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1 **Advantages of a validated UPLC-MS/MS standard addition method for the quantification**
2 **of A-type dimeric and trimeric proanthocyanidins in cranberry extracts in comparison with**
3 **well-known quantification methods.**

4
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22 [†] Dedicated to the memory of our colleague Sandra Apers (° 19/08/1972 - † 05/02/2017)

23

- 1 **Keywords**
- 2 Cranberry
- 3 *Vaccinium macrocarpon*
- 4 LC-MS/MS
- 5 A-type procyanidins
- 6 Urinary tract infections
- 7 Quality control
- 8

1 **Abstract**

2 The berries of *Vaccinium macrocarpon*, cranberry, are widely used for the prevention of urinary
3 tract infections. This species contains A-type proanthocyanidins (PACs), which intervene in the
4 initial phase of the development of urinary tract infections by preventing the adherence of
5 *Escherichia coli* by their P-type fimbriae to uroepithelial cells. Unfortunately, the existing
6 clinical studies used different cranberry preparations, which were poorly standardized. Because
7 of this, the results were hard to compare, which led sometimes to conflicting results. Currently,
8 PACs are quantified using the rather non-specific spectrophotometric 4-
9 dimethylaminocinnamaldehyde (DMAC) method. In addition, a normal phase HPTLC-
10 densitometric method, a HPLC-UV method and three LC-MS/MS methods for quantification of
11 procyanidin A2 were recently published. All these methods contain some shortcomings and
12 errors. Hence, the development and validation of a fast and sensitive standard addition LC-
13 MS/MS method for the simultaneous quantification of A-type dimers and trimers in a cranberry
14 dry extract was carried out. A linear calibration model could be adopted for dimers and, after
15 logarithmic transformation, for trimers. The maximal interday and interconcentration precision
16 was found to be 4.86% and 4.28% for procyanidin A2, and 5.61% and 7.65% for trimeric PACs,
17 which are all acceptable values for an analytical method using LC-MS/MS. In addition, twelve
18 different cranberry extracts were analyzed by means of the newly validated method and other
19 widely used methods. There appeared to be an enormous variation in dimeric and trimeric PAC
20 content. Comparison of these results with LC-MS/MS analysis without standard addition showed
21 the presence of matrix effects for some of the extracts and proved the necessity of standard
22 addition.

23 A comparison of the well-known and widely used DMAC method, the butanol-HCl assay and
24 this newly developed LC-MS/MS method clearly indicated the need for a reliable method able to

1 quantify A-type PACs, which are considered to be the pharmacologically active constituents of
2 cranberry, since neither the DMAC or butanol-HCl assays are capable of distinguishing between
3 A and B-type PACs and therefore cannot detect adulterations with, for example, extracts with a
4 high B-type PAC content. Hence, the combination of the DMAC method or butanol-HCl assay
5 with this more specific LC-MS/MS assay could overcome these shortcomings.

6

7 **List of abbreviations**

8	ANOVA	Analysis of Variance
9	DAD	Diode Array Detection
10	DMAC	Dimethylaminocinnamaldehyde
11	FA	Formic Acid
12	ICH	International Conference on Harmonisation
13	MRM	Multiple Reaction Monitoring
14	PACs	Proanthocyanidins
15	RSD	Relative Standard Deviation
16	TQ	Triple Quad
17	UTI	Urinary Tract Infections

18

1 **1. Introduction**

2 Urinary tract infections (UTIs) are widespread, affecting a large part of the population, and
3 constitute a major medical and economic burden. Because of the increasing resistance of bacteria
4 against antibiotics, such as trimethoprim/sulfamethoxazole, used in the prophylaxis or treatment
5 of UTIs, the search for non-antibiotic prevention and treatment options has become increasingly
6 important [1-7].

7 Nowadays, the berry of *Vaccinium macrocarpon* Aiton (Ericaceae), cranberry, is widely used for
8 the prevention of urinary tract infections. The positive effect of cranberry in the prevention of
9 UTIs is most likely the result of multiple actions. One mechanism, probably the most well-
10 known, is the prevention of the adherence of *Eschericia coli* by their P-type fimbriae to
11 uroepithelial cells, a first step in the development of UTIs [8]. This effect was ascribed to the A-
12 type proanthocyanidins (PACs), which are unusual proanthocyanidins present in a limited
13 number of foods such as cranberry [9-11].

14 A large number of clinical studies has been carried out to evaluate the use of cranberry for the
15 prevention of UTIs. Unfortunately, these studies used different cranberry preparations, which
16 were poorly standardized, making it hard to compare them, and leading sometimes to conflicting
17 results. In order to carry out more reliable clinical studies, new analytical methods for the
18 quantification and standardization of different cranberry preparations are urgently needed.

19 Currently, the rather non-specific spectrophotometric 4-dimethylaminocinnamaldehyde (DMAC)
20 method is used for the quantification of the total amount of PACs. However, this method is
21 unable to differentiate between the different types and degree of polymerization of PACs [12-13].

22 Although a lot of research has been done on the characterization of cranberry proanthocyanidins,
23 much less work has been carried out to quantify A-type proanthocyanidins separately. Recently a

1 normal phase HPTLC-densitometric method, a HPLC-UV method and three LC-MS methods for
2 quantification of procyanidin A2 were published [14-18]. All these methods show some
3 imperfections and errors. The methods published by Boudesocque et al. [14] and Iswaldi et al.
4 [17] describing the quantification of procyanidin A2 in cranberry extracts and syrup by means of
5 HPTLC and HPLC-UV, respectively, most likely will lack the sensitivity and specificity for the
6 quantification of both dimers and trimers. A UPLC-MS/MS method was used to quantify
7 procyanidin A2 in urine samples [18]. This method includes a long and complicated sample
8 preparation. Although an internal standard was used, losses cannot be fully excluded since it
9 concerned quercetin, a monomer, while a dimer is analyzed. This method was validated by
10 analyzing spiked urine from three different persons in triplicate for all precision and accuracy
11 measurements. No interday experiments were performed and the RSD% ranged from 3.85% to
12 11.13% for one concentration level of urine of one person with an accuracy of 91.7%. The two
13 other UPLC-MS/MS methods described the quantification of both dimeric and trimeric
14 proanthocyanidins in berries of different *Vaccinium* spp. including *Vaccinium macrocarpon* and
15 in commercial cranberry products [15-16]. However, these latter two methods were never
16 validated and were found to encounter some problems. For example, one method used the trimer
17 procyanidin C1 to calculate the content of trimeric A-type PACs, and methanol-water (20:80,
18 v/v) with 0.2% HCl as extraction solvent, which can lead to degradation of proanthocyanidins. In
19 addition, the possibility of matrix effects was not taken into consideration. The other method used
20 solid phase extraction as sample clean-up, but without the use of an internal standard the
21 occurrence of losses is overlooked. Matrix effects were investigated by means of a raspberry
22 extract, which did not contain procyanidin A2. However, one cannot expect the matrix of
23 raspberry to be the same as that of cranberry.

1 Therefore, a new fast and sensitive analytical UPLC-MS/MS method for the quantification of the
2 main PACs of interest in a cranberry dry extract, using standard addition, was developed and
3 validated. In addition, twelve cranberry extracts were analyzed using the newly validated method
4 as well as other widely used methods.

5

6 **2. Material and methods**

7 2.1 Standards and reagents

8 The following solvents were used: acetonitrile Far UV (HPLC grade) and methanol (HPLC
9 grade) (Fisher Scientific, Hampton, NH, USA), absolute ethanol (HPLC grade), acetone (HPLC
10 grade), butanol (99%), dichloromethane (HPLC grade) (Acros Organics, Geel, Belgium).

11 Acetic acid (99.8%), formic acid (FA) (99+%), phosphoric acid (p.a. 85%) and sulphuric acid
12 (85%) were provided by Acros. Dimethylaminocinnamaldehyde (DMAC) ($\geq 98\%$), diphenylboric
13 2-aminoethyl ester (98%), gallic acid ($>97\%$) and hydrochloric acid (p.a. 25%) were obtained
14 from Sigma-Aldrich (St. Louis, MI, USA). (+)-Catechin, (-)-epicatechin, procyanidin A2 ($\geq 99\%$),
15 procyanidin B1 ($\geq 80\%$) and procyanidin B2 ($\geq 90\%$) were purchased from Extrasynthese
16 (Genay, Cedex, France).

17

18 2.2 Plant material

19 Several *Vaccinium macrocarpon* extracts were selected for the development of the analytical
20 method, taking into account problems such as peak interference and matrix effects. In total,
21 twelve different extracts were obtained from different manufacturers (Table 1).

22

23 2.3 Equipment

1 An Acquity Ultra Performance LC with sample manager, binary solvent manager, diode array
2 detector (DAD) and triple quadrupole (TQ) detector, equipped with Masslynx software (version
3 4.1) (Waters, Milford, USA) was used. Furthermore, a HPLC-DAD system (Beckmann), an
4 Infinite 200 96 well plate reader (Tecan), a Lambda 35 double beam UV-VIS spectrophotometer
5 (Perkin Elmer), and a Heraeus Labofuge 400 centrifuge (Thermo Scientific, Waltham,
6 Massachusetts, USA) were used. pH measurements were performed on a PHM 92 LAB pH-meter
7 (Radiometer, Copenhagen, Denmark).

