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# High-performance liquid chromatography of phenolic aldehydes with highly selective fluorimetric detection by means of postcolumn photochemical derivatization

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**Abstract**

Postcolumn photochemical derivatization was used in the HPLC with fluorimetric detection of polyphenolic aldehydes in samples which also have present phenolic acids at higher concentrations. This is the most common situation in many food and agricultural samples. Phenolic aldehyde photoproducts give highly sensitive fluorescent signals whereas for some phenolic acids with native fluorescence this property disappears as a consequence of the photoreaction. Individual parameters for each polyphenolic photoproduct (excitation and emission wavelength) were investigated, which allowed the resolution of complex mixtures to be optimized. The selectivity can be improved even further by means of time-programmed changes in the excitation/emission wavelength pairs in the fluorescence detector during the chromatographic elution.

## 1. Introduction

The term "phenolic" or "polyphenol" can be defined chemically as a substance that possesses an aromatic ring bearing one or more hydroxyl substituents, including functional derivatives (esters, methyl ethers, glycosides, etc.) [1]. Phenolic compounds are closely related to the sensory and nutritional quality of fresh and processed plant foods and their analytical control have been of growing interest in recent years because many phenolic compounds in foods seem to have inhibitory effects on mutagenesis and carcinogenesis [2]. The role of such compounds in oenology has been widely demonstrated [3,4]. They affect the quality of wines, in both positive and negative ways.

The determination of such compounds is usually carried out by separation by gradient high-performance liquid chromatography (HPLC) with UV detection [5], although there have been some studies using electrochemical [6–8] and fluorimetric detection [9,10].

In recent years, photochemical postcolumn derivatization had led to improvements in selectivity and sensitivity in the detection of several types of compounds. The advantages of using this type of postcolumn reactor have now been clearly demonstrated [11–17]. Several papers [18–20] have described simple techniques for the construction of these reactors, which are also available commercially [21]. Cela et al. [22] have published data about the applicability of a lab-

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oratory-made photochemical reactor to improve the UV detection and identification possibilities of a large number of polyphenolic species separated by HPLC. These results suggest that many of these species undergo photochemical changes that can be used to improve detectability not only UV absorption [22] but also by fluorimetric and electrochemical methods [23].

In most food and agricultural derived materials, phenolic acids appear in a high concentrations in comparison with phenolic aldehydes. The absorption spectra of both phenolic families are very similar. Normally, the determination of the aldehydes is strongly hampered by overlapping acid peaks when the chromatographic system cannot resolve completely the mixtures in natural samples, which is the most common situation. However, the fluorescence response of phenolic aldehydes generally increases after photochemical reaction, whereas most phenolic acids show the opposite behaviour. Therefore, by optimizing the individual parameters of each type of compound in the fluorimetric detector, this effect can be used to detect and determine aldehydes selectively in the presence of relatively large amounts of acids.

In this paper, the results of such a study covering 36 polyphenolic species (22 phenolic acids and 14 phenolic aldehydes) are presented.

## 2. Experimental

All of the standards of the 36 polyphenolic species (22 acids and 14 aldehydes) used in this study were of purity 97% from Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland). All solutions were prepared in methanol (HPLC grade, Merck)–water [purified with a (Milli-Q Millipore, Milford, MA, USA)] (1:1) in the 10–100 ppm range. Fluorescence spectra of all the standards were measured by means of a Kontron (Zurich, Switzerland) Model SFM23 spectrofluorimeter, sweeping the spectral region between 20 and 30 nm over the absorption maximum wavelength and 700 nm.

Methanol and HPLC-grade water both containing 0.5% formic acid (95%; Merck) were used as solvents in gradient elution. As we have already shown [22], formic acid run better than acetic acid and other acids when photochemical derivatization has to be carried out. Mixtures of the species studied were eluted using the multi-segmented gradient depicted in Table 1; the retention times and capacity factors of the polyphenolic compounds are given in Table 2. The injection volume was always 15 $\mu$l, except for 10 $\mu$l when the flow-injection analysis (FIA) mode was used. All UV chromatograms were recorded over the range 230–450 nm with 2.0-nm resolution.

The experimental set-up consisted of a pump (Waters–Millipore Model 600), a universal injector (Waters Model U6K), a reversed-phase chromatographic column (Waters Novapack $C_{18}$, 15 cm × 3.9 mm I.D.; 4 $\mu$m) and a second injection valve (Model 7010; Rheodyne, Cotati, CA, USA) fitted with a 10-$\mu$l loop for injections in the FIA mode, a laboratory made photoreactor and a pair of detectors in series.

Diode-array (Waters Model 990+) and fluorescence programmable (PU4027, Philips, Cam-

Table 1
Gradient programme used for the elution of complex mixtures of polyphenolic compounds (A = water; B = methanol)

| Time (min) | Flow-rate (ml/min) | A (%) | B (%) | Curve[a] |
|---|---|---|---|---|
| 0 | 1.00 | 95 | 5 | – |
| 5 | 1.00 | 95 | 5 | 10 |
| 20 | 1.00 | 70 | 30 | 9 |
| 30 | 1.00 | 60 | 40 | 9 |
| 40 | 1.00 | 55 | 45 | 9 |
| 50 | 1.00 | 50 | 50 | 9 |

[a] Numbers of gradient curves correspond to pre-programmed gradient profiles on the Waters M600 pump.

Table 2
Retention times and capacity factors of the polyphenolic standards under the elution conditions described under Experimental

