AACC International Approved Methods Technical Committee Report: Collaborative Study on Determination of Total Dietary Fiber (Digestion-Resistant Carbohydrates per Codex Definition) by a Rapid Enzymatic-Gravimetric Method and Liquid Chromatography

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Summary

A method for measurement of total dietary fiber (TDF) (1,2), as defined by the Codex Alimentarius Commission (ALINORM 09/32/26 [4]), was validated for plant materials, foods, and food ingredients. The method measures insoluble dietary fiber (IDF) and total soluble dietary fiber (SDF), including SDF that precipitates from 78% aqueous ethanol (SDFP) and SDF that remains soluble in 78% aqueous ethanol (SDFS). AACCI Approved Method 32-60.01 (Integrated Method for Total Dietary Fiber [9]) is an update on AACCI Approved Method 32-45.01 (Total Dietary Fiber (Codex Alimentarius Definition) [1,7,8]) that is designed to address issues identified by analysts using AACCI 32-45.01 over the past eight years.

Values for higher molecular weight dietary fiber (HMWDF; IDF plus SDFP) were determined gravimetrically for samples that did not contain resistant starch(es) (RS) and were essentially the same as those obtained using AACCI Approved Methods 32-05.01 (Prosky method) (1,12) and 32-07.01 (Lee method) (1,6). The HMWDF values obtained for most samples containing RS were similar to those obtained using AACCI 32-45.01, with the exception of samples containing RS type 2 (RS2; native, high-amylose maize starch) and RS type 4 (RS4; phosphate cross-liked native starches), for which significantly higher dietary fiber values were obtained.

The method was evaluated through an AACC International and ICC collaborative study. Sixteen test samples (eight blind duplicates) with a range of traditional dietary fiber, RS, and nondigestible oligosaccharide contents were assayed by thirteen laboratories. All laboratories returned valid data. In total, only 4 sets of data from the 104 sets submitted were statistically excluded as outliers. Dietary fiber content ranged from 6.79 to 60.6%; within-laboratory variability (s_r) ranged from 0.29 to 0.74 (1.22 to 6.34%, relative); and between-laboratory variability (s_R) ranged from 0.57 to 4.67 (2.64 to 13.38%, relative).

Introduction

A method for measurement of TDF (1,2), as defined by the Codex Alimentarius Commission (ALINORM 09/32/26 [4]), was validated for plant materials, foods, and food ingredients. The Codex definition for dietary fiber adopted in June 2009 (4) includes carbohydrate polymers that are not hydrolyzed by the endogenous enzymes in the small intestine of humans, including RS. This definition also includes oligosaccharides with degrees of polymerization ≥3. The decision on whether to include these oligosaccharides in the dietary fiber value provided on product labels was left to the discretion of national authorities.

A method (7) designed to support implementation of the Codex definition published in 2007 (3) was successfully evaluated in an interlaboratory study (1,8) and approved as AACCI 32-45.01 (1). In this method TDF is measured by summing the quantity of a digestion-resistant food fraction, including IDF and SDF that precipitates in the presence of 78% aqueous ethanol (SDFP), and the SDF that remains soluble in 78% aqueous ethanol (SDFS). Subsequent applications of this method to a range of food products and ingredients identified several issues:

1) Incubation with pancreatic α-amylase (PAA) and amyloglucosidase (AMG) enzymes for 16 hr does not simulate likely physiological conditions. In AACCI 32-45.01 (1), samples are incubated with a solution of PAA and AMG at 37°C and pH 6.0 for 16 hr (the digestion step parallels the incubation conditions employed in AACCI 32-40.01 for measuring RS) (1). A more likely residence time for food in the small intestine is 4 ± 1 hr (McCleary et al. [9,10] and referenced literature).
2) Most commercially available fructooligosaccharides (FOS) contain the trisaccharide fructosyl-β-(2-1)-fructosyl-β-(2-1)-fructose (inulinotriose; F3), which cannot be measured using the Sugar-Pak high-performance liquid chromatography (HPLC) column (Waters).
3) During hydrolysis of products that are high in nonresistant starch, resistant maltodextrins are produced that are incorrectly measured as dietary fiber (9,10).
4) Phosphate cross-linked starch (RS4, e.g., Fibersym [MGP Ingredients]) content is underestimated.
5) Long incubation times with PAA and AMG require the incorporation of sodium azide in buffers to prevent undesirable microbial growth. Although the azide concentration employed is low (0.02%, w/v), it is still considered a health and safety concern for analysts working with the chemical.

