Preparative isolation of apple Flavan-3-ols by pH-zone-refining centrifugal partition chromatography combined with reversed-phase liquid chromatography

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Introduction

Flavan-3-ols (which include procyanidins and condensed tannins) are widespread polyphenols in fruits and edible plants and having numerous properties. They are largely responsible for astringency and bitterness in cider beverages. To study their sensorial, nutritional and biological features, it is essential to recover pure and native flavan-3-ols fractions. A gentle strategy combining pH-zone-refining centrifugal partition chromatography (pH-ZRCPC) and preparative reversed-phase liquid chromatography (Prep-RPLC) was developed to purify hundred milligrams of apple flavan-3-ols fraction in a highly purified state.

Optimization of two-phase solvent system for pH-ZRCPC

The pH-ZRCPC fractionation was optimized from a crude cider apple polyphenol extract (named TotPP) obtained from a non-oxidized juice of the French cultivar Marie Menard (Millet et al. [1]). it contained essentially flavanols monomers and oligomers (46.3%) with an average degree of polymerization close to 3.3, as measured by phloroglucinolysis coupled to reversed-phase HPLC), hydroxycinnamic acid derivatives (35.9 %) and dihydrochalcones (2.6%) (Table 2).

The partition coefficients (K) of the target compounds for a conventional CPC and pH-ZRCPC (by adding of a base or an acid) were determined for four biphasic solvent systems adapted from OKA [2] composed of ethyl acetate:n-butanol:water with 5:0:5, 3:2:5, 2:3:5 and 1:4:5 (v:v) named systems A, B, C and D, respectively.
For successful separation using pH-ZRCPC, it is necessary to respect Kbase << 1 and Kacid >> 1, for an acidic analyte. This allowed to select system B as the best one to discard hydroxycinnamic acid derivatives (HCA)(Table 1).

pH	K	Phenolic compounds in TotPP fraction											
		CQA	PCQ	EC	CAT	PA_B1	PA_B2	PA_B5	PA_C1	DP3	DP4	XPL	PLZ
2.15	Kacid	7.34	14.71	24.51	13.29	4.24	14.90	34.93	16.39	-	15.20	8.47	47.11
4.27	K	3.23	4.16	12.71	14.2	2.79	8.31	-	13.36	-	16.21	8.29	54.81
5.20	Kbase	0.42	0.51	12.27	11.07	3.77	8.38	37.09	13.19	-	16.41	8.14	52.43

Table 1. Partition coefficients of phenolic compounds in TotPP extract according to the solvent system B. CQA: 5'-O-caffeoylquinic acid; PCQ: 4-O-*para*-coumaroylquinic acid; EC: (-)-epicatechin, CAT: (+)-catechin; PA_B1: procyanidin dimer B1; PA_B2: procyanidin dimer B2; PA_B5: procyanidin dimer B5; PA_C1: procyanidin trimer C1; DP3: unknown procyanidin trimer; DP4: procyanidin tetramer; XPL: phloretin 2'-O-xyloglucoside; PLZ: Phloridzin.

pH-ZRCPC + Prep-RPLC procedures

pH-zone-refining CPC was carried out using a FCPC200® apparatus (Kromaton Technologies) (Fig. 1). The collected CPC fractions corresponding to flavan-3-ols (FA), dihydrochalcones (DHC) and flavonols (FO) were immediately acidified to avoid oxidation, and pooled. Prep-RPLC was performed on this intermediate freeze-dried fraction with a Lichrospher 100 RP-18, 12 µm column (Merck, Darmstadt, Germany) and isocratic mode, to recover only flavan-3-ols (Fig.2).

