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A differential scanning calorimetry method to determine the isothermal crystallization kinetics of cocoa butter

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Abstract

This research aimed to reduce the variability on the data obtained from differential scanning calorimetric (DSC) analysis of the isothermal crystallization kinetics of cocoa butter.

To enable transformation of the DSC crystallization peak to a sigmoid crystallization curve, the DSC peak area has to be integrated. Usually, the start and end points of the crystallization peak are determined visually. The result of this visual determination appeared to be very much dependent on the operator, but also differed considerably when the same operator performed the integration several times. By proposing an objective calculation algorithm to determine the start and end points of integration, the variability caused by the operator during the integration procedure could be eliminated. Furthermore, sample preparation and the DSC heating protocol to melt the sample prior to crystallization were studied. Three heating protocols (65 °C for 15 min, 65 °C for 30 min and 80 °C for 15 min) were compared and it was shown that holding at 65 °C for 15 min was sufficient to eliminate any influence of sample history. Two different sample preparation procedures were compared and it appeared that a change in sample preparation procedure had a significant influence on the measured crystallization process. It is thus important to keep this method constant to eliminate the variability caused by it.

Keywords: Cocoa butter; Crystallization kinetics; DSC; Isothermal crystallization

1. Introduction

Thermal analysis methods such as differential scanning calorimetry (DSC) can be used to investigate the reaction kinetics of a broad range of materials, including metals, polymers and glass-forming solids. The two basic approaches to determine reaction kinetics are isothermal and non-isothermal methods. In isothermal experiments, such as the one described in this study, the sample is quickly brought to a predetermined temperature where the thermal analysis instrument monitors the heat flow of the system at constant temperature as function of time [1].

Cocoa butter is the major component of the chocolate fat phase. It is responsible for the texture, gloss and mouth feel of chocolate products. The crystallization behaviour of cocoa butter is thus of paramount importance in chocolate production [2,3].

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DSC has already been used in the past to study the isothermal crystallization kinetics of cocoa butter [3–5] and other natural fats [6–8].

To transform the DSC crystallization peak to a sigmoid crystallization curve representing the amount of heat released as function of time, the DSC peak area has to be integrated. The amount of heat released at a given time t (ΔH_{t_0-t}), is calculated by taking the area enclosed by a baseline and the peak between t_0 (the start point of crystallization) and t. The relative amount of heat released at time t is calculated by taking the ratio of (ΔH_{t_0-t}) and the total heat of crystallization (ΔH), the latter being the integration of the peak between $t_0$and t_e (the end point of crystallization). Evidently, an important element is the determination of t_0 and t_e. Toro-Vazquez et al. [8] mentioned that t_0 (they called it T_i, the induction time for crystallization) is calculated as the time from the start of the isothermal process to the beginning of crystallization (i.e. the time where the heat capacity of the sample has a significant departure from the baseline) using their DSCs software library. However, the authors did not clarify the t_e determination. Kerti [4] and Ziegleder [3] showed a figure in which t_0 and t_e are depicted. However, in these reports as well as in other articles the procedure for the actual calculation of t_0 and t_e was not mentioned. It can be suspected that the determination was done visually, which is a rather subjective method as will be shown in this paper.

Although it is possible that the sample preparation procedure influences the subsequent crystallization process, all above mentioned articles [3–8] lack the necessary information (Is the sample prepared in the liquid or solid state? If the sample is prepared in the liquid state, how is it liquefied? Which tool is used to prepare the sample?).

In general, the time–temperature program used in these investigations [3–8] consisted of three stages: first the samples are heated and kept at a high temperature for some time to destroy all homogeneous crystal nuclei, then the samples are cooled at a specified rate to the isothermal crystallization temperature and finally the samples are kept at that temperature until crystallization is complete. In particular, for cocoa butter the heating protocol used to melt the sample prior to crystallization varied quite drastically, i.e. 80 °C for 5 min [5], 60 °C for 30 min [4] and 65 °C for 15 min [3]. Unfortunately no justification for the use of a specific time–temperature protocol has been given. On the other hand, the cooling rate to the isothermal crystallization temperature also varied from ‘maximum’ cooling rate [3] to 1.2 °C/min [4], or 100 °C/min from 80 °C down to 50 °C followed by an isothermal period of 3 min and a further cooling at 100 °C/min [5].

It was the aim of this research to reduce the variability on the data obtained from isothermal crystallization experiments with cocoa butter monitored by means of differential scanning calorimetry. This study presents an objective calculation algorithm for the determination of t_0 and t_e in the integration procedure eliminating the variability caused by the operator during the integration procedure. Furthermore, this research studied the possible influence of the sample preparation method and the melting protocol.

2. Experimental

2.1. Cocoa butter

The cocoa butter used was a standard factory product supplied by Barry-Callebaut, Wieze, Belgium.

2.2. Sample preparation

To study the influence of sample preparation on the isothermal crystallization kinetics of cocoa butter, two different preparation methods (A and B) were used:

- cocoa butter was taken from the cooling chamber (4 °C), only in method B it was put in a desiccator to equilibrate to room temperature (to prevent humidity to condense on the cocoa butter);
- a sample (approximately 10–20 g) was taken (material cleaned with ethanol in method A, with acetone in method B) and put in a beaker which was put at 60 °C for 15 min;
- a drop of cocoa butter was taken with a plastic micropipette (method A) or with a glass, hot Pasteur pipette (method B).

