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Study of the polyphenol content of red and white grape varieties by liquid chromatography–mass spectrometry and its relationship to antioxidant power[☆]

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Abstract

There is considerable interest in the analysis of phenolic compounds due to their involvement in the organoleptic characteristics of foods and drinks, and their contribution to protection against cardiovascular diseases and cancers through their antioxidant activity. This article describes studies of the polyphenol content, both total (by means of the Folin–Ciocalteu method) and individual (by means of HPLC, LC–MS and LC–MS–MS) and the antioxidant power, of six varieties of grape over the course of their maturation. A good correlation is found to exist between the total polyphenol content of the samples and their antioxidant power, and both seem diminish along the maturation.
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1. Introduction

Polyphenol compounds, present in all plants, are of great importance for the food and drink products derived from plants, since these compounds are responsible for their organoleptic properties. As a consequence, they are closely related to the quality of such products, which makes their analysis of considerable interest.

Moreover, in recent years, numerous research studies have associated the consumption of foods rich in polyphenols with the prevention of cardiovascular diseases, certain types of cancer and other diseases related to aging, thanks to their antioxidant properties [1,2]. The great advantage of wine as a matrix for polyphenols in the diet is that in wines they are present in the soluble state and are hence more biologically available, in contrast with plant foods that contain their polyphenol compounds in polymeric, insoluble or strongly bonded forms, and are thus less available for absorption. In fact, the World Health Organization (WHO) recommends the

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moderate consumption of wine accompanying meals as part of a healthy and balanced diet [3]. All this makes the study of polyphenol content and its relationship with the antioxidant power important for the food industry in general, and for the wines and grape derivatives sector in particular.

It is known that wine receives the greater part of its polyphenol content from its raw material, the grape. In addition, each variety of grapes presents a different composition in polyphenols, and this evolves as the fruit forms, grows and matures [4]. Therefore, the monitoring of this evolution during the course of grape maturation should be informative and useful. What is novel in the present article is that it approaches the study of the polyphenol content by considering six different varieties of grape, white and red, which are monitored during the course of maturation, and that the findings are related to the evolution of antioxidant power of these varieties. Most of the past research dealing with the analysis of polyphenols has traditionally used high-perfromance liquid chromatography (HPLC), but recently the coupled technique of liquid chromatography–mass spectrometry (LC–MS) is being used for this purpose [5–8], and this is the technique employed in the research described here.

2. Experimental

2.1. Reagents

The solvents employed for the extraction of the samples were water purified in a Milli-Q system (Millipore, Bedford, MA, USA) and methanol (Scharlau, Barcelona, Spain).

The Folin reagent (Sigma–Aldrich, Madrid, Spain) and sodium carbonate (Panreac, Barcelona, Spain) were employed for the measurement of the Folin–Ciocalteau total polyphenol index. The calibration curve was constructed with gallic acid (Merck, Darmstadt, Germany).

For the antioxidant activity measurement, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, 98% purity) (Sigma–Aldrich) in a phosphate buffer medium (pH 6; ionic strength 0.05), prepared from solutions of KH_2PO_4 and Na_2HPO_4 (Fluka, Buchs, Switzerland), and $Zn(CH_3COO)_2$ and KCl (Panreac) were used. The calibration curve was constructed with 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 97% purity) supplied by Sigma–Aldrich.

The solvents employed for the HPLC analysis and the LC–MS analysis were prepared with methanol and acetic acid of HPLC quality (Scharlau, Barcelona, Spain) and distilled water (Milli-Q quality). The solutions were filtered through cellulose acetate membranes (solvent A) and PTFE membranes (solvent B) of 0.45 μm pore size (Micron Separation, Westboro, MA, USA) and were degasified in an ultrasound bath.

Calibration curves for HPLC were constructed for the following polyphenols: catechin and epicatechin (Sigma–Aldrich), epicatechingallate (Extrasynthèse, Genay, France), *trans-p*-coumaric acid (Merck) and caffeic acid (Fluka). Caftaric, *cis*-coutaric and *trans*-coutaric acids are not commercialized, so the former was quantified using the caffeic acid calibration curve and the other two using the *trans-p*-coumaric acid one [9].

