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Determination of some structural features of procyanidins and related compounds by photodiode-array detection

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Abstract

In this work we have examined the use of a photodiode-array detector to identify unknown peaks corresponding to procyanidins and related compounds. The type of flavan-3-ol unit can be recognised from the absorption maximum (278.9 nm for catechin and epicatechin, and 270.6 nm for epigallocatechin), whereas the number of flavan-3-ol units does not affect this parameter. The min–max distance (distance between the minimum and the maximum in the original spectrum) decreases by 1.3 nm for each catechin or epicatechin unit. Gallates of flavan-3-ol can be detected by reference to the value of the convexity interval (distance between the inflection points before and after the maximum in the original spectrum).

Keywords: Photodiode-array detector; Procyanidins

1. Introduction

Condensed tannins or polymers of proanthocyanidins are phenolic polymers consisting of flavan-3-ol units (Fig. 1) with C_4–C_8, C_4–C_6 and C_2–C_7 bonds. The importance of these compounds in determining the sensory characteristics of food, such as astringency and bitterness, and their potential negative effects on food utilization have been studied by many workers [1–3]. Among the large number of condensed tannins, oligomeric procyanidins, mainly composed of (+)-catechin and/or (−)-epicatechin units, are usually responsible for the properties attributed to the presence of condensed tannins [4].

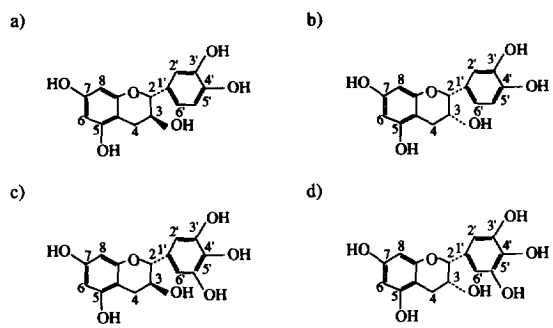

Fig. 1. Commonly occurring flavan-3-ols. (a) (+)-Catechin, (b) (−)-epicatechin, (c) (+)-gallocatechin, (d) (−)-epigallocatechin.

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Since these properties are related to the chemical structure of procyanidins (type and number of units, and bond position), analysis of the individual procyanidins is necessary. Separation of procyanidins has been achieved by HPLC coupled with UV detectors [5–9]. However, identification of the chromatographic peaks requires previous isolation and characterization of the compounds. Procyanidin extracts have been successfully fractionated by Sephadex LH-20 [10–13] and counter-current chromatography [14], and characterized by chemical degradation [10,12,13,15,16] and by NMR spectroscopy [17].

The development of photodiode-array detectors has increased the advantages of HPLC in the study of phenolic compounds. UV-Vis spectra, provided by these detectors, are used for identification of peaks by comparison with standards and to check peak purity. Knowledge of the spectra of unknown chromatographic peaks can also give a preliminary idea about the chemical structure of the corresponding compounds as shown in studies using non-procyanidin-type phenolic compounds [18,19]. However, this second feature requires the existence of data about the spectral properties of the different compounds.

The purpose of the present work is to investigate the possible use of a photodiode-array detector for the determination of the chemical structure of unknown peaks corresponding to procyanidins and related compounds. To this aim, we have studied the spectral properties of different monomers, dimers, trimers and tetramers of flavan-3-ol. Since these compounds commonly occur linked to gallic acid, the spectral properties of some gallates of flavan-3-ol have also been studied. Previously used spectral parameters (wavelengths of the spectrum maxima and convexity interval [20]) and novel ones (min–max distance) were determined for all these standards and the relationships between the spectral properties and the chemical structures of these compounds are discussed. The results have been successfully applied to the analysis of these compounds in an extract of grape seeds.

2. Experimental

2.1. Standards

(+)-Catechin (C) was purchased from Sigma (Deisenhofen, Germany), (−)-epicatechin (E) from Fluka (Buchs, Switzerland), and (−)-epigallocatechin (EGC), (−)-epicatechin 3-O-gallate (E-G) and (−)-epigallocatechin 3-O-gallate (EGC-G) from Extrasynthèse (Genay, France).