AACCI 32-60.01 (9) employs the same basic biochemistry and enzymes (PAA, AMG, and protease) that are used in AACCI 32-45.01 (1), but it resolves each of the issues described above. In particular, with an incubation time of 4 hr, it more closely simulates likely physiological conditions. Additionally, HPLC is performed using TSKgel® G2500PW$_{XL}$ col-

https://doi.org/10.1094/CFW-63-2-0080

umns (Tosoh Bioscience LLC) for gel permeation chromatography (5) with in-line deionization (11).

Precollaborative Ruggedness Testing

To allow the analysts to familiarize themselves with the method, evaluate the protocol, and identify potential problems, four samples were sent to each collaborator with the request that they perform a single analysis on each sample. All key reagents, including the components of the Rapid Integrated Total Dietary Fiber assay kit (Megazyme), anion and cation resins, and the required polypropylene tubes, were supplied to each analyst together with the samples. The key steps and processes required to perform the method effectively were highlighted, e.g., preferred incubation bottles, methods for suspension of samples during incubations, and HPLC equipment. To minimize calculation errors, collaborators were asked to use a Mega-Calc Excel (Microsoft Corp.) spreadsheet (Megazyme) to compute results. The results obtained for the TDF content of the four samples, including statistical analysis, are shown in Table I. The between-laboratory variability (s_R) ranged from 1.10 to 3.40% TDF (RSD_R = 5.64 to 9.28%), which is consistent with statistics reported for analyses of similar samples utilizing other dietary fiber methods (13).

As results were received from the collaborators, it became clear there were some problems and misunderstandings associated with the method protocol, particularly with regard to measurement of the SDFS fraction using HPLC. To clarify the reasons for these problems, the collaborators were surveyed regarding

a) The method used to shake or stir the samples during the 4 hr incubation with PAA and AMG: 2mag submersible stirrer (2mag AG) with stirring in bottle; shaking water bath in orbital motion; or shaking water bath in linear motion with containers held at an ~45° angle to ensure that all sample was continually suspended.
b) The HPLC columns used: TSKgel® $G2500PW_{XL}$ columns or other—if other, which columns. A copy of the HPLC trace for sample 4 (Heinz baked beans) was requested so separations, etc. could be checked.
c) The internal standard used: if a glycerol internal standard was not used, how was the SDFS quantified?
d) Any changes made to the method: details were requested so deviations in the results could be explained and the flexibility of the method could be determined.
e) Any particular problems experienced with the method: details were requested so they could be considered and addressed before the full study was initiated.

Table I. Study data for precollaborative evaluation of AACCI Method 32-60.01 for determination of total dietary fiber[a]

	Total Dietary Fiber (%, w/w)			
Laboratory[b]	A	B	C	D
1	12.46	62.32	20.46	22.24
2	10.93	66.0	19.34	21.5
3	9.95	51.32	18.56	21.88
4	13.1	62.9	19.7	23.2
5	11.40	60.39	19.64	23.23
6	11.48	58.95	18.00	21.25
7	12.53	62.67	21.00	21.22
8	13.04	57.74	19.52	22.17
9	13.04	60.46	19.79	22.78
10	12.34	61.17	19.57	22.74
11	11.57	59.83	19.63	23.57
12	12.28	60.23	20.97	16.85
13	9.86	60.38	16.58	20.07
Mean of lab averages	11.85	60.33	19.44	21.75
s_R	1.10	3.40	1.20	1.77
RSD_R (%)	9.28	5.64	6.15	8.13

[a] Samples: A = whole meal bread; B = high-amylose maize starch (Hylon VII, Ingredion); C = carrots lyophilized; D = baked beans (Heinz) washed and lyophilized.

[b] s_R = reproducibility standard deviation; RSD_R = reproducibility relative standard deviation.

For the gravimetric determinations of HMWDF, no specific problems were identified by the collaborators. Measurement of SDFS was more challenging. Collaborators were asked to prepare standardized solutions from supplied glycerol and D-glucose prepared by the collaborator. The response factors (D-glucose and glycerol) varied between laboratories, so the decision was made to provide the D-glucose and glycerol solution in a stable, ready-to-use form for the full collaborative study. The response factors obtained with glycerol at 10 mg/mL and D-glucose at 5, 10, or 20 mg/mL were essentially the same. Thus, all standardization was subsequently performed with solutions of D-glucose at 10 mg/mL and glycerol at 10 mg/mL.

