FOOD COMPOSITION AND ADDITIVES

Single-Laboratory Validation for Determination of Total Soluble Proanthocyanidins in Cranberry Using 4-Dimethylaminocinnamaldehyde

MARSHA SINTARA and LIN LI
International Chemistry Testing, 258 Main St, Suite 202, Milford, MA 01757
DAVID G. CUNNINGHAM
Ocean Spray, 1 Ocean Spray Dr, Lakeville, MA 02349
RONALD L. PRIOR
University of Arkansas, Department of Food Science, 214 Lakeshore Dr, Searcy, AR 72143
XIANLI WU[1]
U.S. Department of Agriculture, Agricultural Research Service, Beltsville Human Nutrition Research Center, Nutrient Data Laboratory, 10300 Baltimore Ave, Beltsville, MD 20705
TONY CHANG
International Chemistry Testing, 258 Main St, Suite 202, Milford, MA 01757

American cranberry (*Vaccinium macrocarpon*) is native to Eastern North America. Recent studies have suggested that the A-type proanthocyanidins (PACs) in cranberries are effective in preventing urinary tract infection. To meet the growing interest in the cranberry market, an accurate, reliable, and simple method to determine PAC concentration is needed. In this study, a modified method using 4-dimethylaminocinnamaldehyde to quantify total PACs in cranberry products was validated. Cranberry juice extract powder, cranberry capsules containing juice extract, and cranberry juice concentrate were used as the samples in this study. With the modified method, the calibration curves for proanthocyanidin A2 had correlation coefficients (r^2) of >0.99. The recoveries of two different concentrations after spiking were 97.1 and 99.1%, and the RSDs for repeatability and reproducibility were <2.7 and <1.6%, respectively.

American cranberry (*Vaccinium macrocarpon*), a shrub native to Eastern North America, was introduced by Native Americans to the first settlers (1). Native Americans prepared the cranberries by cooking and sweetening them to make sauce, and cranberry sauce has since become a traditional recipe served at Thanksgiving Day feasts. Cranberries were also used by Native Americans to treat wounds to stop the bleeding.

Cranberries gained interest in the 1920s when researchers found that the consumption of cranberries made urine more acidic, and it was assumed that the acidity of cranberries might prevent urinary tract infection (UTI; 2). However, later studies showed that the acidity is not the reason that cranberries prevented UTI (3, 4). *Escherichia coli* is the bacterial species associated with UTI. The adhesion of *E. coli* to uroepithelial cells is the first and critical step of a UTI (5). Without this adhesion, the bacteria cannot grow and do not infect the urinary tract. In 1980s, it was found that the adhesion of *E. coli* to uroepithelial cells was inhibited by the compounds in cranberries (6–9). Therefore, the proposed mechanism of UTI prevention by cranberries was the inhibition of bacterial adhesion, not the killing of bacteria (10, 11). There is no concern for bacterial resistance toward cranberry compounds (12).

Later studies found that the compounds responsible for preventing the adhesion of *E. coli* are proanthocyanidins (PACs), specifically A-type PACs (11, 13). There are also several clinical trials that support the effectiveness of PACs in the prevention of UTI (14, 15). PACs are also known as condensed tannins, with flavan-3-ol monomers, mainly catechins and epicatechins, as the building blocks. There are two common types of proanthocyanidin dimers based on their C–C linkage position. The A-type is linked at the C4–C8 position and has an ether bridge at the C2–C7 position, whereas the B-type has only a C4–C6 or a C4–C8 linkage. Foods that contain only B-type PACs have not been found to bear the inhibitory effects for the prevention of UTI (12).

The effectiveness of cranberry juice to prevent UTI has since been shown in various clinical trials (16–19). With the growing interest of cranberry PACs on the market and the demand for a valid QC method, a unified way to quantify PACs is needed. There are many methods for PAC quantification, such as the vanillin method (20), the acid butanol assay (21), HPLC (22), and the 4-dimethylaminocinnamaldehyde (DMAC) method (23). The vanillin and acid butanol methods are not very accurate because anthocyanins interfere in the reading, leading to overestimation of the PAC value (24, 25). The HPLC method is more accurate, but so far, there are no applicable reference standards commercially available for each of the cranberry PAC oligomers. Quantification of B-type PACs in cocoa can be achieved using (−)-epicatechin-based relative response factors for 2–10 degrees of polymerization (26). However, no response factors have yet been developed specifically for A-type PACs. The cocoa oligomers have different response factors compared to those in cranberry. Therefore, the quantification using HPLC is more difficult to perform and less accurate when using the response factors for B-type PACs.