2.3. DSC

The isothermal crystallization experiments were performed on a 2010 CE DSC (Texas Instruments, New Castle, DE, USA) with a refrigerated cooling

system (Texas Instruments, New Castle, DE, USA). The DSC was calibrated with indium (TA Instruments, New Castle, DE, USA), azobenzene (Sigma–Aldrich, Bornem, Belgium) and undecane (Acros Organics, Geel, Belgium) prior to analyses. Nitrogen was used to purge the thermal analysis system. Fat (7.0–13.6 mg) was sealed into hermetic aluminium pans and an empty pan was used as a reference.

2.4. Time–temperature protocol

The first part of the time–temperature protocol was the melting part, which must eliminate any persisting crystals of cocoa butter (homogeneous nuclei). Three different melting protocols were compared: holding at 65 °C for 15 min, holding at 65 °C for 30 min and holding at 80 °C for 15 min. For each melting protocol two repetitions were performed using sample preparation method A and three repetitions using sample preparation method B. After this melting period the sample was cooled at 8 °C/min to the isothermal crystallization temperature of 17.2 ± 0.1 °C and kept at that temperature until crystallization was completed (i.e. when the curve has returned to the baseline). A cooling rate of 8 °C/min was chosen taking into account the following considerations. On the one hand, the cooling rate must be high enough to prevent the melt from forming a lamellar structure in the liquid state, which would influence the subsequent crystallization process. Toro-Vazquez et al. [9] showed that this effect takes place when using a cooling rate of 1 °C/min when compared to 10 °C/min. On the other hand, the apparatus must be able to keep up with the cooling rate under all circumstances, since otherwise the reproducibility will be lowered. The possibility to maintain a specified cooling rate is checked by evaluating the plot of the actual temperature versus time (which should be a straight line) and by looking for a warning signal given by the apparatus when the specified cooling rate cannot be kept. The highest possible cooling rate was determined to be 8 °C/min. To get more insight in the effect of cooling rate an experiment was designed in which three different cooling rates (1, 5 and 8 °C/min) were compared. For each cooling rate three repetitions (three different cups of the same cocoa butter) were performed. It could immediately be seen from the DSC curves that when cooling at 1 °C/min, some crystallization already occurred during the cooling phase which is unwanted since it was the aim to study isothermal crystallization. The isothermal crystallization data after cooling at 5 and 8 °C/min were fitted to the Avrami model. No significant differences ($\alpha = 0.05$) between the parameters could be found (detailed results not shown) despite the fact that in literature [9,10] an effect of cooling rate has been found. It has to be remarked, however, that the cooling rates used in this study were much closer to each other than these in literature. Since in theory one should quench cool to the isothermal temperature, it was decided to use the highest cooling rate attainable by the apparatus, thus 8 °C/min.

2.5. DSC crystallization curves

The amount of heat released up to time t was determined by calculating the area between a horizontal sigmoid baseline and the DSC peak between t_0 (the start point of crystallization) and t with t varying between t_0 and t_e (the end point of crystallization). When t equals t_e the total heat of crystallization (ΔH) was obtained. Thus

$$\Delta H_{t_0-t} = \int_{t_0}^{t} \frac{\mathrm{d}Q}{\mathrm{d}t}\mathrm{d}t \tag{1}$$

for all t between t_0 and t_e and

$$\Delta H = \int_{t_0}^{t_e} \frac{\mathrm{d}Q}{\mathrm{d}t}\mathrm{d}t \tag{2}$$

in which $\mathrm{d}Q/\mathrm{d}t$ is the heat flow as experimentally determined by DSC.

The determination of t_0 and t_e was performed using an objective calculation algorithm as described in detail in the Section 3. The amount of heat released was calculated at 5-min intervals. The integration was performed using the Universal Analysis software version 2.5 H (Texas Instruments, New Castle, DE, USA).

2.6. Parameter estimation

The data series were fitted to the Avrami model, a model frequently used in literature to describe the crystallization kinetics of fats.

The Avrami model was developed in the 1940s for the description of the isothermal phase transformation kinetics. This equation is given as [11]

$$f(t) = a \times (1 - \exp(-k \times t^n)) \tag{3}$$

where $f(t)$ is the released crystallization heat (J/g) up to time t (h), a (J/g) the value for $f(t)$ as t approaches infinity, k (h^{-n}) a crystallization rate constant which depends primarily on the crystallization temperature and n ( ) is the Avrami exponent, which is a combined function of the time dependence of nucleation and the number of dimensions in which crystal growth takes place [12].

Parameter estimations were performed by non-linear regression using the Sigmaplot 2000 software (SPSS Inc., Chicago, IL, USA). This software uses the Levenberg–Marquardt algorithm to find the parameters that give the 'best fit' between the models and the data, expressed as the sum of squared errors.

