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Improved method for measuring hydrolyzable tannins using potassium iodate

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Until now, the recommended procedure for measuring hydrolyzable tannins has been to mix the reagent and sample, subject the mixture to a certain amount of chill, allow the reaction to continue for some time at a higher temperature and then measure the absorbance of the colored solution. The effect of different chill conditions has been studied by many workers but the effect of conditions after the chill period has not been studied. In this work, the effects of changing certain reaction variables during the increased temperature time period were examined and it was found that small changes in the reaction conditions resulted in large changes in the reaction process. Additionally, the behavior of the reaction depended on the variety of plant from which the hydrolyzable tannins were extracted and the time of the season when the leaves were collected. It is recommended that no chill be used in the colorimetric procedure but, instead, the following procedure should be used: extract the plant material with 70% acetone and take 1 ml of the extract plus 5 ml of 2.5% potassium iodate and place in a test-tube. Vortex mix the mixture and immediately place the test-tube in a 25 °C water bath. When the optimum length of time has passed (which should be determined for each type of plant material and must be precisely measured), remove the sample from the water-bath and measure the absorbance at 550 nm.

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The ability to measure the total concentration of all tannins in the hydrolyzable group is very important because tannins affect plant selection by many different species of animals, from ants[1] to elephants.[2] In fact, the response of plants to herbivory has been identified as one of the most prominent topics in the last 14 years in the field of ecology and environment.[3] Our laboratory has been concerned with the relationship between the concentration of hydrolyzable tannins (HT) in oak leaves and gypsy moth defoliation. It has been shown that the concentration of HT is higher in leaves from red oak trees that have undergone significant levels of defoliation by gypsy moth than in leaves from undefoliated trees.[4,5] Higher concentrations of HT have been shown to decrease fecundity, growth and development of herbivores such as the gypsy moth[4,6–8] and the winter moth, *Operophtera brumata*.[9] Higher concentrations also reduce the efficacy of gypsy moth predators.[10–12] For these reasons, and also the fact that the concentration of HT is important in many other plant–herbivore interactions, a good method is necessary for measuring the various forms of tannins.

The traditional procedure for the measurement of HT has been as follows: mix the sample and potassium iodate, cool the mixture in an ice–water bath, remove from the bath and measure the absorbance at 550 nm.[13] This procedure has been criticized because precipitation occurs when measuring HT in the extract of certain plants or when using certain solvents. Inoue and Hagerman[14] claimed that this method is 'difficult to perform with reproducibility'. When discussing methods for measuring HT, Waterman and Mole[13] stated that 'no suitable analytical technique exists'. One facet of the procedure for which there seems to be no agreement is the chilling of the reaction mixture. Haslam[15] recommends chilling at 0 °C for 40 min. Bate-Smith[16] observed that when chilled at 0 °C the optimum time for measurement is 90 min. According to Bate-Smith,[16] the reaction proceeds faster at higher temperatures, but at temperatures greater than 15 °C the maximum absorbance decreases, and a temperature of 15 °C was recommended.

There are several reasons to study the method: the recommendation regarding chill temperature and time are contradictory, varying from 0 to 15 °C and from 40 to 90 min, respectively; there is no recommendation regarding post-chill conditions which, as this paper shows, is very critical; and there is the claim that 'no suitable analytical technique exists'.

Experimental

Reagents

A 2.5% m/v aqueous solution of potassium iodate was prepared. Standard solutions were prepared by dissolving commercially available tannic acid from either Sigma (St. Louis, MO, USA) (Lot 93H0268) or Fisher (Pittsburgh, PA, USA) (Lot 776028) in 70% v/v aqueous acetone. A 0.1 mol l^{-1} solution of iron(III) ammonium sulfate [$FeNH_4(SO_4)_2$] was prepared by dissolving 4.82 g of the dodecahydrate salt in a mixture of 80 ml of water and 10 ml of 1.0 mol l^{-1} HCl. This solution was diluted to 100 ml with water and stored in a brown bottle. The solution was pale yellow. A 0.008 mol l^{-1} solution in water of potassium hexacyanoferrate(III) [$K_3Fe(CN)_6$] was prepared and stored in a brown bottle.

