Evaluation of co-solvent fraction, pressure and temperature effects in analytical and preparative supercritical fluid chromatography

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Abstract

A chemometric approach is used for studying the combined effect of temperature, pressure and co-solvent fraction in analytical and preparative supercritical fluid chromatography (SFC). More specifically, by utilizing design of experiments coupled with careful measurements of the experimental conditions the interaction between pressure, temperature and co-solvent fraction was studied with respect to productivity, selectivity and retention in chiral SFC. A tris-(3,5-dimethylphenyl) carbamoyl cellulose stationary phase with carbon dioxide/methanol as mobile phase and the two racemic analytes trans-stilbene oxide (TSO) and 1,1'-bi-2-naphthol (BINOL) were investigated. It was found for the investigated model system that the co-solvent fraction and pressure were the parameters that most affected the retention factors and that the co-solvent fraction and column temperature were most important for controlling the selectivity. The productivity in the preparative mode of SFC was most influenced by the co-solvent fraction and temperature. Both high co-solvent fraction and temperature gave maximum productivity in the studied design space.

Keywords: Supercritical Fluid Chromatography; Methanol; Temperature; Pressure; Design of experiments

1 Introduction

Supercritical fluid chromatography (SFC) is continuing to gain momentum and has undoubtedly established itself as an important chromatographic technique, especially for preparative chiral separations [1,2]. The use of low viscosity carbon dioxide as the main solvent enables operation at higher flow rates compared to LC and the low toxicity of the mobile phase and its ease of recycling have created another incentive for SFC.

The type of stationary phase chemistry is the most important factor governing the retention and selectivity in SFC [3–9]. The mobile phase composition used in combination with CO_2 also influences the retention and selectivity [3,4,10]. The stationary phase and the mobile phase composition are often selected early in the method development. In the later stage, operating parameters such as the co-solvent fraction, pressure and temperature are used to optimize the separation.

In most cases, retention of polar solutes in SFC is more sensitive to the co-solvent fraction in the mobile phase compared to LC, making co-solvent fraction the most important operating parameter [11–16] after a stationary phase has been selected. The temperature can affect the retention and selectivity in different ways [2,3,13,17]. By changing the temperature, the density and hence the elution strength of the mobile phase is changed, but the temperature also has the same effect as in LC, i.e. shortening the retention time. Density variations due to temperature are more pronounced at low co-solvent fractions and LC like temperature effect dominates at high co-solvent fractions [13]. The pressure mostly affects the retention of the solutes while the selectivity is mostly independent of pressure [11,18].

However, the combined effect of simultaneously changing two or more of these parameters is not well known for two reasons: (i) the majority of the work found in the literature is concerned with the study of one parameter at a time while the others are kept constant [12,13,18–21] and (ii) the actual values of these parameters are almost always unknown because of a lack of pressure and temperature data inside the column, which is the only relevant data [22–24]. If there is a large pressure drop over the column, for example due to a high volumetric flow rate, there could be significant gradients of pressure, temperature and density along the column which cannot be ignored when modeling the system [25]. Failing to recognize such gradients in SFC may lead to unpredictable method transfer, both between analytical instruments, when changing volumetric flow, connecting capillaries, particle size, column dimensions and when scaling up separations to preparative scale [26].

The aim of this study is to investigate the combined effect of the operating parameters co-solvent fraction, pressure and temperature on the separation of two racemic compounds on a fixed chiral stationary phase, not necessarily explain them. To study the interacting effect of these parameters and quantify each parameter's contribution, a chemometric design of experiments (DOE) approach is used. External sensors of mass flow, pressure and temperature are used throughout the study to ensure that near isocratic, isobaric and isothermal conditions are present. Using additional sensors could also give valuable insight into when such sensors might be unnecessary, which could improve the usability of SFC in general. We have previously employed this approach together with adsorption isotherm determination when studying the adsorption behavior of 1-phenyl-1-propanol in pure CO_2 and at very low fractions of methanol [16].

2 Experimental

2.1 Chemicals

HPLC grade methanol (Fischer Scientific, Loughborough, UK) and CO_2 (> 99.99%, AGA Gas AB, Sweden) were used as mobile phase. (±)-1,1'-Bi-2-naphthol "BINOL" (99%), (±)-trans-stilbene oxide "TSO" (98%) and 1,3,5-tri-tert-butyl-benzene "TTBB" (97%) purchased from Sigma-Aldrich (St. Louis, MO, USA) were used as solutes. All solutes were dissolved in methanol and filtered through a 0.45 µm PTFE syringe filter prior to injection. Nitrous oxide (99.998%), also from Sigma-Aldrich, bubbled through methanol for 2 minutes before injection, was used to measure the column hold-up volume. The nitrous oxide was detected at 192 and 200 nm at 2.5, 5 and 7.5 v% methanol. The column was a Kromasil Cellucoat 100 × 4.6 mm column (AkzoNobel Eka, Bohus, Sweden), with average particle diameter equal to 5 µm.

2.2 Instrumentation

The experiments were performed with a Waters UPC^2 system (Waters Corporation, Milford, MA, USA) equipped with a 50 µL loop. The column inlet and outlet temperatures were measured with two PT-100 4-wire resistance temperature detectors with an accuracy of ± 0.2°C (Pentronic AB, Gunnebo, Sweden). The temperature detectors were attached 1.5 cm from the actual column inlet and outlet, respectively, with a thermal adhesive from Arctic Silver Inc. (Visalia, CA, USA). The inlet and outlet pressures were measured using two absolute pressure transmitters of model EJX530A (Yokogawa Electric Corporation, Tokyo, Japan), respectively, each with an accuracy of ± 1 bar. Total and methanol mass flow were measured using two Bronkhorst mini CORI-FLOW model M12 Coriolis flow meters (Bronkhorst High-Tech B.V., Ruurlo, Netherlands) with an accuracy of ± 0.2% of the mass flow. Pressure, temperature and mass flow were continuously recorded during all experiments; see Appendix A for additional information.

2.3 Selection of factors and design space

A full factorial design in three levels with two center points was selected because; (i) the purpose of the chemometric modelling was modelling of the responses in the design region, (ii) the retention factor has a quadratic relationship with the methanol fraction [12–15,24] which leads to the need for a design that can fit quadratic terms, and (iii) interaction terms are expected.

When defining the design space, both physical restrictions such as instrument constraints and chromatographic limitations such as reasonable retention times must be taken into account. In this work, the column limited the maximum allowed temperature to 40°C, and a temperature range of 24-36°C was therefore chosen. The pressure range 120-200 bar (specified with the back pressure regulator) was determined to cover the common operation pressures [4] and last the methanol fraction was determined so that the retention factor for both compounds for all experimental conditions were between 1.5 to 8.

2.4 Experiments

The backpressure regulator was set to 120, 160 and 200 bar; the column oven was set to 24, 30 and 36°C while the methanol content was set at 2.5, 5 and 7.5%, v/v for TSO and 15, 20 and 25% v/v for BINOL. The column void volume was studied at 160 bars and 30°C by injecting 2 µL of methanol through which N_2O had been bubbled, only methanol or TTBB. The flow rate was set to 0.7 mL/min in all experiments. Analytical injection were performed by injecting 2 µL of a 0.1 g/L solution of BINOL or TSO while overloaded injections were done by injection of 12, 14, 16, 18, 20 and 22 µL of a 40 g/L solution of TSO. All injections were at least done in duplicates.

2.4 Data analysis

The regression analysis was performed with MODDE 7 (Umetrics AB, Sweden). The factors were orthogonally scaled prior to the regression. The measured values of temperature, pressure and methanol fraction were used in the modelling, not the set ones. The responses were retention factor, selectivity and productivity, where retention factor and selectivity was log transformed. Through empirical observations a logarithmic relationship has been found between the retention factor and temperature/co-solvent fraction [12–15,24,27]. All experimental data was corrected for the system void volume from the auto sampler to the detector. The retention factor was calculated with the column void time measured with N_2O and corrected for the variations in volumetric flow rate due to different operating conditions (see Sec. 3.2).

The productivity is defined as [28];

$$P_r = \frac{V_{inj} C^0 Y}{\Delta t_c} \qquad (1)$$

where V_{inj} is the injection volume, C^0 is the sample concentration, Y is the recovery yield and Δt_c is the cycle time. The productivity was studied only for TSO, because BINOL had too low selectivity to give valuable insight, and the sample concentration was chosen as the maximum solubility of TSO in methanol. The injection volume was chosen so that "touching bands" were achieved, i.e. $Y \approx 1$. This was done by choosing the injection volume that best satisfied this condition from the discrete set of experimental band profiles with different injection volumes.

