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Analysis of Functional Compounds in Matcha by Quantitative Nuclear Magnetic Resonance Spectroscopy

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Abstract: Matcha is reported to have high content of some bioactive components such as catechins, theanine, and caffeine, and its consumption is increasing worldwide. Several analytical methods have been established for matcha powder and bioactive compounds, but most of them are based on HPLC methods. This study focused on NMR as an analytical method for simple quantitative analysis of the functional components of matcha. The analytical conditions were established by preparing extract and solvent fractions, evaluating hygroscopicity, and examining quantitative NMR parameters. The analytical performance was evaluated, and the developed analytical condition was also applied to matcha powder by directly mixing in NMR solvent without pre-extraction. Caffeine, epicatechin, epicatechin-3-*O*-gallate, epigallocatechin-3-*O*-gallate, epigallocatechin, gallocatechin-3-*O*-gallate, and gallocatechin were quantified. Analysis of matcha and normal green tea powder suggested the possibility of distinguishing between matcha and green tea powder by the ratio of caffeine content and total catechins content. The qNMR method can be adopted for the simple analysis of the amount of caffeine and catechin compounds in the powders and extracts.

Keywords: matcha; green tea; *Camelia sinensis*; epigallocatechin gallate; caffeine; qNMR

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1. Introduction

The young leaves of the tea plant (*Camellia sinensis* (L.) Kuntze, Syn.: *Thea sinensis* L., Theaceae) were used to prepare various tea formulations ancient times [1–5]. Tea formulations are consumed not only as a simple drink, but also for their health beneficial effects due to presence of various bioactive compounds such as caffeine, catechins, and amino acids [1,3]. Commonly available tea formulations such as green, oolong, black, etc., vary according to their processing methods [6]. Another such tea formulation that is gaining popularity in recent years is matcha. For the production of matcha, tea plants are cultivated under shade (about 90% shade) by covering the cultivation areas/fields with different materials [1,7]. Then, the tea leaves are picked, washed, dried, and then ground using stone mills to make the powder known as matcha [8]. It has been reported that the catechins and caffeine content of matcha are different from those of green tea and other teas due to the unique cultivation method and processing of matcha [1]. Matcha powder, its extracts, and isolated compounds have shown some promising biological activities in in vitro and animal studies [1,7,9–11]. There is also an increasing demand in the market for their use in various beverages and snacks. However, the quality of matcha and the quantity of the components in matcha powder differs among the commercial samples, and

it is difficult to standardize. Various analytical methods are reported, but most of them are based on HPLC [3]. However, a reference standard, which is either the analyte itself or that has a known response factor relative to the analyte, is necessary for HPLC. This can often work to the disadvantage of cost and measurement time. There are some counterfeit matcha products in the market, and it has been confirmed that powdered green tea, rooibos tea, and other teas are being labeled as matcha [12]. In order to prevent the distribution of such inferior or counterfeit products, the International Organization for Standardization (ISO) has issued a technical report on the definition of matcha (ISO TR 21380) [13].

In recent years, quantitative nuclear magnetic resonance (qNMR) has been attracting attention as a method for the quantitative analysis of organic compounds. As described in the Japanese Pharmacopoeia [14], qNMR is used as a test method for reagents and test solutions used in crude drug purity tests. qNMR is also used to cross-check the mass balance method of certified reference material (CRM) purity calculation. qNMR has been applied and widely used for high purity compounds and for the main components of the mixtures. There are only a few methods and reports that target the analysis of not only the main components, but also multiple components and trace constituents from mixtures and extracts. qNMR has been used for screening for counterfeited corticosteroids in topical applications [15], quality evaluation of *Cordyceps sinensis* [16], and many other plant extracts [17]. ^{1}H-NMR-based profiling has been reported for various tea varieties in recent years [18–20]. Napolitano et al. have reported qNMR and LC-MS/MS based analytical methods for green tea catechins [21]. In this study, we aimed to develop a method for the analysis of main components (Figure 1) of matcha extract, fractions, and powder using qNMR.

