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Applicability of a postcolumn photochemical reactor in the high-performance liquid chromatography of 34 polyphenolic compounds with UV detection

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ABSTRACT

The utility of postcolumn photoderivatization for a better and more reliable identification and discrimination of 34 polyphenolic compounds of interest in oenology was studied. The procedure, which is functionally very simple, allows spectral relationships to be obtained between the compounds and their photoproducts which make them easier to identify. These relationships are presented for cases in which a diode-array detector is not available. The variables affecting the experimental device (open-tubular reactor length, pH, nature of the modifier and acids used in the mobile phase) were optimized and their relative importance is discussed.

INTRODUCTION

The importance of polyphenolic compounds in the vegetable world, and particularly in oenology, has been widely demonstrated [1,2]. These compounds play an active role in organoleptic characteristics and also in various reactions that affect the quality of such products, both positively and negatively. In addition for wines they could actually become a highly valuable 'fingerprint' for classification and characterization.

The analysis of such compounds is usually carried out by means of their separation by gradient high-performance liquid chromatography (HPLC) with UV detection [3]. However, the large number of these compounds present in samples at very different concentration levels (which are nonetheless always low) makes it extremely difficult to resolve them and numerous overlaps involving two or more species are observed. Even when diode-array detectors are used and working with first or second derivative spectra [4], the identification of such species is very difficult in the case of overlaps, owing to the similarity of the spectra of many of these compounds.

On the other hand, the use of postcolumn photochemical reactors has, in recent years, led to improvements in selectivity and sensitivity in the detection of highly diverse types of compounds. The advantages of using this type of postcolumn reactor has now been clearly demonstrated [5–7]: selectivity of the reactions; absence of derivatization reagents, and consequently of interfering residues or decomposition products from the reagent; the fact that light can be introduced without additional pumps or mixing devices, and without using an additional solvent (thus, avoiding analyte dilution); rapidity of the photochemical reactions, allowing the use of shorter reactors (which limits analyte dispersion and band broadening); stability of the light sources as compared with chemical derivatization reagents in solution; no limitations between eluent selection and reagent solubility, etc. Also, several workers [8–

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10] have described simple techniques for the construction of the reactors, which are also available commercially.

This paper presents the results of a study on the applicability of such photoreactors in order to improve UV detection and identification possibilities for 34 polyphenolic species (22 phenolic acids and 12 aldehydes) having oenological interest and separated by HPLC.

EXPERIMENTAL

The experimental device is outlined in Fig. 1. It consists of a pump (Waters Model 600), a universal injector (Waters Model UK6), a reversed-phase chromatographic column (Waters Novapak C_{18}) and a second injection valve (Rheodyne Model 7010) fitted with a 10-μl loop which was used for injections in the flow-injection analysis (FIA) mode, a photoreactor made in the laboratory and a diode-array detector (Waters Model 990+) with a system for control and data acquisition.

The photoreactor was constructed according the method described by Poulsen *et al.* [10], by preparing a 1.5-m open-tubular reactor (OTR) with a 0.3 mm I.D. PTFE tube (Supelco). This coil was placed around a 10-W germicide lamp (Osram, HNS10/Uofr), operated by manual switch. The lamp and coil assembly was located inside a shiny aluminium cylinder with a diameter that is large enough for the efficient ventilation of the reactor, which is powered by a fan.

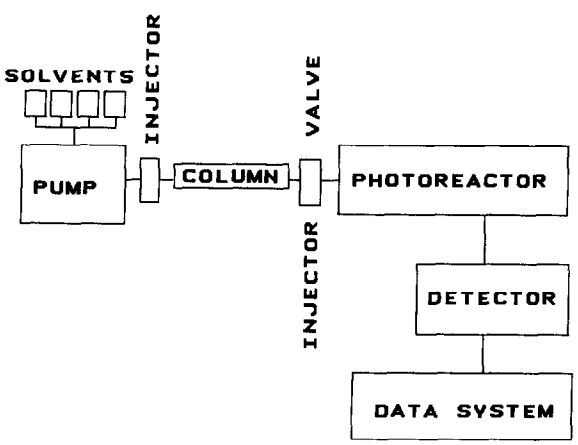

Fig. 1. Schematic view of the experimental system.

