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Technical note

Measuring the specific surface area (SSA) of freeze-dried biologics using inverse gas chromatography

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ABSTRACT

The specific surface area (SSA) of freeze-dried biologics (FD) is usually measured via a Brunauer–Emmett–Teller (BET) analysis of volumetric nitrogen adsorption isotherms. However, this technique has accuracy limitations for materials < 0.5 m^2/g, requires dry samples, must be measured at 77 K and has slow sample preparation times (drying/degassing). Inverse gas chromatography (IGC) is chromatographic characterization technique which can be used to analyse the SSA (down to ≈0.1 m^2/g) of various solid-state materials including powders using organic molecules such as octane at ambient temperatures/pressure for a range of relative humidities. This study presents a comprehensive comparison between the N_2 BET adsorption versus octane BET adsorption using IGC methods for determining the SSA's for a range of freeze dried biological materials. These include IgG 5% w/w, an influenza antigen standard, sucrose 5% w/w and trehalose 5% w/w. IGC provided comparable SSA values to the N_2 BET method with better reproducibility (lower RSDs %). Large variations in average SSA within manufactured FD batches were observed for both IGC and volumetric determinations. IGC was also used to measure the change in SSA with increasing humidity, with FD cakes shrinking and losing their structural integrity with increasing moisture content. Such information highlights the importance of moisture content in determining the physical stability of FD cakes as exemplified by their SSA. In conclusion, IGC is a suitable alternative method for determining the SSA of freeze-dried biological materials which are generally strongly dependent on their moisture content.

1. Introduction

Lyophilisation, also known as freeze-drying (FD), is a process in which water is removed through sublimation under vacuum from a frozen solution containing a dissolved species [1]. Some biologics preparations are unstable as liquid formulations as they are prone to a host of solution chemical reactions which can lead to a loss of activity. Freeze drying is able to extend the shelf life of these biologics and increase their stability via the formation of a solid state final product form [2]. These FD materials present themselves as 3 D porous solids and are often referred to as FD cakes. Specific surface area (SSA) is a property of solid state materials and is defined as the total surface area per unit of mass. It is a highly relevant and common descriptor for all porous solids. The most commonly used method to determining the SSA of a freeze dried material is the Brunauer–Emmett–Teller (BET) analysis using typically nitrogen, or krypton, adsorption [3–8]. Beech et al. used the BET nitrogen adsorption to measure the effect of the cooling profile/annealing on the SSA of BSA and mAB1 formulations [9]. Rambhatla et al. used krypton adsorption (with better sensitivity) to measure cake shrinkage during freeze-drying [10]. The improved sensitivity of krypton adsorption relative to nitrogen can also be overshadowed by the increased adsorbate costs resulting in its infrequent use. N_2 adsorption methods have disadvantages in regards to requiring large amounts of samples for accurate determinations, lengthy experimental times and having significant accuracy limitations for areas below 0.5 m^2/g [3,11]. Furthermore, the need to fully dry the sample to run at 77 K for N_2 BET analysis means that changes in cake morphology or structure may occur during this process compared to a room temperature determination at a non-zero humidity. An alternate method for determining the SSA is Inverse Gas Chromatography (IGC) which can easily determine BET isotherms by using alkane vapour adsorption at real world conditions; room temperature and room humidity [11,12].

IGC is a chromatographic characterisation technique which analyses the surface properties of solid state materials including powders, fibres or films [13,14]. In IGC the roles of the stationary (solid) and mobile phase (gas/vapour) are inverted compared to traditional analytical GC.