2.7. Statistical analysis

To estimate whether the parameters differ significantly between groups of experiments and thus estimate whether there was a significant effect of the melting protocol and the sample preparation method an adapted t-test was developed. This adapted test takes into account that the calculated parameters of the models are themselves estimations. In particular, it is assumed that each estimator $\hat{\beta}_{ji}$ has expectation β_{ji} and variance $\sigma^2_{\beta_{ji}}$, and that the parameter β_{ji} is also a random variable with expectation β_j and variance σ^2_j. This specifies a hierarchical model. The test statistic is calculated as

$$t_w = \left| \frac{\overline{\beta_{(1)}} - \overline{\beta_{(2)}}}{\sqrt{s^2_{(1)} + s^2_{(2)}}} \right| \tag{4}$$

with

$$\overline{\beta_{(j)}} = \frac{\hat{\beta}_{j1} + \hat{\beta}_{j1} + \cdots + \hat{\beta}_{jn}}{n_j} \quad j = 1, 2 \tag{5}$$

with $\hat{\beta}_{ji}$ the parameter value as estimated by Sigmaplot for repetition i ($i = 1, \ldots, n$) and

$$s^2_{(j)} = \frac{s^2_j}{n_j} + \frac{s^2_{j1} + s^2_{j2} + \cdots + s^2_{jn}}{n^2_j} \quad j = 1, 2 \tag{6}$$

Eq. (6) calculates an estimator of the variance of $\bar{\beta}_{(j)}$, with s^2_{ji} being the estimator of the variance $\sigma^2_{\beta_{ji}}$ of $\hat{\beta}_{ji}$ as calculated by Sigmaplot for repetition i and s^2_j the sample variance of the n_j parameter estimates $\hat{\beta}_{ji}$ for one specific condition, i.e. s^2_j is an estimator of σ^2_j. Note that $\bar{\beta}_{(j)}$ is an unbiased estimator for β_j. Since the parameter estimators $\hat{\beta}_{ji}$ are asymptotically normally distributed, the test statistic is asymptotically standard normal under the null hypothesis $\beta_1 = \beta_2$. Though, for finite sample sizes, its null distribution is better approximated by a t-distribution. Since the exact number of degrees of freedom is not straightforward to determine, the use of a lower bound, $n_1 + n_2 - 2$, was chosen. This choice will result in a slightly conservative test. The test statistic has thus to be compared with the threshold value $t_{n_1+n_2-2,\,0.05}$ under the student t-distribution where n_1 is the number of repetitions for group 1 and n_2 is the number of repetitions for group 2.

3. Results and discussion

3.1. Calculation algorithm for t_0 and t_e

The calculation algorithm presented below was developed using crystallization data of different samples of cocoa butter at different crystallization temperatures (19–23 °C). The algorithm works well for all these data sets.

The amount of crystallization heat released as function of time was calculated using formula (1). To enable calculation of these values of released heat, t_0 and t_e have to be determined. In most articles [3,4] the procedure to determine t_0 and t_e is not explicitly mentioned, but it can be suspected that this is done visually, with t_0 and t_e being the visually decided start and end points of crystallization or the moment where the heat flow curve deviates from and returns to the baseline, respectively.

At the authors' laboratory an experiment was organised in which eight DSC-experienced people were independently asked to visually determine the start and end points of a crystallization peak for three different samples of the same cocoa butter (different DSC cups) at three different moments in time. Using these visually determined integration limits the total area of the peak between start and end points (ΔH) was calculated using a horizontal sigmoid baseline. Table 1 gives the results of this experiment (only the values of ΔH are given). It occurred that the coefficient of variation between the different determinations by the

Table 1
Visual determination of start and end points of crystallization as determined by eight persons on three different samples of the same cocoa butter at three different moments in time

Sample (time of integration)	Operators								Mean value using visual determination	Value using calculation algorithm
	1	2	3	4	5	6	7	8		
1 (1)	47.73	46.58	44.14	39.44	44.35	44.95	42.75	47.61		
1 (2)	46.90	47.70	47.53	46.76	46.63	47.55	21.63	44.57	44.61	46.47
1 (3)	46.64	46.00	**49.38**	41.50	46.95	46.79	39.11	47.49		
2 (1)	44.70	45.22	44.19	38.23	43.53	39.57	40.83	44.00		
2 (2)	45.88	42.12	44.95	44.27	43.63	46.98	31.90	44.55	43.33	48.60
2 (3)	48.36	47.68	**49.38**	43.86	42.73	47.95	30.44	45.13		
3 (1)	44.74	50.45	46.35	41.08	44.94	42.50	40.74	43.65		
3 (2)	48.23	47.30	47.53	42.12	41.47	45.98	41.72	47.65	45.22	49.84
3 (3)	50.39	45.46	**51.22**	42.08	42.29	47.78	41.57	47.92		
Mean coefficient of variation (%)[a]	3.74	4.44	5.66	6.10	2.82	6.34	16.79	3.37		

The calculated values of ΔH (J/g) are displayed. The underlined and bold values are the minimum and maximum values obtained for one sample by the different operators at different moments in time.

[a] The mean coefficient of variation is the mean value of the coefficients of variation calculated for each of the three samples.

same operator (the mean value of the coefficients of variation for the three samples was used) of t_0 varied between 1 and 2.7% while the difference between the lowest and the highest value obtained by one operator for one sample ranged between 0.04 and 2.37 min (detailed results not shown). For t_e, the differences were even bigger: the coefficient of variation ranged from 4.9 to 17.2% and the difference between the lowest and the highest value obtained by one operator for one sample varied between 1.61 and 46.09 min (detailed results not shown). Evidently, this spreading on the values of t_0 and t_e influences the area of the crystallization curve: for ΔH the coefficient of variation ranged between 2.8 and 16.8% while the lowest and highest values obtained by one operator for one sample differed by 0.9–21.12 J/g (Table 1). When the different operators are taken into account the overall coefficient of variation (one sample integrated by eight operators at three different moments in time) of t_0 varied around 3%, while the difference between the lowest and the highest value obtained for one sample varied around 4 min (detailed results not shown). For t_e, the coefficient of variation varied around 17% and the difference between the lowest and the highest value obtained for one sample varied between 58 and 96 min (detailed results not shown). Most important, the coefficient of variation for ΔH varied between 7 and 12.5%, while the difference between the lowest and the highest value for one sample ranged between 10 and 28 J/g (Table 1). Since the values of ΔH vary dramatically also the values of ΔH_{t_0-t} and consequently also the kinetic model parameter values will differ depending on the integration when a visual determination of the start and end points is performed. To eliminate this source of variability during the integration procedure an objective calculation algorithm for determining t_0 and t_e was developed (Figs. 1 and 2).