Most of the experiments described here (optimization studies) were carried out in the FIA mode, injecting fixed volumes of standard solutions (in the 10–100 ppm range) of the different compounds studied: gallic, 2,4,6-trihydroxybenzoic, 3,4-dihydroxybenzoic, α-, β- and γ-resorcylic, gentistic, *m*- and *p*-hydroxybenzoic, vanillic, 2,6-dimethoxybenzoic, caffeic, veratric, ferulic, *o*- and *m*-coumaric, sinapic, 2,4-dimethoxybenzoic, 3,5-dimethoxybenzoic and 3,4,5-trimethoxybenzoic acids (Fluka), syringic acid (Eastman) and *p*-coumaric acid (Merck) and 2,5-dihydroxybenzaldehyde, 3,5-dimethoxy-4-hydroxybenzaldehyde, 3,5-dimethoxybenzaldehyde, 2,4-dimethoxybenzaldehyde, 3,4,5-trimethoxybenzaldehyde, 3-hydroxybenzaldehyde, 4-hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, vanillin, isovanillin, orthovanillin and veratraldehyde (Fluka).

When mixtures of the species under study were injected, isocratic mobile phases consisting of methanol or acetonitrile and water modified with acetic acid (1%), formic acid (0.5%) or sulphuric acid (0.3%) were used in different proportions depending on the retention of compounds in the column. The complex mixtures of the species studied were eluted using a multi-segmented gradient described elsewhere [3]. In all instances, chromatograms were recorded over a range of 200–450 nm with 2.0-nm resolution.

RESULTS AND DISCUSSION

Initial experiments

A study was first done to evaluate the effect of the photoreactor on the compounds under study. For this purpose a mixture of these compounds was injected and eluted under methanol–water gradient conditions [3], with the photoreactor turned off. A second injection under the same conditions, but with the photoreactor turned on, showed that many of the chromatographic peaks were greatly modified by photoreaction. Using a 280-nm chromatogram as a reference, some of the peaks were seen to increase in size, whereas others decreased. Fig. 2 shows a comparison of the chromatograms for the different compound mixtures. All this led us to believe that photoderivatization does not affect all compounds in the same way, and therefore not only would it be possible to improve the detection sensi-

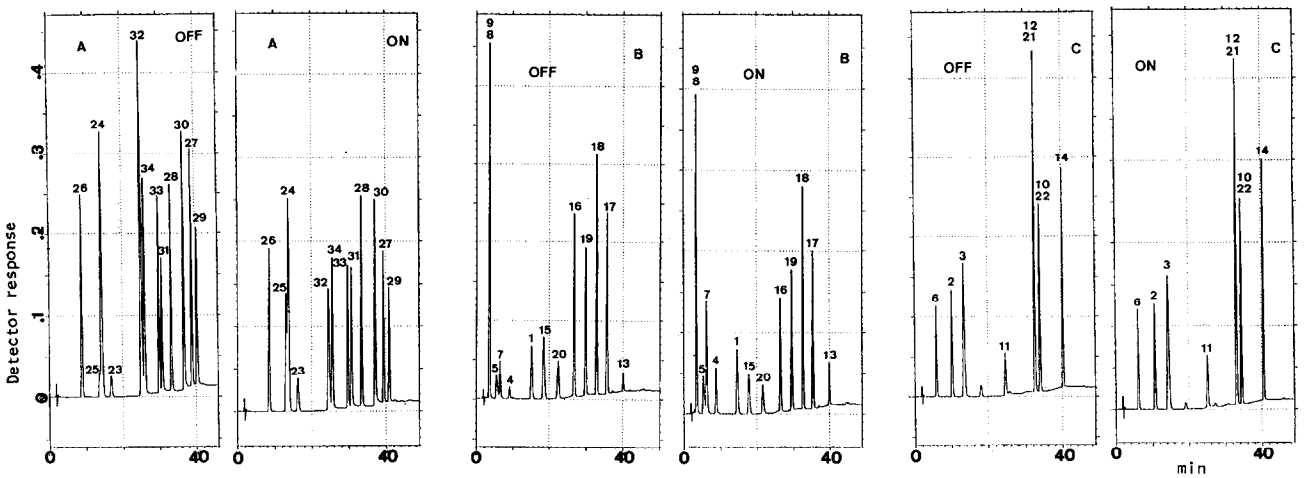

Fig. 2. Comparative chromatograms for three different mixtures of the species under study. (A) Phenolic aldehydes mixture; (B) and (C) phenolic acids mixtures. OFF = chromatograms obtained with photoreactor turned off; ON = chromatograms with photoreactor turned on. For peak identification, see Table I.

tivity in some instances but also it would be feasible to develop work plans for the selective detection or easier identification of some or most of these compounds. However, given that the 34 species could not be resolved in only one elution, an individual study of the photoderivatization of each was deemed necessary.

Optimization of the photoreactor

Before starting the individual study of each species, the operational variables of the photoreactor were optimized. The following variables were studied: coil length, pH of the mobile phase, percentage and nature of the organic modifier and nature of the acid used. All these studies were carried out in the FIA mode, injecting the compounds through the secondary fixed loop injection valve, inserted into the circuit (see Fig. 1). In these instance the chromatographic column was replaced with a damper to avoid deterioration.