| No. | Compound | $t_R$ (min)[a] | $k'$ |
|---|---|---|---|
| 1 | 3,4,5-Trihydroxybenzoic (gallic) acid | 2.94 | 1.16 |
| 2 | 2,4,6-Trihydroxybenzoic (protocatechuic) acid | 3.19 | 1.35 |
| 3 | 2,6-Dihydroxybenzoic (γ-resorcylic) acid | 4.55 | 2.35 |
| 4 | 3,4-Dihydroxibenzoic acid | 5.18 | 2.81 |
| 5 | 3,5-Dihydroxybenzoic (α-resorcylic) acid | 6.26 | 3.60 |
| 6 | 3,4-Dihydroxybenzaldehyde (protocatechualdehyde) | 7.52 | 4.53 |
| 7 | 4-Hydroxybenzoic acid | 9.02 | 5.63 |
| 8 | 2,5-Dihydroxybenzoic (gentisic) acid | 9.9 | 6.28 |
| 9 | 2,5-Dihydroxybenzaldehyde | 10.82 | 6.96 |
| 10 | 4-Hydroxybenzaldehyde | 11.59 | 7.52 |
| 11 | 3-Hydroxybenzoic acid | 12.67 | 8.32 |
| 12 | 3-Hydroxybenzaldehyde | 12.97 | 8.54 |
| 13 | 2,4-Dihydroxybenzoic (β-resorcylic) acid | 14.3 | 9.51 |
| 14 | 4-Hydroxy-3-methoxybenzoic (vanillic) acid | 16.63 | 11.23 |
| 15 | 2,6-Dimethoxybenzoic acid | 21.47 | 14.79 |
| 16 | 2-Hydroxybenzaldehyde (salicylaldehyde) | 21.47 | 14.79 |
| 17 | 3,4-Dihydroxycinnamic (caffeic) acid | 22.64 | 15.65 |
| 18 | 4-Hydroxy-3-methoxybenzaldehyde (vanillin) | 23.51 | 16.29 |
| 19 | 3-Hydroxy-4-methoxybenzaldehyde (isovanillin) | 25.66 | 17.87 |
| 20 | 4-Hydroxy-3,5-dimethoxybenzoic (syringic) acid | 28.81 | 20.18 |
| 21 | 2-Hydroxy-3-methoxybenzaldehyde (*o*-vanillin) | 30.29 | 21.27 |
| 22 | 3,5-Dimethoxy-4-hydroxybenzaldehyde | 30.94 | 21.75 |
| 23 | 4-Hydroxycinnamic (*p*-coumaric) acid | 30.94 | 21.75 |
| 24 | 3-Hydroxycinnamic (*m*-coumaric) acid | 32.67 | 23.02 |
| 25 | 4-Hydroxy-3-methoxycinnamic (ferulic) acid | 33.24 | 23.44 |
| 26 | 3-Methoxybenzaldehyde (*m*-anisaldehyde) | 33.47 | 23.61 |
| 27 | 3,4-Dimethoxybenzaldehyde (veratraldehyde) | 33.47 | 23.61 |
| 28 | 3,4-Dimethoxybenzoic (veratric) acid | 33.59 | 23.70 |
| 29 | 2,4-Dimethoxybenzoic acid | 33.59 | 23.70 |
| 30 | 3,5-Diemthoxy-4-hydroxycinnamic (sinapic) acid | 34.59 | 24.43 |
| 31 | 2-Hydroxycinnamic (*o*-coumaric) acid | 34.59 | 24.43 |
| 32 | 3,4,5-Trimethoxybenzaldehyde | 35.59 | 25.17 |
| 33 | 2,4-Dimethoxybenzaldehyde | 37.3 | 26.43 |
| 34 | 3,5-Dimethoxybenzaldehyde | 38.81 | 27.54 |
| 35 | 3,5-Dimethoxybenzoic acid | 39.45 | 28.01 |
| 36 | 3,4,5-Trimethoxycinnamic acid | 40.83 | 29.02 |

[a] Dead time for column ($t_0$) = 1.36 min.

bridge, UK) having independent data acquisition and control systems. The specific conditions for each analysis are specified in Section 3 or in the figure captions. A complete scheme of this experimental device has been published elsewhere [22,23], and it is reproduced in Fig. 1.

The 6 m × 0.3 mm I.D. PTFE (Supelco, Bellefonte, PA, USA) tubing knitted coil photoreactor was constructed according the method of Poulsen et al. [20] as described elsewhere [22]. The knitted coil photoreactor was placed inside a mirror prism which allowed good light reflection and efficient air-cooling ventilation.

## 3. Results and discussion

### 3.1. Preliminary experiments

As a first step, the absorption and fluorescence spectra of all the compounds under study were measured. In Fig. 2a and b the spectra for

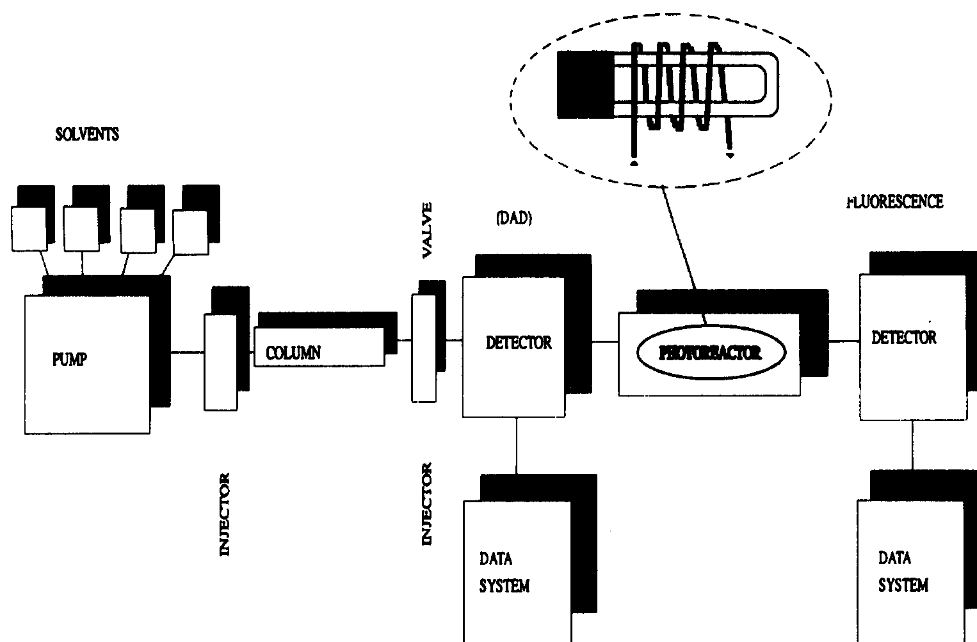


Fig. 1. Scheme of the experimental device with detail of the lamp shape.

salicylaldehyde (taken as a typical example in order to depict the complete process) are shown.

The next step was to determine each individual compound in the FIA mode, connecting the pump to the photoreactor and the fluorescence detector (Fig. 2c). The carrier was water–methanol (70:30) at a constant flow-rate of 1 ml/min. Higher proportions of methanol should be avoided because interferences appear in some spectral regions.

Each standard was injected four times, as can be seen in Fig. 2c. In the first two injections the photoreactor was turned off and in the last two injections it was turned on. This process was repeated for each excitation/emission maxima pairs for standards having more than one excitation/emission pair. Some of the species studied, seven acids (gallic, 3-hydroxybenzoic, 2,4,6-tridroxybenzoic, 4-hydroxycinnamic, 2,6-dihydroxybenzoic, *m*-coumaric and caffeic) and two aldehydes (2,5- and 3,5-dihydroxybenzaldehyde) neither showed native fluorescence nor acquired it after undergoing the photoreaction. Four different patterns were observed for the remaining compounds: some fluorescent species lost this property; others with no natural fluorescence acquired it; and the remaining showed both negative and positive changes in their original fluorescence intensity. The results obtained in this step are summarized in Table 3, showing only those excitation and emission wavelengths for which more significant changes were observed.

