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Development of spectrophotometric and chromatographic methods for the determination of flavanols in food samples.

Desarrollo de métodos espectrofotométricos y cromatográficos para la determinación de flavanoles en muestras alimenticias.

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REPORT

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1. SUMMARY

"A-type" proanthocyanidins (PACs) are bioactive substances present in cranberries (*Vaccinium Macrocarpon*). They are useful to prevent urinary tract infections by inhibiting the adhesion of bacteria to the bladder wall. The bioactive behaviour is attributed to the presence of "A-type" interflavan bonds.

A recent increase in cranberry consumption and the development of food supplements which praise to have a high content of "A-type" PACs opens a new field of research which consists of finding a reliable method to determine the concentration of bioactive molecules in a product.

In this work, a reaction of flavanols with 4-(dimethylamino)-cinnamaldehyde (DMAC) was initially performed. Afterwards, analyses were completed using spectrophotometry and liquid chromatography.

In the spectrophotometric method, advantage was taken of the fact that the condensation products of DMAC with flavanols show a maximum absorbance at 640 nm while the rest of flavonoids do not react. This leads to a selective method to identify the substances of interest and also study their chemical behaviour by modifying several variables which have an effect on the reaction.

In the chromatographic method, a Kinetex C18 reversed-phase column with an UV-Vis detector was used in order to achieve a quantitative determination. With the derivatization, interferences were avoided when signals were recorded at 640 nm. Moreover, by selecting other wavelengths it was possible to study secondary peaks of PACs which did not present "A-type" interflavan bonds and peaks corresponding to the reagent as well.

A process of optimization and analysis of standards was carried out to select the most adequate working conditions. Afterwards, samples of different types were studied to check if the method was satisfactory to quantify bioactive PACs.

Keywords: Flavanol, proanthocyanidin, 4-(dimetylamino)-cinnamaldehyde, interflavan bond, cranberry, spectrophotometry, chromatography.

2. RESUMEN

Las proantocianinas (PACs) de tipo A son moléculas con actividad biológica presentes en los arándanos (*Vaccinium Macrocarpon*). Resultan útiles como método de prevención de las infecciones del tracto urinario evitando la adhesión de las bacterias a la vejiga. La actividad biológica de estos compuestos se debe a la presencia de enlaces interflavanoides de tipo A.

El reciente incremento en el consumo de arándanos y la apuesta por el desarrollo de suplementos alimenticios con un alto contenido en PACs de tipo A abre un nuevo campo de investigación consistente en encontrar un método fiable para determinar la concentración de moléculas bioactivas en una muestra.

En este trabajo se llevó a cabo inicialmente un estudio de la derivatización de los flavanoles con 4-dimetilaminocinamaldehído (DMAC). Posteriormente se realizaron los análisis utilizando las técnicas de espectrofotometría y cromatografía de líquidos.

En el método espectrofotométrico se aprovechó que los productos de condensación del DMAC con los flavanoles absorben a 640 nm. Esta reacción no se produce para el resto de flavonoides. Esto permite hacer una determinación selectiva de los analitos y estudiar su evolución ante la modificación de ciertos factores de la reacción.

En el método cromatográfico, se trabajó con una columna Kinetex C18 de fase reversa con un detector UV-Vis para llevar a cabo una separación y determinación cuantitativa de los analitos. Con la derivatización, se evitó la presencia de interferencias realizando el registro de la señal a una longitud de onda de 640 nm. Además, mediante la selección de otras longitudes de onda se pudieron estudiar picos secundarios de PACs que no presentaban enlaces de tipo A o los picos correspondientes al reactivo.

Se completó un proceso de optimización para encontrar las condiciones de trabajo más adecuadas. Una vez terminado, se procedió al estudio de muestras de diferentes tipos para evaluar si el método resultaba satisfactorio para cuantificar las PACs bioactivas.

Palabras clave: Flavanol, proantocianina, 4-dimetilaminocinamaldehido, enlace interflavanoide, arándano, espectrofotometría, cromatografía.

3. INTRODUCTION

Urinary tract infections are one of the most pervasive bacterial infections and it is estimated that 50% of women will have to deal with this pathology at least once in a lifetime (1). Only in the United States, there are over 7 million visits to hospitals per year. This implies an annual cost of over 2 billion dollars (2).

The ingestion of American cranberries (*Vaccinium macrocarpon*) is increasing as a method to treat the infection. At first, it was believed that the beneficial effect of the consumption of cranberries was derived from their capability to acidify urine, but in the last 20 years further research has been done and this effect has been attributed to proanthocyanidins (PACs), which are configured by oligomers or polymers of flavan-3-ol units, that inhibit the adhesion of bacteria.

Specifically, the substances that are responsible for this beneficial effect are "A-type" PACs. These PACs inhibit the adhesion of P-fimbriated *Escherichia Coli* to uroepithelial cells which are located in the bladder wall (3). Moreover, they are responsible for a decrease in the uropathogenic reservoir in the gastrointestinal tract and a lowering in the inflammation attached to the infection process (1). In this way, PACs eradicate the infection in its first step, without the necessity to kill bacteria, which reduces the possibility of an emergence of strains which are resistant to other treatments such as the use of antibiotics.

A study in 2009 evaluated the optimal dose of "A-type" PACs to inhibit the adhesion of bacteria properly. Even though there is a strong dependence between the quantity ingested and the effect, a dose of 36 $mg \cdot day^{-1}$ should be enough to avoid the emergence of the infection (4).

3.1 FLAVONOIDS

Flavonoids are natural substances with a large variety of phenolic structures. They have many applications in the pharmaceutical, medical and cosmetic fields due to the fact that they have anti-oxidative, anti-mutagenic, anti-inflammatory and anti-carcinogenic properties (5).

In nature it is possible to find flavonoids in plants. Thus, these compounds will also be found in different foods and beverages which are of plant origin such as wine, beer, juices, jams, etc. Flavonoids are responsible for their colour and their aroma. Moreover, they act as defensive agents and provide the plant with an effective UV filter. In animals and human beings further research is taking place but for the moment it is believed that the main application of flavonoids is to prevent and treat different pathologies.

Flavonoids have a basic skeleton configured by 15 carbon atoms which are distributed following an established pattern (see Figure 1). Firstly, there are two phenyl rings (A and B) and a heterocyclic ring (C) (6). It is possible to subdivide flavonoids into different categories depending on the carbon of the C ring on which the B ring is attached and the degree of unsaturation and oxidation of the C ring (5).

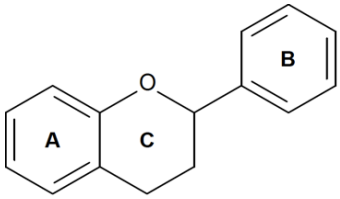

Figure 1: Basic skeleton of a flavonoid *(Christian Becker, 14/03/18, via Wikimedia Commons, Creative commons attribution).*

3.1.1 Flavan-3-ols and proanthocyanidins (PACs)

It is possible to classify flavonoids into many subgroups. One of those groups is called flavan-3-ols. These substances have a very diverse nature and they are usually multisubstituted. Moreover, their name comes from the fact that there is a hydroxyl group which is always bond to position 3 on the C ring (5).

Flavan-3-ols have an important application which is to prevent and treat chronic cardiovascular pathologies. Important sources of these compounds are green tea (approximately an 80% of the total flavonoid content is composed of flavan-3-ols) and red wine (7).

PACs are oligomers and polymers of flavan-3-ol units. Currently over 200 PACs with a degree of polymerization (DP) >5 have been described. However, it is also possible to find other PACs with a bigger DP which have not been characterized yet.

A large number of pharmacological effects have been attributed to PACs (anti-HIV, anti-oxidative, anti-tumour…). Furthermore, these molecules play a crucial role in the defence mechanisms of plants. This property derives from the fact that PACs have a repellent taste which makes them unpalatable to predators and they are also able to form insoluble complexes with proteins in the digestive tract of herbivores which make these substances nutritionally unavailable (8).

