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Development of a reversed phase high performance liquid chromatography method based on the use of cyclodextrins as mobile phase additives to determine pterostilbene in blueberries

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ABSTRACT

In this work, a reversed phase high performance liquid chromatography (RP-HPLC) method was developed for the determination of pterostilbene in food samples. The novel method is based on the addition of cyclodextrins (CDs) to the mobile phase where the complexation of pterostilbene by CDs is carried out. In order to select the most suitable conditions for the RP-HPLC method, the effect of several physico-chemical parameters on the complexation of pterostilbene by CDs was studied. Our results show that the addition of 12 mM HP-β-CD to a 50:50 (v/v) methanol:water mobile phase at 25 °C and pH 7.0 significantly improves the main analytical parameters. In addition, it was seen that pterostilbene forms a 1:1 complex with HP-β-CD, showing an apparent complexation constant of $251 \pm 13\,M^{-1}$. Finally, in order to study the validity of the proposed method, blueberries were analyzed and the concentration of pterostilbene has been determined.

1. Introduction

In recent years, many works have been published on pterostilbene (*trans*-3,5-dimethoxy-4′-hydroxystilbene) (Fig. 1 *inset*), a stilbenoid compound with many beneficial health effects [1–5]. Pterostilbene is a naturally occurring phytoalexin which has been identified in some plant species and is found in different sources such as the leaves of *Vitis vinifera* [6], in infected grape berries [7] and in healthy and immature berries of *var*. Pinot Noir and Gamay [8]. Moreover, pterostilbene is present in the heartwood of sandalwood (*Pterocarpus santalinus*) [9] and *Pterocarpus marsupium* [10] or in the berries of some *Vacciunium* species [8]. Furthermore, this potent antioxidant also appears to be a constituent of the bark of *Guibourtia tessmanii* [11] and, finally, high levels of pterostilbene were found in darakchasava [12].

In spite of the beneficial properties for the health of pterostilbene, some problems concerning the identification of this potent antioxidant, such as the low concentrations present in different foods or the poor sensitivity of the methods used in its determination, have led fewer papers being published on the occurrence of this stilbenoid in other foods than those mentioned above. Pterostilbene has previously been quantified by gas chromatography after methylation and mass spectrometry [13,14]. However, few publications have reported the analysis of pterostilbene by HPLC [3,12,15–17]. Indeed, the works that have been published mention several disadvantages such as the long analysis times required, the high organic dissolvent concentration used in the mobile phase or the poor RP-HPLC analytical parameters obtained in the method, including poor linearity, precision and sensitivity or high detection and quantitation limits. For these reasons, we have developed a new method for analyzing pterostilbene in blueberries using RP-HPLC based on the addition of cyclodextrins (CDs) in the mobile phase.

CDs are a group of structurally related natural products formed during the bacterial digestion of starch [18]. The most important functional property of CDs is their ability to form inclusion complexes with a wide range of organic guest molecules. Among the guest molecules which have been complexed by CDs, several works about the complexation of different stilbenoids with CDs have been recently published [19–23]. However, very few applications of these complexes have been reported. In this work, the use of the pterostilbene/CD complexes in the chromatographic analysis field is presented for first time.

Although the use of CDs as components of the HPLC mobile phases has been reported in several works, the principal aim of those papers was to determine the complexation constants of the guest/CD complexes under different physico-chemical conditions or to improve the separation of different enantiomers [24,25]. How-

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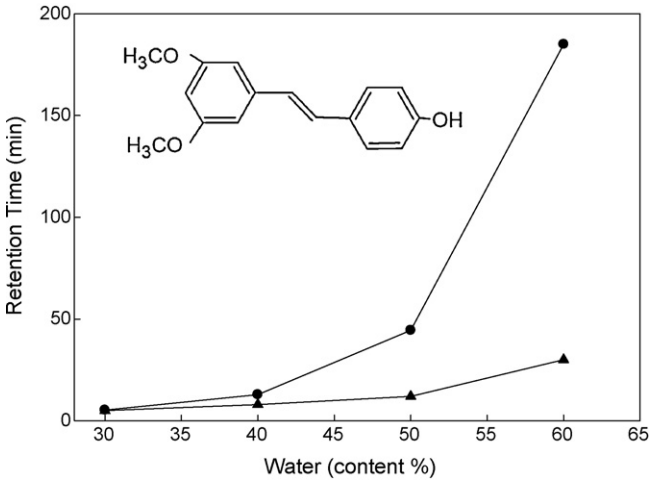

Fig. 1. Effect of the water content (%) on the pterostilbene retention time (flow-rate, 1.00 ± 0.01 mL/min, temperature, 25 °C ± 1 °C). (●) Without HP-β-CD in the mobile phase, (▲) with 8 mM HP-β-CD in the mobile phase. Each data point is the mean of 3 replicates. *Inset*: Structure of pterostilbene.

ever, the use of CDs as additives to improve an HPLC analysis method that permits the rapid analysis of low levels of a compound has not previously been reported up date.

Bearing in mind the above, this work set five principal objectives: (i) to study the complexation of pterostilbene by several type of CDs under various experimental conditions using HPLC method; (ii) to select the optimum conditions of pH, temperature, organic dissolvent and type of CD for use in RP-HPLC; (iii) to develop a new pterostilbene RP-HPLC method with high levels of precision and linearity, low detection and quantification limits and low percentage of organic solvent based on the addition of CDs to the mobile phase using two types of detectors, UV–vis and fluorescence; (iv) to calculate the apparent formation constant of the pterostilbene/HP-β-CD complexes and (v) to apply the new RP-HPLC method for analyzing the pterostilbene concentration in blueberries variety *Duke*.

