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SOP TITLE: DETERMINATION OF TOTAL PROANTHOCYANIDINS BY REACTION WITH DIMETHYLAMINOCINNAMALDEHYDE (DMAC)	
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INTRODUCTION

The DMAC method measures total PACs content in cranberry products by reaction with DMAC. The method shows less interference and seems to be a more reproducible method when compared to the previously published BL-DMAC method[1]. This method is applicable to straight cranberry products only.

PRINCIPLE

DMAC is protonated at it's carbonyl oxygen forming an electrophilic carbocation in the presence of a strong acid solution . This carbocation reacts with *meta* oriented di- or tri hydroxyl phenols to produce a colored carbonium ion which has a max at 640 nm. The most common standard for the DMAC method is procyanidin A2. This method shows increased accuracy because anthocyanins do not interfere with the max of the reaction. The DMAC method has also been known to be approximately 5 higher in its sensitivity as compared to the Vanillin Assay.

EQUIPMENT AND SUPPLIES

1. Microplate reader – operating at 640 nm.
2. Adjustable pipetter (20-200 µL and 100-1000 µL)
3. Centrifuge
4. Balance – readability of 0.01 mg
5. Sonicator
6. Grinder
7. Polystyrene 96-well microtitration plates (reading plate), 360ul flat-bottom (Costar no. 3370; VWR cat. no. 25381-056).

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REAGENTS

1. Millipore De-ionized (DI) water
2. HPLC Grade Methanol (Alfa Aesar cat. no. 22909)
3. P-dimethylaminocinnamaldehyde (DMAC) (Sigma cat. no. EC-228-267-0).
4. Procyanidin A2 (Extrasynthese cat. no. 0985S).
5. Sulfuric acid H_2SO_4 (Sigma cat. no. 339741 Aldrich)

WORKING REAGENT PREPARATION

1. Proanthocyanidin extraction solvent-Methanol
2. Reaction solvent- 0.4N H2SO4 in Methanol. Carefully measure 12 mL H2SO4 into a 50 mL erlenmyer flask. Slowly transfer H2SO4 by using a glass pasteur pipette in a 1L volumetric flask with 500mL methanol. Add a stir bar and allow to stir at least 30 minutes and until the heat generated by addition of H2SO4 is completely released. After the temperature of method reaches room temperature, fill to volume with methanol and allow to stir for a minimum 10 min. Transfer to a glass bottle (1 L or greater capacity) labeled as 0.4N H2SO4. Stable for 1 year at 18-25 °C.
   Note: prepare in a fume hood, wear safety glasses, gloves, lab coat and full-foot cover shoes!
3. 0.1% DMAC solution- Weigh out 0.05g of 4-(Dimethylamine) cinnamaldehyde (DMAC) in a 50mL-tube and add 50ml of reaction solvent to dissolve DMAC by sonicating for 30 minutes. This will yield 50ml of DMAC working solution. Make fresh daily.
4. Calibration Standard-100µg/ml Procyanidin A2 as the stock standard solution. Carefully weigh 5mg procyanidin A2 on a weigh paper. Quantitatively transfer to a 50 mL volumetric flask with 50 mL methanol. Aliquot and store the solution at – 80o C.
5. Control-80 µg/ml Procyanidin A2. Transfer 800 µL of 100µg/ml Procyanidin A2 into a 1.5 mL vial, add 200 uL methanol and vertex. Make fresh daily.

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SAMPLE PREPARATION

Use a labeled 50ml conical tube for each sample

1. For cranberry extracts containing high PACs
   a. Weigh about 0.01g
   b. Add 20 ml of methanol
   c. Sonicate at room temperature for 30minutes
   d. Place on shaker for 1 hour to extract
   e. Centrifuge at 5900 rpm at 20° C for 10 minutes and take the supernatant for assay

2. For cranberry powders
   a. Weigh about 0.1g
   b. Add 20 ml of methanol
   c. Sonicate at room temperature for 30minutes
   d. Place on shaker for 1 hour to extract
   e. Centrifuge at 5900 rpm at 20° C for 10 minutes and take the supernatant for assay

3. For cranberry capsules and tablets- need to be combined and homogenized to get a representative sample
   a. Weigh about 0.1~0.5g
   b. Follow steps 1b-e as above.

4. For fresh cranberry
   a. Grind clean fruit properly;
   b. Weight approximate 2g puree into a labeled 50 ml conical tube;
   c. Follow steps 1b-e as above.

5. For cranberry juice samples
   a. Add 1ml of juice to a C18 column (1cc, 100mg);
   b. Wash them with water 1ml twice to remove some added ingredients;
   c. Elute with 1ml methanol twice (dilution factor 2) and collect the 2ml eluent for assay.

96-WELL PLATE LAYOUT

The plate reader protocol is set to read the absorbance (640 nm) of each well in the plate every minute for 10 min at room temperature. The plate included blanks, standards, controls, and unknowns at serial dilutions of 1-, 2-, 4-, 8-, 16-, and 32-fold as appropriate.

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ANALYSIS

An automatic liquid handler or an adjustable pipetter is used to dispense into wells of a 96 -well plate one of the following: (1) 20 µL of 0.4N H2SO4 in methanol for blanks; or (2) 20 µL of control, standard, and samples. The DMAC solution (200 µL) is added using a multichannel pipetter into all 96 wells (containing blanks, standards, controls, and samples). The final volume is 220 µL well. The microplate is read for 10 min.

CALCULATIONS AND STATISTICAL ANALYSES

The maximum absorbance readings is used for calculation, which generally occurred before 6 min, depending on the dilution of the sample. Corrected absorbencies are calculated by subtracting the average blank absorbance and a calibration curve are generated from the standards. PAC concentrations are calculated by using a regression equation (Y = a + bX) between procyanidin A2 concentration (Y) (µg) and the maximum absorbance minus the blank (X). Concentrations of sample extracts are calculated as total PACs= (C × D × V)/(1000 × S), where the total PACs are in mg g−1; C is the concentration of PACs in a sample extract, in g L−1; D is the dilution factor; V is the extraction volume, in milliliters; and S is the sample size, in grams. Data are expressed as milligrams of procyanidin A2 equivalents per gram or per 300 mL (juice) of sample. The means and relative standard deviation (%RSD) for replicate analyses are calculated for each sample.

CRITICAL POINTS

1. Standard preparation: Procyanidin A2 needs to be accurately weighed.

2. Water Content Of Sample: Because the DMAC assay is very dependent on the acid concentration to catalyze the reaction, small increases in water content could have a significant effect on the pH of the procyanidin A2 standard and thus on the final color produced. For this reason absolute methanol should be utilized in the DMAC assay. Methanol has also been found to give better results as compared to ethanol or butanol because it accelerates the reaction more efficiently. We recommend the use of absolute methanol in the assay. If water in the sample is unavoidable, it is recommended that the standard used contains the same concentration of water to avoid underestimation of the compounds.

3. Interfering substances: Products containing low PACs may encounter with background interference caused by excipients, it is recommended that 0.2 g or more should be weighed to decrease the interference.

REFERENCE CITED

1. J Sci Food Agric. 2010 Jul;90(9):1473-8

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APPENDIX A

Rationale Of The Improvement of the extraction solvent

Acid Nature And Concentration: The DMAC assay should be carried out in an acidic medium because of the catalytic role of the acid in the reaction of procyanidins and DMAC. The previous protocol has utilized HCl in preparation of the DMAC reagent for UV-Vis spectrophotometric assays and the use of H2SO4 has not been explored1. We found 0.4N H2SO4 increased color development, resulting in higher sensitivity by allowing for better detection between smaller changes in concentration.