Procyanidin dimers B1 [(−)-epicatechin-(4$\beta \rightarrow$8)-(+)-catechin], B2 [(−)-epicatechin-(4$\beta \rightarrow$8)-(−)-epicatechin] and B5 [(−)-epicatechin-(4$\beta \rightarrow$6)-(−)-epicatechin], and trimers (named as in Ref. [21]) C1 [(−)-epicatechin-(4$\beta \rightarrow$8)-(−)-epicatechin-(4$\beta \rightarrow$8)-(−)-epicatechin], T2 [(−)-epicatechin-(4$\beta \rightarrow$8)-(−)-epicatechin-(4$\beta \rightarrow$8)-(+)-catechin], and T3 [(−)-epicatechin-(4$\beta \rightarrow$6)-(−)-epicatechin-(4$\beta \rightarrow$8)-(−)-epicatechin], were isolated from apple pulp and identified as described previously [13]. Dimers B3 [(+)-catechin-(4$\alpha \rightarrow$8)-(+)-catechin] and B4 [(+)-catechin-(4$\alpha \rightarrow$8)-(+)-epicatechin], and tetramer T4 (whose structure was not completely determined), were obtained from grape seeds following the same method (Pérez-Ilzarbe, unpublished results). Variable amounts of each compound were injected, giving a response between 0.01 and 0.2 AU at 280 nm at peak apex, i.e. 0.25–5.00 μg referred to (−)-epicatechin. When the concentration of the compound was changed, no variations in the values of the spectrum parameters were observed. Peak purity was also checked using the software of the photodiode-array detector. The computer extracts spectra from the upslope, the apex and the downslope of the peak, normalises the spectra and calculates a match. The computer assigns a value of 1000 to peaks with spectra which it judges to match perfectly. All standards showed values over 990, which confirmed their purity.

2.2. Grape sample

Grape seeds of *Vitis vinifera* var. Airen, were milled to a particle size of less than 0.5 mm. A 6-g amount of sample was macerated for 12 h at

room temperature, three times with 50 ml of a synthetic wine solution (pH 3.5) containing tartaric acid (0.7 g/l), potassium bitartrate (1.11 g/l) in water–ethanol (80:20, v/v). The three combined macerates were concentrated to 35 ml using a rotatory evaporator, always keeping the bath temperature below 35°C. The concentrated solution was extracted four times with ethyl acetate (20 ml). The organic solutions were combined, dried for 30 min with anhydrous sodium sulphate, filtered and evaporated to dryness. The residue was dissolved in 1 ml of methanol–water (50:50, v/v) and 5 μl were injected onto the HPLC apparatus.

2.3. Apparatus

A Waters (Milford, USA) HPLC system equipped with a 600E pump, a U6K injector, and a 991 photodiode-array detector, was used. A data station was used for data storage, comparison and mathematical manipulation of the acquired spectra.

2.4. Column and chromatographic procedure

The column used was a reversed-phase Nova Pak C_{18} (30 cm × 3.9 mm I.D.) with 4 μm packing (Waters, Milford, USA). Elution of procyanidins and related compounds was carried out with an acetonitrile–water gradient as described previously [22]. Solvent A was water–acetic acid (98:2, v/v) and solvent B was water–acetic acid–acetonitrile (78:2:20, v/v). The concentration of solvent A was decreased from 100% to 10% over a 75-min period, using flow-rates of 0.6 (0–20 min), 0.5 (20–50 min) and 0.8 ml/min (50–75 min).

2.5. Spectra acquisition

Detection was performed by scanning from 210 to 400 nm. A resolution of 0.2 nm was used. Spectra were recorded after subtracting the solvent absorption.

Different spectrum parameters were studied: some of them (wavelengths of the spectrum maxima and wavelengths of the second-derivative spectrum maxima) were given by the software of the photodiode-array detector; another parameter (the convexity interval [20]) was calculated as the difference between the values of the maximum and the minimum of the first-derivate spectrum, both also given by the software of the photodiode-array detector.

3. Results and discussion

3.1. Study of the standards

Following the chromatographic procedure described above, it was possible to detect and separate the monomers, dimers, trimers, tetramers and gallates of flavan-3-ol listed in Table 1. Some relationships could be established between retention times (or capacity factors) of these compounds and their chemical structures. Compounds containing (−)-epigallocatechin eluted faster than the ones containing (−)-epicatechin (monomers and gallates, Table 1), due to the additional hydroxy group in the B-ring of the former. The (+)-catechin-type configuration in monomers and oligomers gave a faster elution compared to the (−)-epicatechin-type configuration. Gallates of flavan-3-ol showed higher retention times than the corresponding monomers. No relationship was found between other features of the chemical structure of the compounds (such as number of flavan-3-ol units or bond positions) and elution order under these reversed-phase chromatographic conditions. This is in agreement with the previous finding that only normal-phase chromatographic conditions were successful in separating procyanidins on a molecular mass basis [9].