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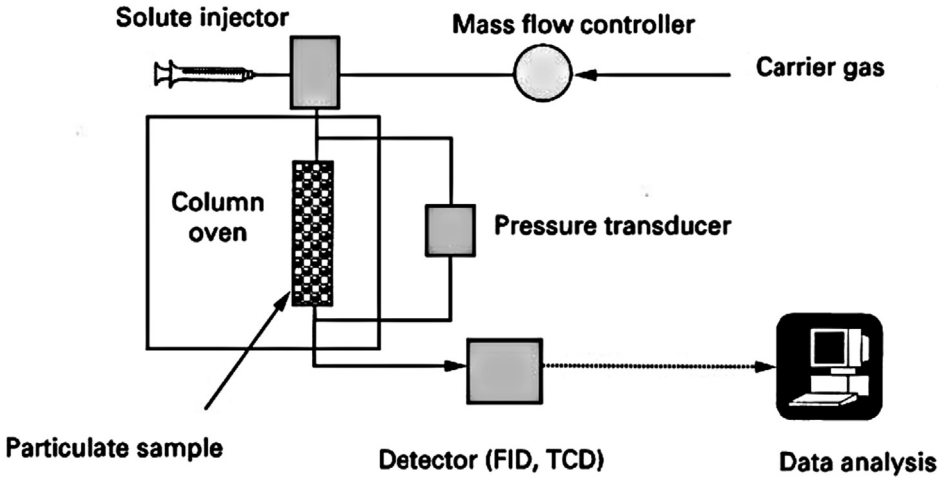

Fig. 1. Schematic diagram of an inverse gas chromatograph [13].

In analytical GC a reference column, containing a known solid stationary phase, is used to separate various unknown vapour or gaseous species into separate components. Whilst in IGC a single known vapour or gas probe molecule is injected into a packed column with the unknown solid sample to be characterised [14]. Sample masses are packed inside a glass column for IGC analysis (Fig. 1). A flame ionisation detector (FID) is commonly used to measure this retention time based on the detection of ions that form during the flame combustion of organic solutes.

The BET theory was originally developed with N_2 adsorption in mind for high surface area porous samples, but is also applicable to other inert gases or vapour adsorbates. The BET equations used to analyse IGC isotherms [11] and to calculate the SSA are given below:

$$\frac{P}{n(P_0 - P)} = \frac{C-1}{n_m C}\left(\frac{P}{P_0}\right) + \frac{1}{n_m C} \quad (1)$$

where P is the solvent or gas partial pressure in gas phase (Torr), P_0 is the saturated solvent vapour pressure (Torr), n_m is the monolayer capacity (Mol g^{-1}), n is the amount of gas absorbed (Mol g^{-1}) and C is the BET constant. The monolayer capacity n_m, can be determined from the slope and intercept of a linearized BET equation fitted to experimental data. The BET SSA ($m^2\,g^{-1}$) is given by:

$$S_{BET} = aN_A n_m \quad (2)$$

where a is the cross-sectional area of the adsorbate, N_A is the Avogadro constant and n_m is the monolayer capacity in moles per gram of adsorbent.

Measuring and detecting changes in surface properties such as the SSA of FD cakes might have a number of potential applications and uses including:

(a) may be a quality attribute which might track batch to batch variations in final products
(b) may correlate with FD cake biological stability over time
(c) could assist in formulation design and predicting re-constitution time of cakes
(d) would directly relate to average pore size distribution
(e) could predict variations in mechanical performance of FD cakes

As such determining the SSA more easily and quickly might allow it to be considered as a critical quality attribute (CQA) for lyophilised biological products [15]. IGC has successfully measured the SSA and surface energy for a wide range of materials such as polymers, fibres and pharmaceuticals [11,14,16]. However, there has been no work focused on using IGC to measure the SSA of FD materials. This study investigates the use of IGC as a suitable alternative method at determining the SSA for a range of FD biologics and excipients compared with traditional N_2 BET adsorption.

2. Materials and methods

2.1. Materials and lyophilisation cycle

5 ml volume screw capped vials (41.5 × 18 mm i.d. Schott VC005 (Adelphi Tubes, Haywards Heath UK) were filled to a fill-volume of 1 ml using an automated multi-pipette stream (Eppendorf, UK). The following materials were freeze-dried:

- IgG 1–5% w/w (NIBSC, Potters Bar UK)
- Influenza antigen B/Phuket 14/252 (formulated with 1% or 20% w/w sucrose and PBS) (NIBSC, Potters Bar UK)
- Lysozyme 1% w/w formulated with 1% w/w sucrose (L6876-5G, Sigma Aldrich, Dorset, UK)
- Sucrose (S5016-1 kg, Sigma Aldrich, Dorset, UK)
- Trehalose (Cargill 16400, Minneapolis, MN, USA)

Solutions of IgG and B/Phuket were FD on the Telstar LyoBeta 15 (Azbil-Telstar SpA, Terrassa, Spain), while lyzosyme, sucrose and trehalose were FD on a Virtis Advantage Plus (Biopharma Process Systems Ltd, Winchester, UK). The freeze-drying cycle run for all material batches are show in Table 1. After the cycle had finished the vials were backfilled with N_2 gas to atmospheric pressure before stoppering down on the 14 mm diameter igloo halobutyl stoppers (Adelphi Group, Haywards Heath, UK). Afterwards the vials were capped by hand, labelled and stored at −20 °C.

