Assessment of experimental factors affecting the sensitivity and selectivity of the spectrophotometric estimation of proanthocyanidins in foods and nutraceuticals

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Abstract

The assessment of a reliable method for the determination of active proanthocyanidins (PACs) in functional foods, nutraceuticals and foodstuffs is still a pending analytical challenge. In general, pharmaceutical and food companies accept the assay based on 4-dimethylaminocinnamaldehyde (DMAC) as an overall index of PACs, although these values provided confuse information of the actual bioactivity of the product. Besides, obtaining accurate and precise results is difficult because of the complexity of the sample matrices and the differential selectivity of analytes. Here, we have investigated in detail those experimental factors contributing to the PAC-DMAC derivatives and we have explored the possibilities for increasing the selectivity of the reaction to detect the fraction of PAC molecules with antibacterial properties. In this regard, the use of harsh conditions, especially working at high temperature, contributes to the formation of the desired derivatives and the degradation of the undesired ones. Unfortunately, the selective detection of A-type species based on DMAC derivatization was not realistic. Another goal of the study was the development of a spectrophotometric assay for the determination of the total content of flavanols. In this case, experimental conditions were chosen to reach high responses for all the compounds. Working under mild

conditions of temperature and reaction time (20^{o}C and 20 min), the method was suitable to estimate the overall flavanol content of food supplements, nutraceuticals, and commercial fruit juices.

Keywords: Proanthocyanidins; DMAC assay; selectivity enhancement; nutraceuticals; fruit juices.

Introduction

Proanthocyanidins (PACs) are secondary metabolites of plants (Granato et al. 2016) which consist of oligomers or polymers of flavanols such as catechin and epicatechin. Flavonoids, and PACs in particular, are abundant in fresh fruits, especially in berries such as cranberry, blueberry, raspberry, grape, etc. Owing to their polyphenolic nature, PACs provide several beneficial effects on health as anti-oxidative, anti-inflammatory, anti-mutagenic and anti-carcinogenic properties. They have multiple applications in pharmaceutical, medicinal and cosmetic fields (Côté et al. 2010; Panche et al. 2016). There are some structural variations according to the degree of polymerization, bond type and monomeric constituents that contribute to the diversity of PACs. The simplest and most common interflavan bond is the B-type which consists of the linkage between the C4 of the upper monomer and the C6 or C8 of the lower monomer. In contrast, A-type is less abundant due to the complexity of the additional bond, that is formed by the linkage between the C2 of the upper monomer with the hydroxyl group of C7 or C5 of the lower monomer (C2-O-C7 or C2-O-C5) (Fig. 1) (Wang et al. 2016; Lucci et al. 2017). The concomitance of B- and A-type linkages affords a conformational rigidity that makes the molecules more resistant to harsh conditions, such as heating and extreme pH values. Furthermore, the A-type PACs have biological properties useful for the prevention and treatment of UTIs (Howell et al. 2005, Prior et al. 2010, Krueger et al. 2013). Preclinical studies comparing placebo and cranberry-based extract preparations statistically demonstrated the suitability of these products to prevent UTIs (Occhipinti et al. 2016).

Nowadays, the pharmaceutical industry produces and commercializes a wide range of nutraceutical products made of cranberry for UTI prophylaxis or treatment. Accordingly, reliable analytical methods are needed to control the content of active ingredients in the pharmaceutical forms. Pioneering studies focused on the development of PAC indexes proposed various colorimetric methods based on the vanillin-acid and n-butanol hydrochloride and 4-dimethylaminocinnamaldehyde (DMAC) assays (Li et al. 1996; Wallace et al. 2010; Granato et al. 2016). Among them, DMAC seemed to be more recommendable from the point of view of drawbacks and occurrence of interferences from colored species of the sample matrix. Prior and coworkers validated an improved DMAC assay using procyanidin A2 standards to obtain more reliable results and Wallace et al. evaluated the influence of some experimental variables on the

reaction (Prior et al. 2010; Wallace et al. 2010). Hence, DMAC is currently used for the global determination of PACs in food products, dietary supplements, and pharmaceuticals, thanks to some advantages, such as simplicity, short analysis time, use of relatively low-cost reagents and inexpensive equipment (Muceniece et al. 2019).

Some recent studies have been proposed to try to improve the performance of DMAC assays for the determination of flavanols and PACs (Sintara et al. 2018). For instance, Payne et al. have developed a 96-well plate version for increasing the sample throughput and studied selectivity issues regarding other phenolic compounds (Payne et al. 2010). In another study, Feliciano and coworkers compared the use of different standard compounds and extract mixtures for a more accurate estimation of PAC contents (Feliciano et al. 2012). The influence of structural variations, as well as the degree of polymerization in the spectra and sensitivity of DMAC derivatives, was thoroughly investigated by Wang et al. (2016). Unfortunately, DMAC provides a rough estimation of the total flavanols, but specific A-type PAC data cannot be obtained by single spectrophotometric analysis (Wallace et al. 2010; Granato et al. 2016; Van Dooren et al. 2018; Ma et al. 2019). As a result, separation techniques such as liquid chromatography may be required to discriminate among A- and B-type compounds (Hummer et al. 2014; Lu et al. 2017; Van Dooren et al. 2018; Bakhytkyzy et al. 2018; Bakhytkyzy et al. 2019). Besides, DMAC has also been used as a staining reagent to develop thin-layer chromatograms of flavanol mixtures (Glavnik et al. 2009; Glavnik et al. 2011). In this case, quantitative determination of components has been accomplished by densitometry at 655 nm. Further research based on high-resolution thin layer chromatography (HRTLC) allowed a better separation and detection of a wide range of oligomers, from monomers up to decamers (Glavnik et al. 2017; Glavnik et al. 2019).

The main aim of this research is to re-evaluate exhaustively the derivatization of flavanols with DMAC, identifying those variables that affect the reaction and characterizing the kinetic behavior under two different perspectives: (i) Improving the overall sensitivity of the reaction to try to establish a general index expressing the total flavanol content; (ii) Improving the selectivity of the reaction towards A type species to achieve a more reliable estimation of active compounds for UTI prophylaxis or treatment. Experimental design approaches have been used for a better understanding of relevant factors influencing on the reactions and their potential interactions. Studies have been focused on several model compounds such catechin, epicatechin and

procyanidins, with a special emphasis on procyanidin A2. Results have shown that although a quite selectivity A2 derivatization can be achieved, the need of a long reaction time hinders the development of a proper analytical method. On the other hand, general conditions for the detection of the total content of flavanols have been successfully stablished leading to new spectrophotometric method. The revisited DMAC assay has been applied to determine the content of PACs in nutraceutical and juice samples.