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[1] Corresponding author's e-mail: Xianli.Wu@ars.usda.gov
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Table 1. Acceptance criteria for various validation parameters

Validation parameter	Acceptance criteria
Linearity	r^2 ≥0.955
Repeatability	RSD ≤3.0%
Intermediate precision	RSD ≤5.0%
Accuracy	Recovery between 90.0 and 100%
Robustness	≤3.0% versus standard conditions

In the presence of strong acid, the aldehyde group in DMAC is protonated, and the carbonyl oxygen forms a strongly reactive carbocation (27). This carbocation then reacts specifically with meta di- or trihydroxy phenols in PACs to produce a green-colored complex that has a maximum absorbance at 640 nm (28, 29). The DMAC spectrophotometric assay method is more accurate compared to the vanillin and acid butanol methods because it excludes the maximum absorbance from anthocyanins at around 500–550 nm (30).

A study was done to validate the DMAC method (31). It is unfortunate that this method suffered low reproducibility (with SDs up to 16%). As such, the previous method is not suitable for QC purposes (the high SDs were due to the bleaching effect of the color produced when water was introduced to the sample preparation). In this study, we performed a single-laboratory validation for the optimized DMAC method that we made simpler, faster, and more reproducible so that it can be used for QC purposes.

METHOD

Sample Material

Cranberry juice extract powder, cranberry capsules containing juice extract powder, and cranberry juice concentrate were provided by Pharmatoka (Rueil Malmaison, France).

Apparatus

(a) *Volumetric flask.*—Used for all reagent preparations and sample extractions.

(b) *Cuvette.*—1 cm path length.

(c) *UV-Vis spectrophotometer.*—Capable of measuring kinetic reactions at 640 nm.

Reagents

(a) *Solvents and chemicals.*—HPLC grade methanol; American Chemical Society grade sulfuric acid; DMAC (≥98% purity; Alfa Aesar, Ward Hill, MA); and proanthocyanidin A2 standard (Indena S.p.A., Milan, Italy).

(b) *Reaction solvent.*—0.4 N H_2SO_4 in methanol: add 12 mL of H_2SO_4 to 1 L volumetric flask and dilute with methanol.

(c) *DMAC solution.*—1 mg/mL: weigh 25 mg DMAC into a 25 mL volumetric flask and dilute to volume with reaction solvent.

(d) *Proanthocyanidin A2 standard solutions.*—(*1*) *Stock solution.*—90 µg/mL: accurately weigh 4.5 mg proanthocyanidin A2 into a 50 mL volumetric flask and dilute to volume with methanol.

(*2*) *Working standard solutions.*—Dilute stock standard solution to 10, 15, 20, 25, and 30 µg/mL with methanol using volumetric flask.

Sample Extraction

(a) *Cranberry extract powder.*—Weigh 50 mg sample to 100 mL volumetric flask and add 80 mL methanol. Shake at 200 rpm for 30 min. Dilute to volume with methanol. Dilute another five times with methanol before analysis.

(b) *Cranberry capsules.*—Weigh 500 mg sample into a 100 mL volumetric flask and add 80 mL methanol. Shake at 200 rpm for 30 min. Dilute to volume with methanol. Dilute another five times with methanol before analysis.