Tube length used in the construction of the OTR

The optimization of this parameter was carried out by varying the mobile phase flow-rate in such a way as to make it possible to control the residence time of the compounds in the OTR effectively, simulating variations in length. All the compounds studied were injected several times with a mobile phase [methanol–water–acetic acid (50:49:1)] flow-rate of 1.5 ml/min with the photoreactor turned off. The average spectrum obtained from these injections was used in each instance as a reference point. Next, species were injected (minimum in duplicate for each established flow value) with the photoreactor turned on and for flow-rates of 1.5, 1.0, 0.8, 0.6, 0.4 and 0.2 ml/min. The graphs in Fig. 3 give the results obtained for vanillic acid. The left part refers to the reference chromatogram (in the FIA mode) at 280 nm, alongside which are superimposed the spectra corresponding to each pair of injections carried out for each value of the mobile phase flow-rate. These spectra reveal the advance of the photochemical reaction as the residence time of the compound in the OTR increases. In this particular case, the photoreaction results in the disappearance of the absorption bands at 260 and 295 nm. Similarly, it can be seen that, below 0.4 ml/min, the photoreaction is complete, but owing to the dead volumes in the system, for 0.2 ml/min the peak is noticeably split. With most of the compounds studied, the photoreaction was complete with a 0.6 ml/min flow-rate. It was appreciable at high flow-rate (1.5 and 1.0 ml/min) for some species (3,5-dimethoxybenzaldehyde, 4-hydroxybenzaldehyde and α-resorcylic acid). Only with a few of the species was it necessary to reduce the flow-rate to as low as 0.4