To obtain PACs, the starting point is one of the basic flavan-3-ol units which are catechin and epicatechin. These molecules are joined by interflavan carbon-carbon bonds. These bonds can be formed in two different ways (see Figure 2).

In “B-type” linkage it is possible to observe bonds between C4 and C8 which is the most common situation, or bonds between C4 and C6 which leads to a less stable state. In case there are two bonds between the flavan-3-ol units the dimer will be described as an “A-type” PAC. In these structures there is an extra interaction between C2 of the lower unit and C7 of the upper unit (this bond also involves an oxygen atom). A study has reported the fact that it is possible that “B-type” PACs turn into “A-type” PACs via radical oxidation at pH 7 or via oxidation with oxygen (9).

Apart from the properties that have been mentioned before which are common for all PACs, structures containing “A-type” linkages develop a bigger resistance to harsh conditions such as heating at extreme pH values. Moreover, these molecules are responsible for the bacterial anti-adhesion effect and, thus, for an antibacterial activity (10).

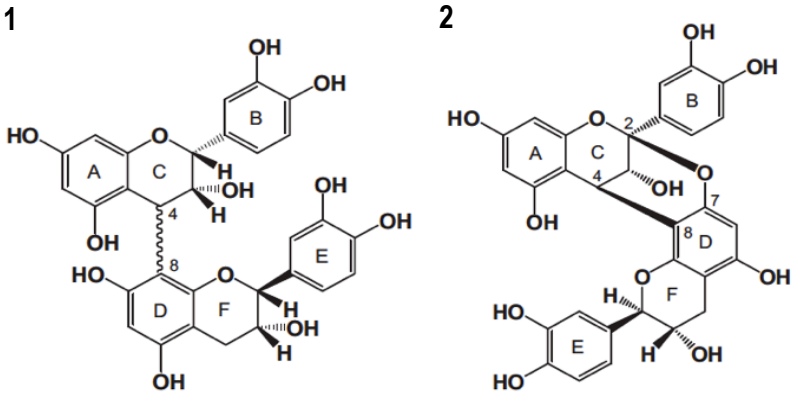

Figure 2: Example of (1) B-type (2) A-type PAC linkage *(Image extracted from Bakhytkyzy, I. ref. 6).*

3.2 METHODS FOR THE DETERMINATION OF PACs

The process of extraction is a complex step in the determination of PACs in samples. Firstly, the selection of the extraction solvent may be complicated because PACs with a small DP are

soluble in organic solvents whereas oligomers and polymers with a higher DP are soluble in water. Thus, it is generally recommended to work with aqueous organic solvents such as acidified acetone (9). Furthermore, conditions during extraction have an important effect on the quantity of PACs which can be extracted. In order to preserve the substances of interest, it is important to keep the samples stored in the freezer and in the dark.

Once the samples are properly prepared there are two main procedures to determine PAC content:

3.2.1 Conventional methods

The most traditional method to determine PACs is the acid butanol assay which is a colorimetric reaction. PACs are hydrolysed into extension and terminal units by applying acid and heat. The extension unit is a carbocation which rapidly converts to individual anthocyanins by autoxidation while the terminal unit is a flavan-3-ol (11). Anthocyanins are detected at 550 nm to do a quantification of the PAC content of a sample. This method has a major disadvantage since the chemical structure of each individual PAC influences the yield and the kinetics of the reaction (12).

Another method which was developed is the vanillin assay. This experiment uses the vanillin aldehyde that reacts with meta-oriented hydroxyl groups on the flavanol A-ring (13). This reaction leads to a coloured product that has an absorbance maximum at 510 nm. Nevertheless, it ought to be taken into account that the absorbance shown by oligomers and polymers is bigger than the one shown by monomers due to the fact that the reaction does not only take place in terminal positions (13). Thus, this method is not very reliable.

The third conventional method is based on the 4-(dimethylamino)-cinnamaldehyde (DMAC) assay. The principle of this technique is the condensation of the A-ring with an aldehyde which provides a coloured product detectable at 640 nm. As shown in the scheme of Figure 3, under acidic conditions, the oxygen atom of the carboxyl group that belongs to the aldehyde is protonated and forms an electrophilic carbocation which can bond with the terminal units of PACs. The reaction mechanism is similar to that of the vanillin assay with the difference that DMAC only reacts with the terminal units. Nonetheless, as a major advantage, the DMAC assay is five times more sensitive than the vanillin one and it is affected by less interferences. Moreover, this procedure is easier and can be carried out at room temperature (9).

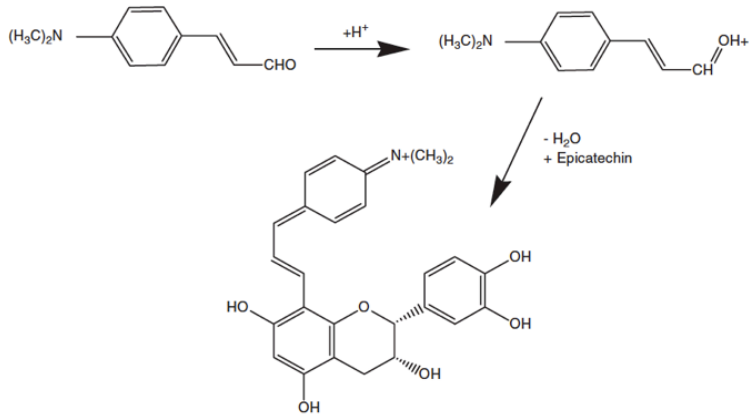

Figure 3: Scheme of the DMAC reaction with a flavan-3-ol unit *(Image extracted from Bakhytkyzy, I. ref. 6).*

3.2.2 Chromatographic methods

Low molecular mass flavan-3-ols can be determined using either reversed or normal-phase HPLC. Nonetheless, determining polymeric PACs is highly difficult due to the fact that there is an exponential increase in the variety of isomers as the DP grows (14).

Another aspect which should be taken into account is that the peak capacity of the stationary phase is reduced while samples get more complex. That results in a hump of the baseline which is impossible to interpret.

In RP mode, if the attention is set on monomers, dimers and trimers, the main reason which will be responsible for the order of elution is not the DP but the overall polarity of each substance.

After the separation has been completed it is very usual to use UV or fluorescence detection. Nevertheless, it is important to consider the fact that the detection limits using fluorescence are nearly one hundred times lower than those with UV detection (15).

3.3 CHEMOMETRIC METHODS

During an optimization process, it is common to rely on a trial-and-error approach. Nevertheless, this method might be inefficient to provide the most satisfactory working conditions. An alternative to this unstructured work method is the use of chemometrics. In this way it is possible to apply a design of experiments (DOE) in order to find the optimal experimental conditions. DOE allows related variables which have an influence on a process to be simultaneously evaluated at various levels following a pre-established scheme of assays (16). One of the most representative methodologies in order to optimize an experimental process is the use of factorial designs (17).

3.3.1 Factorial design

A factorial design allows the simultaneous study of two or more factors (see Figure 4). A factor is an independent experimental variable (such as time, temperature, pH or analyte concentration) which might have an influence on the studied process and could be analysed through chemometrics. Each of these variables ought to be assayed at two or more levels depending on the accuracy that is expected from the optimization process. Factors can be divided into two categories: Variables which can possess any value within a range are considered continuous whilst those that can only take specific values are known as discrete (16).

