

Automatic optosensing device based on photo-induced fluorescence for determination of piceid in cocoa-containing products

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Abstract Piceid (3,4',5-trihydroxystilbene-3- β -D-glucoside) is a stilbene which occurs naturally in various families of plants and has been shown to protect lipoproteins from oxidative damage and to have cancer chemopreventive activity. This paper deals with the determination of piceid in cocoa-containing products by using photo-induced fluorescence and the aid of a multicommutated continuous-flow assembly which was provided with an on-line photoreactor. A strongly fluorescent photoproduct is generated from piceid when it is irradiated under UV light for 30 s, which is retained on Sephadex QAE A-25 and directly monitored on this active solid support at 257/382 nm ($\lambda_{exc}/\lambda_{em}$, respectively). The pre-concentration of the photoproduct of piceid on the solid support greatly improves both sensitivity and selectivity. The influence of different experimental parameters, both chemical (pH, ionic strength) and hydrodynamic (irradiation time, flow rate, photoreactor length, sampling time), was tested. The sample pre-treatment included delipidation with toluene and cyclohexane, stilbene extraction with ethanol/water (80:20, v/v) and clean-up by solid-phase extraction on C₁₈ cartridges and methanol/water (40:20, v/v) as eluting solution. This procedure allowed the elimination of the aglycon of piceid, resveratrol and other potential interfering species and a recovery of about a 90% piceid. The method was applied to the analysis of piceid in cocoa powder, dark chocolate and milk chocolate. The quantification limits were 1.4, 1.1 and 0.09 mg kg⁻¹, respectively. Relative standard deviations ranged from 1.8%

to 3.1%. This is the first reported non-chromatographic method for determination of piceid in these foods.

Keywords Piceid · Multicommutation · Optosensor · Photo-induced fluorescence · Cocoa powder · Chocolate

Introduction

It is well known that a diet rich in fruit and vegetables helps to prevent cardiovascular problems, certain types of cancer, atherosclerosis and ageing-related disorders. This effect can be attributed to their content in polyphenols, which constitute a family of compounds whose antioxidant properties are widely documented [1, 2]. In the last years, much attention has been paid to stilbenes, a kind of polyphenol which occurs naturally in a number of plant families and can be synthesised after a stress, being considered, in this case, as phytoalexins [3]. In particular, resveratrol (3,4',5-trihydroxystilbene), which is mainly present in grapes and red wine, has demonstrated interesting physiological effects such as a cardioprotective action due to inhibition of platelet aggregation [4] and oxidation of human low-density lipoprotein (LDL) [5]; inhibition of cellular events associated with tumour initiation, promotion and propagation [6, 7]; anti-inflammatory activities [8]; prevention and control of the lipid peroxidation in membranes [9] and preventive Alzheimer's disease [10]. Resveratrol can also occur in a glycosylated form, called piceid. Both resveratrol and piceid can occur as the *cis* or *trans* stereoisomers, with the *trans* form being the dominant form.

A number of studies have demonstrated that piceid has biomedical properties similar to those mentioned above for resveratrol. Piceid isomers have been shown to be also active in inhibiting eicosanoid synthesis [11] and reducing the

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elevation of lipid levels [12]. *trans*-Piceid inhibits oxidation of human LDL [13] and platelet aggregation [14] and acts as a tumour and metastatic carcinoma inhibitor [15]. The absorption of some phenols from the diet is enhanced by conjugation with glucose [16], and then piceid could be more efficiently absorbed than its aglycone, resveratrol. Piceid has been reported in wines [17, 18], cranberries [19], peanuts [20], hop [21] and *Polygonum cuspidatum*, a traditional medicinal herb [22], and it is probably the most abundant form of resveratrol in nature [23]. Recently, *trans*-piceid was identified in chocolate and cocoa [24, 25] extracts, coming from the seeds of *Theobroma cacao* L. (Sterculiaceae), which are well known to be rich in polyphenols [26]. *trans*-Piceid concentrations ranged from 2.4 to 5 times higher on average than the level of *trans*-resveratrol in the same product categories. Therefore, cocoa-containing products such as chocolate and cocoa powder, which are consumed widely, can be considered as significant dietary sources of piceid. In this context, dark chocolate with high content of cocoa has been recognised as an important alternative antioxidant in the diet [27].