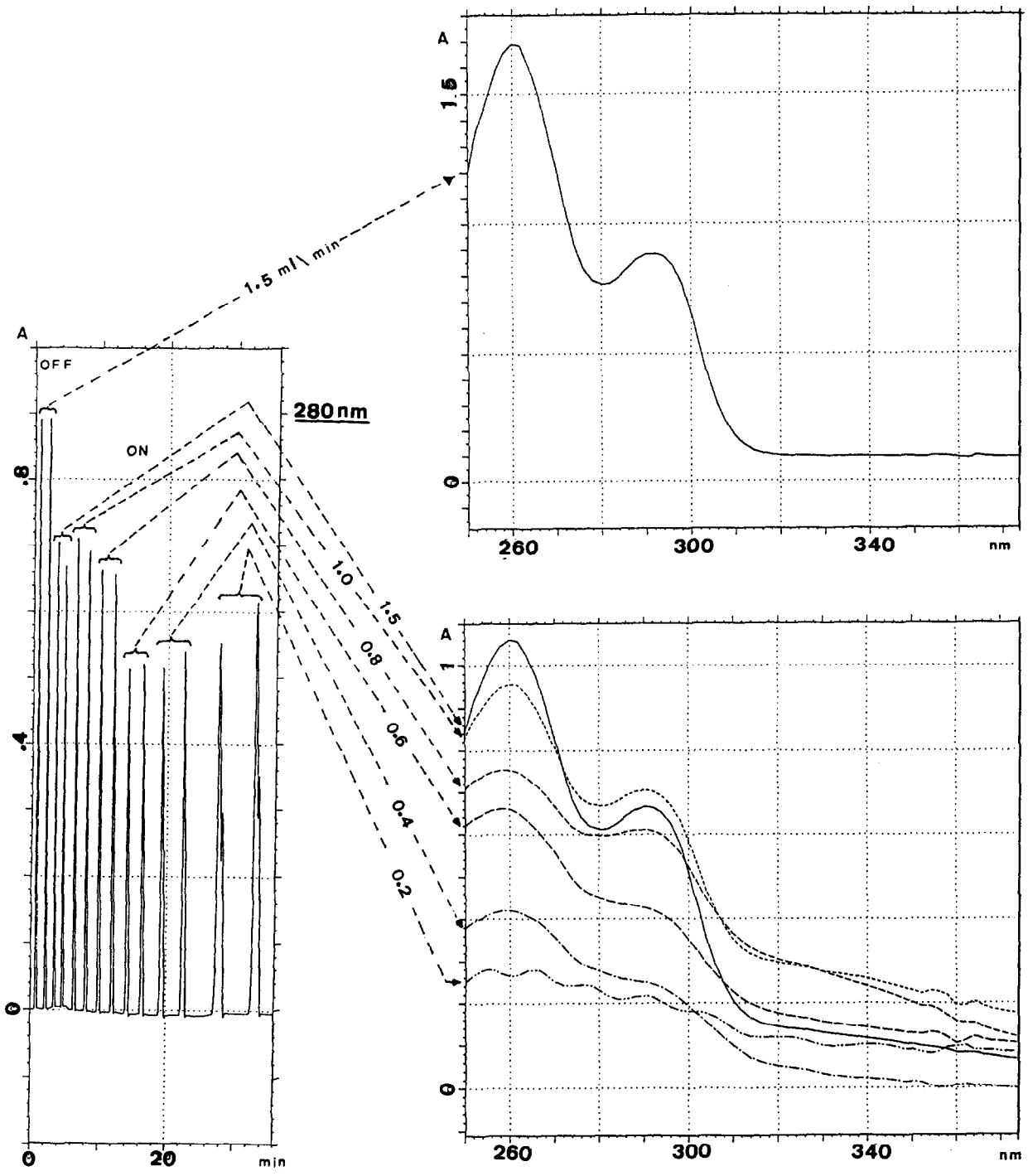

Fig. 3. Influence of the OTR tube length on the completeness of vanillic acid photodegradation (mobile phase acidified with acetic acid).

ml/min (vanillic acid, orthovanillin and vanillin) in order to obtain a complete reaction and so that gentistic and syringic acid would not undergo any appreciable changes in their spectra for any of the flow-rates tested.

It was determined that a flow-rate of 0.5 ml/min was optimum for the whole group of compounds, which is equivalent to a tube length of 4.5–5 m.

Percentage and nature of the organic modifier used

The spectral differences observed in the products from photoreaction with respect to the original species suggest that diverse photoreaction mechanisms are involved. With the aldehydes, a possible hypothesis could be the photoreduction towards the corresponding alcohol, and consequently the nature and proportion of the organic modifier used in the mobile phase could be an influencing factor. In order to verify this influence, two series of injections were made for the species under study. In the first series the proportion of methanol and the mobile phase flow-rate were modified so that for each species the spectra both with the reactor turned on and off for flow-rates of 1.0 and 0.6 ml/min and methanol contents of 20, 40, 60 and 80% were obtained. In no instance were significant modifications observed.

In the second series of injections, methanol was replaced with acetonitrile (in both instances in the presence of 1% acetic acid). The experiment was planned to be identical with the previous one. No significant modifications were observed in the spectra here either, which led to the conclusion that neither the nature of the modifier (at least for the usual solvents in RP-HPLC for these species) nor its percentage has a notable effect on the photoreactions undergone by the species under study. These findings are important from a practical point of view, in that the results of the postcolumn photoderivatization of these species can be compared with each other, regardless of the composition of the mobile phase used and also for gradient elutions.

pH of the mobile phase

Up to this point, all the experiments and injections had been carried out with acidified mobile phases (1% acetic acid, pH 2.7), as this is necessary for the separation of the species under study by RP-HPLC. However, in order to study the influence of the pH of the mobile phase on the photoreactions, two series of injections respective mobile phases of pH 4.6 and 9.1 were carried out.

No noteworthy modifications were observed in the spectra of these species or their photoproducts. Based on these results, acidified mobile phases continued to be used.

Nature of the acid used

Acetic acid is commonly used in the preparation of the mobile phases for the separation of phenolic compounds. In order to find out if the nature of the acid used has any influence on photoreactions, two series of injections were made in which the mobile phase was acidified using formic or sulphuric acid instead of acetic acid in sufficient proportions to obtain a pH that would be low enough to guarantee that all the species to be separated would be in the protonated form. All injections were made with a 0.6 ml/min flow-rate in order to ensure complete reactions. As in previous experiments, the results were deduced from the comparison of the spectra obtained with injections where the photoreactor was turned off and on. The graphs in Fig. 4 serve as typical examples of the series of results obtained in this study. The spectra in Fig. 4a, allow for the comparison of the extension of the photoreaction for 2,5-dihydroxybenzaldehyde, depending on the acid used. It can be seen that the spectra for the products without photodecomposition (solid lines) are similar in mobile phases acidified with acetic and formic acid and slightly different when sulphuric acid is used. In contrast, when the photoreactor is turned on (spectra of the photoproducts in broken lines), the extension of the photoreaction is clearly greater in mobile phases acidified with formic acid, in which event the spectrum of the photoproducts only shows a band having a maximum of 295 nm. When acetic acid is used, this maximum is also visible, but also those related to the original product. Since they are postcolumn reactions, there is no separation between the photoproducts and the non-photodegraded original species, which justifies the spectrum obtained when the photoreaction is not complete. Finally, when sulphuric acid is used, photoreaction does not appear to take place.