Spectra of all the standards were recorded by the photodiode-array detector. UV spectra of monomers (Fig. 2a) and oligomers (Fig. 2b) of flavan-3-ol showed two bands (named band 1 and band 3 [20]), that correspond to the aromatic rings of the flavan-3-ol structure. UV spectra of gallates of flavan-3-ol should have presented a third band (named band 2_1 [20]) due to the

Table 1
Values of chromatographic and spectral parameters obtained using photodiode-array detection

	t_R	k	Spectrum maxima (nm)			Convexity interval (nm) B3	2nd Derivate maximum (nm) λ_1	Min–max distance (nm)
			B1	$B2_1$	B3			
Monomers								
C	31.3	7.4	234.7 ± 0.3		278.9 ± 0.3	20.7 ± 1.0	250.3 ± 0.2	28.6
E	41.3	10.0	234.7 ± 0.2		278.9 ± 0.2	20.3 ± 1.0	250.3 ± 0.2	28.6
EGC	29.6	6.9	234.7 ± 0.5		270.6 ± 0.3	20.0 ± 0.8	251.1 ± 0.3	19.5
Dimers								
B1[E-(4β→8)-C]	27.1	6.2	234.7 ± 0.3		278.9 ± 0.2	20.2 ± 1.0	251.6 ± 0.2	27.3
B2[E-(4β→8)-E]	35.8	8.6	234.7 ± 0.3		278.9 ± 0.1	20.6 ± 1.0	251.6 ± 0.3	27.3
B3[C-(4β→8)-C]	26.7	6.1	234.7 ± 0.3		278.9 ± 0.2	20.5 ± 1.0	251.6 ± 0.3	27.3
B4[C-(4β→8)-E]	33.9	8.0	234.7 ± 0.2		278.9 ± 0.3	20.4 ± 1.1	251.6 ± 0.2	27.3
B5[E-(4β→6)-E]	59.0	14.7	234.7 ± 0.3		278.9 ± 0.3	20.5 ± 1.1	251.6 ± 0.3	27.3
Trimers								
C1[E-(4β→8)-E-(4β→8)-E]	44.3	10.8	234.7 ± 0.4		278.9 ± 0.2	20.3 ± 1.7	252.9 ± 0.2	26.0
T2[E-(4β→8)-E-(4β→8)-C]	30.3	7.1	234.7 ± 0.2		278.9 ± 0.3	20.1 ± 1.6	252.9 ± 0.3	26.0
T3[E-(4β→6)-E-(4β→8)-E]	34.0	8.1	234.7 ± 0.3		278.9 ± 0.1	20.4 ± 1.7	252.9 ± 0.2	26.0
Tetramers								
T4	46.7	11.5	234.7 ± 0.2		278.9 ± 0.3	20.0 ± 1.0	254.2 ± 0.3	24.7
Gallates								
EGC-G	44.0	10.7	234.7 ± 0.9	(*)	274.5 ± 0.4	29.7 ± 1.7	249.8 ± 0.6	24.7
E-G	55.7	13.9	234.7 ± 0.9	(*)	278.5 ± 0.3	29.1 ± 1.4	249.8 ± 0.3	28.6

t_R = Retention time (min); k = capacity factor; B1 = band 1; $B2_1$ = band 2_1; B3 = band 3; C = (+)-catechin; E = (−)-epicatechin; EGC = (−)-epigallocatechin; G = gallate.
[a] Overlapping with band 3.

carboxyl group of the gallate structure. However, only two bands were detected (Fig. 2c), because of overlapping of bands 2_1 and 3.

The "wavelengths of the spectrum maxima", which is the most commonly-used parameter in spectrum studies, are shown in Table 1. Differences were only found in the position of the maximum of band 3. This wavelength value was much lower for (−)-epigallocatechin (270.6 nm) than for (+)-catechin, (−)-epicatechin and procyanidins (278.9 nm), which was attributed to the hypsochromic effect of the additional hydroxy group in the B-ring of the former [23]. The same pattern was found for the 3-O-gallates of (−)-epigallocatechin and (−)-epicatechin (274.5 nm and 278.5 nm, respectively).