Experimental section

Reagents and solutions

Flavanol standards were as follows: procyanidin A2 (≥99%, Phytolab, Vestenbergsgreuth, Germany), procyanidin B2 (≥98%, Chengdu Biopurify Phytochemicals LTD, China), procyanidin C1 (≥99%, Phytolab, Vestenbergsgreuth, Germany), catechin (≥98%, Sigma-Aldrich, St Louis, USA), epicatechin (≥98%, Sigma-Aldrich, St Louis, USA), epigallocatechin (≥98%, Carbosynth, Berkshire, UK), epigallocatechin gallate (≥98%, Carbosynth, Berkshire, UK). Other model polyphenols considered for selectivity assays were gallic acid, vanillic acid, caffeic acid, ferulic acid, quercetin, hesperidin, resveratrol, luteolin, and oenin chloride, all of them from Sigma-Aldrich, St Louis, USA.

4-Dimethylaminocinnamaldehyde (>98% w/w, Tokyo Chemical Industry, Tokyo, Japan) was used as the derivatization reagent. Other reagents and solvents used for sample treatment and derivatization were hydrochloric acid (37% w/w, Panreac Chemistry SA, Castellar del Vallès, Barcelona, Spain), formic acid (≥95% w/w, Sigma-Aldrich, St Louis, USA), methanol UHPLC (99.9%, Supergradient Panreac AppliChem, Darmstadt, Germany) and Milli-Q Water, purified using an Elix 3 coupled to a Milli-Q system (Bedford, USA) and filtered through a 0.22 µm Nylon filter integrated into the Milli-Q System.

Stock standard solutions of each compound were prepared in methanol at a concentration of 1000 mg L^{-1}. Working solutions were obtained by further dilution with H_2O/MeOH (75:25 *v:v*). The reagent solution (1.75% *w:v* DMAC with 5 mol L^{-1} HCl) was prepared daily, and was kept in an amber vial to minimize its degradation.

Instruments and apparatus

Spectroscopic measurements were carried out with a Lambda19 double beam UV–VIS–NIR spectrophotometer (Perkin Elmer, Waltham, MA, USA) using QS quartz glass high-performance macro cells of 10 mm optical path length (Hellma, Müllheim, Germany). Spectra were recorded in the wavelength range 400 to 750 nm (rate scan 420 nm min^{-1}) and the absorbance at 640 nm was taken as the analytical response.

Complementary chromatographic assays were carried out with an Agilent Series 1100 HPLC chromatograph (Agilent, Technologies, Palo Alto, CA, USA) equipped with a binary pump (G1312A), an autosampler (G1379A), a degasser system (G1379A) and diode array (DAD, G1315B) detector. The analytical column consisted of a Kinetex C18 (100 mm length × 4.6 mm I.D, 2.6 µm particle size) with a pre-column Security Guard C18, (4.00 mm length x 3.00 mm I.D), both from Phenomenex, Torrance, California, USA.

Auxiliary equipment for extraction and derivatization procedures consisted of a IKA RCT basic heater (IKA-Werke, Staufen, Germany), a LyoQuest lyophilizer (Telstar, Terrasa, Spain), a Genius 3 Vortex mixer (IKA, Staufen, Germany), a PB1502-L analytical balance (Mettler-Toledo, Columbus, OH, USA), a Branson 5510 ultrasonic bath (Branson Ultrasonic corporation, Danbury, CT, USA) and a Rotanta 460 RS centrifuge (Hettich, Tuttlingen, Germany).

Samples

Food supplements presented as gelatin capsules or pills to be analyzed were as follows: 5 samples corresponded to cranberry extracts mainly prescribed for the treatment of cystitis; 2 combinations of cranberry with other plant ingredients (nettle and vine); 3 raspberry-based extracts; 5 artichoke extracts; and 2 antioxidant preparations mainly based on vine and pomegranate. Commercial juices from different fruit origins were as follows: 3 cranberry, 2 blueberry, 1 pomegranate, 1 pineapple, 2 orange; 3 apple, 2 strawberry, 3 grape, and 9 varied multi-fruit blends.

Sample treatment

Food supplements and nutraceutical samples were first crushed and homogenized in a glass mortar. Then, ca. 0.1 g of powder were weighed on an analytical balance and extracted with 5 mL of MeOH/H_2O/HCl (70:29:1 *v:v:v*) in

conical centrifuge tubes. The recovery of analytes was assisted by sonication for 30 min. Subsequently, the extracts were centrifuged for 15 min at 3500 rpm. Finally, the supernatant solutions were filtered through nylon membranes of 0.45 µm particle size (20 mm diameter, Macherey-Nagel, Düren, Germany). The extractions were carried out in triplicate.

Juice samples were just centrifuged for 15 min at 3500 rpm and filtered through nylon membranes. The resulting solutions were prepared in triplicate.

Spectrophotometric assay

This method is based on the reaction of flavanols with DMAC. In strongly acid conditions, DMAC leads to the formation of an electrophilic carbocation which reacts with the C8 of the terminal A-ring (see scheme in Fig. 1). The reaction product has an intense green coloration, with an absorption maximum at 640 nm. Therefore, any possible interference with other absorbing compounds such as anthocyanidins is avoided (Wallace et al. 2010; Feliciano et al. 2012).

100 µL of DMAC reagent solution (1.75% *w/v* DMAC and 5 mol L^{-1} HCl) were mixed with appropriate volumes of sample (ranging from 20 to 1000 µL, depending on the flavanol concentration) or standards, and diluted up to a final volume of 2.5 mL with H_2O/MeOH (75:25 *v:v*). The reaction was developed for 1 h at room temperature and the absorbance at 640 nm was then recorded with the double beam spectrophotometer using the blank of the reaction as a reference (100 µL of DMAC reagent solution up to a final volume of 2.5 mL with 75:25 (*v:v*) H_2O/MeOH). In the case of the nutraceutical samples, the sample blank, recorded in front of the solvent as a reference, was subtracted from the gross absorbance of the reaction.

Kinetic evaluation

In order to go in depth in the kinetic behavior of the derivatization process, the composition of reaction mixtures containing DMAC reagent and standards of flavanols at 50 mg L^{-1} were analyzed over time. 250 µL of the reacting mixtures were withdrawn at preselected times to be analyzed chromatographically. Assays at room temperature were developed for a period of 24 h and reacting mixtures were withdrawn in steps of 20 min. Assays at 90ºC room temperature were developed for 6 h and reacting mixtures were withdrawn in steps of 30 min.

The concentrations of reagent, derivatives, and degradation products were monitored over time using a chromatographic method with UV-vis detection. Compounds occurring in the mixture were separated by reversed-phase mode using 0.1% *v/v* HCOOH and MeOH as the components of the mobile phase. The elution gradient was as follows: 15% to 95% MeOH, from 0 to 15 min (linear increase); 95% MeOH from 15 to 20 min. Derivatives were recorded at 640 nm.