Upon completion of the precollaborative study and implementation of necessary method protocol adjustments, the full collaborative study was initiated.

Collaborative Study Protocol

Eight food samples were selected for the collaborative study, and because the main focus of the study was to evaluate complex food samples containing RS and nondigestible oligosaccharides, samples high in these components were chosen. The samples included legumes, phosphate cross-linked starch (RS4), whole grain products, and food products enriched with RS and nondigestible oligosaccharides. Moist samples were freeze-dried. All samples were ground to the method-specified size, homogenized, and mixed thoroughly before being subdivided into glass vials that were then sealed and capped. Samples, copies of the method, electronic report sheets, Excel-based calculators, sample storage instructions, and an adequate supply of enzymes, reference standards, and resins were shipped to collaborating laboratories using express overnight shipment.

Thirteen laboratories completed the study and reported a full set of results. Two laboratories advised the study director that they lacked access to TSKgel® $G2500PW_{XL}$ HPLC columns. These collaborators completed all the steps through concentration of the SDFS fractions and then shipped the concentrates to the study director's laboratories, where the concentrates were deionized and chromatographed on TSKgel® $G2500PW_{XL}$ columns. The results were then submitted back to the collaborators for calculation and reporting.

Statistical Analysis

Data from the collaborative study were evaluated statistically according to AACC International protocols using software supplied by AOAC International. Of the 104 valid pairs of assay results reported for TDF content, laboratories 1, 2, 4, 5, 6, 7, 8, 9, 10, and 11 had no statistical outliers. Laboratories 3 and 13 had one statistical outlier each, and laboratory 12 had two statistical

Table II. Interlaboratory study results for determination of total dietary fiber in foods

	Sample[a,b]															
Lab	**A**	**D**	**B**	**F**	**C**	**J**	**E**	**H**	**G**	**N**	**I**	**M**	**K**	**O**	**L**	**P**
1	58.70	59.44	25.26	24.42	30.82	29.56	6.07	6.31	16.91	17.33	19.59	19.91	21.03	21.23	10.36	10.60
2	68.04	69.30	23.82	25.67	30.64	31.19	7.19	7.97	17.26	17.24	21.84	21.48	22.23	21.29	10.00	10.71
3	55.02	54.89	24.11	24.48	28.52	28.73	8.16[c]	6.40[c]	15.12	14.21	17.49	18.38	21.60	21.65	11.84	10.45
4	62.17	61.36	23.87	22.92	28.70	28.40	6.73	6.74	15.42	15.32	19.45	19.74	20.00	21.15	11.29	11.38
5	62.07	62.25	23.46	24.46	29.26	29.21	7.21	6.79	15.94	16.30	18.78	18.69	20.74	20.67	10.49	11.33
6	62.37	62.94	23.02	23.23	29.42	29.59	7.15	6.44	16.40	16.52	20.12	20.40	21.22	21.46	10.37	10.52
7	67.56	69.00	23.78	23.88	28.74	28.90	6.39	6.45	15.78	15.86	17.83	17.12	21.17	20.14	11.44	10.81
8	56.91	55.42	22.78	24.39	28.88	29.34	6.31	6.32	16.26	15.35	20.28	20.05	20.52	19.83	8.98	9.39
9	62.83	60.75	24.49	24.66	30.16	30.12	8.10	8.34	17.37	16.71	20.13	20.66	21.80	21.54	11.60	12.32
10	56.43	56.00	23.61	23.79	29.53	29.69	7.36	7.86	16.46	16.79	18.45	18.41	21.02	21.32	10.20	12.79
11	61.16	60.12	22.25	23.69	29.17	28.39	6.40	5.79	16.75	16.47	21.13	21.08	21.14	21.13	11.25	10.61
12	54.98	55.28	21.81	21.37	31.90[c]	28.07[c]	4.88	5.03	15.16	14.86	15.01	15.33	18.24[g]	18.66[g]	10.11	10.91
13	65.83[c]	61.37[c]	23.19	23.82	28.70	29.11	7.54	7.65	16.56	15.45	20.03	19.79	21.44	20.74	9.92	10.03

[a] Samples: A and D = phosphate cross-linked starch (Fibersym, MGP Ingredients); B and F = kidney beans (canned, washed, and lyophilized); C and J = bran cereal; E and H = defatted cookies containing FOS (fructooligosaccharides); G and N = oat bran; I and M = defatted cookies containing polydextrose and RS2 (resistant starch type 2); K and O = dark rye crispbread; L and P = whole meal bread.

