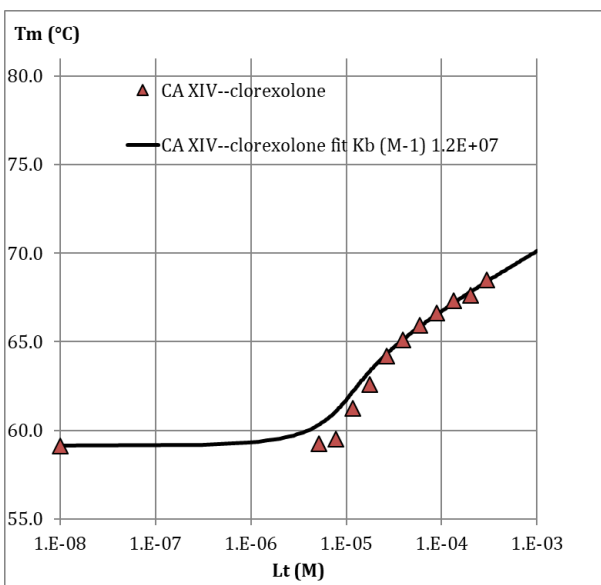
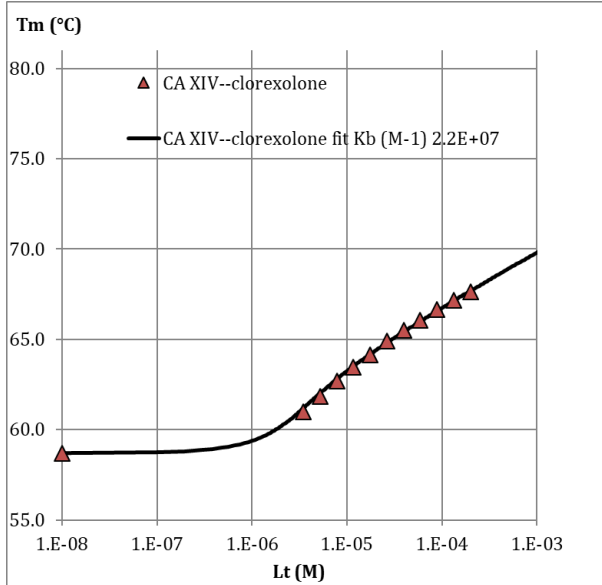
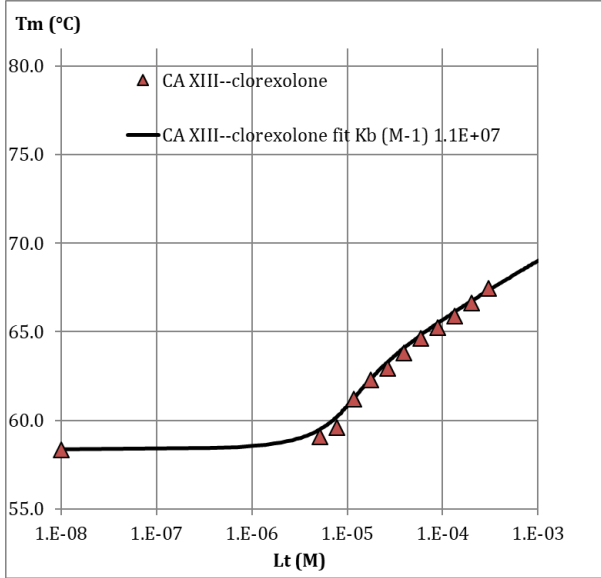
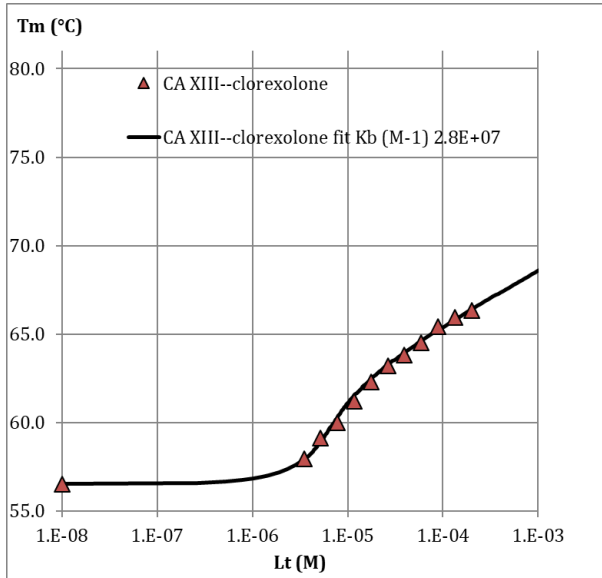
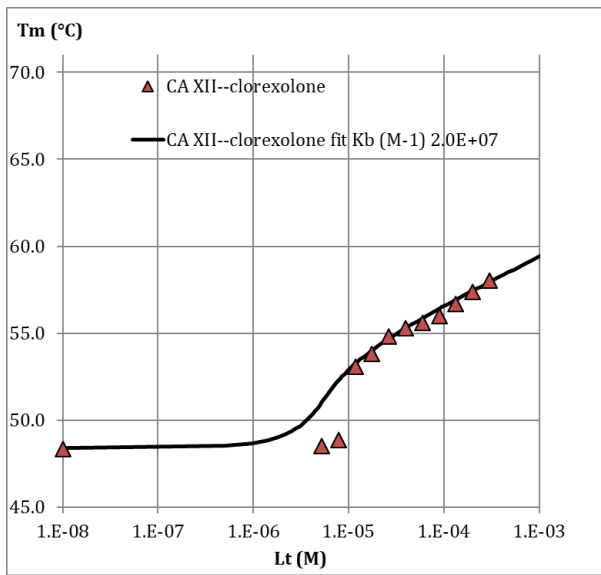
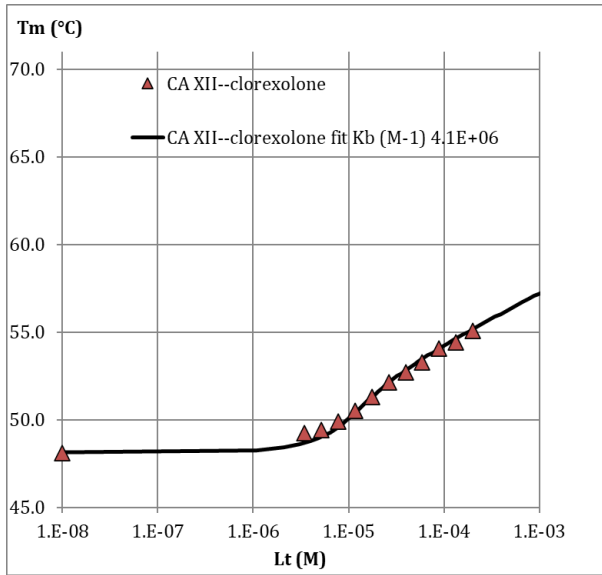
# 17. Clorexolone

