

## **Validation of Method for Identification of Green Tea by HPTLC Fingerprint**

This method was developed for the purpose stated below by Frutarom Ltd, Switzerland<sup>1</sup>.

There is no official method for the separation of Tea polyphenols.

### **1. Purpose of method to be validated:**

The method for identification of Green Tea by HPTLC fingerprint is suitable to identify a given sample of plant material as Green Tea (*Camellia sinensis*) based on its polyphenol fingerprint.

The method may be used to identify an extract as derived from Green Tea (*Camellia sinensis*), provided that the material was made from a single herb and intended to contain the constituent profile seen in Green Tea.

### **2. General acceptance criteria:**

The method is valid if:

- A botanically authenticated sample of Green tea (*Camellia sinensis*) yields a fingerprint which is similar to that shown in section 4.10 of the method with respect to number, position, color, and intensity of bands **and**
- All acceptance criteria specified in sections 5.2 to 5.6 are met **and**
- Any deviation from the expected result doesn't exceed those deviations seen under section 5.7 (Robustness).

1) Reich, E., Schibli, A., Widmer, V., Jorns, R., Wolfram, E., DeBatt, A.: [HPTLC Methods for Identification of Green Tea and Green Tea Extract](#). J. Liq. Chromatogr. Related Technol. 29 (2006) 2141 - 2151.

### **3. Personnel**

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#### **4. Description of method**

##### **4.1 Preparation of test solutions**

0.1 g of milled sample; or 40 mg of dry extract, or enough product equivalent to that amount are sonicated for 10 min with 10 mL ethanol-water 80:20. The solution is centrifuged and the supernatant is used as test solution. These solutions must be stored below -20°C.

##### **4.2 Preparation of reference solutions**

Botanical reference solution: as 4.1

Chemical reference solution: 0.5 mg of each substance ((-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin gallate, (-)-epigallocatechin gallate) are individually dissolved in 10 mL methanol each. These solutions must be stored below -20°C.

##### **4.3 Preparation of derivatizing reagent**

140 mg Fast Blue salt B are dissolved in 10 mL water, 140 mL methanol are added, followed by 50 mL dichloromethane. This reagent must be prepared freshly every week and stored at 4°C in the dark.

##### **4.4 Stationary phase**

10x10 cm (or 20x10 cm) glass plates HPTLC silica gel 60 F<sub>254</sub> (Merck).

##### **4.5 Sample application**

1 µL of test solution, 1 µL of botanical reference solution, and 5 µL of chemical reference solution are applied each as 8 mm bands, at least 2 mm apart, 8 mm from the lower edge and at least 15 mm from left and right edges of the plate.

##### **4.6 Temperature and Humidity**

Record temperature and humidity in the laboratory.

##### **4.7 Chromatography**

Chamber type: 10x10 cm (or 20x10 cm) Twin Trough Chamber  
Configuration: Unsaturated  
Developing solvent: Toluene, acetone, formic acid (4.5:4.5:1); 5 mL  
(respectively 10 mL) developing solvent in developing trough.  
Developing distance: 60 mm from lower edge of plate (52 mm from application position).  
Drying: 5 min with cold air (hair dryer)

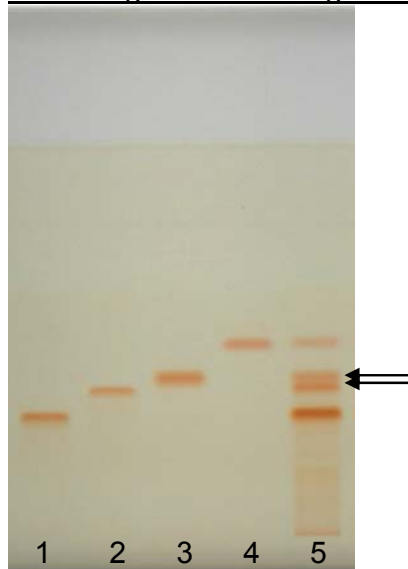
##### **4.8 Derivatization**

The plate is heated at 100°C for 2 min, then while still warm immersed into reagent for 1 s and dried in the fume hood.

##### **4.9 Documentation**

Immediately after derivatization under white light (reflection and transmission).

#### 4.10 Image of chromatograms



- 1: (-)-epigallocatechin gallate  
2: (-)-epigallocatechin  
3: (-)-epicatechin gallate  
4: (-)-epicatechin  
5: Botanical reference material (BRM; Green Tea leaves)

#### 4.11 Evaluation of results:

The chromatograms of the four standards (tracks 1-4 and 7) show a brownish-orange zone for each substance with the following Rf values: epigallocatechin gallate (track 1): Rf=0.38, epigallocatechin (track 2): Rf=0.48, epicatechin gallate (track 3): Rf=0.52, epicatechin (track 4): Rf: 0.62.

The BRM shows brownish-orange zones at the same positions. The lowest zone is the most intense. The upper zone is the faintest.

#### 4.12 System suitability test:

The result obtained in the test is suitable for evaluation if the fingerprint of the test solution shows two distinct bands for epigallocatechin and epicatechin gallate, see arrows in Fig. 4.10.