The convexity interval (Fig. 3), defined as the distance (in nm) between the inflection points before and after the maximum of band 3 [20], showed no differences among the monomers and oligomers of flavan-3-ol (Table 1). However, gallates gave a remarkably higher value, which agreed with previous studies [20,24] showing that this parameter was an indicator of overlapping of spectral bands.

Derivate spectra can enhance differences between spectra [25,26]. The maximum of the second-derivate spectrum λ_1 (Fig. 3), coincided with the minimum between band 1 and band 3 [20]. For monomers and oligomers of (+)-catechin and (−)-epicatechin, the wavelength of λ_1 increased with the number of units forming the compound (Table 1). For a better understanding, we calculated the difference between the wavelength of the maximum of band 3 and λ_1; this parameter was named "min–max dis-

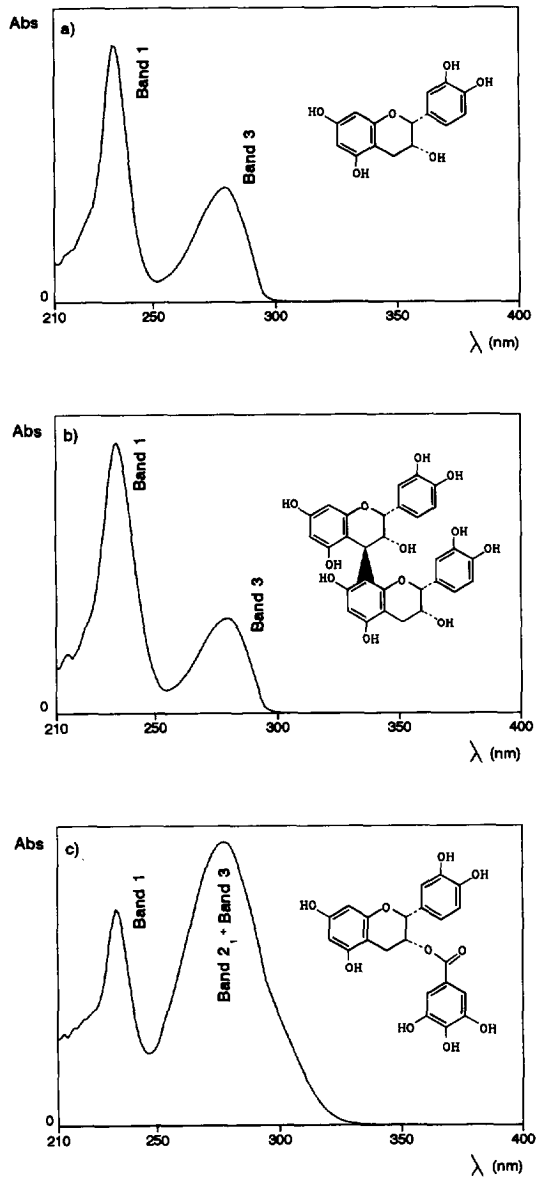

Fig. 2. UV spectra of some of the compounds studied. (a) (−)-Epicatechin, (b) B2 [(−)-epicatechin-(4β→8)-(−)-epicatechin], (c) (−)-epicatechin 3-O-gallate.

