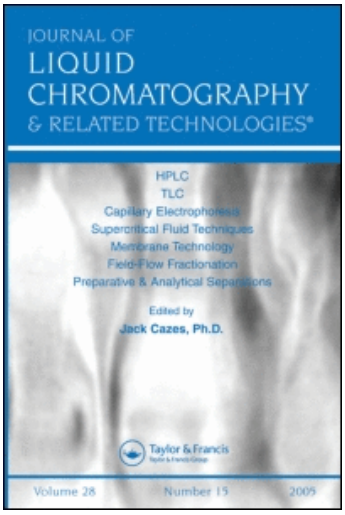

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HPTLC Methods for Identification of Green Tea and Green Tea Extract

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Abstract: For centuries, the leaves of *Camellia sinensis* have been used to make green, black, and special types of tea; it has been the basis for one of the most popular beverages all over the world. More recently, green tea has become the raw material for extracts which are used in various beverages, health foods, dietary supplements, and cosmetic items. In view of the Good Manufacturing Practice (cGMP) for dietary supplements, mandating proper identification of raw materials and finished products, it is very important to have the necessary analytical tools at hand.

This paper presents HPTLC methods for identification of green tea and green tea extract. On silica gel 60 with ethyl formate, toluene, formic acid, water (30:1.5:4:3) as mobile phase, the flavonoid fingerprint of green tea can give information about the geographical origin of the material. The mobile phase toluene, acetone, formic acid (9:9:2) allows the discrimination of green tea from black and other specialty teas, based on the polyphenol pattern. The latter method has been validated, addressing specificity, stability, reproducibility, and robustness. For additional quality control of extracts, ethyl acetate, methanol, and water (20:2.7:2) can be used as mobile phase to investigate the alkaloid profile, whereas, 1-butanol, acetone, acetic acid, water (7:7:2:4) provides an amino acid profile.

Keywords: *Camellia sinensis*, Fingerprint, Flavonoids, Green tea, Polyphenols, Validation

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INTRODUCTION

Tea, a hot water infusion made from leaves of *Camellia sinensis*, is a beverage that has been consumed for centuries all over the world. Green tea, the minimally fermented (oxidized) preparation of the tea leaf, may show certain health benefits[1] and has, therefore, gained increased popularity. Today, in addition to their traditional use for making tea, the leaves of *Camellia sinensis* are industrially processed. Extracts are used in dietary supplements, and are added to an increasing range of products, such as beverages, nutrition bars, ice cream, and even topical skin creams.

To guarantee the quality and safety of these products, effective controls are of importance. FDA's cGMPs for dietary supplements[2] will force the industry to provide identification of all botannical raw material that went into a product.

For the analysis of green tea constituents, a variety of analytical methods have been published during the last five years. Mainly HPLC,[3–8] sometimes in combination with mass spectrometry,[9,10] was used for the simultaneous analysis of polyphenols, flavonoids, purine alkaloids, and amino acids. A few papers deal with electrophoretic methods[8,11] or micellar electrokinetic capillary chromatography.[12]

HPLC has also been used for the analysis of flavonoids in particular,[13] and in numerous publications on the determination of polyphenols/catechins.[14–17] Catechins were analyzed with HPTLC (High Performance Thin-Layer Chromatography) on cellulose layer[18] and with a stability indicating HPTLC method on silica gel.[19] The determination of caffeine in tea was discussed in several papers which shall not be detailed further.

In this paper, we are taking a strictly qualitative approach. HPTLC fingerprint methods have been developed for polyphenols (catechins), flavonoids, amino acids (theanine), and purine alkaloids (caffeine, theobromine). It was the aim of our work to provide to the industry a rapid and reliable tool for identification of green tea raw material and for monitoring product consistency.

EXPERIMENTAL

Material

Tea samples were obtained from tea stores in Basel, Hong Kong, Tokyo, and Mumbai. The investigated tea extract was a production sample of Frutarom Ltd. (Wädenswil, Switzerland). Chemical reference substances and general reagents were purchased from Sigma (St. Louis, MO, USA), Roth (Karlsuhe, Germany), and Merck (Darmstadt, Germany).

HPTLC plates of silica gel 60 F_{254} were manufactured by Merck. Solvents of p.a. grade were purchased from ACROS (Geel, Belgium) or

Merck. Chromatographic equipment (twin trough chamber 20 × 10 cm, glass sprayer, immersion device, automatic TLC sampler 4, digital documentation system, automatic developing chamber ADC2, winCATS 1.3.4 software) were made by CAMAG (Muttenz, Switzerland). Other equipment included a centrifuge and diverse glassware.