## 5. Validation

### 5.1 Materials

#### 5.1.1 Chemicals and solvents

Name	Manufacturer	Quality
Ethanol	Merck	p.a
Methanol	Acros	p.a
Fast blue salt B	Fluka	--
Toluene	Merck	p.a
Acetone	Merck	p.a
Formic acid 98-100%	Merck	p.a
Dichloromethane	Merck	p.a
Water	In house demineralization (ion exchange)	--

#### 5.1.2 Samples and Reference materials

##### Botanical reference material

Name	Source / Lot	Authentication
Green Tea ( <i>Camellia sinensis folium</i> )	Removed - proprietary information	Yes

##### Additional samples

Name	Source / Lot	Authentication
Lung Ching Grade 1 China	Removed - proprietary information	No
Mingqian Green Tea		No
Longjing Green Tea		No
Chinese Sencha		No
Pi Lo Chun China		No
Gunpowder "Temple of Heaven" China		No
Yong Xi Hou Qing China		No
Pao Chung Pouchong China		No
Thé vert au Jasmin		No
Gyokuro Japan		No
Japan Sencha Tea		No
Special Japan Sencha.		No
Bancha Japan		No
Bancha Japan		No
Green tea powder Japan		No
Green Tea		No
Sencha Japan		No
Sencha with citronella Japan		No
Japan Green Tea with rose and Jasmin flowers		No
Hoji Cha, roasted Green tea Japan		No
Java Green Tea.		No
Indian green tea #1		No
Indian green tea #2		No
Special Assam India Green Tea STGFOP 1		No

**Adulterants (Black, Oolong and Red teas)**

Name	Source / Lot	Authentication
Formosa Topest Superieur Fancy Oolong Tea Butterfly of Taiwan	Removed - proprietary information	No
Oolong Tee (halbfermentiert) mit Jasminblüten.		No
Finest Spring Jade Oolong		No
Gaoshan Oolong Tea		No
Oolong Tea		No
Yunnan Imperial Tea Black Tea		No
Superieur Yunnan China Black Tea		No
Superieur Keemun Ning Hong Jing Hao China Black Tea		No
Keemun China Black Tea		No
Yunnan- and Assam Pekoe China and India		No
Lapsang Souchong Tea China Smoken Black Tea		No
Pure Tarry Lapsang Souchong Tea China Smoken Black		No
King of Pu-Erh-Tea China Red Tea. Storage 60 years		No
Pu-Erh-Tea China Red Tea Storage 25 years		No
Pu-Erh Orange. Storage 25 years		No
Pu-Erh Tuo Cha China Red Tea (no fermentation) Storage 20 years.		No
Darjeeling SOOM STGFOP 1 First Flush		No
Darjeeling Castleton Muscatel STGFOP 1		No
TUMSONG Second Flush Darjeeling		No
Badamtan Black Tea 2004 India		No
Chamong Black Tea 2004 India		No
India Black Tea		No
Assam Shantipur Black Tea 2004		No
Hatimara Assam Tea FTGFOP1 India Black Tea		No
Doomur Black Tea India		No
Melong Black Tea India		No
Darjeeling Tea		No
Assam Tea		No
Darjeeling Tea		No
Lemon flavored Black tea		No
Ceylon Black Tea		No
Extra Choicest Ceylon Pekoe Black Tea		No
Ceylon Black Tea		No
Fine Ceylon Fannings Black Tea		No
Ceylon Flowery Orange Pekoe Black Tea		No
Royal Ceylan (Black tea)		No
Boh Cameronian Garden Tea (Black tea)		No
Boh Cameronian Garden Tea (Black tea)		No
Kenya GFBOP 1 Marynin Black Tea		No

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Golden Nepal Tea Maloom FTGFOP 1 Himalaja Black Tea		No
Sikkim Temi FTGFOP1 Himalaja Black Tea		No

### Processed materials

Name	Source / Lot	Authentication
Camellia sinensis folium extrakt siccum	Removed - proprietary information	no
Ice tea powder, green tea + vitamine C+E, 4.5% green tea extract		no

### Standards (marker compounds, chemical references)

Name	Source
(-)-epicatechin	Roth 48525533
(-)-epigallocatechin	Sigma 38H1540
(-)-epicatechin gallate	Sigma 128H0969
(-)-epigallocatechin gallate	Sigma 68H0844

### 5.1.3 Plates

TLC plate	Size	Source	Batch
Glass plates HPTLC Si 60 F254	10x10 cm	Merck	OB473743
Glass plates HPTLC Si 60 F254	20x10 cm	Merck	OB464935

### 5.1.4 Instruments

Instrument	Manufacturer	Serial Number
Automatic TLC Sampler 4	CAMAG	061104
DigiStore using Canon G5 camera	CAMAG	070705
TTC 20x10 cm	CAMAG	n. a.
TTC 10x10 cm	CAMAG	n. a.
ADC2	CAMAG	Prototype
TLC Plate Heater III	CAMAG	981109
Immersion Device III	CAMAG	090301
Mill KB5/10	IKA	00.183107
Centrifuge EBA21	Hettich	0000799-01-00
Ultrasonic Bath TPC25	Telsonic	2003043
Balance AG245	Mettler-Toledo	1114402254

### 5.1.5 Software

Software	Manufacturer	Version
WinCATS	CAMAG	1.3.2-1.3.3
VideoScan	CAMAG	1.02.00

## **5.2 Stability**

### **5.2.1 Stability of analyte in solution and on the plate**

#### **Description of experiment:**

A portion of the BRM is extracted according to section 4.2. 1  $\mu\text{L}$  of this solution are applied onto a 10x10 cm plate according to sections 4.4-4.5. The sample and the plate with the applied sample (wrapped in aluminum foil) are set aside. After 3 hours another portion of the same BRM is extracted according to section 4.2. Two times 1  $\mu\text{L}$  of this solution are applied according to section 4.5 next to the first sample on the set-aside plate, followed by 1  $\mu\text{L}$  of the set-aside sample (see illustration below).

The 4 samples on the plate represent the following: (A) Sample on the plate for 3 hours prior to chromatography, (B) fresh sample applied immediately prior to chromatography (twice), (C) sample prepared 3 hours prior to chromatography (in solution).

The plate is treated according to sections 4.6 to 4.9.

#### **Acceptance criteria:**

The sample is stable for at least 3 hours in solution and 3 hours on the plate prior to chromatography.

#### **Results:**

No difference is seen in any of the chromatograms. The sample is stable on the plate and in solution for at least 3 hours.

