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Fluorescent labeling of cranberry proanthocyanidins with 5-([4,6-dichlorotriazin-2-yl]amino)fluorescein (DTAF)

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ABSTRACT

A novel methodology was developed to elucidate proanthocyanidins (PAC) interaction with extra-intestinal pathogenic *Escherichia coli* (ExPEC). PAC inhibit ExPEC invasion of epithelial cells and, therefore, may prevent transient gut colonization, conferring protection against subsequent extra-intestinal infections, such as urinary tract infections. Until now PAC have not been chemically labeled with fluorophores. In this work, cranberry PAC were labeled with 5-([4,6-dichlorotriazin-2-yl]amino) fluorescein (DTAF), detected by high-performance liquid chromatography with diode-array detection and characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). We report single and double fluorescent-labeled PAC with one or two chlorine atoms displaced from DTAF in alkaline pH via nucleophilic substitution. Fluorescent labeling was confirmed by fragmentation experiments using MALDI-TOF/TOF MS. Fluorescent labeled PAC were able to promote ExPEC agglutination when observed with fluorescence microscopy. DTAF tagged PAC may be used to trace the fate of PAC after they agglutinate ExPEC and follow PAC-ExPEC complexes in cell culture assays.

1. Introduction

Cranberry proanthocyanidins (PAC) interact with extra-intestinal pathogenic *Escherichia coli* (ExPEC) inducing bacterial agglutination and reducing bacterial invasion in the gut (Feliciano, Meudt, Shanmuganayagam, Krueger, & Reed, 2013). Although previous studies demonstrated that cranberry PAC affect *E. coli* virulence factors (Johnson et al., 2008; Liu et al., 2008; Liu, Pinzón-Arango, Gallardo-Moreno, & Camesano, 2010), an understanding of the interaction between cranberry PAC and ExPEC is still lacking.

E. coli has been tagged with fluorescent probes (Loh & Ward, 2012) but a better insight on ExPEC-host cell interactions requires fluorescent labeled PAC. Cranberry PAC exhibit fluorescence but have a very narrow excitation and emission wavelength in the ultraviolet region, 276 and 310 nm, respectively (Adamson et al., 1999), and therefore cannot be used in fluorescence microscopy. The use of fluorescent probes to label PAC has not been described until now. On the contrary, fluorescence labeling of proteins has been widely used for cell biology and biochemistry applications (Toseland, 2013). Fluorescein isothyocianate (FITC), the most widely used fluorochrome, is reactive towards nucleophiles, such as amine groups in proteins (Blakeslee & Baines, 1976; Lalljie & Sandra, 1995) but does not react with molecules with multiple hydroxyl groups, such as in cranberry PAC.

5-([4,6-Dichlorotriazin-2-yl]amino) fluorescein (DTAF) has the advantage of reacting with amines (Blakeslee & Baines, 1976; Krahn, Bouten, van Tuijl, van Zandvoort, & Merkx, 2006; Liu, Hu, Ma, & Lu, 2004; Molina & Silva, 2002; Siegler, Sternson, & Stobaugh, 1989; Wadsworth & Sloboda, 1984; Wang, Li, Li, Hu, & Chen, 2006; Xiao et al., 2007) and hydroxyls in alkaline aqueous solutions (Abitbol, Palermo, Moran-Mirabal, & Cranston, 2013; Ahmed, Alexandridis, & Neelamegham, 2001; Schumann & Rentsch, 1998; Weyermann et al., 2004; Wicks & Li, 2004). The DTAF labeling reaction with hydroxyl groups follows a nucleophilic aromatic substitution by an addition–elimination pathway (Ahmed et al., 2001). In this work, we investigated the possibility

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of labeling PAC with DTAF after optimization of reaction parameters.

We report the detection of fluorescent-labeled PAC with DTAF using high-performance liquid chromatography with diode-array detection (HPLC-DAD), followed by structural confirmation with matrix-assisted laser/desorption ionization time-of-flight (MALDI-TOF MS). Fluorescent-labeled PAC were mixed with ExPEC and bacterial agglutination with emission of fluorescence was detected using fluorescence microscopy.

2. Materials and methods

2.1. Chemicals

Water, methanol, acetone (HPLC grade), acetic acid glacial, trifluoroacetic acid, sodium carbonate and sodium chloride were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ethanol (200 proof) was obtained from Decon Labs Inc (King of Prussia, PA, USA). Triethylamine was purchased from Acros Organics (Geel, Belgium). Sterilized water, thyamin hydrochloride, 2,5-dihydroxybenzoic acid (DHB) and the Folin–Ciocalteu reagent were purchased from Sigma–Aldrich, (St. Louis, MO, USA). Sephadex LH-20 was purchased from GE Healthcare (Uppsala, Sweden). 5-([4,6-Dichlorotriazin-2-yl]amino)fluorescein was obtained from Invitrogen (Carlsbad, CA, USA). HyClone Dulbecco's PBS without calcium and magnesium was obtained from Thermo Scientific (South Logan, UT, USA). Tryptose and dextrose were obtained from BD (Sparks, MD, USA).

2.2. Extraction and isolation of proanthocyanidins

Cranberry fruits *(Vaccinium macrocarpon* Ait. cv. 'Stevens') were obtained from the United Cranberry Growers Cooperative (Wausau, WI, USA). The fruits were homogenized to a fine powder by blending with liquid nitrogen. The fruit powder was stored at −80 °C until extraction.

The PAC extraction procedure was adapted from our previous work (Feliciano, Shea et al., 2012) using a total weight of 1314 g of cranberry powder (151.15 g of dry matter). For each 100 g of cranberry powder, 400 mL of 70% aqueous acetone were added and extracted in an ultrasonic bath for 15 min. The extract was centrifuged at 400*g* at 15 °C for 10 min and the supernatant was collected. The extraction was repeated two additional times and the supernatants combined. After filtration with cellulose paper, acetone was removed by evaporation under vacuum at 35 °C and the remaining suspension was solubilized in ethanol. The extract was then centrifuged at 13,416*g* at 0 °C for 10 min to eliminate insoluble material. The supernatants were used to isolate PAC by chromatography on Sephadex LH-20.