2.2. IGC SSA determination

IGC-SEA instrument (Surface Measurement Systems, London, UK) (Fig. 1) was used to obtain the SSA for FD materials. FD samples (between 100 and 400 mg which equated to 4–8 pooled vials from a batch) were lightly crushed into pieces, funnelled and packed into a 30 cm × 6 mm × 4 mm id Glass Columns (Analytical Columns, Croydon, UK) with the ends plugged by silanized glass wool (20411, Sigma Aldrich, Dorset, UK). Helium was used as the carrier gas and methane was used for the dead volume determination. The retention times of the adsorption solute octane and methane were determined using a flame ionization detector (FID). All experiments were conducted under identical conditions: at 293 K with a flow rate of 10 sccm (standard cubic centimetres per minute) with 0% relative humidity (RH), unless otherwise specified. A preconditioning step of 2 h at 303 K was chosen with a maximum elution time of 5 min for each injection

Table 1
Freeze drying cycle for each material.

Material	Freezing Temperature (°C)	Freezing Ramp Rate (°C/min)	Freezing Hold Time (min)	Primary Drying Ramp Rate (°C/min)	Primary Drying Temperature (°C)	Primary Drying Pressure (mTorr)	Primary Hold Time (min)	Secondary Drying Ramp Rate (°C/min)	Secondary Drying Temperature (°C)	Secondary Drying Pressure (mTorr)	Secondary Hold Time (min)
Sucrose (5% w/w)	−50	0.20	240	0.3	−35	70	2400	0.05	25	20	960
Trehalose (5% w/w)	−50	0.20	240	0.3	−35	70	2400	0.05	25	20	960
IgG (1–5% w/w)	−40	1.00	120	0.8	−15	150	1200	0.15	30	20	600
Lysozyme (1% w/w)	−50	0.78	300	0	−50	20	3765	0.12	15	20	1100
Influenza antigen B/ Phuket 14/252(1% w/w sucrose)	−50	0.30	240	0.45	−35	100	2400	0.07	25	20	1200
Influenza antigen B/ Phuket 14/252 (20% w/w sucrose)	−50	0.30	240	0.45	−35	100	2400	0.07	25	20	1200

which corresponded to a specific total fractional surface coverage point (which was adjusted accordingly to each sample). For the accurate determination of the IGC BET SSA, the peak maximum method was used, as the retention time at that peak maximum can give a better representation of the elution time of the monolayer probe molecules. The peak maximum chromatogram data was analysed using the BET equation. As shown in Eq. (1), the linear region of the BET equation was fitted using equilibrium partial pressures P/P_0 between 0.05 and 0.35. The monolayer capacity n_m was obtained from the slope and intercept which was then used to work out the BET SSA using Eq. (2). In a few cases data points near 0.05 and $0.35 P/P_0$ contributed to non-linearity of the isotherms and were excluded, with typical BET resultant coefficients of determination $R^2 > 0.995$. For sample reproducibility all isotherms and BET surface areas was calculated using the SMS-Surface Energy Analysis Software (v1.4.2.0, Surface Measurement Systems, London, UK).

2.3. Nitrogen (N_2) BET SSA determination

N_2 adsorption method was used to determine the BET surface area and compare with the IGC based method. FD samples were degassed overnight at 303 K under vacuum, loaded into glass tubes and then placed into a 3Flex Physisorption Analyser (Micromeritics, Norcross, GA, U.S.A) run at 77 K. N_2 isotherms were acquired using a P/P_0 range of 0.01–0.99. BET data analysis (P/P_0 0.05–0.35) was performed using the 3Flex software (Micromeritics, Norcross, GA, USA).

