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Production and Analysis of RM 8403 Cocoa Flavanol Extract

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U.S. Department of Commerce
Wilbur L. Ross, Jr., Secretary

National Institute of Standards and Technology
Walter Copan, NIST Director and Undersecretary of Commerce for Standards and Technology

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Abstract

NIST Reference Material 8403 is intended for use in harmonizing methods for the determination of cocoa flavanols monomers and their oligomers up to a degree of polymerization (DP) of 7 units. RM 8403 is a free-flowing powder containing cocoa flavanols and procyanidins sealed in aluminized mylar stick packs. A unit of RM 8403 consists of five stick packs each containing approximately 2 g of the cocoa extract powder. This publication documents the production, measurement results, and statistical analysis in realizing this product.

Key words

catechin, epicatechin, procyanidin, cocoa, degree of polymerization (DP) flavanol, NIST Reference Material (RM)

Technical Information Contact for this RM

Please address technical questions about this SRM to srms@nist.gov where they will be assigned to the appropriate Technical Project Leader responsible for support of this material. For sales and customer service inquiries, please contact srminfo@nist.gov.

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1. Introduction

Flavanols are found primarily in plants and natural products such as chocolate, tea, wine and berries. The consumption of these flavanol-containing foods is frequently cited as being associated with positive cardiovascular effects iIn most of these these foods, including in cocoa, the compounds include the simple flavanol forms of (−)-epicatechin, (+)-epicatechin, (−)-catechin, (+)-catechin, and oligomers of the catechin and epicatechin monomers (Figure 1). The oligomers from degree of polymerization (DP) 2 to DP7 (dimers to heptamers) are termed procyanidins. The heterogeneity of the oligomer structure increases exponentially with the increase in number of conjugated monomeric units.

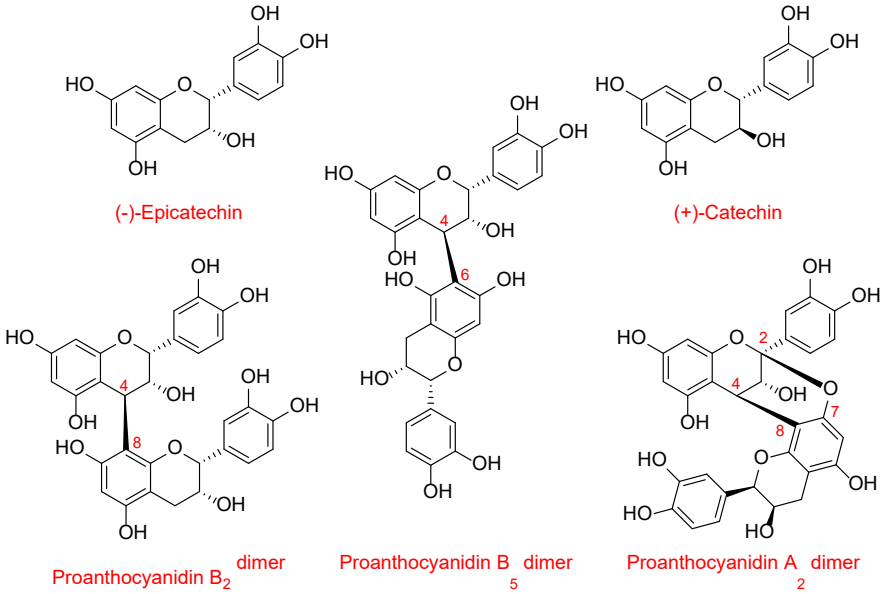


Figure 1. Structures of (−)-epicatechin, (+)-catechin and Selected Procyanidin Dimers.
Reproduced from Bussy et al., 2020 [1] with permission.

Currently, the only pure standards that are commercially available for the cocoa flavanols and procyanidins are limited, including primarily the four flavanol monomers, and a small selection of the dimers. Typically, analytical measurements to determine flavanol and procyanidin content are performed by separating the oligomers by liquid chromatography with fluorescence detection (LC-FL) and comparing the response with a monomeric calibrant.

The fluorescence response to increasing DP is not the same as it is to the monomer, which can lead to poor estimates of procyanidin content. As a result of this limitation, a new method was developed, recently published [1] and accepted as an AOAC official method [2] using a standard that was compositionally defined and well-characterized in its procyanidin content; the details on this standard are described herein

Working in partnership with the United States Department of Agriculture (USDA) and the National Institute of Standards and Technology (NIST) since 2017, Mars EDGE (Germantown, MD USA) has isolated individual oligomer standards and evaluated them for chemical purity by liquid chromatography with UV and mass spectrometric detection (LC-MS) and nuclear

magnetic resonance spectroscopy (NMR). As part of this effort, the oligomers in candidate RM 8403 Flavanols in Cocoa Extract were quantified with the LC-FL method using these well characterized individual calibrants.

Candidate RM 8403 Flavanols in Cocoa Extract will allow for the development of consistent validated methods for procyanidins in cocoa-based products. It will also allow for a consistent comparison of the levels and DPs of procyanidins in a variety of flavanol-containing natural products. These measurements will also help the clinical community to better relate the potential health benefits of flavanol and procyanidin-containing foods to procyanidin content, composition, and/or plant source.

1.1. Material Preparation

Candidate RM 8403 Flavanols in Cocoa Extract was manufactured by Mars EDGE (Germantown, MD). The Cocoa Extract was prepared by extracting flavanols and procyanidins from cocoa cake prepared from unfermented, dried cocoa beans using an aqueous acetone solution. The acetone was removed, and the remaining solution spray-dried to create a dry, free flowing purple powder. The cocoa extract was designed to be freely soluble in acetone/water and methanol/water mixture. For convenience of use, the spray-dried extract was mixed to ensure homogeneity and packaged into 10,000 single aluminized mylar stick packs (approx. 2 g) with dimensions of 55 mm × 11 mm × 4 mm. The filled stick packs were packed into 20 separate storage boxes. The order of fill was not recorded.

2. Homogeneity Measurements

Analysts at Mars EDGE determined the cocoa flavanols and procyanidins for measurands DP1 to DP7 in twenty (20) separate stick packs units (with two replicates, A and B) of candidate RM 8403 Flavanols in Cocoa Extract using the liquid chromatography with the fluorescence detection (LC-FL) method described in [1]. A single stick pack was pulled from each box for analysis. The mass fraction results are provided in Section 4; Figure 2 provides a graphical overview.

A slight run order bias is observed and was attributed to drift in the LC-FL measurement system rather than from between-box material heterogeneity.

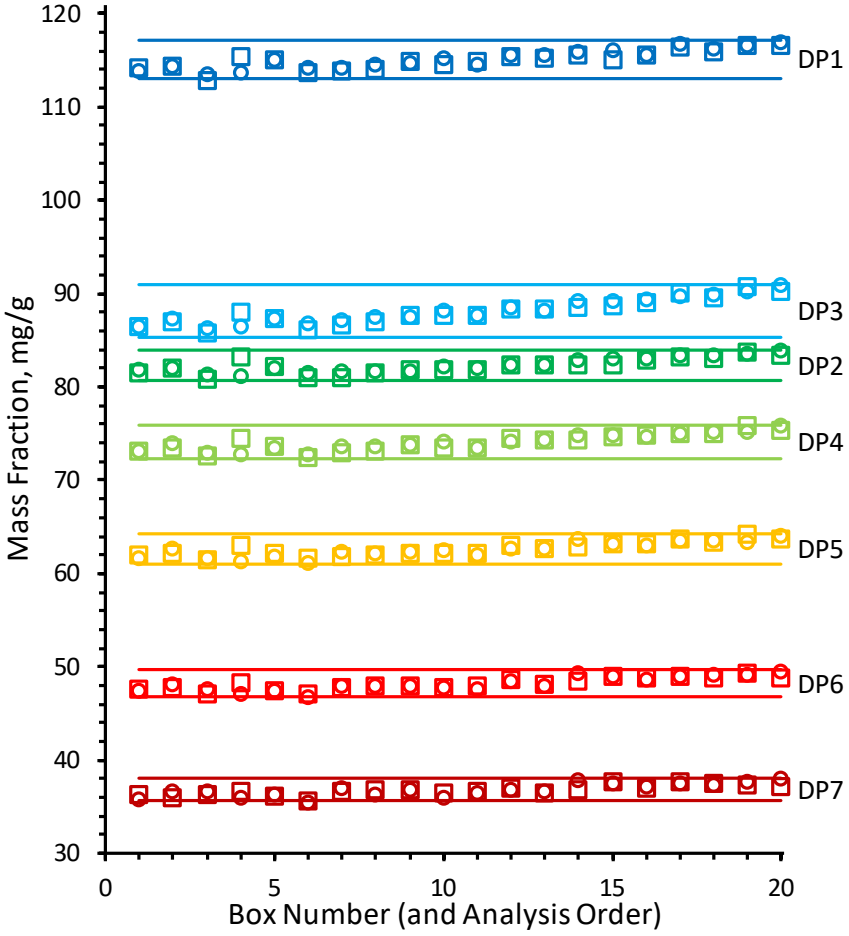


Figure 2. Summary of Homogeneity Measurements.

