New technology for large preparation of a series of bioactive polyphenols from by-products of vinification

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Abstract. Grape pomaces or vinification by-products are abundant and rich in polyphenols, particularly proanthocyanidins. During the last three decades, grape and wine polyphenols, particularly catechins (CATs) and oligomeric proanthocyanidins (OPCs) have attracted considerable attention of the international scientific community, due essentially to their potential health-beneficial effects, related to their protective action towards cardiovascular disease and the oxygen free radical scavenger capacity. Such phenolic compounds have been proved to be the key components responsible for the health-beneficial effects of red wine. Furthermore, although the most widely recognized antioxidants are Vitamins A, C, and E, scientific research has shown that OPCs are likely the most powerful antioxidants known. Although various OPCs have been available in the market, they are generally very expensive due to the high cost of their production. As a consequence, the development of efficient methods for large-scale preparation of these bioactive compounds have still a challenging task for scientists. In this communication, we will present a new and efficient technology for large preparation of bioactive polyphenols from vinification by-products (grape seed and grape skin). On one hand, polymeric proanthocyanidins were transformed into a series of low- molecule-weight bioactive compounds using various degradation methods. On the other hand, High-Speed Countcurrent Chromatography (HSCCC), as a relative new separation technology for natural product preparation, was applied to large and efficient isolation of these bioactive compounds.

1 Introduction

Proanthocyanidins (PAs) are oligomers and polymers of flavan-3-ol units presented essentially in epidermal tissue of plants, leaf, bark, solid parts of fruits, so some agricultural residues or agro-industrial by-products, such as fruit pomaces, are often rich sources of these compounds [1]. According to the hydroxylation pattern of flavan-3-ols, PAs can be classified into various sub-groups, including essentially procyanidins, prodelphinidins, propelargonidins, profisetinidins, and prorobinetinidins.

PAs, as the fifth class of plant biopolymers, was still not familiar thirty years ago, but during the last two decades, some spectacular advances in scientific research on these compounds have been achieved. The explosion of research activities on PAs is mainly due to the discovery of various biological activities of their low-molecular compounds, i.e., flavan-3-ol monomers (commonly catechins) and oligomers (OPCs), which are likely the most powerful antioxidants known: they are 50 times more powerful than vitamin E and 20 times than vitamin

C [2]. Today various OPCs have been available but very expensive. This is mainly because over 90% PAs in natural plants are present in the polymeric form (PPCs), which are structurally-complex and non-nutritive [3-4]. This fact would be the main reason why the majority of published works in the literature were only those related to catechins and OPCs.

However, from enological point of view, both OPCs and PPCs are important phenolic compounds responsible for sensory properties of red wine, so during the last twenty years, one important topic of our research works has been the study on grape and red wine OPCs and PPCs, including their fractionation [5-6], isolation and purification [6-9], identification [7-13], quantification [14-15], condensation reactions [10-12], and biological activities [15-19]. Among these, one of the interesting works was the acidic degradation of grape and wine PPCs in the presence of nucleophiles, releasing flavan-3-ols (catechins) and flavan-3-ol-nucleophile derivatives [5, 20]. This is a unique method allowing to determine the structural composition of highly polymerized proanthocyanidins, which can not be achieved by commonly-used methods such as HPLC, ESI-MS and NMR.

On the other hand, high-speed countercurrent chromatography (HSCCC) has seen a renaissance in natural product preparation. Our recent works have shown that HSCCC is especially suitable for large and efficient separations of catechins, PAs and anthocyanins [7, 8-10, 20-22].

This communication will present our recent work about large preparation of a series of bioactive polyphenols and their derivatives from grape seeds and grape skins – the major components of grape pomaces, firstly by degradation of PPCs in the presence of nucleophiles, followed by preparative separation of the degraded products using HSCCC.

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2 Degradation methods for PPCs

Acidic degradation in the presence of the nucleophiles has been often used for structural analysis of plant polymeric proanthocyanidins. Numerous nucleophiles used include thiolactic acid, benzene-p-sulphinic acid, benzenethiol, benzyl mercaptan, 2-mercaptoethanol and phloroglucinol [23]. All these nucleophilic reagents allow degrade polymeric proanthocyanidins into their constitutive units or derivatives, but with different degradation efficiencies [23].