8

9 2.4 Method development

10 2.4.1 Extraction

11 Extraction was developed using extract 3. Different solvent mixtures were evaluated for their
12 extraction power of proanthocyanidin dimers and trimers. The use of water, methanol and
13 acetone, with and without adding acid, has been described for the extraction of proanthocyanidins
14 from various sources, including cranberry. These solvents are mostly only used in binary solvent
15 mixtures, sometimes combined with acid [19-27]. In this work, both binary and ternary solvent
16 mixtures of water, methanol and acetone were tested (Table S1).

17

18 2.4.2 Chromatographic conditions

19 Different columns, mobile phases and gradients were tested. Two different columns were
20 compared: an HSS T3 (2.1 x 100 mm; 1.8 μ m) column and a BEH C18 (2.1 x 100 mm; 1.8 μ m)
21 column (Waters). As mobile phases, water and acetonitrile with 0.1 and 1% formic acid were
22 used in combination with three gradients. The optimized gradient was compared with the slightly
23 adapted gradient systems published earlier [15-16]. Gradient 1 started at 0 min with 5% B; 1 min,
24 5% B; 8 min, 20% B; 10 min, 40% B; 12 min, 100% B; 14 min, 100% B; 15 min, 5% B; 17 min,

1 5% B. Gradient 2 was based on Sanchez-Patan et al. [15] and was as follows 0 min, 2% B; 8.56
2 min, 16.3% B; 8.86 min, 18.4% B; 11.36 min, 18.4% B; 11.46 min, 99.9% B; 12.86 min, 99.9%
3 B; 12.96 min, 2% B; 14.36 min, 2% B. Gradient 3 was based on Jungfer et al. [16] and started at
4 0 min with 5% B; 1 min, 5% B; 20 min, 18.4% B; 21 min, 100% B; 22 min, 100% B; 23 min, 5%
5 B; 25 min, 5% B. A quick screening was done for all extracts with gradient 1. In order to have a
6 thorough evaluation of the separation, extracts with different profiles are evaluated with all
7 systems mentioned above.

8

9 2.4.3 Mass spectrometric conditions

10 Tuning was done with a standard of procyanidin A2 in negative ion mode. Source parameters
11 were as follows: capillary voltage 2.5 kV, extractor voltage 3 V, RF lens 0.1 V. The source
12 temperature was set at 150 °C and the desolvation temperature was 450 °C. Desolvation gas flow
13 was 900 L/h and the cone gas flow was 50 L/h. The analyzer parameters were 15.0 for LM
14 Resolution 1 and HM Resolution 1 and the ion energy was set at 1.0 in MS/MS mode. The
15 entrance and exit voltages were respectively 1 and 0.5 V. LM Resolution 2 and HM Resolution 2
16 were set at 14.0 and the ion energy at 1.0. The gain was 1.0. The collision gas flow was set at 0.1
17 mL/min.

18 The extract was analyzed in full scan and single ion monitoring mode to locate the dimers and
19 trimers. For each dimer or trimer, a product ion scan was done at different collision energies to
20 obtain an idea of the fragmentation pattern of the compounds. From these product ion scan
21 spectra, the most abundant product ion and another specific product ion were selected, as
22 quantifier and qualifier, respectively, for the development of the MRM (multiple reaction
23 monitoring) method. For every compound, the optimal cone voltage and collision energy were
24 obtained by evaluating different cone voltages and collision energies.

1
2 2.4.4 Matrix effects
3 In order to evaluate possible matrix effects a regression line with the standard procyanidin A2
4 (0.082 – 11.42 µg/mL) and a standard addition regression line of a solution containing 1.0
5 mg/mL sample, in this case extract 3, 4 or 5 and different concentrations of the standard solution
6 of procyanidin A2 (0.082 – 11.42 µg/mL) were constructed. The slope of both lines was
7 evaluated. In addition, the area of the sample was subtracted from the area of the standard
8 addition line and plotted.

9 In addition, different dilutions of the extracts, i.e. extract 1 (1.6 - 25 mg/mL), 2 (0.2 – 2.0
10 mg/mL) and 3 (0.075 – 1.8 mg/mL) were made. The areas of procyanidin A2 and the different
11 trimeric proanthocyanidins were plotted as a function of their concentration.

12 2.4.5 Final method

13 2.4.5.1 Preparation of standard solutions

14 A standard solution of procyanidin A2 was prepared by dissolving 5.0 mg in 10.0 mL methanol.
15 From this solution 4.0 mL was diluted to 100.0 mL with methanol-water (4.5:5.5, v/v).

16
17 2.4.5.2 Sample preparation
18 125.0 mg of the cranberry extract was weighed and 25.0 mL of the extraction solvent (methanol-
19 acetone-water (1:2:1, v/v/v)) was added. This mixture was sonicated for 30 min and filtrated
20 through a nylon membrane filter (0.45 µm). To 2.5 mL of the test solution, 0, 2.0, 5.0, and 9.0
21 mL, respectively, of the procyanidin A2 standard solution was added and diluted to 25.0 mL with
22 methanol-water (4.5:5.5, v/v).

1

2 2.4.5.3 Analysis

3 Separation was performed on an Acquity BEH 18 column (100 mm x 2.1 mm, 1.8 μ m). The
4 solvents were H₂O and acetonitrile, both with 0.1% formic acid. 5 μ L sample was injected, while
5 the column was kept at 40 °C and the sample manager was kept at 4 °C. The detection was done
6 with MRM in the negative ion mode, using the transitions and collision energies/cone voltages as
7 shown in Table 2.

8

9 2.5 Method validation

10 The developed method was fully validated according to the ICH guidelines [28]. For the
11 validation of the method, extract 3 was used.

12

13 2.5.1 Calibration model

14 The calibration model of procyanidin A2 was investigated. For this purpose, five concentrations
15 of a stock solution of procyanidin A2 of 0.02 mg/mL were added to a 1/10 dilution of both 100%
16 sample (125.0 mg, high concentrations) and 50% sample (62.5 mg, low concentrations). The final
17 concentrations of procyanidin A2 ranged from 0.25 μ g/mL to 10.3 μ g/mL. All solutions were
18 injected twice. The percentage of procyanidin A2 and other dimers was calculated by standard
19 addition. For trimers, the logarithm of the area of procyanidin A2 after subtraction of the area
20 procyanidin A2 from the sample itself, was plotted against the logarithm of the concentration of
21 procyanidin A2 added. In this way, trimers are calculated and expressed as procyanidin A2.
22 Regression analysis was performed using Excel 2010.

23

24 2.5.2 Precision

1 Precision experiments were performed on four independent samples (about 125.0 mg extract)
2 which were analyzed on four different days using standard addition. Standard procyanidin A2
3 was added to three of the four dilutions. In addition, different concentration levels were tested,
4 i.e. 62.5 mg (50%) and 187.5 mg (150%) of the sample.

5 The mean, the standard deviation; and the RSD% were calculated for each day and each
6 concentration level. The overall mean, standard deviation and RSD% were calculated for the
7 three days and also for the three concentration levels. The repeatability and intermediate
8 precision were evaluated by an ANOVA single factor.

9 Before performing the ANOVA single factor, a Cochran test and/or Levene's test was done.

10

11 2.5.3 Accuracy

12 The accuracy could not be tested since no blank sample exists and no standards of all trimeric
13 proanthocyanidins of the extract are commercially available.

14

15 2.5.4 Specificity

16 The specificity could not be tested since no blank sample exists and no standards of all trimeric
17 proanthocyanidins of the extract are available. By using the MRM technique the highest level of
18 specificity possible for the apparatus is used, taking into account that a specific transition for
19 these compounds was selected.

20 2.6 Comparison of twelve cranberry extracts

21 Twelve cranberry extracts were analyzed using the validated LC-MS method, the butanol-HCl
22 assay, the DMAC assay, normal phase HPLC, and HPTLC-densitometry.

1 The spectrophotometric method described in the Eur. Ph. for Hawthorn berries (*Crateagi fructus*)
2 [29], also known as the butanol-HCl assay, was used to quantify the proanthocyanidin content
3 expressed as cyanidin chloride. Depending on the amount of PACs present in the sample, the
4 amount of sample and the dilutions steps were adjusted. In addition, after the first reflux
5 extraction, the residue was washed with 20 mL of ethanol-water (7:3, v/v) and refluxed again for
6 15 min. Extracts 4 and 5 were first fully dissolved in water and afterwards the ethanol was added.
7 Since extracts 4 and 5 blocked the filtration paper, centrifugation (2397 g – 10 min.) was used
8 instead.

9 PACs were quantified with the slightly adapted 4-dimethylaminocinnamaldehyde (DMAC)
10 colorimetric method as described by Prior et al. [13]. Briefly, extracts were weighed and
11 extraction solvent (40% of the volume of the measuring flask) (acetone-water-acetic acid
12 (75:24.5:0.5, v/v/v)) was added. For extracts 1, 4, 5 and 7, samples were firstly fully dissolved in
13 water (with 0.5% acetic acid) before the acetone was added to ensure complete extraction of
14 PACs.