Each symbol represents a measurement of one of the DP1 to DP7 measurands in a stick pack taken from one of the twenty boxes. The open circles represent the "A" replicate; the open squares represent the "B" replicate. The horizontal lines bracket the mean ± 2 standard deviations for each of the DPs.

3. Interlaboratory Study

A nine-participant interlaboratory study (ILS) assessed the cocoa flavanol and procyanidin (F/PC) content of candidate RM 8403 Cocoa Flavanol Extract and SRM 2384 Baking Chocolate. The measurands were the mass fraction, reported in mg/g, of the F/PC oligomers with degree of polymerization (DP) from one to seven (monomer to heptamer): DP1, DP2, DP3, DP4, DP5, DP6, and DP7. These flavanol oligomer mass fractions were determined using high-performance liquid chromatograph with fluorescence detection (LC-FL) after the cocoa extract powder was solubilized and extracted with acetone:water:acetic acid (70:30:1).

Participants in this ILS included analysts at the following laboratories which performed measurements that contributed to the value assignment of cocoa flavanols and procyanidins in RM 8403: Waters Corporation (Columbia, MD), Waters Corporation (Milford, MA), United States Department of Agriculture (Beltsville, MD), Mars Wrigley Confectionary (Chicago, IL), Mars Wrigley Confectionary (Hackettstown, NJ), Mars EDGE (Germantown, MD), UC-Davis (Department of Nutrition, Davis, CA), Kay Lab-North Carolina State University (Kannapolis, NC) and Eurofins Supplement Analysis Center (Petaluma, CA).

This report only describes the process and results for the candidate RM 8403 material.

3.1.1. Instructions to participants

Thank you for accepting the invitation to implement and evaluate the new method to determine flavanols and procyanidins (sum total of DP1-7) in cocoa extract and defatted chocolate by LC-FL. It is important to remember that this is a test of the methodology involved and not a test of the individual laboratories or their personnel. In this regard, it is very important to follow these directions and the enclosed method exactly. If for any reason you are not able to complete the study, contact project facilitator [redacted] and copy to project lead [redacted] If you deviate from the instructions provided for this study, please report it in the result spreadsheet attached.

Process Sequence

Your participation will consist of three parts:

The first will be acknowledgment of receipt of the collaborative study package. There are many items – in addition to the samples and standards that are being provided. Please check content of package and the documents provided to you and confirm receipt, so that you are equipped to perform the study.

The second step will be analysis of samples. In this step, you will be taking the samples throughout the entire method from sample preparation, to quantitation and determination of content. Therefore, calibration curves at pre-determined detector gain settings are required for this stage. Note that a single detector gain must be used to allow valley-to-valley integration.

The third phase will consist of the data formulation of the actual test materials that will comprise your portion of the study. Report results in the spreadsheet attached.

Deviations

If there are general questions about the study itself, please contact project facilitator and project lead for assistance.

If there are, in your opinion, questions about the methodology that you feel will seriously jeopardize the study, you may contact project facilitator and project lead.

If possible, it is recommended that a second experienced analyst take part in an initial review of the method and be consulted prior to any course of action with regard to interpretation and subsequent deviations.

In the event that parts of the method appear to be subject to interpretation, take the most reasonable course, then note the step or instruction that was in question. Record what exactly was done and indicate why this step or instruction was thought to be ambiguous. Providing notes concerning ambiguous instructions will help produce a clearly written final report and improve the method.

Method, Materials and Resources

A package will be shortly sent to you (contact project facilitator to obtain tracking information). Please verify that the package you receive contains:

3 cocoa extract samples	☐
20 syringes	☐
20 polytetrafluoroethylene (PTFE) syringe filters	☐
1 Torus column 3.0 x 100 mm 1.7 µm	☐ *
1 Cocoa extract calibrant container (≈1.0 g)	☐ *

*if you have participated to our multi-laboratory implementation study, please use the reference material and column provided previously.

Documents included in the email:

Analytical method	☐
Cocoa extract calibrant certificate	☐

All samples contain ~ 1 g of defatted material. Due to the variability in the levels of flavanols and procyandins (from 0.1-60%), sample weight and dilution are indicated in the method.

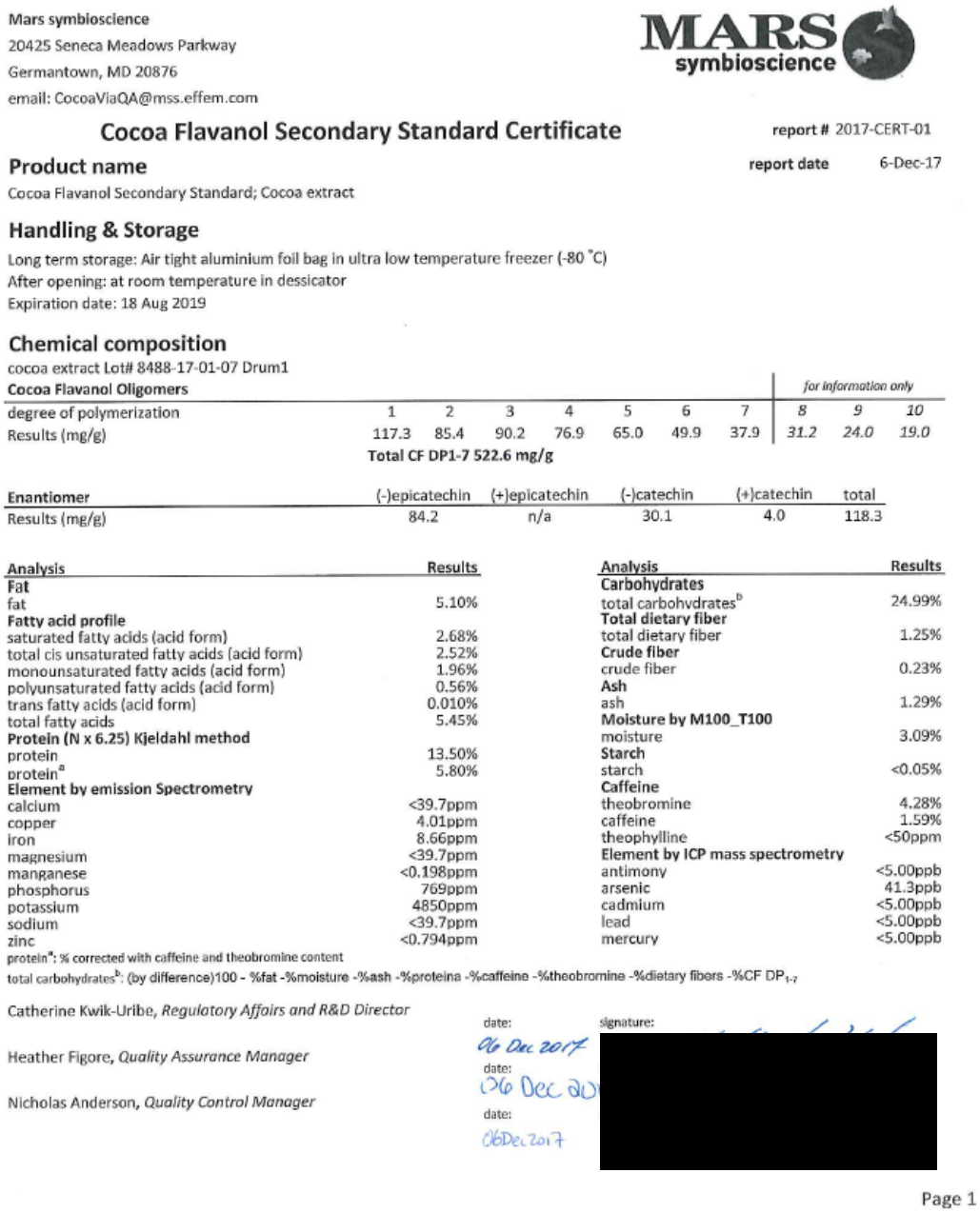
A minimum of approximately 2 analyst-days or more should be resourced for this study (e.g., preparation and analysis of flavanols and procyanidins on the first day and analysis on the second). It is recommended that, due to the time and expense involved in conducting this study, an experienced analyst should be assigned. The analyst should be competent in manual integration, calibration and should also be skilled at interpreting and implementing new procedures.

