Critical Factors of Vanillin Assay for Catechins and Proanthocyanidins

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Several parameters mostly affecting the precision and accuracy of vanillin assay were reexamined and optimized. The reexamination was performed both by vanillin reaction with catechins and by vanillin reaction with purified proanthocyanidins. In addition to the acid nature and concentration, the reaction time, the temperature, and the vanillin concentration, other factors such as the water content, the presence of interfering substances, and the standard utilized, for both vanillin reaction with catechins and vanillin reaction with proanthocyanidins, were also important. However, the kinetics of the two types of reactions were markedly different. For estimating accurately catechins or proanthocyanidins that exist simultaneously in plant tissues, it is necessary to preliminarily separate them from each other.

Keywords: *Catechins; proanthocyanidins; vanillin assay*

INTRODUCTION

Proanthocyanidins (PA) are oligomeric and polymeric flavans or flavans-3-ols. They play an important role in determining the nutritional quality (Butler, 1989) and organoleptic properties (Lea and Arnold, 1978; Haslam and Lilley, 1988; Cheynier et al., 1997) of food products derived from plant sources. Recently, catechins and some low molecular weight PA have received considerable attention owing to their various biological activities, in particular their effects on arteriosclerosis (Masquellier, 1988) and their oxygen free radical scavenger ability (Ricardo-da-Silva et al., 1991).

Different methods used for PA estimation have been proposed; those most commonly used were vanillin assay (Goldstein and Swain, 1963; Broadhurst and Jones, 1978; Price et al., 1978), depolymerization in HCl/BuOH (Porter et al., 1986), *p*-dimethylaminocinnamaldehyde reaction (McMurrough and Dowell, 1978; Vivas et al., 1994), and Folin−Ciocalteu (Singleton and Rossi, 1965).

The Folin−Ciocalteu method is based on the reducing power of phenolic hydroxyl groups. It is not very specific and detects all phenols with varying sensitivity.

The method of depolymerization with HCl/BuOH is based on the transformation of PA into anthocyanidins in hot mineral acid solutions (Porter et al., 1986). The formed anthocyanidins assume a red color with absorbance maxima around 550 nm. This method is very specific for PA estimation but has many shortcomings. First, the transformation of PA into anthocyanidins is not complete; the yields of colored anthocyanidins depend on both structure and polymerization degree of PA (Scalbert, 1992). Second, side reactions are common during the transformation and lead to the formation of red-brown polymers absorbing around 450 nm (Scalbert, 1992). This fact may undoubtedly result in estimation error, and the application of this method for quantitative analysis of PA is limited.

The vanillin assay for PA is more attractive and preferred because of its sensitivity, specificity, and simplicity (Deshpande et al., 1986). It is quite specific to a narrow range of flavanols (monomers and polymers) and dihydrochalcones that have a single bond at the 2,3-position and free meta-oriented hydroxy groups on the B ring (Sarkar and Howarth, 1976). For many years the vanillin assay was extensively used as a standard colorimetric method for flavanols (Swain and Hillis, 1959; Broadhurst and Jones, 1978; Price et al., 1978). However, lack of reproducibility of this method was often reported (Maxson et al., 1972). For this reason, many investigators attempted to improve it (Broadhurst and Jones, 1978; Price et al., 1978).

Some authors preferred the use of *p*-dimethylaminocinnamaldehyde as an alternative to vanillin (McMurrough and Dowell, 1978; Vivas et al., 1994). This reagent offered the same specificity as vanillin, but the color formed was not stable.

The purpose of the present work is to study some factors mostly affecting the vanillin assay, to ensure its precision and accuracy.

MATERIALS AND METHODS

Materials. (+)-Catechin and (−)-epicatechin were purchased from Fluka AG (Buchs, Switzerland). The vanillin was furnished by BDH Chemicals Ltd. (Poole, England). Oligomeric PA (degree of polymerization ranging from 2 to 12−15) and polymeric PA (degree of polymerization >12−15) were isolated from grape seeds by column chromatography on Lichroprep RP-18, as described earlier (Sun et al., 1995, 1998). The purity of both oligomeric and polymeric PA was >92%, determined by combination of various methods, including formaldehyde−HCl precipitation, HCl degradation−PVP, elemental analysis, diode array HPLC, and degradation with toluene-α-thiol. The details in the determination of the purity of these PA will be described in a future paper.

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10.1021/jf980366j CCC: $15.00
Published on Web 09/24/1998

Preparation of the Standard Solution. The (+)-catechin, (−)-epicatechin, purified oligomeric PA, and polymeric PA were dissolved respectively in methanol to give stock solutions of 120 mg/L. Dilution of each stock solution was made to establish standard curves.