## Fluorecence-based thermal shift assay



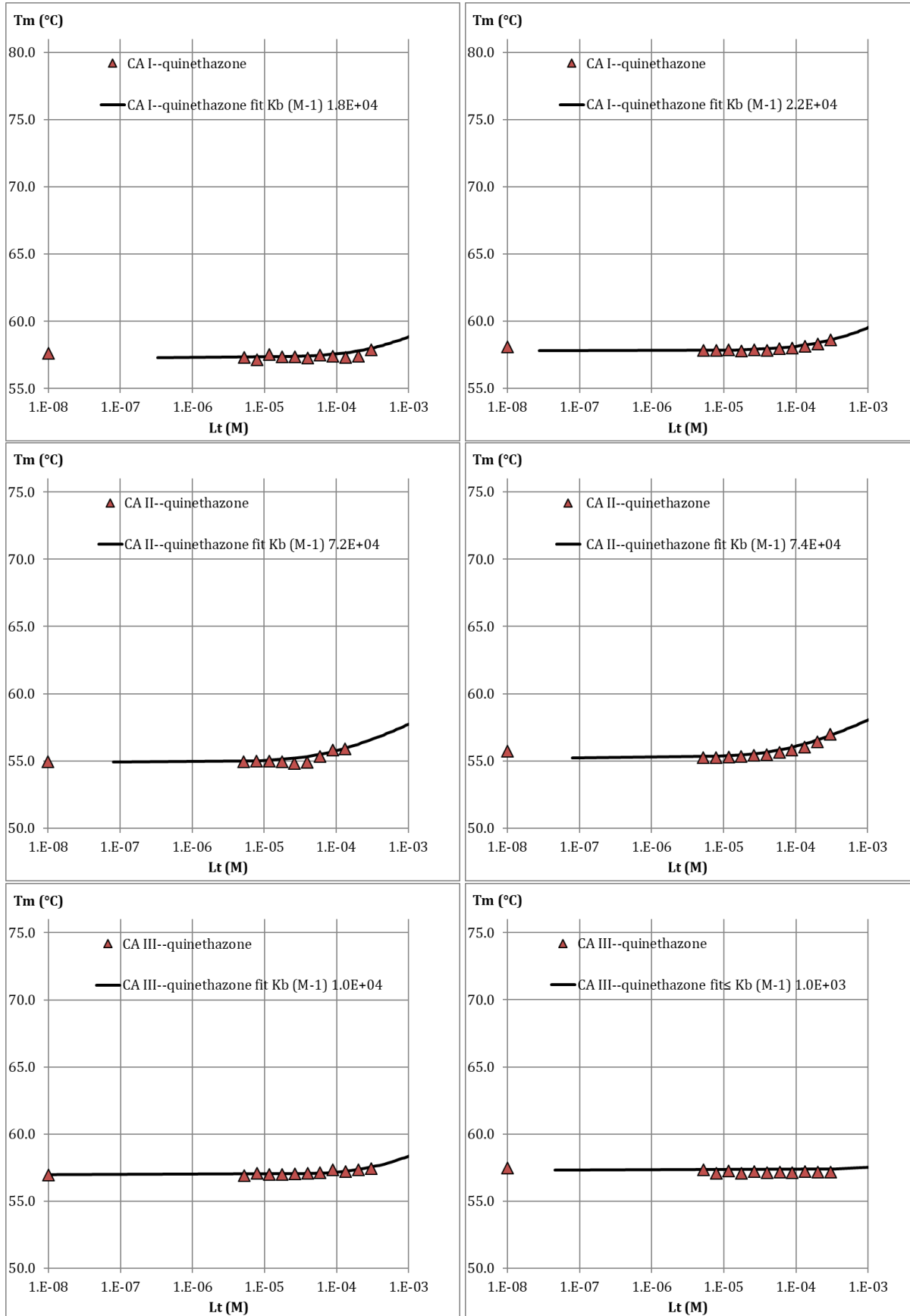




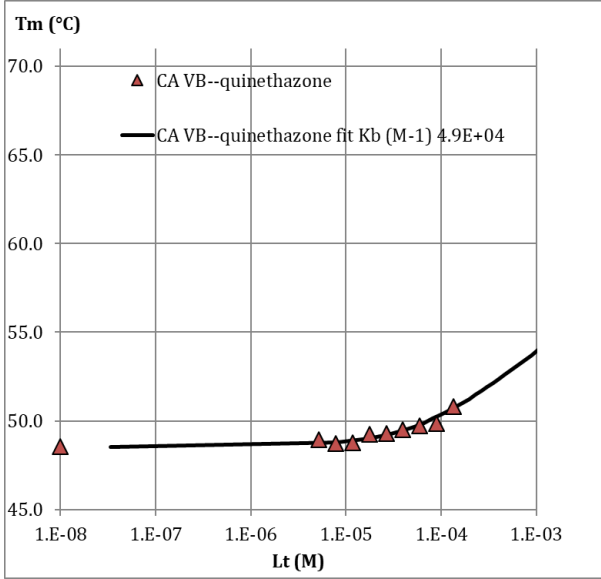
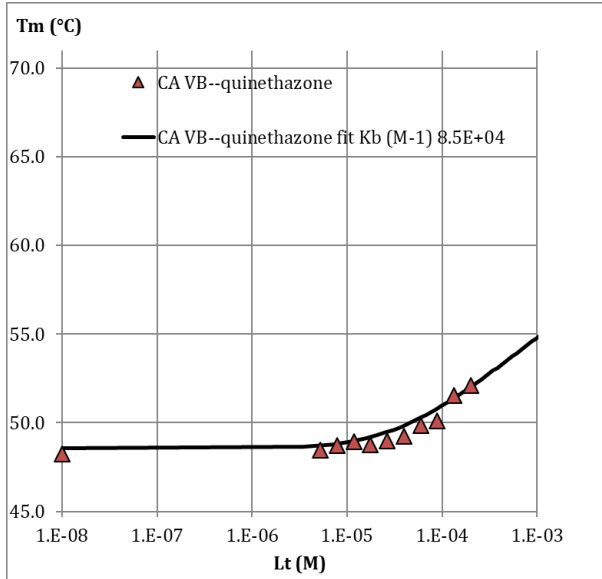
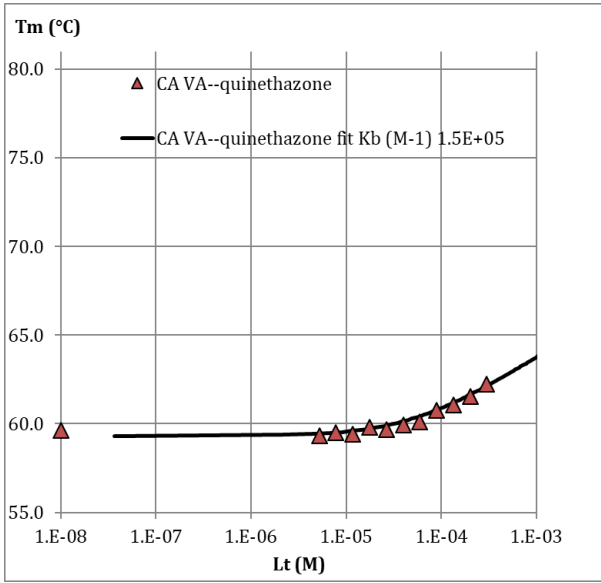
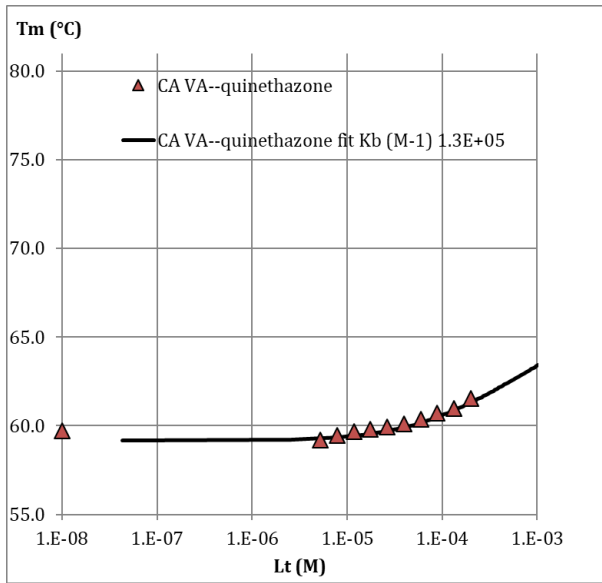
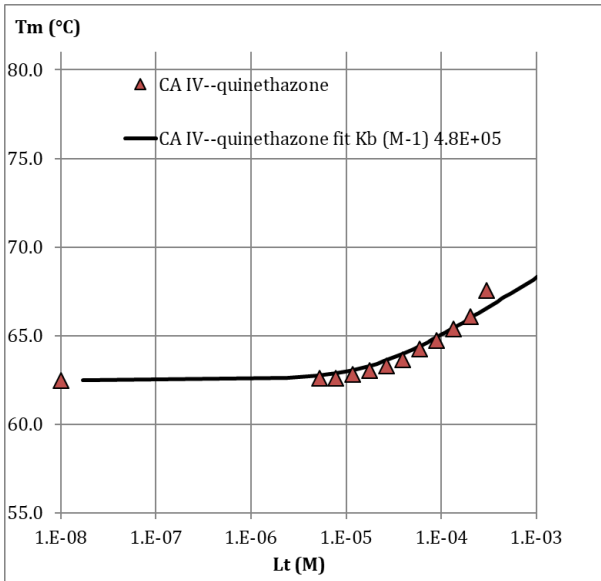
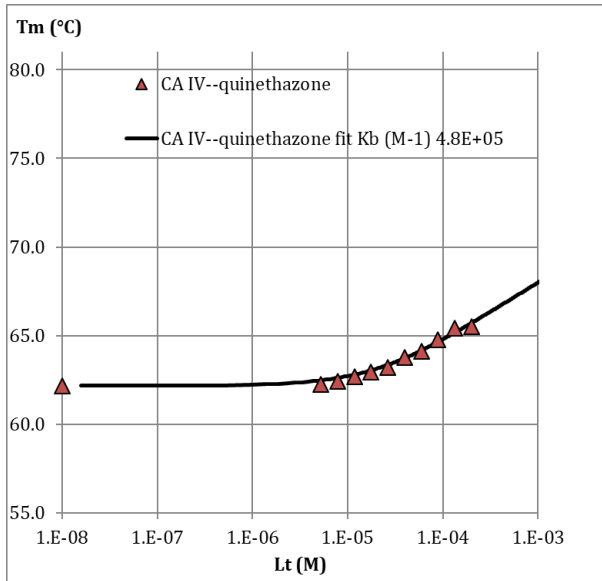


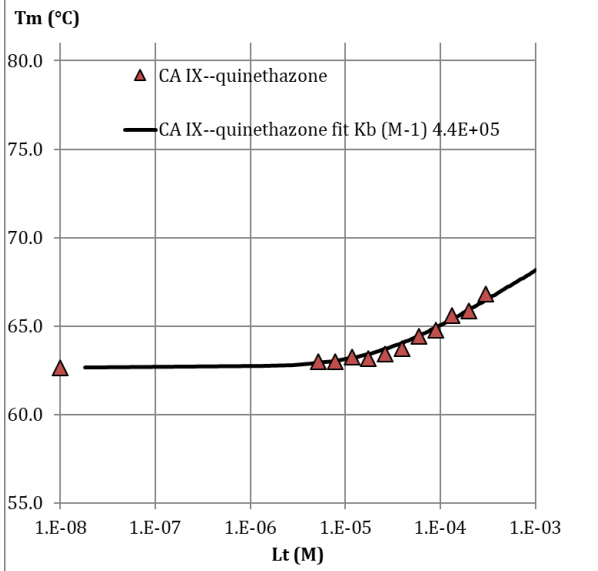
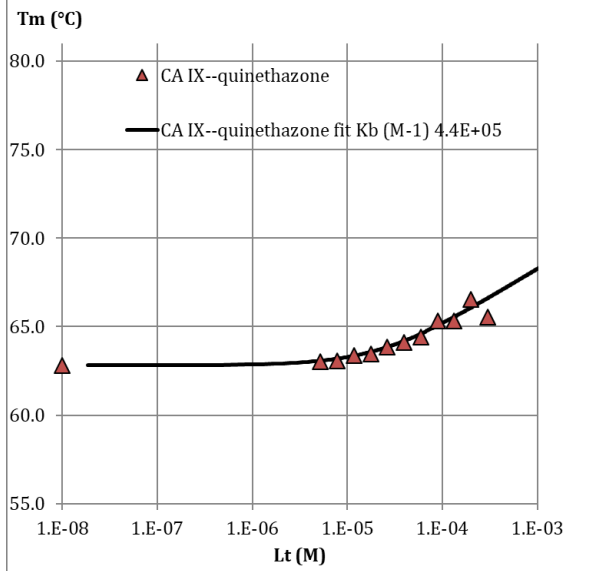
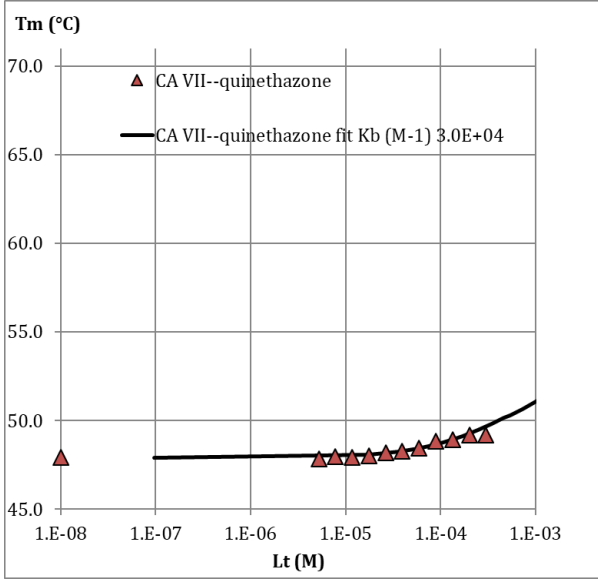
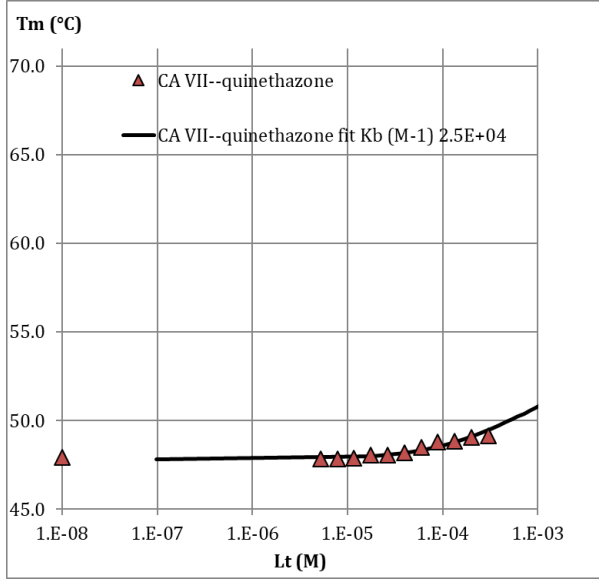
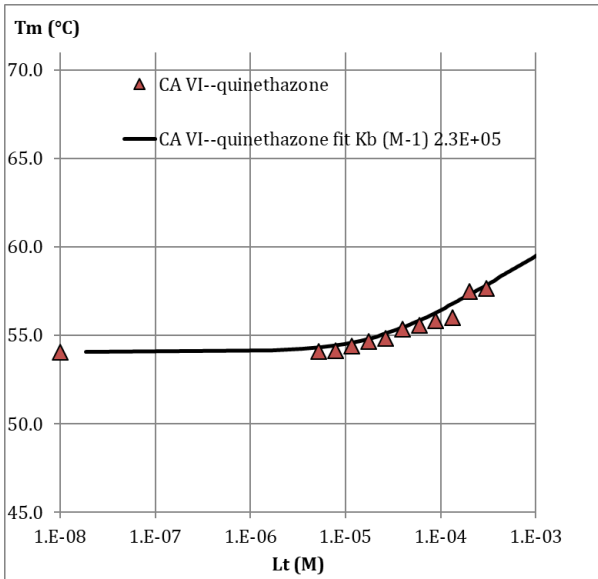
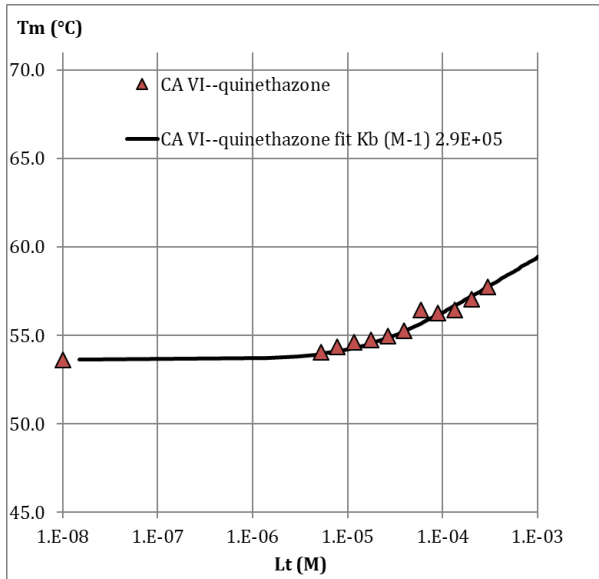
# 18. Quinethazone

