DEG Bioresearch

Spin-off company

Center for Interdisciplinary Biosciences
Technology and Innovation Park TIP-UPJŠ

APPLICATION NOTE

TCORE: TOOL FOR RELIABLE DETERMINATION OF MELTING TEMPERATURES AND INFLECTION POINTS FROM PROTEIN DENATURATION CURVES

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Objectives

Reliable determination of melting temperatures or inflection points from thermal transitions of proteins monitored by the ratio of spectroscopic signals.

Summary

In this application note, we introduce and validate TCORE software tool, which provides a model-free correction of the melting temperatures of proteins determined by the ratio of spectroscopic signals.

Keywords

thermal denaturation, ratio, fluorescence intensity, unfolding, temperature, melting, proteins, biomolecules, antibodies, methods, analysis, two-state model, ligand, drug discovery, candidate, analysis, derivative, transitions, GOX - glucose oxidase, lysozyme, ADH - alcohol dehydrogenase, mechanism, tryptophan

Introduction

Monitoring spectroscopic signals of proteins, particularly fluorescence of intrinsic tryptophan residues, is a widely-used approach in analysis of unfolding transitions (induced by temperature, chemical denaturant, and pH) in proteins. Tryptophan fluorescence provides several suitable parameters such as steady-state fluorescence intensity, apparent quantum yield, mean fluorescence lifetime, position of emission maximum that are often utilized for observation of conformational/unfolding transitions of proteins. In addition, fluorescence intensities ratio at different wavelengths (usually at 330 nm and 350 nm) becomes increasingly popular parameter for evaluation of thermal transitions. In our recent paper [1], we demonstrated that, under certain conditions, the use of this parameter for the analysis of unfolding transitions leads to determination of incorrect thermodynamic parameters characterizing unfolding transitions in proteins (e.g. melting temperature, inflection points). Melting temperature of a protein is used for variety applications, e.g. protein quality control, in formulation development, and in drug discovery.

An analysis of unfolding transitions, induced by temperature, chemical denaturants, and pH, provides important information regarding protein stability and its dependence on the external conditions and ligand binding. This information consequently plays a critical role for further development, pharmacological and biotechnological utilizations of the protein. Among numerous biophysical techniques (methods of optical spectroscopy, differential scanning calorimetry), available for the determination of protein stability, spectroscopic techniques have certain advantages. The main advantages of the methods such as absorption, circular dichroism and fluorescence spectroscopy in protein studies are ease of sample preparation, a high sensitivity (leading to requirement of only low amount of precious protein), a high rate of measurements, and relative simplicity in analysis of the obtained data.

Intrinsic tryptophan (Trp) fluorescence is often used as a probe for thermal and chemical denaturations and in high-throughput analysis. Usually such analysis focuses on the changes in the melting temperature as an indicator for ligand binding or the measure of the stability of protein therapeutics in different formulations.

It has been previously pointed out that the critical condition to use of any of these signals is whether the signal is proportional to the population of macrostates. In fact, out of the commonly used fluorescence signals, this criterion is fulfilled only for the fluorescence emission intensity measured at different excitation and emission wavelengths. Other, above mentioned, fluorescence signals have to be analyzed in more complex way, e.g. they have to be corrected by weighting both the fraction of states and by the fluorescence quantum yield of each state and/or as it has been proved useful to analyze these signals in combination.

Here, we demonstrate that the ratio of fluorescence emission at two different wavelengths, which are used in thermodynamic analysis of conformational transitions of proteins, needs to be corrected. Therefore, we introduce a new program for correct determination of melting temperatures of proteins **TCORE**.

Experimental part

Consumables and sample preparation

- Alcohol dehydrogenase from bakers yeast, Sigma, A-7011
- Glucose oxidase from Aspergillus niger, Sigma Aldrich, G2133-type VII
- Lysozyme from chicken egg white, Sigma 62970-1G-F
- Buffers and salts from Sigma-Aldrich

Proteins were dissolved in the buffer (20 mM sodium cacodylate, pH 7.0) and used immediately for experiments. The pH of the samples was determined using a HI 9017 pH meter coupled to a HI 1330 pH electrode (Hanna Instruments Srl, Padova, Italy).