Reaction of Catechins or PA with Vanillin. The total volume of reaction medium was fixed at 6 mL, comprising 1 mL of sample or standard, 2.5 mL of reagent a (vanillin solution in methanol), and 2.5 mL of reagent b (HCl or H_2SO_4 solution in methanol), as described earlier (Price et al., 1978; Révilla et al., 1991). The vanillin concentration in reagent a, acid concentration in reagent b, reaction temperature, reaction time, and water content of reaction medium were varied to verify the influence of each of these factors on vanillin reaction. Furthermore, HCl or H_2SO_4 concentration in reagent b ranges from 1.8 to 9.0 N, vanillin concentration in reagent a from 0.5 to 2% (w/v), reaction temperature from 5 to 35 °C, reaction time from 0 to 30 min, and water content of reaction medium from 0 to 8% (v/v). When one factor was varied, others were fixed as follows. For (+)-catechin and (−)-epicatechin: reagent a, 1% (w/v) vanillin in methanol; reagent b, 9 N H_2SO_4 in methanol; reaction temperature, 30 °C; reaction time, 15 min. For purified oligomeric proanthocyanidins and polymeric proanthocyanidins: reagent a, 1% (w/v) vanillin in methanol; reagent b, 9.0 N H_2SO_4 in methanol; reaction temperature, room temperature; reaction time, time reaching maximum value of absorbance at $\lambda = 500$ nm (A_{500}). The A_{500} value was measured by a Shimadzu UV-265 spectrophotometer (Japan).

RESULTS AND DISCUSSION

The influence of various factors, that is, acid nature and concentration, reaction time, temperature, water content, vanillin concentration, diffuse sunlight, reference standard, and presence of interfering substances, on the reaction of vanillin with (+)-catechin ($R_{van/cat}$), on the reaction of vanillin with (−)-epicatechin ($R_{van/epicat}$), on the reaction of vanillin with oligomeric PA ($R_{van/oligo}$), and on the reaction of vanillin with polymeric PA ($R_{van/polym}$) was respectively studied. Little difference was observed between $R_{van/cat}$ and $R_{van/epicat}$. On the other hand, $R_{van/oligo}$ was similar to $R_{van/polym}$, whereas $R_{van/cat}$ or $R_{van/epicat}$ does differ significantly from $R_{van/oligo}$ or $R_{van/polym}$. For this reason, we present here only the results obtained from $R_{van/cat}$ and $R_{van/oligo}$.

Influence of Acid Nature and Concentration. The vanillin reaction with catechins or PA should be carried out with acid medium, in which the acid played a catalytic role. Both hydrochloric acid and sulfuric acid were used at various concentrations (Goldstein and Swain, 1963; Pompei and Peri, 1971; Broadhurst and Jones, 1978; Price et al., 1978; Scalbert et al., 1989; Revilla et al., 1991).

First, we examined the influence of these two acids with different normalities ranging from 1.8 to 9.0 N in the solution of reagent b on $R_{van/cat}$. The results have shown that, for all normalities tested, both hydrochloric acid and sulfuric acid gave good linearity ($R^2 > 0.998$) (Figures 1 and 2); increasing the acid normality strengthened A_{500}.

However, for the same acid normality, the A_{500} obtained by using H_2SO_4 was much higher than that obtained by using HCl. In other words, when H_2SO_4 was used, the sensitivity of the method was higher than when HCl was used. These results agreed with those reported previously (Scalbert et al., 1989). It is evident that higher acid concentrations could increase the color intensity. However, as the acid concentration increases, slow self-reaction of vanillin and even PA decomposition may occur (Broadhurst and Jones, 1978; Beart et al., 1985), so the use of acid with very high concentration should be avoided.

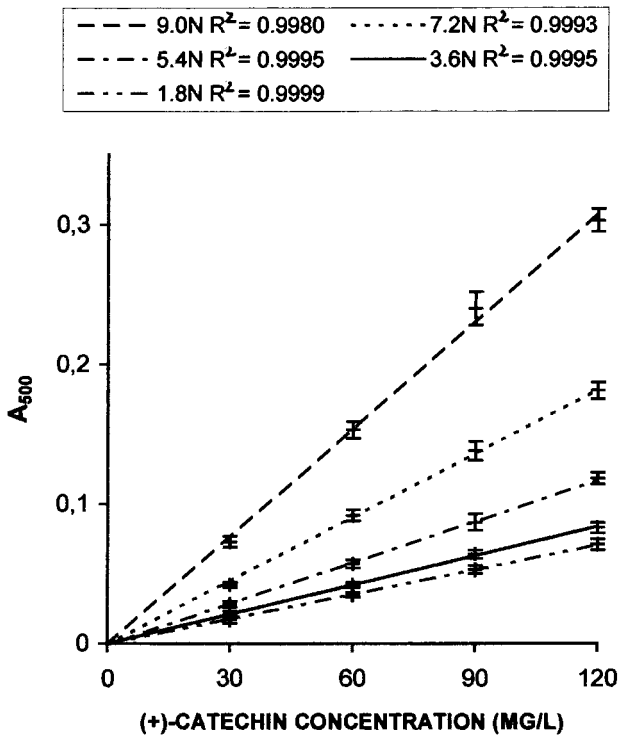

Figure 1. Standard curves of (+)-catechin for vanillin assay established at various HCl concentrations. Each point is a mean of three replicate measurements ± SD.