[b] Values followed by "c" were removed based on Cochran's test; values followed by "g" were removed based on the lowest average in the single Grubb's test.

Table III. Interlaboratory study results for determination of total dietary fiber in foods[a]

	Sample[b]							
Parameter	**A and D**	**B and F**	**C and J**	**E and H**	**G and N**	**I and M**	**K and O**	**L and P**
Number of labs/analysts	12	13	12	12	13	13	12	13
Mean (%)	60.62	23.70	29.37	6.79	16.15	19.28	21.09	10.76
s_r	0.74	0.67	0.36	0.29	0.39	0.29	0.43	0.68
s_R	4.67	0.99	0.78	0.91	0.85	1.74	0.57	0.86
RSD_r (%)	1.22	2.81	1.22	4.32	2.41	1.51	2.05	6.34
RSD_R (%)	7.70	4.17	2.64	13.38	5.29	9.01	2.72	8.02

[a] Statistical evaluation performed according to AOAC International statistics format. s_r = within-laboratory variability; RSD_r = within-laboratory relative variability; s_R = between-laboratory variability; RSD_R = between-laboratory relative variability.

[b] Samples: A and D = phosphate cross-linked starch (Fibersym, MGP Ingredients); B and F = kidney beans (canned, washed, and lyophilized); C and J = bran cereal; E and H = defatted cookies containing FOS (fructooligosaccharides); G and N = oat bran; I and M = defatted cookies containing polydextrose and RS2 (resistant starch type 2); K and O = dark rye crispbread; L and P = whole meal bread.

outliers, for a total of four statistical outlier pairs. The raw and statistically paired data from the blind duplicate results for TDF are shown in Tables II and III, respectively.

Results and Discussion

Raw data for the dietary fiber collaborative study, with Cochran and Grubbs outliers noted, are shown in Table II. Results of statistical analysis, after the removal of outliers, are shown in Table III. The samples tested in this collaborative study were chosen to be challenging, with an emphasis on analyzing complex products containing RS and nondigestible oligosaccharides. As shown in Table III, within-laboratory variability (s_r) for TDF ranged from 0.29 to 0.74, and between-laboratory variability (s_R) ranged from 0.57 to 4.67. Comparison of statistical analyses showed the level and range of variability in results for the current method were similar to those for previously adopted dietary fiber methods (Table IV) and were most likely influenced in all cases by the significant number of technique-dependent manual operations performed (13). For the current method, repeatability, reproducibility, and Horwitz ratio (HorRat) values were within the range of performance characteristics typically obtained with other dietary fiber methods. In previously adopted methods, between-laboratory variability (s_R) ranged from 0.04 to 9.49, and between-laboratory relative variability (RSD_R) ranged from 1.58 to 66.25 (Table IV).

In AACCI 32-60.01, as in AACCI 32-45.01 (1), food digestion in the small intestine is simulated by gentle shaking or stirring of the sample, with enzymatic digestion at 37°C and pH 6.0. The major difference between AACCI 32-45.01 and 32-60.01 is a reduction from 16 to 4 hr for incubation with the PAA and AMG solution to better simulate the likely residence time for food in the small intestine. RS is the most difficult dietary fiber component to measure accurately because the value obtained is dependent on the incubation conditions—time, temperature, pH, and enzyme concentrations. These variables were optimized for AACCI 32-45.01 to assure the values obtained for samples containing RS were in agreement with values obtained for ileostomy studies (7,8). Experience with the method since adoption of AACCI 32-45.01 has shown that values obtained for phosphate cross-linked starches (RS4) are underestimated when using these conditions. To ensure that values obtained for samples containing RS (RS2, RS3, and RS4) when using a 4 hr incubation were in agreement with known values obtained for ileostomy studies, the concentrations of both PAA and AMG were increased to levels above which further increases in activity (as much as fourfold) produced no further decreases in the levels of measured RS (9). PAA was increased from 2 to 4 kU/test, and AMG was increased from 0.14 to 1.7 kU/test. Under these conditions, the dietary fiber values for many RS-containing samples using AACCI 32-60.01 were similar to those obtained using

Table IV. Comparable AACC International and AOAC International method data[a]