2. Experimental

2.1. Chemical and reagents

Pterostilbene was from Sequoia Research Products Limited (Pangbourne, United Kingdom) and was used without further purification (Purity of 98.1% determined by HPLC). Natural (α-CD, β-CD) and modified CDs (HP-β-CD and methyl-β-CD were purchased from Sigma–Aldrich (Madrid, Spain) The water and methanol used in this study, of HPLC grade, were purchased from Análisis vínicos, S.A. (Tomelloso, Spain). Anhydrous D-glucose was supplied by Prolabo (Fontenoy-Sous-Bois, France).

2.2. Equipment and experimental procedure

Twenty microlitres of pterostilbene prepared in methanol, at the concentration mentioned in the text, were injected for HPLC analysis using a Shimadzu LC-10A (Shimadzu Corporation, Kyoto, Japan) unit equipped with an RF-10AXL fluorescence detector (Shimadzu) and a SPD-M10A photodiode array detector (Shimadzu) was used. For the aqueous mobile phase studies, a commercially available C8 column Sunfire (Milford, USA) (150 mm × 4.6 mm I.D. 5 μm particle size) was used. For all experiments the mobile phase flow-rate was systematically controlled at 1.00 ± 0.01 mL/min, the UV–vis detector was operated at 306 nm and the fluorescence detector used an excitation wavelength of 330 nm and emission wavelength of 435 nm. Mobile phases were prepared according López Nicolás and García-Carmona [23].

2.3. Temperature and pH studies

In the temperature studies, the retention factor was determined at the following temperatures: 15.0, 20.0, 25.0, 30.0 and 37.0 ± 0.1 °C. To study the effect of mobile phase pH on the retention time of pterostilbene, buffers were used in the aqueous composition of the mobile phase: 0.1 M sodium acetate buffer for pH 5.0, 0.1 M sodium phosphate for pH 5.5–8.5 and 0.1 M sodium borate for pH 9.0–11.0.

2.4. HPLC method validation

2.4.1. Determination of precision

To evaluate precision, a 2 μg/ml pterostilbene solution was brought to 10 ml with 10% methanol in a volumetric flask, and injected at 20 μl for a total of 10 times. The precision of the method was expressed by the relative standard deviations (RSD) or coefficient of variation of the data set. The mean concentrations of pterostilbene were calculated while SD and RSD were calculated using equations described by Snyder et al. [26].

2.4.2. Determination of linearity

The linearity of the method was evaluated by injecting 20 μl of the seven samples of pterostilbene standards, prepared at 0.2, 0.5, 1, 2, 4, 8 and 12 μg/ml. This set of standards was analyzed in triplicate. The correlation coefficient (r) between peak area and the concentration of pterostilbene was analyzed for each of the seven standards used.

2.4.3. Determination of LOD and LOQ

The LOD and LOQ were determined by analyzing pterostilbene solutions that were sequentially diluted in a series with methanol to obtain the lowest level of analyte that gave a measurable response with a signal-to-noise ratio of 3 and 10, respectively.

2.5. Stilbene extraction and analysis

Blueberries were purchased from a local supermarket. The protocol used for stilbene extraction was adapted from the method previously described by Langcake and Pryce [27] and Jeandet et al. [28]. Blueberries analysis was performed using both the photodiode array and fluorimetric detector. Concentrations of pterostilbene in samples were measured using the external standard method. Response factors (amount of standard/peak area) were calculated with data from the standard calibration curve.

3. Results and discussion

3.1. Selection of organic solvent of mobile phase

In this work, the use of the non-polar stationary phase did not allow determination of pterostilbene when water alone was used as mobile phase, as it involved very long retention times accompanied by the associated experimental error; it was therefore necessary to use an organic modifier in the mobile phase. In the selection of an organic solvent for a reversed-phase system, the retention value of the sample solute and the formation constant of inclusion complexes of the solute are important parameters since they depend on the type of organic solvent and its content in the mobile phase (López-Nicolás et al. [22]).

Acetonitrile, ethanol, methanol and 1-propanol are the most widely used organic solvents in RP-HPLC. In this work, ethanol and 1-propanol were discarded for use as organic solvents because the

strong interaction between these compounds and CDs can interfere in the complexation of pterostilbene by CDs [22]. Indeed, ethanol presented a constant K_m (which describes the affinity of the organic modifier for the β-CD cavity) of 0.93 M^{-1}, and 1-propanol presented a K_m = 3.71 M^{-1} [29]. Several authors have selected acetonitrile [3,12,15,17] or methanol [16] to identify pterostilbene in different samples. These two organic dissolvents have the lowest K_m values of the above mentioned the organic solvents [29]. To choose between these two dissolvents for our investigations, pterostilbene was analyzed using two different mobile phases in the presence of equal HP-β-CD concentrations. Theoretically, and as a result of host–guest interactions, the retention time of the guest will decrease when complexation occurs in the mobile phase and increase when it takes place in the stationary phase. These changes in the retention behaviour are closely related to the stability constants of the complexes formed. In our experiment, when a MeOH:water:HP-β-CD (50:50:8 mM) mobile phase was used, the retention time (*Rt*) of pterostilbene was reduced by about 70% compared with the same mobile phase in the absence of HP-β-CD. However, a reduction of only 10% was observed when ACN:water:HP-β-CD (50:50:8 mM) mobile phase was used. Finally, several environmental problems have been associated with the use of acetonitrile. For these reasons, we selected methanol as the most suitable organic solvent to be used in the new RP-HPLC method described in this work.