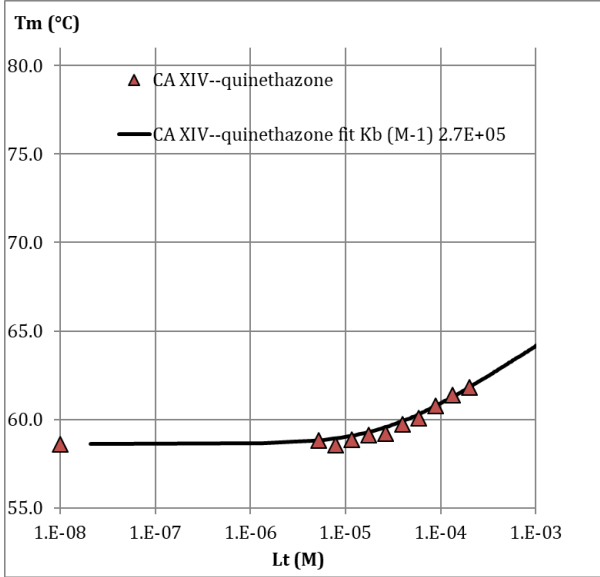
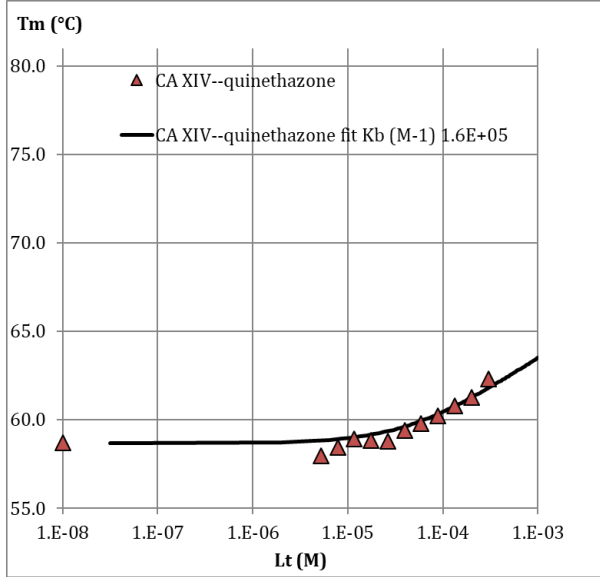
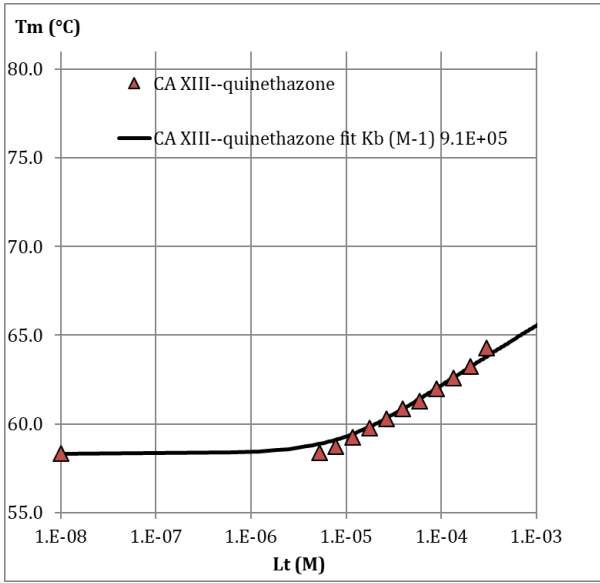
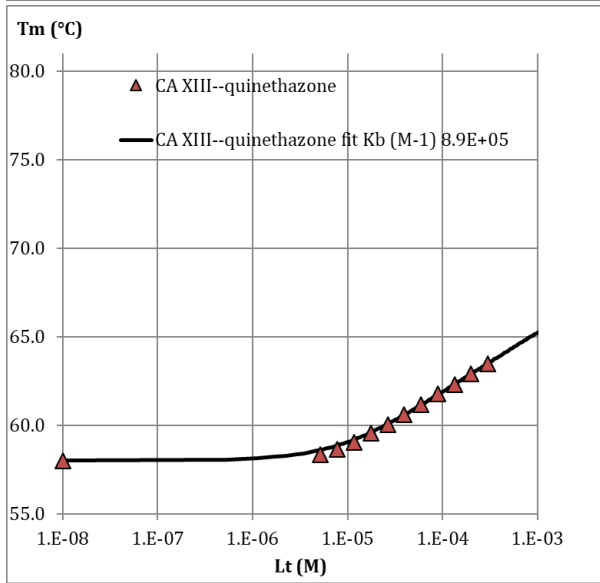
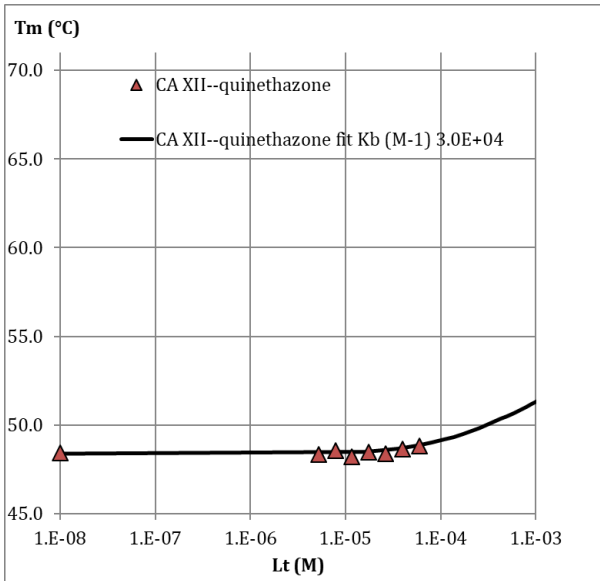
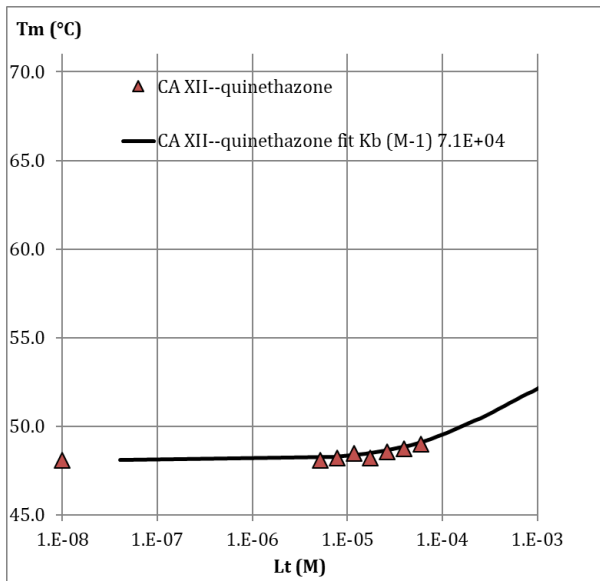
## Fluorecence-based thermal shift assay