It is strongly recommended that a single analyst and a single instrument be dedicated (especially since detector gain needs to be determined prior to running the method) to the study for a continuous period of time. The use of multiple analysts, instruments or time intervals should be avoided within a laboratory.

All work should be completed within one month of receipt of materials, unless other prior arrangements are made. If this schedule cannot be met, project facilitator and project lead should be notified as soon as possible.

3.1.2. Calibrant

Each of the nine laboratories was provided with cocoa extract calibrant and its associated certificate of analysis showing cocoa F/PC content for DP1 to DP7. Individual concentrations for F/PC DP1 to DP7 were used to demonstrate system suitability, create calibration curves and determine NIST materials. Figure 3 displays the certificate of analysis of the supplied calibrant.



Mars symbioscience
20425 Seneca Meadows Parkway
Germantown, MD 20876
email: CocoaViaQA@mss.effem.com

MARS symbioscience

Cocoa Flavanol Secondary Standard Certificate

report # 2017-CERT-01

report date 6-Dec-17

Product name

Cocoa Flavanol Secondary Standard; Cocoa extract

Handling & Storage

Long term storage: Air tight aluminium foil bag in ultra low temperature freezer (-80 °C)
After opening: at room temperature in dessicator
Expiration date: 18 Aug 2019

Chemical composition

cocoa extract Lot# 8488-17-01-07 Drum1

Cocoa Flavanol Oligomers								*for information only*		
degree of polymerization	1	2	3	4	5	6	7	*8*	*9*	*10*
Results (mg/g)	117.3	85.4	90.2	76.9	65.0	49.9	37.9	*31.2*	*24.0*	*19.0*

Total CF DP1-7 522.6 mg/g

Enantiomer	(-)epicatechin	(+)epicatechin	(-)catechin	(+)catechin	total
Results (mg/g)	84.2	n/a	30.1	4.0	118.3

Analysis	Results
Fat	
fat	5.10%
Fatty acid profile	
saturated fatty acids (acid form)	2.68%
total cis unsaturated fatty acids (acid form)	2.52%
monounsaturated fatty acids (acid form)	1.96%
polyunsaturated fatty acids (acid form)	0.56%
trans fatty acids (acid form)	0.010%
total fatty acids	5.45%
Protein (N x 6.25) Kjeldahl method	
protein	13.50%
protein[a]	5.80%
Element by emission Spectrometry	
calcium	<39.7ppm
copper	4.01ppm
iron	8.66ppm
magnesium	<39.7ppm
manganese	<0.198ppm
phosphorus	769ppm
potassium	4850ppm
sodium	<39.7ppm
zinc	<0.794ppm

Analysis	Results
Carbohydrates	
total carbohydrates[b]	24.99%
Total dietary fiber	
total dietary fiber	1.25%
Crude fiber	
crude fiber	0.23%
Ash	
ash	1.29%
Moisture by M100_T100	
moisture	3.09%
Starch	
starch	<0.05%
Caffeine	
theobromine	4.28%
caffeine	1.59%
theophylline	<50ppm
Element by ICP mass spectrometry	
antimony	<5.00ppb
arsenic	41.3ppb
cadmium	<5.00ppb
lead	<5.00ppb
mercury	<5.00ppb

protein[a]: % corrected with caffeine and theobromine content

total carbohydrates[b]: (by difference)100 - %fat -%moisture -%ash -%proteina -%caffeine -%theobromine -%dietary fibers -%CF DP_{1-7}

Catherine Kwik-Uribe, *Regulatory Affairs and R&D Director*
date: 06 Dec 2017 signature: [redacted]

Heather Figore, *Quality Assurance Manager*
date: 06 Dec 2017

Nicholas Anderson, *Quality Control Manager*
date: 06Dec2017

Page 1

Figure 3. Certificate of Analysis for Supplied Calibrant.

3.1.3. Samples

Nine laboratories were provided with all the samples, consumables, and documents necessary to the implementation and analysis using the LC-FL method described below. The study materials were submitted for analysis in blind triplicates. The remaining sample material from the 20 stick packs of the cocoa extract used or the homogeneity analysis were combined and mixed together in a zip-lock bag. The powder was then aliquoted in 27 sealed bags each containing approximately 1g.

3.1.4. Method

All laboratories received detailed instruction for sample preparation, instrument setup, data acquisition and analysis. The method was published in 2020 [1]; the details presented here are as provided to the participants. Laboratories were instructed to strictly follow the methodology provided and document eventual deviation from the written protocol.

3.1.4.1. Common-use solvents and reagents.

- Acetonitrile. Highly flammable, toxic, liquid irritant. Store in flammable liquid storage cabinet. Harmful if inhaled, swallowed, or absorbed through the skin. Use appropriate personal protective equipment and engineering controls such as laboratory coat, safety glasses, rubber gloves, and fume hood. Dispose of acetonitrile and solutions according to federal, state, and local regulations.
- Glacial acetic acid. Corrosive, flammable liquid. Store in acid storage cabinet. Causes severe burns. Use appropriate personal protective equipment and engineering controls such as laboratory coat, safety glasses, face shield, heavy rubber gloves, and fume hood when working with concentrated solutions. Dispose of acid and solutions according to federal, state, and local regulations.
- n-Hexane. Flammable, toxic, liquid irritant. Store in a flammable liquid storage cabinet. Harmful if inhaled, swallowed, or absorbed through the skin. Use appropriate personal protective equipment and engineering controls such as laboratory coat, safety glasses, rubber gloves, and fume hood. Dispose of n-hexane and solutions according to federal, state, and local regulations.
- Methanol. Flammable, toxic, liquid irritant. Store in a flammable liquid storage cabinet. Harmful if inhaled, swallowed, or absorbed through the skin. Use appropriate personal protective equipment and engineering controls such as laboratory coat, safety glasses, rubber gloves, and fume hood. Dispose of methanol according to federal, state and local regulations.
- Acetone. Flammable, toxic, liquid irritant. Store in a flammable liquid storage cabinet. Harmful if inhaled, swallowed, or absorbed through the skin. Use appropriate personal protective equipment and engineering controls such as laboratory coat, safety glasses, rubber gloves, and fume hood. Dispose of acetone and solutions according to federal, state, and local regulations.

3.1.4.2. Equipment

- HPLC system. HPLC with column oven, fluorescence detection and auto-sampler with temperature control, or equivalent.
  - Chromatography data acquisition software.
  - HPLC column. Torus Diol (3.0 mm x 100 mm, 1.7 µm, 130 Å), (Waters; Milford, MA; #186007611), or equivalent.
  - Sonic bath.Capable of sonication and heating to at least 50 °C (VWR, West Chester, PA; Model 150D), or equivalent

- Sample preparation and consumables
  - Class A- Volumetric flasks, 10 mL, 25 mL and 50 mL.
  - Syringe filters. PTFE, 0.45 µm, 13 mm (Nalgene, Rochester, NY; #187-1345), or equivalent.

- HPLC vials/caps. VWR (#608216-1232), or equivalent.
- Vacuum manifold.24 position (Phenomenex; #AH0-6024), or equivalent.
- Syringes with slip tip (not Luer lock). 3 mL (VWR; #BD309586), or equivalent. Only plunger portion is used.
- Disposable centrifuge tubes. 15 and 50 mL (VWR; #21008-210 and #21008-240), or equivalent.
- Centrifuge. Sorval RC33 plus, or equivalent (3000 rpm or 314 rad/s or 2567 × g_n).
- Vortex. Fisher Scientific (#02-215-365), or equivalent.
- Analytical balance. Readability 0.1 mg.
- Graduated cylinder. Fisher Scientific (#08552-4F), or equivalent.

- Reagents
  - Water. Millipore quality (Millipore, Bedford, MA), or equivalent.
  - Methanol. HPLC grade (Fisher A454-4), or equivalent.
  - Acetone. HPLC grade (Fisher A929-4), or equivalent.
  - Acetonitrile. HPLC grade (Fisher A998-4), or equivalent.
  - Acetic acid. Glacial (Mallinckrodt Baker, Inc., Phillipsburg, NJ; #953433), or equivalent.
  - Calibration standard. Cocoa extract calibrant (CEC) Cert-01.
  - Extraction solution. Acidified aqueous acetone solvent system (AWAA). On a 1 L basis, combine 700 mL acetone, 300 mL purified water, and 10 mL glacial acetic acid (70 + 30 + 1). AWAA is used for calibration standards, as well as for extraction of flavanols and procyanidins from test samples. Scale up as needed.