To date, only two high-performance liquid chromatography (HPLC) methods have been reported for the measurement of *trans*-piceid in cocoa-containing products using mass spectrometry [24] and diode array detection [25], respectively. In both methods, samples were previously delipidated, and stilbenes were extracted with ethanol/water (80:20, v/v) [24], previous hydrolysis for 8 h, and ethyl acetate [25]. A potential limitation of these chromatographic methods in the determination of *trans*-piceid is the risk of photoisomerisation of *trans*- to *cis*-isomer [28], which makes necessary conducting all procedures in low-light environments to minimise light-induced degradation. In addition, at the present time there are no commercially available standards for *cis*-isomers. Although this latter can be obtained from *trans*-isomer by exposure to UV radiation [29], this photoisomerisation reaction is very dependent on experimental conditions and can only be used for quantitative production of *cis*-isomers under very well-controlled experimental conditions [30]. These problems can be overcome when the total amount of piceid (*cis*- plus *trans*-isomers) is determined.

The aim of the present work was to develop an automatic fluorimetric method for the analysis of total piceid in chocolate and cocoa powder as an alternative to its chromatographic determination. The method is based on the on-line photoconversion of *trans*-piceid in a fluorescent compound by using a multicommutated assembly [31, 32]. Although *trans*-piceid itself shows a weak fluorescence, intense UV irradiation gives rise to its conversion in the *cis*-isomer, which quickly disappears in favour of strongly fluorescent photoproducts [33]. The generated photoproduct is monitored when retained on an active solid support packed in a flow cell placed in the detection area of the flow system. Solid-phase

extraction (SPE) was used to extract selectively piceid and separate it from its aglycon (resveratrol) and other interfering substances present in the samples analysed. The method was successfully applied to the determination of piceid in cocoa powder, dark chocolate and milk chocolate. To our best knowledge, for the first time a fluorimetric method is proposed for the analysis of piceid in these products.

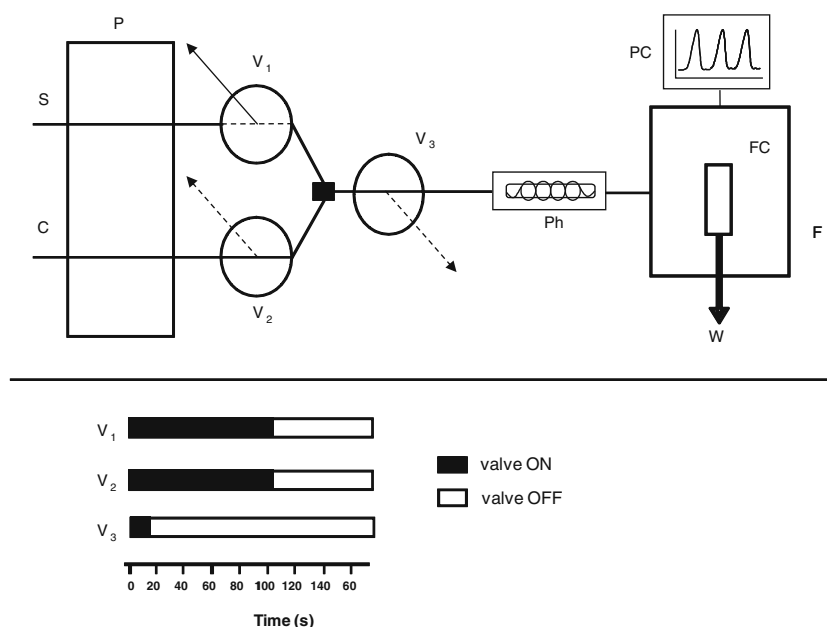
Materials and methods

Apparatus, instrumentation and manifold

Fluorescence measurements and spectra acquisition were performed on a Cary Eclipse spectrofluorimeter (Varian Inc., Mulgrave, Australia) controlled by a microprocessor fitted with the Cary Eclipse software package. The instrumental parameters established were 10 and 10 nm for excitation and emission slits, respectively, and 670 V for the voltage of photomultiplier tube. The excitation and emission wavelengths were established at 257 and 382 nm, respectively. UV-visible spectra were recorded with a Varian Cary 50 Spectrophotometer (Madrid, Spain) controlled by means of a PC fitted with the Varian computerised spectroscopy software, WIN-UV. A Hellma cell 100-QS with a light path length of 10 mm was employed.