A French research group firstly characterized the structure of grape polymeric proanthocyanidins by thiolysed degradation using benzyl mercaptan as nucleophile followed by HPLC analysis [24-26]; Using the same degradation approach, red wine polymeric proanthocyanidins [5] and apple highly-polymerized procyanidins [27] were also structurally characterized.

Matthews et al. [23] compared the degradation methods for estimation of proanthocyanidins in the presence of the two most widely used nucleophiles, benzyl mercaptan and phloroglucinol. These authors recommended the use of benzyl mercaptan rather than phloroglucinol due to the higher yields of depolymerization. However, Kennedy and Jones [28] reported that the degradation method for proanthocyanidins under the proposed analytical conditions using phloroglucinol was more effective and favourable to that using benzyl mercaptan. In fact, phloroglucinol was preferred is also due to its odorless while benzyl mercaptan is very smelly and toxic and probably for this reason, phloroglucinol has been mostly used as nucleophile for analysis of structural composition of proanthocyanidins in various plant tissues. More recently, other non-toxic and bioactive nucleophiles, i.e., captopril, L-cysteine, and tiopronin were also tested for degradation of grape and wine PPCs in our laboratory.

3 HSCCC technology for separation of polyphenols

High-speed countercurrent chromatography (HSCCC) is based on continuous liquid–liquid partitioning, which enables one to eliminate irreversible adsorption on solid supports. As a relative new separation technology for natural plants, HSCCC is especially suited to large and efficient separations of polar compounds like polyphenols and their derivatives [7-9, 20-22, 29]. Compared to conventional liquid–solid separation methods, HSCCC offers several advantages, as it does not suffer from irreversible analyte adsorption and features better loading capacity, higher sample recovery, lower sample denaturation risk, minimised tailing, and decreased solvent consumption [29-31].

Several factors can affect the separation efficiency of HSCCC, including solvent system, elution mode, revolution speed and column temperature. Among these factors, the most important one is the suitable selection of a two-phase solvent system, which provides the ideal partition coefficient (*K*) for the target compounds. Therefore, the partition coefficient (*K*) of one compound is critical for its successful isolation and separation by HSCCC. Another important parameter is the separation factor (α), which indicates the peak resolution between the target compounds. Both partition coefficient (*K*) value and separation factor (α) were used as evaluation parameters for selection of the two-phase solvent system [32]. The partition coefficients (*K*) were determined by HPLC. The *K* values of target components were calculated according to the equation K = AU/AL, where AU and AL are the peak areas of target compounds in the upper phase and lower phase respectively and the separation factor (α) was defined as the ratio of partition coefficients between two compounds ($\alpha = K1/K2$, $K1 \geq K2$).

4 Preparation of bioactive polyphenols from grape seeds

Degradation methods for polymeric proanthocyanidins in the presence of nucleophiles have been used only for their structural analysis. In our laboratory, the degradation methods were, for the first time, employed for the purpose of producing bioactive polyphenols. Thus, by selection of suitable nucleophile, the abundant non-nutritive polymeric proanthocyanidins, from grape and wine or other plant tissues, were effectively transformed into low-molecular-weight bioactive molecules.

Grape seed, one of the richest sources in procyanidins, is the important component of grape pomaces from vinification. Zhang et al. [20] used phloroglucinol as nucleophile for acidic degradation of polymeric proanthocyanidins from a grape seed into monomer catechins and their nucleophilic derivatives. Each of the phloroglucinolysis products was successfully separated and isolated in large amount by semi-preparative HSCCC technique under the optimized conditions based on a selection of suitable solvent system. The optimized solvent system consisted of *n*-hexane–ethyl acetate–water (1:80:80, v/v/v) with a combination of head–tail and tail–head elution modes. By only one-step HSCCC separation, the purity of each obtained phloroglucinolysis product, including monomer catechins and their nucleophile derivatives was above 76%, verified by UPLC. Figure 1 presents the HSCCC chromatogram on the separation of phloroglucinolysis products using head to tail (H–T) and tail to head (T–H) elution modes.

The selection of phloroglucinol as nucleophile for degradation of waste grape seed polymeric procyanidins to produce low-molecular-weight bioactive compounds was because this reagent is a secondary metabolite that occur naturally in certain plant species, non-toxic and present antioxidant activity [20]. Under the optimized degradation and HSCCC conditions, high purity and yield of several phloroglucinolysis products can be achieved by only one-step HSCCC separation; all products obtained by phloroglucinolysis possessed potent antioxidant activities, being catechin-nucleophile derivatives higher than free catechins, and galloylated forms higher than non-galloylated ones [20].