The regression model for each response was evaluated with analysis of variance. The model was refined by first removing statistical outliers and then removing any statistically insignificant terms from the polynomial at a 95% confidence level. All regression models had excellent R^2 and Q^2 values which indicate that the model could explain all variations seen in the responses, see Appendix A for details.

3 Results and discussion

3.1 Measuring the system properties

What the effect of pressure, temperature and density gradients in the column is in SFC is not yet fully understood [2]. For example, when we increased the volumetric flow rate from 0.7 mL/min to 4.0 mL/min, the retention volume of TSO (at 5% methanol, set back pressure 160 bars, 30°C) increased 12%, i.e. the retention volume was dependent on the volumetric flow rate. At 0.7 mL/min the pressure drop over the column was 4 bar and at 4 mL/min the pressure drop was 51 bar, while the temperature gradient was negligible (<0.3°C).

In order to give a clear definition of the column pressure in the chemometric modeling and avoid any unforeseen complications due to pressure gradients, all experiments were performed at a flow rate that gave negligible pressure drop over the column. For all experiments, the measured pressure gradient was between 4-6 bar and the temperature gradient was between 0.0-0.3°C. For all the experiments, the calculated density gradient varied between 0.1-0.9%. All sensor traces for the BINOL experiments are presented in Appendix A.

The actual volumetric flow rate was calculated from the corresponding density of the mobile phase fluid according to ref. [24]. The deviation between the set and calculated volumetric flow rate was found to be varying between 1-15% from the set value. This observation alone motivates the use of additional sensors to control the input parameters in the analysis of variance.

3.2 The column void volume

The column void volume can be measured with static or dynamic methods. In LC, the preferred static method is pycnometry [29] and the most common dynamic method is an unretained marker [30]. There is limited information about unretained markers in SFC in general and chiral SFC in particular. In chiral, normal phase LC, TTBB is generally used us an unretained marker [31,32]. In SFC the first negative baseline disturbance from acetonitrile has been suggested as an unretained marker on ODS columns [33]. Following these results, the first negative baseline disturbance from methanol has been assumed to be unretained and give the apparent void volume for chiral stationary phases in SFC [9,13,18]. Guiochon et al. recently showed that methanol adsorb on both silica and ODS stationary phases at low (<5%) co-solvent fractions [34] while N_2O was shown to be unretained at low co-solvent fractions.

We conclude that there is insufficient experimental data in the literature to determine which void volume marker would be most appropriate in our case. So, pycnometry, TTBB, methanol and N_2O were compared as void markers for methanol fractions in the mobile phase ranging from 2.5 to 7.5 %. The calculated volumetric flow rate was used for each experimental condition. Due the low pressure drop over the column (4-6 bar) the density change inside the column was small and the calculated volumetric flow rate was close to the set one of 0.7 mL/min. Pycnometry was performed by filling the column first with pure methanol and then with pure CO_2 (i.e. the column was empty). This was done at atmospheric pressure in the same way as it is done in LC [29].

The result is presented in Fig. 1 and it can be seen that while the determination with methanol changed considerably with the co-solvent fraction in the mobile phase, the determination with N_2O and TTBB did not. The TTBB void volume was significantly retained, but only somewhat affected by the co-solvent fraction. Pycnometry gave a lower void volume than the others (dashed line), which previously has been observed in normal phase LC and SFC [29,34]. From these results we selected to use the average void volume obtained from N_2O. A more thorough study of void volume in SFC is beyond the scope of this investigation. For co-solvent fractions of more than 7.5%, the methanol and N_2O peaks co-elute, therefore the much smaller N_2O peak could not be discerned above this methanol fraction. The actual volumetric flow rate in the column depends on pressure, temperature and co-solvent fraction [2,23]. Therefore, the void *time* for each experiment was calculated from the constant void volume obtained from N_2O and the calculated volumetric flow rate specific for that experiment.

3.2 Response evaluation

The chromatograms for the center point and the extreme condition, i.e. lowest/highest pressure, temperature, methanol fraction, are shown in Fig. 2a for TSO and in Fig. 2b for BINOL. In Fig. 2c overloaded elution profiles of TSO are shown. For TSO the measured conditions corresponds to (low) 127 bar, 23.8°C, 2.33 v% (center) 168 bar, 29.6°C, 4.71 v% and (high) 208 bar, 35.2°C, 7.10 v%. In the case of BINOL the measured conditions were (low) 131 bar, 24.1°C, 14.7 v% (center) 172 bar, 29.5°C, 19.5 v% and (high) 213 bar, 35.2°C, 24.2 v%.

3.2.1 Retention factors

Before a successful method development can be performed in SFC it is essential to have knowledge about and to predict how the retention factor is affected by a certain change of the operating parameters. Centered and normalized coefficients for the regression models of the retention factor are presented in Fig. 3. The volumetric methanol fraction (C_M) was the most important parameter in the studied design space which is represented in the figure by the largest coefficient value for methanol. The methanol fraction also had a large quadratic term, meaning that the methanol dependence is not linear and here best described by a relationship of the type

$$\ln(k) = \ln(k_0) - SC_M + dC_M^2 \quad (2)$$

where S and d are constants. This relationship agrees with previous findings in both chiral [12,13] and achiral [14,15,24] SFC, where a quadratic relationship was needed to describe the logarithm of the retention factor as a function of the co-solvent fractions.

Pressure was the second most important factor in the studied design space and the retention factors all had similar pressure dependencies with a slight nonlinearity. In Fig. 3, it can also be seen that the interaction terms are all significant. These interaction terms would be missed if one factor at a time were varied.

Because the temperature range was rather small due to stationary phase limitations, the effect of temperature in the investigated region was low for both compounds; however, it was slightly larger for BINOL.

Fig. 4 contains contour plots obtained from the regression models illustrating the dependency of the retention factors on methanol fraction and pressure for TSO and BINOL, respectively, at the fixated column temperature 30°C. It is evident that the retention factors for both solutes have similar trends when studied in the methanol-pressure plane. See Appendix A for the isopycnic plot of the entire range of methanol-pressure fractions.

3.2.2 Selectivity

The selectivity is the most important factor for the resolution between two chromatographic peaks [35] in analytical scale separations. Fig. 5 shows how the selectivity changed with pressure, temperature and co-solvent fraction for the two investigated solutes, TSO and BINOL, respectively.

In the studied design space, the most important factor for BINOL was the temperature and no interaction or quadratic terms were significant, Fig. 5a. Changing the methanol fraction gave very minor effects on the selectivity, Fig. 5c, and we see that the increase in selectivity quite clearly follows the decrease in temperature. For TSO, unlike BINOL, the methanol fraction was the most important factor and a number of other terms were significant. In this case, an increase in selectivity is achieved by increasing the temperature. It should be mentioned that TSO showed a relatively larger variance in the center point values for selectivity as compared to BINOL which resulted in a larger uncertainty in the coefficients (Fig. 5); however all TSO experiments were well described by the regression model.

Since the P and T terms had coefficients with opposite sign for BINOL and TSO, opposite optimal condition when maximizing the selectivity will be found. This highlights the difficulty to draw any general conclusions from these results because of the complexity of chiral stationary phases (CSPs) and the limited number of solutes and CSPs investigated in this work. However, this work aims at presenting a detailed, reliable methodology for extracting information for a specific separation system. To extract more general conclusions, the methodology developed by West and Lesellier could be applied [5–9].

3.2.3 Productivity

In preparative chromatography, it is important to purify the desired components at the desired purity and at a maximal productivity, i.e. amount purified product per unit time. In a separation of one of the enantiomers of a racemate, the first step is to find suitable separation systems. This is achieved by screening combinations of CSPs and co-solvents. Typically, selectivity is maximized while k_1 is minimized.

Loading studies utilizing maximum sample concentration on suitable candidate systems can then be performed [36,37]. In this study, the dependency and variation of productivity on co-solvent, pressure and temperature was studied. Either of the enantiomers of TSO was selected as target. The optimal chromatogram was chosen to maximize injection volume while maintaining touching-band separation, i.e. having a yield of 100%. The optimal chromatogram for the center-point of the design space is shown in Fig. 2c. The productivity was calculated by assuming that stacked injections could be performed. Fig. 2c shows the chromatogram for optimum injection volume (16 µL) for the separation of either enantiomer of TSO at 30°C, 160 bar and 5% MeOH, calculated by Eq. (1) (the time between the dashed lines is the cycle time).

The trends in productivity were very clear and analysis of parameter importance revealed that the most important factor to increase productivity was to increase the fraction co-solvent followed by an increase of temperature; see Fig. 6a. Therefore, by simultaneously increasing methanol content and temperature, the productivity could in this case be efficiently maximized, see Fig. 6b. By decreasing pressure the productivity could further be increased but only marginally, data not shown. It should be noted that a complete optimization also would entail the volumetric flow and or concentration of the sample but that is beyond the scope of this study. Interestingly, the selectivity for the separation of TSO increases with decreasing methanol content. Hence, maximizing selectivity will yield lower productivity.