2.2. Samples

We studied grapes from three red varieties, Cabernet Sauvignon (CS), Syrah (SY) and Tempranillo (TE) and three white ones, Palomino Fino (PF), Pedro Ximenez (PX) and Moscatel (MO). The samples, cultivated under non-irrigated conditions, were taken every week from the beginning of the ripening until the harvest in 1999 and 2000. All of them were obtained from the Agricultural Research Centre ‘‘Rancho de la Merced’’ (Jerez de la Frontera, Spain).

2.3. Extraction procedures

2.3.1. Pressurized liquid extraction (PLE)

After collection of the samples, the grape is cut off at the base of the peduncle, washed, dried and frozen. To proceed with its study, it is cut open and introduced into a cell of the extraction equipment, where it is subjected to two cycles of extraction of 10 min each, the temperature being held at 100 °C and the pressure at 100 atm (1 atm=101.325 Pa). The solvent employed in the process is a 1:1 methanol–water mixture. The extraction process is

performed in an ASE 200 unit of Dionex (Sunnyvale, CA, USA). The volume of the resulting extract is completed up to 10 ml.

2.3.2. Solid-phase extraction (SPE)

In continuation, 5 ml of the extract previously obtained is submitted to an SPE process. This extraction is performed in a vacuum device Visiprep SPE Vacuum Manifold of Supelco (Bellefonte, PA, USA) using LiChrolut EN cartridges (Merck) that contain 200 mg of a polymeric adsorbent based on polystyrene–divinylbenzene. The method is described in Table 1. It consists of passing the liquid sample through the cartridge, where the polyphenols are retained. Next these are re-extracted in two fractions (A and B), with the polyphenols being separated as a function of their polarity.

2.4. Total polyphenol index: Folin–Ciocalteu method

Observing the sequence specified here, the following are introduced into a calibrated 25 ml flask: 250 μl of sample, 12.5 ml of distilled water, 1250 μl of Folin–Ciocalteu reagent, 5 ml of a solution of sodium carbonate at 20%, and distilled water to make up the total volume of 25 ml. The solution is agitated to homogenize it and left to stand for 30 min for the reaction to take place and stabilize. The absorbance at 750 nm is determined in a cuvette of 1 cm [10].

For the measurement of the A fraction, a dilution of 1:2 is employed, and for the B fraction, a dilution of 1:10. The calibration curve is performed with gallic acid, and the results are expressed as equivalents mg of gallic acid (GAE) per g of grape extracted. Calibration analytical parameters were calculated with the ALAMIN program [11] and resulted in: $R=0.9992$, detection limit=41.665 mg/l and linearity=98.60%.

Table 1
Solid-phase extraction (SPE) method

Operation	Solvent
Solvation	4 ml methanol
Condition	4 ml water
Sample elution	5 ml sample
Fraction A elution	2.5 ml methanol–water (1:9)
Fraction B elution	2.5 ml methanol

2.5. Measurement of the antioxidant activity

This is carried out by means of an electrochemical method developed previously by our research group [12]. In an electrolytic device are placed: on the anode, 150 ml of ABTS 50 μ*M* (pH 6) and 150 μl of sample, and on the cathode, 500 ml of saturated zinc acetate; the saline bridge that joins the two recipients is filled with saturated KCl. Holding the intensity constant at 10 mA, the absorbance at 414 and 734 nm is recorded. As response function, the coulombs consumed in the oxidation of the sample are measured, and the antioxidant of reference for the calibration is Trolox. The results are expressed as equivalent concentration of Trolox in m*M*/mg of grape extracted. The calibration analytical parameters of this method, calculated with the ALAMIN program [11], are: $R=0.9922$, detection limit=8.652 m*M* and linearity=97.49%.

2.6. Analysis by LC–MS

The qualitative study of the polyphenol content was performed in a Finnigan LCQ coupled LC–MS system, of Finnigan MAT (Thermo Electron, San Jose, USA). This equipment is fitted with a Spectra System 2000 model gradient pump (Thermo Separation Products, Fremont, USA) and a mass detector (model LCQ) that consists of an electrospray interface and an ion trap mass analyzer. The software for the control of the equipment, and the acquisition and treatment of data is Xcalibur, version 1.2.