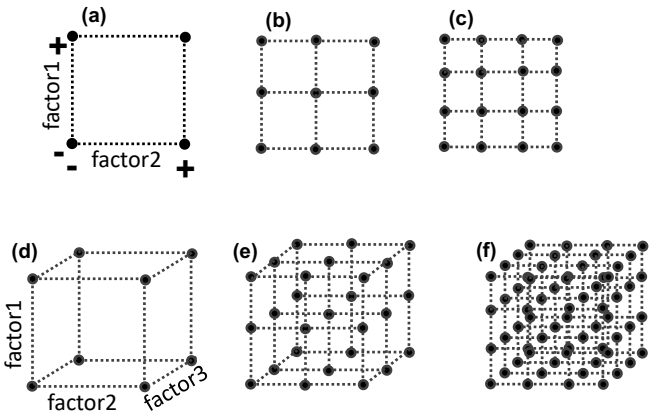

Figure 4: Scheme of some representative full factorial designs for the evaluation of 2 and 3 factors. (a) 2-factor at 2-level; (b) 2-factor at 3-level; (c) 2-factor at 4-level; (d) 3-factor at 2-level; (e) 3-factor at 3-level; (f) 3-factor at 4-level. *(Image extracted from Saurina, X., ref. 16).*

Considering that the experimental cost of a factorial design grows exponentially with the number of factors studied, it is thoroughly recommended that variables undergo a preliminary screening process so that the irrelevant ones are obviated and time and resources are saved. After the selection process, significant parameters are studied more thoroughly. In case the attention is set on 2 or 3 variables it is convenient to work with full factorial designs due to the fact that they provide more precise information. This procedure explores all factors f at various levels $\mathcal{L}$, being $\mathcal{L}^f$ the total number of experiments performed. In case attention is set on a larger number of variables it is a better option to work with a fractional factorial design which significantly reduces the number of assays required.

4. OBJECTIVES

American cranberries (*Vaccinium macrocarpon*) are commonly consumed as a method to prevent and treat urinary tract infections. This phenomenon is attributed to the presence of A-type PACs. While other flavonoids such as anthocyanidins or isoflavones are well described, further investigation is required in case of PACs in order to develop a method to evaluate the authenticity and efficacy of cranberry-based products. Nevertheless, this is highly difficult because of the lack of commercially available standards and the structural variety of oligomeric and polymeric PACs.

The main objective of this project was to evaluate the effectiveness of the spectrophotometric and the chromatographic methods to identify and quantify PACs with a previous reaction of derivatization with the reagent DMAC. In order to get satisfactory results several steps were followed:

- Optimization of the reaction conditions including concentration of the reagent, concentration of acid, temperature and time using UV-Vis spectrophotometric detection. The study was based on experimental design.
- Analysis of juice samples with the spectrophotometric method in order to check if a quantitative determination was possible to achieve.
- Study of the separation of flavan-3-ols (catechin, epicatechin, dimers A2 and B2 and trimer C1) derivatives using RP-HPLC.

- Analysis of juice and pharmaceutical samples using HPLC in order to perform a quantitative determination.

5. EXPERIMENTAL SECTION

5.1 REAGENTS, STANDARDS AND SOLVENTS

The solvents and reagents used for the DMAC spectrophotometric assay were:

- Methanol UHPLC PAI-ACS (SuperGradient Panreac, Castellar del Vallès, Barcelona).
- Hydrochloric acid (37% (w/w), Merck, Darmstadt, Germany).
- 4-(Dimethylamino)-cinnamaldehyde (DMAC) (98% (w/w), Powder, Sigma-Aldrich, St Louis, USA).
- Dimethyl sulfoxide (USP, BP, Ph. Eur.) Pharma grade (Panreac, Castellar del Vallès, Barcelona, Spain).

The polyphenolic standards were prepared from the following sources:

- Catechin (≥ 98% (w/w), Sigma-Aldrich, St Louis, USA).
- Epicatechin (≥ 98% (w/w), Sigma-Aldrich, St Louis, USA).
- Procyanidin A2 (≥ 99% (w/w), PhytoLab, Vestenbergsgreuth, Germany).
- Procyanidin B2 (>98% (w/w), Chengdu Biopurify Phytochemicals LTD., China).
- Procyanidin C1 (≥ 99% (w/w), PhytoLab, Vestenbergsgreuth, Germany).

The standard solutions were dissolved in dimethyl sulfoxide and stored in amber glass vials in the fridge. This was helpful to minimize the decomposition process of the standards throughout time.

Other solvents and reagents used in the HPLC in order to prepare the mobile phase were:

- Formic acid (>96% (w/w), Sigma-Aldrich, St Louis, USA).
- Mili-Q Water filtered through a 0.22 nylon filter.
- Acetonitrile for UHPLC, Supergradient, ACS (Panreac, Castellar del Vallès, Barcelona, Spain).

5.2 SAMPLES

A total of 28 juices of different fruits were analysed (8 cranberry juices, 3 pomegranate juices, 3 orange juices, 4 apple juices, 2 strawberry juices, 7 grape juices and 1 pineapple juice). Moreover, to carry out the chromatographic studies three medicaments to treat and prevent urinary tract infections were analysed as well.

The first treatment that samples underwent was centrifugation, in order to work with solutions that were as transparent as possible to minimize matrix effects. Moreover, the juices were kept in the fridge so that the decomposition processes were slowed down. This also applied to the standards and DMAC solutions, which decomposed rapidly when maintained at room temperature.

5.3 INSTRUMENTATION

The UV-Vis study of the DMAC reaction was carried out with Perkin Elmer UV/VIS/NIR Spectrometer Lambda 19.

The chromatographic study of the DMAC reaction was carried out with an Agilent Series 1100 chromatograph (Agilent Technologies, Palo Alto, California, USA) with a binary pump (G1311A), a degasser (G1322A), an automatic injection system (G1329), a diode array detector (G1315B) and a fluorescence detector (G1321A). All the elements mentioned above belonged to the 1100 series except for the automatic injector which belonged to the 1200 series.

5.4 SPECTROPHOTOMETRIC METHOD

In order to extract information from the spectrophotometric measurements, spectra were recorded in wavelength range from 750 to 400 nm with a step interval of 1 nm. Appropriate volumes of standard/sample, reagent and H_2O/MeOH (75:25 (v/v)) solvent were directly added to a standard quartz cuvette. Solutions were mixed and the cuvette was placed in the sample holder of the spectrophotometer to carry out the spectrophotometric measurements.

During the preliminary assays the kinetics of the reaction was followed for 45 minutes, recording a spectrum every five minutes, whilst when samples were studied the reaction was developed for 1 hour and then the corresponding spectrum was recorded.

Independently of the substance studied, a constant concentration of 0.16% (w/v) DMAC and 0.2M HCl was added.

5.5 CHROMATOGRAPHIC METHOD

Chromatographic analyses during the work were completed using a C18 reversed-phase Kinetex column (Phenomenex, Torrance, California, USA) with length 100 mm, internal diameter 4.6 mm, pore size 100 Å and particle size 2.6 μm.

In order to achieve a chromatographic separation of catechin, epicatechin, procyanidin A2, procyanidin B2 and procyanidin C1 derivatives, 0.1% formic acid in water (v/v) (solvent A) and methanol (solvent B) were used as the components of the mobile phase. The flow rate was 0.4 $mL \cdot min^{-1}$ and the injection volume was 5 μL.

The elution gradient used to separate the different substances was as follows (see Table 1):

Time (min)	Solvent A (%)	Solvent B (%)
0-2	85	15
2-5	85-5	15-95
5-15	5	95
15-15.1	5-85	95-15
15.1-20	85	15

Table 1: Elution gradient used for chromatographic studies (solvent A: 0.1% formic acid in water (v/v); solvent B: Methanol).

UV detection was carried out at 640, 590, 544, 420 and 320 nm (16 nm bandwidth) using a reference wavelength of 700 nm with a bandwidth of 50 nm.

6. DISCUSSION OF RESULTS

6.1 UV-VIS ANALYSIS

6.1.1 Preliminary studies by experimental design

Several tests were made in order to determine the optimal concentration of each standard to work with adequate peak signals. A concentration of 0.08% (w/v) DMAC and 0.12M HCl was fixed according to studies carried out by other researchers (13).