It must be emphasized that the lamp power plays a very important role in the photoreaction of phenolic species. As far as we know, all commercial photoreactors mount 8-W low-pressure mercury tube lamps. Although some commercial devices mount the knitted coil in front of the lamp [24], often the knitted coil is fitted around the tube lamp in order to exploit the maximum lamp radiation and minimize the coil length. The laboratory-made photoreactor used in all the experiments described in this paper has an Osram HSN10/Uafr lamp (Perez Antolin, Santiago, Spain) having a nominal power of 10 W with the shape depicted in Fig. 1. Also laboratory-made knitted coils were used used in all instances. However, when the knitted coil has to be replaced with a new one, we have occasionally encountered significant differences in the results obtained. In these instances the new coil

was discarded and a new one fitted in order to guarantee consistent performance of the photoreactor. In an attempt to avoid coil-to-coil variations we decided to try a commercial knitted tubing coil (Astec 89502; Tecknokroma, Barcelona, Spain), but it cannot be fitted over the lamp owing to its inner hole size. In fact, this knitted reactor only allows the use of the nar-

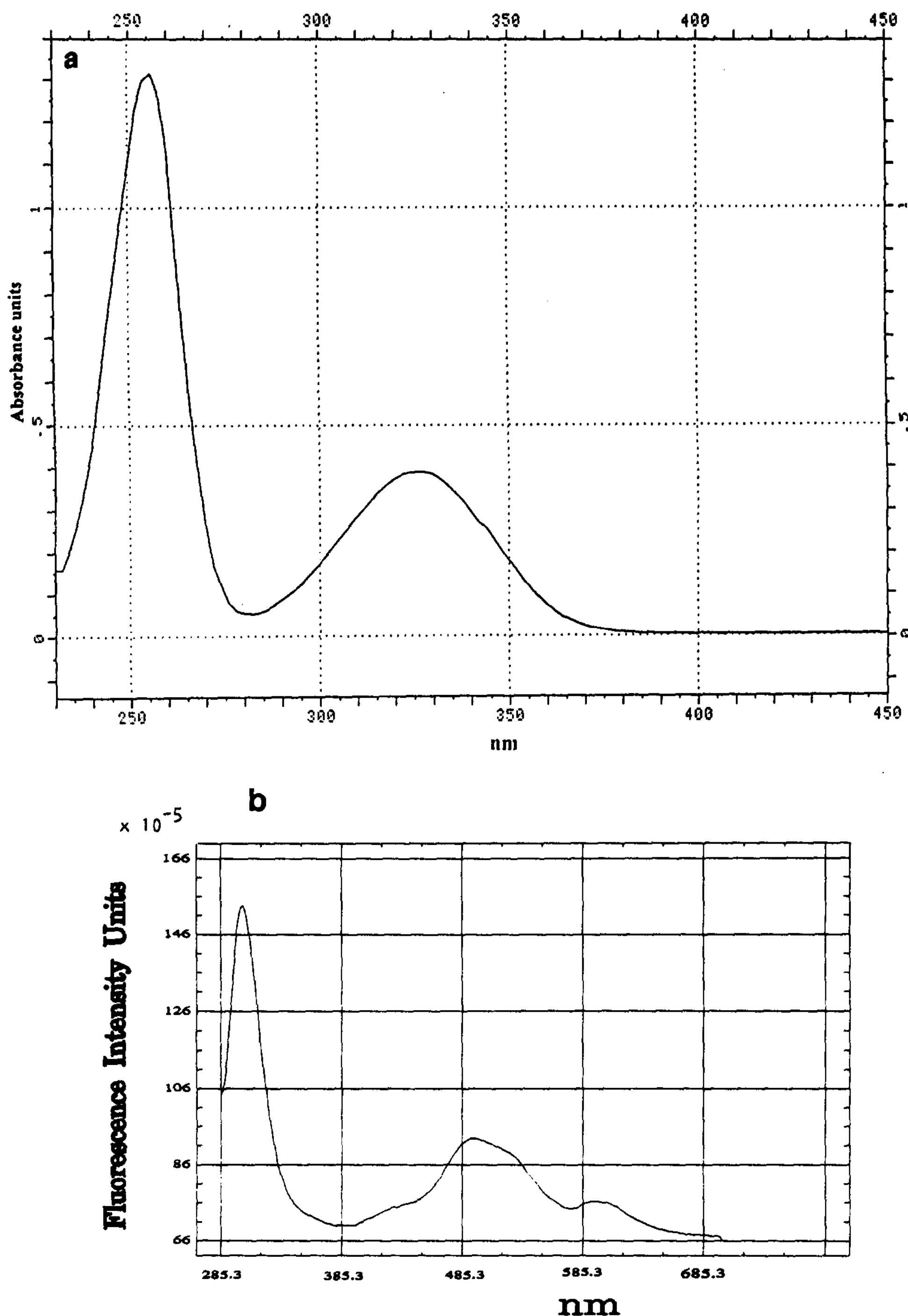


Fig. 2. (Continued on p. 36)