15 The samples were vortexed for 30 s and sonicated for 30 min. Subsequently all samples were
16 stirred for 1 h and centrifuged at 2000 g for 10 min. Dilutions of the supernatant were made using
17 ethanol-water (73:17, v/v). A stock solution of standard procyanidin A2 (100 µg/mL) was
18 prepared in ethanol 91% and dilutions were made with the same solvent ranging from 10 to 50
19 µg/mL. 300 µL 0.1% DMAC solution was mixed with either 900 µL dilution solution, standard
20 solution or sample dilution in a test tube and shortly vortexed. The absorption (640 nm) of the
21 mixture was then analyzed every minute during 50 min using a UV-VIS double beam
22 spectrophotometer. Results were calculated using a calibration curve constructed with standard
23 dilutions of procyanidin A2.

1 A profile of the proanthocyanidins present in the extracts was created by normal phase HPLC-
2 UV (Beckmann). This method was partially based on the analysis described by Romani et al.
3 [30].

4 To 100.0 - 1000.0 mg sample (depending on the sample), 2 parts of absolute ethanol were added
5 and the solution was sonicated for 30 min. Thereafter, 8 parts of dichloromethane were added and
6 the solution was homogenized and filtered (0.45 μ m). Samples were analyzed on a Purospher
7 Star Si-column (250 x 4 mm, 5 μ m). Mobile phase A was methanol-formic acid-water (97:2:1,
8 v/v/v) and mobile phase B dichloromethane-methanol-formic acid (83:15:2, v/v/v). The
9 following gradient was used: from 100% B to 89% B in 20 min – from 89% B to 100% B in 10
10 min – stay at 100% B for 10 min. 15 μ L of each sample was injected. The flow rate was set at
11 0.75 mL/min and detection was performed at 280 nm. Catechin, epicatechin, gallic acid and
12 procyanidins B1 and B2 were used as standards.

13 The normal phase HPTLC method as described by Boudesocque et al. [14] was used to quantify
14 the procyanidin A2 content in all cranberry extracts. Different procyanidin A2 standard solutions
15 (0.07, 0.1, 0.3, and 0.5 mg/mL) and extracts dissolved in methanol (70 mg/mL) were applied to
16 normal phase HPTLC plates by an automatic TLC sampler and developed with CH₂Cl₂-ethyl
17 acetate-formic acid (6:10:1, v/v/v) in an automatic developing chamber. After drying, the plates
18 were dipped in a HCl solution containing 1% vanillin and heated for 2 min at 110 °C.
19 Densitometric detection was done by scanning at 500 nm using a TLC Scanner 3.

20

21 **3. Results and discussion**

22 3.1 Method development

23 3.1.1 Extraction

1 Binary solvent experiments showed that more than 50% of organic modifier was needed to
2 extract PACs. Based on these findings, all ternary solvent mixtures were composed of maximum
3 40% of water. Eight out of nine ternary mixtures gave better extraction results than binary solvent
4 mixtures, except for binary mixture 13 which was as good as ternary mixtures (Fig. 1). Within the
5 ternary mixtures, there were almost no differences except for mixture 9, which only gave partial
6 extraction of PACs. In order to be able to extract PACs from different cranberry extracts, a
7 ternary mixture was chosen over a binary mixture because of its robustness. Based on overall
8 extraction yield and variation, water-aceton-methanol (25:50:25, v/v/v) was chosen as extraction
9 solvent, which combined a high yield with low variation.

10

11 3.1.2 Chromatographic conditions

12 Preliminary screening of all extracts indicated that only one extract (extract 4) showed a different,
13 more complex chromatographic profile. All other extracts resulted in similar chromatograms
14 showing a maximum of one dimer and four trimer peaks. Extract 3, a scientifically based extract
15 used in a cranberry preparation claiming an intake of 36 mg PACs per day, was selected for
16 further method development. All experiments using the HSS T3 column resulted in
17 chromatograms with bad peak shape and a lot of noise, while the BEH C18 column gave a more
18 optimal chromatographic profile (Supporting information - Fig. S1). Based on these results, the
19 BEH C18 column was selected.

20 Separation of extract 3 was achieved with all three gradients (Figs. 2 and 3) but the separation of
21 the more complex sample 4 was better using gradient 3. In comparison with gradient 1 and 2, the
22 resolution of some peaks was higher, but it is the most time consuming of all gradients.

23 The last parameter that was evaluated was the concentration of acid in the mobile phase. Based
24 on literature, concentrations of 0.1% and 1% were compared. Keeping in mind that PACs are not

1 stable in acidic environment ($\text{pH} < 2$), since no influence on the peak shape or resolution was
2 observed for extract 3, and since the separation of extract 4 was better for some peaks and worse
3 for others (Fig. S2), the lower concentration of 0.1% formic acid ($\text{pH} > 2$) was preferred [21].
4 Because of the instability of the procyanidin A2 standard at room temperature, the autosampler
5 temperature was set at 4 °C, which kept the standard solution stable for at least 24 h.

6

7 3.1.3 Mass spectrometric conditions

8 The optimal collision energy and cone voltage for the transitions used for quantitative as well as
9 for qualitative purposes for each compound, giving maximal intensity, are shown in Table 2.

10

11 3.1.4 Matrix effects

12 The regression line of procyanidin A2, the standard addition line (sample + procyanidin A2) and
13 the regression line constructed with the difference between sample and standard addition line are
14 plotted in Fig. S3. When no matrix effects are present, this latter regression line should be
15 identical to the regression line of procyanidin A2. For extract 3 the slope of the regression line of
16 procyanidin A2 (equation: $y = (1956.8 \pm 32.1)x + (368.8 \pm 179.9)$) was steeper than that of the
17 standard addition line (equation: $y = (1381.3 \pm 29.5)x + (12222.0 \pm 165.7)$), suggesting matrix
18 effects, more specifically ion suppression. This effect was not observed for extracts 4 (not shown)
19 and 5 (Fig. S3), meaning that there were no matrix effects.

20 When quantifying procyanidin A2 in the dilution experiment, the same effect was observed for
21 all extracts tested (1-3). When matrix effects were present, a deviation of the linear regression
22 line was visible, i.e. when ions were suppressed the area decreased and when ion formation was
23 enhanced the area of the analyte increased. For these extracts, a flattening of the curve was

1 observed when the concentration increased, indicating ion suppression at higher concentrations
2 (Fig. S4) for extract 2. In the lower concentration region, the constituents causing matrix effects
3 are highly diluted so they do not influence the ionization. This flattening of the curve was not
4 observed for trimeric PACs, but as already mentioned above, these findings could rule out
5 possible matrix effects for trimeric PACs.

6 Theoretically, when the dilution of the sample is sufficient, the results will not be influenced by
7 ion suppression. In routine analysis without standard addition, this implies that before analyzing a
8 sample, the dilution where no matrix effect is present should be chosen. In addition, other reasons
9 that favor a standard addition method are the fact that the sample cannot be endlessly diluted,
10 since next to dimeric PACs also the trimers should be measurable. For example, for extract 3,
11 areas of trimers are smaller than procyanidin A2, creating a challenge. Bearing in mind that this
12 test was only performed on three samples, i.e. crude extracts but no finished products, it is not
13 sure that matrix effects could always be solved by diluting. Hence, it was decided to develop a
14 standard addition method in order to be able to analyze all possible types of cranberry products.

15 3.1.5 Validation

16 3.1.5.1 Calibration model

17 Inspection of all calibration curves and their corresponding residuals plots showed that these were
18 linear. The standard addition calibration curves for procyanidin A2 added to 100% sample (Fig.
19 S5) and 50% sample resulted in regression equations $y = (2570206 \pm 37745)x + (8716.3 \pm 206.4)$
20 (for 100%) and $y = (2378.6 \pm 21.7)x + (3675.6 \pm 76.3)$ (50%). All correlation coefficients were
21 higher than 0.99 and the slopes of the regression lines were significant. All residuals were
22 randomly scattered and showed no heteroscedasticity (Figs. S6 and S7). Since it is not possible to

1 compare the maximal residual value with the area of the expected value (100% sample) when
2 using standard addition, the maximal residual value was compared with the middle and the lowest
3 point of the standard addition curve in order to estimate the deviation. For procyanidin A2 (a
4 dimer) none of the residuals was higher than 6.82% of the middle or 13.96% of the lowest
5 concentration of procyanidin A2 of the regression line. These values are still acceptable for LC-
6 MS methods.

7 For the calculation of trimeric proanthocyanidins, calibration curves were constructed by plotting
8 the logarithm of the concentrations and corresponding logarithm of the areas after subtracting the
9 area of procyanidin A2 in the sample without procyanidin A2 addition from the area of
10 procyanidin A2 at the different addition levels. These calibration curves, obtained with 100%
11 (Fig. S8) and 50% sample, were linear and resulted in linear regression equations $y = (1.010x \pm$
12 $0.025) x + (3.369 \pm 0.014)$ and $y = (6.118 \pm 0.069)x + (0.860 \pm 0.026)$, respectively. Their correlation
13 coefficients were larger than 0.99. The maximum residual value was 3.46% of the lowest
14 concentration of the regression line, as shown in Figs. S9 and S10.

15

16 3.1.5.2 Precision

17 3.1.5.2.1 Procyanidin A2 – dimeric proanthocyanidins

18 The results are shown in Table S2. In case of different days, the calculated Cochran values were
19 smaller than the critical C-value, implying that the variances were not significantly different and
20 an ANOVA single factor could be carried out. The calculated F-value that was higher than the
21 critical F-value, meaning that the results between the days were significantly different.