2.4. Moisture content

Residual moisture content analysis in the FD cakes was performed using Automated Coulometric Karl Fischer titration (Mitsubishi CA-200, A1-Environsciences Ltd, Blyth, UK). FD samples were transferred into HPLC auto sampler vials (Code: 10027364, Thermo Scientific, UK). All samples were manipulated under low humidity (< 5% RH) within a pyramid dry bag (Captair Pyramid, 2200A, #12847CN, Cole Parmer, London, UK) that had been purged with dry N_2 gas to avoid any environmental moisture uptake. A total of three repeats was measured for all samples (n = 3).

3. Results and discussion

In Table 2, the reproducibility (RSD %), operating temperature, time and pressure for FD samples run in both IGC and N_2 methods are compared. Three repeat runs were repeated on the same sample column/tube. These higher RSD's for N_2 adsorption determinations are likely due to the relatively larger dead volumes within the volumetric instrument which becomes critical for lower surface area solids. Dead volumes are critical for volumetric systems, but less critical for flow systems such as IGC-SEA. Previous studies have also corroborated the reproducibility within the IGC technique. Legras et al. investigated the IGC reproducibility of the natural fibres within the same columns [11]. They showed that relative standard deviation ranged between 0.1 and 3.5%, highlighting the excellent reproducibility. An advantage of using IGC is that it can run at significantly higher temperatures (including

Table 2
Comparison of IGC and N_2 adsorption methods used for SSA Determinations in this study.

	N_2 Adsorption Volumetric	Octane Adsorption IGC
Sample Mass (g)	0.1–0.4	0.1–0.4
RSD (%)	3–8	1–4
Temperature (K)	77	293
Pressure (Atm)	0.001–0.01	1
Relative Humidity (%RH)	N/A	0–90%