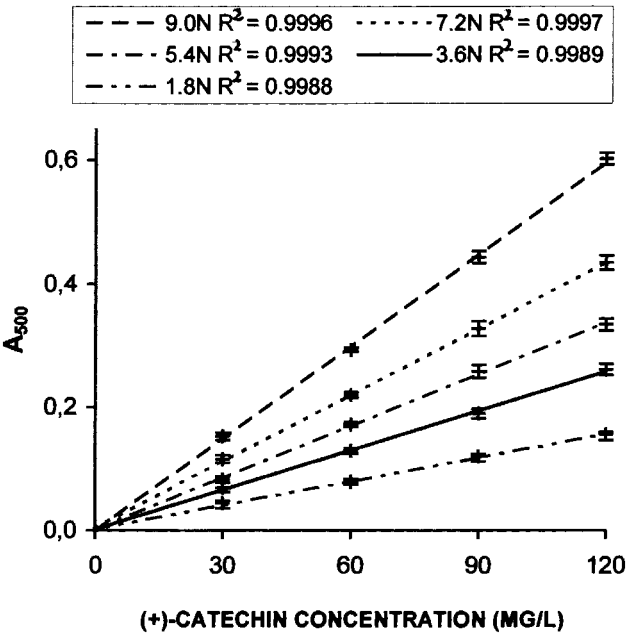

Figure 2. Standard curves of (+)-catechin for vanillin assay established at various H_2SO_4 concentrations. Each point is a mean of three replicate measurements ± SD.

Although similar results were also obtained from $R_{van/oligo}$, Figures 3 and 4 clearly show that the $R_{van/oligo}$ was less sensitive to both acid nature and acid concentration than $R_{van/cat}$.

Because the sensitivity was always higher with H_2SO_4 than with HCl for both types of reactions, the latter tests were performed only with H_2SO_4.

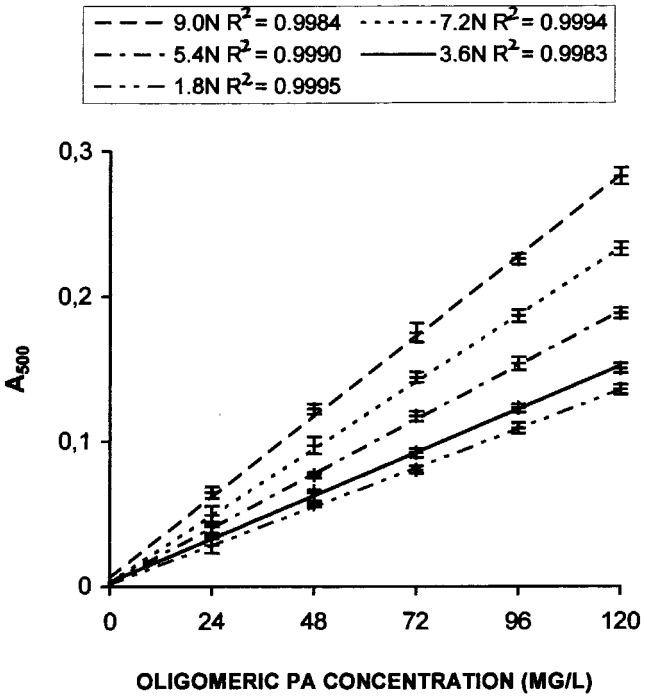

Figure 3. Standard curves of purified oligomeric PA for vanillin assay established at various HCl concentrations. Each point is a mean of three replicate measurements ± SD.

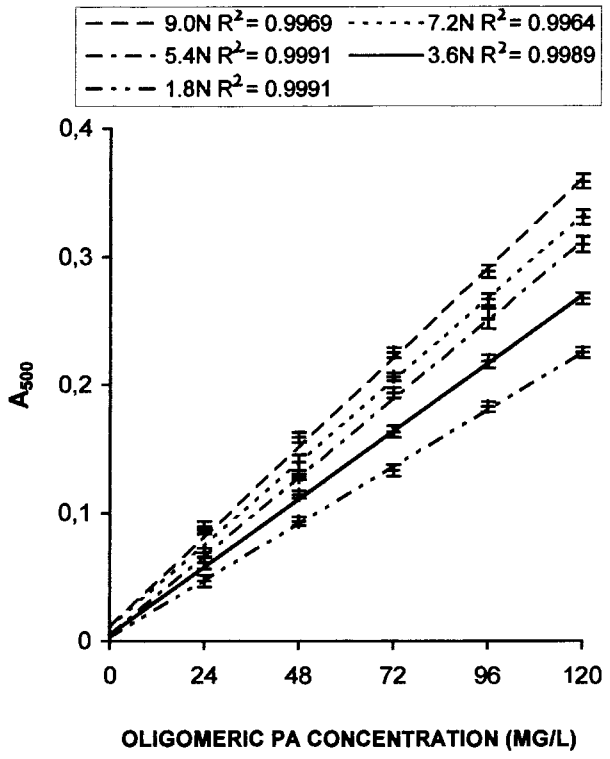

Figure 4. Standard curves of purified oligomeric PA for vanillin assay established at various H_2SO_4 concentrations. Each point is a mean of three replicate measurements ± SD.

Butler et al. (1982) proposed the use of glacial acetic acid as solvent instead of methanol to improve the reaction sensitivity and color stability. Our experimental results suggested that the increase of the reaction sensitivity and color stability should be partially due to the increase of acidity in the reaction medium. Considering that PA could not be dissolved in glacial acetic acid and that high sensitivity of the reaction and stability of color products could also be obtained in methanol/H_2SO_4 medium, glacial acetic acid was not used in this work.

