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Theaflavin Analysis of Black Tea—Problems and Prospects

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ABSTRACT

The Flavognost assay for measurement of theaflavin in black tea is discussed, and experimental evidence presented to explain why some parts of the assay can cause within and between laboratory variation. The major problems raised are concerned with the temperature of the infusion, and with the proportion of the total theaflavin present in the tea leaf that is actually extracted. The role of the leaf's endogenous aluminium in theaflavin analysis is discussed, and it is shown that it might be possible to replace the expensive Flavognost reagent with aluminium chloride, which is less than 1% of the price.

INTRODUCTION

Over the last 10 years a great deal of interest has been shown in the theaflavin content of plain black tea. It has become generally accepted that in plain black teas the theaflavin level is correlated with price (Ellis & Cloughley, 1981; Owuor *et al.*, 1985, and references therein). Partly for this reason, it has been suggested that a minimum theaflavin level be incorporated into any future Minimum Standards Agreement (Ellis & Cloughley, 1981). As a necessary prelude to this, a series of ring tests have been carried out to determine the suitability of the Flavognost assay as an International Standard. This has been under the auspices of the International Standards Organisation (ISO/TC 34/SC8). The Tea

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Research Foundation of Kenya has recently started to participate in these ring tests and as a result has begun to study the assay to find possible areas of within and between laboratory variation. The results of these experiments are presented in this paper.

MATERIALS AND METHODS

All teas used in these experiments were of unorthodox commercial production.

The Flavognost assay used was based on that of Hilton (1973) as modified for Ring Tests for the International Standards Organisation (ISO/TC 34/SC8—Ellis, personal communication). Details are given below.

The percentage of dry matter (DM) in the sample is determined by oven-drying. A tea infusion is made with 375 g (ml) of boiling water, preferably added from an overhead boiler into a tared flask, and 9 g of tea. The flask is shaken for 10 min, the infusion filtered through cotton wool, and 10 ml pipetted into 10 ml of IBMK (isobutylmethylketone; or 4-methyl pentan-2-one). The mixture is shaken for 10 min and allowed to stand until the layers separate. Two millilitres of the upper layer are pipetted into a test tube followed by 4 ml ethanol and 2 ml Flavognost reagent (2 g diphenylboric acid-2-aminoethyl ester dissolved in 100 ml ethanol). The contents are mixed and the colour allowed to develop for 15 min. The optical density (OD) at 625 nm is read against an IBMK/ethanol (1:1 vol/vol) blank.

$$\text{Theaflavin } (\mu\text{mol/g}) = \text{OD}_{625} \times \frac{47{\cdot}9}{\left(\frac{\text{DM}}{100}\right)}$$

The same analysis has also been used, but with a different infusion procedure. Water and IBMK are heated in a boiling water bath, and 10 ml of each are added to 0·24 g tea and shaken for 10 min. The mixture is filtered and the layers are then allowed to separate. The IBMK layer is analysed as above. This method gives the same final ratios of tea to IBMK as does the standard infusion and extraction method.

In some of our experiments the Flavognost reagent was replaced by 2 ml of aqueous aluminium chloride of various concentrations, and the optical density measured at 525 nm (see Results and Discussion).

RESULTS AND DISCUSSION

One of the first problems to be raised is the question of how much water to use in the tea infusion. The method as suggested requires the addition of 375 g (375 ml) of boiling water. At the temperature of boiling water these two are not the same. Although the difference is slight, it would be preferable to standardise on either one or the other.

A more serious matter is that of the temperature of the water used. Roberts & Smith (1963), Spiro & Siddique (1981) and Reeves & Gone (1984) have published data showing that less theaflavin is extracted at lower temperatures. Because of the altitude of Kericho (2178 m amsl) the maximum temperature of boiling water that can be obtained is 94 °C. Thus the data shown in Fig. 1, obtained from temperature curves of 10 different teas, suggests that there is an average of 5% less theaflavin extracted from a tea measured at Kericho than there would be at sea level.

