S. L. J. Tea Sci. **62** (1), 32-37, 1993. *Printed in Sri Lanka.*

HIGH–PERFORMANCE LIQUID CHROMATOGRAPHY OF CHLOROPHYLLS IN TEA *(CAMELLIA SINENSIS)*

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An HPLC method has been developed to separate Chl a and Chl b in tea. Five tea clones were used in the present study. The HPLC separation was performed on a C_{18} – column using acetone – methanol (40:60) as mobile phase. The components were detected by visible (650 nm) light absorption. The values obtained by spectrophotometric analysis were comparable to that obtained by HPLC.

INTRODUCTION

Chlorophyll (Chl), which is the major plant pigment is known to contribute to the "blackness" of made tea, an important criteria in the commercial evaluation of tea. However, with regard to high grown teas, emphasis is given mainly to the flavour of the liquor rather than the appearance of the made tea which is important in the case of the low growns. Chlorophyll is a complex molecule possessing a central Mg atom, $C_{20}H_{39}$ side chain known as phytol, and a porphyrin ring which is the main skeleton (Fig. 1). During black tea manufacture the chlorophylls break down to yield two compounds known as phaeophytin which is black in colour, and phaeophorbide which is brown. Phaeophytin is formed with the removal of magnesium from the chlorophyll molecule – a process which occurs under acidic conditions. Phaeophorbide is formed concurrently during this process (Wickremasinghe and Perera, 1966). This reaction takes place in two steps, where the phytol is first removed by the action of an enzyme called chlorophyllase to yield an olive green compound known as chlorophillide, which itself loses its Mg atom during firing to yield phaeophorbide. Therefore, the blackness or browness of teas could be dependant on the proportions of phaeophytin and phaeophorbide present in the made tea. In order to monitor the formation of transformation products of chlorophylls during various stages of black tea manufacture and to study the model systems in the conversion of chlorophylls to its degradation products it is essential to establish a rapid and an effective procedure for the separation, isolation and quantification of these pigments.

Separation of chlorophyll and its transformation products on tea have so far been carried out only on thin–layer chromatography (Wickremasinghe and Perera, 1966). In general, separation procedures such as conventional column chromatography and thin-layer chromatography are not very effective in separating plant pigments due to a long operation time and exposure of the pigments to air, which could result in the formation of artifacts. In recent years the HPLC technique has been widely applied