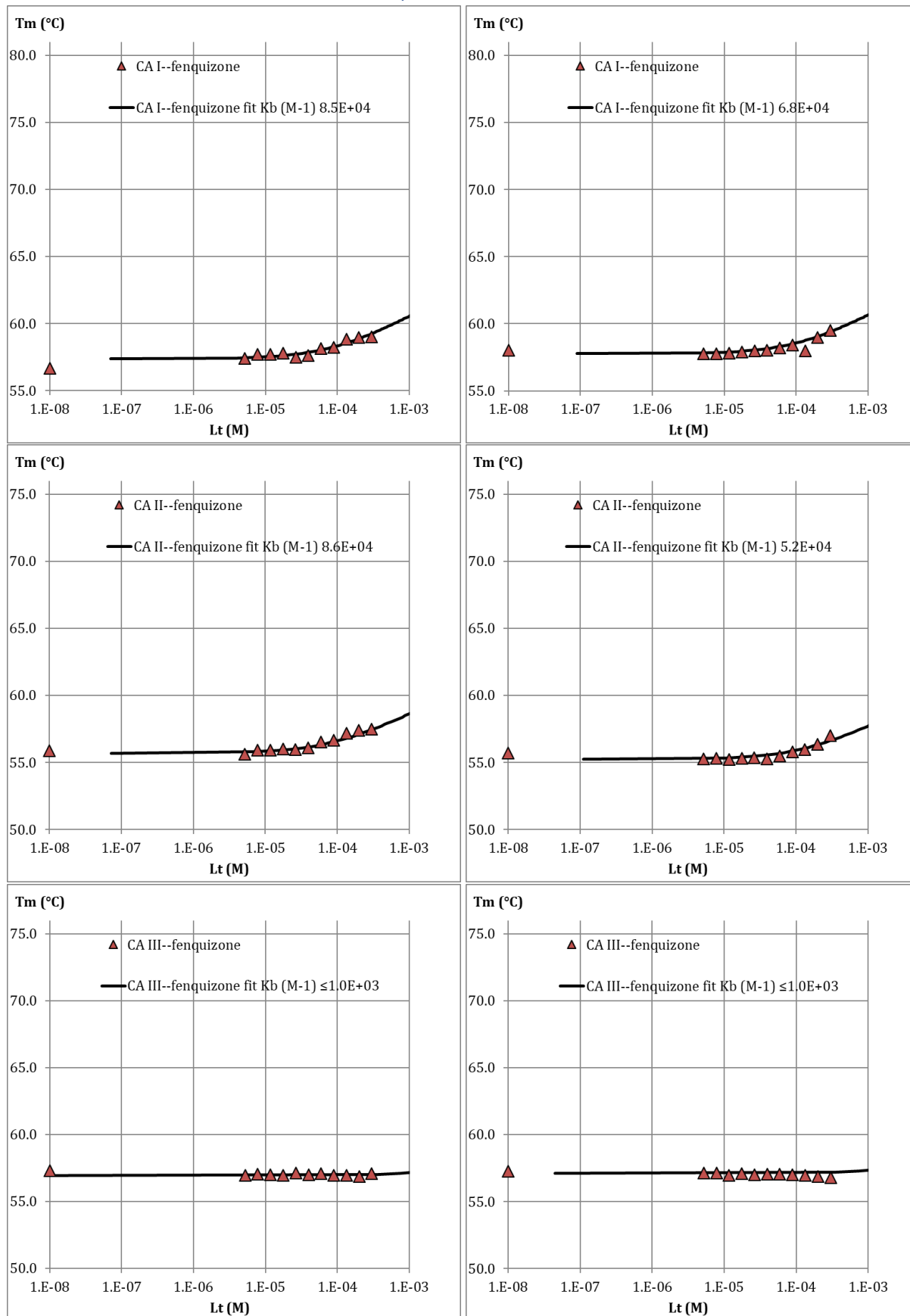


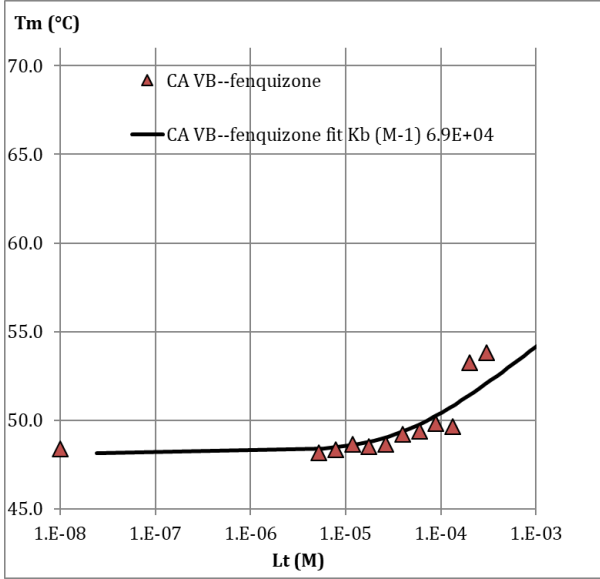
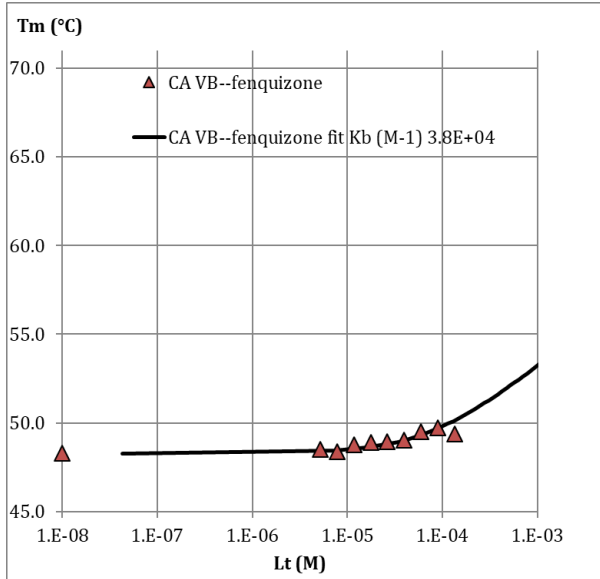
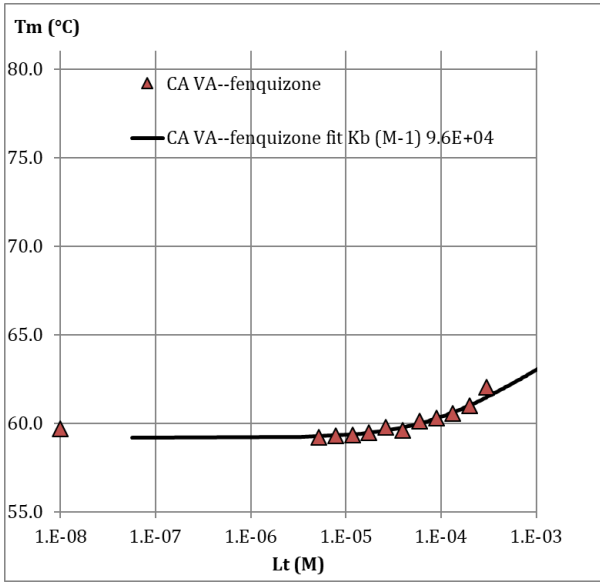
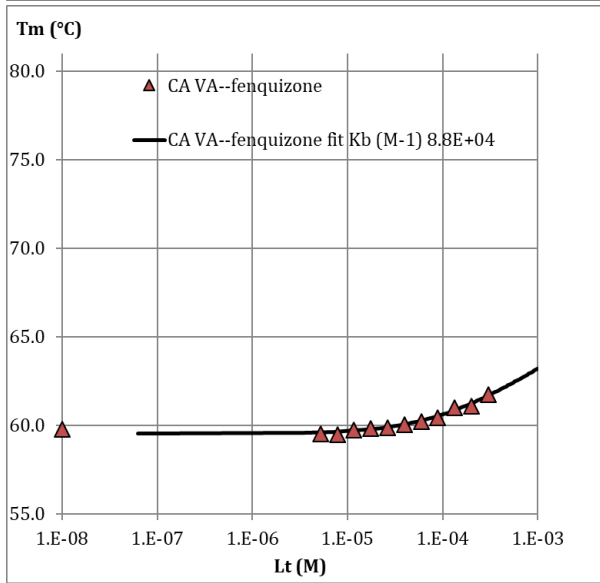
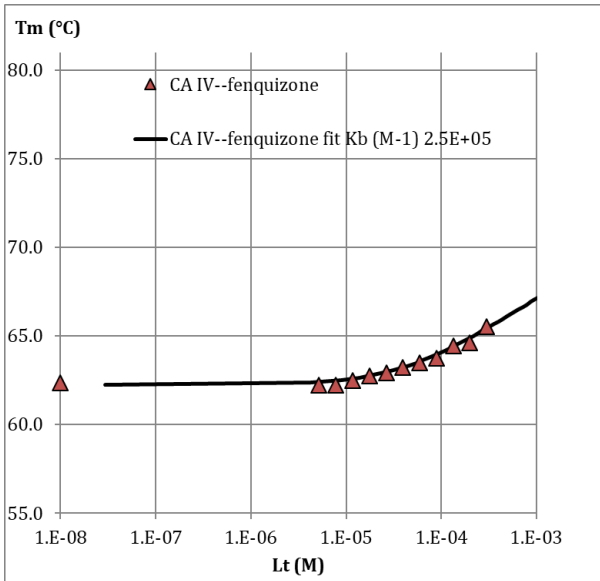
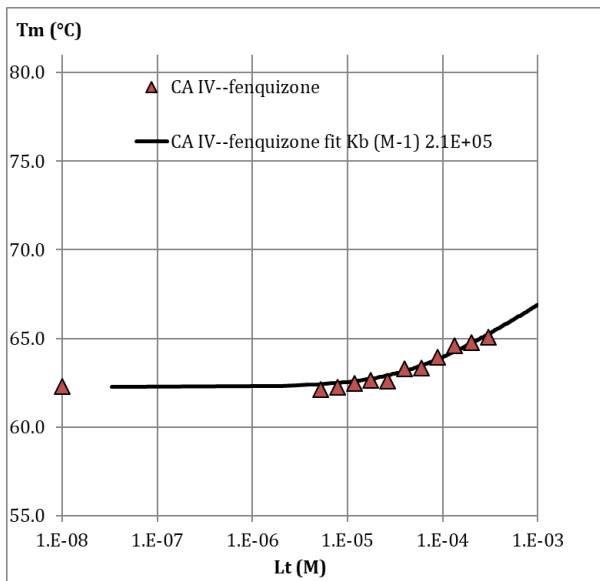