Another aspect of temperature lies in exactly how the water is dispensed. Hilton (1973) suggested dispensing the water from an overhead boiler, and using a tared balance to deliver 375 g. When this method is used the water is never removed from the heating element, and all flasks receive the same temperature water. If, however, an electric kettle is used, then the power has to be turned off and the water will cool

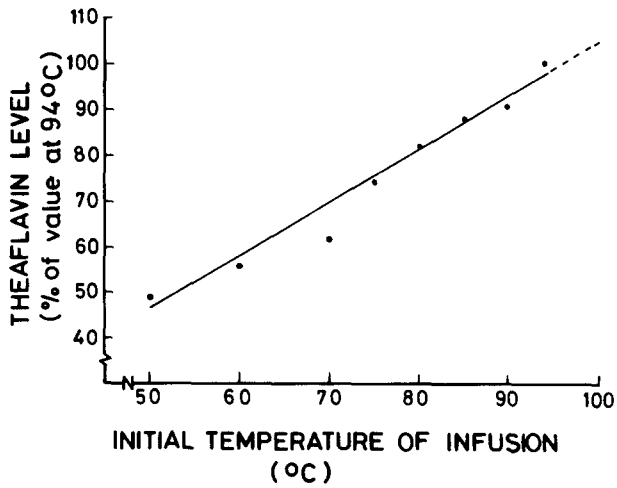

Fig. 1. The effect of initial infusion temperature on theaflavin extraction in tea infusions. For details see text. The line plotted was calculated by linear regression analysis, $r = 0{\cdot}98$.

slightly between the first and last infusion. For a batch of 12 teas this can be a matter of several minutes. The situation is worse if a measuring cylinder is used to measure 375 ml. First, the cylinder is accurate at 20 °C. Secondly, during the preparation of several samples the cylinder will heat up and expand, thus causing variation, both in volume and temperature, between infusions. For these reasons we favour the method of Hilton (1973) and Ellis (ISO TC 34/SC8), which uses an overhead boiler and 375 g of water, and this has been used throughout unless otherwise stated. We believe it is the fastest and most consistent method. For example, 30 infusions were made, 15 using 375 g boiling water from an overhead boiler, and 15 using 375 ml boiling water from a kettle. The average theaflavin value for the former was 17·9 μmol/g, standard deviation 0·53, and for the latter the figures were 18·1 and 1·46, respectively.

A final point on this topic is the size and geometry of the flask used. During the 10 min shaking period the infusion cools down, so there is a temperature gradient from start to finish. The size of air space above the liquid and the shape of the flask can both affect this gradient, and possibly the extraction of the theaflavin.

After shaking the infusion it is filtered and the theaflavins extracted into IBMK. In an experiment to study this step, the IBMK extraction was carried out at various stages during cooling of the infusion. Results suggested that slightly more theaflavin came out in cooled extractions. We feel that in order to reduce variation, the infusion should be extracted as soon as possible after filtration, as long as temperature conditions can be standardised. Allowing the infusion to cool to a standard temperature would be more time-consuming and difficult.

As a demonstration of the difficulties of the infusion and extraction steps, five infusions were made from the same tea, and each infusion sampled 10 times and extracted into IBMK. As can be seen in Table 1, the within infusion variation was much less than the between infusion variation. If a single IBMK extract was repeatedly sampled, almost all the variation disappeared, as would be expected.

It has been reported that extracting tea into boiling water in a boiling water bath removes all the theaflavin from the leaves, and maintaining the infusion above 85 °C extracts 90 % of theaflavin (Roberts & Smith, 1963; Hilton, 1973). During a 10 min infusion in thermos flasks, we have found the temperature of the infusion drops from 94 °C to approximately 70 °C. Consequently we investigated the percentage extraction we were obtaining.

TABLE 1
A Comparison of Within and Between Infusion Variation

Replicate	*Measured theaflavin level (μmol/g)*				
	Infusion A	*Infusion B*	*Infusion C*	*Infusion D*	*Infusion E*
1	16·9	19·8	17·2	18·7	21·9
2	17·2	19·8	16·4	18·2	20·3
3	17·2	19·8	16·9	18·2	21·9
4	16·7	19·3	16·9	18·2	22·1
5	16·7	19·8	17·2	19·3	21·9
6	16·7	19·5	17·7	17·7	22·1
7	16·7	19·3	17·2	17·2	22·1
8	15·1	19·8	17·4	18·7	17·7
9	16·7	19·5	16·7	19·0	19·8
10	17·2	19·5	17·2	19·0	21·9
Mean	16·7	19·6	17·1	18·4	21·2

Using 375 ml of boiling water, measured from a kettle, five infusions were made from a single sample of tea. From each infusion 10 aliquots of 10 ml were extracted into IBMK, and the IBMK layer assayed for theaflavin using the Flavognost reagent.