Fig. 1 displays an example of a DSC curve (dQ/dt as function of time) and its slope as function of time. The first part of the DSC curve is the period where the temperature decreases and equilibrates to the isothermal crystallization temperature (= equilibration). The main part of the DSC curve is the exothermal crystallization peak and after the crystallization the heat flow returns to the baseline (= end phase).

Taking into account the DSC heat flow equation:

$$\frac{dQ}{dt} = C_p \times \frac{dT}{dt} + f(T, t) \quad (7)$$

in which dQ/dt is the heat flow, C_p the heat capacity, dT/dt the rate of change of the temperature and $f(T, t)$ the heat flow from the crystallization process, one can assume that under isothermal conditions ($dT/dt = 0$) the slope of the heat flow curve will be zero when

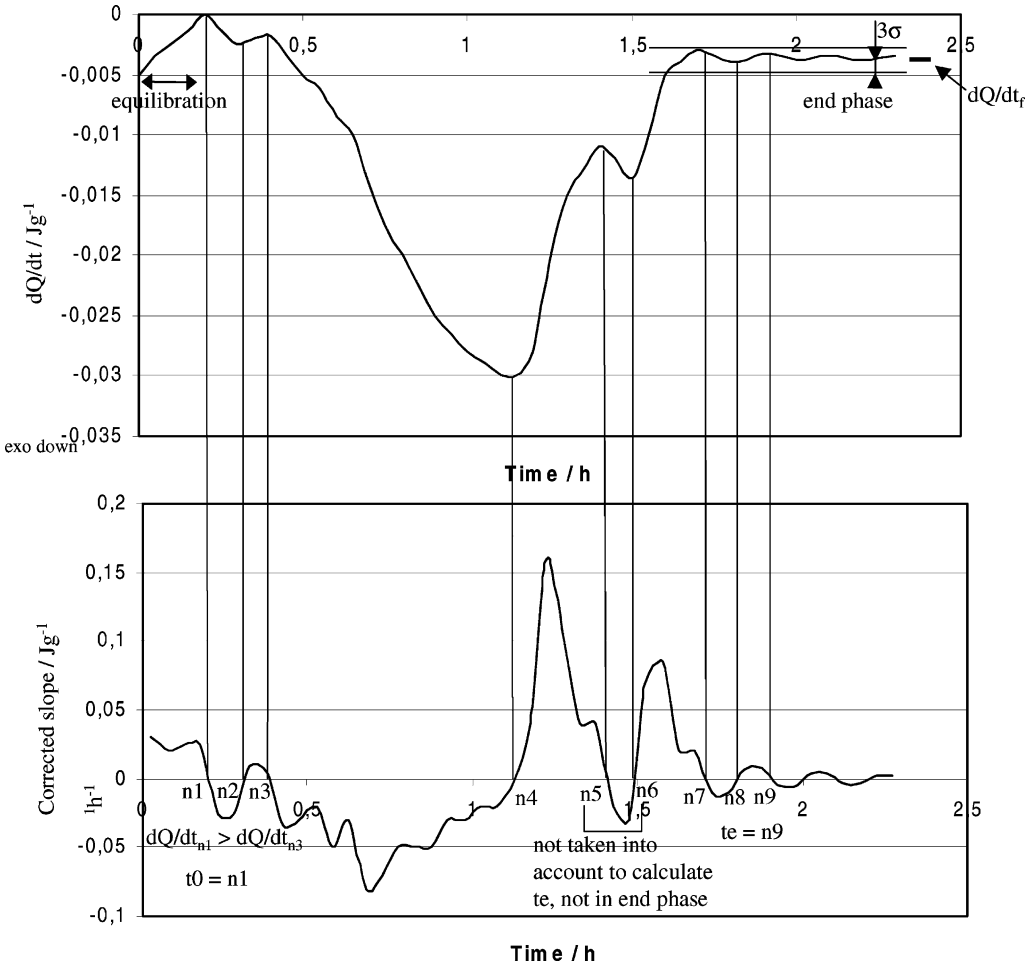

Fig. 1. Calculation algorithm for determining t_0 and t_e.

no crystallization ($f(T, t) = 0$) is occurring. For non-isothermal measurements, however, this reference slope will not be zero. Thus, in the latter case this reference slope will first have to be estimated.

During the equilibration period an exothermal decreasing heat flow and thus a positive slope can be seen (Fig. 1). This is caused by the change in dT/dt in this period. Once the temperature is stabilised while the crystallization has not yet started, the slope is, in theory, zero. However, in practice, this is never exactly the case. Once crystallization starts, the heat flow will start to increase to negative values (if exothermic events are plotted downwards) and the heat flow curve will show a certain negative slope. After the crystallization peak maximum the slope becomes positive. At the end of crystallization the heat flow curve returns to the baseline and as such, in theory, the slope changes from positive to zero. However, due to noise the heat flow curve may vary around the final value with alternating positive and negative slopes (Fig. 1).