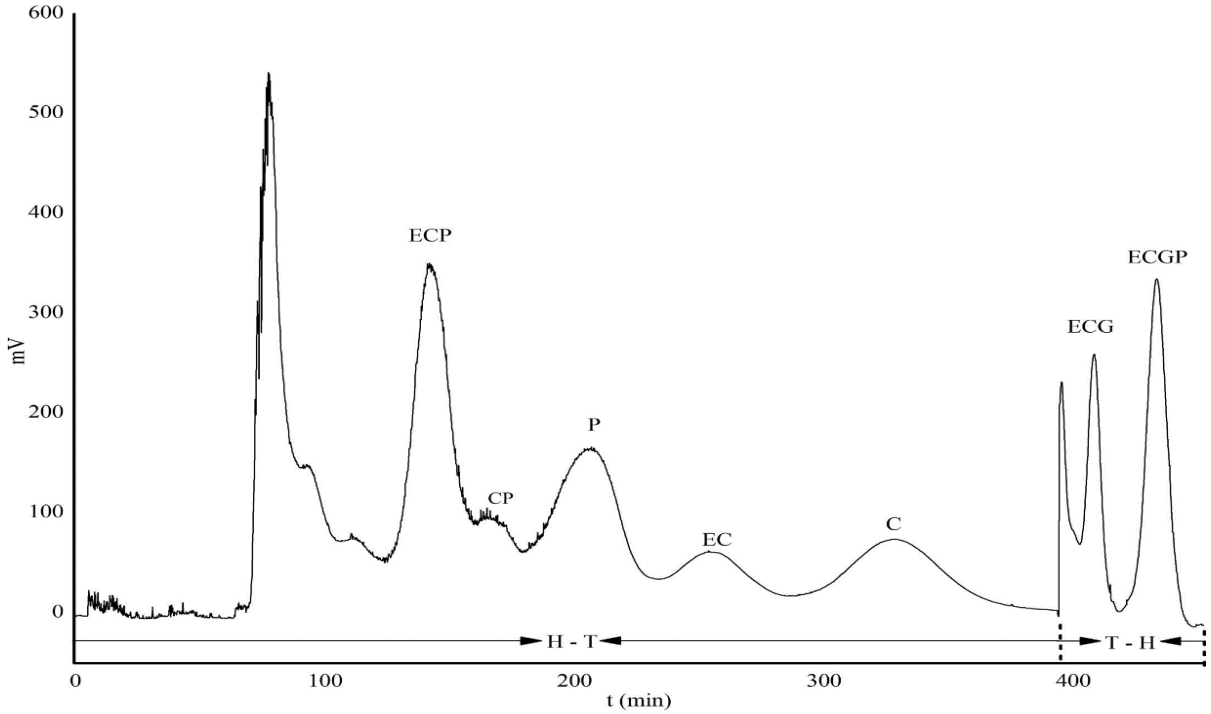

Figure 1. HSCCC chromatogram on the separation of phloroglucinolysis products using head to tail (H–T) and tail to head (T–H) elution modes. ECP, (–)-epicatechin-phloroglucinol derivative; CP, (+)-catechin-phloroglucinol derivative; P, phloroglucinol; EC, (–)-epicatechin; C, (+)-catechin; ECG, (–)-epicatechin-3-O-gallate; ECGP, (–)-epicatechin-3-O-galloyl-phloroglucinol (adopted from Zhang et al. [20]).

In addition to phloroglucinol, tiopronin was lately experimented, in our laboratory, as a novel thiol-containing nucleophile for depolymerizing grape seed polymeric proanthocyanidins, with the purpose of producing low-molecular-weight bioactive compounds [21]. Tiopronin is a well-known diffusible antioxidant, an antidote to heavy metal poisoning and a radioprotective agent and has powerful protective effects on carbon tetrachloride-induced hepatotoxicity [33]. By using this nucleophile, grape seed polymeric proanthocyanidins could be converted into catechins and flavan-3-ol−tiopronin derivatives, each of which could be further isolated by HSCCC, with or without the aid of semi-preparative HPLC. Direct HSCCC could successfully isolate epicatechin gallate, epicatechin-tiopronin conjugate and epicatechin gallate-tiopronin conjugate, with the yields of 6.5, 29.8, and 10.3 mg and purity of 90.3, 93.7, and 89.5%, by each run. Higher purity of these compounds could be obtained by further semipreparative HPLC. Furthermore, all these degradation products were verified to possess potent antimicrobial activity, with epicatechin gallate the strongest.