First we looked at the water layer left after an IBMK extraction. A standard infusion was made from each of 10 teas, 10 ml extracted into IBMK, and the IBMK layer analysed for theaflavin. The water layer was then removed and re-extracted with a fresh aliquot of IBMK. We found that the re-extraction contained theaflavin, and the levels were 10–16% (average 14%) of the first extraction. However, the optical densities recorded were very low (less than 0·1) so this measurement was subject to a high degree of error.

It was then decided to extract theaflavin directly into IBMK/water mixtures (see Materials and Methods). The mixture was used because only about 30% of the theaflavin (as compared to the standard infusion method) was extracted into pure IBMK. If water was added, then much higher levels were extracted. The results for 10 teas are given in Table 2. The IBMK/water extraction gave results 30–65% higher than the standard infusion. This suggests that the standard infusion only extracts a maximum 60–75% of the theaflavin present in the tea. Presumably the theaflavin is extracted into the aqueous phase as in a standard infusion, but then partitions directly into the IBMK phase.

The large increase in theaflavin level using an IBMK/water extraction

TABLE 2
A Comparison of Standard and IBMK Extractions of Theaflavin

Measured theaflavin (μmol/g)		*Increase due to IBMK/water extraction* (%)	*Standard infusion as a % of IBMK water infusion* (%)
Standard infusion	*IBMK/water infusion*		
27·1	38·9	44	70
21·9	31·0	42	71
20·0	28·6	43	70
19·1	25·7	35	74
16·1	26·5	65	61
16·1	24·8	54	65
15·2	21·3	40	71
12·8	20·5	60	62
11·1	17·7	60	63
9·6	12·4	29	77

From each of 10 teas two infusions were made. The first was by the standard method (using 375 g water from an overhead boiler) and the second using IBMK/water (see Materials and Methods). Both extractions were assayed for theaflavin using the Flavognost reagent.

technique compared to the standard infusion can be easily explained. The extraction of theaflavin into water is not simply the dissolving of a chemical. The dry tea leaves take up water and swell, and the theaflavin equilibrates between the swollen leaf phase of the system and the water phase. Using the theoretical model system developed by Spiro & Siddique (1981), and their measured partition coefficients, it can be calculated that at a constant infusion temperature of 94 °C approximately 73 % of the theaflavin should be extracted. (This assumes equilibrium is reached, i.e. the rates of diffusion and reabsorption are equal.) Moreover, because of the temperature sensitivity of the partition coefficient, an infusion at 80 °C would only be expected to extract 61 % of the theaflavin.

However, when IBMK is present in the extraction, the theaflavin will partition between the water phase and the IBMK phase, thus constantly reducing the concentration of theaflavin in the water towards zero. This will alter the equilibrium between the swollen leaf and the water, causing more theaflavin to partition into the water. Thus, in an ideal system, most of the theaflavin would leave the leaf.

The situation is complicated by the fact that, in both the standard

infusion technique and the IBMK water extracts, a temperature gradient occurs. In the standard infusion, the maximum level of theaflavin is extracted after 2–4 min (Reeves, unpublished observations and Ellis, personal communication). As the infusion then cools over the rest of the extraction period it is possible that reabsorption will occur, as the partition coefficient decreases with decreasing temperature (Spiro & Siddique, 1981). Thus, whatever infusion technique is used, it is essential to have consistent initial temperatures, temperature gradients and infusion times.

We are not suggesting that the standard infusion should be replaced with extraction into hot IBMK/water. The method has less temperature control than the standard method, and uses a much smaller sample of tea, thus possibly introducing greater sampling error. Moreover, it must be pointed out that it is not an absolute requirement for an international method for theaflavin analysis to extract 100 % of the theaflavin. It merely has to extract a consistent proportion.