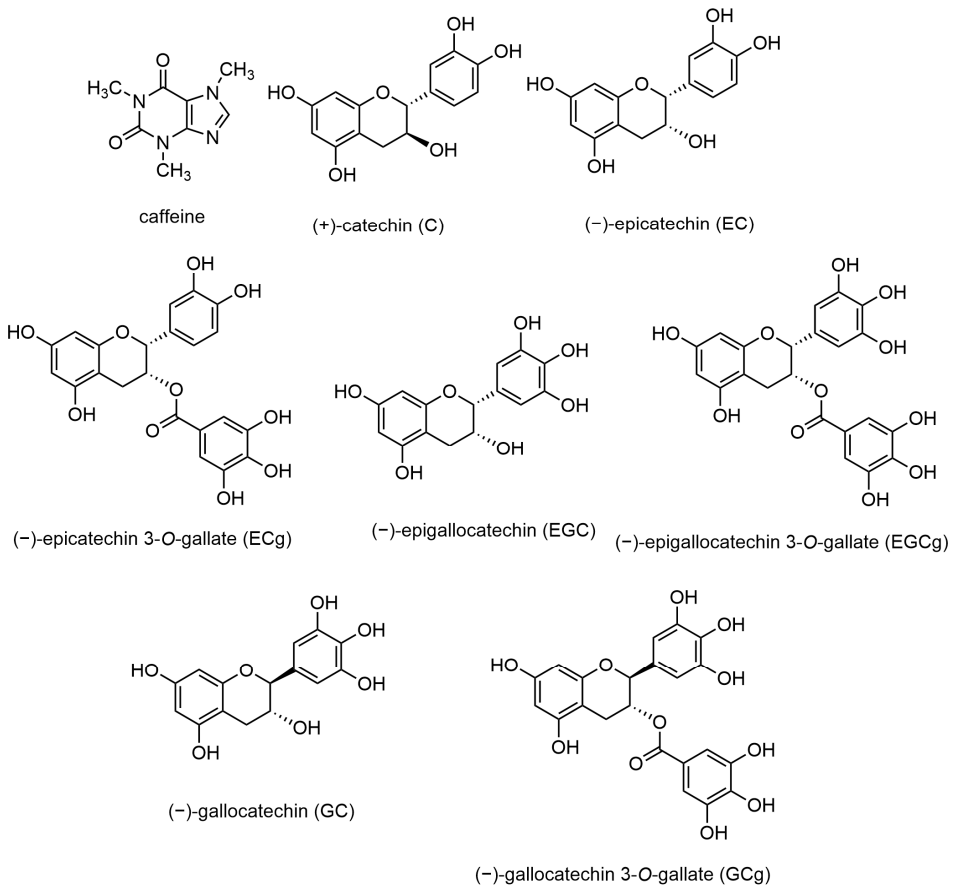

Figure 1. Chemical structures of the components analyzed.

2. Materials and Methods

2.1. Materials

^{1}H-NMR spectra were measured on an AVANCE-III-HD 600 NMR Spectrometer with BBO Probe, TopSpin 3.5pl7 (^{1}H-NMR: 600 MHz, Bruker Inc., Billerica, MA, USA) and an AVANCE NEO 500 NMR Spectrometer/DCH CryoProbe, TopSpin 4.0.7 (^{1}H NMR: 500 MHz, Bruker Inc., Billerica, MA, USA). Sample weights for qNMR were measured on an XPR6UD5 (Mettler-Toledo International Inc., Greifensee, Switzerland). Sample weights for dynamic vapor sorption were measured on an XP205DRV (Mettler-Toledo International Inc., Greifensee, Switzerland). Dynamic vapor sorption was measured on a Q5000SA (TA Instrument Inc., New Castle, DE, USA). Centrifugation was performed on a WSC-2700 MyMiniSpin (ATTO CORPORATION Co., Ltd., Tokyo, Japan). The 1,4-bis(trimethylsilyl)benzene-d_4 (1,4-BTMSB-d_4) reference material was purchased from Fujifilm Wako Pure Chemical Inc. (Lot.No. ESJ5567, purity: 100.0%, was used for experiments for extracts, and Lot.No. DLH0494, purity 99.9% was used for experiments with matcha powders). (Osaka, Japan). Hexadeuterodimethyl sulfoxide (DMSO-d_6, D 100.0%) was obtained from ACROS ORGANICS, Thermo Fisher Scientific Inc., (Waltham, MA, USA). Matcha samples for main analysis was provided from AIYA Co., Ltd. (Aichi, Japan). Single-origin matchas (Gokou, Samidori, Asahi and Okumidori) were purchased from Suhari Co., Ltd. (Kyoto, Japan). Green tea powder 1 was purchased from Harada Tea Processing Co., Ltd. (Shizuoka, Japan). Green tea powder 2 was grown in Yatsushiro (Kumamoto, Japan).