Preparation of Samples and Reference Solutions

Tea sample (100 mg) or 40 mg of tea extract were mixed with 10 mL of ethanol–water 8:2 and extracted by sonication for 5–10 min. The mixtures were then centrifuged and the supernatants were used as test solutions. Test solutions for analysis of polyphenols were stored in the freezer at −20°C; all other test solutions were stored at 6°C.

Chemical reference substances (rutin, hyperoside, isoquercitrin, astragalin, chlorogenic acid, gallic acid, epigallocatechin gallate, epigallocatechin, epicatechin gallate, epicatechin, caffeine, theobromine, theanine, glutamic acid, aspartic acid, and tyrosine) were individually dissolved in methanol to yield concentrations of about 0.5 to 5 mg/mL. Solutions of polyphenols were stored at −20°C; all other solutions were stored at 6°C.

Preparation of Derivatizing Reagents

Natural Products Reagent (NP Reagent)

Diphenylborinic acid aminoethylester (1 g) was dissolved in 200 mL of ethyl acetate.

Macrogol Reagent

Polyethylene glycol 400 (macrogol, 10 g) were dissolved in 200 mL of dichloromethane.

Fast Blue Salt B Reagent

Fast Blue Salt B (140 mg) was dissolved in 10 mL of water, diluted with 140 mL of methanol and 50 mL of dichloromethane. The reagent was prepared freshly every week and stored at 4°C in the dark.

Ninhydrin Reagent

Ninhydrin (400 mg) was dissolved in 200 mL of methanol.

Mobile Phases

Flavonoids: ethyl formate, toluene, formic acid, water (30:1.5:4:3).
Polyphenols: toluene, acetone, formic acid (9:9:2).
Alkaloids: ethyl acetate, methanol, water (20:2.7:2).
Amino Acids: 1-butanol, acetone, acetic acid, water (7:7:2:4).

Chromatography

The general standard operating procedure (SOP) for HPTLC, as previously published,[20] was followed with some deviations: One to 15 microliters of test and reference solutions were applied as 8 mm bands, 8 mm from the lower edge of the plate. Plates were developed over a distance of 70 mm (flavonoids), 60 mm (polyphenols) or 50 mm (alkaloids, amino acids) from the lower edge of plate, using a twin trough chamber saturated for 30 min (flavonoids), or unsaturated (polyphenols, alkaloids, amino acids).

Derivatization

Flavonoids: The plate was heated at 100°C for 2 min and dipped in the NP reagent while hot, and dried in the fume hood. The plate was then dipped in the Macrogol reagent and dried in the fume hood. Evaluation was performed under UV 366 nm.

Polyphenols: The plate was heated at 100°C for 2 min, then dipped in Fast Blue Salt B reagent while hot. The plate was then dried in a fume hood for 5 min after derivatization. Evaluation was performed under white light.

Alkaloids: No derivatization. Evaluation was performed under UV 254 nm.

Amino acids: The plate was dipped into ninhydrin reagent, then heated at 110°C for 3 min. Evaluation was performed under white light.

Documentation

Digital images of the plates were captured in various illumination modes (UV 254 nm, UV 366 nm, and white light).

RESULTS AND DISCUSSION

A total of 80 tea samples from Chinese, Japanese, Indian, and other provenances has been analyzed. Aside from green tea, also black, white, Oolong, Pu-Erh, and other specialty teas were included in the study.

Differentiation of the Geographical Origin of Green Tea

Tea leaves produce a characteristic flavonoid fingerprint when developed on silica gel as stationary phase with ethyl formate, toluene, formic acid, water (30:1.5:4:3) as mobile phase. After derivatization with Natural Product/Macrogol reagent, the flavonoids can be evaluated under UV 366 nm. The fingerprint can be described using (with increasing R_F) rutin, chlorogenic acid, hyperoside, and gallic acid as chemical reference substances. Our investigation of many samples has shown quite some variation in the relative intensities of the separated zones, but there seem to exist three general patterns (type I, II, and III). Although we were not able to link those patterns to the different kinds of tea (green, black, white, etc.), we found a correlation with the geographical origin of the samples, particularly when looking only at green teas (Fig. 1). Most type I samples originated from China, most type II samples from Japan, and type III samples from India. Many of the investigated samples of black tea were also of type III.