The ethanolic cranberry extract was loaded on glass columns (2.5 cm I.D. × 60 cm length, Kontes, Chromaflex) packed with Sephadex LH-20 that were previously swollen and washed in water and equilibrated with ethanol for 45 min at a flow rate of 4 mL/min (Feliciano et al., 2013). The column was eluted with ethanol, ethanol:methanol (1:1) and 80% aqueous acetone. The 80% aqueous acetone fraction was evaporated to dryness and dissolved in 67% aqueous methanol, and defined as "unlabeled proanthocyanidins" or UL-PAC. The extraction of PAC from 151.2 g of cranberry powder dry matter, derived from whole fruits, generated 2.43 g of PAC dry matter. The cranberry fruit had a moisture content of 88.5% and a PAC yield of 1.6%.

2.3. Synthesis of fluorescent labeled cranberry proanthocyanidins

UL-PAC (0.858 mL, 90.1 mg dry matter/mL) was evaporated and solubilized in 50 mL of 80% aqueous acetone under nitrogen. The pH of the solution was raised to ~10.5 with the addition of 0.8 mL of triethylamine (0.935 M in 80% aqueous acetone). DTAF (36 mg) was added to the solution and incubated at room temperature for 30 min with a magnetic stirrer. The reaction was quenched after 30 min with the addition of 0.050 mL of 8 M acetic acid. The acetone was removed by evaporation and the remaining water was diluted 1:1 with ethanol. This reaction mixture was loaded onto a column that contained 20 g of Sephadex LH-20 and was previously washed thoroughly with 400 mL of water and equilibrated with 100 mL of 50% aqueous ethanol. The column was eluted with 100 mL of 50% aqueous ethanol (eluate A), 500 mL of ethanol (eluate B) and 200 mL of 80% aqueous acetone. While eluate A and B were evaporated to dryness and solubilized in water with at least 50% of methanol, the 80% aqueous acetone fraction was evaporated to dryness and solubilized in 75% aqueous methanol and defined as "fluorescently labeled proanthocyanidins" or "FL-PAC".

2.4. Characterization of proanthocyanidins

2.4.1. High-performance liquid chromatography

The PAC content of UL-PAC and FL-PAC was quantified by the Folin–Ciocalteu method and results were expressed as gallic acid equivalents (GAE/mL). UL-PAC was diluted 1:10 in 50% aqueous methanol to a final concentration of 6.99 mg GAE/mL. Eluates A, B and FL-PAC were diluted with 50% aqueous methanol to a final concentration of 0.19 mg GAE/mL in order to monitor the reaction by HPLC. All samples were filtered with a 0.45 μm PTFE membrane before analysis by high-performance liquid chromatography with diode array detection (HPLC-DAD). Samples were analyzed on an Agilent 1100 HPLC (Agilent, Santa Clara, CA, USA) with an autosampler, a quaternary solvent pump and a photodiode array detector. Chromatography was performed with a Luna C_{18} column (5 μm, 10.0 × 250 mm) (Phenomenex, Torrance, CA, USA). The elution solvents were 0.1% trifluoroacetic acid in water (Solvent A) and 0.1% trifluoroacetic acid in methanol (Solvent B). The elution profile (flow rate of 2 mL/min) started at 10% solvent B and increased by 1% every min up to 80% of solvent B then ramped to 100% of solvent B in 1 min and held constant for 5 min. The gradient was reverted to 10% solvent B for 10 min to equilibrate the column. The elution was monitored at 280 and 443 nm, using Open LAB CDS ChemStation Edition C.01.05 software to collect and analyze chromatograms.

2.4.2. Matrix-assisted laser/desorption ionization time-of-flight

Previously developed MALDI-TOF MS methods (Feliciano, Krueger, Shanmuganayagam, Vestling, & Reed, 2012) were applied to characterize UL-PAC and FL-PAC. An aliquot of each fraction was evaporated to dryness and suspended in ethanol to a final concentration of approximately 20 mg GAE/mL. An aliquot (1.0 μL) of each fraction was mixed with 8 μL of DHB (50.0 mg/mL in ethanol) and placed in three different wells on the stainless steel MALDI target. Mass spectra were collected on a Bruker ULTRAFLEX-III™ MALDI-TOF/TOF (Billerica, MA, USA), equipped with a Smart Beam™, a two-stage gridless reflection, and a LIFT™ cell. Mass spectra acquired in positive reflection mode are the sum of spectra from different locations in each well with the deflection set at 550 and 1000 Da for UL-PAC and FL-PAC, respectively. MSMS spectra were collected using the LIFT™ cell. For UL-PAC between degree of polymerization (DP) 3 and 8, the monoisotopic ion was specified, and for FL-PAC between 1000 and 1500 Da, the most intense ions in each cluster were specified. Compass™ v1.3 software controlled the instrument. A standard PAC preparation was used for calibration as previously described (Feliciano, Shea et al., 2012). FlexControl and FlexAnalysis (Bruker Daltonik GmbH, Bremen, Germany,

version 3.0) were used for data acquisition and data processing, respectively.

2.4.3. Fluorescence microscopy

We used an ExPEC strain isolated from a woman with recurrent UTI that was found in both the gastrointestinal and urinary tracts and expressed fimH and papA, the adhesins of type 1 and P fimbriae, respectively (Feliciano et al., 2013). The bacterial culture was done under static culture conditions in tryptose broth (10 g of tryptose, 2.5 g of sodium chloride, 0.5 g of dextrose, and 0.0025 g of thiamin hydrochloride in 500 mL of deionized water) at 37 °C and washed twice with PBS by centrifugation at 1840*g* for 10 min (Feliciano et al., 2013). The optical density of the ExPEC inoculum suspension at 450 nm was used to calculate and adjust the bacterial cell density to 1.0×10^{10} colony forming units (CFU)/mL using a previously established bacterial density–absorbance curve (Feliciano et al., 2013). Three treatments were performed to investigate the ability of FL-PAC to agglutinate ExPEC. In each treatment, 357 μL of ExPEC inoculum (1.0×10^{10} CFU/mL) were mixed with 60 μL of DTAF (0.9 mg/mL) (negative control), 60 μL of UL-PAC (0.7 mg GAE/mL) (positive control: sample A) and 60 μL of FL-PAC (0.7 mg GAE/mL) (sample B). All treatments had a final ExPEC concentration of 8.6×10^9 CFU/mL. UL-PAC and FL-PAC had a final PAC concentration of 100 μg GAE/mL. All samples were incubated at 37 °C for 4 h and diluted 1:1000 in PBS. One hundred microliters of each sample was placed in MatTek P35G-1.5-14C glass bottom culture dishes and observed in wide-field with a Zeiss 200 M Axiovert inverted microscope using a 100× oil-immersion objective equipped with an Ex. 450–490 nm, Em. 510-565 filter cube. The images were captured on the Zeiss AxioCam HRm and analyzed using Zeiss AxioVision Rel. 4.8.1 software.