#### **Image:**



1. Sample on the plate for 3 hours prior to chromatography (A)
2. Fresh sample applied immediately prior to chromatography (B)
3. Sample prepared 3 hours prior to chromatography (in solution) (C)
4. Fresh sample applied immediately prior to chromatography (identical with 2) (B)

**Accepted: YES**



## 5.2.2 Stability of analyte during chromatography

### Description of experiment:

A portion of the BRM is extracted according to section 4.2. 5  $\mu\text{L}$  are applied as spot at the lower right corner of a 10x10cm plate (10 mm from each edge). The plate is developed and dried according to section 4.6. The plate is now turned 90° to the right and developed a second time according to section 4.6 with a fresh portion of developing solvent.

The plate is derivatized and documented according to section 4.8 and 4.9.

### Acceptance criteria:

The sample is stable during chromatography if all zones are located on the diagonal connecting the application position with the intersection of the two solvent fronts.

### Results:

No zone is located aside of the diagonal. The sample (Green Tea, *Camellia sinensis*) is stable during chromatography.

### Image:



Accepted: YES

### 5.2.3 Stability of derivatization/result

#### Description of experiment:

The botanical reference solution (4.2) is chromatographed according to section 4.3-4.8. After documentation under white light (4.9), the plate is observed for 1 hour. An image is taken after 2, 5, 10, 20, 30 min, and 1h. The images are compared visually and with the help of video-densitometry.

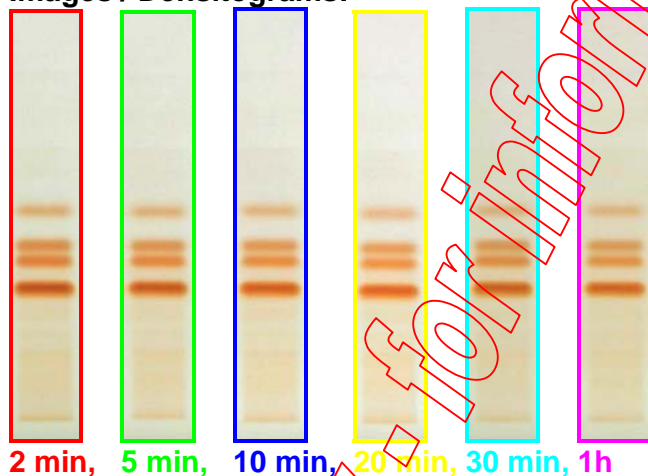
#### Acceptance criteria:

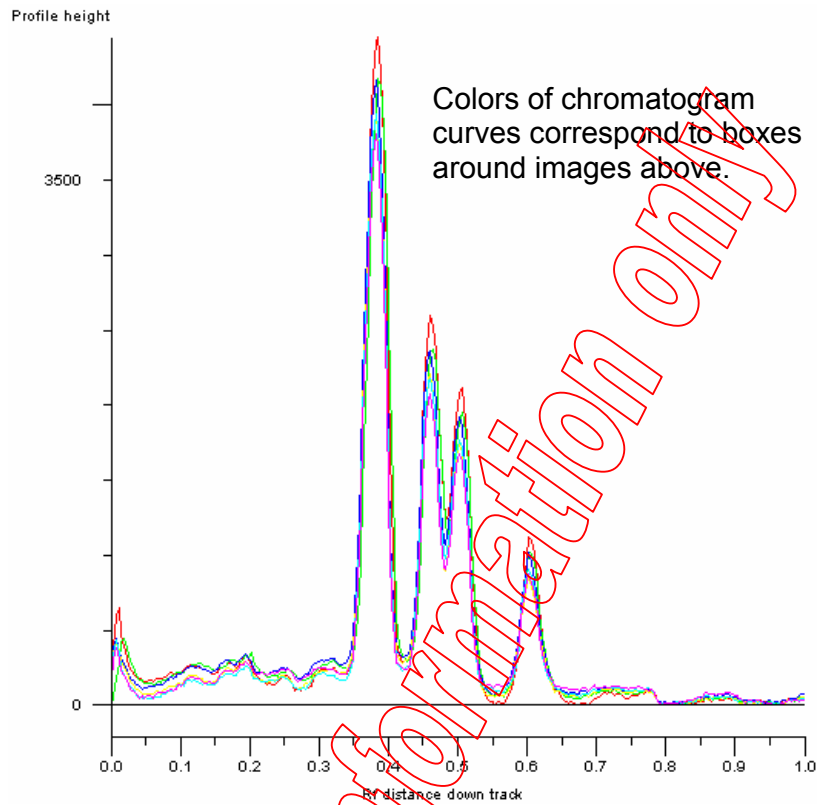
The derivatization yields a stable result, if there is no significant change in the image within 30 min.

#### Results:

The color and intensity of zones remains constant over time, however, the coloration of the plate background intensifies with time. The plate should not be documented and evaluated after 30 min, because the color of the background becomes too intense.

#### Images / Densitograms:





Accepted: YES

### **5.3 Specificity**

#### **5.3.1 Identification of Green Tea samples by comparison to the Botanical Reference Material (BRM) and chemical references**

##### **Description of experiment:**

Test solutions are prepared according to section 4.1. The BRM of *Camellia sinensis* and the chemical references are prepared according to section 4.2. All samples are applied onto the same plate according to section 4.5. Following chromatography (section 4.7) and derivatization (section 4.8) the plate is documented (section 4.9) and the results compared to those shown in section 4.10.

##### **Acceptance criteria:**

The method is specific if the fingerprints obtained with the test solutions representing Green Tea are similar to that shown in section 4.10 of the method with respect to number, position, color, and intensity of bands matching the chromatogram of the BRM and samples of other identity, if present, yield different fingerprints.