Method selectivity

The selectivity of the reaction of flavanols in front of other flavonoids and phenolic acids was assessed at 10 mg L^{-1} each compound. Other experimental conditions were 0.16% *w/v* DMAC, 0.2 mol L^{-1} HCl, 60 min reaction time and 20ºC. For each compound, the absorbance of the reaction with respect to the DMAC blank was used as the response.

Design of Experiments (DoE) for the evaluation of reaction conditions

The influence of DMAC, HCl concentration and reaction time on the reaction of derivatization of model flavanols was evaluated by a 3-factor at 2-level design. Levels selected were 0.08 and 0.16% (w/v) DMAC, 0.12 and 0.20 mol L^{-1} HCl and 15 and 45 min reaction time. The working temperature was 20ºC. Compounds assayed were catechin, epicatechin, procyanidin A_2 and procyanidin B_2 as the preliminary studies indicated that they were representative model cases of the behavior of the rest of flavanols. Working concentrations were 100 mg L^{-1} catechin, 50 mg L^{-1} epicatechin, 400 mg L^{-1} procyanidin A2 and 20 mg L^{-1} procyanidin B2.

The main effect of each factor and the interactions among the variables were accounted from the absorbance at 640 nm as the response for the set of runs of the experimental plan. Hence, each effect (or interaction) was calculated as the average of the outcomes at the high levels minus the average of the outcomes at the low levels. Results were presented in bar graphs plotting the magnitude of each effect and interaction. Their significance was evaluated statistically from the variance of replicate experiments at the center of the design based on t of Students' and ANOVA tests. Excel spreadsheet from Microsoft was used for the calculation.

Results and Discussion

Study of the reaction conditions

Recommended conditions reported in the literature to carry out the DMAC assay consisted of mixing 210 µL of reagent solution (0.1% *w:v* DMAC in 4.5% *v:v* HCl acidified ethanol solution) and 70 µL of sample/standard with 25 min reaction time at 25^{o}C (Prior 2010). Other researchers applied different conditions regarding DMAC concentration, acidity, reaction time, etc. (Prior et al. 2010; Payne et al. 2010; Feliciano et al. 2012; Granato et al. 2016; Wang et al. 2016). All these studies revealed some selectivity and sensitivity drawbacks so a wrong estimation of A-type species could be produced because DMAC was a general reagent for flavanol compounds. On the other hand, using catechin as a model, it was found that the absorbance depended on the water percentage in the reaction medium (Wallace et al. 2010); the decay in the signal with increasing the water content was noticeable in the range 0 to 3% *v/v* water, especially at high catechin concentrations. In our case, preliminary studies recommended a percentage of water (ca. 25% *v/v*) for a better recovery of the analytes so we assume that a certain bleaching effect will occur since we cannot avoid water in the reacting mixture.

The selectivity of the reaction was assessed spectrophotometrically by comparing the absorbances resulting from the reaction of DMAC with some flavanols with respect to those from other flavonoids and phenolic acids. Model polyphenols were selected as representative examples of the most relevant families, including cinnamic acids (caffeic and ferulic acids), benzoic acids (gallic and vanillic acids), anthocyanins (oenin chloride, flavones (luteolin), flavanones (hesperidin), flavonols (quercetin), and stilbenes (resveratrol). Results in Fig. 2, showing the absorbance at 640 nm under the spectrophotometric assay at a concentration of 10 mg L^{-1} each compound, demonstrated that the reaction with DMAC is essentially specific to flavanol analytes while other families of polyphenolic species attained negligible responses. Besides, it was observed that the sensitivity depended on the analyte, being quite high for procyanidins B_2 and C_1, moderate for the monomeric species and much lower for procyanidin A_2. Then, the overall response of PACs does not depend exclusively on their concentrations but also on the type of compounds.

As a result, despite being the DMAC assay commonly used by the pharmaceutical industry to check the amount of PACs, these results show a low sensitivity for some polyphenolic compounds, in particular procyanidin A2, that is the biologically active PAC against UTIs (de Llano et al, 2015). Hence, the re-optimization of the method intended to focused on two main issues, namely: (i) improving the selectivity of A-type species reaction in front of other flavanols for a better assessment of active compounds thus avoiding any overestimation of the therapeutic effects; (ii) enhancing the sensitivity of the general detection of flavanols for establishing a proper overall PAC index.

The influence of DMAC, HCl concentration and reaction time on the reaction of derivatization of model flavanols (catechin, epicatechin, procyanidin A_2 and procyanidin B_2) was evaluated by factorial design as explained in the experimental section. Bar plots (see Fig. 3) representing the intensity of main factors and interactions were accounted from the absorbance values of the experimental runs. It was found that, in general, time, acidity and DMAC concentration exhibited noticeable effects with positive signs, thus contributing to the formation of derivatives when increasing these factors. The influence of DMAC concentration on the reaction of procyanidin A2 was the only exception. The most remarkable interaction was between HCl-DMAC that provided large values for catechin and epicatechin while it was rather negligible when the dimers were analyzed. Because of the common DMAC and HCl interaction, these variables were assessed simultaneously while the reaction time showed a quite independent behavior. Anyway, just to summarize, it was concluded that the relevance of each factor on the reaction was highly dependent on the nature of the analyte.

The significance of each main factor and interaction was assessed statistically from a t-Student test with $t_{cal} = |\text{Effect}|/s_{error}$, being Effect the magnitude of the effect (or the interaction) and s_{error} the standard deviation of replicates (n =3). As a result, the DMAC and HCl concentration were significant in all the cases with p values > 0.05; the reaction time was relevant for catechin (p =0.0043) and epicatechin (p =0.013). Regarding interactions, DMAC-HCl was significant for catechin (p =0.01) and epicatechin (p =0.023), and time-DMAC for B2 (p =0.025). In the other cases, main factors and interaction were not significant (α = 0.05). These results confirmed the complexity of the nature of the DMAC reaction with different the compounds so that

additional experiments were carried out as follows to better understand each analyte behavior.

Kinetic studies of flavanol-DMAC reactions

Preliminary kinetics of the reactions of flavanols with DMAC were carried out at 20ºC under the experimental methodology given in the section of the kinetic evaluation. Studies published by Sintara and Wallace indicated that the reaction achieved a steady state in 10 min, approx. (Sintara et al. 2018; Wallace and Giusti, 2010). However, studies carried out here under the other experimental conditions showed slower processes (Fig. 4a). The fastest reactions were obtained with procyanidin C_1 and B_2, while the slowest process corresponded to procyanidin A_2. This finding was attributed to the fact that B-type molecules could rotate freely around the B-bonds between monomeric units while A-type molecules were more restricted, meaning more steric impediment and less reactivity than the other compounds. Catechin and epicatechin displayed an intermediate behavior. These results were the basis of further studies focused on general (i.e., maximizing the overall derivatization of flavanols) or selective conditions (i.e., achieving a more specific reaction for A_2).