The separation was performed in a Luna Synergi column of 250×2 mm I.D., 4 μm particle size, 80 Å pore size (Phenomenex, Torrance, CA, USA). The chromatographic conditions were: flow-rate of 0.15 ml/min, sample injection volume of 20 μl and mobile phases A (1% methanol, 1% acetic acid, 98% water) and B (90% methanol, 2% acetic acid, 8% water). The gradient program is specified in Table 2.

The interface conditions were: negative ionization mode, temperature of the capillary, 250 °C; spray voltage, 4.5 kV; capillary voltage, −5 V; focus gas

Table 2
Elution gradient program for LC–MS

Time (min)	Solvent A (%)	Solvent B (%)
0	100	0
10	100	0
40	50	50
50	50	50
60	0	100
65	0	100
70	100	0

flow, 80 (arbitrary units) and auxiliary gas flow, 10 (arbitrary units).

The mass detection was performed in the base peak mode, for m/z between 100 and 1000.

For the LC–MS–MS study an energy of activation of 25% was applied.

2.7. Analysis by HPLC

The quantitative analysis of the polyphenol content was performed using a Waters HPLC system (Waters/Millipore, Milford, MA, USA) consisting of a model 616 pump, a model 600S gradient controller, a model 717 automatic sampler, and a model 996 photodiode detector.

The separation of the polyphenols was conducted in a LiChrospher 100 RP-18 column (Merck), 250× 3 mm I.D., 5 μm particle size, 100 Å pore size. The chromatographic conditions were: 0.4 ml/min flow-rate, 40 μl injection volume, eluents: A (10% methanol, 2% acetic acid, 88% Milli-Q water) and B (90% methanol, 2% acetic acid, 8% Milli-Q water). The gradient program is shown in Table 3. The detection by UV absorption was conducted by scanning between 250 and 600 nm, with a resolution of 1.2 nm, and the quantification were conducted at 320 nm for the derivatives of cinnamic acid and at 280 nm for the rest of the polyphenols. The data acquisition and treatment were conducted using the Millenium 2010, version 2.21 software.

Table 3
Elution gradient program for HPLC

Time (min)	Solvent A (%)	Solvent B (%)	Curve
0	100	0	0
25	85	15	9
45	50	50	9
60	50	50	9
75	0	100	6
85	0	100	6
90	100	0	6
100	100	0	6

The analytical parameters of the calibration curves were calculated with the ALAMIN program [11]. Coefficient of correlation, detection limit (in mg/l) and linearity were, respectively: 0.9848, 6.054 and 82.38% for catechin, 0.9958, 3.513 and 95.43% for epicatechin, 0.9998, 0.628 and 98.52% for epicatechingallate, 0.9998, 0.660 and 98.92% for caffeic acid and 1.0000, 0.245 and 99.61% for *trans*-*p*-coumaric acid.

3. Results and discussion

All the samples were submitted to an initial extraction process with pressurized solvent. Next, to separate and concentrate the polyphenol content in the resulting extracts, they were submitted to a second extraction process in solid-phase, being separated into two fractions, A and B.

The total polyphenol index (TPI) was measured by the Folin–Ciocalteu method for all the extracts finally obtained. It was found that in all the cases, the fraction A gave TPI values some 10 times lower than those of the fraction B (Figs. 1 and 2). This clearly indicates that the solvent B (methanol) extracts a greater quantity of polyphenols than solvent A (10% methanol), which is more polar. At the same time, it is confirmed that the red varieties have a somewhat greater polyphenol content than the white. During

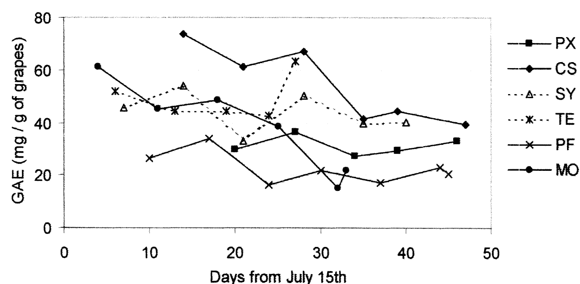

Fig. 1. Fraction A total polyphenol index along maturation.