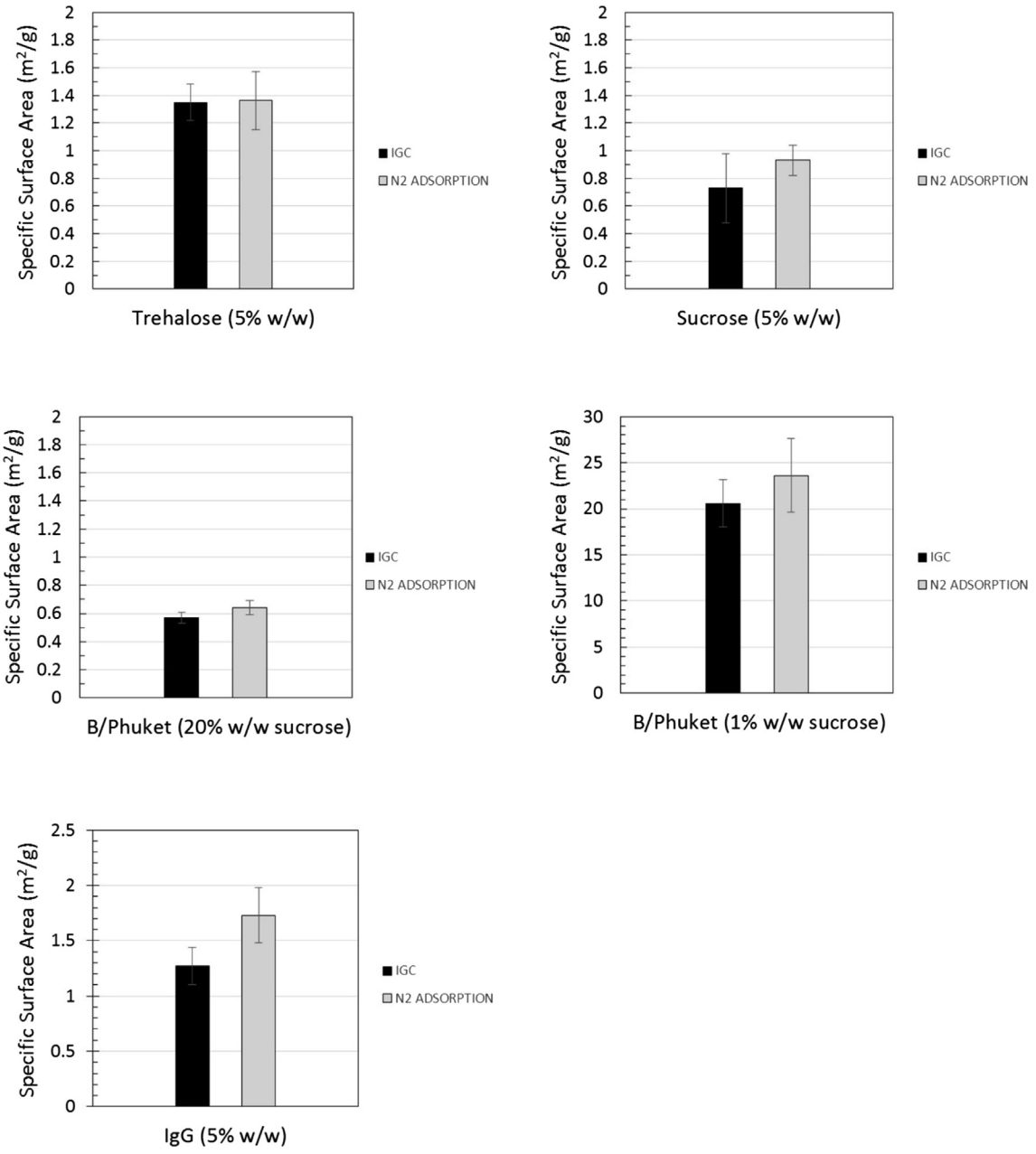

Fig. 2. Comparison in SSA (m^2/g) of pooled vials from a batch of the freeze-dried material as measured by Octane IGC adsorption at 293 K and 0% RH or N_2 adsorption at 77 K. A total of 3 columns or glass tubes were tested for each material (n = 3). (Error bars represent ± 95% confidence intervals).

ambient temperatures) and atmospheric pressure compared to typical gas adsorption methods such as N_2 as shown in Table 2. Additionally, another advantage of IGC experiments is that it can be operated at any sample humidity between 0 and 90% RH.

Fig. 2 shows the measured average SSA (m^2/g) for both the octane IGC method and the N_2 volumetric adsorption via BET analysis (error bars representing 95% confidence intervals.) Several sample vials were taken randomly from the same manufactured batch pool to examine SSA consistency across a batch. Three different packed columns or samples tubes for both IGC and N_2 BET methods were tested (n = 3). IGC provided comparable SSA values to N_2 adsorption technique for all samples. FD excipients, trehalose 5% and sucrose 5% exhibited IGC measured SSA values of 1.35 ± 0.13 and 0.73 ± 0.25. FD biologics, IgG 5%, B/Phuket 1% and B/Phuket 20% had IGC measured SSA values of 1.31 ± 0.18, 20.56 ± 2.57 and 0.57 ± 0.04 respectively. B/Phuket with 1% w/w sucrose has the highest SSA and exhibited the greatest variability. The 1% w/w sucrose formulation has the greatest porosity and appeared as a more 'fluffy' brittle cake, which made it more difficult for column/tube packing. Increasing the sucrose content to 20% w/w decreased the resulting SSA dramatically by a factor ≈ 20 and made the cake significantly stiffer. The observed sample variability within a specific manufactured batch shown by both techniques is well outside of both instruments reproducibility, and could arise from a range of factors including [17–20]:

I. Cake heterogeneity variances in each vial due to process parameters or tray location within the freeze drier
II. Inaccuracies introduced from physical manipulation, extraction and loading of FD cake from the vials for the BET analysis
III. Vial to vial variations in moisture content associated with I.

These factors would benefit from a detailed investigated in future work, however a preliminary discussion follows. It has been suggested that the silanized glass wool used to hold the powders inside the IGC

Table 3
Bound moisture measured in FD samples by coulometric Karl Fischer Titration (n = 3).

Sample	Moisture Content (w/w %)
Sucrose (5% w/w)	0.78 ± 0.07
Trehalose (5% w/w)	0.96 ± 0.19
IgG (5% w/w)	0.11 ± 0.05
Influenza antigen B/Phuket 14/252 (1% w/w sucrose)	0.13 ± 0.07
Influenza antigen B/Phuket 14/252 (20% w/w sucrose)	1.67 ± 0.18

columns could possibly also have an effect for samples with very low surface area [21]. However, glass wool has a surface area of 0.27 m^2/g [21]. Since there is < 20 mg of glass wool packed in the column with the FD sample, it typically accounts for a small % of the total surface area and therefore is negligible. Another point worth considering is that process of sample loading onto the column or glass vials, could affect the SSA. While loading, the sample is exposed to the environmental atmosphere for a short period. For extremely delicate materials that are sensitive to moisture, structural changes might occur before complete transfer of sample to column/tube in order to have degassing or pre-conditioning step to remove any water present.