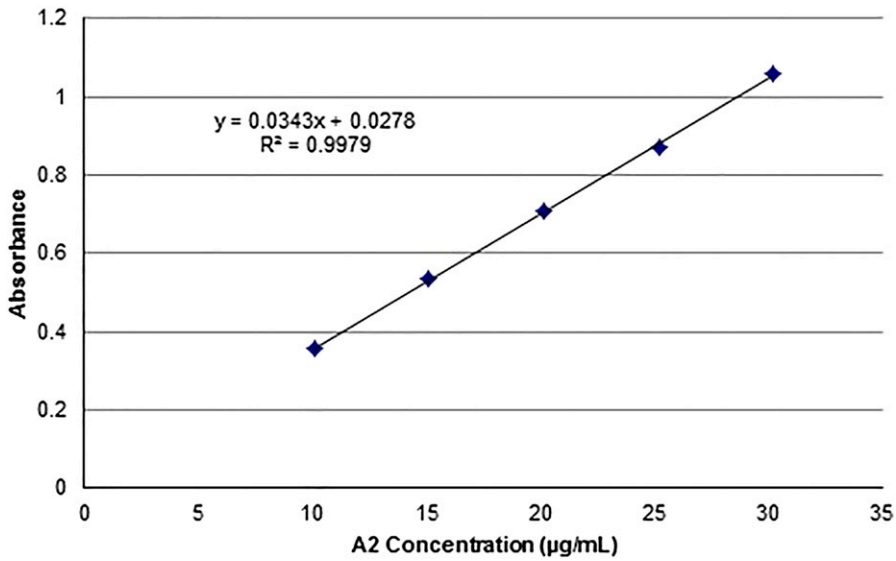

Figure 1. Representative standard curve of proanthocyanidin A2 concentration versus absorbance.

Table 2. Calibration curves of proanthocyanidin A2

Calibration curve No.	r^2	Slope	Intercept
1	0.9984	0.0352	0.0882
2	0.9978	0.0338	0.0324
3	0.9979	0.0343	0.0278
4	0.9999	0.0313	0.0226
5	0.9990	0.0317	0.0258
6	0.9990	0.0328	0.0751

(c) *Cranberry juice concentrate*.—Add 2 mL cranberry juice concentrate to a 100 mL volumetric flask. Add 20 mL methanol and shake at 200 rpm for 30 min to extract. Dilute to volume with methanol. The sample is ready for analysis.

Analysis

Mix 1 mL DMAC reagent with 200 µL diluted sample and read the mixture at 640 nm with a UV-Vis spectrophotometer every 5 s for 5 min.

Calculations

The highest absorbance observed over the 5 min measurement period was determined and used for the calculation of PAC concentration against the standard curve. Total PAC concentration was quantified using the following equation:

$$\text{Total PACs (mg/g)} = \frac{(Abs_{\text{spl}} - Abs_{\text{blank}}) - c}{m} \times \frac{V}{W} \times \frac{D}{1000}$$

where Abs_{spl} = the maximum absorbance of the sample; Abs_{blank} = the average absorbance of the blank; c = the intercept of the calibration curve; m = the slope of the calibration curve; V = the volume of the test solution (mL); W = the weight of the sample (g); and D = the dilution factor. Data were expressed as milligrams of proanthocyanidin A2 equivalents per gram of sample.

Method Validation and Statistical Analysis

The validation of this method was conducted according to the International Conference on Harmonization Harmonized Tripartite Guideline *Validation of Analytical Procedures: Text and Methodology Q2 (R1)*. Table 1 shows the acceptability criteria for various validation parameters.

Table 3. Method precision using cranberry extract, capsules, and juice concentrate as the samples analyzed by the same operator on the same day

	Proanthocyanidin, mg/g		
Replicate	Cranberry extract	Cranberry capsules	Cranberry juice concentrate
A	182.5	126.6	5.766
B	189.2	124.2	5.905
C	181.2	121.5	5.723
D	182.5	126.7	5.805
E	183.0	125.1	5.675
F	183.0	123.8	5.763
Mean	183.6	124.6	5.773
RSD, %	1.55	1.57	1.35

Table 4. Day-to-day repeatability of cranberry extract analyzed on 4 different days and by two different operators

	Proanthocyanidin A2 found, mg/g	
Replicate	Operator 1	Operator 2
Day 1A	180.6	
Day 1B	183.9	
Day 2A		187.7
Day 2B		176.5
Day 3A	182.5	
Day 3B	189.2	
Day 4 A		180
Day 4 B		175.7
Mean	184.1	179.98
RSD, %	2.00	3.04
Total mean	182.0	
Total RSD, %	2.66	

Results and Discussion

Linearity

Five concentrations of the proanthocyanidin A2 standard (10, 15, 20, 25, and 30 µg/mL) were used, and the absorbance of each concentration was measured to make the standard curve (Figure 1). The correlation coefficients, slopes, and intercepts of the proanthocyanidin A2 standard at those five concentrations is shown in Table 2. The regression coefficients of the six different standard curves were all greater than 0.995.