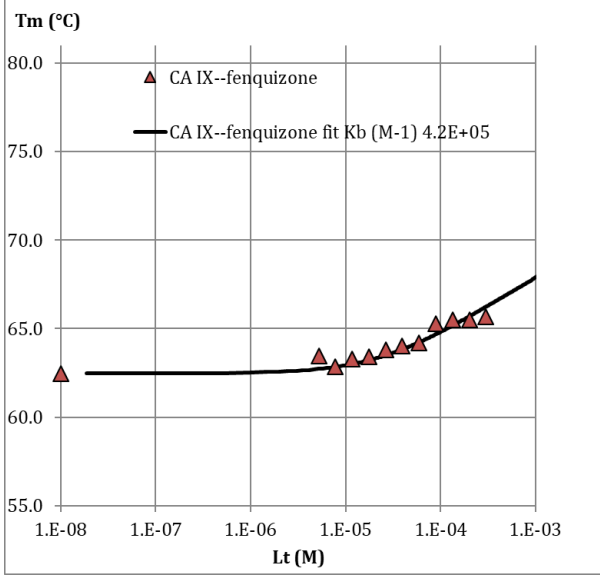
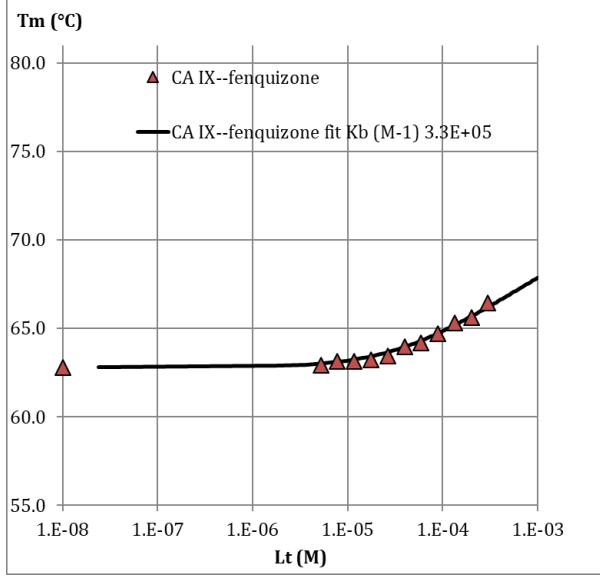
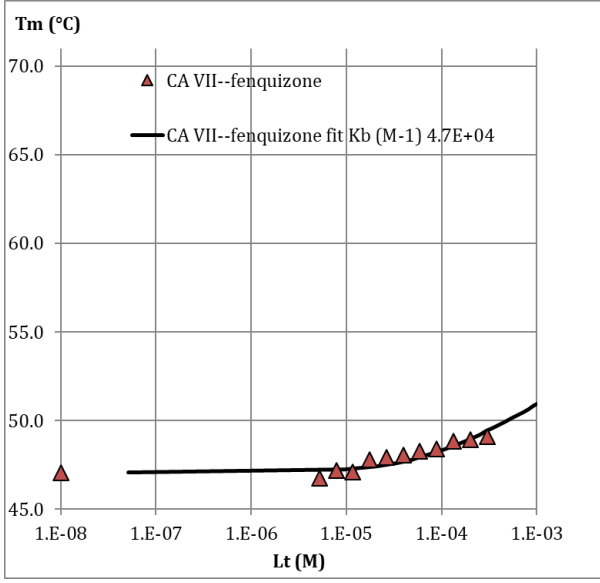
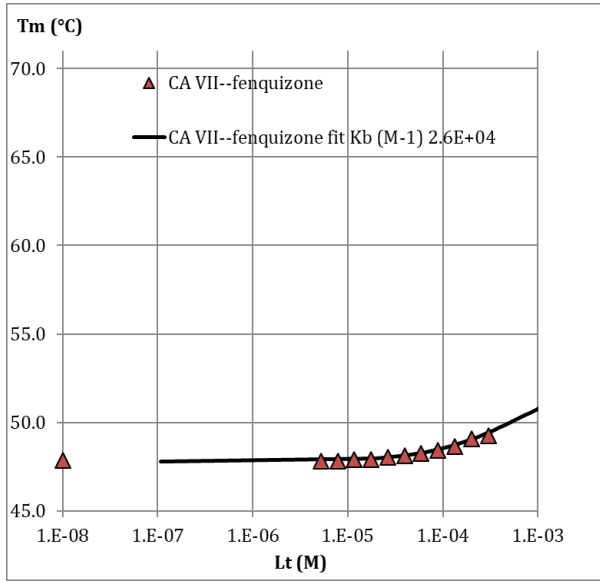
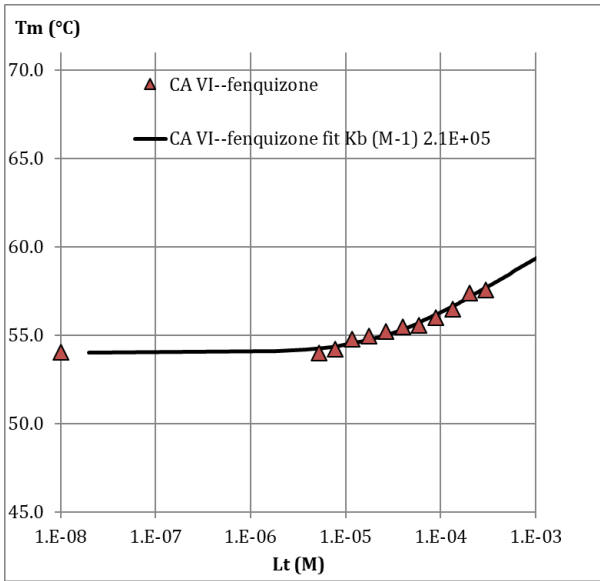
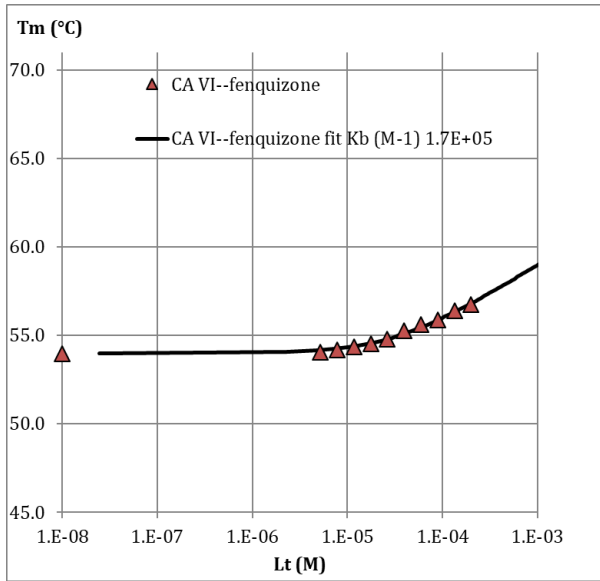


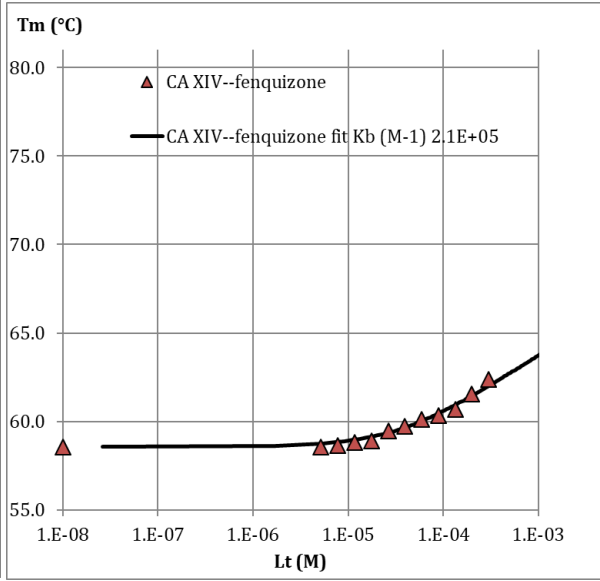
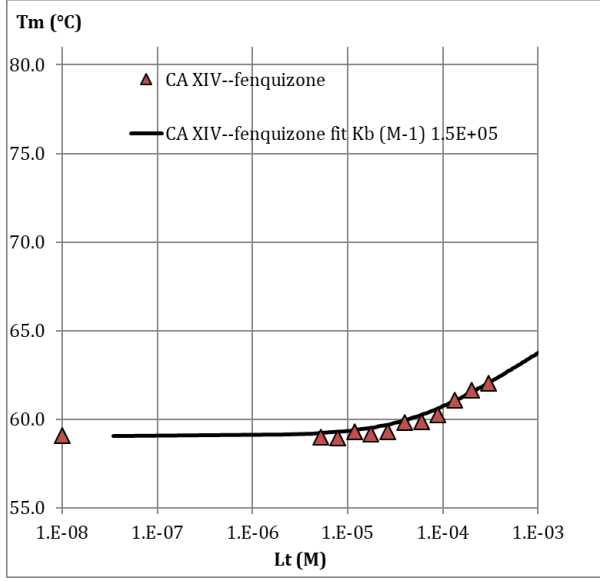
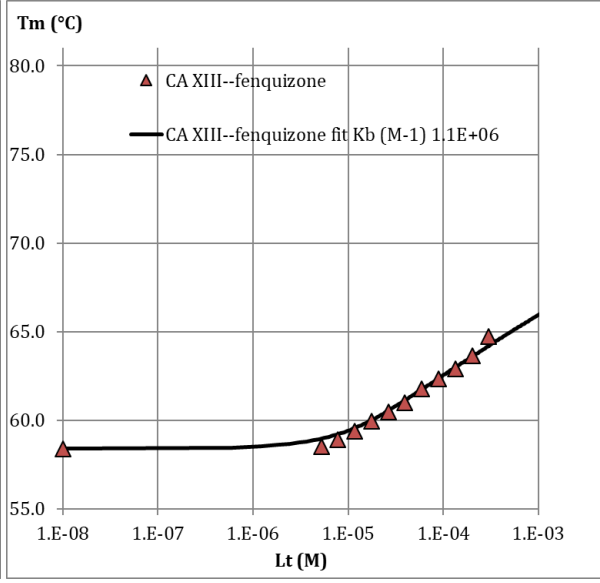
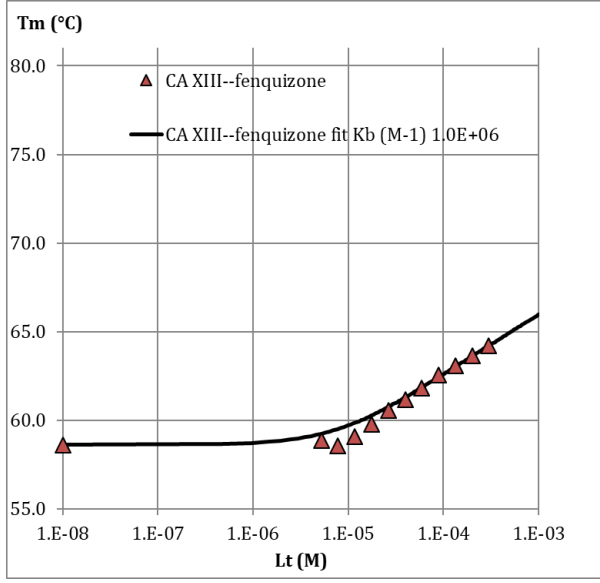
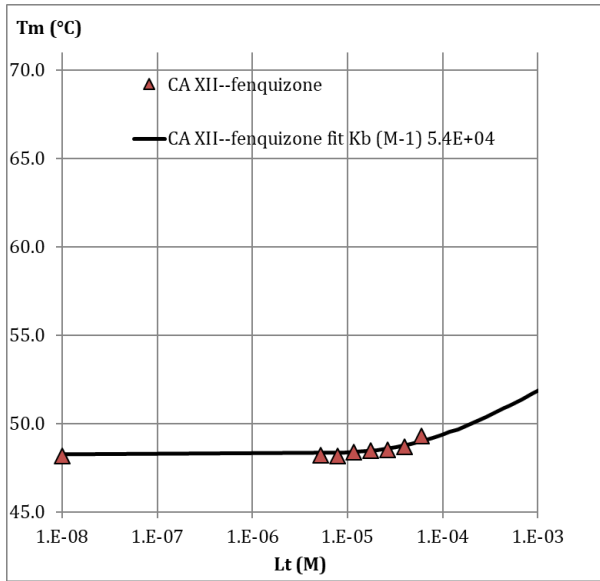
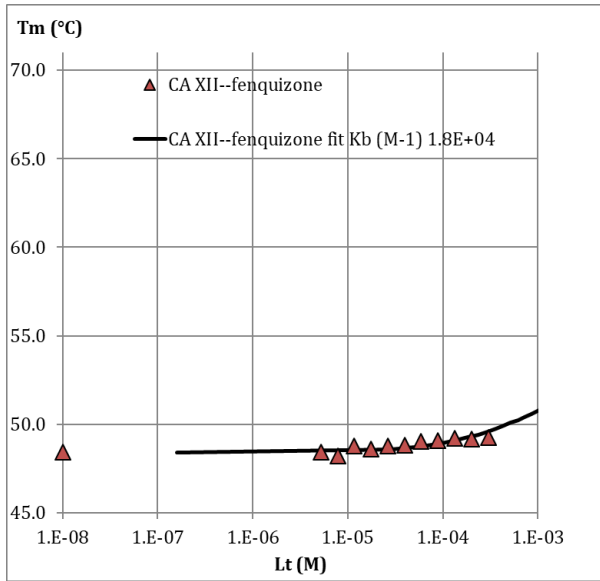
# 19. Fenquizone