2.5. Data and statistical analysis

mMass version 3.9.0 (Strohalm, Kavan, Novak, Volny, & Havlicek, 2010) was used for mass spectra analysis.

3. Results and discussion

3.1. Rationale for method optimization

Nucleophilic attack by PAC hydroxyl groups on DTAF can only occur at alkaline pH values. The increase in pH allows ionization of the most acidic hydroxyl groups. The first ionizable hydroxyl group corresponds to the hydroxyl group attached to C3′ which is slightly more acidic than C4′. The second group to be ionized corresponds to hydroxyls on positions C5 and C7 (Herrero-Martinez, Sanmartin, Roses, Bosch, & Rafols, 2005) (Fig. 1). The monomeric units of cranberry PAC, catechin and epicatechin, have p*K*a values of 8.7 and 8.9, respectively, for the hydroxyl groups on position C3′ (Herrero-Martinez et al., 2005). Theoretical calculations using computer software (MarvinView 5.11.4) showed that in a PAC trimer with 1 "A-type" interflavan bond, the lowest p*K*a is 8.8. Thus, the pH of the reaction mixture needs to be raised, at least above 8.7 to cause ionization of the hydroxyl group. Our preliminary work showed a marked decrease in the efficiency of the reaction for pH values between 9.0 and 9.7 when compared to 10.5 (data not shown). However, if the pH is too high, PAC undergo depolymerization (White, Howard, & Prior, 2010) or form highly reactive quinones that can convert "B-type" into "A-type" interflavan bonds (Poupard, Sanoner, Baron, Renard, & Guyot, 2011). Furthermore, DTAF degrades if the pH of the solution is near the p*K*a of the bridging amine of the DTAF molecule at 10.82 (Siegler et al., 1989). As a consequence, there is a narrow window of pH values, between 9.7 and 10.5, to conduct the labeling reaction. In this work, we calculated the amount of base that was necessary to yield a pH of approximately 10.5 in an aqueous solution as an approximation to the 80% aqueous acetone solution used, which was necessary to solubilize PAC. We used an organic base (triethylamine) (Petrossian, Kantor, & Owicki, 1985), instead of borate (Liu et al., 2004) or carbonate buffers (Wang et al., 2006) to simultaneously solubilize PAC and reduce the amount of water that could compete with PAC in terms of nucleophilic activity (Wicks & Li, 2004).

Previous work showed that DTAF needs to be in excess compared to the nucleophile to increase the yield of the reaction products (Molina & Silva, 2002). Ephedrine and pseudoephedrine were labeled with DTAF with a molar ratio of DTAF to substrates of 20:1 (Wang et al., 2006). Excess DTAF was removed by size exclusion chromatography Sephadex G-50 (Ahmed et al., 2001), dialysis against water (Weyermann et al., 2004), centrifugation (Helbert, Chanzy, Husum, Schülein, & Ernst, 2003) or thin-layer chromatography (Petrossian et al., 1985). In our work due to the structural complexity of PAC we used Sephadex LH-20. Our preliminary data showed that high amounts of DTAF caused long chromatography separations to remove excess DTAF on Sephadex LH-20, poor chromatographic resolution and no yield increase (data not shown). Since the reaction was conducted with excess DTAF, we prevented DTAF degradation after 30 min of reaction by quenching the mixture with acetic acid to lower the pH to approximately 4.5.

By optimizing reaction conditions, ionized hydroxyl groups act as weak nucleophiles towards the electrophilic carbon on the triazine ring to which the chlorine is bound. The reaction is promoted by the strong electron withdrawing nitrogen atoms in the triazine ring (Ahmed et al., 2001), which leads to the formation of ether bonds between PAC and DTAF (Krahn et al., 2006). We hypothesized that the aromatic nucleophilic substitution would lead to the displacement of one chlorine atom generating MTAF (5-([monochlorotriazin-2-yl]amino)fluorescein) or two chlorine atoms generating TAF (5-([triazin-2-yl]amino)fluorescein) labeled PAC. Since PAC have multiple hydroxyl groups, a second DTAF molecule could react with the same PAC leading to double fluorescent-labeled PAC: MTAF–MTAF–PAC, MTAF–TAF–PAC and TAF–TAF–PAC (Fig. 1). This double labeling mechanism was shown for polymers with two terminal hydroxyl groups (Ahmed et al., 2001).

3.2. High-performance liquid chromatography

Unlabeled proanthocyanidins, UL-PAC, eluted as a single hump with maximum absorbance at 280 nm (Fig. 2A). PAC complex structural heterogeneity (Feliciano, Shea et al., 2012) is responsible for this poor resolution on a C18 reverse-phase HPLC column (Krueger, Dopke, Treichel, Folts, & Reed, 2000; Prodanov et al., 2013).

When eluates A and B from Sephadex LH-20 were analyzed by HPLC-DAD they did not display a major hump. Whereas eluate A (50% aqueous ethanol fraction) contained mainly DTAF (retention time = 78 min), eluate B (ethanol fraction) displayed DTAF and a very small hump with absorbances at 280 and 443 nm (data not shown). These wavelengths were chosen because DTAF has two absorbance maxima at 275 and 443 nm and PAC have a maximum absorbance at 280 nm.

