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A Critical Investigation into the Flavognost Method for Theaflavin Analysis in Black Tea

A. Robertson & M. N. Hall

Campden Food and Drink Research Association,
Chipping Campden, Gloucestershire, GL55 6LD, UK

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ABSTRACT

There are several critical stages in the Flavognost method for theaflavin analysis in black tea. These have been identified and their significance determined experimentally. During sample preparation, the particle size of the ground material, and the type of grinder used to produce it, directly influence the level of extracted theaflavin. The temperature of the water used and the time over which infusion is carried out are also very significant as is the method of shaking. Failure to control conditions at these critical stages can give rise to a fourfold variation in results. Recommendations are given for possible incorporation into standard protocols.

INTRODUCTION

The role of theaflavin in tea quality is well recognised, contributing brightness and astringency to the infused liquor (Bradfield & Penney, 1944; Roberts & Smith, 1961, 1963). Hilton and Ellis (1972) showed good correlations between the theaflavin level of Malawi-grown teas and London auction prices and much research has been devoted to chemically characterising the theaflavins (Collier *et al.*, 1973), elucidating the mechanism of synthesis and ultimately determining the factors which affect their formation (Robertson, 1983*a*, *b*). There is now a considerable knowledge of the steps which can be taken, both in the field and in the factory, to produce higher levels of theaflavin in the product.

The need to measure theaflavin has, until recently, been limited to the Tea

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Research Institutes and Foundations, perhaps to the tea-producing companies, and to those carrying out research into this area. However, consideration is now being given to the use of TF levels as an international standard for monitoring the quality and/or authenticity of teas. As a pre-requisite, such a method should be reproducible and provide consistent results within and between laboratories. Ring tests set up to examine the repeatability of the Flavognost method have shown gross inconsistencies between laboratories with differences in measured theaflavin of up to 3-fold on the same sample. The Campden Food and Drink Research Association participated in the first ring test in 1982 organised under the auspices of the International Standards Organisation (ISO/TC 34/SC8) and noted that the accompanying protocol of the Flavognost method was poorly defined and open to misinterpretation.

The method is comprised of three stages; (a) sample preparation, (b) infusion and (c) partitioning and colour development. At each of these stages errors can be introduced. Reeves *et al.* (1985) investigated some of the problems of theaflavin analysis and concluded that control of infusion temperature is of prime importance.

As a result and under the joint funding of MAFF and the UK tea industry, the CFDRA has undertaken research to identify the factors responsible for the wide variation in results. The results of this work are reported in this paper and will enable modification to existing protocols.

Experimental samples were supplied by the tea industry and consisted of single teas from Kenya, Sri Lanka and India with grades of Dust, Broken Orange Pekoe (BOP) and Pekoe Dust, respectively.

MATERIALS AND METHODS

Flavognost Method

Investigations were based on the Flavognost method of Hilton (1973) as modified by Ellis (ISO/TC 34/SC8—pers. comm.). The stages of this procedure were as follows.

(a) Sample Preparation. Tea was ground in accordance with ISO 1572-1980 to produce particles of less than 0·5 mm diameter.

(b) Infusion. Ground tea (9 g) was infused in boiling water (375 g) for 10 min. The infusion was carried out in a Thermos flask with continuous mechanical shaking.

(c) Partition/ Colour Development. After filtration through glasswool, a 10 ml aliquot of the infusion was shaken for 10 min with 10 ml of iso-butylmethyl ketone (IBMK) and the two layers allowed to separate. An

aliquot of the upper layer (2 ml), 4 ml of ethanol and 2 ml of Flavognost reagent (2% w/v diphenylboric acid-2- aminoethyl ester in ethanol—supplied by Sigma Chemical Co. Ltd) were mixed and allowed to stand for 15 min. The absorbance was then measured at 625 nm in 1 cm path length cuvettes using a Pye Unicam PU8800 spectrophotometer against an IBMK/ethanol (1:1 v/v) blank. Results were calculated on a dry weight basis by the formula:

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

where E is optical density and DM is dry matter content of the tea sample.

Dry matter determination

Dry matter was determined as mass at 103°C in accordance with ISO 1573-1980.

RESULTS AND DISCUSSION

Each stage of the procedure was investigated to determine where variability could be introduced and the magnitude of associated errors.

