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Photodiode array detection for elucidation of the structure of phenolic compounds

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ABSTRACT

A photodiode array detector was employed to obtain spectral data and to study a set of derived parameters in order to assign some structural features (functional group conjugated with aromatic ring, degree of substitution, position of substituents, etc.) to unknown chromatographic peaks of low-molecular-mass phenolics.

INTRODUCTION

Phenolic compounds are characteristic of vegetable tissues and are of great interest in the study of food and beverages of that origin [1–3]. Identification of these compounds by chromatographic techniques requires the availability of commercial standards or the extraction and purification of the product from natural extracts, prior to the use of other analytical techniques (NMR, mass spectrometry, hydrolysis etc.) [4,5].

The photodiode array detector has led to considerable improvements in the HPLC analysis of phenolic compounds, as not only the retention time but also the UV spectrum can be used for identification purposes. Quantification also becomes more exact owing to the simultaneous recording of different wavelengths and the possibility of peak purity checking [6–8].

In only a few instances [9–12] has the UV spectrum obtained by diode array detection been used for the identification of unknown chromatographic peaks, by comparison with standards giving similar spectra. It was considered necessary, therefore, to make a detailed study of the possibilities and methodology for the use of this chromatographic detector in the elucidation of the structures of unknown phenolic compounds.

In this work we studied different parameters obtained from spectral data with the software of the equipment. The parameters studied were those useful in assigning structural features, *e.g.*, functional groups and number and position of substituents, to low-molecular-mass phenolics such as phenols, benzyl alcohols, benzoic acids, benzaldehydes, cinnamic acids, cinnamyl alcohols, phenylacetic acids, phenethyl alcohols, 3-phenyl-1-propionic acids, 3-phenyl-1-propanols and mandelic acids.

We chose a wide variety of commercial standards of phenolic compounds that are present in vegetable foods. Our aim was to obtain the information to infer rapidly the structure of unknown compounds present in food extracts and eluted under the same chromatographic

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conditions. According to the literature [13], the UV spectra of these compounds are composed of two bands (called E and B) corresponding to the benzene ring, and a third band (called K) that appears when there is a substituent conjugated with the aromatic ring.

EXPERIMENTAL

Standards

Standards of phenolic compounds from Merck (Darmstadt, Germany), Fluka (Buchs, Switzerland), Sigma (Deisenhofen, Germany) and Aldrich (Steinheim/Albuch, Germany) of high purity were used: eight phenols, five benzyl alcohols, seventeen benzoic acids, twelve benzaldehydes, six cinnamic acids, one cinnamyl alcohol, seven phenylacetic acids, three phenethyl alcohols, two 3-phenyl-1-propionic acids, one 3-phenyl-1-propanol and four mandelic acids. The structures are shown in Fig. 1.

HPLC analysis

Solutions of 0.25 mg/ml in acetonitrile–water (4:1, v/v) were used. Variable amounts from 0.1 to 0.7 μg were injected into a Waters (Milford, MA, USA) chromatograph equipped with a Model 600E pump system controller, a U6K universal injector and a Model 991 photodiode-array detector.

The column was a reversed phase Nova-Pak C_{18} (300 × 3.9 mm I.D.). The gradient elution conditions are given in Table I; solvent A was water–acetic acid (98:2, v/v) and solvent B was water–methanol–acetic acid (68:30:2, v/v). Detection was performed by scanning from 210 to 400 nm with an acquisition speed of 1 s.

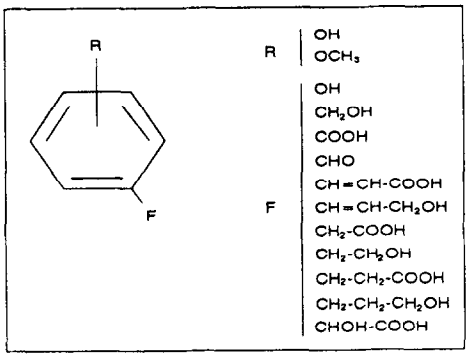

Fig. 1. Structure of the compounds studied.

TABLE I

MOBILE PHASE GRADIENT COMPOSITION AND FLOW-RATE

Time (min)	Flow-rate (ml/min)	A (%)	B (%)
0	0.8	100	0
59	0.8	20	80
70	0.8	20	80

RESULTS AND DISCUSSION

Assignment of spectral bands

The bands denoted E, K and B in the literature will be referred to as 1, 2 and 3, respectively (Fig. 2). Band 2, due to substituents conjugated with the aromatic ring, will be called 2_1 if it is a carboxyl group, 2_2 if it is a carbonyl group or 2_3 if there is a C=C double bond conjugated with the benzene ring.