The FL-PAC had absorbance maxima at 280 and 443 nm and eluted as a single hump at higher retention times than for UL-PAC (Fig. 2B). The increase in retention time of FL-PAC compared to UL-PAC is due to decreased polarity of FL-PAC. The fluorescein moiety of these molecules increases their affinity to the non-polar C18 stationary phase, increasing the retention time of the hump. The polarity of a compound dictates the extent of its affinity to organic solvents and water. This property has been used to measure the octanol–water partition coefficient (log*P*) of tannins

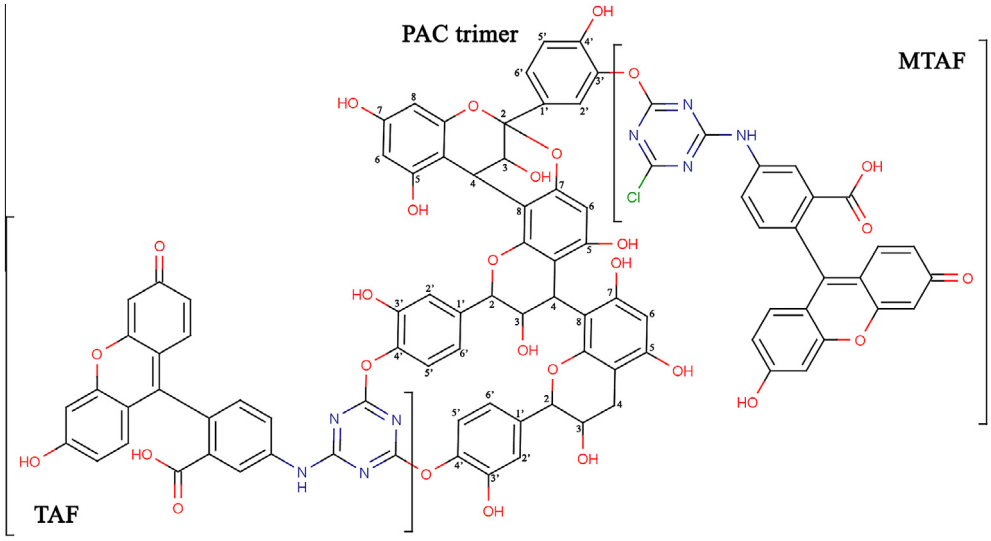

Fig. 1. Hypothetical structure of a proanthocyanidin (PAC) trimer with 1"A-type" interflavan bond covalently linked to 5-([monochlorotriazin-2-yl]amino)fluorescein (MTAF) and to 5-([triazin-2-yl]amino)fluorescein (TAF).

(Mueller-Harvey et al., 2007). For instance, log*P* of procyanidin A2 is 3.59 whereas log*P* values for MTAF-procyanidin A2, TAF-procyanidin A2, MTAF-MTAF-procyanidin A2 and MTAF-TAF-procyanidin A2 are at least 2–3 times higher (log*P* = 8.02, 8.15, 12.99 and 12.59, respectively, as calculated by MarvinView 5.11.4), showing the higher hydrophobicity of PAC fluorescent molecules when compared with unlabeled PAC with the same DP.

The hump detected in FL-PAC had combined spectral characteristics of PAC and DTAF displaying absorbance maxima at 277 and 443 nm indicating that PAC were effectively labeled with DTAF. However, the HPLC-DAD results also indicate that some PAC might have not reacted completely with DTAF since between 30 and 40 min the absorbance at 443 nm is, on average, 18 times lower than at 280 nm, while in the hump, between 40 and 80 min, the absorbance at 443 is, on average, only 4 times lower than at 280 nm. Moreover PAC elute up to 60 min in UL-PAC, which overlaps with the hump detected for FL-PAC corroborating the presence of unreacted PAC in FL-PAC.

3.3. Matrix-assisted laser/desorption ionization time-of-flight

3.3.1. Analysis of unlabeled proanthocyanidins

The mass spectrometry analysis of UL-PAC showed peaks at m/z = 615, 903, 1191, 1479, 1768, 2056, 2344, 2632, 2920, 3209 and 3495, which are consistent with potassium adducts of PAC with DP between 2 and 12 (Fig. 3A), according to the equation $m/z = 290 + 288d - 2A + c$, where 290 represents the molecular weight of the terminal catechin/epicatechin unit, d is the number of catechin/epicatechin extension units, A is the number of "A-type" interflavan bonds and c is the molecular weight of potassium cations (Feliciano, Krueger et al., 2012; Krueger, Vestling, & Reed, 2004). PAC with 1 "A-type" interflavan bond were the most intense peaks between DP 2 and 12. This PAC distribution is comparable to our previously published mass spectrometry data on PAC isolated from cranberry fruits (Feliciano et al., 2013) and press-cake (Feliciano, Krueger et al., 2012).

MSMS experiments were conducted between DP 3 and 8 for UL-PAC (Supplementary information – Table 1). The fragmentation pattern of these molecules was characterized by losses of 288 atomic mass units (amu), characteristic of a catechin/epicatechin monomeric unit. Moreover, the fragmentation of a PAC octamer with 1 "A-type" interflavan bond yielded other intermediary peaks, which can be seen on the MSMS spectrum (Supplementary information – Fig. 1). These intermediary peaks differ 170 amu from each main PAC peak and correspond to retro-Diels–Alder rearrangements (−152 amu), followed by elimination of a water molecule (−18 amu) as described by other authors for fragmentation experiments conducted with PAC isolated from cinnamon (Mateos-Martín, Fuguet, Quero, Pérez-Jiménez, & Torres, 2012).

3.3.2. Analysis of fluorescent-labeled proanthocyanidins

Unlabeled PAC with DP between 4 and 9 (Fig. 3B) were detected by MALDI-TOF MS in the FL-PAC indicating that the reaction was not complete, even with excess of DTAF reagent, which is in agreement with the HPLC-DAD results.