Very recently, other non-toxic and bioactive nucleophiles, i.e., captopril and L-cysteine were also tested for degradation of grape seed PPCs in our laboratory [22]. L-cysteine is a nature amino acid inhibiting insulin release from the pancreatic *β*-cell [34]; captopril is orally active antihypertensive agent [35-36]. By using these new bioactive nucleophiles in degradation of grape seed PPCs, followed by HSCCC and semi-preparative under the optimized separation conditions, a series of new antioxidants including free catechins and their conjugates were effectively separated, each of them with high purity and high yield, as shown in Tables 1–2 [7-8].

Table 1. Purity and yield of L-cysteine degradation products by HSCCC separation and semi-preparative HPLC (adopted from Tian et al. [22]).

Compounds	Purity (%)	Yield (mg)
C	93.2±2.5	13.1±1.3
EC	94.6±1.7	8.9±0.7
ECG	91.5±2.6	9.2±1.0
C-Cys	93.8±1.1	8.8±0.9
EC-Cys	94.5±1.8	33.1±1.8

C, (+)-Catechin; EC, (–)-epicatechin; ECG, (–)-epicatechin-3-*O*-gallate; C-Cys, (+)-Catechin-4*β*-cysteine; EC-Cys, (–)-epicatechin-4*β*-cysteine; ECG-Cys, (–)-epicatechin-3-*O*-gallate-4*β*-cysteine

Table 2. Purity and yield of Captopril degradation products by two-steps HSCCC separation (adopted from Tian et al. [22]).

Compounds	Purity (%)	Yield (mg)
C	95.4±2.6	15.41±1.5
EC	94.1±1.7	9.62±0.9
EC-C	94.7±1.6	30.80±2.5
ECG	92.8±2.1	8.90±0.8
ECG-C	93.2±1.9	14.33±1.3

C, (+)-Catechin; EC, (–)-epicatechin; ECG, (–)-epicatechin-3-*O*-gallate; ECC, (–)-epicatechin-4*β*-captopril methyl ester; ECGC, (–)-epicatechin-3-*O*-gallate-4*β*-captopril methyl ester.

Furthermore, all these degradation products presented high antioxidant activities of which the most potent are those galloylated due to presence of a gallate group, leading that the activities of hydroxies were more reactive than others [34].

In a word, grape seed procyanidins are presented essentially in the polymeric form, which are non-nutritive.

The proposed methods permitted transform efficiently such polymers into low-molecular-weight bioactive polyphenols and their derivatives.

5 Preparation of bioactive polyphenols from grape skins

Quantitatively, grape skins are the most important part of grape pomaces, particularly white grape pomaces from vinification. Polyphenols in grape skins are more complex and diverse than in grape seeds. Grape skins contain not only procyanidins (as grape seeds), but also other phenolic compounds such as flavonols, flavanonols, flavones, derivatives of cinnamic acids and tartaric acid [37]; for grape skins from red varieties, they contain also large amounts of anthocyanins [38]. The mean polymerization degree of proanthocyanidins in grape skins may reach 80 [25]. As compared with grape seeds, this value is between 25-30 [5, 24]. Because the disposal of grape skins may also cause environmental problems during wine-making process, the recovery of phenolic compounds from these waste materials is of undoubtedly economic and social significance.

Luo et al. [7] developed an efficient method for large preparation of various individual polyphenols from white grape skins, firstly using preparative HSCCC to separate grape skin polyphenols into various fractions (Fig. 2), followed by preparative HPLC to isolate each of phenolic compounds (Figs. 3 and 4).