On one hand, regarding an enhancement of the sensitivity as a type general PAC index, a reaction time of 1 h was selected for the determination of the overall quantity of flavanols present in the samples. At that time, most of the compounds reached the highest derivatization yields while the degradation process was still limited. On the other hand, it was observed that the absorbance of A_2 derivative was approximately maintained for a long time and then decreased slowly. This finding could be exploited to increase the selectivity to differentiate A_2 from other standards. For example, after 16 h of reaction, the signal of most of the derivatives was almost negligible while that of A_2 was quite high.

Kinetics were also developed at 90ºC to speed up the processes as well as to infer in the modulation of the selectivity towards A_2 molecules. As expected, in comparison with assays at 20ºC, the maximum of signal was obtained at lower reaction times (Fig. 4b). The compared behavior of procyanidin A_2, B_2, and C_1 at 90ºC was analogous to the previous case, meaning that the reaction of A_2 was slower because of the A-type bond restrictions.

The decay in the kinetic curves of the analytes was attributed to the instability of the oligomer derivatives under the harsh reaction conditions, leading to the breakdown of links between the monomeric moieties. Differences of stability among B- and A-type oligomers were related to the occurrence of single or double interflavan links, respectively. As a result, the decrease in the concentration of B_2 and C_1 derivatives (with one C-C bond between the flavanol units) was faster than that of A_2 (with one C-C bond and one C-O-C bridge between the flavanol units, see Fig. 1). Exceptionally, the epigallocatechin gallate derivative was higly resistant due to the protective effect of gallate moiety that slowed down the breaking of the DMAC-flavanol link. The combination of high temperature and time provided quite selective derivatization conditions for procyanidin A2. This finding was due to the fact that the degradation of other derivatives was almost completed so that they contribution to the gross response measured was negligible (i.e., the resulting absorbance was mainly attributed to A-type species). Unfortunately, these conditions are unrealistic as such long reaction times would make the method unfeasible for practical purposes. Since the selective detection of procyanidin A2 based on DMAC derivatization was not possible, the research was refocused on the development of an application for the determination of the total content of flavanols.

The comparison of results at 20ºC and 90ºC indicated that higher absorbances were obtained at 20ºC so this temperature was chosen to establish an overall PAC index under more stable and reproducible results. The influence of reagent concentration on the reaction of catechin was studied in the range 0.07 to 0.45% *w:v* DMAC. The concentration of HCl also varied from 0.2 to 0.6 mol L^{-1}. Fig. 5 shows the influence of these factors on the kinetics, suggesting that a reagent composition of 0.07% DMAC and 0.2 mol L^{-1} HCl was highly suitable for the development of the reaction. As can be seen, the reaction of catechin reached a steady state in ca. 15 min, thus being highly recommendable for the implementation of an analytical method for total flavanol determination. Although more concentrated reagents provided higher absorbances, responses decayed quickly thus leading to more unstable readouts and, probably, more variability in the results. Hence, conditions recommended corresponded to 20 min reaction time, 0.07% *w/v* DMAC, 0.2 mol L^{-1} HCl in the reaction medium. Calibration curves of three representative species (catechin, epicatechin and procyanidin A_2) are given as an example in Fig. 6. It should be noted that, again, the sensitivity for the

different analytes was different, meaning that the total index of PACs in complex sample mixtures depended on both type and concentration of species.

Figures of merit

The spectroscopic index of the overall flavanol content was established at 640 nm using catechin as the standard. As indicated above, the reaction was developed at 20ºC for 20 min and the absorbance was recorded using the blank of reagents as a reference.

The linearity was studied in the range of concentrations from 1 to 400 mg L^{-1} catechin using 12 standard solutions. Each concentration was assayed in triplicate (n = 3). The absorbance was linear in the range 1 to 80 mg L^{-1} catechin, with a sensitivity of 12.6 L mg^{-1} and determination coefficient r2 of 0.996. The quantification of the PAC index in the nutraceutical and juice samples (see below) was carried out by proper sample dilution to work within the desired concentration range. The limit of detection (LoD) and quantification (LoQ) were estimated statistically at a signal-to-noise ratio of 3 and 10, respectively, from 10 independent replicates at 1 mg L^{-1} catechin. Using the sensitivity at this concentration level, LoD was 0.3 mg L^{-1} and LoQ 1.0 mg L^{-1}. The repeatability of the methods was first established from 10 solutions of derivatized catechin (n = 10) at a concentration of 5 mg L^{-1}. The relative standard deviation (RSD) of A_{640} was 2.1%. A similar assay using a cranberry extract provided an RSD of 2.6%, thus showing a slightly higher value because of the matrix complexity.

Determination of PACs in nutraceutical products and fruit juices

DMAC assay was used to quantify the global content of flavanols in the 17 food supplement, antioxidant or detox products; results were expressed as catechin equivalents (mg $_{catechin}$ g^{-1} $_{sample}$). Determinations were carried out in triplicate (see Fig. 7). The samples made of cranberry (sample 5), raspberry (sample 7 and 8) and the mixtures of pomegranate with vine (sample 16) and cranberry with vine (sample 15) showed a higher index of PACs than the other samples. In some cases, the concentration of the analytes was lower than the LoD, such as for artichoke extracts (sample 9-13).

PAC indexes were also estimated in 26 juice samples and results were expressed in mg L^{-1} using catechin as the reference. Determinations were carried out in triplicate. As can be seen in Table 1, red grape juice and a mixture of different fruits (samples 10 and 19) showed higher PAC indexes. The samples made of pomegranate, strawberry, cranberry, blueberry had similar concentrations, while PAC content in orange, apple, and pineapple juices was irrelevant (1, 2, 3, 12, 13 and 14), with values below the LoD.