In the lower third of the chromatogram (Figure 1) of green tea below the position of rutin, an orange and a green zone may be located. These zones are weak in type I, strong in type II, and very faint or missing in type III. All samples show a reddish violet zone at the position of rutin. Just above, or co-eluting with this zone, type I and II samples feature a weak or strong blue fluorescing zone, which is not seen in type III samples. There is a prominent blue fluorescing zone slightly above the position of chlorogenic acid in samples of types I and III. Type II samples lack this zone and exhibit several weak reddish violet zones instead. Slightly above the position of hyperoside, all samples show a reddish violet zone. In type I samples, this zone may be covered up by a blue fluorescent zone. In the

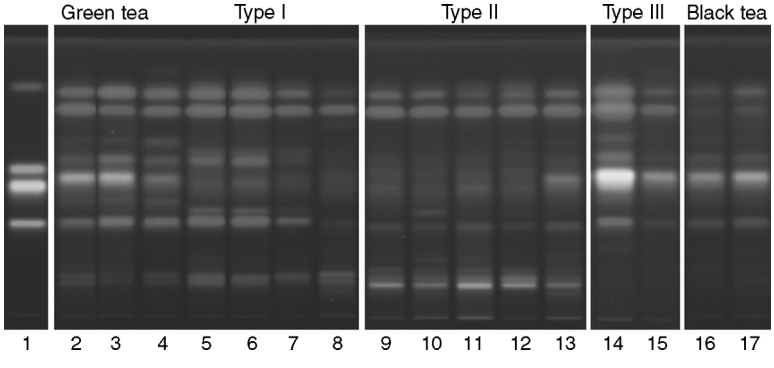

Figure 1. HTPLC fingerprint (flavonoids) of green tea samples representing different geographic origins. Track assignment: 1 reference substances with increasing R_F: rutin, chlorogenic acid, hyperoside, gallic acid; 2–8 samples from China; 9–13 samples from Japan; 14–15 samples from India, 16–17 black tea from Ceylon. For details see text. Note: for comparison tracks are taken from different plates.

upper portion of the chromatogram, at the position of gallic acid, and just below two prominent blue fluorescent zones, can be found in all samples.

Because all samples were obtained from regular tea shops, it is not possible to address the effects of other factors such as exact plant part, age, storage conditions, and harvest quality of the tea, which may also contribute to the formation of the different types. In any case, the flavonoid pattern can be used conveniently by a manufacturer to compare batches of green tea in order to ensure raw material consistency and possibly even correct provenience.

Discrimination of Green Tea from Other Types of Tea

During the processing of tea leaves, fermentation (oxidation) takes place to a defined degree. The polyphenol pattern (relative content of epigallocatechin gallate, epigallocatechin, epicatechin gallate, and epicatechin) was found to be affected (Fig. 2).

Chromatography on silica gel 60 with toluene, acetone, formic acid (9:9:2) as mobile phase, in an unsaturated chamber, allows baseline separation of the target compounds. The polyphenol profile can be visualized with Fast Blue Salt B reagent. While the profiles of the investigated samples of white and oolong samples are inconsistent and may not be representative, black tea either shows no polyphenol zones at all or strong zones of epigallocatechin gallate and epigallocatechin, and a weak zone for epicatechin. Only green tea features four zones, of which the epigallocatechin gallate zone is strongest. Such pattern was found for all samples of green tea (Fig. 3). Quality control of the botanical industry can use this fingerprint to identify, with certainty, a sample as green tea or derived from it.

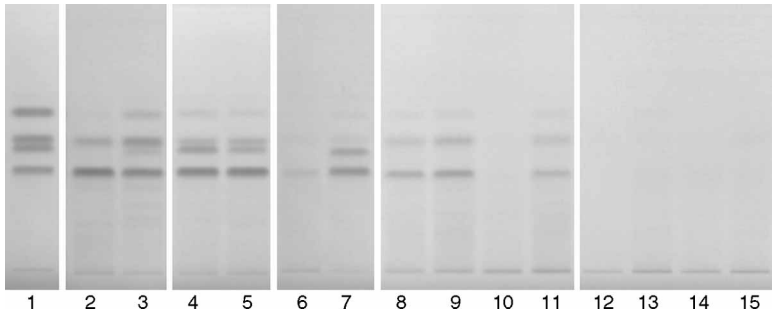

Figure 2. HTPLC fingerprint (polyphenols) of tea samples representing different types. Track assignment: 1 reference substances with increasing R_F: epigallocatechin gallate, epigallocatechin, epicatechin gallate, and epicatechin; 2–3 white tea from China; 4–5 green tea from China; 6–7 Oolong tea from china (sample 7 is very little fermented); 8–11 black tea from India; 12–15 black tea from China. For details see text. Note: for comparison tracks are taken from different plates.