3.2. Effect of mobile phase additives on pterostilbene retention

To validate the use of CDs as additives in the mobile phase for developing a new method of HPLC analysis it was first necessary confirm that the effect of CDs on the pterostilbene *Rt* is not due to the glucidic nature of the CDs but to their ability to complex hydrophobic compounds. To do this, we have studied the possible reduction of the pterostilbene *Rt* due to the presence of D-glucose in the reaction medium because glucose is a molecule included in the CD structure. Thus, various amounts of D-glucose (14 and 84 mM), corresponding to 2 and 12 mM of β-CD and HP-β-CD as regards the number of glucose units (each molecule of β-CD contains seven units of D-glucose in a ring), were added to the 50:50 (v/v) (methanol:water) mobile phase and the *Rt* of pterostilbene was checked. The *Rt* of pterostilbene in the absence of any additive was 44.62 min. Moreover, the *Rt* of pterostilbene decreased in the presence of β-CD at 2 mM (41.27 min) and HP-β-CD at 12 mM (10.6 min), whereas the addition of D-glucose did not alter the retention times (44.83 min) even though the concentration of D-glucose was the same as that of β-CD and HP-β-CD as regards the number of glucose units. Several conclusions can be deduced from these results. Firstly, the addition of CD to the mobile phase reduces the pterostilbene *Rt* due to its capacity to complex hydrophobic substances since no glucose/pterostilbene complexes exist. Moreover, the possible elution modifications observed in the presence of β-CD cannot therefore be attributed to modifications in solvent strength.

3.3. Effect of methanol concentration on pterostilbene retention in the presence and absence of cyclodextrin

The next step in our investigation was to analyze the effect of the concentration of the organic solvent in the mobile phase on the capacity factor. Fig. 1 shows, in both the absence and presence of 8 mM HP-β-CD, pterostilbene retention increased exponentially with increasing percentages of water, in the mobile phase. As shown in Fig. 1, at methanol concentrations below 50% the pterostilbene analysis leads to very long *Rt* which can result in substantial experimental errors. On the other hand, at a high methanol percentage (more than 50% v/v in binary methanol–water mixtures), a substantial amount of methanol can interact with HP-β-CD, leading to competition with pterostilbene complexation. This high degree competition between the organic solvent and the guest molecule for the CD cavity means that the decrease in the *Rt* in the presence of HP-β-CD was not significant compared with that observed in the absence of this glucidic molecule. As can be seen in Fig. 1, at these high methanol concentrations the decrease in the mobile phase polarity provokes a decrease in both complexation and *Rt*. The amount of methanol present provides a less polar mobile phase, in which the non-polar solutes become more soluble; as a consequence, the solute affinity for the hydrophobic cavity of HP-β-CD diminishes and part of the driving force for inclusion is removed.

Table 1
Comparison of the retention times, type and percentage of solvent organic used to determine pterostilbene in different works.

Type of organic solvent	% Organic solvent	Retention time (min)	Reference
Methanol	50	10.6	This work
Acetonitrile	5–80	50.0	17
Acetonitrile	10–85	21.2	15
Acetonitrile	60	20.7	12
Acetonitrile	50	15.0	3
Methanol:formic	50	26.3	16

Furthermore, the use of only 50% methanol in the mobile phase rather than the high percentages of methanol or acetonitrile used by other researchers (up to 85%, see Table 1) provides environmental benefits that only serve to confirm the advantages of the method presented in this work with respect to those reported previously.

For this reason, we chose 50% as the optimum methanol percentage to be used in our method.

3.4. Effect of pH on pterostilbene retention

Selection of the pH of the mobile phase is one of the most important steps in the development of a new HPLC method. For this reason, the pterostilbene *Rt* was studied in a wide range of pH (5.0–11.0). As shown in Fig. 2A the *Rt* of this potent antioxidant remains constant at pH values below pH 9.0. However, when the mobile phase pH is increased from pH 9.0 to 10.0, the *Rt* passes from a stable value of about 44.6 min to about 25 min in just one pH unit, as happens during the titration of a weak ionizable group. This strong decrease in the pterostilbene *Rt* may be due to the deprotonation of the hydroxyl group of this stilbenoid as has described for the complexation of *trans*-resveratrol by CDs [22,23]. Indeed, the significant decrease in the *Rt* value between pH 9.0 and 10.0 coincides with the pH region where stibenoids usually suffer the deprotonation of their hydroxyl groups [23]. A likely explanation for the dependence of the pterostilbene *Rt* on the pH is that when the reaction medium pH is above the pK_a of the guest molecule, the number of hydrogen bonds between the CDs and several compounds are lower than when the guest molecules are protonated. For this reason, the guest/CDs complexation constants also decrease at pH values higher than the pK_a values of the complexed molecule. For this reason a pH of 7.0 was selected for our experiments.

3.5. Effect of temperature on pterostilbene retention

As is known, an increase in the system temperature generally leads to a decrease in the *Rt* of the analyte. However, also it is known that this increase in the temperature can weaken the interactions between CDs and the guest molecules. In this study the effect of temperature on the *Rt* was studied for pterostilbene at five different temperatures: 15, 20, 25, 30 and 37 °C. As can be seen in Fig. 2B, an increase in the system temperature reduced the *Rt* from 65 min at 15 °C to 25 min at 37 °C. According to these results,