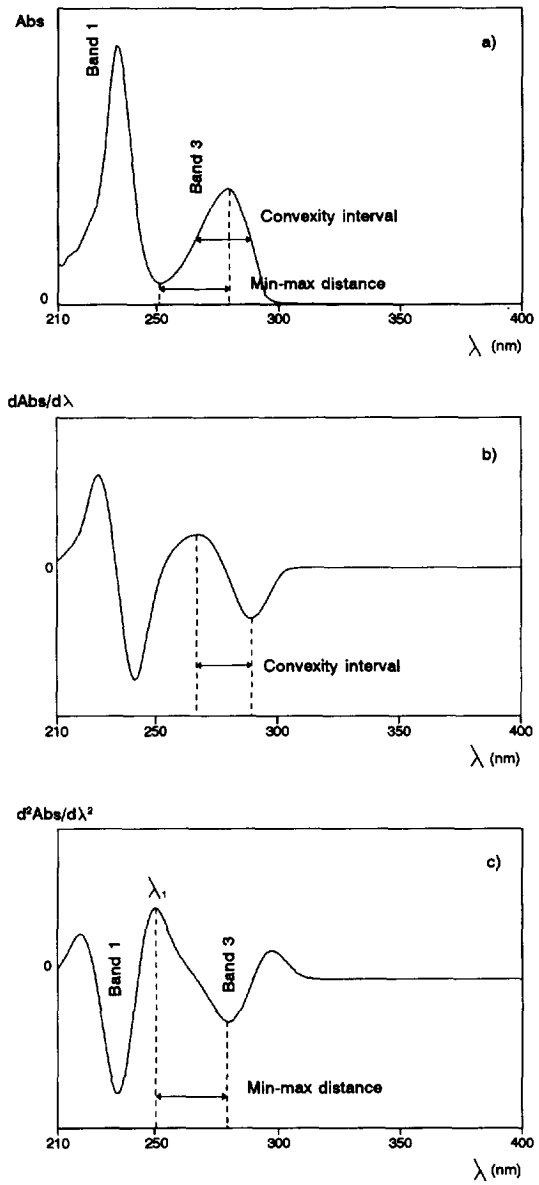

Fig. 3. (+)-Catechin. (a) Original spectrum, (b) first-derivative spectrum, (c) second-derivative spectrum.

tance" because it coincided with the distance between the minimum and the maximum in the original spectrum (Fig. 3). This new parameter gave information about the shape of band 3 and showed different values for monomers (28.6 nm), dimers (27.3 nm), trimers (26.0 nm) and tetramers (24.7 nm) of (+)-catechin and (−)-epicatechin, and for (−)-epigallocatechin (19.5 nm). It is important to note that the study of the spectrum derivatives must be carried out with care: if spectra are not noise free, incorrect results could be obtained [27]. The absorption spectrum due to the solvent at the peak elution time was first considered to be the same as that

at a point immediately preceding the eluting peak. Although this could be applied to standards, unclear results were obtained with samples due to the complexity of the chromatograms. Hence, the absorption due to the solvent was successfully considered to match that observed at the same retention time in a blank chromatogram, and thus spectra were recorded after subtracting the solvent absorption.

All these results indicate that there are important differences amongst the spectral properties of these compounds. Thus, this work could be very useful in the determination of the chemical structure of unknown chromatographic peaks corresponding to procyanidins and related compounds. Only peaks with a value higher than 21 nm for the convexity interval of band 3 could be assigned to gallates of flavan-3-ol. Since procyanidins exhibit the same value of the convexity interval as (+)-catechin and (−)-epicatechin, as well as (−)-epigallocatechin, this statement should also be true for gallates of procyanidins and prodelphidins [oligomers of (+)-gallocatechin and/or (−)-epigallocatechin].

The band 3 maximum in spectra of (+)-catechin and (−)-epicatechin at 278.9 nm differs from that of (−)-epigallocatechin at 270.6 nm. In addition there is no variation in the value of this parameter with the number of units of (+)-catechin and/or (−)-epicatechin that form the procyanidin. Thus this parameter potentially can be used to distinguish between groups of compounds. Those corresponding to condensed tannin polymer mixtures of both types would have an intermediate value.

Our data suggest that oligomers of (+)-catechin and/or (−)-epicatechin up to tetramers can be identified by reference to the value of the min–max distance, decreasing by 1.3 nm for each unit. It would be interesting to study this parameter in larger polymer procyanidins as well as in other condensed tannin oligomers.

3.2. Applicability

A chromatogram of an extract from grape seeds (*Vitis vinifera* var. Airen) is shown in Fig. 4. Identification of peaks (Table 2) was carried out by comparing the retention times and spectral data with those of standards. Peaks 6, 17, and 18 were classified as procyanidin dimers according to their spectral characteristics. Since other procyanidin dimers such as B6 [(+)-catechin-(4α→6)-(+)-catechin] and B7 [(+)-catechin-(4α→6)-(−)-epicatechin] had previously been determined in different samples of *Vitis vinifera* grape seeds [28], it was suggested that these compounds could belong to the (4→6) procyanidin dimer series.