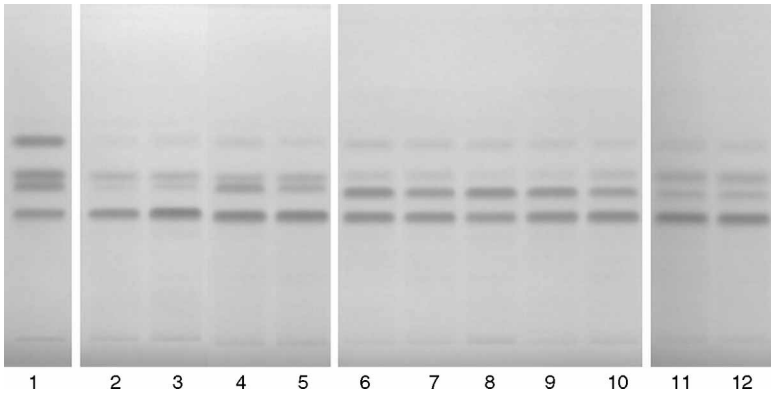

Figure 3. HTPLC fingerprint (polyphenols) of green tea samples representing different geographical origin. Track assignment: 1 reference substances with increasing R_F: epigallocatechin gallate, epigallocatechin, epicatechin gallate, and epicatechin; 2–5 Chinese samples; 6–10 Japanese samples; 11–12 Indian samples. Note: for comparison tracks are taken from different plates. The separation of epigallocatechin and epicatechin gallate (2 middle zones) is affected by humidity changes and is not exactly the same on all plates.

Comprehensive Characterization of a Tea Extract

In addition to the methods described above, two other methods can be utilized to evaluate the constituents of green tea (Fig. 4). The resulting set of fingerprints allows a manufacturer not only to compare batches of raw material and finished products against a botanical reference material (BRM), but also to demonstrate that, during production, the entire spectrum of compounds has been transferred from raw material to product. The alkaloid profile developed on silica gel 60 with ethyl acetate, methanol, water (20:2.7:2) as the mobile phase shows several quenching zones, the most prominent of which is due to caffeine. A weaker zone, just below, is due to theobromine. The amino acid theanine is a compound characteristic for tea leaves. In the amino acid profile obtained on silica gel 60 with 1-butanol, acetone, acetic acid, water (7:7:2:4) as mobile phase, a red zone due to theanine is the most prominent feature. Just below, a zone due to aspartic acid is seen.

Validation of the Method for Polyphenols

The method for identification of green tea based on its polyphenol profile was validated according to the concept published by Koll et al.[21] using a botanically authenticated sample of green tea as BRM. The following validation parameters were addressed.