The basic study mentioned above provided that the appropriate concentration of each standard to work with was:

- Catechin: 100 $mg \cdot L^{-1}$
- Epicatechin: 50 $mg \cdot L^{-1}$
- Procyanidin A2: 400 $mg \cdot L^{-1}$
- Procyanidin B2: 20 $mg \cdot L^{-1}$

The experiments for catechin and epicatechin were performed in a cuvette with a capacity of 2.5 mL (cuvette a) whereas measurements for PACs A2 and B2 were carried out in a cuvette that had a capacity of 0.5 mL (cuvette b) in order to save expensive reagents. Even though the concentration of DMAC and HCl remained the same in all assays, the experimental values could not be used to estimate the differences in the sensibility of the standards because the behaviour of the two cuvettes was considerably different. At the same concentrations, cuvette b provided larger signals and saturation was often reached, whilst cuvette a could be used to work with a wider range of concentrations.

When it comes to catechin and epicatechin derivatives, the peak at 640 nm (working with cuvette a) reached absorbance values between 0.6 and 0.8 (see Figure 5), which was an advantage because in this interval of absorbances the error attached to the signal was minimal. Nonetheless, when PACs A2 and B2 derivatives were analysed in cuvette b the maximum absorbances that could be registered oscillated between 0.1 and 0.3 because at higher concentrations saturation was reached. The fact that the signals had such low intensity lowered the precision of the results because the spectrophotometer became unable to register small variations in the concentration.

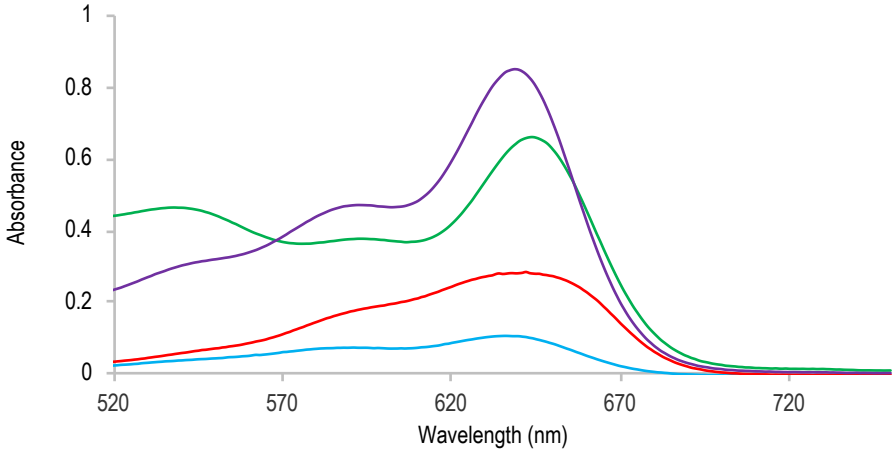

Figure 5: Shape of the absorbance spectra of flavan-3-ol derivatives using 0.2M HCl and 0.16% (w/v) DMAC. Assignation: ●50 mg·L^{-1} epicatechin derivative, ●100 mg·L^{-1} catechin derivative, ●400 mg·L^{-1} procyanidin A2 derivative, ●20 mg·L^{-1} procyanidin B2 derivative.

After fixing the optimal concentrations of each standard, simultaneous influence of DMAC and HCl concentration was studied using a 2-factor at 2-level design with replicates at the central point (see Table 2):

	DMAC level	HCl level	DMAC (%)	HCl (M)
Experiment 1	-	-	0.08	0.12
Experiment 2	-	+	0.08	0.2
Experiment 3	+	-	0.16	0.12
Experiment 4	+	+	0.16	0.2
Central point			0.12	0.16

Table 2: Concentrations of DMAC and HCl inside the cuvettes during the experiments to evaluate the simultaneous influence of those variables in the reaction of derivatization.

Once this previous study was completed, dependence with temperature was studied by performing assays with epicatechin at 10 and 40ºC. Moreover, the reaction of catechin and epicatechin was studied overnight to obtain information on their kinetic behaviour to further establish an index of the content of equivalents of catechin in 28 juice samples.

6.1.2 Evaluation of factors that can affect 4-(dimethylamino)-cinnamaldehyde (DMAC) reaction

As the conditions under which the reaction took place were varied, different responses for each standard were observed (see Figure 6).

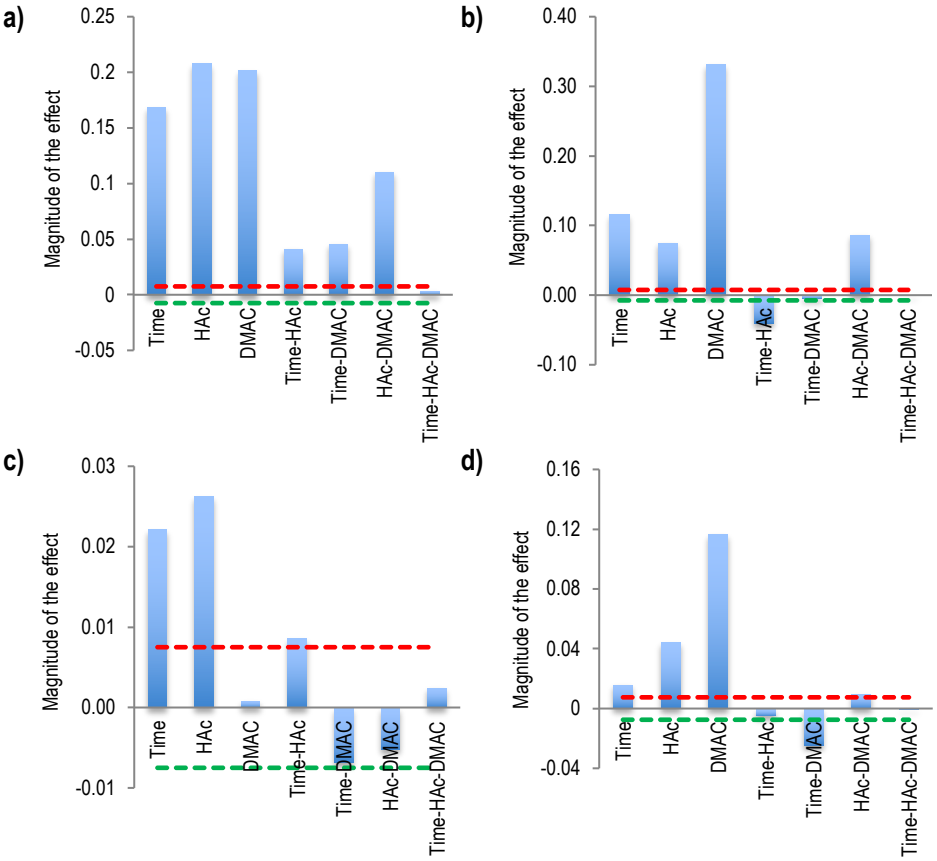

Figure 6: Evaluation of effects and interactions by factorial design (see Table 2) for the derivatization of flavanols with DMAC. Assignation: a) catechin, b) epicatechin, c) procyanidin A2, d) procyanidin B2. Dotted lines correspond to the standard deviation estimated from the replicated central point.

The study was based on the factorial design of Table 2. Besides, spectra were recorded at 15 and 45 minutes so the influence of time was also investigated. Four graphics are shown to clarify the variations in the behaviour of the substances. In these plots, apart from the changes in the response of each standard as time, DMAC concentration and acidity increased, the interactions between these parameters were studied as well.

After the experiments were fulfilled certain conclusions were extracted. First of all, an increase in the magnitude of the response as acidity and time enlargened was observed for all compounds. Moreover, when the concentration of DMAC increased, the response was more intense except in case of procyanidin A2 where it was barely impossible to notice any effect.