Sample preparation

For comparison of different teas and grades the ISO theaflavin method stipulates an initial grinding procedure followed by a simple particle size separation, removing particles greater than 0·5 mm diameter. This procedure should, in theory, reduce all teas to a common and uniform particle size and character. In practice this was not found to be the case.

Three laboratory grinders, differing in cutting or shearing action, were studied. These were a hammer mill, a revolving blade grinder (typified by modern coffee grinders of the Moulinex type) and an adjustable hand-operated coffee grinder.

Particle size

Full particle size analysis was carried out by sieving. Results showed that the hammer mill and hand grinder (medium setting) both produced particle size distributions with the main fractions around 0·1 to 0·4 mm. However, the revolving blade grinder produced a much larger proportion of very fine particles less than 0·13 mm in diameter (Fig. 1).

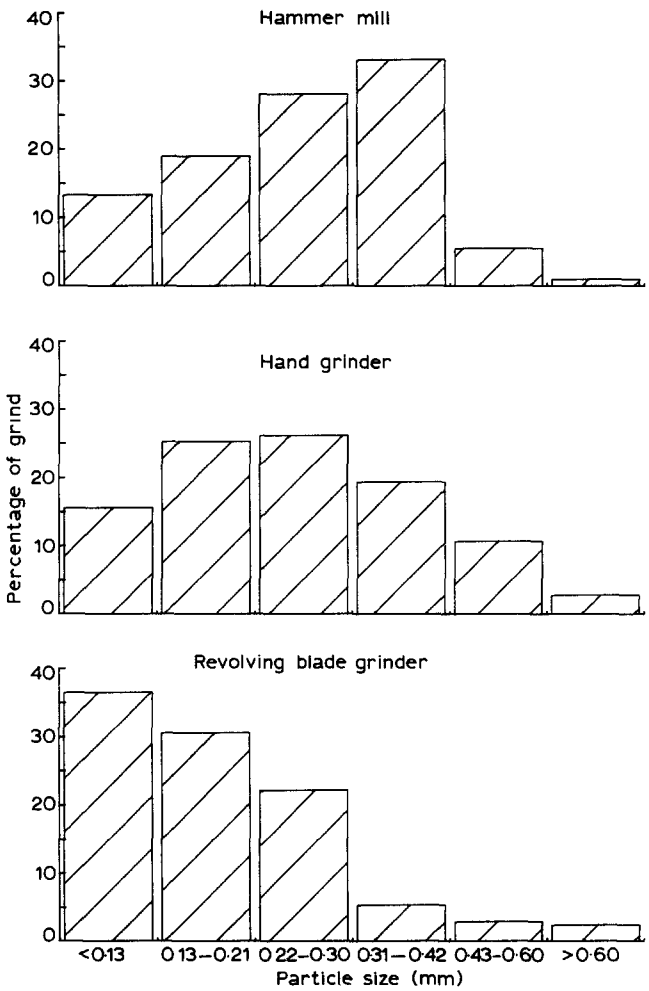


Fig. 1. Particle size distribution from different grinders.

All samples of the whole unsieved grind from each grinder, together with an unground control, were then infused and the theaflavin levels determined. (Fig. 2 and Table 1). The rate of theaflavin extraction, as a function of particle size, has been investigated by Price and Spiro (1985) and found to increase with surface area. Differences in extracted theaflavin between the different grinder types in this work,* however, were not statistically significant.

This failure to show a significant difference was largely due to the variation in results, particularly in the revolving blade grinder. The relatively high deviation in results with this type of grinder is probably a reflection of the broader distribution of particle sizes less than 0·5 mm and in particular

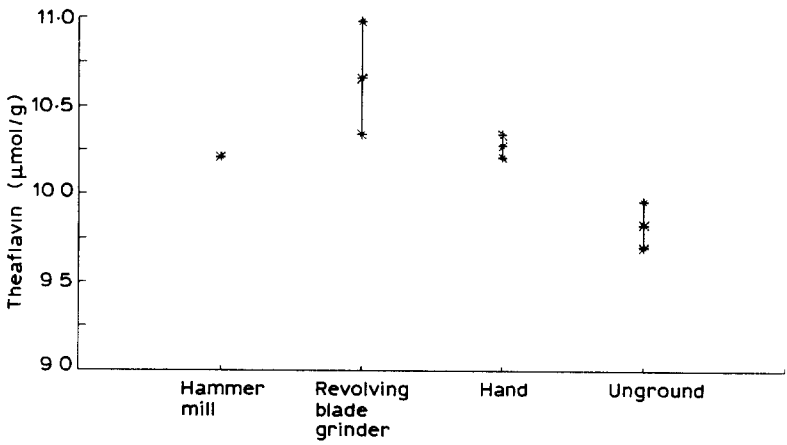


Fig. 2. Theaflavin content of samples produced by different grinding methods (bars represent range of results).