Extraction of biological material

A sample extract was prepared from freeze-dried foliage that was ground with a Cyclone Mill and passed through a 0.2 mm screen. A 50 mg amount of this material was placed in a test-tube along with a triangular stirring vane (Fisher, Catalog No. 14-511-95A) and 10 ml of 70% v/v aqueous acetone. The test-tube was placed in a Reacti-Therm heater–stirrer module (Pierce, Rockford, IL, USA) and heated at 45 °C for 15 min. The stirring vane was removed, the mixture centrifuged and the supernatant saved and refrigerated for the colorimetric procedure described below.

The extraction procedure just described is almost identical with that of Hunter and Schultz[17] in that the same temperature (45 °C) was used. We deviated from their procedure by using a time of only 15 min rather than 3 h, and by leaving the sample dissolved in 70% acetone, which presented no turbidity problem, rather than changing the solvent to water. Preliminary experiments indicated that results were the same as those obtained using the procedure of Hunter and Schultz.[17]

Colorimetric procedure

For each replication of standard or plant extract, 5 ml volumes of KIO_3 were placed in 150 × 18 mm test-tubes, which were placed in a 25 °C water-bath. At 1 min intervals, we added 1 ml of sample or standard, vortex mixed and placed the tube back in the water-bath. After the optimum time had passed (see below), we measured the absorbance of the first tube at 550 nm. At 1 min intervals, we measured the absorbance of each succeeding sample. In this way, the reaction time was kept constant for each sample.

A calibration curve was prepared using standards of commercially available tannic acid. The concentration of each sample was calculated using this calibration curve and expressed as mg equivalents ml^{-1}.

Determination of optimum reaction time

The optimum time for each type of biological material being analyzed was determined, by placing several test-tubes, each containing 5 ml of KIO_3, in a 25 °C water-bath. When the temperature of the solution in the tube had equilibrated, 1 ml of sample was added to the tube; it was vortex mixed and returned to the water-bath, keeping a constant time interval between tubes. At measured time intervals, such as every 2 min, a tube was removed and the absorbance measured. In this way, for each tube, the reaction time (time at 25 °C) and the absorbance were obtained. The absorbance was plotted as a function of reaction time and the time at which maximum absorbance occurred was determined to be the optimum reaction time.

Measurement of chill requirement

Two sets of identical samples were analyzed using an identical procedure except that one set was chilled and the other not.

For set one, test-tubes, reagent and test solution were pre-chilled in an ice–water bath kept in a refrigerator. When the temperature of the materials had become constant, the sample and reagent were mixed, vortex mixed and returned to the ice–water bath in the refrigerator. The temperature of the samples for this set, measured after the temperature had stabilized, was 4 °C. After samples had cooled for the desired time (either 1 or 4 h), they were removed from the cold water-bath and placed in a warm water-bath (25 °C). The second set, which was not chilled, was placed in the warm water-bath immediately after the reagents had been mixed and vortex mixed. For both sets, samples were removed from the water-bath, one at a time, at 2 min intervals and the absorbance measured.

Purification procedure

An alternative method for determining HT uses HPLC, which requires that a sample is first purified (to remove any monomers that might be present). We wanted to test this procedure to see if there was any value in purifying the samples first. Purification procedures using an anion exchanger[18] and the adsorbent Sephadex LH-20[19] have been described. We used a slight modification of the latter procedure. The plant extract, with its tannins and other impurities dissolved in 70% acetone, was purged of acetone by directing a stream of nitrogen over the solution. The acetone that was removed was replaced with an equal volume of ethanol (95% or absolute). This was then passed through a column of Sephadex LH-20. When the tannin-containing solution had passed through the column, monomers were eluted using 95% ethanol until a negative Prussian Blue spot test was obtained. The Prussian Blue spot test was based on a procedure described by Price and Butler.[20] Specifically, each day a 1 + 1 iron(III) ammonium sulfate–potassium hexacyanoferrate(III) solution was prepared from stock solutions. One drop of this reagent was added to one drop of the column eluate. If the color turned blue immediately it indicated that phenolics were present. Polymers were then eluted using 70% acetone until a negative Prussian Blue spot test was obtained. The monomers were discarded and the polymers were saved and used for testing purified tannins.

Results and discussion

Effect of chilling

Because of the many conflicting recommendations regarding chilling requirements, we evaluated the effect of chilling at different temperatures and for different lengths of time. The results are shown in a plot of absorbance *versus* time, beginning when the samples were placed in the 25 °C water-bath (Fig. 1).