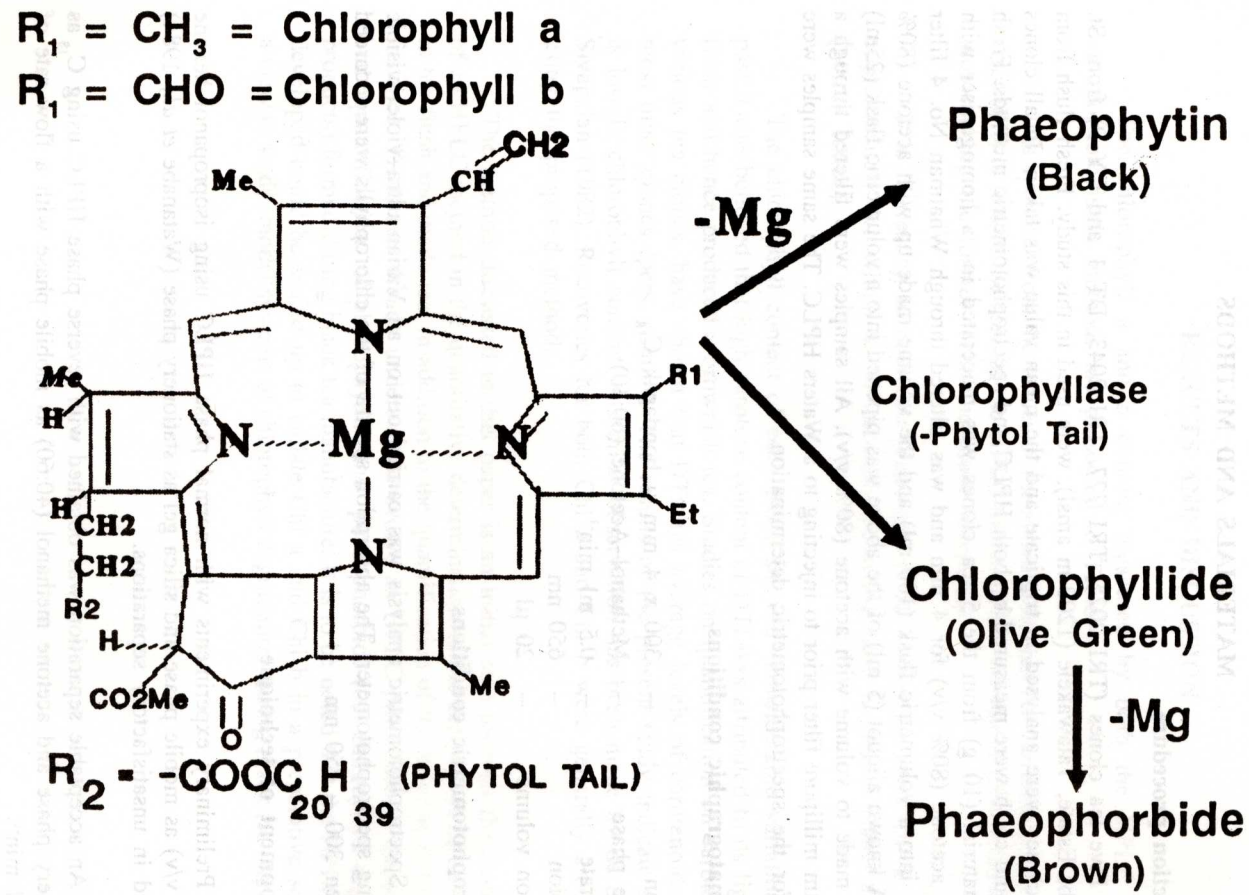

Fig. 1–*The chlorophyll molecule and its breakdown products.*

for the separation of plant pigments. Therefore, as a preliminary investigation an HPLC technique was developed to separate and quantify the levels of Chl a and b in several tea clones. As a comparison spectrophotometric analysis (Vernon, 1960) of Chl a and b was carried out in parallel on the same samples.

MATERIALS AND METHODS

Extraction procedure

Five tea clones (TRI 2025, TRI 777, TRI 2043, DT 1 and CY 9) from St. Coombs Estate, Talawakele (1200 m amsl), were used in this study. Fresh flush from these clones were analysed in triplicate and the mean value was taken. In all clones chl a and chl b were measured by both HPLC and spectrophotometric methods. Fresh leaf material (10 g) from the 5 tea clones was macerated in a homogeniser with 50 ml acetone (80% v/v) for 4 min and was filtered through Whatman No. 4 filter paper into a volumetric flask (100 ml) and the volume made up with acetone (80% v/v). A known aliquot (5 ml) of the above was pipetted into a volumetric flask (25ml) and made to volume with acetone (80% v/v). All samples were filtered through a 0.45 µm millipore filter prior to injecting to a Waters HPLC. The same samples were used for the spectrophotometric determination.

Chromatographic conditions

Column	–	300 x 4 mm µ bondapak C_{18}
Mobile phase	–	Methanol–Acetone (60:40)
Flow rate	–	0.5 ml min^{-1}
Detection	–	650 nm
Injection volume	–	20 µl

Spectrophotometric conditions

Spectrophotometric analysis was carried out on an Varian ultra–violet–visible scanning spectrophotometer. The absorption spectra of the chlorophylls were recorded between 300 – 750 nm.

Development of technique

Preliminary experiments with normal phase HPLC using isopropanol–hexane (3:97, v/v) as mobile phase and silica gel as stationery phase (Watanabe *et al.*, 1984) resulted in unsatisfactory separations.

An acceptable separation was obtained with reverse phase HPLC using C_{18} as stationery phase and acetone methanol (40:60) as mobile phase with a flow rate of 0.5 ml min^{-1}.

Figure 2 illustrates a typical chromatogram obtained under the latter conditions. Chl a and b elute as two individual peaks at retention times corresponding to the respective standards (Sigma Chemical Co., U.S.A.). It is evident from Fig. 2 that a > 1.0, which is the value needed for a base line separation.