Repeatability

Six replicates of the samples were analyzed by the same operator on the same day to test the precision of the method (Table 3). All the samples had an RSD value of <3%, which

Table 5. Spike and recovery of cranberry extract spiked with 180 and 324 mg/g proanthocyanidin A2

Spike level, mg/g	Proanthocyanidin A2 found, mg/g	Recovery, %
0	170.6	—
180		
Replicate A	345.1	96.9
Replicate B	345.7	97.2
Replicate C	345.7	97.2
Mean	345.5	97.1
324		
Replicate A	490.9	98.9
Replicate B	489.8	98.5
Replicate C	494.2	99.9
Mean	491.6	99.1

Table 6. Method robustness using different extraction times, DMAC suppliers, and spectrophotometers

Parameter	Proanthocyanidin, mg/g	Difference versus the standard condition, %
Extraction time, min		
25	178.3	0.24
30	177.9	Standard condition
35	176.0	−1.07
DMAC reagent brand		
TCI	177.7	−0.10
Alfa Aesar	177.9	Standard condition
Fluka	177.0	−0.49
Spectrophotometer brand		
Jasco	181.0	Standard condition
Agilent	184.7	0.38

satisfied the acceptance criteria. The highest RSD was 1.57%, which is lower compared to the previous method's intralaboratory RSD of 6.1%. The results proved that this method is more reproducible compared to the previous one. Some changes that were made to the old method could have contributed to the smaller RSD. First, the extraction solution was changed from acetone, water, and acetic acid to pure methanol. This change not only made the procedure simpler but eliminated water from the sample preparation step. According to Wallace and Giusti (32), >1% water content in the reaction produced a bleaching effect of the DMAC. Because this bleaching effect would affect the pH value of the sample and, therefore, reduce the reproducibility of the assay, we used methanol for the extraction solution instead of using the standard acetone, water, and acetic acid extraction method for PACs.

Second, the DMAC was dissolved in 0.4 N sulfuric acid in methanol instead of hydrochloric acid in ethanol. This change provided a lower pH for the DMAC so that it would be more protonated and more readily available. Therefore, when it reacts with the PACs from the sample, it will reach the maximum absorption at a faster time (5 min) compared to that of the old method (15 min).

Intermediate Precision

To evaluate intermediate precision, the samples were analyzed in duplicate on 4 different days by two different operators (eight independent analyses). It can be seen in Table 4 that the RSD for all of the samples was <2.7%.

Accuracy

The accuracy of the method was evaluated by spiking the cranberry extract sample with proanthocyanidin A2 at two levels: 180 and 324 mg/g. The analysis was done in triplicate on the same day. The recoveries of all samples were between 90 and 100% (Table 5).

Robustness

Method robustness was evaluated with respect to extraction time, DMAC reagent supplier, and spectrophotometer. Three different extraction times were performed: 25, 30, and 35 min. The DMAC reagent was purchased from three different suppliers: TCI, Alfa Aesar, and Fluka. The analysis was done using the same extract reacted with DMAC reagents from different suppliers. In addition, two different brands of spectrophotometers, the Jasco V-530 and the Agilent Cary UV-Vis 60, were used to evaluate the robustness. The results for the standard conditions using different extraction times, suppliers, and spectrophotometers were all <3% (Table 6).

Conclusions

We introduced three modifications to the original DMAC method: (*1*) the change of sample extraction solution from a mixture of acetone, water, and acetic acid to methanol (for better reproducibility); (*2*) the change of DMAC solution from hydrochloric acid in ethanol to sulfuric acid in methanol (for higher sensitivity); and (*3*) the change of plate reader to a UV-Vis