The position and height of the maxima depend on the main functional group of the ring and also

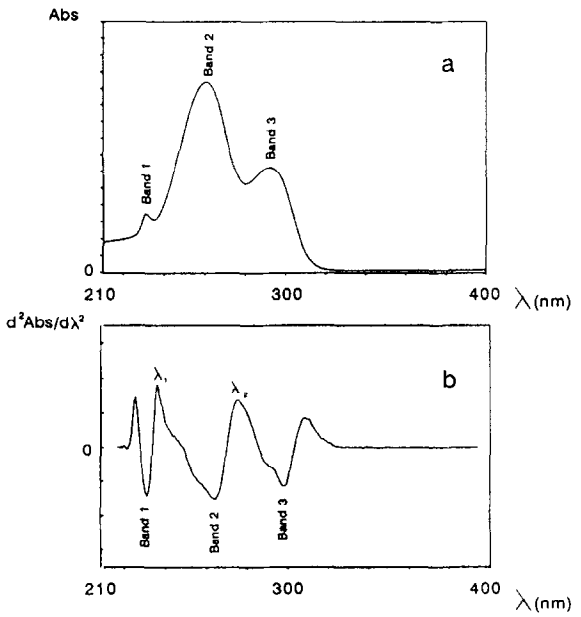

Fig. 2. Spectral bands: (a) Original spectrum; (b) second-derivative spectrum. Abs = Absorbance.

on the nature, number and positions of the remainder of the substituents. In all the compounds, the maxima vary in the ranges 219–235 nm for band 1, 236–298 nm for band 2 and 266–358 nm for band 3 (Table II). It has been demonstrated that changes in the composition of mobile phase during the gradient have no effect on the position and height of the maxima.

Simple phenols. The UV spectrum of phenols and benzyl alcohols is constituted by two bands, 1 and 3 (Fig. 3a and b). The same applies to phenylacetic acids, phenethyl alcohols, 3-phenyl-1-propionic acids, 3-phenyl-1-propanols and mandelic acids, as none of them has a double or triple bond conjugated with the aromatic ring (Fig. 3g, h, i, j and k, respectively).

Benzoic acids. The spectrum of benzoic acids (Fig. 3c) shows three bands. The position of band 2 (2_1) varies with the substituents on the ring, with the possibility of overlapping band 1 (with 3-hydroxy, 2-hydroxy and 2,5-dihydroxy substituents) or band 3 (as with 4-hydroxybenzoic, 3,4,5-trihydroxybenzoic and 3,5-dimethoxy-4-hydroxybenzoic acids) (Table II).

In the 3-hydroxybenzoic acid spectrum, the overlap is due to the hypsochromic effect of the *meta* substituents (deactivating position). The spectrum of 3,5-dihydroxybenzoic acid shows three distinct bands, as the effect of the substituents is offset by the bathochromic effect of the high symmetry of the molecule.

The hypsochromic shift of the carboxylic band in the 2-hydroxybenzoic acid spectrum may be due to the formation of hydrogen bonds between the carboxyl and the hydroxyl groups in *ortho* positions. The bands do not overlap in the 2,6-dihydroxybenzoic acid spectrum because the hypsochromic effect of the *ortho* position is counteracted by the bathochromic effect of the molecule symmetry.

The effects of *ortho* and *meta* positions are combined in the 2,5-dihydroxybenzoic acid spectrum, so the bands 1 and 2_1 overlap. However, the 2,3-dihydroxybenzoic acid spectrum has three bands because of the hydrogen bond between substituents, which compensates for their hypsochromic effect.

The shift in band 2_1 that causes overlap with band 3 (4-hydroxybenzoic, 3,4,5-trihydroxybenzoic and 3,5-dimethoxy-4-hydroxybenzoic acids) is due to the delocalizing effect of the *para* substitution and to the symmetry of the molecules.

Benzaldehydes. The UV spectrum of benzaldehydes (Fig. 3d) also shows three bands and, as happens to benzoic acids, bands 2_2 and 3 are overlapped when there are 4-hydroxy and 3,5-dimethoxy-4-hydroxy substituents.

Cinnamyl alcohols. The spectrum of cinnamyl alcohols (Fig. 3f) contains three bands, band 2_3 corresponding to the C=C double bond conjugated with the aromatic ring.