below 0·13 mm. These findings suggest that the particle size distribution below 0·5 mm due to the grinding method could be an important factor affecting the variation in theaflavin results between laboratories. A lower cut-off mesh of possibly 0·18 mm as well as that for the upper limit of 0·5 mm, already written into the ISO protocol, is therefore recommended.

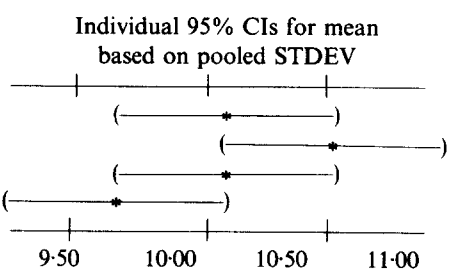
Particle character

Theaflavin levels were determined on the 0·21 to 0·30 mm fractions from the hammer mill and revolving blade grinder. Results were higher from the blade grinder (16·8 μmol/g) than the hammer mill (15·7 μmol/g) suggesting an effect of the type of grinder on the infusion characteristics of the particles.

TABLE 1
Analysis of Variance of Results

Source	DF	SS	MS	F
Factor	3	0·693 1	0·231 0	3·74
Error	4	0·247 0	0·061 8	
Total	7	0·940 2		

	N	Mean	STDEV
Hammer mill	2	10·210	0·000
Moulinex	2	10·660	0·453
Hand	2	10·275	0·092
Unground	2	9·830	0·184
Pooled STDEV =		0·249	



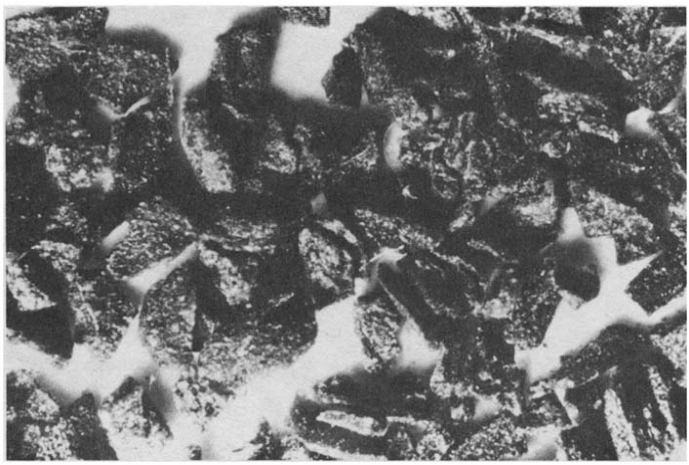


Fig. 3a. Particles from hammer mill showing clean angular fragments of uniform size.

On microscopic examination, the particles from the hammer mill were found to consist of separate, clean and angular fragments of fairly consistent size and appearance. (Fig. 3a). Those from the revolving blade grinder, however, consisted of much smaller fragments clumped together with a large amount of dust carried over (Fig. 3b). These characteristics were probably the result of electrostatic charge produced during grinding in this type of machine and also explain the higher levels of theaflavin extracted from material produced by this method of grinding.

Fig. 3b. Particles from revolving blade grinder showing smaller fragments, clumped together with a considerable amount of dust particles adhering.

Infusion

The second area considered to be a possible source of major variation in the Flavognost method, was that of infusion where time, temperature and shaking of the infusion were studied. The water used in all the subsequent experiments was freshly distilled.

Infusion temperature

The level of theaflavin extraction from samples of the same tea infused at temperatures between 75° and 100°C was examined. The results showed that theaflavin level increased linearly with infusion temperature (Fig. 4), and indicate that variation could occur if laboratories are unable to control infusion temperature.

The maintenance of infusion temperature close to boiling is dependent, in part, upon speed of operation, and is likely to be best in those laboratories where the method is used routinely. The use of vacuum flasks is recommended in the ISO protocol, but optimal handling of materials to maintain the best infusion temperature possible is not considered.