- Prepare working standards (WS)
  - Weigh 0.100 g ± 0.010 g of cocoa extract calibrant (CEC) in a 50 mL volumetric flask.
  - Dissolve and dilute to volume using AWAA to obtain working standard 5 (WS5).
  - Prepare WS1, WS2, WS3, and WS4 by pipetting 2.5 mL, 4.0 mL, 5.0 mL and 8.0 mL of WS5 into 10 mL flasks.
  - Filter WS1 to WS5 into autosampler HPLC vials using PTFE 0.45 µm syringe filters.

- Check working standard (CWS) solution
  - Weigh 50 mg ± 5.0 mg of CEC into a 50 mL volumetric flask. Record the weight to the nearest 1 decimal place.
  - Dilute to volume with AWAA diluent and invert to mix.
  - Using an appropriate syringe, draw up sample.
  - Attach a 0.45 µm PTFE filter to tip and filter sample into labeled HPLC vial and cap tightly
- Prepare Cocoa extract samples
  - Weigh 0.050 g ± 0.005 g of cocoa extract in a 50 mL volumetric flask. Dissolve and dilute to volume using AWAA.
  - Filter sample solution into autosampler HPLC vial using a PTFE 0.45 µm syringe filter.

3.1.4.3. Method Validation

- Fluorescence detector sensitivity/dynamic range optimization and reproducibility:
  - Fluorescence detector sensitivity performances can vary across hardware manufacturer, model or unit. Prior to any experiments, the gain or sensitivity of the detector must be adjusted to select the highest gain possible that allow sensitive detection of DP7 (smallest peak intensity) without compromising the peak shape of DP1 (highest peak intensity).
  - New column must be conditioned for at 30 minutes by running 50:50 mobile phase A and B followed by five consecutive blank injections.
  - Flush column with acetonitrile (no acetic acid additive) for long term storage

- Gain optimization
  - Prepare a stock solution of CEC in AWAA at 2.0 mg/mL by weighing accurately 100 mg CEC into a 50 mL volumetric flask. Dilute to volume with AWAA solution. Prepare fresh. Do not store.
  - Select an appropriate starting sensitivity level (i.e., gain setting) on the fluorescence detector of the HPLC; often one can begin at instrument default.
  - Analyze WS5 of CEC using the HPLC conditions specified in Section 3.1.4.4.
  - Observe whether DP1 peak is of normal shape and on scale. If the peak shape is saturated or distorted, repeat previous step at a lower gain. If the peak shape is not acceptable, repeat at a higher gain. Repeat the process until optimal gain is defined. The optimal gain is defined as the highest gain that allows repeatable detection of DP1 (relative standard deviation, RSD, on signal area ≤ 2 %) with an acceptable peak shape.

- Evaluate repeatability of WS5 injection results.
  - Inject WS5 three times.
  - DP1 peak areas from the three injections should have RSD < 2 %.
  - Evaluate sensitivity of WS5 injection results. Calculate limit of quantifications (LOQs) for DP1 to DP7 as ten times the standard deviation of the signal area across triplicates of WS5 divided by the slope. The LOQ for each DP must be below WS5 concentrations.
  - Because of valley-to-valley integration, detection conditions (e.g., gain) are identical for DP1 to DP7.

- Evaluate precision
  - Inject check CWS five times.
  - Evaluate precision on DP1 determination. Relative standard deviation on signal area must be ≤5% across five replicates.

3.1.4.4. HPLC Parameters

  - The column is a Waters Torus Diol 3.0 mm x 100 mm, 1.7 µm, 130 Å. Hold the column temperature at 50 °C. The flow rate is 1 mL/min, and typical injection volume is 2 µL. Set the autosampler to, and hold at, 5 °C.
  - The mobile phase is a binary gradient (solvents A and B) consisting of acidic acetonitrile [(A) Acetonitrile:Acetic Acid, 98: 2 volume fraction] and acidic aqueous

methanol [(B) Methanol:Water:Acetic Acid, 95:3:2 volume Fraction Total run time is 13.1 min. Post run equilibration is 3.0 min. The gradient is

- 0 min to 0.37 min, 0 % B
- 0.37 min to 10.40 min, 45 % B
- 10.40 min to 13.00 min, 95 % B
- 13.00 min to 13.1 min, 0 % B

3.1.4.5. Detection

- Conduct fluorescence detection with an excitation wavelength of 230 nm and emission at 321 nm
- To maximize precision performances, the detector lamp should always be on (use of economy mode or lamp on only during analysis are prohibited) and the lamp energy reference should be in use. Set photomultiplier gain to the optimized level prior to conducting analyses.

3.1.4.6. Integration

- Integration is performed valley-to-valley as shown in Figure 4. Magnifying the chromatograms is mandatory to accurately position the valley-to-valley integration and determine signal area appropriately.

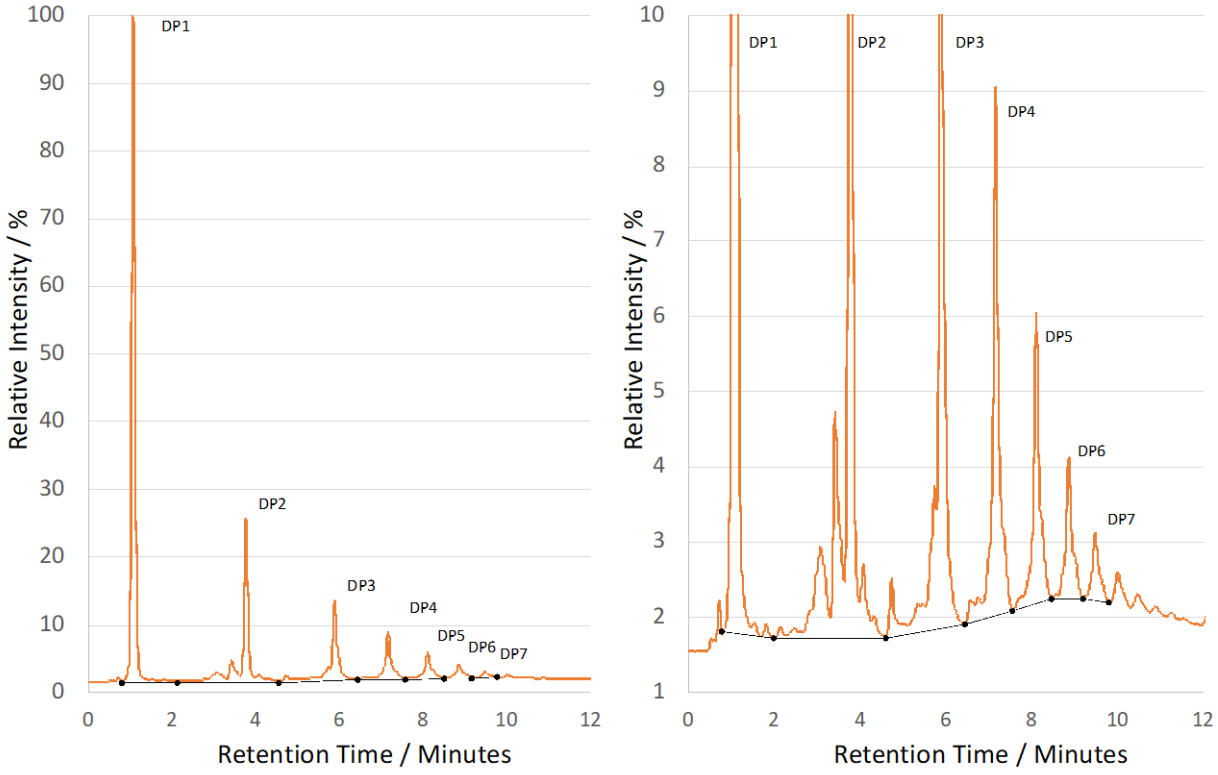


Figure 4. Exemplar Fluorescence Chromatogram of Cocoa Extract
Left: full scale chromatogram showing DP1 to DP7 valley-to-valley integration. Right: magnified view of valley-to-valley integration for DP1 to DP7 signals. Chromatogram is for working standard WS3.

3.1.4.7. Analysis sequence

- The sample injection sequence must be organized as follows:
  - Blank
  - CWS
  - Blank
  - WS1 to WS5
  - Blank
  - Samples
  - CWS
  - Blank
- The signal areas for the bracketing CWS must be within 90 % to 110 % of the average signal area observed for the five precision injections.
- Build calibration curves by plotting peak area as a function of concentration for DP1 top DP7. The coefficient of determination (r^2) for each curve must be ≥0.99.