As Bate-Smith[16] discusses, and as Fig. 1 shows, when HT and KIO_3 are mixed, two reactions take place. The first is the colorimetric reaction that produces the red species which then degenerates to a yellow species. After chilling for 1 h at 4 °C, the first reaction had not gone to completion, as indicated by the rising portion of the curve. Even after 4 h at 4 °C, the first reaction had not gone to completion, as indicated by the slight rise of the curve. The question we address is whether there is any reason to chill samples. To answer this question, we examined the absorbance plots shown in Fig. 1. For samples not chilled or chilled for only 1 h, the plots had a relatively sharp peak, suggesting that it is necessary to time the reaction very precisely, regardless of which condition is chosen. If samples were chilled for 4 h, the curve was flatter and this procedure provided an opportunity for better repetition if the timing and temperature control of the 25 °C water-bath were not precise . However, a 4 h chill would result in a sharp decrease in the number of samples analyzed per day, has about 30% lower sensitivity and, if good results can be obtained with no chilling, there would be no need to require the additional 4 h. The question of whether chilling is necessary or not was tested, additionally, in four ways: reproducibility, effect of interferents, sensitivity and linearity of the calibration curve. The results of these tests are described below, along with results of other tests necessary to establish the validity of the method.

Effect of temperature

Two sets of samples were analyzed as described in the recommended procedure except that one set was placed in a warm water-bath maintained at 25 °C and the other in a warm water-bath maintained at 30 °C. The absorbance as a function of the time that samples were in the water-bath was measured (Fig. 2).

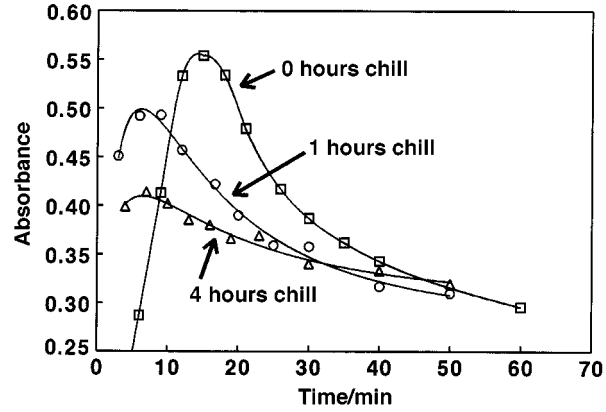

Fig. 1 Absorbance as a function of reaction time in a 25 °C water-bath for samples not chilled or chilled for 1 or 4 h at 4 °C. Samples tested were 1 mg ml^{-1} of commercially prepared tannic acid dissolved in 70% acetone. Each data point is the absorbance for a solution prepared as described in the section Measurement of chill requirement.

In both cases, the absorbance increased steadily until it reached a maximum, after which it decreased. Samples in the 30 °C water-bath reached the maximum absorbance quicker and had a higher maximum absorbance than those in the 25 °C water-bath. This sharper peak makes the timing more critical and we therefore recommend the lower temperature. However, if an autoanalyzer or any other device that precisely controls timing is being used, higher temperatures may be advantageous.

Effect of concentration

HT solutions that are the same except for concentration respond differently, as shown in the example of two solutions of commercially available tannic acid for which the absorbance was plotted as a function of time (Fig. 3). In this case, the solution with the higher concentration reached an absorbance maximum quicker and had a sharper peak. Ideally, when analyzing a set of samples, one should choose a reaction time equal to the time required for the absorbance to reach a maximum. However, as Fig. 3 shows, this is impossible. For the two concentrations shown here, the reaction time would be either 7 or 13 min depending on whether the HT concentration was 1.5 or 0.13 mg ml^{-1}. To illustrate the consequences of using the two different reaction times, a calibration curve was prepared using each of the suggested reaction times (Fig. 4). The resulting calibration curves have one significant difference, namely linearity. In this case, we recommend the reaction time of 7 min since it gives a more linear calibration curve.

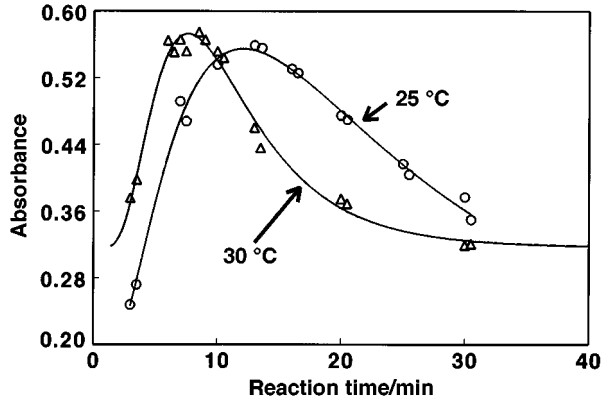

Fig. 2 Absorbance as a function of reaction time for two sets of standards, one equilibrated in a 25 °C and the other in a 30 °C water-bath. The procedure for determining absorbance values is as described under Determination of optimum reaction time.