As such, it was decided to determine t_0 as the point where the slope changes from a positive (equilibration period) to a negative (crystallization) value and t_e as the point where the slope changes sign for the yth time after the peak maximum.

Fig. 2 gives an overview of this algorithm that can easily be programmed in a spreadsheet.

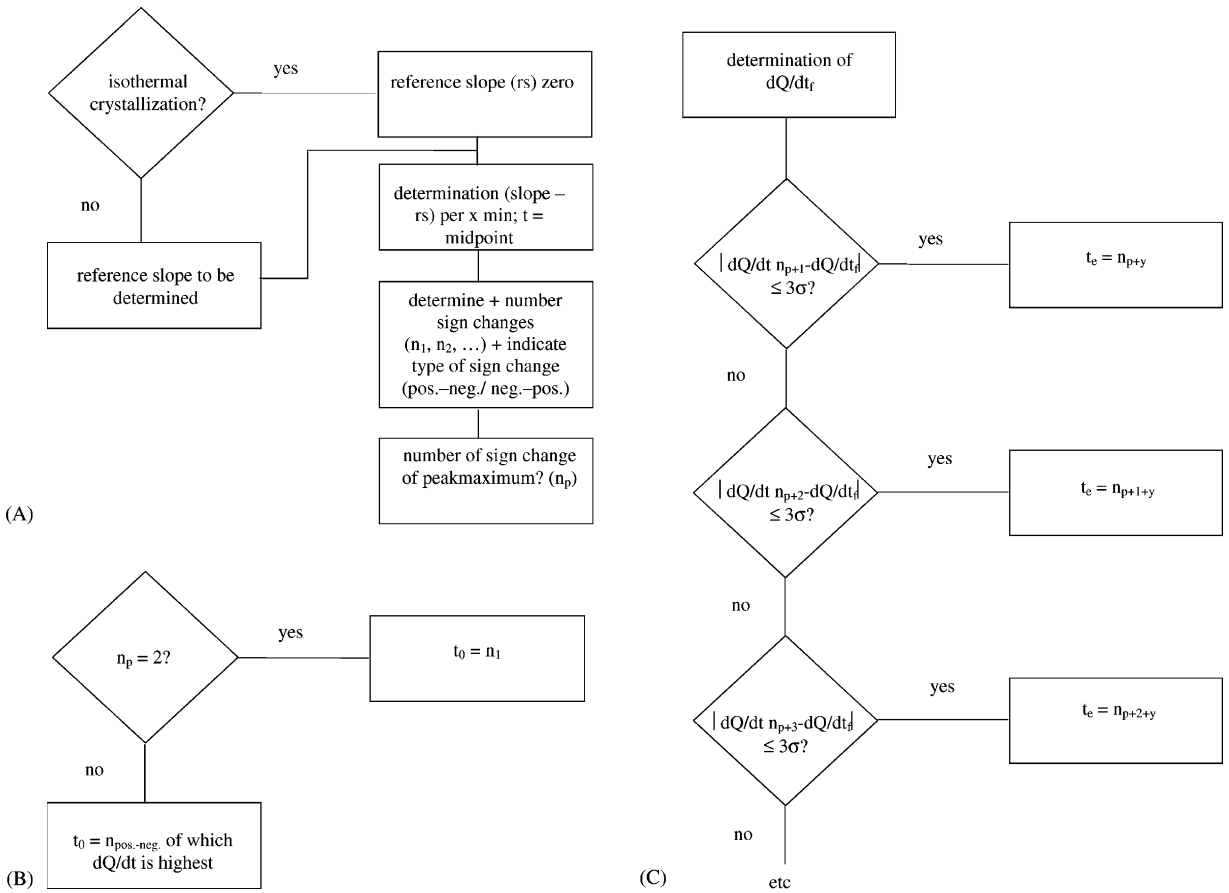

Fig. 2. Calculation algorithm for determination of t_0 and t_e (A) preparation step, (B) determination of t_0, (C) determination of t_e.

Part A of the algorithm (Fig. 2A) consists of some preparation steps to determine the sign changes in the corrected slope and to number them. The corrected slope is calculated for periods of x min.

Part B of the algorithm (Fig. 2B) determines t_0. In the most straightforward situation t_0 is determined as the time where the slope changes sign from positive to negative before the peak maximum. However, it is possible that several sign changes exist before the peak maximum (in that case the peak maximum does not correspond to the second sign change). This can be caused by a bump (i.e. a deviation from the normal course of the heat flow curve) in the equilibration phase or in the first part of the crystallization curve. In this case the time corresponding to the sign change with the highest heat flow value (when exothermic events are plotted downwards) is taken as t_0. However, only the sign changes from positive to negative are taken into account. In the example in Fig. 1, the peak maximum corresponds to the fourth sign change. Two sign changes (n_1 and n_3) qualify for t_0 as sign changes from positive to negative but n_1 is selected because it corresponds to a higher heat flow value.