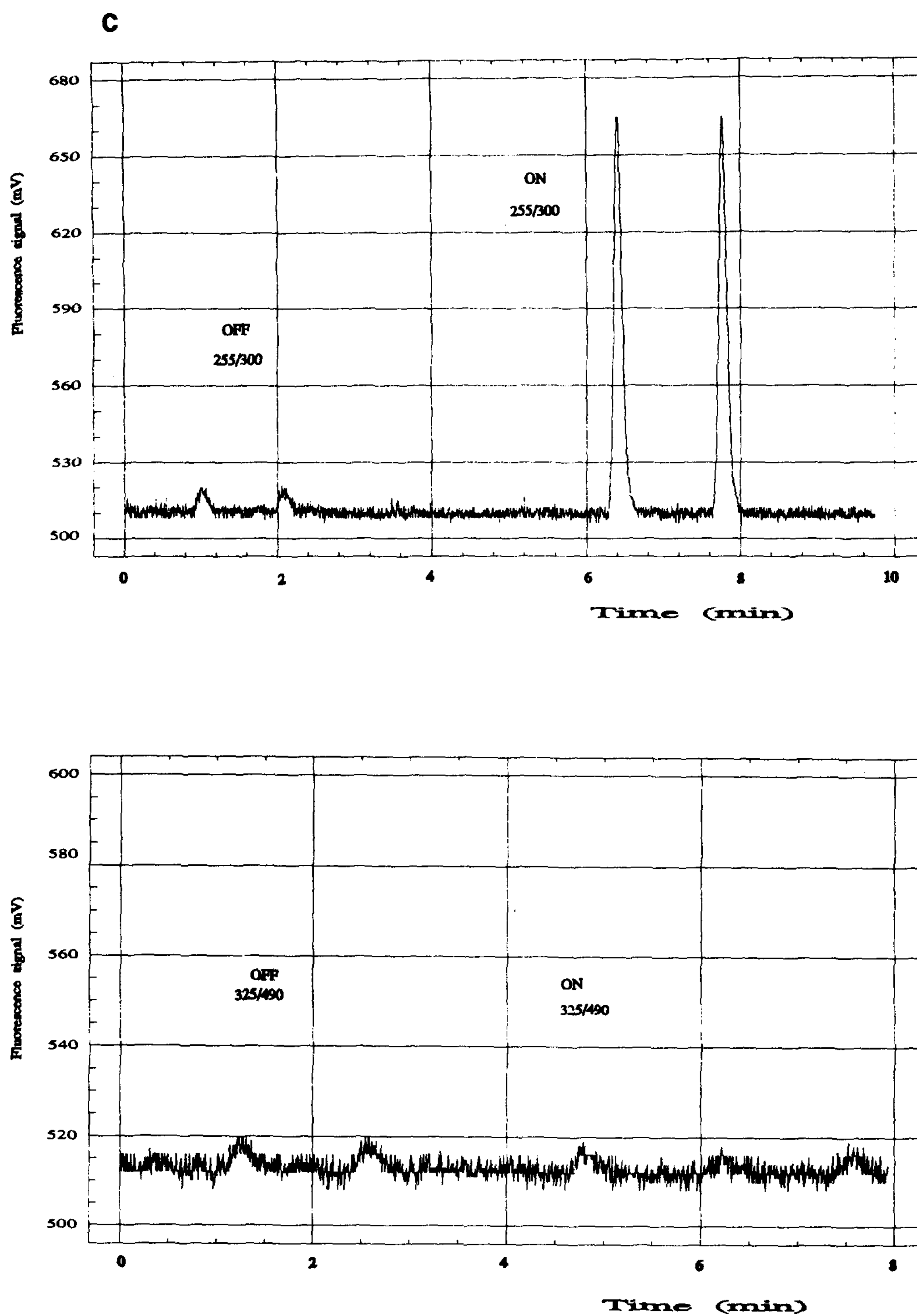


Fig. 2. Example of the steps followed in the analysis of polyphenolic standards 2-hydroxybenzaldehyde (salicylaldehyde). (a) Absorption spectrum; (b) emission spectrum; (c) fluorescence recordings obtained in the FIA mode turning the photoreactor 'on'/'off'. Mobile phase, water–methanol (70:30); flow-rate, 1 ml/min; wavelength pairs as shown in (c).

Table 3
Effect of the postcolumn photochemical reactor (PCR) on the fluorescence behaviour of the studied phenolic compounds

| Compound | $\lambda_{ex}$ (nm) | $\lambda_{em}$ (nm) | Fluorescence response[a] |
|---|---|---|---|
| 3,4,5-Trihydroxybenzoic (gallic) acid | 275 | 345 | ■ |
| 2,4,6-Trihydroxybenzoic (protocatechuic) acid | 290 | 326 | ■ |
| 2,6-Dihydroxybenzoic (γ-resorcylic) acid | 245 | 392 | ↓ |
| 3,4-Dihydroxibenzoic acid | 260 | 344 | ↓ |
| 3,5-Dihydroxybenzoic (α-resorcylic) acid | 250 | 388 | ↓ |
| 3,4-Dihydroxybenzaldehyde (protocatechualdehyde) | 280 | 311 | + |
| 4-Hydroxybenzoic acid | 255 | 320 | ↓ |
| 2,5-Dihydroxybenzoic (gentisic) acid | 335 | 439 | ↓ |
| 2,5-Dihydroxybenzaldehyde | 235 | 330 | ■ |
| 4-Hydroxybenzaldehyde | 280 | 310 | ↑ |
| 3-Hydroxybenzoic acid | 240 | 349 | ■ |
| 3-Hydroxybenzaldehyde | 255 | 302 | ↑ |
| 2,4-Dihydroxybenzoic (β resorcylic) acid | 260 | 384 | ↓ |
| 4-Hydroxy-3-methoxybenzoic (vanillic) acid | 260 | 351 | ↓ |
| 2,6-Dimethoxybenzoic acid | 280 | 376 | ↓ |
| 2-Hydroxybenzaldehyde (salicylaldehyde) | 255 | 300 | ↑ |
| 3,4-Dihydroxycinnamic (caffeic) acid | 245 | 426 | ■ |
| 4-Hydroxy-3-methoxybenzaldehyde (vanillin) | 280 | 313 | + |
| 3-Hydroxy-4-methoxybenzaldehyde (isovanillin) | 275 | 312 | ↑ |
| 4-Hydroxy-3,5-dimethoxybenzoic (syringic) acid | 275 | 359 | ↓ |
| 2-Hydroxy-3-methoxybenzaldehyde (o-vanillin) | 265 | 313 | ↑ |
| 3,5-Dimethoxy-4-hydroxybenzaldehyde | 270 | 464 | − |
| 4-Hydroxycinnamic (p-coumaric) acid | 310 | 406 | ■ |
| 3-Hydroxycinnamic (m-coumaric) acid | 280 | 405 | ■ |
| 4-Hydroxy-3-methoxycinnamic (ferulic) acid | 295 | 432 | ↓ |
| 3-Methoxybenzaldehyde (m-anisaldehyde) | 227 | 296 | ↑ |
| 3,4-Dimethoxybenzaldehyde (vertraldehyde) | 235 | 308 | ↑ |
| 3,4-Dimethoxybenzoic (veratric) acid | 260 | 349 | ↓ |
| 2,4-Dimethoxybenzoic acid | 255 | 339 | ↓ |
| 3,5-Dimethoxy-4-hydroxycinnamic (sinapic) acid | 325 | 430 | − |
| 2-Hydroxycinnamic (o-coumaric) acid | 275 | 439 | − |
| 3,4,5-Trimethoxybenzaldehyde | 285 | 438 | − |
| 2,4-Dimethoxybenzaldehyde | 235 | 339 | + |
| 3,5-Dimethoxybenzaldehyde | 310 | 418 | ■ |
| 3,5-Dimethoxybenzoic acid | 255 | 361 | − |
| 3,4,5-Trimethoxycinnamic acid | 300 | 435 | − |

[a] ↓ = The fluorescence detector signal decreases under PCR action; ↑ = the fluorescence detector signal increases under PCR's action; + = non-fluorescence compound that acquires this property by PCR action; − = fluorescence compound that loses this property under PCR action; ■ = non-fluorescent compound or photoproduct.

rower 8-W tube lamps, so we decided to change the lamp also, mounting a General Electric G8T5. With this new photoreaction device a series of experiments were carried out showing that the photoreactions do not take place at all for most of the compounds studied. The conclusion was that at least a 10-W lamp is needed in order to obtain the results described in this paper, although we are currently investigating this effect in more detail in connection with the photochemical mechanisms involved in polyphenol photoreactions.