Conclusions

The method developed here aimed at the determination of flavanols in nutraceutical and juice samples, represented as PACs index, giving relevance to the content of procyanidin A2 as the representative species with preventive antibacterial activity. The DMAC reaction was found to be selective to flavanols in relation to the other polyphenolic families studied. Anyway, the flavanols have different sensitivity as a function of their nature, with B-type oligomers displaying higher responses and A-type species providing the lowest sensitivity. Assays at low temperature and/or low reaction time led to an overestimation of procyanidin A2 because other oligomeric and monomeric species strongly contributed to the overall response. Instead, stronger conditions were more recommendable for a more selective determination focused on A-type species. This finding was attributed to the higher physicochemical resistance of procyanidin A2 derivative compared with the other compounds. Anyway, experimental conditions for a more selective detection of A-type species were, in practice, not feasible as required long reaction times. The complementary research focused on the determination of the total content of flavanols allowed a new method to be developed using mild temperature and time conditions. The updated method was successfully applied to determine PAC indexes in food supplements, nutraceuticals, and commercial fruit juices. It was found that samples made of different berries (cranberry, blueberry, strawberry, and raspberry), pomegranate and vine were rich in these components while preparations from orange, apple, pineapple, and artichoke contained quite poor quantities of flavanols. Further studies will be focused on achieving a better compromise in the derivatization conditions of a wide range of species as well as on modulating cross selectivities among them.

Compliance with Ethical Standards

Conflict of Interest Oscar Vidal-Casanella declares that he has no conflict of interest. Oscar Nuñez declares that he has no conflict of interest. Santiago Hernández-Cassou declares that he has no conflict of interest. Javier Saurina declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with humans and animals performed by any of the authors.

Informed Consent Not applicable

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Figure captions

Figure 1. Derivatization reaction mechanism of procyanidin A2 with DMAC.

Figure 2: Study of selectivity of the DMAC assay for different polyphenols using the spectrophotometric method. Absorbance values recorded at 640 nm using the QS quartz glass cells of 10 mm optical path length for 10 mg L^{-1} standard solutions of each polyphenol. Conditions: DMAC, 0.16% *w/v*; HCl 0.2 mol L^{-1}; reaction time, 60 min; temperature, 20ºC.

Figure 3. Evaluation of effects and interactions for the derivatization of flavanols with DMAC by a 3-factor at 2-level experimental design. Compound assignation: a) catechin, b) epicatechin, c) procyanidin A2, d) procyanidin B2. Dotted lines correspond to the standard deviation estimated from the replicated central point. Effect/Interaction: T, time; A, acidity; R, DMAC concentration; TA, time-acidity interaction; TR, time–DMAC interaction; AR, acidity–DMAC interaction; TAR, time–acidity–DMAC interaction. Levels selected: DMAC (% w/v), 0.08 (–) and 0.16 (+); HCl (mol L^{-1}) 0.12 (–) and 0.20 (+); reaction time (min) 15 (–) and 45 min (+). Flavanol concentrations: 100 mg L^{-1} catechin, 50 mg L^{-1} epicatechin, 400 mg L^{-1} procyanidin A2 and 20 mg L^{-1} procyanidin B2.

Figure 4. Kinetic evaluation of the derivatization of flavanols with DMAC recorded at 640 nm: (a) assay at 20ºC and (b) assay at 90ºC using the chromatographic method. Compound assignment: 1, procyanidin C1; 2, procyanidin B2; 3, epicatechin; 4, catechin; 5, procyanidin A2; 6, epigallocatechin gallate. Flavanol concentrations: 50 mg L^{-1} each.

Figure 5. Influence of the DMAC and HCl concentration on the kinetics of the catechin reaction. Conditions: temperature, 20ºC; Catechin concentration 20 mg L^{-1}. Assignment: 1, 0.07% *w/v* DMAC HCl 0.2 mol L^{-1}; 2, 0.32% *w/v* DMAC HCl 0.4 mol L^{-1}; 3, 0.48% *w/v* DMAC HCl 0.6 mol L^{-1}.

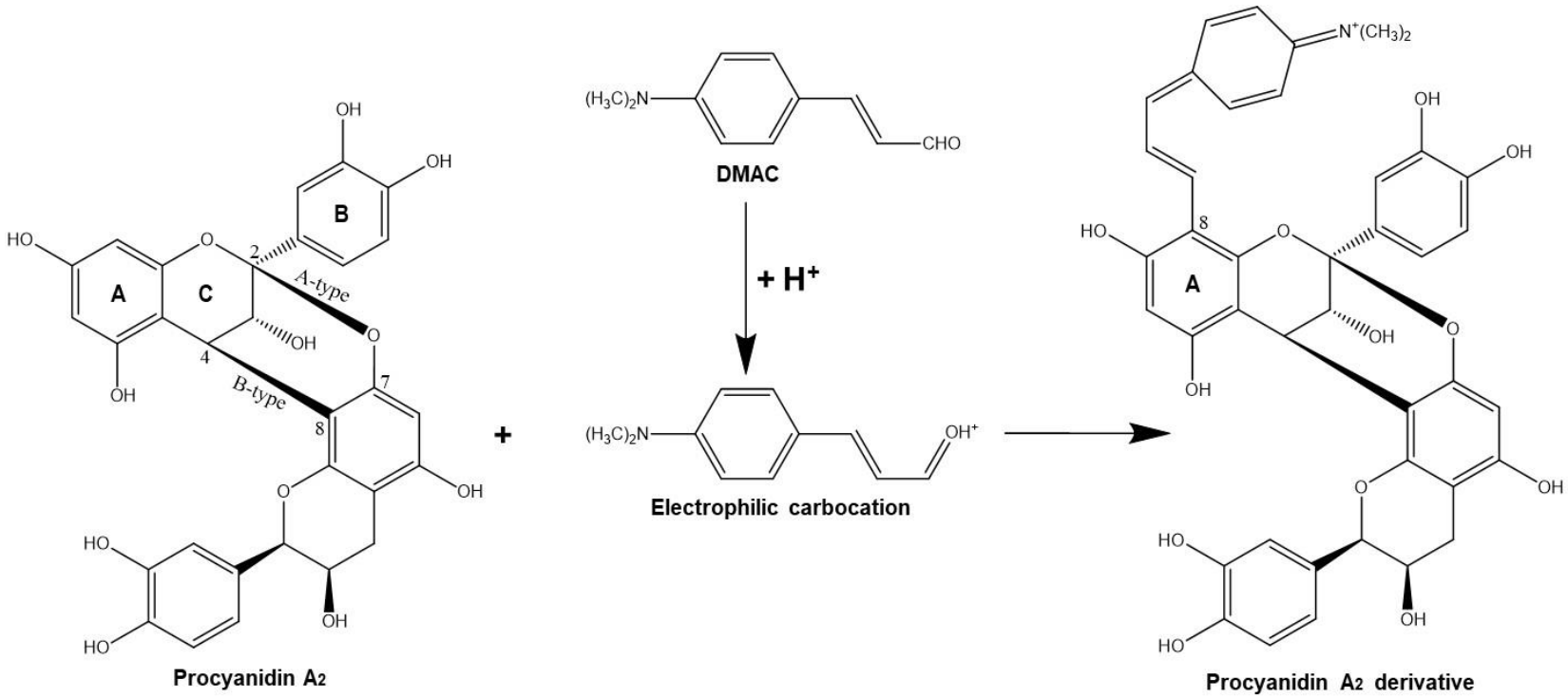
Figure 6. Calibration curves under the selected working conditions for epicatechin (1), catechin (2) and procyanidin A2 (3).