4. Conclusions

The combined effect of co-solvent fraction in eluent, temperature and pressure was studied using chemometrical tools to understand their impact on the retention factor, selectivity and productivity in SFC. All input parameters were carefully measured using external sensors. In this study, the measurements of pressure and mass flow was found to necessary to correct for variations in the volumetric flow rate with the experimental conditions and for correct pressure inputs in the analysis of variance. The temperature measurements agreed with the set temperature making them unnecessary in this case.

Two racemic probes, TSO and BINOL, were investigated using the proposed methodology. Retention of both probes was found to be strongly dependent of amount of methanol and pressure. Selectivity of TSO was strongly dependent on the methanol content which was not the case of BINOL where temperature had the largest effect. Temperature was found to have opposite effect on selectivity for TSO and BINOL. For preparative purification of either enantiomer of TSO, the most important parameters controlling productivity were found to be methanol content and temperature. The selectivity for the separation of TSO increases with decreasing methanol content. Hence, maximizing selectivity will yield lower productivity in this case.

With the presented approach, information about the relative importance of the parameters in the design space could be obtained. Such insight in SFC can serve to (i) show if a factor is important or not to control, (ii) aid reliable method transfer and (iii) perform fundamental studies aimed at explaining variations with these parameters.

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Figure Captions

Fig. 1: The void volume determinations using pycnometry (dashed line), and injections of nitrous oxide, TTBB and MeOH (bars), are presented for 2.5, 5.0 and 7.5 v/v % MeOH. The volumetric flow rate was set to 0.7 mL/min but the elution volume was calculated from the actual estimated volumetric flow rate for each experiment from the measured mass flow and density of the mobile phase. The back pressure was set to 160 bar and the temperature was set to 30°C.

Fig. 2: Example of chromatograms used in the calculations. In a) TSO analytical separation at: (1) 120 bar at 24 °C and 2.5% MeOH, (2) 160 bar 30 °C and 5% MeOH, and (3) 200 bar 36°C and 7.5% MeOH. In b) BINOL separation at: (1) 120 bar at 24°C and 15% MeOH, (2) 160 bar at 30 °C and 20% MeOH, and (3) 200 bar at 25°C and 25% MeOH. In c) the optimum touching-band overloaded injection (16 µL) of TSO at 30°C, 160 bar and 5% MeOH is presented. The time between the dashed lines represent the cycle time.

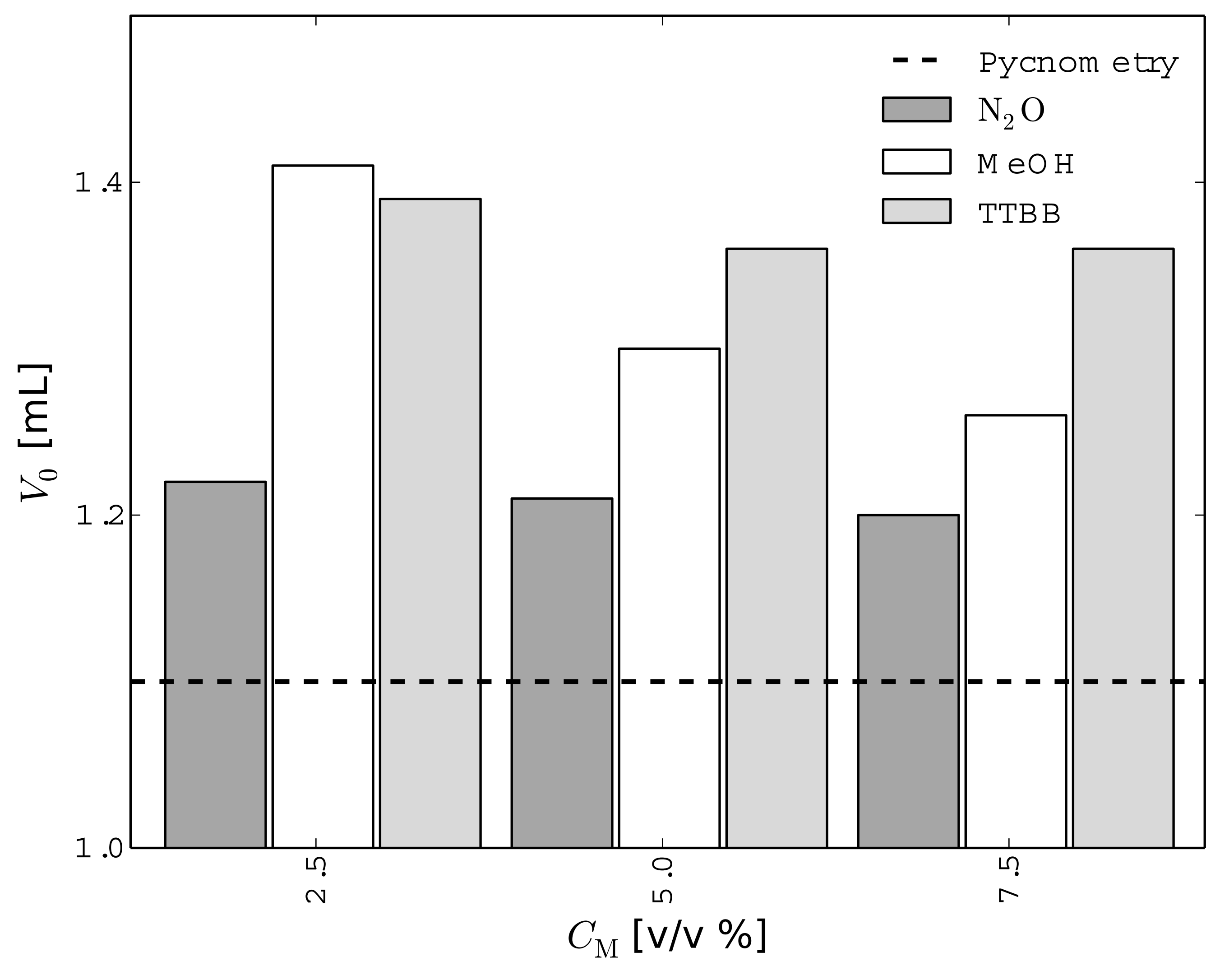
Fig. 3: Centered and normalized coefficients from the model fit for the first and second retention factor, respectively, for: a) TSO and b) BINOL. The error bars represent the 95% confidence interval of the coefficients.

Fig. 4: The contour plot shows the retention factor (*k*) as a function of pressure and amount of methanol in the eluent. a) k_1 for TSO, b) k_2 for TSO, c) k_1 BINOL and d) k_2 BINOL. For the density variations, the reader is referred to Appendix A.