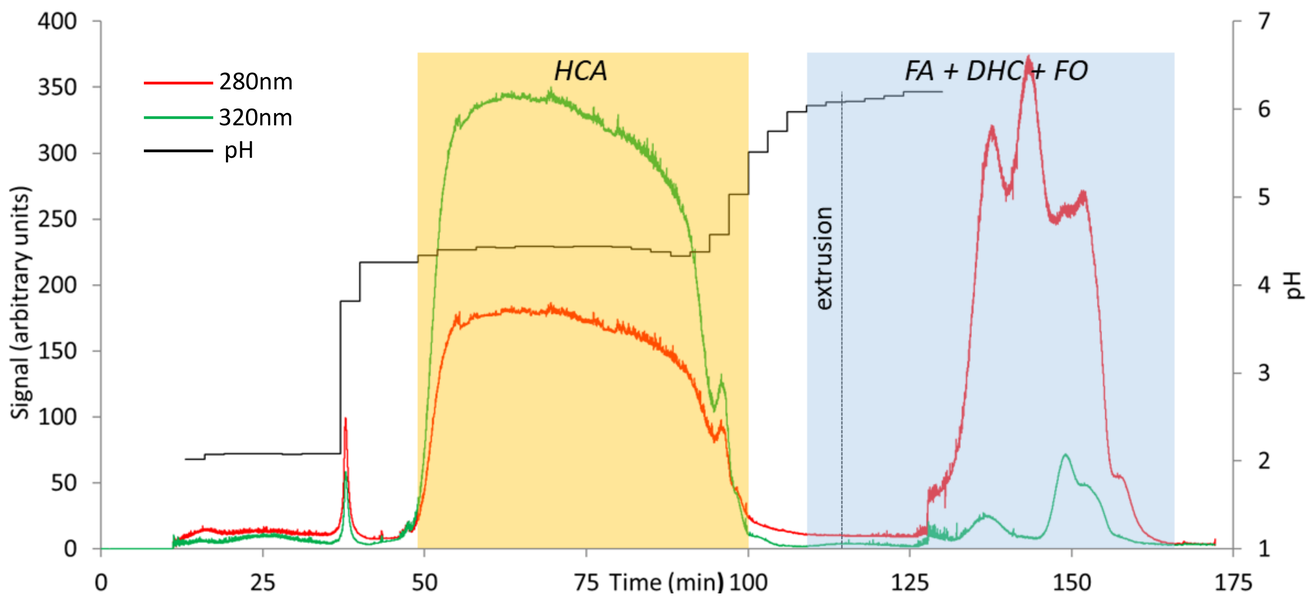

Fig 1. pH-ZRCCC chromatogram of the crude sample from 1 g TotPP extract. solvent system used: ethyl acetate-*n*-butanol-water (3:2:5); flow rate: 5 mL/min; revolution: 1200 rpm. Retainer (TFA) and eluter (NaOH) at 10 mM in the stationary and mobile phases, respectively.

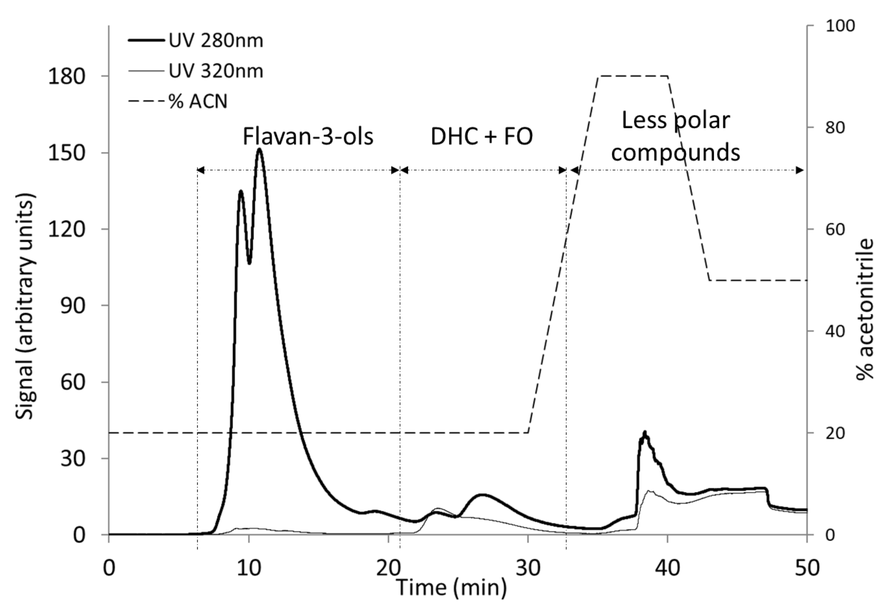

Fig.2. Reversed-Phase Preparative HPLC chromatogram of pH-ZRCPC Fraction

Analyses of fractions using UPLC-UV/MS

The composition of the collected fraction (called MM-FA) was characterized and compared to that of the crude extract (Fig. 3) showing that it contained only flavan-3-ols (Table2).