Part C of the algorithm determines t_e. One has to make sure that only the sign changes at the real end of crystallization are taken into account and not the sign changes caused by a bump in the second part of the crystallization peak. Therefore, the heat flow value (dQ/dt) at each sign change after the peak maximum is compared to the last available heat flow values (dQ/dt_f). To eliminate the influence of an outlier, the median of the last five data points (i.e. the last 5 min) is taken as dQ/dt_f. If the difference between dQ/dt and dQ/dt_f is bigger than three times the noise

on the baseline (σ), the sign change still belongs to the crystallization peak and is thus not taken into account to determine t_e. In the example in Fig. 1, n_5 and n_6 are not taken into account because the difference between their corresponding heat flow value and dQ/dt_f is larger than 3σ. These sign changes are caused by the bump in the heat flow curve. Sign change 7 is the first which is in the end phase and as such t_e corresponds to n_9. The value of σ is determined as follows. First, a run or part of a run where no kinetic processes are happening (i.e. a straight baseline) is considered and a linear regression is performed on these data. The regression provides the sum of squared errors (SSE). The value of σ is given by Eq. (8):

$$\sigma = \sqrt{\frac{\text{SSE}}{z-2}} \tag{8}$$

where z is the number of data points used to calculate the regression.

The value of σ may thus be different for different instruments. In this study, a value of 0.000154 J/g was obtained.

The optimum values for x (the length of the period for which the slope is determined) and y (the number of the sign change after the peak maximum which is taken as t_e) may also change from instrument to instrument. In our research, the values of x and y were 7 and 3 min, respectively.

When the values of the mathematical determination were compared with those of the visual determination, the following conclusions could be drawn: for two of the three samples the mathematical value for t_0 was situated between the minimum and maximum value obtained by visual determination (by different operators at different moments in time), for the other sample it was slightly lower than the lowest visual value. For two samples, the mathematical value for t_e was higher than the highest visual value, for the other sample there were some visual values that were higher than the mathematical value. An explanation for the latter could be that when the crystallization curve is not enlarged enough for visual determination of t_e, it can appear that the curve has already returned to the baseline at a specific point, which is in reality not the case. The consequence for the integration was that the value of ΔH determined by using the mathematical values for t_0 and t_e was on the average about 9% higher than the mean of the values determined by using the visual integration limits (Table 1). However, for two of the three samples some values obtained by using visual limits were higher than the value using mathematical limits.

The variability caused by the visual determination of t_0 and t_e was compared with the sample variability, i.e. the variability originating from using different samples of the same cocoa butter. To do so, the total variability obtained in the experiment described in the beginning of this section was split-up in the sample variability, the inter-operator and the intra-operator variability. The inter-operator variability is the variability originating from different operators performing the integration of the same DSC curve, while the intra-operator variability is the variability when the same operator performs the integration of the same DSC curve several times. The different variabilities were determined using an ANOVA analysis with sample and operator as random factors. The effect of the factor sample allows to calculate the sample variability, the effect of the factor operator allows to calculate the inter-operator variability and the residual error allows to calculate the intra-operator variability. The standard deviations corresponding to these variabilities are quoted in Table 2. From this table, it can be seen that the variability caused by the visual determination of t_0 and t_e (inter- and intra-operator) is considerably larger than the sample variability, which shows that it is worth to use the calculation algorithm to determine t_0 and t_e of the crystallization curves.

3.2. Influence of melting protocol

The main aim of the melting part of the time–temperature protocol is to eliminate all homogeneous crystal nuclei so they cannot have any effect on the subsequent crystallization. As a reference, holding at 65 °C for 15 min (as used by Ziegleder [3]) was

Table 2
Split-up of the total variability in sample, inter-operator and intra-operator variability

Type of variability	S.D. (J/g)
Sample	0.77
Inter-operator	3.32
Intra-operator	3.24

The standard deviations corresponding to the variabilities are quoted.