If attention was paid to the interaction between different parameters, the most remarkable information was that the interaction between acidic medium and DMAC resulted in a larger signal for catechin and epicatechin. Nevertheless, the interaction was rather neglectable when the dimers were being analysed.

Once the results of the preliminary assays were obtained, it was utterly important to choose a set of reaction conditions that provided the largest response. Thus, concentrations of 0.2M HCl and 0.16% (w/v) DMAC were chosen to study samples afterwards. Nonetheless, this information was not enough to achieve proper experimental results so further research was done on additional variables such as temperature and time.

To test the effect that temperature had in the evolution of the reaction three experiments were carried out using 50 $mg \cdot L^{-1}$ epicatechin as a standard (see Figure 7). The reaction developed in a faster way when it took place at room temperature. This can be explained by different reasons. At 10ºC, the reaction developed at a lower rate due to the fact that the molecules of analyte and reagent found more difficulties in colliding because of the low energy that the system had. Nonetheless, when temperature rose to 40ºC the absorbance maximum started to decrease after fifteen minutes. This happened mainly because a DMAC derivative is a rather unstable compound that tends to decompose at higher temperatures. Moreover, an increase in the standard deviation was expected because methanol evaporation is harder to minimize at higher temperatures. As a result, room temperature was selected to carry out further studies.

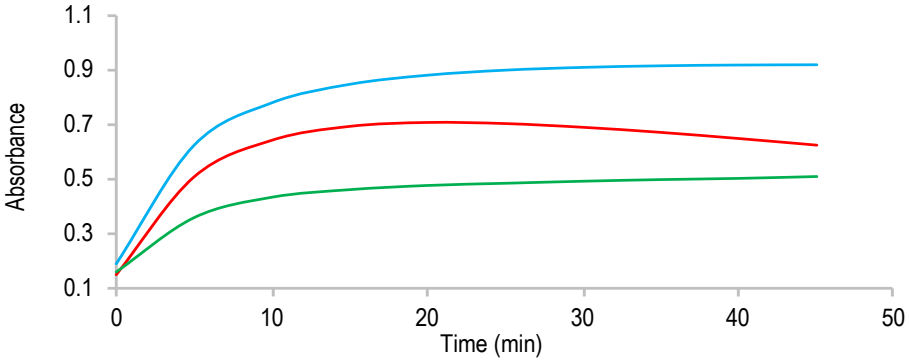

Figure 7: Variation of the absorbance at 640 nm with temperature using epicatechin as a standard. Assignation: ● room temperature, ● 40ºC, ● 10ºC.

Even though in previous assays it was confirmed that a solution containing 0.16% (w/v) DMAC and 0.2M HCl provided the best results, a last study was carried out to check if a further increase in acidity and DMAC concentration could maximize the absorbance even more. As it is shown in Figure 8 the response was higher using 0.48% (w/v) DMAC and 0.6M HCl. Nevertheless, the more increase there was, the more abrupt the decrease got. The fact that a plateau was not reached was a major drawback because it minimized the time during which it was possible to make any measurements. Therefore, an increase in acid and DMAC concentration was dismissed.

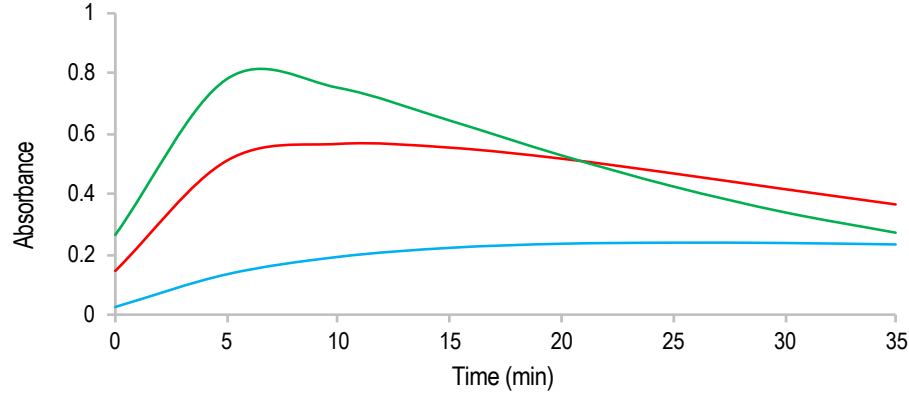

Figure 8: Evolution of the reaction of 50 mg·L^{-1} epicatechin. Assignation: ● 0.16% (w/v) DMAC 0.2M HCl, ● 0.32% (w/v) DMAC 0.4M HCl, ● 0.48% (w/v) DMAC 0.6M HCl.

In order to study the samples it was important to determine the time range where the signal was maximum. That information was extracted from the data obtained in the study of catechin and epicatechin kinetics overnight (see Figure 9).

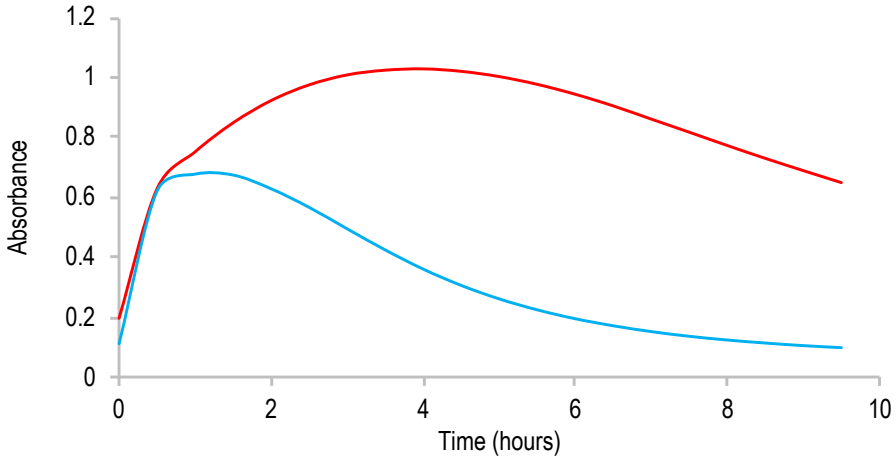

Figure 9: Evolution of the absorbance of the derivatives as a function of time. Assignation: ● 100 mg·L^{-1} catechin derivative and ● 50 mg·L^{-1} epicatechin derivative.

Both substances reached the maximum absorbance at different times. Thus, it was impossible to work at optimal conditions for both analytes. Nonetheless, it was more appropriate to pay extra attention to epicatechin due to the fact that once it reached the maximum absorbance the decrease was rather abrupt. Taking into account all of this information it was decided to record measurements one hour after the reaction had begun.

6.1.3 Index of catechin equivalents in juice samples

To determine the concentration of the analytes in the samples a process of calibration was required. As all of the analytes showed maximum absorbance at 640 nm, it was decided to make a calibration using the catechin derivative and express the total flavanol content of the samples in catechin equivalents. Catechin derivative solutions with concentrations of 100 mg·L^{-1}, 50 mg·L^{-1}, 25 mg·L^{-1}, 10 mg·L^{-1} and 5 mg·L^{-1} were prepared to build the curve of calibration (see

Figure 10). Taking into account the absorbances at 640 nm shown in Figure 5, no solutions of a higher concentration were prepared because the signal would be close to saturation.