Figure 1 shows the manifold employed in the system development. A four-channel Gilson Minipuls-3 (Villiers Le Bell, France) peristaltic pump with rate selector and pump tubes type Solvflex (Elkay Products, Shrewsbury, MA, USA) were used for propelling solutions. An electronic interface based on ULN 2803 integrated circuit was used to control the three 161 T031 NResearch three-way solenoid valves (Neptune Research, MA, USA). The valves were operated at an electric potential of 12 V and a direct current of 100 mA. The software for system control was developed in Visual Basic 6.0 by our research group. For the photochemical on-line conversion, a home-made photoreactor was prepared by loosely coiling 189 cm of polytetrafluoroethylene tubing (0.8 mm i.d.) around a low-pressure mercury lamp (15 W, 254 nm). The UV lamp was placed in an aluminium box for maximum light reflection and heat dissipation, the use of a cooling device not being necessary. The photoreactor was placed between V_3 and the detection area. All the experiments were carried out at room temperature. Sephadex QAE A-25 resin was used as active support placed in a 176.752 Hellma QS (Müllheim, Baden, Germany) flow cell (inner volume, 25 μ L; optical length, 1.5 mm). The flow-cell outlet was locked with glass wool to avoid bead movement and allow continuous flow. The cell was filled with an aqueous slurry of the solid support just up to a height which enabled the light beam to pass completely through the solid phase. Higher levels would

Fig. 1 Multicommutated flow-injection system. *S* sample (buffered with 0.02 mol L^{-1} NaAc/HAc buffer solution, $\text{pH}=5.5$); *C* carrier solution ($0.06 \text{ M NaCl}/0.001 \text{ M HNO}_3$, $\text{pH}=3.0$); *P* peristaltic pump; V_1 , V_2 and V_3 three-way solenoid valves; *Ph* photoreactor; *F* spectrofluorimeter; *FC* flow cell (filled with Sephadex QAE A-25); *W* waste; *PC* computer. For each solenoid valve, the solid and dotted lines refer to “Off” and “On” positions, respectively. Flow rate, 1.9 mL min^{-1} . The scheme at the lower part shows the valve time program (sampling time, 90 s)



cause the analyte pre-concentration on the upper part of the solid support, above the excitation light beam in the detection area, and the consequent decreasing signal; lower levels would cause a decreasing signal too, due to the light beam that would pass partially or completely through the flowing solution.

A vacuum system 12-port Visiprep SPE Vacuum Manifold (Supelco, Bellefonte, PA) with octadecyl (C_{18}) Bakerbond SPE cartridges of 6 mL with 500 mg of packing material (J.T. Baker, Phillipsburg, NJ, USA) and a Heidolph rotary evaporator (Selecta, Barcelona, Spain) ($40\text{--}50 \text{ }^\circ\text{C}$ water bath) were also used.

Reagents and solutions

All reagents were analytical reagent grade, and Milli-Q (Millipore, Bedford, MA) water was used throughout.

trans-Piceid (Sigma-Aldrich, St. Louis, MO, USA) stock solution, 200 mg L^{-1} , was prepared by dissolving the appropriate amount in ethanol (Panreac, Barcelona, Spain). It was kept away from light with an aluminium foil and stored at $4 \text{ }^\circ\text{C}$ in a refrigerator, remaining stable for at least 4 weeks. Working standard solutions were prepared daily by taking an aliquot of the stock solution and diluting with 0.02 mol L^{-1} $\text{NaCH}_3\text{COO}/\text{HCH}_3\text{COO}$ (NaAc/HAc) buffer solution, $\text{pH}=5.5$. The working solutions were stable for at least 8 h, when protected from light, which was enough to make the daily experiments.

The carrier stream consisted of 0.06 mol L^{-1} NaCl/ 0.001 mol L^{-1} HNO_3 solution ($\text{pH}=3.0$). It was prepared by dissolving the required weight of NaCl in water and setting the pH to 3.0 with HNO_3 solution (both obtained of Panreac) with the aid of a pH-meter.

Sephadex QAE A-25 ($40\text{--}120 \text{ }\mu\text{m}$ average particle size) (Sigma, Alcobendas, Madrid, Spain) was used as active solid support for filling the flow cell. Other cation and anion exchangers (Sephadex SP C-25, Sephadex CM C-25 and Sephadex DEAE A-25, all of them having $40\text{--}120 \text{ }\mu\text{m}$ average particle size) (Sigma) and C_{18} bonded phase silica gel beads ($55\text{--}105 \text{ }\mu\text{m}$ average particle size) (Waters, Milford, MA, USA) were also tested for retention of the target compounds. Methanol, ethanol, nitric acid, toluene and cyclohexane were obtained from Panreac.