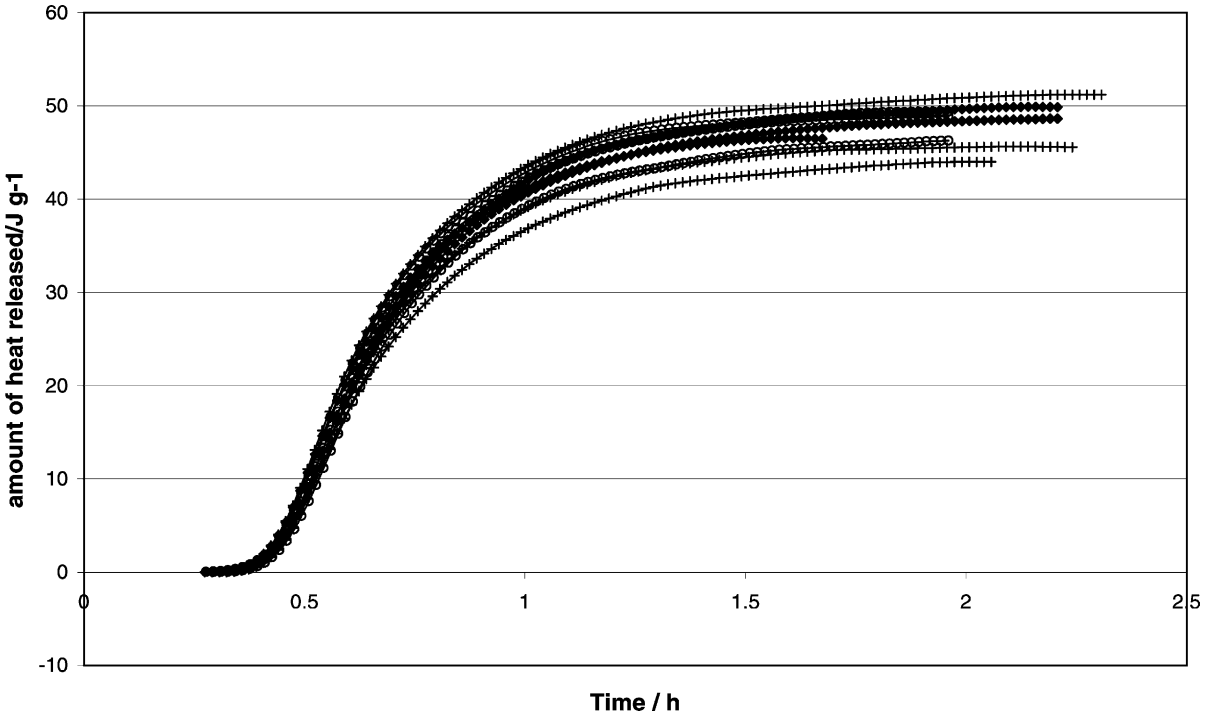

Fig. 3. Influence of melting protocol on the isothermal crystallization of cocoa butter (sample preparation method B). Legend: (◆) 65 °C for 15 min; (+) 65 °C for 30 min; (○) 80 °C for 15 min.

chosen. To check whether this time–temperature combination was adequate to melt all homogeneous crystal nuclei, it was compared with holding at 65 °C for 30 min and holding at 80 °C for 15 min. To compare the different crystallization processes, the parameters of the Avrami model were used.

To study the effect of the melting protocol, only the experiments performed with sample preparation procedure B were taken into account. Fig. 3 shows the crystallization curves for the different melting protocols. It can be seen that no obvious difference between the three melting protocols can be detected.

Table 3 gives an overview of the mean values and the standard deviations calculated for the three crystallization parameters for each of the melting protocols. Significance of the differences was checked using the adapted *t*-test. The three melting protocols were compared two by two and for none of the three crystallization parameters any significant difference ($\alpha = 0.05$) could be detected. Thus, it seems that holding at 65 °C for 15 min is enough to melt all homogeneous nuclei and eliminate any memory effect on the crystallization process. This result confirms the results of Van Malssen et al. [13] and Hachiya et al. [14] who reported that to eliminate memory effects, the temperature has to be raised only slightly above the melting point.

It has to be remarked that whether a specific time–temperature combination is sufficient to melt all

Table 3
Influence of heating protocol on the isothermal crystallization of cocoa butter (mean ± S.D. for three repetitions)

Crystallization parameter/heating protocol	Avrami *a* (J/g)	Avrami *k* (h^{-n})	Avrami *n* ( )
65 °C/15 min	47 ± 2[a]	2.7 ± 0.6[a]	3.4 ± 0.2[a]
65 °C/30 min	46 ± 4[a]	2.5 ± 0.1[a]	3.3 ± 0.1[a]
80 °C/15 min	47 ± 2[a]	2.7 ± 0.2[a]	3.5 ± 0[a]

The letter a in superscript indicate whether the sample preparation method has a significant effect; means with the same letter are not significantly different at $\alpha = 0.05$.

nuclei is independent of the temperature at which the following isothermal crystallization is performed. As such, the result obtained at 17.2 °C can be generalised to other isothermal crystallization temperatures. This could be illustrated by comparing the same melting protocols for dark chocolate (with cocoa butter as the fat phase) as a substrate and 20 °C as the isothermal crystallization temperature which led to the same result (detailed results not shown).

It has to be remarked that this study was conducted on cocoa butter and that for other fats the necessary melting protocol might be different. It is thus important to check whether the melting protocol has an influence on the crystallization process before isothermal DSC experiments are performed on a particular fat.

3.3. Influence of sample preparation method

Fig. 4 shows the influence of the sample preparation method on the crystallization curves. For clarity reasons, only the crystallization curves taken up with a melting protocol of 65 °C for 15 min are shown. It can clearly be noticed that the sample preparation method has an influence on the crystallization curve: when using sample preparation method A the induction time seems to be longer and the final value seems to be higher. Table 4 gives an overview of the mean values and the standard deviations calculated for the three crystallization parameters for each sample preparation method and this for the three melting protocols separately and irrespective of the melting protocol. Using the adapted *t*-test, it was investigated which crystallization parameters were significantly influenced by the sample preparation method. First, the influence of the sample preparation method was studied irrespective of the melting protocol used. Significant differences could be found for both the *a* and *k* parameters ($\alpha = 0.05$)). The higher values for *a* when using sample preparation method A are also illustrated clearly in Fig. 4.

The adapted *t*-test was also performed on the data for each melting protocol separately. No significant