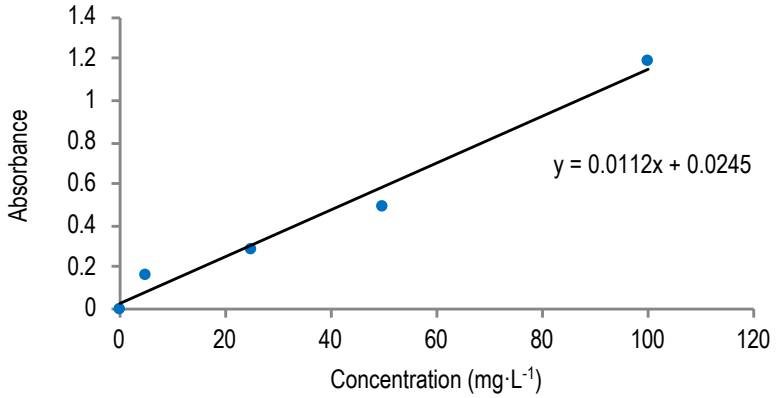

Figure 10: Catechin derivative calibration curve using 0.16% (w/v) DMAC and 0.2M HCl. The reaction was developed for 1 hour and then the corresponding spectrum was recorded. The data given in the plot corresponds to absorbance at 640 nm.

As it can be seen in Figure 10, the linearity of the calibration curve was quite poor even though 5 independent replicated measurements were registered for each concentration. This can be attributed to the fact that both DMAC and catechin are rather unstable substances which can undergo degradation processes if slight variations in the reaction media occur.

Conditions finally selected to carry out the determination of a flavanol index based on the DMAC reaction were applied to the analysis of juice samples. In particular these conditions were as follows: 0.16% (w/v) DMAC, 0.2M HCl, room temperature and time 1 hour. In order to determine the volume of juice that ought to be introduced in cuvette a (which had a capacity of 2.5 mL), several assays were done. Eventually, it was decided that the mixture would include 500 µL of juice sample. Even though the intensity of the response using this volume was lower than desired, higher concentrations implied too large matrix effects.

In Table 3, an index of flavan-3-ol content expressed in catechin equivalents is presented:

	Name of the juice	Catechin equivalents ($mg \cdot L^{-1}$)	RSD (%)
Sample 1	Ocean spray – Cranberry classic	6 (± 1)	17
Sample 2	The berry company – Superberries	-	50*
Sample 3	Ekolo Bio zumo - Pomegranate	11 (± 4)	36
Sample 4	Don Simon – Orange	-	-
Sample 5	Disfruta – Apple	-	-
Sample 6	Lambda – Apple	-	-
Sample 7	We squeeze – Strawberry	16 (± 1)	6
Sample 8	Zumosol – Berries, grape, raspberry	68 (± 4)	6
Sample 9	Compal – Grape, apple, raspberry, strawberry, cranberry, gooseberry	6 (± 1)	17
Sample 10	El Corte Inglés – Pomegranate, red grape juice	-	67*
Sample 11	Juice Murillo blueberry	6 (± 1)	17
Sample 12	Ocean spray – Cranberry light	14 (± 4)	28
Sample 13	Ocean spray – Cranberry pomegranate	3.7 (± 0.4)	11
Sample 14	El Corte Inglés – Cranberry	6.5 (± 0.9)	14
Sample 15	Lambda – Cranberry, blackcurrant	15 (± 3)	20
Sample 16	The berry company – Blueberry	8 (± 2)	25
Sample 17	El Corte Inglés – Pineapple	-	-
Sample 18	Don Simon – Apple	-	-
Sample 19	Happy day – Strawberry	7 (± 2)	29

Sample 20	Lambda – Grape	12 (± 2)	17
Sample 21	Mosto greip – White	3.4 (± 0.7)	21
Sample 22	Mosto greip – Red	59 (± 10)	17
Sample 23	We squeeze – Grape, cherry, pomegranate	11 (± 1)	9
Sample 24	Minute maid – Orange, raspberry, carrot, grape, blackcurrant	1.3 (± 0.2)	15
Sample 25	Disfruta – Grape, apple, raspberry	11 (± 1)	9
Sample 26	Happy day – Apple, grape, blackcurrant, cherry, lemon, raspberry	1.4 (± 0.4)	29
Sample 27	El Corte Inglés – Red grape, cherry, blackcurrant, raspberry, strawberry	2.3 (± 0.5)	22
Sample 28	Zumosol - Orange	-	-

Table 3: Content of catechin equivalents of juice samples 1 to 28 expressed in $mg \cdot L^{-1}$. (±) means the standard deviation from 3 independent replicated measurements. *A quantification was not achieved for samples 2 and 10 due to the large RSD values obtained. -: The concentration of the analytes was lower than the limit of detection.

The standard deviation of the absorbance measurements of each sample was not neglectable showing an RSD between the 6% and the 67%. Thus, the spectrophotometric method was found to be imprecise to do a quantitative analysis. Nonetheless, it provided some interesting qualitative data.

6.2 CHROMATOGRAPHIC ANALYSIS

As it was mentioned in the experimental section, the aim of these experiments was to find a set of conditions which enabled the proper separation of the substances under study. To accomplish that, the following solutions of reaction media were prepared in small amber vials:

- Catechin derivative solution: 50 $mg \cdot L^{-1}$ catechin, 0.16% (w/v) DMAC and 0.2M HCl.
- Epicatechin derivative solution: 50 $mg \cdot L^{-1}$ epicatechin, 0.16% (w/v) DMAC and 0.2M HCl.
- Procyanidin A2 derivative solution: 200 $mg \cdot L^{-1}$ procyanidin A2, 0.16% (w/v) DMAC and 0.2M HCl.

- Procyanidin B2 derivative solution: 8 mg·L^{-1} procyanidin B2, 0.16% (w/v) DMAC and 0.2M HCl.
- Procyanidin C1 derivative solution: 5 mg·L^{-1} procyanidin C1, 0.16% (w/v) DMAC and 0.2M HCl.

These solutions were immediately injected into the HPLC after being prepared. The injection volume was 5 μL.

The optimization of the separation was considered using aqueous solutions of 0.1% formic acid in water (v/v) and methanol or acetonitrile as the components of the mobile phase.

6.2.1 Chromatographic assays using acetonitrile

Acetonitrile is a solvent with lower polarity than methanol due to its organic nature. Thus, the studied compounds were expected to show smaller retention times in acetonitrile than methanol (when using the same solvent percentages). The gradient that was used while working with this solvent is shown in Table 4.

Time (min)	Solvent A (%)	Solvent B (%)
0-5	85-5	15-95
5-12	5	95
12-12.1	5-85	95-15
12.1-15	85	15

Table 4: Elution gradient used for chromatographic studies (solvent A: 0.1% formic acid in water (v/v); solvent B: Acetonitrile).

In Figure 11, the chromatograms obtained using this method are shown. In these chromatograms a very important level of noise could be observed, which was a major a source of error. Moreover, the peak symmetry was poor. Eventually, this method was discarded because it was not accurate and the peaks corresponding to the analytes appeared overlapped.

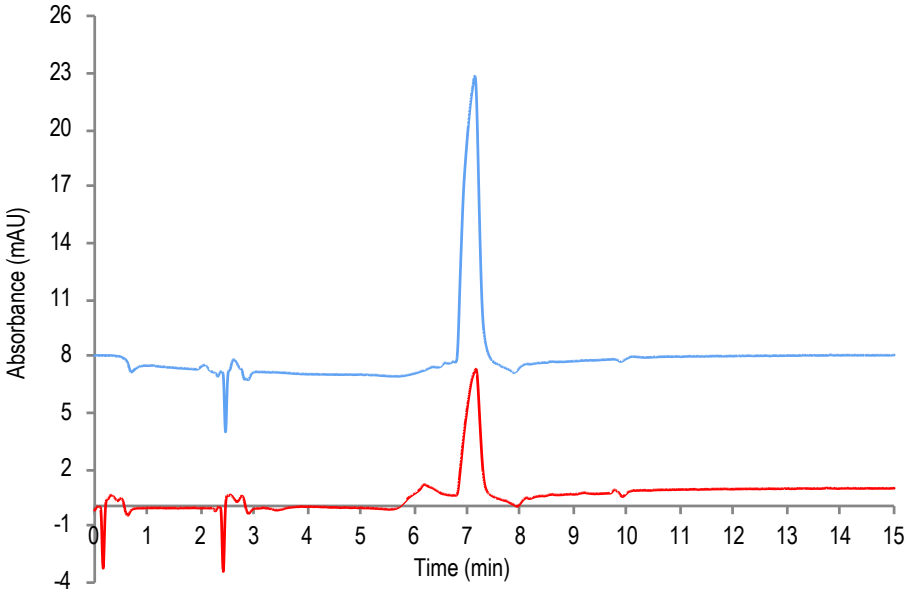

Figure 11: Chromatograms obtained using acetonitrile as solvent B. Assignation: ●50 mg·L^{-1} catechin derivative and ●50 mg·L^{-1} epicatechin derivative.