The graphs in Fig. 4b show another typical situation. In this instance the photoreaction takes place independent of the nature of the acid, and its

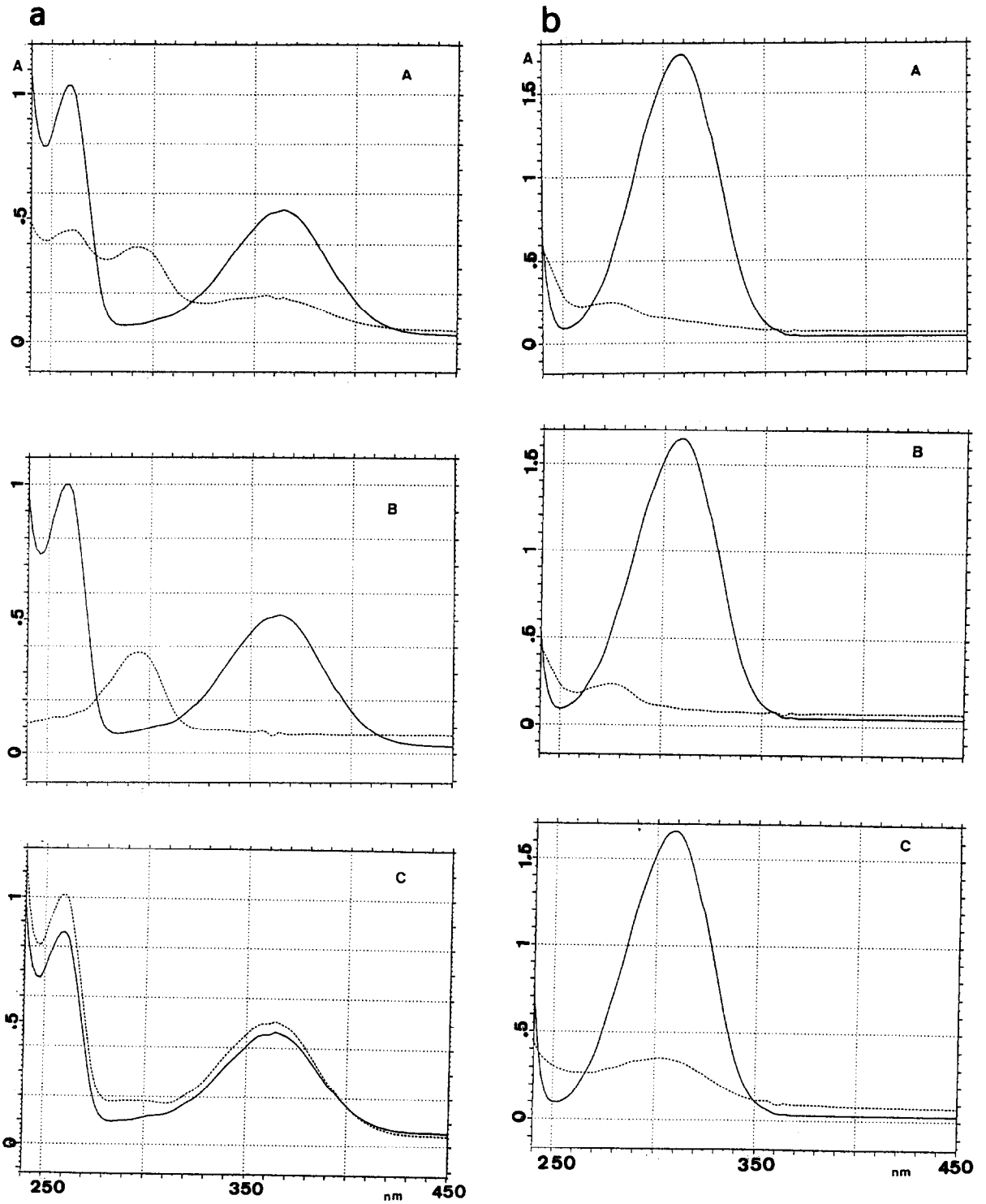

Fig. 4. Influence of the nature of the acid used in mobile phase preparation on photodegradation for (a) 2,5-dihydroxybenzaldehyde and (b), 3,5-dimethoxy-4-hydroxybenzaldehyde. Spectra with solid lines correspond to photoreactor turned off and those with dashed lines to photoreactor turned on. Mobile phases prepared with (A) 1% acetic acid, (B) 0.5% formic acid and (C) 0.3% sulphuric acid.

TABLE I

INFLUENCE OF THE NATURE OF THE ACID USED IN MOBILE PHASE PREPARATION ON THE PHOTODEGRADATION OF POLYPHENOLIC SPECIES

Key: − = no photoreaction; ± = slight or very slow photoreaction; + = partial or slow photoreaction; + + = rapid and complete photoreaction.