Based on the equation presented for PAC we were able to develop a new equation to calculate the expected m/z for fluorescent labeled PAC: $m/z = 290 + 288d - 2A + n\text{DTAF} - n\text{H} - n\text{Cl} + c$, where 290 represents the molecular weight of the terminal catechin/epicatechin unit, d is the number of catechin/epicatechin extension units, A is the number of "A-type" interflavan bonds, nDTAF represent the molecular weight of DTAF multiplied by the number of molecules that participated in the reaction (n = 1 or 2 for single or double fluorescent-labeled PAC, respectively), nH and nCl are the number of hydrogen atoms and chlorine atoms lost during the nucleophilic substitution multiplied by their corresponding atomic weights [n = 1 (MTAF), n = 2 (TAF and MTAF–MTAF), n = 3 (MTAF–TAF) or n = 4 (TAF–TAF)], and c is the molecular weight of sodium or potassium cations. The displacement of the first chlorine atom on the dichlorotriazin ring makes the second chlorine atom less reactive and therefore the first Cl substitution is more facile than the second Cl substitution (Blakeslee & Baines, 1976).

We detected 34 fluorescent labeled PAC with 1 "A-type" interflavan bond between 1000 and 4150 Da (Fig. 3B), including MTAF–PAC with DP between 2 and 11, TAF–PAC with DP between 3 and 10, MTAF–MTAF–PAC with DP between 5 and 11 and MTAF–TAF–PAC with DP between 2 and 9 (Table 1). No peaks with m/z corresponding to TAF–TAF–PAC with a signal-to-noise ratio >3.0 were detected. Single-labeled PAC were more prevalent in the spectrum than the double-labeled PAC probably due to the higher steric hindrance of a subsequent attack of a single-labeled PAC on the DTAF molecule.

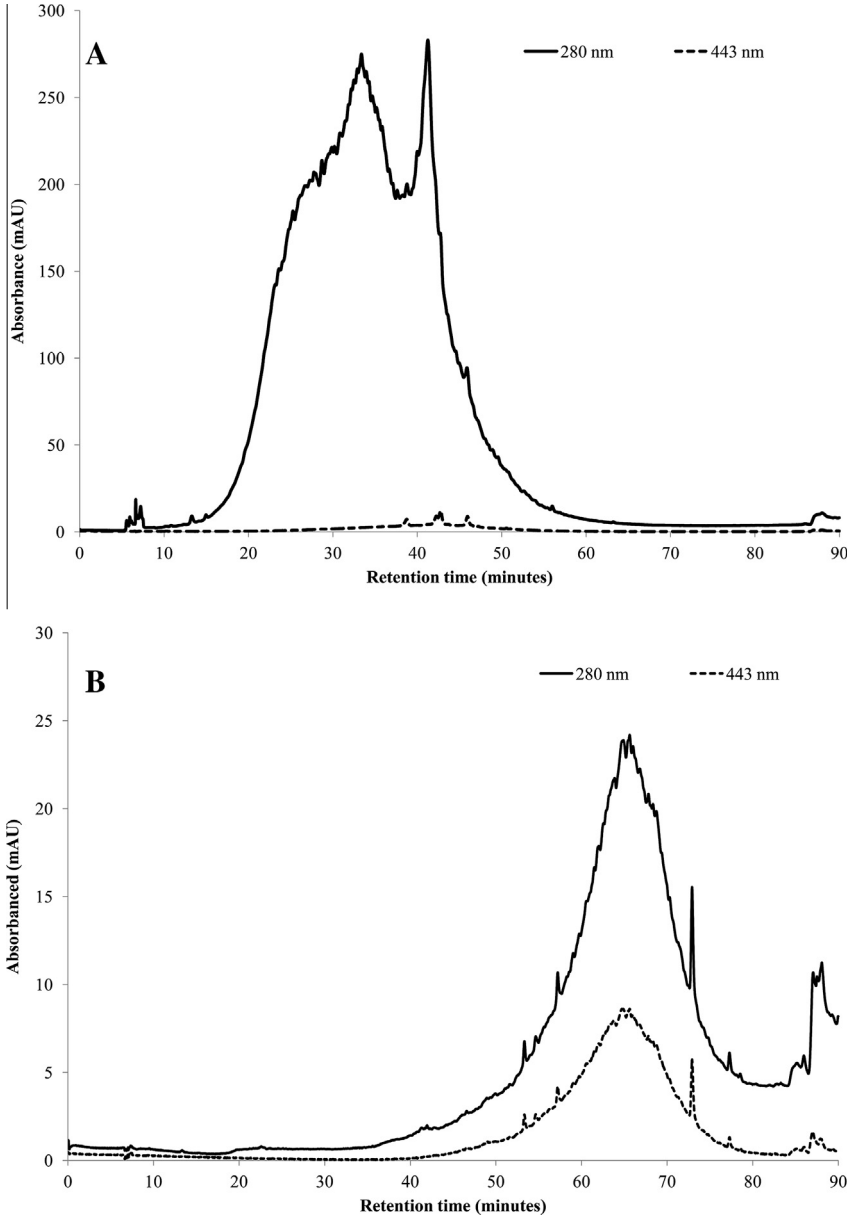

Fig. 2. HPLC chromatograms at 280 and 443 nm for (A) unlabeled proanthocyanidins (UL-PAC) and (B) fluorescent labeled proanthocyanidins (FL-PAC).

We chose a subset of the MALDI-TOF MS spectrum to illustrate and explain each type of labeled PAC. Peaks 19, 20, 21 and 22 (Fig. 3B) were enlarged in Fig. 3C and two sets of peaks can be visualized. The first one corresponds to single-labeled PAC octamers with TAF (m/z = 2750.0) and MTAF (m/z = 2785.0) and the second one corresponds to double-labeled PAC heptamers with MTAF–TAF (m/z = 2920.2) and MTAF–MTAF (m/z = 2955.2). The ions detected were less than 0.7 Da from the predicted m/z using the equation previously described (Table 1). The 35 amu difference between TAF and MTAF corresponds to the mass of one chlorine atom, whereas the 170 amu difference between either TAF and MTAF–TAF or MTAF and MTAF–MTAF corresponds to the combination of an additional fluorescent moiety (+459 amu) and one less monomeric unit (−288 amu) in the PAC chain (Fig. 3C).

