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Proceeding Paper

Validation of an HPLC Method for the Determination of Epigallocatechin Gallate, Caffeine and Chlorogenic Acid in Coffee Leaves †

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Abstract: An existing reference HPLC method (ISO 14502-2) was applied and validated for the matrix of coffee leaves (genus *Coffea*), which can be used to prepare a tea-like infusion. This method is usually used to measure conventional *Camellia sinensis* teas with high-performance liquid chromatography (HPLC). For the purposes of method validation, robustness, applicability, measurement precision and linearity were investigated. The focus was kept on the ingredients epigallocatechin gallate (EGCG), caffeine and chlorogenic acid. It was possible to determine a high level of robustness, applicability and measurement precision of this method through the measurement of caffeine and chlorogenic acid in authentic sample matrices. The repeatability standard deviation of caffeine was lower than 0.09 g/100 g, and that of chlorogenic acid was lower than 0.08 g/100 g. The repeatability coefficient of variation (CV) of caffeine ranged between 0.93% and 4.52%, and that of chlorogenic acid was between 1.20% and 21.10%. The reproducibility standard deviation of caffeine was lower than 0.03 g/100 g, and that of chlorogenic acid was lower than 0.03 g/100 g. The reproducibility CV of caffeine was between 0.68% and 2.92%, and that of chlorogenic acid was between 0.91% and 5.36%. These results show a high level of robustness and measurement precision in each case. However, the used samples were unsuitable for determining the robustness, applicability and measurement precision of EGCG because its amount in coffee leaves was too low or even undetectable. A detection limit (LOD) for EGCG in coffee leaf of 0.02 g/100 g, and a quantification limit (LOQ) of 0.04 g/100 g were determined. Overall, this method was judged as suitable for the regulatory analysis of coffee leaves.

Keywords: coffee byproducts; coffee leaves; infusion; tea; analysis; HPLC; food control; novel food; caffeine; chlorogenic acid; epigallocatechin gallate

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1. Introduction

The leaves of the coffee plant have long been used in coffee-origin countries for the preparation of tea-like beverages [1]. Especially in West Sumatra, Ethiopia, Jamaica, Yemen, India, Java and South Sudan, the infusion is consumed as a traditional food [2,3]. Coffee leaves can be processed, e.g., by steaming, rolling, fermenting and drying the leaves. So-called coffee leaf tea is an infusion beverage where the tea-like beverage is obtained by infusing it with hot water. It is usually made from the leaves of the coffee species *Coffea arabica* or *Coffea canephora*. Depending on processing, the taste of coffee leaf infusion can be compared to those of black tea and green tea. It has a slightly sweet note and is not particularly bitter. The color of this drink is usually green-brownish. The smell of coffee leaf infusion is similar to that of green tea [4].

So-called coffee leaf tea is a tea-like infusion drink approved in the European Union (EU) since July 2020 [5]. During this approval, the EU set the limits for some ingredients, based on an EFSA assessment, as follows: chlorogenic acid < 100 mg/L, caffeine < 80 mg/L and epigallocatechin gallate (EGCG) < 700mg/L [6].

This work is about testing the applicability of a method for measuring coffee leaves using high-performance liquid chromatography (HPLC). The existing method ISO 14502-2 [7] is usually used for the determination of green tea. The method is suitable, in principle, for coffee leaves as well [8], and this study provides a full method validation for this matrix. For this purpose, the focus is on the ingredients chlorogenic acid, epigallocatechin gallate and caffeine, as these are the major ingredients with possible human health risk [9].

2. Materials and Methods

In principle, the procedures and analyses were always carried out according to the ISO 14502-2 standard [7]. During this work, some deviations from the standard were deliberate, e.g., to determine the robustness of the method under varying conditions.