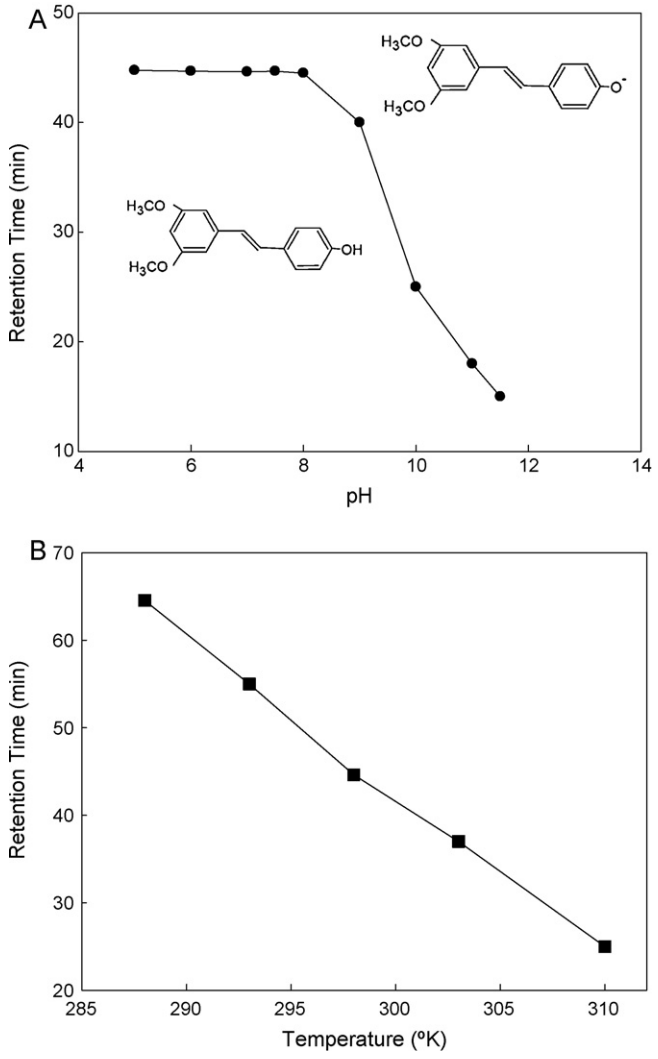

Fig. 2. Effect of pH (A) and temperature (B) on the pterostilbene retention time. Each data point is the mean of 3 replicates.

the optimum temperature to determine pterostilbene would be 37 °C because the analysis times are lower than at high temperatures. However, according to the results of several researchers [23], temperature values higher than 25 °C are not recommendable for analyzing guest molecules by HPLC in the presence of CDs because the complexes between CDs and guest molecules may be dissociated by the rupture of the interaction bonds. For this reason, a temperature of 25 °C was selected for our experiments.

3.6. Effect of different types of CD on pterostilbene retention

The next step was to determine the best type of CD to be used in the new method. Four types of natural (α-CD and β-CD) and modified CDs (methyl-β-CD and hydroxypropyl-β-CD) were used to this end and the effect of the addition of 8 mM of α-CD, methyl-β-CD and HP-β-CD and 2 mM of β-CD (this low concentration is due to the poor solubility of β-CD) on the pterostilbene *Rt* was tested.

The two types of natural CD have GRAS status and have been approved as additives in the European for food use, and the corresponding E-numbers assigned are E-457 for α-CD and E-459 for β-CD.

As is shown, the *Rt* remained practically constant when β-CD was added to the mobile phase (43.2 min) respect to the observed in the absence of CD (44.6 min). Moreover, when a α-CD was used

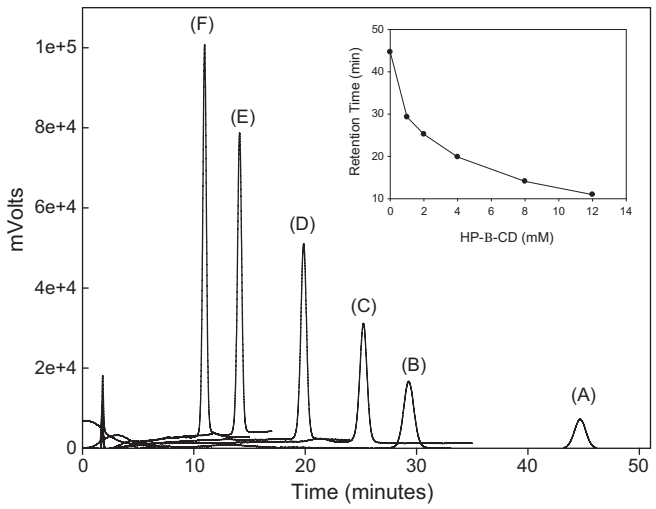

Fig. 3. HPLC chromatograms of pterostilbene at different HP-β-CD concentrations: (A) no CD, (B) 1 mM, (C) 2 mM, (D) 4 mM, (E) 8 mM, (F) 12 mM. *Inset*: Effect of HP-β-CD concentration on the pterostilbene retention time.

a slight decrease in *Rt* was observed (38.9 min). As the new HPLC method proposed in this work is based in the use of increasing concentrations of CDs to reduce the *Rt* of pterostilbene, these data led us to two conclusions: (i) The interaction of pterostilbene with natural CDs is very weak at the concentration tested due to the poor formation of the pterostilbene/α-CD or pterostilbene/β-CD complexes and (ii) natural CDs cannot be recommended to be used as additives in the mobile phases to develop a rapid method for determining pterostilbene by RP-HPLC.

On the other hand, different studies have reported that modified CDs can complex stilbenoids better than natural CDs. For this reason, and due to these above results, we used modified CDs such as HP-β-CD and methyl-β-CD in this work. Our results show a strong decrease in the *Rt* of pterostilbene when 8 mM HP-β-CD or methyl-β-CD were added to the reaction medium. Thus, HP-β-CD reduced the *Rt* from 44.6 min to 14.1 min and methyl-β-CD to 17 min. Although similar data were obtained when both modified CDs were used, the fact that HP-β-CD presents the lowest *Rt* of all the CDs tested and because several studies indicate that this type of CD presents a high complexation constant for other stilbenoid/CD complexes led us to select HP-β-CD as the most suitable type of CD in our investigation.

3.7. Effect of HP-β-CD concentration on pterostilbene retention

After the RP-HPLC conditions were determined in the presence of CDs, validation tests were performed to check retention time, precision, linearity, range and limit of detection and quantitation.