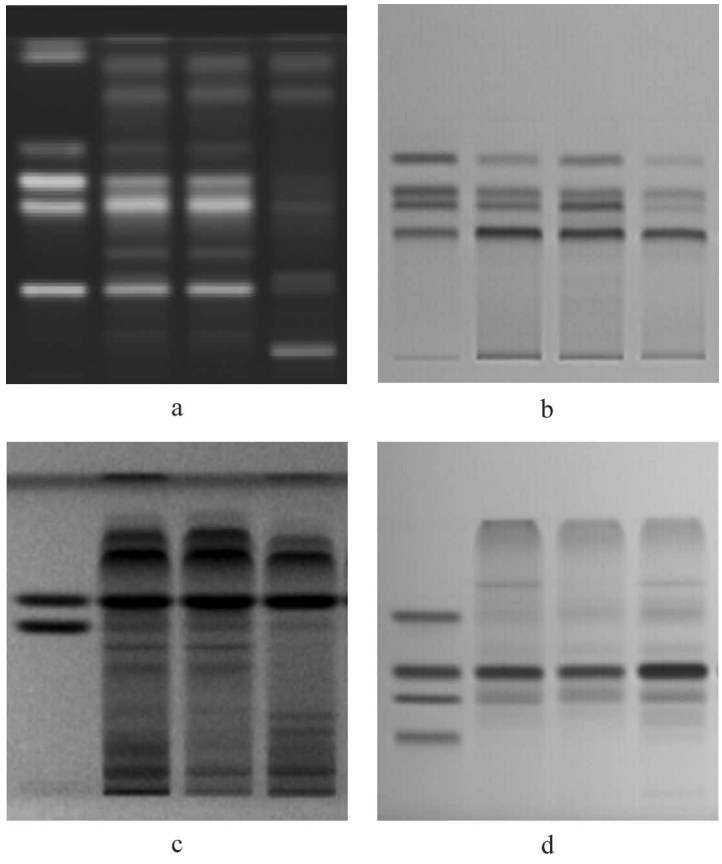

Figure 4. HPTLC fingerprints of green tea and green tea extract. a) Flavonoids; b) Polyphenols; c) Alkaloids; d) Amino acids. Track assignment: 1 reference substances with increasing R_F-values [a) rutin, chlorogenic acid, isoquercitrin, astragalin, and caffeic acid; b) epigallocatechin gallate, epigallocatechin, epicatechin gallate, and epicatechin; c) theobromine and caffeine; d) aspartic acid, glutamic acid, theanine, and tyrosine], 2 green tea BRM; 3 green tea extract; 4 green tea commercial product.

Specificity: The method clearly discriminates green tea from other types of tea. Only green tea shows four zones. The method works also for green tea extract and further processed materials, such as ice tea powder (green tea). There is no interference from other components such as sugar and citric acid.

Stability: The sample (green tea BRM) is stable on the HPTLC plate and in solution for at least 3 hours. The sample is also stable during chromatography, as shown by 2D chromatography. After derivatization, the chromatogram is stable up to 30 min. After that time, the plate's background darkens significantly.

Precision: Repeatability was evaluated by individually preparing three portions of the BRM and chromatographing three aliquots of each preparation

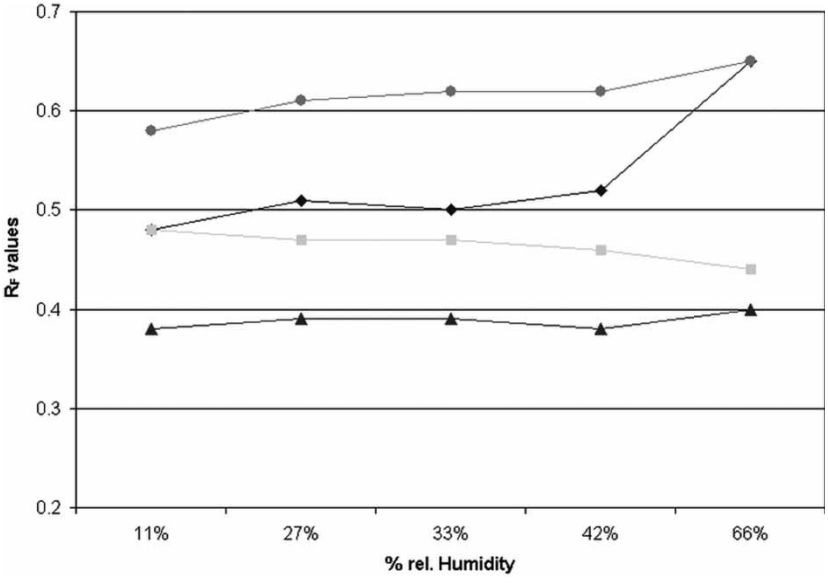

Figure 5. R_F values of polyphenols in green tea as function of relative humidity.

on three plates. The average R_F values of the four polyphenols on each plate ($n = 9$) varied less than 0.02 R_F units. Intermediate precision was determined on three days. Average R_F values of the four polyphenols on each plate ($n = 9$) varied less than 0.03 R_F units. Reproducibility was established in a collaborating laboratory by performing the repeatability experiment. Between labs, the R_F values of the four polyphenols ($n = 9$) varied less than 0.03 R_F units.

Robustness: If chromatography is performed in a horizontal developing chamber, in sandwich mode, similar results as in a twin trough chamber are obtained. The R_F values are higher by about 0.04 units. Extending the developing distance from 60 to 70 mm (from lower edge of plate) does not affect the result. The method is sensitive to changes in relative humidity (Fig. 5). Only between 27 and 40% RH all four polyphenols are separated.

A copy of the comprehensive validation protocol can be obtained from the corresponding author.

CONCLUSIONS

HPTLC offers a rapid and cost efficient possibility for quality control of green tea raw material and green tea products. The validated method for identification, based on the polyphenol profile, allows discrimination of green tea from differently processed tea leaves (*Camellia sinensis).* The flavonoid

profile can be used to ensure batch-to-batch consistency of green tea raw material. The method may be used to address quality issues related to the geographical origin of the material. With four HPTLC fingerprints evaluating flavonoids, polyphenols, alkaloids, and amino acids, the constituents of green tea extracts can be comprehensively compared to those of the corresponding raw material.

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