2.1. Preparation of the Samples

For the preparation, dried, ground and homogenized coffee leaf samples were always used, which were stored in air- and light-impermeable bags. This ensures that the ingredients of the samples do not change due to these factors. First, 0.200 g of the coffee leaf sample was weighed into an extraction tube with a screw cap, and the weight was noted. Methanol/water mixture (70 + 30, *v*/*v*) in the dosing unit was tempered to 70 °C for at least 30 min in a water bath. The extraction tube with the sample was then also briefly placed in the water bath, 5 mL of the methanol/water mixture was added to the sample, and the extraction tube was closed and mixed on the vortex mixer. The sample was then placed back in the water bath and mixed again on the vortex mixer after 5 min. The sample was again placed in the water bath for 5 min and then mixed again on the vortex mixer. Afterwards, the sample was cooled down to room temperature and centrifuged for 10 min at 3500/min. The supernatant (the extract) was decanted into a 10 mL volumetric flask, and the extraction procedure was repeated with the remaining sample in the extraction tube. At the end, the second extract was also added to the 10 mL volumetric flask. The 10 mL volumetric flask was filled with the methanol/water mixture. In all, 1.0 mL of the extract from the 10 mL volumetric flask was pipetted into another extraction tube with screw cap using a piston-stroke pipette. Further, 4 mL of the stabilizing solution (10% acetonitrile with 500 µg/mL ethylenediaminetetraacetic acid (EDTA) and 500 µg/mL ascorbic acid) was added with a multipipette. This solution was filtered through a 0.45 µm membrane filter using a 3 mL syringe, placed in an HPLC vial and sealed with a lid. This solution was then submitted for measurement by HPLC.

2.2. HPLC

An Agilent (Waldbronn, Germany) HPLC 1200 Series with diode array detector (DAD detector) was used, including degasser G1379B, pump G1312A, column oven G1316A and autosampler G1329A. A polar reversed phase (RP) separation column from Phenomenex (Aschaffenburg, Germany) was used, the Synergy Polar/RP separation column with dimensions 250 mm × 2 mm and a particle size of 4 µm. In addition to the polar RP column, a C18 RP column, AJ0-4286, 4/2 mm from Phenomenex was used as a pre-column. These columns were thermostated to 35 °C during the measurement. Mobile phase A, 9% acetonitrile, 2% acetic acid with 20 µg/mL EDTA. Mobile phase B, 80% acetonitrile, 2% acetic acid with 20 µg/mL EDTA. The gradient was 100% A (0–10 min), 100% > 68%A (10–25 min), 68% A (25–35 min), 68% > 100% A (35–40 min). Further measuring conditions: flow at 0.2 mL/min, and injection volume at 5 µL. The evaluation of the measurement was carried out via the chromatogram, which was recorded at the measuring wavelength of 278 nm. To identify the individual substances, the spectra of the sample were compared with the spectra from a reference standard library.

2.3. Data Evaluation

When evaluating the chromatograms of the samples, first, the peaks were identified according to their retention time. Furthermore, the peaks (especially in the case of uncertainties) were evaluated and identified with the help of the spectra. With the help of the retention times and the spectra, a reliable qualitative statement can be made about the samples. For the quantitative evaluation of the HPLC measurements, the areas of the individual peaks were determined by the HPLC software. Additionally, an external calibration series with caffeine was also measured for each series of measurements. From the peak areas of the external calibration series and the concentrations of these calibration points, a calibration curve and a linear function were determined by the HPLC software. With the help of the slope and intercept of the function, the sample weight, the dilution steps, the respective peak areas and a relative response factor (RRF) value, the respective content of a substance in the sample, can be calculated. The RRF indicates the ratio of a peak area of an investigated substance in relation to the peak area of caffeine. This allows peaks of other substances to be quantitatively evaluated with the aid of a calibration curve in which only caffeine was used as an external standard.

3. Results

3.1. Method Validation Results

3.1.1. Extraction Temperature

The samples were each processed according to Section 2.1; only the temperature of the water bath was varied. Each standard sample was processed once with water bath temperatures of 50 °C, 60 °C, 65 °C, 75 °C and 80 °C (instead of 70 °C). The water bath temperature was not increased further due to concerns about exceeding the boiling temperature of the methanol–water mixture. After sample preparation, each extract was analyzed once by HPLC according to Section 2.2. No significant difference was found in the measurement results. Only at a water bath temperature of 50 °C was less chlorogenic acid detected. This is probably because chlorogenic acid is only completely extracted at higher temperatures (>50 °C). However, this difference was not significant. Thus, a change in the temperature of the water bath during extraction does not lead to a significant change in the measurement results. Nevertheless, a dark discoloration suggests a change in some ingredients. However, no clearly discernible differences were observed in the HPLC chromatograms. It is possible that some components are altered by the temperature change of the water bath. However, these have no effect on the chromatograms as they are removed by the membrane filtration.

3.1.2. Number of Extractions

The number of extractions was varied (1×, 2×, 4×). The aim was to determine whether the usual number of extractions (2×) is sufficient for the sample matrix coffee leaf. It was confirmed that a double extraction is sufficient, since more extractions do not extract significantly more of the substances. For EGCG, the measurement signal thus falls even further below the limit of detection, and the measurement thus becomes more inaccurate at 4× extraction.