One final problem with the Flavognost assay is of a 'non-scientific' nature. In many of the tea-producing countries the Flavognost reagent can be difficult to obtain, both because of availability of the reagent and availability of foreign exchange.

The reaction of aluminium salts with theaflavins is known (Edmonds & Gudnason, 1979; Chang & Gudnason, 1982), and this reaction was studied further with a view to replacing the Flavognost reagent with a simple aluminium salt that would be more easily available, and less than 1 % of the cost. Chang & Gudnason (1982) showed that certain aluminium salts would complex with theaflavin to produce a red coloration. Using a standard infusion and extraction into IBMK, it is possible to mix 2 ml of the IBMK layer, 4 ml ethanol and 2 ml of aqueous aluminium chloride to produce a red aluminium–theaflavin complex. The colour takes about 15 min to form, and is then stable for several hours. In this assay, the aqueous aluminium replaces the Flavognost reagent. Figure 2 shows the effect of increasing the final concentration of aluminium chloride on the optical density (525 nm) of the resultant solution. The two teas had theaflavin contents, as measured by the Flavognost method on a standard infusion, of approximately 10 and 20 μmol/g. This means that in the aliquot taken from the IBMK layer they would have approximately 0·5 and 1·0 μmol of theaflavin, respectively. As can be seen, the reaction is saturated by the time 100 μmol of aluminium have been added (a similar molarity to that used in the Flavognost assay).

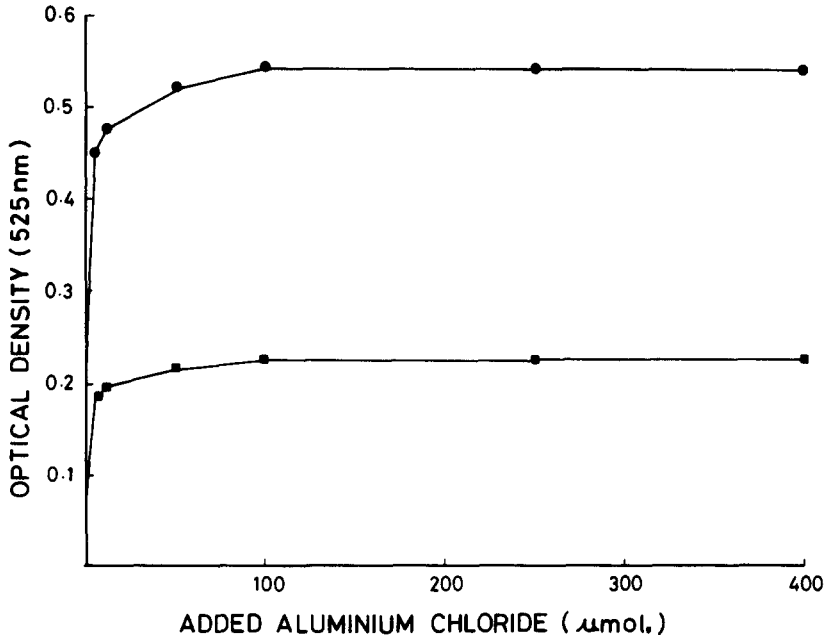

Fig. 2. The effect of added aluminium chloride at high levels on the optical density of an IBMK extract of a tea infusion; ● = tea containing 20 μmol theaflavin/g; ■ = tea containing 10 μmol theaflavin/g. For details see text.

The response to low levels of added aluminium is shown in Fig. 3. Even the addition of equimolar amounts of aluminium causes considerable colour development.