The different pterostilbene chromatograms obtained at increasing concentrations of HP-β-CD (from 0 to 12 mM) in the mobile phase are presented in Fig. 3. As is shown in Fig. 3 *inset*, a strong reduction from 44.6 to 10.6 min (around 75%) in the pterostilbene *Rt* was observed when this antioxidant was determined in a C8 Sunfire column using 50:50 water–methanol proportions containing 12 mM HP-β-CD in mobile phase. These results are due to the ability of CD to reduce the *Rt* of pterostilbene because the inclusion complexes between this stilbenoid and HP-β-CD enhances guest solubility in the mobile phase and reduced its residency time in the column. Moreover, the *Rt* presented in this work (10.6 min in the presence of 12 mM HP-β-CD) are the lowest times reported for the analysis of pterostilbene (up to 50 min) [3,12,15–17].

This reduction in the *Rt* of several compounds when different types of CDs are added to the mobile phases has been used by

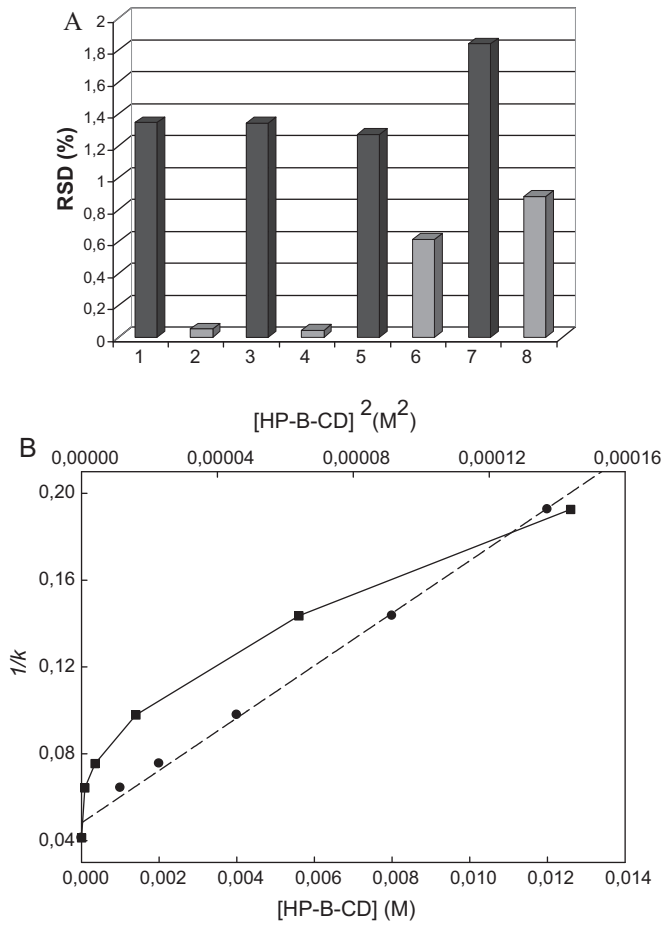

Fig. 4. (A) Relative standard deviations (RSD) for the pterostilbene measurements in the presence and absence of HP-β-CD using different detectors: (1) Rt, UV–vis, no CD; (2) Rt, UV–vis, 12 mM HP-β-CD; (3) Rt, fluorescence, no CD; (4) Rt, fluorescence, 12 mM HP-β-CD; (5) Area, UV–vis, no CD; (6) Area, UV–vis, 12 mM HP-β-CD; (7) Area, fluorescence, no CD; (8) Area, fluorescence, 12 mM HP-β-CD. (B) Effect of total HP-β-CD concentration on the reciprocal of retention factor (k) of pterostilbene for determining the stoichiometry of pterostilbene HP-β-CD complexes: $1/k$ vs. [HP-β-CD] (assumption of 1:1 complex) (filled circles), (b) $1/k$ vs. [HP-β-CD]^2 (hypothesis of 1:2 complex) (filled squares). Methanol–water (50:50%) mobile phase. Each data point is the mean of 3 replicates.

some researchers to determine the complexation constants of the guest/CD complexes. However, no work has suggested that this decrease in the *Rt* might be used to develop new method to analyse antioxidant compounds. With this in mind the effect of adding CDs on the main HPLC parameters, such as precision, linearity, reproducibility, detection and quantification limits of the analyte was studied for the first time.

3.8. Effect of HP-β-CD addition on the method precision

One of the most important factors to be borne in mind when an RP-HPLC method is developed is precision. In this work, the precision of the method was expressed by the relative standard deviations (RSD) of ten analysis of area and *Rt* of 2 μg/ml pterostilbene solutions using both UV–vis and fluorescence detection in the presence and absence of 12 mM HP-β-CD. Very interesting results for the RSD values can be observed in Fig. 4A. As regards the analysis of the pterostilbene *Rt*, no significant differences in RSD values were found between the detectors. However, reductions of 96% and 97% in the RSD values for UV–vis and fluorescence *Rt* determinations were observed when 12 mM HP-β-CD was added to the mobile phase. Indeed, this is the first study to show an increase in the precision parameter due to the presence of HP-β-CD in the RP-HPLC mobile phase.

As regards the area measurements, precision parameter differed substantially between UV–vis and fluorescence detection. In both the absence and presence of HP-β-CD, the RSD values obtained using the fluorescence detector was higher than those obtained using UV–vis. Furthermore, the precision of the area determinations was again higher when 12 mM HP-β-CD was added to the mobile phase than when this complexant agent was not present.

These results confirm that the use of 12 mM HP-β-CD in the mobile phase not only reduces the analysis time but increases the precision of the method when both UV–vis and fluorescence detection were used for the pterostilbene analysis.