3.1.3. Extraction Time

The extraction time, i.e., the time the sample solutions are exposed in the 70 °C water bath, was varied. The samples were extracted for 2 × 2 min, 2 × 7 min and 2 × 10 min (instead of 2 × 5 min) in the water bath per extraction, respectively. Different extraction times have no significant influence on the measurement results. This means that measurement results obtained in sample preparations where the extraction time differs are comparable with each other.

3.1.4. Sample Stabilization

This experiment aimed to determine how deviations in the preparation of the stabilizing solutions affect the measurement results. This was to determine the robustness of the method in the event of any deviations from the specification, e.g., due to errors on the part of the technical personnel. The extracts were each diluted in stabilizing solutions with different concentrations. The concentrations were:

- High EDTA concentration: 0.6 g/L EDTA;
- High ascorbic acid concentration: 0.6 g/L ascorbic acid;
- High acetonitrile concentration: 12% acetonitrile;
- Low EDTA concentration: 0.4 g/L EDTA;
- Low ascorbic acid concentration: 0.4 g/L ascorbic acid;
- Low acetonitrile concentration: 8% acetonitrile;
- Standard stabilizing solution: 0.5 g/L EDTA and ascorbic acid, 10% acetonitrile.

The deviations in the stabilizing solutions did not have a significant influence on the measurement results.

3.1.5. Stability of Extract

In this experiment, it was determined how long the extract of the samples obtained after extraction can be kept in the refrigerator (4 °C). It was also determined whether the extracts of the samples can be kept for a longer period in the refrigerator and how this storage affects the measurement result quantitatively and qualitatively. The extracts obtained in the sample preparation were diluted with the stabilizing solution on the same day, after 3 days, after 7 days and after 2 weeks, respectively. No decay of the ingredients could be observed in the extracts, even those stored in the refrigerator at 4 °C for 14 days.

3.1.6. Linearity

Internal (addition method) and external calibration curves were established. The method was found to have sufficient linearity for the determination of all compounds in coffee leaves ($R^2 > 0.998$ in all cases). Furthermore, it could be observed that the internal and external calibration curves of EGCG and caffeine, which were created, overlap very strongly. Thus, it can be assumed that these substances can be almost completely extracted during the processing of coffee leaves according to this method, and thus, losses or decomposition of these substances are not expected. When determining the linearity of chlorogenic acid, it was observed that less of the substance could be detected in the internal calibration curves than in the external calibration curves. Thus, it can be assumed that chlorogenic acid cannot be completely extracted when coffee leaves are processed by this method. The portion, which is not extracted, is lost or decomposes in this case ranges between 0.05 and 0.262 g/100 g. Generally, however, this method shows high linearity in the determination of caffeine, EGCG and chlorogenic acid in coffee leaves.

3.1.7. Limit of Detection

For EGCG, an internal calibration curve with very low concentrations was created to determine the limit of detection (LOD). An LOD of 0.7 mg/L was determined (at 3 times the signal/noise ratio), which corresponds to an EGCG content of 0.02 g/100 g. The limit of quantification (LOQ) of EGCG results in 1.7 mg/L (at 9 times the signal/noise ratio), corresponding to an EGCG content of 0.04 g/100 g.

3.1.8. Precision and Applicability

Each sample was repeatably measured by HPLC, with a time interval of several hours, which was followed by the same series of measurements under repeatability conditions conducted by four different operators on several different days (Table 1). This method has a high measurement precision in the analysis of coffee leaves. Especially in the determination of caffeine and chlorogenic acid, this can be well demonstrated. However, it is difficult to make a well-founded statement about the measurement precision of the method in the

determination of EGCG, since the EGCG contents of the coffee leaf samples are in a very low range. However, it must then be questioned how relevant a high measurement precision is at such low levels, since the limit set by the EU is much higher (700 mg/L infusion).

Table 1. Precision of HPLC measurement of 4 samples of coffee leaves.