##### **Results:**

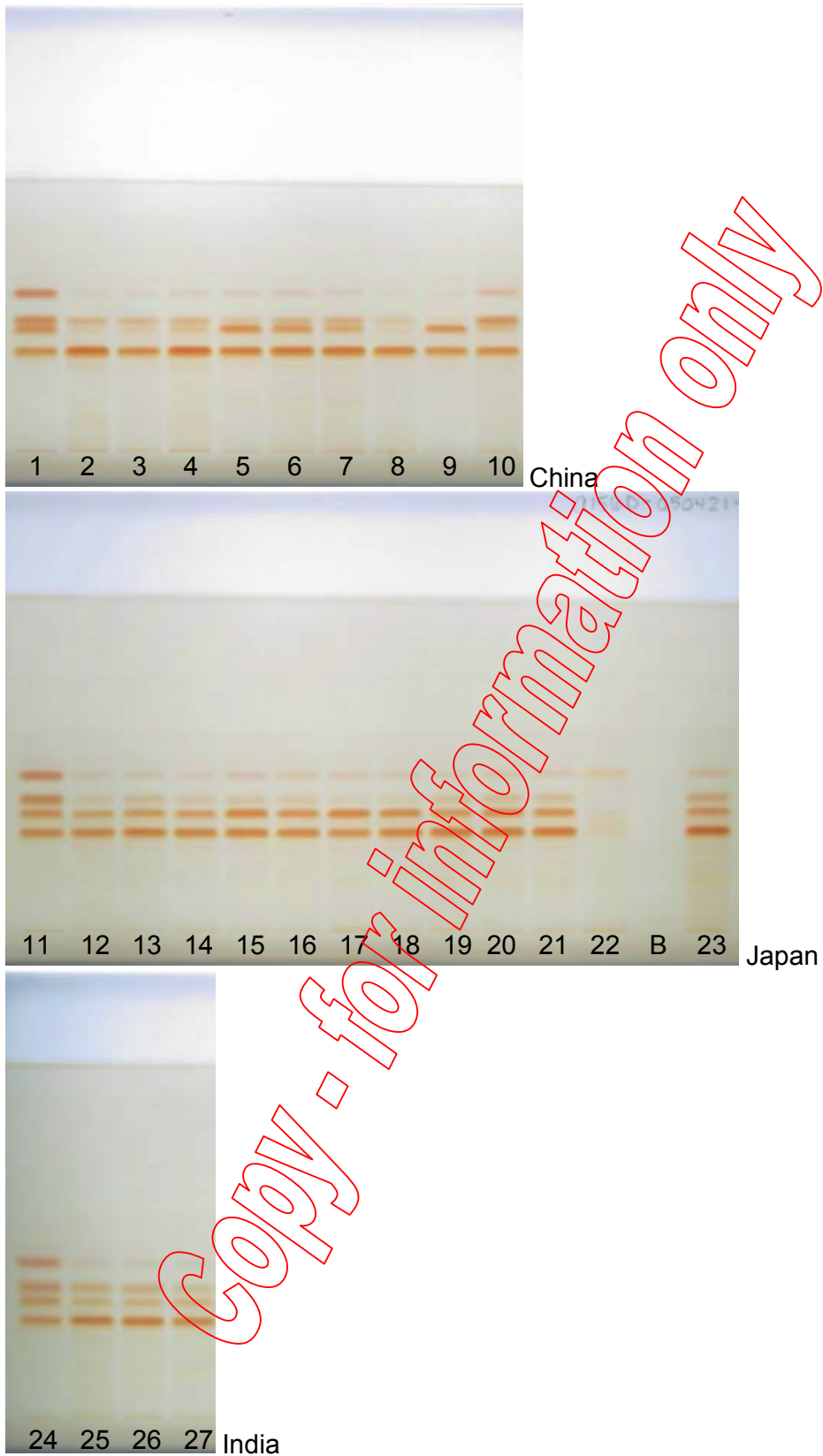
The content of polyphenols in samples varies greatly with the age of leaves and the drying and fermentation process. Most of the green tea samples show bands corresponding to the four standards, but the relative content is not constant. The fingerprints of almost all samples show a similar sequence of bands and can be identified as that of green tea.

All samples show a strong band corresponding to epigallocatechin gallate and a faint one corresponding to epicatechin. The content of epigallocatechin is high in some of the Chinese, all Japanese, and Indian samples. Epicatechin gallate is somewhat fainter.

Sample on track 9 shows almost no epicatechin gallate and epicatechin. Sample on track 10 shows only very faint epigallocatechin and high epicatechin gallate.

The samples from China show greater variation than the samples from Japan and India. The Japanese sample on track 22 is totally different due to the roasting process.

##### **Images:**



1. Epigallocatechin gallate, epigallocatechin, epicatechin gallate, epicatechin
2. Lung Ching Grade 1.

3. Mingqian Green Tea
  4. Longjing Green Tea
  5. Chinesischer Sencha Green Tea
  6. Pi Lo Chun
  7. Green Tea Gunpowder "Temple of Heaven"
  8. Yong Xi Hou Qing Green Tea
  9. Pao Chung Pouchong
  10. Thé vert au Jasmin
  11. Epigallocatechin gallate, epigallocatechin, epicatechin gallate, epicatechin
  12. Gyokuro.
  13. Japan Sencha Tea
  14. Special Japan Sencha
  15. Bancha
  16. Bancha
  17. Green tea powder, Japan
  18. Green Tea, Japan
  19. Sencha Green Tea
  20. Sencha Green Tea with citronella
  21. Japan Green Tea with rose and Jasmin flowers
  22. Hoji Cha, geröstet, Japan
  23. Java Green Tea
  24. Epigallocatechin gallate, epigallocatechin, epicatechin gallate, epicatechin
  25. Indian green tea #1
  26. Indian green tea #2
  27. Special Assam India Green Tea STGFOP 1
- B: Blank

**Accepted: YES**

**Fail: samples on tracks 9, 10, and 22.**

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### 5.3.2 Detection of adulteration

#### Description of experiment:

Authenticated adulterants (in this case other preparations of *Camellia sinensis*, such as Oolong, Black, and Red Tea) are prepared according to section 4.1. The BRM and chemical references are prepared according to section 4.2. All samples are applied onto the same plate according to section 4.5. Following chromatography (section 4.7) and derivatization (section 4.8) the plate is documented (section 4.9) and the results compared to those shown in section 4.10.