Treatment of samples

The samples were purchased from local markets. Protection against light was kept during all the analysis, and the samples were stored in darkness at $4 \text{ }^\circ\text{C}$. A previous lipid removal with toluene and cyclohexane was carried out to improve the subsequent extraction. Chocolate samples were blinded and reduced to powder. The cocoa powder samples did not require grinding.

One gram (cacao powder/dark chocolate) or 2 g (milk chocolate) of sample was spiked with an appropriate amount of *trans*-piceid and treated under gentle stirring in consecutive 3-min steps, first with 5 mL of toluene (three times) and then with 5 mL of cyclohexane (three times). At the end of each step, the mixture was centrifuged for 5 min at $4,200\times g$, and the supernatant was discarded. Finally, the defatted sample was dried under vacuum ($30 \text{ }^\circ\text{C}$) to get rid of residual solvent.

The delipidated pellet was extracted three times with 4 mL of ethanol/water ($80:20, v/v$) under gentle stirring, each step for 5 min. At the end of each extraction, the sample was centrifuged for 5 min at $4,200\times g$, and the supernatant was

collected. Then the combined supernatants were dried under vacuum (30 °C) to remove any residual solvent.

A C₁₈ cartridge was conditioned by passing consecutively 3 mL of methanol and 6 mL of water. Subsequently, stilbenoid residual was passed through the cartridge at a flow rate of 3–4 mL min⁻¹ under vacuum. Then, the cartridge was washed with 6 mL of water in order to remove the most polar interfering substances. Finally, the elution of *trans*-piceid retained in the cartridge was carried out with 3×2 mL of methanol/water (40:60, v/v), and the eluate was concentrated to dryness under a gentle stream of nitrogen. The final residue was solubilised in 10 mL of 0.02 mol L⁻¹ NaAc/HAc buffer solution, pH=5.5.

Procedure

Firstly, the peristaltic pump and the UV lamp were turned on, and the carrier stream was propelled at 1.9 mL min⁻¹ to wash the whole flow system (Fig. 1) until a stable baseline was recorded, all the valves being turned off. Thus, the carrier solution flowed through the system, while the sample solution was recycled to its vessel.

The determination of piceid was carried out by following the next steps. First, valves V₁, V₂ and V₃ were turned on simultaneously for 105, 105 and 15 s (dashed lines in Fig. 1), respectively. Thus, for the first 15 s, the sample solution was directed towards the waste through V₃, thus cleaning the tubing between V₁ and V₃ with the new sample solution, whereas for the next 90 s the sample plug was pumped towards the detection area. Meanwhile, the carrier solution was recirculated to its recipient. Once all the valves were again turned off (solid lines in Fig. 1) and the sample plug reached the photoreactor, placed between V₃ and the detector, the photochemical conversion of piceid into a fluorescent photoproduct took place, and this latter was driven to the detection area, where it was temporarily retained onto the solid support and monitored (257/382 nm, λ_{ex}/λ_{em}). The solid support was regenerated by the carrier solution itself, and the system was prepared for a next sample insertion.

Every sample was inserted in the system in triplicate, and the maximum fluorescence intensity of the peak was used as analytical parameter for quantitative purposes.

Results and discussion

Optimisation of *trans*-piceid extraction

A new extraction procedure was developed to extract *trans*-piceid from cocoa powder and chocolate. Removal of fats and stilbene extraction was carried out by modifying the method previously described by Callemien et al. for the

analysis of stilbenes in hop pellets [21]. Sample clean-up procedures are generally being used to clean up polyphenol extracts prior to further analysis by HPLC [34]. In our case, the stilbene extracts also had to be purified in order to eliminate the aglycon of *trans*-piceid (*trans*-resveratrol), which is also present in cocoa and chocolate [24, 25]. In the working conditions, *trans*-resveratrol was converted into a fluorescent photoproduct, which was also retained on the solid support, Sephadex QAE A-25 (277/382 nm, λ_{ex}/λ_{em}), thus competing with the fluorescent photoproduct from *trans*-piceid for the active sites and giving rise to the spectral overlapping of both compounds. The removal of *trans*-resveratrol was carried out by using SPE on C₁₈ silica gel. Several solvents for the rinsing and elution steps were assayed: water, methanol, ethanol, ethyl acetate and diethyl ether. With this purpose, the washout fractions were monitored with the UV–visible spectrophotometer from 250 to 425 nm. A first rinsing step with water allowed the elimination of most polar compounds from the matrix as sugars. The optimum water volume was studied in the range 2–10 mL and found to be 6 mL. The elution of *trans*-piceid without eluting *trans*-resveratrol was only possible by using aqueous solutions containing different methanol percentages (20% to 50%). A methanol/water (40:20, v/v) solution allowed the elution of a 90% *trans*-piceid. In these conditions, *trans*-resveratrol remained retained on the C₁₈ beads (Fig. 2). The rest of solvents tested did not allow the sequential elution of *trans*-piceid and *trans*-resveratrol.