Cinnamic acids. The spectrum of cinnamic acids (Fig. 3e) contains a fourth band in addition to the three of the cinnamyl alcohols owing to the carboxyl group that is conjugated with the double bond and hence conjugated with the benzene ring. This band is also called 2_1 (it is due to a carboxyl group) and overlaps with band 1, except for 4-hydroxy-3-methoxycinnamic acid. Bands 2_3 and 3 are separated in every spectrum, except that of 3,5-dimethoxy-4-hydroxycinnamic acid, owing to the described effects of *para* substitution and the high symmetry that cause overlapping.

Study of parameters

The photodiode array detector permits of the different parameters in the UV spectra (Table II) to be measured to establish the relationship between structure and UV spectrum. These parameters are not dependent on the amount injected for the range of concentrations considered.

Retention time. The retention time depends on the main functional group and on the substituents of the benzene ring, so it is difficult to infer the structure of a compound solely from its retention time.

Position of maxima. For the same substitution pattern of the benzene ring, the maximum wavelength of band 2 decreases in the order cinnamic acids (2_3) > benzaldehydes (2_2) > benzoic acids (2_1). Cinnamyl alcohols are not included owing to insufficient data. In the same way, the maximum wavelength of band 3 decreases in the order cinnamic acids > benzaldehydes > benzoic acids > phenols > compounds with a saturated

TABLE II

VALUES OF PARAMETERS OBTAINED WITH THE PHOTODIODE ARRAY DETECTOR

t_R = Retention time (min); M1 = absorbance maximum (nm) of band 1; $M2_1$, $M2_2$, $M2_3$ = absorbance maxima (nm) of band 2 (see text); M3 = absorbance maximum (nm) of band 3; $C2_1$, $C2_2$, $C2_3$ = convexity intervals (nm) of band 2; C3 = convexity interval (nm) of band 3; 260/320, 270/300 = ratios between absorbances of different wavelengths (nm); λ_1, λ_2 = absorbance maxima (nm) of second-derivative spectra. Spectra were recorded after correcting for solvent absorption.