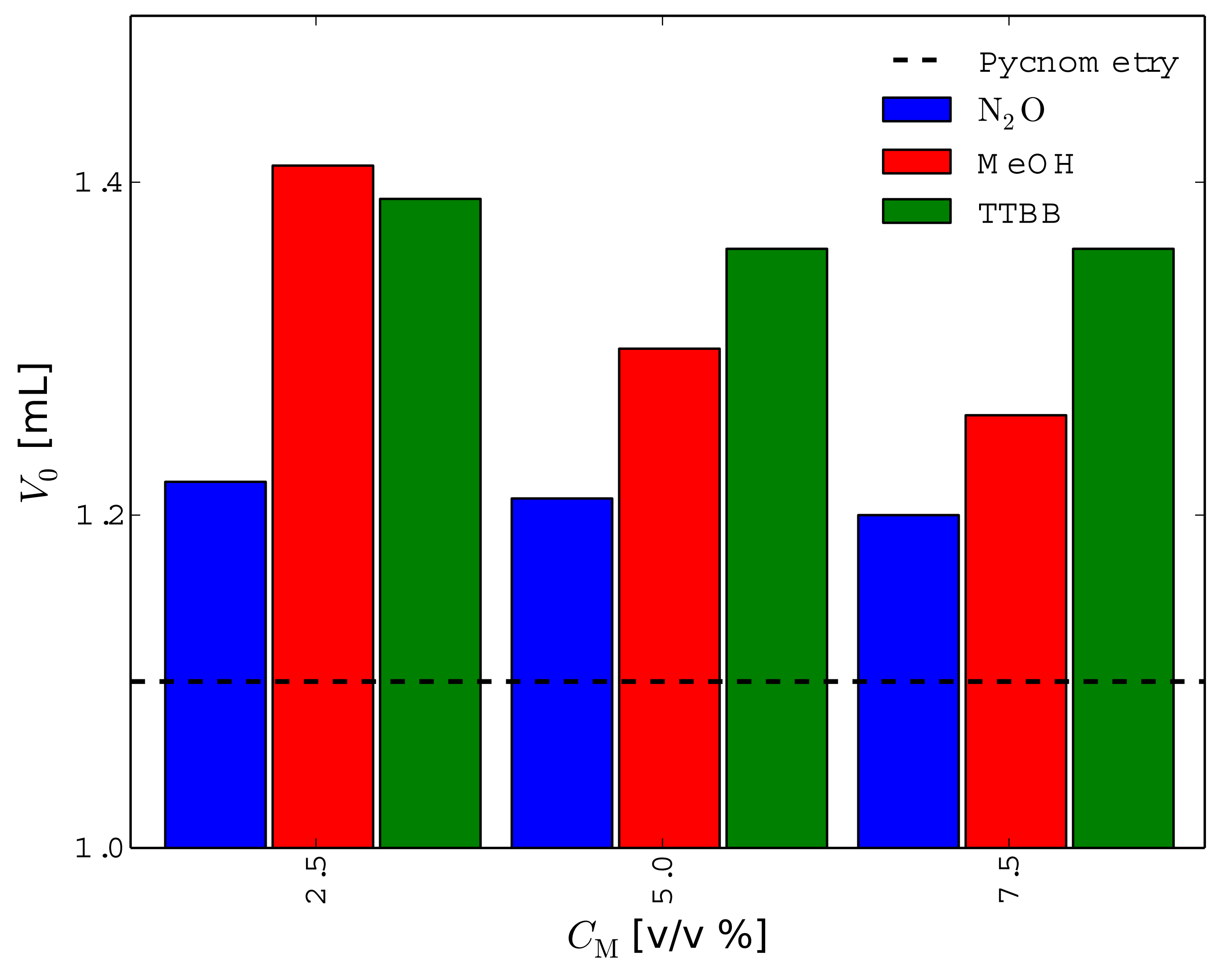
Fig. 5: a) Centered and normalized coefficients with 95 % confidence interval from the model fit to the selectivity for TSO and BINOL is plotted. In b) and c) the selectivity is plotted as a function of amount of modifier in the eluent and the temperature for b) TSO and c) BINOL.

Fig. 6: a) Centered and normalized coefficients with 95 % confidence interval from the model fit to the productivity for the optimum touching-band chiral separation of TSO is plotted. In b) the productivity is

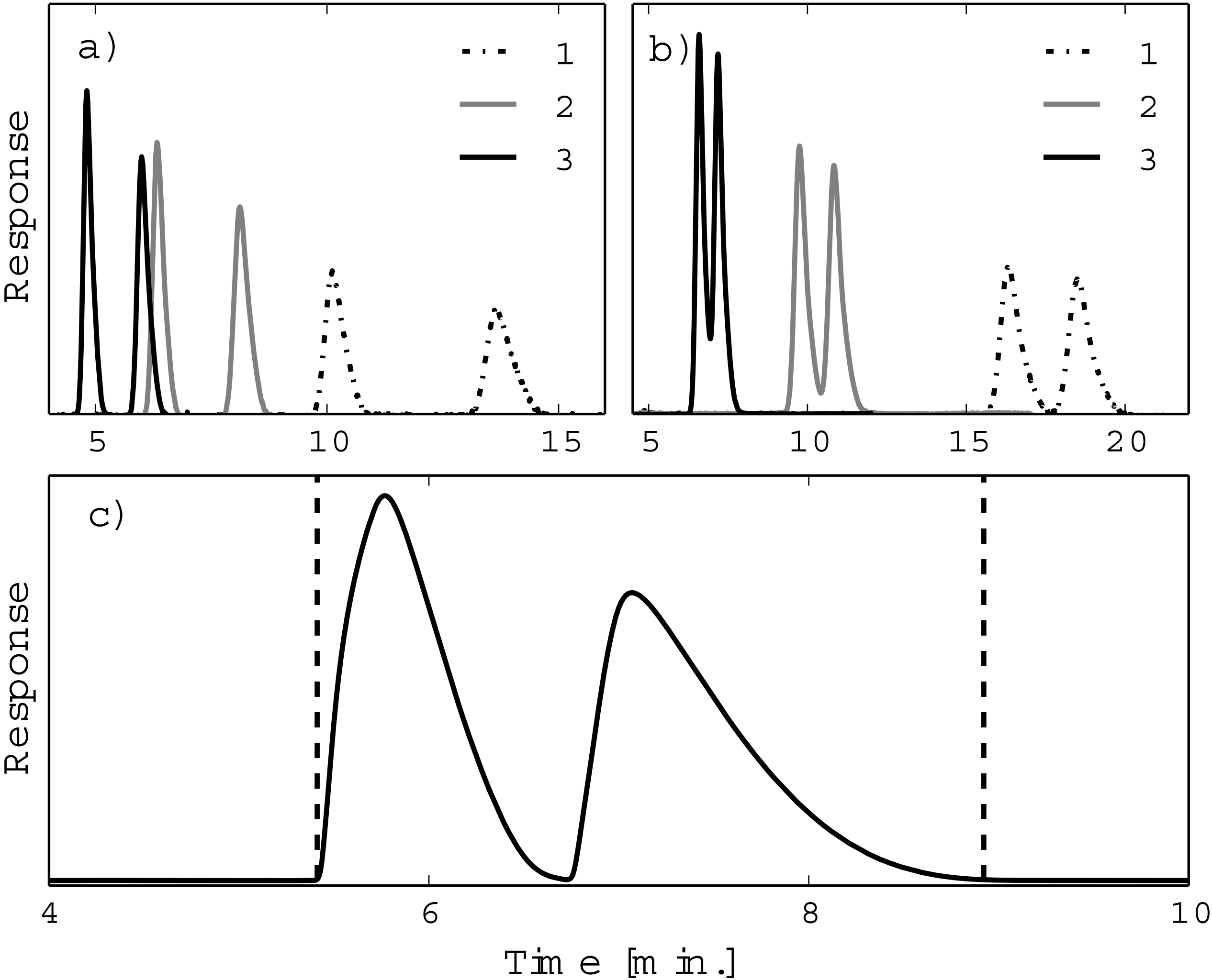
plotted as a function of amount of modifier in the eluent and the temperature.



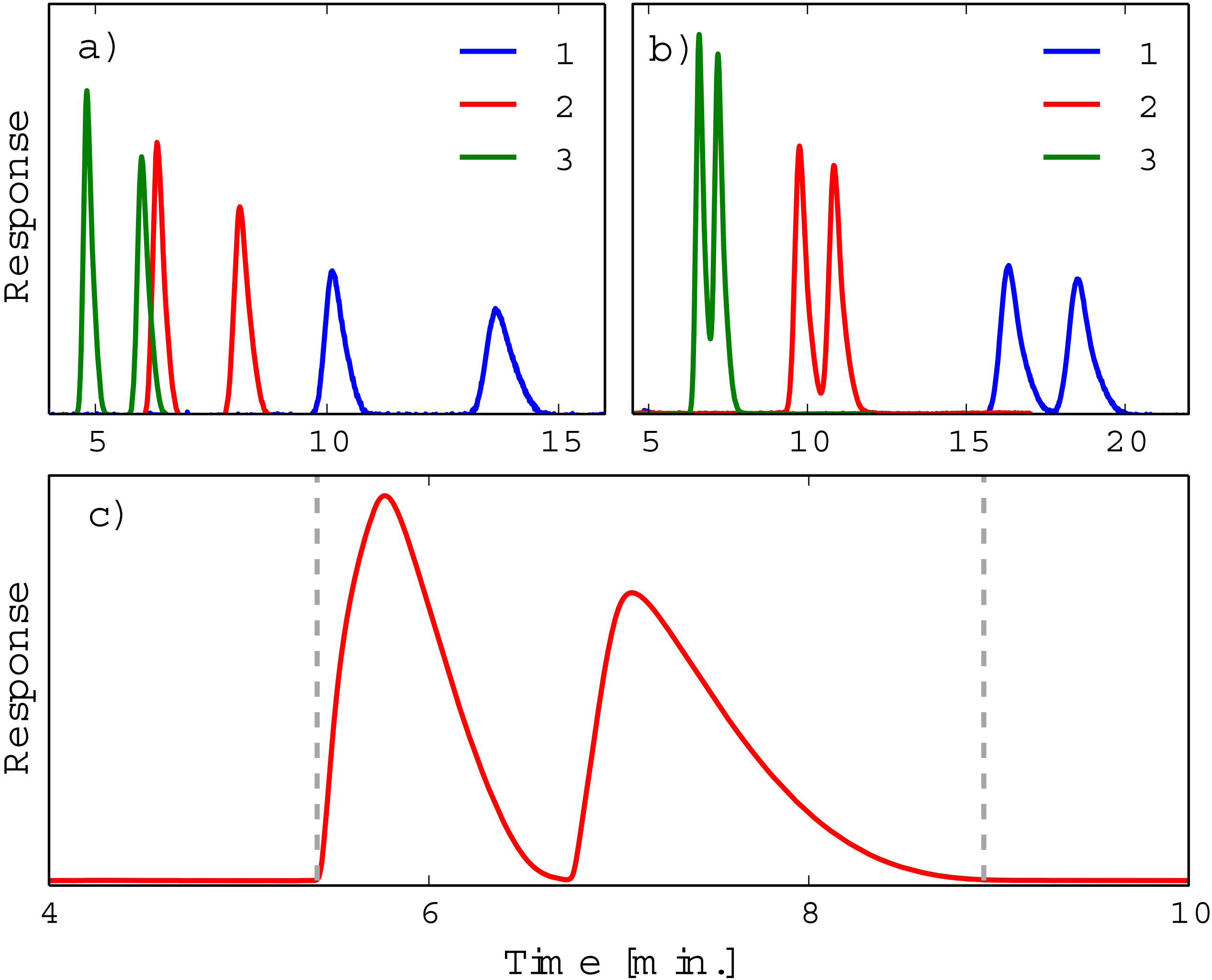
Pycnometry
N_2O
MeOH
TTBB
1.4
1.2
1.0
2.5
5.0
7.5
V_0 [mL]
C_M [v/v %]