### 3.2. Spectral characteristics of the photoproducts

The spectral data obtained from pure compounds without connecting the photoreactor

could be used to find the optimum detection conditions only if the compounds to be analysed show native fluorescence. The chromatogram depicted in Fig. 3 corresponds to the gradient elution of a 50 μg/ml each of the 36 species under study in a mixture showing that most of the species do not give any fluorescence signal. This result is not surprising because it is well known [25–27] that electron-withdrawing substituents (such as COOH and COH) in the aromatic ring tend to decrease fluorescence. However, the data in Table 3 indicate that the fluorescence characteristics of the photoproducts could be used for detection purposes. Moreover, if the spectral characteristics of the acid and aldehyde photoproducts are sufficiently different, a selective procedure to detect aldehydes in the presence of acids could be developed. In any case, it is clear that the spectral characteristics of these photoproducts of unknown nature have to be recorded in order to optimize the detector settings. However, keeping in mind that the system must operate in HPLC, it is clear that one must choose a limited number of excitation/emission pairs that can be programmed into the detector.

In Table 3 it can be seen that no excitation/emission pair can be selected as a general solution. Therefore, it was decided to register several emission spectra, fixing the excitation wavelengths in a broad range (230–320 nm) around the absorption maximum of each photoproduct.

Fig. 4a shows the absorption spectrum of the salicylaldehyde photoproduct and Fig. 4b its emission spectrum. It should be noted that some of these photoproducts are unstable and also have different photoreaction kinetics, which implies a large influence of the flow-rate [22]. This means that emission scans cannot be registered from a single injection of the compound with stopped flow when the chromatographic band reaches the detector cell. Hence, once the working flow-rate is fixed, the emission spectra have to be registered by means of several successive injections, keeping the absorption wavelength fixed at the selected value with the postcolumn photochemical reactor in the 'on' position. These injection series produce a group of ten ASCII data files for each species, which, once adequately formatted for the SURFER program (Golden Software, Golden, CO, USA), allow one to obtain a three-dimensional view or contour plot of the emission spectrum as depicted in Fig. 4b.

From a comparison of all the registered alde-

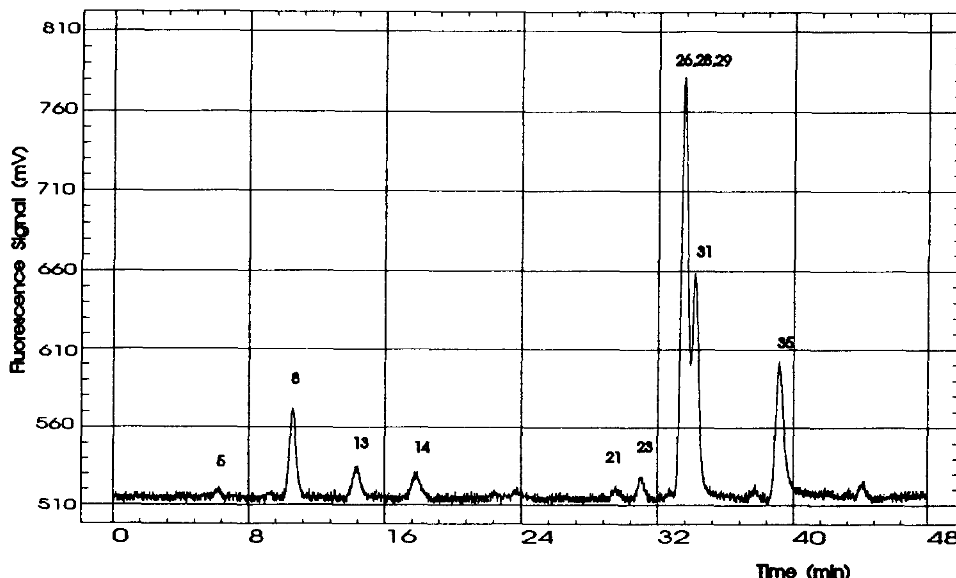


Fig. 3. Fluorescence chromatogram of a mixture of 36 polyphenolic compounds (50 μg/ml each) with photoreactor in 'off' position. Gradient conditions in Table 1. $\lambda_{ex} = 270$ nm; $\lambda_{em} = 310$ nm.