Preliminary tests of sorption

Piceid shows a very weak fluorescence and is photosensitive. Nevertheless, when aqueous, ethanolic or hydroethanolic *trans*-piceid solutions are UV-irradiated, it is converted first into its *cis*-isomer, which very quickly disappears to lead to the formation of a highly fluorescent photoproduct [33, 35].

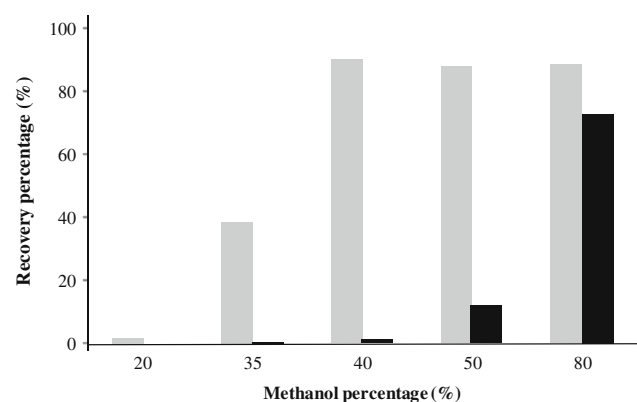


Fig. 2 Recovery percentages in SPE for *trans*-piceid and *trans*-resveratrol, according to the methanol percentage in the eluting solution (methanol/water, v/v). *trans*-Piceid (gray bars), *trans*-resveratrol (black bars); 240 ng mL⁻¹ *trans*-piceid; 240 ng mL⁻¹ *trans*-resveratrol

Therefore, although only *trans*-piceid has been identified in cocoa-containing products [24, 25], the proposed method, based on photo-induced fluorescence, allows the determination of total piceid (*trans*-isomer plus *cis*-isomer).

With the purpose of improving both sensitivity and selectivity in the determination of piceid by measuring the fluorescence of its photoproduct photochemically generated, anion exchangers (Sephadex QAE A-25 and Sephadex DEAE-25), cation exchangers (Sephadex SP C-25 and Sephadex CM C-25) and a non-ionic support (C_{18} silica gel) were tested, at different pH values, to retain this compound. The studies proved that C_{18} silica gel and both anion exchangers allowed the retention of both *trans*-piceid and its photoproduct, Sephadex QAE A-25 being the solid support that, at weakly acidic pH value, provided the maximum analytical signal.

No spectral changes were observed for aqueous *trans*-piceid solutions spectra retained on Sephadex QAE A-25, in the range of pH studied (2.0 to 9.0), showing an excitation maximum at 332 nm and an emission maximum at 392 nm. In the same pH range, the photoproduct of *trans*-piceid presented excitation and emission maxima at 257 and 382, respectively. In both cases, the fluorescence signal was greatly improved by the sorption of *trans*-piceid or its photoproduct on the solid support and their consequent pre-concentration on a little amount of this latter. When comparing the spectra obtained for *trans*-piceid and *trans*-piceid photoproduct solutions in the flow system (Fig. 1) with and without solid support in the flow cell, an improvement in the analytical signal of 12- and 40-fold, respectively, is obtained. Taking into account this enhancement in sensitivity and the complexity of the samples to analyse, the measurement of the photo-induced fluorescence was chosen for the development of the method.

Nature of sample and carrier solutions

pH was shown to be an essential variable since the analytical signal changed significantly with the pH of the sample and carrier solutions. It is necessary to point out that the optimum working pH value will be that providing the maximum conversion of *trans*-piceid in the fluorescent photodegradation product, the maximum fluorescence of the photoproduct generated and the maximum sorption of this latter on the solid support in the detection area.