Compound	t_R	M1	$M2_1$	$M2_2$	$M2_3$	M3	$C2_1$	$C2_2$	$C2_3$	C3	260/320	270/300	λ_1	λ_2
Phenols														
Phenol	26.8	229.5				270.2				19.6	307	388	249.1	
4-Hydroxy-	7.0	231.2				288.1				23.9	1560	0.7	255.0	
3-Hydroxy-	9.5	231.2				273.8				19.3	1601	3437	251.7	
2-Hydroxy-	14.6	231.0				275.1				20.8	178	167	253.0	
2-Methoxy-	38.1	232.5				275.4				19.5	196	233	248.1	
2,6-Dimethoxy-	50.7	228.5				268.6				19.7	113	104	250.4	
2,3-Dihydroxy-	6.2	230.0				266.0				19.3	138	241	250.4	
3,5-Dihydroxy-	5.4	229.1				266.0				17.9	212	52	250.4	
Benzyl alcohols														
4-Hydroxy-	14.9	235.0				273.8				21.5	1120	356	253.2	
4-Methoxy-	43.0	232.5				272.8				21.3	298	254	246.8	
2-Hydroxy-	21.4	227.0				273.8				21.8	220	254	251.7	
4-Hydroxy-3-methoxy-	21.9	233.8				279.3				20.7	139	72	252.0	
3,4-Dimethoxy-	39.8	233.5				277.7				19.6	151	132	251.7	
Benzoic acids														
4-Hydroxy-	21.5	226.0	255.6			–[a]	37.4			–[a]	407	143	236.4	–
3-Hydroxy-	29.4	–[a]	237.7			295.9	–			27.9	1.4	0.3	–	256.9
2-Hydroxy-	57.3	–[a]	242.0			301.1	–			29.5	0.2	0.2	–	255.6
2,6-Dihydroxy-	17.8	230.0s[b]	246.5			306.3	16.7			29.7	0.9	0.1	–	266.3
2,6-Dimethoxy-	39.6	223.5s[b]	242.6			280.3	–			18.7	14	2.2	–	263.4
2,5-Dihydroxy-	20.6	–[a]	236.4			327.1	–			40.9	0.1	0.1	–	256.0
2,4-Dihydroxy-	26.1	226.0	255.6			294.6	18.2			20.9	32	1.1	236.4	273.8
2,4-Dimethoxy-	61.7	225.0	255.6			292.0	18.5			18.6	30	1.4	232.0	275.1
2,3-Dihydroxy-	29.2	228.0s	245.2			314.1	–			38.5	0.8	0.1	–	271.2
3,4-Dihydroxy-	10.1	228.6	258.2			294.6	20.4			18.5	38	1.5	241.3	277.7
4-Hydroxy-3-methoxy-	30.9	231.0	259.8			292.3	20.7			17.0	77	1.8	239.0	278.0
3,4-Dimethoxy-	56.3	235.0	259.5			292.3	20.8			16.9	94	1.9	242.6	277.7
3,5-Dihydroxy-	13.0	231.0	249.1			306.3	18.3			31.3	2.2	0.5	244.5	271.2
3,5-Dimethoxy-	<70	224.7	249.4			305.3	22.0			30.3	2.2	0.6	232.5	274.1
3,4,5-Trihydroxy-	6.5	231.0	271.2			–[a]	37.7			–[a]	27	2.8	246.5	–
3,5-Dimethoxy-4-hydroxy-	36.4	223.4	275.1			–[a]	42.3			–[a]	31	2.2	247.8	–
2,4,6-Trihydroxy-	10.2	231.2	255.6			293.3	17.9			17.8	41	2.1	241.6	275.1
Benzaldehydes														
4-Hydroxy-	27.8	228.0		284.2		–[a]		40.8		–[a]	11	1.5	242.9	–
3-Hydroxy-	31.2	228.6		254.3		315.4		19.6		35.3	3.6	1.7	237.7	276.4
2-Hydroxy-	49.8	229.0		255.6		324.5		18.1		43.8	3.3	1.6	237.0	273.8
2,5-Dihydroxy-	27.5	230.0		258.2		358.3		16.2		43.7	6.4	8.6	245.2	277.7
2-Hydroxy-5-methoxy-	62.3	229.0		258.2		357.0		16.8		42.8	5.9	7.9	245.2	277.2