No.[a]	Compound	Acetic acid	Formic acid
	Acids		
1	3-Hydroxybenzoic	−	−
2	4-Hydroxybenzoic	+	+ +
3	2,4-Dihydroxybenzoic (β-resorcylic)	−	−
4	2,5-Dihydroxybenzoic (gentisic)	+ +	+
5	2,6-Dihydroxybenzoic (γ-resorcylic)	−	−
6	3,4-Dihydroxybenzoic	−	+
7	3,5-Dihydroxybenzoic (α-resorcylic)	+	+ +
8	2,4,6-Trihydroxybenzoic (protocatechuic)	−	±
9	3,4,5-Trihydroxybenzoic (gallic)	−	−
10	2,4-Dimethoxybenzoic	−	+
11	2,6-Dimethoxybenzoic	−	+ +
12	3,4-Dimethoxybenzoic (veratric)	−	+ +
13	3,5-Dimethoxybenzoic	+	+ +
14	3,4,5-Trimethoxycinnamic	−	+ +
15	4-Hydroxy-3-methoxybenzoic (vanillic)	+	+ +
16	4-Hydroxy-3,5-dimethoxybenzoic (syringic)	+	+ +
17	2-Hydroxycinnamic (*o*-coumaric)	−	+ +
18	3-Hydroxycinnamic (*m*-coumaric)	−	+ +
19	4-Hydroxycinnamic (*p*-coumaric)	−	+ +
20	3,4-Dihydroxycinnamic (caffeic)	−	+ +
21	4-Hydroxy-3-methoxycinnamic (ferulic)	−	+ +
22	3,5-Dimethoxy-4-hydroxycinnamic (sinapic)	+	+ +
	Aldehydes		
23	3-Hydroxybenzaldehyde	−	+
24	4-Hydroxybenzaldehyde	+	+ +
25	2,5-Dihydroxybenzaldehyde	+	+ +
26	3,4-Dihydroxybenzaldehyde (protocatechualdehyde)	−	+
27	2,4-Dimethoxybenzaldehyde	−	+
28	3,4-Dimethoxybenzaldehyde (veratraldehyde)	−	+
29	3,5-Dimethoxybenzaldehyde	+	+ +
30	3,4,5-Trimethoxybenzaldehyde	+	+
31	2-Hydroxy-3-methoxybenzaldehyde (*o*-vanillin)	+	+ +
32	4-Hydroxy-3-methoxybenzaldehyde (vanillin)	+	+
33	3,5-Dimethoxy-4-hydroxybenzaldehyde	+	+
34	3-Hydroxy-4-methoxybenzaldehyde (isovanillin)	−	+

[a] Peak assignments in figures.

extension is similar in mobile phases acidified with acetic and formic acid. It takes place with a smaller extension for phases acidified with sulphuric acid. Table I summarizes the results of this series of experiments with the use of acetic and formic acid, as it was found that in the presence of sulphuric acid, photoreactions always take place with a smaller extension or not at all. It is clear from Table I that the nature of the acid used is a highly important parameter, especially as regards the extension of the photoreactions. A typical example can be seen in the spectra in Fig. 5, which gives a simultaneous view of the influence of the acid used and flow-rate for the photoreactions of α-resorcylic acid. It formic acid is used, the spectra of the photoproducts barely change when the mobile phase flow-rate is varied,

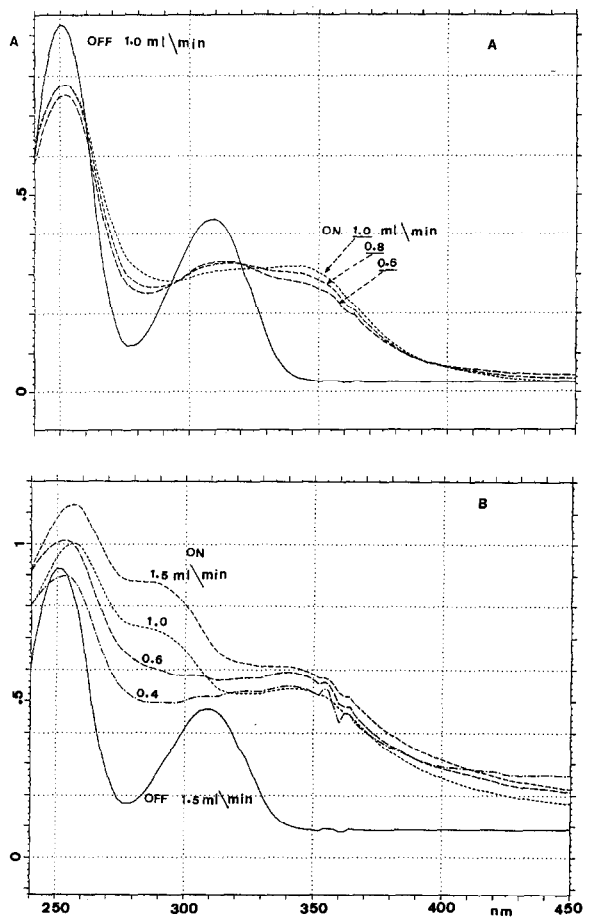

Fig. 5. Influence of the nature of the acid and flow-rate on the photodegradation of α-resorcylic acid. Mobile phases prepared with (A) 0.3% formic acid and (B) 1% acetic acid. Flow-rates as indicated on curves. Spectra with solid lines correspond to photoreactor turned off and are included for comparative purposes.