The study on the influence of pH of the sample was performed by varying it between 2.0 and 9.0, using HNO_3 or $NaOH$ solutions. It was observed that for values above 7.0, signals were not obtained. A drastic decrease in the fluorescence intensity occurs in the narrow range of 5.5–7.0 (Fig. 3). The maximum fluorescence was obtained at pH 5.5. Lower pH values are also traduced in a big decrease in the fluorescence signal. Therefore, a pH value of 5.5 was selected for the next experiments. Several buffer

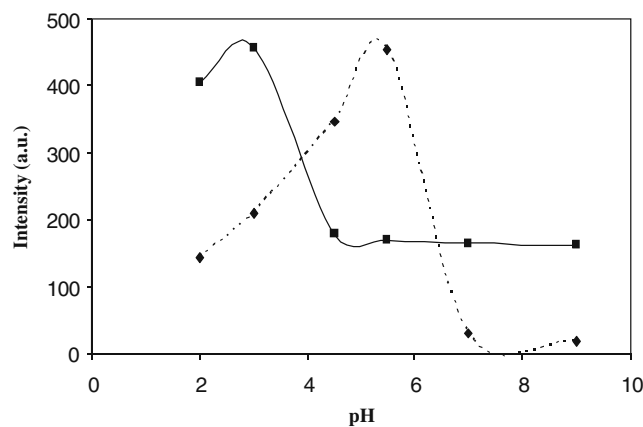


Fig. 3 Influence of pH of the sample (*dashed line*) and carrier (*continuous line*) solutions. *trans*-Piceid, 240 ng mL⁻¹; carrier solution, 0.001 mol L⁻¹ HNO_3 ; irradiation time, 30 s

solutions at pH 5.5 were tested in order to adjust the pH of the sample solution (citric acid/sodium hydroxide; sodium dihydrogen phosphate/sodium hydroxide; sodium acetate/acetic acid), and the best results were achieved by using NaAc/HAc solution. The influence of its concentration on the analytical response was assayed in the range 0.005–0.05 mol L⁻¹, and finally, a 0.02-mol L⁻¹ concentration was chosen for buffering the sample since it provided the best analytical signal. Buffer concentrations below 0.02 mol L⁻¹ originated lower signals. This was attributed to the strong sorption of the photodegradation product of piceid in an area of the solid support placed just above the light beam and to the consequent arrival of a more diluted sample plug to the irradiated area. Higher concentrations caused a lower fluorescence signal too, probably due to the competition between buffer ions and the photodegradation product for the active sites in the ionic solid support.

The influence of pH of the carrier solution was studied by inserting a 240-ng mL⁻¹ piceid solution (pH 5.5) into different aqueous solutions adjusted at pH values ranging from 1.0 to 9.0 with HNO_3 (Fig. 3). The maximum fluorescence signal was obtained at pH 3.0. For pH values higher than 3.0, a drastic decay in the fluorescence signal was obtained, this latter remaining constant and negligible from 4.5 to 9.0. The fluorescence signal also decreased for pH values lower than 3.0, and in addition, overpressure problems were observed due to the compaction of the solid support. Finally, a pH of 3.0 for the carrier solution was chosen as optimum. None of the tested aqueous solutions provided the elution of the photoproduct of *trans*-piceid in a reasonable time.

The introduction of an electrolyte in the carrier solution provided a quicker elution of the *trans*-piceid photoproduct from the solid support, so increasing the throughput. Different electrolytes ($NaCl$, KCl , $NaNO_3$, Na_2CO_3) were added to the aqueous carrier solution (pH 3.0) in concen-

trations ranging from 0.01 to 0.2 mol L⁻¹. The best results were obtained when using a 0.06 mol L⁻¹ NaCl concentration (Fig. 4). This solution provided the highest analytical signal, and a rapid and complete elution of the species was monitored, so regenerating completely this one and avoiding the use of an additional eluting solution. Concentrations higher than 0.06 mol L⁻¹ provided a quicker elution of the photoproduct, but they also originated a significant decrease in the analytical signal, probably due to the competition between the species monitored and the electrolyte anions by the active sites of the solid support. Therefore, that concentration was selected as a compromise between analytical signal and peak width. Lower concentrations produced similar analytical signals to that obtained with NaCl 0.06 mol L⁻¹ but wider peaks, decreasing the sampling frequency. The assay of different buffer solutions to adjust the pH of the carrier solution at 3.0 did not provide a significant improvement in the analytical signal or in the time of elution of the photoproduct. Therefore, the carrier solution consisted of 0.06 mol L⁻¹ NaCl/0.001 mol L⁻¹ HNO₃ (pH=3.0).

Taking into account that in multicommutated flow systems there is a very little mixing between both the sample and carrier solutions, the pH of the sample probably primarily influences the photodegradation of *trans*-piceid, whereas the pH of the carrier solution establishes the optimum conditions for the retention of the fluorescent photoproduct on the solid support.