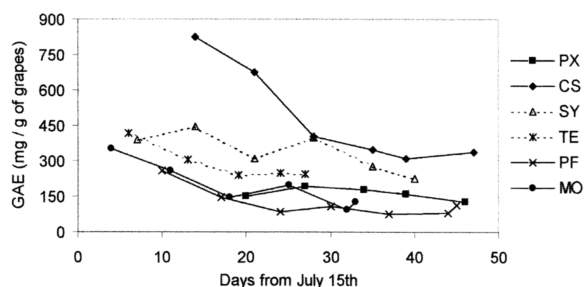

Fig. 2. Fraction B total polyphenol index along maturation.

the course of the maturation, in general, the tendency followed is irregular and different for each variety. Neither a progressive increase nor a decrease of the TPI is observed. What is apparently observed is that the first samples taken of each variety have a greater polyphenol content. This finding could be explained taking into account that, according to what has been reported, most of the polyphenols are found in the skin and seeds of the grape. Considering that, at the start of the maturation, the grapes are smaller, the ratio of skin and seeds to the pulp is higher, with the polyphenols therefore being more concentrated.

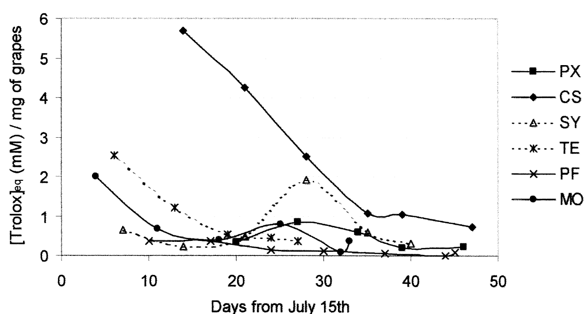

Fig. 3. Fraction B antioxidant power along maturation.

In continuation, the antioxidant power (AP) of fraction A was measured in the samples that had presented a higher TPI, and this always gave a result below the limit of detection of the method. Thus it was decided to continue the study with only fraction B, which appeared to contain the greater part of the polyphenols in the samples. The AP of all the extracts was measured (Fig. 3), and it was found that, the same as for the TPI, the red varieties have a greater AP than the white, and that the first samples taken of each variety are those with the greater AP. Over the course of the maturation, the antioxidant power appears to diminish, although the tendency is not at all clear.

Then the qualitative study of the polyphenol content was performed by LC–MS. Since both the AP and the TPI were generally found to be higher at the start of the maturation, the first two samples taken of each variety were chosen for this study. In all the samples analyzed, nine phenolic compounds were clearly identified: catechin, epicatechin, epicatechingallate, caftaric acid, *cis*-coutaric acid, *trans*-coutaric acid, quercetin glucoside, quercetin methylglucoside and luteolin-7-*O*-β-glucuronide. In addition, miricetinglucoside was found in the red

Table 4
Molecular ions and fragments of the identified compounds in the LC–MS analysis and base peaks of the LC–MS–MS analysis

Compound	*t*R (min)	LC–MS (NI)		LC–MS (PI)		LC–MS–MS (NI)
		$[M-H]^-$	Fragments	$[M-H]^+$	Fragments	Base peak
Caftaric acid	32.7	311	179			179
Catechin	35.0	289		291		245
cis-Coutaric acid	37.3	295	163			163
trans-Coutaric acid	38.6	295	163			163
Epicatechin	40.0	289		291		245
Epicatechingallate	44.0	441	289	443	291	289
Miricetin glucoside	47.5	479	317	481	319	
Quercetin glucoside	52.3	463	301	465	303	
Quercetin methylglucoside	53.5	477	301	479	303	
Vitexin	57.5	447	285	449	287	285
Luteolin-7-*O*-β-glucuronide	58.4	447	285	449	287	285

Key: NI, negative ionization; PI, positive ionization.