2.2. Extraction and Fractionation of Matcha

The matcha powder (100 g) was extracted with 80% EtOH (1 L) for two days at room temperature. The extract was filtrated and evaporated under reduced pressure at 50 °C to give 21.9 g of extract (TS-2021-E). From the extract, 18.5 g was suspended in water (300 mL), extracted with hexane (300 mL), and then divided. The extraction with hexane was repeated four times. The fractions of hexane were then combined and evaporated under reduced pressure to give 2.2 g (hexane soluble fraction). The hexane insoluble fraction of the water was extracted with ethylacetate (EtOAc, 300 mL), and then divided similarly 4 times. The fractions of EtOAc were then combined and evaporated under reduced pressure to give 8.7 g (EtOAc soluble fraction). The aqueous layer was evaporated under reduced pressure to give 8.1 g (water soluble fraction).

2.3. Hygroscopicity Measurement

A moisture adsorption/desorption capacity of the extract and fractions were evaluated as hygroscopicity of samples can be a problem in qNMR. Ten mg of each samples was weighed, and then relative humidity was increased in 5%RH ration from 5%RH to 95%RH, and then the humidity was decreased again in 5%RH steps down from 95%RH to 5%RH at 25 °C.

2.4. Parameters of qNMR Method

The choice of NMR solvent is important in qNMR. The NMR solvent selected should have sufficient solubility in the sample, and the signal derived from the non-deuterated residual peak of the solvent does not overlap with the signal of the compound to be measured. Therefore, DMSO-d_6 was selected as the most appropriate solvent, as it has good solubility for the extract and other solvent fractions. It is important that the reference material be separated from the signal to be measured as well as the non-deuterated residual peak of the solvent. It is also necessary to select a substance that does not react with the sample and is stable. To assure the accuracy and precision of the quantitative values obtained, it is preferable to select a certificated product. 1,4-BTMSB-d_4 was selected as the

internal standard material because of its solubility in DMSO-d_6 and compatibility with the sample.

For 80% ethanol extract and solvent fractions, about 10 mg of sample and 1 mg 1,4-BTMSB-d_4 were dissolved in 0.75 mL of DMSO-d_6. For analysis of matcha and green tea powders, about 15 mg of sample and 1 mg of 1,4-BTMSB-d_4 were directly dissolved in 0.75 mL of DMSO-d_6. Centrifugation samples were performed at up to 6000 rpm for 10 min.

The following analysis parameters were used: Apparatus: A nuclear magnetic resonance spectrometer having ^{1}H resonance frequency of 600 MHz or 500 MHz; target nucleus: ^{1}H; digital resolution after zero filling: 0.18 Hz/pt (600 MHz) or 0.15 Hz/pt (500 MHz); the number of data points to 65k; acquisition time: 2.7 s (600 MHz) or 5.0 s (500 MHz); measuring spectrum range: 20 ppm; including at least −2 ppm to 10 ppm; spinning: off; pulse angle: 90°; pulse widths: 10 µs (600 MHz) or 12 µs (500 MHz); ^{13}C decoupling: off (600 MHz) or on (500 MHz: CPDPRG: mpf9.noise); relaxation time: 60 s; scanning times: 8; dummy scanning: 2; measuring temperature: 25 °C; Lorentzian window function (line broadening: 0.2 Hz (600 MHz) 0.3 Hz (500 MHz)). All of the NMR spectra were manually phased, and baseline corrections were also performed manually. The receiver gain was automatically corrected for each measurement.