which points to a rapid and complete photoreaction. However, for mobile phases acidified with acetic acid, the spectra obtained for 0.6 and 0.4 ml/min flow-rates are very similar to those obtained using formic acid. This is not true for higher flow-rates, where a 290-nm absorption band was seen, which is probably due to an intermediate photoproduct that can only be appreciated if the exposure time to luminous radiation is relatively short.

Of the three acids considered, formic acid is the most and sulphuric acid is the least conducive to photoreactions, with acetic acid in an intermediate position. As many of the species considered do not

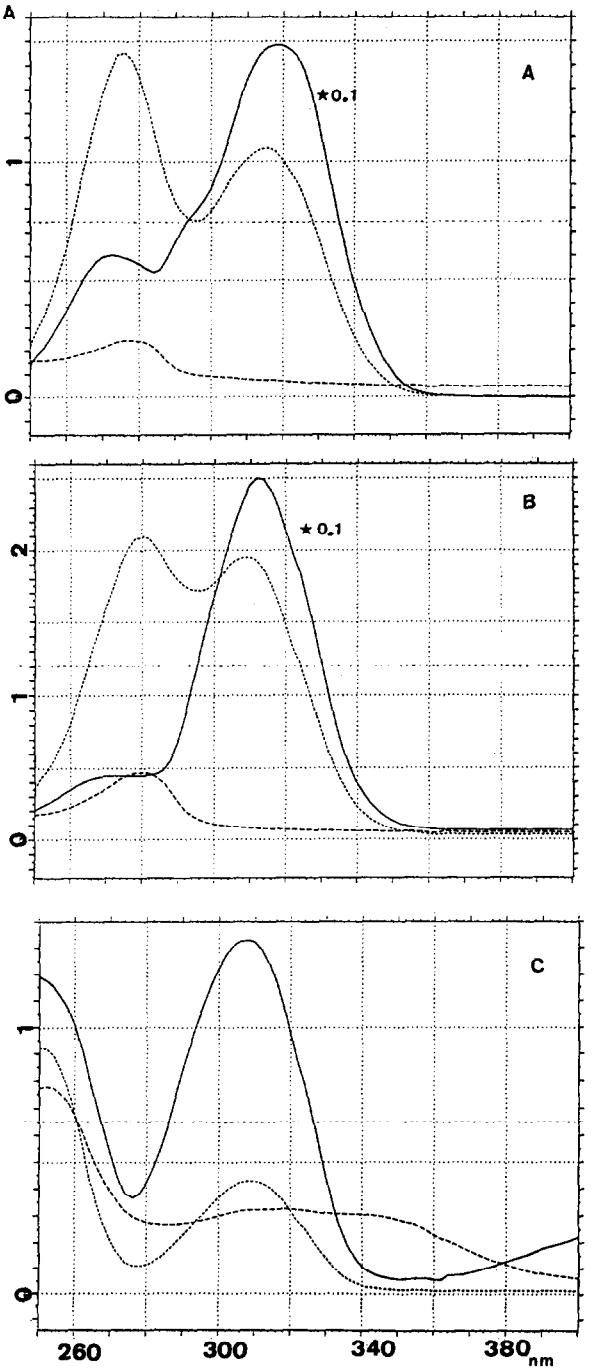

Fig. 6. Ratio spectra (solid lines) for some polyphenolic compounds (dotted lines) and their respective photoproducts (dashed lines). (A) 2,4-Dimethoxybenzaldehyde, (B) vanillin and (C) α-resorcylic acid. Ratio spectra have been multiplied by the indicated factor in order to fit on the absorbance scale for their respective species and photoproduct spectra.