#### Acceptance criteria:

The method is specific for Green Tea (*Camellia sinensis*) if the fingerprints of other Tea preparations (Black, Red, Oolong Tea) are significantly different from those of the BRM with respect to number, position, color, and intensity of bands.

#### Results:

Oolong Tea (tracks 2-6): epicatechin gallate and epicatechin are very faint. Profiles resemble either Green Tea or Black Tea.

Chinese Black Tea (tracks 8-11): show almost no polyphenols.

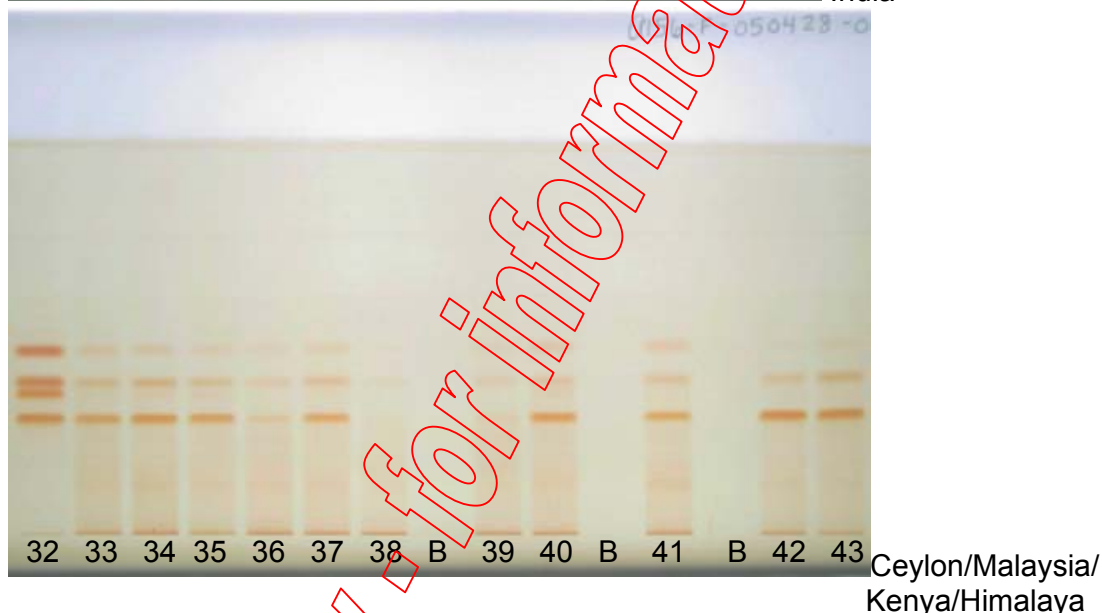
Smoked Black Tea samples (tracks 12 and 13): almost no polyphenols

Red Tea (14-17): the fermented samples show almost no polyphenols, the unfermented Red Tea (track 17) shows strong bands. Samples on tracks 15 and 16 show epicatechin.

Other Black Tea (tracks 18-31, 33-43): epigallocatechin is very faint, but the other polyphenols are present in most of the samples.

Green Teas show a different ratio of the polyphenols than the Black Teas.





1. Epigallocatechin gallate, epigallocatechin, epicatechin gallate, epicatechin
2. Formosa Topest Superieur Fancy Oolong Tea "Butterfly of Taiwan
3. Oolong Tee (halbfermentiert) mit Jasminblüten
4. Finest Spring Jade Oolong Tea
5. Gaoshan Oolong Tea China
6. Oolong Tea
7. Yunnan Imperial Tea Black Tea
8. Superieur Yunnan China Black Tea
9. Superieur Keemun Ning Hong Jing Hao China Black Tea
10. Keemun China Black Tea
11. Yunnan- and Assam Pekoe. China and India
12. Lapsang Souchong Tea China Smoken Black Tea
13. Pure Tarry Lapsang Souchong Tea China Smoken Black
14. King of Pu-Erh-Tea China Red Tea. Storage 60 years
15. Pu-Erh-Tea China Red Tea. Storage 25 years
16. Pu-Erh Orange. Storage 25 years



17. Pu-Erh Tuo Cha China Red Tea (no fermentation). Storage 20 years
  18. Darjeeling SOOM STGFOP 1 First Flush
  19. Darjeeling Castleton Muscatel STGFOP 1
  20. TUMSONG Second Flush Darjeeling
  21. Badamtan Black Tea 2004 India
  22. Chamong Black Tea 2004 India
  23. India Black Tea
  24. Assam Shantipur Black Tea 2004
  25. Hatimara Assam Tea FTGFOP1 India Black Tea
  26. Doomur Black Tea India
  27. Melong Black Tea India
  28. Darjeeling Tea
  29. Assam Tea
  30. Darjeeling Tea
  31. Lemon flavored Black tea
  32. Epigallocatechin gallate, epigallocatechin, epicatechin gallate, epicatechin
  33. Ceylon Black Tea
  34. Extra Choicest Ceylon Pekoe Black Tea
  35. Ceylon Black Tea
  36. Fine Ceylon Fannings Black Tea
  37. Ceylon Flowery Orange Pekoe Black Tea
  38. Royal Ceylan (Black tea)
  39. Boh Cameronian Garden Tea (Black tea) Malaysia Tea
  40. Boh Cameronian Garden Tea (Black tea) Malaysia Tea
  41. Kenya GFBOP 1 Marynin Black Tea
  42. Golden Nepal Tea Maloom FTGFOP 1 Himalaja Black Tea
  43. Sikkim Temi FTGFOP1 Himalaja Black Tea
- B: Blanks

**Accepted: YES. Black and Red Tea can be distinguished; Oolong Tea may be difficult to identify.**

### 5.3.3 Identification of processed materials and finished products

#### Description of experiment:

Samples of extracts and/or finished products are prepared according to section 4.1. The BRM of *Camellia sinensis* and chemical references are prepared according to section 4.2. The excipients are prepared in similar concentration as in the finished product. All samples are applied onto the same plate according to section 4.5. Following chromatography (section 4.7) and derivatization (section 4.8) the plate is documented (section 4.9) and the results compared to those shown in section 4.10.