## Fluorecence-based thermal shift assay



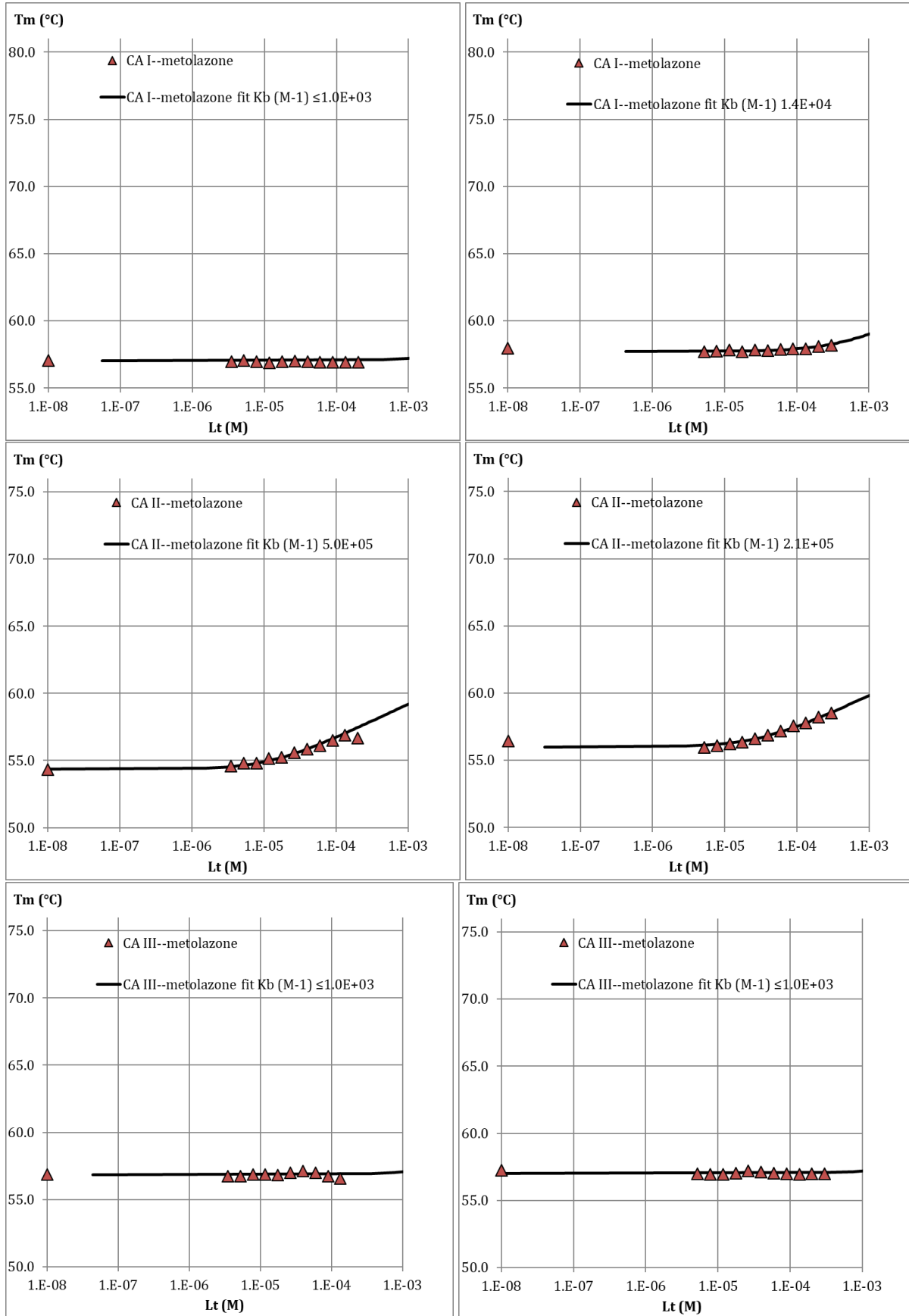




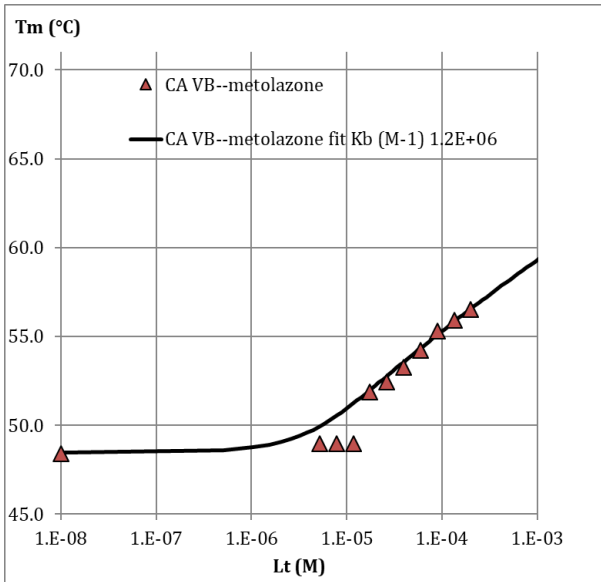
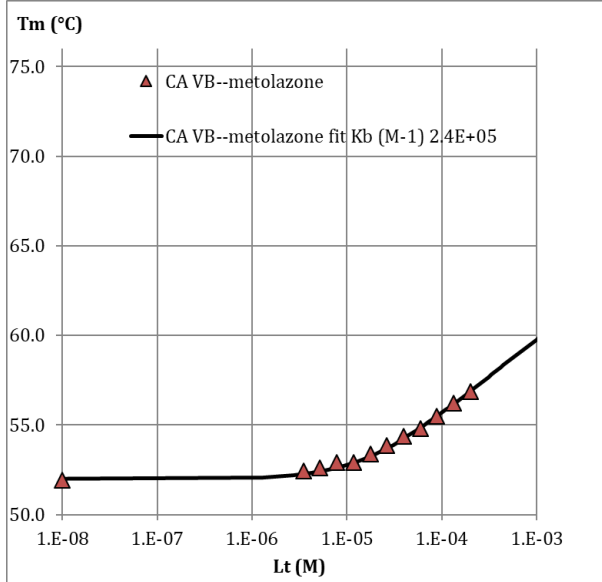
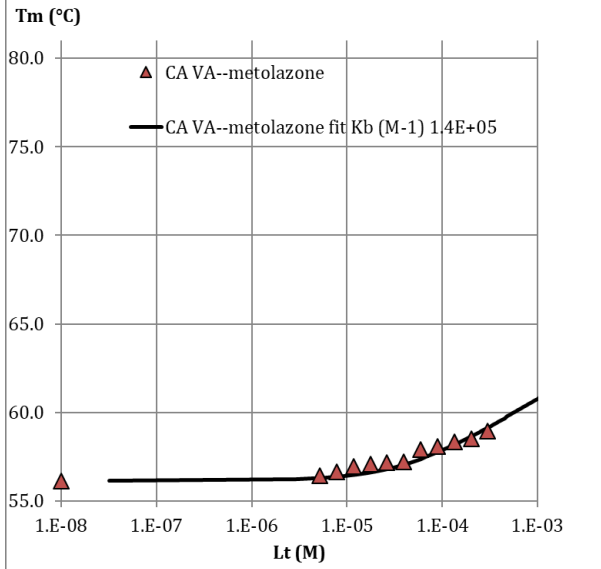
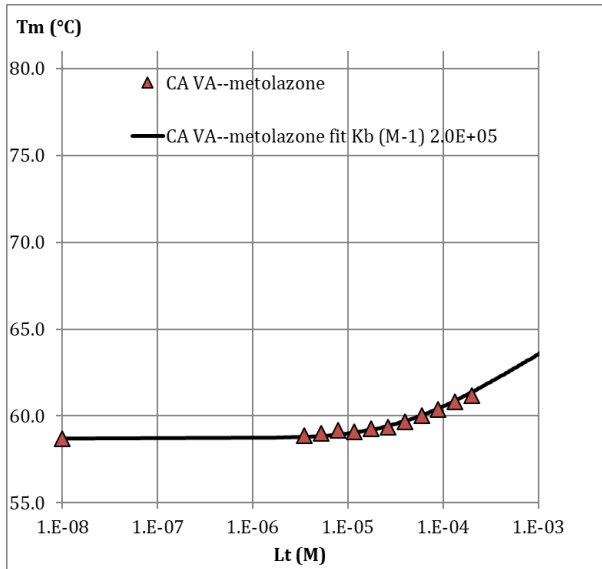
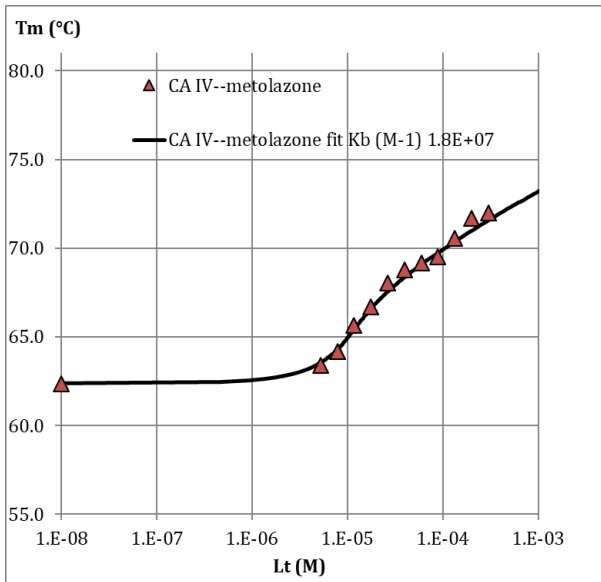
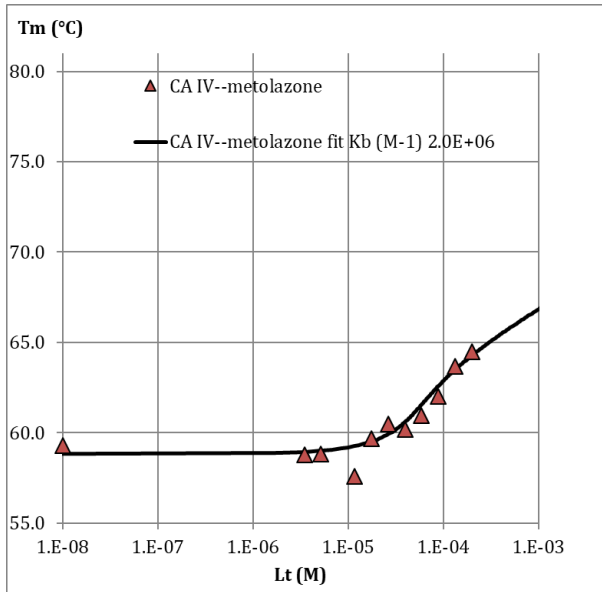