The calculation of the concentration of each component was carried out using following formula and Table 1:

$$\text{Amount of each component (mg/g of sample)} = (Hs/H) \times (I/Is) \times (M/Ms) \times (Ws/W) \times P \times 10$$

Hs: number of protons of 1,4-BTMSB-d_4 reference material (18).
H: number of protons of each compound for quantification signal.
I: intensity of quantification signal for each compound.
Is: intensity of 1,4-BTMSB-d_4 reference material.
M: molecular weight of each compound.
Ms: molecular weight of 1,4-BTMSB-d_4 reference material (226.50 g/mol).
Ws: amount (mg) of 1,4-BTMSB-d_4 reference material.
W: amount (mg) of sample.
P: purity (%) of 1,4-BTMSB-d_4 reference material.
10: unit correction form % to mg/g

Table 1. Profiles of compounds for qNMR calculation [21].

Compound	Molecular Weight	Chemical Shift [ppm] [a,b]	Position	No. of Protons
Caffeine	194.19	8.01	H-8	1
(+)-Catechin (C)	290.27	6.71	H-2′	1
(−)-Epicatechin (EC)	290.27	6.88	H-2′	1
(−)-Epicatechin-3-*O*-gallate (ECg)	442.37	6.74	H-6′	1
(−)-Epigallocatechin-3-*O*-gallate (EGCg)	458.37	6.39	H-2′, H-6′	2
(−)-Epigallocatechin (EGC)	306.27	6.37	H-2′, H-6′	2
(−)-Gallocatechin-3-*O*-gallate (GCg)	458.37	6.26	H-2′, H-6′	2
(−)-Gallocatechin (GC)	306.27	6.24	H-2′, H-6′	2
1,4-BTMSB-d_4	226.50	0.23	-	18

[a] δ (ppm) from TMS; [b] the variation of ±0.01 ppm was allowed for identification.

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3. Results

3.1. Results of Hygroscopicity Test

The matcha powder was extracted with 80% EtOH to obtain the extract. As in our previous study, where we performed the anti-anxiety activity evaluation of the extract and its hexane, ethyl acetate, and water-soluble fractions [7], a similar procedure was applied to make three fractions, i.e., hexane, ethyl acetate and water-soluble fractions. We measured the hygroscopic behavior of the extract using the standard method. The 80% EtOH extract was hygroscopic, and showed a weight gain of about 30% under high humidity (25 °C/95%RH) (Figure S1). Each fraction also showed hygroscopicity, with a weight increase of 60% for water soluble fraction, 20% for EtOAc soluble fraction, and 15% for hexane soluble fraction under high humidity. Therefore, weighing operations and storage must be performed in a low-humidity environment, or, if environmentally difficult, operations must be performed to shorten the exposure time to humidity.

3.2. Results of qNMR Method Development

In this study, we applied qNMR to the matcha extract, targeting multiple components such as caffeine, (+)-catechin (C), (−)-epicatechin (EC), (−)-epicatechin-3-*O*-gallate (ECg), (−)-epigallocatechin-3-*O*-gallate (EGCg), (−)-epigallocatechin (EGC), (−)-gallocatechin-3-*O*-gallate (GCg), (−)-gallocatechin (GC). Selection of the NMR solvent, internal reference materials, signals for quantitation of each compound, and validation items were made.

The validation items were selected with reference to ICH Q2 [22], and the results were used to evaluate the level of the test method. For the specificity and selectivity, the signals used for quantification were selected based on the NMR spectra of catechins in previous studies [21], which provide a reference for the specificity of catechins. Although there are several similar compounds in the matcha extract, the signals with specificity were selected as shown in Table 1 and Figure S2. Chemical structures of the analyzed components are provided in Figure 1. The assigned peaks in the NMR of the extract are presented in Figures 2 and S3.