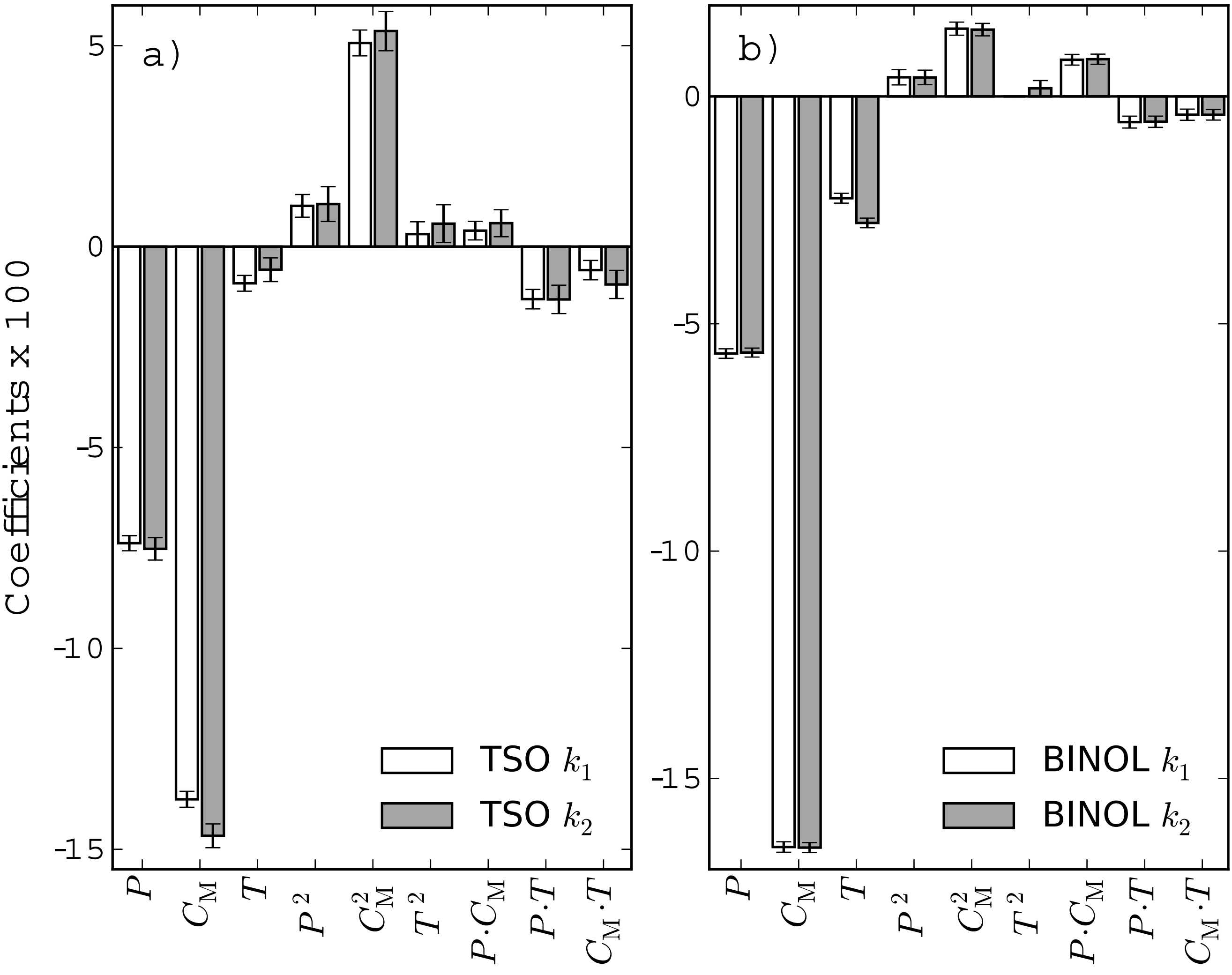
Pycnometry
N_2O
MeOH
TTBB
1.4
1.2
1.0
V_0 [mL]
2.5
5.0
7.5
C_M [v/v %]

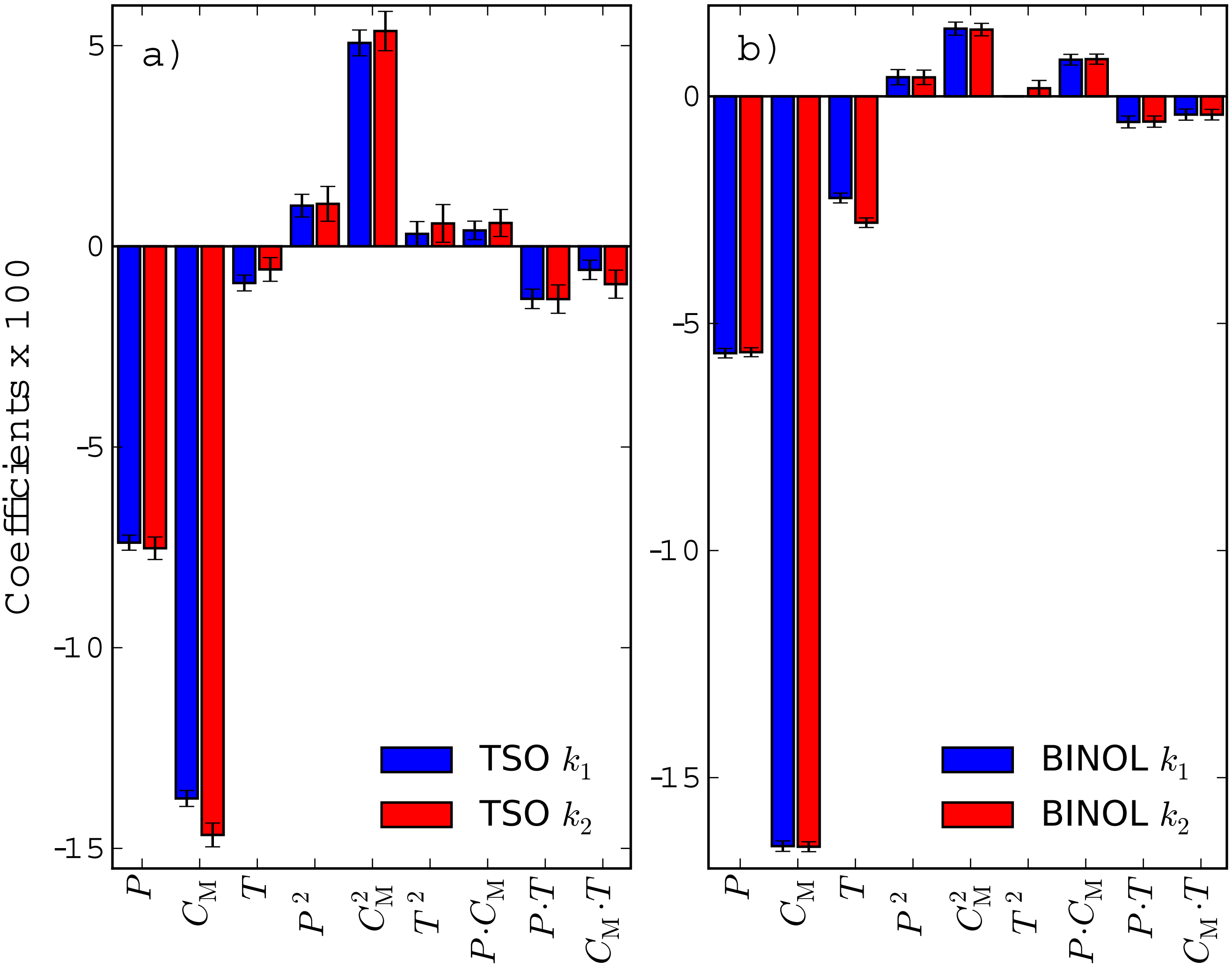


a)
1
2
3
Response
5
10
15
b)
1
2
3
5
10
15
20
c)
Response
4
6
8
10
Time [min.]