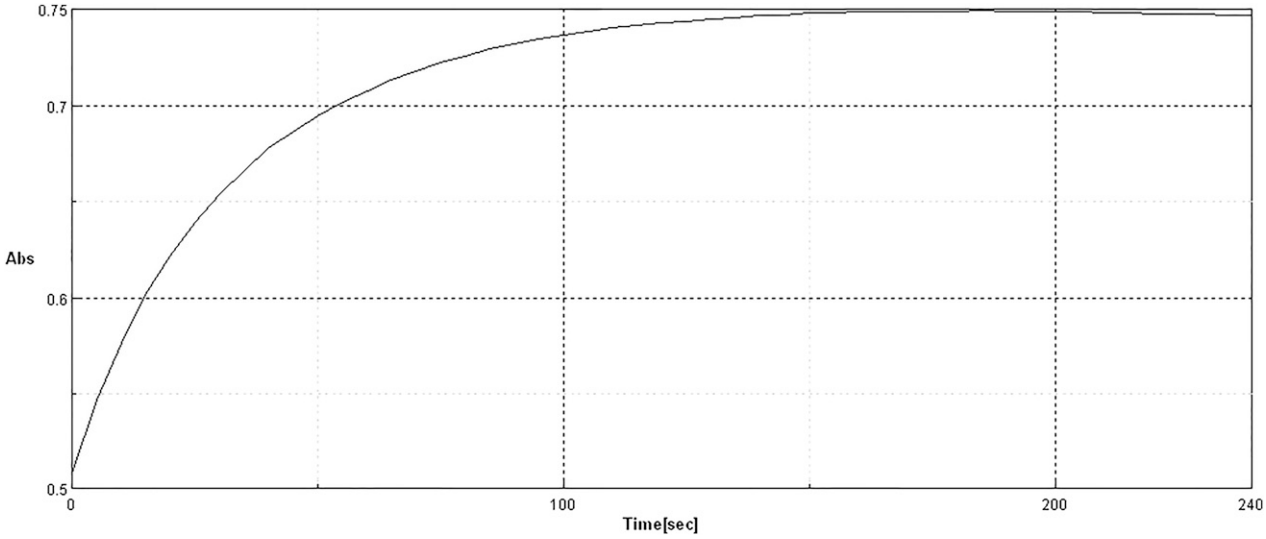

Figure 2. Representative kinetic curve of the sample reacted with DMAC.

spectrophotometer (for better accessibility). The modified method to determine the total proanthocyanidins in different cranberry products was validated, and the results of the validation satisfied the criteria listed in Table 1. The RSD was improved from 6.1 to 1.57%, and the reaction time was also shortened from 15 to 5 min (Figure 2). With this simple colorimetric assay, any laboratory can perform this modified DMAC method for the QC of their products. For further study, we recommended that a multilaboratory validation study be performed for this method.

References

(1) Guay, D.R. (2009) *Drugs* **69**, 775–807. doi:10.2165/00003495-200969070-00002

(2) Blatherwick, N.R.L., & Louisa, M. (1923) *J. Biol. Chem.* **57**, 815–818

(3) Sobota, A.E. (1984) *J. Urol.* **131**, 1013–1016. doi:10.1016/S0022-5347(17)50751-X

(4) Walsh, B.A. (1992) *J. ET Nurs.* **19**, 110–113

(5) Beachey, E.H. (1981) *J. Infect. Dis.* **143**, 325–345. doi:10.1093/infdis/143.3.325

(6) Avorn, J., Monane, M., Gurwitz, J., Glynn, R.J., Choodnovskiy, I., & Lipsitz, L. (1994) *JAMA* **271**, 751–754. doi:10.1001/jama.1994.03510340041031

(7) Haverkorn, M.J., & Mandigers, J. (1994) *JAMA* **272**, 590. doi:10.1001/jama.272.8.590a

(8) Papas, P.N., Brusch, C.A., & Ceresia, G.C. (1966) *Southwest. Med.* **47**, 17–20

(9) Walker, E.B., Barney, D.P., Mickelson, J.N., Walton, R.J., & Mickelson, R.A.J. (1997) *J. Fam. Pract.* **45**, 167–168

(10) Howell, A.B., Vorsa, N., Der Marderosian, A., & Foo, L.Y. (1998) *N. Engl. J. Med.* **339**, 1085–1086. doi:10.1056/NEJM199810083391516

(11) Gupta, A., Dwivedi, M., Mahdi, A.A., Gowda, G.A.N., Khetrapal, C.L., & Bhandari, M. (2012) *Urol. Res.* **40**, 143–150. doi:10.1007/s00240-011-0398-2

(12) Howell, A.B., Reed, J.D., Krueger, C.G., Winterbottom, R., Cunningham, D.G., & Leahy, M. (2005) *Phytochemistry* **66**, 2281–2291. doi:10.1016/j.phytochem.2005.05.022