6.2.2 Chromatographic assays using methanol

6.2.2.1 Preliminary assays

Based on experimental procedures done by other researchers (6), the following elution gradient (see Table 5) was the first one used to separate the substances of interest:

Time (min)	Solvent A (%)	Solvent B (%)
0-5	95-10	5-90
5-15	10	90
15-15.1	10-95	90-5
15.1-20	95	5

Table 5: Elution gradient used for the preliminary chromatographic studies (solvent A: 0.1% formic acid in water (v/v); solvent B: Methanol). This elution gradient was extracted from ref. 6.

As it is shown in Figure 12, peaks corresponding to PACs A2 and B2 appeared completely overlapped. This was a major drawback due to the fact that it was impossible to do a quantitative analysis of PAC A2 that is a bioactive compound. Moreover, if attention was set on the peaks corresponding to catechin and epicatechin, not only they were overlapped but they also had a very poor symmetry.

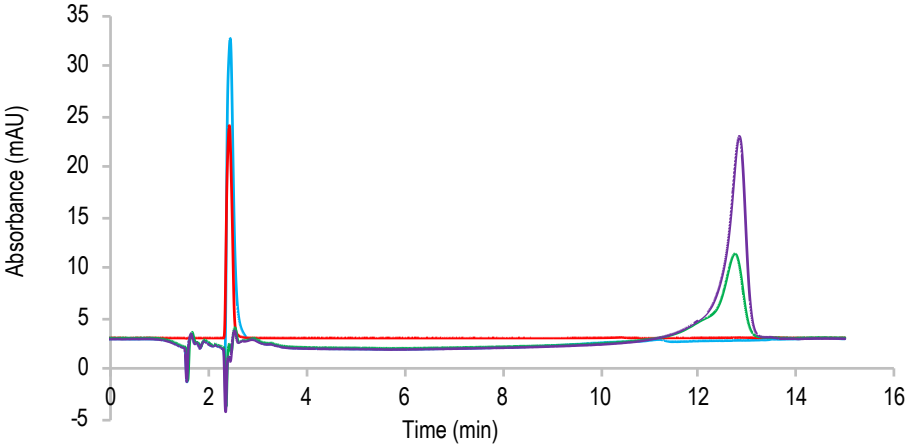

Figure 12: Chromatograms of the preliminary studies using methanol as solvent B. The reaction was developed for 1 hour before the measurements were made. Assignation: ●50 mg·L^{-1} catechin derivative, ●50 mg·L^{-1} epicatechin derivative, ●50 mg·L^{-1} procyanidin B2 derivative and ●50 mg·L^{-1} procyanidin A2 derivative.

The elution gradient of Table 5 applied in these preliminary studies showed another peculiarity (see Figure 13). To study the kinetics of the reaction of each derivative a chromatogram was registered every fifteen minutes. When PAC A2 derivative was being analysed it was found that the position of the peak varied with time. At the beginning of the reaction, the peak showed a retention time of 2.45 minutes. Fifteen minutes after the peak became an undetectable hump and eventually when the process was developed for 30 minutes the retention time for PAC A2 derivative was 11.26 minutes. This behaviour was not repeated when other elution gradients were used, and so, for all the reasons mentioned above the method was discarded.

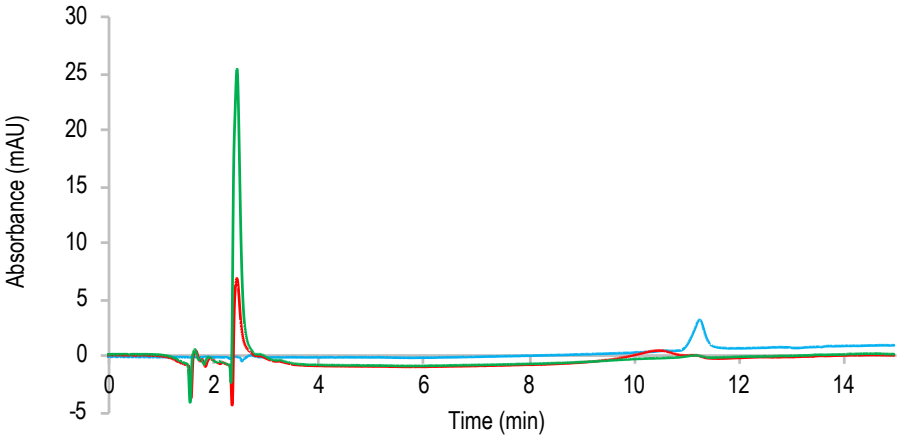

Figure 13: Evolution of the chromatogram of dimer A2 derivative over time using the preliminary gradient. Assignation: ●After 0 minutes, ●After 15 minutes, ●After 30 minutes.

6.2.2.2 Optimized method

Chromatograms for each standard were registered using the gradient mentioned in the experimental section (see Table 1), and the results are shown in Figure 14. As it is shown in this plot, PAC A2, PAC B2 and PAC C1 derivatives were properly separated. This is a relevant result as PAC A2 is responsible for the antibacterial properties of cranberry-based products and it was utterly important to be able to do a precise determination of the quantity of this substance present in a product. Moreover, the shape of the peaks was very symmetric and they did not experiment an excessive broadening which would make quantitative analyses less reliable.

On the other hand, it was not possible to separate the peaks corresponding to catechin and epicatechin even though several modifications of the elution conditions were tried. That means that the determination of these two substances should be done using alternative methods.

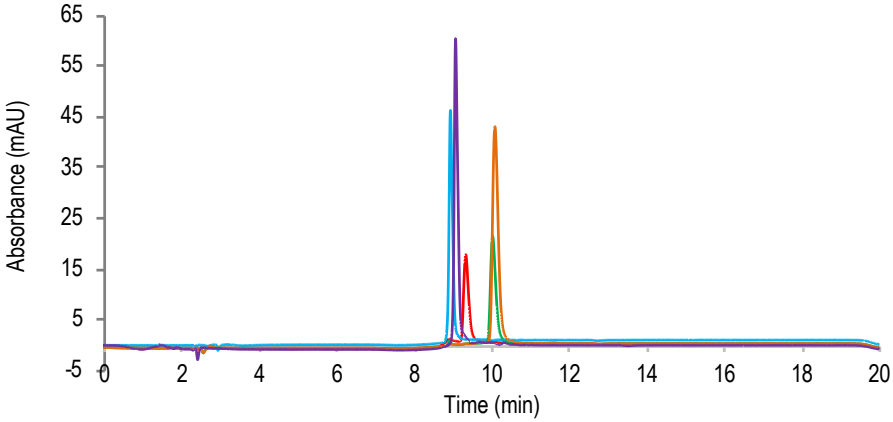

Figure 14: Chromatogram of flavanol derivatives recorded at 640 nm. Assignation: ●catechin, ●epicatechin, ●PAC A2, ●PAC B2 and ●PAC C1 derivatives.

A mixture of the five standards was prepared and analysed to check if it was possible to do a quantitative determination of the dimers and the trimer (see Figure 15).