3.1.4.8. Calibration

Individual calibration curves are built using WS1 to WS5 for DP1 to DP7 using the model:

$$A_{ij} = \alpha_i + \beta_i C_{ij}$$

where: i indexes the measurands, DP1 to DP7.
j indexes the working standards, WS1 to WS5.
α_i intercept of linear function for DPi.
β_i slope of linear function for DPi.
A_{ij} signal area for DPi in WSj.
C_{ij} concentration of DPi in WSj.

The concentration C_{ij} is calculated:

$$C_{ij} = \frac{W_{\text{CEC}}\ \text{DP}i}{V_{\text{WS}j}} \frac{V_{WS5}}{V_{\text{WS}j}}$$

where: W_{CEC} mass (g) of CEC in WS5. W_{CEC} should be ≈0.1 g.
DPi DPi content (mg/g) in cocoa extract calibrant as specified in the Certificate of Analysis.
$V_{\text{WS}j}$ volume (mL) of WSj. V_{WS1} to V_{WS4} should be 10 mL; V_{WS5} should be 50 mL.
$V_{\text{WS}5}$ volume (mL) of WS5 used to prepare WSj. The volumes for {WS1, WS2, WS3, WS4, and WS5} should be {2.5, 4.0, 5.0, 8.0, and 50} mL.

The regression should not force the curve to go through the origin.

3.1.4.9. Quantification

The concentration of each DPi in a sample is calculated as:

$$C_{s,i} = \frac{(A_{s,i} - \alpha_i) \times V_s}{\beta_i \times W_s}$$

where: $A_{s,i}$ signal area for DPi in a sample
Vs volume (mL) of the sample solution
Ws mass (g) of the sample in the solution

3.1.5. Collection of data

Each laboratory transcribed sample and standard weights alongside signal areas in a Microsoft Excel Spreadsheet provided with instructions. Out of nine participating laboratories, all returned a complete set of data. All laboratories reported acceptable system performances for linearity and precision. Precision, determined as the %RSD of DP1 signal area on five replicate injection of cocoa extract calibrant, was measured between 0.4 % and 3.8 %. Linearity was demonstrated with coefficients of determination systematically higher or equal to 0.99. System performances were also assessed along each sequence with bracketing standards that did not deviate from the initial five precision injections by more than 5 %.

3.1.6. Within- and Between Precision of the Method

The complete interlaboratory study results are provided in Section 4. Table 1 summarizes within- and between-laboratory precision for DP1 to DP7 as estimated by one-factor analysis of variance (anova) [3].

Table 1. Within- and Between-Laboratory Imprecision [a]

Measurand	$\bar{x}$ mg/g	s_{wth} mg/g	s_{btw} mg/g	$s_{wth}/\bar{x}$ %	$s_{btw}/\bar{x}$ %
DP1	115.40	2.61	2.82	2.3	2.4
DP2	83.25	2.03	2.25	2.4	2.7
DP3	87.74	2.04	2.18	2.3	2.5
DP4	74.90	1.72	2.62	2.3	3.5
DP5	63.19	1.63	2.12	2.6	3.4
DP6	48.51	1.42	1.68	2.9	3.5
DP7	36.94	0.77	1.67	2.1	4.5

a $\bar{x}$, arithmetic mean
s_{wth}, pooled within-laboratory precision
s_{btw}, between-laboratory precision
$s_{wth}/\bar{x}$, relative pooled within-laboratory precision expressed as percent, $100 s_{wth}/\bar{x}$
$s_{btw}/\bar{x}$, relative within-laboratory precision expressed as percent, $100 s_{btw}/\bar{x}$

Assuming that one laboratorian or team of laboratorians in each laboratory made all three replicate measurements, $s_{wth}/\bar{x}$ estimates the expected relative repeatability precision of the measurement process. Since all participants were supplied with the same calibrant, many of the consumables, were instructed to use equivalent equipment, and make all measurements within a two-day period, the $s_{btw}/\bar{x}$ estimates short-term between laboratory intermediate precision. These estimates provide a lower-bound on the reproducibility precision of the method.

4. Data

The following pages detail the measurement data used to value assign the mass fraction of the DP1 to DP7 measurands. Tables 2 to 8 list both the Mars EDGE LC-FL homogeneity measurements and the results from the interlaboratory study. These Tables use the following terms:

Box	The assigned number of the storage box the samples number
Rep	For the homogeneity measurements, RepA and RepB are replicate instrumental evaluations of the same sample preparation. For the interlaboratory study, RepA, RepB, and RepC are independent preparations of three samples.
Mean	Arithmetic mean
SD	Standard deviation
s_{mean}	Standard deviation of the mean, $\text{SD}/\sqrt{N}$, where N is the number of replicate values.
U_{mean}	95 % expanded uncertainty about the mean of the homogeneity measurements: $t_{0.95,19}\ \text{SD}/\sqrt{20}$.
Median	Median of the interlaboratory study results.
MAD_E	Median absolute deviation from the median of the interlaboratory study results, adjusted to estimate the standard deviation for normally distributed data.
U_{median}	95 % expanded uncertainty about the median of the interlaboratory study results: $1.24\ t_{0.95,8}\ \text{MAD}_\text{E}/\sqrt{9}$. The factor 1.24 adjusts for the increased variability of the median relative to the mean for normally distributed data.

Figures 5 to 11 summarize the two sets of measurement results for measurands DP1 to DP7, displaying both on the same mass fraction scale to facilitate comparison.

Table 2. Homogeneity and Interlaboratory Study Results for DP1

Homogeneity

Box	RepA	RepB	Mean	s_{mean}
1	113.89	114.31	114.10	0.21
2	114.34	114.46	114.40	0.06
3	113.57	112.95	113.26	0.31
4	113.74	115.46	114.60	0.86
5	115.03	115.16	115.10	0.06
6	114.25	113.67	113.96	0.29
7	114.28	113.93	114.11	0.17
8	114.66	114.07	114.37	0.30
9	114.70	114.93	114.82	0.12
10	115.34	114.56	114.95	0.39
11	114.66	114.86	114.76	0.10
12	115.66	115.45	115.56	0.10
13	115.53	115.34	115.44	0.09
14	115.99	115.61	115.80	0.19
15	116.09	115.06	115.58	0.52
16	115.69	115.64	115.67	0.02
17	116.86	116.49	116.68	0.19
18	116.31	115.93	116.12	0.19
19	116.61	116.68	116.65	0.04
20	116.90	116.63	116.77	0.14
		Mean:	115.14	
		SD:	0.98	
		U_{mean}:	0.46	

Interlaboratory Study

Lab	RepA	RepB	RepC	Mean	s_{mean}
A	105.09	112.82	113.88	110.60	2.77
B	112.90	111.14	111.55	111.86	0.53
C	115.91	113.14	115.19	114.75	0.83
D	115.34	115.68	114.28	115.10	0.42
E	113.88	115.54	113.40	114.27	0.65
F	114.88	114.41	114.42	114.57	0.16
G	119.48	118.67	118.05	118.73	0.41
H	121.65	115.21	119.22	118.69	1.88
I	120.26	124.69	115.23	120.06	2.73
			Median:	114.75	
			MAD_E:	4.28	
			U_{median}:	4.08	