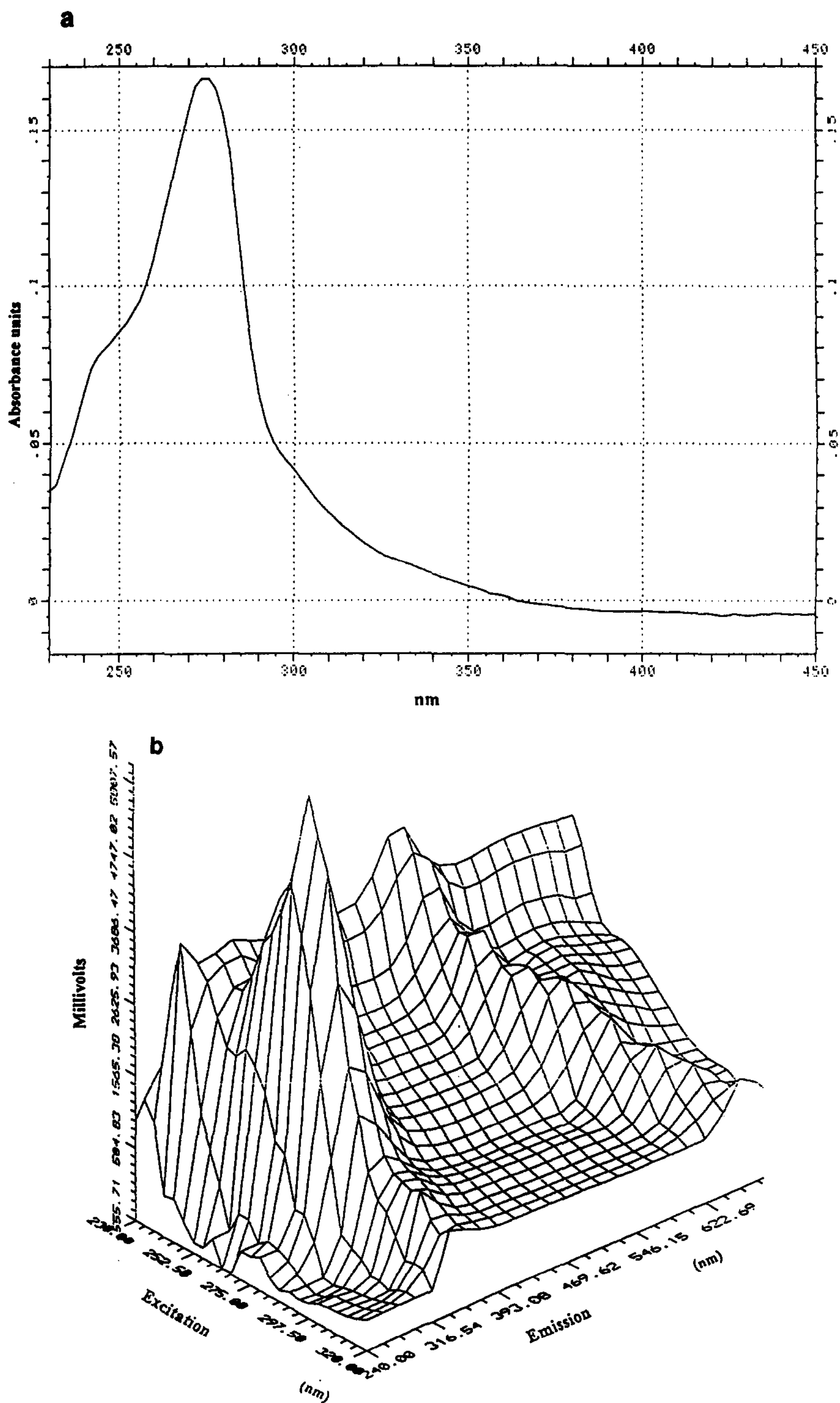


Fig. 4. Example of the steps followed in the analysis of polyphenolic photoproducts: 2-hydroxybenzaldehyde (salicylaldehyde) photoproducts. (a) Absorption spectrum; (b) experimentally reconstructed scanning emission spectrum.

hyde and acid photoproduct fluorescence spectra, it was decided that 270/310 nm is the most convenient excitation/emission wavelength pair. This pair permits the very sensitive detection of all the polyphenolic aldehydes studied except for those not photoreacting, such as 2,5-dihydroxybenzaldehyde and 3,5-dimethoxybenzaldehyde. Most of the polyphenolic acids considered do not interfere under these operating conditions, with the exception of 2,6-dimethoxybenzoic, syringic, 4-hydroxycinnamic, veratric, ferulic, 2,4-dimethoxybenzoic, *o*-coumaric and sinapic acids, which give fluorescence signals and overlap some of the aldehyde peaks.

Fig. 5 shows the UV chromatogram for the mixture of 36 polyphenolic compounds at a 50 $\mu$g/ml concentration of each. In this instance the concentration levels of polyphenolic acids and aldehydes are similar. However, it is evident that for mixtures where the concentration level of each species approaches those in real food and agricultural samples (e.g., wines), the aldehyde peaks could not be integrated owing to overlapping of the acid peaks.

The chromatogram in Fig. 3 corresponds to the same injection as depicted in Fig. 5 when the signal of the fluorescence detector was monitored with the photoreactor 'off'.

Fig. 6 shows the fluorescence chromatogram for a similar mixture with an aldehyde concentration level of 10 $\mu$g/ml each. In this instance, only the aldehyde peaks (and the above-mentioned interfering acids, of course) appear with an excellent signal-to-noise ratio. The more critical region in the chromatogram is that in the range 31–35 min. In this region ten compounds of the complex mixture eluted. In the UV chromatograms only five peaks can be distin-

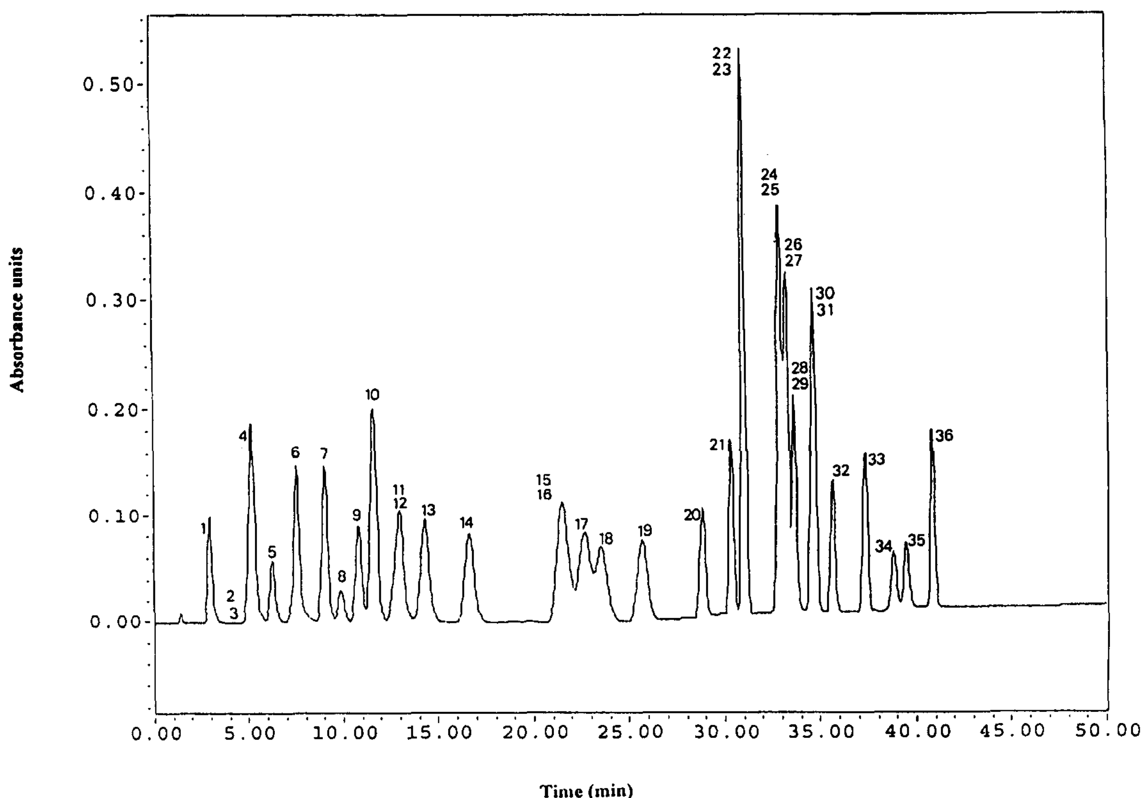


Fig. 5. UV chromatogram of a mixture of 36 polyphenolic compounds (50 $\mu$g/ml each) with photoreactor in 'off' position. Gradient conditions in Table 1. $\lambda_{ref}$ = 280 nm; resolution = 2 nm.