It is important to notice that, coincidently, MTAF–TAF–PAC molecules have the same m/z as potassium adducts of unlabeled PAC. For instances, a PAC with DP = 5 with 1 "A-type" interflavan bond, which ionizes as a potassium adduct [PAC with DP = 5 + K]$^+$, has an exact mass of 1479.28, whereas a MTAF–TAF–PAC with DP = 2 with 1 "A-type" interflavan bond that ionizes as a sodium adduct [MTAF–TAF–PAC with DP = 2 + Na]$^+$ has an exact mass of 1479.22. Fluorescent labeled PAC have a more complex isotopic distribution than PAC due to the presence of N and Cl atoms. This effect is particularly important for Cl due to ^{37}Cl isotope abundance being approximately 24%. However, the lower abundance and resolution of the peaks with m/z = 1479 in the spectrum did not allow an unambiguous identification of these peaks just based on their isotopic distribution. The difference between potassium adducts of unlabeled PAC with DP = n and sodium adduct of labeled PAC with DP = n − 3 can be evaluated by high-resolution techniques, such as matrix-assisted laser desorption/ionization–Fourier transform ion cyclotron resonance mass spectrometry (MALDI–FT-ICR-MS) (Feliciano, Shea et al., 2012) or by MALDI–TOF/TOF MS. In order to unambiguously identify these peaks, fragmentation experiments using MALDI–TOF/TOF MS were conducted.

MSMS experiments revealed a different fragmentation pattern for FL-PAC, comparatively to UL-PAC, which was consistent with PAC structures fluorescently labeled. Based on absolute intensity,

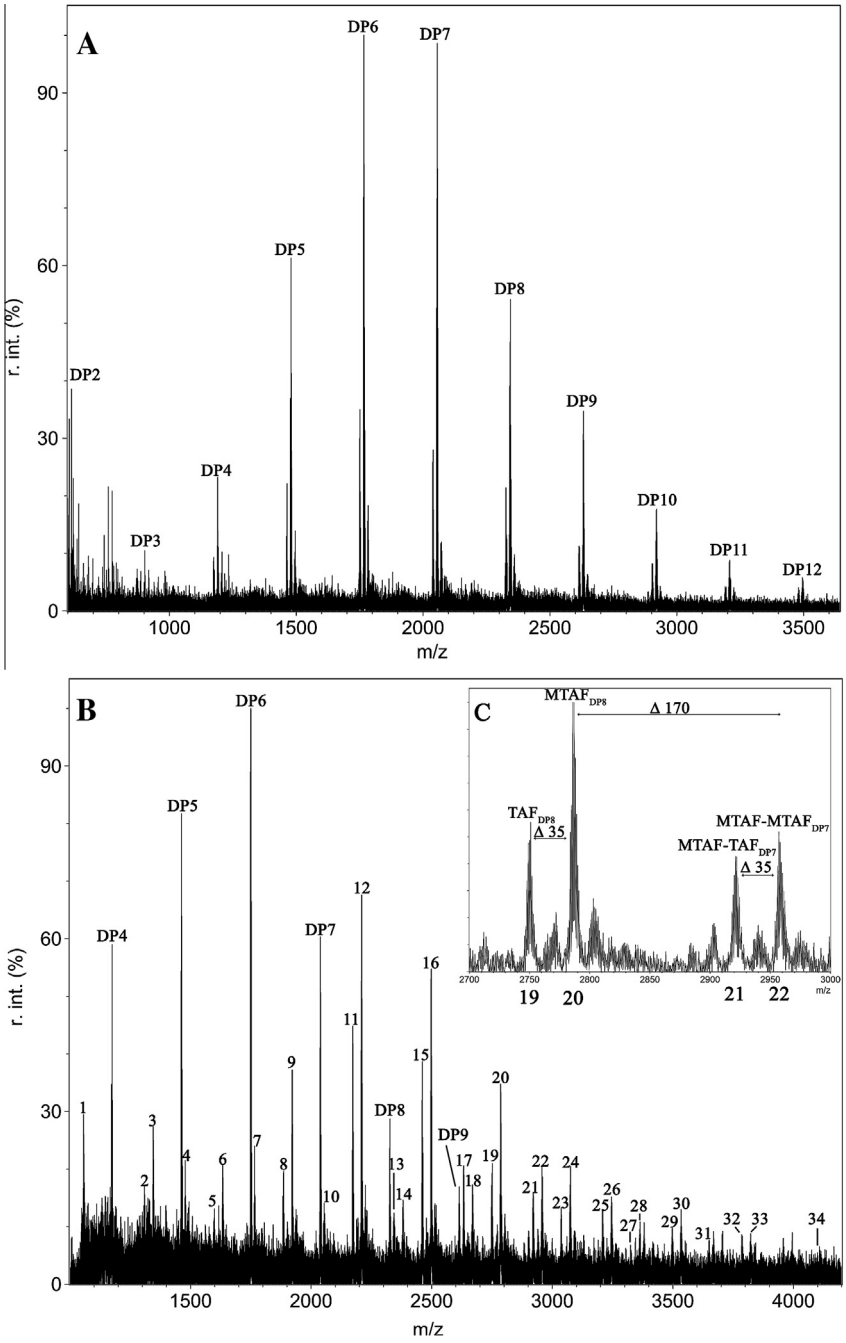

Fig. 3. Mass spectra obtained with MALDI-TOF MS in positive reflectron mode for (A) unlabeled proanthocyanidins (UL-PAC) between 550 and 3650 Da and (B) fluorescent labeled proanthocyanidins (FL-PAC) between 1000 and 4150 Da. Fluorescent tagged PAC are labeled from 1 to 34 and their identification is described in Table 1. (C) Inset – enlarged spectrum between 2700 and 3000 Da of peaks 19, 20, 21 and 22 in FL-PAC. "MTAF" stands for (5-([monochlorotriazin-2-yl]amino)fluorescein and "TAF" stands for 5-([triazin-2-yl]amino)fluorescein, respectively. "r.int." and "DP" correspond to relative intensity and degree of polymerization, respectively.