Additionally one of the other considerations and limitations of this (and most studies with gas adsorption) when conducting BET surface area analysis of lyophilised materials is that they require extraction, manipulation and breakage of cakes from the vials to place inside sample tubes or columns. These largely unstandardized cake extraction approaches could give rise to errors and variability. Micromeritics 'Application Note 161' compared the SSA of lyophilized sucrose with non-intact crushed cakes versus fully intact cakes with a custom sample holder using krypton adsorption method [18]. Their data showed that the SSA of fully intact cakes was 35–55% lower than manipulated/crushed non-intact cakes. Future studies should further investigate to what extent that cake manipulation, packing and sample mass might have on the SSA values determinations. It is not clear in this study whether the freeze dried cakes were lightly crushed, or heavily crushed resulting in a micronized sample. Another significant factor is likely to be vial to vial variations in the batch due the manufacturing process or vial location in the freeze drier. Detection of lot-to-lot variations in lyophilised protein formulations have been discussed and reviewed by Hirakura et al.[19] and Wahl et al.[17], whilst Hancock et al., have communicated about heterogeneity in cakes from possible various amorphous forms emerging out of complex FD processes [20]. Nevertheless, the current study shows that IGC and N_2 adsorption methods give satisfactory agreement for SSA values for a wide range of FD cake