2,3-Dihydroxy-	29.5	228.3		266.0		345.3		24.8		47.8	7.2	17	245.2	288.1
2-Hydroxy-3-methoxy-	50.3	228.0		264.7		344.0		23.8		47.1	5.9	14	245.2	286.2
3,4-Dihydroxy-	19.8	232.5		280.3		310.2		26.4		24.9	0.7	1.2	251.7	297.2
3-Hydroxy-4-methoxy-	36.6	231.0		279.0		311.5		25.8		25.3	0.7	1.2	250.4	295.9
4-Hydroxy-3-methoxy-	36.4	235.1		280.3		308.9		24.9		24.6	0.6	1.0	250.4	295.9
3,4-Dimethoxy-	60.7	233.0		277.7		308.9		21.4		23.4	0.7	1.1	247.8	294.6
4-Hydroxy-3,5-dimethoxy	44.5	229.5		307.6		–[a]		46.4		–[a]	0.1	0.3	250.4	–

TABLE II *(continued)*

Compound	t_R	M1	$M2_1$	$M2_2$	$M2_3$	M3	$C2_1$	$C2_2$	$C2_3$	C3	260/320	270/300	λ_1	λ_2
Cinnamic acids														
4-Hydroxy-	47.7	–[a]	232.5		298.0s	308.9	–		–	52.3	0.2	0.4	251.7	308.2s[b]
3-Hydroxy-	54.7	–[a]	235.1		279.0	325.0s[b]	–		29.8	16.1	2.5	2.0	251.7	303.7
2-Hydroxy-	65.4	–[a]	233.5		276.4	323.2	–		31.8	30.6	1.2	2.1	249.1	301.1
3,4-Dihydroxy-	27.5	–[a]	233.8		290.0s[b]	323.2	–		–	25.4	0.3	0.4	259.5	307.6
4-Hydroxy-3-methoxy-	55.0	219.0	236.0		292.0s[b]	323.5	–		–	31.7	0.2	0.4	260.8	306.3
3,5-Dimethoxy-4-hydroxy-	58.7	–[a]	237.5		323.2	–[a]	–		–[a]	51.1	0.1	0.2	258.2	–
Cinnamyl alcohol														
4-Hydroxy-3-methoxy-	44.0	231.2			262.4	304.0s[b]			23.2	19.8	22	2.5	242.9	281.9
Phenylacetic acids														
4-Hydroxy-	28.6	233.5				275.1				20.5	150	155	251.7	
4-Methoxy-	59.7	233.8				274.1				19.6	172	180	249.4	
3-Hydroxy-	30.9	231.2				272.8				21.0	172	222	246.8	
2,5-Dihydroxy-	9.9	232.5				291.0				23.5	10	0.4	249.4	
3,4-Dihydroxy-	17.9	233.8				280.6				19.6	75	27	252.0	
4-Hydroxy-3-methoxy-	35.6	233.8				280.6				19.5	68	29	251.0	
3,4-Dimethoxy-	55.2	235.1				278.0				19.3	159	99	250.0	
Phenethyl alcohols														
4-Hydroxy-	24.1	231.2				276.0				20.7	189	186	249.5	
4-Methoxy-	58.3	231.2				275.4				19.7	199	237	246.8	
4-Hydroxy-3-methoxy-	31.2	233.8				279.3				19.5	90	56	249.4	
3-Phenyl-1-propionic acids														
4-Hydroxyphenyl-	40.1	233.5				276.4				21.6	165	151	251.7	
3,4-Dihydroxyphenyl-	27.9	233.8				280.6				19.7	72	26	249.4	
3-Phenyl-1-propanol														
4-Hydroxyphenyl-	37.2	231.2				276.7				20.4	135	128	266.3	
Mandelic acids														
4-Hydroxy-	5.1	233.8				274.1				18.5	31	49	251.2	
4-Methoxy-	12.6	235.1				279.3				18.3	95	52	249.0	
4-Hydroxy-3-methoxy-	7.3	235.1				279.3				18.4	78	54	249.0	
3,4-Dihydroxy-	3.2	235.1				280.6				19.6	79	56	250.7	