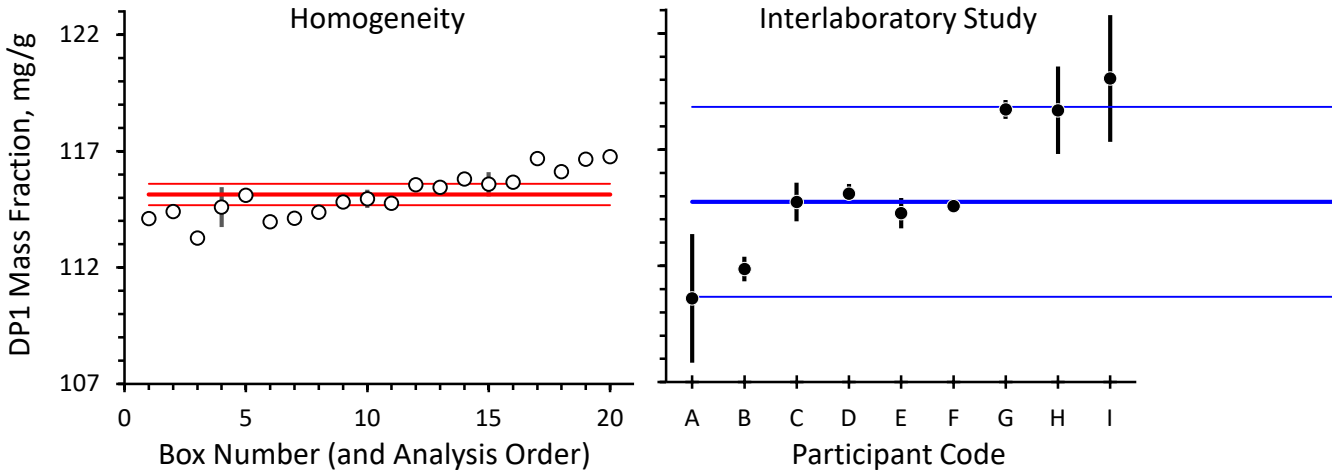


Figure 5. Graphical Summary of DP1 Results

Table 3. Homogeneity and Interlaboratory Study Results for DP2

Homogeneity

Box	RepA	RepB	Mean	s_{mean}
1	81.81	81.60	81.71	0.11
2	82.11	81.99	82.05	0.06
3	81.30	80.92	81.11	0.19
4	81.28	83.32	82.30	1.02
5	82.02	82.20	82.11	0.09
6	81.53	80.97	81.25	0.28
7	81.66	81.03	81.35	0.31
8	81.79	81.55	81.67	0.12
9	81.70	81.94	81.82	0.12
10	82.20	81.82	82.01	0.19
11	82.04	81.89	81.97	0.08
12	82.44	82.33	82.39	0.05
13	82.49	82.48	82.49	0.00
14	82.86	82.42	82.64	0.22
15	83.03	82.38	82.71	0.33
16	83.08	82.88	82.98	0.10
17	83.35	83.32	83.34	0.02
18	83.43	83.10	83.27	0.17
19	83.57	83.84	83.71	0.14
20	83.98	83.48	83.73	0.25
		Mean:	82.33	
		SD:	0.78	
		U_{mean}:		

Interlaboratory Comparison

Lab	RepA	RepB	RepC	Mean	s_{mean}
A	74.47	81.92	82.12	79.50	2.52
B	81.46	79.76	81.01	80.74	0.51
C	82.74	79.57	81.82	81.38	0.94
D	82.57	82.31	82.14	82.34	0.13
E	84.02	84.65	83.91	84.19	0.23
F	83.24	82.65	82.49	82.79	0.23
G	86.59	86.01	85.74	86.11	0.25
H	87.23	83.03	85.73	85.33	1.23
I	86.69	90.07	83.86	86.87	1.80
			Median:	82.79	
			MAD_E:	3.04	
			U_{median}:	2.90	

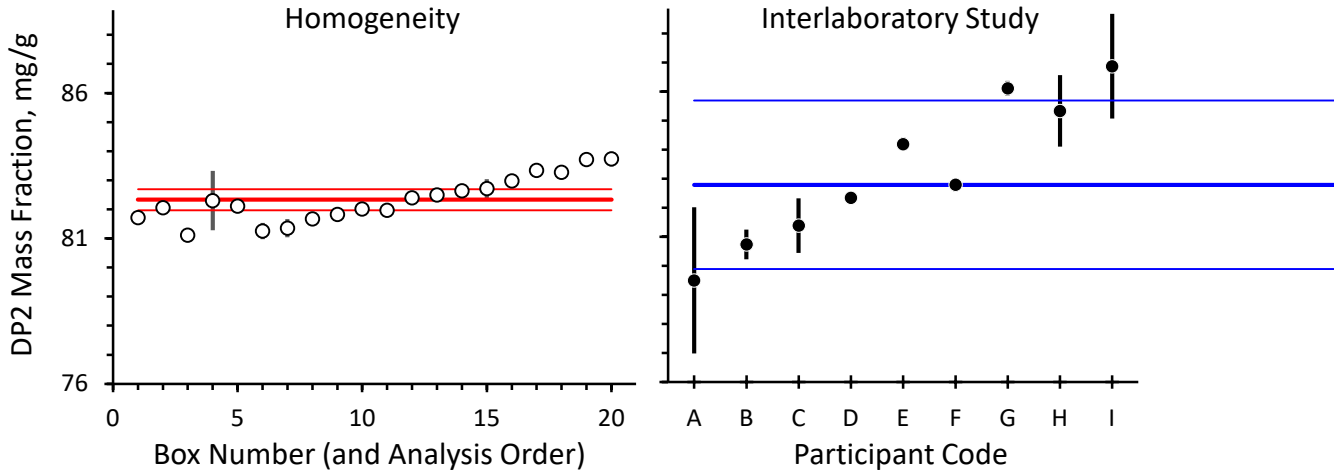


Figure 6. Graphical Summary of DP2 Results

Table 4. Homogeneity and Interlaboratory Study Results for DP3

Homogeneity

Box	RepA	RepB	Mean	s_{mean}
1	86.48	86.48	86.48	0.00
2	87.37	86.98	87.18	0.20
3	86.31	85.79	86.05	0.26
4	86.57	88.04	87.31	0.74
5	87.32	87.31	87.32	0.00
6	86.78	86.11	86.45	0.34
7	87.14	86.61	86.88	0.27
8	87.48	87.07	87.28	0.21
9	87.48	87.69	87.59	0.10
10	88.30	87.71	88.01	0.30
11	87.70	87.76	87.73	0.03
12	88.65	88.34	88.50	0.16
13	88.30	88.33	88.32	0.02
14	89.31	88.51	88.91	0.40
15	89.22	88.75	88.99	0.23
16	89.41	89.10	89.26	0.16
17	89.81	90.08	89.95	0.13
18	89.97	89.51	89.74	0.23
19	90.21	90.74	90.48	0.27
20	90.96	90.33	90.65	0.31
		Mean:	88.15	
		SD:	1.37	
		U_{mean}:		

Interlaboratory Comparison

Lab	RepA	RepB	RepC	Mean	s_{mean}
A	81.44	86.79	88.58	85.60	2.14
B	84.90	84.36	85.10	84.79	0.22
C	87.37	83.79	86.94	86.03	1.13
D	86.81	86.48	86.79	86.69	0.11
E	86.30	87.54	85.93	86.59	0.49
F	88.24	87.46	87.33	87.68	0.28
G	89.78	89.85	89.88	89.84	0.03
H	91.78	88.32	90.97	90.36	1.04
I	91.77	96.22	88.35	92.11	2.28
			Median:	86.69	
			MAD_E:	1.62	
			U_{median}:	1.54	

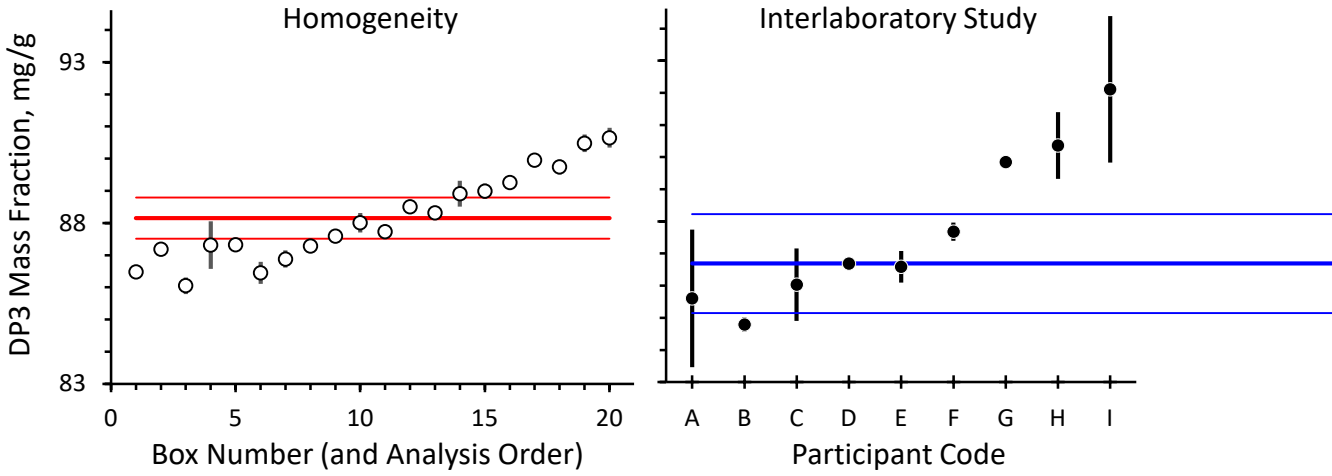


Figure 7. Graphical Summary of DP3 Results

Table 5. Homogeneity and Interlaboratory Study Results for DP4

Homogeneity

Box	RepA	RepB	Mean	s_{mean}
1	73.12	73.13	73.13	0.00
2	74.02	73.52	73.77	0.25
3	73.02	72.73	72.88	0.14
4	72.75	74.56	73.66	0.91
5	73.55	73.69	73.62	0.07
6	72.85	72.54	72.70	0.15
7	73.66	73.05	73.36	0.31
8	73.61	73.24	73.43	0.19
9	73.79	73.80	73.80	0.00
10	74.15	73.54	73.85	0.31
11	73.46	73.57	73.52	0.05
12	74.20	74.56	74.38	0.18
13	74.40	74.39	74.40	0.01
14	74.92	74.31	74.62	0.31
15	74.79	74.62	74.71	0.09
16	74.63	74.88	74.76	0.13
17	75.09	75.13	75.11	0.02
18	75.23	75.08	75.16	0.08
19	75.30	75.97	75.64	0.34
20	75.97	75.32	75.65	0.33
		Mean:	74.11	
		SD:	0.88	
		U_{mean}:		