3.9. Effect of HP-β-CD addition on the method linearity

The linearity of the response was examined by analyzing solutions in a range of concentration between 0.2 and 12 μg/ml. The linearity calibration curves were constructed for seven concentrations of each reference compound in triplicate. The regression equation was calculated in the form of $y = ax + b$, where y and x were the values of peak area and concentration of each reference compound, respectively. The results of the regression analyses and the correlation coefficients (r) are shown in Table 2, which illustrates the substantial differences found in the absence and presence of HP-β-CD. Firstly, the results obtained show that the correlation coefficients of the linear regression of the standard curves are greater for the fluorescence detector than for the UV–vis detector in the absence (0.9985 vs. 0.9977) and presence of HP-β-CD (0.9999 vs. 0.9992). Moreover, in the presence of 12 mM HP-β-CD the new method presents higher correlation coefficient values ($r > 0.999$) than in the absence of the complexant agents for both detectors: UV–vis (0.9992 vs. 0.9977) and fluorescence (0.9999 vs. 0.9985). These results lead us to affirm that the combination of fluorescence detector and the presence of HP-β-CD significantly increases the linearity of pterostilbene determination by RP-HPLC.

3.10. Effect of HP-β-CD addition on the LOD and LOQ

To complete the study of the validation of the new method in the presence of CD, the limit of detection (LOD) was determined with a signal-to-noise ratio of 3, and the limit of quantitation (LOQ) with signal-to-noise ratio of 10. Our results show that LOD and LOQ showed different behaviours in the absence and presence of HP-β-CD for both detectors (UV–vis and fluorescence). Although they both showed high sensitivity in all chromatographic conditions assayed, two main conclusions can be extrapolated from the data obtained: (i) in the absence and presence of HP-β-CD, LOD and LOQ presented lower values for the fluorescence detector than for UV–vis; (ii) in the presence of 12 mM HP-β-CD both limits were strongly reduced, reflecting a significant increase in the sensitivity of the method described in this work. Thus, when 12 mM HP-β-CD was added to the reaction medium, the LOD was reduced from 3.41 to 0.95 μM in the case of fluorescence detector and from 4.33 to 2.35 μM for UV–vis detector, representing increases in sensitivity of 72% and 32%, respectively, calculated at a signal-to-noise ratio of 3. A similar behaviour was observed for LOQ. Without any agent in the mobile phase, the value for this limit was 10.84 and 13.49 μM for the fluorescence and UV–vis detectors. However, the addition of HP-β-CD reduced these limits to 2.11 and 7.71 μM, respectively. These results confirm the increased sensitivity of 80% and 43% in the LOQ for the UV–vis and fluorescence detectors, respectively.

This is the first time that where a decrease in the LOD and LOQ has been demonstrated for a compound when CDs are added to the mobile phase, confirming, once again, the validity of the new

Table 2
Correlation coefficients, limits of detection (LOD) and limits of quantitation (LOQ) for pterostilbene RP-HPLC analysis in the presence and absence of HP-β-CD.

Detector	Calibration		Detection limits			
	No HP-β-CD	12 mM HP-β-CD	No HP-β-CD		12 mM HP-β-CD	
	Corr. Coeff.	Corr. Coeff.	LOD (μM^{-1})	LOQ (μM^{-1})	LOD (μM^{-1})	LOQ (μM^{-1})
Fluorescence	0.9985	0.9999	3.41	10.84	0.95	2.11
UV–vis	0.9977	0.9992	4.33	13.49	2.35	7.71

method proposed in this work to measure pterostilbene by RP-HPLC.

3.11. Study of the complexation of pterostilbene by cyclodextrins. Stoichiometry of the complexes and determination of the apparent complexation constant

One advantage of the method proposed in this work is the possibility of calculate the apparent complexation constant (K_F) between the molecule analyzed (pterostilbene) and the optimum CD selected (HP-β-CD). In view of the results obtained in previous sections, a temperature of 25 °C, a pH of 7.0 and a modified type of CD such as HP-β-CD have been selected as the best physicochemical conditions to determine the K_F between pterostilbene and HP-β-CD.

To determine the K_F value for the pterostilbene/HP-β-CD complex, equation 1, which relates the capacity factor, k, and the HP-β-CD mobile-phase concentration, [CD], is proposed [24]. In this equation we have assumed two conditions: (1) the complex presents a 1:1 stoichiometry and (2) interaction of the pterostilbene/HP-β-CD complex with the stationary phase is negligible.

$$\frac{1}{k} = \frac{1}{k_o} + \frac{K_F}{k_o}\,[CD] \quad (1)$$

where k is the capacity factor of the solute, k_0 the solute capacity factor in the absence of CD, K_F is the apparent formation constant of the inclusion complex and [CD] is the HP-β-CD mobile-phase concentration.

Although several authors have claimed that stilbenes can not form CD complexes with a 1:2 stoichiometry, López-Nicolas et al. [21] demonstrated that trans-stilbene can able to be complexed by two molecules of HP-β-CD. For this reason, we studied the possible formation of a 1:2 pterostilbene/HP-β-CD complex via a precursor 1:1 complex. Eq. (2) is an extension of Eq. (1) and includes a second-order term that accounts for the possibility of 1:2 pterostilbene–HP-β-CD complex formation:

$$\frac{1}{k} = \frac{1}{k_o} + \frac{K_{F_1}}{k_o}\,[CD] + \frac{K_{F_1}K_{F_2}}{k_o}\,[CD]^2 \quad (2)$$

where k_0 is the capacity factor of pterostilbene in the absence of HP-β-CD modifier, K_{F1} is the apparent formation apparent for the 1:1 pterostilbene/HP-β-CD complex and K_{F2} is the apparent formation constant for the 1:2 pterostilbene/HP-β-CD complex.