It was found in this laboratory that a higher infusion temperature could be obtained by:-

(i) prefilling the vacuum flasks with boiling water.
(ii) pre-weighing and wrapping the tea samples into single ply tissue (*cf.* sweet wrapper). This also had the advantage of improved accuracy compared with rapid weighing directly into a wet vacuum flask.

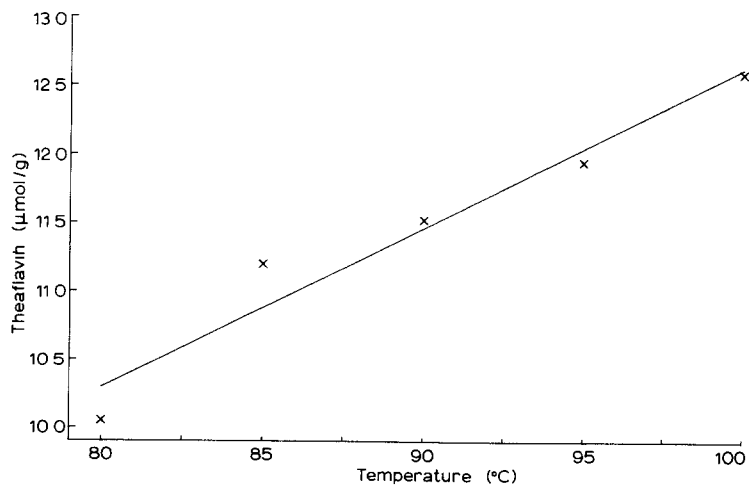


Fig. 4. Theaflavin extraction with infusion temperature.

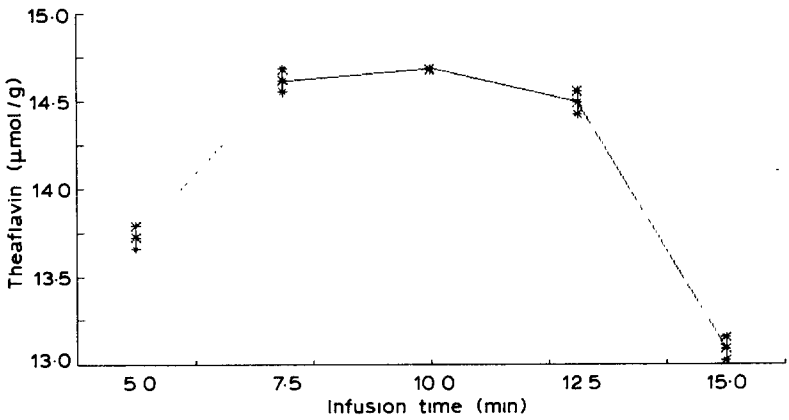


Fig. 5. Theaflavin extraction with different infusion times (bars represent range of results).

(iii) emptying out the boiling water from the flask, addition of wrapped tea samples and a new fill with fresh boiling water. This procedure is very rapid, reproducible and does not allow the flask to cool down significantly. The tissue wrapper unfolded immediately on addition of the boiling water, releasing the tea for normal infusion.

Infusion time

It is well known that theaflavin is labile and particularly sensitive to further oxidation to thearubigins. This occurs during its production in fermentation, continues at low level in dry tea during storage, and there is every reason to believe that it will occur during high temperature infusion.

TABLE 2
Analysis of Variance of Results

Source	DF	SS	MS	F
Factor	4	3·838 16	0·959 54	141·94
Error	5	0·033 80	0·006 76	
Total	9	3·871 96		

	N	Mean	STDEV
5	2	13·725 0	0·091 9
7·5	2	14·615 0	0·091 9
10	2	14·680 0	0·000 0
12·5	2	14·485 0	0·091 9
15	2	13·085 0	0·091 9

Pooled STDEV = 0·082 2

Individual 95% CIs for mean based on pooled STDEV

13·20 13·80 14·40 15·00

Consequently the effect of infusion time on the theaflavin was studied over a period of 5 to 15 min.

The results showed (Fig. 5 and Table 2) that theaflavin did break down over this period of infusion with maximum extraction after about 10 min. It is probable that theaflavin was degrading from zero infusion time, although it was not observed until the rate of extraction decreased below the rate of breakdown. The decrease was very rapid after around 12·5 min.

When analysing large numbers of samples, it is perhaps reasonable to assume that a laboratory unfamiliar with the method could allow over-infusion to occur.