Figure 7. Determination of PAC index as catechin equivalents in nutraceutical and juice samples. Error bars indicate the standard deviation from 3 independent replicates.

Table 1. Determination of PAC index as catechin equivalents in juice samples.

Sample	Composition	Catechin equivalents (mg L^{-1})	Standard deviation (mg L^{-1})
1	Apple	*nd*	-
2		*nd*	-
3		*nd*	-
4	Blueberry	6	1
5		8	2
6	Cranberry	6	1
7		6.5	0.9
8		14	4
9	Grape	12	2
10	Grape red juice	59	10
11	Grape white juice	3.4	0.7
12	Orange	*nd*	-
13		*nd*	-
14	Pineapple	*nd*	-
15	Pomegranate	11	4
16	Strawberry	16	1
17		7	2
18	Apple, grape, blackcurrant, cherry, lemon, raspberry	1.4	0.4
19	Berries, grape, Raspberry	68	4
20	Cranberry, blackcurrant	15	3
21	Cranberry, pomegranate	3.7	0.4
22	Grape, apple, raspberry	11	1
23	Grape, apple, raspberry, strawberry, cranberry, gooseberry	6	1
24	Grape, cherry, pomegranate	11	1
25	Orange, raspberry, carrot, grape, blackcurrant	1.3	0.2
26	Red grape, cherry, blackcurrant, raspberry, strawberry	2.3	0.5

Fig. 1


DMAC
+ H+
Electrophilic carbocation
Procyanidin A2
Procyanidin A2 derivative
A-type
B-type

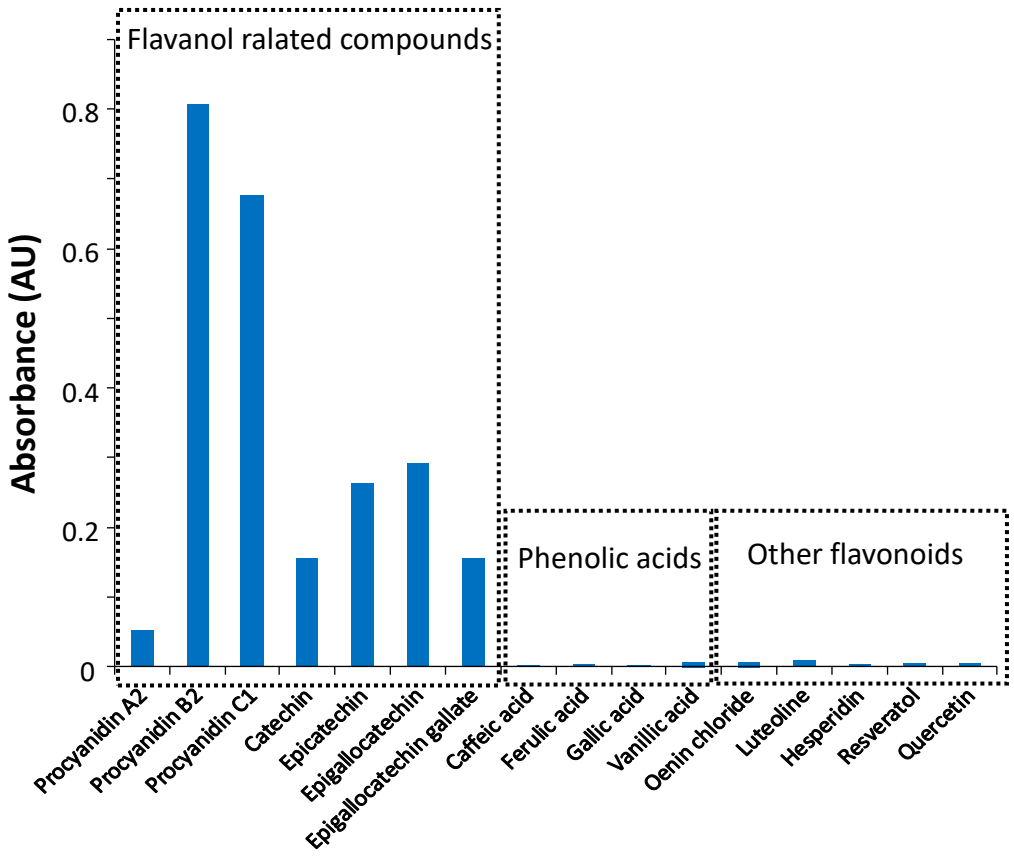

Flavanol ralated compounds
Phenolic acids
Other flavonoids
Absorbance (AU)
0
0.2
0.4
0.6
0.8
Procyanidin A2
Procyanidin B2
Procyanidin C1
Catechin
Epicatechin
Epigallocatechin
Epigallocatechin gallate
Caffeic acid
Ferulic acid
Gallic acid
Vanillic acid
Oenin chloride
Luteoline
Hesperidin
Resveratol
Quercetin