Method[b]	Title	s_r	RSD_r	s_R	RSD_R	HorRat
AACCI 32-05.01	Total Dietary Fiber	0.15–0.99	0.56–66.25	0.27–1.36	1.58–66.25	0.76–17.46
AACCI 32-20.01	Insoluble Dietary Fiber	0.41–2.82	0.86–10.38	0.62–9.49	3.68–19.44	1.73–8.68
AACCI 32-07.01[c]	Soluble, Insoluble, and Total Dietary Fiber in Foods and Food Products	0.36–1.06	1.50–6.62	0.41–1.43	1.58–12.17	0.74–4.66
AACCI 32-06.01	Total Dietary Fiber—Rapid Gravimetric Method	0.18–1.01	1.48–14.73	0.22–2.06	4.13–17.94	1.84–4.62
AOAC 993.19	Soluble Dietary Fiber in Food and Food Products	0.49–1.15	1.74–5.93	0.79–2.05	2.41–7.01	1.13–2.83
AACCI 32-25.01	Total Dietary Fiber—Determined as Neutral Sugar Residues, Uronic Acid Residues, and Klason Lignin (Uppsala Method)	0.32–2.88	1.80–6.96	0.52–4.90	4.80–11.30	2.32–4.20
AACCI 32-41.01	Total Dietary Fiber in Foods Containing Resistant Maltodextrin—Enzymatic-Gravimetric Method and Liquid Chromatography Determination	0.02–1.63	1.33–6.10	0.04–2.37	1.79–9.39	0.77–3.32
AACCI 32-40.01	Resistant Starch in Starch Samples and Plant Materials	0.08–2.66	1.97–4.12	0.21–3.87	4.48–10.90	1.44–3.74
AACCI 32-45.01	Total Dietary Fiber (Codex Alimentarius Definition)	0.41–1.43	1.65–12.34	1.18–5.44	4.70–17.97	1.91–6.49
AACCI 32-50.01	Insoluble, Soluble, and Total Dietary Fiber (Codex Definition) by an Enzymatic-Gravimetric Method and Liquid Chromatography	0.47–1.41	2.43–8.60	0.95–3.14	6.85–14.48	2.85–5.51
AACCI 32-60.01[d]	Integrated Method for Total Dietary Fiber	0.29–0.74	1.28–6.69	0.58–4.59	2.65–13.42	1.08–4.46

[a] s_r = within-laboratory variability; RSD_r = within-laboratory relative variability; s_R = between-laboratory variability; RSD_R = between-laboratory relative variability; HorRat = Horwitz ratio.
[b] Sources: AACC International (1) and AOAC International (2).
[c] Samples were not dried and/or were desugared only.
[d] Current method.

AACCI 32-45.01. The notable exceptions were native, high-amylose maize starch (e.g., Hylon VII, Ingredion), for which measured TDF increased from ~46 to ~60%, and phosphate cross-linked native wheat starch (Fibersym), for which measured TDF increased from ~30 to ~60%.

It is essential to ensure that increased levels of enzyme, especially AMG, do not lead to hydrolysis of other dietary fiber components, such as FOS, galactooligosaccharides, or resistant maltodextrins. Studies confirming this were reported previously (9,10). After incubation with PAA and AMG, the pH of the incubation mixture was increased to ~8.2 followed by temporary heating of the sample to ~100°C to inactivate the PAA and AMG and promote protein denaturation, ensuring efficient protein hydrolysis by protease after cooling of the solution to 60°C. The fraction containing HMWDF was recovered gravimetrically after alcohol precipitation of the SDFP, and combining this result with the water–alcohol soluble fiber (SDFS) content determined by HPLC completed the assay.

Reducing the incubation time with PAA and AMG from 16 to 4 hr has the added advantage of removing the risk of microbial contamination of the sample during extended incubation and alleviating the need to add sodium azide to the incubation buffer. Although the PAA and AMG in the current method are dissolved in buffer containing sodium azide, its presence is not essential and can be omitted if the enzyme solution is kept on ice before use and is used soon after its preparation. The use of sodium azide is still recommended, however, for the maltodextrin chromatographic standard and the glucose and glycerol reference solutions, because these solutions may be prepared and stored for several years before use.

In AACCI 32-60.01, the concentrates containing SDFS from the samples are analyzed using TSKgel® $G2500PW_{XL}$ gel permeation HPLC columns preceded by in-line removal of anions and cations. The in-line deionizing cartridges (Bio-Rad) have a limited capacity and are only able to deionize 25–30 samples before they are exhausted. To reduce the cost associated with the expensive cartridges, the concentrates are deionized in a polypropylene tube containing anion and cation exchange resins prior to use, resulting in 90–95% removal of ions and extension of the life of the HPLC deionization cartridges by 10–20 times the usual number of injections.