Coffee Leaf Sample	Sample #1	Sample #2	Sample #3	Sample #4
(a) Repeatability conditions	(n = 22)	(n = 22)	(n = 22)	(n = 15)
Repeatability standard deviation, caffeine (g/100 g)	0.09	0.02	0.03	0.01
CV, caffeine	3.74%	2.35%	4.52%	0.93%
Repeatability standard deviation, EGCG (g/100 g)	0.00	0.01	0.01	0.00
CV, EGCG	n/a	111.16%	64.86%	12.84%
Repeatability standard deviation, chlorogenic acid (g/100 g)	0.00	0.08	0.07	0.02
CV, chlorogenic acid	21.10%	9.27%	8.93%	1.20%
(b) Intra-laboratory reproducibility conditions	(n = 16)	(n = 16)	(n = 16)	(n = 16)
Reproducibility standard deviation, caffeine (g/100 g)	0.03	0.02	0.02	0.01
CV, caffeine	1.08%	2.92%	2.81%	0.68%
Reproducibility standard deviation, EGCG (g/100 g)	0.00	0.01	0.01	0.00
CV, EGCG	n/a	138.81%	235.24%	30.99%
Reproducibility standard deviation, chlorogenic acid (g/100 g)	0.00	0.03	0.01	0.02
CV, chlorogenic acid	5.36%	2.95%	1.58%	0.91%

CV: Coefficient of variation.

The applicability of the method was found to be sufficient as demonstrated by precise results determined by four different technicians, independent of slight variations in handling and setup.

4. Discussion

The repeatability standard deviation of caffeine was lower than 0.09 g/100 g, and that of chlorogenic acid was lower than 0.08 g/100 g. The repeatability coefficient of variation (CV) of caffeine ranged between 0.93% and 4.52%, and that of chlorogenic acid was between 1.20% and 21.10%. The reproducibility standard deviation of caffeine was lower than 0.03 g/100 g, and that of chlorogenic acid was lower than 0.03 g/100 g. The reproducibility CV of caffeine was between 0.68% and 2.92%, and that of chlorogenic acid was between 0.91% and 5.36%.

When determining the linearities of the individual substances, no abnormalities could be observed. It was possible to demonstrate a high linearity for all substances when the calibration curves were generated.

It was not possible to meaningfully and reliably verify the applicability of the method in the determination of EGCG in coffee leaves, as no sufficient amount of EGCG could be detected in any sample. The EGCG content of the samples, which could be detected in the individual measurements to determine the robustness, measurement precision and applicability, is in each case below the detection limit or determination limit. Thus, a reliable statement about the robustness, measurement precision and applicability is not possible in this case. However, in this case, a determination of the robustness, measurement precision and applicability of the method is not necessary for measuring EGCG in coffee leaves, because in this sample matrix, according to the current knowledge, no, or only a very low EGCG content is to be expected [4]. Thus, it is only of minor importance how precisely this substance can be detected. Additionally, the analysis of coffee leaves is only intended

to check whether the samples exceed the limit, and this is set relatively high for EGCG. Generally, this method has high robustness, measurement precision and applicability in the determination of caffeine and chlorogenic acid in coffee leaves.

When processing the samples, care must be taken to maintain the temperature of the water bath, the number of extraction steps and the ascorbic acid concentration of the stabilizing solution (in practice, however, this does not usually pose any problems). It was found that the higher the water bath temperature during extraction, the darker the extract. This is probably because some ingredients change at higher temperatures. However, this has no influence on the measurement and on the results, as no significant changes were observed here. Nevertheless, attention should be paid to the temperature during extraction to obtain comparable results.

Furthermore, comparable results are produced with an extraction time between 2 × 2 min and 2 × 10 min; the extracts can be stored at 4 °C for at least 14 days and do not noticeably change, and a slight change in the EDTA and acetonitrile concentration of the stabilizing solution does not have a major effect on the measurement results.

No detection limit could be determined for the substances of caffeine and chlorogenic acid. This was because these substances are always present in this sample matrix. Thus, no usable blank sample could be found, which would have been necessary for the determination of the LOD and LOQ. It would have been possible to determine these with a different sample matrix, such as a fruit tea. However, this would not allow for the influence of the sample matrix of the coffee leaves on the individual substances, which would have produced a result that would not have been informative. Furthermore, the determination of the LOD of chlorogenic acid and caffeine was omitted, since these substances (especially caffeine) are usually present in larger amounts in coffee leaves. Additionally, caffeine and chlorogenic acid contents are rather irrelevant in the low range, since the limits specified by the EU are significantly higher for these substances. In comparison, the LOD of EGCG was needed to better reflect the measurement results in determining robustness, applicability and measurement precision. This was not necessary for chlorogenic acid and caffeine.

5. Conclusions

In conclusion, the method used for the analysis of coffee leaves is suitable and can be applied in the future to regulatorily analyze EGCG, chlorogenic acid and caffeine in coffee leaves qualitatively and quantitatively.

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