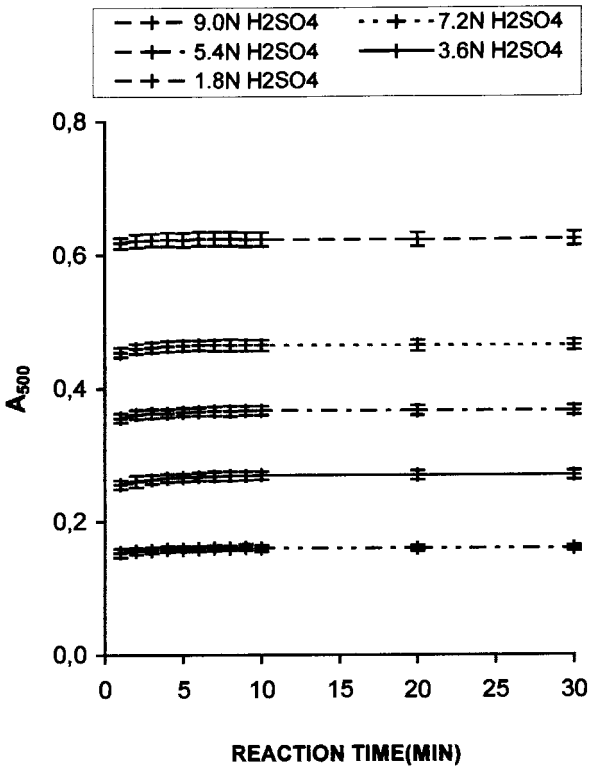

Figure 5. Effect of reaction time on A_{500} of vanillin reaction with (+)-catechin. (+)-Catechin concentration = 120 mg/L. Each point is a mean of three replicate measurements ± SD.

Influence of Reaction Time. The influence of reaction time on A_{500} $R_{van/oligo}$ at experimental conditions is presented in Figures 5 and 6, respectively.

For $R_{van/cat}$ at all H_2SO_4 concentrations tested, the A_{500} increased very slowly, reached maximum value at 5−10 min, and, after that, stabilized during >20 min. In the case of PA, the A_{500} increased slowly until maximum value obtained and, after that, decreased gradually. The A_{500} obtained at higher acid concentrations (>5.4 N), either by $R_{van/cat}$ or by $R_{van/oligo}$, appeared to be more stable than that at lower acid concentrations. In addition, the maximum A_{500} was found to be linearly related to the concentration of (+)-catechin or oligomeric PA. These results indicated that it was important to utilize more concentrated acid as reagent b and the maximum A_{500} should be taken as the measured value. In fact, the A_{500} presented in Figures 1−4 was the maximum value obtained.

Furthermore, the fixation of reaction time at 15 or 25 min as described by previous works (Pompei and Peri, 1971; Scalbert et al., 1989) is suitable for vanillin assay for catechin but not suitable for purified PA because the values measured may be not the maximum A_{500}. For this reason, we also recommend fixing the reaction time at 15 min for catechin estimation. However, for proanthocyanidin estimation, the maximum A_{500} should be taken as the measured value.

Influence of Temperature. The influence of reaction temperature on the vanillin assay for both (+)-catechin and oligomeric PA is presented in Figure 7.

It has been shown that the maximum A_{500} obtained from $R_{van/cat}$ was related to the reaction temperature. On the other hand, when the maximum A_{500} of $R_{van/cat}$

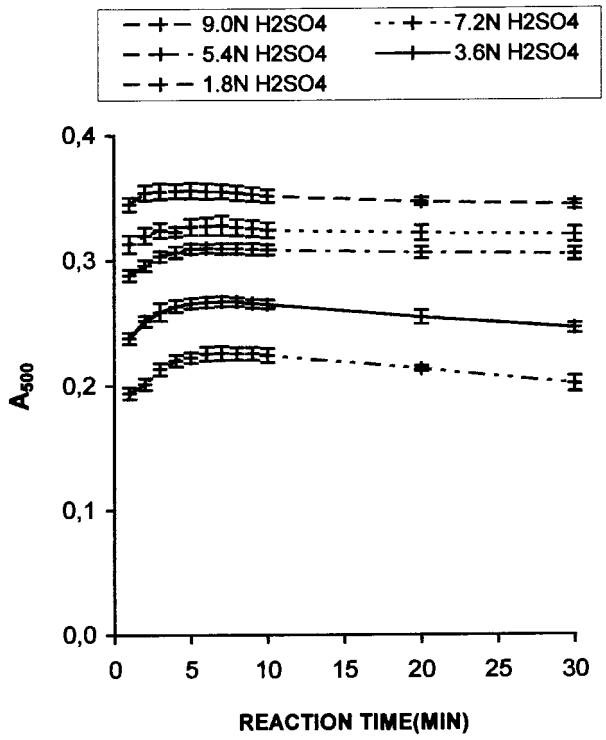

Figure 6. Effect of reaction time on A_{500} of vanillin reaction with purified oligomeric PA. Purified oligomeric PA concentration = 120 mg/L. Each point is a mean of three replicate measurements ± SD.