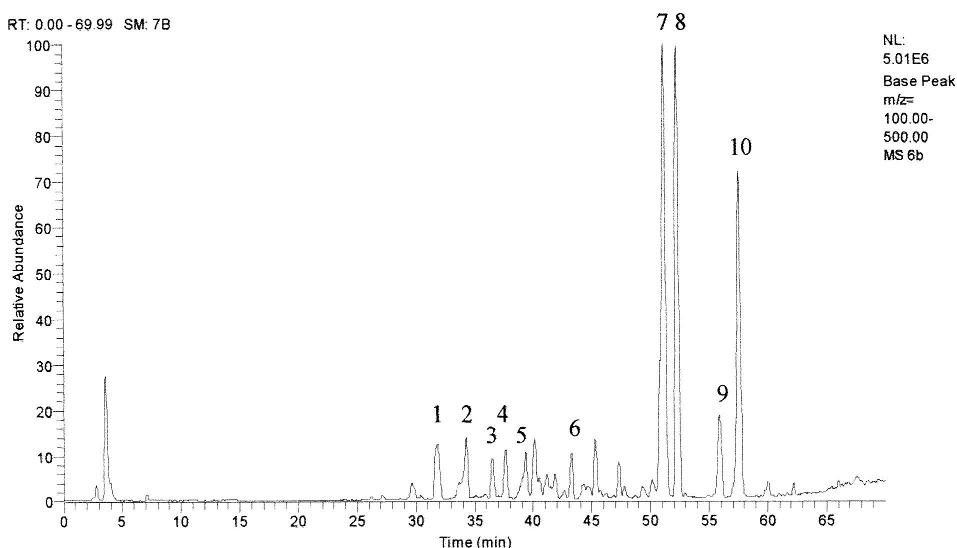

Fig. 4. Chromatogram (in negative ionization mode) corresponding to the sample PX-1. (Key: 1, caftaric acid; 2, catechin; 3, *cis*-coutaric acid; 4, *trans*-coutaric acid; 5, epicatechin; 6, epicatechingallate; 7, quercetin glucoside; 8, quercetin methylglucoside; 9, vitexin; 10, luteolin-7-*O*-β-glucuronide).

grapes and vitexin in the white. Table 4 shows the molecular ions and fragments corresponding to the studies carried out with LC–MS by negative and positive ionization, together with the base peaks of the study carried out with LC–MS–MS with negative ionization. As examples, the chromatograms by negative and positive ionization of the first taken sample of Pedro Ximenez variety (PX-1) are shown (Figs. 4 and 5).

In continuation, the quantitative study was per-

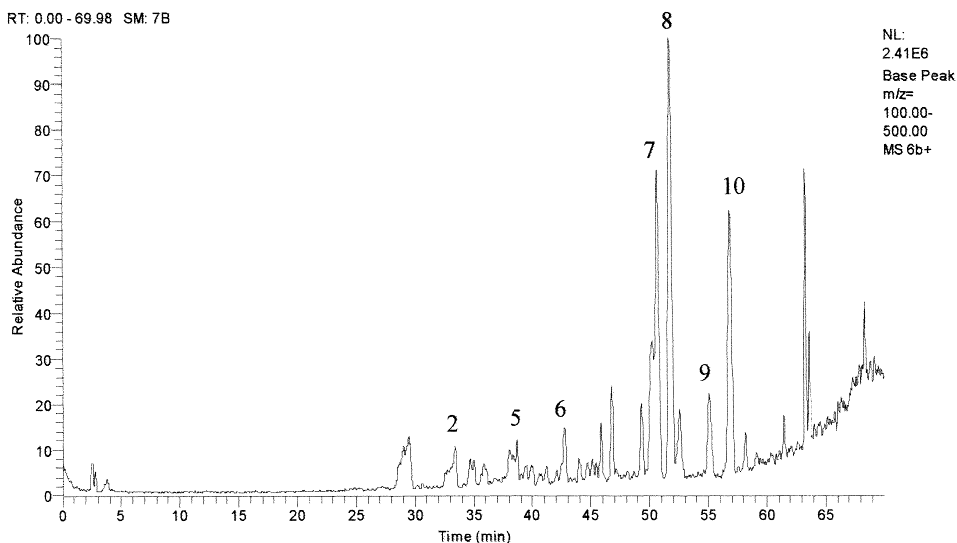

Fig. 5. Chromatogram (in positive ionization mode) corresponding to the sample PX-1. (Key: 2, catechin; 5, epicatechin; 6, epicatechingallate; 7, quercetin glucoside; 8, quercetin methylglucoside; 9, vitexin; 10, luteolin-7-*O*-β-glucuronide).