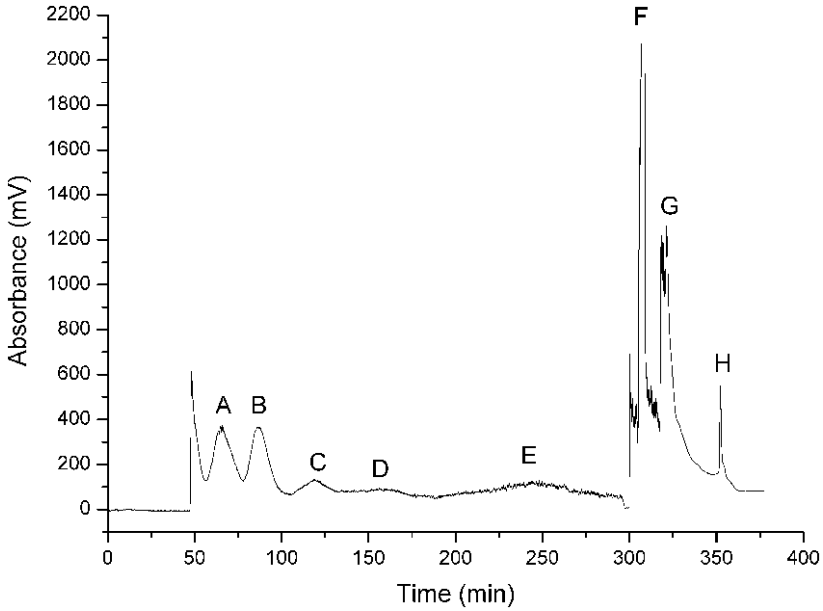

Figure 2. HSCCC chromatogram on the separation of grape skin extract using tail to head (0-300 min) and head to tail (300 min-end) elution modes (adopted from Luo et al. [7]).

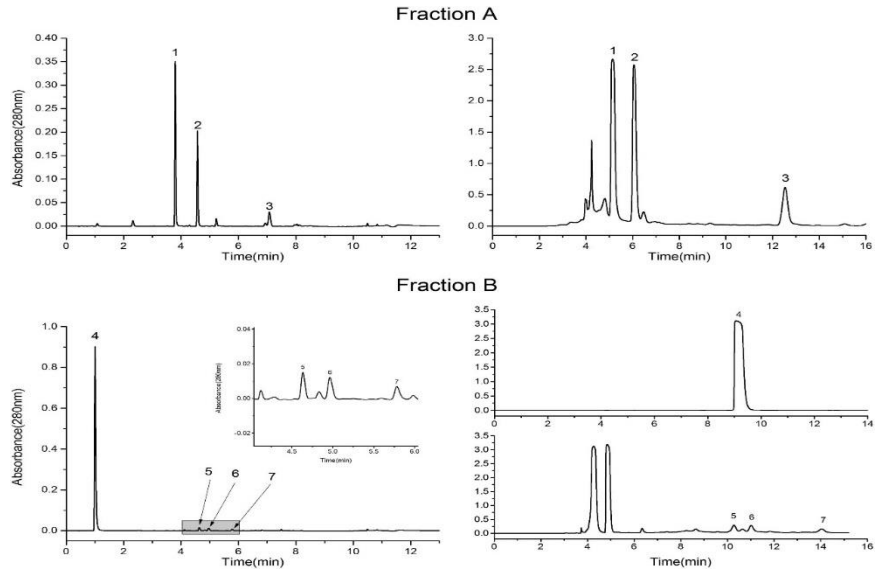

Figure 3. Fraction A UPLC analysis (Fraction A, left side) and prep-HPLC isolation (Fraction A, right side) of individual phenolic compounds isolated from HSCCC of grape skin extract; peak 1 = catechin, peak 2 = epicatechin, peak 3 = astilbin. Fraction B UPLC analysis (Fraction B, left side) and prep-HPLC isolation (Fraction B, right side) of individual phenolic compounds isolated from HSCCC of grape skin extract; peak 4 = gallic acid, peak 5 = procyanidin dimer B2-3-*O*-gallate, peak 6 = procyanidin dimer B1-3-*O*-gallate, peak 7 = procyanidin B2-3-*O*-gallate (adopted from Luo et al. [7]).