Sample preparation for thermal denaturation

Fluorescence measurements were performed using a Varian Cary Eclipse fluorescence spectrophotometer (Varian Australia Pty Ltd) equipped with a Peltier multicell holder. Details of the measurement protocol are placed in Table 1.

I. THEORETICAL MODELS FOR VALIDATION TCORE

Results and Discussion

Among many possibilities, fluorescence intensity ratio is the widely used for thermal denaturation of proteins and, hence, using experimental and theoretical examples the application of our correction software tool will be shown for fluorescence intensities. To validate our software tool **TCORE**, first, we conducted theoretical modeling of the irreversible thermal denaturation of proteins and calculate melting temperatures from the individual signals and from the ratio of the signals as well.

Test #1: a two-state irreversible model

$$N \rightarrow U$$

$$f_{Native} = exp\left(-\frac{1}{v}\int_{T_0}^{T} exp\left(\frac{E_a}{R}\left(\frac{1}{T}-\frac{1}{T_k}\right)\right)dT\right)$$

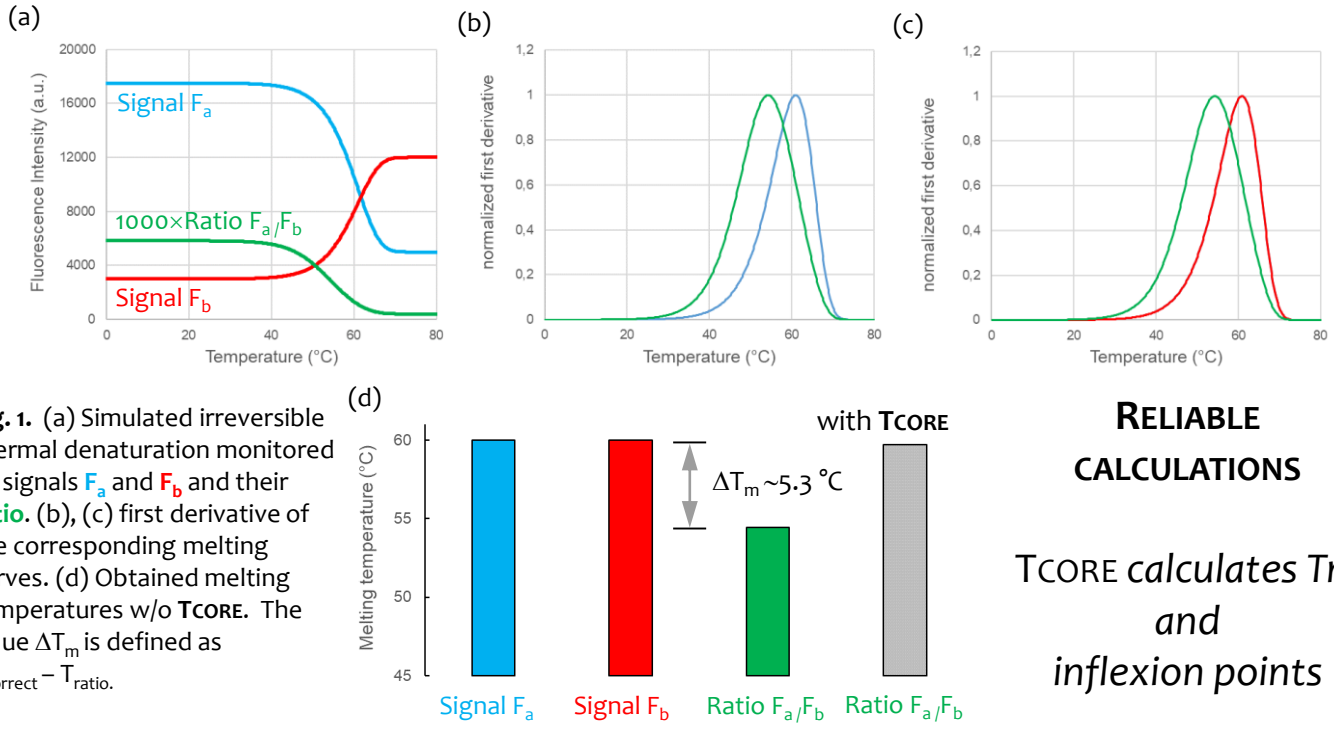


Fig. 1. (a) Simulated irreversible thermal denaturation monitored by signals F_a and F_b and their **ratio**. (b), (c) first derivative of the corresponding melting curves. (d) Obtained melting temperatures w/o **TCORE**. The value ΔT_m is defined as $T_{correct} - T_{ratio}$.