three labeled PAC (MTAF–PAC with DP = 2, MTAF–PAC with DP = 3, and MTAF–TAF–PAC with DP = 2: peaks 1, 3 and 4, respectively from Fig. 3B) were chosen to be fragmented using MALDI-TOF/TOF MS. We were not able to perform MSMS experiments on peak 2 due to its low absolute intensity. The fragmentation pattern of these fluorescent-labeled PAC showed commonalities exhibiting fragments at m/z = 373, 395, and 598 (data not shown). The first two fragments, correspond to the fragmentation of the triazine ring, forming a stable nitrile group and detected as $[M+H]^+$ (m/z = 373) and $[M+Na]^+$ (m/z = 395) (Supplementary information – Fig. 2). The third fragment (m/z = 598) is characteristic of a PAC dimer after loss of a single or double fluorescent label. Nonetheless, other fragments could be found on MSMS spectra. For instance, MSMS spectrum of MTAF–PAC with DP = 2 (peak 1) showed fragments with m/z = 1021, 896, 769, 746, 633, 447, 309 and 287 (Fig. 4). The fragment with m/z = 1021 corresponds to the loss of the chlorine (−36 amu). A subsequent heterocyclic ring fission leads to the loss of a phloroglucinol ring (−126 amu), displaying a peak at m/z = 896. The major fragment corresponds to the loss of one catechin/epicatechin unit (−288 amu) that ionized as $[M+H]^+$ (m/z = 746) and $[M+Na]^+$(m/z = 769), respectively. We also detected a retro-Diels Alder rearrangement (Li & Deinzer, 2007) that led to fragments ionized as $[M+Na]^+$ at m/z = 447 and 633 (Supplementary Information – Figs. 3 and 4). At m/z = 309 and

Table 1
Fluorescent labeled proanthocyanidins (PAC) with 1 "A-type" interflavan bond after reaction with 5-([4,6-dichlorotriazin-2-yl]amino)fluorescein (DTAF)[a].

Peak	Fluorescent moiety	DP	Expected	Observed	S/N
1	MTAF	2	1057.2	1057.2	12.1
2	TAF	3	1309.2	1309.3	3.7
3	MTAF	3	1345.2	1345.2	7.7
4	MTAF–TAF	2	1479.2	1479.3	4.5
5	TAF	4	1597.3	1597.3	3.7
6	MTAF	4	1633.3	1633.3	7.2
7	MTAF–TAF	3	1767.3	1767.3	5.2
8	TAF	5	1885.4	1885.5	6.9
9	MTAF	5	1921.4	1921.4	13.3
10	MTAF–TAF	4	2055.3	2055.5	4.5
11	TAF	6	2173.4	2173.6	15.0
12	MTAF	6	2209.4	2209.6	22.4
13	MTAF–TAF	5	2343.4	2343.6	4.6
14	MTAF–MTAF	5	2379.4	2379.8	5.1
15	TAF	7	2461.5	2461.8	13.8
16	MTAF	7	2497.5	2497.7	18.5
17	MTAF–TAF	6	2631.5	2631.9	7.1
18	MTAF–MTAF	6	2667.4	2667.9	6.0
19	TAF	8	2749.5	2750.0	6.9
20	MTAF	8	2785.5	2785.0	12.7
21	MTAF–TAF	7	2919.5	2920.2	5.6
22	MTAF–MTAF	7	2955.5	2955.2	4.5
23	TAF	9	3037.6	3037.4	5.1
24	MTAF	9	3073.6	3074.1	6.3
25	MTAF–TAF	8	3207.6	3207.6	5.4
26	MTAF–MTAF	8	3243.6	3244.6	5.8
27	TAF	10	3325.7	3325.7	3.0
28	MTAF	10	3361.6	3361.8	3.9
29	MTAF–TAF	9	3495.6	3495.9	3.9
30	MTAF–MTAF	9	3531.6	3531.8	4.3
31	MTAF	11	3649.7	3649.9	3.8
32	MTAF–TAF	10	3783.7	3784.4	3.4
33	MTAF–MTAF	10	3819.7	3820.3	4.0
34	MTAF–MTAF	11	4107.7	4107.8	3.9

[a] MTAF" stands for (5-([monochlorotriazin-2-yl]amino)fluorescein , "TAF" stands for 5-([triazin-2-yl]amino)fluorescein, and "S/N" corresponds to signal-to-noise ratio, respectively.

287, the monomeric catechin can be detected as $[M+Na]^+$ or $[M+H]^+$, respectively. These experiments indicated that peak 1 was identified as $[MTAF–TAF–PAC \text{ with } DP = 2 + Na]^+$ instead of $[PAC \text{ with } DP = 5 + K]^+$.

3.4. Fluorescence microscopy

Three samples were observed under brightfield and fluorescence microscopy using a FITC filter. The negative control showed no agglutination after 4 h demonstrating that ExPEC do not agglutinate in the presence of DTAF (data not shown). As far as sample A is concerned (positive control), we were able to detect ExPEC agglutination in the presence of UL-PAC, which is in agreement with our previously developed spectrophotometric method (Feliciano et al., 2013). When FL-PAC were mixed with ExPEC (sample B), bacterial agglutination was detected with fluorescence on the ExPEC clump suggesting that the fluorescent labeling of PAC did not prevent PAC agglutination activity (Fig. 5).

Future work will include the use of fluorescence microscopy to visualize the interaction between fluorescent-labeled PAC and fluorescent-labeled ExPEC and to study the kinetics of this interaction in the context of gut colonization and subsequent prevention of UTI. ExPEC can be fluorescently tagged using plasmids that carry genes for the expression of red autofluorescent proteins, such as *mcherry* (Lagendijk, Validov, Lamers, de Weert, & Bloemberg, 2010). Phagocytosis of fluorescent ExPEC bound to fluorescent labeled PAC will allow the visualization of finer details of PAC interaction with ExPEC.