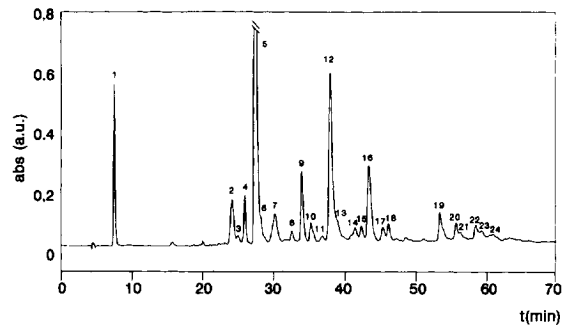

Fig. 4. Chromatogram recorded at 280 nm of the grape seed extract. Peaks: 1 = gallic acid; 2 = B3; 4 = B1; 5 = (+)-catechin; 7 = B4; 9 = B2; 12 = (−)-epicatechin; 14 = C1; 19 = (−)-epicatechin 3-O-gallate; 22 = B5.

Peaks 11, 13, 15, and 23 exhibited a min–max distance value representative of procyanidin dimers (27.3 nm), although the value for the convexity interval was higher (~26 nm). In the same way, peaks 16 and 24 exhibited a min–max distance value proper of procyanidin trimers (26.0 nm), the value for the convexity interval being higher (~24 nm). These features suggested that we may be dealing with gallates of both procyanidin dimers and trimers–with values for the min–max distance of 27.3 and 26.0 nm, respectively–, which agreed with other reports on the presence of different procyanidin gallates in *Vitis vinifera* grape seeds [29]. Following the same argument, peak 20–with 29.8 nm as the convexity interval value and 27.3 nm as the min–max distance value–and peak 21–with 25.4 nm as the convexity interval value and 26.0 nm as the min–max distance value–could correspond to di- or trigalloylated dimer and trimer respectively,

Table 2
Values of spectral parameters for the peaks detected in the chromatogram of an extract of grape seeds (*Vitis vinifera* var. Airen)

Peak No.	Spectrum maximum B3 (nm)	Convexity interval C3 (nm)	Min–max distance (nm)	Compound
1	271.2	37.7	24.7	gallic acid
2	278.9	20.6	27.3	B3
3	278.9	20.9	28.6	
4	278.9	20.4	27.3	B1
5	278.9	20.6	28.6	C
6	278.9	20.7	27.3	procyanidin dimer
7	278.9	20.7	27.3	B4
8	278.9	21.1	28.6	
9	278.9	20.6	27.3	B2
10	274.5	29.6	24.7	
11	278.9	26.3	27.3	gallate of procyanidin dimer
12	278.9	20.5	28.6	E
13	278.9	26.1	27.3	gallate of procyanidin dimer
14	278.9	20.7	26.0	C1
15	278.9	26.2	27.3	gallate of procyanidin dimer
16	278.9	24.3	26.0	gallate of procyanidin trimer
17	278.9	20.7	27.3	procyanidin dimer
18	278.9	20.5	27.3	procyanidin dimer
19	278.9	28.8	28.6	E-G
20	278.9	29.8	27.3	di or trigallate of procyanidin dimer
21	278.9	25.4	26.0	di or trigallate of procyanidin trimer
22	278.9	20.7	27.3	B5
23	278.9	25.8	27.3	gallate of procyanidin dimer
24	278.9	23.9	26.0	gallate of procyanidin trimer

whose occurrence has been reported by the same authors.

Peak 10 exhibited the same spectral characteristics as (−)-epigallocatechin 3-O-gallate, but differed in retention time. This could possibly correspond to (+)-gallocatechin 3-O-gallate. Since peaks 3 and 8 showed the same spectral characteristics as monomers of procyanidins but with different retention times, they could correspond to (+)-catechin or (−)-epicatechin esterified with a non-phenolic acid.

4. Conclusions

Spectral parameters have been found to be related to some features of the chemical structure in procyanidins and related compounds. The spectrum maximum is different for epigallocatechin and for catechin and/or epicatechin units. The min–max distance varies with the number of units that form the procyanidin. Gallates of flavan-3-ol show higher values for the convexity interval than the corresponding flavan-3-ols.

These relationships between spectral parameters and chemical structure have been proved to be very useful in the identification of unknown chromatographic peaks corresponding to condensed tannins which are not be commercially available or have not yet been identified.

This simple procedure, suitable for different chromatographic separation conditions, is complementary to other identification techniques. Once procyanidin extracts are fractionated, the study of the spectrum of an individual compound can give a preliminary idea about its chemical structure which can be sufficient for certain

studies, or which can facilitate the choice of the right method (acid hydrolysis, phloroglucinol degradation,..) for the complete identification of the compound.