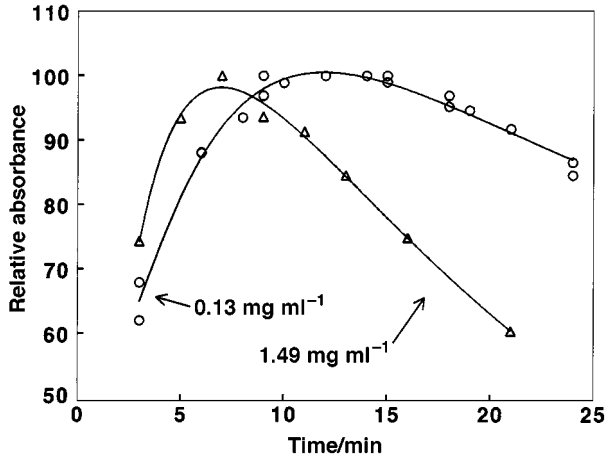

Fig. 3 Relative absorbance as a function of reaction time for tannic acid standards having concentrations of 0.13 and 1.49 mg ml^{-1}. The graphs shown are for commercially prepared tannic acid from Sigma. Each data point is the absorbance of the solution prepared as described in the section Determination of optimum reaction time, relative to the maximum absorbance for that HT concentration.

Sensitivity of chilled versus *unchilled samples*

Calibration curves were plotted for commercially prepared tannic acid, comparing the results obtained using the procedures with and without chilling. The calibration curve using the chill procedure had slightly more curvature, which can be illustrated by the following data. Concentrations less than 0.8 mg ml^{-1} essentially had equal absorbance for the two calibration curves. For concentrations greater than 0.8 mg l^{-1} the absorbance for the chill procedure gradually decreased, so that at 1 mg l^{-1}, the absorbance was 4% less. The equation for each curve was $y = 0.00963x^2 + 0.7336x - 0.149$ ($r = 0.99987$) without chilling and $y = 0.0253x^2 + 0.7168x - 0.058$ ($r = 0.99970$) with chilling.

Comparison of different biological materials

Commercially available tannic acid from two different companies, Fisher and Sigma, was tested in addition to the extracts of red, white, black, and pin oak leaves and hemlock needles. Fundamental differences were found between these extracts, which are illustrated by plotting the absorbance as a function of reaction time for the different biological materials (Fig. 5). For example, commercial tannic acid from either Sigma or Fisher and white oak had much sharper peaks than any other plant material tested. Hemlock needles had a very flat peak. The difference in plant materials can also be illustrated by comparing the time until maximum absorbance. On comparing red (not shown), pin, white, and black (not shown) oak leaves with hemlock needles, white oak had the shortest reaction time (7 min) and hemlock the longest (80 min).

The fact that white oaks are known to contain a higher concentration of ellagitannins than gallotannins[21] and red oaks contain a high concentration of gallotannins[14] suggests that the sharp peak at the beginning of the reaction is probably due to ellagitannin, which deteriorates much more rapidly than gallotannins. The latter portion of the peak that is flatter and slower to develop is probably due to gallotannins. The higher

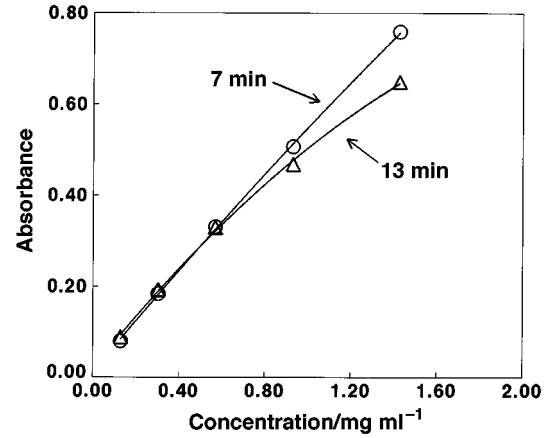

Fig. 4 Calibration curve for a set of standards analyzed twice using an equilibration time of 7 min for one test and 13 min for the other. The line for the equation representing the 7 min reaction time has the equation $y = -0.0120 + 0.5624x - 0.02787x^2$ ($r = 0.99945$) and that for the 13 min reaction time has the equation $y = 0.018 + 0.5937x - 0.1078x^2$ ($r = 0.9994$). Error bars are smaller than the size of the symbols and are therefore not shown. The procedure used was that described in the section Determination of optimum time.