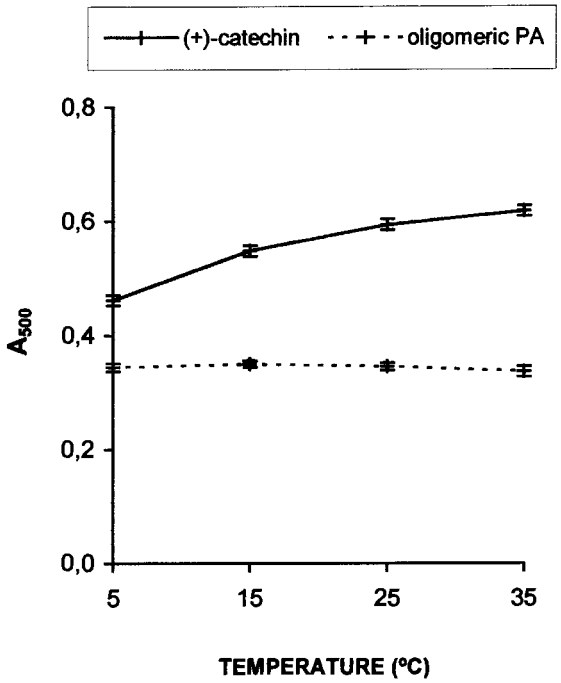

Figure 7. Effect of reaction temperature on A_{500} of vanillin reaction with (+)-catechin and purified oligomeric PA. Concentrations: (+)-catechin = 120 mg/L; purified oligomeric PA = 120 mg/L. Each point is a mean of three replicate measurements ± SD.

was obtained at a given temperature, incubation of this medium at different temperatures for 10 min gave different A_{500} values. Interestingly, this relationship between maximum A_{500} and the temperature of the reaction medium was identical to that shown in Figure 7. In other words, for $R_{van/cat}$ the dependence of A_{500} on

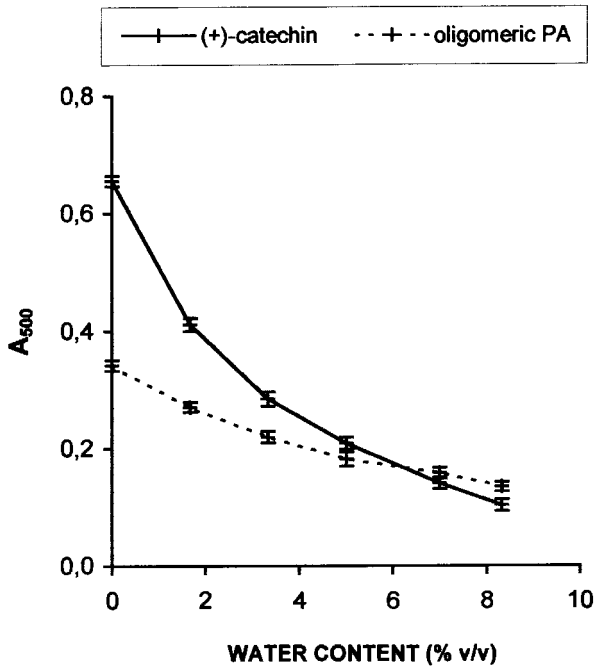

Figure 8. Effect of water content in reaction medium on A_{500} of vanillin reaction with (+)-catechin and purified oligomeric PA. Concentrations: (+)-catechin = 120 mg/L; purified oligomeric PA = 120 mg/L. Each point is a mean of three replicate measurements ± SD.

reaction medium temperature, ranging from 5 to 35 °C, was reversible. These results agreed with those already reported (Dalby and Shuman, 1978). Because increasing temperature increased the A_{500} value, 25−35 °C is recommended for catechin estimation.

On the contrary, the maximum A_{500} of $R_{van/oligo}$ depended seldom on the reaction temperature. Figure 7 shows that the $R_{van/oligo}$ performed at different temperatures gives nearly the same A_{500}.

In other words, for catechin estimation, the reaction temperature should be well controlled, whereas there was no problem for PA estimation when the temperature was not significantly changed, and therefore PA estimation could be performed at room temperature.

Influence of Water Content. The influence of water content on both $R_{van/cat}$ and $R_{van/oligo}$ is shown in Figure 8. The A_{500} decreased rapidly along with the increase of water content, for both types of reactions. Only 3% of water in the sample could decrease A_{500} by 56−59% in the case of $R_{van/cat}$ and by 33−38% in the case of $R_{van/oligo}$.

The sharp decline of A_{500} along with the increase of water content may be partially explained by the theory of Levasseur (Cabannes, 1953). According to Levasseur, the real pH value depends strongly on the water content in the sample of organic solvent media. Because the A_{500} also depends on the acid concentration or, more properly said, the pH, a change of water content modifies undoubtedly the A_{500}. In the present work, absolute methanol was used as a solvent and the reaction medium was free of water. This not only stabilized the color but increased significantly the sensitivity. As already described above, for the same normality, H_2SO_4 gave much higher A_{500} than HCl. This may be explained by the fact that a considerable amount of water is present in concentrated HCl solution.

However, the Levasseur theory cannot explain completely our experimental results. The influence of water