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References

[1] R.A Arnold, A.C. Noble and V.L. Singleton, J. Agric. Food Chem., 28 (1980) 675.
[2] E. Haslam and T.H. Lilley, CRC Rev. Food Sci. Nutr., 27 (1988) 1.
[3] A.E. Hagerman and L.G. Butler, in G. Rosenthal (Editor), Herbivores: Their Interactions with Secondary Plant Metabolites, Vol. I, The Chemical Participants, Academic Press, New York, 1991, p. 355.
[4] E. Haslam, Plant Polyphenols, Vegetable Tannins Revisited, Cambridge University Press, Cambridge, 1989.
[5] A.G.H. Lea, J. Sci. Food Agric., 30 (1979) 833.
[6] J. Jerumanis, Proc. Eur. Brewery Conv., (1979) 309.
[7] A.W. Jaworski and C.Y. Lee, J. Agric. Food Chem., 35 (1987) 257.
[8] J.M. Ricardo da Silva, J.P. Rosec, M. Bourzeix and N. Heredia, J. Sci. Food Agric., 53 (1990) 85.
[9] J. Rigaud, M.T. Escribano-Bailon, C. Prieur, J.M. Souquet and V. Cheynier, J. Chromatogr. A, 654 (1993) 255.
[10] A.G.H. Lea, P. Bridle, C.F. Timberlake and V.L. Singleton, Am. J. Enol. Vitic., 30 (1979) 289.
[11] J. Oszmianski and J.C. Sapis, J. Agric. Food Chem., 37 (1989) 1293.
[12] J.A. McCallum and J.R.L. Walker, Cereal Chem., 67 (1990) 282.
[13] F.J. Pérez-Ilzarbe, V. Martínez, T. Hernández and I. Estrella, J. Liq. Chromatogr., 15 (1992) 637.
[14] L.J. Putman and L.G. Butler, J. Chromatogr., 318 (1985) 85.
[15] M.J. Brandon, L.Y. Foo, L.J. Porter and P. Meredith, Phytochemistry, 21 (1982) 2953.
[16] J. Rigaud, F.J. Pérez-Ilzarbe, J.M. Ricardo da Silva and V. Cheynier, J. Chromatogr., 540 (1991) 401.
[17] L.J. Porter, in J.H. Harbone (Editor), Methods in Plant Biochemistry, Vol. I, Plant Phenolics, Academic Press, New York, 1989, p. 389.
[18] R.G. Bailey, I. McDowell and H.E. Nursten, J. Sci. Food Agric., 52 (1990) 509.
[19] V. Hong and R.E. Wrolstad, J. Agric. Food Chem., 38 (1990) 708.
[20] B. Bartolomé, M.L. Bengoechea, M.C. Gálvez, F.J. Pérez-Ilzarbe, T. Hernández, I. Estrella and C. Gómez-Cordovés, J. Chromatogr. A, 655 (1993) 119.
[21] V. Cheynier and J.M. Ricardo da Silva, J. Agric. Food Chem., 39 (1991) 1047.
[22] F.J. Pérez-Ilzarbe, T. Hernández and I. Estrella, Z. Lebensm. Unters. Forsch., 192 (1991) 551.
[23] K.R. Markham, Techniques of Flavonoid Identification, Academic Press, London, 1982, p. 36.
[24] B. Bartolomé, M.L. Bengoechea, F.J. Pérez-Ilzarbe, T. Hernández, I. Estrella and C. Gómez-Cordovés, J. Chromatogr. A, 664 (1994) 39.
[25] H. Ping-kay and F. Wai-kwong, Analyst, 116 (1991) 751.
[26] D.E.H. Palladino and K.A. Cohen, J. Chromatogr. Sci., 29 (1991) 91.
[27] P. Lehtonen and H. Vuorela, J. Liq. Chromatogr., 17 (1994) 1245.
[28] M.T. Escribano-Bailón, Ph.D. Thesis, Universidad de Salamanca, Salamanca, 1993.
[29] T. Escribano-Bailón, Y. Gutiérrez-Fernández, J.C. Rivas-Gonzalo and C. Santos-Buelga, J. Agric. Food Chem., 40 (1992) 1794.