22 The RSD_{between} was 4.86% and was higher than the calculated maximum RSD%, based on
23 RSD_{Horwitz} . In case of the different concentration levels, the calculated C-value was smaller than

1 the critical C-value. The ANOVA single factor test showed a significant difference for results on
2 the concentration levels since the calculated F-value was higher than the critical F-value. The
3 RSD_{between} value was 4.28% and was also higher than the calculated maximum RSD%. Since
4 RSD_{between} values, for different days and on different concentration levels were still smaller than
5 5%, which is generally accepted in complex analyzes, the method can still be considered as
6 precise. The graphical representation of the results (Fig. 4) revealed no trend between the results
7 obtained at different concentration levels, proving the completeness of extraction. The overall
8 mean procyanidin A2 content was 0.62%.

9 3.1.5.2.2 Trimeric proanthocyanidins

10 A summary of the results is shown in Table S3. Calculations were done for all trimeric PACs
11 separately and for the total content of trimeric PACs. The calculated Cochran values were smaller
12 than the critical C-value, implying that the variances obtained on different days were not
13 significantly different and an ANOVA single factor could be carried out. The factor “day” had no
14 significant effect on the results since all calculated F-values were lower than the critical F-value.
15 Although all RSD_{between} values were higher than the maximal RSD, the highest RSD_{between} was
16 5.61% which is acceptable for an LC-MS/MS method. In case of different concentration levels,
17 all calculated C-values, except for trimer 3, were slightly higher than the critical C- values. A
18 Levene’s test was executed and this test did not confirm unequal variances. The subsequently
19 performed ANOVA single factor test did not show a significant effect of concentration levels on
20 the results (Fig. 5). As for the different days, the RSD_{between} was higher than the calculated
21 maximum RSD% and the highest RSD_{between} values was 7.65%.

22 3.1.6 Comparison of twelve cranberry extracts

1 The results obtained with all analytical techniques discussed are summarized in Table 3.
2 Apparently the optimal extraction mixture for extract 3, used for development and validation of
3 the LC-MS method, was not applicable to all other extracts. When the extraction mixture was
4 added to extracts 1, 4, 5 and 7, aggregates were formed. During dilution of extracts 6, 10, and 11,
5 which were whole berry extracts and cranberry fibers, respectively, the solution became opaque.
6 Therefore, the extraction solvent was optimized for the different extracts. This resulted in water
7 as extraction solvent for extracts 1, 5 and 7 since all three extracts were fully soluble in water,
8 while extraction with methanol was the best alternative for extract 4, the only extract that was
9 manufactured using water and ethanol. A mixture of water and methanol (50:50, v/v) was optimal
10 for extracts 10 and 11, and a mixture of water and acetone (50:50, v/v) was chosen for extract 6.
11 Results for dimers were as follows: extract 2, showed a concentration higher than 1%, i.e. 1.12%;
12 extracts 3, 4, 8 and 12 had a concentration between 0.1 and 1%; and all other extracts had a
13 concentration below 0.1% with extract 7 showing a concentration even lower than 0.01%. With
14 regard to trimers, extract 4 showed the highest concentration with 1.44% and extracts 5, 7 and 11
15 had the lowest content (<0.01%), extracts 1, 6, 9 and 10 ranged between 0.01 and 0.1% and
16 extracts 2, 3, 8 and 12 between 0.1 and 1%.

17 The ratio between dimeric and trimeric PACs ranged from 52 to 273%. In addition, extract 4
18 showed a totally different profile of dimers and trimers. This could be caused by the extraction
19 solvent, ethanol/water, used by the manufacturer to produce the extract, which differed from all
20 other extracts. During the analysis of different commercial cranberry products, most preparations
21 showed only four trimeric PACs, while some preparations also showed a more complex profile
22 [15].

23 The dimer content of all extracts was also analyzed without standard addition and compared with
24 results obtained with standard addition (Tables 4 and 5). For a majority of the extracts, a similar

1 trend could be observed, namely, a higher percentage procyanidin A2 was found when using the
2 standard addition method, suggesting potential matrix effects (Fig. 6a and 6b). This trend was
3 more profound for extracts 1, 2, 4, 7, and 12.

4 For all extracts, with the exception of extract 12 a normal phase HPLC profile was obtained (Fig
5 S11 and S12). The ratio of polymers versus monomers was visually interpreted and was the
6 highest for extracts 2, 3 and 8, followed by extract 9. An intermediate ratio was observed for
7 extracts 1, 4 and 7 and almost no oligomeric and polymeric PACs were present in extracts 6, 10,
8 and 11. Extract 5 hardly showed any signal neither for monomers nor for polymers.

9 During sample preparation using the butanol-HCl assay, ethanol-water (70:30, v/v) caused
10 problems. When added to extracts 1, 4, 5 and 7, an aggregate was formed. Therefore, minor
11 adjustments were made to the sample preparation as described in 2.6. Results are shown in Table
12 3 and ranged from 0.134 to 7.57%.

13 As for the other methods the extraction solvent of the DMAC method was not optimal for all
14 extracts and again aggregation was observed for extracts 1, 4, 5, and 7. Therefore, the sample
15 preparation step was slightly adjusted (see 2.6).

16 All samples were measured using a double beam UV/VIS spectrophotometer. This resulted in
17 calibration curves with correlation coefficients larger than 0.99. PAC concentrations ranged from
18 0.16 to 32.77% (Table 3).

19

20 3.1.7 Comparison of different techniques

21 A correlation of R^2 0.74 – R 0.86 was observed between the butanol-HCl assay and the total
22 content of dimers and trimers obtained with LC-MS/MS, meaning that when a higher
23 concentration of A-type PACs was found by LC-MS the % PACs expressed as cyanidin HCl also

1 roughly increased. Deviations between these two techniques can be explained by the fact that the
2 butanol-HCl assay does not distinguish between A-type and B-type PACs and is biased by the
3 interference of anthocyanidins. Samples containing a high amount of anthocyanidins and/or B-
4 type PACs but a low amount of A-type PACs will result in a high proanthocyanidin content. In
5 addition, because the LC-MS assay only gives the content of dimeric and trimeric A-type PACs
6 and not higher polymers, the % PACs measured with the butanol-HCl assay of samples with a
7 similar dimer and trimer content measured by LC-MS can differ depending on their polymeric
8 PAC content.

9 A correlation coefficient of 0.88 was found between the LC-MS assay and DMAC assay.
10 Deviations between both methods can be explained by the fact that the DMAC assay, as the
11 butanol-HCl assay, cannot distinguish between A and B-type PACs. In addition, this technique
12 also measures monomers which can overestimate the amount of PACs. On the other hand, an
13 underestimation is also possible for samples with a high content of polymers with a high degree
14 of polymerization since DMAC only reacts with one PAC molecule. This implies that a high
15 PAC content can be obtained for a sample containing mainly B-type monomers, and a lower PAC
16 content for a sample that mainly consists of high-molecular weight A-type PACs. This problem
17 could be overcome by combining the DMAC assay with the LC-MS assay.

18 The correlation between the DMAC and butanol-HCl assay was 0.65. Extract 4 deviated largely
19 from the regression line. When this result was eliminated, the correlation coefficient increased to
20 0.89. A possible explanation could be the higher content of monomers present for extract 4 in
21 comparison with extracts 2, 3, 8 and 12, which was found by analysis with normal phase HPLC-
22 UV. These monomers are also measured with the DMAC assay but not with the butanol-HCl
23 assay. Another remarkable deviation was observed for extract 7 (combination with *Vaccinium*
24 *oxycoccus*) which has a very low PAC content according to the DMAC method compared to

1 extracts 1, 5, 6, 10 and 11, whereas it showed a much higher PAC content than the other extracts
2 according to the butanol-HCl assay. Since the solution of this extract was extremely colored due
3 to the presence of anthocyanidins, this might have led to an overestimation of the PAC content
4 when using the butanol-HCl assay.

5 All samples were analyzed with the method described by Boudesocque et al. [14] but,
6 unfortunately, this method was not sufficiently sensitive to quantify the procyanidin A2 content
7 of the crude cranberry extracts.

8
9 A comparison of the well-known and widely used DMAC method, the butanol-HCl assay and the
10 newly developed LC-MS/MS method clearly indicated the need for a reliable method able to
11 quantify A-type PACs, which are considered to be the pharmacologically active constituents of
12 cranberry. Neither the DMAC or butanol-HCl assay are capable to distinguish between A and B-
13 type PACs and therefore cannot detect adulterations with, for example, extracts with a high B-
14 type PAC content. Hence, the combination of the DMAC method or butanol-HCl assay with this
15 more specific LC-MS/MS assay could overcome these existing shortcomings.

16

17 **4. Conclusions**

18 A standard addition LC-MS/MS method for the simultaneous quantification of A-type
19 proanthocyanidin dimers and trimers in cranberry extracts was developed and validated. A linear
20 calibration model could be adopted for dimers and, after logarithmic transformation, for trimers.
21 The maximal interday and interconcentration precision was found to be 4.86% and 4.28% for
22 procyanidin A2 5.61% and 7.65% for trimeric PACs. Analysis of twelve different extracts using
23 the LC-MS standard addition method highlighted the enormous variation in dimeric and trimeric
24 PAC content. Comparison of these results with LC-MS/MS analysis without standard addition

1 showed the presence of matrix effects for some extracts and proved the necessity of standard
2 addition.