Fig. 3

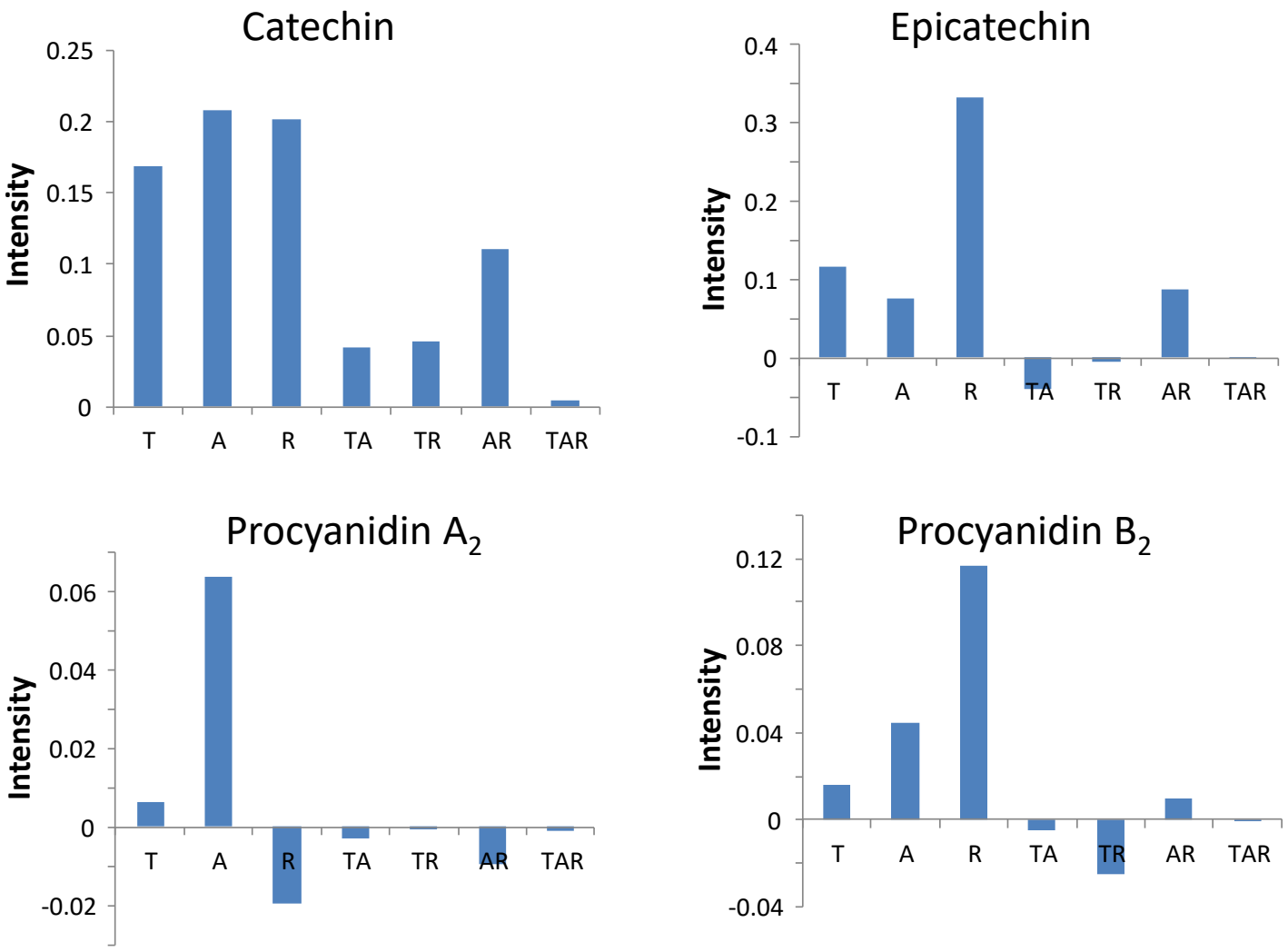

Catechin
Intensity
0.25
0.2
0.15
0.1
0.05
0
T
A
R
TA
TR
AR
TAR
Epicatechin
Intensity
0.4
0.3
0.2
0.1
0
-0.1
T
A
R
TA
TR
AR
TAR
Procyanidin A2
Intensity
0.06
0.04
0.02
0
-0.02
T
A
R
TA
TR
AR
TAR
Procyanidin B2
Intensity
0.12
0.08
0.04
0
-0.04
T
A
R
TA
TR
AR
TAR

Fig. 4

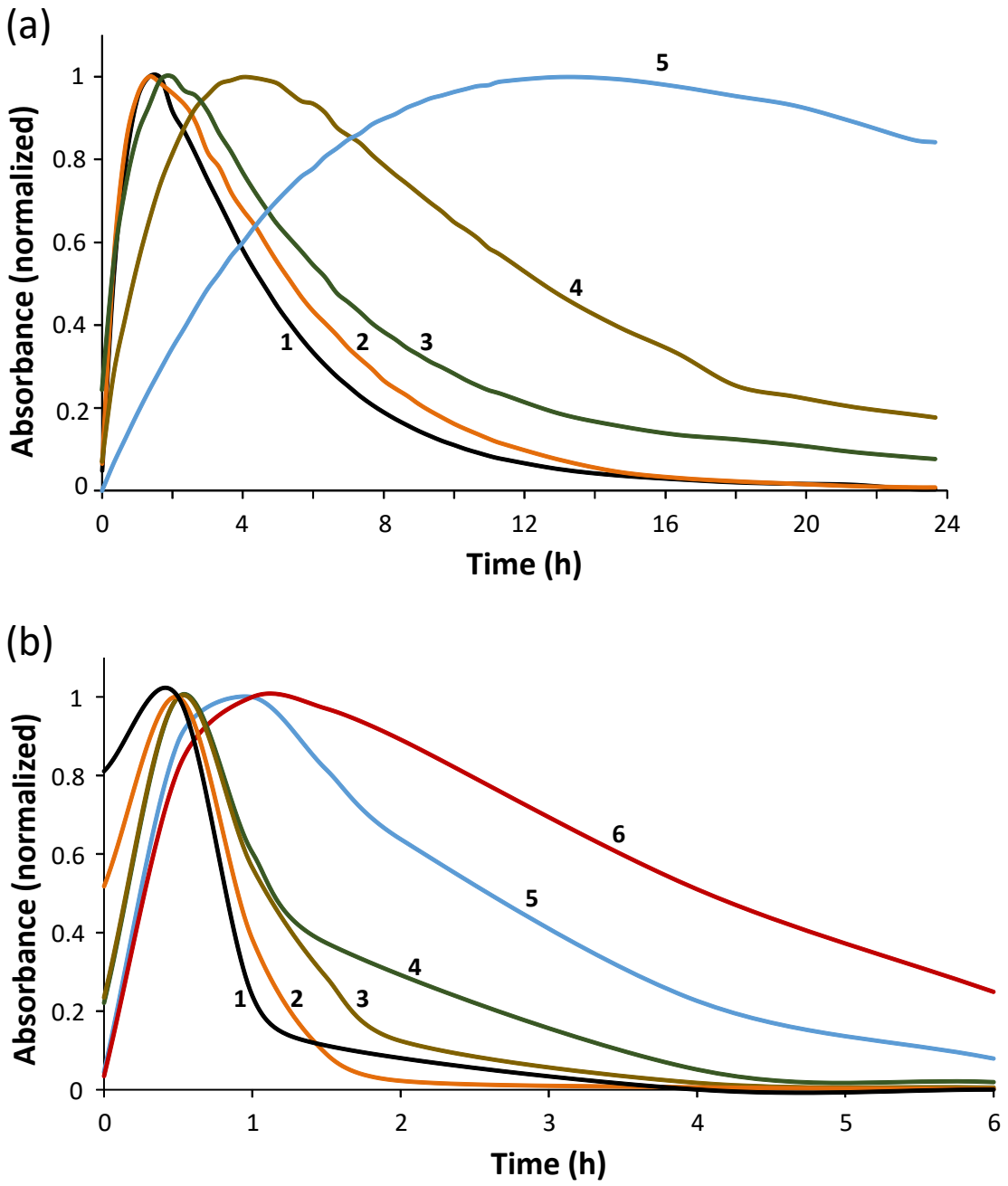

(a)
Absorbance (normalized)
Time (h)
1
2
3
4
5
(b)
Absorbance (normalized)
Time (h)
1
2
3
4
5
6