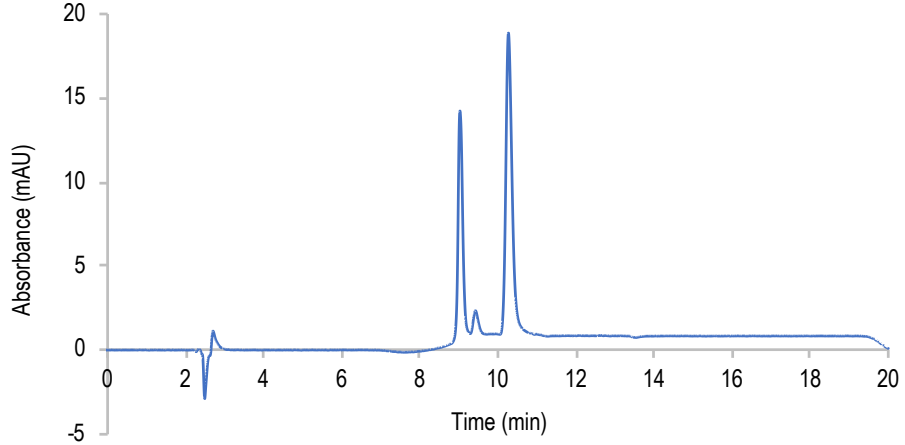

Figure 15: Chromatogram of a mixture containing 50 mg·L^{-1} catechin, 50 mg·L^{-1} epicatechin, 200 mg·L^{-1} procyanidin A2, 8 mg·L^{-1} procyanidin B2 and 5 mg·L^{-1} procyanidin C1.

As it is seen, it was possible to observe precisely the signal of catechin, epicatechin and PAC A2 derivatives. Nonetheless, the signal corresponding to the dimer B2 derivative appeared completely imbibed within the C1 peak. It was thus concluded that this separation should be

improved to enhance the resolution overlapping. Unfortunately, there was not enough time for further studies.

Once the separation conditions had been selected, the analysis of commercial samples was considered. Firstly, a study of juice samples 1 to 28 was completed. Nonetheless, as the content in the studied substances was so low, a relevant response could only be detected for samples 8 and 22 (see Figure 16).

In order to provide more interesting results and check if the method being applied was useful, three medicaments which higher concentrations in the substances of interest were analysed (see Figure 17).

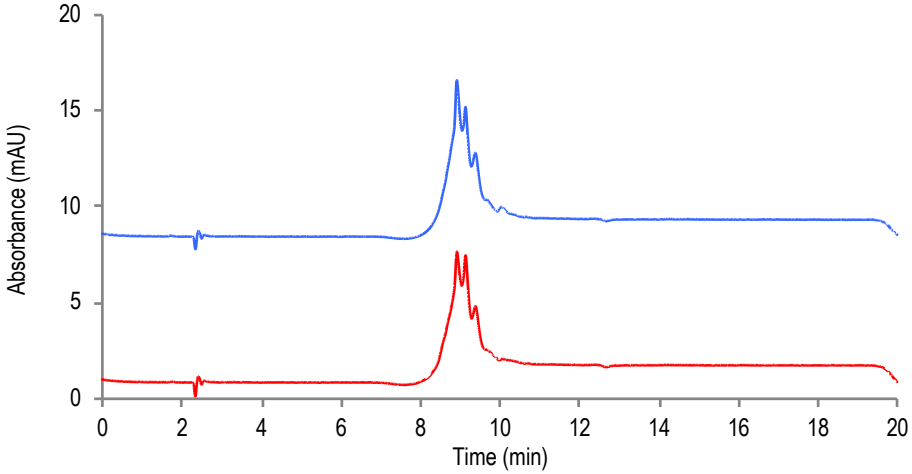

Figure 16: Chromatograms of juice samples. Assignation: ● Sample 8, ● Sample 22.

As it can be seen in the plots, a quantitative determination could not be done for any of the samples. If attention was paid to the juice samples, overlapping of the peaks corresponding to PACs A2, B2 and C1 derivatives could be observed. Thus, the studies could only provide qualitative data.

In the chromatograms corresponding to the medicaments some other factors could be highlighted. Firstly, taking into account the much larger response given by PAC C1 derivative compared to those of PACs A2 and B2, it could be concluded that this signal overshadowed the adjacent ones.

Moreover, medicaments are complex substances and the presence of oligomeric and polymeric species was expected. This could lead to a chromatogram showing unresolved humps such as the ones that were obtained.

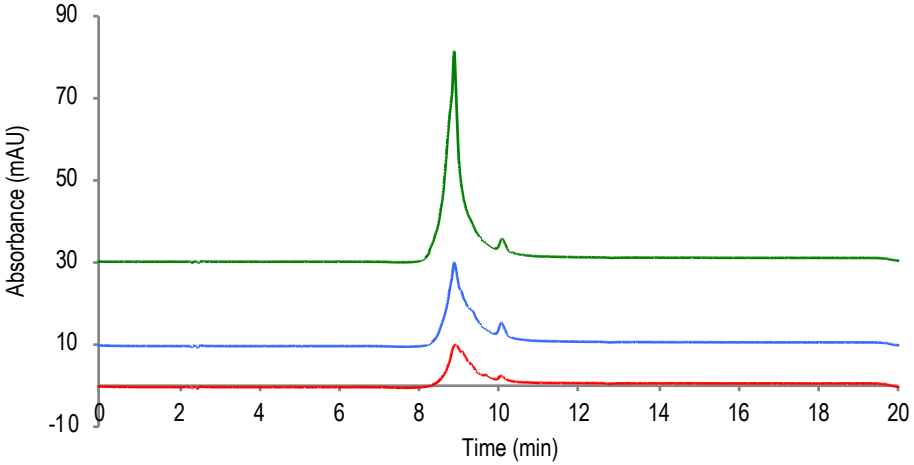

Figure 17: Chromatograms of pharmaceutical samples. Assignation: ●C02, ●C10 and ●C12.

7. CONCLUSIONS

During this work, spectrophotometric and chromatographic analyses were fulfilled in order to determine PACs in juice and pharmaceutical samples which presented complex matrices. The extracted conclusions were the following:

1. Spectrophotometric method:

    - Flavan-3-ols (catechin, epicatechin, PAC A2, PAC B2 and PAC C1) presented different behaviours when they reacted with DMAC, due to their differences in structure and type of bond. Nonetheless, as the maximum of the spectra remained constant for all of the derivatized products it was not possible to determine the substances of interest separately and only a global response could be registered.
    - Spectrophotometric studies provided better results when the analysis was done at room temperature and for 1 hour of reaction time. Other reaction conditions established were 0.16% (w/v) DMAC and 0.2M HCl. Nonetheless, the global quantifications had large standard deviations due to the fluctuations in the measurements.

2. Chromatographic method:

    - Methanol proved to be a more adequate solvent than acetonitrile when separating flavanol derivatives. The lower organic nature of acetonitrile lead to an important level of noise and the loss of symmetry of the peaks.
    - Chromatographic studies showed some improvements in the separation of the previously derivatized standards. The dimers and the trimer could be separated but the peaks corresponding to catechin and epicatechin appeared completely overlapped. Moreover, the peaks were symmetrical and narrow, which is an indicator that the elution gradient being used was adequate.
    - In the chromatographic studies the signal corresponding to PAC C1 showed a very high intensity. When the concentration of this substance was relevant the signals of PACs A2 and B2 were completely overshadowed. This drawback made it impossible to perform a quantitative analysis due to the fact that it was not possible to determine which part of the signal corresponded to each of the substances of interest.

- During the project, samples with complex matrices were studied. Oligomers and polymers of flavan-3-ols were also a part of the matrices and underwent derivatization with DMAC.

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9. ACRONYMS

DMAC: 4-(dimethylamino)-cinnamaldehyde.

DOE: Design of Experiments.

DP: Degree of Polymerization.

HPLC: High-Performance Liquid Chromatography.

PAC: Proanthocyanidin.

RP: Reversed-Phase.

UHPLC: Ultra High-Performance Liquid Chromatography.