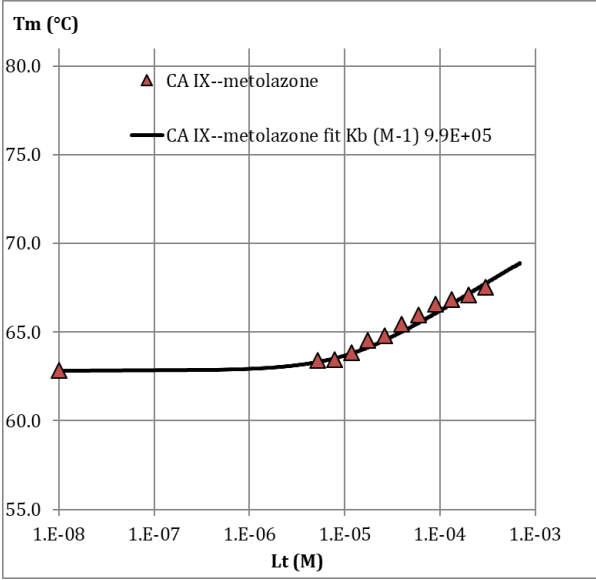
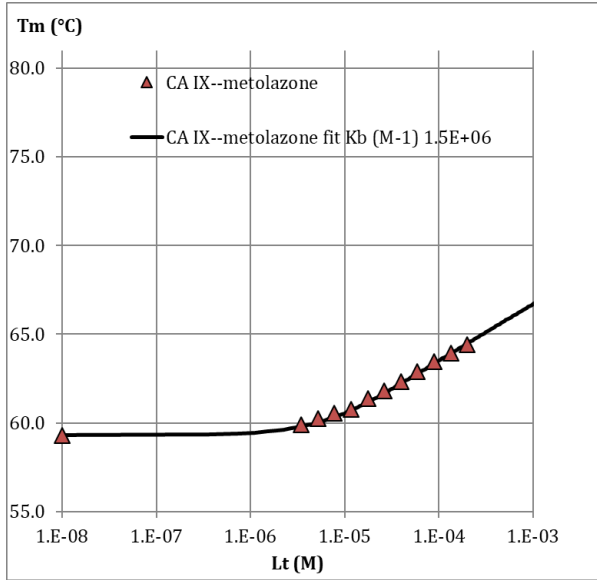
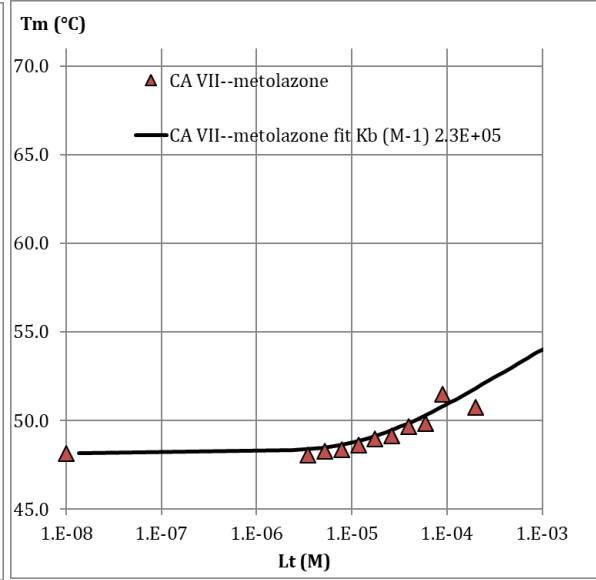
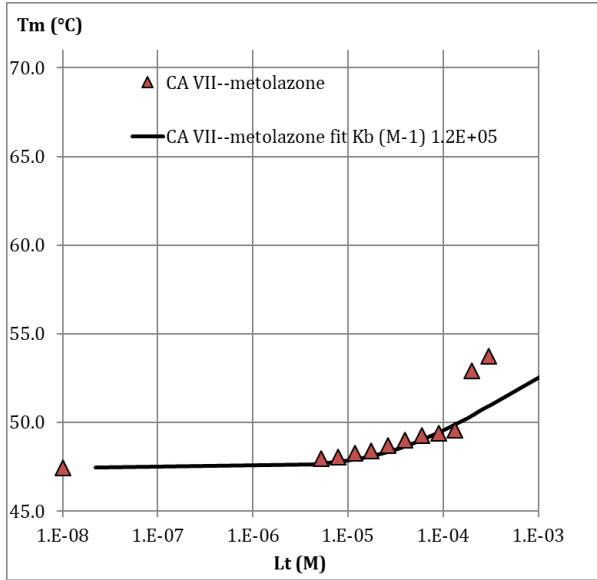
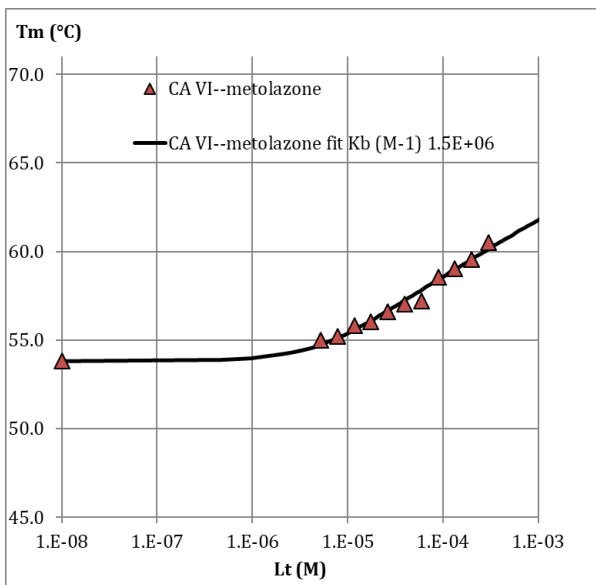
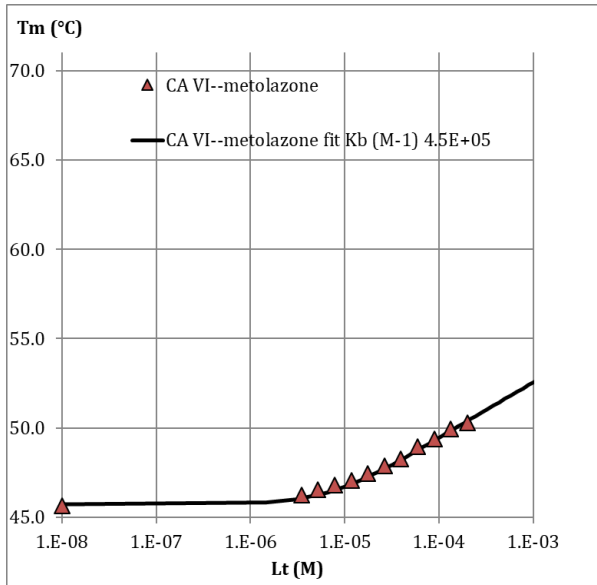
## 20. Metolazone

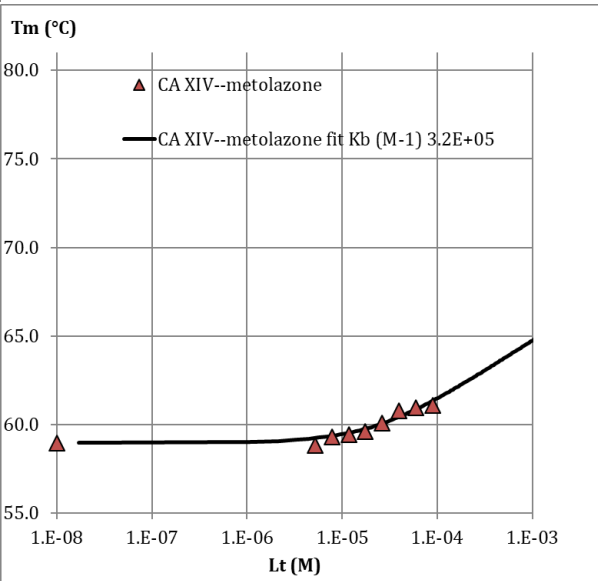
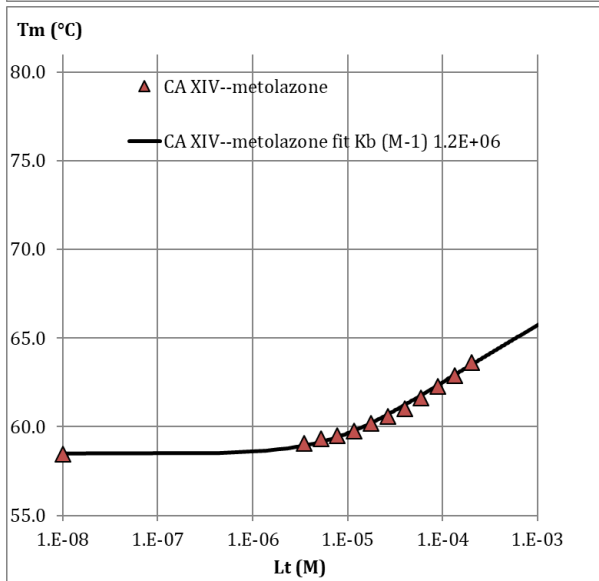
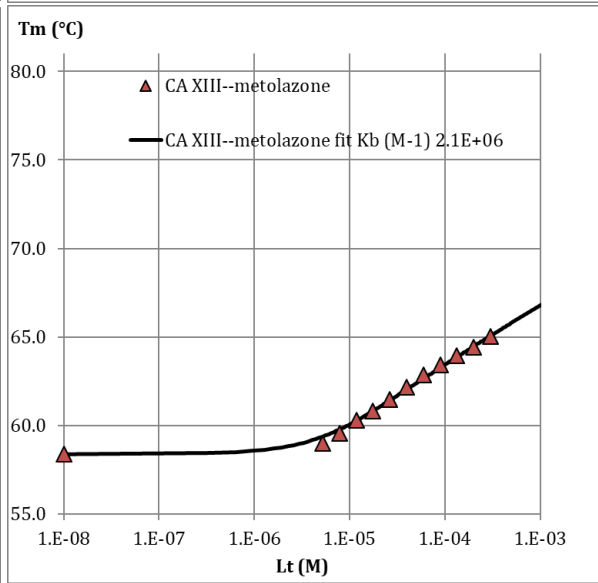
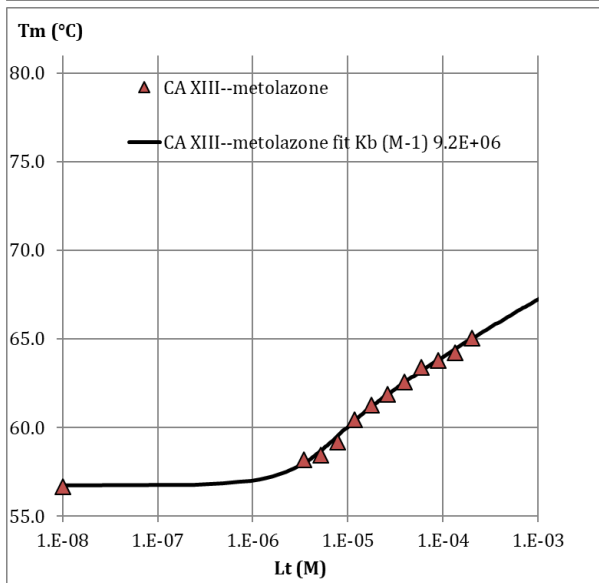
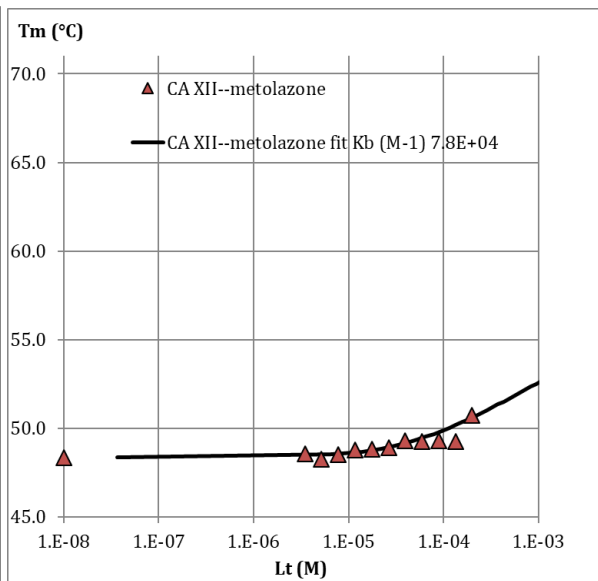
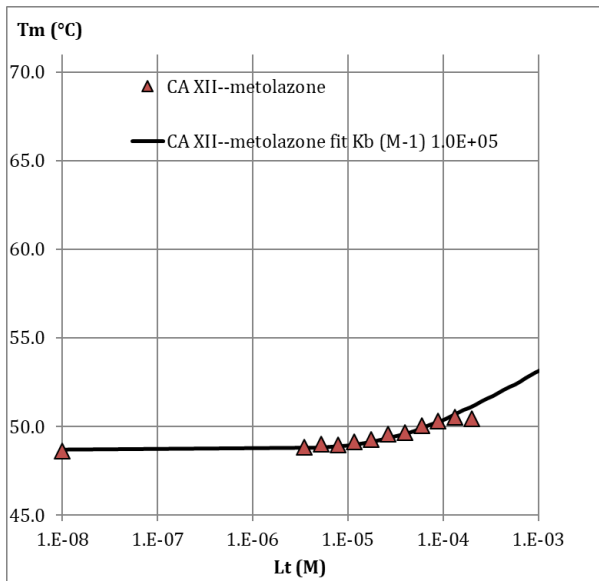
### Fluorecence-based thermal shift assay