Using Eq. (1) and the data obtained from the chromatogram presented in Fig. 3 in the presence of increasing concentrations of HP-β-CD, a plot of the reciprocal of k vs. [HP-β-CD] should give a straight line, indicating the formation of 1:1 pterostilbene/HP-β-CD complex. However, in the case of a 1:2 pterostilbene/HP-β-CD complex formation, a plot of reciprocal of k vs. [HP-β-CD] should give a parabolic curve that fits Eq. (2). In our study, a plot of $1/k$ vs. [HP-β-CD] gave a straight line with a linear correlation higher than 0.99, indicating that the presumed stoichiometry of the pterostilbene/HP-β-CD complexes formed was 1:1 (Fig. 4B, filled circles). On the other hand, when $1/k$ was plotted against $[\text{HP-β-CD}]^2$, a non-linear relationship was obtained (linear correlation of 0.82) (Fig. 4B, filled squares), which indicates that the stoichiometry of the inclusion complex is not 2:1. Fitting the data obtained to Eq. (1), the K_F value was calculated as $251 \pm 13\ M^{-1}$.

This K_F value determined at a methanol concentration of 50% is lower that the reported ($17520 \pm 981\ M^{-1}$) by our group for the interaction between pterostilbene and HP-β-CD in aqueous solution [21] determined by fluorimetric techniques. The strong dependence of apparent K_F values on the methanol concentration is due to the existence of a strong competition on the part of methanol and solute for the HP-β-CD cavity reported by different authors [24]. Moreover, the decrease in K_F values can be interpreted is interpreted by reference to hydrophobic interactions, which are known to play a key role in the inclusion process. The transfer of a solute containing a hydrophobic moiety from a polar solvent to the hydrophobic HP-β-CD cavity, leads to a large decrease in solute free energy and favours complexation. As the mobile phase increases in polarity, the polarity difference between the HP-β-CD cavity and the eluent will become more intense. Consequently, complex formation will be even more strongly favoured.

3.12. Analysis of pterostilbene in blueberries and recovery study

In order to study the validity of the proposed method, it was applied to the determination of pterostilbene in blueberries. Although different varieties of blueberries present high concentrations of pterostilbene, other varieties not show significant values of this antioxidant [13]. For this reason it was of interest to determine whether the stilbene pterostilbene, which is also reported to have antioxidant activities, is present in blueberries variety *Duke*.

The use of photodiode array or fluorimetric detection meant that it was necessary to study the possible existence of a matrix effect. Fig. 5 shows the chromatograms obtained for blueberries samples using the photodiode array detector in the absence (II) and presence (I) of 12 mM HP-β-CDs in the mobile phase selected in the previ-

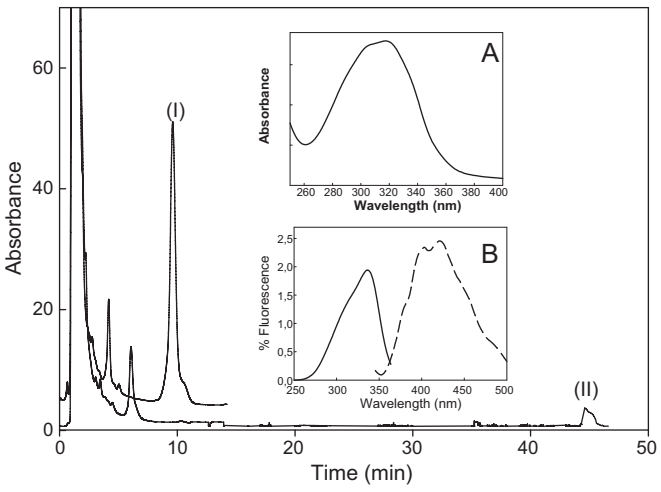

Fig. 5. HPLC analysis of blueberries in the absence (II) and presence (I) of 12 mM HP-β-CD concentrations (wavelength of monitoring: 306 nm). *Inset*: (A) Absorption spectra of pterostilbene. (B) Excitation and emission spectra of pterostilbene.

ous sections (50:50 (v/v) methanol:water mobile phase at 25 °C and pH 7.0). As can be seen, in the absence of 12 mM HP-β-CDs, the presence of pterostilbene in blueberries is practically imperceptible because of its low levels and the poor limits of both detection and quantification of traditional chromatographic methods (note the very low peak with poor definition at 44.6 min). However, due to the complexation of pterostilbene by CDs described previously, when 12 mM HP-β-CDs was added to the mobile phase, a well-defined peak emerges at 10.6 min. The peak was identified by: (a) comparing the retention data obtained for blueberries samples, the standard and the blueberries spiked with the standards under identical conditions; (b) using the photodiode array detector to continuously measure the pterostilbene UV–visible spectrum while the solute passed through the flow-cell; (c) comparing the information obtained using a fluorimetric detector.

Good agreement was found when the retention time of the pterostilbene peak obtained in the absence (44.6 min) and presence (10.6 min) of 12 mM HP-β-CDs in the mobile phase for the standard, blueberries sample and the spiked sample were compared. In addition, the absorption spectra obtained for the three samples also showed a good agreement. Fig. 5 *inset* A shows the absorbance spectrum of the pterostilbene found in blueberries samples. Absorption spectrum showed a single absorption band around 300 nm with a bandwidth of 30 nm and two small maxima centred at 304 and 335 nm. Finally, fluorimetric detection confirmed the identity of the compounds since the chromatographic profiles of blueberries and same sample spiked with the standard was identified. Good agreement was obtained for the fluorescence spectra of the chromatographic peak (Fig. 5 *inset* B). The excitation and emission spectra of pterostilbene showed a single excitation band at around 335 nm and a principal emission wavelength at around 420 nm, followed by another lower intensity emission wavelength at 400 nm and a shoulder at 378 nm.