Irradiation time

Irradiation time is, together with pH, a key variable for the generation of fluorescent photoproducts. To establish the optimum irradiation time, a 120 ng mL⁻¹ *trans*-piceid solution was inserted into the system (sampling time, 70 s; flow rate, 1.9 mL min⁻¹), the flow was stopped just when the whole plug of sample was within the photoreactor (440 cm), and the sample was irradiated for increasing periods of time (5 to 70 s). The results showed that the kinetic of photodegradation of *trans*-piceid in the working conditions is very quick. The fluorescence signal increased with the irradiation time to reach a maximum value corresponding to the optimum value of this variable (30 s), and thereafter a decrease in fluorescence intensity was obtained (Fig. 5). The shape of the curve obtained suggests a two-step photolysis mechanism, consisting of the formation of a strongly fluorescent photoproduct and the posterior photodegradation of the latter compound into non-fluorescent product(s). The length of the transport system between the photochemical reactor and the flow cell was the minimum, allowing both units to be connected.

The residence time of *trans*-piceid in the photochemical reactor and, consequently, the irradiation time can be controlled by the flow rate of the carrier solution and/or the

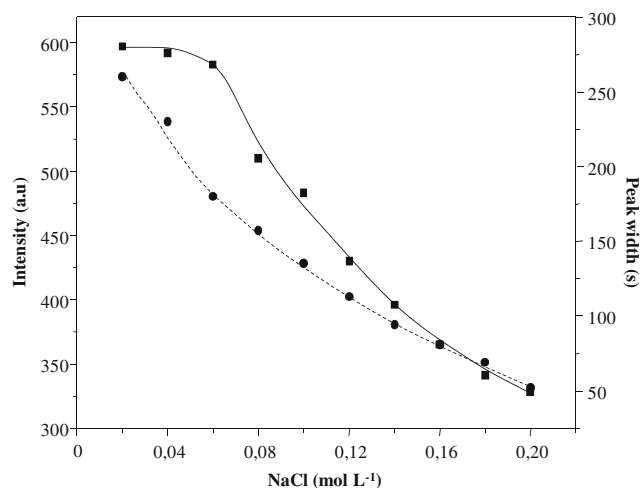


Fig 4 Influence of NaCl concentration in the carrier solution. *trans*-Piceid, 240 ng mL⁻¹; *continuous line* fluorescence intensity; *dashed line* peak width

length of the tubing around the lamp. The optimum irradiation time (30 s) was established by using the maximum flow rate allowed by the flow system, 1.9 mL min⁻¹, combined with a 189-cm photoreactor. Longer photoreactor lengths involved higher flow rates and the consequent overpressure problems. Shorter lengths allowed working at lower flow rates, although it caused a significant reduction in throughput, without providing a higher analytical signal. For these latter tests, the sampling time was conveniently varied for providing a same amount of sample.

Sampling time

In flow injection–solid phase systems, it is possible to increase the analytical signal (keeping constant the analyte concentration) by increasing the sample volume injected or, in case of multicommutated systems, by increasing the insertion time of samples. This fact is the consequence of the sorption of higher

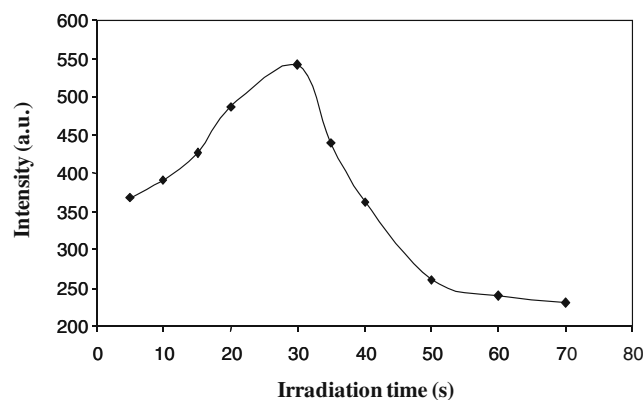


Fig. 5 Influence of irradiation time. *trans*-Piceid, 240 ng mL⁻¹; sampling time, 70 s; flow rate, 1.9 mL min⁻¹; photoreactor length, 440 cm