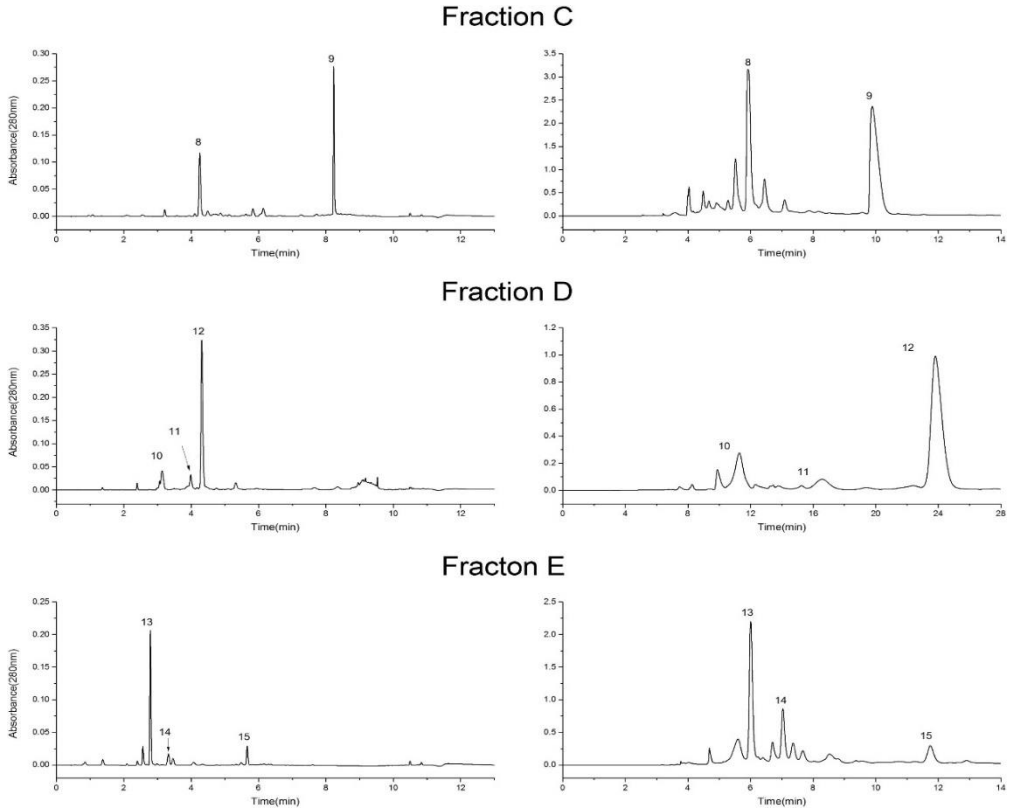

Figure 4. Fraction C UPLC analysis (Fraction C, left side) and prep-HPLC isolation (Fraction C, right side) of individual phenolic compounds isolated from HSCCC of grape skin extract; peak 8 = trans-coutaric acid, peak 9 = quercetin-3-glucuronide. Fraction D UPLC analysis (Fraction D, left side) and prep-HPLC isolation (Fraction D, right side) of individual phenolic compounds isolated from HSCCC of grape skin extract; peak 10 = procyanidin dimer B3, peak 11 = procyanidin dimer B4, peak 12 = procyanidin dimer B2. Fraction E UPLC analysis (Fraction E, left side) and prep-HPLC isolation (Fraction E, right side) of individual phenolic compounds isolated from HSCCC of grape skin extract; peak 13 = procyanidin dimer B1, peak 14 = procyanidin trimer T2, peak 15 = procyanidin trimer C1 (adopted from Luo et al. [7]).

As shown in Figure 2, grape skin polyphenolic extract could be successfully separated, under the optimized HSCCC conditions, into 8 fractions during less than 400 min of elution. For each run, 300 mg of sample loading yielded total of 260.6 mg of different phenolic fractions, with recovery over 86%.

From each of these fractions, a preparative-HPLC separation was applied to isolate individual polyphenols. As shown in Figures. 3 and 4, total of fifteen individual compounds were obtained, most of which presented high yields and purity (all over 90%).

It should be mentioned that the last three fractions from HSCCC (Fig. 2) were those composed of polymeric proanthocyanidins. From this point of view, it is suggested that these fractions can also be degraded using suitable nucleophiles, followed by re-chromatography on HSCCC and/or with preparative HPLC, for the purpose of producing low-molecular-weight bioactive polyphenols.

6 Conclusion

It is for the first time that, in our laboratory, the different non-toxic and bioactive nucleophiles were applied in degradation of grape seed polymeric procyanidins for the purpose of producing low-molecular-weight bioactive polyphenols. The proposed technology appeared feasible and cost-efficient which can also be applicable for grape skin polymeric proanthocyanidins. The new bioactive compounds obtained are expected to have the possibility for their use in functional food, pharmaceutical and/or cosmetic sectors.

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