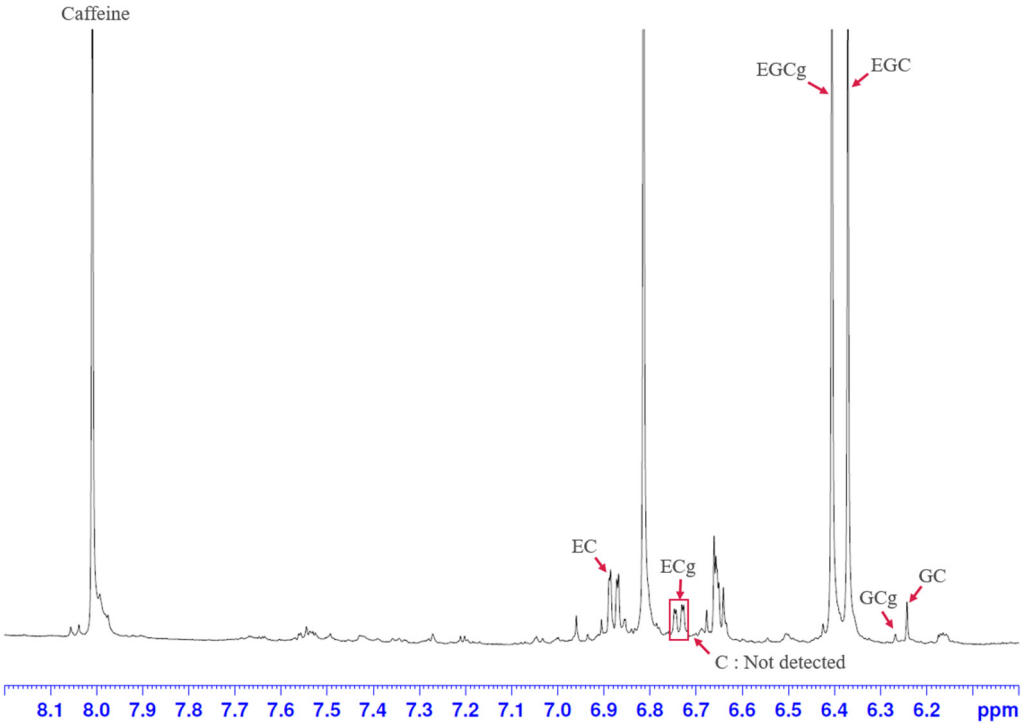

Figure 2. NMR spectrum of 80% EtOH extract of matcha (6.0–8.2 ppm).

For the measurement of accuracy, the results were evaluated for recovery rate and relative standard deviations (RSDs). The recovery rate and its RSD were calculated for 15 mg/0.75 mL and 5 mg/0.75 mL relative to the sample concentration (10 mg/0.75 mL) (Table S1). For the precision, the entire test method was repeated six times at the sample concentration (10 mg/0.75 mL). The RSDs were calculated from the content repeated six times at the sample concentration (Table S2).

For the working range, the result of specificity, accuracy and precision was considered. The limit of quantification required for practical use was set based on accuracy, precision and signal/noise (S/N) ratio. The acceptance criterion of the S/N ratio was set to 10 with reference to ICH Q2 [22]. The RSD of the limit of quantification was set to 25% as the acceptance criterion. The upper limit of the quantitative value was not evaluated in this study, as the gain was adjusted appropriately by the setup before the measurement for receiver saturation. And, in general, the larger the area intensity, the more the variability is low.

To test the system suitability, the results of RSD are shown for three instances of one preparation and six measurements (Table S3). The reason for the smaller RSD than the results for accuracy and precision may be due to the small preparation scale and the effect of variation due to weighing. Based on the results, the following system suitability was established. The following requirements are considered to provide a simple confirmation of the capability of a validated analytical method: the test for required detectability: the S/N ratio of the objective signal should be not less than 10; system performance: the objective signal is not overlapped with any obvious signal of foreign substance; and system repeatability: the RSD of the objective amount should be no more than 25% (n = 6).

For the stability data of the sample solution of the extract and other solvent fraction, since a decreasing trend with time was observed for caffeine, epicatechin-3-*O*-gallate, and epigallocatechin contents, it was decided to prepare the sample solutions just before the analysis (Table S4). On the other hand, the matcha powder sample solution took 1 day to extract and precipitate, but showed a decrease in the compounds to be analyzed (Table S5). Care should be taken to avoid suspending matcha powder, even after precipitation has occurred, as this will worsen the resolution (Figure S4). To solve this problem, centrifugation was performed, which resulted in a good spectrum (Figure S5). However, extraction was not sufficient immediately after preparation, and the content of compounds were generally less than that of the day 1 data (Table S6). Therefore, it takes 1 day to extract the compounds, but if it takes longer than that, decomposition will progress, so 1 day is considered appropriate. Also, if the precipitation does not progress sufficiently and the resolution is poor, centrifugation is recommended.