3

4 **Conflict of interest**

5 The authors declare they have no conflict of interest.

6

7 **Acknowledgements**

8 Dedicated to the memory of our colleague Sandra Apers (° 19/08/1972 - † 05/02/2017).

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11

12 **References**

- 13 [1] R.G. Jepson, G. Williams, J.C. Craig, Cranberries for preventing urinary tract infections, *Cochrane Database*
14 *of Systematic Reviews* 2012.
- 15 [2] S. Micali, G. Isgro, G. Bianchi, N. Miceli, G. Calapai, M. Navarra, Cranberry and recurrent cystitis: more
16 than marketing?, *Crit. Rev. Food Sci. Nutr.* 54 (2014) 1063-1075.
- 17 [3] A. Aydin, K. Ahmed, I. Zaman, M.S. Khan, P. Dasgupta, Recurrent urinary tract infections in women, *Int.*
18 *Urogynecol. J.* 26 (2015) 795-804.
- 19 [4] A. Occhipinti, A. Germano, M.E. Maffei, Prevention of urinary tract infection with Oximacro (R), a cranberry
20 extract with a high content of A-type proanthocyanidins: A pre-clinical double-blind controlled study, *Urol. J.*
21 13 (2016) 2640-2649.
- 22 [5] C.G. Krueger, J.D. Reed, R.P. Feliciano, A.B. Howell, Quantifying and characterizing proanthocyanidins in
23 cranberries in relation to urinary tract health, *Anal. Bioanal. Chem.* 405 (2013) 4385-4395.
- 24 [6] G.C. Schito, K.G. Naber, H. Botto, J. Palou, T. Mazzei, L. Gualco, A. Marchese, The ARESC study: an
25 international survey on the antimicrobial resistance of pathogens involved in uncomplicated urinary tract
26 infections, *Int. J. Antimicrob. Agents* 34 (2009) 407-413.
- 27 [7] J. Bien, O. Sokolova, P. Bozko, Role of uropathogenic *Escherichia coli* virulence factors in development of
28 urinary tract infection and kidney damage, *Int. J. Nephrol.* Article ID 681473 (2012).

- 1 [8] K. Gupta, M.Y. Chou, A. Howell, C. Wobbe, R. Grady, A.E. Stapleton, Cranberry products inhibit adherence
2 of P-fimbriated *Escherichia coli* to primary cultured bladder and vaginal epithelial cells, *J. Urol.* 177 (2007)
3 2357-2360.
- 4 [9] L.Y. Foo, Y.R. Lu, A.B. Howell, N. Vorsa, A-type proanthocyanidin trimers from cranberry that inhibit
5 adherence of uropathogenic P-fimbriated *Escherichia coli*, *J. Nat. Prod.* 63 (2000) 1225-1228.
- 6 [10] A.B. Howell, Bioactive compounds in cranberries and their role in prevention of urinary tract infections, *Mol.*
7 *Nutr. Food Res.* 51 (2007) 732-737.
- 8 [11] L.Y. Foo, Y.R. Lu, A.B. Howell, N. Vorsa, The structure of cranberry proanthocyanidins which inhibit
9 adherence of uropathogenic P-fimbriated *Escherichia coli in vitro*. *Phytochemistry* 54 (2000) 173-181.
- 10 [12] L. Krenn, M. Steitz, C. Schlicht, H. Kurth, F. Gaedcke, Anthocyanin- and proanthocyanidin-rich extracts of
11 berries in food supplements - analysis with problems, *Pharmazie* 62 (2007) 803-812.
- 12 [13] R.L. Prior, E. Fan, H. Ji, A. Howell, C. Nio, M.J. Payne, J. Reed, Multi-laboratory validation of a standard
13 method for quantifying proanthocyanidins in cranberry powders, *J. Sci. Food Agric.* 90 (2010) 1473-1478.
- 14 [14] L. Boudesocque, J. Dorat, J. Pothier, A. Gueiffier, C. Enguehard-Gueiffier, High performance thin layer
15 chromatography-densitometry: A step further for quality control of cranberry extracts, *Food Chem.* 139
16 (2013) 866-871.
- 17 [15] F. Sanchez-Patan, B. Bartolome, P.J. Martin-Alvarez, M. Anderson, A. Howell, M. Monagas, Comprehensive
18 assessment of the quality of commercial cranberry products. Phenolic characterization and *in vitro* bioactivity,
19 *J. Agric. Food Chem.* 60 (2012) 3396-3408.
- 20 [16] E. Jungfer, B.F. Zimmermann, A. Ruttkat, R. Galensa, Comparing procyanidins in selected *Vaccinium* species
21 by UHPLC-MS(2) with regard to authenticity and health effects, *J. Agric. Food Chem.* 60 (2012) 9688-9696.
- 22 [17] I. Iswaldi, A.M. Gomez-Caravaca, D. Arraez-Roman, J. Uberos, M. Lardon, A. Segura-Carretero, A.
23 Fernandez-Gutierrez, Characterization by high-performance liquid chromatography with diode-array
24 detection coupled to time-of-flight mass spectrometry of the phenolic fraction in a cranberry syrup used to
25 prevent urinary tract diseases, together with a study of its antibacterial activity, *J. Pharm. Biomed. Anal.* 58
26 (2012) 34-41.
- 27 [18] J.M. Walsh, X.B. Ren, C. Zampariello, D.A. Polasky, D.L. McKay, J.B. Blumberg, C.Y.O. Chen, Liquid
28 chromatography with tandem mass spectrometry quantification of urinary proanthocyanin A2 dimer and its
29 potential use as a biomarker of cranberry intake, *J. Sep. Sci.* 39 (2016) 342-349.
- 30 [19] L.W. Gu, M.A. Kelm, J.F. Hammerstone, G. Beecher, J. Holden, D. Haytowitz, R.L. Prior, Screening of
31 foods containing proanthocyanidins and their structural characterization using LC-MS/MS and thiolytic
32 degradation, *J. Agric. Food Chem.* 51 (2003) 7513-7521.
- 33 [20] R.L. Prior, S.A. Lazarus, G.H. Cao, H. Muccitelli, J.F. Hammerstone, Identification of procyanidins and
34 anthocyanins in blueberries and cranberries (*Vaccinium* spp.) using high-performance liquid
35 chromatography/mass spectrometry, *J. Agric. Food Chem.* 49 (2001) 1270-1276.
- 36 [21] W. Hummer, P. Schreier, Analysis of proanthocyanidins. *Mol. Nutr. Food Res.* 52 (2008) 1381-1398.

- 1 [22] S. Gómez-Alonso, E. García-Romero, I. Hermosín-Gutiérrez, HPLC analysis of diverse grape and wine
2 phenolics using direct injection and multidetection by DAD and fluorescence, *J. Food Compos. Anal.* 20
3 (2007) 618-626.
- 4 [23] T.C. Wallace, M.M. Giusti, Extraction and normal-phase HPLC-fluorescence-electrospray MS
5 characterization and quantification of procyanidins in cranberry extracts, *J. Food Sci.* 75 (2010) C690-6.
- 6 [24] M. Karonen, J. Loponen, V. Ossipov, K. Pihlaja, Analysis of procyanidins in pine bark with reversed-phase
7 and normal-phase high-performance liquid chromatography–electrospray ionization mass spectrometry. *Anal.*
8 *Chim. Acta* 522 (2004) 105-112.
- 9 [25] P.J. Sarnoski, J.V. Johnson, K.A. Reed, J.M. Tanko, S.F. O'Keefe, Separation and characterisation of
10 proanthocyanidins in Virginia type peanut skins by LC-MSⁿ, *Food Chem.* 131 (2012) 927-939.
- 11 [26] M. Naczki, F. Shahidi, Extraction and analysis of phenolics in food, *J. Chromatogr. A* 1054 (2004) 95-111.
- 12 [27] F. Travaglia, M. Bordiga, M. Locatelli, J.D. Coisson, M. Arlorio, Polymeric proanthocyanidins in skins and
13 seeds of 37 *Vitis vinifera* L. cultivars: a methodological comparative study, *J. Food Sci.* 76 (2011) C742-749.
- 14 [28] International Conference on Harmonization. Validation of analytical procedures: text and methodology Q2
15 (R1). 2005
- 16 [29] Council Europe. Hawthorn berries. In: European Pharmacopeia 7.6 [Online]; Council of Europe: Strasbourg,
17 2016. <http://online6.edqm.eu/ep706> (accessed March 1, 2013).
- 18 [30] A. Romani, F. Ieri, B. Turchetti, N. Mulinacci, F.F. Vincieri, P. Buzzini, Analysis of condensed and
19 hydrolysable tannins from commercial plant extracts, *J. Pharm. Biomed. Anal.* 41 (2006) 415-420.
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1 **Table 1 Overview of different cranberry extracts**