TABLE II

SPECTRAL RATIOS (ABSORBANCE RATIOS FOR SELECTED WAVELENGTHS) FOR POLYPHENOLIC COMPOUNDS SUBJECTED OR NOT TO PHOTOREACTION

Compound	Absorbance ratio[a]		
	260 nm	280 nm	320 nm
Acids			
3-Hydroxybenzoic[b]	0.6	0.9	0.7
4-Hydroxybenzoic	1.5	1.0	0.2
2,4-Dihydroxybenzoic (β-resorcylic)[b]	1.0	0.9	0.9
3,5-Dihydroxybenzoic (α-resorcylic)	1.0	0.4	1.0
2,5-Dihydroxybenzoic (gentisic)	0.5	0.6	0.9
2,6-Dihydroxybenzoic (γ-resorcylic)[b]	0.9	0.8	1.0
3,4-Dihydroxybenzoic	1.4	1.3	0.7
2,4,6-Trihydroxybenzoic (protocatechuic)[b]	0.9	0.9	0.9
3,4,5-Trihydroxybenzoic (gallic)[b]	1.1	1.1	1.5
2,4-Dimethoxybenzoic	2.0	1.5	0.2
2,6-Dimethoxybenzoic	1.9	3.9	0.6
3,4-Dimethoxybenzoic (veratric)	2.8	1.9	0.3
3,5-Dimethoxybenzoic	1.2	0.6	0.8
3,4,5-Trimethoxycinnamic	3.9	12.2	147.8
4-Hydroxy-3-methoxybenzoic (vanillic)	2.7	2.0	0.2
4-Hydroxy-3,5-dimethoxybenzoic (syringic)	1.3	1.5	0.3
3-Hydroxycinnamic (*m*-coumaric)	8.8	10.5	42.1
2-Hydroxycinnamic (*o*-coumaric)	10.3	9.3	61.8
4-Hydroxycinnamic (*p*-coumaric)	2.9	7.2	116.5
3,4-Dihydroxycinnamic (caffeic)	4.7	2.9	147.7
4-Hydroxy-3-methoxycinnamic (ferulic)	3.4	2.9	53.7
3,5-Dimethoxy-4-hydroxycinnamic (sinapic)	2.2	3.7	131.2
Aldehydes			
3-Hydroxybenzaldehyde	6.7	0.4	7.2
4-Hydroxybenzaldehyde	7.4	10.0	2.3
2,5-Dihydroxybenzaldehyde	8.0	0.4	2.7
3,4-Dihydroxybenzaldehyde (protocatechualdehyde)	2.1	4.0	10.9
2,4-Dimethoxybenzaldehyde	3.3	5.1	10.8
3,4-Dimethoxybenzaldehyde (veratraldehyde)	4.8	4.8	42.8
3,5-Dimethoxybenzaldehyde	3.9	2.9	6.2
3,4,5-Trimethoxybenzaldehyde	1.9	6.0	5.3
2-Hydroxy-3-methoxybenzaldehyde (*o*-vanillin)	7.2	1.6	3.0
4-Hydroxy-3-methoxybenzaldehyde (vanillin)	3.8	5.7	51.7
3-Hydroxy-4-methoxybenzaldehyde (isovanillin)	3.1	3.6	13.8
3,5-Dimethoxy-4-hydroxybenzaldehyde	0.7	7.8	22.5

[a] Absorbance at the peak apex for the indicated wavelength with photoreactor turned off divided by absorbance at the peak apex at the same wavelength with photoreactor turned on.

[b] Compounds not affected by photoreactions (see Table I and text).

completely photodegrade in mobile phases acidified with acetic acid, and as the photoreactions take place in the postcolumn mode, its usefulnesss for identification purposes is relatively small. Owing to the lack of research on the influence of other acids, formic acid is recommended for these purposes.

Spectral relationships

The phenomena described above will make it possible for a new qualitative dimension to be available for the analysis of these types of compounds. The inspection of spectra associated with peaks obtained with a photoreactor turned off or on in two

identical, consecutive injections will allow a more reliable identification of species in complex samples. If such a possibility is available, by creating a library of spectra of photoreaction products, the task of identification and discrimination will be greatly simplified. A library could be created where ratio spectra (with the photoreactor turned off and on) could be stored in order to make identification even easier. In fact, these ratio spectra are characteristic for the different species studied. An example of this possibility can be seen in Fig. 6, which shows spectra corresponding to 2,4-dimethoxybenzaldehyde, vanillin and α-resorcylic acid.

If a diode-array detector is not available but a wavelength-programmable detector is, it is possible to determine some spectral relationships for the same purposes. In our study we found that absorbance ratios at 260, 280 and 320 nm allow typical relationships to be determined for each species, and are able to discriminate the species studied here. These relationships are summarized in Table II, where several compounds (see footnote) are considered to be unaffected by photoreactions. In fact, this is not exactly true because, as can be seen from the absorbance ratios, slight modifications have been produced. Hence the criterion was to consider a photoreaction to be significant when one or more characteristic absorption bands in the spectrum were clearly modified and not when the whole spectrum was slightly decreased but conserved all of its typical features. Obviously this fact cannot be seen properly if only the absorbance ratios at the three selected wavelengths are considered.

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