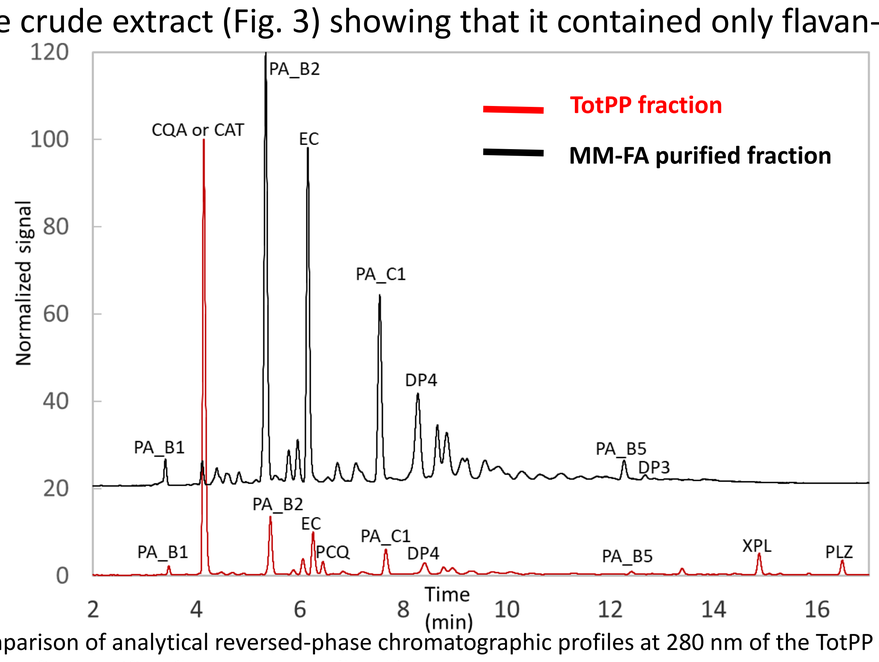

Fig.3. Comparison of analytical reversed-phase chromatographic profiles at 280 nm of the TotPP extract (red) and the final purified flavanol extract (black).

Phenolic compounds	TotPP	MM-FA extract
CQA	34.4	-
PCQ	14.9	-
EC	68.7	137.6
CAT	4.3	7.1
PA_B1	10.3	9.8
PA_B2	105.7	204.9
PA_B5	5.7	10.7
PA_C1	43.4	90.2
DP3	1.5	2.6
DP4	25.5	63.2
XPL	18.1	-
PLZ	8.0	-
Other procyanidins assayed after phloroglucinolysis	198.2	298.9
Total polyphenols purity (%)	84.8	82.5
Total flavan-3-ols purity (%)	46.3	82.5
Structural characterization of flavan-3-ols:		
Extension units (%)	70	67.5
Terminal units (-)-epicatechin (%)	26.8	29.1
Terminal units (+)-catechin (%)	3.2	3.4
aDP	3.3	3.1

Table 2. Composition of phenolic compounds in TotPP and MM-FA extracts (results expressed in g/Kg of dry extract) and structural characterization of flavan-3-ols.

Conclusion

Starting from a crude apple polyphenol extract, our experimentations demonstrate the relevance of using CPC combined with a pH-displacement mode to efficiently separate flavan-3-ols from hydroxycinnamic acid derivatives at a preparative scale. The objective was successful through optimisation of solvent systems with suitable conditions for pH-ZRCPC and an attention paid to avoid autoxidation. Finally, preparative reversed-phase chromatography allowed the separation of the flavan-3-ols with satisfactory purity (82%) and recovery (73%).

REFERENCES
[1] Millet, M, et al., *Food Chem.*, **2019**, 276, 797.
[2] Oka, F, et al., *Journal of Chromatography*, **1991**, 538, 99.