In the current method, SDFS is analyzed on TSKgel® $G2500PW_{XL}$ gel permeation columns with a glycerol internal standard. If the sample being analyzed contains glycerol, diethylene glycol is a suitable alternative internal standard. In AACCI 32-45.01, a Sugar-Pak column is employed; however, with this column a significant component of hydrolyzed fructans (i.e., inulinotriose) elutes at the same point as disaccharides and, thus, is not measured as dietary fiber. FOS are completely separated and measured on TSKgel®$G2500PW_{XL}$ columns.

Based on the HPLC chromatographic traces supplied by the collaborating laboratories, several of the HPLC systems did not operate optimally, as evidenced by the significant upward slant of the baseline of the chromatogram during a run. This indicates that the column was partially blocked, and operating pressure was likely higher than the recommended level. Backwashing the column more than 24–48 hr prior to its continued use would reoptimize column performance.

Collaborator Comments

No negative comments were received concerning the ease of use of the method; however, one collaborator did notice an allergic reaction to the PAA and AMG powder mixture. Therefore, the protocol now includes an option whereby an analyst who is not allergic can suspend the enzyme powder mixture in an ammonium sulfate solution to produce a stabilized liquid form that reduces the risk to susceptible analysts. One collaborator did not have access to the deionizing precolumns, so they deionized the samples according to the specifications in AACCI 32-45.01. The same collaborator did not have a water bath with an orbital motion and, thus, was advised to position the incubation containers at an ~45° angle, relative to the direction of shaking, to ensure that the sample did not settle to the bottom of the

container during incubation. One collaborator asked for further advice on where to distinguish between SDFS and disaccharides on the HPLC pattern.

Acknowledgments

The study directors wish to thank the collaborators for all their efforts in completing this study: Nancy Ames, Sean Austin, Jodi Cox, Delphine Curti, Saskia Iilians, Yulai Jin, Susan McCarthy, Vincent McKie, Helena Pastell, David Plank, Hayfa Salman, Toyohide Nishibata, Peter Sanders, Alessandro Santi, and Mikihiko Yoshida. The study directors also thank all of the organizations that supported this effort: Agriculture and Agri-Food Canada (Canada), CRDS (Central Department for Research & Development Services) (Belgium), Eurofins Food Testing (Netherlands), Finnish Food Safety Authority Evira (Finland), AEGIC (Australian Export Grains Innovation Centre) (Australia), Japan Food Research Laboratories (Japan), Kellogg Company (U.S.A.), Matsutani Chemical Company (Japan), Medallion Laboratories/General Mills (U.S.A.), Megazyme (Ireland), and Nestlé Research Centre (Switzerland). Finally, the study directors thank the chairs and members of the AACCI Dietary Fiber and Other Carbohydrates Technical Committee for valuable editing of this report and the associated AACCI method document.

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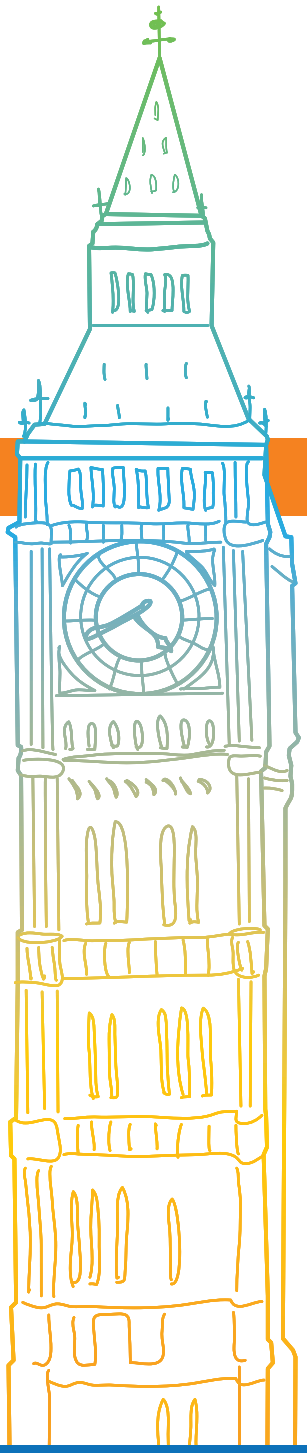

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