Table 5
Quantified compounds in white varieties (mg/g of grape) in the HPLC analysis

Sample	Cat	Epi	ECG	Caft	c-Cut	t-Cut
PX-1	31.64	9.73	3.73	1.29	0.20	0.31
PX-2	36.50	10.87	5.37	2.09	0.21	0.38
PX-3	42.02	8.60	5.25	1.47	0.19	0.32
PX-4	16.16	4.17	1.55	0.43	0.13	0.08
PX-5	22.44	8.95	1.13	0.26	0.09	0.07
PF-1	36.89	9.81	4.50	29.09	1.84	8.08
PF-2	16.98	4.98	1.77	13.55	1.17	3.68
PF-3	15.39	4.35	1.60	2.99	0.43	1.26
PF-4	17.25	4.47	1.46	1.99	0.33	0.79
PF-5	14.11	3.30	0.91	0.25	0.14	0.13
PF-6	7.44	2.20	0.90	3.23	0.45	1.11
PF-7	5.99	1.41	0.60	5.21	0.84	1.73
MO-1	84.71	14.03	19.77	39.88	1.68	6.37
MO-2	87.18	13.88	8.03	6.57	0.40	0.85
MO-3	52.57	7.38	5.41	3.32	0.25	0.44
MO-4	50.63	6.85	0.50	3.79	0.30	0.80
MO-5	21.12	3.94	1.05	1.18	0.15	0.23
MO-6	45.33	9.39	1.86	0.24	0.11	0.06

Key: Cat, catechin; Epi, epicatechin; ECG, epicatechingallate; Caft, caftaric acid; c-Cut, *cis*-coutaric acid; t-Cut, *trans*-coutaric acid.

formed for all the samples, by means of HPLC, in respect of those compounds for which a commercial standard was then available (Tables 5 and 6). The results are expressed as mg/g of grape. What seems to be observed is that the concentration of each compound is higher in the initial samples and that it then decreases over the course of the maturation, which could be explained by the fact already commented on, that the grape swells during the maturation, effectively diluting all its content. It can be stated that, in all the varieties tested, catechin is the compound present in the greatest quantity and, together with epicatechin, is notably above all the rest of the polyphenols.

Finally a correlation analysis was made between the AP, the TPI, and the individual polyphenols quantified. A good correlation was found between the TPI and AP ($R=0.8864$), but no specific compound showed a correlation with the AP. In previous studies [12,13], similar results have been found for wines and byproducts derived from white and red grapes. Thus it can be concluded that the AP is determined by the global polyphenol content of the samples, to which each individual polyphenol contri-

Table 6
Quantified compounds in red varieties (mg/g of grape) in the HPLC analysis

Sample	Cat	Epi	ECG	Caft	c-Cut	t-Cut
CS-1	586.86	137.12	41.73	6.97	0.45	1.23
CS-2	373.19	93.47	19.38	2.65	0.22	0.45
CS-3	174.19	48.73	4.02	0.96	0.10	0.17
CS-4	121.14	33.05	2.22	0.43	0.08	0.17
CS-5	100.10	28.14	1.88	0.81	0.15	0.15
CS-6	118.42	32.38	1.96	0.51	0.14	0.16
SY-1	159.28	73.18	15.63	15.88	1.38	4.89
SY-2	118.96	63.06	14.57	12.55	0.93	3.74
SY-3	68.70	43.81	4.77	1.71	0.15	0.60
SY-4	114.79	55.06	8.75	4.10	0.36	1.23
SY-5	57.04	32.10	2.00	1.06	0.25	0.52
SY-6	42.61	28.64	1.12	0.37	0.19	0.28
TE-1	175.29	68.45	18.69	6.80	1.09	4.88
TE-2	96.49	29.96	6.09	2.53	0.46	2.15
TE-3	37.85	22.87	3.63	0.94	0.33	1.56
TE-4	25.15	18.84	1.64	1.90	0.44	2.06
TE-5	22.87	12.05		0.85	0.26	0.90

Key: Cat, catechin; Epi, epicatechin; ECG, epicatechingallate; Caft, caftaric acid; c-Cut, *cis*-coutaric acid; t-Cut, *trans*-coutaric acid.

butes in a different way, but none of them exerts a determining influence.

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