In order to compare the use of aluminium and the Flavognost reagent, infusions were made of 10 teas with theaflavin contents ranging from 10 to 25 μmol/g. The IBMK layers from these infusions were analysed either with the Flavognost reagent or by using 200 μmol aluminium chloride in 2 ml water. The results are shown in Fig. 4. The correlation between the two methods is excellent ($r = 0{\cdot}998$), but the line of best fit does not pass through the origin. This is because the IBMK extract absorbs slightly at 525 nm (see Figs 2 and 3), whereas it does not do so at 625 nm. However, because of the linearity of the aluminium assay against the Flavognost assay, the latter can be used for calibration. It can be calculated that

$$\text{Theaflavin } (\mu\text{mol/g}) = \text{OD}_{525} + \frac{33}{\left(\frac{\text{DM}}{100}\right)}$$

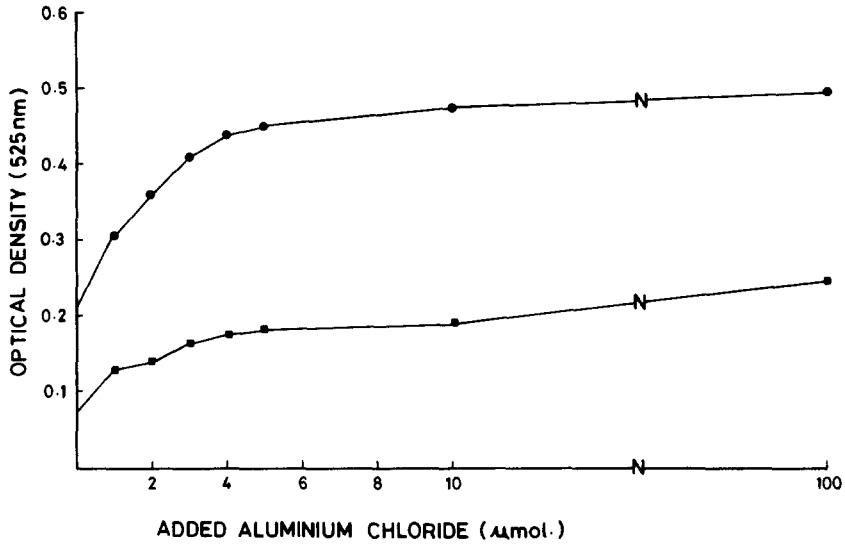

Fig. 3. The effect of added aluminium at low levels on the optical density of an IBMK extract of a tea infusion; ● = tea containing 20 μmol theaflavin/g; ■ = tea containing 10 μmol theaflavin/g. For details see text.

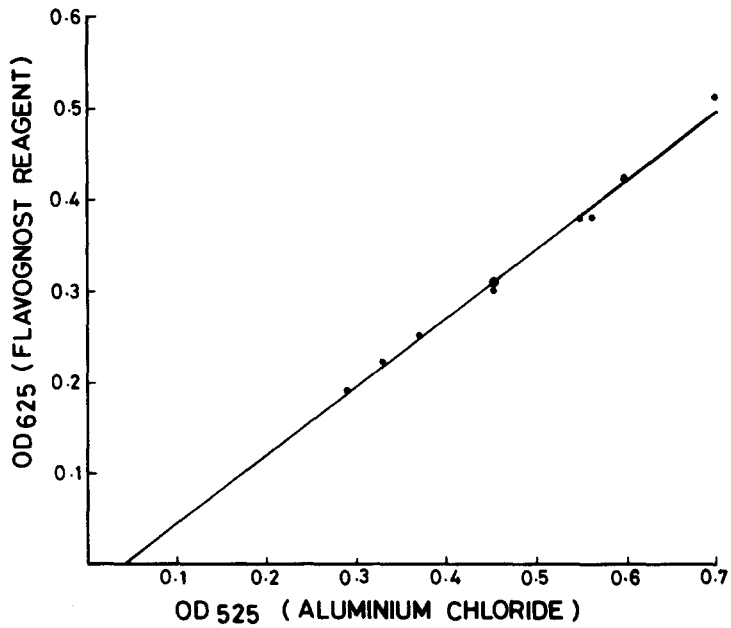

Fig. 4. A comparison of the Flavognost reagent and aluminium chloride for estimation of theaflavin in an IBMK extract of a tea infusion. For details see text. The line plotted was calculated by linear regression analysis, $r = 0{\cdot}998$.

A more accurate calculation could be carried out using pure theaflavins, and any differences between the four major theaflavins noted. Unfortunately we are not yet in a position to synthesise these, and thus cannot carry this out.