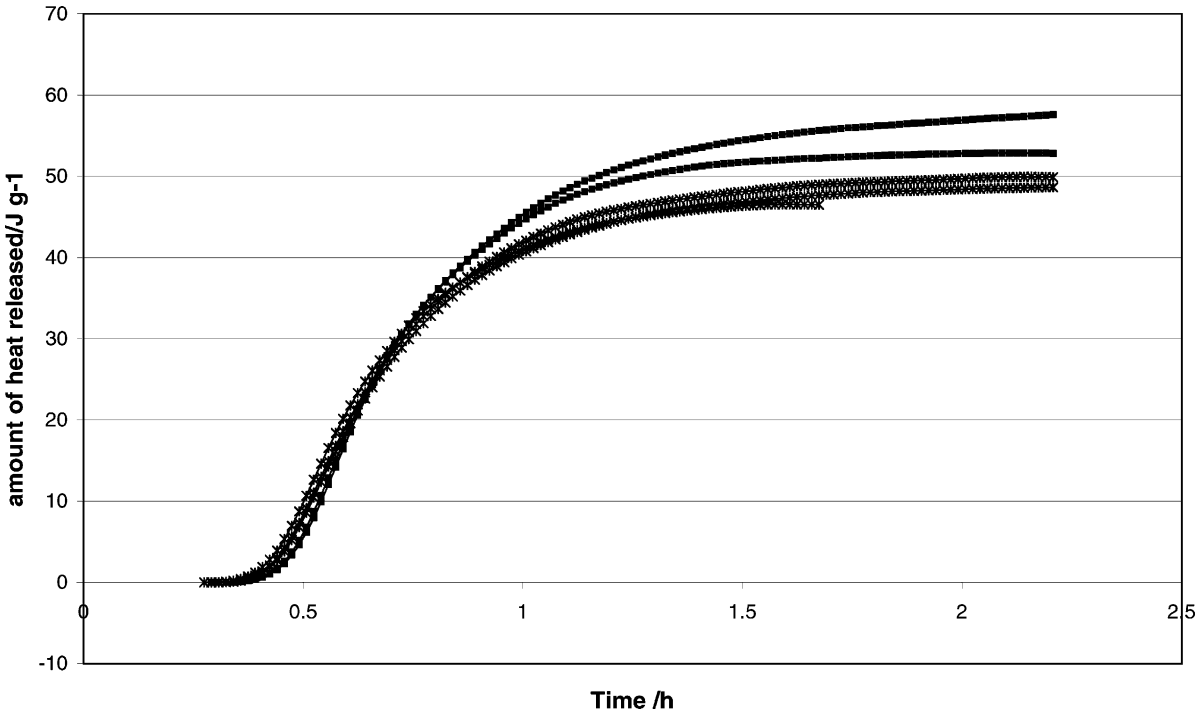

Fig. 4. Influence of sample preparation method on the isothermal crystallization of cocoa butter (melting protocol 65 °C for 15 min). Legend: (■) sample preparation method A; (✖) sample preparation method B.

Table 4
Influence of sample preparation method on the isothermal crystallization of cocoa butter (mean ± S.D. for two repetitions of method A and three repetitions of method B)

Crystallization parameter/heating protocol	Sample preparation method	Avrami a (J/g)	Avrami k (h^{-n})	Avrami n ( )
65 °C/15 min	A	54 ± 3^a	2.1 ± 0.4^a	3.4 ± 0.2^a
	B	47 ± 2^a	2.7 ± 0.6^a	3.4 ± 0.2^a
65 °C/30 min	A	51 ± 2^a	2.2 ± 0^a	3.45 ± 0.07^a
	B	46 ± 4^a	2.5 ± 0.1^a	3.3 ± 0.1^a
80 °C/15 min	A	49.8 ± 0.3^a	2.35 ± 0.07^a	3.55 ± 0.07^a
	B	47 ± 2^a	2.7 ± 0.2^a	3.5 ± 0^a
All heating protocols	A	51 ± 2^a	2.2 ± 0.2^a	3.5 ± 0.1^a
	B	46 ± 2^b	2.6 ± 0.2^b	3.4 ± 0.1^a

The letters a and b in superscript indicate whether the sample preparation method has a significant effect; means with the same superscripts are not significantly different at $\alpha = 0.05$.

differences could be found. However, when studying the data in Table 4, one notices the same trends (higher a values and lower k values) for each melting protocol separately. The lack of significance observed when the melting protocols were considered separately is probably related to the smaller number of repetitions, which reduces the power of the statistical methods.

It was expected that prevention of humidity condensing on the sample by using a desiccator to equilibrate the sample to room temperature and the more careful cleaning of all material would have led to less heterogeneous nuclei (foreign materials, e.g. dust particles) and thus to a longer induction time when using sample preparation method B. This could however not be observed (Fig. 4). Another difference between the preparation methods is the possibility of fractionation in method A caused by the use of cold, plastic pipettes. When using this kind of pipettes it is possible that high-melting triglycerides crystallize on the inner surface of the pipette, which is prevented by using hot, glass pipettes. Thus, in method B, where no fractionation is possible, a higher amount of high-melting triglycerides could be present in the final sample, which could possibly explain the shorter induction time observed when method B is used (Fig. 4). For the difference in the a and k parameters no reasonable explanation could be found. All in all, it is important to notice that the sample preparation method can have a significant influence on the crystallization process.

Although the experiments were only performed at one crystallization temperature and on one type of fat, one can expect that also for other temperatures and other fats the sample preparation method may have a significant influence on the crystallization process. Consequently, it is very important to keep the sample preparation method constant when preparing DSC samples. Otherwise a difference between two groups could be wrongfully attributed to a difference between the groups while the real reason is a difference in the sample preparation method. By eliminating the variability caused by a difference in sample preparation method, the variability on the data can be lowered and as such the quality of the data will increase.

4. Conclusions

When the start and end points of crystallization are determined visually, the result will strongly depend on the operator and will also differ when the same operator performs the integration several times. To eliminate the high variability caused by visually determining the integration limits, an objective calculation algorithm for the start and end points was developed.

Further, there is no significant difference between the melting protocols 65 °C for 15 min, 65 °C for 30 min and 80 °C for 15 min. Thus, 65 °C for 15 min is sufficient to melt all homogeneous nuclei of cocoa butter and can be used as a melting protocol in further experiments.

Finally, it is important to keep the sample preparation method constant since it can influence the

subsequent crystallization process and as such increase the variability on the data.

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