[a] Overlapping with the nearest band.
[b] s: Considered as a shoulder.

side-chain (benzyl alcohols, phenylacetic acids, phenethyl alcohols, 3-phenyl-1-propionic acids, 3-phenyl-1-propanols and mandelic acids).

The effect of the substituents on the UV spectrum depends more on their position and interactions between them than on their nature. The *ortho* and *meta* positions give hypsochromic shifts of band 2 and bathochromic shifts of band 3. However, the effect of the substituents is not additive, owing to possible interactions between substituents. The 2,5- and 2,3-substituents correspond to the most separated wavelengths for band 2 (very low with respect to the other substitutions) and for band 3 (very high). The symmetry of the molecule (2,6- and 3,5-substituents) compensates for the effects of *ortho* and *meta* positions.

The effect of *para* substitution is opposite to that of *meta* and *ortho* substitution, *i.e.*, a bathochromic effect on band 2 and a hypsochromic effect on band 3.

Width of convexity interval. The convexity interval is defined for bands 2 and 3 as the distance (in nm) between the inflection points before and after the maximum. These inflection points are the maximum and minimum, respectively, of the first-derivative spectrum.

For the same substitution pattern of the aromatic ring, the convexity interval is larger in benzaldehydes than in benzoic acids for both

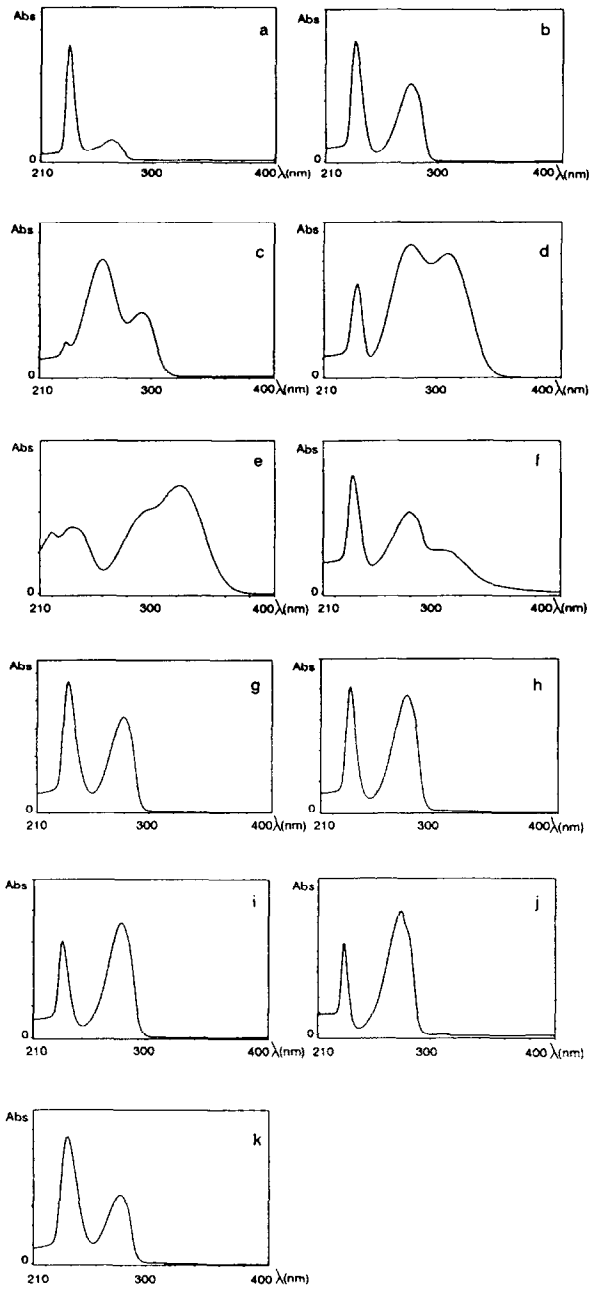

Fig. 3. Spectra of some of the compounds. (a) 3,5-Dihydroxyphenol; (b) 4-hydroxy-3-methoxybenzyl alcohol; (c) 4-hydroxy-3-methoxybenzoic acid; (d) 4-hydroxy-3-methoxybenzaldehyde; (e) 4-hydroxy-3-methoxycinnamic acid; (f) 4-hydroxy-3-methoxycinnamyl alcohol; (g) 4-hydroxy-3-methoxyphenylacetic acid; (h) 4-hydroxy-3-methoxyphenethyl alcohol; (i) 3-(3,4-dihydroxyphenyl)-1-propionic acid; (j) 3-(4-hydroxypheny)-1-propanol; (k) 4-hydroxy-3-methoxymandelic acid.

band 2 and band 3. There are no significant differences in the convexity interval of band 3 of the compounds with a saturated side-chain. Substituents in the 2,5- and 2,3-positions give the highest values of the convexity interval for band 3 with any type of functional group.

The position of the maximum and the convexity interval of the bands are related: a bathochromic shift of the maximum corresponds to an increase in the convexity interval. The study of these two parameters can be used to determine the presence, in an unknown compound, of a group conjugated with the aromatic ring (*i.e.*, if it is a benzoic acid, benzaldehyde, cinnamic acid or cinnamyl alcohol). In this instance, the spectrum will be composed of three distinct bands or two bands, that of highest wavelength having a convexity interval greater than 25 nm (because of overlapping of bands).

Absorbance ratios. The photodiode array detector permits the rapid calculation of absorbance ratios between different wavelengths. Several ratios were tested in order to classify the spectra by functional groups or by other criteria (number of substituents, relative position, etc.). Only the ratios A_{260}/A_{320} and A_{270}/A_{300} can partially differentiate the studied functional groups. In this way, a value of A_{270}/A_{300} of less than 17 indicates the presence of a double bond conjugated with the aromatic ring (benzoic acid, benzaldehyde, cinnamic acid or cinnamyl alcohol). The only exceptions are 4-hydroxyphenol, 4-hydroxybenzoic acid and 2,5-dihydroxyphenylacetic acid. The ratio A_{260}/A_{320} establishes very similar separation limits.

Position of maxima in the second-derivative spectrum. The maxima of the second-derivative spectrum (λ_1 and λ_2) correspond to the minima of the original spectrum (Fig. 2b). Thus, the maximum λ_1 corresponds to the minimum between bands 1 and 2 of the original spectrum. Maximum λ_2 corresponds to the minimum between bands 2 and 3 (if the latter exists), or between bands 2_1 and 2_3 of the cinnamic acids.

These parameters are related to the nature and position of the substituents on the ring. In compounds without a double bond conjugated with the aromatic ring (phenols, benzyl alcohols, phenylacetic acids, phenethyl alcohols, 3-phenyl-

1-propionic acids, 3-phenyl-1-propanols and mandelic acids), λ_1 takes higher values when there are hydroxyl groups far from the main functional group (3- and 3,4-positions). Methoxyl groups have a hypsochromic effect.

For compounds in which λ_2 is defined (benzoic acids, benzaldehydes, cinnamic acids and cinnamyl alcohols), the substituents that are far from the functional group (4- or 3,4-positions) have a very marked bathochromic effect, whereas the positions 2-, 2,5-, 2,6- and 3-positions lead to low values of λ_2. The 2,3-, 2,4- and 3,5-positions give intermediate values (see Table II).

The possibility of using all these parameters to develop an algorithm for establishing the possible structures of phenolic compounds is being studied.

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