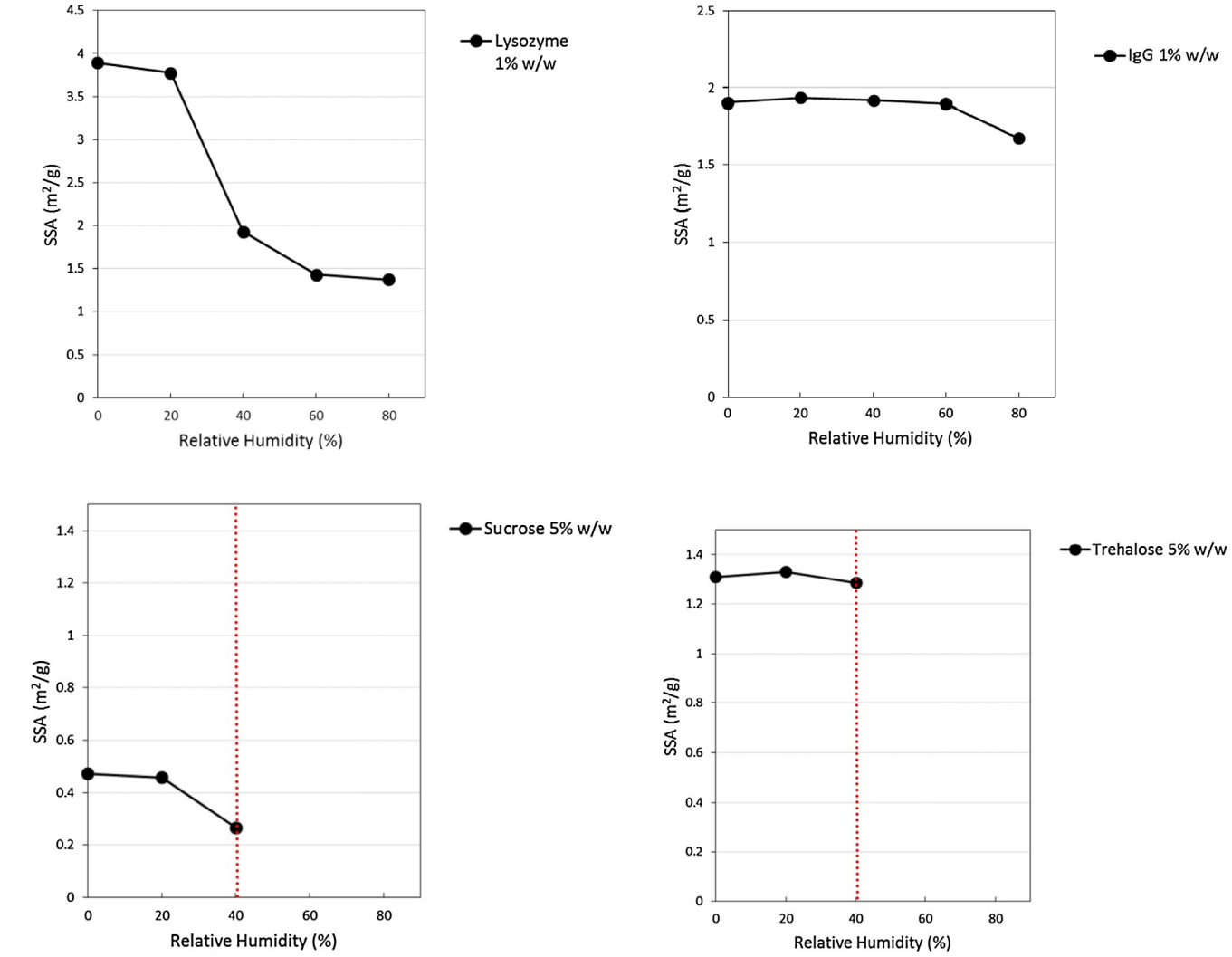

Fig. 3. Changes in SSA (m^2/g) of FD materials, conditioned at different RH (%) (held for 2 h at each step). The red dotted lines represents the last SSA determination after which BET sample analysis gave poorly fitted data. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

materials.

The FD cake moisture content (w/w %) was measured using coulometric Karl Fischer titration. The FD samples were all tested in triplicate from single vials and the water content is shown in Table 3. All FD samples have a moisture content of less than 2% w/w, which for this study, provides a context when quoting their specific surface areas. Most FD material are extremely hygroscopic and over time can display moisture increase even during storage. Water is a known plasticiser and once the moisture content exceeds a critical threshold, the glass transition point (T_g) is reduced. The amorphous solids then undergoes a transition from a brittle glassy state to a more rubbery state with increase molecular flexibility. In the case of FD cakes such moisture uptake events could result in changes in the SSA. In a moisture uptake study by Duralliu et al, they showed visually that with increasing humidity (and moisture gained) the FD cake will shrink and eventually collapse after exceeding a certain critical moisture content [22]. Certainly when considering the SSA for any FD material, the moisture content is a vital property for the FD cakes, however here there are no reports on the dependency of cake SSA on cake moisture content. IGC allows the FD packed column to be conditioned and tested at various relative humidity's (%) and any major changes in SSA to be measured. Fig. 3 shows the impact of holding for 2 h at a fixed humidity (and thus gain in moisture content) on the SSA of several FD samples. From 0% RH to 40% RH lysozyme 1% w/w has lost almost 50% of its original SSA value. After the final 80% RH conditioning step, IgG 1% w/w loses roughly 13% of its original SSA. Excipients trehalose 5% w/w and sucrose 5% w/w, between 0% RH and 20% RH, have a drop of SSA by 3% and 43% respectively. The SSA values could not be determined above 40% RH for sucrose and trehalose since there was significant non-linearity in the BET analysis. This indicates that sufficient partial collapse or possibly a phase transition of the sample inside the IGC columns had occurred which was confirmed visually. FD sucrose has been shown to exhibit phase transition (such as crystallization) at or above 40% RH [23].

4. Conclusions

IGC has been used to determine the SSA of FD materials. The instrument reproducibility of the IGC technique has here been shown to be better than N_2 BET adsorption method. IGC SSA values were agreed with those determined by the N_2 adsorption method. There was a significant vial to vial variability in SSA measurements using both techniques, confirming a significant vial to vial variation in the SSA within the manufactured batch of FD vials. Other advantages of IGC include the ability to vary the RH (%) the FD cakes is exposed to whilst also working at ambient temperature/pressure. For all FD materials studied, conditioning at low humidities resulted in very little change in SSA, compared to the larger drops seen at higher humidities, where for some formulations, complete structural cake collapse was observed. This information further highlights the practical importance of moisture content in determining the physical stability of FD cakes as exemplified by their SSA.

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