3.3. Results for Amounts of Major Constituents in Matcha

The developed method was applied first to analyze the concentration of components in the 80% extract and fractions (Table 2). The results of the 80% EtOH extract showed similar trends to those reported in previous studies [1]. Then, it was again applied to analyze the compounds in various matcha powders and green tea powders. For this, matcha powders or green tea powders were directly mixed in DMSO-d_6 without any pre-extraction. The results of matcha powders form DMSO-d_6 extraction showed the higher content as compared to the ethanol extract (Table 3).

Table 2. Contents of chemical compounds in extract and fractions.

	80% EtOH ext. (mg/g of Matcha Powder [a])	80% EtOH ext. (mg/g of Extract)	Water Soluble Fraction (mg/g of Fraction)	EtOAc Soluble Fraction (mg/g of Fraction)	Hexane Soluble Fraction (mg/g of Fraction)
Caffeine	31.38	143.28	249.60	199.40	50.59
C	ND [b]	ND	ND	10.22	ND
EC	7.27	33.18	ND	60.65	18.25
ECg	15.69	71.66	ND	91.01	42.20
EGCg	36.54	166.85	ND	318.50	93.40
EGC	20.59	94.00	92.15	126.45	37.73
GCg	1.39	6.33	ND	9.51	ND
GC	1.88	8.60	13.41	11.97	ND

[a]: Corrected from the amount of 80% EtOH extracted form matcha power in 2.2; [b] ND: not detected or less than limit of quantification.

Table 3. Contents of chemical compounds (mg/g) in matcha and green tea powder [a].

	M1	MG	MS	MA	MO	GT1	GT2
Caffeine	101.77	93.78	93.11	106.34	114.22	36.32	50.91
EC	27.56	11.96	9.12	15.76	17.93	18.57	22.31
ECg	60.04	40.01	33.42	48.03	48.63	35.42	37.30
EGCg	121.09	80.99	62.38	106.16	99.58	76.88	107.79
EGC	64.27	25.30	20.33	42.87	32.36	56.98	65.73
GCg	8.28	ND [b]	ND	ND	ND	ND	4.36
GC	9.34	NA [c]	NA	NA	NA	3.86	6.92

[a] (+)-catechin excluded from the evaluation because of low content; [b] ND: not detected or less than limit of quantification; [c] NA: not applicable; the objective signal is overlapped with any obvious signal of other compounds. Matcha samples: M1: matcha from AIYA; MG: Gokou; MS: Samidori; MA: Asahi; MO: Okumidori. Green tea powder samples: GT1: green tea powder 1; GT2: green tea powder 2.

When analyzing signals with a small S/N ratio, a consistent procedure is necessary, because the phase correction, baseline correction, and integration range taken can lead to large variations. Since matcha and its extracts are hygroscopic, the variation in weighing may have a significant impact on the quantification of the major components.

For the analysis of matcha powder with our pre-extraction, further exploration of conditions for optimal extraction and precipitation time and solution stability is required, and data reflecting the effects of different varieties and processing is needed to accumulate. At present, it is possible that differences in lots may result in insufficient precipitation, which may worsen the quantification from resolution (Figure 2). Analysis of the total catechin and caffeine contents can be used to identify the differences between matcha and other tea varieties, such as green tea powder, based on a total catechins/caffeine ratio (Figure S6, Table S7).

4. Discussion

The results for solvent fractions may be better than those of the above validation because of the reduction of the interrupting substance. If the validation result and system suitability of this study are not at the purposed level, pre-treatment of the sample solution (e.g., preparative purification) or other should be considered. Even if the qNMR parameters differ from this study, if the equivalent spectra are obtained and system suitability is met, then the equivalent analytical method performance is expected. If a large amount of phenolic acid is included, there is a risk of the specificity of the signal from B-ring (substructure of catechins) will be lost, and there is a possibility of overestimation. Similarly, there is a risk of the specificity of the signal of caffeine will be lost because of interrupting from methylxanthines. However, previous studies have shown that the amounts are small, and the impact on quantitative performance is thought to be small [1]. The reason for the

short stability of the solution is thought to be due to isomerization caused by oxidation (theaflavinization) of catechins [23].