In their studies on aluminium, Chang & Gudnason (1982) reported that if aluminium was added to a solution of instant tea, the aluminium–theaflavin complex formed was insoluble in ethyl acetate. This meant that if aluminium was added to an instant tea solution, and this was extracted with ethyl acetate, the level of theaflavin measured in the ethyl acetate layer was greatly reduced. When saturating levels of aluminium were added, the theaflavin was reduced to approximately 15% of the original level.

However, when excess aluminium is added to an infusion from tea leaves, and IBMK used for extraction, there is a slightly different result. There is an obvious increase in redness in the infusion, but this cannot be measured spectrophotometrically, as when saturating amounts of aluminium are used the solution turns turbid. Furthermore, when mixed with IBMK an emulsion forms. When the emulsion is centrifuged to separate the layers, analysis of the IBMK layer indicates that there is only 20% of the theaflavin remaining in the water layer. When aluminium is approximately equimolar with theaflavin, there is very little emulsion formation, and there is no measurable reduction in measured theaflavin in the IBMK layer. This suggests the Flavognost–theaflavin complex is formed preferentially to the aluminium–theaflavin complex.

The formation of a fairly easily broken emulsion is not uncommon when carrying out theaflavin analyses. Certain teas have consistently done this in the past. It is possible that these were teas with a high endogenous free aluminium level.

The question of the effect of endogenous aluminium on the infusion process is relevant because tea is known as an 'aluminium accumulator' (Eden, 1976) and can have very high levels present in the leaf (Sivasubramarian & Talibudeen; 1971, Kulasegaram & Kathiravetpillai, 1983).

It is thought that the young leaves of the tea bush, plucked to make tea, can contain as much as 1500 ppm aluminium (Sivasubramarian & Talibudeen, 1971). If it is assumed that no aluminium is lost during processing, then black tea will have similar levels of aluminium. Thus black tea could have up to approximately 50 μmol/g of aluminium compared to the 10–20 μmol/g of theaflavin routinely found in tea. If

most of this aluminium were to be extracted in the tea infusion, then there could be up to four times as much aluminium present as theaflavin. The net result of this would mean that some of the red colour of a tea infusion could be due to the aluminium–theaflavin complex.

S. S. Chang & G. V. Gudnason (personal communication) have found that aluminium extraction into an infusion is highly variable, giving an average of 50 % of the total aluminium present. This means that the levels of aluminium would be too low to affect subsequent extractability of theaflavin into the IBMK layer or to affect theaflavin measurement by the Flavognost reagent, but could be large enough to affect the colour of the infusion. It might thus be possible to have two teas of identical theaflavin content but very different 'free' aluminium contents. The tea with the higher aluminium content would be a redder colour, and might well be adjudged to be of higher quality. It is possible for the range of aluminium contents in an infusion from black tea to be from 0·5 to 4·0 μmol aluminium per μmol theaflavin. Addition of aluminium at this level causes a change of colour of an infusion, and increases the valuation of the tea (Edmonds & Gudnason, 1979; Reeves, unpublished observations). We do not currently know what the free aluminium levels are in tea, nor how they are affected by environment, cultural practices or processing methods, but this could be of economic importance.

CONCLUSIONS

It must be emphasised that the object of this paper is not to demonstrate that the Flavognost assay is not suitable for routine use or as an international standard. Rather it is to highlight points that can cause within and between laboratory variation.

The main problem with the assay appears to lie in the temperature, due to the sensitivity of theaflavin's partition coefficient to temperature. Thus, for good reproducibility, it is necessary to be able to control the initial temperature and the temperature gradient during the infusion. At the moment it appears that the use of boiling water in a thermos flask fails in this respect.

The aluminium–theaflavin interactions discussed are of importance on two counts. First, there is the possibility of replacing the Flavognost reagent with aluminium chloride, which is much cheaper and more easily available. Secondly, it provides a possible explanation for the erratic correlation of the price of Kenyan teas with the theaflavin content.

ACKNOWLEDGEMENTS

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NOTE ADDED IN PROOF

Since writing this paper, the authors have received a personal communication (M. Spiro & W. E. Price, *The Analyst* (in press)) that confirms that, during partitioning of theaflavin from the filtered infusion into IBMK, about 10% of the theaflavin remains in the water layer.