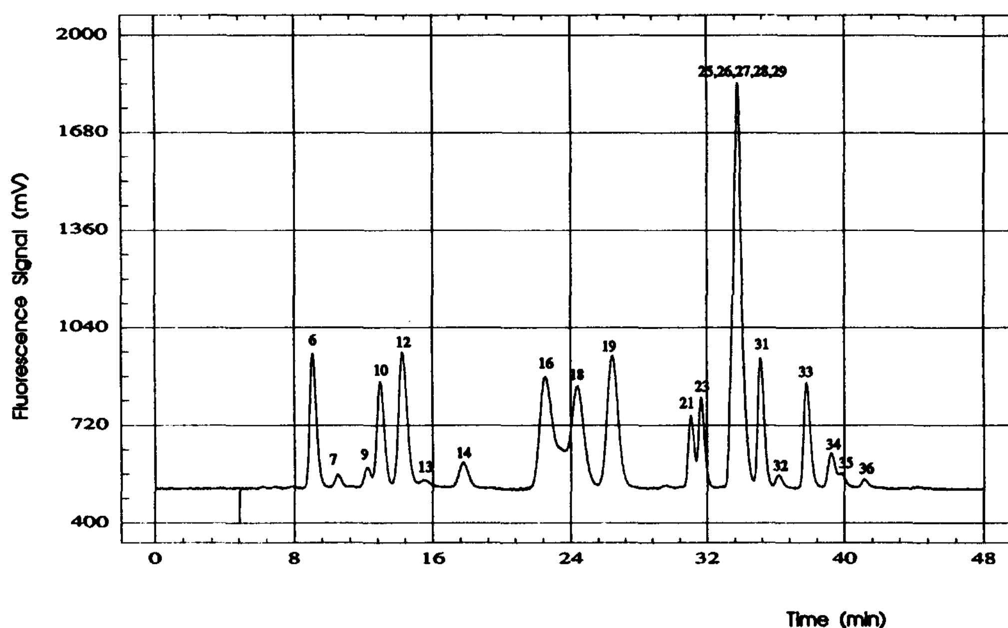


Fig. 6. Fluorescence chromatogram of mixture of 36 polyphenolic compounds (10 $\mu$g/ml each) with photoreactor in 'on' position. Gradient conditions in Table 1. $\lambda_{ex} = 270$ nm; $\lambda_{em} = 310$ nm.

Table 4
Absorption and emission maxima for phenolic aldehydes and the interfering acid photoproducts

| Photoproduct of: | $\lambda_{abs}$ (max.) (nm) | $\lambda_{em}$ (max.) (nm) |
|---|---|---|
| 3,4-Dihydroxybenzaldehyde (protocatechualdehyde) | 232/282 | 321 |
| 2,5-Dihydroxybenzaldehyde | 294 | 350 |
| 4-Hydroxybenzaldehyde | 232/276 | 310 |
| 3-Hydroxybenzaldehyde | 274 | 306 |
| 2,6-Dimethoxybenzoic acid | 255 | 260 |
| 2-Hydroxybenzaldehyde (salicylaldehyde) | 277 | 310 |
| 4-Hydroxy-3-methoxybenzaldehyde (vanillin) | 234/280 | 312 |
| 3-Hydroxy-4-methoxybenzaldehyde (isovanillin) | 234/278 | 316 |
| 4-Hydroxy-3,5-dimethoxybenzoic (syringic) acid | 275 | 400 |
| 2-Hydroxy-3-methoxybenzaldehyde (*o*-vanillin) | 278 | 313 |
| 3,5-Dimethoxy-4-hydroxybenzaldehyde | 232/276 | 340 |
| 4-Hydroxycinnamic (*p*-coumaric) acid | 280 | 354 |
| 4-Hydroxy-3-methoxycinnamic (ferulic) acid | 235/280 | 354 |
| 3,4-Dimethoxybenzoic (veratric) acid | 260/290 | 374 |
| 3-Methoxybenzaldehyde (*m*-anisaldehyde) | 243/277 | 305 |
| 3,4-Dimethoxybenzaldehyde (veratraldehyde) | 234/278 | 302 |
| 2,4-Dimethoxybenzoic acid | 255/339 | 300 |
| 3,5-Dimethoxy-4-hydroxycinnamic (sinapic) acid | 240/325 | 270 |
| 2-Hydroxycinnamic (*o*-coumaric) acid | 275 | 316 |
| 3,4,5-Trimethoxybenzaldehyde | 236/274 | 335 |
| 2,4-Dimethoxybenzaldehyde | 234/276 | 310 |
| 3,5-Dimethoxybenzaldehyde | 236/274 | 335 |

guished. Several attempts to develop a gradient curve, allowing a better separation of this region, led to the conclusion that more powerful separation techniques (e.g., multi-dimensional LC) are needed to resolve this group of very similar compounds; seven of them are 3- or 4-methoxy-substituted and the other three are structural isomers. In the fluorescence chromatogram an enormous peak appears in this region. In this peak, ferulic acid, veratric acid, veratraldehyde, *m*-anisaldehyde and 2,4-dimethoxibenzoic overlapped. If the aim of the separation process, as in this instance, is the selective detection of the aldehydes, it is possible to avoid the interference of the overlapping acids in this peak by means of an adequate programming of the excitation/emission pairs. The data in Table 4 show that careful selection of the absorption/emission pairs could help to minimize the signal of the acids in the 31–35-min band.

The chromatogram in Fig. 7 shows the result of an injection of the same mixture but programming the detector to change the excitation/emission pair from 270/310 nm to 240/305 nm at 32.5 min, returning to 270/310 nm at 35 mins. In this instance no signal of the acids was obtained in the 33–35-min band, thus allowing a more accurate integration of the aldehyde peaks.