RESULTS AND DISCUSSION

The chlorophyll a and b content measured by both the HPLC and the spectrophotometric methods are presented in Table 1.

It is apparent from the values of the above table that the variation in the standard error (SE) is less in the case of the HPLC method vis-a-vis spectrophotometric method thus making the former technique more acceptable.

It is interesting to note that practically in all clones the ratio Chl a: Chl b is approximately 3:1 which is the naturally occurring ratio in higher plants and algae (Merck Index).

The chlorophyll content of each clone showed considerable variation. In the past it was believed that high carotene content of DTI was attributed to its light green flush and hence produced a brownish tea. Studies on quantification on carotenoids in various tea clones, have shown that DTI has average levels of carotenoids as with most other clones (pers. comm.). The results of the present study indicate that DT1 is low in chlorophyll content. A similar observation has been made by de Silva and Sivapalan (1982). Recoveries for both Chl a and Chl b were generally around 98% using the proposed method.

The method discussed in this paper is an initial step towards the development of an HPLC method in the quantitative determination of the derivatives of chlorophylls in tea flush and made tea, in addition to the quantification of the latter as in the present instance. Trials are already underway to evolve an HPLC procedure in the separation and quantification of phaeophytin and phaeophorbide (i.e. chlorophyll degradation products) formed during black tea manufacture. It is to be noted that the HPLC method developed could be scaled up to isolate Chl a and Chl b in a preparative scale with a view to study model systems of chlorophyll degradation during black tea manufacture.

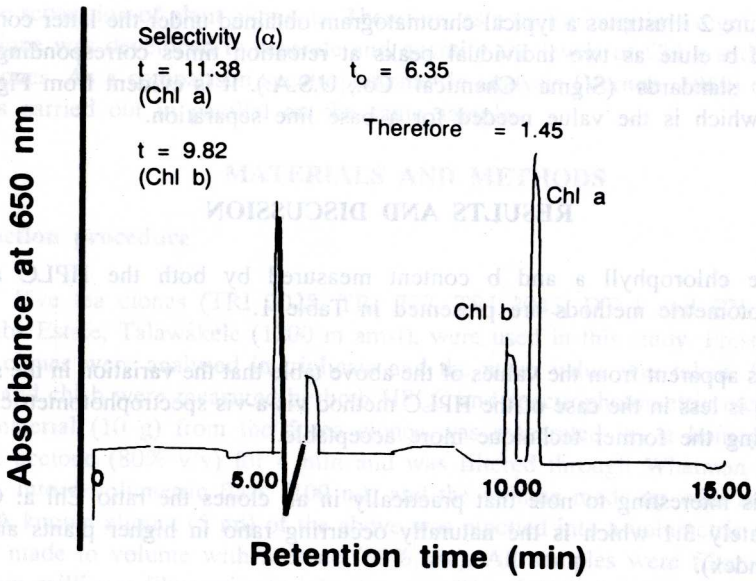

Fig. 2–Separation of chlorophylls by HPLC method

$t_{Chl\ a}$ and $t_{Chl\ b}$ retention times of Chl a and Chl b respectively. t_o = solvent front

TABLE 1–Chlorophyll a and b content (mean ± SE) obtained by the HPLC and spectrophotometric methods*

	HPLC method Chl a mg g^{-1}	Chl b mg g^{-1}
Clone		
TRI 2025	0.5389±0.0012	0.1827±0.0010
TRI 777	0.6384±0.0064	0.2024±0.0009
CY 9	0.3711±0.0003	0.1407±0.0003
TRI 2043	0.5548±0.0013	0.1907±0.0003
DT1	0.1904±0.0001	0.0707±0.0003
	Spectrophotometric method Chl a mg g^{-1}	Chl b mg g^{-1}
Clone		
TRI 2025	0.5922±0.0038	0.1943±0.0012
TRI 777	0.5794±0.0060	0.1971±0.0014
CY 9	0.4308±0.0056	0.2355±0.0040
TRI 2043	0.4825±0.0022	0.2208±0.0030
DT1	0.2669±0.0001	0.1075±0.0030

* on a fresh weight basis

ACKNOWLEDGEMENTS

We thank Dr N. L. Herath for his critical reading of the manuscript and Dr A. Anandacoomaraswamy for the assistance given with the statistical analysis.

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