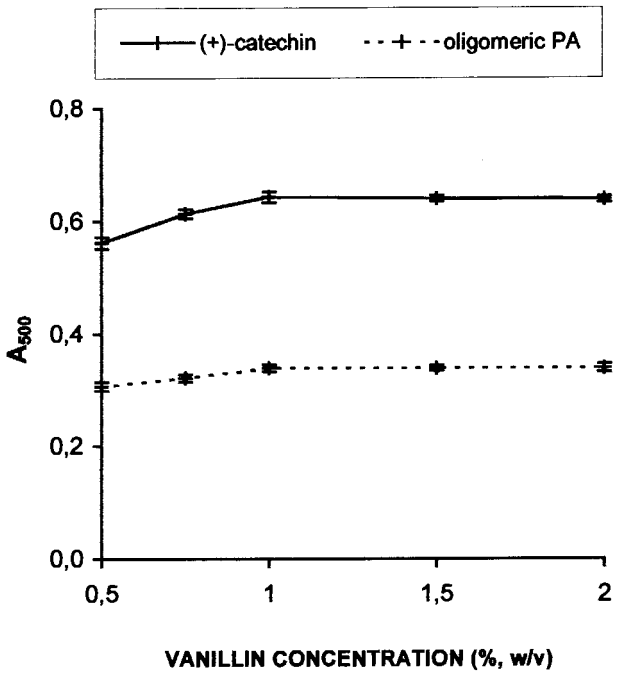

Figure 9. Effect of vanillin concentration in reagent a on A_{500} of vanillin reaction with (+)-catechin and purified oligomeric PA. Concentrations: (+)-catechin = 120 mg/L; purified oligomeric PA = 120 mg/L. Each point is a mean of three replicate measurements ± SD.

content on the color intensity of $R_{van/cat}$ was different from that of $R_{van/oligo}$. In other words, the colored products formed by $R_{van/oligo}$ were more resistant or less sensitive to water than those formed by $R_{van/cat}$ (Figure 8).

Influence of Vanillin Concentration. For the vanillin assay, the vanillin, as reagent a, must be excessive so that the reaction is complete. The influence of vanillin concentration is presented in Figure 9.

It has been shown that the A_{500}, both for $R_{van/cat}$ and for $R_{van/oligo}$, did not change when vanillin concentration was >10 g/L. In other words, the vanillin concentration used in the vanillin assay should be at least 10 g/L. Therefore, a vanillin concentration between 10 and 12 g/L in reagent a was recommended. Higher vanillin concentration should be avoided as the vanillin molecules might be slowly self-condensed in acidic medium to yield colored products (Broadhurst and Jones, 1978).

Influence of Reference Standard. (+)-Catechin is currently used as a reference standard of the vanillin assay (Swain and Hillis, 1959; Price and Butler, 1977; Price et al., 1978; Revilla et al., 1991). However, this reference standard cannot express correctly PA content in the sample because reactivity of vanillin with catechin is different from that of vanillin with proanthocyanidins (Deshpande et al., 1986; Scalbert, 1992).

In fact, as can be seen in Figures 2 and 4, for the same concentration of (+)-catechin and oligomeric PA and at a given H_2SO_4 concentration, the A_{500} obtained by $R_{van/cat}$ is different from that by $R_{van/oligo}$.

To elucidate the effect of reference standard, we have performed the vanillin assay using two samples of grape seed methanol extracts with different PA concentrations separated preliminarily from catechins (Sun et al., 1998). The PA contents in each sample were expressed, respectively, as (+)-catechin and purified oligomeric PA. The results are presented in Table 1.

Table 1. PA Content of Grape Seed Extracts Expressed by Different Reference Standards

			calcd concn (mg/L)	
sample	H_2SO_4 concn (N) in reagent b	A_{500}[a]	using oligomeric PA as standard	using (+)-catechin as standard
1	1.8	0.201 ± 0.006	106.6 ± 3.2	147.7 ± 4.6
	3.6	0.230 ± 0.002	102.2 ± 0.9	100.7 ± 0.9
	5.4	0.248 ± 0.008	94.7 ± 3.2	81.0 ± 2.6
	7.2	0.266 ± 0.009	96.1 ± 3.5	68.5 ± 2.4
	9.0	0.294 ± 0.007	98.0 ± 2.5	56.0 ± 1.4
2	1.8	0.415 ± 0.005	222.1 ± 2.7	310.2 ± 3.8
	3.6	0.474 ± 0.003	216.8 ± 1.4	208.7 ± 1.3
	5.4	0.508 ± 0.006	198.3 ± 2.4	166.5 ± 2.0
	7.2	0.553 ± 0.009	208.6 ± 3.5	143.7 ± 2.4
	9.0	0.613 ± 0.011	214.1 ± 4.0	117.6 ± 2.1

[a] Mean values of triplicate determinations ± SD.

It shows clearly that if the purified oligomeric PA is used as a reference standard, change of H_2SO_4 concentration seldom modifies the results. On the contrary, if (+)-catechin is used as a reference standard, the measured values decrease significantly along with the increase of H_2SO_4 concentration. Clearly, the purified oligomeric PA expresses more correctly PA contents in the sample.

As described above, the A_{500} depends on not only the substrate (catechin or PA) concentration but also the H_2SO_4 concentration. So we have

$$A_{500} = f([C][H^+]) \quad (1)$$

where [C] = (+)-catechin or PA concentration (mg/L) and $[H^+]$ = H_2SO_4 concentration in reagent b [5−25% (v/v), corresponding to 1.8−9.0 N].