Extract	Description
1	spray drying of the concentrated juice of the cranberry fruits/ used for manufacturing capsules
2	obtained from industrial producer of natural ingredients
3	concentrate of the juice from cranberry fruits / used for manufacturing capsules
4	produced by extraction of the cranberry fruits with ethanol and water/ used for manufacturing capsules
5	concentrated juice extract/ used in a solid dosage form, namely, tablets and contained a maltodextrin carrier
6	whole berry-derived extract produced by an industrial natural ingredient manufacturer
7	extract of the fruits of <i>Vaccinium macrocarpon</i> and <i>Vaccinium oxycoccus</i> produced by a manufacturer specialized in the development of active plant extracts, by extraction of the fruits with water and containing a carrier of maltodextrin
8	a dehydrated 100% cranberry extract, obtained from a natural ingredient manufacturer
9	obtained from a natural ingredient manufacturer
10	whole fruit extract from a manufacturer of value-added ingredients
11	cranberry fibers, produced by a food ingredient manufacturer
12	obtained from a manufacturer of fruit, vegetables and medicinal extracts

2
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4

1 **Table 2 Overview of the optimal collision energy and cone voltage for every transition.**

Transition	Optimal collision energy (eV)	Optimal cone voltage (V)
574.9 → 285.2	27	45
574.9 → 449.2	21	50
863.0 → 575.2	24	45
863.0 → 711.2	18	40
863.0 → 411.2	32	50
863.0 → 573.2	22	55

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1 **Table 3 PAC content of the all extracts using different analytical techniques.**

Extract	LC-MS		Butanol-HCl assay	DMAC assay	NP-HPLC profile
	% procyanidin A2	% trimeric procyanidins expressed as procyanidin A2	% procyanidins expressed as cyanidin HCl	% procyanidins expressed as procyanidin A2	Ratio polymers/monomers
1	0.0570 ± 0.0037	0.0503 ± 0.0026	0.344 ± 0.020	0.654 ± 0.037	+/-
2	1.121 ± 0.033	0.839 ± 0.054	4.955 ± 0.034	14.82 ± 0.29	+
3	0.614 ± 0.015	0.655 ± 0.016	4.63 ± 0.19	18.03 ± 0.16	+
4	0.0287 ± 0.0035	1.090 ± 0.079	4.87 ± 0.13	32.77 ± 0.83	+/-
5	0.0147 ± 0.0010	0.00618 ± 0.00035	0.1340 ± 0.0053	0.155 ± 0.010	-
6	0.0409 ± 0.0037	0.0226 ± 0.0031	0.2720 ± 0.0075	0.654 ± 0.027	-
7	0.001587 ± 0.000090	0.001842 ± 0.000053	1.115 ± 0.026	0.223 ± 0.017	+/-
8	0.575 ± 0.012	0.484 ± 0.020	6.93 ± 0.23	15.897 ± 0.099	+
9	0.0982 ± 0.0025	0.0359 ± 0.0011	1.56 ± 0.11	2.880 ± 0.084	+
10	0.05185 ± 0.00042	0.0237 ± 0.0036	0.2083 ± 0.0068	0.6397 ± 0.0090	-
11	0.01559 ± 0.00045	0.00577 ± 0.00039	0.160 ± 0.012	0.337 ± 0.017	-
12	0.846 ± 0.030	0.792 ± 0.011	7.58 ± 0.29	15.71 ± 0.49	n.a.

2 -: low ratio of polymers/monomers; +/-: intermediate ratio of polymers/monomers; +: high ratio of polymers/monomers.

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1 **Table 4 Results for procyanidin A2 measured with and without standard addition.**

	With standard addition	Without standard addition
Extract 1		
<x> ± s	0.0570 ± 0.0037	0.0515 ± 0.0010
Extract 2		
<x> ± s	1.121 ± 0.033	1.0578 ± 0.0090
Extract 3		
<x> ± s	0.614 ± 0.015	0.581 ± 0.020
Extract 4		
<x> ± s	0.0287 ± 0.0025	0.0242 ± 0.0015
Extract 5		
<x> ± s	0.0147 ± 0.0010	0.01408 ± 0.00034
Extract 6		
<x> ± s	0.0409 ± 0.0037	0.0375 ± 0.0006
Extract 7		
<x> ± s	0.001587 ± 0.000090	0.00126 ± 0.00013
Extract 8		
<x> ± s	0.575 ± 0.012	0.56 ± 0.12
Extract 9		
<x> ± s	0.0982 ± 0.0025	0.0960 ± 0.0019
Extract 10		
<x> ± s	0.05185 ± 0.00042	0.0523 ± 0.0016
Extract 11		
<x> ± s	0.01559 ± 0.00045	0.01605 ± 0.00069
Extract 12		
<x> ± s	0.846 ± 0.030	0.690 ± 0.012

2

3

1 **Table 5 Overview of trimeric PAC content.**

	Trimer 1	Trimer 2	Trimer 3	Trimer 4	Total
Extract 1					
$\langle x \rangle \pm s$	0.0113 ± 0.0010	0.0191 ± 0.0010	0.0111 ± 0.0012	0.0089 ± 0.0012	0.0503 ± 0.0026
Extract 2					
$\langle x \rangle \pm s$	0.195 ± 0.012	0.299 ± 0.018	0.182 ± 0.012	0.163 ± 0.013	0.839 ± 0.054
Extract 3					
$\langle x \rangle \pm s$	0.1492 ± 0.0040	0.2306 ± 0.0069	0.1642 ± 0.0036	0.1114 ± 0.0039	0.655 ± 0.016
Extract 4					
$\langle x \rangle \pm s$	0.3473 ± 0.0058*	1.090 ± 0.074*			1.437 ± 0.079
Extract 5					
$\langle x \rangle \pm s$	0.00323 ± 0.00011	0.001049 ± 0.000070	0.000993 ± 0.000093	0.000914 ± 0.000087	0.00618 ± 0.00035
Extract 6					
$\langle x \rangle \pm s$	0.0074 ± 0.0010	0.00626 ± 0.00083	0.00492 ± 0.00062	0.00401 ± 0.00065	0.0226 ± 0.0031
Extract 7		Not applicable			
$\langle x \rangle \pm s$	0.001842 ± 0.000053				0.001842 ± 0.000053
Extract 8					
$\langle x \rangle \pm s$	0.1651 ± 0.0049	0.1437 ± 0.0058	0.0922 ± 0.0044	0.0835 ± 0.0045	0.484 ± 0.020
Extract 9					
$\langle x \rangle \pm s$	0.01935 ± 0.00048	0.00582 ± 0.00024	0.00546 ± 0.00014	0.00527 ± 0.00029	0.0359 ± 0.0011
Extract 10					
$\langle x \rangle \pm s$	0.09957 ± 0.00092	0.00538 ± 0.00095	0.00383 ± 0.00085	0.00454 ± 0.00091	0.0237 ± 0.0036
Extract 11					
$\langle x \rangle \pm s$	0.00205 ± 0.00017	0.001270 ± 0.000062	0.001170 ± 0.000067	0.001270 ± 0.000095	0.00577 ± 0.00039
Extract 12					
$\langle x \rangle \pm s$	0.1344 ± 0.0029	0.2934 ± 0.0038	0.2149 ± 0.0042	0.1489 ± 0.0023	0.792 ± 0.011

2 * More trimeric PACs with transition of 863 → 411 were present in extract 4 and trimeric PACs with transition 863 → 575 were
3 different from those of the other extracts.

4

5

1 Figure 1 Overview of extraction results obtained with water/acetone/methanol mixtures: 1:
2 10/50/40; 2: 25/35/40; 3: 10/35/55; 4: 40/35/25; 5: 25/65/10; 6: 40/50/10; 7: 25/50/25; 8:
3 10/10/80; 9: 20/20/60; 10: 0/80/20; 11: 0/50/50; 12: 0/20/80; 13: 20/0/80; 14: 20/80/0.

4
5 Figure 2 Chromatograms of cranberry extract 3 with gradient 3 for trimeric proanthocyanidins
6 (863 > 411 and 863 > 575) and dimeric proanthocyanidins (575 > 285).

7
8 Figure 3 Chromatograms of cranberry extract 4 with gradient 3 for trimeric proanthocyanidins
9 (863 > 411 and 863 > 575) and dimeric proanthocyanidins (575 > 285).

10
11 Figure 4 Precision and intermediate precision results for procyanidin A2.