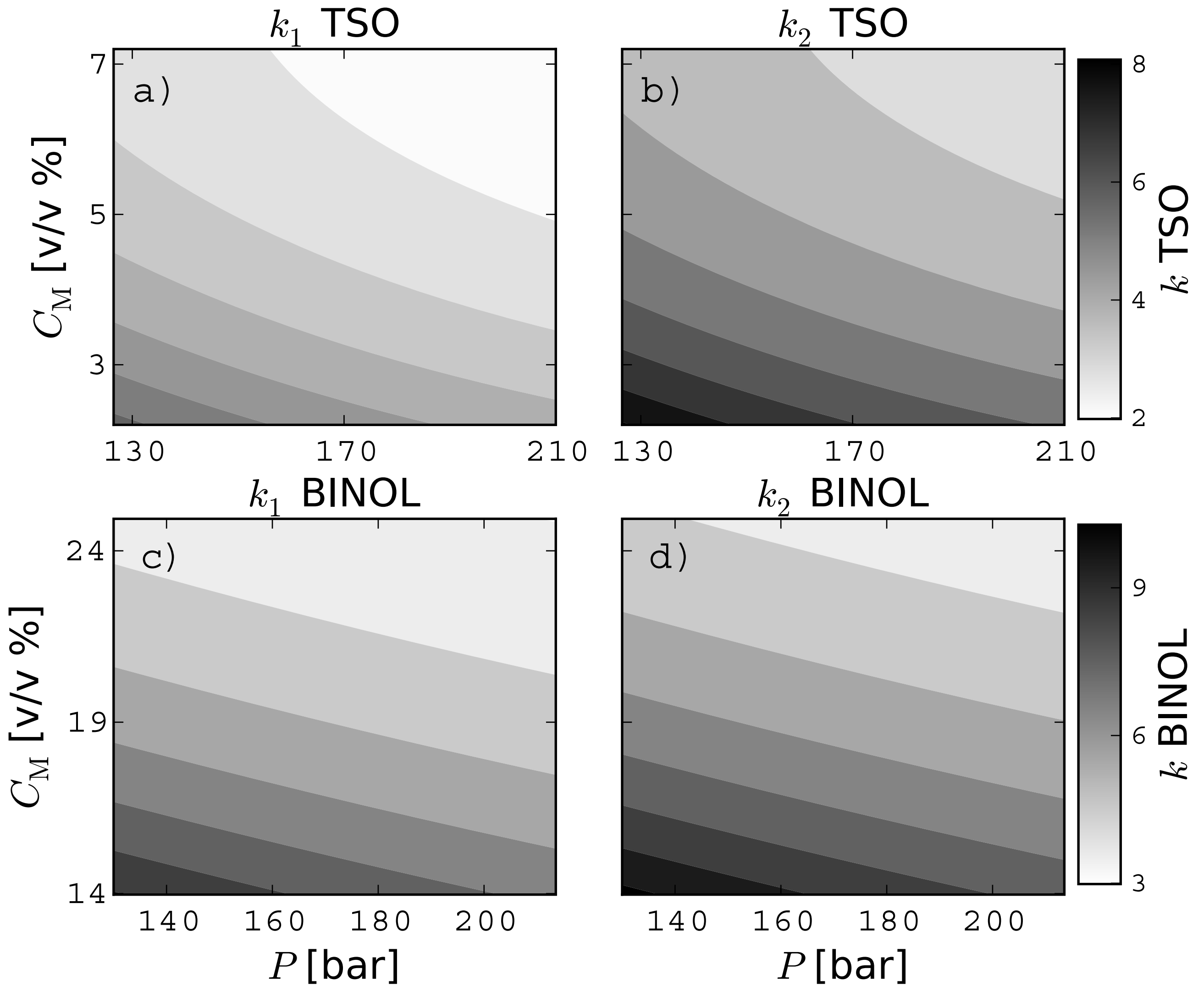


a)
b)
c)
1
2
3
Response
5
10
15
20
4
6
8
Time [min.]

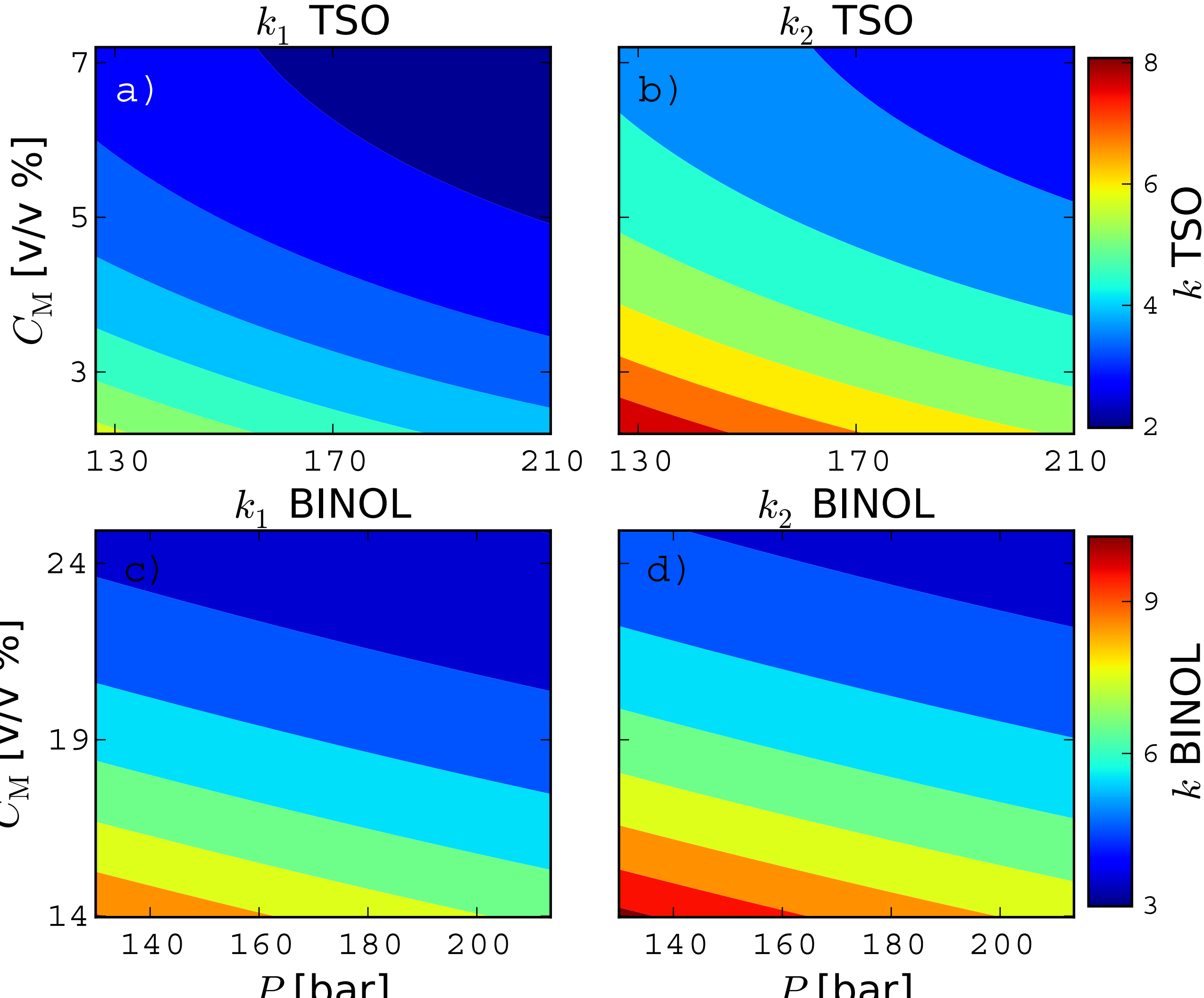

a)
b)
Coefficients x 100
5
0
-5
-10
-15
TSO k_1
TSO k_2
BINOL k_1
BINOL k_2
P
C_M
T
P^2
C_M^2
T^2
$P \cdot C_M$
$P \cdot T$
$C_M \cdot T$