Interlaboratory Comparison

Lab	RepA	RepB	RepC	Mean	s_{mean}
A	69.56	73.73	74.28	72.52	1.49
B	71.79	72.15	71.70	71.88	0.14
C	73.48	70.78	73.42	72.56	0.89
D	74.05	73.43	73.85	73.78	0.18
E	73.66	74.52	73.39	73.86	0.34
F	76.16	75.12	74.86	75.38	0.40
G	76.35	75.94	76.42	76.24	0.15
H	79.00	74.77	77.99	77.25	1.28
I	80.52	84.12	77.26	80.63	1.98
			Median:	73.86	
			MAD_E:	2.25	
			U_{median}:	2.15	

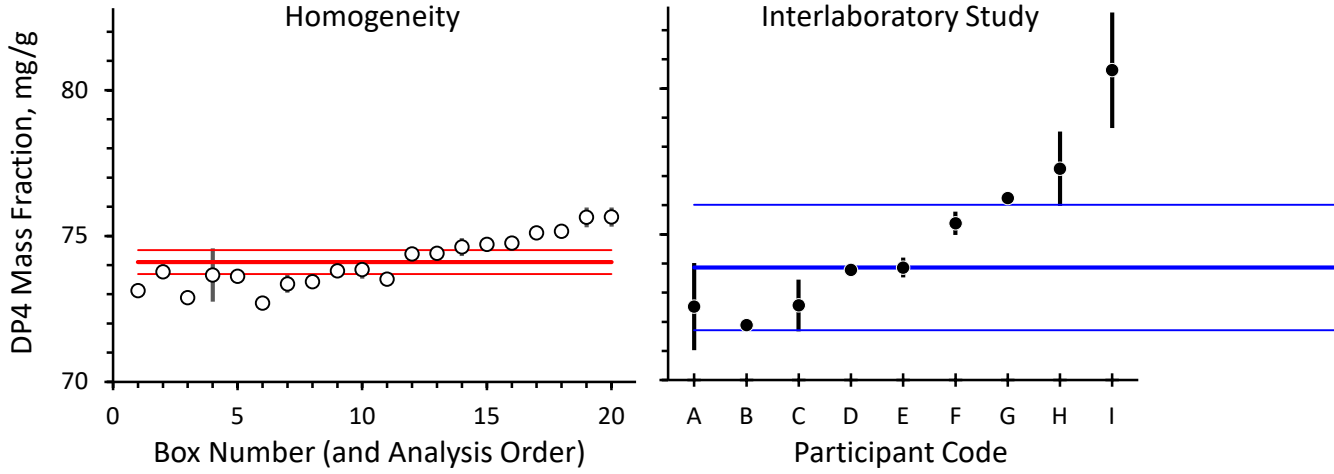


Figure 8. Graphical Summary of DP4 Results

Table 6. Homogeneity and Interlaboratory Study Results for DP5

Homogeneity

Box	RepA	RepB	Mean	s_{mean}
1	61.68	62.10	61.89	0.21
2	62.74	62.14	62.44	0.30
3	61.70	61.44	61.57	0.13
4	61.36	63.04	62.20	0.84
5	61.91	62.15	62.03	0.12
6	61.22	61.61	61.42	0.20
7	62.39	61.88	62.14	0.25
8	62.14	61.98	62.06	0.08
9	62.35	62.26	62.31	0.05
10	62.58	62.19	62.39	0.20
11	61.96	62.17	62.07	0.11
12	62.77	63.10	62.94	0.16
13	62.74	62.72	62.73	0.01
14	63.68	62.81	63.25	0.43
15	63.22	63.22	63.22	0.00
16	63.05	63.25	63.15	0.10
17	63.62	63.76	63.69	0.07
18	63.66	63.36	63.51	0.15
19	63.45	64.18	63.82	0.37
20	64.17	63.67	63.92	0.25
		Mean:	62.64	
		SD:	0.76	
		U_{mean}:		

Interlaboratory Comparison

Lab	RepA	RepB	RepC	Mean	s_{mean}
A	57.03	62.08	63.45	60.85	1.95
B	60.54	61.48	60.78	60.93	0.28
C	61.23	59.70	61.68	60.87	0.60
D	62.42	62.03	62.40	62.28	0.13
E	62.14	63.43	62.69	62.75	0.37
F	65.21	64.38	64.17	64.59	0.32
G	63.95	63.66	64.18	63.93	0.15
H	66.31	62.58	65.36	64.75	1.12
I	67.70	70.39	65.29	67.79	1.47
			Median:	62.75	
			MAD_E	2.73	
			U_{median}:	2.60	

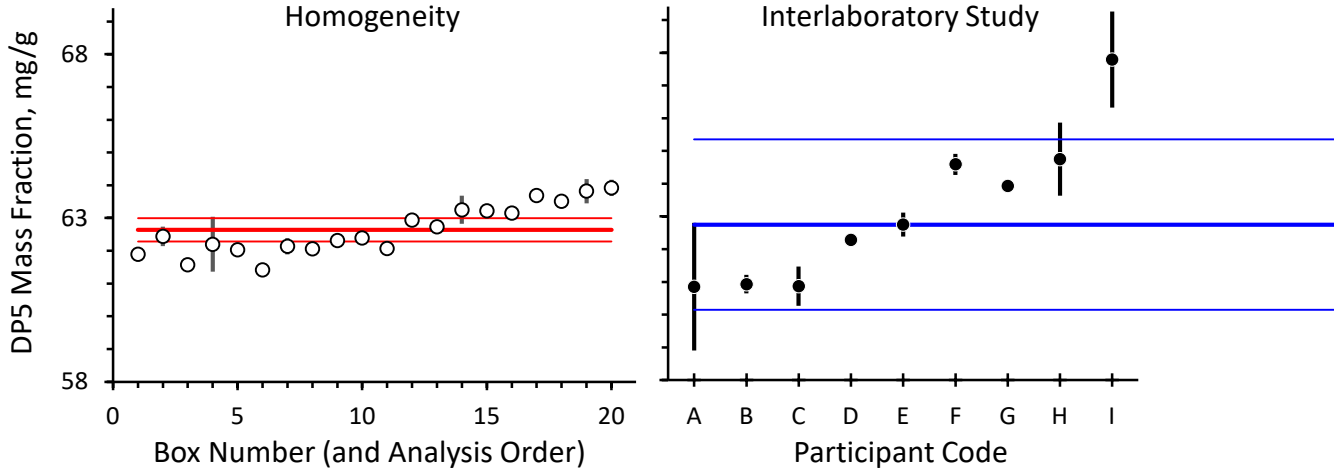


Figure 9. Graphical Summary of DP5 Results

Table 7. Homogeneity and Interlaboratory Study Results for DP6

Homogeneity

Box	RepA	RepB	Mean	s_{mean}
1	47.45	47.66	47.56	0.10
2	48.20	47.84	48.02	0.18
3	47.71	47.23	47.47	0.24
4	47.19	48.32	47.76	0.57
5	47.50	47.48	47.49	0.01
6	46.79	47.14	46.97	0.18
7	47.94	47.80	47.87	0.07
8	47.99	47.92	47.96	0.04
9	48.03	48.03	48.03	0.00
10	47.81	47.87	47.84	0.03
11	47.74	47.94	47.84	0.10
12	48.46	48.72	48.59	0.13
13	47.99	48.14	48.07	0.07
14	49.29	48.46	48.88	0.41
15	48.97	48.95	48.96	0.01
16	48.77	48.89	48.83	0.06
17	48.96	49.11	49.04	0.07
18	49.17	48.88	49.03	0.15
19	49.16	49.45	49.31	0.15
20	49.60	48.92	49.26	0.34
		Mean:	48.24	
		SD:	0.69	
		U_{mean}:		