#### Acceptance criteria:

The method is suitable for the identification of processed materials of Green Tea (*Camellia sinensis*) if the fingerprints of the samples are similar to that shown in section 4.10 of the method with respect to number, position, color, and intensity of bands matching the profile of the BRM.

The fingerprints of the excipients don't show any zone that interferes with the fingerprint of the BRM.

#### Results:

The extract on track 7 shows the same profile as the BRM. The Ice tea powder on track 7 shows zones for all standards, however the epigallocatechin zone is very faint. This profile resembles more some of the Chinese and Japanese Green Tea samples seen above (5.3.1). The excipients of the Ice tea powder don't interfere with the detection of the polyphenols.



1. (-)-Epigallocatechin gallate
2. (-)-epicatechin gallate
3. (-)-epigallocatechin
4. (-)-epicatechin
5. *Camellia sinensis* folium (BRM)
6. *Camellia sinensis* folium extrakt siccum
7. Ice tea powder, green tea + vitamine C+E, 4.5% green tea extract
8. Citric acid
9. Saccharose
10. Fructose

**Accepted: YES**

## 5.4 Repeatability

### Description of experiment:

Three portions of the BRM are individually prepared according to section 4.2. Onto three 20x10 cm plates, three aliquots of 2  $\mu$ L of each reference solution are applied according to section 4.5. The plates are chromatographed (section 4.7) subsequently using the same chamber but fresh portions of the developing solvent and fresh filter paper. The plates are derivatized and documented according to 4.8-4.9.

The results across each plate and from plate to plate are evaluated. The average Rf values of the four polyphenols are determined for each track on each plate.

### Acceptance criteria:

The Repeatability of the method is acceptable if:

All fingerprints on each plate are identical with respect to number, position, color, and intensity. Across each plate the zones – due to the same compounds – form parallel lines with no disturbance (waves or curves) **and**

The Rf values for each of the four zones on the three plates don't vary more than 0.02.

### Results:

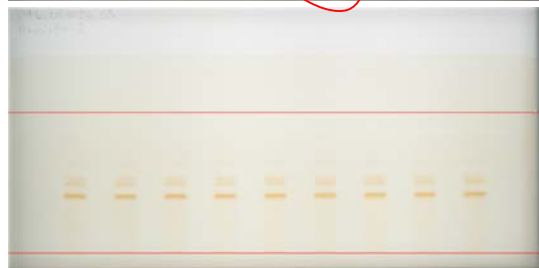
All chromatograms look very similar with respect to number, position, color, and intensity of zones. No disturbances are seen.

Rf	P76_050126_02	P76_050126_03	P76_050126_04	$\Delta$ Rf
epicatechin	0.62	0.64	0.63	0.02
epigallocatechin	0.51	0.53	0.52	0.01
epicatechin gallate	0.48	0.49	0.48	0.01
epigallocatechin gallate	0.39	0.40	0.40	0.01

### Images:



P76\_050126\_02



P76\_050126\_03



P76\_050126\_04

**Accepted: YES**

### **5.5 Intermediate precision**

#### **Description of experiment:**

Repeat the experiment described under 5.4 on 2 other days.

The average Rf values of the four polyphenols are determined for each track on each plate (one plate prepared during experiment 5.4, 2 plates on different days) and variations from plate to plate are evaluated.

#### **Acceptance criteria:**

The intermediate precision of the method is acceptable if:

All fingerprints on each plate are identical with respect to number, position, color, and intensity. Across each plate the zones – due to the same compounds – form parallel lines with no disturbance (waves or curves) **and**

The average Rf values for each of the four zones on the three plates do not vary more than 0.05.

#### **Results:**

All chromatograms look very similar with respect to number, position, color, and intensity. No disturbances are seen.

Rf	P76_050126_02	P76_050127_01	P76_050128_01	$\Delta$ Rf
epicatechin	0.62	0.6	0.59	0.03
epigallocatechin	0.51	0.5	0.49	0.02
epicatechin gallate	0.48	0.47	0.45	0.03
epigallocatechin gallate	0.39	0.38	0.37	0.02

**Images:**



P76\_050126\_02



P76\_050127\_01



P76\_050128\_01

**Accepted: YES**

**5.6 Reproducibility**

**Description of experiment:**

The confirmatory lab repeats the experiment described under 5.4 (Repeatability).

**Acceptance criteria:**

The reproducibility of the method is acceptable if:

All fingerprints on each plate are identical with respect to number, position, color, and intensity. Across each plate the zones – due to the same compounds – form parallel lines with no disturbance (waves or curves) **and** the average Rf values for each of the four zones on the three plates don't vary more than 0.02.

The reproducibility is acceptable if the Rf obtained in this test are not significantly different from those in obtained in section 5.4 ( $<0.05$  if using plate from the same manufacturer,  $<0.07$  for plates of different manufacturers).

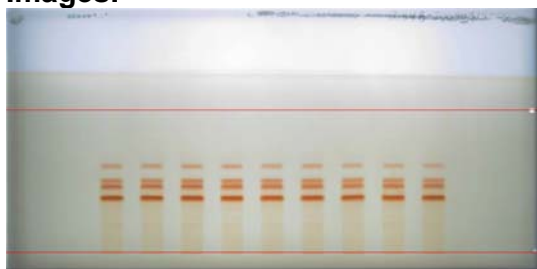
**Results:**

All chromatograms developed by the confirmatory lab look very similar with respect to number, position, color, and intensity of zones. No disturbances are seen.

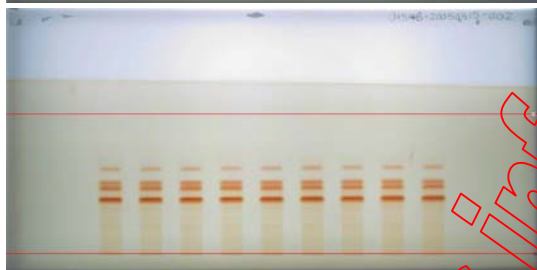
The color/brightness of the image differs slightly from the image made in the primary lab. This could be due to different documentation devices and settings.