Shaking during infusion

Although mechanical shaking of the infusing leaf is stipulated in the ISO method, no further information is given. The effect of shaking was therefore studied in more depth by using the following methods.

(i) Control—standing unshaken for 10 min.
(ii) Purpose built rotary shaker consisting of four Thermos flasks continuously rotating about a central axis at 50 rpm for 10 min.
(iii) Commercial vibratory shaker for 10 min.
(iv) Hand shaken by single inversion every 30 s over the 10 min infusion.

The results for extracted theaflavin (Fig. 6 and Table 3) using each method showed that the vibrating shaker resulted in very poor extraction, with no improvement over no-shaking at all. It is considered that when using this shaker, taking into account the cylindrical dimensions of a vacuum flask, there is little agitation in the liquor, resulting in poor suspension of the leaf.

By comparison, methods which inverted the infusion, and thus

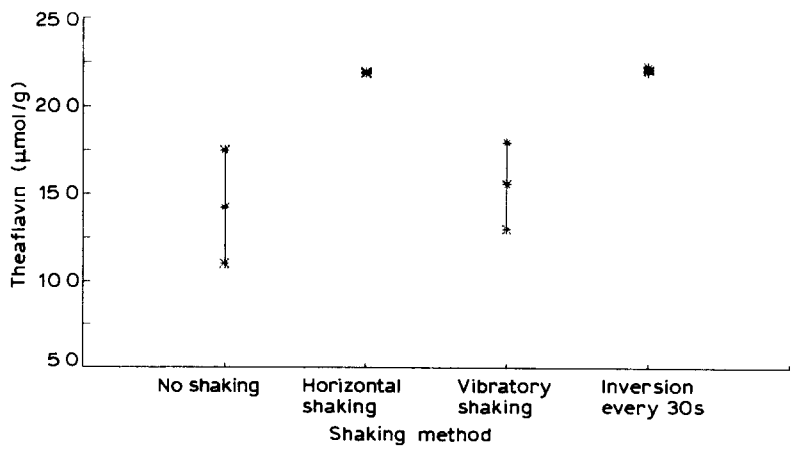
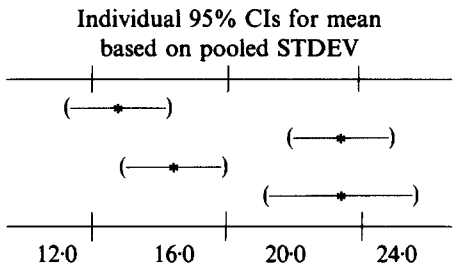


Fig. 6. Theaflavin extraction with different shaking methods.

TABLE 3
Analysis of Variance of Results

Source	DF	SS	MS	F
Factor	3	172·24	57·41	14·64
Error	12	47·05	3·92	
Total	15	219·29		

	N	Mean	STDEV
Control	2	14·250	4·596
Horizontal	2	21·950	0·071
Vibrating	8	15·625	1·923
Standing	4	22·125	0·126
Pooled STDEV =		1·980	



maintained suspension of the leaf, appeared to be extremely efficient and reproducible, resulting in significantly higher theaflavin levels. To this end, occasional but regular inversion of the flask by hand gave similar results to those from continuous rotation. Few laboratories will have the type of shaker necessary for this procedure if theaflavin analysis is not carried out routinely. This should be borne in mind in future ring tests and perhaps hand inversion should be included in the ISO standards as a better alternative to unspecified mechanical shaking.

Partitioning/colour development

Solvent extraction of TF

After infusion, the hot liquor was filtered and the filtrate extracted with an equal volume of IBMK. Hot extraction with a volatile solvent was considered to present handling problems and invariably led to the formation of an emulsion which had to be broken by centrifugation. The infusions were therefore allowed to cool prior to extraction. This resulted in little or no difference in the final theaflavin value compared with the hot extraction method, but slightly better overall reproducibility between replicates. This apparent insensitivity to temperature is consistent with reports from other workers (Spiro & Price, 1986).