RELIABLE CALCULATIONS

TCORE calculates Tm and inflexion points

In the first test, we simulated non-equilibrium, irreversible thermal denaturation of a protein using following values: activation energy, E_a = 175 kJ·mol^{-1} and T_k = 70 °C (temperature at which k=1 min^{-1}) and heating rate v = 1 °C·min^{-1}. The simulated curves are shown in Fig. 1a for a thermal profile for signal F_a (blue lines), signal F_b (red lines)and their ration (green lines). The normalized absolute value of the first derivative of the thermal profiles of individual signals (Fig. 1b- red and Fig.1c-blue lines) yields a maximum at 60 °C which is the expected

However, the first derivative of the signal ratio shows maximum (green lines in Fig1b and 1c), which is different compared to individual signals. Hence, signal ratio is not a reliable parameter for melting temperature determination. Our newly developed software tool, **TCORE** can utilize the information in the shape of the temperature profile of the signal ratio and can determine the correct melting temperature value (Fig.1d), even when the deviation is very high for the ratio alone.

I. THEORETICAL MODELS FOR VALIDATION TCORE

Results and Discussion

In our first example, we analyzed theoretical thermal denaturation of a protein undergoing irreversible transition to unfolded state(s). In fact, proteins can denature reversibly upon heating as well. In the next step, we focused on whether **TCORE** can determine melting temperatures from reversible denaturation transitions which are described by the enthalpy change ΔH and melting temperature T_m.

Test #2: a two-state reversible model

$$N \leftrightarrow U$$

$$f_{Native} + f_{Unfolded} = 1$$

$$f_{Native} = \frac{1}{1 + exp\left(\frac{\Delta H}{R}\left(\frac{1}{T} - \frac{1}{T_m}\right)\right)}$$