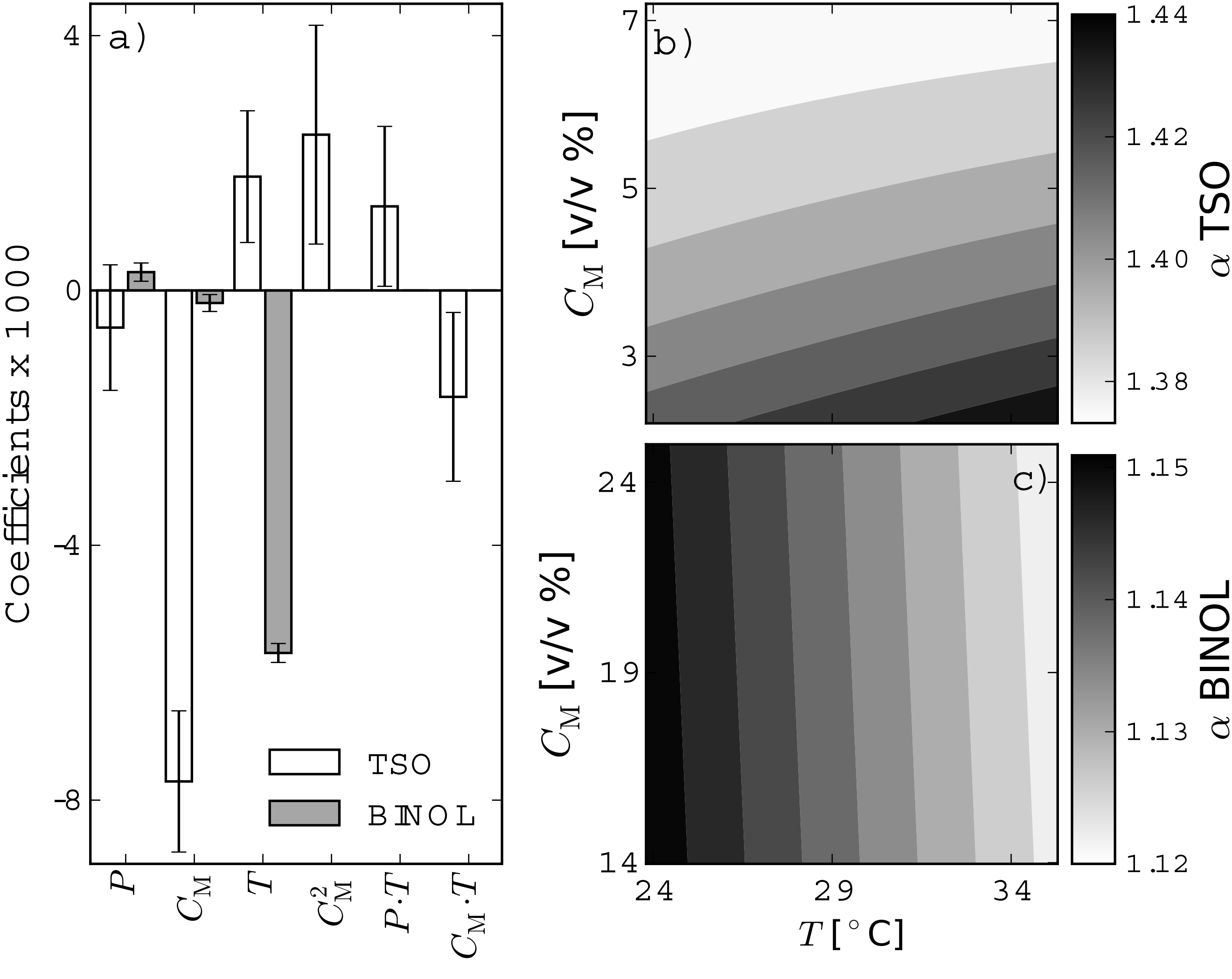
a)
b)
Coefficients x 100
5
0
-5
-10
-15
TSO k_1
TSO k_2
BINOL k_1
BINOL k_2
P
C_M
T
P^2
C_M^2
T^2
$P \cdot C_\mathrm{M}$
$P \cdot T$
$C_\mathrm{M} \cdot T$

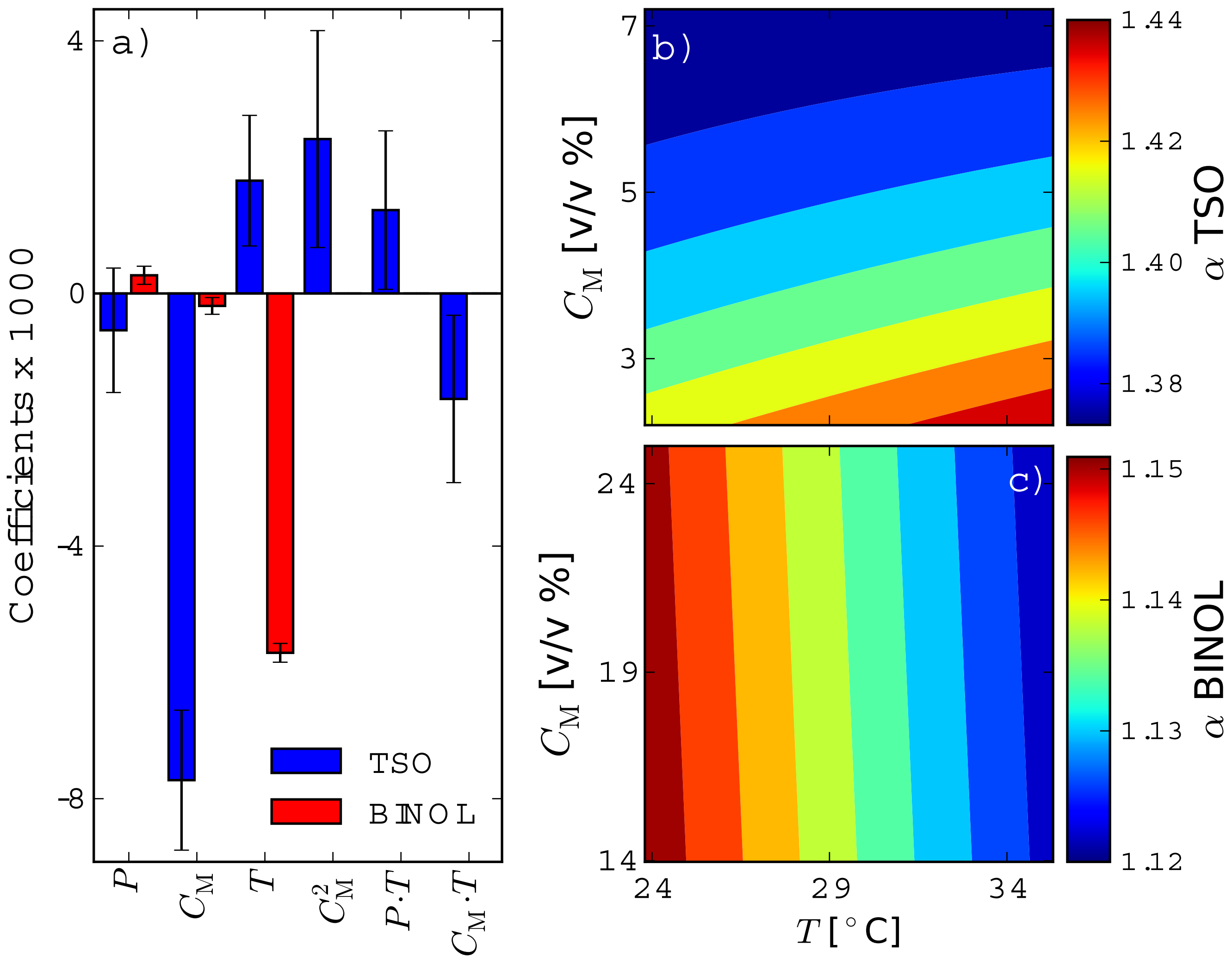


k_1 TSO
k_2 TSO
a)
b)
C_{M} [v/v %]
7
5
3
130
170
210
k TSO
8
6
4
2
k_1 BINOL
k_2 BINOL
c)
d)
24
19
14
140
160
180
200
P [bar]
k BINOL
9
6
3