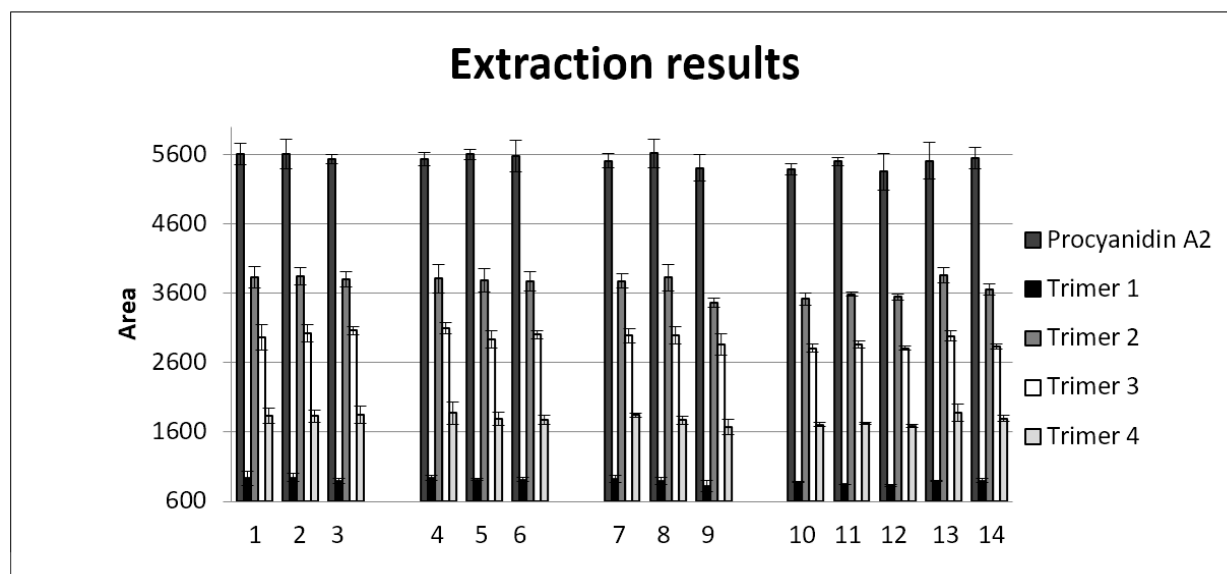
12
13 Figure 5 Precision and intermediate precision results for trimeric PACs.

14
15 Figure 6 a) % PAC A2 measured without and with standard addition. For each extract the results
16 are shown in the following order: without and with standard addition. The number of the
17 extract is written below. b) Close up of extracts with a lower procyanidin A2 content:
18 extracts 1, 4, 5, 6, 7, 9, 10, and 11.

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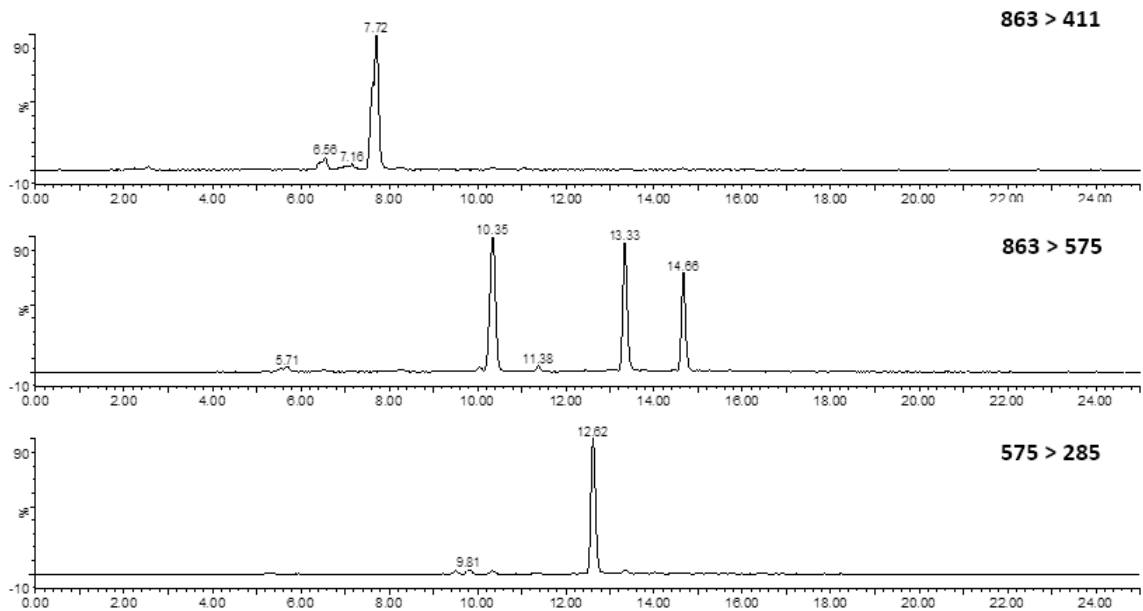
4 Figure 1 Overview of extraction results obtained with water/acetone/methanol mixtures: 1:

5 10/50/40; 2: 25/35/40; 3: 10/35/55; 4: 40/35/25; 5: 25/65/10; 6: 40/50/10; 7: 25/50/25; 8:

6 10/10/80; 9: 20/20/60; 10: 0/80/20; 11: 0/50/50; 12: 0/20/80; 13: 20/0/80; 14: 20/80/0.

7

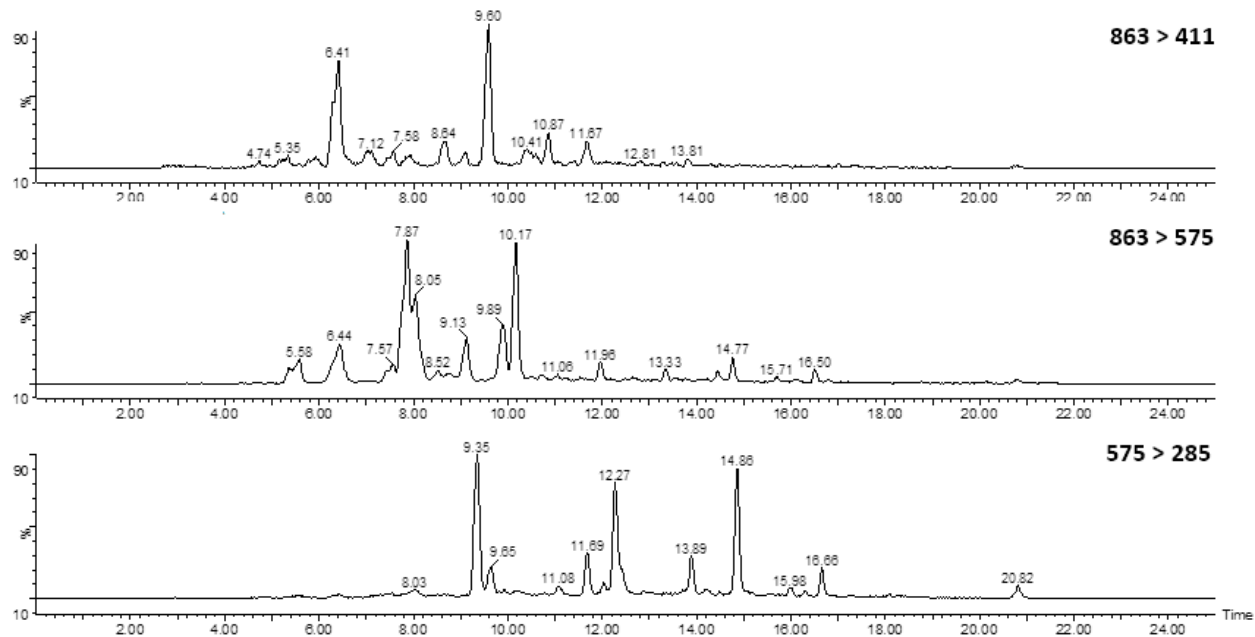
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Figure 2 Chromatograms of cranberry extract 3 with gradient 3 for trimeric proanthocyanidins (863 > 411 and 863 > 575) and dimeric proanthocyanidins (575 > 285).

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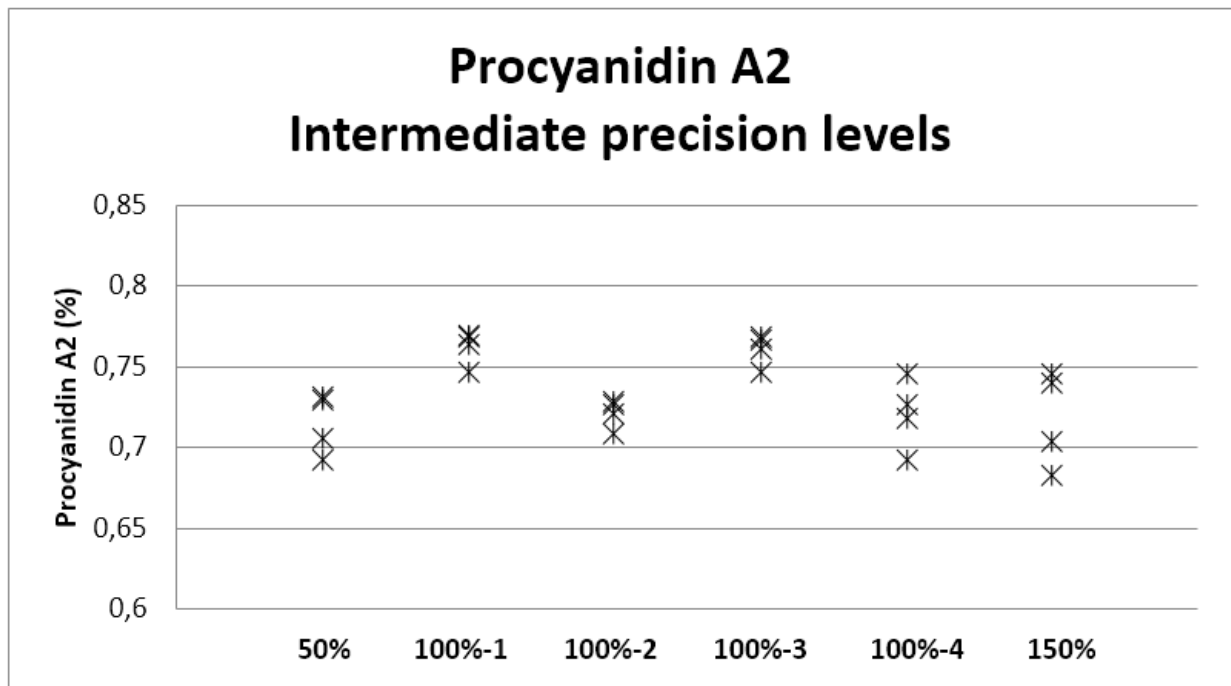
4 Figure 3 Chromatograms of cranberry extract 4 with gradient 3 for trimeric proanthocyanidins

5 (863 > 411 and 863 > 575) and dimeric proanthocyanidins (575 > 285).