The efficiency of both the extraction and analysis methods was confirmed by performing a recovery study. The samples were spiked at the beginning of the extraction procedure, and then spiked and unspiked samples were treated as described in the Experimental procedures before being analyzed. Absolute recoveries were evaluated by comparing the concentrations found in blueberries samples spiked with a known amount of pterostilbene standard. These were submitted to the chromatographic procedure developed in this work and the concentrations were obtained using the calibration graphs. When the spike and recovery data were combined, an average recovery ± SD ($n = 6$) of 97.89 ± 1.2% was obtained.

Once the pterostilbene was identified and the absence of a matrix effect had been confirmed, the stilbene in blueberries samples was quantified. Blueberries showed the presence a pterostilbene concentration of 110 ng/g dry sample. This concentration is similar to the obtained by Rimando et al. [13] in rabbit eye blueberry. Moreover, pterostilbene has been detected in fungos-infected grapes at levels of 0.2–4.7 μg/g fresh weight of skin [7] and also in healthy grape berries var. Gamay and Pinot Noir al levels of 14–74 ng/g and 120–530 ng/g fresh berries respectively [8].

These data confirm the validity of the novel RP-HPLC method described which uses cyclodextrins as mobile phase additives to determine pterostilbene in food samples.

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References

[1] A.M. Rimando, M. Cuendet, C. Desmarchelier, R.G. Mehta, J.M. Pezzuto, S.O. Duke, J. Agric. Food Chem. 50 (2002) 3453.
[2] A. Kathryn, K.A. Roupe, C.M. Remsberg, J.A. Yáñez, N.M. Davies, Pharmacology 1 (2006) 81.
[3] C.M. Remsberg, J.A. Yáñez, K.A. Roupe, N.M. Davies, J. Pharm. Biomed. Anal. 43 (2007) 250.
[4] R. Pezet, V.J. Pont, Phytopathology 129 (1990) 19.
[5] C.M. Remsberg, J.A. Yáñez, Y. Ohgami, K.R. Vega-Villa, A.M. Rimando, N.M. Davies, Phytother. Res. 22 (2008) 169.
[6] P. Langcake, C.A. Cornford, R.J. Pryce, Phytochemistry 18 (1979) 1025.
[7] M. Adrian, P. Jeandet, A.C. Douillet-Breuil, A.L. Tesson, R. Bessis, J. Agric. Food Chem. 48 (2000) 6103.
[8] R. Pezet, V.J. Pont, Plant Physiol. Biochem. 26 (1988) 603.
[9] R. Seshadri, Phytochemistry 11 (1972) 81.
[10] R. Maurya, A.B. Ray, F.K. Duah, D.J. Slatkin, P.L. Schiff, J. Nat. Prod. 47 (1984) 179.
[11] V. Fuendjiep, J. Wandji, F. Tillequin, D.A. Mulholland, H. Budzikiewicz, Z.T. Fomum, A.M. Nyemba, M. Koch, Phytochemistry 60 (2002) 803.
[12] B. Paul, I. Masih, J. Deopujari, C. Charpentier, India J. Ethnopharm. 68 (1999) 71.
[13] A.M. Rimando, K. Wilhelmina, J.B. Magee, J. Dewey, J.R. Ballington, J. Agric. Food Chem. 52 (2004) 4713.
[14] A.C. Douillet-Breuil, P. Jeandet, M. Adrian, R. Bessis, J. Agric. Food Chem. 47 (1999) 4456.
[15] P. Jeandet, A.C. Breuil, M. Adrian, L.A. Weston, S. Debord, P. Meunier, G. Maume, R. Bessis, Anal. Chem. 69 (1997) 5172.
[16] R. Pezet, V.J. Pont, P. Cuenat, J. Chromatogr. A 663 (1994) 191.
[17] O. Mikes, N. Vrchotová, J. Tríska, M. Kyselákova, J. Smidrkal, Czech J. Food Sci. 26 (2008) 182.
[18] J. Szetjli, Budapest, Akademial Kiado, 1982.
[19] J.M. López-Nicolás, F. García-Carmona, Food Chem. 118 (2010) 648.
[20] J.M. López-Nicolás, P. Rodríguez-Bonilla, F. García-Carmona, J. Agric. Food Chem. 57 (2009) 10175.
[21] J.M. López-Nicolás, P. Rodríguez-Bonilla, L. Méndez-Cazorla, F. García-Carmona, J. Agric. Food Chem. 57 (2009) 5294.
[22] J.M. López-Nicolás, E. Núñez-Delicado, A.J. Pérez-López, A. Carbonell, P. Cuadra-Crespo, J. Chromatogr. A 1135 (2006) 158.
[23] J.M. López-Nicolás, F. García-Carmona, Food Chem. 109 (2008) 868.
[24] I. Clarot, D. Cledat, S. Battu, P.J.P. Cardot, J. Chromatogr. A 903 (2000) 67.
[25] T. Cserháti, E. Forgacs, Cyclodextrins in Chromatography, Royal Society of Chemistry, London, 2003, pp. 47–78.
[26] L.R. Snyder, J.J. Kirkland, J.L. Glajch, Practical HPLC method development, John Wiley & Sons Inc., New York, 1997.
[27] P. Langcake, R.J. Pryce, Physiol. Mol. Plant Pathol. 9 (1976) 77.
[28] P. Jeandet, R. Bessis, B. Gautheron, Am. J. Enol. Vitic 42 (1991) 41.
[29] M. Matsui, K. Mochida, Bull. Chem. Soc. Jpn. 52 (1979) 2808.