Rf	A154B-2005031-5-001	A154B-2005031-5-002	A154B-2005031-5-003	$\Delta Rf$	P76_050126_02 (Comparison)	$\Delta Rf$
epicatechin	0.61	0.60	0.61	0.02	0.62	0.02
epigallocatechin	0.50	0.51	0.51	0.01	0.51	0.01
epicatechin gallate	0.45	0.46	0.47	0.02	0.48	0.03
epigallocatechin gallate	0.39	0.38	0.38	0.01	0.39	0.01

**Images:**



A154B-20050315-001



A154B-20050315-002



A154B-20050315-003



P76\_050126\_02 (Comparison)

**Accepted: YES**

## **5.7. Robustness**

### **5.7.1 Chamber type**

#### **Description of experiment:**

The method is executed according to section 4 using the BRM. Instead of a Twin Trough Chamber a Horizontal Chamber (sandwich configuration) of comparable size is used.

#### **Acceptance criteria:**

The fingerprints obtained in both chambers are similar with respect to number, position, color, and intensity of zones. The Rf values obtained in this test are not significantly different from those described in section 4.10 ( $<0.05$ ). In the case of differences between the results the use of a Horizontal Chamber (sandwich configuration) must be excluded.

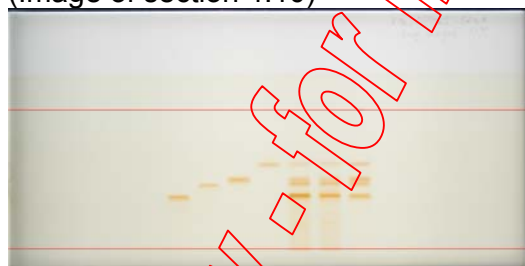
#### **Results:**

No significant difference is seen when the plate is developed in a Horizontal Chamber (sandwich configuration). Slightly higher Rf values are observed.

Rf	Image 4.10	P76_050125_02	$\Delta$ Rf
epicatechin	0.62	0.66	0.04
epigallocatechin	0.51	0.54	0.03
epicatechin gallate	0.48	0.51	0.03
epigallocatechin gallate	0.38	0.42	0.04

#### **Images:**

Twin Trough Chamber  
(Image of section 4.10)



Horizontal Chamber (sandwich configuration)



**Accepted: YES**

### 5.7.2 Developing distance

#### Description of experiment:

The method is executed according to section 4 using only the BRM.  
The developing distance is increased to 70 mm from the lower edge of plate.

#### Acceptance criteria:

The fingerprints obtained with different developing distances are similar with respect to number, position, color, and intensity of zones. The Rf values obtained in this test are not significantly different from those described in section 4.10 ( $<0.05$ ). In the case of differences between the results the developing distance of more than 60 mm yields invalid results.

#### Results:

The separation is not affected by the increased developing distance

Rf	Image 4.10	P76_050125_04	$\Delta Rf$
epicatechin	0.62	0.62	0.00
epigallocatechin	0.51	0.51	0.00
epicatechin gallate	0.48	0.48	0.00
epigallocatechin gallate	0.38	0.39	0.01

#### Images:

60 mm

70 mm

(Image of section 4.10)



Accepted: YES

### 5.7.3 Waiting times

Because the sample is stable on the plate, in solution, and during chromatography, and the derivatization is not critical, this experiment was not performed in this example. For details see section 5.2.1-5.2.3.



### 5.7.4 Relative humidity

#### Description of experiment:

Six plates are prepared according to section 4 using only one BRM. Prior to chromatography (4.6), the plates are conditioned over salt solutions or using a molecular sieve for adjusting different relative humidity. Relative humidity covering a range of about 10-70% rH should be tested.

Alternatively, results of plates developed under different relative humidity are compared.

#### Acceptance criteria:

The fingerprints obtained under different relative humidity are similar with respect to number, position, color, and intensity. In this case the relative humidity does not affect the result. In the case of differences of the results, the method may require the control of relative humidity.

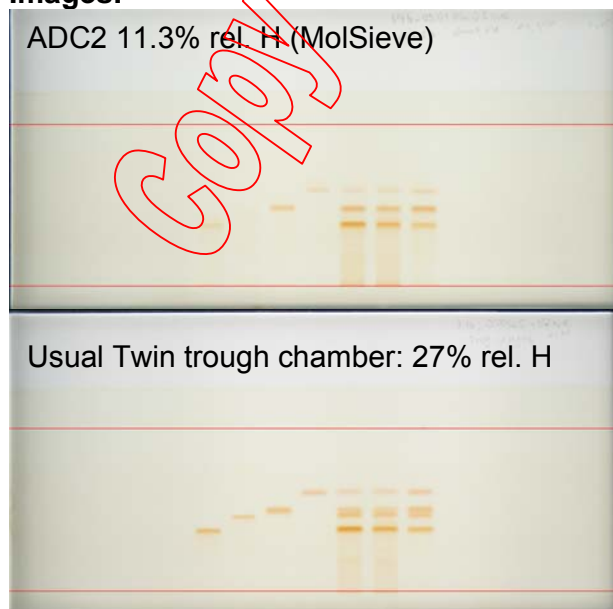
#### Results:

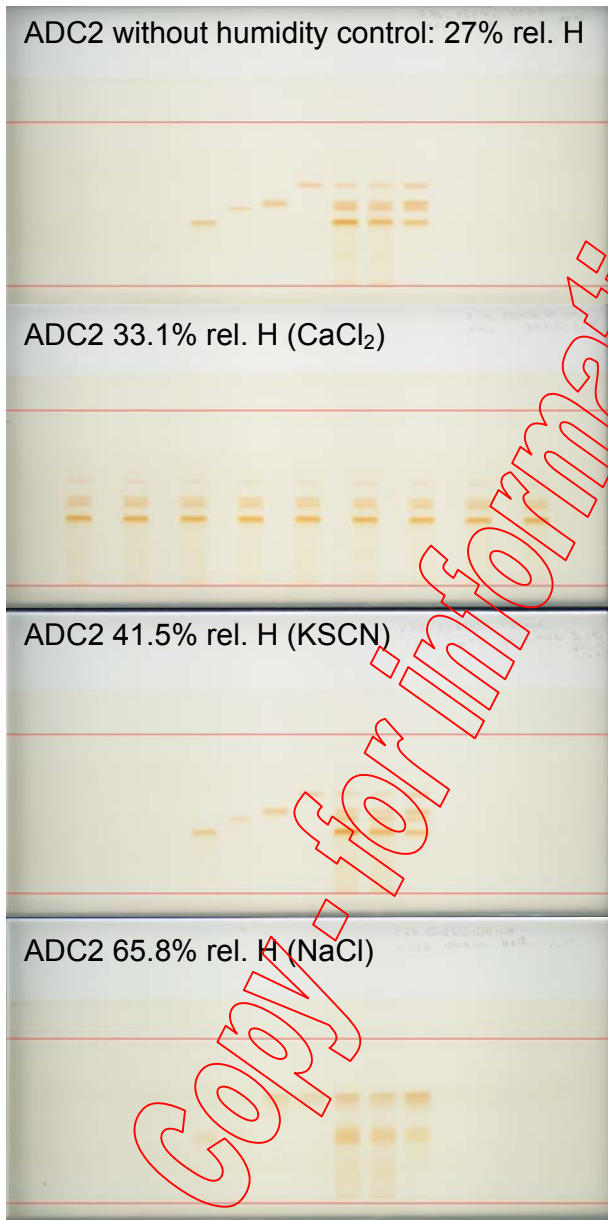
This experiment was performed in a prototype of an automatic developing chamber with humidity control (ADC2 prototype).

Only small variations in Rf values are seen at medium rel. humidity (27-42%). Band broadening and separation difference are seen at low humidity (11%) and high humidity (>66%). The chromatogram should not be developed without humidity control when the surrounding relative humidity is below 25% or over 50%.

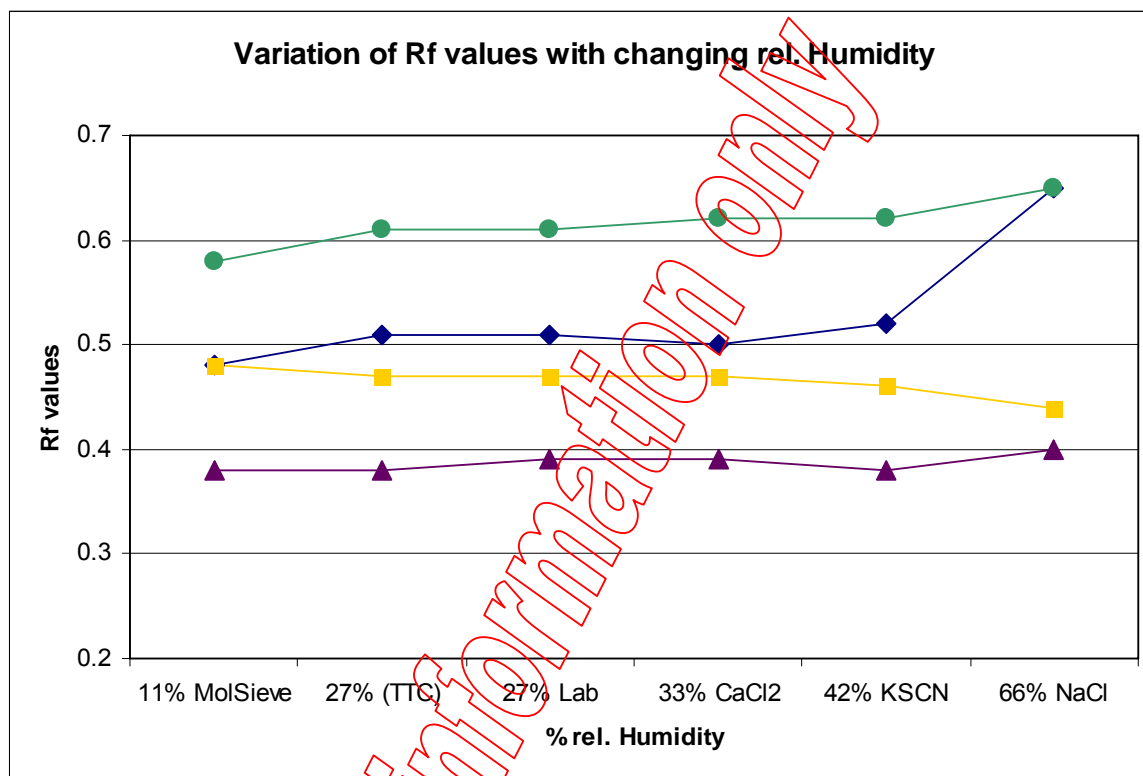
Rf	11% MolSieve	27% (TTC)	27% Lab	33% CaCl2	42% KSCN	66% NaCl
epicatechin	0.58	0.61	0.61	0.62	0.62	0.65
epigallocatechin	0.48	0.51	0.51	0.5	0.52	0.65
epicatechin gallate	0.48	0.47	0.47	0.47	0.46	0.44
epigallocatechin gallate	0.38	0.38	0.39	0.39	0.38	0.40

#### Images:





Copy for information only



**Accepted: YES**

**6. Conclusions, Approvals, and Signatures**

**6.1 Conclusions of primary lab**

Date:

Analyst of primary lab:

**6.2 Conclusions of substantiating lab**

Date:

Analyst of substantiating lab:

**6.3. Final approval of study director**

Date:

Study director: