Method Development and Optimisation for the Characterisation and the Quantification of A-Type Proanthocyanidins - Application to Cranberry Extracts and Tissues

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INTRODUCTION: Among polyphenols, proanthocyanidins (PAC) (*i.e.* condensed tannins) are widespread plant and food components which are largely involved in some crucial organoleptic qualities including color, bitterness and astringency. In addition, the nutritional properties have been extensively studied in the two last decades showing that these components may exhibit various activities as antioxidant, agents and urinary tract infections [1]. Proanthocyanidins, in cranberry are flavanol oligomers and polymers characterized by the presence of A-type and type B interflavan linkages (Figure 1) [2]. Acid depolymerization in the presence of a nucleophilic agent is well-adapted to their complete characterization (nature of the flavanol units, the average degree of polymerization) and their quantification. However, the A-type links are resistant to depolymerization. This work aims to develop and optimize the extraction steps, the phloroglucinolysis reaction and the HPLC profiling for A-type proanthocyanidins in cranberry extracts.

PROANTHOCYANIDINS AND PHLOROGLUCINOLYSIS

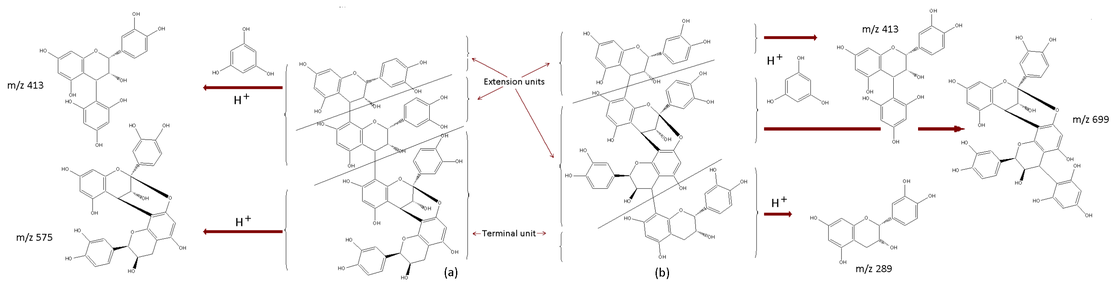

Figure 1: Structure of proanthocyanidins showing A-type links as terminal unit (a) or as extension units (b), the phloroglucinolysis reaction gives four products : epicatechin (m/z 289), the epicatechin phloroglucinol adduct (m/z 413), the A2 dimer (m/z 575) and the phloroglucinol adduct of the A2 dimer (m/z 699)[3].

OBJECTIVES

Development of a reliable qualitative and quantitative analytical method for procyanidins in cranberry and its derivatives including A-type procyanidins even in highly polymerized state.

- Sample preparation (extraction/purification)
- Complete validation of the method (calibration, LOD, LOQ ...)
- Transposed to industrial scale

HPLC PROFILING

First investigation of the polyphenols profile of a Cranberry extract was performed by high performance liquid chromatography (HPLC) in tandem with an ion trap mass spectrometer (MS) and a PDA UV-visible detector. A mixture of acetone/water/formic acid (70/29/1 v/v/v) enabled efficient extraction of proanthocyanidin oligomers in cranberry, including those of the A-type.

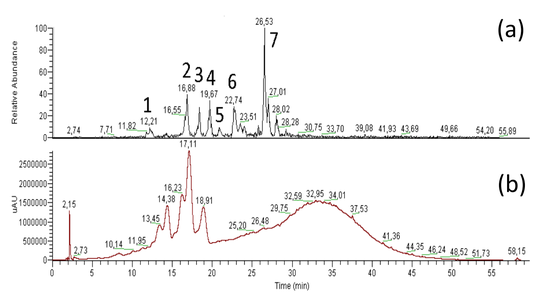

Figure 2: Proanthocyanidins extracted ions MS chromatogram (a) and UV-Visible 520 nm chromatogram (b) showing overlapping between proanthocyanidins and anthocyanidins.

Table 1: identification of molecules of interest.

	Analytes	Retention Time (min)	λ_{max} (nm)	Precursor Ion	Daughter Ion
1	tetramer A	12,2	278	1151	863/575/289
2	Procyanidin B2	16,9	280	577	425/289
3	(-) Epicatechin	18,14	278	289	
4	trimer A	19,58	278	863	575
5	trimer B	20,4	277	865	577
6	trimer A	22,8	278	863	575
7	Procyanidin A2	26,46	277	575	423/289

PURIFICATION/QUANTIFICATION

The quantification of the total PAC fraction is performed by a depolymerization reaction: phloroglucinolysis reaction in acid environment and in the presence of a nucleophilic agent in excess.
Noticeably, some of the acidolysis products (*i.e*. products with molecular ions at m/z 289 and m/z 699, corresponding to epicatechin and procyanidin A2 phloroglucinol adduct, respectively) gave a problematic overlapping with anthocyanins which rendered impossible accurate UV-visible quantifications (figure 2).

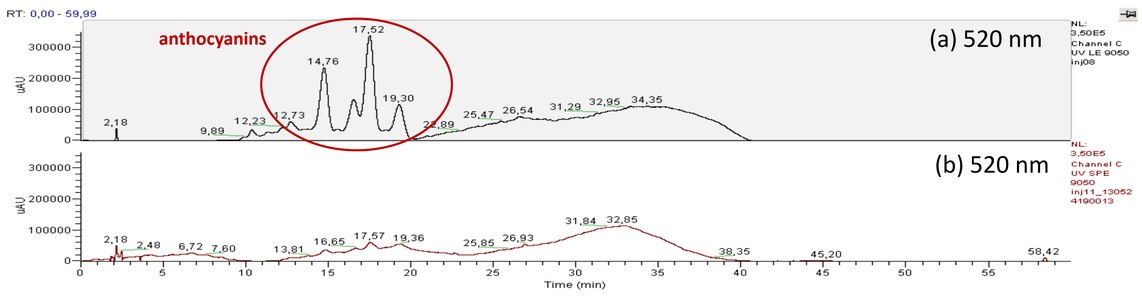

Figure 3: chromatogram at 520 nm of a sample before (a) and after (b) SPE purification.

Therefore, a further purification step was optimized in order to remove anthocyanins. Solid phase extraction (SPE) on cation exchange resin was used to remove anthocyanins efficiently (figure 3).

INFLUENCE OF SPE ON PAC QUANTIFICATION

The following products of the phloroglucinolysis reaction were quantified : epicatechin (EC), the phloroglucinol adduct of epicatechin (EC-PLG), the A2 dimer (A2) and the phloroglucinol adduct of A2 dimer (A2-PLG). In addition we have verified that no loss of proanthocyanidins occurred after SPE purification (Figure 4).

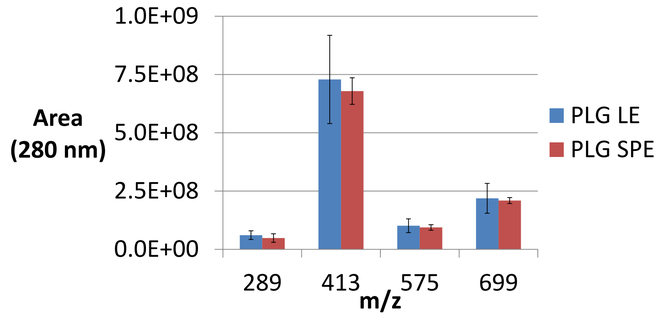

figure 4: HPLC-UV analysis of the phloroglucinolysis reaction products. Comparison between solvent extraction (LE) and solvent extraction followed by SPE extraction.

Excepted for A2-PLG, all reaction products were quantified according to their own calibration curve. The method allows the quantification of total proanthocyanidins and the overall characterization of the nature and proportion of the structural units (Table 2) [3].

APPLICATION TO COMMERCIAL EXTRACTS AND CRANBERRY TISSUES

The use of the phloroglucinolysis reaction coupled to HPLC analysis allows to estimate the composition, degree of polymerization (DP*n*) and the nature of the constituent units [3] (table 2).

Table 2: A-type proportion, average degree of polymerization of proanthocyanidins in dried cranberries powder.

Cranberry extracts	DPn	%A
DN1	6.9 ± 0.4	28.1±0.2
DN2	5.0 ±0.6	20.2±0.5
DN3	12.6±0.1	24.6±0.1
DN4	6.5±0.0	31.4±0.0

Flesh :
DP*n* = 6.0 ± 0.1
%A = 25.7 ± 0.4
Total PAC (g/kg**) =** 1,0 ± 0,1

Skin:
DP*n* = 9.7 ± 0.5
%A = 18.5 ± 0.8
Total PAC (g/kg) = 6.5 ± 0,7

Seeds :
DPn = 4,1 ± 0.4
%A = 27.4 ± 1.0
Total PAC (g/kg) = 6.9 ± 0,7

CONCLUSION

- Development of a qualitative and quantitative method adapted to cranberry proanthocyanidins containing A-type linkages: phloroglucinolysis reaction, SPE removal of anthocyanins, optimization of HPLC separation and detection.

PROSPECTS

- Additional validation (LOD, LOQ, …)
- Application to fruit samples and juices
- Calibration of the adduct phloroglucinol of dimer A2
- Fractionation of A-type proanthocyanidins according to the degree of polymerization
- Study of fruit tissue distribution of PAC and their varietal differences

References :
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[2] Foo, L. Y., Y. R. Lu, A. B. Howell and N. Vorsa (2000). "The structure of cranberry proanthocyanidins which inhibit adherence of uropathogenic P-fimbriated Escherichia coli in vitro." Phytochemistry 54(2): 173-181.
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