(13) Foo, L.Y., Lu, Y., Howell, A.B., & Vorsa, N. (2000) *J. Nat. Prod.* **63**, 1225–1228. doi:10.1021/np000128u

(14) Burleigh, A.E., Benck, S.M., McAhran, S.E., Reed, J.D., Krueger, C.G., & Hopkins, W.J. (2013) *Nutr. J.* **12**, 139. doi:10.1186/1475-2891-12-139

(15) Howell, A.B., Botto, H., Combescure, C., Blanc-Potard, A.B., Gausa, L., Matsumoto, T., Tenke, P., Sotto, A., & Lavigne, J.P. (2010) *BMC Infect. Dis.* **10**, 94. doi:10.1186/1471-2334-10-94

(16) Stothers, L. (2002) *Can. J. Urol.* **9**, 1558–1562

(17) Valentová, K., Stejskal, D., Bednář, P., Vostálová, J., Číhalík, Č., Večeřová, R., Koukalová, D., Kolář, M., Reichenbach, R., Škňouřil, L., Ulrichová, J., & Šimánek, V. (2007) *J. Agric. Food Chem.* **55**, 3217–3224. doi:10.1021/jf0636014

(18) Lavigne, J.P., Bourg, G., Combescure, C., Botto, H., & Sotto, A. (2008) *Clin. Microbiol. Infect.* **14**, 350–355. doi:10.1111/j.1469-0691.2007.01917.x

(19) Greenberg, J.A., Newmann, S.J., & Howell, A.B. (2005) *J. Altern. Complement. Med.* **11**, 875–878. doi:10.1089/acm.2005.11.875

(20) Sarkar, S.K., & Howarth, R.E. (1976) *J. Agric. Food Chem.* **24**, 317–320. doi:10.1021/jf60204a041

(21) Porter, L.J., Hrstich, L.N., & Chan, B.G. (1986) *Phytochemistry* **25**, 223–230. doi:10.1016/S0031-9422(00)94533-3

(22) de Pascual-Teresa, S., Treutter, D., Rivas-Gonzalo, J.C., & Santos-Buelga, C. (1998) *J. Agric. Food Chem.* **46**, 4209–4213. doi:10.1021/jf980255k

(23) Cunningham, D.G., Vannozzi, S., O'Shea, E., & Turk, R. (2002) in *Quality Management of Nutraceuticals*, C.T., & Ho Q.Y. Zheng (Eds), American Chemical Society, Washington, DC, pp 151–166

(24) Schofield, P., Mbugua, D.M., & Pell, A.N. (2001) *Anim. Feed Sci. Technol.* **91**, 21–40. doi:10.1016/S0377-8401(01)00228-0

(25) Waterhouse, A.L., Ignelzi, S., & Shirley, J.R. (2000) *Am. J. Enol. Vitic.* **51**, 383–389

(26) Robbins, R.J., Leonczak, J., Li, J., Johnson, J.C., Collins, T., Kwik-Uribe, C., & Schmitz, H.H. (2012) *J. AOAC Int.* **95**, 1153–1160. doi:10.5740/jaoacint.12-162

(27) Krueger, C.G., Reed, J.D., Feliciano, R.P., & Howell, A.B. (2013) *Anal. Bioanal. Chem.* **405**, 4385–4395. doi:10.1007/s00216-013-6750-3

(28) McMurrough, I., & McDowell, J. (1978) *Anal. Biochem.* **91**, 92–100. doi:10.1016/0003-2697(78)90819-9

(29) Treutter, D. (1989) *J. Chromatogr. A* **467**, 185–193. doi:10.1016/S0021-9673(01)93963-9

(30) Walton, M.F., Haskins, F.A., & Gorz, H.J. (1983) *Crop Sci.* **23**, 197–200. doi:10.2135/cropsci1983.0011183X002300020001x

(31) Prior, R.L., Fan, E., Ji, H., Howell, A., Nio, C., Payne, M.J., & Reed, J. (2010) *J. Sci. Food Agric.* **90**, 1473–1478. doi:10.1002/jsfa.3966

(32) Wallace, T.C., & Giusti, M.M. (2010) *J. Food Sci.* **75**, C619–C625. doi:10.1111/j.1750-3841.2010.01734.x