Table 1 Analytical figures of merit

	Parameter	Sample		
		Cocoa powder	Dark chocolate	Milk chocolate
	Linear dynamic range/mg kg ⁻¹	1.4–50	1.1–25	0.09–2.0
	Calibration graph			
	Intercept±σ ^a	44.8±0.5	44.5±0.3	42.6±0.6
	Slope±σ ^a (kg mg ⁻¹)	8.0±0.2	14.5±0.4	124±1
	Correlation coefficient	0.9993	0.9996	0.9996
Matrix-matched standard method; 90 s sampling time	Detection limit/mg kg ⁻¹	0.4	0.3	0.03
	Quantification limit/mg kg ⁻¹	1.4	1.1	0.09
^a Standard deviation	Relative standard deviation (%)	2.1 ^b	1.8 ^b	3.1 ^b
^b <i>trans</i> -Piceid concentration, 120 ng mL ⁻¹ ; n=5	Throughput (h ⁻¹)	20	20	20

amount of the species of interest on the solid support, causing an enhancement in the system sensitivity, which could allow the sample dilution in order to reduce matrix effects [36].

The influence of sampling time was tested by inserting into the flow system a 120 ng mL⁻¹ *trans*-piceid solution for sampling times ranging from 5 to 120 s. The fluorescence signal increased linearity until the value of 90 s, whereas higher sampling times did not show a significant increment in the signal. Taking into account that the flow rate and sampling time were established at 1.9 mL min⁻¹ and 90 s, respectively, it corresponded with a sample volume of 2,850 μL, which was finally selected.

Figures of merit

Quantification was carried out by using peak height as analytical signal, and data were fitted by standard least-squares treatment. Analytical parameters are shown in Table 1. A negative matrix effect was noticed, and so, the calibration curves were constructed with matrix-matched standards. With this purpose, increasing volumes of a

standard solution of *trans*-piceid were added to different aliquots of a wine sample before applying the whole analytical procedure on it. International Union of Pure and Applied Chemistry detection [37] and quantification [38] limits were calculated as the average of ten determinations.

Analytical applications

The proposed method was applied to the determination of *trans*-piceid in cocoa powder, dark chocolate and milk chocolate by following the procedure described above. A recovery study at three concentration levels was also performed on the samples analysed, and in all the cases, good recoveries were achieved. The applicability of the proposed method to the analysis of these products was demonstrated by comparison with a reference method [24]. In all the cases, the concentrations found were in good agreement with those obtained by the chromatographic method. The results obtained are shown in Table 2. The analysis of the samples by the reference method allowed to establish that, as previously reported, only the *trans*-isomer of piceid is

Table 2 Determination of *trans*-piceid in cocoa-containing products

Sample	Proposed method		Reference method ^b
	Added (mg kg ⁻¹)	Found±σ ^a (mg kg ⁻¹)	Found±σ ^a (mg kg ⁻¹)
Cocoa powder	0	5.6±0.5	5.9±0.2
	10	15.7±0.9	16.1±0.5
	20	25.3±0.5	26.0±1.1
	40	46.1±0.4	45.6±0.7
Dark chocolate	0	3.0±0.4	2.8±0.4
	10	13.5±0.2	12.5±0.6
	15	18.3±0.3	17.6±1.2
	20	23.5±1.0	23.0±0.8
Milk chocolate	0	0.7±0.3	0.8±0.6
	0.8	1.5±1.3	1.4±0.2
	1.2	2.0±0.3	1.9±1.1
	1.8	2.4±0.5	2.7±0.9

^a Average of three replicates

^b Reference method [24]

present in cocoa powder and chocolate [24, 25]. Cocoa powder had the highest level of *trans*-piceid (5.6 mg kg⁻¹), followed by dark chocolate (3.0 mg kg⁻¹) and milk chocolate (0.7 mg kg⁻¹). The same pattern has been observed in a previous survey of cocoa-containing products [25].

Conclusions

For the first time, a non-chromatographic method for the analysis of piceid in cocoa powder and chocolate is reported. The highly fluorescent photoproduct generated by on-line photochemical conversion of piceid is strongly retained on an active solid support filling the flow cell, which provides remarkable analytical features such as an enhancement in sensitivity and selectivity, when comparing with conventional photo-induced fluorescence methods. The elimination of the aglycon of piceid, resveratrol, which shows a very similar behaviour in the flow system, is carried out by SPE, selectively eluting piceid from solid support. The implementation of multicommutation principles contributes with low chemical consumption, simplicity in the handle and an enhancement in reproducibility, as well as a higher sampling throughput. The proposed method can be considered as an interesting alternative to the chromatographic determination of piceid in these food products since it provides higher throughputs and lower cost of instrumentation. In addition, although the proposed fluorimetric method is not able to distinguish between *trans*- and *cis*-piceid, the determination of total piceid overcomes the serious problem present in chromatographic methods due to the risk of photoisomerisation of *trans*-piceid.

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