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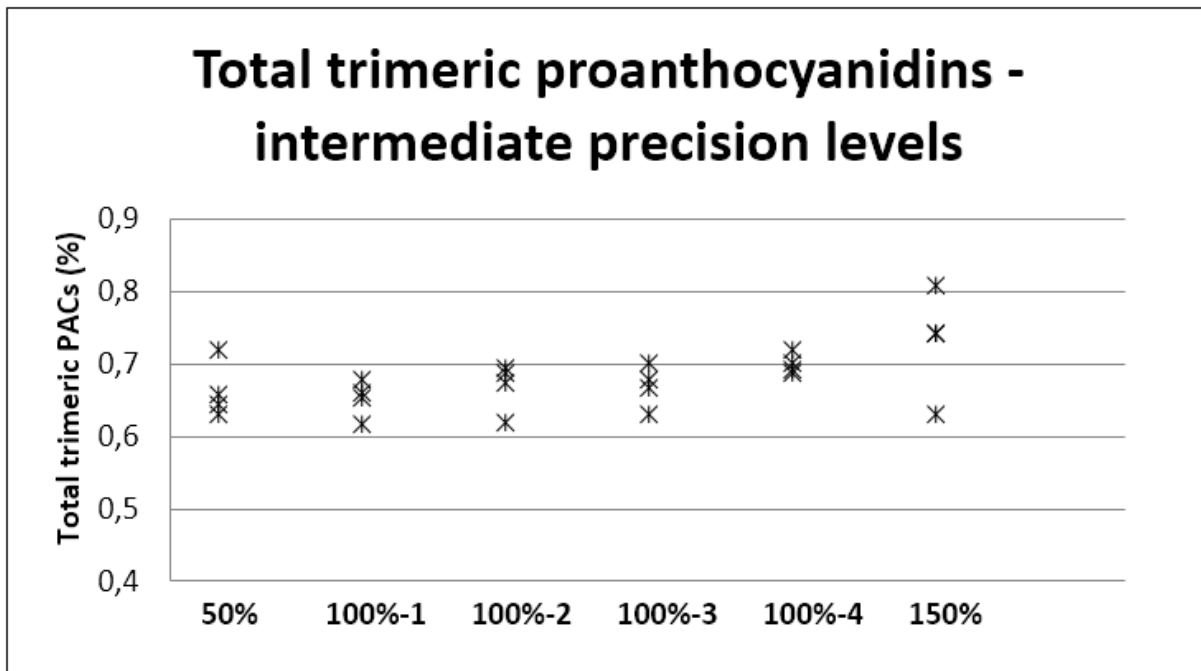
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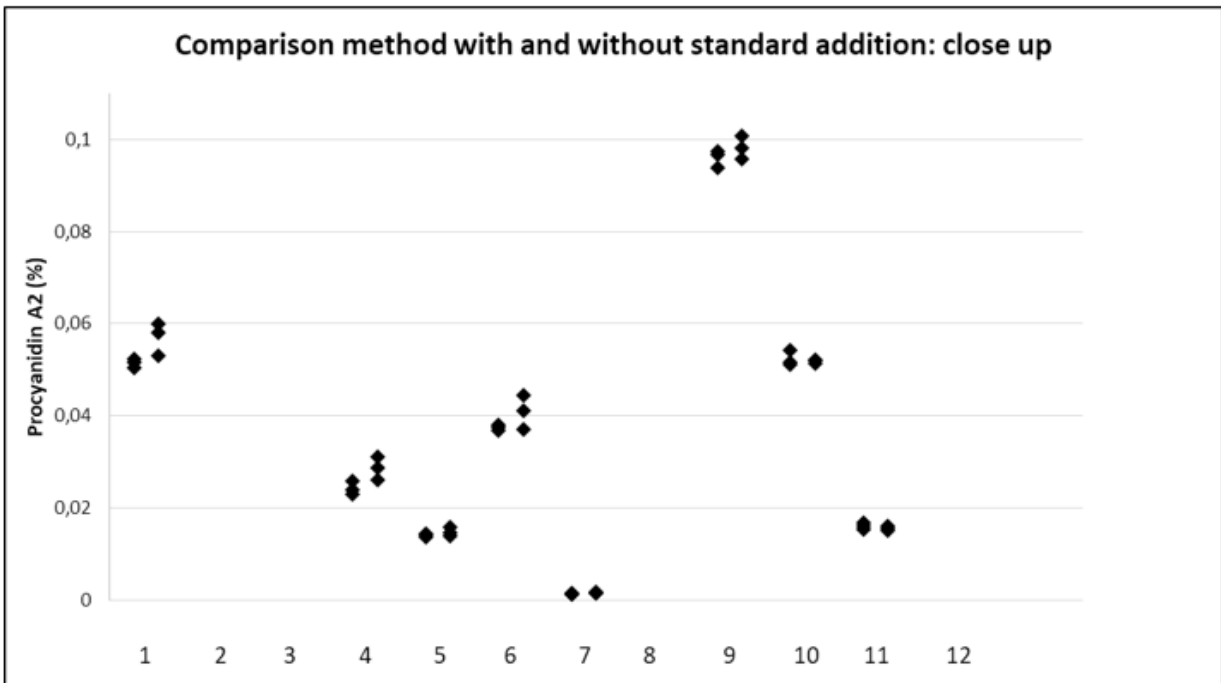
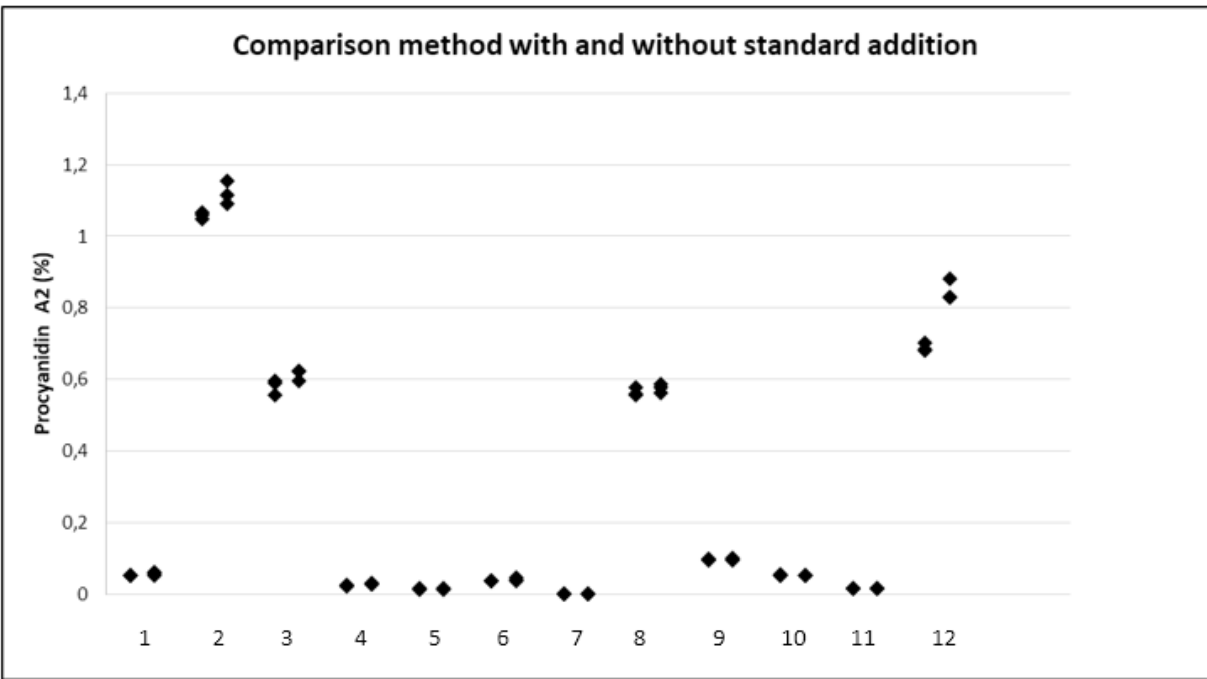
Figure 4 Precision and intermediate precision results for procyanidin A2.

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Figure 5 Precision and intermediate precision results for trimeric PACs.



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 2 Figure 6 a) % PAC A2 measured without and with standard addition. For each extract the results
 3 are shown in the following order: without and with standard addition. The number of the
 4 extract is written below. b) Close up of extracts with a lower procyanidin A2 content:
 5 extracts 1, 4, 5, 6, 7, 9, 10, and 11.