Interlaboratory Comparison

Lab	RepA	RepB	RepC	Mean	s_{mean}
A	42.46	47.74	48.60	46.27	1.92
B	45.61	46.78	46.84	46.41	0.40
C	46.58	46.34	46.88	46.60	0.16
D	48.19	47.93	47.79	47.97	0.12
E	48.30	48.72	48.81	48.61	0.16
F	50.72	50.04	49.79	50.18	0.28
G	48.80	49.60	49.09	49.16	0.23
H	49.66	48.11	51.45	49.74	0.97
I	51.48	53.51	49.93	51.64	1.04
			Median:	48.61	
			MAD_E:	2.33	
			U_{median}:	2.22	

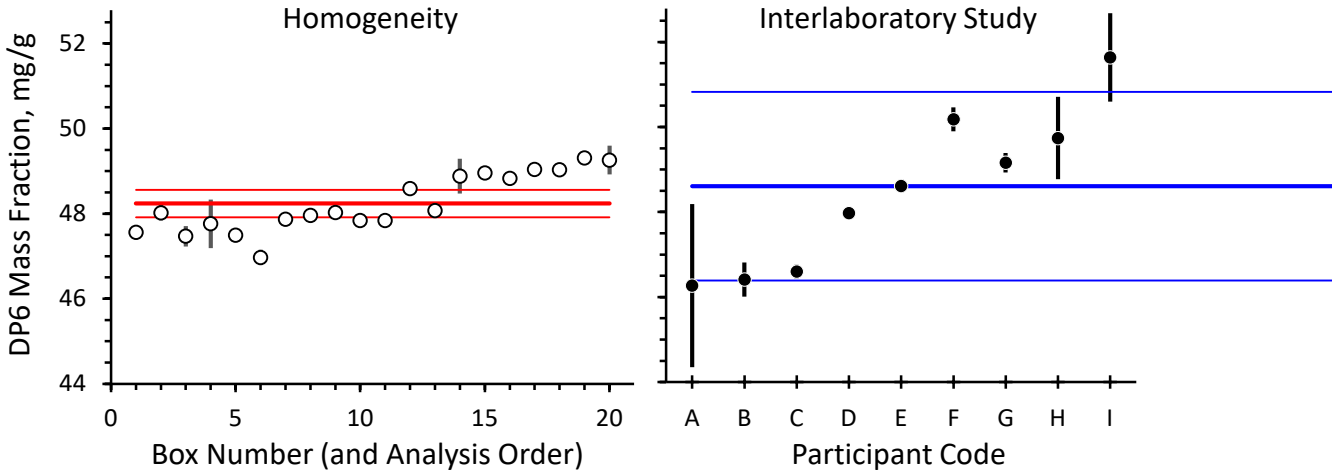


Figure 10. Graphical Summary of DP6 Results

Table 8. Homogeneity and Interlaboratory Study Results for DP7

Homogeneity

Box	RepA	RepB	Mean	s_{mean}
1	35.87	36.29	36.08	0.21
2	36.72	36.02	36.37	0.35
3	36.72	36.34	36.53	0.19
4	35.99	36.75	36.37	0.38
5	36.34	36.23	36.29	0.06
6	35.51	35.69	35.60	0.09
7	36.99	36.76	36.88	0.12
8	36.43	36.82	36.63	0.20
9	36.95	36.84	36.90	0.05
10	36.09	36.61	36.35	0.26
11	36.56	36.68	36.62	0.06
12	36.90	37.06	36.98	0.08
13	36.64	36.60	36.62	0.02
14	37.86	36.95	37.41	0.45
15	37.57	37.68	37.63	0.05
16	37.16	37.12	37.14	0.02
17	37.50	37.67	37.59	0.09
18	37.37	37.54	37.46	0.09
19	37.75	37.37	37.56	0.19
20	38.10	37.25	37.68	0.43
		Mean:	36.83	
		SD:	0.59	
		U_{mean}:		

Interlaboratory Comparison

Lab	RepA	RepB	RepC	Mean	s_{mean}
A	34.43	35.97	34.63	35.01	0.48
B	34.44	35.49	35.25	35.06	0.32
C	34.84	35.08	35.67	35.20	0.25
D	36.63	36.13	36.92	36.56	0.23
E	36.41	36.63	36.32	36.45	0.09
F	39.49	38.52	38.26	38.76	0.37
G	36.95	37.60	37.99	37.51	0.30
H	37.54	37.48	39.42	38.15	0.64
I	39.78	41.25	38.35	39.79	0.84
			Median:	36.56	
			MAD_E:	2.22	
			U_{median}:	2.12	

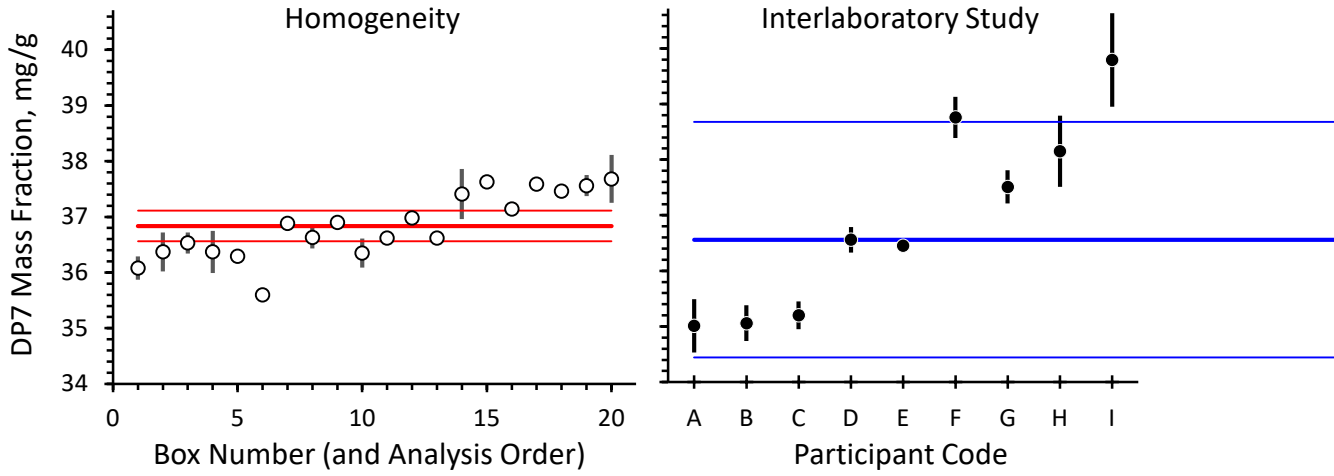


Figure 11. Graphical Summary of DP7 Results

4.1. Data Analysis

4.1.1. Homogeneity

Each analyte had 40 measurements (duplicate measurements from 20 boxes). There was a similar pattern of positive correlation between the box number and the measurement level for all analytes. According to the analyst, the box numbers indicate the order in which the samples were run and not the order they were produced (which is not exactly known). Thus, the chemical experts consider it likely that this association is due to some drift in the measurement system rather than from differences in the material.

Due to the clear association of box number with analyte level, the results will be based on using the set of box means as the measurements set. Since each box has two measurements for each analyte, the mean of the box means equals the mean of all measurements for each analyte. An uncertainty component related to possible drift in the system, based on the standard deviation of the box means, is incorporated into the uncertainty of each result.

4.1.2. Interlaboratory study

For the interlaboratory study the method estimate for each analyte is the weighted median of the individual laboratory means for that analyte, where the weights are based on a Laplace random effects model [5]. For this SRM, the weighted median is equal to or extremely close to the unweighted median of laboratory means for all analytes. The uncertainty of the weighted median is estimated using a bootstrap procedure based on a Laplace random effects model for the between-lab and within-lab effects [4,5,6,7,8].

4.1.3. Assignment of values and uncertainties:

For each analyte, the reference value is the mean of the method estimates available for that analyte. The uncertainty of the combined mean is estimated using a bootstrap procedure based on a Gaussian random effects model for the between-method effects [4-7].

Table 9. Assigned Values, mg/g

Analyte	Value	U_{95}(Value)
DP1	114.94	1.69
DP2	82.56	1.40
DP3	87.42	1.86
DP4	73.98	1.48
DP5	62.69	1.27
DP6	48.42	1.12
DP7	36.70	0.96

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