Fig. 5

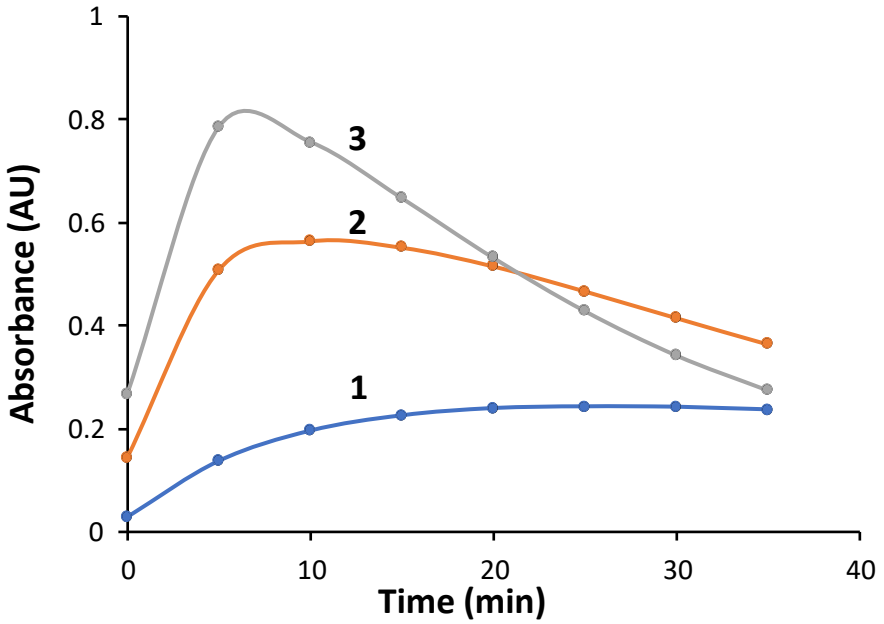


Fig. 6

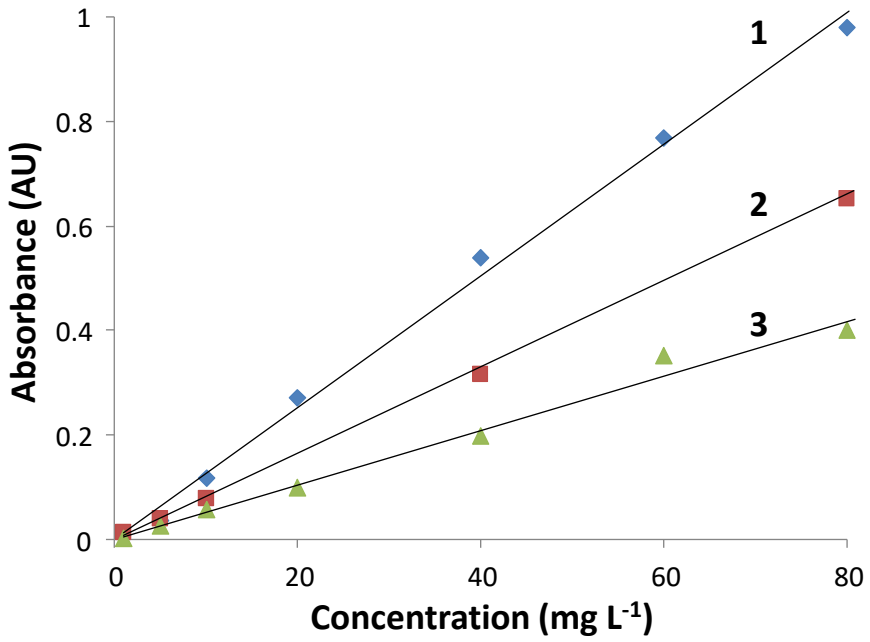
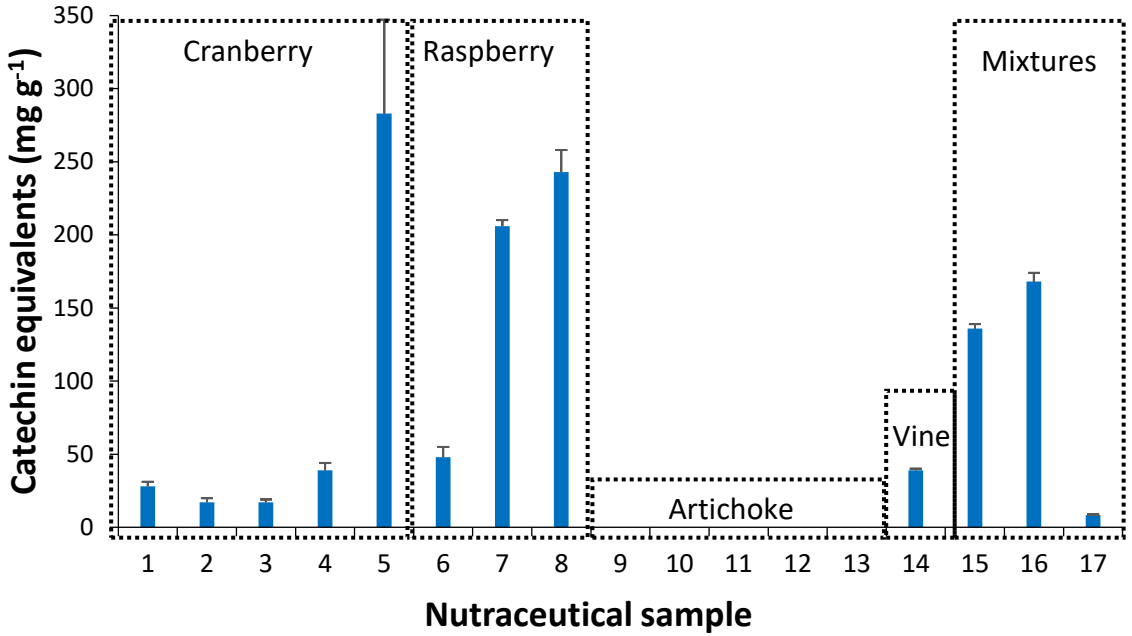

1
0.8
0.6
0.4
0.2
0
Absorbance (AU)
0
20
40
60
80
Concentration (mg L-1)
1
2
3

Fig. 7


Catechin equivalents (mg g-1)
350
300
250
200
150
100
50
0
Cranberry
Raspberry
Artichoke
Vine
Mixtures
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
Nutraceutical sample