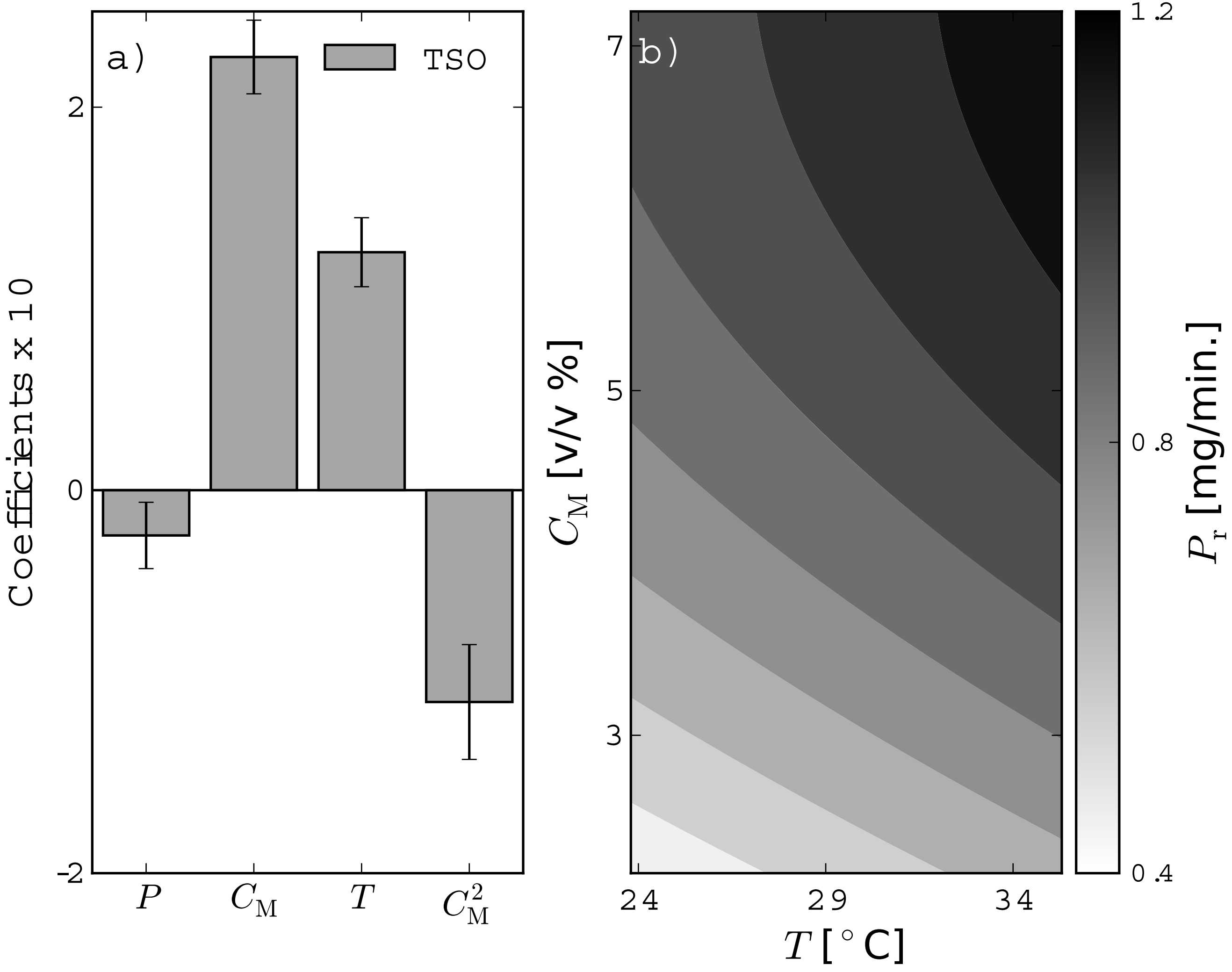


k_1 TSO
k_2 TSO
k_1 BINOL
k_2 BINOL
a)
b)
c)
d)
C_{M} [v/v %]
P [bar]
k TSO
k BINOL
7
5
3
130
170
210
24
19
14
140
160
180
200
2
4
6
8
3
6
9

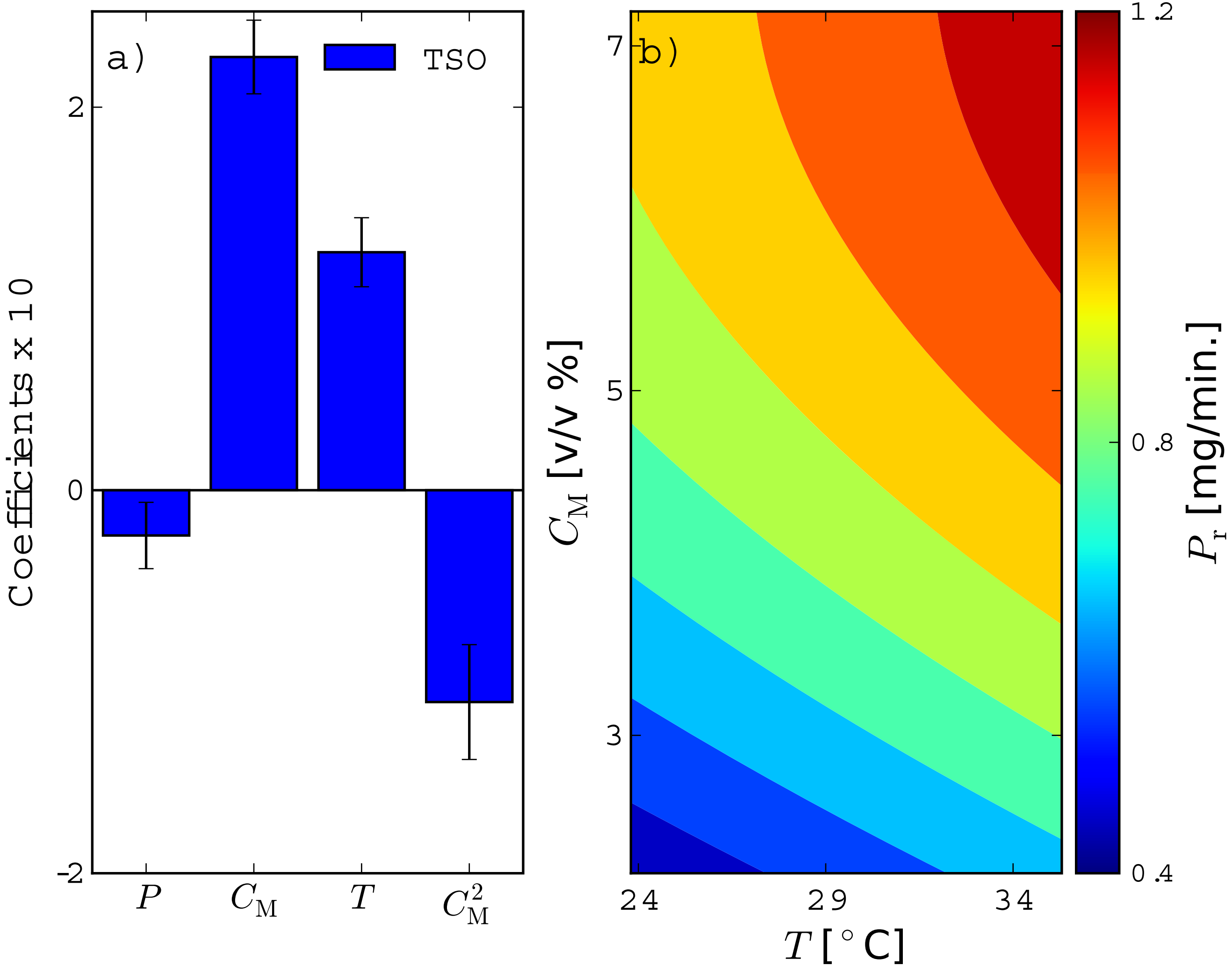

a)
b)
c)
coefficients x 1000
4
0
-4
-8
TSO
BINOL
P
C_M
T
C_M^2
$P{\cdot}T$
$C_\mathrm{M}{\cdot}T$
C_M [v/v %]
7
5
3
24
19
14
29
34
T [°C]
1.44
1.42
1.40
1.38
α TSO
1.15
1.14
1.13
1.12
α BINOL



a)
b)
c)
Coefficients x 1000
TSO
BINOL
P
C_M
T
C_M^2
$P \cdot T$
$C_M \cdot T$
C_M [v/v %]
T [°C]
α TSO
α BINOL



a)
TSO
Coefficients x 10
2
0
-2
P
C_M
T
C_M^2
b)
7
5
3
C_M [v/v %]
24
29
34
T [°C]
1.2
0.8
0.4
P_r [mg/min.]



a)
TSO
Coefficients x 10
2
0
-2
P
C_M
T
C_M^2
b)
7
5
3
C_M [v/v %]
24
29
34
T [°C]
1.2
0.8
0.4
P_r [mg/min.]