**Note:**  $T_m$  variations for the same protein depend on exact protein concentration in the experiment and vary within approx.  $\pm 3$  degrees, except for CA VI – in this case, protein preparations produced in bacterial (less stable) or eucaryotic (more stable) expression systems were used [1].

## Equations used to fit the fluorescence-based thermal shift assay (FTSA) data

Equations used to fit the fluorescent thermal shift assay (FTSA) data are provided in this section describe the model for ligand-induced protein stabilization against thermal denaturation. Fluorescence yield,  $f$ , upon protein unfolding is

$$f = f_N X_N + f_U X_U, \quad (1)$$

where  $f_N$  and  $X_N$  are the fluorescence yield and fraction of the native form of a protein,  $f_U$  and  $X_U$  are those of the unfolded form. We consider native (N) and unfolded (U) states of a protein. The molar fractions of native and unfolded protein

$$X_N = \frac{[N]}{[N] + [U]}, \quad (2)$$

$$X_U = \frac{[U]}{[N] + [U]} \quad (3)$$

allow to obtain the following equation of fluorescence yield:

$$f = f_N + \frac{f_U - f_N}{1 + \frac{[N]}{[U]}}. \quad (4)$$

The fluorescence yields in the native and unfolded states are usually temperature-dependent and can be approximated by linear equations:

$$f_N = f_{N,T_m} + m_N(T - T_m), \quad (5)$$

$$f_U = f_{U,T_m} + m_U(T - T_m), \quad (6)$$

where  $m_N$  and  $m_U$  are slopes in the pre- and post-transition states, while  $f_{N,T_m}$  and  $f_{U,T_m}$  are the levels of fluorescence yields of the native and unfolded protein states at the melting temperature,  $T_m$ . Substituting  $f_N$  and  $f_U$  terms in Equation (4) by their temperature dependencies (Equations (5) and (6)) we obtain the final equation for the fluorescence yield:

$$f = f_{N,T_m} + m_N(T - T_m) + \frac{f_{U,T_m} - f_{N,T_m} + (m_U - m_N)(T - T_m)}{1 + \frac{[N]}{[U]}}. \quad (7)$$

In the Fluorescent Thermal Shift Assay (FTSA), a protein stabilization is described by the shift in its melting temperature upon addition of a ligand. Mathematically this phenomenon requires the equation that relates the concentration of added ligand with the changes in the Gibbs energy, enthalpy, and entropy of protein unfolding and protein-ligand binding. To derive this relationship, we start with four equations:

$$[P]_t = [N] + [P \cdot L] + [U], \quad (8)$$

$$[L]_t = [L] + [P \cdot L], \quad (9)$$

$$K_U = \frac{[U]}{[N]}, \quad (10)$$

$$K_b = \frac{[P \cdot L]}{[N][L]}, \quad (11)$$

where  $K_U$  and  $K_b$  are equilibrium constants of protein unfolding and protein-ligand binding, respectively;  $[P \cdot L]$ ,  $[P]_t$  and  $[L]_t$  denotes the molar concentrations of protein-ligand complex, initial protein

and initial ligand, respectively. Unbound ligand molarity,  $[L]$ , in the Equation (9) can be substituted by  $[L] = \frac{[P \cdot L]}{K_b[N]}$  from Equation (11). We also make use of the relation  $[N] = \frac{[U]}{K_U}$  from Equation (10), which leads to

$$[L]_t = \frac{K_U[P \cdot L]}{K_b[U]} + [P \cdot L] = [P \cdot L] \left( \frac{K_U}{K_b[U]} + 1 \right). \quad (12)$$

Equations (8) and (10) are combined to obtain the molarity of protein-ligand complex

$$[P \cdot L] = [P]_t - [U] - \frac{[U]}{K_U}. \quad (13)$$

Writing the  $[P \cdot L]$  term into Equation (12) results in:

$$[L]_t = \left( [P]_t - [U] - \frac{[U]}{K_U} \right) \left( \frac{K_U}{K_b[U]} + 1 \right). \quad (14)$$

We describe the temperature dependencies of equilibrium constants  $K_U$  and  $K_b$  in terms of the standard Gibbs energies of protein unfolding,  $\Delta G_U^\circ$ , and protein–ligand binding,  $\Delta G_b^\circ$ :

$$K_U(T) = \exp\left(-\frac{\Delta G_U^\circ}{RT}\right), \quad (15)$$

$$K_b(T) = \exp\left(-\frac{\Delta G_b^\circ}{RT}\right), \quad (16)$$

where  $R$  is the molar gas constant. The change in Gibbs energy has two constituent parts: enthalpy ( $\Delta H$ ) and entropy ( $\Delta S$ ). Both terms are temperature-dependent and their standard values at a particular temperature,  $T$ , can be calculated using equations

$$\Delta H_x^\circ = \Delta H_{x,T_r}^\circ + \int_{T_r}^T \Delta C_{p,x} dT, \quad (17)$$

$$\Delta S_x^\circ = \Delta S_{x,T_r}^\circ + \int_{T_r}^T \left( \frac{\Delta C_{p,x}}{T} \right) dT, \quad (18)$$

where  $T_r$  is a reference (initial) temperature,  $\Delta H_{x,T_r}^\circ$  and  $\Delta S_{x,T_r}^\circ$  – standard enthalpy and entropy at  $T_r$ ,  $\Delta C_{p,x}$  – the change in constant pressure heat capacity. The subscript  $x$  in Equations (17) and (18) stands for either unfolding or binding process. If  $\Delta C_p$  is constant in the temperature range from  $T_r$  to  $T_m$ , we can integrate Equations (17) and (18) and the resulting Gibbs energy is

$$\begin{aligned} \Delta G_x^\circ &= \Delta H_x^\circ - T_m \Delta S_x^\circ = \\ &= \Delta H_{x,T_r}^\circ + \Delta C_{p,x}(T_m - T_r) - T_m \left( \Delta S_{x,T_r}^\circ + \Delta C_{p,x} \ln \frac{T_m}{T_r} \right). \end{aligned} \quad (19)$$

Folded and unfolded fractions of a protein are equal at melting temperature ( $[U] = \frac{[P]_t}{2}$  at  $T_m$ ), thus Equation (14) simplifies, and together with Equations (15) and (16) provides the mathematical background of the FTSA:

$$\left\{ \begin{array}{l} [L]_t = (K_U - 1) \left( \frac{1}{K_b} + \frac{[P]_t}{2K_U} \right), \\ K_U = \exp \left( -\frac{\Delta H_{U,T_r}^\circ + \Delta C_{p,U}(T_m - T_r) - T_m \left( \Delta S_{U,T_r}^\circ + \Delta C_{p,U} \ln \frac{T_m}{T_r} \right)}{RT_m} \right), \\ K_b = \exp \left( -\frac{\Delta H_{b,T_0}^\circ + \Delta C_{p,b}(T_m - T_0) - T_m \left( \Delta S_{b,T_0}^\circ + \Delta C_{p,b} \ln \frac{T_m}{T_0} \right)}{RT_m} \right). \end{array} \right. \quad (20)$$

## Equation used to fit stopped-flow enzymatic inhibition assay (SFA) data

This equation is also known as Morrison's equation [2,3] or Greco & Hakala equation [4,5], which is used when enzyme concentration in the assay is comparable with inhibition constant, as is in the case of carbonic anhydrase inhibition:

$$CA \text{ activity} = 1 - \frac{([CA] + [I] + K_d) - \sqrt{([CA] + [I] + K_d)^2 - 4[CA][I]}}{2[CA]}$$

Where [CA] is the total concentration of CA, [I] is the total concentration of added inhibitor, and  $K_d$  is the apparent inhibition constant, which is equal to the protein – ligand dissociation constant in 1:1 binding model.

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