The effect of time and temperature on colour formation

After addition of the Flavognost reagent to the IBMK extract, colour formation is allowed to develop at room temperature over a period of 15 min. This reaction is likely to be temperature-dependent and the

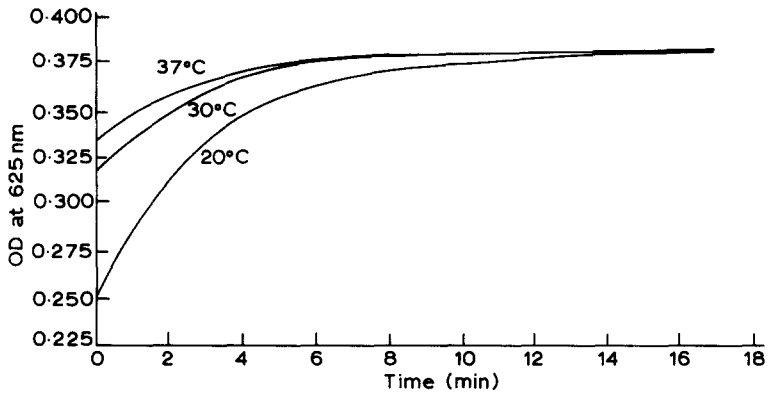


Fig. 7. Rate of development of colour at different temperatures.

possibility exists that variation in the absorbance readings could occur if it has not reached completion or if colour loss occurs after prolonged incubation. Figure 7 shows the time course of absorbance development at 625 nm after addition of the Flavognost reagent. The incubation was carried out at three temperatures nominally 20, 28 and 40°C and continued over 25 min. The results showed that, although increased temperature of incubation decreased the reaction time, after about 10 min, all incubations ended up with a similar final absorbance value which did not change with extended incubation. It is concluded that this step in the methodology will not contribute to the variability within and between laboratories.

Instrumental measurement of colour

Variations in spectrophotometer response could be a possible major source of error, particularly in countries where routine maintenance and calibration of laboratory instruments is infrequent. A standard calibration curve based on a standard absorbing material at 625 nm is required for the calibration of any spectrophotometer. This procedure should be written into the method.

Additive effect of different laboratory practice

The additive effects of a combination of poor practices on two samples A and B of the same tea, assessed for theaflavin content using different laboratory procedures, are shown below. In this exercise both deviation from the method and different interpretations of the protocol have been considered.

	Sample *A*	Sample *B*
Infusion temp. (°C)	75	100
Infusion time	15	10
Grinding	Hammer mill	Rotary blade
Shaking	Vibratory	Rotary
Pre-warming flask	None	Yes

Sample A yielded TF levels of 4·4 μmol/g whereas sample B yielded 20·1 μmol/g, a difference of over 450%. Although it is assumed that no laboratory would be guilty of such gross deviations from prescribed methods of practice the results demonstrate the level of variation introduced if certain points are not considered.

CONCLUSIONS AND RECOMMENDATIONS

The general method for determination of theaflavin is sound and reproducible. There are, however, instructions in the present method which are either ambiguous or imprecise.

The critical stages and recommended amendments to the protocol are shown below.

Flavognost assay	*Critical areas*	*Recommendations*
TEA	Different grinding methods cause variations in particle size profile and surface character of resultant particles. Both affect extractability	(1) Standardisation of mill/grinder (2) Definition of upper and lower particle size limits
INFUSION 9 g ground tea + 375 g boiling water. Shake 10 min. Filter	Quantity, pH and temperature affect extraction and solubility of theaflavin. Agitation is important to remove localised concentration build up around particles, which also prevents formation of temperature differentials in infusion vessel. Infusion time is important to optimise extraction vs degradation of TF	(1) Weigh boiling H_2O into a tared flask (2) Use standard water e.g. distilled/deionised (3) Pre-heat flask (4) Pre-weigh tea into a single ply tissue to enhance speed of transfer (5) Standardise vacuum flask/thermos (6) Use a large bench top stainless steel water boiling vessel (7) Hand inversion of flask (time interval specified) or tumbler shaker (8) Stress importance of infusion time (10 min)

Flavognost assay	*Critical areas*	*Recommendations*
PARTITION Mix 10 ml aqueous infusion + 10 ml IBMK for 10 min. Allow to separate. Take 2 ml upper layer + 4 ml ethanol + 2 ml Flavognost reagent. Shake for 2 min. Stand for 15 min.	Temperature of extract. Hot extract often produces emulsion which has to be ethanol-broken by centrifugation	(1) Cool extract quickly in cold water. Partition cold extract with IBMK
COLOUR MEASURE-MENT OD 625 nm	Spectrophotometer calibration	(1) Standard OD 625 nm absorbing material

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