The ethyl acetate fraction has been reported as a fraction with strong anxiolytic activity [7], and the results of this study revealed that it contained the largest amount of catechins and caffeine. A simple comparison of the amounts alone did not clarify the correlation, and the effects of other components or combinations could not be fully investigated in this study.

The amounts of major components were higher in directly analyzed matcha powders (mixing with DMSO-d_6) as compared to that of 80% EtOH extract (Table 2). This suggests that extraction with DMSO-d_6 may be more efficient than commonly used solvents such as alcohol and water [1]. However, there have been no reports of extract extraction and measurement with DMSO and DMSO-d_6, and further investigation is required, considering the possibility of an increase in the quantitative values due to the deterioration of resolution due to matcha particles.

The results of this study were based on the evaluation of one brand of matcha powder from one manufacturer, as well as data on the solvent fraction extracted from the matcha powder, and also included samples of one lot of each of four varieties from one manufacturer. Therefore, further investigation is needed into differences between manufacturers, blending methods, and lots. On the other hand, it may be possible to add a scientific method option to the quality evaluation of matcha, which has been classified and judged by sensory tests, and it is considered to be beneficial for quality control. In addition, although the initial investment and maintenance costs of NMR are higher than those of HPLC, compared to the ISO [13] and HPLC method, NMR has the advantage of being faster, and does not require standard samples of the analyte being measured, thereby reducing experimental time and reagent procurement costs. Since the qNMR method is simple, and the sample solution can be easily prepared using a small amount of matcha powder, we believe that this method can be used in a practical manner to evaluate large quantities of commercially available lots.

In cases where more precision and accuracy are required (such as when values on the μg/g order are required), it is difficult to ensure sensitivity, so it is difficult to evaluate using these conditions. It is advisable to change the NMR parameters and other analytical methods, depending on the purpose. It is expected that this study will be useful for brief quality evaluations of matcha, and for the development of the qNMR test method for compounds in mixtures.

5. Conclusions

In this experiment, we developed a qNMR method for the quantitative analysis of major components in matcha powder, extract, and fractions. The analysis of matcha and green tea suggested the possibility of distinguishing between matcha and green tea by the ratio of caffeine content and total catechins.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/scipharm93010011/s1, Table S1: Recovery rate (%) in accuracy for 80% EtOH extract (600 MHz); Table S2: Results of precision (mg/g of 80% EtOH extract, 600 MHz); Table S3: Results of system reputability (RSD%) (600 MHz); Table S4: Results of stability of 80% EtOH extract solution at room temperature (mg/g of 80% EtOH extract, 600 MHz); Table S5: Results of stability of matcha powder solution at room temperature (mg/g of matcha powder, 600 MHz); Table S6: Results (mg/g) of comparison of matcha powder solution immediately after preparation (Day 0) and after 1 day (Day 1) with and without centrifugation at room temperature (500 MHz); Table S7: Comparison of total catechin/caffeine ratio of matcha powder (500 MHz) Figure S1: Adsorption isotherm of 80% EtOH extract and each fraction against relative humidity; Figure S2: Atom

number of the caffeine and catechins; Figure S3: Typical NMR spectrum of 80% EtOH extract solution (500 MHz); Figure S4: NMR spectra overlay of matcha powder solution for the comparison of effects of precipitation (600 MHz); Figure S5: Expanded NMR spectra overlay of matcha powder solution for the comparison of effect of centrifugation (600 MHz); Figure S6: NMR spectra overlay of matcha powder solution of different varieties and green tea powder (500 MHz); Figure S7: Expanded NMR spectra overlay of matcha powder solution of different varieties and green tea powder (500 MHz).

Author Contributions: Conceptualization, K.H. and H.P.D.; methodology, K.H.; formal analysis, K.H. and H.P.D.; investigation, K.H.; resources, K.H., Y.K. and H.P.D.; writing—original draft preparation, K.H. and H.P.D.; writing—review and editing, K.H., Y.K., S.K. and H.P.D.; supervision, H.P.D. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: One of the authors Kengo Hori is the employee of Astellas Pharma Inc. Other authors declare no conflicts of interest.

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