absorbance of samples of white oak leaves compared with those of other trees does not mean that white oaks have a higher concentration of HT; it only suggests that ellagitannins have a higher sensitivity to KIO_3 and hence, a higher absorbance.

In addition to the variations among different biological materials, there was considerable variation with the time of the season when the plant material was collected. An example of this is shown for red oak (Fig. 6), comparing leaf material collected in June with that collected in October from the same tree. The peak that was present for the June collection was missing for the October collection, suggesting that the ratio of ellagitannins to gallotannins had decreased considerably from June to October.

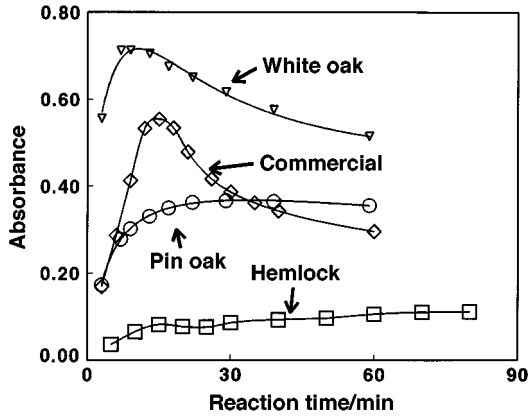

Fig. 5 Absorbance–reaction time plots for commercially prepared tannic acid from Sigma, pin oak and white oak leaves and hemlock needles. The procedure used was that described in the section Determination of optimum reaction time. Other materials that were tested but are not shown were commercial tannic acid from Fisher, which had a plot similar to the tannic acid from Sigma, red oak, which was similar to pin oak, and black oak, which was similar to white oak.

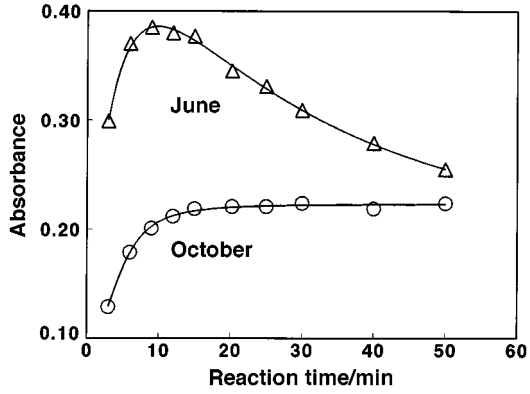

Fig. 6 Comparison of red oak leaves collected in October 1994 and June 1995. Data points were measured using the procedure described in the section Determination of optimum reaction time.

Interference study

Several compounds that are present in plant materials, or that have oxidizing or reducing properties, were tested for interference using the recommended procedure described in the Experimental. A 1 ml volume of test solution that contained 0.83 mg equivalents of HT and 0.17 mg of interferent was used in each case. The results are given in Table 1.

The data suggest that any compound with oxidizing or reducing capabilities may interfere. The important question is whether or not such compounds are in the extracting solution in sufficient concentration to interfere significantly. To test this possibility, a spiking test was performed in which the HT concentration was first measured in the extract of three different oak species. A known amount of HT was then added to each extract. The resulting mixtures were analyzed using the procedures with and without chilling. The final concentration was compared with the theoretical concentration (determined by adding the original concentration of HT plus the amount of HT added). The results (Table 2) indicate that no interference occurred, regardless of whether the samples were measured using the procedure with chilling (40 min at 4 °C) or without chilling. The equilibration time of each test is shown, since it differs for each plant species and measurement technique. In each case, the measurement time is a compromise of the optimum values for the plant extract, the standard tannic acid solution and the mixture containing both standard and plant extract.

Reproducibility

The reproducibility was determined for both procedures. Five solutions, each containing a different concentration of HT, were analyzed. The average RSD was 0.7% using the procedure with chilling and 0.9% using that without chilling. Based on this, we conclude that both procedures are very reproducible.