Test #2: a two-state reversible model

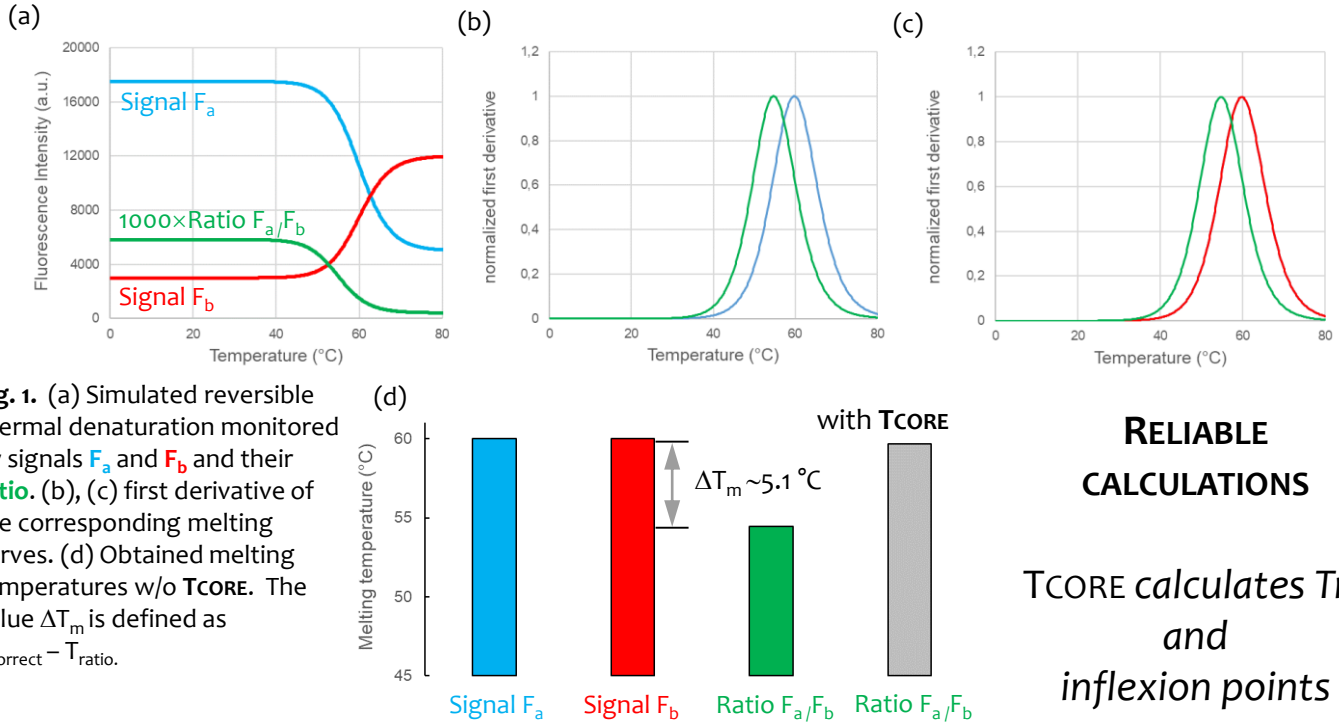


Fig. 1. (a) Simulated reversible thermal denaturation monitored by signals **F_a** and **F_b** and their **ratio**. (b), (c) first derivative of the corresponding melting curves. (d) Obtained melting temperatures w/o **TCORE**. The value ΔT_m is defined as $T_{correct} - T_{ratio}$.

RELIABLE CALCULATIONS

TCORE *calculates Tm and inflexion points*

In the second test, we simulated equilibrium, reversible thermal denaturation of a protein using following values: enthalpy of denaturation, ΔH=250 kJ·mol^{-1} and T_m = 60 °C. The simulated curves are shown in Fig. 2a for a thermal profile for signal F_a (blue lines), signal F_b (red lines)and their ratio (green lines). The normalized absolute value of the first derivative of the thermal profiles of individual signals (Fig. 2b- red and Fig.2c-blue lines) yields a maximum at ca. 60 °C which is the value expected from the simulation

Again, the first derivative of the signal ratio shows maximum (green lines in Fig. 2b and 2c) which is completely different compared to individual signals. Hence, signal ratio is not a reliable parameter for melting temperature determination. Our newly developed tool, TCORE can utilize hidden information in the shape of the temperature profile of the signal ratio and can determine the correct melting temperature value (Fig.2d) even when the deviation is very high for the ratio alone.

II. EXPERIMENTAL THERMAL DENATURATIONS OF PROTEINS

Results and Discussion

The presented theoretical approach validate application of **TCORE** as a tool for the correct determination of melting temperatures of proteins. Next, we measured thermal denaturation of three unrelated proteins: glucose oxidase, lysozyme, and alcohol dehydrogenase and determined melting temperatures from the fluorescence emission intensities at 350 nm (F_a) and 330 nm (F_b) and their ratio F_a/F_b. Our selection of proteins was based on their availability and their unrelated sequence and structure of the proteins and hence unique local tryptophan environment– which is confirmed by their distinct thermal profiles.

TABLE 1. **DETAILS OF THE MEASUREMENT PROTOCOL**

PROTEIN CONCENTRATION	2.4 μM (GOX) 2.6 μM (lysozyme) 2.1 μM (ADH)
BUFFER	20 mM sodium cacodylate. pH 7,0
TEMPERATURE RANGE	25-92°C
SCANNING RATE	1 °C/min
EMISSION WAVELENGTH	330 and 350
EMISSION SLITS	5 nm
EXCITATION WAVELENGTH	290 nm
EXCITATION SLITS	5 nm