From Fig. 7 it is evident that low detection limits for phenolic aldehydes can be achieved by the proposed procedure. The data in Table 5 show the absolute detection limits obtained for all the compounds studied showing an enhanced fluorescence signal when submitted to photochemical derivatization. Also, repeatability data for five successive injections of 5 $\mu$l of a standard mixture of these compounds with a concentration of 0.1 $\mu$g/ml each are included in Table 5. It can be seen that except for those compounds for which the concentration levels are very close to

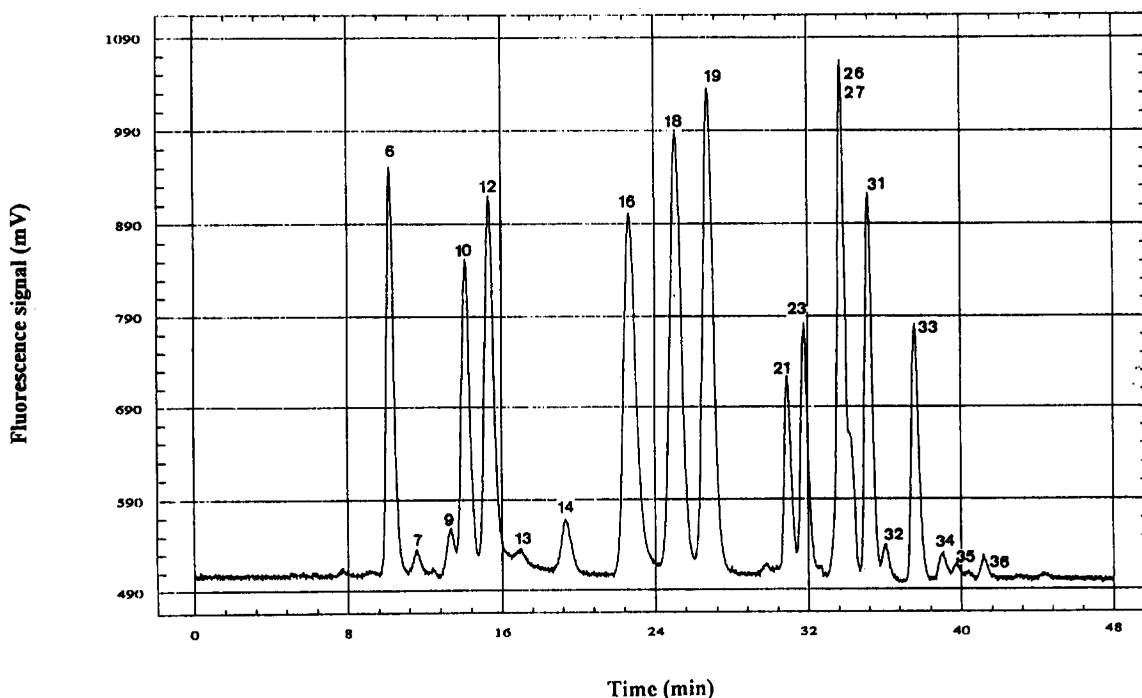


Fig. 7. Fluorescence time-programmed chromatogram of a mixture of 36 polyphenolic compounds (10 $\mu$g/ml each) with photoreactor in 'on' position. Gradient conditions in Table 1. Wavelength programme: 0–32.5 min, $\lambda_{ex}$ = 270 nm, $\lambda_{em}$ = 310 nm; 32.5–35 min, $\lambda_{ex}$ = 240 nm, $\lambda_{em}$ = 305 nm; 35–50 min, $\lambda_{ex}$ = 270 nm, $\lambda_{em}$ = 310 nm.

Table 5
Absolute limits of detection (signal-to-noise ratio = 3), peak height (mV) and relative standard deviation (R.S.D.) of the peak height for the species producing signals in the fluorescence detector under the action of the photochemical reactor

| Compound | Limit of detection (ng) | Peak height (*)[a] | R.S.D. (%) |
|---|---|---|---|
| 3,4-Dihydroxybenzaldehyde (protocatechualdehyde) | 1.2 | 83 | 15 |
| 4-Hydroxybenzoic acid | 10.0 | 4 | 42 |
| 2,5-Dihydroxybenzaldehyde | 3.3 | 16 | 29 |
| 4-Hydroxbenzaldehyde | 0.8 | 119 | 10 |
| 3-Hydroxybenzaldehyde | 0.7 | 135 | 7 |
| 2,4-Dihydroxybenzoic (β-resorcylic) acid | 10.0 | 4 | 35 |
| 4-Hydroxy-3-methoxybenzoic (vanillic) acid | 5.0 | 7 | 35 |
| 2-Hydroxybenzaldehyde (salicylaldehyde) | 0.6 | 151 | 3 |
| 4-Hydroxy-3-methoxybenzaldehyde (vanillin) | 0.8 | 123 | 4 |
| 3-Hydroxy-4-methoxybenzaldehyde (iosvanillin) | 0.8 | 129 | 4 |
| 2-Hydroxy-3-methoxybenzaldehyde (*o*-vanillin) | 2.5 | 10 | 12 |
| 4-Hydroxycinnamic (*p*-coumaric) acid | 2.0 | 19 | 19 |
| 4-Hydroxy-3-methoxycinnamic (ferulic) acid | 2.0 | 25 | 16 |
| 3-Methoxybenzaldehyde (*m*-anisaldehyde) | 0.6 | 185 | 8 |
| 3,4-Dimethoxybenzaldehyde (veratraldehyde) | 0.5 | 446 | 6 |
| 3,4-Dimethoxybenzoic (veratric) acid | 0.5 | | |
| 2,4-Dimethoxybenzoic acid | 0.5 | | |
| 2-Hydroxycinnamic (*o*-coumaric) acid | 0.6 | 158 | 9 |
| 3,4,5-Trimethoxybenzaldehyde | 10.0 | 5 | 20 |
| 2,4-Dimethoxybenzaldehyde | 1.0 | 107 | 3 |
| 3,5-Dimethoxybenzaldehyde | 2.4 | 11 | 52 |
| 3,5-Dimethoxybenzoic acid | 5 | 8 | 48 |
| 3,4,5-Trimethxoycinnamic acid | 10.0 | 4 | 50 |

[a] Average from five chromatograms.

their detection limits, the relative standard deviations obtained are satisfactory for quantitative purposes.

On the other hand, the photoreaction mechanisms for the considered compounds are still obscure. Taking into account the variety of compounds considered here, probably no unique mechanism could be responsible for the behaviour exhibited by all the species. Apparently several mechanisms could take place as a function of the lamp power. Although some data have appeared in the literature regarding the photochemistry of cinnamic acids [28] and we have made several attempts to elucidate the nature of some photoproducts by means of LC–MS and GC–MS, so far we can draw no reliable conclusions about the photoreaction mechanisms.

## 4. Conclusions

The opposite behaviour in the fluorescence response of phenolic aldehydes and acids after undergoing photochemical reactions can be exploited to improve the selectivity and sensitivity in the fluorimetric detection of the former group. Moreover, programming different pairs of excitation/emission wavelengths throughout the analysis time, interferences from the acids can be avoided, thus improving the selectivity of the aldehyde detection even further. Further research is in progress in order to elucidate the photoreaction mechanisms taking place for each species or group of compounds and to establish the effect of the lamp power on these mechanisms and the kinetics of photoreactions. Once having this knowledge, it will be possible to

exploit fully the analytical possibilities of the proposed technique.

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