According to the experimental results, we obtain by linear regression analysis

for $R_{van/cat}$

$$A_{500} = 0.02[C]_{cat}[H^+] + 0.015 \quad (R^2 = 0.989,\ n = 20) \quad (2)$$

for $R_{van/oligo}$

$$A'_{500} = 0.00575[C]_{pro}([H^+] + 0.2625) + 0.014 \quad (R^2 = 0.995,\ n = 20) \quad (3)$$

For the same concentrations of (+)-catechin and PA, that is to say $[C]_{cat} = [C]_{pro} = [C]$, we have

$$d(A_{500})/d([H^+]) = 0.02[C] \quad (4)$$

and

$$d(A'_{500})/d([H^+]) = 0.00575[C] \quad (5)$$

From eqs 4 and 5, we have

$$\frac{d(A_{500})/d[H^+]}{d(A'_{500})/d[H^+]} = 3.48 \quad (6)$$

Equations 4 and 5 give, respectively, the sensitivity of $R_{van/cat}$ and of $R_{van/oligo}$ to change of H_2SO_4 concentration. Furthermore, eq 6 shows that $R_{van/cat}$ is nearly 3.5 times as sensitive to change of H_2SO_4 concentration as $R_{van/oligo}$.

Table 2. Effect of Diffuse Sunlight on Vanillin Assay

	A_{500}[a]							
	reaction of vanillin with (+)-catechin				reaction of vanillin with oligomeric PA			
light condition	max value (0−10 min)	at 10 min	at 20 min	at 30 min	max value (0−10 min)	at 10 min	at 20 min	at 30 min
tubes in dark	0.647 ± 0.002	0.643 ± 0.003	0.640 ± 0.002	0.642 ± 0.003	0.502 ± 0.003	0.500 ± 0.008	0.497 ± 0.007	0.491 ± 0.002
diffuse sunlight	0.652 ± 0.006	0.651 ± 0.010	0.647 ± 0.002	0.649 ± 0.009	0.505 ± 0.003	0.503 ± 0.008	0.499 ± 0.002	0.496 ± 0.006

[a] Mean values of triplicate determinations ± SD.

On the other hand, from eqs 2 and 3, we can also calculate the acid concentration $[H^+]^*$, in which condition the same concentrations of (+)-catechin and oligomeric PA give the same A_{500}. That is

$$\frac{A_{500}}{A'_{500}} = \frac{0.020[C]_{cat}[H^+]^* + 0.015}{0.00575[C]_{pro}([H^+]^* + 0.2625) + 0.014} \quad (7)$$

where $[C]_{cat} = [C]_{pro}$, $A_{500} = A'_{500}$,

$$1 = \frac{0.02[H^+]^*}{0.00575([H^+]^* + 0.2625)} \quad (8)$$

so

$$[H^+]^* = 0.106 = 10.6\% \quad (9)$$

Equation 9 shows if the H_2SO_4 concentration of 10.6% is chosen, equivalent concentrations of (+)-catechin and PA give the same A_{500}. If the H_2SO_4 concentration is <10.6%, utilization of (+)-catechin as reference standard overestimates PA content. If the H_2SO_4 concentration is >10.6%, (+)-catechin underestimates PA content.

Hence, for total analysis of catechins and PA in one sample containing these two types of compounds, without preliminarily separating them from each other, we should use H_2SO_4 with a concentration of ~10%; in this case, (+)-catechin could be taken as reference standard, and the measured value should be approximately true concentration in sample. The results presented in Table 1 show that for the same sample, the PA concentration expressed as (+)-catechin is nearly equal to that expressed as purified oligomeric PA, at 10% (v/v) (3.6 N) H_2SO_4.

However, the properties and the reactivity of catechins and that of PA are very different, so knowledge of the proportion between these two types of compounds in a sample, especially in grapes and wine, is important. On the other hand, from the point of view of color stability or sensitivity of method, preliminarily separating catechins from PA and quantifying them separately using H_2SO_4 of higher concentration are often necessary.

Price et al. (1978) reported that the use of (+)-catechin as reference standard overestimated PA content. On the contrary, Scalbert et al. (1989) and Scalbert (1992) reported that (+)-catechin underestimated PA content. These apparently contradictory confirmations may be explained by the fact that the extreme acid concentrations were used by the two authors; the former used very diluted acidic medium [≅3.3% (v/v) HCl final concentration] and the latter, very concentrated acidic medium [≅46.7% (v/v) H_2SO_4 final concentration]. Moreover, when HCl is used, overestimation of PA content by (+)-catechin standard may be partially due to the presence of water in concentrated HCl solution because vanillin reaction with catechins is more sensitive to water content of reaction medium than that with proanthocyanidins, as described above. In other words, our experimental results are in agreement with both confirmations.

Effect of Diffuse Sunlight. Table 2 presents the effect of diffuse sunlight on the vanillin assay. Analysis of variance showed that, both for (+)-catechin and for oligomeric PA, there were no significant differences at the 5% level between vanillin reaction undergone in the dark and vanillin reaction undergone in diffuse sunlight. For this reason, all of our experiments were performed in diffuse sunlight.

It has been reported that the stability of color products formed by vanillin reaction was significantly affected by direct sunlight and, to a lesser extent, by diffuse sunlight (Broadhurst and Jones, 1978). In fact, according to published data by Broadhurst and Jones (1978), the effect of diffuse sunlight was obviously observed only after 40 min of the reaction, that is, a 5% decrease in absorbance maximum, whereas the reaction times in our work ranged from 0 to 30 min.

Interference Substances. Ascorbic acid or ascorbate interfered with the vanillin assay in H_2SO_4 medium due presumably to the oxidative nature of H_2SO_4 (Broadhurst and Jones, 1978). Obviously, the ascorbate or ascorbic acid, if it exists in the sample, should be preliminarily separated from catechins and PA. In the case of wine or grape extract, this problem could be easily resolved by fractionation with C18 Sep-Pak cartridges as described earlier (Sun et al., 1998).