Thermal denaturation of proteins: experiments

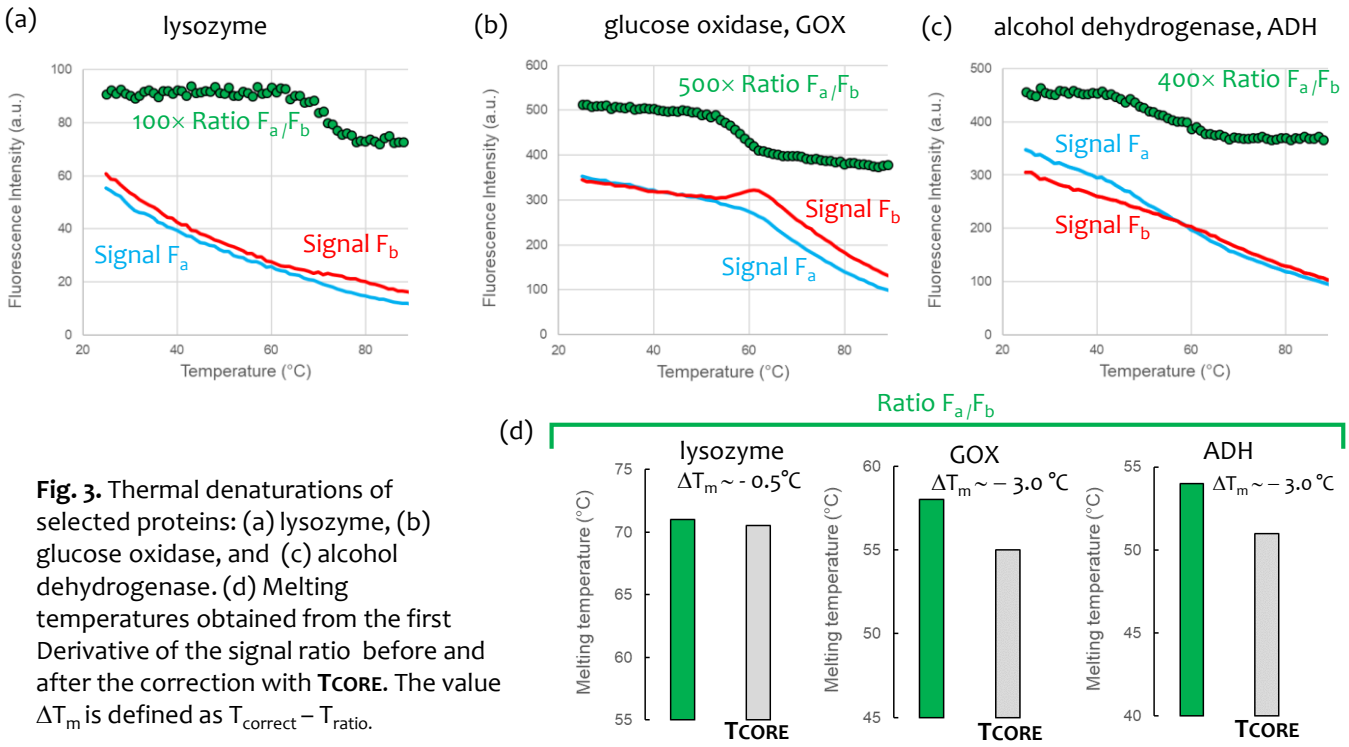


Fig. 3. Thermal denaturations of selected proteins: (a) lysozyme, (b) glucose oxidase, and (c) alcohol dehydrogenase. (d) Melting temperatures obtained from the first Derivative of the signal ratio before and after the correction with **TCORE**. The value ΔT_m is defined as $T_{correct} - T_{ratio}$.

Figure 3 shows thermal denaturation curves of three selected proteins – lysozyme, glucose oxidase, and alcohol dehydrogenase, measured at two different wavelengths 330 nm (blue line) and 350 nm (red lines) independently as well as the ratio of the signals (green lines). Obviously, compared to the signal ratio, the individual thermal profiles measured at 330 nm and 350 nm do not yield a good clear signal of thermal denaturation; except glucose oxidase, the thermal profiles of lysozyme and ADH display barely observable transitions.

Thus, the signal ratio provides valuable and clear thermal profile for the analysis. After applying the first derivative of the thermal profile, melting temperature can be obtained in a model-free way as maximum or minimum of the derivative curve. Fig. 3d shows are melting temperatures obtained using such first derivative and melting temperatures obtained by the **TCOR**. While for lysozyme, the correction is relatively small, ca. 0.5 °C, for other proteins, GOX and ADH, T_m has to be corrected by ca. 3 °C.