Table 1 Interference of various compounds in the measurement of hydrolyzable tannins

Interferent	Absorbance	Relative error (%)
Control	0.432	—
Starch	0.436	+1.0
Dextrose	0.430	−0.5
Sucrose	0.433	+0.4
Phenol	0.456	+5.0
Iron(II) ammonium sulfate	0.766	+77.0
trans-Cinnamic acid	0.467	+8.2
Iron(III) ammonium sulfate	0.600	+38.8

Table 2 Comparison of results for samples spiked with standard tannic acid. Concentration units are mg ml^{-1}

Oak variety	Original concentration	Concentration added	Theoretical concentration	Measured concentration	Relative error (%)	Time/min*
With chilling†—						
White	0.564	0.468	1.033	1.059	2.5	5
Red	0.317	0.470	0.788	0.799	1.4	9
Pin	0.438	0.527	0.965	0.919	−4.8	5
No chilling—						
White	0.498	0.438	0.936	0.931	−0.6	13
Red	0.319	0.484	0.803	0.793	−1.2	11
Pin	0.353	0.490	0.842	0.834	−1.1	14

* The time is the number of minutes for which samples were equilibrated at 25 °C. † Samples that were chilled were chilled for 40 min at 4 °C.

Effect of purification

An alternative to the colorimetric method of determining HT, as described in this paper, is liquid chromatography.[18] When using chromatography, it is necessary first to purify or remove monomers from the sample being tested. We wanted to determine if samples needed to be purified when using this procedure. We plotted absorbance *versus* time for a purified sample and compared it with a sample that was not purified. As shown in Fig. 7, the sharp peak at the beginning of the plot was not present in the purified sample. Apparently, the ratio of ellagitannins to gallotarnins had decreased during the purification process. No other difference was observed and we found no evidence to indicate that purification is necessary.

Limitation of the method

There is one limitation to this procedure, namely that the reagent is differentially sensitive to various types of HT, which means that it is impossible to obtain true concentrations. However, it does show changes from one day to another or between plants of the same species but treated differently. For this reason, it is a very valuable tool for ecological experiments.

Conclusion

It is not necessary to chill samples prior to placing them in a constant-temperature water-bath. The results are about the same whether samples are chilled or not, and there are distinct advantages in the elimination of this very time-consuming step, simplification of the procedure and elimination of one potential source of error. Our conclusion regarding the equality of the results was based on five observations: (1) reproducibility, as measured by the RSD, was equally good; (2) sensitivity, as shown by the calibration curve, was the same; (3) linearity of the calibration curve was not significantly different; (4) accuracy, as measured by spiking extracts of plant material with standard tannic acid, was equal; and (5) it was equally necessary to determine the optimum time to leave samples in a warm water-bath and then, very accurately, to adhere to that time when analyzing each sample, whether the samples were chilled or not.

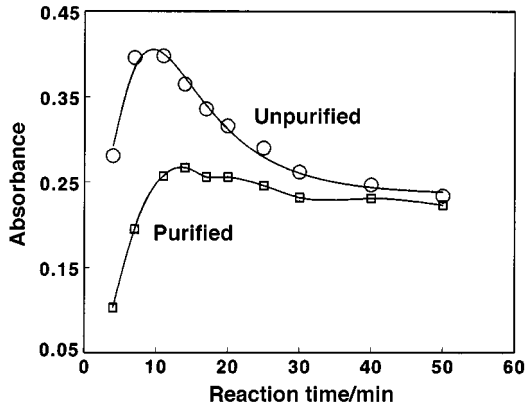

Fig. 7 Comparison of purified and unpurified commercially obtained tannins. Data points were measured using the procedure described in the section Determination of optimum reaction time.

When analyzing samples from the same plant species, a preliminary experiment should be performed to measure the absorbance as a function of reaction time. This shows the time required to obtain maximum absorbance and, thus, the recommended reaction time to use in measuring each sample.

Data from purified extracts were not fundamentally different from those for unpurified extracts and therefore it is not necessary to purify sample extracts.

Of the two kinds of HT (ellagitannins and gallotannins), ellagitannins react faster with potassium iodate forming a red compound, which is the species being measured, and then deteriorate faster than the gallotannins.

The techniques described in this paper can be used to help identify a plant species or the time of the season when the plant sample was collected.

It is our opinion that the present work provides a suitable analytical technique for hydrolyzable tannins, a need identified by Waterman and Mole[13] in their recent review on the subject.

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