Chlorophyll, existing in some plant samples such as grape stem extract, can interfere with the vanillin assay. In this case, after plant tissue extract is transferred into aqueous solution, chlorophyll in it can readily be extracted by hexane without modifying catechins and PA (Sun et al., unpublished data).

Broadhurst and Jones (1978) reported that anthocyanins, as the majority of other non-flavanols, do not react with vanillin at low concentration. This was also confirmed in the present work by testing the reactivity of vanillin with malvidin 3-glucoside. However, in acidic conditions, anthocyanins absorb at 490−540 nm, which coincides with the colored product (500 nm) of the vanillin assay. This interference can be simply eliminated using a suitable blank that is the same as the reaction medium but in the absence of vanillin (Broadhurst and Jones, 1978).

The reactivity of vanillin with some other non-flavanol compounds often encountered in plant tissues, that is, phenolic acids (cinnamic acid, *p*-hydroxybenzoic acid, caffeic acid, gallic acid, *p*-coumaric acid, syringic acid), flavonols (quercetin dihydrate, kaempferol, myricetin, rutin), was also reexamined in this work. As expected, none of these compounds either reacted with vanillin or interfered with the vanillin reaction with catechins or PA.

CONCLUSIONS

The critical evaluation of vanillin assay is summarized as follows: (1) separation of catechins from PA in the sample and quantifying each of them separately; (2) use of absolute methanol as solvent both for the sample and for the reagents; (3) choice of H_2SO_4 [7.2−9.0 N or 20−25% (v/v)] as reagent b with strict control of its concentration, preferably using the same lot of reagent b both for establishing standard curves and for estimating catechins and PA in the sample; (4) use of purified PA issue of the source itself and (+)-catechin or (−)-epicatechin as reference standards, for PA estimation and catechin estimation, respectively; (5) elimination of interfering substances (chlorophyll, ascorbic acid, . . .) and correction of anthocyanins (in the cases of red wine or extract of red grape skins) by suitable blank; (6) for catechin estimation the reaction temperature (between 25 and 35 °C) should be strictly controlled and the reaction time fixed at 15 min; PA estimation can be performed at room temperature, and the maximum ΔA_{500} should be taken as a measured value.

However, for two sample issues of different plant origins, the methods used for separating catechins from proanthocyanidins may be different. In addition, one sample may contain some unpredictable interfering substances but another does not. So the procedures of the vanillin assay for different samples may be not always identical. The following example is given to illustrate the application of the modified vanillin assay for grape and wine samples.

−Wine or grape extract (2-4 mL, the volume depending on the richness of phenolic compounds in the sample) was passed through the C18 Sep-Pak cartridges and separated using different organic solvents into three fraction containing, respectively, monomeric catechins, oligomeric PA, and polymeric PA, as recently described (Sun et al., 1998). Each fraction was evaporated to dryness under vacuum at 30 °C, and the residue was dissolved in methanol to give a desired concentration. One milliliter of this methanolic solution was mixed first with 2.5 mL of 1% (w/v) vanillin in methanol and then with 2.5 mL of 25% (v/v) H_2SO_4 in methanol to undergo vanillin reaction. The blank was simultaneously prepared in the same way except that 1% (w/v) vanillin in methanol was substituted by methanol. For young red wine, methanol-insoluble substances in the catechin fraction were sometimes found. In this case, the methanolic solution was filtered using a 0.45 μm filter before undergoing the vanillin reaction. The vanillin reaction with catechin fraction was carried out in a 30 °C water bath for 15 min, and the measurement of A_{500} was also performed at 30 °C. For oligomeric PA and polymeric PA fractions, both the vanillin reaction and the measurement of A_{500} were performed at room temperature and the maximum A_{500} was taken as the measured value. Three standard curves should be conducted using (+)-catechin, purified oligomeric PA, and purified polymeric PA, to express, respectively, catechin content, oligomeric PA content, and polymeric PA content in grape extracts or wines.

NOTE ADDED

Attempts have been also made in our laboratory to verify the effect of different factors on the vanillin reaction with individual procyanidins—B_1 and B_2. However, the amount of pure procyanidins B_1 and B_2 isolated from grape seeds was not sufficient to test all factors mentioned above, so only two the most important factors, that is, H_2SO_4 concentration and reaction temperature, were verified. As expected, the kinetic behavior of the vanillin reaction with either procyanidin B_1 or procyanidin B_2 is quite similar to that with purified proanthocyanidins. In other words, the vanillin reaction with either procyanidin B_1 or procyanidin B_2 was much less sensitive to change of H_2SO_4 concentration and reaction temperature, compared with $R_{van/cat}$. Isolation of various individual procyanidins to obtain adequate amounts of each compound for testing all factors mentioned is now being done in our laboratory. Such studies will help in determining the ideal reference standard of the vanillin assay for plant PA, that is, using individual procyanidin instead of the mixture of oligomeric or polymeric proanthocyanidins, which may permit meaningful comparisons of PA analyses issue from various sources within and between laboratories.

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Received for review April 10, 1998. Revised manuscript received July 31, 1998. Accepted July 31, 1998. B.S. thanks the Fundação Oriente and the Fundação para a Ciência e a Tecnologia (Portugal) for their grants.

JF980366J