Conclusions

Determination of the melting temperatures of proteins is central to many research areas including hit identification in the campaigns, protein quality control approaches and accelerated studies for the formulation development. Often, researchers use ratio of fluorescence intensities for monitoring thermal profiles which is, however, not always reliable parameter for determining melting temperatures. In this application note, we suggest that the correction is necessary and possible by using our software tool **TCORE**. We validated **TCORE** using theoretical curves describing models for reversible and irreversible thermal denaturations.

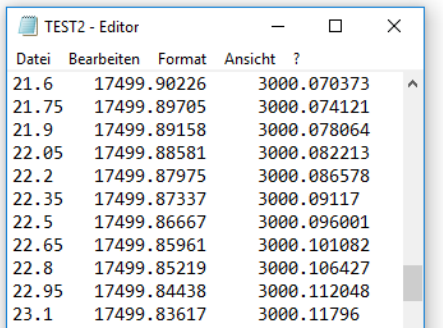
References

[1] Žoldák G, Jancura D, Sedlák E (2017) The fluorescence intensities ratio is not a reliable parameter for evaluation of protein unfolding transitions.
Protein Sci. 26(6), 1236-1239.

III. STEP-BY-STEP APPLICATION OF **TCORE**

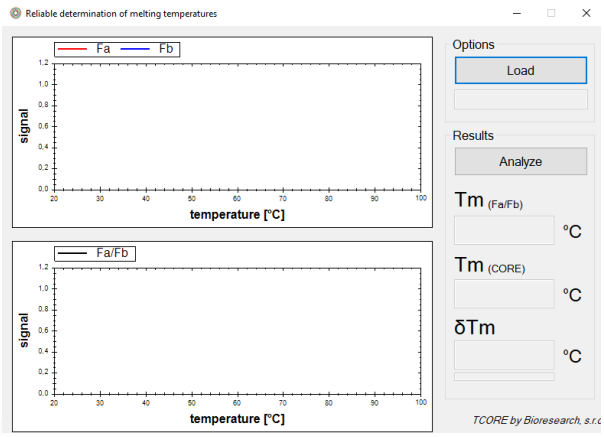
1. Data format

- Experimental or theoretical data *.txt format
- Temperature, F_a and F_b separated by space
- Temperature (in K or °C)
- See example below

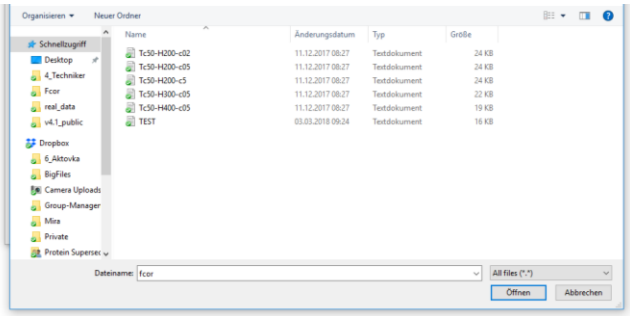


2. Running TCORE

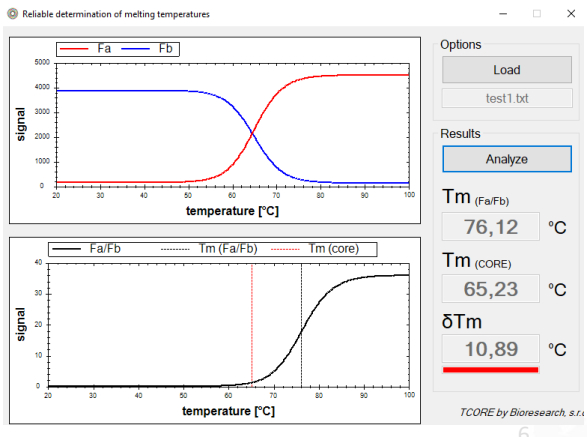
- After the start, click on load data



- Select a file for the analysis (e.g. test2.txt)



After data loading, thermal profiles of the individual signals F_a and F_b are shown in the upper graph. Press Analyze. On the right panel, an uncorrected value of the melting temperature is shown in the box. Below the box, the corrected value is displayed as well as the difference between corrected and uncorrected melting temperatures.