We used reverse phase chromatography to monitor the appearance of labeled PAC after reaction with DTAF, not to separate PAC. Another approach would be to isolate PAC by normal phase HPLC for oligomers up to DP 10 (Gu et al., 2002) and react the oligomers with DTAF. However, there are still a large number of isomers at each DP and oligomers that differ by 2 or more amu because of the presence of "A-type" interflavan bonds in the oligomers. We hypothesize that PAC oligomers at a specific DP will not have the

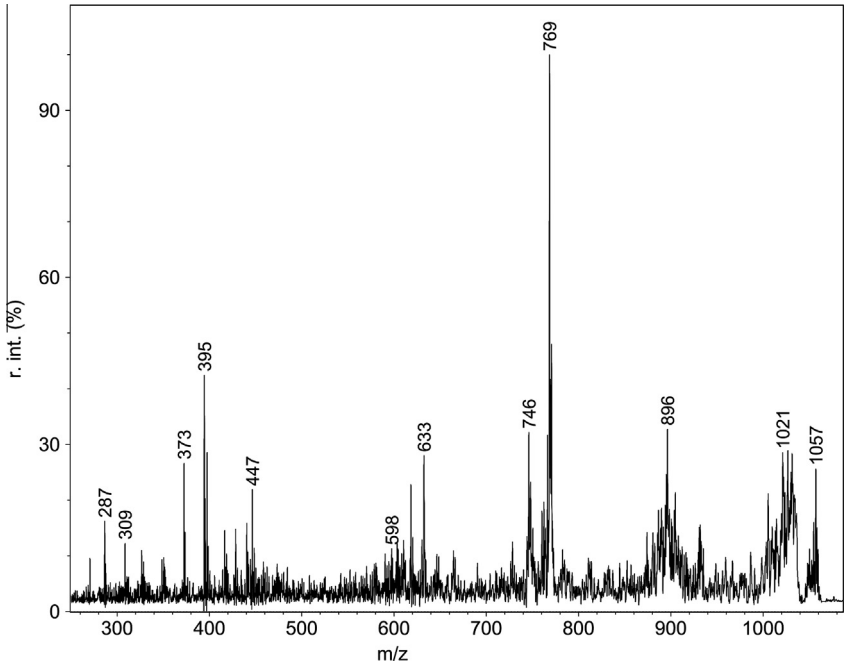

Fig. 4. Mass spectra obtained with MALDI-TOF/TOF MS in positive reflectron mode for peak 1 $[MTAF\text{-}PAC \text{ with } DP = 2 + Na]^+$ using $m/z = 1057.2$ as parent ion. "r.int." correspond to relative intensity.

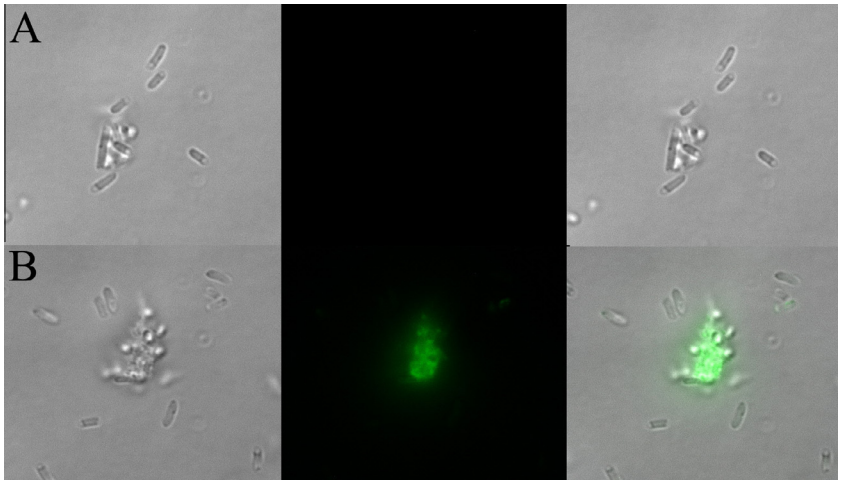

Fig. 5. Interaction between (A) ExPEC and unlabeled proanthocyanidinds (UL-PAC) and (B) ExPEC and fluorescent labeled proanthocyanidins (FL-PAC). Left images show bacterial agglutination obtained with brightfield, middle images were obtained with FITC filter and the images on the right represent an overlay of both images with a magnification 1000×.

same bioactivity as the total PAC fraction. This would be an interesting hypothesis to test. The fluorescent-labeled PAC provides a tool to study their interactions in vitro and it opens a new exciting field of research for the application of this methodology in vivo. This methodology can also be applied ex vivo to investigate the effect of fluorescent labeled PAC on gastrointestinal tissues, similarly to what our group previously described with unlabeled PAC (Pierre et al., 2012).

4. Conclusion

In this work, we report a novel method of fluorescent labeling of PAC with DTAF, which is more stable than FITC (Beard, Edel, & deMello, 2004; Lalljie & Sandra, 1995). Although DTAF was used to label glycyrrhizin and isoliquiritigenin from licorice (Wicks & Li, 2004), alkaloids from *Ephedra* (Wang et al., 2006), amino acids (Liu et al., 2004; Xiao et al., 2007), proteins (Wadsworth & Sloboda, 1984) and more recently cellulose nanocrystals (Abitbol et al., 2013), prior to our research, DTAF has not been reported for use in labeling PAC. To our knowledge, this is the first time that a fluorescent probe was used to react with PAC for use in microscopy.

The characterization of DTAF labeled products was generally done by fluorescence spectrophotometry (Luensmann & Jones, 2010), which does not distinguish between unreacted DTAF and DTAF labeled products. As far as we know, mass spectrometry was used only once to confirm the covalent bond between DTAF and a peptidyl-phosphatidylethanolamine (Petrossian et al., 1985). Our work reinforced the relevance of MALDI-TOF MS in evaluating PAC structural features by allowing characterization of fluorescent labeled PAC. Moreover, we were able to elucidate fluorescent-labeled PAC by MSMS using TOF/TOF MS. MALDI TOF/TOF MS has been widely used in proteomics but only recently was used to elucidate PAC structures in cinnamon (Mateos-Martín et al., 2012).

5. Notes

The authors declare the following competing financial interest(s): Jess D. Reed and Christian G. Krueger have ownership interest in Complete Phytochemical Solutions, LLC and in full disclosure their affiliation with this company is acknowledged in the author affiliation.

6. Role of the funding source

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.06.031.

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