IV. VALIDATION TCORE USING MONTE-CARLO APPROACH

two-state model → **Set of parameters:**
variable: {$F_{a,}$, F_b for native state, F_b unfolded state, ΔH}
constant: T_m value, F_a unfolded state,
noise-free signals
→ **F_a/F_b vs. temperature**
Inflexion points
(1^{st} derivative calculations)

FOR ALL SAMPLES T_M WAS CONSTANT (65 °C)

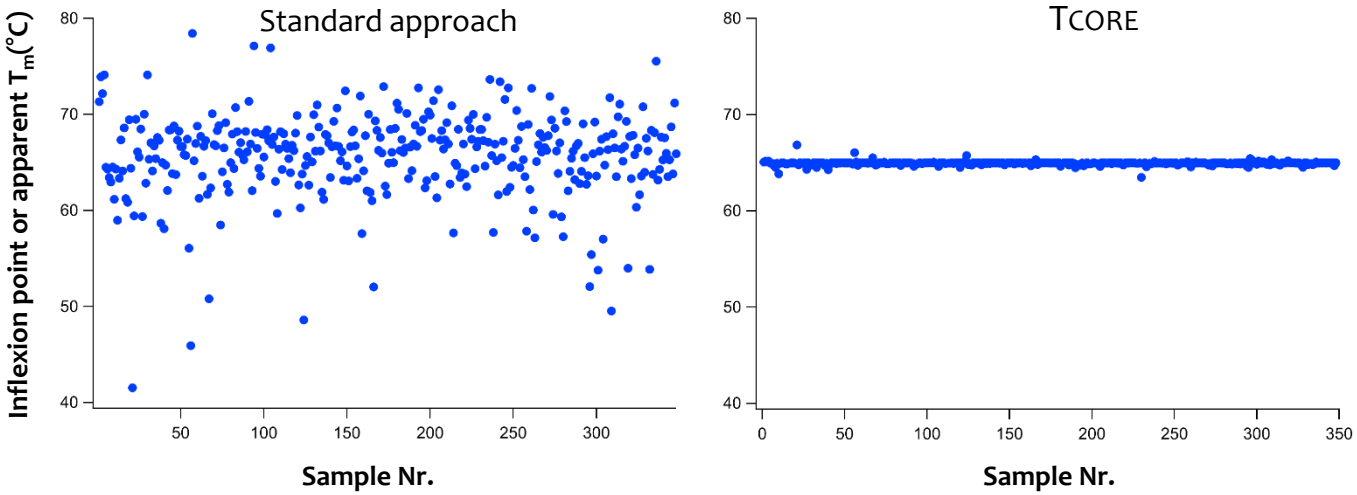


Fig. 4. Standard approach vs. TCORE for the inflection point determination. Thermal denaturation curve (sample) was obtained using randomisation procedure. Parameters: T_m= 65°C, ΔH (120-840 kJ/mol), $F_{a,\ native}$ (0.1-10x$F_{a,unfolded}$), $F_{a,\ unfolded}$= 5000 a.u., $F_{b,\ native}$ (0.1-10x$F_{a,unfolded}$), $F_{b,\ unfolded}$ (0.1-10x$F_{a,unfolded}$).

To validate our Tcore algorithm, we applied the Monte-Carlo approach where fluorescence intensities and enthalpy change were randomized between values commonly found in protein unfolding experiments. After calculating a thermal denaturation curve at two wavelengths (a, b), data were analyzed by a standard approach. Inflection point was determined from the first derivative (F_a/F_b vs. temperature) of experimental data. This inflection point is considered to correspond to the melting temperature of the protein. Next, the F_a/F_b

Data were analyzed using Tcore algorithm, and new values were recorded. For all samples, the melting temperature was set constant. We generated 349 independent thermal denaturation curves (Fig. 4). The standard approach leads to large variations and scatters of the inflection points. Tcore algorithm, using the same input values, determine inflection point reliably with significant less scatter. Only 2 out of 349 points deviate from the expected value for melting temperature, which is, however, due to the fundamental mathematical difference between an inflection point and T_m value.

SUMMARY

TCORE PERFORMS RELIABLE CALCULATIONS OF INFLEXION POINTS AND TM UNDER LARGE VARIATIONS OF INPUT CONDITIONS.